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A RAPID ASSAY PROCEDURE FOR ATP:L-METHIONINE ADENOSYLTRANSFERASE

TING-CHAO CHOU AND J. B. LOMBARDINI

Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University School of Medicine, Baltimore, Md. 21205 (U.S.A.)

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SUMMARY

A new assay technique for ATP:L-methionine *S*-adenosyltransferase activity (EC 2.5.1.6) is described. The procedure eliminates the use of ion exchange resin columns which have been routinely used in the past. The principle of the assay depends upon the separation of *S*-adenosyl-L-[*Me*-^{14}C]methionine from L-[*Me*-^{14}C]-methionine by differential adsorption on cellulose phosphate ion exchange discs. The cellulose phosphate disc method is considerably simpler, more rapid, and less expensive than the column procedure while retaining high sensitivity and precision.

INTRODUCTION

The discovery of (—)-*S*-adenosyl-L-methionine and formulation of its structure by Cantoni[1] in 1952 were the culmination of over a decade of investigation into the mechanism by which adenosine triphosphate and L-methionine participate in enzymatic methylation reactions. The chemical properties and stability of *S*-adenosyl-L-methionine as well as the stereospecificity at the sulfonium atom have been described[2–4].

The formation of *S*-adenosylmethionine involves the transfer of the adenosyl moiety of ATP to one pair of free electrons of the sulfur atom of L-methionine with the formation of a high energy, positively charged, sulfonium compound which is energetically capable of donating its methyl group, or following decarboxylation, its propylamine group to appropriate acceptors. The resultant enzyme-bound tripolyphosphate, formed by complete dephosphorylation of ATP, is asymmetrically hydrolyzed to pyrophosphate and inorganic phosphate[5,6].

The enzymatic synthesis of *S*-adenosyl-L-methionine which occurs in animal, plant and microbial systems is metabolically important for three reasons: first, as a methyl donor[7,8]; secondly, as a propylamine donor[9–11]; and thirdly, as a regulator of certain enzymatic reactions[8,12].

In the light of the central importance[8] of the ATP:L-methionine *S*-adenosyltransferase (EC 2.5.1.6) reaction for a variety of metabolic pathways it became

clearly desirable to develop a rapid, convenient and inexpensive assay method for this enzyme. The assay system initially described by Cantoni[13] measured the amount of liberated inorganic phosphate. A spectrophotometric assay[14,15] devised in 1957 utilized Dowex-1(chloride) resin which removed ATP from the incubation mixture while allowing *S*-adenosylmethionine to remain in solution. The absorbance of the supernatant fluid at 260 nm was then used to measure the *S*-adenosylmethionine formed[15].

Mudd *et al.*[16] further improved the assay by employing radioactive L-[*Me*-^{14}C]-methionine. The enzymatically synthesized *S*-adenosyl-L-[*Me*-^{14}C]methionine was then separated from the starting materials by adsorption onto a cation exchanger (Dowex AG 50W-X2) and was quantitatively eluted from the resin with 10 ml of 3 M NH_4OH. After evaporation of the ammonium hydroxide, the radioactivity of the *S*-adenosylmethionine (and its decomposition products) was determined.

Lombardini *et al.*[17] introduced minor modifications of the procedure of Mudd *et al.*[16] by eluting the radioactive *S*-adenosylmethionine from the cation exchange columns into scintillation vials with two 5-ml aliquots of concentrated NH_4OH and then directly counting the *S*-adenosylmethionine after addition of 15 ml of Bray's scintillation solution[18]. Radioactive ATP could similarly be used as a tracer for the synthesis of *S*-adenosylmethionine.

In this paper we describe a new assay procedure based on the separation of *S*-adenosyl-L-[*Me*-^{14}C]methionine from L-[*Me*-^{14}C]methionine by cellulose phosphate ion exchange paper. This method has the same precision and sensitivity as the column assay systems when using radioactive substrates.

EXPERIMENTAL PROCEDURE

Materials

All solutions were prepared from reagent grade chemicals in deionized glass-distilled water. L-Methionine was supplied by Mann. L-[*Me*-^{14}C]methionine (53.6 μCi/μmole) was purchased from Amersham/Searle and was purified by passage through Dowex AG 50W-X2 (NH_4^+) columns (0.6 cm $\times$ 12 cm) at neutral pH. *S*-Adenosyl-L-[*Me*-^{14}C]methionine (52.3 μCi/μmole) was purchased from New England Nuclear. The cation exchange Dowex resin designated AG 50W-X2 (100–200 mesh) was supplied by Bio-Rad, Richmond, Calif. 23 mm diameter cellulose phosphate ion exchange paper discs, catalogue No. P 81, and paper impregnated with Amberlite IR-120 resin, catalogue No. SA-2, were purchased from Reeve Angel, Clifton, N.J.

Preparation of cation exchange columns

The Dowex AG 50W-X2 resin which was initially in the hydrogen form was packed into columns (6 mm $\times$ 30 mm) and was converted to the ammonium form by addition of 25 ml of 4 M NH_4OH, and then washed with water until neutral.

Preparation of cellulose phosphate ion exchange paper

The ion exchange paper initially purchased in the ammonium form was converted to the hydrogen from by immersion in 4% acetic acid for 20 min and then washed with water 5 times by decantation. The paper discs were separated and dried before use.

Preparation of bakers' yeast ATP:L-methionine S-adenosyltransferase

The enzyme was prepared as described by Lombardini *et al.*[17].

Enzymatic assay

The conversion of L-methionine to *S*-adenosyl-L-methionine was carried out in one of two systems, designated as reaction mixture A[16,17] or B[19] (Table I), which differ markedly with respect to ionic strength and pH. Since studies in this laboratory have utilized both reaction mixtures, we have examined the efficiencies of the ion exchange paper to retain radioactive *S*-adenosylmethionine and not to retain radioactive L-methionine under a variety of conditions. Partially purified yeast adenosyltransferase (specific activity: 7.0 μmoles *S*-adenosylmethionine formed per mg protein per 30 min) was used to initiate the reaction. Incubations were carried out at 37 °C with agitation for time periods and with the amounts of enzyme protein specified. For comparison the quantity of *S*-adenosylmethionine synthesized in each

TABLE I

COMPOSITION OF REACTION MIXTURES USED FOR ASSAY OF ATP:L-METHIONINE *S*-ADENOSYLTRANSFERASE

For enzymatic assays the reaction mixture (final volume 250 μl) also received L-[*Me*-^{14}C]methionine, 650 000 cpm. For non-enzymatic experiments L-[*Me*-^{14}C]methionine, 800 000 cpm, or *S*-adenosyl-L-[*Me*-^{14}C]methionine, 180 000–300 000 cpm, were also added to the reaction mixture.

Reaction mixture A[16,17]	*Concn (mM)*	*Reaction mixture B*[19]	*Concn (mM)*
Tris–HCl (pH 7.6)	160	Tris–histidine (pH 9.0)	90
KCl	200	KCl	100
$MgCl_2$	300	$MgCl_2$	5
Glutathione	8	2-Mercaptoethanol	5
L-Methionine	5	L-Methionine	5
ATP	20	Mg-ATP	10

reaction vessel was determined by both the column assay technique[17] and by the cellulose phosphate disc method. For these purposes, the enzymatic reaction was terminated by two different procedures: (a) A 50-μl aliquot of the incubation mixture was removed by means of a disposable micropipette and applied to the uppermost of three cellulose phosphate ion exchange discs previously placed in close apposition on a pin, inserted into a cork ring, and dried under an infrared lamp. The dry papers were then washed on Gooch crucibles by suction filtration with 200 ml water to remove the unreacted L-[*Me*-^{14}C]methionine from the *S*-adenosyl-L-[*Me*-^{14}C]-methionine which remains adsorbed to the cellulose phosphate. The ion exchange papers were then placed in scintillation vials and counted with 7 ml of Bray's scintillation fluid[18] in a liquid scintillation spectrometer; (b) to the remaining 200 μl of incubation mixture 10 ml of cold water was added and the total mixture was applied to the Dowex AG 50W-X2 columns. A minimum of 100 ml of water was used to wash the unreacted L-[*Me*-^{14}C]-methionine through the columns. The adsorbed *S*-adenosyl-L-[*Me*-^{14}C]methionine was eluted from the columns with two 5-ml aliquots of concentrated NH_4OH and each counted for radioactivity with 15 ml of Bray's scintillation fluid[18].

Calculations

In order to calculate the quantities of *S*-adenosylmethionine formed during the reaction it is necessary to prepare standards of L-[*Me*-^{14}C]methionine that can be counted under exactly the same conditions as employed for measuring the radioactivity of *S*-adenosylmethionine in the two assays. (a) In the Dowex column assay the standard is prepared by adding an aliquot (200 μl) of the reaction mixture (without enzyme, and containing 1.00 μmole of L-methionine and 512 000 cpm; see Table II)

TABLE II

TYPICAL COLUMN ASSAY FOR ATP:L-METHIONINE ADENOSYLTRANSFERASE

Details of the assay conditions and calculations are described under Experimental Procedure. Incubation 15 min at 37 °C. An aliquot (200 μl) of the total reaction mixture A (250 μl) was processed in the assay. The controls were also incubated and contained all components of the complete system except ATP. Control values were averaged and subtracted.

Reaction system	*Radioactivity (cpm)*				*Amount of S-adenosylmethionine (nmoles)*	
	First eluate	*Second eluate*	*Total of first and second eluates (cpm)*	*Total corrected for control** (cpm)*	*synthesized in 200 µl aliquot*	*Total incubation system*
Experimental						
1	62 341*	672	63 013	62 632	122	153
2	61 272	616	61 888	61 507	120	150
Control (*minus* ATP)						
1	362	12	374			
2	381	7	388			

* The digits shown in some entries represent direct readings obtained from the scintillation counter (corrected for background).

** Corrected by subtraction of the average of the two controls (381 cpm).

TABLE III

TYPICAL CELLULOSE PHOSPHATE DISC ASSAY FOR ATP:L-METHIONINE ADENOSYLTRANSFERASE

Details of the assay conditions and calculations are described under Experimental Procedure. Incubation 15 min at 37 °C. An aliquot (50 μl) of the total reaction mixture A (250 μl) was processed in the assay. The controls were also incubated and contained all components of the complete system except ATP (the same controls as in column assays).

Reaction system	*Radioactivity (cpm)*	*Total corrected for control** (cpm)*	*Amount of S-adenosylmethionine (nmoles) synthesized in*	
			50 µl aliquot	*Total incubation system*
Experimental				
1	14 769*	14 291	27.9	140
2	14 530	14 052	27.5	137
Control (*minus* ATP)				
1	531			
2	424			

* The digits shown in some entries represent direct readings obtained from the scintillation counter (corrected for background).

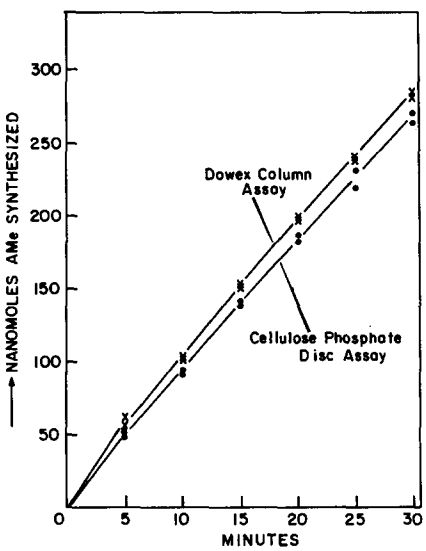
** Corrected by subtraction of the average of two controls (478 cpm).

to a mixture of 5 ml of concentrated NH_4OH and 15 ml of Bray's scintillation fluid. (b) The standard for the cellulose phosphate ion exchange paper assay consists of an aliquot (50 μl) of the reaction mixture (without enzyme, and containing 0.25 μmole of L-methionine and 128 000 cpm; see Table III) which is placed with the aid of a micropipette onto the discs of cellulose phosphate paper (3 thicknesses), dried, and then counted with 7 ml of Bray's scintillation fluid[18].

RESULTS AND DISCUSSION

Comparison of assays

Typical experimental results are presented in Tables II and III. Control reaction vessels containing all reactants, except ATP, were incubated for 5, 15 and 30 min in order to correct for any positively charged spontaneous or enzymatic decomposition products or contaminants of radioactive L-methionine which would be retained on either the Dowex column or the ion exchange paper. Controls with different time periods of incubations showed no increases of radioactivity with time. These values were then averaged and subtracted from the experimental results. The total quantity of *S*-adenosyl-L-methionine synthesized as measured by the Dowex column and the cellulose phosphate ion exchange paper assays does not differ by more than 6–8% at each time interval of incubation (Fig. 1). Strict proportionality of product formation



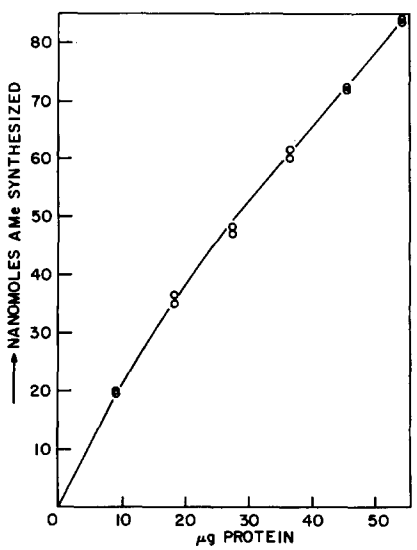


Fig. 1. Comparison of the time course of formation of *S*-adenosyl-L-methionine (AMe) measured by the Dowex column and cellulose phosphate disc methods. Incubations (reaction mixture A) were carried out at 37 °C with partially purified yeast adenosyltransferase (43.8 μg). For conditions see Experimental Procedure. A 50-μl aliquot of the reaction system was used for the disc assay and a 200 μl aliquot for the column assay. The quantity of *S*-adenosylmethionine synthesized is calculated for the entire reaction volume (250 μl). Sample data are presented in Tables II and III.

Fig. 2. Relation between formation of *S*-adenosyl-L-methionine (AMe) and protein concentration, measured by the cellulose phosphate disc method. Incubations (reaction mixture A) were conducted for 8 min at 37 °C with partially purified yeast adenosyltransferase. For conditions see Experimental Procedure. A 50-μl aliquot of the reaction system was assayed and the total quantity of *S*-adenosylmethionine synthesized is calculated for the entire reaction volume (250 μl).

with respect to time is not observed in either assay system. This phenomenon is an intrinsic property of the enzyme and not related to the assay procedure[12]. The quantity of *S*-adenosylmethionine formed as a function of protein concentration is shown in Fig. 2.

Initial problems

Some of the initial problems encountered in developing the assay procedure were the following:

Choice of ion exchange paper and ionic form. The two types of ion exchange papers that were tested were cellulose phosphate (a strong cation exchanger attached to the cellulose molecule by an ester linkage) and Amberlite IR-120 (strong cation exchange resin which is processed with cellulose pulp to form a homogeneous paper). Both types of ion exchange papers were examined in three ionic forms (Na^+, H^+ and NH_4^+) for their ability to adsorb quantitatively from the reaction system *S*-adenosyl-L-[*Me*-^{14}C]methionine and not to retain L-[*Me*-^{14}C]methionine after thorough washing with water. The cellulose phosphate (P 81) ion exchanger in the hydrogen form gave the maximum retention of *S*-adenosylmethionine and a minimum adsorption of L-methionine (Table IV).

TABLE IV

COMPARISON OF THE EFFICIENCY OF VARIOUS IONIC FORMS OF CATION EXCHANGE PAPERS IN THE ASSAY

The papers were compared for their ability to retain *S*-adenosylmethionine and not adsorb L-methionine after washing with distilled water. Cellulose phosphate ion exchange paper, supplied in the NH_4^+ form was converted to the H^+ form by treatment with 4% acetic acid for 20 min and then washed with distilled water to neutral pH; or converted to Na^+ form by treatment with 1 M NaOH for 20 min and then washed with distilled water to neutral pH. Amberlite IR-120 ion exchange paper, supplied in the Na^+ form, was converted to the H^+ form with 4% acetic acid as above, or converted to the NH_4^+ form by treatment with 4% NH_4OH for 20 min and then washed with distilled water to neutral pH. Reaction mixture A or B (Table I) contained either radioactive L-methionine (0.1 μmole, 59 000 cpm) or radioactive *S*-adenosylmethionine (0.02 μmole, 25 000 cpm). 20 μl of either reaction mixture were applied to a single cation exchange paper disc with the aid of a micropipette, dried under infrared lamp and washed with water. The exchange paper was then placed in a scintillation vial and counted with 7 ml of Bray's scintillation fluid. Results are expressed as percent retention of radioactivity after washing with distilled water.

Type of cation exchange paper	*Ionic form of cation exchange paper*	*Radioactive label in*	*% Retention of radioactivity after washing with distilled water*	
			Reaction mixture A	*Reaction mixture B*
Cellulose phosphate (P 81)	H^+	L-Methionine	0.17	0.07
		S-Adenosylmethionine	77.1	94.6
	NH_4^+	L-Methionine	0.12	0.07
		S-Adenosylmethionine	60.4	75.6
	Na^+	L-Methionine	0.11	0.07
		S-Adenosylmethionine	4.90	1.96
Amberlite IR-120 (SA-2)	H^+	L-Methionine	25.8	38.1
		S-Adenosylmethionine	98.1	99.7
	NH_4^+	L-Methionine	0.72	1.26
		S-Adenosylmethionine	93.1	94.4
	Na^+	L-Methionine	0.67	1.06
		S-Adenosylmethionine	91.1	92.9

Thicknesses of ion exchange paper. It was found that a single thickness of paper disc did not retain quantitatively the radioactive *S*-adenosylmethionine after water-washing especially in reaction mixture A which has high ionic strength. Moreover, comparison of results obtained by the column and the ion exchange paper assays always resulted in lower yields for the latter when a single cellulose phosphate disc was used. This difficulty could be corrected by using three thicknesses of paper discs which resulted in retention of more than 90% of the *S*-adenosylmethionine after washing (cellulose phosphate discs of different thicknesses are not commercially available). In one experiment, the total radioactivity of 4 paper discs prepared from a reaction system as described was 13 200 cpm. The radioactivity (cpm) was distributed as follows: first disc (11 154); second disc (1530); third disc (300) and fourth disc (30). The radioactivity remains quantitatively adsorbed to the exchange paper after addition of Bray's scintillation fluid. This eliminates any problems in counting technique which may arise from variations in the radioactivity distributed between the paper and the solution.

Wash procedure for separation of L-[*Me*-^{14}C]*methionine from S-adenosyl*-L-[*Me*-^{14}C]*methionine.* Various buffers of low ionic strength (5 mM sodium acetate, potassium phosphate, Tris–HCl, Tris–histidine, or sodium carbonate), and ranging in pH values from 5–10.7 were tested for their efficiency in washing away the unreacted L-[*Me*-^{14}C]methionine from the cellulose ion exchanger. Each of the above buffers was either no more effective than distilled water in lowering blank values or had a detrimental effect, *i.e.* also removed radioactive *S*-adenosylmethionine from the ion exchange paper. Consequently, water was routinely used as the wash medium.

It was also found that the passage of the water wash through the paper by suction filtration (minimum 200 ml) was much more effective in removing radioactive L-methionine than by placing the paper in a beaker of water and decanting several times.

Advantages of the cellulose phosphate disc method

There are three principal advantages that characterize the cellulose phosphate disc method in comparison to the Dowex column procedure. First, the cellulose phosphate disc method eliminates the time required for the preparation of the columns, the protracted water wash and the two elutions with 5 ml of NH_4OH which are required for the column procedure. Secondly, the disc procedure does not require a fume hood as there is no need to pipette and elute with NH_4OH. Thirdly, the disc procedure is less expensive, since it eliminates the cost of the columns and uses only 7 ml of Bray's scintillation fluid rather than 30 ml. The cellulose phosphate disc procedure does not sacrifice accuracy or sensitivity as shown by the duplicates (Table III) and by comparison with the Dowex column procedure (Table II). The results shown in Tables II and III were obtained by the two procedures on the same reaction mixtures.

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