AN EXCEPTION TO MUTUAL EXCLUSION OF THE CILIARY ANTIGENS IN *PARAMECIUM AURELIA*¹

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THE system of ciliary antigens of *Paramecium aurelia* has provided abundant material for the study of the inheritance of diverse phenotypes by organisms of identical genotype. Each animal of any one stock of *P. aurelia* has the genetic potential for the expression of a series of serologically different alternative ciliary antigens (SONNEBORN 1948, 1950a; SONNEBORN and LESUER 1948; BEALE 1954). Single genes have been shown to control the serological specificities of each of the antigens. Allelic genes have been found to control the specificities of serologically corresponding serotypes of different stocks of variety 4, in all cases thus far investigated (SONNEBORN 1950a, 1951; BEALE 1954). Normally only one antigen is detectably present at any one time in a single animal. The identity of the antigen present on the cilia of an animal is referred to as its ciliary antigenic type or serotype. Serotypes can be inherited through varying numbers of vegetative and sexual reproductions. SONNEBORN and LESUER (1948) have shown that the cytoplasm plays an important role in serotype inheritance, by crossing serotypically different clones of the same stock. If no cytoplasm was exchanged the lines of descent from the exconjugants retained the serological differences of the conjugants, despite the fact that they must have become genically identical. However, if large amounts of cytoplasm are exchanged, often the lines of descent from both exconjugants of a pair have the same serotype, sometimes that of one exconjugant and sometimes that of the other.

Which serotype will be expressed is determined by the interaction of the genes controlling the specificities of the different antigens and various factors such as temperature and growth rate (SONNEBORN 1947, 1950a; BEALE 1948, 1954; SKAAR 1952; SONNEBORN, OGASAWARA and BALBINDER 1953; MARGOLIN 1955). Changes in these factors, without any change in genotype, may cause transformations from one serotype to another. During the period of transition, usually lasting only about one to two fissions, it is often possible to detect as present at the same time, both the disappearing initial serotype and the new serotype coming to expression (BEALE 1948, 1954; MARGOLIN 1955). Normally this is the only time at which two different antigens, whose specificities are controlled by nonallelic genes, are to be found detectably present at the same time in a single animal.

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The expression of any one serotype to the exclusion of all others potentially expressible has been called mutual exclusion (SONNEBORN 1950a; BEALE 1954). The primary basis for the exclusion is not differences in optimal conditions of culture or temperature for the different serotypes; pure cultures of different serotypes may be maintained side-by-side under the same conditions of culture. This phenomenon of mutual exclusion provides one of the most interesting aspects of the antigen system in *P. aurelia* from the point of view of developmental genetics. The present report describes an apparent exception to mutual exclusion in stock 172 of *P. aurelia*, variety 4.

NOMENCLATURE, MATERIALS AND METHODS

The serotypes are designated by capital letters to which stock numbers are prefixed since corresponding serotypes from different stocks may differ serologically. The homologous antisera are given the same designations as the serotypes against which they were obtained. Thus 172D antiserum is the homologous serum of antigenic type D of stock 172. A corresponding antiserum for a serotype is one obtained against the corresponding serotype of a different stock. Thus 172D antiserum is a corresponding antiserum for serotype D of stock 32. Small letters are used to designate the genes controlling the serological specificities of the serotypes and the stock designations are given as superscripts. For example, d^{172} designates the gene controlling the serological specificity of serotype D in stock 172.

The majority of the work described here was carried out on stock 172 (MARGOLIN 1955) of variety 4, *P. aurelia*. The use of another stock for some portions of the work will be indicated in the appropriate places. The two culture media used were a dehydrated lettuce (Difco) infusion and a dehydrated rye leaf (Cerophyl) infusion. The medium used will be indicated in the appropriate places in the description of the results. Both media were inoculated with *Aerobacter aerogenes*, incubated for 24 hours at 27°C and finally adjusted to approximately pH 6.4 before use (SONNEBORN 1950b).

The presence of a specific antigen in animals being tested is indicated by a retardation of the forward swimming movement in the appropriate antiserum. In serum of high enough concentration, following a sufficient time lapse, complete immobilization of the test animals will result. At the concentrations used for testing (SONNEBORN 1950b), the unabsorbed antisera are remarkably specific, usually having no visible effects on animals of non-homologous or non-corresponding antigenic types. A 10% Ringers solution was used as the diluent for all the sera used in these investigations.

The antisera used were obtained by following, in general, the procedures described by SONNEBORN (1950b). Four anti-D sera and 2 anti-M sera were used as well as antisera against several other antigenic types. The specific antisera used will be indicated in the appropriate places in the section describing the results.

Maximal fission rates were maintained in slide depressions by means of daily reisolations into fresh culture medium (SONNEBORN 1950b). Specific fission rates in test tubes were maintained by daily addition of appropriate quantities of culture medium (PREER 1948; SONNEBORN 1950b).

RESULTS

Simultaneous expression of two antigens, its characteristics and its maintenance

The first indication of the simultaneous expression of two antigens was noted as a decreased reactivity to 51D antiserum of serotype 172D animals. The animals were being grown in slide depressions at maximal fission rate at 31°C by means of daily reisolations into fresh Difco culture medium. Animals from the same depressions reacted somewhat weakly to 51D serum and also to both 32M and 131M antisera. The reaction to 131M antiserum, a variety 8 serum, was stronger than to 32M antiserum which had been obtained against serotype M of the variety 4 stock 32. It could be readily demonstrated that the same animals reacted to both D and the M antisera. One half of the animals from a clone consisting of the progeny of one animal produced in the course of one day were placed in 51D antiserum and the remaining animals placed in 131M antiserum. All the animals in both the sera showed a reaction by having a greatly reduced rate of swimming after 30 minutes. At the dilutions of antisera used, none of the animals in either serum was completely immobilized. Normally serotype 172D animals are immobilized within 15 minutes in such a dilution of 51D antiserum. The animals which were originally in D serum were then placed in M serum and vice versa. After 30 more minutes all the animals in both sera were completely immobilized. Thus, the conclusion that both the D and M antisera affect the same animals, which was initially indicated by the fact that both sera affected 100% of the animals in samples from the same clone, is further supported by the fact that animals showing a partial reaction to one of the sera show an additional reaction in the other serum. This suggested that both the D and the M antigens were being expressed simultaneously in the same animals.

Initially it was suspected that animals seeming to express both D and M antigens simultaneously also could at the same time express antigen J, but this proved otherwise. The DM animals also reacted to a 29J rabbit antiserum. However they did not react either to a 29J chicken antiserum or to a 51J rabbit antiserum, although no significant serological difference has been found between the J antigens of stocks 29 and 51. Moreover a pure J serotype occurs in stock 172 and these 172J animals react normally and strongly to both of the antisera (29J and 51J) to which the DM animals failed to react. It seems likely that the reaction to the original J serum was in reality a cross-reaction unique to that serum and not indicative of the simultaneous presence of the J antigen along with the D and M antigens in the animals tested. For our present purposes we may then disregard the possibility of a triple DMJ type and return to the problem of the double expression of D and M in single animals, specifically, is this to be considered as a true breakdown of the principle of mutual exclusion?

Such a breakdown would not exist on any one of three possible explanations. The most obvious of these, that the tests were made on animals which were in the process of transformation in the usual manner from serotype D to serotype M, could be eliminated immediately. Daily tests of the reisolation lines showed that the reactivity to both D and M sera could be maintained for more than 30 fissions, whereas com-

plete antigenic transformations, as has been mentioned previously, normally occur within one to two fissions in animals that are well fed. The other two explanations are based on the fact that cross-reactions are known to occur between antisera and serotypes which are neither homologous nor corresponding (SONNEBORN and BEALE 1949; BEALE 1954; MARGOLIN 1955). In other words, double reactivity could be due to either a cross-reaction of serotype 172D with M antisera or of serotype 172M with D antiserum.

The possibility that 172D animals merely cross-react to M antisera is easily ruled out. The DM animals described above react comparatively weakly to anti-D serum and also react to anti-M serum. There occur in stock 172 animals that are clearly type D only; they react strongly to anti-D serum and do not cross-react to anti-M serum at all so that the DM animals are clearly different from 172D animals. The second of the two possible cross-reactions, serotype M with D antiserum, could explain the decreased activity of D antiserum, but it is eliminated by some of the following observations.

Animals of stock 172 reactive to anti-M sera never fail to react also to anti-D sera. This of course is consistent with the interpretation that the latter reaction represents a cross-reaction rather than dual expression of two antigens. However, a cross-reaction should be constant. That is, if only a single antigen, M, were present and cross-reacting with D antiserum, the strength of the reactions to both the M and D antisera should remain the same from clone to clone and day to day. In order to discover whether such constancy exists, attempts were made to obtain the double-reacting animals under diverse conditions and to ascertain whether the reactions were identical under all conditions that yield it at all. Series of six lines, initially pure type D, were carried at maximal fission rate by daily reisolutions at 35°, 31°, and 27°C. Similar sets of lines were grown at 19°C with reisolutions every second day and at 14°C with reisolutions every fourth day. The greater intervals between reisolutions at these latter two temperatures served to compensate for the slower growth at low temperature and to provide sufficient numbers of animals for testing from each discard depression following reisolution. Half the animals remaining in each discard depression at every temperature were tested in 51D antiserum at an effective 1/200 dilution and half in 131M antiserum at an effective 1/100 dilution. The tests were examined after two hours. The results were then classified as to whether the animals were reactive to only one of the antisera or to both. In the latter case they were further classified as to whether the degree of the reaction was greater in the D serum, greater in the M serum or not significantly different for both sera. Figure 1 shows, as examples, results for two of the lines at 27°C and two of the lines at 31°C. It can readily be seen that the reactivity to the two sera varies from time to time for the same line, sometimes D serum being more effective, sometimes M serum being more effective and sometimes the two sera being equally effective. This sort of variation is definitely not consistent with the view that a cross-reaction of the pure M antigen is being detected with the D antiserum, as was suggested above. A pure M antigen would have shown a consistently similar reaction from day to day to both the corresponding M serum with which it was reacting and the D serum with which it was cross-reacting. The only simple remaining explanation for such a reaction to

FIGURE 1.—Comparisons of the strengths of reactions to 51D and 131M antisera of two daily reisolation lines of initially 172D animals grown at 27°C and two grown at 31°C.

two different sera is that two antigens are present on the cilia at the same time and that the mutual exclusion mechanism is either not operating or its effects are being subverted in some manner. The variation in the effectiveness of the two sera supports the conclusion that the two antigens are present at the same time since DIPPELL (1953) in variety 4 and BEALE (1952, 1954) in variety 1 have found that such variations occur in heterozygotes where, with dominance being absent, two allelically controlled but serologically different antigens are expressed simultaneously.

The simultaneous expression of the D and M antigens was obtained at most temperatures tested, but under no conditions was the M antigen detected alone. The 14°C lines were discarded after approximately 35 fissions without any M antigen ever being detected, but three of the lines eventually transformed to serotype G. At 19°C only some of the lines showed a reaction to M serum and then only sporadically and weakly. At the three higher temperatures all the lines at one time or another showed strong reactions to the M antiserum. The higher the temperature the fewer fissions before all the lines expressed M. At 35°C all the lines showed a reaction to the M antiserum by the 7th fission and the reaction to M began to disappear by about the 50th fission. The disappearance of the M reaction was accompanied by a marked decrease in fission rate and 5 of the 6 lines died out shortly afterward. At

31°C the lines all reacted to M antiserum by the 10th fission although some of the lines reacted earlier. The M reaction began to disappear shortly after the 80th fission and the disappearance was again accompanied by a marked decrease in fission rate followed by death of most of the lines shortly afterward. At 27°C not all the lines were showing reactions to M antiserum until the 40th fission, although one line showed it as early as the 5th fission. All the lines continued to express M throughout the course of the experiment, lasting about 100 fissions for these lines. There was no marked decrease in the fission rate of these 27°C lines.

From the above description it seems that the appearance and maintenance of the M antigen in stock 172 is related to a high metabolic rate. The higher the temperature and the higher the fission rate, the more rapidly it comes to expression and the more readily it is maintained. Growth in depressions, when held to low fission rates of approximately one fission per day by limiting the food supply, does not bring about the expression of the M antigen. If the M antigen is already present when growth at such low fission rate is begun, it disappears. Under no conditions could the presence of the M antigen be maintained in test tube cultures, not even at maximal fission rate at 31°C.

A second set of daily reisolation lines was grown at 31°C and tested periodically in a series of dilutions of both the D and M antisera to determine the immobilization titres in these two sera. The immobilization titre is defined as the greatest dilution

FIGURE 2.—Immobilization titres in 51D and 131M antisera of 4 daily reisolation lines at 35°C and 4 at 31°C, showing the negative correlation of the variation in titre in these two sera. Dilutions corresponding to those represented by the numbers 1 to 6 are 1/12.5, 1/25, 1/50, etc. to 1/400, respectively, each being half the concentration of the preceding one.

which causes immobilization in two hours (SONNEBORN 1950b). Comparisons of the titres in the two sera do not indicate quantitative ratios of the two antigens since the two sera are not necessarily equivalent in their ability to immobilize. For example, a higher titre in M serum does not necessarily mean that there is more M antigen present than D antigen. It may merely mean that the M serum is a stronger serum than the D serum. However, comparison of the variations in titre of the M serum with variation in titre of the D serum serves to indicate relative variations in the quantities of the two antigens on the animals. There is found a negative correlation between the variations in the titres in the two sera: the higher the titre in the D antiserum, the lower the titre in the M antiserum. When the titre in M antiserum rises the titre in D antiserum falls. This is shown by figure 2 which compares the immobilization titres in the D and M sera. The values in figure 2 represent single observations. The differences between the lines at each temperature do not remain constant since the D and M titres of each line vary with time. The variations in titre most likely indicate variations in the quantities of each of the antigens, as suggested above. If the simultaneous expression of the two antigens is due to a breakdown of the mechanism of mutual exclusion then the negative correlation may indicate some sort of competitive relationship between the antigens. Such competition could either be for the antigen sites on the cilia or for some common substrate or precursor which is in limited supply.

Attempts to establish simultaneous double expression of serotypes other than D and M

If one attempts to conceive of a relationship between two serotypes which might be conducive to their simultaneous expression, similarity in conditions for stability is one which immediately comes to mind. It is not possible to determine whether or not such a relationship exists in the case of D and M since the conditions for maintenance of a pure M serotype have never been found. However, to determine if other double types could be established when such a relationship is known to exist, the following two experiments were carried out.

For the first experiment serotypes 172B and 172G were chosen because both were found to be very stable under the same conditions of culture. Lines of these two types were grown for more than 50 fissions in daily reisolutions at 27°C; they very rarely transformed to any other serotypes during this period of growth at maximal fission rate. But each line also remained pure for the initial type, either B or G, never showing simultaneous expression of both. There still remained the possibility that if animals, which were already expressing both types because they were in the midst of a transformation from one to the other, were placed under these conditions, the double type might be maintained. It had been found previously that B animals transformed primarily to G at 14°C, following exposure to immobilizing antiserum (MARGOLIN 1955). Serotype B animals were exposed to antiserum and washed as previously described (MARGOLIN 1955) and then isolated into fresh Difco medium and placed at 14°C. Every 4 hours during the next 12 hours groups of six isolations were transferred to 27°C. Two additional groups were later transferred at eight hour intervals. The transferred lines were then grown at maximal fission rate at 27°C by daily reisolutions. Each day's discards were tested for the double expression of B and

G. No such double expression was encountered in any of the lines. Some lines remained B, some transformed to G and some to other serotypes.

A second experiment which attempted to determine if similar conditions for stability could be a basis for simultaneous expression of two serotypes made use of cytoplasmic exchange to initiate a mixed cytoplasm. As mentioned previously, it had been found that the cytoplasm plays a part in determining which serotype is expressed. It was felt that an exchange of small amounts of cytoplasm between two serotypes having the same conditions for stability, followed by growth under these conditions might initiate and maintain a double expression. Serotypes 172B and 172D were chosen as the two types. Type B, as mentioned above, is quite stable in daily reisolation at 27°C. Type D is also stable under these conditions in the sense that at least some D antigen continues to be expressed. The M antigen normally also appears along with D under these conditions indicating that D is at least amenable to double expression, and bringing up the possibility of obtaining a triple type, BDM.

The cytoplasms of D and B animals were mixed by obtaining an exchange of cytoplasm during conjugation. The process has been described by SONNEBORN (1950b). The length of delay in complete separation of conjugants and the broadness of cytoplasmic bridges which may develop between the separating conjugants are taken as indices of the amounts of cytoplasm exchanged. In this experiment no broad bridges were formed and the delays in separation ranged from 5 to 44 minutes among 12 conjugating pairs. Under these conditions only comparatively small amounts of cytoplasm can be expected to have been exchanged. All the exconjugants were then carried as daily reisolation lines at 27°C for approximately 18 fissions. Each day's discards were all tested with B, D and M antisera. In no case was the B antigen found expressed simultaneously with any other antigen. It would appear from the results of this and the preceding experiment that similarity in conditions for stability alone is not a sufficient basis for the simultaneous expression of two antigens.

There remains one additional type of relationship known to exist between some serotypes which might conceivably serve as a basis for the maintenance of double antigenic expression. If two serotypes which readily and preferentially transform to each other could be maintained in a state of constant, rapid transformation back and forth, the detectable results would be the simultaneous expression of the two antigens. This sort of an explanation for double expression has in its favor that it does not require a breakdown of the mutual exclusion mechanism. SONNEBORN (unpublished) had found that in stock 131 of variety 8 the above described sort of relationship existed between serotypes E and H. At a low fission rate type E transformed readily to type H, whereas at a high fission rate H transformed to E, all at 27°C. To determine whether or not at some intermediate fission rate a state of continuous transformation from one serotype to the other could be maintained a series of test tube cultures, initially pure for 131H, were grown at various fission rates. Average fission rates of $\frac{1}{4}$, $\frac{1}{2}$, $\frac{3}{4}$, 1, 2 and 3 fissions per day were maintained by daily adding appropriate quantities of Difco culture medium and duplicate cultures were grown at each rate.

After three weeks of growth one sample from each tube was tested with E antiserum and a second sample with H antiserum. The $\frac{1}{4}$, $\frac{1}{2}$ and $\frac{3}{4}$ fissions per day tubes were found to have remained 98% or more pure for type H. About half the non-H animals were type E and the remainder were not identified as to serotype. The remaining tubes were mixtures of animals which were primarily of types E and H. The higher the fission rate, the higher the percentage of type E animals found, ranging from an average of 9% of type E in the 1 fission per day tubes to an average of 55% of type E in the 3 fissions per day tubes. In all cases the percentage of E animals from a tube plus the percentage of H animals from that tube always added up to approximately 100%, when allowance was made for a sampling error and a small percentage of animals which were neither E nor H. This would indicate that E and H were not being expressed simultaneously in the same animals. Such a double expression would have been expected if a state of continuous back and forth transformation between E and H existed. Apparently a readiness for two serotypes to transform to each other preferentially is not alone sufficient to support such a state.

Genetic analysis

The experiments described in the previous section indicate that the two sorts of relationships which were considered, similarity of conditions of stability for two serotypes and preferential transformation back and forth between two serotypes, do not support simultaneous expression of two antigens. This suggests that some special relationship may exist between 172D and 172M which has not been recognized. Since the relationship itself is not at present discernible the next approach would be to attempt to determine at least on what level it exists. That is, whether it occurs only for the specific D and M serotypes of stock 172, or for D and M serotypes of any stock or for any serotypes which have the necessary proper characteristics. The relationship can be divided into two parts; the role of the M serotype and the role of the D serotype. The evidence suggests that some specific characteristic of the M serotype of stock 172 is involved. It is not a general characteristic of M antigens in *P. aurelia* as indicated by the fact that serotype M of stock 32, variety 4, always appears as a single antigenic type. Serotype 172M not only differs from all the serotypes except D by showing the capacity for dual expression, but it even differs from D in never showing the capacity for single expression under any of the conditions used. There thus seems to be something very special and, thus far, unique about antigen 172M that makes double expression possible.

But there is more to the story than this, for 172M is expressed only with D, not with any other serotype,—not even with any serotype that is stable under conditions in which M is expressed. Thus when serotypes 172B or 172G are grown at maximal fission rate at 27°C serotype M does not come to expression. However if 172D is the initial type under these conditions type M does come to expression. The question then arises as to whether the ability to be expressed simultaneously with the 172M antigen is a general characteristic of all the D antigens or if it is a unique characteristic of the 172D serotype. Stock 32 has both D and M serotypes but they have never been found expressed simultaneously. Type 32M perhaps does not have the proper

characteristics to permit such double expression. Would the 32D serotype come to double expression with 172M if the d^{32} gene were substituted for the d^{172} gene in the stock 172 genome?

Fortunately serotype 172D is serologically distinguishable from serotype 32D, so that the above question can be answered. The sera used to distinguish these two serotypes from each other were 138D and 32D. The immobilization titres of each of the serotypes were determined in each of the antisera using the methods described by SONNEBORN (1950b). It was found that 138D antiserum had a clearly higher titre against 172D animals (immobilization titre: 1/200 dilution) than against 32D animals (immobilization titre: 1/50 dilution), whereas the titre of 32D antiserum was higher against 32D animals (immobilization titre: 1/200 dilution) than against 172D animals (immobilization titre: 1/50 dilution). Dilutions of these two sera were chosen which readily distinguished the two antigens from each other by the differences in the strengths of their reactions in each of the sera, following exposure for two hours. In this case, at the dilutions of sera used, the heterozygote could not be distinguished from the 32D homozygote. This does not necessarily indicate dominance of the d^{32} gene. DIPPEL (1953) has found in a similar case in variety 4 that the heterozygote could be distinguished from the homozygote which it resembled if enough different sera were tried. All the work described below was carried out in Cerophyl culture medium.

Eight backcrosses to stock 172 were made so as to approach isogenicity. The F_2 obtained by autogamy from the final backcross gave a ratio of 42:41 for the 32D antigen and 172D antigen, respectively (four F_2 lines died before testing). This was clearly a 1:1 ratio, indicating allelism of the d^{32} and d^{172} genes. The numbers obtained are too small to distinguish between alleles and closely linked genes, but no cases of serologically corresponding serotypes have ever been found to be non-allelic in variety 4 (SONNEBORN 1950a, 1951, unpublished; BEALE 1954).

One of the F_2 lines, homozygous for d^{32} , obtained from the eighth backcross described above was crossed once again to 172D. The exconjugants, which were then heterozygous for the two alleles for the D serotype, were grown in daily reisolation lines at maximal fission rate at 31°C. Controls consisted of crossing the 172D animals used in the above cross to another clone of 172D animals of the complementary mating type. The exconjugants from this control cross, which were homozygous for the d^{172} gene, were grown under the same conditions as the experimental heterozygotes. Ten exconjugants of each sort were cultured as described and animals in discard depressions were periodically tested with 32D, 172D and 131M antisera. The 172D antiserum was used in place of 138D antiserum. These two sera act similarly in distinguishing between the two D serotypes. The M antigen was detected in both the experimental and the controls and as expected it was always accompanied by the presence of some D antigen. The presence of a single dose of the d^{32} gene did not prevent the simultaneous expression of the D and M antigens but it did have an effect. All the homozygous 172D lines manifested some M antigen before even one of the heterozygotes was manifesting it. In fact one of the heterozygote lines never showed any M antigen during the 54 fissions it was kept. Another effect which was noted was that the quantity of M antigen, as indicated by the strength of the reaction

in the M antiserum, never became as great in the strongest M reacting heterozygotes as it did in the strongest M reacting lines which were homozygous for the d^{172} gene. The single d^{32} gene, while not completely preventing the double expression of the D and M antigens, clearly had the effect of retarding and diminishing the expression of the M antigen.

The next step consisted of testing the effect of the homozygous d^{32} condition. An F_2 was obtained by autogamy from one of the heterozygotic lines used for the experiment above. Five of these F_2 's, homozygous for d^{32} and five homozygous for d^{172} were tested for the presence of the M antigen. All were found to be expressing only the D serotype. Three subcultures from each of these lines were then grown at maximal fission rate in daily reisolutions at 31°C. The 15 reisolation lines homozygous for d^{32} and the 15 homozygous for d^{172} were tested periodically with 32D, 172D and 131M antiserum. The same general effect of the presence of 32D was found as had been found in the heterozygotes. The expression of the M antigen was retarded but not always completely suppressed. The degree of retardation of development of the M antigen was not significantly greater in the presence of the double dose of the d^{32} gene than in the heterozygotes containing only a single dose of the gene.

In the two experiments described above the D antigen which was detected when the M antigen was present in the lines having d^{32} genes, always reacted with the two D antisera used in the same way that the pure 32D serotype reacted. That is, the reaction was distinctly stronger in 32D antiserum than in 172D antiserum. The reverse was always true in the lines which were homozygous for the d^{172} gene. This is shown clearly in table 1. The 32M antigen is readily distinguishable from the 172M antigen using 32M and 131M antisera. Serotype 32M reacts more strongly than serotype 172M in the 32M antiserum and the reverse is true for 131M antiserum.

TABLE 1

Reactions to 131M, 32D and 172D antisera of samples of eight animals exposed for two hours, showing that the 32D and 172D antigens can be distinguished even when being expressed simultaneously with 172M

Lines tested		Antisera used and effective dilutions		
		131M, 1/50	32D, 1/100	172D, 1/100
I	1	+++	++	+
	2	++	+++	++
II	1	+++	-	++
	2	++	-	+++
III	32D	-	+++	++
	172D	-	+	+++

- no visible effect of antiserum.

+ least definitely perceptible retardation of movement.

++ very sluggish movement.

+++ complete immobilization.

I Two lines homozygous for the d^{32} gene and approximately isogenic with stock 172. Grown in daily reisolutions at 31°C.

II Two lines homozygous for the d^{172} gene and grown in daily reisolutions at 31°C.

III Samples from stock tube cultures of 172D and 32D grown at one fission per day at 27°C.

The M antigen which was expressed in the lines containing the d^{32} genes was tested in appropriate dilutions of these two sera and could always be identified as 172M. This was, of course, to be expected in approximately isogenic lines.

Since 32M and 172M were readily distinguishable an attempt was made to create a stock containing the m^{32} gene but essentially isogenic with stock 172. Great difficulty was encountered following the initial cross between stocks 32 and 172. Serotype 32M was found to be extremely unstable in the hybrid under all conditions which were tried. No means was discovered which would cause 32M to come to expression in the F_2 lines obtained by autogamy from this first cross. The lines were then grown for many months in tubes at one fission per day at 27°C and tested periodically for the presence of 32M animals. Finally one such line was discovered and used for the first backcross to stock 172. The same difficulty with 32M was encountered in this cross and the same means used to overcome it. A total of three backcrosses was finally obtained by this method. Fifteen F_2 lines obtained by autogamy from the third backcross, all expressing the 172D serotype, were grown at maximal fission rate by daily reisolations at 31°C and periodically tested for simultaneous expression of the 32M and 172D antigens. All the lines were grown for approximately 40 fissions under these conditions but in no case was such double antigenic expression encountered. One line transformed to 32M after about 25 fissions and continued to express the M antigen for the remainder of the experiment without exhibiting any detectable trace of the D antigen. The 32M antigen does not appear to be compatible with double expression even in the presence of the d^{172} gene and what was primarily a 172 genetic background. This confirms the conclusion, previously mentioned, that the capability for double antigenic expression was a specific characteristic of the 172M serotype and not a generic characteristic of the variety 4 M serotypes.

DISCUSSION

The above results demonstrate clearly that under appropriate conditions two antigens controlled by non-allelic genes can be expressed simultaneously and this dual expression is maintained as long as the conditions remain the same. This immediately raises the question as to whether or not this represents an actual lack of functioning on the part of the mechanism which normally maintains mutual exclusion of such antigens. The only simple explanation not requiring such a lack of functioning is that a state of continuously unstable transformation is being maintained between serotypes D and M. That is, serotype D transforms to serotype M which immediately transforms back to D and so on. Evidence presented in the results section indicated that a readiness of two antigens to transform to each other preferentially is not alone sufficient to maintain such a state. However, it is possible to visualize additional relationships between two serotypes which might be expected to sustain it.

For example, two antigens exhibiting the following relationships could be maintained in a state of continuous transformation under the proper conditions. The two antigenic types, as mentioned before, would have to show a very strong preference to transform to each other rather than to any of the other potential serotypes. In addition they would have to be mutually exclusive on the basis of the conditions of culture which allow their expression. That is, two serotypes, each stable in different

portions of a range of conditions of culture and each completely unstable under the conditions favoring the other. It would be necessary that there be no zone of overlapping of ranges of stability; the stability ranges must be contiguous and mutually exclusive. If animals expressing one of the two serotypes in question were then grown under conditions which approximated the boundary dividing the stability ranges of the two serotypes, slight fluctuations would cause transformations back and forth as the conditions shifted from one side of the boundary to the other. It is interesting to note that the conditions necessary for the origin and maintenance of the DM double type, maximal growth rate in the continued presence of excess food, are the very conditions which can be maintained most constantly, subject only to the minor fluctuations such as are hypothesized above. Furthermore, the M antigen is never expressed under the same conditions as the D antigen except in the very narrow range of conditions where they are expressed as a double type. The assumption, on the basis of the above hypothesis, would then be that, in the medium used, maximal growth rate just reaches a threshold value above which the M antigen can be expressed but not the D antigen. Below this threshold the D antigen can be expressed but not the M antigen. Assuming further that D and M transform to each other preferentially, the result of minor fluctuations above and below the threshold would be a continuous series of transformations between D and M.

The above explanation can also account for the retarding effect, previously mentioned, of 32D on the expression of 172M. SONNEBORN and BALBINDER (unpublished) have obtained evidence indicating that the genes for the specificities of all the serotypes in a genome interact in determining the relationship of any one serotype to environmental conditions, with respect to both stability and origin. Therefore the presence of the d^{32} gene may have shifted upward the threshold value needed for the expression of M. Fewer peaks in fluctuation would be sufficiently great to cross this higher threshold so that D would transform to M less often and the M expression would be of shorter duration and retarded. Thus by means of a formal explanation the simultaneous expression of the D and M antigens and the characteristics of this double expression can be accounted for without resorting to non-functioning of the mutual exclusion mechanism; but the latter possibility is not thereby excluded.

One somewhat inconclusive fact points to the actual breakdown of the mutual exclusion mechanism in this case. The mechanism is known to breakdown regularly with respect to allelic serotypes: a heterozygote expresses both allelic serotypes (BEALE 1952, 1954; DIPPELL 1953; MARGOLIN 1955). In a different sense alleles are also involved in the DM case. Double expression with M occurs when either of two d alleles are present, d^{172} and d^{32} . On the other hand, replacement of m^{172} by m^{32} results in failure to obtain DM, as does also the combination $d^{32}m^{32}$. The argument therefore is rather weak, but nevertheless it must be kept in mind.

In sum, both alternative explanations, repeated transformations and breakdown of the mechanism of mutual exclusion, are equally compatible with the observations. Actual criteria that would differentiate the two explanations operationally are lacking. Both would give exactly the same detectable results, namely, the simultaneous presence of both D and M antigens.

Here, however, a distinction must be made between mutual exclusion as a mech-

anism and mutual exclusion as an observation. The two explanations offered above attempt to interpret the observed breakdown of the mutual exclusion phenomenon from the point of view of the mechanism by which it is normally maintained. The observation of mutual exclusion refers simply to the fact that usually only one ciliary antigen can be detected in any animal at any one time. Two sorts of exceptions to the observation have previously been known: one is the case of allelic serotypes in a heterozygote, mentioned above; the other is the transient state of transformation, narrowly localized in time, between two relatively stable and different single serotypes. The present DM case is a third sort of exception to the observation of mutual exclusion: a persistent condition in which two non-allelic serotypes are continually expressed.

SUMMARY

Contrary to the usual condition of mutual exclusion existing among non-allelic ciliary antigens of *P. aurelia*, stock 172, variety 4 is regularly capable of expressing simultaneously two ciliary antigens, D and M. This dual expression can be maintained through many reproductive cycles when the animals are grown at maximal fission rate by means of daily reisolutions at 35°, 31° and 27°C. Antigenic type D is often expressed under different conditions as a single antigen, in the normal manner, but the M antigen when detectably present is always accompanied by the D antigen. In any one line of descent expressing DM the ratios of the quantities of the two antigens vary from time to time. There is a negative correlation in the variations in quantity of the two antigens, an increase in one usually being correlated with a decrease in the other. This suggests a competitive relationship of some sort.

Attempts to initiate similar dual expression of other antigens on the basis of similarity in conditions for stability or a readiness for preferential transformation to each other of two antigenic types failed. The capacity for dual expression appears to be due to some unique quality of the 172M antigenic type. The M antigenic type of stock 32, variety 4, does not show this capacity either in the stock 32 genome or when the m^{32} gene is substituted for the m^{172} gene in a genome that was primarily that of stock 172. However, when the d^{32} gene is substituted for the d^{172} gene in a genome which is approximately isogenic with stock 172, the 32D antigen does show a capacity for dual expression with 172M, although the expression of M is somewhat retarded when compared to that which is found with the 172D antigen.

The observations can be explained as due to either a non-functioning of the mutual exclusion mechanism or a state of continuously unstable transformations between the D and M antigenic types. Operationally these two explanations are identical since they both lead to the continuous simultaneous expression of two non-allelic antigens. The persistence of the DM state and the fact that it involves non-allelic antigens, together distinguish this exception from the two previously known classes of exceptions to mutual exclusion.

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