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Production of the Oocyte Maturation-Inducing Substance of Starfish by Heat Treatment of S-Adenosylmethionine

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ABSTRACT—1-Methyladenine (1-MeA) has been identified as the oocyte maturation-inducing substance (MIS) in starfish, but little is known about its biosynthesis. This study showed that starfish MIS activity was present in a reactant derived from S-adenosylmethionine (SAM) by heat treatment. *In vitro* MIS production was markedly dependent on the temperature of the SAM solution: it increased as the temperature was raised, and reached a plateau within 5 min upon boiling, although hardly only MIS was observed upon incubation below 20°C. MIS production was also dependent on the solution pH. Analyses by high-performance liquid chromatography and thin-layer chromatography showed that the MIS was 1-MeA, though the maximum amount of 1-MeA obtained from SAM by boiling was only 0.3% of the initial SAM amount. Furthermore, use of S-[¹⁴C-methyl]SAM showed that a methyl group of 1-MeA was transferred from the SAM. Thus, it is possible that 1-MeA may be produced from SAM *in vivo*.

INTRODUCTION

In most animals, oocytes in the ripe ovary do not proceed beyond the end of the first prophase stage of meiosis. Such immature oocytes are not fertilizable. In starfish oocytes, the resumption of meiosis is induced by 1-methyladenine (1-MeA) (Kanatani, 1969, 1973, 1985; Kanatani *et al.*, 1969; Schuetz, 1971), which is produced by ovarian follicle cells following stimulation by a gonad-stimulating substance (GSS) released from the radial nerves (Hirai and Kanatani, 1971; Hirai *et al.*, 1973). Previous studies have shown that the 1-MeA produced under the influence of GSS is newly synthesized, rather than being pre-stored within follicle cells (Shirai, 1972; Mita, 1991a) or a breakdown product of some 1-MeA-containing substance, such as ribonucleic acid (Shirai, 1972).

It has also been shown that GSS-dependent 1-MeA production is enhanced by methionine and suppressed by ethionine (Shirai *et al.*, 1972; Shirai, 1973; Mita, 1991b). In addition, the [¹⁴C]methyl group of methionine is converted to 1-MeA upon GSS stimulation (Shirai *et al.*, 1972; Tarr, 1985). Thus, GSS may stimulate a methylation process during 1-MeA production. Recently, we reported that there was an increase in

the levels of radiolabelled S-adenosylmethionine (SAM) during incubation of isolated follicle cells with [methyl-¹⁴C] methionine (Mita *et al.*, 1997). It has also been demonstrated that SAM acts as a direct methyl donor (Mudd and Cantoni, 1964), suggesting that SAM is related to 1-MeA biosynthesis in starfish follicle cells. However, the details of 1-MeA biosynthesis still remain unknown. The present study was designed to examine *in vitro* 1-MeA production from SAM. The results obtained showed that 1-MeA is produced from SAM by heat treatment.

MATERIALS AND METHODS

Reagents

SAM, S-adenosylhomocysteine (SAH), 5'-deoxy-5'-methylthioadenosine (MTA), 1-MeA and 1-methyladenosine (1-MeAdo) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Since commercially available SAM was crude, the SAM used in the present experiments was purified by high-performance liquid chromatography (HPLC). 1-Methyladenosine monophosphate (1-MeAMP) was synthesized according to the method of Griffin and Reese (1963) with slight modification by Shirai and Kanatani (1973). S-[¹⁴C-methyl]SAM (1.96 GBq/mmol) was obtained from Amersham (Buckinghamshire, U.K.). All other reagents were of analytical grade.

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Incubation of S-adenosylmethionine

The standard reaction mixture contained 1 mM purified SAM and 50 mM Tris-HCl, pH 7.5, in a total volume of 0.1 ml. After boiling, the reactant adjusted to pH 8.2 was used for MIS assay. A pH is generally influenced by temperature. In the case of 50 mM Tris-HCl, the pH of 7.5 at 20°C decreased slightly to 6.0 at 90°C. In an experiment for pH dependency, although each pH in buffer solution composed of sodium citrate, sodium phosphate and sodium borate was determined at 20°C, the pH value maintained a small change at high temperatures.

For determination by radioactivity, the reaction mixture was consisted of 2 mM S-¹⁴C-methyl]SAM (37 kBq), 8 mM purified SAM and 50 mM Tris-HCl, pH 7.5, in a total volume of 0.1 ml. After boiling, the reactants were used for HPLC and thin-layer chromatography (TLC) analyses.

Assay of maturation-inducing substance

MIS was measured by biological assay using oocytes of the starfish, *Asterina pectinifera*. In the assay MIS is detectable at concentrations of more than 0.01 μM equivalent to 1-MeA (Shirai, 1974, 1986). Briefly, oocytes were isolated from the ovaries of *A. pectinifera* and washed with modified van't Hoff's artificial seawater (ASW) at pH 8.2 (Kanatani and Shirai, 1970). Isolated oocytes were placed in a small amount of test solution (usually about 200-300 oocytes in 0.2 ml) and the rate of germinal vesicle breakdown (GVBD) was observed after 1 hr at 20°C. In some cases, authentic 1-MeA dissolved in ASW at various concentrations was used as a reference standard, and the concentration of MIS was expressed as the 1-MeA equivalent.

High-performance liquid chromatography

The HPLC system used comprised a Pharmacia LKB pump 2248, a VMW 2141-UV detector and a LCC 2252 control system. A Shodex DE-613 column (6.0 × 150 mm, Showa Denko, Tokyo, Japan) used for reverse-phase analysis was equilibrated in 10 mM sodium phosphate buffer, pH 4.5. After injection of the sample (40 μl), the bound fraction was eluted with the same buffer for 20 min at a flow rate of 1.0 ml/min, and then for another 20 min with a linear gradient of acetonitrile (0-50%) in 10 mM sodium phosphate buffer, pH 4.5.

For the radioactive sample, the radioactivity was monitored by a Beckman 171 radioisotope detector.

Thin-layer chromatography

TLC plates (silica gel 60, E. Merck, Darmstadt, Germany) were used for analysis of reactants of S-¹⁴C-methyl]SAM. The samples (2 μl) were developed with a solvent system of 10% NaCl:isopropanol (2 : 3, v/v). The spots of radioactivity were detected by an image analyzer BAS-2000 (Fuji Film, Tokyo, Japan).

RESULTS

When crude SAM solution (1 mM) at pH 7.5 was boiled for 10 min, the reactant obtained induced starfish oocyte maturation accompanied GVBD and polar body formation (Fig. 1). About 2.4 μM MIS equivalent to 1-MeA was produced from 1 mM SAM. The production of MIS was not influenced by the addition of adenine (Ade) and adenosine (Ado). Without heat treatment, no MIS activity was found in the crude SAM solution, suggesting that the heat treatment of SAM induces MIS production. However, the commercially available SAM was not pure, because HPLC analysis indicated that about 70% SAM (retention time 7.1 min) was present in the crude solution and was contaminated by about 5% SAH (retention time 13 min) and about 22% MTA (retention time 34 min) (Fig. 2). 1-MeA was not contained in the crude solution, since no peak

of 1-MeA (retention time 7.8 min) was observed. Nevertheless, MIS production also occurred in the solution upon boiling of HPLC-purified SAM (1 mM) for 10 min, (Fig. 3). In contrast to SAM, heat treatment of SAH and MTA did not result in MIS production.

Previous studies have shown that 1-MeAdo and 1-MeAMP may be intermediates in 1-MeA biosynthesis (Kanatani and Shirai, 1971; Shirai and Kanatani, 1973). To determine whether 1-MeA is produced from 1-MeAdo and 1-MeAMP, the latter two substances at a concentration of 0.1 mM at pH 7.5 were boiled for 10 min. However, no MIS activity was observed in the boiled solution of 1-MeAdo and 1-MeAMP, suggesting that neither 1-MeAdo nor 1-MeAMP induces 1-MeA production upon heat treatment. In contrast, the

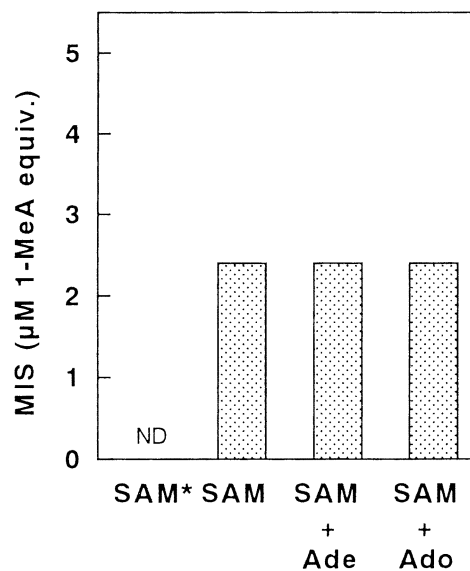


Fig. 1. Production of MIS by heat treatment of SAM. Crude SAM solution (1 mM) at pH 7.5 was boiled for 10 min in the absence and presence of Ade (1 mM) or Ado (1 mM), except *, zero time control. MIS activity was measured by biological assay. The values shown are means for duplicate determinations. ND, not detectable.

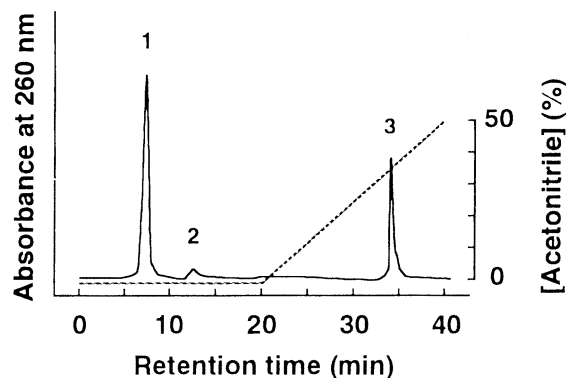


Fig. 2. High-performance liquid chromatogram of crude SAM using a reverse-phase column. Solid and dotted lines show absorbance at 260 nm and acetonitrile concentration, respectively. Peak 1 was identified to SAM and peaks 2 and 3 were to SAH and MTA, respectively.

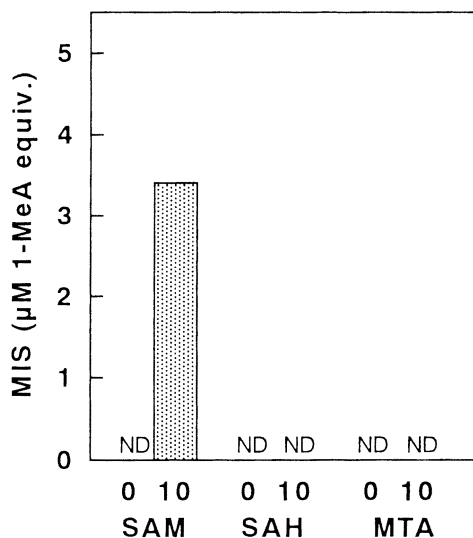


Fig. 3. Effect of heat treatment of SAM, SAH and MTA on MIS production. Before (0) and after boiling of purified SAM, SAH and MTA solutions (1 mM) at pH 7.5 for 10 min (10), MIS activity was measured by biological assay. The values shown are means for duplicate determinations. ND, not detectable.

Table 1. Effect of heat treatment of 1-MeA related compounds on 1-MeA production

Chemicals	Condition	1-MeA (µM)
1-MeA (0.1 mM)	Control	100
	Boiling for 10 min	100
1-MeAdo (0.1 mM)	Control	ND
	Boiling for 10 min	ND
1-MeAMP (0.1 mM)	Control	ND
	Boiling for 10 min	ND

1-MeA concentrations were measured by biological assay before and after boiling of 1-MeA, 1-MeAdo and 1-MeAMP solution (0.1 mM) at pH 7.5 for 10 min. ND, not detectable.

concentration of 1-MeA was unchanged at 100 µM before and after heat treatment (Table 1), indicating that 1-MeA did not decompose during heat treatment.

The production of MIS increased in parallel with the dose of SAM (Fig. 4). The amount of MIS produced by boiling SAM increased as the SAM concentration rose from 0.1 to 10 mM, and the longer the time of heat treatment, the greater the amount of 1-MeA produced. The maximum amount of MIS produced was obtained after 10 min of boiling, although it was almost the same as that after 5 min of boiling.

MIS production was also influenced by the temperature of SAM treatment. When SAM solution (1 mM) was incubated for 1 hr at 0°C and 20°C, no MIS was produced (Fig. 5). SAM treatment at a temperature above 40°C induced MIS production; the higher the temperature, the greater the amount of MIS that was produced. Incubation of SAM at 80°C for 10 min or boiling for 5 min resulted in a plateau level of MIS production.

Since polar groups such as NH_3^+ , COO^- and S^+CH_3 are

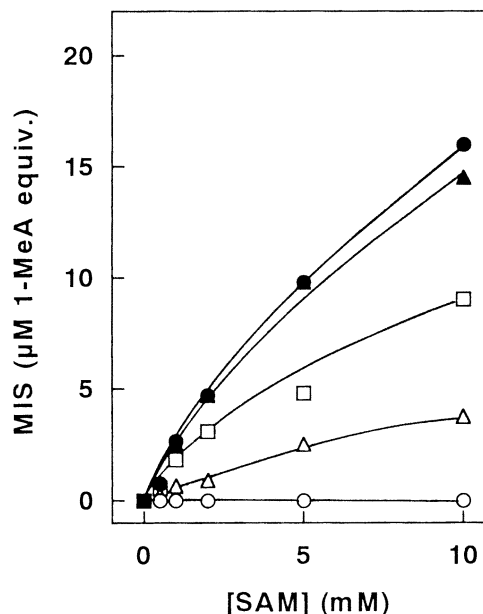


Fig. 4. Effect of heat treatment of SAM on MIS production. Various concentrations of purified SAM solution (0.1-10 mM) at pH 7.5 were boiled for 0 min (○), 1 min (△), 2 min (□), 5 min (▲) and 10 min (●), and MIS activity was measured by biological assay. The values shown are means for duplicate determinations.

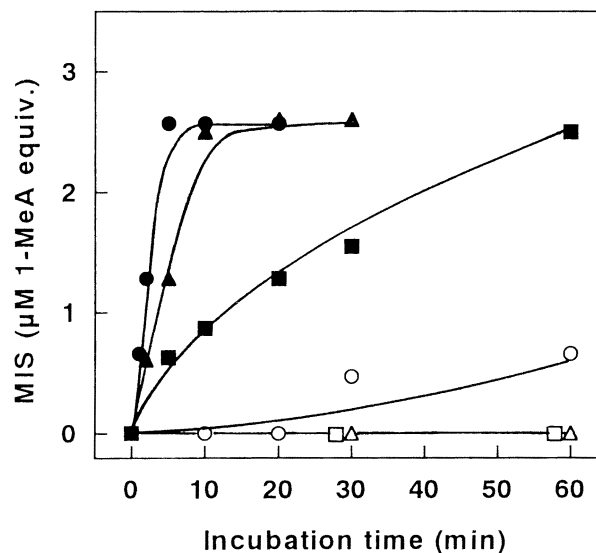


Fig. 5. Effect of temperature on MIS production from SAM. Purified SAM solution (1 mM) at pH 7.5 was incubated for the indicated times at 0°C (□), 20°C (△), 40°C (○), 60°C (■), 80°C (▲) and boiling (●), and MIS activity was measured by biological assay. The values shown are means for duplicate determinations.

present in the molecular structure of SAM, methylation associated with 1-MeA production should be influenced by pH. To determine whether MIS production is influenced by the pH of the SAM solution, SAM was boiled for 10 min in sodium citrate buffer (pH 3-6), sodium phosphate buffer (pH 6-7.5), Tris-HCl buffer (pH 7-8.5) and sodium borate buffer (pH 7.5-9).

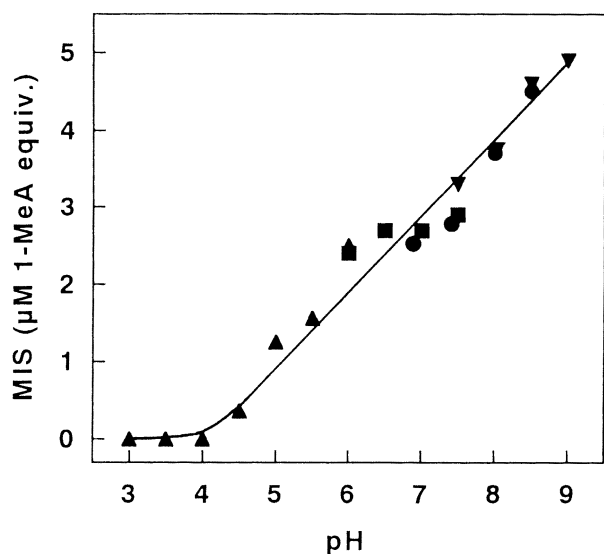


Fig. 6. Effect of pH on MIS production from SAM. Purified SAM solution (1 mM) at various pH values at 20°C buffered by 10 mM of sodium citrate (▲), sodium phosphate (■), Tris-HCl (●) and sodium borate (▼) was boiled for 10 min, and after the pH in each sample was adjusted to 8.2 with NaOH or HCl, MIS activity was measured by biological assay. The values shown are means for duplicate determinations.

The MIS production due to heat treatment of SAM was markedly dependent on pH. Although MIS production did not occur below pH 4, the amount of MIS produced increased linearly as the pH rose from 4.5 to 9.0 (Fig. 6). Data could not be obtained under more alkaline conditions, since SAM easily decomposes to MTA above pH 9 (Cantoni, 1957).

It is important to determine whether the MIS produced by heat treatment of SAM corresponds to 1-MeA, which is the natural MIS present in starfish gonads (Kanatani *et al.*, 1969). An experiment was therefore carried out to confirm this, and the reactant produced from SAM upon heat treatment was analyzed by HPLC and TLC. After crude SAM has been purified by HPLC (Fig. 7a), the purified SAM solution (10 mM) at pH 7.5 was boiled for 20 min, and the reactant was applied to a HPLC system using a reverse-phase column. The absorbance peak of SAM at 260 nm (retention time 7.1 min) mostly disappeared after boiling for 20 min (Fig. 7b), and instead several separate absorbance peaks were obtained. In comparison with the standards, a large peak at 34 min was identified as MTA, upon peaks at 7.8 and 18.6 min were identified as 1-MeA and Ade, respectively. In contrast, MIS activity confirmed by biological assay was observed at a retention time of 7.8 min. The retention time of MIS was consistent with that of 1-MeA, although the MIS peak at 260 nm was small (Fig. 7b).

To obtain further information on 1-MeA production from SAM, S-[¹⁴C-methyl]SAM was used. After boiling S-[¹⁴C-methyl]SAM for 20 min, several radioactive peaks, such as MTA (retention time 34 min), 6-methyladenine (retention time 29 min) and methionine sulfoxide (Met(O)) (retention time 3.8 min), were observed by HPLC (Fig. 7c) and TLC (Fig. 8).

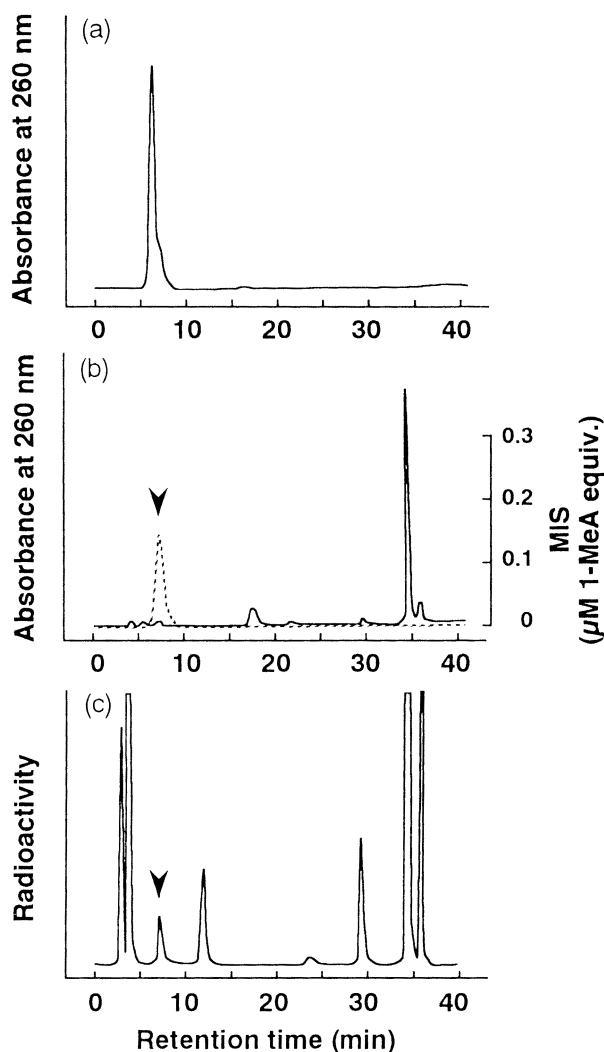


Fig. 7. (a) High-performance liquid chromatogram of purified SAM using a reverse-phase column. Absorbance was monitored at 260 nm. (b) High-performance liquid chromatogram of a reactant after heat treatment of SAM. The purified SAM solution (10 mM) at pH 7.5 was boiled for 20 min. Absorbance (solid line) was monitored at 260 nm and MIS activity (dotted line) was measured by biological assay. Arrow head shows the peak of 1-MeA. (c) High-performance liquid chromatogram of the reactant after heat treatment of S-[¹⁴C-methyl]SAM. A mixture containing S-[¹⁴C-methyl]SAM (2 mM) and purified SAM (8 mM) at pH 7.5 was boiled for 20 min, and radioactivity was monitored by a radioisotope detector. Arrow head shows the peak of 1-MeA.

Among these metabolites, radioactive 1-MeA (retention time 7.8 min) was also recognized, though some peaks (retention times 3.0 min, 12 min and 37 min) remained unknown. Following boiling the solution, the level of S-[¹⁴C-methyl]SAM decreased rapidly (Fig. 9a), whereas the levels of MTA (Fig. 9a) and 1-MeA (Fig. 9b) increased. Although the amount of [¹⁴C-methyl]1-MeA produced was only 0.2-0.3% of the initial amount of S-[¹⁴C-methyl]SAM, this result strongly suggested that 1-MeA is produced by transfer of a methyl group of SAM to the N-1 site of the purine base.

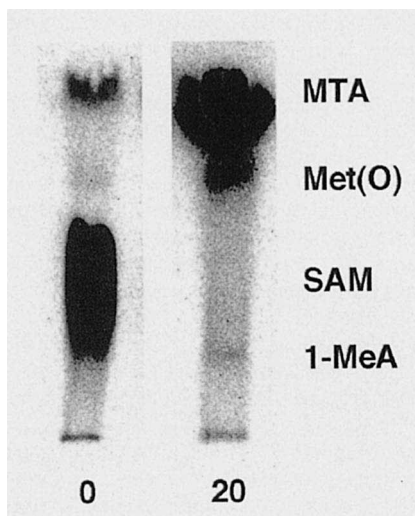


Fig. 8. Thin-layer chromatograms of S-[^{14}C -methyl]SAM before and after heat treatment using a silica gel plate. Before (0) and after boiling of a mixture containing S-[^{14}C -methyl]SAM (2 mM) and purified SAM (8 mM) at pH 7.5 for 20 min (20), each sample was applied to a TLC plate, and radioactivity was analyzed by an image analyzer.

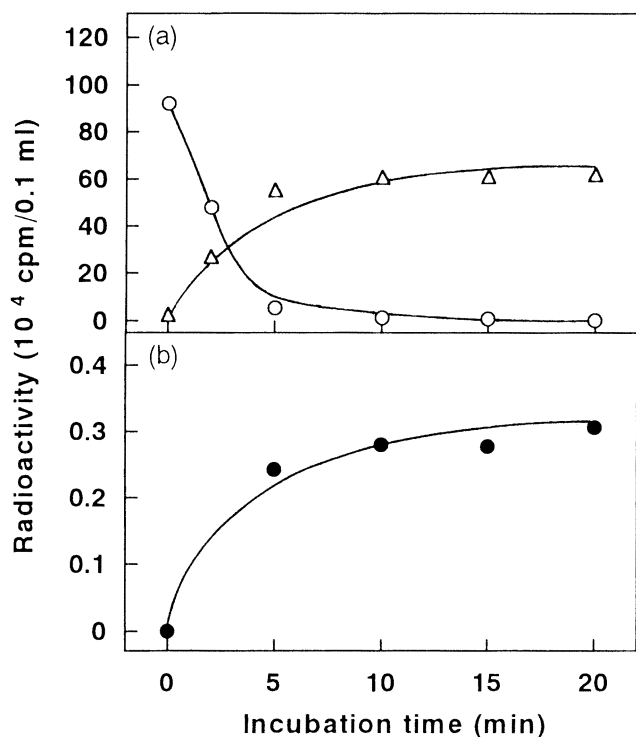


Fig. 9. Change in the radioactivity of (a) SAM, MTA and (b) 1-MeA following heat treatment of SAM. A mixture containing S-[^{14}C -methyl]SAM (2 mM) and purified SAM (8 mM) at pH 7.5 was boiled for the indicated times. Radioactivities of SAM (\circ), MTA (\triangle) and 1-MeA (\bullet) were analyzed by HPLC using a reverse-phase column. The values shown are means for duplicate determinations.

DISCUSSION

The present study has demonstrated that 1-MeA, the natural MIS in starfish gonads, is produced by heat treatment of SAM. Since the 1-MeA production from SAM was not dependent on the presence of Ade or Ado, the methyl group of SAM is considered to be transferred to the N-1 site of the purine base of SAM molecule. However, it is unclear whether the methylation process is based on an inner- or intra-transformation of SAM molecule. Upon the inner- or intra-transformation of SAM, N1-methyl-S-adenosyl-homocysteine (N1-MeSAH) or N1-methyl-S-adenosylmethionine (N1-MeSAM) should be produced, respectively. Unfortunately, neither N1-MeSAH nor N1-MeSAM was identified in this study, but it is possible that N1-MeSAM is produced by heat treatment of SAM, because the N1-site is distant from S-methyl group within SAM molecule. Since any N1-MeSAM is probably unstable in the N-glycoside linkage between base and sugar, N1-MeSAM produced would be hydrolyzed rapidly to 1-MeA. Thus, 1-MeA appears to be produced by way of methylated SAM.

In this study, 1-MeA production was found to require high temperature and high pH. However, the maximum amount of 1-MeA produced was less than 0.3% that of SAM. This was caused by the decrease in the level of SAM following heat treatment, since most (about 95%) was decomposed to MTA and Met (O).

It is important to consider whether the chemical 1-MeA production from SAM observed in this study is associated with the biological 1-MeA production by starfish ovarian follicle cells. Unfortunately, little is known about the biosynthesis of 1-MeA. A process of methylation has been reported to be involved in GSS-dependent biosynthesis (Shirai *et al.*, 1972; Shirai, 1973; Tarr, 1985; Mita, 1992). Previous studies have shown that the formation of SAM as a methyl donor is involved in 1-MeA production (Kanatani *et al.*, 1976; Mita, 1991b, Mita *et al.*, 1997). Recently, GSS has been shown to cause a reduction in the intracellular level of ATP following 1-MeA production (Mita *et al.*, 1996). Thus, ATP may possibly be used for the formation of SAM. SAM is a possible candidate for a methyl donor in 1-MeA biosynthesis. However, there is no information about the methyl acceptor involved. In this study, 1-MeA was found to be produced from SAM alone, suggesting that SAM is available for use as a methyl acceptor as well as a methyl donor for 1-MeA production.

The action of GSS on 1-MeA production in follicle cells is mediated by its receptor, guanine nucleotide-binding regulatory proteins and adenylyl cyclase (Mita and Nagahama, 1991). It has been reported that upon incubation of follicle cells with GSS, there is a dose-related increase in adenosine 3',5'-cyclic monophosphate (cAMP) production, coincident with an increase in 1-MeA production (Mita *et al.*, 1987, 1989; Mita and Nagahama, 1991). The methylation process in 1-MeA biosynthesis has also been shown to be associated with elevation of the cAMP level (Mita, 1992). It has been demonstrated that methylation using SAM as a methyl donor is catalyzed by a methyltransferase (Cantoni, 1957). Recently, cAMP-

dependent protein kinase (PKA) has been found in starfish follicle cells (Mita *et al.*, 1996). Since the effect of cAMP is mediated by PKA (Walsh *et al.*, 1968; Beavo *et al.*, 1974), it is possible that an unknown methyltransferase related to methylation between SAM and SAM is activated by PKA.

It has been reported that the enzyme 1-MeAdo ribohydrolase is present in follicle cells (Shirai and Kanatani, 1972; Tarr, 1973). 1-MeAdo ribohydrolase is capable of hydrolyzing 1-MeAdo to 1-MeA and ribose, but does not catalyze Ado. It is possible that N¹-MeSAM synthesized from SAM is catalyzed by 1-MeAdo ribohydrolase to 1-MeA. Further studies on the enzyme system related to the methylation of SAM *in vivo* should provide additional insight into 1-MeA biosynthesis by starfish ovarian follicle cells.

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