Immunochromical Analysis of Serologically Active Lipids of *Mycoplasma pneumoniae*¹

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The major complement-fixing antigen of *Mycoplasma pneumoniae* is found in the lipid fraction of the organism. When the lipids of *M. pneumoniae* were fractionated by column chromatography on silicic acid, serological activity against both rabbit and human immune sera was found in two fractions, B and D. Fraction B, eluted with chloroform–methanol (9:1), was a minor component in terms of total complement-fixing activity and contained a complex of lipids which were detected in the region characteristic of phosphatidic acids by thin-layer chromatography on Silica Gel G. Fraction D, eluted with ethyl acetate–methanol (3.5:2), had approximately the same complement-fixing antigen titer as the original lipid extract and appeared as a "comet-shaped" spot between phosphatidylethanolamine and phosphatidylcholine on Silica Gel G plates charred with sulfuric acid. However, by thin-layer chromatography on Silica Gel H impregnated with sodium tetraborate, it was demonstrated that fraction D did contain multiple components, all but one of which were carbohydrate-containing lipids (giving positive reactions when sprayed with orcinol-sulfuric acid reagent). Fraction D was found to contain glycerol and phosphate in equimolar ratios but did not contain nitrogen. Two sugars were detected which migrated on paper chromatograms with glucose and galactose.

The serological activity of *Mycoplasma pneumoniae*, as measured by complement fixation against both human and rabbit immune sera, is found primarily in the lipid fraction (13). *M. pneumoniae* is a major cause of atypical pneumonia and nearly all patients infected with this human pathogen show an antibody response measurable by complement fixation with lipid preparations (6, 7).

Lipids can easily be separated from other cellular components, proteins, and polysaccharides. This factor and the relative chemical stability of lipids present advantages for characterization of serologically active components, particularly from a group of organisms such as the *Mycoplasta*ceae, which can be cultivated only at considerable expense with very small yields. Current knowledge indicates that lipid antigens are rare in nature, and an organism in which the major antigenic determinant is lipid would be unique and commend itself to study (24). [Antigen, as used in this paper, refers to a serologically active component without necessarily implying immunogenicity or any particular size of the reactive complex, since the serologically reactive lipid is probably not the single molecule but is in the form of a micelle of relatively large molecular weight (8, 26).] The partial characterization of two distinct serologically active fractions recovered from *M. pneumoniae* is presented in this report.

MATERIALS AND METHODS

Cultivation of *M. pneumoniae*. *M. pneumoniae* strain AP-164 (13), *M. fermentans* PG-18, and *M. laidlawii* strain B were cultivated in a dialysate medium derived from dried active yeast and soy peptone (12). The dialysate medium was supplemented with 12% "salpha" horse serum. Antibacterials were penicillin (200 units/ml) and thallium acetate (0.5 mg/ml). The strain of *M. pneumoniae* used in these experiments was employed between the 9th and 12th transfers on artificial medium from the patient. The 800-ml cultures were agitated with magnetic spin bars and were incubated 4 to 5 days at 37°C (2 to 3 days for *M. laidlawii* at 32°C). The culture medium was acidic at this point. The organisms were concentrated from the medium by centrifugation at 10,000 × g. The pellet was washed three times with phosphate-buffered saline. The organisms were suspended in water and were lyophilized. Each liter of culture yielded approximately 20 mg (dry weight) of organisms.

Extraction and fractionation of lipids. The total lipid, comprising 14 to 18% (dry weight) of the organisms, was extracted from 200 to 300 mg of lyophi-

¹ Some of the data in this paper were presented in a preliminary communication, Bacteriol. Proc. p. 94, 1967.
lized mycoplasmata by suspending the dried organisms in 200 ml of chloroform and 100 ml of methanol overnight at 4°C. The extract was washed with 75 ml of 0.1 M KCl (5). Chloroform-methanol (2:1) was used because it was more effective than neutral solvents for complete extraction of the serologically reactive components from M. pneumoniae (13). The organic solvent was evaporated in vacuo with a rotary evaporator and the lipid residue was reconstituted in 5 ml of chloroform.

This lipid extract, containing 400 to 700 µg of lipid phosphorus, was applied to a siliceic acid chromatographic column composed of 2 g of activated siliceic acid (100 mesh; Mallinckrodt, St. Louis, Mo.) and 1 g of Hyflo-supercel. The resulting column (1 x 9 cm) had a void space of 3 ml. Neutral lipids, choleseterol-esters, triglycerides and diglycerides, free fatty acids, and cholesterol were eluted with 50 ml of chloroform (0.5 ml/min). Polar lipids or phospholipids were then eluted sequentially with solvents of increasing polarity. Fraction A was eluted with chloroform–methanol (20:1), fraction B with chloroform–methanol (9:1), fraction C with chloroform–methanol (6:1), fraction D with ethyl acetate–methanol (3.5:2), fraction E with chloroform–methanol (1:1), and fraction F with chloroform–methanol (1:20). The elution volume for each solvent (fraction) was 30 ml, collected in 5-ml subfractions. Two 1-ml samples were removed from each tube for duplicate nitrogen analysis and 0.5 ml was analyzed for total phosphorus. The contents remaining in each tube were dried under a stream of nitrogen and suspended in 1 ml of absolute ethyl alcohol for assay of serological activity by complement fixation.

The lipids eluted in each fraction were examined by thin-layer chromatography on Silica Gel G (E. Merck AG, Darmstadt, Germany). Sugar-containing phospholipids were separated by thin-layer chromatography on Silica Gel H impregnated with sodium tetraborate. The plates were prepared by the method of Hakomori and Strycharz (9). Samples were applied in 20 µl of chloroform to layers 200 µm thick, and the plates were developed in chloroform–methanol–water (95:35:4 or 65:35:8). The solvent was allowed to flow 16 cm from the origin. Lipids were detected with iodine vapor or by spraying with 5 N sulfuric acid followed by charring 5 min in a muffle oven. Phospholipids were detected with molybdate spray reagent (4), and nitrogenous lipids were detected with 0.2% ninhydrin in acetone. Lipids containing carbohydrates moieties were detected by spraying with 0.25% orcinol in 2 M sulfuric acid and exposing the plates to 120°C for 10 to 15 min.

Phosphatidycholine and phosphatidylethanolamine purified from yeast extracts were kindly supplied by D. J. Hanahan (University of Arizona, Tucson). Lyso phosphatidycholine from egg lecithin and spingomyelin from bovine brain were obtained from Sigma Chemical Co., St. Louis, Mo. Phosphatidyglucose from M. laidlawii strain B was the gift of P. F. Smith (University of South Dakota, Vermillion).

Analytical procedures. Total phosphorus was determined by the method of Bartlett (1) after digesting the samples with 70% perchloric acid. Nitrogen was assayed by nesslerization (14) as modified by Wells and Dittmer (33). Glyceraldehyde analysis was by the method of Hanahan and Olley (10) in which the formaldehyde produced by oxidation of glyceral with periodate is reacted with chromotropic acid in a strong sulfuric acid solution. The periodate oxidation was carried out directly on the 1 N HCl hydrolysates in the modified procedure of Wells and Dittmer (34).

Lipid samples were analyzed for carbohydrate content after hydrolysis with 4 N HCl at 100°C for 3 hr in screw-capped tubes. The HCl was evaporated with a jet of air and the residue was dissolved in water. Hydrolysates were applied to Whatman no.1 paper and chromatographed for 18 hr by descending technique, with ethyl acetate-pyridine-water (12:5:4) as the solvent (11). Carbohydrates on the chromatograms were detected with alkaline silver nitrate reagent (32).

Infrared absorption spectra were obtained with a Beckman IR4 double-beam spectrophotometer. Samples were examined as liquid films on press sintered zinc sulfide (Itran 2, Connecticut Instrument Co., Wilton, Conn.) A scale expansion apparatus was employed.

Antiserum. Antisera were prepared by immunizing rabbits with M. pneumoniae strain AP-164, M. laidlawii strain B, and M. fermentans PG-18 cultivated in dialysate broth supplemented with agama calf serum. Specific details of the immunization procedure are described elsewhere (12). Human M. pneumoniae antiserum were obtained from convalescent pneumonia patients from whom M. pneumoniae had been isolated (6).

Complement-fixation technique. The complement-fixation test employed was a modification of the Kolmer technique (18) scaled for use with microtiter equipment (27). Antigens and antibody were determined by block titration of twofold dilutions of antigen against twofold dilutions of antisera. Two full units of complement were employed. Fixation of complement to the antigen-antibody complex was facilitated by incubation at 4°C for 18 hr prior to the addition of sensitized erythrocytes. End points were designated as that initial dilution containing the least amount of antigen or antibody which completely prevented hemolysis of the sensitized sheep red blood cells. Crude lipid extracts and subfrations eluted from the siliceic acid column were titrated as ethyl alcohol suspensions against rabbit and human antiserum. The ethyl alcohol suspension was serially diluted in complement-fixing diluent. Ethyl alcohol did not give false-positive or negative reactions even at a dilution of 1:6 (i.e., the first dilution).

RESULTS

Extraction of M. pneumoniae lipids. Chloroform-methanol extraction of the lipids of M. pneumoniae completely partitions the serologically active components of the organism into the solvent phase, with no complement-fixing activity remaining in either the residue or the 0.1 M KCl when tested against human sera. Overnight
extraction with chloroform-methanol in the cold did not appear to result in an increased formation of degradation products when compared with less prolonged exposure of the lyophilized organisms to the solvent; no additional products were observed on thin-layer chromatograms, and complement-fixation titers were unchanged. However, the possibility that degradation products are represented in the lipids extracted cannot be ruled out, because the broth cultures were acidic during the last 24 to 48 hr of growth.

Fractionation of *M. pneumoniae* lipids. The lipids of *M. pneumoniae* were fractionated by silicic acid column chromatography. The major phospholipids were found in fractions D, E, and F; fractions B and C contained minor phospholipid components (Fig. 1 and 2). When these fractions were examined by thin-layer chromatography on Silica Gel G plates, A and B were found to contain several unidentified lipids. Fraction C contained a very minor component which migrated with phosphatidylethanolamine as well as a component chromatographically identical to fraction D. Fraction D appeared as a single “comet-shaped” spot on plates charred with sulfuric acid. Fractions E and F contained two lipids which migrated with phosphatidylcholine and sphingomyelin.

Serological activity. Significant complement-fixing activity against human antisera was found in fractions B, C, and D. Complement-fixing activity was found to coincide with peak phosphate levels in subfractions of fraction D (Fig. 1). This coincidence was not so clear for fraction B. The complement-fixing activity found in fraction C was found to be the result of overloading the column with respect to fraction D. This was evident both on rechromatography on silicic acid and thin-layer chromatography on Silica Gel G. The relative complement-fixing activity of the fractions was compared on a molar basis by calculating the titer of a solution containing 1 µmole of phosphorus per ml (CF/P ratio). The CF/P ratio for fraction B was 400; the CF/P ratio for fraction D was 1,000. (This difference was found in several experiments but may not be significant because of the twofold dilution scheme used for complement-fixing assay.) Fraction D was further characterized, but fraction B was not because considerably smaller amounts of lipid were available for analysis. The relative complement-fixing antigen titers of fraction D and crude lipid extract were approximately equal; hence, complement-fixing activity was not lost as a result of the fractionation process. Furthermore, the CF/P ratio did not change when the silicic acid column was eluted with acetone prior to the polar solvents, thus lessening the possibility that neutral glycolipids (not containing phosphorus) contributed to the serological activity of fractions B and D.

Serological specificity. Since mycoplasmae are usually cultivated in media containing protein supplements, contamination of the concentrated organisms with extraneous antigens is quite likely. The lipid composition of the *Mycoplasmataceae* frequently mimics to some degree the lipid composition of the medium (28). To establish that the complement-fixing activity was derived from the organism, the lipid component of agamma horse serum (extracted from 4 g of lyophilized serum, as described for *M. pneumoniae*) was compared with *M. pneumoniae* lipids by thin-layer chromatography (Fig. 3). By thin-layer chromatography, *M. pneumoniae* lipids did not contain lysophosphatidylcholine and contained very little phosphatidylethanolamine in contrast to their relative abundance in horse serum lipids. Fraction D was distinctly synthesized by the organism,
Another control measure for insuring that fraction D contained a specific antigen produced by \( M. \) pneumoniae was the comparison of \( M. \) pneumoniae with two other fermentative species, \( M. \) fermentans and \( M. \) laidlawii strain B, since whatever components might have been selectively adsorbed and concentrated by one organism might well be taken up by another species. The lipid fraction of \( M. \) fermentans is serologically active and distinct from \( M. \) pneumoniae (12), whereas \( M. \) laidlawii is a saprophyte which does not incorporate lipids from media to the extent shown by other Mycoplasma species (28). \( M. \) fermentans and \( M. \) laidlawii lipids were extracted and fractionated in the same manner as those of \( M. \) pneumoniae on silicic acid columns. Elution patterns by phosphate are shown in Fig. 4 and 5. Both species contained major phosphate peaks in their D fractions and they contained strikingly less phosphatidylcholine and sphingomyelin than \( M. \) pneumoniae.

Fractions \( D_{PN} \) (fraction D, \( M. \) pneumoniae), \( D_F \) (fraction D, \( M. \) fermentans), and \( D_{LB} \) (fraction D, \( M. \) laidlawii strain B) were compared by complement-fixing block titration against rabbit antisera to the three organisms. Fraction \( D_{PN} \) had approximately four times the complement-fixing activity of \( D_{F} \) and \( D_{LB} \) when CF/P ratios

since no corresponding spot was found among horse serum lipids. Fractions A and B, represented by that group of unidentified lipids just below the neutral lipids at the solvent front, also contained at least one distinct component which was not detected in horse serum lipids.

Lipids extracted from lyophilized agamma horse serum and from dialyseate broth showed only minimal complement-fixing antigen titers against human antisera (1:4). These lipids were employed in concentrations equivalent to the mycoplasmal lipids. (If the mycoplasma had adsorbed all the serologically active lipids in the medium, the resulting complement-fixing titer from medium lipids would have been 1:4, whereas the \( M. \) pneumoniae lipid titer was 1:128.) The serological activity of both \( M. \) fermentans and \( M. \) pneumoniae lipids could be distinguished from medium lipids in a previous study (12).
were compared (Table 1) and could be clearly distinguished by antigen titer. Sera from patients with primary atypical pneumonia from whom \textit{M. pneumoniae} had been isolated were also used in block complement-fixing titrations against fractions D\textsubscript{PN}, D\textsubscript{F}, and D\textsubscript{LB} (Table 2). Only fraction D\textsubscript{PN} reacted with these sera, indicating that the complement-fixing antigen in fraction D\textsubscript{PN} was specific.

Since the distribution of serological activity in lipid fractions of \textit{M. fermentans} and \textit{M. laidlawii} has not yet been clearly worked out, these data should not be taken to imply that the major lipid complement-fixing antigen of either organism is located in the D fraction. For \textit{M. laidlawii}, the system was complicated by the fact that pooled guinea pig complement was generally markedly anticomplementary, and guinea pig sera had to be selected individually for lack of anticomplementary activity against this antigen. Accordingly, fractions B\textsubscript{PN}, D\textsubscript{PN}, and the crude lipid of \textit{M. fermentans} were compared by complement-fixing block titration against rabbit antisera to \textit{M. pneumoniae} and \textit{M. fermentans} (Table 3). The crude lipids could be distinguished more clearly than fractions D\textsubscript{PN} and D\textsubscript{F} (Table 1), presumably because fraction D\textsubscript{F} did not appear to be the major specific lipid antigen of \textit{M. fermentans}. Hence, \textit{M. pneumoniae} lipid fractions B and D, which are chromatographically distinct, can both be distinguished serologically from the antigen(s) contained in the lipids of \textit{M. fermentans}.

\textbf{Characterization of fraction D\textsubscript{PN}.} A comparison of the elution patterns of \textit{M. pneumoniae} lipids from the silicic acid column, as determined by phosphorus and nitrogen (Fig. 2), shows that fraction D contained phosphorous but not nitrogen. The infrared spectrum of a liquid film of fraction D\textsubscript{PN} (Fig. 6) showed predominant absorption in the 3-\mu m region characteristic of hydroxyl groups, carbon-hydrogen bonding at 3.5 \mu m, ester linkage at 5.8 \mu m, and phosphate ester and ethers in the region of 9 to 9.5 \mu m. This spectrum suggests the presence of carbohydrate residues and confirms that fraction D\textsubscript{PN} does not contain nitrogen, since significant absorption was observed neither in the 6.0 to 6.8 region characteristic of the NH group nor at 8.25 \mu m characteristic of secondary amides.

Fraction D\textsubscript{PN} was then hydrolyzed and analyzed for carbohydrate content by paper chromatography. Two sugars were detected which migrated with glucose and galactose (standards obtained as NRC grade from General Biochemicals, Chagrin Falls, Ohio), respectively. Glycerol analysis of fraction D\textsubscript{PN} hydrolysates indicated that glycerol and phosphorus were present in equimolar amounts, 0.25 \mu M glycerol to 0.22 \mu M phosphorus.

The preceding analyses were based on the assumption that fraction D\textsubscript{PN} was homogeneous.
Indeed, fraction $D_{PN}$ showed a single comet-shaped spot on Silica Gel G (developed by char-ring with sulfuric acid) with a nominal $R_f$ value of 0.41 (Fig. 7) which migrated between phos-

A table follows:

<table>
<thead>
<tr>
<th>Lipid fraction*</th>
<th>$M.$ fermentans</th>
<th>$M.$ pneumoniae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antigen titer</td>
<td>Antibody titer</td>
</tr>
<tr>
<td>$M.$ fermentans (crude lipid)</td>
<td>128</td>
<td>64</td>
</tr>
<tr>
<td>$M.$ pneumoniae (crude lipid)</td>
<td>&lt;8</td>
<td>&lt;8</td>
</tr>
<tr>
<td>$M.$ pneumoniae (fraction D1)*</td>
<td>&lt;8</td>
<td>&lt;8</td>
</tr>
<tr>
<td>$M.$ pneumoniae (fraction B9)</td>
<td>&lt;4</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

* Lipid fractions obtained as described from organisms cultivated in dialyzed broth supplemented with agamma horse serum (12).
* Antisera from rabbits immunized with washed organisms cultivated in dialyzed broth supplemented with agamma calf serum.
* Titer is given as the reciprocal of the highest initial antigen (or antibody) dilution giving complete fixation of complement in a block titration.
* Samples taken from first 5-ml fraction of D.

Phosphatidylethanolamine ($R_f = 0.52$) and phosphatidylcholine ($R_f = 0.24$). Similar comet-shaped spots were observed in the D fractions of $M.$ fermentans and $M.$ laidlawii on Silica Gel G. However, when the comet-shaped spot of fraction $D_{PN}$ was sprayed with orcinol sulfuric acid reagent, it was found to be heterogeneous (Fig. 8). It appeared possible to further resolve the sugar-containing components by Hakomori and Strycharz's method of thin-layer chromatography on borate-impregnated Silica Gel H (9), where carbohydrate-containing lipids are retarded proportionately to sugar residue content. Fractions $D_F$, $D_{LB}$, and $D_{PN}$ were compared by this method (Fig. 9). Fractions $D_F$ and $D_{LB}$ both separated into two components, one of which gave a positive hexose reaction when sprayed with the orcinol-sulfuric acid reagent and migrated with phosphatidylglucose. $M.$ pneumoniae fraction $D_{PN}$ contained only a minor component which migrated with phosphatidylglycerol but showed three larger spots, presumably containing di-, tri-, and tetrahexoses, because they were retarded proportionately. The least polar component, common to all three fractions, gave a negative reaction with the orcinol-sulfuric acid reagent.

**DISCUSSION**

The results presented in this paper lead to the conclusion that the complement-fixing antigen in the most reactive lipid fraction of $M.$ pneumoniae

**FIG. 6. Infrared spectrum of fraction $D_{PN}$ isolated from M. pneumoniae strain AP-164. (One micron is one micrometer.)**
COMPLEMENT-FIXING LIPIDS OF M. PNEUMONIAE

Fig. 7. Thin-layer chromatography of fraction $D_{PN}$ and standards on Silica Gel G with chloroform-methanol-water (95:35:4). (1) Lysophosphatidylcholine (lysolecithin); (2) sphingomyelin; (3) phosphatidylcholine (lecithin); (4) fraction $D_{PN}$; and (5) phosphatidylethanolamine.

(strain AP-164) is a non-nitrogenous lipid and possibly a glycopospholipid. It is a unique finding that a lipid should account for most of the serological activity of an organism and that it is the component to which humans regularly respond by the formation of antibody. A second, less active fraction, chromatographically distinct from the first, was also present. The chemical and serological relationships between the two active fractions is not yet known.

This study was complicated by our initial assumption that fraction $D_{PN}$ was homogeneous; in fact, many of the assay procedures were done on that assumption. Only late in the study was a system found which would resolve the comet-shaped spot of fraction $D_{PN}$ observed on thin-layer chromatographic plates charred with sulfuric acid. The use of Silica Gel H plates impregnated with sodium tetraborate to retard sugar-containing lipids, as well as the orcinol-sulfuric acid spray reagent for detection of glycolipids, made it possible to establish the fact that fraction $D_{PN}$ was heterogeneous. It would seem to contain at least five components, four of which appear to be a family of compounds containing carbohydrate moieties. This heterogeneity may be analogous to the multiplicity of glycolipids carrying a common carbohydrate determinant in the blood group glycolipids (9).

Additional evidence in support of the role conceivably played by sugar residues in determining the serological specificity of lipid extracts of M. pneumoniae was provided by Lemcke et al. (17).

They have reported that the complement-fixing activity of a chloroform-methanol extract was reduced by treatment with periodate and also by mixed carbohydrazes. They consequently suggested that the active component could be glycolipid. We can confirm this in that we found the serological activity in fraction $D_{PN}$ periodate labile. Also interesting in this regard is their demonstration of a serological cross-reaction between a lipid extract from M. pneumoniae and a galactan preparation from M. mycoides (19).

A serologically active lipid reportedly was isolated by silicic acid fractionation of an unwashed acetone extract of M. pneumoniae by Prescott et al. (22) and Sobeslavsky et al. (30). They described the active fraction as a nitrogenous glycerophospholipid which did not contain carbohydrate. The nitrogen was presumably in the form of amino acids rather than amino sugars (31). Thus, the composition reported for that compound appears in sharp contrast to that of
the active components in fraction $D_{PN}$. It is possible that their serologically active fraction corresponds to the serological activity we found in fraction B. Since they extracted the lipids from the pellet of sonically treated organisms with acetone (23), it is possible that: (i) lipids were lost by solubilization in the form of mlecules during sonic disruption (26), and (ii) the more polar components, including fraction $D_{PN}$, may not have been efficiently extracted by acetone, a relatively neutral solvent. Also, different strains of *M. pneumoniae* were employed, and these might have differed in their relative amounts of serological activity in fractions B and D.

The crude lipid extract of *M. pneumoniae* strain AP-164 has been employed as test antigen in a complement-fixation test for diagnosis of *M. pneumoniae* infections in a pneumonia incidence study of a civilian population (6, 7). Nearly all patients, over a 5-year period, from whom *M. pneumoniae* had been isolated, showed antibody increase or high titers in paired sera to this test antigen. Since the AP-164 strain was isolated in 1963, it appears that substantial strain variation with regard to the major lipid antigen had not occurred in Seattle by 1967. Strain AP-164 was deliberately chosen because it was a wild strain (isolated from an ill patient) which had been transferred a minimal number of times on artificial medium and hence had less opportunity for alteration from the virulent state.

A lipid antigen for *M. mycoides* has been reported (3) which accounted for the majority of the complement-fixing activity of *M. mycoides* but did not cross-react with lipid preparations of *M. pneumoniae* (19). The nature of the serologically active lipid of *M. mycoides* is unknown; Plackett (21) reported that the main glycolipid synthesized by *M. mycoides* was a neutral lipid which contained fatty acid esters, glycerol, and galactose in the ratio of 2:1:1. Glycerolphosphorylglycerol and glycerolphosphorylglycerol-phosphorylglycerol deacylation products of phospholipids were also recovered. The serological activity of these fractions was not reported.

A limited survey of six other *Mycoplasma* species (12) showed that only *M. fermentans* possessed a serologically active lipid component as the major complement-fixing antigen. Hence, the possession of serologically active lipids is not a sine qua non for membership in the order *Mycoplasmatales*.

Hexose-containing phospholipids have only recently been reported in microorganisms. Smith and Henrikson (29) have demonstrated the presence of phosphatidylglycerol in *Mycoplasma laidlawii* strain B. *Mycobacterium tuberculosis* and *Mycobacterium phlei* have been found to contain a family of mycosesesol phospholipids with various amounts of D-mannose substituted on the mycosesol ring (16). Conversely, glycolipids which contain no phosphorus have been observed in the nervous system and other tissues, examples of which include cytoplasm H (ceramide-lactose) (25) and globoside (ceramide-glucose-galactose-N-acetylgalactosamine) (35). Gysglycolipidcides from a number of the gram-positive bacteria were described by Brundish et al. (2). The glycolipids of acid-fast bacteria have been reviewed by Lederer (15).

The study of the immunological characteristics of lipids is still a relatively new field of research. Immunochemical and serological methods have been difficult to apply to lipid systems for at least two reasons: (i) the limited solubility of lipids in aqueous medium, and (ii) a lack of insight con-
cerning the physical and chemical nature of lipid-lipid interactions, owing to their amphipathic character in aqueous systems.

To date, complement-fixation has been the method giving the most consistent results in assaying serologically active lipids. Complement-fixation antigen titers of lipids are frequently greatly increased when a mixture of lecithin and cholesterol (equal parts by weight) is added to the purified lipid at ratios of up to 100:1, as reported by Rapport and Rapport et al. (24, 25) in studies with cytolipin H and by Pangborn (20) with cardiolipin. Lecithin and cholesterol react with the lipid antigen to form stable micelles which, behaving as macromolecules (8), are then capable of reacting with homologous antibody to fix complement. Consequently, serological activity may diminish as purification proceeds because of the separation of auxiliary lipids from the active component (24).

Fraction D_{2N} did not appear to require auxiliary lipids for serological activity: (i) the complement-fixing antigen titer of fraction D_{2N} was approximately equal to that of the starting crude lipid extract, and (ii) the titer was not increased on attempts to complex it to bovine albumin. Therefore, it appears that the serological activity of fraction D_{2N} depends, in part, on its ability to form micelles in the absence of additional lipids.

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