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The Penicillin-Binding Proteins in *Streptococcus faecalis* ATCC 9790

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Streptococcus faecalis ATCC 9790 possesses seven membrane-bound penicillin-binding proteins. They have been characterized with respect to their apparent molecular weights, relative abundance, specificity profiles for 15 different β-lactam antibiotics and stability under various conditions. In water and at 37 °C, all the native penicillin-binding proteins have half-lives longer than 20 h except protein 3b (half-life of about 600 min) and protein 4 (half-life of about 175 min). The short-lived 80000-M_r protein 4 is spontaneously converted into a 73000-M_r water-soluble, penicillin-binding protein 4*. Similarly, the short-lived 82000-M_r protein 3b seems to be the protein from which the 72000-M_r water-soluble protein X spontaneously originates during incubation of the membranes. Release of both proteins 4* and X from the membrane is maximal under alkaline conditions; it is not inhibited by various protease inhibitors. After exposure to trypsin, the 43000-M_r membrane-bound penicillin binding protein 6 (a DD-carboxypeptidase) gives rise to a 30000-M_r water-soluble protein 6*. Like the parent protein, protein 6* exhibits both DD-carboxypeptidase activity and penicillin-binding ability. With proteins 6 and 6*, low dose levels of *p*-chloromercuribenzoate prevent both enzyme activity and combination with penicillin, thus strongly suggesting that a thiol group is involved in the enzyme active center. We have shown previously [Coyette et al. in *Eur. J. Biochem. 88*, 297–305 (1978) and *75*, 231–239 (1977)] that the DD-carboxypeptidase protein 6 fragments the benzylpenicillin molecule with formation of phenylacetylglycine. Breakdown of the complex formed between [^{14}C]benzylpenicillin and the 140000-M_r membrane-bound protein 1 is also 'enzyme-catalysed'. Most likely, however, the released product is penicilloate. With all the other penicillin-binding proteins whose molecular weights are intermediate between those of proteins 1 and 6, breakdown of the complexes formed with [^{14}C]benzylpenicillin results from proteolysis and is not due to the release of the bound metabolite. None of the penicillin-binding proteins behaves, by itself, as a lethal target for β-lactam antibiotic action on the living cells.

As shown in previous studies [1], the isolated membranes of *Streptococcus faecalis* ATCC 9790 possess several proteins which form, with [^{14}C]benzylpenicillin, complexes that are stable enough to be detected by autoradiography after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. These penicillin-binding proteins have apparent molecular weights ranging from 40000 to 140000. Altogether, they confer on the membrane the property to bind about 100 pmol penicillin/mg total proteins [2]. The only penicillin-binding protein known to be an enzyme has an apparent molecular weight of 43000. It represents more than one third of the total penicillin-binding proteins. It has been isolated in a water-soluble form (in the presence of a suitable non-ionic detergent) and has been characterized as a DD-carboxypeptidase (standard reaction: Ac_2LLys-DAla-D-Ala + H_2O → Ac_2LLys-DAla + DAla). It is also able to catalyse exchange reactions where the Ac_2LLys-DAla moiety of the donor tripeptide is transferred to amino compounds such as glycine, D-alanine or glycyl-glycine [3]. The DD-carboxypeptidase (E) reacts with benzylpenicillin (I) in a way which is compatible with a three-step mechanism $E + I \overset{K}{\rightleftharpoons} EI \xrightarrow{k_3} EI^* \xrightarrow{k_4} E +$ degraded products [1,2]. The low k_4 value ($3-4 \times 10^{-5}$ s^{-1} at 37 °C) confers on complex EI* a rather long half-life so that, when labelled with [^{14}C]benzylpenicillin, the enzyme can be visualized in the form of the radioactive 43000-M_r complex EI*. Spontaneous breakdown (at pH 7.5) of complex EI* formed with the purified enzyme causes enzyme regeneration with release of free phenylacetylglycine and *N*-formyl-D-penicillamine, indicating that the processing of the penicillin molecule involves both the opening of the C(7)-N(4) amide bond and the rupture of the C(5)-C(6)

Enzyme. DD-Carboxypeptidase (EC 3.4.17.8).

linkage of the β-lactam ring [1,2]. Spontaneous breakdown (at pH 7.5) of the complexes formed between [^{14}C]benzylpenicillin and the isolated membrane (thus involving all the penicillin-binding proteins present) also causes regeneration of the membrane-bound DD-carboxypeptidase activity [2]. At about 90% enzyme recovery, 39% of the total radioactivity is released in the form of phenylacetylglycine, 29% remains bound to the membranes, 30% is released as a soluble material which is immobile on paper electrophoresis at pH 6.5, and 8% as benzylpenicilloate. These observations indicate that among the various penicillin-binding proteins present in *S. faecalis*, the 43000-M_r DD-carboxypeptidase is probably the only one which is able to perform fragmentation of the penicillin molecule. The relative ability of several β-lactam antibiotics to immobilize the DD-carboxypeptidase in the form of complexes EI* is apparently related to the relative effectiveness of the same antibiotics to inhibit cellular growth, suggesting that this enzyme might be an important target for penicillin action *in vivo* [1]. However, the relationship between the two parameters is complex and does not apply to all the antibiotics tested. Obviously, a better understanding of the mechanism through which penicillin exerts its lethal effect on *S. faecalis* requires a better knowledge of all the penicillin-binding proteins present in this organism. Results obtained along this line are reported in this paper.

MATERIALS AND METHODS

Protein Estimation

The technique of Lowry et al. [5] modified as described in [1] was used.

Polyacrylamide Slab Gel Electrophoresis in Sodium Dodecyl Sulfate

The electrophoreses were carried out in 50 mM Tris/383 mM glycine buffer pH 8.3 containing 0.1% sodium dodecyl sulfate. The technique was essentially that of Laemmli and Favre [6] and the experimental conditions were those described previously [1] except that (a) the slab gel was 25-cm long, (b) the separation gel was 7.5% polyacrylamide (and not 10% as in [1]); and (c) the electrophoreses were carried out at 100 V, constant voltage, for 1 h and then at 350 V, constant voltage, for 4–5 h. Bromophenol blue migrated 18–20 cm toward the anode. These modified conditions permitted a better resolution of the 70000–90000-M_r proteins. Staining of the proteins with Coomassie brilliant blue and destaining were performed as in [1]. The following standard proteins were used: β-galactosidase (130000-M_r and 65000-M_r subunits), phosphorylase *a* (94000), bovine serum albumin (68000), catalase (60000), leucine aminopeptidase (53000), ovalbumin (43000) and carbonic anhydrase (29000).

[^{14}C]Benzylpenicillin

[^{14}C]Benzylpenicillin was purchased from Radiochemical Center, Amersham. The specific radioactivity was 59 Ci/mol with the radioactive label on the CO group of the phenylacetyl substituent.

Non-radioactive β-Lactam Antibiotics

The following antibiotics were used: ampicillin, carbenicillin, methicillin, cloxacillin (from Beecham Pharmaceuticals, UK); benzylpenicillin (Rhone Poulenc, France) and phenoxymethylpenicillin (Imperial Chemical Industries Ltd, UK), all representatives of the penicillin family. Mecillinam (Leo Pharmaceutical Products, Denmark) is an amidinopenicillin. Cephalosporin C, cephalothin and cephalexin (Eli Lilly and Co. Benelux) are representatives of the Δ^3-cephalosporin family. Cefoxitin (Merck Sharp and Dohme, NJ, USA) is a 7α-methoxy-Δ^3-cephalosporin. Thienamycin (Merck Sharp and Dohme, NJ, USA) is a 1-carba-Δ^2-penemcarboxylate derivative. Clavulanic acid (Beecham Pharmaceuticals, UK) is an oxapenicillin and nocardicin A (Fusijawa Pharmaceutical Co., Ltd, Japan) a monocyclic β-lactam. These antibiotics were gifts from Pharmaceutical Industries.

Growth Conditions and Minimal Growth Inhibitory Concentrations of β-Lactam Antibiotics

Streptococcus faecalis ATCC 9790 was grown in the yeast extract/peptone medium as described in [4]. The minimal growth inhibitory concentrations of the non-radioactive β-lactam antibiotics listed above were those which inhibited cellular growth of *S. faecalis* under the conditions described in [4].

Membranes

The membranes were prepared as described in [3]. In this procedure, cellular lysis is carried out in 5 mM phosphate buffer pH 7.0 containing egg-white lysozyme, DNase and RNase, and is complete after 1 h of incubation at 37 °C. The final preparations (20 mg membrane protein/ml) were in water. They could be stored at −25 °C for months without any major alteration of the pattern of penicillin-binding proteins and without loss of DD-carboxypeptidase activity.

Binding of [^{14}C]Benzylpenicillin to Intact Cells. Saturation Experiments

Growing cells collected from 1 l of a log-phase culture (absorbance at 550 nm = 1.0) were washed

twice with cold water by centrifugation and resuspended in 10 ml water. The suspension contained 27 mg (dry weight) cells/ml. Part of the cell suspension (5 ml) was treated with 0.5 ml of an aqueous solution of 0.1 M non-radioactive benzylpenicillin for 15 min at 37 °C; the cells were washed twice by centrifugation with cold water and resuspended in 5 ml water. Samples (400 µl) of both the original cell suspension (A samples) and the cell suspension previously treated with non-radioactive benzylpenicillin (B samples) were supplemented with various concentrations of [^{14}C]benzylpenicillin (0.01 – 100 µM in 4 µl water), incubated for 15 min at 37 °C and then supplemented with 40 µl of an aqueous solution of 0.1 M non-radioactive benzylpenicillin. The cells were washed twice by centrifugation with 1 ml of an aqueous solution of 1 mM non-radioactive benzylpenicillin, resuspended in 0.2 ml water and dissolved with the help of 1 ml Lumasolve and 9 ml Lipoluma (Luma Systems A.G., Basel). The radioactivity of the resulting solutions was then measured. The amounts of [^{14}C]benzylpenicillin specifically fixed on the cells were determined by subtracting the radioactivities of the samples B from those of the corresponding samples A. At the highest [^{14}C]benzylpenicillin concentration used (100 µM), 0.16% of the total radioactivity was found associated with the pellet of the control B sample. In an alternative procedure, the cells were precipitated with 10% trichloroacetic acid and collected on glass-fiber filters. Both procedures yielded similar results.

Binding of [^{14}C]Benzylpenicillin to Isolated Membranes. Saturation Experiments

The technique was that described previously [2].

Affinity of [^{14}C]Benzylpenicillin for the Penicillin-Binding Proteins in Intact Cells

Samples (20 ml) of a growing culture of *S. faecalis* collected at the mid-logarithmic phase (absorbance at 550 nm = 0.5) and whose cell contents corresponded to about 150 µg membrane proteins, were supplemented with [^{14}C]benzylpenicillin at concentrations of 0.01 – 100 µM, incubated for 5 min at 37 °C and then supplemented with 200 µl of an aqueous solution of 0.1 M non-radioactive benzylpenicillin. The cells were washed by centrifugation with 40 ml 40 mM phosphate buffer pH 6.8 containing 1 mM $MgCl_2$, resuspended in 1 ml 40 mM phosphate buffer pH 6.8 containing 1 mM $MgCl_2$, 4 µg DNase, 2 µg RNase, 200 µg egg-white lysozyme and 10 µg of crystalline *N*-acetylmuramidase M1 (from *Streptomyces globisporus* 1829; a gift from Dr K. Yokogawa, Dianippon Pharmaceutical Co., Japan [7]). Cellular lysis occurred within 10 min at 37 °C. The pellets (membranes) were collected by centrifugation (for 10 min at 4 °C and 40000 × *g*), treated with a mixture of 25 µl water and 25 µl denaturing buffer (i.e. 120 mM Tris-HCl buffer pH 6.8 containing 2% sodium dodecyl sulfate, 20% glycerol, 10% mercaptoethanol and 0.002% bromophenol blue). After boiling for 1 min, the samples were subjected to polyacrylamide slab gel electrophoresis in sodium dodecyl sulfate as described above and the penicillin-binding proteins were detected by fluorography using preflashed Kodak X-omat XL-1 films [1]. The time of exposure was 10 days at – 70 °C. The penicillin-binding proteins were then estimated by microdensitometry of the fluorograms with the help of a Joyce Loebl Mk3CS densitometer.

Affinity of [^{14}C]Benzylpenicillin for the Penicillin-Binding Proteins in Isolated Membranes

Membrane samples (20 µl) containing 125 µg of total protein and various concentrations of [^{14}C]-benzylpenicillin (0.01 – 500 µM) were incubated in water for 15 min at 37 °C. After reaction, the samples were supplemented successively with 2 µl of an aqueous solution of 0.1 M non-radioactive benzylpenicillin (or 5 µl if the concentrations of radioactive penicillin used in the preceding step were higher than 100 µM), and 25 µl of the same denaturing buffer as above. The samples were boiled for 1 min, subjected to polyacrylamide gel electrophoresis in sodium dodecyl sulfate and the penicillin-binding proteins estimated by densitometry of the fluorograms.

Affinity of Non-radioactive β-Lactam Antibiotics for the Penicillin-Binding Proteins in Isolated Membranes. Competition Experiments

Membrane samples (20 µl) containing 125 µg of total proteins and various concentrations (1 nM to 1 mM) of each of the non-radioactive β-lactam antibiotics listed above (including benzylpenicillin) were incubated in water for 15 min at 37 °C. The membrane suspensions were then supplemented with 100 µM (final concentration) [^{14}C]benzylpenicillin, incubated at 37 °C for another period of 15 min, and finally supplemented with 2 µl of an aqueous solution of 0.1 M non-radioactive benzylpenicillin and 25 µl of denaturing buffer. The samples were boiled, subjected to polyacrylamide slab gel electrophoresis and the penicillin-binding proteins estimated by densitometry of the fluorograms.

DD-Carboxypeptidase Activity Measurements

With the membranes and the substrate preparations used in the present studies, the optimal conditions for DD-carboxypeptidase activity were in > 0.1 M

phosphate buffer pH 7.0. Routinely, the assays were performed as follows: membranes or the solubilized enzyme and 4.25 mM (final concentration) Ac_2LLys-DAla-DAla were mixed together in 125 mM phosphate buffer pH 7.0 (20 µl, final volume) and incubated for 30 min at 37 °C. The released D-alanine was estimated enzymatically using the D-amino acid oxidase/peroxidase/*o*-dianisidine procedure, modified as described in [3].

Inhibitors

The following inhibitors were used: *p*-chloromercuribenzoate (Sigma; stock solution: 1 mM in water; the pH was brought to 9.0 by addition of NaOH); *N*-ethylmaleimide (Koch Light Laboratories; stock solution: 100 mM in water); phenylmethylsulfonyl fluoride (Boehringer; stock solution: 20 mM in methanol); diisopropylfluorophosphate (Merck; stock solution: 20 mM in water).

RESULTS

Binding of [^{14}C]Benzylpenicillin to Intact Cells and Isolated Membranes

Saturation curves indicated that maximal binding of [^{14}C]benzylpenicillin to growing cells (using the conditions described in Materials and Methods) and to isolated membranes (as described in [2]) occurred at 20 µM and 5 µM [^{14}C]benzylpenicillin concentrations, respectively. Maximal binding resulted in the fixation of about 100 pmol [^{14}C]benzylpenicillin/mg protein of the isolated membranes or about 7 pmol/mg (dry weight) cells. The isolated membranes hydrolysed 0.023 µmol Ac_2LLys-DAla-DAla × min^{-1} × mg protein^{-1} (under the conditions described in Materials and Methods). Complete inhibition of the DD-carboxypeptidase activity of the isolated membranes also occurred at about 100 pmol [^{14}C]benzylpenicillin bound/mg protein.

Multiplicity and Relative Abundance of the Penicillin-Binding Proteins

The patterns (Fig. 1 A and B) and relative abundance (Table 1) of the penicillin-binding proteins were determined using 100 µM concentration [^{14}C]benzyl-

Table 1. *Apparent molecular weight and relative abundance of the penicillin-binding proteins in* S. faecalis *ATCC 9790 under conditions of saturation by [^{14}C]benzylpenicillin (except for protein 5)*
The values for isolated membranes were obtained from 15 different fluorograms prepared under identical conditions. Protein 5 has a very low penicillin affinity (it is not saturated at 500 µM [^{14}C]benzylpenicillin) and is therefore underestimated

Protein	Apparent molecular weight	Percentages found in	
		isolated membranes	intact cells
1	140000	11.7 ± 1.7	10
2	90000	9.7 ± 0.8	6.3
3a	85000	± 5	very low
3b	82000	19 ± 3.2	15
4	80000	14 ± 2.8	16
5	75000	2.2 ± 0.4	very low
4*	73000	3.2 ± 1.0	absent
6	43000	39.1 ± 5.1	52

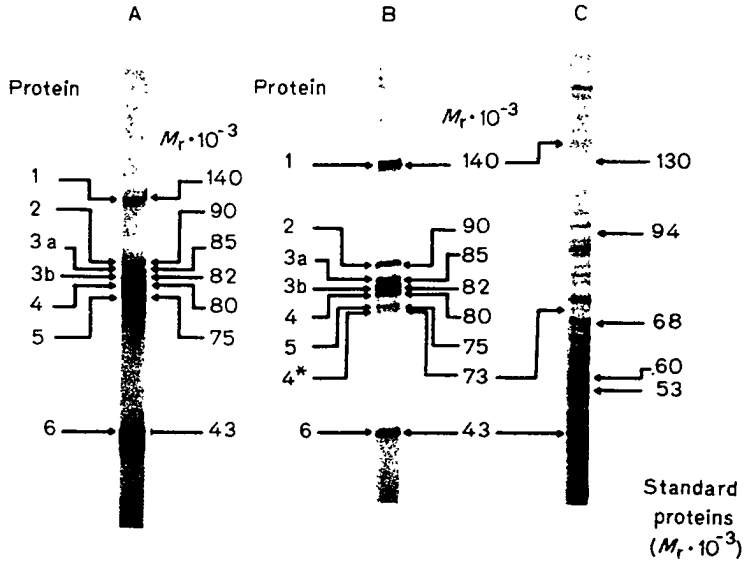

Fig. 1. *Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of the penicillin-binding proteins of* S. faecalis *ATCC 9790.* (A) Fluorogram. Growing cells were first labelled with 100 µM [^{14}C]benzylpenicillin and the membranes isolated and analysed. (B) Fluorogram. Membranes were first isolated from growing cells then labelled with 100 µM [^{14}C]benzylpenicillin and analysed. (C) Coomassie staining of gel B. For conditions and standard proteins, see text

penicillin (i.e. under conditions of saturation). The results were very similar whether the treatment with [^{14}C]benzylpenicillin was carried out on intact cells (Fig. 1 A) or on previously isolated membranes (Fig. 1 B), suggesting that the set of penicillin-binding proteins found in the membranes, as they were prepared, was a fair reflection of the proteins which together constitute the target of benzylpenicillin in *Streptococcus faecalis*. Both Fig. 1 A, obtained with intact cells, and Fig. 1 B, obtained with isolated membranes, showed the occurrence of five major penicillin-binding proteins exhibiting apparent molecular weights of 140000, 90000, 82000, 80000 and 43000 (designated as proteins 1, 2, 3b, 4 and 6, respectively) and two minor penicillin-binding proteins exhibiting apparent molecular weights of 85000 and 75000 (designated as proteins 3a and 5, respectively). Fig. 1 B, obtained with isolated membranes, shows one additional minor 73000-M_r penicillin-binding protein, designated as protein 4*. (For an explanation, see further). As revealed by Coomassie staining (Fig. 1 C), the gel portion where all the penicillin-binding proteins were located, except protein 6, had a low protein density. If the membranes were first treated for 5 min at 100 °C, further treatment with [^{14}C]benzylpenicillin failed to reveal any penicillin-binding protein. Affinity studies showed that all the penicillin-binding proteins were saturated by [^{14}C]benzylpenicillin at concentrations lower than 100 μM, except, however, protein 5 (see further). Protein 5 is characterized by a very low penicillin affinity and consequently protein 5 was underestimated.

Affinities of β-Lactam Antibiotics for the Penicillin-Binding Proteins

The affinities of [^{14}C] benzylpenicillin for all the penicillin-binding proteins (except protein 5) were expressed by the antibiotic concentrations at which the extent of protein labelling was 50% of the maximal value. Within the limits of experimental error, identical results were obtained whether the determinations were carried out on the isolated membranes or on intact cells. In turn, the affinities of the non-radioactive β-lactam antibiotics (including non-radioactive benzylpenicillin) for the same penicillin-binding proteins were expressed by the antibiotic concentrations at which the extent of protein labelling, by further treatment with 100 μM [^{14}C]benzylpenicillin, was 50% of the maximal value. All these competition experiments were carried out on isolated membranes. With nonradioactive benzylpenicillin, they yielded the same affinity values as those previously obtained by direct labelling with [^{14}C]benzylpenicillin.

Within the limits of experimental errors (Table 2), the three penam derivatives, benzylpenicillin, phenoxymethylpenicillin and ampicillin could not be distinguished from each other with respect to their affinities for the penicillin-binding proteins. The antibiotic concentrations required to achieve half-saturation of the proteins were 0.03 – 0.12 μM for protein 3(a + b); 0.1 – 0.35 μM for protein 2; 0.2 – 0.62 μM for protein 1; and 0.4 – 2.7 μM for proteins 4, 4* and 6. In parallel to this, benzylpenicillin, phenoxymethylpenicillin and ampicillin inhibited cellular growth (under the conditions described in Materials and Methods) at the same, 1 – 2 μM, minimal concentrations.

Fig. 2 shows the specificity profiles of the penicillin-binding proteins for the β-lactam antibiotics. Each protein is characterized by a pattern of three peaks obtained by plotting on a log scale and, in a defined order, the affinities (as defined above) of the various penicillins (gels 1 – 7), Δ^3-cephalosporins (gels 8 – 11) and non-classical β-lactam compounds (gels 12 – 15) studied. Fig. 2 also shows the pattern obtained by plotting the minimal inhibitory concentrations of the antibiotics on the same log scale and in the same order. Benzylpenicillin, phenoxymethylpenicillin and ampicillin were the most effective β-lactam antibiotics with

Table 2. *Affinities of β-lactam antibiotics expressed as the concentrations required to achieve 50% binding to the proteins*
Techniques: (A) competition between non-radioactive antibiotic and [^{14}C]benzylpenicillin; isolated membranes; (B) direct binding of [^{14}C]benzylpenicillin; isolated membranes; (C) direct binding of [^{14}C]benzylpenicillin; intact cells

Antibiotics	Technique	Concentration for 50% binding to protein					
		1	2	3 (a + b)	4	4*	6
		μM					
Benzylpenicillin	A	0.55	0.26	0.075	0.42	0.40	1.2
	B	0.50	0.35	0.125	2.0	1.50	1.6
	C	0.20	0.10	0.030	–	–	1.0
Ampicillin	A	0.32	0.20	0.040	0.45	0.60	0.8
	A	0.62	0.31	0.040	–	–	0.6
Phenoxymethylpenicillin	A	0.22	0.30	0.035	2.70	1.60	0.6

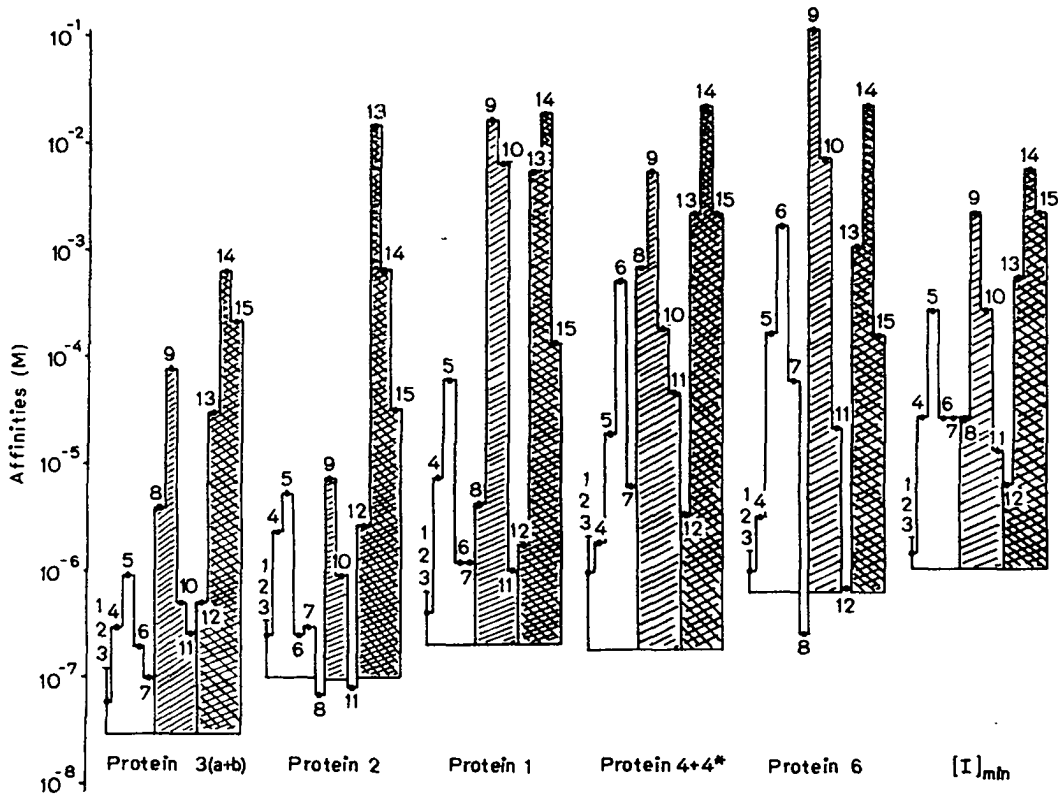

Fig. 2. *Specificity profiles of the penicillin-binding proteins for the β-lactam antibiotics.* The patterns of the proteins 3 (a + b), 2, 1, 4-4* and 6 were obtained by plotting on a log scale the antibiotic concentrations necessary to achieve 50% binding to the relevant proteins. The following order was used: 1–3, benzylpenicillin, phenoxymethylpenicillin and ampicillin (see text and Table 2); 4, carbenicillin; 5, methicillin; 6, cloxacillin; 7, oxacillin; 8, cefoxitin; 9, cephalosporin C; 10, cephalexin; 11, cephalothin; 12, thienamycin; 13, mecillinam; 14, nocardicin A; 15, clavulanic acid. The pattern of minimal inhibitory concentrations ($[I]_{min}$) on the extreme right was obtained by plotting on the same log scale the minimal growth inhibitory concentrations of the same β-lactam antibiotics

respect to their minimal growth inhibitory concentrations and their capacities to combine with all the penicillin-binding proteins except, however, the affinities of cefoxitin and cephalothin for protein 2 and those of cefoxitin and thienamycin for protein 6 were at least as high as those of benzylpenicillin, phenoxymethylpenicillin or ampicillin.

Inhibition of cellular growth by cloxacillin, cephalosporin C, and cephalexin occurred at antibiotic concentrations at least 10-fold lower than those required to achieve 50% binding to protein 6. The same observations applied to cloxacillin and cefoxitin with proteins 4 and 4*, to cephalexin, cephalosporin C, and mecillinam with protein 1, and to mecillinam with protein 2. In fact, protein 3(a + b) was the only penicillin-binding protein to which 50% binding of any of the antibiotics tested occurred at an antibiotic concentration lower than the corresponding minimal growth inhibitory concentration. Yet the differences between the two concentrations under consideration varied widely (10–1000-fold) depending upon the antibiotic. These observations suggest that inhibition of cellular growth can be obtained under circumstances where either one of proteins 1, 2, 4, 4* or 6 (and of course 5) is untouched by the antibiotic and that inactivation of protein 3(a + b) alone is probably not sufficient to inhibit cellular growth.

Stability (in Water and at 37 °C) of Penicillin-Binding Proteins and Their Complexes with [^{14}C]Benzylpenicillin

In order to study the stability of the native penicillin-binding proteins, a membrane suspension (1250 µg total proteins in 200 µl water) was maintained at 37 °C. Samples (20 µl, containing 125 µg total proteins) were removed after increasing times of incubation (up to 24 h), treated with 100 µM [^{14}C]benzylpenicillin and the penicillin-binding proteins analysed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate followed by fluorography. In order to study the stability of the complexes formed between the penicillin-binding proteins and [^{14}C]benzylpenicillin, a membrane suspension (2500 µg total proteins in 100 µl water) previously supplemented with 100 µM [^{14}C]benzylpenicillin, incubated for 15 min at 37 °C and then supplemented with 100 µl of an aqueous solution of a 20 mM non-radioactive benzylpenicillin solution, was maintained at 37 °C. In turn, samples (10 µl containing 125 µg total proteins) were removed after

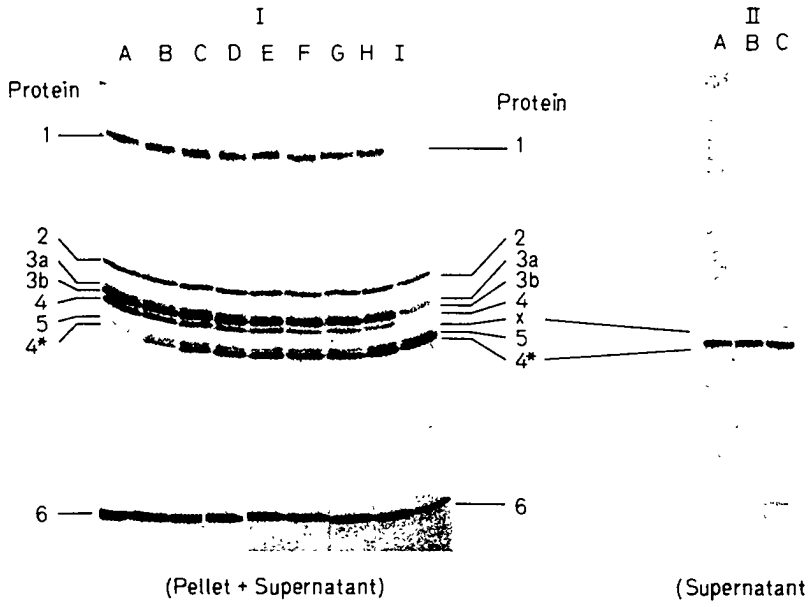

Fig. 3. *Fluorograms after polyacrylamide gel electrophoresis in sodium dodecyl sulfate.* (I) Membrane suspensions previously treated with [^{14}C]benzylpenicillin were analysed after incubation in water and at 37 °C for 0 h (A), 1 h (B), 2 h (C), 3 h (D), 4 h (E), 5 h (F), 6 h (G), 7 h (H) and 24 h (I). (II) Membrane suspensions were incubated in phosphate buffer pH 6.9 (A), in phosphate buffer pH 8.0 (B) and in carbonate buffer pH 9.1 (C) for 1 h at 37 °C (for all buffers $I = 0.05$ M), and the supernatant fractions obtained after centrifugation were treated with [^{14}C]benzylpenicillin and analysed for their contents in penicillin-binding proteins

increasing times of incubation and the penicillin-binding proteins analysed. Fig. 3, part I, illustrates such an experiment. The following observations were made (Table 3).

Proteins 2, 3(a + b) and 5. These proteins underwent proteolysis as shown by the loss of their ability to bind [^{14}C]benzylpenicillin. The processes were first-order reactions and, on this basis, the half-life values of the proteins ranged between 700 min to more than 4000 min. In turn, the radioactive complexes formed between [^{14}C]benzylpenicillin and the same proteins 2, 3(a + b) and 5 underwent spontaneous breakdown as shown by the loss of bound radioactivity. The processes were also first-order reactions, indicating half-life values identical or at least very similar, within the limits of experimental error, to those of the corresponding proteins. Hence, breakdown of the complexes formed between [^{14}C]benzylpenicillin and these proteins was due to proteolysis and not to the release of the radioactive label from the intact proteins.

Protein 4. The native 80000-M_r protein 4 also underwent proteolysis (half-life about 200 min) but its disappearance was paralleled by an equivalent increase of the native 73000-M_r protein 4*. Similarly, the disappearance of the radioactively labelled protein 4 was paralleled by the appearance of increasing amounts of the radioactively labelled protein 4* (Fig. 3, part I, and Fig. 4B). In addition, the sum of the native proteins 4 and 4* and the sum of the radioactively labelled proteins 4 and 4* (Fig. 4A), decreased at the same low rate as a function of time, indicating that protein 4* had a half-life of about 1350 min and that breakdown of the radioactive complex formed between protein 4* and [^{14}C]benzylpenicillin was also due to proteolysis and not to the release of the radioactive label.

Table 3. *Stability in water and at 37 °C of the proteins in relation to their ability to bind [^{14}C]benzylpenicillin and stability of the corresponding complexes formed with [^{14}C]benzylpenicillin*
All the proteins are membrane-bound except protein 4* which originates from protein 4 and is water-soluble

Protein	Half-life of the	
	native protein	complex formed with [^{14}C]benzylpenicillin
	min	
1	2000	350
2	1700	1900
3(a + b)	700	500
4	200	150
5	very stable	4000
4*	1300	1400
6	> 4000	500

Proteins 1 and 6. The rates at which the native proteins 1 and 6 underwent proteolysis were significantly lower than the rates at which the corresponding complexes formed with [^{14}C]benzylpenicillin broke down (Table 3). With protein 6 (i.e. the DD-carboxypeptidase), it has been established that complex breakdown is enzyme-catalysed and results in the fragmentation of the benzylpenicillin molecule into phenylacetylglycine and *N*-formyl-D-penicillamine (see the

introduction). It seems highly probable that breakdown of the complex formed between protein 1 and [^{14}C]benzylpenicillin generates penicilloate, based on the facts that breakdown of the radioactive complex formed with protein 1 also appears to be 'enzyme-catalysed', and that phenylacetylglycine and benzylpenicilloate are released from membranes previously saturated with [^{14}C]benzylpenicillin in the proportion of 5 to 1 (see the introduction), which is the relative abundance of proteins 6 and 1 in the membranes. Thus protein 1 would behave as a β-lactamase of low efficiency. Another conclusion was that the unidentified materials (either still membrane-bound or immobile on paper electrophoresis) that were detected after a prolonged incubation at 37 °C of the [^{14}C]benzylpenicillin-membrane complex (see the introduction) probably originate through spontaneous proteolysis of the complexes formed between [^{14}C]benzylpenicillin and the proteins 2, 3(a + b), 4* and 5.

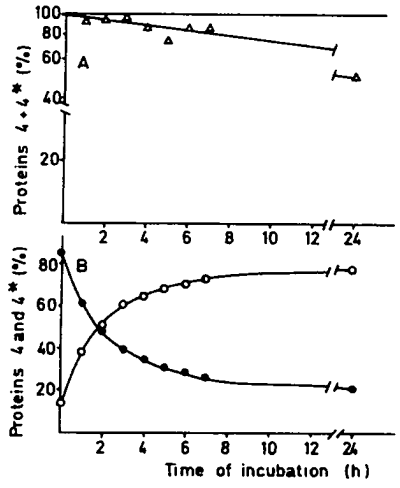

Fig. 4. *Time course experiments showing the progressive disappearance of radioactively labelled protein 4 (●), the progressive appearance of radioactively labelled protein 4* (○), and the decay of the total radioactivity of both labelled proteins 4 and 4* (△).* (A) The sums of proteins 4 + 4*, expressed as a percentage of the amount found at zero time, were plotted on a log scale against increasing times of incubation. Fig. 5 is the quantitative expression of Fig. 4, part I. (For details, see text.) (B) The individual amounts of proteins 4 and 4*, expressed as a percentage of the total amount found after each time of incubation, were plotted on an arithmetic scale against increasing times of incubation

Protein X. This protein had an apparent molecular weight intermediate between those of the penicillin-binding proteins 4 and 5 (Fig. 3, part I). It was detected in trace amounts only after prolonged incubation of the membrane (in water and at 37 °C).

Spontaneous Conversion of Membrane-Bound Penicillin-Binding Proteins into Water-Soluble Penicillin-Binding Proteins

In the experiments described in the preceding section, the membrane suspensions had been maintained at 37 °C and then the complete preparations (pellet + supernatant) analysed after reaction with [^{14}C]benzylpenicillin. In subsequent experiments, the pellet and the supernatant fractions were analysed separately. Most of the protein 4* and most of the protein X were found in the supernatant fraction (after centrifugation at 40000 × *g* for 15 min). Conditions for maximal release of both proteins 4* and X were investigated.

*Protein 4**. Table 4 shows both the pH effects, from 5.8 to 10.3 with low ionic strength, and the salts effects at pH 6.8, on the solubilization of the membrane-bound protein 4 in the form of protein 4* (after 1 h of incubation of the isolated membranes at 37 °C).

Table 4. *Conversion of membrane-bound protein 4 into water-soluble protein 4**
Membranes were incubated at 37 °C for 1 h in the buffers indicated below. After centrifugation (10 min at 40000 × *g*), both the pellet and the supernatant fractions were treated with [^{14}C]benzylpenicillin and analysed for their penicillin-binding proteins by polyacrylamide gel electrophoresis followed by fluorography and densitometry measurements. The results are expressed as a percentage of total proteins 4 and 4* estimated after each individual treatment

Buffer			Supplement to buffer	Pellet		Supernatant protein 4*
Type	pH	*I*		protein 4	protein 4*	
		M				
PO_4	5.8	0.05	–	82.7	8.3	9
PO_4	6.6	0.05	–	43.5	10.9	45.6
PO_4	6.9	0.05	–	24.5	13.5	62
PO_4	8.0	0.05	–	11.4	12.5	76.1
CO_3	9.1	0.05	–	11.4	13.9	74.7
CO_3	10.3	0.05	–	15.6	15.6	68.9
PO_4	6.6	0.05	10 mM $MgCl_2$	82.1	9.9	8
PO_4	6.6	0.05	0.1 M NaCl	65.6	25	20.5
PO_4	6.6	0.05	1 M NaCl	54.5	34.4	0
Control (water)				66.9	11.7	21.4

Release was maximal at pH 8–9 and low ionic strength (I = 0.05 M). Addition of 10 mM $MgCl_2$ at pH 6.6 inhibited the release to a great extent (Table 4). Inhibition was also observed at high NaCl concentrations. In the presence of 1 M NaCl, protein 4* appeared to remain associated with the membrane, probably by salting out. From the foregoing, it thus follows that the spontaneous elimination from the 80000-M_r protein 4, of a 7000-M_r segment not involved in penicillin binding results in the solubilization of this particular protein in the form of the 73000-M_r protein 4*. This mechanism (which occurs in water, although at a slow rate) provided an explanation for the observation reported above that membranes freshly prepared from radioactively prelabelled cells were free of penicillin-binding protein 4* (in contrast to isolated membrane preparations which were used after more or less prolonged storage times).

Protein X. At pH 6.9, the release of protein 4* was a specific process, all the other penicillin-binding proteins remaining membrane-bound (Fig. 3, part II). At alkaline pH, however, substantial amounts of the 77000 M_r penicillin-binding protein X were also detected in the supernatant fractions. As the pH of the membrane suspensions increased from 6.9 to 10.3, the increased amounts of protein X found in the supernatant fractions compensated, at least at first sight, for the observed decrease of the 82000-M_r membrane-bound protein 3b. If, as suggested, proteins 3b and X were related by precursor-product relationships, then after 1 h of incubation at pH 10.3 and 37 °C, about 35% of protein 3b underwent solubilization in the form of protein X.

Effects of Inhibitors on the Release of Proteins 4 and X.* The effects of the inhibitors listed in Materials and Methods were studied as follows. Membrane samples (25 µl) containing 500 µg total proteins and 100 µM [^{14}C]benzylpenicillin were incubated together in water for 15 min at 37 °C. After addition of 500 µl water the membranes were collected by centrifugation and resuspended either in 40 µl water (control samples) or in 40 µl 1 mM solution of inhibitor. The various suspensions were then incubated for 60 min at 37 °C, supplemented with 40 µl 0.05 M carbonate buffer pH 9.0 (to favor the release of the proteins) and further incubated for 3 h at 37 °C. Analysis by polyacrylamide gel electrophoresis in sodium dodecyl sulfate followed by fluorography showed that the inhibitors had no detectable effect on the release of protein 4*, nor on that of protein X.

*Trypsin-Catalysed Conversion of Membrane-Bound Protein 6 into Water-Soluble Protein 6**

In all experiments described above, the penicillin-binding protein 6 (i.e. the DD-carboxypeptidase) exhibited very high stability. The effect of trypsin was then studied under the following conditions. Membrane samples containing 12500 µg protein and 600 µg trypsin, in 1 ml 0.2 M $NaHCO_3$ pH 8.3, were incubated at 37 °C. After increasing times (up to 180 min), 100-µl samples (containing 1250 µg protein) were removed, supplemented with 150 µl of an aqueous solution containing 500 µg soybean trypsin inhibitor (Sigma, type I-S), centrifuged for 15 min at 40000 × g and the pellets resuspended in 240 µl water. The DD-carboxypeptidase activity was measured on 4-µl samples, and the penicillin-binding proteins were estimated on 25-µl samples of both the pellet and the supernatant fractions. Control samples consisted of membrane suspensions treated as above but in the absence of trypsin.

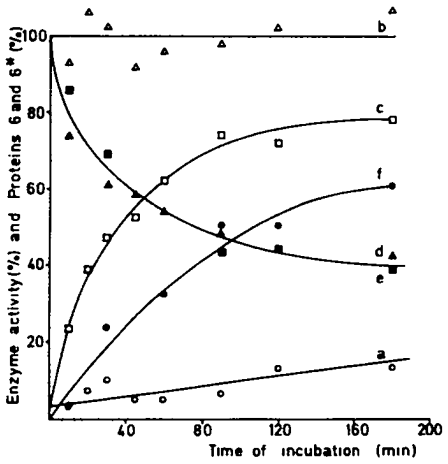

Fig. 5. *Time course experiments showing the effects of trypsin on the solubilization of the membrane-bound* DD-*carboxypeptidase activity and the conversion of the membrane-bound protein 6 into water-soluble protein 6**. (a–d) Enzyme activity measurements (expressed as a percentage of the activity of the membranes at zero time): (a) supernatant fractions obtained in the absence of trypsin; (b) pellet fractions (membranes) obtained in the absence of trypsin; (c) supernatant fractions obtained after trypsin treatment; (d, ■——■) pellet fractions (membranes) obtained after trypsin treatment. (e, f) Estimation of penicillin-binding proteins (expressed as a percentage of the amounts of protein 6 present in the membranes at zero time): (e; curve ▲——▲) protein 6 in the pellet fractions (membranes) obtained after trypsin treatment; (f) protein 6* in the supernatant fractions obtained after trypsin treatment

In the absence of trypsin, the level of DD-carboxypeptidase activity of the membrane fractions remained unchanged (Fig. 5, curve b) and that of the supernatant fractions increased very slowly as a function of the incubation time (Fig. 5, curve a). The pattern of penicillin-binding proteins was little affected (not shown in Fig. 5) if one adjusts for the formation of some amounts of protein 4* (from protein 4) and protein X (presumably from protein 3b).

In the presence of trypsin (as shown in Fig. 6) all the membrane-bound penicillin-binding proteins underwent rapid proteolysis, except the 43000-M_r protein 6 which was relatively more stable. Moreover, the process yielded various water-soluble penicillin

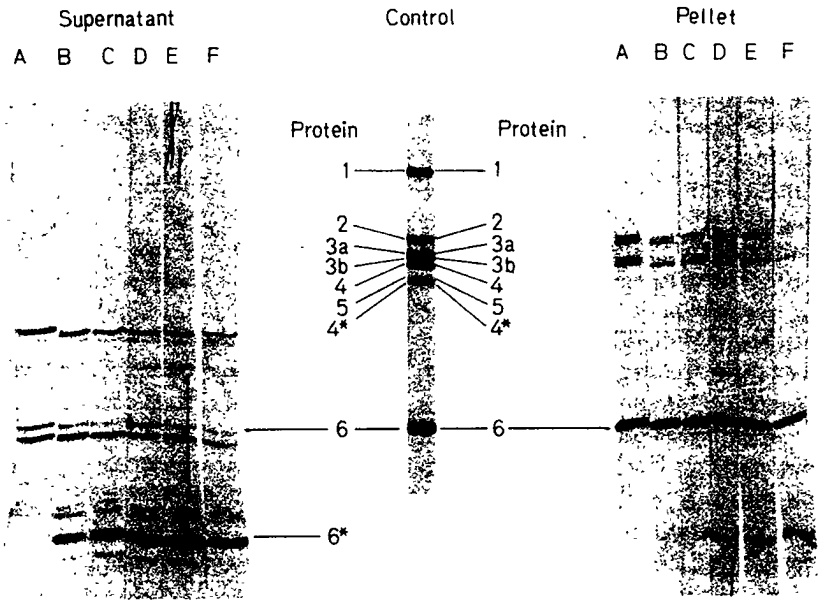

Fig. 6. *Fluorograms of polyacrylamide gel electrophoresis in sodium dodecyl sulfate showing the conversion by trypsin of membrane-bound protein 6 into water-soluble protein 6**. The experimental conditions were those of Fig. 5. (A) 10 min; (B) 30 min; (C) 60 min; (D) 90 min; (E) 120 min; (F) 180 min

binding proteins, in particular a 30000-M_r protein 6*. That the membrane-bound protein 6 and the water-soluble protein 6* were related by precursor-product relationships was established as follows. Time course experiments showed that the disappearance of protein 6 from the membrane fractions (Fig. 5, curve e), and the appearance of the 30000-M_r protein 6* in the supernatant fractions (Fig. 5, curve f) were strictly concomitant events. In parallel to this, the DD-carboxypeptidase activity, which was initially totally membrane-bound, progressively underwent solubilization (Fig. 5, curves d and c). The gain in soluble DD-carboxypeptidase activity, however, somewhat exceeded the loss in the corresponding membrane-bound DD-carboxypeptidase activity. Such an unmasking effect might be the expression of increased enzyme activity when the DD-carboxypeptidase is in the form of protein 6*.

Finally, one should add that treatment with trypsin of the genapol-solubilized penicillin-binding protein 6 [1], under conditions where 87% of the DD-carboxypeptidase activity remained intact, also gave rise to various degraded protein fragments, the major one exhibiting the same mobility as the protein 6* under consideration.

From the foregoing, it thus appeared that the elimination of a 13000-M_r fragment from the membrane-bound protein 6 by trypsin action resulted in the solubilization of this particular protein in the form of protein 6* without alteration of the enzyme activity or its penicillin-binding capacity. Both protein 6* and the associated DD-carboxypeptidase activity remained water-soluble after centrifugation for 1 h at 150000 × *g*. Further alteration of protein 6*, however, which is due to a slow proteolytic effect of trypsin, may occur. Thus, by using a ratio of membrane proteins to trypsin of 5 to 1 (instead of 20 to 1) and after 24 h of incubation at 37 °C, the DD-carboxypeptidase activity found in the supernatant fraction and that found in the pellet fraction represented only 40% and 17%, respectively, of the enzyme activity of control samples incubated under the same conditions but in the absence of trypsin.

Effects of Inhibitors on the Ability of the Proteins to Bind [^{14}C]Benzylpenicillin

In order to study the effects of the inhibitors listed in Materials and Methods, membrane samples (40 µl containing 500 µg total proteins) were incubated for 1 h at 37 °C in the presence of the inhibitors at concentrations of 0.001 – 1 mM. After addition of 2 ml water, the membranes were collected by centrifugation and suspended in 80 µl water. Samples (20 µl containing 125 µg membrane proteins) were then treated with 100 µM [^{14}C]benzylpenicillin and the penicillin binding proteins analysed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, followed by fluorography. None of the inhibitors had any detectable effect on the ability of the membrane-bound proteins to bind penicillin except *p*-chloromercuribenzoate which completely prevented penicillin binding to only protein 6. Densitometry measurements of the fluorogram indicated that a concentration of *p*-chloromercuribenzoate of 0.04 mM was required to inhibit by 50% penicillin binding to protein 6. In another series of experiments, membrane samples (125 µg protein in 25 µl water)

were supplemented with *p*-chloromercuribenzoate at concentrations of 0.001 – 1 mM and incubated for 1 h at 37 °C. DD-Carboxypeptidase activity measurements carried out on 5-µl samples showed that the enzyme activity was inhibited by 50% at 0.03 mM *p*-chloromercuribenzoate.

The effects of *p*-chloromercuribenzoate were also investigated on the supernatant fractions obtained after trypsin treatment of membrane samples. The reagent concentration required to inhibit by 50% [^{14}C]benzylpenicillin binding to protein 6* and that required to inhibit by 50% the soluble DD-carboxypeptidase activity were identical and equal to 0.007 mM (in 0.1 M $NaHCO_3$).

DISCUSSION

By using [^{14}C]benzylpenicillin as the labelling reagent, *Streptococcus faecalis* ATCC 9790 has been shown to possess at least seven distinct membrane-bound penicillin-binding proteins. Their molecular weights range from 43000 to 140000. On the basis of their specificity profiles (as determined by competition experiments between [^{14}C]benzylpenicillin and 15 different non-radioactive β-lactam antibiotics), the various proteins under consideration exhibit decreasing affinities for β-lactam antibiotics in the following order: protein 3 (a + b) > protein 2 > proteins 1 and 4 > protein 6 ⩾ protein 5. With few exceptions, benzylpenicillin, phenoxymethylpenicillin and ampicillin are the most effective antibiotics with respect to their abilities to combine with all the penicillin-binding proteins. Remarkably, however, the affinities of cefoxitin and cephalothin for protein 2 and the affinities of cefoxitin and thienamycin for protein 6 are at least as high as those of benzylpenicillin, phenoxymethylpenicillin and ampicillin for the corresponding proteins. None of the penicillin binding proteins behaves, by itself, as a lethal target for β-lactam antibiotic action on the living cells. Inactivation of the most penicillin-sensitive protein 3(a + b) alone does not appear to be sufficient to cause inhibition of cellular growth and, conversely, inhibition of cellular growth by various antibiotics can be obtained under circumstances where any one of the proteins 1, 2, 4, 5 or 6 escapes inactivation. It may be that several penicillin-binding proteins fulfil similar physiological functions with the result that such 'spare' or 'detour' enzymes might compensate for the lack (by inactivation) of one of them. Such compensatory mechanisms have been evoked in other instances [8 – 10].

The complexes formed between [^{14}C]benzylpenicillin and both the 43000-M_r protein 6 (previously characterized as a DD-carboxypeptidase; see the introduction) and the 140000-M_r protein 1 (of unknown enzyme activity) undergo breakdown at much faster rates than the corresponding native proteins undergo spontaneous proteolysis (at 37 °C and in water), so that complex breakdown appears to be 'enzyme-catalysed'. As shown in previous studies [1,2], protein 6 fragments the benzylpenicillin molecule into phenylacetylglycine and *N*-formyl-D-penicillamine. The present studies strongly suggest that protein 1 simply hydrolyses benzylpenicillin into benzylpenicilloate and thus behaves as a β-lactamase of low efficiency. With all the other penicillin-binding proteins [2, 3(a + b), 4* and 5] whose molecular weights are intermediate between those of proteins 1 and 6, breakdown of the complexes formed with [^{14}C]benzylpenicillin results from proteolysis and is not due to the release of the radioactive label.

Proteins 4 and 3b are characterized by very short half-lives (about 175 and 600 min, respectively, at 37 °C and in water). Clearly, the 80000-M_r membrane-bound protein 4 undergoes spontaneous conversion into the water-soluble 73000-M_r protein 4*. Similarly, it seems highly probable that the water-soluble 77000-M_r protein X originates from the membrane-bound 82000-M_r protein 3b. In both cases, maximal release occurs at alkaline pH values. Although the inhibitors tested have no effect on the process, it is likely that the release of the water-soluble proteins 4* and X results from some endogenous proteolytic activity. Whatever the mechanism, the 5000 – 7000-M_r segments that are eliminated from the original proteins probably consist, at least in part, of the hydrophobic portions through which the relevant proteins are anchored in the plasma membranes.

All the penicillin-binding proteins of *S. faecalis* are very sensitive to trypsin action except the 43000-M_r protein 6 (i.e. the DD-carboxypeptidase). This latter protein is degraded relatively slowly in the form of a water-soluble 30000-M_r protein 6*. In spite of the fact that one third of the original protein molecule has been eliminated, protein 6* is still a penicillin-sensitive DD-carboxypeptidase and a penicillin-binding protein. The penicillin-binding proteins of *Bacillus subtilis* are also very sensitive to proteases action [11]. Removal by trypsin action of a 2000 – 3000-M_r hydrophobic membrane-anchoring segment from the C terminal of some DD-carboxypeptidases has been reported [12]. These slightly shortened and water-soluble enzymes also exhibit the same enzymic properties as the parent ones but unlike the latter, they cannot be reconstituted into liposomes and do not bind detergent micelles [12].

Proteins 6 and 6* are inhibited by low dose levels of *p*-chloromercuribenzoate with respect to both their DD-carboxypeptidase activity and their penicillin-binding capacity. Like the proteases which are known to operate by at least four different mechanisms [13], DD-carboxypeptidases of different classes exist. The R61 and the *Bacilli* DD-carboxypeptidases (for a review, see [14]) may be considered as the counter-

parts of the serine proteases and the G enzyme (O. Dideberg, P. Charlier, J. M. Frère and J. M. Ghuysen, unpublished results) as the counterpart of a metalloprotease. Further work is required in order to establish whether the *S. faecalis* enzyme is a thiol DD-carboxypeptidase or not. The involvement of an SH group in enzyme action has been postulated for the *Escherichia coli* DD-carboxypeptidase 1A [15]. In this latter case, however, *p*-chloromercuribenzoate inhibits the enzyme activity but not the penicillin-binding ability. The interpretation has been that this SH-group reagent does not affect enzyme acylation by penicillin or a natural substrate analogue but prevents the acyl-enzyme complexes thus formed from being deacylated.

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