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Biosynthesis of Polyketides[1] The Synthesis of 6-Methylsalicylic Acid and Triacetic Acid Lactone in *Penicillium patulum*[2]

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An enzyme fraction from *P. patulum* has been prepared which will synthesize 6-MSA[5] and fatty acids from acetyl CoA, malonyl CoA and NADPH. The 6-MSA synthetase was purified threefold by ammonium sulphate fractionation and catalyses the formation of 0.86 nmoles of 6-MSA per minute per mg of protein. Chemical degradation of ^{14}C-6-MSA biosynthesized from 1,3-^{14}C-malonyl CoA, acetyl CoA and NADPH gave a ratio of 2.39/1 for the radioactivity in the aromatic ring to that in the terminal carboxyl group. This is consistent with the acetate–polymalonate origin of 6-MSA; the deviation from the theoretical value of 2/1 is probably due to malonyl CoA decarboxylase activity leading to the formation of 1-^{14}C-acetyl CoA. The rate of synthesis of 6-MSA was generally about ten times faster than the rate of fatty acid synthesis, but this ratio varied with the preparation. The 6-MSA synthetase is inhibited by iodoacetamide and *N*-ethymaleimide, which suggests that the synthetase activity contains sulphydryl groups in the active sites.

The 6-MSA synthetase activity is inhibited by the following acetylenic thioesters: 3-pentynoyl-NAC, 3-hexynoyl-NAC and 2-hexynoyl-NAC. The activity is not inhibited by 3-hexynoic acid. The synthesis of 6-MSA can be abolished at a concentration of 10^{-4} *M* 3-hexynoyl-NAC without affecting the synthesis of free palmitic and stearic acid. The synthesis of 6-MSA by *P. patulum* under *in vivo* conditions is also inhibited by 3-hexynoyl-NAC.

The syntheses of 6-MSA and TAL (in the presence of NADPH) are inhibited to comparable extents by 5×10^{-5} *M* 3-hexynoyl-NAC while the synthesis of fatty acids is not inhibited below 10^{-4} *M*. The mechanism of 6-MSA synthesis is discussed in relation to the formation of TAL and to the possible mode of action of the acetylenic thioester inhibitors.

6-Methylsalicylic acid (6-MSA), a metabolite of *P. patulum*, is a typical example of polyketide-derived aromatic metabolites occurring in fungi, lichens, and higher plants (*1–3*). The polyacetate origin of these metabolites was suggested independently by Collie (*4*) in 1907, Birch (*5*) in 1952, and confirmed by experiment (*1*, *5–7*). Further development showed that the biosynthesis of penicillic acid (*8*) and 6-methylsalicylic acid (*9*) involved the condensation of a starter acetyl group with chain elongating

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[5] The abbreviations used are: NAC, *N*-acetylcysteamine; 6-MSA, 6-methylsalicylic acid; TAL, triacetic acid lactone.

malonyl units, thus illustrating the formal relationship to the mechanism of synthesis of the fatty acids (*10, 11*).

Studies on the detailed enzymology of systems for the synthesis of polyketide-derived aromatic compounds have lagged behind the progress made in the study of fatty acid biosynthesis (*12*). Partially purified systems have been obtained that will synthesize such compounds as alternariol (*13*), orsellinic acid (*14*), 6-methylsalicylic acid (*15–17*), and 5-methylorsellinic acid (*18*) but the general instability of the enzymes involved has hindered progress both in the purification of the enzymes and in the detection of intermediates.

Previous work in our laboratories has been concerned[6] with the development of the chemistry of several of the putative intermediates of polyketide biosynthesis. In order to relate this chemistry with the intermediary metabolism of aromatic biosynthesis, we began in 1965 the study of cell free biosynthesis based on the work of Lynen and Tada (*17*). After much experimentation a system was developed which has allowed us to examine inhibition of an intermediate stage of aromatic biosynthesis, i.e., before the actual formation of 6-MSA. This work also served to confirm the results of Lynen and Tada (*17*) and of Light (*15, 16*). More recently the purification and properties of 6-MSA synthetase were reported by Dimroth, Walter and Lynen (*19*). Certain aspects of the mechanism of 6-MSA synthesis as adumbrated in the latter work have been examined during our studies on the inhibition of the synthetase using acetylenic thioesters.

This paper describes the isolation of an ammonium sulphate protein fraction from *P. patulum* which synthesizes 6-methylsalicylic acid, triacetic acid lactone and fatty acids. The synthesis of triacetic acid lactone is maximal in the absence of NADPH but is also shown to occur at concentrations of NADPH which are optimal for the synthesis of 6-methylsalicylic acid. In this respect the synthesis of triacetic lactone by this system is similar to the *E. coli* system described by Brock and Bloch (*20*) but different from the fatty acid synthetases from pigeon liver (*21*) and yeast (*22*) which effect the synthesis of triacetic acid lactone in the absence of NADPH. The implication of this result is discussed in terms of a possible mechanism for the synthesis of 6-methylsalicylic acid. The effect of inhibitors, which have been shown to act specifically on dehydrase enzymes that lead to the formation of β,γ-*cis*-enoates, has been studied with the 6-methylsalicylic acid synthetase. In this context 3-hexynoyl-NAC, 2-hexynoyl-NAC and 3 pentynoyl-NAC inhibit the synthesis of 6-methylsalicylic acid at concentrations which do not affect the synthesis of saturated fatty acids. The possible role of these inhibitors is discussed in relation to the synthesis of 6-methylsalicylic acid and compared to their action of β-hydroxydecanoylthioester dehydrase (*23*) whose mechanism of action has been elucidated (*24*).

EXPERIMENTAL PROCEDURE

Materials

Experimental materials were obtained from the following sources. Acetyl CoA, malonyl CoA and NADPH were procured from P-L Biochemicals and 1,3-^{14}C-malonyl CoA from New England Nuclear Corporation. Tris base, β-mercaptoethanol, ammonium sulphate (enzyme grade), palmitic, stearic and oleic acid were purchased from

[6] The results of these synthetic experiments have been published in preliminary form (for a review see A. I. Scott, *Chimia*, **22**, 1968) and are detailed by A. I. Scott, H. Guilford and D. Skingle, *Tetrahedron* **27**, 3039 (1971).

Mann Research Laboratories. Bovine serum albumen was purchased from Calbiochem. EM-Reagent precoated thin layer plates were obtained from Brinkman Instruments Inc. 6-Methylsalicylic acid was synthesized by the method of Eliel et al. (*25*). Triacetic acid lactone was a gift from Dr. H. Guilford. 3-Hexynoyl, 3-pentynoyl and 2-hexynoic acid were from the Chemical Sample Company. 3-Pentynoic acid and 3-hexynoic acid were prepared from the corresponding alcohols by the method of Knight and Diamond (*26*). *N*-Acetyl cysteamine was prepared by the method of Kuhn and Quadbeck (*27*) and purified as described by Basford and Huennekens (*28*). A culture of *Penicillium patulum* NRRL 2159A (RL-17, early MSA strain) was used in this study and was a generous gift from Dr. R. Light. Liquid scintillation counting was performed using Brays solution (*29*) in a Packard model liquid scintillation counter. Protein was determined by the Biuret method of Gornall, Bardawill and David (*30*) using Bovine serum albumen as internal standard.

Culturing Methods

Cultures were maintained on a malt agar medium and grown in a germinating medium with transfer to a Czapek–Dox medium as described by Light (*15*). In a typical experiment the spores from five culture slopes were added to ten, 250-ml Erlenmeyer flasks each containing 100 ml germinating medium and grown on a shake table at 28°C for 4 hr. The mycelium was harvested by filtration and macerated in 200 ml Czapek–Dox medium in a Sorvall Omnimixer for 10 sec. The resulting suspension was used as inoculum for twenty, 250-ml Erlenmeyer flasks, each containing 100 ml Czapek–Dox medium. The culture was grown as before for 4–6 hr after which the mycelium was filtered and washed with 0.25 *M* sodium chloride to give about 15 g.

Preparation of Enzyme Extract

Generally, 15 g mycelium was ground at 4°C for 10 min in a mortar with 30 g acid-washed sand and 50 ml 0.2 *M* Tris containing 10^{-3} *M* β-mercaptoethanol, 10^{-3} *M* EDTA, 0.25 *M* sodium chloride and 20% glycerin, pH 7.5. The cell mass was centrifuged at 18 800*g* for 15 min and the supernatant recentrifuged at 104 000*g* for 1 hr. The supernatant was brought to 35% saturation with solid ammonium sulfate and spun at 18 800*g* for 10 min. The pellet was discarded and the supernatant made 50% in ammonium sulfate, followed by centrifugation for 20 min as described above. The resulting pellet was dissolved in either 0.2 *M*, 0.05 *M* or 0.005 *M* potassium phosphate buffer pH 7.5 containing 10^{-3} *M* β-mercaptoethanol and 10^{-3} *M* EDTA. Slightly higher activities were obtained when 0.005 *M* buffer was used. The enzyme was unstable to storage except when dissolved in the above buffers containing 20% glycerin and stored at +4°C when most of the enzyme activity was retained for up to 24 hr.

Estimation of Enzyme Activity

The assay mixture contained 12 n*M* acetyl CoA, 66 n*M* 1,3-^{14}C-malonyl CoA, 200 n*M* NADPH, 40 n*M* 6-MSA, 0.2 μ*M* EDTA, 0.2 μ*M* β-mercaptoethanol, 40 μ*M* potassium phosphate buffer pH 7.5 and enzyme (0.4–0.8 mg) in a final volume of 0.42 ml. Samples were assayed at 28°C for 10 min after the addition of enzyme. The incubations were terminated by the addition of 0.05 ml 60% (w/v) perchloric acid, followed by 0.05 ml ethanol containing 0.5 mg each of 6-MSA and TAL and 0.1 mg each of palmitic, stearic and oleic acid. The mixture was extracted with 3 × 4 ml ether which was dried (Na_2SO_4) and evaporated to 0.2 ml under a stream of nitrogen. The extracts were chromatographed on silica gel plates (Brinkman F254, 20 × 20 cm and 0.2-mm thick) developed with benzene–dioxan–acetic acid (90:25:4). The 6-MSA and TAL were

detected by exposure to uv light and the oleic acid detected by exposure to iodine vapour. The R_f values obtained were: palmitic, stearic and oleic acid, 0.78; 6-MSA, 0.53; TAL, 0.3. The silica gel containing the carrier compounds was recovered and assayed for radioactivity in Brays solution (*29*).

The Synthesis and Identification of 6-MSA, TAL and Fatty Acids

6-MSA. A large-scale incubation for the synthesis of 6-MSA contained 0.144 μM acetyl CoA, 0.864 μM 1,3-^{14}C-malonyl CoA (sp act 5316 dpm/nM, 1.98 μM NADPH, 0.60 μM of 6-MSA in a volume of 1.56 ml. The reaction was started by the addition of 10.8 mg protein contained in 1.2 ml of the 104 000g supernatant, and incubated at 28°C for 1 hr. The incubation was terminated and the 6-MSA extracted into ether and chromatographed as described in the assay procedure. The synthetic 6-MSA was diluted with 50 mg carrier 6-MSA and crystallized to constant radioactivity from chloroform. The ^{14}C-6-MSA was diluted with carrier 6-MSA and converted to tribromo-*m*-cresol and CO_2 by the method of Light et al. (*31*). The tribromo-*m*-cresol was purified by preparative thin-layer chromatography on silica gel F254 developed in hexane–benzene–acetic acid (70:30:1), R_f 0.50. The material was eluted and crystallized to constant specific activity. The CO_2 was collected as $BaCO_3$ which was dried and reconverted to CO_2 by acidification with 0.7 N sulphuric acid. The CO_2 was collected in 6 ml hyamine solution contained in three Baryta tubes. The hyamine was assayed for radioactivity in Brays solution and the efficiency of counting determined from a calibration curve.

TAL

(*a*) *Synthesized in the absence of NADPH.* Radioactive TAL was synthesized in an incubation mixture containing 0.12 μM acetyl CoA, 0.66 μM 1,3-^{14}C-malonyl CoA (sp act 4142 dpm/nM), 0.35 mm potassium phosphate buffer pH 7.5, 1.7 μM EDTA, 1.7 μM β-mercaptoethanol and 6.4 mg of protein (35–50% ammonium sulfate fraction solubilized in 5×10^{-3} M phosphate buffer containing 10^{-3} M EDTA and 20% glycerin) in a total volume of 4.0 ml. The incubation was run at 28°C for 12 min and worked up as described in the assay section after adding 2.5 mg carrier TAL. The ethereal extract of the incubation mixture was chromatographed on silica gel F254 chromatogram plates and developed in chloroform–acetic acid (9:1). The TAL, R_f 0.3 was eluted and further treated with 50 mg carrier and recrystallized from ethyl acetate to constant radioactivity.

(*b*) *Synthesized in the presence of NADPH.* The TAL was obtained from twenty incubations, each containing 11 nM acetyl CoA, 66 nM 1,3-^{14}C-malonyl CoA (sp act 2580 dpm/nM) 0.2 μM NADPH 40 nM 6-MSA, 30 μM potassium phosphate buffer pH 7.5, 0.15 μM EDTA, 0.15 μM β-mercaptoethanol and 0.82 mg protein (35–50% ammonium sulfate fraction solubilized in 5×10^{-2} M phosphate buffer containing 10^{-3} M EDTA and 20% glycerin) in a volume of 0.41 ml. The incubations were run at 28°C for 10 min and the reaction terminated by the addition of 0.05 ml 60% (w/v) perchloric acid and 0.05 ml solution of 100 mg 6-MSA, 100 mg TAL, 20 mg each palmitic, stearic and oleic acid in 10 ml ethanol. The TAL was isolated as described above, diluted with 50 mg carrier and recrystallized from acetate to constant radioactivity.

Fatty Acids

The incubation mixture for the isolation of the fatty acids contained 0.12 μM acetyl CoA, 0.66 μM 1,3-^{14}C-malonyl CoA (sp act 4570 dpm/nM), 2.0 μM NADPH, 0.4 μM

6-MSA, 0.3 mm phosphate buffer pH 7.5, 1.5 μM EDTA, 1.5 μM β-mercaptoethanol and 4.92 mg enzyme (35–50% ammonium sulfate fraction solubilized in 0.2 M phosphate buffer pH 7.5 containing 10^{-3} M EDTA) in a volume of 4.6 ml. The incubation was carried out at 20°C for 10 min, and the reaction stopped by the addition of 0.5 ml perchloric acid, followed by the addition of 0.5 ml ethanol containing 5 mg 6-MSA and 1 mg each palmitic, stearic and oleic acid. The incubation mixture was extracted with 3×4 ml ether and the concentrated ethereal extract chromatographed as described in the assay section. The silica gel from the region of the fatty acids was eluted with ether, 1 mg each of the acids added and the solution treated with diazomethane. Aliquots of the esterified acids were assayed by gas–liquid chromatography on a Varian Autograph using a column of 15% ethyleneglycol succinate on a support of acid–base washed Celite, mesh 80–120 at 150°C using helium gas at a flow rate of 100 ml/min. Compounds emerging from the column were trapped in U tubes in a dry ice–acetone bath. Samples were eluted from the traps with 15 ml Brays solution which was assayed for radioactivity in a liquid scintillation counter.

Dependence of 6-MSA Synthesis on Time, Protein Concentration, Acetyl CoA, Malonyl CoA and NADPH

The protein used for the experiments was a 35–50% ammonium sulfate fraction solubilized in 0.2 M phosphate buffer pH 7.5 containing 10^{-3} M EDTA, 10^{-3} M β-mercaptoethanol and 20% glycerin. The incubations were performed as described in the assay section, with the exception of the substrate under study which was varied over the ranges shown in the requisite figures. Each incubation was duplicated and contained 0.5 mg protein, with the exception of the experiment which showed dependence on protein concentration.

Inhibition of Synthetases with Iodoacetamide and N-Ethylmaleimide

A series of tubes containing 0.73 mg protein on 0.1 ml 0.05 M phosphate buffer pH 7.5 containing 10^{-3} M EDTA and 20% glycerin were incubated independently with either iodoacetamide or N-ethylmaleimide to final concentrations of 0, 10^{-5}, 10^{-4}, 10^{-3} and 10^{-2} M in a total volume of 0.2 ml, at 28°C for 10 min. Each sample was treated with 0.225 ml of a solution containing 12 nM acetyl CoA, 66 nM 1,3-^{14}C-malonyl CoA, 40 nM 6-MSA, 200 nM NADPH, 20 μM phosphate buffer, 100 nM EDTA, and 100 nM β-mercaptoethanol pH 7.5. The enzyme was incubated with the substrates at 28°C for 10 min and the 6-MSA was isolated as described in the assay section.

Dependence of TAL Synthesis on Time

The rate of synthesis of TAL was determined both with and without NADPH.

(a) *With NADPH.* A series of tubes containing 0.64 mg enzyme (35–50% ammonium sulfate fraction) in 0.2 ml of 2.5×10^{-3} M phosphate buffer pH 7.5 containing 5×10^{-4} EDTA and 10% glycerin were preincubated at 28°C for 10 min. At this time 0.22 ml of a mixture containing 12 nM acetyl CoA, 70 nM 1,3-^{14}C-malonyl CoA, 200 nM NADPH, 40 nM 6-MSA, 30 μM phosphate buffer pH 7.5, 0.15 μM EDTA and 0.15 μM β-mercaptoethanol were added and the reactions stopped at time intervals of 3, 6, 9 and 12 min. The TAL was isolated and assayed for radioactivity as described in the assay procedure.

(b) *Without NADPH.* The enzyme was preincubated as above and incubated with 0.22 ml of a mixture containing 12 nM acetyl CoA, 70 nM 1,3-^{14}C-malonyl CoA, 35 μM phosphate buffer pH 7.5, 0.175 μM EDTA and 0.175 μM β-mercaptoethanol, at 28°C for times of 3, 6, 9 and 12 min and stopped as described before. The ethereal

extracts of the incubation mixtures were chromatographed on silica gel F254 plates developed with chloroform–acetic acid (9:1) and the TAL (R_f 0.3) was removed and assayed for radioactivity.

3-Hexynoic acid. This was prepared from 3-hexynol by the method of Knight and Diamond (*26*). The crude product was distilled to give 3-hexynoic acid bp 102–4°C/4.0 mm, mp 57–58°C [lit. mp 59–61°C (*31*)]; ir ν_{max} 2250 (—C≡C—), 1710 (CO_2H) cm^{-1}; nmr ($CDCl_3$) 3.30 (2 H, t, $J = 2$ Hz, C≡C—*CH_2*—CO), 2.20 (2 H, m, $J = 8$ and 2 Hz, CH_3—*CH_2*—C≡C—) and 1.15 (3 H, t, $J = 8$ Hz, *CH_3*—CH_2—).

3-Hexynoyl-NAC. This was synthesized from 3-hexynoic acid and the lead salt of *N*-acetyl-cysteamine (*28*) by the method of Bloch et al. (*32*) and crystallized from pentane at –20°C, mp 52–54°C; ir ν_{max} 3300 (NH), 1930 (v. weak, CH=C=CH), 1675 (—CO—S—), 2240 (—C≡C—), 1650, 1565 (amide I and II band) cm^{-1}; uv λ_{max} (methanol) 229 nm ($\epsilon = 3858$); nmr ($CDCl_3$) δ 6.30 (I H, broad s, *NH*) 3.40 (4 H, m, C≡C—*CH_2*—CO and —*CH_2*—CH_2), 3.15 (2 H, m, —CH_2—*CH_2*—), 2.25 (2 H, m, CH_3—*CH_2*—C≡C), 2.0 (3 H, s, *CH_3*CO), 1.15 (3 H, t, $J = 8$ Hz, CH_3—CH_2); a trace of allene representing 8% was detected at δ 5.90. tcl R_f 0.26 in methylene chloride–methanol (98:2).

3-Pentynoic acid. This was prepared as described for 3-hexynoic acid. The product was crystallized from hexane to give needles mp 107–109°C [lit. mp 102–4°C (*31*)] ir ν_{max} 2240 (—C≡C—) and 1710 (CO_2H) cm^{-1}.

3-Pentynoyl-NAC. This was synthesized by the method of Bloch et al. (*38*) and crystallized from pentane at –20°C giving needles mp 59–62°C; ir ν_{max} 3300 (NH), 2240 (—C≡C—, weak), 1940 (CH= C= CH, v. weak), 1700 (—CO—S—), 1630, 1550 (amide I and II band) cm^{-1}; uv λ_{max} (MeOH) 232 nm ($\epsilon = 3550$); nmr ($CDCl_3$) 3.45 (4 H, m, C≡C—*CH_2*—CO and —*CH_2*—CH_2—), 3.15 (2 H, m, —CH_2*CH_2*—), 2.03 (3 H, s, *CH_3*CO) 1.9 (3 H, t, $J = 2.5$ Hz, *CH_3*—C≡C—CH_2—), no allenic absorption was detected in the nmr; TLC R_f 0.24 in methylene chloride–methanol (98:2).

2-Hexynoyl-NAC. This was prepared by the method referenced above and crystallized from pentane at –20°C to give pale yellow crystals mp 51.5–53°C; ir 3300 (NH), 2230 (—C≡C—), 1675 (COS and amide I band) and 1550 (amide II band) cm^{-1}; uv λ_{max} (Methanol 226 ($\epsilon = 5587$) and 260 ($\epsilon = 5985$) nm; tlc R_f 0.26 in methylene chloride–methanol (98:2).

Inhibition Studies with N-*Acetyl-Cysteamine Derivatives of Acetylenic Acids*

(a) *The inhibition of 6-MSA and fatty acid synthesis by 3-hexynoic acid and 3-hexynoyl-NAC.* A series of incubations containing 0.5 mg enzyme (35–50% ammonium sulfate fraction) and either 3-hexynoic acid or 3-hexynoyl-NAC at final concentrations of 0, 10^{-6}, 10^{-5}, 5×10^{-5}, 10^{-4} and 10^{-3} *M* in a final volume of 0.2 ml 0.1 *M* phosphate buffer pH 7.5 containing 5×10^{-4} *M* EDTA, were incubated at 28°C for 10 min. At 10 min each sample was treated with 0.2 ml of a mixture of 12 n*M* acetyl CoA, 66 n*M* 1,3-^{14}C-malonyl CoA, 40 n*M* 6-MSA and 200 mμ of NADPH in the above buffer containing 5×10^{-4} *M* β-mercaptoethanol and incubated for a further 10 min. The production of 6-MSA and fatty acids were assayed as previously described and the inhibition measured relative to the control.

(b) *Inhibition of 6-MSA synthesis by 2-hexynoyl-NAC.* This experiment was performed under the same conditions as described in (a) with the exception that the quantity of enzyme used per incubation was 0.47 mg. A parallel experiment using 3-hexynoyl-NAC was carried out for comparison.

(c) *Inhibition of 6-MSA synthesis by 3-pentynoyl-NAC.* A series of tubes containing 0.61 mg enzyme (35–50% ammonium sulfate fraction solubilized in 0.05 *M* phosphate

buffer pH 7.5 containing 10^{-3} *M* EDTA and 20% glycerin) was incubated with 0 *M* or 5×10^{-5} *M* 3-pentynoyl-NAC or 3-hexynoyl-NAC in a volume of 0.2 ml, at 28°C for 10 min. Each series of incubations was then further incubated with 0.21 ml of a solution containing 12 n*M* acetyl CoA, 66 n*M* 1,3-^{14}C-malonyl CoA, 200 n*M* NADPH, 40n*M* 6-MSA, 20 μm potassium phosphate buffer pH 7.5, 0.1 μm EDTA and 0.1 μm β-mercaptoethanol at 28°C for times of 3, 6, 9 and 12 min. The reactions were stopped and the 6-MSA isolated as described before. The inhibition was calculated from the rates of synthesis with and without inhibitor.

(d) *Inhibition of 6-MSA synthesis by 3-hexynoyl-NAC.* A series of 0.64 mg enzyme samples was incubated with a solution of 3-hexynoyl-NAC (to a final concentration of 0, 5×10^{-5} and 10^{-4} *M*) in 0.2 ml of 2.5×10^{-3} *M* phosphate buffer pH 7.5 containing 5×10^{-4} *M* EDTA and 10% glycerin at 28°C for 10 min. After 10 min each sample was treated in duplicate with 0.2 ml of a solution containing 12 n*M* acetyl CoA, 70 n*M* 1,3-^{14}C-malonyl CoA, 200 n*M* NADPH and 40 n*M* 6-MSA in 0.15 *M* phosphate buffer pH 7.5 containing 7.5×10^{-4} *M* EDTA and β-mercaptoethanol, and the reactions stopped at time intervals of 3, 6, 9 and 12 min in the usual way. The assay of the 6-MSA was carried out as previously described.

The inhibition of 6-MSA synthesis in vivo. The spores from two slopes of *P. patulum* were distributed between 400 ml of germinating medium (*15*) in four 250-ml Erlenmeyer flasks and grown for 24 hr under the previously documented conditions. The mycelium was harvested by filtration resuspended in 80 ml Czapek–Dox medium (*15*), homogenized for 15 sec in a Sorvall Omnimixer and distributed equally between eight 250-ml flasks containing 100 ml of the same medium. The organism was grown on a shake table at 180 rpm and a separate set of flasks in duplicate were assayed for 6-MSA production every hour, for up to 4 hr by aseptically removing 5 ml of the cell suspension and adding it to 0.5 ml of 1 *N* HCl which was then extracted with 5 ml ether and the 6-MSA assayed by measuring the optical density at 312 nm. After 4.5 hr, three sets of duplicates were treated to (a) nothing, (b) 10 ml of a solution containing 1 mg of cycloheximide, (c) same as (b) and in addition 10 ml of a 10^{-3} *M* solution of 3-hexynoyl-NAC. Each set of duplicates was again sampled as before at times of 5, 6, 7 and 9 hr. The measured optical densities obtained were corrected for volumetric differences incurred by the addition of inhibitors.

RESULTS

Unit of enzyme activity. Enzyme activity is expressed in milliunits (mU). A milliunit of enzyme activity is defined as the amount of enzyme required to catalyse the formation of 1 nmole 6-MSA per min under the conditions of the assay. Specific activity is expressed as mU of enzyme activity per mg protein.

Preparation and stability of the enzyme. The method used for the preparation of an enzyme fraction containing the 6-MSA, TAL and fatty acid synthetases is shown in Table 1. The initial 104 000*g* supernatant rapidly lost activity for 6-MSA synthesis when stored at 0 + 4°C. In contrast the ammonium sulfate fraction retained about 75% of the initial activity for 40 hr when stored in 0.2 *M* phosphate buffer pH 7.5 containing 10^{-3} *M* EDTA, 10^{-3} *M* β-mercaptoethanol and 20% glycerin, but slightly higher initial activities were obtained when the same fraction was stored in either 0.2 *M* or 5×10^{-3} *M* phosphate buffer pH 7.5 again in the presence of EDTA and β-mercaptoethanol but without glycerin. The enzyme was stable to treatment with calcium phosphate gel but this did not lead to further purification. The use of Polyclar–AT, at a

ratio of 2 g/15 g mycelium for the preparation of the initial extract, led to an inactive preparation.

TABLE 1

PREPARATION OF ENZYME EXTRACT FROM P. PATULUM FOR THE SYNTHESIS OF 6-MSA[a]

Fraction	Volume	Total protein	Specific activity[b]	Total milliunits	Recovery
104 000*g* supernatant	38 ml	350 mg	0.29	102	100%
30–50% ammonium sulfate precipitate	12 ml	61.2 mg	0.86	52.6	46%

[a] The experimental details are given in the text.
[b] Expressed as mU per mg protein.

Rate of synthesis of 6-MSA. The rate of 6-MSA synthesis was linear up to 10 min when 0.5 mg protein was used. On the other hand, the synthesis of fatty acids was linear up to 25 min but the rate of synthesis was ten times slower than 6-MSA synthesis, although this ratio varied with different preparations.

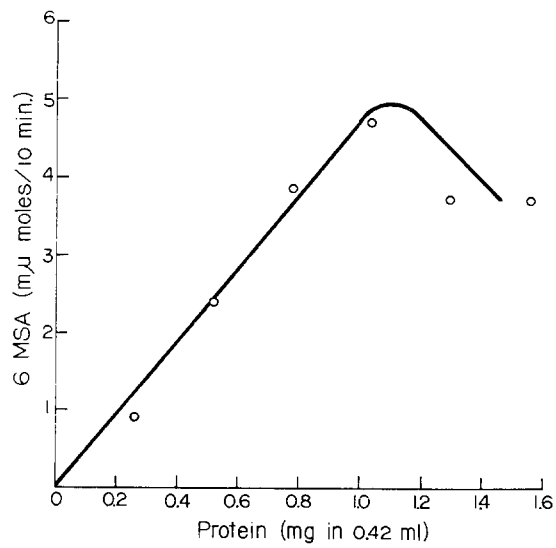


FIG. 1. The effect of protein concentration on the synthesis of 6-MSA. The assays were performed as described in the text using protein from the 35–50% $(NH_4)_2SO_4$ fraction.

Dependence of 6-MSA synthesis on protein concentration. The synthesis of 6-MSA was linear with protein concentrations up to 1 mg/0.42 ml under conditions described in the assay. At higher protein concentration there was a decrease in rate. The nature of this effect has not been investigated but all studies were carried out with levels of protein concentration which were on the linear part of the curve (Fig 1).

Dependence of 6-MSA synthesis on substrates and cofactors. The dependence of 6-MSA synthesis on malonyl CoA and NADPH is shown in Figs. 2 and 3, respectively. The synthetase shows a requirement for both malonyl CoA and NADPH. The depend-

ence on malonyl CoA is linear up to 70 nmoles (in the standard assay) above which there is substrate inhibition. The synthesis shows a linear dependence on NADPH up to a maximum level of 120 nmoles. The rate of synthesis proved to be maximal with an acetyl CoA concentration of 10 nmoles/0.42 ml.

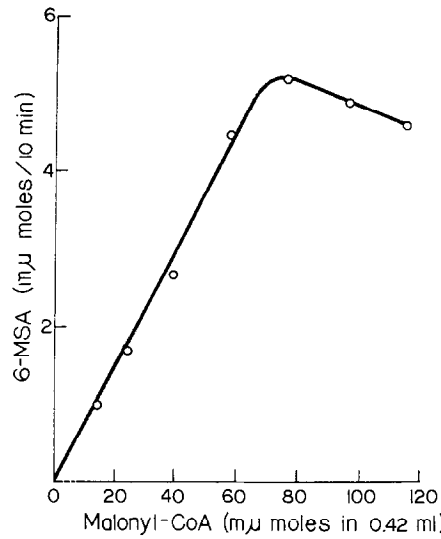


FIG. 2. The effect of malonyl CoA on the synthesis of 6-MSA. The assays were performed as described in the text using 0.49 mg enzyme (35–50% ammonium sulphate fraction) except 1,3-^{14}C-malonyl CoA was varied over the concentration range shown.

Chemical degradation of ^{14}C-6-MSA. The results of the degradation of ^{14}C-6-MSA are shown in Table 2 and reveal that 6-MSA synthesized from acetyl CoA 1,3-^{14}C-malonyl CoA and NADPH contains 29.5% of the total activity in the terminal carboxyl group. The formation of 6-MSA from a starter acetyl group with three ^{14}C-malonyl

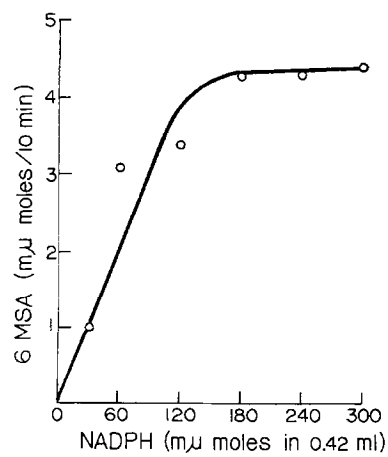


FIG. 3. The effect of NADPH concentration on the synthesis of 6-MSA. The components of each incubation were the same as in the text with the exception of NADPH which was varied over the concentration range shown.

groups requires that the terminal carboxyl group contains 33.3% of the radioactivity with the remaining 66.7% equally distributed between the two malonate units in the aromatic ring. The value of 70.5% for the radioactivity in the ring compared to 29.5% in the carboxyl group is consistent with a ratio of 2.39/1 which is in agreement with the condensation of a starter acetate with three malonate functions. However the deviation from the theoretical value of 2/1 represents 11.5% radioactivity in the aromatic ring,

TABLE 2

DEGRADATION OF 6-MSA BIOSYNTHESIZED FROM 1,3-^{14}C-MALONYL CoA[a]

Recrystallization to constant specific activities

Compound	Specific activity (dpm/mg) in successive recrystallizations
6-Methylsalicylic acid	(1) 940 (2) 931 (3) 943 (4) 936
Tribromo-*m*-cresol	(1) 104 (2) 101 (3) 103

Distribution of radioactivity in 6-MSA, Tribromo-*m*-cresol and Carbon dioxide

Compound	Specific activity (dpm/m mole)	% Radioactivity
6-Methylsalicylic acid	50 099	100
Tribromo-*m*-cresol	35 294	70.5
Carbon dioxide[b]	14 805	29.5
Carbon dioxide	12 000	23.9

[a] The components of the incubation mixture for the synthesis of 6-MSA, the isolation of 6-MSA and the method of degradation to Tribromo-*m*-cresol are given in the experimental section.

[b] This value is calculated from the difference between the specific radioactivity of 6-MSA and Tribromo-*m*-cresol.

not originating in a malonate function. This 11.5% may be associated with a portion of the starter acetyl function originating from 1,3-^{14}C-malonyl CoA by means of malonyl CoA decarboxylase activity. The latter hypothesis is substantiated by a report by Bu'Lock et al. (*9*), that 6-MSA obtained from *P. urticae* Bainier, grown in the presence of 2-^{14}C-diethyl malonate, contained 8% of the overall radioactivity in the acetate derived starter unit.

The identification of the fatty acids. The components of the free fatty acids formed in the incubations of enzyme with acetyl CoA, 1,3-^{14}C-malonyl CoA and NADPH were assayed by glc. The mixture of radioactive fatty acids was converted to its methyl esters and mixed with carrier amounts of the methyl esters of palmitic, stearic and oleic acid prior to analyses by glc. The profile of the glc effluent is shown in Fig. 4 and shows that 80% of the radioactivity is associated with the esters of palmitic and stearic acid, while 20% corresponded to methyl oleate. Thus the saturated nature of the fatty acids formed is similar to the fatty acids obtained from the yeast (*33*) and pigeon liver (*34*) multienzyme complexes.

The effect of sulphydryl inhibitors on the synthesis of 6-MSA and fatty acids. The synthesis of 6-MSA and the saturated fatty acids is inhibited by iodoacetamide and *N*-ethylmaleimide (Table 3). The inhibition of both syntheses is effected most efficiently by *N*-ethylmaleimide. A comparison of the two enzyme systems showed that the 6-

MSA synthetase is the most susceptible to inhibition by both sulphydryl inhibitors, thereby illustrating that the synthesis of fatty acids and 6-MSA involve different sulphydryl sites, probably on two distinct enzyme systems. Lynen has recently purified

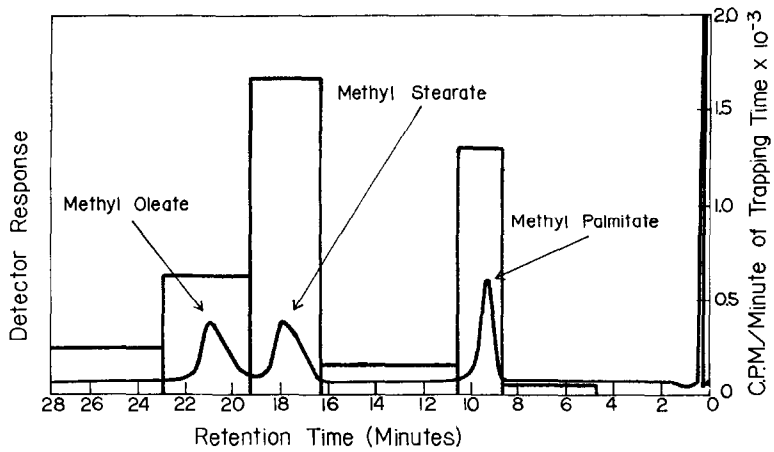


Fig. 4. Gas–liquid chromatography of the methyl esters of palmitic, stearic and oleic acids. The large-scale incubation for the synthesis of fatty acids and the procedures used for their isolation, conversion to methyl esters and assay by gas–liquid chromatography is described in detail in the text.

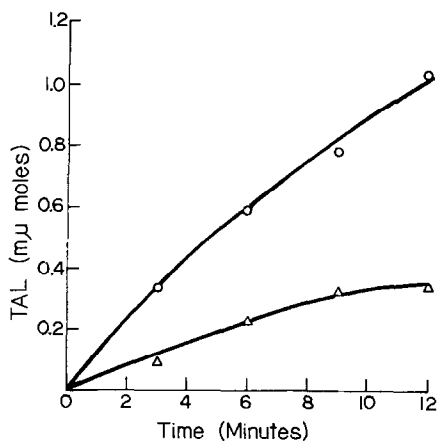


FIG. 5. Rate of synthesis of TAL. (a) In the presence of NADPH (△). (b) Without NADPH (○). The incubation mixture for series (a) and (b) are the same as described under assay in the text, containing 0.64 mg enzyme. The assays were performed for the times shown in the figure and worked up as described in the text.

the F.A. synthetase from *P. patulum* and indeed shown it to be a different enzyme from 6-MSA synthetase (*50*).

The synthesis of TAL. The synthesis of TAL is effected by omitting NADPH from the incubation system, described in the assay section. However, it is shown in Fig. 5 that TAL is also synthesized in the presence of NADPH, but the omission of NADPH from the incubation mixture leads to a threefold increase in rate under the conditions

TABLE 3

EFFECT OF SULPHYDRYL INHIBITORS ON THE FORMATION OF 6-MSA AND FATTY ACIDS[a]

Inhibitor concentration	Inhibition %	
	6-MSA	Fatty acids
Iodoacetamide		
10^{-2} *M*	98	95
10^{-3} *M*	96	92
10^{-4} *M*	50	0
10^{-5} *M*	15	*
N-Ethylmaleimide		
10^{-2} *M*	100	85
10^{-3} *M*	100	86
10^{-4} *M*	98	61
10^{-5} *M*	29	*

* The enzyme activity was stimulated by 10^{-5} *M* iodoacetamide and *N*-ethylmaleimide by about 60% and 22%, respectively.

[a] The enzyme was preincubated with the given level of inhibitor at 28°C for 10 min and assayed for the synthesis of 6-MSA and fatty acids, in a fixed time assay of 10 min as described in the experimental section.

TABLE 4

CRYSTALLIZATION OF TAL TO CONSTANT SPECIFIC RADIOACTIVITY

TAL synthesized in the absence of NADPH[a]	
Number of crystallizations	dpm/mg
1	352.0
2	354.0
3	348.0
4	359.0
TAL synthesized in the presence of NADPH[a]	
1	138
2	116
3	114
4	114
5	114

[a] The components of the incubation mixtures used for the synthesis of TAL are given in the experimental section.

used. The TAL from both sources was characterized by recrystallization to constant specific radioactivity, the results of which are shown in Table 4. On a relative basis, the rate of synthesis of 6-MSA is about twelve times faster than TAL under conditions of synthesis from acetyl CoA, malonyl CoA and NADPH (see Figs. 5 and 7). The effect of increasing the NADPH concentration to twice that reported in the conditions

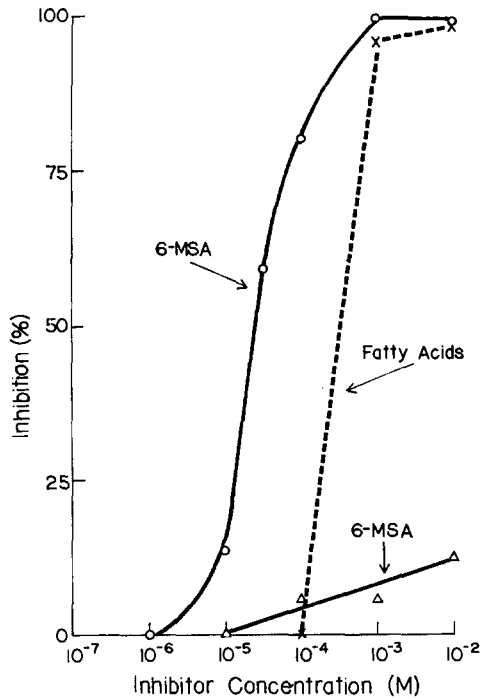


FIG. 6. Comparison of 3-hexynoic acid and 3-hexynoyl-NAC as inhibitors of 6-MSA and fatty acid synthetases. Incubation tubes containing enzyme 0.5 mg (35–50% ammonium sulfate fraction) and either 3-hexynoic acid or 3-hexynoyl NAC at final concentrations of 0, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} *M* in a volume of 0.2 ml potassium phosphate buffer pH 7.5 and EDTA, 5×10^{-4} *M* were preincubated at 28°C for 10 min. At 10 min each sample underwent the standard assay described in the text. The 6-MSA and fatty acids were isolated as described in the text. The curves represent the inhibition of 6-MSA (—⊙—) and fatty acid(– – × – –) synthesis by 3-hexynoyl-NAC and the inhibition of 6-MSA (—△—) synthesis by 3-hexynoic acid.

of assay did not lead to any increase in the quantity of either 6-MSA or fatty acids and also lead to the production of the same quantity of TAL. On the basis that each molecule of 6-MSA and TAL contains 3 and 2 malonate units respectively, it is estimated from the rates of synthesis shown in Figs. 5 and 7 that the system synthesizes 6-MSA at about four times that of TAL, the latter in the absence of NADPH.

The comparative inhibition of 6-MSA and fatty acid synthesis by 3-hexynoyl-NAC and 3-hexynoic acid. Figure 6 shows the comparative inhibition of 6-MSA and fatty acids with increasing concentrations of 3-hexynoyl-NAC. The results were obtained by preincubating the enzyme and inhibitor for 10 min followed by a fixed time assay of 10 min. The 6-MSA synthetase is inhibited by 3-hexynoyl-NAC at 10^{-5}–10^{-4} *M*, while the fatty acids require a tenfold increase in inhibitor concentration to effect the same degree of inhibition. It is also shown in Fig. 6 that the corresponding 3-hexynoic acid

only affords about 12% inhibition of 6-MSA at a concentration of 10^{-2} *M* and illustrate the need for a thioester moiety for the inhibition.

Inhibition of 6-MSA by 3-hexynoyl-NAC. Figure 7 shows the rates of synthesis of 6-MSA with and without inhibitor. The experiment was performed by incubating the enzyme with inhibitor for 10 min and thereafter measuring the rate of synthesis.

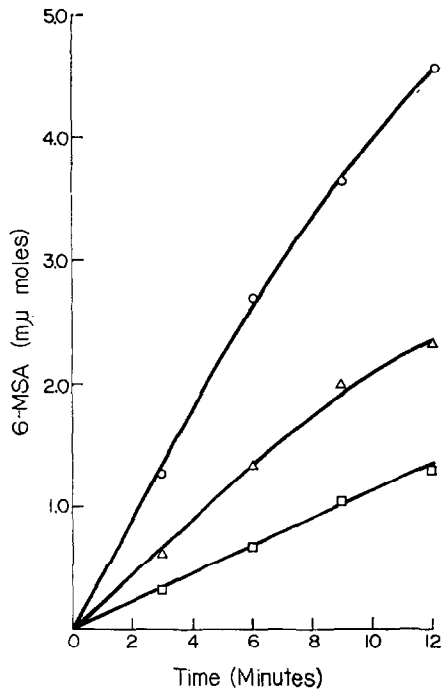


FIG. 7. Inhibition of 6-MSA synthesis by 3-hexynoyl-NAC. A series of incubation mixtures containing enzyme, 0.34 mg and 3-hexynoyl-NAC at final concentrations of 0 (⊙), 5×10^{-5} *M* (△), and 10^{-4} *M* (□) in 0.2 ml of 2.5×10^{-3} *M* potassium phosphate buffer pH 7.5, 5×10^{-4} *M* EDTA and glycerin 10% were incubated at 28°C for 10 min. Each series of samples containing the requisite concentration of inhibitor underwent the standard assay for the time intervals shown in the figure. The isolation of 6-MSA is described in the text.

Preliminary experiments showed that maximal inhibition was achieved under these conditions. The results illustrated in Fig. 7 show that the rate of synthesis of 6-MSA is inhibited 75% by 10^{-4} *M* 3-hexynoyl-NAC and 50% at 5×10^{-5} *M*.

Inhibition of 6-MSA synthetase with 2-hexynoyl-NAC and 3-pentynoyl-NAC. Table 5 shows a comparison of hex-3-ynoyl-NAC with 2-hexynoyl-NAC and 3-pentynoyl-NAC. When the synthetase was inhibited by either the NAC derivative of 2-hexynoic acid and 3-hexynoic acid and the rate of 6-MSA synthesis compared in a fixed time assay of 10 min, it is seen that the 2-hexynoyl-NAC is approximately 5.7 times better as an inhibitor than 3-hexynoyl-NAC, at a concentration of 10^{-4} *M*, the values becoming almost equal at higher concentrations. The effect of chain length on the level of inhibition is not so marked, the levels of inhibition being comparable for 3-pentynoyl-NAC and 3-hexynoyl-NAC, when used at 5×10^{-5} *M*.

The effect of 3-hexynoyl-NAC on the synthesis of 6-MSA in vivo. The synthesis of 6-MSA is inhibited when 3-hexynoyl-NAC is added to cultures of *P. patulum* which are

TABLE 5

COMPARISON OF 2-HEXYNOYL-NAC, 3-PENTYNOYL-NAC AND 3-HEXYNOYL-NAC AS INHIBITORS OF 6-MSA SYNTHETASE

	Inhibition %	
Inhibitor concentration	2-Hexynoyl-NAC	3-Hexynoyl-NAC
10^{-6} M^a	11	14
10^{-5} M^a	57	10
10^{-4} M^a	84	80
10^{-3} M^a	96	98
	3-Pentynoyl-NAC	3-Hexynoyl-NAC
5×10^{-5} M^b	56.0	61.0

[a] The inhibition was calculated in a fixed time assay of 10 min as described in the experimental section.

[b] The inhibition was calculated from the initial velocity measurements. The experimental conditions are described in the text.

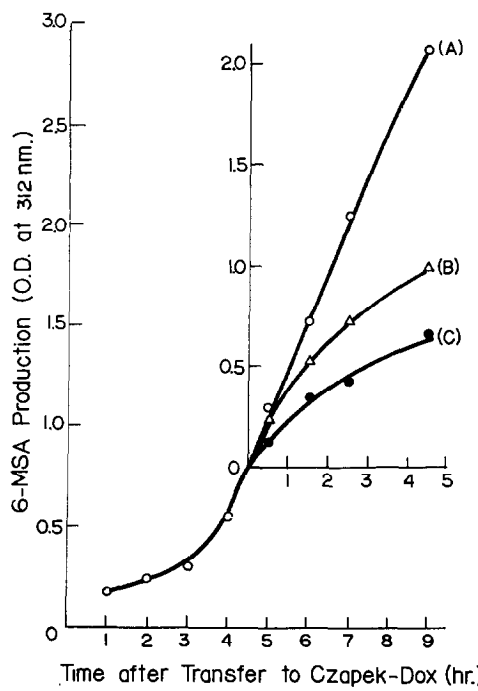
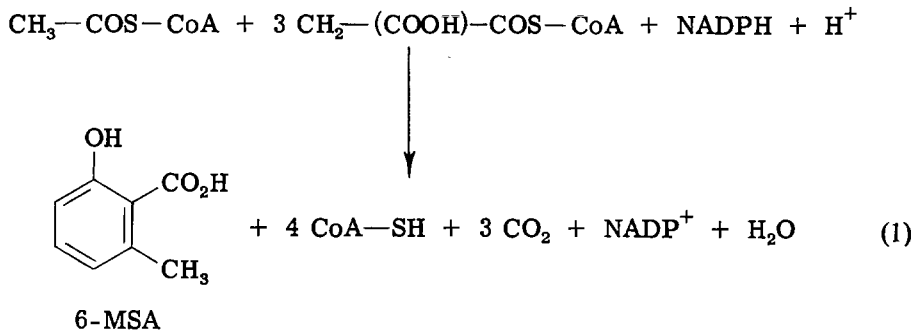


FIG. 8. The effect of 3-hexynoyl-NAC on the production of 6-MSA by *P. patulum in vivo*. Curve (A) represents the production of 6-MSA in cultures of *P. patulum* grown in Czapek-Dox medium. Curve (B) represents the production of 6-MSA in the same cells which were treated to cycloheximide, 10 μg/ml, and curve (C) represents the production of 6-MSA in cells which were treated with both cycloheximide, 10 ug/ml and 3-hexynoyl-NAC at 10^{-4} *M*. The experimental details are described in the text.

producing 6-MSA. Figure 8 shows the rate of 6-MSA synthesis in Czapek–Dox medium (curve A) under normal conditions. After 4.5 hr the protein synthesis inhibitor cycloheximide was added to a final concentration 10 μg/ml, and the rate of 6-MSA production was reduced (curve B). This is in agreement with the data published by Light (*35*) who reported that this level of cycloheximide abolished protein synthesis in this organism. This led to approximately a 55% reduction in the level of 6-MSA accumulated in 4.5 hr after addition of inhibitor. The effect of 10^{-4} *M* 3-hexynoyl-NAC is seen in curve C, where the level of 6-MSA is now reduced to approximately 60% of the control containing cycloheximide alone. This means that 10^{-4} *M* 3-hexynoyl-NAC is inhibiting the endogenous 6-MSA synthetase to about 40% in complete cultures, compared to a value of 74% under *in vitro* conditions.

DISCUSSION

The biosynthesis of the fungal metabolite 6-MSA is formally accomplished according to Scheme 1 (*17*).



This scheme differs from the known mechanism of fatty acid synthesis (*10*, *11*, *36*) in that the enzymes involved in 6-MSA synthesis need only one complement of β-keto-reductase and β-hydroxy dehydrase for three condensation steps. A salient feature of the mechanism then involves the question as to whether the reduction and dehydration occurs at the level of a 3,5-diketohexanoyl entity or at the level of a 3,5,7-triketo octanoyl intermediate. One apparent difference between the synthesis of 6-MSA and saturated fatty acid synthesis is that the latter involves the formation of α,β-*trans* enoyl intermediates (*33*, *37*, *38*) while the geometry of the 6-MSA molecule would necessitate the formation of an intermediate containing a *cis* double bond.

In order to gain information on the mechanism of synthesis of 6-MSA, we have studied its biosynthesis in relation to that of TAL and fatty acids.

The data presented shows that an ammonium sulphate fraction of the supernatant from *P. patulum* catalyses the formation of 6-MSA, TAL and fatty acids, in the presence of malonyl-CoA, acetyl CoA and NADPH. The rate of synthesis of 6-MSA is about ten times faster than fatty acids and about twelve times faster than the synthesis of TAL (Figs. 5 and 7) under the conditions used. A partial degradation of the 6-MSA obtained from 1,3-^{14}C-malonyl CoA shows that only 29.5% of the total radioactivity is located in the carboxyl group compared with the required 33.3%, if the molecule is synthesized from 1 acetate and 3 malonate units. This deviation from the theoretical value may be due to the presence of malonyl CoA decarboxylase activity in the extract. This would account for the aromatic ring having a higher specific activity due to the

formation of 1-^{14}C-acetyl CoA. Similar results were obtained by Bu'Lock (*9*) on feeding 2-^{14}C-malonate to cultures of *P. patulum*.

Both the 6-MSA and fatty acid synthetase activity is inhibited by sulphydryl blocking reagents. The results of comparative inhibition (Table 3) studies show that *N*-ethylmaleimide is a better inhibitor than iodoacetamide for both systems. Further both inhibitors act on the aromatic synthetase at lower concentrations than that required for a comparable inhibition of the fatty acid synthetase. This provides evidence that the active sites of the individual enzymes functioning in 6-MSA synthesis are sulphydryl in nature and may be similar to the fatty acid synthetases that contain 4-phosphopantetheine and cysteine (*10*, *11*, *37*). This result is in agreement with a report by Dimroth et al. (*19*) that the purified 6-MSA synthetase contains two sulphydryl sites, as indicated by a pH dependent inhibition with *N*-ethylmaleimide and a pH independent inhibition with iodoacetamide.

The synthesis of 6-MSA can be selectively inhibited by the acetylenic inhibitor 3-hexynoyl-NAC. This inhibitor at concentrations of 5×10^{-5} *M* gives a 50% inhibition of 6-MSA synthesis (Fig. 7) while not affecting the synthesis of palmitic and stearic acid (Fig. 6) below 10^{-4} *M*. The same pattern of inhibition of 6-MSA synthesis was obtained with 3-pentynoyl-NAC and 2-hexynoyl-NAC (Table 5) but the free 3-hexynoic acid was not inhibitory. This result parallels the results obtained by Kass and Bloch (*39*) who showed that 3-decynoyl-NAC at low concentrations specifically inhibited the synthesis of unsaturated fatty acids by the fatty acid synthetase of *E. coli*. The mode of action of this inhibitor involves the inhibition of the β-hydroxydecanoyl thioester dehydrase (*23*), an enzyme that catalyses the formation of both α,β-trans and β,γ-*cis*-decenoyl thioester (*32*, *24*) Bloch et al. (40) have shown that 3-decynoyl-NAC acts by binding to a histidyl site. Furthermore, recent studies have shown that the β-hydroxydecanoyl-thioester dehydrase probably functions by isomerizing the inhibitor 3-decynoyl-NAC to the 2,3-decadienoyl-NAC which in turn is the active entity which binds to the enzyme (*41*). The enzyme therefore acts on the inhibitor and substrates, e.g., β-hydroxydecanoyl thioester in the same manner by first abstracting the α hydrogen to form the α,β-*trans* derivative which is then isomerized to the β,γ-*cis* isomer. In this context the mode of inhibition of the 6-MSA synthetase by 3-hexynoyl-NAC may by similar, although there is as yet no evidence to rule out the possibility that the latter is functioning as a sulphydryl inhibitor. However it should be noted that the inhibitory properties of 3-decynoyl-NAC toward the production of unsaturated fatty acids in *E. coli* was reported with the complimentary fatty acid synthetase enzymes (*39*), which contain sulphydryl sites in the β-keto-acyl-ACP synthetase (*42*, *43*), acetyl-CoA and malonyl-CoA transacylases (*44*, *45*) and a dehydrase which is specific for β-hydroxyacyl-ACP derivatives of chain length C_4–C_8 which is also sulphydryl in nature (*38*). Further evidence that acetylenic thioesters are not inhibitory to sulphydryl sites is found in a report by Kass and Bloch (*39*) that the inhibition of saturated fatty acids by 10^{-4} *M* 3-decynoyl-NAC leads to the formation of β-hydroxydecanoate, a process which involves enzymes that are sulphydryl in nature. It is therefore possible that the 6-MSA synthetase contains a dehydrase enzyme that leads to the production of a β,γ-*cis* enoate such as *cis*-5-keto-3-hexenoate. The inhibitor 3-hexynoyl-NAC is also inhibitory towards 6-MSA synthesis under *in vivo* conditions as is shown in Fig. 8. The cells were first treated to cycloheximide in order to arrest protein synthesis. Under these conditions the inhibitor at 10^{-4} *M* gave an inhibition of approximately 40%, which is lower than the value obtained with an enzyme preparation.

The enzyme fraction which was used for the synthesis of 6-MSA and the fatty acids was also capable of TAL synthesis, both in the presence and absence of NADPH

(Fig. 5). The rate of synthesis of TAL without NADPH was about threefold greater than when NADPH was present. There are previous reports of the synthesis of TAL by the fatty acid synthetases from yeast (*22*) and pigeon liver (*21*) when NADPH was omitted from the incubation mixture. Furthermore, it has been shown by Bloch (*20*) that the fatty acid synthetase from *E. coli* is capable of TAL synthesis in the presence of NADPH. It is reported that TAL is produced by the *E. coli* condensing enzyme which utilizes ACP derivatives in conjunction with a sulphydryl site on the condensing enzyme (*12*). The enzyme responsible for the production of TAL in the current investigation has not been characterized, but the production of TAL could be related to the 6-MSA synthetase activity. There are other reports of the production of TAL by organisms that produce polyketide compounds, among them being the reported synthesis of ^{14}C-TAL from ^{14}C-acetate in *P. patulum* (*46*), the formation of TAL and tetraacetic acid lactone by cultures of *P. stipitatum* which had been inhibited for tropolone production by ethionine (*47*) and the isolation of the methyl derivative of TAL (3,6-dimethyl-4-hydroxy-pyran-2-one) from another strain of the same organism (*48*).

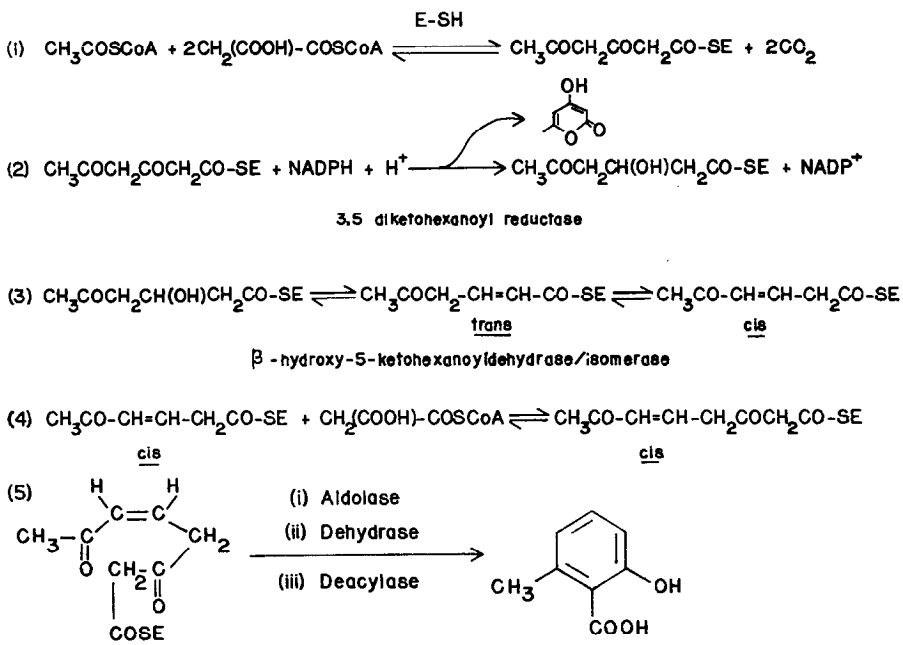


FIG. 9. Proposed mechanism for the synthesis of 6-MSA. (After Lynen *et al*, ref. 19).

The evidence discussed above is outlined in schematic form in Fig. 9. The first step is the condensation of one acetate and two malonate thioester units with the formation of enzyme bound 3,5-diketohexanoyl enzyme. This compound is suggested to play a central role in that it is reduced to form enzyme bound 3-hydroxy-5-ketohexanoyl enzyme or lactonized to form triacetic acid lactone. The reduced intermediate is then dehydrated to give the *cis*-5-keto-3-hexenoyl enzyme, by a dehydrase which has a mode of action similar to the β-hydroxydecanoyl thioester dehydrase (*24*) which functions in the biosynthesis of *cis* unsaturated fatty acids in *E. coli*. A further condensation between *cis*-5-keto-3-hexanoyl enzyme and malonyl CoA (enzyme bound) gives a further enzyme bound intermediate which undergoes aldol condensation and a further

dehydration to give enzyme bound 6-MSA which, in turn, is acted upon by a thiolase to produce the free acid. The feasability of the final aldolase step has recently received support from an *in vitro* model (*49*) which has been used to generate the diketo–octenoic acid (Eq. (4); Fig. 9) *in situ*. This species readily undergoes dehydrative aromatization to afford 6-MSA.

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