Immunogenetic analysis of proteins of *Paramecium*

VI. ADDITIONAL EVIDENCE FOR THE EXPRESSION OF SEVERAL LOCI IN ANIMALS OF A SINGLE ANTIGENIC TYPE*

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1. INTRODUCTION

The ciliary antigens of *Paramecium aurelia* can be grouped into several classes on the basis of their solubility in ammonium sulfate, electrophoretic mobility and immunological cross-reactivity (Preer, 1959c; Steers, 1961; Bishop & Beale, 1960). Serum against one of the serotypes may immobilize types other than that injected. A previous study has provided evidence that such an incompletely specific antibody response is due, at least in part, to the presence of two antigens within single cells rather than to the existence of a mixed culture with two serotypes. The two antigens examined, E and G, were serologically related, a fact which not only proved a technical handicap but which suggested that this cross-reactivity might be the cause for this 'exception' to the rule of general exclusion of all other immobilization antigens from animals of a single serotype.

Therefore immobilization serotypes have now been re-examined for the presence of antigens that are distinct by all the immunologic techniques used thus far and so considered unrelated to the surface antigen expressed. Animals of serotype C have been shown to possess concurrently G antigen not detectable by the immobilization test. The amount of this secondary antigen varies considerably within a stock and from stock to stock. The secondary antigen is indistinguishable from the G antigen isolated from animals of the G serotype and is probably located primarily in or on the cilia, as is the primary antigen, although differences in distribution may exist.

2. MATERIALS AND METHODS

(i) *Stocks and cultures*

Stocks 72, 7, 30 and 83 of syngen 2 were raised in bacterized infusions of 0·15% Cerophyl (Cerophyl Laboratories, Kansas City, Mo., U.S.A.) (see Finger, 1957, for detailed procedures). Typically the paramecia were maintained at room temperature in 20 l. jugs, although occasionally cultures were placed at 17° C. Cultures were

harvested by passing them though a DeLaval Gyro-Tester centrifuge, recovering the 400 ml. of residue in the rotor, and recentrifuging this for 2 min. at maximum speed in an International oil-testing centrifuge.

(ii) *Purification of antigens*

Preer's method (homogenization of entire animals, followed by acid precipitation of non-ciliary proteins and salting out of the neutralized supernatant) was used to extract and purify antigens (Preer, 1959 *b*). Three classes of ammonium sulfate concentrations—0–30%, 30–50%, 50–70%—served to separate roughly those immobilization antigens of primary interest, C and G. G antigen was predominant in the lower and middle cuts, C in the higher per cent. The precipitates obtained from 12×10^6 animals were resuspended in 0·6 ml. of 0·9% saline, unless otherwise indicated.

(iii) *Serotype determination*

Animals were tested for serotype using the standard immobilization tests of Sonneborn & LeSuer (1948).

(iv) *Sera and titration*

The procedures for preparing antigen for injection, the course of immunization, the methods of bleeding and handling blood, and the titration of sera for immobilization antibodies were as described by Finger & Heller (1962 *a*).

(v) *Gel diffusion in tubes*

Amounts of antigens were estimated in small tubes by diffusion in agar of antigen and antiserum according to the method devised by Preer (1956). The distance (between antigen and serum reservoirs) of the band precipitated varies with the ratio of concentrations of antigen and antibody and thus can be used as a quantitative measure of antigen (Preer, 1956; Finger & Kabat, 1957). Details of making these estimates are presented in the main text. A 0·3% distilled water solution of Oxoid Agar Agar No. 3 to which was added merthiolate (1:10,000) and glycine (0·5 M) was used in all gel methods.

(vi) *Gel diffusion on slides*

For identification of unknown antigens by a comparison with a standard antigen of band interactions at junctions of precipitates, a microscope slide adaptation of Ouchterlony's Petri dish technique was used (Hartmann & Toilliez, 1957).

(vii) *Immunoelectrophoresis*

Electromigration of antigens through an agar supporting medium (substituting for distilled water 0·2 M veronal buffer of pH 9·0) was carried out on microscope slides. A current of 35 milliamps per tray of 12 slides was passed for 90 min. in the cold or at room temperature (Finger, Heller & Green, 1962).

(viii) *Absorption*

One test for the presence of antigens involved the absorption of specific immobilizing and precipitating antibodies. Purified antigen was added to serum in equal volumes and the mixture placed at 31° C. for 1 hour, then 8° C. overnight. The mixtures were centrifuged at 850 G for 20 min. in the cold and the supernatants saved and frozen.

When the supernatants were to be assayed in gel diffusion tubes for loss of antibody, the antigen was sometimes placed as the bottom-most layer with one or two crystals of sucrose added to increase the density and allow the warm agar to overlay evenly. At other times the sucrose was added to the serum (previously diluted by the absorbing antigen) with the usual sequence of layers: bottom layer of serum, then agar, and finally antigen.

(ix) *Sucrose density gradient*

A gradient of 10–40% sucrose was set up in a 5 ml. plastic centrifuge tube using a mixing chamber. Atop 4·5 ml. of the sucrose solution was added about 0·5 ml. of purified antigen from 4×10^7 animals. After centrifugation in a free-swinging head of the Spinco Model L for 22 hours at 35,000 r.p.m., 0·3 ml. samples were carefully removed from the uppermost portion of the fluid in the tube using a 0·25 ml. tuberculin syringe and a 26-gauge needle. The first sample removed, containing the slowest sedimenting molecules, was called tube No. 1. For assay in gel diffusion tubes, samples could be used directly as the bottom layer because of their high density.

(x) *Separation of sub-cellular fractions*

To obtain cell fractions, the procedure devised by Preer & Preer (1959) was slightly modified. A concentrate of animals ($10^6/0·1$ ml.) was suspended in 8 c.c. of salt-alcohol and worked vigorously through a macropipette at intervals during a half-hour period. The suspension was centrifuged at 5000 r.p.m. in a Servall RC-2 (rotor SS-34); this and subsequent centrifugations lasted 10 min. The top layer of cilia and trichocysts was saved, the deciliated cells were resuspended in 10 c.c. of 0·9% saline, homogenized, and centrifuged at 13,000 r.p.m. The precipitated debris was removed, the supernatant centrifuged at 20,000 r.p.m. the resulting pellet was called the mitochondria and small granule fraction. The remaining supernatant was centrifuged in a Spinco Model L centrifuge for 3 hours at 35,000 r.p.m. yielding 'ribosomal' and soluble supernatant fractions. All fractions collected were resuspended in a volume of 0·1 ml. 0·9% saline per 2×10^6 animals in the original population and frozen.

3. RESULTS

(i) *Presence of G antigen in animals of C immobilization serotype*

The complete lack of cross-reactivity of C and G can be shown by the failure of either serotype to be immobilized, or even retarded, by most sera prepared against either one of these serotypes as found in stock 30 (Finger, 1956). We have also shown

with the syngen 2 serotypes (Finger, 1956), as has Preer (1959) using syngen 4 sero-
types, that the two are distinct as precipitins in gel diffusion. This specificity, added
to the marked tendency of both C and G animals to retain their serotypes under a
wide range of cultural conditions when grown in large carboys at ambient temper-
ature, made these serotypes particularly useful for these studies.

(a) Cross-reactions of C antisera

The presence of G antibodies in a C antiserum may be an index to the presence of
G antigen in C animals so situated that the antigen-antibody complex is not detect-
able *in vivo*. Or it may simply be evidence of the presence of some G animals in the
otherwise C culture that was used as the immunizing antigen. In the case of G–E
relationship previously reported (Finger & Heller, 1962; Balbinder & Preer, 1959),
cross-reacting sera were common due probably to a combination of circumstances:
structural cross-reactivity, contaminating animals (partly as a result of transfor-
mation between G and E), and true secondary, 'internal' antigens. With C sera the

Table 1

Serum number	Immunizing antigen	Immobilization titers		Serum number	Immunizing antigen	Immobilization titers	
		C	G			C	G
Rf 101	83C brei	4	3+	RW 9	83C brei	3+	—
Rf 102	83C brei	3	2	RW 10	83C brei	3+	—
Rf 116	83C brei	3+	2	Cl 46	7C brei	4+	—
FR 3	7C brei	5+	2+	RW 14	30C cilia	6	—
Rf 114	7C brei	4+	4+	RW 13	30C cilia	7	—
Rf 111	7C brei	7	3	Cl 49	30C brei	6	—
RW 11	72C brei	3	3+	Rf 119	30C brei	4+	—
RW 12	72C brei	4	3+	Rf 120	30C brei	6+	—
				Rf 121	30C brei	5+	—

The figures in the body of the table represent the lowest concentration of serum that will
immobilize the animals in 1 hour at room temperature. Where two figures are given, the second
figure represents the lowest concentration that will retard cells. Number 1 is a dilution of
1:12·5, number 2 is 1:25 and so on in a series of two-fold dilution steps. +means that, in the
concentration indicated, all animals are immobilized and in the next more dilute concentration
most are immobilized, and the remainder strongly retarded.

influence of most of the factors is minor and the relative non-specificity of sera
against various C serotypes is of greater interest. 30C antisera generally had little
or no G reacting antibodies (stock 30 antisera were the basis for stating above that G
and C were immunologically unrelated), while stocks 83, 72 and 7 gave rise to C
antisera which often affected G animals (Table 1). It was later observed that this
rough ordering is correlated with the propensity of these stocks to possess G second-
ary antigens, although fluctuations in quantities of G and C antigens from cells of the
same stock harvested at different times were frequent (Table 4).

(*b*) *Absorption of G antibodies with* $(NH_4)_2SO_4$ *fractions of C animals*

Because relative antibody concentration is a highly unreliable guide to antigen concentration in the immunizing substance, G antigen in C animals was looked for directly. C animals grown to a population of about $12 \times 10^6/15$ l. were harvested and immobilization antigen extracted. The purity of the culture was assayed by testing a sample of at least 200 paramecia with C and G antisera. If more than 1% of the cells were G, the culture was discarded. Most cultures used were 100% C by this criterion.

Table 2. *Presence of G antigen in homogenates of C serotypes of various stocks, as shown by lowering in titer of G antiserum by addition of various ammonium sulfate precipitates from C serotypes*

	Number of cultures with G antigen			
	Total number of cultures assayed			
	$(NH_4)_2SO_4$ cut			
Stock	0–40%	40–50%	50–60%	above 60%
30C	3/8	1/2	0/2	0/1
83C	4/5	0/3	1/3	0/1
7C	6/9	3/5	3/5	0/1
197C	2/2	1/2	1/2	0/1
Totals	15/24	5/12	5/12	0/4

Identification of G antigen as a constituent of C paramecia was made by determining the absorptive capacity of the soluble supernatant of the debris of homogenized cells and that of the various ammonium sulfate cuts—0–30%, 30–50%, 50–70%. The individual ammonium sulfate fractions were also tested in agar diffusion tubes for the presence of antigen that would form a band with G antisera. Drop in antibody concentration of these antisera was determined by reduced immobilization titer and by changes in the Preer tube band position of the antigen-antibody precipitate compared with a standard G primary antigen preparation. Table 2 records the frequence of any changes in antibody titer of a G antiserum after addition of antigen isolated in various cuts of ammonium sulfate precipitations of C serotypes of several stocks (following homogenization, acid precipitation of non-immobilization antigens, and neutralization). The lower per cent ammonium sulfate cuts (0–50) are richest in the secondary G antigen, as is true with primary G (30G) (Finger & Heller, 1962). The distinctions between the various fractions are somewhat blurred because of the inclusion of stock 30C, a stock from which G antigen rarely can be isolated even using extracts of entire C animals.

Although the differences between controls (unabsorbed serum) and experimentals are sometimes slight, they were reproducible. Table 3 also suggests the extremes of variability that can be encountered when different cultures are purified and assayed.

Thus when antigen was purified from 7C-1 the 40–50% fraction was the richest in secondary G antigen, while purification of 7C-2 yields a 50–60% fraction high in G antigen. The G antigens isolated were indistinguishable. Some idea of the specificity

Table 3. *Absorption of serotype G and E antibodies with ammonium sulfate fractions of extracts from C animals*

Source of extract	G-titers of supernatants of $(NH_4)_2SO_4$ cuts		
	0–40%	40–50%	50–60%
7C-1	4+, 6	−, 2	3, 5
7C-2	5, 7	2, 3	−, 2
30C-1	4+, 6	5, 7	5, 7
Unabsorbed serum	5, 7		

	G and E titers of supernatants of $(NH_4)_2SO_4$ cuts			
	0–40%		40–50%	
	E	G	E	G
7C-3	4+, 6	2+, 4	5, 6	−, 2
30G	4+, 6	−, 2	4+, 6	−, 2
Unabsorbed serum	6, 7	6, 7		

7C-1, 7C-2 and 7C-3 refer to three cultures of serotype 7C grown, harvested and purified separately. See also legend, Table 1.

of absorption can be obtained by comparing the ability of a preparation of 7C to absorb 30 C antibodies and E antibodies. Marked decreases in G titers are accompanied by relatively modest decreases in E titer, decreases accountable solely by G–E cross-reactivity, especially in view of the absence of *e* genes in stocks 30 and 7 (Finger & Heller, 1964).

(c) Direct demonstration of G antigen by precipitation in agar

Confirmation of the existence of G antigens in preparations from C animals comes also from the formation of a band in Preer tubes with G antisera (Table 4). The

Table 4. *Variation in amounts of precipitating G antigen in C animals as determined by band position in Preer tubes*

Source of antigen	Primary G	Source of antigen	Secondary G
30G-2	640	72C	84
30G-3	500	7C-5	9
30G-4	690	7C-6	35
		7C-7	44
		7C-8	0

position of the band in the agar colum was used to determine relative amounts of each antigen. The preparation which yields antigen forming a band farthest down the agar has the highest concentration of antigen, and all other antigens were compared with this according to the following formula:

$$\text{Number of units of X antigen} = \frac{1000}{\text{antilog}\left(\dfrac{\Delta}{Z}\log 2\right)}$$

where the most concentrated antigen = 1000 units;

Δ = (band position with most concentrated antigen) − (band position with X antigen);

Z = change in band position with halving of concentration (from calibrated curve, Z = about 10 in this system).

It is apparant that the stock from which the antigens were extracted was not a reliable guide to whether secondary G antigen would be found. Neither, however, was the distribution among the stocks random; for instance, 30 rarely had G antigen while 83 and 72 were quite likely to possess it. The minimum amounts of secondary antigen when found are generally considerably in excess of what would be expected from even the most grossly contaminated (1%) preparation.*

(ii) *Comparison of primary and secondary G antigens*

Complete identification of primary G (i.e. *in vivo* determined) with secondary G (i.e. not recognizable by *in vivo* tests) would require, at the very least, digestion of each antigen and chromatographic and electrophoretic analysis of peptide fragments ('finger-printing') (Steers, 1962; Jones & Beale, 1963). The amounts of antigen required together with the variability in quantity within a stock made such an effort prohibitive. However, certain other criteria provide evidence that the two antigens if not identical are markedly similar. For example, the same purification procedure suffices for the isolation of G and G animals and C animals. Also, gel-diffusion patterns reveal no immunologic differences (Fig. 1). Complete fusion of secondary antigen with primary 30 G antigen in Ouchterlony type patterns, as well as similar cross-reaction patterns with E antigens, (cf. Finger & Heller, 1962) indicated antigenic identity.

Two other parameters were also examined: net charge and sedimentation constant. With an immunoelectrophoretic procedure (Scheidegger, 1955) that can distinguish the cross-reacting antigens G and E, as well as allelic forms of E and allelic forms of C (Wilcox & Finger, unpublished), primary and secondary G's migrated at the same rate. In sucrose gradient centrifugation the percentages of recovery for the two G's at different densities were very much alike (Table 5).

However, this technique as used was unable to distinguish primary and secondary G, G and E antigens from each other, and is probably sensitive to only rather gross differences in sedimentation rates. Also there was considerable variation between

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* The 95% confidence interval for 2 out of 200 is 3·6%.

runs. For instance in Fig. 1, the major share of a primary immobilization antigen appeared in tubes 8 and 9, rather than in the customary 10th and 11th tubes. What this experiment shows particularly well is the validity of using any of three criteria
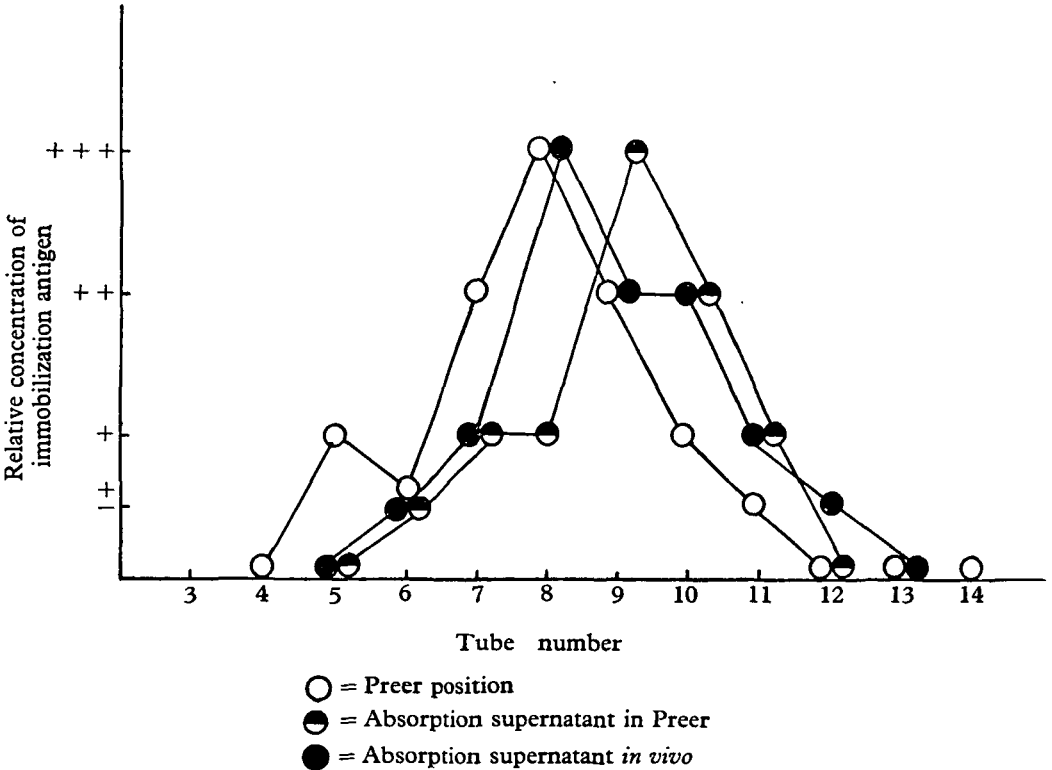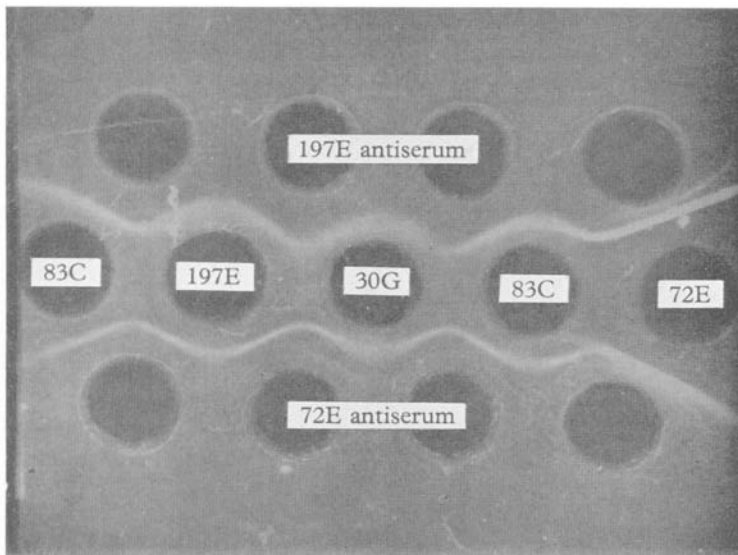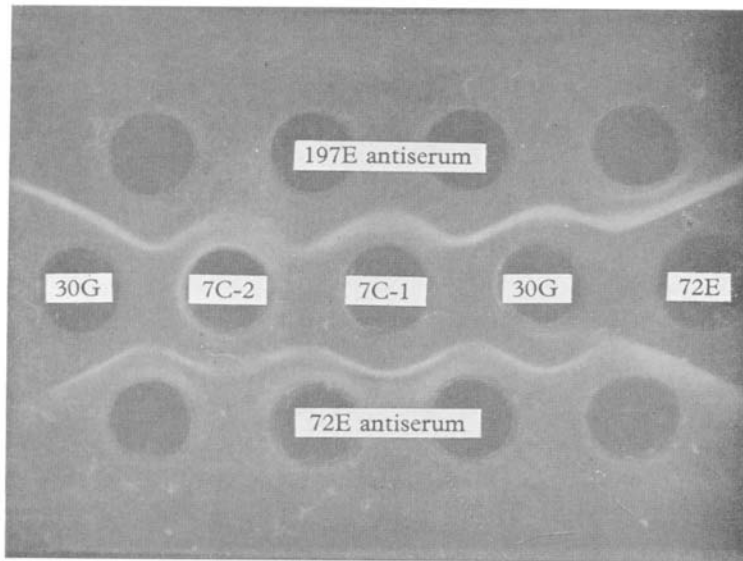

Fig. 1. Centrifugation of primary immobilization antigen G in a sucrose density gradient.

for assaying immobilization antigen—tests of supernatants of absorbed sera in gel diffusion tubes or *in vivo*, and direct analysis of antigen in tubes—and confirms Preer's (1959a) similar observations.

Table 5. *Sucrose gradient fractions in which highest concentration of immobilization antigen occurs*

Source of 0–40% (NH₄)₂SO₄ antigen	Serum	Tube numbers with maximum amount of antigen
7C	C antiserum	10, 11
7C	G antiserum	10, 11
30G	G antiserum	10, 11
72E	E antiserum	10, 11

Gel diffusion patterns comparing primary and secondary G antigens. The top slide shows a reaction of identity with 30G and two preparations of 7C secondary antigen and 72E with 197E antiserum. Only 72E antiserum produces a reaction of partial identity (spur) with 30G and 72E; this confirms the identification of the band as G antigen-antibody precipitate (cf. Finger & Heller, 1962). The bottom Ouchterlony pattern also demonstrates that a secondary antigen from 83C animals is G. The spurs formed by 197E with 30G using 197E antiserum are barely visible. Neither antiserum possessed C antibodies.

J. R. SEED, S. SHAFER, I. FINGER AND C. HELLER   (*Facing p.* 144)

(iii) *Location within cell of secondary antigens*

Since it did not seem that the basis for the differences in concentration and detectability between primary and secondary antigens lay in the structure of the antigens, it was thought that perhaps the distribution within a paramecium was unique for the two kinds of antigens. From several previous studies it was known that fractions of breis separated by differential centrifugation into cilia, trichocysts, mitochondria, etc., could be extracted for antigen (Preer & Preer, 1959; Finger *et al.*, 1960), and that data obtained in this way agreed well with the localization of antigen determined on whole animals using fluorescent antibodies (Beale & Kacsar, 1957; Beale & Mott, 1962).

Table 6. *Distribution of primary and secondary immobilization antigens in 83C animals as determined by gel diffusion in tubes*

	Band position in per cent		Per cent of total antigen	
	Anti G	Anti C	G	C
Cilia	69	68	49	90
Debris	55	28	28	6
Small granules	—	0	0	0
Ribosomes	—	12	0	1
Soluble supernatant	58	18	23	3

Cells were therefore broken up and the antigen isolated from cytoplasmic organelles, debris and the supernatant solution. Preer tubes with C antiserum and G antiserum were then used to determine the relative amounts of antigen in each fraction. As a final step in arriving at the figures of Table 6, the figure for each fraction was converted to a proportion of total antigen extractable. Cilia, debris and the material present in solution in the supernatant represent the greatest repositories of antigen, although to different degrees for primary and secondary antigens. Since the antigens are prone to leak out of structures during the preparation of fractions (cf. Preer & Preer, 1959), it is difficult to know what reliance to place on the differences between the two kinds of antigens in relative amounts associated with each fraction. This difficulty is accentuated by the variation from culture to culture of a single stock in the total amounts of secondary antigen found (Table 4), as well as differences between duplicate cultures in distribution of even the primary antigen, a situation illustrated in Table 7. Although both preparations of 7C had equal concentrations of antigen in the cilia fraction, one had much more C antigen in the ribosome fraction. This same 7C culture (7C-10) had less antigen in the supernatant. The formula used to arrive at the figures in Table 4 was used in Table 6 except that the most concentrated antigen was arbitrarily set at 100 units. The debris fraction in these runs was frozen for 24 hours and thawed, rather than extracted as soon as the debris was recovered, and this undoubtedly accounts for the large amounts of absorptive material present in this fraction as compared with the proportion recorded for freshly prepared material.

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Table 7. *Absorption of C and G antibodies by subcellular fractions of C animals*

	Cilia		Stored debris		Small granules	
	Anti C	Anti G	Anti C	Anti G	Anti C	Anti G
Unabsorbed serum	6+	4	6+	4	6+	4
7C-9	3+, 5	3+, 6	—, 7	—	6+	4, 6
7C-10	3+, 6	3, 6	—, 7	—	6+	4, 6
197C-1	5+, 7	5, 7	—, 7	—	6+	3+, 6
197C-2	4+, 6	4, 6	—, 7	—	6+	4, 6

	Ribosomes		Soluble supernatant	
7C-9	5, 7	4, 7	3+, 5	4, 7
7C-10	2+, 6	4, 6	4+, 6	4, 6
197C-1	4, 6	4, 6	6, 7	4, 7
197C-2	5+, 7	4, 6	6, 7	5, 7

See legend, Table 1.

Not only may G animals possess E as a secondary antigen (and vice versa) (Finger, Heller, & Green, 1962), and C animals have G, but experiments like those above have shown that two (and conceivably more) antigens may be present simultaneously as secondary antigens, none of which reveal themselves by immobilization tests.

4. DISCUSSION

The extension of the finding of more than one immobilization antigen in animals of a single serotype to immunologically unrelated antigens, requires that the phenomenon of mutual exclusion of immobilization antigens be cast in new terms. Rather, than as has been previously assumed, may one gene be turned on by a specific cytoplasmic state and other genes simultaneously turned off, all loci concerned with specifying the various immobilization antigens may be functioning continually. The part played by the cytoplasm may be that of regulating the quantity of each kind of information available to the cells synthetic machinery or even of providing an environment uniquely favourable to the utilization of a particular bit of information. Since the immobilization antigens are composed of subunits (Finger & Heller, 1963), perhaps the cytoplasm acts by selecting a particular combination of subunits as most suited to coat the paramecium.

Apparently there is no unique sequestering within a cell of secondary antigens as contrasted with primary antigens. Both appear to be distributed to the same organelles, with the cilia being one of the prime sites equally of the two kinds of antigens. Thus the topology of structures of the cell is unlikely to be the determining factor as to which antigen will be dominant, although Sonneborn (personal communication) has evidence that with one of the secondary antigens only relatively drastic treatment will reveal its presence.

Our finding that both primary and secondary antigens are associated with the ribosomal fraction is at variance with the suggestion of Beale & Mott (1962) that

no immobilization antigen is to be found internally, but consistent with the view that this fraction is the major site of protein synthesis in all cells. Although there is some non-specific absorption of antigen on ribosomes, the considerable variation in quantities of antigen associated with this fraction between cultures with the same total amount of antigen suggests that this localization may be *bona fide*.

As to the generality of the observation that cells possess multiple immobilization antigens, we have previously reported on the existence in syngen 2 of G in E animals (and vice versa), Margolin (1956) has described the coincident expression of D and M antigens on the surface of animals of syngen 4, Van Wagdentonk & Tijn (1953) have some indirect evidence consistent with the occurrence of secondary antigens in the same syngen, Bishop (1963) has reported some weakly absorptive antigen in animals of D and G serotypes in syngen 1 and many workers have noted sera with cross-reacting antibodies after immunization with cultures of a single serotype. On the other hand, for example, Balbinder & Preer (1959) were unable to detect any precipitating immobilization antigen other than the one recognized by immobilization tests in syngen 4. Also it is quite true that the vast majority of sera are highly specific (Pringle, 1956; Finger, 1957; Preer, 1959; Sonneborn & Le Suer, 1948). In view of this spectrum of observations and our own that the same serotype found in one particular stock may or may not possess measurable amounts of a secondary antigen, no unequivocal statement can be made as to whether the presence of secondary antigens in a cell is the rule or the exception. Considering the mounting evidence for the existence of these antigens, experiments assuming complete absence in animals of any immobilization antigen other than that detected by *in vivo* tests should certainly be viewed with caution.

Adding to the difficulties of interpreting evidence indicating absence of an antigen is the occurrence of sera with antibodies that precipitate closely related antigen but fail to retard at all an animal with this second antigen on its surface (Balbinder & Preer, 1959; Finger, Heller & Green, 1962). Also indicative of the possible importance of the relationship of the antigen to the surface configuration of the cell is the demonstration in some instances of a considerable quantity of secondary antigen extractable from cilia, yet undetectable by *in vivo* reaction. An alternative explanation would invoke multiple forms of antibodies, some capable of precipitating, but not of immobilizing.

SUMMARY

A paramecium generally bears on its surface but one immobilization antigen from among the many it may have the potentiality for expressing. By assaying soluble extracts of whole cells, it had been demonstrated previously that animals of the E serotype may possess the cross-reacting G antigen. Now the existence of these secondary antigens—immobilization antigens undetected by *in vivo* tests—has been extended to include unrelated antigens, e.g. G antigen in animals of C serotype.

The amount of these cryptic immobilization antigens varies from stock to stock; also within a stock cultures harvested at different times may have quite different quantities.

The secondary and primary G antigens appear to be indistinguishable by the criteria of antigenic specificities, immunoelectrophoretic mobilities sedimentation rates and ammonium sulfate solubilities. As to their distribution within the cell, both secondary and primary antigens are located primarily on or within the cilia.

REFERENCES

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

BEALE, G. H. & KACSER, H. (1957). Studies on the antigens of *Paramecium aurelia* with the aid of fluorescent antibodies. *J. gen. Microbiol.* **17**, 68–74.

BEALE, G. H. & MOTT, M. R. (1962). Further studies on the antigens of *Paramecium aurelia* with the aid of fluorescent antibodies. *J. gen. Microbiol.* **28**, 617–623.

BISHOP, J. O. (1963). Immunological assay of some immobilizing antigens of *Paramecium aurelia*, variety 1. *J. gen. Microbiol.* **30**, 271–280.

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

FINGER, I. (1956). Immobilizing and precipitating antigens of *Paramecium*. *Biol. Bull.*, *Wood's Hole*, **111**, 358–363.

FINGER, I. (1957). Immunological studies of the immobilization antigens of *Paramecium aurelia*, variety 2. *J. gen. Microbiol.* **16**, 350–359.

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. (1964). Immunogenetic analysis of proteins of *Paramecium*. V. Detection of specific determinants in strains lacking a surface antigen. *Genet. Res.* **5**, 127–136.

FINGER, I. & KABAT, E. A. (1958). A comparison of human antisera to purified diphtheria toxid with antisera to other purified antigens by quantitative precipitin and gel diffusion techniques. *J. exp. Med.* **108**, 453–474.

FINGER, I., HELLER, C. & GREEN, A. (1961). Immunogenetic analysis of proteins of *Paramecium*. II. Coexistence of two immobilization antigens within animals of a single serotype. *Genetics*, **47**, 241–253.

FINGER, I., KABACK, I., KITTNER, P. & HELLER, C. (1960). Immunological studies of isolated particulates of *Paramecium aurelia*. I. Antigenic relationships between cytoplasmic organelles and evidence for mitochondrial variations as demonstrated by gel diffusion. *J. biophys. biochem. Cytol.* **8**, 591–601.

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

JACOB, F. & MONOD, J. (1961). On the regulation of gene activity. *Cold Spr. Harb. Symp. quant. Biol.* **26**, 193–209.

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

MARGOLIN, P. (1956). An exception to mutual exclusion of the ciliary antigen in *Paramecium aurelia*. *Genetics*, **41**, 685–699.

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. (1959a). Studies on the immobilization antigens of *Paramecium*. I. Assay methods. *J. Immunol.* **83**, 276–283.

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

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

PREER, J. R., Jr. & PREER, L. B. (1959). Gel diffusion studies on the antigens of isolated cellular components of *Paramecium*. *J. Protozool.* **6**, 88–100.

PRINGLE, C. R. (1956). Antigenic variation in *Paramecium aurelia*, variety 9. *Z. indukt. Abstamm. u. VererbLehre*, **87**, 421–430.

SCHEIDEGGER, J. J. (1955). Une microméthode de l'immuno-électrophorèse. *Intern. Arch. Allergy Appl. Immunol.* **7**, 103–110.

SONNEBORN, T. M. & LESUER, A. (1948). Antigenic characters in *Paramecium aurelia* (variety 4): determination, inheritance and induced mutations. *Amer. Nat.* **82**, 69–78.

STEERS, E. (1961). Electrophoretic analysis of immobilization antigens of *Paramecium aurelia*. *Science*, **133**, 2010-2011.

STEERS, E. (1962). A comparison of the tryptic peptides obtained from immobilization antigens of *Paramecium aurelia*. *Proc. natl. Acad. Sci., Wash.*, **48**, 867–874.

VAN WAGTENDONK, W. J. & VAN TIJN, B. (1953). Cross reaction of serotype 51 A, 51 B and 51 D in *Paramecium aurelia*, variety 4. *Exp. Cell Res.* **5**, 1–9.

WILCOX, H. B. & FINGER, I. (unpublished).