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Penicillinacylase

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INTRODUCTION

The penicillin molecule is susceptible to hydrolytic cleavage not only at the β-lactam bond (the process which is carried out by the enzyme penicillinase, or by treatment with dilute alkali), but also at the peptide linkage (*see* Fig. 1) by which the side chain (R group) of the penicillin (I) is joined to the "nucleus," 6-aminopenicillanic acid (6APA, II).

The latter reaction (equation 1, Fig. 2) can be brought about enzymatically; the enzyme responsible has been given several different names (13), including "penicillin amidase," "penamidase," "benzylpenicillin acylase," "penicillin splitting and synthesizing enzyme," "acyltransferase," and, perhaps the least satisfactory name of all, the official (Enzyme Commission) nomenclature "benzylpenicillin amidohydrolase" (EC 3.5.1.11). This last name is unsatisfactory for two main reasons: (i) it is too similar to the name for penicillin β-lactamase (EC 3.5.2.6), which is "penicillin amidohydrolase," and (ii) it suggests that benzylpenicillin is the best, or even the only, substrate, a fact which, as will be shown below, is totally erroneous. The enzyme will be referred to here simply as "acylase." Acylase is an enzyme of great commercial importance, as it is because of its existence that 6APA is available in amounts large enough to enable the large-scale production of semisynthetic penicillins to be an economic proposition (19).

HISTORICAL BACKGROUND

Acylase activity was first described by workers in Japan (48), in *Penicillium chrysogenum* Q 176; they reported that the mycelia of this strain were capable of converting benzylpenicillin into phenylacetic acid and a compound that they called "penicin." Further details of this reaction were subsequently given by Murao (42), who showed that penicin was degraded by penicillinase to "penicic acid," which was strongly ninhydrin-positive; the same author reported that "penicin" had a melting point of 157 to 160 C. Meanwhile, it had been suggested (34) that penicin was in fact the penicillin nucleus 6APA. The matter rested here for some years until the report by Batchelor and his colleagues (4) at the Beecham Research Laboratories to the effect that 6APA had been detected in a fermentation brew of *P. chrysogenum* W51.20 to which no side chain precursors had been added; the identity of this 6APA was proved by these workers by virtue of the fact that, on phenylacetylation, benzylpenicillin was produced. Purification and isolation of 6APA from the brew were described in great detail in a subsequent paper (6). The melting point of 6APA was given by Batchelor et al. (4) as 208 to 209 C (decomposition), which does not agree with the figure given by Murao (42). There was further doubt in many minds as to whether the "penicin" described by Murao and his colleagues was indeed 6APA because,

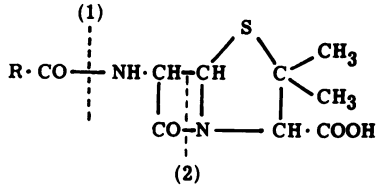


FIG. 1. *Linkage of penicillin side chain to 6-aminopenicillanic acid residue. (1) Site of action of penicillin acylase (splitting the amide linkage). (2) Site of action of penicillinase (giving rise to penicilloic acid).*

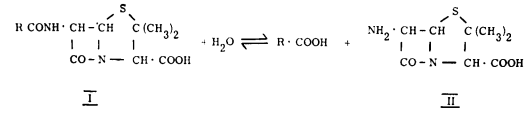


FIG. 2. *Equation 1. Side chain (R group) of the penicillin (I) is irreversibly removed from the "nucleus" 6-aminopenicillanic acid (II).*

although acylase activity had been demonstrated independently in 1960 by several groups of workers (11, 30, 36, 46) in various different microbial species (see subsequent sections), no one had succeeded in repeating the experiments performed by the Japanese workers; i.e., despite detailed searches, no acylase activity could be found in *P. chrysogenum* Q 176. However, the Japanese workers were vindicated when, in 1961, both Erickson and Bennett (22) and Murao and Kishida (43) succeeded in demonstrating that this strain is capable of hydrolyzing benzylpenicillin to 6APA. Erickson and Bennett (22) also suggested reasons for the failure of previous workers to repeat the original work.

PROPERTIES OF ACYLASES

Acylases differ, broadly speaking, in their properties according to whether they come from bacteria, or from yeasts, molds, or fungi. The distinction is not clear-cut; thus, to avoid confusion and contradictions, the properties of the various acylases will be subsumed under type I and type II, according to the nomenclature of Claridge, Luttinger, and Lein (12).

Type I Acylases ("Fungal" Type)

The chief characteristic of type I acylases is that they hydrolyze phenoxymethylpenicillin much more rapidly than benzylpenicillin. The enzyme has been described in many species of actinomycetes, filamentous fungi, and yeasts, the following genera having been reported to exhibit acylase activity: *Alternatia, Aspergillus, Botrytis, Cephalosporium, Cryptococcus, Emericellopsis, Epicoccum, Epidermophyton, Fusarium, Mucor, Penicillium, Phoma, Trichoderma, Trichophyton,* and *Trichosporon* (7, 10, 12, 14, 15, 46, 48, 55, 58).

The enzyme of *Streptomyces lavendulae* BRL 198 has been studied in detail by the Beecham team (7,46; U.S. Patent 3,014,845). Heptylpenicillin (penicillin K), pentylpenicillin (penicillin dihydroF), phenoxymethylpenicillin (penicillin V), monosubstituted phenoxymethylpenicillins, alkoxy- and alkenoxy- methylpenicillins, and similar compounds in which the oxygen atom between the methylene group and the benzene ring has been replaced by a sulfur atom (e.g., allylmercaptomethylpenicillin, penicillin O) are readily hydrolyzed, whereas benzylpenicillin is hydrolyzed at only 1% of the rate of phenoxymethylpenicillin. This enzyme is largely extracellular, is not inducible, and has a *p*H optimum of 10 for the hydrolytic reaction; for the synthetic reaction (i.e., acylation of 6APA), however, the *p*H optimum lies between 5.0 and 5.5. The K_m for phenoxymethylpenicillin was found to be about 12.5 mM.

Cole (14) studied certain properties of intracellular acylases from nine fungal strains (five representatives of *Penicillium*; one each of *Aspergillus, Trichophyton, Epidermophyton,* and *Cephalosporium*). All hydrolyzed phenoxymethylpenicillin, and two of the strains hydrolyzed benzylpenicillin in addition, but much more slowly. When grown in the presence of 0.1% phenoxyacetic acid, four of the strains investigated (two *Penicillium* species, *A. ochraeus,* and *T. mentagrophytes*) possessed an increased activity against phenoxymethylpenicillin but not against benzylpenicillin. The author suggests that activity against phenoxymethylpenicillin and against benzylpenicillin may be due to the presence of two separate enzymes, and, in addition, reports that a third type of acylase activity (methylpenicillin acylase) exists in a yeast strain. The finding of Claridge and his colleagues (12) that the acylase of *Cephalosporium* CMI 49137 hydrolyzes phenoxymethylpenicillin but not benzylpenicillin was confirmed by Cole (14). Uri et al. (55) describe an acylase derived from certain dermatophytes for which phenoxymethylpenicillin is also a better substrate than is benzylpenicillin; the activity was largely intracellular and was inducible (56).

P. chrysogenum has been reported to produce acylase: strains Q 176 (22, 43, 48), A 9342 (12), Wis 49.408 and P.5009 (23) have this ability. The enzyme appears to be intracellular and noninducible, and has optimal activity at *p*H values between 8 and 8.5. It is capable of hydrolyzing benzylpenicillin, phenoxymethylpenicillin, Δ^2-pentenyl-penicillin (penicillin F), heptylpenicillin,

phenethicillin, and semisynthetic penicillins containing either carboxyl or amino groups in the side chain; methicillin, cephalosporin C, and α-aminoadipyl-penicillin (penicillin N) are not hydrolyzed.

The acylase of *Fusarium semitectum*, which has been reported to hydrolyze phenoxymethylpenicillin preferentially, has a molecular weight of about 65,000 and contains 2 atoms of zinc per molecule (58).

On the basis of the rather scanty enzymological findings concerning the properties of type I acylases, fungal acylases in general appear to have a similar specificity pattern; irrespective of their species of origin, they hydrolyze phenoxymethylpenicillin more rapidly than benzylpenicillin. Although it is most commonly found in fungi, molds, and yeasts, type I acylase has also been reported in a strain (NCIB 9424) of *Achromobacter* (13). Type I acylase activity has been found in sources other than microbial ones. Cole (13) reported that a commercial pig kidney powder was capable of hydrolyzing phenoxymethylpenicillin, and Weitnauer (U.S. Patent 3,070,511) found that the particulate fraction of homogenates prepared from the kidney, liver, spleen, or lung of cattle and pigs possessed similar activity. In contrast, English, Huang, and Sobin (20) were unable to detect acylase activity in the following mammalian tissues: mouse kidney, rat spleen, and rabbit liver, lung, and brain. Holt and Stewart (28) reported that a crude aminopeptidase preparation from pig gastric mucosa hydrolyzed benzylpenicillin to 6APA. It seems clear that the deacylation of penicillins by animal tissue extracts is not brought about by a specific penicillinacylase, but by an "amidase" of broad specificity. Kidney preparations such as those used are well known to be a potent source of relatively nonspecific deacylating enzymes, active against a variety of N-acylated L-amino compounds.

Alburn, Grant, and Clark (U.S. Patent 3,032,473) found that preparations of ficin, a proteolytic enzyme obtained from trees of the genus *Ficus*, hydrolyzed phenoxymethylpenicillin to 6APA.

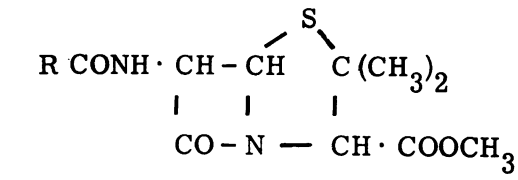
Type II Acylases ("Bacterial" type)

The bacterial species in which acylase activity has been most widely studied is *Escherichia coli*, particularly the strain designated ATCC 9637 (10, 12, 35, 36, 45, 46, 49). The enzyme, which is both inducible and intracellular, requires an alkaline medium for greatest hydrolytic activity. It is produced in greater amounts when the organism is cultured at temperatures below 31 C, and also in the absence of fermentable carbohydrates. Benzylpenicillin is hydrolyzed much more rapidly than is phenoxymethylpenicillin. The work of Cole (13), Kaufmann and Bauer (37), and Lucente, Romeo, and Rossi (41) has shown conclusively that the specificity of the bacterial acylases is directed at the phenylacetyl group, rather than at the penicillin nucleus. These workers found that acylases were capable of removing phenylacetyl groups from a variety of phenylacetyl-L-amino acids and related compounds (no activity was displayed against the diastereoisomeric D compounds). It is not yet apparent whether more than one acylase enzyme is present in bacteria; it has been suggested (45) that several acylases of differing specificities may be produced by the same bacterium. Indeed, from the work of Holt and Stewart (28), it is apparent that many gram-negative species contain enzymes capable of hydrolyzing L-leucyl-β-naphthylamide to free naphthylamine, and L-leucinamide to free leucine; from considerations of specificity (13, 37, 41), it seems unlikely that either of these enzymes is identical with penicillinacylase. Szentirmai (53) and Kaufmann (35) have both made the interesting observation that low concentrations of certain biologically active penicillins inhibit the hydrolytic activity of *E. coli* acylase.

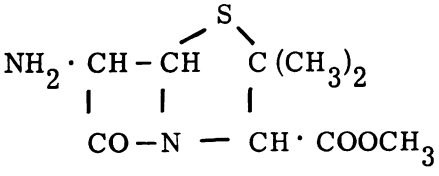
Type II acylase activity has been found in members of the following genera: *Aerobacter*, *Alcaligenes*, *Bordetella*, *Cellulomonas*, *Corynebacterium*, *Erwinia*, *Escherichia*, *Flavobacterium*, *Micrococcus*, *Nocardia*, *Proteus*, *Pseudomonas*, *Salmonella*, *Sarcina*, and *Xanthomonas* (11, 30, 31, 36, 43, 44, 46). Thus, with the exception of *Nocardia* (which, although being classified in the fungi, nevertheless produces a type II acylase), it is bacteria, and most commonly gram-negative species, that elaborate type II acylase.

A detailed study has been made by Huang, Seto, and Shull (31) of the specificity of the type II acylase produced by *Nocardia* and *Proteus rettgeri*. They found that the activity of the acylase against penicillin substrates was decreased by substitution in the side chain benzene ring or in the α-methylene group, or by insertion of either an oxygen atom or, particularly, a sulfur atom between the methylene group and the benzene ring. Modifications to the penicillin nucleus, however, proved to have little effect upon the activity of the acylase; the methyl esters of penicillins (III, Fig. 3) were hydrolyzed to the methyl ester of 6APA (IV, Fig. 3) as rapidly as were the free parent penicillins, and N-acyl derivatives of 7-aminocephalosporanic acid were as susceptible as the same derivatives of 6APA.

The structure of the "nucleus" (i.e., the group



III



IV

FIG. 3. *Methyl ester of penicillin (III); methyl ester of 6APA (IV).*

to which the phenylacetyl radical is attached) does, however, have some part to play in determining the ease with which the phenylacetyl group is removed. Pruess and Johnston (45) found that the penicilloic and penilloic acids of benzylpenicillin were hydrolyzed as a considerably slower rate than was benzylpenicillin itself. Cole (13) pointed out that phenylacetylglycine, phenacetylaminophenylacetic acid, and phenylacetamide were more rapidly hydrolyzed than was benzylpenicillin, and that benzylpenicilloic acid was hydrolyzed at little over one-fifth the rate of its parent compound. Lucente et al. (41) reported that phenylacetylalanine was a much better substrate than phenylacetylvaline. All these studies were carried out with the *E. coli* acylase.

The type II acylase produced by a strain of *Micrococcus roseus* (ATCC 516) is apparently metal-activated, as its activity is destroyed by ethylenediaminetetraacetic acid (EDTA) (45).

A summary of the properties of acylases of both types is given in Table 1.

Synthetic Ability of Acylases

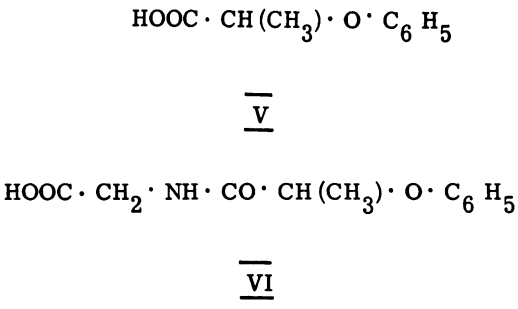
Most of the interest in acylase activity has concentrated upon the hydrolytic activity, i.e., the catalysis of the forward reaction shown in Fig. 2, and the production of 6APA and a free side chain acid from penicillins. However, as shown in Fig. 2, the enzyme is capable of bringing about the back (synthetic) reaction also. The synthetic reaction occurred (7) at acid *p*H values, with the *S. lavendulae* enzyme, if the concentration of

TABLE 1. *Properties of penicillinacylases*

Type	Source	Substrate[a] specificity	Inducibility	Cellular location[b]	*p*H optima		References
					Hydrolysis	Synthesis	
I	*Streptomyces lavendulae*	G, 1% of V; V, K, and FH_2, "rapidly"	No	LE	10	5.0–5.5	7
	Dermatophytes	V, "rapidly"; G, "less rapidly"	Yes	LI			14, 55, 56
	Penicillium chrysogenum	V, 100%; K, 58%; F, 42%; G, 17%; methicillin, cephalosporin C, and penicillin N not hydrolyzed	No (23) Yes (14)	I	8.5		12, 14, 23
II	*Escherichia coli*	G, 100%; X, 100%; V, 20%; ampicillin, 83%; *p*-aminobenzylpenicillin, 75%; oxacillin, 0%	Yes	I	8.0	5.5	38, 39, 45, 46, 53, 53*a*
	Nocardia, Proteus	G, 100%; V, 20–50%; O, 20–50%; X, 20–50%; FH_2, 5–20%; K, 1–5%		I	8.0		31

[a] Substrates: G, benzylpenicillin; V, phenoxymethylpenicillin; K, heptylpenicillin; F, Δ^2-pentenylpenicillin; FH_2, pentylpenicillin; X, *p*-hydroxybenzylpenicillin; O, allylmercaptomethylpenicillin; N, α-aminoadipylpenicillin.

[b] LE, largely extracellular; LI, largely intracellular; I, intracellular.



V

VI

FIG. 4. *α-Methylphenoxyacetic acid (V); α-methylphenoxyacetylglycine (VI).*

6APA was raised (mass action effect). Kaufmann, Bauer, and Offe (39) studied the synthetic reaction using *E. coli* acylase. They found that this reaction can be brought about, in certain cases, much more rapidly by using an "energy-rich" side chain acid derivative in place of the side chain acid alone. Examples of such "energy-rich" derivatives are phenylacetylglycine and phenoxyacetylglutamate (for the synthesis of benzyl- and phenoxymethylpenicillins from 6APA, respectively). Whereas neither α-methylphenoxyacetic acid (V, Fig. 4) nor α-methylphenoxyacetylglycine (VI, Fig. 4) will react to any great extent with 6APA, it was found that α-methylphenoxyacetylthioglycollic acid (VII) will rapidly acylate 6APA with the formation of phenethicillin (VIII) and thioglycollic acid (*see* equation 2, Fig. 5).

Two factors militate against the successful use of enzymatic methods for the commercial production of semisynthetic penicillins: the reversibility of the process and the fact that considerable product inhibition can occur. Thus, the chemical processes for the N-acylation of 6APA have proved to be more economical, rapid, and reliable.

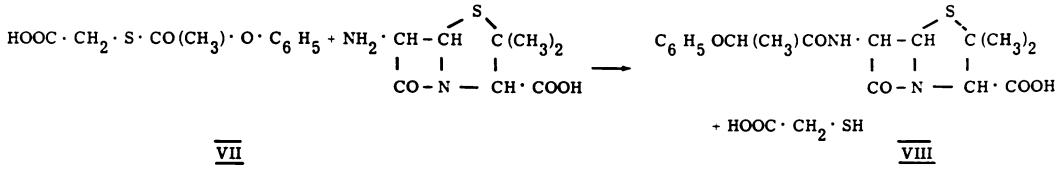
ROLE OF ACYLASES IN MICROBIAL ECONOMY

It is possible that acylase may have a role in the biosynthesis of penicillins by *P. chrysogenum*. Although the exact biosynthetic pathways have not yet been worked out, the studies of Arnstein and his co-workers (2, 59) suggest that the final step in the biosynthesis of benzylpenicillin may be a transacylation between either penicillin N or isopenicillin N (16, 24) and phenylacetic acid, yielding benzylpenicillin and either D- or L-α-aminoadipic acid. It seems likely that such a reaction would be catalyzed by a "transacylase." This enzyme, however, is probably not identical with the acylase in *P. chrysogenum* whose hydrolytic ability was studied by Erickson and Bennett (23), because the latter enzyme did not hydrolyze penicillin N. It is significant that Cole (14) found that all the penicillin-producing fungi he studied possessed acylase activity.

It has been suggested (21) and implied (28, 29) that acylase may play an important part in the resistance of coliform organisms to penicillins, notably benzylpenicillin and ampicillin. A similar suggestion has been made (55) regarding the resistance of dermatophytes to penicillins. Although *prima facie* this may appear to be an attractive hypothesis, it cannot stand up to a detailed examination, for the following reasons. (i) Both the type I (7) and the type II (10) acylases have low affinities for their substrates; thus, although penicillins may be rapidly deacylated under laboratory conditions, at concentrations which are attained in vivo the activity of the enzyme will be greatly reduced. (ii) Both types of enzyme operate most effectively at appreciably alkaline *p*H values (7, 39), so that, again, the activity in vivo will be less than maximal. (iii) The bacterial enzyme is inhibited by therapeutically attainable levels of certain biologically active penicillins (35, 53). (iv) Production of the enzyme is repressed by growth of the organisms above 31 C, and also by the presence of fermentable carbohydrates (17, 38, 53); both of these adverse conditions will obtain in vivo. (v) The product of the enzyme's action, 6APA, is frequently as active as benzylpenicillin against coliform organisms (18, 21, 25, 47, 50).

Cole and Sutherland (17), in a careful investigation involving 148 clinical strains, showed that acylase activity can be discounted almost completely as a determinant of resistance to penicillins in vitro.

It has been shown that certain bacteria which produce acylase are capable of utilizing phenylacetic acid as a carbon source (33, 53). Thus, in



VII VIII

FIG. 5. *Equation 2. Acylation of 6APA by α-methylphenoxyacetylthioglycollic acid (VII), forming phenethicillin (VIII) and thioglycollic acid.*

the presence of benzylpenicillin, such bacterial strains will have an advantage, in terms of nutrition, over similar strains that do not possess acylase activity.

Kaufmann (35) has suggested, in view of the widespread nature of bacterial acylases and the fact that many biologically active penicillins inhibit acylase activity at relatively low concentrations, that the membrane-bound acylase may be a target for the antibacterial action of penicillins.

QUANTITATIVE ASPECTS

Acylase appears to be distributed widely among different genera of microorganisms; indeed, it seems that it is almost as widespread as is penicillinase (1). What is not clear at this stage is the extent to which individual strains of the same species produce the enzyme. There have been few quantitative surveys on the incidence of acylase activity in different species. Holt and Stewart (28) claimed that of 436 gram-negative strains studied (most of which were *E. coli*) 154 (35%) produced acylase; however, for reasons discussed below (*see* Assay Methods), there is strong reason to doubt the truth of this finding. Cole and Sutherland (17) also question the validity of Holt and Stewart's findings. They tested three of the strains claimed by the latter to produce acylase, and found only β-lactamase activity; further, they found only 10 acylase-producing strains (4 *E. coli* and 6 *P. rettgeri*) in their investigation of 148 clinically isolated coliform strains. Ayliffe (3) did not find any acylase activity among 109 coliform strains, and J. T. Smith and Hamilton-Miller (*unpublished data*) were unable to detect any acylase in 33 clinically isolated strains of gram-negative bacteria. More large-scale surveys are needed on the incidence of acylase-producing bacteria.

COEXISTENCE OF ACYLASE AND β-LACTAMASE (PENICILLINASE)

Penicillinase and acylase can both be produced by the same microbial strain, e.g., *E. coli* ATCC 9637 (45), a *Nocardia* strain (44), *Streptomyces lavendulae* BRL 198 (7). Cole and Sutherland (17) have reported this phenomenon in coliform organisms, but English et al. (21) were not able to find the two enzymes coexistent in any strain. The consequences of the coexistence of the two enzymatic activities in one strain have to be considered carefully. Benzylpenicillin is hydrolyzed to its penicilloic acid (IX, Fig. 6), whereas the acylase acts upon both the intact penicillin and the penicilloic acid, although the latter is hydrolyzed more slowly than the former (12, 45).

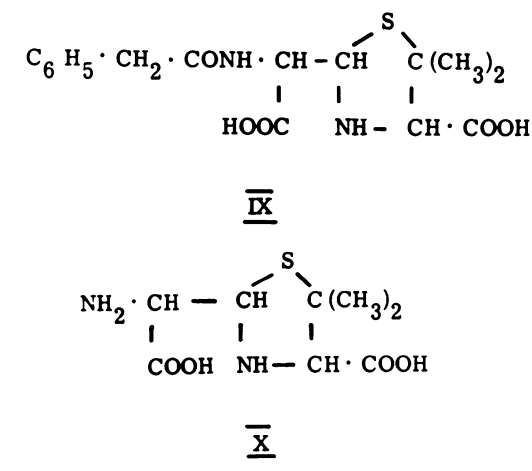


FIG. 6. *Penicilloic acids of benzylpenicillin (IX) and 6APA (X).*

When IX is deacylated, the product is the penicilloic acid of 6APA, the "penicic acid" of Sakaguchi and Murao (48), or, to give it its correct chemical name, D-4-carboxyl-5,5-dimethyl-α-amino-2-thiazolidineacetic acid (X, Fig. 6). Meanwhile, the acylase is hydrolyzing the intact benzylpenicillin to 6APA. The fate of this compound in the presence of a penicillinase depends upon the specificity of the latter enzyme. Should the penicillinase be of the broadly specific type that is typical of *K. aerogenes* and *A. cloacae* (51), 6APA will be hydrolyzed to give X; if the penicillinase is of the type that is found in *E. coli*, *P. morgani*, and *P. rettgeri* (27), the 6APA will remain as such. Thus, short-term incubation of benzylpenicillin with a strain of *E. coli* which produces both acylase and β-lactamase would be expected to result in a mixture of unchanged benzylpenicillin together with benzylpenicilloic acid and 6APA (there may also be a small amount of X, which could arise owing to the deacylation of benzylpenicilloic acid); with a similar strain of *K. aerogenes*, a mixture of benzylpenicillin, benzylpenicilloic acid, 6APA, and X would be found. Clearly, the accurate quantitative estimation of acylase activity in the presence of β-lactamase activity is a task of great magnitude.

ASSAY METHODS

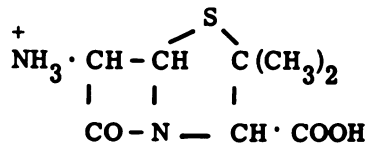
Workers in this field have been plagued by the fact that, until recently, there has been no rapid, easy, and quantitative assay for acylase activity (26). Many methods have been employed; some of the more widely used will be reviewed briefly below, and an attempt will be made to point out the merits and demerits of each.

Biological Methods

Basically, this method consists of noting a loss in biological activity against a suitable indicator strain (which should be a gram-positive strain) when benzylpenicillin has been incubated with the bacterial strain under investigation. Biological activity should be restored when the inactive incubation mixture is treated with phenylacetyl chloride [*see* Batchelor et al. (4) for details of this process]. This method, if properly carried out, is highly specific, but takes some time to perform (particularly if chromatography precedes the biological assay), and must be accompanied (as for all biological assay methods) by careful and complete controls. The most serious disadvantage of this method is that, if the strain being tested has a penicillinase as well as an acylase, and if this penicillinase is capable of hydrolyzing 6APA to its penicilloic acid (X), phenylacetylation of the products of the reaction will yield the inactive benzylpenicilloic acid rather than the biologically active benzylpenicillin. Hence, the test is not suitable for the detection of acylase activity in penicillinase-producing strains of *K. aerogenes*. The method has been widely used (3, 4, 11, 12, 17, 20–22, 23, 31). Holt and Stewart (28, 29) used a simple biological technique to detect acylase activity. It appears likely, from a careful consideration of their techniques, that many of the strains reported by them to produce acylase were penicillinase-producers, because in only three cases was the presence of 6APA in incubation mixtures confirmed by the use of the phenylacetylation method. Furthermore, Holt and Stewart assume that a strain that destroys benzylpenicillin but not 6APA is an acylase-producer; this is precisely the sort of behavior one expects from a penicillinase-producing *E. coli* strain. Cole and Sutherland (17) criticize this simple assay method, and find that it gives misleading results.

Butyl Acetate Extraction Technique

The incubation mixture (bacterial suspension plus benzylpenicillin) is adjusted to *p*H 2 and extracted with butyl acetate. At this *p*H value, benzylpenicillin carries no charge, and is thus extracted into the organic phase. However, 6APA is present as a cation (XI, Fig. 7) and remains in the aqueous phase, in which it is subsequently estimated by the hydroxylamine assay [*see* (7) for details]. This technique has been used by Kaufmann and Bauer (36) as well as by the Beecham team. Again, if a penicillinase capable of hydrolyzing 6APA is present, the presence of an acylase will go undetected, because, although compound X remains in the aqueous phase, it does not react with hydroxylamine as it does not have an intact β-lactam ring.



XI

FIG. 7. *Structure of 6APA when it is present as a cation during butyl acetate extraction.*

Use of Radioactive Substrate

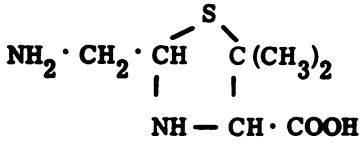
Pruess and Johnston (45) used benzylpenicillin labeled with S^{35} as substrate for the acylase. The reaction mixture was chromatographed, and areas of radioactivity corresponding to the various breakdown products of enzymatic action were detected and identified. Benzylpenicillin was shown to be converted to 6APA, and benzylpenicilloic acid was hydrolyzed to X, which was then degraded to its "penillic aid," D-4-carboxy-5,5-dimethyl-2-aminomethylthiazolidine (XII, Fig. 8). This is an excellent method, as it caters for all combinations of penicillin-destroying enzymes, and positively identifies the breakdown products. However, it requires a rather complex substrate and a chromatographic procedure and, hence, would not be suitable for all purposes.

Detection of X After Treating Reaction Mixture with Penicillinase

This is the method used by Sakaguchi and Murao (48). X, being an α-amino acid, is strongly ninhydrin-positive, whereas 6APA is only weakly ninhydrin-positive, giving a yellow coloration. Hence, treating 6APA with a suitable penicillinase preparation greatly increases the ninhydrin positivity. Huang et al. (30) also used this method. X was separated from the rest of the reaction mixture by means of paper chromatography, ninhydrin was added, and the intensity of the color was read by use of a spectrophotometer. This is a useful method, but care must be taken, particularly if it is being used quantitatively, that the incubation with the penicillinase is sufficient for complete hydrolysis of the 6APA to occur.

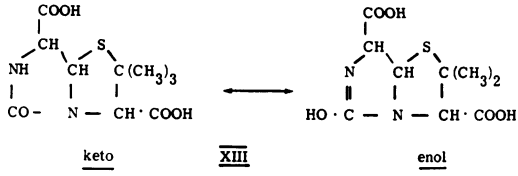
Liberation of Ammonia or Amino Groups from Substrates Other than Benzylpenicillin

Cole (13) used an ingenious assay method, for which various phenylacetylated amino compounds were used as substrates. Acylase activity was determined by a colorimetric assay of the



XII

FIG. 8. *D-4-Carboxy-5,5-dimethyl-2-aminomethylthiazolidine.*



XIII

FIG. 9. *8-Hydroxypenillic acid (XIII).*

liberated amino group (ninhydrin assay). When benzylpenicillin was used as substrate, the liberated phenylacetic acid was determined titrimetrically. This assay seems to be the one best suited for plotting progress curves and for kinetic studies on acylases.

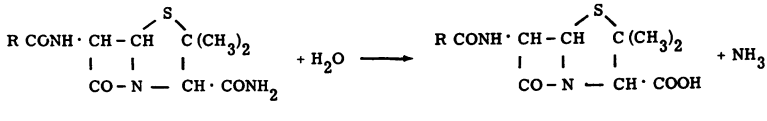
Direct Estimation of 6APA

Bomstein and Evans (9) devised a specific assay for 6APA, which depends on the reaction of the 6-amino group with *p*-dimethylaminobenzaldehyde to form a colored Schiff base, which is estimated colorimetrically. The chromogen is stable for a very short time, and the assay method is considered to be suitable only for use with automatic equipment. It would appear that this method could be very useful for the assay of acylase, especially since intact penicillins do not react to form a chromogen (it is not stated whether penicillins which contain a free amino group, such as ampicillin and penicillin N, or penicillinamides, will form a Schiff base). The assay could be applied directly to the reaction mixture without an extraction step being necessary. The penicilloic acid of 6APA (X) also forms a chromogen, a fact which, although regarded as a disadvantage by the original authors, tends to make this assay method potentially more useful as regards measuring acylase activity, because it could be used in the presence of a β-lactamase. It is therefore hoped that a modification of this method can be devised that will enable its use by laboratories not in possession of automatic equipment.

Some methods that have been published do not appear to be very satisfactory, e.g., the manometric assay used by Vinze, Borkar, and Sen (57). It is very difficult to correlate their findings (that the presence of 6APA during the manometric assay does not cause retention of CO_2) with the work of Batchelor, Gazzard, and Nayler (8) and of Johnson and Hardcastle (32), which showed clearly that CO_2 reacts with 6APA to form 8-hydroxypenillic acid (XIII, Fig. 9), indicating that CO_2 retention must occur. Similarly, some of the results of Brandl (10), who also used a manometric method, must be regarded with caution. Other methods, such as those described by Szentirmai (53) and Nyiri (44), in which a mixture of 6APA and benzylpenicillin is assayed by a hydroxamic method and an iodometric method, cannot be repeated on the basis of the descriptions given by the authors. It is presumed that an extraction step must be included in these assays to differentiate between benzylpenicillin and 6APA.

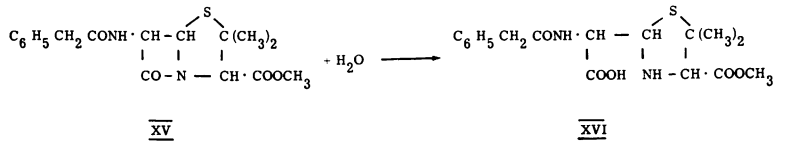
PENICILLINAMIDASE

In view of the fact that the enzyme (called acylase in this review) that hydrolyzes penicillins to 6APA has also been called penicillin-amidase, in particular by the Beecham team (5–7, 46), it seems worthwhile to point out that a true "penicillinamidase" does exist. As its name implies, this enzyme is specific for penicillinamides (XIV), which are hydrolyzed to the free penicillin as shown in equation 3 (Fig. 10). Such penicillinamidases have been found in *Rhodotorula gracilis* and *Torulopsis* spp. (31), and will hydrolyze the amides of both benzyl- and phenoxymethyl penicillins. It should be noted that type II acylases would convert penicillinamides such as XIV into the amide of 6APA.



XIV

FIG. 10. *Equation 3. Hydrolysis of penicillinamides (XIV) to the free penicillin.*



XV XVI

FIG. 11. *Equation 4. Hydrolysis of the methyl ester of benzylpenicillin (XV) to the β-methyl ester of benzylpenicilloic acid (XVI).*

Penicillinamidase thus belongs to the small group of enzymes that are specific for penicillins substituted in the 3-carboxyl position. This group includes the "penicillinesterase" found in the blood of the guinea pig, rat, and mouse (54), which hydrolyzes certain esters of benzylpenicillin (40) to free benzylpenicillin, and the ester-specific penicillin β-lactamase present in liver homogenates from the guinea pig, mouse, and rat (52), which hydrolyzes the methyl ester of benzylpenicillin (XV) to the β-methyl ester of benzylpenicilloic acid (XVI), as shown in equation 4 (Fig. 11).

RETROSPECT AND PROSPECT

The discovery of penicillinacylase opened the door to the production of the semisynthetic penicillins. Rarely is a single enzyme responsible for so great a revolution in therapeutic practice. It would probably be no exaggeration to say that the introduction of methicillin, the first commercially available semisynthetic penicillin to be practically unaffected by staphylococcal penicillinase, changed the status of the benzylpenicillin-resistant hospital staphylococcus overnight from "public enemy number one" to a mere nuisance. The drug companies that invested large sums of money in basic research into the penicillin molecule have been amply and deservedly rewarded for their enterprise. Thus from the point of view of both the medical practitioner and the pharmaceutical industry, the story of penicillinacylase may be considered to have come to a happy ending. From the academic viewpoint, however, there remain several fascinating problems concerning the enzyme still awaiting clarification. What is the physiological role of acylase? It seems from the present state of knowledge that, in penicillin-producing molds, acylase has at least an indirect part to play in the biosynthesis of penicillins. The full answer to the problem, however, must await a fuller understanding of the biosynthetic pathways involved. From the information we have, it is difficult to believe that acylase makes an important contribution to the day-to-day life of bacteria. We have seen that it plays no real part in the mechanisms of resistance to penicillins. It seems to confer a slight nutritional advantage in the presence of some phenylacetylated substrates, but the distribution of the latter in nature cannot be wide enough for this to make more than a marginal contribution to the bacterial economy. One is left with the theory of Kaufmann (35), that membrane-bound acylase is the site of action of penicillins in gram-negative bacteria, which is an interesting hypothesis deserving further thought. It is conceivable that acylase could act as a "detoxification" mechanism, analogous to those occurring in more highly developed forms of life. Or again, following the suggestion of Abraham (1) regarding microbial penicillinase, it may be that acylase activity is an incidental property of some protein molecule which has a completely separate main function (which could be structural).

There have been few reports of attempts to purify acylase (7, 43, 53a, 58), which could be due to the fact that most acylase-producing organisms do not seem to make large amounts of the enzyme. However, it is likely that the drug companies which manufacture semisynthetic penicillins have obtained high-yielding constitutive mutants, which would be a suitable starting point for a purification procedure. A detailed study of acylases from different sources would be a rewarding project; not only would it throw light upon differences between type I and type II acylases, but it would also show whether the bacterial acylases had a common structure. This fact could in turn answer the question whether acylases form part of the bacterial membrane (35). Another question that remains to be settled is whether it is possible, as suggested by Cole (14), for one microbial strain to possess more than one type of acylase.

No acylase has yet been reported to be capable of removing the side chain from the naturally occurring compound cephalosporin C [7-(5-amino - 5 - carboxyvaleramido)cephalosporanic acid]. The discovery of such an enzyme would greatly facilitate the production of the semisynthetic cephalosporins, and it is therefore hoped that such an acylase will be found in the near future.

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