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Penicillins and Cephalosporins are Active Site-Directed Acylating Agents: Evidence in Support of the Substrate Analogue Hypothesis

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Penicillins and cephalosporins are active site-directed acylating agents: evidence in support of the substrate analogue hypothesis

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Penicillin and related β-lactam antibiotics are known to exert their bactericidal effects by inhibiting the cross-linking step (transpeptidation) of bacterial cell wall biosynthesis. Evidence is presented in support of the hypothesis that this inhibition results from covalent modification of the active site of sensitive enzymes as a consequence of the structural similarity between penicillin and the acyl-D-alanyl-D-alanine terminus of nascent peptidoglycan strands. Several predictions of this proposal have been verified experimentally. Penicillin-sensitive enzymes are inactivated, with the formation of a covalent, stoichiometric penicilloyl-enzyme complex *in vitro*. Acylenzyme intermediates have been trapped with several of these enzymes by using cell wall-related substrates. Sequence analysis of the peptides derived from active site-labelled enzymes has established that both penicilloyl and an acyl moiety derived from substrate are covalently bound to the same site, as an ester of serine 36, as predicted by the substrate analogue hypothesis. Sequences near the active site serine are homologous to sequences found in four β-lactamases, supporting the proposal that penicillin-sensitive D-alanine carboxypeptidases and penicillin-inactivating β-lactamases are evolutionarily related. Structural features important for the specific and potent inhibitory properties of β-lactam antibiotics are discussed in terms of the original substrate analogue hypothesis.

Introduction

The accidental discovery of penicillin by Fleming (1929) opened the era of modern pharmacology. No other drug has resulted in the alleviation of as much illness as has penicillin. This reason alone would account for the intense interest which scientists have shown in elucidating the mechanism by which this clinically effective compound kills bacteria while leaving the animal host untouched. In addition, penicillins and the related cephalosporins have been indispensable tools in studying bacterial cell wall biosynthesis, the primary target of these β-lactam antibiotics.

A number of early studies led to the conclusion that penicillin is a selective inhibitor of bacterial cell wall biosynthesis. Fleming himself realized that penicillin killed bacteria by lysis. Gardner (1940) observed that at low concentrations of antibiotic, organisms do not lyse but are converted to filaments. Early morphological studies by Duguid (1946) indicated that penicillin interfered with the synthesis of an unknown surface structure of bacterial cells. These results were confirmed and extended by Lederberg (1957) who observed that penicillin induced formation of spheroplasts of *Escherichia coli* in hypertonic medium and by Park & Strominger (1957) who reported that the uridine nucleotide which accumulated within penicillin-treated cells (Park & Johnson 1949) had both a sugar and amino acid composition similar to that of the recently discovered bacterial cell wall. By this time Cooper (1956) and others had used radioactive penicillin to demonstrate that the antibiotic was bound to specific targets in the membranes of bacterial cells. The studies by Schepartz & Johnson (1956) suggested that penicillin was bound to these sites as the penicilloyl moiety.

During the next decade the chemical nature of the bacterial cell wall and the complex mechanisms by which it is synthesized were elucidated (for a recent review, see Tipper & Wright 1979). Of the several constituents in the cell wall, it is the peptidoglycan that determines cell shape and that imparts the rigidity necessary to protect the bacterium from osmotic rupture. The peptidoglycan sacculus is a large polymer composed of long, linear polysaccharide chains of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid extending in one direction, cross-linked by short peptides in a second direction. Peptidoglycan structure was largely deduced by use of bacteriolytic enzymes, including various carbohydrases and peptidases (Strominger & Ghuysen 1967). The cross-linking of tetrapeptide subunits derived from pentapeptide precursors was found to be a fundamental feature of all bacterial cell walls. Peptidoglycan biosynthesis can be divided into several stages: (1) synthesis of two uridine nucleotide precursors by cytoplasmic enzymes, (2) translocation of these precursor molecules across the bacterial membrane, (3) their subsequent polymerization to form a linear peptidoglycan polymer and finally (4) incorporation of the linear polymer into existing peptidoglycan via a cross-linking of the peptide side chains.

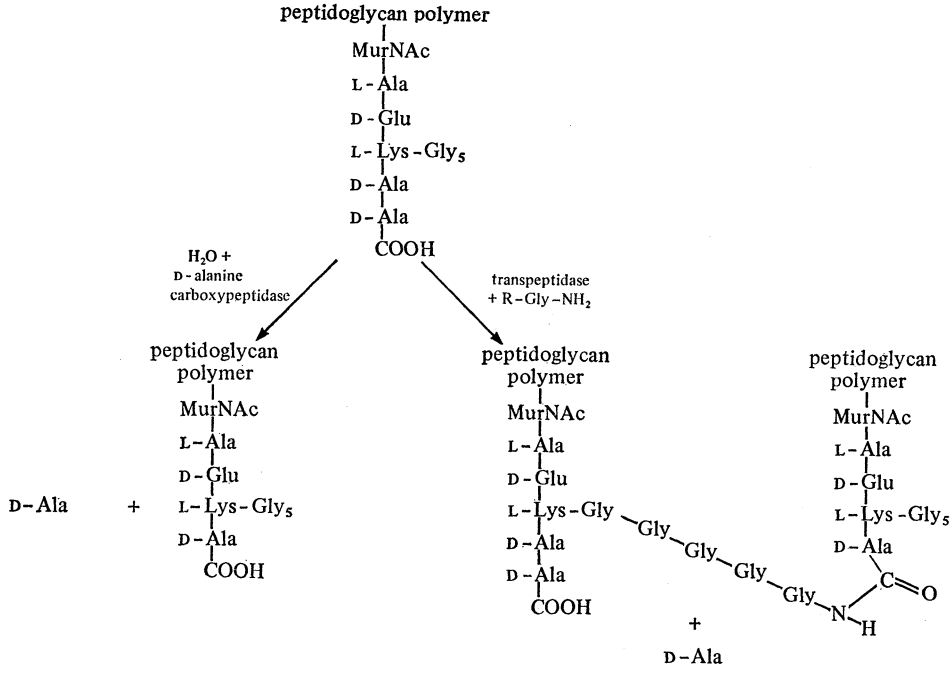

FIGURE 1. Transpeptidase and D-alanine carboxypeptidase reactions.

Wise & Park (1965) and Tipper & Strominger (1965) independently showed that formation of this cross-link is the penicillin-sensitive step in bacterial cell wall biosynthesis. Peptidoglycan transpeptidase catalyses this reaction in which a free amino group of a cross-bridge in one peptidoglycan strand attacks the peptide bond of the D-alanyl-D-alanine terminus of another strand with the release of the terminal D-alanine and the formation of a peptide cross-link (figure 1). Shortly after this discovery, penicillin-sensitive transpeptidation catalysed by *E. coli* membranes was demonstrated *in vitro* (Izaki *et al.* 1968). A second, related penicillin-sensitive D-alanine carboxypeptidase (CPase) activity was also discovered (Izaki *et al.* 1968).

CPase removes the terminal D-alanine residue from a strand that is not cross-linked, preventing further extension of the peptidoglycan by transpeptidation (figure 1). However, it is not certain that CPase actually serves this role *in vivo*.

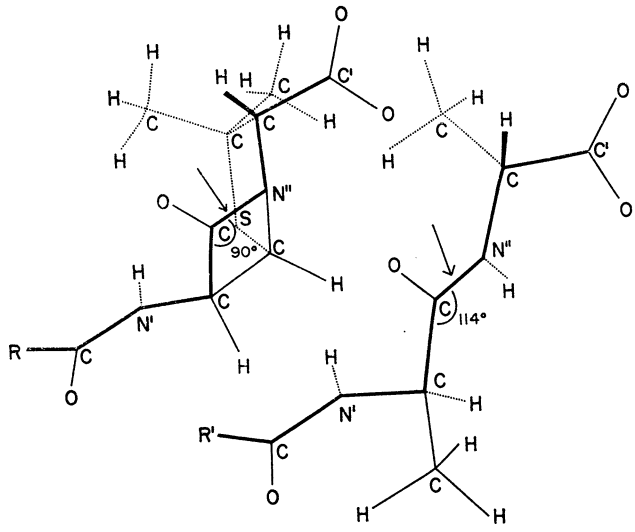

FIGURE 2. Dreiding stereo-models illustrating the proposed structural similarity between penicillin and acyl-D-Ala-D-Ala. The backbone of the dipeptide is drawn in a conformation that best aligns with the analogous portions of penicillin. Arrows indicate the CO—N bonds cleaved during catalysis or during inactivation by penicillin (see figure 3). Bond angles and other structural differences that have a bearing on the mechanism of penicillin action are discussed in the text.

SUBSTRATE ANALOGUE HYPOTHESIS

Tipper & Strominger (1965) proposed that penicillin inactivates peptidoglycan transpeptidase by acting as a structural analogue of the dipeptide terminus of nascent peptidoglycan strands. Dreiding stereo-models suggested that one of the possible conformations of the acyl-D-alanyl-D-alanine terminus of peptidoglycan is quite similar to that of penicillin (figure 2). In the conformations shown, the free carboxyl group and the terminal asymmetric centres, necessary for activity of both penicillin and peptide substrates, are aligned. Moreover, the highly reactive —CO—N— bond in the β-lactam ring of penicillin is in the same position as the peptide bond cleaved during transpeptidation. It was suggested that the transpeptidase reacts with its substrate to form an acyl-enzyme intermediate, with the elimination of D-alanine (Tipper & Strominger 1965) (figure 3). Subsequent reaction with the free amino group of a second cross-bridge would lead to formation of a cross-link and regeneration of the enzyme. Alternatively, if the acyl-enzyme intermediate were attacked by water, the sequence would correspond to a D-alanine carboxypeptidase reaction. Penicillin, acting as a structural analogue of the substrate, would bind to the enzyme (transpeptidase or carboxypeptidase) with its β-lactam bond positioned at the active site. A relatively facile acylation of the catalytically active amino acid residue would then occur with the opening of the β-lactam ring, forming an inactive penicilloyl-enzyme (figure 3) (Tipper & Strominger 1965).

There are several predictions of this proposal for the mechanism of action of penicillin. One is the existence of a covalent penicilloyl-enzyme as the inactive form of peptidoglycan transpeptidase. A second prediction is the occurrence of an acyl-enzyme as a catalytic intermediate

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of the uninhibited reaction. Finally, the model predicts that the acyl group derived from the substrate will be substituted on the same amino acid residue as the penicilloyl moiety derived from penicillin. This paper reviews the evidence substantiating these three predictions for two penicillin-sensitive enzymes, CPases from bacilli.

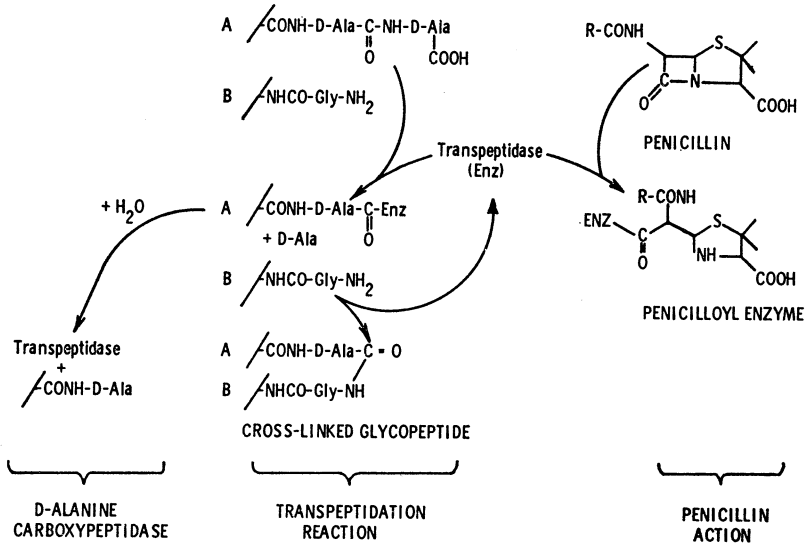
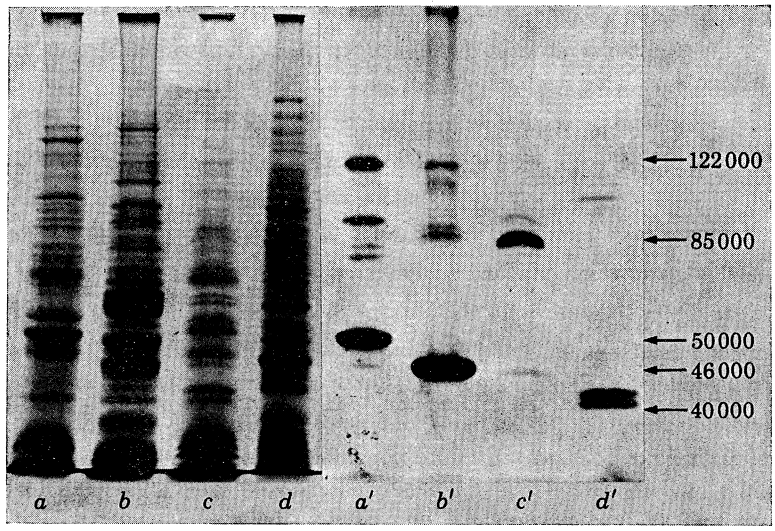


FIGURE 3. Proposed mechanism of inhibition of transpeptidation by penicillins: A represents the end of the main peptide chain of the glycan strand; B represents the end of the pentaglycine substituent from an adjacent strand. If the acyl-enzyme intermediate can react with water instead of the amino acceptor (left), the enzyme would be regenerated and the substrate released, effecting hydrolysis of the terminal D-alanine residue of the substrate (D-alanine carboxypeptidase activity). Carboxypeptidase (left) and transpeptidase (centre) reactions can be catalysed by separate enzymes, perhaps by similar mechanisms. Transfer of the covalently bound penicilloyl moiety (right) to a free amino group or to water is slow, due (at least in part) to continued occupancy of the acceptor site by the thiazolidine ring of the antibiotic.



(*a*) *B. subtilis* (*c*) *S. aureus*
(*b*) *B. stearothermophilus* (*d*) *E. coli*

FIGURE 4. Penicillin-binding proteins (PBPs) detected in bacterial membranes. Membranes from the four indicated bacterial species were incubated with [^{14}C]penicillin G and the acetone precipitated protein then analysed by sodium dodecyl sulphate gel electrophoresis and fluorography. Left (lanes *a*–*d*), Coomassie Blue stained protein; right (lanes *a′*–*d′*), penicillin-binding proteins detected by fluorography. Apparent molecular masses are shown. Note: PBPs 2 and 3 of *E. coli* are too faint to appear in this photograph (lane *d′*).

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Penicillin-binding proteins

Attempts to purify and identify a penicillin-sensitive peptidoglycan transpeptidase have failed. Unlike CPase, which is stable to non-ionic detergents, transpeptidase activity *in vitro* is lost upon solubilization of bacterial membranes. The problem is compounded by the fact that bacterial membranes contain not one, but several proteins that bind penicillin covalently (figure 4) (Suginaka *et al.* (1972); reviewed in Blumberg & Strominger (1974) and Strominger *et al.* (1978)). Genetic, biochemical and immunological evidence indicate that the multiple penicillin-binding proteins (PBPs) are independent proteins, not related by precursor–product relations. Most PBPs are integral membrane proteins that require non-ionic detergents (e.g. Triton X-100) for solubilization in an active form. They interact with penicillin and other β-lactams to form covalent complexes which can be enzymatically broken, either in the presence of amino acceptors, such as neutral hydroxylamine, or in their absence (Kozarich *et al.* 1977; Tamura *et al.* 1976; Kozarich & Strominger 1978; Hammarstrom & Strominger 1975; Frère *et al.* 1975; Ghuysen 1976; Waxman & Strominger 1979*b*). Much effort has gone into studying the functions *in vivo* of the various PBPs. Studies of *E. coli* PBPs indicate that the multiple PBPs may mediate various penicillin-sensitive reactions. One PBP (PBP 1a, b) functions as the transpeptidase active in cell elongation, another (PBP 3) functions in septation and yet a third (PBP 2) is responsible for maintenance of the characteristic rod shape (Spratt 1977; Spratt, this symposium).

PBPs have been purified from detergent-solubilized membranes by covalent β-lactam affinity chromatography (Blumberg & Strominger 1974; Strominger *et al.* 1978). The PBPs are acylated by the penicillin or cephalosporin bound to the affinity resin and can be eluted with neutral hydroxylamine. By using this technique, the lowest molecular mass PBP has been purified from several bacterial species, including *B. subtilis*, *B. stearothermophilus* and *S. aureus*. The purified PBPs catalyse the specific hydrolysis of the terminal D-alanine residue from cell wall-related substrates, i.e. they have CPase activity *in vitro*. These CPases, often the major PBPs in bacterial membranes, catalyse transpeptidation *in vitro* in the presence of a sufficiently high concentration of amino acceptor, suggesting that they might be transpeptidases *in vivo* that have been 'uncoupled' during purification (for a more complete discussion, see Yocum *et al.* 1980). The function of these CPases *in vivo* is not yet known. As the CPases purified from membranes of *B. subtilis* and *B. stearothermophilus* catalyse penicillin-sensitive reactions with the use of cell wall-related substrates, and as these PBPs can be purified in milligram quantities, they were chosen as model enzymes for studying the mechanism of penicillin action at the molecular level.

Interaction of penicillin with CPase

The binding of penicillin by CPases from *Bacillus* is dependent upon specific structural features of the antibiotic, including the free carboxyl, D-asymmetric centre and, as an absolute requirement, an intact β-lactam bond. Mild heat or ionic detergent treatment abolishes this penicillin-binding activity, indicating that it is not simply the result of chemical acylation of an active nucleophile, but rather the consequence of specific interactions between penicillin and the native enzyme. Stoichiometric penicillin–CPase complexes can be isolated by gel filtration after incubation of penicillin and enzyme under saturating conditions (table 1) (Waxman & Strominger 1979*a*). Loss of D-alanine carboxypeptidase activity can be directly correlated with the extent of complex formation (Lawrence & Strominger 1970*b*).

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[^{14}C]Penicillin G is covalently bound to these enzymes via a linkage that is sufficiently stable to boiling with sodium dodecyl sulphate to permit detection after polyacrylamide gel electrophoresis and radioautography. That a near stoichiometric complex can be recovered after denaturation by any one of several methods, including treatment with 6 M guanidine HCl or 70% formic acid, indicates that a covalent complex most probably exists in the native enzyme. It is generally believed that penicillin is covalently bound via its β-lactam carbonyl as the penicilloyl moiety in the native complex. This is supported by the fact that these CPases catalyse hydroxylaminolysis of the covalently bound penicillin, resulting in restoration of enzymatic activity concomitant with release of penicilloyl hydroxamate (Lawrence & Strominger 1970*b*; Kozarich *et al.* 1977). Related CPases from *E. coli* and *S. aureus* also catalyse conversion of the bound penicillin to penicilloic acid (Tamura *et al.* 1976; Kozarich & Strominger 1978). It is likely that these release reactions occur with transfer of a penicilloyl moiety to appropriate nucleophilic acceptors (hydroxylamine or water) consistent with the occurrence of a bound penicilloyl moiety in the native complex. In addition, chemical release of penicilloic acid by mild base treatment (unpublished observations) and recent nuclear magnetic resonance data (Degelaen *et al.* 1979) indicate that a penicilloyl moiety is covalently bound in the denatured complex. Thus, although not yet rigorously proved, it is most likely that a covalent penicilloyl-enzyme is the inactive form of CPase.

TABLE 1. STOICHIOMETRY OF PENICILLOYL-CPASE COMPLEXES

([^{14}C]Penicilloyl-enzyme complexes were isolated by gel filtration through Sephadex G-50 equilibrated in 10 mM Na cacodylate (pH 6.0) at 4 °C. The stoichiometry of penicillin binding was determined by quantitation of radioactivity and protein content.)

	radioactivity content†		protein content		
	count/min	nmol	total amino acids‡/nmol	protein nmol§	penicillin: CPase‖
B. subtilis CPase	13300	0.133	60	0.137	0.97
B. stearothermophilus CPase¶	16400	0.165	83	0.203	0.81

† By liquid scintillation counting, assuming 85% counting efficiency and 53 μCi/μmol of [^{14}C]penicillin G.
‡ By amino acid analysis after acid hydrolysis.
§ Assuming 440 residues per molecule (*B. subtilis* CPase) or 409 residues per molecule (*B. stearothermophilus* CPase).
‖ Calculated on a molecular basis.
¶ Data taken from Waxman & Strominger (1979*a*).

ACYL-ENZYME INTERMEDIATES

It had been postulated that acyl-enzyme intermediates occur in the reactions catalysed by penicillin-sensitive enzymes (Tipper & Strominger 1965). Much evidence supports the existence of acyl-enzymes as catalytic intermediates in reactions mediated by CPases. Kinetic data, i.e. a marked rate acceleration by hydroxylamine with some substrates, have indicated that the CPases from *B. subtilis* and *B. stearothermophilus* form an acyl-enzyme intermediate in the reaction with substrate (Nishino *et al.* 1977). The simplest kinetic scheme for such a reaction is

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E\cdot S \xrightarrow{k_2} E{-}S \xrightarrow{k_3} E + P,$$

where E·S is the non-covalent enzyme–substrate complex and E—S the putative acyl-enzyme intermediate. With the synthetic substrate diacetyl-L-Lys-D-Ala-D-Ala, the acylation step (k_2)

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results in formation of diacetyl-L-Lys-D-alanyl-enzyme (E—S) and free D-alanine. Deacylation (k_3) releases the carboxypeptidase product, diacetyl-L-Lys-D-Ala and regenerates free enzyme.

With the *S. aureus* CPase, acylation (k_2) is more rapid than deacylation (k_3) so that the acyl-enzyme intermediate accumulates and can be trapped by rapid denaturation (Kozarich & Strominger 1978). For CPase IA from *E. coli*, k_2 and k_3 are roughly equal, as indicated by the ability to trap only small amounts of acyl-enzyme. Treatment of the *E. coli* enzyme with *p*-chloromercuribenzoate slows down the deacylation step without significantly affecting acylation, favouring accumulation of acyl-enzyme (Curtis & Strominger 1978). No acyl-enzyme could be trapped with the CPases from *Bacillus*, suggesting that k_3 is much greater than k_2 and that, consequently, the E—S intermediate exists in steady-state concentrations.

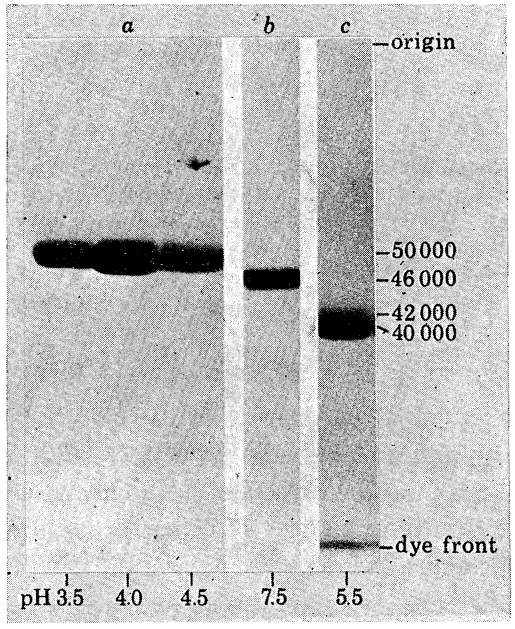

FIGURE 5. Trapping of acyl-enzyme intermediates (E—S) by using the ester substrate. Fluorographs of SDS-polyacrylamide gels showing purified D-alanine carboxypeptidases from (*a*) *B. subtilis* (PBP 5), (*b*) *S. aureus* (PBP 4) and (*c*) *E. coli* (PBPs 5 and 6) incubated at the indicated pH with 0.9 mM [^{14}C]diacetyl-L-Lys-D-Ala-D-lactate, followed by acetone precipitation. Taken from Rasmussen & Strominger (1978).

It is well known that α-chymotrypsin and other peptidases often hydrolyse synthetic ester substrates much more readily than the corresponding amides, owing to a more facile acylation (corresponding to k_2) by the esters (Hess 1971). With this in mind, Rasmussen & Strominger (1978) synthesized the depsipeptide diacetyl-L-Lys-D-Ala-D-lactate with the goal of improving the kinetics to favour acyl-enzyme trapping. This depsipeptide was shown to be a good substrate for several penicillin-sensitive CPases. As expected, k_2 was markedly accelerated and acyl-enzymes were readily trapped with the CPases purified from *B. subtilis*, *S. aureus* and *E. coli* (figure 5) (Rasmussen & Strominger 1978). Thus it is clear that acyl-enzymes form and can be trapped with these penicillin-sensitive enzymes.

Penicilloyl-CPase complexes catalyse the release of the bound penicilloyl moiety, either in the presence of added nucleophiles or in their absence, in which case a slow ($t_{\frac{1}{2}}$ = 80–200 min at 37 °C) fragmentation of the bound penicilloyl moiety yields reactivated enzyme, phenylacetylglycine and dimethylthiazoline carboxylate (or its hydrolysis product, *N*-formyl

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D-penicillamine) (Kozarich *et al.* 1977; Hammarstrom & Strominger 1975; Frère *et al.* 1975; Ghuysen 1976). That CPase can enzymatically release or fragment the bound penicillin indicates that the antibiotic is fixed at a catalytically active site. Is the acyl group derived from the substrate bound to the same catalytically active site, as had been predicted by the Tipper–Strominger hypothesis? The efficient trapping of acyl-enzyme intermediates by using the depsipeptide substrate made it feasible to approach this question directly by isolating and sequencing [^{14}C]penicillin- and [^{14}C]substrate-labelled peptides.

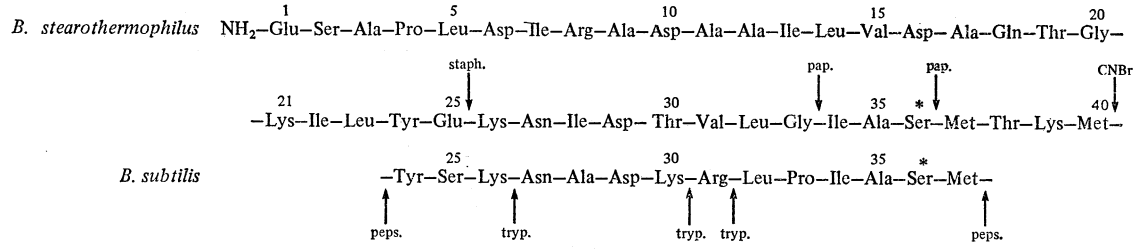

FIGURE 6. Active site sequences for *B. stearothermophilus* and *B. subtilis* CPases. CPases were labelled with [^{14}C]-penicillin or [^{14}C]diacetyl-L-Lys-D-Ala-D-lactate, cleaved with CNBr (*B. stearothermophilus*) or pepsin (*B. subtilis*) and the labelled peptides were purified and sequenced. Both active site labels were bound in ester linkage to serine 36 (*) determined from further cleavage studies by using staphylococcal protease, papain and trypsin, with cleavage at the indicated sites.

ACTIVE SITE PEPTIDES OF CPASE

Penicilloyl-enzymes were prepared by incubating the CPases purified from *B. subtilis* and *B. stearothermophilus* with a twofold molar excess of [^{14}C]penicillin G. The corresponding acyl-enzymes were prepared by trapping the substrate, [^{14}C]diacetyl-L-Lys-D-Ala-D-lactate, at the enzymes active site by rapid denaturation at 0 °C. These procedures yielded 0.9 mol penicillin or 0.7–0.9 mol substrate covalently bound per mole of CPase. (For further details of these experiments see Yocum *et al.* (1979).) Peptides were generated from the *B. subtilis* CPases by hydrolysis with pepsin after guanidine denaturation and from the *B. stearothermophilus* CPase by chemical cleavage with CNBr. Labelled peptides were subsequently purified by combinations of gel filtration, ion exchange and preparative thin layer chromatography; amino acid sequences were obtained by automated Edman degradation. The amino acid sequences of the penicillin- and substrate-labelled peptides were identical (figure 6). Further enzymatic cleavage of these peptides identified the site labelled by the [^{14}C]penicilloyl or [^{14}C]-diacetyl-L-Lys-D-alanyl moiety as serine 36 for both penicillin-sensitive CPases (figure 6). Mild base treatment of the labelled peptides released either penicilloic acid or diacetyl-L-Lys-D-Ala, indicating that the corresponding penicilloyl and acyl moieties were covalently bound to serine 36 via an ester linkage (Yocum *et al.* 1979). Studies of [^{14}C]cefoxitin-labelled *B. subtilis* CPase have demonstrated that this antibiotic also binds covalently to serine 36 (Waxman & Strominger 1980). These findings prove that penicillin acylates the active site amino acid residue of these penicillin-sensitive enzymes, as originally predicted by the Tipper–Strominger hypothesis.

Earlier work had shown that neutral hydroxylamine, H_2O_2 or thiols could reverse penicillin binding to CPase in a crude membrane fraction from *B. subtilis* (Lawrence & Strominger 1970*a*). Because ester and amide bonds would not be susceptible to cleavage under these conditions, it was suggested that penicillin was bound to a cysteine residue of CPase via a thioester

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linkage. However, it has more recently been established that hydroxylaminolysis of the bound penicilloyl moiety is an enzymatically catalysed reaction (Kozarich *et al.* 1977). Thus, the penicilloyl-enzyme bond is catalytically activated such that it is less stable than the analogous ester bond in a non-catalytic system. That the ester linkage was found for both penicillin and substrate by using several methods of denaturation and cleavage suggests that this linkage is present in the native complex, i.e. it is unlikely that a base-catalysed acyl migration occurred during peptide generation and purification.

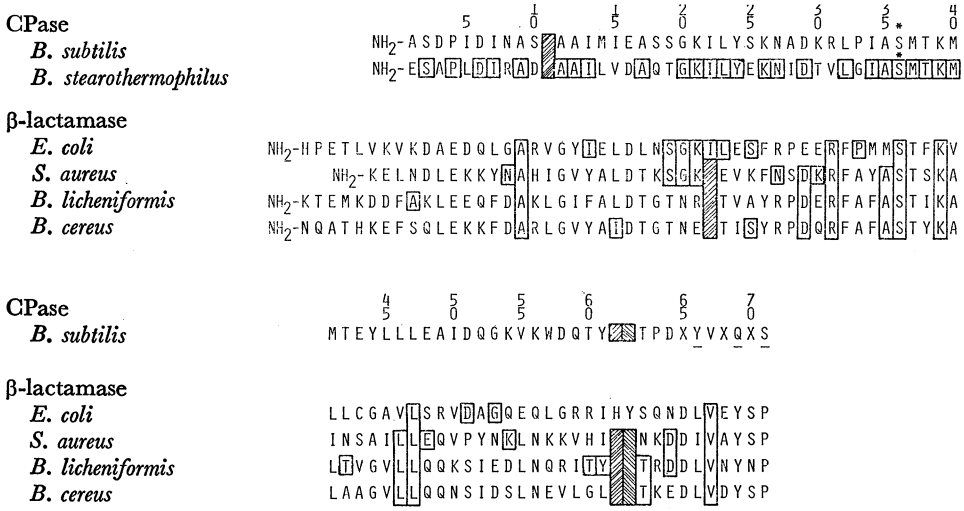

FIGURE 7. Sequence homology between CPases and β-lactamases. The NH_2-terminal 71 amino acid residues of the *B. subtilis* CPase (Waxman & Strominger 1980) are compared with the NH_2-terminal 40 residues of the *B. stearothermophilus* CPase (Yocum *et al.* 1979) and the NH_2-terminal portions of four β-lactamases of known primary structure (Ambler, this symposium); the single letter amino acid code is used. Gaps introduced in the β-lactamase sequences (at residue 22 and after residue 61–numbering system of CPase) were those described previously (Ambler, this symposium); one additional gap is inserted after residue 10 of CPase (Waxman & Strominger 1980). Amino acids identical to those of the *B. subtilis* CPase are in boxes. Sequence homology between the CPases and β-lactamases is most extensive in the region containing the active site residue (serine 36, *) of the CPases. This active site serine is correctly aligned with the serine residue likely to be important for β-lactamase catalysis (Knott-Hunziker *et al.* 1979), adding significance to the observed sequence homology.

HOMOLOGY TO β-LACTAMASES

It had been suggested that β-lactamases, which hydrolyse the β-lactam of penicillins to form the bactericidally inactive penicilloic acid, may have evolved from penicillin-sensitive enzymes of cell wall biosynthesis (Tipper & Strominger 1965). The finding that the CPases from *E. coli* and *S. aureus* are active in the conversion of penicillin G to penicilloic acid (Tamura *et al.* 1976; Kozarich & Strominger 1978) supports this hypothesis. To determine whether an evolutionary relation between CPases and β-lactamases might be reflected by homology at the level of primary structure, additional sequence data were obtained for the CPases. [^{14}C]penicillin-labelled CNBr and trypsin fragments and the NH_2-terminus of the *B. subtilis* CPase were each sequenced. The two CPases studied were thus shown to be greater than 60% homologous in their NH_2-terminal 40 amino acids (figure 7). Moreover, a computer search for homologous proteins indicated significant sequence homology between the active site of CPases and each

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of four β-lactamases of known primary structure (figure 7). That the active site serine of the CPases (serine 36) is conserved in all four β-lactamases suggests that this residue might be involved in β-lactamase catalysis. In fact, it was recently reported that β-bromopenicillanic acid, an active site-directed β-lactamase inhibitor, covalently binds as an ester to the corresponding serine (serine 44) in the *B. cereus* β-lactamase (Knott-Hunziker *et al.* 1979). Studies with the *E. coli* β-lactamase and the inhibitor quinacillin sulphone similarly indicate that an inhibitor-derived moiety covalently labels an octapeptide which includes the equivalent serine (J. R. Knowles, personal communication). The sequence homology and occurrence of the same active site serine lend strong support to the idea that penicillin-sensitive CPases and penicillin-inactivating β-lactamases are evolutionarily related and suggests that the catalytic mechanisms of these two groups of enzymes might be similar.

Crystallization studies

One approach to the question of structural similarity between β-lactams and acyl-D-Ala-D-Ala would be to co-crystallize a CPase with either penicillin or substrate bound at the active site and to determine the three-dimensional structure of the complexes. This type of study would also yield information on the structural relatedness of CPases and β-lactamases. Knox *et al.* (1979) and Dideberg *et al.* (1979) have recently crystallized exocellular CPases from two species of *Streptomyces*. For the albus G CPase, both a cephalosporin and a competitive inhibitor (a substrate equivalent) were recently localized to the same region of the molecule (J.-M. Ghuysen, personal communication). Although highly desirable, crystallization of the membrane-derived CPases from *Bacillus* sp. would also be difficult because these enzymes are dissolved in detergent micelles. However, Waxman & Strominger (1978, 1979) have proteolytically removed a 2000–3000 molecular mass hydrophobic membrane-anchoring segment from the COOH terminus of *B. stearothermophilus* and *B. subtilis* CPases. These slightly shortened CPases retain full enzymatic and penicillin-binding activities, but unlike the parent enzymes they cannot be reconstituted into phospholipid vesicles and do not bind detergent micelles. These active, water-soluble fragments should be amenable to crystallization. Current procedures for trapping substrate at the active site required denaturation, so it is not yet clear if a crystalline enzyme–substrate complex can be obtained. Perhaps a native enzyme specifically blocked at the deacylation step (Curtis & Strominger 1978) will be useful for crystallizing an acyl-enzyme intermediate.

β-Lactams *v.* acyl-D-Ala-D-Ala: an evaluation

In order for β-lactam antibiotics to be effective active site inhibitors of cell wall biosynthesis, they must have (1) structural similarity to physiological substrates that is sufficient to permit recognition by essential cell wall enzymes, (2) a highly reactive β-lactam bond to facilitate acylation of the enzyme's active site, and (3) structural features that effectively prevent removal of the bound penicilloyl moiety by cell wall amino acceptors, i.e. the inhibition must be slowly reversible or irreversible. This last condition is possibly met because the cleaved bond was part of a cyclic amide and thus the thiazolidine (or dihydrothiazine) ring remains covalently bound and can sterically block incoming nucleophiles.

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To what extent are β-lactam antibiotics structural analogues of acyl-D-Ala-D-Ala? What structural features enable them to rapidly acylate sensitive enzymes? These questions can, in part, be answered by examining several important structural differences between penicillins and acyl-D-Ala-D-Ala. (1) The angle about the β-lactam carbonyl (C—CO—N) is approximately 90°, indicative of the strained β-lactam ring present in many penicillins and cephalosporins, in contrast to the corresponding angle in a typical peptide which is closer to 114°. (2) The dihedral angle about the β-lactam bond of penicillin, 136°, is almost 45° less than the corresponding peptide dihedral angle, 180°. (3) The L-asymmetric centre at C-6 (C-7) of penicillins (cephalosporins) contrasts with the D-configuration at the penultimate residue of acyl-D-Ala-D-Ala. (4) Portions of both rings of penicillin have no analogy in the dipeptide.

Several of these differences can be explained in terms of structural analogy between penicillin and a possible transition state structure formed during the enzyme-mediated cleavage of the D-Ala-D-Ala peptide bond (Tipper & Strominger 1965; Lee 1971). This possibility is supported by molecular orbital calculations which indicate a better conformational analogy of β-lactam antibiotics with the tetrahedral transition state of a dipeptide than with the dipeptide itself (Boyd 1979). Affinity of the enzyme for such a transition state would facilitate compression of the peptide dihedral angle by twisting about the —CO—N— amide bond, resulting in the loss of double bond character and a weakening of the peptide bond. The energy required for this distortion would reduce the net energy of binding of substrate to enzyme. In contrast, the non-planarity of the bicyclic ring system of penicillins (and cephalosporins) already imparts significant single bond character to the corresponding β-lactam bond, thus favouring penicillin binding to the enzyme.

Do penicillin-sensitive enzymes in fact bind penicillins more tightly than normal substrates? Constants for binding ($K_D = k_{-1}/k_1$) and acylation (k_2) have been determined for various β-lactam antibiotics (Umbriet & Strominger 1973; Ghuysen 1976) and can be compared directly with K_m and k_{cat} values obtained with various CPase substrates (table 2).† These data indicate that the CPases do *not* bind β-lactams with significantly higher affinity (lower K_D) than substrate. Thus, if certain structural features of β-lactams favour binding by mimicking the tetrahedral transition state of a dipeptide undergoing cleavage, other features, including side chain substituents and portions of the thiazolidine (dihydrothiazine) ring with no analogy in the dipeptide, might decrease enzyme recognition such that, overall, substrate and antibiotic bind with similar affinity.

One possible criticism of the structural analogue hypothesis is the reduced activity of 6-α-methylpenicillins and 7-α-methylcephalosporins (Firestone *et al.* 1972). Indeed, the original proposal (Tipper & Strominger 1965) suggested that these compounds might have enhanced activity as a result of a closer structural analogy to acyl-D-Ala-D-Ala. The inactivity of synthetic penicillins with a D-centre at C-6 also seems to conflict with the proposed similarity of β-lactams to the D-D-dipeptide. Virudachalam & Rao (1977) have resolved these difficulties by analysing the conformational similarity of several penicillins, cephalosporins and dipeptides. They found that the L-centre of penicillins and cephalosporins (at C-6 and C-7 respectively) would *not* be able to assume a conformation similar to the penultimate residue of acyl-D-Ala-D-Ala, except for

† Assuming a rapid binding equilibrium ($k_{-1} \gg k_2$), K_m equals K_D and k_{cat} equals k_2 if deacylation (k_3) is much more rapid than acylation (k_2). In the case where k_2 equals k_3, $K_m = \frac{1}{2}K_D$ and $k_{cat} = \frac{1}{2}k_2$. Kinetic data (Nishino *et al.* 1977), and the inability to trap acyl-enzyme intermediates (E—S) unless k_2 is accelerated by use of the depsipeptide diacetyl-L-Lys-D-Ala-D-lactate (Rasmussen & Strominger 1978), make it unlikely that $k_2 \gg k_3$ for the enzymes and substrates listed in table 2.

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TABLE 2. COMPARISON OF BINDING AFFINITIES AND ACYLATION RATES OF CPASES BY SUBSTRATES AND BY β-LACTAMS

β-lactam	K_D/mM	k_2/s^{-1}	k_2/K_D/(M^{-1} s^{-1})
(i) *B. subtilis* CPase†			
penicillin G (4 °C)	0.10	0.12	1200
penicillin V (4 °C)	0.13	0.06	460
ampicillin	0.73	0.08	110
propicillin	2.0	0.42	210
cloxacillin	4.6	0.02	4.3
cephalothin	28	0.10	3.6
(ii) *Streptomyces* R61 CPase‡			
penicillin G (25 °C)	13	180	13700
penicillin V	—	—	1500
carbenicillin	0.109	0.091	830
ampicillin	7.2	0.77	107
cephaloglycin	0.4	0.0085	21

substrate	K_m/mM	V_{max}/s^{-1}	V_{max}/K_m/(M^{-1} s^{-1})
(i) *B. subtilis* CPase†			
UDP-muramylpenta-peptide (pH 5.5, 37 °C, 15 mM Zn^{2+})	0.26	0.015	58
diacetyl-L-Lys-D-Ala-D-Ala (25 °C)	6.7	0.17	25
(ii) *Streptomyces* R61 CPase‡			
diacetyl-L-Lys-D-Ala-D-Ala	12	9.4	783
N-acetyl-L-Lys-D-Ala-D-Ala	15	0.21	14
UDP-muramyl-pentapeptide	11	0.032	2.9

† Data from Umbreit & Strominger (1973) and Rasmussen & Strominger (1978). Measurements were made at pH 6, 37 °C, unless indicated otherwise.
‡ Data from Ghuysen (1976). Measurements were made at pH 7.5, 37 °C, unless indicated otherwise.

the fact that closure of the β-lactam ring fixes the conformation about C-6 or C-7 such that the antibiotic's L-centre adopts a conformation similar to a D-residue, rather than to an L-residue. Furthermore, the relative inflexibility of the conformation at this centre does not permit the antibiotic's 6(7)-acylamino group to overlap with the wider range of possible conformations of acyl-D-Ala-D-Ala if C-6 is substituted with an α-methyl group or if it has a D-configuration. In contrast, placement of an α-methoxy group at C-7, such as in the active cephamycins (e.g. cefoxitin), does yield an antibiotic with a conformation similar to certain allowed conformations of acyl-D-Ala-D-Ala (Virudachalam & Rao 1977). Although the 6-α-hydroxyethyl group of thienamycin is even more bulky than a 6-α-methyl group, one would not expect difficulty in overlapping its conformation with that of acyl-D-Ala-D-Ala (as was encountered with the 6-α-methylpenicillins) because this antibiotic lacks a 6-aminoacyl substituent. Thus the D-Ala-like conformation at C-6 or C-7, maintained by an intact β-lactam ring, seems to be as important as an intact β-lactam bond for antibacterial activity.

The high reactivity of the β-lactam bond in many penicillins and cephalosporins is likely to be important for the rapid acylation of target enzymes. This reactivity largely results from a combination of the strain imparted by the four-membered ring and from the loss of normal amide resonance due to steric constraints that prevent coplanarity of the three substituents of the β-lactam nitrogen. This is reflected by a greater C=O stretching frequency, shorter C=O bond length and longer CO—N bond length in active penicillins and cephalosporins compared with unstrained β-lactams (Woodward 1949; Sweet 1972). Enamine resonance acts to further decrease amide resonance in Δ^3-cephalosporins (Morin *et al.* 1969). β-Lactam reactivity and biological activity can both be increased significantly by substitution of a good leaving group at the C-3 methylene of cephalosporins (see, for example, Boyd & Lunn 1979). This modification permits expulsion of the methylene substituent with formation of an exocyclic double bond at C-3 upon cleavage of the β-lactam. The importance of the reactive β-lactam bond is further supported by the biological inactivity of the Δ^2-cephalosporin derivatives. These compounds are more closely isosteric to penicillin than the active Δ^3-cephalosporins (Sweet 1972), yet they are significantly less active, presumably as a consequence of their low chemical reactivity. Conversely, penicillin G is a strong inhibitor of the *Streptomyces* R61 CPase because of its rapid acylation of the enzyme (k_2 is 20–6000 times greater than k_2 for the usual CPase substrates) and not because of a particularly high affinity for the enzyme (as reflected by a K_D value which is comparable to other CPase substrates) (table 2). Thus the high potency and specific inhibitory properties of β-lactam antibiotics are derived from two important features: a relatively favourable (or at least tolerable) recognition by enzyme and a strong acylating ability. These facts are reflected in a high k_2/K_D ratio (table 2) which, when coupled with the stability of the covalent β-lactam–enzyme complex (very small k_3), enables these antibiotics to efficiently inactivate sensitive enzymes of cell wall biosynthesis.

The interactions of β-lactam antibiotics with target enzymes have proved to be enormously complex. Future research should lead to a better understanding of structure–activity relations for these antibiotics. Structural studies of enzyme–substrate and enzyme–antibiotic complexes will help to determine how β-lactams are recognized by and how they inactivate sensitive enzymes. Finally, a combination of genetic and biochemical techniques should help elucidate the function *in vivo* of each of the penicillin-binding proteins and lead to a better understanding of the modes of action of β-lactam antibiotics.

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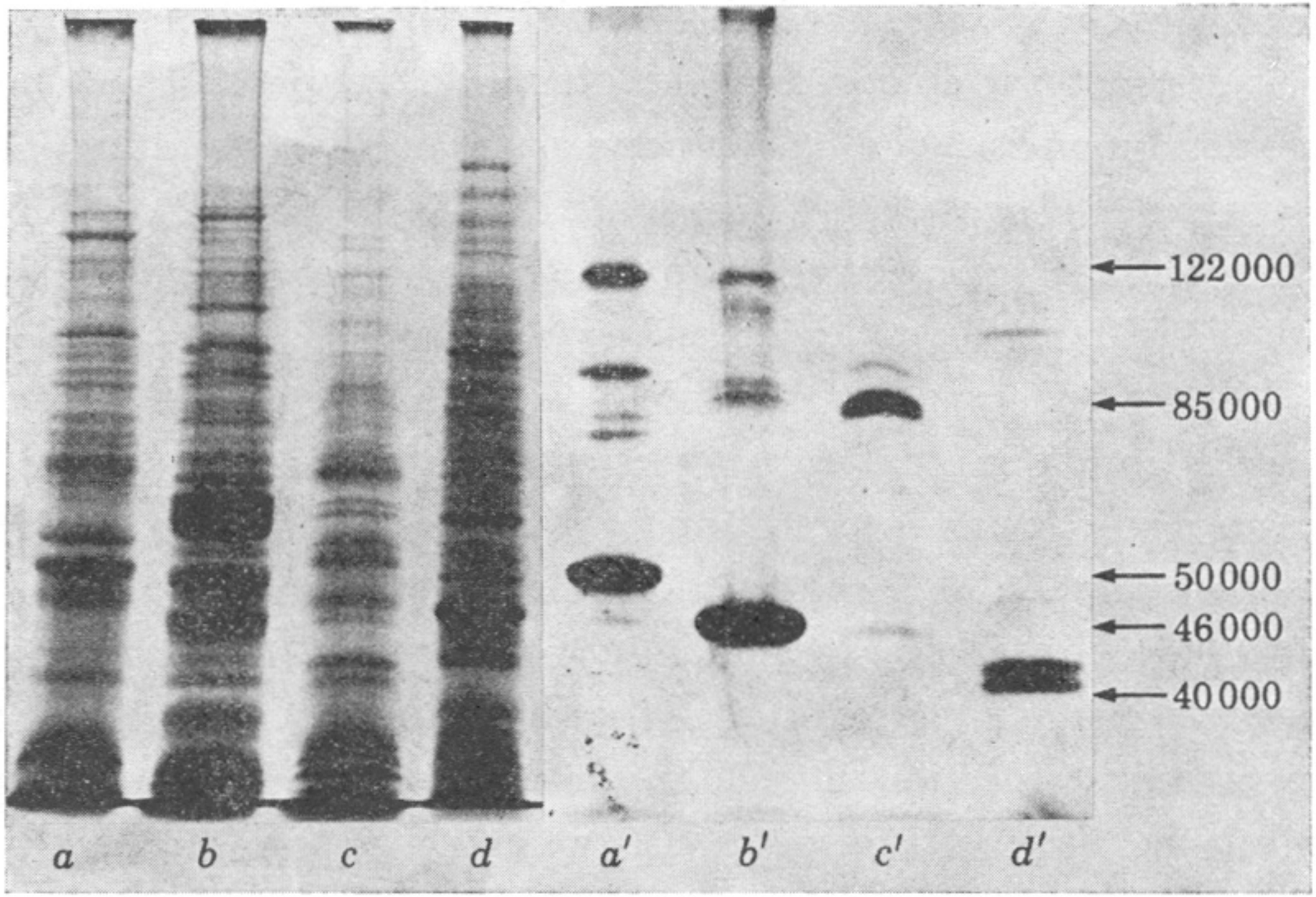

(*a*) *B. subtilis*
(*b*) *B. stearothermophilus*
(*c*) *S. aureus*
(*d*) *E. coli*

FIGURE 4. Penicillin-binding proteins (PBPs) detected in bacterial membranes. Membranes from the four indicated bacterial species were incubated with [^{14}C]penicillin G and the acetone precipitated protein then analysed by sodium dodecyl sulphate gel electrophoresis and fluorography. Left (lanes *a*–*d*), Coomassie Blue stained protein; right (lanes *a*′–*d*′), penicillin-binding proteins detected by fluorography. Apparent molecular masses are shown. Note: PBPs 2 and 3 of *E. coli* are too faint to appear in this photograph (lane *d*′).

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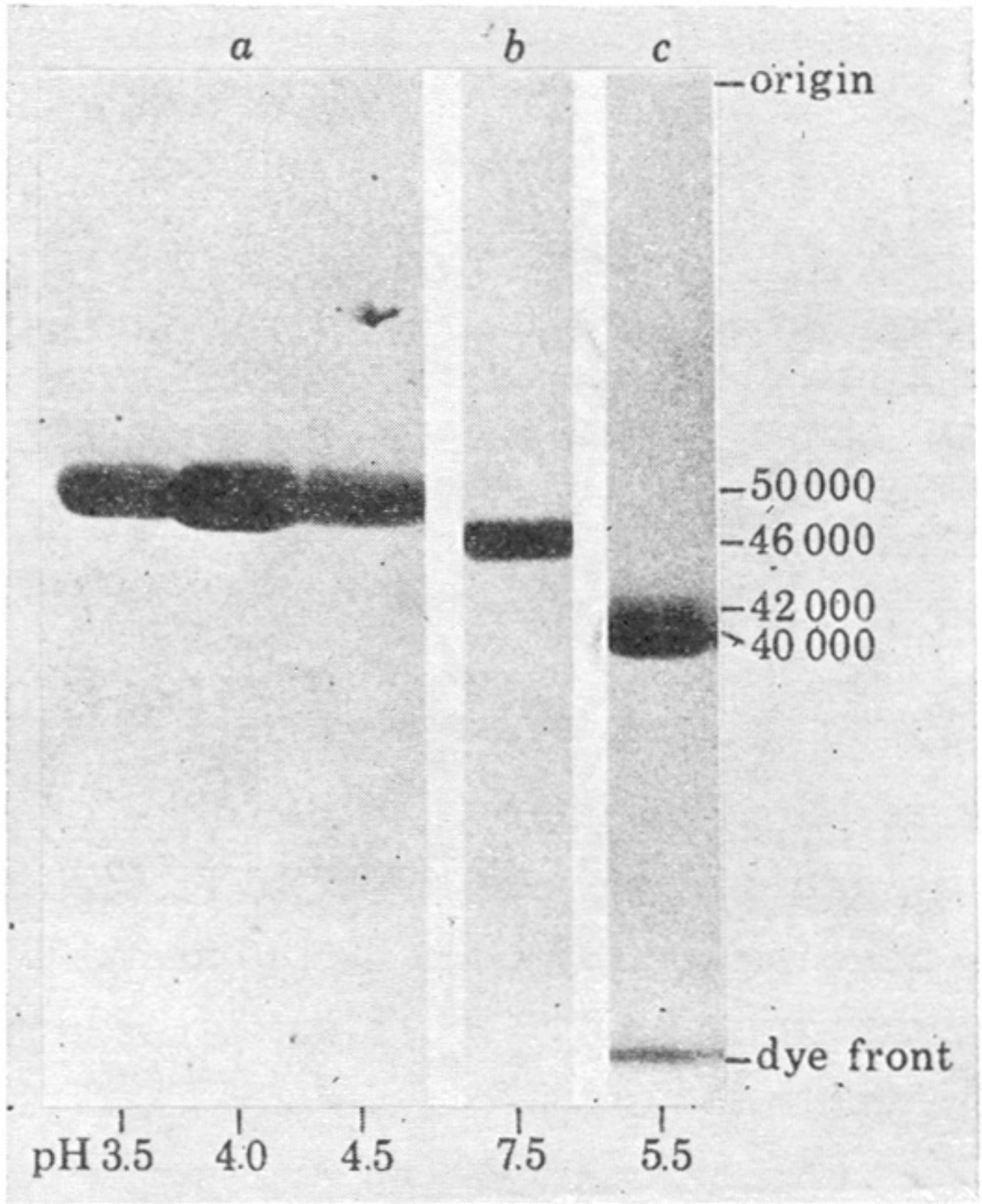

FIGURE 5. Trapping of acyl-enzyme intermediates (E—S) by using the ester substrate. Fluorographs of SDS-polyacrylamide gels showing purified D-alanine carboxypeptidases from (*a*) *B. subtilis* (PBP 5), (*b*) *S. aureus* (PBP 4) and (*c*) *E. coli* (PBPs 5 and 6) incubated at the indicated pH with 0.9 mM [^{14}C]diacetyl-L-Lys-D-Ala-D-lactate, followed by acetone precipitation. Taken from Rasmussen & Strominger (1978).