Distribution and Substrate Specificity of Benzylpenicillin Acylase[1]

H. T. Huang, T. A. Seto, and G. M. Shull

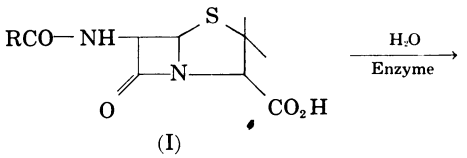
Fermentation Research Department, Chas. Pfizer & Co., Inc., Groton, Connecticut

Received for publication 18 June 1962

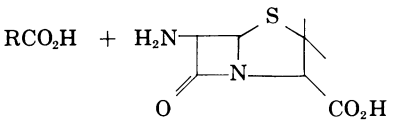
Abstract

Huang, H. T. (Chas. Pfizer & Co., Inc., Groton, Conn.), T. A. Seto, and G. M. Shull. Distribution and substrate specficity of benzylpenicillin acylase. Appl. Microbiol. **11**:1–6. 1963.—Benzylpenicillin acylase, which hydrolyzes benzylpenicillin to 6-aminopenicillanic acid, was found to be widely distributed among members of the *Schizomycetes*, particularly in gram-negative bacteria, and in the genus *Nocardia*. The hydrolysis of a series of biosynthetic and semisynthetic penicillins by freeze-dried cells of a strain of *Nocardia* and of *Proteus* was studied. Benzylpenicillin was the preferred substrate; all departures from the benzylpenicillin side-chain structure led to reduction of substrate activity (the greater the departure, the greater the reduction in activity). Penicillin amides and methyl esters were also hydrolyzed, as were suitable *N*-acyl derivatives of 7-aminocephalosporanic acid. Occurrence of an enzyme activity which hydrolyzes benzylpenicillinamide to benzylpenicillin was detected in certain strains of yeasts.

Sakaguchi and Murao (1950), and later Murao (1955*a*, *b*), reported the occurrence of an enzyme in *Penicillium chrysogenum* Q176, which hydrolyzes benzylpenicillin (I; R = $C_6H_5CH_2$) to phenylacetic acid, and a substance called "penicin" with the structure of 6-aminopenicillanic acid (6-APA; II). However, the properties they described



(I)



(II)

for "penicin" do not agree with those of authentic 6-APA, later isolated by Batchelor et al. (1959). Efforts to reproduce the results of Sakaguchi and Murao (1950) have been unsuccessful (e.g., Claridge, Gourevitch, and Lein, 1960).

[1] Presented in part at the 137th National Meeting (St. Louis, Mo.), March 27–30, 1961, and the 140th National Meeting (Chicago, Ill.) September 4–8, 1961, of the American Chemical Society.

The occurrence of an enzyme in bacteria and *Nocardia* species which hydrolyzes benzylpenicillin readily to 6-APA was reported by us (Huang et al., 1960), and also independently by others (Claridge et al., 1960; Rolinson et al., 1960; Kaufmann and Bauer, 1960). Rolinson et al. (1960) also described the occurrence, in streptomycetes and fungi, of a different type of enzyme which hydrolyzed phenoxymethylpenicillin (I; R = $C_6H_5OCH_2$) readily but had very poor activity on benzylpenicillin. More recently, Erickson and Bennett (1961) and Murao and Kishida (1961) were able to confirm the earlier data of Sakaguchi and Murao (1950) by demonstrating the hydrolysis of benzylpenicillin to 6-APA by *P. chrysogenum* Q176. The enzyme in this culture was apparently of the type specific for phenoxymethylpenicillin. Its relative lack of activity on benzylpenicillin may explain earlier failures to reproduce the same result.

Sakaguchi and Murao (1950) had called the enzyme which hydrolyzes benzylpenicillin to 6-APA, penicillinamidase. We have preferred to call it penicillin acylase, since it hydrolyzes an *N*-acyl group from an *N*-acyl-6-APA derivative (I), an action analogous to that of kidney acylases (Greenstein, 1954), which specifically hydrolyze the *N*-acyl group of *N*-acyl-L-amino acids. We further propose that the two types of penicillin acylases, now known to exist, be distinguished according to their specific substrates; thus, the bacterial type should be called benzylpenicillin acylase, and the fungal type, phenoxymethylpenicillin acylase.

The present paper records in detail our data on the distribution of benzylpenicillin acylase among microorganisms, and the substrate specificity of the enzymes from *Nocardia* and from *Proteus* species. We also report the occurrence in yeast of a true penicillin amidase activity, which hydrolyzes penicillin amides to the free penicillin.

Materials and Methods

Substrates. Benzylpenicillin (sodium salt) and phenoxymethylpenicillin (potassium salt) were commercial products. All other penicillins were prepared by reaction of the appropriate acid chloride with 6-APA in acetone-water at neutral pH, and were isolated as the potassium salts. Most of the products were crystalline, and all gave greater than 80% purity by hydroxylamine assay with benzylpenicillin as the standard (Boxer and Everett, 1949).

Benzylpenicillinamide was prepared as described by

Johnson (1953); prisms from benzene, mp 158 to 158.5 C, $[\alpha]_D^{23}$ = +320° (C, 1.0 in acetone). Calculated for $C_{16}H_{19}N_3O_3S$: C, 57.64; H, 5.74; N, 12.60. Found: C, 57.38; H, 5.70; N, 12.61. Phenoxymethylpenicillinamide was prepared in the same way; needles from benzene, containing ⅓ mole of solvent of crystallization, soften at 125 C, melt at 136.5 C, $[\alpha]_D^{23}$ = + 225° (C, 1.0 in acetone). Calculated for $C_{16}H_{19}N_3O_4S$ ⅓ C_6H_6: C, 57.45; H, 5.64; N, 11.19. Found: C, 57.11; H, 5.69; N, 11.31.

Benzylpenicillin methyl ester was prepared by reacting the penicillin-free acid with diazomethane; rods from ethylacetate-hexane, mp 98.5 C, $[\alpha]_D^{23}$ = + 243° (C, 1.0 in acetone); reported by Wintersteiner et al. (1949), mp 97 to 98 C, $[\alpha]_D$ = + 328° (methanol). Phenoxymethylpenicillin methyl ester, prepared by the same method, gave prisms from ethylacetate-hexane, mp 68 to 70 C, $[\alpha]_D^{25}$ = + 156° (C, 1.0 in acetone). Calculated for $C_{17}H_{20}O_5N_2S$: C, 56.03 H, 5.53; N, 7.69. Found: C, 55.88; H, 5.13; N, 7.48. *N*-Phenoxyacetyl- and *N*-phenylmercaptoacetyl-7-amino-cephalosporanic acids were prepared by acylation of 7-aminocephalosporanic acid (Loder, Newton, and Abraham, 1961; prepared by Edward Martin) with the appropriate acid chlorides. Both compounds were isolated as the potassium salt and were estimated to be at least 90% pure by their ultraviolet absorption at 263 mμ.

Cephalosporin N (synnematin B) was a preparation from Abbott and Company and cephalosporin C was prepared by fermentation (Abraham, Newton, and Hale, 1954).

6-Aminopenicillanamide (6-APA amide) and methyl 6-aminopenicillanate (6-APA methyl ester) were prepared from the corresponding trityl derivatives (kindly supplied by B. K. Koe).

Screening procedure. Screening of cultures for penicillin acylase activity was carried out as follows. Cultures were grown in 300-ml Erlenmeyer flasks containing 100 ml of medium incubated at 28 C on a rotary shaker moving at 230 cycles/min. Each culture was tested in at least two of the following media (in g/liter): (A) corn steep liquor, 20.0; beet molasses, 20.0; glycerol, 10.0; magnesium sulfate, 1.0; adjusted to pH 7.5 with NH_4OH before autoclaving; (B) yeast extract (Difco), 4.0; malt extract (Difco), 10.0; dextrose, 4.0; pH 7.3 before autoclaving; and (C) minimal salts of Davis and Mingioli (1950); NZ-amine B (Sheffield Chemical, Norwich, N.Y.), 5.0; yeast extract (Difco), 0.5; pH 7.3 before autoclaving. After 18 to 24 hr when good growth was obtained, the broth was adjusted to pH 7.0 to 7.5 with 50% KOH, mixed with 0.2 g of sodium benzylpenicillin and 2 ml of toluene, and again incubated on the shaker for 3 to 18 hr. The presence of 6-APA in the broth was detected by paper chromatography, phenylacetylation, and bioautograph.

Screening of cultures for penicillin-amidase activity was carried out in the same manner, except that pure benzylpenicillinamide replaced sodium benzylpenicillin as the added substrate. The reaction was then analyzed by paper chromatography-bioautograph for the presence of benzylpenicillin.

Preparation of freeze-dried cells. For freeze-drying, cultures were grown in a series of 4-liter glass fermentors (Shull and Kita, 1955) containing 2 liters of medium. *Nocardia* FD 4697 was grown in medium B and *Proteus rettgeri* FD 13424 in medium C. The fermentors were inoculated with a 5% vegetative flask culture grown in the same medium as described in the screening procedure. They were stirred at 1,750 rev/min at 28 C for 16 to 20 hr, under vigorous aeration (1 vol per vol^{-1} per min^{-1}). The cells were collected by centrifugation, washed with 0.07 M potassium phosphate buffer, resuspended in distilled water, and freeze-dried. Yields of dry weight of cells varied from 2 to 3 g/liter. These freeze-dried preparations could be kept in a refrigerator up to 6 months without significant loss of acylase activity.

Substrate specificity studies. Substrate specificity of *Nocardia* and *Proteus* enzymes was studied in a test system containing 10 mg of penicillin, 10 mg of freeze-dried cells, and 0.1 ml of toluene in 5 ml of 0.07 M potassium phosphate buffer at pH 7.0. After shaking at 28 C for 16 hr, the supernatants were analyzed for 6-APA by paper chromatography, phenylacetylation, and bioautograph. The amount of 6-APA formed was estimated by comparing the size of zones of samples with those given by 6-APA standards selected to correspond to 1, 5, 20, 50, and 90% hydrolysis of benzylpenicillin. Each substrate was tested at least twice with both cell preparations. Under the condi-

TABLE 1. *Summary of chromatography systems and R_F values of substrates and products*

Compound	R_F in system*			
	A	B	C	D
Benzylpenicillin	0.9	0.9	0.8	0.0
Other penicillins (Tables 3 to 5)	0.8–0.9			
6-Aminopenicillanic acid (6-APA)	0.5	0.5		
Benzylpenicillinamide		0.9		
Phenoxymethylpenicillinamide		0.9		
6-APA amide		0.4		
Methyl benzylpenicillinate				0.8
Methyl phenoxymethylpenicillinate				0.9
6-APA methyl ester				0.65
Cephalosporin C	0.1		0.1	
Cephalosporin N	0.25		0.15	
Phenoxyacetyl-7-ACA	0.8		0.7	
Phenylmercaptoacetyl-7-ACA	0.8		0.75	
7-Aminocephalosporanic acid (7-ACA)	0.25		0.25	

* A = butanol-acetic acid-water (5:1:4; v/v); running time, 5 hr. B = methyl isobutyl ketone-acetone-formic acid-water (80:6:2:12; v/v); running time, 1.5 hr. C = *n*-propanol-water (8:2; v/v); running time, 16 hr. D = benzene saturated with formamide paper impregnated with 32% formamide in acetone; running time, 1 hr.

tions stated, hydrolysis of benzylpenicillin was usually 50 to 90%. *N*-acyl-7-aminocephalosporanic acids were tested in the same manner. In experiments with penicillin amides and methyl esters, the amount of substrate in the test system was reduced to 5 mg.

Paper chromatography. The procedure employed in running paper chromatograms and bioautographs on *Bacillus subtilis* plates is described in detail by Lees, DeMuria, and Boegemann (1961). 6-APA and related compounds (i.e., 6-APA amide, 6-APA methyl ester, and 7-ACA) on fully developed chromatograms were made bioactive by phenylacetylation (Batchelor et al., 1959). The major solvent systems used and the R_F values of substrates and products are summarized in Table 1.

Results and Discussion

Distribution of acylase activity. The distribution of benzylpenicillin acylase activity among cultures screened is given in Table 2. From a total of 392 *Schizomycetes* from 36 genera (from 14 families), 55 cultures from 14 genera (from 7 families) showed activity. Inasmuch as the species identification of many of the cultures was incomplete, a breakdown of the number of species involved could not be accurately made.

Except for one genus (*Nocardia*) from one family (*Actinomycetaceae*), all the cultures listed in Table 2 are true bacteria and belong to the orders *Pseudomonadales* (*Pseudomonadaceae*) and *Eubacteriales* (*Rhizobiaceae* to *Bacillaceae*) as defined in *Bergey's Manual* (7th ed., 1957). It is clear that benzylpenicillin acylase occurs widely among bacteria. Active cultures are found in both gram-positive and gram-negative species, although our data tend to suggest a greater frequency of occurrence among gram-negative bacteria, particularly members of the *Enterobacteriaceae*. In general, these results are consistent with the distribution of penicillin acylase activity in bacteria recorded by Murao and Kishida (1961).

TABLE 2. *Distribution of benzylpenicillin acylase activity among schizomycetes*

Family	No. screened		No. found active	
	Genera	Isolates	Genera*	Isolates
Pseudomonadaceae	5	103	2	14
Rhizobiaceae	2	3	0	0
Achromobacteraceae	3	13	2	6
Enterobacteriaceae	6	76	5	29
Brucellaceae	2	2	1	1
Micrococcaceae	3	59	2	2
Neisseriaceae	1	2	0	0
Brevibacteriaceae	1	4	0	0
Lactobacteriaceae	5	7	0	0
Propionibacteriaceae	2	2	0	0
Corynebacteriaceae	4	11	2	2
Bacillaceae	1	69	0	0
Actinomycetaceae	1	41	1	6
All families	36	392	15	60

* Genera in which benzylpenicillin acylase occurred were, in order of the families listed, *Pseudomonas*, *Xanthomonas*, *Alcaligenes*, *Flavobacterium*, *Escherichia*, *Aerobacter*, *Erwinia*, *Serratia*, *Proteus*, *Bordetella*, *Micrococcus*, *Sarcina*, *Corynebacterium*, *Cellulomonas*, and *Nocardia*.

We believe that the distribution of activity among bacteria is wider than indicated by the data in Table 2, for two reasons. First, among each species there exists a high degree of specificity with regard to enzyme production. Thus, for example, both active and inactive strains are found in *Escherichia coli*, *Proteus vulgaris*, and *Pseudomonas aeruginosa*, as illustrated in previously published data (English, McBride, and Huang, 1960). Secondly, we cannot rule out the possibility that many cultures may produce both penicillin acylase and penicillinase. In these cases, any 6-APA formed will be degraded further to penicic acid (Sakaguchi and Murao, 1950) and thus escape detection. Consequently, many of the genera which do not yield acylase-positive cultures in our screening program may, quite possibly, contain active strains.

The occurrence of benzylpenicillin acylase is, of course, not limited to bacteria. In fact, one of the most active of all cultures screened is a strain of *Nocardia* FD 4697. It is interesting to note that the taxonomically related *Streptomyces* do not produce benzylpenicillin acylase but rather phenoxypenicillin acylase (Rolinson et al., 1960; Batchelor et al., 1961), indicating that in this particular biochemical activity the *Nocardia* resemble bacteria more closely than the *Streptomyces*.

General properties of benzylpenicillin acylase. Two of the most active cultures tested, *Nocardia* FD 4697 and *P. rettgeri* FD 13424, were selected for further study. Under screening conditions, both cultures gave nearly complete hydrolysis of benzylpenicillin to 6-APA in 18 hr. In view of the considerable taxonomical separation between these two genera, it was thought of interest to investigate whether any differences in the enzyme activities of these organisms would emerge as their properties become better known.

The enzyme activity of whole broth, centrifuged supernatants, and washed cells resuspended in 0.07 M potassium phosphate buffer (pH 7.5), was compared. With both cultures, there was no activity in the supernatant, although the cell suspension was as active as the original broth. Thus, the enzymes are entirely intracellular.

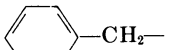
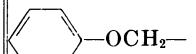
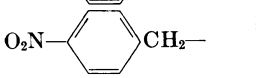
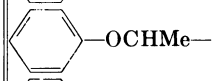
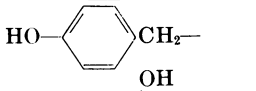
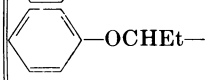
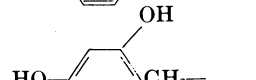
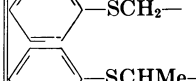
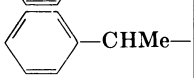
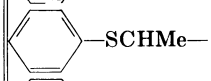
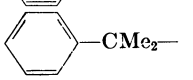
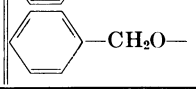
The activity of whole broth adjusted to pH 5.0, 6.0, 7.0, and 8.0 was also compared. Although precise determinations were not carried out, it was clear from qualitative paper-chromatographic data that with both *Nocardia* and *Proteus* preparations activity increased with increasing pH, reaching a maximum at pH 8.0. However, production of 6-APA at pH 5.0 was at least 60% as much as that at pH 8.0.

Thus, with regard to both site of localization and pH of optimal activity, the acylase from the *Nocardia* was indistinguishable from that of *Proteus*.

Substrate specificity and side-chain variations. The action of freeze-dried cells of *Nocardia* and *Proteus* on a variety of biosynthetic and semisynthetic penicillins was studied under conditions described in Materials and Methods. All the substrates were substituted acetyl derivatives of 6-APA and thus differed from each other only in respect to the structure of the side chain. In general, within the range of precision obtainable, the results for both *Nocardia* and *Proteus* preparations appear to be indistinguishable. The data reported in Tables 3 to 5 thus apply to both the *Nocardia* and *Proteus* enzymes.

The actions of the two enzymes on a series of structural modifications of benzylpenicillin are summarized in Table 3. Three effects may be noted. First, substitution in the phenyl ring of benzylpenicillin tends to reduce substrate activity (compare benzylpenicillin with its *p*-nitro, *p*-hydroxy, and *o*,*p*-dihydroxy derivatives). Secondly, substitution on the α-methylene group also reduces activity. Thus, α,α-dimethyl benzylpenicillin is less readily hydrolyzed than the α-mono-substituted compound. Thirdly, the insertion of an oxygen and particularly a sulfur atom between the phenyl ring and the methylene carbon also reduces activity. Phenoxymethylpenicillin is a poorer substrate and phenylmercaptomethylpenicillin is a much poorer substrate than benzylpenicillin. Further indication of the weakened substrate activity of these compounds is shown by the fact that α substitution of phenoxymethylpenicillin and of phenylmercaptomethylpenicillin practically eliminates all substrate activity. It may also be pointed out that insertion of an oxygen between benzyl and the carboxamido group of benzylpenicillin, as in benzyloxypenicillin, leads to complete loss of substrate activity.

TABLE 3. *Hydrolysis of benzylpenicillin and structural modifications by Nocardia and Proteus acylase (RCO-6-APA ⟶ RCO_2H + 6-APA)*

R	Hydrolysis*	R	Hydrolysis*
	+++		++
	++		< tr
	++		< tr
	++		±
	++		< tr
	±		< tr

* Estimated per cent hydrolysis: +++, 50–90; ++, 20–50; +, 5–20; ±, 1–5; tr, ≃ 1.

The effect of the number of methylene groups between phenyl and the carboxamido group is shown in Table 4. When $n = 1$, we have the reference substrate, benzylpenicillin. When $n = 0$, no activity was detected. When $n = 2$, activity fell sharply, although it appears to remain at a constant level till $n = 4$.

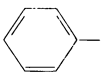
It is clear from these data (Tables 3 and 4) that maximal substrate activity is associated with the benzyl side chain, that is, with the structure of benzylpenicillin. Any departure from this structure leads to reduction in substrate activity (the greater the departure, the greater the loss in activity).

The above generalization appears to be followed even in simple alkyl penicillin series, as in Table 5. Methyl and ethyl penicillins are not hydrolyzed. Substrate activity emerges with *n*-propylpenicillin, reaches a maximum at C_5 and C_6 with the *n*-amyl and *n*-hexyl side-chains, and again declines with further increase in chain length. The *n*-amyl group is, of course, nearest in size to the benzyl group. Similarity in size to the benzyl group may also explain the relatively high susceptibility to hydrolysis of allylmercaptomethylpenicillin, in contrast to the poor activity of phenylmercaptomethyl penicillin indicated in Table 3.

Substrate specificity and modifications in the nucleus. A limited number of compounds with modifications in the 6-APA nucleus was also available for investigation. Results

TABLE 4. *Hydrolysis by Nocardia and Proteus acylase of substrates**

n	Hydrolysis†
0	0
1	+++
2	+
3	+
4	±

* With the general formula,  —$(CH_2)_n$CO-6-APA.

† Estimates of per cent hydrolysis to 6-APA as in Table 3.

TABLE 5. *Hydrolysis of alkyl and substituted-alkyl penicillins by Nocardia and Proteus acylase (RCO-6-APA ⟶ RCO_2H + 6-APA)*

R	Hydrolysis*
CH_3	< tr
C_2H_5	< tr
C_3H_7	tr
C_4H_9	±
C_5H_{11}	+
C_6H_{13}	+
C_7H_{15}	±
$CH_3S—CH_2—$	±
$C_2H_5S—CH_2—$	+
$CH_2=CH\cdot CH_2SCH_2—$	++

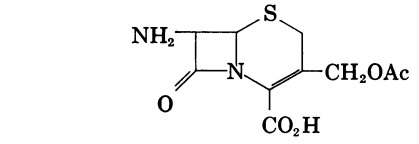
* Estimates of per cent hydrolysis as in Table 3.

on the amides and the methyl esters of benzyl- and phenoxymethylpenicillins are presented in Table 6. They conclusively indicate that modifications of the carboxylic acid group in the nucleus can be made, under favorable conditions, with only a partial loss of susceptibility to acylase action. Again, we note the superior substrate activity of the benzyl side chain as against the phenoxymethyl side chain. The reaction products (6-APA amide and 6-APA methyl ester) are easily detected and estimated on paper chromatograms by the emergence of bioactive zones after phenylacetylation.

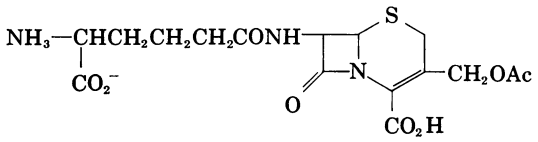
It should be noted that, in going from a benzyl to a phenoxymethyl side chain in 6-APA amides and methyl esters, the *Nocardia* preparation appears to suffer a proportionately greater loss of substrate activity than does the *Proteus* preparation. This observation was confirmed in experiments in which fresh culture broths were employed. It may reflect a real, intrinsic difference between the *Nocardia* and *Proteus* enzymes, or perhaps indicate the existence of a permeability barrier for hydrophobic substrates to the site of enzyme localization in the *Nocardia* cells.

In earlier experiments with penicillin amides, a small amount of 6-APA was usually observed in the reaction mixture, suggesting enzymatic hydrolysis of these substrates to the free penicillins and subsequently to 6-APA. This effect was finally found to be due to traces of the parent penicillins, which occur as impurities in the substrate preparations. When pure substrates, completely free from penicillins, were employed, no 6-APA was detected in the reaction mixture.

Recently, Loder et al. (1961) described the preparation of 7-aminocephalosporanic acid (III; 7-ACA) from the antibiotic cephalosporin C (IV; Abraham and Newton,



(III)



(IV)

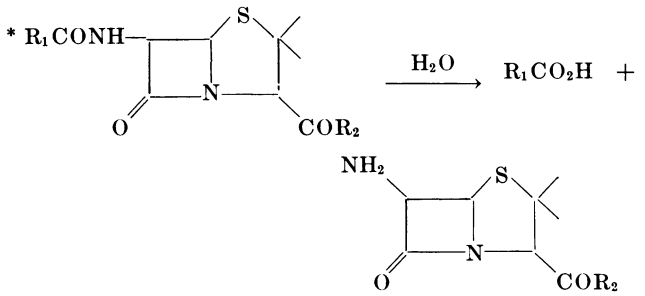
1954). 7-ACA is the nucleus of cephalosporin C, analogous to 6-APA as the nucleus of the penicillins. It is, in fact, a naturally occurring structural modification of 6-APA. We prepared the *N*-phenoxyacetyl and *N*-phenylmercaptoacetyl derivatives of 7-ACA and compared their susceptibility to *Nocardia* and *Proteus* acylase with that of the corresponding 6-APA derivatives. 7-ACA was detected and estimated in the same way as 6-APA. The results (Table 7) indicate clearly that substitution of 6-APA in phenoxymethylpenicillin and phenylmercaptomethylpenicillin by 7-ACA had only a minor effect on substrate activity. Furthermore, as with 6-APA derivatives, the side-chain specificity for the *Nocardia* enzyme parallels that for the *Proteus* enzyme. These data are consistent with those of Abraham and Newton (*personal communication*), who observed the hydrolysis of phenylacetyl-7-ACA by cells of *Alcaligenes faecalis*. We have also included in Table 7 data on the two naturally occurring derivatives of 6-APA and 7-ACA (i.e., cephalosporin N and cephalosporin C) that contain the same highly polar side chain derived from D-α-aminoadipic acid. Neither was hydrolyzed to the nucleus to a detectable extent.

Penicillin acylase and Penicillin amidase. We have preferred to call the enzyme which hydrolyzes penicillins to 6-APA a "penicillin acylase," even though the name "penicillin amidase" proposed by Sakaguchi and Murao (1950) has been widely used. The term "penicillin acylase" describes with reasonable accuracy the reaction that is catalyzed, whereas "penicillin amidase" may be misleading for two reasons.

First, penicillin amidase, by the generally accepted rules of enzyme nomenclature (Hoffmann-Ostenhoff, 1953), tends to suggest that penicillin amide is the major substrate for this enzyme, and that the amide group is in-

TABLE 6. *Hydrolysis of penicillin amides and methyl esters by Nocardia and Proteus acylase**

Substrate		Hydrolysis†	
R_1	R_2	*Nocardia*	*Proteus*
$C_6H_5CH_2$—	—NH_2	++	++
$C_6H_5CH_2$—	—OCH_3	+	+
$C_6H_5OCH_2$—	—NH_2	tr	+
$C_6H_5OCH_2$—	—OCH_3	tr	±



† Estimates of per cent hydrolysis as in Table 3.

TABLE 7. *Hydrolysis of N-acyl derivatives of 6-APA and 7-ACA by Nocardia and Proteus acylase*

Side-chain R	RCO-6-APA*		R-CO-7-ACA*	
	Nocardia	*Proteus*	*Nocardia*	*Proteus*
$C_6H_5OCH_2$—	++	++	++	++
$C_6H_5SCH_2$—	±	±	±	±
$^{+}NH_3$—$CHCH_2CH_2CH_2$— CO_2^-	< tr	< tr	< tr	< tr

* RCO-6-APA ⟶ 6-APA; RCO-7-ACA ⟶ 7-ACA. Estimates of per cent hydrolysis as in Table 3.

volved in the enzymatic reaction. Neither suggestion is consistent with the facts. The enzyme we have studied hydrolyzes a wider variety of penicillins and penicillin derivatives, including amides. When it acts on benzylpenicillin amide, the product is not benzylpenicillin but 6-APA amide: the amide group is actually untouched. Secondly, we have found that certain yeasts (e.g., *Rhodotorula gracilis* and species of *Torulopsis*) can hydrolyze benzylpenicillin amide (and phenoxymethylpenicillin amide) to benzylpenicillin (and phenoxymethylpenicillin). The activity is entirely intracellular and is presumably enzymatic in nature. This enzyme should correctly be known as penicillin amidase.

ACKNOWLEDGMENT

We thank Bernice Cummings for carrying out the paper chromatography and bioautograph analyses involved in the substrate specificity studies.

LITERATURE CITED

ABRAHAM, E. P., G. G. F. NEWTON, AND C. W. HALE. 1954. Purification and some properties of cephalosporin N, a new penicillin. Biochem. J. **58**:94–102.

BATCHELOR, F. R., E. B. CHAIN, M. RICHARDS, AND G. N. ROLINSON. 1961. 6-Aminopenicillanic acid. VI. Formation of 6-aminopenicillanic acid from penicillin by enzymic hydrolysis. Proc. Roy. Soc. London Ser. B **154**:522–531.

BATCHELOR, F. R., F. P. DOYLE, J. H. C. NAYLER, AND G. N. ROLINSON. 1959. Synthesis of penicillin: 6-aminopenicillanic acid in penicillin fermentations. Nature **183**:257–258.

BOXER, G. E., AND P. M. EVERETT. 1949. Colorimetric determination of benzylpenicillin. Anal. Chem. **21**:670–673.

CLARIDGE, C. A., A. GOUREVITCH, AND J. LEIN. 1960. Bacterial penicillin amidase. Nature **187**:237–238.

DAVIS, B. D., AND E. S. MINGIOLI. 1950. Mutants of Escherichia coli requiring methionine or vitamin B_{12}. J. Bacteriol. **60**:17–28.

ENGLISH, A. R., T. J. MCBRIDE, AND H. T. HUANG. 1960. Microbial resistance to penicillin as related to penicillinase or penicillin acylase activity. Proc. Soc. Exptl. Biol. Med. **104**:547–549.

ERICKSON, R. C., AND R. E. BENNETT. 1961. Degradation of penicillins to 6-aminopenicillanic acid by *Penicillium chrysogenum*. Bacteriol. Proc., p. 65.

GREENSTEIN, J. P. 1954. The resolution of racemic α-amino acids. Advan. Protein Chem. **9**:121–202.

HOFFMANN-OSTENHOFF, O. 1953. Suggestions for a more rational classification and nomenclature of enzymes. Advan. Enzymol. **14**:219–260.

HUANG, H. T., A. R. ENGLISH, T. A. SETO, G. M. SHULL, AND B. A. SOBIN. 1960. Enzymatic hydrolysis of the side chain of penicillins. J. Am. Chem. Soc. **82**:3790–3791.

JOHNSON, D. A. 1953. Carboxy derivatives of benzylpenicillin. J. Am. Chem. Soc. **75**:3636–3637.

KAUFMANN, W., AND K. BAUER. 1960. Enzymatische Spaltung und Resynthese von Penicillin. Naturwissenschaften **47**:474–475.

LEES, T. M., P. J. DEMURIA, AND W. H. BOEGEMANN. 1961. Paper chromatographic separation of multi-component antibiotic mixtures. J. Chromatog. **5**:126–130.

LODER, B., G. G. F. NEWTON, AND E. P. ABRAHAM. 1961. The cephalosporin C nucleus (7-aminocephalosporanic acid) and some of its derivatives. Biochem. J. **79**:408–416.

MURAO, S. 1955*a*. Studies on penicillin amidase. II. Research on conditions of producing penicillin amidase. J. Agr. Chem. Soc. Japan **29**:400–403.

MURAO, S. 1955*b*. Studies on penicillin amidase. III. Research on mechanism of penicillin amidase action on sodium penicillin G. J. Agr. Chem. Soc. Japan **29**:404–407.

MURAO, S., AND Y. KISHIDA. 1961. Studies on penicillin-amidase. J. Agr. Chem. Soc. Japan **35**:607–615.

ROLINSON, G. N., F. R. BATCHELOR, D. BUTTERWORTH, J. CAMERON-WOOD, M. COLE, G. C., EUSTACE, M. V. HART, M. RICHARDS, AND E. B. CHAIN. 1960. Formation of 6-aminopenicillanic acid from penicillin by enzymatic hydrolysis. Nature **187**:236–237.

SAKAGUCHI, K., AND S. MURAO. 1950. A preliminary report on a new enzyme, "penicillin-amidase." J. Agr. Chem. Soc. Japan **23**:411.

SHULL, G. M., AND D. A. KITA. 1955. Microbiological conversion of steroids. I. Introduction of the 11 β-hydroxyl group into C_{21} steroids. J. Am. Chem Soc. **77**:763–764.

WINTERSTEINER, O., W. R. BOON, H. C. CARRINGTON, D. W. MACCORQUODALE, F. H. STODOLA, J. L. WACHTEL, R. D. COGHILL, W. C. RISSER, J. E. PHILIP, AND O. TOUSTER. 1949. Isolation and characterization of the various penicillins, p. 93. *In* H. T. Clark, J. R. Johnson, and R. Robinson, The chemistry of penicillin. Princeton University Press, Princeton, N.J.