INBREEDING DEGENERATION IN TETRAHYMENA[1]

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Received August 30, 1956

INBREEDING degeneration has been observed in many higher plants and animals and its physical basis and its bearing on the genetic economy of these organisms have been widely discussed (GOWEN 1952; LERNER 1954). While some genetic problems such as gene action, mutagenesis and genetic recombination have been actively and profitably pursued with microorganisms, relatively little work on inbreeding degeneration and its converse, hybrid vigor, has been undertaken with the smaller and more readily studied forms. The chief reason for this neglect is that most microorganisms are haploid for a major portion of their life cycles, and both heterosis and inbreeding degeneration are characteristic of diploids. Among the haploids only the inbreeding effects in yeast (WINGE and LAUSTSEN 1940) and heterocaryosis in ascomycetes (DODGE 1942; BEADLE and COONRADT 1944) bear a similarity to such phenomena in higher organisms and these similarities may be superficial. Even the organisms with predominantly diploid life cycles often undergo autogamy or some other form of intensive inbreeding which assures a high degree of homozygosity and for this reason have an "essentially haploid" economy free from the disadvantages of inbreeding and from certain of the advantages of crossbreeding observed in other diploids.

Many, but not all, of the Ciliated Protozoa do, however, show a type of inbreeding degeneration similar to that in higher organisms (JENNINGS 1944a, 1944b). Recently work on *Tetrahymena pyriformis* has demonstrated that this Ciliate has a genetic system essentially like that in other crossbreeding diploids and that it may provide a useful model system for studying the effects of inbreeding. It undergoes conjugation, but autogamy has not been observed; it shows deterioration upon inbreeding and vigor is restored on outcrossing. The occurrence of inbreeding degeneration is not, of course, an unmixed blessing and presents problems for a genetic program in which homozygosis is a valuable tool. Fortunately, strains can be obtained which survive the various inbreeding crises and return to approximately the state of vitality observed in the original crossbred strains.

The Ciliated Protozoa in general are useful in a comparative study of the types of genetic economy since closely related forms may show great differences in their breeding patterns and genetic structure (SONNEBORN 1956). The observations to be reported here have been acquired incidentally in the course of studying other traits in Tetrahymena and do not constitute a definitive analysis. A thorough evaluation of the phenomena reported must await more extensive study.

[1] This work was supported by grants from the National Science Foundation. The author wishes to acknowledge the helpful technical assistance of MISS PATRICIA CAUGHEY and MRS. A. TEFANKJIAN LENEY.

MATERIALS AND METHODS

Inbreeding effects were first observed in *T. pyriformis* during attempts to establish reasonably homozygous strains from two wild cultures of variety 1, WH-6 (mating type I) and WH-14 (mating type II), supplied by ELLIOTT. The cross between these strains yields few viable progeny which have completed the conjugation process, but these few can be crossed and yield progeny with good viability and a total of seven different mating types (NANNEY and CAUGHEY 1953). Two different inbred series have been developed which differ in the mating types produced at conjugation; "Family A" produces types I, II, III, V and VI in characteristic frequencies and "Family B" regularly yields types II, III, IV, V, VI and VII. Genetic studies (NANNEY, CAUGHEY and TEFANKJIAN 1955) show that the differences in mating type potentialities in these families are controlled by a single pair of genes; Family A is homozygous for a gene $mt^{(IV,\ VII)-}$ and Family B is homozygous for an allele $mt^{(I)-}$. Within a family the mating types are distributed more or less at random among the caryonides (first fission products, each with a single new macronucleus) after conjugation so that more often than not a single exconjugant yields two mating types (NANNEY 1956). Since these caryonides have the same genetic constitutions but different mating types, close inbreeding, equivalent to selfing in other forms, can be carried out. The cytological events occurring at conjugation (ELLIOTT and HAYES 1953; NANNEY 1953; RAY 1956) and the general methods used in these studies (NANNEY and CAUGHEY 1955; NANNEY, CAUGHEY and TEFANKJIAN 1955) have been described previously and will not be presented here.

TYPES OF INBREEDING ANOMALIES

In establishing the inbred strains by crosses of sister caryonides, inbreeding degeneration became apparent after three or four generations and took a number of forms which need to be distinguished.

Death at conjugation

In most of the crosses to be reported the exconjugants and the first fission products (caryonides) were isolated into separate containers and were allowed to give rise to cultures. In some instances the exconjugants undergo no fissions, however, and the first fission products cannot be separated; in other cases the exconjugants divide a few times and then die. In general the pairs can be separated into two classes—those whose progeny fail to give rise to cultures in the first depression slide and those whose progeny grow indefinitely. Occasionally one of the exconjugants or one of the caryonides fails to give rise to a culture, but a high intrapair correlation is observed and many of the exceptions are certainly due to injury of the cells during isolation. The class "dead at conjugation" is made up of those pairs, none of whose progeny undergoes ten fissions.

Nonconjugation

When normal conjugation occurs and new macronuclei are formed, the progeny show a period of sexual immaturity lasting usually about sixty fissions. Moreover, new mating types arise and these are distributed approximately at random among

the caryonides. Some of the pairs in certain crosses, however, separate and yield cultures only of the two parental types (one parental type from one mate and the other parental type from the other mate) and without an immature period. These pairs have been interpreted as pairs in which the normal reorganization process was incomplete and in which new macronuclei did not develop. The nonconjugant category, therefore, includes pairs showing cytological irregularities during conjugation. Careful cytological examinations of many crosses showing nonconjugation have not yet been carried out and different kinds of cytological irregularities may emerge when this is done.

One may not conclude, however, that all "nonconjugation" is due to cytological aberrations. If pairs are isolated shortly after conjugation is initiated in a mixed culture, a large fraction will separate without even initiating a reorganization. Even when pairs are isolated several hours after the beginning of mating, a small percentage will be of this type, for pairs are formed continuously in a mating mixture. These nonconjugant pairs can often be distinguished from those undergoing a partial reorganization since the separated cells undergo fission without the lag normally accompanying conjugation. No such distinction is made, however, in the following tabulations; pairs were generally isolated about twelve hours after the initiation of conjugation when the latter type of nonconjugants should be infrequent.

Delayed micronuclear anomalies

A third type of anomaly (in some ways the most interesting) has a late onset, rarely being manifested earlier than the 40th postzygotic fission and occasionally not appearing until after 100 or more fissions. Clones showing this aberration are designated as "semi-amicronucleate" (S.A.) for reasons to be described shortly. The first visible sign of this condition is the appearance, at first in low frequency, of small rounded crinkled cells lying at the bottom of a depression culture as it begins to starve. The frequency of these cells rises gradually in subsequent transfer cultures until nearly all the cells are of this type. When the crinkled cells are isolated they are incapable of initiating a new culture and the clones can be maintained in serial single cell transfers only through isolations of the apparently normal cells; selection of normal cells does not, however, result in the establishment of normal cultures. Since in our work the isolations are usually made when the cells have not yet starved and when the two types of cell are difficult to distinguish, the S.A. cultures are not maintained for many transfers.

A cytological examination of these cultures reveals that nearly all of the visibly abnormal cells lack micronuclei and that most of the normal cells contain at least one and often several micronuclei; hence, the term "semi-amicronucleate" for the cultures. Even when micronuclei are present, however, they may be grossly abnormal; some are many times as large as normal micronuclei and others may be as small as 10 percent of the normal size. The facts that the amicronucleate cells never give rise to cultures and that cells with any of several anomalous micronuclear conditions can give rise to cultures suggest that the micronuclei in these strains are necessary for continued growth, but that the precise content of the micronucleus may not be important in this respect.

The derivation of the micronuclear anomalies was studied by examining stained

preparations of the S.A. cultures during their growth period. The chief aberration (loss of micronuclei from some cells) appears to result from a failure to coordinate the divisions of the micronucleus with the divisions of the cell. In normal cells the micronucleus migrates to a mid-point in the cell and has completed its division by the time the cell begins to constrict. In S.A. cultures dividing cells may be found in which the micronucleus has either remained near one end of the cell and has not divided, or has begun to divide in an excentric position so that the entire spindle or a large portion of it lies within one of the presumptive daughter cells. Thus, following cell division one of the daughter cells is amicronucleate. The origins of the minute micronuclei and of the very large micronuclei have not yet been clearly established.

Although continued growth is not possible in the absence of a micronucleus, the stained preparations include dividing cells in which no micronuclei are apparent. Hence, amicronucleate cells are capable of undergoing at least one cell division before they die.

When the S.A. cultures are sexually mature they may be crossed with either normal cultures or other S.A. cultures showing a different mating type. A cytological examination of conjugation under these circumstances demonstrates two things. First, at least some of the amicronucleate cells can pair with cells of another mating type, and in mixtures of two S.A. cultures pairs may be found in which both members are amicronucleate. These cells are, however, the phenotypically normal amicronucleates and may have been recently derived; the grossly abnormal crinkled cells have not been observed to form pairs. Secondly, even those cells with micronuclei usually do not behave properly in conjugation. As in cell division, many of the micronuclei fail to make the important nuclear migrations and conjugation is halted at intermediate stages. In any event, conjugation involving an S.A. culture has always led to death. This fact must be qualified slightly. On a few occasions crosses have been made with clones which were "presumptive" S.A. clones, i.e. clones in which the S.A. condition appeared later, and from these crosses viable exconjugants were obtained. Some of these surviving exconjugants, moreover, appeared to be free from the S.A. stigma in further growth. These facts suggest that the establishment of a new nuclear constitution is in some instances capable of rescuing otherwise doomed clones, but that this rescue must occur before the clone has expressed the first signs of deterioration. Unfortunately, little information is available about this aspect of the problem since sexual maturity and the S.A. condition usually have their onset at about the same time.

The evidence that the S.A. condition has a genetic basis comes from a consideration of its distribution among related clones. In a given cross all the progeny of some pairs will be normal and all the progeny of other pairs will become S.A. When observations are discontinued at 100 fissions, as in most of the experiments to be reported, a few pairs will include progeny of both types, but these may be interpreted as S.A. pairs in which the S.A. condition had not yet appeared in some cultures at the time observations were stopped. This interpretation was examined more critically for one cross by expanding the progeny of a number of pairs until each pair was represented by 16 cultures, all of which were maintained in daily isolations until they had undergone 150 fissions. By 100 fissions most of the pairs gave either all or

no S.A. cultures and by 120 fissions complete concordance was observed. Hence, the S.A. condition is a "pair" characteristic and is probably determined by events occurring at conjugation, most reasonably by the establishment of a genetic constitution at that time.

A condition similar to that described above may also be observed under certain other circumstances, but the causal factors are clearly different. When normal clones are kept under continued growth for very long periods of time (500 to 1500 fissions) changes occur similar to the aging effects reported in other Ciliates (See SONNEBORN 1954). Among these changes are micronuclear anomalies resulting again in S.A. cultures. No confusion with these cultures is possible in the experiments reported since the observations were discontinued long before the aging effects become apparent, but the existence of this other kind of S.A. culture suggests that what is being studied may be some kind of premature aging effect.

THE COURSE OF INBREEDING DEGENERATION

Although the various signs of inbreeding deterioration appeared during the initial breeding experiments, useful quantitative data are not available for this period; proper screening techniques for nonconjugants had not yet been adopted and the S.A. condition was not always recognized. The information to be presented, therefore, concerns the events occurring after an intercross between the two inbred families. A fourth generation Family A caryonide was crossed with a sister caryonide from the same family to give an F_5; similarly a third generation Family B caryonide was crossed with a sister caryonide to yield an F_4. (It should be pointed out, perhaps, that these Family A and Family B clones were not chosen at random but were selected on the basis of their ability to produce a reasonably high frequency of viable progeny.) One of the Family A caryonides was then crossed with one of the Family B caryonides to yield the intercross generation (I_1) from which all the subsequent cultures were derived. The results of these first crosses are given in table 1.

The method of scoring the frequencies of the anomalies requires some comment. First, the frequency of the S.A. cultures is meaningful only in terms of the total pairs which could have shown this condition, i.e., those pairs which were maintained long enough to manifest the trait; this automatically excludes both the pairs that died and the pairs that failed to complete conjugation and were discarded. This treatment permits the frequency of S.A. pairs to vary from 0 to 100 percent, re-

TABLE 1

Initial crosses in the inbreeding experiments

Parents	Total pairs	Normal	Died	Viable	Non-conjugant	Viable conjugants	S.A.
Fam. A; $F_4 \times F_4$ (F_5) A	45 (100%)	20 (45%)	15 (33%)	30 (100%)	3 (10%)	27 (100%)	7 (30%)
Fam. B; $F_3 \times F_3$ (F_4) B	45 (100%)	20 (45%)	16 (36%)	29 (100%)	5 (17%)	24 (100%)	4 (17%)
Fam. A $F_4 \times$ Fam. B F_3 (I_1)	59 (100%)	57 (97%)	0 (0%)	59 (100%)	2 (3%)	57 (100%)	0 (0%)

gardless of the fraction of the pairs which died at conjugation or were nonconjugant. Similarly, the test for nonconjugation can be applied only to those pairs which survive long enough to be examined and the frequency of nonconjugants is expressed as a fraction of the viable pairs. The frequency of dead conjugants and the frequency of normal conjugants are expressed as fractions of the total pairs isolated.

These results show a fairly high incidence of anomalies in both the inbred strains when crossed among themselves. Less than half the pairs isolated gave rise to normal cultures. Secondly they show the elimination of these anomalies in the intercross. Except for a few pairs in the nonconjugant class, which may have been pairs which were disturbed in the early stages of conjugation, all the pairs gave rise to normal cultures. This high viability is not permanent, however, and gradually disappears when inbreeding is resumed.

The general features of the inbreeding effects can be demonstrated by a presentation of the history of one inbred series. The upper graph in figure 1 depicts the frequencies of pairs which failed to survive conjugation in successive generations of crosses between sister caryonides. In the first and second intercross generations no death was observed, but the two separate crosses made for the I_3 generation showed 8 percent and 36 percent respectively. Progeny from the more viable of these crosses were selected and four different I_4 crosses were made; all yielded some inviable progeny with frequencies ranging from 11 percent to 92 percent. Although some viable progeny were produced in each of these crosses, many of the survivors were either nonconjugant or S.A. For the I_5 generation one cross was made using a pair from the most viable of the previous crosses; 28 percent of these pairs failed to survive. In the I_6 generation two crosses were made, one of which yielded 40 percent death at conjugation and one of which showed no death, the first instance in

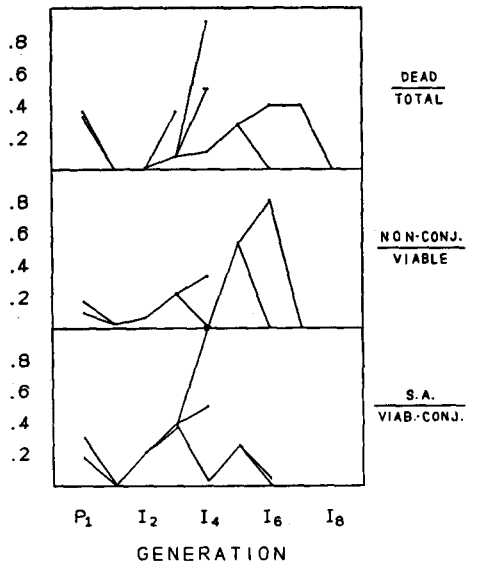

FIGURE 1.—The course of inbreeding degeneration and recovery in strains of *Tetrahymena pyriformis* initiated from moderately inbred parents (P_1) and inbred through crosses of selected sister caryonides for eight generations.

which viability improved. At this point the selection procedure was altered and the I_7 was derived from the less viable cross in the previous generation; again 40 percent inviability was obtained. Finally, a single I_8 cross was made and all the pairs survived.

The frequencies of non-conjugating pairs in this same series are given in the middle graph in figure 1 and show the same general trends. The inbreeding effect becomes progressively more noticeable for several generations and then begins to disappear. Similarly, the frequency of the S.A. condition (lower graph, figure 1) rises with inbreeding, but with selection for normal parents gradually decreases until it has been entirely lost. Hence, all three indices of inbreeding degeneration rise in time and are then progressively reduced until, after 6 to 8 generations of selfing with constant selection, strains are obtained which are approximately as free from defects as the I_1 generation.

Two other inbred series of about the same size have been studied and with approximately the same results. The series differ in the times at which the inbreeding crises became most acute and in the relative and absolute contributions of the different kinds of anomalies, but all three show a peak for each kind of abnormality followed by a dropping off. A detailed analysis of these data is not indicated since great variability is encountered in the various subseries and sufficient subseries were not studied. Moreover, larger samples would be required for the individual crosses; most of the qualitative conclusions drawn are based on sample sizes of only about 30 pairs. It is reasonably clear, however, that the various kinds of degeneration are at least partially independent of each other; any one may be prevalent in a cross in which the others are negligible.

THE PHYSICAL BASIS OF INBREEDING EFFECTS

Two general explanations of inbreeding deterioration may be considered in light of these observations. One explanation holds that the deleterious effects of inbreeding are due to the exposure of harmful recessive genes normally carried as heterozygotes in a crossbreeding population; according to this interpretation a population should upon inbreeding pass through a series of crises as the recessive genes are brought into homozygous condition and eliminated. After this "purification" procedure the strains should return to normal if a strict enough selection can be practiced to prevent the fixation of deleterious genes. A second type of explanation attributes heterosis primarily to heterozygosity accompanied by overdominance; the heterozygote is superior to either of the homozygotes. This interpretation leads to the expectation of deterioration upon inbreeding but does not predict a return to normal vitality unless some special mechanism is evolved to maintain heterozygosity. When a return to normal vitality is not observed, distinctions between the two hypotheses are difficult, but when vitality is restored after intensive inbreeding the evidence strongly supports the first hypothesis. The observations on Tetrahymena suggest that at least for the traits studied overdominance is not a major factor. Perhaps when other characteristics of the inbred lines are studied (such as growth rate or resistance to harmful environmental agents) this conclusion will have to be modified.

Although an explanation based on harmful recessive genes explains the observa-

tions in a general way, efforts to detect genes with individually significant effects have been unsuccessful. If many of the signs of degeneration were due to individual genes, the greatest deterioration would be expected in the I_2. The I_1 generation would be the most heterozygous of the generations and each lethal gene should be homozygous in one fourth of the I_2; if two lethals were present seven sixteenths of the I_2 should be affected, etc. With selection against these homozygotes the frequencies of abnormal pairs should decrease upon further inbreeding. Yet none of the indices reached 25 percent in the I_2 and all continued to rise for several more generations. Hence, the most reasonable explanation for these effects is the accumulation in homozygous condition of many genes (polygenes) individually insignificant, but in the aggregate (perhaps after reaching a threshold level) capable of severely damaging the cell.

The available data do not permit a precise evaluation of the number of such genes, but suggest a large number. The relative independence of the various kinds of abnormalities suggests at least three different groups of genes concerned with different kinds of cellular functions, and the number of genes in each group must be fairly large. This is shown best, perhaps, by examining the results of crosses within and between strains in an intermediate stage of inbreeding. Five different sets of sister caryonides in the I_2 generation were chosen for such an analysis; all were selected to be homozygous for the $mt^{(IV,\ VII)-}$ allele and on the basis of progeny tests which showed about the same level of S.A. pairs among their progeny. *1a* and *1b* were derived from a single I_1 pair; *2* was derived from a different I_1 pair and *3a* and *3b* came from a third. Each of these sets was crossed with itself and also with each of the others and all the indices of degeneration were calculated. Only those for the S.A. characters will be given since they illustrate the kinds of results

TABLE 2

The frequency of the S.A. condition among the progeny of crosses of selected I_2 parents. 1a and 1b are sets of sister caryonides derived from the same I_1 cross; 2 is from a second I_1 cross and 3a and 3b are from a third

Parents	S.A. progeny	Pairs examined
1a × 1a	.39	50
1b × 1b	.36	50
2 × 2	.23	50
3a × 3a	.30	50
3b × 3b	.51	50
1a × 1b	.36	50
1a × 2	.00	30
1a × 3a	.00	50
1a × 3b	.04	80
1b × 2	.36	80
1b × 3a	.08	50
1b × 3b	.04	30
2 × 3a	.07	30
2 × 3b	.07	30
3a × 3b	.43	50

observed (table 2). When crossed with themselves each set yields a moderate frequency of S.A. progeny—from 23 percent to 51 percent. Most of the intercrosses yield a lower frequency than either parent when crossed with itself, suggesting that the two parents differ in the factors responsible for the trait. Some exceptions to this rule are observed, however, for *1a* and *1b* yield about the same frequency of S.A. pairs when intercrossed as when selfed. Similarly *3a* and *3b* yield about the same results when intercrossed as when selfed. *1a* and *1b* can be distinguished, however, on the basis of crosses to *2*; *1a* in this cross produced no S.A. cultures and *1b* produced 36 percent. Hence, among these five sets of I_2 strains, showing similar rates of S.A. production when selfed, at least four different genetic constitutions can be distinguished for the S.A. trait alone. Since other selfing crosses yield all intermediates from 0 to 100 percent S.A. progeny, a large number of genotypes and hence genes must be expected.

SUMMARY AND CONCLUSIONS

The information presented demonstrated that variety 1 of *Tetrahymena pyriformis* possesses the type of genetic economy usually associated with crossbreeding diploids. During intensive inbreeding various signs of inbreeding degeneration appear; these signs include death at conjugation, failure to complete nuclear reorganization, and delayed micronuclear anomalies. Each of these kinds of aberration rises gradually for several generations, but with constant selection for normal progeny, strains may be derived which have passed each of the crises and which are apparently as vigorous as the original crossbred strains. A preliminary genetic analysis suggests that the anomalies are due to the accumulation in homozygous condition of combinations of genes which individually have little or no detectable effect.

These observations by no means settle all the questions relating to the breeding system of these strains in nature. In particular they do not explain the high incidence of death and nonconjugation encountered in crosses taken from the wild. A possible explanation may however be attempted. JENNINGS (1944a) observed that clones of *Paramecium bursaria* underwent a regular sequence of changes during their life cycles. Following conjugation they entered a period of sexual immaturity lasting for a variable, but usually long, period of time. Following this period the cultures became capable of mating and usually bred true for a particular mating type. If crosses were made between two young clones, they often resulted in many viable progeny, but if the same cross was repeated from time to time as the cultures grew older, the frequency of the viable progeny dropped gradually to zero. SONNEBORN (1954) observed similar aging effects with clones of *Paramecium aurelia* and further discovered that gross chromosomal rearrangements accompanied the aging processes. Hence, it is possible that the F_1 mortality observed in many crosses with Tetrahymena is due to the use of aged clones in which chromosomal aberrations have been established. According to this interpretation the initial death at conjugation is due to age-correlated chromosomal aberrations acting as dominant lethals. In any case, it is certainly clear that chromosomal rearrangements are common in some of the stocks that yield inviable progeny (RAY 1955) and that Tetrahymena stocks can become senile in the laboratory (ALLEN and NANNEY unpublished).

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