ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1975, p. 721-732
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Vol. 8, No. 6
Printed in U.S.A.

Biosynthesis of Puromycin in *Streptomyces alboniger*: Regulation and Properties of *O*-Demethylpuromycin *O*-Methyltransferase

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Received for publication 29 July 1975

Mechanisms for regulation of puromycin biosynthesis in *Streptomyces alboniger* were studied by measuring the levels of *S*-adenosyl-L-methionine:*O*-demethylpuromycin *O*-methyltransferase. The enzyme was released in soluble form from mycelia by 3 to 5 min of sonication at 4 C. Maximal specific activities of 0.7 and 0.1 nmol/min per mg of protein were found in cells grown in corn steep liquor-corn starch and Hickey-Tresner media, respectively. In both media, the *O*-methyltransferase activity rose from low levels to a maximum during mid-logarithmic growth and then declined or disappeared completely (in Hickey-Tresner medium) during stationary phase. Either glucose (1%) or ethidium bromide (5 μM) reduced *O*-methyltransferase formation to very low levels with no effect on overall growth. Complete glucose repression of antibiotic formation occurred on agar. Cells grown in the presence of ethidium bromide continued to produce low enzyme levels after regrowth in the absence of dye, but formed normal amounts of puromycin on Hickey-Tresner agar. The *O*-methyltransferase, either crude or purified, was rapidly inactivated at 37 C. Each substrate alone, or both together at lower concentrations, protected against this loss of activity. Puromycin inhibited the transferase. Regulation of *O*-methyltransferase synthesis in *S. alboniger* includes (i) induction early in growth that is susceptible to catabolite repression and differential inhibition by ethidium bromide, and (ii) protection of the enzyme from inactivation by increased intracellular levels of its substrates. The *O*-methyltransferase was purified 30- to 40-fold by a combination of protamine sulfate precipitation, ammonium sulfate fractionation, adsorption and gradient salt elution from diethylaminoethyl-cellulose and Sephadex G-200 gel filtration. The enzyme was very unstable, even at low temperatures, upon purification beyond the salt fractionation step, but was stabilized by the addition of *S*-adenosyl-L-methionine during later stages of purification.

Streptomyces alboniger produces puromycin, an antitumor agent and protein synthesis inhibitor (29). A specific enzyme which catalyzes the transfer of methyl groups from *S*-adenosyl-L-methionine (SAM) to *O*-demethylpuromycin (ODMP) has been found in soluble extracts of *S. alboniger* (26) (SAM:ODMP *O*-methyltransferase). This reaction is presumed to be the terminal biosynthetic step in the formation of the antibiotic. ODMP has been synthesized chemically (26) and is also found in trace quantities, together with the other demethyl derivatives, in commerical puromycin samples (23).

We have followed the activity of the *O*-methyltransferase as a marker during the growth cycle of the organism under varied cultivation conditions to ascertain the regulatory mechanisms involved in puromycin biosynthesis. The peculiar time sequence of appearance and disappearance of the *O*-methyltransferase prompted us to study in detail the effects of glucose and intercalating dyes on enzyme formation and antibiotic production, and of substrates on the stability of the *O*-methyltransferase. Our results indicate that *O*-methyltransferase synthesis and puromycin production are susceptible to catabolite repression and that ethidium bromide inhibits formation of the transferase. We have also found that either substrate protects the *O*-methyltransferase against inactivation and have utilized this property to purify the enzyme to a greater degree than previously reported.

MATERIALS AND METHODS

Chemicals. ODMP was prepared chemically (26). SAM chloride, grade II (80% pure), azocasein, azoalbumin, bovine serum albumin, acridine orange, acri-

flavine, blue dextran, phenylmethanesulfonyl fluoride, sodium lauryl sulfate, Pronase, rabbit muscle lactic dehydrogenase (LDH) (type I), and ethylenediaminetetraacetate (EDTA) were purchased from Sigma Chemical Co.; ethidium bromide, grade B, and dithiothreitol were from Calbiochem; Tween 80 was from Fisher; 2-mercaptoethanol was from Eastman; and protamine sulfate and puromycin dihydrochloride were from Nutritional Biochemicals. Subtilisin was from Novo Industries, Copenhagen, Denmark; enzyme grade ammonium sulfate was from Schwarz/Mann; diethylaminoethyl (DEAE)-cellulose was from Whatman; Sephadex G-200 was from Pharmacia; and Diaflo PM30 membrane was from Amicon. [*Methyl*-^{3}H]SAM was from New England Nuclear, and [5-^{14}C]methyltetrahydrofolate (Ba salt) was from Amersham/Searle. Yeast extract, malt extract, nutrient broth, tryptic soy agar, and broth were from Difco Laboratories. All other chemicals were of analytical grade.

Distilled water was used for preparation of media and distilled, deionized water in all enzyme assays.

Organisms. *S. alboniger* strains, ATCC 12461 and 12462, were maintained on Hickey-Tresner (HT) agar slants (pH 7.3; 11). Cultures were incubated at 28 C until sporulation occurred and then stored at 4 C.

Staphylococcus aureus (ATCC 6538 P) was maintained on tryptic soy agar and stored at 4 C.

Media. Liquid media were HT (11), final pH of 7.3, 6% corn steep liquor-2% corn starch, pH 6.4 (CSL-CS), and Perlman (24). Agar media were: glucose-yeast extract-malt extract (25), nutrient broth-yeast extract supplemented with glycerol (19) or 1% glucose, and HT (11).

Media were autoclaved at 121 C for 15 min. Glucose was autoclaved separately and added aseptically.

Cultivation of S. alboniger. Spores from HT agar slants, suspended in sterile water or medium, or 0.2-ml stationary phase cell suspensions were inoculated into 25 ml of medium in 125-ml Erlenmeyer flasks and grown at 28 C for 2 days in a gyrotory shaker. Five milliliters of this culture was inoculated into 25 ml of fresh medium and grown for 2 additional days. The contents of these flasks served as inoculum for 500 ml of medium in 2-liter flasks. Two milliliters of 2.5% sterile Dow-Corning Antifoam suspension in water was added to the medium during growth. Two milliliters of a sterile 20% $CaCO_3$ suspension was regularly added to CSL-CS medium cultures at 0 and 24 h of growth. Cells (20 ml of culture) were harvested at different time intervals by centrifugation at 4 C, washed twice with equal volumes of water, and stored at −20 C.

Estimate of sporulation in cultures. Spores were easily visualized as gram-positive bodies attached to hyphae, which changed in staining characteristics from gram positive to gram negative as the extent of sporulation and age of the culture increased.

Separation of spores from hyphae. *S. alboniger* cells were suspended in 0.1% Tween 80 and vortexed for 1 to 2 min. This procedure lysed most of the hyphae and released the spores intact and viable.

Assay for antibiotic production on agar. Suitable volumes of *S. alboniger* cultures in stationary growth phase were spread on HT agar plates and incubated at 28 C for 2 days. Individual colonies were then transferred, spaced about 1 inch (2.54 cm) apart, to agar plates with sterile toothpicks and incubated at 28 C for 4 days. An agar plug (6-mm diameter) of each colony was then placed on tryptic soy agar plates (150 by 25 mm) seeded with *S. aureus*. The antibiotic was allowed to diffuse into the agar for 30 to 60 min at 4 C, the plates were incubated at 37 C overnight, and the zones of inhibition were measured. A filter disk (10-mm diameter) containing 8 μg of puromycin was used as standard.

Growth of S. alboniger on cellophane disks. Cellophane disks (150 mm in diameter), cut out of dialysis tubing and sterilized at 121 C for 15 min in water, were placed on the agar surface, and washed samples of cells from stationary phase cultures were inoculated onto the cellophane. Plates were incubated for 3 days at 28 C and the cells were collected by scraping.

Enzyme assays. *O*-methyltransferase assays were carried out as described earlier (27). [*Methyl*-^{3}H]SAM (80 μM), ODMP (1 mM), and enzyme were incubated in 0.1 M sodium phosphate, pH 7.5, at 37 C in a final volume of 20 μl. The reactions were stopped by the addition of 0.1 M sodium borate-5 M NaCl buffer (pH 9), and the labeled product was extracted directly into Spectrafluor. A unit of enzyme was defined as the amount which formed 1 nmol of product per min, and specific activity was defined as units per milligram of soluble protein.

Inactivation studies. Enzyme was incubated at 30 or 37 C in 0.1 M sodium phosphate (pH 7.5) without or with substrates in 15- to 100-μl volumes, and aliquots were assayed for enzyme activity.

Preparation of cell-free extracts. Frozen cell pellets were thawed; an approximate 10% (wt/vol) suspension in a known final volume of 0.1 M sodium phosphate (pH 7.5) was quantitatively transferred and disrupted in an ultrasonic disintegrator (Measuring and Scientific Equipment, Ltd., London, 60 W, 0.75-inch [ca. 1.91-cm] probe) for 3 to 5 min, with cooling between 1-min bursts. The cell suspension was cooled by circulating ice water during sonication.

Growth was estimated from the amount of total protein in a cell sonic extract and expressed as milligrams per milliliter of culture. For enzyme assays and determination of soluble protein, samples were centrifuged at 32,000 × *g* for 30 min at 4 C to remove cell debris and other particulate matter. Protein was determined by the procedure of Lowry et al. (17) with bovine serum albumin as standard.

Purification of O-methyltransferase. Our original studies on *O*-methyltransferase were carried out in extracts from a mutant strain of *S. alboniger*, ATCC12462 (26). Higher specific activities could be obtained with the wild-type organism, ATCC12461, from CSL-CS medium by harvesting cells during mid- or late-logarithmic growth (48 to 72 h) and shortening the time of sonication.

All purification procedures were carried out at 0 to 4 C. Centrifugations were usually for 15 min at 36,000 × *g*. Results of a typical purification are summarized in Table 1.

Step 1: Removal of nucleic acids and salt frac-

TABLE 1. *Purification of O-methyltransferase*

Fraction	Volume (ml)	Units	Protein (mg)	Recovery (%)	Specific activity (U/mg)
A. Crude extract	15.9	43.0	76.3	100	0.56
B. Protamine sulfate supernatant	17.1	40.6	67.5	94	0.60
C. Ammonium sulfate precipitate, (45–70%)	1.3	25.2	23.6	59	1.1
D. DEAE-cellulose column I, fractions 66–84	38	17.5		41	
E. Amicon 12 concentrate	1.04	12.9		30	
F. DEAE-cellulose column II, Amicon 12 concentrate	0.82	7.5	1.53	17	4.9
G. Sephadex G-200, Amicon 12 concentrate	1.4	7.1	0.43	17	16.5

tionation. Cell-free supernatants with specific activity >0.5 were pooled and used as the starting material (fraction A). EDTA was added to a final concentration of 1 mM. Nucleic acids were removed by the dropwise addition of a 2% solution of protamine sulfate (pH 7) (0.11 ml per ml of extract). The turbid solution was stirred for 30 min and centrifuged to remove the precipitate. To this supernatant (fraction B), solid ammonium sulfate was gradually added to 45% saturation (0.277 g/ml). The precipitate was removed by centrifugation and the concentration of ammonium sulfate was increased to 70% saturation by the addition of more salt (0.171 g/ml). This precipitate, which contained most of the enzyme activity, was collected by centrifugation and dissolved in 1.3 ml of 5 mM sodium phosphate-0.1 mM EDTA-10 μM SAM, pH 7.5 (PES) (fraction C). (Upon further purification, the *O*-methyltransferase was found to be unstable in dilute solution. Inclusion of 10 μM SAM in all solutions used for column chromatography prevented this inactivation [see below].)

Step 2: DEAE-cellulose chromatography. Fraction C was placed on a column of DEAE-cellulose (Whatman DE-52, 5 g, 0.9 by 13 cm) previously equilibrated with PES buffer. The column was washed with PES and eluted with a concave salt gradient at a flow rate of 16 ml/h. A Technicon 1-liter capacity Autograd was used to form the gradient. The first two chambers each contained 70 ml of 0.1 M NaCl in PES and the third chamber contained 70 ml of 0.6 M NaCl in PES. Fractions of 2 ml were collected and assayed for enzyme activity. The methyltransferase appeared as one symmetrical peak in fractions 66–84 (fraction D; Fig. 1). This material was pooled, concentrated in an Amicon 12 ultrafiltration unit with a PM 30 membrane, and washed and concentrated twice with 9-ml volumes of PES (fraction E).

The DEAE-cellulose chromatography procedure was repeated as described above with fraction E. Pooled fractions 58–76 were concentrated as above (fraction F; Fig. 1).

Step 3: Sephadex G-200 gel filtration. Fraction F was placed on a Sephadex G-200 column (2.5 by 40 cm) equilibrated with PES and eluted at a flow rate of 15 ml/h with PES. Two-milliliter fractions were collected, and the methyltransferase was eluted as

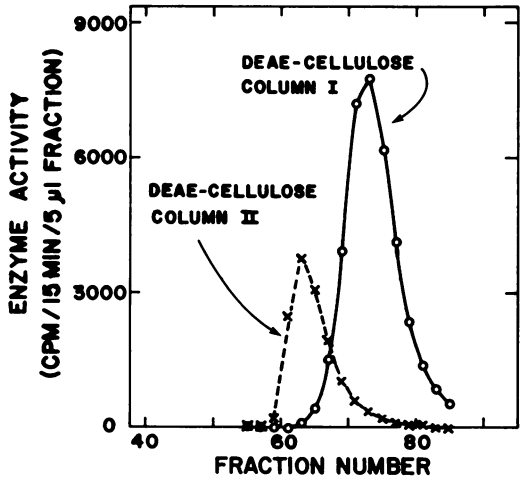


FIG. 1. *DEAE-cellulose chromatography of methyltransferase. See text for details.*

one peak in fractions 46–55 (Fig. 2). This material was pooled, concentrated by ultrafiltration as above, and stored at −65 C (fraction G; specific activity, 16.5).

In another preparation, where Sephadex gel filtration was carried out before DEAE-cellulose chromatography, the final specific activity was 26.

From these results and sucrose gradients run without or with 10 μM SAM, the molecular weight appears to be closer to that of the LDH marker (150,000) (Fig. 2) than the lower estimate of 68,000 previously reported (26).

RESULTS

Effect of time of sonication on the release of O-methyltransferase activity and protein. A systematic study of the release of enzyme from *S. alboniger* cells as a function of time of sonication showed that maximal enzyme activity was present in soluble form within 5 min (Fig. 3). The *O*-methyltransferase was slowly inactivated with longer periods of sonication,

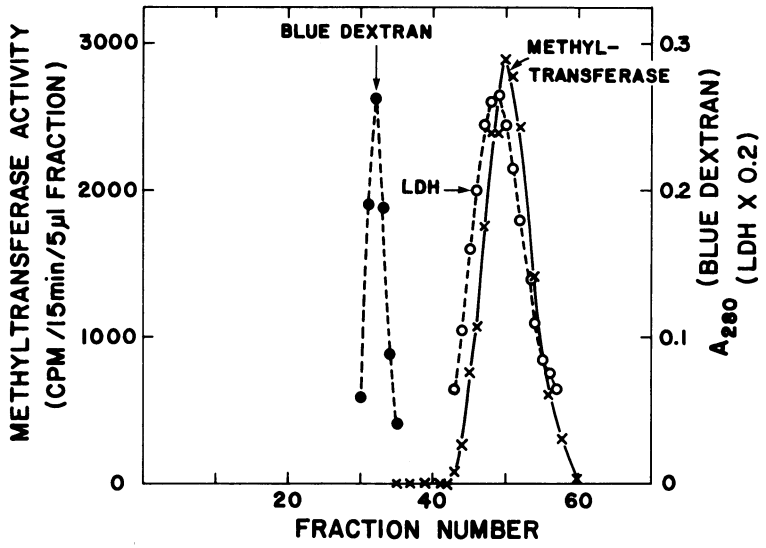


FIG. 2. *Sephadex G-200 gel filtration of methyltransferase. See text for details. Blue dextran, 3 mg in 0.5 ml of phosphate-EDTA buffer; rabbit muscle LDH, 10 mg in 0.4 ml of PES. For these markers, fractions from the column were passed through an LKB 8300 Uvicord II photometer coupled to a Fisher concentration computer (model CDR). Absorbancy values at 280 nm (A_{280}) at the termination of collection of each fraction were recorded on a Fisher printer (model DG).*

although more protein was solubilized. Routinely, samples of cells were disrupted by sonication for 3 to 5 min, with cooling between 1-min bursts.

Growth and O-methyltransferase yield in liquid media. In CSL-CS medium, the *O*-methyltransferase activity was low during the early stages of growth, then increased rapidly during the mid- or late-logarithmic stages, and again declined to lower levels in the stationary phase (Fig. 4a). Similar results were obtained in HT medium, designed to enhance sporulation in *Streptomyces* (11). The formation of spores was qualitatively estimated in liquid media by Gram staining (see above). Spores started appearing in HT medium at 48 h and continued to increase in number with further growth. The change in enzyme specific activity followed a bell-shaped curve with time, and the enzyme activity completely disappeared after a high degree of sporulation had occurred (Fig. 4b). In agreement with these observations, methyltransferase activity could not be detected in sonic extracts of spores obtained from HT agar. The enzyme specific activity varied with the two strains tested, and both enzyme levels and growth yield depended on the medium used for cultivation of cells. There was no obvious relationship between growth and enzyme activity. Thus, in three media tested, growth decreased in order from CSL-CS > Perlman > HT medium, whereas the enzyme specific activity was maximum in cells grown in CSL-CS followed by HT medium, and lowest in Perlman medium. About a 4- to 10-fold difference in maximal specific activity was observed for strain 12461 in CSL-CS and HT media and about a 20-fold difference between CSL-CS and Perlman media. Even with the same strain, a twofold variation in enzyme specific activity with the same medium was not uncommon in different experiments.

We were particularly intrigued by the unusual nature of *O*-methyltransferase formation in HT medium (Fig. 4b): the rapid rise in activity during log-phase growth, the complete disappearance during stationary phase and sporulation, and the apparent symmetry of the process. These observations suggested a different control of enzyme formation than could be explained by classical mechanisms of regulation through enzyme induction and catabolite repression.

Glucose repression of O-methyltransferase induction. When cells from strain 12461 were grown on cellophane disks placed over glucose-yeast extract-malt extract agar, no antibiotic was detectable in the underlying agar, and the *O*-methyltransferase activity of the cells was very low (Table 2, experiment 1). These observations suggested the possibility of catabolite repression of both puromycin and enzyme production and prompted us to test the effect of glucose addition on these processes in

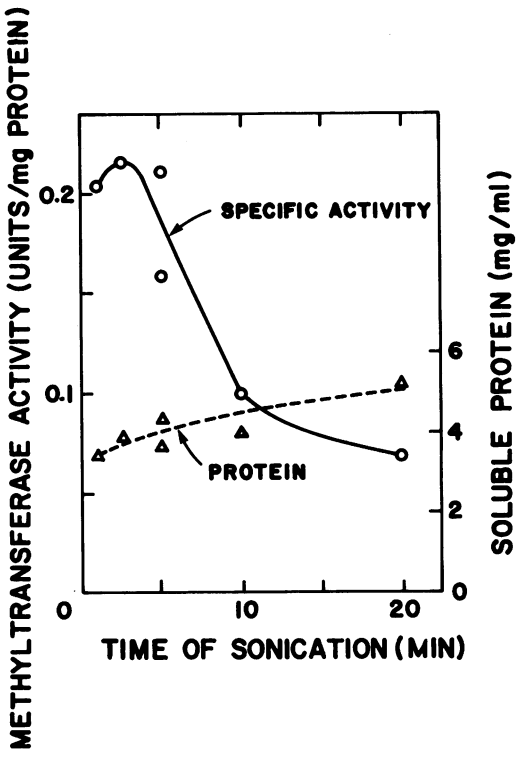


FIG. 3. *Effect of time of sonication on release of O-methyltransferase activity and protein. S. alboniger cells (strain 12462) were suspended in 0.1 M sodium phosphate (pH 7.5) (10% wt/vol), disrupted by sonication for varying time periods and centrifuged, and enzyme activity and protein were determined in the supernatants. Details are described under Materials and Methods.*

other media. The addition of 1% glucose to HT agar completely prevented the formation of antibiotic (Table 2, experiment 2). Also, in nutrient broth-yeast extract agar, puromycin was produced by both strains of *S. alboniger* in the presence of glycerol but not with glucose as added carbon source (Table 2, experiment 3).

The major source of carbohydrate in CSL-CS and HT media are complex polysaccharides, and the rapid rise in enzyme activity during logarithmic growth could have been caused by a release from catabolite repression. In liquid culture, the presence of glucose markedly suppressed *O*-methyltransferase formation in HT (Fig. 5a) and CSL-CS media (28). Glucose had no effect on the growth rate of strain 12461 in either medium, and the cells sporulated normally in HT medium containing 1% glucose. The low level of methyltransferase found in Perlman synthetic medium (with glucose as carbon source; 24) may also be attributed to catabolite repression. When 1% glucose was added to *S. alboniger* cells after growth for 30 h in CSL-CS medium, further *O*-methyltransferase synthesis was stopped and the already induced enzyme activity decayed with time (Fig. 5b). Glucose was not detectable in the medium 18 h after addition.

Complete repression by glucose of puromycin production on agar was found in buffered HT agar over the pH range 6.0 to 7.5 and therefore was not simply caused by organic acid production and a lowering of pH (10). Also, in all of the liquid studies, $CaCO_3$ was added to cells growing in CSL-CS and HT containing 1% glucose and was found to maintain the pH above 7.

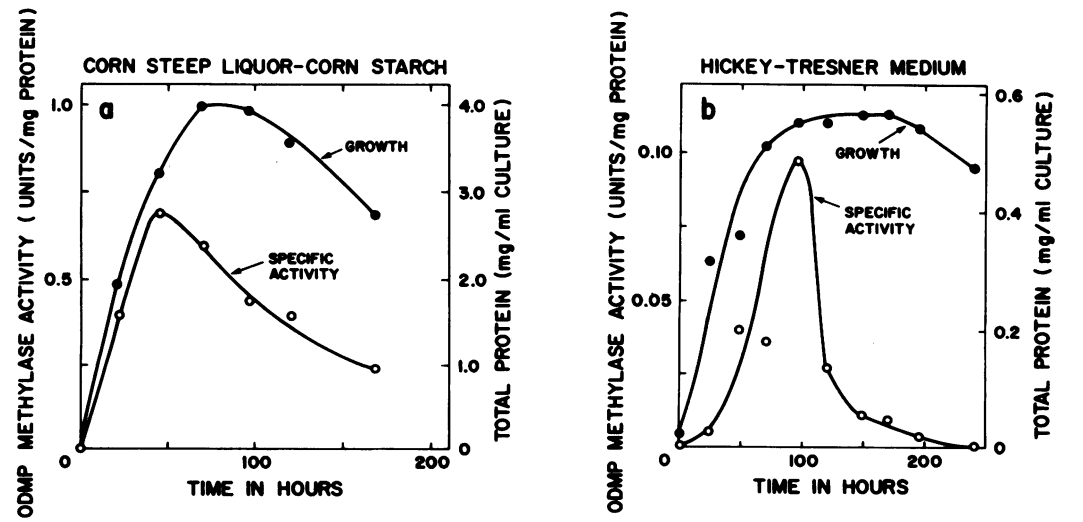


FIG. 4. *Changes in O-methyltransferase activity during growth of S. alboniger strain 12461.*

TABLE 2. *Glucose repression of puromycin and O-methyltransferase formation on agar*

Expt	Cells	Agar medium	Mean zone of inhibition (mm) ±SD[a]		Enzyme specific activity (U/mg of protein)
			Control	Plus 1% glucose	
1.	Control[b]	Glucose-yeast extract-malt extract (3 days)	0[c]		0.002[c]
	Grown in 15 μM EB[d]	Glucose-yeast extract-malt extract (3 days)	0[c]		0.001[c]
2.	Control[b,e]	HT (4 days)	11 ± 1	0	0.119[c]
	Grown in 15 μM EB[d,e]	HT (4 days)	11 ± 1	0	0.018[c]
3.	Control (strain 12461)[e]	Glycerol-nutrient broth-yeast extract (4 days)	9 ± 1	0[f]	
	Control (strain 12462)[e]	Glycerol-nutrient broth-yeast extract (4 days)	8 ± 1	0[f]	

[a] SD, Standard deviation.
[b] *S. alboniger* strain 12461 previously grown in HT medium.
[c] Cells grown on cellophane. Agar plugs taken for antibiotic assay after removing cells.
[d] Strain 12461 grown in HT plus 15 μM EB.
[e] Cells vortexed in 0.1% Tween 80 before plating for antibiotic assay.
[f] Glucose added in place of glycerol.

Inhibition of O-methyltransferase induction by EB. Among the early suggestive evidence for the existence of extrachromosomal deoxyribonucleic acid (DNA) in streptomycetes were reports by Gregory and Huang (9) and Okanishi et al. (22) that treatment with acridine dyes resulted in the elimination of tyrosinase formation in *Streptomyces scabies* and the "curing" of both antibiotic and aerial mycelia production in *Streptomyces kasugaensis*. When the effects of the intercalating dye, ethidium bromide (EB), were tested on *S. alboniger* grown on HT (Fig. 6a) or CSL-CS medium (28), enzyme induction was specifically blocked under subinhibitory conditions of growth. Cells grown in the presence of EB and their sonicated supernatants were pink colored in contrast to the pale yellow color of normal cells, indicating that a significant amount of EB was bound to the cells. However, the presence of an enzyme inhibitor in the EB cell sonic extracts was ruled out by mixing experiments; and, even at 50 μM EB, no significant inhibition of *O*-methyltransferase was found (Table 3). Increasing levels of EB further lowered the maximal *O*-methyltransferase specific activity attained during a growth cycle (Fig. 6b). Acriflavine (3 μg/ml) showed a similar inhibitory effect (to 19% of maximal control specific activity), whereas 10 μM acridine orange did not significantly change the level of enzyme induction (85% of control).

Our attempts to clarify the mechanism of EB inhibition of *O*-methyltransferase formation, namely, either (i) a "curing" of postulated plasmids involved in production of the enzyme or (ii) a differential inhibition of induction of a catabolite-susceptible enzyme, have led to somewhat ambiguous results. Because of the similarity of repression by glucose and EB, we first extended these studies with *Escherichia coli* and have shown unambiguously that intercalating dyes can differentially inhibit the induction of catabolite-susceptible enzymes under conditions where growth is not affected (28). The most likely explanation is an increased binding of dye to specific promoter regions and thus an inhibition of initiation of transcription.

If the decrease in enzyme activity in *S. alboniger* cells exposed to EB was the result of permanent damage to extrachromosomal DNA similar to that seen in petite mutagenesis in yeast or the elimination of plasmids in many procaryotes, then cells exposed to EB and regrown in the absence of the dye should have had very low or no enzyme activity. On the other hand, if only inhibition of chromosomal gene expression was occurring, then regrowth of cells in the absence of EB should have resulted in full recovery of ability to form *O*-methyltransferase. Thus, with *E. coli*, a complete recovery of enzyme inducibility occurs after removal of the intercalating dyes. As may be seen in Fig. 6a and Table 4, *S. alboniger* cells exposed to a growth cycle in EB did have significantly lower *O*-methyltransferase than cells grown in its ab-

sence. In all of these experiments *S. alboniger* cells were first passed through at least two 2-day cycles of growth in the complete absence of dye. It is to be noted that the maximum growth reached was consistently higher in EB-grown cells than their controls. Also, EB cells grown on cellophane placed above HT agar had 15% of the activity found with control cells (Table 2, experiment 2). The maximum recovery of enzyme activity (84%) was found in EB-grown cells passed through HT agar and then grown in CSL-CS medium as described above.

The amounts of puromycin formed on agar were only slightly lower with *S. alboniger* colonies grown in the presence of EB on agar or with those previously grown in liquid culture with dye, even when individual spores were first obtained by treatment with sodium lauryl sulfate or Tween 80 before plating.

Properties of the enzyme. (i) Substrate specificity. The enzymatic methylation reaction is very specific for ODMP as methyl acceptor (26), and so it was of interest to test the specificity of SAM as methyl donor. No incorporation of radioactivity into puromycin was found with [5-^{14}C]methyltetrahydrofolate as substrate.

(ii) Product inhibition. Puromycin inhibited the purified *O*-methyltransferase, and a sigmoidal curve was obtained (Fig. 7).

(iii) Enzyme inactivation and substrate protection. The rapid loss of *O*-methyltransferase activity during later growth stages and sporulation suggested that a mechanism existed in vivo for inactivation of enzyme. Incubation of crude extracts of *S. alboniger* at higher temperatures resulted in loss of activity, much more rapidly at 37 than at 30 C (Table 5). As is illustrated in Fig. 8, crude supernatant, first dialyzed overnight against 0.1 mM EDTA (pH 7.5), rapidly lost activity at 37 C. The addition of either SAM (80 μM) or ODMP (1 mM) during this incubation period resulted in almost full protection against inactivation. The presence of both substrates appeared to offer even greater protection, and this point was confirmed using lower concentrations of each compound (Table 6). Almost full protection was provided when both substrates were present at their K_m values (10 μM SAM, 0.21 mM ODMP [26]). Other general stabilizers of enzyme activity including thiols (2-mercaptoethanol, dithiothreitol), bovine serum albumin, and EDTA offered no protective effect at 37 C. Also, enzyme activity, once lost, was not recovered by further incubation with either thiol compound.

Inactivation of the *O*-methyltransferase was not due to proteolytic degradation. Although many proteases have been characterized in culture media from *Streptomyces* (21), we could find no evidence for proteolytic activity in crude sonicates. No hydrolysis of azoalbumin (31), casein (21), or azocasein (2) was found nor any increase in trichloroacetic acid-soluble absorbancy material (280 nm) after incubation of the whole sonic extract. Also, phenylmethanesulfonyl fluoride, a serine protease inhibitor (5), did not prevent loss of enzyme activity at 0.1 and 1 mM levels.

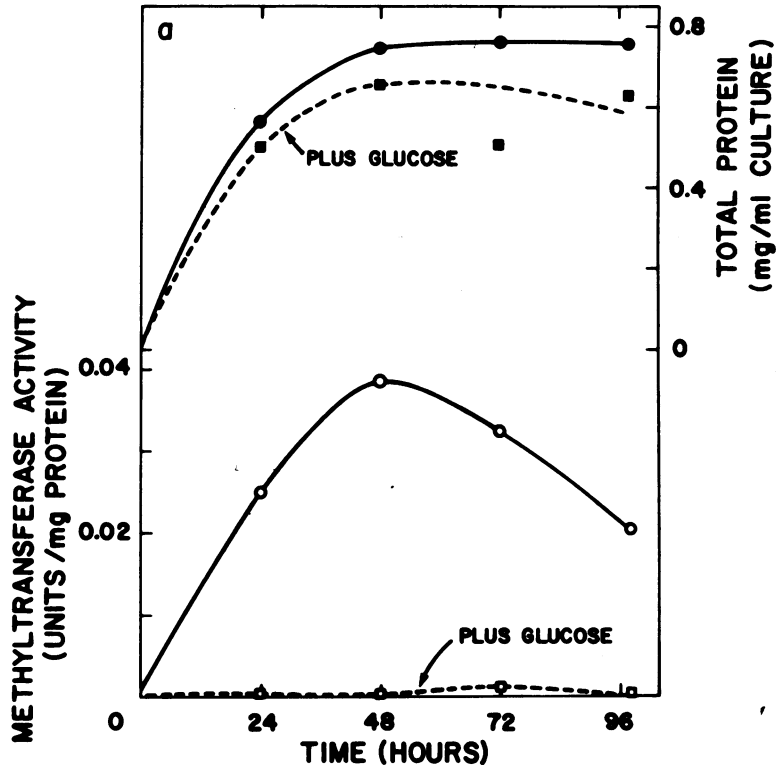
As further evidence that proteolytics were not involved in the inactivation, we tested the stability of crude and purified enzyme (fraction F) under identical conditions in the absence and presence of 80 μM SAM (Table 5). The amount of inactivation and protection by SAM were essentially the same, indicating that no inactivating protein had been removed during the purification. However, the presence of SAM did protect the *O*-methyltransferase against inactivation by Pronase and, to a lesser extent, by subtilisin (Table 7).

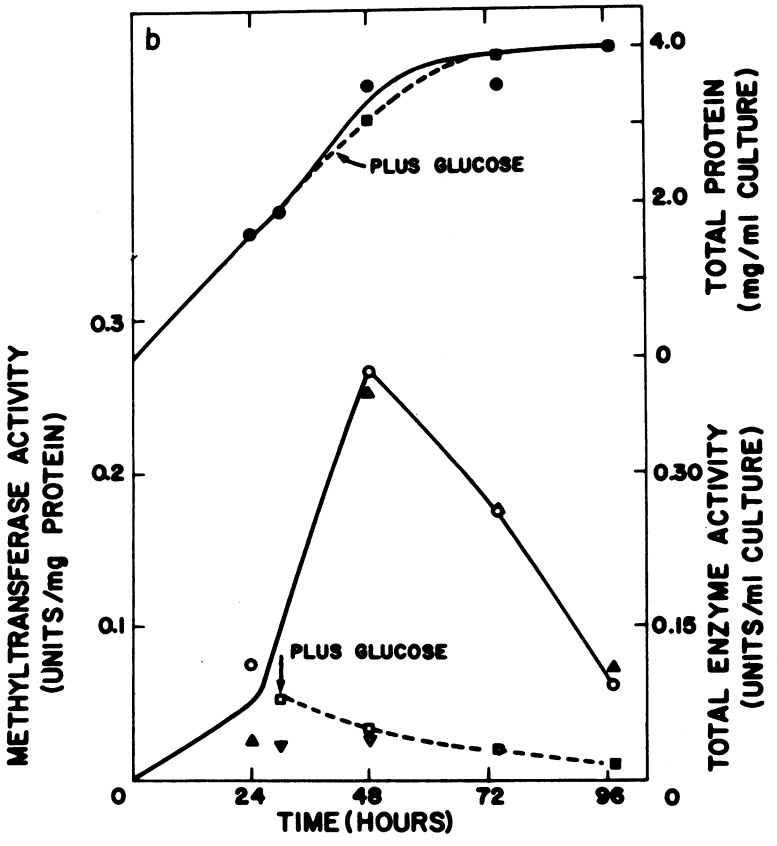
Advantage was taken of stabilization of activity by SAM during purification of the *O*-methyltransferase. Enzyme purified through the ammonium sulfate precipitation step was completely stable when stored at −20 C. However, further purification or dialysis of the enzyme resulted in loss of activity. Thus, dilute, purified fractions of enzyme eluted from DEAE-cellulose or Sephadex G-200 columns lost most of their activity upon freezing and thawing. Including 10 μM SAM in buffers used for elution and concentration of the dilute purified fraction by membrane ultrafiltration protected the enzyme from inactivation and resulted in very high recoveries of activity.

DISCUSSION

The regulatory model that comes out of the present studies to explain the appearance and disappearance of the *O*-methyltransferase may be summarized as follows.

Induction and catabolite repression. In *S. alboniger*, the specific inducer for *O*-methyltransferase synthesis is not known. During the early phase of growth, the formation or accumulation of a specific metabolic product probably initiates the transcription and translation necessary for *O*-methyltransferase accumulation. An attractive possibility for the inducer is an adenine derivative, which could also be a precursor for the antibiotic. It has been reported that addition of uric acid, adenine, or other purine bases to puromycin fermentations increases yield by as much as 160% (30). In our


a
PLUS GLUCOSE
PLUS GLUCOSE
METHYLTRANSFERASE ACTIVITY
(UNITS/mg PROTEIN)
TOTAL PROTEIN
(mg/ml CULTURE)
0.04
0.02
0
0.8
0.4
0
24
48
72
96
TIME (HOURS)


b
PLUS GLUCOSE
PLUS GLUCOSE
METHYLTRANSFERASE ACTIVITY
(UNITS/mg PROTEIN)
TOTAL PROTEIN
(mg/ml CULTURE)
TOTAL ENZYME ACTIVITY
(UNITS/ml CULTURE)
0.3
0.2
0.1
4.0
2.0
0
0.30
0.15
0
24
48
72
96
TIME (HOURS)

laboratory (P. A. Redshaw and P. A. McCann, unpublished data), it has been confirmed that adenine and, to a lesser extent, adenosine increase yields of antibiotic on HT agar. The pattern of appearance of the O-methyltransferase during growth resembles that found for novobiocic acid synthetase in *Streptomyces niveus* (16) and phenoxazinone synthase in *Streptomyces antibioticus* (7).

The induction of O-methyltransferase and its further synthesis, and puromycin biosynthesis as well, are completely blocked by the addition of glucose in what appears to be a classical example of catabolite repression. However, a role for cyclic adenosine 3',5'-monophosphate in any *Streptomyces* has yet to be established. The high yield of enzyme and antibiotic obtained on CSL-CS probably is caused in part by the low steady-state level of glucose using corn starch as the major source of carbohydrate. This behavior is in contrast to the inhibition of aerial hyphae formation by glucose in *Streptomyces* (Redshaw and McCann, unpublished data) which appears to be more similar to the example described by Haavik (10), where the formation of un-ionized organic acids from glucose in *Bacillus licheniformis* inhibits bacitracin formation. Catabolite repression by glucose of the synthesis of antibiotic specific enzymes, such as the α-D-mannosidase which hydrolyzes mannosidostreptomycin (4) and phenoxazinone synthase involved in actinomycin formation (7), has been reported. In the latter study, repression by glucose of actinomycin formation also occurred. Inhibition by glucose of the synthesis of many other antibiotics, including penicillin (3, 14), siomycin (15), neomycin (18), kanamycin (1), and indolmycin (13), has been described.

Disappearance. The presence of high steady-state levels of SAM or ODMP, or both,

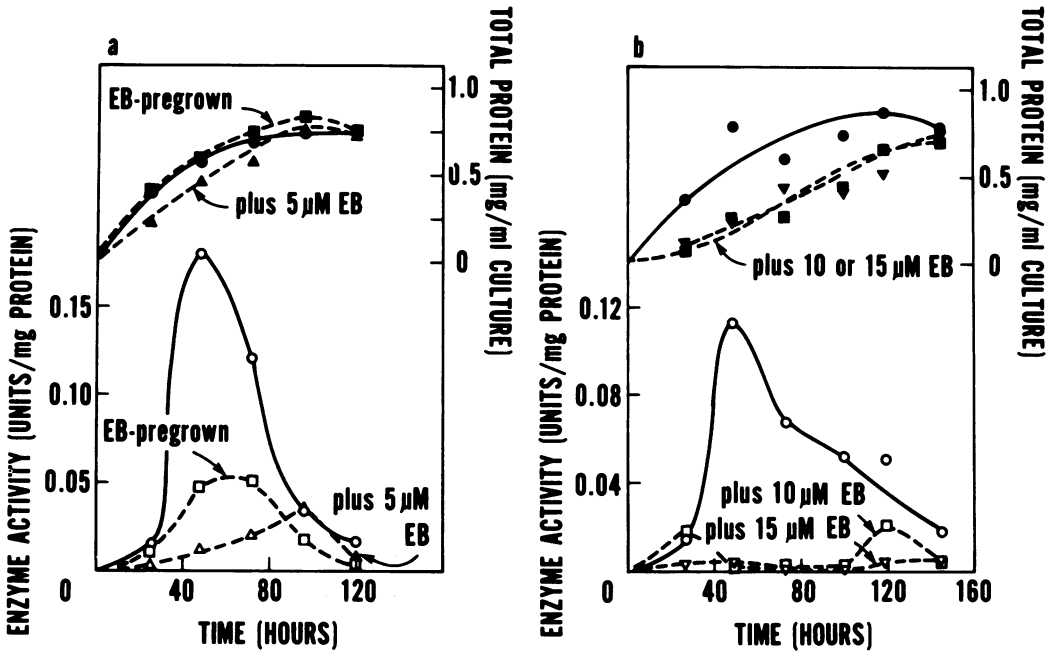


FIG. 6. *Effect of ethidium bromide on O-methyltransferase formation by S. alboniger strain 12461 in HT medium. Growth, closed symbols; enzyme specific activity, open symbols. (a) Cells were grown in the absence (○, ●) or presence (△, ▲) of 5 μM EB. (□, ■) Cells, grown in 5 μM EB for 10 days, were then regrown in the absence of EB through two growth cycles and processed as described in the text; (b) cells were grown in the absence (○, ●) or presence of 10 μM EB (□, ■), or 15 μM EB (▽, ▼).*

FIG. 5. *Effect of glucose on O-methyltransferase formation by S. alboniger strain 12461. (a) Flask contained 500 ml of HT medium ±1% glucose at the start of growth. Two milliliters of 20% $CaCO_3$ suspension was added to the control at 24 h and to the culture with glucose at 24 and 74 h to maintain neutral pH. (b) After 30 h of growth in 400 ml of CSL-CS medium, the culture was divided in half and 1% glucose was added to one portion. Final pH was 8.1 at 128 h of growth in both cultures. Growth: control (●), plus glucose (■); specific activity: control (○), plus glucose (□); total activity: control (△), plus glucose (▽).*

TABLE 3. *Effect of EB and extract from cells grown in ethidium bromide on O-methyltransferase activity*

Sample (0.01 mg of protein per 20 μl of assay)	Counts/min in puromycin[a]	Relative activity (%)
Control supernatant[b]	1,552	
EB-grown supernatant[c]	201	
Control supernatant plus EB-grown supernatant	1,586	91
Control supernatant[b]	1,640	100
+0.5 μM EB	1,507	92
+5 μM EB	1,553	95
+50 μM EB	1,472	90

[a] 30-min incubation.
[b] 37,000 × *g* supernatant from *S. alboniger* strain 12461 cells grown in HT medium for 96 h in the absence of 5 μM EB.
[c] 37,000 × *g* supernatant from *S. alboniger* strain 12461 cells grown in HT medium for 96 h in the presence of 5 μM EB.

TABLE 4. *O-methyltransferase activity in EB-treated cells[a] regrown in the absence of the dye*

Cells grown in:	Growth medium	Maximum enzyme activity[b] (%)	Maximum growth[b] (%)
0	HT	100	100
5 μM EB	HT	28	112
15 μM EB	HT	10	109
30 μM EB	HT	19	136
25 μM EB	CSL-CS	52	114

[a] *S. alboniger* strain 12461.
[b] Results are from different experiments but compared to a control minus EB from the same experiment.

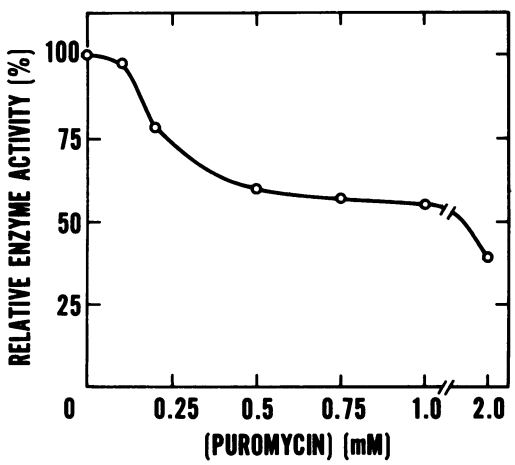


FIG. 7. *Inhibition of O-methyltransferase activity by puromycin. Reaction mixtures contained 1.5 μg of purified enzyme (specific activity, 17.3 U/mg) and indicated contractions of puromycin; 20-min incubation.*

TABLE 5. *Thermal inactivation of O-methyltransferase and protection by SAM*

Temperature (C)	Activity[a] (% of control)			
	Dialyzed crude extract[b]		Dialyzed purified enzyme[b,c]	
	No addition	Plus 80 μM SAM	No addition	Plus 80 μM SAM
30	74	86	57	
37	7	49	2	58

[a] Inactivation for 30 min; enzyme assays, 15 min.
[b] Dialysis against 5 mM sodium phosphate-0.1 mM EDTA, pH 7.5, for 2 h at 4 C.
[c] Enzyme with specific activity of 26 U/mg of protein. See text.

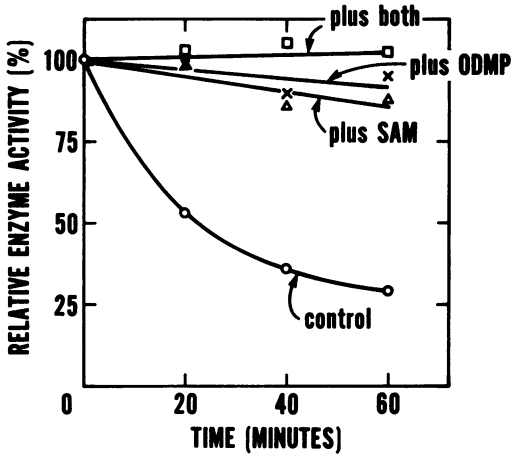


FIG. 8. *Protection by substrates against loss of O-methyltransferase activity. Dialyzed crude supernatant was first incubated at 37 C in the absence or presence of one or both substrates (80 μM SAM, 1 mM O-demethylpuromycin); aliquots were removed and assayed for enzyme activity (15 min, 16-μl final volume). Corrections for the extra substrates in assays were applied and the final concentrations were maintained as above.*

during logarithmic growth probably prevents the *O*-methyltransferase from inactivation. A drop in the levels of these metabolites in the stationary phase and during spore formation may account for the rapid loss of *O*-methyltransferase during this period. The specific mechanism for loss of enzyme activity has not been established. Substrates protect the *O*-methyltransferase against both thermal and proteolytic inactivation, so either of these processes could be operative in the cells. We have not been able to find any evidence for proteolytic activity in extracts of *S. alboniger*, but large quantities of several proteolytic enzymes (Pronase) are excreted by *Streptomyces* species (21). It is possible that specific inhibitors of proteo-

lytic enzymes, such as have been described in detail by Holzer and co-workers (12), may exist in *Streptomyces*. The transition from logarithmic phase to stationary phase could result in the activation of a proteolytic enzyme in a similar manner to the process which results in the loss of tryptophan synthase in *Neurospora crassa* and *Saccharomyces cerevesiae*. The complete loss of *O*-methyltransferase activity after exponential growth in HT medium (Fig. 4b), in sharp contrast to the small change in total protein, requires the action of a relatively specific proteolytic activity. In this regard, an analogous situation has been described in studies by Molano and Gancedo (8, 20). A rapid loss of fructose 1,6-bisphosphatase activity in *S. cerevisiae* was found in vivo upon addition of glucose, but no inactivation could be demonstrated in vitro. They have recently shown that a relatively specific proteolytic enzyme that inactivates the phosphatase is activated after removal of an inhibitor by treatment at pH 5 (20). Thermal inactivation is also possible and could be aided by specific metabolites in a manner similar to the catabolite inactivation by pyruvate of threonine dehydratase in *E. coli*, recently described by Feldman and Datta (6). Even more probable is that the loss of *O*-methyltransferase activity in the presence of low SAM and ODMP levels, with resulting conformational changes and dissociation into subunits, makes the enzyme more susceptible to in vivo proteolysis.

TABLE 6. *Effect of substrates on O-methyltransferase inactivation*

Determinants	Relative activity after incubation[a] (%)
1. Enzyme[b] alone	12
+10 μM SAM	26
+0.2 mM ODMP	50
+1.0 mM ODMP	93
+10 μM SAM +0.1 mM ODMP	51
+10 μM SAM +0.2 mM ODMP	88
2. Enzyme[b] alone	13
+5 μM SAM	25
+10 μM SAM	31
+0.1 mM ODMP	33
+0.2 mM ODMP	41
+5 μM SAM +0.1 mM ODMP	71
+5 μM SAM +0.2 mM ODMP	79
+10 μM SAM +0.1 mM ODMP	75

[a] Compared to unincubated controls. Preincubations, 30 min at 37 C; enzyme assays, 15 min.

[b] Crude extract dialyzed against 5 mM sodium phosphate-0.1 mM EDTA, pH 7.5, for 2 h at 4 C.

TABLE 7. *Proteolytic inactivation of O-methyltransferase and protection by SAM*

Determinants	Incubation time (min)	Counts/min in puromycin	
		Minus SAM	Plus SAM
Control[a]	0	4,493	4,569
	30	2,792	3,492
+ Pronase	0	4,541	4,562
	30	2,114	3,612
+ Subtilisin	0	3,960	3,887
	30	24	414

[a] Partially purified enzyme from strain 12462 (114 μg; specific activity, 0.5 U/mg of protein) incubated at 38 C without or with 1.2 μg of Pronase or 1.3 μg subtilisin in the absence or presence of 80 μM SAM in a final volume of 50 μl of 0.1 M sodium phosphate, pH 7.5. Assays, 15 min.

Possible role of extrachromosomal DNA. Although the peculiar behavior of *S. alboniger* cultures after treatment with EB can be explained partially by differential inhibition of transcription from the chromosome, the participation of a plasmid may also be involved in the formation of the *O*-methyltransferase. Further studies are now underway to obtain more definitive evidence that extrachromosomal DNA is indeed involved in the regulation of puromycin biosynthesis.

ACKNOWLEDGMENTS

This work was supported by a Public Health Service grant (CA-12080) from the National Cancer Institute, and by a grant from Eli Lilly and Co.

We wish to thank S. V. K. Narasimha Murthy for his assistance in some of these studies.

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