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Biosynthesis of Polyketides.[1] Purification and Inhibition Studies of 6-Methylsalicylic Acid Synthase

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6-MSA[3] synthase has been purified 190-fold with 33% yield. The purification was found to be dependent on the presence of glycerol. The acetylenic inhibitors 3-pentynoyl- and 2-hexynoyl-NAC completely inhibit 6-MSA production at concentrations in which fatty acid synthesis, TAL production as well as NADPH oxidation are only partially affected. These results confirm earlier studies on the specificity of inhibition by acetylenic inhibitors and support a mechanism wherein the NADPH-mediated reduction step occurs on a 6-carbon rather than on an 8-carbon intermediate.

6-Methylsalicylic acid (6-MSA), a typical fungal metabolite (but see Refs. *1* and *2*), is one of the earliest examples of polyketide-derived aromatic metabolites occurring in fungi, lichens, and higher plants (*3–5*). The enzyme responsible for its biosynthesis was extensively purified by Lynen (*6*) and was shown by sucrose gradient centrifugation to be a single particle of MW $1.1–1.5 \times 10^6$.

Previous studies in this laboratory with the partially purified 6-MSA synthase (former name: 6-MSA synthetase) have shown that the enzyme is inhibited by 5- and 6-carbon acetylenic thioesters (*7*). Based on these findings, it was suggested that one of the components of 6-MSA synthase may act in a manner analogous to that postulated for the *E. coli* dehydrase (*8*).

The present study was undertaken in order to investigate the action of acetylenic inhibitors on the purified enzyme. The specificity of inhibition was further utilized in determining whether the NADPH-mediated reduction does indeed occur, as had been suggested in the past (*6–7*), on a 6- rather than an 8-carbon intermediate.

EXPERIMENTAL

Materials

Acetyl CoA, malonyl CoA and NADPH were obtained from P-L Biochemicals; [1,3-^{14}C]- and [2-^{14}C]-malonyl CoA from New England Nuclear Corporation; Tris base, β-mercaptoethanol (β-ME), ammonium sulphate (enzyme grade), palmitic,

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[3] The abbreviations used are: ACP, acyl carrier protein; BSA, bovine serum albumin; β-ME, β-mercaptoethanol; NAC, *N*-acetylcysteamine; NEM, *N*-ethylmaleimide; 6-MSA, 6-methylsalicylic acid; TAL, triacetic acid lactone.

stearic and oleic acids from Mann Research Laboratories; bovine serum albumin from Sigma; EM-reagent precoated thin-layer plates from Brinkman Instruments Inc.; Bacto-Peptone, malt extract and Bacto-agar from Difco Laboratories; and glass beads from Fisher Scientific. 3-Hexynoic acid, 3-hexynoly-NAC, 3-pentynol-NAC, and 2-hexynoyl-NAC were a generous gift of Dr. G. T. Phillips. *Penicillium patulum* NRRL2151A (RL-17, early MSA strain) was kindly provided by Dr. R. Light.

Miscellaneous Methods

The culturing methods were essentially those of Light (*9*) except that fermentations were carried out in 14 liter jars in an MF214 New Brunswick microfermenter with 6 liters/min aeration and 400 rpm agitation. Radioactivity was determined by a Packard model scintillation counter using Bray's scintillation cocktail (*10*). Protein was determined by the Biuret method of Gornall, Bardawill, and David (*11*) or the method of Warburg and Christian (*12*).

Estimation of 6-MSA Synthase Activity

6-MSA synthase activity was routinely assayed by Lynen's radiochemical method [hereafter indicated as Method (a)] (*6*). Purified 6-MSA activity could also be estimated optically, by the decrease in OD at 340 nm [hereafter indicated as Method (b)] (*6*). The second method was a valuable tool in dissecting the activities of the multienzyme complex (see Results). In cases where fatty acid activity was to be simultaneously determined, saponification of the incubation mixture was performed [hereafter indicated as Method (c)] (*6*). In both methods (a) and (c) the previously described tlc system (*7*) was used. All calculations of 6-MSA synthase activity assumed the following stoichiometry (*6*, *9*, *13*, *14*):

$$1\ [\text{acetyl CoA}] + 3\ [\text{malonyl CoA}] + 1\ [\text{NADPH}] + [\text{H}^+]$$
$$\downarrow$$
$$1\ [\text{6-MSA}] + 4\ [\text{CoA}] + 3\ [\text{CO}_2] + 1\ [\text{NADP}^+] + 1\ [\text{H}_2\text{O}].$$

RESULTS

Enzyme Purification

The purification of 6-MSA synthase from *Penicillium patulum* NRRL2159A (RL-17, early MSA strain) was modeled after and gives results similar to Lynen's purification of the same enzyme from *P. patulum* NRRL679 (*6*). Briefly, the procedure involves: (i) preparation of "crude extract" by centrifugation at 100,000*g*; (ii) formation of acetone powder; (iii) 25–40% ammonium sulphate fractionation; (iv) high speed centrifugation (4 hr; 104,000*g*); (v) 5–20% sucrose gradient centrifugation. However, some modifications of the published procedure were found necessary, notably the inclusion of glycerol in all purification steps. Reasons for this are discussed below.

A summary of the purification is shown in Table 1. The enzyme was purified 192-fold with 33% recovery of activity.

Comments on the Purification

The purification of 6-MSA synthase could not be carried out beyond the 25–40% ammonium sulphate fractionation in the absence of glycerol. In fact, a higher specific

TABLE 1

PURIFICATION OF 6-MSA SYNTHETASE

Step	Total protein[a] (mg)	Total activity[b] (mU)	Specific activity (mU/mg)	Relative yield (%)	Purification (-fold)
Crude extract from centrifugation at 100,000*g*	3080	1400	0.456	100	1
Acetone powder	2190	1380	0.630	98	1.38
25–40% ammonium sulphate precipitation	533	1400	2.63	100	5.77
Resuspended pellet from centrifugation at 144,000*g*	68.2	629	9.22	45	20.2
Active fractions from 5 to 20% sucrose gradients	5.4	467	86.4	33	189
0–50% ammonium sulphate precipitation	5.25	461	87.8	33	192

[a] Protein was determined by the Biuret method, except for the sucrose gradient fractions, which were determined by the method of Warburg and Christian (*12*).

[b] Enzyme activity was assayed by radioactive method (a).

activity and a better yield were obtained even in the early stages of the purification if the buffer contained glycerol. In order to recover activity from the 144,000*g* pellet, it was necessary to do the centrifugation in buffer containing glycerol; the addition of glycerol to an inactive pellet did not restore activity (*15*). In the sucrose gradient centrifugation, sucrose could not completely substitute for glycerol; the recovery of activity was tenfold higher in the gradients containing glycerol (*15*).

As can be seen in Figs. 1 and 2, in the sucrose gradients not containing glycerol the protein peak which is associated with 6-MSA activity (fractions 11 and 12) is greatly diminished, while higher molecular weight peaks (fractions 5, 6 and 9) are greatly

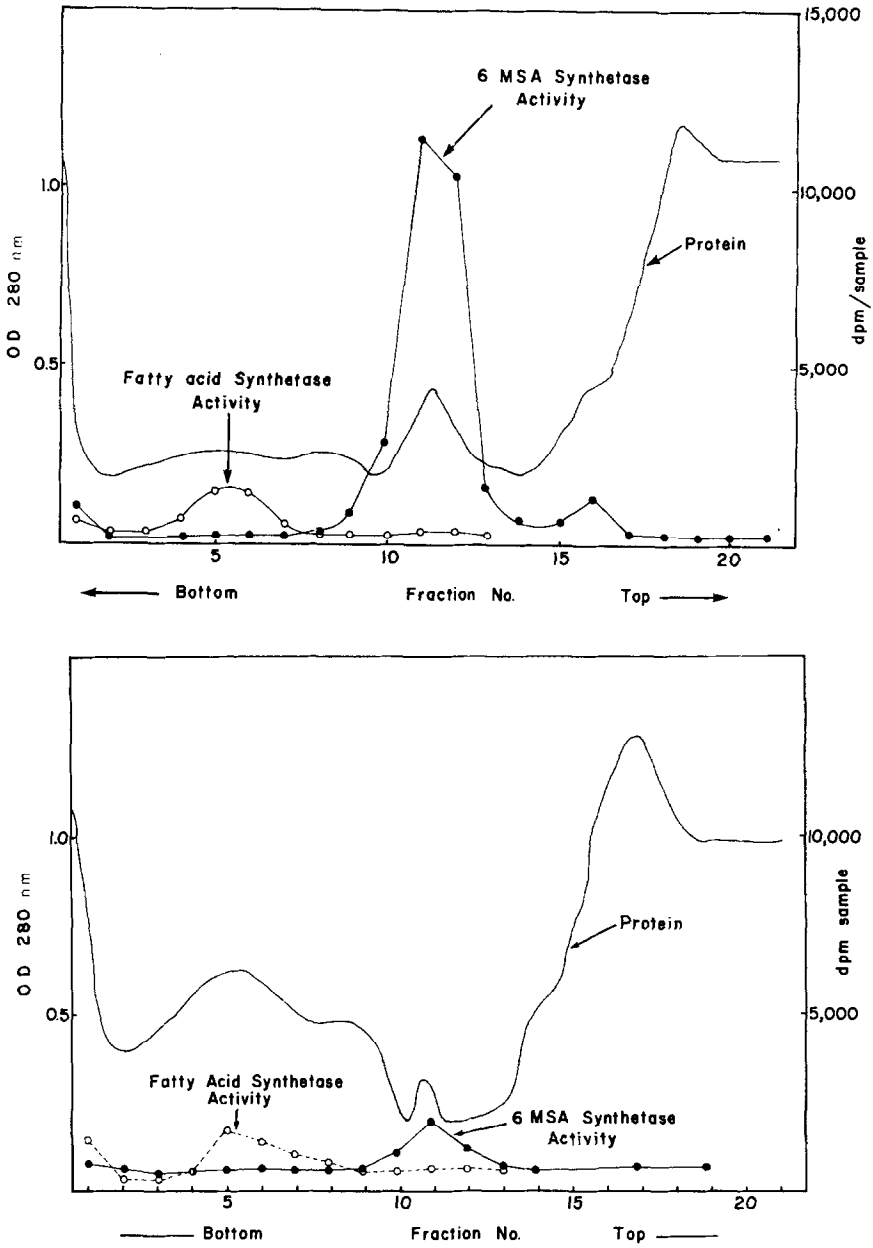
TABLE 2

CO-PURIFICATION OF FATTY ACID SYNTHETASE

Step	Total protein[a] (mg)	Total activity[b] (mU)	Specific activity (mU/mg)	Relative yield (%)	Purification (-fold)
Crude extract from centrifugation at 100,000*g*	890	341	0.383	100	1
Acetone powder	638	325	0.509	95	1.3
25–40% ammonium sulphate precipitation	220	125	0.567	37	1.5
Resuspended pellet from centrifugation at 144,000*g*	21	46	2.19	14	5.7
Active fractions from 5 to 20% sucrose gradients	0.9	13.1	14.6	4	38.1

[a] Protein was determined by the Biuret method, except for the sucrose gradient fractions, which were determined by the method of Warburg and Christian (*12*).

[b] Enzyme activity was assayed by radioactive method (a).



FIGS. 1 and 2. Sucrose Gradient. (—) Protein concentration, determined by uv absorption at 280 nm (Uvicord II). (●), 6-MSA synthetase activity, assayed by procedure (a). (○), Fatty acid synthetase activity, assayed by procedure (c).

increased. A very similar pattern was obtained in gradients containing glycerol when more than 20 mg of protein were layered on the gradient tube. Dialysis of the higher molecular peaks against 0.1 *N* K-phosphate buffer (pH 7.6) containing 2×10^{-3} *M* EDTA, 1×10^{-3} *M* β-ME and 15% glycerol did not restore activity.

Centrifugation in a 10–35% glycerol gradient also yielded active 6-MSA synthase and the elution profile was similar to that of a glycerol-containing sucrose gradient. However, due to the fact that both specific and total activity were approximately three-fourths of that with the glycerol-containing sucrose gradient, the glycerol gradient was not used.

Separation of Fatty Acid Synthase Activity

Fatty acid synthase co-purified with 6-MSA synthase up to the sucrose gradient step (Table 2). Recovery of fatty acid synthase activity from the sucrose gradients was low and inconsistent. However, fractions 5 and 6 did contain some activity in most preparations.

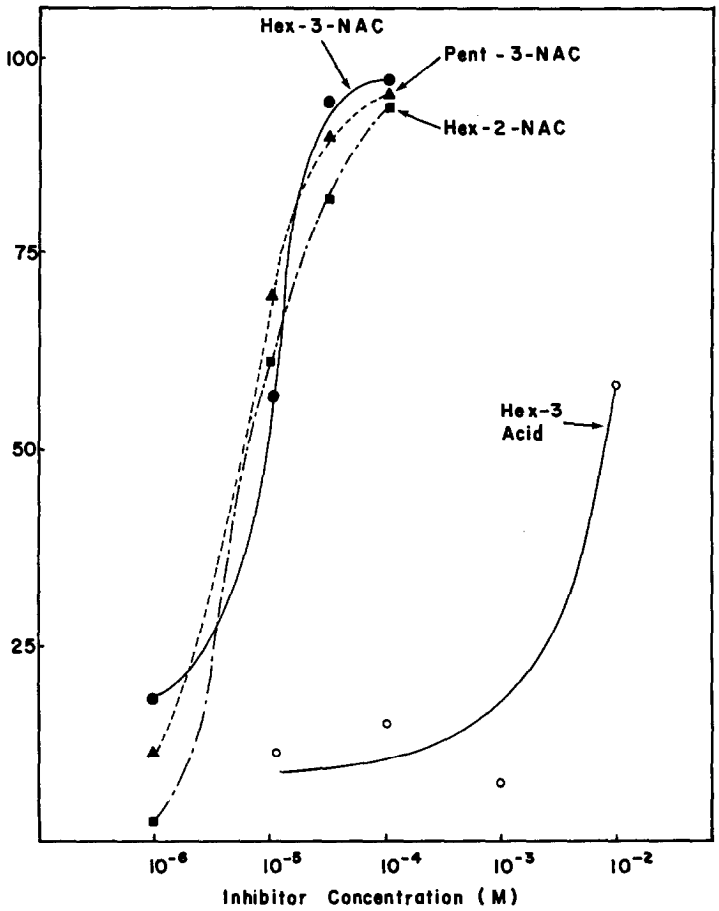


FIG. 3. Inhibition by Acetylenic Thioesters. The incubation mixture contained in a final volume of 1.0 ml: 200 μM K-phosphate pH 7.6, 0.15 μM [2-^{14}C] malonyl CoA, 0.035 μM acetyl-CoA, 0.1 μM 6-MSA, inhibitor to give the desired concentration, and 0.025 mg purified enzyme. The components minus the [2-^{14}C]-malonyl CoA were preincubated for 10 min at 30°C, then the incubation started by the addition of the [2-^{14}C]-malonyl CoA. The reaction was stopped after 10 min and assayed by procedure (a) of the assay methods.

Inhibition of 6-MSA Synthase with Acetylenic Thioesters

The acetylenic thioesters 3-hexynoyl-NAC, 2-hexynoyl-NAC and 3-pentynoyl-NAC inhibit 6-MSA synthase almost completely at 10^{-4} *M* concentrations (Fig. 3). The same inhibitors inhibit 6-MSA synthase approximately 60% at 10^{-5} *M* concentration, whereas free 3-hexynoic acid inhibits to the same extent (i.e., 60%) at 10^{-2} *M* concentration (Fig. 3).

In Fig. 4, the conversion of NADPH to $NADP^+$ by the acetylenic thioester-inhibited 6-MSA synthase is compared to the uninhibited enzyme, and the NEM-inhibited enzyme. Under conditions that led to 96% inhibition of 6-MSA production (10^{-4} *M* concentration of the acetylenic thioester; 10 min preincubation), the initial velocity

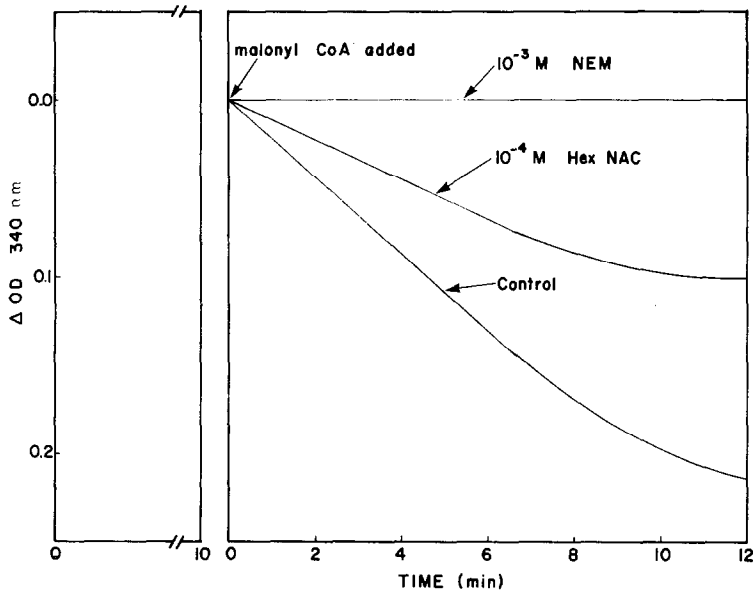


FIG. 4. Oxidation of NADPH by Inhibited 6-MSA Synthetase. The control cuvette contained in a total volume of 1.0 ml: 100 μM NADPH, 0.035 μM acetyl CoA, 50 μg enzyme. $d = 1$ cm, $T = 25°C$. After a preincubation period of 10 min, the reaction was started by the addition of 0.1 μM malonyl CoA and the decrease in OD at 340 nm was followed in a Cary 14 uv spectrophotometer. The other cuvettes contained, in addition to the above components, 3-hexanoyl-NAC in a final concentration of 10^{-4} *M* or NEM in a final concentration of 10^{-3} *M*. The inhibitor was preincubated with components of the incubation mixture and enzyme for 10 min, then the reaction was started by the addition of 0.1 μM malonyl CoA and followed as in the control.

of NADPH oxidation by the enzyme was reduced by only 40% and the 10 min fixed time assay was 50% that of the control. Under the same assay conditions, a concentration of NEM (10^{-3} *M*) which inhibited 6-MSA production 100% (*15*) inhibited NADPH oxidation 100% too. Furthermore, addition of acetylenic inhibitors (10^{-4} *M*) to an already reacting system decreased the reaction velocity by only 25%, whereas addition of NEM (10^{-3} *M*) resulted immediately in complete inhibition. However, when the concentration of acetylenic thioester was increased to 10^{-3} *M*, NADPH oxidation was completely inhibited. This indicates that acetylenic inhibitors may in fact react with sulf-

hydryl groups (but at a slower rate), a possibility which was pointed out in an earlier paper (*7*). Thus, the specificity of the dehydrase/isomerase inhibition bears an inverse relation to the concentration of the acetylenic inhibitors. Similar results were obtained when TAL production was examined under different concentrations of the acetylenic inhibitors (*15*).

DISCUSSION

The preservation of activity during the purification of 6-MSA synthase from *P. patulum* NRRL2159A (RL-17, early 6-MSA strain) requires the presence of glycerol in the buffers used. The stabilizing effect of glycerol was noted in previous work in this laboratory, when the enzyme was partially purified. (*7*) However, in Lynen's published procedure there is no indication that glycerol was used in the steps subsequent to the formation of acetone powder. This discrepancy is more apparent than real; in a very recent personal communication regarding the matter we were informed that the aforementioned group did in fact always use buffers containing glycerol.

The stabilizing effect of polyhydroxy compounds on various proteins has been known for many years and studies aimed at its explanation have been made (for example, Ref. *16*; however, in our case sucrose could not substitute for glycerol). The effect of glycerol seems to be most pronounced in the steps that involved handling the enzyme for somewhat prolonged periods of time (e.g., sucrose density centrifugation; duration 16 hr).

Comparison of the eluting patterns of the sucrose gradients with and without glycerol (Figs. 1 and 2) provides some information as to the nature of the stabilizing effect of glycerol on 6-MSA synthase. Thus, it seems that glycerol prevents the aggregation of the multienzyme complex with itself and/or other proteins resulting from nonspecific interaction of the macromolecules. Interestingly enough, fatty acid synthase does not exhibit a similar behavior (cf. fatty acid activity in Figs. 1 and 2).

6-MSA biosynthesis, although similar in many respects to the known mechanism of fatty acid biosynthesis, differs in that it needs only one reduction for three condensation steps. Also, the geometry of the 6-MSA molecule necessitates the formation of an intermediate after dehydration containing a *cis* double bond rather than the α, β-*trans* enoyl intermediate that occurs in saturated fatty acid biosynthesis (*17*). Therefore, a prime consideration of any proposed mechanism involves the question as to whether the reduction and dehydration occur on a 6-carbon intermediate, a 3,5-diketohexanoyl entity, or an 8-carbon intermediate, a 3,5,7-triketooctanoyl entity. Dimroth, Walter, and Lynen have proposed a detailed mechanism of 6-MSA biosynthesis (*6*) based on their experimental results and the similarity of 6-MSA synthesis to fatty acid synthesis (*18*). According to this mechanism, the action of 6-MSA synthase can be presented schematically as in Fig. 5.

As is shown in Fig. 5, there are distinct "central" and "peripheral" sulfhydryl groups quite analogous to those of fatty acid synthase (*18*)[4]. The synthetic process is initiated by the transfer of an acetyl residue from acetyl CoA to the "peripheral" sulfhydryl group and malonyl CoA to the "central" sulfhydryl group. The next step is the conden-

[4] However, contrary to (yeast) fatty acid synthase (19) neither an ACP-like polypeptide nor a 4′ phosphopantetheine moiety have yet been isolated from 6-MSA synthase.

sation of enzyme-bound acetyl and malonyl groups forming acetoacetyl-enzyme and liberating CO_2. The acetoacetyl group is transacetylated to the peripheral sulfhydryl, another malonyl CoA is loaded onto the central sulfhydryl group and a second condensation takes place forming 3,5-diketohexanoyl-enzyme. At this point, reduction of the 3-keto group by NADPH to form 3-hydroxy-5-keto-hexanoyl-enzyme occurs. The reduced intermediate is then dehydrated to give the *cis*-5-keto-hex-3-enoyl-enzyme, by a dehydrase whose mode of action is similar to the β-hydroxydecanoyl thioester dehydrase which functions in the anaerobic biosynthesis of *cis*-unsaturated fatty acid in

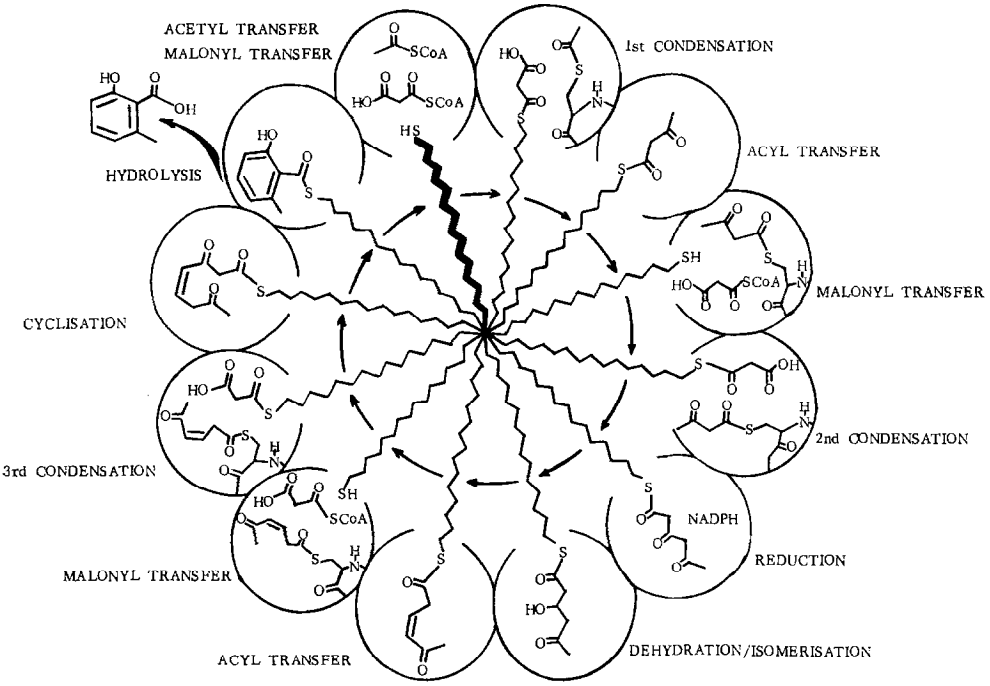


FIG. 5. Hypothetical scheme of 6-MSA biosynthesis.

E. coli (*20*). A third condensation with malonyl-enzyme gives an 8-carbon enzyme-bound intermediate, 3,7-diketo-oct-5-enoyl-enzyme, which undergoes an internal aldoltype condensation between the C-2 and C-7 positions followed by further dehydration to give enzyme-bound 6-MSA. A thiolesterase then releases the product, free 6-MSA.

The experimental evidence supporting the above mechanism is as follows:

1. The presence of two sulfhydryl sites is suggested by the fact that the inhibition of 6-MSA synthase by NEM is pH dependent, whereas the inhibition by iodoacetamide is pH independent (*21*). Thus, 6-MSA synthase is very similar to yeast fatty acid synthase (*22*), which has been shown to contain two distinct sulfhydryl sites (*18*).

2. The omission of NADPH from the enzyme incubation mixture leads to the formation of the 6-carbon compound TAL (*6*). This has been taken as evidence that the

reduction occurs at the triacetic acid level, before the third condensation with malonyl CoA.

3. The model intermediate, ethyl 3,5-diketohexanoate, was reduced by the enzyme in the presence of NADPH (*23*), further indicating that the reduction occurs on a 6-carbon intermediate. However, neither the nature of the product nor its conversion to 6-MSA was described.

Regarding the second piece of evidence, it should be noted that fatty acid synthase from pigeon liver (*24*) and yeast (*25*) effect the synthesis of TAL in the absence of NADPH. Also, the condensing enzyme involved in fatty acid formation in *E. coli* effects TAL synthesis even in the presence of NADPH (*26*). Since the first reduction step in fatty acid biosynthesis is known to occur on a 4-carbon intermediate, the production of TAL by these systems may indicate that TAL is a stable "shunt" metabolite released from enzyme systems that condensate acetate reduced by the enzyme. In line with this possibility are other reports of TAL production by organisms that produce polyketide compounds (e.g., TAL formation and tetraacetic acid lactone formation by cultures of *P. stipitatum* when inhibited in tropolone production by ethionine (*27*); production of a methyl derivative of TAL, 3,6-dimethyl-4-hydroxy-pyran-2-one, by another strain of the same organism (*28*). Lynen *et al.* (*6*) and Scott *et al.* (*7*) demonstrated that TAL is formed by 6-MSA synthase even in the presence of NADPH, suggesting that TAL may be a normal "shunt" or "derailment" product of 6-MOA synthase.

The synthesis of 6-MSA by the purified 6-MSA synthase is inhibited by the acetylenic thioesters 3-hexynoyl-NAC, 3-pentynoyl-NAC, and 2-hexynoyl-NAC (Fig. 3). The inhibitors at a concentration of 10^{-5} *M* give a 60% inhibition of 6-MSA synthesis. This inhibition is slightly greater than that previously reported for the partially purified enzyme (*7*). The free 3-hexynoic acid inhibited to the same extent (60%) only at 10^{-2} *M* concentration. This indicates that secondary interactions between the NAC moiety and specific amino acid residues in the active site may be important for efficient binding of the acetylenic inhibitors. Our results parallel those obtained by Kass and Bloch, who showed that 3-decynoyl-NAC at low concentrations specifically inhibited the synthesis of unsaturated fatty acid synthase of *E. coli* (*29*). The mode of action of this inhibitor involves the inhibition of the β-hydroxydecanoyl thioester dehydrase (*30*), an enzyme that catalyzes the formation of both α, β-*trans*, and β, γ-*cis*-decenoyl thioester (*8*, *31*). Bloch *et al.* have shown that 3-decynoyl-NAC acts by covalent binding to a histidyl site (*32*). Recent studies have indicated that the β-hydroxydecanoyl-thioester dehydrase probably functions by isomerizing the inhibitor 3-decynol-NAC to the 2,3-decadienoyl-NAC, the latter being the active entity which binds to the enzyme. By analogy, the mechanism of action on the assumed intermediate and on the acetylenic analog can be drawn as shown in Fig. 6.

In order to clarify the mode of action of the acetylenic inhibitors on 6-MSA synthase, the oxidation of NADPH by the enzyme in the presence of inhibitor was examined. At a concentration of acetylenic inhibitor (10^{-4} *M*) which completely inhibited 6-MSA synthesis, there was a significant oxidation of NADPH, the initial rate being 60% of the control without inhibitor (Fig. 4). In an identical experiment with *N*-ethylmaleimide at a concentration (10^{-3} *M*) which completely inhibited 6-MSA production, the NADPH oxidation was also completely inhibited (Fig. 4). This indicates that the acetylenic inhibitors are acting after the reduction step. The partial inhibition of NADPH oxi-

dation suggests that some inhibition of sulfhydryl groups *is* taking place.[5] Thus, 6-MSA synthase contains a dehydrase activity which leads to the production of a *β,γ-cis*-enoate in a manner similar to *β*-hydroxydecanoyl dehydrase as suggested by Lynen (*6*) and Scott, *et al.* (*7*).

The extent and mechanism of enzyme control over the final steps of 6-MSA biosynthesis is unknown. An *in vitro* model compound, 5,6-dihydrotetracetic acid lactone, has

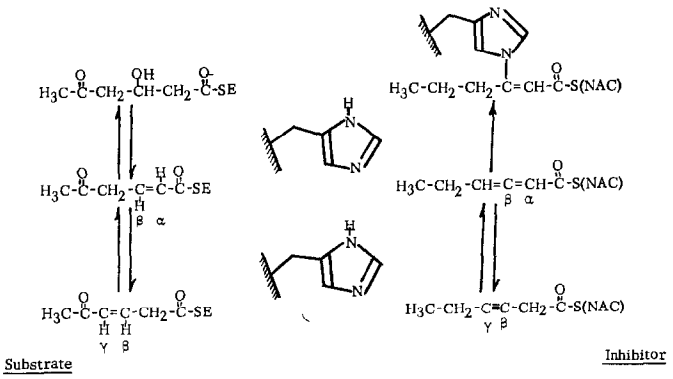


FIG. 6. Possible mechanism of action of the dehydrase component of 6-MSA synthetase, by analogy to *E. coli* *β*-hydroxydecanoyl thioester dehydrase.

been used to generate *in situ* diketo-octenoic acid (*33*), which is the free acid of the final 8-carbon unsaturated enzyme-bound intermediate in Fig. 5. This species readily undergoes dehydrative aromatization to yield 6-MSA. The experiment suggests that once the 8-carbon *cis* unsaturated entity is formed, it could very easily undergo the internal aldol, dehydration and aromatization needed to form 6-MSA with little enzyme control. A thiolesterase then hydrolyzes the thioester bond to form the final product, 6-MSA.

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[5] An alternative explanation is that the observed partial inhibition is due to conformational changes originating in the dehydrase component as a direct result of the action of acetylenic inhibitors. These changes would be transmitted, by heterologous interactions, to enzymic compounds responsible for reactions earlier in the Scheme (Fig. 5). However, in view of the fact that fatty acid activity also shows some inhibition in the presence of the acetylenic inhibitors, sulfhydryl inhibition is most likely to be the case.

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