Biological Breakdown of Benzylpenicillin by Preformed Mats of Penicillin-Producing Organisms

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1. *Penicillium chrysogenum* and *Aspergillus flavus* degraded benzylpenicillin in the same way. 2. Degradation of the antibiotic was initiated by the cleavage of phenylacetic acid from 6-aminopenicillanic acid. 3. Phenylacetic acid was left unchanged whereas 6-aminopenicillanic acid was degraded into cysteine and valine. This reaction is probably complex. 4. Cysteine was not utilized but valine was cleaved into acetone and glycine. Catabolism of valine by the preformed mats of the two moulds confirms this result. This step probably involves the formation of propan-2-ol. 5. Cleavage of benzylpenicillin into phenylacetic acid and 6-aminopenicillanic acid was performed through the activity of a cellular-bound enzyme.

Sakaguchi & Murao (1950) reported the presence of 6APA* in the enzymic hydrolysate of benzylpenicillin by the mould *Penicillium*, but they did not follow up the various chemical changes that took place.

In the present work the degradation of benzylpenicillin by preformed mats of two penicillin-producing organisms was followed quantitatively by using paper chromatography for the assay of the various metabolites.

EXPERIMENTAL

The sodium benzylpenicillin used throughout the present study had a potency of 1650 units/mg. The rest of the chemical reagents were all of the A.R. grade. The various degradation products were quantitatively assayed by the paper-chromatographic zone-strip technique described by Shimi and co-workers (Shimi, Nour El Dein & Imam, 1961; Shimi, Imam & Dewedar, 1961; Shimi & Imam, 1965). We used 0.5 cm.-wide strips of Whatman no. 1 filter paper and the solution of the sample to be analysed was loaded in a 2 mm.-wide zone near the upper end of the strip. The developing solvent was allowed to flow down for a sufficient length and subsequently the zones occupied by the compounds were located by suitable reagents. Concentrations of these compounds were calculated from the following formula:

$$l = \log c \cdot \log d \cdot k$$

where l is the length in cm. of the zone occupied by the compound, c is the concentration in $\mu\text{g./ml.}$, d is the distance in cm. travelled by the developing solvent and k is a constant specifying each compound under controlled experimental conditions. The different k values of the compounds assayed in the present work are listed in Table 1.

* Abbreviation: 6APA, 6-aminopenicillanic acid.

A non-pigmented ultraviolet-induced mutant of *Penicillium chrysogenum* (N.R.R.L.Q176) and a local strain of *Aspergillus flavus* were used throughout the present study. The former secretes the G type whereas the latter produces the F and H₂F types of penicillins.

The two moulds were allowed to develop their mats on a medium of the following composition (g./l.): sucrose, 30; NaNO₃, 3.0; KH₂PO₄, 1.0; KCl, 0.5; MgSO₄·7H₂O, 0.5; FeSO₄, 0.005. The incubation period lasted for 3 days at 24°, when fully-grown mycelial mats were obtained. The metabolism solutions were poured off and replaced by the

Table 1. k values of the compounds investigated

The k value is defined in the text. Acetone 2,4-dinitrophenylhydrazone was chromatographed with butan-1-ol-water (1:1, v/v) as developing solvent. The loaded zones were located with u.v. light. The rest of the compounds were assayed chromatographically with butan-1-ol-acetic acid-water (4:1:5, by vol.) as developing solvent. The zones occupied by organic acids were located with bromophenol blue and subsequent treatment with dilute CuSO₄ solution (Reio, 1959). The amino acids were located with ninhydrin solution in acetone (Reio, 1959). 6APA was assayed by the method described by Alicino (1961) and frequently checked by the paper-chromatographic zone-strip technique. Differences never exceeded $\pm 2\%$. The development temperature was 25–27°.

Compound	k value
Acetone 2,4-dinitrophenylhydrazone	1.00 $\pm$ 0.02
Phenylacetic acid	0.79 $\pm$ 0.01
Gluconic acid	0.66 $\pm$ 0.03
Cysteine	1.15 $\pm$ 0.03
Glycine	1.36 $\pm$ 0.04
Valine	1.12 $\pm$ 0.02
6APA (freshly prepared)	0.80 $\pm$ 0.02

same volume of a salt solution that had the same composition as the growth medium except that sucrose was lacking. This solution kept the mats in physiologically active condition. The cultures were incubated at 24° for 24 hr. and the solutions were then decanted and finally replaced by equal volumes of penicillin solution (200 mg./100 ml.) in phosphate buffer, pH 6.2. A quintuplicate set of 250 ml. conical flasks, each containing 50 ml. of the antibiotic solution, was always used for each strain. Also, a set of control flasks that lacked the antibiotic was set up and incubated with the rest of the flasks. To examine the stability of 6APA, 0.2 g. was dissolved in 100 ml. of the buffer and kept at 24° for 24 hr. Subsequently, the utilization of valine by the preformed mats was investigated. The same experimental steps were adopted with a solution of 0.05 g. of valine/100 ml. of the buffer. Finally the liquid contents of the control flasks (lacking the added antibiotic) were separated from the mats and centrifuged at 5000 *g*, to free them from the suspended cell debris. The clear solutions were then supplemented with benzylpenicillin (200 mg./100 ml.) and incubated at 24° for 72 hr. Samples were withdrawn from the different sets at intervals and assayed for their contents of organic acids, amino acids, ketones and aldehydes by the zone-strip technique.

Identification of the different metabolites was simultaneously conducted during their assay by the zone-strip technique. The metabolism solutions were spotted on Whatman no. 1 filter sheets in line with and in between two spots of the authentic compound suspected to exist. These sheets were separately developed with different solvent

systems (Block, Durrum & Zweig, 1958). These developing solvents were selected in such a way that would produce low, moderate and high *R_F* values for each compound. A compound was considered to exist in the metabolism solution if its spot lay in between the two spots of the corresponding authentic sample with the different developing solvents. The presence of glycine was further confirmed by spraying the corresponding areas with acetone solution of *o*-phthalaldehyde (Curzon & Giltrow, 1954). Acetone was characterized by isolating its 2,4-dinitrophenylhydrazone derivative from the metabolism solution with ethyl acetate and subsequent crystallization from ethanol. The yellow needles had m.p. 128° and the mixed m.p. with authentic sample was not altered. The crude hydrazones isolated from the culture fluids were usually separated into acidic and non-acidic fractions by treatment with warm *N*-NaHCO₃ solution. The acidic fraction was obtained from the bicarbonate solution by acidification and extraction with ethyl acetate and was then analysed.

The presence of cysteine and valine was confirmed by isolating the two amino acids from the metabolism solution. A 10 l. portion of the metabolism solution was removed from below the mats after 72 hr. subsequent to replacement. The solution was evaporated to dryness *in vacuo*. The residue was first extracted with diethyl ether to remove phenylacetic acid. Subsequently the remaining solid was repeatedly extracted with aq. 10% (v/v) propan-2-ol, which was then concentrated *in vacuo*. Sheets of Whatman no. 3 chromatographic paper were loaded with the alcoholic solution and developed with butan-1-ol-acetic acid-water (4:1:5 by vol.).

Table 2. *Biological degradation of valine by preformed mats of P. chrysogenum and A. flavus*

All calculations are based on 500 ml. of medium. Traces of α -oxoglutaric acid could be detected in cultures of *A. flavus* after 9 hr.

Incubation period (min.)	Concn. of compound (g./500 ml.)				
	Valine	Glycine	Acetone	Pyruvic acid	Acetoacetic acid
<i>P. chrysogenum</i>					
0	0.250	—	—	—	—
30	0.235	0.005	0.005	—	—
60	0.221	0.006	0.011	—	—
120	0.213	0.007	0.018	—	—
180	0.204	0.007	0.021	—	—
240	0.194	0.006	0.022	—	—
300	0.180	0.005	0.028	—	—
360	0.165	0.003	0.039	—	—
420	0.155	0.003	0.047	Trace	Trace
480	0.130	0.004	0.052	0.003	0.004
540	0.120	0.005	0.058	0.004	0.005
<i>A. flavus</i>					
0	0.250	—	—	—	—
30	0.220	0.004	0.012	—	—
60	0.180	0.005	0.031	—	—
120	0.165	0.003	0.038	—	—
180	0.141	0.005	0.045	0.005	—
240	0.124	0.003	0.053	0.007	—
300	0.111	0.003	0.061	0.009	—
360	0.102	0.004	0.070	0.012	—
420	0.081	0.003	0.075	0.015	0.004
480	0.063	0.003	0.087	0.016	0.009

The developed chromatograms were dried and two lateral 1 cm.-wide strips were cut from each sheet. The two strips were sprayed with ninhydrin reagent, heated until the characteristic colour of the amino acids developed and finally placed back in their original positions. Zones in between the located amino acids of the strips were cut and eluted with aq. 10% (v/v) propan-2-ol. The different fractions were evaporated *in vacuo* and the melting points of the crystalline products were determined. Cysteine was transformed into its hydrochloride, m.p. 178° (decomp.), by eluting with warm dilute HCl and subsequently with warm water and then evaporating the eluate under a stream of N_2 to incipient crystallization. Valine had m.p. 314–316° (decomp.). Mixed melting points of the isolated pure metabolites with L-valine or L-cysteine hydrochloride were the same as those of the individual amino acids. Thus L-valine and L-cysteine did exist in the metabolism solution. The 6APA and phenylacetic acid were also isolated and characterized. All the results given in Table 2 and Figs. 1 and 2 refer to 500 ml. of the culture fluids.

RESULTS AND DISCUSSIONS

Benzylpenicillin was completely inactivated by the preformed mats of the two moulds, the process being more pronounced in cultures of *P. chrysogenum*. The antibiotic molecule was cleaved into phenylacetic acid and 6APA at the amide linkage of the side chain by hydrolysis (Scheme 1, step *a*). Phenylacetic acid was not attacked by the moulds, but 6APA was degraded by the mycelial cells. About 43% and 35% of 6APA (calculated on basis of the original content of penicillin) were left unchanged in cultures of *P. chrysogenum* and *A. flavus* respectively. That 6APA remained unchanged in the flasks devoid of the mycelial mats excludes the possibility of its spontaneous decomposition.

Further utilization of 6APA took place through

Fig. 1. Biological breakdown of benzylpenicillin by preformed mats of *P. chrysogenum*: (a) during the first 15 min.; (b) during the subsequent 72 hr. Curve 1, benzylpenicillin; curve 2, 6APA; curve 3, phenylacetic acid; curve 4, valine; curve 5, cysteine; curve 6, glycine; curve 7, acetone.

Fig. 2. Biological breakdown of benzylpenicillin by preformed mats of *A. flavus*: (a) during the first 4 hr.; (b) during the subsequent 72 hr. Curve 1, benzylpenicillin; curve 2, 6APA; curve 3, phenylacetic acid; curve 4, valine; curve 5, cysteine; curve 6, glycine; curve 7, acetone.