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The production of ^{15}N-Labelled S-Adenosylmethionine and Adenine by Yeast Biosynthesis *

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SUMMARY

A method for the biosynthesis of ^{15}N-labelled S-adenosylmethionine from $^{15}NH_4^+$ and L-methionine by yeast is reported. Culture conditions suitable for economy in the utilization of the isotope were developed, and the use of γ-irradiated yeast (1 Mrad) for this purpose was explored. The irradiated yeast proved as effective as non-irradiated yeast, but it did not offer any advantage. ^{15}N-Adenine was obtained from S-adenosylmethionine by hydrolysis; its isolation by ion exchange chromatography is described.

^{15}N-Labelled purines have played an important part in the exploration of nucleoside, nucleotide, and nucleic acid metabolism [1–3]. Two methods are available for the preparation of the labelled bases : the first involves chemical synthesis from suitable ^{15}N-labelled precursors, mainly by the procedure of Traube [4–5]. Its advantage is that the ^{15}N-label can be placed in selected positions of the purine ring according to the needs of the contemplated metabolic experiments. The steps of the synthesis, however, are numerous and time-consuming [6]. The second method is the biosynthesis of the purines from $^{15}NH_4^+$ by yeast. The product is adequate for many experiments in which uniform labelling of the purine is sufficient. The ease of management of the cultures and the high ribonucleic acid content of yeast have facilitated the isolation of uniformly labelled material [7].

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The present report concerns a modification of the latter procedure, particularly for the synthesis of ^{15}N-S-adenosylmethionine (S-AM) and subsequent hydrolysis of the sulfonium compound to ^{15}N-adenine. The importance of the latter compound in ^{15}N-labelled form is obvious from many reports in the literature [6]. ^{15}N-labelled S-adenosylmethionine is required for studies of the nitrogen metabolism of its purine ring.

The method is based on the stimulation of intracellular ATP synthesis by providing a supplement of methionine in the culture medium (Scheme I). In the cells, methionine intercepts ATP by the reaction :

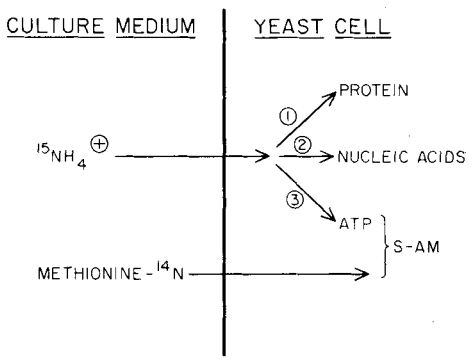
L-Methionine + ATP → S-Adenosylmethionine + PP + P (I)

A high level of the sulfonium compound is reached, and the product is transferred into the vacuole for storage [8,9]. Under favorable conditions the concentration of S-AM attained in yeast is as high as that of the combined values of all nucleotides and nucleic acids [10].

In the present investigation yeast cultures with L-methionine supplement and $^{15}NH_4^+$ as a source of nitrogen have been used to produce S-adenosylmethionine labelled with ^{15}N in the adenine part. Procedures for the isolation of ^{15}N-adenine from the sulfonium compound are described, and incidental observations on the distribution of $^{15}NH_4^+$ in the yeast culture are reported.

MATERIALS AND METHODS

$^{15}NH_4^+$ was obtained as the sulfate or chloride from the International Chemical and Nuclear Corporation, California. *Saccaromyces cerevisiae* in the form of activated dry bakers' yeast has been used. This commercial product which is sold for baking purposes has the advantage over moist yeast cakes of being stable at low temperature for several years. For the



SCHEME 1. The assimilation of ammonia and methionine by yeast.

present experiments, the material was obtained from the National Yeast Corporation, Chicago. No significant difference from other commercial products has been observed.

The medium contained the following ingredients : NH_4^+ or $^{15}NH_4^+$, as sulfate or chloride, 10 or 15 mM, as specified in the experiments; KH_2PO_4, 75 mM; K_2HPO_4, 30 mM; trisodium citrate, 3.5 mM; Mg^{++}, 0.15 mM; Ca^{++}, 0.09 mM; Mn^{++}, 0.03 mM; Zn^{++}, 0.03 mM; glucose, 85 mM; L-methionine, 5 to 10 mM, as indicated in the experiments. Sufficient quantities of trace metal ions and vitamins (biotin) are contained in the commercial yeast preparations. Sterilization of the medium is not necessary. A maximum rate of aeration is of utmost importance [11,12]. This was attained by using 100 ml of medium in 500 ml Erlenmeyer flasks to which 0.75 g of dry bakers' yeast (about 3 g in the hydrated form) was added. A rotary shaker (Gyrotory Shaker model G 22, New Brunswick Scientific Co., New Brunswick, New Jersey) was used, and the conventional cotton plugs were replaced by a double layer of gauze.

The culture period usually was 40-48 hours. Since the initial amount of yeast added to the system is large and the quantity of nutrients is limited, growth remains restricted. The initial period of intense metabolism produces the desired synthesis of ATP and S-AM [12]. The cells were harvested by centrifugation, washed twice with cold water, and the entire yield (3 to 4 gm) of each culture flask was extracted with 25 ml of 1.5 N perchloric acid. Extraction for 1 hour at room temperature is sufficient [12].

The isolation of S-AM was carried out by chromatography on Dowex 50-H^+ resin, as described earlier [13]. The product is contaminated by S-adenosylhomocysteine. If pure S-AM is desired, a Dowex 50 column in Na^+ form can be used [14] for removal of S-adenosylhomocysteine (see also Table III). An alternative is the isolation of S-AM as the Reinecke salt [15].

The concentration of S-AM was determined by spectrophotometry [14] at 256 mμ if in acid solution (A = 14,700), and at 260mμ if in water (A = 15,400). U. V. spectrophotometry was employed also for assay of other adenine compounds. Ninhydrin was used for amino acid determination, the nitroprusside test [16] for methylthioribose, and the orcinol pentose test [17] for S-pentosylmethionine assay. Ammonia was collected by microdiffusion using the Seligson-Conway technique [18].

For analysis of the ^{15}N-content, the eluates were concentrated in a flash evaporator and the resulting material subjected to the Kjeldahl procedure in an Aminco apparatus. The digest was distilled after addition of NaOH containing 5 % $Na_2S_2O_3.5H_2O$ and collected in 0.5 NH_2SO_4. This technique, when tested with adenine, adenosine, S-AM, or amino acids, gave full recovery of the nitrogen. Ammonia was converted to N_2 by reaction with sodium hypobromite in Rittenberg flasks [19]. A mass spectrometer (Ital-elettronica, SP-21-F) was used for determination of the percentage of ^{15}N.

RESULTS AND DISCUSSION

Biosynthesis of ^{15}N-labelled S-Adenosylmethionine.

For efficiency in the conversion of $^{15}NH_4^+$ into ^{15}N-S-AM, it was desirable to select the concentration of ammonium salt as low as was compatible with high production of S-AM. Furthermore, in order to avoid the diversion of nitrogen into protein and nucleic acid biosynthesis (reactions 1 and 2 of Scheme 1), and to favor instead the synthesis of ATP (reaction 3 of Scheme 1), two variations of the conventional method of yeast culture were employed. First, instead of cultivating the yeast from a small inoculum to the final yield of 3 to 4 g of cells per 100 ml of medium, nearly the full amount of bakers' yeast was added at the outset [12] since cellular growth is not a prerequisite for the formation of S-AM. Second attempts were made to curtail protein and nucleic acid biosynthesis by pre-irradiation of the yeast. Several experiments, therefore, were carried out with irradiated as well as non-irradiated yeast (Tables I and II). Preliminary experiments indicated that a concentration of 10 to 15 μmoles NH_4^+ per ml of medium is most economical in the incorporation of ammonia into the adenine of S-AM. In earlier experiments [12] with 30 mM NH_4^+ it had been found that the maximum level of S-AM is reached with 5 mM

TABLE 1. Effect of γ-irradiation on the biosynthesis of S-Adenosylmethionine in *Saccharomyces cerevisiae.*

Additions to the culture medium		Yield of cells		Yield of S-adenosylmethionine	
NH_4^+	L-Methionine	Irradiated yeast	Non irradiated yeast	Irradiated yeast	Non irradiated yeast
mM		g/100 ml		μmoles/culture	
0	5	3.3	3.6	26.4	24.7
10	5	3.6	3.8	67.8	53.1
10	10	3.8	4.2	63.4	60.3
15	5	3.2	3.5	68.3	69.8
15	—	3.1	—	0.8	—

Each flask contained 100 ml of salts and glucose medium with L-methionine, and NH_4^+ as indicated; 0.75 g of dehydrated bakers' yeast was added. The culture period was 48 hrs at 30° C. The γ-irradiation of the yeast was carried out with the dry material prior to the experiment; 1 Mrad was provided from a ^{60}Co source. Further details are given under Materials and Methods.

L-methionine in the medium. It appeared possible that a higher concentration of methionine would be needed for the conversion in presence of only 10 mM $^{15}NH_4^+$. The effect of doubling of the concentration of methionine was tested, but no advantage was found with 10 mM L-methionine as supplement (Table 1). In the absence of L-methionine, the usual [12, 13] low concentration of S-AM was found. On the other hand, the effect of L-methionine in the absence of added NH_4^+ indicates that the yeast contains considerable reserves of nitrogen.

On the basis of the results given in Table 1, experiments were designed with $^{15}NH_4^+$ as precursor. The data (Table II)show that the percentage of ^{15}N of the ammonium salt added to the culture is not attained in the adenine part of S-AM. The nitrogen reserves of the yeast and an intracellular pool of purine compounds may account for the dilution. No difference in the ^{15}N dilution was found between non-irradiated and irradiated yeast. Table III shows that the dilution of the isotope in the culture medium occurred rapidly after addition of the dry yeast. Nitrogenous compounds other than NH_4^+ are released by the cells as indicated by the lower value of ^{15}N in the total nitrogen compared with that in NH_4^+ in the culture medium. There is also a significant decrease in the concentration of the isotope in the total nitrogen after 24 hours of incubation. Apparently, autolytic processes release further nitrogen compounds into the medium. The 0.1 N NaCl eluate (Table III) contains nucleotides and S-adenosylhomocysteine, and the presence of ^{15}N in this fraction suggests the possibility of isolating other bases as well.

The present experiments show that the nitrogen reserves of the yeast employed here are considerable. The hydration of 0.75 g of dry bakers' yeast usually leads to 2.5 g of hydrated cells. With yields of 3 to 4 g of moist cells after the culture period, it is obvious that growth is limited under the experimental conditions. Gamma-irradiated yeast (1 Mrad), which is non-viable as judged by the inability to form colonies after plating, still shows high metabolic activity as evidenced by undiminished synthesis of S-AM (see also [20]). The somewhat higher yield of cell centrifugate from irradiated yeast may indicate a difference in hydration. Since the yield of labelled S-AM was not improved, no advantage was found in the use of irradiated yeast. The continued metabolic activity of lethally irradiated yeast has been reviewed recently by James and Werner [21, 22].

Isolation of Adenine-^{15}N

For the isolation of adenine from S-AM several procedures are available. The base can split by treatment with alkali [17]. In contrast to nucleotides and related adenine-containing compounds, S-AM is rather stable toward acid [9]. If acid hydrolysis for isolation of its adenine is preferred, the sulfonium group is eliminated first by hydrolysis to homoserine and

TABLE II. Synthesis of ^{15}N-S-Adenosylmethionine from $^{15}NH_4^+$ and unlabelled methionine by yeast

Type of yeast	Supplements in the medium : $^{15}NH_4^+$ (5.89 % ^{15}N)	Supplements in the medium : L.Methionine	Yeast obtained	S-Adenosyl-methionine obtained	Efficiency in ^{15}N incorporation [a]	^{15}N content of S-AM
	mM	mM	g/100 ml	μmoles/culture	%	%
Non-irradiated	0	5	3.7	26.0	—	0.37
	10	5	3.8	65.1	18.3	2.77
	10	10	3.9	61.2	15.5	2.65
Gamma irradiated (1 Mrad)	0	5	4.3	25.0	—	0.37
	10	5	4.3	54.5	15.4	2.78
	10	10	4.5	60.3	17.1	2.82

The experimental details were the same as those in TABLE I; analytical techniques are described under Materials and Methods. The values are averages of similar results obtained from three separate experiments.

[a] These values are calculated on the basis of ^{15}N added as $^{15}NH_4^+$, and ^{15}N found in S-adenosylmethionine.

TABLE III. The distribution of ^{15}N in various fractions of yeast cultures

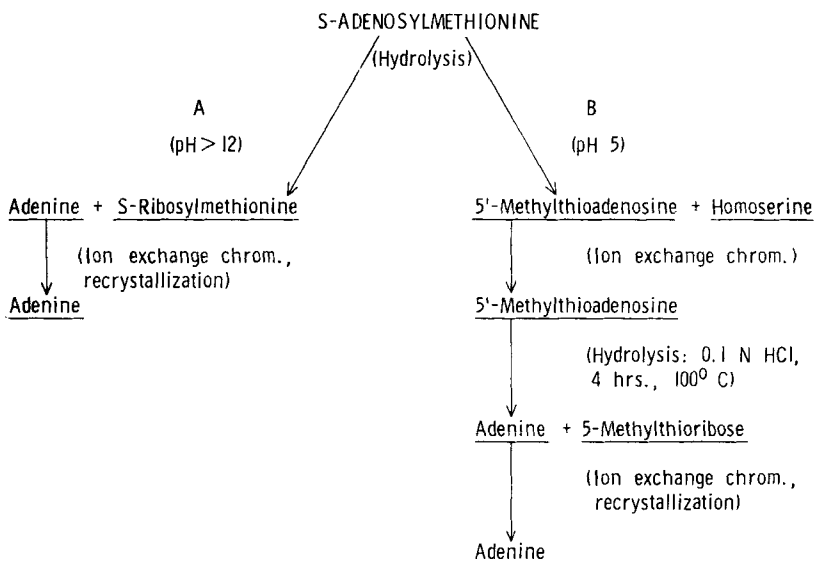
Incubation time	Percentage of ^{15}N in :				
				Chromatographics eluates	
	Total N of culture medium	Ammonia of culture medium	Perchloric acid extract of cells	0.1 N NaCl [b]	6 N HCl [c]
5 min [a]	5.77	8.05	1.50	—	—
8 hrs	5.33	7.50	4.48	4.40	5.30
24 hrs	4.78	7.80	4.86	4.50	4.51
48 hrs	1.80	7.00	4.82	4.20	4.61

Each flask contained 100 ml of medium with 10 mM NH_4^+ (19.8 % ^{15}N), 5 mM L-methionine, and cells derived from the initial amount of 0.75 g dehydrated bakers' yeast. At the times specified, samples were centrifuged, and cells and supernatant fluid were analyzed as described under Materials and Methods.

[a] Centrifugation of the sample was begun after 5 minutes.
[b] This eluate contains S-adenosylhomocysteine, nucleotides, and related compounds.
[c] S-Adenosylmethionine.

5′-methylthioadenosine at pH 5-6, 100° C, for 30 minutes. 5′-Methylthioadenosine does not have a sulfonium group and behaves toward acid like other adenine nucleosides and nucleotides; 4 hours of hydrolysis at 100° C in 0.1 N HCl splits it into adenine and 5-methylthioribose [17] which are separated with ease in a cation exchange column (see Scheme II). The choice of the procedure will depend on the hydrolytic products that are desired in addition to adenine.

The following is an example of the preparation of ^{15}N-S-AM, its alkaline hydrolysis, and the isolation of ^{15}N-adenine (Proc. A, Scheme II). Six 500 ml Erlenmeyer flasks containing a total of 600 ml of medium with 15 mM NH_4^+ (30 % ^{15}N), L-methionine (7 mM) and a total of 4.5 g of dehydrated active bakers' yeast gave, after a fermentation period of 48 hrs at 30° C, a total of 24.2 g of washed cell centrifugate. Extraction with



SCHEME II. The isolation of adenine from S-adenosylmethionine.

1.5 N perchloric acid at room temperature and chromatography with Dowex 50 H^+ resin [13] gave 433 μmoles of S-AM, contaminated by small quantities of S-AH [14]. The product was purified by precipitation as Reinecke salt [15]. Adenine was split off by treatment with 0.1 N $Ba(OH)_2$ for 5 hrs in an ice bath [11]. After removal of Ba^{++} by H_2SO_4, spectrophotometry showed the presence of 343 μmoles of adenine. The hydrolyzate was applied to a Dowex 50 H^+ column of 1.6 cm diameter and 10 cm length.

Elution was begun with 200 ml of 0.1 N HCl, followed by 300 ml 0.5 N HCl. The release of adenine was accomplished with 900 ml of 2 N HCl; the recovery in this step was quantitative. After evaporation of HCl under reduced pressure, the dry residue was taken up in a few ml of H_2O and neutralized with a small quantity of solid $KHCO_3$ to pH 7. Impure adenine crystallyzed slowly from the brown solution. Apparently, S-pentosylmethionine and methionine, which are not separated quantitatively, impede the crystallization. After concentration and two recrystallizations from H_2O, a yield of 37.5 mg of adenine (278 μmoles) was obtained. The material was pure as judged by spectrophotometry and paper chromatography with 1-butanol-acetic acid-water (12 : 3 : 5, v/v); U. V. quenching and ninhydrin spray were used for the detection of the spots. Mass spectrometry showed the presence of 17.8 % ^{15}N in the final product.

The procedure B (Scheme II) is exemplified by the following experiment. S-AM (238 μmoles) obtained from a culture with 10 mM $^{15}NH_4^+$ (96.1 % ^{15}N) and 5 mM L-methionine was hydrolyzed at pH 5, for 30 minutes at 100° C. The hydrolyzate was applied to a Dowex 50 H^+ column, 1 cm diameter and 8 cm length. Elution with 2 N HCl released homoserine in the first 60 ml; thereafter, 5′-methylthioadenosine appeared and was eluted by 200 ml of 2 N HCl (229 μmoles). After removal of HCl by evaporation under reduced pressure, the dry residue was taken up in 10 ml of 0.1 N HCl. For conversion of 5′-methylthioadenosine into adenine and 5′-methylthioribose, the solution was heated in a boiling water bath for 4 hours. The hydrolyzate was applied to a Dowex 50 H^+ column as specified above : 5′-Methylthioribose was eluted with 40 ml of 0.1 N HCl, adenine with 100 ml of 4 N HCl. The latter was evaporated under reduced pressure. The dry, somewhat discolored residue was taken up in a few ml of H_2O, neutralized to pH 7, and stored at low temperature for crystallization. After several recrystallizations from small quantities of H_2O and concentration of the supernatant fluids, 20.1 ml (183 μmoles) of pure adenine was obtained. Mass spectrometry showed 40.6 % ^{15}N in the product.

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