Growth Medium Constituents Contaminating Mycoplasma Preparations and their Role in the Study of Membrane Glycoproteins in Porcine Mycoplasmas

By J. NICOLET,^{1*} PH. PAROZ¹ AND B. KRISTENSEN²

*Institute of Veterinary Bacteriology¹ and Institute of Clinical Immunology, Inselspital,²
University of Berne, 3012 Berne, Switzerland*

(Received 15 October 1979; revised 4 January 1980)

Several *Mycoplasma* and *Acholeplasma* species chosen at random and solubilized with sodium dodecyl sulphate showed a common periodic acid-Schiff positive band with an apparent molecular weight of about 64000, when examined by polyacrylamide gel electrophoresis. Another more cathodic minor band was detected in *M. hyopneumoniae* and *M. flocculare*. The common periodic acid-Schiff positive band appeared when a precipitate of serum constituents of the uninoculated growth medium after incubation was examined. The minor band was identified as a serum glycoprotein contaminating mycoplasmas grown in the presence of swine serum. We draw attention to the compounds as a possible source of error in serological tests or in the lymphocyte stimulation response. After lithium diiodosalicylate solubilization and aqueous phenol extraction, polyacrylamide gel electrophoresis showed a periodic acid-Schiff positive band in membranes from *M. hyopneumoniae* (molecular weight 75000) and *M. hyorhinis* (molecular weight 80000), suggesting the presence of a membrane glycoprotein. Such a glycoprotein was absent from *A. granularum*. Since the common periodic acid-Schiff positive band was not extracted by aqueous phenol, this growth medium constituent did not contaminate the preparations of membrane glycoproteins. However, the minor band was present in glycoprotein preparations of *M. hyopneumoniae* grown in the presence of swine serum.

INTRODUCTION

The propagation of mycoplasma cells is usually achieved in complex growth media containing up to 20% whole animal serum. The absorption of foreign proteins to the mycoplasma surface is a potential hazard. Jordan & Kulasegaram (1968) reported that *Mycoplasma gallisepticum* picked up protein from their growth medium. Bradbury & Jordan (1971) showed that the pH of the culture medium influenced the adsorption of medium protein to the organisms. Schiefer *et al.* (1974) suggested that the electron-dense material on the outer membrane of several mycoplasmas may be adsorbed from the growth medium containing blood group substances or yeast extract. In *M. pneumoniae*, Schütten *et al.* (1978) identified by crossed immunoelectrophoresis two precipitin lines as medium constituents. Using complement fixation and immunodiffusion techniques, Clark *et al.* (1969) concluded that mycoplasma preparations can carry serum antigens and blood group substances. Williams *et al.* (1970) reported the non-specific uptake of IgG from the culture medium by *M. fermentans*. Rottem *et al.* (1973) found that at pH values below 5 a large amount of serum albumin was bound to *Acholeplasma laidlawii* cell membranes. By immunizing a rabbit with *M. gallisepticum*, Bradbury & Jordan (1972) found antibodies to growth medium serum constituents, particularly to immunoglobulins, transferrin and a pre-albumin. When examining the electrophoretic pattern of various mycoplasmas by poly-

acrylamide gel electrophoresis (Paroz *et al.*, 1976), we observed at least two fractions common to all *Mycoplasma* and *Acholeplasma* species studied, suggesting a contamination from the growth medium. Staining of the gel with periodic acid-Schiff reagent (PAS) revealed PAS-positive bands (Paroz & Nicolet, 1978) which may be misinterpreted as mycoplasma membrane glycoproteins.

The presence of genuine glycoproteins in the membrane of mycoplasmas was first recognized in *M. pneumoniae* by Kahane & Marchesi (1973) who postulated a structure and localization similar to that seen in red blood cells. Goel & Lemcke (1975) succeeded in isolating and characterizing a glycoprotein fraction from membranes of *M. gallisepticum*. The presence of a membrane glycoprotein was also suspected in *M. hominis* (Hollingdale & Lemcke, 1972); the authors suggested, however, that this component could be a peptide with periodate-sensitive amino acids. Johansson (1978) reported the isolation of a glycoprotein in *A. laidlawii* with a molecular weight of 140000 which had antigenic determinants exposed on the cytoplasmic face of the membrane. Recently, Kahane & Brunner (1977) isolated a glycoprotein with an apparent molecular weight of about 60000 from the membrane of *M. pneumoniae*.

The aim of the present study was to define the properties of the PAS-positive fractions of the growth medium contaminating mycoplasma preparations and to distinguish them from glycoproteins which were obtained after aqueous phenol extraction of mycoplasma membrane preparations.

METHODS

Organisms. The following *Mycoplasma* strains were used. Human strains: *M. hominis* PG 21 (H 50) and *M. pneumoniae* FH. Bovine strains: *M. mycoides* subsp. *mycoides* PG 1, *M. bovis genitalium* PG 11 (B 2), *M. bovirhinis* PG 43 (S M 331), *M. bovis* Donetta (PG 45), *M. dispar* 462/2, *M. bovoculi* M 165/69, *M. alkalescens* D 12 (PG 51) and *M. verecundum*. Caprine and ovine strains: *M. mycoides* subsp. *capri* PG 3, *M. agalactiae* subsp. *agalactiae* PG 2, *M. conjunctivae* HRC 581, *M. arginini* G 230, *M. capricolum* California Kid, *M. putrefaciens* KS-1 and *M. ovipneumoniae* Y 98. Porcine strains: *M. hyorhinis* BTS-7, *M. hyopneumoniae* JF 184 (kindly supplied by P. Whittlestone, School of Veterinary Medicine, Cambridge), *M. hyosynoviae* S 16 and *M. flocculare* Ms 42 (kindly supplied by N. F. Friis, Royal Veterinary and Agricultural University, Copenhagen, Denmark). Rodent strain: *M. pulmonis* Ash (PG 34).

Acholeplasma strains used were: *A. laidlawii* PG 8 (Sewage a), *A. granularum* BTS-39, *A. axanthum* S-743, *A. modicum* Squire (PG 49) and *A. oculi* 19 L.

All *Mycoplasma* and *Acholeplasma* strains except *M. hyopneumoniae* and *M. flocculare* were kindly supplied by Professor E. A. Freundt, FAO/WHO Collaborating Centre for Animal Mycoplasmas, Institute of Medical Microbiology, University of Aarhus, Denmark.

Media. Goodwin-Whittlestone medium (Goodwin *et al.*, 1965) was used for the propagation of *M. hyopneumoniae*; this contains 30 % (v/v) Hartley's digest broth (Oxoid PM1185), 20 % (v/v) pig serum, 0.5 % (w/v) lactalbumin hydrolysate (Difco), 50 % (v/v) Hanks balanced salt solution (Oxoid BR19) and 0.05 % (w/v) yeast extract (Fleischman's active dry yeast).

Friis medium (Friis, 1971) used for the propagation of *M. flocculare* contains both horse and pig serum.

Bern medium (Bannerman & Nicolet, 1971) was used for propagating all other mycoplasmas examined; this contains PPLO medium (Difco) enriched with 20 % (v/v) horse serum, 2.5 % (w/v) yeast extract (Fleischman's active dry yeast), 1 % (w/v) glucose and 0.005 % DNA.

To reduce contamination with growth medium constituents (for the lymphocyte stimulation assay), sterile whole serum was preincubated for 5 d at 37 °C and centrifuged at 20000 g before addition to the growth medium.

Culture conditions. All broth cultures were incubated at 37 °C without shaking, except *M. hyopneumoniae* which was shaken at 70 rev. min⁻¹. Cells were harvested in the exponential phase of growth; in the case of glucose-fermenting strains the pH of the medium was about 6.8 to 7.0 at harvest. The cells were thoroughly washed three times in 0.25 M-NaCl by centrifugation at 20000 g for 20 min at 4 °C. The resuspended cells were designated as whole cells or were used for membrane preparation. *Mycoplasma pneumoniae* was grown on the surface of a plastic tissue culture flask and harvested as described by Kahane & Brunner (1977).

Sodium dodecyl sulphate (SDS) solubilization. Whole cells, membranes or serum precipitate were solubilized with 12.5 mM-phosphate buffer pH 7.2 containing 1 % (w/v) SDS and 1 % (w/v) β -mercaptoethanol [1 ml (mg cell protein)⁻¹] and boiled for 3 min, as described previously (Paroz *et al.*, 1976).

Isolation of cell membranes. Two procedures described by Razin & Rottem (1976) were used: osmotic lysis in deionized water for *A. granularum* and digitonin lysis for *M. hyopneumoniae*, *M. hyorhinis* and *M. pneumoniae*. To prevent contamination with ribosomes and DNA, the washed membrane suspensions were treated with RNAase and DNAase (Serva) for 15 min at 37 °C and after washing were resuspended in 1/20 diluted β -buffer (Razin & Rottem, 1976).

Extraction of membrane glycoprotein. Kahane & Brunner's (1977) modification of the procedure described by Marchesi & Andrews (1971) was followed, i.e. treatment with lithium diiodosalicylate and subsequent extraction with fresh aqueous phenol and delipidation with chloroform/methanol.

SDS-polyacrylamide gel electrophoresis. We used the phosphate-buffered 7.5 % (w/v) gel system described by Morowitz & Terry (1969), containing 1 % (w/v) SDS, with the flat gel slab method in an apparatus from Biowerk (Biozentrum, Basel, Switzerland). Samples (20 to 40 μ l containing 1 mg cell protein ml⁻¹) were layered with 30 % (v/v) glycerol and subjected to electrophoresis. The slabs were stained for proteins with Coomassie Brilliant Blue R (Chrambach *et al.*, 1967) and for glycoproteins with the periodic acid-Schiff reagent by the method described by Zacharius *et al.* (1969). To reveal membrane glycoproteins better the following modification was used. Immediately after electrophoresis the gels were immersed in 12.5 % (w/v) trichloroacetic acid for 45 min and then oxidized with 0.8 % (w/v) periodic acid for 3 h. The gels were then washed by shaking 10 times for 10 min with water. These steps were all carried out at room temperature. The gels were then immersed in freshly prepared Schiff reagent and stored in the dark at 4 °C for 10 h. Subsequently, they were rinsed three times for 10 min in 0.5 % (w/v) sodium metabisulphite in the dark and three times for 10 min with water in the light. The gels were preserved in sodium metabisulphite.

Molecular weight determination. Apparent molecular weight was determined by comparing the electrophoretic mobility with a standard calibration curve constructed by running in parallel a low molecular weight calibration kit (Pharmacia).

Chemical analyses. Protein content was measured by the Hartree (1972) modification of the Lowry method, using bovine serum albumin as the standard. Carbohydrates were estimated by measuring total hexose using the anthrone procedure (Troy *et al.*, 1953) with galactose as standard. Phospholipid content was determined as described by Bartlett (1958).

Immunization of rabbits. Young rabbits weighing 2 to 3 kg were immunized with washed mycoplasma suspensions or serum precipitates mixed with an equal volume of complete Freund's adjuvant according to the schedule described previously (Bannerman & Nicolet, 1971).

Double-diffusion test. The test was performed by the method of Ouchterlony (1967) in its micro-modification. An agarose layer (0.5 mm) was poured with the use of a matrix on precleaned slides. A 6 mm thick plastic block with six peripheral conical holes and one central hole of 1 mm diameter was then applied to the agarose and the wells were filled with antiserum or antigen. Optimal precipitin lines were detected after 48 h incubation at room temperature in a moist chamber.

Lymphocyte stimulation. The technique used was based on that described by Lazary *et al.* (1974), with the following modifications: pig lymphocytes were isolated from peripheral blood by centrifugation on a Ficoll-isopaque gradient (Ficoll-isopaque, 400) and cultured in a micro-tissue culture plate (Micro Test II TM, Falcon, Oxnard). The results were reported as counts min⁻¹ or, for purposes of comparison, as stimulation index (SI).

Affinity chromatography on concanavalin A-Sepharose. Affinity chromatography was carried out using concanavalin A-Sepharose gel (Pharmacia) in a column (K 16/20, Pharmacia) with a bed volume of 2.2 ml. The gel was equilibrated with 0.025 M-Tris/HCl buffer, pH 7.2, containing 0.2 M-NaCl at a flow rate of 4.2 ml h⁻¹. After application of 1.8 ml horse or swine serum, unbound protein was eluted with the same buffer, and elution of adsorbed material was achieved by including 0.3 M-methyl- α -D-mannoside (Fluka, Buchs, Switzerland) in the buffer. Fractions (1 ml) were collected in a LKB 7000 Ultrarac fraction collector. For processing a greater amount of serum (60 ml), we used a larger column (K 50/60, Pharmacia) with a bed volume of 230 ml and a flow rate of 30 ml h⁻¹.

RESULTS

Electrophoretic pattern of SDS-solubilized whole cells and membranes

All strains listed in Methods were submitted to polyacrylamide gel electrophoresis. The protein pattern of a random selection of mycoplasmas from different origins is illustrated in Fig. 1(a). The overall picture is specific for each species, but two fractions (arrowed) seem to have similar migration patterns in all the strains examined.

Staining the gels with periodic acid-Schiff reagent (PAS) showed that a common broad

Fig. 1. Flat gel SDS-polyacrylamide electrophoresis of SDS-solubilized whole cells of various mycoplasmas. (a) Protein pattern: Coomassie Blue stain (arrows indicate fractions with similar migration). (b) Periodic acid-Schiff reagent (1, minor band; 2, common band). A, *M. pneumoniae*; B, *M. hominis*; C, *M. ovipneumoniae*; D, *M. agalactiae* subsp. *agalactiae*; E, *M. hyopneumoniae*; F, *M. hyorhinis*; G, *M. mycoides* subsp. *mycoides*; H, *M. arginini*; I, *M. bovis*; J, *M. pulmonis*. Positions of bands from molecular weight standards are shown on the left.

PAS-positive band with a similar mobility, but with some differences in intensity, was obtained with all strains tested (Fig. 1b). A minor PAS-positive band was detected only in *M. hyopneumoniae* and *M. flocculare*.

Preparation of membranes by osmotic or digitonin lysis with subsequent SDS-solubilization eliminated neither the common broad PAS-positive band nor the minor band of *M. hyopneumoniae*.

Fig. 2. Flat gel SDS-polyacrylamide electrophoresis, stained with periodic acid-Schiff reagent. Comparison between fresh growth media or serum and serum precipitates sedimented at 20000 *g* and *M. hyopneumoniae* (1, minor band; 2, common band). A, Goodwin-Whittlestone medium; B, Bern medium; C, swine serum; D, horse serum; E, PPLO serum fraction (Difco); F, *M. hyopneumoniae* (SDS-solubilized whole cells grown in A); G, serum precipitate from Goodwin-Whittlestone medium; H, serum precipitate from Bern medium; I, serum precipitate from swine serum; J, serum precipitate from horse serum; K, serum precipitate from PPLO serum fraction.

Electrophoretic pattern of growth media and serum constituents

In the search for contamination of our mycoplasma preparations by growth medium constituents, we investigated the electrophoretic pattern of different uninoculated fresh growth media and of horse and swine sera. With the exception of the minor band, which seems to be similar to that seen in the mycoplasma preparations grown in media containing swine serum, the growth media (Fig. 2A, B), whole sera (Fig. 2C, D) and PPLO serum fraction (Fig. 2E) (Difco) all had PAS-positive bands that migrated at a different rate from those found in the mycoplasma preparations (Fig. 1*b*, Fig. 2F). Furthermore, when uninoculated growth media, or sera (swine, horse or PPLO serum fraction) were incubated for a few days at 37 °C and the barely visible precipitates sedimented at 20000 *g* were dissolved in SDS, the preparations gave a common PAS band (Fig. 2G to K) similar to that observed in the mycoplasma preparations.

Properties of the common PAS-positive band

The common PAS-positive band from precipitates also migrated as a single band shown by Coomassie Blue staining. Comparison of the mobility of this band and those of standard proteins gave an apparent molecular weight of 64000. The gross chemical analysis of the precipitates indicated that they consisted of proteins (40%) and phospholipids (60%). Carbohydrates were also present, as indicated by the PAS stain, but no quantitative analysis was made.

Fig. 3. Agar gel diffusion patterns. (a) 1, Antiserum to precipitates from swine serum; 2, *M. hyopneumoniae* grown in Goodwin-Whittlestone medium (swine serum); 3, precipitates from swine serum; 4, *M. hyorhinis* grown in Bern medium (horse serum). (b) 1, Precipitates from swine serum; 2, antiserum to precipitates from swine serum; 3, antiserum to swine IgG; 4, antiserum to horse IgG.

Effect of the common PAS-positive band on serological investigation

A double-diffusion test between antiserum to swine serum precipitates and *M. hyopneumoniae*, *M. hyorhinis* and swine serum precipitates as antigens gave at least three precipitin lines. One of these lines was common to *M. hyopneumoniae* and serum precipitates, the second was specific for serum precipitates and the third was common to all three antigens tested. This suggests that at least two of the serum precipitate antigens contaminate the *M. hyopneumoniae* preparation (growth medium containing swine serum) and one of them cross-reacts with a *M. hyorhinis* preparation grown in a medium containing horse serum (Fig. 3a). Moreover, Fig. 3(b) indicates that the swine serum precipitate contained swine IgG that did not cross-react with anti-horse IgG.

Removal of the serum precipitates

Passage of sera through a concanavalin A-Sepharose column for elimination of the glycoproteins removed, among others, the minor PAS band, but did not prevent the formation of the common band after incubation. Additionally, such sera depleted of glycoproteins no longer permitted the growth of mycoplasmas. Attempts to adapt *M. hyopneumoniae* to growth in a medium containing serum from colostrum-deprived new-born piglets failed. We finally removed quantitatively the common PAS-positive band by incubating the whole sterile serum for 5 d at 37 °C and centrifuging at 20000 g before adding it to the growth medium. The minor PAS-positive band, however, was not eliminated by this procedure.

Effect on lymphocyte stimulation assay

The blastogenic effect of *M. hyopneumoniae* grown in a medium with preincubated and centrifuged serum was significantly lower than with antigen contaminated with serum precipitates (Table 1).

Demonstration of membrane glycoproteins after treatment with lithium diiodosalicylate and aqueous phenol extraction

The electrophoretic pattern after extraction of the membrane glycoproteins from *M. hyopneumoniae*, *M. hyorhinis* and *A. granularum* is shown in Fig. 4, together with the whole peptide pattern of SDS-solubilized cells of *M. hyopneumoniae*. The extracted fractions of *M. hyopneumoniae* revealed two bands with R_F values of 0.08 (molecular weight > 94000) and 0.3 (molecular weight 75000; mean from several runs). The extract of membranes of *M. hyorhinis* revealed only one peptide with an R_F value of 0.33 (molecular weight 80000). In both strains the PAS stain (modified) showed a band with a pattern similar to the one given with Coomassie Blue protein stain.

Table 1. *Blastogenic effect of M. hyopneumoniae antigens on peripheral blood lymphocytes of pigs*

Whole cell antigen was cultured in medium pre-incubated at 37 °C for 5 d and centrifuged at 20000 *g* (without contaminants) or under conditions described in Methods (with contaminants). The results are expressed as counts min⁻¹ (mean values of triplicate cultures) ± standard deviation and as stimulation index (SI) calculated relative to unstimulated lymphocytes. For comparison, the results of the stimulation of lymphocytes with phytohaemagglutinin P (PHAP) are given.

Animal no.	Unstimulated cultures	Cultures stimulated with antigen (20 µg ml ⁻¹) (without contaminants)		Cultures stimulated with antigen (20 µg ml ⁻¹) (with contaminants)		Cultures stimulated with PHAP (8 µg protein ml ⁻¹)
		Counts min ⁻¹	SI	Counts min ⁻¹	SI	
3394*	7312 ± 2016	6797 ± 309	0.9	12603 ± 911	1.7	54297 ± 11290
3395†	5916 ± 594	9259 ± 2546	1.6	36414 ± 5088	6.2	64429 ± 5061
3396†	2251 ± 838	7671 ± 1051	3.4	10412 ± 1552	4.6	57672 ± 10525
3397†	761 ± 247	2407 ± 473	3.2	6048 ± 966	7.9	63062 ± 6327
3398†	1188 ± 247	465 ± 130	0.4	907 ± 253	0.8	46565 ± 8164
3399†	3545 ± 334	1836 ± 505	0.5	4840 ± 226	1.4	64338 ± 10159
3400*	614 ± 57	538 ± 144	0.9	874 ± 132	1.4	69776 ± 8350
3401*	1390 ± 1077	2014 ± 361	1.4	6159 ± 619	4.4	71080 ± 11806

* Pigs vaccinated with a *M. hyopneumoniae* vaccine in Freund's adjuvant.

† Spontaneous infection with *M. hyopneumoniae*.

Fig. 4. Flat gel SDS-polyacrylamide gel electrophoresis, stained with Coomassie Blue (1 to 5) or with the periodic acid-Schiff reagent (6 to 8). 1, Molecular weight standards; 2, *M. hyopneumoniae* (SDS-solubilized whole cells). Membrane glycoprotein extracts from: 3 and 6, *M. hyopneumoniae*; 4 and 7, *M. hyorhinis*; 5 and 8, *A. granularum*.

For comparison, we examined the membrane glycoprotein of *M. pneumoniae* according to the method of Kahane & Brunner (1977). A single band corresponding to a molecular weight, estimated in our gel system, of 65000, was obtained with both stains. Using the same extraction procedure and applying a tenfold higher concentration, no peptide band was observed in the *A. granularum* membrane extract. This shows, at least, that the common PAS-positive band as a growth medium contaminant was not extracted by this method.

The carbohydrate component of the extracted membrane glycoproteins was estimated as $230 \mu\text{g (mg protein)}^{-1}$ (23 %) for *M. hyopneumoniae* and $110 \mu\text{g (mg protein)}^{-1}$ (11 %) for *M. hyorhinis*.

Aqueous phenol extraction of growth medium precipitates did not show any bands; extraction of whole pig serum, however, showed the presence of a minor band with a molecular weight of more than 94000, similar to that observed in SDS-solubilized whole cells of *M. hyopneumoniae* and *M. flocculare*.

DISCUSSION

The fact that mycoplasmas grown *in vitro* may be contaminated by constituents of the growth medium is well known. Few reports, however, have dealt with the nature of these contaminants. During an investigation of membrane glycoproteins of different mycoplasma preparations we observed that whole cells or membranes solubilized with SDS showed, after polyacrylamide gel electrophoresis and PAS staining, at least one common broad PAS-positive band (Fig. 1*b*) with a molecular weight estimated at 64000. Since this band was not evident in fresh growth media and whole sera (Fig. 2*A* to *E*), we concluded that some serum constituents precipitated during prolonged incubation (Fig. 2*G* to *K*).

The chemical nature of such precipitates, including the presence of carbohydrates (PAS stain), protein (40 %), phospholipids (60 %) and IgG, suggested a complex from different serum constituents, among them lipoproteins, possibly glycolipids and/or aggregates of glycoproteins.

Another PAS-positive growth medium constituent, designated as the minor band, has been identified as a glycoprotein present in serum (Fig. 2*A* to *D*) and as a contaminant of mycoplasma cultures grown in swine serum (Fig. 1*b*, Fig. 2*F*). Since this minor band seemed to be present in the whole horse serum as well (Fig. 2*D*), but not in the different precipitates (Fig. 2*G* to *K*), it appeared that this fraction in swine serum has a certain affinity for the mycoplasma surface. Preliminary results obtained by ultracentrifugation in a sucrose density gradient confirmed this hypothesis. The presence of such contaminants is obviously highly undesirable; it leads, for instance, to confusing serological cross-reactions, at least with rabbit antisera (Fig. 3), and to enhancement of the blastogenic effect of antigens used in the lymphocyte transformation assay (Table 1).

Attempts to eliminate the contaminating serum constituents by chromatographic methods were not successful. At present the only way to eliminate these contaminants effectively is to preincubate the whole sterile serum (or serum fraction) and to discard the precipitate obtained after centrifugation.

In the study of membrane glycoproteins using aqueous phenol extraction these growth medium contaminants are of less importance. The common PAS-positive band was not extracted by this method, confirmation being afforded by the lack of bands in *A. granularum* extracts (Fig. 4). However, the minor PAS-positive band shown in *M. hyopneumoniae* (Fig. 4) may be a glycoprotein from the swine serum contained in the growth medium and extractable by aqueous phenol. Bearing in mind these considerations, we suggest that the bands revealed by Coomassie Blue and PAS stains after aqueous phenol extraction with molecular weights of 75000 for *M. hyopneumoniae* and 80000 for *M. hyorhinis* are genuine membrane glycoproteins.

For comparison, we confirmed the observations of Kahane & Brunner (1977) with *M. pneumoniae*, except that in our gel system the molecular weight appeared to be 65000. This suggested a varying chemical structure for these mycoplasma membrane glycoproteins. Unfortunately, no support for this hypothesis is provided by the carbohydrate composition, since the higher content of *M. hyopneumoniae* may be due to the contamination by the swine serum glycoprotein.

The variability of the intensity of the PAS-stained band is a major problem in detecting

the carbohydrate content of the extracted band. With *M. pneumoniae* we did not encounter real difficulties. However, with *M. hyopneumoniae* and *M. hyorhinis* we found that the gel wash and oxidation conditions could be modified to ensure that the removal of periodic acid was complete without allowing any significant loss of PAS-staining material through sample solubilization because of the reversibility of fixation with trichloroacetic acid (Kapitany & Zebrowski, 1973). Kahane & Brunner (1977) further suggest that carbohydrate content may vary with the age of the culture.

Although we cannot yet achieve a more complete chemical characterization of the glycoproteins observed in the porcine strains of mycoplasma, we nevertheless confirm similar observations recorded for *M. pneumoniae* (Kahane & Marchesi, 1973), *M. gallisepticum* (Goel & Lemcke, 1975) and *M. hominis* (Hollingdale & Lemcke, 1972), including the lack of such a membrane component in *Acholeplasma* (Kahane & Brunner, 1977). We certainly cannot offer more proof in favour of the fact that we are dealing with true glycoproteins which are rare constituents of prokaryote membranes, but we refer to the very convincing arguments offered by Kahane & Brunner (1977).

This investigation was supported by grant no. 3.036.076 of the Swiss National Foundation for Scientific Research.

REFERENCES

- BANNERMAN, E. & NICOLET, J. (1971). Isolation and identification of porcine mycoplasma in Switzerland. *Schweizer Archiv für Tierheilkunde* **113**, 697–710.
- BARTLETT, R. (1958). Phosphorus assay in column chromatography. *Journal of Biological Chemistry* **234**, 466–471.
- BRADBURY, J. M. & JORDAN, F. T. W. (1971). The adsorption of gamma-globulins to *Mycoplasma gallisepticum* and the possible role in non-specific serological reactions. *Veterinary Record* **89**, 318.
- BRADBURY, J. M. & JORDAN, F. T. W. (1972). Studies on the adsorption of certain medium proteins to *Mycoplasma gallisepticum* and their influence on agglutination and haemagglutination. *Journal of Hygiene* **70**, 267–278.
- CHRAMBACH, A., REISFELD, R. A., WYCKOFF, M. & ZACCARI, J. (1967). A procedure for rapid and sensitive staining of protein fractionated by polyacrylamide gel electrophoresis. *Analytical Biochemistry* **20**, 150–154.
- CLARK, H. W., BAILY, J. S. & MCBROWN, T. (1969). Variations on mycoplasma antigenic activity. In *Mycoplasma Diseases of Man*, Proceedings of an International Symposium, Reinhardtsbrunn Castle. Edited by M. Sprössig & W. Witzleb. Jena: Gustav Fischer.
- FRIS, N. F. (1971). Mycoplasmas cultivated from the respiratory tract of Danish pigs. *Acta veterinaria scandinavica* **12**, 69–79.
- GOEL, M. C. & LEMCKE, R. M. (1975). Dissociation of *Mycoplasma gallisepticum* membranes with lithium diiodosalicylate and isolation of a glycoprotein. *Annales de Microbiologie* **126B**, 299–312.
- GOODWIN, R. F. W., POMEROY, A. P. & WHITTLESTONE, P. (1965). Production of enzootic pneumonia in pigs with a mycoplasma. *Veterinary Record* **77**, 1247–1249.
- HARTREE, E. F. (1972). Determination of protein; a modification of the Lowry method that gives a linear photometric response. *Analytical Biochemistry* **48**, 422–427.
- HOLLINGDALE, M. R. & LEMCKE, R. M. (1972). Membrane antigens of *Mycoplasma hominis*. *Journal of Hygiene* **70**, 85–98.
- JOHANSSON, K. E. (1978). Production and utilization of monospecific antisera against membrane proteins from *Acholeplasma laidlawii*. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene (Abteilung I, Originale A)* **241**, 199–200.
- JORDAN, F. T. W. & KULASEGARAM, P. (1968). Non-specific antibodies in chickens inoculated intratracheally with *Mycoplasma gallisepticum*. *Journal of Comparative Pathology* **78**, 407.
- KAHANE, I. & BRUNNER, H. (1977). Isolation of a glycoprotein from *Mycoplasma pneumoniae* membranes. *Infection and Immunity* **18**, 273–277.
- KAHANE, I. & MARCHESI, V. T. (1973). Studies on the orientation of proteins in mycoplasma and erythrocyte membranes. *Annals of the New York Academy of Sciences* **225**, 38–45.
- KAPITANY, R. A. & ZEBROWSKI, E. J. (1973). A high resolution PAS stain for polyacrylamide gel electrophoresis. *Analytical Biochemistry* **56**, 361–369.
- LAZARY, S., RIVERA, E., DE WECK, A. L., GERBER, H. & NICOLET, J. (1974). In vitro stimulation of bovine leucocytes by phytohaemagglutinin and other mitogens. *Research in Veterinary Science* **17**, 344–350.
- MARCHESI, V. T. & ANDREWS, E. P. (1971). Glycoproteins: isolation from cell membranes with lithium diiodosalicylate. *Science* **174**, 1247–1248.
- MOROWITZ, H. J. & TERRY, T. M. (1969). Characterization of the plasma membrane of *Mycoplasma laidlawii*. *Biochimica et biophysica acta* **183**, 276–294.
- OUCHTERLONY, O. (1967). Immunodiffusion and immunoelectrophoresis. In *Handbook of Experimental Immunology*, pp. 655–706. Edited by D. M. Weir. Oxford: Blackwell Scientific Publications.

- PAROZ, PH. & NICOLET, J. (1978). Glycoproteins in mycoplasmas. Contamination with serum aggregates from growth media. *Experientia* **34**, 1668.
- PAROZ, PH., KRAWINKLER, M. & NICOLET, J. (1976). Verwendung der Polyacrylamid-Gel-Elektrophorese in der Diagnostik von Nutztier-Mycoplasmen. *Zentralblatt für Veterinärmedizin, B* **24**, 668–677.
- RAZIN, S. & ROTTEM, S. (1976). Techniques for the manipulation of mycoplasma membranes. In *Biochemical Analysis of Membranes*, pp. 3–26. Edited by A. H. Maddy. London: Chapman and Hall.
- ROTTM, S., HASIN, M. & RAZIN, S. (1973). Binding of proteins to mycoplasma membranes. *Biochimica et biophysica acta* **298**, 876–886.
- SCHIEFER, H. G., GERHARDT, U., BRUNNER, H. & KRUEPE, M. (1974). Studies with lectins on the surface carbohydrate structure of mycoplasma membranes. *Journal of Bacteriology* **120**, 81–88.
- SCHÜTTEN, H. J., MOGENSEN, H. H. & LIND, K. (1978). *Mycoplasma pneumoniae* antigens demonstrated by crossed immunoelectrophoresis. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene (Abteilung I, Originale A)* **241**, 240.
- TROY, A., SCOTT, J. & MELVIN, E. (1953). Determination of dextran with anthrone. *Analytical Chemistry* **25**, 1656–1661.
- WILLIAMS, M. H., BROSTOFF, J. & ROITT, J. M. (1970). Possible role of *Mycoplasma fermentans* in pathogenesis of rheumatoid arthritis. *Lancet* **2**, 277–288.
- ZACHARIUS, R. M., ZELL, T. E., MORRISON, J. H. & WOODLOCK, J. J. (1969). Glycoprotein staining following electrophoresis on acrylamide gels. *Analytical Biochemistry* **30**, 148–152.