homozygotes. Moreover, all wild types studied so far have been heterozygotes and both homozygotes, +/+ and p/p, produced in breeding experiments show low viability. In addition, in all crosses where the end results were homozygotes and heterozygotes, the ratios are always skewed toward the latter. This investigation seems to support the thesis that in populations of *T. pyriformis* an outbreeding economy is operating.

The fact that the heterozygotes do grow slowly through several transfers, if care is taken to increase the transfer time to 5 or 6 days, means that they can synthesize small quantities of pyridoxine. If it is assumed that pyridoxine synthesis is controlled by a single gene, it would appear that the recessive gene has some effect in the heterozygous condition. It might also be assumed that the wild type contains a dominant suppressor gene which inhibits the effective operation of the gene controlling pyridoxine synthesis. The mutant, containing no suppressor gene, would then be able to synthesize sufficient pyridoxine for its own use. If this were true the present interpretation would need to be altered. Until more data is available these questions cannot be answered.

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Genetic Studies of the Serine Mutant in Variety Nine of *Tetrahymena pyriformis**

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SYNOPSIS. Crosses between serine requiring and non-requiring clones from natural habitats give rise to progeny that are numerically equally distributed between the two categories. Most of the progeny from crosses between two serine requiring parental clones require serine with a few segregating out that do not need the amino acid. These data indicate that the gene or genes controlling the serine non-requirement are recessive to the wild type which requires serine. Growth of the F_1 progeny was highly variable. Some clones failed to survive on serine deficient media and were therefore tentatively assigned the genotype +/+. The remaining clones were of two classes: one grew slowly on serine deficient media and was given the genotype of the heterozygote, +/s; the other grew without serine, hence was given the double recessive genotype, s/s. The F_2 progeny from matings of s/s and s/s need no serine whereas the cross of +/s and s/s gave equal numbers of serine requiring and serine non-requiring progeny. The cross of two heterozygotes, +/s and +/s, yield progeny approximating the 3:1 ratio in favor of serine requiring clones. Crosses at the F_3 level produced non-viable offspring in all cases except one. Progeny from this cross with the genotype s/s were mated giving rise to F_4 progeny all of which grew without serine. The data support an outbreeding economy for this organism with selection in favor of the heterozygote. Although the data seem to favor a single-gene hypothysis, suppressor genes may be involved. With selection favoring the heterzygote, distorted genetic ratios make the data difficult to analyze.

A living cell is a highly organized unit characterized at the biochemical level by a capacity for carrying out a remarkable number of chemical reactions. Current evidence indicates that hereditary units play an important role in the control of these reactions. The application of genetic methods in studies of metabolic pathways has been fruitful and may eventually lead to a better understanding of the chemical interrelationships that exist among a large number of cellular components involved in metabolism.

Various aspects of these problems have been attacked by studying a rather wide variety of organisms including vertebrates, insects, plants, fungi, and bacteria(13). Up to the present the protozoa, exclusive of the chlorophyll-bearing forms, have not been used in this type of study probably because of the many presently insurmountable problems encountered when attempting to culture these fastidious organisms. However, the ciliated protozoan, *Tetrahymena pyriformis*, which has become a valuable organism in nutritional studies may also be useful in studies of biochemical genetics. With the discovery of sexuality in this organism(2,3), coupled with what is already known about its biochemistry, its value as an experimental tool becomes apparent. From tropical habitats several sexually active clones were found that grew without serine, a normal constituent of the diet of the wild type (5). With these sharp nutritional differences occurring within one variety and among diverse mating types, the possibility for genetic studies was available. The purpose of this report is to describe our efforts in attempting genetic analysis of the genes controlling serine synthesis.

MATERIAL AND METHODS

Among 77 sexually active clones isolated from habitats in Mexico, Panama, and Colombia, 6 grew without serine(4); all others (2500) collected in these areas as well as in the United States and Canada require the amino acid for normal growth. Hence we may speak of the serine-requiring strains as the wild type as opposed to those that grow without serine, the serine mutants.

The serine-requiring strains used in this study were: TC 105, TC 110, and TC 148. A single serine non-requiring strain, TC 89, was the only one available of the proper mating type. The four strains belonged to variety 9. All stock cultures were maintained on a 1% proteose-peptone-tryptone medium and carried through at least three transfers before matings were made. Prior to mating, the cells were washed in three changes of sterile double-distilled water and mixed in depression slides. Conjugation usually occurred within 24-48 hours and the process reached the anlagen stage (when the two new macronuclei are visible 36-48 hours later). When stained samples revealed that at least 60-70% had reached the anlagen stage, pairs were isolated into drops of stock medium in depression slides. From 50 to 100 pairs were isolated, the number depending on the cross being made. In the original crosses where the viability was poor, only pairs were isolated; these were permitted to grow into depression cultures and were finally transferred to tubes containing 2 ml. of media. They were later transferred to tubes containing 5 ml. of media and subcultured in such tubes. The small amount of media in the initial tubes fostered better growth than where larger amounts of media were used. When viability improved at the F_2 and F_3 levels, exconjugants and caryonides were isolated and carried to cultures. Each isolated clone was checked for mating type and for possible bacterial contamination by plating on nutrient agar. It was then screened for its nutritional requirement on serine deficient media as well as on complete chemically defined media(3). The latter precaution was necessary because occasionally a clone would fail to grow even when all the nutritional requirements were available signifying other deficiencies which were of no immediate interest.

A close form of inbreeding was followed throughout this program with crosses being made between diverse mating types arising from a single cross. Whenever possible crosses were made between caryonidal or exconjugant clones if more than one mating type was derived from a single isolate pair.

RESULTS

In this investigation 42 crosses were made, 6-7000 pairs isolated and over 700 clones obtained, all of which were tested for their capacity to grow on serine deficient and complete media. If any showed growth on serine deficient media they were carried through at least eight transfers. In all crosses no immaturity period(3) was found after true conjugation and nuclear exchange between the conjugants had occurred. This immaturity period has proven useful in other varieties where it serves as a rather reliable test for the occurrence of nuclear exchange during conjugation. In varieties 1 and 2, for example, if conjugation has occurred the resulting exconjugant clones are sexually inactive until they have undergone a certain number of fissions. In variety 2 the immaturity period exists for at least 250 fissions in some crosses. The lack of an immaturity period in variety 9 has proven unfortunate in that no convenient method of proving the existence of true conjugation in each cross is presently available. The method now employed is to isolate pairs only when a high percentage (70% or better) of the mated cells are in the anlagen stage which assures that nuclear exchange has occurred. One is thus certain that only an occasional pair is selected which separates without undergoing nuclear exchange.

Crosses of clones from nature. When the original

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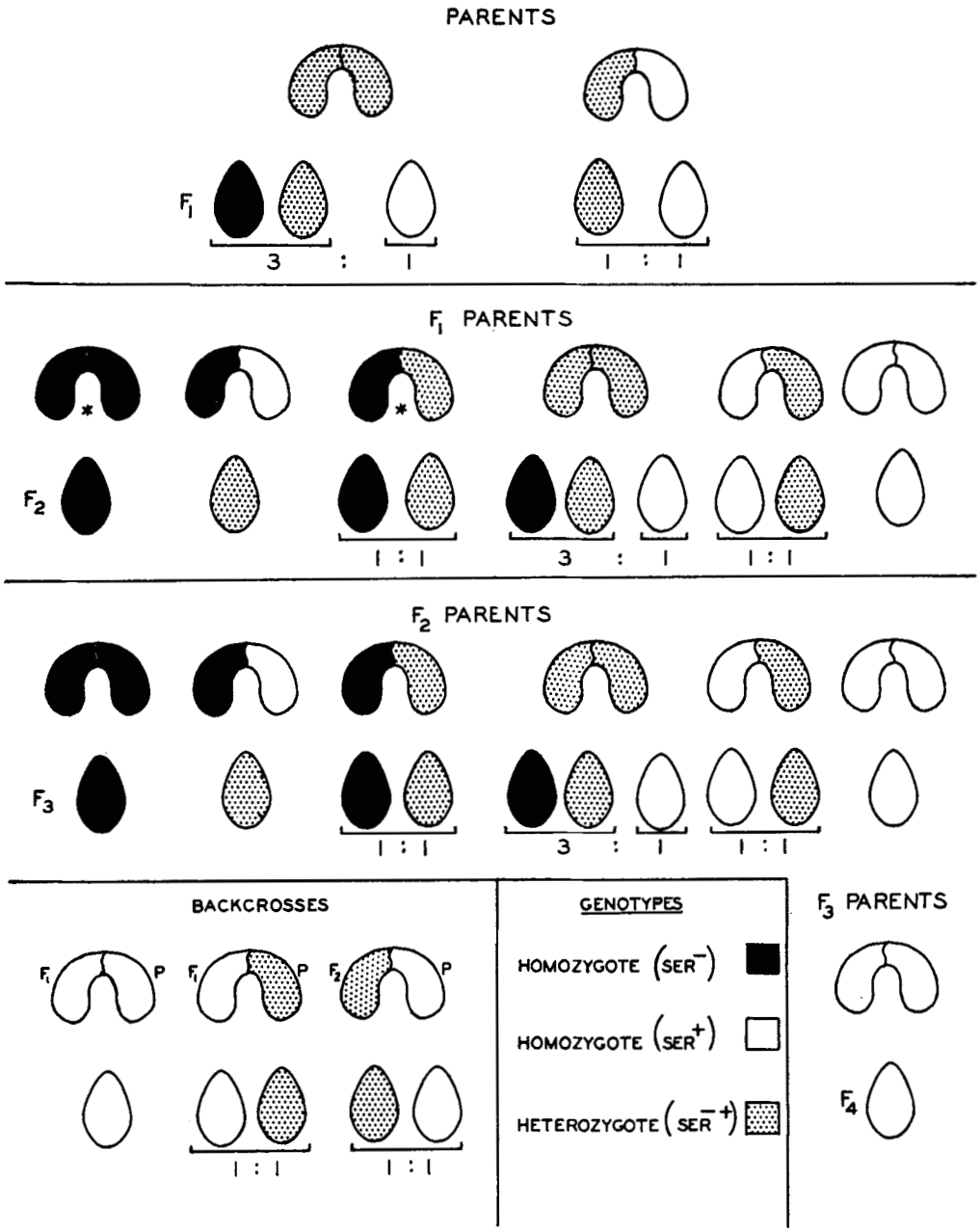

Fig. 1. A schematic representation of the crosses made through three generations, together with backcrosses. The asterisk indicates the expected results although no progeny were actually obtained. ($SER^- = +/+$, $SER^+ = s/s$, $SER^{-+} = +/s$)

clones collected from natural habitats were mated the resulting survival of the exconjugants was exceedingly poor, ranging from 3.3 to 6.4%. Such reduced viability precluded the possibility of isolating exconjugant clones in any numbers, hence clones were established from paired cells. For each cross, however, clones were isolated from each culture and used for the next cross. Where diverse mating types arise from a single pair, crosses were made on the exconjugant or caryonidal level. Where this was not possible matings were made between opposite mating types originating from one cross.

Crosses between two serine requiring strains, TC 110 x TC 148, yielded a high percentage of clones requiring serine with a few segregating out that grew without the amino acid (Fig. 1). Crosses between serine requiring and serine non-requiring strains, TC 110 x TC 89, TC 148 x TC 89, and TC 105 x TC 89, gave rise

to progeny that were numerically equally distributed between the two categories. These data indicated that the factors controlling the serine non-requirement are recessive to the wild type which requires serine. The wild type was, therefore, assigned the tentative genotype +/s and the mutant strains s/s. From a cross of +/s x +/s several classes of individuals segregate out. Some clones failed to grow on serine deficient media, dying out in one or two transfers, although they grew normally on the complete chemically defined medium indicating that their only deficiency was the lack of serine synthesizing capacity. The remainder of the clones fell into two categories. Clones of the s/s genotype grew rapidly without serine and were labelled fast growers (FG) for convenience. Most of the serine requiring segregated clones, however, grew at variable rates on serine deficient media and could be maintained only if the interval between transfers was lengthened to 5 days rather than the customary 3-day interval for the FG clones. These clones were classed as slow growers (SG) and they constituted the majority of the genotypes. These purely arbitrary categories were essential since growth on deficient media was the criterion used to determine genetic ratios. It is important to emphasize the difficulty that is encountered when attempting to analyse the data. For example, very often clones of the s/s genotype grow poorly on the deficient media and it is not until further crosses are made that the s/s and the +/s genotypes can be confirmed. Subsequent F_2 crosses have demonstrated that selection is toward the heterozygote (+/s) over either of the homozygotes (+/+ and s/s) resulting in distorted ratios. These factors forced an arbitrary decision in the assignment of categories for the different questionable cases.

Lethality following conjugation was a constant source of difficulty throughout this investigation, particularly during the earlier work. This may be accounted for in part by the fact that *T. pyriformis* probably exists in nature with many if not all genes in the heterozygous state. Signs of lethality were apparent from the very first breeding experiments. In crosses in which low viability was noted, pairs usually did not separate after reaching the anlagen stage. If they did succeed in separating the usual consequence was death of both exconjugants in one or two fissions. Even in some cases where clones were succesfully established a high percentage of the vegetative cells became spherical in shape and settled to the bottom of the depressions, ultimately dying. However, in these cases the culture was maintained by the few apparently normal cells that were able to divide. Lethality was also observed in later work when the isolations were carried to the caryonidal level. In these cases often times three of the caryonides would die, the culture being derived from the one remaining.

F_1 backcrosses. Backcrosses of F_1 progeny to the original parental stocks resulted in poor viability (0-26%). However, sufficient data were obtained to confirm tentative genotypes. In one cross of an F_1 of the probable genotype s/s to the parental TC 89, all resulting clones grew without serine confirming the tentative genotype of this parental stock as homozygous recessive (s/s). However, after several weeks on peptone media all of these clones failed to survive. Backcrosses of F_1 s/s clones to the parental stock TC 105 yielded viabilities ranging from 10.9 to 26% with equal numbers of serine requiring and serine non-requiring clones segregating out, thus confirming the tentative genotype of the parental stock as +/s. After several weeks of serial transfers on the stock medium these likewise were lost. The same lethal condition was encountered when F_3 crosses were made where all the progeny died out with the exception of those from one cross which will be discussed later. Whether or not lethality distorts the ratios reported here is difficult to say. The consistency of the results after many crosses seems to indicate that the conclusions stated are correct.

F_1 crosses. Fifteen crosses of F_1 clones were made including all combinations as indicated in Table I and Figure 1. No progeny survived from crosses +/+ x +/+ and +/s. In all of these crosses varying degrees of lethality were exhibited by failure of the mates to separate, resulting in death after three or four days. Occasionally the pair would become a huge multinucleated cell which failed to divide. The highest viability (50%) resulted when two SG clones, +/s x +/s, were crossed. Fifty-five clones were obtained from this cross of which 42 required serine and 13 grew without the amino acid. This ratio closely approximates 3:1. Of the 42 serine requiring clones, 30 grew slowly without serine when transferred every 5 days. It seems that the heterozygote can sometimes synthesize small amounts of serine, sufficient to maintain the culture at a very low level through several transfers although it eventually dies out.

Four crosses were made utilizing the genotypes +/s x s/s. Viability of the progeny from these crosses ranged from 9.4 to 50%. From a total of 164 clones isolated and screened, 97 required serine and 67 grew without the amino acid. Here again this ratio is skewed toward the heterozygote indicating that lethality was differentially acting against the homozygote. However, the data do not differ significantly from a 1:1 ratio.

Only one cross was made between the genotypes +/+ and s/s, which resulted in 48% viability. All of the 24 clones obtained needed serine, although a few

TABLE I. A summary of crosses made and the results obtained.

Generation	Genotypes	No. of crosses made	No. of clones obtained	No. of serine-requiring progeny	No. of serine-non-requiring progeny
P	+/s × +/s	1	12	9	3
	+/s × s /s	3	98	60	38
F_1	+/+ × +/+	1	0	0	0
	+/+ × s /s	1	24	24	0
	+/+ × +/s	1	0	0	0
	+/s × +/s	1	55	42	13
	s /s × +/s	4	164	97	67
	s /s × s /s	7	85	3	82
F_2	+/+ × +/+	4	48	48	0
	+/+ × s /s	4	71	71	0
	+/+ × +/s	1	0	0	0
	+/s × +/s	1	24	21	3
	s /s × +/s	1	0	0	0
	s /s × s /s	4	133	0	133
F_3	s /s × s /s	4	44	0	44
Backcrosses	F_1 s /s × P s /s	1	11	0	11
	F_1 s /s × P +/s	2	28	18	10
	F_2 +/s × P s /s	1	0	0	0

were able to continue through several transfers in the deficient media indicating that the recessive gene or genes in some cases influences the serine synthetic capacity of the organism. In order to rule out the possibility that what was seen here was adaptation, strain E was used in parallel experiments. This strain has a strict serine requirement, always failing to grow beyond the second transfer in serine deficient media. The results of such controlled experiments left no doubt but that some heterozygotes were actually synthesizing small amounts of serine sufficient to sustain them for some period of time.

Seven crosses were made between the genotypes s/s and s/s, where the parents in all cases grew well without serine. The viability varied from 15 to 20%. Of the 85 clones that were isolated from these crosses 82 grew without serine, the remaining three seemed to need the amino acid. These three clones demonstrated clear signs of morphological abnormalities although they were able to maintain themselves both in stock and complete chemically defined media. Aside from the possibility of back mutations, an explanation of the behavior of these three clones is not immediately evident.

Some conclusions can be drawn concerning viability as a result of these crosses. It seems quite clear that viability is highest when the end products of a cross are heterozygous. This might be expected in an organism where almost all clones taken from nature are heterozygotes. Another consistent observation was that if, upon mating two clones, conjugation occurs in most, lethality is apt to be high in the progeny whereas if the process is sparse the resulting progeny have a better chance for survival. Similar observations have been made in variety 2. Just what this means is conjectural. Many experiments were performed to improve viability. No success was obtained by backcrossing F_2 clones to F_1 clones. In one such cross (F_2 s/s to an F_1 s/s), 11 clones were obtained from 50 pairs isolated (viability 22%), all of which grew through several transfers on serine deficient media and then died when maintained at room temperatures. However, the parallel cultures which were in stock media and maintained at 16° C. were still actively growing. When these were incubated at 25° C. and transferred 3-4 times they too died. In the latter case death was merely delayed by the lower temperature which slowed fission rate. Apparently macronuclear abnormalities became apparent only after a certain number of fissions had occurred, assuming that the macronucleus controls the metabolic processes involved in growth.

F_2 crosses. F_2 parents were used in 50 crosses involving all possible combinations; these resulted in 276 clones with which screening procedures were initiated. Conjugation was poor in most crosses and in some the level was so low that pair isolations were not attempted. Of the 50 crosses it was possible to isolate pairs from only 15 which included the following genotypes: +/+ x +/+, +/+ x s/s, +/+ x +/s, +/s x +/s, +/s x s/s, s/s x s/s (Table I, Fig. 1). Initially the viability ranged from 0 to 96.8%, but the progeny from all but one cross (17a) were lost after several transfers. The cross, s/s x s/s, resulted in the highest viability, namely 96.8%, whereas the cross +/+ x +/s yielded no viable offspring. In the one cross (17a), involving the genotypes s/s and s/s, initial viability was 96.8%. After a variable number of fissions 37.6% of the clones survived. These remaining clones have been screened on deficient media

and grow well without serine and are being utilized for further crosses.

F_3 crosses. Four crosses have been made using the F_3 progeny of the exceptional cross mentioned above (17a). Viability of the progeny (F_4) resulting from this cross range from 7.1 to 62.5%. It may be that other crosses would result in better viability. Backcrosses of the F_4 generation to the F_3, F_2, and F_1 may allow for the recovery of diverse genotypes and mating types which are essential for further study.

DISCUSSION

In most plants and animals where their nutritional needs have been carefully analyzed, serine is synthesized in sufficient quantities to preclude an exogenous requirement. When taken from its natural habitat, the classical fungus, *Neurospora crassa,* synthesizes sufficient serine for its own needs, although mutant strains have been induced which require the amino acid (8). Considerable difficulty has been encountered in studying the nutritional requirements of protozoa owing to the fact that only a few (exclusive of the chlorophyll-bearing forms) have been succesfully cultured in axenic media. Unless protozoa can be grown free from other microorganisms a critical analysis of their dietary needs is impossible.

Among the ciliated protozoa that have been grown axenically, *Paramecium aurelia*(12) and *P. multimicronucleatum*(10) require serine for normal growth. Most of the classical amicronucleate strains of *T. pyriformis* and *T. vorax*(1,11) also require the amino acid. Strain W (*T. pyriformis*) has a limited capacity to synthesize serine. This requirement can be spared by glycine in this strain but not in strain E(9). Hence strain differences in regard to serine synthesis might be suspected and the present investigation seems to lend support to this notion. The present study leaves little doubt but that under natural conditions the vast majority of *T. pyriformis* must obtain serine from their immediate environment in order to grow at their optimal rate assuming that the maximal yield seen in test-tube cultures occurs in nature. Only occasionally an organism is found that possesses adequate enzymes to manufacture sufficient serine to supply its own needs. In an earlier study six such rare ciliates were found in a small river system in Panama in 1954(4). They all belong to mating type V in variety 9 and could well have been members of the same clone even though they were taken from different places within the same river system. No others were found among 2500 clones taken from many parts of the United States and Canada(7) as well as from Mexico, Panama, and Colombia. It hardly seems possible that such serine mutants exist only in the tropics and only in one variety and mating type. The fact that strain W possesses some capacity to synthesize serine lends support to the idea that probably others exist elsewhere in nature.

The finding of the serine mutant was an essential step before any genetic experimental work could be done. Even after the mutant strains were discovered considerable preliminary information was necessary before the most elementary genetical studies could be undertaken. The mating type system of variety 9 had to be understood before satisfactory crosses could be made. The growth characteristics and mating behavior of many strains had to be examined in order to find the ones most suitable for the contemplated experiments. These and many other procedural difficulties account for the slow progress in these studies.

The rarity of such mutant strains leads one to speculate about the evolution of the nutrition of this ciliate. It is generally agreed that primitive organisms were able to synthesize all, or nearly all, of their nutritional needs. With the passing of time they found themselves in environments rich in essential nutrilites placed there by the constant degradation of other organisms which had evolved in the meantime. Since a vast array of enzymes were necessary to manufacture these nutrilites, which must have caused a considerable drain on the energy reserves of these organisms, there was a gradual selection toward bypassing the synthesis of certain nutrilites which were abundantly rich in the immediate environment. This loss in synthetic ability varies in different organisms and for most strans of *T. pyriformis* it included 11 amino acids, 7 B-vitamins, and several other substances. Since the serine mutant can still manufacture sufficient serine for its own needs it might be thought of as a relict member of the species which exists in limited areas of the earth today and may ultimately become extinct.

The question arises as to whether or not adaptive enzymes are revealed by these experiments and that what is observed is actually adaptation rather than a genetic effect. It has been reported(6) that the serine requiring strain E, if grown through several transfers on complete chemically defined media, would slowly synthesize serine. However, if transferred directly from a stock proteose-peptone medium to serine deficient media no growth occurred signifying complete failure to synthesize the amino acid. This was interpreted as a possible adaptive effect, although the occurrence of a mutation followed by selection was not ruled out. The present investigation includes no experiments that would answer the question of the occurrence of adaptation since routine experiments involved the transfer of small inocula (*0.1 ml*) directly from stock media to the serine deficient chemically defined media. If growth failed the clone was consid-

ered to require the amino acid. It is altogether possible that some, if not all, of the serine requiring clones could be forced to synthesize small amounts of serine if the same procedure was undertaken as in the previous report. However, such time consuming experiments were impossible when great numbers of clones had to be analyzed.

Selection for the traits studied was essential in this investigation and must be taken into account. The nature of the screening procedure introduced an element of selection toward the serine requiring and serine non-requiring clones. Breeding stocks were originally, and throughout all succeeding generations, selected for their ability either to grow well without serine or to fail altogether in the absence of the amino acid. Moreover, clones, were also selected for their mating behavior and for progeny survival. Such selection is inherent in all genetic experiments and just how much bias is introduced into the data is in the realm of speculation.

The acquisition of a nutritional requirement in *Neurospora* is accompanied by a variety of physiological changes. A very common one is reduced viability (13). This was also observed in the present study where crosses involved serine non-requiring clones. The poor viability observed on the F_1 level gradually improved through the F_2 and F_3 generations. Consistent with the evidence that selection is toward the heterozygote in the population, crosses involving the genotypes +/s x +/s and +/+ x s/s resulted in the highest viability. The heterozygote grows uniformly well on the stock and complete chemically defined medium. In contrast the clones of the genotype +/+ could be derived only in small numbers and they were difficult to maintain on either stock or chemically defined media. Conjugation, however, was high in all clones that were used. Clones of the genotype s/s usually grew well on the stock and chemically defined media, both with and without serine, and most conjugated readily. The few that grew poorly on the above media, still mated satisfactorily but showed extreme lethality. The number of homozygotes was almost always smaller than the expected ratio in all matings indicating again a selection for the heterozygote. The evidence presented here, as well as that from a study of the pyridoxine mutant point to an *outbreeding economy* in *T. pyriformis*.

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