Deacylation of Acylamino Compounds other than Penicillins by the Cell-Bound Penicillin Acylase of *Escherichia coli*

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1. The action of the penicillin acylase enzyme of *Escherichia coli* N.C.I.B. 8743 on non-penicillin substrates suggests that the enzyme is an amidohydrolase. 2. The rates of hydrolysis for a small group of penicillins closely parallel those for a corresponding series of *N*-acylglycines. 3. For a series of *E. coli* strains, ability to cause rapid hydrolysis of phenylacetyl-glycine is correlated with ability to hydrolyse benzylpenicillin. 4. Amides and *N*-acylglycines are hydrolysed to the corresponding acids. The phenylacetyl group is hydrolysed most readily. Benzamide and β -phenylpropionamide are not substrates. In a series of aliphatic acylglycines only valeryl- and hexanoyl-glycine are substrates. 5. Acylated L- but not D- α -amino acids are hydrolysed. D- α -Hydroxyphenylacetamide is a better substrate than the L compound.

In the preceding paper (Cole, 1969) the deacylation of penicillins, penicillin derivatives and cephalosporins by *Escherichia coli* penicillin acylase was described. This same enzyme preparation was observed to catalyse the hydrolysis of many other acyl compounds. These reactions are described in the present paper along with evidence of substrate specificity that strongly suggests that only one acylase enzyme, or a group of very closely related enzymes, is responsible for all of these reactions. Deacylation of non-penicillin acylamino acids by the penicillin acylase was first reported by Kaufmann & Bauer (1964) and Cole (1964). These observations are further supported by the work of Lucente, Romeo & Rossi (1965) and Pruess & Johnson (1965).

MATERIALS AND METHODS

Enzyme substrates and analytical standards. The penicillin substrates, analytical standards and phosphate buffers were described in the preceding paper (Cole, 1969).

The following acylamino acids were prepared by reacting the respective commercially available acid chloride or anhydride with ammonia, glycine or other required amino acid; where necessary the acid chlorides were prepared from commercially available acids by reaction with thionyl chloride: *N*-phenylacetyl-glycine, m.p. 143–144°; *N*-phenoxycetyl-glycine, m.p. 119–120°; *N*- α -phenoxypropionyl-glycine, m.p. 105–106°; *N*-valeryl-glycine, m.p. 81–82°; *N*-hexanoyl-glycine, m.p. 92–92.5°; *N*-heptanoyl-glycine, m.p. 95.5–96°; *N*-octanoyl-glycine, m.p. 106–106.5°; *N*-phenylacetyl-D- α -aminophenylacetic acid, m.p. 134–134.5°; *N*-phenylacetyl-L- α -aminophenylacetic acid, m.p. 128–130°; *N*-phenylacetyl-DL- α -aminophenylacetic acid, m.p. 90–91°; *N*-phenylacetyl- ϵ -aminocaproic acid, m.p.

77°; *N*-propionyl-glycine, crude preparation; *N*-butyryl-glycine, crude preparation; *N*-phenylacetyl-*p*-aminobenzoic acid, m.p. >236°; valeramide, m.p. 102–103°; hexamide, m.p. 98–99°; heptamide, m.p. 93–94°; 3-phenylpropionamide, m.p. 99–100°.

N-(D- α -Aminophenylacetyl)-glycine, *N*-(DL- α -hydroxyphenylacetyl)-glycine, D-, L- and DL-mandelamide, phenylmalonamide and D-, L-, and DL- α -aminophenylacetamide were produced by the Chemistry Department, Beecham Research Laboratories, along with the compounds listed in Table 4. Acetyl-glycine, benzoyl-glycine and acetamide were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks. and benzamide and phenylacetamide from British Drug Houses Ltd., Poole, Dorset.

Preparation of penicillin acylase and assay of rates of hydrolysis of penicillins. The cell-bound penicillin acylase of *E. coli* was prepared exactly as described in the preceding paper (Cole, 1969), which also gave details of the measurement of the rates of penicillin hydrolysis based on liberation of 6-APA*.

Quantitative determination of ammonia and amino acids. The ammonia or amino acid liberated when *E. coli* cells were incubated with amides or *N*-acyl derivatives of amino acids was measured by the ninhydrin (indanetrione hydrate; British Drug Houses) method of Moore & Stein (1948). Standard curves were prepared in the range 0–2 mg./ml. with NH₄NO₃ or the appropriate amino acid. Cells were removed by centrifugation before assay. Controls consisted of *E. coli* cells incubated in 0.05 M-phosphate buffer, pH 8, and blanks for the ninhydrin assay consisted of the test substrates incubated in buffer without enzyme. Colorimetry was performed with an EEL colorimeter (Evans Electro-selenium Ltd., Halstead, Essex) with a green OGRI filter (approx. 530 nm.).

Amino acids on chromatograms. Paper chromatography

* Abbreviation: 6-APA, 6-aminopenicillanic acid.

was performed as described by Cole (1969). Amino acids were detected on paper chromatograms by dipping in ninhydrin reagent (0.2% ninhydrin in butan-1-ol or 0.2% ninhydrin in acetone containing 1% pyridine).

Assay of p-aminobenzoic acid. To 1.0 ml. of cell-free sample, suitably diluted, 0.5 ml. of 1M-HCl and 0.5 ml. of 1% NaNO₂ was added. The mixture was kept in an ice-bath for 3 min. before addition of 1 ml. of 1% β -naphthol, previously made up in 1M-NaOH. After thorough shaking 13 ml. of water was added and the colour intensity measured

in an EEL colorimeter with a blue-green no. 623 filter (495nm.). Controls and blanks were as used in the ninhydrin assay. Yields were determined by reference to a standard curve (0–100 μ g./ml.) prepared with *p*-aminobenzoic acid.

RESULTS

Hydrolysis of penicillins and related acylamino acids. It has been shown (Cole, 1969) that cells of *E. coli* N.C.I.B. 8743 can remove the acyl group from penicillins, penicillin derivatives and even cephalosporins. Results are presented below that show that the cells of this bacterium also rapidly remove the acyl group from *N*-acyl derivatives of glycine. The results in Table 1 suggest, on the basis of substrate specificity and relative hydrolysis rates, that one and the same enzyme catalyses the deacylation of *N*-acyl-6-APA (penicillins) and *N*-acylglycines when the acyl side chains are common. This is further supported by the results of an examination of the deacylation activities of other strains of *E. coli*, as shown in Table 2.

The rates of hydrolysis of *N*-phenylacetyl derivatives of various amino acids compared with phenylacetyl-6-APA (benzylpenicillin) have been published (Cole, 1964) for *E. coli* N.C.I.B. 8743 (B.R.L. 1040). These results demonstrated that the nature of the amino acid that was acylated does have a profound effect on the rate of reaction, phenylacetyl-DL-valine being hydrolysed at one-tenth of the rate for phenylacetyl-glycine, which in turn was hydrolysed at nearly twice the rate of phenylacetyl-6-APA.

Hydrolysis of N-acyl derivatives of glycine. The effect of keeping the amino acid constant and varying the nature of the *N*-acyl group is shown in Table 3 for a series of *N*-acyl derivatives of glycine. The best substrate was phenylacetyl-glycine.

Table 1. *Relative rates of hydrolysis of certain N-acyl derivatives of glycine and 6-APA by E. coli N.C.I.B. 8743*

Reaction mixtures were shaken at 30° and consisted of 2mg. of substrate/ml. in 0.05M-phosphate buffer, pH 8.0, and *E. coli* N.C.I.B. 8743 cells resuspended at the same cell density as that in the growth medium. Controls consisted of cells in the same phosphate buffer. At $\frac{1}{2}$ hr. and 3 hr. glycine was determined by the ninhydrin method and 6-APA by the hydroxylamine method after solvent extraction of residual substrate.

Substrate	Percentage hydrolysis		Rate relative to corresponding phenylacetyl compound	
	over $\frac{1}{2}$ hr.	over 3 hr.	over $\frac{1}{2}$ hr.	over 3 hr.
Phenylpenicillin	0	0	0	0
Benzylpenicillin	46.6	68.3	100	100
Phenoxyethylpenicillin	1.9	15.0	4.1	22
α -Phenoxyethylpenicillin	0	0	0	0
Benzoylglycine	0	0	0	0
Phenylacetyl-glycine	79.0	95.5	100	100
Phenoxyacetyl-glycine	3.9	24.8	4.9	26
α -Phenoxypropionyl-glycine	0	0	0	0

Table 2. *Rates of hydrolysis of phenylacetyl-glycine and benzylpenicillin for various strains of E. coli*

The composition of the reaction mixtures and the analytical methods were as described in Table 1. Cultures (100 ml.) were shaken in conical flasks (500 ml.) on a rotary shaker (240 rev./min., radius 1 in.) at 24° for 18 hr.

Strain of <i>E. coli</i> (B.R.L. culture no.)	Cultures grown in 2% (w/v) 'Yeast' yeast extract + 0.05% sodium phenylacetate, all at pH 7.0		Cultures grown in 1.5% (w/v) corn-steep liquor + 0.05% sodium phenylacetate, all at pH 7.0	
	Hydrolysis of phenylacetyl-glycine (% conversion over $\frac{1}{2}$ hr.)	Hydrolysis of benzylpenicillin (% conversion over 1 hr.)	Hydrolysis of phenylacetyl-glycine (% conversion over $\frac{1}{2}$ hr.)	Hydrolysis of benzylpenicillin (% conversion over 1 hr.)
1009	73.5	41.0	66.0	47.0
1360	80.5	50.0	63.0	34.0
1162	68.0	54.0	26.5	10.0
1029	5.0	0	6.0	0
1262	4.5	0	1.0	0
1016	5.5	0	1.0	0
1158	5.0	0	1.0	0

Table 3. *Rate of hydrolysis of various N-acyl derivatives of glycine by E. coli N.C.I.B. 8743*

The reaction mixtures consisted of a final concentration of 2 mg. of substrate/ml., except for acetylglycine (1.5 mg./ml.), in 0.05M-phosphate buffer, pH 8.0, plus *E. coli* cells resuspended to give a cell density equal to that in the cultivation medium. The mixtures were shaken at 35° and assayed for glycine content by the ninhydrin method at $\frac{1}{2}$ hr. and 3 hr. A control reaction mixture consisted of cells in phosphate buffer. Blanks were obtained by incubating substrate in the absence of enzyme.

N-acyl derivatives of glycine	% hydrolysis at	
	$\frac{1}{2}$ hr.	3 hr.
Acetyl	0	0
Propionyl	0	0
Butyryl	0	0
Valeryl	6	33
Hexanoyl	7	36
Heptanoyl	1	9
Octanoyl	0	1
Benzoyl	0	0
Phenylacetyl*	79	95
D- α -aminophenylacetyl*	31	85
DL- α -hydroxyphenylacetyl*	43	63
Phenoxyacetyl	4	25
α -Phenoxypropionyl	0	0

* In a duplicate experiment with these substrates at 10 mg./ml., glycine was detected by paper chromatography. No glycine was observed when acetylglycine and benzoyl-glycine were used.

Table 4. *Derivatives of glycine that were not substrates for the acylase of E. coli N.C.I.B. 8743*

The composition of the reaction mixtures and the method of analysis was the same as that described in Table 3.

Table 5. *Rate of hydrolysis of various amides by E. coli N.C.I.B. 8743*

The reaction mixtures consisted of final concentrations of 2 or 4 mg. of substrate/ml. in 0.05M-phosphate buffer, pH 8.0, plus *E. coli* N.C.I.B. 8743 cells resuspended to give a density equal to that in the cultivation medium. The mixtures were shaken at 35° for 4 hr. before removal of the cells by centrifugation and assay of the liberated ammonia by the ninhydrin method.

Substrate	% hydrolysis after 4 hr.	
	Substrate concn. 2 mg./ml.	Substrate concn. 4 mg./ml.
Acetamide	0	—
Benzamide*	0	—
Phenylacetamide*	100	84
Phenylpropionamide*	0	—
Phenylmalonamide	14	—
L-Mandelamide	28	25.5
D-Mandelamide	100	52.5
DL-Mandelamide	64	48
Valeramide	—	1.5
Hexamide	—	8
Heptamide	—	0

* These three amides at 10 mg./ml. were incubated at 40° and pH 5.0 with *E. coli* cells (N.C.I.B. 8743) at ten times the cell density in the cultivation medium and examined by paper chromatography (Whatman no. 1) with Bromocresol Green spray for carboxylic acids. The only amide to show hydrolysis to the corresponding acid was phenylacetamide, a zone of phenylacetic acid being detected at R_F 0.65 (butan-1-ol-pyridine-water, 1:1:1, by vol.)

α -Substituted forms of this compound were also good substrates. For aromatic compounds any deviation from the phenylacetyl structure is associated with marked loss of suitability as a substrate. In the aliphatic series of *N*-acylglycines, an acyl group containing five or six linear carbon atoms was optimum for enzymic hydrolysis; this represents the length most closely approximating to that of the phenylacetyl group. The compounds listed in Table 4 were not substrates, a fact consistent with the theory that the enzyme of *E. coli* only hydrolyses a $\text{---CH}_2\cdot\text{CO}\cdot\text{NH}\text{---}$ linkage.

Hydrolysis of amides. The cells of *E. coli* N.C.I.B. 8743 produce an amidase enzyme capable of hydrolysing simple amides. The substrate spectrum is shown in Table 5. As in the series of acylglycine compounds, it is compounds containing the phenylacetyl group, or substitutions of it, that are the best substrates. For aromatic amides, the amidase shows very high specificity. Removing the α -methylene group of phenylacetamide or inserting a β -methylene group abolishes activity. For aliphatic amides a six-carbon chain is optimum, but this does not give such a good substrate as phenylacetamide.

Table 6. *Other phenylacetyl compounds hydrolysed by E. coli N.C.I.B. 8743*

The reaction mixtures consisted of final concentrations of 2 mg. of substrate/ml. in 0.05M-phosphate buffer, pH 8.0, plus *E. coli* cells resuspended at the same cell density as that in the cultivation medium. The reaction mixtures were shaken at 35° and assayed for ϵ -aminocaproic acid content by the ninhydrin method or for *p*-aminobenzoic acid content by diazotizing and coupling to β -naphthol.

Substrate	% hydrolysis at		
	$\frac{1}{2}$ hr.	2 hr.	4 hr.
<i>N</i> -Phenylacetyl- <i>p</i> -aminobenzoic acid	21	53	65
<i>N</i> -Phenylacetyl- ϵ -aminocaproic acid	14	51	81

The close parallel between the substrate spectrum for amides, *N*-acylglycines and *N*-acyl-6-APA compounds (penicillins) suggests that the amidase and amidohydrolase activities are properties of one enzyme. The effect of asymmetry at the α -carbon atom is illustrated by using the isomers of α -hydroxy-phenylacetamide (mandelamide) as substrates. The *D*-configuration is clearly more suitable as a substrate, which correlates with the finding that α -hydroxybenzylpenicillin with the side chain in the *D*-configuration is a better substrate than that with the *L*-configuration (Cole, 1969) although the results become complicated with long reaction times.

Hydrolysis of other *N*-acyl compounds. The *E. coli* acylase system can also remove the phenylacetyl group from aromatic and aliphatic amino acids that have amino groups distant from the carboxyl group, as shown in Table 6.

The *E. coli* acylase can also hydrolyse esters of phenylacetic acid. This was readily demonstrated by incubating 1% *p*-aminophenylacetic acid methyl ester with the cells of *E. coli* N.C.I.B. 8743 at pH 5 and 40°. The reaction mixture was examined by paper chromatography (butan-1-ol-acetic acid-water, 12:3:5, by vol.) zones being detected by diazotization (spray with 0.2% potassium nitrite in cold 0.1M-hydrochloric acid) and coupling with α -naphthylamine (spray with 0.2% α -naphthylamine in 50% ethanol). This chromatogram revealed a rapid conversion of the ester (R_F 0.84) into *p*-aminophenylacetic acid (R_F 0.61). *p*-Aminophenylacetamide (R_F 0.45) was also hydrolysed, but more slowly. The hydrolysis of the methyl ester of *D*- α -aminophenylacetic acid was also demonstrated by paper-chromatographic analysis (by detection with ninhydrin) of a reaction mixture containing this compound and the *E. coli* cells, but the rate of hydrolysis was slower than for the *p*-aminophenylacetic acid methyl ester.

Stereospecificity of the *E. coli* acylase. The results

Table 7. *Stereochemical specificity of hydrolysis of N-phenylacetyl derivatives of α -aminophenylacetic acid by E. coli N.C.I.B. 8743*

The reaction mixtures consisted of a final concentration of 2 mg. of substrate/ml. in 0.05M-phosphate buffer, pH 8.0, plus *E. coli* cells resuspended at a cell density equal to that in the cultivation medium. The reaction mixtures were shaken at 30° and samples assayed for their α -aminophenylacetic acid content by the ninhydrin method.

Substrate	% conversion at		
	$\frac{1}{2}$ hr.	1 hr.	2 hr.
<i>N</i> -Phenylacetyl-DL- α -aminophenyl-acetic acid	37.5	41	45.5
<i>N</i> -Phenylacetyl-L- α -aminophenyl-acetic acid ('L')	90	93	100
<i>N</i> -Phenylacetyl-D- α -aminophenyl-acetic acid ('D')	0	0	0
Mixtures:			
25% 'L', 75% 'D'	17	23	25
50% 'L', 50% 'D'	42	44.5	51
75% 'L', 25% 'D'	68	73	77.5

given in Table 3 of the preceding paper (Cole, 1969) and in Table 5 of the present paper show that the stereo configuration at the α -carbon atom in the acyl group does influence the rate of reaction to some extent. However, there is an absolute requirement for the α -amino acid that is acylated to be in the *L*-configuration, as is shown by the results for the deacylation of the phenylacetyl derivatives of *D*- and *L*- α -aminophenylacetic acid (Table 7). The *N*-phenylacetyl derivative of DL-leucine was found to be hydrolysed only to the extent of 50%. *N*-phenylacetyl-glycine, having no asymmetric centre, was 100% hydrolysed.

DISCUSSION

It appears, from results presented in the present and preceding papers (Cole, 1969), that the enzyme in *E. coli* cells that hydrolyses penicillin will also hydrolyse the *N*-acylglycines with the corresponding acyl side chains. Of the acylamino acids examined in the present paper, the phenylacetyl group is most easily hydrolysed. In the aliphatic range only valeryl and hexanoyl groups are removed, these being close in length to the phenylacetyl group. The substrate specificity for the hydrolysis of a series of amides by *E. coli* also closely parallels that for *N*-acylglycines and penicillins even down to the effects of asymmetry at the α -carbon atom in the acyl group, the *D*-configuration always being hydrolysed more readily.

The *E. coli* enzyme appears to be primarily

specific for the acyl structure but the nature of the amino compound that is acylated is also of importance, particularly its stereochemical configuration. The effect of varying the amino group in a series of phenylacetyl amino compounds has been described by Cole (1964), and is further exemplified in the present paper. The *E. coli* acylase system will also hydrolyse esters such as *p*-aminophenylacetic acid methyl ester. The hydrolysis of non-penicillin compounds by *E. coli* N.C.I.B. 8666 (A.T.C.C. 9637) has also been demonstrated by Kaufmann & Bauer (1964), Lucente *et al.* (1965) and Pruess & Johnson (1965). Kaufmann & Bauer (1964) also reported hydrolysis of esters and thioesters.

Stereospecificity appears to be virtually absolute for deacylation of α -amino acids; the latter must be in the L-configuration for hydrolysis to occur. In this respect the enzyme resembles pig kidney acylase (Birnbaum, 1952) and other microbial acylases (Patent, 1965). *N*-acylamino acids with no asymmetry are completely hydrolysed whereas *N*-acyl derivatives of DL-amino acids are only 50% hydrolysed, thus providing a method for resolving the isomers of amino acids. It is fortunate for the antibiotics industry that benzylpenicillin is an acylated L-amino acid and is therefore completely hydrolysed to 6-APA. Asymmetry in the acyl group has only a slight effect, compounds with the α -carbon atom in the D-configuration being slightly more readily deacylated than those with the L-configuration.

The theory that one and the same enzyme is responsible for the hydrolysis of penicillins and non-penicillin *N*-acylamino acids is further supported by the findings of Szentirmai (1966), who reported that his 40-fold purified penicillin acylase of *E. coli* hydrolysed *N*-phenylacetyl amino acids and did so stereospecifically. This enzyme preparation also both synthesized and hydrolysed penicillins. Preliminary results in the author's laboratory with a small amount of benzylpenicillin acylase isolated from *E. coli* N.C.I.B. 8743A (100-fold purification) have also shown that the

penicillin acylase activity is still associated with high phenylacetyl glycine-hydrolysing activity.

Nyiri (1967) has shown that throughout the cultivation of *E. coli*, benzylpenicillin acylase activity parallels deacylase activity against phenylacetyl glycine and Chiang & Bennett (1967) have shown that their purified enzyme from *Bacillus megaterium* hydrolysed both benzylpenicillin and phenylacetamide, although the latter was hydrolysed at one-quarter of the rate. Walton (1964) has used adipamide as a substrate in a soil-enrichment system for isolating bacteria that would hydrolyse *N*-adipoyl-*p*-nitroaniline and would liberate 7-aminocephalosporanic acid from cephalosporin C. Walton also used the liberation of yellow *p*-nitroaniline from colourless *N*-phenylacetyl-*p*-nitroaniline as a screening method and assay system for organisms that produced benzylpenicillin and benzylecephalosporin acylase (i.e. phenylacetylase).

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