Genet. Res., Camb. (1964), **5**, *pp.* 127–136
With 1 *text-figure and* 1 *plate*
Printed in Great Britain

Immunogenetic analysis of proteins of *Paramecium*

V. DETECTION OF SPECIFIC DETERMINANTS IN STRAINS LACKING A SURFACE ANTIGEN*

BY IRVING FINGER AND CAROL HELLER
Biology Department, Haverford College, Haverford, Pennsylvania, U.S.A.

(*Received* 1 *October* 1963)

1. INTRODUCTION

The immunologically diverse proteins that comprise the immobilization antigens and form the basis for the classification of *Paramecium* into discrete serotypes are remarkably similar in molecular weights and other physical characteristics (Preer, 1959; Bishop & Beale, 1960). So much so that the same general purification procedures can be used for all serotypes of syngens 4 and 2 with only the ammonium sulfate concentration being varied for final precipitation (Preer, 1959; Finger & Heller, 1962). The ease of isolation has rekindled interest in the control of two aspects of the physiology of the antigens: (1) the ready and reversible transformation of one serotype into another, and (2) the presence of one ciliary antigen precluding the simultaneous existence of a secondary antigen on the surface. Possibly related to this latter phenomenon is the failure of a number of strains ever to manifest certain immobilization antigens.

Generally every clone is capable of expressing any of a spectrum of immobilization antigens. However, some stocks appear to be deficient in their potentialities. A serotype missing from several stocks of syngen 2 of *Paramecium aurelia* is that of E (at one time called 29). As has been previously shown with type F in syngen 1, (Sonneborn, 1948), a single gene difference appears to be responsible, the ability to manufacture the antigen being dominant (Finger, 1957). With the advances in knowledge of the properties of the antigens, questions about deficient strain can now be asked in more precise terms. For example, is the entire protein missing, or are all the components of the protein made, the genetic defect having to do with either quaternary structure or with the placement on the cell surface? Or are there modifying genes that do not permit the antigen to be expressed?

Although the specific example of the E serotype proved to be complicated by the coincident presence in all stocks examined of a locus determining a cross-reacting antigen (G), the experiments to be described show that the E-deficient strains

* These studies were supported by grants from the National Science Foundation and the National Institutes of Health.

indeed do possess a gene for making an E cross-reacting determinant, perhaps as a hapten. Hybrids between 'deficient' and 'normal' cells make complete antigen which has a specificity distinguishing it from the antigen of the 'normal' parent. The possible relationship between these findings and the genetic control of quaternary structure as well as to the mutual exclusion of immobilization antigens is discussed.

2. MATERIALS AND METHODS

(i) *Culture*

Stocks 72, 197, 30, 83 and 7 of syngen 2 were grown in 0·15% Cerophyl infusion (Finger, 1957) at temperatures ranging from 17° C. to 31° C., the lower temperature being used prior to and immediately after conjugation. Only stocks 72 and 197 had ever expressed the E serotype. Generally mass cultures were maintained at room temperature, 27° C., or occasionally 31° C.

(ii) *Genetic methods*

The techniques used for inducing conjugation and handling pairs and exconjugants were those described by Sonneborn (1950).

(iii) *Purification of antigens*

Preer's (1959) procedure of ammonium sulfate purification of the antigens of syngen 4 was followed as slightly modified for syngen 2 serotypes (Finger & Heller, 1962).

(iv) *Immunological methods*

(*a*) In vivo

Samples of cultures were placed for 1 to 2 hours in appropriate dilutions of sera prepared against clones pure for each of several specific serotypes (Sonneborn & LeSuer, 1948). Paramecia completely immobilized by one serum and unaffected by the others were classified as being of the general serotype used in the preparation of this serum. Two sub-types of serotype E, 197E and 72E, were used as sources of sera for these experiments.

(*b*) In vitro—*Gel diffusion methods*

1. *Tubes.* Preer's method (1956) for the detection of antigen-antibody precipitates (serum-agar-antigen serially layered in tubes) was employed. Further characterization and identification of specific E antigens utilized the 'profile' technique (Finger & Heller, 1962). In this method the antigen to be identified is allowed to diffuse against a number of sera from different rabbits immunized with homologous or crossreacting antigen and the position of the band in the agar column formed with each serum is represented by a point. When joined by a line, the points form a profile, and this profile can be compared with one previously obtained with a standard antigen. Identical, single antigens will give superimposable profiles.

2. *Slides.* Ouchterlony (1949) patterns (wells of antigen and antibody separated from each other by agar) were arranged on microscope slides according to the modification of Hartmann & Toilliez (1957) using a brass template. Wells were 3·5 mm. in diameter, with the nearest borders 2·4 mm. apart.

(*c*) *Sera*

The volumes and concentrations of antigen used as well as the course of injection, etc., were those previously described (Finger & Heller, 1962). Absorptions were also carried out according to the protocols given in this paper.

3. RESULTS

(i) *Gel diffusion profiles of F_1s*

Initially, the profile method (see Materials and Methods) previously used to detect antigenic distinctions between closely related proteins was applied to the antigens isolated from hybrids of stocks 197 or 72 with stocks 7, 30 or 83. Profiles of purified antigen extracted from cultures of F_1s immobilized by either 197 or 72E antiserum were compared with profiles of purified 197E and 72E antigen. In theory, if the 'non-E' stocks (7, 30, 83) contributed nothing in the way of E antigenic determinants to form a hybrid E molecule, the gel diffusion pattern formed by the band positions in tubes should be indistinguishable from the pattern found with the parental E. Moreover, all hybrid clones should present a homogeneous collection of identical patterns.

When compared with individual parental E profiles, differences between heterozygotes and an E control were readily apparent and, furthermore, the heterozygous profiles were not uniform. However, while the heterogeneity of profiles was genuine, its value as a criterion for diverse antigens proved problematical; even repeated stock 197E profiles were not uniform, with considerable variation often appearing from culture to culture. Simulation of some of these profiles could be accomplished by adding cross-reacting immobilization antigen, G, to the purified E preparations. The profiles of the E antigen were then altered, and a different profile could be plotted for each amount of G added (Fig. 1). With larger amounts of G two bands would form with some sera, indicated by the dotted lines extending to two points. Apparently one, and perhaps the major, cause for the different profiles observed among heterozygotes and E parents is the fluctuation in amounts of G antigen present in E animals.

It was the existence of this secondary, cross-reacting antigen in paramecia classified as E on the basis of *in vivo* tests that also prevented the use of standard immunological techniques for the detection of E-like determinants in non-E strains. All of these strains were capable of producing G antigens and thus such methods as hapten inhibition in liquids and gels, absorptions, etc., were of limited value.

(ii) *F_1 antisera*

To overcome the difficulties raised with the G–E system, the hybrids of E × non-E strains were injected into rabbits in the hope of eliciting antibodies specific for the

hybrid. Analysis of the products formed by heterozygotes from two stocks, each of which made a distinguishable form of an allelic antigen, had previously suggested that individual hybrid molecules may combine antigenic determinants contributed by both parents (Finger & Heller, 1963). Should the same process of assortment of 'subunits' occur in E × non-E heterozygotes, it was reasoned that perhaps the hybrid

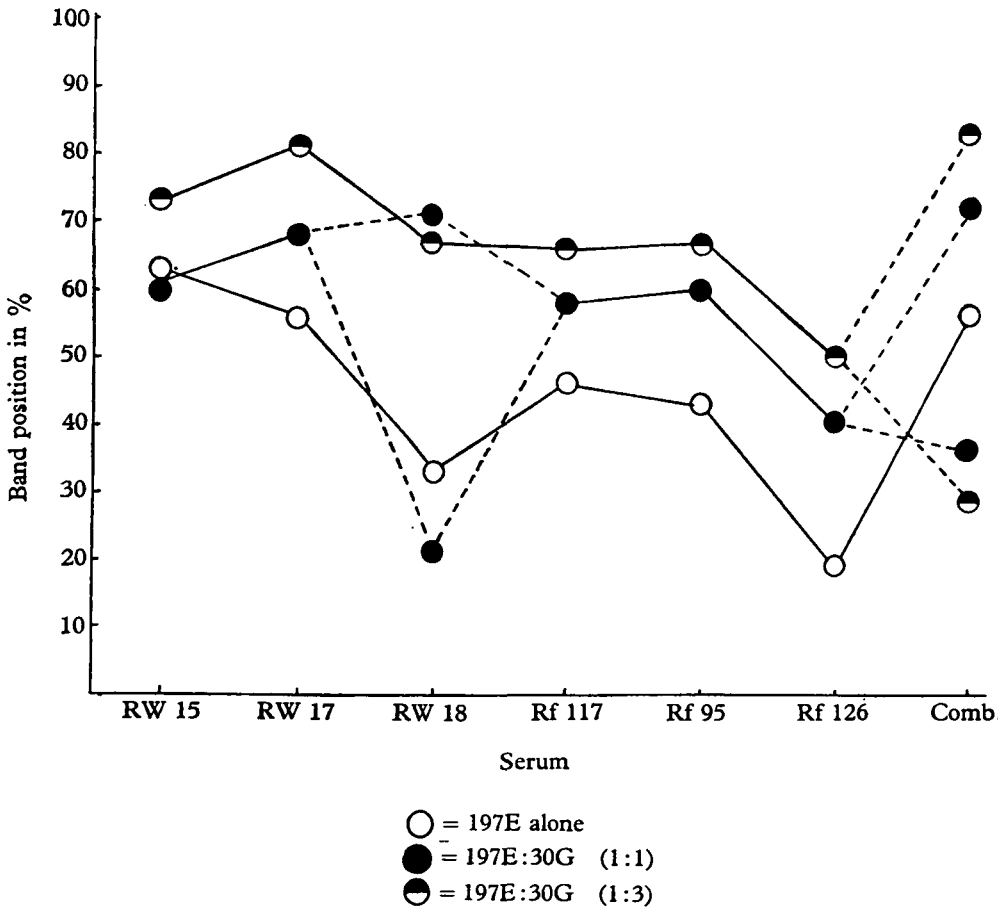

Fig. 1. Band positions in Preer tubes of 197E and mixtures of 197E and 30G. Six tubes, each having a different G or E antiserum, were used in the plotting of the separate profiles. Band positions were measured and converted to per cent traveled down the length of the agar column, with 100 representing the bottom agar-serum miniscus and O the top agar-antigen miniscus.

cells would incorporate any E-like determinants encoded by the non-E parent and thus yield an antigen with a new specificity.

Eight rabbits were injected with homogenates of heterozygotes of either 197 or 72 crossed with stocks 30 or 83. Each rabbit was immunized with the progeny of a single exconjugant. Of these, two produced sera of such low E titer that the sera were not examined further. Because by the time bleedings were made no paramecia

https://doi.org/10.1017/S0016672300001075 Published online by Cambridge University Press

of the original cultures remained to be tested as heterozygotes, immobilization titers against homologous paramecia could not be contrasted with the E parent titer.

Aliquots of the antigen purified from the original F_1 clones used as immunogens were on hand, however, and the F_1 antisera were tested by gel diffusion methods to determine whether antibodies were present capable of reacting only with F_1 antigen. Two techniques were used. One was the tube method of Preer in which two antigens are mixed. If the antigens are cross-reacting and heterologous antigen is in excess, two bands will form when the serum used to develop bands has antibodies which can distinguish the antigens. In the other method, the two antigens to be distinguished are placed in wells forming two corners of a triangle in a gel layer on a slide, with antisera in the third corner. Serum with antibodies, some of which are capable of reacting with one of the antigens, will precipitate bands with both antigens, with the one nearest the homologous antigen well forming an extension or spur at the point of fusion (Finger & Heller, 1960).

None of these F_1 antisera, all of which had high E titers, reacted any differently than did sera prepared against the antigen from the E stock.

(iii) *Gel diffusion patterns on slides*

Up to this point there was no evidence at all that heterozygotes were in any way different from the E parent. However, when the gel diffusion experiments were repeated using antigen from additional F_1 clones and serum prepared against the E parent alone, spurs appeared. Although few in number, 4 clones of 28 tested, some F_1 antigens (as well as antigens from backcross and F_2 clones) produced spurs when compared with antigen isolated from the E stock parent (Table 1). Significantly, from the point of view of determining whether these may not have been artifacts of precipitation in agar, all spurs were extensions of the E parent antigen-antibody

Table 1. *Gel diffusion reactions of antigens from hybrids of E × E-deficient stocks*

Neighbouring wells		No. of tests	Anti-serum	Reaction of identity with parent	Spur with E parent
A	B				
F_1	Parent				
197 × 7	197 E	10	197 E	9	1
197 × 30	197 E	1	197 E	0	1
197 × 83	197 E	6	197 E	6	0
72 × 30	72 E	7	72 E	5	2
72 × 83	72 E	4	72 E	4	0
Backcrosses and F_2's					
(197 × 7) × (7)		9	197 E	9	0
(197 × 7) × (197 × 7)		8	197 E	6	2

In the comparisons between F_1's and parents each test represents an individual clone. With the backcrosses and F_2's one clone may have been used in several tests so the number of tests represents a very small number of clones.

band. In other words, the antigen derived from a heterozygote reacted as though missing a determinant present in its E parent.

Adding to this evidence for heterogeneity among heterozygote clones—some identical with and others distinguishable from the E parent—was the occurrence of spurs when heterozygotes were compared with each other (Table 2). F_1's of 72E × 83 and 72E × 30 compared with each other using 72E antiserum also showed spurs. Control patterns in which various 197E (and 72E) preparations were compared with each other, using homologous antisera, always showed reactions of identity.

Table 2. *Gel diffusion reactions among F_1's, backcrosses, and F_2's*

Neighboring wells		Anti-197E		Anti-72E	
F_1	F_1	Reaction of identity	Spur	Reaction of identity	Spur
(197 × 30)	(197 × 83)	2	1	0	0
(197 × 83)	(197 × 83)	2	0	2	0
(197 × 30)	(197 × 30)	0	1	0	0
(72 × 30)	(72 × 30)	—	—	3	0
(72 × 83)	(72 × 83)	5	0	10	1
(72 × 83)	(72 × 30)	0	1	0	4
(197 × 83)	(72 × 30)	—	—	0	2
(197 × 83)	(72 × 83)	—	—	0	3
Backcross (197 × 7) × (7)	Backcross (197 × 7) × (7)	—	—	9	1
Backcross (197 × 7) × (7)	F_2 (197 × 7) × (197 × 7)	—	—	7	1

In these patterns a single clone may have been used in several tests. Therefore the number of clones is fewer than the number of tests. — indicates that no test was run.

Apparently several hybrids differed from each other by failing to possess determinants shared with a heterologous E antigen. For example, 72E heterozygotes formed spurs with each other when tested with 197E antiserum. This indicated that this serum—and, by extrapolation, the immunizing antigen—had at least two kinds of E complementaries: those distinguishing 197E from 72E and those which were shared with several E's. Added to the determinant known to be previously shared by the G antigens (Balbinder & Preer, 1959; Finger, Heller & Green, 1962), the E immobilization antigen therefore has a minimum of three specificities.

(iv) *Serum with 'deficient-E' specificity*

Advantage was taken of this finding that the E molecule is composed of multiple determinants and of the previous observation that the immobilization antigens are composed of subunits which reassort in heterozygotes. A fresh attempt was made to

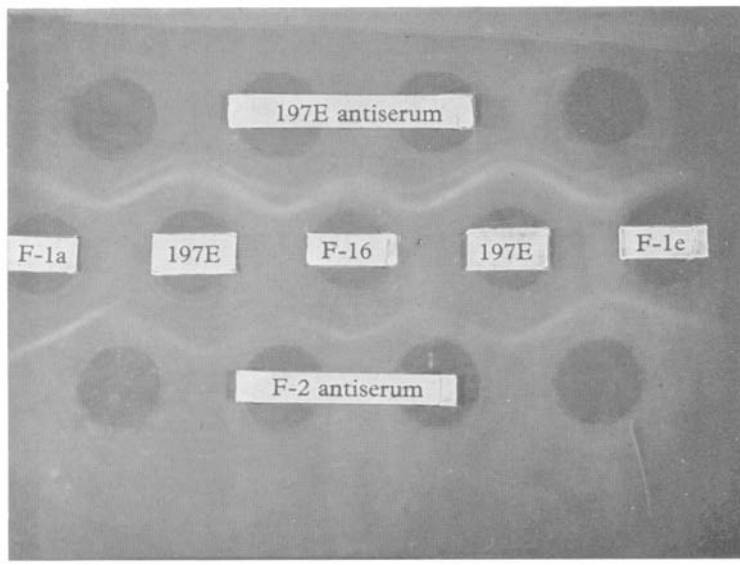

Plate I. Gel diffusion patterns comparing several F_1's with parental antigens using F_2 segregant antiserum and 197E antiserum. The thin second band formed with 197E antiserum is due to an antigen other than E immobilization antigen.

https://doi.org/10.1017/S0016672300001075 Published online by Cambridge University Press

obtain serum specific for the presumed contribution to an E antigen by a deficient stock. This time antigen was injected from a hybrid clone that had 'incomplete' E specificity. An F_2 segregant was selected from the descendants of an original cross of 197E × 7 that possessed the following characteristics:

(*a*) Precipitated with 197 antiserum.

(*b*) Failed to precipitate with the same serum after absorption with 72E antigen.

An antigen reacting in this fashion would be expected were stock 7 to introduce an E determinant shared by 72E and 197E as well as an E determinant specific for stock 7, one that could replace the 197 stock-specific E determinant. Serum made against such an antigen should distinguish 197E from the hybrid E, the spur forming in gel diffusion this time being an extension of the band formed in front of the hybrid antigen well.

Table 3. *Gel diffusion reactions with serum prepared against segregant from 197 × 7 F_2 generation*

Neighboring wells		No. of tests	Reaction of identity	Spur with	Spur with
F_1 (197 × 7)	Parent 197	12	10	F_1 2	197 0
Backcross (197 × 7) × (7)	Backcross (197 × 7) × (7)	10	7	Spur 3	
Backcross (197 × 7) × (7)	F_2 (197 × 7) × (197 × 7)	14	6	8	

In these patterns a single clone may have been used in several tests. Therefore the number of clones involved is fewer than the number of tests.

This is precisely what was observed when a rabbit was immunized with an antigen meeting the requirements listed above (Plate I and Table 3). As would also be expected, this serum readily distinguished various backcross clones from each other and from F_2 clones. At least one E-'deficient' strain, then, can be said to possess a gene determining the specificity of an E-like determinant. The nature of the mutation that prevents this stock from making an E antigen detectable by immobilization tests is still unknown.

4. DISCUSSION

Many mutations which lead to defective proteins have been described. If the defective proteins are not functional and the wild-type protein is essential, the mutation may be lethal. Biochemical analysis may only be practicable if the defect is reparable or can otherwise be substituted for.

No function is known for the immobilization antigens, which are proteins, but several observations suggest that they are vital. A paramecium completely devoid

of these surface antigens has not been found; furthermore, the presence of a large array of substitutable proteins available to a single cell provides a large 'margin of safety' should a 'loss' mutation occur. In one case it has been shown that a selective advantage is conferred on paramecia of a specific serotype (Austin *et al.*, 1956). Also an animal left in homologous antiserum sufficiently strong to immobilize often dies. And, lastly, a cell whose protein-synthesizing systems are rendered non-functional will die if its surface antigen is complexed with dilute specific serum at the same time, as though the animal has been irreparably deprived of a vital substance (Seed, Finger & Heller, unpublished).

In *Paramecium* we may have in the E-deficient stocks a situation analogous to that observed with enzymatic mutants in other forms with the added 'advantage' that deficiency mutants are not inviable. Disadvantages do exist however: the mutants may be difficult to recognize as such due to the complications of spontaneous serotype transformation; and antigenic mutants generally require specific serum for their recognition especially if they involve drastic changes in specificity. The difficulties ensuing from this last requirement are well illustrated in these studies.

With the demonstration that 'absence' stocks possess at lease some genetic information for making a specific antigen, questions arise as to the nature of the deficiency:

Is only a small portion of the antigen made, large enough to recombine (i.e. complement) in a heterozygote but too small to be immunogenic (i.e. a hapten)? Sedimentation studies and other experiments are planned to determine the size of the protein made.

Or are all of the necessary subunits of the antigen synthesized without being put together to form complete antigen? In other words, is the defect due to a mutation at a locus other than the one determining specificity? Appropriate genetic analysis should provide an answer to this question. If the specification of amino acid sequence were all that is needed to determine the complete structure of a protein, than an additional structural locus should not be required.

Are all absence strains deficient from the same cause? Of what significance is the fact that most serotypes are ubiquitous while a few, such as E in syngen 2 and F in syngen 4 appear to be prone to loss?

Is the antigenic component made by these stocks only produced in a hybrid or is it being continuously produced in the stock itself? If the latter is correct, then a repressor mutation perhaps can be ruled out as an explanation for the deficiency. Beale (1957) has reported an instance in syngen 1 a stock that was incapable of forming serotype G until genes from another stock were introduced which allowed a stable G state to be made. If a similar situation is responsible for the E-deficient stocks, further genetic analysis should reveal this.

Certain other results of these studies require additional comment. Rather unexpected was the failure of rabbits injected with extracts of F_1's to make antibody specific for the heterozygotes. Apart from the unlikely possibility that these were unresponsive rabbits for this determinant (unlikely because the E titers of the sera were high), another possible explanation is that only a very small portion of the

molecule is specified by deficient strains and that this may only rarely be incorporated into complete antigen in a heterozygote. At the same time the sera were made, profiles were the major criterion for distinguishing F_1's from the E parent and so the F_1 antigens injected may not have been hybrid antigens. After all true hybrid antigens turned out to be rather rare. Perhaps the F_1 antigens were not hybrid because the matings were necessarily between E and non-E cells. Previous studies have shown that the kind of E expressed in such a cross (where both mates possess 'E' genes) will be primarily that of the E expressing mate (Finger & Heller, unpublished).

The abandonment of the profile technique as a guide for discriminating among E antigens does not invalidate its general usefulness. For this particular situation where cells might possess a number of related antigens simultaneously (e.g. E and secondary G) profiles were of limited utility because differences in amounts of secondary G) profiles were of limited utility because differences in amounts of secondary G alone could alter a profile. In order to use the profile technique with E hybrids, sera would first have to be absorbed with G antigen. Conclusions arrived at in a previous study—that hybrid antigens exist and that immobilization antigens are comprised of subunits—which utilized the E serotype and profiles extensively are, however, still valid since other serotypes and method were used coincidentally. Indeed, the evidence presented in this paper of gel diffusion spur patterns further supports the view that hybrid antigens are made in heterozygotes.

SUMMARY

Several stocks of *Paramecium aurelia* that had never expressed an E serotype were examined for E-reacting material. The regular presence of the cross-reacting antigen G in these stocks ruled out the use of hapten inhibition and other standard immunological techniques. Consequently, a genetic approach was attempted in which hybrids of the E-deficient strains and non-E stocks were bred, and antigen isolated and tested for the existence of molecules with a specificity differing from that of the E-containing parent.

Gel diffusion analysis suggested that E antigen from a hybrid indeed could differ from the 'normal' E. One of these hybrid clones which behaved as though its E antigen may have possessed a determinant contributed by the E-deficient strain was used as an immunizing antigen. The antibodies elicited possessed specificity unique for the hybrid. Thus the possession of E-like determinants by E-deficient stocks was confirmed.

REFERENCES

Austin, M. L., Widmayer, D. & Walker, L. M. (1956). Antigenic transformation as adaptive response of *Paramecium aurelia* to patulin; relation to cell division. *Physiol. Zoöl.* **19**, 261–287.

Balbinder, E. & Peer, J. (1959). Gel diffusion studies on serotype and serotype transformation in *Paramecium*. *J. gen. Microbiol.* **21**, 156–167.

Beale, G. H. (1957). The antigen system of *Paramecium aurelia*. *Int. rev. Cytol.* **6**, 1–23.

BISHOP, J. O. & BEALE, G. H. (1960). Genetical and biochemical studies of the immobilization antigens of *Paramecium aurelia*. *Nature, Lond.*, **186**, 734.

FINGER, I. (1957). The inheritance of the immobilization antigens of *Paramecium aurelia*, variety 2. *J. Genet.* **55**, 361–374.

FINGER, I. & HELLER. C. (1960). Gel diffusion analysis of cross-reactions of a protein-hapten conjugate. *J. Immunol.* **85**, 332–336.

FINGER, I. & HELLER, C. (1962). Immunogenetic analysis of proteins of *Paramecium*. I. Comparison of specificities controlled by alleles and by different loci. *Genetics*, **47**, 223–239.

FINGER, I. & HELLER, C. (1963). Immunogenetic analysis of proteins of *Paramecium*. IV. Evidence for the presence of hybrid antigens in heterozygotes. *J. mol. Biol.* **6**, 190–202.

FINGER, I., HELLER, C. & GREEN, A. (1962). Immunogenetic analysis of proteins of *Paramecium*. II. Coexistence of two immobilization antigens within animals of a single serotype. *Genetics*, **47**, 241–253.

HARTMANN, L. & TOILLIEZ, M. (1957). Micro-méthode d'étude en gélose de la réaction antigène-anticorps (Variance du procédé d'Ouchterlony). *Rev. franç., Etudes clin. biol.* **2**, 197–199.

JONES, I. G. & BEALE, G. H. (1963). Chemical and immunological comparisons of allelic immobilization antigens in *Paramecium aurelia*. *Nature, Lond.*, **197**, 205–206.

OUCHTERLONY, O. (1949). Antigen-antibody reactions in gels. *Ark. Kemi* **B26**, 1–19.

PREER, J. R., Jr. (1956). A quantitative study of a technique of double diffusion in agar. *J. Immunol.* **77**, 52–60.

PREER, J. R., Jr. (1959*a*). Studies on the immobilization antigens of *Paramecium*. II. Isolation. *J. Immunol.* **83**, 378–384.

PREER, J. R., Jr. (1959*b*). Studies on the immobilization antigens of *Paramecium*. IV. Properties of the different antigens. *Genetics*, **44**, 803–814.

SONNEBORN, T. M. (1948). The determination of hereditary antigenic differences in genically identical *Paramecium* cells. *Proc. nat l. Acad. Sci. Wash.*, **34**, 413–418.

SONNEBORN, T. M. (1950). Methods in the general biology and genetics of *Paramecium aurelia*. *J. exp. Zool.* **113**, 87–148.