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Enzymic Synthesis of the Aromatic Product Alternariol

STEN GATENBECK and SVENNE HERMODSSON

Institute of Biochemistry, University of Lund, Lund, Sweden

An enzyme solution with the capacity to synthesize the diphenyl derivative alternariol has been prepared from *Alternaria tenuis*. The enzyme has been purified about 30-fold and malonyl coenzyme A and acetyl coenzyme A found to be precursors. Malonyl coenzyme A could be substituted with malonyl pantetheine or S-malonyl N-caprylcysteamine. The formation of alternariol has been shown to proceed through polycondensation of malonate with the participation of only one acetate unit.

Isotope technique applied to whole cells of molds has revealed the formation of many aromatic compounds by head-to-tail condensations of acetate. "The polyacetate theory" as it was introduced by Birch [1] has recently undergone a modification in that malonate formed by carboxylation of acetate has been found to be the polycondensing reagent. The present view on the reactions leading to the formation of aromatic compounds from C_2 units is that activated malonate, *i.e.* malonyl CoA,* is subject for polycondensations initiated by acetyl CoA. This means, that every product formed from one sequence of malonate condensations will contain one acetate unit appearing at the terminal end of the hypothetical polyketomethylene compound. This has been demonstrated in several cases by observing the low incorporation of ^{14}C labelled malonate into the supposed acetate derived portion of the formed aromatic compound. In the clear-cut case this portion should be non-labelled from malonate but the decarboxylation of malonate is in practice giving rise to some radioactive acetate which will be available for incorporation into the aromatic compound. It is obvious that the described reactions for the biosynthesis of aromatic compounds show many similarities with the sequence of reactions participating in fatty acid formation and it is very possible that the studies in one field will furnish important information to the other one. Enzyme studies of fatty acid synthesis have gone on for some years but still there are many gaps in our knowledge of the mechanism of the malonate condensations.

* CoA = coenzyme A

In the series of aromatic compounds the first attempts to cell free studies of their formations have been made by Lynen and Tada [2] and by Tanenbaum and Bassett.[3] The formers confirmed in their experiment the formation of 6-methylsalicylic acid from 1 acetyl CoA and 3 malonyl CoA by using an enzyme solution sligthly enriched by ammonium sulphate precipitation between 45—80 % saturation. Tanenbaum and Bassett were able to get incorporation into tropolones from various radioactive substances in crude cell free extracts from *Penicillium stipitatum*. Both groups have prepared their crude cell free solutions by extraction of the mycelia with dilute ammonia. The procedure described for the preparation of the cell free solutions seems to be rather hazardous as it has never been successful in our hands. To be able to study the mechanism of the aromatic synthesis a reproducible system is a necessity. This paper will describe a procedure for cell free preparation and about 30-fold purification of the enzyme responsible for the synthesis of alternariol, a diphenylcarboxylic

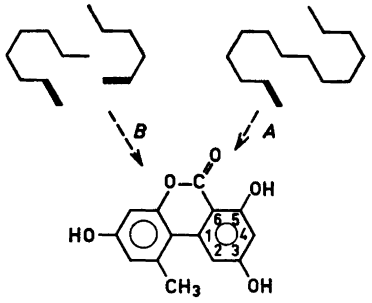

Fig. 1. Possible biosynthetic pathways for the formation of alternariol. The thick lines indicate acetate units.

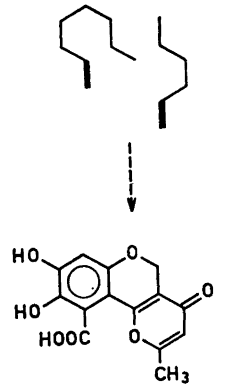

Fig. 2. The biosynthetic pathway for the formation of citromycetin. The thick lines indicate acetate units.

acid produced by *Alternaria tenuis* (Fig. 1). Thomas [4] has shown that alternariol is built up from polyacetate but there is no reason to doubt that the acetate-polymalonate system is involved in the biosynthesis. There are, however, at least two different theoretical pathways to obtain alternariol from polymalonate which have already been discussed by Thomas. On the one hand the whole molecule of alternariol could be derived by intramolecular cyclization of a single polyketomethylene chain thus containing only one acetate residue (CH_3-C in alternariol) on the other hand alternariol could arise by condensation of two separately formed polyketomethylene chains as outlined in Fig. 1; the resulting molecule carrying two acetate residues (CH_3-C and C2, C3). The latter case would be analogous to the formation of citromycetin [5] in *Penicillium frequentans* (Fig. 2) and in fact identical intermediates for the compounds were possible. The two outlined pathways are practically impossible to distinguish experimentally by using whole cells as acetate is very efficiently carboxylated to malonate and the carbon atoms in positions 2 and 3

are not available by chemical degradations. Structurally, however, alternariol is very convenient for studies of the condensing enzyme(s) as no other enzymic transformations should be required for its synthesis. The lactone formation is very likely a spontaneous reaction.

It was found that mechanical disruption of the cells of *A. tenuis* by grinding with sand at pH 8.0, followed by centrifugation yielded a crude cell free solution with the ability to synthesize alternariol after incubation with acetyl CoA and malonyl CoA.

By using ^{14}C labelled malonyl CoA the identity of alternariol was shown by paper chromatogram scanning. The radioactive compound with R_F value corresponding to alternariol was eluted from the paper and without change of specific radioactivity repeatedly recrystallized after addition of non-labelled alternariol.

The crude extract was in another experiment incubated with labelled acetyl CoA and non-labelled malonyl CoA. The formed radioactive alternariol was isolated as described and diluted with non-labelled alternariol. Determination of the total radioactivity, supplemented by Kuhn-Roth oxidation and decarboxylation of the alternariol, demonstrated that 83 % of the radioactivity was located in the CH_3-C positions. The remaining 17 % of radioactivity was most likely equally distributed between the other six C_2 units as the carboxyl group contained 2.3 % of the radioactivity (Table 1). These results conclusively indicate that the alternariol molecule is formed from a single sequence of malonate condensations; thus following pathway A in Fig. 1. The small amount of radioactivity appearing in the six C_2 units is a result of a carboxylation to malonyl CoA of the added radioactive acetyl CoA in the crude extract.

A systematic investigation of the conditions for obtaining cell free solutions capable to perform aromatic synthesis demonstrated big differences between cultures inocculated and grown under similar conditions. On average two out of three cultures were found to yield active enzyme solutions. The mycelia were harvested at the time for intense alternariol production as checked with the ferric chloride reaction. Because of the variations in enzyme activity, to which we have no explanation, every harvested mat of mycelium has been tested for its enzyme activity in a small scale cell free preparation to avoid wasting expensive materials. An extensive study of the influence of buffer properties on the preparation procedure revealed an alkaline solution

Table 1. Labelling pattern of alternariol from acetyl-1-^{14}C CoA.

	cpm/mmole	%
Alternariol	5.23×10^4	100
Alternariol minus CH_3-C	0.9×10^4	17
Alternariol carboxyl group	0.12×10^4	2.3
CH_3-C (calculated by difference)	4.39×10^4	83

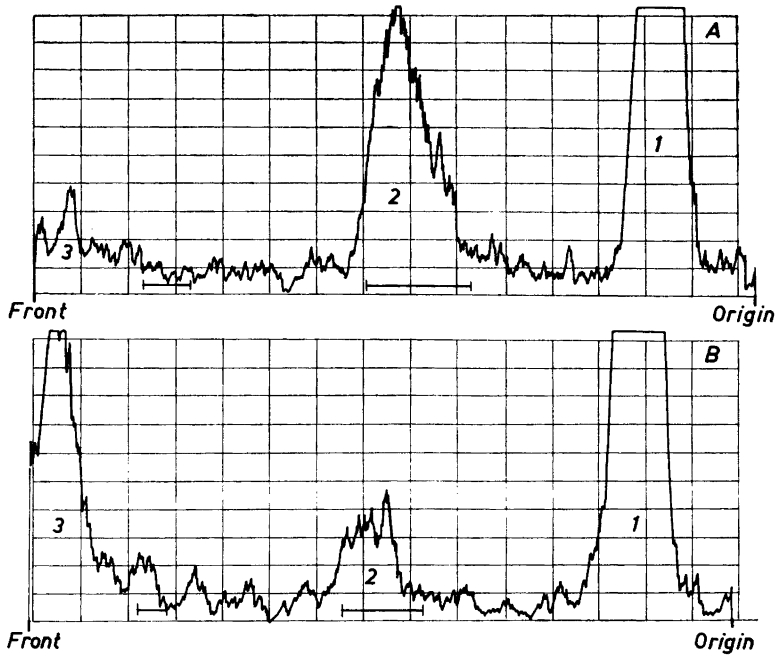

Fig. 3. The scanning of the paper chromatogram for the products of alternariol and "lipid" synthesis after the condensation of malonyl-2-^{14}C pantetheine and acetyl CoA in buffers, of high (A) and low (B) ionic strengths. The peaks correspond to: 1. unreacted malonate 2. alternariol, 3. "lipids".

(pH about 8.0) to be the most effective, and furthermore the result was found to depend on the ionic strength of the buffer solution. A high ionic strength significantly favoured the preparation of an active enzyme solution. The crude cell free preparation also had the capacity to synthesize lipid material (probably fatty acids) when supplied with $NADPH_2$.* The lipid formation was dominating when using buffer solutions of low ionic strength whereas little or no lipids were found under the conditions for optimal alternariol production (Fig. 3).

The crude enzyme solution was further purified by centrifugation at 100 000 *g* for 30 min followed by precipitation with ethanol of the supernatant solution, the fraction obtained between 15 to 24 % of ethanol being collected. By subsequent gel filtration of the redissolved precipitate an about 30-fold purified enzyme solution was obtained (Table 2).

In the assay used on the purified enzyme no radioactive substance other than alternariol could be observed, supporting the hypothesis advanced by Ehrensvärd and Gatenbeck [6] of an enzyme-bound formation of the primary

* $NADPH_2$ = dihydronicotinamide-adenine dinucleotide phosphate.

Table 2. Purification of the alternariol forming enzyme. Incubation conditions are described in enzyme assay. 240 000 cpm of malonyl-2-^{14}C pantetheine added.

Fraction	Total protein/ mg	Alternariol formed cpm	Cpm/mg protein
Supernatant, 100 000 *g*	40.4	4 732	117
Ethanol precipitate (15—24 %)	6.6	7 745	1 175
Sephadex effluent	4.2	15 275	3 640

structures of aromatic compounds. The enzyme activity was found to be inhibited by iodoacetamide indicating the presence of important SH groups in the enzyme.

It was found that malonyl pantetheine and S-malonyl N-caprylcysteamine could substitute for malonyl CoA in the aromatic synthesis and in the lipid formation as well and even exceed the CoA thiolester as precursors. Acetate seemed to require more specifically CoA as the thiol moiety for its function as a precursor (*cf.* rows 2 and 4 in Table 3).

Some evidence, however, was obtained for the possibility to substitute acetyl CoA with other monoacyl CoA derivatives as propionyl-1-^{14}C CoA together with malonyl CoA gave rise to a radioactive product with paper chromatographic behaviour expected for the higher homologue of alternariol. Table 3 further demonstrates that alternariol was formed to some extent when using malonyl CoA as the sole precursor. This is probably due to a malonyl CoA decarboxylase activity in the enzyme solution, giving rise to acetyl CoA.

The purified enzyme solution also contained O-methyltransferase activity as the methyl group in S-adenosyl methionine was transferred to a phenolic hydroxyl group in alternariol giving rise to the 3-methylether of alternariol previously isolated and described as a natural product in *A. tenuis* by Raistrick *et al.*[7] The methylating enzyme was very effective and acted on alternariol added to the incubation mixture as well as on alternariol synthesized *de novo* from malonyl CoA and acetyl CoA. In the latter case all alternariol formed was converted to the methylether under the conditions used.

Table 3. Substrate specificity of the enzyme.

Substrate	Alternariol formed cpm
Acetyl CoA + malonyl-2-^{14}C CoA	5 310
Acetyl CoA + malonyl-2-^{14}C pantetheine	7 280
Acetyl pantetheine + malonyl-2-^{14}C CoA	5 150
Acetyl pantetheine + malonyl-2-^{14}C pantetheine	1 230
Malonyl-2-^{14}C CoA	3 370
Malonyl-2-^{14}C pantetheine	860

EXPERIMENTAL

Culture conditions. Alternaria tenuis L. S. H. T. M. 108 was grown as surface cultures in 2 l Fernbach flasks each containing 500 ml of a modified Czapek-Dox medium of the following composition: $NaNO_3$, 1.0 g; NH_4Cl, 0.25 g; KH_2PO_4, 1.0 g; $MgSO_4$ $7H_2O$, 0.5 g; KCl, 0.25 g; NaCl, 0.25 g; $ZnSO_4$ 7 H_2O, 0.01 g; $FeSO_4$ 7 H_2O, 0.01 g; yeast extract, 1.0 g; glucose, 40 g and distilled water, 1000 ml. The cultures were incubated at 23–25° and harvested after 7–9 days, *i.e.* at the time when alternariol production is most active.

Enzyme assay. 2 ml of the enzyme solution, pH 8.0, were incubated with 100 µl (0.4 µmole) of acetyl CoA and 200 µl (1.2 µmoles, 0.25 µC) of malonyl-2-^{14}C CoA or malonyl 2-^{14}C pantetheine for 1 h at 29°C. The reaction was stopped by addition of 0.25 ml N NaOH and heated for 20 min at 40°C to hydrolyse the thiolesters. After acidification with 2 N HCl the solution was extracted with 3 × 3 ml of ether. The residue obtained after evaporation of the ether phase was dissolved in a minimal volume of acetone and chromatographed on paper using as solvent propanol/butanol/2 N ammonia (6:1:3) (R_F = 0.45). The area on the paper containing the radioactive alternariol was cut out and the alternariol eluted with ethanol and the solution rechromatographed in methanol/ 2 N HCl (5:1) (R_F = 0.65). The alternariol was isolated from the paper as described and its radioactivity measured in a liquid scintillation counter after dissolving in 5 ml of 0.5 % diphenyloxazole in toluene.

Preparation of the enzyme. The mycelium from one flask was washed twice with 0.02 M phosphate buffer, pH 6.5 and then carefully dried by pressing between filter papers. After freezing to −20° the cell mass (21 g) was ground in a mortar with sand (44 g) and 36 ml buffer solution, pH 8.0, (0.05 M phosphate + 0.2 M Tris + 0.2 M NaCl + 10^{-3} M EDTA) at 4°. (The frozen cell mass can be stored for several days without losing its enzyme activity). The cell debris and the sand were separated from the liquid by centrifugation at 25 000 *g* for 6 min. (When pretesting the organism on enzyme activity about 1/10 of the given amounts has been used and the activity tested at this stage). The liquid fraction was recentrifuged at 100 000 *g* for 30 min at 4° yielding 26.5 ml of supernatant solution (20.1 mg of protein per ml). To 20.0 ml of the supernatant fraction 8.5 ml of 50 % ethanol were added at −5° to −3° followed by centrifugation at 1600 *g* for 7 min at 5°. The precipitate was discarded and the addition of further 9.5 ml of 50 % ethanol to the clear supernatant at −7° to −5° yielded a precipitate carrying the enzyme activity. After dissolving in 7.5 ml of the pH 8.0 buffer this solution (3.3 mg of protein per ml) was passed through a Sephadex column (G 25, coarse). The effluent contained 2.1 mg of protein per ml.

Malonyl CoA was prepared as described by Lynen.[8] Malonyl pantetheine was obtained by the same method after reduction of pantethin in aqueous solution with $NaBH_4$. Acetyl CoA and acetyl pantetheine were synthesized by using the method of Simon and Shemin.[9] The preparation of propionyl CoA was performed from the mixed anhydride of propionic acid and ethyl carbonate following the directions given by Seubert[10] for preparation of capryl CoA.

Degradation of alternariol labelled from acetyl-1-^{14}C CoA. The paper chromatographically isolated radioactive alternariol was recrystallized with 130 mg carrier alternariol to constant specific radioactivity from a mixture of ethanol and water. The radioactivity of the alternariol was determined after combustion of a portion of the material with van Slyke and Folch oxidizing solution. The obtained CO_2 was trapped in aqueous $Ba(OH)_2$ and an accurate weight of the $BaCO_3$ formed was suspended in 10 ml of 0.5 % diphenyloxazole in toluene with the aid of 400 mg of "Aerosil". Another portion of the radioactive alternariol was oxidized to acetic acid and CO_2 with chromic acid. The radioactivity of the CO_2 was determined as described; 46 mg of alternariol were decarboxylated by refluxing for several hours in freshly distilled quinoline with copper chromite as catalyst. The CO_2 evolved was collected as $BaCO_3$ and the radioactivity measured as above (Table 1).

Formation of alternariolmethylether. The methylether of alternariol was formed when the incubation mixture prepared as in "enzyme assay" was supplied with 0.5 µmole of S-adenosyl methionine. Radioactive ether could also be obtained by substituting malonyl-2-^{14}C CoA (or pantetheine) and acetyl CoA with nonlabelled alternariol and S-adenosyl methionine-$^{14}CH_3$ (0.5 µmole, 100 000 cpm).

The effect of $NADPH_2$ on the synthesizing system. The lipid formation was studied in relation to alternariol production using the following buffer solutions: (a) the pH 8.0 buffer described in "preparation of the enzyme", (b) 0.1 M Tris + 10^{-3} M EDTA (pH 8.0). The enzyme solution was incubated with "the enzyme assay mixture" and 200 μl of a solution containing 2 mg of glucose-6-phosphate, 2 mg of NADP and glucose-6-dehydrogenase (commercial) in 0.1 M Tris pH 8.0). The latter solution was preincubated for 5 min at 29° before the addition.

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