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Penicillin-Binding Proteins of Penicillin-Susceptible and Intrinsically Resistant *Neisseria gonorrhoeae*

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The penicillin-binding proteins (PBPs) of *Neisseria gonorrhoeae* were investigated by using [^{3}H]benzylpenicillin of high specific activity. This made it possible to label the PBPs both in cytoplasmic membranes and in the membranes of actively growing cells (in vivo labeling). A total of 20 strains isolated from different geographic locales showed the same pattern of three major PBPs, which had molecular weights of approximately 90,000, 63,000, and 48,000. Five clinical isolates of intrinsically penicillin-resistant gonococci each exhibited reduced penicillin binding of PBPs 1 and 2. The construction of an isogenic set of transformants with increasing levels of penicillin resistance indicated that the *penA* gene was associated with a decrease in penicillin binding to PBP 2. Decreased binding to PBP 1 is likely to accompany the newly reported *pem* and *tem* genes, which govern the highest level of penicillin resistance.

Strains of *Neisseria gonorrhoeae* have shown marked increases in resistance to penicillin, necessitating larger doses to avoid treatment failures (24). The majority of resistant strains do not produce β-lactamase, but rather are termed "intrinsically resistant." This resistance can result in minimal inhibitory concentrations (MICs) of 1 to 2 μg of penicillin per ml, which contrasts with the low MICs for susceptible strains, which are in the range of 0.007 to 0.03 μg/ml.

The genetic basis of intrinsic resistance to penicillin has been investigated in detail (25). The biochemical basis for increased resistance is less well defined. A decreased cellular binding of radioactive penicillin was noted in several strains of intrinsically penicillin-resistant gonococci (19). Very few differences were found in the overall chemical compositions of the cell envelopes of susceptible and resistant strains (34). Studies have found an increased cross-linkage of the peptidoglycan and an increase in the quantity of a minor outer membrane protein in resistant cells (4, 7). At present, it is difficult to assess the relationship of cell envelope changes to increased resistance.

Penicillin-binding proteins (PBP) are presumably penicillin-sensitive bacterial enzymes involved in the terminal stages of cell wall biosynthesis (30), and comparative examinations of the PBPs of antibiotic-susceptible and -resistant bacteria could conceivably provide clues about the biochemical basis of resistance. We report here the results of studies on the PBPs of gonococci. Benzylpenicillin ([^{3}H]penicillin) of high specific radioactivity was used to identify PBPs, either by allowing the radioactive penicillin to react with the binding proteins of live bacteria (in vivo labeling) or by labeling binding proteins in isolated membranes. Distinct decreases in the penicillin affinities of PBPs 1 and 2 were observed in intrinsically penicillin-resistant gonococci. Studies with isogenic genetic transformants indicated that the change in PBP 2 accompanied acquisition of resistance to intermediate levels of penicillin (*penA*). The change in PBP 1 was associated with the highest level of resistance and may be controlled by the newly reported *pem* and *tem* genes (32).

MATERIALS AND METHODS

Cultures. *N. gonorrhoeae* cultures were obtained from recently isolated strains at The New York Hospital; clinical isolates of highly resistant strains were obtained from the Center for Disease Control, Atlanta, Ga., and from the culture collections of several investigators. The sources and pertinent properties of the strains are listed in the legend to Fig. 3. Cells were cultured on GCBA agar (BBL Microbiology Systems) containing a supplement identical in composition to IsoVitaleX (BBL Microbiology Systems). Cells were passed daily and incubated at 37°C in 5% CO_2. Liquid cultures were grown in gonococcal broth base (28) (GCBB) with 1% supplement and $NaHCO_3$ (420 μg/ml). All liquid cultures were grown at 37°C with shaking, and cell density was measured with a Coleman model 9 nephelo-colorimeter (6). Cells were stored at −70°C in GCBB containing 15% glycerol.

Susceptibility tests. MICs were determined by the agar dilution method. The desired concentrations of antibiotics were mixed with melted GCBA agar (52°C) in plates. Colonies from 16- to 18-h cultures were suspended in broth, the turbidity of each suspension was adjusted, and 5 μl (5×10^4 colony-forming units) was spotted onto the surface of each plate. The plates were air dried and incubated at 37°C in 5% CO_2

for 24 h. The MIC was the lowest concentration of antibiotic that inhibited colony formation completely.

Transformation. Transformation was performed in liquid GCBB, and the results were assayed by using double agar overlays, as described previously (6). Several concentrations of penicillin were incorporated into the plates, and the transformants were tested against these levels. Rather sharp cutoffs were observed, with several hundred transformants at one penicillin level and essentially no colonies at a twofold-higher level. Analogous to the findings of Maier et al. (14) and Sparling et al. (25), we found several levels of penicillin resistance (see Table 1 for derivation and properties of the transformant cells).

Penicillin-binding assays. (i) In vivo assay. Exponentially growing cultures of gonococci (approximately 2×10^8 cells per ml) in GCBB were exposed in 1-ml volumes to [^{3}H]penicillin at varying concentrations for 15 min at 37°C. Each reaction was terminated with 10 μl of a 20% Sarkosyl (NL-97) detergent solution. This treatment rapidly terminated penicillin binding and lysed the cells. After incubation at room temperature for 5 min, an equal volume of acetone was added to each suspension, and the mixtures were placed at 4°C for 20 min. The precipitates were collected by centrifugation at 3,500 × *g* for 10 min, the supernatant fluids were removed, and the pellets were dried in a stream of air. The pellets were solubilized in 75 μl of sample buffer (3) and boiled for 5 min. The entire sample volume was loaded onto a polyacrylamide gel and electrophoresed, as described below.

(ii) In vitro assay (membrane binding). Cells were grown in 500-ml volumes of GCBB containing IsoVitaleX and bicarbonate at 37°C with agitation. When the cultures reached an N of 400 to 500 (Nephelos units; 3×10^8 cells per ml), the cells were collected by centrifugation at 10,000 × *g* for 10 min at 4°C. The cells were washed twice with ice-cold 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, pH 7.4. The cell pellets were frozen in a dry ice-acetone bath and stored overnight at −70°C. Cell membranes were prepared by rupture in a French pressure cell (12,000 lb/in^2; Aminco) and differential centrifugation, as described by Walstad et al. (31). The initial 45,000 × *g* pellet, which had a white color, contained approximately 65% of the outer membrane, as measured by determination of the amount of 2-keto-3-deoxyoctonate per milligram of protein. The markedly red membrane pellet (225,000 × *g*) was washed once, suspended in 500 μl of 10 mM phosphate buffer (pH 6.8) and stored frozen at −70°C. All membrane preparations of penicillin-susceptible and -resistant strains were prepared in parallel. The protein yields were virtually identical for these matched samples.

Thawed membranes (200 μg of protein) were added to different [^{3}H]penicillin concentrations in 10 mM phosphate buffer (pH 6.8) to give a total volume of 100 μl. The samples were incubated for 15 min, and the reactions were terminated by adding 10 μl of 20% Sarkosyl. (Early experiments included addition of a 500-fold excess of unlabeled penicillin; however, subsequent work demonstrated that Sarkosyl was a highly efficient reaction quencher by itself and that addition of excess penicillin was not necessary.)

The samples were centrifuged at 40,000 × *g* for 40 min, and 50 μl of each supernatant fluid was removed and added to sample buffer containing mercaptoethanol (8); these mixtures were then boiled for 2 min and loaded onto polyacrylamide gels.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The system used was essentially that of Laemmli (10), as described for slab gels by Ames (1) (5% stacking gel, 10% separating gel; ratio of acrylamide to bisacrylamide, 30:0.8). The gels were stained in a Coomassie blue solution, destained with multiple changes of methanol–acetic acid–water, photographed, and prepared for fluorography by the method of Bonner and Laskey (2). The PPO (2,5-diphenyloxazole)-impregnated gels were dried with a Bio-Rad model 224 gel dryer and exposed to presensitized Kodak X-Omat R X-ray film (11) for 5 to 8 days at −70°C. Gels were scanned by using a Gelman ACD-15 computing densitometer. Molecular weights were determined by using known proteins as standards (33).

Assays. Protein was determined by the method of Lowry et al. (13), using bovine serum albumin as the standard. Determination of 2-keto-3-deoxyoctonate was by the method of Osborn et al. (18). Authentic 2-keto-3-deoxyoctonate (Sigma Chemical Co., St. Louis, Mo.) was used as a standard. Tests for β-lactamase production were performed by using the chromogenic cephalosporin nitrocefin (Glaxo Laboratories, Greenford, England). A penicillinase-producing *N. gonorrhoeae* strain gave a strongly positive test within 2 min. All resistant strains in this study were negative, even with prolonged (1-h) incubations.

Antibiotics and reagents. [4-^{3}H]benzylpenicillin (ethylpiperidinium salt; 25 Ci/mmol) was the kind gift of E. O. Stapley, Merck Sharp & Dohme, Rahway, N.J.

The [^{3}H]benzylpenicillin used in this study reliably labeled the PBPs of *Escherichia coli* and gave the same pattern as previously reported (26). The high specific activity of this antibiotic allowed a substantial reduction in fluorograph exposure compared with the time used with [^{14}C]penicillin; it also allowed us to label growing cells in vivo. Benzylpenicillin (potassium salt) was obtained from Eli Lilly & Co., Indianapolis, Ind. All reagents used for electrophoresis were purchased from Bio-Rad Laboratories, Rockville Center, N.Y. All other chemicals were commercially available reagent grade.

RESULTS

Some general properties of the gonococcal PBPs. Membranes of *N. gonorrhoeae* N.Y.H. strain 52 were exposed to 0.5 μg of [^{3}H]-penicillin, and the reaction between the antibiotic and the binding proteins was terminated by adding excess unlabeled penicillin and 2% Sarkosyl (26). Three major PBPs were detected after separation by gel electrophoresis and fluorography (Fig. 1). Pretreatment of membranes with either excess unlabeled penicillin (ethylpiperidinium or potassium salt), 2% Sarkosyl, or trypsin (100 μg/ml for 10 min) abolished subsequent binding of labeled penicillin. The apparent molecular weights of the PBPs determined by sodium dodecyl sulfate gel electrophoresis were

90,000 (PBP 1), 63,000 (PBP 2), and 48,000 (PBP 3).

Turnover of the PBP-penicillin complex by deacylation was studied by incubating membranes with 0.5 μg of [^{3}H]penicillin for 15 min and then adding a thousandfold excess of unlabeled penicillin. At intervals, samples were removed and treated with 2% Sarkosyl. No significant loss of label as measured by densitometry was observed over a 90-min chase period (data not shown). The addition of either 2-mercaptoethanol or neutral hydroxylamine did not appear to stimulate turnover.

Membranes were incubated with [^{3}H]penicillin for 1, 2, 5, 10, 15, and 20 min. An exposure time of 15 min was necessary to saturate the PBPs at 37°C, and at lower temperatures of incubation (30 and 20°C) longer exposure times were necessary for saturation. Figure 2 shows the saturation kinetics at selected time points.

Previous reports in the literature (16, 20) suggested that the PBPs of gonococci may vary from strain to strain. In contrast, we found that the PBP patterns of the strains that were routinely used were highly reproducible. To study this point further, a number of local strains, as well as isolates from geographically diverse areas, were subjected to in vivo PBP labeling. Figure 3 shows that the profiles of the binding proteins did not vary in the isolates tested. *Neisseria* strains taxonomically related to gonococci also showed a similar number of PBPs, and the electrophoretic mobilities of these PBPs were similar to the mobilities of *N. gonorrhoeae* PBPs.

PBPs of susceptible and intrinsically resistant gonococcal isolates. A comparison of a penicillin-susceptible strain (FA 19; MIC, 0.007 μg/ml) and a penicillin-resistant strain (CDC 77-124615; MIC, 2.0 μg/ml) of *N. gonorrhoeae* was performed to measure possible differences in the binding of radioactive penicillin to the PBPs. The assays were performed in the following two ways: (i) with logarithmically growing, intact cells (in vivo labeling) and (ii) with isolated crude plasma membrane preparations (see above). Cells and membranes were exposed to a wide range of concentrations of [^{3}H]penicillin. Figures 4 and 5 illustrate the results. It is clear that labeling of PBPs 1 and 2 of the resistant cells required substantially higher antibiotic concentrations than labeling of the corresponding binding proteins of the susceptible cells. Since this was observed both by in vivo labeling and by labeling the PBPs of isolated membranes, the changes probably represented a decrease in the penicillin affinities of PBPs 1 and 2.

An additional change involving PBP 3 was also observed. This binding protein appeared to be more strongly labeled in the membrane prep-

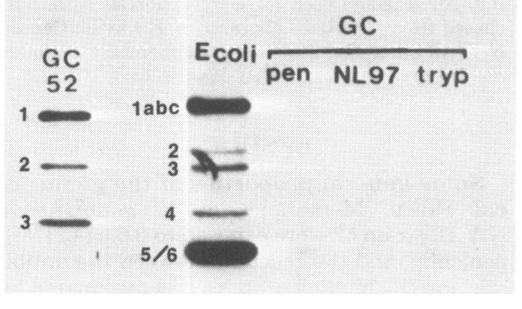

FIG. 1. *General properties of gonococcal PBPs. A 100-μl volume of cell membranes (200 μg of protein) from N.Y.H. strain 52 (MIC, 0.25 μg/ml) was treated with 5 μg of benzylpenicillin (lane 3), 2% Sarkosyl NL-97 (lane 4), or 10 μg of trypsin (lane 5). Then these preparations were incubated along with untreated strain 52 (lane 1) with 0.5 μg of [^{3}H]benzylpenicillin for 15 min. An E. coli membrane preparation was included to verify the [^{3}H]penicillin label (lane 2).*

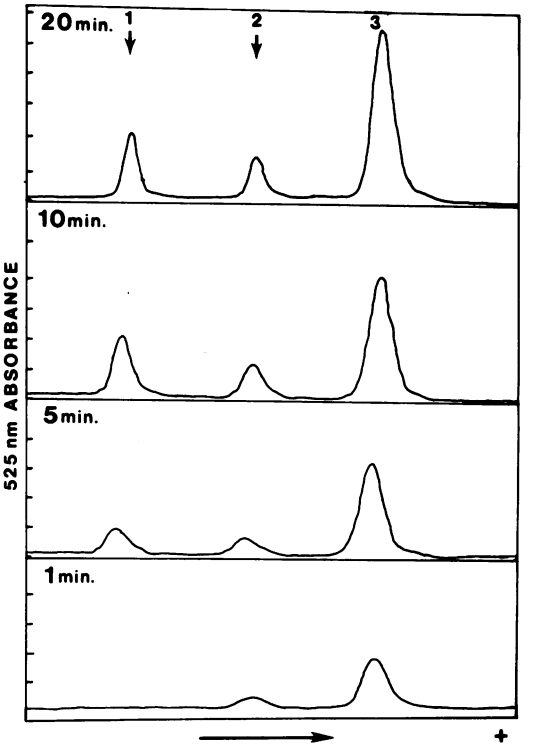

FIG. 2. *Kinetics of penicillin binding at 37°C. Membranes of strain N.Y.H. 52 were incubated with [^{3}H]benzylpenicillin for the times indicated, and the reactions were terminated by adding 2% Sarkosyl. After electrophoresis and fluorography, the gels were scanned at 525 nm with a Gelman ACD-15 densitometer. Units are relative optical density.*

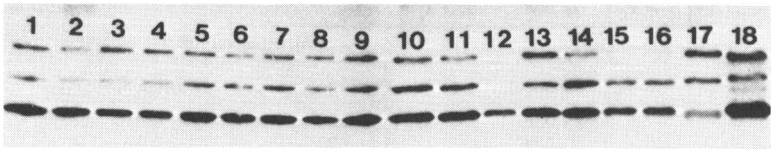

FIG. 3. *PBPs of gonococci from different sources. Cultures of N. gonorrhoeae were grown in GCBB and exposed to 2 μg of [^{3}H]penicillin for 15 min using the in vivo assay. Strains and sources (lanes 1 through 18, respectively) were as follows: N.Y.H. strains 59 (disseminated gonococccal infection isolate), 1563, 1355, 4408B, 846, 1939, and 4408 (all endocervical isolates from The New York Hospital); FA 19 pen B (transformant of FA 19); FA 19 (P. F. Sparling); 8403, 8404, and 8405 (Jacques Acar, Paris, France); M1 and M2 (L. de Repentigny, Montreal, Canada); CDC 77-124615 and CDC 78-002802 (B. W. Catlin, Milwaukee, Wis.); N. meningitidis (E. C. Gotschlich, Rockefeller University); and N. sicca (American Type Culture Collection). Quantitative differences in penicillin binding reflect the degree of resistance of a given strain.*

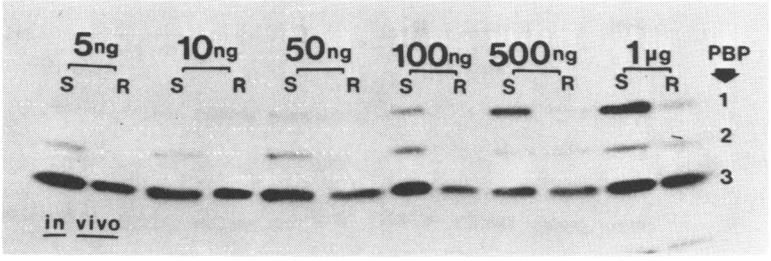

FIG. 4. *Binding of [^{3}H]penicillin to growing cells (in vivo assay). Actively growing cultures (1 ml) of FA 19 and CDC 77 were exposed to the indicated concentrations of [^{3}H]benzylpenicillin for 15 min at 37°C. The reaction was terminated with Sarkosyl, and the cells were processed as described in the text. Lanes S, FA 19 (penicillin MIC, 0.007 μg/ml); lanes R, CDC 77 (penicillin MIC, 2.0 μg/ml).*

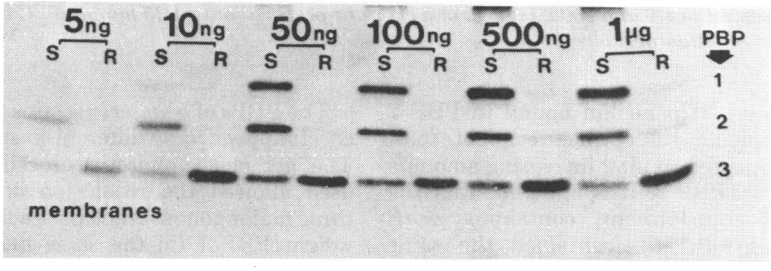

FIG. 5. *Binding of [^{3}H]penicillin to cell membranes. Crude cytoplasmic membranes (200 μg of protein) of FA 19 (lanes S) and CDC 77 (lanes R) were incubated for 15 min at 37°C with different [^{3}H]benzylpenicillin concentrations. Membranes were treated with Sarkosyl and prepared for electrophoresis and fluorography as described in the text.*

arations of resistant bacteria, whereas no differences were evident when PBP 3 of susceptible bacteria and PBP 3 of resistant bacteria were compared after in vivo labeling.

Although Figures 4 and 5 only document the case of a single penicillin-resistant isolate (CDC 77-124615; MIC for benzylpenicillin, 2.0 μg/ml), virtually identical changes were observed (data not shown) in the PBPs of four other resistant gonococcal clinical isolates (CDC 78-002802, CDC 78-067051, CDC 78-032868, and CDC 78-013076, kindly provided by C. Thornsberry, Center for Disease Control; MICs, between 1.0 and 8.0 μg of benzylpenicillin per ml).

PBPs in isogenic penicillin-resistant strains constructed by genetic transformation. Table 1 shows the origins and properties of penicillin-resistant strains constructed by genetic transformation, using the deoxyribonucleic acid of penicillin-resistant strain CDC 77 and strain FA 19 as the penicillin-susceptible recipient. Transformants representing three distinct levels of penicillin resistance (corresponding to MICs of 0.06, 0.12, and 0.5 μg of penicillin per ml) were selected and purified, and their PBPs were examined in membrane preparations. Figure 6 shows that the *penA*, *mtr*, and *penB* penicillin-resistant transformant classes

TABLE 1. *Properties of deoxyribonucleic acid donor, recipient, and transformant strains*[a]

Strain	MIC (μg/ml) of:			
	Penicillin	Erythromycin	Rifampin	Tetracycline
FA 19	0.007	0.30	0.07	0.15
TR1 *penA*	0.06	0.30	0.07	0.15
TR2 *penA mtr*	0.12	1.25	0.30	0.15
TR3 *penA mtr penB*	0.5	1.25	0.30	0.63
CDC 77-124615[b]	2.0	0.63	0.30	1.25

[a] Transformant strains were constructed by selection on penicillin plates. Deoxyribonucleic acid was extracted from CDC 77 and used at saturating concentrations in the transformation system. TR1 is CDC 77 × FA 19. TR2 is CDC 77 × TR1, and TR3 is CDC 77 × TR2. Levels of resistance to penicillin, erythromycin, rifampin, and tetracycline were measured on agar plates, and genetic loci were assigned on this basis (25).

[b] Deoxyribonucleic acid donor.

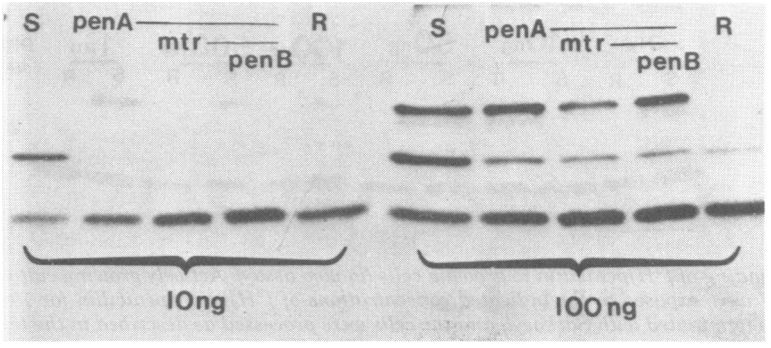

FIG. 6. *Binding of [^{3}H]penicillin to cell membranes prepared from penicillin-resistant transformants. Cytoplasmic membranes from FA 19, CDC 77, and the constructed transformants (Table 1) were incubated with labeled penicillin (10 and 100 ng/100 μl of membrane preparation) for 15 min at 37°C and prepared for electrophoresis and fluorography.*

had much less [^{3}H]penicillin bound to PBP 2 than the penicillin-susceptible recipient cells. They also appeared to bind increasing amounts of penicillin to PBP 3. It is apparent that the highest-level transformant, containing *penB* (MIC, 0.5 μg/ml), still contained the same amount of PBP 1 as the more susceptible strains. In contrast, CDC 77 (MIC, 2.0 μg/ml) exhibited a greatly reduced binding of [^{3}H]penicillin to PBP 1.

DISCUSSION

Membrane preparations of a number of gram-negative bacteria have been reported to yield PBP patterns of considerable similarity. Thus, strains of *E. coli* (15, 26), *Pseudomonas*, *Enterobacter*, *Klebsiella*, *Proteus* (5, 17), *Serratia* (26), and *Salmonella* (15) each have seven to nine major PBPs of similar although not identical molecular weight ranges. Each of the strains shows characteristic morphological responses to a group of beta-lactams, and these beta-lactams show selective affinity for one or more PBPs. Furthermore, low-molecular-weight PBPs (PBPs 5 and 6) exhibit rapid release of the penicilloyl group in each of the strains (29).

The PBPs of *Neisseria* strains (gram-negative cocci) appear to be different in several respects. The gel electrophoretic procedure which we used allowed the resolution of the following three major gonococcal PBPs: a high-molecular-weight PBP 1 (in the same molecular weight range as *E. coli* PBPs 1a, b, and c), PBP 2 (molecular weight close to that of *E. coli* PBP 3), and PBP 3 (similar in molecular weight to *E. coli* PBP 4). Our electrophoretic procedure applied to *E. coli* membranes (as controls) reproduced the usual pattern of PBPs, with PBPs 1a, b, and c and PBPs 5 and 6 unresolved (Fig. 1). The same three gonococcal PBPs were detected whether the labeling was performed in vitro (with membrane preparations) or in vivo (by labeling live bacteria with radioactive penicillin). An increase in the concentration of [^{3}H]penicillin above 5 μg/ml caused the appearance of additional radioactive bands on the fluorograms; most of these had molecular weights lower than the molecular weight of gonococcal PBP 3. However, all of these extra bands seemed to be due to nonspecific labeling since they were not eliminated by pretreatment of the gonococcal mem-

branes with excess nonradioactive penicillin or by denaturation with detergent or heat. As expected, all of these treatments eliminated the three "true" gonococcal PBPs (Fig. 1). For these reasons the maximum concentration of [^{3}H]penicillin used in our studies was 2 μg/ml.

An additional difference between the PBPs of gonococci and those of the other gram-negative bacteria seems to be the relative stability of the penicilloyl derivatives of gonococcal PBPs, inasmuch as no detectable turnover was observed after a 90-min incubation with unlabeled penicillin. The addition of thiol reagents or nucleophilic donors did not stimulate enzymatic deacylation of any of the PBPs, in contrast to the findings reported in other systems (9). The extreme stability of the penicillin-enzyme complex will simplify studies of PBP resynthesis in vivo after brief penicillin exposures.

The same PBP pattern was observed in a number of isolates deliberately selected from geographically diverse sites. One of the isolates tested belonged to the group of gonococci capable of causing disseminated infections. Similarly, preparations from the taxonomically related species *Neisseria menigitidis* and *Neisseria sicca* yielded PBP patterns similar to the pattern of gonococci. We did not observe strain-to-strain variation in the PBP pattern (16); differences in the details of the methodologies used (growth of cells, electrophoretic procedure) may account for this.

Rodriguez and Saz have reported that intrinsically penicillin-resistant gonococcal isolates and membranes prepared from such bacteria bind less radioactive penicillin than susceptible cells (19, 20). In the experiments described above, actively growing penicillin-susceptible and -resistant cells were exposed to [^{3}H]penicillin; substantial reductions in the binding of radioactive penicillin to PBPs 1 and 2 of the resistant bacteria were observed. The same results were obtained when membranes isolated from the resistant gonococci were used, implying that the outer membrane penetration barrier (present during in vivo studies but not in membranes) is not the basis of the observed decreases in labeling. Results of titration experiments (Fig. 5) suggested that the decreased penicillin binding was caused by decreased affinities of PBPs 1 and 2 for the antibiotic. Further studies will be needed to test whether a decrease in the cellular concentration of these binding proteins may not also contribute to the observed decreased labeling of PBPs 1 and 2.

Further investigations were made by comparing the PBPs of isogenic strains constructed by genetic transformation. The studies with transformants allowed us to tentatively associate the decreases in the penicillin affinities of PBPs 1 and 2 with two of the multiple genetic events that are known to control penicillin resistance in this bacterium (25). A decrease in the penicillin affinity of PBP 2 was first noted in genetic transformants that acquired low-level resistance (increase in the MIC from 0.007 to 0.06 μg/ml) controlled by the *penA* locus. *penA* mutants are also known to have a small increase in murein cross-linking (7, 21). No further change in PBP 2 affinity was observed in transformants of the next higher level of resistance (MIC, 0.12 μg/ml; controlled by the *mtr* locus).

Our data indicate that membranes isolated from transformants exhibiting the second and third level of penicillin resistance (*mtr* and *penB*) possess increased amounts of PBP 3. The interpretation of this finding is not clear since the apparent increase in the labeling of PBP 3 was not noticed during in vivo labeling of resistant bacteria. The increased labeling may have been due to the relatively poor recovery of this binding protein during the preparation of the membranes from the penicillin-susceptible bacteria. An alternative explanation (namely, the selective increase in the cellular concentration of PBP 3 in the resistant bacteria) cannot be excluded at present. Such an increase might be masked during in vivo labeling due to the presence of a superimposed change in the permeability barrier.

The decreased affinity of PBP 1 observed in the highly resistant cells (MIC, 2.0 μg/ml) must occur during acquisition of resistance levels higher than an MIC of 0.5 μg/ml, since transformants showing the third level of resistance still had normal PBP 1 affinity. The recently described loci *pem* and *tem*, which modify the expression of penicillin resistance, may be involved in the change in PBP 1 affinity (32). We are currently constructing transformants carrying these loci.

The changes observed in the PBPs of penicillin-resistant gonococci are reminiscent of similar alterations described recently in the penicillin-resistant pneumococci, as well as in cloxacillin-resistant mutants of *Bacillus subtilis* (3, 8, 35). It seems that in gram-negative bacteria intrinsic beta-lactam resistance has so far always been associated primarily with changes in outer membrane permeability (12, 36), although a similar instance of decreased affinity in PBP2 has been reported in *E. coli* (27). It is clear from prior studies that mutations at the *mtr* locus and possibly the *penB* locus can cause decreased permeability of gonococcal cells to antibiotics (14, 25). However, Scudamore et al. have measured the permeability properties of both *E. coli* (22) and *N. gonorrhoeae* (23). They found that,

in contrast to *E. coli*, gonococci possess a relatively poor permeability barrier to penicillin in the penicillin-resistant mutants. They postulated that factors in addition to an outer membrane permeability barrier were responsible for the increased resistance observed. The observed decreases in PBP affinities appear to be another mechanism that contributes to intrinsic β-lactam resistance in *N. gonorrhoeae*.

ACKNOWLEDGMENTS

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