H SEROTYPE DIFFERENTIATION IN TETRAHYMENA¹

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THE *H* locus, controlling the specificity of the H immobilizing-antigens in variety 1 of *Tetrahymena pyriformis*, is involved in a system of "allelic repression" (NANNEY and DUBERT 1960; NANNEY 1963c). Cells heterozygous for genes at this locus yield heterogeneous clones, some sublines of which manifest one allele, and others of which manifest the other allele. The clonal differentiation proceeds progressively as larger and larger fractions of the cells become irreversibly monotypic. The basis for this differentiation is believed to be an inactivation of one of the alleles in each of the macronuclear subunits during the development of macronuclei at conjugation. The extended and progressive clonal differentiation is interpreted as due to an assortment of the differentiation subnuclei, which occurs in precisely the same manner as the assortment of subnuclei differentiated for mating types (ALLEN and NANNEY 1958; SCHENSTED 1958; NANNEY 1960). Similar systems of mutual exclusion in allelic expression have been discovered for esterases and phosphatases (ALLEN 1960, 1961; ALLEN and MISCH 1962).

The mechanisms of allelic repression are not understood, but some information has been acquired about the conditions affecting the establishment of particular states of exclusion. This communication documents the fact that the particular allelic combination established in a heterozygote is a major factor determining the direction of allelic repression. Efforts to modify allelic repression by treatment of differentiating cells are briefly reported.

MATERIALS AND METHODS

Most of the materials and methods employed in this study are described in a preceding paper (NANNEY 1963d). Most of the data were obtained from a series of 48 crosses among inbred strains of variety 1. Of these, 25 were found to show evidence of genetic irregularities and were not included in the assessment of allelic interactions. Except where indicated, no more than three sublines were derived from a single pair so that the minimum number of pairs sampled may be estimated by dividing the number of sublines by three. The immunological procedures were those described by MARGOLIN, LOEFER and OWEN (1959); NANNEY and DUBERT (1960); and LOEFER and OWEN (1961). Some special techniques are described later.

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To permit comparisons between crosses, the serotypes of the sublines from a cross (usually no more than three from any one pair) were determined and then reduced to an index designated as the "output ratio." The calculation of the output ratio requires a brief explanation and justification. If two specificities, say Hx and Hy, are represented in a cross, individual sublines may express either or both of the specificities. If both specificities are expressed, individual cultures may be relatively strongly affected by one or the other antiserum. Cultures may also differ in the distribution of serum responses; in some cultures most of the cells respond to both antisera, but in other cultures many of the cells respond to only one antiserum. An additional complication arises from the fact that many cultures contain varying proportions of cells which are resistant to both the parental antisera and which are probably expressing serotype specificities controlled by other genes (see NANNEY 1962). Hence, sublines from a cross may be classified into a large number of categories. A simpler procedure would be to determine simply whether or not a particular culture reacted to an appreciable extent with each of the specific antisera. This would yield only three classes of sublines in each cross: Hx, Hxy and Hy, and these could be readily reduced to an output ratio.

Another complication in determining the output ratios involves the time at which the population is assessed. Initially a population of heterozygous sublines has a large fraction of lines showing intermediate responses (Hxy), and only after 100 or more fissions are the large majority monotypic. The question arises as to whether output ratios determined for "young" populations can be equated with those from more mature populations. The early assessment of output ratios is of course highly desirable. To examine this problem output ratios were computed from data previously published (NANNEY and DUBERT 1960) on a population which was serotyped at each transfer interval. The procedure used was the following. Each subline was classified as Hx, Hxy or Hy. The output for Hx was then determined by the formula $100 \times (2Hx + Hxy)/2N$ where N is the number of lines typed. The output ratio for the other specificity may be calculated in a comparable way, or simply by subtraction since $100 - Hx = Hy$. This treatment will give comparable results at any two time intervals if the Hxy clones have approximately equal probabilities of differentiating to the two pure types. A bias is expected in the early samples since the rates of stabilization to the two alternative types may be very different initially, and only gradually approach equality over an extended period of time (ALLEN and NANNEY 1958; SCHENSTED 1958). Since the rate of stabilization is initially higher toward the majority type, the assumption of equality of rates would lead to an underestimate of the majority type. That this bias is not a serious factor, however, is shown by calculating the output ratios for the single population at various fission intervals (Table 1). The calculated output ratios show little variation between 30 and 120 fissions, as the fraction of intermediate clones declines from over 40 percent to about 15 percent. Hence, at least for eccentricities of 3:1 or less, the output ratios may be approximated reasonably accurately as early as 30 fissions. Most

TABLE 1

*The determination of output ratios in populations of sublines at various times after conjugation**

Age in fissions	Subline serotypes			N	Output ratio
	Hx	Hxy	Hy		
18	0	11	5	16	34:66
31	15	100	123	238	27:73
44	17	100	120	227	30:70
57	20	95	126	231	29:71
70	29	71	139	239	27:73
83	42	48	144	234	28:72
96	41	51	147	239	28:72
109	40	50	149	239	27:73
122	41	38	157	236	26:74

* Calculated from Table 4 of NANNY and DUBERT 1960.

of the data were in fact obtained from populations which had undergone 80 or more fissions, but some populations were typed as early as 50 fissions.

RESULTS

Output ratios under standard conditions. The first studies on clonal differentiation of serotypes (NANNY and DUBERT 1960) revealed that output ratios vary from cross to cross. Although most heterozygous clones manifest some degree of response to antisera against either parent when they are tested ten to 15 fissions after conjugation, these responses are often unequal. Although a strict quantitative comparison between the initial responses and the later assessed output ratios was not possible, a qualitative agreement could be seen.

The first studies also demonstrated that the output ratios for different macro-nuclei produced in the same cross, and even in the same pair, could be different. To determine the relationship between the genotype generated in a cross and the output ratio of that cross requires therefore that many caryonides be examined; to determine an output ratio for one or a few caryonides could be misleading. Most of the data to be presented were collected in a manner designed to minimize the contributions of individual caryonides; usually only two or three isolation lines were started from each pair culture and relatively few caryonides are represented by more than one or two sublines in the total population. In some of the crosses output ratios were determined for a number of individual caryonides and subcaryonides; in these cases the output ratios of the total populations are given, but a consideration of the distribution of output ratios in cell lineages will be deferred for a later paper.

Originally 16 crosses under standard conditions (26°C in *Aerobacter-Cerophyl*) remained after screening for genetic aberrations. These have been supplemented by a few additional crosses and are presented along with previously published data (Table 2). Although some variation is apparent in crosses with the

TABLE 2
Serotype output ratios in heterozygotes under standard conditions

Genotypes	Parents	Cross no.	Serotype distribution			Total	Output ratio
			Hx	Hxy	Hy		
H^A/H^C	*	*	103	21	9	133	85:15
	A, D1	60-201	51	11	9	71	81:19
		61-23	74	6	3	83	93: 7
		62-50	38	9	4	51	83:17
		62-72	31	20	5	56	73:27
	Total		297	67	30	394	84:16
H^A/H^D	*	*	112	3	5	120	95: 5
	A, B	61-73	42	14	0	56	87:13
		61-74	163	32	5	200	90:10
		62-75	193	37	50	280	76:24
	A, E	62-65	41	20	5	66	77:23
	Total		551	106	65	722	84:16
H^A/H^E	A, C	61-20	11	10	69	90	18:82
		62-70	11	12	36	59	29:71
	Total		22	22	105	149	22:78
H^C/H^D	*	*	41	38	157	236	15:75†
	*	*	156	110	53	319	66:34†
	B, D1	62-23	29	6	16	51	63:37
		62-24	19	13	39	71	36:64
		62-64	230	223	244	697	49:51†
		62-65	170	82	260	512	41:59†
		62-73	17	12	27	56	41:59
		62-74	147	165	862	1174	20:80†
		62-77	17	9	63	89	24:76
	Total		826	658	1721	3205	36:64
H^C/H^E	B1, C	60-187	8	2	109	119	8:92
H^D/H^E	A1, C	60-185	13	9	62	84	21:79

* Crosses with early inbred strains reported by NANNY and DUBERT (1960).

† Ratios computed from samples including many lines from each caryonide.

same H genotype, certain patterns are clear. In each cross in which the H^E allele is present, the corresponding serotype (He) is the predominant phenotype in the progeny. Similarly, when H^A is present (except with H^E), the Ha serotype is the predominant phenotype in the heterozygotes. The situation with regard to the H^C/H^D heterozygotes is less regular. The output ratios are usually less extreme than in the other cases, but either the Hc or the Hd serotypes may be predominant types in different experiments.

Output ratios under modified conditions. Evidence from cell lineage studies to be presented later indicates that serotype differentiations occur during the development of new macronuclei at conjugation. Hence, the period immediately following conjugation should be a critical period in modifying output ratios. In hopes of specifically altering the differentiations a number of kinds of treatments

have been tried in preliminary experiments. Thus far the treatments have yielded negative or inconclusive results and these will be presented only briefly.

1. *Temperature variations.* To determine whether the temperature at which conjugation occurs is significant in serotype differentiation, a number of crosses have been carried out at 19°C and 32°C, as well as at 26°. Pairs were induced at these temperatures, isolated at room temperature, and returned to the lower or higher temperatures until they had undergone at least one post-zygotic fission. Thereafter the progeny were brought to room temperature and maintained there until the experiment was completed.

A large fraction of the crosses at these temperatures had to be rejected for cytogenetic irregularities (NANNEY 1963d), but some data were obtained for each of the six genotypes. Although some variation occurs between crosses run at different temperatures (Table 3), the variation appears not to be greater than that between crosses generating the same genotype at the same temperature. The same general pattern of allelic expression is observed in these crosses as in those conducted at room temperature. If temperature is a significant variable in serotype differentiation, more extensive studies will be required to establish it.

2. *Antiserum effects.* In *Paramecium aurelia* (see BEALE 1954, 1957) homologous antiserum has been found effective in promoting alterations in expressed antigens. Moreover, similar antigenic changes have been described (MARGOLIN *et al.* 1959) in *Tetrahymena*. Although in each of these cases the shifts in antigenic expression appear to involve nonallelic genes, the possibility that antiserum against one of the allelic specificities in a heterozygote might promote the expression of the alternative type seemed worthy of exploration.

TABLE 3
Temperature effects on output ratios

Genotypes	Parents	Cross no.	Temperature (Degrees C)	Serotype distribution			Total	Output ratio
				Hx	Hxy	Hy		
H^A/H^C	A, D1	62-72	19	30	12	9	51	71:29
			26*	31	20	5	56	73:27
			32	44	27	13	84	68:32
H^A/H^E	A, C	61-20	26*	11	10	69	90	18:82
			32	16	13	64	93	24:76
H^C/H^D	B, D1	62-73	19	7	7	34	48	22:78
			26*	17	12	27	56	41:59
		62-77	26*	17	9	63	89	24:76
			32	17	11	56	84	27:73
H^C/H^E	B1, C	60-187	26*	8	2	109	119	8:92
	D1, C	62-59	32	2	4	42	48	8:92
H^D/H^E	B, C	62-71	19	2	10	20	32	22:78
	A1, C	60-185	26*	13	9	62	84	21:79
	B, C	60-202	32	5	10	70	85	12:88

* Crosses reintroduced from Table 2.

Antiserum effects were sought in a cross between inbred series A and B, which yields the H^A/H^D heterozygote. Matings were carried out at room temperature and pairs were isolated either into Cerophyl-Aerobacter media or into antiserum diluted with de-ionized water. In the first experiment three antisera against three different H specificities were employed at concentrations (1/100) just beyond their immobilization range. MARGOLIN *et al.* (1959) had shown that such low concentrations were capable of inducing transformations in vegetative cells, and low concentrations might be expected to minimize any selective effects which would complicate the interpretation. However, no significant effects of the treatment were apparent (Table 4) ($P = 0.4$).

One other attempt to demonstrate an antiserum effect was made, using a similar cross. In this case non-immobilizing sera were used and at a higher concentration (1/10). One serum was prepared against an H^D homozygote which had been grown in peptone (Anti-P); another against an H^D homozygote grown at 15°C in Aerobacter-Cerophyl; the other two antisera were against H specificities which were not involved in the cross. Such experiments might reveal effects on antigenic components other than the immobilizing antigens which could secondarily modify the course of serotype differentiation. The data again, however, reveal no significant consequences of antiserum treatment on serotype differentiation ($P = 0.05$ to 0.10).

Brei effects. Serotype differentiation at conjugation in *Paramecium aurelia* (see BEALE 1954, 1957) involves the transfer of information from the parental cell to the daughter cells through the mediation of the cytoplasm. In Tetrahymena, on the other hand, critical evidence for information transfer under these conditions has not been obtained (NANNEY 1963b). A suggested basis for this difference between the two forms is that agents of information transfer are present in both Ciliates and may play an important role in the vegetative persistence of serotype differentiations, but that these agents are either used up or destroyed at conjugation in Tetrahymena prior to macronuclear differentiation. In this case, an agent capable of directing serotype differentiation in Tetrahymena might be present in vegetative cells.

TABLE 4
Antiserum effects on output ratios

Cross	Treatment of pairs	Serotype distributions			Total	Output ratio
		Ha	Had	Hd		
61-73	None	42	14	0	56	87:13
	Anti-Ha (1/100)	91	9	3	103	93:7
	Anti-Hd (1/100)	55	4	3	62	92:8
	Anti-Hc (1/100)	98	13	11	122	86:14
61-74	None	163	32	5	200	90:10
	Anti-P (1/10)	163	32	4	199	90:10
	Anti-L (1/10)	191	17	1	209	94:6
	Anti-Hc (1/10)	174	28	2	204	92:8
	Anti-He (1/10)	154	42	4	200	87:13

TABLE 5

Brei effects on output ratios

Cross	Treatment of pairs	Serotype distributions			Total	Output ratio
		Ha	Had	Hd		
61-73	None	42	14	0	56	87:13
	Ha brei	31	2	1	34	94:6
	Hd brei	174	3	7	184	95:5
	Hc brei	155	4	1	160	98:2

Cells were grown in Cerophyl-Aerobacter media, harvested by centrifugation and frozen. After thawing, the packed cells were diluted in four parts of de-ionized water and pairs were isolated from a cross of A $\times$ B into the suspension. The results (Table 5) indicate that the brei treatment was significant in increasing the frequency of Ha differentiations. However, no specificity in this effect was apparent; the most marked effect was in fact obtained from a brei prepared from cells producing an H antigen not involved in the cross at all. If informative agents were present in the brei, they were inactivated or failed to gain entry into the conjugating cells.

RNA effects. Although crude breis failed to yield specific effects on serotype differentiation, the possibility existed that cellular extracts presented to the differentiating cells under other conditions would be effective. In view of the suggestion (see NANNEY 1963a, 1963c) that internuclear communication might be accomplished by ribonucleic acid, an attempt was made to ascertain whether purified ribonucleic acid had significant effects.

For this purpose cells were grown in bacteria free medium (0.4 percent yeast extract and 0.4 percent nutrient broth) at 30°C in a shaker culture. Growth was followed by optical methods and by cell counts; cultures were harvested as they approached plateau. Checks for bacterial and mold contaminants were made by plating samples. The cells were centrifuged in the cold and resuspended in Tris buffer (10^{-2} M, pH 7.3) containing Mg (5×10^{-3} M). DNAase at a final concentration of 30 μ g/ml was added and the mixture rapidly frozen in methanol and dry ice. To break up the cells they were rapidly thawed and frozen again three times. Dupanol was added to a final concentration of 0.4 percent and the lysates shaken at room temperature for five minutes. The lysates were then chilled and the RNA was extracted according to the method of GIERER and SCHRAMM (1956). After removal of the ether, the RNA was thrice precipitated with 2.5 volumes of cold ethanol and redissolved in Tris buffer. Samples of the RNA were examined for size distribution by centrifugation in a linear sucrose gradient (NOMURA, HALL and SPIEGELMAN 1960). The centrifugation was carried out at 23,000 rpm for 12 hours at 15°C in a SW25 rotor. Fractions were collected by piercing the tubes and were examined by determining the ultraviolet absorption at 260 m μ . Three distinct peaks were observed and little RNA breakdown was indicated.

The RNA extracts were diluted to 80 μ g/ml in sterile Cerophyl, and single pairs were isolated into 0.5 ml aliquots. Penicillin and streptomycin were added

TABLE 6
RNA effects on output ratios

Cross	Treatment of pairs	Serotype distributions			Total	Output ratio
		Ha	Had	Hd		
62-75	None	193	37	50	280	76:24
	<i>H^A</i> RNA-Total	233	15	53	301	80:20
	<i>H^D</i> RNA-Total	157	29	38	224	77:23

to reduce the hazard of bacterial contamination. After two to three days, single cells were isolated into bacterized Cerophyl. The clones were screened for non-conjugation and tested for serotypes after eight to ten transfers. The results of this study (Table 6) are suggestive of a slight effect of the treatment. A chi-square test for homogeneity on the entire table yields a P value of about 0.01. However, this inhomogeneity is almost entirely the consequence of the partitioning of the classes Ha and Had in the cells treated with *H^A* RNA. A chi-square test on the output ratios (using the total cells tested as *N*) yields a P value of about 0.50. Since the output ratios are not affected, this particular treatment is not shown to have modified significantly the serotype differentiation.

DISCUSSION

This paper reports preliminary attempts to define the conditions under which H serotype differentiation occurs in variety 1 of *T. pyriformis*, and to regulate the course of that differentiation. Thus far the efforts to modify allelic repression in the serotype system by temperature, by antiserum, by breis and by RNA extracts have yielded either negative or inconclusive results.

The only significant determinants of output ratios thus far defined are the allelic combinations themselves. In all combinations thus far tried, the allele *H^E* makes the major contribution in phenotypic expression in the heterozygotes. In all combinations other than with *H^E*, *H^A* predominates in expression. Alleles *H^C* and *H^D* occupy the lowest position in the sequence and are approximately equally likely to predominate in combinations with each other.

The conclusion that allelic combinations are the critical factors in determining output ratios actually requires a brief justification. Several general methods for establishing the significance of allelic combinations are available. The first consists of creating heterozygotes by crosses among a number of different inbred series. If the output ratios are clearly characteristic of the allelic combinations and are in no way dependent upon the sources of those alleles, several alternative explanations may be ruled out. To illustrate the alternatives, consider the following facts. The allele *H^E* is only established in one inbred series (C). Perhaps cells of inbred series C have the capacity to "activate" any *H* allele they possess, so that it will have a high probability of expression in a heterozygote. In this case, the predominance of *H^E* in the phenotypes of heterozygotes would not be an intrinsic property of the allele, but rather would reflect some kind of "maternal

effect" mediated by C cells. It should be noted that the allele H^A is also available in only one inbred series—A. Moreover, since inbred series B1 has become genetically unreliable (NANNEY 1963d), the allele H^C is now available only in inbred series D1. The only allele available in a number of functional inbred series is H^D (in A1, B, D, E, and F). Hence, except for one apparently normal cross with inbred B1, the only variable which can be introduced systematically in the crosses is the source of the H^D allele.

The data available for this kind of analysis are limited. The heterozygote H^C/H^E has been generated once by a cross of B1 $\times$ C at 26°C and once by a cross of D1 $\times$ C at 32° (Table 3). In both cases the H^E specificity was predominant. Similarly, H^D/H^E has been established by crosses of A1 $\times$ C and B $\times$ C, again at different temperatures; in both cases the H^E expression was predominant. Finally, the output ratio of the H^A/H^D heterozygote from a cross of A $\times$ E is within the ranges of the outputs for crosses of A $\times$ B. Thus, though fragmentary, the crosses between inbred strains yield results consistent with a primary significance of allelic combinations.

Another approach to this problem involves an examination of the consequences of inbreeding on the behavior of strains in the various inbred series. If the terminal members of an inbred series differ from earlier members in regard to their "activating potential" for H alleles, these differences would probably be referable to differences at other genetic loci. The genetic constitutions of the inbred series might be expected to vary during the inbreeding program until homozygosis was achieved for the relevant loci. The data recorded in Tables 2 and 3 include results obtained in 1958 and 1959 (the data from NANNEY and DUBERT 1960) when all of the inbred series were in much earlier stages of inbreeding and some had been inbred for only three or four generations. Crosses numbered 60-xx were carried out in 1960; those numbered 61-xx in 1961, and those numbered 62-xx in 1962; during this interval all the inbred series were carried through a number of inbreeding generations. Yet, throughout this period from 1958 to 1962, the basic patterns of predominance have not changed. Some of the variability from cross to cross may reflect variations in genetic backgrounds, but the major patterns of exclusion appear to have been established coincidentally with the establishment of homozygosis at the H locus.

One other kind of evidence bears on the significance of the allelic combinations. If genes other than the H genes are of primary significance in determining patterns of exclusion, these should assort in the F_2 following crosses of inbred strains and new patterns should be established. This has not yet been systematically explored, but some data have been obtained incidentally in other studies in this laboratory. The only published results are those reported (NANNEY 1962) for the F_2 of a cross between an aberrant (H_r) strain and a member of series C. The H^E allele was preferentially expressed in both the F_1 and the F_2 .

Hence, all the available data are consistent with the hypothesis that the major factor in determining output ratios in H serotype heterozygotes is the particular combination of alleles involved.

The meaning of the allelic interactions has not been established. Allelic re-

pression implies that the activity of one allele in a subnucleus is antagonistic to the activity of another. Since different alleles are active in different subnuclei within the same macronucleus, the antagonism is probably effected very close to the allele being repressed and not at a site distant from the chromosomal material. The simplest interpretation possible is that the production of messenger RNA by one gene directly or indirectly prevents the production of messenger RNA by its allele. The reciprocity and indeterminism of the allelic interactions may be considered in light of this general hypothesis.

According to one specific formulation of this hypothesis, the RNA transcribed from a gene might complex with the partially homologous DNA of the allele and render it inactive. Utilizing the simplest assumptions (e.g. that two alleles are equally effective in producing messenger RNA in the period prior to differentiation) allelic repressions should be completely reciprocal; regardless of the degree of homology between the RNA of one gene and the DNA of a particular allele, the same homology would exist between the DNA of that gene and the RNA of the allele; the probabilities of repressing the two alleles by random encounters between semi-homologous RNA and DNA should be equal. Once established, a repression could be maintained by a feedback system; the inhibited allele would be incapable of producing RNA to repress the other. This expectation is not met; output ratios are commonly eccentric. Either the hypothesis of repression by RNA is incorrect or the assumptions are oversimplified.

Although special assumptions (e.g. that the intrinsic rates of activity of the alleles differ in the period prior to differentiation) might rescue the hypothesis, no great virtue is attached to the exercise of elaborating them, particularly in view of the negative results obtained with RNA extracts. An alternative hypothesis is equally tenable and is more consistent with studies on other repression systems (see MONOD, CHANGEUX and JACOB 1963). Instead of RNA being the direct agent of repression, a protein coded by the RNA might complex with the DNA of an allele and interfere with its function. In this case no reciprocity of repression is specified. The failure to obtain an effect on allelic repression by cellular breis cannot be considered serious evidence against this interpretation since the proteins might be inactivated in the crude preparations, and they would probably be incapable in any case of approaching the critical intranuclear sites.

SUMMARY

The patterns of allelic repression in H serotype differentiation have been studied for six heterozygotes in variety 1 of *T. pyriformis*. The allele H^E is preferentially expressed over H^A , H^C and H^D . Allele H^A is expressed in a majority of the progeny when combined with H^C or H^D . The H^C/H^D heterozygote is variable; in some crosses a majority of the progeny express H^C and in others the majority express H^D . No significant effects of temperature, antiserum, cell lysates, RNA extracts or genetic background have been established in preliminary studies.

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