ACTA CHEMICA SCANDINAVICA 21 (1967) 547–551

Enzymatic Hydrolysis of Some Penicillins and Cephalosporins by *Escherichia coli* Acylase

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The deacylation rate of penicillins and cephalosporins by a strain of *Escherichia coli* has been studied. For both types of compounds the pH for optimal activity was about 9.0. Structurally corresponding penicillins and cephalosporins showed a similar rate of cleavage.

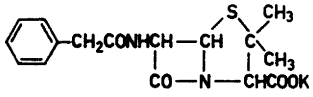
Extensive work has previously been reported on the splitting of penicillins by deacylating enzymes produced by various microorganisms.[1–14] Particular attention has been devoted to the deacylating enzymes from *Escherichia coli*, which are used industrially in the production of 6-aminopenicillanic acid. It has been shown by Huang *et al.*[10] that cephalosporins with phenoxyacetyl and phenylmercaptoacetyl as side chains are deacylated by enzymes from *Nocardia* and *Proteus*, but no studies seem to have been made of the effect of *E. coli* acylases on semisynthetic cephalosporins. The purpose of the present study was to compare the activity of the enzymes produced by a strain of *E. coli* (BRL 351) on some penicillins and cephalosporins.

It was reported by Sakaguchi and Murao [1] that *Penicillium chrysogenum Q* 176 can produce an enzyme that hydrolyses benzylpenicillin in phenylacetic acid and a compound that was later recognized [15] as the nucleus of the penicillin molecule, 6-aminopenicillanic acid. Bacteria and *Nocardia* species are able to produce acylases with the property of splitting benzylpenicillin.[2–6] On the other hand, acylases with a marked property of splitting phenoxymethylpenicillin have been found to be produced by a number of streptomycetes and fungi.[2,7,9]

In their study of the substrate specificity of acylase from different sources Claridge *et al.*[9] have found one strain of *Alcaligenes faecalis* and one of *E. coli* that produced acylase which was effective in splitting benzylpenicillin, but which also exerted a fairly powerful effect on α-aminobenzylpenicillin and phenoxymethylpenicillin. On the other hand, penicillin N, cephalosporin C, and 2,5-dimethoxyphenylpenicillin were completely resistent to the action of the enzyme. In recent investigations on the specificity of an acylase produced

by a strain of *E. coli* (BRL 1040) Cole [12] found the enzyme to be specific for splitting off the phenylacetyl group from a large number of phenylacetylated compounds — even ones structurally distinct from penicillin. The rate of hydrolysis, however, was clearly dependent on the structure of the acylated part of the molecule. Phenylacetylglycine, for example, was split at twice the rate of benzylpenicillin. Because of the higher specificity of the enzyme for the phenylacetyl group than for the penicillin nucleus, Cole proposed that it be regarded as phenylacetylase. Kaufmann and Bauer,[13] too, found that the specificity of enzymes from *E. coli* is dependent more on the side chain of penicillins than on the nucleus.

It was considered of interest to compare the activity of our acylase from *E. coli* in splitting the 2-thienylacetyl group — the side chain of the two cephalosporins used in medical practice — and the phenylacetyl group. The substrates used were benzylpenicillin (I), 2-thenylpenicillin (II), penicillin N (III), benzylcephalosporin (IV), cephalothin (V), cephaloridine (VI), and cephalosporin C (VII).



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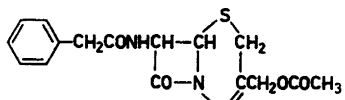

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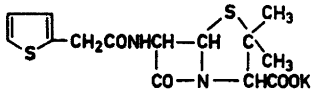

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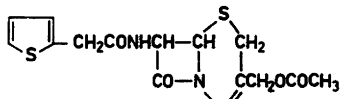

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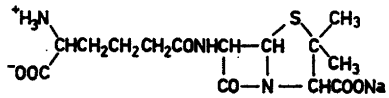

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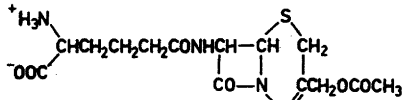

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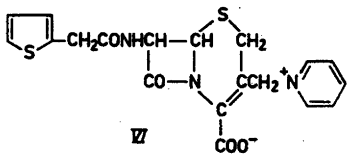

VI

MATERIALS AND METHODS

The bacterial cell suspension was obtained from an ordinary factory batch of *E. coli* (BRL 351), which is derived from the BRL strain 1040 used by Cole.[12] Prior to use the cells were washed by centrifuging, suspended in about two volumes of deionized water, and recentrifuged. This procedure was repeated. Benzylpenicillin, penicillin N (Salmotin, Abbott), cephalothin (Keflin, Lilly), cephaloridine (Ceporin, Glaxo), and cephalosporin C were all commercial products. 2-Thenylpenicillin and benzylcephalosporin (7-phenylacetamidocephalosporanic acid) were prepared in our laboratories.

Reaction velocities. The rates of enzymatic cleavage of the amide bond in the penicillins and the cephalosporins were measured at constant pH with a pH-stat (Radiometer Titrigraph type SBR 2c connected to a TTT Automatic Titrator) as described by Jeffery *et al.*[16] 10 ml of a solution of the substrate in water (1 — 100 mM) was placed in a water-jacketed vessel thermostatically controlled at 37°C. The vessel was covered with a lid through which passed a glass electrode, a calomel reference electrode, a polyethene tube carrying a stream of nitrogen to maintain a carbon dioxide free atmosphere, a stirrer, and a narrow polyethene tube from a 0.5 ml syringe containing 0.05 N sodium hydroxide. After hydroxide had been added from the syringe to adjust the pH of the solution to the predetermined value, more was added to neutralize acid groups liberated by hydrolysis of the β-lactam ring. The amount of hydroxide added was recorded against time, and the rate of the non-enzymatic hydrolysis of the substrates was calculated. After the non-enzymatic hydrolysis had been measured for about 5 min, 1.0 ml of cell suspension was added (washed cell suspension diluted 1:10 with water; pH adjusted to that of the substrate). The consumption of hydroxide was measured for a further 10 min; by subtracting the rate of the non-enzymatic hydrolysis the rate of enzymatic amide hydrolysis was obtained, as both the hydrolytic processes involve a monomolecular liberation of acid groups and a monomolecular consumption of hydroxyl groups. The results presented in Tables 1 and 2 are means of at least three separate determinations.

Table 1. Influence of pH on the acylase activity, determined on benzylpenicillin and cephaloridine. Substrate concentration 10 mM; reaction temperature 37°C.

pH	Rate of hydrolysis (μmole/min)			Rate referred to pH 9.0 (%)
	Total	Non-enzymatic	Due to acylase	
		Benzylpenicillin		
7.5	8.2	0.2	8.0	82
8.0	8.9	0.2	8.7	90
8.5	9.7	0.4	9.3	96
9.0	10.4	0.7	9.7	100
9.5	10.9	1.6	9.3	96
10.0	12.4	3.8	8.6	89
		Cephaloridine		
7.5	6.8	0.4	6.4	82
8.0	7.5	0.5	7.0	90
8.5	8.2	0.6	7.6	97
9.0	9.1	1.3	7.8	100
9.5	11.4	3.8	7.6	97
10.0	17.6	10.5	7.1	91

RESULTS

Determination of optimal pH for the acylase activity. The effect of pH on the acylase activity of *E. coli* was studied on benzylpenicillin and cephaloridine. The determinations were performed as described above with substrate concentrations of 10 mM, a reaction temperature of 37°C, and pH ranging from 7.5 to 10.0. The results are presented in Table 1.

As this table shows, the pH for optimal activity of the *E. coli* acylase was the same for benzylpenicillin and cephaloridine. Though the optimal pH was around 9.0, we preferred to carry out the determinations at pH 8.0, where the acylase activity was as high as 90 % of the optimal and the non-enzymatic hydrolysis still low.

Deacylation rates of some penicillins and cephalosporins for E. coli *amidase.* The rates of deacylation of 2-thenylpenicillin, penicillin N, benzylcephalosporin, cephalothin, cephaloridine, and cephalosporin C relative to that of benzylpenicillin were determined at pH 8.0 and 37°C. Two substrate concentrations were chosen, 1 and 100 mM. The results are shown in Table 2.

Table 2. Deacylation rate of penicillins and cephalosporins at pH 8.0 and 37°C. Substrate concentrations 1 and 100 mM.

	Substrate conc. 1 mM		Substrate conc. 100 mM	
	Initial rate of deacylation (μmole/min)	Rate referred to pcG (%)	Initial rate of deacylation (μmole/min)	Rate referred to pcG (%)
Benzylpenicillin	7.9	100	13.5	100
2-Thenylpenicillin	5.9	75	8.0	59
Penicillin N	0.3	4[a]	—	—
Benzylcephalosporin	6.3	70	—	—
Cephalothin	4.6	58	7.5	56
Cephaloridine	6.9	87	11.3	84
Cephalosporin C	0.0	0	—	—

[a] The penicillin N used contained only 23% of active penicillin. The low acylase activity may be due to the presence of impurities which can be utilized as substrates.

DISCUSSION

It was concluded by both Cole [12] and Kaufmann and Bauer [13] that the enzyme produced by *E. coli* is specific for the phenylacetyl group but not for the penicillins. It would be expected that a group bearing a close resemblance to phenylacetyl, *e.g.* thienylacetyl, would behave in a similar way. Our results show that the enzyme from *E. coli* splits off the 2-thienylacetyl group from 6-aminopenicillanic acid at only a slightly lower rate than the phenylacetyl group. The relation between the rates of cleavage was about the same for 7-aminocephalosporanic acid. Penicillin N and cephalosporin C were unaffected by the acylase from our strain of *E. coli*, as would be expected in view of the similar results obtained by Claridge *et al.*[9]

Cole [12] found that the rate of hydrolysis was greatly affected by structural and steric differences in the acylated part of the molecule. Steric factors around the amide bond appear to be of major importance. Phenylacetyl-L-α-aminophenylacetic acid was thus split more readily than penicillin G, while phenylacetyl-α-D-aminophenylacetic acid was not affected at all.[12] Corresponding penicillins and cephalosporins have closely similar steric environments at the 6 and 7 positions, respectively.[17] Replacement of the acetoxyl group in cephalothin (V) with the pyridinium group to form cephaloridine (VI) would greatly modify the charge distribution in the molecule, but would not give rise to any steric changes around the amide bond. This structural modification also resulted in only a slight change in the rate of hydrolysis, cephaloridine being cleaved slightly more rapidly than cephalothin (Table 2).

REFERENCES

1. Sakaguchi, K. and Murao, S. *J. Agr. Chem. Soc. Japan* **23** (1950) 411.
2. Rolinson, G. N., Batchelor, F. R., Butterworth, D., Cameron-Wood, J., Cole, M., Eustace, G. C., Hart, Marian V., Richards, M. and Chain, E. B. *Nature* **187** (1960) 236.
3. Claridge, C. A., Gourevitch, A. and Lein, J. *Nature* **187** (1960) 237.
4. Huang, H. T., English, A. R., Seto, T. A., Shull, G. M. and Sobin, B. A. *J. Am. Chem. Soc.* **82** (1960) 3790.
5. Kaufmann, W. and Bauer, K. *Naturwiss.* **47** (1960) 474.
6. Borkar, P. S., Vinze, V. L. and Sen, G. *Hindustan Antibiot. Bull.* **4** (1961) 48.
7. Batchelor, F. R., Chain, E. B., Richards, M. and Rolinson, G. N. *Proc. Roy. Soc. (London) Ser. B.* **154** (1961) 522.
8. Cole, M. and Rolinson, G. N. *Proc. Roy. Soc. (London) Ser. B.* **154** (1961) 490.
9. Claridge, C. A., Luttinger, J. R. and Lein, J. *Proc. Soc. Exptl. Biol. Med.* **113** (1963) 1008.
10. Huang, H. T., Seto, T. A. and Shull, G. M. *Appl. Microbiol.* **11** (1963) 1.
11. Holt, R. J. and Stewart, G. T. *Nature* **201** (1964) 824.
12. Cole, M. *Nature* **203** (1964) 519.
13. Kaufmann, W. and Bauer, K. *Nature* **203** (1964) 520.
14. Pruess, D. L. and Johnson, M. J. *J. Bacteriol.* **90** (1965) 380.
15. Batchelor, F. R., Doyle, F. P., Nayler, J. H. C. and Rolinson, G. N. *Nature* **183** (1959) 257.
16. Jeffery, J.d'A., Abraham, E. P. and Newton, G. G. F. *Biochem. J.* **81** (1961) 591.
17. Abraham, E. P. and Newton, G. G. F. *Biochem. J.* **79** (1961) 377.

Received November 9, 1966.