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The Radiopurity of S-Adenosylmethionine and S-Adenosylethionine Preparations *

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SUMMARY

In the biosynthesis of labeled S-adenosylmethionine and S-adenosylethionine from the corresponding ^{14}C-labeled sulfur amino acids by yeast, some radioimpurity occurred by non-specific labeling of the sulfonium compounds. This is explained by partial degradation of methionine and ethionine in the yeast cells, followed by utilization of the randomized fragments in the biosynthesis. The degree of mislabeling remains low because endogenous methionine synthesis from metabolic pool constituents is suppressed by the excess of methionine and ethionine supplement in the culture medium. Hydrolysis of the sulfonium compounds and analysis of the fragments showed that the misplaced isotope amounts to approximately 0.5% in the adenine, and 0.2% in the homoserine part compared with the total radioactivity of the sulfonium compounds (100%).

INTRODUCTION.

The biological methyl donor, S-adenosylmethionine, and its analogue, S-adenosylethionine, are prepared for experimental purposes almost exclusively by biosynthesis from methionine or ethionine with growing or metabolizing yeast [1, 2]. The site of the label in the precursor determines its principal location in the sulfonium compound. The recovery of the isotope in the final product, however, rarely exceeds 20% [3]. In most instances, only a small quantity of the labeled precursor is left in the spent culture medium. Some of the methionine is incorporated into cellular protein, and a large part is metabolized in non-specific ways after degradation. It appears possible, therefore, that some fragments of methionine may find their way into a metabolic

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pool, and from there into various positions in S-adenosylmethionine. When using labeled methionine as the precursor, this would result in a limited amount of labeling at sites other than those intended. Similar considerations apply to ethionine and S-adenosylethionine.

The purpose of the present investigation was to examine the extent of non-specific labeling of the biosynthetic sulfonium compounds and to evaluate the preparations obtained with regard to their usefulness in transmethylation and transethylation experiments.

EXPERIMENTAL PROCEDURES AND MATERIALS.

The conditions of biosynthesis of S-adenosylmethionine and S-adenosylethionine from the corresponding ^{14}C-labelled sulfur amino acids have been described earlier [2-4]. The level of the sulfur amino acid in the present experiments was 3.0 μmoles per ml of culture medium for metabolizing *Saccharomyces cerevisiae* (dried activated baker's yeast) and growing cells of *Candida utilis* (formerly termed *Torula* or *Torulopsis utilis*). In experiments with *S. cerevisiae*, dried activated baker's yeast (0.75 gm) was added to 100 ml of supplemented medium [3]. For growing *C. utilis*, a 24 hour culture was transferred into 100 ml of supplemented medium. Intense aeration was provided in both types of experiment by agitation of the flasks on a rotary shaker with about 100 oscillations per minute. After 40 to 48 hours at 30°, the cells were harvested by centrifugation, washed twice with water, and weighed. The yield from each culture (2.0 to 3.5 gm of moist cells) was extracted with 25 ml of 1.5 N perchloric acid at room temperature for one hour or longer [3]; the sulfonium compounds are stable in acid.

The extracts were purified by chromatography on Dowex 50 H^+ resin with 1 N, 2 N, and 6 N H_2SO_4 [5]. The latter elutes S-adenosylmethionine (S-adenosylethionine) and small quantities of S-adenosylhomocysteine. Removal of S-adenosylhomocysteine from the final product was accomplished by precipitation of the sulfonium compound as the Reinecke salt, $[(SCN)_4(NH_3)_2Cr]^-$, and by isolation as described previously [4]. The concentration of the product was determined by spectrophotometry, and its radioactivity by scintillation counting.

Hydrolytic products of the sulfonium compounds were secured by heating at pH 5 to 6 in a boiling water bath for 30 minutes. Homoserine and 5′-methylthioadenosine (5′-ethylthioadenosine) are the principal products under these conditions [6]. It was not possible to assay the fragments of the sulfonium compounds by simple paper chromatography and radioscanning, or elution of the spots and scintillation counting. Small amounts of various secondary products overlap with the principal spots on the chromatograms. Depending on the initial purity of the preparation and the mode of hydrolysis and further treatment, the interfering substances include methionine sulfoxide, 5′-methyl-

thioadenosine sulfoxide, S-pentosylmethionine, 5-methylthioribose and its sulfoxide, and perhaps other substances. The following procedure was adopted, therefore. For preliminary separation of 5′-methylthioadenosine (5′-ethylthioadenosine) and homoserine, paper chromatography of bands with guiding spots was performed with Whatman No. 1 paper and 1-butanol-acetic acid-water (60 : 15 : 25, v/v). Care was taken that the quantity of hydrolysate did not exceed 0.2 μmoles of each compound per cm of band at the site of application. After development and drying, the bands were located by the ultraviolet quenching effect of 5′-methylthioadenosine (Rf 0.65) or 5′-ethylthioadenosine (Rf 0.75), and by ninhydrin spray of the guiding spots for homoserine (Rf 0.25). The bands were cut and eluted with water in descending arrangement; the first 10 to 20 ml contained the desired material.

Homoserine (5 to 10 μmoles) was applied to a column of Dowex 50 Na^+ resin, 1 cm in diameter and 8 cm long, conditioned with citrate buffer at pH 2.2; elution was begun with 50 ml of the same buffer and continued with citrate buffer at pH 3.1 [7]. Homoserine usually appeared after 30 to 40 ml of the latter buffer had passed the column. The material was confined to 2 or 3 fractions of 10 ml each; its concentration was determined by quantitative ninhydrin tests [8], and the radioactivity was assayed by a Beckman scintillation counter.

The paper eluate of 5′-methylthioadenosine (5′-ethylthioadenosine) was adjusted with H_2SO_4 to a concentration of 0.1 N and hydrolyzed in a boiling water bath for 4 hours [9]. The hydrolysate was applied to a Dowex 50 H^+ column, 1 cm diameter and 8 cm long, and developed with 100 ml 1 N H_2SO_4, followed by 2 N H_2SO_4. 5-Methylthioribose (5-ethylthioribose) passed the column without delay; adenine appeared in the 2 N acid eluate in a rather broad band; it was assayed by spectrophotometry and by liquid scintillation counting.

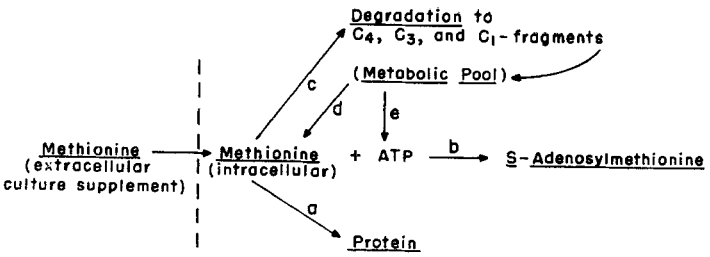
Results.

The endogenous biosynthesis of S-adenosylmethionine in yeast can be summarized as follows:

$$SO_4^- \rightarrow \rightarrow \rightarrow \text{Methionine} \qquad (1)$$

$$\text{Methionine} + \text{ATP} \rightarrow \text{S-Adenosylmethionine} + \text{P} + \text{PP} \qquad (2)$$

Under suitable conditions, *C. utilis* produces 0.8 to 1.7 μmoles of the sulfonium compound per gram of yeast centrifugate. *S. cerevisiae* accumulates less; 0.2 to 0.5 μmoles per gram is usually found in absence of an extraneous source of methionine. For preparation of large quantities of the sulfonium compound, one supplies methionine in the culture medium [3, 5]. The sulfur amino acid gets into the cells, intercepts endogenous ATP, and produces S-adenosylmethionine according to Reaction 2 in quantities ranging up to 20 μmoles per gram of yeast.



SCHEME 1. Some features of methionine metabolism in yeast.

After entry of methionine into the cell, however, Reaction 2 is not the only route of metabolism. The more important reactions are represented by Scheme I. Various products in the metabolic pool may participate in the endogenous synthesis of methionine and adenine. Undesired, non-specific labelling of S-adenosylmethionine results if the metabolic pool contains breakdown products from labelled extraneous methionine which are used in a random way for the synthesis of endogenous methionine (Scheme I, *d*). The labelled fragments (Scheme I, *c*) could participate also in the synthesis of the purine ring system of ATP (Scheme I, *e*); their occurrence in the carbohydrate part of S-adenosylmethionine, however, is virtually precluded, because glucose, which is present in large quantity in the medium, serves as the source of ribose. Thus, the misplaced label should be confined to adenine and the C_4-amino acid part of the sulfonium compound.

Similar considerations apply to ethionine and S-adenosylethionine except that the endogenous formation of ethionine in analogy to Reaction I occurs, if at all, at a very low rate [10, 11]; methionine would be the more probable product derived from the metabolic pool. The incorporation of ethionine into protein (Scheme I, *a*) remains at a level lower than that of methionine [12, 13].

The chances of mislabelling of S-adenosylmethionine by randomization of labelled precursors in the cellular metabolic pool (Reactions *c*, *d*, *e* in Scheme I) are decreased, if the extracellular supply of methionine suppresses endogenous methionine biosynthesis. This question was examined by the use of ^{35}S-labelled sulfate in combination with non-labelled methionine in the culture medium (Table I). If Reaction 1 (or *d* in Scheme I) is suppressed by the addition of external methionine, little or no ^{35}S should be found in S-adenosylmethionine.

The assay of total radioactivity in the sulfonium compound (Table I) shows a low value in cultures of *C. utilis* supplemented with L-methionine. If D-methionine is supplied in the culture medium, the endogenous synthesis of ^{35}S-adenosylmethionine remains considerable. In *S. cerevisiae*, the incorporation of ^{35}S from $^{35}SO_4^-$ is not much depressed by the supplement of methionine and ethionine; this indicates endogenous formation of methionine and

TABLE I. The incorporation of ^{35}S from $^{35}SO_4^-$ into S-adenosylmethionine by yeast.

Type of yeast and supplement added to culture medium	Yield of yeast	Yield of S-adenosylsulfonium compound	Radioactivity in S-adenosylsulfonium compound fraction
	gm/100 ml	μmoles	10^6c.p.m.
Candida utilis			
No supplement	2.93	4.65	11.48
L-Methionine (0.6 mM)	2.99	6.00	3.15
L-Methionine (1.0 mM)	2.56	7.63	0.30
L-Methionine (2.0 mM)	2.48	12.08	0.08
L-Methionine (3.0 mM)	2.33	14.55	<0.02
D-Methionine (3.0 mM)	2.76	19.85	1.80
Saccharomyces cerevisiae			
No supplement	3.44	0.85	1.55
L-Methionine (3.0 mM)	3.55	59.4	1.25
L-Ethionine (3.0 mM)	2.94	45.3	0.93

Each flask contained 100 ml of culture medium of low sulfate content (0.6mM) as specified earlier.[4, 14] In the experiments with *C. utilis*, 1.74×10^8 c.p.m. per flask were present, and 1.0 ml of 24 hour culture was added as inoculum. The cultures with *S. cerevisiae* (dried activated baker's yeast) contained 1.65×10^8 c.p.m., and 0.75 gm of dry yeast was added at the outset. The latter corresponds to nearly 3 gm of hydrated cells, and growth remains limted under these circumstances. The nutrients of the medium merely permit a period of intense metabolism. Analytical details are described under Experimental Procedures.

the sulfonium compound in addition to the assimilation of exogenous material. D-Methionine was not tested with *S. cerevisiae*, because in contrast to *C. utilis* this organism does not form S-adenosyl-D-methionine [2, 5]; conversely, ethionine was not tested with *C. utilis* because it inhibits growth [15, 16]. The low incorporation of the isotope in *S. cerevisiae* compared with that in *C. utitis* is explained mainly by the rather limited endogenous synthesis of S-adenosyl-methionine by *Saccharomyces* under the experimental conditions employed here.

A detailed examination of the radiopurity of the sulfonium compounds derived from labelled methionine or ethionine was carried out. Table II lists the radioactivity of homoserine and of adenine obtained by hydrolysis from methyl-^{14}C-, ethyl-^{14}C-, and α-^{14}C-labelled adenosylfulsonium compounds. The specific activity of the parent material is listed for reference, and the concentration of the culture supplements is given. Absence of measurable radioactivity in the hydrolytic products would indicate radiopurity of the molecule in these parts. In the present experiments, the radioactivity in the adenine part of

TABLE II. Examination of S-adenosylmethionine for radiopurity.

Precursor(s) in the culture medium	Yeast employed	Radioactivity of S-adenosyl-methionine	Radioactivity of hydrolytic homoserine	Radioactivity of hydrolytic adenine
		10^5c.p.m./μmole	*10^3c.p.m./μmole*	*10^3c.p.m./μmole*
L-Methionine-$^{14}CH_3$ (3 mM)	*C. utilis*	1.27	0.18	0.61
L-Methionine-$^{14}CH_3$ + adenine (3 mM each)	*C. utilis*	1.32	0.17	0.54
L-Methionine-$^{14}CH_3$ + adenosine (3 mM each)	*C. utilis*	1.33	0.17	0.84
DL-Methionine-2-^{14}C (3 mM)	*C. utilis*	1.35	—	0.79
L-Methionine-$^{14}CH_3$ (3 mM)	*S. cerevisiae*	1.14	0.19	0.17
L-Methionine-$^{14}CH_3$ + adenine (3 mM each)	*S. cerevisiae*	1.30	0.24	0.68
L-Methionine-$^{14}CH_3$ + adenosine (3 mM each)	*S. cerevisiae*	1.30	0.18	0.44
DL-Methionine-2-^{14}C (3 mM)	*S. cerevisiae*	1.35	—	0.88
DL-Ethionine-ethyl-1-^{14}C (2 mM)	*S. cerevisiae*	1.13 [a]	0.22	0.54

The biosynthesis of S-adenosylmethionine by *C. utilis* was carried out in growing cultures with a final yield of 2.5 to 3.5 gm of cells per 100 ml of medium. In the experiments with *S. cerevisiae*, 0.75 gm of dry baker's yeast (about 3 gm of cells after hydration) was added to 100 ml of medium; growth remains limited under these conditions, but sufficient ATP is synthesized in the cells to convert the methionine or ethionine into the sulfonium compounds. Experimental details are given in the text.

[a] S-Adenosylethionine was formed in this experiment.

S-adenosylmethionine averaged 0.5%, and that of homoserine 0.2% of that found in the parent material. A similar result was obtained after hydrolysis of S-adenosylethionine. In this case, the stray isotope in the homoserine was presumably derived from endogenous S-adenosylmethionine, because the biosynthesis of ethionine from metabolic pool constituents is improbable. Adequate methods for separation of S-adenosylmethionine and S-adenosylethionine by cation exchange resins have not yet been found. Supplementation of the culture medium with adenine or adenosine in addition to methyl-^{14}C-labelled L-methionine did not lower the level of undesired isotope in the adenine part of S-adenosylmethionine; apparently, the endogenous purine synthesis is not significantly suppressed.

DISCUSSION.

The relatively low level of radioimpurities in S-adenosylmethionine derived from *C. utilis* is caused by the suppression of endogenous methionine biosynthesis by the addition of this amino acid to the culture medium (Table I); this effect is less in *S. cerevisiae*. In both yeasts, the large quantity of sulfonium compound with the desired label (Reaction 2) dilutes the mislabelled product which is formed by endogenous synthesis after randomization of the precursor in the cellular metabolic pool (Scheme I).

The level of radioimpurities in the adenine (0.5%) and homoserine moiety (0.2%) is too low to warrant concern in enzymatic experiments with currently available analytical techniques. Some decomposition products are present in most preparations at the outset and are formed also during experimentation with solutions near neutrality [6]; their level exceeds that of the radioimpurities observed presently by an order of magnitude. The analytical methods available for transmethylation and transethylation studies are not yet accurate within a fraction of one percent [17-19].

The present study of radioimpurities in S-adenosylmethionine suggests analytical surveys in other instances of microbial biosynthesis in which an excess of labelled precursor is used for the production of a complex product.

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