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Alterations in Kinetic Properties of Penicillin-Binding Proteins of Penicillin-Resistant *Streptococcus pneumoniae*

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Earlier studies have shown that the highly penicillin-resistant South African strains of pneumococci contain altered penicillin-binding proteins (PBPs) (S. Zighelboim and A. Tomasz, Antimicrob. Agents Chemother. 17:434–442, 1980). We now describe a detailed quantitative characterization of the reaction of radioactively labeled penicillin with the PBPs of the penicillin-susceptible and penicillin-resistant pneumococci and several intermediate-resistance-level genetic transformants as well. The altered binding of the antibiotic by the PBPs of resistant cells appears to be due to a combination of two factors: lower drug affinity and change in the cellular amounts of PBPs. No alteration in the rates of deacylation of the penicilloyl-PBPs of the resistant cells was detected.

Altered penicillin-binding proteins (PBPs) associated with intrinsic resistance to beta-lactam antibiotics have been detected in both laboratory mutants (2, 11, 13, 16, 23) and clinical isolates of a number of bacterial species, including most of the major human invasive pathogens (1, 4, 6, 8, 12, 17, 21, 22, 26). Although in most cases the altered PBPs of these mutants have been described as having lower antibiotic affinities, a rigorous demonstration of this fact has not been made in any of the cases reported, and alternative mechanisms, such as change in the cellular concentration of the PBP or increase in the rate of deacylation of the penicilloyl-PBP complex, have not been excluded. In experiments in which the altered PBPs have been characterized only in live, growing bacteria (27), the less effective binding of the antibiotic may be caused by an increased intracellular concentration of the PBP's natural substrates, which compete more effectively with the beta-lactam molecules. Clearly, further understanding of the basis of penicillin resistance in these strains requires a more careful quantitative evaluation of the reaction between the antibiotic and the PBPs.

The purpose of the present study was to distinguish between these mechanisms by a detailed comparative study of the reaction between radioactive penicillin and the PBPs of susceptible and resistant pneumococci.

MATERIALS AND METHODS

Bacteria. *Streptococcus pneumoniae* R6 is a derivative of the unencapsulated Rockefeller University strain R36A which has an MIC of 0.006 μg/ml for benzylpenicillin. Strain 8249 is a penicillin-resistant clinical isolate with an MIC of 6 μg/ml. It was obtained from H. Koornhof of Johannesburg, South Africa. Strains pen 0.05, pen 0.1, and pen 1.6 represent transformants obtained from crossing the DNA of strain 8249 into that of strain R6 (27) and selecting for bacteria with MICs of 0.05, 0.1, and 1.6 μg/ml, respectively. Details of the genetic transformation and selection procedures have been described elsewhere (27).

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Cultures were grown in a casein hydrolysate medium (19) supplemented with yeast extract (0.2% [wt/vol] final concentration; Difco Laboratories, Detroit, Mich.) (c+y) at 37°C without aeration and were stored at −70°C in c+y with 10% glycerol. In the experiments to determine deacylation rates, a chemically defined medium lacking leucine (cden-leu) (A. Tomasz, Bacteriol. Proc., p. 29, 1964) was used to prevent lysis or antibiotic-induced secretion of PBPs into the medium (18). Growth was monitored by spectrophotometry (Sequoia-Turner Instruments, Mountainview, Calif.). Viability counts were determined by surface plating of samples of diluted cultures onto tryptose soy blood agar base (Difco) supplemented with 5% defibrinated sheep blood (Wilfer Laboratories, Stillwater, Minn.).

In vivo labeling of PBPs. The experimental data were all obtained by labeling live bacteria with radioactive penicillin. [^{3}H]benzylpenicillin (ethylpiperidinium salt) with a specific activity of 27 Ci/mmol was provided by Merck & Co., Inc., Rahway, N.J., and was stored in acetone at −70°C. Acetone was evaporated immediately before use, and the penicillin was suspended in an equal volume of 50 mM potassium phosphate buffer (pH 7.0). Samples (1 ml each) of early-logarithmic-phase cultures of equal optical density (representing 10^8 CFU/ml and 80 to 100 μg/ml of total protein) were incubated for 10 min at 37°C with appropriate dilutions of [^{3}H]penicillin, and the reaction was stopped by transfer to an ice bath and the addition of excess unlabeled penicillin (final concentration, 200 μg/ml). Cells were recovered by centrifugation (6,000 × *g* for 10 min) at 4°C and were suspended in 50 μl of 50 mM potassium phosphate buffer (pH 7.0). Cells were lysed by the addition of Sarkosyl NL-97 (final concentration, 2%) followed by incubation for 10 min at 37°C. A buffer mixture (30 μl) was added to yield final concentrations of 3.3% glycerol, 1.7% mercaptoethanol, 1% sodium dodecyl sulfate, 0.0017% bromphenol blue, and 0.011 M Tris hydrochloride (pH 6.8), and samples were boiled for 3 min. The final sample volume was 100 μl.

In experiments to determine deacylation rate, the procedure was modified to prevent cell lysis or secretion of PBPs into the medium (14, 18). Cells were grown in c+y medium to early logarithmic phase, recovered by filtration (membrane filter, 45-nm pore diameter; Millipore Corp., Bedford, Mass.), and gently suspended in cden-leu. [^{3}H]penicillin was

TABLE 1. Kinetics of PBP binding: fraction of maximal binding as a function of time[a]

Strain	PBP	Fraction of maximal binding at t (min)			
		10	20	30	80
R6	1a	0.55	0.55	0.88	1.00
	1b	0.10	0.98	1.00	1.00
	2a/b	0.12	0.83	0.89	1.00
	3	0.37	0.92	1.00	1.00
pen 0.1	1c	0.35	0.50	0.71	1.00
	2a/b	0.48	0.51	0.69	1.00
	3	0.54	0.70	0.77	1.00

[a] Data from a representative experiment. The [^{3}H]penicillin concentration used was 0.02 μg/ml.

added for 10 min, after which the cells were chilled, recovered by centrifugation at 4°C, washed, and suspended in cden-leu containing excess unlabeled penicillin (200 μg/ml). Cultures were returned to a 37°C bath, and 1-ml samples were removed at 30-min intervals for sample preparation as described above.

In several experiments, samples were removed just prior to [^{3}H]penicillin labeling for viability plating to confirm equal numbers of CFU per sample; the mean count was 10^8 CFU/ml. Samples were also removed after lysis, and the protein concentration was determined by the method of Lowry (20) to confirm that cellular protein per sample was identical.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gels were prepared as previously described (25), with final concentrations of acrylamide-bisacrylamide (Bio-Rad Laboratories, Richmond, Calif.) of 10 to 0.16% in the separating gel and 7 to 0.116% in the stacking gel. Electrophoresis was carried out at 60 mV through the stacking gel and 110 mV through the separating gel and continued for 60 to 90 min after the electrophoretic front had run off the gel. Gels were stained with Coomassie blue (Bio-Rad) and destained with methanol-acetic acid-water (30:10:60%). Gels were impregnated with 2,5-diphenyloxazole (Amersham Corp., Arlington Heights, Ill.) as previously described (25), dried, and exposed to prefogged XAR-2 film (Eastman Kodak Co., Rochester, N.Y.) at −70°C for 7 days. Molecular weights were estimated from comparison of stained gels and fluorographs with molecular weight standards obtained from Sigma Chemical Co., St. Louis, Mo.

Quantitative determinations. Autoradiographs were scanned with a Quick Scan Jr. densitometer with a 545-nm filter and a 0.3- by 3-mm slit (Helena Laboratories, Beaumont, Tex.) interfaced with an AIM 65 microcomputer (Rockwell International, Newport Beach, Calif.) programmed to integrate curves. Density readings were corrected for variation between gels by comparison with samples of the sensitive strain R6 exposed to four to six difference concentrations of [^{3}H]penicillin that were included on each gel. After preliminary experiments to define the most appropriate range of antibiotic concentrations, standardized samples of each strain were exposed to sets of six to eight different concentrations of [^{3}H]penicillin. Each one of these titrations was repeated four to eight times, and data were used to generate statistically significant values for penicillin binding. The optical density values were plotted against penicillin concentration, and the results were expressed as mean density in arbitrary units. Binding was also compared by plots of reciprocal concentration versus density; slopes and y intercepts were obtained by linear regression, and absolute values of slopes were used to compare penicillin "affinity." S_{50}, the concentration of [^{3}H]penicillin at which each PBP was 50% saturated, was obtained from visual inspection of the density versus concentration curves. For deacylation experiments, the percentage of PBP bound was determined by dividing density at a given time by density at time zero, and $t_{1/2}$ values were determined from plots of log percentage PBP bound versus time.

Scintillation counting of gels. In several experiments, scintillation counting was used to confirm the reliability of scanning densitometry. Slices of stained and PPO-impregnated gels (1 cm by 2 mm) were placed in scintillation vials, and 0.4 ml of a 1:9 mixture of water-protosol tissue solubilizer (New England Nuclear Corp., Boston, Mass.) was added. After overnight incubation at 37°C, Econofluor (New England Nuclear) was added, and the vials were wrapped in foil to minimize light exposure and refrigerated overnight. Samples were counted in a Mark II Nuclear-Chicago scintillation counter. The efficiency of counting of gel slices in the protosol-Econofluor system was 43%, as determined with a tritium standard in toluene. The background for each sample was determined by counting of a gel slice from the same lane. Results are expressed as counts per minute after subtraction of the background from each sample.

Membrane preparation. Logarithmic-phase cultures growing in c+y medium were chilled rapidly to 4°C, and cells were recovered by centrifugation (4,000 × g for 10 min). Cells were washed, suspended in 10 mM potassium phosphate buffer (pH 8.0), and broken by agitation with 100-μm glass beads in a Mickle apparatus (Gomshall, Surrey, England) for 2 h. Unbroken cells and cell walls were removed by centrifugation (27,000 × g for 10 min).

Membranes were recovered by ultracentrifugation (200,000 × g for 90 min), washed, and suspended in 10 mM potassium phosphate buffer (pH 8.0). All steps in preparation were performed at 4°C. Protein concentration was determined by the method of Lowry (20) with bovine serum albumin as a standard. Membranes were stored at a concentration of 3 to 5 mg/ml at −70°C until use.

Membrane labeling. Samples of membrane suspensions containing 150 to 200 μg of protein were diluted to a final volume of 100 μl in 50 mM potassium phosphate buffer. [^{3}H]penicillin was evaporated, diluted, and added in concentrations of 1 to 10 μg/ml. Samples were incubated at 37°C for 10 min. Unlabeled penicillin, Sarkosyl, and sample buffer

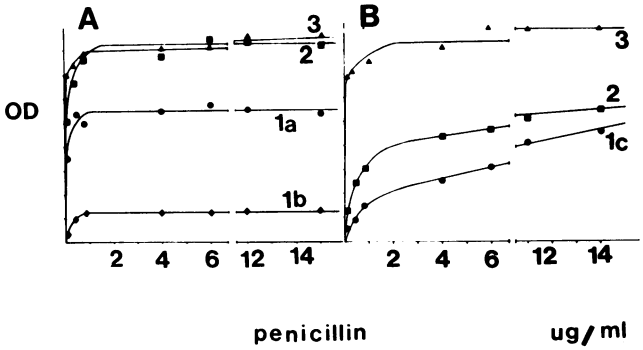

FIG. 1. PBP affinity: density (in arbitrary units) versus concentration of [^{3}H]penicillin. (A) PBPs of strain R6. (B) PBPs of strain 8249. OD, Optical density.

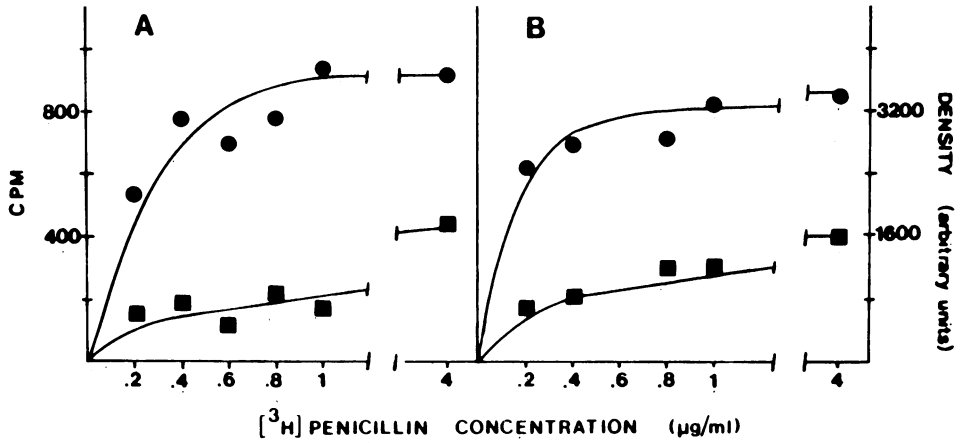

FIG. 2. Comparison of scanning densitometry and scintillation counting. (A) Affinity of PBP group 2 of strains R6 (●) and 8249 (■) as measured by the mean counts per minute (cpm) of PBPs cut from three gels. (B) Results as determined by scanning densitometry.

were added as described above, and the samples were boiled for 3 min.

RESULTS

For the interpretation of the observed differences in penicillin binding, it was important to ascertain that under the standard conditions of the experiments, the radioactive penicillin used remained in excess, and thus an increased binding observed at higher concentrations of the antibiotic did not simply involve titration of the amounts of PBPs (3, 7). The results of such a control study are summarized in Table 1. Membrane preparations from strains R6 and pen 0.1 were exposed to a low concentration of [^{3}H]penicillin (0.02 μg/ml), and samples were removed after various lengths of time to determine the fractions of the different PBPs acylated. It can be seen that full saturation required prolonged incubation times (30 to 80 min), far more than the incubation time used in the standard binding assay (10 min). Similar results were obtained with strain 8249.

Quantitative characterization of the reaction of penicillin with the PBPs of penicillin-susceptible and resistant cells. The rates and maximum degrees of [^{3}H]penicillin binding to the PBPs of strains R6 and 8249 were determined by exposing samples of standardized pneumococcal cultures or membrane preparations to various concentrations of the radioactive antibiotic for 10 min and determining the amounts of penicillin bound by scanning densitometry (Fig. 1). Experimental values were derived from a large number (four to eight) of parallel determinations for each concentration of the antibiotic, and differences in grain density of individual gels were corrected for by internal standards run on each gel, as described in Materials and Methods. The total amount of a PBP present (in arbitrary density units) was determined from the density at the plateau of the concentration versus density curves. The highest concentration of [^{3}H]penicillin routinely used was 20 μg/ml. However, in several experiments, higher concentrations (50 to 100 μg/ml) as well as longer exposure times (up to 2 h) were also tested without a

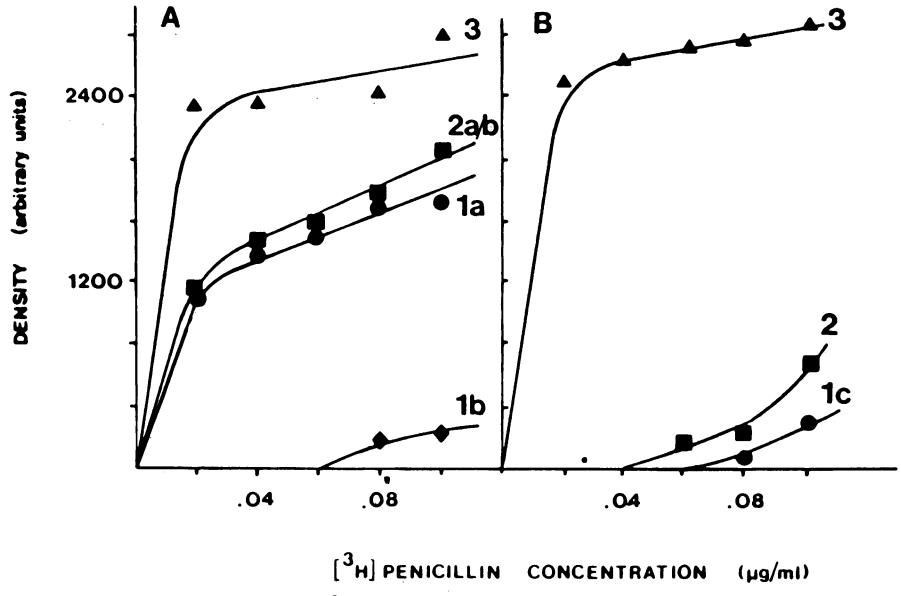

FIG. 3. PBP affinity: density versus concentration of [^{3}H]penicillin of the parent strains 8249 and R6. Plot showing the marked differences in binding at a very low concentration of [^{3}H]penicillin. PBPs of strains R6 (A) and 8249 (B).

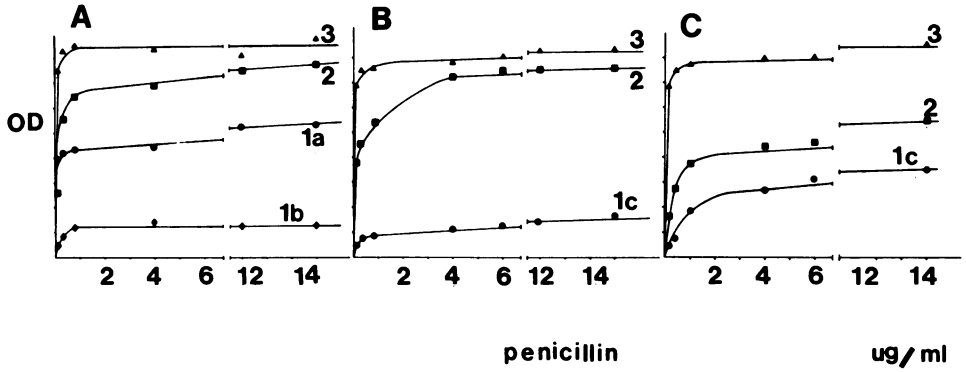

FIG. 4. PBP affinity: density versus concentration of [^{3}H]penicillin. PBPs of strains pen 0.05 (A), pen 0.1 (B), and pen 1.6 (C). OD, Optical density.

detectable increase in band density. Exposure to these concentrations, however, resulted in high background nonspecific binding.

To test the reliability of the densitometric assay for the quantitation of the binding data, several experiments with the PBPs of strains R6 and 8249 were also evaluated by direct scintillation counting of the [^{3}H]penicillin bound to the various PBPs. Figure 2 shows the results of one such experiment. Four lanes from each of three identical gels were cut into slices for this comparison. Figure 2A shows the results obtained by scintillation counting, and Fig. 2B shows the same experiment evaluated by densitometry. Comparison of the two methods by analysis of covariance with log transformation of the data showed no significant differences. However, PBPs 1a and 1b could be more reliably separated by scanning densitometry than by cutting gels for scintillation counting. Figure 3 shows an enlarged portion of the penicillin concentration against density plots for the PBPs of strains R6 and 8249 to demonstrate more clearly the striking differences between binding of the antibiotic to the susceptible and resistant PBPs at low penicillin concentrations.

Figure 4 shows the results of similar penicillin-binding assays with the three intermediate-level genetic transformants pen 0.05, pen 0.1, and pen 1.6 (Fig. 4A, B, and C, respectively).

Stability of the penicilloyl-PBP complexes of susceptible and resistant pneumococci. The decrease in penicillin binding observed in the resistant PBPs may be due to an increased rate of deacylation of the PBP-penicillin complexes. However, an experimental test of this possibility yielded negative results. The PBPs of strains R6 and 8249 were labeled with saturating concentrations of [^{3}H]penicillin, and the rate of loss of radioactive label after incubation was monitored under conditions described in Materials and Methods. Figure 5 shows a plot of the amounts of radioactive penicillin associated with the PBP complexes 1 (panels A and B), 2 (panels C and D), and 3 (panels E and F) of the susceptible and resistant strains as a function of time. No increase in the loss of antibiotic from the resistant PBPs is apparent. The estimated half-lives of the penicilloyl-PBPs 1a (strain R6) and 1c (strain 8249) were 75 and 95 min, respectively. The half-lives for the penicillin-PBP 2 and penicillin-PBP 3 complexes of strains R6, pen 0.1, and 8249 were 50, 50, and 80 min and 70, 75, and 75 min, respectively.

Table 2 is a summary of all the quantitative data derived from Fig. 1, 3, and 4. The S_{50} value was used in Table 2 as a simple numerical index of the reactivity (affinity or acylation rate) of the PBP. Relative amounts of PBPs were determined from the density data at the plateau of the concentration versus density curves.

DISCUSSION

Penicillin-susceptible pneumococci contain a set of five PBPs (1a, 1b, 2a, 2b, and 3) when examined with the currently used sodium dodecyl sulfate-polyacrylamide gel electrophoresis fluorographic method. In contrast, the two highly penicillin-resistant isolates examined in some detail, strains 8249 and D20, contain only the following three PBPs: 1c, a protein with a smaller molecular size (92,000 daltons) than either 1a or 1b, a second PBP with approximately the molecular size of 2a, and PBP 3. In addition to these differences in molecular size, PBPs 1c and 2a of the resistant strains are also less effective in binding radioactive penicillin than are PBPs 1a and 2a of the susceptible laboratory reference strain (24, 27). Less effective binding of the antibiotic by the PBPs was also demonstrated in low- or intermediate-resistance-level genetic transformants constructed by crossing the DNA of highly penicillin-resistant transformants into susceptible recipient cells (24, 27).

To gain a better understanding of these complex changes, we examined in detail the nature of the interaction between radioactive penicillin and the PBPs of the following strains: penicillin-susceptible R6 strain (recipient in the genetic transformation experiments; MIC, 0.006 μg/ml), strain 8249 (DNA donor; MIC, 6.0 μg/ml), and three transformants named pen 0.05, pen 0.1, and pen 1.6. The corresponding MICs of penicillin for these strains were 0.1, 0.2, and 3.2 μg/ml, respectively.

Studies by Ghuysen and his colleagues (9) on the mechanism of interaction of model substrates and beta-lactam antibiotics with penicillin-sensitive transpeptidases and D,D-carboxypeptidases have recognized the following multistep process:

$$E + I \underset{}{\overset{K}{\rightleftharpoons}} EI \xrightarrow{k_2} EI^* \xrightarrow{k_3} E + P(s)$$

where E is the enzyme, I is the inhibitor, and EI* is the enzyme-inhibitor complex detected by PBP assay and P(s) indicates the degraded penicillin products. Decreased binding of the inhibitor (penicillin), i.e., a decreased concentration of EI* detected at a given penicillin concentration, might correspond to several possibilities: (i) an increase in the dissociation constant K, (ii) a decrease in the acylation rate, k_2, (iii) an increase in the deacylation rate, k_3, or (iv) a

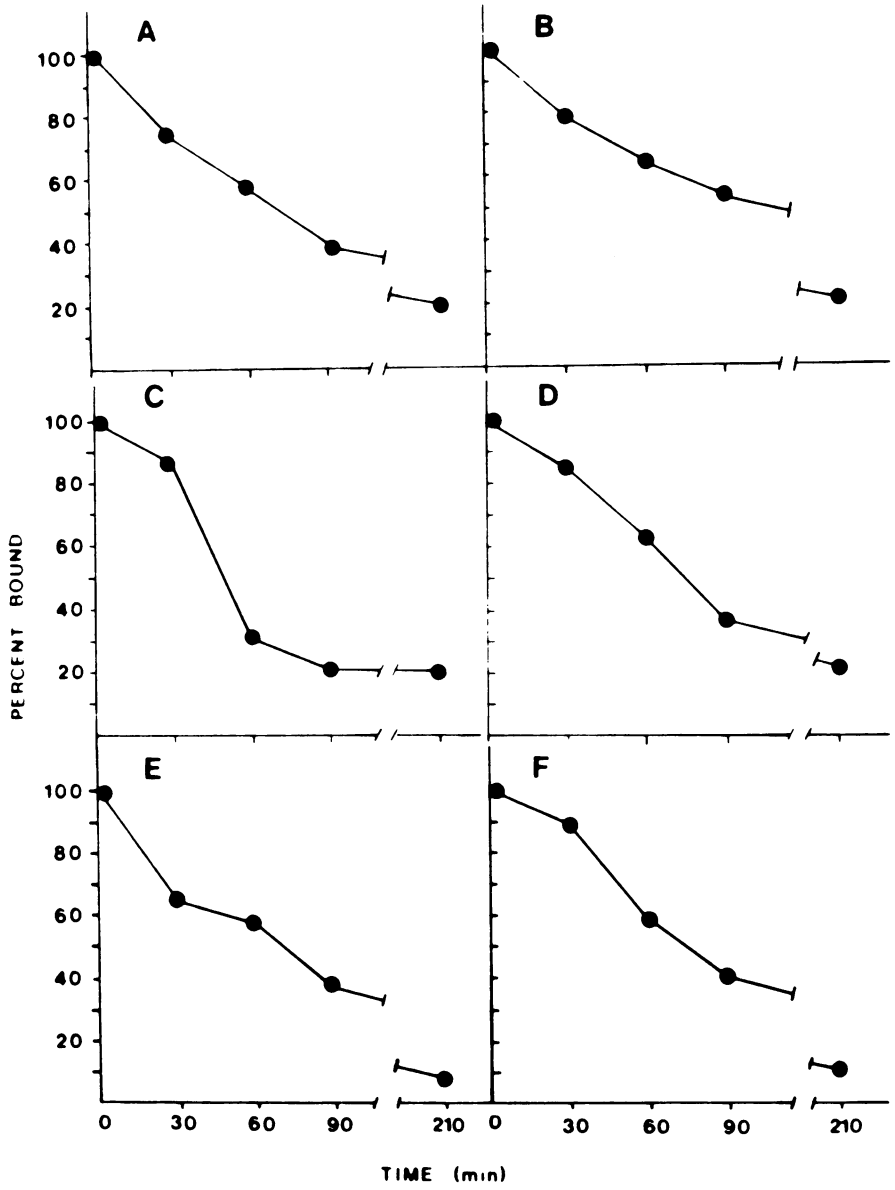

FIG. 5. Deacylation rates of strains R6 and 8249. (A, C, E) PBPs 1, 2, and 3, respectively, of strain R6; (B, D, F) PBPs 1c, 2, and 3, respectively, of strain 8249.

change in the concentration of enzyme available. In studies in which the interaction between radioactive penicillin and the PBPs was examined only in live, growing bacteria (27), an additional possibility (v) has to be considered; namely, that in the resistant cells, intracellular concentration of the natural substrate(s) of PBPs is increased, causing a more effective competitive inhibition in the binding of the antibiotic molecules. Our experiments address each of these possibilities. This last mechanism does not appear to be a major factor, since the key experimental findings were also demonstrable with membrane preparations. Possibility iii is ruled out, since there was no detectable increase in the rate of loss of penicillin bound to any of the PBPs of the resistant cells. Our results indicate that the factor mainly responsible for the observed decrease in antibiotic binding in resistant cells involves a decrease in the affinity (K) or the rate of acylation (k_2) of PBPs by penicillin or both. This result is consistent with studies of the penicillin-sensitive strain R61 transpeptidase-carboxypeptidase in which the inhibitory potency of beta-lactam antibiotics was found to parallel the second-order rate constant k_2/K (9), indicating that mechanisms i and ii were operable. Since the methodology used here detects only the EI* complex, alterations in affinity caused by mechanisms i and ii could not be separately distinguished.

Another process apparently operative between the intermediate and high levels of resistance is mechanism iv, i.e., a change (gradual increase) in the relative copy number of the low-affinity PBP 1c along with increasing resistance. This mechanism may be seen by comparing the relative amounts (i.e., plateau-level bindings) of PBP 1c in strains pen 0.1, pen 1.6, and 8249 (MIC, 6.0); the corresponding relative amounts of PBP 1c in these strains were estimated as 0.33, 0.78, and 1.0 (Table 2). PBPs 2a and 2b were not always resolved in

TABLE 2. PBP affinity and copy number

PBP	PBP affinity (μg/ml) and maximum amt in tests with strain									
	R6		pen 0.05		pen 0.1		pen 1.6		8249	
	S_{50}	Amt[a]	S_{50}	Amt	S_{50}	Amt	S_{50}	Amt	S_{50}	Amt
1a	<0.02	1.0	0.06	1.0						
1b	0.10	1.0	0.10	1.0						
1c					0.15	0.33[b]	0.8	0.78[b]	4.0	1.0[b]
2a/b	0.05	1.0	0.10	1.0	0.15	1.0	0.4	0.66	0.5	0.66
3	<0.01	1.0	0.01	1.0	0.01	1.0	0.01	1.0	0.01	1.1

[a] Maximum band density obtained at saturating concentrations of penicillin. The data are expressed relative to the figures derived for the PBPs of the susceptible R6 strain.
[b] Amounts relative to PBP 1a of strain R6.

the experiments, and the decreased plateau binding in the PBP 2 region may simply represent the disappearance of PBP 2b from the gels. Of course, the apparent absence of a PBP may simply mean that due to the low affinity for penicillin, the protein is no longer detectable by the [^{3}H]penicillin-binding technique.

It is not yet clear precisely how many genetically and physiologically definable steps exist in the acquisition of resistance between the susceptible and highly resistant strains. In previous studies it was shown that in the lowest-level penicillin-resistant transformants (MIC range, 0.012 to 0.025), the binding capacity of PBP 1a decreased (27). In transformant pen 0.05, decreased affinities of PBPs 1a and 2a are observable, and a gradual decrease in the antibiotic affinity of the PBP 2 complex continued through a wide range of resistance levels (see S_{50} values in Table 2). These data suggest that the genetic determinants of PBPs 1a, 1b, 2a, and 2b in the resistant DNA donor strain carry multiple mutations affecting the affinity of these proteins for the antibiotic molecule and that each of these binding proteins performs physiologically indispensable functions. All experimental results so far obtained indicate that the high-affinity PBP 3 (a protein exhibiting D,D-carboxypeptidase activity) (15) does not undergo detectable changes along with penicillin resistance. During selection for penicillin-resistant transformants of gradually increasing resistance levels, the order in which the individual PBPs of the susceptible strain are changed towards lower affinity reflects the order of relative penicillin affinities of the pneumococcal PBPs, i.e., 1a > 2a > 1b > 2b (25). This result suggests that the apparent orderliness of PBP alterations observed in previous studies (24, 27) can be explained as follows. If pneumococci contain four potential penicillin "killing target" PBPs, the affinity of the most penicillin-susceptible PBP will first decrease during acquisition of the lowest level of resistance. In such a strain, a second PBP may now become the relatively most susceptible target. Thus, acquisition of the next incremental level of resistance would require a tune-down in the affinity of this second PBP and, possibly, a further decrease in the affinity of the first PBP as well. Further stepwise increases in resistance may require simultaneous affinity decreases in two or three of the PBPs. Peptide mapping data indicate the PBPs 1a, 1b, 2a, and 2b are distinct proteins, (5) and presumably, similarly to the PBPs of other bacteria, they have distinct genetic determinants. The simultaneous alteration in two or more PBPs needed for intermediate resistance would then require the uptake of several DNA molecules by the same recipient cell. This requirement may be the main factor responsible for both the multistep nature and the gradually decreasing efficiency of transformation of penicillin resistance in these bacteria (24, 27).

The mechanism of the striking switchover from five PBPs to the triad of PBPs characteristic of the highly resistant strain is not clear. The most peculiar feature of the switchover phenomenon is that the appearance of the low-drug-affinity PBP 1c is always accompanied by the disappearance of PBP 1a from the fluorograms. As a working hypothesis, we have proposed that PBP 1c may be derived from 1a (24). The gradual accumulation of mutational changes (amino acid replacements) in PBP 1a may at one point be recognized by the cells as a processing signal resulting in the splitting of 1a (molecular size, 98,000) to a molecular size corresponding to that of 1c (92,000) (24). Experiments are in progress to test this hypothesis. It is interesting that both the spectrum of changes in the relative penicillin affinity and the amount of PBP 1a are a continuum with those of PBP 1c when the properties of these proteins in strains pen 0.1, pen 1.6, and 8249 are compared. The amount of 1c (measured in arbitrary density units) in the most resistant strain, 8249, approaches the amount of PBP 1a seen in the susceptible R6 strain. The lack of detection of PBP 1b in the fluorograms of resistant strains may be due to decreased antibiotic affinity. The small quantities of this protein have made more detailed studies difficult.

The greatly lowered penicillin affinity of PBPs raises the question of whether or not these enzymes also have decreased affinities for their physiological substrates in cell wall synthesis. The highly penicillin-resistant pneumococci can grow with normal doubling times in synthetic media, and these bacteria show no symptoms of defective cell wall synthesis (24). It is possible that the molecular changes resulting in lower penicillin affinity are clustered within a domain of the PBP molecules which is primarily involved with the (noncovalent) binding of the antibiotic only. Such separate binding sites for beta-lactam antibiotics and for substrates in the penicillin-susceptible transpeptidases have been postulated (10).

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