Enzymatic Studies on the Polyketide Hypothesis

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The polyketide hypothesis proposes that many natural products are derived biosynthetically by cyclization of poly-β-carbonyl (polyketo) acids. Recent versions of this hypothesis propose that the polyketo acid chains can be formed by condensation of one acetyl and several malonyl thiol esters in a manner similar to the condensation reaction of fatty acid biosynthesis. Properties of three crude synthases which support this hypothesis are reviewed: 6-methylsalicylic acid (6-MSA) synthase, alternariol synthase, and orsellinic acid synthase. 6-MSA synthase has been purified about 100-fold and has been shown to be a multi-enzyme complex. Principal problems in studying polyketide synthases include instability of the enzyme preparation and difficulties in reproducibly obtaining cultures with active enzyme. 6-MSA synthase has been stabilized by acetone precipitation, and activity of this enzyme in cultures can be enhanced by low concentrations of cycloheximide.

The polyketide hypothesis dates back to the turn of the century when Collie (1907) first postulated that a number of natural products could be derived from head to tail acetate polymers or polyketides. This notion lay dormant for almost 50 years when it was put forward again by Birch and Donovan (1953) who related the hypothesis to a number of known structures and who subsequently began to provide a valid experimental basis for the proposal.

Several features of the hypothesis are illustrated in Figure 1. The polyketide, or β-polyketoacid shown here, is a linear chain of four acetyl units with the acid derivative left unspecified for the moment. An aldol condensation between the methylene carbon-2 and the carbonyl carbon-7 (path a) would lead to orsellinic acid (I). A Claisen type acylation of methylene carbon-6 by the carboxyl carbon-1 (path b) would lead to acetylphloroglucinol (II). Minor modifications of the chain would produce other known compounds. For example, a reduction of the carbonyl carbon-5 is necessary for the formation of 6-methylsalicylic acid (6-MSA) (IV), and alkylation of methylene carbon-6 by a methyl group is necessary for the formation of 5-methyl orsellinic acid (III). A second example is illustrated for compounds of the anthraquinone group, found in many angiosperm orders as well as in fungi (Mathis, 1966; Shibata, 1967) (Figure 2). In this case a 16 carbon octaacetyl chain is the presumed intermediate of both emodin (V) and chrysophanol (VI). Notice that a single reduction of carbonyl carbon-9 is necessary in the formation of chrysophanol. A second point is illustrated here. The compound isolated from nature seldom represents the initial cyclization product of a polyketide chain. In this case both a decarboxylation and oxidation at position 9 of the anthraquinones have occurred. By varying the chain length and the path of cyclization many natural products are derivable by the polyketide mechanism.

The first evidence put forward for this hypothesis was purely structural, and included the recognition of such things as the positioning of the phenolic groups beta to each other on a large variety of aromatic compounds. In fact, the hypothesis allowed the correct assignment of structure to be made in several ambiguous cases. Birch *et al.* (1955) provided the first experimental evidence by chemically degrading radioactive 6-MSA (IV) which was formed from incorporation of either methyl or carboxyl labeled acetic acid. The alternating pattern found was consistent with head to tail condensations of acetate as required by the polyketide structure. Since then incorporation experiments have verified the acetate labeling pattern for many natural products. An experiment by Gatenbeck and Mosbach (1959) demonstrated that the phenolic oxygens of orsellinic acid were derived from the oxygen of acetate which was labeled with ^{18}O.

COMPARISON WITH FATTY ACID SYNTHESIS

The fatty acids might be considered as a special case of a fully reduced polyketide chain, and so it is not surprising that a slight modification of the polyketide hypothesis was made

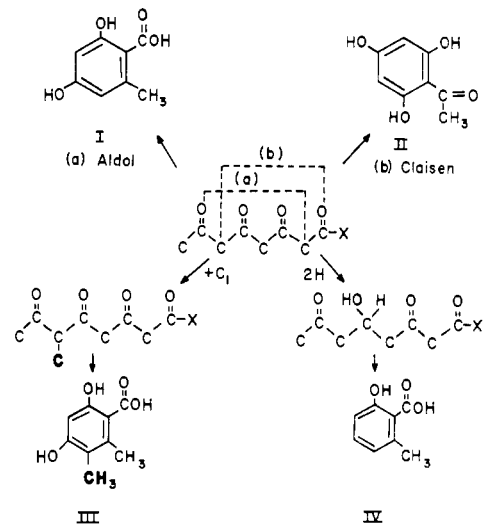

Figure 1. Several polyketides derivable from a tetraacetyl chain

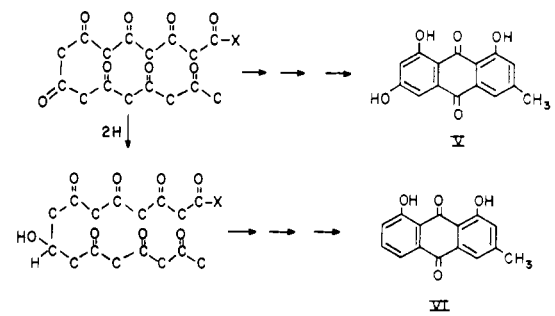

Figure 2. Anthraquinones derivable from an octaacetyl chain

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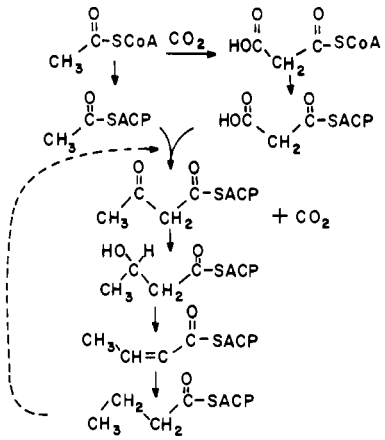


Figure 3. Mechanism of fatty acid biosynthesis

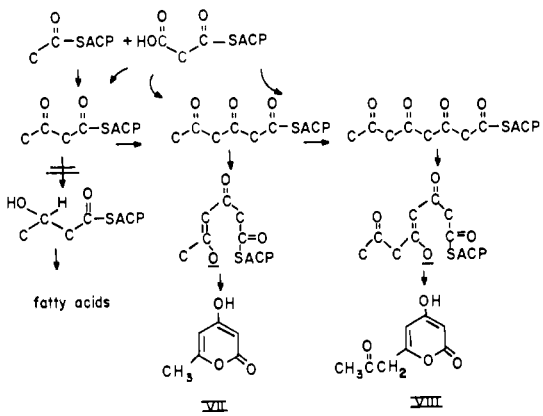


Figure 4. Polyketide byproducts of fatty acid synthases

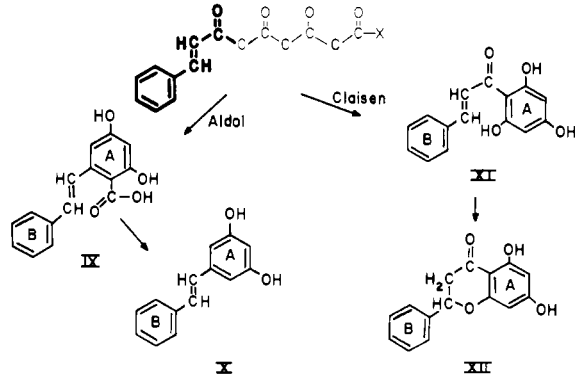


Figure 5. Polyketides with a cinnamoyl starter group

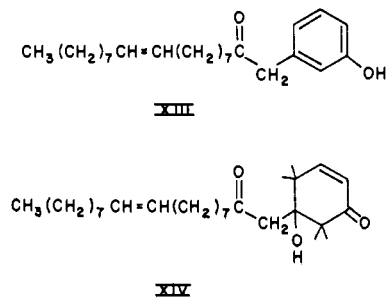


Figure 6. Tigaso oil constituents from *Campnosperma brevipetiolata* (Dalton and Lamberton, 1958)

as understanding of the mechanism of fatty acid biosynthesis developed. Figure 3 shows a summary of this mechanism as derived from contributions of many laboratories (see recent review by Majerus and Vagelos, 1967). An acetyl thiol ester forms the starting point of the fatty acid chain, and condensation occurs with a malonyl thiol ester to form a β-keto thiol ester. Reduction, dehydration, and reduction steps occur to form a saturated (butyryl) thiol ester, and the process is repeated, each cycle adding two carbons to the chain. The intermediates are bound as thiol esters to a protein called acyl carrier protein (ACP) (Majerus *et al.*, 1964), this linkage being first formed by an exchange reaction between acetyl-CoA and malonyl-CoA with the acyl carrier protein. In *Escherichia coli*, the ACP and the enzymes catalyzing each step can be separated and studied individually (see Majerus and Vagelos, 1967). In yeast (Lynen, 1961) and pigeon liver (Hsu *et al.*, 1965), all components as well as the acyl carrier protein are part of a multienzyme complex which has not yet been successfully taken apart and reconstituted.

The fatty acid synthases demonstrate that the polyketide producing condensation reactions are possible in principle (Figure 4). In the absence of NADPH, both the purified pigeon liver (Nixon *et al.*, 1968) and yeast (Yalpani *et al.*, 1969) synthases and the crude *E. coli* synthase (Brock and Bloch, 1966) will carry out a second malonyl addition to the β-keto thiol ester. The resulting diketoester apparently cyclizes spontaneously as shown in Figure 4, as triacetic acid lactone (VII) is the principal product in all cases. The rate of triacetic acid lactone production by the yeast synthase is only 1% of that of fatty acid production (Yalpani *et al.*, 1969). The *E. coli* condensing enzyme, purified free of the β-keto acyl ACP reductase, will produce lactone VII even in the presence of NADPH. Tetraacetic acid lactone (VIII) is also formed, indicating a third condensation is possible. Octanoyl ACP will react with two moles of malonyl ACP to produce the corresponding lactone (Soucek and Bloch, 1969). These lactonization reactions also illustrate the inherent instability of polyketide thiol esters and make it necessary to assume that enzyme surfaces provide a means of stabilizing longer polyketide chains. Lactones VII and VIII do not themselves serve as intermediates in the biosynthesis of other polyketides (Light *et al.*, 1966; Bentley and Zwitkowitz, 1967).

Tracer experiments with ^{14}C-malonate (Bentley and Keil, 1961) first provided evidence that penicillic acid was formed from one acetyl and three malonyl groups. Since then the "acetyl-polymalonyl" labeling pattern has been established for many polyketides. Tracer experiments with ^{3}H-acetate showed similar tritium retention in the fatty acid chain and in 6-MSA (IV), consistent with similar condensation mechanisms (Light, 1965).

A structural feature of some polyketides also supports this analogy with fatty acid biosynthesis. Several starter groups other than acetate are possible as indicated by the examples shown in Figures 5, 6, and 7. Addition of three malonyl units to a cinnamoyl starter group (Figure 5) could produce a polyketide which would give chalcone XI and then compounds of the flavonoid series such as flavanone XII by a Claisen type cyclization, or compounds of the stilbene series such as pinosylvic acid (IX) and pinosylvin (X) by an aldol cyclization. Again labeling experiments support this view; although the cinnamoyl group (ring B) is derived from compounds related to shikimic acid, ring A is derived from acetate condensations (for example, Hillis and Ziegler, 1962; Under-

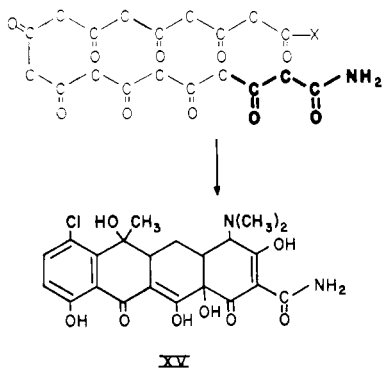


Figure 7. Proposed polyketide precursor of 7-chlorotetracycline (McCormick, 1965)

hill *et al.*, 1957). Biosynthesis of the flavonoid compounds has been recently reviewed by Grisebach (1965), who has made many contributions to understanding the relationships between compounds in this series. Campnospermol (XIII) and its hydrated analog (XIV) (Figure 6), plant products from the sapwood of *Campnosperma brevipetiolata* (Dalton and Lamberton, 1958) probably utilize oleic acid as a starter group. A malonamide starter group (Figure 7) has been postulated for tetracyclines (McCormick, 1965). Many of the structural relationships illustrated in Figures 1, 2, 5, and 6 were first noted by Birch and Donovan (1953).

ENZYMATIC STUDIES

The experimental foundations for the polyketide hypothesis, both structural agruments and isotope incorporation data, have been extensively reviewed in many places (for example, Bentley and Campbell, 1968; Birch, 1968; Bu'Lock, 1965; Richards and Hendrickson, 1964; Shibata, 1967). The purpose of this review is to summarize experiments from several laboratories over the last few years which attempt to verify the hypothesis at the enzymatic level. This field has lagged far behind the mainstream of intermediary metabolism in terms of establishing a metabolic pathway by characterizing the enzymes responsible for each step of the pathway.

It must be recognized that there are at least three and sometimes four separate processes to be considered in attempts to isolate polyketide biosynthetic enzymes. These are illustrated in Figure 8 for the case of 6-MSA (IV). The first process is the generation of the hypothetical polyketide chain, where —X would represent a thiol ester linkage to a protein molecule if the analogy with fatty acid synthesis holds. In some cases evidence indicates that a modification of the chain must occur prior to cyclization. This is illustrated here by reduction of one carbonyl group. Other examples would include alkylation by a methyl group at one or more of the methylene positions, or perhaps at oxygen. Whether chain modification occurs before the chain is completed, after it is completed, or after it is released from the thiol ester linkage can only be answered by enzymatic studies. The third process, cyclization, might occur before or after the chain is released from the thiol ester linkage. The fourth process illustrated in Figure 8 has nothing to do with the polyketide hypothesis *per se*, but has considerable influence on what compounds are actually isolated from natural sources. In the case of the fungus *Penicillium patulum*, enzymatic modifications of 6-MSA lead to a family of phenolic substances including gentisaldehyde (XVI) shown here, and patulin (XVII), which under cer-

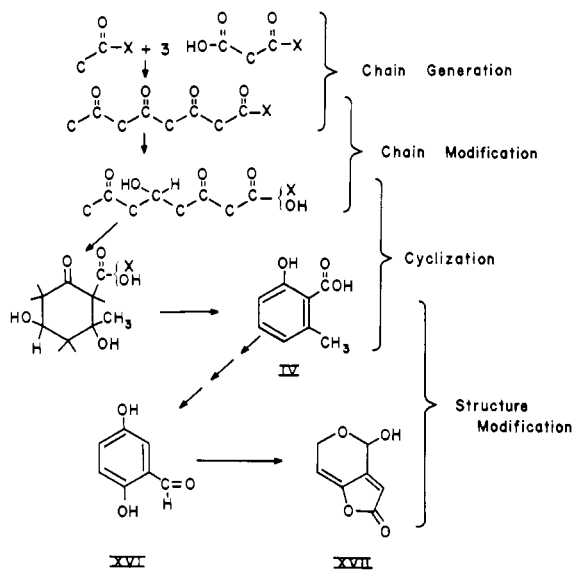


Figure 8. Synthetic processes in polyketide formation illustrated with the 6-methylsalicylic acid family

tain conditions can be the major metabolic product (Tanenbaum and Bassett, 1959).

Structure modifying reactions are of relatively few basic types, and include decarboxylation, hydroxylation, alkylation by methyl or isopentenyl groups, oxidation, aromatic ring cleavage, and phenol coupling. Enzymes catalyzing some of these reaction types have been isolated and partially characterized. For example, decarboxylases for 2,3-dihydroxybenzoic acid (Terui *et al.*, 1952), stipitatonic acid (Bentley and Thiessen, 1963), orsellinic acid (Petterson, 1965), and 6-MSA (Light, 1969) have been reported. Bu'Lock *et al.* (1965) have discussed the evidence that these modifying enzymes may be sequentially induced following appearance in cultures of the initial cyclic polyketide. Study of these enzymes and their induction may certainly provide important information about control mechanisms in organisms other than bacteria.

Two reports from Tanenbaum and Bassett indicate very complex cell free activity converting glucose and other precursors to patulin (Bassett and Tanenbaum, 1960) and to stipitatic acid (Tanenbaum and Bassett, 1962). These systems must involve not only all four processes cited above, but enzymes metabolizing glucose to the polyketide precursors as well. The final test of the polyketide hypothesis, however, must come from enzymatic studies which focus on the mechanism of the chain generation and cyclization steps, and which hopefully can identify the intermediate polyketide chain.

6-MSA SYNTHASE

Lynen and Tada (1961) reported a cell free preparation, similar to Bassett and Tanenbaum's (1960) from surface pads *Penicillium patulum*, which incorporated ^{14}C-acetyl-CoA into 6-MSA. The incorporation required both malonyl-CoA and NADPH and was inhibited by sulfhydryl reagents, all properties which supported the fatty acid analogy. Unfortunately several laboratories, including our own, encountered difficulty in obtaining active preparations by the reported method. Eventually we were able to obtain active preparations from submerged cultures of *P. patulum* (Light,

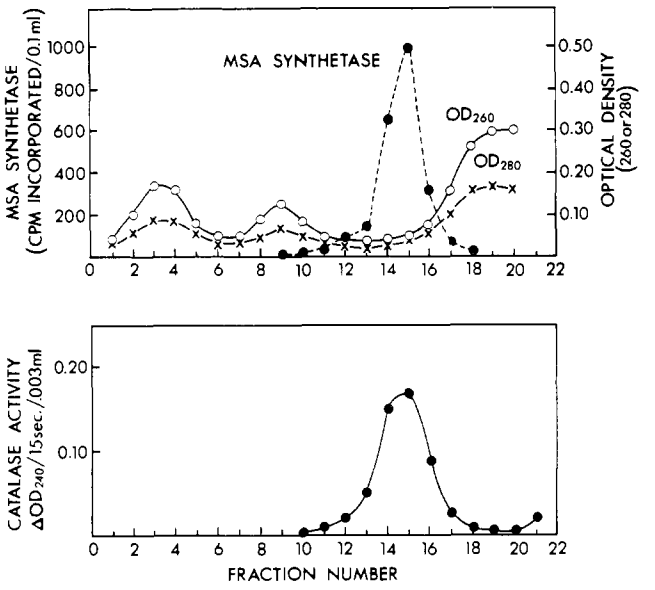


Figure 9. Sucrose density gradient centrifugation of 6-MSA synthase activity in *Penicillium patulum* crude extracts using catalase as a marker (Light and Hager, 1968)

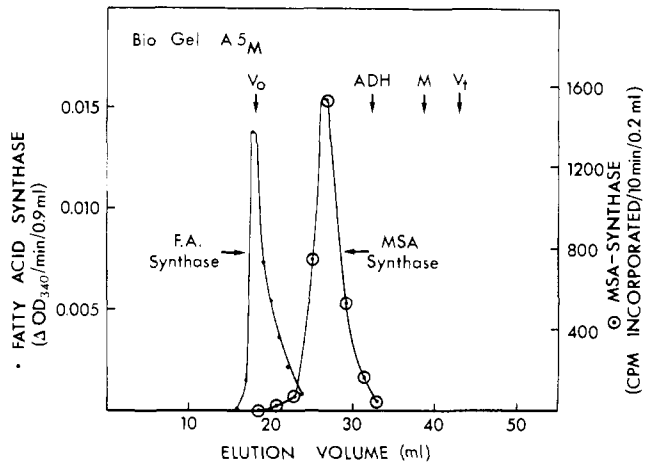


Figure 10. Gel filtration of crude *Penicillium patulum* extracts on Bio Gel A5m

Reference standards: V_0 (void volume), blue dextran; ADH, alcohol dehydrogenase; M, myoglobin; V_t (total volume), glucose. The column (1.5 × 27 cm) was packed in buffer (0.2*M* phosphate, 0.1*M* "TES" [*N-tris*-(hydroxy methyl) methyl-2-amino-ethanesulfonic acid] 1m*M* EDTA. 0.1m*M* dithiothreitol, pH 7.7), 0.75 ml of crude extract applied, and fractions of 1.04 ml were collected and assayed for fatty acid synthase (Colowick and Kaplan, 1962) or 6-MSA synthase (Light, 1967b)

1967b). These crude preparations confirmed the requirement for malonyl-CoA and NADPH. When carboxyl labeled malonyl-CoA was employed as substrate, the product 6-MSA contained one third of the radioactivity in the carboxyl group as would be expected from a condensation of one acetyl and three malonyl groups. In collaboration with Hager (Light and Hager, 1968), we demonstrated that the 6-MSA biosynthetic activity has another feature in common with fatty acid synthase. It behaves as a high molecular weight complex, both on Sephadex gel filtration and sucrose density gradient centrifugation experiments. In Figure 9 is shown the sedimentation of 6-MSA synthase in a sucrose density gradient with catalase as a marker. This experiment gives an estimate of about 10.4 S for the sedimentation constant. Gel filtration on Sephadex G-200 provided an

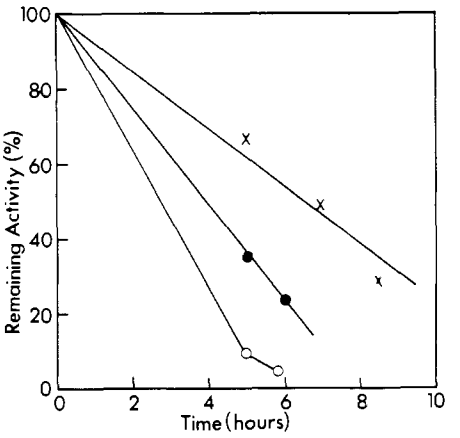


Figure 11. Stability of 6-MSA synthase in crude extracts in different buffers at pH 7.6: (○) 0.02*M* phosphate; (●), 0.05*M* phosphate, 0.2*M* TRIS, 0.2*M* sodium chloride, 10^{-3}*M* EDTA; (×), 0.05*M* phosphate, 0.2*M* TRIS, 0.2*M* sodium chloride, 10^{-3}*M* EDTA, 0.08*M* ammonium sulfate

estimate of 85 Å for the Stokes radius. The molecular weight calculated from these two values depends upon the partial specific volume of the complex. Assuming the average protein value of 0.725 cm.^{3} we obtain a molecular weight of 3.7 × 10^5 and an unusually large frictional ratio of 1.8. The high frictional ratio indicated either a very high axial ratio or a much higher partial specific volume than our estimated value. This complex is not as large as the fatty acid synthase from the same organism, and the two activities can be separated on a Bio Gel A5m column (Figure 10).

Studies with crude extracts present several difficulties. In these preparations there are at least two competing activities present besides the fatty acid synthase. One is a malonyl-CoA decarboxylase and the other a 6-MSA decarboxylase. Interference by these activities can be minimized by using high enough concentrations of malonyl-CoA and by adding trapping quantities of carrier 6-MSA, but interpretation of results must take them into account. For example, it is not possible to demonstrate a dependence on acetyl-CoA because of the malonyl-CoA decarboxylase. A much worse problem, however, lies in the instability of the preparation, illustrated by the data in Figure 11. High salt gives a slight improvement adequate for the gel filtration and centrifugation studies, but not adequate for purification.

Recent work by Dr. Dimroth in Lynen's laboratory may have provided a key to this problem (Dimroth, 1968). He achieved considerable stability of 6-MSA synthase by making an acetone powder of the crude extract. The stabilized preparation was then purified about 100-fold with a yield of 24% as shown in Table I. The purified preparation appeared about 90% homogeneous in the ultracentrifuge and was free of fatty acid synthase, NADPH oxidase, and malonyl-CoA decarboxylase. It was relatively stable, losing about 50% of its activity in 10 days when stored in 50% glycerol at −10° C. The purified synthase had a sedimentation constant of about 30 S using a sucrose density gradient with catalase as a marker (Figure 12). This value is considerably higher than ours, but provides the same conclusion that the activity is associated with a multi-enzyme complex. There are several possible explanations for the discrepancy. Dimroth employed a different strain of *P. patulum* and considerably different growth conditions. His acetone treatment may have caused either aggregation of the complex or may have removed lipid and thereby reduced the partial specific volume

Table I. Purification of 6-MSA Synthase[a]

Purification Step	Protein, mg.	Total Activity, milli-units	Specific Activity, milli-units/mg.	Yield, %
Crude extract	5870	7360	1.2	100
Acetone precipitation	4280	7310	1.7	99
Ammonium sulfate (0.25–0.4)	810	7100	8.8	96
Ultracentrifuge sediment (4 hr. 144,000 × g.)	160	3750	23.4	51
Gradient centrifugation	20	2220	111	30
Ammonium sulfate (0–0.5)	13.5	1780	131	24

[a] One enzyme unit corresponds to synthesis of 1 μmole of 6-MSA per minute. Taken from Dimroth's doctoral thesis (Dimroth, 1968).

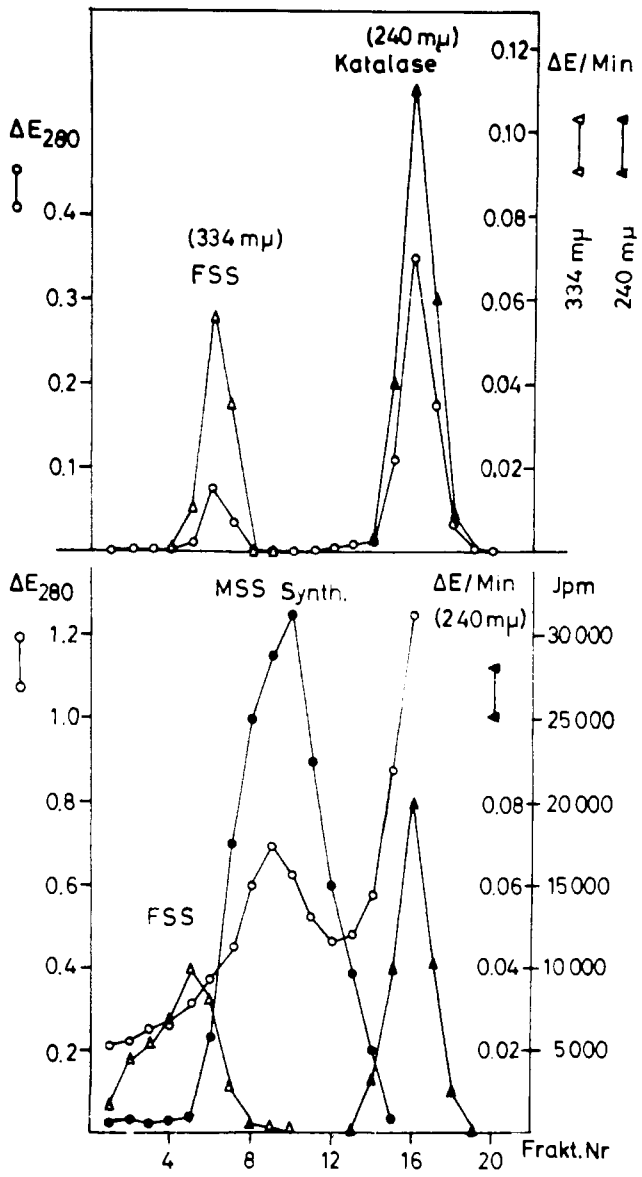


Figure 12. Sucrose density gradient centrifugation of purified 6-MSA synthase activity using catalase and the *Penicillium patulum* fatty acid synthase as markers in the lower curve

Upper curve shows catalase and purified yeast fatty acid synthase in a similar gradient. The figure was taken from Dimroth's doctoral thesis (Dimroth, 1968)

of the complex. Aggregation may have resulted from the higher enzyme concentration possible with purified enzyme. And finally our preparation was a very unstable crude extract which could have presented some unknown difficulty.

Characterization of the purified 6-MSA synthase revealed further similarities with the fatty acid synthase (Dimroth, 1968). The involvement of at least two sulfhydryl groups was indicated by a pH dependent inhibition with *N*-ethylmaleimide and a pH independent inhibition with iodoacetamide. A K_m of $2 \times 10^{-5}M$ was obtained for both acetyl-CoA and malonyl-CoA. The pH optimum was 7.6, identical to that of the fatty acid synthase from *P. patulum*. And finally, the 6-MSA synthase produced triacetic acid lactone in the absence of NADPH, at a rate of one tenth that of 6-MSA production.

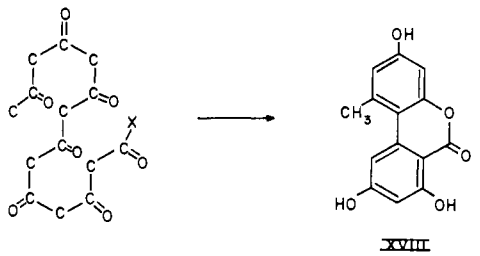
ALTERNARIOL SYNTHASE

A second polyketide synthesizing system has been partially characterized by Gatenbeck's group (Gatenbeck and Hermodsson, 1965; Sjöland and Gatenbeck, 1966). Figure 13 shows the structure of alternariol (XVIII) which is produced by the fungus *Alternaria tenuis*. Notice that no chain modifying reactions are necessary, so that this system involves only chain generation and cyclization. Extracts of *A. tenuis* catalyzing the synthesis of alternariol could be purified about 30-fold by ethanol precipitation and gel filtration on Sephadex G-25. The extracts were unstable, and considerable variability in yield of activity was noted among cultures grown under similar conditions.

Acetyl-CoA was incorporated specifically into the two carbons which correspond to the methyl end of the polyketide chain. A K_m of $1.8 \times 10^{-5}M$ was obtained for acetyl-CoA. It could be replaced by acetyl-pantetheine which had a K_m of $5 \times 10^{-4}M$. Both acetyl-CoA and malonyl-CoA inhibited at higher concentrations, as did free CoA which showed a K_m of $8 \times 10^{-5}M$. Malonylpantetheine and *S*-malonyl-*N*-caprylcysteamine could substitute for malonyl-CoA. No other product besides alternariol was detected which suggests that all intermediates are enzyme bound. The enzyme was inhibited by Zn^{2+}, Cu^{2+}, and Mn^{2+}, and by sulfhydryl inhibitors. A slight inhibition by acetylacetone and ethyl acetoacetate suggested competition with the polyketide chain for an enzyme site. The pH optimum was 7.9, similar to that for 6-MSA synthase.

ORSELLINIC ACID SYNTHASE

Still another cell free system has been reported by Gaucher and Shepherd (1968). They obtained orsellinic acid synthase activity from *P. madriti*. The activity was purified about fivefold by pelleting in the ultracentrifuge at 210,000 × g. for 60 minutes. Incorporation of malonyl-CoA into orsellinic acid required acetyl-CoA. Again, variability in activity



XVIII

Figure 13. Possible polyketide cyclization producing alternariol

from different cultures and instability of the activity was noted and probably hindered further work.

EFFECT OF CULTURE CONDITIONS ON ACTIVITY

Aside from instability of the enzyme preparations, one of the biggest headaches which has seemed to plague investigators in this field has been the variability of activity isolated from the cultures. Bu'Lock *et al.* (1965) found the onset of MSA production in carefully controlled cultures to vary between 24 and 36 hours of growth. Dimroth (1968) found a similar but not identical production period. We have found, however, there can be considerable strain variation and changes in cultures during their normal maintenance in the laboratory. A compilation of data from our own laboratory might help illustrate this point (Figure 14). Here we were attempting to study the time of appearance of *in vitro* 6-MSA synthase activity after transfer of a mycelial inoculum from a germinating medium into Czapek-Dox, a medium containing glucose and salts.

Our problem was simplified when we studied single colony isolates of our cultures and discovered at least two different strain behaviors (Figure 15) (Light, 1967b). One strain produced 6-MSA synthase in high though slightly variable quantity and very soon after the transfer to the Czapek-Dox medium (early strain). The other produced activity at some later time which was still variable, and the activity was lower (late strain). The late strain behavior more closely corresponds to that found by Bu'Lock *et al.* (1965).

An additional procedure for obtaining high levels of activity was discovered during a study of the effect of protein synthesis inhibitors (Light, 1967a). Cycloheximide at 0.3–1 μg/ml., a concentration which only partially blocks protein synthesis, stimulates the early formation of 6-MSA synthase in transfer cultures. Stimulated levels were highest with the early strain, but stimulation was also obtained with the late strain and with either strain growing in germinating medium. Similar stimulation was obtained with the amino acid analogs *p*-fluorophenylalanine and 4-methyltryptophan at concentrations of 10–100 μg/ml.

Our routine procedure for obtaining 6-MSA synthase makes use of this stimulation. The early strain is grown for twenty-four hours in a germinating medium and transferred into Czapek-Dox containing 0.3 μg/ml. cycloheximide. The mycelium is harvested five to seven hours later. The procedure works for both shake and fermentor cultures (Light and Hager, 1968). The more highly aerated fermentor cultures sometimes produce excessive acid which must be controlled by titration or by substitution of sucrose for glucose in the medium (Light, 1969). Whether the stimulation by cycloheximide will be generally applicable to other polyketide systems remains to be seen.

The interpretation of the protein synthesis inhibitor effects is not yet clear. At higher concentrations (10 μg/ml.), early formation of 6-MSA synthase is blocked, but 6-MSA synthase activity does appear later after 24 hours (Table II). Bu'Lock *et al.* (1969) also found that several protein synthesis inhibitors did not block the late (24 hour) appearance of 6-MSA in culture media, while appearance of enzymes metabolizing 6-MSA was blocked. Therefore, it is not possible to be certain that 6-MSA synthase formation represents unmasking of a precursor protein or formation of a new protein. In either case, however, the process appears related to a partial disruption of protein synthesis. Such a partial disruption could occur during transition to a stationary growth phase (Bu'Lock *et al.*, 1965), during step-down culture from a rich to a poor medium (Light, 1967b) or by low concentrations of cycloheximide (Light, 1967a).

Table II. Effect of Cycloheximide on 6-MSA Synthase in Early Strain[a]

Concentration of Cycloheximide, μg/ml.	Radioactivity Incorporated by Extracts,[b] mμmoles/10 min./mg. protein	
	5 Hours	24 Hours
0	1.1	0.8
0.3	2.3	1.0
10.0	0.2	2.2

[a] Mycelium (0.8 g., wet weight) from a 24-hour germinating culture of the early strain of *P. patulum* was suspended in Czapek-Dox medium (50 ml.) in the presence of the indicated concentration of cycloheximide. The cultures were shaken in 125-ml. Erlenmeyer flasks for the indicated period of time, harvested, and assayed for 6-MSA synthase activity as described elsewhere (Light, 1967a, 1967b). Activity is expressed as mμmoles of ^{14}C-malonyl-CoA incorporated into 6-MSA. [b] Average of duplicate incubations.

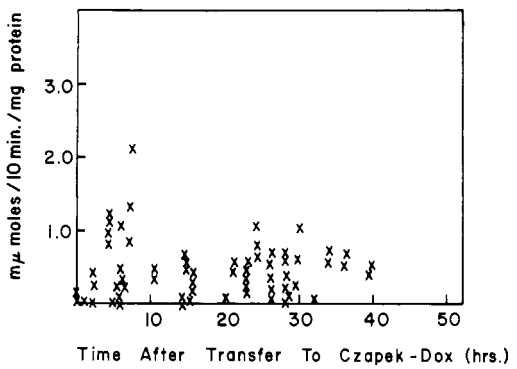


Figure 14. Variability of 6-MSA synthase activity in cultures of *Penicillium patulum* strain 2159A

Mycelium was transferred from a germinating medium to Czapek-Dox shake cultures for the specified period of time. Conditions for growth and assay were the same as indicated in Figure 15, which has been reported (Light, 1967b)

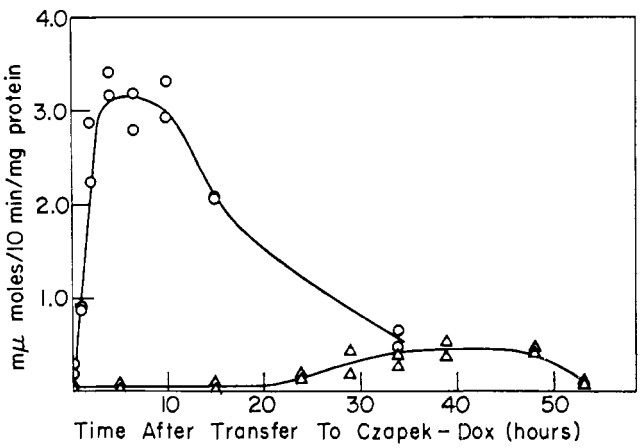


Figure 15. Production of 6-MSA synthase activity in single colony isolates of *Penicillium patulum* 2159A showing a strain with "early" (○) and one with "late" (△) producing behavior

Conditions of germination and transfer of mycelium and of extraction and assay of 6-MSA synthase activity have been described (Light, 1967b). Activity is expressed as the rate of incorporation of ^{14}C-malonyl-CoA into 6-MSA

CYCLIZATION OF FREE POLYKETO ACIDS

In the three synthase systems discussed so far, the implication has been that cyclization of the polyketide chain pre-

cedes or is concerted with hydrolysis of the putative thiol ester linkage to protein. Unfortunately we know very little about the chemistry of the free polyketo acids. Several groups have attempted to study the cyclization reactions of these acids by generating them from "masked" forms (See, for example, Bram, 1967; Crombie and James, 1966; Guilford *et al.*, 1968; Money *et al.*, 1967; Money *et al.*, 1966). The intermediate polyketo acids were not isolated in the reactions, however. Harris and coworkers (Harris and Carney, 1967; Howarth *et al.*, 1969) have succeeded in synthesizing a series of triketo acids by carboxylation of the corresponding triketone trianions. Cyclizations of both the aldol and Claisen type were demonstrated for these acids and their esters. Of particular interest is the finding that tetraacetic acid cyclized very easily to orsellinic acid when dissolved in acetate buffer at pH 5.0 (Howarth *et al.*, 1969).

Availability of these compounds should enable us to establish whether or not they are free intermediates in polyketide biosynthesis. Two recent reports indicate this might be the case. Hillis and Ishikura (1969) obtained extracts from leaves of *Eucalyptus sideroxylon* which catalyzed the conversion of cinnamoyltriacetic acid into pinosylvin (X) (Figure 5). This reaction presumably involves pinosylvic acid (IX) as an intermediate, and it is difficult to tell if they have ruled out spontaneous cyclization followed by an enzymatic decarboxylation. They proposed that this enzymatic activity could play a role in determining whether products of the stilbene type or the flavonoid type (XI and XII) are produced.

Gatenbeck *et al.* (1969) have recently proposed that tetraacetic acid can serve as a methyl acceptor in the biosynthesis of 5-methylorsellinic acid (III). They obtained cell free extracts from *Aspergillus flaviceps* which incorporated methyl-^{14}C-*S*-adenosylmethionine into the product, identified as 5,6-dimethylresorcinol after chemical decarboxylation. Orsellinic acid (I) was not the methyl acceptor, but an acceptor could be isolated by ether extraction of urea treated enzyme preparations. The availability of synthetic tetraacetic acid (Howarth *et al.*, 1969) should now allow the identity of this endogenous acceptor to be checked. Of course, the methylating enzyme might work as well on a free polyketo acid as on an enzyme bound polyketide chain, making more difficult analysis of the cyclization stage. If so, however, purification and characterization of such an enzyme should provide a powerful tool for studying the protein bound intermediates.

FUNCTION ?

Perhaps the most puzzling question of polyketide biosynthesis remains unanswered. These compounds belong to a class known as secondary metabolites, compounds with restricted distribution and no known function in the mainstream of intermediary metabolism (Bentley and Campbell, 1968; Bu'Lock, 1965). Nature is surely not working solely in the interest of organic chemistry by providing this vast array of interesting chemical structures. And so we ask, "Do polyketides have a function in the cells producing them?" Many polyketides are antibiotics, and could offer a selective advantage, but many more are not. I suspect we may be asking the wrong question. Instead we should ask, "Do the enzymes forming polyketides have a function in the cell?" Perhaps 6-MSA synthase occurs as an "inactive" precursor which is normally active in some other way. Perhaps 6-MSA synthase represents an incompletely formed complex whose normal function is something different. The answer to such speculation, and to the question of function, can come only from studies of the purified enzymes.

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