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Studies on the adsorption of certain medium proteins to *Mycoplasma gallisepticum* and their influence on agglutination and haemagglutination reactions

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SUMMARY

Serum proteins from *Mycoplasma gallisepticum* culture medium could be detected on the organisms as a result of incubation at low pH. Only certain of the serum proteins, including IgG and IgM, were found, and the adsorption appears to depress the haemagglutinating activity of the organisms. There was no obvious effect on slide agglutination (SA) sensitivity but an incidental finding was that brief acid treatment enhanced the SA sensitivity of an antigen prepared from a young culture.

INTRODUCTION

The adsorption of protein to cell surfaces is a well-recognized phenomenon (King, 1968). In many cases the reaction appears to depend on specific protein reacting with specific cells, e.g. the fixation of protein hormones to sensitive cells, and the adsorption of cytophilic antibody to macrophages. In other cases, however, adsorption is less specific, such as the adsorption of protein to tanned red blood cells.

Hamburger, Pious & Mills (1963) demonstrated that mammalian cells in tissue culture adsorbed serum protein components from the growth medium, and, after some initial studies by Smith, Dunlop & Strout (1966), Jordan & Kulasegaram (1968) reported that *Mycoplasma gallisepticum* organisms also picked up proteins from their growth medium. In neither case could the contaminating proteins be removed by extensive washing, and inoculation of the washed cells or mycoplasmas into animals resulted in the production of antibody to the adsorbed proteins. Thus, false results may be obtained in serological tests using mycoplasmas if reactions between adsorbed proteins and their homologous antibodies should occur. Adsorbed proteins may also cause complications that are less readily recognized than the addition of new antigenic determinants. For example they may physically mask some of the existing determinants and thus reduce the specific reactivity of the mycoplasma antigens. These effects could be of considerable importance when such antigens are used for the serological diagnosis of *M. gallisepticum* infection in poultry flocks.

This communication describes (*a*) a study of the conditions of culture of *M. gallisepticum* that give rise to adsorption of medium proteins, (*b*) preliminary identification of the adsorbed proteins, and (*c*) a study of their effect on *M. gallisepticum* antigens in agglutination and haemagglutination tests.

MATERIALS AND METHODS

M. gallisepticum *strain*

A 514 originally obtained from Dr H. Chu, University of Cambridge, passaged numerous times in culture in this laboratory, was used.

Mycoplasma growth media

Broth medium (*BM*). This was prepared as described by Bradbury & Jordan (1971*a*) with 15% (v/v) heat-inactivated pooled swine serum in place of horse serum and a glucose concentration of 0·1%.

Buffered broth medium (*BBM*). BM was buffered with 0·2 M phosphate buffer, pH 7·5.

Chicken infusion broth (*CIB*). This was similar to that described by Jordan & Kulasegaram (1968) except that the infusion was autoclaved at 10 lb./in.^{2}, 114° C. for 30 min., and the pH indicator was phenol red.

Antisera

Rabbit sera. Two types of antisera were prepared: (1) two rabbits were immunized with BM and (2) four rabbits were immunized with a 24 hr. culture of *M. gallisepticum* that had been grown in BM, washed three times in 0·02 M phosphate buffered saline (PBS), pH 7·0, and adjusted to a standard optical density (Bradbury & Jordan, 1971*b*). The inoculation procedure was as described by Jordan & Kulasegaram (1968) but a multiple emulsion (Herbert, 1967) replaced the single emulsion adjuvant.

Turkey sera were obtained from natural infections with *M. gallisepticum*. All sera were stored at 0–4° C. and heated at 56° C. for 30 min. before use.

Preparation and standardization of M. gallisepticum *antigens*

These procedures have been described by Bradbury & Jordan (1971*b*). Inocula were 10% (v/v) of a 24 hr. broth culture unless otherwise stated, and cultures were incubated at 37° C.

Detection of adsorbed medium proteins

The antiserum raised in rabbits against BM was used to detect adsorbed protein by a slide agglutination or a gel diffusion test.

Slide agglutination (*SA*), *haemagglutination* (*HA*) *and gel diffusion* (*GD*) *tests*

These were all performed as described by Bradbury & Jordan (1971*b*).

Table 1. *The influence of pH of the medium and incubation time on the adsorption of medium proteins to* M. gallisepticum (*Expt.* 1)

Incubation time (hr.)	pH of medium	Reactions of antigens with antiserum to BM: SA	Reactions of antigens with antiserum to BM: Gel precipitation
24	6·8	−	−
48	5·2	−	−
72	5·1	+	−
120	5·1	+ + +	+
168	5·1	+ + +	+

SA: +, Positive reaction in 2 min.; + +, positive reaction in 1 min.; + + +, strong positive reaction within 30 sec.

Table 2. *The effect of culture in buffered broth medium (BBM) compared with BM on the adsorption of medium proteins to* M. gallisepticum (*Expt.* 2)

Incubation time (hr.)	Antigens from BBM: pH of medium	Antigens from BBM: SA with antiserum to BM	Antigens from BM: pH of medium	Antigens from BM: SA with antiserum to BM
48	6·9	−	5·1	+
168	6·3	−	5·2	+ +

Immunoelectrophoretic analysis (IEA)

This was carried out by the microtechnique of Scheidegger (1955) using LKB* equipment. One per cent agar in veronal buffer (I = 0·025, pH = 8·6) was used on the slides and the electrode vessels were filled with veronal buffer (I = 0·1, pH = 8·6).

RESULTS

The influence of pH on the adsorption of media proteins

Experiment 1

One-litre volumes of BM were inoculated with *M. gallisepticum* and, after 24, 48, 72, 120 and 168 hr. incubation, the pH was measured, the organisms harvested and standardized antigens were prepared. The antigens were tested for the presence of medium protein by SA and GD using rabbit antiserum to BM and the results are shown in Table 1.

The pH of the medium had fallen to 5·2 by 48 hr. and medium proteins were detectable by SA after 72 hr. incubation when the pH of the medium had been low for at least 24 hr. After 120 hr. incubation the agglutination reaction was more rapid, with larger floccules, and a single precipitin line was seen in gel diffusion.

* Stockholm, Sweden.

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Table 3. *The influence of pH of the medium on the adsorption of medium proteins to non-viable* M. gallisepticum *organisms* (*Expt.* 3)

pH of medium	SA reaction of antigens with antiserum to BM
7·0	−
6·0	±
5·0	+
4·0	+ + +
3·0	+ + +

±, Doubtful positive reaction.

Experiment 2

To investigate further the influence of pH, *M. gallisepticum* was grown in BM and BBM, and after incubation periods of 48 and 168 hr. antigens were prepared and tested as above.

Table 2 shows that although the pH of the buffered medium did fall, at the time of harvest it was considerably higher than the unbuffered medium. Antigens prepared from the buffered medium showed no detectable medium constituents in contrast to those from BM.

Experiment 3

To examine the effect of pH on the adsorption of protein to dead organisms, antigen was prepared from BBM after 2 days incubation and then stored at 0–4° C. until the organisms were no longer viable. Medium proteins could not be detected by SA. Equal volumes (1 ml.) of antigen were then added to 25 ml. BM that had been adjusted to the following pH values: 7·0, 6·0, 5·0, 4·0, 3·0. After incubation for 24 hr. at 37° C. the organisms were harvested and prepared in the usual way as serological antigens. Table 3 gives the results of slide agglutination tests with these antigens and shows that acid pH caused adsorption of medium proteins to the dead organisms.

Identification of the adsorbed proteins

Experiment 4

(i) To determine which of the medium constituents are antigenic, serum from rabbits immunized with BM was examined by GD against BM and against each individual component of BM that was likely to be antigenic (i.e. swine serum, peptone, beef heart infusion, yeast extract). BM and swine serum were the only materials that gave a positive reaction (Plate 1 A). The precipitin lines developed by whole BM showed reactions of identity with those of swine serum indicating that swine serum proteins were the only constituents of this medium to elicit a detectable precipitin response.

(ii) To determine which of the antigenic constituents of BM attach to the *M. gallisepticum* organism, rabbits were immunized as described earlier with washed organisms grown in BM. The resulting sera were examined by GD tests against BM and the possible antigenic components of BM. Plate 1 B shows three

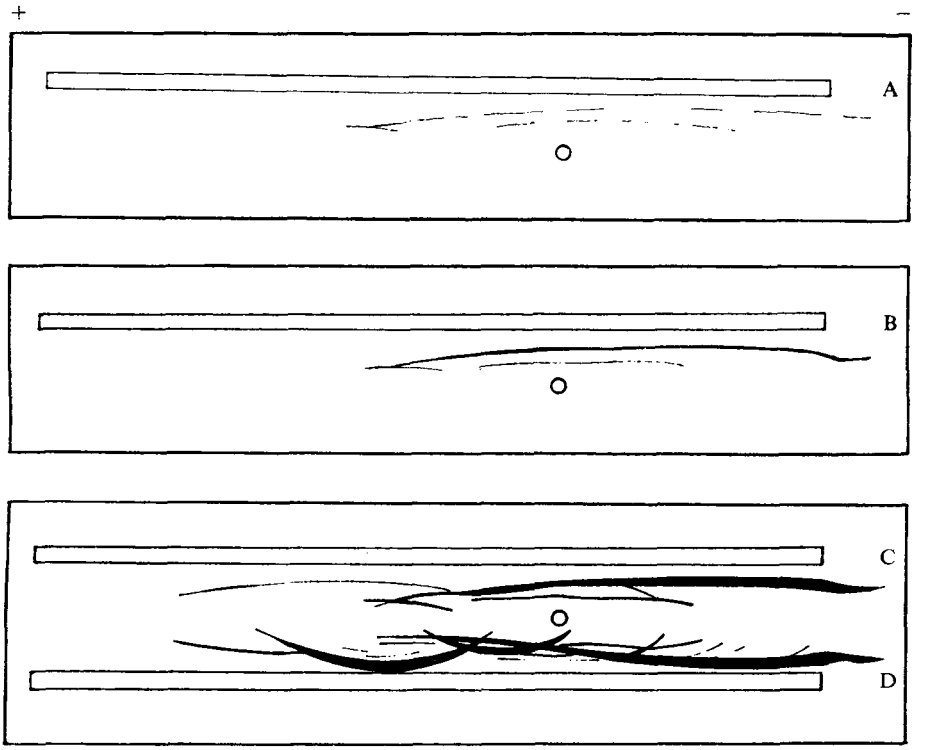

Fig. 1. Immunoelectrophoretic identification of swine serum proteins adsorbed to *M. gallisepticum*. The wells contained normal swine serum and troughs A, B and C contained rabbit antiserum to washed *M. gallisepticum* organisms. Trough D contained rabbit antiserum to whole swine serum.

precipitin bands, indicating that at least three medium constituents had resulted in antibody production; it further shows that the same three precipitin bands were developed against swine serum. Precipitating antibodies to the other medium constituents were not detected.

(iii) Immunoelectrophoresis was used to identify the swine serum proteins that attach to *M. gallisepticum*. Normal swine serum was separated by electrophoresis and two troughs were cut, one on either side of the path of separation. One trough was filled with a rabbit antiserum to washed organisms and the other with a rabbit antiserum to whole swine serum* for reference. The rabbits (six in number) had been immunized with washed organisms. Numbers 1–3 had received one immunizing course and the others, 4–6, had received two courses (Nutor, 1969).

Fig. 1A shows that sera from rabbits 1–4 had antibodies to three swine serum proteins. These were: (1) IgG, (2) a protein resembling IgM; (3) a protein of α-globulin mobility. Serum from rabbit 5 (Fig. 1B) showed antibody to the same three proteins but the precipitin arc in the α-globulin region was more readily seen. Serum from rabbit 6 (Fig. 1C) had antibody to two more proteins – one of prealbumin mobility and one of β-globulin mobility, possibly transferrin (Tormo, Chordi, Rodrigues-Burgos & Diaz, 1967).

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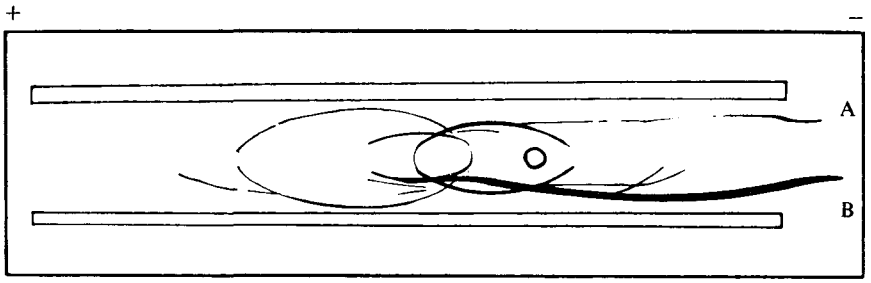

Fig. 2. Immunoelectrophoretic identification of swine serum proteins adsorbed to *M. gallisepticum*. The well contained normal swine serum and trough A contained rabbit anti-swine serum that had been incubated with *M. gallisepticum* organisms carrying adsorbed swine serum proteins. Trough B contained rabbit antiserum to whole swine serum.

Table 4. *The influence of pH of the medium and incubation time on the adsorption of chicken serum proteins to* M. gallisepticum (*Expt.* 5)

Incubation time (hr.)	pH of medium	SA reaction of antigens with antiserum to chicken serum proteins
18	6·0	−
48	5·0	±
168	5·0	+ + +

The slides were stained for lipoprotein with Oil Red O (Crowle, 1961) and the α-globulin showed a weak positive reaction.

(iv) To confirm the identity of the attached serum protein *M. gallisepticum* was cultured for 168 hr. in BM and standardized antigen was prepared. It was strongly agglutinated by antiserum to BM. A 1 ml. sample of this antigen was centrifuged at 3000*g* for 40 min., the supernatant was removed and 0·15 ml. rabbit anti-swine serum was added and left overnight at 0–4° C. so that any swine serum proteins on the antigen surface would absorb the corresponding antibody from the antiserum. After further centrifugation at 3000*g* for 40 min. the supernatant 'absorbed rabbit anti-swine serum' was used for analysis of electrophoresed normal swine serum. Unabsorbed antiserum was also used as a reference.

Fig. 2 shows that much antibody to IgG had been absorbed by the mycoplasmas. In addition antibody to two other proteins in the β–γ-globulin region had been absorbed. One of these corresponded to the IgM-like protein shown by all the sera in part (iii) above and the other corresponded to the probable transferrin shown by rabbit serum number 6 above. Antibody to pre-albumin had also been absorbed.

Experiment 5

To determine whether serum protein from a different species would adsorb to the organisms under similar conditions, *M. gallisepticum* was cultured in chicken infusion broth supplemented with chicken serum (CIB). Organisms were harvested, and standard antigens prepared after 18, 48 and 168 hr. incubation. The antigens

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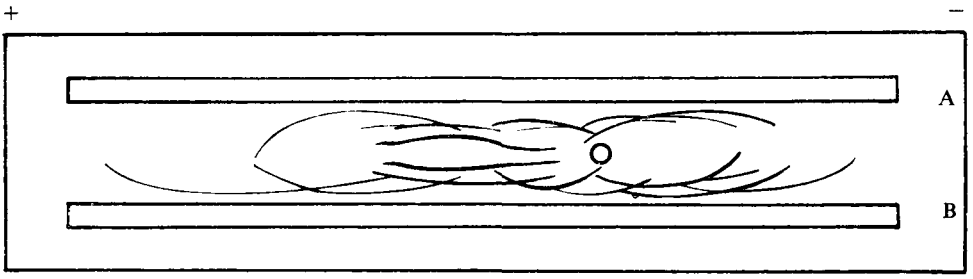

Fig. 3. Immunoelectrophoretic identification of chicken serum proteins adsorbed to *M. gallisepticum*. The well contained normal chicken serum and trough A contained rabbit anti-chicken serum that had been incubated with *M. gallisepticum* organisms carrying adsorbed chicken serum proteins. Trough B contained rabbit antiserum to whole chicken serum.

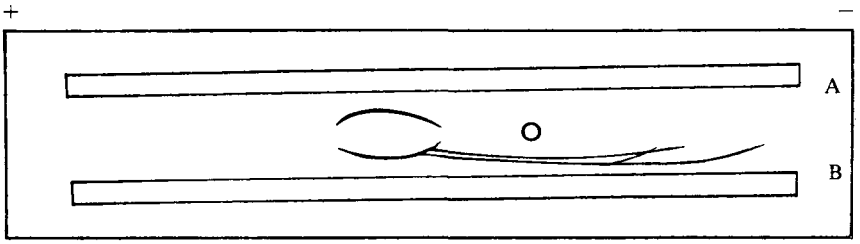

Fig. 4. Immunoelectrophoretic identification of chicken serum proteins adsorbed to *M. gallisepticum*. The well contained normal chicken serum and trough A contained rabbit antiserum to 4* chicken serum proteins and had been incubated with *M. gallisepticum* organisms carrying adsorbed chicken serum proteins. Trough B contained rabbit antiserum to 4* chicken serum proteins. * = IgG, IgM, transferrin and an α-globulin.

were then tested for the presence of adsorbed chicken serum proteins by slide agglutination with rabbit antiserum to chicken serum proteins.

Table 4 shows that chicken serum proteins were detected with increasing duration of incubation at low pH.

Experiment 6

To identify the attached chicken serum proteins an experiment similar to Expt. 4 part (iv) was carried out with antigen prepared after 168 hr. incubation in CIB. Rabbit anti-chicken serum was used instead of rabbit anti-swine serum for IEA and a rabbit serum with antibody to chicken IgG, IgM, transferrin and an α-globulin was also used.

Figs. 3 and 4 show that the proteins that were adsorbed probably correspond to some of those that were adsorbed from swine serum, i.e. IgG, IgM, transferrin and pre-albumin.

Table 5. *Reciprocal SA and HA titres of* M. gallisepticum *antigen after incubation in various media* (*Expt.* 7)

Incubation time (hr.)...	0	8	24	48	96	0	8	24	48	96
Serum	Antigen from medium A					Antigen from medium B				
SA 1	5	1	1	1	1	10	5	5	1	1
2	1	1	1	1	1	10	5	5	5	5
3	1	0	0	0	0	10	1	1	1	1
4	10	10	5	5	5	20	10	10	10	10
HA	32	16	8	4	2	16	8	4	4	4
	Antigen from medium C					Antigen from medium D				
SA 1	5	1	1	1	1	10	5	5	1	1
2	1	1	0	1	1	10	5	1	1	1
3	1	0	0	0	0	10	1	1	1	1
4	10	10	5	5	1	20	10	10	10	10
HA	64	64	32	32	32	32	32	64	64	32

Serum	Untreated antigen
SA 1	1
2	1
3	1
4	5
HA	64

Medium A = BM, pH 7·5; medium B = BM, pH 5·0; medium C = BM without swine serum, pH 7·5; medium D = BM without swine serum, pH 5·0.

The influence of adsorbed medium proteins on SA and HA reactivity of M. gallisepticum

Experiment 7

Antigen for this experiment was prepared from an *M. gallisepticum* culture after 24 hr. incubation in BM and medium proteins were not detectable by agglutination. Standardized antigen was inoculated in 1 ml. samples into 5 × 25 ml. volumes of each of the following media: (A) BM, pH 7·5; (B) BM, pH 5·0; (C) BM without swine serum, pH 7·5; (D) BM without swine serum, pH 5·0. The broths at pH 7·5 contained 0·4 M phosphate buffer so that pH change on incubation would be minimal. The other broths (B and D) were adjusted to pH 5·0 with lactic acid to simulate acid conditions produced by the mycoplasmas (Tourtellotte & Jacobs, 1960; Neimark & Pickett, 1960). Since 5·0 was the minimum pH reached by any *M. gallisepticum* culture in BM, it was not necessary to buffer these broths.

Standardized antigen was prepared immediately (0 hr.) from one sample of each type of broth. The other broths were incubated at 37° C. and antigen was prepared from one of each type after 8, 24, 48 and 96 hr. incubation.

All these antigens, together with a sample of the untreated antigen, were compared for sensitivity in SA tests with turkey sera from a naturally infected flock, and in HA tests.

The results, presented in Table 5, show that the antigen sensitivities in SA and

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Table 6. *Reciprocal SA and HA titres of* M. gallisepticum *antigen* after *incubation in various media* (*Expt.* 8)

Incubation time (hr.)...	0	8	24	48	96	0	8	24	48	96
Serum	Antigen from medium A					Antigen from medium B				
SA 5	10	10	10	10	10	10	10	10	5	1
6	5	1	1	1	1	1	1	1	1	0
7	5	5	5	5	5	5	5	5	5	1
8	5	5	5	10	5	10	10	5	10	1
HA	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2
	Antigen from medium C					Antigen from medium D				
SA 5	10	10	10	5	10	10	10	10	10	10
6	5	1	1	1	1	5	1	1	1	1
7	5	5	5	5	5	5	5	5	5	5
8	10	10	10	10	10	10	10	10	10	10
HA	2	4	2	< 2	2	2	2	2	< 2	2

Untreated antigen	
Serum	
SA 5	10
6	1
7	5
8	5
HA	4

Medium A = BM, pH 7·5; medium B = BM, pH 5·0; medium C = BM without swine serum, pH 7·5; medium D = BM without swine serum, pH 5·0.

HA tests are affected differently by the different media and by the duration of incubation.

For the slide agglutination test the sensitivity of the antigen was enhanced by lowered pH (B and D) although this declined on continued incubation. Swine serum appeared to be without effect.

For haemagglutination the sensitivity of the antigen was immediately reduced and progressively reduced on incubation in the presence of swine serum. The pH seemed to have little effect except that a slightly more rapid decline in titre was seen at pH 5·0 (medium B).

Experiment 8

This experiment was undertaken to confirm the earlier observations by Bradbury & Jordan (1971*b*) that antigens prepared from cultures after prolonged incubation at low pH reached a minimum of SA and HA sensitivity, and, therefore, unlike those from young cultures, would not be expected to be much affected by further treatment.

The experiment was therefore similar to Expt. 7 except that the antigen was from a 168 hr. culture that had been at low pH (5·0–5·1) for 120 hr. The results are shown in Table 6.

The SA sensitivity was not obviously altered by treatment with any of the four

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media except for a slight decrease after 96 hr. in medium B (with swine serum, at pH 5·0). The HA titres were all low, but those antigens treated with media containing swine serum (A and B) showed consistently lower titres than antigens from media without serum (C and D).

DISCUSSION

Our results have shown that the pH of the culture medium influences the adsorption of medium proteins to the organisms. In Expt. 1 adsorbed proteins were detected after the pH had become low (5·1–5·2) for at least 24 hr, and the role played by low pH was further demonstrated in Expt. 2, in which prolonged incubation of the organisms in buffered broth did not result in adsorption while incubation for similar periods in unbuffered broth did. The pH of the medium was the only variable in Expt. 3 since the organisms used were non-viable. This experiment clearly confirmed that medium proteins attach in acid pH. They could not be detected after incubation of the organisms in broth of neutral pH.

Jordan & Kulasegaram (1968) found that, in broth medium, the proteins of the serum supplement were the only demonstrable precipitinogens. Expt. 4 confirmed this since rabbits that had been repeatedly inoculated with BM had detectable precipitins to swine serum but not to any of the other medium constituents.

Rabbits that had been inoculated with washed *M. gallisepticum* organisms also had precipitins to swine serum proteins but to no other medium constituents. It is relevant to note that these organisms had been in culture for only 24 hr. and yet had picked up sufficient serum protein to elicit specific antibody. It seems likely therefore that most *M. gallisepticum* antigen preparations will be carrying some serum proteins from the medium although they may be in amounts too small to be detectable even by agglutination. Other studies (unpublished observations) suggest that proteins may also become attached at other-than-acid pH, but again in amounts too low for detection by agglutination. It is not known whether the protein attaches by a physical bond or by an enzyme-mediated reaction, but the attachment appears to be irreversible and the proteins are not removed by extensive washing (Jordan & Kulasegaram, 1968). It is interesting in this context to note that Hirata & Stashak (1965) were able to adsorb bovine serum albumin onto formalin-treated erythrocytes at pH 5·0 and that the adsorbed protein was not removed by three washes in buffer at pH 7·2. Ovalbumin was found to adsorb at pH 4·0 but not at pH 5·0, which indicates that different proteins may require different pH values for adsorption. There was no likelihood of enzyme involvement in these reactions.

Immunoelectrophoretic analysis of the rabbit antisera to washed *M. gallisepticum* organisms revealed that only certain swine serum proteins including IgG and IgM had elicited an antibody response. It is possible that all the serum proteins had become non-selectively attached to the organism and that the rabbits made a better immune response to some than others; however, this in unlikely in view of the absence of antibody to albumin, which is a powerful antigen and accounts for approximately one-third of the total protein of swine serum. A more likely

explanation is that certain swine serum proteins were adsorbed preferentially. In similar experiments Hamburger *et al.* (1963) identified horse IgG as the major contaminating protein on washed HeLa H cells that had been grown in medium containing horse serum, and in 1967 Beernink & Steward, studying the attachment of guinea-pig serum proteins to *Escherichia coli*, were able to demonstrate the presence of seven globulins on the organism. Of these, IgG, IgM and a β-globulin were most consistently found.

The attachment of serum proteins to the *M. gallisepticum* organisms is not confined to swine serum. In Expt. 5 chicken serum proteins were detected in increasing amounts with incubation and accompanying fall in pH. As with swine serum, the proteins were adsorbed selectively. It is of some significance that these proteins include immunoglobulins which could perhaps play a contributory role to the false positive reactions that are frequently encountered in serological tests involving *M. gallisepticum* and avian sera (Bradbury & Jordan, 1971*c*).

Asmar (1965), in a study of chicken and turkey agglutinating antibodies to *M. gallisepticum*, found that certain proteins in non-agglutinating sera were adsorbed to the organism. The proteins were detected after incubation of the sera with antigen in buffer at pH 8·0 and the bound chicken serum proteins were characterized by Asmar as gamma, beta 2 M, beta 1 C and alpha 1 globulins. The first three may well correspond to the IgG, IgM and transferrin picked up by *M. gallisepticum* in culture in our studies. Asmar's findings support the suggestion that serum proteins adsorb to the organism at other than acid pH.

Expt. 7 revealed an association between the presence of swine serum proteins and the HA properties of the *M. gallisepticum* organism, for when an antigen was prepared from a 24 hr. culture in broth with swine serum and then further incubated in fresh broth of similar constitution, the HA titre was progressively diminished. This apparent blocking effect was not seen when such an antigen was incubated in broth without swine serum. The adsorption of serum protein may thus explain why *M. gallisepticum* organisms in routine culture lose their HA titre on continued incubation. In contrast, the SA sensitivity of the antigen in this experiment was not apparently influenced by the presence or absence of swine serum but the results did confirm an earlier observation by Bradbury & Jordan (1971*b*) that brief incubation of the organisms at low pH actually enhances their SA sensitivity although it declines on further incubation. This aspect is under further investigation in view of the wide application of the SA test for the detection of small amounts of antibody in poultry flocks.

When an antigen was prepared from a 168 hr. culture and then treated as in the previous experiment, the sensitivity in SA and HA tests was not noticeably affected by either the presence of swine serum or the pH. This was probably because the sensitivity had already declined following the long (168 hr.) culture period (Bradbury & Jordan, 1971*b*).

Since these studies have shown that the HA reactivity of *M. gallisepticum* is reduced by prolonged incubation in serum-supplemented medium, it would be of interest to know whether other properties of the organism are similarly affected.

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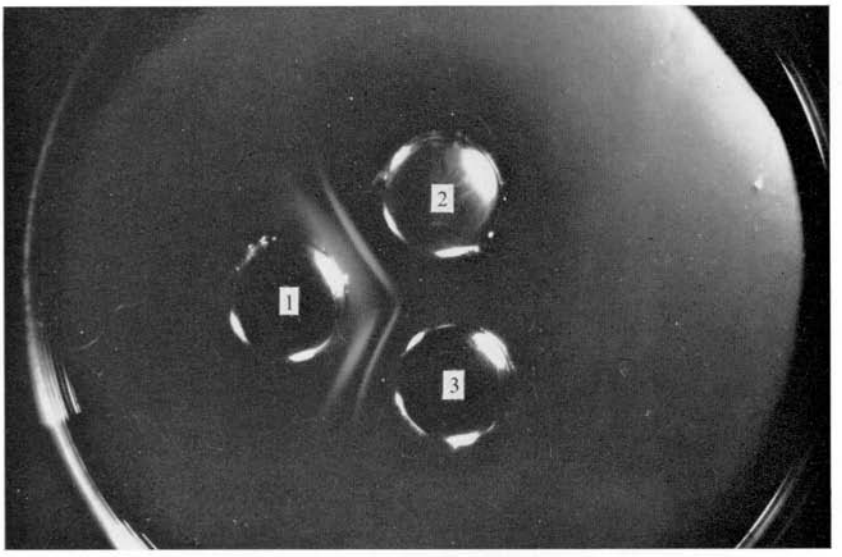
EXPLANATION OF PLATE

A. Precipitin reactions of rabbit antiserum to broth medium (1) with broth medium (2) and swine serum (3).

B. Precipitin reactions of rabbit antiserum to washed *M. gallisepticum* organisms (1) with broth medium (2) and swine serum (3).



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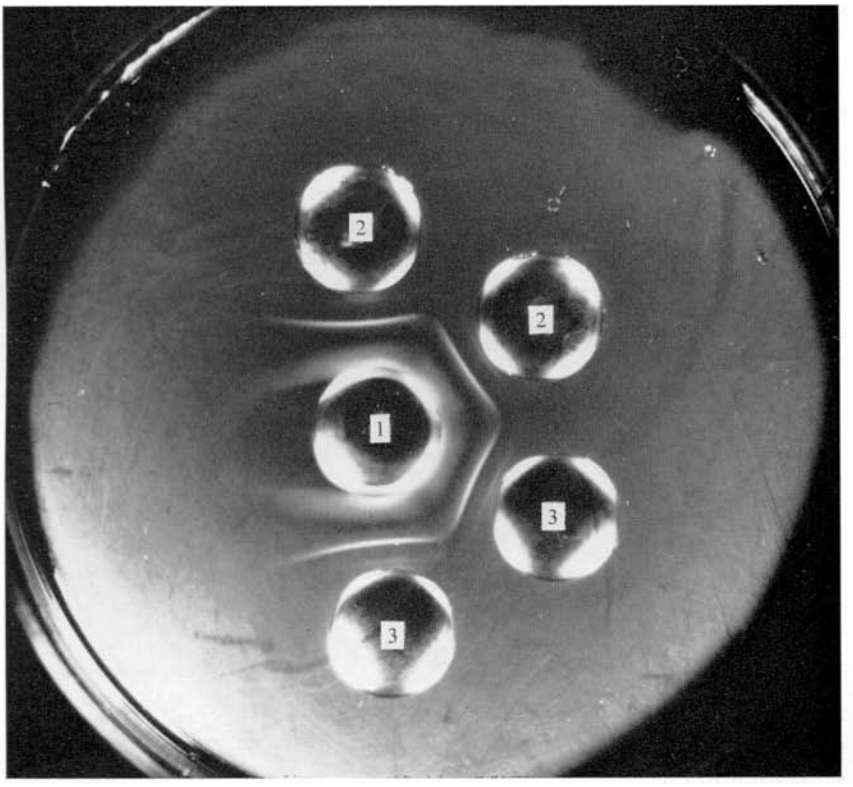

B

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