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Hydrolysis of Penicillins and Related Compounds by the Cell-Bound Penicillin Acylase of *Escherichia coli*

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1. A method is given for the preparation of penicillin acylase by using *Escherichia coli* N.C.I.B. 8743 and a strain selected for higher yield. The enzyme is associated with the bacterial cells and removes the side chains of penicillins to give 6-aminopenicillanic acid and a carboxylic acid. 2. The rates of penicillin deacylation indicated that *p*-hydroxybenzylpenicillin was the best substrate, followed in diminishing order by benzyl-, DL-α-hydroxybenzyl-, 2-furylmethyl-, 2-thienylmethyl-, D-α-aminobenzyl-, *n*-propoxymethyl- and isobutoxymethyl-penicillin. Phenylpenicillin and DL-α-carboxybenzylpenicillin were not substrates and phenoxymethylpenicillin was very poor. 3. Amides and esters of the above penicillins were also substrates for the deacylation reaction, as were cephalosporins with a thienylmethyl side chain. 4. For the deacylation of 2-furylmethylpenicillin at 21° the optimum pH was 8·2. The optimum temperature was 60° at pH 7. 5. By using selection A of N.C.I.B. 8743 and determining reaction velocities by assaying yields of 6-aminopenicillanic acid in a 10 min. reaction at 50° and pH 8·2, the K_m for benzylpenicillin was found to be about 30 mM and the K_m for 2-furylmethylpenicillin, about 10 mM. The $V_{max.}$ values were 0·6 and 0·24 μmole/min./mg. of bacterial cells respectively.

Penicillin acylase is used on an industrial scale to catalyse the hydrolytic removal of the side chain of penicillins to give the nucleus, 6-APA*. This nucleus is the starting material for the synthesis of the new β-lactamase-stable and broad-spectrum penicillins (semi-synthetic), which are prepared by acylation of the 6-amino group. The naturally occurring penicillins, such as benzylpenicillin and phenoxymethylpenicillin synthesized by *Penicillium chrysogenum*, are used as substrates for the penicillin acylase.

Two main types of penicillin acylase have been found, one that readily deacylates phenoxymethylpenicillin and usually occurs in moulds and actinomycetes and the other that readily deacylates benzylpenicillin and usually occurs in bacteria. The present paper is concerned with the latter enzyme and in particular with the hydrolytic activity of the enzyme occurring in *Escherichia coli*.

In *E. coli* the enzyme is largely bound to the cell and, because it is readily available, stable and easy to handle in this form, killed *E. coli* cells have been used as the enzyme preparation throughout this work. It is recognized that such an enzyme preparation must contain other enzymes. However, the substrate-specificity results show such a narrow range of suitable structures that it seems reasonable to conclude that in *E. coli* a single enzyme is responsible for the reactions under investigation. The *E. coli* cells were always grown in the presence of phenylacetic acid as inducer.

The bacterial enzymes that catalyse the liberation of 6-APA from penicillin have been given several names, and it now seems to be generally agreed that the name penicillin acylase is the most suitable, preferably prefixed by the name of the side chain of the chief penicillin substrate (see reviews by Hamilton-Miller, 1966; Cole, 1967). The International Commission on Enzyme Nomenclature has given the enzyme the systematic name penicillin amidohydrolase (EC 3.5.1.11) but still retains the early, less satisfactory, trivial name penicillin amidase.

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

MATERIALS AND METHODS

Enzyme substrates and analytical standards. Pure sodium benzylpenicillin (Glaxo Crystapen; 1670 i.u./mg.) was used as a standard for the assay of other penicillins by the hydroxylamine procedure with molecular-weight correction (Boxer & Everett, 1949; Batchelor, Chain, Hardy, Mansford & Rolinson, 1961*a*). Pure potassium benzylpenicillin (Glaxo Laboratories Ltd., Greenford, Middlesex) was used for K_m determinations. Potassium 2-furylmethylpenicillin (90% pure) was prepared by fermentation, with 2-furylacetic acid as precursor, with *P. chrysogenum* (Patent,

1968). Reprecipitated material was used for the K_m measurements, the results being corrected for purity. The 6-APA was pure crystalline material obtained by recrystallization of bulk material (Beecham Research Laboratories Ltd., Betchworth, Surrey) by the method of Batchelor *et al.* (1961*a*).

α-Aminobenzylpenicillin (ampicillin, free acid), potassium α-phenoxyethylpenicillin (phenethicillin), sodium α-carboxybenzylpenicillin (carbenicillin) and sodium 2,6-dimethoxyphenylpenicillin (methicillin) were all the products of Beecham Research Laboratories. Potassium phenoxymethylpenicillin (penicillin V), benzylpenicillin diethylaminoethyl ester HI (penethamate hydriodide) and 2-thienylmethylcephalosporin pyridine (cephaloridine) were obtained from Glaxo. 2-Thienylmethylcephalosporin was cephalothin from Eli Lilly and Co., Indianapolis, Ind., U.S.A. 7-Aminocephalosporanic acid was prepared by enzymic removal of the thienylacetyl side chain of cephalothin by the *E. coli* acylase enzyme, as in the deacylation of benzylpenicillin to give 6-APA. Paper chromatography showed that a small amount of deacetyl-7-aminocephalosporanic acid was also present.

Potassium *p*-hydroxybenzylpenicillin was prepared from 6-APA and *p*-hydroxyphenylacetic acid by enzymic synthesis with the *E. coli* enzyme (Cole, 1969). Potassium α-hydroxy benzylpenicillin with D or L side chain was prepared from 6-APA and D- or L-α-hydroxyphenylacetamide by enzymic coupling by using the *E. coli* penicillin acylase (Patent, 1963). Benzylpenicilloic acid was prepared by treating 1% benzylpenicillin with 5M-NaOH for 2hr. at 37° and adjusting the pH to 8·5 to give a solution ready for use as a substrate. *N*-(Benzylpenicilloyl)-ε-aminocaproic acid, prepared as described by Batchelor, Dewdney & Gazzard (1965), was supplied by Dr F. R. Batchelor. The remaining penicillins and penicillin derivatives were prepared by members of the Chemistry and Development Departments of Beecham Research Laboratories.

Phosphate buffer. The 0·5M stock buffer (pH7·0) contained 24·3g. of KH_2PO_4 and 56·0g. of K_2HPO_4 in 1l. of water, and was freshly prepared. Lower or higher pH values were obtained by adding 5M-HCl or 5M-NaOH.

Preparation of E. coli *cells containing penicillin acylase.* The following method of preparation of the stock suspension particularly relates to *E. coli* N.C.I.B. 8743A, which was the preferred culture. The procedure for *E. coli* N.C.I.B. 8743 and B.R.L. 1360 was very similar, the only difference being that the final medium contained 0·08% phenylacetic acid (sodium salt, pH7) for N.C.I.B. 8743 and 0·1% for B.R.L. 1360 in both cases added initially instead of as a feed.

(1). Stock and working cultures. Agar slopes in test tubes (Oxoid CM55 blood–agar base, 35g./l.) were inoculated from a freeze-dried culture and incubated for 24hr. at 27° before storing at 5° for not more than 1 month. A working agar slope was inoculated from the stock slope and incubated for 24–48hr. at 27°.

(2). Shaken-flask inoculum stage. Medium (100ml.) was dispensed in 500ml. conical flasks which were then closed with cotton-wool plugs and autoclaved at 15lb./in.^{2} for 15min. The medium at pH7 before autoclaving consisted of 2·5% (w/v) yeast extract (Yeatex standard grade; The English Grain Co. Ltd., Burton-on-Trent, Staffs.) and 0·08% phenylacetic acid (converted into sodium salt with NaOH and adjusted to pH7 before addition to the medium). Each flask was inoculated with 0·5ml. of a suspension of *E. coli* cells prepared by adding 5ml. of sterile nutrient broth to a working agar slope. The flasks were shaken on a rotary shaker (1·25in. radius circle at 240rev./min. for 24hr. at 25°).

(3). Final cultivation stage (100l. fermenter). A 50l. batch of 2·5% (w/v) yeast extract as above and 10ml. of 20% silicone RD antifoam in water (Midland Silicones, London S.W.1) at pH7 before sterilization were sterilized in a 100l. fermenter at 120° for 15min. The medium was inoculated with the contents of three shaken flasks and stirred at 430rev./min. (impeller 7·5in. diam.) with an air flow of 20l./min. and temperature 24°. Starting at 6hr. and continuing up to 17hr., hourly additions of 100ml. of a 15% solution of phenylacetic acid (prepared as sodium salt in water and adjusted to pH7) were made. This represented a feed rate of 0·03% (w/v) phenylacetic acid/hr. Silicone antifoam was added when necessary. The cultivation stage was terminated at 19hr. or when the pH rose above 8·0.

(4). Collection of *E. coli* cells. *n*-Butyl acetate was added to the culture to give a final concentration of 1% (v/v) and stirring continued for 20min. to kill the cells. The cells were then collected by passing the culture through an air-driven Sharples centrifuge and suspended in deionized water (5% of original vol.). This stock was stored at 5° in the presence of 1% butyl acetate.

Routine test for penicillin acylase activity and the determination of the rates of deacylation of various penicillins. For routine assays on various batches of *E. coli* culture the penicillin acylase activity was expressed in terms of the yield of 6-APA in a suitable reaction mixture. This reaction mixture consisted of *E. coli* cells collected from the cultivation medium by centrifugation and thoroughly suspended in 3% potassium benzylpenicillin in 0·1M-phosphate buffer, pH8·5, so as to give a cell density half that in the cultivation medium. The reaction mixture was shaken at 40° for 1·5hr. before determining the yield of 6-APA by the following procedure. The *E. coli* cells were centrifuged (4000*g* for 15min.) from the reaction mixture and a suitable volume of supernatant was adjusted to pH2 with 5M-HCl. The unconverted benzylpenicillin and the phenylacetic acid product were then removed by shaking with 2×1vol. of *n*-butyl acetate and rejecting the acetate phase. The aqueous phase was returned to pH7 and after dilution to a known final volume the 6-APA content was assayed by the hydroxylamine method as described by Batchelor *et al.* (1961*a*), but with pure 6-APA as a standard.

The rates of deacylation of various penicillins listed in Table 1 were also determined by assaying 6-APA production by the above method except in the following instances. The rate of ampicillin deacylation was measured by bioassay of the resultant 6-APA after separation from unconverted ampicillin by paper chromatography by the method described by Cole & Sutherland (1966). Methicillin and carbenicillin reaction mixtures were examined for 6-APA formation by the same method, but as no zones of 6-APA were detected the rate was recorded as zero.

Methods for K_m and $V_{max.}$ experiments. Solutions (2%) of potassium benzylpenicillin or potassium 2-furylmethylpenicillin in 0·05M-phosphate buffer, pH8·2, were prepared. Cells of *E. coli* N.C.I.B. 8743A required for K_m determinations were taken from the stock suspension and washed with 0·05M-phosphate buffer, pH8·2, 20ml. of stock suspension being centrifuged down at 26000*g* and the cells resuspended

in 20ml. of fresh phosphate buffer. These cells were again centrifuged down and resuspended in 200ml. of 0·05M-phosphate buffer, pH 8·2. Then 25ml. of this cell suspension was mixed with 25ml. of penicillin solution to give final concentrations of penicillin of 1·0, 1·25, 1·5, 2·0, 2·5 and 4·0mg./ml. The final *E. coli* cell density was similar to that in the original growth medium, the final dry weight (after 24hr. at 90°) of *E. coli* cells in the reaction mixture being 4·55mg./ml.

The reaction mixtures were stirred at 50° for 10min. The pH was automatically controlled at pH 8·2 by the addition of 0·25M- or 1M-NaOH with a Pye autotitrator. At the end of 10min. the pH was rapidly lowered to 2·0 with 5M-HCl. The whole of the reaction mixture was then shaken with 100ml. of cold *n*-butyl acetate saturated with water and centrifuged at 26000*g*. A 50ml. portion of the 6-APA-containing aqueous layer was then removed and added to a further 100ml. of cold *n*-butyl acetate and shaken, the phases were allowed to separate and 30ml. of the aqueous 6-APA-containing layer was readjusted to pH 7 with a few drops of 1M-NaOH. This aqueous solution was then assayed for 6-APA content by using the hydroxylamine assay with NaOH blank as described by Batchelor *et al.* (1961*a*), the results being expressed in terms of a pure 6-APA standard assayed in the same experiment. Bioassay of 6-APA solutions obtained after the above extraction procedure showed them to be free of penicillin.

Paper-chromatographic examination of reaction mixtures. Penicillins and 6-APA were separated on 1cm.-wide Whatman no. 1 paper strips with various solvent systems (see the tables). Penicillin zones on these chromatograms were detected by the bio-autographic procedure with *Bacillus subtilis* as seed organism. Zones of 6-APA were detected in a similar fashion except that the chromatograms were phenylacetylated before application to the seeded agar as described by Batchelor *et al.* (1961*a*). Similar procedures were used for cephalosporins and 7-amino-cephalosporanic acid. Penicilloic acids were detected on paper chromatograms by using the starch–iodine spray described by Thomas (1961).

RESULTS

Penicillin acylase-producing bacteria. The bacterial strains used in the present study were discovered as a result of screening for penicillin deacylation (Cole & Sutherland, 1966). *E. coli* strain B.R.L. 1360, one of the first selected for study, produced a smooth type of colony and was replaced by strain B.R.L. 1040 (N.C.I.B. 8743, rough colony), which was equally active but more easily centrifuged. For the most recent experiments a more active selection

Table 1. *Relative rates of deacylation of a series of penicillins by* E. coli

Each penicillin was compared with potassium benzylpenicillin in a separate experiment. Reaction mixtures at pH 8·5 and 37° contained a final concentration of 5mg. of penicillin (potassium salt)/ml. in 0·1M-phosphate buffer and *E. coli* cells (N.C.I.B. 8743 or N.C.I.B. 8743A) resuspended at the same cell density as that in the cultivation medium. Samples withdrawn at various times were assayed for 6-APA content by the hydroxylamine assay after removal of unconverted penicillin by solvent extraction. The percentage conversion/unit time for benzylpenicillin varied slightly from experiment to experiment, and hence the rates for other penicillins are expressed relative to the rate for benzylpenicillin. Typical values for the hydrolysis of potassium benzylpenicillin to 6-APA by *E. coli* N.C.I.B. 8743 were: ½hr., 29%; 1hr., 47%; 2hr., 74%; and for N.C.I.B. 8743A: ½hr., 85%; 1hr., 92%.

Penicillin	*E. coli* strain (N.C.I.B. no.)	Relative rate of conversion of penicillin into 6-APA at ½hr.	1hr.	2hr.
Benzylpenicillin (Penicillin G)	8743 and 8743A	100	100	100
2-Furylmethylpenicillin	8743A	84	91	—
2-Thienylmethylpenicillin	8743	72	80	78
DL-α-Hydroxybenzylpenicillin	8743	78	88	78
p-Hydroxybenzylpenicillin (Penicillin X)	8743	156	150	—
D-α-Aminobenzylpenicillin	8743	49	50	—
n-Propoxymethylpenicillin	8743A	—	—	40†
Isobutoxymethylpenicillin	8743A	—	—	29†
Phenoxymethylpenicillin	8743	—	5·5 (7·6)*	6·5
α-Phenoxyethylpenicillin	8743	—	<5 (2·3)*	—
Phenylpenicillin	8743	—	<5 (1·8)*	—
Heptylpenicillin	8743	—	<5 (0·6)*	—
α-Carboxybenzylpenicillin	8743A	—	<5	—
2,6-Dimethoxyphenylpenicillin	8743	—	<5	—

* Because of a very slow reaction, the percentage conversion in 4hr. was used to calculate the percentage conversion at 1hr., which was in turn related to percentage conversion for benzylpenicillin at 1hr. to give the relative rate.

† Measured with [S] at 10mg./ml.; results are relative to a benzylpenicillin control also at 10mg./ml.

Table 2. *Rates of deacylation of isomers of α-hydroxybenzylpenicillin*

α-Hydroxybenzylpenicillin (2mg./ml. on a pure-material basis) in 0·05M-phosphate buffer, pH 8·0, was shaken at 35° with *E. coli* N.C.I.B. 8743 cells resuspended to give a final cell density one-quarter of the density in the culture medium. Concentrations of 6-APA were measured by extracting unconverted penicillin with solvent and assaying the 6-APA in the aqueous phase by the hydroxylamine method.

Configuration of side chain in α-Hydroxybenzylpenicillin	% conversion into 6-APA at ½hr.	2hr.
D	23·0	53·8
L	20·0	47·8
DL	20·6	45·5

from strain N.C.I.B. 8743 was used (N.C.I.B. 8743A) having been obtained after prolonged incubation in a corn-steep-liquor medium. No differences were noted in the properties of the acylase enzyme in the various *E. coli* strains mentioned above and almost all the enzyme was associated with the bacterial cells. None of the strains produced significant amounts of penicillinase (β-lactamase) when cultivated under conditions suitable for high penicillin acylase production.

Enzyme activity. The benzylpenicillin acylase activities of the cells in 1ml. of culture were as follows: N.C.I.B. 8743, dry weight of cells 3·5mg., yield of 6-APA in routine assay, 2·14mg.; N.C.I.B. 8743A, dry weight 5·36mg., 6-APA 5·18mg. Thus N.C.I.B. 8743A produced more cells with a higher intrinsic activity than N.C.I.B. 8743.

Deacylation of penicillins: substrate spectrum. The relative rates of deacylation of a series of penicillins (Table 1) show that penicillins with side chains closely related in size or character to the phenylacetyl side chain of benzylpenicillin are the best substrates. Hydroxylation of benzylpenicillin in the *p*-position provides an even better substrate than benzylpenicillin. In benzylpenicillin, insertion of an oxygen atom between the α-carbon atom and the benzene ring of the side chain or deletion of the α-methylene group in this side chain gives compounds that are very poor substrates. Of the aliphatic compounds tested, heptylpenicillin (penicillin K) is a very poor substrate, but isobutoxymethyl- and *n*-propoxymethyl-penicillin are better substrates. Variation of the configuration of the hydroxy substituent on the α-carbon atom of the benzylpenicillin side chain has little effect, as is shown in experiments at lower cell density (Table 2). The enzyme does not seem to be specific for acyl derivatives of 6-APA because the acyl side chain is also readily removed from penicillin derivatives and even cephalosporins to give 6-APA derivatives and 7-aminocephalosporanic acid respectively, as illustrated by the examples in Table 3.

Effect of pH *on the hydrolysis of 2-furylmethylpenicillin.* Cells of *E. coli* N.C.I.B. 8743A, washed in phosphate buffer, pH 7, were resuspended in 1% (w/v) potassium 2-furylmethylpenicillin to give a cell density twice that in the culture medium. The reaction mixture was kept stirred at 21° and constant pH by the automatic addition of 0·1M-sodium hydroxide with a Pye autotitrator. The rate of reaction at various pH values was determined by measuring the rate of addition of sodium hydroxide and the yield of 6-APA at ½hr. by the hydroxylamine assay after solvent extraction of unconverted substrate. The pH optimum was found to be 8·2 by both methods, although the sodium hydroxide titration method gave higher rates because of the release of acidic material from the bacterial cells and the neutralization of a small amount of penicilloic acid that was also formed. The pH curve was quite broad, the rate at pH 7·6 and 8·8 (23·5μg./min./ml.) being only slightly lower than that at the optimum, pH 8·2 (28·6μg./min./ml.; results by hydroxylamine method). The pH optimum was similar when the reaction mixtures contained phosphate. The temperature used was kept low at 21° to minimize the formation of penicilloic acid at the alkaline pH used. A very similar pH optimum was obtained with benzylpenicillin as substrate.

Effect of temperature on the hydrolysis of 2-furylmethylpenicillin. Cells of *E. coli* N.C.I.B. 8743A were resuspended, at a cell density half that in the cultivation medium, in 2% (w/v) potassium 2-furylmethylpenicillin in 0·1M-phosphate buffer, pH 7. The rate of reaction was determined by measuring the yield of 6-APA at ½hr. by the hydroxylamine assay after solvent extraction of unconverted substrate. The rate of formation of 6-APA at 30°, 40°, 50°, 55°, 60° and 70° was respectively 25, 49, 67, 80, 123 and 28μg./min./ml. In a comparable experiment with potassium benzylpenicillin the following rates were obtained for the same series of temperatures: 33, 72, 111, 153, 130 and 46. A pH of 7 was chosen for this experiment so as to minimize the formation of penicilloic acid, which can become appreciable at alkaline pH values.

K_m and $V_{max.}$ for the penicillin acylase of E. coli *N.C.I.B. 8743A.* The velocity of the penicillin-deacylation reaction may be determined by measuring the rate of disappearance of the penicillin or the rate of appearance of carboxylic acid, both relatively non-specific, or the rate of appearance of 6-APA. The third method, although laborious, was used here. K_m and $V_{max.}$ values were determined by Lineweaver–Burk plots and are shown in

Table 3. *Penicillin derivatives and related substances hydrolysed by* E. coli *N.C.I.B. 8743A*

Substrate concentrations varied between experiments but were in the range 2–10mg./ml. The cells of *E. coli* N.C.I.B. 8743A were resuspended at a final cell density twice that in the growth medium. Rate of reaction was judged from size of substrate and product zones on chromatograms of samples taken between 1 and 5hr. Chromatography solvents: (1) butan-1-ol–ethanol–water (4:1:5, by vol., top phase); (2) butan-1-ol–pyridine–water (1:1:1, by vol.); (3) butan-1-ol–acetic acid–water (12:3:5, by vol.); (4) butan-1-ol–acetic acid–water (8:3:8, by vol.); (5) paper-strip electrophoresis for 2·5 hr. at 17 v/cm. at pH 4·5 (pyridine–acetic acid buffer).

Substrate	R_F in solvent no.	Reaction conditions	Products	R_F in solvent no.	Rate and degree of conversion
Benzylpenicillin amide†	0·9 (1)	35°, pH 8·0	6-APA amide*	0·62 (1) 0·88 (2)	Rapid, complete
			also some of this compound with β-lactam ring opened	0·36 (2)	
Benzylpenicillin methyl ester†	0·95 (1)	30°, pH 7·0	6-APA methyl ester* also small amount of 6-APA*	0·8 (1) 0·11 (1)	Rapid, complete
Benzylpenicillin cyanomethyl ester†	0·92 (3)	35°, pH 7·0	6-APA cyanomethyl ester* also a very small amount of 6-APA*	0·65 (3) 0·4 (3)	Rapid, complete
Benzylpenicillin acetoxymethyl ester†	0·92 (1)	30°, pH 6·0	6-APA acetoxymethyl ester*	0·75 (1)	Fairly rapid, complete
Benzylpenicillin diethylaminoethyl ester HI	0·78 (1)	35°, pH 6·0	6-APA diethylaminoethyl ester* also some 6-APA* present	0·37 (1) 0·14 (1)	Fairly rapid
Benzylpenicillin phenacyl ester†	0·92 (1)	30°, pH 7·0	6-APA phenacyl ester*	0·84 (1)	Slow
Benzylpenicillin acetonyl ester†	0·94 (3)	30°, pH 7·0	6-APA acetonyl ester* and some 6-APA*	0·67 (3)	Fairly rapid
Benzylpenicillin thiomethyl ester† (contaminated with some benzylpenicillin)	0·94 (1)	30°, pH 7·0	6-APA thiomethyl ester* and some 6-APA*	0·8 (1) 0·14 (1)	Rapid, complete
Benzylpenicilloic acid	0·51 (2)	37°, pH 8·5	Deacylpenicilloic acid‡, i.e. 6-APA with β-lactam ring opened	0·31 (2)	Slow
N-Phenylacetyl-cyclic-DL-cysteinyl-D-valine	0·7 (2)	37°, pH 8·0	cyclic-cysteinylvaline§ (suspect only L-cysteinyl-D-valine is formed)	0·5 (2)	Rapid, incomplete (50%)
N-(Benzylpenicilloyl)-ε-aminocaproic acid	4cm. to +ve (5)	37°, pH 8·0	*N*-(deacylpenicilloyl)-ε-aminocaproic acid‡§	0cm. (5)	Slow
2-Furylmethylpenicillin methyl ester†	0·89 (1)	37°, pH 7·0	6-APA methyl ester* and some 6-APA*	0·8 (1)	Rapid, complete
n-Propoxymethylpenicillin cyanomethyl ester†	0·85 (3)	35°, pH 7·0	6-APA cyanomethyl ester* and some 6-APA*	0·7 (3)	Slow
2-Thienylmethylcephalosporin	0·32 (1) 0·71 (2) 0·75 (3)	37°, pH 8·0	7-aminocephalosporanic acid* (7-ACA)	0·06 (1) 0·39 (2) 0·25 (3) 0·7 (4)	Rapid, complete
			also some deacetyl 7-ACA* (suspect acetylesterase present in *E. coli* preparation)	0·1 (3) 0·5 (4)	
2-Thienylmethylcephalosporin pyridine	0·26 (1) 0·63 (2) 0·45 (3)	37°, pH 7·5	7-aminocephalosporanic acid pyridine*	0·03 (1) 0·2 (2) 0·05 (3)	Fairly rapid, complete

* Only bioactive after phenylacetylation of paper chromatograms. Zones were detected by contact with agar seeded with *B. subtilis*. Zones of penicillins and cephalosporins were detected similarly but without phenylacetylation.

† Dissolved or suspended compound in ethanol. The final concentration of ethanol in the reaction mixture was 5–10%; dimethylformamide was used in place of ethanol for benzylpenicillin cyanomethyl ester; acetone was used in place of ethanol for *n*-propoxymethylpenicillin cyanomethylester.

‡ Detected by spraying chromatogram with starch–iodine spray (Thomas, 1961).

§ Detected by dipping chromatogram in ninhydrin reagent (0·1% indanetrione hydrate in butan-1-ol).

Table 4. *K_m and $V_{max.}$ for penicillin acylase* (E. coli *N.C.I.B. 8743A*) *against benzyl- and 2-furylmethylpenicillins*

The reaction mixtures for these determinations consisted of washed *E. coli* N.C.I.B. 8743A cells resuspended at the final concentration of 4·55mg. dry wt. of cells/ml. in 0·05M-phosphate buffer and various concentrations of substrate in the range 1–10mg./ml. Reactions were maintained at 50° and pH8·2 with an autotitrator. The yield of 6-APA at 10min. as determined by hydroxylamine assay was used to calculate the initial velocities. The potassium 2-furylmethylpenicillin used in these experiments was 90% pure by the hydroxylamine assay compared with potassium benzylpenicillin, but the K_m values have been corrected to refer to 100% purity. $V_{max.}$ and K_m were calculated from plots of $1/v$ against $1/[S]$.

		K_m		$V_{max.}$		
Substrate	Expt. no.	(mg./ml.)	(mM)	(μg. of 6-APA/min./ml.)	(μmoles of 6-APA/min./ml.)	(μmole of 6-APA/min./mg. of cells)
Potassium benzylpenicillin (100% pure)	1	11·49	30·9	625	2·89	0·614
	2	11·11	29·9	588	2·72	0·578
Potassium 2-furylmethylpenicillin	1	3·53	9·75	250	1·16	0·246
	2	3·60	9·95	238	1·10	0·234

Table 4 for benzylpenicillin and 2-furylmethylpenicillin.

By using the $V_{max.}$ values for potassium benzylpenicillin to calculate the units of enzyme activity, a value of 2·8units/ml. of culture or 0·6unit/mg. dry wt. of *E. coli* cells was obtained. For this calculation 1unit of enzyme was taken as the amount catalysing the hydrolysis of 1μmole of potassium benzylpenicillin/min. at 50° and pH8·2.

DISCUSSION

It is unlikely that the penicillin acylase is the only enzyme present in the killed cells of *E. coli* N.C.I.B. 8743 and it could be argued that the presence of other enzymes would confuse any substrate-specificity work. However, the cells of this strain have a remarkably narrow substrate range, activity being confined to compounds containing the acyl group $R \cdot CH_2 \cdot CO-$, where R is phenyl, furyl, thienyl or an unbranched carbon chain of three or four carbon atoms, or *n*-propoxy. Substitution with an amino or hydroxyl group on the α-carbon atom is associated with a fall in hydrolysis rate, and α-carboxybenzylpenicillin is not a substrate. It is noteworthy that *p*-hydroxybenzylpenicillin is hydrolysed more rapidly than benzylpenicillin. Insertion of an oxygen atom between phenyl and $-CH_2 \cdot CO-$ groups, as in phenoxymethylpenicillin, is associated with a marked fall in the rate of hydrolysis, in contrast with the big increase in hydrolytic rate observed with the penicillin acylase of moulds and *Streptomyces* (Rolinson *et al.* 1960; Cole, 1966). The *Streptomyces lavendulae* enzyme also hydrolyses aliphatic penicillins (Batchelor, Chain, Richards & Rolinson, 1961*b*), and significantly the carbon chains of the acyl side chain of the best substrate for this enzyme are longer than in the best substrates for the *E. coli* acylase.

The penicillin substrate spectra for the *Nocardia* and *Proteus rettgeri* enzymes (Huang, Seto & Shull, 1963) are indistinguishable and although similar to that for *E. coli* there are certain differences. For the *Proteus* and *Nocardia* enzymes the hydrolysis rate does not fall nearly so much as with the *E. coli* enzyme when going from benzylpenicillin to phenoxymethylpenicillin. Also, the hydrolysis rate falls with *p*-hydroxy substitution whereas it increases with the *E. coli* enzyme. The best alkyl penicillin is structurally very similar to *n*-propoxymethylpenicillin, which is rapidly hydrolysed by the *E. coli* enzyme. Phenylpenicillin is a very poor substrate for all of these enzymes. The substrate specificities of the penicillin acylases of *Bacillus megaterium* (Chiang & Bennett, 1967) and *Alcaligenes faecalis* (Claridge, Luttinger & Lein, 1963) are also similar to that for *E. coli*.

Penicillin amides and esters and cephalosporins are also substrates for the *E. coli* penicillin acylase provided that the acyl side chain is phenylacetyl, thienylacetyl or a closely related structure. Sjöberg, Nathorst-Westfelt & Örtengren (1967) have also reported that *E. coli* will rapidly hydrolyse cephalosporins but not cephalosporin C. Walton (1964) and also Huang *et al.* (1963) have reported that acylases from other bacteria hydrolyse not only the side chain from penicillins but also the side chains from cephalosporins, again illustrating the specificity of these enzymes for the acyl group and not the nucleus.

There are very few literature K_m values for penicillin acylase with which to compare those for *E. coli* N.C.I.B. 8743A. Two that have been reported, 4·5mM for *B. megaterium* (Chiang & Bennett, 1967) and 1·35–1·59mM for *E. coli* (Brandl,

1965), both with soluble enzyme and benzylpenicillin as substrate, are much lower than the 30mM reported here for the cell-bound enzyme of *E. coli* N.C.I.B. 8743A. The difference could be the result of a permeability barrier for the latter enzyme but comparison is made difficult because assay procedures vary considerably and a lower K_m was obtained with 2-furylmethylpenicillin (K_m = 10mM). The K_m values reported for soluble fungal penicillin acylases with phenoxymethylpenicillin were 2·5–2·75mM for *Fusarium semitectum* (Brandl, 1965) and 10·3mM for *Streptomyces lavendulae* (Batchelor *et al.* 1961*b*).

The optimum pH and temperature for the hydrolysis of 2-furylmethylpenicillin by *E. coli* N.C.I.B. 8743A are 8·2 and 60° respectively, similar results being obtained with benzylpenicillin. These values are similar to previous results (Rolinson *et al.* 1960; Huang *et al.* 1963; Brandl, 1965; Szentirmai, 1966*a*; Chiang & Bennett, 1967), except that the optimum temperature was somewhat higher, probably as a result of different methods being used.

Penicillin acylases are reversible enzymes (Rolinson *et al.* 1960; Kaufmann, Bauer & Offe, 1960; Batchelor *et al.* 1961*b*), but in the region of pH 8 the hydrolytic reaction is predominant, low concentrations of penicillin being completely converted into 6-APA. At very high substrate concentrations, end products accumulate to such a great extent that the reaction slows and comes to an equilibrium at about 60–80% conversion, depending on the pH and substrate concentration. Chiang & Bennett (1967) have reported that phenylacetic acid (K_i very high) is a competitive inhibitor and 6-APA a non-competitive inhibitor of the penicillin acylase of *B. megaterium*. Kaufmann (1964) reported that carboxylic acids could act as inhibitors of the the hydrolysis of penicillins by *E. coli* penicillin acylase, the effect being more pronounced when ampicillin was used as substrate. Szentirmai (1966*b*) also reported that phenylacetic acid, phenoxyacetic acid and phenoxymethylpenicillin inhibited benzylpenicillin hydrolysis.

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