Biosynthesis of Penicillins

I. Isolation of a 6-Aminopenicillanic Acid Acyltransferase from *Penicillium chrysogenum*

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The penicillins are structurally built up from two moieties, a heterocyclic ring-system known as 6-aminopenicillanic acid (6-APA) and an acyl group. 6-APA is a constituent of all penicillins whereas the variations in the nature of the acyl group constitute the different penicillins. Consequently, in the studies of the biosynthesis of penicillins two main problems can be distinguished. One of them is concerned with the formation of 6-APA and the other one deals with the mechanism of the introduction of the various acyl groups into 6-APA.

In 1960 Arnstein *et al.*[1] isolated a tripeptide, δ-amino-adipylcysteinylvaline, from the mycelium of the penicillin producing mould *Penicillium chrysogenum*. As cystein and valine are involved in the synthesis of 6-APA, the discovery of the tripeptide corresponding to penicillin N gave rise to the hypothesis that penicillin N formed a common intermediate for the synthesis of 6-APA and all penicillins. According to this hypothesis 6-APA is formed by deacylation of penicillin N, the penicillins being products of transacylation reactions between penicillin N and the proper acyl entity.

Recently Pruess and Johnsson[2] demonstrated the occurrence of a penicillin acyltransferase in *Penicillium chrysogenum*. The enzyme catalyzes an exchange of the acyl groups of penicillin V, G, K, X and dihydro F with 6-APA. However, the demonstration of this enzyme does not give any support to the described hypothesis of penicillin formation as the enzyme does not act on penicillin N. It may be questioned if the acyltransferase actually is involved in the penicillin biosynthesis. It is possible, however, that the enzyme has a broad specificity, and that the penicillins tested do not represent the natural substrates for the enzyme. If this is the case, another primary acylated intermediate could be the true donor of the acyl group to 6-APA. When investigating this possibility we have been able to demonstrate a direct acylation of 6-APA by the coenzyme A derivatives of some acids using a purified enzyme preparation from *Penicillium chrysogenum*. In a recent publication Brunner *et al.*[3] describe a similar system observed in homogenates and crude extracts from *P. chrysogenum*. The similarity of this system as compared to ours prompts us to report the observations we have made in our investigations.

The acyltransferase was released from the cells by grinding with sand and the purification of the enzyme as given in Table 1 yielded a 150-fold enriched preparation. A solution of the purified enzyme can be stored in frozen state for 2 days without significant loss of activity. The enzyme activity is stimulated by the presence of

Table 1. Purification of the 6-aminopenicillanic acid transacylase.

	Total vol. ml	Total prot. mg	Spec. act. U/mg[a]	Total U	Recovery %
Supernatant (30 000 *g*)	240	2640	0.6	1584	100
Ammonium sulfate	30	144	14.0	2016	127
Hydroxylapatite	4	14.4	17.5	252	16
Sephadex G 200	7	0.7	91.0	64	4

[a] 1 U = 1 mμmole of acylated product formed under the conditions described in the assay method.

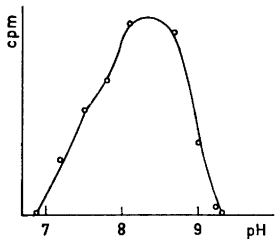

Fig. 1. Effect of pH on enzyme activity.

thiol compounds such as dithiothreitol. A pH optimum of the activity was observed at app. pH 8.5 when using phenylacetyl-CoA as acyl substrate (Fig. 1). At pH 7 the activity of the enzyme was *nil* in contrast to the system of Brunner *et al.* which exhibited an optimal rate at pH 7.

In addition to phenylacetyl-CoA some other CoA derivatives were tested as acyl substrates. From Table 2 can be seen that

Table 2. Acyl substrate specificity of the enzyme.

Substrate	Rel. eff. %
Phenylacetyl-CoA	100
p-Methoxyphenylacetyl-CoA	50
DL-α-Phenoxypropionyl-CoA	0
Phenoxyacetyl-CoA	100
Octanoyl-CoA	80
Lauryl-CoA	0

the efficiency of phenoxyacetyl-CoA as substrate in the acylation reaction is comparable to that of phenylacetyl-CoA. α-Phenoxypropionyl-CoA, on the other hand, is not utilized in the reaction. Determinations of the efficiencies of the free acids as precursors of respective penicillins on the cell level showed that phenoxyacetic acid was much more effectively utilized than phenylacetic acid. α-Phenoxypropionic acid, however, did not give rise to the corresponding penicillin. This agreement between the results on the cell level and in the enzymatic reaction might indicate that the studied enzyme system is, in fact, involved in the biosynthesis of penicillin *in vivo*. Of the two aliphatic CoA derivatives tested only octanoyl-CoA was utilized, yielding the natural penicillin K. The enzyme preparation did not show any amidase activity when tested against penicillin G.

Experimental. Penicillum chrysogenum was grown on a shake table (1″ stroke, 250 rpm) at 28°C in 500 ml conical flasks each containing 150 ml of substrate medium (KH_2PO_4 3.0 g, Na_2SO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.25 g, $ZnSO_4 \cdot 7H_2O$ 0.02 g, $MnSO_4 \cdot H_2O$ 0.02 g, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ 0.1 g, $CuSO_4 \cdot 5H_2O$ 0.005 g, $CaCl_2 \cdot 2H_2O$ 0.05 g, yeast extract 1.0 g, NH_4-acetate 3.5 g, NH_4-lactate 6.0 g, glucose 10 g, lactose 30 g, and distilled water to 1000 ml). After 4 days of growth the mycelia from 12 flasks were filtered off and washed with buffer solution (0.2 M NaCl, 0.2 M Tris, 0.05 M phosphate, 0.001 M EDTA, pH 7.8). The cell mass was ground with sand in the same buffer at +4°C for 10 min and the homogenate centrifuged at 30 000 *g* for 20 min at 4°C. Solid ammonium sulfate was added to the supernatant solution and the protein fraction obtained at 30–51 % of saturation was collected by centrifugation. The precipitate was redissolved in 10 ml of 0.02 M phosphate buffer, pH 7.8, and the solution passed through a column of Sephadex G 25, coarse. The eluate was then fixed on a hydroxylapatite (7.0 g) column. The gel was washed with 20 ml of 0.02 M phosphate buffer, pH 7.8, followed by 10 ml of 0.04 M phosphate buffer, pH 7.8, before the enzyme was solubilized with 25 ml of 0.075 M phosphate buffer, pH 7.8. After concentrating the eluate from the hydroxylapatite column to a volume of 3–4 ml by using an ultrafilter, the enzyme was further fractionated in a column of Sephadex G 200 (diameter and height 3.0 and 70.0 cm, resp.) equilibrated with a buffer of 0.2 M NaCl, 0.2 M Tris, 0.05 M phosphate, 0.001 M EDTA, pH 7.8. The purification scheme is given in Table 1 and a fractionation diagram from the Sephadex G 200 column in Fig. 2.

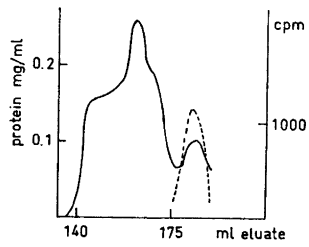

Fig. 2. Elution from Sephadex G 200. Solid line — protein concentration, dotted line — enzyme activity.

Assay method. 100 μl of the enzyme solution were incubated at 30°C with 0.2 mg 6-aminopenicillanic acid, 10 μl 0.25 M dithiothreitol, 25 μl (0.25 mg, 200 000 dpm) phenylacetyl-1-^{14}C-CoA and 100 μl buffer (0.2 M NaCl, 0.2 M Tris, 0.05 M phosphate, 0.001 M EDTA, pH 8.4). After 40 min of incubation 10 μg of carrier penicillin G were added to each incubation mixture and 25 mg of penicillinase were added to the control mixtures and the incubation continued for 15 min at 30°C. The total mixtures were spotted on paper chromatogram strips after adjusting the pH of the solutions to 5.5. The chromatograms impregnated with citrate buffer, pH 5.7, were developed in ether saturated with water. The radioactive penicillin G formed was detected by running the paper strips in a radiochromatogram scanner. The identity of penicillin G was established by confirming the localization of the penicillin G on the paper by spraying with alkali and iodine solution as described by Thomas.[4] The disappearance of the radioactivity and the negative colour reaction in the penicillinase incubated mixtures further confirmed the identity of penicillin G. By cutting out the radioactive area from the paper chromatogram and by dipping the paper into a solution of BBOT in toluene-methanol (1:1) and measuring the radioactivity in a scintillation spectrometer the amount of penicillin G formed was determined. When the specificity of the enzyme was tested phenylacetyl-CoA and penicillin G were substituted in the assay mixture with other ^{14}C labelled acyl-CoA compounds and their corresponding penicillins.

The pH dependence of the reaction was tested over a range from pH 6.5 to 9.5 at 30°C using the following buffer systems: pH 6.5–7.8 0.2 M phosphate buffer, pH 7.8–9.5 0.2 M Tris buffer. The enzyme preparation obtained after the hydroxylapatite treatment in the purification procedure was used for this experiment as well as for the specificity tests.

The following CoA derivatives were synthesized and tested as substrates: octanoyl-1-^{14}C-CoA, lauryl-1-^{14}C-CoA, *p*-methoxyphenylacetyl-1-^{14}C-CoA, phenoxy-^{14}C-acetyl-CoA and DL-α-phenoxy-^{14}C-propionyl-CoA. The synthesis of the thiolesters were in all cases performed by reacting the mixed anhydride of the labelled acid and carbonic ester with coenzyme A. Ring labelled phenoxyacetic acid and DL-α-phenoxypropionic acid were prepared by condensing uniformly ^{14}C-labelled phenol with α-chloroacetic acid and α-bromopropionic acid, respectively.

As the phenoxy acids have about the same R_F values as their corresponding penicillins in the paper chromatographic system used in the described assay, these penicillins were separated by thin-layer chromatography. The radioactivities of the penicillins formed were measured after scraping out the radioactive spots and suspending the powder in a gel of carbosil in BBOT in toluene-methanol.

The efficiencies of phenylacetic acid, phenoxyacetic acid, and DL-α-phenoxypropionic acid as precursors of penicillins when added to producing cultures were determined by incubating the cultures with the labelled aromatic acids for 48 h as described by Erickson and Dean.[5] The radioactive penicillins were extracted into butylacetate and 0.5 % $NaHCO_3$ solution and purified by thin-layer chromatography. The radioactivities were measured as above.

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