Penicillins and other Acylamino Compounds Synthesized by the Cell-Bound Penicillin Acylase of *Escherichia coli*

By M. COLE

Beecham Research Laboratories Brockham Park, Betchworth, Surrey

(Received 20 May 1969)

1. The penicillin acylase of *Escherichia coli* N.C.I.B. 8743 is a reversible enzyme. Reaction rates for the two directions have been determined. 2. Measurements of the rates of enzymic synthesis of penicillins from 6-aminopenicillanic acid and various carboxylic acids revealed that *p*-hydroxyphenylacetic acid was the best substrate, followed by phenylacetic, 2-thienylacetic, substituted phenylacetic, 3-hexenoic and *n*-hexanoic acids. 3. The rate of synthesis of penicillin improved when amides or *N*-acylglycines were used; α -aminobenzylpenicillin and phenoxymethylpenicillin were only synthesized when using these more energy-rich compounds. 4. Phenylacetyl-glycine was the best substrate for the synthesis of benzylpenicillin compared with other derivatives of phenylacetic acid. 5. The enzyme was specific for acyl-L-amino acids, benzylpenicillin being synthesized from phenylacetyl-L- α -aminophenylacetic acid but not from phenylacetyl-D- α -aminophenylacetic acid. 6. α -Phenoxyethylpenicillin was synthesized from 6-aminopenicillanic acid and α -phenoxypropionylthioglycollic acid non-enzymically, but the rate was faster in the presence of the enzyme. 7. The *E. coli* acylase catalysed the acylation of hydroxylamine by acids or amides to give hydroxamic acids, the phenylacetyl group being the most suitable acyl group. The enzyme also catalysed other acyl-group transfers.

The hydrolytic properties of the penicillin acylase enzyme of *E. coli* N.C.I.B. 8743 have been described in the preceding papers (Cole, 1969*a,b*). This reaction is readily reversible, with the result that acylamino acids such as benzylpenicillin are synthesized in reaction mixtures containing amino compound, carboxylic acid and enzyme. The enzymic synthesis of penicillin, which was first described by Kaufmann & Bauer (1960) and Rolinson *et al.* (1960), lends itself to the study of substrate specificity because of the availability of a wide range of carboxylic acids and the specificity of assay and chromatographic procedures for penicillins. The present paper describes this substrate specificity for synthetic reactions and shows that there is a close parallel with the substrate specificity for hydrolytic reactions and that the synthetic reactions are not confined to the synthesis of penicillins. The enzyme of *E. coli* is best viewed as an acylase that can catalyse the transfer of an acyl group to a suitable hydroxyl or amino group.

MATERIALS AND METHODS

Enzyme. The enzyme used throughout the present paper was the cell-bound penicillin acylase of *E. coli* N.C.I.B. 8743 or N.C.I.B. 8743A. The cultures were selected and grown

for enzyme production as previously described (Cole, 1969*a*). The enzyme was stored at 5° in the form of a dense suspension of *E. coli* cells plus 1% *n*-butyl acetate as preservative until required for experiments.

Substrates and reagents. Benzylpenicillin, buffers and 6-APA* were as previously described (Cole, 1969*a*). Hydroxylamine hydrochloride was the laboratory grade reagent from BDH (Chemicals) Ltd., Poole, Dorset. The following compounds were supplied by the Chemistry Department of Beecham Research Laboratories: butylthioacetic acid, *N*-phenylglycine, phenylmercaptoacetic acid, α -methylphenoxyacetic acid, 2-thienylacetic acid, 1- and 2-naphthylacetic acids, 2,6-dimethoxybenzoic acid, 2,6-dimethoxybenzoylglycine, α -phenoxypropionylglycine, DL- α -phenylphenoxyacetyl-glycine, α -phenoxypropionylthioglycollic acid, 2,6-dimethoxybenzoylthioglycollic acid, DL- α -phenylphenoxyacetamide, DL- α -phenoxypropionamide, 2,6-dimethoxybenzamide, D-, L- and DL-mandelamides, D- α -aminophenylacetamide hydrobromide and the following substituted phenylacetic acids: α -oxo-, DL- α -ethyl-, DL- α -methoxy-, DL- α -carboxy-, D- and L- α -amino-, *m*-amino-, *p*-methoxy-, 3,4-dichloro- and *p*-amino-phenylacetic acids, its amide and ethyl ester. Compounds in Tables 4, 5 and 6 were synthesized from the respective amino acid or ammonia and acid chloride or anhydride (Cole, 1969*b*). *N*-Phenylacetyl-DL-valine, m.p. 113.5°, and *N*-phenylacetyl-DL-leucine, m.p. 110°, were also made this way. All other

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

carboxylic acids or derivatives were obtained from commercial sources.

Determination of the rate of enzymic synthesis of various penicillins. The rate of formation of benzylpenicillin, α -hydroxybenzylpenicillin, *o*-, *m*- and *p*-hydroxybenzylpenicillin and the penicillins in Table 2, 5 and 6 were determined by the hydroxylamine assay after separation of the resultant penicillin from unconverted 6-APA by solvent extraction. At suitable times a 5 ml. sample of each reaction mixture was centrifuged to stop the reaction and remove the enzyme-containing cells, and 4 ml of the supernatant was diluted to 10 ml. with water. This solution was then adjusted to pH 2.0 with HCl and extracted twice with 10 ml. of cold *n*-butyl acetate. The extracts were shaken with 10 ml. of 0.5% NaHCO₃ and the bicarbonate solution was assayed for penicillin content by the hydroxylamine assay, as described by Batchelor, Chain, Hardy, Mansford & Rolinson (1961). Pure potassium or sodium benzylpenicillin standards were assayed alongside the bicarbonate extracts. Results had to be expressed in terms of benzylpenicillin because pure preparations of the many penicillins synthesized were not available.

Experience gained from assaying large numbers of semi-synthetic penicillins has shown that in most cases equimolar concentrations of different penicillins give similar colorimeter readings in the hydroxylamine assay. A notable exception is α -aminobenzylpenicillin (ampicillin) which has a higher molar extinction than benzylpenicillin. However, as this penicillin and *p*-aminobenzylpenicillin are not readily extractable into the solvent at low pH values, they could not be separated from 6-APA and were therefore not assayed by the above hydroxylamine procedure. 3,4-Dihydroxybenzylpenicillin could not be assayed because of an interaction with the ferric reagent in the hydroxylamine assay method. Evidence of synthesis of these compounds was based on paper chromatography. The solvent-extraction efficiencies for the different penicillins listed in Tables 2, 5 and 6 varies somewhat from compound to compound but are believed to be in excess of 80% for a double extraction. Most of the penicillins synthesized in the substrate-spectrum experiments were acid-labile, but the loss occurring during the solvent-extraction procedure at pH 2 was minimized by lowering the pH to 2.0 while rapidly stirring in the presence of cold solvent, in which penicillins are much more stable. The above limitations mean that the yields of penicillins in Tables 2, 5 and 6 provide only an approximate quantitative guide, but the substrate-specificity spectrum is probably correct and comparisons are much more satisfactory than those based on the sizes of the zones on the paper chromatograms, size being a function of activity as well as of quantity.

Paper chromatography of penicillins. The penicillins present in various reaction mixtures were separated by descending chromatography at 5° overnight, on 1 cm.-wide strips of Whatman no. 1 paper. Penicillin zones were located by contact with agar seeded with spores of *Bacillus subtilis* A.T.C.C. 6633 (bio-autography) and incubation overnight at 30°. The water-saturated ether solvent system was that described by Karnovsky & Johnson (1949). Where authentic preparations of penicillins were available these were used to prepare marker chromatograms. 6-APA was detected by phenylacetylation of one of a pair of chromatograms before bio-autography.

Detection of *p*-aminophenylacetic acid and its derivatives on paper chromatograms. Paper chromatograms were

sprayed with freshly prepared 0.2% KNO₂ in cold 1 M-HCl, heated to 50° and then sprayed with 0.2% β -naphthol in 1 M-NaOH or 0.2% α -naphthylamine in 50% ethanol.

Detection of amino acids on paper chromatograms. Chromatograms were dipped in a solution of 0.2% ninhydrin in butan-1-ol.

Detection of phenylacetic acid, phenylacetamide and phenylacetyl-glycine on paper chromatograms. After chromatography of the samples on 1 cm.-wide strips of Whatman no. 1 paper the strips were dried, sprayed with a freshly prepared mixture of 1 mg. of 6-APA/ml. and the penicillin acylase-containing cells of *E. coli* N.C.I.B. 8743 (final cell density twice that in the growth medium) in 0.05 M-potassium phosphate buffer at pH 5.5. The strips were then hung for 1 hr. in a tank with an atmosphere saturated with water vapour to allow the enzymic synthesis of benzylpenicillin from 6-APA and the various phenylacetyl compounds on the paper. The strips were then dried and laid on agar seeded with *B. subtilis* as in the bio-autographic procedure for penicillins. By this method, 1.8 μ g. of phenylacetic acid gave an antibacterial zone of 22 mm. at *R_F* 0.66 (solvent butan-1-ol-pyridine-water, 1:1:1, by vol.). Besides phenylacetic acid, α -hydroxy-, α -amino- and *p*-amino-phenylacetic acids and their amides, esters, thioesters etc., can be readily detected. Benzoic acid and β -phenylpropionic acid and their derivatives are not detected by this method.

Assay of hydroxamate. Samples were withdrawn from the reaction mixtures and centrifuged, and 1 ml. of supernatant was diluted to 8 ml. with water before addition to 2 ml. of the ferric ammonium sulphate reagent as used in the hydroxylamine assay for penicillin (200 g. of ferric ammonium sulphate, 93.4 ml. of H₂SO₄/l.). Extinctions were measured with an EEL colorimeter with a no. 623 filter (490 nm). A standard curve for *p*-aminophenylacetic acid was obtained by reacting for 3 min. in a boiling-water bath 1 ml. of each of a series of concentrations of aqueous solutions of *p*-aminophenylacetic acid ethyl ester with 1 ml. of saturated hydroxylamine hydrochloride in methanol and 1 ml. of 5 M-NaOH. Next 4 ml. of water, 1 ml. of 5 M-HCl and 2 ml. of ferric ammonium sulphate reagent were added in that order before the extinction was read in an EEL colorimeter with no. 623 filter. There was a straight-line relationship over the range 0–1 mg. of the ethyl ester/ml. An extinction of 4.6 on the EEL colorimeter corresponded to 1 mg. of *p*-aminophenylacetic acid/ml.

RESULTS

Reversible nature of penicillin acylase. When the concentration of benzylpenicillin exceeded 3–4% (w/v) the deacylation reaction catalysed by the *E. coli* acylase enzyme appeared to stop before the reaction was complete, suggesting that an equilibrium was being set up between forward and reverse reactions. Such a reverse reaction was demonstrated by incubating equimolar solutions of 6-APA and phenylacetic acid with the enzyme, when benzylpenicillin was formed. This synthetic reaction proceeded most rapidly in the region of pH 5 whereas the hydrolytic reaction had optimum pH about 8.0. The rates of reaction in the two directions for high and low concentrations of enzyme are shown in Table 1.

Table 1. *Reversible enzyme reaction: comparison of the rate of hydrolysis of benzylpenicillin with the rate of synthesis of this penicillin from 6-APA and phenylacetic acid or phenylacetyl glycine*

For benzylpenicillin hydrolysis the reaction mixture consisted of a final concentration of 2.08% (w/v) potassium penicillin G in water (giving 1% 6-APA if completely hydrolysed) plus *E. coli* cells at the concentrations shown. The mixture was stirred at 37° and maintained at pH 8.5 by automatic addition of 0.5M-NaOH. The amount of NaOH added over the first 10 min. of reaction was used as a measure of the initial rate of reaction.

For benzylpenicillin synthesis the reaction mixture consisted of final concentrations of 1% 6-APA + 0.63% phenylacetic acid or 0.89% phenylacetyl glycine (equimolar proportions) plus *E. coli* cells at the concentrations shown. The mixture was stirred at 37° and pH 5.0. The yield of benzylpenicillin over the first 10 min. of reaction was assayed by the hydroxylamine method after solvent extraction and was used as a measure of the initial rate of reaction.

'Concentration of enzyme' refers to the final densities of *E. coli* N.C.I.B. 8743 cells in the reaction mixtures (1× or 10× that in the growth medium).

	Concn. of enzyme	Rate of hydrolysis or synthesis (μmole/min./ml.)	Rate of hydrolysis/ rate of synthesis
Benzylpenicillin hydrolysis (pH 8.5)	10.0	1.00	—
	1.0	0.105	—
Benzylpenicillin synthesis from 6-APA and phenylacetic acid (pH 5)	10.0	0.12	8.3
Benzylpenicillin synthesis from 6-APA and phenylacetyl glycine (pH 5)	10.0	0.81*	1.2
	1.0	0.117	0.9

* Rate slightly lower at pH 7.0

Synthesis of benzylpenicillin also takes place when a derivative of phenylacetic acid is used, e.g. phenylacetyl glycine. Under these conditions the phenylacetyl group is transferred from the amino group of glycine to the 6-amino group of 6-APA. However, if no 6-APA is present in the reaction mixture the phenylacetyl group reacts with water to give phenylacetic acid and glycine, i.e. there is simple hydrolysis. The results in Table 1 show that the rate of synthesis of benzylpenicillin from phenylacetyl glycine and 6-APA is about the same as the rate of hydrolysis of benzylpenicillin.

Enzymic coupling of carboxylic acids to 6-APA; substrate spectrum. A wide range of carboxylic acids was examined for ability to be linked to the 6-amino group of 6-APA in the presence of the cell-bound *E. coli* acylase. Tests for the formation of penicillin in such reaction mixtures consisted of paper-chromatographic examination with two solvent systems (Table 2). The presence of a penicillin zone in a position different from those in the control or of a size greater than those of the control was taken as evidence of the incorporation of the carboxylic acid into a penicillin. The control contained only 6-APA and the cells of *E. coli* N.C.I.B. 8743, and chromatography at 3 hr. with the butan-1-ol-ethanol-water system showed a 6-APA zone (11 mm. wide) at R_F 0.10–0.12, a 'factor 1' zone at R_F 0.03 (16 mm.

wide) (Batchelor, Cole, Gazzard & Rolinson, 1962) and occasionally a zone at R_F 0.4 (celcillin). In the ether system the control showed 6-APA and 'factor 1' at the origin and other small zones at 4 cm. and beyond (celcillin zones). Presumably these celcillin zones arise by acylase-catalysed coupling of traces of various cellular carboxylic acids on to 6-APA. As the *E. coli* enzyme was induced with phenylacetic acid it is very likely that one of the celcillin zones is benzylpenicillin.

Relative rates of utilization of the carboxylic acids were deduced from the amounts of penicillin synthesized at 1.5 and 3 hr. in selected reaction mixtures. The effect of changing the position of a hydroxyl group in the benzene ring of phenylacetic acid is shown in Table 3. The hydroxylamine assays assume similar colour production per mole for benzylpenicillin and the various hydroxy-penicillins. The relative bioactivities of these penicillins against *B. subtilis* are also given, the figures being based on the hydroxylamine assay.

Enzymic synthesis of benzylpenicillin from 6-APA and a variety of phenylacetic acid derivatives. As phenylacetic acid is one of the best acyl substrates for the penicillin acylase of *E. coli* and its derivatives are readily made, a study was made of the effect of using such derivatives for the enzymic acylation of 6-APA to yield benzylpenicillin (Table 4). Reaction

Table 2. *Enzymic synthesis of penicillins from 6-aminopenicillanic acid and various carboxylic acids*

Reaction mixtures consisted of a final concentration of 0.05 M-phosphate buffer, 1% 6-APA, an equimolar concentration of carboxylic acid, (both dissolved with the aid of dil. NaOH, then adjusted to pH 5.0) and *E. coli* cells (N.C.I.B. 8743) resuspended at ten times the cell density in the growth medium. The reaction mixtures were adjusted to pH 5.0 and shaken at 37°. The control reaction mixture had the same composition except that the carboxylic acid was omitted. The penicillin contents of selected reaction mixtures were determined by hydroxylamine assay after solvent extraction and are expressed in terms of potassium benzylpenicillin. The butan-1-ol-ethanol-water solvent was 4:1:5, by vol. (top phase). All reaction mixtures were examined by paper chromatography the penicillin zones being detected by bio-autographic means. Zones that were common to the control and test reaction mixtures are not recorded in the table.*

Carboxylic acid	Concn. of penicillin (mg./ml.) in reaction mixture at		Paper chromatography of reaction mixtures at 3 hr.			
			Butan-1-ol- ethanol-water		Water-saturated ether, pH 6.2	
	1.5 hr.	3.0 hr.	Zone width (mm.)	<i>R_F</i>	Zone width (mm.)	Migration (cm.)
Propionic	—	—	0	—	0	—
<i>n</i> -Butyric	<0.05	<0.05	Trace	0.36	12	3.0
<i>n</i> -Valeric	0.2	0.3	25	0.46	36	8.0
<i>n</i> -Hexanoic	0.7	1.2	28	0.49	29	15.5
3-Hexenoic	1.1	1.9	30	0.46	31	13.0
<i>n</i> -Heptanoic	0.1	0.3	23	0.54	24 + 21	12 + 15.5
<i>n</i> -Octanoic	0.1	<0.05	Trace	0.56	11	11.0
<i>n</i> -Nonanoic	—	—	0	—	0	—
<i>n</i> -Decanoic	—	—	0	—	0	—
Palmitic	—	—	0	—	0	—
Glutaric	—	—	0	—	0	—
Adipic	—	—	0	—	0	—
Pimelic	—	—	0	—	13	5.0
γ -Amino- <i>n</i> -valeric	—	—	0	—	12	4.8
ϵ -Aminocaproic	—	—	0	—	12	4.5
DL-Leucine	—	—	0	—	0	—
α -Hydroxyisocaproic	—	—	0	—	18	4.2
Butylthioacetic	—	—	24	0.53	23	10.0
Benzoic	<0.05	<0.05	0	—	0	—
Phenylacetic	2.7	3.3	34	0.47	35	6.5
β -Phenylpropionic	<0.05	<0.05	0	—	0	—
γ -Phenylbutyric	<0.05	<0.05	0	—	0	—
Cinnamic	—	—	0	—	0	—
<i>N</i> -Phenylglycine	—	—	16	0.37	19	3.0
α -Ketophenylacetic	—	—	Trace	0.37	15	2.5
DL- α -Hydroxyphenylacetic	0.5	0.8	29	0.40	32	3.4
DL- α -Aminophenylacetic	—	—	0	—	0	—
DL- α -Ethylphenylacetic	<0.05	0.1	12	0.43	0	—
DL- α -Methoxyphenylacetic	<0.05	<0.05	19	0.41	17	3.5-6.5
DL- α -Carboxyphenylacetic	—	—	0	—	0	—
<i>p</i> -Hydroxyphenylacetic	3.4	3.9	34	0.29	34	0.5
<i>p</i> -Aminophenylacetic	—	—	29	0.21	28	0
<i>m</i> -Aminophenylacetic	—	—	32	0.22	31	0
<i>p</i> -Methoxyphenylacetic	1.0	1.7	32	0.39	32	4.2
3,4-Dichlorophenylacetic	<0.05	<0.05	16	0.57	19	6.4
3,4-Dihydroxyphenylacetic	—	—	26	0.24	29	0
<i>p</i> -Hydroxy- α -hydroxyphenylacetic	1.1	1.4	29	0.32	30	0
α -Methyl- α -hydroxyphenylacetic	—	—	Trace	0.46	13	6.9
Homogentisic acid lactone	0.2	0.3	17	0.31	24	0
Phenylmercaptoacetic	—	—	0	—	0	—
Phenoxyacetic	<0.05	<0.05	0	—	14	7.0
<i>p</i> -Chlorophenoxyacetic	—	—	0	—	14	6.5
α -Phenoxypropionic	—	—	0	—	0	—
2-Thienylacetic	1.3	1.9	34	0.40	36	5.3
1-Naphthylacetic	<0.05	0.1	14	0.48	16	6.0
2-Naphthylacetic	<0.05	0.3	13	0.52	13	7.4
Control	<0.05	<0.05	*	*	*	*

* For paper chromatography of the control see the text.

pH values of 5.0 for phenylacetic acid and 7.0 for phenylacetic acid derivatives were chosen because these were closest to the respective optima, but at pH 7 benzylpenicillin was broken down to phenylacetic acid and 6-APA. In the phenylacetamide reaction mixture the release of ammonia would

result in an upward drift in pH and increased rate of breakdown of the benzylpenicillin; this probably accounted for the very low yield at 80 min. The yields at 10 min. gave the best indication of the relative rates of synthesis from the various substrates. The reactions are transfer reactions, the

phenylacetyl group being transferred from one amino compound to a second (6-APA), with ammonia, glycine, L-amino acid etc. being liberated.

Amides as a source of acyl groups for the enzymic acylation of 6-APA; substrate spectrum. The substrate spectrum for the enzymic formation of penicillin from a series of carboxylic acid amides was compared to that for the series of free carboxylic acids shown in Table 2. The results in Table 5

suggest that it is similar and the amide derivatives of benzoic acid and β -phenylpropionic acid are not substrates. However, α -aminophenylacetamide does support the synthesis of α -aminobenzylpenicillin whereas this penicillin is not synthesized from the free carboxylic acid, possibly because of its very low solubility.

N-acyl derivatives of glycine as a source of acyl groups for the enzymic acylation of 6-APA; substrate spectrum. Various *N*-acyl derivatives of glycine were examined for ability to support the synthesis of penicillin by enzymic transfer of the acyl group to 6-APA, the methods being similar to those used for amides in the previous section. The results in Table 6 show that the rate of synthesis of penicillin is clearly greater than that obtained with amides (Table 5) or carboxylic acids (Table 2). The substrate spectrum is essentially the same, but is broadened slightly to include heptanoyl- and phenoxyacetyl-glycine. *N*- α -Phenoxypropionylglycine gives a small amount of α -phenoxyethylpenicillin (phenethicillin) in a very slow reaction whereas the α -phenoxypropionic acid does not give phenethicillin. Ampicillin was synthesized from the α -aminophenylacetyl-glycine, probably because of the improved water-solubility of this compound compared with the α -aminophenylacetic acid (cf. the amide). The major zone at R_F 0.24 (Table 6) is ampicillin; the identity of the other zone is not known.

Non-enzymic acylation of 6-APA. Reaction mixtures containing final concentrations of 1% 6-APA and 1% carboxylic acid or derivative, all at

Table 3. *Enzymic synthesis of o- m- and p-hydroxybenzylpenicillins*

Reaction mixtures at pH 5.0 and 35° consisted of 1% *o*-, *m*- or *p*-hydroxyphenylacetic acid, 2% 6-APA and *E. coli* cells (N.C.I.B. 8743) resuspended at 15 times the cell density in the growth medium. Yields of penicillin were determined by hydroxylamine assay of solvent extracts of the reaction mixture and expressed in terms of sodium benzylpenicillin. The R_F values refer to the butanol-ethanol-water solvent as described in Table 2. The relative biological activities for solutions giving equal values in the hydroxylamine assay were expressed in terms of the relative dilutions required to give a 25 mm. zone of inhibition in a hole-in-plate bioassay with *B. subtilis* as seed organisms.

Substituted phenylacetic acid	Penicillin (mg./ml.) synthesized in 3 hr.	R_F of penicillin	Relative biological activity
<i>o</i> -hydroxy	0.6	0.42	1.0
<i>m</i> -hydroxy	4.2	0.36	1.3
<i>p</i> -hydroxy	6.6	0.32	1.6

Table 4. *Effect of type of derivative of phenylacetic acid on the rate of enzymic synthesis of benzylpenicillin*

The reaction mixtures had the same composition and treatment as those described in Table 2 except that the 6-APA was dissolved at pH 7.0 and the final pH of the reaction mixtures was 7.0. The phenylacetic acid control reaction mixture was at pH 5.0. Penicillin assays were carried out as described in Table 2 and are expressed as mg. of potassium benzylpenicillin/ml.

Phenylacetic acid derivative	Potassium benzylpenicillin (mg./ml.) in reaction mixture at			
	10 min.	20 min.	40 min.	80 min.
Phenylacetic acid (pH 5)	0.47	1.02	1.81	2.8
Phenylacetamide	1.2	1.4	1.37	0.1
<i>N</i> -Phenylacetyl-glycine	3.3	3.3	2.7	1.0
<i>N</i> -Phenylacetyl-ethanolamine	0.38	—	0.49	0.5
<i>N</i> -Phenylacetyl- <i>p</i> -aminobenzoic acid	0.31	—	0.44	0.5
<i>N</i> -Phenylacetyl-DL-valine	0.32	0.54	0.81	0.92
<i>N</i> -Phenylacetyl-DL-leucine	0.49	0.58	0.58	0.68
<i>N</i> -Phenylacetyl- ϵ -aminocaproic acid	1.08	1.14	—	—
<i>N</i> -Phenylacetyl-D- α -aminophenylacetic acid	< 0.05	< 0.05	0.16	< 0.05
<i>N</i> -Phenylacetyl-L- α -aminophenylacetic acid	2.76	3.52	3.9	2.06
<i>N</i> -Phenylacetyl-DL- α -aminophenylacetic acid	2.02	1.74	1.25	0.59
Benzylpenicilloic acid	0.22	0.24	0.31	0.35
Control (6-APA and enzyme only)	< 0.05	< 0.05	< 0.05	< 0.07

Table 5. *Enzymic synthesis of penicillins from 6-aminopenicillanic acid and various amides*

The reaction mixtures had the same composition and were treated in the same way as those described in Table 2 except that 6-APA was dissolved at pH 7.0 and the final pH of the reaction mixture was 7.0. The phenylacetic acid control reaction mixture was at pH 5.0. Penicillin assays and paper chromatography were carried out as described in Table 2. The zones on the chromatogram common to the control (no amide) and test reaction mixtures have not been recorded. Penicillin yields are expressed in terms of potassium benzylpenicillin.

Amide	Concn. of penicillin (mg./ml.) in reaction mixture at			Paper chromatography of reaction mixtures at 1.5 hr.			
				Butan-1-ol-ethanol-water		Water-saturated ether, pH 6.2	
				Zone width (mm.)	R_F	Zone width (mm.)	Migration (cm.)
	0.25 hr.	0.5 hr.	1.5 hr.				
Acetamide	< 0.05	< 0.05	< 0.05	0	—	0	—
Valeramide	< 0.05	0.1	0.2	24	0.49	24	7.5
Hexanamide	0.1	0.3	0.4	26	0.55	25	13.5
Heptanamide	< 0.05	< 0.05	< 0.05	19.5	0.58	20	15.5
Benzamide	< 0.05	< 0.05	< 0.05	0	—	0	—
Phenylacetamide	1.2	1.3	1.0	31	0.5	30	7.2
Phenylacetic acid control (pH 5)	0.7	1.2	2.6	34	0.5	32	7.5
β -Phenylpropionamide	< 0.05	< 0.05	< 0.05	0	—	0	—
<i>p</i> -Aminophenylacetamide	—	—	—	31	0.23	28	0
DL-mandelamide	0.9	1.1	1.4	29	0.48	30	4.5
D- α -aminophenylacetamide	—	—	—	29	0.25	28	0
DL- α -phenoxypropionamide	< 0.05	< 0.05	< 0.05	14	0.58	16	16
DL- α -phenylphenoxyacetamide	< 0.05	< 0.05	< 0.05	0	—	0	—
2,6-Dimethoxybenzamide	< 0.05	< 0.05	< 0.05	0	—	0	—

pH 6 in 0.05 M-phosphate buffer, were shaken at 37°. An examination for penicillin formation was made at $\frac{1}{2}$ or 4 hr. by paper chromatography with the butan-1-ol-ethanol-water solvent system and the bio-autographic detection method with *B. subtilis*. The expected penicillins were also chromatographed as markers. Penicillin synthesis was not observed in the absence of *E. coli* cells for the following carboxylic acids and their derivatives: phenylacetic acid, its amide and phenylacetyl-glycine; DL- α -hydroxyphenylacetic acid its amide and α -hydroxyphenylacetyl-glycine; D- α -aminophenylacetic acid, its amide and D- α -aminophenylacetyl-glycine; DL- α -phenoxypropionic acid, its amide and DL- α -phenoxypropionyl-glycine; 2,6-dimethoxybenzoic acid, its amide, 2,6-dimethoxybenzoyl-glycine and 2,6-dimethoxybenzoylthioglycollic acid.

Synthesis of α -phenoxyethylpenicillin from benzylpenicillin or 6-APA and α -phenoxypropionylthioglycollic acid. In contrast with the above results some non-enzymic synthesis of phenethicillin and ampicillin was obtained in reaction mixtures containing 6-APA + DL- α -phenoxypropionylthioglycollic acid and 6-APA + D- α -aminophenylacetylthioglycollic acid respectively, although more penicillin was synthesized in the presence of the *E. coli* enzyme. The composition of the reaction mixtures

and the yields of phenethicillin are shown in Table 7. The yields of phenethicillin were determined by bioassay (*B. subtilis*) after separation from benzylpenicillin or 6-APA by chromatography using the water-saturated ether solvent system.

Enzymic acylation of hydroxylamine. *E. coli* acylase also catalyses the transfer of an acyl group to hydroxylamine. The results in Table 8 show chromatographic evidence for the formation of *N*-(*p*-aminophenylacetyl)-hydroxylamine from *p*-aminophenylacetic acid or its amide. The zone at R_F 0.78 was positive both to the ferric ammonium sulphate spray and to the β -naphthol spray (after diazotization) and had the same R_F value as synthetic *N*-(*p*-aminophenylacetyl)-hydroxylamine.

The optimum pH for the formation of hydroxamate was determined in a further experiment in which 0.5% *p*-aminophenylacetic acid was reacted at various pH values (range 5–9) with 1% hydroxylamine hydrochloride in 0.05 M-phosphate buffer (final concentrations of substrates given) in the presence of *E. coli* cells (N.C.I.B. 8743) resuspended at five times the cell density in the culture medium. Reaction mixtures were shaken at 35° and the amounts of *p*-aminophenylacetic acid coupled to the hydroxylamine were obtained by colorimetry with the ferric ammonium sulphate reagent. The

Table 6. *Enzymic synthesis of penicillins from 6-APA and various N-acyl derivatives of glycine*

The reaction mixtures had the same composition and were treated in the same way as those described in Table 2, except that the 6-APA was dissolved at pH 7.0 and the final pH of the reaction mixture was 7.0. The phenylacetic acid control reaction mixture was at pH 5.0. Penicillin assays and paper chromatography were carried out as described in Table 2. Chromatogram zones common to the control (no *N*-acyl compound) and test reaction mixtures have not been recorded. As in Table 2, zero concentrations of penicillin were detected in the hydroxylamine assays of the control reaction mixture consisting of only 6-APA and *E. coli* cells. Penicillin yields are expressed in terms of potassium benzylpenicillin.

<i>N</i> -acyl derivatives of glycine	Concn. of penicillin (mg./ml.) in reaction mixture at			Paper chromatography of reaction mixture at 1.5 hr.			
				Butan-1-ol-ethanol-water		Water-saturated ether, pH 6.2	
	0.25 hr.	0.5 hr.	1.5 hr.	Zone width (mm.)	<i>R_F</i>	Zone width (mm.)	Migration (cm.)
Acetyl glycine	—	—	—	0	—	0	—
Propionyl glycine	< 0.05	< 0.05	0.05	0	—	0	—
Butyryl glycine	0.05	0.07	0.07	0	—	0	—
Valeryl glycine	0.38	0.71	1.27	27	0.51	40	7.5
Hexanoyl glycine	0.46	0.82	1.44	29	0.55	30	14.0
Heptanoyl glycine	0.16	0.18	0.56	26	0.58	13	10.5
						28	16.0
Octanoyl glycine	0.07	0.07	< 0.05	12	0.62	14	25
Benzoyl glycine	< 0.05	< 0.05	< 0.05	0	0	0	0
Phenylacetyl glycine	3.25	3.0	0.82	30	0.50	32	8.0
Phenoxyacetyl glycine	0.75	1.1	2.06	32	0.50	33	12.0
DL- α -phenoxypropionyl glycine	0.04	0.15	0.18	22	0.54	23	14.5
DL- α -hydroxyphenylacetyl glycine	4.1	3.22	2.28	39	0.45	30	5.0
D- α -aminophenylacetyl glycine	—	—	—	27	0.03	30	0
				32	0.24		
DL- α -phenylphenoxyacetyl glycine	0.08	0.08	< 0.05	0	—	0	—
Phenylacetic acid control (pH 5.0)	0.63	1.2	2.31	31	0.50	34	8.0
Phenylacetamide control (pH 7.0)	1.41	1.56	1.22	30	0.50	35	8.5

Table 7. *Synthesis of α -phenoxyethylpenicillin (phenethicillin)*

The quantity of phenethicillin was determined by using a graph of the diameter of the zones on phenethicillin standard chromatograms against the logarithm of the phenethicillin concentrations. The reaction mixture had final pH 7; compositions are given as final concentrations.

Composition of reaction mixture			Yield of phenethicillin at 0.5 hr. (mg./ml.)	
Benzylpenicillin (0.33%)	+ α -Phenoxypropionylthioglycollic acid (0.2%)	+ <i>E. coli</i> * cells	1.70	(0.00)†
		No cells	0.00	(0.00)†
6-Aminopenicillanic acid (0.2%)	+ α -Phenoxypropionylthioglycollic acid (0.2%)	+ <i>E. coli</i> * cells	2.40	(1.80)†
		No cells	0.03	(0.00)†
6-Aminopenicillanic acid (1.0%)	+ α -Phenoxypropionylthioglycollic acid (1.0%)	No cells	1.40	—

* Cells of *E. coli* N.C.I.B. 8743A were resuspended to give a final cell density in the reaction mixtures ten times that in the growth medium.

† Results with *N*- α -phenoxypropionylglycine in place of α -phenoxypropionylthioglycollic acid.

optimum pH was found to be 6.0, the concentrations of *p*-aminophenylacetic acid coupled at pH 5.0, 6.0, 7.0 and 8.0 being respectively 0.22, 0.38, 0.23 and

0.12 mg./ml. at 2 hr. The control (without enzyme) gave a value of 0.04 mg./ml. at pH 7 in the same assay system.

Table 8. *Enzymic acylation of hydroxylamine using E. coli N.C.I.B. 8743*

Reaction mixtures were shaken at pH 6.0 and 35° for 1 hr. before examination by paper chromatography using butan-1-ol-pyridine-water (1:1:1, by vol.) as solvent system. The hydroxamic acid zone at R_F 0.78 was revealed by spraying with the ferric ammonium sulphate reagent as used in the hydroxylamine assay of penicillin. *p*-Aminophenylacetic acid (R_F 0.56) and *p*-aminophenylacetamide (R_F 0.73) were detected by diazotization and coupling with β -naphthol. Such treatment also detected the zone at R_F 0.78. *E. coli* cells were resuspended at ten times the cell density in the culture medium.

Composition of reaction mixture					Chromatography Zone at R_F 0.78
<i>p</i> -aminophenylacetic acid	<i>p</i> -aminophenylacetamide	2% hydroxylamine hydrochloride	<i>E. coli</i> cells		
+	—	+	+		+
—	+	+	+		+
+	—	+	—		—
—	+	+	—		—
—	—	—	+		—
<i>p</i> -aminophenylacetic acid ethyl ester + hydroxylamine hydrochloride + NaOH					+

Substrate spectrum for enzymic acylation of hydroxylamine. Various carboxylic acids or their amides were examined to establish the preferred acyl group for the acylation of hydroxylamine. The results in Table 9 clearly show that of the series benzoic acid, phenylacetic acid and β -phenylpropionic acid, only phenylacetic acid was a good substrate, phenylacetamide being even more suitable.

Enzymic synthesis of N-(p-aminophenylacetyl)-glycine by acyl transfer from an ester. A reaction mixture consisting of 2% *p*-aminophenylacetic acid ethyl ester, 1% glycine, 0.05 M-phosphate buffer and *E. coli* cells (N.C.I.B. 8743) resuspended to give a density five times that in the cultivation medium was adjusted to pH 7 and shaken at 35°. The reaction mixture was examined by paper chromatography with the butan-1-ol-acetic acid-water system (12:3:5, by vol.) at zero time and at intervals up to 60 min. Zones were revealed by diazotization and coupling with β -naphthol or α -naphthylamine. The chromatograms showed the formation of a new zone at R_F 0.33, which, as described below, was shown to be *p*-aminophenylacetyl-glycine. Another zone (R_F 0.56) that appeared during the reaction was presumed to be *p*-aminophenylacetic acid, with which it had an identical R_F when chromatographed side by side. The *p*-aminophenylacetic acid ethyl ester had R_F 0.82 and glycine had R_F 0.12.

A preparation of material with R_F 0.33 was made by chromatography on Whatman 3MM paper of 2 ml. of the supernatant from a 1 hr. reaction mixture. After development with the butan-1-ol-acetic acid-water system and drying at 40°, the band visible at R_F 0.33 was cut out and extracted with 1 ml. of phosphate buffer, pH 8, treated with *E. coli* cells (N.C.I.B. 8743) at 35° for 0.5 hr. before removal

Table 9. *Enzymic synthesis of hydroxamic acids by E. coli N.C.I.B. 8743*

The reaction mixtures consisted of 1% carboxylic acid or amide, 2% hydroxylamine hydrochloride, 0.05 M-phosphate buffer and *E. coli* cells resuspended at five times the cell density in the culture medium. The reaction mixtures at pH 6.0 and 35° were shaken. Hydroxamic acid formation was detected by reaction of centrifuged samples with the ferric ammonium sulphate reagent and measurement of the extinction in an EEL colorimeter with no. 623 filter (490 nm). Results expressed, as colorimeter readings at the reaction times given, were corrected for readings on samples taken from both no-enzyme and no-substrate controls.

Carboxylic acid or amide	Yield of hydroxamic acid		
	0.5 hr.	2.0 hr.	3.5 hr.
Benzoic acid	0	0	0
Benzamide	0	0	0
Phenylacetic acid	0.7	2.8	4.0
Phenylacetamide	3.4	6.4	8.8
β -Phenylpropionic acid	0.1	0.1	0.2
β -Phenylpropionamide	0	0	0
D-Mandelamide	1.6	3.0	3.1
L-Mandelamide	0.8	2.3	3.3
DL-Mandelamide	1.3	2.7	3.0

of the cells and examination of the supernatant by paper chromatography with the butan-1-ol-acetic acid-water system. Before treatment with *E. coli* the extract was shown to give one zone at R_F 0.35. After treatment this zone completely disappeared and two new zones appeared, having the same R_F values as *p*-aminophenylacetic acid and glycine respectively.

Enzymic synthesis of phenylacetyl-glycine and N-(α -aminophenylacetyl)-glycine by acyl transfer

from an amide. A reaction mixture consisting of 1% phenylacetamide, 0.05 M-phosphate buffer, 1% glycine and *E. coli* cells (N.C.I.B. 8743) resuspended to give a cell density ten times that in the cultivation medium was adjusted to pH 7 and shaken at 35°. The reaction mixture was examined by paper chromatography with the solvent system butan-1-ol-ethanol-water (4/1/5, by vol.; top phase), the zones being revealed by bio-autography after spraying with a mixture of 6-APA and *E. coli* penicillin acylase. At 1 and 2 hr. a new zone was detected at R_F 0.21 and corresponding in R_F value to a marker of phenylacetyl-glycine (phenylacetamide R_F 0.81). Large amounts of phenylacetic acid were also released in the reaction. There was no phenylacetyl-glycine zone from a reaction mixture at pH 5 containing phenylacetic acid in place of phenylacetamide.

In an analogous reaction involving D- α -amino-phenylacetamide the synthesis of *N*-(D- α -amino-phenylacetyl)-glycine was demonstrated chromatographically (with detection by ninhydrin).

DISCUSSION

It seems very likely that the acylation and deacylation reactions catalysed by *E. coli* N.C.I.B. 8743 are brought about by the same acylase enzyme. In the presence of the enzyme the acyl groups that are readily removed from *N*-acylamino compounds are the same as those that readily acylate amino compounds. This is revealed when the acyl substrate spectra described in this paper are compared with the substrate spectra for hydrolytic reactions already described (Cole, 1969*a,b*). Thus *p*-hydroxy-phenylacetyl-6-APA was the most readily hydrolysed penicillin, and of a wide range of carboxylic acids, *p*-hydroxyphenylacetic acid gave the highest rate of enzymic acylation of 6-APA. Phenylacetyl-6-APA (benzylpenicillin) was the second most readily deacylated penicillin and phenylacetic acid was the second most reactive compound for acylation of 6-APA. α -Hydroxy- or α -amino-phenylacetyl compounds follow in order of reactivity. It is noteworthy that provided an activated form of phenylacetic acid such as phenylacetyl-glycine is used for enzymic acylation, rates of acylation can be as fast as rates of deacylation (see Table 1) when the pH for the acylation is 5.0 and that for deacylation, pH 8.5.

Kaufmann, Bauer & Offe (1960) gave details of various types of acyl-transfer reaction, but did not describe a substrate spectrum for synthetic reactions or give reaction rates. Experiments described in the present paper confirm the statements by Kaufmann *et al.* (1960) that penicillin synthesis proceeds more rapidly when carboxylic acid derivatives are used instead of the free acid. Such

derivatives are more energy-rich and thus associated with less energy change than that occurring when using a carboxylic acid; in this connexion it is surprising that the acylation of 6-APA by *p*-hydroxyphenylacetic acid or phenylacetic acid proceeds a rapidly as it does.

The substrate-specificity results in Tables 2, 5 and 6 show that the preferred acyl group of the enzyme is the phenylacetyl group and its *p*-hydroxy, *p*-methoxy, *p*-amino etc., derivatives. Benzoic acid and β -phenylpropionic acid are not substrates, nor are α -substituted phenylacetic acids such as α -amino, α -ethyl- and α -carboxy-phenylacetic acids, with the exception of the α -hydroxy compound. As was shown by Kaufmann (1964) α -amino-phenylacetic acid becomes a substrate when it is used in the form of its amide hydrobromide or *N*-acylglycine derivative, possibly because such compounds are very much more water-soluble than the free amino acid. This situation is not true for α -carboxyphenylacetic acid, i.e. carbenicillin is not synthesized when the monoamide of α -carboxy-phenylacetic acid is used in an enzymic coupling reaction with 6-APA.

The 2-thienylacetyl, valeryl, hexanoyl, hexenoyl and heptanoyl derivatives, being somewhat similar in size to the phenylacetyl compounds, are also substrates but somewhat poorer. The use of *N*-acylglycine derivatives of straight-chain carboxylic acids does not seem to broaden the substrate profile. Phenoxymethylpenicillin and phenethicillin are not synthesized from the carboxylic acids with corresponding side-chains but are synthesized when the more energy-rich forms of these compounds, such as *N*-glycine derivatives, are used, indicating that these acyl groups are on the edge of the substrate spectrum. Using the more energy-rich thioester of α -phenoxypropionic acid, Kaufmann *et al.* (1960) reported that phenethicillin was readily synthesized enzymically. This has been confirmed in the present paper but this ester is so reactive that some acylation of 6-APA takes place in the absence of the acylase enzyme. Thus it is probably not surprising that phenethicillin can be synthesized from benzylpenicillin in the presence of this thioester, an observation first made by Kaufmann *et al.* (1960) and confirmed in the present paper. As soon as any benzylpenicillin is deacylated to 6-APA it would be rapidly reacylated enzymically and non-enzymically by transfer of the α -phenoxypropionyl group from the thioester.

Considerable variations in the rate of enzymic synthesis of benzylpenicillin occur with different derivatives of phenylacetic acid but there is an absolute stereochemical requirement. Thus the phenylacetyl group is not transferred from the amino group of a D-amino acid but is readily transferred from an L-amino acid (see Table 4).

This correlates precisely with the findings previously reported (Cole, 1964, 1969b) that only phenylacetyl-L-amino acids are deacylated by the enzyme. The presence of 6-APA in the reaction mixture in no way alters the absolute requirement.

The substrate spectrum for the *E. coli* enzyme with respect to acylation of hydroxylamine appears to parallel that for the acylation of 6-APA, and also parallels that for the hydrolysis of amides and substituted amides (Cole, 1969b). The enzyme can also catalyse the transfer of the phenylacetyl group to glycine, but the reaction requires an energy-rich derivative such as an amide or ester and unlike the acylation of hydroxylamine does not take place with the free carboxylic acid. Enzymic transfer of an acyl group from an aliphatic amide to hydroxylamine has been described by Brammar & Clarke (1963) and Kelly & Kornberg (1964) for an enzyme from *Pseudomonas aeruginosa*. Similar enzyme activity has also been reported in *Mycobacterium smegmatis* (Draper, 1967) and *Pseudomonas fluorescens* (Jakoby & Fredericks, 1964). Kelly & Kornberg (1962, 1964), when purifying their *Pseudomonas* enzyme, could not separate the amidase activity from the acyltransferase activity (to hydroxylamine) although they were able to distinguish between the two activities by selective enzyme inhibition. They concluded that if the two reactions were brought about by the same enzyme then the enzyme protein must have more than one catalytic site. However, Draper (1967), while studying the *Mycobacterium* amidase, was able to remove the transferase activity from it, which suggests that in this case there are two enzymes. Whether or not there are two separate enzymes in *E. coli*, one catalysing the hydrolysis of amides and acylamino compounds and the other catalysing the transfer of acyl groups to compounds such as 6-APA and hydroxylamine, will not be known until extensive enzyme-purification and inhibition studies have been carried out.

The *Pseudomonas* enzyme hydrolyses aliphatic amides and transfers aliphatic acyl groups, whereas the *E. coli* enzyme hydrolyses and transfers longer-chain aliphatic and aromatic groups. Further, the *E. coli* enzyme will hydrolyse substituted amides such as phenylacetyl-glycine, whereas the *Pseudo-*

monas enzyme does not. A point of similarity between the *E. coli* and *Pseudomonas* enzymes appears to be their ability to hydrolyse esters and transfer acyl groups from esters to amino compounds (McFarlane, Brammar & Clarke, 1965; Cole, 1969b; Kaufmann & Bauer, 1960; Kaufmann, 1964). It is interesting to speculate that the *Pseudomonas* aliphatic acyltransferase might be able to catalyse the transfer of an acyl group such as the propionyl group from propionamide to give propionyl-6-APA, i.e. ethylpenicillin.

The author thanks Mr P. F. Doyle and the staff of the Chemistry Department for the supply of many of the compounds used, Dr G. N. Rolinson and Dr M. O. Moss for help in various aspects of the work and Mrs M. V. Hart and Mr R. Payne for technical assistance.

REFERENCES

- Batchelor, F. R., Chain, E. B., Hardy, T., Mansford, K. R. L. & Rolinson, G. N. (1961). *Proc. Roy. Soc. B*, **154**, 498.
Batchelor, F. R., Cole, M., Gazzard, D. & Rolinson, G. N. (1962). *Nature, Lond.*, **195**, 954.
Brammar, W. J. & Clarke, P. H. (1963). *J. gen. Microbiol.* **32**, *Proceedings* (iii).
Cole, M. (1964). *Nature, Lond.*, **203**, 519.
Cole, M. (1969a). *Biochem. J.* **115**, 733.
Cole, M. (1969b). *Biochem. J.* **115**, 741.
Draper, P. (1967). *J. gen. Microbiol.* **46**, 111.
Jakoby, W. B. & Fredericks, J. (1964). *J. biol. Chem.* **239**, 1778.
Karnovsky, M. L. & Johnson, M. J. (1949). *Analyt. Chem.* **21**, 1125.
Kaufmann, W. (1964). *Proc. 3rd int. Congr. Chemotherapy, Stuttgart 1963*, **2**, 1248.
Kaufmann, W. & Bauer, K. (1960). *Naturwissenschaften*, **47**, 474.
Kaufmann, W., Bauer, K. & Offe, H. A. (1960). *Anti-microbial Agents Annual 1960*, p. 1. New York: Plenum Press.
Kelly, M. & Kornberg, H. L. (1962). *Biochim. biophys. Acta*, **64**, 190.
Kelly, M. & Kornberg, H. L. (1964). *Biochem. J.* **93**, 557.
McFarlane, N. D., Brammar, W. J. & Clarke, P. H. (1965). *Biochem. J.* **95**, 24c.
Rolinson, G. N., Batchelor, F. R., Butterworth, D., Cameron Wood, J., Cole, M., Eustace, G. C., Hart, M. V., Richards, M. & Chain, E. B. (1960). *Nature, Lond.*, **187**, 236.