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A Comparative Study on the Biosynthesis of Palmitic and Orsellinic Acids in *Penicillium baarnense*

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The composition of the fatty acids produced by the fungus *Penicillium baarnense* was determined by gas liquid chromatography of the corresponding methyl esters. The major components were shown to be palmitic, oleic, and linoleic acid. The esters were preparatively fractionated according to their degree of unsaturation by thin-layer chromatography on silica gel plates impregnated with silver nitrate.

In a representative series of experiments the formation of fatty acids was compared to that of secondary metabolites by supplying cultures of the fungus with a pulse of $CH_3{}^{14}COONa$ for 4 h. Special reference was given to the acetate-polymalonate derived compounds, palmitic acid representing saturated fatty acids, and to the more oxidized orsellinic acid (2-methyl-4,6-dihydroxybenzoic acid) as a representative of secondary metabolites. These incorporation studies were conducted on two different media: A. (Czapek-Dox) and B. (Czapek-Dox without nitrate). It was found that the incorporation of radioactive acetate into palmitic acid was considerably higher on the medium lacking nitrate. Conversely, the found radioactivity in orsellinic acid was higher with the medium containing nitrate. This finding indicates that the ratio between palmitic and orsellinic acid formed is not constant but subject to regulation. The possible regulating effect of the $NADPH_2$ pool is discussed.

The biosynthesis of saturated fatty acids has been shown in a number of systems such as yeast,[1] bacteria,[2] and plants [3] to proceed by condensation of one "starter" unit acetyl-CoA and a varying number of malonyl-CoA units. For example, the formation of palmitic acid involves the condensation of one acetyl-CoA with seven malonyl-CoA units. This route of formation, conveniently called the acetate-polymalonate pathway, also operates in the biosynthesis of a large number of aromatic compounds particularly found in microorganisms. These latter compounds, many of which have considerable commercial importance, have seldom any direct recognizable function for the organism and are therefore designated secondary metabolites. One of its simplest representative is orsellinic acid (2-methyl-4,6-dihydroxybenzoic acid) which has been shown to arise by direct condensation of one acetyl-CoA and

three malonyl-CoA units.[4-6] Using the same building units, acetyl-CoA and malonyl-CoA, the only apparent difference is the requirement for reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$) in the synthesis of fatty acids and similar reduced polyketides as compared to the synthesis of aromatic polyketides with no or small $NADPH_2$ requirement. It could be that this difference in coenzyme requirement might be of importance as a factor regulating the balance between these two types of polyketides. The other alternative would be that the relative proportion of the different polyketides formed is constant and not affected by $NADPH_2$ supply.

Studies on the biosynthesis of secondary metabolites have with few exceptions been directed towards a search for possible precursors so that today our knowledge of this aspect in some cases is very detailed. Only few reports, however, have appeared in which the formation of these compounds has been related to the general metabolism of the organism.[7-9]

The starting-point for the present investigation was the well established finding observed in microorganisms that a suboptimal level of nitrogen for growth is usually favorable to maximum fatty acid accumulation.[10] Similarly, anthraquinone production in a *Penicillium* species has been shown to increase under such conditions.[7,8] We were interested in comparing the production of both fatty acids and secondary metabolites within one organism under similar conditions.

EXPERIMENTAL

Test organism. Penicillium baarnense van Beyma was used in the comparative study described.

Media and culture conditions. A. Czapek-Dox medium with the following composition: $NaNO_3 = 2.0$ g, $KCl = 1.0$ g, $KH_2PO_4 = 1.0$ g, $MgSO_4 \cdot 7H_2O = 0.5$ g, $FeSO_4 \cdot 7H_2O = 10$ mg, $CuSO_4 \cdot 7H_2O = 5$ mg, $ZnSO_4 \cdot 7H_2O = 10$ mg, glucose = 50 g and distilled water to make 1000 ml. B. The modified Czapek-Dox medium is as above except without $NaNO_3$. Spores from agar slants were used to inocculate the culture in 2 l Fernbach flasks containing 0.5 l of Czapek-Dox medium. The fungus was grown as a surface culture at 27°. After incubation for seven days the mycelial pad was carefully removed from the culture medium and washed several times with distilled water. Two uniform discs averaging 3 cm^2 were cut from the pad and floated on 30 ml of either medium A or B in 250 ml Erlenmeyer flasks. To each flask a pulse of 12.5 μC of $CH_3{}^{14}COONa$ was given for 4 h.

Isolation of fatty acids by thin-layer chromatography. The mycelial disc was filtered from the medium and thoroughly washed with distilled water. The lipid material was extracted from the dried pulverized mycelium over night at 4° using 10 ml of a solution of chloroform-methanol (2:1, v/v). The solvent mixture was equilibrated with 4 ml of a 2 % (w/v) aqueous KH_2PO_4 solution. The chloroform phase containing the lipid material was dried *in vacuo* and methanolyzed by heating in 4 ml of a solution of sulfuric acid-methanol (2.5:97.5, v/v) at 65° for 5 h in stoppered tubes. After addition of 1 ml of water the esters were extracted with hexane. The solvent was removed by evaporation and the esters redissolved in hexane. They were then separated by TLC. Samples were applied in 3 cm long streaks to 0.35 mm thick adsorbent layers. These were prepared from a mixture of Silica Gel G— (E. Merck A. G., Darmstadt, Germany)—$AgNO_3$—H_2O (10:40:80, w/w/w).[11]

The developing system used was diethyl ether-petroleumether (b.p. 40—60°) (10:90, v/v). The bands were visualized under ultraviolet light after spraying with a 0.2 % (w/v) solution of 4,5-dichlorofluorescein in ethanol. The R_F values obtained were 0.50 for palmitic and stearic acid, 0.35 for oleic acid, and 0.25 for linoleic acid. The individual bands were scraped off and eluted by repeated washings on a sintered glass filter with

diethyl ether. After transferring to scintillator vials the solvent was evaporated and the esters assayed for ^{14}C activity.

Gas liquid chromatography. The fungal fatty acid methyl esters were prepared as described above. Before submitting to GLC they were purified by TLC on 0.35 mm thick Silica Gel G in a hexane-benzene system (1:1, v/v). The esters were then analyzed by GLC using as a stationary phase ethylene glycol succinate polyester, 10 % on washed Celite, 100—200 mesh.

Isolation of secondary metabolites. After filtering the mycelium the medium was acidified and extracted several times with diethyl ether. The solvent was then evaporated and one half of the extract submitted to paper chromatography in the system chloroform-methanol-4 % formic acid (10:1:1, v/v/v). The R_F values of the two major components orsellinic acid and penicillic acid were 0.47 and 0.85, respectively.[12] The other half of the extract was assayed for radioactivity in a liquid scintillation counter.

Radioactive analysis. The radioactive measurements were performed in a liquid scintillation counter with the samples dissolved in a toluene solution containing 0.5 % (w/v) of 2,5-diphenyloxazole. The activities of the residues obtained from the ether extracts of the media and the methyl esters were counted in the same way. Any quenching was compensated for by use of standards. The paper chromatograms were scanned in a strip counter and the radioactive peaks obtained integrated.

RESULTS AND DISCUSSION

Table 1 shows the fatty acid composition of *P. baarnense* as identified by gas chromatography. The predominant fatty acids were found to be palmitic, oleic, and linoleic acid with the saturated acids comprising about 30 % of the total. An investigation on the fatty acid content of *P. griseofulvum* revealed a somewhat similar picture, the saturated fatty acid fraction however representing only 20 % of the total.[13] No conclusive variation in the fatty acid distribution on different media listed in the table could be observed.

Table 1. Fatty acid composition of *Penicillium baarnense* mycelia (weight %).

Fatty acid	A	B	C
16:0	23.6	26.4	23.9
18:0	6.8	5.6	5.2
18:1	31.6	28.0	26.3
18:2	37.8	40.0	44.4
	99.8 %	100.0 %	99.8 %

A, B, and C denote mycelia obtained from fungi kept for 4 h on fresh Czapek-Dox medium, on modified Czapek-Dox medium, and on the original medium, respectively.

In Table 2 three representative experiments from a large series are listed showing the incorporation of ^{14}C-labeled acetate into fatty acids and secondary metabolites on nitrate containing and nitrate free medium. The total activity found in the two classes of compounds is denoted 100 %. The actual incorporation into fatty acids and secondary products of the acetate added averaged 3—5 % after 4 h of incubation corresponding to 0.75—1.25 $\times$ 10^6 cpm/extract from each mycelial disc. As is seen from this table the activity found in the total

Table 2. Distribution in per cent of $CH_3{}^{14}COONa$ incorporated into fatty acids and secondary metabolites.

Med.	Fatty acids Total	16:0*	18:1	18:2	Secondary metabolites Total	ors.a.	penc.a.	Ratio $\frac{\text{F.A.}}{\text{sec.m.}}$	Ratio $\frac{\text{16:0*}}{\text{ors.a.}}$
A	38	11	11	11	62	22	29	0.6	0.5
B	52	32	10	10	48	5	14	1.1	6.4
A	46	20	13	13	54	31	16	0.9	0.7
B	69	34	20	14	31	16	15	2.2	2.1
A	33	13	9	10	67	56	10	0.5	0.2
B	79	44	13	20	21	5	10	3.8	8.8

* The values given for 16:0 represent the total saturated fatty acid fraction which besides palmitic acid consists of about 25 % of stearic acid.

A and B denote Czapek-Dox and Czapek-Dox without nitrate medium, respectively.

fatty acid fraction is considerably higher in the medium lacking nitrate. The major increase of activity is in the saturated fatty acid fraction, 75 % of which consists of palmitic acid. A corresponding increase of activity in the secondary metabolites here largely represented by orsellinic and penicillic acid cannot be observed. On the contrary a decrease in the total activity is found which is mainly due to orsellinic acid whereas penicillic acid, a tetronic acid believed to be derived from orsellinic acid by a series of transformations including ring-fission,[14] shows insignificant variations in its activity.

The quotients fatty acids/secondary metabolites as well as palmitic-stearic acid/orsellinic acid are invariably higher in the nitrate free medium. In other words, the formation of the primary metabolites fatty acids and of the secondary metabolites orsellinic and penicillic acid, does not occur in a constant ratio but may be subject to alterations.

The reasons for the differences seen here may be manyfold. It seems possible to think of orsellinic acid synthetase in contrast to fatty acid synthetase appearing transiently, thus being active in the nitrate free medium for only a short time. Another alternative explaining the lower activity found in fatty acids and the higher found in secondary metabolites on sodium nitrate containing medium would be that sodium and nitrate ions *per se* inhibit fatty acid production and/or favor secondary metabolite formation. We feel, however, the size of the pool of $NADPH_2$ to be responsible for this change as outlined in Fig. 1.

On nitrate containing medium two major biosynthetic pathways compete for the $NADPH_2$ pool present. One is represented by the different fatty acid synthetases, the other by the enzymes involved in nitrate reduction. Palmitate synthetase for example requires for each mole of palmitate formed 14 moles of $NADPH_2$. For the reduction of nitrate several reduction equivalents are required before its assimilation into amino acids. The first step involved is catalyzed by a nitrate reductase which in the case of *Neurospora* has been

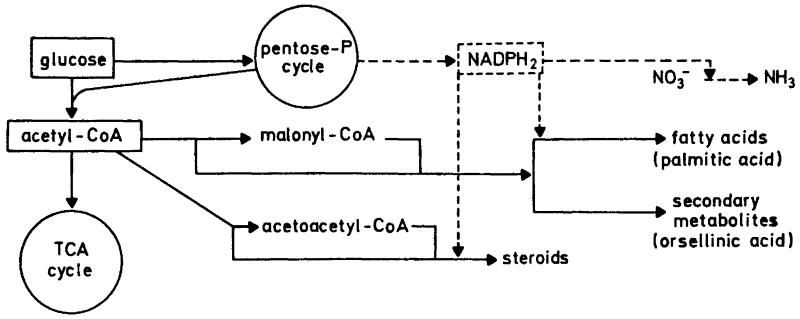

Fig. 1. Simplified scheme showing the different pathways leading to the formation of palmitic and orsellinic acids in *P. baarnense*.

shown to be relatively specific for $NADPH_2$ as hydrogen donor.[15] Even the successive reductive steps catalyzed by the enzymes nitrite and hydroxylamine reductases have in some cases been shown to require $NADPH_2$.[16,17] Finally the ammonia formed has to be converted to amino acids through enzymic reaction with glutamate dehydrogenase the latter again linked to $NADPH_2$.[18] This withdrawal of $NADPH_2$ by these different processes will limit the supply of reduced coenzymes needed in fatty acid formation thus channeling the acetyl- and malonyl-CoA units present into polyketide synthesis of less reduced structures. On nitrate free medium this drain from the pool is not occurring thus giving a larger supply of reducing equivalents.

Even taking into account that part of the nitrate reduction steps may not be $NADPH_2$ specific but can be linked to $NADH_2$, through the action of transhydrogenases catalyzing a hydrogen transfer from $NADPH_2$ to NAD the overall picture on nitrate containing medium will again be that of a paucity of reduced NADP. Our results obtained from similar experiments in which casein hydrolysate has been added to the nitrate medium are in good agreement with this interpretation. Here a considerably higher incorporation of labeled acetate into the fatty acid fraction was observed as compared to plain nitrate medium suggesting that the amino acids present could be directly assimilated and thus not competing for reduction equivalents.

We therefore believe the pool of reduced coenzyme to be of importance as a factor regulating the balance between these two classes of compounds. In this context a comparative study of the production of orsellinic acid as compared to that of a secondary metabolite of more reduced nature such as barnol (5-ethyl-4,6-dimethylpyrogallol)[19] under the conditions outlined above would be of interest.

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