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The influence of pH of the culture medium on the sensitivity of *Mycoplasma gallisepticum* antigens for use in certain serological tests

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SUMMARY

Mycoplasma gallisepticum antigens were prepared from organisms cultured in broth medium with glucose. The influence of period of growth, pH of the medium and duration of incubation at low pH (5·0) on the sensitivity of these antigens was determined in certain tests. The most sensitive antigen for the serum plate test was harvested after no more than 8 hr. incubation at pH 5·0. Sensitivity in serum plate, haemagglutination and gel diffusion tests was impaired if organisms were incubated at pH 5·0 for long periods. Antigens prepared from buffered broth medium were found to be at least as sensitive as those from unbuffered medium for the haemagglutination and gel precipitation tests, but considerably less so for the serum plate test.

INTRODUCTION

Serological tests currently used for the diagnosis of *Mycoplasma gallisepticum* infection in poultry include the haemagglutination inhibition (HI), tube agglutination (TA), whole blood agglutination (WB) and serum plate agglutination (SP) tests.

None of these can be regarded as entirely satisfactory. The HI test yields a result within a few hours but normal chicken and turkey sera sometimes give false positive reactions (White, Wallace & Alberts, 1954; Roberts, Olesiuk & Van Roekel, 1967; Kulasegaram, 1967). Measures taken to avoid these non-specific HI titres may reduce the sensitivity of the test (Jordan & Kulasegaram, 1968*a*; Thornton, 1969). The TA test may be difficult to read (Jacobs, Jungherr, Luginbuhl & Gianforte, 1953), false positive reactions may occur (Roberts *et al.* 1967; Kulasegaram, 1967) and the results in one well-recognized method cannot be read for 24–48 hr (Mayeda & Lewis, 1969). The WB and SP tests have found wide application for the detection of *M. gallisepticum* antibodies because they are simple to perform and yield rapid results. However, such tests should be interpreted with caution since false positive reactions can occur due to a variety of factors (Thornton, 1969; Roberts, 1970).

Conflicting reports on the sensitivity of these tests (Jungherr, Luginbuhl, Tourtellotte & Burr, 1955; Leach & Blaxland, 1966; Jordan & Kulasegaram, 1968*a*; Thornton, 1969) suggest that they must be influenced both by the method of performing the tests and by the sensitivity of the antigen used. Considerable variations in sensitivity have been noted between *M. gallisepticum* agglutinating

antigens prepared in different laboratories (Barber, 1962; Halen & Schyns, 1969) and even between batches prepared in the same laboratory (Coller, Strout & Dunlop, 1955; Dunlop & Strout, 1956). Factors reported to influence the sensitivity of such antigens include the strain of the organism (Adler & DaMassa, 1965), the concentration of organisms (Halen & Schyns, 1969) and the diluents and preservatives used (Hromatka & Adler, 1969).

Other factors that may be of considerable importance are the type of medium and conditions of culture. Although various media have been used for the production of *M. gallisepticum* antigens, little has been reported on the effects of different media constituents or different conditions of growth on the subsequent sensitivity of the antigen. Adler & DaMassa (1968) found that *M. gallisepticum* antigens grown in broth with glucose, where the pH became acid, were less sensitive in the SP test than antigens grown in broth without glucose where the pH did not fall below 7·1. They concluded that the low pH had damaged the antigenic properties of the organism. In their experiments cultures were incubated for 7 or 8 days but the influence of the duration of incubation at low pH was not investigated.

Our own preliminary work had indicated that duration of incubation might influence the sensitivity of the resulting antigen, and we here report the results of further investigation into the effects of pH of the culture medium with particular reference to duration of incubation. We have also examined the sensitivity of antigen grown in buffered medium, as suggested by Adler & DaMassa in 1968.

MATERIALS AND METHODS

Mycoplasma gallisepticum *strains*

The following two strains were used.

A 514 obtained from Dr H. Chu, University of Cambridge. This strain had undergone numerous passages in artificial medium in this laboratory.

S 6 obtained from Dr D. H. Roberts, Central Veterinary Laboratory, Weybridge.

Culture media

Broth medium (BM). The broth used for routine maintenance of cultures and for growth of the antigens was that described by Bradbury & Jordan (1971) except that it contained 15% heat-inactivated swine serum instead of horse serum. The medium contained 0·1% glucose, and the initial pH was 7·5.

Buffered broth medium (BBM). BM was supplemented with 0·2 M phosphate buffer, a molarity necessary to maintain the pH of the culture above 6·7 for 3 days and allow good growth of the organism.

Agar medium (AM). This was similar to BM except that it contained 1% ion agar No. 2,* and phenol red was omitted.

Antigen preparation

BM, warmed to 37° C., was inoculated with one-tenth of its volume of a 24 hr. culture of *M. gallisepticum* in BM. The culture was incubated at 37° C. and in some experiments the number of viable organisms was determined just before

* Oxoid Ltd., London.

harvest. Organisms were harvested by centrifugation at 3000g and the pH of the culture medium was measured. The organisms were washed three times in 0·02 M phosphate-buffered saline pH 7·0 (PBS), and in the earlier experiments the total yield of antigen was determined as described below. Antigens were tested for sterility and standardized to a concentration of 75 times an optical density of 40 on an E.E.L. nephelometer. This optical density was selected as being approximately equivalent to 15 times the turbidity of Brown's opacity scale number 4 (Jordan & Kulasegaram, 1968*a*).

Viable counts

These were performed by the method of Miles & Misra (1938). Serial tenfold dilutions of the culture were prepared in BM and five 0·02 ml. drops were immediately transferred from each dilution to AM, using one plate per dilution. Plates were incubated at 37° C. for at least 1 week before colonies were counted.

Total yield of antigen

This was measured in terms of turbidity on an E.E.L. nephelometer. Harvested antigens, after washing, were made up in PBS to 2·5 times their original concentration in BM. This was to bring the turbidity within the range of the nephelometer.

Chicken and turkey sera

These were from field outbreaks of *M. gallisepticum* infection. They were stored at 0–4° C. Serial dilutions of the sera for the SP test were prepared in PBS pH 7·0.

Rabbit sera

Serum was obtained from rabbits immunized with the A 514 strain of *M. gallisepticum.* The immunizing antigen was prepared from an 18 hr. broth culture, harvested and standardized as outlined above. The inoculation procedure was as described by Jordan & Kulasegaram (1968*b*) except that a multiple emulsion adjuvant (Herbert, 1967) was used instead of Freund's complete adjuvant.

SP tests

Tests were carried out at room temperature on clean grease-free microscope slides. A Marburg* micropipette was used to deliver 0·02 ml. of serum, or serum dilution and 0·02 ml. of the test antigen was similarly added. The slide was gently rocked for a standard reaction time of 2 min. for chicken sera and 3 min. for turkey sera (Jordan & Kulasegaram, 1968*a*). A known negative homologous serum was included in each series of tests. In each experiment antigens were compared by testing against four or five chicken sera.

HA tests

Serial twofold dilutions of the standardized antigen under test were made in PBS in Microtitre† disposable 'V' plates using microdiluters.† An equal volume

* Eppendorf, V. A. Howe, London.
† Flow Laboratories, Irvine, Scotland.

(0·05 ml.) of a 0·75 % suspension of turkey red blood cells was added to each cup. A cell control containing 0·05 ml. PBS and 0·05 ml. red blood cell suspension was included in each test. The plate was shaken, allowed to stand at room temperature and results were read when the control cells had settled. The HA titre of the antigen was taken as the highest dilution of antigen giving complete haemagglutination.

Precipitin tests

Double diffusion in 1 % agar gel was used to examine the precipitation reaction between the antigens and the serum from immunized rabbits, having first carried out tests to ensure that the reaction was specific, and not due to antigenic components of the medium (Jordan & Kulasegaram, 1968*b*). The gel was prepared from Noble agar (Difco) and contained 0·15 M sodium chloride. Results were photographed after incubation at 37° C. in a moist atmosphere for 48 hr.

RESULTS

Experiment 1

To study the effects of duration of incubation and consequent pH change of the medium on the sensitivity of *M. gallisepticum*, the A 514 strain was grown in BM and harvested after 1, 3, 5 and 7 days incubation. At the time of harvest a viable count was performed, the pH of the medium was measured and the total yield of

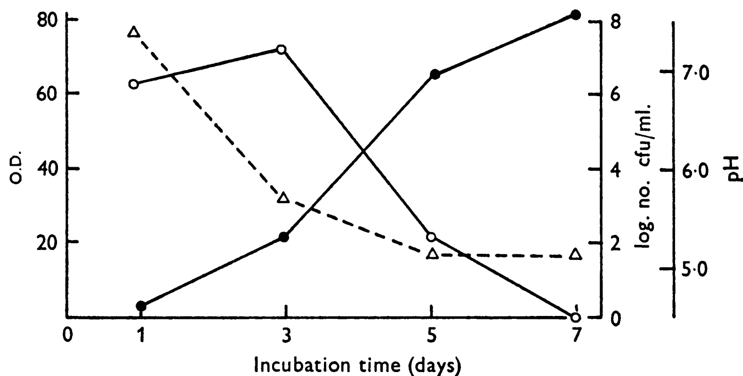

Fig. 1. The effect of incubation time on the viability, pH and antigen yield of an *M. gallisepticum* A514 culture (Expt. 1). O.D. = optical density; cfu = colony forming units. ●, O.D.; ○, cfu; △, pH.

antigen was determined (Fig. 1). The turbidity of the culture increased with incubation time; the number of viable organisms declined rapidly between the third and fifth day by which time the pH had fallen to 5·1. The pH was unchanged at day 7.

The antigen yield was insufficient for the SP and HA tests after only 1 day's incubation but the results for the other antigens (Table 1) showed a decline in both agglutinating sensitivity and HA activity with increasing incubation time.

Table 1. *Reciprocal SP and HA titres of* M. gallisepticum *A* 514 *antigens prepared by harvesting after different periods of incubation* (*Expt.* 1)

		Serum	Incubation time (days) 3	5	7
SP	Chicken sera	1	80	20	10
		2	40	10	5
		3	10	5	5
		4	20	10	10
		5	20	20	10
	Turkey sera	6	40	20	20
		7	160	160	80
		8	20	20	20
		9	10	10	5
		10	10	10	10
HA			32	8	4

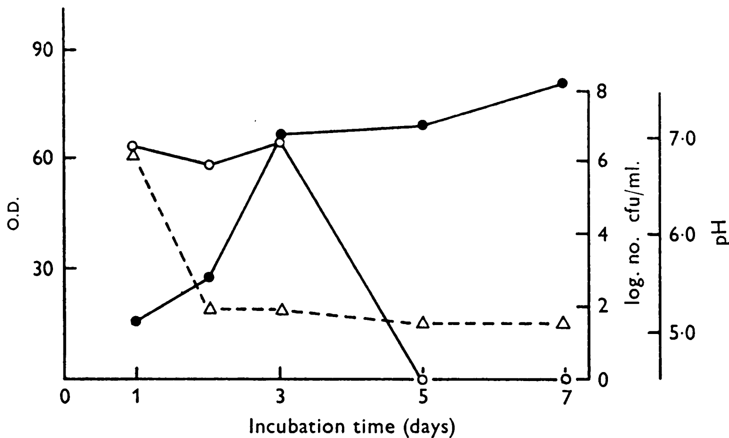

Fig. 2. The effect of incubation time on the viability, pH and antigen yield of an *M. gallisepticum* A514 culture (Expt. 2). O.D. = optical density; cfu = colony forming units. ●, O.D.; ○, cfu; △, pH.

Experiment 2

This experiment was similar to Expt. 1 but antigen was also harvested after 2 days incubation. The pH fell more rapidly than in Expt. 1 and reached 5·2 by the second day (Fig. 2). As in the previous experiment, the turbidity of the culture increased steadily with incubation time even after day 5 when no viable organisms could be detected. In the SP and HA tests maximum sensitivity was shown by the antigen harvested after 2 days incubation (Table 2) and a decline in sensitivity with increased incubation time was again noted. Plate 1A shows the result of the gel diffusion test. The antigens harvested after 1 and 2 days showed a strong reaction but this decreased both in intensity and number of precipitin lines with increasing duration of incubation.

Table 2. *Reciprocal SP and HA titres of* M. gallisepticum *A* 514 *antigens prepared by harvesting after different periods of incubation* (*Expt.* 2)

		Serum	Incubation time (days) 1	2	3	5	7
SP	Chicken sera	11	5	20	10	10	5
		12	5	5	5	5	1
		13	20	80	40	20	10
		14	N	1	1	1	1
		15	1	10	5	5	1
	Turkey sera	16	160	640	160	80	80
		17	40	160	80	20	20
		18	20	40	40	20	20
		19	40	40	40	20	20
		20	40	160	40	40	40
HA			64	512	16	8	4

N = negative.

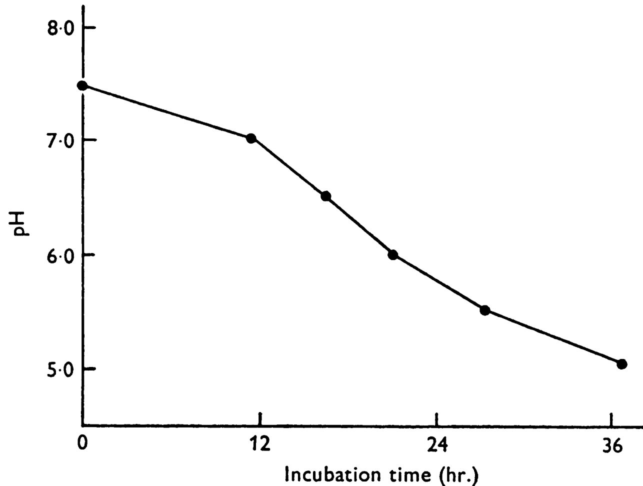

Fig. 3. The effect of incubation time on the pH of an *M. gallisepticum* A 514 culture (Expt. 3).

Experiment 3

Although Expts. 1 and 2 indicated that prolonged incubation at low pH diminished the sensitivity of the antigen in all three tests, it was not possible to determine if there was a critical pH below which sensitivity was impaired. Therefore, in this experiment organisms were harvested as soon as the medium had fallen to pH values 7·0, 6·5, 6·0, 5·5, and 5·0. A recording meter* was used to monitor pH. The incubation times at which these pH values were reached are shown in Fig. 3.

The SP and HA titres of the antigens showed no definite pattern (Table 3) but the total incubation time of this experiment was relatively short.

* Analytical Measurements Ltd., Richmond.

There was little difference in the precipitin reactions of the antigens (Plate 1B). All showed a wide, intense precipitation band approximately midway between antigen and serum but with antigen harvested at pH 5·5 or 5·0, only one other line was visible near the outer wells while several were visible with the other antigens.

Table 3. *Reciprocal SP and HA titres of* M. gallisepticum *A* 514 *antigens prepared by harvesting at different pH values* (*Expt.* 3)

		Serum	pH 7·0	pH 6·5	pH 6·0	pH 5·5	pH 5·0
SP	Chicken sera	21	10	10	10	10	10
		22	20	10	10	20	20
		23	1	N	N	N	1
		24	1	N	N	N	1
		25	5	N	N	N	1
	Turkey sera	26	10	10	10	20	20
		27	10	5	5	5	5
		28	20	20	20	20	40
		29	1	1	1	1	1
		30	1	1	1	1	1
HA			128	256	128	128	128

N = negative.

Table 4. *Reciprocal SP and HA titres of* M. gallisepticum *A* 514 *antigens prepared by harvesting after varying incubation times at pH* 5·0 (*Expt.* 4)

		Total incubation time (hr.)	46	54	62	70	78	86
		Serum / Incubation time at pH 5·0 (hr.)	8	16	24	32	40	48
SP	Chicken sera	31	5	NT	5	5	5	5
		32	40	NT	20	10	10	10
		21	5	NT	5	5	5	5
		22	40	NT	40	10	10	10
	Turkey sera	26	160	NT	80	80	80	80
		28	160	NT	40	40	40	40
		33	320	NT	80	80	80	80
		34	80	NT	40	40	40	40
HA			64	64	8	8	4	4

NT = not tested because antigen became contaminated.

Experiment 4

To investigate the effects of continued incubation at low pH, a culture was grown until the pH reached 5·0, a period of 38 hr. being required. Antigens were harvested after incubation for 8, 16, 24, 32, 40 and 48 hr. at pH 5·0.

A decline in SP sensitivity of the antigens was observed between 8 and 32 hr.

(Table 4). The HA titre of the antigens fell rapidly between 16 and 24 hr. The decline in SP titre appeared to be more gradual, although the antigen harvested after 16 hr. incubation at pH 5·0 was not included in these tests owing to bacterial contamination. (The HA and gel diffusion tests were conducted before the antigen became contaminated.) Examination of the antigens by agar gel diffusion also showed fewer precipitin lines with prolonged incubation of the culture (Plate 2C).

Experiment 5

To extend the previous experiment, cultures were grown to pH 5·0 (38 hr.) and organisms harvested after 48, 56, 64 and 72 hr. incubation at that pH. There was little difference in the SP or the HA titres of the four antigens (Table 5). Two of the chicken sera showed a slight fall in titre with the 64 and 72 hr. antigens but all other SP titres were constant.

Table 5. *Reciprocal SP and HA titres of* M. gallisepticum *A* 514 *antigens prepared by harvesting after varying incubation times at pH* 5·0 (*Expt.* 5)

			Total incubation time (hr.)			
			86	94	102	110
			Incubation time at pH 5·0 (hr.)			
		Serum	48	56	64	72
SP	Chicken sera	31	5	5	1	1
		32	10	10	5	5
		21	5	5	5	5
		22	10	10	10	10
	Turkey sera	26	20	20	20	20
		28	20	20	20	20
		33	40	40	40	40
		34	20	20	20	20
HA			16	8	8	8

On examination by gel diffusion (Plate 2E) all the antigens showed a diffuse precipitin line together with a less distinct line nearer the antigen wells. Both lines were more clearly defined with 48 hr. antigen than with the other three.

Experiment 6

In order to determine whether prolonged incubation of culture at pH 5·0 would affect the sensitivity of the antigen if a different strain of organism were used, Experiment 4 was repeated with the S 6 strain. The pH of the culture had fallen to 5·0 by 30 hr. and antigens were harvested after 8, 16, 24, 32, 40 and 48 hr. incubation at that pH. Since the pH of the medium fell more rapidly with this strain of *M. gallisepticum* than A 514, the total incubation times were shorter.

The SP and HA titres of the S 6 antigens fell with increasing incubation time at pH 5·0, the greatest loss in sensitivity occurring between 24 and 32 hr. (Table 6).

On gel precipitation these antigens gave at least four lines although those with the 48 hr. antigens were less intense than the others (Plate 2D).

Table 6. *Reciprocal SP and HA titres of* M. gallisepticum *S6 antigens prepared by harvesting after varying incubation times at pH* 5·0 (*Expt.* 6)

			Total incubation time (hr.)					
			38	46	54	62	70	78
			Incubation time at pH 5·0 (hr.)					
		Serum	8	16	24	32	40	48
SP	Chicken sera	31	40	40	40	1	1	1
		32	40	40	40	10	10	10
		21	10	10	10	5	5	5
		22	40	40	40	20	20	20
	Turkey sera	35	40	40	40	10	10	10
		36	80	80	80	80	40	40
		37	80	80	80	40	40	40
		38	10	5	5	5	5	5
HA			16	16	16	8	8	8

Experiment 7

Adler & DaMassa (1968) associated loss of *M. gallisepticum* S6 antigen sensitivity with low pH of the medium because they found that a more sensitive SP antigen was obtained from medium without glucose, in which a fall in pH did not occur.

In an attempt to confirm these findings using A514, the organism was passaged ten times in broth without glucose, but even after the tenth passage the pH of the medium fell to 5·0–5·1. Similar results were obtained in broth containing horse serum in place of swine serum. It was concluded therefore that the sera contained fermentable carbohydrates and that the work of Adler & DaMassa (1968) could not be repeated.

Experiment 8

This experiment was designed to compare the sensitivity of antigens prepared from buffered broth medium (BBM) with those prepared from broth medium (BM). *M. gallisepticum* A514 was cultured in BBM and in BM. The experiment was conducted four times in all, twice with an incubation time of 48 hr. (i and ii) and twice with an incubation time of 72 hr. (iii and iv). The pH values of the media at the time of harvest are shown in Table 7.

Antigens prepared from BBM were more easily suspended in PBS and gave a finer suspension than those from BM. The SP and HA titres of 48 hr. antigens from (i) and (ii) are shown in Table 8 and those of 72 hr. antigens from (iii) and (iv) in Table 9. All the antigens from BBM were less sensitive in the SP test than those from unbuffered broth. There was no significant difference in HA titres of the antigens in (i) and (ii) but in (iii) and (iv) antigens prepared from BBM showed considerably higher titres than those from BM.

The antigens from (iv) were examined by gel diffusion (Plate 1A) and that from the BBM (3*b*) showed 2 intense precipitin bands and at least one other band while the antigen from BM (3) showed only two faint bands.

Table 7. *pH of the media at harvest* (*Expt.* 8)

Expt. ...	(i)		(ii)		(iii)		(iv)	
Incubation time (hr.) ...	48		48		72		72	
Medium	BBM	BM	BBM	BM	BBM	BM	BBM	BM
pH	6·8	5·1	6·9	5·0	6·7	5·7	6·7	5·1

BBM = buffered broth medium. BM = broth medium.

Table 8. *Reciprocal SP and HA titres of* M. gallisepticum *A* 514 *antigens prepared from cultures grown in buffered broth medium* (*BBM*) *and broth medium* (*BM*) *for* 48 *hr*. (*Expt.* 8)

			i			ii	
		Serum	BBM	BM	Serum	BBM	BM
SP	Chicken sera	39	5	20	49	5	10
		40	1	5	50	N	1
		41	20	160	51	N	1
		42	N	5	52	N	1
		43	1	10	53	N	1
	Turkey sera	44	40	160	54	40	160
		45	20	80	55	20	80
		46	10	10	56	10	40
		47	20	20	57	20	40
		48	20	40	58	20	40
HA			32	32		32	16

N = negative.

Table 9. *Reciprocal SP and HA titres of* M. gallisepticum *A* 514 *antigens prepared from cultures grown in buffered broth medium* (*BBM*) *and broth medium* (*BM*) *for* 72 *hr*. (*Expt.* 8)

			iii			iv	
		Serum	BBM	BM	Serum	BBM	BM
SP	Chicken sera	1	5	80	11	5	10
		2	1	40	12	1	5
		3	1	10	13	10	40
		4	1	20	14	N	1
		5	1	20	15	N	5
	Turkey sera	6	10	40	16	40	160
		7	160	160	17	20	80
		8	10	20	18	10	40
		9	5	10	19	10	40
		10	5	10	20	20	40
HA			128	32		512	16

N = negative.

DISCUSSION

The results of Expts. 1 and 2, which clearly show that loss of *M. gallisepticum* antigen sensitivity is associated with low pH of the culture medium, confirm the findings of Adler & DaMassa (1968).

In Expt. 1 the sensitivity of the antigens in SP and HA tests declined with increasing incubation time. However, in addition to a fall in the pH of the medium there was a decrease in the number of viable organisms and it was thought that both factors might have contributed to the decrease in antigen sensitivity.

In a similar experiment (2) a decline in antigen sensitivity with increasing incubation time was again noted in SP, HA and in gel precipitation tests. This decline was particularly evident between 2 and 3 days of incubation, when the number of viable organisms was fairly stable suggesting that death of the organism was not responsible for lowered sensitivity. A more likely cause was the acid pH of the medium. This had fallen rapidly and to a minimum by day 2 and, therefore, by day 3 the organisms had been at low pH for at least 24 hr. The inhibitory effects of low pH have also been noted by Pollock, Somerson & Senterfit (1969) in connexion with *M. pneumoniae* antigen sensitivity. They found that antigens harvested from medium which had become acid lost their ability to induce antibody production when inoculated into animals, and to act as antigens in immunodiffusion tests.

The next experiments investigated the effects of pH in more detail. In Expt. 3 organisms harvested as soon as the pH of the medium had fallen to specific values between 7·0 and 5·0 showed no obvious loss in sensitivity, suggesting that reduced sensitivity was probably associated with continued incubation of cultures after the pH had fallen to 5. Expts. 4 and 5 studied the effects of continued incubation on the antigenic properties of the organism. Cultures harvested at 8-hourly intervals after the pH of the medium had reached 5·0–5·1 exhibited a loss in sensitivity with increasing time. Clearly, therefore, *M. gallisepticum* organisms intended for use as antigens should not remain in medium of low pH for more than a few hours.

It seems likely that there may be an optimal time at which to harvest *M. gallisepticum* for SP antigens. Evidence for this view is provided by Expts. 2, 3 and 4. In Expt. 2 the antigen prepared after 1 day's incubation was not as sensitive in SP tests as that from 2 days incubation, although the pH was low by day 2. In Expts. 3 and 4 the titres of the two turkey sera (Nos. 26 and 28) that were common to both experiments were four- to eightfold higher with the antigen harvested after 8 hr. incubation at pH 5·0 than that from organisms harvested as soon as the pH had reached 5·0.

Thus it appears that, while incubation of A 514 antigens at low pH for more than 8 hr. will damage antigenic properties, a shorter period of incubation at low pH may actually enhance them. There is no ready explanation for this. It is possible that the first effect of low pH on the antigen suspension is to reduce its stability so that it is more readily agglutinated by addition of antibody. Another influencing factor might be the morphology of the organisms. This is known to vary with pH

(Freundt, 1969; Anderson, 1969) and might also influence agglutination properties. An electron microscope study of morphology in relation to antigenic sensitivity might prove of value in this context.

Loss in sensitivity with increasing incubation time at pH 5·0 is clearly not confined to the A 514 strain of *M. gallisepticum* since the S 6 strain of the organism exhibited the same property.

The experiment (7) designed to repeat the conditions for antigen production with little fall in pH, by the omission of glucose (Adler & DaMassa, 1968) was unsatisfactory, since omission of glucose from the medium failed to prevent such a fall. Therefore, organisms were cultured in BBM so that the pH of the medium did not fall below 6·7 on incubation for up to 72 hr. Antigens prepared from this medium proved to be considerably less sensitive in SP tests than those prepared from unbuffered medium although HA titres were the same or higher and the number and intensity of gel precipitation lines were greater. An explanation for the SP and HA results may be that antigens prepared from BBM, being in a fine suspension, might have required a larger number of antibody molecules to produce a visible SP agglutination than antigens from BM which were already partially clumped. On the other hand, a fine suspension of antigen would favour HA reactions since there would probably be more HA sites available. The improved gel precipitation reaction is not easily explained but it is interesting that Pollack *et al.* (1969) could prevent the loss of *M. pneumoniae* antigen reactivity in gel tests caused by acidity of the medium by growing the organism in buffered broth.

The adverse effect of low pH with increasing duration of incubation remains unexplained but it is almost certainly associated with some irreversible alteration of the cell membrane. Pollack *et al.* (1969) concluded that, while low pH was an important factor in influencing *M. pneumoniae* sensitivity, the effect was probably not solely a hydrogen ion effect because incubation of organisms in buffers of low pH did not affect their reactivity in gel diffusion tests. Loss in reactivity may, as these authors suggest, be the result of enzymic action, but results in this laboratory (Bradbury & Jordan, in preparation) suggest that proteins from the culture medium may also play some role in altering antigen sensitivity. These proteins become firmly attached to the surface of the organism with increasing incubation time in acid medium, and could, in theory, affect antigenicity by masking antigenic sites. Results from Expts. 1 and 2 suggest that the antigens become increasingly contaminated with non-specific material since turbidity increased steadily even when there were no longer any detectable viable organisms. A similar observation was made by Eng (1969) in the production of *M. pneumoniae* antigen.

On the basis of our experimental findings certain suggestions can be made for the production of sensitive *Mycoplasma gallisepticum* antigens.

1. For serum plate tests, organisms should be grown in unbuffered medium and harvested after no more than 8 hr. at low pH.

2. For HA and gel precipitation tests, antigens may be prepared as above, or, more conveniently, organisms may be grown in buffered medium where the time of harvest is not critical.

3. Although our observations have not included other serological tests it is

possible that antigen sensitivity may be similarly affected and therefore merits further investigation.

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EXPLANATION OF PLATES

PLATE 1

A. Gel precipitation reactions of *M. gallisepticum* A514 antigens prepared by harvesting after different periods of incubation (Expt. 2). The central well contained rabbit antiserum to *M. gallisepticum* and the outer wells contained the antigens. 1, 2, 3, 5, 7 = number of days incubation. 3*b* = antigen prepared from buffered broth (Expt. 8).

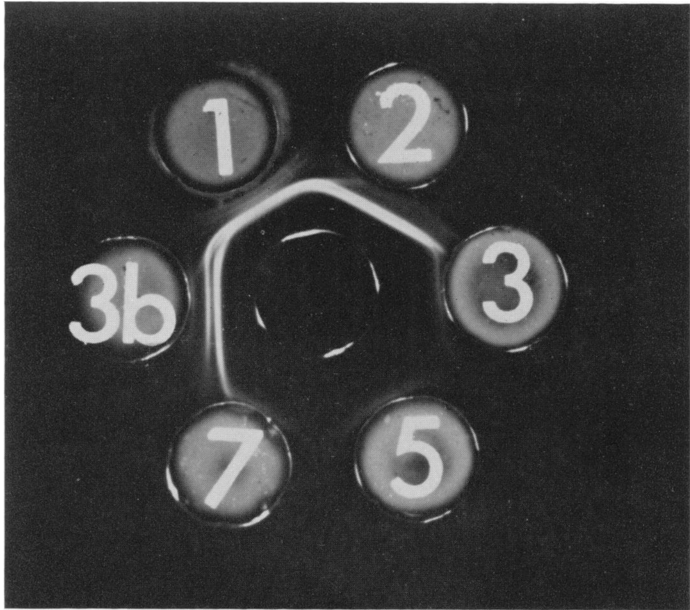
B. Gel precipitation reactions of *M. gallisepticum* A514 antigens prepared by harvesting at different pH values (Expt. 3). The central well contained rabbit antiserum to *M. gallisepticum* and the outer wells contained the antigens. 7·0, 6·5, 6·0, 5·5, 5·0 = pH at which organisms were harvested.

PLATE 2

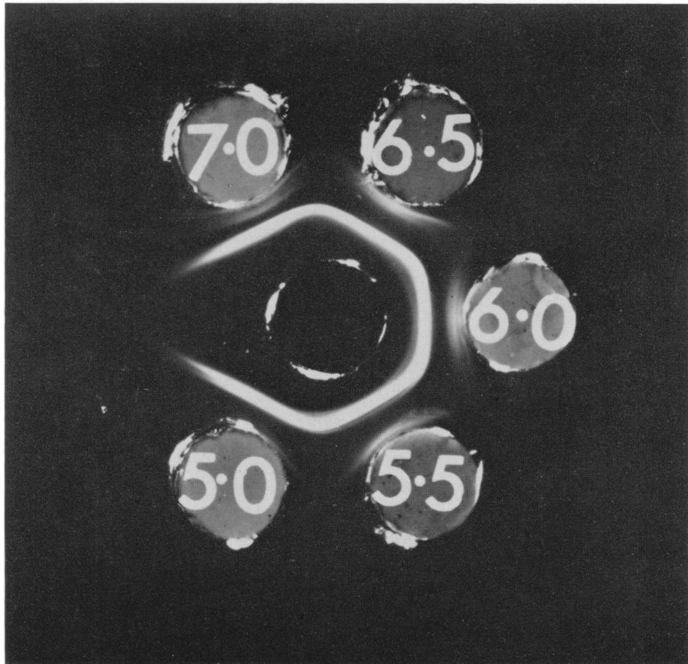
C. Gel precipitation reactions of *M. gallisepticum* A514 antigens prepared by harvesting after varying incubation times at pH 5·0 (Expt. 4). The central well contained rabbit antiserum to *M. gallisepticum* and the outer wells contained the antigens. 8, 16, 24, 32, 40, 48 = number of hours incubation at pH 5·0.

D. Gel precipitation reactions of *M. gallisepticum* S6 antigens prepared by harvesting after varying incubation times at pH 5·0 (Expt. 6). The central well contained rabbit antiserum to *M. gallisepticum* and the outer wells contained the antigens. 8, 16, 24, 32, 40, 48 = number of hours incubation at pH 5·0.

E. Gel precipitation reactions of *M. gallisepticum* A514 antigens prepared by harvesting after varying incubation times at pH 5·0 (Expt. 5). The central well contained rabbit antiserum to *M. gallisepticum* and the outer wells contained the antigens. 48, 56, 64, 72 = number of hours incubation at pH 5·0.



A



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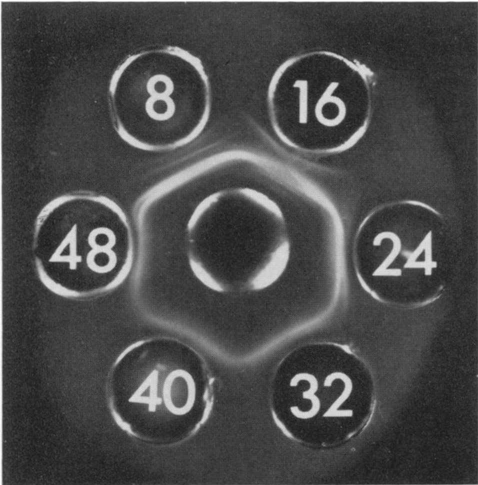

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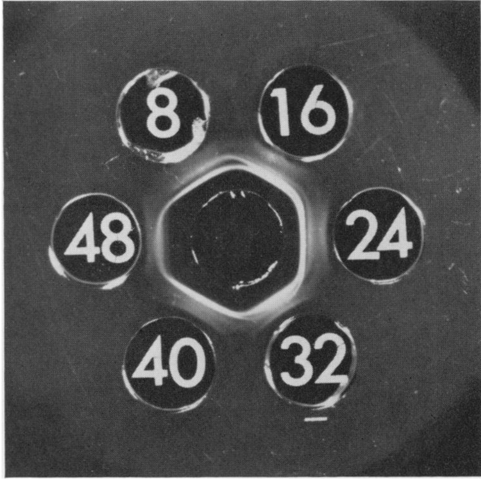

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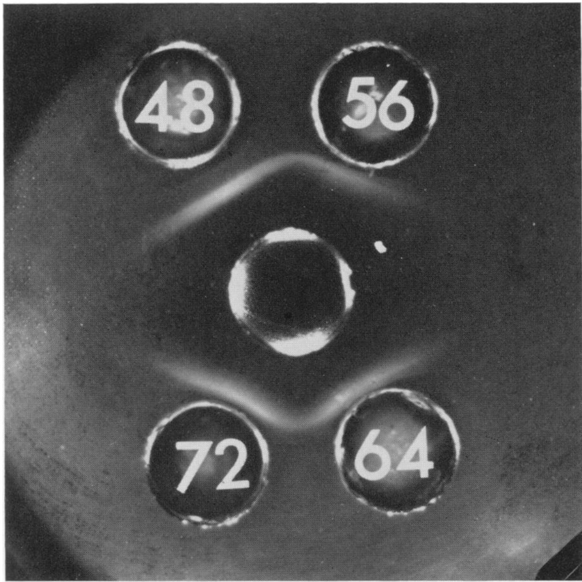

E

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