Isolation and Characterization of Fractions of *Mycoplasma pneumoniae*

I. Chemical and Chromatographic Separation

B. PRESCOTT, O. SOBELAVSKY,¹ G. CALDES, AND R. M. CHANOCK

Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, U.S. Public Health Service, Bethesda, Maryland, and World Health Organization International Reference Centre for Respiratory Virus Diseases Other than Influenza, Bethesda, Maryland

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ABSTRACT

PRESCOTT, B. (National Institute of Allergy and Infectious Diseases, Bethesda, Md.), O. SOBELAVSKY, G. CALDES, AND R. M. CHANOCK. Isolation and characterization of fractions of *Mycoplasma pneumoniae*. I. Chemical and chromatographic separation. J. Bacteriol. 91:2117-2125. 1966.—Fractionation of *Mycoplasma pneumoniae*, cultured on a beef heart infusion-horse serum-yeast extract medium, was carried out by chemical and chromatographic procedures. The chemical method yielded eight fractions consisting of lipid, carbohydrates, and proteins. Four protein-rich fractions were isolated by chromatographing a supernatant fluid of sonically treated organisms on Sephadex G-25. The 12 fractions were tested for serological and antigenic activity in vitro and in vivo. The lipid fraction was serologically active and the relative order of activity of the protein fractions appeared to depend on the amount of lipid present in the molecule. The highly serologically active Sephadex G-25 protein fraction 1 prepared chromatographically contained 15% lipid in the molecule, whereas the less serologically active protein fraction 2 prepared by chemical means contained 2% lipid. The acetone-extracted lipid fraction was chromatographed on thin-layer chromatography plates and found to consist of nine fractions. Serological activity was associated with only the first three spots above the origin. Lipid extracted from the protein fractions seemed to be similar to the acetone-extracted lipid from the sediment of the sonically treated organisms.

Mycoplasma pneumoniae is an important respiratory tract pathogen of man (2). For this reason, we were interested in fractionating the organism and determining the nature of the antigens which are reactive in different serological tests. For this purpose, we employed chemical and physicochemical procedures which had been used successfully in isolating antigenic components from bacteria and animal tissues (5, 8). In this report, we will describe the results of our preliminary experiments. It should be emphasized that our major interest was in recovering serologically active fractions and not in describing the chemical composition of the organism.

MATERIALS AND METHODS

M. pneumoniae strain. The FH strain of *M. pneumoniae* was isolated in embryonated eggs by Liu from

a patient with atypical pneumonia (6). The organism was subsequently passed 305 times in artificial cell-free media in this laboratory (3). Harvests from the 295th through the 305th passage were used for this study.

Cultivation medium for preparation of crude starting material. *M. pneumoniae* was grown in 5-liter Povitsky bottles, in a broth medium (7) consisting of seven parts of PPLO broth (Difco), two parts of unheated horse serum, and one part of 25% fresh yeast extract (3). The medium was supplemented with 1% glucose, 0.002% phenol red, 1,000 units of penicillin, and thallium acetate at a final dilution of 1:2,000. Each bottle contained 1,000 ml of the medium.

The bottles were inoculated with 10 ml of an *M. pneumoniae* culture containing approximately 5×10^6 to 5×10^7 colony-forming units (CFU)/ml. They were then stoppered with cotton plugs and incubated in a stationary position for 6 to 8 days at 34°C under aerobic conditions. The organisms were then sedimented from the medium by centrifugation in the continuous-flow rotor of a Sorvall RC-2 centrifuge at $30,000 \times g$. The sediment was washed three times in 0.15 M phosphate-buffered saline, pH 7.2 (PBS) by

¹ World Health Organization Fellow from the Institute of Epidemiology and Microbiology, Prague, Czechoslovakia.

further centrifugation in a Spinco model L centrifuge at $30,000 \times g$. The pellet was suspended in deionized water and then subjected to sonic treatment at 10 kc in a Raytheon sonic oscillator for 2 hr at 4 C. The material was then centrifuged in a Spinco model L centrifuge at $63,000 \times g$ for 1 hr. The resulting supernatant fluid was chromatographed on Sephadex G-25 columns, and the sediment was extracted by various chemical procedures.

Control medium. Uninoculated growth medium was studied to determine whether it contributed to any of the serological reactions detected with the *M. pneumoniae* fractions. One liter of uninoculated growth medium was incubated for 8 days at 34 C. This material was lyophilized and then subjected to the same chemical and chromatographic procedures as the *M. pneumoniae* organisms.

Chemical extraction and precipitation. The exact procedure of the chemical extraction of *M. pneumoniae* is shown in Fig. 1. Briefly, sediment from a suspension of *M. pneumoniae* organisms which had been sonically treated for 2 hr and centrifuged at $63,000 \times g$ for 1 hr was extracted with 3 volumes of acetone. The acetone-soluble material was evaporated, and the residue was dissolved in 1 volume of ether, filtered through a no. 50 Whatman filter paper and evaporated. This extract represented the lipid fraction.

The acetone-insoluble residue from lipid extraction was treated with 0.25 N NaOH and centrifuged; the soluble material was precipitated with cold alcohol-ether (3:1). The resulting supernatant fluid was acidified with acetic acid to pH 5.0. The precipitate which formed represented protein fraction 1, and the evaporated supernatant contained the polysaccharide fraction 1.

The precipitate which formed after 0.25 N NaOH treatment and cold alcohol-ether (3:1) precipitation was dissolved in distilled water and the solution precipitated with 1 volume of 15% trichloroacetic acid. This precipitate was dissolved in distilled water and the pH was adjusted to 7.4. This material constituted protein fraction 2.

The supernatant fluid, after trichloroacetic precipitation, was treated with cold alcohol-ether (3:1), and the precipitate which formed was dissolved in distilled water and the pH was adjusted to 7.2. This constituted polysaccharide fraction 2. The water-insoluble residue after preparation of protein fraction 2 was treated with 1 N NaOH, and the procedures for protein fraction 2 and polysaccharide fraction 2 extraction were repeated. The fractions which resulted from this treatment were designated protein fraction 3 and polysaccharide fraction 3.

Polysaccharide fraction 4 was obtained by evaporating the supernatant fluid which formed when polysaccharide fraction 2 was precipitated.

Column chromatography. Column chromatography was used for fractionation of the supernatant fluid of the sonically treated *M. pneumoniae* suspension as well as for additional fractionation of the chemically extracted lipid fraction and protein fraction 2.

Sephadex G-25 chromatography. The supernatant extract from sonically treated organisms was applied

to a column (80 by 5.9 cm) packed with coarse Sephadex G-25, allowed to adsorb overnight at 4 C, and then eluted with distilled water at 4 C. A total of 144 fractions (25 ml each) were collected by means of an automatic fraction collector at a rate of 10 ml/min. The column was then washed with 1 M NaCl to elute residual highly bound material. Absorbance values of the effluent samples were measured at 280 m μ in a Beckman DU spectrophotometer.

Chemically extracted protein fraction 2 was also subjected to chromatography on Sephadex G-25 by the same method.

Sephadex G-25 fine chromatography. The chemically extracted lipid fraction was subjected to chromatography on fine-grained Sephadex G-25 gel. The procedure followed was that of Wells and Dittmer (10). A 200-mg amount of dried lipid fraction was dissolved in 15 ml of a mixture of chloroform-methanol-water (60:30:4.5, v/v) and passed through a column (60 by 1 cm) of fine-grained Sephadex G-25 at a flow rate of 0.5 ml/min. The column was then washed with 25 ml of chloroform-methanol mixture (2:1). The effluent was pooled with the first effluent and dried in vacuo. The resulting material was designated Sephadex G-25 fine fraction 1. The nonlipid material was eluted from the columns by washing with 40 ml of methanol-water mixture (1:1), and the eluate was evaporated. This fraction was designated Sephadex G-25 fine fraction 2.

Thin-layer chromatography. The chemically extracted lipid fraction was subjected to thin-layer chromatography separation on silica gel uniplates (Custom Service Chemicals Inc., Wilmington, Del.). The lipid fraction was dissolved in a chloroform-methanol mixture (2:1) and applied to the plates which had been previously heated overnight at 80 C and washed with ethylene dichloride-methanol (98:2) to remove substances which gave background color. The fractions were then developed with *n*-propanol-12.5% NH₄OH mixture (80:20) for 105 min and dried. After that they were further developed with ethylene dichloride-methanol (98:2) mixture for 105 min and dried. The third developing solvent was a chloroform-96% acetic acid (95:5) mixture which was applied for 75 min. The lipid spots were then located by exposing the dried plate to iodine vapor (9).

Lipid extraction from Sephadex G-25 fraction 1 and protein fraction 2. Lipids from these fractions were extracted by means of a 95% alcohol-ammonium hydroxide mixture. The solvent was evaporated, and the solid residue was dissolved in ether, which was then filtered and evaporated in vacuo. These lipids were subjected to further analysis on thin-layer chromatographic plates by the same procedure employed for the lipid extracted from the sediment of the sonically treated organism suspension.

Paper chromatography. Amino acid analysis of protein fraction 2 and Sephadex G-25 fraction 1 was performed by means of descending paper chromatography with the use of Whatman no. 3 paper and spraying with a 2% solution of ninhydrin. Carbohydrates were identified according to the method of Colombo et al. (4) with the use of 2.5% aniline hy-

FIG. 1. Flow sheet for extraction procedure of fractions from *Mycoplasma pneumoniae*.

drogen phthalate in water-saturated *n*-butanol as a spray (4).

Amino acid analysis. Quantitative determination of amino acids was performed according to the procedure of Wolfe (11), with the ninhydrin reagent of Barrolier (1) substituted for the reagent used by Wolfe. The acid hydrolysate was prepared by refluxing a 20-mg sample in 20 ml of 6 N HCl for 18 hr, evaporating to dryness in vacuo to remove acid, and redissolving the residue in 5 to 10% isopropanol.

Carbohydrate analysis. Carbohydrates were assayed by heating a sample of material in 1 N H₂SO₄ in a boiling-water bath for 1 hr. The solution was then neutralized with barium carbonate by use of congo red as an indicator. The precipitate was filtered, the supernatant fluid was evaporated to dryness in vacuo, and the residue was dissolved in 10% *n*-propanol.

RESULTS

Fractions obtained by chemical procedures. The chemical fractionation scheme shown in Fig. 1 was employed to obtain fractions from the sediment of the sonically treated organism suspension. A total of eight fractions were obtained. These fractions represented approximately 74% of the material subjected to the fractionation procedure. Weight relationships among the various fractions prepared by chemical means are shown in Table 1. The three protein fractions represented approximately one-half of the total yield.

Fractions obtained by chromatography. Figure 2 shows typical results of absorbance versus effluent volume obtained when a water-soluble extract of sonically treated *M. pneumoniae* was applied onto a column of Sephadex G-25 (SG 25). A standard pattern was obtained with the solution separating into four fractions of different chromatographic behavior. Two main peaks were evident when the eluates were measured for absorbance at 280 mμ in a Beckman DU spectrophotometer. Yields

FIG. 2. Chromatography of a water-soluble extract of sonically treated *Mycoplasma pneumoniae* on Sephadex G-25.

from the chromatographic separation are tabulated in Table 2. The pattern shown in Fig. 2 was reproduced when other water-soluble extracts of *M. pneumoniae* were subjected to chromatography on Sephadex G-25.

A sample of Sephadex G-25 fraction 1 shown in Fig. 2 was rechromatographed on Sephadex G-100 by means of the same procedure used for Sephadex G-25. The results (Fig. 3) demonstrate the complex nature and heterogeneity of the Sephadex G-25 fraction 1.

Proteins. The major protein fraction obtained by chemical extraction was protein fraction 2, and the major protein fraction obtained by chromatography was the Sephadex G-25 fraction 1. On the assumption that chemical composition determines immunological activity, analytical studies were performed to determine the relationship of the chemical composition of certain fractions to antigenicity. The Sephadex G-25 fraction 1 was found to be different from protein fraction

TABLE 1. Yields of fractions by chemical procedure

Material	Dry wt*	Per cent of starting material
	g	
Sediment of sonically treated organisms	6.54	100
Lipid	1.015	15.5
Polysaccharide fraction 1	0.025	0.38
Polysaccharide fraction 2	0.035	0.46
Polysaccharide fraction 3	0.870	13.30
Polysaccharide fraction 4	0.340	5.21
Protein fraction 1	0.067	1.02
Protein fraction 2	2.145	32.80
Protein fraction 3	0.320	4.90
Total lipid, polysaccharide, and protein	4.812	73.57

* From 10.24 g of dry whole organisms.

TABLE 2. Yields of fractions by chromatographic procedure

Material	Dry wt*	Per cent of starting material
	g	
Starting material, supernatant fluid of centrifuged sonically treated organisms	3.7	100
Sephadex G-25 fraction 1, pool from tubes 23-31	1.075	29.0
Sephadex G-25 fraction 2, pool from tubes 32-67	1.120	30.3
Sephadex G-25 fraction 3, pool from tubes 68-73	0.210	5.7
Sephadex G-25 fraction 4, pool from tubes 74-136	0.030	0.8
Total	2.435	65.8

* From 10.24 g of dry whole organisms.

FIG. 3. *Rechromatography of fraction 1 in Fig. 2 on Sephadex G-100. Rate of flow was 10 ml/hr.*

2 in composition. Certain chemical differences are shown in Table 3. It is evident from the data that the total nitrogen and carbohydrate contents of the two preparations were different. When samples of these materials were hydrolyzed and paper chromatographed, the carbohydrate components of the Sephadex G-25 fraction 1 were found to consist of glucose and mannose, whereas those of protein fraction 2 were glucose and galactose. In addition, the most striking difference in the two fractions was detected in the lipids extracted from them. Only 2% of the total weight of protein fraction 2 could be extracted as lipid with alcoholic-ammonium hydroxide, whereas more than 15% lipid was extractable from Sephadex G-25 fraction 1. The high content of lipid in Sephadex G-25 fraction 1 indicates clearly the lipoprotein character of the preparation.

The Sephadex G-25 fraction 1 and protein fraction 2 were each hydrolyzed with 6 N HCl for 18

TABLE 3. *Comparison of proteins derived from Mycoplasma pneumoniae*

Constituent	Chromatographic procedure SG 25 fraction 1*	Chemical procedure protein fraction 2
	%	%
Nitrogen.....	12.20	15.20
Phosphorus.....	0.41	0.42
NH ₃ -N.....	0.61	0.92
Carbohydrate†.....	2.06	0.82
Lipid.....	15.28	2.10
Ash.....	2.62	4.30

* SG = Sephadex.

† Calculated as glucose.

TABLE 4. *Difference in amino acids between proteins derived from Mycoplasma pneumoniae*

Amino acid	Prepared by chromatography, Sephadex G-25 fraction 1		Prepared by chemical procedure, protein fraction 2	
	Per cent* amino acid	Per cent† nitrogen	Per cent amino acid	Per cent nitrogen
Alanine.....	4.21	0.66	4.28	0.62
Arginine.....	5.25	1.69	5.79	1.86
Aspartic acid.....	7.22	0.76	12.48	1.31
Cysteine and cystine.....	ND	—	0.58	0.07
Glutamic acid.....	12.98	1.23	15.12	1.44
Glycine.....	9.10	1.70	6.24	1.16
Histidine.....	NM	—	1.51	0.41
Isoleucine.....	1.63	0.18	2.60	0.28
Leucine.....	3.67	0.39	6.55	0.70
Lysine.....	6.19	1.18	5.70	1.09
Phenylalanine.....	2.48	0.21	2.80	0.24
Proline.....	3.80	0.46	3.68	0.45
Serine.....	3.92	0.52	5.03	0.67
Threonine.....	3.65	0.43	5.39	0.64
Tyrosine.....	2.06	0.16	3.00	0.23
Valine.....	3.62	0.43	5.87	0.70
Total.....	69.80	10.00	86.62	11.87
NH ₃ -N.....		0.61		0.92
Humin-N.....		0.08		0.08
Total nitrogen.....		10.69		12.87

* On the basis of dry weight. ND = not detectable; NM = not enough to measure.

† Amino acid nitrogen is expressed as a percentage of the total nitrogen present in the sample after hydrolysis.

hr at 120 to 130 C and chromatographed on paper; the ninhydrin-reacting spots were then assayed for amino acid concentration. The data presented in Table 4 indicate that there were several differences in the overall amino acid composition of the two proteins. Glutamic acid was present in both fractions in larger amounts than any of the other amino acids; there was more glutamic acid in the protein 2 than in the Sephadex G-25 fraction 1. Other differences between the fractions were found in the amounts of aspartic acid, glycine, leucine, serine, threonine, and valine. Cysteine, methionine, and histidine were not detectable in Sephadex G-25 fraction 1, but were present in very small amounts in protein fraction 2. Amino acids accounted for 86.6% of the weight of protein fraction 2 and 69.8% of the weight of Sephadex G-25 fraction 1.

Polysaccharides. Chemical and analytical studies on the polysaccharide fractions indicated that these materials consisted entirely of carbo-

hydrate. Protein nitrogen was not detectable, and lipid could not be found in hydrolyzed samples of the material. In preliminary experiments, only polysaccharide 1 stimulated moderate levels of complement-fixing antibodies in rabbits. No further attempts were made to define any of these fractions.

Lipids. Lipid was removed from the sediment of the sonically treated organism suspension by extraction with acetone. This lipid material possessed a high level of specific *M. pneumoniae* complement-fixing activity (12). The use of lipid solvents other than acetone was not investigated systematically. In several experiments, chloroform-methanol (2:1) extracted serologically active lipid from an acetone-insoluble residue. However, in each of these experiments, the amount of serologically active lipid extracted by chloroform-methanol represented only one-third of that initially extracted by acetone.

Before detailed investigations were undertaken on the composition of the acetone-soluble lipid, preliminary analytical studies showed that the material was nitrogen-free, and contained 0.76% phosphorus and 0.94% carbohydrate in the form of glucose. No other carbohydrates were detectable in the lipid. Attention was then directed to the possible heterogeneity of the material. Non-lipid contaminants were removed from the lipid on a column of fine-grained Sephadex G-25. Two factors were released from the column by stepwise changes of eluting solvent, the first with a mixture of chloroform-methanol (2:1) which yielded 50.9% of the sample applied to the column and the second with methanol-water (1:1) which yielded 7.9%. The behavior of the first fraction on rechromatography on a column similar to the original showed that this fraction was still a mixture.

Thin-layer chromatography on silica gel-G plates was found to be a convenient way to achieve a preliminary fractionation of the complex lipid mixture. When this method was applied to the lipids of *M. pneumoniae*, chromatographic heterogeneity of the sample was observed. The elution pattern produced by this procedure is shown in Fig. 4. The material appeared to resolve into at least nine distinct components. After the spots were located with iodine vapor and marked, duplicate spots without iodine were scooped into tubes and extracted with chloroform-methanol (2:1) for yields and for analytical and biological studies. The first three components were the only ones which showed serological activity; these fractions represented a significant portion of the total lipid complex (19 to 28%). The first three fractions did not contain cholesterol, which is a

FIG. 4. Chromatography of lipid from whole organisms on silica gel G.

major lipid constituent of mycoplasmas which require cholesterol for growth. Cholesterol was present in the sixth thin-layer chromatographic fraction. Several large-scale experiments were performed to separate bands of the first three fractions for further studies. Similar results of total recoveries of pooled (1 to 3) fractions were obtained in three experiments, and a slight variation in quantity of yield in one (27.9; 26.4; 25.9 and 19.3%). Whether this last result was due to variation in technique or variation in the content of these fractions in the lipid complex awaits further study.

The serologically active lipid fractions came from *M. pneumoniae* and not from the growth medium. Fractions of lyophilized concentrated uninoculated growth medium yielded an acetone-soluble lipid fraction; however, this fraction did not fix complement with *M. pneumoniae* antisera. Similarly, the protein, polysaccharide, and Sephadex G-25 fractions of the uninoculated medium were not reactive with *M. pneumoniae* antisera. In addition, serological studies failed to detect growth medium components in the acetone-extracted lipid of *M. pneumoniae*. This lipid reacted to high titer with *M. pneumoniae* antiserum (1,024 units of complement-fixing activity per mg), whereas it failed to fix complement with a

hyperimmune serum prepared against normal medium components.

Comparisons of lipids extracted from protein fractions with lipids chemically extracted from the whole organism. Further studies were performed on the major protein fractions—Sephadex G-25 fraction 1 and protein fraction 2—to determine the nature of the lipids present in these materials. Samples of each material were resuspended in 100 ml of absolute ethyl alcohol. To the suspensions was added 2 ml of concentrated ammonium hydroxide. The mixture was refluxed on a steam bath for 2 hr and cooled to room temperature, and the contents of the flasks were filtered. The filtrate was evaporated to a residue and extracted with anhydrous ether. The ether-soluble material was taken to dryness. Lipids derived from the two major protein fractions and a sample of the lipid extract from the whole-organism suspension tested as a control were subjected to thin-layer chromatography on silica gel-G plates. Several interesting similarities were noted between the derived lipids and the lipids from the whole organism. Figure 5 demonstrates the patterns obtained when these samples were chromatographed in parallel. Five fractions were separable from the lipids of the Sephadex G-25 fraction 1 and six fractions were separable from the lipids of protein fraction 2. In addition, the lipids from the Sephadex G-25 fine fraction 1 yielded 7 discrete spots on the silica gel chromatogram.

The relationship of the fractions which correspond to the first three fractions of the whole-organism lipid are of greatest interest, since only these fractions exhibit serological activity. The first and second fractions of the Sephadex G-25 fraction 1 lipids corresponded in chromatographic position to the first and third fractions of the whole-organism lipid. Similarly, the first and second fractions from the lipids of protein fraction 2 corresponded in position to the first fraction of the whole-organism lipid, whereas the third fraction of the former corresponded to the third fraction of the latter.

The first thin-layer chromatographic fraction of the whole-organism lipid was not homogeneous, since rechromatography on silica gel yielded four fractions. Rechromatography of the first fraction obtained from the lipids of Sephadex G-25 fraction 1 yielded three discrete fractions. These corresponded in position to three of the fractions obtained when fraction 1 of the whole-organism lipid was rechromatographed on silica gel. The chromatographic similarity of these three components was established by mixing silica gel fraction 1 of the whole-organism lipid and the chromatographically similar fraction 1 from the

FIG. 5. Comparison of whole organism lipid with lipids derived from Sephadex G-25 fraction 1 and from protein fraction 2 on silica gel (composite).

Sephadex G-25 material. The mixture of the two fractions yielded a chromatographic pattern which indicated that the three components of the Sephadex G-25 material were similar to the corresponding fractions of the whole-organism lipid.

DISCUSSION

In this study, a number of chemical and chromatographic fractions were separated from a sonically treated suspension of *M. pneumoniae*. Since the organisms were grown in a broth medium containing beef heart infusion, yeast extract, and horse serum, the possibility that the various fractions were contaminated with growth medium constituents cannot be excluded. To minimize such contamination, the organisms were washed three times by centrifugation prior to fractionation; however, some degree of contamination may still have occurred.

Our major interest in fractionating *M. pneumoniae* was in determining the chemical nature of its various antigens. Thus, we were interested in determining the nature of the antigens which react in the complement-fixation, indirect-hemagglutination, and growth-inhibition reactions. Although the various fractions obtained in this study may not have been completely free from broth medium constituents, it is still possible to interpret the data in a preliminary fashion as to the general nature (protein, lipid, or polysaccharide) of the various antigens. In one instance, it seems clear that the serologically reactive lipids of *M. pneumoniae*, i.e., the first three thin-layer chromatographic fractions, were derived from

the organism and not from medium constituents. This also holds true for the serologically reactive lipids separated from protein fraction 2 and Sephadex G-25 fraction 1.

Chemical extraction of the sediment of sonically treated organisms yielded three fractions which were predominantly protein in nature, one fraction which consisted of lipids, and three polysaccharide fractions. The major protein fraction recovered by this technique was protein fraction 2. This fraction was not wholly protein, since it also contained approximately 2% lipids. In addition, it contained small quantities of glucose and galactose.

The major chromatographic fraction of the supernatant fluid from the sonically treated organisms was obtained after the use of Sephadex G-25, and was predominantly protein; however, lipids (15% by weight), glucose, and mannose were also present. In addition to the difference in the quantity of lipids present in the chemically and chromatographically prepared protein fractions, quantitative differences in amino acid composition were also detected. These differences indicate that the chemical method of protein extraction yielded an end product substantially different from the protein prepared by chromatography. In the second paper of this series, it will be shown that the chromatographically prepared protein (lipoprotein) was more active serologically and immunogenically than was the chemically extracted protein fraction. This is not surprising, since the chemical extraction procedure offers a number of opportunities for denaturation to occur, whereas the chromatographic separation is considerably more gentle in this respect.

The complex nature of *M. pneumoniae* is evident from the large number of chromatographically distinct lipids which were identified. On preliminary analysis, the lipids extracted from the sediment of sonically treated whole organisms separated into nine discrete fractions on silica gel. The first three of these fractions were of special interest, since they exhibited complement-fixing activity (12). The major serologically active material was present in the first fraction. When this fraction was rechromatographed on silica gel, four separate fractions were detected. Thus, the lipid extract contained at least 12 discrete fractions, and this must represent a minimal estimate, since only the first fraction was rechromatographed. It is of interest that the three serologically active fractions did not contain cholesterol, which is a major constituent of sterol-requiring mycoplasmas. Preliminary studies indicate that the first three fractions contain phospholipids.

The lipids extracted from the Sephadex G-25

fraction 1 and from protein fraction 2 exhibited some chromatographic similarities to the lipids extracted from the sediment of the organisms. The former lipids yielded five or six fractions on silica gel, and these fractions corresponded in position to a number of the fractions of the lipid extract of the sediment of the organism. The lipids Sephadex G-25 fraction 1 and protein 2 yielded fractions which corresponded to the serologically active first and third fractions of the lipids of the organism sediment. This finding is of some interest, since the early fractions obtained from the Sephadex G-25 fraction 1 lipids were also shown to exhibit complement-fixing activity (12). This suggests that chromatographic behavior of *M. pneumoniae* lipids may prove helpful in the separation and tentative identification of the serologically reactive lipid constituents. The present study represents only a preliminary inquiry into the number and nature of such reactive lipid materials. Further separation and chemical identification of these substances are clearly indicated.

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