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Specificity of Penicillin Acylase of *Fusarium* and of *Penicillium chrysogenum*

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Extracts containing penicillin acylase were obtained by shaking the mycelium of *Fusarium avenaceum* and of *Penicillium chrysogenum* in 0.2 M sodium acetate or sodium chloride solution. The optimum *p*H for conversion of penicillin V into 6-aminopenicillanic acid (6-APA) by the enzyme of *Fusarium* was about 7.5, and the reaction velocity was increased by a rise in temperature from 27 to 37 C. Penicillin G and penicillins with an aliphatic side chain were cleaved much less readily than was penicillin V. With the enzyme preparation obtained from a nonpenicillin-producing strain of *P. chrysogenum*, the reaction rate was higher at *p*H 8.5 than at *p*H 7.5 and *p*H 6.5. The acylase of *P. chrysogenum* hydrolyzes penicillin V more readily than penicillin G. In a series of aliphatic penicillins, the amount of 6-APA formed through the action of this enzyme increased with the number of carbon atoms of the side chain. Penicillins with a glutaryl or an adipyl group as side chain were unaffected by the enzyme of *Fusarium* and of *Penicillium*. No reaction was observed upon incubation of penicillin N (with a D-aminoadipyl side chain) or isopenicillin N (with an L-aminoadipyl side chain) with *Fusarium* and *Penicillium* extract. When the carboxy group of the side chain of these penicillins was esterified, formation of 6-APA was observed upon incubation with *Penicillium* extract, whereas no 6-APA or only very small amounts were obtained by acylase of *Fusarium*.

The enzyme that cleaves penicillin into 6-aminopenicillanic acid (6-APA) and the side-chain acid has been given different names (13). It will be referred to here as "acylase." Two types of enzyme have been distinguished: the "bacterial" type, which hydrolyzes benzylpenicillin more readily than phenoxymethylpenicillin, and the "fungal" type, which acts preferentially on phenoxymethylpenicillin (13, 17).

We have studied the properties and the substrate specificities of two enzymes of the second type, one produced by *Fusarium* and the other obtained from *Penicillium chrysogenum*. We examined several species of *Fusarium*, such as *F. oxysporum*, *F. semitectum*, and *F. avenaceum*, but selected *F. avenaceum* because our strain of this species gave the highest yield of acylase. We also used *P. chrysogenum* Wis. 49-408 because this strain, which does not produce penicillin or 6-APA, has been shown to give results similar to those obtained with a penicillin-producing strain (10).

MATERIALS AND METHODS

Substrates. Benzylpenicillin (penicillin G) and phenoxymethylpenicillin (penicillin V) were obtained from R.I.T. (Genval, Belgium). Methylpenicillin, *n*-propylpenicillin, *n*-pentylpenicillin (penicillin FH_2) *n*-heptylpenicillin (penicillin (K), and *n*-nonylpenicillin and *n*-undecylpenicillin (sodium salts) were prepared by reaction of the appropriate acid chlorides with 6-APA. The sodium salts of 3-carboxypropyl- and 4-carboxybutylpenicillin (glutaryl- and adipyl-6-APA) were obtained by hydrogenolysis at constant *p*H of the corresponding benzyl-esters. Penicillin N and isopenicillin N (potassium salts) were prepared by hydrogenation of D- and L-4-benzyloxycarbonyl-4-azido-*n*-butylpenicillin or of D- and L-4-benzyloxycarbonyl-4-carbobenzyloxyamino-*n*-butylpenicillin (H. Vanderhaeghe, M. Claesen, and A. Vlietinck, in preparation). The purity of the penicillins, estimated by iodometric assay, was at least 75%. Penicillin N (Synnematin B) of a purity of 230 μg/mg, obtained from W. E. Grundy, Abbott Laboratories, was also used.

Cultures and growth conditions. A strain of *F. avenaceum*, isolated in our laboratory by P. Van Dijck, was used. Spore inoculates were obtained by growing the cultures at 26 C for 5 days on Potato Dextrose Agar medium (Difco). The spores were removed from the surface of the slant and used to inoculate four 250-ml Erlenmeyer flasks containing 50 ml of the following medium (in g/liter): corn steep powder, 25.0; saccharose, 20.0; $CaCO_3$, 5.0; $Na_2S_2O_3 \cdot 5H_2O$, 0.2, adjusted to *p*H 5.5 with 10% NaOH before autoclaving. The flasks were shaken for 3 days at 26 C and

150 rev/min on a rotary shaker. These precultures were added to 20 250-ml Erlenmeyer flasks (5 ml per flask) containing 50 ml of Jarvis and Johnson medium, to which 2 mg of phenoxyacetic acid per ml had been added (15, 20). After incubation at 26 C for 3 days on a rotary shaker, the mycelium from the Erlenmeyer flasks was filtered on a Buchner funnel, washed with 10 liters of water, and washed four times with 0.5 liter of cold (−30 to −40 C) acetone. The mycelium was kept in the air until the odor of acetone had disappeared and then was stored in a deepfreeze. A total of 14 to 20 g of dried mycelium was obtained from 20 Erlenmeyer flasks.

P. chrysogenum Wis. 49-408, which produces no detectable amounts of penicillin or 6-APA, was obtained from R. Erickson (10). The mycelium was grown under the conditions described for *Fusarium*, except that the incubation time in the Jarvis and Johnson medium was only 2 days. From 20 Erlenmeyer flasks, 60 g (46 to 82 g) of wet mycelium or 17 g (15 to 20 g) of dry mycelium was obtained.

Enzyme extracts. Dried mycelium of *F. avenaceum* was suspended in 0.2 M sodium acetate (600 ml for 18 g of mycelium). After adjustment of the *p*H to 6.0 with acetic acid, the suspension was shaken in Erlenmeyer flasks (60 ml in a 250-ml flask) at 26 C for 16 hr. The mycelium was then filtered in a Buchner funnel and the filtrate was put into dialysis bags (Dialysierschlauch 28, Kalle AG, Wiesbaden-Biebrich, Germany). After dialysis in the cold room for 24 hr against 10 liters of distilled water, the water was replaced and the operation was repeated for another 24 hr. The dialyzed filtrate was concentrated by submerging the dialysis bags in Carbowax G25000. When the volume was reduced to $\frac{1}{10}$, the dialysis bags were put into distilled water for 24 hr and the solution was freeze-dried. The mycelium that had been extracted was washed with cold (−30 to −40 C) acetone and used for a second extraction and eventually a third.

Wet and acetone-dried mycelium of *P. chrysogenum* was extracted with 0.2 M sodium acetate and with 0.2 M sodium chloride. The filtrate was dialyzed, concentrated, and lyophilized as described for *Fusarium*.

Determination of 6-APA and of the reaction velocities. The penicillin (usually 0.25 mmole) was dissolved in 20 ml of distilled water, a certain amount of mycelium or enzyme extract was added, and the suspension or solution was stirred in a vessel kept in a water bath at constant temperature. The *p*H was kept constant with a *p*H-stat (Radiometer Titrigraph type SBR 2c connected to a TTT Automatic Titrator). From the consumption of alkali (0.5 or 0.1 N KOH), the amount of side chain acid and hence the amount of penicillin transformed into 6-APA could be determined (18). This method will be called the *p*H-stat method.

In many experiments, samples were taken at different time intervals, and volumes of 1 to 8 μliters (sometimes up to 40 μliters) were put on strips (1 × 40 cm) of Whatman no. 1 paper. Descending chromatography was performed with the system pyridine-*n*-butyl alcohol-water, 1:1:1 (v/v) for penicillins having a carboxy group in the side chain or with the top phase of *n*-butyl alcohol-ethyl alcohol-water, 4:1:5 (v/v) for the other penicillins. After chromatography, the paper strips were dried under a hood and sprayed with 1% sodium bicarbonate solution. After drying, the strips were treated with a 1% solution of phenoxyacetyl chloride in acetone. After evaporation of the solvent, they were put on agar plates seeded with *Micrococcus pyogenes* var. *aureus* ATCC 6538 P, which were incubated overnight at 37 C. The amount of 6-APA was determined by comparing the areas of the inhibition zones with those obtained with known amounts (0.25, 0.5, 1.0, and 2.0 μg) of 6-APA treated in the same manner as the samples (6, 10). Amounts of 0.5 to 4.0 μg of 6-APA were used in the chromatography system containing pyridine.

An 0.5% conversion of penicillin into 6-APA could be assayed in the first chromatography system, and 0.2% in the second. This method will be called the microbiological method.

Penicillin acylase unit. The amount of enzyme that produces 25.2 μmoles of 6-APA from penicillin V in 1 hr at *p*H 7.5 and 37 C was adopted as the penicillin acylase unit. This unit fits the definition of Waldschmidt-Leitz and Bretzel (20), except that these authors measured the enzyme activity at 32 C.

RESULTS

Fusarium extract. Different fermentations of *F. avenaceum* gave acetone-dried mycelia with a penicillin acylase activity of 0.005 to 0.025 unit per mg. These mycelia were extracted with 0.2 M sodium acetate at *p*H 6.0 (20). The results of a typical experiment are given in Table 1.

In several experiments, a yield of 15 to 23% was obtained for the first extraction and of 4 to 8% for the second extraction. The enzyme preparations of several extractions were pooled and used for all further studies.

Effect of reaction conditions. The effect of *p*H on the rate of formation of 6-APA from penicillin V was determined (Fig. 1). The optimum *p*H of the reaction was about 7.5. The influence of temperature on the hydrolysis of penicillin V to 6-APA is shown in Fig. 2. Similar results were obtained when the mycelium of *F. avenaceum*

TABLE 1. *Extraction of acylase enzyme from the mycelium of F. avenaceum*[a]

Extraction	Amount of lyophilized extract	Acylase activity[b]	Total units in extract	Yield of extraction
	g	*units/mg*		%
1	1.552	0.050	78.11	23.3
2	0.894	0.018	16.27	4.8
3	0.570	0.012	7.20	2.1

[a] Acetone-dried mycelium (14.2 g) with an activity of 0.0236 unit/mg was extracted with 473 ml of 0.2 M sodium acetate, *p*H 6.0.

[b] *p*H-stat method.

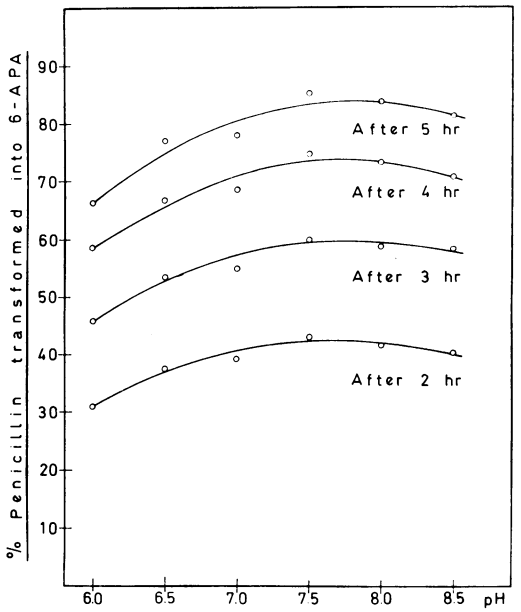

FIG. 1. *Influence of pH on the formation of 6-APA from penicillin V. Potassium penicillin V (0.25 mmole) and 60 mg of F. avenaceum extract (0.0364 unit/mg) in 20 ml of water were incubated at 37 C and different pH values.*

was used. In further studies, all experiments were performed at *p*H 7.5 and 37 C. A temperature of 37 C was preferred for practical reasons and also because it has been shown that there is no marked increase of the formation of 6-APA from penicillin V with *F. semitectum* when the temperature is raised from 40 to 50 C (3).

All results given in Fig. 1 and 2 were obtained with the *p*H-stat method. The data were compared in several experiments with those obtained with the microbiological method, and a good correlation was observed.

Influence of the side chain of the penicillin. The deacylation of penicillin G and V and of a series of penicillins with an aliphatic side chain by the enzyme of *F. avenaceum* was also examined (Table 2). Only the data obtained with the microbiological method are shown. The *p*H-stat method gave higher figures, the discrepancy increasing with the number of carbon atoms of the side chain. The *p*H-stat method did not seem to give valid results with penicillins that are poorly cleaved by the acylase enzyme. Penicillin G and the penicillins with an aliphatic side chain are much less susceptible than penicillin V to penicillin acylase of *Fusarium*.

A certain maximum can be observed in the series of aliphatic penicillins when six carbon atoms are present in the side chain. A similar observation was made with *Nocardia* and *Proteus* acylases, which are of the "bacterial" type (14).

Penicillium extract. In the first extractions in which 0.2 M sodium acetate was used, only a low acylase activity was obtained. The extraction

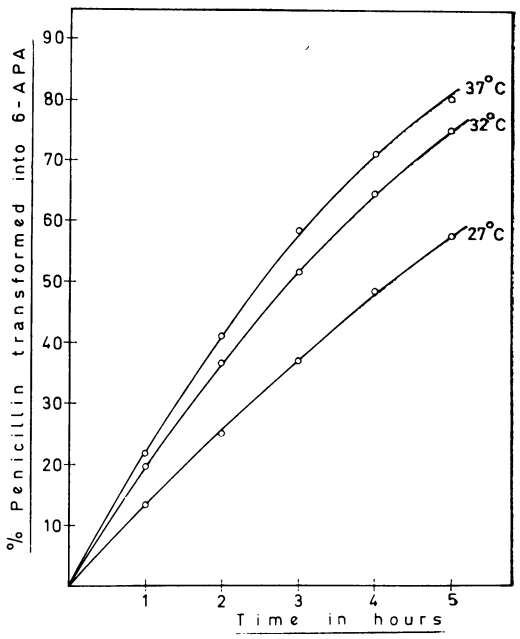

FIG. 2. *Effect of temperature on the formation of 6-APA from penicillin V. Potassium penicillin V (0.25 mmole) and 60 mg of F. avenaceum extract (0.0364 unit/mg) in 20 ml of water were kept at pH 7.5 and different temperatures.*

TABLE 2. *Influence of the structure of the side chain of penicillin on the formation of 6-APA by Fusarium acylase*[a]

Type of penicillin	Penicillin transformed into 6-APA at			
	2 hr[b]	4 hr	8 hr	10 hr
	%	%	%	%
n-Propylpenicillin (C_4)[c]	0.8	1.1	0.8	1.3
n-Pentylpenicillin (C_6)	2.3	3.9	5.3	5.1
n-Heptylpenicillin (C_8)	0.5	0.8	1.4	
n-Nonylpenicillin (C_{10})		0.2	0.2	
n-Undecylpenicillin (C_{12})	0.2	0.2	0.4	
Penicillin G	Trace	0.2	0.2	
Penicillin V	41.2	70.16		

[a] With 0.25 mmole of penicillin and 60 mg of *F. avenaceum* extract (0.0364 unit/mg) in 20 ml of water at 37 C, *p*H 7.5. Microbiological method.

[b] Hours of incubation.

[c] C_4, C_6, etc., indicate the number of carbon atoms of the aliphatic side chain of the penicillin.

could not be improved by sonic treatment or by disruption of the mycelium with a Mickle cell disintegrator (Braun). Extracts with sufficient activity could be prepared by shaking the mycelium in 0.2 M sodium chloride. No marked difference in the activity of the extract was observed between wet and acetone-dried mycelium. It is not possible to state the yield of the extraction because the activity was determined with the *p*H-stat method. It was observed subsequently that this method gives much higher values than those obtained with the microbiological method. These discrepancies are due to the presence of varying amounts of penicillinase in some extracts. Evidence of the presence of this enzyme could be detected by paper chromatography of penicilloic acid in the incubation mixture. All further results were obtained by the microbiological assay of 6-APA, and only extracts with a low penicillinase content were used.

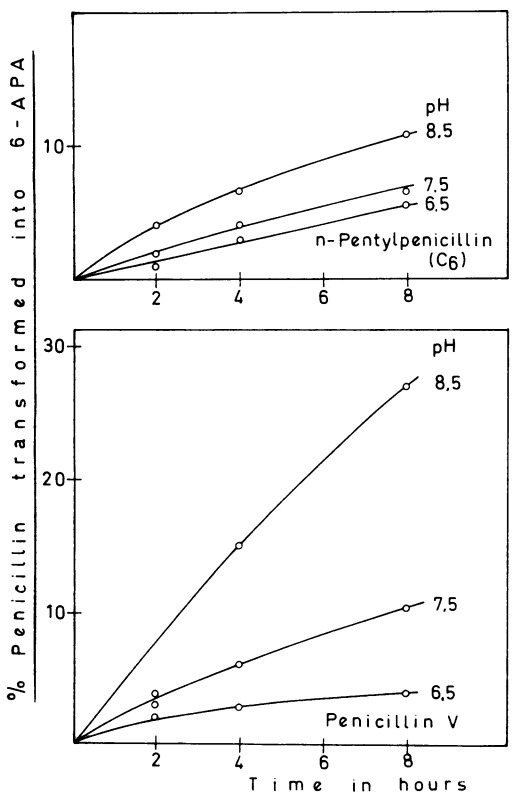

FIG. 3. *Influence of pH on the formation of 6-APA from phenoxymethyl-(V) and from n-pentylpenicillin (FH_2). Penicillin V or penicillin FH_2 (0.25 mmole) and 120 mg of P. chrysogenum extract (0.0013 unit/mg) in 20 ml of water were incubated at 37 C. The pH was kept constant by means of a pH-stat. Microbiological method.*

TABLE 3. *Influence of the structure of the side chain of penicillin on the formation of 6-APA by Penicillium acylase*[a]

Type of penicillin	Penicillin transformed into 6-APA at		
	2 hr[b]	4 hr	8 hr
Methylpenicillin (C_2)[c]	ND[d]	ND	ND
n-Propylpenicillin (C_4)	Tr[e]	Tr	Tr
n-Pentylpenicillin (C_6)	2.2	3.7	6.6
n-Heptylpenicillin (C_8)	6.4	9.1	17.8
n-Nonylpenicillin (C_{10})	11.2	13.7	33.4
n-Undecylpenicillin (C_{12})	16.7	23.5	39.2
Penicillin G	0.3	0.4	0.5
Penicillin V	3.3	6.7	11.8

[a] With 0.25 mmole of penicillin and 120 mg of *P. chrysogenum* Wis. 49-408 extract (0.0013 unit/mg) in 20 ml of water at 37 C, *p*H 7.5. Microbiological method.

[b] Hours of incubation.

[c] C_2, C_4, etc., indicate the number of carbon atoms of the aliphatic side chain of the penicillin.

[d] Not detectable.

[e] Trace of 6-APA < 0.1%.

Effect of reaction conditions and the structure of the side chain. The effect of *p*H on the rate of deacylation of penicillin V and *n*-pentylpenicillin (penicillin FH_2) was examined (Fig. 3). A marked increase of the reaction rate was observed when the *p*H of the incubation mixture was increased. The influence of the structure of the side chain on the formation of 6-APA is shown in Table 3. From these data it can be concluded that only small amounts of 6-APA are formed from penicillin G but that the acylase of *P. chrysogenum* efficiently hydrolyzes penicillins with an aliphatic side chain. The reaction rate increases with the chain length and can even be higher than the velocity observed with penicillin V.

Penicillins with a polar group in the side chain. No 6-APA was formed when 3-carboxypropylpenicillin (Ia) or 4-carboxybutylpenicillin (Ic) was incubated with *P. chrysogenum* extract. When the carboxy group of the side chain of these penicillins was esterified, cleavage by the acylase enzyme could be observed (Ib, Id, Ie, Table 4).

Similarly, no formation of 6-APA took place when both epimers of 4-carboxy-4-amino-*n*-butylpenicillin, i.e., penicillin N (IIa) or isopenicillin N (IIb), were treated with penicillin acylase. When the starting products of the synthesis of isopenicillin N (IIc and IId, Table 4) were used, a slight conversion into 6-APA was obtained.

All penicillins shown in Table 4 were also treated with *F. avenaceum* extract under the same

TABLE 4. *Transformation of penicillins having a polar group in the side chain into 6-APA*[a]

Type of penicillin	Penicillin transformed into 6-APA at		
	2 hr[b]	4 hr	8 hr
Ia $HOOC-(CH_2)_3-CO-R$[c]	Tr[d]	Tr	Tr
Ib $C_6H_5CH_2OOC-(CH_2)_3-CO-R$	4.1	4.4	6.4
Ic $HOOC-(CH_2)_4-CO-R$	Tr	Tr	Tr
Id $C_6H_5CH_2OOC-(CH_2)_4-CO-R$	1.3	2.9	6.6
Ie $C_2H_5OOC-(CH_2)_4-CO-R$	1.6	2.0	2.4
IIa $HOOC-CH(NH_2)-(CH_2)_3-CO-R$ D-isomer	Tr	Tr	Tr
IIb L-isomer	Tr	Tr	Tr
IIc L-$C_6H_5CH_2OOC-CH(NHCOOCH_2C_6H_5)-(CH_2)_3-CO-R$	0.6	1.3	1.5
IId L-$C_6H_5CH_2OOC-CH(N_3)-(CH_2)_3-CO-R$	0.3	0.5	1.1

[a] With 0.025 mmole of penicillin and 12 mg of *P. chrysogenum* Wis. 49-408 extract (0.0013 unit/mg) in 2 ml of 0.15 M phosphate buffer, *p*H 7.5, at 37 C. Microbiological method.
[b] Hours of incubation.
[c] R = 6-aminopenicillanic acid residue.
[d] Only traces or no 6-APA formed.

conditions described in Table 2, but no 6-APA or only very small amounts were obtained.

DISCUSSION

The effects of *p*H and temperature on the deacylation of penicillin by the mycelium and the enzyme extract of *F. avenaceum* (Fig. 1 and 2) correspond to the data published for *F. semitectum* (3, 20). We also found that the influence of *p*H on the formation of 6-APA by an extract of *P. chrysogenum* (Fig. 3) was the same as that observed when mycelium was used (10). Some difference was observed for the rate of cleavage of the aliphatic penicillins by *Penicillium* extract compared to the results of Erickson and Bennett (10), who used a mycelial suspension. This difference may be due to slightly different conditions of incubation (temperature, *p*H) and also to our use of an enzyme extract rather than the mycelium. Previous authors could not obtain active cell-free extracts from viable or acetone-dried mycelium of *P. chrysogenum* (10).

The penicillin acylases of *Fusarium* and of *Penicillium* are considered to be of the "fungal" type, because they split penicillin V more efficiently than penicillin G (13). The differences observed in our studies concerning the influence of *p*H (Fig. 1 and 3) and the structure of the side chain of aliphatic penicillins (Tables 2 and 3) indicate that marked differences exist between these two enzymes.

In our experiments, no 6-APA could be detected when 3-carboxypropylpenicillin or 4-carboxybutylpenicillin was incubated with the enzyme of *Fusarium* or *Penicillium*. These results are different from those reported by Claridge et al. (5), who observed formation of 6-APA from several penicillins having a carboxy group in the side chain when cells of *P. chrysogenum* were used. When the carboxy group of the side chain was replaced by an esterfunction, cleavage of the penicillin occurred (Table 4). Negative results were also obtained with penicillin N and isopenicillin N, whereas a certain amount of 6-APA was formed upon incubation of some related esters with *P. chrysogenum* extract (Table 4). All these data indicate that a free carboxy group inhibits the cleavage of the side chain by this type of pencillin acylase. With *F. avenaceum* extract, no significant formation of 6-APA was

observed with penicillins having a free carboxy group or an esterfunction in the side chain. This result was not unexpected, because this enzyme cleaves poorly all penicillins with an aliphatic side chain (Table 2).

Our results with penicillin N confirm that this penicillin, which has a D-aminoadipyl side chain, is resistant to penicillin acylase present in several bacteria and fungi (5, 10, 14, 18). The observation that no 6-APA is formed from isopenicillin N by our *P. chrysogenum* extract is more significant. Isopenicillin N, which has an L-aminoadipyl side chain, has been isolated from *P. chrysogenum* (7, 12) and has been considered as an intermediate in the biosynthesis of penicillin (1, 9). It has been suggested that 6-APA, which is present in small amounts in most penicillin fermentations (2, 8), is formed by hydrolysis of isopenicillin N (9). The results of our experiments are not in agreement with this hypothesis, although the possibility cannot be excluded that isopenicillin N will be transformed to 6-APA by another strain of *P. chrysogenum*. A possible origin of 6-APA is the deacylation of aliphatic penicillins (penicillin F, FH_2, K) by penicillin acylase of *P. chrysogenum*, which has been observed by Erickson and Bennett (10) and during the present study. The formation of 6-APA from other intermediates of the biosynthesis of penicillin, e.g., cysteinylvaline, is possible (9), but no experimental evidence is available for this hypothesis.

The last step of the penicillin biosynthesis, the acylation of 6-APA, has been described recently. Several authors have observed that benzyl- and phenoxymethylpenicillin may be formed through acylation of 6-APA by the corresponding acids, catalyzed by an enzyme present in the mycelium and in extracts of *P. chrysogenum* (4, 11, 19). The significance of another enzyme, acyltransferase, which catalyzes the exchange of the side chain, e.g., between benzyl- and phenoxymethylpenicillin, is not clearly understood at the moment (16). As no exchange between the side chain of penicillin N and the two other penicillins was observed (16), a similar study with isopenicillin N is important for a better understanding of the role of this product in the penicillin biosynthesis.

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