Immunology, 1959, **2**, 112.

Serological Reactions in *Eimeria stiedae* Infection of the Rabbit

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Summary. Circulating antibodies to *Eimeria stiedae* have been demonstrated in rabbit serum using precipitation in gel and liquid media and complement fixation tests.

Sera prepared by subcutaneous injections of antigen were similar to those obtained after oral infection with oocysts of *E. stiedae*.

Antigens prepared from crushed oocysts and the exudate of infected bile ducts had similar properties, although a constituent of the bile duct antigen was absent or only present in very small amounts in the oocyst antigen.

Antigens obtained on different days after infection from bile ducts showed differing precipitation patterns in gel; the maximum number of bands was given by antigens obtained 17–19 days after infection, antigens taken before and after this period reacted to give fewer precipitation bands.

INTRODUCTION

RESISTANCE to reinfection with their parasitic Coccidia has been demonstrated for many species of animals. A very solid resistance in dogs and cats has been reported by Andrews (1926); in chickens by Beach and Corl (1925), Johnson (1927), Tyzzer (1929) and Horton-Smith (1947); in rabbits by Smith (1910), Chapman (1929) and Bachman (1930); in guinea pigs by Henry (1931); in rats by Becker, Hall and Hager (1932); in pigs by Biester and Schwarte (1932); and in calves by Senger, Hammond and Thorne (1957).

As this resistance resulted from the entrance into the host animal and, in some cases, the migration through its tissues of a foreign organism, it seemed likely that antibodies would be produced by the host animal and be demonstrable in its serum. However, attempts to demonstrate antibodies have not been conclusive. Paterson (1932), Chapman (1929), Bachman (1930) and Henry (1931) investigated the immunological responses of animals to infection with Coccidia, using tests which included complement fixation, precipitation, agglutination and skin sensitivity, all with negative, inconclusive or unreliable results. Attempts by Tyzzer (1929), Becker and Hall (1933) and Becker (1935) to protect animals from infection by the passive transfer of immunity failed. McDermott and Stauber (1954) reported the agglutination of merozoites of *E. tenella* with sera from infected chickens. Itagaki and Tsubokura (1955) showed agglutination of merozoites of a chicken coccidium with antisera prepared in rabbits.

The present work on the infection and reinfection of rabbits with *E. stiedae* has shown that, after a single infection, rabbits become resistant, in varying degrees, to further infection.

In order to demonstrate the presence of antibody in the sera of rabbits infected with *E. stiedae*, the technique of precipitation in agar medium (Ouchterlony, 1949) was used, as this method, in contrast to other serological techniques, does not require a purified antigen. This method also gives an opportunity for comparing the antigens obtained at different stages of infection and for determining the identity of antigens obtained by

different methods and of the antibodies of sera prepared in different ways. The criteria used for determining the presence of common constituents of various antigens and antisera are the 'reactions of identity, non-identity and partial identity' of Ouchterlony (1953). The results obtained were supplemented by complement fixation and quantitative precipitin tests.

MATERIALS AND METHODS

ANTIGENS

1. *Bile Duct Antigen*

This was the first material to be used as an antigen because of the high concentration of parasites in infected bile ducts and because bile has been used for extracting antigens from micro-organisms (Avery and Heidelberger, 1923). The antigen was the exudate collected from the bile ducts of infected livers. Very little fluid, if any, can be withdrawn before the ninth day; before the twelfth day the exudate is slight, difficult to extract from the bile ducts and usually colourless, but, after this time, the bile ducts are grossly dilated and a clear, yellow fluid containing particulate material is obtained.

After removal from the bile ducts the fluid was frozen and kept, in small quantities, at −20°C. Only the clear supernatant was used in Ouchterlony plates, while, for complement fixation reactions, the fluid was centrifuged before withdrawing the supernatant. All bile duct antigens were given the prefix 'B' to distinguish them from the oocyst saline antigen. They were further identified by a number corresponding to the day after infection on which the antigen was removed from the bile ducts, e.g. B17 represents bile duct antigen removed from the liver of a rabbit killed seventeen days after infection. If more than one antigen obtained on the same post-infection day was used, this was further identified by small letters, e.g. B17a. In all, eighteen bile antigens were used, including one called PBA, which was a pool of all available bile antigens. In addition to being test antigens in Ouchterlony plates, these antigens were used as immunizing antigens in the preparation of antisera.

2. *Antigen from oocysts.*

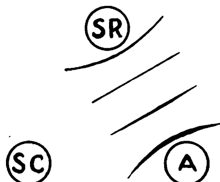
Packed oocysts, obtained from the heavily infected livers of rabbits killed between the twenty-second and twenty-sixth days after infection, were washed many times with water and, finally, with 0·9 per cent saline. They were assessed for purity by microscopical examination, suspended in saline to give a final concentration of 10 per cent v/v packed oocysts, and the suspension subjected to rapid freezing and thawing. It was then ground in a glass tissue grinder, the *p*H adjusted to 7·6, and the solution centrifuged at 2,000 r.p.m. for five minutes. The milky supernatant was taken off and stored, in small quantities, at −20° C. to avoid deterioration. This antigen is called antigen 'A' in the experiments described below.

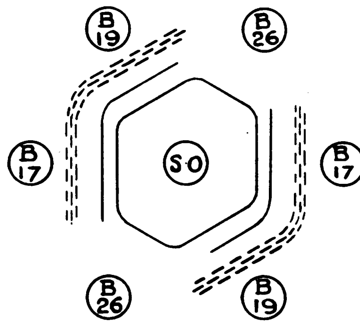
ANTISERA

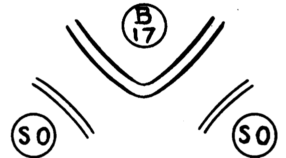
1. Antisera were obtained from rabbits which had received repeated oral infections with sporulated, viable oocysts of *E. stiedae*. These animals showed no clinical signs of the infection and did not pass oocysts in their faeces. These antisera are designated 'SO'.

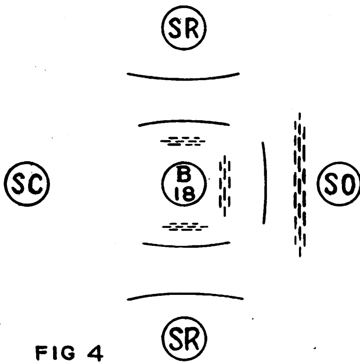
2. Serum obtained from an animal recovering from a very heavy, almost fatal, infection was used and is called 'SR'.

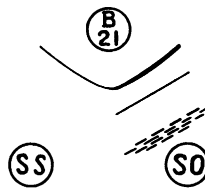
3. Sera were also prepared in rabbits by the subcutaneous injection of bile duct antigen with and without alum precipitation, according to the method of Proom (1943). Two animals, R47 and R51, were each given three injections totalling 6 ml. of alum precipitated antigen B21 at intervals of five and seven days respectively. This was followed, nineteen days later, by a further 6 ml. of alum precipitated B21 antigen. These antisera are labelled 'SA1' and 'SA2'. A third animal, B/W, was given the same number of injections of untreated B21 antigen, amounting to a total volume of 12 ml. This serum is labelled 'SU'. The three animals were bled from the ear vein fourteen days after the last injection.

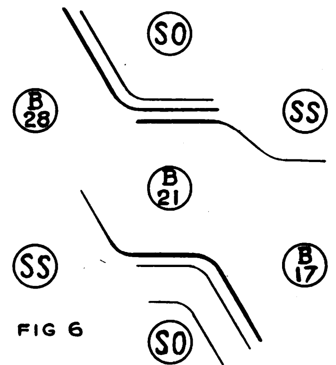


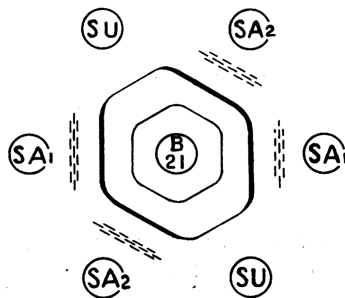


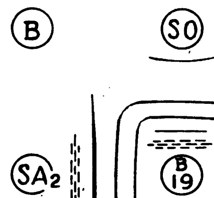












FIGS. 1 to 17. Ouchterlony plates with *Eimeria stiedae* antigens and antisera.

4. Control sera were obtained from two animals, kept apart from other rabbits and under strict conditions of hygiene, in the hope of keeping them free from infections with *E. stiedae*. Control serum is labelled 'SC'.

5. Sera taken from stock rabbits (adult rabbits in the animal house) were tested and labelled 'SS'.

6. Serum samples obtained from rabbits on different days after infection were labelled S11, S14, S24, etc., the numerals referring to the day after infection on which they were obtained.

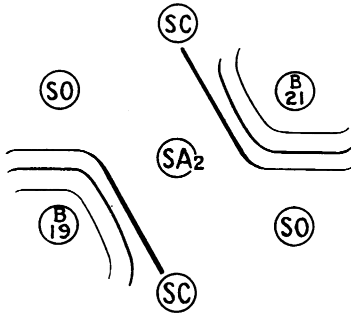


FIG 9

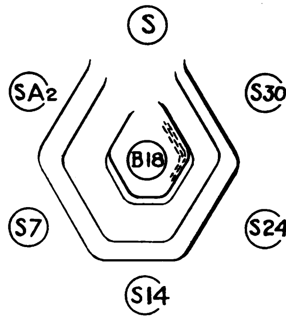
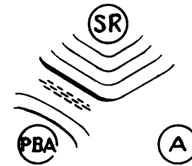


FIG 10



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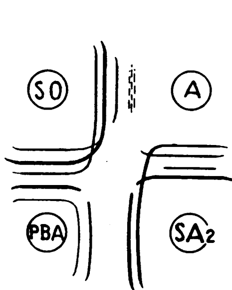

FIG 12

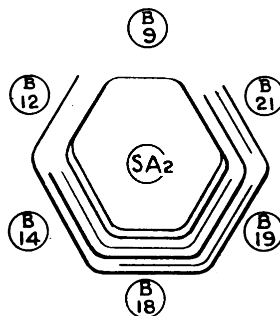

FIG 13

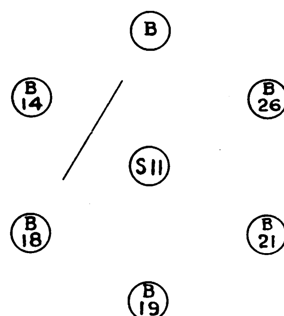

FIG 14

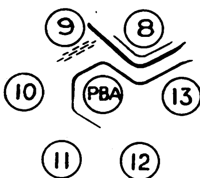

FIG 15

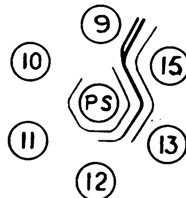

FIG 16

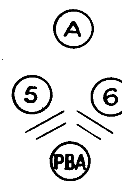

FIG 17

PRECIPITATION IN AGAR GEL

Plates were prepared as described by Datt and Orlans (1958). A useful modification when the plates were to be stained was to pour the agar directly on to lantern slides, using a square perspex mould. The distance between wells (ranging from 10–17 mm. centre to centre) was adjusted according to the particular requirements of the system under investigation. With the weaker systems, where lines failed to appear after 24 hours, the wells were refilled, usually three times. Drawings were made as the lines appeared and developed and photographs were taken when the lines reached their maximum strength and complexity. In the latter part of this work plates were dried and stained with azocarmine and amidoschwarz, as described by Uriel and Grabar (1956).

QUANTITATIVE PRECIPITIN TESTS

Quantitative precipitin tests were performed as described by Kabat and Mayer (1948), the precipitates estimated colorimetrically using the Folin-Ciocalteu phenol reagent and protein values obtained by calibration of the Folin method with rabbit globulin whose nitrogen content had been determined by micro-Kjeldahl analysis (Kabat and Mayer, 1948; Conway, 1947).

COMPLEMENT FIXATION TESTS

Complement fixation tests were carried out using the 50 per cent end point technique, based on the method described by Wadsworth, Maltaner and Maltaner (1931); the results being expressed as the value of the ratio $\frac{IS+A}{IS}$ where IS+A was the titre of the 50 per cent end point of the immune serum in the presence of antigen and IS the titre of the 50 per cent end point of immune serum alone. The percentage haemolysis was measured in a portable 'EEL' colorimeter using a green filter ($\lambda = 530$ mμ) and the activity of complement determined using conversion factors (Kabat and Mayer, 1948) calculated from the equation of von Krogh (1916).

RESULTS

Strong, multiple precipitation bands were obtained with sera from recovering animals, from those which had received repeated infections and from rabbits injected subcutaneously with antigens. Occasionally fainter reactions were found with sera from stock adults (Figs. 5 and 6). Sera from control animals gave no precipitation bands, but were able to deflect a band given by positive sera (Fig. 9).

REACTIONS OF SERA

The reaction between the serum, SR, of a rabbit recovering from a heavy infection with *E. stiedae* and antigen prepared from crushed oocysts, A, is shown in Fig. 1. Serum SC from a non-infected, carefully housed animal was used as a negative serum control. Four precipitin bands were formed between SR and A denoting at least four antigen antibody complexes (Oudin, 1952). No bands were formed between the antigen and the serum control.

The reaction of serum, SO (centre well), of an animal receiving repeated infections with antigens obtained from infected bile ducts is shown in Fig. 2. It can be seen that

B17 and B19 are identical, forming one diffuse and two sharp bands; B26 contains only one constituent of the three demonstrated in B17 and B19. Fig. 3 shows the same serum reacting with antigen B17 and a normal bile control, B. When the distance between the wells was slightly greater than 17 mm., four precipitin bands were demonstrated, two of them being very close together. No bands were seen with the normal bile control. When sera SR and SO were tested against the same antigen stronger reactions were obtained with SR, indicating that the antibody levels are higher in rabbits newly recovered from infection than in those given repeated large doses of oocysts. This observation was confirmed by the results of complement fixation and quantitative precipitin tests. In Fig. 4 the reactions given by SR, SC and SO with bile antigen B18 can be compared.

Antisera (SA_1, SA_2 and SU) prepared by the injection of alum precipitated and untreated bile antigen were tested against the immunizing antigen, B21. Fig. 7 shows that much stronger precipitin bands were obtained with sera prepared by the injection of alum precipitated antigen than with that prepared by injecting untreated antigen. The pattern of lines obtained very closely resembled that seen with this antigen and SO sera.

The serum obtained from orally infected animals was compared with that from rabbits injected with alum precipitated antigen to determine whether these two types of serum possessed antibodies in common. This experiment is illustrated in Figs. 8 and 9, in which SO and SA2 are reacting with bile antigens B19 and B21 with SC as serum control and B as normal bile control. The pattern of the bands shows that these sera have at least two and probably three antibodies in common, represented by the common precipitin bands. These were much stronger with SA antiserum. Here, the control serum, SC, gave a slight deflection of the precipitin band nearest the antigen wells B21 and B19. As a result of these and similar tests, SA was used for comparing antigens as it gave much stronger bands than sera SO. (SA has been used in complement fixation tests with results roughly comparable to those obtained with the sera of orally infected rabbits.)

The effect of oral infection with *E. stiedae* oocysts on an animal previously injected with alum precipitated antigens was demonstrated by the reactions of its sera with bile antigen, B18. The rabbit from which SA2 had been obtained was infected orally with a massive dose of *E. stiedae* oocysts fourteen days after the last injection of antigen, to determine the effect on the serum antibodies. The rabbit had been bled before and after immunization and was bled 7, 14, 24 and 30 days after infection; the corresponding sera are S, SA2, S7, S14, S24 and S30. The reactions of the sera are shown in Fig. 10. No bands were given with S. Serum SA2 gave three bands, at this well distance, which corresponded to those given by S7. These three bands were also given, but more strongly, by S14, S24 and S30, together with an additional faint band which remained near the antigen well.

REACTIONS OF ANTIGENS

Fig. 11 shows the comparison of the pooled bile antigen, PBA, with saline oocyst extract A, using serum SR from a rabbit recovering from a severe infection. This plate showed that PBA and A contained at least four common constituents.

Fig. 12 shows an arrangement of reagents whereby the reactions of the two antigens with sera SO and SA2 were compared. The bands given by the two sera with antigen A were fainter and took longer to develop than those given by them with antigen PBA, but they did occur and were common to both antigens. This indicated that the two antigens had similar properties, although the constituents possessed in common were present in greater

amounts in PBA than in A. This observation was confirmed, to a certain extent, by the results of complement fixation tests with these antigens; bile antigen at a dilution of 1:60 gave results roughly comparable to saline oocyst antigen at a dilution of 1:6 (Table 1). Confirmation of this observation was also given by the results of the quantitative precipitin tests.

TABLE 1

RESULTS OF COMPLEMENT FIXATION TESTS WITH VARIOUS SERA AND ANTIGENS

Serum	*Antigen*	*Antigen dilution*	$\frac{IS+A}{IS}$ *Activity of serum*
SO	PBA	1:60	14
SO	A	1:6	39
SR	A	1:6	127
SR	PBA	1:60	60
SC	A	1:6	5
SC	PBA	1:60	2
SA_2	PBA	1:60	81
SA_2	A	1:6	123
SS	PBA	1:60	3
S	PBA	1:60	2
S_7	PBA	1:60	92
S_{14}	PBA	1:60	340
S_{24}	PBA	1:60	468
S_{30}	PBA	1:60	490
Pooled SO	PBA	1:20	15
Pooled SO	A	1:20	2

Comparison of bile antigens obtained at different stages of infection, as illustrated in Figs. 6 and 2, using sera from both orally infected and subcutaneously immunized animals, had shown that more precipitin bands were given by antigens obtained at 17–19 days after infection than by those obtained at any other time. To investigate this further, a plate was set up as in Fig. 13, using SA2 as test antiserum and bile antigens Bs 9, 12, 14, 18, 19 and 21. A very complex pattern of bands was obtained and again the maximum number was found with B18 and B19; with antigens taken from the bile ducts earlier or later in infection, fewer bands were present. The findings are summarized in Table 2, where the number of crosses is a rough indication of the intensity of the bands. To aid in description the bands are arbitrarily numbered from the centre well outwards. The intensity of bands was greatest with B18 and B19.

A serum, S11, taken from an animal eleven days after its infection with a moderate dose of *E. stiedae* oocysts, was tested against bile antigens Bs 14, 18, 19, 21 and 26. Only the earliest of these, B14, gave a precipitin band with this serum (Fig. 14).

QUANTITATIVE PRECIPITIN TESTS

Quantitative precipitin tests were carried out using pooled bile antigen 'PBA' and oocyst antigen 'A', with pooled serum 'SO' from rabbits orally infected with *E. stiedae* oocysts.

Constant amounts of serum, mixed with increasing amounts of antigen as shown in Table 3, were allowed to react at room temperature for 1 hour and then kept at 4° C. for

TABLE 2
COMPARISON OF BILE ANTIGENS (see Fig. 14)

Antigen	*Band* 1	*Band* 2	*Band* 3	*Band* 4	*Band* 5	*Band* 6
9 day, B_9		±				
12 day, B_{12}		+++				++
14 day, B_{14}	+++	+++	++	+++		+++
18 day, B_{18}	+++	+++	++	+++	+	+++
19 day, B_{19}	+++	+++	++	+++	+++	+++
21 day, B_{21}		+++		+	++	++

one week, the tubes being agitated gently every day. The tubes were centrifuged, the precipitates carefully washed three times with chilled saline, and measured by tyrosine estimation according to Kabat and Mayer (1948). Results are shown in Table 3 and the curves, in which the precipitate in mg. of protein (per ml. of serum) is plotted against antigen added, are shown in Fig. 18.

TABLE 3
THE REAGENTS USED AND RESULTS OBTAINED IN THE QUANTITATIVE PRECIPITIN TESTS

Tubes	*Pooled serum, 'SO' ml.*	*Antigen 'A' ml.*	*Saline ml.*	*Protein in precipitate mgm.*
1	0·75	0·4	2·6	0·17
2	0·75	0·75	2·25	0·26
3	0·75	1·5	1·5	0·26
4	0·75	2·0	1·0	0·26
5	0·75	2·5	0·5	0·39
6	0·75	3·0	—	0·39
7	—	1·5	2·25	—
8	0·75	—	3·0	—
		Antigen 'PBA' ml.		
9	0·75	0·4	2·6	0·4
10	0·75	0·75	2·25	0·45
11	0·75	1·5	1·5	0·58
12	0·75	2·0	1·0	0·72
13	0·75	2·5	0·5	0·77
14	0·75	3·0	—	0·79
15	—	1·5	2·25	—

The maximum amount of protein precipitated from pooled 'SO' by antigen 'A' was 0·53 mg./ml. serum, and by antigen 'PBA' 1·0 mg./ml. serum, although the precipitates given by the bile antigen were much slower to come down.

Residual antibody and antigen in the supernatant fluids retained from tubes 9, 10, 11, 12 and 13 of the 'PBA' precipitin test were demonstrated by setting up the plates shown in Figs. 15 and 16, using material from tubes 8 and 15 as serum and antigen controls respectively. The presence of antibody was shown in the supernatant fluids from tubes 8, 9, 10 and 11 (very slight) (Fig. 16), and excess antigen was shown in the supernatant fluids from tubes 10 and 11 (both very slight), 12 and 13 (Fig. 16).

The presence of residual antibody in serum 'SO' after precipitation with oocyst antigen 'A' was demonstrated by setting up the supernatant fluids from tubes 5 and 6 against bile antigen 'PBA' and oocyst antigen 'A' as shown in Fig. 17. No bands of precipitation were

obtained between these supernatant fluids and 'A', but 'PBA' reacted with them to give two bands. This confirmed the results of the quantitative precipitin tests in which twice as much protein was precipitated from 'SO' by 'PBA' than by 'A'.

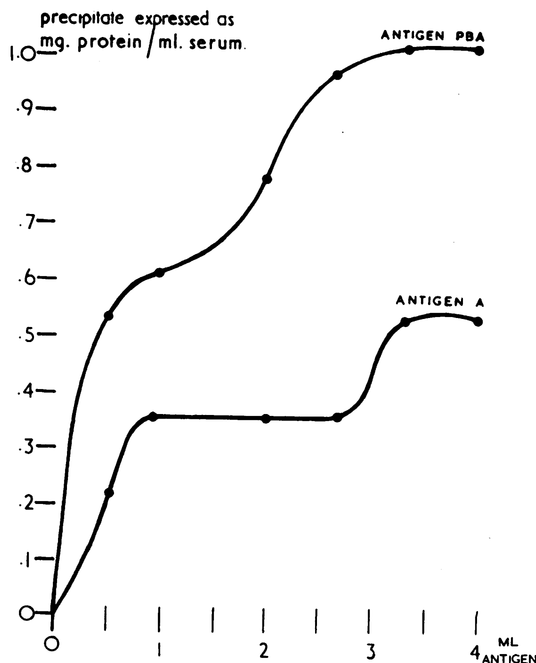

FIG. 18. Precipitin curves with pooled antisera 'SO' and oocyst antigen 'A' and pooled bile antigen 'PBA'.

DISCUSSION

The number of precipitin bands given with the sera and antigens described varied from three, in the case of sera from orally infected rabbits, to six with sera prepared by the immunization of animals with alum precipitated bile antigens. The number of bands, however, varied with the strength of the reagents, the distance between wells and the relative proportions between well diameter and well distance. With unknown systems, it was found advisable to set up several test plates of varying dimensions to determine the optimum conditions for those particular reagents.

The strongest reactions in gel were given by sera prepared by the subcutaneous injection of alum precipitated bile antigens both with homologous antigen and with oocyst antigen. In the case or orally infected rabbits, the strongest reactions were given by sera obtained from rabbits recovering from a heavy infection, usually 40–60 days after infection. Sera taken from adult rabbits given repeated doses of infective oocysts did not produce such strong bands of precipitation with the same antigens. In complement fixation tests the same pattern of responses was obtained, the highest values being given by serum SA2, followed by serum SR, with serum SO giving the lowest values. Repeated reinfection had little effect on the $\frac{IS+A}{IS}$ ratio.

The production of a satisfactory convalescent serum was not easy, as the infective doses of oocysts had to be as heavy as possible without causing the death of the animal. Variation in the viability of oocysts and the ability of individual animals to withstand infection caused the difficulty.

From the results of precipitation tests in agar gel and in liquid medium and complement fixation tests, the antibodies produced by the subcutaneous injection of alum precipitated antigens appeared to be essentially similar to those produced by the oral infection of rabbits. Gel precipitation tests, however, showed an extra band in addition to the intensification of already existing bands when an oral infection was superimposed upon a 'vaccinated' animal. No protection was demonstrated.

Antigens prepared from crushed oocysts and from the exudate from infected bile ducts had similar constituents. The bile duct antigens gave stronger precipitation bands in gel and precipitated twice as much antibody from a given serum as the oocyst antigen and, in complement fixation tests, gave higher values for $\frac{IS+A}{IS}$ when used at the same concentration. The shapes of the precipitation curves and also the Ouchterlony Plate, Fig. 17, show that an antigenic constituent is either lacking, or present in much smaller amounts, in the oocyst antigen. These curves also showed that with both antigens at least two different antigen/antibody reactions were involved.

A comparison of bile antigens obtained on different days after infection showed that the greatest number of precipitation bands was obtained with 17–19 day antigens, suggesting that the greatest number of different antigens was present at this time in the life cycle. Microscopical examination of the infected bile ducts on these days showed that both schizogony and gametogony were occurring. It appears that the development of the maximum number of precipitation bands was related to the phase of the infection associated with the maximum number of various developmental stages of the parasite. Confirmatory evidence was given by the reaction of a serum taken early in infection (on the eleventh day) with an early bile duct antigen, B_{14}; no reactions were obtained with later bile duct antigens.

Comparable results were obtained with precipitation in gel, complement fixation and quantitative precipitin tests, using both antigenic systems. The particular advantage of the Ouchterlony method is that qualitative comparisons can very easily be made; it may therefore be of use in demonstrating reactions of identity or non-identity between different species of the Coccidia, with the possibility of distinguishing between species when morphological methods are unreliable.

ACKNOWLEDGMENTS

I should like to thank Dr. E. S. Orlans of this Department for suggesting the use of the Ouchterlony gel diffusion and quantitative precipitin techniques and for helpful advice and criticism in carrying out the work and preparing the manuscript. I am grateful to Dr. E. J. L. Soulsby, also of this Department, for suggesting the problem, providing the original oocysts and criticizing the manuscript. Thanks are also due to Miss Jill Mizen for technical assistance and Miss J. Minns for photographic assistance.

The work was performed during the tenure of an Animal Health Trust Research Training Scholarship.

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