INFECTION AND IMMUNITY, Sept. 1970, p. 326-339

Vol. 2, No. 3
Printed in U.S.A.

Isolation, Characterization, and Immunogenicity of *Mycoplasma pneumoniae* Membranes

J. DENNIS POLLACK, NORMAN L. SOMERSON, AND LAURENCE B. SENTERFIT[1]

Departments of Medical Microbiology and Pediatrics, Ohio State University, Children's Hospital, Columbus, Ohio 43205, and Department of Pathology, St. John's Mercy Hospital, St. Louis, Missouri 63141

Received for publication 12 March 1970

Membrane and soluble fractions of *Mycoplasma pneumoniae*, *M. pulmonis*, and *M. laidlawii* B were prepared by hypotonic lysis of whole cells. The membranes of *M. pneumoniae* and *M. laidlawii* B contained, as percentage of dry weight: 34 to 37% protein, 59 to 61% lipid, 3 to 4% carbohydrate as hexose, and 0.2% ribonucleic acid as ribose. $NADH_2$ and $NADPH_2$ oxidase activities were localized in the soluble fractions of *M. pneumoniae* and in the membrane fraction of *M. laidlawii* B. $NADH_2$ oxidase activity was localized in the soluble fraction of *M. pulmonis*. The lipids of *M. pneumoniae* were labeled when the organism was grown in the presence of either radioactive palmitic acid, oleic acid, cholesterol, or glycerol. The lipids were not labeled when grown in the presence of radioactive acetate. Palmitic acid radioactivity was found in neutral lipid, glycolipid, and phosphatide fractions. Immunodiffusion analyses of whole cells and membrane fractions demonstrated three reactive antigens. Two immunodiffusion antigens were localized in the membrane fraction. One of these apparently contains lipid. A third antigen, also considered lipoidal, was found in whole cells. Membrane and soluble fractions of *M. pneumoniae* were immunogenic. The immunogens eliciting metabolic-inhibiting antibodies were localized in the membrane. The membrane preparation also induced the formation of antibodies which fixed complement with an antigen extracted with lipid solvent. The soluble fraction contained a distinct immunogen which induces antibodies reactive in complement fixation with an antigen prepared by phenol extraction.

Membranes of several *Mycoplasma* species have been isolated by osmotic lysis and examined for their gross chemical and enzymatic content (21, 22, 27, 28). The human respiratory pathogen *M. pneumoniae* was not included in these studies, and there is only one report of the preparation of membranes of *M. pneumoniae* (53). In the latter study, cellular fractions were identified as membranes by lack of viability and by electron micrography.

Virtually all of the lipids of mycoplasmas are presumably localized in their membranes (29, 42). Because the antigenic components of *M. pneumoniae* are reported to be lipoidal in nature (1, 10, 13, 17, 20, 25), it can be inferred that they are contained in the membranes. Lipoidal components of *M. pneumoniae* elicit (44, 45, 53) as well as block (25, 45) antibodies reacting in metabolic inhibition tests, indicating that the membrane contains immunogen which stimulates the production of such antibodies. In other reports, this antibody in man has been correlated with prior exposure to the organism and with resistance to naturally acquired infection (3, 26, 41, 47, 48).

This work reports the isolation and the chemical and immunological characterization of *M. pneumoniae* membranes.

MATERIALS AND METHODS

Organisms. *M. pneumoniae* 65-2053 (24) was used after the 31st laboratory passage. This strain was then adapted to grow in a medium containing added oleic acid. *M. laidlawii* B (PG-9) and *M. pulmonis* (N3) were originally obtained from D. G. ff. Edward of Wellcome Laboratories and J. Nelson of Rockefeller University, respectively.

Media. The broth medium was modified from a formula reported earlier (24) and is designated SSR4. It contains: 10.5 g, PPLO dehydrated broth (Difco); 40 ml, 25% fresh yeast extract solution (Microbiological Associates, Inc., Bethesda, Md.); 4.8 g, powdered Eagle's Minimum Essential Medium (Grand Island Biological Co., Grand Island, N.Y.); 20 ml, PPLO Serum Fraction (Difco); 5 g, glucose; 20 mg, phenol red; 11.90 g, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES; Calbiochem, Los Angeles,

[1] Present address: Department of Microbiology, Cornell University Medical College, New York, N.Y.

Calif.); and distilled water to make 1000 ml. Oleic acid (Applied Science Laboratories, State College, Pa.), as a solution of 1.5 mg/ml of 95% aqueous ethanol (v/v), was added to give a final concentration of 6 μg of oleic acid per ml of medium. Preliminary experiments indicated that the yield of mycoplasmas as measured by protein was greater in a medium supplemented with oleic acid. *M. laidlawii* B was grown in SSR4 lacking both PPLO Serum Fraction and exogenously supplied oleic acid; in some experiments it was grown in tryptose broth (21). Mycoplasmas were also grown in SSR4 containing ^{3}H- and ^{14}C-labeled compounds. The final concentration of radioactive compounds, in μCi per ml of medium, was thymidine-*methyl*-^{3}H and uridine-*5*-^{3}H, 0.50; palmitic acid-*9,10*-^{3}H, cholesterol-*7*-^{3}H, acetic acid-*GL*-^{3}H, glycerol-*GL*-^{3}H, 1.00; palmitic acid-*1*-^{14}C and oleic acid-*1*-^{14}C, 0.02–0.10; and ^{14}C-algal hydrolysate, 0.10. Radioactive glycerol was supplied by Isotopes Inc., Westwood, N.J. All other radioactive compounds were obtained from New England Nuclear Corp., Boston, Mass.

When either thymidine-*methyl*-^{3}H or uridine-*5*-^{3}H was used, the level of fresh yeast extract solution was reduced to 30 ml per liter to obtain maximum incorporation of radioactivity. In earlier experiments, *M. pneumoniae* was grown in the presence of thymidine-*methyl*-^{3}H in SSR4 containing from 1 to 5% yeast extract solution. Both the maximum yield of cells measured by protein and the counts per minute per milligram of cell protein occurred with 3% fresh yeast extract solution after 2 days of incubation. The counts per minute per milligram of cell protein was lower after 1 or 3 days of incubation. Identical experiments with uridine-*5*-^{3}H produced comparable responses.

Reagents and solutions. Kappa buffer (κ) was composed of: 0.15 M NaCl, 0.001 M HEPES, and 0.10 mM 2-mercaptoethanol, adjusted to *p*H 7.5. A scintillation cocktail was composed of reagent toluene; 25.15 mM 2-(4′-*tert*-butylphenyl)-5-(4′-biphenylyl)-1, 3, 4-oxadiazole (Butyl-PBD) and 2.87 mM *p*-bis-(*o*-methylstyryl)-benzene (bis-MSB), both supplied by Packard Instrument Co., Inc., Downers Grove, Ill.; and 4.49 mM 2, 6-di-*tert*-butyl-4-methylphenol (BHT; Aldrich Chemical Co., Milwaukee, Wisc.). A deoxyribonucleic acid (DNA) solution contained 0.01% (w/v) calf thymus DNA, highly polymerized sodium salt (Mann Research, Westbury, N.Y.) dissolved in 0.15 M NaCl in 0.10 M NaOH.

Ribonuclease (bovine pancreas, 5× crystallized, 90 K units per mg) was obtained from Sigma Chemical Co., St. Louis, Mo. Deoxyribonuclease (DP, beef pancreas, salt-free) was obtained from Mann Research.

Samples of phosphatidylglycerol, phosphatidylglucose, monoglucosyl diglyceride, diglucosyl diglyceride, and cholesteryl glucoside, all isolated from *Mycoplasma* spp., were kindly supplied by Paul F. Smith, University of South Dakota, Vermillion. Other lipid standards were obtained from Applied Science Laboratories; Analabs, Hamden, Conn.; or Supelco, Bellefonte, Pa.

In some experiments, a lipid-free grade of methanol was used (Lipopure, Applied Science Laboratories).

All chemicals were reagent grade. In some experiments, corrections were made for contaminating substances found in reagent grade solvents.

Growth and harvest of cells. *M. pneumoniae* and *M. pulmonis* were grown for 2 days in 200 ml of SSR4 contained in 2-liter Povitsky bottles by the procedure of Somerson et al. (46). In all experiments, prior to harvesting, the overlaying culture fluids were discarded, and the mass of organisms adherent to the glass was washed three to four times with 100 ml of cold κ buffer solution. Occasionally, κ buffer was replaced by 0.15 M phosphate-buffered saline (PBS), *p*H 7.3. A rubber-tipped rod was used to scrape the organisms from the glass into κ buffer or PBS. The resultant suspension was designated "whole cells." *M. laidlawii* B was grown in broth culture in Erlenmeyer flasks (21), collected by centrifugation, and washed with κ buffer.

Chemical assays. The protein content of samples was determined either by the procedure of Lowry et al. (14) or by the differential absorbance between 215 and 225 nm after Waddell, as described by Murphy and Kies (18), with bovine crystalline albumin as a standard. Protein values were corrected for the presence of HEPES. Ribose was assayed by the method of Ceriotti (2) by using D-(-)-ribose as a standard. Cholesterol or cholesteryl esters were quantitated by the technique of Rosenthal et al. (33), with the addition of 0.523 M acetic anhydride to the acetic acid reagent. Cholesterol and cholesteryl palmitate were used as standards. Carbohydrate was assayed by the anthrone procedure (36) but was modified by increasing the heating time at 100 C to 10 min and by using (+)-glucose as a standard. Glyceride was determined by the technique of Van Handel and Zilversmit (49), with tripalmitin as a standard. Regression analysis showed that equimolar samples of tripalmitin, 1,2-dipalmitin, 1,3-dipalmitin, or 1-monopalmitin over the range of 2.4 to 219.3 nmoles gave identical absorbance [coefficient of correlation is 0.986 (52)].

Separation and assay of lipids. Mycoplasmas were suspended in chloroform–methanol (CM; 2:1, v/v) for 4 to 18 hr at room temperature. The suspension was filtered through small CM-extracted paper filters, and the extract was reduced to dryness under nitrogen. The dried extract was separated from water-soluble nonlipid contaminants by Sephadex chromatography (35). Only the Sephadex chloroform–methanol (19:1, water-saturated fraction) eluate was used for subsequent lipid analysis. The residue remaining after solvent removal was designated "total lipid." Total lipid was assayed gravimetrically.

Total lipid samples were fractionated by silicic acid column chromatography. To separate low polarity (neutral), intermediate polarity (glycolipid), and polar (phosphatide) lipids, a Unisil column technique (35, 50), which was modified to incorporate 1% (v/v) acetic acid in the methanol solvent, was used. Alternatively, a modification of the column procedures of Hirsch and Ahrens (8) was used to resolve neutral lipids from each other and from a fraction containing both glycolipids and phosphatides. Both column procedures were occasionally used in sequence.

Silicic acid column effluents were rechromatographed by thin-layer chromatography (TLC) on

plates coated with Adsorbosil-1 (Applied Science Laboratories) or on commercially precoated aluminum sheets of Silica Gel G (type F-254, Brinkmann Instruments, Inc., Westbury, N.Y.). TLC plates impregnated with borate were prepared by the procedure of Plackett et al. (20). Neutral lipids were chromatographed by TLC in two to four dimensions by using benzene–diethyl ether–acetic acid (50:40:0.2, v/v), benzene–ethyl acetate (5:2, v/v), benzene-heptane (44:56, v/v), and heptane in the first, second, third, and fourth dimensions, respectively. Glycolipids were chromatographed by TLC in one dimension in acetone–acetic acid–water [100:2:1, v/v (7)] or chloroform–methanol–water [65:25:4, v/v (51)]. Phosphatides were resolved by TLC in one dimension in either diisobutyl ketone–acetic acid–water [40:25:3.7, v/v (19)], chloroform–methanol–acetic acid–water [80:13:8:0.3, v/v (40)], chloroform–methanol–28% aqueous ammonia [65:35:5, v/v (35)], or chloroform–acetone–methanol–acetic acid–water [50:20:10:10:5, v/v (35)]. Compounds were visualized with reagent sprays containing Rhodamine 6G, molybdenum (6), alpha-naphthol (39), or chromate-sulfuric acid (35). Spots were extracted with either chloroform, diethyl ether, CM, or methanol–acetic acid–water (100:1:0.1, v/v).

$NADH_2$ and $NADPH_2$ oxidase assay. $NADH_2$ and $NADPH_2$ oxidase activity was measured spectrophotometrically as previously described (21). The reaction mixture (3 ml) contained: 0.105 to 1.155 mg of cell protein, as dictated by enzyme activity, and the following (in micromoles): NaCl, 23.4; HEPES (*p*H 7.4), 7.5; and 2-mercaptoethanol, 1.5. The reaction was started with 3.6 μmoles of $NADH_2$ or $NADPH_2$. Results are expressed as μmoles of $NADH_2$ or $NADPH_2$ oxidized per minute per milligram of cell protein.

Gravimetric assay. Dry-weight determinations of whole cells and subcellular fractions were made after the sample had been washed in 0.155 M ammonium acetate (*p*H 7.0) to remove nonvolatile salts. The washed residues were exposed to continuous evacuation over phosphorus pentoxide for at least 1 week to remove contaminating traces of volatile ammonium acetate and water. Total lipids were weighed in Teflon cups (Ventron Instrument Corp., Paramount, Calif.). All gravimetric assays were conducted to within 10^{-7} g by using a microbalance (Cahn, model G-2, Ventron Instrument Corp.) with the substitution weighing technique.

Immunology and serology. Immunogenicity was examined by injecting guinea pigs with *M. pneumoniae* samples and testing their sera for the development of tetrazolium reduction inhibition (TRI) antibody (37). The double-diffusion gel precipitation technique of Conant et al. (5) was employed for examining antigenic relations of cellular fractions. Washed and dried immunodiffusion slides were stained with modified Crowle's Triple Stain (24) or with 0.5% Sudan Black B (C. I. 26150, National Aniline) in propylene glycol (w/v) for 1 hr (11) for evidence of lipid. Excess dye was removed by washing for 15 min with 15% water in propylene glycol (v/v). Complement fixation (CF) tests were performed by the procedure of Sever (38). Animal sera were tested with three CF antigens prepared from *M. pneumoniae* FH by phenol treatment (4), chloroform-methanol extraction (10), and untreated whole cells (46).

Radioassay. Samples to be assayed for soluble trichloroacetic acid-precipitable material were filtered through nonpadded 0.45-μm membrane filters (HAWP; Millipore Corp., Bedford, Mass.). To 1 ml of filtrate held in an ice bath were added 10 μg of DNA as carrier and trichloroacetic acid to give a final concentration of 5%. After 1 hr, the opalescent reaction mixture was filtered through a Bactoflex filter B-6 (Carl Schleicher and Schuell Co., Keene, N.H.). The filters containing trichloroacetic acid-precipitable residues were washed with cold 5% aqueous trichloroacetic acid. Washed filters were dried in liquid scintillation vials prior to the addition of liquid scintillation cocktail.

The distribution of radioactivity in cellular fractions was examined in 35 to 60% linear sucrose gradients prepared in κ buffer. The gradients were overlaid with approximately 7 mg of cell protein in κ buffer containing 35% sucrose. The gradients were centrifuged for 19 hr at 19,000 rev/min in an SW 25.1 rotor of a Spinco model L ultracentrifuge (Beckman Instrument Co., Palo Alto, Calif.). Twenty-drop samples were collected and filtered through Bactoflex filters B-6 (Carl Schleicher and Schuell). The filters and their cellular residues were washed with κ buffer which was also 0.01 M $MgCl_2$. Washed filters and their washed residues were assayed for radioactivity.

Radioautographs were made of thin-layer plates with X-ray film (No-Screen Medical X-ray film, NS-54T, Kodak, Rochester, N.Y.) by procedures previously reported (23).

Radioactivity was assayed in a liquid scintillation spectrometer (model 3310S, Packard Instrument Co., Inc.) with bialkali photomultiplier tubes and automatic external standardization mode. Efficiencies were determined for ^3H and ^{14}C from previously prepared standard curves by using nitromethane (Matheson Coleman and Bell, Norwood, Ohio) as an internal quencher and toluene as a radioactive source. Efficiencies for single-labeled experiments at minimal quenching were 86.0% for ^{14}C and 54.5% for ^3H. Efficiencies for double-labeled experiments at minimal quenching were 55.0% for ^{14}C, 25.2% for ^3H, and 3.8% for ^{14}C in ^3H channel. Each sample was counted for 10 to 50 min or at a preset count of 5,000.

Disruption of mycoplasmas. Mycoplasmas were disrupted by sonic oscillation while held in a cold bath with a Sonifer (Heat Systems Co., New York, N.Y.) operating at 65 w output for 2 min. The observed sample temperatures never exceeded 4 C. Experiments involving disruption by hypotonic shock were kept at 37 C.

Electron microscopy. Material was either negatively stained or fixed in buffered glutaraldehyde, treated with osmic acid, embedded in Epon 812, sectioned, and treated with uranyl acetate by procedures described by Maniloff et al. (16).

Final procedure for preparation of membrane and soluble fractions of M. pneumoniae. Mycoplasmas were grown, harvested, and washed as described above.

Suspensions of washed whole cells were concentrated by centrifugation in a GSA head of an RC-2B centrifuge (Ivan Sorvall Inc., Norwalk, Conn.) at 9,000 rev/min for 20 to 30 min. Pelleted whole cells were mixed quickly by pipette with 2.5 ml of κ buffer diluted 1:1,000 with deionized water (κ 1:1,000) for every harvested Povitsky bottle and incubated at 37 C. After 15 min, another equal quantity of κ 1:1,000 was added. After 15 min, deoxyribonuclease was added (2.5 mg/100 ml), and the mixture was further incubated for 30 min. Ribonuclease was then added (1.0 mg/100 ml), and the mixture was incubated an additional 30 min. *All further centrifugations for the preparation of membrane fractions were conducted in an SS34 head (Ivan Sorvall Inc.).* The mixture, designated "crude lysate," was centrifuged at 3,000 rev/min for 2.5 min. The pellet was discarded. The supernatant was centrifuged at 19,000 rev/min for 40 min. The resultant supernatant was designated "crude soluble fraction." The pellet was resuspended in a volume of κ 1:1,000 equal to that of the crude lysate and was again mixed with ribonuclease (1.0 mg/100 ml). After 60 min of incubation, the suspension was centrifuged at 2,000 to 3,000 rev/min for 1.5 to 2.5 min, and the pellet was discarded. The supernatant was centrifuged at 19,000 rev/min for 40 min, and the supernatant discarded. The pellet was washed by resuspension in no less than 250 volumes of κ 1:1,000 and collected by centrifugation. The pellet was washed to a total of four to eight times and designated "membrane fraction."

Crude soluble fractions were further treated by centrifugation in a no. 40 rotor of a Spinco model L ultracentrifuge (Beckman Instruments, Inc.) at 40,000 rev/min for 2 hr, discarding the residue; by filtration through a 0.20 μm pore filter (no. 120; Nalgene, Rochester, N.Y.); or by both methods. The supernatant or filtrate is designated "soluble fraction."

RESULTS

Lysis of M. pneumoniae by hypotonic shock. The successful lysis of other *Mycoplasma* spp. by hypotonic shock to obtain membranes prompted these similar experiments with *M. pneumoniae*. Mycoplasmas were grown in medium containing thymidine-*methyl*-^3H and oleic acid. The cells were pooled, concentrated by centrifugation, and washed with κ buffer. One sample was mixed with κ buffer and another sample with κ 1:1,000. Both suspensions were adjusted with appropriate buffers so that their concentrations of protein (determined by the Lowry method) were within 7% of each other. At intervals, samples were assayed for soluble trichloroacetic acid-precipitable material. Data obtained from the κ 1:1,000 series were corrected at each sampling interval for radioactive material released in the undiluted buffer series. Data are expressed as the ratio of radioactivity in trichloroacetic acid-precipitable material from cell-free filtrates to that amount detected at the beginning of the experiment (Fig. 1). The radioactivity is presumed to be DNA which was localized within the mycoplasma. These results showed that approximately 80% of the radioactive DNA could be released by hypotonic shock.

To show that proteins are released by hypotonic shock, we performed the following experiment. Cells were grown in SSR4 containing ^{14}C-algal hydrolysate and collected as described above. One sample was mixed with PBS and another identical sample was added to PBS diluted 1:1,000 with deionized water. At intervals, samples were filtered through a 0.20-μm filter (no. 120 Nalgene). Cell-free filtrates were assayed for both radioactivity and protein. There was a significant increase in both the ultraviolet-absorbing material and the radioactivity found in the hypotonic filtrate (Fig. 2). The data indicated that soluble proteinaceous material located within the mycoplasma could be released by hypotonic shock.

Incorporation of radioactivity into M. pneumoniae lipids. To identify the fraction containing membranes, we added radioactive lipid precursors to the growth medium. *M. pneumoniae* was grown in media containing oleic acid and one of the following radioactive compounds: tritiated palmitic acid, cholesterol, glycerol, sodium acetate, or oleic acid-*1*-^{14}C. Isolated cells grown in the presence of radioactive acetate contained little radioactivity. Washed cells contained 29, 38, and 81% of the total radioactivity added to the media

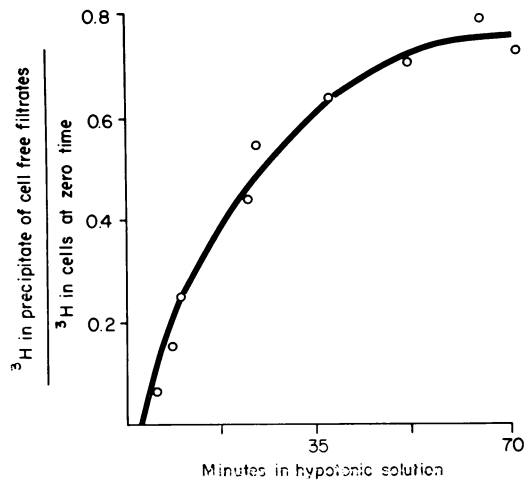

FIG. 1. *Release of radioactive material from cells grown in the presence of thymidine-methyl-*^3H *and subjected to hypotonic shock. Approximately equal quantities of cells were suspended in isotonic κ buffer or κ buffer diluted 1:1,000 with deionized water. Cell-free filtrates were assayed for radioactive trichloroacetic acid-precipitable material. Data in the hypotonic series were corrected for radioactive material found in the isotonic series.*

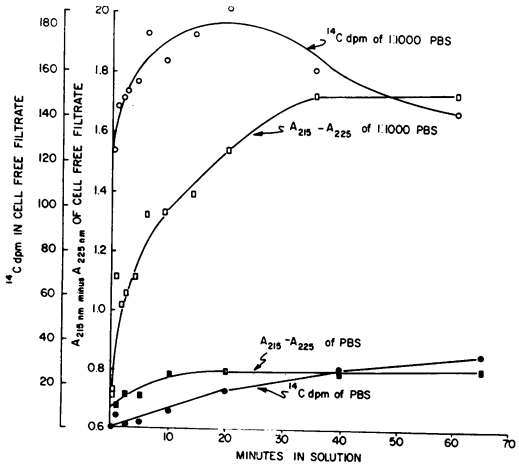

FIG. 2. *Release of proteinaceous material from cells grown in the presence of ^{14}C-algal hydrolysate and subjected to hypotonic shock. Approximately equal quantities of cells were suspended in isotonic phosphate buffered saline (PBS) or PBS diluted 1:1,000 with deionized water. Cell-free filtrates were examined for radioactivity (round symbols) and ultraviolet-absorbing material (square symbols). The upper curves (open symbols) represent the hypotonic condition; the lower curves (closed symbols) represent the isotonic condition.*

as either oleic acid, palmitic acid, or cholesterol, respectively.

At least 98% of the radioactivity detected in unextracted whole cells grown in the presence of radioactive palmitic or oleic acids, glycerol, or cholesterol was recovered in the total lipid fraction. About 75% of the radioactivity in cells grown in the presence of labeled cholesterol was recovered in a free cholesterol fraction. The remaining radioactivity could not be conclusively identified.

These experiments showed that radioactive lipid precursors were incorporated into *M. pneumoniae* lipids.

Incorporation of palmitic acid into neutral lipid. The following series of experiments showed that the radioactivity in the total lipid fraction from palmitic acid-*9,10-^{3}H*-grown cells was found in neutral lipid other than free fatty acids.

The total lipid from cells grown in tritiated palmitic acid was extracted. The total lipid had 2×10^6 ^{3}H counts per min. Cholesterol-*4-^{14}C* (New England Nuclear Corp.) was added as an internal standard. The mixture was applied to a silicic acid column to resolve neutral lipid components and eluates assayed for ^{3}H and ^{14}C. The radioactivity in each sample is given in Fig. 3A as the percentage of total counts per minute recovered. About 97% of the ^{3}H counts/minute applied to the column were recovered by this procedure. Radioactive neutral lipid peaks were tentatively identified by comparison of their elution patterns to those obtained with a standard mixture (Fig. 3B). Samples containing the radioactivity peaks were pooled and co-chromatographed with nonradioactive standards by TLC. Neutral lipid spots were then eluted from TLC plates and assayed for radioactivity. After TLC, approximately 67% of the radioactivity initially applied to the silicic acid column was identified as co-chromatographing with neutral lipid standards. On the average (n = 3), 42% of the ^{3}H radioactivity in the neutral lipid fraction, excluding palmitic acid, was identified as co-chromatographing in TLC with the diglycerides 1,3- and 1,2-dipalmitin.

To provide evidence that the organism converted radioactivity from palmitic acid to diglyceride, a mixture was prepared which contained: tetracosane, cholesteryl palmitate, tripalmitin, cholesterol, palmitic acid, 1,3- and 1,2-dipalmitin, 1-monopalmitin, monogalactosyl diglyceride, lecithin, a crude lipid fraction from *M. pneumoniae*, and palmitic acid labeled *1-^{14}C* or *9,10-^{3}H*. This mixture was incubated with CM, and either fractionated on silicic acid columns, or by four dimensional TLC, or both. In all experiments, no more than 0.8% (n = 8) of the total recovered counts per minute co-chromatographed with both dipalmitins. To correct for the radioactivity from fatty acid appearing in diglyceride, we assumed that all counts per minute found in the neutral lipid fraction were in palmitic acid, and 1% of this radioactivity was subtracted from the amount found in the subsequently isolated diglyceride fraction. We concluded that radioactive palmitic acid could be used to label neutral lipids of *M. pneumoniae*.

Incorporation of palmitic acid into glycolipid and phospholipid. To confirm that glycolipid and phospholipid were labeled by palmitic acid, the peak E fraction (Fig. 3A) containing approximately 6.5×10^5 ^{3}H counts/min was assayed by Unisil column technique for neutral lipid, glycolipid, and phosphatide. Approximately 92% of the ^{3}H counts/minute applied to the column were recovered. The chloroform eluate contained 0.6% of the recovered radioactivity. Thus, neutral lipids were not present in peak E. The acetone and methanol-acetic acid eluates were radioactive and were individually co-chromatographed with phosphatidylglycerol by TLC with an acetone–acetic acid–water (100:2:1 v/v) solvent. In this solvent system, phosphatidic acid migrates slightly ahead of phosphatidylglycerol. The TLC plates were assayed for phosphorus by molybdenum spray and for radioactivity by radioautography or by counting zone scrapings. About 70% of the

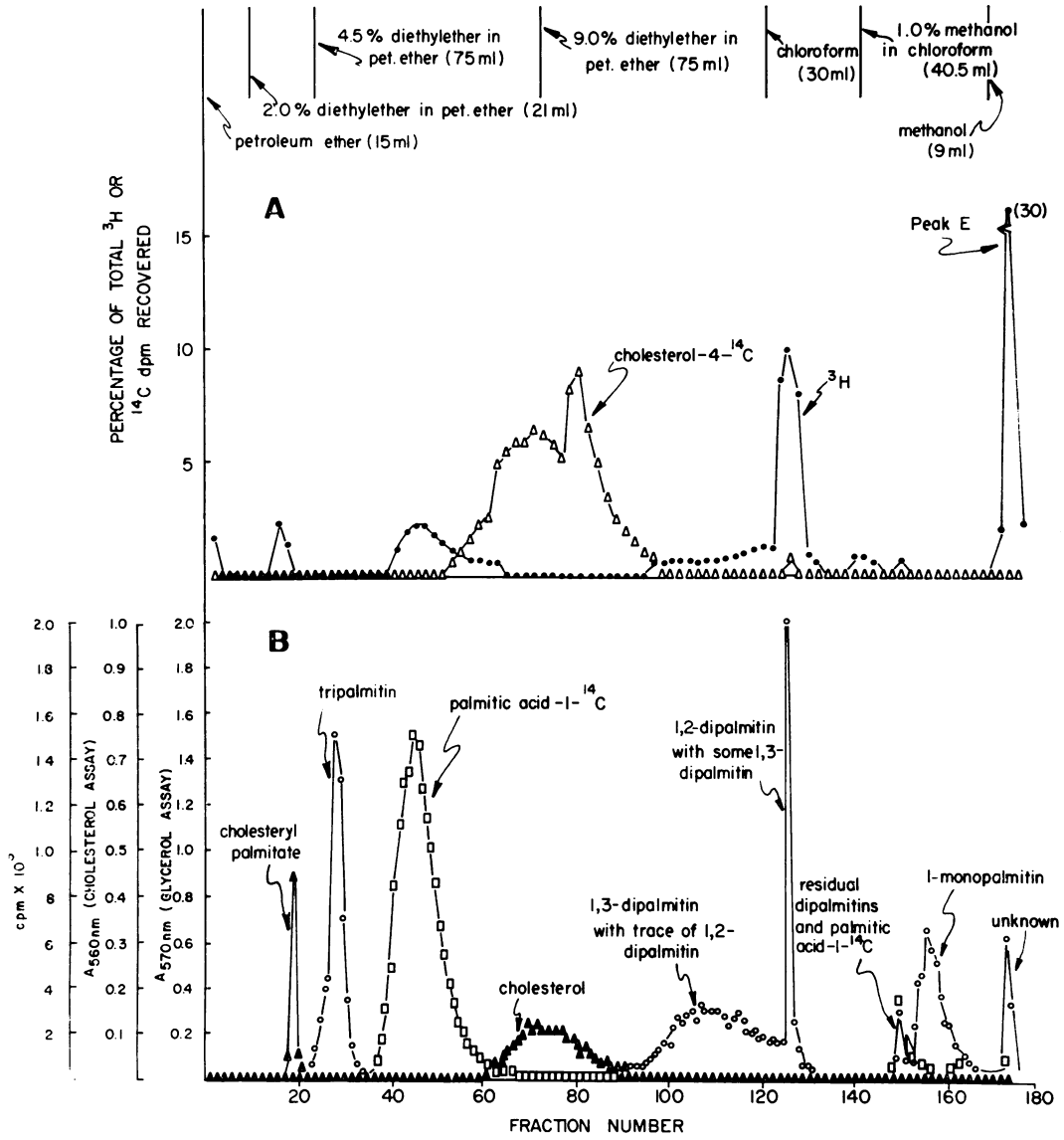

FIG. 3. *Separation of neutral lipids on silicic acid columns after Hirsch and Ahrens (8). (A) Radioactivity in eluates of fractionated total lipids from M. pneumoniae grown in the presence of palmitic acid-9,10-^{3}H. Before application to the column, cholesterol-4-^{14}C was added as an internal standard. The data are expressed as: the percentage of the total ^{3}H counts per minute, ●; or ^{14}C counts per minute, △, recovered. (B) Chromatographic separation of a mixture of seven lipid standards. Fractionation as in A. Each fraction was assayed for radioactivity, □; glyceride, ○; and cholesterol, ▲.*

radioactivity in the acetone eluate migrated near the front and was considered glycolipid (Fig. 4A). Seventy-eight per cent of the methanol-acetic acid eluate radioactivity remained near the origin and was considered phosphatide (Fig. 4B). With the latter eluate, we detected an unidentified phosphorus-containing spot, not migrating with phosphatidylglycerol but containing radioactivity. Further chromatography of the same methanol-acetic acid eluate in four different phosphatide solvent systems indicated the presence of at least six radioactive components not detectable by molybdenum spray. Subsequent chromatography of the acetone eluate from the silicic acid column was done on borate-impregnated plates by using a glycolipid solvent system. Two radioactive com-

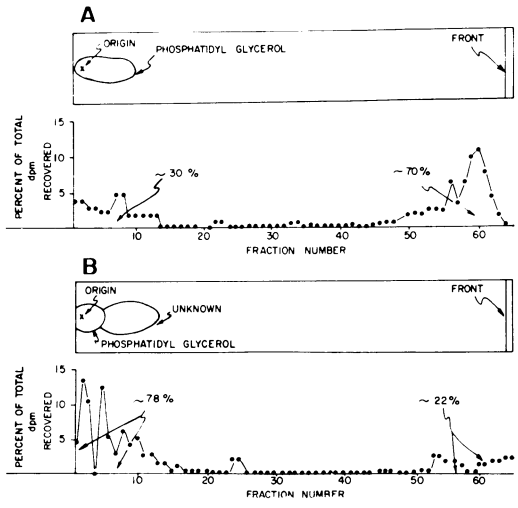

FIG. 4. *TLC of subfractions of peak E shown in Fig. 3A. Peak E was rechromatographed on a Unisil column (35, 50). The loaded column was first eluted with chloroform, then acetone, and finally methanol-acetic acid (100:1, v/v). In 4A, the Unisil acetone eluate was mixed with phosphatidylglycerol, as an internal standard, and chromatographed by TLC on Silica Gel G with acetone-acetic acid-water (100:2:1, v/v). After spraying with molybdenum reagent (upper drawing), zonal scrapings were assayed for radioactivity (upper graph). In 4B, the Unisil methanol-acetic acid eluate was treated, chromatographed, and examined as in A (lower drawing and lower graph).*

ponents were detected. We concluded that radioactive palmitic acid could be used to label glycolipids and phospholipids of *M. pneumoniae*.

From these and other experiments, it was calculated that approximately 25, 9, and 66% of the isolated radioactivity were found in the neutral lipid, glycolipid, and phosphatide fractions, respectively. Thus, palmitic acid can be used as a marker for a variety of *M. pneumoniae* lipids and presumably for the identification of membranes.

Isolation of membrane-rich fraction. Since cells of *M. pneumoniae* release DNA and protein when subjected to hypotonic shock, this technique was investigated as a means of producing membrane-rich fractions. Palmitic acid was used as a marker for cell lipid and membrane, and thymidine was a marker for internal cellular components.

M. pneumoniae was grown in the presence of both thymidine-*methyl*-^{3}H and palmitic acid-*1*-^{14}C. Washed cells were suspended in approximately 100 ml of κ 1:1,000. After 30 min, 10 mg of deoxyribonuclease was added. Thirty minutes later, nine 12-ml portions of the mixture were placed in separate 50-ml centrifuge tubes in an ice bath. All incubations were at 37 C. Each sample was centrifuged for 3 min at a different speed, between 2,500 and 9,500 rev/min, and the sediments were discarded. The supernatants were recentrifuged at 19,000 rev/min for 40 min. The sediments were washed four times in cold κ 1:1,000 containing 0.01 M $MgCl_2$ and resuspended to equal volumes; samples were then assayed for ^{3}H and ^{14}C. A parallel experiment was performed with washed cells disrupted by sonic oscillation. The data are expressed as the ratio of the ^{14}C counts per minute to the ^{3}H counts per minute in each sample (Fig. 5). The ratio in sonically disrupted cells remained relatively constant. Exposure to sonic oscillation produced a suspension whose components could not be qualitatively resolved by centrifugation in the 2,500 to 8,500 rev/min range. The ratio from hypotonically shocked samples, however, was directly proportional to the speed of the initial centrifugation. This indicated that the supernatants obtained at higher initial centrifugation contained fewer whole cells and proportionately more membrane. The second centrifugation at 19,000 rev/min separated sedimentable membranes containing palmitic acid-*1*-^{14}C from such nonsedimentable substances as ^{3}H-DNA, ribosomes, and soluble substances.

These experiments showed that by differential centrifugation a membrane-rich fraction could be isolated from hyptonic lysates.

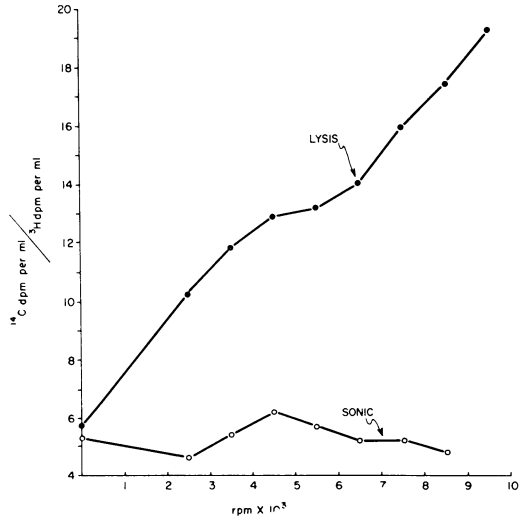

FIG. 5. *Isolation of membrane enriched fractions. Cells were grown in the presence of both palmitic acid-1-^{14}C and thymidine-methyl-^{3}H and subsequently subjected to either hypotonic lysis or sonic disruption. Samples of each preparation were centrifuged (SS34 head, Ivan Sorvall, Inc.) at speeds from 2,500 to 10,000 rev/min. All the resultant supernatants were recentrifuged at 19,000 rev/min for 40 min. The pellets were washed by centrifugation, suspended to equal volumes, and assayed for radioactivity.*

Ribonucleic acid (RNA) content of membrane-rich fractions. To assess the level of RNA in membrane-rich fractions, *M. pneumoniae* was grown in the presence of both uridine-*5-*^{3}H and palmitic acid-*1-*^{14}C, harvested, and subjected to hypotonic shock. But 30 min after the addition of deoxyribonuclease, 5 mg of ribonuclease was added, and incubation was continued for an additional 30 min. Samples were centrifuged for 3 min at a different rev/min between 1,000 and 9,000. The supernatants were recentrifuged, as described above at 19,000 rev/min, washed, resuspended to equal volumes, and assayed for ^{3}H and ^{14}C. The data in Fig. 6 show that the ratio of ^{14}C to ^{3}H increased until 4,000 rev/min and then dropped. Thus, initial centrifugation at 4,000 rev/min for 3 min appears to be optimal for the separation of a membrane-rich fraction from the hypotonic mixture. Supernatants centrifuged at higher initial speeds apparently lost membrane material into the pellet.

We also found that supernatants obtained after centrifugation at 4,000 rev/min yielded residues at 19,000 rev/min containing ^{3}H and ^{14}C which could not be separated on a sucrose gradient (Fig. 7). Such ^{3}H radioactivity most likely represents membrane bound ^{3}H-RNA not removed by ribonuclease. Electron microscopy of thin sections of membrane fractions taken before passage through sucrose gradients showed some apparently whole cells and cells containing internal material (Fig. 8). Further, the great majority of empty cells were not collapsed or visibly ruptured. On the other hand, negative staining of these same preparations showed collapsed cells apparently devoid of all contents.

Enzyme activity of membrane and soluble fractions. Cellular fractions were prepared from

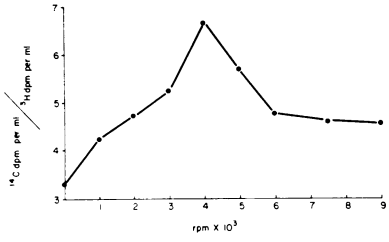

FIG. 6. *RNA content of membrane fractions. Cells were grown in the presence of both palmitic acid-1-*^{14}C *and uridine-5-*^{3}H *and subsequently subjected to hypotonic lysis. Lysates were treated with deoxyribonuclease and ribonuclease. Samples were centrifuged (SS34 head, Ivan Sorvall, Inc.) at various speeds from 1000 to 9,000 rev/min. All the resultant supernatants were recentrifuged at 19,000 rev/min for 40 min. The pellets were washed by centrifugation, suspended to equal volumes, and assayed for radioactivity.*

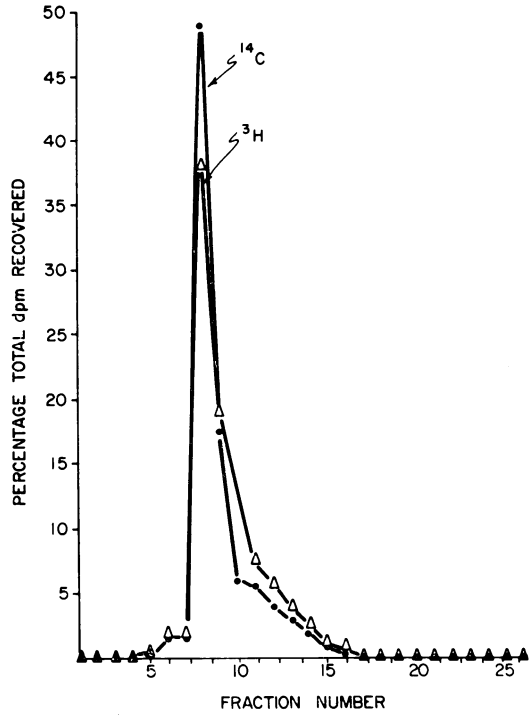

FIG. 7. *Sucrose gradient sedimentation of isolated membrane fraction. Cells were grown, lysed, and treated as in Fig. 6. The lysate was centrifuged at 4,000 rev/min for 3 min. The supernatant was recentrifuged (SS34 head, Ivan Sorvall, Inc.) at 19,000 rev/min for 40 min. The residue, now called membrane fraction, was washed four times with* κ *1:1,000. The washed membrane fraction was centrifuged in 35 to 60% linear sucrose gradient. Twenty-drop fractions were filtered. The washed filter membranes were dried and assayed for radioactivity.*

M. pneumoniae and *M. pulmonis*. *M. laidlawii* B was fractionated as reported elsewhere (21). Whole cells and soluble and membrane fractions were assayed for $NADH_2$ oxidase activity (Table 1). The $NADH_2$ oxidase activity is localized in the soluble fractions of *M. pneumoniae* and *M. pulmonis* and in the membrane fraction of *M. laidlawii* B. These findings support earlier conclusions (21, 22) which associated $NADH_2$ oxidase activity with the soluble fraction of all sterol-requiring *Mycoplasma* spp. examined. The relative difference in $NADH_2$ oxidase activity between the soluble and membrane fractions also reflects the degree of separation and purity of these preparations.

Fractions of *M. pneumoniae* were also examined for $NADPH_2$ oxidase activity. The μmoles of $NADPH_2$ oxidized per minute per milligram of cell protein were: whole cells, 6.4; membrane fraction, 3.2; and soluble fraction, 110.9.

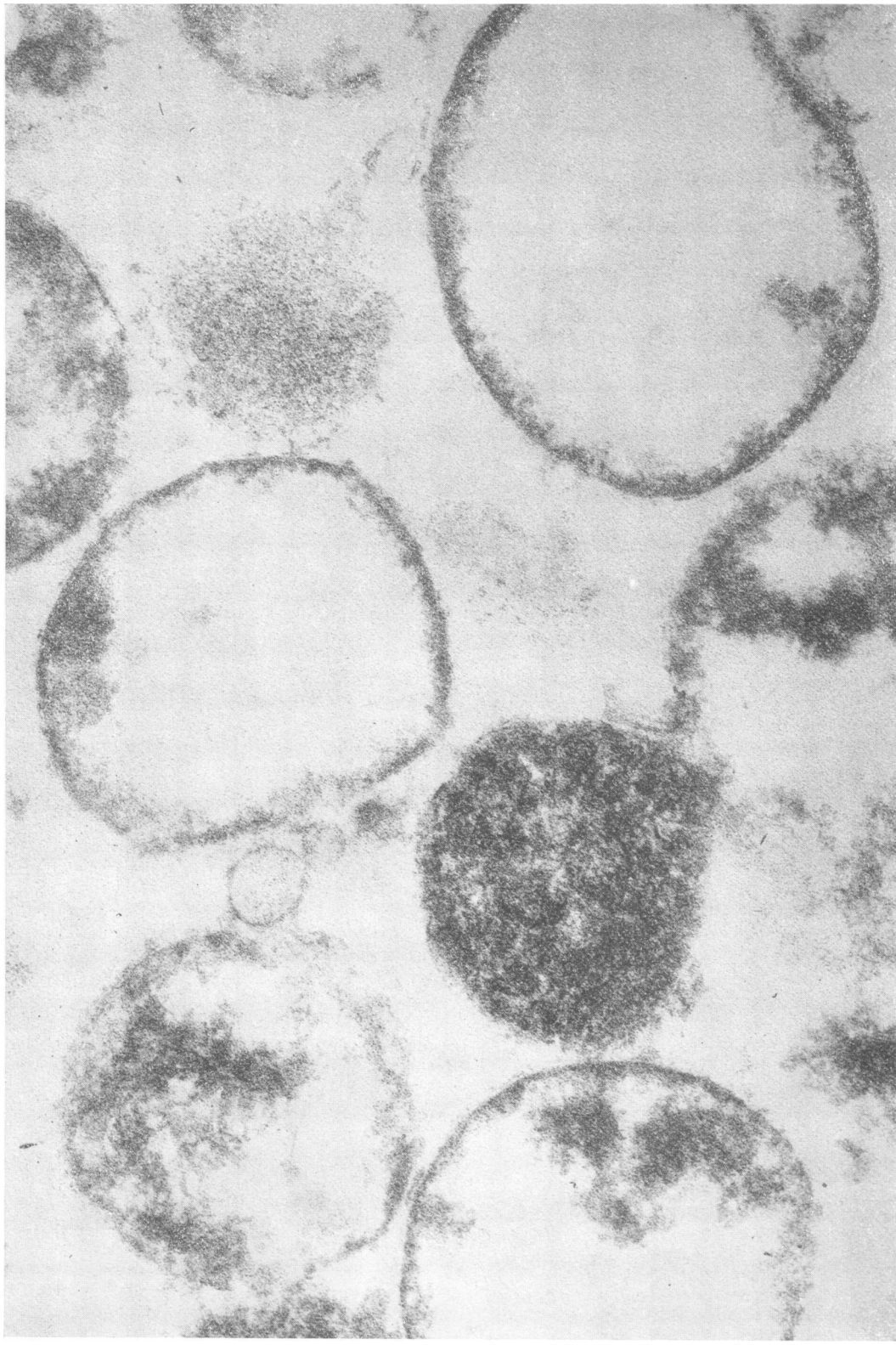

FIG. 8. *Electron micrograph of a thin section of a membrane preparation of M. pneumoniae 65-2053.*

We concluded that *M. pneumoniae* membranes showed the same localization of $NADH_2$ and $NADPH_2$ enzyme activities as other sterol-requiring *Mycoplasma*.

Gross chemical composition of M. pneumoniae and M. laidlawii B membranes. Membrane fractions of *M. pneumoniae* and *M. laidlawii* B were prepared. Dried fractions were weighed and assayed for protein (14), total lipid, carbohydrate, and RNA (Table 2). The chemical composition of membrane fractions from both organisms was similar. The relatively low level of RNA in the *M. pneumoniae* preparations (2.1%) is in agreement with other reports (27).

Immunodiffusion analysis of whole cell and membrane fractions. To determine the relationship of the *M. pneumoniae* membrane antigens to whole cell antigens, preparations of each were diffused simultaneously against two human convalescent sera in immunodiffusion (ID) tests. Homologous strain 65-2053 and strain FH ID antigens, prepared as described elsewhere (5), were used as controls.

The whole cell suspension reacted with serum D (Fig. 9) to produce a band which reacted with identity to a band formed with control antigens. This band is designated A. In addition, a second band was observed, designated B, which was not detectable in the reactions with either control antigen. In a similar ID test, membrane fraction was substituted for whole cell suspension (Fig. 10). Dense lines of precipitation fused in a reaction of identity between membrane fraction and both sera. A second, less dense, band, designated C, was observed between membrane antigen and serum P.

A comparison was made of antigens detectable in whole cell, membrane fraction, and the control FH antigen when reacted against the two sera (Fig. 11, A and B). One antigen (band B) was common to each preparation. Apparently, this is the antigen associated with the B band shown in Fig. 10. Whole cell and control FH antigen preparations contain a second antigen evidenced by band A, which appears to be the A band of Fig. 10. This antigen was not detected with membrane fraction. A third antigen was demonstrated in the reaction between membrane fraction and serum P (band C). This third antigen is also present in

TABLE 1. *$NADH_2$ oxidase activity of fractions of Mycoplasma spp.*

Organism	Amt of $NADH_2$ oxidized per min per mg of protein (μmoles)		
	Whole cell	Membrane	Soluble
Mycoplasma pneumoniae	13.0	5.8	294.0
M. pulmonis	<1	<1	39.1
M. laidlawii B	1.5	308.6	10.2

TABLE 2. *Composition of Mycoplasma membrane fraction as percentage of dry weight ± standard error of mean*

Organism	Protein	Lipid[a]	Carbohydrate[b]	RNA
M. pneumoniae	37.2 ± 8.8	58.5 ± 4.0	3.2 ± 0.8	2.1 ± 0.2
M. laidlawii B	33.5 ± 5.4	60.6 ± 1.1	4.0 ± 0.4	0.1 ± 0.0

[a] Weight of water-washed chloroform-methanol (2:1) soluble matter.
[b] Lipid was not extracted.

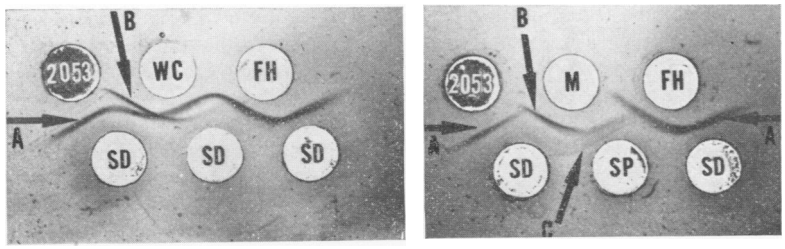

FIG. 9. *Gel diffusion tests performed on a whole cell suspension of M. pneumoniae 65-2053 placed in well WC. Wells 2053 and FH contained, respectively, control immunodiffusion antigen of M. pneumoniae strains 65-2053 or FH prepared as described by Conant et al. (5). Well SD contains human convalescent phase serum D.*

FIG. 10. *Gel diffusion tests performed on a membrane fraction suspension of M. pneumoniae 65-2053 placed in well M. Wells 2053, FH, and SD as in Fig. 9. Well SP contains human convalescent phase serum P, used in earlier work (24).*

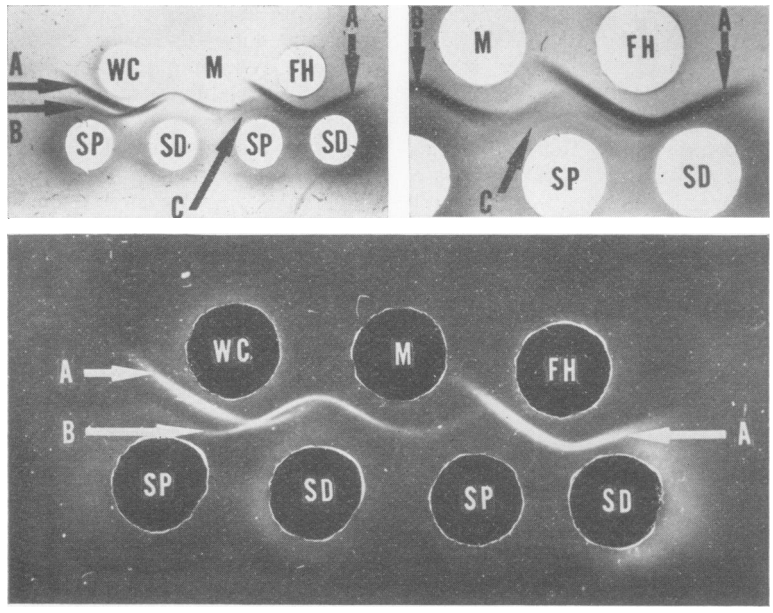

FIG. 11. *(A) Gel diffusion tests performed on suspensions of a membrane fraction and whole cells of M. pneumoniae 65-2053. The membrane suspension in well M was derived from whole cells placed in well WC. Wells FH, SP, and SD as in Fig. 10. (B) Magnified view of portion of Fig. 11A.*

FIG. 12. *Sudan Black B stained preparation of gel diffusion test shown in Fig. 11A.*

the control FH antigen preparation as shown by a reaction of identity. Both A and B bands of the preparation seen in Fig. 11A were stained by Sudan Black B (Fig. 12).

These experiments show that there are at least three reactive immunodiffusion antigens in our preparations of *M. pneumoniae* 65-2053. The B and C antigens, one of which probably contains lipid (B), can be localized in the membrane fraction. A third antigen (A), also presumed to be lipoidal, is found in whole cells but is not demonstrable in membrane fractions.

Immunogenicity of fractions of M. pneumoniae. We wanted to determine the immunogenic potential as well as the antigenic relationships of membrane fractions, soluble fractions, and whole cells. We could also use measurements of the immunogenicity of such fractions (i) to assess the homogeneity of the immunizing preparations and (ii) to obtain evidence about the antigenic composition of the fractions. Our objectives could be accomplished if the antibody responses to immunization with membranes and soluble fractions were different from those of animals receiving whole cells.

Animals were immunized with these preparations. The antisera were tested for TRI antibody as well as in CF tests by using different CF antigen preparations.

Sera from animals immunized with the whole cell or membrane fractions contained growth-inhibiting (TRI) antibody (Table 3). The highest TRI titers were produced with membrane fractions. In CF tests, with whole cells or chloroform-methanol antigens, sera from animals immunized with membranes had mean titers significantly higher ($P < 0.05$) than those from animals immunized with whole cells. By using phenol-treated antigens, sera from animals receiving whole cells had mean titers significantly higher ($P < 0.05$) than those injected with membranes.

The soluble fraction did not elicit growth-inhibiting antibody or CF antibody detected with lipid antigen. Low levels of CF activity were detected with these sera when whole cells were used as antigen. With phenol-treated CF antigens, sera from animals receiving soluble fractions gave a higher CF titer, similar to that obtained when whole cells served as immunogen.

Our results indicated that the membrane fraction contained antigens that stimulated the production of growth-inhibiting antibodies, as well as antibodies reactive in CF with either lipid or untreated whole cell antigens. The soluble fraction contains antigens which stimulate the production of the antibodies detected mainly in CF with phenol-treated antigen. Immunization with whole cells yielded results intermediate to those with the membrane and soluble fractions.

Interestingly, the results obtained in the TRI

TABLE 3. *Immunological response of animals receiving fractions of M. pneumoniae*[a]

Serum of animals receiving immunogenic fraction	Serological test			
	Growth inhibition by TRI test[b]	Complement fixation		
		Whole cell antigen[c]	Chloroform-methanol antigen[d]	Phenol-treated antigen[e]
Whole cells	59 ± 12.3[f]	30 ± 1.6[f]	58 ± 9.3[f]	32 ± 4.1[f]
Membrane	118 ± 20.1[f]	80 ± 19.8[f]	93 ± 12.1[f]	4 ± 1.4
Soluble	1 ± 0.0	6 ± 1.8[g]	1 ± 0.2	25 ± 5.4[f, g]

[a] Each entry represents the mean titer of sera from 10 animals ± standard error of mean.
[b] Tetrazolium reduction inhibition test.
[c] After Somerson et al. (46).
[d] After Kenny and Grayston (10).
[e] After Chanock et al. (4).
[f] Statistical difference of paired groups, t test: TRI test results, whole cells and membrane, $t = 2.58$ ($P < 0.05$); CF results with chloroform-methanol antigen, whole cells and membrane, $t = 2.30$ ($P < 0.05$); CF results with phenol-treated antigen, whole cells, and soluble, $t = 0.99$ ($P > 0.05$); CF results with whole cell antigen, whole cells, and membrane, $t = 2.50$ ($P < 0.05$).
[g] Statistical difference of paired group, t test: $t = 3.37$ ($P < 0.01$).

test correlated closely with those of the CF test with the lipid antigen.

DISCUSSION

The distribution of radioactivity from palmitic acid into neutral lipid, glycolipid, and phosphatide fractions of *M. pneumoniae* is compatible with the findings of Plackett et al. (20). In our work, the neutral lipid to phospholipid ratio of radioactivity from *M. pneumoniae* was higher than that reported with *M. laidlawii* (30). In particular, we found that the diglycerides were significantly labeled. Smith (43) had indicated that diglycerides may be intermediates leading to glycosyl diglycerides. He has shown that membrane fractions of *M. laidlawii* B synthesized monoglucosyl diglyceride from 1,2-diglyceride and uridine-5′-diphosphoglucose. Since lipases are present in many mycoplasma species (34), enzymatic degradation of glycolipids or phosphatides could also account for the high level of labeled diglycerides we observed.

The gross chemical content of membrane preparations of *M. pneumoniae* and *M. laidlawii* B are quite similar. Our data support the findings of Razin et al. (27) and Razin and Boschwitz (28), who showed that the membranes of three *M. laidlawii* strains and *M. bovigenitalium* and *M. mycoides* var. *capri* are chemically similar. However, our percentages of protein were lower and the lipid content higher for the membranes of both *M. pneumoniae* and *M. laidlawii* B than the values they reported. We attribute these differences to our use of another growth medium and to the addition of deoxyribonuclease and ribonuclease in the final preparative steps. The fatty acid content of the membrane of *M. laidlawii* B can be markedly altered by changing the fatty acid content of the growth medium (15, 30, 32). Further, in preparing membranes as described elsewhere (21), deoxyribonuclease and ribonuclease removed Lowry-positive material (Pollack, *unpublished data*). All these factors would be reflected in our data as a higher percentage of lipid and a lower percentage of membrane protein.

The localization of $NADH_2$ oxidase activity in the soluble fractions of hypotonically shocked *M. pneumoniae* and *M. pulmonis* supports evidence that this enzyme activity is uniquely localized in the nonmembranous portion of sterol-requiring *Mycoplasma* spp. (21, 22). Rodwell reported that $NADH_2$ oxidase activity is localized in the soluble fraction of *M. mycoides* (31), and this observation can be extended to include nine species of sterol-requiring *Mycoplasma*.

In immunodiffusion assays, we have shown that isolated membranes not treated with detergent react with human convalescent phase sera. With whole cell preparations, we detected two distinct gel diffusion bands which stained by Sudan Black B, a lipid stain. With membrane preparations, only one of two bands seen stained with Sudan Black B. The reason for the formation of only one lipid-staining band with membrane preparations is uncertain. Perhaps lipoidal elements reactive in immunodiffusion, e.g., glycolipids or lipoproteins, are present in the nonmembranous portion of the cell. More likely, hypotonic lysis might release lipoidal components from the membrane as a nonsedimentable fraction or, if still membrane-bound, become nonreactive in immunodiffusion.

Our studies showed that animals immunized with membrane fractions produced antibodies reacting in TRI test or with lipid antigen in complement fixation tests. A comparison of the titers induced by soluble and membrane fractions showed that the immunizing antigens were localized in the membrane. Williams and Taylor-Robinson (53) have presented evidence that a membrane preparation of *M. pneumoniae* FH induced a metabolic inhibition titer at least eight times higher than a "cell content" preparation. Also Kahane and Razin (9) have compared the ability of membrane and soluble fractions of *M. laidlawii* OR and *M. gallisepticum* A5969 to stimulate the production of metabolic inhibition antibody. These workers indicated that the immunogen inducing metabolic inhibition antibodies was localized in membrane fractions.

Animals immunized with membranes or whole cells developed antibody measurable by both the TRI and lipid-CF tests. Those animals immunized with membrane fractions had significantly higher titers. This probably reflects the greater amount of lipoidal mass present as immunogen. The data support the view that the immunogens which induce antibody reactive in the TRI or lipid-CF tests are localized in the membrane. The correlation of our results in the TRI and lipid-CF tests suggests that both assays may be used to detect the presence of membrane immunogens. In the soluble fraction, we localized immunogens which stimulated the production of high levels of antibodies detected by CF by using a phenol-treated antigen. Apparently, in contrast to other suggestions (9), all antigens of *M. pneumoniae* are not lipid in nature or membrane associated. In chloroform-methanol extracted residues of *M. pneumoniae*, Lemcke et al. (12) found a phenol-extractable component reactive with human sera in double immunodiffusion and in CF. They suggested that this antigen represented a component distinct from that recovered in their chloroform-methanol extracts. Our data support the view that at least two CF antigens exist and are located at different sites within the cell. Antigen extracted by phenol is in the soluble fraction, and the antigen extracted by chloroform-methanol is located in the membrane. This distinction might apply to other *Mycoplasma* spp., allowing specific identification of membrane and internal components by serological techniques.

ACKNOWLEDGMENTS

We thank Donna Clark, Elisabeth Angrick, Evelyn Blackman, and Antoinette Schenk for excellent technical assistance. We also thank Robert M. Conant for his advice and help in the immunodiffusion assays.

This investigation was supported by Public Health Service contracts PH 43-67-78 and PH 43-68-1323 from the Vaccine Development Branch of the National Institute of Allergy and Infectious Diseases, and by Public Health Service General Research Support grant FR 05504 from the Children's Hospital Research Foundation.

LITERATURE CITED

1. Beckman, B. L., and G. E. Kenny. 1968. Immunochemical analysis of serologically active lipids of *Mycoplasma pneumoniae*. J. Bacteriol. **96**:1171–1180.
2. Ceriotti, G. 1955. Determination of nucleic acids in animal tissues. J. Biol. Chem. **214**:59–70.
3. Chanock, R. M., H. H. Fox, W. D. James, R. R. Gutekunst, R. J. White, and L. B. Senterfit. 1967. Epidemiology of *M. pneumoniae* infection in military recruits. Ann. N.Y. Acad. Sci. **143**:484–496.
4. Chanock, R. M., W. D. James, H. H. Fox, H. C. Turner, M. A. Mufson, and L. Hayflick. 1962. Growth of Eaton PPLO in broth and preparation of complement fixing antigen. Proc. Soc. Exp. Biol. Med. **110**:884–889.
5. Conant, R. M., N. L. Somerson, and L. B. Senterfit. 1968. Immunodiffusion reactions between human sera and *Mycoplasma pneumoniae*. Proc. Soc. Exp. Biol. Med. **129**:401–407.
6. Dittmer, J. C., and R. L. Lester. 1964. A simple, specific spray for the detection of phospholipids on thin-layer chromatograms. J. Lipid Res. **5**:126–127.
7. Gardner, H. W. 1968. Preparative isolation of monogalactosyl and digalactosyl diglycerides by thin-layer chromatography. J. Lipid Res. **9**:139–141.
8. Hirsch, J., and E. H. Ahrens, Jr. 1958. The separation of complex lipid mixtures by the use of silicic acid chromatography. J. Biol. Chem. **233**:311–320.
9. Kahane, I., and S. Razin. 1969. Immunological analysis of mycoplasma membranes. J. Bacteriol. **100**:187–194.
10. Kenny, G. E., and J. T. Grayston. 1965. Eaton pleuropneumoniae like organism (*Mycoplasma pneumoniae*) complement-fixing antigen. J. Immunol. **95**:19–25.
11. Lansink, A. G. W. 1968. Thin layer chromatography and histochemistry of Sudan Black B. Histochemie **16**:68–84.
12. Lemcke, R. M., B. P. Marmion, and P. Plackett. 1967. Immunochemical analysis of *Mycoplasma pneumoniae*. Ann. N.Y. Acad. Sci. **143**:691–702.
13. Lemcke, R. M., P. Plackett, E. J. Shaw, and B. P. Marmion. 1968. Immunochemical analysis of *Mycoplasma pneumoniae*. II. Properties of chloroform-methanol extract from *M. pneumoniae*. Aust. J. Exp. Biol. Med. Sci. **46**:123–139.
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. I. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
15. McElhaney, R. N., and M. E. Tourtellotte. 1969. Mycoplasma membrane lipids: variations in fatty acid composition. Science **164**:433–434.
16. Maniloff, J., H. J. Morowitz, and R. J. Barrnett. 1965. Studies of the ultrastructure and ribosomal arrangements of the pleuropneumonia like organism A 5969. J. Cell Biol. **25**:139–150.
17. Marmion, B. P., P. Plackett, and R. M. Lemcke. 1967. Immunochemical analysis of *Mycoplasma pneumoniae*. I. Methods of extraction and reactions of fractions from *M. pneumoniae* and from *M. mycoides* with homologous antisera and with antisera against *Streptococcus M G*. Aust. J. Exp. Biol. Med. Sci. **45**:163–182.
18. Murphy, J. B., and M. W. Kies. 1960. Note on the spectrophotometric determination of proteins in dilute solutions. Biochim. Biophys. Acta **45**:382–384.
19. Nichols, B. W. 1963. Separation of the lipids of photosynthetic tissues: improvements in analysis by thin-layer chromatography. Biochim. Biophys. Acta **70**:417–422.
20. Plackett, P., B. P. Marmion, E. J. Shaw, and R. M. Lemcke. 1969. Immunochemical analysis of *Mycoplasma pneumoniae*. III. Separation and chemical identification of serologically active lipids. Aust. J. Exp. Biol. Med. Sci. **47**:171–195.

21. Pollack, J. D., S. Razin, and R. C. Cleverdon. 1965. Localization of enzymes in *Mycoplasma*. J. Bacteriol. 90:617–622.
22. Pollack, J. D., S. Razin, M. E. Pollack, and R. C. Cleverdon. 1965. Fractionation of mycoplasma cells for enzyme localization. Life Sci. 4:973–977.
23. Pollack, J. D., and M. E. Tourtellotte. 1967. Synthesis of saturated long chain fatty acids from sodium acetate-*1*-C^{14} by *Mycoplasma*. J. Bacteriol. 93:636–641.
24. Pollack, J. D., N. L. Somerson, and L. B. Senterfit. 1969. Effect of *p*H on the immunogenicity of *Mycoplasma pneumoniae*. J. Bacteriol. 97:612–619.
25. Prescott, B., G. Caldes, W. D. James, and R. M. Chanock. 1967. Phospholipid hapten of *Mycoplasma pneumoniae*. Ann. N.Y. Acad. Sci. 143:497–500.
26. Purcell, R. H., D. Wong, R. M. Chanock, D. Taylor-Robinson, J. Canchola, and J. Valdesuso. 1967. Significance of antibody to *Mycoplasma* as measured by metabolic-inhibition techniques. Ann. N.Y. Acad. Sci. 143:664–675.
27. Razin, S., M. Argaman, and J. Avigan. 1963. Chemical composition of mycoplasma cells and membranes. J. Gen. Microbiol. 33:477–487.
28. Razin, S., and C. Boschwitz. 1968. The membrane of the *Streptobacillus moniliformis* L-phase. J. Gen. Microbiol. 54:21–32.
29. Razin, S., and R. C. Cleverdon. 1965. Carotenoids and cholesterol in membranes of *Mycoplasma laidlawii*. J. Gen. Microbiol. 41:409–415.
30. Razin, S., M. E. Tourtellotte, R. N. McElhaney, and J. D. Pollack. 1966. Influence of lipid components of *Mycoplasma laidlawii* membranes on osmotic fragility of cells. J. Bacteriol. 91:609–616.
31. Rodwell, A. W. 1967. The nutrition and metabolism of Mycoplasma: progress and problems. Ann. N.Y. Acad. Sci. 143:88–109.
32. Rodwell, A. W. 1968. Fatty-acid composition of *Mycoplasma* lipids: biomembrane with only one fatty acid. Science 160:1350–1351.
33. Rosenthal, H. L., M. L. Pfluke, and S. Buscaglia. 1957. A stable iron reagent for determination of cholesterol. J. Lab. Clin. Med. 50:318–322.
34. Rottem, S., and S. Razin. 1964. Lipase activity of mycoplasma. J. Gen. Microbiol. 37:123–134.
35. Rouser, G., G. Kritchevsky, and A. Yamomoto. 1967. Column chromatographic and associated procedures for separation and determination of phosphatides and glycolipids, p. 99–162. *In* G. V. Marinetti (ed.), Lipid chromatographic analysis. Marcel Dekker, Inc., New York.
36. Scott, T. A., Jr., and E. H. Melvin. 1953. Determination of dextran with anthrone. Anal. Chem. 25:1656–1661.
37. Senterfit, L. B., and K. E. Jensen. 1966. Antimetabolic antibodies to *Mycoplasma pneumoniae* measured by tetrazolium reduction. Proc. Soc. Exp. Biol. Med. 122:786–790.
38. Sever, J. L. 1962. Application of a microtechnique to viral serological investigation. J. Immunol. 88:320–329.
39. Siakotos, A. N., and G. Rouser. 1965. Analytical separation of nonlipid water soluble substances and gangliosides from other lipids by dextran gel column chromatography. J. Amer. Oil Chem. Soc. 42:913–919.
40. Skipski, V. P., M. Barclay, E. S. Reichman, and J. J. Good. 1967. Separation of acidic phospholipids by one-dimensional thin-layer chromatography. Biochim. Biophys. Acta 137:80–89.
41. Smith, C. B., R. M. Chanock, W. T. Friedewald, and R. H. Alford. 1967. *Mycoplasma pneumoniae* infections in volunteers. Ann. N.Y. Acad. Sci. 143:471–483.
42. Smith, P. F. 1968. The lipids of *Mycoplasma*, p. 69–105. *In* R. Paoletti and D. Kritchevsky (ed.), Advances in lipid research. Academic Press Inc., New York.
43. Smith, P. F. 1969. Biosynthesis of glucosyl diglycerides by *Mycoplasma laidlawii* strain B. J. Bacteriol. 99:480–486.
44. Sobeslavsky, O., B. Prescott, W. D. James, and R. M. Chanock. 1966. Isolation and characterization of fractions of *Mycoplasma pneumoniae*. II. Antigenicity and immunogenicity. J. Bacteriol. 91:2126–2138.
45. Sobeslavsky, O., B. Prescott, W. D. James, and R. M. Chanock. 1967. Serological and immunogenic activities of different fractions of *Mycoplasma pneumoniae*. Ann. N.Y. Acad. Sci. 143:682–690.
46. Somerson, N. L., W. D. James, B. E. Walls, and R. M. Chanock. 1967. Growth of *Mycoplasma pneumoniae* on a glass surface. Ann. N.Y. Acad. Sci. 143:384–389.
47. Steinberg, P., S. L. Fuld, R. J. White, R. Gutekunst, R. M. Chanock, and L. B. Senterfit. 1969. Ecology of *Mycoplasma pneumoniae* infections in marine recruits at Parris Island, South Carolina. Amer. J. Epidemiol. 89:62–73.
48. Taylor-Robinson, D., R. H. Purcell, D. C. Wong, and R. M. Chanock. 1966. A colour test for the measurement of antibody to certain mycoplasma species based upon the inhibition of acid production. J. Hyg. 64:91–104.
49. Van Handel, E., and D. B. Zilversmit. 1957. Micromethod for the direct determination of serum triglycerides. J. Lab. Clin. Med. 50:152–157.
50. Vorbeck, M. L., and G. V. Marinetti. 1965. Separation of glycosyl diglycerides from phosphatides using silicic acid column chromatography. J. Lipid Res. 6:3–6.
51. Wagner, H., L. Hörhammer, and P. Wolff. 1961. Dünnschichtchromatographie von Phosphatiden und Glykolipiden. Biochem. Z. 334:175–184.
52. Walker, H. M., and J. Lev. 1953. Statistical inference. Henry Holt and Co., New York.
53. Williams, M. H., and D. Taylor-Robinson. 1967. Antigenicity of mycoplasma membranes. Nature (London) 215:973–974.