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Binding of Progesterone to *Neisseria gonorrhoeae* and Other Gram-Negative Bacteria

RICHARD D. MILLER AND STEPHEN A. MORSE*

Department of Microbiology and Immunology, University of Oregon Health Sciences Center, Portland, Oregon 97201

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The binding of [1,2-^{3}H]progesterone to progesterone-sensitive *Neisseria gonorrhoeae* CS-7 and the progesterone-insensitive *Neisseria mucosa*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium* (rough and smooth strains) was investigated. The kinetics of binding to *N. gonorrhoeae* CS-7 demonstrated that the majority of the progesterone binding occurred and equilibrium was reached within the first 30 min. Despite the rapid binding of progesterone, only about 20% of the added steroid was bound at the cell concentration used throughout this study. Whole cells of progesterone-insensitive bacteria bound progesterone less efficiently than the progesterone-sensitive *N. gonorrhoeae* CS-7. *N. mucosa* bound low amounts of this steroid (20% of that bound by *N. gonorrhoeae* CS-7) whereas the other gram-negative bacteria exhibited little progesterone binding (<3% of that bound by *N. gonorrhoeae* CS-7). The outer membrane permeability of *N. gonorrhoeae* CS-7, as measured by crystal violet uptake and inhibition, was similar to the deep rough mutant of *S. typhimurium* TA 1535. The latter organism neither bound nor was inhibited by progesterone. However, isolated cell envelopes of *N. gonorrhoeae* and progesterone-insensitive bacteria all bound progesterone equally well. Cortisone and cholesterol, although structurally similar to progesterone, were not inhibitory to *N. gonorrhoeae* and did not bind to whole cells as well as progesterone. The major site of progesterone binding appeared to be the cytoplasmic membrane, which bound four times more progesterone than the outer membrane. In addition, isolated cytoplasmic membrane proteins bound more than three times more progesterone per milligram of protein than the intact membrane.

Gonadal steroids and related azasteroids inhibit the growth of gram-positive bacteria, but generally do not inhibit the growth of gram-negative bacteria (4, 21, 23, 28, 29). Recently, Morse and Fitzgerald (13) demonstrated that the growth of several species of the genus *Neisseria*, particularly *N. gonorrhoeae* and *N. meningitidis*, was inhibited by progesterone despite their classification as gram-negative bacteria. These authors suggested that inhibition was due, in part, to an inhibition of electron transport. However, the differences in sensitivity between *N. gonorrhoeae* and other gram-negative bacteria have not been explained. The purpose of the present investigation was to study the binding of progesterone and related steroids to *N. gonorrhoeae* and other gram-negative bacteria, and to determine the site of progesterone binding in the gonococcus.

MATERIALS AND METHODS

Organisms. *N. gonorrhoeae* strains CS-7 (type 4) and JW31 (type 4) were used in this study. The criteria for identification and specific properties of these strains were previously described (12, 14). *N. mucosa* was obtained from D. Kellogg, Center for Disease Control, Atlanta, Ga. *Salmonella typhimurium* DB-21, a prototrophic strain of LT-2, was obtained from D. Botstein, Massachusetts Institute of Technology, Cambridge, Mass. *S. typhimurium* strain TA 1535 was obtained from B. N. Ames, University of California, Berkeley, Calif. *S. typhimurium* TA 1535 is a deep rough mutant of strain LT-2 (3). This mutant has an abbreviated outer membrane lipopolysaccharide, lacking all those components exterior to the 2-keto-3-deoxyoctonate core. *Pseudomonas aeruginosa* PS-7 was obtained through the courtesy of H. Fisher, Portland State University, Portland, Ore. *Neisseria* spp. were stored and maintained as previously described (12, 14). *S. typhimurium* DB-21 and *P. aeruginosa* PS-7 were maintained on Trypticase soy agar (Difco) slants stored at 4°C. *S. typhimurium* TA 1535 was stored at −70°C in nutrient broth containing 0.5% NaCl and 7.5% dimethyl sulfoxide.

Media and growth conditions. The basal medium contained the following per liter: proteose peptone no. 3 (Difco), 15 g; K_2HPO_4, 4 g; KH_2PO_4, 1 g; NaCl,

5 g; and soluble starch, 1 g. The final pH of the medium was 7.2. Glucose (5 g/liter), $NaHCO_3$ (42 mg/liter), and a growth factor supplement, identical in composition to IsoVitalex enrichment (BBL) but lacking glucose, were added after autoclaving.

A 1% inoculum of a suspension of cells from an overnight culture was used to inoculate the growth medium. All liquid cultures were incubated at 37°C in a model G-76 gyratory water bath shaker (New Brunswick Scientific Co., New Brunswick, N.J.).

Chemicals and radioisotopes. Progesterone (Δ^4-pregnen-3,20-dione), 2,5-diphenyloxazole, phenazine methosulfate (PMS), 2,5-diphenyl-3-(3,4-dimethyl-2-thiazolyl)monotetrazolium bromide (thiazolyl blue), Tween 80, Pronase, and tris(hydroxymethyl)aminomethane (Tris) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Sodium deoxycholate was from ICN Pharmaceuticals, Inc. (Cleveland, Ohio). [1,2-^{3}H]progesterone (specific activity 55.7 Ci/mmol), [1,2-^{3}H]cortisone (specific activity 43.6 Ci/mmol) and [1,2-^{3}H]cholesterol (specific activity 60 Ci/mmol) were products of New England Nuclear Corp. (Boston, Mass.). All other reagents were of analytical grade and obtained from standard commercial sources.

Analytical procedures. Protein was assayed by the method of Lowry et al. (11) with bovine serum albumin as standard. Total lipid phosphorus was measured by the procedure of Ames and Dubin (2). Samples (0.01 to 0.1 ml) were added to 0.05 ml of 10% $Mg(NO_3)_2 \cdot 6H_2O$ and ashed over a hot flame. After cooling, 0.6 ml of 0.5 N HCl was added and the samples were boiled for 15 min. Inorganic phosphate was determined by addition of 1 part 10% ascorbic acid and 6 parts 0.42% $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in 1 N H_2SO_4. Samples were then incubated at 37°C for 60 min and the absorbancy at 800 nm was measured in a Beckman Acta CIII spectrophotometer. Unknown values were calculated from a standard curve prepared with KH_2PO_4. Heptose was determined by the cysteine-H_2SO_4 procedure as described by Osborn (16). Radioactivity of samples (0.1 ml) was measured in a liquid scintillation spectrometer (Beckman Instrument Corp.) after addition of 15 ml of Tritosol scintillation fluid (17).

Miscellaneous measurements. Turbidity was measured by Klett-Summerson colorimetry at 540 nm. Dry weights were obtained by drying aliquots of preparations to constant weight in tared aluminum dishes at 80°C in a vacuum oven. Buoyant densities of the sucrose gradient fractions were determined by weighing 10-μl samples in tared micropipettes (Dade Diagnostics, Inc., Miami, Fla.).

Enzyme assays. Succinate dehydrogenase (EC 1.3.99.1) and D-lactate dehydrogenase (EC 1.1.1.28) were measured by coupling the enzymes via PMS to the reduction of thiazolyl blue. Incubation mixtures contained 60 mM phosphate buffer (pH 7.5), 10 mM NaCN, 30 μg of thiazolyl blue, 10 μg of PMS, 25 mM succinate or 2.5 mM D-lactate, and membrane-rich sample (5 to 100 μg of protein) in a volume of 1.0 ml. Increase in the absorbance at 550 nm was followed in a Beckman model 25 recording spectrophotometer at 25°C.

Crystal violet uptake and sensitivity. Log-phase cultures were harvested by centrifugation at 4°C (10,000 × *g*, for 15 min) and suspended in fresh basal medium at a turbidity of 275 Klett units (approximately 1 mg [dry wt]/ml). Crystal violet (1 mg/ml in 95% ethanol) was added to the cells to a final concentration of 2.5 μg/ml, and the suspension was incubated at 37°C for 15 min. After centrifugation (10,000 × *g* for 15 min), the optical density (590 nm) of the supernatant was measured. The amount of dye remaining in the supernatant was calculated from a standard curve prepared with known concentrations of crystal violet. The relative sensitivity of the organisms to crystal violet was determined by spreading 0.1 ml of a log-phase culture over the surface of a GC agar (Difco) plate. After drying for 30 min at 37°C, a filter paper disk (6.35 mm in diameter) containing 10 μg of crystal violet was placed on the surface of the agar. The diameter of the zone of inhibition surrounding the disk was measured after 24 h of incubation at 37°C in a CO_2 incubator (5% CO_2).

Binding of labeled steroids to cell suspensions. Late log-phase cultures were harvested by centrifugation at 4°C (10,000 × *g* for 10 min), washed once, and suspended to 470 Klett units (approximately 2 mg [dry wt]/ml) in 25 mM potassium phosphate buffer (pH 7.2) containing 0.5% NaCl, 0.5% glucose, and 0.01% Tween 80. Aliquots (1 ml) were transferred to screw-top test tubes and warmed to 37°C. An equal volume of labeled steroid (0.1 μCi/ml diluted in the same buffer) was added to each tube, and incubation continued at 37°C. At 30-min intervals, tubes were removed and centrifuged at 1,200 × *g* for 15 min to sediment the cells. The radioactivity remaining in the supernatant was determined by liquid scintillation spectrometry.

Preparation of cell membrane. Cell envelopes were isolated by sonic disruption of whole cells. Stationary-phase cultures were harvested by centrifugation at 4°C (10,000 × *g* for 20 min), washed once, and suspended in 50 mM Tris-hydrochloride buffer (pH 7.4) containing 1 mM ethylenediaminetetraacetic acid (EDTA). Cells were disrupted by sonic oscillation (Bronwill Biosonik IV, Rochester, N.Y.) in an ice bath for a total of 3 min in 30-s pulses, each followed by a 2-min cooling period. The resulting suspension was centrifuged (23,500 × *g* for 15 min) to remove unbroken cells and cell debris. The supernatant was then centrifuged at 100,000 × *g* for 90 min (SW41 rotor, Beckman model L5-65 ultracentrifuge). The membrane pellet was suspended in 50 mM Tris-hydrochloride buffer (pH 7.4) containing 1 mM EDTA, and dispersed by sonic oscillation for 3 to 5 s prior to use.

Outer and inner membrane fractions were isolated by the method of Wolf-Watz et al. (26). Outer membrane components were removed from plasmolyzed cells by lysozyme-EDTA treatment and aggregated by lowering the pH to 5.0. These membranes were collected by centrifugation (10,000 × *g* for 10 min) and washed four times in cold 5 mM EDTA. Spheroplasts formed as a result of the lysozyme-EDTA treatment were then osmotically shocked and sonically oscillated for 6 min to disrupt the remaining cells. The resulting suspension was centrifuged

at low speed (23,500 × g for 15 min) to remove unbroken cells. Cytoplasmic membranes were then collected from the resulting supernatant by centrifugation at 100,000 × g for 90 min. Both cytoplasmic and outer membrane preparations were further purified by isopycnic centrifugation on 15 to 65% (wt/wt) sucrose density gradients at 250,000 × g for 4 h (SW41 rotor, Beckman model L5-65 ultracentrifuge).

Lipid and protein depletion of cell membrane. Whole envelopes were depleted of lipid by chloroform-methanol (2:1) extraction of the membrane pellet. The extracted membrane suspension was centrifuged at 4°C (30,000 × g) for 1 h, and the extraction procedure was repeated. The final pellet was washed once and suspended in 50 mM Tris-hydrochloride buffer (pH 7.4) containing 1 mM EDTA, and then sonically oscillated briefly (1 to 5 s) to disperse the pellet. Cell envelopes were depleted of proteins by digestion with Pronase. Pronase (200 μg/ml) was added to a suspension of the whole cell envelopes in 50 mM Tris-hydrochloride buffer (pH 7.4) containing 1 mM EDTA. After incubation at 37°C for 3 h, the membranes were collected by centrifugation (30,000 × g for 1 h), and the resulting pellet was washed once before suspension in 50 mM Tris-hydrochloride buffer (pH 7.4) with 1 mM EDTA. These procedures resulted in the depletion of lipid or protein by approximately 80 and 65%, respectively.

Binding of progesterone to cell membranes. Cell membrane preparations (2 to 5 mg of protein per ml) were suspended in 50 mM Tris-hydrochloride buffer (pH 7.4) containing 1 mM EDTA and 0.01% Tween 80. Aliquots (1 ml) were transferred to glass centrifuge tubes and prewarmed to 37°C. An equal volume of labeled progesterone (0.1 μCi/ml in the same buffer) was then added to each tube. At intervals thereafter, tubes were removed and centrifuged at 30,000 × g for 1 h to remove the membranes. The radioactivity remaining in the supernatant was determined by liquid scintillation spectrometry.

Membrane solubilization by sodium deoxycholate. Whole cell envelope or cytoplasmic membrane preparations were solubilized by the addition of sodium deoxycholate (21 mg/mg of membrane protein) followed by incubation at 37°C for 3 to 6 h. The membrane proteins and lipids were then separated by gel filtration in a Bio-Gel A-0.5m (Bio-Rad Laboratories, Richmond, Calif.) column (1 by 30 cm) equilibrated with 0.05 M sodium carbonate buffer (pH 9.6) containing 10 mM sodium deoxycholate. Fractions (1 ml) were collected and analyzed for protein (absorbance 280 nm) and lipid phosphorus.

RESULTS

Binding of [1,2-^{3}H]progesterone to cell suspensions of *N. gonorrhoeae*. The rate of progesterone binding (Fig. 1) was rapid at 37°C and virtually complete within the first 30 min. Since the kinetics of binding were similar for all three cell concentrations tested, a cell concentration of 470 Klett units (approximately 2 mg [dry wt]/ml) was used in all subsequent studies. The effect of varying the concentration of labeled progesterone is shown in Fig. 2. Typical binding kinetics for each of the four progesterone concentrations tested were observed (Fig. 2, insert). When the amount of progesterone bound was plotted as a function of the progesterone added (0 to 1.6 ng/mg [dry wt]), a linear relationship was observed. It should be noted that, although the binding was very rapid in these studies, the data from Fig. 2 indicate that at a cell concentration of 470 Klett units only about 20% of the added progesterone was bound to the cells. Subsequent studies (data not presented) have shown that approximately half of the bound progesterone could be removed by washing the cells; however, the remaining progesterone (approximately 10% of the added progesterone) was tightly bound to the cells and could not be removed even after incubation for 3 h in the presence of high concentrations of unlabeled progesterone.

Binding of [1,2-^{3}H]progesterone to cell suspensions of progesterone-insensitive bacteria. The binding of [1,2-^{3}H]progesterone to several progesterone-insensitive bacteria (Fig. 3) indicates that a relationship exists between progesterone binding and progesterone inhibition of growth. *N. mucosa* bound significantly less progesterone than *N. gonorrhoeae* CS-7 although similar kinetics were observed. *P. aeruginosa* and *S. typhimurium* strains DB-21 and TA 1535 bound little, if any, progesterone.

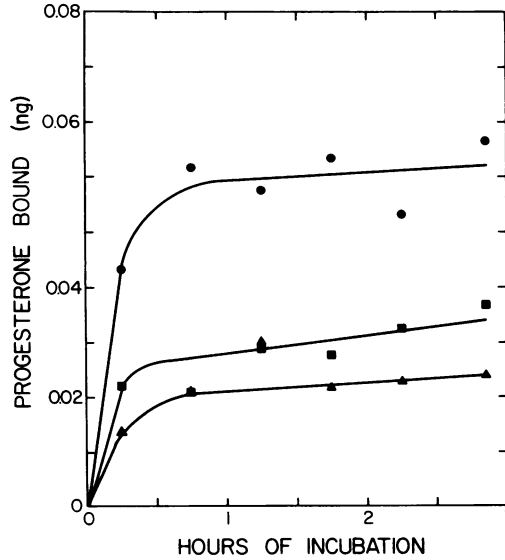

FIG. 1. *Binding of progesterone to N. gonorrhoeae CS-7 at different cell concentrations. (Solid circles) Original suspension of 470 Klett Units (approximately 2 mg [dry weight]/ml); (solid squares and triangles) 1:2 and 1:4 dilutions, respectively.*

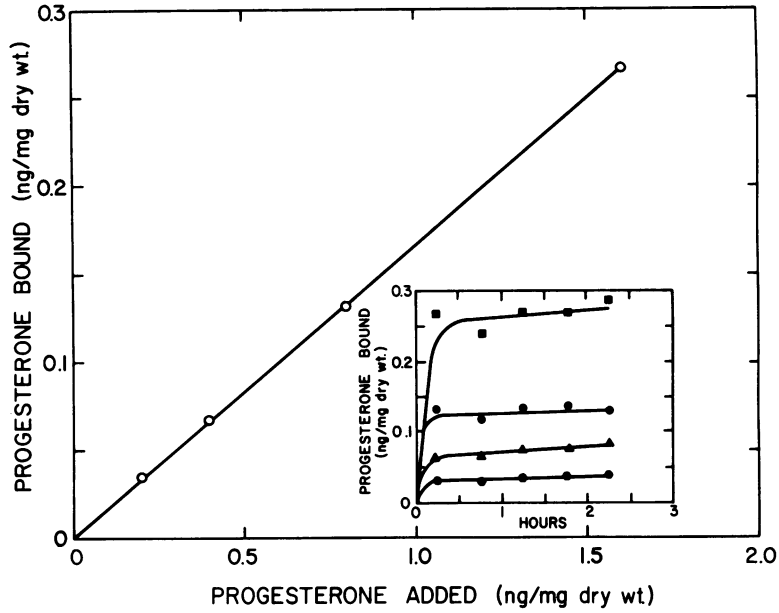

FIG. 2. *Binding of progesterone to N. gonorrhoeae CS-7 using different progesterone concentrations. Large graph shows progesterone bound as a function of progesterone added. Insert shows progesterone bound as a function of time. Symbols: ■, 1.6; ●, 0.8; ▲, 0.4; ⬢, 0.2 ng of progesterone per mg (dry weight), respectively.*

Comparison between crystal violet uptake and inhibition and progesterone binding and inhibition. The resistance of gram-negative organisms to many antibiotics and dyes such as crystal violet has been attributed to the outer membrane permeability barrier (6). Outer membrane mutants with abbreviated lipopolysaccharide chains (rough strains) often have increased permeability to these agents (1, 9, 10). A comparison between crystal violet uptake and inhibition and progesterone binding and inhibition for *N. gonorrhoeae* and other gram-negative bacteria is shown in Table 1. *N. gonorrhoeae* CS-7 exhibited significant crystal violet uptake and inhibition and was sensitive to progesterone. *P. aeruginosa* and *S. typhimurium* DB-21 showed no inhibition by crystal violet and bound only 30% of the added dye, which has been shown to remain bound to the cell surface (9). Both of these organisms were insensitive to progesterone. *S. typhimurium* TA 1535 was very permeable and sensitive to inhibition by crystal violet. However, neither binding nor growth inhibition by progesterone was observed.

Binding of [1,2-^{3}H]progesterone to isolated cell envelopes. The binding of [1,2-^{3}H]progesterone to isolated cell envelopes (1 to 2 mg of protein per ml) of *N. gonorrhoeae* and several progesterone-insensitive bacteria is shown in Table 2. Within experimental variation, there

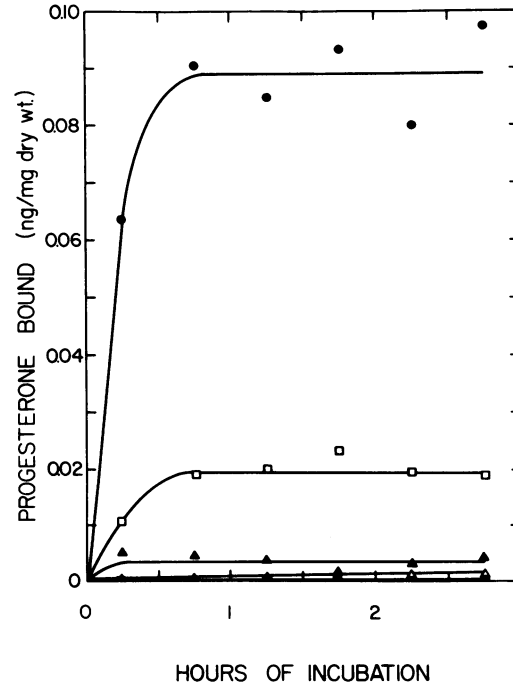

FIG. 3. *Comparison of binding of progesterone to N. gonorrhoeae CS-7 and other gram-negative organisms. Symbols: ●, N. gonorrhoeae CS-7; □, N. mucosa; ▲, P. aeruginosa; △, S. typhimurium TA 1535; ■, S. typhimurium DB-21.*

TABLE 1. *Comparison between crystal violet uptake and inhibition and progesterone binding and inhibition*

Organism	Crystal violet		Progesterone	
	Uptake (%)[a]	Zone of Inhibition (cm)[b]	Binding[c]	Inhibition (% of control)[d]
Neisseria gonorrhoeae CS-7	75	1.49	0.089	3
N. mucosa	50	0.71	0.019	95
Pseudomonas aeruginosa	ND[e]	0	0.003	100
Salmonella typhimurium DB-21	30	0	0.001	100
S. typhimurium TA 1535	88	0.96	0.001	98

[a] Percent of dye added; 15 min of incubation; 37°C.
[b] Diameter of zone of inhibition; filter paper disk containing crystal violet (10 μg).
[c] [1,2-^{3}H]progesterone bound (nanograms per milligram [dry weight]).
[d] Percent of control turbidity; progesterone concentration, 40 μg/ml.
[e] ND, Not determined.

TABLE 2. *Binding of [1,2-^{3}H]progesterone to cell envelopes of N. gonorrhoeae and other gram-negative organisms*

Organism	Progesterone bound		
	μg/mg of protein[a]	%[b]	% after washing[c]
Neisseria gonorrhoeae CS-7	0.154	28	14
N. mucosa	0.132	26	19
Pseudomonas aeruginosa PS-7	0.142	28	19
Salmonella typhimurium DB-21	0.146	29	20
S. typhimurium TA 1535	0.135	26	18

[a] Average of five determinations.
[b] Percent of total progesterone added.
[c] Cell envelopes were washed twice with 50 mM Tris-hydrochloride buffer (pH 7.4) containing 1 mM EDTA and 0.01% Tween 80.

was little difference in progesterone binding by *N. gonorrhoeae* and the progesterone-insensitive bacteria examined in this study. It should be noted that the kinetics of binding to isolated cell envelopes (data not shown) is similar to that of whole cells, and only about 20 to 30% of the added progesterone was bound (Table 2). As was observed with whole cells, approximately half of the progesterone bound to cell envelopes of *N. gonorrhoeae* could be removed by repeated washing of the membranes (Table 2). Progesterone-insensitive bacteria also gave similar results (Table 2), indicating that the binding to these membranes was not totally the result of loose, superficial binding.

Binding of [1,2-^{3}H]cortisone and [1,2-^{3}H]cholesterol. Cortisone and cholesterol, although structurally similar to progesterone, have no inhibitory effect on *N. gonorrhoeae* (unpublished data). Therefore, the binding of [1,2-^{3}H]cortisone and [1,2-^{3}H]cholesterol to cell suspensions of *N. gonorrhoeae* CS-7, *N. mucosa*, and *P. aeruginosa* was examined. [1,2-^{3}H]cortisone showed no binding to any of these organisms (data not shown), whereas binding of [1,2-^{3}H]cholesterol was observed (Fig. 4). The rate of [1,2-^{3}H]-cholesterol binding to *N. gonorrhoeae* was linear over the 150-min incubation period and slower than the rate of progesterone binding. The rate of [1,2-^{3}H]cholesterol binding to *N. mucosa* and *P. aeruginosa* was significantly slower than the rate observed with *N. gonorrhoeae*.

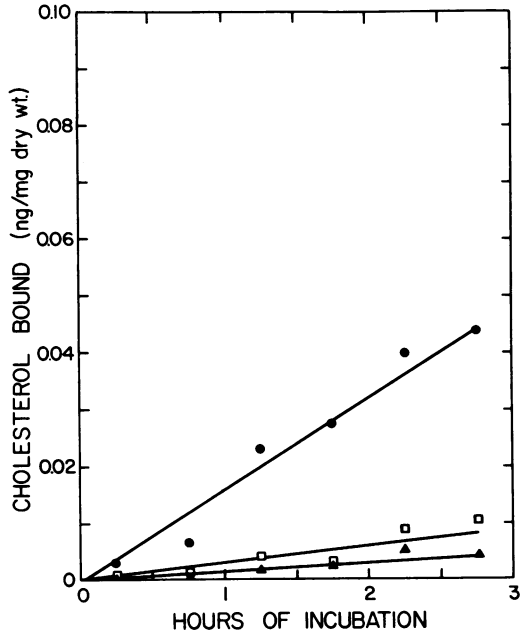

FIG. 4. *Comparison of binding of cholesterol to N. gonorrhoeae CS-7 and other gram-negative organisms. Symbols: ●, N. gonorrhoeae CS-7; □, N. mucosa; ▲, P. aeruginosa.*

Site of progesterone binding. Morse and Fitzgerald (13) determined that progesterone bound to the cell membrane. However, no dif-

ferentiation was made between outer and inner (cytoplasmic) membrane binding. To determine the location of the bound progesterone, the inner and outer membranes were isolated from cells of *N. gonorrhoeae* CS-7 previously labeled with [1,2-^{3}H]progesterone. These membrane preparations were centrifuged to equilibrium on linear sucrose density gradients (15 to 65%), and the radioactivity associated with each of the membranes was counted. These results are shown in Table 3. When compared on the basis of protein concentration, the cytoplasmic membrane bound four times more progesterone than did the outer membrane. Buoyant densities of these membranes were close to reported values. Lactate and succinate dehydrogenase activity (cytoplasmic membrane markers) and heptose analysis (outer membrane marker) demonstrated less than 5% cross contamination.

Progesterone is a hydrophobic molecule and can bind to either the lipid or protein portion of the membrane (5, 22, 24). To determine which component of the membrane was involved in progesterone binding, membranes (approximately 5 mg of protein per ml) were treated to reduce the concentration of lipid or protein. Whole membrane preparations depleted of 80% of their lipid by chloroform-methanol extraction or 65% of the membrane protein by Pronase digestion still bound progesterone (Table 4). In fact, Pronase-treated membranes showed a slight increase in binding over the untreated membranes. Progesterone bound to the untreated membranes after 2 h of incubation was not released with subsequent Pronase treatment (data not shown), indicating that progesterone does not bind to the more hydrophilic extrinsic membrane proteins.

Membrane proteins can be separated from their lipid matrix by treatment with a detergent. Using this approach, cytoplasmic membranes were solubilized in sodium deoxycholate and then chromatographed on a Bio-Gel A-0.5m column. The protein eluted as a major peak in the void volume (indicating a molecular weight of >500,000) followed by a smaller peak (Fig. 5). Lipid phosphorus was completely separated from the major protein peak, eluting as a single peak in fractions 10, 11, and 12. The pooled proteins from fractions 4, 5, and 6 were then tested for their ability to bind [1,2-^{3}H]progesterone. These large membrane proteins appeared as particulate aggregates in the elution buffer and were isolated by centrifugation and tested for progesterone binding according to the procedure described for whole membranes. The amount of progesterone bound to these mem-

TABLE 3. *Incorporation of [1,2-^{3}H]progesterone into outer and cytoplasmic membranes of N. gonorrhoeae CS-7*

Determination	Outer membrane	Cytoplasmic membrane
Progesterone bound (ng/ml of protein)	0.033	0.132
Buoyant density (g/cm)^3	1.246	1.189
Lactate dehydrogenase[a]	<0.1	5.46
Succinate dehydrogenase	1.42	29.47
Heptose analysis (μg/mg of protein)	2.5	<0.05

[a] All enzyme activities are expressed as nanomoles of thiazolyl blue reduced per minute per milligram of protein.

TABLE 4. *Binding of [1,2-^{3}H]progesterone to protein- and lipid-depleted membranes of N. gonorrhoeae JW 31*[a]

Membrane treatment	Progesterone bound (ng)	
	30 min	150 min
Untreated membrane	0.176	0.179
Pronase treatment[b]	0.215	0.229
Chloroform-methanol extraction[c]	0.159	0.172

[a] Amount of lipid in the Pronase-treated membrane suspension was adjusted to the amount in the untreated membrane, while the amount of protein in the lipid-depleted system was adjusted to the amount of protein in the untreated membrane suspension.

[b] Protein depleted by 65%.

[c] Lipid depleted by 80%.

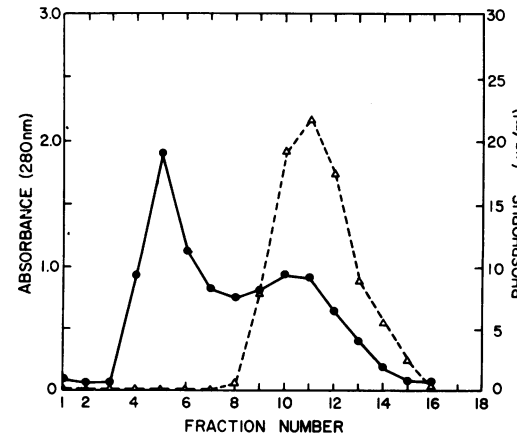

FIG. 5. *Separation of membrane protein and lipid on a Bio-Gel A-0.5m column (1 by 30 cm) equilibrated with 0.05 M sodium carbonate buffer (pH 9.6) containing 10 mM deoxycholate. Membranes were solubilized in deoxycholate (21 mg/mg protein) for 6 h at 37°C. Symbols: ●, absorbance at 280 nm; △, lipid phosphorus.*

brane proteins (0.13 ng/mg of protein) was more than three times greater than the intact membrane (0.04 ng/mg of protein).

DISCUSSION

These studies demonstrate that the binding of progesterone to cell suspensions of *N. gonorrhoeae* is a rapid process. The kinetics of binding indicate that the majority of the progesterone binding occurred and an equilibrium was reached by the time the first sample had been centrifuged. Yet, in spite of the rapid binding, only about 20% of the added progesterone was bound at the cell concentration used throughout the study. Approximately 50% of the bound progesterone appeared to be bound superficially and could be washed off the cells. The low percentage of bound progesterone was apparently a function of an equilibrium between bound and free progesterone and not due to a saturation of the binding sites. Increasing the concentration of labeled progesterone increased the amount bound, yet even at the highest concentration only 20% of the added progesterone was bound.

The low percent of progesterone bound may be due to the Tween 80 added to the binding buffer to prevent binding of the steroid to the glass surfaces. Gershfeld et al. (8) have shown that 0.01% Tween 80, while eliminating cholesterol adsorption to glass, also decreased the rate of binding of cholesterol to membranes of *Acholeplasma laidlawii*. At concentrations of 0.01%, Tween 80 exists almost completely as miscelles. The binding of progesterone represents a partition of these hydrophobic molecules between the Tween 80 miscelles and the hydrophobic region of the cytoplasmic membrane. The equilibrium that is reached would be independent of the progesterone concentration, but would depend upon the concentration of the cells or membranes. The progesterone concentrations used in this study were extremely low (<1 ng/ml). However, we have observed (unpublished data) that the 20% binding equilibrium exists even with inhibitory concentrations of progesterone (>10 μg/ml).

In the present study we have demonstrated that whole cells of progesterone-insensitive bacteria bind progesterone less efficiently than *N. gonorrhoeae*. Although *N. mucosa* bound low amounts of this steroid, the other gram-negative bacteria tested exhibited little, if any, progesterone binding.

Preliminary results suggested the possibility that differences in progesterone susceptibility were related to the permeability of the cell envelope. To evaluate this possibility, progesterone binding and inhibition was compared with crystal violet uptake and inhibition for each organism. Outer-membrane mutants of *E. coli* (9), *S. minnesota* (1), and *N. gonorrhoeae* (10) exhibit increased sensitivity to antibiotics and increased permeability to crystal violet. The progesterone-sensitive *N. gonorrhoeae* CS-7 exhibited a marked uptake and sensitivity to crystal violet, thus confirming the increased permeability of the outer membrane of *N. gonorrhoeae* over that of other gram-negative bacteria (25). However, the crystal violet-sensitive, deep rough mutant *S. typhimurium* TA 1535 exhibited neither progesterone sensitivity nor binding. These results would seem to suggest that the sensitivity to progesterone is not due to increased outer membrane permeability, at least as measured by crystal violet uptake and sensitivity. However, since crystal violet and progesterone differ considerably in their hydrophobicity, different mechanisms of uptake may be involved for a hydrophobic molecule such as progesterone than for the more hydrophilic crystal violet molecule. Nikaido (15) has postulated two mechanisms for transmembrane diffusion of molecules. Hydrophilic molecules would diffuse through aqueous pores, whereas more hydrophobic molecules would penetrate by dissolving into the hydrocarbon interior of the outer membrane. The binding of progesterone to isolated cell envelopes (Table 2) has shown that membranes of progesterone-insensitive bacteria bind progesterone as well as *N. gonorrhoeae*. These data indicate that the lack of progesterone binding by whole cells of progesterone-insensitive bacteria is due to the inability of this molecule to penetrate the outer membrane. The outer membrane of *S. typhimurium* TA 1535 which exhibits an increased permeability to crystal violet is still sufficiently impermeable to progesterone to prevent binding of this steroid.

Cortisone and cholesterol, although structurally similar to progesterone, are not inhibitory to the growth of *N. gonorrhoeae* and do not bind as well as progesterone. Cortisone, a slightly more polar molecule than progesterone, may be unable to penetrate the outer membrane, or perhaps may find no compatible areas of the cytoplasmic membrane to which it can bind. Cholesterol is able to bind to *N. gonorrhoeae* although at a much slower rate. The requirement for cholesterol of various mycoplasmas is well known (17). However, it has recently been shown that *Proteus vulgaris* and *Micrococcus lysodeikticus* are also capable of incorporating appreciable quantities of cholesterol into their membranes (18). Cholesterol may thus be capable of binding to limited areas of the mem-

branes of *N. gonorrhoeae* while causing no inhibition of growth.

The site of progesterone binding and the exact nature of growth inhibition in *N. gonorrhoeae* has not been adequately determined. Morse and Fitzgerald (13) observed inhibition of NADH oxidase and (cytochrome *b*) L-lactate dehydrogenase by progesterone in cell membrane preparations of *N. gonorrhoeae* and suggested that the steroid bound at or adjacent to membrane-bound enzymes involved in electron transport. Inhibition of electron transfer in mitochondria by progesterone has also been demonstrated in several studies (22, 24, 27). The results of the present study have shown conclusively that progesterone binds to the cytoplasmic membrane of *N. gonorrhoeae* CS-7. The limited binding of progesterone to the outer membrane of *N. gonorrhoeae* may be due to a lack of specific membrane receptors, or perhaps to observed physical differences between outer and cytoplasmic membranes of gram-negative bacteria such as a higher viscosity of the outer membrane lipid domain or the resistance of outer membrane phospholipids to extraction with aqueous acetone (20).

Whether progesterone binding in the gonococcus occurs to cytoplasmic membrane proteins or adjacent phospholipids has not been conclusively determined. In eucaryotic systems, progesterone has been shown to bind to either protein (5) or phospholipids (22, 24). Removal of 65% of the membrane protein or 80% of the membrane lipid did not decrease the binding of progesterone to membranes of *N. gonorrhoeae*. The slight increase in binding exhibited after Pronase treatment could be due to destruction of extrinsic hydrophilic regions of the membrane, thus exposing more hydrophobic regions to progesterone. Or perhaps these results merely reflect a selective digestion of the nonbinding hydrophilic regions of the membrane, thus increasing the progesterone-to-protein ratio. These data are in contrast to the work of Razin et al. (19) who studied cholesterol uptake in membranes of *A. laidlawii*. They reported that cholesterol uptake was unaffected by removal of 75% of the membrane protein by Pronase treatment, whereas lipid depletion by aqueous acetone extraction greatly reduced the amount of cholesterol taken up. The binding of progesterone to gonococcal membranes appears to be of a different nature.

Our results also show that isolated cytoplasmic membrane proteins which were separated from the lipid portion of the membrane by detergent solubilization and gel filtration bound progesterone three times more efficiently than the native membranes. Preliminary sodium dodecyl sulfate polyacrylamide gel electrophoresis of the solubilized membrane proteins (unpublished data) has shown a protein profile similar to that of the native membrane. It remains to be shown, however, whether binding to these membrane proteins, as shown in vitro, is an accurate representation of the binding that takes place in the intact organism. Studies to elucidate the specific protein or phospholipid binding sites for progesterone are in progress.

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LITERATURE CITED

1. **Alexanian, S., S. Schlecht, and O. Westphal.** 1974. Investigations on the effect of crystal violet on Salmonella R mutants. I. Communication: binding experiments. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. 226:207-214.
2. **Ames, B. N., and D. T. Dubin.** 1960. The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. J. Biol. Chem. 235:769-775.
3. **Ames, B. N., F. D. Lee, and W. E. Durston.** 1973. An improved bacterial test system for the detection and classification of mutagens and carcinogens. Proc. Natl. Acad. Sci. U.S.A. 70:782-786.
4. **Casas-Campillo, C., D. Balandrano, and A. Galarza.** 1961. Steroids CLIX. Antimicrobial properties of 21,21-dimethoxyl progesterone and other progesterone analogues. J. Bacteriol. 81:366-375.
5. **Ciavatti, M., G. Michel, B. Chataing, and A. Jouvenceaux.** 1974. Preservation of human erythrocyte membrane proteins during cold storage with addition of progesterone. Biochem. Med. 9:293-300.
6. **Costerton, J. W., J. M. Ingram, and K.-J. Cheng.** 1974. Structure and function of the cell envelope of gram-negative bacteria. Bacteriol. Rev. 38:87-110.
7. **Fricke, V.** 1975. Tritosol: a new scintillation cocktail based on Triton X-100. Anal. Biochem. 63:555-558.
8. **Gershfeld, N. L., M. Wormser, and S. Razin.** 1974. Cholesterol in mycoplasma membranes. I. Kinetics and equilibrium studies of cholesterol uptake by the cell membrane of *Acholeplasma laidlawii*. Biochim. Biophys. Acta 352:371-384.
9. **Gustafsson, P., K. Nordstrom, and S. Normark.** 1973. Outer penetration barrier of *Escherichia coli* K-12: kinetics of the uptake of gentian violet by wild-type and envelope mutants. J. Bacteriol. 116:893-900.
10. **Guymon, L. F., and P. F. Sparling.** 1975. Altered crystal violet permeability and lytic behavior in antibiotic-resistant and -sensitive mutants of *Neisseria gonorrhoeae*. J. Bacteriol. 124:757-763.
11. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
12. **Morse, S. A., and L. Bartenstein.** 1974. Factors affecting autolysis of *Neisseria gonorrhoeae*. Proc. Soc. Exp. Biol. Med. 145:1418-1821.
13. **Morse, S. A., and T. J. Fitzgerald.** 1974. Effect of progesterone on *Neisseria gonorrhoeae*. Infect. Immun. 10:1370-1377.
14. **Morse, S. A., S. Stein, and J. Hines.** 1974. Glucose

metabolism in *Neisseria gonorrhoeae*. J. Bacteriol. 120:702–714.

15. **Nikaido, H.** 1976. Outer membrane of *Salmonella typhimurium* transmembrane diffusion of some hydrophobic substances. Biochim. Biophys. Acta 433:118–132.
16. **Osborn, M. J.** 1963. Studies on the gram-negative cell wall. I. Evidence for the role of 2-keto-3-deoxyoctonate in the lipopolysaccharide of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. U.S.A. 50:499–506.
17. **Razin, S.** 1975. The mycoplasma membrane, p. 257–312. *In* J. F. Danielli, M. D. Rosenberg, and D. A. Cadenhead (ed.), Progress in surface and membrane science, vol. 9. Academic Press Inc., New York.
18. **Razin, S.** 1975. Cholesterol incorporation into bacterial membranes. J. Bacteriol. 124:570–572.
19. **Razin, S., M. Wormser, and N. L. Gershfeld.** 1974. Cholesterol in mycoplasma membranes. II. Components of *Acholeplasma laidlawii* cell membranes responsible for cholesterol binding. Biochim. Biophys. Acta 352:385–396.
20. **Rottem, S., M. Hasin, and S. Razin.** 1975. The outer membrane of *Proteus mirabilis*. II. The extractable lipid fraction and electron-paramagnetic resonance analysis of the outer and cytoplasmic membranes. Biochim. Biophys. Acta 375:395–405.
21. **Smith, R. F., D. E. Shay, and N. J. Doorenbos.** 1964. Relationship of surfactant properties of some synthetic steroids to bactericidal action. J. Pharm. Sci. 53:1214–1216.
22. **Stoppani, A. O. M., C. M. C. de Brignone, and J. A. Brignone.** 1968. Structural requirements for the action of steroids as inhibitors of electron transfer. Arch. Biochem. Biophys. 127:463–475.
23. **Varricchio, F., N. J. Doorenbos, and A. Stevens.** 1967. Effect of azasteroids on gram-positive bacteria. J. Bacteriol. 93:629–635.
24. **Varricchio, F., and D. R. Sanadi.** 1967. Inhibition of mitochondrial respiration by progesterone and an azasteroid. Arch. Biochem. Biophys. 121:187–193.
25. **Wolf-Watz, H., T. Elmros, S. Normark, and G. D. Bloom.** 1975. Cell envelope of *Neisseria gonorrhoeae:* outer membrane and peptidoglycan composition of penicillin-sensitive and -resistant strains. Infect. Immun. 11:1332–1341.
26. **Wolf-Watz, H., S. Normark, and G. D. Bloom.** 1973. Rapid method for isolation of large quantities of outer membrane from *Escherichia coli* K-12 and its application to the study of envelope mutants. J. Bacteriol. 115:1191–1197.
27. **Yielding, K. L., and G. M. Tomkins.** 1959. Inhibition of the enzymatic oxidation of DPNH by steroid hormones. Proc. Natl. Acad. Sci. U.S.A. 45:1730–1739.
28. **Yotis, W. W., and R. Stanke.** 1966. Bacteriostatic action of progesterone on staphylococci and other microorganisms. J. Bacteriol. 92:1285–1289.
29. **Yotis, W. W., and J. Waner.** 1968. Antimicrobial properties of testosterone and its intermediates. Antonie van Leeuwenhoek J. Microbiol. Serol. 34:275–286.