The induction of gene mutation and chromosome aberration in *Chlamydomonas eugametos* by a phenylalanine analog*

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1. INTRODUCTION

In a study of the sensitivity of *Chlamydomonas eugametos* to an array of analogs and antimetabolites (paper in preparation), the phenylalanine analog, 2-amino-3-phenylbutanoic acid (APBA, 3-methylphenylalanine), was found to be an effective inhibitor of growth of wild-type cells. Mutants resistant to the analog were recovered.

This paper describes the unexpected mutagenic properties of APBA when cells of *C. eugametos* are grown in its presence. Edelson & Keeley (1963) reported that APBA was not toxic to the bacterium *Escherichia coli* W, but did inhibit growth of *Leuconostoc dextranacum* 8086. No report of mutagenic action of APBA has been described.

2. MATERIALS AND METHODS

The wild-type stocks of *C. eugametos* were originally obtained from the Culture Collection of Algae at Indiana University (Starr, 1964). The basic minimal medium of Gowans (1960) was used. For solid media 1·2 % Difco agar was added. The techniques used in genetic analyses were those described by Gowans (1960) and Nakamura & Gowans (1965).

Fluctuation tests (Luria & Delbrück, 1943) were utilized to study mutation to APBA resistance. Each test was started from a reisolated colony arising from a single sensitive cell. The cells were grown in liquid minimal medium and an aliquot diluted to inoculate 25–30 tubes (independent cultures). Fewer than 100 cells were placed in each tube containing 0·5 ml liquid minimal medium. These independent cultures were grown for approximately 100 h in continuous light at 25 °C. The entire culture from each tube was then plated on medium containing the analog. Control platings (samples from the same culture) of progeny of the original APBA-sensitive isolate were also made in each test. All resistant colonies were counted 14 days after plating.

3. RESULTS

Most cells of *C. eugametos* do not form colonies on medium that contains more than 50 μg APBA per ml. Substantial numbers of resistant clones can, however, be recovered from cells plated on media containing lethal levels of the analog. The frequency of such clones increases with time after plating. Table 1 depicts this increase with time, and shows a decrease in numbers of resistant colonies on higher analog levels. This latter observation is consistent with genetic data. Three APBA-resistant genes (designated *mpa* for methylphenylalanine), each of which is genetically independent of the others and confers a different level of resistance, have been analysed (unpublished data). One gene, *mpa-2*, confers low-level resistance (less than 200 μg/ml); a second, *mpa-3*, provides resistance to 200 but not 500 μg/ml; while the third, *mpa-1*, permits growth on plates containing more than 500 μg/ml. Therefore, the plates containing 100 μg APBA/ml allow growth of all three types, whereas *mpa-2* mutants are unable to grow on the 200 μg/ml plates; 500 μg APBA/ml permits growth of only *mpa-1* mutants.

Table 1. *Appearance of APBA-resistant colonies with time on different APBA concentrations*

(Resistant colonies per 10^6 cells plated.)

APBA (μg/ml)	Days after plating		
	6	10	18
100	5·0	41·2	79·0
200	5·2	12·5	25·3
500	1·6	4·7	8·0

Fluctuation tests (Luria & Delbrück, 1943) were done to explore further the high frequency and concomitant increase with time in numbers of resistant colonies. Table 2 presents data from five such tests.

Spontaneous mutations to APBA resistance in the absence of the analog should result in much higher variance values from independent cultures than from samples from one culture. This is not the case. Variance values are similar in sections A and B (Table 2). One test in each section does have a significant P value, but neither variance is extremely large.

Results, as in sections A and B, could occur from either plating populations containing a very similar number of pre-existing resistant cells or plating sensitive cells which became resistant after exposure to the analog. Support for this latter possibility is given in section C (Table 2), in which cells were grown in sublethal levels of APBA. This treatment increased both the number of resistant colonies and the variance.

The resistant colonies appearing on the APBA plates in these fluctuation tests could be resistant due to mutations of *mpa-1*, *mpa-3*, or other genes conferring resistance, or they could be physiological adaptations (phenocopies) to the presence of APBA in the medium. A survey of resistant colonies isolated from APBA plates

has shown that 90 % (27 of 30 tested) have a permanent inherited change to APBA resistance. Resistance segregated in a Mendelian manner when these isolates were crossed with sensitive strains. Only 10 % of the isolates tested proved, on subsequent testing, to be sensitive to APBA (unpublished data).

The absence of large fluctuations among independent cultures is not due to differences in growth rates. The growth rates of *mpa-1*, *mpa-2* and *mpa-3* strains in minimal medium are not different from each other or from wild type. Reconstruction experiments, in which equal numbers of APBA-resistant, and wild-type cells were grown in mixed culture, showed no competitive advantage of either type.

Table 2. *Fluctuation analyses*

Test	APBA concentration in plates (μg/ml)	No. of samples	Average no. resistant colonies per sample	Variance
		A. Samples same culture		
1	500	23	0·27	0·21
2	200	30	6·32	2·51
3	200	28	4·00	9·88*
4	200	25	7·08	9·64
5	500	25	1·58	1·73
		B. Independent cultures (grown in minimal medium)		
1	500	24	3·03	3·89
2	200	30	6·33	10·09
3	200	27	2·25	3·53
4	200	25	6·17	5·15
5	500	25	1·60	4·33*
		C. Independent cultures (grown in 10 μg APBA/ml)		
4	200	25	12·48	43·44*
5	500	25	2·68	9·89*

* P value (χ^2) less than 0·01.

Experiments involving mutation to antibiotic resistance as well as to two levels of APBA resistance are given in Table 3. Wild-type cells were grown either in liquid minimal medium or in liquid minimal medium containing 10 μg APBA per ml for 100 h. Aliquots were spread on plates containing either neamine, streptomycin, or APBA at one of two concentrations. The cultures grown in the presence of the analog had significantly higher numbers of cells resistant to each antibiotic as well as to APBA.

Meiotic product lethality occurred when some APBA-resistant isolates were crossed with wild type. Five of thirty-one crosses gave lethality patterns which could best be interpreted as resulting from the presence of heterozygous reciprocal translocations.

Perkins (1966) has given the expected frequencies of various patterns of ascospore survival within unordered (shot) asci of *Neurospora crassa* that have developed from diploid cells heterozygous for different types of chromosome aberrations. The expected tetrad patterns in *C. eugametos* (converting from *Neuro-*

spora) for a cross containing a heterozygous reciprocal translocation can be summarized with regard to ratios of survivors:non-survivors as follows: equal frequencies of the 4:0 and 0:4 tetrad classes (a result of alternate or adjacent type segregation respectively, with no exchanges between break points and centromeres); and a 2:2 class that reflects single exchanges within the interstitial segments. The frequency of this latter class is a function of distances involved and may range from nil to at least two-thirds. On the basis of this model, tetrads of the 3:1 and 1:3 types are not expected (Perkins, 1966).

Table 3. *Mutation to antibiotic and APBA resistance*

	Growth medium			
	Minimal		Minimal + APBA (10 μg/ml)	
Selection medium,	No. cells plated ($\times 10^6$)	Resistant colonies per 10^6 cells	No. cells plated ($\times 10^6$)	Resistant* colonies per 10^6 cells
Experiment 1				
APBA (200 μg/ml)	21·2	18·0	9·0	230·0
APBA (500 μg/ml)	22·0	9·3	9·0	90·0
Neamine (50 μg/ml)	22·0	11·2	9 0	20·1
Streptomycin (50 μg/ml)	22·0	2·2	9·0	7·1
Experiment 2				
Streptomycin (50 μg/ml)	28·4	2·5	27·9	5·7

* All differences between treated and untreated cells significant at $P = 0.01$ (*t*).

Survival data from crosses of the five strains carrying putative reciprocal translocations to normal strains are presented in Table 4. A comparison of the frequencies of the 2:2 types indicates that at least three distinct translocations are present. All strains, except T 5, were selected as APBA-resistant colonies. T 5 was isolated as a streptomycin-resistant mutant following growth of an *mpa-2* strain in a sublethal concentration of APBA. The presence of a number of the 3:1 and 1:3 tetrad types in some crosses may reflect additional alterations.

Four other isolates (in addition to those reported in Table 4), when crossed with wild type, exhibited meiotic product lethality that could be interpreted as resulting from heterozygous inversions or complex chromosome aberrations, whereas the remaining twenty-two isolates produced little, if any, lethality among the meiotic products.

Several points discount any association of APBA resistance and the chromosomal aberrations. (1) The original strains of *mpa-1*, *mpa-2* and *mpa-3* (as well as the twenty-two isolates described above) have never exhibited lethality in crosses. (2) Of those isolates having apparent reciprocal translocations, T 5 was derived

from an *mpa-2* strain, and it remained resistant at the *mpa-2* level; T 1, T 2, and T 4 are resistant to different levels of the analog, whereas T 3, isolated as an APBA-resistant clone, proved sensitive to the analog (a phenocopy). (3) The reciprocal translocation most studied (T 4) shows 2:2 segregation of both APBA resistance and the ability to produce lethality in crosses. The two, resistance and product lethality (reciprocal translocation) are separable in progeny tests.

Table 4. *Survival patterns of five putative reciprocal translocations when crossed with wild type*

| | Number of tetrads* | | | | | 2:2 |
Cross	4:0	3:1	2:2	1:3	0:4	frequency
wt × wt	93	1	1	0	0	—
T 1 × wt	43	5	3	0	27	0·04
T 2 × wt	28	5	95	20	26	0·55
T 3 × wt	33	4	71	7	26	0·50
T 4 × wt	43	7	105	12	44	0·50
T 5 × wt	26	4	29	2	25	0·34

* Survivors:non-survivors.

4. DISCUSSION

The high frequency of mutation to APBA resistance, results of fluctuation tests, mutation to antibiotic resistance as well as to APBA resistance following growth in sublethal concentrations of APBA, and the presence of apparent chromosome abnormalities after APBA treatment, all support the hypothesis that this phenylalanine analog affects genetic material.

The two vitamin analogs, 3-acetylpyridine (Nakamura & Gowans, 1965) and pyrithiamine (McBride & Gowans, 1967), have been previously studied for their effects on *C. eugametos* and no evidence was found for such effects on either genes or chromosomes.

There have been reports of a variety of cytogenetic and genetic effects being induced in fungi by another phenylalanine analog, *para*-fluorophenylalanine. This analog has been reported to induce haploidization of diploid *Aspergillus niger* (Lhoas, 1961), aneuploid production in diploid *Saccharomyces* (Emeis, 1966), and mitotic crossing-over, non-disjunction, aneuploidy, and gene conversion in diploid *Saccharomyces cerevisiae* (Strömnaes, 1968). Growth of *C. eugametos* is not inhibited by *para*-fluorophenylalanine in concentrations as high as 1 mg/ml.

The three APBA-resistant genes reported here segregate in Mendelian manner in crosses and each shows a consistent frequency of second-division segregation. These second-division segregation frequencies are interpreted in tetrad analysis as measures of gene-centromere distances of chromosomally located genes. This contrasts with the induction of 'non-chromosomal' streptomycin resistance in *C. reinhardii* by streptomycin, where uniparental inheritance is exhibited (Sager & Tsubo, 1962; Gillham, 1965).

It would appear, then, that APBA is inducing mutations in nuclear genes. The concomitant effect of APBA on chromosome integrity might be taken as further evidence of this conclusion.

The mechanisms involved in induction of mutations and aberrations by APBA are not known. The apparent chromosome abnormalities may result from incorporation of the analog into chromosome-associated proteins. Gene mutations may result from donation of the methyl group of APBA to DNA bases either directly or indirectly following analog degradation. It does not seem unlikely that APBA could be incorporated into DNA polymerase resulting in altered DNA in subsequent generations. The high frequency of mutational change and chromosome breakage even admits the possibility that APBA might induce controlling elements similar to those observed in maize (McClintock, 1965).

SUMMARY

Evidence is presented which indicates that the phenylalanine analog 2-amino-3-phenylbutanoic acid (APBA) induces mutation to APBA, streptomycin, and neamine resistance in the green alga *Chlamydomonas eugametos*. Apparent chromosome abnormalities (reciprocal translocations and inversions) were also found among some clones recovered after APBA treatment. The two, mutation and chromosome aberration, are separable.

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