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Effect of *p*H on the Immunogenicity of *Mycoplasma pneumoniae*

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

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

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Mycoplasma pneumoniae harvested from media which had become acid lost the ability both to induce formation of tetrazolium reduction inhibition antibody and to act as antigens in immunodiffusion against human convalescent-phase sera. Incorporation of *N*-tris(hydroxymethyl)-2-aminoethane sulfonic acid and *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid buffers into a new medium containing PPLO Serum Fraction instead of horse serum delayed the *p*H decline. Tris(hydroxymethyl)aminomethane, triethanolamine, and 3,6-endomethylene-1,2,3,6-tetrahydrophthalic acid buffers inhibited growth. Mycoplasmas obtained from buffered cultures retained antigenicity as measured by immunodiffusion and could stimulate tetrazolium reduction inhibition antibody formation in animals.

Mycoplasma pneumoniae, the etiological agent of cold agglutinin-positive primary atypical pneumonia, has been shown to grow more rapidly in a medium containing dextrose (2). In the present studies, emphasis was placed on producing maximal amounts of mycoplasmas for use in serological tests. The possible deleterious effect of lowered *p*H or the accumulation of metabolic products was not investigated. We have studied the cultural characteristics of *M. pneumoniae* and some of the factors that influence the production and quality of immunogenic and antigenic materials. Our report details the damaging effect of low *p*H on the immunogenicity of antigens produced with this organism. In addition, the formulation of a new medium with a high buffer capacity suitable for mass culture of *M. pneumoniae* is detailed.

MATERIALS AND METHODS

Organisms. *M. pneumoniae* strain FH was originally isolated from material obtained by C. Liu (12). This strain was used between the 306th and the 317th passages on artificial medium. *M. pneumoniae* strain 65-2053 was reisolated by us from a clinical specimen supplied by Jose Canchola, Children's Hospital, Washington, D.C., and was used between the 5th to 7th passages.

Media. Two formulas were used in preparing broth media. The first, designated "standard," was made by following Hayflick's procedure (8). It contained 20% horse serum and a yeast extract supplement, but was modified by addition of glucose to 1% and phenol red to 0.002% final concentrations. The second medium, designated SSR1, was composed of: PPLO broth, dehydrated (Difco), 10.5 g; 25% fresh yeast extract solution (Microbiological Associates, Inc., Bethesda, Md.), 50 ml; Eagle's Minimum Essential Medium powdered (Grand Island Biological Co., Grand Island, N.Y.), 4.8 g; PPLO Serum Fraction (Difco or Microbiological Associates), 50 ml; glucose, 5 g; phenol red, 20 mg; penicillin G, 500,000 units; distilled water to make 1,000 ml. In some experiments, the medium contained 25 mg of thallium acetate per liter. The final *p*H of all media was 7.5.

Growth of cells. Mycoplasmas were grown as described by Somerson et al. (20) in 500 ml of media contained in 2-liter Povitsky bottles. The medium in each bottle was inoculated with approximately 1 ml containing 10^6 to 10^7 viable mycoplasma particles. Cultures were incubated at 37 C. Within 1 to 2 days, a confluent layer of organisms attached to the glass surface. In all experiments, the overlaying culture fluids were discarded and the mass of organisms adhering to the glass was washed four times, 100 ml per wash, with sterile 0.15 M phosphate-buffered saline (PBS), *p*H 7.3. Washing was effective in removing culture medium constituents, since the fourth PBS wash, after centrifugation at 37,000 × *g* for 30 min, contained less than 2 μg of protein per ml as measured by the method of Lowry et al. (13). A rubber-tipped rod was used to scrape the organisms from the glass into 20 ml of PBS. The *M. pneumoniae* suspensions were divided into portions and stored at −60 C.

Quantitation of viable mycoplasma particles. To determine the number of mycoplasmas, three to five samples from each suspension were titrated by serial 10-fold dilutions in the standard or SSR1 medium. Titrations were conducted in 1-dram screw-capped vials which were tightly closed. Vials were kept at 37 C for at least 16 days. The titration end point was determined colorimetrically against a standard adjusted to *p*H 6.9. All vials on the acid side of the *p*H 6.9 stand-

ard were considered positive for the presence of organisms. The number of mycoplasmas in broth cultures was calculated by the method of Reed and Muench (15) as acid-forming units per milliliter (AFU_{50}).

Assessment of immunogenicity. The immunogenicity of *M. pneumoniae* preparations was assessed by injecting animals with suspensions and examining sera for the presence of antibody. Preliminary experiments had indicated that adequate antibody responses were obtained by intramuscular injections. As a routine procedure, mycoplasmas were inctivated with 1:4,000 Formalin, the preparation was diluted to contain 18 μg of nitrogen per ml, and 1 ml was injected intramuscularly into either a young adult hamster or a guinea pig. After 4 to 6 weeks, sera were obtained by cardiac puncture.

Tetrazolium reduction inhibition (TRI) antibody test. TRI antibody tests were performed as described by Senterfit and Jensen (17, 18). The test was modified by supplementing the culture medium with 1% glucose (w/v) and 2% (v/v) fresh normal guinea pig serum which had been pretested for the presence of inhibitors.

Gel diffusion. An assessment of the antigenic quality of *M. pneumoniae* suspensions was obtained by double-diffusion gel precipitation using the micromethod of Conant et al. (3). All reagent wells were 5 mm in diameter and diffusion distance between wells was 2 to 3 mm. Suspensions of organisms were placed in outer wells and diffused against human convalescent-phase *M. pneumoniae* serum. Tests were read after incubation at 33 C for at least 16 hr. Reactions were recorded photographically after washing, drying, and staining. Slides were stained with Crowle's Triple Stain (4) modified by substituting 0.005% (w/v) Coomassie Brilliant Blue-R250 (Mann Research Laboratories, N.Y.) for amidoswarz 10B. Slides were stained for 7.5 min and destained in 2% aqueous acetic acid (w/v) until background stain was reduced.

Disruption of mycoplasmas. Organisms were disrupted by either nine rapid freeze and thaw cycles between −20 C and at 30 C or by sonic oscillation in an ice bath using a Sonifer (Heat Systems Co., New York, N.Y.) at 65 w output for 3 min.

Assays. The turbidity of mycoplasma suspensions was assessed by spectrophotometric examination at 400 nm and 625 nm. The protein content was assessed by the procedure of Lowry et al. (13) using bovine crystalline albumin as a standard, and total nitrogen was assessed by micro-Kjeldahl or by the method of Johnson (10).

Buffers. The following buffers were used: *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES) and *N*-tris(hydroxymethyl)-2-aminoethane sulfonic acid (TES), both supplied by Calbiochem, Los Angeles, Calif.; Tris (hydroxymethyl)aminomethane (Tris) from Sigma Chemical Co., St. Louis, Mo.; 3,6-endomethylene-1,2,3,6-tetrahydrophthalic acid (EMTA) from Aldrich Chemical Co. Inc., Milwaukee, Wis.; and triethanolamine from Matheson Coleman and Bell, Norwood, Ohio.

RESULTS

Immunogenicity of M. pneumoniae grown in standard medium. *M. pneumoniae* strain FH was grown in standard medium and harvested at various intervals beginning at day 4 and concluding at day 15 (Fig. 1). At these intervals, the *p*H of culture fluids, total nitrogen of glass-attached mycoplasma, and immunogenicity were determined. The initial *p*H of 7.6 dropped to approximately 5.5 by day 7 and remained relatively constant throughout the remainder of the experiment. The nitrogen level decreased from approximately 1,600 μg at day 4 to 400 μg at day 10, and then rose to 700 μg at day 15.

Mycoplasmas harvested at intervals over the 15-day period were suspended in PBS, and the nitrogen content was adjusted to 17 to 18 μg per ml in all preparations. Hamsters and guinea pigs were immunized with these suspensions. Sera from animals immunized with cells harvested at 4 and 7 days had much higher TRI antibody titers than those immunized with cells taken at 10 to 15 days. The lower TRI antibody titers in animals immunized with 10- to 15-day cell suspensions were associated with a reduction in the *p*H of the culture from which the organisms were harvested. These results indicate that the antigenicity of mycoplasmas harvested from cultures only a few days old is different from organisms harvested later.

Effect of medium replacement on the maintenance of cell nitrogen and viability. The association of decreased TRI antibody response of *M. pneumoniae* antigens with a decrease in the *p*H of the culture fluids from which they were harvested prompted a study of the immunogenicity of cells grown under *p*H control. In these experiments, the *p*H of cultures was monitored and adjusted throughout a 21-day period. In one series, the overlaying culture fluids were replaced with fresh medium when the *p*H approached 7 (medium-change series); in the other series, the medium was not changed. The *p*H of the unchanged series went below 7 at day 4, and remained around 5.5 (Fig. 2A).

Cell nitrogen and AFU_{50} were determined on suspensions of organisms from these cultures. In the unchanged medium series, the cell nitrogen increased from day 1 through day 4 and, in the medium-change series, through day 5 (Fig. 2B). Approximately 50% more cell nitrogen could be recovered at day 5 in the medium-change series (in which the *p*H was above 7.0) than in the unchanged series. The nitrogen content of both series remained relatively stable after reaching these maxima.

A pronounced difference in AFU_{50} was observed

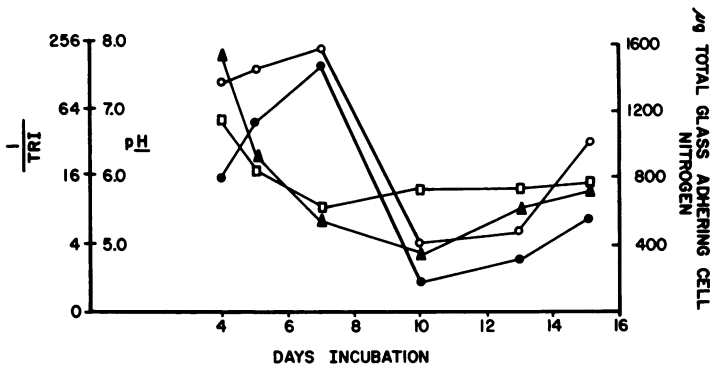

FIG. 1. *Relation of pH of medium, total cell mass measured as nitrogen, and TRI antibody production to time of harvest of M. pneumoniae FH grown in standard medium. Symbols: ○, TRI response in guinea pigs; ●, TRI response in hamsters; □, pH of culture fluids; ▲, cell nitrogen.*

between the two series (Fig. 2C). The AFU_{50} of the medium-change series remained relatively constant after reaching a peak at 3 days. The AFU_{50} in the unchanged series was lower at day 5 and decreased for 10 days, at which time no organisms were detected by titration. Thus, medium replacement gave increased growth, as measured by nitrogen, and maintained metabolic activity as determined by AFU.

Maintenance and assessment of the importance of pH by the addition of KOH to cultures. Results of experiments in which alkaline *p*H was maintained by medium replacement did not eliminate the possibility that continued growth and metabolic activity was due to replenishment of nutrients or removal of inhibitory waste products. The hypothesis that *p*H maintenance alone without medium replacement would support continued growth and metabolic activity was examined by the addition of KOH to continuously growing cultures. In these experiments, the *p*H of the cultures was adjusted at frequent intervals by addition of KOH (KOH-add series) and compared to cultures with medium change or cultures left unattended (Fig. 3A). In the KOH-add series, the *p*H was maintained between 7.0 and 7.5 with 1.0 N KOH. The control of *p*H in this series required approximately 36 meq of KOH during a 10-day period (Fig. 3D). In both the KOH-add and medium-replacement series, higher levels of both cell nitrogen and AFU_{50} were obtained than when the *p*H was allowed to drop below 7 (Fig. 3B and 3C). It appeared that the addition of KOH produced effects comparable to medium change. This experiment confirmed the observation of the deleterious effect of low *p*H upon both cell nitrogen and AFU_{50} levels.

Guinea pigs were immunized with mycoplasma suspensions from the KOH-add series, whose

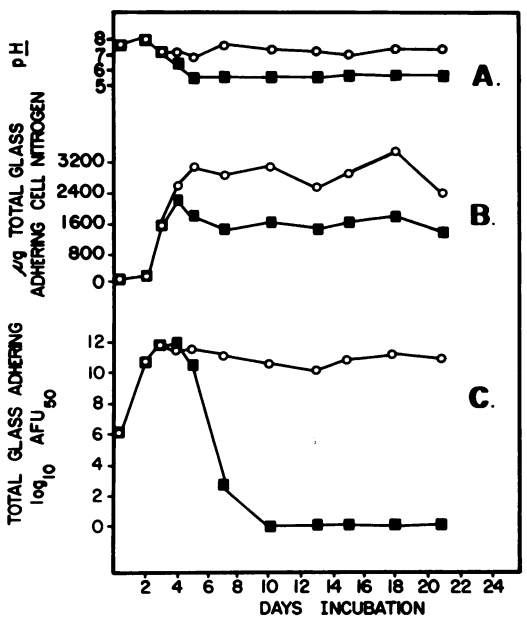

FIG. 2. *Relation of pH of medium (A), total cell mass measured as nitrogen (B), and acid-forming units (C) to time of harvest of M. pneumoniae FH grown in standard medium. Organisms were harvested at intervals from cultures left unchanged throughout the experiment, control series (■), or from cultures in which the overlaying fluids were replaced with fresh standard medium when the pH approached 7, medium-change series (○).*

neutral or slightly alkaline *p*H had been maintained for 13 days. The TRI antibody titer was 16 when animals were injected with organisms harvested at 3 days, and gradually increased when animals were injected with older harvested organisms. Organisms harvested at 13 days induced a TRI antibody titer of 1,024. These results con-

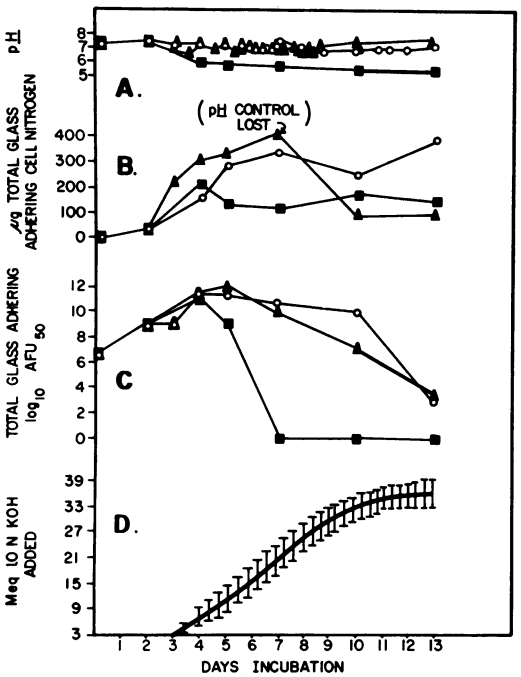

FIG. 3. *Relation of pH (A), total cell mass measured as nitrogen (B), and acid-forming units (C) to time of harvest of M. pneumoniae FH grown in standard medium. Organisms were harvested at intervals from three series. Control (■) and medium-change series (○) are as in Fig. 2; in a third series, KOH-add (▲), the pH of overlaying fluids was adjusted to 7 with 1.0 N KOH as it approached 6.5. The average sum of milliequivalent KOH added to maintain cultures at pH 7 is recorded in D.*

trasted with lower decreasing titers obtained upon immunization with cells harvested from periods between 7 and 13 days without *p*H control (Fig. 1). These data confirm the relationship of *p*H and immunogenicity, i.e., the antigenic potential of mycoplasma suspensions is increased by maintaining cultures at a *p*H near neutrality.

Immunodiffusion reactions of M. pneumoniae organisms harvested at different time intervals. In addition to the TRI test, immunodiffusion reactions with human convalescent-phase sera were used as a measure of the antigenic potential of mycoplasma suspensions. Cells were grown in standard medium and harvested at daily intervals over 4 days. At day 3, the culture fluids were replaced; at day 4, the medium was acid. Suspensions prepared from 2- and 3-day-old cells were reactive in immunodiffusion (Fig. 4). The absence of a gel band with the day 4 preparation parallels the loss of immunogenicity (Fig. 1) associated with acid *p*H.

Growth and antigenic potential of M. pneumoniae strains grown in medium free of horse serum. The effect of *p*H on the growth and antigenicity of *M. pneumoniae* was further substantiated when the FH or the 65-2053 strains were grown in a new mycoplasma broth medium (SSR1). As in earlier experiments, cultures incubated for 3 days or longer showed a sharp *p*H drop, with a concomitant decrease in cell nitrogen and AFU_{50}. These results are similar to those previously obtained with the FH strain grown in standard medium.

We compared the immunogenicity of *M. pneumoniae* preparations grown by our procedure with that of an experimental pooled vaccine PC821S (9) produced by another laboratory. We grew *M. pneumoniae* strain 65-2053 in SSR1 medium for 3 days, at which time the culture fluids were still alkaline. The cells were prepared for injection as described in Materials and Methods. The experimental pooled vaccine PC821S (9) had been produced from FH organisms grown for 6 to 12 days in a medium containing calf serum and which had become acid. The nitrogen content of both preparations was adjusted to 17 to 18 μg of nitrogen per ml, and 1 ml was injected into each of 10 guinea pigs. The geometric mean titer of the TRI antibody response to 65-2053 was 10-fold greater than that of animals receiving PC821S (Table 1). These results indicate that significant TRI titers were achieved only with organisms harvested from alkaline media.

Growth of M. pneumoniae in the presence of buffers. The damaging effect of acid *p*H on the growth and antigenicity of *M. pneumoniae* suspensions prompted a search for a suitable buffer. HEPES, TES, Tris and triethanolamine buffers at 0.10 M were added to SSR1 media and adjusted to *p*H 7.5 before autoclaving. As controls, we used two unbuffered cultures, one in which the medium was replaced when the *p*H of the culture fluids approached 7 (medium-change series) and one in which the culture fluids were not changed. The inoculum was an actively growing culture of FH which had been passed in unbuffered SSR1 5 times after 317 passes in standard medium. The growth of *M. pneumoniae* FH in SSR1 buffered with TES or HEPES was comparable to that in the medium-change series (Fig. 5), as measured by protein assay. The *p*H of culture fluids in the presence of these buffers remained above 6.9 for 8 days. The yield of cells was negligible when triethanolamine or Tris was present. In a similar experiment with 3-6-endomethylene-1,2,3,6-tetrahydrophthalic acid (EMTA) buffer (14) at 0.05 M, the amount of cell protein was also low. Cell nitrogen and turbidity measurements in all of these studies paralleled data obtained from protein determinations. Our results indicate that 0.10

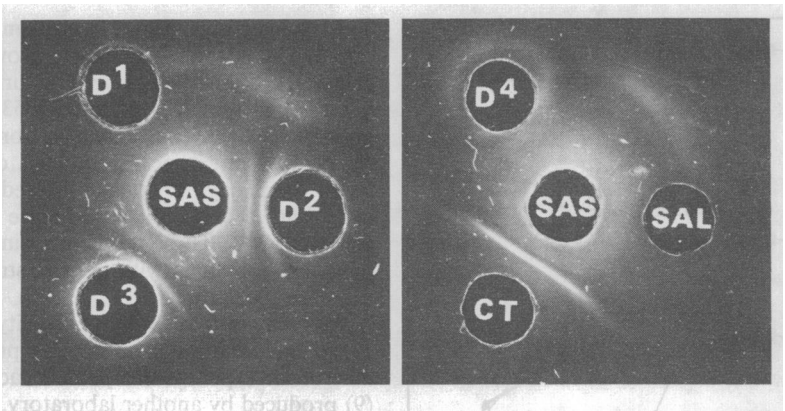

FIG. 4. *Gel diffusion tests performed on suspensions of M. pneumoniae FH grown in standard medium. Wells D^1, D^2, D^3, and D^4 contain organisms harvested after 1, 2, 3, and 4 days, respectively; well SAL, phosphate-buffered saline; well CT, control immunodiffusion antigen (3); and well SAS, human convalescent-phase serum.*

TABLE 1. *Comparison of immunogenicity of Formalin-inactivated M. pneumoniae suspensions prepared by different processes*[a]

Antigen	Guinea pig no.	1/TRI antibody
Vaccine lot PC821S[b]	1	0
	2	0
	3	4
	4	0
	5	0
	6	1
	7	2
	8	0
	9	2
	10	0
	Gm[c]	2.0
Strain 65-2053[d]	11	8
	12	8
	13	16
	14	32
	15	32
	16	8
	17	64
	18	128
	19	2
	20	256
	Gm	22.6

[a] One intramuscular injection, at day 0, of 1 ml containing 17 to 18 μg of nitrogen. All guinea pigs were TRI antibody-negative at time of injection. Sera were obtained 4 weeks postimmunization.

[b] Prepared as described in reference 9 from glass-adherent *M. pneumoniae* FH grown on medium containing bovine serum.

[c] Gm = geometric mean (1/TRI antibody values of 0 and 1 treated as 1.77).

[d] Prepared as described in Materials and Methods.

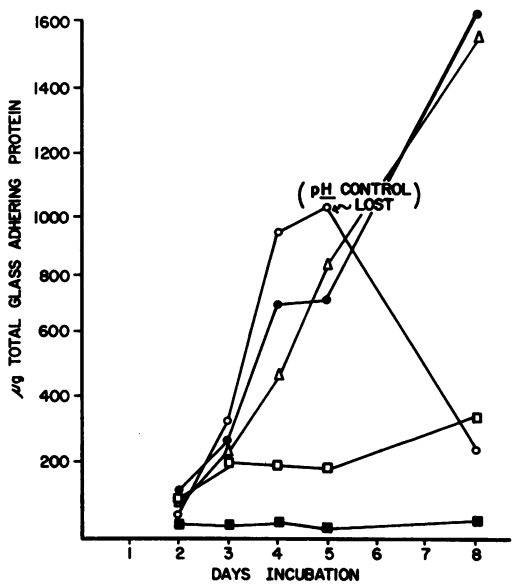

FIG. 5. *Recovery of M. pneumoniae FH, measured as protein, grown in SSR1 medium in the presence or absence of buffers. Control series (□) and medium-change series (○) are as in Fig. 2. SSR1 medium containing 0.05 M HEPES (●), TES (△), Tris, or triethanolamine (■).*

M TES and HEPES are suitable buffers in SSR1 medium. The yields of mycoplasmas, as measured by protein, nitrogen, and turbidity, were comparable to findings in the series where *p*H was controlled by medium replacement. Thus, the use of HEPES- or TES-buffered SSR1 permitted maintenance of neutral or alkaline *p*H over prolonged incubation periods.

Immunodiffusion reactions obtained with M. pneumoniae FH grown in HEPES-buffered SSR2. We examined the effect of HEPES on the antigenicity of *M. pneumoniae* suspensions. Strain FH was subcultured five times in SSR1 medium, which had been modified to contain 3% PPLO Serum Fraction (SSR2). The sixth subculture was made in SSR2 and SSR2 containing 0.05 M HEPES. Mycoplasmas were harvested daily starting at day 2 through day 6. In cultures with HEPES, the *p*H remained above 6.4 over the entire 6 days. In the absence of HEPES, the *p*H dropped from 7.5 to 5.7 by day 3, and then remained relatively constant. Suspensions of mycoplasma were stored at 4 C until all collections were completed.

For immunodiffusion studies, each suspension was adjusted to contain 422 μg of nitrogen per ml. Immunodiffusion reactions obtained between these antigenic suspensions and human convalescent-phase sera are seen in Fig. 6. An *M. pneumoniae* antigen previously shown to form gel diffusion lines when reacted against the same sera (3) served as a control for serum reactivity.

All five suspensions prepared from HEPES-buffered media reacted with human convalescent sera S and V to form precipitation bands (Fig. 6A and 6B).

When mycoplasmas grown in unbuffered SSR2 were examined, only two suspensions reacted to form immunodiffusion lines (Figs. 6A and 6B). This observation can be associated with the marked *p*H drop between days 2 and 4 in this series, as already noted. These findings indicate that mycoplasmas lose antigenic reactivity, as assayed in immunodiffusion, when the culture fluids from which they are harvested have become acid.

DISCUSSION

Our results indicate that the TRI antibody response of animals injected with *M. pneumoniae* suspensions is related to the *p*H of the culture fluids from which the organisms are harvested As the *p*H of the culture fluids decreases, the im

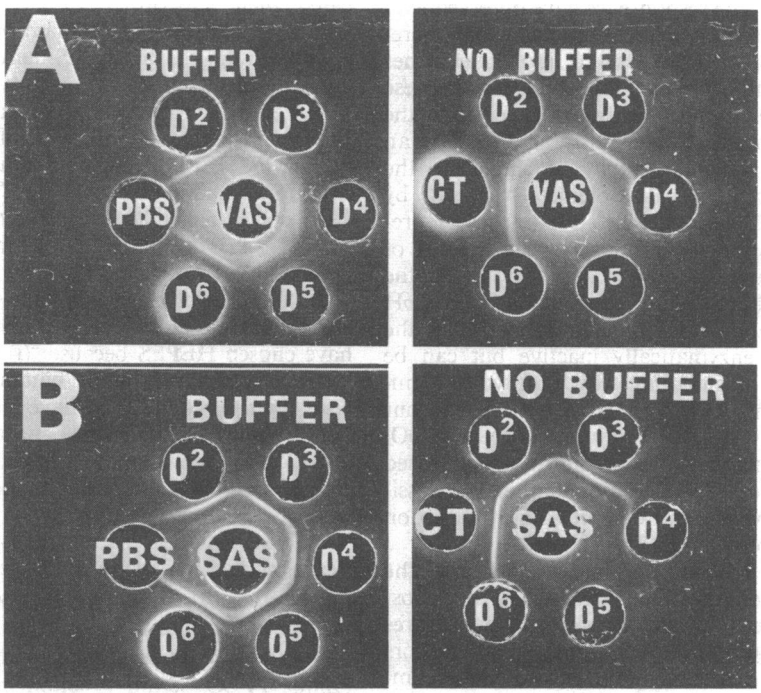

FIG. 6. *Gel diffusion tests performed on suspensions of M. pneumoniae FH grown in SSR1 modified to contain 3% PPLO Serum Fraction, with or without 0.05 M HEPES. Mycoplasma suspensions were prepared daily starting at day 2 and ending at day 6. These suspensions were adjusted to contain 422 μg of nitrogen per ml and were placed in wells labelled D^2 through D^6. In A, preparations were tested against human convalescent-phase serum VAS, and in B, against serum SAS. Wells PBS, contained phosphate-buffered saline; wells CT, control immunodiffusion antigen (3).*

munogenicity of the organisms, as measured by TRI antibody response, also decreases. Organisms harvested from cultures which had become acid also lose gel reactivity against human convalescent-phase sera as measured in immunodiffusion tests. This acid lability can be demonstrated with organisms grown in media containing either PPLO Serum Fraction or horse serum. The antigenic reactivity of *M. pneumoniae* isolates in these two media maintained at neutral or alkaline *p*H supports the view that *p*H rather than the composition of the medium is a critical factor in the production of immunogenic or gel-reactive preparations in *M. pneumoniae*. Adler and DaMassa (1) reported that acid production appears to affect the quality of *M. gallisepticum* antigens produced for use in a slide agglutination test.

We cannot demonstrate a loss of gel reactivity following incubation of *M. pneumoniae* organisms in various buffers over the *p*H range 3.5 to 6.5 for periods up to 38 hr at 37 C (*unpublished data*). Therefore, the loss of reactivity does not point to a direct hydrogen ion effect on the organisms used to elicit TRI antibody response in animals or in gel reactivity. The response we have observed with cells harvested from acid culture fluids may represent the action of proteolytic or lipolytic enzymes present in the growing mycoplasmas. These enzymes could be released from the cells into the culture fluids and subsequently activated by an acid *p*H. This sequence may be similar to the precursor streptococcal proteinase reported by Elliott (5). He showed that an extracellular precursor was optimally produced when the *p*H of the culture fluid was 5.5 to 6.5, but not when the *p*H of the culture fluid was maintained above *p*H 7.0. Elliott and Dole (6) also showed that the precursor is enzymatically inactive but can be activated by incubation with sulfhydryl compounds. Such a change might be effected in our experiments with older cultures where the pO_2 would presumably be lower due to an increased oxygen demand of a dense population. The possibility that low *p*H can derepress the production of lytic enzymes cannot be excluded.

Smith and Koostra (19) recently reported the appearance of O-amino acid esters of phosphatidyl glycerol in exogenously acidified cultures of sterol-requiring mycoplasmas. The authors questioned the natural occurrence of these compounds in *Mycoplasma* strains which generally do not grow at a *p*H lower than 7.0. They postulated, in part, that their formation was an attempt of the organism to raise the exogenously lowered *p*H by blocking free carboxyl groups. As shown in this work, *M. pneumoniae* grows at a *p*H below 7.0. Also, Conant et al. (3) and Lemcke et al. (11) indicated that the involvement of lipid in serologically active preparations of *M. pneumoniae*. In unpublished work, we have shown that immunodiffusion reactivity is lost after treatment of *M. pneumoniae* suspensions with porcine lipase or detergent. By inferring the mechanism proposed by Smith and Koostra, we would hypothesize that O-amino acid esterification of lipids, perphaps phosphatidyl glycerol, may block immunodiffusion reactivity or the induction of TRI antibody.

Apparently, *M. pneumoniae* strains grow in the presence of HEPES or TES buffers without a detectable adaptation or lag period. Growth of mycoplasma not adapted to any buffer gave higher yields of organisms in the presence of HEPES or TES rather than Tris, triethanolamine, or EMTA. Earlier work had also indicated that Tris was inhibitory to the growth of *M. pneumoniae* FH in medium of Hayflick's formula (8). Incorporation of buffers into media delayed the *p*H decline.

The use of HEPES or TES as biological buffers was first advocated by Good et al. (7), who indicated their superiority to Tris or phosphate buffers. More recently, Williamson and Cox (21) advocated the use of HEPES buffer in animal cell cultures infected with viral agents. These authors observed no cytotoxic effects attributable to the buffer and reported that virus infectivity in cells grown in HEPES was comparable to that in medium containing bicarbonate-CO_2 buffer. Also, Richter (16) has indicated the advantage of using TES over Tris in a plaque assay for Eastern equine encephalitis virus.

At the present time, our data do not indicate clearly the superiority of HEPES over TES. We have chosen HEPES because: (i) this compound has a lower metal buffer binding constant for Cu^{+2} than TES (7); (ii) we have recovered slightly higher yields of organisms; and (iii) Williamson and Cox(21) grew animal cells in the presence of HEPES or TES and reported higher yields of cells when HEPES was used.

M. pneumoniae isolates are usually grown in media containing relatively large quantities of horse serum (8). However, horse serum cannot be present in vaccines used for human injection. We have grown *M. pneumoniae* FH in a medium containing PPLO Serum Fraction instead of horse serum. The organisms were harvested at *p*H above 6.8 and teated with formaldehyde. This preparation was tested by National Institutes of Health scientists for its immunogenicity in man and was effective in eliciting significant TRI antibody response (*in preparation*).

ACKNOWLEDGMENTS

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