limpet encounters D-fucose residues in its diet, but the possibility that one or other of the two enzymes under discussion may hydrolyse α -L-arabinosides has still to be studied (see Wallenfels & Malhotra, 1960).

SUMMARY

1. The limpet, *Patella vulgata*, contains an enzyme that hydrolyses β -D-fucosides. This enzyme and β -D-galactosidase can be separated from each other by heat inactivation at suitable pH values.

2. Both enzymes are inhibited by galactonolactone and fuconolactone, but inhibition is much more pronounced when the inhibitor and substrate are derived from the same sugar.

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Penicillinase from *Klebsiella aerogenes*

A COMPARISON WITH PENICILLINASES FROM GRAM-POSITIVE SPECIES

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The introduction of α -aminobenzylpenicillin (Penbritin, ampicillin), an antibiotic active against Gram-negative organisms (Rolinson & Stevens, 1961*a*), has revived interest in the penicillinases [penicillin amidohydrolases, EC 3.5.2.6, called β -lactamases by Pollock (1961)] produced by members of the Enterobacteriaceae.

During a clinical trial of ampicillin carried out by Trafford *et al.* (1962) several penicillinase-producing coliform organisms were isolated, one of which is the subject of the present paper. The penicillinase from this organism was markedly different from the penicillinases of Gram-positive bacteria.

METHODS AND MATERIALS

Reagents. The following penicillins were used: sodium penicillin G, obtained from Glaxo Laboratories, Greenford, Middlesex; penicillin V (phenoxymethylpenicillin), phenoxethylpenicillin (Broxil, phenethicillin), phenoxypentylpenicillin (propicillin), 2,6-dimethoxyphenylpenicillin (Celbenin, methicillin), α -aminobenzylpenicillin (Penbritin, ampicillin), 4-isoxazolyl-5-methyl-3-phenylpenicillin (BRL 1400) and 6-aminopenicillanic acid, all gifts from Beecham Research Laboratories, Brockham Park, Betchworth, Surrey; and phenoxymethylpenicillin (Penspek), a gift from The Distillers Co. (Biochemicals) Ltd., Speke.

Phosphate-buffered saline was made by diluting 100 ml. of 0.2M-phosphate buffer, pH 7.4 [81 ml. of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (35.6 g./l.) plus 19 ml. of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (31.2 g./l.)], with 300 ml. of 0.9% NaCl and 400 ml. of water.

Organisms. A motile variant of *Klebsiella aerogenes* (laboratory number 418) was isolated from a patient with a post-operative chest infection. It gave a positive reaction in the filter-membrane test of Knox & Smith (1961*a*) and a negative penicillin-amidase test (carried out by the butyl acetate-extraction technique of Batchelor, Chain & Rolinson, 1961*b*), indicating that it produced a penicillinase. The organism produced acid and gas from the following compounds: glucose, lactose, sucrose, maltose, mannitol, salicin, adonitol and inositol. Dulcitol was not fermented, nor was H_2S or indole produced. It was weakly urease-positive, Voges-Proskauer-negative, and methyl red- and malonate-positive. Growth occurred on citrate as the sole carbon source. Gelatin was not liquefied.

Bacillus cereus NRRL 569 and the following strains of penicillinase-producing *Staphylococcus aureus* were used as representative of Gram-positive penicillinase-producing organisms: E3 (Knox, 1960), 13137 (Jevons, 1961), 8354/6/1 (Gorrell, 1957) and 110 (a recently isolated clinical strain).

Organisms were maintained on agar slopes and stored at 4° between subcultures.

Penicillinase preparations. Organisms were inoculated into infusion broth (Southern Group Laboratories, Hither Green, London, S.E. 13) and incubated at 37° overnight

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without shaking. Sodium *p*-chloromercuribenzoate (1 mM) was added to stop induction (Steinman, 1961), and organisms were removed by centrifuging at 2000g for 15 min. Supernatant fluids from *B. cereus* cultures were Seitz-filtered and stored at 4°. Sedimented pellets of *K. aerogenes* and *S. aureus* were washed in phosphate-buffered saline, and resuspended to the required concentration either in phosphate-buffered saline or in 0.9% NaCl. *K. aerogenes* suspensions were disrupted at 3000 lb./in.² in the liquid-extrusion press of Milner, Lawrence & French (1950), as modified by Wimpenny (1962); the treated suspensions were centrifuged at 2000g for 15 min., after which the penicillinase activity was found in the opalescent supernatant; the latter was clarified by centrifuging at 20000g for 50 min., and this supernatant, containing all the penicillinase activity, was the 'cell-free' enzyme preparation.

Unless otherwise stated, penicillinase of *S. aureus* was induced by growing the cells in the presence of 0.5 µg. of methicillin/ml.; this increased the enzyme activity about 30-fold. *B. cereus* was grown in 5 µg. of the same inducing agent/ml., which resulted in a 100-fold increase in total penicillinase activity.

Bacillus licheniformis penicillinase was obtained from the Wellcome Research Laboratories, Beckenham, Kent. It was a cell-free culture filtrate.

Penicillinase assay. The hydroxylamine assay as described by Knox & Smith (1962) was used.

The spectrophotometric assay was essentially as described by Saz, Lowery & Jackson (1961): 1 cm. cuvettes containing 0.5 ml. of 25 mM-phosphate buffer, pH 7.4, 0.25 ml. of 0.008% phenol red solution, with substrate, enzyme and water to 3 ml., were read at intervals in a Unicam SP.500 spectrophotometer at 558 mµ and 22°.

A colorimetric modification of this method was used with slower reactions. Tubes containing 1.5 ml. of 25 mM-phosphate buffer, pH 7.4, and 1.5 ml. of 0.008% phenol red solution, plus enzyme solution, substrate and any other additional requirements to a final volume of 9 ml., were incubated at 37° and read at intervals in an EEL colorimeter (filter no. 625).

The spectrophotometric method was used for K_m studies (except with 6-aminopenicillanic acid); the colorimetric modification was used to measure inhibition of penicillin G hydrolysis, and the hydroxylamine assay for all other studies.

Chromatography. Washed thick suspensions of organisms in phosphate-buffered saline were incubated at room temperature with 20 mg. of penicillin G/ml. for 1 hr.; the organisms were removed by centrifuging and 5 µl. of each supernatant was applied to Whatman no. 1 paper, with markers of penicillin G, 6-aminopenicillanic acid and their cognate penicilloic acids (which were made by dissolving the parent compounds in *N*-NaOH, allowing them to stand for 30 min. and then neutralizing with *N*-HCl). Papers were developed for 16 hr. at room temperature in descending butan-1-ol-acetic acid-water (4:1:5, by vol.), dried, dipped in 0.2% (w/v) ninhydrin in acetone containing 1% (v/v) of pyridine, and heated at 80° for 10 min.

Antibiotic sensitivities. Dilutions of the different penicillins were made in 1 ml. of infusion broth, inoculated with 0.02 ml. of a tenfold-diluted overnight culture and incubated overnight at 37°. The lowest concentration of each penicillin that prevented growth was taken as the minimum inhibitory concentration.

Induction. Organisms were grown overnight in the presence of sub-inhibitory concentrations of the various penicillins; the actual concentrations used in these experiments were the highest that would allow adequate growth after overnight culture, except for those penicillins whose minimum inhibitory concentrations were 1 mg./ml. or greater, when 500 µg./ml. was used in the interests of economy. Cultures were inactivated with 1 mM-sodium *p*-chloromercuribenzoate, spun down, washed, resuspended to equal turbidities on the EEL colorimeter and assayed against penicillin G.

RESULTS

Amount of penicillinase present in Klebsiella aerogenes cultures

Cells from overnight broth cultures of *K. aerogenes* and the four strains of uninduced *S. aureus* were concentrated approximately tenfold in phosphate-buffered saline, adjusted to the same turbidity (4.8 units) on the EEL colorimeter (filter no. 625) and assayed against penicillin G. The *K. aerogenes* suspension contained 25–30% as much enzyme as did equally turbid suspensions of the three uninduced strains of *S. aureus* known to be 'good' penicillinase producers (E3, 13137 and 8354/6/1), and the same amount as an uninduced *S. aureus* strain (110/10) known to be a 'poor' penicillinase producer.

Characteristics of Klebsiella aerogenes enzyme

Chromatographic identification as a β-lactamase. Spots with the same R_F (0.72) as that of the marker benzylpenicilloic acid were obtained after incubation of penicillin G (R_F 0.80) both with a known penicillinase and with *K. aerogenes*. Incubation of penicillin G with a known penicillin-amidase preparation (an *Escherichia coli* strain obtained from Beechams Research Laboratories) gave a compound with the same R_F (0.53) as that of the marker 6-aminopenicillanic acid, but this was absent from the *K. aerogenes* hydrolysate.

The minimum amounts that could be detected were: penicillin G and its cognate penicilloic acid, 50 µg.; 6-aminopenicillanic acid, 20 µg.; and the penicilloic acid of 6-aminopenicillanic acid (R_F 0.33), 3 µg.

Action of possible inhibitors and activators. The activity of the cell-free penicillinase was completely destroyed by overnight incubation at 37° with 0.1% papain [activated with 1% (w/v) thioglycolic acid] or with 0.2% trypsin, both in the presence of 25 mM-phosphate buffer, pH 7.4; heating at 100° for 5 min. and treatment with 1 mM- Cu^{2+} ion also inactivated the enzyme. A 77% inactivation was obtained by treatment with 5% (w/v) trichloroacetic acid at 4° for 10 min., but treatment with the following compounds had no effect on activity: 1 mM-calcium nitrate, 1 mM-

Table 1. Comparison of rates of breakdown of various penicillins by four penicillinases

Experimental details are given in the text. Absolute rates ($\mu\text{g. of penicillin G/ml./min./10}^9$ organisms, at pH 7.4 and 37°) are shown in parentheses. The values for *S. aureus* and *B. cereus* refer to methicillin-induced cultures (Smith, 1963); the *B. licheniformis* preparation was a 1:144 dilution of commercially obtained enzyme (see the Methods and Materials section).

Enzyme source	...	Penicillinase activity			
		<i>S. aureus</i> E3	<i>B. cereus</i> 569	<i>B. licheniformis</i>	<i>K. aerogenes</i>
Penicillin					
Penicillin G		100 (696)	100 (500)	100	100 (1.9)
Penicillin V		110	117	88	136
Phenethicillin		100	103	64	61
Propicillin		71	56	40	55
Phenoxybenzyl		167	91	96	52
Ampicillin		198	156	110	193
Methicillin		1	1	7	< 10
BRL1400		1	1	9	< 10
6-Aminopenicillanic acid		1	3	3	127

manganous chloride, 1 mM-magnesium chloride, 0.1 mM-ferric ammonium sulphate, 0.1 mM-ferrous ammonium sulphate, 10 mM-thioglycollate, 1 mM-sodium *p*-chloromercuribenzoate and 0.1 mM-EDTA.

Extraction from cells. The extrusion press was found to be the most effective way of breaking cells to obtain active enzyme preparations. The only other method used which released enzyme from the cells was shaking with Ballotini beads (grade 11) for 15 min. on a Microid shaker. No lysis (judged by decrease in extinction at 600 $m\mu$) or release of enzyme was brought about by incubating suspensions for 90 min. at 22° with either 1% (v/v) Teepol or egg-white lysozyme (1 mg.), EDTA (372 $\mu\text{g.}$) and 50 mM-tris buffer, pH 7.5 (167 μmoles) (total volume 3 ml.); nor did grinding a thick suspension in a glass Potter-type homogenizer or suspending the organism in water release penicillinase.

Purification. Cells from 500 ml. of overnight culture were washed and concentrated 15-fold in phosphate-buffered saline, disrupted in the press and centrifuged at 20000*g* for 100 min. The supernatant fraction, diluted to 60 ml., was adjusted to pH 5 with *N*-hydrochloric acid and allowed to stand for 10 min., after which the precipitate was removed by centrifuging. The supernatant fraction was adjusted to pH 7.4 with *N*-sodium hydroxide and fractionally precipitated with crystalline ammonium sulphate. Fractions were assayed against penicillin G after protein determination (Layne, 1957); the fraction that was precipitated at 90% saturation contained half of the total enzymic activity.

Effect of pH on activity. The hydrolysis of penicillin G was followed at pH values from 3 to 8 in half-strength citrate-phosphate buffer (McIlvaine, 1921); the results are shown in Fig. 1.

Specificity pattern. Table 1 shows the specificity patterns of penicillinases from three Gram-positive bacteria and also that from *K. aerogenes* (whole

Fig. 1. Effect of pH on the activity of the penicillinase of *K. aerogenes* 418 in McIlvaine's (1921) buffer. Experimental details are given in the text.

cells). Initial penicillin concentrations were 2 mg./ml. The values for the staphylococcal and *B. cereus* enzymes are taken from Smith (1963).

Affinities of penicillins for enzyme. Michaelis constants for penicillins G and V, phenethicillin, propicillin and phenoxybenzylpenicillin were all less than 130 μM ; for 6-aminopenicillanic acid and ampicillin the values were 11 mM and 600 μM respectively.

Enzyme inhibition. Penicillinase preparations of *K. aerogenes* (cell-free), *S. aureus* E3, *B. licheniformis* and *B. cereus* were adjusted so that all hydrolysed 2 $\mu\text{g. of penicillin G/min.}$ under identical assay conditions (Smith, Hamilton-Miller & Knox, 1962). The rates at which these preparations hydrolysed penicillin G alone, and in the presence of an equal concentration (500 $\mu\text{g./ml.}$) of methicillin and of BRL 1400, were measured by using the colorimetric modification of the spectrophotometric assay. The results (Table 2) show that staphylococcal penicillinase was not inhibited, that *K. aerogenes* penicillinase was the most susceptible and that methicillin was the better inhibitor.

Activity of disrupted cells

A suspension of cells was taken, half was disrupted in the press, and samples of the intact- and broken-cell preparations were assayed against various penicillins. Against 6-aminopenicillanic acid at an initial concentration of 1.5 mg./ml. the activities of both suspensions were almost identical, but against all the other penicillins tested the disrupted preparations showed considerably greater activities than intact preparations. The ratio of the rate of hydrolysis by intact cells to that by disrupted cells was different for each penicillin; further, this ratio appeared to be connected with the anti-

bacterial activity of the penicillin concerned: Fig. 2 shows the correlation between these two parameters.

Smith (1963) reported that the rate of hydrolysis of penicillin G by a strain of *E. coli* can be increased, not only by disruption of cells, but also by shaking the organisms with toluene. However, after intact cells of *K. aerogenes* 418 had been treated with toluene, no increase in the rate of penicillin G hydrolysis was observed, and on cell-free preparations toluene had an inhibitory effect on penicillinase activity.

Induction experiments

Growth in the presence of the following penicillins did not increase the penicillinase concentration in intact organisms [values in parentheses are concentrations ($\mu\text{g./ml.}$) in culture medium at inoculation]: penicillin G (125), phenethicillin (500), ampicillin (70), BRL 1400 (500) and phenoxybenzylpenicillin (250). Growth in methicillin (500) caused clumping of the organisms to such an extent that turbidimetric measurements could not be made, and so this compound could not be tested. After growth in the presence of penicillin V (250) and 6-aminopenicillanic acid (63) intact organisms showed a fourfold increase in penicillinase, and growth in propicillin (500) caused a doubling of the concentration of the enzyme. But when the latter three cultures were disrupted in the press, it was found that they contained the same amount of penicillinase as did a disrupted untreated culture.

DISCUSSION

A penicillin-destroying enzyme in *K. aerogenes* 418 has been positively identified as a penicillinase (β -lactamase) by several independent methods. The enzyme was labile to proteolytic enzymes and to heat, unaffected by the addition of compounds that either contain sulphhydryl groups or that block such groups, and insensitive to EDTA and several bivalent cations. In these ways it resembles the penicillinases of *B. cereus* 569 and *S. aureus* (Henry & Housewright, 1947; Leitner & Cohen, 1962), but it retains more activity at acid pH values than they do (see Batchelor, Cameron-Wood, Chain & Rolinson, 1961a).

The enzyme of *K. aerogenes* 418 differs in four major respects from penicillinases of Gram-positive bacteria.

(1) Localization. After disruption of *K. aerogenes* cells in the press, the formerly intracellular penicillinase is released in a form not sedimented by centrifuging at 20000g for 100 min. Other intracellular penicillinases, however, are particulate in nature: Saz *et al.* (1961) reported that penicillinase can be sedimented from disrupted *S. aureus* by

Table 2. Inhibitory action of methicillin and BRL 1400 on the hydrolysis of penicillin G

Experimental details are given in the text. All enzyme preparations originally hydrolysed 2 $\mu\text{g.}$ of penicillin G/min. at pH 7.4 and 37°.

Enzyme source	Inhibition of penicillin G hydrolysis (%)	
	Methicillin	BRL 1400
<i>K. aerogenes</i> 418	89	63
<i>S. aureus</i> E3	0	0
<i>B. licheniformis</i>	52	42
<i>B. cereus</i>	65	29

Fig. 2. Correlation between antibacterial power of various penicillins against *K. aerogenes* 418, and their ease of access through the bacterial permeability barrier. A suspension of cells was taken, and half was disrupted in the press. Intact and broken preparations were then assayed against the various substrates, and the percentage increase in activity resulting from the disruption was found for each substrate. Experimental details are given in the text. Minimum inhibitory concentrations were found as described in the Methods and Materials section. 1, Phenoxyethylpenicillin; 2, phenoxypropylpenicillin; 3, phenoxymethylpenicillin; 4, phenoxybenzylpenicillin; 5, benzylpenicillin; 6, α -amino-benzylpenicillin; 7, 6-aminopenicillanic acid.

144000g for 15 min., and Sabath & Finland (1962) have made similar findings; Kushner & Pollock (1961) sedimented penicillinase from disrupted *B. subtilis* preparations by centrifuging at 20000g for 10 min.

(2) Specificity. As Table 1 shows, the main difference between the specificity of the *K. aerogenes* penicillinase and the penicillinases of the Gram-positive bacteria is that 6-aminopenicillanic acid is a good substrate for the former but a very poor one for the latter; Batchelor *et al.* (1961a) reported rapid hydrolysis of this compound by *S. aureus* and *B. cereus* penicillinases only at acid pH values and at high substrate concentrations.

(3) Inducibility. Geronimus & Cohen (1958) and Housewright & Henry (1947) reported that the penicillinases of *S. aureus* and *B. cereus* 569 are inducible, and Knox & Smith (1962) showed that enzyme production in *S. aureus* E3 can be increased about 50-fold by induction. In all these cases induction was brought about by penicillin G, even though the inducing agent is rapidly hydrolysed by the enzyme it induces. Further, Knox & Smith (1962) showed that ampicillin, phenethicillin and penicillin V also act as inducing agents for staphylococcal penicillinase, although they are hydrolysed at least as rapidly as penicillin G. In addition, the isoxazolyl series of penicillins are excellent inducing agents (Smith *et al.* 1962), but when BRL 1400 was tested against *K. aerogenes* 418 no induction was observed; it is clear then that the penicillinase of this organism cannot be induced in the same way as that of Gram-positive organisms. It might be argued that the permeability barrier in *K. aerogenes* 418 will so limit the rate of entry of a potential inducing agent that it is destroyed by the penicillinase already present as soon as it enters the cell, and before reaching the inducing site. However, the experiments with 6-aminopenicillanic acid [which is known to induce staphylococcal penicillinase (Batchelor *et al.* 1961a; Knox & Smith, 1962)] seem to point to the penicillinase of this organism being non-inducible. The penicillinase of a strain of *E. coli* has also been shown to be non-inducible under these conditions (Smith, 1963).

(4) Permeability barrier. The increase of penicillinase activity brought about by disrupting *K. aerogenes* suspensions can be explained by the existence of a differential permeability barrier against penicillins. No such permeability barrier has been found in *B. subtilis* (Pollock, 1961), but there are conflicting reports concerning *S. aureus* (Geronimus & Cohen, 1958; Novick, 1962); Smith (1963) has shown the existence of such a barrier in *E. coli*.

Loss of the permeability barrier does not inflict serious enough damage on the bacterial cells to cause death; overnight cultures reach no greater turbidity in plain broth than in broth containing

sufficient penicillin V or 6-aminopenicillanic acid to render them completely permeable (evidenced by the absence of any increase in enzymic activity against penicillin G, after subsequent disruption of such cultures). Subculture from such 'permeable-cell' cultures on a solid medium gave colonies indistinguishable from those produced from the control cultures. Prestidge & Pardee (1957) have also reported damage to cell barriers caused by penicillin.

6-Aminopenicillanic acid was the most potent antibacterial agent against *K. aerogenes* 418; Rolinson & Stevens (1961b), English, MacBride & Huang (1960) and Smith (1963) have reported other members of the Enterobacteriaceae more susceptible to 6-aminopenicillanic acid than to penicillin G. The results of the present experiments suggest that the efficiency of 6-aminopenicillanic acid as an antibacterial agent could be due, in part at least, to its ease of penetration into the organism. Further, the relatively high Michaelis constant (34 times as large as the minimum inhibitory concentration) of 6-aminopenicillanic acid for the penicillinase of this organism means that at inhibitory concentrations it will be hydrolysed slowly. The failure of the easily hydrolysed penicillins to pass freely into the bacterial cell, combined with the fact that their Michaelis constants are at least ten times as large as their minimum inhibitory concentrations, will diminish their antibacterial potential.

The part played by penicillinase in the acquisition of resistance of organisms to penicillin therapy is difficult to assess. The presence of a penicillinase does not automatically provide an explanation for resistance to penicillin (see Knox & Smith, 1961b; Smith, 1963); however, there is no doubt that the possession of an enzyme capable of destroying ampicillin at inhibitory concentrations contributes a great deal towards the resistance of this *K. aerogenes* to ampicillin.

SUMMARY

1. A penicillinase-producing *Klebsiella aerogenes* resistant to ampicillin has been isolated during clinical studies.

2. The penicillinase has been characterized, and some of its physical and chemical properties are described.

3. The enzyme differs both qualitatively and quantitatively from penicillinases produced by *Staphylococcus aureus*, *Bacillus cereus* and *Bacillus licheniformis*, particularly with regard to behaviour towards 6-aminopenicillanic acid.

4. The enzyme is not inducible.

5. A differential permeability barrier exists in whole cells against the penicillins. The possible effects of the barrier on antibacterial potency are

discussed, together with the part played by penicillinase in the production of resistance to ampicillin.

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The Metabolism of Progesterone by Animal Tissues *in vitro*

5. INHIBITION OF CONJUGATE FORMATION BY ETHANOL AND PROPYLENE GLYCOL DURING THE METABOLISM OF [4-¹⁴C]PROGESTERONE BY RAT LIVER *IN VITRO**

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Cooke & Taylor (1963) presented evidence that water-soluble conjugates (mainly glucuronides and sulphates) are formed when [4-¹⁴C]progesterone is incubated with female-rat-liver homogenate at tissue:steroid ratios of 56 000:1. During the investigations it was found that ethanol and propylene glycol inhibit this conjugate formation. These alcohols have been used extensively in studies *in vitro* to dissolve steroids before addition to incubation media because of the low solubility of steroids in water [e.g. Ofner (1955), Wiest (1959) and Dominguez, Acevedo, Huseby & Samuels (1960) used ethanol; Taylor (1954), Wotiz & Lemon

(1954) and Sie & Fishman (1957) used propylene glycol]. Therefore it was decided to investigate the degree and possible mechanisms of this inhibition of conjugate formation by the alcohols. A preliminary report of this work has been published (Cooke & Taylor, 1961).

MATERIALS AND METHODS

Ethanol (The Distillers Co. Ltd.) that had stood over sodium hydroxide pellets overnight was distilled and then redistilled in all-glass apparatus through a 14 in. column packed with Raschig rings. Propylene glycol (propane-1,2-diol) (Laboratory Reagent, British Drug Houses Ltd.) was used without further purification. Assay of radioactivity was carried out as described by Taylor & Scratcherd (1961).

* Part 4: Cooke & Taylor (1963).

† Present address: British Drug Houses Ltd., Graham Street, London, N. 1.