according to results shown in Table III.

In Figure 2 some of the chromatograms of the extracts obtained from water, must, and white wine are presented. No significant interferences of the background were found under the working chromatographic conditions (see Figure 2D).

Analyses of Must and Wine. Vineyards (Chardonnay and Cabernet Sauvignon v.vs.) were treated with the above-mentioned fungicides in the periods and at doses shown in Table IV.

Must and wine from the Chardonnay grapes were allowed at pH 6 and 5, respectively, prior to being subjected to the analyses described above.

Wine from Cabernet Sauvignon grapes was allowed at pH 5 and then analyzed.

In Table V some of our earlier results are shown. Residues are very low, and at these levels we found some interferences in the area of carbendazim and metalaxyl, especially with red wine (see Figure 3). Evidence of the presence of the corresponding compounds in the chromatogram was obtained by using spiked samples. Fungicide residues are at higher levels in the red wine than in the white, which is a logical result considering the conditions of the vinification process. In white vinification, must residues are quite similar to the wine ones with a little decrease of carbendazim and metalaxyl levels during alcoholic fermentation.

Carbendazim was the fungicide found at higher levels (about 0.4 mg/L in must, 0.25 mg/L in white wine, and 1.5 mg/L in red wine). Residue levels of propiconazole and metalaxyl are lower (less than 0.05 mg/L in must and white wine and 0.7 mg/L in red wine). Folpet was not detectable at quantifiable levels in our analyses.

Further studies on the influence of vinification technology on residue levels of some of these fungicides are under way.

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Registry No. Carbendazim, 10605-21-7; metalaxyl, 57837-19-1; folpet, 133-07-3; propiconazole, 60207-90-1.

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Microbiological Screening of Mevalonate-Suppressive Minor Plant Constituents[1]

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Halobacterium halobium is an extremely halophilic bacterium whose survival in a high-salt environment rests on its capacity to synthesize via the mevalonate pathway the diether phytanyl phosphatidyl glycerol phosphate constituent of its cell membranes. We report that a variety of monoterpenes exert a dose-dependent, mevalonate-reversible suppression of *H. halobium* growth. In companion feeding trials, the monoterpenes suppressed hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase activity. These results suggest that the mevalonate-suppression action of plant metabolites can be screened by monitoring their effects on *H. halobium* growth.

Halobacterium halobium is an extremely halophilic bacterium whose survival is due to its capacity to synthesize, via the mevalonate pathway, the diether phytanyl phosphatidyl glycerol phosphate constituent of its cell membranes (Kates, 1978). Mevalonate metabolism in *H. halobium* appears to be regulated through the modulation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) concentration rather than of HMG-CoA reductase activity (Watson et al., 1983; Cabrera et al., 1986).

The inverse association between the intake of plant products and plasma cholesterol levels is strongly supported by epidemiological studies. Early evidence pointed to the cholesterol-lowering action of a diet rich in plant constituents including linoleic acid, various types of fiber, and phytosterols and poor in cholesterol and saturated fatty acids (Anderson et al., 1973). Another prospective cholesterol-lowering action of this diet, consistent with the Brown and Goldstein (1980) concept of the multivalent

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feedback regulation HMG-CoA reductase activity, is that of a variety of non-sterol, post-mevalonate plant metabolites acting in the regulation of HMG-CoA reductase activity at the non-sterol site (Qureshi et al., 1985). Numbered among these metabolites are the monoterpenes which, according to the report of Clegg et al. (1980), suppress the transcription of the reductase gene.

In this report, we propose that the suppression of *H. halobium* growth by diverse plant metabolites and by crude lipophilic extracts of plant materials can serve as a means to evaluate their mevalonate-suppressive actions.

METHODS AND MATERIALS

Materials were obtained from the following sources: *H. halobium*, American Type Culture Collection (ATCC 29341), Rockville, MD; $NADP^+$, glucose-6-phosphate dehydrogenase, *dl*-3-hydroxy-3-methylglutaryl coenzyme A, acetyl coenzyme A, acetoacetyl coenzyme A, coenzyme A, dithiothreitol (DTT) and ethylenediaminetetraacetic acid (EDTA), Sigma Chemical Co., St. Louis, MO; monoterpenes, Aldrich Chemical Co., Milwaukee, WI; *dl*-3-hydroxy-3-[*methyl*-^{3}H]glutaryl CoA, *dl*-3-hydroxy-3-[^{14}C]methylglutaryl CoA, and sodium [1-^{14}C]acetate, New England Nuclear, Boston, MA; white Leghorn chicks, Sunnyside Hatchery, Oregon, WI; Master Mix Starter Ration, Bowar Feed Mill, Cross Plains, WI.

***H. halobium*.** A stock culture of *H. halobium* was grown in Erlenmeyer flasks filled to 40% capacity with a medium consisting of (per liter) 156 g of NaCl, 13 g of $MgCl_2 \cdot 6H_2O$, 20 g of $MgSO_4 \cdot 7H_2O$, 1 g of $CaCl_2 \cdot 2H_2O$, 4 g of KCl, 0.2 g of $NaHCO_3$, 0.5 g of NaBr, 5 g of yeast extract, and 1 g of glucose with the pH adjusted to 7.0. The cultures were incubated in the dark at 37 °C in a controlled-environment gyrorotary shaker (120 rpm). Growth was monitored by the determination of absorbance at 530 nm and alternatively by following the incorporation of label by cells grown in media containing 5–10 μCi of sodium [1-^{14}C]-acetate/100 mL. At intervals, duplicate 100-μL aliquots were placed on filter disks that were washed three times with 5% trichloroacetic acid and twice with acetone. The air-dried disks were transferred to scintillation vials containing 5 mL of toluene with 0.8% diphenyloxazole (PPO) and the radioactivity was counted.

***H. halobium* Assay.** In all experiments, growth of *H. halobium* was monitored spectrophotometrically at 530 nm. Stock cultures of *H. halobium*, grown to 0.025–0.035 absorbance unit, were distributed into 250-mL Erlenmeyer flasks filled at 40% capacity. Components to be tested were added to the test flasks. Test and control cultures were grown to 0.20–0.30 absorbance unit. The time required for the test culture growth, expressed as percent of time required for the control culture growth, inversely reflects the suppressive potency of the test material.

***H. halobium* Enzyme Assays.** *HMG-CoA Reductase.* Cells, harvested by centrifugation (24000 *g*, 15 min) from 100-mL cultures grown to 0.35 absorbance unit, were suspended in buffer (1 vol of packed cells in an equal volume of buffer consisting of 0.1 M K_3PO_4, 4 mM $MgCl_2$, 1 mM EDTA, and 2 mM DTT, pH 7.4), frozen, and held at −20 °C. Prior to the assay, the lysed cell suspensions, diluted with buffer, were centrifuged (24000 *g*, 15 min) and the supernatant fluid (<1 mg of protein/mL) collected and assayed for HMG-CoA reductase activity by a modified Phillipp and Shapiro (1979) procedure. The supernatant fluid (100–200 μg of protein) was incubated with 1 unit of glucose 6-phosphate dehydrogenase in a 400-μL Beckman microfuge tube for 20 min at 37 °C. Then, a cofactor–substrate mixture was added to provide 1 μmol of $NADP^+$, 5 μmol of glucose-6-phosphate, 0.45 μmol of DTT, and 50 nmol 1 *dl*-3-hydroxy-3-[*methyl*-^{3}H]glutaryl coenzyme A (3900 dpm) in a final volume of 200 μL. After incubation for 20 min at 37 °C, the reaction was terminated with the addition of 25 μL of 6 N HCl while the tube was vortexed. The mixture was held at 37 °C for 30 min to permit lactonization of the mevalonic acid. The samples were deprotenized by centrifugation for 2 min in the Beckman 152 microfuge. A 100-μL aliquot of the supernatant was added to 5 mL of scintillation counting fluid, the mixture was shaken vigorously, and the phases were permitted to separate. The radioactivity in the toluene phase was determined with use of a liquid scintillation counter.

HMG-CoA Synthetase. Harvested cells were suspended in 10 vol of buffer [0.3 M Tris (Cl^-), 0.2 mM EDTA, pH 8.0], the lysed cell suspensions were centrifuged (24000*g*, 15 min), and the supernatant fluid was collected and assayed for HMG-CoA synthetase activity (Clinkenbeard et al., 1975b). A base-line recording of the change in absorbance (300 nm) of a mixture consisting of 100 μmol of Tris (Cl^-), 0.1 μmol of EDTA, 0.05 μmol of acetoacetyl CoA, with or without 20 μmol $MgCl_2$, and 180–250 μg of supernatant protein in a volume of 1 mL was taken during a 2-min incubation at 30 °C. Acetyl CoA (0.2 μmol) was added to initiate the reaction. The rates of the disappearance of acetoacetyl CoA in the presence of Mg^{2+} ($E_{300} = 16.1 \times 10^3\ M^{-1}$) and absence of Mg^{2+} ($E_{300} = 3.6 \times 10^3\ M^{-1}$) inversely reflect the rates of HMG-CoA synthesis in sterolgenic and ketogenic pathways, respectively.

Acetoacetyl CoA Thiolase. The supernatant fluid used in the HMG-CoA synthetase assay was assayed for acetoacetyl CoA thiolase activity as described by Clinkenbeard et al. (1975a). A mixture consisting of 100 μmol of Tris (Cl^-), 0.1 μmol of EDTA, 0.12 μmol of acetoacetyl CoA, and 180–250 μg of supernatant protein in a volume of 1 mL was monitored at 300 nm for 3 min at 30 °C to establish a base-line disappearance of acetoacetyl CoA. The reaction was initiated with the addition of 0.09 μmol of coenzyme A. The rate of the cleavage of acetoacetyl CoA in the absence of Mg^{2+} was calculated by using the factor $E_{300} = 3.6 \times 10^3\ M^{-1}$.

Avian Studies. The suppression of mevalonate biosynthesis by diverse dietary monoterpenes was demonstrated in an avian model. Groups of six white Leghorn chicks (1 day to 4 weeks of age) were fed a commercial starter/grower mash with or without monoterpenes or extracts of plant materials for 3–4 weeks. In each study, the control group conformed with the experimental groups in terms of age, sex, and length of feeding. The chicks were fasted for 36 h and refed for 48 h prior to sacrifice at which time livers were taken. This regimen permits us to examine the effects of the plant materials on mevalonate synthesis under conditions that maximize HMG-CoA reductase activity.

Extraction of Plant Constituents. The petroleum ether soluble fraction (PESF) and methanol soluble fraction (MESF) of high-protein barley flour (HPBF) were extracted as described by Burger et al. (1984). Alfalfa meal (1 kg) was stirred into petroleum ether and the mixture allowed to stand at room temperature overnight. The supernatant fluid was decanted and concentrated to dryness under vacuum at 50–60 °C. The MESF was similarly extracted from the residue. To partition the PESF, methanol was added to the PESF, the mixture was transferred to a separatory funnel, and the contents were allowed to separate into distinct phases that were collected. Each fraction was evaporated to dryness under vacuum at 50–60 °C. One kilogram of alfalfa meal yielded 4.93 g of PESF, which partitioned into 1.46 g of PESF-PE solubles and 3.87 g of PESF-ME solubles.

Hepatic HMG-CoA Reductase. Livers removed from decapitated chickens were rinsed in chilled phosphate buffer (0.1 M, pH 7.4), blotted, and weighed. A weighed portion of the liver was minced and suspended (1:2, w/v) in the buffer. Homogenization, 15 s, 0–4 °C, was done with a Polytron set at 6. The homogenate was centrifuged at 15000*g* for 10 min. The resulting supernatant fluid was passed through cheesecloth and then centrifuged at 100000*g* for 60 min. The precipitate (microsomal fraction) was suspended in 1 mL of buffer and stored at −20 °C. Prior to the assay, a modification of the Phillipp and Shapiro (1979) procedure, the microsomal suspensions were thawed and diluted in buffer (<15 mg of protein/mL). An aliquot (200 μg of protein) was transferred to a 400-μL Beckman microfuge tube, glucose-6-phosphate dehydrogenase (0.3 unit) added, the volume corrected to 70 μL, and the mixture incubated at 42 °C for 10 min. To this mixture was added 80 μL of a cofactor–substrate solution containing 4.5 μmol of glucose-6-phosphate, 450 nmol of $NADP^+$, 25 nmol of *dl*-3-hydroxy-3-[*methyl*-^{3}H]glutaryl coenzyme A (2×10^5 dpm), 0.3 μmol of DTT, and 5 μmol of potassium phosphate buffer, pH 7.4. After a 15-min incubation at 42 °C, the reaction was terminated with the addition of 25 μL of 10 N HCl while the contents of the tube were mixed with a vortex mixer. The mixture was held at 42 °C for 30 min to permit complete lactonization of the mevalonic acid. The assay mixture was deproteinized by centrifugation for 2 min in a Beckman 152 microfuge. The su-

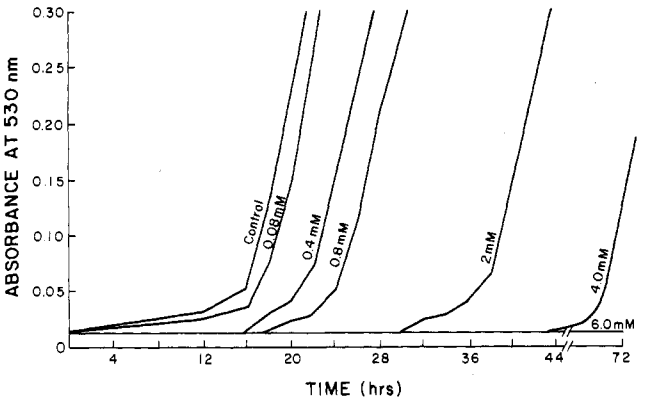

Figure 1. Suppression of *H. halobium* growth by the monocyclic monoterpene, menthone. A culture grown to A = 0.025 was distributed into 250-mL Erlenmeyer flasks (100 mL/flask). Menthone was added (0.08–4.0 mM) to the culture which was incubated at 37 °C in a gyrorotary shaker (120 rpm). Culture growth was measured by monitoring its optical absorbance (530 nm).

pernatant fluid was collected and saturated with anhydrous Na_2SO_4. The contents of the tube were extracted three times with 1 mL of toluene containing 0.8% PPO. The three washes were added to scintillation vials containing 3 mL of counting solution, and radioactivity was determined.

Protein. Protein was assayed according to Lowry et al. (1951).

RESULTS

Both morphological changes and changes in cell number are reflected in changes in optical absorbance. Therefore, we first establish that the increase in optical absorbance during the linear growth phase of the culture paralleled the increase in radiolabeled acetate incorporation into cellular constituents under diverse growth conditions (r = 0.81, $P < 0.01$).

In order to test our hypothesis that plant constituents that suppress mevalonate synthesis will suppress the growth of *H. halobium*, cultures were incubated with two cyclic monoterpenoids that suppress rat liver HMG-CoA reductase activity. Menthone, a cyclic ketone, effected a concentration-dependent (0.08–0.8 mM) suppression of culture growth. The effect was not cytotoxic as slow growth was observed in cultures with 4.0 and 6.0 mM (at 96 h) menthone (Figure 1). Borneol, a bicyclic alcohol, also effectively suppressed *H. halobium* growth. The time required for the culture to grow to A_{530} = 0.2 was increased by 50% with 0.5 mM borneol. As anticipated, the growth-suppressive action of borneol was reversed by 0.1 mM mevalonolactone (Figure 2). To further confirm the concentration-dependent suppression of *H. halobium* growth by plant constituents, cultures were grown with a bicyclic ketone, fenchone (Figure 3). Clegg et al. (1980) recorded the effects of a series of monoterpenes on rat liver HMG-CoA reductase activity. On Figure 4 we show the concentration-dependent effects of those monoterpenes on the growth of *H. halobium*. With the single exception of cineole, an effective inhibitor of the enzyme, the relative potencies of the terpenoids as suppressors of mevalonate synthesis estimated by two systems were similar. When tested in both the microbial and avian systems, limonene and camphene, the hydrocarbon counterparts of menthol and borneol, were less effective than the alcohols.

The influences of mevalonolactone and borneol on HMG-CoA metabolism were examined with the supernatant fraction of lysed cells grown to 0.30A_{530} unit. The results (Table I) appear to confirm the observation of Cabrera et al. (1986), namely that, in *H. halobium*, mevalonate production is regulated by the availability of

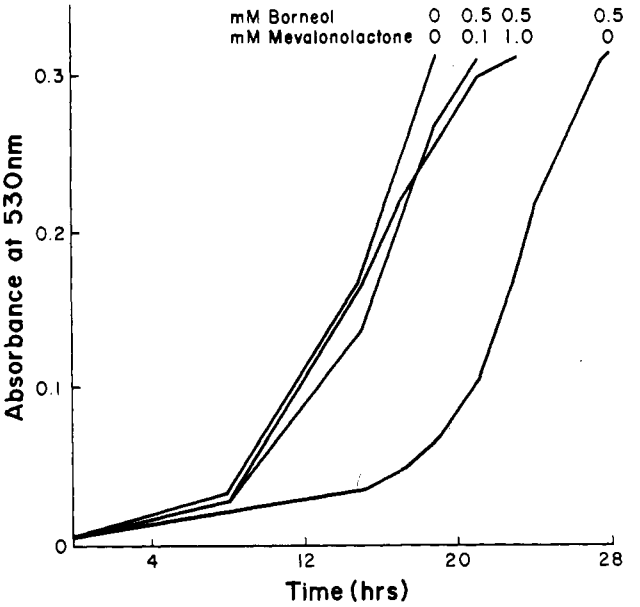

Figure 2. Suppression of *H. halobium* growth by the bicyclic monoterpene alcohol borneol and reversal of growth suppression by mevalonic acid. The mevalonolactone was added at 0 time.

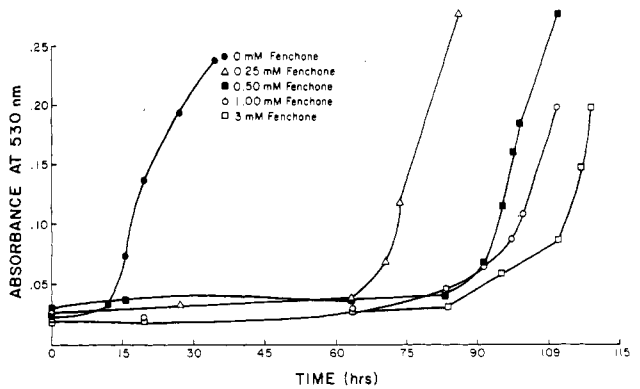

Figure 3. Suppression of *H. halobium* growth by the bicyclic monoterpene fenchone. For details, see Figure 1.

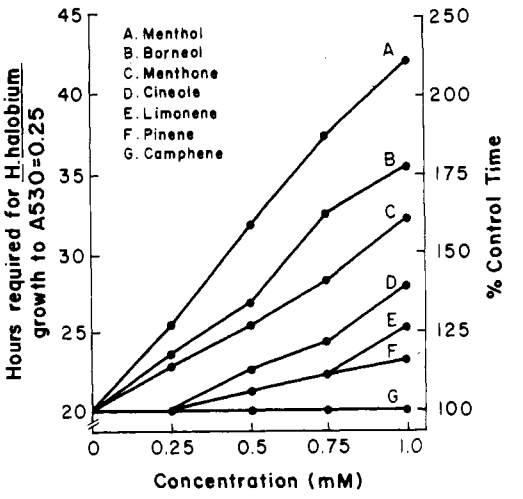

Figure 4. Concentration-dependent suppression of *H. halobium* growth by terpenoids that have been shown to suppress rat liver HMG-CoA reductase activity (Clegg et al., 1980).

HMG-CoA rather than by changes in HMG-CoA reductase activity. Neither HMG-CoA reductase nor acetoacetyl-CoA thiolase activity changed consistently in response to mevalonolactone or borneol. Under the conditions of this experiment HMG-CoA synthetase activity was suppressed

Table I. Influence of Mevalonolactone and Borneol on *H. halobium* HMG-CoA Metabolism

mevalonolactone, 0.1 mM	borneol, 0.5 mM	acetoacetyl-CoA thiolase[a]	HMG-CoA synthetase[a]	HMG-CoA reductase[a]
-	-	35	12.0	0.183 ± 0.057
+	-	37	6.2	0.169 ± 0.016
-	+	66	5.4	0.184 ± 0.040
+	+	33	2.4	0.180 ± 0.022

[a] Nanomoles of product per minute per milligram of protein.

Table II. Influence of Monoterpenes on Avian Hepatic 3-Hydroxy-3-methylglutaryl CoA Reductase Activity and *H. halobium* Growth

treatment	n	hepatic HMG-CoA reductase, pmol/mg microsomal protein per min	*Halobium* growth, % of control
control	6	666 ± 1,3	100
monoterpenes			
acyclic			
citral	12	477 ± 123	>400
geraniol	6	505 ± 177	>400
monocyclic			
menthone	6	481 ± 89	130
menthol	6	512 ± 104	165
bicyclic			
fenchone	6	503 ± 92	359
fenchyl alcohol	6	544 ± 157	>400

[a] The diets and culture media contained 50 ppm and 0.5 mM terpenoids, respectively.

by 50% when the additives were tested individually and by 80% when they were tested in combination.

The impacts of oxygenated acyclic, monocyclic, and bicyclic monoterpenoids on avian hepatic HMG-CoA reductase activity and on *H. halobium* growth are listed on Table II. HMG-CoA reductase activities in the livers of birds fed diets containing 50 ppm monoterpenoid were on the average 25% lower than the control activity. Acyclic and bicyclic monoterpenoids were the more potent suppressors of *H. halobium* growth. Differences in solubility, permeability, or metabolic products of the diverse monoterpenes may influence their growth-suppressive potencies in the *H. halobium* system.

We have reported that constituents of barley (Burger et al., 1984) and ginseng root (Qureshi et al., 1983b) suppress mevalonate synthesis. Extracts of these materials also suppress *H. halobium* growth (Table III). We also found that a cholesterol-suppressive constituent of alfalfa meal (Malinow et al., 1980) could be extracted with petroleum ether. Partition of the alfalfa constituents in the petroleum ether extract between petroleum ether and methanol revealed that the less polar fraction contains the more concentrated or more potent inhibitor of mevalonate synthesis (Table III).

Avian cholesterol synthesis is suppressed when the plant growth retardant AMO 1618 (Qureshi et al., 1983a) or cholesterol (Brown and Goldstein, 1980) is fed. Although both compounds inhibit HMG-CoA reductase activity, their inhibitory actions occur at sites (Douglas and Paleg, 1972; Brown and Goldstein, 1980) distinct from the proposed post-mevalonate, non-sterol site of action (Watson et al., 1985; Cabrera et al., 1986; Havel et al., 1986). While both cholesterol and AMO 1618 suppressed HMG-CoA reductase activity, neither influenced *H. halobium* growth (Table III).

We have tested the effects of other plant materials on *H. halobium* growth (unpublished data). Constituents of cumin, sassafras, fenugreek seed, and nutmeg retard *H. halobium* growth. The effect of these materials as well as that of the monoterpenes on microbial growth is not due to their cytotoxicity. This lack of cytotoxicity is apparent in the delayed growth pattern of cells exposed to high concentrations of the various compounds. Furthermore, growth suppression by monoterpenes was reversed by the addition of mevalonate to the culture medium.

Table III. Parallels in Suppression of Avian Hepatic HMG-CoA Reductase Activity and *H. halobium* Growth

additive	HMG-CoA reductase: diet, mg/kg	HMG-CoA reductase: % control	*H. halobium* media, μg/100 m	*H. halobium* growth, % control
HPBF[a]	200000	67		
PESF[b]	7000	64	30.0	200
MESF[c]	8000	51	40.0	147
ginseng	2840	32		
PESF	300	32	12.5	>400
MESF	790	59	40.0	>400
alfalfa meal	50000	35		
PESF-PE[d]	82	74	12.5	166
PESF-ME[d]	280	72	40.0	106
MESF	7000	105	500.0	106
cholesterol	10000	82	0.25	101
			0.50	104
AMO 1618	2.5	86	0.014	106
	7.5	74	0.081	104
	15.0	55		

[a] High-protein barley flour. [b] Petroleum ether extract. [c] Methanol extract. [d] Partitioned into PE and ME solubles.

DISCUSSION

The established role of mevalonate in the sterolgenic pathway (Brown and Goldstein, 1980) coupled with the renewed interest in the unique regulation of its synthesis in neoplastic tissue (Siperstein and Fagan, 1964) led us to develop a microbiological assay for screening plant materials for constituents that might act as dietary suppressors of mevalonate biosynthesis. The assay appears to detect in test materials the presence of non-sterol, post-mevalonate products that suppress mevalonate synthesis. Our data, confirming the report of Cabrera et al. (1986), suggest that, in *H. halobium*, the regulation of mevalonate synthesis is dependent on the regulation of a single activity, HMG-CoA synthetase. In animal tissues, mevalonate biosynthesis is regulated by coordinated changes in the activities of at least three enzymes, acetoacetyl-CoA thiolase, HMG-CoA synthetase, and HMG-CoA reductase (Clinkenbeard et al., 1983). More recently, changes in the mRNA levels for the latter pair of enzymes were observed under conditions that altered their activities (Clarke et al., 1987). Although not tightly clustered, both cytosolic HMG-CoA synthetase and microsomal HMG-CoA reductase genes reside on the same chromosome (Mehrabian et al., 1986). The genes have in common a 26-nucleotide sequence located in their 5′-untranslated regulatory regions (Gil et al., 1986). The coordinate regulation of the two activities may be traced to a non-sterol isoprenoid signal molecule (Cabrera et al., 1986; Watson et al., 1985) acting as a physiological regulator of the transcription of both genes. *H. halobium* is useful as a nonpathological tool for screening plant materials for the presence of non-sterol, post-mevalonate constituents that appear to act in the down-regulation of mevalonate production. Besides providing a rapid, economical means of screening plant material, this model avoids the expenditure of animal resources.

Registry No. HMG-GA, 1553-55-5; AMO 1618, 2438-53-1; acetoacetyl-CoA thiolase, 9027-46-7; HMG-CoA synthetase,

9027-44-5; HMG-CoA reductase, 9028-35-7; mevalonate, 150-97-0; phytanylphosphatidylglycerol phosphate, 2679-48-3; cholesterol, 57-88-5; menthone, 89-80-5; borneol, 507-70-0; fenchone, 1195-79-5; mevalonolactone, 503-48-0; citral, 5392-40-5; geraniol, 106-24-1; menthol, 89-78-1; fenchyl alcohol, 1632-73-1; cineole, 470-82-6; limonene, 138-86-3; pinene, 1330-16-1; camphene, 79-92-5.

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