JOURNAL OF BACTERIOLOGY, May 1980, p. 608-614
0021-9193/80/05-0608/07$02.00/0

Vol. 142, No. 2

Stimulation of Yeast Ascospore Germination and Outgrowth by S-Adenosylmethionine

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The supplementation of S-adenosylmethionine (SAM) to germination medium stimulated the accumulation of [^{14}C]uracil from the medium into germinating cells, as well as its incorporation into ribonucleic acid during germination and outgrowth of ascospores of *Saccharomyces cerevisiae*. In addition to uracil, the accumulation of leucine, cytosine, serine, and methionine was also stimulated by the extracellular addition of this sulfonium compound. The SAM-stimulatory effect was dose dependent; half-maximal stimulation was observed at about 50 μM. The effect exerted by SAM supplementation appeared to be specific for SAM and for germination and outgrowth. In the absence of SAM biosynthesis (in the presence of cycloleucine), spores were inhibited in their ability to accumulate label, whereas the supplementation of SAM completely reversed the cycloleucine-induced inhibition of accumulation. In addition to accumulation and incorporation, the kinetics of bud formation during outgrowth were also stimulated by exogenous SAM. The stimulation of budding by SAM was amplified in an ethionine-resistant strain. These observations suggest that SAM may be essential for the initiation of cell division during the breaking of spore dormancy.

The synthesis of S-adenosylmethionine (SAM) has been suggested to be required for the initiation of germination and outgrowth of ascospores of *Saccharomyces cerevisiae* (4). Because of the numerous reports linking the onset of rapid cellular proliferation with increased polyamine biosynthesis (1, 3, 7, 11, 15), the synthesis of these cations from SAM was assumed to be essential for the initiation of the cell division cycle in resting ascospores. The syntheses of spermidine and spermine, however, were found not to be a requisite for either macromolecule biosynthesis or germination and outgrowth of *S. cerevisiae* spores (5). Recently, the synthesis of putrescine was also shown to be nonessential for the breaking of spore dormancy, although cell divisions subsequent to the initial one after the breaking of dormancy were found to proceed more slowly in the absence of continual polyamine biosynthesis (2).

Since the synthesis of SAM is essential for germination and outgrowth, whereas polyamine biosynthesis is not an absolute requirement, an alternative function for SAM in the initiation of cell division during germination is suggested. To further investigate the role played by SAM in this process, we studied the effect of extracellular supplementation of SAM on germination and outgrowth. In this paper, we report that (i) exogenous SAM stimulates the accumulation of a variety of metabolites from the germination medium into the cells, (ii) the SAM-mediated enhancement is dose dependent and appears to be specific for SAM, as well as for germinating cells, and (iii) this effect is also apparent when the kinetics of budding during the first cell cycle are followed.

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MATERIALS AND METHODS

Organisms and culture conditions. A diploid *S. cerevisiae* prototroph (ATCC 7752) and an ethionine-resistant diploid R3720 × RJB-21 (α Ura$^-$ Eth^r × a Ade$^-$ Eth^r) were used in this study. The latter strain was provided by K. D. Spence, Washington State University. The preparation of single spore populations was essentially as described by Tingle et al. (21). Diploid cells were grown in YPA medium (1% yeast extract, 2% peptone, 1% potassium acetate) to a density of about 3×10^7 cells/ml at 30°C at 300 rpm. The cells were centrifuged at 8,000 × *g*, washed with sterile water, recentrifuged, and suspended in a volume of sporulation medium (1% potassium acetate) equivalent to the volume of vegetative medium in which the cells were originally grown. The cells were shaken in sporulation medium for about 72 h. The asci were centrifuged and washed as described above. The pellet from 1 liter of medium was mixed with 3.2 ml of spore buffer and 0.3 ml of glusulase (Endo Laboratories). Spore buffer contained 0.067 M potassium phosphate buffer (pH 6.8), 1% Tween 80, 0.14 M 2-mercaptoethanol, and 20 μg of tetracycline per ml. When smaller quantities of asci were processed, the volumes were all

scaled down accordingly. This suspension was shaken for 3 h at 30°C at 150 rpm; 100 ml of 1% Tween 80 was added to the suspension, and the cells were centrifuged at 250 × *g* for 15 min. The pellet was washed three times with 1% Tween 80, suspended in 7 ml of spore buffer, and passed through an Aminco French pressure cell at 8,000 lb/in^2. This preparation was free of vegetative cells and intact asci.

Spores were routinely germinated at a concentration of 2 × 10^6 spores/ml in Wickerham minimal medium (22) with 2% glucose and 0.5% ammonium sulfate added as sole sources of carbon and nitrogen, respectively. Germination and outgrowth were monitored by spectrophotometry of the change in absorbance at 600 nm (14) and by phase-contrast microscopy. Budding was determined by direct microscopic observation of at least 400 cells.

Measurement of accumulation and incorporation. The accumulation of radioactive compounds was determined by the withdrawal of duplicate samples from the growth media at various times. The samples were added to tubes containing 3 ml of cold (4°C) water and rapidly collected on membrane filters (HAWP 25 mm, 0.45 μm; Millipore Corp.), using a multifiltration apparatus (New Brunswick Scientific Co.). The filters were washed with 10 ml of cold water and dried. Radioactivity was determined in 3 ml of scintillation fluid consisting of Triton X-100 and toluene (1:2) with 0.4% 2,5-diphenyloxazole, using an LS 250 liquid scintillation counter (Beckman Instruments, Inc.).

The incorporation of radioactive precursors into trichloroacetic acid-insoluble material was used to measure RNA synthesis. Duplicate samples were removed from the growth media and added to 5 ml of cold 10% trichloroacetic acid. The precipitates were collected on Whatman GF/C filters with a multifiltration apparatus (New Brunswick Scientific Co.). The precipitates were washed three times with 5 ml of 5% trichloroacetic acid and once with 95% ethanol. The filters were dried, and radioactivity was determined as described above.

Chemicals. SAM was purchased from Boehringer Mannheim Corp., and cycloleucine was obtained from Calbiochem. All radioactively labeled compounds were from Amersham Corp. *S*-Adenosyl-D-methionine and 5′-dimethylthioadenosine were the gifts of Fritz Schlenk.

RESULTS

Kinetics of germination and outgrowth. Germination is defined as the transition period from a refractile to a nonrefractile spore, whereas outgrowth is the development of a budded cell from a phase-dark cell (21). The kinetics of germination and outgrowth of strains 7752 (prototroph) and R3720 × RJB-21 (ethionine resistant) are shown in Fig. 1. Spores of the prototrophic strain germinated more rapidly than did spores of the ethionine-resistant strain. The maximum decrease in light absorbance occurred after about 1.5 h in strain 7752 and after 3 h in strain R3720 × RJB-21. During the outgrowth period of both strains, initial bud formation began shortly after the maximal decrease in absorbance was attained (2 to 2.5 h for strain 7752; 3.5 to 4 h for strain R3720 × RJB-21). Detailed characteristics of germination and outgrowth of strain 7752 in Wickerham minimal medium have previous been described (2).

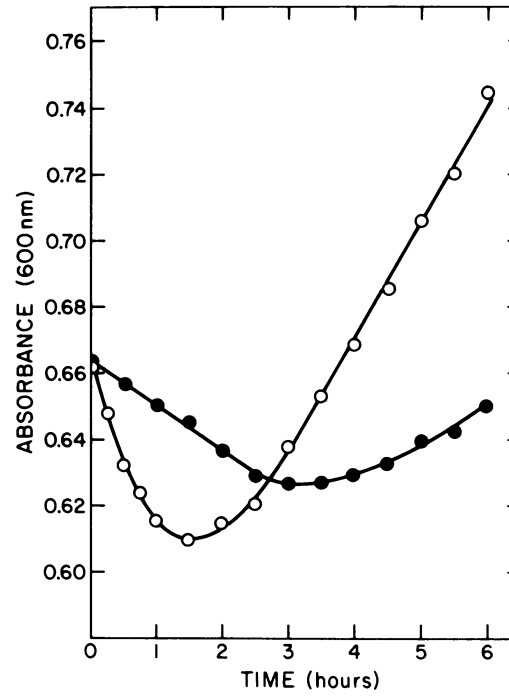

FIG. 1. *Kinetics of germination and outgrowth of spores of S. cerevisiae strains 7752 (○) and R3720 × RJB-21 (●). Spores were germinated in Wickerham minimal medium, and the absorbance at 600 nm was determined at the specified times.*

Effect of SAM on uracil accumulation and incorporation. The addition of 250 μM SAM at 0 h created a significant stimulation in both the accumulation of [^{14}C]uracil within the spores and its incorporation into RNA (Fig. 2). Similar quantities of [^{14}C]uracil accumulated within the cells as were incorporated into RNA, indicating that most of the uracil taken up by the germinating cells was incorporated into RNA. At 5 h, the SAM-treated culture showed a stimulation of 315% over the control when the accumulation of [^{14}C]uracil was measured and a stimulation of 295% over the control when RNA synthesis was measured. At 1, 2, 3, and 4 h, smaller but significant increases in accumulation and incorporation were also found. These time periods encompass both the process of germination and outgrowth. Since the accumulation of labeled uracil and its incorporation into RNA were similar, only the accumulation of [^{14}C]uracil was used to

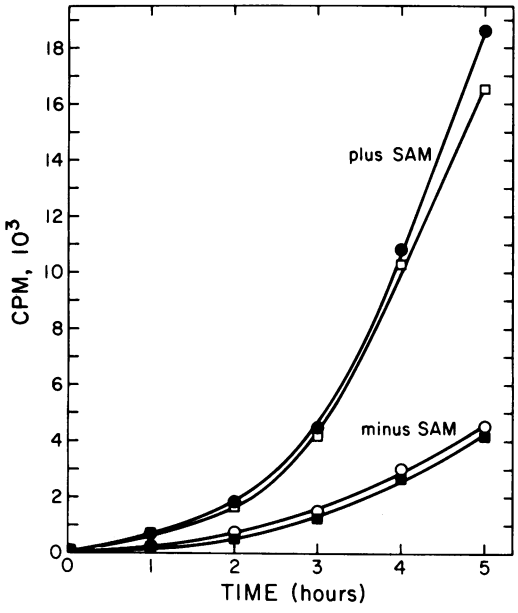

FIG. 2. *Effect of SAM on the accumulation and incorporation of [^{14}C]uracil during germination and outgrowth. Spores were germinated in the presence and absence of SAM (250 μM), and the accumulation of label within the cells and the incorporation of label into acid-insoluble material was measured at the designated times. Symbols:* ○, *control, accumulation;* ●, *plus SAM, accumulation;* ■, *control, incorporation;* □, *plus SAM, incorporation. [^{14}C]uracil (1.3 μCi/ml) and SAM were added to the germination medium at* t_0.

measure the stimulatory effect exerted by SAM in further experiments. In addition to uracil, the accumulation of other metabolites was also stimulated by SAM. For example, at 250 μM, SAM stimulated the accumulation of leucine (70%), cytosine (142%), serine (31%), and methionine (24%) (Table 1).

The degree of stimulation of uracil accumulation was measured at several concentrations of SAM to determine any dose dependence of this effect (Fig. 3). SAM and [^{14}C]uracil were added 4 h after the initiation of germination, and the degree of stimulation was measured at 6 h. The data indicated that the stimulation of [^{14}C]uracil accumulation by exogenous SAM was dose dependent. An increase of about 180% in the accumulation of [^{14}C]uracil was elicited over this time period by 500 μM SAM. Half-maximal stimulation was observed at about 50 μM SAM. A SAM concentration of 250 μM, which stimulated nearly as well as 500 μM, was adopted for further studies. Similar results were obtained when SAM and [^{14}C]uracil were added at t_0, and the degree of stimulation was measured at t_2 (data not shown).

TABLE 1. *Effect of SAM on the accumulation of various compounds during germination and outgrowth of ascospores of strain 7752*

Supplement[a]	Accumulation (cpm)		% Stimulation
	Control	Plus SAM[b]	
Leucine	12,742	21,674	70
Cytosine	21,502	52,136	142
Serine	18,781	24,660	31
Methionine	8,380	10,426	24

[a] All supplements were added to the germination medium at t_0 and measured for accumulation at t_2. The radioactively labeled compounds added were: leucine (0.67 μCi/ml), cytosine (1 μCi/ml), serine (4 μCi/ml), and methionine (1 μCi/ml).

[b] SAM was added at a concentration of 250 μM at t_0.

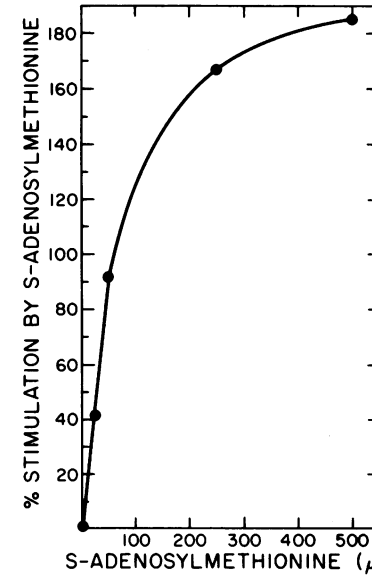

FIG. 3. *Dose-dependent stimulation of [^{14}C]uracil accumulation by SAM during germination and outgrowth. Spores were germinated with [^{14}C]uracil (1.3 μCi/ml) in the presence and absence of various concentrations of SAM. Both label and SAM were added at* t_4, *and the percent stimulation was determined at* t_6.

Specificity for SAM. A variety of analogs and metabolites of SAM were tested for their ability to stimulate [^{14}C]uracil accumulation during germination of ascospores of strain 7752 (Table 2). The data reveal that, of the compounds tested, only SAM was effective in enhancing [^{14}C]uracil accumulation. In contrast to the L-isomer, *S*-adenosyl-D-methionine did not cause any stimulation, suggesting a stereospecific requirement for the stimulatory molecule. 5′-Dimethylthioadenosine and *S*-adenosylethionine (sulfonium compounds), the diamine

TABLE 2. *Effect of SAM and SAM-related compounds on the accumulation of [^{14}C]uracil in germinating ascospores of strain 7752*

Supplement[a]	% Stimulation[b]
None	0
S-Adenosyl-L-methionine	231
S-Adenosyl-D-methionine	−4
S-Adenosyl-L-homocysteine	−4
S-Adenosyl-L-ethionine	26
5′-Methylthioadenosine	−10
5′-Dimethylthioadenosine	−19
Methionine	0
Homocysteine	10
Putrescine	−15
Spermidine	−11
Spermine	−17

[a] SAM (250 μM), all other supplements (500 μM), and [^{14}C]uracil (1.3 μCi/ml) were added at t_0 and measured for accumulation at t_4.

[b] The percent change from unsupplemented cultures. In the absence of any supplement, 2,810 cpm accumulated, and the cells were 30% budded by t_4.

putrescine, and polyamines spermidine and spermine were also found to be ineffective, suggesting that the stimulation did not result from neutralization of negative charges. The possibility that the stimulation was exerted via a contaminant in the SAM preparation is unlikely since both hydrolyzed SAM and the known contaminants found in commercially available SAM (methionine, 5′-methylthioadenosine) were found to be ineffective. In addition, the SAM utilized was greater than 98% pure as determined by paper chromatography and high-pressure liquid chromatography. None of the SAM analogs was tested for its effect on the process of germination and outgrowth.

Specificity of SAM stimulatory effect for germination. To examine any time-dependent specificity of the stimulatory effect exerted by SAM, it was added at various times during germination and outgrowth. SAM and [^{14}C]uracil were added at 0, 2, 4, 6, 8, 10, and 12 h after the initiation of germination and outgrowth. The accumulation of label in the control and SAM-treated cultures was then measured 1, 3, and 5 h after each addition (Fig. 4). The data showed that stimulation was greatest during the early hours of germination and outgrowth. A culture treated with SAM at 0 h exhibited a stimulation of over 300% at 5 h, whereas cells treated with SAM at 2 h showed increased uracil accumulation by about 250% 5 h later. The greatest increase measured over the first hour was seen when SAM was added at 6 h (80%). At later times, the addition of SAM evoked less stimulation, whether measured 1, 3, or 5 h later. At 8 h, stimulation was very low, and by 12 h the

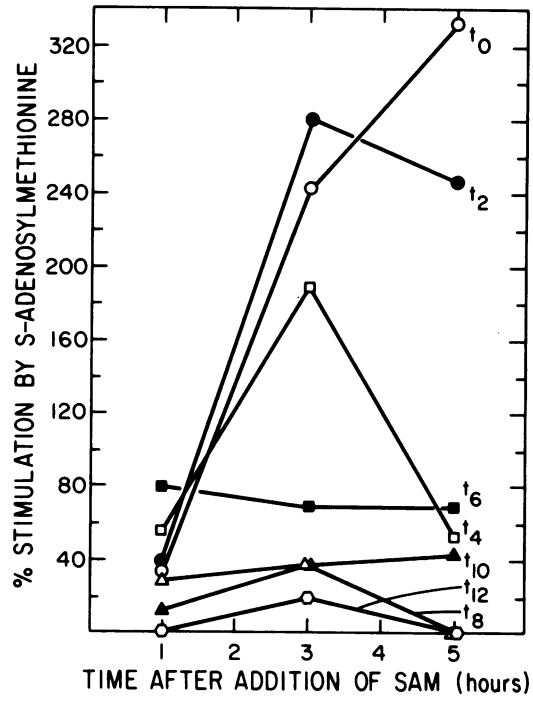

FIG. 4. *Stimulation of [^{14}C]uracil accumulation by SAM when supplemented to the medium at various times during germination and outgrowth. SAM (250 μM) and [^{14}C]uracil (1.3 μCi/ml) were added to the germination medium at t_0 (○), t_2 (●), t_4 (□), t_6 (■), t_8 (△), t_{10} (▲), and t_{12} (○), and the accumulation of label in the presence and absence of SAM was determined 1, 3, and 5 h after each time of addition.*

stimulation caused by SAM was negligible. Thus, it appeared that the SAM-induced stimulation of accumulation of uracil was specific for the early hours of germination and outgrowth of yeast ascospores.

To further examine this specificity, exponential-phase diploid 7752 cells and stationary diploid 7752 cells were inoculated into fresh media, and the accumulation of uracil was measured, in the presence and absence of 250 μM SAM. Both SAM and [^{14}C]uracil were added at 0 h, and [^{14}C]uracil accumulation was measured 5 h later. In each case, the medium was the same as that used for germination. The effect of SAM in each instance was minor; a 10% stimulation in [^{14}C]uracil accumulation was observed with exponential cells, whereas only an 8% stimulatory effect was noted with the stationary cells (data not shown).

Effect of SAM on germination and outgrowth. Since SAM enhances the accumulation of a variety of compounds, it was possible that the increase in available nutrients might stimulate cell division in this system. Formation of

buds, an event that occurs at the time of DNA synthesis (10), was monitored in spores germinated in the presence and absence of SAM (Fig. 5). Although bud formation began at about the same time in both cultures, the SAM-supplemented culture exhibited a greatly increased rate of budding from 3.5 to 5 h. At 4 h, the number of budded cells in the culture supplemented with SAM was almost double that in the control culture. Viability was not affected since both cultures reached similar final values for bud formation. The data suggest that the stimulation of accumulation shown with a variety of nutrients may lead to an accelerated initial cell division during outgrowth. These results have shown that exogenous SAM can stimulate the accumulation of a variety of compounds, as well as stimulate budding in a prototrophic yeast strain.

It is known that SAM is actively taken up by yeast cells (20). If exogenous SAM were acting in the same manner as internally synthesized SAM, the inhibition of SAM formation would be expected to cause effects opposite to those observed with the addition of exogenous SAM. Cycloleucine has been shown to be a specific inhibitor of SAM formation (6). [^{14}C]uracil accumulation was measured from 0 to 4 h and 4 to 6 h in cells treated with cycloleucine, SAM, and a combination of SAM and cycloleucine (Table 3). Cycloleucine (1 mM) inhibited uracil accumulation over both of these time periods and, in each case, 250 μM SAM overcame the inhibition. Over the 0- to 4-h period, in particular, SAM completely overcame the cycloleucine-induced inhibition of uracil accumulation.

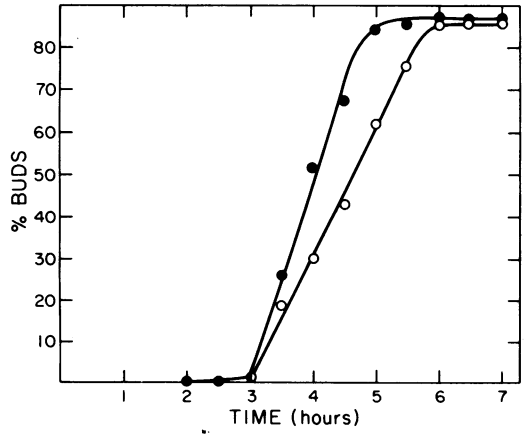

FIG. 5. *Effect of SAM on budding during outgrowth of spores of strain 7752. SAM (250 μM) was added to the medium at t_0. The percentage of cells containing buds was determined in cultures undergoing outgrowth in the presence (●) and absence (○) of SAM.*

TABLE 3. *Effect of cycloleucine and SAM on the accumulation of [^{14}C]uracil in germinating ascospores of strain 7752*

Supplement[a]	[^{14}C]uracil accumulation[b]			
	0–4 h		4–6 h	
	cpm	% Stimulation	cpm	% Stimulation
None	2,710	0	19,665	0
SAM	6,419	137	69,962	256
Cycloleucine	922	−66	12,508	−36
SAM + cycloleucine	7,098	162	44,140	12

[a] SAM and cycloleucine were added at 250 μM and 1 mM, respectively.

[b] SAM, cycloleucine, and [^{14}C]uracil were supplemented to the germination medium at t_0 and measured for accumulation of label at t_4; in a similar experiment, they were added at t_4, and accumulation of label was measured at t_6.

If the synthesis of SAM were critical for cell division and accumulation of metabolites, as is suggested by the data, the effect of exogenous SAM might be magnified in a strain of yeast deficient in the ability to synthesize SAM. The ethionine-resistant diploid strain R3720 × RJB-21 has been shown to possess a methionine adenosyltransferase (SAM synthetase) with very low enzymatic activity and to synthesize low quantities of SAM (13). The accumulation of labeled serine and uracil was measured during germination in the presence and absence of SAM in this strain (Table 4). The stimulation of serine accumulation elicited by SAM was over 100%, whereas the enhancement of uracil accumulation was about 200%. Budding was also monitored in the ethionine-resistant strain in control cells and in the presence of SAM (Fig. 6). Budding of control ethionine-resistant cells was much slower than the budding of 7752 spores. Moreover, the ethionine-resistant strain appeared to bud in a stepwise manner. SAM, however, greatly accelerated the rate of budding. At 7 h, when only 27% of control cells were budded, essentially all the SAM-treated cells had formed buds. Over 50% of the SAM-treated spores contained buds at 5 h, whereas the control cultures only possessed 13% buds.

DISCUSSION

The addition of SAM to germination medium stimulates the accumulation and incorporation of a variety of metabolites from the medium into germinating yeast cells, and the concentrations which elicit this response are within the physiological levels for cells of *S. cerevisiae* (9, 16, 17). The specificity may be because the mechanisms responsible for the SAM stimulatory effect are

TABLE 4. *Effect of SAM on the accumulation of [^{14}C]uracil and [^{14}C]serine during germination and outgrowth of ascospores of strain R3720 × RJB-21*

Supplement[a]	Accumulation (cpm)		% Stimulation
	Control	Plus SAM[b]	
Serine	2,496	5,602	124
Uracil	1,062	3,255	206

[a] Serine (2 μCi/ml) and uracil (1 μCi/ml) were added to the germination medium at t_0 and measured for accumulation at t_2.

[b] SAM was added at a concentration of 250 μM at t_0.

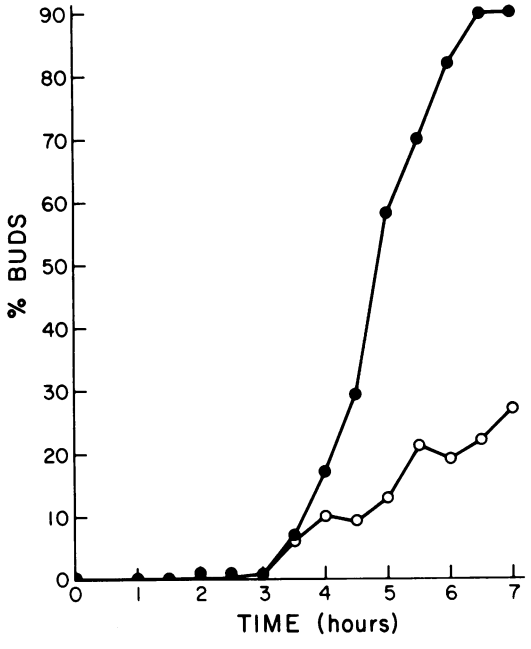

FIG. 6. *Effect of SAM on budding during outgrowth of spores of strain R3720 × RJB-21. SAM (250 μM) was added to the medium at t_0. The percentage of cells containing buds was determined in cultures undergoing outgrowth in the presence (●) and absence (○) of SAM.*

only present in germinating spores or because the processes stimulated by SAM are already operating at maximal rates in yeast cells in phases of growth other than germination and outgrowth.

In contrast to the stimulatory effect observed here, the extracellular supplementation of SAM to yeast cells has also been shown to be inhibitory. Law and Ferro (12) found that SAM inhibits RNA synthesis during glucose-derepression in *S. cerevisiae* and that cAMP acts as an antagonist to the SAM-mediated inhibition. The supplementation of cAMP to germinating yeast ascospores had no effect on uracil accumulation or incorporation into RNA, nor did it affect the stimulation caused by SAM (unpublished observation).

In addition to a stimulation of accumulation and incorporation, ascospores extracellularly supplied with SAM also exhibit an increased rate of budding. Bud initiation marks the end of the G1 interval of the cell division cycle and marks the beginning of the S phase (10), whereas the first doubling in cell number in *S. cerevisiae* strain 7752 during germination and outgrowth occurs about 12 h after the initiation of germination (2). Since bud initiation begins at about 3 h in strain 7752, and the greatest stimulation of uracil accumulation was noted during the first 5 h after the initiation of germination, it appears that SAM may be exerting its stimulatory effect during the G1 or S phase or both of the cell division cycle.

An enhancement of the kinetics of bud formation by SAM was observed during germination and outgrowth of the ethionine-resistant strain. The ethionine-resistant strain used in this study possesses a defective methionine adenosyltransferase and, therefore, in the presence of ethionine synthesizes only low levels of *S*-adenosylethionine (13). Our data show that the absence of normal methionine adenosyltransferase activity results in a very slow rate of bud formation during germination and outgrowth. The supplementation of SAM to the germination medium completely restores the kinetics of bud formation to a rate comparable to wild-type strains. Recently, Singer et al. (19) showed that ethionine causes arrest of yeast cells within the G1 phase of the cell cycle and specifically at "start." Given that ethionine competes with methionine for the methionine adenosyltransferase (13), that methionine is rapidly converted to SAM in yeast cells (16), and that methionine is capable of reversing the ethionine-induced inhibition of cell growth (8) and G1 arrest (19), it is possible that the synthesis of SAM, in addition to being required for germination and outgrowth, is also essential for the start of the cell division cycle in *S. cerevisiae*. It is noteworthy that adenine and methionine, both precursors of SAM, are essential for the initiation of germination (4), as well as for the regulation of the rate of cell cycle initiation in auxotrophic strains of *S. cerevisiae* (18).

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant GM 26194 from the National Institutes of Health. A.J.F. is a recipient of a Public Health Service Research Career Development Award (CA00617) from the National Institutes of Health.

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