

STUDIES ON THE IMMOBILIZATION ANTIGENS OF PARAMECIUM. IV. PROPERTIES OF THE DIFFERENT ANTIGENS¹

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EACH stock, or strain, of *Paramecium* can produce a number of antigenic types or serotypes. Each serotype is immobilized by its homologous antiserum, but not by antiserum made against other serotypes. The complex cytoplasmic, genic, and environmental factors that determine which of the possible serotypes is manifested in a given animal were discovered by SONNEBORN (1948). The inheritance of serotypes in *Paramecium* has been reviewed recently by BEALE (1957). Animals of each serotype contain a single characteristic protein or immobilization antigen which is localized primarily in the cilia and body wall. The specificity of most, if not all, of the immobilization antigens is determined by the alleles at several different loci—one locus being responsible for the specificity of each serotype. Since individual cells generally show only one serotype at a time, only one genetic locus comes to expression at a time. Within a stock serotypic differences are determined either by the environment or by cytoplasmic inheritance. In the first paper of this series (PREER 1959a) it was shown how the immobilization antigens can be followed quantitatively *in vitro* using antigen-antibody precipitation in agar gel. PREER (1959b) has recently shown how the antigens can be isolated chemically, and finally PREER (1959c) gave an account of the properties of antigen A of stock 51. It was found that the A antigen is a large asymmetric protein molecule with a molecular weight of around a quarter of a million. The present and last paper of the series deals with a comparative study of several of the immobilization antigens of syngens (varieties) 2 and 4 of *Paramecium aurelia*. Serology, solubility in ammonium sulfate, and, to a lesser extent, sensitivity to proteolytic enzymes have been studied.

MATERIALS AND METHODS

Stocks of *Paramecium aurelia*, culture methods, the method of isolating the immobilization antigens, and serological methods have already been described (PREER 1959a; PREER and PREER 1959). A few points, as well as some special methods, require comment.

Each stock of *P. aurelia* exhibits a series of serotypes designated A, B, C, etc. When the serotypes of different stocks are compared, many are found to be

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indistinguishable or closely related. Within a syngen, all homologous serotypes are designated by the same letter. Thus 51 A of syngen 4 (stock 51, serotype A) is closely related to 29 A of syngen 4 and 51 B is indistinguishable from 29 B. The serotypes in each syngen, however, have been given letter designations somewhat independently of each other, and corresponding letters indicate homologies only in certain cases. We have utilized serotypes A, B, C, D, E, G, H, J, M, and Q of syngen 4, and also serotypes E and G of syngen 2. The two E serotypes are completely unrelated (do not cross react) while the two G serotypes are closely related. Since most of the work has been done on syngen 4, the letter designations used in this paper refer to syngen 4, unless otherwise indicated.

In the serological technique of double diffusion in agar used here (PREER 1956), a layer of agar is established in a small tube with a layer of serum below the agar and a layer of antigen above. Where the diffusing antigens and antibodies meet within the agar, bands of precipitation are formed. The position of a given band is dependent upon the relative diffusion coefficients and relative concentrations of antigen and antibody. Since these are rarely the same for independent antigen-antibody systems, each antigen usually forms a separate band.

The bands of precipitation formed when both heterologous and homologous cross-reacting antigens are present in the antigen layer present a special problem. In general, it can be expected that if the homologous antigen is sufficiently in excess, it will absorb all the antibodies, and the heterologous antigen cannot form a band of precipitation behind (nearest the antigen-agar interface) the band produced by the homologous antigen. On the other hand, if the heterologous antigen is present in sufficient excess two bands of precipitation will be formed. The first (farthest from the antigen-agar interface) will be produced by the reaction of the heterologous antigen with those antibodies with which it can combine, and the second will be produced by the reaction of the homologous antigen with the remaining antibody.

The determinations of ammonium sulfate solubility were made as described earlier (PREER 1959b, c). Animals of the appropriate serotype were extracted in four volumes of one part 30 percent alcohol plus one part of buffered saline (0.15 M sodium chloride in 0.01 M sodium phosphate buffer, pH 7.0) for one hour at 2°C. The pH was reduced to 2.0 with 0.2 N HCl and the precipitate removed by centrifugation at 25,000 G for three minutes. The pH was raised to 7.0 with 0.2 N NaOH and the precipitate again removed and discarded. Fractional precipitation with ammonium sulfate was then carried out as follows. Sufficient saturated (23°C) ammonium sulfate (pH 7.5) to reach the first desired level of saturation was added. The precipitate was removed by centrifugation, washed in 75 percent saturated ammonium sulfate, and redissolved in buffered saline. The supernatant was then raised to a higher level of saturation by adding more saturated ammonium sulfate, and the process continued until the desired final level of saturation was attained.

Immobilization antigen was determined in each fraction quantitatively by gel diffusion according to the procedure described earlier (PREER 1959a). The

method is based upon the fact that, for constant antibody concentration, the position of the band (measured as the distance from the antigen-agar interface to the band, divided by the total agar length) is linearly related to the logarithm of the antigen concentration. An estimate of total protein was obtained spectrophotometrically using the method of WARBURG and CHRISTIAN (1941). Values obtained by the method of WARBURG and CHRISTIAN were multiplied by the factor 0.85 as recommended by PREER (1959c).

Tests for the action of proteolytic enzymes were made by exposing either crude extract or purified antigen to the enzymes. After a suitable incubation period, the mixture was added to antiserum, and then paramecia were added to see if the antigen had absorbed antibody from the serum or whether its capacity to react with antibody had been destroyed by the enzyme. The detailed procedure, including appropriate controls, was as follows. Crystalline trypsin (Nutritional Biochemicals Co.) was dissolved in 0.01 M tris (hydroxymethyl) aminomethane buffer, pH 7.6, containing 0.04 M CaCl_2 . Four preparations were made in performing a test: (1) Two parts buffer (2) One part enzyme, one part buffer (3) One part antigen (containing about 300–400 micrograms per milliliter), one part buffer (4) One part enzyme, one part antigen. Each of the four preparations was incubated two to three hours at 37°C, and then to each was added two parts of antiserum diluted with buffered saline. The antiserum was 25 times as strong as the greatest dilution giving complete immobilization in one half hour. After five minutes the mixtures were diluted by adding 36 parts of ten percent Ringer's solution and ten parts of a culture containing primarily animals of the serotype being studied (homologous) as well as a few animals of a non-homologous serotype. After one half hour the animals were observed under the microscope. In all mixtures the non-homologous animals should be unaffected, showing that the constituents are nontoxic. In preparation (1) the homologous animals should be immobilized, demonstrating active specific antibodies. In mixture (2) animals should be immobilized showing that the enzyme has no harmful effect on the antibodies. In preparation (3) the animals should be unaffected, demonstrating the activity of the unimpaired antigen in absorbing antibodies. In preparation (4) the homologous animals are immobilized if the enzyme has destroyed the antigen, thereby leaving the antibodies to react with the animals, and unaffected if the antigen has not been destroyed by the enzyme.

Crystalline chymotrypsin (salt free, Nutritional Biochemicals Co.) was dissolved in 0.1 N sodium phosphate buffer, pH 7.6, and tests were conducted in the same way as those on trypsin.

Crystalline pepsin (Nutritional Biochemicals Co.) was dissolved in 0.06 N HCl. The antigen preparations were adjusted to pH 2.0 with 0.2 N HCl. The usual four preparations (see above) were made, using 0.06 N HCl in place of buffer, and incubated in the usual way. Then each of the preparations was neutralized by adding one part of 0.2 M Na_2HPO_4 to two parts of each mixture. Serum, ten percent Ringer's solution, and animals were then added in appropriate amounts taking into account the dilution introduced by adding the Na_2HPO_4 .

RESULTS

Serology

Purified immobilization antigens in buffered saline were adjusted to a concentration of 85 micrograms per milliliter and double diffusion tubes were set against both homologous and heterologous sera. Some of the tubes are shown in Figure 1.

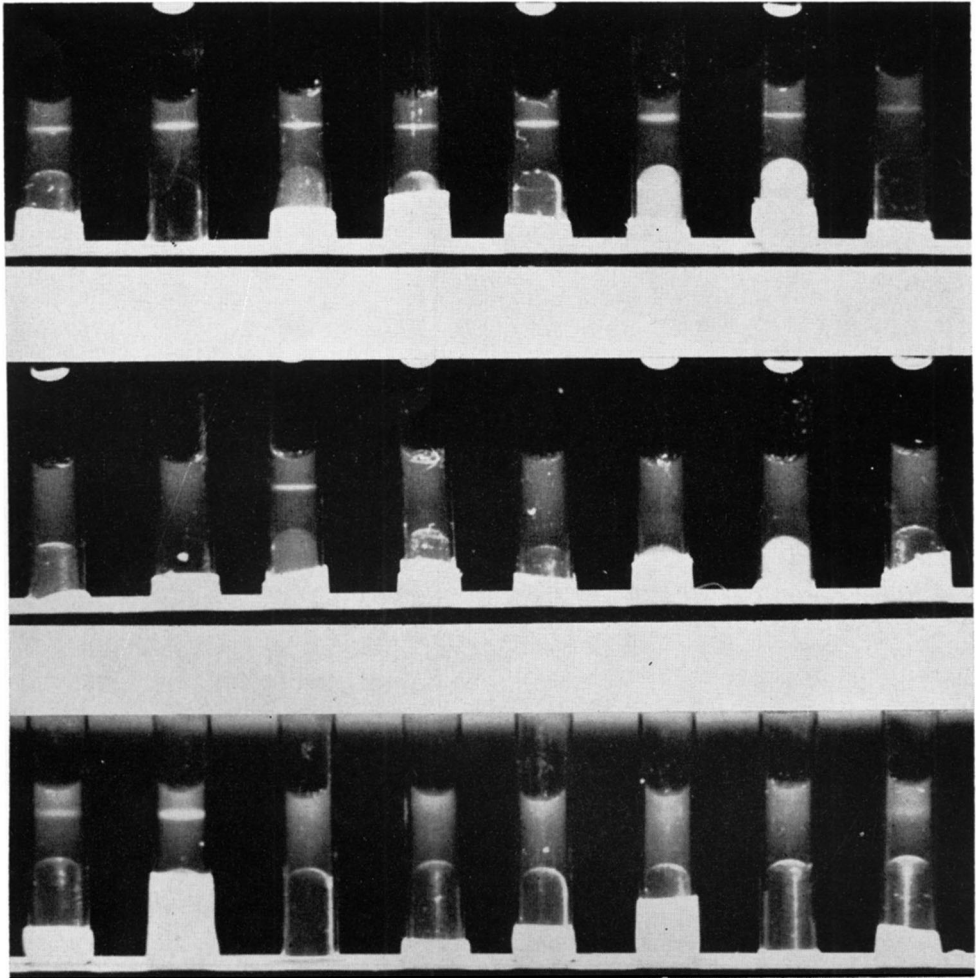


FIGURE 1.—Reactions of purified antigens with various sera. The top layer in each tube is the antigen solution; the middle layer is agar and contains the precipitation zones; the bottom layer is serum; a paraffin plug is visible at the bottom of some of the tubes. The eight tubes in each figure were set with eight sera prepared against whole homogenates; the same eight sera were used in each set; reading from left to right, they were (1) 51 A, (2) 51 B, (3) 51 C, (4) 51 D, (5) 51 E, (6) 51 H, (7) 131 M, (8) syngen 2 E. a. Homologous reactions; each tube set with the corresponding antigen. b. Complete specificity of the C antigen which was used in all tubes. c. The B antigen was used in all tubes and cross-reacts only with the A and syngen 2 E sera in tubes (1) and (8).

Figure 1a shows homologous combinations. Since the sera were prepared against homogenized whole animals, antibodies against many nonimmobilization antigens were present. But since the antigen preparations were purified, only a single band of precipitation is found in each tube. Figure 1b shows the 51 C (stock 51, serotype C) antigen set against the same series of sera. 51 C is completely specific and reacts only with its homologous antiserum. Figure 1c shows 51 B set against the sera. In this case, in addition to the strong homologous reaction, somewhat weaker bands are found with the 51 A and 4 E (syngen 2) sera. It will be shown presently that these weak reactions are true cross reactions caused by the reaction of the B antigen with antibodies against other serotype antigens. In Table 1 the results of setting the antigens with a number of sera are

TABLE 1

Reaction of purified immobilization antigens with various sera in gel diffusion. Twenty-one sera (designated by number in the first row of the table) prepared against the indicated serotypes were diffused against each purified antigen. + indicates a single band of precipitation; no entry indicates no reaction

Antigens	Sera																					
	C		E		H		D		J		M		A		B		Q	syn. 2 E		G		
	24	64	17	54	58	61	74	123	75	78	43	81	56	110	45	119	117	132	149	116	28	
C	+	+	+
E	+	+
H	+	+
D	+	+	+	+	+	+
J	...	+	+	+	+	+	+	+	+	...	+	+	...
M	+	+	+	+	+	+
A	+	+	+	+	+	+	+	+	+	...
B	+	...	+	+	+	+	+	+	+	+	+	+	+
syn. 2 E	+	+	+	+	+	+	+	+	+	+

given. All sera were from the collection of PROF. T. M. SONNEBORN except #132 and #149 which were prepared by the author. It is noted that each antigen (except Q) was set against two sera prepared against each serotype, and that all combinations were made. In the table, a + indicates that a band was obtained; no designation means that no band was found. Homologous bands were invariably more intense than heterologous bands. Inspection of the table shows that, on the basis of the pattern of cross reaction, D, J, and M form one group, and A, B, Q, syngen 2 E, and G form another, with C, E, and H separate from each other and the groups. The arrangement of serotypes in the table has been made so as to emphasize these relations. The evidence indicates that these groups consist of serologically related antigens. A few cross reactions appear outside the groups sporadically, but they are not sufficiently frequent to disturb the general scheme of grouping.

Cross reactions can also be seen in immobilization tests, i.e., when animals are treated with diluted antiserum. Table 2 contains the results of immobilization tests on the same serotypes and sera given in Table 1. Immobilization tests were

TABLE 2

Reaction of living paramecia with various sera in immobilization tests. Sera (same as those in Table 1) were tested against paramecia of each serotype. + indicates immobilization or strong retardation of paramecia in a dilution of 1/12.5 serum after 2 hours at 27°C; no entry indicates no reaction

Serotypes of paramecia	C		E		H		D		J		Sera M		A		B		Q	syn. 2 E	G		
	24	64	17	54	58	61	74	123	75	78	43	81	56	110	45	119	117	132	149	116	28
C	+	+	+	+	+
E	+	+	+	+
H	+	+	+
D	+	+	...	+	...	+	+	...
J	+	+	+	+	+	+
M	+	+	...	+	+	+
A	+	+	...	+	+	+
B	+	...	+	+	...	+	+	+	+	...	+	+	+
syn. 2 E	+	+	+	+	+	...

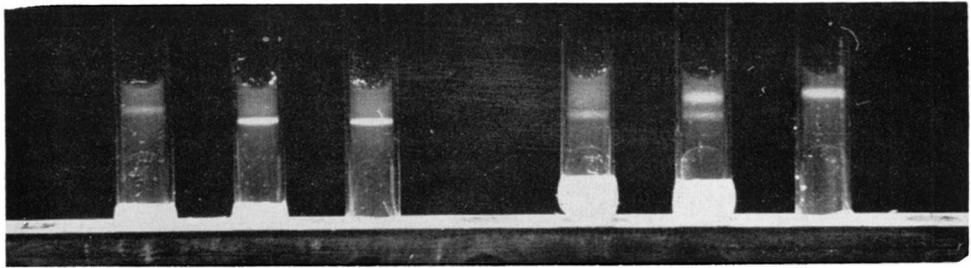


FIGURE 2.—Serological analysis of the cross reaction between A and B. All six tubes are set with B serum. Tube (1) is set with a low concentration of A antigen; tube (3) has a high concentration of B; tube (2) has a mixture of the two, and contains only one band showing all the antibodies can react with the homologous antigen. Tube (4) has a high concentration of A; tube (6) has a low concentration of B. Tube (5) contains the mixture and shows two bands, revealing that some of the antibodies may react with B.

performed as prescribed by SONNEBORN (1950) and a + in the table indicates that animals were either immobilized or markedly retarded in their swimming by the serum in concentrations of 1/12.5 or less. It is noted that the cross reactions are not all the same as observed in the gel diffusion tests, but that the same general pattern is followed.

The cross reaction between 51 A and 51 B was chosen for special study. Each gives a strong band with homologous serum and a weak band with heterologous. Information about the nature of the cross reaction is obtained by setting mixtures of the A and B antigens against the sera. Figure 2 shows the results of the tests on serum S#45 (51 B). Tube (1) shows the relatively weak band produced by using 47 micrograms per milliliter of the heterologous 51 A antigen. Tube (3) was set with 425 micrograms per milliliter of homologous 51 B antigen and gives a strong band lower in position. Tube (2) was set with a mixture containing 47

micrograms of A and 425 micrograms of B per milliliter. Only one band is obtained, showing that the concentrated B antigen has absorbed the antibodies from the serum before it could reach the more dilute A.

When the ratio of A to B is reversed, a very different result is obtained. The antigen layer of tube (4) contains 375 micrograms of A per milliliter and forms a weak heterologous band near the center of the agar. Tube (6) contains 95 micrograms of B per milliliter and forms an intense homologous band just within the agar. Tube (5) contains 375 micrograms of A per milliliter and 95 micrograms of B per milliliter and two bands appear. The heterologous A band forms first, reacting with some of the antibody, and the remaining antibody, being unable to react with the heterologous A, diffuses further into the agar and reacts with B to form the characteristically intense homologous band. All these results were obtained with anti-B serum. When the experiment was repeated with the anti-51 A serum S#110, analogous results were obtained. One band is obtained in mixtures in which the homologous antigen is in excess, two bands are obtained when the heterologous antigen is in excess. These experiments show that sera S#45 (B) and S#110 (A) have some antibodies which can react with the heterologous as well as the homologous antigens, but that all antibody can react with the homologous antigens. The data provide no evidence that cross reactions are the result of small amounts of heterologous antigen present in the extracts of each serotype. If the extracts from each serotype contained mixtures of antigens, more than one band should be seen, but they are not. Instead, some of the antibodies formed in response to one antigen can also react with closely related antigens.

Solubility

Solubility of the antigens in ammonium sulfate was determined in one experiment by measuring the amount of antigen precipitated from acid treated extracts (see above). Ammonium sulfate was added until the solution reached 38 percent saturation. The precipitate was removed, redissolved, and assayed for immobilization antigen (using gel diffusion) and protein (spectrophotometrically). The ammonium sulfate concentration was now increased stepwise to 47 percent, 56 percent, 65 percent, and 74 percent, the precipitates being assayed for antigen and protein at each step. One half to two milliliters of packed cells were used for each extraction, and fractionations of antigen from each serotype were done in duplicate on separate cultures of animals. All serotypes within a stock were recently derived from the progeny of one animal. The results are shown graphically in Figure 3. The determinations of the amounts of antigen and protein in each fraction are given in percent of the total in all five fractions. It is noted that the data on antigen are very closely paralleled in every case by the data on protein. There is a tendency for the protein to exceed the antigen in fractions in which there is only little antigen (particularly in the higher ammonium sulfate concentrations), and a corresponding tendency for the measure of antigen to exceed protein where antigen is high. This discrepancy would be expected if the major protein were the antigen, and if a small amount of contaminating protein is also present.

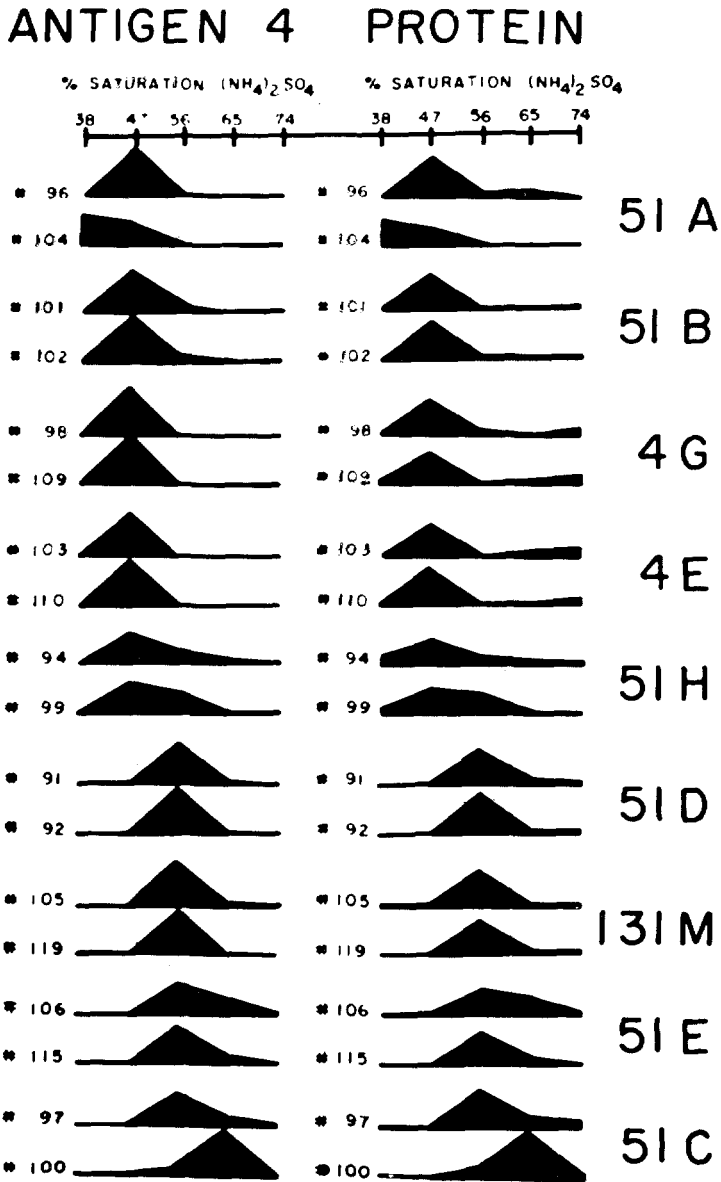


FIGURE 3.—Ammonium sulfate solubility of immobilization antigens of different serotypes in partially purified extracts. The percent precipitated by increasing the salt concentration to the indicated percent levels of saturation was obtained on two antigen preparations from different serotypes. Measurements on antigen 4 (the immobilization antigen) by gel diffusion are given in the left hand column, while measurements based on total protein measured spectrophotometrically are given in the right hand column.

It should be noted that the parallel between antigen and protein is maintained even when duplicate runs do not agree, and even when different antigens with different solubilities are studied.

The data of Figure 3 also show that the solubilities of the different antigens are different. The discrepancy between duplicate runs seen in certain cases results from the very great differences in antigen solubility in ammonium sulfate solutions of only slightly different concentrations. A very steep curve results when solubility is plotted against ammonium sulfate concentration (See PREER 1959c for a solubility curve for antigen 51 A). As a result, great differences in duplicate runs can be seen if one of the five levels of ammonium sulfate concentration used here happens to be critical for a given antigen. 51 A is a good example, for in the antigen concentrations used in the experiments, 38 percent saturation is critical. A very small change in solubility or in ammonium sulfate concentration can result in the precipitation of virtually all of the antigen, or in most of it remaining in solution. It is seen that 51 A, 51 B, 4 G (syngen 2), and 4 E (syngen 2) show the lowest solubilities. 51 H is slightly higher, 51 D and 131 M are next highest, and 51 C and 51 E are still higher. Extensive data other than those shown in Figure 3 on the solubility of immobilization antigen from cilia, and on both crude and purified antigen from whole homogenates, confirm and extend these conclusions. Four and possibly five solubility groups are found. (1) 51 A, 51 B, 51 Q, 4 G (syngen 2), and 4 E (syngen 2) constitute one group; (2) 51 H is slightly, but significantly different; (3) 51 D, 29 J, and 131 M constitute another group; (4) 51 C and 51 E are the most soluble, and there are indications that 51 C is slightly more soluble than 51 E. The data given above on cross reactions parallel these findings remarkably well. The serotypes in group (1) are all serologically related; those in group (3) are also related; while 51 H, 51 C and 51 E are unrelated.

Sensitivity to proteolytic enzymes

The method of conducting the tests is described above. Antigen was assayed by its ability to absorb or block immobilization antibody and thereby prevent the immobilization of paramecia. Enzymatic activity was demonstrated if the enzyme was able to prevent antigen from reacting with immobilizing antibody. Appropriate controls indicated that the sera did contain active immobilizing antibodies, that the unimpaired antigen was able to absorb antibodies, that the enzymes did not destroy the antibodies, and that no substances injurious to the living animals (other than specific antibody) were present in the test. Controls were satisfactory even when the enzymes were used in concentrations as high as 0.2 percent in the incubation mixtures.

In one experiment 51 D antigen #35 (237 micrograms per milliliter, purified from a homogenate after overnight extraction at 2°C) was completely inactivated in three hours in 0.2 percent trypsin, and partially inactivated in 0.05 percent trypsin. 0.2 percent trypsin was able to destroy 51 D in numerous other experiments. Inactivation (usually complete) was obtained not only in purified antigen,

but in antigen in ciliary extracts, and in crude extracts of whole homogenates, either freshly prepared, or after overnight extraction. The 51 D antigen #35 was completely destroyed in concentrations as low as 0.05 percent chymotrypsin, and was partially inactivated in 0.01 percent. Chymotrypsin proved very active on various other antigen preparations. Pepsin was even more active than chymotrypsin; complete inactivation of antigen #35 was obtained in 0.0015 percent pepsin, and partial inactivation at even lower concentrations. Other purified and crude antigen extracts were tested in pepsin concentrations of 0.05–0.01 percent pepsin and were completely inactivated. Tests showed that pepsin acted very quickly, the stronger concentrations destroying the antigen in minutes.

Antigen 51 A was also inactivated by chymotrypsin and pepsin. However, unlike 51 D, it was not inactivated by trypsin even in the highest concentrations used (0.2 percent). The resistance to trypsin was observed in purified preparations as well as in fresh extracts of homogenates. Other serotypes were not tested.

DISCUSSION

The serological analysis of the cross reaction between the 51 A and 51 B antigens reveals that although all of the immobilization antibodies in each serum can react with their homologous antigen, a portion can also react with the heterologous antigen. BALBINDER and PREER (1959) reached the same conclusion in a study of the cross reacting serotypes G and E of stock 28, syngen 2. The other cases of cross reactions were not similarly tested. Nevertheless, since no more than one band was produced in any gel diffusion tube, it is probable that the remaining cross reactions are of the type analyzed, rather than being caused by a mixture of immobilization antigens. Consequently the reaction of antibody against one antigen with a heterologous antigen is evidence of structural similarity of the molecules. Antigens may thus be placed into related groups: (1) A, B, G, Q, and syngen 2 E; (2) D, J, and M. The other antigens studied, C, E, H, are unrelated. The exceptional cross reactions in gel diffusion may be ascribed to a more remote relationship, and to the variability of rabbits in responding to similar molecules. Discrepancies between the cross reactions of gel diffusion and those of immobilization tests are not surprising in view of the fact that the activity of serum is only partly correlated with its activity in immobilization (FINGER 1956). The explanation for this fact is unknown, but possibly is explained by differing efficiencies of antibody in these two activities. This same phenomenon may also explain the greater variability of immobilization cross reactions as compared to gel diffusion cross reactions. The possibility of immobilization reactions dependent on antigens other than the usual (antigen 4) family of immobilization antigens is perhaps worth consideration. The only other soluble ciliary antigen known, however, is antigen 5 (PREER 1959b), and a serum (P#183) against purified antigen 5 does not immobilize.

The grouping of antigens given here can be supported on other grounds. D and M in some cases (MARGOLIN 1956) can coexist in single animals, constituting an exception to the rule that the different serotypes found in homozygous lines are

mutually exclusive. Somewhat related to this situation is the relative ease of transformation of one type into another. 51 H when brought from 12°C to 27° often transforms to D and J and often to D through J (SCHNELLER, personal communication). Serotypes G and E of syngen 2 fall into the same cross reacting group and are easily transformed one into the other. AUSTIN *et al.* (1956 and personal communication) report that when various serotypes are treated with the antibiotic patulin and other agents, transformation is to B, A, N, and Q—all cross-reacting types. Such facts must be interpreted with caution, however, for *all* types in stock 51 go to A at high temperatures. Furthermore within one related group, A and syngen 2 E are more stable at high temperatures, while B and G are stable at low temperatures.

The solubilities of the antigens in ammonium sulfate parallel the groupings based on cross reactions remarkably well. The relation between A, B, Q, G, and syngen 2 E is confirmed, as well as the relation between D, J, and M. Even the fact that H, C, and E are unrelated to other types is suggested by the data on solubility.

The data reported here provide very strong evidence that the immobilization antigen, the antigen observed in gel diffusion, and the protein are all identical, and have indeed been purified. The experiments on proteolytic enzymes reinforce the conclusion that the antigens are proteins and show that the different antigens (D and A) may differ in their response to trypsin.

The different immobilization antigens are similar substances. They are all represented in the cell in about the same quantity, have about the same cellular distribution and diffusion coefficients, and are purified by the same procedures. They are similar in their unusually strong ability to induce antibody in the rabbit. Furthermore, their parallel cellular distributions and other properties suggest that their function, whatever it may be, is probably very similar in all serotypes. Nevertheless in some respects many of the immobilization antigens are very different—some show no serological cross reaction, they may have markedly different solubilities in ammonium sulfate and different sensitivities to trypsin. What these differences mean in terms of molecular structure, whether they reflect fundamental differences in amino acid sequence or in more gross patterns of cross-linkage and folding, is unknown. Since the differences in the antigens result from the expression of different genes, further elucidation of the nature of the differences between the antigens should yield information on the role of genes in determining protein structure.

SUMMARY

The immobilization antigens from different serotypes of *Paramecium aurelia* are closely related but distinct proteins. While many of the antigens are completely specific, others show cross reactions with other immobilization antigens. Such cross reactions are caused by the fact that although all of the antibody elicited by a given antigen can react with that antigen, some of it also can react with other serotype antigens. The pattern of cross reactions provides a means of

grouping related antigens. The immobilization antigens also differ in ammonium sulfate solubility. Furthermore, the pattern of solubility parallels the groupings based on serological specificity. The antigens differ in their sensitivity to trypsin.

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