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GONOCOCCAL BACTERIOCINS

Inhibition of gonococci by lysophosphatides and free fatty acids of N. gonorrhoeae and by inhibitory substances from other bacteria; analysis of N. gonorrhoeae phospholipase activity

Final Scientific Report

GEO. F. BROOKS, M.D.

November, 1976

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U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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Analysis of previous data, as outlined in the initial proposal of January, 1974, suggested the gonococcal inhibitory substances produced by N. meningitidis and N. gonorrhoeae were bacteriocin-like materials, possibly the protein complement of the lipopolysaccharide of the Neisseria cell wall. The inhibitors can be extracted quantitatively from meningococcal or gonococcal cells using chloroform. The inhibitors are heat stable and are not degraded by proteolytic enzymes. The inhibitors are not proteins, but appear to be lysophosphatides and possibly free fatty acids. Preparation of these

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substances on silica gel thin layer plates after $CHCl_3$ extraction from gonococci gave solutions which killed gonococci in liquid medium, inhibited growth on solid medium, and inhibited uptake of ^{3}H-adenine. Analysis of phospholipid composition of lysophilized cells showed: phosphatidylethanolamine, 69-75%; phosphatidylglycerol, 16%; cardiolipin, 2-3%; and lysophosphatidylethanolamine (LPE), 6-11%. However, the amount of LPE was only 1% when freshly grown cells in growth medium were extracted. Phospholipase A activity was associated with the cell membranes. There were no significant differences between cells from gonococci colony types 1 or 4.

The urethras of a group of human male volunteers were cultures to determine the aerobic and anaerobic flora. A large number of bacterial species were found. Of those, Staphylococcus epidermidis and a hemolytic streptococci were inhibitory to N. gonorrhoeae.

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Summary

Analysis of previous data, as outlined in the initial proposal of January, 1974, suggested the gonococcal inhibitory substances produced by N. meningitidis and N. gonorrhoeae were bacteriocin-like materials, possibly the protein complement of the lipopolysaccharide of the Neisseria cell wall. The inhibitors can be extracted quantitatively from meningococcal or gonococcal cells using chloroform. The inhibitors are heat stable and are not degraded by proteolytic enzymes. The inhibitors are not proteins, but appear to be lysophosphatides and possibly free fatty acids. Preparation of these substances on silica gel thin layer plates after $CHCl_3$ extraction from gonococci gave solutions which killed gonococci in liquid medium, inhibited growth on solid medium, and inhibited uptake of ^3H-adenine. Analysis of phospholipid composition of lysophilized cells showed: phosphatidylethanolamine, 69-75%; phosphatidylglycerol, 16%; cardiolipin, 2-3%; and lysophosphatidylethanolamine (LPE), 6-11%. However, the amount of LPE was only 1% when freshly grown cells in growth medium were extracted. Phospholipase A activity was associated with the cell membranes. There were no significant differences between cells from gonococcal colony types 1 or 4.

The urethras of a group of human male volunteers were cultured to determine the aerobic and anaerobic flora. A large number of bacterial species were found. Of those, Staphylococcus epidermidis and α hemolytic streptococci were inhibitory to N. gonorrhoeae.

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Final Report: Gonococcal Bacteriocins
DAMD-17-75-C-5007

I. Funding and Performance

Official notification for the first year of funding for this project was received on August 19, 1974. The contract was renewed and abruptly terminated on October 31, 1975, three months into the second year of investigation. The work was continued after the termination of contract using in part resources from the contract and limited funds from other sources.

Frances Issac was assigned as the full-time technician for the project. She had been the technician for the project when the initial experiments were done, between July, 1972, and June, 1973.

II. Analysis of Inhibitory Substances

A. As outlined in the initial proposal the initial experiments were done using meningococcus strain 51 (m51) as the strain producing the most active inhibitor and gonococcus strain 18 (g18) as the indicator or sensitive strain. The same experiments done for m51 were completed using gonococcus strain 11 (g11) as the producing strain. Extracts or specimens to be tested for activity were titered with serial two-fold dilutions and 0.02 ml of each dilution were dropped on lawns of g18 using Mueller-Hinton agar plus IsoVitale-X (MHI agar); the g18 lawns were made from broth dilutions containing about 10^8 colony forming units (CFU) per ml. The following experiments were done:

1. Extraction of m51 and g11 at neutral and alkaline pH: Neisseria meningitidis strain 51 (M51) was extracted with phosphate buffered saline (PBS) at pH 7.4 and at pH 12 and g11 were extracted with PBS at pH 7.4. The pH 12 extracts were neutralized before determination of activity. Results of tests of activity are summarized in Table 1 ($\geq$ 2+ activity-obvious inhibition is considered significant). This experiment has been repeated several times with comparable results each time. Saline extraction of m51 at pH 7.4 yields an extract with more inhibitory activity when compared to g11 extract. Also, alkali does not enhance extraction of active inhibitor from m51.

2. Phenol-water extraction of m51: A total of 62.7g wet weight of m51 were mixed with 88% phenol (4 ml/g of bacteria) and water (2 ml/g of bacteria), shaken at room temperature for 10 minutes, and centrifuged at 18,000 x g for 20 minutes. Three phases developed: water, debris, and phenol.

These were removed separately and each was dialyzed against water and saline to remove the phenol. None of the three phases contained material active against g18. This experiment was done twice.

3. Heat stability of m51 extract: M51 extracts were incubated at the temperatures indicated for various periods of time and then titered for activity. The results are indicated in Table 2 (the starting extract for this experiment was less active than some other m51 extracts). Autoclaving the m51 extract for 15 minutes did not change the activity when compared with the unautoclaved extract. The m51 extract was heat stable; heating could have enhanced activity or caused release of an inhibitor from the extract.

4. Enzyme treatment of m51 extract: The results of various enzyme treatments (60 minutes, 37°C) of the m51 extract are outlined in Table 3. None of the enzyme controls showed activity against g18.

Enzyme treatment of m51 extract did not reduce the inhibitory activity. Proteolytic enzymes may actually have enhanced release of activity from the extract.

5. Bactericidal activity of m51 extract: Several experiments designed to confirm the bactericidal activity of the m51 extract were completed. Inhibition of growth of g18 was in liquid media using a 1:10 dilution of m51 extract. Four liquid media were used: MHI broth; MHI broth containing dialysate of yeast extract; a media outlined by Mayer, et al (1); and MHI broth plus 10% calf serum. Results of typical experiments with the first three media are in Figures 1 and 2. The data from several experiments were similar. Titering of the m51 extract showed activity at a 1:15 dilution with minimal activity at a 1:20 dilution. Addition of 10% calf serum to MHI broth reversed the bactericidal effect of extract.

6. Inhibition of ^{14}C-adenine uptake of gonococcal inhibitory substances: The same assay system outlined in Figures 1 and 2 was used for these experiments. The incubation mixture contained: 4.0 ml MHI broth; 0.5 ml of a saline extract of m51; 0.05 ml of ^{14}C-adenine solution (500 μc/ml); and 10^8 colony forming units (0.5 ml) of the g18 sensitive strain (log phase cells from MHI broth cultures). At times 0, 1, 2½, and 4 hour samples were taken for colony counts and for determination of ^{14}C-adenine incorporation. The incorporation of ^{14}C-adenine into nucleic acid was determined by pipetting a 0.1 ml sample of the broth mixture into 2.5 ml of 2.5% cold trichloracetic acid (TCA). The TCA insoluble ^{14}C material was collected on a small millipore filter and washed with 10 ml cold saline. ^{14}C activity was determined using a Packard Tri-carb liquid

scintillation counter. Results of one experiment are plotted in Figure 3. In the control tube (without m51 extract) both the CFU and the ^{14}C incorporated into precipitable material increased over the 4 hour sampling period. In the test system the CFU fell to 10^2 after 2.5 hours; and no increase in ^{14}C incorporation was observed over a period of 4 hours. This experiment showed significant bactericidal activity of the inhibitor as well as inhibition of incorporation of ^{14}C-adenine. The determination of viable colony counts was more sensitive, at least in these early experiments. However, the difference at 4 hours between 10,000 cpm/ml and 100,000 cpm/ml in the test and control tubes, respectively, is also very significant. This type of experiment was repeated several times with comparable results.

7. Chloroform-methanol extract of m51 and g11: A modified Bligh-Dyer procedure (2,3) was used for chloroform-methanol extraction of the test producing strains. After extraction and centrifugation three phases were evident: lower chloroform phase; middle cellular debris; and upper methanol-water phases. Material from the phases was spotted on MHI agar plates, evaporated to dryness and lawns of g18 placed over the spots. Results are outlined in Table 4.

All inhibitory activity was associated with the chloroform phase. Analysis of these data and results of the other experiments suggested that the inhibitor was a lipid component both in the m51 and in the g11 strains.

8. Standard chloroform - methanol extraction of lipid inhibitory substances from N. gonorrhoeae: A standardized method for extraction of gonococci with chloroform-methanol by a modification of the method of Bligh and Dyer (2,3) is depicted in Figure 4. This method has been used to extract producer strains of gonococci in order to test for differential activity against other strains of gonococci.

9. Liquid medium assay for differential inhibition of gonococci by chloroform extracts of producer strains: Three strains of gonococci were picked to test for the production of inhibitors which are $CHCl_3$ extractable. The three strains were:

1. g11, strain previously shown to yield extracts active on solid medium and in liquid medium assay experiments;

2. g163, strain thought to produce an inhibitor with the greatest spectrum of activity using the solid medium assay system; and

3. 2686, strain which did not show inhibitory activity against 60 strains of gonococci using the solid medium assay.

The chloroform extracts were prepared as outlined in section 8 (Figure 4). The liquid media bactericidal assay system is as follows:

1. 0.05 ml of the $CHCl_3$ extract was placed in a glass tube and gently heated to evaporate the chloroform. A control tube containing 50 λ of $CHCl_3$ was treated similarly as was a control tube with no $CHCl_3$.

2. 4.5 ml of MHI broth was added to each tube and the residue from the extract was suspended in the broth.

3. 0.5 ml of suspension of the test strain of gonococci (10^8 CFU from log phase cultures) was added to give a final concentration of about 10^7 CFU/ml.

4. Samples for colony counts were taken at 0, 1, and 3 hours, diluted in MHI broth and plated on MHI agar.

The three $CHCl_3$ extracts were initially tested for activity against 12 strains of gonococci divided into five arbitrary subgroups:

1. previously sensitive (possibly sensitive) to most saline extracts; g18, g46, g68.

2. previously resistant (possibly resistant) to most saline extracts: g15, g9, g20.

3. isolates from patients with asymptomatic gonococcal infections; g590, g578.

4. isolates from patients with symptomatic gonococcal infections; g593, g596.

5. isolates from patients with disseminated gonococcal infections (DGI): g681, g779.

The results of three assay experiments are shown in Figures 5-7. A positive assay is defined as a decrease of > 1 $\log_{10}$ of the test strain as compared to the controls. Figure 5

shows the test strain (g593) was killed by $CHCl_3$ extracts of all three producer strains. Figure 6 shows killing of g681 by two of the three extracts while Figure 7 shows killing of g590 by one of the three extracts.

The results of 36 of the liquid medium assay experiments are summarized in Table 5. These data indicate that at a 1:100 dilution the chloroform extracts of three strains of gonococci differentially killed other strains of gonococci. The chloroform extract of g11 killed all but three of the test strains; that of g163 did not kill one test strain; and that of 2686 killed all but one of the test strains. The patterns of bactercidal inhibition were different for the three extracts.

10. Solid medium assay for differential inhibition of gonococci using chloroform extracts of producing strains: Two of the chloroform extracts (g163 and 2686) used in the liquid medium assay experiments were titered using the solid medium assay procedure outlined in section 7. The results are summarized in Table 6. For purposes of comparison the format in Tables 5 and 6 and 7 are the same.

    Using extracts of g163 the results obtained on the solid medium were roughly comparable to those in the liquid medium assay (i.e., g15 was not killed using either system). In contrast, the 2686 extract showed no inhibition on solid media whereas all but one of the 12 indicator strains were inhibited in the liquid medium assay, suggesting the inhibitor produced by 2686 was bound or inactivated by materials present in the agar but not in broth, e.g., starch. Starch inactivation of fatty acid inhibitors was noted by others and we have noted reversal of inhibitor activity by serum (see section 5).

11. Activity of lysophosphatidylethanolamine (Lyso PE) against 12 strains of gonococci: Lyso PE was purchased commercially (Sigma Chemical Co.) and dissolved in chloroform (20 mg/ml). The solution was titered using two-fold dilutions and tested for activity against the 12 indicator strains of gonococci using the solid media assay. The results are summarized in Table 7. At this concentration Lyso PE was active against all but three strains: g18, g15 and g779.

The activity of lyso PE, and results of the solid and liquid media assays of g11, g163, and 2686 extracts showed correlation with lack of activity against indicator strain g15; also, a few indicator strains were sensitive to Lyso PE or extracts by each assay method.

12. Silica gel thin layer chromatography of chloroform extracts of m51, g11, g163 and 2686: Chloroform extracts were prepared from m51, g11, g163, and 2686 as previously outlined (2,3) except that the chloroform extract was not concentrated 10 fold. Each extract (0.05 ml) and authentic standards and appropriate controls were spotted on silica gel thin layer chromatography plates (Brinkman Co.) and chromatographed using a solvent system of 65 parts $CHCl_3$, 25 parts CH_3OH, and 4 parts H_2O (3). After development with iodine vapor the spots were marked and the plates were developed with ninhydrin to detect compounds that contain free amino groups. Results are schematically depicted in Figure 8. Each of the four extracts had significant amounts of lyso PE, PE, and phosphatidylglycerol. Also,spots probably representing free fatty acidswere present for each extract.

III. Analysis of Phospholipase A Activity

Subsequent to the development of the data outlined in Section II and to the publication by Walstad, et al (4), it was apparent the gonococcal inhibitors being studied were primarily lysophosphatides and long chain fatty acids. The elaboration of large amounts of lysophosphatidylethanolamine indicated that N. gonorrhoeae might have highly active, cell wall-associated, phospholipases. Subsequently, a series of experiments were performed in collaboration with Drs. Leah Senff, W.S. Wegener, W.R. Finnerty and R.A. Makula. The results of the analysis of phospholipid composition and phospholipase A activity are summarized below and detailed in the attached publication (reference 5, appendix I). In addition, portions of this data were presented at two different meetings (6,7).

A. Phospholipid Composition and Phospholipase A Activity of Neisseria gonorrhoeae.

Exponential-phase cells of Neisseria gonorrhoeae 2686 were examined for phospholipid composition and for membrane-associated phospholipase A activity. When cells were harvested by centrifugation, washed, and lyophilized before extraction,

approximately 74% of the total phospholipid was phosphatidylethanolamine, 18% was phosphatidylglycerol, 2% was cardiolipin, and 10% was lysophosphotidylethanolamine. However, when cells still suspended in growth medium were extracted, the amount of lysophosphatidylethanolamine decreased to approximately 1% of the phospholipid composition. This suggests that a gonococcal phospholipase A may be activated by conditions encountered during centrifugation and/or lyophilization of cells preceding extraction. Phospholipase A activity associated with cell membranes was assayed by measuring the conversion of tritiated phosphatidylethanolamine to lysophosphatidylethanolamine. Optimal activity was demonstrated in 10% methanol at pH 8.0 to 8.5, in the presence of calcium ions. The activity was both detergent sensitive and thermolabile. Comparisons of gonococcal colony types 1 and 4 showed no significant differences between the two types with respect to either phospholipid content or phospholipase A activity.

IV. Inhibition of Neisseria gonorrhoeae by other Bacteria Present in the Human Male Urethra

Twenty-three male patients, attending the Marion County Venereal Disease Clinic, were cultured to determine the normal flora of the anterior urethra. Specimens were obtained by insertion of a calcium alginate-tipped urethrogenital swab (Calgiswab, Inolex) into the urethra. The swabs were streaked first on sheep blood agar for cultivation of anaerobes and then on chocolate agar for cultivation of aerobes. Anaerobic isolates were maintained in cooked meat broth medium and aerobic isolates on trypticase soy agar slants or were frozen in skim milk.

Isolates were screened for inhibitory activity against N. gonorrhoeae by the basal streak-cross streak or basal streak-overlay methods. Basal streaks of aerobic organisms were grown on Mueller Hinton or on brain heart infusion agar plates and those for anaerobic organisms were grown on sheep blood sugar.

Table 8 outlines the spectrum of organisms recovered from the anterior urethra. Staphylococcus epidermidis was the aerobic organism most frequently found, while members of the genus Peptococcus were the most frequently recovered anaerobes. Several of these isolates have been examined for the ability to inhibit N. gonorrhoeae in vitro. Table 9 shows the results of these studies. Among the aerobes tested, all of the Staphylococcus epidermidis and alpha hemolytic streptococcus strains were inhibitory to the gonococcus. None of the anaerobic species tested was inhibitory.

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6. Makula RA, Senff LM: Characterization of phospholipids and phospholipase A activity of Neisseria gonorrhoeae. Presented Annual Meeting American Society for Microbiology, Atlantic City, May, 1976.

7. Senff LM, Brooks GF, Wegener WS: Membrane associated phospholipase A activity of Neisseria gonorrhoeae. Presented Spring Meeting, Indiana Branch, American Society for Microbiology, Indianapolis, Indiana, April, 1976

Table 1

Gonococcal Inhibition by Neutral and Alkaline pH Extracts of Meningococcus 51 and Gonococcus 11

Extract	Titer and Activity					
	1	1:2	1:4	1:8	1:16	1:32
pH 7.4						
m51	4+	4+	4+	4+	2+	$\pm$
g11	4+	4+	3+	-	-	$\pm$
pH 12						
m51	4+	4+	4+	4+	2+	-

Table 2

Heat Stability of m51 Extract

Temperature	Time (Min)	Titer and Activity					
		1	1:2	1:4	1:8	1:16	1:32
-17°C	Storage	3+	1+	+	-	-	-
Room Temp.	60	4+	4+	$\pm$	+	-	-
37°C	30	4+	4+	3+	$\pm$	+	+
	60	4+	4+	3+	$\pm$	$\pm$	$\pm$
56°C	15	4+	4+	1+	1+	$\pm$	$\pm$
	30	4+	3+	2+	+	$\pm$	$\pm$
	60	4+	4+	2+	$\pm$	$\pm$	$\pm$
80°C	10	4+	3+	2+	+	$\pm$	-
	20	4+	3+	2+	$\pm$	-	$\pm$
100°C	10	4+	4+	2+	$\pm$	-	-

Table 3

Enzyme Activity Against Extracts of Meningococcus Strain 51

Enzyme	Titer and Activity					
	1	1:2	1:4	1:8	1:16	1:32
Control	ND*	4+	4+	3+	1+	+
Neuraminidase	ND	4+	4+	4+	2+	$\pm$
Trypsin	ND	4+	4+	3+	3+	$\pm$
Protease	ND	4+	4+	4+	4+	$\pm$
Papain	ND	4+	4+	4+	4+	$\pm$

*ND = not done

Table 4

Inhibitory Activity of Chloroform-Methanol Extracts of Meningococcus 51 and Gonococcus 11

meningococcus 51 extract phase	1	1:2	1:4	1:8	1:16	1:32	1:64
chloroform	4+	4+	4+	4+	4+	4+	±
debris	-	-	-	-	-	-	-
methanol	-	-	-	-	-	-	-
gonococcus 11 extract phase							
chloroform	4+	4+	2+	-	-	-	-
debris	-	-	-	-	-	-	-
methanol	-	-	-	-	-	-	-
Controls							
chloroform	-						
methanol	-						

Table 5

Liquid Media Assay: Inhibition of 12 Strains of Neisseria gonorrhoeae by Chloroform Extracts of 3 Inhibitor Producer Strains

Group and Test Strain	Chloroform Extract of g11	g163	2686
	(Logs of Test Strain Killed in 3 Hours)		
Possibly sensitive			
g18	3	4	4
g46	2	5	2
g68	3	3	4
Possibly Resistant			
g15	0	0	3
g9	2	7	5
g20	1	5	3
Asymptomatic			
g590	0	6	0
g578	3	4	3
Symptomatic			
g593	5	6	5.5
g596	3	3	2
DGI			
g681	0	5	3
g779	1.5	3	2

Table 6

Solid Media Assay: Inhibition of 12 Strains of Gonococci by Chloroform Extracts of 2 Inhibitor Producer Strains

Group and Test Strain	Chloroform Extract of g163 (Titer yielding ≥2+ inhibition)	Chloroform Extract of 2686 (Titer yielding ≥2+ inhibition)
Possibly sensitive		
g18	1:8	-
g46	1:32	-
g68	1:2	-
Possibly resistant		
g15	-	-
g9	No growth	No growth
g20	1:8	-
Asymptomatic		
g590	No growth	No growth
g578	1:32	-
Symptomatic		
g593	1:2	-
g596	1:32	-
DGI		
g681	1:32	-
g779	≥1:1	-

Table 7

Solid Media Assay: Inhibition of 12 Strains of Gonococci by Lysophosphatidylethanolamine (Lyso PE)*

Group and Test Strain		Lyso PE**
Possibly Sensitive		
	g18	-
	g46	1:4
	g68	1:2
Possibly Resistant		
	g15	-
	g9	1:2
	g20	1:2
Asymptomatic		
	g590	1:4
	g578	1:32
Symptomatic		
	g593	1:32
	g596	1:8
DGI	g681	1:4
	g779	-

* Initial concentration Lyso PE 20 mg/ml
** Titer yielding >2+ Inhibition

Table 8

Aerobic and Anaerobic Bacteria Isolated from the Human Male Anterior Urethra

Aerobic Isolates	Number found/Number tested
Staphylococcus epidermidis	19/21
diphtheroids	14/21
α hemolytic streptococcus	6/21
H. parainfluenzae	2/21
H. vaginalis	1/21
Gram-negative rod (not identified)	1/21
None	1/21
Anaerobic Isolates	
Peptococcus asacchrolyticus	7/23
P. prevoti	3/23
P. magnus	3/23
Bacteroides melaninogenicus	4/23
Bacteroides sp.	5/23
Eubacterium lentum	1/23
Pseudomonas parvulus	1/23
P. acnes	1/23
Peptococcus sp.	1/23
Veillonella parvulus	1/23
Eubacterium sp.	1/23

Table 9

Isolates Inhibitory* to *N. Gonorrhoeae*

Aerobes		
Staph Epidermidis	10/11**	
α Hemolytic streptococci	2/2	
Diphtheroids	0/8	
Other	0/1	(*H. parainfluenzae*)
Anaerobes		
Peptococcus asacchrolyticus	0/5	
P. prevoti	0/3	
P. magnus	0/2	
Bacteroides sp.	0/1	
Other	0/1	*Eubacterium lentum*
	0/1	*Veillonella parvula*

* Assay either by basal streak/cross streak or basal streak/overlay

** $\frac{\text{No. of isolates which inhibited}}{\text{No. of isolates tested}}$

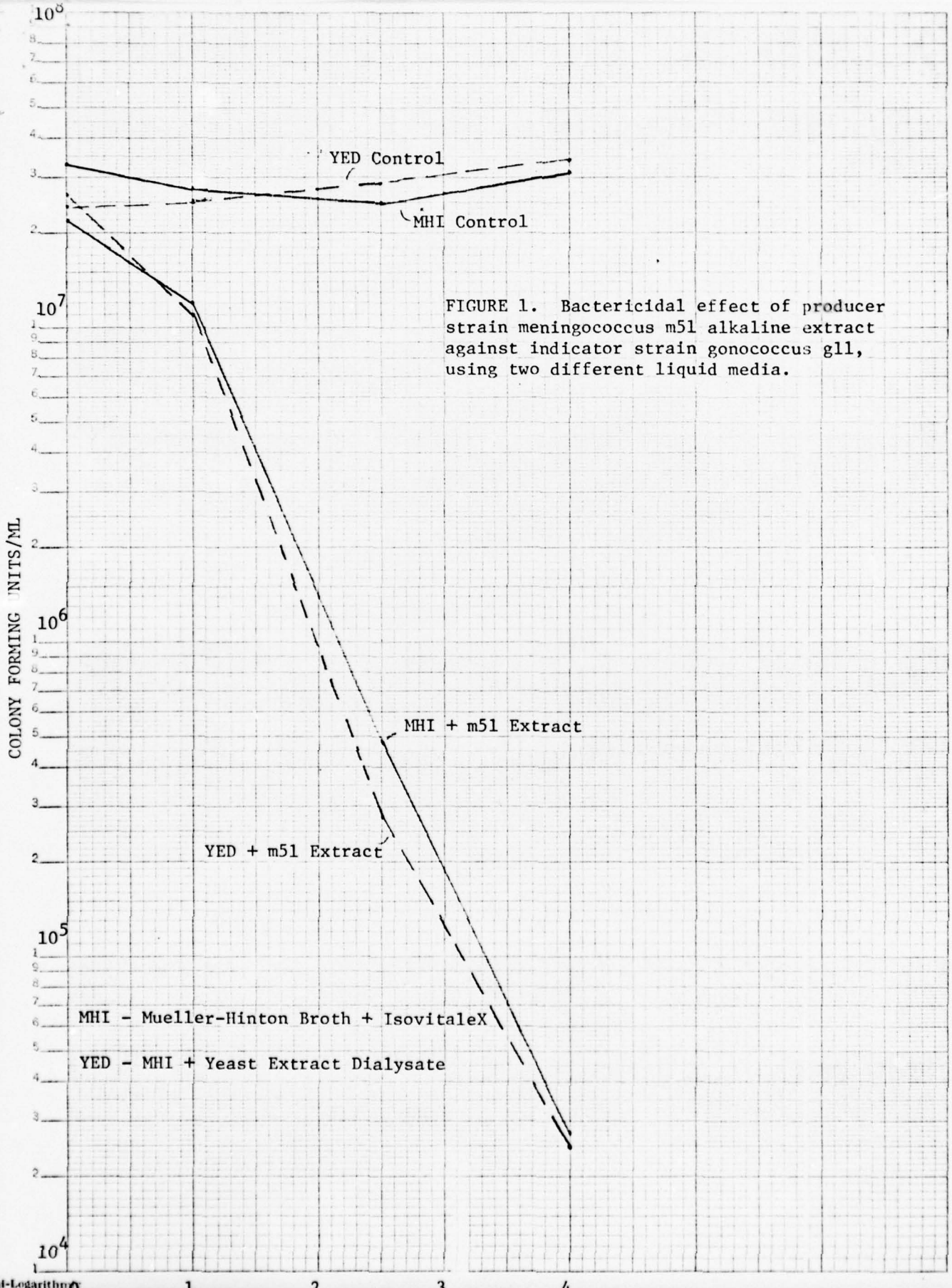
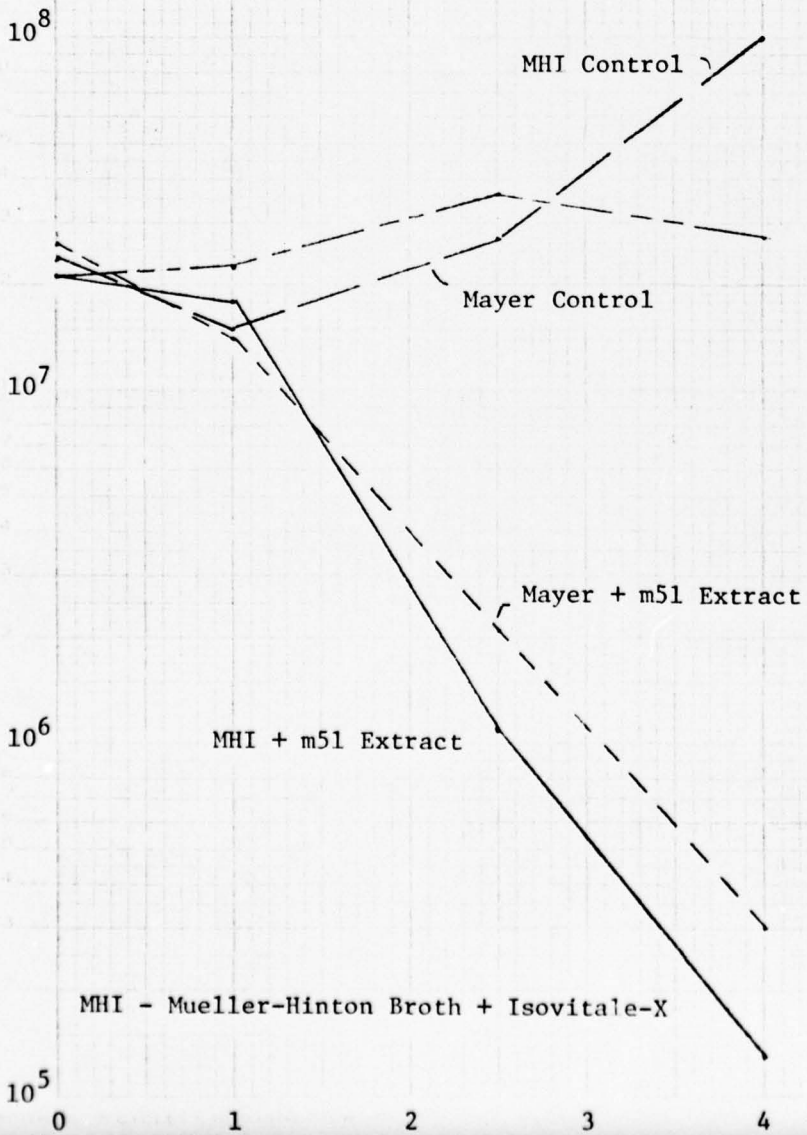


FIGURE 1. Bactericidal effect of producer strain meningococcus m51 alkaline extract against indicator strain gonococcus g11, using two different liquid media.

FIGURE 2. Bactericidal effect of producer strain meningococcus m51 alkaline extract against indicator strain gonococcus g11, using two different liquid media.



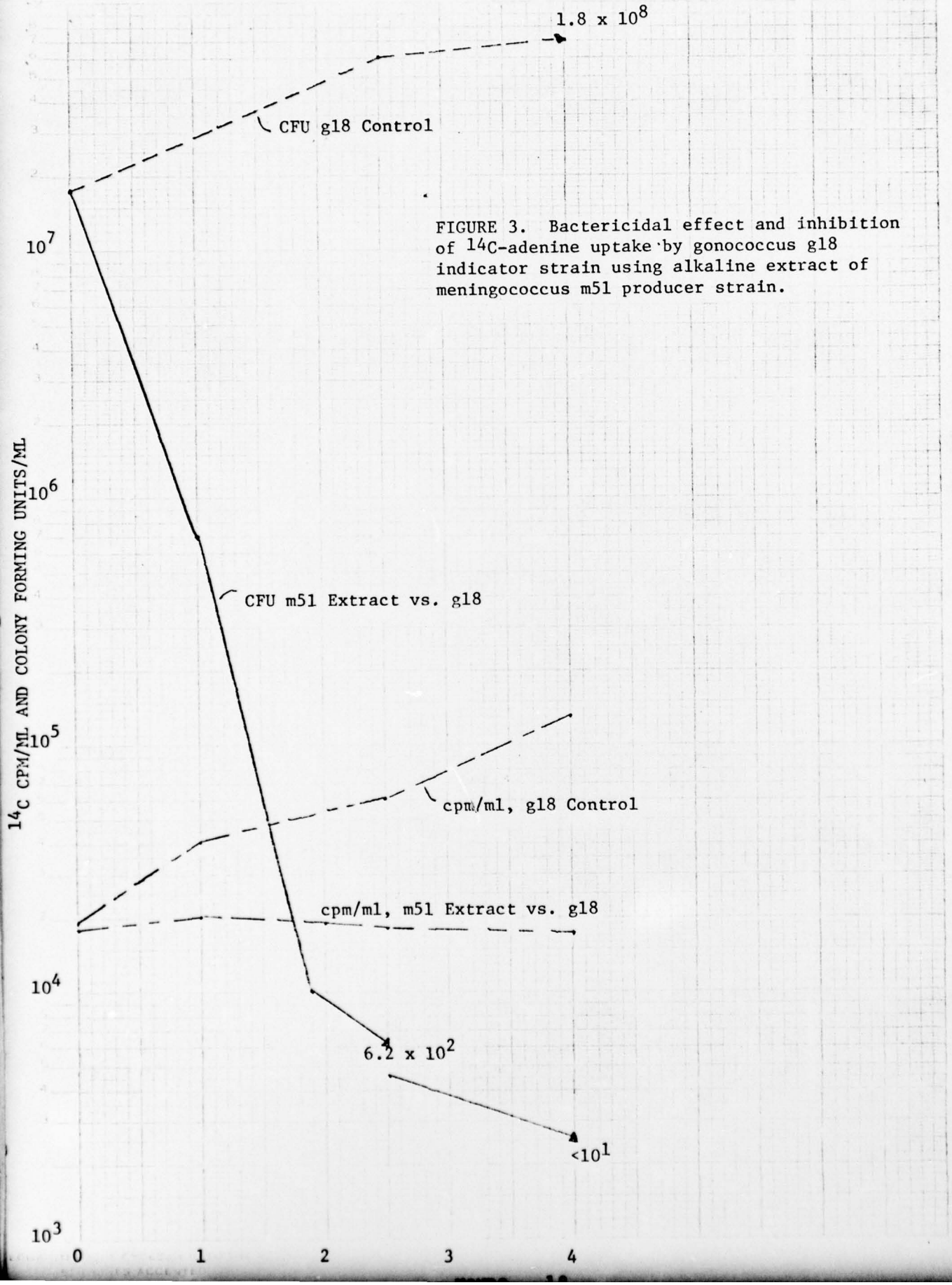
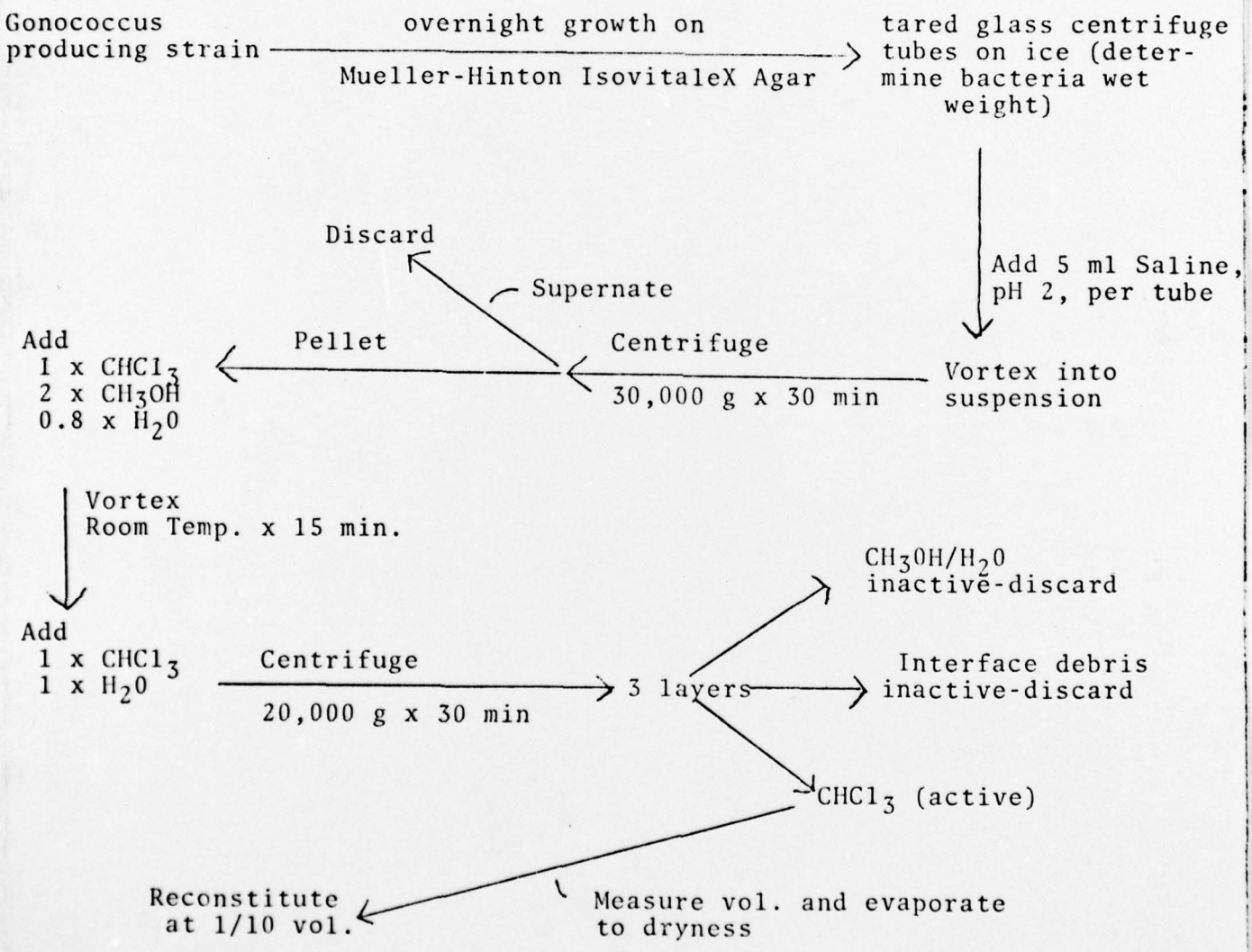


FIGURE 3. Bactericidal effect and inhibition of ^{14}C-adenine uptake by gonococcus g18 indicator strain using alkaline extract of meningococcus m51 producer strain.

Figure 4

Flow Chart for Extraction of Lipids from Neisseria gonorrhoeae



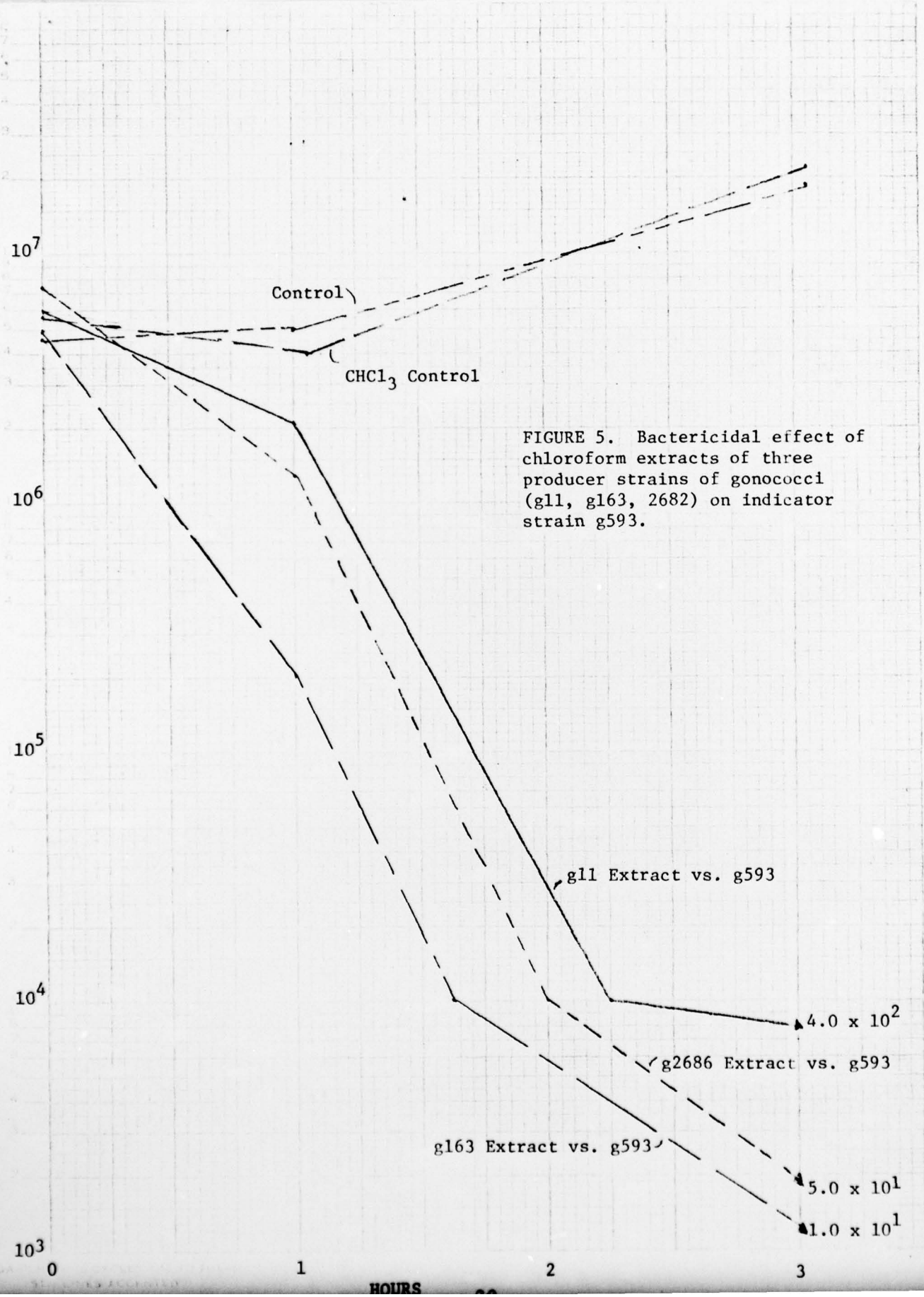


FIGURE 5. Bactericidal effect of chloroform extracts of three producer strains of gonococci (g11, g163, 2682) on indicator strain g593.

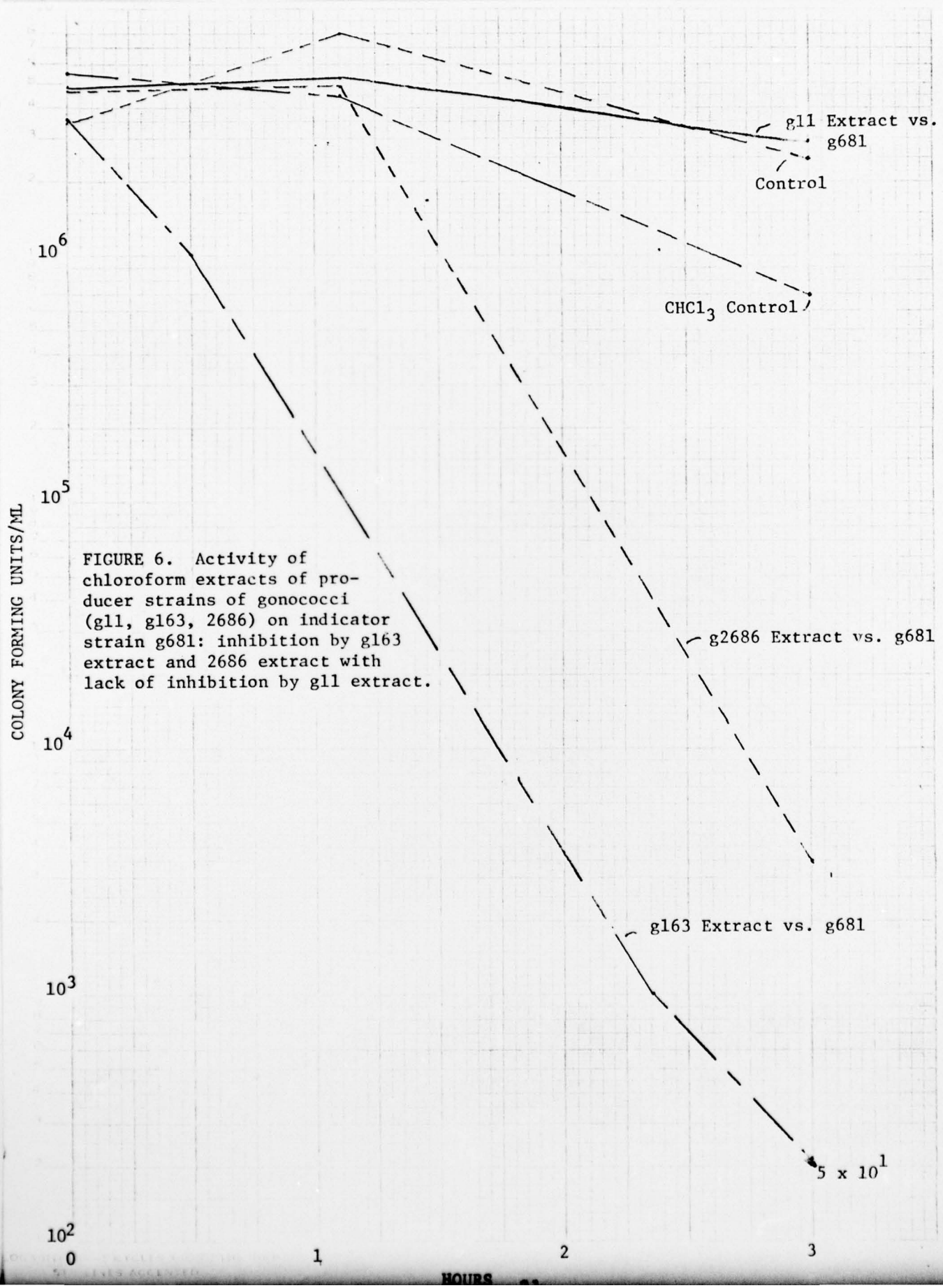


FIGURE 6. Activity of chloroform extracts of producer strains of gonococci (g11, g163, 2686) on indicator strain g681: inhibition by g163 extract and 2686 extract with lack of inhibition by g11 extract.

FIGURE 7. Activity of chloroform extracts of three producer strains of gonococci (g11, g163, 2686) on inhibitor strain g590: inhibition by g163 extract and lack of inhibition by g11 and 2686 extracts.

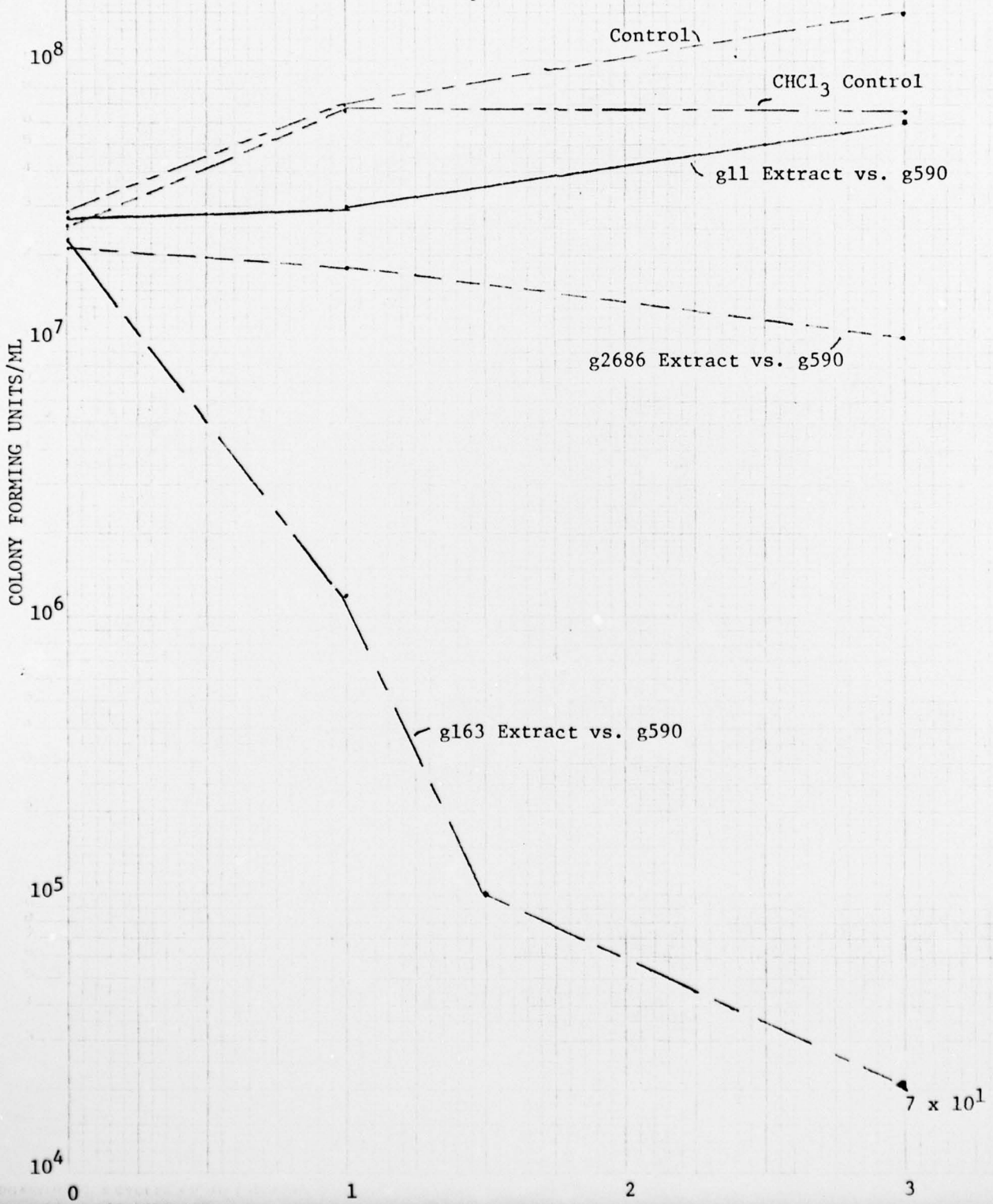
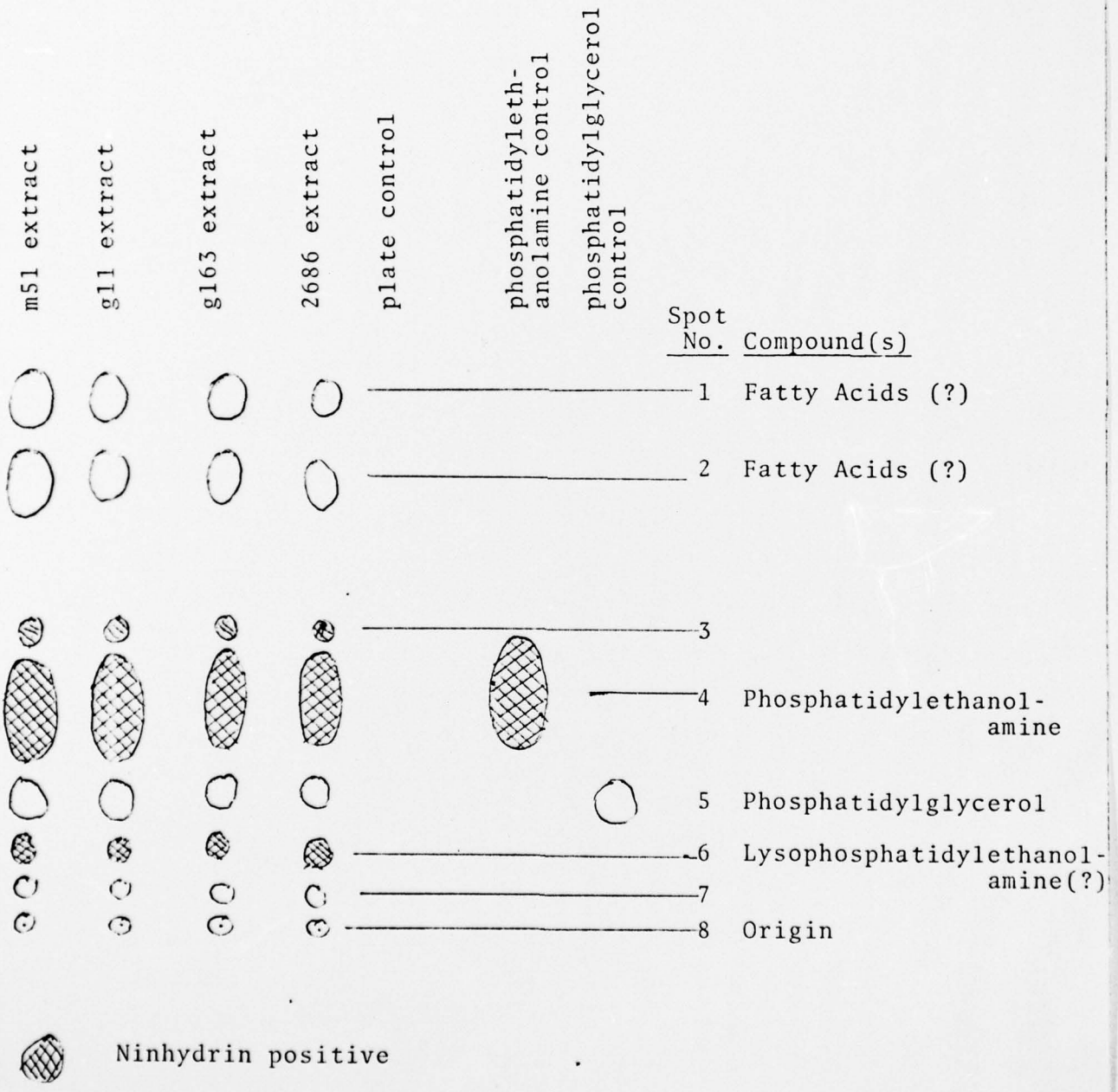


Figure 8

Schematic Diagram of Results of Silica Gel Analysis of Chloroform Extracts of Meningococcus Strain m51 and Gonococcus Strains g11, g163 and 2686



APPENDIX 1

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Phospholipid Composition and Phospholipase A Activity of *Neisseria gonorrhoeae*

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Exponential-phase cells of *Neisseria gonorrhoeae* 2686 were examined for phospholipid composition and for membrane-associated phospholipase A activity. When cells were harvested by centrifugation, washed, and lyophilized before extraction, approximately 74% of the total phospholipid was phosphatidylethanolamine, 18% was phosphatidylglycerol, 2% was cardiolipin, and 10% was lysophosphatidylethanolamine. However, when cells still suspended in growth medium were extracted, the amount of lysophosphatidylethanolamine decreased to approximately 1% of the phospholipid composition. This suggests that a gonococcal phospholipase A may be activated by conditions encountered during centrifugation and/or lyophilization of cells preceding extraction. Phospholipase A activity associated with cell membranes was assayed by measuring the conversion of tritiated phosphatidylethanolamine to lysophosphatidylethanolamine. Optimal activity was demonstrated in 10% methanol at pH 8.0 to 8.5, in the presence of calcium ions. The activity was both detergent sensitive and thermolabile. Comparisons of gonococcal colony types 1 and 4 showed no significant differences between the two types with respect to either phospholipid content or phospholipase A activity.

Several characteristics of *Neisseria gonorrhoeae* suggest that the gonococcal cell envelope is less stable than the cell envelope of most gram-negative bacteria. The gonococcus is more susceptible to penicillin and to inhibition by long-chain fatty acids (20, 36) than are most gram-negative microorganisms. An increased tendency toward lysis has also been observed (7, 16, 26). This apparent cell fragility and/or alteration of membrane permeability could reflect unique envelope structure and composition or could represent the results of enzymatic degradation of envelope constituents.

Phospholipids and phospholipases represent integral components of gram-negative outer membranes (2, 14). Phospholipase A (EC 3.1.1.4) has been implicated in the loss of envelope stability in a variety of bacteria (3, 8, 13, 18, 19, 31). That the gonococcus has an active phospholipase A is suggested by observations that the end products that would result from action of this enzyme are apparently released during in vitro growth of the organism (6, 36). The present studies compare exponential phase gonococci of colonial types 1 and 4 with respect to cellular phospholipid composition and membrane-associated phospholipase A activity.

MATERIALS AND METHODS

Culture conditions. Colonial types 1 and 4 of *N. gonorrhoeae* 2686 were maintained on a solid medium, GCBI, containing GC agar base (Difco) supplemented with 1% IsoVitaleX (BBL), and specific colonial types were serially subcultured (35). For phospholipid analysis or for preparation of cell membranes, gonococci were grown in the broth medium of Mayer et al. (24) containing 2.5% glucose. The medium was dispensed in Erlenmeyer flasks so that 20% of the flask volume was utilized, and cultures were aerated by shaking at 37°C. Cells of the appropriate colonial type were grown for 18 h on GCBI plates, suspended in a small volume of liquid medium, and inoculated into broth cultures to an initial viable count of 3×10^7 to 4×10^7 colony-forming units (CFU) per ml. Growth was monitored as optical density at 600 nm. (For large cultures, ≥800 ml, of type 4 cells only, 200-ml cultures were inoculated as above, grown to the late exponential phase, and used as inocula for the larger volumes of broth.) At the time of cell harvest, viable counts and colonial morphology were assessed on GCBI. Under these conditions of culture, the desired colonial type was maintained (≥90%).

Chemicals. Lysozyme (EC 3.2.1.17), deoxyribonuclease (EC 3.1.4.5), and ribonuclease (EC 3.1.4.22) were purchased from Sigma Chemical Co., St. Louis, Mo. Phospholipid standards were purchased

from Serdary Research Laboratory, London, Ontario, Canada. Silica gel G containing $CaSO_4$ binder and silica gel H were obtained from Brinkman Instruments Inc., Westbury, N.Y. Aquasol and [2-^{3}H]glycerol (specific activity, 8.81 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass.

Lipid extraction. Cells were harvested during exponential growth (5×10^8 to 7×10^8 CFU/ml; optical density of 0.47 at 600 nm) by centrifugation at 10,000 $\times$ *g* for 10 min at 15°C, washed once with 0.05 M HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) buffer, pH 7.4, and lyophilized. Lyophilized cells (0.5 to 2.0 g) were extracted as previously described (23). The recovery of lysophosphatidylethanolamine (LPE) by this extraction method was 88%, as determined by the addition of purified radioactive LPE. The lipid composition of cells before physical manipulation was determined by direct extraction according to Bligh and Dyer (5) of exponential-phase cultures. The recovery of LPE under these conditions was 94%. Procedures used for column chromatography, mild alkaline methanolysis, and quantitation of phospholipid by thin-layer chromatography (TLC) have been previously reported (23). Recoveries of lipid phosphorus applied to the TLC plates were 95 to 97%.

TLC. Glass plates with either silica gel G or silica gel H impregnated with 1 mM sodium tetraborate were made 0.4 mm thick, activated at 110°C, and used within 30 min. Development of TLC plates was in the following solvent systems: (i) solvent A, chloroform-methanol-water (95:35:5, vol/vol/vol) for borate-impregnated plates; and (ii) solvent B, chloroform-methanol-5 N NH_4OH (65:30:5, vol/vol/vol) for silica gel G plates. Lipids were visualized with the following spray reagents in the sequence specified: (i) total lipid was visualized with iodine vapor, (ii) amino nitrogen was visualized with 0.2% ninhydrin in acetone followed by heating at 110°C for 5 min, and (iii) organic phosphorus was visualized by the phosphate spray reagent of Dittmer and Lester (10).

Isolation of membranes. Cells were harvested as described and suspended at a concentration of 1 g (wet weight) of cells per 10 ml of 0.05 M HEPES buffer, pH 7.4, containing 0.6 M sucrose. Lysozyme was added at a concentration of 10 mg per g (wet weight) of cells and incubated at room temperature for 15 min with stirring. Cells were lysed by the addition of 10 volumes of ice-cold distilled water. After incubation for 60 min at 4°C, the suspension was centrifuged at 65,000 $\times$ *g* for 90 min at 4°C, and the resulting membrane pellet was suspended in 0.05 M sodium phosphate buffer, pH 7.5 (10 ml/g of original cells [wet weight]). This preparation was sheared by two passages through an 18-gauge needle, incubated with deoxyribonuclease and ribonuclease (2 mg/g of original cells [wet weight]) for 30 min at 0°C, and centrifuged at 65,000 $\times$ *g* for 90 min at 4°C. The membrane pellet was washed once by centrifugation and suspended in phosphate buffer by sequential passage through 18- and 20-gauge needles.

The crude membrane fraction was further purified by sucrose density gradient centrifugation in a Spinco model L ultracentrifuge with an SW25.1 rotor. Gradients were prepared by layering 12 ml of 24% (wt/wt) sucrose over 15 ml of 55% (wt/wt) sucrose. A 3.0-ml sample of the crude membrane preparation was layered on the top of each gradient. After centrifugation at 51,500 $\times$ *g* for 10 h at 4°C, the resulting membrane material that banded at a buoyant density of 1.26 g/cm^3 was collected by syringe, diluted five- to sixfold in 0.05 M sodium phosphate buffer, pH 7.5, and centrifuged at 65,000 $\times$ *g* for 90 min at 4°C. The pellet was washed once and suspended in phosphate buffer at a protein concentration of approximately 6 mg/ml and stored at 0°C. The phospholipase A activity of such preparations declined gradually over a 2-week period. For longer periods of storage, preparations were held at −15°C and thawed just before assay.

Phospholipase A assay. The substrates employed for assay of phospholipase activity were purified ^{3}H-labeled phospholipids obtained by chloroform-methanol extraction of *Acinetobacter* HOI-N grown on nutrient broth-yeast extract in the presence of [2-^{3}H]glycerol (22). All assays were performed under conditions of linearity with respect to time (5 to 30 min) and protein concentration (for type 1, up to 100 μg; for type 4, up to 150 μg). The usual assay mixture contained in a final volume of 1.0 ml: 300 nmol of [^{3}H]phosphatidylethanolamine (PE; 80 cpm/nmol); 10% methanol; 100 mM HEPES buffer, pH 8.0; and 5 mM $CaCl_2$. The substrate, dissolved in chloroform-methanol (2:1, vol/vol) was added to the reaction tube and evaporated to dryness in vacuo. Methanol (0.1 ml) was added and the mixture was agitated vigorously on a Vortex mixer to suspend the substrate. The other assay components were added, and the reaction was initiated by the addition of the membrane (50 to 100 μg of protein). All enzyme assays were performed in duplicate. Controls that contained all of the ingredients except the membrane suspension or that contained heat-inactivated membranes (100°C, 5 min) were included to determine nonenzymatic hydrolysis of substrate. The background activity obtained with either type control was the same. In addition to PE, [^{3}H]cardiolipin (CL) and [^{3}H]phosphatidylglycerol (PG) were tested as substrates. Reaction mixtures were as described for PE, except that 140 nmol of CL (240 cpm/nmol) or 130 nmol of PG (183 cpm/nmol) was added.

The reaction mixture was incubated at 37°C with shaking for 20 min, and the reaction was terminated by the addition of 2.0 ml of methanol. The mixture was blended with a Vortex mixer and immediately chilled on ice. The following additions were made sequentially: 1.0 ml of chloroform, with Vortex blending; 0.8 ml of water plus 1.0 ml of chloroform, with Vortex blending. This mixture was chilled on ice and centrifuged (200 $\times$ *g* for 4 min, 25°C) to facilitate separation of the organic and aqueous phases. The aqueous layer was removed and extracted with an additional 2.0 ml of chloroform. The

combined chloroform layers were evaporated to dryness under a stream of nitrogen and dissolved in a known volume of chloroform-methanol-water (2:1:0.1, vol/vol/vol). Unlabeled LPE was added as carrier and the entire sample was spotted on borate-impregnated TLC plates and developed in solvent A. Spots were visualized with iodine vapor and those areas corresponding to LPE were scraped into scintillation vials. One milliliter of methanol-water (2:1, vol/vol) and 10 ml of Triton X-100 scintillation fluid (1) were added, and the radioactivity was determined in a liquid scintillation spectrometer (Mark II, Nuclear-Chicago Corp.). The recovery of LPE under these conditions was 93% as determined by the addition of radioactive LPE to duplicate assays containing heat-inactivated membranes and unlabeled PE. The radioactivity in the aqueous phase after chloroform extraction was determined on 1.0-ml samples mixed with 10 ml of Aquasol for PG hydrolysis, since lysophosphatidylglycerol (LPG) partitions into the aqueous layer under the above extraction conditions (31). Control reactions demonstrated less than 0.2% of the labeled PG partitions into the aqueous layer. Specific activity was expressed as nanomoles of substrate converted per minute per milligram of membrane protein.

Analytical methods. Lipid phosphorus was measured by the method of Bartlett as quoted in reference 11. Protein was estimated by the method of Lowry et al. (21) with bovine serum albumin as a standard. Acyl ester was determined by the method of Snyder and Stephens (32).

RESULTS

Characterization of the phospholipids. TLC analyses (Fig. 1) of the polar lipids obtained from *N. gonorrhoeae* enabled tentative identification of the phospholipids as well as the quantitative determination of the individual phospholipid species.

N. gonorrhoeae exhibited a typical gram-negative phospholipid pattern, with PE comprising the major phospholipid (Table 1). In addition to PG and CL, LPE was detected in both type 1 (6%) and type 4 (11%) cells. Trace amounts of LPG and lysocardiolipin (LCL) were also observed in type 4 cells. Qualitative analysis of cells collected in the stationary phase of growth demonstrated the same major and minor phospholipid components in both colony types. No appreciable differences were found between type 1 and type 4 cells with respect to the amount of lipid phosphorus per gram (dry weight) of cells.

Further identification of the phospholipids was carried out after purification of the individual phospholipids by diethylaminoethyl (DEAE)-cellulose column chromatography and preparative TLC.

PE. PE and LPE, which were eluted in the same column fraction of DEAE-cellulose columns, were separated by preparative TLC with silica gel H containing 1 mM sodium tetraborate and solvent A as the developing solvent. The isolated PE was pure as determined by TLC and demonstrated chromatographic identity with authentic PE in solvent systems A and B. PE was deacylated by mild alkaline methanolysis, and the water-soluble product co-chromatographed with authentic glycerylphosphorylethanolamine in two solvent systems (23). Acid hydrolysis of the isolated PE in 6 N HCl at 100°C resulted in a water-soluble product that was identified as ethanolamine-HCl on an amino acid analyzer (Beckman Instruments, Inc.).

LPE. LPE co-chromatographed with authentic LPE in solvent systems A and B and gave a positive test for amino nitrogen with the ninhydrin spray reagent. The water-soluble product resulting from mild alkaline methanolysis was the same as found for PE. The ester-to-phosphorus ratio obtained on the intact lipid was 0.9:1 (theoretical ratio, 1:1).

CL. CL and PG, along with the trace amounts of LCL and LPG, were obtained in the acidic fraction from DEAE-cellulose column chromatography. These were purified to homogeneity by TLC using solvent system A. Chromatographic identity with authentic CL was established using solvent systems A and B. CL was deacylated by mild alkaline methanolysis and the water-soluble product, glycerylphosphorylglycerylphosphorylglycerol (GPGPG), was shown to co-chromatograph with authentic GPGPG derived by mild alkaline methanolysis of known CL.

PG. The purified PG was shown to be chromatographically identical to authentic PG in solvent systems A and B. Mild alkaline methanolysis of known PG and PG purified from *N. gonorrhoeae* yielded the water-soluble product, glycerylphosphorylglycerol.

LCL and LPG. LCL and LPG were present in low levels, and criteria for identification were based on chromatographic identity by TLC. ^{3}H-labeled LCL and LPG standards were derived by treatment of known [^{3}H]CL and [^{3}H]PG, respectively, with pancreatic lipase (9). The unknown components from *N. gonorrhoeae* lipid extracts were purified by TLC and were found to co-chromatograph with the standard [^{3}H]LCL and [^{3}H]LPG in both solvent systems A and B. In addition, the water-soluble product derived by mild alkaline methanolysis of LCL was GPGPG.

Direct extraction of cultures. Since lysophospholipids are not usually found in significant amounts in gram-negative organisms, the pos-

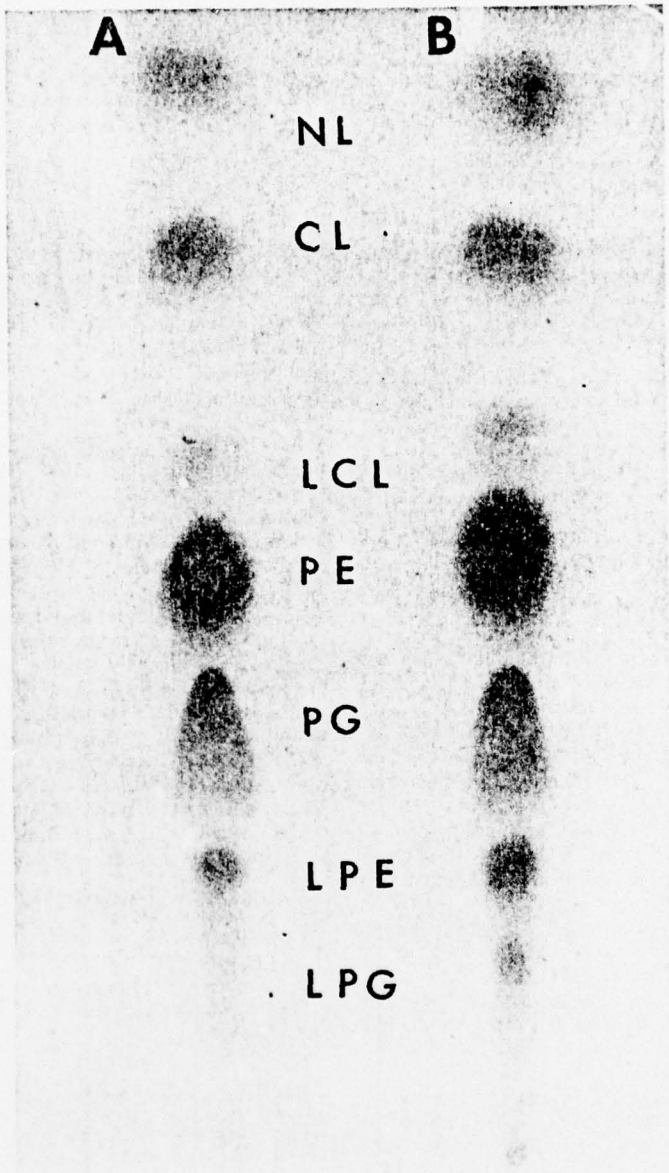

FIG. 1. *TLC of the phospholipids obtained from N. gonorrhoeae type 1 (A) and type 4 (B). Lipids were resolved on TLC plates containing 1 mM borate-impregnated silica gel H in solvent system A. The lipids were visualized by charring with 50% H_2SO_4 at 180°C. NL, Neutral lipids. For other abbreviations, see text.*

sibility exists that these compounds result from phospholipase action during manipulation of the cells (i.e., harvesting, washing, and lyophilization). To test this possibility, 200 ml of exponential-phase cultures was extracted directly according to the procedure of Bligh and Dyer (5). With this extraction procedure, a marked decrease in the amount of LPE was observed, and LPG and LCL could not be detected (Table 1).

TABLE 1. *Phospholipid composition of exponential-phase cells of N. gonorrhoeae, colony types 1 and 4*

Phospholipid	Type 1		Type 4	
	Lyophilized cells	Freshly extracted cells	Lyophilized cells	Freshly extracted cells
PE	75[a]	77	69	76
LPE	6	2	11	1
PG	16	19	16	22
CL	3	2	2	1
LPG	ND[b]	ND	1	ND
LCL	Trace	ND	1	ND
Total lipid phosphorus	87[c]		97	

[a] Percentage of total micromoles of lipid phosphorus.

[b] ND, Not detected.

[c] Micromoles of lipid phosphorus per gram of lyophilized cells.

Phospholipase A activity associated with cell membranes. Phospholipase A activity was present in the membrane preparations of gonococci. Optimal enzyme activity was obtained with 10% methanol; increasing concentrations were increasingly inhibitory (Table 2). Ethanol and isopropanol substituted for methanol but yielded only 75 and 23%, respectively, of the activity obtained with 10% methanol. *N*-propanol, *n*-butanol, and isobutanol were inactive when substituted for methanol. Phospholipase A was inhibited by 0.1% Triton X-100 as well as by 0.1% cutscum, tergitol, ammonyx, and sodium deoxycholate. Nonenzymatic hydrolysis of PE was less than 3% in 10% methanol; increasing concentrations resulted in greater nonenzymatic hydrolysis.

Enzyme activity required the presence of Ca^{2+}; Mg^{++} could not be substituted for calcium. The enzyme exhibited an optimal Ca^{2+} requirement of 5 mM (Fig. 2A) and an optimum pH range between 8.0 and 9.0 (Fig. 2B). The enzyme activity showed a marked decrease at pH values of less than 8.0. The treatment of membrane preparations at 60°C for 5 min and at 100°C for 5 min resulted in the loss of 40 and 100%, respectively, of the enzyme activity. This is in contrast to membrane preparations obtained from other microorganisms, which show heat activation of phospholipase (12, 31).

A comparison of phospholipase A activity against PE, CL, and PG for membranes obtained from type 1 and type 4 *N. gonorrhoeae* is shown in Table 3. The specific activity of the phospholipase A for PE was greater than that observed for CL or PG. Phospholipase A activity against PG exhibited similar requirements and reaction conditions as determined for PE including an obligate Ca^{2+} requirement and inhibition by detergents. Whether one phospholipase A with activity towards PE, CL, and PG is present or different activities with specificity to particular phospholipids is impossible to assess at this point.

DISCUSSION

Several characteristics of *N. gonorrhoeae* suggest that its cell envelope is functionally different from that of other gram-negative bacteria. The sensitivity of the organism to penicillin and to long-chain fatty acids is greater than usually found for gram-negative microorganisms (20, 36). Increased fragility and spontaneous lysis appear characteristic of the gonococci under a variety of conditions (7, 16, 26). This cellular instability does not appear related to phospholipid composition, as evidenced by this study and related work (33, 38). This work has, however, provided evidence for the stimulation and/or activation of phospholipase activity in the gonococci.

The phospholipid composition of *N. gonorrhoeae* was similar to that reported for other gram-negative microorganisms (2, 14). Sud and Feingold (33) recently reported the presence of phosphatidylcholine (PC) in type 3 gonococci. We did not detect PC in either exponential- or stationary-growth-phase cultures of *N. gonorrhoeae* 2686 type 1 and type 4. We also examined a fresh clinical isolate of colony type 1 and failed to find PC even when employing extraction and chromatographic techniques similar to those described by Sud and Feingold (33).

TABLE 2. *Conversion of [^{3}H]PE to LPE by membrane preparations of type 1 N. gonorrhoeae*

Assay mixture	Sp act[a]
Complete system[b] (10% methanol)	12.9
0% Methanol	Variable[c]
20% Methanol	9.6
30% Methanol	4.0
40% Methanol	0.6
10% Ethanol (methanol omitted)	9.8
10% Isopropanol (methanol omitted)	3.0
0.1% Triton X-100 (methanol omitted)	0.0
0.1% Triton X-100 (methanol present)	0.0
Calcium omitted	0.0

[a] Nanomoles per minute per milligram of protein.

[b] The complete system contained in 1.0 ml: 300 nmol of PE dissolved in 0.1 ml of methanol; 0.1 M HEPES, pH 8.0; 0.005 M $CaCl_2$; and 65 μg of protein of a type 1 membrane preparation. Modifications were made as indicated. The assay mixture was incubated 20 min at 37°C, and the formation of LPE from PE was determined as described in Materials and Methods.

[c] Specific activity in absence of methanol varied from 5.1 to 12.3 nmol/min per mg of protein.

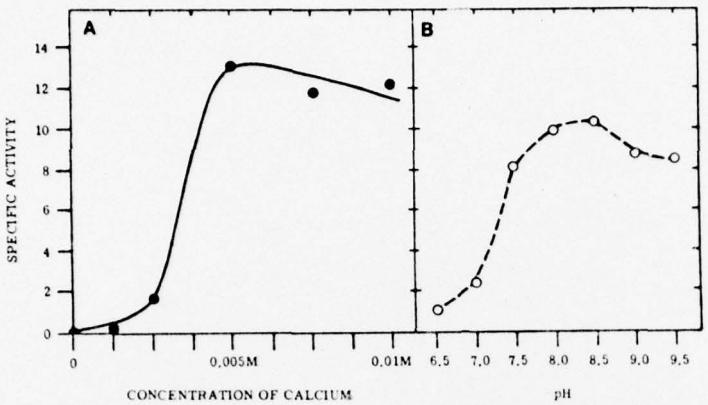

FIG. 2. *Effect of calcium concentration and pH on the conversion of [^{3}H]PE to LPE by membrane preparations of N. gonorrhoeae, type 4. The assay mixture was as described in Table 2 except that $CaCl_2$ concentration (A) or pH (B) was varied. Buffers employed were HEPES (pH 6.5 to 8.5) and glycine/NaOH (pH 9.0 to 9.5). Amount of membrane suspension added to the assay mixture was 87 μg of protein (A) or 115 μg of protein (B).*

TABLE 3. *Hydrolysis of PE, CL, and PG by membrane preparations of N. gonorrhoeae*

Substrate	Hydrolytic end product	Sp act[a]	
		Type 1	Type 4
PE	LPE	13.3	10.6
CL	LCL	4.3	5.0
PG	LPG	2.0	1.3

[a] Values represent averages of two separate membrane preparations for each of the colonial types. Assay conditions were as described in Materials and Methods.

In contrast to the phospholipid composition reported by Wolf-Watz et al. (38) and Sud and Feingold (33), the present study documents the presence of LPE (6 to 11%) in lyophilized exponential-growth-phase cells of colony types 1 and 4. However, direct extraction of the cellular lipids from actively growing cells showed the presence of only 1 to 2% LPE. This relationship indicates that such manipulations as centrifugation, cell suspension, or lyophilization increase the amount of lysophospholipid subsequently found in the lipid extracts.

The physiological role for phospholipases in bacteria remains unclear. Examples of the bacteriolytic effects of phospholipases have been noted, including those encountered during T4 phage infection of *Escherichia coli* (8, 31), megacin A-mediated lysis of *Bacillus megaterium* (28), and complement-mediated lysis of *E. coli* (4) and of group A streptococcal L forms (13).

Phospholipase A activity associated with the cell membranes of *N. gonorrhoeae* was similar to the membrane-bound phospholipases previously described for *E. coli* (31), *Bacillus subtilis* (18), and *Mycobacterium phlei* (27) in that its requirements for optimal activity include alkaline pH and calcium ions. It differed in that it was thermolabile and detergent sensitive. A thermolabile, detergent-sensitive phospholipase A has been reported in *E. coli* (12), but this phospholipase was specific for PG, had a pH optimum of 6.5 to 7.0, and was soluble, as determined by centrifugation at 105,000 × *g* for 2 h.

The relationship of phospholipase A to the autolysis of gonococci is unknown. Interestingly, the pH and temperature optima for cell membrane-associated phospholipase A are similar to the optima reported for gonococcal autolysis (16).

Although phospholipase A activity does not appear to be a correlate of virulence (i.e., we found no significant difference in activity of phospholipase A from type 1 and type 4 cells), the enzyme could contribute to the pathogenesis of gonococcal infections. Specific end products resulting from the action of phospholipase A exhibit a variety of effects on mammalian cells, including the release of cytoplasmic enzymes (30) and the alteration of membrane-bound glycosyltransferases (15, 25). Virulent gonococci have the ability to adhere to host cells (17, 29, 34, 35, 37). Thus, if the enzyme is active in vivo, its end products could be released in proximity to host cell surfaces and might influence host cell function.

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