A convenient procedure for the biosynthesis and isolation of ^{35}S-adenosylmethionine

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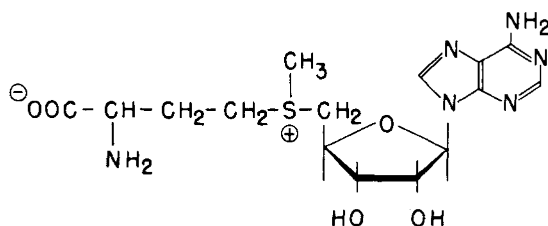
SUMMARY

^{35}S-(5'-Deoxyadenosine-5')-L-methionine (^{35}S-adenosylmethionine, $C_{15}H_{23}N_6O_5S^+$) has been isolated in useful amounts from yeast cells which were cultivated in a medium containing $^{35}SO_4^{--}$. Procedures for chromatographic purification and analysis are reported.

INTRODUCTION

L-Cystine-^{35}S and L-methionine-^{35}S are usually produced from labeled sulfate by biosynthesis with yeast [(1)]. Other sulfur-containing compounds are formed under these conditions, but so far they have found less attention than the labeled sulfur amino acids. We have observed that yeast is an excellent source of S-adenosylmethionine (Formula 1); its content of the sulfonium compound is higher than that of other micro-organisms, plant, and animal tissues[(2)].



FORMULA 1. — S-ADENOSYLMETHIONINE

Without special supplementation of the culture medium, *Saccharomyces cerevisiae* contains 0.2 to 0.5 μ moles of S-adenosylmethionine, and *Candida utilis* (*Torulopsis utilis*) 0.5 to 2.0 μ moles per g of moist cells. These concentrations

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are similar to the values reported for DPN, TPN, and ATP. It appeared possible therefore, to isolate ^{35}S-adenosylmethionine from yeast that had been cultivated in the presence of $^{35}SO_4^{--}$. Labeled modifications of this important metabolite of methionine have been rather inaccessible so far, with the exception of the methyl-^{14}C-labeled material.

The ^{35}S-labeled form of S-adenosylmethionine can be employed for transmethylation experiments by determination of the quantity of ^{35}S-adenosylhomocysteine which is formed in the process. The sulfur-labeled material has been indispensable in studies concerning the metabolism and regeneration of the sulfonium compound [3-6].

Experimental procedures and materials

Candida utilis ATCC 9950 was used in our experiments. Several other strains were found to be as suitable for the production of S-adenosylmethionine [7]. The organism was maintained on wort agar slants. In the culture medium chlorides or acetates were used instead of sulfates. The final concentrations per liter were as follows : 10 g KH_2PO_4, 5 g K_2HPO_4, 2.35 g ammonium acetate, 1.0 g trisodium citrate, 0.02 g $MgCl_2 \times 6\,H_2O$, 0.01 g $MnCl_2 \times 4H_2O$, 0.01 g $ZnCl_2$, and 0.01 g $CaCl_2$; glucose, 15 g was sterilized separately.

The cultures were carried out in two steps. First, a starter culture was obtained with 20 ml of medium in a 125 ml Erlenmeyer flask; for unimpeded growth of the yeast, this was supplemented by the addition of 0.3 mmole of K_2SO_4. A loop of organisms was transferred, and growth was heavy after 24 hours on a rotating shaker at 30°C. In the second step, 1.0 ml quantities of starter culture were transferred into 100 ml of sulfate-free medium in 500 ml Erlenmeyer flasks which were supplemented with $^{35}SO_4^{--}$ and SO_4^{--} as indicated in the experiments. A culture time of 48 to 72 hours with shaking at 30°C was usually employed. The cells were harvested by centrifugation and washed twice with cold water. After centrifugation in a tared tube, the yeast was weighed; the yield ranged between 2.5 and 3.5 g per 100 ml of culture medium. The cells were extracted three times by adding 3 ml, 2 ml, and 1 ml of 1.5 N perchloric acid per gram. Occasional agitation at room temperature for a minimum of one hour in each step completes the extraction. After centrifugation, the extracts were combined, and S-adenosylmethionine was isolated by the procedures described in the following part.

For chromatography, Whatman No. 1 paper and Eastman Chromagram thin layer sheets without fluorescent indicator were used with 1-butanol, water, acetic acid (60 : 25 : 15, v/v) as the developing solvent. Thin layer chromatograms gave better resolution of S-adenosylmethionine and S-adenosylhomocysteine than paper. The radioactivity scanning was carried out with the instrument developed in this laboratory [8], and a Packard Scintillation Spectrometer was used for counting of solutions. The efficiency of counting was 40%.

Lyophilized snake venom (*Agkistrodon piscivorus*), purchased from Ross

Allen's Reptile Institute, Silver Springs, Florida, was used as a source of L-amino acid oxidase [9, 10]. Reinecke salt [11], $NH_4[Cr(SCN)_4(NH_3)_2]$, for precipitation of the sulfonium compound was obtained from Eastman Organic Chemicals, Rochester, New York. A 0.06 N solution was prepared freshly for each experiment by dissolving the salt in water with stirring at room temperature. A small quantity of insoluble material was filtered off after 1 hour. Freshly precipitated $BaCO_3$ was used for neutralization of the preparations in dilute sulfuric acid. It was obtained by treatment of a 0.3 N $Ba(OH)_2$ solution with an excess of CO_2 (dry ice).

Results

Earlier experiments on the production of S-adenosylmethionine without L-methionine supplement in the medium had shown that the quantity of the sulfonium compound in the resulting cells was variable [2, 12]. The choice of *C. utilis* in preference to *S. cerevisiae* was made because this organism consistently yielded higher quantities of S-adenosylmethionine.

Under our usual conditions of culture [5, 13] the medium contains 15 mmoles of sulfate per liter in the form of ammonium sulfate. Only a fraction of this is incorporated into the organic components of the yeast, and therefore, dilution of this medium with $^{35}SO_4^{--}$ would be uneconomical. The sulfate-free medium specified under Methods was used, therefore, with minimal sulfate supplementation. Graded amounts of K_2SO_4 were added to determine the lower limit of sulfate concentration that not only supports growth of the yeast, but also yields a satisfactory quantity of intracellular S-adenosylmethionine.

The data in Table I show that even the lowest quantity of sulfate tested is sufficient to yield an undiminished crop of cells. At this level, however, the

Table I. The Influence of Sulfate Concentration on the Culture of *Candida utilis*

Concentration of sulfate in the culture medium	Yield of yeast	Yield of S-adenosylmethionine
mM	g/100 ml	μmoles/g
15	3.45	0.81
5	3.30	1.04
0.9+	3.47	0.95
0.3+	3.41	0.32
0.15+	3.33	0.20

The cultures were incubated for 48 hours at 30°C with aeration by vigorous rotary shaking. Perchloric acid extracts of the cells were analyzed by chromatography on Dowex 50 cation exchange resin (7) and spectrophotometry.

The quantities of sulfate specified in these experiments include the transfer of 15 μmoles of SO_4^{--} with 1.0 ml. of the starter culture.

yield of the sulfonium compound is curtailed. A sulfate concentration between 0.6 and 0.9 mmoles was chosen, therefore, in the labeling experiments. A culture period of 48 to 72 hours was found most suitable. The addition of a vitamin supplement [14] did not improve the yield.

For the preparation of the sulfonium compound, millicurie quantities of ^{35}S-sulfate were added to 100 ml of medium. Four parallel cultures were arranged in most experiments. The cells were harvested, washed with water and pooled for extraction [15]. The experimental conditions, yield, and distribution of radioactivity in several experiments are shown in Table II. Only

TABLE II. The Incorporation of Sulfur-(35) from Sulfate into S-Adenosylmethionine in Yeast Cultures

	Experiment No.				
	1	2	3	4	5
Conditions of culture :					
Volume of culture medium, ml	100	400*	400*	400*	100
Concentration of $^{35}SO_4^{--}$, mM	0.90	0.75	0.60	0.60	0.60
Culture period, hours	48	44	48	65	50
Yield of cells, gm	3.65	13.56	9.85	10.3	2.7
Yield of S-adenosylmethionine, μmoles/gm	1.45	1.10	1.95	1.92	1.75
*Survey of Radioactivity, c.p.m. × 10^{-9}***					
Incorporated into the culture medium	2.87	3.50	4.00	3.83	1.29
Recovered from spent medium and washes	1.41	1.40	1.01	1.21	0.42
In perchloric acid extract of cells	0.30	0.652	0.83	1.01	0.28
In S-Adenosylmethionine fraction	0.030	0.129	0.176	0.246	0.061
Recovery of radioactivity in S-adenosylmethionine fraction, per cent	1.04	3.7	4.4	6.4	4.7

* Four 100 ml. cultures were combined in these experiments for isolation of the sulfonium compound.

** The efficiency of counting was 40%.

part of the $^{35}SO_4^{--}$ that is incorporated into the cells can be extracted with perchloric acid. The balance of it is present in cellular proteins and other acid-insoluble fractions. Radioactivity measurement of dilute suspensions of

the cell residues in a scintillation spectrometer accounted for most of the isotope deficiency; this procedure of counting, however, is only an approximation. Losses of sulfur-35 during the culture period in the form of volatile sulfur compounds were not detectable.

The isolation of S-adenosylmethionine from the perchloric acid extracts is simple, because the strong cationic properties of the sulfonium compound and its characteristic ultraviolet absorbancy facilitate ion exchange chromatography and spectrophotometric analysis. For all operations it has to be borne in mind that S-adenosylmethionine is very stable in acid, but exceedingly sensitive to alkali [2].

Procedure I. S-Adenosylmethionine is precipitated as the Reinecke salt followed by purification on a polystyrene carboxylic type cation exchange column.

The perchloric acid extracts of the yeast are combined, and 2 to 10 micromoles of non-labeled S-adenosylmethionine per gram of yeast may be added as carrier material. To the solution, one third volume of freshly prepared 0.06 M Reinecke salt is added. The precipitation of S-adenosylmethionine is completed by keeping the mixture at low temperature for some hours or overnight. S-Adenosylhomocysteine, nucleotides, nucleosides, and other impurities remain in solution [16]. The precipitate is collected at low temperature by filtration through a medium grade sintered glass filter. The filter cake is suspended in 5 to 10 ml of H_2O. To avoid losses, the filter is rinsed with 1 to 2 ml of acetone-water (1 : 1), and this is added to the suspension. The Reinecke acid is extracted by a few 10 ml. charges of 1-butanol. Sufficient water is added to maintain a water phase in this process, and the layers are separated by brief centrifugation. This is followed by a few extractions with ether to remove dissolved 1-butanol and acetone. A stream of nitrogen, directed toward the surface of the solution, removes remnants of ether. The solution is adjusted to pH 4 with small quantities of solid $KHCO_3$. After an hour in an ice bath, a few crystals of $KClO_4$ may appear. They are removed before application of the solution to the ion exchange column.

Amberlite, IRC 50 resin, 50-100 mesh size, is used. A column of 1 cm diameter and 10 cm length is suitable for quantities up to 20 μmoles of S-adenosylmethionine. The column is washed with 100 ml of 1 N H_2SO_4, followed by water until the effluent shows pH 4 or higher. The solution is now applied, followed by water (about 100 to 300 ml) until the absorbancy (260 mμ) of the effluent is < 0.05. S-adenosylmethionine is eluted by 0.1 N H_2SO_4. Small fractions (3-5 ml) are collected, and a total of 25 ml usually completes the process.

Spectrophotometry (A (260 mμ) = 15,400) and counting reveal the concentration of S-adenosylmethionine. The best fractions are adjusted to pH 3 with freshly prepared $BaCO_3$, the precipitate is removed, and the solutions are examined by paper or thin layer chromatography. The material is stored at or

below pH 3 in the frozen state. Neutralization for enzyme experiments is carried out by adding buffer or bicarbonate shortly before use.

Procedure II. S-Adenosylmethionine is purified by chromatography with a strongly cationic sulfonic acid resin. It is precipitated from the eluates as Reinecke salt.

The perchloric acid extracts may be diluted with carrier material as described under Procedure I. The acid solution is applied to a Dowex 50 H^+ column, 8% cross-linked, 100-200 mesh size. A resin bed of 1 cm diameter and 10 cm depth is ample for 20 µmoles of the sulfonium compound. The elution of undesired material is accomplished with 2 N H_2SO_4 (200 to 400 ml) until the effluent shows an absorbancy of < 0.1 at 256 mµ [5,7]. Small quantities of S-adenosylhomocysteine remain associated with S-adenosylmethionine. Both are eluted by 6 N H_2SO_4 in 25 ml fractions, but S-adenosylhomocysteine remains in the supernatant fluid in the precipitation with Reinecke salt. For this, the fractions with an absorbancy (256 mµ) higher than 0.3 are combined and treated with one fifth volume of 0.06 M Reinecke solution. A bulky precipitate appears immediately. This consists of free Reinecke acid and its S-adenosylmethionine sulfonium salt. The precipitation is completed overnight at low temperature. The crystals are very fine, and centrifugation rather than filtration may be expedient. The removal of the Reinecke acid and neutralization by $BaCO_3$ are carried out as described under Procedure I.

RADIOACTIVITY SCANNING OF THIN LAYER CHROMATOGRAM OF ^{35}S-ADENOSYLMETHIONINE.

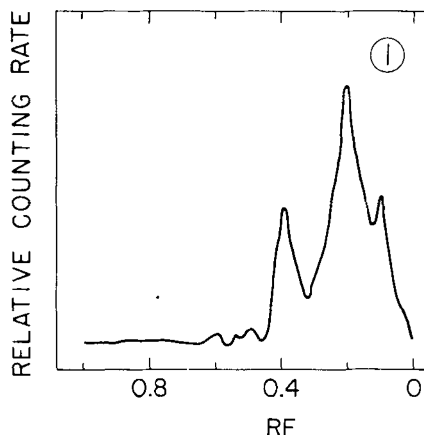

FIG. 1. — Perchloric extract of yeast, after neutralization to pH 3 with $KHCO_3$; the quantity (5 µl) corresponds to 0.73 mg of yeast. The principal peak of radioactivity is caused by ^{35}S-adenosylmethionine; the shoulder at Rf 0.1 may indicate cystine and glutathione. At Rf 0.39, S-adenosylhomocysteine is noted, and the small peak near Rf 0.6 may be attributable to methionine. 5′-Methylthioadenosine (Rf 0.9) is not seen.

The choice of the purification procedure will depend on the purpose for which the preparation is to be used. Procedure I is particularly simple and rapid while somewhat higher purity is achieved by Procedure II. The losses do not exceed 10 to 20% in either method.

Analysis of S-adenosylmethionine

Criteria of purity and analytical procedures have been reported earlier (2, 5, 7, 12). Radioactivity scanning of thin layer chromatograms was of particular importance in the present experiments. The results are illustrated in Figures 1 to 3.

RADIOACTIVITY SCANNING OF THIN LAYER CHROMATOGRAM OF ^{35}S-ADENOSYLMETHIONINE.

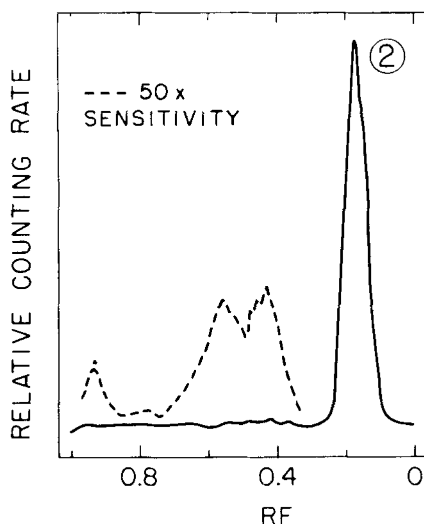

Fig. 2. — Perchloric acid extract of yeast, purified by chromatography on Amberlite IRC 50 (Procedure I). A quantity of 5 μl (2.7 mμmoles of ^{35}S-adenosylmethionine) of the 0.1 N H_2SO_4 eluate fraction 2 was applied after adjustment to pH 3 with $BaCO_3$. Radioactivity scanning with increased sensitivity of the instrument shows the contaminants in more detail. The concentration of S-adenosylhomocysteine has been reduced to less than 1%.

In addition to the conventional tests, we have included an analysis of the amino acid configuration. The biologically active form is S-adenosyl-L-methionine (17). In contrast to other cells examined to this date, *C. utilis* is able to convert extraneously supplied D-methionine into S-adenosyl-D-methionine (7, 12). There is no indication that this organism itself can produce D-methionine, but a test seemed desirable to ascertain that the material isolated by us has the biological L-configuration. For this, the compound was hydrolyzed at 100 °C

with 0.1 N $Ba(OH)_2$ for 15 minutes to yield methionine. The solution was treated with a small excess of H_2SO_4 to remove Ba^{++}. It was neutralized with $KHCO_3$ and mixed with an equal part of L-amino acid oxidase. For the latter, lyophilized venom of the water moccasin was dissolved in 0.015 M K_2HPO_4 to a concentration of 10 mg per ml; the final pH was 7.4. After incubation for 2 and 4 hours at 30°C, samples were spotted on Whatman No. 1 paper and the chromatograms developed by 1-butanol-water-acetic acid (60 : 25 : 15, v/v). Ninhydrin spray and radioactivity scanning showed that less than 5% of the original radioactivity in the methionine spot was left after 2 hours, and less than 2% after 4 hours. Thus, the amino acid component of the sulfonium compound has the L-configuration. This is important, because S-adenosyl-D-methionine was found inactive in all enzyme systems that have been examined to this date [17].

RADIOACTIVITY SCANNING OF THIN LAYER CHROMATOGRAM OF ^{35}S-ADENOSYLMETHIONINE.

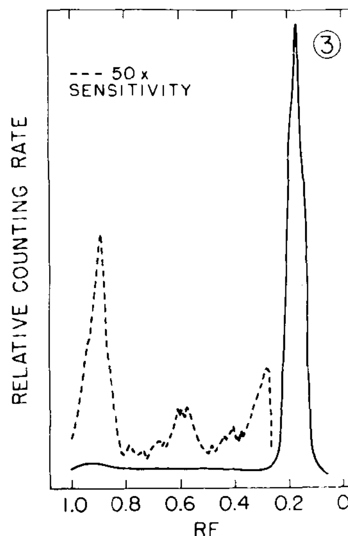

Fig. 3. — Chromatogram after Procedure II with carrier material. The region from Rf 0.25 to 1.0 was evaluated also with increased sensitivity of the scanning instrument. Small quantities of S-pentosylmethionine (Rf 0.3), methionine (Rf 0.6), and 5′-methylthioadenosine (RF 0.9) are seen. All of them amount to less than 1% of the total radioactivity. S-Adenosylhomocysteine (Rf 0.4) is the least conspicuous contamination.

Of particular importance for the utility of sulfur-labeled S-adenosylmethionine is the absence or low level of other sulfur containing compounds in the preparations. In the yeast extracts, no significant quantities of methionine and 5′-methylthioadenosine were detected. The concentration of S-adenosylhomo-

cysteine may be as high as 20%, but this material is removed by the purification procedures.

Inappropriate manipulation of the sulfonium compound during isolation may lead to the formation of sulfur-containing breakdown products. Exposure to alkali leads to S-pentosylmethionine and methionine [18]. Prolonged maintenance near neutrality, especially at elevated temperature, leads to 5′-methylthioadenosine. These impurities are readily detected by paper or thin layer chromatography, ultraviolet light quenching, ninhydrin spray, or by radioactivity scanning. S-Adenosylmethionine is stable at pH 3 or below, especially when it is stored in the frozen state [2].

The radioactivity of the present preparations ranged from 6.5 to 14.5 μc per μmole, and the purity obtained was not surpassed by any currently available S-adenosylmethionine.

Discussion

The yield of S-adenosylmethionine obtained by the present procedure is smaller than that in the methods employing L-methionine as a supplement in the culture [13]. However, a comparison of $^{35}SO_4^{--}$ with L-methionine-^{35}S as a precursor shows the following : The isotope recovery in S-adenosylmethionine may be as high as 20% when L-methionine-^{35}S is used in the culture medium [13]. With $^{35}SO_4^{--}$, an incorporation of 4% has been realized. A comparison of the price of the labeled precursors reveals the economy of $^{35}SO_4^{--}$ as starting material. Furthermore, L-methionine-^{35}S usually is not available with as high specific activity as sulfate. The steps of culture and isolation are virtually the same in both instances. Dilution with carrier sulfonium compound facilitates the isolation of ^{35}S-adenosylmethionine. The economical aspect of the procedure can be improved further by isolation of the labeled sulfur amino acids from the protein of the same yeast. Conversely, the present procedure can be made a part of established routines for the isolation of ^{35}S-labeled cystine and methionine.

The demands on the purity of the preparations depend on the type of experiment that is to be performed with them. The use of highly purified preparations is futile in experiments with crude enzymes for prolonged incubation periods near or above pH 7. Spontaneous decomposition under these circumstances may accumulate quantities of breakdown products far in excess of those present originally.

The stability of S-adenosylmethionine has been reviewed elsewhere [2]. In the dry state, the compound undergoes gradual decomposition [19]. It is very stable in acid solution below pH 3, and it can be kept in the frozen state for periods in excess of the useful life of the sulfur isotope. An incidental advantage of this is that radiodecomposition is kept at a minimum. No unusual sensitivity of the sulfonium compound in this respect has been noted.

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