3-Hydroxy-3-methylglutaryl CoA reductase and mevalonate kinase of *Neurospora crassa*

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Abstract Two enzymes of polyisoprenoid synthesis, 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase (mevalonate:NADP oxidoreductase [acylating CoA], EC 1.1.1.34) and mevalonate kinase (ATP:mevalonate 5-phosphotransferase, EC 2.7.1.36), are present in the microsomal and soluble fractions of *Neurospora crassa*, respectively. HMG CoA reductase specifically uses NADPH as reductant and has a K_m for DL-HMG CoA of 30 μM. The activities of HMG CoA reductase and mevalonate kinase are low in conidia and increase threefold during the first 12 hr of stationary growth. Maximum specific activities of both enzymes occur when aerial hyphae and conidia first appear (2 days), but total activities peak later (3–4 days). Addition to the growth media of ergosterol or β-carotene, alone or in combination, does not affect the specific or total activity of either enzyme. The mevalonate kinase of *N. crassa*, purified 200-fold to a specific activity of 5 μmoles/min/mg, is free from HMG CoA reductase, phosphomevalonate kinase, ATPase, adenylate kinase, and NADH oxidase activities. Mevalonate kinase specifically requires ATP as cosubstrate and exhibits a marked preference for Mg^{2+} over Mn^{2+}, especially at high ratios of divalent metal ion to ATP. Kinase activity is inhibited by *p*-hydroxymercuribenzoate, and this inhibition is partially prevented by mevalonate or MgATP. Optimum activity occurs at pH 8.0–8.5 and at about 55°C. The *Neurospora* kinase, like that of hog liver, has a sequential mechanism for substrate addition. The Michaelis constants obtained were 2.8 mM for DL-mevalonate and 1.8 mM for $MgATP^{-2}$. Geranyl pyrophosphate is an inhibitor competitive with MgATP (K_i = 0.11 mM).

Supplementary key words biosynthesis of carotenoids · biosynthesis of sterols · biosynthesis of isoprenoids · comparative biochemistry of *Neurospora*

Advantages of using *Neurospora crassa* for investigations of differential gene expression include the brevity and discrete morphological phases of the asexual life cycle. Activation of the spores, which exhibit low overall metabolic activity, initiates RNA and protein synthesis and a rapid increase in respiratory activity (1, 2). The vegetative phase, which terminates with spore formation, is characterized by significant changes in enzymic composition. The activities of most *Neurospora* enzymes are either low in spores and high in mycelia (succinate dehydrogenase [3–5], aldolase [4], tryptophan synthetase [4], glucose-6-phosphate dehydrogenase [5], and malate dehydrogenase [5]) or high in spores and low in mycelia (invertase [6], trehalase [6, 7], and NADPase [5]). Except for tryptophan synthetase, all are associated with energy metabolism.

We present data on two biosynthetic enzymes. One, HMG CoA reductase (mevalonate:NADP oxidoreductase [acylating CoA], EC 1.1.1.34), regulates sterol synthesis in liver (8) and probably also ergosterol synthesis in yeast (9–11). The other, mevalonate kinase (ATP:mevalonate 5-phosphotransferase, EC 2.7.1.36), may also fulfill a regulatory role in mammalian liver sterol synthesis (12). HMG CoA reductase activity has not previously been reported in *Neurospora*. Mevalonate kinase occurs in organisms and tissues that contain sterols but apparently not in blue-green algae or photosynthetic bacteria that contain carotenes but not sterols (13). Mevalonate kinase from hog liver (14) and *Sarcophaga bullata* larva (15) has been purified to an apparently homogeneous state and has been partially purified from yeast (16), rabbit liver (17), pumpkin seedlings (18), latex (19), and rat ovary (20). The reaction catalyzed by the hog liver kinase (12, 14, 21) is ordered sequential, and physiological concentrations of geranyl and farnesyl pyrophosphates are potent inhibitors competitive with MgATP. These compounds have been proposed as regulators of mevalonate kinase activity in vivo (12).

EXPERIMENTAL PROCEDURES

Chemicals

Chemicals were from the following sources: DL-mevalonic acid lactone, ATP, CTP, GTP, ITP, UTP,

Abbreviations: HMG, 3-hydroxy-3-methylglutaric acid; TLC, thin-layer chromatography.

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NAD, NADP, 2-mercaptoethanol, phosphoenolpyruvate, glucose-6-phosphate, *p*-hydroxymercuribenzoate, 3,3′-dimethylglutarate, pyruvate kinase, glucose-6-phosphate dehydrogenase, alkaline phosphatase, and cytochrome *c*, Sigma Chemical Co., St. Louis, Mo.; DL-3-hydroxybutyrate, 3-hydroxy-3-methylglutarate, DL-mevaldate, and enzyme-grade $(NH_4)_2SO_4$, Schwarz/Mann, Orangeburg, N.Y.; ergosterol and β-carotene, Nutritional Biochemicals Corp., Cleveland, Ohio; triethanolamine and hydroxylamine, Matheson Coleman & Bell, Cincinnati, Ohio; coenzyme A and dithiothreitol, Calbiochem, Los Angeles, Calif.; 4-hydroxy-4-methyl-2-pentanone and 1,3-butanediol, Aldrich Chemical Co., Inc., Milwaukee, Wis.; Tween 40, J. T. Baker Chemical Co., Phillipsburg, N.J.; 2,5-diphenyloxazole, Arapahoe Chemicals, Boulder, Col.; bovine serum albumin, Pentex Research Div., Miles Laboratories, Kankakee, Ill.; DEAE cellulose (Cellex D, capacity 0.95 meq/g), Bio-Rad Laboratories, Richmond, Calif.; yeast alcohol dehydrogenase, Worthington Biochemical Corp., Freehold, N.J.; chemicals for disc electrophoresis, Canalco, Inc., Rockville, Md.; Sephadex G-200, Pharmacia Fine Chemicals, Inc., Piscataway, N.J.; DL-[2-^{14}C]mevalonic acid lactone, Amersham/Searle Corp., Arlington Heights, Ill.; and Glusulase, a snail gut preparation containing glucuronidase and sulfatase actvities, Endo Laboratories, Inc., Garden City, N.J. Ansco nonscreen X-ray film was used for radioautography.

[3-^{14}C]HMG CoA was prepared from [3-^{14}C]HMG (Schwarz/Mann) as described by Hilz et al. (22). CTP, GTP, ITP, and UTP exhibited the reported spectrophotometric constants (23) and were free of detectable ATP when examined by cellulose TLC in isobutyric acid–15 N NH_4OH–H_2O 66:1:33 (v/v) (23). Geranyl pyrophosphate, a gift of Prof. John W. Porter, was judged to be 92% homogeneous by cellulose TLC in isopropyl alcohol–isobutyl alcohol–15 N NH_4OH–H_2O 40:20:1:39 (v/v) (24) and by determination of total phosphorus (25) and of inorganic orthophosphate in the presence of labile phosphorus esters (26).

Culture

Neurospora crassa, a cross of wild types E 5297a and E 5256A (27), was a gift of Prof. F. W. Quackenbush. Stock cultures were maintained on solid Wainwright's medium (28), and conidia were transferred biweekly.

Production of conidia

Conidia from a 5–7-day-old slant suspended in sterile water were distributed over solid Wainwright's medium in an aluminum foil-wrapped 2.8-l Fernback flask (29). After 3 days at 30°C, the foil was removed, the cotton plug was replaced with a special cover designed to permit improved aeration (29), and incubation was continued for an additional 3 days at room temperature. Conidia suspended in sterile water by shaking with sterile marbles were filtered through cheesecloth. The conidial density was estimated from a calibration curve relating absorbance at 700 nm to number of conidia, determined by counting in a hemocytometer chamber. The curve was linear from 0.5 to 5.0 × 10^6 conidia/ml. One flask yielded from 7 to 15 × 10^9 conidia. Conidial suspensions were used directly or stored aseptically at 4°C for 1–4 days prior to use.

Growth of cultures

For stationary growth in continuous light at 25°C, 250-ml flasks containing 50 ml of 1.5% sucrose in Fries minimal medium (30) were inoculated with 1 × 10^8 conidia. Shaken 50-ml cultures in 250-ml flasks, or 500-ml cultures in fluted, long-necked 2-l flasks, were grown for 14–16 hr at 30°C on a New Brunswick Gyrotory shaker. Mycelia were filtered through cheesecloth and washed with water, and excess fluid was expressed manually. Growth was estimated by drying weighed portions of damp, washed mycelia to constant weight at 85°C. The residual mycelia were used for enzymic analyses.

For production of mycelia for purification of mevalonate kinase, 1 l of 1.5% sucrose in Fries basal medium (30) in a 2-l fluted flask was inoculated with 2 × 10^9 conidia and shaken at 30°C for 10–12 hr. The resulting mycelia were transferred to 15 l of fresh medium in a 20-l carboy. Growth with forced aeration (10 l of air/min) was at 30°C for 20–24 hr. The mycelia were poured onto four layers of cheesecloth on a Büchner funnel and washed with several liters of water, and the excess water was expressed manually. Damp mycelia were weighed (yield, 150–200 g/carboy), wrapped in aluminum foil, and stored at −20°C. Frozen mycelial preparations retain mevalonate kinase activity for at least 6 months.

Preparation of cell-free extracts

Extracts were prepared either by grinding with an abrasive or by enzymic lysis. Mycelia were ground to a smooth paste with twice their damp weight of acid-washed glass beads or sand in a chilled mortar. A volume of buffer, 0.1 mM EDTA–10 mM 2-mercaptoethanol–50 mM potassium phosphate, pH 7.5, equal to three times the damp weight of the mycelia was added, in portions, with grinding between additions. The resulting slurry was centrifuged at 1,500 *g* for 10 min, and the supernatant liquid was retained as the cell extract. Alternatively, 10 g of damp mycelia was suspended in 200 ml of 0.63 M sorbitol–30 mM 2-mercaptoethylamine·HCl–0.4 mM EDTA–0.10 M potassium citrate, pH 5.8 (31), and 2.0 ml of Glusulase suspension was added. The mixture was incubated at 30°C for 1 hr then centrifuged at 500 *g* for 5 min. The pellet was washed twice with 100 ml of cold 0.90 mM sorbitol, suspended in 30 ml of 0.25 M sucrose–10 mM EDTA–10 mM Tris·HCl, pH 7.2, and homogenized with five strokes of a Teflon–glass Potter-Elvehjem ho-

mogenizer. The homogenate was centrifuged at 1,500 *g* for 10 min, and the supernatant liquid was retained as the cell-free extract.

Determination of mevalonic acid

Solutions of potassium DL-mevalonate, pH 8.0, prepared from DL-mevalonic acid lactone, were analyzed for mevalonate concentration by the method of Lynen and Grassl (32).

Assay for HMG CoA reductase activity

HMG CoA reductase activity was quantitated by the radioisotopic TLC method of Shapiro, Imblum, and Rodwell (33). Incubation mixtures contained, in 1.0 ml, 25 μmoles of potassium phosphate, pH 7.0, 10 μmoles of 2-mercaptoethanol, 5.0 μmoles of EDTA, 3.0 μmoles of NADP, 30 μmoles of glucose-6-phosphate, 2.0 IU of glucose-6-phosphate dehydrogenase, 10 μmoles of DL-mevalonate, 3.0 μmoles of DL-[3-^{14}C]HMG CoA (sp act, 430 cpm/nmole), and 0.6–0.9 mg of protein. Incubations were at 30°C for 5 min. Mevalonolactone formed after acidification was extracted into ether (32), isolated by TLC, and counted (33). One enzyme unit (mU) of HMG CoA reductase is that catalyzing formation of 1 nmole of mevalonate/min under the above conditions.

Assays for mevalonate kinase activity

Concentrations of $MgCl_2$ and of ATP required to generate the desired concentrations of MgATP plus a 1 mM excess of free Mg^{2+} were calculated from the stability constant for $MgATP^{-2}$ at pH 8.0 of 70,000 M^{-1} (34). Mevalonate kinase activity was assayed by measuring either the incorporation of isotope from DL-[2-^{14}C]mevalonate into 5-phosphomevalonate (16) or the rate of ADP production in a coupled spectrophotometric assay (12, 16, 20). The coupled assay was unsuitable for preparations cruder than the DEAE fraction due to the presence of interfering enzymes. For either assay, one enzyme unit (mU) of mevalonate kinase is that catalyzing formation of 1 nmole of 5-phosphomevalonate/min at 30°C.

For the radioisotopic assay, reaction mixtures contained, in 0.2 ml, 20 μmoles of triethanolamine·HCl, pH 8.0, 1.9 μmoles of potassium DL-[2-^{14}C]mevalonate (sp act, 1.4–1.6 × 10^5 cpm/μmole), 4.0 μmoles of ATP, 4.2 μmoles of $MgCl_2$, and 2–125 enzyme units of mevalonate kinase. All components except enzyme were incubated for 3 min at 30°C, and the reaction was initiated by adding 10 μl of kinase solution. Incubation at 30°C was terminated after 2 min by immersion in a boiling water bath. All data are corrected for any phosphomevalonate formed during heating to a temperature sufficient for heat denaturation. Precipitated protein was removed by centrifugation, and a 100-μl aliquot of the solution was applied over a distance of 4 cm to a 20 × 20 cm cellulose TLC sheet (Eastman) ruled vertically into five channels. Development was in 1-butanol–formic acid–water 77:10:13 (v/v) in a large-volume chamber (16). R_F values were: mevalonate, 0.84; 5-phosphomevalonate, 0.23; and 5-pyrophosphomevalonate, 0.03. The region between the origin and R_F 0.33 was removed, transferred to 10 ml of scintillation fluor (5.0 g of 2,5-diphenyloxazole and 100 g of naphthalene plus dioxane to 1 l), and counted in a Beckman model CPM-100 liquid scintillation spectrometer. The counting efficiency was 90%. For fractions cruder than the calcium phosphate gel fraction, the observed radioactivity represents both 5-phosphomevalonate and 5-pyrophosphomevalonate.

TABLE 1. Intracellular distribution of HMG CoA reductase and of mevalonate kinase

Fraction	HMG CoA Reductase[a]		Mevalonate Kinase[b]	
	Specific Activity	Total Activity	Specific Activity	Total Activity
	mU/mg	*mU*	*mU/mg*	*mU*
1,500 *g* supernatant liquid	0.71	142	675	15
8,000 *g* supernatant liquid	0.84	154	9	2
8,000 *g* pellet	0.28	22	713	17
70,000 *g* supernatant liquid	0.01	2.0	9	1
70,000 *g* pellet	1.5[c]	90[c]	697	20

[a] A homogenate prepared from 10 g of mycelia by Glusulase treatment was diluted to 80 ml with homogenizing buffer and fractionated by successive centrifugation for 10 min at 1,500 *g*, 20 min at 8,000 *g*, and 60 min at 70,000 *g*. Supernatant liquids and pellet fractions were resuspended in homogenizing buffer and assayed for HMG CoA reductase activity under standard conditions.

[b] Mycelia were extracted in 10 mM 2-mercaptoethanol–1.0 mM EDTA–50 mM potassium phosphate, pH 7.5. The extract was then fractionated by centrifugation as described above and assayed for mevalonate kinase activity. The data are mean values for duplicate determinations.

[c] These data represent minimum values because one-half of the DL-HMG CoA present was converted to mevalonate.

For the coupled spectrophotometric assay, reaction mixtures contained, in 1.0 ml, 100 μmoles of triethanolamine·HCl, pH 8.0, 0.50 μmole of dithiothreitol, 0.50 μmole of sodium phosphoenolpyruvate, 162 nmoles of NADH, 25 IU of lactate dehydrogenase, 20 IU of pyruvate kinase, 1.0 μmole of ATP, 2.0 μmoles of $MgCl_2$, and 0.8–8.0 enzyme units of mevalonate kinase. The reaction was initiated by adding 2.5 μmoles of potassium DL-mevalonate, pH 8.0, and the change in absorbancy at 340 nm due to oxidation of NADH was observed at 30°C in a Cary model 15 recording split-beam spectrophotometer equipped with an expanded scale.

Assay of other enzymes

To test for the presence in purified mevalonate kinase of other enzyme activities, we employed an amount of kinase at least 10-fold greater than that used to detect kinase activity. All assays were at 30°C. Adenylate kinase activity

TABLE 2. Absence of HMG CoA reductase from the mitochondrial fraction[a]

Enzyme Assayed	Specific Activity[b]		
	8,000 *g* Pellet	Washed 8,000 *g* Pellet	Peak Fractions from Sucrose Gradient
Cytochrome *c* oxidase	1.1	1.6	2.3
HMG CoA reductase	0.26	0.12	0.026

[a] Mitochondria were isolated essentially as described by Luck (38). A 1.0-ml portion of the 8,000 *g* pellet fraction (Table 1) suspended in homogenizing medium, 10 mg of protein/ml, was layered on a 4.0-ml linear 0.58–1.0 M sucrose gradient containing 1.0 mM EDTA and was centrifuged at 40,000 rpm for 5 hr in an SW-39 rotor of a Spinco model L ultracentrifuge. 18-drop fractions were assayed for protein by the method of Lowry et al. (39) and for cytochrome *c* oxidase activity (40). The fraction containing maximal cytochrome oxidase activity was then assayed for HMG CoA reductase activity.

[b] For cytochrome *c* oxidase, decrease in A_{550}/min/mg. For HMG CoA reductase, mU/mg.

was assayed by substituting 1.0 mM AMP for mevalonate in a standard spectrophotometric assay for mevalonate kinase. Formation of ADP was measured as above at 340 nm. ATPase activity was measured by incubating 20 mM ATP and 22 mM $MgCl_2$ in 100 mM triethanolamine·HCl, pH 8.0, for 60 min in the absence or presence of mevalonate kinase. A portion, 50 μl, was assayed for ADP by the standard spectrophotometric assay. NADH oxidase activity was measured by incubating 25 μM 2,3′,6-trichlorophenolindophenol and 0.165 mM NADH in 100 mM triethanolamine·HCl, pH 8.0, in the absence or presence of mevalonate kinase. The disappearance of NADH was measured at 340 nm.

Determination of protein

Bovine serum albumin was used as a standard. For fractions cruder than the DEAE fraction, protein was determined by a biuret method (35) modified to eliminate interference by carotenoids, which absorb at 540 nm (36). For the DEAE and subsequent fractions, protein was determined by the spectrophotometric method of Waddell (37).

RESULTS

Detection of HMG CoA reductase activity

Since HMG CoA reductase activity has not previously been reported in *Neurospora*, we attempted to detect its presence by techniques suitable for its analysis in yeast, bacteria, and mammalian tissues (33). Extracts of cultures were fractionated by differential centrifugation, and the fractions were assayed for HMG CoA reductase activity (Table 1). The major portion of the apparent HMG CoA reductase activity was associated with the fraction sedimenting at 70,000 *g*. Each fraction was also assayed for reductase activity after substituting 3.0 μmoles of $[NAD]^+$ for $NADP^+$ and 40 μmoles of ethanol and 3.0 IU of alcohol dehydrogenase for the NADPH-generating system. Activity in all fractions was barely detectable (sp act, 0.01 mU/mg; total activity, 2 mU), indicating that $NADP^+$ rather than NAD^+ is the cofactor for *Neurospora* HMG CoA reductase. To establish that the observed counts indeed represented reductase activity, we next characterized the reaction product as mevalonate.

Characterization of mevalonic acid

Neurospora microsomes incubated with DL-[3-^{14}C]HMG CoA and appropriate cofactors formed a single radioactive product with an R_F value in the TLC system used for analysis of reductase activity (33) equal to that of mevalonolactone. This product was recovered and its chromatographic properties were compared with that of authentic mevalonate in two additional solvent systems. Radioactive compounds present were visualized by radioautography for 7 days. Only one compound was detected in either solvent system. The R_F values of the reaction product after cellulose TLC in a basic solvent (*n*-propanol–15 N NH_4OH 7:3 [v/v]; R_F = 0.66) and in an acidic solvent (isobutyric acid–15 N NH_4OH–H_2O 66:1:33 [v/v]; R_F = 0.82) coincided exactly with those of authentic mevalonate. The sole product thus is mevalonate, and *Neurospora* microsomes thus catalyze the HMG CoA reductase reaction.

TABLE 3. Summary of a typical purification[a]

Fraction	Volume	Total Activity	Total Protein	Specific Activity	Recovery of Activity
	ml	*units*	*mg*	*units/mg*	%
54K *g* supernatant fraction	1,570	104,000	9,420	11[b]	100
38–48% $(NH_4)_2SO_4$ precipitate	93	64,000	2,380	27	62
DEAE fraction	145	43,000	145	300	41
Calcium phosphate gel fraction	2.4	15,500	15.6	990	15
Sephadex fraction	2.8	12,000	5.30	2,260[c]	11

[a] The data are for a preparation from 530 g wet weight of mycelia and were obtained using the radioisotopic method of assay throughout.

[b] Range 10–20 in several preparations.

[c] Range 2,300–5,000 in several preparations.

Absence of HMG CoA reductase from mitochondria

Significant apparent HMG CoA reductase activity (10–20%) was consistently present in the 8,000 *g* pellet fraction or mitochondrial fraction (Table 1). Washing the 8,000 *g* pellet by suspending it in buffer and resedimenting at 8,000 *g* reduced the specific activity of HMG CoA reductase 50% but raised that of cytochrome *c* oxidase 50% (Table 2). Reductase activity removed from the mitochondria by washing was resedimented by centrifugation at 48,000 *g* for 1 hr, indicating that it was still particulate. The washed 8,000 *g* pellet was next layered on a sucrose gradient suitable for purification of mitochondria (38). The specific activity of HMG CoA reductase in the mitochondria peak decreased about fivefold while that of cytochrome *c* oxidase increased (Table 2). Since both the specific activity and the total activity of HMG CoA reductase in purified mitochondria were less than 2% of that found in the microsomal fraction, we conclude that apparent HMG CoA reductase activity in the 8,000 *g* pellet fraction is due principally or entirely to contamination by microsomes.

K_m for DL-HMG CoA

To establish appropriate conditions for assay of *Neurospora* HMG CoA reductase, we examined the effect of HMG CoA concentration on the rate of mevalonate formation. The K_m for DL-HMG CoA is about 30 μM (Fig. 1).

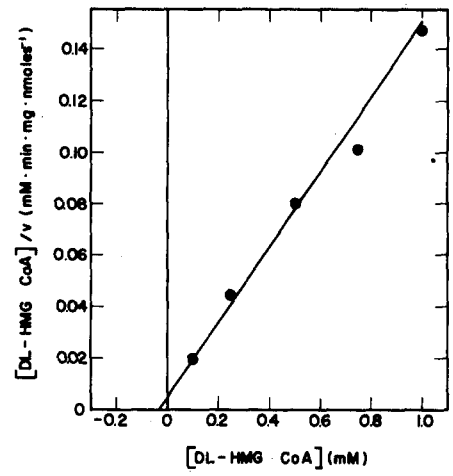

Fig. 1. K_m for DL-HMG CoA. Standard conditions were employed except for HMG CoA concentration, which ranged from 0.1 to 1.0 mM.

Localization of mevalonate kinase activity

Mevalonate kinase activity is associated with the nonsedimentable fraction of *Neurospora* extracts (Table 1). The product is shown below to be 5-phosphomevalonate.

Activities of HMG CoA reductase and mevalonate kinase during the asexual life cycle

Soluble and microsomal protein concentrations increase rapidly after about 1 day of growth and attain peak values at about 4 days (Fig. 2, *top*). Total cell mass increases linearly after 1 day to at least day six. HMG CoA reductase and mevalonate kinase are present in conidia at low activity (0.15 and 4.0 mU/mg of protein, respectively). After 12 hr growth, the specific activities of both enzymes have increased about threefold (Fig. 2, *middle* and *bottom*). Both reductase and kinase specific activities peak at about 2 days, and the total activities of both enzymes peak at 3–4 days. When total HMG CoA reductase and mevalonate kinase activities are plotted as log functions, the lines have similar slopes. Total and specific activities subsequently decline (Fig. 2, *middle* and *bottom*). The specific activities of both enzymes increased at a rate greater than that of protein in either fraction, however.

Since the changes in activity observed during growth might reflect activation and/or subsequent inhibition of existing activities rather than altered enzyme levels, the soluble and microsomal fractions from conidia and from mycelia harvested after 4.5 days of growth were mixed with the corresponding fractions from a 2-day-old culture. The mixed microsomal fractions were assayed for HMG CoA reductase and the soluble fractions for mevalonate kinase activity. The amount of product formed did not differ significantly from that predicted from the sum of the activities of the individual fractions. The rise and fall of both enzyme activities thus appear to reflect real changes in activity rather than artifacts due to interfering enzymes or to nonphysiological activators or inhibitors.

Effect of the presence during growth of ergosterol and β-carotene on HMG CoA reductase and mevalonate kinase activity

Addition of ergosterol and β-carotene to the growth medium, either alone or in combination, at concentrations of 0.50, 2.5, or 5.0 mg/ml of growth medium (shake cultures) during the first 15 hrs of growth had no detectable effect on the activity of either enzyme. Cultures appeared to grow normally in the presence of either compound, as indicated by the equivalent quantities of mycelia present in control and experimental flasks.

Purification of mevalonate kinase

All operations were carried out at about 4°C. Pieces of frozen mycelia, about 500 g, were homogenized for 5 min at low speed in a 4-l Waring blender containing 1,000 g of acid-washed sand and 1.5 l of 1.0 mM EDTA–10 mM 2-mercaptoethanol–50 mM potassium phosphate, pH 7.5. The resulting slurry was centrifuged at 9,000 *g* for 10 min, and the supernatant liquid was decanted and centrifuged at 54,000 *g* for 60 min. The supernatant liquid was retained as the 54K *g* supernatant fraction. Since this fraction loses activity when stored at 5 or −20°C, it was immediately fractionated with ammonium sulfate. Ho-

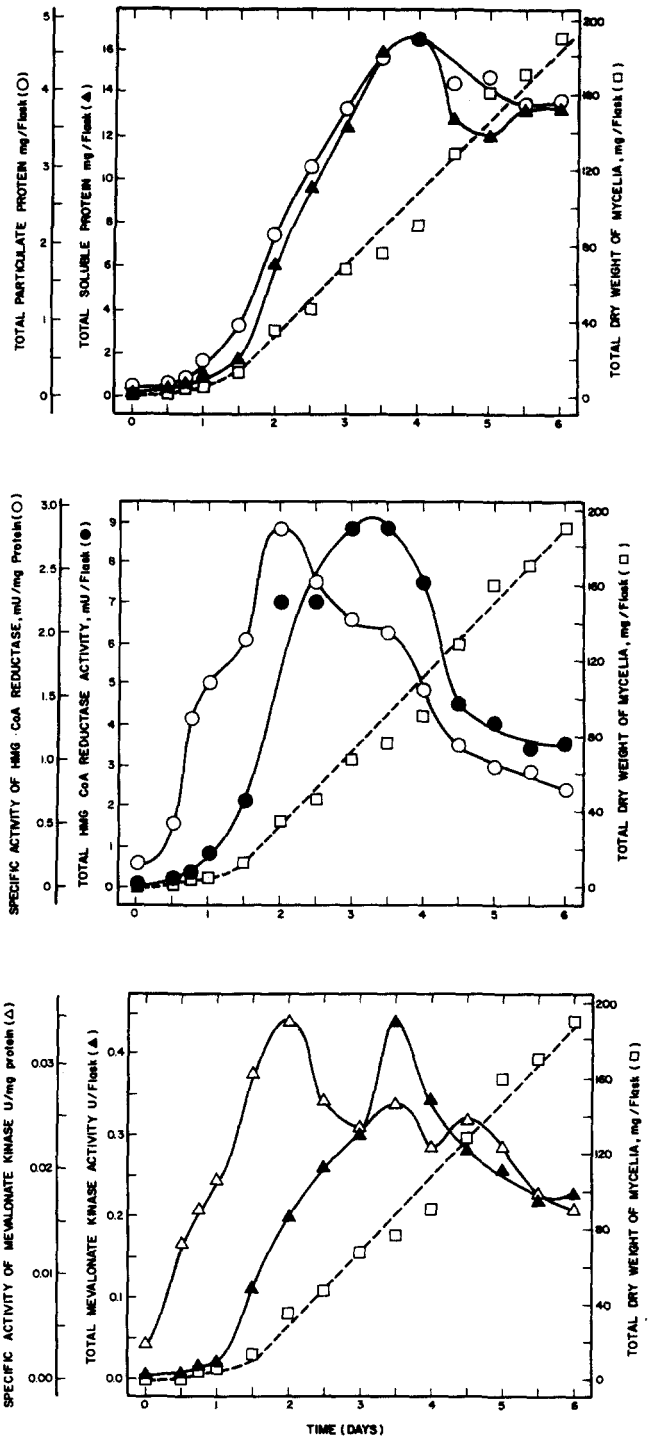

Fig. 2. Total soluble and particulate protein, HMG CoA reductase, and mevalonate kinase during the asexual life cycle. Protein present in the 70,000 *g* pellet and supernatant fractions was assayed by the biuret method *(top)*. For assay of HMG CoA reductase *(middle)* and mevalonate kinase *(bottom)* activities, at least two flasks were used at each time. Cell-free extracts of stationary cultures prepared by grinding with glass beads were centrifuged at 10,000 *g* for 10 min. The 10,000 *g* supernatant solution was then centrifuged at 70,000 *g* for 60 min to give the 70,000 *g* pellet and supernatant fractions. The 70,000 *g* pellet was suspended in 5.0 mM EDTA–10 mM 2-mercaptoethanol–50 mM potassium phosphate, pH 7.0, and assayed for HMG CoA reductase activity. The 70,000 *g* supernatant fraction was assayed for mevalonate kinase activity.

mogenization buffer was added to the 54K *g* supernatant fraction to adjust its protein concentration to 6.0 mg/ml. To each liter of diluted 54K *g* supernatant fraction, 613 ml of $(NH_4)_2SO_4$ solution, pH 7.0, saturated at 25°C, was added. Protein precipitating at 0–38% saturation was removed by centrifugation at 9,000 *g* for 10 min and discarded. $(NH_4)_2SO_4$ solution, 300 ml/l of original 54K *g* supernatant fraction, was added and the mixture was centrifuged as before. The supernatant liquid was discarded. The 38–48% $(NH_4)_2SO_4$ precipitate was dissolved in a minimum volume of 10 mM 2-mercaptoethanol–100 mM potassium phosphate, pH 7.5, and dialyzed for 5 hr against two changes of 3 l of this buffer. At this stage, mevalonate kinase is stable to storage at −20°C for several weeks. To facilitate fractionation on DEAE cellulose, a peristaltic pump was used to maintain a flow rate of 1.0 ml/min. Dialyzed $(NH_4)_2SO_4$ fraction, about 100 ml, was pumped into a column, 2.5 × 50 cm, of DEAE cellulose equilibrated with 10 mM 2-mercaptoethanol–100 mM potassium phosphate, pH 7.5. The column was washed with 2 column volumes (about 500 ml) of this buffer then eluted with a linear 100 to 250 mM phosphate gradient. The mixing chamber contained 1 l of 10 mM 2-mercaptoethanol–100 mM potassium phosphate, pH 7.5, and the reservoir 1 l of 10 mM 2-mercaptoethanol–250 mM potassium phosphate, pH 7.5. The effluent was concentrated about fivefold with an Amicon on-line concentrator to permit use of the spectral assay to monitor the kinase activity of column fractions. The active fractions, which emerged at a mean phosphate concentration of 170–180 mM, were combined and diluted to a phosphate concentration of 100 mM and a protein concentration of 1.0 mg/ml to give the DEAE fraction. The kinase activity of this and succeeding fractions decreased significantly when stored at 5 or −20°C. 0.5 ml of $Ca_3(PO_4)_2$ gel, 1.0 mg dry weight/ml (41), was added to each ml of DEAE fraction. After 5 min, the suspension was centrifuged at 2,000 *g* for 5 min and the precipitate was discarded. $Ca_3(PO_4)_2$ gel, 2.0 ml/ml of original DEAE fraction, was added to the supernatant solution; the mixture was permitted to stand for 5 min and then centrifuged as before. The pelleted gel was washed by resuspension and centrifugation using 12-ml portions of the following buffers at pH 7.5: 10 mM 2-mercaptoethanol–25 mM potassium phosphate (twice) and 10 mM 2-mercaptoethanol–50 mM potassium phosphate (twice). Mevalonate kinase was then eluted by extracting the gel three times with 10 mM 2-mercaptoethanol–100 mM potassium phosphate. The extracts were combined and concentrated to a volume of 2–3 ml in an Amicon ultrafiltration cell with a PM 30 filter to give the calcium phosphate gel fraction. This fraction is free from phosphomevalonate kinase activity. The gel fraction was adjusted to a concentration of 5% sucrose by addition of solid sucrose and layered onto a column, 2.5 × 38 cm, of Sephadex G-200 equilibrated with 0.01

TABLE 4. Stoichiometry of the mevalonate kinase reaction[a]

Substrate or Product	Change in Concentration
	μmoles
Mevalonate	−4.5
ATP	−4.5
5-Phosphomevalonate	+4.5
ADP	+4.3

[a] Sephadex fraction, 400 μg, was incubated, in 1.5 ml, with 75 μmoles of triethanolamine·HCl, pH 8.0, 375 nmoles of dithiothreitol, 27 μmoles of ATP, 30 μmoles of $MgCl_2$, and 9.0 μmoles of DL-[2-^{14}C]mevalonate (sp act, 158 cpm/nmole). After 55 min at 30°C, the reaction mixture was placed in a boiling water bath for 5 min and centrifuged to remove precipitated protein, and the supernatant liquid was applied to a column, 1.5 × 15 cm, of DEAE cellulose equilibrated with 20 mM NH_4HCO_3. The column was eluted at 5°C with a linear NH_4HCO_3 gradient (375 ml of 20 mM NH_4HCO_3 in the mixing chamber and 375 ml of 200 mM NH_4HCO_3 in the reservoir) (42). 7.0-ml fractions were collected at a flow rate of 36 ml/hr. The absorbancy at 260 nm and the radioactivity present in 0.1-ml portions of all fractions were determined. Two peaks containing radioactivity (fractions 9–12 and 43–47) and two containing material that absorbed at 260 nm (fractions 57–63 and 65–78) were observed. Peaks 3 and 4 were identified as ADP and ATP, respectively, from their ultraviolet absorption spectra and R_F values after cellulose TLC in isobutyric acid–15 N NH_4OH–H_2O 66:1:33 (v/v). The quantities of ATP and ADP present were determined spectrophotometrically at 259 nm. The two radioactive peaks, which together contained over 99% of the initial radioactivity, were identified as mevalonic acid and 5-phosphomevalonic acid, respectively, by their R_F values after cellulose TLC in 1-butanol–formic acid–H_2O 77:10:13 (v/v) and in 2-methyl-2-propanol–formic acid–H_2O 20:5:8 (v/v) (43). Material tentatively identified as 5-phosphomevalonic acid was converted to mevalonic acid and inorganic phosphate when incubated with alkaline phosphatase. The quantities of mevalonate consumed and of 5-phosphomevalonate formed were calculated from the observed radioactivity in each peak and the specific radioactivity of the mevalonate used. The quantities of ADP and ATP present were calculated from spectral data.

mM EDTA–0.5 mM dithiothreitol–100 mM triethanolamine·HCl, pH 7.5. The column was eluted with the triethanolamine buffer at a flow rate of 0.2 ml/min. Fractions containing mevalonate kinase were combined, concentrated to about 2 ml in an ultrafiltration cell, and diluted with an equal volume of 50% glycerol in the above buffer to give the Sephadex fraction. The entire procedure requires about 3.5 days. Table 3 summarizes a typical purification. Approximately one-fourth of the initial activity of the purified enzyme was lost after 3 wk storage at −20°C in 25% glycerol. 1.0 M sucrose or 0.50 mM MgATP also stabilizes the purified kinase to storage at −20°C to about the same extent as glycerol.

Stoichiometry

1 mole each of 5-phosphomevalonate and ADP were produced for each mole of mevalonate and of ATP consumed (Table 4). The equation for the reaction is thus: mevalonate + ATP = 5-phosphomevalonate + ADP.

TABLE 5. Effect of divalent cations on mevalonate kinase activity[a]

Addition	Specific Activity of Mevalonate Kinase Measured at a Cation/ATP Ratio of: 0.1	1.0	1.5
	nmoles/min/mg		
None	5	5	5
$MgCl_2$	95	750	780
$MnCl_2$	190	100	60
$FeSO_4$	37	37	26
$CaCl_2$	10	47	37
$Co(NO_3)_2$	26	10	5
$Ni(NO_3)_2$	10	10	5
$Cu(NO_3)_2$	5	5	5
$ZnSO_4$	0	5	<1

[a] The effect of divalent cations on mevalonate kinase activity was measured at molar ratios of cation to ATP of 0.1, 1.0, and 1.5. Incubation mixtures containing, in 0.2 ml, 20 μmoles of triethanolamine·HCl, pH 8.0, 0.1 μmole of dithiothreitol, 1.9 μmoles of DL-[2-^{14}C]mevalonate, 2.0 μmoles of ATP, and either 0.2, 2.0, or 3.0 μmoles of the indicated metal salts were incubated at 30°C for 2 min prior to addition of 19 μg of mevalonate kinase. Kinase activity was determined by the radioisotopic assay.

Homogeneity

Electropherograms of the Sephadex fraction on 7.5% acrylamide gels at pH 8.5 revealed multiple bands when stained with Coomassie blue. When sections of unstained gel were macerated in 0.50 mM dithiothreitol–100 mM Tris·HCl, pH 7.5, and assayed, mevalonate kinase had a mobility relative to the tracking dye of 0.32. Although the most highly purified preparation contained several proteins, the contaminants were without effect on the substrates or products of the mevalonate kinase reaction. The Sephadex fraction (mevalonate kinase sp act, 5,000 nmoles/min/mg) was free from NADH oxidase (sp act, < 0.3 nmole/min/mg), ATPase (sp act, < 1.3 nmoles/min/mg), and adenylate kinase activities (sp act, < 2 nmoles/min/mg). Even crude fractions are free from HMG CoA reductase activity, which is associated with the fraction sedimenting at 70,000 *g* (Table 1), and the calcium phosphate gel and Sephadex fractions are free from detectable phosphomevalonate kinase activity as determined by radioautography. The recovery of substrates and products in the stoichiometry experiment (Table 4) confirms the absence from the purified kinase of enzymes acting on mevalonate, 5-phosphomevalonate, ATP, or ADP.

Requirement for a divalent metal ion

Although kinase activity is significantly stimulated by Mg^{2+}, Mn^{2+}, Fe^{2+}, Ca^{2+}, and Co^{2+}, Mg^{2+} and Mn^{2+} were most effective at all ratios of metal ion to ATP tested (Table 5). Mg^{2+} is far more effective at equimolar concentrations or when present in 50% excess over ATP. The ability of Mn^{2+}, but not of Mg^{2+}, to stimulate ki-

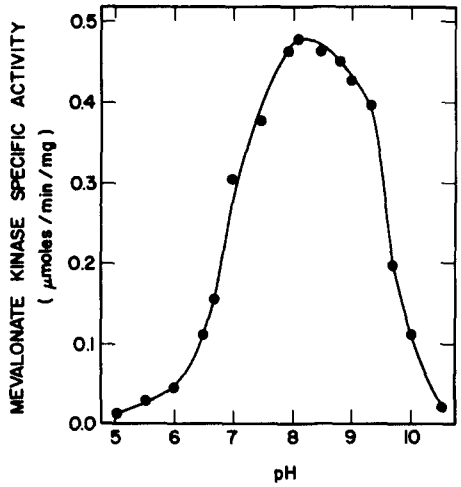

Fig. 3. Effect of pH on velocity of the mevalonate kinase reaction. Reaction mixtures contained, in 0.2 ml, 0.1 μmole of dithiothreitol, 4.0 μmoles of ATP, 2.0 μmoles of $MgCl_2$, 1.9 μmoles of DL-[2-^{14}C]mevalonate, 10 μg of kinase protein, and 40 μmoles of potassium acetate (pH 5.0–5.5), potassium phosphate (pH 5.5–7.5), triethanolamine (pH 7.5–8.8), or glycine (pH 8.8–10.5) buffer. Analysis of kinase activity was by the isotopic method. The recorded pH values are those measured after incubation.

nase activity decreased as more metal ion was added. Although low concentrations of Mn^{2+} activate mevalonate kinase, the preferred activator thus appears to be Mg^{2+}.

Effect of pH and temperature

Optimal activity was observed at pH 8.0–8.5 (Fig. 3). The same pH optimum was obtained using 25 mM DL-mevalonate and 50 mM ATP with either 25 mM or 5.0 mM $MgCl_2$. Optimal activity occurred at 50°C, and an activation energy of about 9.4 kcal (39.5 J)/mole was calculated from a graph of log v vs. $1/T$.

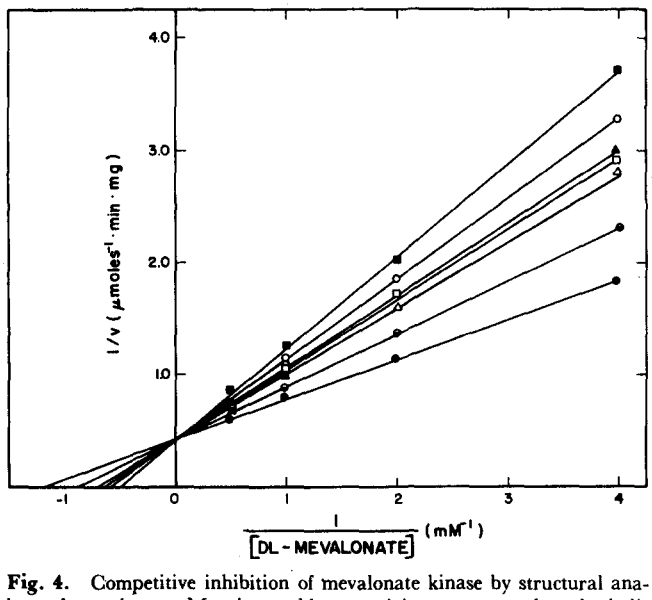

Fig. 4. Competitive inhibition of mevalonate kinase by structural analogs of mevalonate. Mevalonate kinase activity was assayed at the indicated concentrations of DL-mevalonate in the presence and absence of the indicated additions under otherwise standard conditions for the spectrophotometric assay. Additions: ●, none; ■, 0.8 mM DL-mevaldate; ○, 20 mM 3-hydroxy-3-methylglutarate; △, 40 mM 3,3′-dimethylglutarate; □, 50 mM DL-3-hydroxybutyrate; ▲, 50 mM 4-hydroxy-4-methyl-2-pentanone; and ◒, 100 mM DL-1,3-butanediol.

TABLE 6. Inhibition of mevalonate kinase by analogs of mevalonate[a]

Inhibitors	K_i	K_i/K_m
	mM	
DL-Mevaldate	0.73	0.88
3-Hydroxy-3-methylglutarate	14	17
4-Hydroxy-4-methyl-2-pentanone	56	66
DL-3-Hydroxybutyrate	70	84
3,3′-Dimethylglutarate	80	96
DL-1,3-Butanediol	333	400

[a] Mevalonate kinase activity was assayed with four concentrations of DL-mevalonate as the variable substrate in the presence and absence of inhibitors under otherwise standard spectrophotometric assay conditions. The concentrations of inhibitors used were 0.80 mM DL-mevaldate, 20 mM 3-hydroxy-3-methylglutarate, 40 mM 3,3′-dimethylglutarate, 50 mM DL-3-hydroxybutyrate, 50 mM 4-hydroxy-4-methyl-2-pentanone, and 100 mM DL-1,3-butanediol. K_i values for the active isomers were calculated from the slopes and intercepts of a plot of $1/v$ vs. 1/mevalonate. All lines intersected on the $1/v$ axis, indicating all were inhibitors competitive with mevalonate.

Ability of other nucleoside triphosphates to replace ATP

MgUTP, MgCTP, MgITP, and MgGTP, tested at 1.5 and 15 mM concentration, were less than 4% as effective as MgATP as a phosphate donor. Nevertheless, since these other nucleoside triphosphates were free from detectable ATP, the observed activities appear to represent a limited ability of these nucleoside triphosphates to replace ATP.

Inhibition by *p*-hydroxymercuribenzoate

Mevalonate kinase is inhibited by *p*-hydroxymercuribenzoate, and this inhibition is prevented by dithiothreitol. The extent of inhibition is significantly decreased if either mevalonate or MgATP is present. MgATP protects more effectively than mevalonate. $MgCl_2$ at concentrations of 5 or 25 mM has no protective effect.

Inhibition by structural analogs of mevalonate

Several compounds structurally related to mevalonate inhibited competitively with mevalonate (Fig. 4). Of those tested, DL-mevaldate was by far the most effective (Table 6). The only primary alcohol tested, 1,3-butanediol, did not replace mevalonate as substrate, as determined by its inability to form ADP in the standard spectrophotometric assay.

Initial rate studies

The initial rate of the mevalonate kinase reaction was measured at four different concentrations each of DL-mev-

alonate and MgATP. When the data were plotted with either mevalonate (Fig. 5) or MgATP as the variable substrate, a pattern of lines intersecting below the negative x axis was obtained. This is the pattern for a sequential mechanism (44). When the data were fitted to the two-substrate equation for a sequential initial velocity pattern (44), the Michaelis constants for DL-mevalonate and $MgATP^{-2}$ were 2.8 ± 0.4 and 1.8 ± 0.2 mM, respectively. We conclude that *Neurospora* mevalonate kinase undergoes sequential addition of substrates.

Inhibition by geranyl pyrophosphate

Mevalonate kinase activity was measured in the presence of four concentrations each of geranyl pyrophosphate and MgATP. A double-reciprocal plot characteristic of competitive inhibition was obtained (Fig. 6). The K_i for geranyl pyrophosphate was 0.11 mM.

DISCUSSION

Mevalonate kinase of *Neurospora crassa,* like that of yeast (16), mammalian liver (14), *Sarcophaga bullata* (45), and *Euglena gracilis* (13), resides in the cytosol. By contrast, over 98% of the HMG CoA reductase activity is associated with the microsomal fraction. Reductase activity initially sedimenting in the mitochondrial fraction is separable from a mitochondrial marker enzyme, and thus probably reflects microsomal contamination of the mitochondria. In yeast, HMG CoA reductase is mitochondrial and is induced when cultures are switched from an anaerobic to an aerobic environment (46). Rat intestine appears to contain both mitochondrial and microsomal HMG CoA reductase (47). The reductase of *Neurospora,* however, appears to resemble more closely that of rat liver, which is present solely in the microsomal fraction (48, 49).

Neurospora HMG CoA reductase, in common with all known HMG CoA reductases except that of *Pseudomonas* (50), utilizes NADPH rather than NADH as reductant, a specificity typical of biosynthetic pathways in eukaryotes. It is thus of interest that *Neurospora* glucose-6-phosphate dehydrogenase (5), a major source of NADPH, exhibits an activity–time profile similar to that of HMG CoA reductase.

The first detectable increases in HMG CoA reductase and mevalonate kinase activities occur during or shortly after spore germination. Both activities continue to rise until conidiation begins, at which time both activities decrease. That the observed increases in activity represent neither relief of inhibition nor activation of preexisting enzyme is suggested by the mixing experiment. We therefore infer that these increases in activity probably represent synthesis of new enzyme protein. Despite their occurrence in different cell fractions, the synthesis of HMG CoA reductase and mevalonate kinase appears to be temporally

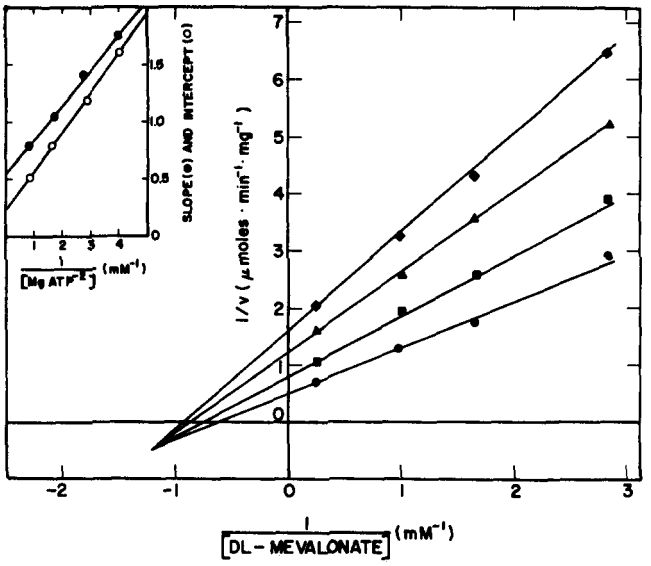

Fig. 5. Initial velocity pattern of mevalonate kinase as a function of DL-mevalonate concentration at four concentrations of $MgATP^{-2}$. Standard spectrophotometric assay conditions were used except for DL-mevalonate concentration and $MgCl_2$ concentration, which was always 2 mM in excess of $MgATP^{-2}$. $MgATP^{-2}$ concentrations: ●, 1.20 mM; ■, 0.60 mM; ▲, 0.35 mM; and ◆, 0.25 mM. The inset represents the slopes and intercepts of the data in the main figure.

coordinated. *Neurospora* (51) and other fungi (2) accumulate ergosterol early in development, and the rapid appearance of orange pigment in mycelia exposed to air during filtration suggests that carotenoids and their colorless C_{40} precursors are present even during early growth. All the enzymes of ergosterol and carotenoid synthesis may thus be expressed more or less coordinately early in the

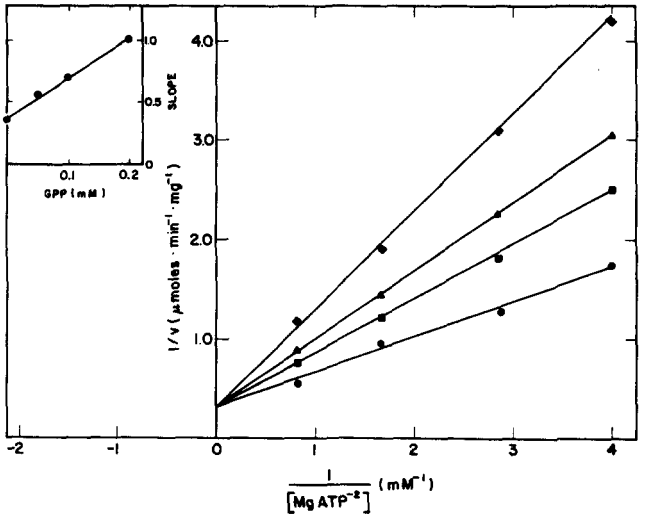

Fig. 6. Inhibition of mevalonate kinase by geranyl pyrosphosphate competitive with $MgATP^{-2}$. Standard conditions for the spectrophotometric assay were used except for DL-mevalonate concentration (10 mM) and $MgATP^{-2}$ concentration. $MgCl_2$ concentration was always 2 mM in excess of $MgATP^{-2}$. Concentrations of geranyl pyrophosphate (GPP): ●, none; ■, 0.049 mM; ▲, 0.098 mM; and ◆, 0.196 mM. The inset represents the slope of the lines in the main figure.

TABLE 7. Properties of mevalonate kinases from various sources

Source	Purification	Maximal Specific Activity[a]	Optimal pH	K_m	
				ATP	DL-Mevalonate
	fold	*nmoles/min/mg*		*mM*	
Hog liver (14, 21)	600	17,000	7.3	0.30	0.019
Neurospora crassa	200	5,000	8.0–8.5	1.8	2.8
Hevea latex (19)	140	1,600	7.5	2.0	0.13
Yeast (16)	50	600	6.4–6.7		
Rat ovary (20)		120[b]	7.0–7.5	0.12	0.0036
Rabbit liver (17)	100	110[c]	7.8		5.1
Sarcophaga bullata (15)	110	30	7.1	4.7	0.62
Pinus radiata (54)		5	6.0		
Euglena gracilis (13)	8			6.0	0.06
Cucurbita pepo (18)	3		5.5–6.0		

[a] Assayed at 30°C in the presence of Mg^{2+} except where otherwise noted.
[b] Assayed at 25°C.
[c] Assayed at 35°C.

growth cycle when amphibolic intermediates are in ample supply.

From 36 to 48 hr after inoculation, stationary cultures cover the surface of the medium and aerial hyphae and conidia appear. The total activities of both HMG CoA reductase and mevalonate kinase continue to increase, but at a rate less than that of total cellular protein. This suggests that the organism switches from synthesis of proteins associated with germination and vegetative growth (e.g., HMG CoA reductase and mevalonate kinase) to synthesis of those associated with conidiation. This view is consistent with the observation of Urey (52) that the synthesis of specific enzymes is initiated when conidiation begins.

In *Phycomyces,* exogenous ergosterol is reported to inhibit formation of ergosterol from acetate, HMG, and mevalonate (53), and addition of 10 μg of ergosterol/ml of growth medium inhibits incorporation of acetate into ergosterol in yeast (9). This inhibition occurs at the reaction catalyzed by HMG CoA reductase (9). However, addition of the "end products," ergosterol or β-carotene, at levels as high as 100 μg/ml failed to depress the activities of *Neurospora* HMG CoA reductase or mevalonate kinase. Therefore, end product regulation of ergosterol synthesis may not occur in *Neurospora.* This hypothesis is supported by the observation that *Neurospora* accumulates crystalline ergosterol (51). Organisms such as *Neurospora* that synthesize both steroids and carotenoids might regulate their biosynthesis at the level of farnesyl pyrophosphate, the branch-point compound of the two pathways. Alternatively, regulation of polyisoprenoid synthesis might occur via elimination of enzymes of the polyisoprenoid pathway when conidiation begins and they appear to be no longer needed.

While not physically homogeneous, *Neurospora* mevalonate kinase is free from enzymes acting on the substrates or products and has a specific activity greater than that from any source other than hog liver (Table 7). Attempts to achieve further purification using affinity columns of HMG, ATP, or ADP attached to Sepharose-4B were unsuccessful.

Although all mevalonate kinases tested preferentially utilize MgATP as phosphate donor, their specificity with respect to other nucleoside triphosphates varies widely. Yeast mevalonate kinase (16), and to a lesser extent that from pumpkin seedlings (18), exhibits broad nucleoside triphosphate specificity. Others, for example that from hog liver, which utilizes ITP in addition to ATP (21), exhibit intermediate specificity. That from *Neurospora* appears to be highly specific for ATP.

Mevalonate kinases typically require either Mg^{2+} or Mn^{2+}. Other divalent metals may give small amounts of activity. Frequently, Mn^{2+} is best at low and Mg^{2+} at high concentrations. This is the case for the *Neurospora* kinase, which is inhibited by high concentrations of Mn^{2+}.

The pH optimum of 8.0–8.5, the highest for any mevalonate kinase, matches more closely that of mammalian tissues than that of yeast (Table 7). Using concentrations of ATP and $MgCl_2$ calculated to give equivalent concentrations of $MgATP^{-2}$ at pH values as low as 6.0 gave the same pH optimum. The kinase is active at pH 6 but is inactivated below pH 5.5.

Of the mevalonate analogs tested, only mevaldate was a potent inhibitor. This compound most closely resembles the substrate. 3-Hydroxy-3-methylglutarate, which bears a negatively charged group at both ends, is 20 times less effective as an inhibitor. Replacement of the 3-hydroxy group by a methyl group results in a further sixfold decrease in inhibitory activity. All other compounds that were weak competitive inhibitors have some structural characteristics of mevalonate. This suggests that the enzyme is relatively specific for its natural substrate.

Although hog liver mevalonate kinase appears to be the most readily inactivated in the absence of sulfhydryl re-

agents, all kinases tested are inactivated by mercury compounds. The hog liver kinase is protected from both *p*-hydroxymercuribenzoate and monoiodoacetamide inactivation by mevalonate or ATP (14). That from *Neurospora* is protected from *p*-hydroxymercuribenzoate inhibition by either substrate, but MgATP is far more effective than mevalonate. Possibly, substrate binding protects a sulfhydryl at or near the active site or induces a conformational change elsewhere that buries a previously exposed sulfhydryl group.

The initial rate pattern for *Neurospora* mevalonate kinase, which indicates sequential addition of substrates, is characteristic of phosphotransferases such as creatine kinase (55), yeast hexokinase (56), *Escherichia coli* galactokinase (57), and brain pyridoxal kinase (58) that have two dissimilar substrates. Nucleotide diphosphate kinase, for which both substrates are similar, has a ping-pong mechanism (59, 60).

Geranyl pyrophosphate is an inhibitor competitive with MgATP, as was found for mevalonate kinase from hog liver (12) and from rat ovary (20). Although for the hog liver kinase the apparent K_i to K_m ratio is about 4×10^{-3}, the ratio for the *Neurospora* kinase is about 6×10^{-2}. The hog liver enzyme is thus far more strongly inhibited by geranyl pyrosphosphate.

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