Membrane-Bound Transpeptidase and Penicillin Binding Sites in *Streptomyces* Strain R61

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High-affinity penicillin binding sites from which the antibiotic could not be removed by washings at 4 °C in 0.017 M K_2HPO_4 or 0.05 M Tris-HCl pH 7.5, were shown to occur in the isolated membranes of *Streptomyces* R61. These sites caused the attachment of 25 picomoles of [^{14}C]benzylpenicillin per milligram membrane protein. Penicillins and cephalosporins competed for the same binding sites. The antibiotic concentrations which excluded [^{14}C]benzylpenicillin from 50% of the binding sites were those which inhibited by 50% the membrane-bound transpeptidase. The same rate constant (about 1×10^{-4} s^{-1}) for the dissociation of the benzylpenicillin membrane complex at 37 °C and in 0.017 M K_2HPO_4, was calculated either from the release of the radioactivity (using [^{14}C]benzylpenicillin) or from the recovery of the transpeptidase activity. These observations supported the conclusion that the high-affinity binding sites in the isolated membranes were the transpeptidase molecules. All the complexes formed between the membranes and the various penicillins and cephalosporins examined were reversible at 37 °C and in 0.017 M K_2HPO_4 at least with regard to the transpeptidase. Depending upon the antibiotics, the rate constants for the dissociation of these complexes varied from 3.3×10^{-3} to 0.73×10^{-4} s^{-1}. The radioactivity released through the dissociation of [^{14}C]benzylpenicillin membrane complex occurred mainly in the form of a compound which behaved as [^{14}C]benzylpenicilloic acid both by paper electrophoresis and thin-layer chromatography. It was impossible to choose between several possible mechanisms for the release of the antibiotic molecule.

The isolated membranes of *Streptomyces* R61 were shown to catalyse transpeptidation reactions in which the dipeptide N^α,N^ε-diacetyl-L-lysyl-D-alanine was transferred from the tripeptide donor N^α,N^ε-diacetyl-L-lysyl-D-alanyl-D-alanine to various amino acceptors such as glycine, D-amino acids and peptides with glycine or D-alanine at the N-terminal [1]. With glycyl-glycine as an acceptor, the transpeptidation product was the tetrapeptide N^α,N^ε-diacetyl-L-lysyl-D-alanyl-glycyl-glycine, a compound which was a structural analogue of the peptide crosslinking made *in vivo* during the biosynthesis of the wall peptidoglycan of *Streptomyces* R61 [2]. Penicillins and cephalosporins inhibited the transpeptidase activity of the isolated membranes at those concentrations which inhibited the cell growth [1]. These properties strongly suggested that the membrane-bound transpeptidase, as it was

revealed by the above model transpeptidation reaction, was the killing target of the β-lactam antibiotics. These studies were extended by investigating the probable relationships between this specific enzymatic target and the binding sites for penicillin of the isolated membranes. The results are presented in this paper.

MATERIALS AND METHODS

Membranes

Membranes were prepared as described previously [1]. The final membrane suspensions, either in 0.017 M K_2HPO_4 or in 0.05 M Tris-HCl pH 7.5, contained about 25 mg protein per ml. The suspensions were stored at 4 °C in the presence of thymol.

Transpeptidase Activity

The tripeptide donor N^α,N^ε-diacetyl-L-lysyl-D-alanyl-D-alanine (1.35 mM) and the dipeptide acceptor

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Enzyme. β-Lactamase or penicillinase (EC 3.5.2.6).

Definitions. LD$_{50}$, the antibiotic concentration which caused 50% cell survival; ID$_{50}$, antibiotic concentration which decreased by 50% the rate of transpeptidation.

Table 1. *Competition between non-radioactive β-lactam antibiotics and [¹⁴C]benzylpenicillin for the high-affinity binding sites of the membrane*

Excl. D_{50} values: antibiotic concentrations which excluded [¹⁴C]benzylpenicillin from 50% of the binding sites. LD_{50} values: antibiotic concentrations which caused 50% cell survival (Materials and Methods and [1]). ID_{50} values: antibiotic concentrations which inhibited by 50% the membrane-bound transpeptidase activity (Materials and Methods and [1]). Experimental conditions: Membranes (200 μg protein) and various concentrations of each of the non-radioactive antibiotics were incubated together for 30 min at 30 °C in 200 μl of 0.05 M Tris-HCl buffer pH 7.5—0.005 M MgCl₂. The membranes were washed three times by centrifugation at 4 °C (37000 ×g; 30 min) with 2.5 ml of cold buffer, resuspended in 100 μl of buffer and treated with 20 μM [¹⁴C]benzylpenicillin, final concentration (*i.e.* at saturating concentration). Blanks consisted of the same reaction mixtures except that non-radioactive benzylpenicillin (20 mM, final concentration) was added to the reaction mixtures before treatment with [¹⁴C]benzylpenicillin. All the membrane suspensions were then incubated for 30 min at 30 °C, the membranes were washed 3 times by centrifugation at 4 °C with cold buffer as above and the radioactivity was estimated

Antibiotics	Excl. D_{50} values	ID_{50} values	LD_{50} values	Excl. D_{50} / ID_{50}	Excl. D_{50} / LD_{50}
	μM				
Benzylpenicillin	3	2	5	1.50	0.60
Penicillin V	4	18	5	0.22	0.80
Ampicillin	9	8	5	1.10	1.80
6-Aminopenicillanic acid	13	80	18	0.16	0.72
Carbenicillin	30	34	33	0.90	0.91
Cephaloglycine	34	62	29	0.55	1.16
Methicillin	37	35	44	1.06	0.84
Cloxacillin	45	39	50	1.16	0.90
Oxacillin	65	88	47	0.74	1.40
Cephalothin	65	70	14	0.93	4.64
Cephalexin	78	60	21	1.30	3.70
Cephalosporin C	220	510	112	0.43	2

[¹⁴C]glycylglycine (13.5 mM) were incubated at 37 °C with the isolated membranes in 35 μl of 0.017 M K₂HPO₄ (or 0.05 M Tris-HCl pH 7.5 buffer) and in the presence of 0.3% (v/v) Triton X-100. The transpeptidation product $N^α,N^ε$-diacetyl-L-lysyl-D-alanyl-[¹⁴C]glycyl-glycine was separated from the excess of [¹⁴C]glycylglycine by paper electrophoresis at pH 5.6 and estimated as described previously [1]. The yield of transpeptidation carried out by 500 μg of membrane proteins and expressed as conversion of the donor tripeptide, was 30—40% after 16 h of incubation at 37 °C [1]. Triton X-100 did not cause solubilization of the membrane-bound transpeptidase and had no effect on the rates of transpeptidation, but it was added to the reaction mixtures because it improved the isolation of the transpeptidation product by paper electrophoresis. In the absence of glycyl-glycine, the tripeptide $N^α,N^ε$-diacetyl-L-lysyl-D-alanyl-D-alanine was not hydrolysed into D-alanine and $N^α,N^ε$-diacetyl-L-lysyl-D-alanine, *i.e.* the membranes had no detectable carboxypeptidase activity.

Antibiotics, ID_{50} and LD_{50} Values

6-Methylpenicillin V was a gift from Prof. Vanderhaeghe (Katholiek Universiteit, Leuven, Belgium). The other antibiotics were those used previously [1]. [¹⁴C]Benzylpenicillin (45 mCi/mmol) was purchased

from The Radiochemical Centre, Amersham. The LD_{50} values were the antibiotic concentrations which caused 50% cell survival, as determined by measuring the effect of the antibiotics on the ability of conidia to form "single cell" colonies [1]. The LD_{50} and ID_{50} values are reported in Table 1. The ID_{50} values were the antibiotics concentrations which decreased by 50% the rate of transpeptidation catalysed by the isolated membranes when the reaction was carried out under the standard conditions (see above). The same ID_{50} values were obtained irrespective of the amount of transpeptidation product formed in the absence of the antibiotic at least up to a maximal conversion of the donor tripeptide of about 25%.

Separation of [¹⁴C]Benzylpenicillin and [¹⁴C]Penicilloic Acid

This was carried out either by thin-layer chromatography or by paper electrophoresis. Silica-gel plates (4×20 cm) were developed at room temperature with 1-butanol—water—ethanol—acetic acid (10:4:3:3, v/v/v/v), dried at 120 °C for 1 h and scanned with the help of a Packard radiochromatogram scanner. The radioactive spots were transferred to counting vials and mixed with 1 ml of 0.017 M K₂HPO₄. After 1 h, 10 ml of Bray's scintillation liquid was added and after sedimentation of the silica-gel powder, the radioactiv-
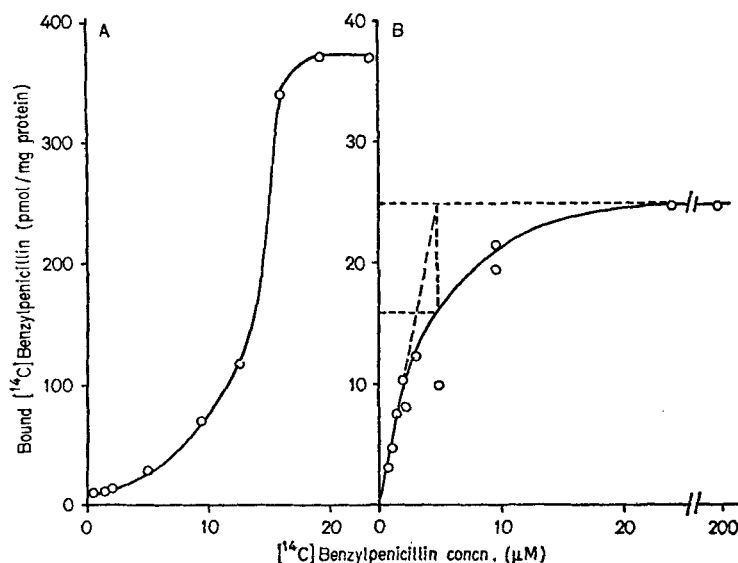
Fig. 1. *Binding of [^{14}C]benzylpenicillin to isolated membranes of* Streptomyces *R61*. (A) Total binding sites; (B) high-affinity binding sites, *i.e.* the sites from which bound [^{14}C]benzylpenicillin could not be removed by washings with cold 0.017 M K$_2$HPO$_4$ or 0.05 M Tris-HCl buffer pH 7.5. The high affinity binding sites fixed a maximum of 25 pmol [^{14}C]benzylpenicillin per mg membrane protein. Based on a specific radioactivity for the [^{14}C]benzylpenicillin of 45 mCi/mmol, the [^{14}C]benzyl-penicillin-saturated membranes had a low specific radioactivity corresponding to about 2250 counts $\times$ min^{-1} $\times$ mg protein^{-1}. The experiments were carried out on suspensions containing 200 μg of membrane proteins in final volumes of 200 μl (for other conditions, see text). Hence, the suspensions containing the [^{14}C]benzylpenicillin-saturated membranes were 25 nM in bound benzylpenicillin and by

equating the maximum binding to total binding sites and to total transpeptidase molecules (see text), the total enzyme concentration was 25 nM. The linear rise from the origin and the final portion of the graph (dotted lines) intersected at a point from which a free enzyme concentration [E] of 9 nM, an enzyme-benzylpenicillin complex concentration [EP] of 16 nM and a total benzylpenicillin concentration of 5 μM could be calculated. By subtracting the amount of benzylpenicillin bound to all sites (24 nM; see total binding sites), the free penicillin concentration [P] was 4.976 μM. Assuming a simple mechanism benzylpenicillin + membrane-bound transpeptidase $\rightleftarrows$ benzylpenicillin-membrane-bound transpeptidase, the association constant for the complex, K_A = [EP]/([E] [P]) was found to be equal to 3.58×10^5 l mol^{-1}

ity was measured with a Packard Tri-Carb liquid scintillation spectrometer. Thr R_F values were 0.70 for benzylpenicillin and 0.60 for penicilloic acid. Electrophoreses were carried out on strips of Whatman No 3MM paper at pH 6.5 (collidine—acetic acid—water, 9.1:2.65:1000) for 60 min at 60 V/cm, using a Gilson high-voltage (10000 V) electrophorator model DW. The radioactive spots were located on the strips with the Packard radiochromatogram scanner and the radioactivity was estimated as described by Pollock *et al.* [3] with the Packard Tri-Carb spectrometer. The migrations towards the anode were 13 cm for benzyl-penicillin and 22 cm for benzylpenicilloic acid.

Penicillinase

Penicillinase was purchased from Calbiochem (B grade) and from Riker Laboratories, England (Neu-trapen). One penicillinase unit was expressed in μmol benzylpenicillin hydrolysed into benzylpenicilloic acid per min at pH 7.0 and 25 °C.

RESULTS

*Total Binding Sites
of Membranes for [^{14}C]Benzylpenicillin*

Membranes (200 μg protein) and various concentrations of [^{14}C]benzylpenicillin were incubated together for 30 min at 30 °C in 200 μl (final volumes) of either 0.017 M K$_2$HPO$_4$ or 0.05 M Tris-HCl buffer pH 7.5. Blanks consisted of the same mixtures except that the membrane suspensions were first mixed with non-radioactive benzylpenicillin in amounts 1000 times higher than those of [^{14}C]benzylpenicillin. The membranes were centrifuged at 37000 $\times g$ for 30 min at 4 °C, the pellet resuspended in 250 μl of buffer and the radioactivity was immediately estimated (Fig. 1 A). Saturation of the binding sites occurred at 20 μM [^{14}C]benzylpenicillin under which conditions 370 pmol antibiotic was fixed per mg membrane protein. Half saturation occurred at 16 μM [^{14}C]benzyl-penicillin.

High-Affinity Binding Sites of Membranes for [14C]Benzylpenicillin

In these experiments, the [14C]benzylpenicillin-treated membranes were washed three times by centrifugation, at $37000 \times g$, for 30 min at 4 °C with 2.5 ml of cold buffer before the radioactivity was estimated (Fig. 1B). Further washes removed essentially no more radioactivity. Under these conditions, saturation also occurred at 20 μM [14C]benzylpenicillin but only 25 pmol antibiotic was fixed per mg membrane protein. Half saturation occurred at 3 μM [14C]benzylpenicillin, a concentration which was virtually identical to the concentration of benzylpenicillin which inhibited by 50% the membrane-bound transpeptidase (ID_{50} value: 2 μM; Table 1). It should also be noted that 25 pmol [14C]benzylpenicillin was always fixed per mg membrane protein irrespective of the concentrations of the membrane suspensions used, at least between 1 and 10 mg protein/ml.

Competition between Non-Radioactive β-Lactam Antibiotics and [14C]Benzylpenicillin for the High-Affinity Binding Sites of the Membrane

Experiments were carried out in order to determine the effect of a prior treatment of the membranes with non-radioactive β-lactam antibiotics on the subsequent fixation of [14C]benzylpenicillin on the high-affinity binding sites. The relative effectiveness of each non-radioactive antibiotic as a competitor of [14C]benzylpenicillin was expressed as the concentration which was required to exclude [14C]benzylpenicillin from 50% of its high-affinity binding sites. The experimental conditions and results are given in Table 1. The striking conclusion of these experiments was that the antibiotic concentrations which inhibited by 50% the binding of [14C]benzylpenicillin were close to those which inhibited by 50% the activity of the membrane-bound transpeptidase (ID_{50} values) or those which caused 50% cell survival (LD_{50} values). This correlation was true for antibiotics with ID_{50} or LD_{50} values ranging from 2—500 μM and 5—110 μM, respectively.

The Benzylpenicillin-Membrane Complex

The complex formed between the benzylpenicillin molecules and the high-affinity sites of the membrane (in short, the benzylpenicillin-membrane complex) readily dissociated when, after removal of the unbound benzylpenicillin, it was resuspended in 0.017 M K_2HPO_4 (or 0.05 M Tris-HCl buffer pH 7.5) and maintained at 37 °C for a few hours. The dissociation was estimated by measuring both the recovery of the transpeptidase activity and the release of the radioactivity (using [14C]benzylpenicillin).

Recovery of Transpeptidase Activity

Membranes treated with a large excess of benzylpenicillin were either first washed to remove the unbound antibiotic or placed directly in the transpeptidation assay system already described, and incubated at 37 °C. As shown in Fig. 2, the membrane-bound transpeptidase remained completely inhibited when it was incubated with the transpeptidation substrates in the presence of the unbound benzylpenicillin (curve III), but it progressively recovered its activity when the unbound benzylpenicillin was removed from the reaction mixture by washing (two washings were in fact sufficient; see curve II) and eventually, after 5 h of incubation in 0.017 M phosphate at 37 °C, it exhibited a transfer activity identical to that of the untreated membranes (compare curves I and II). Hence, with regard to the catalysed transfer reaction, the dissociation of the benzylpenicillin-membrane complex resulted in a full recovery of the enzyme activity. Penicillinase had no effect on the kinetics of the recovery. This was true whether the initial benzylpenicillin-membrane mixture (after 30 min at 30 °C) was treated with 3.4 units of penicillinase for 15 min at 37 °C before the washings, or 3.4 units of penicillinase were present during the incubation of the isolated benzylpenicillin-membrane complex in 0.017 M phosphate at 37 °C.

Sensitivity of the Recovered Membrane-Bound Transpeptidase to Benzylpenicillin

The membrane-bound transpeptidase not only recovered its enzymic activity but it also recovered its initial sensitivity to benzylpenicillin. This property was checked on membranes which after treatment with benzylpenicillin and removal of unbound benzylpenicillin, had recovered by incubation in 0.017 M K_2HPO_4 for 5 h at 37 °C. At this stage, the membranes were collected by centrifugation and their transpeptidase activity was measured in the absence and in the presence of increasing concentrations of benzylpenicillin (see Materials and Methods). The ID_{50} value thus found was 2.7 μM, to be compared with a value of 2 μM for the untreated membranes.

Release of Radioactivity

Membranes incubated with excess of [14C]benzylpenicillin were washed in cold 0.017 M K_2HPO_4 and then reincubated in the same buffer at 37 °C. [14C]Benzylpenicillin remaining bound to the membranes
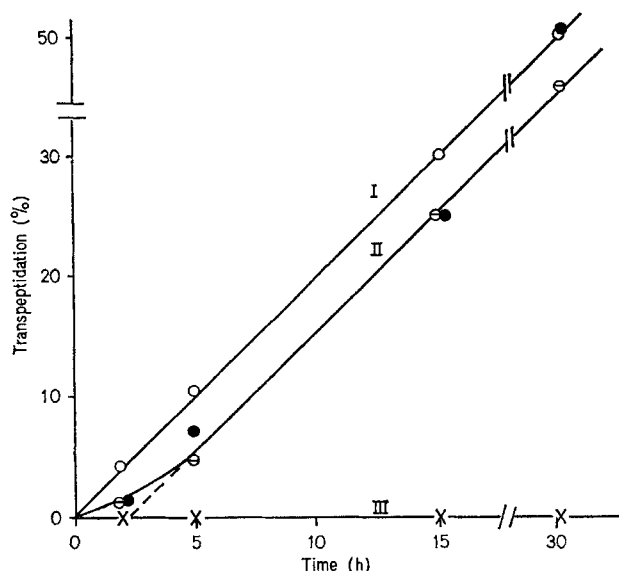
Fig. 2. *Kinetics of dissociation of the benzylpenicillin-membrane complex in 0.017 M K₂HPO₄ and at 37 °C as revealed by the recovery of the transpeptidase activity.* Membranes (2500 µg protein in 250 µl, final volumes, of 0.017 M K₂HPO₄) were incubated for 30 min at 30 °C in the absence and in the presence of 2 mM and 20 mM non-radioactive benzylpenicillin (*i.e.* 100 times and 1000 times the ID₅₀ value). The membrane samples which had not been treated by benzylpenicillin and some of the anti-biotic-treated samples were washed twice by centrifugation at 4 °C with 2.5 ml of cold buffer and resuspended in 190 µl of 0.017 M K₂HPO₄ containing Nᵅ,Nᵋ-diacetyl-L-lysyl-D-alanyl-D-alanine (1.35 mM), [¹⁴C]glycyl-glycine (13.5 mM) and Triton X-100 (0.3%, v/v). The other membrane samples which had been treated with benzylpenicillin were not washed but were directly supplemented with the peptide substrates and Triton X-100 at the same final concentrations as above. All the membrane samples were then incubated at 37 °C and the transpeptidation product Nᵅ,Nᵋ-diacetyl-L-lysyl-D-alanyl-[¹⁴C]glycyl-glycine formed after increasing times was estimated on 35-µl aliquots by paper electrophoresis (Materials and Methods). Results are expressed as the percentage of peptide donor converted into transpeptidation product. (I) Membranes not treated with benzylpenicillin; (II) membranes treated with benzyl-penicillin at concentrations equivalent to 100 times the ID₅₀ value (⊖) and to 1000 times the ID₅₀ value (●). After treatment, the unbound benzylpenicillin was removed by washings at 4 °C; (III) membranes treated with benzyl-penicillin at a concentration equivalent to 100 times the ID₅₀ value and from which the unbound benzylpenicillin was not removed by washings. The intercept of the straight line portion of curve II with the abscissa gave a *t* value of about 2.5 h. From this value, a rate constant for the dissociation ($k = 1/t$) of 1.1×10^{-4} s⁻¹ was calculated

was taken to be that which remained at the origin after paper electrophoresis or thin-layer chromatography (Fig. 3A). Although the amount of radioactivity initially bound to the membranes did not significantly depend upon the technique used for its estimation,
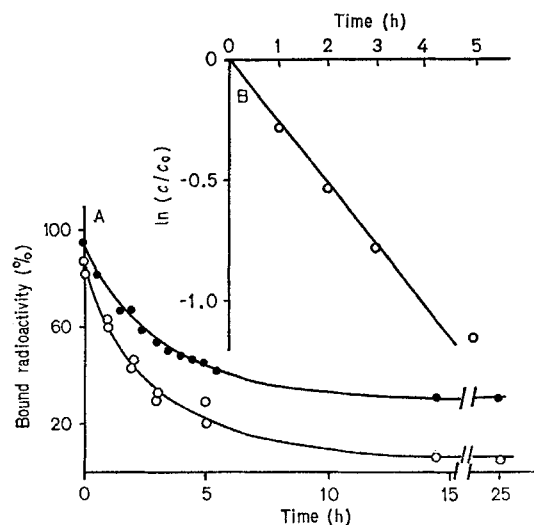
Fig. 3. *Kinetics of dissociation of the [¹⁴C]benzylpenicillin-membrane complex in 0.017 M K₂HPO₄ at 37 °C as revealed by the release of the radioactivity.* Membranes (10 mg protein per ml, final volume) were incubated with 20 µM [¹⁴C]benzylpenicillin (*i.e.* a saturating concentration) for 30 min at 30 °C in 0.017 M K₂HPO₄, washed three times by centrifugation at 37000×g and 4 °C with 2.5 ml of cold buffer, and resuspended in 1 ml of phosphate buffer. Aliquots (200 µl) were analysed immediately and after increasing times of incubation at 37 °C, by paper electrophoresis at pH 6.5 and thin-layer chromatography in 1-butanol—water—ethanol—acetic acid (Materials and Methods). (A) Measurement of the bound-radioactivity, *i.e.* the radioactivity remaining at the origins of the electrophoretograms (●──●) and of thin-layer chromatograms (O──O). (B) First-order kinetic plot of the release of the radioactivity based on the thin-layer chromatographic data. c₀ and c: radioactivity at the origin of the chromatograms at time 0 and time *t*, respectively

after incubation at 37 °C more radioactivity remained at the origin of electrophoretograms than on thin-layer chromatograms. The chromatographic results showed that the release of the radioactivity from the [¹⁴C]benzylpenicillin-membrane complex proceeded to completion, as previously observed for the recovery of the enzyme activity.

Rate Constant for the Dissociation of the Benzylpenicillin-Membrane Complex

The rate constant for the dissociation of the benzylpenicillin-membrane complex was calculated both from the release of the radioactivity and from the recovery of the enzyme activity. Based on the data obtained by thin-layer chromatography for the release of the radioactivity (Fig. 3A), the plot ln (c/c₀) vs time (where c₀ and c were the radioactivity remaining at the origin of the chromatograms, *i.e.* the concentrations of the [¹⁴C]benzylpenicillin-membrane complex, at *t* = 0 and after increasing times of incubation) gave

rise to a straight line (first-order reaction; Fig. 3B) from which a rate constant of $0.33\,h^{-1}$ (or $0.92 \times 10^{-4}\,s^{-1}$) could be calculated.

The recovery of the enzyme activity was estimated (curve II in Fig. 2) under conditions where both the reversion of the penicillin-inhibited enzyme into active enzyme, and the transpeptidation reaction occurred concomitantly:

$$EP \xrightarrow{k} E \xrightleftharpoons{+A+D} [EAD] \rightarrow E + \text{transpeptidation}$$
$$[\text{product}$$
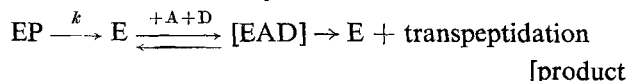
where EP is the penicillin-inhibited enzyme, E the active enzyme, k the rate constant for the dissociation of the EP complex, A the peptide acceptor and D the peptide donor used as substrates for the transpeptidation reaction. Based on previous enzymic analyses by Frieden [4], it could be demonstrated that the amount of transpeptidation product formed [Q] (at initial velocity) was related to rate constant k by the equation

$$[Q] = KE_0[t - \frac{1}{k}(1 - e^{-kt})]$$

where E_0 was the total enzyme concentration and K contained the various constants relevant to the transpeptidation reaction pathway and the substrate concentrations [A] and [D]. For $e^{-kt} \ll 1$, the above equation simplified to

$$[Q] = KE(t - \frac{1}{k})$$

and the plot of [Q] vs time became a straight line which extrapolated on the abscissa ([Q] = 0) at a t value equal to $1/k$. As shown in Fig. 2, the straight part of curve II, when extrapolated on the abscissa, gave a t value of 2.5 h and from this, a rate constant for the dissociation of $0.4\,h^{-1}$ (or $1.1 \times 10^{-4}\,s^{-1}$). The fact that both the release of the radioactivity and the recovery of the enzyme activity gave a same rate constant value $(0.92 - 1.1 \times 10^{-4}\,s^{-1})$ strongly suggested that the penicillin high-affinity binding sites on the membrane were the transpeptidase molecules. From the value of 25 pmol [^{14}C]benzylpenicillin fixed per mg membrane protein and assuming a molecular weight of 50000 for the transpeptidase and that each molecule of enzyme bound one molecule of penicillin, the transpeptidase would represent about 0.125 % of the total proteins of the membranes (as they were prepared and isolated).

Nature of the Radioactive Compound(s) Released from the [^{14}C] Benzylpenicillin-Membrane Complex

As revealed by paper electrophoresis and thin-layer chromatography, a major part of the radioactivity released from the [^{14}C]benzylpenicillin-membrane complex was in the form of a compound which behaved as [^{14}C]benzylpenicilloic acid and a minor
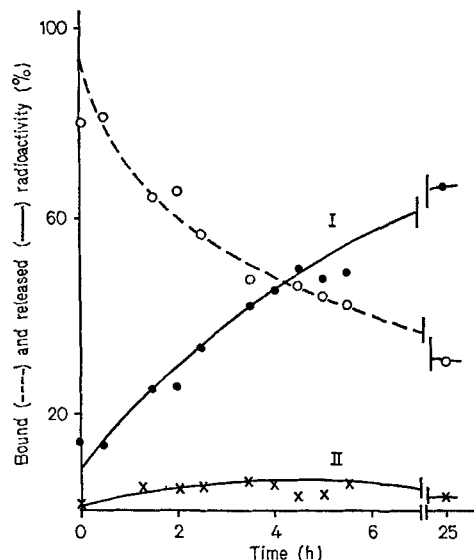

Fig. 4. *Kinetics of dissociation of the [^{14}C]benzylpenicillin-membrane complex in 0.017 M K_2HPO_4 and at 37 °C as revealed by the release of the radioactivity.* Membranes were treated as described in Fig. 3 and separation of the released compounds was carried out by paper electrophoresis. (●——●) Compound migrating with the same mobility as benzylpenicilloic acid; (×——×) compound migrating with the same mobility as benzylpenicillin; (O----O) radioactivity remaining at the origin of the electrophoretograms

part of it, in the form of a compound which behaved as [^{14}C]benzylpenicillin. The pattern of the release in 0.017 M K_2HPO_4 (Fig. 4) suggested that the accumulation of the compound like benzylpenicilloic acid might proceed through the transitory occurrence of the benzylpenicillin-like compound. As expected, the addition of 5 mM non-radioactive benzylpenicillin to the suspension of the [^{14}C]benzylpenicillin-membrane complex (in 0.017 M K_2HPO_4) did not affect the rate of the release of the radioactivity. Unexpectedly, however, this large amount of non-radioactive benzylpenicillin did not cause an increase of the amount of the [^{14}C]benzylpenicillin-like compound transitorily formed. These observations suggested that the benzylpenicillin-like compound might not be genuine benzylpenicillin. Hence, the release of the radioactivity might occur through a mechanism more complex than a simple dissociation of the enzyme-benzylpenicillin complex into its constituents, followed by a spontaneous hydrolysis of the antibiotic into benzylpenicilloic acid. The fact that benzylpenicillin did not appear in the reaction mixture, however, fitted the observation that dissociation of the antibiotic-membrane complex was an irreversible process that proceeded to completion.

Because of the relatively low specific radioactivity of the commercially available [^{14}C]benzylpenicillin

(45 mCi/mmol) and the paucity of the penicillin binding sites on the membrane (25 pmol benzyl-penicillin fixed per mg protein), the [^{14}C]benzyl-penicillin-membrane complex itself had a very low specific radioactivity (corresponding to about 2250 counts × min^{-1} × mg protein^{-1}). Under these conditions, characterization of the released radioactive compounds could not be achieved. It must await the solubilization and the isolation of the penicillin binding sites.

Rate Constants for the Dissociation of β-Lactam Antibiotic-Membrane Complexes

Whatever the exact mechanism for the dissociation of the β-lactam antibiotic-membrane complexes, the rate constants were determined from kinetics of recovery of the transpeptidase activity as described for non-radioactive benzylpenicillin. Each antibiotic was used at a concentration at least equivalent to 100 times the corresponding ID$_{50}$ value. Extrapolation of the straight line portion of the plots [Q] vs time (Fig. 5) gave t values which, depending upon the antibiotics, varied from 5 to 225 min. From these values, the corresponding rate constants were calculated (Table 2). There was a complete lack of correlation between these rate constant values and the ID$_{50}$ and LD$_{50}$ values for the corresponding antibiotics (Table 1).

Effects of —SH-Group Reagents on Binding of [^{14}C]Benzylpenicillin to Membranes and on the Activity of the Membrane-Bound Transpeptidase

Membranes (1250 μg protein in 200 μl, final volumes, 0.017 M K$_2$HPO$_4$) were incubated for 30 min at 30 °C in the absence and in the presence of various concentrations of iodoacetate, iodoacetamide or N-ethylmaleimide. After two washings by centrifugation at 4 °C and 37000×g with cold buffer, the membranes were resuspended in 100 μl water. The high-affinity binding sites for [^{14}C]benzylpenicillin were estimated as already described. In another series of experiments, membranes (2500 μg protein in 150 μl, final volumes) were treated and washed as above, resuspended in 190 μl of 0.017 M K$_2$HPO$_4$ containing N^α,N^ε-diacetyl-L-lysyl-D-alanyl-D-alanine (1.35 mM), [^{14}C]glycyl-glycine (13.5 mM) and Triton X-100 (0.3%, v/v) and the transpeptidation product formed after 16 h of incubation at 37 °C was estimated on 35-μl aliquots.

A 50% inhibition of the membrane transpeptidase activity and of [^{14}C]benzylpenicillin binding required pretreatments of the membranes with iodoacetate and iodoacetamide at concentrations as high as 0.1 M. N-Ethylmaleimide was a more potent inhibitor.
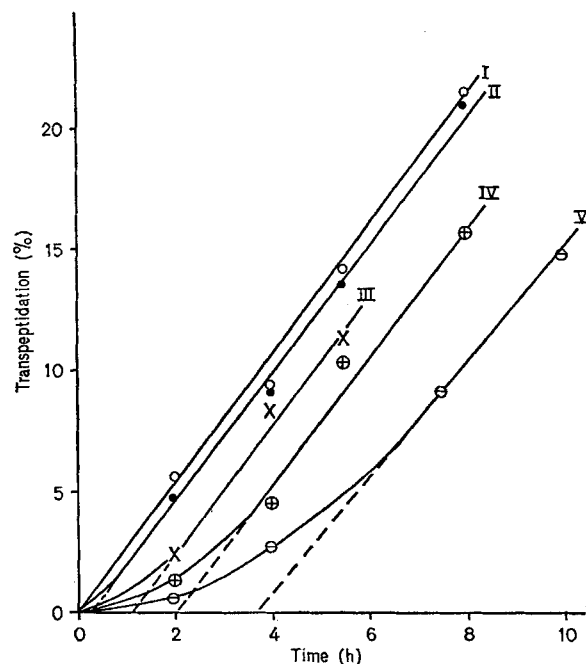
Fig. 5. Kinetics of dissociation of several β-lactam antibiotic-membrane complexes in 0.017 M K$_2$HPO$_4$ and at 37 °C, as revealed by the recovery of the transpeptidase activity. The experiments were performed as described in Fig. 2. Untreated membranes (I). Membranes previously saturated with ampicillin (II), penicillin V (III), methyl-penicillin V (IV) and carbenicillin (V). The t values obtained by extrapolating the straight line portions of the curves on the abscissa were used for the estimation of the rate constants for the dissociation of the complexes (Table 2)

Table 2. Dissociation of the complexes between β-lactam antibiotics and membrane-bound transpeptidase in 0.017 M K$_2$HPO$_4$ at 37 °C
For conditions, see text. Results were expressed in t values as obtained by extrapolating on the abscissa the straight portions of the plots transpeptidation vs time (Fig. 2 and 5), and in rate constants ($k = 1/t$)

Antibiotics	t	Rate constants
	min	s^{-1}
Ampicillin	5	3.3 ×10^{-3}
Cephalosporin C	5	3.3 ×10^{-3}
Cephalothin	20	8.3 ×10^{-4}
Penicillin V	60	2.8 ×10^{-4}
Methicillin	80	2.1 ×10^{-4}
6-Methylpenicillin V	100	1.7 ×10^{-4}
6-Aminopenicillanic acid	120	1.4 ×10^{-4}
Cloxacillin	135	1.23×10^{-4}
Benzylpenicillin	150	1.10×10^{-4}
Carbenicillin	225	0.73×10^{-4}

[^{14}C]Benzylpenicillin binding and enzyme activity were inhibited by 50% by pretreatments of the membranes with 2 mM and 0.2 mM N-ethylmaleimide, respectively (Fig. 6). When the time of exposure of
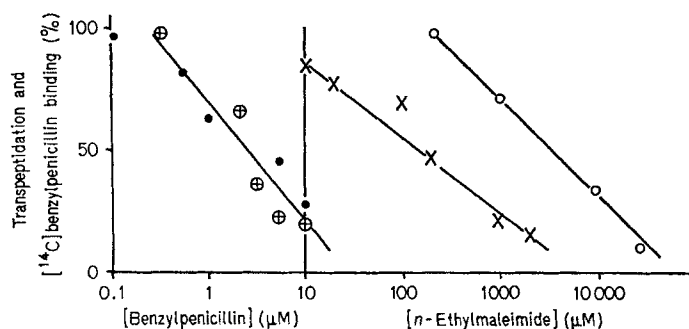
Fig. 6. *Effects of increasing concentrations of benzylpenicillin and N-ethylmaleimide on transpeptidase activity and [14C]benzylpenicillin binding capability of isolated membranes.* Effects of N-ethylmaleimide. For conditions of treatment, estimation of residual transpeptidase activity and residual binding capability (high-affinity binding sites): see text. The results were expressed as a percentage of the transpeptidase activity ($\times$) and binding capability ($\bigcirc$) of untreated membranes. Effects of benzylpenicillin, ($\oplus$) mem-branes were treated with increasing concentrations of benzylpenicillin (in 0.017 M K_2HPO_4, for 30 min at 30 °C), washed twice by centrifugation at 4 °C with cold buffer and the residual binding capability (high-affinity binding sites) was measured as described in the text (*i.e.* determination of Excl. D_{50} value). ($\bullet$) Transpeptidase activity of isolated membranes carried out in the presence of increasing concentration of benzylpenicillin (*i.e.* determination of the ID_{50} value)

the membranes to the reagents was decreased from 30 min to 5 min, N-ethylmaleimide had no effect on the enzyme activity even at a concentration as high as 1 mM. Hence, high concentrations of iodoacetate, iodoacetamide and N-ethylmaleimide and prolonged exposures of the membranes to these reagents were required to impair the enzyme activity and to prevent the fixation of benzylpenicillin. Under these conditions, the reactivity of these reagents is not restricted to —SH groups. Moreover, in view of the extensive changes that the sulphydryl reagents induce in membrane structure [5], the interpretation of the inhibitory effects observed on the functioning of the enzyme and on penicillin binding remains extremely speculative. Since to all appearances, the membrane penicillin binding sites were the transpeptidase molecules (see above), the differences observed between the sensitivity of the enzyme activity and the sensitivity of the penicillin binding toward N-ethylmaleimide (Fig. 6) indicated that the site of acylation affecting penicillin binding must be different from the site affecting transpeptidase activity and hence presumably involved in the binding of the transpeptidation substrates. This provides further evidence for a difference in binding site for substrate and *β-lactam* antibiotics [6].
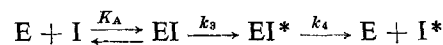
Mechanism of Inhibition
of the Membrane-Bound Transpeptidase
by β-Lactam Antibiotics

Inhibition of the membrane-bound transpeptidase by incubation with penicillins and cephalosporins was fully reversible so far as the enzyme was concerned.

Assuming the following simple mechanism of inhibition

Antibiotic + enzyme $\rightleftharpoons$ Antibiotic-enzyme complex

an association constant of 3.58×10^5 $1/mol^{-1}$ could be calculated for benzylpenicillin from the curve of Fig. 1 A. As a result of its combination with the membrane-bound enzyme, however, penicillin might undergo modification(s) resulting in its inactivation. Hence, the following mechanism of inhibition might also occur:

$$E + I \underset{\longleftarrow}{\overset{K_A}{\longrightarrow}} EI \xrightarrow{k_3} EI^* \xrightarrow{k_4} E + I^*$$

where E is the active enzyme, I the intact penicillin, I* the inactivated penicillin, EI a reversible complex, $K_A = [EI]/[E][I]$, EI* a complex with the inactivated penicillin, k_3 the rate constant for the isomerisation of the penicillin-enzyme complex and k_4 the rate constant for the dissociation of the EI* complex (resulting in the recovery of the enzyme activity). In such a mechanism, the dissociation rate constants for penicillins and cephalosporins shown in Table 2 are k_4 values. The question whether or not the mechanism of inhibition of the R61 membrane-bound transpeptidase fits this proposed model is under current study. A similar mechanism was proposed by Umbreit and Stominger [7] for the inhibition of the DD-carboxy-peptidase of *Bacillus subtilis* by penicillin except that in this latter system, k_3 was considered as the rate constant for the irreversible inactivation of the enzyme, a situation which clearly did not exist with both exo-cellular [6] and membrane-bound transpeptidases of *Streptomyces* R61. A choice of mechanisms, however, must rest upon the complete characterization of the antibiotic after its release from the complex with the

R61 transpeptidases. At the present stage, this question remains open.

DISCUSSION

It has been known for many years that there was a correlation between the sensitivity of bacteria to penicillin and the amount of antibiotic which was "irreversibly" fixed to them [8,9]. "Irreversibly" fixed penicillin was the amount which could not be released from the cells by washing in neutral or acidic buffers, by treatment with penicillinase or (when fixation was carried out with radioactive penicillin) with a large excess of non-radioactive penicillin. However, the membrane-bound transpeptidase which is currently considered as the specific killing target of penicillins might represent only a very small portion of these cell binding sites. It was observed that the great increase in resistance to penicillin exhibited by a series of mutants of Staphylococcus aureus could not be accounted for by a change in the number and/or the reactivity of the penicillin "irreversible" binding sites [10]. When reincubated in a penicillin-free medium, cells of S. aureus that had been previously saturated with benzylpenicillin, were able to recover and to grow while carrying 90% of the penicillin molecules initially fixed [11]. This observation suggested that very few of the total cell penicillin binding sites were relevant to peptidoglycan synthesis and that association of the relevant sites with penicillin could be reversed during recovery. "Irreversible" penicillin binding sites were actually isolated from S. aureus and other bacteria such as Escherichia coli, Bacillus subtilis and Bacillus cereus [12,13]. None of the components so far isolated was characterized as the transpeptidase.

In the experiments described in the present paper, membrane "high-affinity" binding sites for penicillin were defined as the sites from which the antibiotic fixed during a preincubation with the membrane in 0.017 M K_2HPO_4 or 0.05 M Tris-HCl pH 7.5 for 30 min at 30° C, was not removed by two or three washings at 4 °C (by centrifugation at $37000 \times g$ for 30 min and using the same cold buffer). The main purpose of these experiments was to show that (a) the high-affinity binding sites of the isolated membranes of Streptomyces R61 for penicillins and cephalosporins behaved as if they were the membrane-bound transpeptidase molecules; (b) the complexes formed between the membranes and the β-lactam antibiotics were fully reversible at least with regard to the transpeptidase which recovered both its transfer activity and its sensitivity to penicillin and (c) in several respects, the membrane-bound transpeptidase behaved as the purified, exocellular transpeptidase excreted by the same Streptomyces R61. It was not possible, however, to ascertain whether or not the association between the membranes and the β-lactam antibiotics was also reversible for the antibiotic. Experiments suggested that the antibiotic molecule might undergo chemical alteration during its association with the membrane. A choice of mechanisms for the inhibition of the transpeptidase could not be made.

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