

# Biosynthesis of Mycophenolic Acid: Purification and Characterization of *S*-Adenosyl-L-Methionine:Demethylmycophenolic Acid *O*-Methyltransferase

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The final step in the biosynthesis of mycophenolic acid involves the transfer of a methyl group from *S*-adenosylmethionine to demethylmycophenolic acid. The enzyme, *S*-adenosylmethionine:demethylmycophenolic acid *O*-methyltransferase, was isolated from *Penicillium stoloniferum* and purified 2,700-fold by ammonium sulfate fractionation and diethylaminoethyl-cellulose and Sephadex G-200 chromatography. Maximum enzyme activity was achieved at pH 7.5 and a temperature of 27 to 28 C. The apparent  $K_m$  for demethylmycophenolic acid was  $3.1 \times 10^{-6}$  M. The enzyme preparation was 50% inactivated when exposed to 33 C for 15 min. Mycophenolic acid, homocystine, *S*-adenosylhomocysteine, ethanol, and  $Mg^{2+}$  inhibited the methyltransferase. This enzyme appears to be subject to end product inhibition which may regulate the synthesis of mycophenolic acid. The methyltransferase activity was highest during the early phases of the fermentation.

Mycophenolic acid (MPA) was first isolated from a *Penicillium* culture by Gosio in 1896 (15). Since that time several species of *Penicillium*, including *P. brevis-compactum*, *P. stoloniferum*, *P. scabrum*, *P. nagami*, *P. saferi*, *P. patris-mei*, *P. griseobrunneum*, and *P. viridicatum*, were reported to produce MPA (12). This fungal metabolite has been reported to possess the following diverse biological properties: antiviral (2, 26), antifungal (14), antibacterial (1, 14), antitumor (13, 22, 26), immunosuppressive (13, 21), and anti-psoriasis (17).

In an effort to elucidate the biosynthesis of mycophenolic acid, a number of investigators have studied the incorporation of various radioactive precursors (4-11, 16). These labeling studies have implicated several phenolic intermediates derived from a tetraketide chain in the biosynthesis of mycophenolic acid. A proposed biosynthetic scheme based on these labeling studies is shown in Fig. 1. The last step involves the transfer of a methyl group from *S*-adenosyl-L-methionine (SAM) to demethylmycophenolic acid (DMPA). In this study the enzyme, SAM:DMPA *O*-methyltransferase, was isolated from *P. stoloniferum*. In addition, we characterized some of the factors that influence its activity.

## MATERIALS AND METHODS

**Culture and culture conditions.** A mycophenolic acid-producing mutant of *P. stoloniferum* designated AAO-051 was used throughout this study. This mutant was derived by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment of the wild-type culture obtained from the Northern Regional Research Laboratory, Peoria, Ill. The culture used in this study was preserved by conventional liquid nitrogen procedures and was maintained on a complex sporulation medium (GCY-B) which contained: glucose, 1.0%; yeast extract, 0.2%; acid-hydrolyzed casein, 0.5%;  $KH_2PO_4$ , 0.5%;  $NH_4Cl$ , 0.1%;  $MgSO_4 \cdot 7H_2O$ , 0.04%;  $CaCO_3$ , 0.04%; and agar, 2.0%.

The cells for purification of the transferase were grown in a 2.5-liter, mechanically stirred and aerated fermentor containing a defined medium of the following composition: glucose, 10%; glycine, 1.46%; methionine, 0.05%;  $MgSO_4 \cdot 7H_2O$ , 0.1%;  $KH_2PO_4$ , 0.3%;  $FeSO_4 \cdot 7H_2O$ , 0.00022%;  $CuSO_4 \cdot 5H_2O$ , 0.00003%;  $ZnSO_4 \cdot 7H_2O$ , 0.000246%;  $MnSO_4 \cdot 4H_2O$ , 0.000016%; and  $KMoO_4$ , 0.00002%. The fermentor, containing 1.5 liters of medium was inoculated with 160 ml of a 48-h culture grown in 1% glycerol, 1% glucose, and 2.5% cotton seed flour. Incubation was carried out at 25 C for 64 h. At that time major cell mass had been achieved and synthesis of MPA was most active. Cells were harvested by centrifugation at  $13,200 \times g$  for 30 min.

**SAM:DMPA *O*-methyltransferase assay.** The methylating activity was monitored by the transfer of the radioactively labeled methyl group from SAM to

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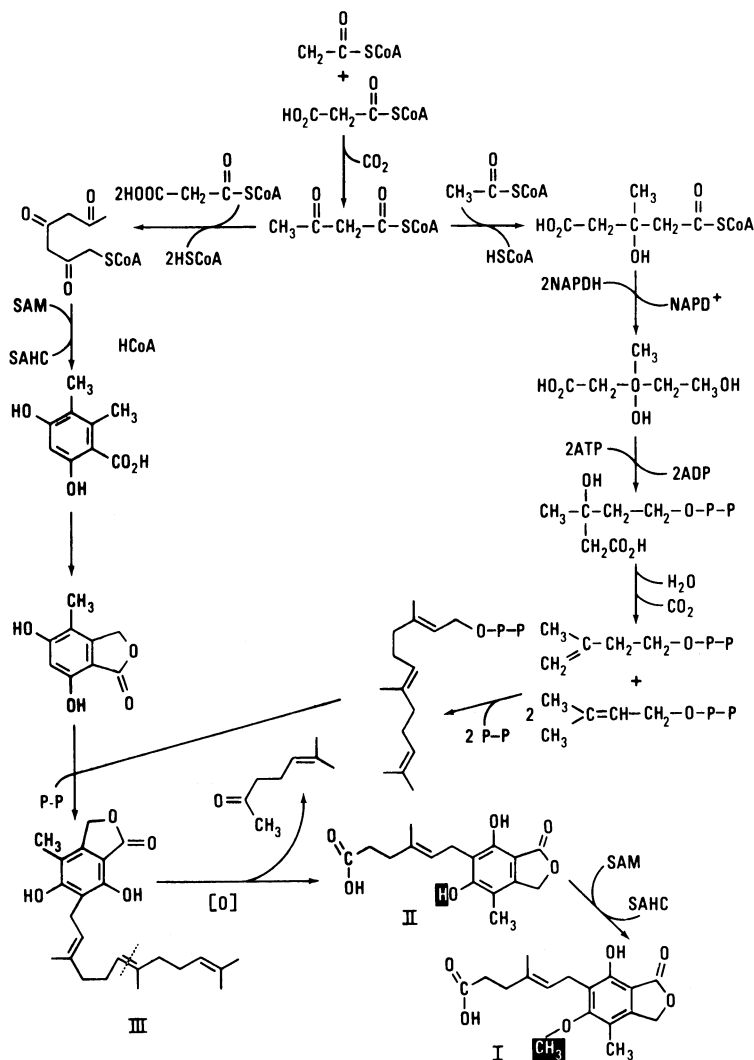


FIG. 1. Proposed biosynthetic pathway for mycophenolic acid.

DMPA. The reaction mixture contained, except when specified in the results, the following constituents in a total volume of 200  $\mu$ l: *N*-tris(hydroxymethyl)-methyl glycine (Tricine buffer, pH 7.5), 1 mmol; DMPA, 0.0984  $\mu$ mol; [*methyl*- $^3$ H]SAM, 1.7  $\mu$ Ci or  $1.39 \times 10^{-4}$   $\mu$ mol; and 50  $\mu$ l of enzyme preparation. In some experiments the [ $^3$ H]SAM was replaced with [*methyl*- $^{14}$ C]SAM, 0.2  $\mu$ Ci or 0.0035  $\mu$ mol. The reactions were incubated in a gyratory waterbath shaker (50 rpm) at 28 C for 30 to 240 min and were terminated by addition of 100  $\mu$ l of 3 N HCl followed by 1 ml of ethyl acetate. Samples were mixed well and allowed to stand for 1 h in order for the phases to separate. A 50- $\mu$ l sample of the ethyl acetate layer was transferred into a scintillation vial containing 10 ml of Bray scintillation solution (Nuclear-Chicago, Chicago, Ill.). The vials were gently shaken and counted for 10 min each in a scin-

tillation counter. The methylating activity was expressed as picomoles of labeled MPA formed per hour per 200  $\mu$ l of reaction mixture. One unit of activity is equivalent to 1 pmol of labeled MPA formed per h. A correction was made for a small amount of solvent-extractable radioactive material formed when the enzyme was incubated without DMPA.

The reaction products were identified by thin-layer chromatography and autoradiography. For this procedure, 1.5 ml of the ethyl acetate layer was taken to dryness in a vacuum oven. The residue was then dissolved in 50  $\mu$ l of ethyl acetate. A 30- $\mu$ l portion of the concentrated sample was spotted on a Silica Gel F 254 plate and developed with chloroform-ethyl acetate-acetic acid (240:90:15). The radioactive compounds were detected by autoradiography using X-ray film and by scraping small regions of the

thin-layer plates for counting.

**Assay procedures.** The protein concentration in various fractions was determined by either the method of Lowry (19) with bovine serum albumin as standard or by the relative absorbance at 260 and 280 nm during the purification (27).

Mycophenolic acid was estimated after adjusting the pH of the whole broth to pH 8 with 3 N sodium hydroxide, removing the cells by filtration, adjusting the pH of the broth to 4.5 with 3 N HCl, and extracting with 10 volumes of amyl acetate. The amyl acetate was diluted appropriately and absorbance at 304 nm was determined. Concentration of MPA was calculated based on the molar extinction coefficient  $E_{cm}^m = 9.8 \times 10^4$  liters/mol per cm.

Growth was monitored by dry cell weight. Cells were removed from a 10-ml sample of whole broth by filtration through a Millipore prefilter, washed with 20 ml of water, dried for 24 h at 100 C, and weighed.

**Enzyme purification.** All steps except the gel filtration were carried out at 5 C. The Sephadex G-200 column was run at 21 C.

**Crude lysate.** The cell paste (approximately 450 g) was suspended in approximately 900 ml of 0.02 M potassium phosphate-saline buffer, pH 7.1. The slurried cells were disrupted in a French pressure cell at 20,000 lb/in<sup>2</sup>. The cell debris was removed by centrifugation at 30,900  $\times g$  for 20 min. Cellular lipid was removed from the supernatant solution by passage through glass wool. Attempts to remove nucleic acids at this point in the purification resulted in extreme loss of activity.

**Ammonium sulfate fractionation.** The crude extract was diluted in the same buffer to approximately 10 mg of protein/ml and then fractionated between 50 and 100% with solid ammonium sulfate. The resulting precipitated protein was dissolved in 0.05 M Tris (Tris-chloride buffer, pH 7.7) and exhaustively dialyzed against two 2-liter changes of the same buffer.

**Diethylaminoethyl (DEAE)-cellulose chromatography.** The ammonium sulfate-treated extract (210 units, 56 ml) was placed on a DEAE-cellulose (DE-52) column (0.5 cm by 40 cm) which had been equilibrated with 0.05 M Tris-chloride buffer, pH 7.7. The column was washed with the equilibration buffer until the absorbance at 280 nm ( $A_{280}$ ) of the eluate was less than 0.1. Then the transferase was eluted with the equilibration buffer containing 0.25 M KCl. Fractions of 11.5 ml were collected at a flow rate of approximately 70 ml/h. Fractions containing the enzyme were pooled and concentrated by ultrafiltration through a Diaflo UM-10 membrane (Amicon Corp., Cambridge, Mass.) using nitrogen as the pressurizing gas. There were approximately 24 distinguishable protein bands upon analytical polyacrylamide gel electrophoresis of a sample of the concentrate from the DEAE-cellulose column.

**Sephadex G-200 filtration.** The concentrated sample (5,590 units) from the DEAE-cellulose column was applied to a column (2.5 by 65 cm) which was packed with Sephadex G-200 supported on silanized 6-mm glass beads according to the technique of Sachs and

Painter (24) and equilibrated with 0.05 M Tris-chloride buffer (pH 7.5). Fractions of 5.4 ml were collected at a flow rate of approximately 50 ml/h. The transferase eluted in the first protein peak after the void volume (Fig. 2). Fractions containing maximal activity (17 through 23) were pooled and concentrated as before. Analysis of the preparation by polyacrylamide gel electrophoresis indicated six distinguishable proteins present in the concentrate. Further attempts at purification resulted in a total loss of activity. A typical purification of SAM:DMPA *O*-methyltransferase is summarized in Table 1. The partially purified enzyme was stable for at least 3 months when stored in the freezer (-10 to -20 C).

## RESULTS

**Requirements for methyltransferase activity.** The methylating activity was monitored by the transfer of the radioactive methyl group from SAM to DMPA. Labeled MPA was identified by radioautography as the only solvent-extractable radioactive product formed during the reaction. Close agreement was found in the amount of radioactivity recovered in the ethylacetate and the radioactive MPA recovered from thin-layer plates.

The requirements for SAM:DMPA *O*-methyltransferase activity were investigated by deleting constituents from the standard reaction mixture and supplementing with possible po-

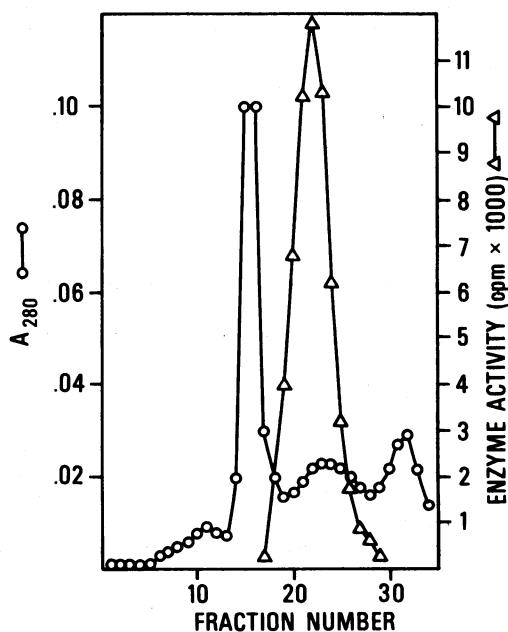


FIG. 2. Elution profile of SAM:DMPA *O*-methyltransferase from Sephadex G-200 column.

TABLE 1. Purification of SAM:DMPA O-methyltransferase from *Penicillium stoloniferum*

Procedure	Vol (ml)	Enzyme concn (U/ml)	Total act (U)	Protein (mg/ml)	Sp act (U/mg of protein)	Purification (fold)
Cell lysate	1,200	4.48	5,380	29.6	0.15	
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (50 to 100%)	56	3.69	210	11.8	0.31	2
After DEAE-cellulose	19	294	5,590	3.4	85.7	570
After Sephadex G-200	14	26.9	380	0.066	408	2,717

tentiators (Table 2). When either the enzyme preparation or DMPA was deleted from the reaction mixture, the methyltransferase activity was reduced by 98 and 93%, respectively. Addition of 0.986  $\mu\text{mol}$  of  $\text{Mg}^{2+}$  to the reaction mixture inhibited the activity by 52%. Further addition of  $\text{Mg}^{2+}$  only slightly affected the formation of labeled MPA. In preliminary experiments DMPA was dissolved in ethanol which was then added to the reaction mixture. Ethanol markedly inhibited the incorporation of label. For example, addition of 20 and 40  $\mu\text{l}$  of ethanol to the reaction mixture reduced the activity by 37 and 81%, respectively.

**Effect of pH.** The effect of pH on the transferase was investigated from pH 5 to 9 (Fig. 3). The standard reaction mixtures were buffered with citric acid buffer at pH 5 to 6, Tricine buffer at pH 6.5 to 8, and Tris buffer at pH 8 to 9, all at a final concentration of 0.07 M. The enzyme possessed a sharp pH optimum at 7.5 with half-maximal activities at pH 7.1 and 8.0.

**Effect of temperature.** The enzyme was active at temperatures ranging from 10 to 45 C (Fig. 4). The optimal activity was observed at a temperature of 27 to 28 C with half-maximal activities at 15 and 39 C. The enzyme had a  $Q_{10}$  of 2.3.

**Temperature inactivation.** The heat inactivation of the methyltransferase was investigated by exposing the buffered enzyme preparation at temperatures ranging from 23 to 45 C for 15 min and then assaying the enzyme at 23 C (Fig. 5). The enzyme was rapidly inactivated when exposed to temperatures greater than 33 C. A maximal activity was observed after exposure to 33 C and half-maximal activity after treatment at 37.5 C.

**Kinetics of methyltransferase.** The kinetics with DMPA are shown in a double reciprocal plot (Fig. 6). The methyltransferase has an apparent  $K_m$  of  $3.1 \times 10^{-6}$  M for DMPA and a  $V_{max}$  of  $6.8 \times 10^{-6}$  M. The plot shows no evidence of sigmoid kinetics.

TABLE 2. Influence of the constituents of the reaction mixture on SAM:DMPA O-methyltransferase

Reaction constituents	Activity (pmol/h per 200 $\mu\text{l}$ )
Complete	11.8
Minus enzyme	0.2
Minus DMPA	0.8
Plus 0.986 $\mu\text{mol}$ of $\text{MgCl}_2 \cdot 7\text{H}_2\text{O}$	5.6
Plus 1.972 $\mu\text{mol}$ of $\text{MgCl}_2 \cdot 7\text{H}_2\text{O}$	5.2
Plus 20 $\mu\text{l}$ of ethanol	7.4
Plus 40 $\mu\text{l}$ of ethanol	2.3

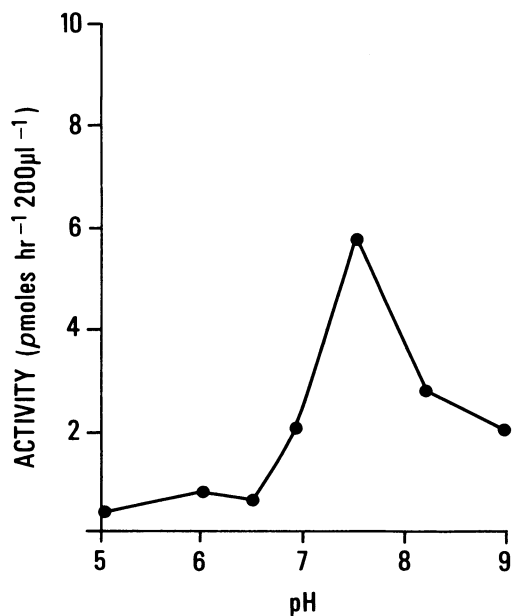


FIG. 3. Effect of pH on SAM:DMPA O-methyltransferase.

**Inhibition of methylating activity.** The products of SAM:DMPA O-methyltransferase were examined as possible feedback inhibitors of the reaction. These data are summarized in

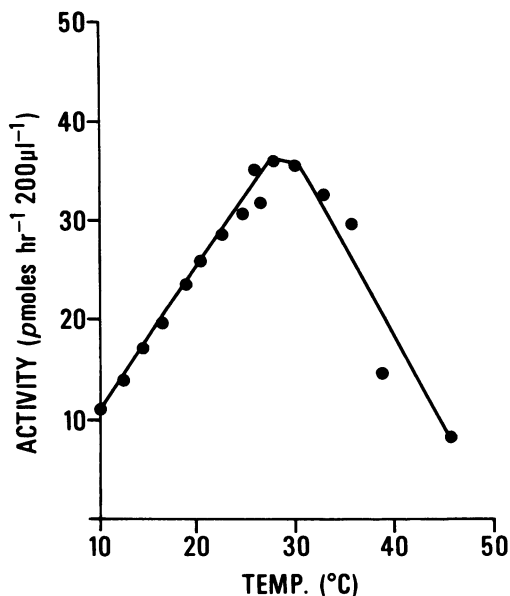


FIG. 4. Effect of temperature on SAM:DMPA O-methyltransferase.

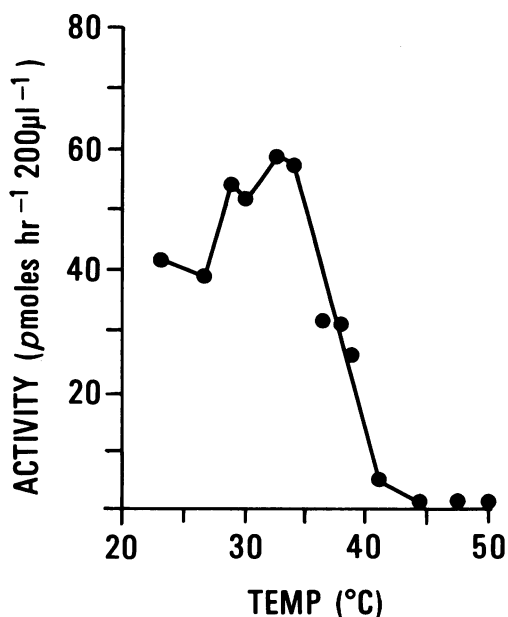


FIG. 5. Inactivation of SAM:DMPA O-methyltransferase by exposure (15 min) to elevated temperatures.

Table 3. S-adenosyl-L-homocysteine (SAHC), DL-homocystine, and mycophenolic acid all inhibited the methylating enzyme. Betaine, an alternative methyl donor, did not inhibit the methyltransferase. The end product inhibition

was investigated further to resolve the nature of the inhibition. The kinetic data are shown in Fig. 7. Both end products show competitive kinetics.

**Methyltransferase and synthesis of MPA.**

In an effort to determine the relationship between the methyltransferase and the accumulation of MPA, both parameters were monitored during the course of the fermentation (Fig. 8). The specific enzyme activity peaked at about 20 h, which is prior to the phase of rapid MPA accumulation. Methyltransferase activity steadily dropped after 20 h to a basal level which was maintained between 45 to 60 h. The

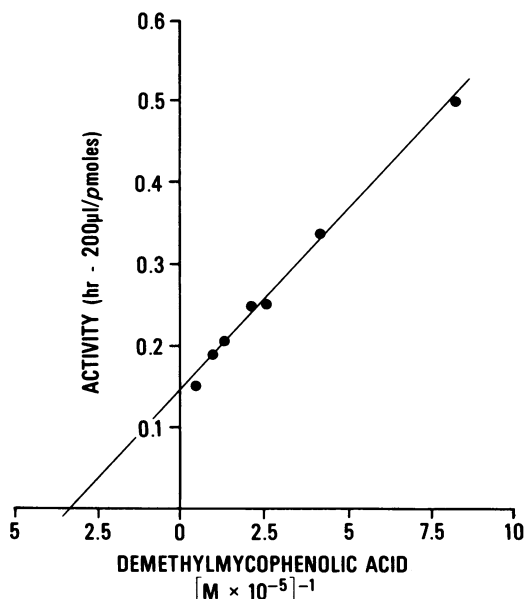


FIG. 6. Lineweaver-Burk plot for DMPA.

TABLE 3. Inhibition of SAM:DMPA O-methyltransferase

Inhibitor	Concn (mM)	Activity (pmol/h per 200 µl)	% Inhibition
None		19.2	0
DL-homocystine	5	11.2	42
	10	1.5	92
Betaine	5	22.2	0
	10	20.1	0
SAHC <sup>a</sup>	5	0.5	97
	10	0.5	97
Mycophenolic acid	6.2	13.9	28
	12.4	12.3	36
	24.8	6.2	68

<sup>a</sup> SAHC, S-adenosyl-L-homocysteine.

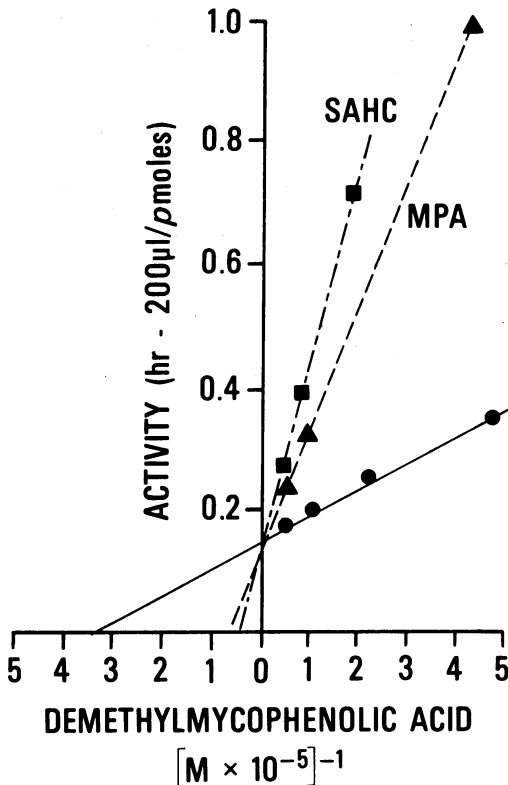


FIG. 7. Kinetic analysis (Lineweaver-Burk plot) of MPA and SAHC inhibition of SAM:DMPA *O*-methyltransferase. The SAHC and MPA were added at final concentrations of 0.5 mM and 0.62 mM, respectively, while SAM was at 17.5  $\mu$ M.

MPA accumulation mimicked the growth of *P. stoloniferum* until 50 h when growth ceased and accumulation of MPA continued.

### DISCUSSION

SAM:DMPA *O*-methyltransferase is the first enzyme in the mycophenolic acid biosynthetic pathway to be purified and partially characterized. One of the cosubstrates, DMPA, appears to be novel, though other possible substrates for the enzyme were not tested due to the difficulty in obtaining them. One possible substrate that should be examined is 6-farnesyl-5,7-dihydroxy-4-methyl phthalide (Fig. 1, compound III), since the order of the reactions in this portion of the sequence has not been definitely established.

The purification of the transferase led to a 2,700-fold increase in specific activity. The recovery of 7% of the original activity might have been higher if the Sephadex G-200 step had been completed in the cold. However, our aim was not primarily to produce high yields of

enzyme. The low total activity recovered from the ammonium sulfate fractionation might be due to the presence of an inhibitor which was subsequently removed during DEAE-cellulose chromatography.

The formation of labeled MPA was dependent on the presence of DMPA and the enzyme in the reaction mixture. The methylating enzyme was inhibited by both ethanol and  $Mg^{2+}$ . In contrast, catechol *O*-methyltransferase from various mammalian tissues has an absolute requirement for divalent cations (3). The potentiation with divalent cations, however, was not observed with the puromycin *O*-methyltransferase from *Streptomyces alboniger* (23). The response to divalent cations may represent a distinguishing feature of various *O*-methyltransferases.

Optimal SAM:DMPA *O*-methyltransferase activity was observed at pH 7.5 and temperature 27 to 28 C. Maximum accumulation of MPA was achieved at 28 C with the *P. stoloniferum* strain used in this study. Other phenolic compounds accumulate at lower temperatures possibly due to reduced methyltransferase activities (unpublished data). The enzyme was rapidly inactivated when exposed to a temperature greater than 33 C for 15 min, which is above the maximum growth temperature of *P. stoloniferum*. Some difficulties were encountered with the temperature studies because SAM is degraded to a solvent-extractable metabolite when exposed to temperatures greater than 45 C. This degradation is further accentuated at a pH of 8 and above. Similar

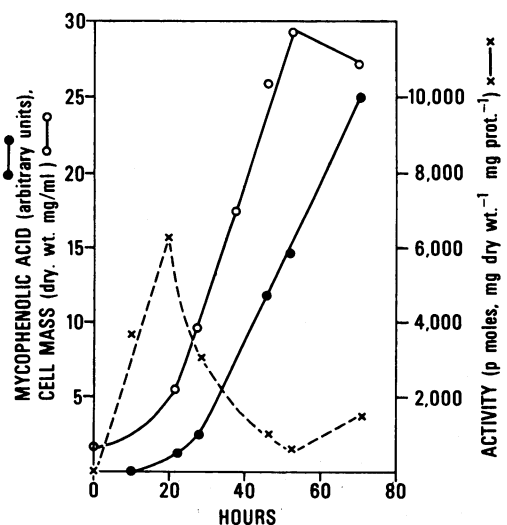


FIG. 8. Relative activity of SAM:DMPA *O*-methyltransferase during the MPA fermentation.

solvent-extractable radioactive degradation products of SAM were observed by Sankaran and Pogell (25).

SAM:DMPA *O*-methyltransferase has an apparent  $K_m$  of  $3.1 \times 10^{-6}$  M for DMPA which would suggest a strong specificity for the substrate. Since radioactive DMPA was not available, kinetic analysis of the cosubstrate, SAM, was not pursued.

The enzyme is competitively inhibited by both end products, SAHC and MPA. It is doubtful whether levels of SAHC ever become high enough to influence the activity of the transferase. On the other hand, there is strong inhibition of MPA biosynthesis in the presence of MPA (unpublished data). Whether the phenomenon is true feedback inhibition or repression is not known.

Methyltransferase achieved a peak activity very early in the fermentation and dropped to a basal level at about 45 h. The maximal level occurred prior to rapid accumulation of MPA. The reduced methyltransferase activity after 25 h is not due to inhibition by carry-over of MPA from the fermentation liquid, since cells were thoroughly washed before preparing cell lysates and intracellular pools are negligible. The limited activity of the SAM:DMPA *O*-methyltransferase during the latter part of the fermentation may account for accumulation of a number of other phenolic compounds (unpublished data). Demethyl precursors of both novobiocin and tetracycline are reported to accumulate under certain culture conditions (18, 20). These demethyl precursors and the presence of SAM:DMPA *O*-methyltransferase and puromycin *O*-methyltransferase in antibiotic-producing cultures support the proposed role of methyltransferases as a terminal step in the biosynthesis of several antibiotics.

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