Acetyl-coenzyme A in patulin biosynthesis

The hypothesis that acetate condenses directly in biological systems to form aromatic compounds was initiated by COLLIE[1], and was further applied by BIRCH[2] to explain the occurrence of many natural products. Experiments with *Penicillia* spp. fed labeled acetate[3,4] demonstrated that 6-methylsalicylic acid is formed by polymerization of C_2 units; and it was also shown that the antibiotic patulin in turn can result from the enzymically catalyzed molecular rearrangement[4] of 6-methylsalicylate.

In light of recent developments on the biosynthesis of fatty acids and of sterols, it appeared most probable that the activated form of acetic acid utilized in the formation of the foregoing extra-mycelial aromatic and related compounds would by acetyl-SCoA. This report deals with the occurrence of moderately large amounts of coenzyme A in *Penicillium patulum*, and describes the preparation of a cell-free extract which can convert acetyl-SCoA into patulin.

Growth of *P. patulum* was carried out as previously described[5]. When the absorbancy of the fermentation beer at 276 mμ was about 1.0 at a 0.005 dilution, the mold was repeatedly washed with cold water to remove mechanically bound metabolites. The mycelia were then suspended in 0.01 *N* NH_4OH (see ref. 6) (50 ml per mycelial mat from 2.8 l fernbach flasks) and stirred for several hours at 3°. After gravity filtration first through muslin and then through Whatman No. 4 filter paper, the extract was centrifuged in the cold at 5000 × *g*. The resultant clear, slightly yellow solution averaged 0.65 mg protein/ml, measured colorimetrically against a γ-globulin standard by the Folin-Ciocalteau method. This crude preparation was capable of transforming glucose, acetyl-SCoA, or 6-methylsalicylic acid to patulin. All active preparations made in this fashion were found to contain coenzyme A.

Other procedures which were used for making cell-free extracts from *P. patulum* included: grinding in the cold with boat sand, with alumina, with powdered glass, and with combinations of these abrasives in various buffers; and treatment in the cold with abrasives in the Waring blendor. Although considerable protein was liberated by these methods, and although many of these extracts could oxidize glucose, as measured manometrically, they were devoid of patulin or 6-methylsalicylate-synthesizing ability.

The presence of coenzyme A in the mycelium of *P. patulum* was demonstrated by concentration of the crude material, using the methods previously applied[7] to isolation of the coenzyme from yeast. Spectrophotometric assay of the initial hot water extract from mycelial mats indicated that about 50 mg of coenzyme A were present/g (dry wt.) of the mold. The crude coenzyme was precipitated from the aqueous extract by the addition of acetone, and was then reprecipitated from solution as its glutathione complex with Cu_2O. After removal of metal with H_2S, the resultant solution of the partially purified coenzyme was lyophilized to give hygroscopic white needles. A comparison of this material with a commercial sample (Pabst) by spectrophotometry in the u.v. at pH 2.5 and at pH 11 showed that the curves were identical. Analysis by paper chromatography demonstrated that the *P. patulum* coenzyme A was resolved into major spots which had the same R_F values and color reactions[8] as did the authentic yeast coenzyme. Finally, the coenzyme A from *P. patulum* exhibited biological activity in the *Escherichia coli* phosphotransacetylase enzyme system[9]. By this

Abbreviations: HSCoA, Coenzyme A.

method it was estimated that the best preparation contained about 133 LIPMANN units/mg.

Patulin which was formed in the experiments using cell-free extracts was removed from the reaction mixture by ether extraction. The ether was then washed with 1% $NaHCO_3$, dried over $MgSO_4$, and passed through a column of Woelm alumina. The eluate was taken to dryness, redissolved in ethanol, and chromatographed serially several times on Whatman No. 1 paper using the butanol–acetic acid–water (4:1:1) solvent system, until constant radioactivity was obtained. Radioactive acetyl-SCoA was synthesized by the method of SIMON AND SHEMIN[10] using [2-^{14}C]acetic anhydride. Labeled 6-methylsalicylic acid was obtained by biosynthesis from an experiment[4] in which *P. patulum* was grown on [2-^{14}C]acetate. [^{14}C]glucose was a uniformly labeled commercial sample.

The results of experiments with these radioactive substrates and the enzyme extract from *P. patulum* are given in Table I. It can be seen from Expts. 1 and 2 that samples of labeled acetyl-SCoA prepared either from the mold or yeast coenzymes were roughly equivalent in their efficiency for incorporation into patulin. Dilution of

TABLE I

5 ml cell-free extract (3.0 mg protein) were diluted with an equal volume of 0.15 *M* phosphate buffer, pH 6.8, containing the appropriate radioactive supplement. After incubation at 35° for 4 h, the contents of the flasks were acidified and worked up as indicated in the text.

Expt. No.	*Substrate*	*Specific activity counts/min/μmole*	*Total activity counts/min*	*Specific activity of isolated patulin counts/min/μmole*
1	Acetyl-SCoA (mold)	3,000	30,000	2,200
2	Acetyl-SCoA (Pabst)	1,000	10,000	600
3	Na-[2-^{14}C]Acetate	60,000	600,000	0
4	Glucose	3,000	30,000	1,130
5	Glucose + 10^{-3} *M* sodium monofluoroacetate	3,000	30,000	0
6	6-methylsalicylic acid	3,750	16,000	3,960

the specific activity of the resultant patulin in these experiments is undoubtedly due to endogenous formation of the antibiotic from acetyl precursors present in the extract. Acetate itself, added in 60-fold higher radioactive concentration, failed to yield radioactive patulin. This demonstrated both that the necessary acetate-activating components were absent from the extract, and that the mechanism of condensation of labeled acetyl-SCoA did not include prior hydrolysis to free acetate. It had previously been shown[4], with intact mycelia, that glucose is transformed into 6-methylsalicylate and patulin by way of dissimilation to "active acetate". This conclusion is now reinforced by the results of Expts. 4 and 5, where the presence of 10^{-3} *M* sodium monofluoroacetate in the enzyme system inhibited isotope incorporation from labeled glucose into patulin. In the final experiment it is seen that the enzyme extract catalyzed the molecular rearrangement of 6-methylsalicylate to patulin, and that the product had a specific activity slightly higher than that of its precursor. This result is in accord with previous replacement experiments using mycelial mats, wherein the loss of the non-labeled carboxyl from the alternately tagged aromatic compound to give patulin with four of its seven carbon atoms was

demonstrated. The results of Expt. 6 also indicate that the presence of 6-methylsalicylate in the reaction mixture repressed the endogenous formation of patulin from acetyl-SCoA precursors. This finding, and the fact that only trace amounts of 6-methylsalicylate itself were detected in Expts. 1 to 5 supports the contention that this aromatic compound is not an obligatory precursor of the antibiotic, but that it is in reversible equilibrium with a common acetyl-SCoA-derived open-chain intermediate.

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An enzymic study on the cellular origin of the DUNNING and the NOVIKOFF hepatomas in the rat

Because of technical difficulties that are difficult to surmount, the literature on the biochemistry of cancer is filled with comparisons of questionable validity. For example, in comparing liver cells and hepatoma cells, any such comparison can be questioned on the basis of whether the hepatoma cells have been derived from parenchymal liver cells*, from bile-duct epithelium, or from still other cells that occur in liver tissue. A number of studies summarized by POTTER[3,4] in relation to the "deletion hypothesis" have shown that several enzymes normally found in liver are either missing or present in very small quantities in the NOVIKOFF hepatoma, which has been regarded by some to be derived from parenchymal liver cells (discussed by NOVIKOFF[5]). Several of these enzymes were later reported by PITOT *et al.*[6] to be present in the DUNNING hepatoma and these authors questioned the significance of the earlier comparisons of NOVIKOFF hepatoma and liver, while further studies by DE VERDIER AND POTTER[7] with the DUNNING hepatoma revealed new differences between this tumor and normal and regenerating liver.

Abbreviations: 3'-Me-DAB, 3'-methyl-dimethylaminoazobenzene; dCMP, deoxycytidylic acid; Tris, tris(hydroxymethyl)aminomethane.

* That the parenchymal cells of the liver lobule differ quantitatively in their enzyme content depending on their anatomical location in the lobule has been shown by SHANK *et al.*[1] and by SCHUMACHER[2]. Such differences, which are small in comparison with the major fluctuations in enzymic activity shown by the liver as a whole, may reflect the blood flow and oxygen tension in various zones of the liver lobule[2].