Factors Affecting the Synthesis of Ampicillin and Hydroxypenicillins by the Cell-Bound Penicillin Acylase of *Escherichia coli*

By M. COLE

Beecham Research Laboratories, Brockham Park, Betchworth, Surrey

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1. The effect of pH, temperature, reactant concentration and reaction time has been investigated for the synthesis of benzylpenicillin, DL- α -hydroxybenzylpenicillin and D- α -aminobenzylpenicillin from 6-aminopenicillanic acid by the penicillin acylase of *Escherichia coli*. 2. Synthesis of penicillins from carboxylic acids proceeds most rapidly at pH 5; with amides the optimum pH is higher (6–7) but the reverse reaction rapidly sets in. This can be counteracted by lowering the pH or adding more amide. Optimum temperatures are 35–40°. 3. Most rapid synthesis of penicillin was obtained with the *N*-acylglycine and methyl ester derivatives of carboxylic acids. Increasing the amide/6-APA ratio above 1:1 raised the rate of synthesis of penicillins. 4. Preferential synthesis of D- α -hydroxybenzylpenicillin takes place in a reaction mixture containing DL-mandelic acid. 5. From D- and L-mandelamide, D- and L- α -hydroxybenzylpenicillins were prepared, the former being more bio-active than the latter. *p*-Hydroxy- and 3,4-dihydroxybenzylpenicillins were also prepared, the latter being more active against some Gram-negative bacteria than benzylpenicillin.

It is well established that the penicillin acylase enzyme of *Escherichia coli* is reversible with the result that benzylpenicillin is formed when 6-APA* and phenylacetic acid are incubated with the enzyme (Kaufmann, Bauer & Offe, 1960; Rolinson *et al.* 1960). There is little published information on factors affecting the synthesis of penicillins in such reactions apart from the note made by the above authors that an acid pH (4–6) favoured the synthetic reaction but an alkaline pH favoured the hydrolysis of benzylpenicillin. Kaufmann *et al.* (1960) also reported that the use of carboxylic acid derivatives such as phenylacetyl-glycine enhanced the rate of synthesis of benzylpenicillin.

In the preceding paper (Cole, 1969c) the substrate spectrum for the synthesis of penicillins was obtained with the acylase enzyme of *E. coli* N.C.I.B. 8743. In the present paper an examination has been made of the various factors that affect such reactions. The synthesis of benzylpenicillin, ampicillin (D- α -aminobenzylpenicillin) (Patents 1962, 1964) and α -hydroxybenzylpenicillin (Patent, 1963) have been investigated with the enzyme from *E. coli* strains N.C.I.B. 8743 and B.R.L. 1360. The synthesis of *p*-hydroxy- and 3,4-dihydroxybenzylpenicillins is also described.

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

MATERIALS AND METHODS

Enzyme. The enzyme used throughout the present paper was the cell-bound penicillin acylase of *E. coli*. The following strains of *E. coli* were used: N.C.I.B. 8743 (B.R.L. 1040), N.C.I.B. 8743A (a selection of N.C.I.B. 8743 producing more enzyme) and B.R.L. 1360 (a strain producing a similar enzyme but having slightly different morphology.) The cultures were grown for enzyme production as previously described (Cole, 1969a) and stored as dense cell suspensions at 5° in the presence of 1% *n*-butyl acetate.

Carboxylic acids, carboxylic acid derivatives, penicillins and buffers. The sources, purities and compositions of these materials have been previously described (Cole, 1969a,b).

Paper chromatography. Four descending solvent systems were used with Whatman no. 1 paper at 4°: butan-1-ol-pyridine-water (1:1:1, by vol.) (B/P/W); butan-1-ol-ethanol-water (4:1:5, by vol.; top phase) (B/E/W); butan-1-ol-acetic acid-water (12:3:5, by vol.) (B/A/W) and ether saturated with water (paper buffered at pH 6.2) (Karnovsky & Johnson, 1949). Penicillins and amino acids were chromatographed and detected as described in a previous paper (Cole, 1969b).

Assay of benzylpenicillin and α -hydroxybenzylpenicillin. The hydroxylamine assay after solvent extraction was used as described previously (Cole, 1969c). The extraction efficiency in this procedure was about 85% and all results were corrected for this and for the small difference in molecular weight between potassium benzylpenicillin (372) and potassium α -hydroxybenzylpenicillin (388).

In the biological assay the biotape method was used as described under 'Assay of ampicillin'. Samples were diluted 1:100 when the reaction mixtures contained 1% 6-APA and 1:300 when they contained 3% 6-APA. In the absence of a pure standard of α -hydroxybenzylpenicillin of known stereochemical configuration sodium benzylpenicillin (Crystapen) was used as an arbitrary standard. The biological-assay results only serve to show changes in the ratio of bioactivity to chemical-assay results during enzyme reactions.

Assay of ampicillin. Being an amino acid ampicillin is not readily separated from 6-APA by solvent extraction at low pH and therefore assay of reaction mixtures containing ampicillin by the hydroxylamine assay could not be used. The traditional hole-in-plate bioassay was also unsuitable because of difficulty in preventing changes in the samples during the assay. A modified bioassay called a 'biotape' assay was used in which samples were dried on strips of chromatography paper to stop the reactions. When these tapes were placed in contact with agar the ampicillin and reactants diffused away rapidly, leaving the enzyme on the paper. For the biotape assay, samples (including *E. coli* cells) were diluted 1:100 and amounts (6 μ l.) applied in quadruplicate and dried with a stream of air at 5 cm. intervals along tapes (1 cm. wide) of Whatman no. 1 filter paper (Reeve Angel 200 m. reels). Ampicillin standard solutions in the range 100–6.25 μ g./ml. (doubling dilutions) were also applied to paper tapes. The tapes were placed in contact with agar seeded with *Bacillus subtilis* for bioassay in the usual way. The logarithm of the concentration of ampicillin was proportional to the zone diameter. 6-APA (1% concentration) gave no zone in the above assay at 1:100 dilution. The ampicillin standard was the monohydrate assaying as 105% pure, by the hydroxylamine method against benzylpenicillin (molecular weight corrected).

Anti-bacterial screening. The minimum inhibitory concentrations of the various hydroxyphenicillins for a range of bacteria were determined by serial dilution in nutrient broth as described by Sutherland (1964). These determinations were carried out by Mr R. Sutherland and his staff.

Preparation of various hydroxybenzylpenicillins. (1) Potassium 6-(*p*- α -hydroxyphenylacetamido)-penicillanate. A reaction mixture (0.5 l.) consisting of (final concns.) 4% 6-APA, 3.5% *p*-mandelamide and *E. coli* N.C.I.B. 8743 cells resuspended at ten times the density in the culture medium was adjusted to pH 6 and stirred for 16 hr. at 23° and pH 6. At 16 hr. the yield of α -hydroxybenzylpenicillin was 2.5 mg./ml. Bacterial cells were removed from the reaction mixture by centrifugation, the filtrate was cooled to 5°, acidified to pH 2 with conc. HCl and extracted with 0.5 vol. of *n*-butyl acetate. The acetate phase was rapidly back-extracted with 0.33 vol. of 0.1 M-phosphate buffer, pH 8.5, the pH being adjusted to 7.5 before separation of the phases. The buffer extract was adjusted to pH 2 and extracted with 0.5 vol. of *n*-butyl acetate, which after separation was dried with anhydrous MgSO₄ and decolorized by addition of 0.5% active charcoal. After the charcoal and MgSO₄ had been centrifuged, the potassium α -hydroxybenzylpenicillin was precipitated from this butyl acetate solution by adding, dropwise, 2 M-potassium 2-ethylhexanoate in *n*-butyl acetate (2-ethylhexanoic acid dissolved in *n*-butyl acetate and the theoretical amount of solid

KOH added; a small amount of undissolved material was filtered off and rejected). The precipitate was collected by centrifuging, washed twice with *n*-butyl acetate and twice with acetone before drying *in vacuo* to yield 0.7 g. of potassium 6-(*p*- α -hydroxyphenylacetamido)-penicillanate, $\alpha_D^{20} + 168^\circ$ and 92% pure by hydroxylamine assay (with potassium benzylpenicillin as standard and molecular weight correction). Bio-autography with *B. subtilis* gave a single zone at R_F 0.55 in B/E/W, R_F 0.9 in B/A/W and R_F 0.8 in B/P/W. The compound had half the migration rate of benzylpenicillin in the ether–water system.

(2) Potassium 6-(*L*- α -hydroxyphenylacetamido)-penicillanate. The preparation was exactly as described above except that *L*-mandelamide was used instead of the *D*-isomer. At 16 hr. the α -hydroxybenzylpenicillin content of a sample was found to be 5.5 mg./ml. by the hydroxylamine assay. Extraction of the whole reaction gave 1.3 g. of potassium 6-(*L*- α -hydroxyphenylacetamido)-penicillanate, 92% pure by hydroxylamine assay, $\alpha_D^{20} + 277^\circ$, and the same chromatographic properties as the *D*-compound above.

(3) Sodium 6-(3,4-dihydroxyphenylacetamido)-penicillanate. A reaction mixture (1 l.) consisting of (final concns.) 2% 6-APA and 0.4% 3,4-dihydroxyphenylacetic acid was prepared by dissolving the 6-APA at pH 7 and lowering the pH to 5.0 before adding the dihydroxyphenylacetic acid. *E. coli* N.C.I.B. 8743 cells were added to give a final density 15 times that in the growth medium and the reaction mixture was saturated with N₂ and stirred under N₂ for 5 hr. at pH 5 and 30°. The cells were removed from the reaction by centrifugation and the chilled supernatant was adjusted to pH 2 with dil. HCl and extracted with 3 $\times$ 0.5 vol. of *n*-butyl acetate. The pooled butyl acetate extracts were back-extracted by stirring with 0.33 vol. of water while under N₂, the pH being maintained at 8 with dil. NaOH. The aqueous phase (final pH 7) was concentrated to a small volume in a rotary vacuum evaporator and freeze-dried to give 2.5 g. of solid with a purity of 50% by iodometric assay (reaction with iodine followed by titration of untreated iodine with Na₂S₂O₃) against a sodium benzylpenicillin standard, with molecular weight correction. After prolonged drying over P₂O₅ the purity rose to 62% by iodometric assay. If care is not taken to avoid high pH and aerobic conditions a certain amount of autooxidation of the 3,4-dihydroxyphenylacetic acid or the resultant penicillin takes place and the product has about half the biological activity against a range of bacteria. The hydroxylamine assay method cannot be used for this penicillin because of a reaction between the phenolic side chain and the ferric reagent, which results in an excessively high blank value. The 3,4-dihydroxybenzylpenicillin can be detected on paper chromatograms by the bio-autographic procedure with *B. subtilis* or *Salmonella typhimurium* as seed organisms (R_F 0.25 B/E/W; R_F 0.85 B/A/W; zero migration in the ether–water system). An aq. 1% FeCl₃ spray also detects the penicillin.

(4) Potassium 6-(*p*-hydroxyphenylacetamido)-penicillanate. A reaction mixture (0.2 l.) consisting of (final concns.) 2% 6-APA, 0.5% *p*-hydroxyphenylacetic acid and *E. coli* (N.C.I.B. 8743 A) resuspended to give a final density ten times that in the growth medium, was adjusted to pH 5 and stirred for 4 hr. at 35° in the presence of 1% *n*-butyl acetate. The cells were centrifuged from the mixture and the supernatant was chilled before adjustment of the pH to 2.0 with HCl. The penicillin was extracted by shaking with

2 × 0.25 vol. of *n*-butyl acetate. After separation the solvent extracts were combined and shaken with 0.5 vol. of cold aq. 1% NaHCO₃. The bicarbonate extract was separated, adjusted to pH 2 and shaken with 2 × 0.4 vol. of *n*-butyl acetate. After separation the combined butyl acetate extracts were dried with anhydrous MgSO₄ and the penicillin was precipitated as the potassium salt by addition of potassium 2-ethylhexanoate, as for α -hydroxybenzylpenicillin. The precipitate was washed with acetone and dried under vacuum to give 710 mg. of potassium *p*-hydroxybenzylpenicillin, 86% pure by hydroxylamine assay in terms of potassium benzylpenicillin, with molecular weight

correction. Bio-autography with *B. subtilis* showed a zone at *R_F* 0.28 in B/E/W and a zone of 1 cm. migration in ether-water (18 hr.)

RESULTS AND DISCUSSION

Enzymic synthesis of benzylpenicillin. Fig. 1 shows progress curves for the synthesis of benzylpenicillin from 6-APA at two pH values and with three different sources of the phenylacetyl group. In the phenylacetic acid reaction mixture the initial rate of synthesis of benzylpenicillin at pH 5 is double that at pH 7. The reaction at pH 7 soon stops whereas at pH 5 the reaction continues with only a slight fall in rate over the first 80 min., suggesting that the reaction reaches equilibrium more rapidly at pH 7 than at pH 5. For phenylacetamide and phenylacetyl glycine the initial rates are very similar for the two pH values but after 40 min. the curves diverge, the reaction at pH 7 showing a rapid loss of penicillin whereas at pH 5 the rate of synthesis of penicillin just slows up and stops. An explanation for these results is that at pH 7 benzylpenicillin, phenylacetyl glycine and phenylacetamide are all rapidly hydrolysed. Initially the concentration of phenylacetyl glycine or phenylacetamide is sufficient at pH 7 to drive the penicillin synthesis faster than the penicillin hydrolysis but this is reversed when the activated forms of phenylacetic acid run out. The final products of the reactions at pH 7 are phenylacetic acid, 6-APA and glycine or ammonia, there being only a transient appearance of benzylpenicillin. At pH 5 the rate of deacylation of benzylpenicillin is slow enough to allow it to accumulate.

Enzymic synthesis of α -hydroxybenzylpenicillin. A more extensive study of the conditions affecting

Fig. 1. Effect of duration of reaction on the enzymic synthesis of benzylpenicillin at pH 5 and pH 7. The reaction mixtures consisted of 1% 6-APA, an equimolar concentration of phenylacetic acid (■, □), phenylacetamide (▲, △), or phenylacetyl glycine (●, ○) prepared in aqueous solution at a final pH of 5 or 7 and *E. coli* cells (N.C.I.B. 8743) resuspended in 0.5M-phosphate buffer at ten times the cell density in the growth medium. The reaction mixtures were adjusted to pH 5 (●, ▲, ■) or 7 (○, △, □) before shaking at 37°. The benzylpenicillin content was determined by hydroxylamine assay after solvent extraction.

Table 1. Effect of pH on the enzymic synthesis of α -hydroxybenzylpenicillin from DL-mandelic acid and DL-mandelamide

The final compositions of the reaction mixtures were as shown in the table plus cells of *E. coli* N.C.I.B. 8743 resuspended in 0.05M-phosphate buffer at ten times the cell density in the growth medium. The reaction mixtures at the final pH values shown were shaken at temperatures near the optimum, assayed for α -hydroxybenzylpenicillin content by bioassay (biotape) and the results were expressed in terms of potassium benzylpenicillin.

Reaction mixture	Reaction time (hr.)	Yield of α -hydroxybenzylpenicillin (mg./ml.) at the following pH values							
		4.0	4.8	5.0	5.2	5.6	6.0	7.0	8.0
2.8% DL-mandelamide	0.5	0.3	—	0.8	—	—	1.2	0.9	0.7
1.0% 6-APA (molar ratio 4:1); temp., 30°	1.0	0.5	—	1.3	—	—	2.0	1.8	1.3
1.4% DL-mandelic acid, 2.0% 6-APA (molar ratio 1:1); temp., 40°	1.0	—	0.4	—	0.5	0.5	0.3	—	—
	5.0	—	1.2	—	1.7*	1.1	0.3	—	—

* With the hydroxylamine assay also the optimum was found to be pH 5.2.

Table 2. *Effect of temperature on the enzymic synthesis of α -hydroxybenzylpenicillin from DL-mandelic acid and DL-mandelamide by E. coli N.C.I.B. 8743*

The reaction mixtures and assays were as described in Table 1, the pH values being adjusted to the optima (pH 6 for mandelamide and pH 5.2 for mandelic acid) and the reaction mixtures being shaken at the temperatures shown. Yields determined by bioassay are expressed in terms of potassium benzylpenicillin.

Source of side chain	Reaction time (hr.)	Yield of α -hydroxybenzylpenicillin (mg./ml.) at the following temperatures					
		20°	25°	30°	35°	40°	45°
2.8% DL-mandelamide	0.5	0.8	0.8	0.9	1.1	1.1	—
1.0% 6-APA, pH 6.0	1.0	1.2	1.5	1.7	1.7	1.8	—
1.4% DL-mandelic acid	3.0	—	—	1.2	1.3	1.8*	1.3
2.0% 6-APA, pH 5.2	5.0	—	—	1.7	2.3	2.4*	2.1

* With the hydroxylamine assay also the optimum temperature was found to be 40° over both 3 and 5 hr.

Table 3. *Enzymic synthesis of α -hydroxybenzylpenicillin from mandelic acid, mandelamide and mandelylglycine*

The final compositions of the reaction mixtures were as shown in the table plus cells of *E. coli* N.C.I.B. 8743 resuspended at ten times the cell density in the cultivation medium. The reaction mixtures were stirred at the approximate optimum pH and temperature for the particular side-chain derivative and the yield of α -hydroxybenzylpenicillin was determined by bioassay and hydroxylamine assay. Both assays were expressed in terms of potassium benzylpenicillin.

	Reaction mixture					Yield of potassium α -hydroxybenzylpenicillin (mg./ml.)				Ratio bioassay/hydroxylamine assay at	
	Side-chain derivative	6-APA	Molar	pH	Temp.	hydroxylamine assay		bioassay		assay at	
	%	%	ratio			2hr.	4hr.	2hr.	4hr.	2hr.	4hr.
DL-Mandelic acid	1.4	2.0	1.1	5.2	40°	1.60	2.71	2.51	3.86	1.57	1.42
DL-Mandelamide	1.4	0.5	4.1	6.0	20	1.72	1.60	1.57	1.25	0.91	0.78
DL-Mandelylglycine	1.94	0.5	4.1	6.0	20	3.32	2.95	2.51	1.46	0.76	0.49

penicillin synthesis was done, with DL- α -hydroxyphenylacetic acid or its amide (DL-mandelic acid and DL-mandelamide) to acylate 6-APA. The product of this reaction, DL- α -hydroxybenzylpenicillin, is somewhat more acid-stable than benzylpenicillin and is readily assayed by the hydroxylamine method or by bioassay. The effect of pH on the coupling of α -hydroxybenzylpenicillin from mandelic acid and mandelamide is shown in Table 1; the optimum for the acid was pH 5 whereas the optimum for mandelamide was pH 6 but the effect of pH was much less pronounced with the latter. These results are similar to those observed for benzylpenicillin synthesis (Fig. 1). The effect of temperature is shown in Table 2, the optimum probably being in the region 35–40°. For mandelamide only very small increases in rate were observed as the temperature was increased, possibly because of product instability. For mandelic acid the rate increased considerably between 30° and 40°, but the lower pH (5.2) would be more favourable for stability of the product.

Table 3 shows a comparison between mandelic acid, its amide and the glycol derivative (equimolar proportions). The conditions for each reaction were chosen to be a combination of the optimum for rate and the optimum for stability of product. Because of the increased efficiency of coupling with derivatives of mandelic acid the 6-APA concentration for these was one-quarter that for the mandelic acid reaction mixture. Even so the rate of coupling with DL-mandelylglycine was twice that for mandelic acid as judged by the hydroxylamine assay over the 2 hr. period, the conversion of 6-APA into α -hydroxybenzylpenicillin at 2 hr. being about 37%. The comparable conversions of 6-APA for mandelamide and mandelic acid were respectively 19% and 4.5% but the latter value may have been depressed by the fact that the ratio of side-chain compound to 6-APA was only 1:1. The bioassay results for the yields of α -hydroxybenzylpenicillin are quite different from the results of the hydroxylamine assay. The bioassay/hydroxylamine assay ratios (Table 3) show that the α -hydroxybenzylpenicillin made from

Table 4. *Effect of ratio of mandelamide or mandelic acid to 6-APA on the enzymic synthesis of α -hydroxybenzylpenicillin*

The reaction mixtures consisted of 6-APA and DL-mandelic acid or DL-mandelamide at the final concentrations shown in the table and *E. coli* cells resuspended at ten times the density of cells in the cultivation medium. The mandelamide reaction mixtures were incubated at 20° and a final pH of 6.0 and the mandelic acid reaction mixtures at 40° and a final pH of 5.2. Reaction mixtures were shaken for 3 hr. before assay of the α -hydroxybenzylpenicillin by the hydroxylamine method.

Reaction mixtures				Yield of potassium α -hydroxybenzylpenicillin at 3 hr. (mg./ml.)	Conversion of 6-APA into potassium α -hydroxybenzylpenicillin (%)
6-APA (%)	DL-Mandelamide (%)	DL-Mandelic acid (%)	Molar ratio		
0.5	0.35	—	1:1	0.35	4.6
0.5	0.7	—	1:2	0.63	8.2
0.5	1.4	—	1:4	1.25	16.3
0.5	2.8	—	1:8	2.0	26.2
2.0	—	0.35	1:0.25	0.42	1.4
2.0	—	0.7	1:0.5	0.84	2.7
2.0	—	1.4	1:1	1.25	4.1
2.0	—	2.8	1:2	1.15	3.8

DL-mandelic acid is more bioactive than that made from DL-mandelamide or DL-mandelylglycine. It was shown by measurement of optical rotation of the α -hydroxybenzylpenicillin from the DL-mandelic acid reaction mixture that it contained a proportion much greater than 50% of the more bioactive D-side-chain isomer.

Table 4 shows the effect of varying the ratio of mandelamide or mandelic acid to 6-APA in a reaction mixture. The concentrations of 6-APA were chosen to give comparable yields of penicillin for the two sets of reaction mixtures. For the mandelic acid reaction about four times the concentration of 6-APA was needed to obtain yields of penicillin comparable with those in the mandelamide reaction. The yields of α -hydroxybenzylpenicillin almost doubled for every doubling in DL-mandelamide concentration, except at the highest values. However, for DL-mandelic acid no increase occurred beyond a mandelic acid/6-APA molar ratio of 1:1, suggesting some kind of inhibition by mandelic acid.

End-product inhibition of α -hydroxybenzylpenicillin synthesis. The enzyme reaction leading to the synthesis of α -hydroxybenzylpenicillin from mandelic acid and 6-APA rapidly slowed down and stopped long before the reaction was complete. When more *E. coli* cells were added to a reaction that had stopped no further synthesis of α -hydroxybenzylpenicillin took place at pH 5.2 and 40°; in fact the penicillin concentration dropped from 1.1 mg./ml. to 0.71 mg./ml. over a period of 3 hr. It appears that enzyme inactivation was not the reason for the reaction stopping. When the α -hydroxybenzylpenicillin was removed from the reaction mixture by solvent extraction at pH 2

before the addition of more enzyme, further synthesis of α -hydroxybenzylpenicillin took place (at pH 5.2 and 40°), the concentration increasing from 0.08 mg./ml. to 0.27 mg./ml. over the 3 hr. after the addition. The low yield of penicillin was probably due to the fact that much of the mandelic acid was also extracted with the α -hydroxybenzylpenicillin at pH 2. Thus the accumulation of end product is associated with the cessation of the reaction, which comes to an equilibrium when only 6.6% of the 6-APA has been converted into α -hydroxybenzylpenicillin (from equimolar concentrations of 6-APA and DL-mandelic acid at pH 5.2 and 40°).

Effect of asymmetry in mandelamide on the rate of formation of α -hydroxybenzylpenicillin. The rate of enzymic transfer of the D- or L- α -hydroxyphenylacetyl group from D-, L- or DL-mandelamide to 6-APA was studied by assaying the yields of α -hydroxybenzylpenicillin by the hydroxylamine and bioassay methods. The results in Table 5 clearly show that the rates of synthesis are not the same for the two isomers. Initially the D- α -hydroxyphenylacetyl group is transferred more rapidly than the L-group but over long reaction times the yields of L- α -hydroxybenzylpenicillin as judged by chemical assay are higher than those of the D-penicillin.

Possible explanations for this are that either the α -hydroxybenzylpenicillin once formed starts to be deacylated, the D-compound being deacylated more readily than the L-compound (Cole, 1969b), or the reaction mixture with the L-compound reaches equilibrium at a higher concentration of α -hydroxybenzylpenicillin than that with the D-compound. The biological activity of the L-hydroxybenzylpenicillin against *B. subtilis* is clearly lower than

Table 5. *Synthesis of α -hydroxybenzylpenicillin from D-, L- or DL-mandelamide by E. coli N.C.I.B. 8743*

The reaction mixtures consisted of (final concns.) 4% 6-APA, 0.7% D-, L- or DL-mandelamide and *E. coli* cells resuspended at ten times the density of cells in the cultivation medium, all at final pH 6.0. The reaction mixtures were shaken at 20° and samples assayed for α -hydroxybenzylpenicillin content by the hydroxylamine method after separating the penicillin by solvent extraction. The yields were expressed in terms of mg. of potassium benzylpenicillin/ml. without correction for molecular weight or for extraction efficiency, as pure samples of the various isomers were not available. Penicillin content was also determined by bioassay (biotope) and the results again expressed in terms of mg. of potassium benzylpenicillin/ml.

Configuration of mandelamide	Yield of α -hydroxybenzylpenicillin (mg./ml.)						Ratio bioassay/hydroxylamine assay		
	hydroxylamine			assay					
				bioassay					
	1 hr.	2 hr.	16 hr.	1 hr.	2 hr.	16 hr.	1 hr.	2 hr.	16 hr.
DL	0.81	1.1	3.6	0.78	1.2	2.8	0.96	1.1	0.78
L	0.48	0.87	5.5*	0.19	0.24	2.3	0.40	0.28	0.42
D	1.1	1.8	2.5†	1.5	2.6	3.3	1.4	1.4	1.3

* Product has $[\alpha]_D^{20} + 277^\circ$.

† Product has $[\alpha]_D^{20} + 168^\circ$.

Table 6. *Enzymic synthesis of ampicillin from D- α -aminophenylacetamide; effect of temperature*

Reaction mixtures consisted of (final concns.) 1% 6-APA and 1.07% D- α -aminophenylacetamide hydrobromide and *E. coli* B.R.L. 1360 cells resuspended at ten times the cell density in the growth medium. The final pH was 6.0 and the reaction mixtures were incubated at the temperatures shown in the table and returned to pH 6 at 1 hr. Ampicillin yields were determined by the biotope assay.

Reaction time (hr.)	Temp. ...	Yield of ampicillin (mg./ml.)				
		10°	20°	30°	40°	50°
1		0.33	0.53	1.1	1.6	1.8
2		0.66	1.3	2.9	3.0	2.9

that of the D-compound, the results for both being expressed in terms of benzylpenicillin. The yields of penicillin obtained for the reaction mixture with the DL-compound were approximately midway between those for the D- and the L-compounds.

Enzymic synthesis of ampicillin. Unlike α -hydroxybenzylpenicillin, ampicillin is only synthesized in trace amounts from the free carboxylic acid side chain and 6-APA. About 50 μ g./ml. was detected in a reaction mixture saturated with α -aminophenylacetic acid and containing 3% 6-APA and *E. coli* acylase at pH 6. This low yield may in part reflect the very low water solubility of α -aminophenylacetic acid. In contrast D- α -aminophenylacetamide hydrobromide and D- α -aminophenylacetyl-glycine are both very water-soluble and give rapid synthesis of ampicillin from 6-APA in the presence of the *E. coli* acylase. No such synthesis

Table 7. *Enzymic synthesis of ampicillin from α -aminophenylacetamide; effect of starting pH on progress of reaction*

Reaction mixtures consisted of (final concns.) 1% 6-APA and 1.07% D- α -aminophenylacetamide hydrobromide (molar ratio 1:1) and *E. coli* B.R.L. 1360 cells resuspended at ten times the density in the growth medium. The starting pH values of the reaction mixtures were adjusted to the values shown in the table. The reaction temperature was 30°. Ampicillin yields were determined by the biotope assay.

Reaction time (hr.)	pH ...	Yield of ampicillin (mg./ml.)			
		6.0	7.0	8.0	8.5
0.75		1.0	2.8	2.8	2.0
1.5		1.2	3.0	3.1	2.8
2.25		1.7	3.6	1.9	2.3
4.5		2.6	2.2	0.6	0.8
6.5		3.1	1.3	0.0	0.0

occurred in the absence of the enzyme. The optimum temperature for the synthesis of ampicillin from D- α -aminophenylacetamide (Table 6) was in the region of 40° at pH 6, a pH favourable to product stability. The effect of pH on the synthesis of ampicillin from the amide is shown in Table 7, the optimum for initial rate being in the region pH 7–8.

As with α -hydroxybenzylpenicillin, rapid product deacylation occurs at higher pH values. The effect of lowering the pH from 7.0 to 6.0 after 1 hr. is shown in Table 8 for two strains of *E. coli*. For this experiment two reactions were started at pH 7 with

Table 8. *Enzymic synthesis of ampicillin from α -aminophenylacetamide: effect of pH change during the reaction for two E. coli cultures*

The reaction mixtures consisted of (final concns.) 1% 6-APA, 8.3% D- α -aminophenylacetamide hydrobromide (molar ratio 1:8) and *E. coli* cells resuspended at ten times the cell density in the growth medium. The final pH was 7.0 in all reaction mixtures. At 1 hr. the pH in one (B) of each pair of reaction mixtures was decreased to 6.0; A denotes the mixture at pH 7.0 throughout. All reaction mixtures were incubated at 30° throughout. The yield of ampicillin was assayed by the biotape method.

Reaction time (hr.)	Yield of ampicillin (mg./ml.) in reaction mixtures containing			
	<i>E. coli</i> N.C.I.B. 8743		<i>E. coli</i> B.R.L. 1360	
	A	B	A	B
0.5	2.3	2.5	2.1	2.3
1.0	4.0	4.0	4.4	4.3
1.5	4.8	5.2	4.6	5.7
2.5	5.0	8.3	5.9	8.0
3.5	5.6	8.7	6.4	8.7

an eightfold excess of D- α -aminophenylacetamide to maintain a prolonged supply of acyl groups. One of each pair of reaction mixtures was adjusted to pH 6 after 1 hr. and it is clear that this gives more favourable conditions for survival of ampicillin and even its continued synthesis.

Table 9 illustrates the synthesis of ampicillin from other derivatives of D- α -aminophenylacetic acid. The rates of synthesis again increase with increase in concentration of derivative except for the thioester, the results for which may be complicated by the non-enzymic synthesis of ampicillin. The methyl ester appears to be one of the best substrates, the yield of 10.1 mg. of ampicillin monohydrate/ml. representing a 60% conversion of the 6-APA. Paper-chromatographic analysis revealed the formation of large amounts of glycine in the reaction with α -aminophenylacetyl-glycine.

Enzymic synthesis of various hydroxybenzylpenicillins. The conditions that were found suitable for the synthesis of α -hydroxybenzylpenicillin from mandelic acid were applied to the enzymic synthesis of *p*-hydroxy- and 3,4-dihydroxy-benzylpenicillins.

Table 9. *Enzymic synthesis of ampicillin with various derivatives of D- α -aminophenylacetic acid*

Reaction mixtures consisted of (final concn.) 1% 6-APA and various concentrations of five derivatives of D- α -aminophenylacetic acid. Each reaction mixture also contained *E. coli* cells (N.C.I.B. 8743) resuspended to give a density ten times that in the growth medium. All reaction mixtures had a final pH of 7.0 and were incubated at 34° for 1 hr. before dilution of samples 1:100 and assay for ampicillin content by the biotape assay.

Molar ratio 6-APA/ α -aminophenylacetic acid derivative	X	Yield of ampicillin at 1 hr. from D- α -aminophenylacetyl-X (mg./ml.)				
		... -NH ₂ ·HBr	-NH·(CH ₂) ₂ OH	-NH·CH ₂ CO ₂ H	-S·CH ₂ CO ₂ H	-OCH ₃ ·HCl
1:1		2.6	<0.4	4.8	5.9	4.5
1:2		5.1	1.0	5.8	8.5	8.1
1:4		8.6	1.9	6.6	7.8	10.1

Table 10. *Minimum inhibitory concentrations for a collection of enzymically prepared hydroxybenzylpenicillins against a range of bacteria*

	Minimum inhibitory concentrations (μ g./ml.)				
	α -Hydroxy-benzylpenicillin (D-side chain)	α -Hydroxy benzylpenicillin (L-side chain)	<i>p</i> -hydroxy benzylpenicillin	3,4-dihydroxy benzylpenicillin	Benzylpenicillin
<i>Escherichia coli</i>	5.0	25.0	25.0	12.5	25.0
<i>Salmonella typhi</i>	2.5	12.5	5.0	0.5	5.0
<i>Salmonella typhimurium</i>	2.5	12.5	5.0	1.25	12.5
<i>Shigella shigae</i>	1.25	12.5	—	—	25
<i>Shigella flexneri</i>	2.5	12.5	25	5.0	25
<i>Klebsiella aerogenes</i>	250	125	250	250	250
<i>Pseudomonas pyocyanea</i>	> 500	> 500	> 500	> 500	> 500
<i>Proteus mirabilis</i>	2.5	5.0	—	2.5	5.0
<i>Bacillus subtilis</i>	0.1	0.25	0.5	0.25	0.1
<i>Staphylococcus aureus</i> (Oxford)*	0.05	0.1	0.05	0.1	0.02
<i>Streptococcus faecalis</i>	1.25	12.5	2.5	5.0	2.5

* The above penicillins are not stable to the penicillinase produced by penicillin-resistant strains of *Staphylococcus*.

The two isomers of α -hydroxybenzylpenicillin were also prepared by enzymic synthesis from D- and L-mandelamide. The resulting penicillins were isolated and their spectra of anti-bacterial activity determined in comparison with that of benzylpenicillin (Table 10).

Anti-bacterial activity of hydroxypenicillins. Enzymic synthesis has provided a route for the preparation of various hydroxypenicillins. The results of anti-bacterial screening given in Table 10 show some of these compounds to be very active, notably 3,4-dihydroxybenzylpenicillin, for which the following minimum inhibitory concentrations ($\mu\text{g./ml.}$) were recorded for various *Salmonella* spp: 1.25 (*typhimurium*), 0.5 (*typhi*), 0.5 (*London*), 1.25 (*cholerae-suis*), 1.25 *paratyphi* A, 0.25 *paratyphi* B. Certain strains of *E. coli* had minimum-inhibitory-concentration values as low as 1.25 $\mu\text{g./ml.}$, but Gram-negative organisms that produced β -lactamase had high values, suggesting instability of this penicillin to Gram-negative β -lactamase. Another disadvantage of the compound was its acid-instability. The screening results for D- α -hydroxybenzylpenicillin is rather similar to that of ampicillin and as with the latter compound the

D-diastereoisomer is more bioactive than the L-form (Gourevitch, Wolfe & Lein, 1962).

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REFERENCES

- Cole, M. (1969a). *Biochem. J.* **115**, 733.
Cole, M. (1969b). *Biochem. J.* **115**, 741.
Cole, M. (1969c). *Biochem. J.* **115**, 747.
Gourevitch, A., Wolfe, S. & Lein, J. (1962). *Antimicrobial Agents and Chemotherapy 1961*. Ed. by Finland, M. & Savage, G. M. Michigan: American Society for Microbiology.
Karnovsky, M. L., & Johnson, M. J. (1949). *Analyt. Chem.* **21**, 1125.
Kaufmann, W., Bauer, K. & Offe, H. A. (1960). *Antimicrobial Agents Annual 1960*, p. 1. New York: Plenum Press.
Patent (1962). *Brit. Patent* 897618 (Bayer).
Patent (1963). *Brit. Patent* 939708 (Beecham).
Patent (1964). *Brit. Patent* 945637 (Bayer).
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.
Sutherland, R. (1964). *J. gen. Microbiol.* **34**, 85.