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Genetic Studies of the Pyridoxine Mutant in Variety Two of *Tetrahymena pyriformis**

ALFRED M. ELLIOTT and GORDON M. CLARK†
Department of Zoology, University of Michigan, Ann Arbor

SYNOPSIS. When two strains of *T. pyriformis* that do not require exogenous pyridoxine are crossed, all progeny grow without the vitamin. Offspring from crosses of two pyridoxine requiring clones require pyridoxine with the exception of a few which will grow without pyridoxine. The ratio is approximately 3:1 favoring the pyridoxine requiring category. In matings involving the homozygous dominant pyridoxine requiring clones with the double recessive mutant, that is +/+ × p/p, all of the resulting progeny need pyridoxine. Test crossing these heterozygotes (+/p) with the parental pyridoxine non-requiring clones (p/p) gives offspring approximating a 1:1 ratio. Matings between two heterozygotes derived from breeding experiments also yield progeny in approximately 3 pyridoxine requiring : 1 pyridoxine non-requiring. All data indicate selection for the heterozygote in the population and a possible selection against either homozygote. The great abundance of heterozygotes and rarity of recessive homozygotes in natural habitats corroborates these findings. The genetic evidence supports a single gene hypothesis although the possibility of multiple closely linked genes cannot be ignored. There is also the possibility that a dominant suppressor gene may function in blocking the activity of the pyridoxine mutant genes. Moreover, if this gene exists it may be incompletely dominant since the heterozygote grows slightly on deficient media.

VERY LITTLE is known about the biosynthesis of pyridoxine and the part this B-vitamin plays in metabolism is still quite unclear. The attack on this problem through *Neurospora* genetics, which has been so fruitful in elucidating the function of other nutrilites in nutrition and metabolism, has yielded little information concerning this vitamin, although pyridoxine mutants have been induced(11). An exogenous source of pyridoxine is a requirement for mammals and some protozoa. The parasitic flagellate, *Crithidia fasciculata,* requires the vitamin(14). Among the ciliates, *Paramecium aurelia* shows a possible requirement(12) and all of the classical amicronucleate strains of *T. pyriformis* so far examined fail to grow without it(1,9). The remarkable consistency in the nutritional requirements of this ciliate is indicated by the fact that from 2500 clones isolated from natural habitats and tested for their capacity to get along without any one of 18 nutrilites, only 6 clones have been found that do not require serine(4) and 41 which grow without pyridoxine(5). Among the pyridoxine non-requiring clones opposite mating types were present which made possible breeding experiments. The purpose of this report is to describe the initial steps taken in attempting to understand the genetics of the gene or genes that control pyridoxine synthesis.

MATERIALS AND METHODS

The wild type, as well as all of the classical strains of *T. pyriformis* that have been tested, require pyridoxine. The strains that do not require this vitamin are here referred to as the mutant strains. Of the 41 pyridoxine non-requiring clones, 28 were collected from natural habitats in Mexico, Panama, and Colombia(3) whereas the remaining 13 were taken in the United States. From this collection the following clones were selected for study: TC 42, TC 117, and TC 78 which are all pyridoxine non-requiring clones. UM 350 and UM 7, both requiring pyridoxine, were also used. All of these clones belonged to variety 2. These particular clones were selected following preliminary studies which indicated that their growth characteristics and

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† Present address: Department of Zoology, University of Toronto, Toronto, Canada.

sexual behavior were best suited to the needs of the contemplated experiments.

The clones were maintained on stock media consisting of 1% Proteose-peptone-Tryptone. At least three transfers at 3-day intervals were made before mating procedures were initiated. This precaution was necessary to insure maximal pairing. Previous to mating, clones were washed in centrifuge tubes through three changes of sterile double-distilled water and mixed in depression slides which were stored in petri dishes as moist chambers. Conjugation usually occurred within 6 to 8 hours at 25° C. and the process proceeded to the anlagen stage (when the two new macronuclei are visible) 14 to 18 hours later. When stained samples (aceto-carmine) demonstrated that at least 70-80% had reached the anlagen stage, pairs were isolated singly into drops of stock media in depression slides. Survival following conjugation of the original parents was so low that it was unprofitable to isolate exconjugant or caryonidal clones, hence only pairs were taken and permitted to develop into clones. In later work where crosses were made at the F_1, F_2 and F_3 levels exconjugants and caryonides were isolated and established in clonal cultures. When a flourishing culture in the depressions had been established the entire drop culture was transferred to tubes containing 2 ml. stock media. Once these cultures were growing well succeeding transfers were made to tubes containing 5 ml. of stock media.

Once the clones were established and had been subcultured through several transfers, the mating type of each was determined by cross-matching with each of the mating types (testers) in variety 2. Each clone was then tested for its pyridoxine requirement by subculturing with 0.1 ml. inoculation to tubes containing chemically defined media deficient in this vitamin. Growth of pyridoxine non-requiring clones usually occurred in all tubes following the first transfer but the pyridoxine requiring clones usually failed to grow after the second or third transfer. The pyridoxine non-requiring clones were carried through 8-10 transfers before it was decided that they could synthesize sufficient pyridoxine for their own needs. At the time this report was written a total of 67 crosses had been made, 8,000 pairs isolated, and 400 clones screened on pyridoxine deficient media. All clones were maintained at 25° C. and routinely checked for possible bacterial contamination by appropriate plating techniques.

RESULTS

Early in this investigation it became apparent that the breeding system in variety 2 is different from both variety 1(13) and 9(7). It is rare to find exconjugant clones derived from one pair that possess opposite mating types, either those of the parents or any other. Approximately 99% of both exconjugants or caryonides derived from a conjugating pair possess the mating type of either one parent or the other. This situation essentially precludes the close form of inbreeding that is desirable, namely, the mating of exconjugant or sister caryonides with identical ancestry. The usual, and often the only, course open then is to outbreed by crossing clones derived from different parents. On exceptional occasions it has been possible to isolate diverse mating types from a single cross and whenever this occurred such clones were used in subsequent breeding experiments. Selfing clones arise from some crosses (less than 1%) but owing to the difficulty in handling these clones they have not been used even though mating such clones would be highly desirable since this would be the closest form of inbreeding.

An immaturity period(2) exists in variety 2 which, in these investigations, varies from a minimum of 60 to a maximum of 250 fissions. This has the advantage of giving reasonable assurance that actual nuclear exchange has or has not occurred during the mating process. This is done by test-mating exconjugant clones with the original parents of the clones. If mating occurs there can be little doubt but that such clones are false exconjugant clones and that nuclear exchange did not occur.

Gene location. Early in this study it was essential to know whether pyridoxine synthesis was gene controlled and if so, where the genes were located, that is were they in the nuclei or in the cytoplasm? In a previous study haploid clones of different mating types had been induced by X-irradiation(6). These were useful in answering this question. Both of the haploid nuclei (migratory and stationary) in the diploid cell possess the same genetic constitution. The haploid is usually unable to undergo fruitful meiosis, hence does not produce normal haploid nuclei and is therefore a genetic blank as far as subsequent progeny are concerned.

When a pyridoxine non-requiring diploid is mated to a pyridoxine requiring haploid, both clones derived from the exconjugants grow without pyridoxine, like the diploid parent (Fig. 1). It seems quite clear that the migratory nucleus carries the gene or genes that control pyridoxine synthesis.

Crosses of clones from nature. The initial breeding experiments included the following crosses: both mates pyridoxine non-requiring and both mates pyridoxine requiring pyr- × pyr-. Crosses between TC 42 and TC 78, and between TC 78 and TC 117, all pyridoxine non-requiring yielded offspring that grew without pyridoxine. Crosses between UM 7 and UM 350, both

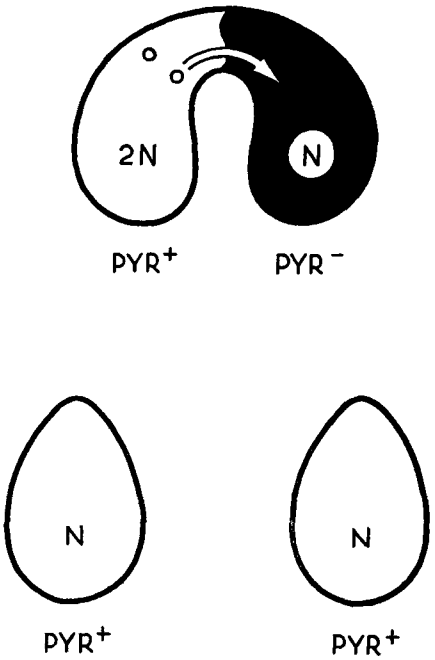

Fig. 1. A schematic representation of the events that follow when a diploid pyridoxine non-requiring (p/p) clone is crossed with a haploid pyridoxine requiring (+/o) clone. The resulting pyridoxine non-requiring progeny carry only the genotype of the diploid parent proving that the gene or genes controlling pyridoxine syntheses are carried in the micronucleus.

pyridoxine requiring, gave rise to a high percentage of clones that required pyridoxine although a few segregated out that grew well without the vitamin. From these data it was tentatively concluded that the factors controlling the pyridoxine non-requirement were recessive to those that control the requirement for the vitamin, that is, the mutant was recessive to the wild type. Based on this evidence the following genotypes were assigned to the strains: TC 78, TC 117, and TC 42 = p/p; UM 7 and UM 350 = +/p.

From the original experiment of 9 crosses involving UM 7 × UM 350 (+/p × +/p), three classes of offspring resulted (Table I, Fig. 2). One class, (+/+), failed to grow in deficient media after one or two transfers. This inability to survive in pyridoxine deficient media equalled that of strain E which has long been known to require this vitamin(1). A second group, (p/p), thrived in the deficient media even after 10 transfers. The third group, (+/p), grew slowly in the absence of pyridoxine and could be maintained for some time if the transfer interval was 5 days instead of the usual three. At this point it must be emphasized that this group of slow growers (SG) which are here classified as heterozygotes, must be a more or less arbitrary group because some of the p/p clones also grew poorly on deficient media. However, later breeding experiments proved conclusively that most of the SG clones were heterozygotes.

The second group of 6 crosses involving the mutants TC 42, TC 117, and TC 78, all p/p, resulted in 26 viable clones that grew without pyridoxine. Their growth in pyridoxine-deficient media is equivalent to that of their parents.

Lethality became evident at the F_1 level and was seen throughout all subsequent breeding experiments. Typical signs of this phenomenon were failure of the conjugants to separate, slow growth, death of the culture after a variable number of fissions, rounding up of the vegetative forms, and the loss of the micronucleus following abnormal fission. This can be accounted for, in part, by the fact that *T. pyriformis*

TABLE I

Generation	Genotypes	No. of crosses made	No. of clones obtained	No. of pyridoxine-requiring progeny	No. of pyridoxine-non-requiring progeny
P	+/p × +/p	9	17	13	4
	p /p × p /p	6	26	0	26
F_1	+/+ × +/+	—	—	—	—
	+/+ × +/p	—	—	—	—
	+/p × +/p	—	—	—	—
	+/+ × p /p	2	53	53	0
	+/p × p /p	11	43	26	17
	p /p × p /p	11	57	0	57
F_2	+/+ × +/+	1	5	5	0
	+/+ × p /p	2	17	17	0
	+/+ × +/p	1	13	13	0
	+/p × +/p	1	11	9	2
	p /p × +/p	4	36	23	13
	p /p × p /p	6	14	0	14
F_3	p /p × +/p	1	9	5	4
	p /p × p /p	2	11	0	11
Backcrosses	F_1 p/p × P_1 p /p	3	5	0	5
	F_1 p/p × F_2 +/p	3	42	25	17

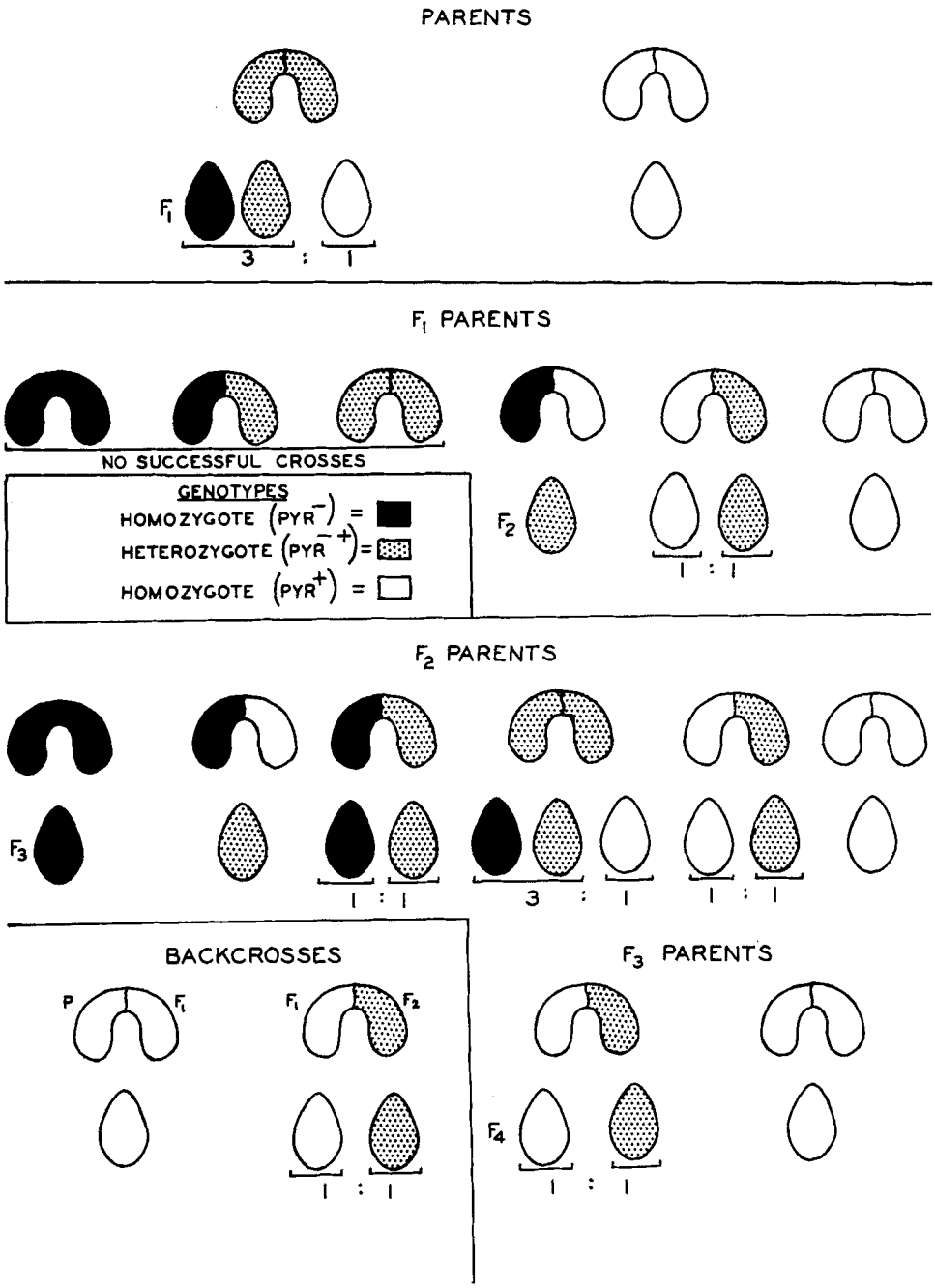

Fig. 2. A schematic representation of the crosses made through three generations, together with backcrosses. ($PYR^- = +/+$, $PYR^{-+} = +/p$, $PYR^+ = p/p$)

probably possesses a large number, if not all, of its genes in the heterozygous state which fosters an outbreeding economy. This tendency to eliminate homozygotes from the population distorts the ratios to some extent. Undoubtedly the segregating out of recessive lethal genes accounts for much of the low viability observed at the F_1 level since the parental stocks isolated from natural habitats are largely heterozygous and whatever recessive, lethal genes they may possess are expressed in the homozygous state.

F_1 backcrosses. When F_1 clones of genotype p/p were backcrossed to the parental strains TC 42, TC 117, and TC 78, (genotype p/p), all of the resulting clones grew without pyridoxine (Table I, Fig. 2). However, viability was low, ranging from 0 to 6%. Such high lethality made any other F_1 backcrosses un-

profitable, hence no more were tried. However, when the F_2 genotype +/p was backcrossed to the F_1 genotype p/p the resulting progeny approximated the 1:1 ratio (25 pyridoxine requiring : 17 pyridoxine non-requiring). In this cross the viability was much improved ranging from 13.0 to 39.1%.

F_1 crosses. The following successful crosses were made using F_1 clones: +/+ x p/p, p/p x +/p, p/p x p/p (Table I, Fig. 2). Unfortunately it was impossible to make the remaining possible crosses owing to the fact that diverse mating types were not available. One critical clone became contaminated with an antibiotic-resistant microorganism which excluded it for mating. Eleven crosses using the genotypes p/p x p/p were made. All of the progeny grew without pyridoxine. The viability ranged from 0 to 26% with a mean of 7.3%. The two crosses of +/+ x p/p resulted in progeny that needed pyridoxine in all instances although approximately 80% of them grew slowly for several transfers in the deficient media when the transfer time was extended to 5 or 6 days. Since these progeny were heterozygotes it might be expected that the survival would be high. This proved to be the case, with a mean viability of 30.5%.

Eleven crosses were made between clones of the genotypes p/p x +/p. The offspring fell into two categories approximating a 1:1 ratio. Here again the ratio was skewed toward the heterozygote, the numbers being 17 of the genotype p/p as against 26 for the heterozygotes.

In some crosses on the F_1 level, there were no surviving progeny. Usually the pairs remained fused until death although a few would separate, undergo a few fissions, then die. Occasionally amoeba-like monsters formed measuring 150 μ across. They could not be isolated with pipettes owing to the fragility of their membranes.

F_2 crosses. Thirty-five crosses, using F_2 genotypes as parents, were attempted of which only 15 gave results sufficiently satisfactory to be reported here. Among the causes for failure was the usual high degree of lethality. Moreover in many crosses, the number of pairs formed was so few that it was fruitless to isolate pairs for study. The reason for this condition, which has occurred throughout all of the experiments, is not clear. In other crosses many cells die and disintegrate supplying food for the remainder which then feed instead of conjugating as would be the case in a starved culture. The unaccountable death has been the cause for failure of many of the crosses.

All possible combinations were made in the 15 successful crosses (Table I, Fig. 2). The resulting progeny from the cross of genotypes +/+ and +/+ all needed pyridoxine. The cross +/+ x +/p gave rise to offspring which all required pyridoxine. When heterozygotes were crossed, +/p x +/p, the resulting progeny approximated the 3:1 ratio favoring pyridoxine requiring clones. Here again the ratio indicated that selection against the homozygote, p/p, was in evidence. Two crosses were made between the two homozygotes, +/+ and p/p, which gave rise to pyridoxine requiring progeny, as might be expected since they were all heterozygotes. The viability was comparatively good (30%). However, most of these clones grew slowly through several transfers without pyridoxine, which is characteristic of the heterozygote. The cross +/p x p/p yielded offspring that approximated the 1:1 ratio with selection again favoring the heterozygote. Six crosses were made involving homozygous recessive parents, p/p x p/p. All of the progeny grew without pyridoxine although growth was variable, some attaining maximal growth in 72 hours whereas others required 96 hours to attain this level. Viability was the poorest of all F_2 crosses with this combination of genotypes (mean 4.8%).

F_3 crosses. At present three crosses involving F_3 parents have been analyzed on the F_4 level. In the cross, +/p x p/p, the resulting progeny fell into two categories approximating the 1:1 ratio. The mean viability was 20%. Crosses of the two homozygous recessives, p/p x p/p, produced all pyridoxine non-requiring progeny and the mean viability was 10.9%.

DISCUSSION

Pyridoxine has been clearly demonstrated to be an absolute requirement for those classical strains of *T. pyriformis* that have been carefully studied(1,9). Moreover, Kidder and Dewey(9) have shown that pyridoxal and pyridoxamine are 500 times more active than pyridoxine in supplying the B_6 requirement for strain W. Although no specific function has been found for pyridoxine itself, pyridoxal phosphate is known to serve as a coenzyme for six different reactions involving amino acids as substrates, hence is thought to be the most versatile of any known coenzyme. Since pyridoxal phosphate readily replaces pyridoxine in the nutrition of *T. pyriformis*(9) the latter vitamin must also play a substantial role in metabolic processes. With this in mind it was surprising to find strains collected from nature that grew without the vitamin. As with the serine studies(8) the pyridoxine mutant may be considered a relict species that is on its way to extinction and exists only in small numbers and in isolated places today.

There are many examples in the literature to support the contention that heterozygotes have a selective advantage over homozygotes in nature(10). The fact that both the serine(4) and the pyridoxine mutants exist in nature as homozygotes and occur so rarely is indirect evidence for selection against the

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**

ALFRED M. ELLIOTT and GORDON M. CLARK†
Department of Zoology, University of Michigan, Ann Arbor

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.