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Isolation, ultrastructure and antigenicity of *Mycoplasma gallisepticum* membranes

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SUMMARY

The cell membrane of *Mycoplasma gallisepticum* was isolated by lysing the cells with digitonin. Chemical and density-gradient analyses and electron microscopy showed the isolated membranes to be relatively free of cytoplasmic contaminants. The density of the membranes exceeded that of other mycoplasma membranes, indicating a higher protein content. Small vesicular extensions seen in the sectioned membranes were interpreted as empty blebs.

The isolated membranes, but not the cytoplasmic fraction, elicited in chickens the production of growth-inhibiting, agglutinating and haemagglutination-inhibition antibodies to *M. gallisepticum* in titres resembling those obtained by injection of whole cells. The peak of the serological response varied with the serological test employed. The rapid slide-agglutination test became positive as early as 3 days after the first injection of only 50 μg. of membrane protein. The haemagglutination-inhibition antibody titre reached its peak at about 10 days after the first injection, while that of the growth-inhibiting antibodies was reached only at about 25 days. The addition of adjuvant to the membrane antigen did not improve the production of the growth-inhibiting antibodies in chickens, but it produced some improvement in rabbits. Our results support the thesis that the chief immunogens of *M. gallisepticum* reside in the cell membrane of this organism.

INTRODUCTION

As is well known, mycoplasmas differ from most other bacteria in that they lack a rigid cell wall, a characteristic which makes them particularly suitable for studies of the cell membrane (Razin, 1973). In some strains, such as *Acholeplasma laidlawii*, isolation of pure cell membranes is achieved by the simple procedure of osmotic lysis, and examination of the antigenic and biochemical properties of the membrane has advanced accordingly (Ne'eman, Kahane, Kovartovsky & Razin, 1972). In other strains, including some of the important pathogenic mycoplasmas, the cells are more refractory to osmotic lysis, and it has proved more difficult to obtain pure cell membranes in sufficient quantity for critical analysis of their antigens. Tech-

niques for preparation of membranes have included alternate freezing and thawing (Williams & Taylor-Robinson, 1967), osmotic lysis of glycerol-loaded cells (Kahane & Razin, 1969), ultrasonic treatment (Argaman & Razin, 1969), and gas cavitation (Hollingdale & Lemcke, 1969), but these lead either to fragmentation of the membranes or to lysis of only part of the cells (Razin, Kahane & Kovartovsky, 1972). In a recent report Rottem & Razin (1972) demonstrated the effectiveness of digitonin for the isolation of pure cell membranes from *Mycoplasma hominis*. The cells remain sensitive to digitonin treatment throughout the growth cycle. In addition, digitonin is effective in the presence of Mg^{2+} or other ions necessary to preserve membrane structure and function. We have examined the sensitivity of *Mycoplasma gallisepticum* to lysis by digitonin and have employed this reagent for the isolation of large quantities of pure membranes. The biochemical properties and the appearance in the electron microscope of these membranes are reported.

Previous communications have indicated that in *M. gallisepticum*, as in the other mycoplasmas studied, the cell membrane is the site of the chief cell antigens (Williams & Taylor-Robinson, 1967; Kahane & Razin, 1969). However, it was then difficult to obtain complete separation of the membrane from the non-membrane antigens. The ability to isolate pure membranes by digitonin has opened the way for a more critical analysis of the localization of antigens in this pathogenic mycoplasma, and for the fractionation of the membrane antigens. In addition, it was considered important to study the immunogenicity of the mycoplasma and its isolated membrane in the natural host – in this case, the chicken. Use of the chicken would permit an examination of the protective effect of the immune response to mycoplasma antigens, an essential step in the development of a vaccine to this or other pathogenic mycoplasmas. Thus, in addition to the details of the isolation of membranes from *M. gallisepticum* by digitonin and the biochemical analysis of these membranes, this report presents various aspects of the immunogenicity of mycoplasma membrane antigens in chickens.

MATERIALS AND METHODS

Organism and growth conditions

Mycoplasma gallisepticum strain A5969 was obtained from Dr M. E. Tourtellotte (The University of Connecticut, Storrs, U.S.A.). Identification of the strain as *M. gallisepticum* was verified by growth inhibition tests (Clyde, 1964) with a standard *M. gallisepticum* anti-serum obtained from the Central Veterinary Laboratory, Weybridge, Surrey. The organisms were grown in 3 l. volumes of a modified Edward medium (Razin, 1963) containing either 10 % (v/v) horse serum or 2 % (v/v) of Difco PPLO serum fraction. For labelling membrane lipids 50 μCi of (9, 10-^{3}H)-oleic acid (The Radiochemical Centre, Amersham, England) were added to each litre of the growth medium. Cultures were usually harvested after 20 hr. of static incubation at 37° C. when turbidity reached 0·2–0·3 O.D. units at 590 nm. The organisms were sedimented by centrifugation at 9000 *g* for 30 min., resuspended and washed once in 0·25 M-NaCl.

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Assessment of sensitivity to lysis by digitonin

The sensitivity of the organisms to lysis by digitonin was tested as described before (Rottem & Razin, 1972) in a series of test tubes containing different concentrations of digitonin in 0·25 M-NaCl. Lysis was assessed by measuring the change in turbidity of the treated cell suspensions at 500 nm after 15 min. incubation at 37° C. Results were expressed as percentage lysis calculated according to the formula

$$\% \text{ lysis} = \frac{\text{O.D. of untreated suspension} - \text{O.D. of treated suspension}}{\text{O.D. of untreated suspension} - \text{O.D. of completely lysed suspension}} \times 100,$$

where 'untreated suspension' represents a control suspension without digitonin and 'completely lysed suspension' one treated with 100 μg./ml. or more digitonin until there was no further decrease in turbidity upon the addition of more digitonin. The remaining turbidity is presumably due to membrane and other insoluble material.

Isolation of cell membranes

The organisms harvested from 3 l. of medium at O.D.590 = 0·20–0·30 were washed once in 0·25 M-NaCl and resuspended in a small volume of the salt solution. The turbidity of this suspension was adapted so that a 1/30 dilution of it in 0·25 M-NaCl gave a reading of 0·5 O.D. unit at 500 nm. One volume of the concentrated cell suspension was added to 30 volumes of 0·25 M-NaCl containing 25 or 50 μg. digitonin/ml. The suspension was incubated in a 37° C. water bath for 15 min. The turbidity of the suspension at the end of the incubation period dropped to 0·05–0·09 O.D. units at 500 nm. The membranes were collected by centrifugation at 35,000 *g* for 30 min. The supernatant fluid was separated and used for the preparation of the cytoplasmic antigens as described in a forthcoming section. The viscous membrane pellet was resuspended in 60—80 ml. of 0·05 M-NaCl in 0·01 M phosphate buffer, pH 7·4, containing 5–10 μg. deoxyribonuclease per ml. (DN-100, Sigma) and 0·01 M-$MgCl_2$. After 30–60 min. incubation at room temperature with constant stirring viscosity disappeared and the membranes were collected by centrifugation at 35,000 *g* for 30 min. and washed six times alternately with deionized water and 0·05 M-NaCl in 0·01 M phosphate buffer, pH 7·4. The washed membranes were resuspended in 1/20 β-buffer (Razin, Morowitz & Terry, 1965) to a concentration of 3–4 mg. membrane protein/ml. and stored at −70° C. until used.

An alternative method of digesting the viscous DNA liberated during cell lysis is to add the deoxyribonuclease and Mg^{2+} to the digitonin solution, so that the enzyme can act during cell lysis. The only disadvantage of this method is that, owing to the large volume of the digitonin solution, considerable quantities of the expensive enzyme are necessary to maintain the level of 10 μg. enzyme/ml.

Analytical methods

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as standard. Membrane lipids were extracted with chloroform + methanol (2 + 1, by vol.) according to Folch, Lees & Sloane-Stanley (1957). Nucleic acids were extracted from the lipid-depleted residue and

determined as described before (Razin, Argaman & Avigan, 1963). Total carbohydrate was estimated by the method of Dubois *et al.* (1956) on the residue remaining after nucleic acid extraction. Radioactivity in membrane lipids was determined in a Packard Tri-Carb liquid scintillation spectrometer using a dioxane-toluene scintillation liquor (Kahane & Razin, 1969). Density-gradient centrifugation was performed as described before (Rottem, Stein & Razin, 1968) using 12 ml. of a linear sucrose gradient of 30 to 60%.

Preparation of antigens

M. gallisepticum cells washed at least four times in 0·25 M-NaCl and suspended in the same solution were used as whole cell antigen. The preparation of membrane antigens was as described above. The cytoplasmic antigens were prepared by freeze-drying of the supernatant fluid obtained after removal of the membranes of cells lysed by digitonin. The dried material was redissolved in physiological saline.

Antigens for immunization were prepared by dilution of the above preparations to the desired protein concentration in phosphate-buffered saline (PBS), pH 7·4. For use with adjuvant, equal volumes of antigen solution and Freund's complete or incomplete adjuvant (Difco) were mixed and treated in an M.S.E. ultrasonic disintegrator (60 W, 20 kc/sec.) for six 30 sec. periods in ice.

Immunization of rabbits

Rabbits were immunized according to the schedule of Kahane & Razin (1969). The rabbits were bled before immunization, at the time of the last injection and 1 week after that.

Immunization of chickens

The chickens used were a cross between White Rock and Cornish breeds from mycoplasma-free flocks. Periodical examination using the rapid slide-agglutination test showed the chickens to be serologically negative to *M. gallisepticum*. The chickens were 11–13 weeks old at the start of the immunization period. The immunization schedule is summarized in Table 1. As with the rabbits one half of the total antigen dose was given on the first day and the rest was distributed over the three subsequent weeks. The chickens were bled from the wing vein before immunization, at the time of each injection and at other time intervals as stated in the Results section.

Serological tests

Rapid slide agglutination test

Chicken and rabbit sera were tested for the presence of antibodies to *M. gallisepticum* by a commercial stained antigen prepared from *M. gallisepticum* strain S6 (De Zeeuw Laboratories, De Bilt, Netherlands). The tests were performed according to the manufacturer's instructions.

Tube agglutination

Agglutination of whole cells was tested according to Bailey *et al.* (1961), but the temperature of incubation was 37° C. instead of 52° C.

Table 1. *Schedule of immunization of chickens by* M. gallisepticum *antigens*

Day of injection	Antigen + adjuvant	Antigen alone	Volume injected (ml.) Each leg IM	Each leg SC	Each side of breast IM	Each side of breast SC	Each wing membrane SC
1	+	—	0·2	0·2	0·2	0·2	0·2
8	—	+	0·1	—	—	—	—
15	—	+	0·1	—	0·1	—	—
22	—	+	0·1	—	0·1	—	—

IM = intramuscular; SC = subcutaneous.

Growth inhibition

The technique of Clyde (1964) was used. The medium was a modified Edward medium (Razin, 1963) containing 10% (v/v) fresh horse serum and 1·5% (w/v) Difco agar. The filter paper disks were saturated with undiluted antiserum. Best results were usually obtained when the inoculum spread on the plate was taken from a 10^{-2} dilution of a logarithmic-phase culture of *M. gallisepticum*.

Metabolic inhibition

The test was carried out in a medium containing glucose as described by Taylor-Robinson, Purcell, Wong & Chanock (1966). In early experiments a 10^{-2} dilution of an actively growing culture was used as an inoculum; later, in an attempt to reduce variability, small samples of a young culture were kept at $-70°$ C. and used at 10^{-1} dilution for each performance of the test.

Haemagglutination inhibition

The antigen for this test was a concentrated suspension of washed *M. gallisepticum* cells prepared in 50% glycerol solution in PBS and stored in small samples at $-20°$ C. Haemagglutination inhibition was tested according to the constant antigen-decreasing antiserum method recommended in 'Methods for Examining Poultry Biologics and for Identifying and Quantifying Avian Pathogens' (published by the U.S.A. National Academy of Science, 1971, p. 229). The test was performed in lucite plates with a reaction volume of 1·0 ml. Dilutions of the antisera in the antigen suspension were allowed to stand for 30 min. at room temperature before the addition of red blood cells. The results were expressed in haemagglutination-inhibition units, which are equivalent to the highest dilution of serum producing complete inhibition of haemagglutination multiplied by the haemagglutination units of the antigen (Kuniyasu & Ando, 1966). A haemagglutination unit (HA) is defined as the highest dilution of the stock antigen which will completely agglutinate the test dose of erythrocytes under the standard conditions of the test. In our experiments 2–8 haemagglutination units were used.

Electron microscopy

For the preparation of thin sections, pellets of sedimented membranes were fixed in 4% glutaraldehyde in 0·1 M phosphate buffer (pH 7·4) for 4 hr. at 0° C., washed in the phosphate buffer, and postfixed in 2%OsO_4 in the same buffer for 16 hr. The material was dehydrated and embedded in Epon by the method of Luft (1961). Sections were stained with uranyl acetate and lead citrate (Reynolds, 1963) and examined in a Phillips EM-300 electron microscope.

RESULTS

Sensitivity of M. gallisepticum *to lysis by digitonin*

M. gallisepticum cells were found to be sensitive to lysis by digitonin. As with *M. hominis* (Rottem & Razin, 1972) the lytic effect of digitonin decreased with the increase of the amount of cells in suspension (Fig. 1). Cultures harvested at various times before their O.D.590 reached 0·3 showed about the same sensitivity to digitonin.

Ultrastructure and composition of M. gallisepticum *membranes*

Plate 1 shows thin sections prepared from the sediment obtained after lysis of *M. gallisepticum* cells with 25 μg. digitonin per ml. The sections demonstrate that the sedimented material consists of membranes having the characteristic trilaminar shape in section with very little evidence of cytoplasmic contaminants, such as ribosomes. Very few cells could be detected in the material examined. The gross chemical composition of the membrane preparation is compared with that of whole *M. gallisepticum* cells in Table 2. The membranes were highly enriched in lipid, but contained much lower amounts of nucleic acids than whole cells. Total carbohydrate content was very low in both cells and membranes. As could be expected the density of the membrane preparation was significantly lower than that of the cells. In a typical preparation about 65 mg. of membrane protein were obtained from 160 mg. of total cell protein.

Immunogenicity of isolated membranes and cytoplasmic fraction

Table 3 shows that all the chickens injected with membranes responded in production of growth-inhibiting, agglutination and haemagglutination-inhibiting antibodies to M. *gallisepticum* in titres resembling those obtained by injection of whole cells. Injection of the cytoplasmic fraction failed to elicit any significant antibody response as measured by the above-mentioned tests (Table 3). In general, pre-immunization sera were negative when tested by the tube agglutination and growth inhibition tests, and were negative or showed only low titres in the haem-agglutination-inhibition test.

The peak of the serological response in the positive responders depended on the serological test employed. Thus, the chickens which were immunized with whole cells or membranes were converted from negative to positive responders in the slide agglutination test as early as 3 days after the first injection. The birds

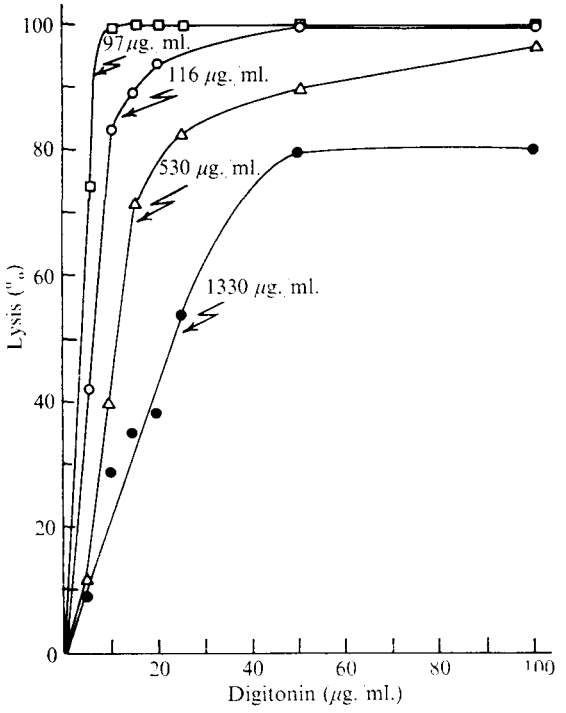

Fig. 1. Effect of the concentration of *M. gallisepticum* cells in suspension on their sensitivity to lysis by digitonin. The concentration of cell protein is given in μg. per ml. of suspension.

Table 2. *Chemical composition of* M. gallisepticum *cells and membranes isolated by digitonin lysis*

	Lipid*		DNA	RNA	Carbohydrate	
Preparation	μg./mg. protein	Counts/min. per mg. protein	(μg./mg. protein)	(μg./mg. protein)	(μg./mg. protein)	Density (g./cm.^{3})
Whole cells	74	261,122	60	130	6	1·230
Membranes	198	700,017	10	10	2	1·199

* Membrane lipids were labelled during growth with [9,10-^{3}H]-oleic acid.

remained positive in this test for at least 6 weeks after the last injection. Those which received the cytoplasmic fraction remained negative throughout the immunization period. Fig. 2 shows that the appearance of haemagglutination-inhibition antibodies was also very fast, reaching a peak-titre at about 10 days after the first injection, while the growth-inhibiting antibodies reached the peak at about 25 days after the first injection.

The effect of adjuvant on the immune response of chickens and rabbits to *M. gallisepticum* membranes can be seen in Table 4. The data in the table indicate that the production of growth-inhibiting antibodies in chickens does not depend on the addition of adjuvant to the membrane antigen. With rabbits, on the other hand, Freund's complete adjuvant appears to improve the immune response. Table 4 also shows that the antisera prepared in rabbits inhibited growth of *M. gallisepticum* more effectively than the antisera prepared in chickens.

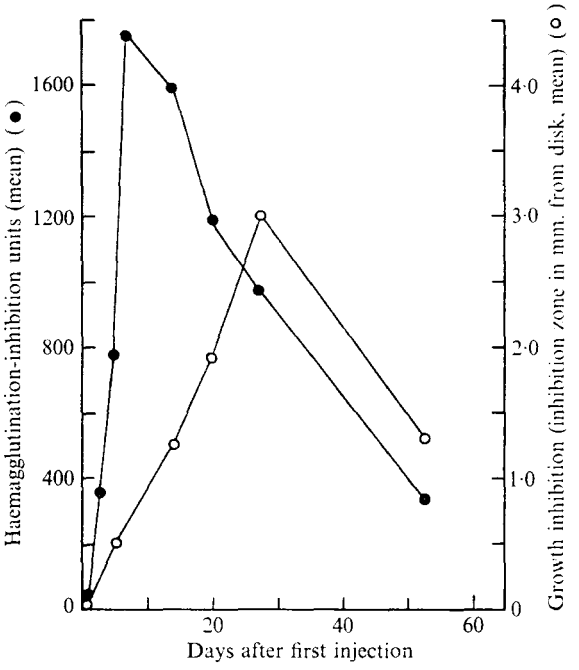

Fig. 2. The development and persistence of haemagglutination-inhibition and growth-inhibition antibodies to *M. gallisepticum* in nine chickens injected with either whole cells or membranes without adjuvant.

Table 3. *Serological response of chickens to whole cells and cell fractions of* M. gallisepticum

Expt.	Antigen*	No. of chickens	Growth inhibition (inhibition zone in mm. from disk, mean and range)	Tube agglutination titre (mean and range of reciprocal)	Haemagglutination inhibition units (mean and range)
1	Whole cells	5	2·1 (1·0–4·0)	33 (20–40)	ND
	Membranes	4	1·6 (1·0–2·0)	45 (10–80)	ND
	Cytoplasmic fraction	4	0	< 5	ND
2	Whole cells	5	ND	20 (10–40)	3710 (640–10240)
	Membranes	5	ND	13 (10–40)	1090 (320–2560)
	Cytoplasmic fraction	4	ND	< 5	160 (0–640)
	Uninoculated chickens	3	ND	< 5	< 10

* The total amount of antigen injected into each chicken contained in the first experiment 5 mg. protein with Freund's incomplete adjuvant and in the second experiment 1 mg. protein with no adjuvant. The data given in the table represent the maximal titres attained. ND = not done.

Table 5 shows the relation between the antigen dose and the immune response. The most striking effect of the decrease in antigen dose was a decrease in the proportion of the inoculated birds showing a positive response. That is, at 5 mg. membrane or whole cell protein all the inoculated birds showed a detectable response. At lower doses the proportion of positive responders in the growth

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Table 4. *Effect of adjuvant on the immune response of chickens and rabbits to* M. gallisepticum *membranes as measured by growth-inhibiting antibodies*

Animal	No. of animals	Adjuvant	Growth inhibition (inhibition zone in mm. from disk, mean and range)
Chickens*	4	Freund's complete	1·6 (1·0–2·0)
	5	Freund's incomplete	1·8 (1·0–3·5)
	4	No adjuvant	1·9 (1·5–2·0)
Rabbits†	4	Freund's complete	5·2 (3·7–6·7)
	3	Freund's incomplete	3·3 (3·0–3·9)
	2	No adjuvant	3·2 (2·6–3·8)

* Chickens were immunized with 5 mg. membrane protein. Maximal titres are presented.
† The titre at 5 weeks after the initial injection.

Table 5. *Relationship between antigen dose and the immune response in chickens*

		Growth inhibition*		Tube agglutination†	
Antigen	Dose (mg. protein)	Proportion of positive responders	Inhibition zone in mm. from disk in positive responders, mean and range	Proportion of positive responders	Reciprocal of titre in positive responders (mean and range)
Membranes	5	4/4	2·0 (1·0–4·0)	4/4	22 (10–40)
	1	2/5	1·5 (1·0–3·0)	5/5	17 (5–40)
	0·1	2/5	2·0 (1·0–3·0)	3/5	30 (10–40)
Whole cells	5	4/4	1·8 (1·0–3·0)	4/4	30 (20–40)
	1	3/5	1·4 (1·0–2·0)	5/5	60 (10–160)
	0·1	3/5	1·8 (0·5–3·0)	4/5	12 (10–20)

* Tested in sera obtained 27 days after the first injection.
† Tested in sera obtained 7 days after the first injection.

inhibition and tube agglutination tests decreased. However, all the birds, even those receiving the lowest dose (0·1 mg. protein) were converted from negative to positive when the rapid slide-agglutination test was employed. Moreover, the slide-agglutination test became positive even after the injection of only 50 μg. of membrane protein. Table 5 also shows that the positive responders to all doses of antigen did not differ significantly in the agglutination and growth inhibition titres.

DISCUSSION

The details of a simple reliable method for the isolation of large quantities of pure membranes from *Mycoplasma gallisepticum* are presented. To achieve lysis the method uses low concentrations of digitonin, a reagent known to form a specific complex with cholesterol and to cause cell lysis of the cholesterol-requiring mycoplasmas (Smith & Rothblat, 1960; Rottem & Razin, 1972). As emphasized by

Rottem & Razin (1972) complete lysis of the mycoplasma cells required a critical ratio between digitonin and cell mass – a point which might be overlooked in a large-scale preparation. Membranes can be readily prepared from cells at the end of the logarithmic phase of growth, the point of greatest cell yield. Further incubation, accompanied by a precipitous drop in the pH of the medium, is considered undesirable because of the possibility of adsorption of medium proteins to the cell membrane (Bradbury & Jordan, 1972; Rottem, Hasin & Razin, 1973) leading to changes in its antigenic structure.

Chemically and ultrastructurally the isolated *M. gallisepticum* membranes resemble the membranes of other mycoplasmas examined so far (Razin, 1973). The isolated membrane is composed almost entirely of protein and lipid. However, as indicated by their relatively high density (Table 2), the *M. gallisepticum* membranes appear to contain a higher percentage of protein than membranes of other mycoplasmas. This corroborates the finding of Rottem *et al.* (1968) that *M. gallisepticum* membranes, isolated by osmotic lysis of glycerol-loaded cells, consisted of about 79% protein and 19% lipid, as against 59% protein and 36% lipid in *Acholeplasma laidlawii* membranes. The idea put forward by Rottem *et al.* (1968) that the high protein content of *M. gallisepticum* membranes results from the presence of the bleb, a unique structure of this mycoplasma (Manniloff, Morowitz & Barrnett, 1965) appears to find support in our study. Thin sections of the isolated *M. gallisepticum* membranes show small vesicular extensions which may represent empty blebs (Plate 1*b*). Similar structures have not been detected so far in sections of isolated membranes from other mycoplasmas.

Our results demonstrate that the membranes isolated with digitonin are potent antigens. Thus immunization of chickens with membranes leads to the production of antibodies causing growth inhibition, agglutination of cells and haemagglutination inhibition. These results bear out the conclusions of earlier investigations with rabbits that the membrane is the site of the major immunogens of the cell (Williams & Taylor-Robinson, 1967; Argaman & Razin, 1969; Kahane & Razin, 1969; Razin *et al.* 1972). In contrast to the results with rabbits, only very low metabolism-inhibiting activity could be demonstrated in the immunized chicken sera. This observation, also noted by other investigators (Jordan & Kulasegaram, 1968; Taylor-Robinson & Berry, 1969), probably occurs because of the presence of factors interfering with the fixation of complement by sera from artificially immunized birds (Rice, 1947). We obtained demonstrable metabolism-inhibiting activity with immune chicken sera, but the titres were very low (only up to 1/32). Under the same experimental conditions rabbit sera produced in response to the same membrane antigen exhibited very high titres of metabolism-inhibiting activity. Attempts to increase this activity in chicken sera by the addition of exogenous complement were not successful, and the results of the test were not included in the present study.

The antibodies measured by the slide-agglutination and the haemagglutination-inhibition tests appeared and reached their peak much earlier in our experiments than the antibodies causing growth inhibition of *M. gallisepticum*. These results are in accordance with previous reports. Thus, Jordan & Kulasegaram (1968)

found that chickens and turkeys infected with *M. gallisepticum* responded by the rapid production of agglutinating antibodies measured by the slide and tube-agglutination tests and by the haemagglutination-inhibition test. Kleven & Pomeroy (1971) found that the first detectable serological response of turkeys infected with *M. meleagridis* was a positive reaction in the slide-agglutination test (as early as 4 days after infection). The peak titre of the growth-inhibiting antibodies was reached much later in the experiments of Ogra & Bohl (1970) working also with turkeys infected with *M. meleagridis*. The finding of Roberts (1969), Kuniyasu (1969) and of Kleven & Pomeroy (1971) that the slide-agglutination test is associated with the early-appearing high molecular weight immunoglobulins (IgM) may provide the explanation for the rapid conversion of immunized chickens and turkeys from negative to positive in this test.

It is clear from the experiments presented in this work that chickens respond to the injection of membranes of *M. gallisepticum* by the production of specific antibodies against the mycoplasma cell. By the use of non-infectious antigens it is possible to distinguish the phenomenon of immunization from that of infection. It is hoped in the continuation of this work to relate the presence of antibodies of the various types to protection against infection by *M. gallisepticum*. It is also planned to examine the response of chickens to purified membrane fractions. Methods for the isolation of membrane fractions highly enriched in immunogenic activity have already been developed for *A. laidlawii* and *M. hominis* (Ne'eman *et al.* 1972; Razin *et al.* 1972; Hollingdale & Lemcke, 1969; 1972). If a similar fractionation of membrane antigens can be made in *M. gallisepticum* it may be possible to separate antigens that specifically induce protective antibodies and to eliminate antigens involved in hyperimmune effects. Furthermore, since chickens are the natural host of *M. gallisepticum* it should be possible to evaluate the protective effect of the membranes and specific antigens contained in it.

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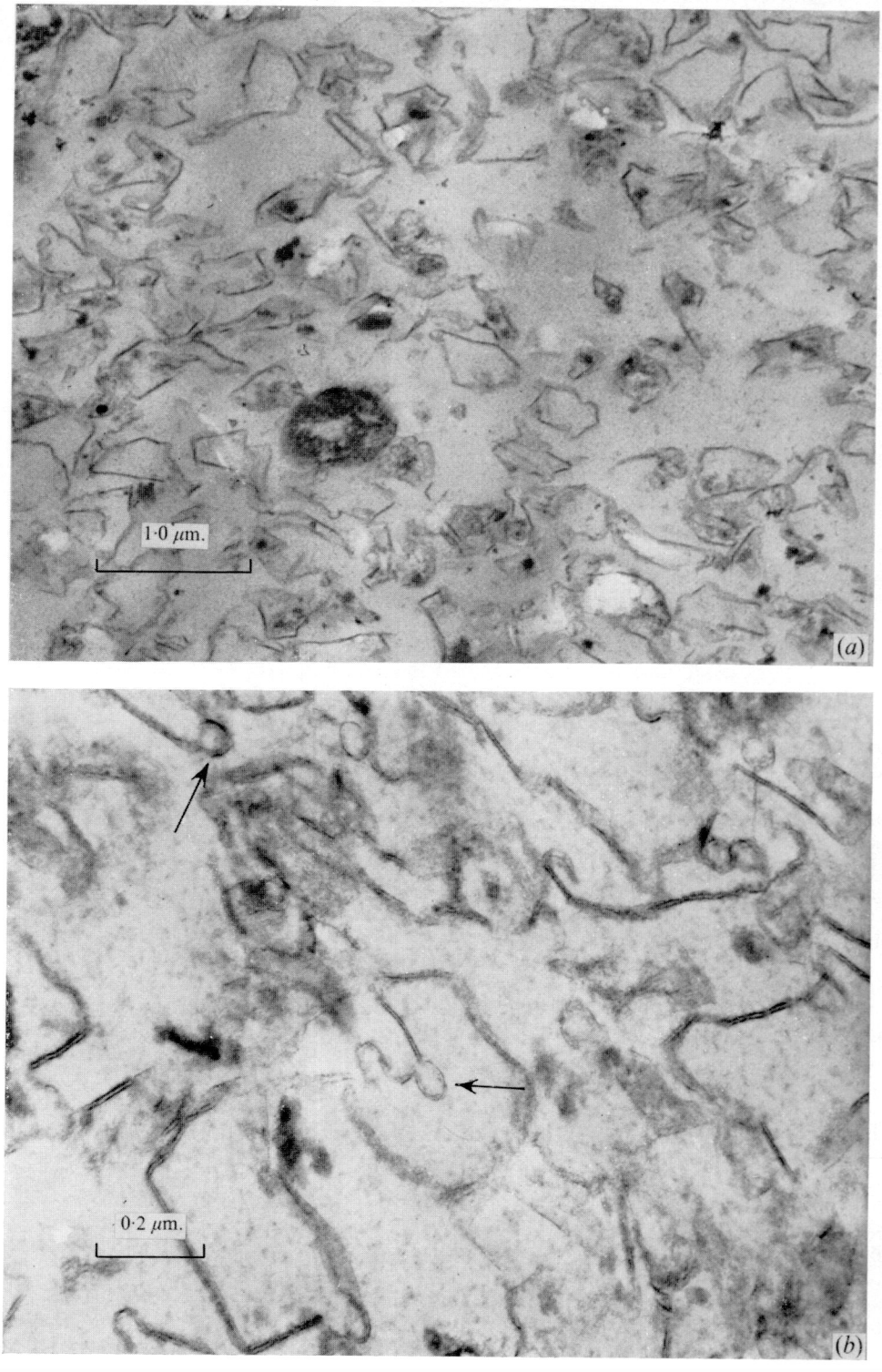

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EXPLANATION OF PLATE

Thin sections of *M. gallisepticum* membranes isolated by digitonin. (a) Low magnification, showing also an unlysed cell. (*b*) High magnification, showing the characteristic trilaminar structure of the membranes and the vesicular extensions marked by arrows.