Immunology, 1968, 15, 429.

Immunological Responses of the Toad, *Xenopus laevis*, to the Antigens of the Ciliate, *Tetrahymena pyriformis*

J. J. Lykakis* and F. E. G. Cox

Department of Zoology, University of London King's College, London, W.C.2

(*Received 27th February* 1968)

Summary. Toads, *Xenopus laevis*, were immunized with the ciliate *Tetrahymena pyriformis* and their sera examined for specific anti-*Tetrahymena* precipitating, immobilizing, agglutinating and lytic antibodies 65–75 days later. Precipitating antibodies were detected in whole antiserum by ring and Ouchterlony tests against soluble antigens of *Tetrahymena*. Immobilizing, agglutinating and lytic antibodies against living ciliates were also detected in whole serum. Antibody activity was localized in the serum fraction corresponding with mammalian γ-globulin after starch block electrophoresis and this was confirmed by immunoelectrophoresis. After fractionation on Sephadex G-200 precipitating antibodies were found to correspond with γG immunoglobulins and non-precipitating antibodies with both γM and γG. These results are comparable with the situation which exists in rabbits immunized against *Tetrahymena pyriformis*.

INTRODUCTION

The history of our knowledge of the immunological responses of poikilotherm vertebrates can be divided into three periods. First came the period during which it was not known that poikilotherms were capable of producing an immune response. Then followed a long period during which it was believed that the immunological responses of poikilotherms differed qualitatively from those of homoiotherms in that the latter responded to an antigen by producing antibodies which were first localized in the γM fraction of the serum and later in the γG whereas poikilotherms were capable of a γM response only. More recently it has been realized that the differences between the immunological responses of poikilotherms and homoiotherms are largely quantitative and that poikilotherms can and do produce γG antibodies but that they do so more slowly than homoiotherms. In poikilotherms, also, the production of antibody is in many cases dependent on temperature and γG antibodies are produced either after a long period or at a relatively high temperature (Evans, 1963).

Various poikilotherms have been used in immunological studies and subjected to a variety of antigens (see for example the papers in the symposium edited by Smith, Miescher and Good, 1965). Some of these antigens have been comparatively simple ones eliciting a single response while others have been bacterial and viral. Nothing is known, however, of the way in which poikilotherms respond to protozoan antigens and the present study was undertaken in order to discover how a poikilotherm, in this case a toad, responds to a protozoan, the ciliate *Tetrahymena pyriformis*. This particular combination,

* Present address: Department of Zoology, University of Athens, Athens, Greece.

Xenopus and *Tetrahymena*, was chosen because the immunological responses of these toads to relatively simple antigens have been thoroughly investigated (Elek, Rees and Gowing, 1962; Lykakis, unpublished observations) and the ways in which mammals respond to *Tetrahymena* are fully documented (Robertson, 1939a, b; Harrison, 1955; Sinclair, 1958; Watson, Alexander and Silvester, 1964) and, at the time these experiments were planned, were being investigated by colleagues in the School of Biological Sciences at King's College. The observations made by Lykakis provided the basic information that the immunological responses of *Xenopus* could be evaluated using agar diffusion techniques, starch gel and starch block electrophoresis, immunoelectrophoresis and separation on Sephadex. The observations on the immunological responses of rabbits to *Tetrahymena* provided the information that such responses could be detected using precipitation, lytic, agglutination and immobilization tests. The implications of the results obtained in relation to the defence mechanisms of toads to protozoan pathogens will be discussed later.

MATERIALS AND METHODS

1. *Animals*

Toads, *Xenopus laevis*, were bought from commercial dealers and kept in the Zoology Department aquarium in order to ensure that they were free from disease. The toads were kept in plastic aquarium tanks and fed twice weekly on chopped fresh meat. The temperature of the water fluctuated between 17° and 23° during the course of the experiments.

2. *Preparation of antigens*

The ciliates, *Tetrahymena pyriformis* strain S, were cultured axenically in 2 per cent proteose peptone with forced aeration according to the method described by Watson *et al.* (1964) and Alexander (1968). The ciliates were concentrated by low speed centrifugation, resuspended and washed three times in saline. They were finally resuspended in distilled water and disrupted by a freezing–thawing cycle. The disrupted ciliates combined with equal quantities of Freund's complete adjuvant were used for immunization. The clear antigen required in the gel diffusion technique consisted of the soluble antigens of ciliates prepared in the manner outlined above but with the particulate material removed by centrifugation.

3. *Preparation of antisera*

Toads were immunized with 0·25 ml *Tetrahymena* antigen plus adjuvant subcutaneously into the hind legs. Four injections were given, three at 2-week intervals and a fourth 1 week after the third. Some of the animals were bled 30 days after the final injection and the remainder 10 days after that. Toads were anaesthetized in 4 per cent urethane in water and bled from the heart into centrifuge tubes. The blood was allowed to clot for 2 hours at room temperature and the serum expelled at 4° overnight prior to centrifugation.

4. *Fractionation of antisera*

The antisera were fractionated by starch block electrophoresis according to the method described by Campbell, Garvey, Cremer and Sussdorf (1963). Ten millilitres of serum were applied to the block and subjected to 9–10 V/cm for 20–24 hours in 0·2 M borate buffer, pH 8·6, at 4°. After electrophoresis the block was cut into 1-cm segments which were eluted with 5 ml borate buffer and the extinction coefficient measured with a Hilger

Uvispeck spectrophotometer at 280 mμ (see Fig. 2). Three peaks were cathodal and the fractions in each of these peaks were pooled, concentrated by dialysis against Carbowax 20M (G. T. Gurr) and tested for antibody activity to soluble antigens of *Tetrahymena* by ring tests. Sera were also fractionated on a Sephadex G-200 column according to the method described by Flodin and Killander (1962). The column measured 90 × 2·5 cm. Fractions of 4–5 ml were collected with the aid of a fraction collector and the extinction coefficient measured at 280 mμ (see Fig. 3). Three peaks were apparent and the fractions in each of these were pooled, concentrated to approximately equal volumes against Carbowax 20M and tested for antibody activity. All fractions, separated on Sephadex, showing antibody activity were further tested for precipitation in agar gel and for the immobilization, agglutination and lysis of *Tetrahymena*.

5. *Double diffusion in agar*

Double diffusion in agar was carried out in Petri dishes according to the method described by Ouchterlony (1958). The agar plates were prepared from 0·85 per cent Ionagar (Oxoid) in 0·05 M veronal buffer at pH 8·2 with 1:10,000 merthiolate added as a preservative.

6. *Immunoelectrophoresis*

Immunoelectrophoresis was carried out according to the method described by Scheidegger (1955) on a microscope slide coated with 0·85 per cent Ionagar in 0·025 M veronal buffer at pH 8·2. The electrophoresis itself was carried out in a Kohn electrophoresis tank (Shandon) at 250–300 V for 45–60 minutes with 0·05 M veronal buffer at pH 8·2 in the tank. Sera from toads immunized with *Tetrahymena* were reacted against rabbit anti-*Xenopus* sera.

7. *Staining of diffusion and immunoelectrophoresis plates*

Plates were thoroughly washed in saline to remove any non-precipitated proteins, dried and stained in acid fuchsin.

8. *Agglutination, immobilization and lytic tests*

These tests are usually classified together as stages in the immobilization test. Serial dilutions of immune sera in saline were set up in agglutination racks so that each well contained 0·5 ml. Ciliates were added to each well, mixed with the serum and left at room temperature for 2 hours. Normal sera and saline were used as controls. The ciliates were examined microscopically for signs of agglutination, immobilization and lysis and the end-point was taken to be the dilution before the one at which the majority of ciliates were undamaged and moving freely.

9. *Procedure*

Nine toads were immunized with *Tetrahymena pyriformis*; three of these were bled 30 days after the last immunizing injection and six 10 days later. Samples of pooled serum were tested for precipitating antibodies against soluble *Tetrahymena* antigens and for agglutinating, immobilizing and lytic antibodies against living ciliates. The pooled serum was fractionated by starch block electrophoresis and on Sephadex G-200 columns and the fractions tested for antibody activity. The serum proteins in whole and fractionated sera were characterized by immunoelectrophoretic methods.

RESULTS

IMMUNOLOGICAL PROPERTIES OF WHOLE ANTISERA

Sera from all the toads immunized with *T. pyriformis* gave positive reactions when tested against the soluble antigens of the ciliates by means of the ring test. Normal toad

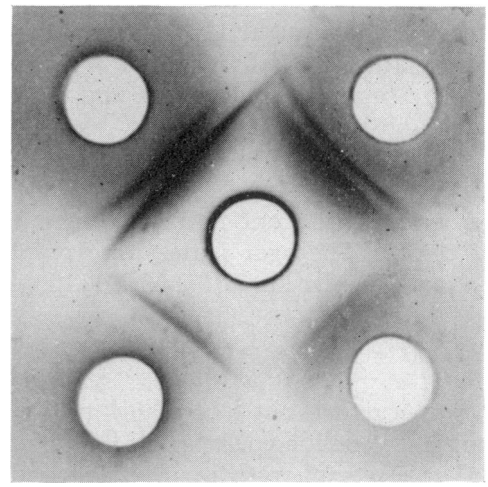

FIG. 1. Ouchterlony plate showing the reactions between *Xenopus* anti-*Tetrahymena* sera and the soluble antigens of *Tetrahymena*. The central well contained the soluble antigens and the four peripheral wells individual *Xenopus* antisera. The variations in the immune reaction are obvious. Similar results were obtained using rabbit anti-*Tetrahymena* sera.

TABLE 1

THE EFFECT OF *Xenopus* ANTI-*Tetrahymena* SERUM ON LIVING *Tetrahymena pyriformis*

Reciprocal of serum dilution	
1	Ciliates agglutinated and immobilized
2	Ciliates agglutinated and immobilized
4	Ciliates agglutinated and immobilized
8	Ciliates lysed
16	Ciliates lysed
32	Ciliates lysed
64	Ciliates lysed
128	Ciliates lysed
256	Ciliates lysed
512	Most ciliates agglutinated and immobilized
1024	No agglutination, a few ciliates immobilized
Normal serum	Ciliates all moving freely
Saline	Ciliates all moving freely

sera produced no such reactions. When these immune sera were tested against the soluble antigens of *Tetrahymena* by the Ouchterlony double diffusion test in agar gels all gave positive precipitin reactions with some variation between individuals. The strength of the reaction varied as did the number of precipitin lines. In all cases two strong lines were

evident and in most cases there were four or five. The reactions of four individual animals are shown in Fig. 1. No precipitin lines were formed with non-immune sera.

When the immune sera were pooled and tested for the presence of immobilizing, agglutinating and lytic antibodies the results shown in Table 1 were obtained. These results showed that normal toad serum had no effect on their motility. At high concentrations of antisera (1:1, 1:2, 1:4) all the ciliates became non-motile and agglutinated. At concentrations of antisera between 1:8 and 1:256 the ciliates lysed and at 1:512 the

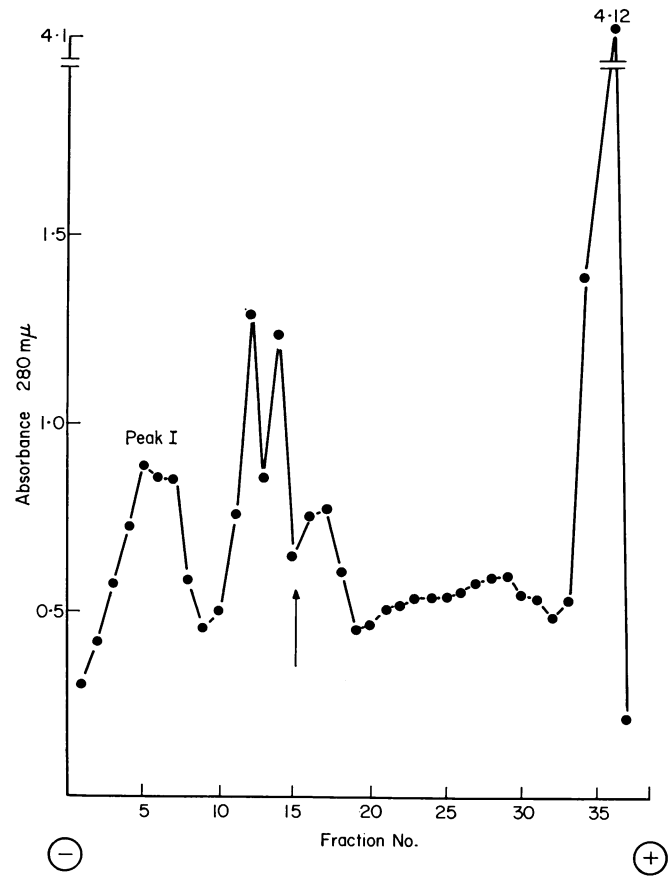

FIG. 2. Graph showing the absorbance at 280 mμ of fractions of *Xenopus* anti-*Tetrahymena* serum eluted from a starch block after electrophoresis. The arrow indicates the point of application of the serum. Antibody activity was detected in the peak which moved furthest towards the cathode (Peak I).

action of the serum became less marked, some of the cilates continued to move freely and none were lysed. At 1:1024 the antiserum had practically no effect.

IMMUNOLOGICAL PROPERTIES OF SERUM FRACTIONS

The results obtained from the fractionation of pooled samples of immune sera by starch block electrophoresis are shown in Fig. 2. This graph represents the absorbancy of each fraction eluted from the starch block read at 280 mμ. A number of peaks representing distinct serum fractions are evident. Antibody activity against the soluble antigens of

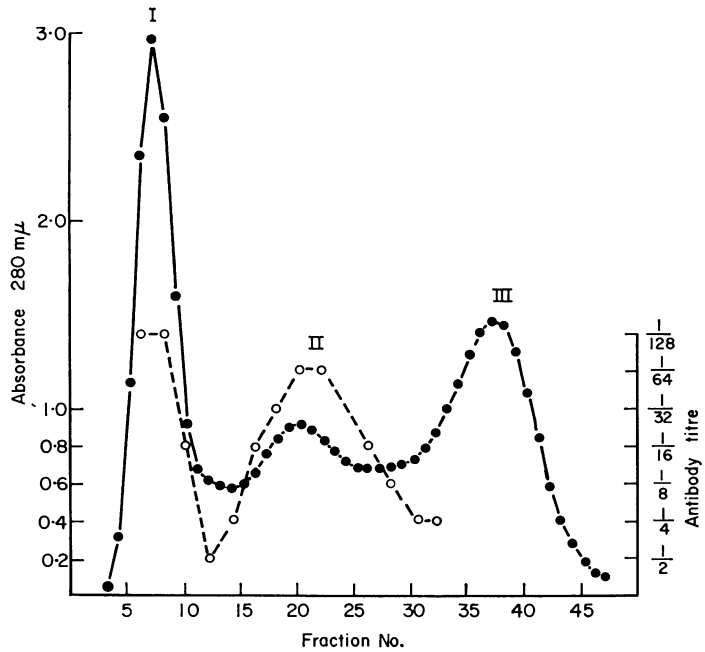

FIG. 3. Graph showing the absorbance (●) at 280 mμ of fractions of *Xenopus* anti-*Tetrahymena* serum separated on Sephadex G-200 with respect to antibody activity (○). Peak I corresponds with mammalian γM immunoglobulin, peak II with γG immunoglobulin and peak III with serum albumin. Agglutinating, immobilizing and lytic antibodies were detected in peaks I and II. Precipitating antibodies were detected in peak II only.

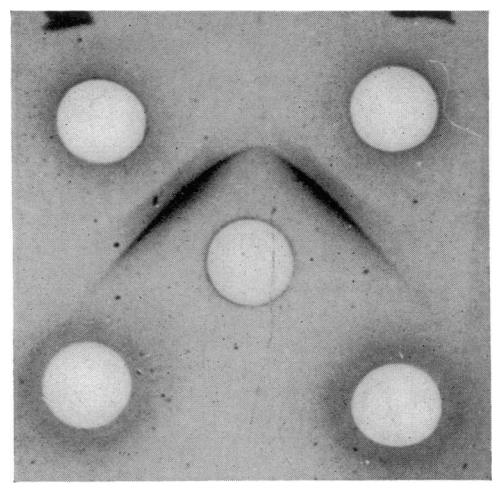

FIG. 4. Ouchterlony plate showing the reaction between fractions of *Xenopus* anti-*Tetrahymena* sera and the soluble antigens of *Tetrahymena*. The central well contained the soluble antigens. The two top wells contained the pooled fractions from the second peak after separation on Sephadex G-200 and the bottom wells the fractions from the first peak.

Tetrahymena, as determined by the ring test, occurred only in the first peak—that is the one which moved furthest towards the cathode. The fractions in this peak correspond with mammalian γ-globulin.

Pooled immune sera were fractionated on Sephadex G-200 columns and three peaks were obtained when the fractions were examined spectrophotometrically. The results are shown in Fig. 3. Precipitating antibodies were found only in the second peak when tested against soluble antigens and these results are shown in Fig. 4. Agglutinating, immobilizing and lytic antibodies were distributed in peaks I and II as shown in Fig. 3. In the first peak the titre was 1:128 and in the second peak it was 1:64. No antibody activity of any kind occurred in the third peak.

The analysis of whole immune sera and various fractions of sera by immunoelectrophoretic methods showed that antibody activity was confined to those parts of the serum corresponding with the mammalian γ-globulins.

DISCUSSION

These experiments have shown that the toad *Xenopus laevis* can recognize the antigens of the ciliate *Tetrahymena pyriformis* and can respond to these antigens by producing specific antibodies against them. These results suggest that all amphibians might well be expected to be able to respond to protozoan antigens. The antibodies produced can be classified under two headings, precipitins against soluble antigens and antibodies against the whole ciliates. Three types of activity were observed within the latter category, agglutination, immobilization and lysis. Whether or not these are manifestations of the same antibody response is uncertain, and this is discussed later.

After starch block electrophoresis the antibody activity was localized in the slow moving serum fractions and these correspond with mammalian γ-globulins; the serum proteins which contain the immunoglobulins. Starch block electrophoresis does not separate the main classes of immunoglobulins but this can be done on Sephadex G-200 columns. Three main peaks can be obtained from mammalian serum: the first contains the γM immunoglobulins and the second the γG. When *Xenopus* serum immune to *Tetrahymena* was fractionated, precipitins were found in the γG fractions only while the immobilizing, agglutinating and lytic antibodies were distributed between the γG and the γM fractions. These results cannot be compared directly with those recorded for mammals. Robertson (1939a, b), Harrison (1955), Watson *et al.* (1964) and Alexander (1968) have immunized rabbits with whole or fractionated *Tetrahymena* and obtained precipitating antibodies and these authors and Sinclair (1958) have also obtained immobilizing antibodies in the same way. In rabbits, however, the aim has been to obtain as intense a reaction as possible and most of the antibody activity has presumably been in the γG immunoglobulin fraction.

Alexander (personal communication) using an early anti-cilia (contaminated with mucocysts) serum detected immobilization at dilutions of 1:512 compared with 1:128 in the present study. Too much significance cannot be placed on these figures but they do show that the immobilizing system operates at the same order of magnitude in toads as it does in rabbits. In the case of the precipitins it seems likely that in mammals and amphibia the antibody activity in hyperimmune animals exists in the γG fraction of the serum. In this study, two to four precipitin lines could be identified in Ouchterlony plates. Robertson (1939a, b) and Watson *et al.* (1964) found more precipitin lines although their antigen consisted of isolated cilia and not whole ciliates. Obviously only a few of the

strongest antigens can be identified in this way and there is no reason to suspect that mammals and toads are qualitatively different with respect to the production of precipitating antibodies.

The immobilizing, agglutinating and lytic antibodies may be different. Robertson (1939a) and Sinclair (1958) have presented evidence to suggest that complement may play an important role in the immobilization and allied reactions. Little is known of complement in *Xenopus* although Gewurz, Finstad, Muschel and Good (1965) suggest that complement systems exist in all animals above the lamprey. Complement may play a role in the immobilization of *Tetrahymena* by the sera of immune toads and if it does it would be expected in peaks I and II on Sephadex filtration. Complement, however, has no effect by itself and negative results were obtained with non-immune but presumably complement-containing sera. A reasonable hypothesis is that anti-*Tetrahymena* antibodies exist in the γM and γG serum fractions and their activity may or may not be mediated by complement. It is impossible to say whether a single antibody has different effects on living *Tetrahymena* or whether agglutination, immobilization and lysis are brought about by different antibodies. Alexander (1968) has recently shown that the immobilization antigen of *Tetrahymena* is probably the mucocyst and it would seem unlikely that any antibody directed against this could also be lytic. In *Xenopus* the situation is still a long way from clear and the results obtained during the present investigation do not contribute to an understanding of this particular problem.

Precipitating antibodies against the soluble antigens of *Tetrahymena* were found in the γG immunoglobulin fraction only, whereas the agglutinating, immobilizing and lytic antibodies occurred in both the γG and the γM fractions. Lykakis (unpublished observations) has found that when toads are immunized with soluble antigens, bovine serum albumin and bovine γ-globulin, γM immunoglobulins are replaced by γG immunoglobulins by the 65th day. The present experiments indicate that this also happens in the case of antibodies against the soluble antigens of *Tetrahymena*. With particulate antigens, in this case living ciliates, γM antibodies can not only be detected between the 65th and 75th days after immunization but the titre is higher than for γG antibodies. This might mean that only small amounts of γM antibody are present, and cannot be detected by precipitation techniques, but that the antibodies are still effective against surface antigens. Robbins, Altemeier and Smith (1965) have demonstrated that in rabbits γM antibodies are more efficient than γG in the agglutination and opsonization of bacteria and the situation may be similar in the case of toads immunized against *Tetrahymena*.

The experiments described in this paper were originally performed in order to obtain information on how a poikilotherm recognizes and reacts to a protozoan antigen. Obviously an ideal laboratory situation would be one in which a poikilotherm, in which the immunological responses had been characterized, was infected with and overcame a protozoan disease. This model does not exist as yet and the alternative model, outlined above, was used. In homoiotherms, little is known about protective immunity to protozoan infections and how the various immunoglobulins act and interact. It was felt that perhaps the reason for this lack of knowledge was that everything happens so quickly in homoiotherms that the relative roles of γM and γG immunoglobulins become obscured. By using a poikilotherm this problem is avoided. The results of these experiments have shown that *Xenopus* can recognize and respond to *Tetrahymena* antigens and that the antibodies produced could be protective. These agglutinating, immobilizing and lytic antibodies occurred in both the γM and γG immunoglobulin fractions. Precipitating antibodies

occurred in the γG fraction only. This means that precipitating and (potentially) protective antibodies are probably not the same and the presence of precipitins does not necessarily indicate protection. This has also been shown to be true in a number of protozoan infections in homoiotherms (Cox, 1968). These experiments have shown that the immunological responses of rabbits and toads to *Tetrahymena* are similar. That these responses occur slowly in the toads has been demonstrated by Lykakis (unpublished observations). It is, therefore, probably a valid assumption that the investigation of immune responses to protozoa in poikilotherms will contribute to our overall understanding of the protective immune response in general.

ACKNOWLEDGMENTS

We wish to thank J. B. Alexander of the University of Salford who provided us with the ciliates used in this investigation and allowed us to draw from information in his unpublished manuscripts. We also wish to thank the Greek Science Foundation which provided the money for one of us (J.J.L.) to work in London.

REFERENCES

ALEXANDER, J. B. (1968). 'The mucocysts of *Tetrahymena pyriformis*: localization of the TCA-ethanol-soluble protein.' *Exp. Cell Res.*, **49**, 425.

CAMPBELL, D. H., GARVEY, J. S., CREMER, N. E. and SUSSDORF, D. H. (1963). *Methods in Immunology*. Benjamin, New York.

COX, F. E. G. (1968). 'Immunity to tissue parasitic protozoa.' *Immunity to Parasites* (Ed. by A. E. R. Taylor), p. 5. Blackwell Scientific Publications, Oxford.

ELEK, S. D., REES, T. A. and GOWING, N. F. C. (1962). 'Studies on the immune response in a poikilothermic species (*Xenopus laevis* Daudin).' *Comp. Biochem. Physiol.*, **7**, 255.

EVANS, E. E. (1963). 'Antibody response in Amphibia and Reptilia.' *Fed. Proc.*, **22**, 1132.

FLODIN, P. and KILLANDER, J. (1962). 'Fractionation of human-serum proteins by gel filtration.' *Biochim. biophys. Acta (Amst.)*, **63**, 403.

GERWUZ, H., FINSTAD, J., MUSCHEL, L. H. and GOOD, R. A. (1965). 'Phylogenetic inquiry into the origins of the complement system.' *Phylogeny of Immunity* (Ed. by R. T. Smith, P. A. Miescher and R. A. Good), p. 105. University Press, Florida.

HARRISON, J. A. (1955). 'General aspects of immunological reactions with bacteria and protozoa.' *Biological Specificity and Growth* (Ed. by E. G. Butler), p. 141. University Press, Princeton.

OUCHTERLONY, O. (1958). 'Diffusion-in-gel method for immunological analysis.' *Progr. Allergy*, **5**, 1.

ROBBINS, J. B., ALTEMEIER, W. A. and SMITH, R. T. (1965). 'The biological activity of immunoglobulins —an approach to the phylogenetic study of immunity.' *Phylogeny of Immunity* (Ed. by R. T. Smith, P. A. Miescher and R. A. Good). p. 76. University Press, Florida.

ROBERTSON, M. (1939a). 'A study of the reactions *in vitro* of certain ciliates belonging to the *Glaucoma–Colpidium* group to antibodies in the sera of rabbits immunised therewith.' *J. Path. Bact.*, **48**, 305.

ROBERTSON, M. (1939b). 'An analysis of some of the antigenic properties of certain ciliates belonging to the *Glaucoma–Colpidium* group as shown in their response to immune serum.' *J. Path. Bact.*, **48**, 323.

SCHEIDEGGER, J. J. (1955). 'Une micro-méthode de l'immuno-électrophorèse.' *Int. Arch. Allergy*, **8**, 103.

SINCLAIR, I. J. B. (1958). 'The role of complement in the immune reactions of *Paramecium aurelia* and *Tetrahymena pyriformis*.' *Immunology*, **1**, 291.

SMITH, R. T., MIESCHER, P. A. and GOOD, R. A. (1965). *Phylogeny of Immunity*. University Press, Florida.

WATSON, M. R., ALEXANDER, J. B. and SILVESTER. N. R. (1964). 'The cilia of *Tetrahymena pyriformis*, Fractionation of isolated cilia.' *Exp. Cell Res.*, **33**, 112.