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Interactions between β-Lactam Antibiotics and Isolated Membranes of *Streptococcus faecalis* ATCC 9790

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The DD-carboxypeptidase–exchange membrane-bound enzyme in *Streptococcus faecalis* ATCC 9790 reacts with β-lactam antibiotics to form complexes with rather long half-lives. Depending upon the antibiotic, the second-order rate constants for complex formation range from 0.75–560 $M^{-1} s^{-1}$ (at 37 °C and in water) and the first-order rate constants for complex breakdown range from 1.3 to 26×10^{-5} s^{-1} (at 37 °C and in 5 mM phosphate buffer pH 7.5). There are about 30 pmol of DD-carboxypeptidase–exchange enzyme per mg of membrane protein. The degradation products arising from benzylpenicillin are phenylacetylglycine and probably *N*-formyl-D-penicillamine. Isolated membranes also contain other penicillin binding sites (about 70 pmol/mg membrane protein). That part of benzylpenicillin which reacts with at least some of these latter sites is slowly degraded into penicilloic acid. Normal functioning of the DD-carboxypeptidase–exchange membrane-bound enzyme is important, if not essential, for cell growth. With the β-lactam antibiotics tested, inhibition of cell growth is mainly related to the rates of formation of the inactive enzyme-antibiotic complexes. The relationship, however, is not a direct one probably due to the competitive effect exerted by the other penicillin binding sites.

Isolated membranes of *Streptococcus faecalis* ATTC 9790 contain at least two activities that may constitute or at least be part of the peptide cross-linking enzyme system involved in cell wall peptidoglycan synthesis [1]: (a) a transfer activity that is revealed by the standard exchange reaction Ac_2-L-Lys-D-Ala-D-Ala + D-[^{14}C]alanine → Ac_2-L-Lys-D-Ala-D-[^{14}C]Ala + D-alanine and which is expressed at high pH values; (b) a DD-carboxypeptidase activity that is revealed by the standard hydrolytic reaction Ac_2-L-Lys-D-Ala-D-Ala + H_2O → Ac_2-L-Lys-D-Ala + D-alanine and which occurs at pH 10 and at pH 6. Previous studies [1] suggested that both hydrolysis and exchange reactions were catalysed by the same enzyme, or at least by two very closely related enzymes. They failed to prove, however, whether or not the above simple exchange reaction was a model of the transpeptidation reaction through which the nascent peptidoglycan undergoes peptide crosslinking. Experiments were therefore designed in order to establish whether the hydrolysis and/or the exchange reaction, as they were revealed by the above standard reactions, were physiologically important and for this purpose, the nature and the mechanism of the interactions between the isolated membranes and several β-lactam antibiotics were investigated.

Abbreviations. ID_{50}, concentration of antibiotic which inhibits the enzyme activity by 50%; $[I]_{min}$, minimal antibiotic concentration which prevented growth after 18 h of incubation at 37 °C (stationary phase cultures).

Enzyme. DD-Carboxypeptidase–exchange enzyme (EC 3.4.12.6).

MATERIALS AND METHODS

Plasma Membranes

The techniques used to prepare the plasma membranes were those previously described [1, 2]. Membrane suspensions were in water at 20 mg protein/ml. In the exchange reaction carried out in 50 mM carbonate buffer pH 10, the $K_{m\,(app)}$ values for Ac_2-L-Lys-D-Ala-D-Ala at infinite concentration of D-alanine and for D-alanine at infinite concentration of Ac_2-L-Lys-D-Ala-D-Ala were virtually identical (6–7 mM), and at saturating concentrations of both substrates the V value was 50 nmol of tripeptide utilized min^{-1} (mg protein)$^{-1}$ [1]. In the DD-carboxypeptidase assay, the K_m and V values were 11 mM and 33 nmol of tripeptide hydrolysed min^{-1} mg^{-1} at pH 10 (in 50 mM carbonate buffer pH 10) and 4 mM and

20 nmol min^{-1} mg^{-1} at pH 6 (in 50 mM cacodylate buffer) [1].

Estimation of Enzyme Activities

Unless otherwise stated, the following procedures were used.

DD-*Carboxypeptidase Activity*. Membranes and 3 mM Ac_2-L-Lys-D-Ala-D-Ala were incubated in 20 µl (final volume) of 50 mM carbonate buffer pH 10 for 30 min at 37 °C, after which time the amount of free D-alanine released was measured as described previously [1].

Exchange Reaction. Membranes and 3 mM Ac_2-L-Lys-D-Ala-D-Ala + 10 mM D-[^{14}C]alanine (0.49 Ci/mol) were incubated in 20 µl (final volume) of 50 mM carbonate buffer pH 10 for 30 min at 37 °C, after which time the amount of Ac_2-L-Lys-D-Ala-D-[^{14}C]Ala formed was measured as described previously [1].

Antibiotics and Minimal Inhibitory Concentrations

The minimal inhibitory concentrations, $[I]_{min}$ which prevented growth after 18 h of incubation at 37 °C (stationary phase cultures). For other conditions, see [2]. Phenoxymethylpenicillin, benzylpenicillin, ampicillin, carbenicillin, oxacillin, cloxacillin and methicillin were used for the present studies. They had increasing $[I]_{min}$ values which, in the order as they are listed, ranged from 1 µM to 250 µM (Table 1). They all belong to the penicillin series.

ID_{50} Values

ID_{50} values were the concentrations of antibiotics which inhibited the enzyme activity by 50 %. The ID_{50} values for the membrane-bound DD-carboxypeptidase activity in 50 mM sodium cacodylate buffer pH 6 were determined previously [2]. Similar experiments were repeated in 50 mM carbonate buffer pH 10 for both the DD-carboxypeptidase activity and the exchange activity. Membranes (40 µg) and substrates were incubated in the absence and in the presence of various concentrations of antibiotics and the residual enzyme activities were estimated (Table 1).

Penicillinase

Penicillinase Riker (Neutrapen) was purchased from Serva. It was used with a specific activity of 0.68 IU/µl. One IU (international unit) hydrolyses 1 µmol of benzylpenicillin/min.

Radioactive Benzylpenicillin and Derivatives

[^{14}C]Benzylpenicillin (with the radioactive label on the carbonyl group of the phenylacetyl side chain; either 16, 25 or 54 Ci/mol) was purchased from The Radiochemical Center, Amersham. [^{14}C]Phenylacetylglycine was prepared by enzymatic degradation of [^{14}C]benzylpenicillin with the help of the exocellular DD-carboxypeptidase–transpeptidase from *Streptomyces* strain R61 [3]. Benzyl[^{3}H]penicillin (with the label on the β-methyl group; 1.56 Ci/mol) was that used previously [4]. Its preparation has been described recently [5]. [^{14}C]Benzylpenicilloic acid and benzyl[^{3}H]penicilloic acid were prepared by treatment of the corresponding radioactive benzylpenicillin with penicillinase.

Separation of Benzylpenicillin Degradation Products on Cation-Exchange Column

The separation was carried out on a cation-exchange column, eluted with a lithium citrate buffer gradient under conditions applied for amino acid analysis [6]. For that purpose, a Technicon amino acid analyser was used with a 140 by 0.6-cm column of Chromobeads type B in the Li^+ form. All samples were buffered with 0.2 M lithium citrate buffer pH 2.2 before loading on the column.

RESULTS

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

Three techniques were used to study the binding of [^{14}C]benzylpenicillin to the isolated membranes. In all cases, binding was carried out in water.

In a first series of experiments, membranes (500 µg protein) and increasing concentrations of [^{14}C]benzylpenicillin in 25 µl (final volumes) of water were incubated for 15 min at 37 °C. Penicillinase (1 µl) was added and the solutions incubated for 5 min more at 30 °C. After addition of Genapol X-100 (20 µl of a 4 % solution made in 20 mM carbonate buffer pH 10) which solubilises the membrane suspensions, the solutions thus obtained were submitted immediately to electrophoresis on strips of Whatman 3MM paper at pH 6.5 (collidine/acetic acid/water, 9.1/2.65/1000, v/v/v) for 1 h at 60 V/cm and the radioactivity remaining at the origin of the strips was counted. Controls consisted of membrane suspensions supplemented with 0.1 M non-radioactive benzylpenicillin. They were incubated for 15 min at 37 °C and after elimination of the excess of non-radioactive benzylpenicillin by centrifugation, they were treated exactly as under the same conditions as above. The radioactivity remaining at the origin of these electrophoretograms was deducted from that found in the corresponding samples in the presence of radioactive penicillin alone. The values thus obtained represented the [^{14}C]benzylpenicillin specifically bound to the membranes.

In a second series of experiments, binding was carried out as above except that samples and controls

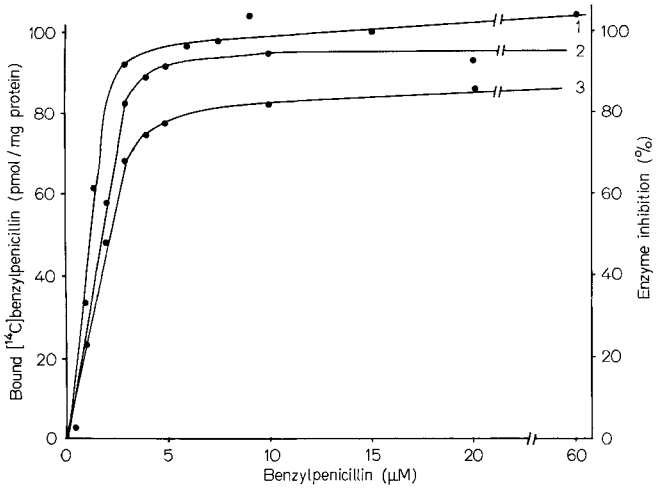

Fig. 1. *Binding of [^{14}C]benzylpenicillin to* S. faecalis *membranes and inhibition of* DD*-carboxypeptidase and exchange activities.* Results were expressed as [^{14}C]benzylpenicillin bound to membrane protein (curve 1), percentage of exchange activity inhibition (curve 2) and percentage of DD-carboxypeptidase activity inhibition (curve 3). The data of curve 1 were obtained by paper electrophoresis. Similar results were obtained by precipitation with trichloroacetic acid (see text)

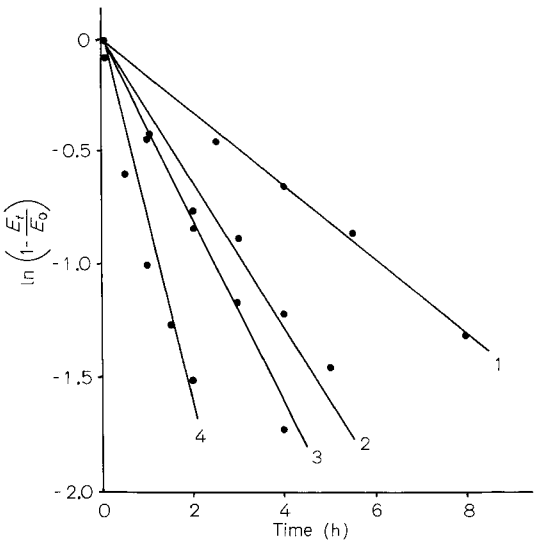

Fig. 2. *Breakdown of the complexes formed between membranes and various β-lactam antibiotics. Reappearance of the exchange activity.* Results were expressed as ln [1 − (E_t/E_0)] were E_t = the concentration of active enzyme present at time t and E_0 = the total (active + inhibited) enzyme concentration. Benzylpenicillin (curve 1); methicillin (curve 2); phenoxymethylpenicillin (curve 3); oxacillin (curve 4)

of 1 mg of membrane protein were used in 50-μl final volumes. After treatment with penicillinase (1 μl), trichloroacetic acid (0.5 ml of an 11% solution) was added to each sample at 0 °C, the precipitates collected on Whatman GF/A filters and washed successively four times with 0.5 ml and twice with 2 ml of the trichloroacetic acid solution and finally once with 5 ml of a mixture of ethanol/water (3/1, v/v). The filters were dried and solubilized in scintillation vials with 0.5 ml Soluene at room temperature for 16 h. Radioactivity was then counted (in 10 ml of Bray scintillation liquid). As above, the counts in the controls were deducted from those of the corresponding samples treated only with radioactive penicillin and the values thus obtained represented the [^{14}C]benzylpenicillin specifically bound to the membranes.

In a third series of experiments, residual DD-carboxypeptidase and exchange activities were followed as a function of the amount of non-radioactive benzylpenicillin added to the membrane suspensions. Membranes (200 μg protein) and benzylpenicillin in 10 μl (final volumes) of water were incubated for 15 min at 37 °C. Controls consisted of membrane suspensions without penicillin. After addition of 1 μl penicillinase and further incubation at 30 °C for 5 min, the membrane suspensions were diluted to 50 μl with water and the enzyme activities were estimated on samples containing 80 μg of membrane protein. These incubation mixtures (20 μl final volumes) contained 50 mM carbonate buffer pH 10, 6 mM Ac_2-L-Lys-D-Ala-D-Ala (DD-carboxypeptidase assays) or 6 mM Ac_2-L-Lys-D-Ala-D-Ala and 10 mM D-[^{14}C]alanine (0.49 Ci/mol) (exchange assays) and the reaction products formed after 30 min of incubation at 37 °C were estimated.

The three techniques used yielded identical results. On the basis of the amount of radioactivity left at the origin of the electrophoretograms and of the amount of radioactivity precipitable by trichloroacetic acid, saturation of the membranes occurred at about 80–100 pmol of [^{14}C]benzylpenicillin bound per mg of protein (Fig. 1). Similarly, inhibition of both DD-carboxypeptidase and exchange activities was complete after fixation of 85–95 pmol benzylpenicillin per mg protein (Fig. 1).

Breakdown of the Complexes Formed between Membranes and Various β-Lactam Antibiotics

Membranes (1.3 mg protein) and 10 mM β-lactam antibiotic were incubated together in 200 μl water (final volumes) for 30 min at 37 °C. Unbound antibiotic was destroyed by addition of 20 μl of penicillinase and further incubation at 30 °C for 10 min. Controls showed that the membrane-bound enzyme activities were completely inhibited. Spontaneous breakdown of the membrane-antibiotic complexes thus formed was followed on the basis of enzyme reactivation. For this purpose, the antibiotic-membrane complexes were diluted to 500 μl in 5 mM sodium phosphate buffer pH 7.5 containing 3 mM

Table 1. *Effects of β-lactam antibiotics on cell growth and on the membrane-bound* DD*-carboxypeptidase–exchange enzyme of* S. faecalis
Cbpase = DD-carboxypeptidase activity; $\overline{ID}_{50}$ (pH 10) = average value of the ID_{50} value for DD-carboxypeptidase and the ID_{50} value for exchange activity. With all the antibiotics, the $k_{breakdown}$ and ID_{50} values are higher in exchange reaction than in DD-carboxypeptidase reaction. Within the limits of the experimental errors, the observed differences are too small to be meaningful

Antibiotic	Growth inhibition, $[I]_{min}$	Enzyme inhibition *in vitro*, ID_{50}				Breakdown of the membrane-antibiotic complexes (37 °C)			Formation of the membrane-antibiotic complexes, k_1			$10^3 \times k_1 \times ID_{50}$ (pH 6)	$10^3 \times k_1 \times \overline{ID}_{50}$ (pH 10)
		Cbpase pH 6	Cbpase pH 10	exchange pH 10	$\overline{ID}_{50}$ pH 10	$10^5 \times k_{breakdown}$		average half-life of the complexes	Cbpase	exchange	average		
						Cbpase	exchange						
	μM					s^{-1}		min	$M^{-1} s^{-1}$			s^{-1}	
1. Phenoxymethylpenicillin	1	1	1.7	2.3	2	7.2	12	130	470	660	560	0.560	1.120
2. Benzylpenicillin	2	1.2	2.5	3.4	3	4.2	4.6	263	480	410	445	0.534	1.335
3. Ampicillin	10	6	5.7	9	7.35	1.3	1.7	820	230			1.380	1.690
4. Carbenicillin	25	40	200	260	230	3.2	4.3	310	22	17	19	0.760	4.370
5. Oxacillin	25	540	420	540	480	21	26	50	4.5			2.430	2.160
6. Cloxacillin	50	420	4500	7500	6000	4.6	7.3	205	0.7	0.8	0.75	0.315	4.500
7. Methicillin	250	1070	1700	3300	2500	5.9	6.7	183	2	1.7	1.85	1.980	4.600

NaN_3 and after increasing times of incubation at 37 °C, 20-μl samples (*i.e.* 52 μg of membrane protein) were removed and used for estimation of enzyme activities. After correction for the fact that breakdown of the membrane-enzyme complexes and enzyme reactivation occur during the 30 min of incubation needed for the estimation of the enzyme activity [7], reappearance of the enzyme activities was seen to proceed as a first-order reaction (Fig. 2). Within the limits of experimental errors, the constant values ($k_{breakdown}$) for a given antibiotic were similar for the hydrolysis and the exchange reaction (Table 1). Depending upon the antibiotics, the $k_{breakdown}$ values ranged from $1.3-26 \times 10^{-5}\ s^{-1}$, corresponding to half-lives of 900 and 45 min, respectively.

The membrane-phenoxymethylpenicillin complex had the same stability in water as in 5 mM phosphate buffer pH 7.5. In 5 mM carbonate buffer pH 10 and at 37 °C, however, the complex formed with benzylpenicillin had a half-life of 600 min (instead of 260 min) and that formed with phenoxymethylpenicillin had a half-life of 200 min (instead of 130 min). Finally, it should be noted that the $k_{breakdown}$ and half-life values shown in Table 1 are average values of 2–4 determinations. Under the standard conditions described above, variations in the $k_{breakdown}$ values of about 25% were observed depending upon the membrane preparations. Alterations in the above procedure such as centrifugations and washings of the membrane-antibiotic complexes formed may also alter their rate of breakdown (see below).

Formation of Membrane-β-Lactam Antibiotic Complexes

These kinetics were determined in water. The reaction mixtures contained at least 1000 pmol of antibiotic per mg of membrane protein. On the basis that 1 mg of membrane protein was saturated when about 100 pmol of [^{14}C]benzylpenicillin were fixed to it, it was assumed that under these conditions, the molar ratio of antibiotic concentration to 'enzyme' concentration was at least 10 to 1. The rate constants k_a for the formation of the complexes at a given antibiotic concentration were calculated from the simplified formula $A_t/A_0 = 1 - e^{-k_a t}$ [7] where A_0 is the activity of the membranes in the absence of antibiotic and A_t is the residual activity after time t of incubation. The use of this simplified formula was justified on the basis that the rates of complex formation were much higher than the rates complex breakdown.

Membranes (400 μg) and various antibiotic concentrations (up to 8 mM) in 100 μl, final volumes, of water were incubated together at 37 °C. At increasing times of incubation, 10-μl samples were removed, supplemented with 10 μl of penicillinase and the residual enzyme activities were estimated after a

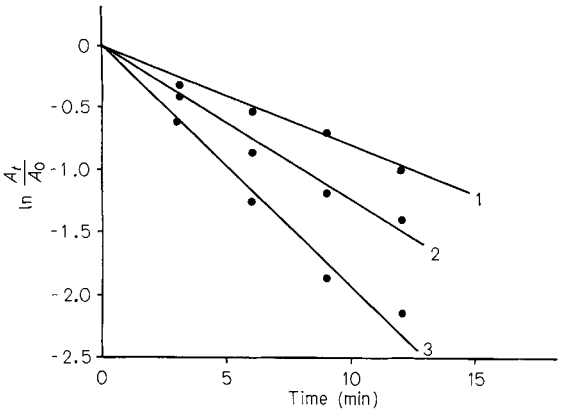

Fig. 3. *Apparent rate constant* k_a *for formation of membrane-benzylpenicillin complex.* Results were expressed as ln (A_t/A_0) where A_0 = the activity of the membranes in the absence of antibiotic, A_t = the residual activity of the membrane after time t of incubation. Concentrations of benzylpenicillin were: 4 μM (curve 1), 6 μM (curve 2), 8 μM (curve 3). The data shown were based on inhibition of exchange activity. Identical results were obtained on the basis of inhibition of DD-carboxypeptidase activity

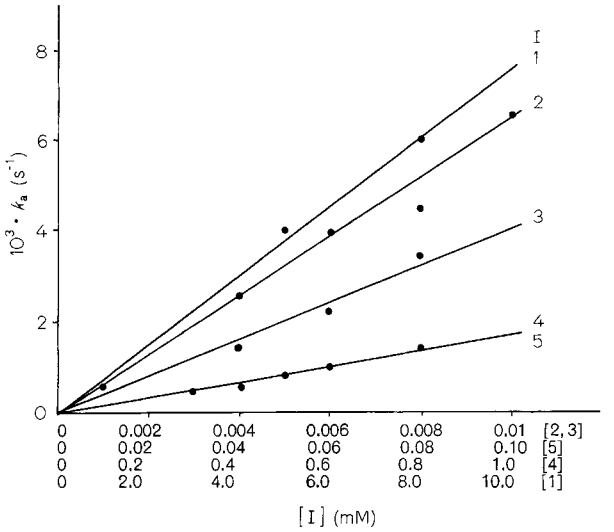

Fig. 4. *Plots of* k_a versus *[I] for various penicillins.* The k_a values obtained as shown in Fig. 3 were plotted against increasing concentrations of antibiotic [I] (different for each antibiotic as shown). k_1 values are the slopes of the lines thus obtained. The antibiotics illustrated here are: (1) cloxacillin, (2) phenoxymethylpenicillin, (3) benzylpenicillin, (4) methicillin, (5) carbenicillin

further incubation with the substrates of 30 min at 37 °C. The data thus obtained were corrected for the breakdown of the membrane-antibiotic complexes occurring during this last incubation [7]. With all the antibiotics tested, the plots ln (A_t/A_0) *vs* t gave rise to straight lines (Fig. 3) and the secondary plot slopes (*i.e.* the rate constants k_a or ln $[A_t/A_0 t]$) *vs* [antibiotic] also gave rise to straight lines passing through the origin of the coordinates (Fig. 4). The observed kinetics were compatible with either a one-step process $E + I \xrightarrow{k_1} EI^*$ or a two-step process $E + I \overset{K}{\rightleftharpoons} EI \xrightarrow{k_3} EI^*$ [7] (I = inhibitor, *i.e.* the antibiotic; EI* = membrane-antibiotic complex characterized by the half-life values determined above; EI = intermediate membrane-antibiotic complex; K = dissociation constant of complex EI; k_1 = second-order rate constant; k_3 = first-order rate constant). In the case of two-step process, deviation from linearity in the plots k_a *vs* [antibiotic] should be observed at high antibiotic concentrations since at the limit, k_a is equal to k_3. Experimentally, however, complex formation had to be slow enough in order to be studied by enzymatic procedures and therefore it had to be carried out at relatively low antibiotic concentrations. Under these conditions, even if the reaction were a two-step process, deviation from linearity could not be observed.

Table 1 gives the second-order rate constants k_1 obtained with the various antibiotics tested. Within the limits of experimental errors, the k_1 values relative to a given antibiotic were virtually identical whether complex formation was followed on the basis of the inhibition of the DD-carboxypeptidase activity or on

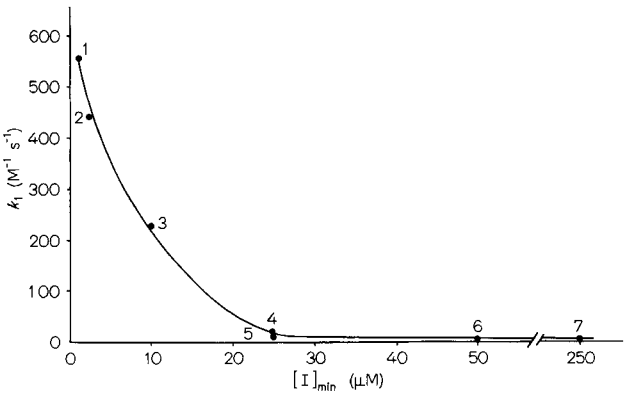

Fig. 5. *Relationship between the* $[I]_{min}$ *values and the second-order constant* k_1 *values for the formation of the enzyme-antibiotic complexes.* The $[I]_{min}$ and k_1 constant values are those of Table 1; the numbered points refer to the antibiotics given in this table

the basis of the inhibition of the exchange activity. Depending upon the antibiotics, however, the k_1 values exhibited large variations from 0.75 – 560 M^{-1} s^{-1}.

Relationship between the Second-Order Rate Constant k_1 *and the* ID_{50} *and* $[I]_{min}$ *Values*

The higher the k_1 value and the smaller the $k_{breakdown}$ value, the better is the antibiotic as an inhibitor of the membrane-bound DD-carboxypeptidase – exchange activity; consequently, the smaller should be its ID_{50} value (*i.e.* the antibiotic concentration which inhibits by 50% the enzyme activities of

the isolated membranes) and, if one assumes that enzyme inactivation causes cessation of cell growth, the smaller should be its $[I]_{min}$ value (*i.e.* the antibiotic concentration which prevents cell growth). Since, however, the half-lives of the various enzyme-antibiotic complexes were either equivalent to or longer than both the incubation time used for the determination of the ID_{50} values and the generation time of the bacterium (*i.e.* 30 min under the conditions where the $[I]_{min}$ values were determined), it thus followed that the observed differences in the ID_{50} and $[I]_{min}$ values should be primarily related to the k_1 values of the corresponding antibiotics. In agreement with this view, the data of Table 1 showed that between the extreme values there was a 1000 – 3000-fold variation for the ID_{50} values, a 800-fold variation for the k_1 values but only a 4 – 8-fold variation for the ($ID_{50} \times k_1$) product values. Hence, at least at first approximation, there is for all the antibiotics tested a direct relationship between the ID_{50} values and the reciprocals of the corresponding k_1 values. Furthermore, a plot of $[I]_{min}$ *vs* k_1 (Fig. 5) showed that, indeed, the higher the k_1 value, the lower was the $[I]_{min}$ value. The relationship, however, was not a simple one, the $[I]_{min}$ values decreasing at the expense of disproportionate increases of the corresponding k_1 values (see Discussion).

Kinetics of Release of the Radioactive Degradation Products Arising from Breakdown of the Membrane-[^{14}C]Benzylpenicillin Complex

Membranes (210 mg protein in 8.4 ml of water) and [^{14}C]benzylpenicillin (50 μM, final concentration; 54 Ci/mol) were incubated for 15 min at 37 °C. The suspension, diluted to 30 ml with 1 mM phosphate buffer pH 7 containing 5 mM $MgCl_2$, was divided into three samples (10 ml each). The samples were centrifuged at 0 °C for 30 min at 40000 × *g* and the pellets washed twice by centrifugation with 10 ml of the phosphate/$MgCl_2$ buffer. Two of the membrane pellets were then resuspended separately in 10 ml of 5 mM phosphate buffer pH 7.5 containing 3 mM NaN_3. One of these suspensions was immediately incubated at 37 °C, whereas the other was heated in a boiling water bath (for 5 min after the temperature in the suspension had reached 90 °C) and then cooled down to 37 °C and incubated at this latter temperature. Finally, the third membrane pellet was resuspended in 10 ml of 5 mM carbonate buffer pH 10 containing 3 mM NaN_3 and the suspension was also incubated at 37 °C. After increasing times of incubation (up to 28 h), estimation of enzyme recovery was performed directly on 20-μl samples and 1.3-ml samples were centrifuged at 40000 × *g* for 30 min. After measurement of the radioactivity (100 μl in 10 ml of Bray scintillation liquid), 1.0 ml of the supernatants was lyophilised and analysed by paper electrophoresis at pH 6.5 (75 min, 60 V/cm, strip of paper Whatman 3MM). Three radioactive compounds were detected. One of these remained at the origin whereas the two others co-migrated with standard [^{14}C]phenylacetylglycine and standard [^{14}C]benzylpenicilloic acid, respectively.

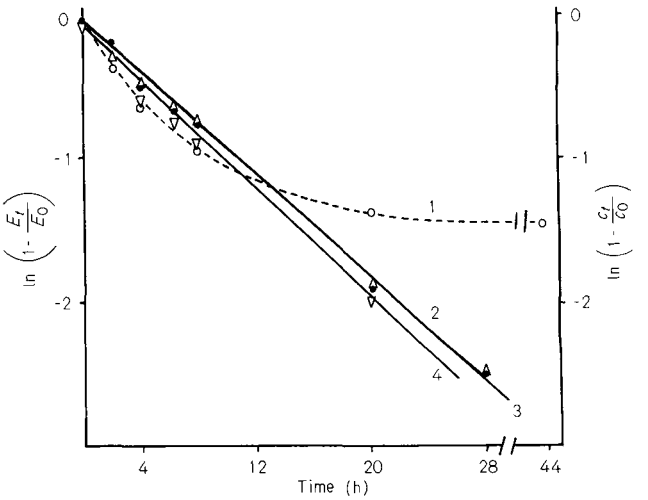

Fig. 6. *Breakdown of membrane-[^{14}C]benzylpenicillin complex. Kinetics of enzyme reactivation and of release of the degradation products.* E_0 *and* E_t: see Fig. 2; c_0 and c_t: see text. Release of [^{14}C]benzylpenicilloic-acid-like compound (curve 1); release of [^{14}C]phenylacetylglycine-like compound (curve 2); recovery of DD-carboxypeptidase activity (curve 3) and of exchange activity (curve 4). Data were corrected for the fact that complex breakdown and enzyme reactivation occur during the incubation needed for the estimation of enzyme activity

In the experiments carried out at pH 7.5 with the non-heated membrane-[^{14}C]benzylpenicillin complex the $k_{breakdown}$ constant values (under the present conditions of membrane manipulations) were 2.60×10^{-5} s^{-1} for the DD-carboxypeptidase activity (half-life: 445 min) and 2.75×10^{-5} s^{-1} for the exchange activity (half-life: 420 min). Kinetics of release of the [^{14}C]-phenylacetylglycine-like compound were determined from plots of $\ln [1 - (c_t/c_0)]$ *vs* t where c_0 was the amount of [^{14}C]phenylacetylglycine-like compound released at full enzyme recovery and c_t the amount of [^{14}C]phenylacetylglycine-like compound formed after increasing times of incubation. On this basis, the release of this compound was a first-order reaction (Fig. 6) characterized by a $k_{breakdown}$ of 2.60×10^{-5} s^{-1} (half-life: 445 min), *i.e.* a value virtually identical to those estimated on the basis of enzyme recovery. By using the same procedure, release of the [^{14}C]benzylpenicilloic-acid-like compound was seen to be more complex and not related to enzyme reactivation (Fig. 6).

In the experiments carried out at pH 7.5 with the heated membrane-[^{14}C]benzylpenicillin complex, en-

Table 2. *Yields of the products released during breakdown of the membrane-[^{14}C]benzylpenicillin complex*
The results are given as percentages based on the radioactivity which remained associated with the membranes after two washings by centrifugation

Buffer	Complex	Time of incubation (37 °C)	Products			
			membrane-bound	immobile on paper electrophoresis	phenylacetylglycine	benzylpenicilloic acid
		h	%			
5 mM phosphate buffer, pH 7.5	non-heated	20	23	30	39	8
5 mM phosphate buffer, pH 7.5	heated	0	61	16	6	17
5 mM phosphate buffer, pH 7.5	heated	20	36	16	6	42
5 mM carbonate buffer, pH 10	non-heated	20	16	19	30	35

zyme reactivation and release of the [^{14}C]phenylacetylglycine-like compound did not occur (as a function of time). The [^{14}C]benzylpenicilloic-acid-like compound, however, was produced in large amounts (Table 2).

In the experiments carried out at pH 10 with the non-heated membrane-[^{14}C]benzylpenicillin complex, the $k_{breakdown}$ constant value was 2.4×10^{-5} s^{-1} (half-life: 480 min) on the bases of both enzyme recovery and release of the [^{14}C]phenylacetylglycine-like compound. The amount of [^{14}C]benzylpenicilloic-acid-like compound released was higher than that observed at pH 7.5 (Table 2).

From the above kinetics and the data of Table 2, the following conclusions could be drawn: (a) the release of the phenylacetylglycine-like compound was specifically related to enzyme reactivation; (b) as suggested by the final yields of phenylacetylglycine-like compound released, the membrane-bound enzyme represented about one third of the total membrane-penicillin binding sites in the membrane; (c) the release of benzylpenicilloic-acid-like compound was irrelevant to enzyme reactivation.

Identification of [^{14}C]Phenylacetylglycine as One of the Degradation Products Released from the Membrane-[^{14}C]Benzylpenicillin Complex

Membranes (65 mg protein in 2.5 ml of water) and [^{14}C]benzylpenicillin (14 μM, final concentration; 25 Ci/mol) were incubated for 15 min at 37 °C. The suspension was centrifuged at $40000 \times g$ for 30 min and the pellet was washed twice by centrifugation at 0 °C with 5 ml of water. In the experiment described here, the membranes were resuspended in 2.5 ml of 5 mM carbonate buffer pH 10 containing 3 mM NaN_3 and the suspension was incubated for 20 h at 37 °C. After centrifugation and two washings of the membranes, the supernatants were pooled and lyophilised and the residues was dissolved in 1 ml of water. Samples (to some of which either standard benzyl[^{3}H]-penicilloic acid or standard [^{14}C]phenylacetylglycine had been added) were saturated with ammonium sulfate at pH 2, extracted by ethyl acetate and the extracts buffered with 0.6 ml of 0.2 M lithium citrate buffer pH 2.2. The solutions were then analysed by chromatography on the cation-exchange column (Materials and Methods). Fig. 7 shows the elution profile of the sample supplemented with exogenous benzyl-[^{3}H]penicilloic acid. The radioactive compound eluted at fraction 40 was [^{14}C]phenylacetylglycine since this peak was seen to increase when exogenous [^{14}C]phenylacetylglycine had been added.

Nature of the Degradation Products Arising from Benzyl[^{3}H]penicillin by Interaction with the Isolated Membranes

A membrane suspension (256 mg protein in 10 ml of 5 mM phosphate buffer pH 7.5 containing 3 mM NaN_3) was supplemented with 240 μl of a 100 μM solution of benzyl[^{3}H]penicillin (1.56 Ci/mol) and incubated at 37 °C. Every 3 h, and for a total period of 48 h, the membrane suspension was supplemented with 60-μl samples of the benzyl[^{3}H]penicillin solution, after which time the suspension was centrifuged at $40000 \times g$ for 30 min, the supernatant lyophilised and the residue dissolved in 1 ml of water.

Part of this latter solution (0.25 ml containing 45000 dis./min) was treated with 5 mg dithiothreitol for 15 min at 22 °C and then supplemented with 0.5 M HCl (50 μl) and 0.2 M lithium citrate buffer pH 2.2 (0.5 ml). After centrifugation, analysis of the supernatant on the cation-exchange column revealed the occurrence of two main tritiated compounds. One of them (36 % of the total radioactivity) had the same retention time as *N*-formyl-D-penicillamine. The other (12 %) had the same retention time as benzylpenicilloic acid. Remarkably, the amount of *N*-formyl-D-[^{3}H]-penicillamine was virtually identical to the amount

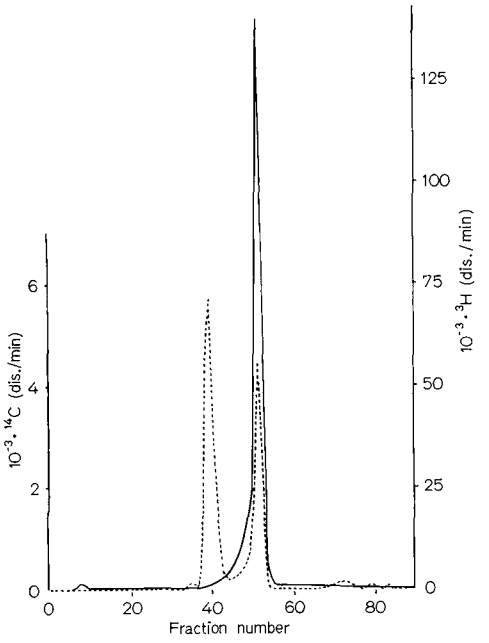

Fig. 7. *Analysis of the ^{14}C-labeled degradation products arising from breakdown of the membrane-[^{14}C]benzylpenicillin complex. Separation of [^{14}C]phenylacetylglycine (fraction 40) and [^{14}C]benzylpenicilloic acid (fraction 52) by chromatography on cation-exchange column.* Cochromatography with added benzyl[^{3}H]penicilloic acid (– ——). (------) ^{14}C. Volume of the fractions: 3 ml

of [^{14}C]phenylacetylglycine recovered from [^{14}C]-benzylpenicillin (Table 2). Similarly, the amount of benzyl[^{3}H]penicilloic acid was virtually equivalent to that of [^{14}C]benzylpenicilloic acid previously formed (Table 2).

DISCUSSION

Isolated membranes of *S. faecalis* ATCC 9790 contain about 100 pmol of benzylpenicillin binding sites per mg protein. About one third of these sites are the enzyme molecules responsible for the DD-carboxypeptidase and exchange activity performed by the isolated membranes. These specific sites react with β-lactam antibiotics to form inactive, rather stable complexes. Inactivation, however, is not irreversible. Spontaneous breakdown of the enzyme-antibiotic complexes leads to enzyme reactivation and, concomitantly, to the release of the antibiotic molecule in a chemically degraded and physiologically inactive form. Irrespective of the antibiotic used, the kinetics of inactivation and reactivation of the DD-carboxypeptidase activity are similar to the kinetics of inactivation and reactivation of the exchange activity, strongly suggesting that one single enzyme performs both activities. Complex formation is a second-order reaction characterized by rate constants which, depending upon the antibiotic, range from 0.75–560 $M^{-1}\,s^{-1}$ (at 37 °C and in water). Complex breakdown is a first-order reaction characterized by rate constants which, also depending upon the antibiotic, range from $1.3-26\times10^{-5}\,s^{-1}$. One of the reaction products arising from interaction with benzylpenicillin is phenylacetylglycine, thus demonstrating that a complete rupture of the β-lactam ring occurs involving the opening of both the amide bond and the C(5)–C(6) linkage. In essence, the above mechanism is qualitatively identical to the one previously proposed for the interaction between benzylpenicillin (and other β-lactam antibiotics) and the exocellular DD-carboxypeptidase–transpeptidase of *Streptomyces* strain R61 [3,4, 7,8]. With this latter enzyme (which has been purified to protein homogeneity), high antibiotic concentrations and rapid kinetic techniques could be used showing that the best model for the formation of the stable enzyme-antibiotic complexes was a two-step process

$$E + I \overset{K}{\rightleftharpoons} EI \overset{k_3}{\rightarrow} EI_{(stable)}$$

where an intermediate EI complex characterized by a dissociation constant K is transitorily formed. Note that the k_3/K values as measured at high antibiotic concentrations are quantitatively identical to the second-order reaction rate constant k_1 as measured at low antibiotic concentrations. By interacting with the exocellular R61 enzyme, benzylpenicillin is also split into two fragments with formation of phenylacetylglycine.

After interaction with both the membrane-bound DD-carboxypeptidase–exchange enzyme of *S. faecalis* and the exocellular enzyme from *Streptomyces* R61, the thiazoline moiety of the benzylpenicillin molecule occurs in the form of *N*-formyl-D-penicillamine. It is possible that dimethylthiazoline carboxylic acid is the primary product formed during the enzymatic reaction. This compound, however, is quickly hydrolysed to *N*-formyl-D-penicillamine in aqueous solutions at pH 4–7 and hence the demonstration of its release may be difficult. The formation of phenylacetylglycine by interaction of benzylpenicillin with the DD-carboxypeptidase isolated from the membranes of *Bacillus stearothermophilus* was demonstrated but the transformation of the other part of the molecule into dimethylthiazoline carboxylic acid was speculative [9].

All the complexes formed between the membrane-bound DD-carboxypeptidase–exchange enzyme of *S. faecalis* and the various antibiotics tested have half-lives which are either almost equal to or, more often, larger than the generation time of the organism (at least when measured under the conditions used for the determination of $[I]_{min}$, *i.e.* 30 min at 37 °C). If the inactivation of the DD-carboxypeptidase–exchange enzyme really causes cessation of cell growth of *S. faecalis*, then the relative efficiency of the β-lactam antibiotics should rest almost entirely upon

their ability to react with the enzyme and to form inactive enzyme-antibiotic complexes and should not be related to the stability of the complexes formed. In fact, it has been shown that the higher the value of the second-order rate constant k_1 for the formation of the inactive complex, the lower is the $[I]_{min}$ value of the corresponding antibiotic, thus suggesting that a normal functioning of the membrane-bound DD-carboxypeptidase–exchange enzyme is important, if not essential, for the *S. faecalis* cells. The relationship, however, is not a direct one. This situation is not surprising since in addition to the DD-carboxypeptidase–exchange enzyme molecules, the *S. faecalis* membranes also contain twice as many other penicillin binding sites. Reaction of penicillin with these latter sites, at least in part, does not lead to irreversible fixation and breakdown of these complexes gives rise to penicilloic acid. Inactivation by penicillin of the membrane-bound DD-carboxypeptidase–exchange activity in *S. faecalis* is thus a competition phenomenon in which a large number of other penicillin binding sites are involved. A better knowledge of these other sites is important for understanding the action *in vivo* of the β-lactam antibiotics. Membranes of *S. faecalis* contain six distinct penicillin binding proteins as revealed by the technique used by Spratt [10] for *Escherichia coli* K12 (Roberta Fontana, unpublished results). These proteins are under current study.

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