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Detection of serum proteins in the electrophoretic patterns of total proteins of mycoplasma cells

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SUMMARY

The contamination of mycoplasma cell preparations by serum proteins originating from culture medium was studied. *A. laidlawii* and *M. arthritidis* cells were grown in the presence of [^{14}C]-aminoacids, and the cells were washed with 0·9% NaCl by threefold centrifugation. Total proteins of the washed cells were analysed by SDS gel electrophoresis. Coomassie-stained electrophoretic patterns were compared with autoradiographs of the same gels. The stained electrophoretic pattern of washed *A. laidlawii* grown without serum was identical with autoradiographs of the same cells grown without or with serum. That of washed *A. laidlawii* grown with serum differed from the corresponding autoradiograph by the presence of extra protein bands I, II, III, and IV with molecular weights of over 160,000, 80,000–87,000, 55,000 and 25,000, respectively. The same extra bands were found in stained electrophoretic patterns of washed: (*a*) *A. laidlawii* cells grown without serum and mixed with serum in the stationary phase, (*b*) *M. arthritidis* cells, as compared with their autoradiographs, (*c*) serum precipitate. The bands III and IV may be due to the heavy and light chains of γ-globulin, the band II might belong to transferrin or to some component of complement. Acidification of serum to pH 5 brought about 100-fold rise of amount of serum precipitate, the number of bands in the electrophoretic pattern of the precipitate being also increased. Stained electrophoretic patterns of cells purified by twofold centrifugation in step sucrose density gradient (1·20–1·27 g./cm.^{3} for *A. laidlawii*, and 1·15–1·25 for *M. arthritidis*) contained no extra bands and matched completely with their autoradiographs.

It was concluded that contamination of washed mycoplasma cells by serum proteins is mainly due to co-precipitation of aggregated serum proteins together with cells during centrifugation rather than to adsorption of serum proteins on the cell surface.

INTRODUCTION

In studying protein composition and antigenic properties of mycoplasma cells grown in the presence of serum investigators frequently faced the problem of contamination of cellular preparations by serum proteins. Thus, for example, the titre of *M. pneumoniae* cells grown in the presence of horse serum diminished by one order at their incubation with rabbit antihorse serum (Sethi & Brandis, 1972). Lipman, Clyde & Denny (1969) concluded that preparations of *M. pneumoniae* cells washed off by threefold centrifugation in 0·9% NaCl contained five serum

proteins. This conclusion does not seem reliable for the bands were revealed only by comparison of electrophoretic patterns of total proteins in preparations with that of serum proteins, the bands with similar electrophoretic mobility being considered identical. It was shown by Chelton, Jones & Walker (1968) that the amino acid composition of *M. mycoides* var. *capri* cells measured in the study of Jones, Terriennson & Walker (1965) is unreliable since the cellular precipitate which was analysed contained over 50% of serum proteins. A decrease in pH occurring during the growth of fermenting mycoplasma enhances the degree of contamination of cells by serum proteins when standard washing from culture medium by centrifugation is used (Bradbury & Jordan, 1971; 1972; Chelton *et al.* 1968; Pollack, Sommerson & Senterfit, 1969). It was found by Rottem, Hasin & Razin (1973) that at pH below 5 a large amount of serum albumin was bound to *A. laidlawii* cell membranes and that the bound albumin was removed by washing in 1 M-NaCl. It was suggested by the authors that other serum proteins with isoelectric points in the acid region can be bound as well to mycoplasma membranes at low pH values in the medium.

The above data show that serum proteins may contaminate mycoplasma cells washed off by repeated centrifugation in saline solution. The contamination may be caused either by their adsorption on cellular membranes, by specific incorporation into cells or by precipitation of aggregated serum component together with mycoplasma cells. In previous studies on mycoplasma species identification by electrophoresis in polyacrylamide gel (Smirnova, Yaguzhinskaya & Balaeva, 1975; Yaguzhinskaya *et al.* 1974) we found protein bands with similar mobility in electrophoretic patterns of all mycoplasma species investigated; the relative intensity of these common bands increased with reduction of mycoplasma titre. The present study shows that the bands are those of serum proteins, the cause of contamination of cells by serum proteins is investigated and a method of its removal from cell preparations has been suggested. Mycoplasma cultures were grown in the presence of [^{14}C]-protein hydrolysate, labelled cellular proteins were subjected to electrophoresis and autoradiograms obtained were used as controls in determining the degree of cell purification.

MATERIALS AND METHODS

Mycoplasma culture media

(1) The medium without serum was obtained from Nutritive Media Division, Institute of Microbiology and Epidemiology, Academy of Medical Sciences of the U.S.S.R., Moscow.

It was made as follows: 9 vol. of tryptic digest of cattle myocardium was added to 1 vol. of yeast extract, then NaCl was added to 0·5 %, pH value was brought to 8 with NaOH, and the mixture was autoclaved at 110° C. for 30 min. Yeast extract preparation: 5 kg. of pressed baker's yeast was added to 20 l. of tap water, the mixture was boiled for 30 min. and filtered hot through linen.

(2) The serum medium contained 90% (v/v) of the medium without serum and 10% (v/v) of non-heated horse serum.

To both media, 100 units/ml. of penicillin were added. The final pH's were 7·65 for the medium without serum and 7·75 for the serum medium.

Non-labelled mycoplasma cell preparations

pH-values were measured with the aid of pH-meter (potentiometer) LPU-01, U.S.S.R., at room temperature (20–22° C.).

Two mycoplasma strains which were used were obtained from the collection of Dr G. Ya. Kagan (Institute of Microbiology and Epidemiology, Academy of Medical Sciences of the U.S.S.R., Moscow): *Acholeplasma laidlawii* str. Köhler was originally obtained from Dr W. Köhler, Institut für Mikrobiologie und experimentelle Therapie, Jena, D.D.R. *Mycoplasma arthritidis* PG6 was originally obtained from Dr E. A. Freundt, Aarhus University, Aarhus, Denmark.

Five ml. of 2- or 4-day *A. laidlawii*, 10^8 c.f.u./ml., grown with serum, were inoculated into 200 ml. of serum medium. Three-day culture (pH 7) with 10^8 c.f.u./ml. was used.

Five ml. of 2-day *A. laidlawii* culture grown without serum (third passage), 10^8 c.f.u./ml., were inoculated into 200 ml. of medium without serum. Three-day culture (pH 6·9) with 10^8 c.f.u./ml. was used.

10% of horse serum was added to 100 ml. of 3-day *A. laidlawii*, culture grown without serum and incubated for 2 hr. at 37° C.

Five ml. of 7- or 3-day culture of *M. arthritidis*, 10^8 c.f.u./ml., were inoculated into 20 ml. of serum medium: 3-7 day culture (pH 7·7) was used.

Standard procedure of washing: the cells were precipitated by centrifugation (8000 *g*, 40 min.) and washed three times with 0·9% NaCl under the same conditions of centrifugation. All these operations were made at 4° C.

Preparations of cells labelled with [^{14}C]-amino acids

Immediately after inoculation 0·05 ml. (1·7 mCi/ml.) of [^{14}C]-protein hydrolysate was added to 1 ml. of mycoplasma culture. After 3–4 days the cells were precipitated by centrifugation (15,000 *g*, 40 min.), resuspended in 0·9% NaCl and precipitated once more in the same conditions.

Isopycnic centrifugation in sucrose density gradient

Linear density gradient (*aqueous sucrose solution*, 15–60%, w/w)

A UPR-8 centrifuge, U.S.S.R., and 5- or 30-ml. tubes were used. The cells were layered on the gradient either in culture medium, the volume of sample being 1 ml. for 5-ml. tubes and 5 ml. for 30-ml. tubes (variant '*a*'), or the cells were concentrated from 100 ml. of culture in 1 ml. of 0·9% NaCl, the volume of sample being 0·1 and 0·3–0·5 ml. respectively (variant '*b*'). The centrifugation conditions were the following: for 5 ml. tubes: rotor RKS-40, 105,000 *g*, 40 min., 20°; for 30 ml. tubes: rotor RKS-25, 96,000 *g*, 2 hr., 20°. The fractions (0·375 ml. for 5-ml. tubes or 1 ml. for 30-ml. tubes) were collected beginning from the bottom of the tube by a peristaltic pump (Perpex, LKB). Refractive index, cell titre and radioactivity were determined in each fraction. For radioactivity measurement, 0·1 ml.

of fraction was placed in 2 ml. of the scintillator (100 g. of naphthalene, 6 g. of PPO (2,5-biphenyl oxazole, scintillation grade, U.S.S.R.), 0·5 g. of POPOP (1,4-bis-[5-phenyl-2-oxazolyl]-benzene, scintillation grade, U.S.S.R.) in 1 l. of dioxane (Lomonosov & Soshin, 1968). For better solubility of sucrose in the scintillator, 0·4 ml. of water was added to each sample. Radioactivity was measured by USS-1 scintillation counter, U.S.S.R.

Step gradient

30-ml. tubes were used. Layer density: 1·27 and 1·20 g./cm.^{3} for *A. laidlawii*, and 1·25 and 1·15 g./cm.^{3} for *M. arthritidis*. Specimen composition, volume (variant '*b*') and centrifugation conditions were the same as that for linear gradient. After centrifugation the turbid part of gradient containing cells was collected manually by a syringe. The collected fraction was diluted with 0·9% NaCl, precipitated at 40,000 ***g***, the precipitate was either immediately converted to the sample for electrophoresis or it was resuspended in 0·9% NaCl solution and repeatedly centrifuged under above conditions.

Electrophoresis in 10% *polyacrylamide gel with sodium dodecyl sulphate* (*SDS*)

This was carried out according to the method of Laemmli (1970) at 4 mA per tube with the modification that the concentration of Tris-HCl buffer in separating gel was increased 1·5 times to improve the quality of electrophoretic patterns (Andreev & Gonikberg, 1974). Non-fixed gels were stained according to Ward (1970) by 0·25% solution of Coomassie-brilliant blue R-250 in the mixture of methanol:acetic acid:water (5:1:5) for 2 hr. and washed by a solution containing 10% methanol and 10% acetic acid.

For the preparation of samples, the cells were sedimented by centrifugation at 15,000 ***g***, 40 min. and the pellet was dissolved in 'final sample buffer' of Laemmli (1970). For non-radioactive mycoplasma, a precipitate corresponding to 4 ml. of culture (about 10^8 c.f.u./ml.) was applied to one gel; for the radioactive mycoplasma that from 0·4 ml. of culture.

For autoradiography, longitudinal sections of 0·5 mm. thickness were cut from the gels. The sections tightly compressed between a polyethylene film and a chromatographic paper were dried at room temperature for 2 days (Andreev & Gonikberg, 1974). Dried sections adhered to the paper were applied to the X-ray film RT–1, USSR, and exposed for 7 days.

RESULTS

Detection of serum proteins in electrophoretic patterns of cell preparations

Plate 1A shows electrophoretic patterns of total proteins in *A. laidlawii* cell preparations grown in various conditions (gels 3–5) as well as autoradiograms of electrophoretic patterns of *A. laidlawii* cells grown in the presence of labelled amino-acids in serum-containing medium (gel 2) and serum-free medium (gel 1). As may be seen from the plate, the patterns 1, 2 and 3 are actually identical, i.e. the set of proteins synthesized by cells does not practically depend on the presence of serum in the culture medium. Gels 4 and 5 (Plate 1A) differ from gels 1–3 by

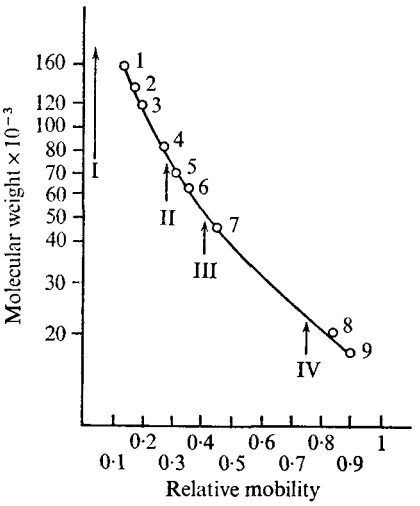

Fig. 1. Determination of molecular weights of admixture serum proteins in mycoplasma electrophoretic patterns. The relative mobilities of protein bands were measured as X/X_0 where (X) distance from the beginning of separation gel to a protein band and (X_0) distance from the beginning of separation gel to the bromphenol blue marker. The calibration curve is plotted with T4 bacteriophage proteins with the known molecular weights (1) P34 (150,000–160,000) (King & Laemmli, (1971), (2) P 7 (135,000) (King & Laemmli, 1973), (3) P37 (120,000) (King & Laemmli, 1971), (4) P10 (88,000) (King & Laemmli, 1973), (5) P18 (68 000–70,000), (6) P20 (63,000), (7) $P23^x$ (46,500), (8) $IPIII^x$ (21,000), and (9) P19 (18,000) (Laemmli, 1970). The arrows show the mobilities of admixture serum proteins I, II, III, IV in mycoplasma electrophoretic patterns.

the presence of additional bands I, II, III, IV. The bands I and II are always well noticeable, the bands III and IV are rather diffuse and were seen only in some preparations. Thus the growth of *A. laidlawii* in the presence of 10 % horse serum (gel 4) or the addition of the same amount of serum to the culture in a stationary phase (gel 5) results in the appearance of several additional protein bands in electrophoretic patterns. Similar comparison of electrophoretic patterns for preparations of *M. arthritidis* (Plate 1B) has shown that the cells grown in the presence of serum contain the same additional bands as in the case of *A. laidlawii* (c.f. gels 1 and 2). The comparison of electrophoretic patterns of serum and broth with yeast extract precipitates (washed as cells) has shown that no specific bands are due to broth precipitate whereas serum precipitate (Plate 1B, gel 5) produces electrophoretic patterns with most intense bands corresponding to bands I, II, III, IV, which arise in electrophoretic patterns of mycoplasmas grown in the presence of serum.

Identification of serum proteins in electrophoretic patterns of mycoplasma cell preparations

The curve of log molecular weight of polypeptide chains versus their relative mobility was plotted (Fig. 1) applying the T4 bacteriophage proteins as molecular weight standards. By using this curve we determined the molecular weights of polypeptides I, II, III, IV from the serum corresponding to additional bands in

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Table 1. *Molecular weights of serum proteins in electrophoretic patterns of mycoplasmas*

Protein	Relative mobility	Molecular weight
I	0·03	> 160,000
II	0·268–0·29	87,000–80,000
III	0·392–0·42	52,000–57,000
IV	0·73–0·79	22,000–25,000

electrophoretic patterns of *A. laidlawii* and *M. arthritidis* (Table 1). For band I only the lower limit of molecular weight can be evaluated since the calibration curve begins at 160,000 daltons. Serum proteins contain much albumin (50%) and γ-globulin (11%). The molecular weight of serum albumin chain is 68,000 (Weber & Osborn, 1969), that of light and heavy γ-globulin chains – 25,000 and 55,000, respectively, i.e. they are close to molecular weights of proteins, II, III and IV. We tried to find out whether they are identical with proteins II, III and IV. For that purpose mycoplasma cell preparations were subjected to electrophoresis in the presence of internal standards – bovine serum albumin and γ-globulin (Plate 1C). As may be seen from the plate, serum albumin is not identical with protein II, hence it cannot noticeably contribute to the electrophoretic patterns of mycoplasma proteins grown in the presence of serum. The γ-globulin bands, however, have mobilities coinciding with the mobility of bands III and IV. Thus the γ-globulin may be one of the serum proteins that contaminate mycoplasma cell preparations.

Electrophoresis of serum precipitate at various pH values

It was important to test the pH dependence of the amount and composition of the serum precipitate in the absence of mycoplasma cells. Plate 1 D shows electrophoretic patterns of serum precipitates washed by the standard technique and collected at pH 5 (gel 1), pH 5·3 (gel 2), pH 6·5 (gel 3) and those of native serum, pH 7·4 (gel 4). The serum was brought to the required pH values by HCl. The precipitate was collected from 5 ml of serum. The amount of the sample applied to gel 1 was 100 times smaller than to gels 3 and 4. The comparison of these gels shows that the amount of precipitate after acidification to pH 5 increases nearly by 2 orders of magnitude. The protein composition of the precipitate also changes, being added by new components. The cultures of *A. laidlawii* and *M. arthritidis* acidified in the stationary phase to pH 5, pH 5·3 and pH 6·5 had the same additional bands in electrophoretic patterns as the serum precipitate at these pH values.

Separation of mycoplasma cells from serum proteins

Since the additional bands in electrophoretic patterns of mycoplasmas grown in the presence of serum coincide with the main bands of serum precipitate we tried to separate the precipitate by preliminary centrifugation of serum. Twofold centrifugation of serum at 8000 g prior to mycoplasma cultivation did not give the desired results. Serum proteins may possibly reaggregate during cultivation. Since protein aggregates not containing much lipids would have a higher density

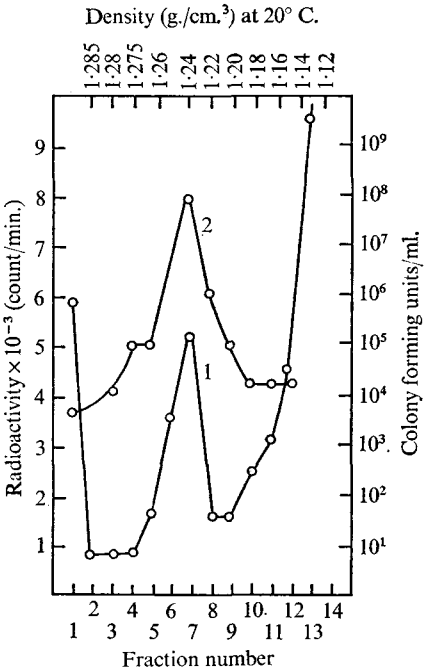

Fig. 2. Isopycnic centrifugation of *M. arthritidis* in linear sucrose density gradient. (Curve 1) radioactivity of fractions. (Curve 2) the number of colony forming units in fractions.

than the cells, we tried to separate them by centrifugation to equilibrium in sucrose density gradient.

For that purpose it was necessary first to determine the buoyant density of mycoplasma cells in sucrose density gradient. Fig. 2 shows the results of centrifugation in linear sucrose density gardient of *M. arthritidis* culture labelled with [^{14}C]-amino acids. As is seen from the figures, the position of peaks of radioactivity and of colony-forming-units coincide and correspond to the density of 1·22–1·25 g./ml. It was found in a similar experiment that the buoyant density of *A. laidlawii* is approximately 1·21 g./ml. These data agree with the values 1·22–1·24 g./ml. of buoyant density in sucrose density gradient that were determined for other mycoplasma species (Todaro, Aaronson & Rands, 1971; Lozinsky *et al.* 1974). In the layer of gradient where the cells were found a sharp, turbid, visible zone was observed.

The concentrated suspension of mycoplasma cells washed by the standard method produced a wide zone of turbidity after isopycnic centrifugation in linear sucrose density gradient. The width increased if the cell suspension was stored before centrifugation was observed with both mycoplasma species. To test whether different parts of the turbidity zone become heterogenous in protein composition the 4-day *M. arthritidis* culture (washed and concentrated by standard method) was subjected to isopycnic centrifugation in step gradient and the fractions corresponding to density intervals 1·150–1·165, 1·165–1·197 and 1·197–1·255 g./ml. were studied electrophoretically. It was found, however, that the

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electrophoretic patterns of these fractions were identical. Thus the cellular material was collected from the turbidity zone corresponding to the interval of densities 1·15–1·25 g./ml. for *M. arthritidis* and 1·20–1·27 g./ml. for *A. laidlawii*.

Plate 1B shows that in the fraction of cells taken from the step gradient (gel 3) the relative intensity of serum protein bands decreased compared with the initial preparation where the cells were washed by standard method (gel 2). After repeated centrifugation the bands of serum proteins completely disappeared (gel 4). If mycoplasma cells were preliminarily washed at 15,000 *g* or at higher *g* values, the precipitate was so compact that it could not be dispersed, and serum components remained even after twofold centrifugation in step gradient. So the mycoplasma cells grown in the presence of serum at neutral pH and washed at 8000 *g* can be separated from serum components.

DISCUSSION

The above results show that the mycoplasma cell preparations grown at neutral pH in a medium containing serum and washed from serum by a standard method (threefold centrifugation and resuspending in 0·9% NaCl) contain an admixture of serum proteins. This admixture is composed of only a few proteins and does not contain albumin, the main serum protein. The protein composition of the admixture does not depend on mycoplasma species. The precipitate of the serum itself contains the same proteins. At culture titre 10^8 c.f.u./ml., the relative content of serum proteins in cellular preparations is not high and only slightly changes electrophoretic patterns, but at lower titres the relative contribution of serum proteins to electrophoretic patterns largely increases. It seems that the quantity of serum proteins in the cellular preparation may be approximately proportional not to the number of cells but to amount of serum in the culture. The facts suggest that cell contamination by serum proteins is caused mainly not by adsorption of serum proteins on the cells but by coprecipitation of serum protein aggregates together with mycoplasma cells during centrifugation of the culture. Of course, some amount of serum proteins may also adsorb on cells. For example, the evidence of Sethi & Brandis (1972) cannot be explained without assuming adsorption of serum proteins on surface of cells. Besides, during the standard washing procedure the cells may adhere to the particles of aggregate serum proteins.

At neutral pH values the admixture of serum proteins in the cellular preparation consists mainly of four types of polypeptide chains. There is not enough evidence for identification of these proteins but some suggestions can be made. The molecular weights of the diffuse bands III and IV coincide with molecular weights of the heavy and light chains of γ-globulin. Thus it may be assumed that chains III and IV belong to γ-globulin.

The denaturated and aggregated γ-globulin is known to bind the complement (Ishizaka, Ishizaka & Borsos, 1961). This process may lead to the formation of a precipitate found in serum. Thus it may be proposed that chains I and II are related to some components of complement. On the other hand the band II may be also related to transferrin since its molecular weight is close to 81,000 daltons, the

molecular weight of transferrin chains (Palmour & Stutton, 1971). It was shown also that γ-globulin can complex with iron-deficient transferrin (Tukachinsky & Badyina, 1974).

Similar serum proteins contaminating mycoplasma cell preparations were also found by other authors. Thus it was found by Bradbury & Jordan (1971) that *M. gallisepticum* cells washed from the culture medium at pH 5·1–5·2 contained five serum proteins (identified by immunoelectrophoresis): (1) IgG, (2) protein similar to IgM, (3) protein with α-globulin mobility, (4) protein with prealbumin mobility, (5) protein with β-globulin mobility, possibly transferrin. Serum proteins similar to IgG, IgM and transferrin were also found in *M. gallisepticum* preparation after incubation of cells with serum at pH 8·0 (Asmar, 1965).

The number of serum proteins in cellular preparations sharply increases at acidification of culture medium to pH 5. The same proteins are found in the precipitate of serum brought to pH 5. The total amount of precipitate at serum acidification also sharply increases. Other authors have also noted that lowering of pH to 5, results in highly increased contamination of mycoplasma cells by serum proteins (Bradbury & Jordan, 1972), but in those studies it was supposed that contamination was caused only by adsorption of serum proteins on cells.

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EXPLANATION OF PLATE 1

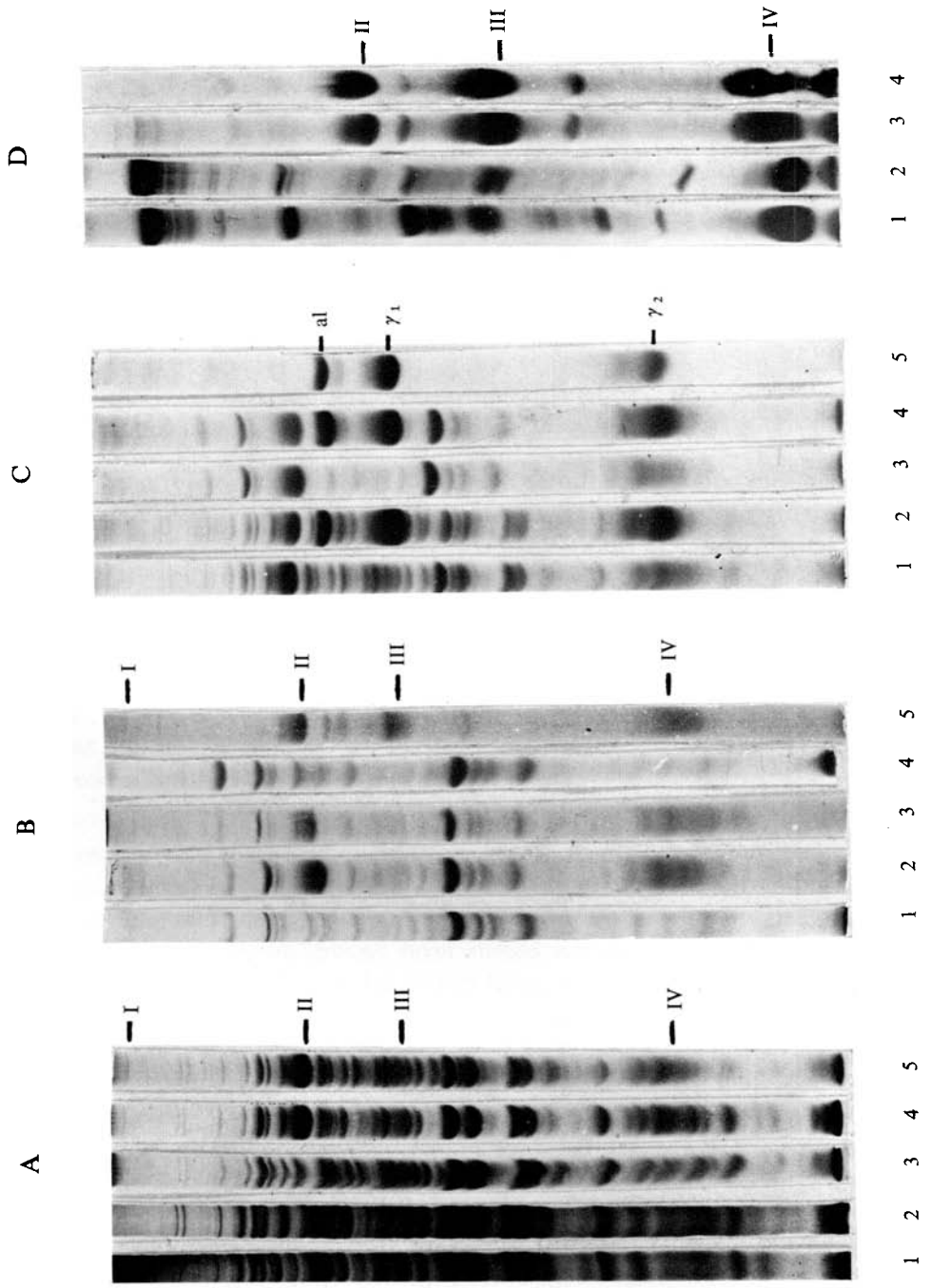
Electrophoretic patterns of proteins in mycoplasma cell preparations

(A) *A. laidlawii*. (1, 2) autoradiograms; (1) culture grown with serum, (2) without serum; (3, 4, 5) total proteins in cell preparations washed by standard method; (3) culture grown without serum, (4) with serum, (5) grown without serum, the serum is added in the stationary phase; gels 3–5 are Coomassie stained.

(B) *M. arthritidis* (gels 1–4). (1) autoradiogram; (2) total proteins in cell preparation washed by standard method; (3, 4) fractions from step gradient; (3) after the first centrifugation, (4) after the second centrifugation; (5) Electrophoretic patterns of the precipitate from 2 ml, of serum washed by standard method. Gels 2–5 are Coomasie stained.

(C) Comparison of electrophoretic patterns of mycoplasma proteins with that of some serum proteins. Mycoplasma cells were washed by standard method. (1) *A. laidlawii*, (2) *A. laidlawii* with 5 μg. of albumin and 50 μg. of γ-globulin; (3) *M arthritidis*; (4) *M. arthritidis* with 10 μg. of albumin and 50 μg. of γ-globulin; (5) 5 μg. of albumin (Al) and 50 μg. of γ-globulin (γ1) heavy and (γ2)-light chains). All gels are Coomasie stained.

(D) Electrophoretic patterns of precipitates from 5 ml. of serum at different pH (electrophoresis in 8·5% gel). The precipitates were washed by standard method: (1) at pH 5, (2) at pH 5·3 (3) at pH 6·4, (4) native serum, pH 7·4.



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