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Immunity to Four Species of *Eimeria* in Fowls

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Summary. Two or three graded infections with oocysts of *Eimeria acervulina*, *E. tenella*, *E. necatrix* and *E. maxima* produced a resistance to further infection with the immunizing species. The oocyst output after the second infection, in each case, was lower than that after the initial dose indicating the substantial immunizing effect of the initial infection. The species could be placed in a descending order of immunizing activity as follows: *E. maxima*, *E. acervulina*, *E. tenella* and *E. necatrix*. A solid immunity to the immunizing species in no way prevented the development of an additional infection, here referred to as 'cross-infection', with any of the species studied.

Serum precipitins were produced in infections with all four species, the response to infection with *E. necatrix* being less marked than to the other species. A first challenge of immune fowls with the immunizing species produced some increase in precipitation in agar whereas a second challenge had no such effect; the significance of this lack of response is discussed. Usually, fowls immunized against one species and then infected with an additional one, produced serum precipitins which reacted only with the antigen of the additional species. But *E. tenella* immunized fowls, when given an additional infection with *E. necatrix*, produced precipitins that reacted with antigens of both species. The same was also true when *E. necatrix* immunized fowls were infected with *E. tenella*.

INTRODUCTION

Fowls, once recovered from coccidial infections have been shown to be resistant to reinfection (Beach and Corl, 1925; Johnson, 1927; Tyzzer, 1929; Horton-Smith, 1947). Attempts to detect serum antibody in fowls that have recovered have not been very successful, although a brief report of the agglutination of *Eimeria tenella* merozoites by the sera of infected fowls was made by McDermott and Stauber (1954). Horton-Smith, Beattie and Long (1961) showed that infection of one caecum with *E. tenella* could confer resistance on its non-infected and isolated partner and later, Pierce, Long and Horton-Smith (1962) demonstrated precipitating antibodies in the sera of birds which had received two or more graded infections with *E. tenella* oocysts.

In the present paper, the antibody responses, measured by precipitation in agar, of fowls infected with *E. acervulina*, *E. tenella*, *E. necatrix* and *E. maxima* are described and the results of some cross-infection experiments are given. The immunizing effects, judged by the number of oocysts passed in the faeces after various doses of each of the four species of

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Eimeria oocysts used, are also given and an attempt is made to relate them to antibody responses. A comparison of the antigens of some *Eimeria* species will be given elsewhere.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS. Twenty R.I.R. × L.S. fowls, 3 weeks old and of mixed sexes, were divided into four groups, each of five chicks. One bird from group 1 was subsequently killed leaving only four in that group. The fowls were kept indoors in electrically heated, wire-floored metal cages for the first 6 weeks of the experiment and then moved out of doors to wire-floored, paraffin-heated brooders. They were finally housed in slatted-floor, wooden houses, raised 2 feet from the ground. This method aimed at a high degree of cleanliness but not strict isolation; all the equipment had been rested for several months before use. The diet was similar to commercial rations but did not contain antibiotics or coccidiostats.

INFECTIVE MATERIAL. The oocyst cultures used were pure lines maintained at this station in birds kept in strict isolation and were all derived from single oocyst infections. The methods used for culturing the oocysts have been described (Horton-Smith and Long, 1959). Oocysts for dosing were counted directly using a Fuchs-Rosenthal counting chamber.

FAECAL EXAMINATIONS. After the first two infections, when large numbers of oocysts were being passed, the total oocyst output of each group was determined using a dilution method (Long and Rowell, 1958). A concentration method, using saturated sodium chloride solution, was used on the faeces obtained after the third infections (groups 2 and 4) and after each of the challenging doses (groups 1–4) when only very small numbers of oocysts, if any, were present.

INFECTION OF THE FOWLS. The choice of the number of *E. tenella* oocysts was based on the work of Pierce *et al.* (1962) but only two of their three doses were given, as it was thought that these might be sufficient to immunize. The lethal doses of oocysts of *E. necatrix* and *E. tenella* are approximately the same (Davies, 1956) but, as less is known of the immunizing power of *E. necatrix*, three doses of oocysts were given. The numbers of *E. maxima* oocysts were based on the results of previous work (Long, 1959). The numbers of oocysts of *E. acervulina* required to produce clinical disease are very much greater than those of other species and the immunizing doses of oocysts of this species were derived from the values for lethal doses given by Morehouse and McGuire, 1958. These graded doses, which were used to produce infections in the fowls, are referred to as 'immunizing doses'. After completion of the immunizing schedules the susceptibility of the fowls in each group to further infection was tested by administering large numbers of oocysts of the relevant species. These are referred to as 'challenge doses'. The challenge doses in each case consisted of at least twice the numbers of oocysts required to produce severe disease or death in susceptible birds.

Groups 1, 2, 3 and 4 were immunized with *E. acervulina*, *E. tenella*, *E. necatrix* and *E. maxima* oocysts respectively. The first doses were given when the birds were 3 weeks old and subsequent doses at weekly intervals. The numbers of oocysts used and the scheme adopted are shown in Table 1.

CHALLENGE DOSES. The first doses were given on the thirty-eighth day after the initial infection with oocysts and consisted of 20,000,000 *E. acervulina* to group 1, and 100,000

oocysts of *E. tenella*, *E. necatrix* and *E. maxima* oocysts to groups 2, 3 and 4 respectively. The second doses, which comprised the same number of oocysts of each species as the first challenge, were given on the eighty-fourth day after the initial infections with oocysts.

CROSS-INFECTION EXPERIMENTS. The infection of fowls, shown to be resistant to the species used for immunization, with another species will be referred to as 'cross-infection'.

TABLE 1

THE IMMUNIZING SCHEDULES FOR GROUPS 1-4

Group no.	*Species of oocysts used*	*Wing-band nos.*	*Number of oocysts given to each bird*		
			First infection	*Second infection*	*Third infection*
1	*E. acervulina*	1-5	500,000	5,000,000	10,000,000
2	*E. tenella*	6-10	500	5,000	—
3	*E. necatrix*	11-15	500	5,000	50,000
4	*E. maxima*	16-20	500	5,000	—

On the 107th day after initial infections, when the birds were 4½ months old, the five birds in Group 2 (*E. tenella* immunized) were given *E. necatrix* oocysts and the five birds in group 3 (*E. necatrix* immunized) were given the same number of *E. tenella* oocysts.

One week later groups 1 and 4 were subdivided, each into two, and each of the resulting four sub-groups was infected according to the scheme given in Table 2.

TABLE 2

CROSS-INFECTION OF FOWLS IN GROUPS 1-4

Species used for original infection					
E. acervulina		*E. tenella*	*E. necatrix*	*E. maxima*	
1*a*	1*b*			4*a*	4*b*
Wingband numbers					
3, 2	4, 1	6–10	11–14	1, 17, 19	18, 20
Species and numbers of oocysts used for cross-infection					
E. maxima	*E. necatrix*	*E. necatrix*	*E. tenella*	*E. acervulina*	*E. necatrix*
1,000,000	200,000	100,000	100,000	20,000,000	200,000

ANTISERA. Birds were bled from the wing vein at intervals, starting 14 days after the first infection. The serum samples harvested were stored at −20° C.

PRECIPITATION IN AGAR. The Ouchterlony double diffusion method was used. Difco Agar was made up to 1.5 per cent in a veronal buffer, pH 7.4, containing 0.1 per cent sodium azide and 8 per cent sodium chloride. This concentration of salt was used to provide the optimum conditions for the precipitation of fowl antibody/antigen complexes

(Goodman, Wolfe and Norton, 1951). Dried and stained preparations (Uriel and Grabar, 1957) of the plates were made after allowing 7–10 days for development of the precipitin bands. After staining, the intensity of the precipitation bands was graded + + +, + +, +, (+) and O (sharp and very distinct to negative) and the number of bands noted. The entire series of plates was graded at the same time to avoid variations; the system used is shown in Fig. 9.

EXAMINATION POST-MORTEM. Gross lesions were noted and smears made from fresh material were examined microscopically.

RESULTS

Quantitative oocyst estimations were carried out on the oocysts produced by the first two infections and a sensitive oocyst detection method was used subsequently. The numbers of oocysts passed in the faeces per oocyst fed to the bird is a measure of the reproduction ability of a species of coccidium and this will be referred to as 'Reproduction Index'. The reproduction index obtained from the determination of oocyst output after the first infection is taken as the standard and those values obtained for the second and subsequent infections can be compared with the standards and used as criteria for assessing resistance. The oocyst output of all four groups of birds after the first and second infections are given in Table 3 together with the results of faecal examinations by the flotation method on the twenty-second day after infection and after each of the two challenges.

The reproduction indices of the oocysts fed in the second infections were much lower than those obtained from the first infection, particularly in the case of *E. maxima*. Single doses of as few as 500 oocysts of *E. tenella*, *E. necatrix* and *E. maxima* reduced the reproduction indices for these species so indicating the development of resistance by the host. If the values obtained for the reproduction indices are taken as criteria, a very substantial resistance was produced after the second infection and this proved to be complete when the birds were challenged.

Very small numbers of *E. acervulina* oocysts were found in the faeces of group 2 birds (*E. tenella* immunized) towards the middle of the experiment and, similarly, small numbers of *E. maxima* oocysts were present in group 3 birds (*E. necatrix* immunized). These findings showed that slight extraneous infections had occurred in birds of these groups.

CROSS-INFECTION EXPERIMENTS

Results of the cross-infection experiment are given in Table 4.

Complete resistance of the birds to one species of *Eimeria* failed to protect them against infection with another species. This was particularly marked in the group 2 birds (immunized with *E. tenella*), two of which died from *E. necatrix* infection; the remaining three had severe infections with this species. Examination, *post-mortem*, of one member from each of group 3, sub-groups 1a, 1b, 2a and 2b, killed on the fourth or fifth days after infection showed the presence in all but one, of severe to moderate infections. One bird from group 4b (immunized with *E. maxima* and cross-infected with *E. necatrix*) showed no evidence of having actually become infected with the species used for cross-infection. A later experiment however, using *E. maxima* resistant birds, showed that these were fully susceptible to infection with *E. necatrix*

TABLE 3

FAECAL EXAMINATIONS AFTER IMMUNIZING AND CHALLENGE INFECTIONS

Group no.	*Species administered*	*Details*	*Examination by counting method*			*Faecal examination by flotation method*		
			After first infection	*After second infection*	*Ratio of reproduction index after first infection to that after the second infection*	*Before challenge* *	*First challenge* X	*Second challenge* X
1	*E. acervulina*	Dose	500,000	5,000,000			20,000,000	20,000,000
		Total oocysts produced	513,000,000	60,000,000				
		Reproduction index	1,026	12	86 : 1	+	−	−
2	*E. tenella*	Dose	500	5,000			100,000	100,000
		Total oocysts produced	32,000,000	23,000,000				
		Reproduction index	64,000	4,600	14 : 1	+	−	−
3	*E. necatrix*	Dose	500	5,000			100,000	100,000
		Total oocysts produced	6,000,000	6,800,000				
		Reproduction index	12,000	1,360	9 : 1	+	−	−
4	*E. maxima*	Dose	500	5,000			100,000	100,000
		Total oocysts produced	15,000,000	26,000				
		Reproduction index	30,000	5.2	5700 : 1	+	−	−

+ = small numbers (not revealed by counting method)

− = negative (concentration method)

* = 8 days after third infection of groups 2 and 4 (15 days after second infection of groups 1 and 3)

X = Examinations were made at times when oocysts would normally be expected

TABLE 4

RESULTS OF CROSS-INFECTION EXPERIMENTS

E. acervulina		*E. tenella*	*E. necatrix*	*E. maxima*	
Sub-groups		Group 2	Group 3	Sub-groups	
1A	1B			4A	4B
Species used for cross-infection					
E. maxima	*E. necatrix*	*E. necatrix*	*E. tenella*	*E. acervulina*	*E. necatrix*
Results of post-mortem examination					
Heavy *E. maxima* infection. Intestinal wall thickened containing orange-red exudate rich in gametocytes and oocysts	Moderate-heavy *E. necatrix* infection. Thickened intestinal wall with numerous lesions. Orange exudate; schizonts and merozoites present	V. severe *E. necatrix* infection. (Fatal in two birds) haemorrhagic intestine, numerous schizonts and merozoites	Moderate *E. tenella* infection. Thickened caecal wall with a few haemorrhagic areas. Gametocytes and oocysts present in smears	Heavy *E. acervulina* infection with copious mucous exudate. Gametocytes and oocysts present	No gross lesions visible — no parasitic stages found in smears

REACTIONS OF SERA

All the birds developed precipitins to the test antigens although there was some variation in the response of individual birds within a group. The weakest bands were found in the sera of birds given *E. necatrix* infections; single bands of low intensity being obtained

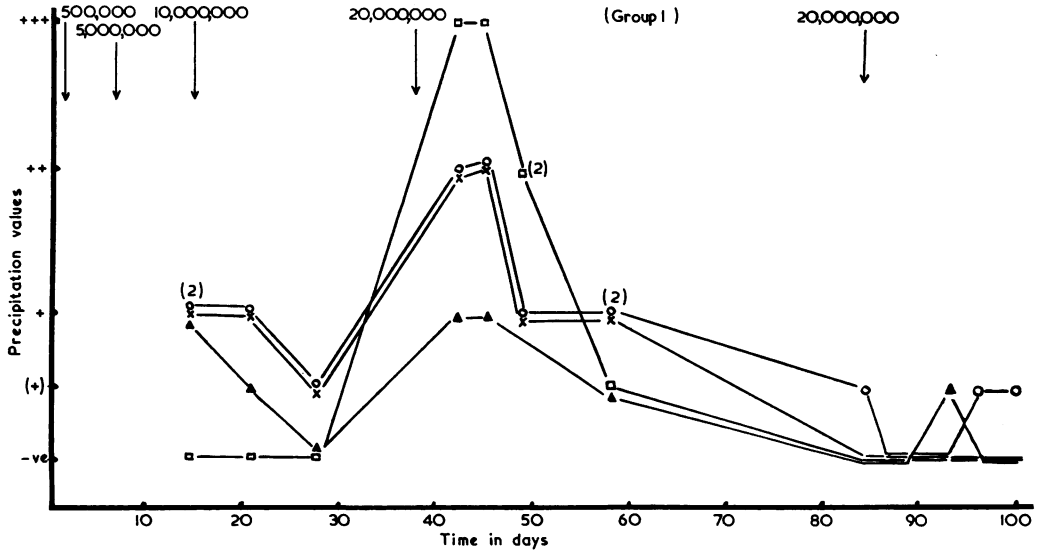


FIG. 1. ○ = Bird 1; ▲ = Bird 2; × = Bird 3; □ = Bird 4.

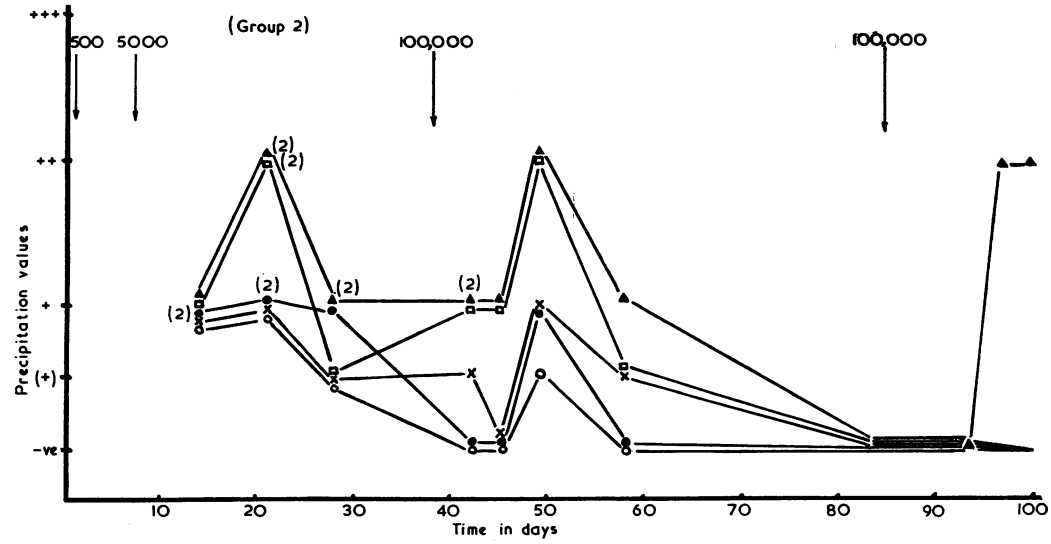


FIG. 2. ● = Bird 6; ○ = Bird 7; ▲ = Bird 8; × = Bird 9; □ = Bird 10.

during a limited period. Response to the other infections was good with many sera reacting to give two bands.

Figs. 1–4 show the variations in the precipitin contents of sera obtained from individual

birds in groups 1–4. These sera were tested only against antigens prepared from the species with which the birds had been immunized.

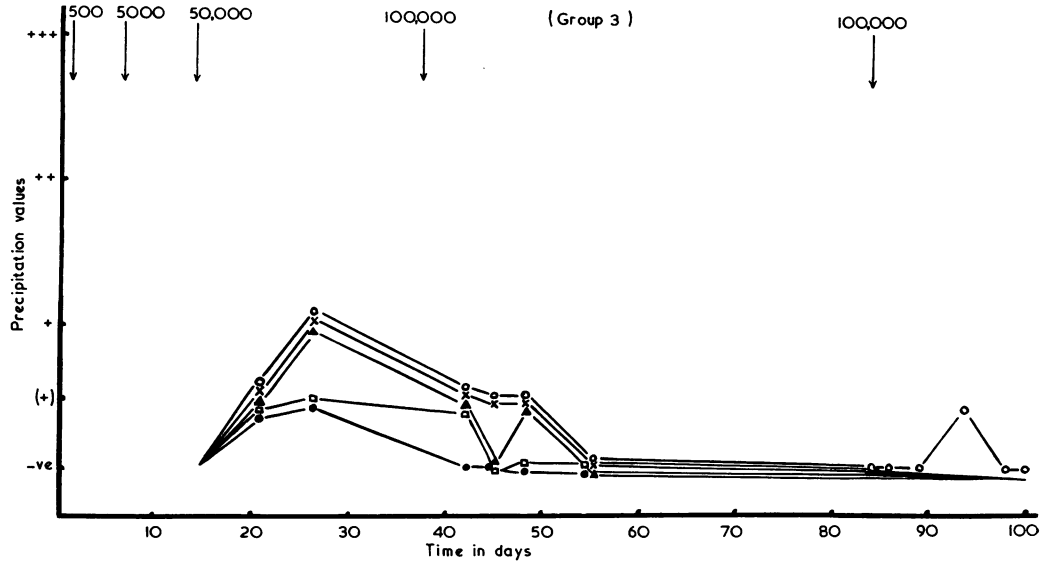


FIG. 3. ● = Bird 11; ○ = Bird 12; ▲ = Bird 13; × = Bird 14; □ = Bird 15.

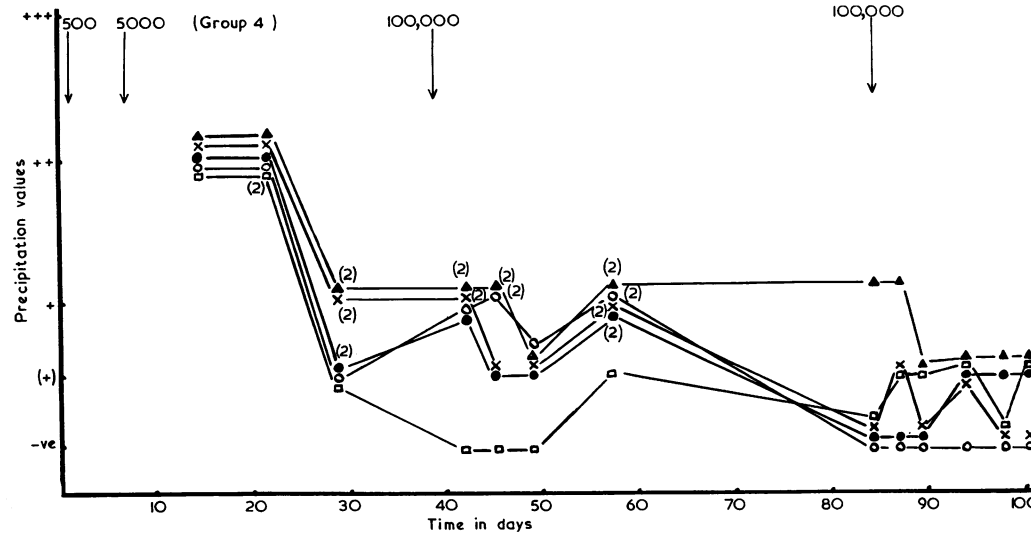


FIG. 4. ● = Bird 16; ○ = Bird 17; ▲ = Bird 18; × = Bird 19; □ = Bird 20.

Initial Infections

Sera obtained from all the birds in groups 2 and 4 and three of the four in group 1 at the first bleeding (14 days after the initial infection) gave single or double bands but group three birds were negative. Maximum responses in groups 1 and 4 were found at the four-

teenth and twenty-first days. Precipitin bands in group two birds were at their maxima on the twenty-first day. No antibody was found in the sera of group three birds until the twenty-first day after infection, when all five sera reacted slightly; the maximum response was obtained on the twenty-seventh day.

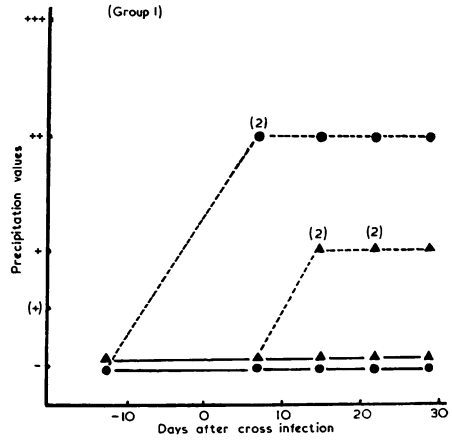


FIG. 5. Results with antigen prepared from immunizing species = *E. acervulina* ●——— = Bird 1. ▲——— = Bird 3.
Results with antigen prepared from cross-infection species *E. necatrix* ●- - - - = Bird 1. *E. maxima* ▲- - - - - - = Bird 3.

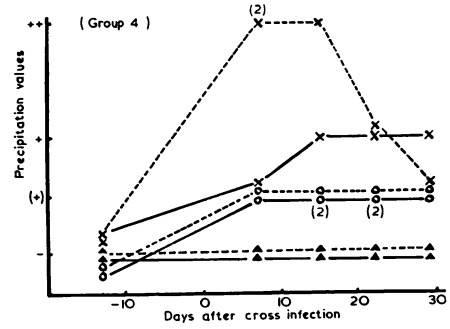


FIG. 6. Results with antigen prepared from immunizing species (*E. maxima*).
× ——— = Bird 19
○ ——— = Bird 17
▲ ——— = Bird 18
Results with antigen prepared from cross-infecting species =
E. acervulina ○- - - - - = Bird 17
× - - - - - = Bird 19
E. necatrix ▲- - - - - - = Bird 18

First Challenge

Birds in groups 1 and 2 responded to a challenging infection as shown by enhanced precipitation in agar. Increased precipitation was found earlier with the sera of group 1 birds (4 and 7 days after challenge) than with sera from group 2 birds (10 days after challenge). None of the birds in the other groups showed increased precipitation.

Second Challenge

The birds in all groups were challenged for a second time on the eighty-fourth day after the sera became negative. Only one bird (No. 8, group 2) reacted appreciably to this challenge, giving a strong single band on the twelfth and fourteenth days after challenge.

Cross-infection Experiments

Sera obtained after the cross-infection of birds were tested both against antigens prepared from the species used for immunization and from the species used for the cross-

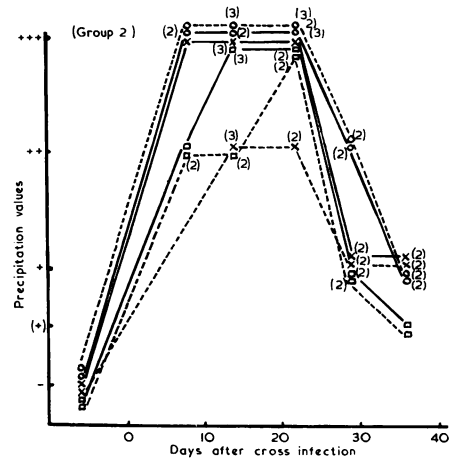


FIG. 7. Results with antigen prepared from immunizing species *E. tenella*————
Results with antigen prepared from cross-infecting species *E. necatrix* - - - - - -
○ Bird 7; × Bird ♀; □ Bird 10.

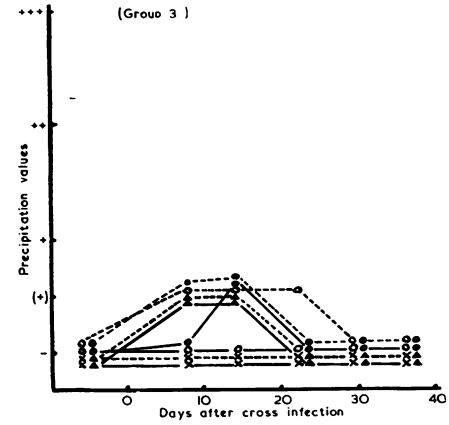


FIG. 8. Results with antigen prepared from immunizing species = *E. necatrix*————
Results with antigen prepared from cross-infecting species = *E. tenella* - - - - - -
○ Bird 12; × Bird 14; ● Bird 11; ▲ Bird 13.

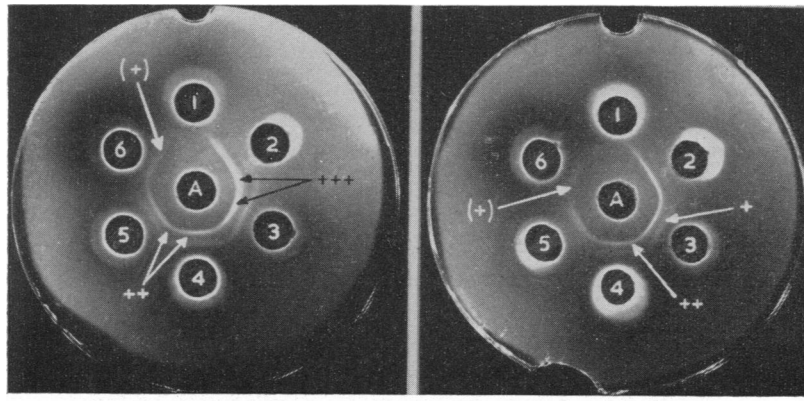
FIGS. 1–4 inclusive. Results of agar-gel precipitin tests on the sera of fowls in Groups 1–4. Infection with oocysts are indicated ↓ and figures in brackets refer to the number of precipitin bands when more than one was obtained.

FIGS. 5–8 inclusive. Results of precipitin tests of sera taken during the cross-infection experiments. Solid lines indicate results obtained with antigen prepared from the immunizing species, dotted lines refer to those given with antigens from the species used for cross-infecting.

infection. They were not tested against the other antigens. Results are shown in Figs. 5–8.

In general, the sera from birds in groups 1 and 4 reacted similarly; precipitins developed with the cross-infecting antigens, but not with the antigen derived from the immunizing species.

Group 1 (Immunized with *E. acervulina*). The sera of two birds in this group were tested, one (No. 1) cross-infected with E. *necatrix* and the other (No. 3) cross-infected with *E. maxima*. Neither serum reacted with the immunizing antigen, but each reacted with antigen prepared from the cross-infecting species, giving strong and moderately strong bands respectively. In contrast to the results obtained when *E. necatrix* was the initial infection (group 3), precipitins were found earlier, two bands being present on the seventh day.



(*a*) (*b*)

FIG. 9. Agar-gel precipitin reactions of the sera of a fowl immunized with *E. tenella* and cross-infected with *E. necatrix* against both *E. tenella* (Fig. 1a) and *E. necatrix* (Fig. 1b) antigen. The precipitation values are indicated.

FIG. 9 (*a*) Test antigen *E. tenella* centre well.

FIG. 9 (*b*) Test antigen *E. necatrix* centre well.

Wells 1–6 (both plates)

Well 1 = serum obtained 6 days before cross-infection with *E. necatrix*
" 2 = " " 8 " after " " " "
" 3 = " " 14 " " " " " "
" 4 = " " 22 " " " " " "
" 5 = " " 29 " " " " " "
" 6 = " " 36 " " " " " "

The sera were obtained from bird No. 7, group 5, immunized with *E. tenella* and cross-infected with *E. necatrix*.

→ indicate precipitin values +++, ++, +, (+).

Serum from No. 3, infected with *E. maxima*, reacted similarly to that obtained from birds in which *E. maxima* was the initial infection (group 4).

Group 4 (Immunized with *E. maxima*). One (No. 18) of the three birds whose sera were tested, did not give precipitation bands with either the original immunizing antigen or with the cross-infecting antigen, *E. necatrix*. Of the remaining two birds cross-infected with *E. acervulina* one (No. 19) reacted strongly with the cross-infecting antigen and weakly with the immunizing antigen, while the other (No. 17) was very weakly positive with both.

The serological responses of groups 2 and 3 differed markedly from those of groups 1 and 4. All the birds in group 2 responded to cross-infection with *E. necatrix* by the production of strong precipitins which reacted with both immunizing antigens (Fig. 7). A greater

response was obtained with these sera than with any others in the experiment, three strong bands being given with many of the bleedings (Fig. 9).

In contrast, group 3 birds (immunized with *E. necatrix*) responded only very slightly to cross-infection with *E. tenella*; one was completely negative with both antigens and the other three reacted to give one very faint band with both antigens on the eighth and/or fourteenth day after cross-infection.

DISCUSSION

The marked reduction in oocyst production after the second infection shows that a high degree of resistance was conferred by the initial infection. This was particularly marked for *E. maxima*, especially in view of the comparatively small numbers of oocysts given in the first infection, and confirms the conclusions of other authors that a prompt resistance is developed (Tyzzer, 1929; Long, 1959). A very high degree of immunity was also produced in response to the first infection with *E. acervulina* although it has been stated (Tyzzer, 1929) that this is not usually the case and that this parasite is well known as the cause of so-called chronic intestinal coccidiosis in fowls. Bearing this in mind, high doses of *E. acervulina* oocysts were used in this experiment and probably account for the results obtained. As might be expected from the similarity in their morphology and life cycles, much the same results were obtained with *E. necatrix* and *E. tenella*. The degree of resistance produced to these two species by one infection was much less than that obtained with the same numbers of *E. maxima*. Nevertheless, a solid resistance existed against all species when the birds were challenged.

The way in which the life cycle is halted in *E. tenella* resistant fowls is not yet fully understood. Tyzzer, Theiler and Jones (1932) found that in birds resistant to *E. necatrix*, the sporozoites of this species invaded the intestinal epithelium but were not able to develop further; similar observations have been made on *E. tenella* in resistant birds by Pierce *et al.* (1962). Nothing is known of these processes in *E. acervulina* and *E. maxima* infections.

The development of resistance was accompanied by circulating antibody in infections with all four species. Although the method of testing for antibody is neither sensitive nor strictly quantitative it seems that initial infections with *E. acervulina*, *E. tenella* and *E. maxima* produced much the same kind of response; with *E. necatrix* infections the response was delayed and slight.

In infections with all four species, the birds were resistant to the final (second) challenges although antibodies were no longer detectable in their sera. In rabbits that had recovered from hepatic coccidiosis (*E. stiedae*) and were resistant to further infection, complement — fixing antibodies persisted at a low level for at least a year (Rose, 1961). This difference may be due to the more sensitive method of testing for antibody used with rabbit serum and/or to the persistence of the parasite (and, hence, of antigen) in the sealed-off lesions usually found in the livers of these rabbits. More sensitive methods for the detection of antibodies including also 'incomplete' ones, may show that antibodies do exist in the resistant birds, either in small amounts or in non-precipitating form. It is known that fowls produce incomplete antibodies (Terzin, 1960; Orlans, Rose and Marrack, 1961) and incomplete antibodies have been detected in parasitic infections (Soulsby, 1960); tests for incomplete antibodies in fowl serum are being investigated. On the other hand, resistance to re-infection in these birds may be due to cell-bound antibody and localized

in the intestinal sub-mucosa. Attempts are being made to extract antibodies from the caeca of resistant birds.

Enhanced precipitation after a challenging infection was found only with groups 1 and 2 (immunized with *E. acervulina* and *E. tenella* respectively), the response occurring more quickly with group 1 (4 days) than with group 2 (12 days). One bird in group 2 responded after the same interval to a second challenge.

The failure of animals once solidly resistant to coccidial infections to respond to challenging infections with enhanced antibody production has already been noted in rabbits (Rose, 1959 and 1961) and in fowls (Pierce *et al.*, 1962). In the two groups of birds in the present experiment, those immunized with *E. acervulina* and *E. tenella*, in which increased precipitation was found after the first challenges, the resistance conferred by the immunizing infection may not have been as complete as that in the other groups.

Where resistance is complete, in the case of *E. tenella* infections the organisms used as challenge cannot be detected in the caecal wall 24 hours after the infection, although similar organisms given to susceptible birds are well established and numerous (Pierce *et al.*, 1962). The failure of an infection to establish itself and hence the lack of antigenic stimulus may account for the absence of a secondary antibody response in the resistant birds. With one exception, complete resistance to infection with one species did not prevent infection with any of the other species used in this experiment. The absence of any cross-immunity confirms earlier observations (Tyzzer *et al.*, 1932; Johnson, 1938). When the birds were cross-infected, they were 4.5 months old and had not developed any resistance which could be attributed to age. That the birds were susceptible to the cross-infection indicated that they had not experienced stray infection of any magnitude although very small numbers of oocysts of species other than those administered to the birds were found in faeces from groups 2, 3 and 4 on one or two occasions. This comparative freedom from extraneous infection can probably be attributed to the precautions taken and to the resting of all equipment before use.

The results obtained from the serological testing of the cross-infected birds in groups 1 and 4 confirm that the antigens involved are not very closely related. In general, the fowls reacted with antigens prepared from the cross-infecting species and not with those prepared from the immunizing species. The very different results obtained with sera of the birds in groups 2 and 3 can probably be explained by the close similarity of the antigens involved. Group 2 birds responded to an antigen prepared from *E. tenella* when cross-infected with *E. necatrix*, but failed to do so when challenged with *E. tenella*. The *E. necatrix* infection was well established in these birds.

These two species have some antigens in common, as shown by cross-reactions using anti-sera to both, in Ouchterlony plates (Rose, unpublished). Group 3 birds responded very poorly, if at all, to cross-infection with *E. tenella* and again those sera which did precipitate did so with both immunizing and cross-infecting antigens. When previously challenged with the immunizing species they had not responded at all. The poorer serological response of group 3, when compared with group 2, may be due to the lesser severity of the cross-infection. It is possible that *E. tenella* produces a less severe cross-infection in *E. necatrix* — immune fowls than vice versa because, in the former case, the caeca, having already experienced a considerable infection with *E. necatrix* gametocytes, are better equipped to resist a further parasitic invasion. If one species truly impeded a cross-infection with the other, this should also be observed when the immunizing and cross-infecting species are reversed as in group 2 fowls.

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