Mycoplasma pneumoniae Phosphatidyl Glycerol (34867)

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Most of the serologic reactivity of *M. pneumoniae*, the etiologic agent of cold agglutinin positive atypical pneumonia, is associated with lipids which can be extracted from the organism with chloroform–methanol or acetone (1–6). Lipid extracts of *M. pneumoniae* do not stimulate an appreciable level of antibody in experimental animals, but when tested by complement-fixation or the metabolism-inhibiting antibody blocking technique such lipids exhibit an unusually high reactivity (1–6). Recent observations by Plackett *et al.* (7), confirmed by Razin *et al.* (8), indicate that the haptens of *M. pneumoniae* are glycolipids which contain glucose and/or galactose.

Initially we had thought the hapten of the organism was a nitrogen free phospholipid (2). However, subsequent studies indicated that our semipurified preparations of hapten contained small quantities of glycolipids and these carbohydrate-containing materials were responsible for the observed serologic activity (8). The semipurified hapten preparations were thus a mixture of predominantly phospholipid and small quantities of glycolipids. In this communication we describe the evidence that the major phospholipid constituent of our preparations was phosphatidyl glycerol. Although phosphatidyl glycerol does not react with specific *M. penumoniae* antibodies, it enhances the serologic reactivity of the glycolipid determinants (8).

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Materials and Methods. Growth, harvesting, and extraction of organisms. The FH strain of *Mycoplasma pneumoniae* was used in its 557th to 607th passage in broth medium (9). The medium for the growth of the organism consisted of Difco PPLO broth, uninactivated horse serum, and 25% fresh yeast extract (7:2:1); this formulation was supplemented with 1% glucose, 0.05% thallium acetate, and penicillin (1000 U/ml) (9, 10). *M. pneumoniae* was grown on a glass surface, and prior to the removal from glass after 3- to 11-days incubation at 37°, the organisms were washed extensively with 0.15 *M* saline to remove constituents of the growth medium (11). The organisms grown in forty 5-liter Povitsky bottles were suspended in distilled water and collected by centrifugation at 34,000*g* for 45 min. The pooled organisms were washed 3 times in a similar manner with distilled water. The packed organisms (10–15 ml) were extracted with 150 ml (2:1) chloroform–methonol (v/v) for 18 hr at room temperature and the mixture was filtered through No. 50 Whatman paper. In one instance the organisms were first extracted with 150 ml of acetone and the acetone insoluble residue was extracted with 150 ml of chloroform–methanol (2:1). The solvent was removed from the lipid by vacuum distillation under nitrogen, and the residue was redissolved in 15 ml of chloroform–methanol (2:1); the nonlipid contaminants in the preparation were removed on a column of Sephadex G25 fine as described by Wells and Dittmer (12).

Column chromatography. Resolution of the chloroform–methanol-soluble material was carried out on a 30 × 1-cm column of silicic acid; lipids were eluted according to the method of Plackett (13) or Beckman and

Kenny (14). The fractions were evaporated, weighed and stored dry in N_2 at 0°; subsequently they were tested for complement-fixing (CF) activity using 4–8 antibody units of a human *M. pneumoniae* convalescent serum and a rabbit hyperimmune *M. pneumoniae* serum (4).

Thin-layer chromatography. Samples (50 μg) of the different lipid fractions were subjected to thin-layer chromatography on silica gel Uniplates (purchased from Analtech Inc., Wilmington, Del.) for 105 min with chloroform–methanol–water (65:25:4, by vol) as the solvent. Lipid spots were detected by spraying the dried plates with a solution of anisaldehyde–glacial acetic acid–sulfuric acid (1:97:2) followed by heating at 120° for 20 min (15).

Hydrolysis and determination of glycerol, fatty acids, and phosphorus. Aliquots of the most serologically active fractions were dissolved in chloroform–methanol 2:1 (v/v), and a portion of this material was used to determine the concentration of phosphorus by the micromethod of Bartlett (16). The remaining material was hydrolyzed with 0.1 *N* tetraethylammonium hydroxide (E_4NOH) in ethanol (60° for 45 min), acidified with an equal volume of 0.1 *N* HCl and the fatty acids were extracted with hexane. Unlike stronger bases, E_4NOH does not hydrolyze phosphate bonds, while under the conditions employed, all acyl ester bonds are cleaved (17). The aqueous phase following hexane extraction contained all of the phosphorus of the sample and virtually no free glycerol or glycerol-3-phosphate. Heating the aqueous phase with 2 *N* HCl (130° for 90 min) yielded free glycerol and some glycerol phosphates. Neutralization of the acidic solution and addition of bacterial alkaline phosphatase (Worthington Chemical Co.) increased the yield of free glycerol by hydrolysis of glycerol phosphates. Glycerol was determined by a fluorimetric modification of the enzymatic procedure of Wieland (17, 18). Theoretical glycerol:phosphorus ratios were obtained by this procedure for lecithin, phosphatidic acid, cardiolipin, and phosphatidyl glycerol (Table III).

Chromatography of E_4NOH deacylation products was adapted from the procedure of Plackett (19). Elution from the Dowex 1 (HCO_3) column was stepwise with 5 ml each of 0, 0.04, 0.05, 0.1 and 0.4 *M* NH_4HCO_3 (Fig. 1).

The method of Nash (20) as modified by De Frietas (21) was used for the determination of the 1,2-glycol structure in the original lipids and their E_4NOH hydrolysates.

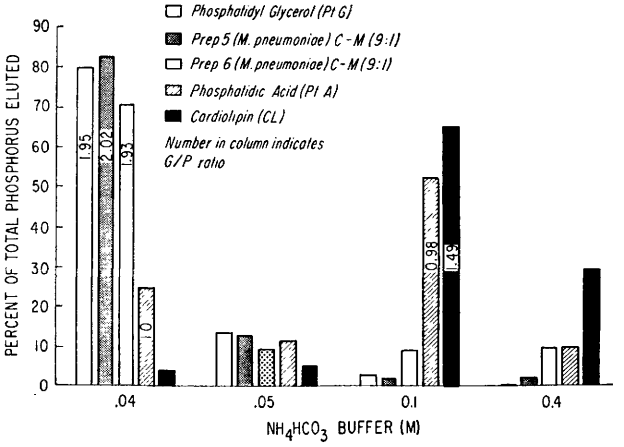

FIG. 1. Ion exchange chromatography of water-soluble deacylation products (0.7–1.0 μmoles of P). The column was packed with 0.8 ml of Dowex 1-X-10 (50–100 mesh) in the bicarbonate form (6 cm long). Stepwise elution with 5 ml of ammonium bicarbonate buffer (pH 8.6) of the molar strengths shown. The lipids shown are (1) phosphatidyl glycerol from *S. aureus* (Lennarz); (2) *M. pneumoniae* hapten preparation no. 5; (3) *M. pneumoniae* hapten preparation no. 6; (4) phosphatidic acid; (5) cardiolipin.

An aliquot of the hexane-soluble solution was titrated with dilute NaOH for fatty acid content and the remainder was used for fatty acid analysis. The methyl esters of the fatty acids were prepared according to the method of Stoffel *et al.* (22) and Farquhar *et al.* (23) and dissolved in *n*-hexane. The individual fatty acids were estimated by gas-liquid chromatography (GLC) on a Barber-Colman model 5000 gas-chromatograph with an 8-ft column of diethylene glycol succinate polyester, 17% by weight, on 80–100 mesh Chromosorb W at 190° with nitrogen carrier gas flow at 60 ml/ min, using a flame ionization detector.

Tests for carbohydrates. Carbohydrate analyses of the active *M. pneumoniae* lipids were performed by the method of Vance and Sweeley (24). After methanolysis in dry, 1.0 *M* methanolic–HCl, and passage through Amberlite CG-4B (OH phase) (Mallinchrodt Chemical Works, St. Louis, Mo.) the samples were silylated. An aliquot of the reaction mixture was injected onto an 8-ft stainless steel column containing 7.5% HI-EFF-2BP + 1.5% SE-30 (80/100 mesh) on Gas Chrom Q (supplied by Applied Science Laboratories, Inc., State College, Pa.) at 165°. In addition, aliquots of the preparation were hydrolyzed with 1 *M* HCl in sealed tubes. The HCl was removed by passage through Amberlite CG-4B resin. The samples were then analyzed for galactose by the method of Sempere *et al.* (25), employing galactostat (Worthington Biochemical Corp., Freehold, N.J.).

Phospholipid standards. Phosphatidic acid (Na salt) and cardiolipin (beef heart) were purchased from General Biochemicals, Chagrin Falls, Ohio. These materials were chromatographically pure. Purified phosphatidyl glycerol prepared from *Staphylococcus aureus* was a gift from Dr. William J. Lennarz, Johns Hopkins University. Dr. Paul Smith, U. of S. Dakota, kindly supplied purified phosphatidyl glycerol derived from *M. laidlawii* B (26).

Serologic tests. The procedure used for determining complement-fixing activity of *M. pneumoniae* and its various fractions has been described previously (3, 4). Briefly, a microtiter complement-fixation technique was used which included overnight incubation at 4° of antigen, 1.7 units of complement, and 4–8 complement-fixing antibody units of either *M. pneumoniae* hyperimmune rabbit serum or convalescent serum from a patient with *M. penumoniae* pneumonia. The antigen titers were generally somewhat higher with the human convalescent serum.

The blocking test described by Sobeslavsky *et al.* (4) was used to determine the capacity of various fractions of *M. pneumoniae* to inhibit the effect of 4 units of growth-inhibiting (metabolism-inhibiting antibody. This antibody was assayed by the tetrazolium reduction inhibition technique of Senterfit and Jensen (27).

Results. Recovery of serologically reactive material. A total of 17 batches of organisms, with an average dry weight of 2.96 g (range 1.23 to 3.96 g), were processed to yield 6 preparations of semipurified serologically reactive material which were analyzed chemically. The organisms used in preparation 1 were first extracted with acetone, and the residue was then extracted with chloroform–methanol (CM), 2:1. The 16 subsequent batches of organisms were extracted only with CM, 2:1. The mixture of lipids removed by these extraction procedures represented approximately 21 to 30% of the dry weight of the original organisms.

The lipid extracts were first freed of nonlipid contaminants and then resolved on a column of silcic acid by performing successive elutions with either chloroform–acetone, 1:1; acetone; CM, 19:1, 9:1; CM, 4:1; and CM, 1:1 [the method of Plackett (13)] or CM, 20:1; CM, 9:1; CM, 6:1; ethylacetate–methanol, 3.5:2; CM, 1:1; and CM, 1:20 [the method of Beckman and Kenny (14)]. In 3 instances the latter method was used, while the remaining 14 batches of organisms were processed by the procedure described by Plackett. When the lipid extract of *M. pneumoniae* was chromatographed by the Beckman and Kenny method, the major share of serological (CF) activity was recovered in the CM, 9:1, eluate. Similarly 10 of 14 batches processed by the Plackett method

TABLE I. Silicic Acid Column Chromatography of Lipids from *M. pneumoniae* Organisms by the Method of Plackett—Summary of Experiment Yielding Hapten Preparation No. 4.

Material[a]	Wt (mg)	% of starting material	Total CF activity ($\times 10^3$) with *M. pneumoniae* serum[b]		Total metabolism-inhibiting antibody blocking activity[b] ($\times 10^3$)—tested with rabbit hyperimmune serum[c]	No. of spots in silica gel thin-layer chromatogram
			Human convalescent	Rabbit hyperimmune		
Dry organisms	2308.5	100.0	29.6	59.1	44.3	
Defatted organisms (extracted with CM, 2:1)	1720.0	74.5	ND[d]	5.5	ND	
Lipid (extracted with CM, 2:1)	588.5	25.5	150.6	37.7	56.5	
Silicic acid column eluates of CM, 2:1, extract						
CA, 1:1	321.5	13.9	ND	ND	1.9	1
A	9.8	0.4	2.5	0.7	0.7	6
CM, 19:1	4.4	0.2	1.1	ND	0.8	4
9:1	16:1	0.7	131.9	4.2	33.0	1
4:1	14.8	0.6	15.2	1.9	0.7	1
1:1	215.9	9.4	ND	ND	ND	2

[a] C = chloroform; M = methanol; A = acetone.

[b] Total activity = reciprocal of highest dilution of suspension which fixed complement or inhibited metabolism-inhibiting antibody × weight (mg).

[c] Determined with 2–4 units of metabolism-inhibiting antibody.

[d] ND = none detected.

exhibited the greatest CF activity in the CM, 9:1, eluate; the most active fraction of the remaining 4 batches was eluted with acetone (3 batches) or CM, 4:1, (1 batch).

Three of the CM, 9:1, silicic acid column eluates produced only on spot on silica gel thin-layer chromatography (TLC) and were thought to be relatively pure. These eluates (preparations 1, 2, and 4) were studied without being subjected to further silicic acid column chromatography. The remaining 14 CM, 9:1, eluates produced 2 to 4 spots on TLC and were rechromatographed using the procedure of Plackett. Preparation 3 was processed by itself, while the other 13 eluates were used to prepare 2 pools which were rechromatographed to yield preparations 5 and 6. Each of the rechromatographed preparations (nos. 3, 5, and 6) produced only one spot on TLC.

Comparison of methods for recovery of serologically reactive material. Lipid extracts from a total of 49 batches of organisms, including the 17 utilized for chemical analysis, were chromatographed on silicic acid columns. Based upon this experience the Plackett system of elution was selected as the standard method for recovery of hapten material from the CM, 2:1, extract of *M. pneumoniae*. Fourteen of 42 lipid extracts (33%) processed by this method yielded CM, 9:1, eluates which produced only one spot on TLC; representative findings from one such experiment are shown in Table I. The CM, 9:1, eluate of 12 other batches produced 2 spots on TLC, 13 eluates produced 3 spots, and the remaining 3 eluates produced 4 spots. In contrast none of the 7 batches processed by the Beckman and Kenny method yielded "single spot" CM, 9:1, eluates; the eluates from 3 batches produced 3 spots, 2 eluates gave 4 spots while the remaining 2 produced 3 spots, 2 eluates gave 4 spots while the remaining 2 preparations produced 5 or 6 spots.

Variation in time of cultivation of *M. pneumoniae* on glass, within the range of 5 to 11 days, did not appear to influence the number of components detected in the CM, 9:1, eluates prepared by the Plackett method. Similarly, the results of a time study indicated that the age of glass grown organisms, over the range of 3 to 11 days, did not appear to influence the purity of CM, 9:1, eluates prepared by the Beckman and Kenny method (14). In this study the total weight of organisms harvested from the surface of Povitsky bottles increased with time of cultivation, but the weight and CF activity of the CM, 9:1, eluates did not increase progressively with time. Unlike the findings reported by Beckman and Kenny, the major share of serologic activity was regularly recovered in the CM, 9:1, eluate, while the ethyl acetate–methanol eluate made only a minor contribution to the total CF activity (14).

Serologic activity. Although the CM, 9:1, eluates prepared by the Plackett method represented only 0.5 to 0.9% of the dry weight of the starting material, in most experiments these eluates exhibited greater CF activity than the original suspension of organisms when tested with antibodies in human serum. In tests with *M. pneumoniae* hyperimmune rabbit sera results were more variable. Representative findings from one experiment are shown in Table I. In this experiment, which is representative of other preparations, there was a similarity in the distribution of metabolism-inhibiting antibody blocking activity and CF activity in the eluates from silicic acid column chromatography. This correlation is in keeping with previous experience which indicated that these two serologic activities were closely related (2).

The serologic reactivity of the 6 semipurified preparations of *M. pneumoniae* hapten, which were studied chemically, is shown in Table II. Each of the preparations exhibited a moderate to high level of CF activity and metabolism-inhibiting antibody blocking activity. Two glycerophospholipids, cardiolipin and phosphatidyl glycerol, which were tested for comparison, lacked reactivity with *M. pneumoniae* antibodies in human and rabbit hyperimmune sera. The specificity of the CF reactivity of the hapten preparations was established by demonstrating that these materials did not react with human serum obtained prior to *M. pneumoniae* infection,

TABLE II. Serologic Reactivity of *M. pneumoniae* Hapten Preparations (CM, 9:1) and Other Phospholipids.

Material	Silicic acid column chromatography	Activity (reciprocal) of 0.1% suspension		Reciprocal of CF antibody titer of pre- and post-*M. pneumoniae* infection sera (2–8 antigen units)	
		CF[b]	Inhibition of metabolism-inhibiting antibody[c]	Patient no. 1 (pre/post)	Patient no. 2 (pre/post)
M. pneumoniae					
Prep. no. 1[a]	1X	2048	1536; 1024	<8/256	<8/64
2[a]	1X	1024	384	<8/512	<8/128
3[a]	2X	2048	384	ND	ND
4[a]	1X	8192	1024; 4096	<8/128	<8/64
5[a]	2X	4096	6144	<8/64	<8/64
6[a]	2X	8192	25,576	<8/64	<8/64
Cardiolipin[e]	ND[d]	<2[f]	<2	ND	ND
	ND	<2[f]	<2	ND	ND
Phosphatidyl glycerol (GPG)					
S. aureus	ND	<2[f]	<2	ND	ND
M. laidlawii B	ND	<2[f]	<2	ND	ND

[a] Produced single spot on silica gel thin-layer chromatogram.

[b] Determined with 4 CF antibody units of convalescent serum from patient with *M. pneumoniae* pneumonia.

[c] Determined with 4 metabolism-inhibiting antibody units of *M. pneumoniae* hyperimmune rabbit serum.

[d] Not done.

[e] Beef heart.

[f] Also <2 when tested after dilution in 25 μg/ml of egg lecithin.

whereas they did react with antibodies in serum collected during convalescence from *M. pneumoniae* pneumonia.

Lemcke *et al.* (5) had reported that auxiliary lipid was required for maximal expression of CF activity by certain semipurified lipid fractions of *M. pneumoniae*. Although the hapten preparations shown in Table II produced only one spot on TLC, their CF activity was not potentiated by the presence of an auxiliary lipid such as egg lecithin at a concentration of 25 μg/ml.

Chemical characterization of semipurified preparations. Preparations 3 and 4 lacked detectable nitrogen (<0.05%). Carbohydrate other than glycerol was detected in preparations 3, 4, and 5 at a level which varied from 0.1 to 0.5%. The quantity of galactose present varied from none detectable to 0.25%.

Analyses of 3 phospholipid standards and the 6 preparations containing hapten for phosphorus, glycerol, and fatty acids are summarized in Table III. The glycerol:phosphorus and fatty acid:phosphorus ratios of the 6 hapten preparations resembled those of phosphatidyl glycerol (GPG). This suggests that the major constituent of the hapten preparations had a structure similar to that of GPG. The low fatty acid content of preparations 5 and 6 probably resulted from partial deacylation occurring during the purification procedure.

Chromatography on Dowex 1 (HCO_3-form) of the aqueous soluble deacylation products of preparations 5 and 6 yielded an elution pattern similar to that shown by GPG (Fig. 1). In contrast, the elution patterns of cardiolipin (GPGPG) and phosphatidic acid (GP) differed from both the hap-

TABLE III. Characterization of *M. pneumoniae* CM, 9:1, Eluates.

Material	Molar ratio	
	Glycerol:P	Fatty acids:P
Phosphatidic acid (GP)	1.0 ± 0.1 (4)[a]	1.0 ± 0.1 (4)[a]
Phosphatidyl glycerol (GPG)		
S. aureus	2.1 ± 0.07 (4)	2.0 ± 0.2 (4)
M. laidlawii B	2.1	2.1
Diphosphatidyl glycerol (GPGPG)[b]	1.5 ± 0.1 (4)	1.9 ± 0.1 (4)
M. pneumoniae CM, 9:1	1.8	2.0
Prep. no. 1	2.0	2.2
2	2.0	2.2
3	2.0 ± 0.1 (5)	2.2 ± 0.1 (5)
4	2.0	1.9
5	2.0 ± 0.05 (3)	1.6 ± 0.08 (3)
6	2.0 ± 0.05 (4)	1.3 ± 0.1 (4)

[a] Mean ± SE (no. of determinations).
[b] Beef heart cardiolipin.

ten preparations and GPG. The elution patterns of hapten preparations 5 and 6 suggested that the major constituent of these materials was a "GPG-like" compound.

Additional evidence for the GPG structure of the major constituent of the hapten preparations was obtained when 1, 2-glycol analysis was performed before and after hydrolysis with E_4NOH. As shown in Table IV the amount of 1, 2-glycol was doubled following deacylation with E_4NOH. A similar result was obtained with the GPG standard. The 1, 2-glycol data show that both fatty acids are present on the same glycerol of the GPG and that the other glycerol has adjacent–OH groups.

The fatty acid composition of preparations 1 and 4 is shown in Table V. This pattern, *i.e.*, a predominance of 16.0, 18.0, and 18.1 fatty acids, resembles that seen in mammalian serum (28). A similarity of this type is not surprising since Rodwell (29) recently has shown that *M. mycoides* incorporates fatty acids directly from the medium.

The water-soluble deacylation product of the preparations and the hexane-soluble fatty acids were without activity when tested by CF.

Antigenicity of CM, 9:1, eluate. A high titered CM, 9:1, eluate of *M. pneumoniae* (65,536 CF units in a 0.1% suspension, *i.e.*, 1 mg/ml) was evaluated for antigenicity in monkeys. Three monkeys each were given 0.01, 0.1, or 1.0 mg of the eluate by the

TABLE IV. 1,2-Glycol Content of Hapten Before and After Hydrolysis with Tetraethylammonium Hydroxide.

Material	Molar ratio (moles/mole of phosphorus)		
	1,2-Glycol		Fatty acids after hydrolysis
	Before hydrolysis	After hydrolysis	
Glycerol-3-PO_4	0.92	0.91	0
Phosphatidic acid	0.0	0.93	2.1
M. pneumoniae CM, 9:1			
Prep. no. 5	0.8	1.9	2.1
6	1.0	2.1	1.6

TABLE V. Fatty Acid Composition of *M. pneumoniae* CM, 9:1, Preparations (chloroform–methanol, 9:1, eluate from silicic acid column) and Cardiolipin.

Fatty acids	Percentage of total fatty acids of indicated type		
	M. pneumoniae CM, 9:1, eluate		Cardiolipin (beef heart)
	Prep. no. 1	Prep. no. 4	
14.0[a]	0.8	0.7	
14.1		0.3	
15.0	0.3	0.5	
16.0	55.7	41.5	0.8
16.1	0.8	1.5	0.8
17.0	1.4	1.2	
18.0	24.9	23.8	
18.1	14.8	26.7	0.7
18.3	1.2	3.8	89.8
20.1			7.3
20.4			trace

[a] No. of carbon atoms to no. of double bonds.

intramuscular route. Neither CF nor growth-inhibiting antibody were detected in serum collected at 2, 3, 4, 5, or 6 weeks after inoculation. Similarly, attempts to stimulate the development of CF antibody were negative when 2 rabbits were given 3.5 mg of CM, 9:1, eluate (8192 CF units in a 0.1% suspension) in a total of 11 intravenous injections at 2-day intervals.

Discussion. Antigens of *M. pneumoniae* have been demonstrated in a number of different serologic tests—CF, indirect hemagglutination (IHA), growth inhibition (GI) and immunodiffusion (IMF). A component(s) extracted from the organism with lipid solvents was shown to be responsible for most of the reactivity with CF and GI antibodies (1–5, 30). *M. pneumoniae* lipid also produced one of the precipitin lines seen in IMF tests and was responsible for the reaction of antigen sensitized tanned red cells with IHA antibody (4). In the latter instance it appeared that lipid was attached to a protein carrier which adsorbed to tanned red cells (4, 30). Although lipid extracts of *M. pneumoniae* were highly reactive in various serologic tests, this material was a very poor antigen (1, 4, 30). In contrast, when lipid hapten remained combined with mycoplasmal protein, it functioned as an effective antigen and was capable of stimulating the development of moderate to high levels of CF, GI, and IHA antibodies (4, 30).

In a previous study, when the lipids of *M. pneumoniae* were separated by silicic acid column chromatography, we found that a major share of CF and GI antibody reactive material was recovered in a CM, 9:1, eluate (2). The pattern of recovery of CF activity in the various eluates was remarkably similar to that of GI antibody blocking activity. In the present study in which another system of solvents was used for silicic acid column chromatography these two activities of *M. pneumoniae* were again closely associated, suggesting that the same moiety of the organism responsible for reactions with both CF and GI antibodies.

In the present study, as before, in most instances the major serologically reactive material was eluated from the silicic acid column with CM, 9:1. One-third of such CM, 9:1, eluates were relatively pure chromatographically, while those preparations which produced two or more spots on thin-layer chromatography produced only one spot after rechromatography. At times other eluates, especially the acetone eluate, contained the major share of serologically reactive material, but such eluates invariably produced multiple spots on thin-layer chromatography. For this reason efforts at chemical analysis were restricted to the relatively purified CM, 9:1, eluates. Glycerol, phosphorus, and fatty acid analysis of 6 preparations of CM, 9:1, eluate indicated that the major constituent of these materials resembled phosphatidyl glycerol (GPG). A 2-fold increase in 1, 2-glycol content following deacylation suggested that both fatty acids were present on the same glycerol molecule.

In view of these findings and the low concentration of carbohydrate containing lipid in the CM, 9:1, eluates it was tempting to ascribe the serological activity of *M. pneumoniae* lipids to phosphatidyl glycerol. However, the findings of Plackett and colleagues

(7), confirmed by Razin *et al.* (8) clearly indicate that the haptens of *M. pneumoniae* are glycolipids which contain glucose and/or galactose. Furthermore, these glycolipids require an auxillary lipid in order to assume a configuration required for serological reactivity. Highly purified glycolipids of *M. pneumoniae* exhibit a low level of serological activity, but when auxillary lipid, reacting with CF antibody and inhibiting the action of GI antibody is added, the haptens show a remarkable degree of activity (7, 8).

If one interprets the present experiments in the light of this information regarding glycolipids and their requirement for auxillary lipid, it would appear that the CM, 9:1, eluates were active because of the presence of small quantities of highly active glycolipids and a large excess of phosphatidyl glycerol which has been shown by Razin *et al.* (8) to act as an effective auxillary lipid for the carbohydrate containing haptens of *M. pneumoniae.*

Summary. When the lipids of *M. pneumoniae* were fractionated by silicic acid column chromatography, the major serologically reactive material was usually eluted with chloroform–methanol, 9:1. Initially or after rechromatography the 9:1 eluates contained primarily a phospholipid moiety which on chemical analysis appeared to be phosphatidyl glycerol. Although this phospholipid did not react with *M. pneumoniae* antibodies, it acted as an auxillary lipid to enhance the activity of the glycolipid haptens which were present in low concentration in the 9:1 eluates.

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