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Use of Haploid Plants as Bioassays for Mutagens

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Results of a pilot program show that suspension cultured polyhaploid *Nicotiana tabacum* cells can be used to bioassay the effects of mutagens. Reproducible survival curves with significant regression coefficients are obtained. Putative mutation conferring resistance to amino acid analogs is significantly more frequent after exposure to mutagens; in contrast, habituants, cytokinin-independent clones, are significantly less frequent (although the variance of clone size increases!). The maximum spontaneous mutation rate is estimated at 3×10^{-8}; the equilibrium frequency of habituant cells in an otherwise nonhabituated culture is estimated at 5×10^{-7}. An evaluation of the system suggests changes in several and further characterization of other of the parameters involved. The use of haploid tobacco as an *in vivo* mutagen screen is briefly described, as is the importance of similar *in vivo* diploid systems for discriminating between various kinds of mutational processes.

Introduction

Mutagenic agents typically effect several mutational processes. In mouse erythoblasts, for example, a "known bacterial frame-shift mutagen" produces chromosome fragments (*1*). Most bioassays for mutagenic activity by use of higher plants simply measure the total number of mutations; the partitioning of this quantitative measurement into categories—sectors due to deletion, due to point mutation, due to gene conversion, and so on—is not, and usually could not, be done. This lack of qualitative characterization severely limits the depth of comparisons of different mutagens or even the evaluation of a single mutagen at different doses. Two mutagens, or doses of one mutagen, could give the same total number of "mutations" in a bioassay system. One mutagen or dose might cause mainly deletions while the other mutagen or dose might cause primarily point mutations. In another biological system, the relative mutagenicity of those two agents or doses will be the same only if the biological system allows for both types of mutations, and at the same sensitivity as the first assay system.

One approach that eliminates the difficulty brought on by different types of mutation being involved is developing assays specific for a single kind of mutational process. Some of the experiments reported in this paper were designed to recover point mutations conferring resistance to amino acid analogs on *Nicotiana tabacum* cells grown in suspension culture. These experiments measured the point mutagenic effect of a mutagen; other experiments were designed specifically to separate out and quantify the "carcinogenic" effects of a mutagen by selection of epigenetically modified, cytokinin-habituated subclones of tobacco cells from suspension culture.

Methodology

Material

Cell lines in these experiments were derived from polyhaploid pith of *Nicotiana tabacum* cv. Wisconsin 38, $n = 2X = 24$. Cursory cytological analysis showed a much higher chromosome number (50+) including the presence of a single huge chromosome. These cells were maintained as suspension cultures at 25°C in standard liquid media [Murishige and Skoog salts (*2*), thiamine, 1 mg/l., sucrose, 30 g/l., IAA, 3 mg/l., 0.3 mg/l. kinetin was added for nonhabituated lines; these media and the cells growing in them are referred to as 3/0 or 3/.3, respectively.].

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Growth Measurement

Growth of suspension cultures is routinely measured by the V_{30}, the volume of settled cells in a 50 ml culture after 30 min. This is easily ascertained with graduated centrifuge tubes and allows for correction of culture volume to 50 ml. Reconstruction experiments show that V_{30} is linearly related to cell number up to a V_{30} of 35 (Fig. 1). Cell counts show there to be $3.8 \pm 0.5 \times 10^5$ cells/ml V_{30}.

Mutagenesis

The chemical mutagen NTG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) as a filter-sterilized aqueous solution was added in appropriate amounts (0 to 200 μg/ml final concentration). Initial experiments exposed cells with agitation for 30 min, followed by settling for 30 min. Removal of mutagen was via repeated decantation and 30-min settlings, after Widholm (*3*). As experiments detected no ill effects of centrifugation at up to 20 min at 810*g*; later experiments exposed cells to NTG for 1 hr with 5-7 min at 600*g* centrifugation to settle cells between washes.

Initial "failure" to recover mutants instigated a series of ultraviolet irradiations. Cultures in quartz flasks were exposed to a germicidal lamp (Sylvania G15T8, 13 cm) while being agitated. Irradiation and transfer to aluminum foil-covered culture flasks was done in very dim light to disallow photorepair.

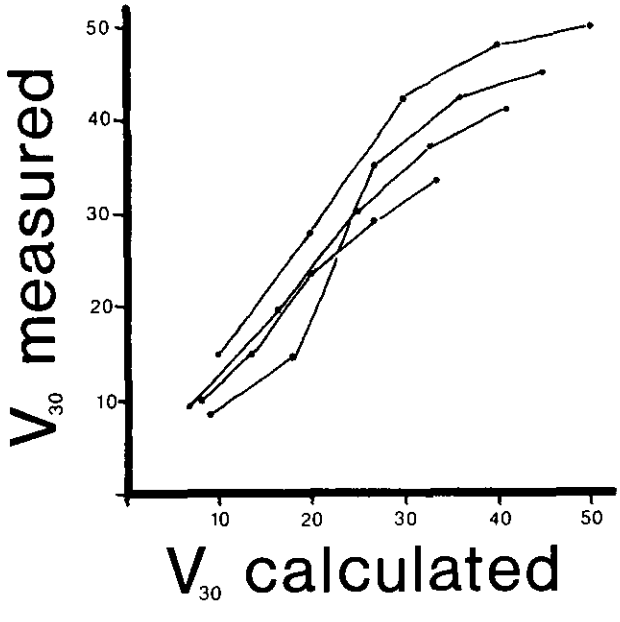

FIGURE 1. Linearity of V_{30} as a measurement of cell number. Cell suspensions were diluted out and the V_{30} measured (the volume of settled cells after 30 min). The V_{30} calculated is the dilution times the measured V_{30} of the undiluted suspension; the relationship is similar if the most dilute preparation is used as the standard. The combined data have a linear regression, $r = 0.96$, 18 d.f., slope = 1.01.

Kill Curves

Per cent survival was estimated by back extrapolation of growth curves (*4*); controls for each experiment were standardized to 100%.

Recovery of Variants

Amino Acid Analog Resistant. Cultures were allowed to grow up after mutagenesis. Initially, two "level scoops"—a standard wire screen implement in our laboratory containing 1.1 ml V_{30} (B. Floyd, personal communication) were placed on each selective plate (9 cm diameter) and spread evenly with a spoonlike spatula (8.4×10^5 cells per plate). Later, larger experiments used the method of our colleague, R. Malmberg: 10 ml of a cell suspension adjusted to a 2:1 ratio of V_{30} to excess media will cover five petri dishes evenly and almost exactly (5.1×10^5 cells/plate). Selective plates were 3/.3 media solidified with 9 g/l. Difco Bacto-Agar and had the amino acid analogs *S*-aminoethylcysteine, methyltryptophan, or ethionine added at 0.1, 0.1, and 1.0 *mM*, respectively. These are analogs for the amino acids lysine, tryptophan, and methionine, respectively.

Habituants. While both 3/.3 and 3/0 cell lines were used in mutagenesis, quantitation of the carcinogenic/epigenetic effects of a mutagen (measured by the production of cytokinin-independent, habituated lines) necessarily were restricted to 3/.3 lines. Cells were mutagenized as above, washed with and left in 3/0 media, grown up to starve the cells for kinetin and then plated *via* Malmberg's "slosh" on 3/0 solid media.

Results

Kill Curve

A careful quantitation of mutagenesis in suspension culture must necessarily include adequate statistical analysis; indeed, we find such analysis uncovers important parameters governing mutagenesis. Table 1 presents parallel regression analyses, survival against NTG added per culture, or per ml V_{30}. In both cases, the variance within each of the four experiments was found to be homogenous, permitting comparisons of the data without transformation; the four regression lines in each analysis were found to be parallel. However, the elevation of the four lines is not significantly

Table 1. Regression analyses of survival curve data.

Source	df	SS_x	SS_{xy}	SS_y	Deviations df	Deviations SS	Deviations MS	
NTG per flask								
within								
7605	4	70.00	− 1.9101	0.06159	3	0.0095	0.0032	
7607	6	1171.43	− 8.8303	0.12737	5	0.0608	0.0122	
7609	9	640.00	−11.5159	0.33773	8	0.1305	0.0163	
7612	13	2685.71	−19.6266	0.42492	12	0.2815	0.0234	
					28	0.4823	0.0172	$\chi^2 = 3.3$, 3 df, N.S.
pooled	32	4567.14	−41.8829	0.95161	31	0.5675	0.0183	
			difference between slopes		3	0.0852	0.0284	$F_{3,28} = 1.6$, N.S.
B	3	1421.66	3.5824	0.11626				
Total	35	5988.89	−38.3005	1.06787	34	0.8229		
			between adjusted means		3	0.2554	0.0851	$F_{3,31} = 4.7, p < 0.05$
$Y = -6.3953X + 0.8838$			$Y = 0.8688e^{-0.008275X}$					
$r = 0.48$			$r = 0.45$					
NTG per V_{30}								
within								
7605	4	0.34571	−0.13209	0.06159	3	0.0111	0.0037	
7607	6	3.28460	−0.45889	0.12737	5	0.0633	0.0127	
7609	9	1.92462	−0.63640	0.33773	8	0.1273	0.0159	
7612	13	3.88199	−0.78046	0.42492	12	0.2680	0.0223	
					28	0.4697	0.0360	$\chi^2 = 2.7$, 3 df, N.S.
pooled	32	9.43685	−2.00784	0.95161	31	0.5244	0.0169	
			difference between slopes		3	0.0547	0.0182	$F_{3,28} = 0.5$, N.S.
B	3	0.74538	0.03143	0.11626				
Total	36	10.18223	−1.97641	1.06787	34	0.6842		
			between adjusted means		3	0.1598	0.0533	$F_{3,31} = 3.2$, N.S.
$Y = -0.1941X + 0.9217$			$Y = 0.9125e^{-0.251218X}$					
$r = 0.59$			$r = 0.57$					

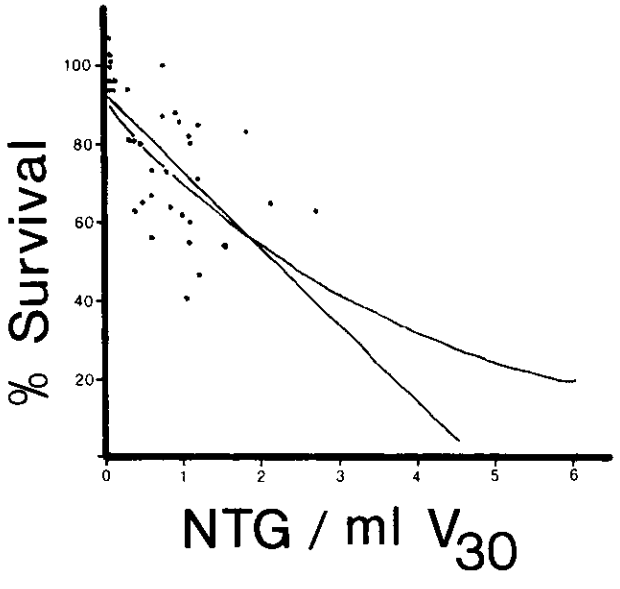

FIGURE 2. Survival of *N. tabacum* suspension cells after exposure to NTG. Cell suspensions (3/0) exposed to NTG for 1 hr. Abscissa is NTG in μg/ml media/ml V_{30}. The linear regression, survival = −0.1941 NTG/ml V_{30} + 0.9217, and the exponential regression, survival = 0.9125 exp {−0.251218 NTG/ml V_{30}}, are both significant, $r = 0.59$ or 0.57, 34 d.f., respectively. Survival was measured by back extrapolation of growth curves, controls taken as 100%.

different only for the regression against NTG per ml V_{30}. The combination of the data to calculate one line regressed against NTG per culture is invalid ($0.05 > p > 0.01$) even though the regression lines, linear and exponential, are significant.

While the regression analysis against NTG per ml V_{30} does permit combination of all the data, such combination results in both significant linear and exponential regression lines. Reference to Figure 2 shows that the difference between the two lines is greatest at high concentrations of mutagen. Despite our efforts, 41% survival was the lowest we could achieve; high mutagen concentrations typically killed off the entire culture, although it has been calculated (*5*) that given the required minimum inoculation density, with a generation time of 41 hr, 24 days are required before recognizable growth occurs; Widholm (*3, 6*) allows cultures to recover for two months. Extrapolation of these two regressions to high doses leads to a paradox—the linear regression predicts zero or negative numbers of surviving cells. As mutant induction or habituation experiments at those doses do show some survivors (see later, Table 2), the exponential regression is taken as a better predictor at high doses.

Amino Acid Analog Resistant Variants

As our "mutants" have not been adequately tested, we will follow the suggestion of Parke and Carlson (*5*) and call them variants or putative mutants. The number of true mutants (meiotically transmissible trait) among variants growing on a selective plate is often quite small (5.8%, 5.0%) (*7, 8*).

The maximum spontaneous mutation rate for resistance to 5-MT, SAEC, or ETH may be calculated from these experiments: what is the greatest mutation rate that gives a χ^2 of 3.841 against 0 variants in 191 plates (or 248 if the UV controls are included, or 174.3 if the NTG controls are corrected via the exponential regression). That maximum rate is $3.9 \pm 1.9 \times 10^{-8}$ (or 3.1 ± 2.0 or $4.3 \pm 1.9 \times 10^{-8}$, respectively). This is in agreement with values calculated by Malmberg (personal communication) for *ts* mutants in these same cell lines, and apparently similar to the values of Sung for cycloheximide or 5-fluorouracil resistance in carrot cells (*4*) or 5-MT resistance in soybean (*9*). This maximum rate may, of course, be an overly large estimate. Nevertheless, mutagen significantly increases the frequency of putative mutants, corrected or uncorrected for percent survival (Table 2).

Confidence in the rates of mutation reported in Table 2 would be greater (that is, the 95% confidence interval would hopefully decrease) if larger numbers of mutant events had been recovered. It is important to distinguish between the frequency of putative mutants—recovered clones of variant cells—and the mutations (*10*). When plating out is done several to many generations after a mutational event, the number of mutant clones recovered will be high. The values in Table 2 are putative mutational events; the frequency of variant recovery was so low that two or more resistant clones on plates from one treated flask are almost certainly derived from the same mutational event. The mutational events were represented by one, two, or three variant clones in eight, four, and two cases, respectively (the total, 14, putative mutational events reported in Table 2). As cultures in Sung's (*4*) experiments were recovered after increasingly long times as mutagen dose increased, her reports of increased frequencies of resistant colonies are an interaction of clonal propagation of variants before plating as well as any increase in frequency of mutation. The methods of Lea and Coulson (*10*) should be used to determine accurate frequencies of mutation when medium to large numbers of mutant clones are recovered. Further investigations quantitating mutation rate in cell suspension culture will hopefully report mutation rate per cell exposed rather than mutations or mutants per cell plated.

Rigorous identification of the variant cell lines

Table 2. Numbers and frequencies of putative mutational events after exposure of *Nicotiana tabacum* cell suspension cultures.[a]

				Plates				
		Dose			Corrected		Rate × 10^{-8}	
Dose	V_{30}	$\frac{\text{Dose}}{V_{30}}$	Variants	Total	Lin.	Exp.	Gross	Exp corrected
NTG[b]								
0	23.3	0.0	0	191	176.0	174.3	0.0	0.0
5	14.5	0.3	0	19	16.2	15.9	0.0	0.0
10	20.3	0.5	0	91	75.2	73.4	0.0	0.0
20	21.6	0.9	1	36	26.7	26.0	5.6 ± 1.0	7.7 ± 1.0[d]
30	26.4	1.1	3	143	100.3	98.1	4.2 ± 1.7	6.1 ± 1.7
40	24.5	1.6	1	18	10.9	10.5	11.1 ± 1.0[d]	19.0 ± 1.0[d]
50	28.1	1.8	2	194	111.8	113.2	2.1 ± 1.4	3.5 ± 1.4
80	31.2	2.6	0	138	58.5	66.1	0.0	0.0
100	32.1	3.1	1	125	39.6	52.1	1.6 ± 1.0	3.8 ± 1.0
150	29.6	5.1	0	24	Neg	6.1	0.0	0.0
200	34.0	5.9	1	103	Neg	21.4	1.9 ± 1.0	9.3 ± 1.0[d]
UV[c]								
0			0	57			0.0	
5			3	69			8.7 ± 1.7[d]	
10			1	33			6.1 ± 1.0	
15			1	33			6.1 ± 1.0	
				Maximum spontaneous			3.1 ± 2.0	4.3 ± 1.9

[a] Values in the table represent a combination of data from experiments with both 3/0 and 3/.3 cell lines; variant clones grew on selective plates containing either methyltryptophan, aminoethylcysteine or ethionine at 0.1, 0.1 or 1.0 *mM*, respectively; numbers are totals for all three selective regimes (homogeneity χ^2, cell type, 2.9, 1 d.f., selective regime, 3.5, 2 d.f., both N.S.).

[b] NTG in μg/ml media.

[c] UV, min at 13 cm, Sylvania #G15T8 germicidal lamp.

[d] Differs from the maximum spontaneous rate by more than the sum of the two standard deviations.

selected on plates (or in liquid culture) as mutant lines (as opposed to epigenetically modified lines) requires regeneration of plants and a demonstration of the transmission of the character in a sexual cross; putative subclones of the same mutation will, of course, map at the same point and will not complement. These properties can resolve any residual uncertainty as to whether two variants recovered from one treated culture represent one or two mutations.

This test of regeneration and transmission has been applied only to a few "mutants" produced in cell culture (*7, 8, 11, 12*) and never in a quantitative study of mutagenesis.

Habituant Production

A given agent may have other than mutagenic action; the carcinogenic or teratogenic activity of a mutagen is often of greater immediate importance. The phenomenon of habituation in plant cell culture, an epigenetic event giving auxin, or especially, cytokinin independence and its parallels with crown gall tumors and to other cancers is well discussed (*13, 14*). One estimate of its frequency of occurrence spontaneously is 10^{-3} per cell doubling (*15*); we initiated some experiments to measure the incidence of habituation, both spontaneously and after exposure to NTG.

The six cultures not exposed to NTG all produced at least one variant, cytokinin-independent clone (from one to five); after exposure to mutagen, however, the frequency of cultures with at least one habituant declined (Table 3). Yet, the average number of clones recovered per culture with variants increased. This latter is usually taken as evidence for a causal relationship between an agent and a phenomenon; the former is a definite contraindication. The increased clone size could also be explained as the result of more doublings of habituated cells in a culture recovering from mutagen-mediated cell killing. If there were, on the average, only a few habituants in a flask at the time of treatment, coincidence of mutagen-mediated killing could also explain recovered cultures devoid of habituants. In fact, the numbers of cultures with habituants are not significantly different from those expected on the basis of a standard number of 3/0 cells per ml V_{30} in a 3/.3 culture, given the survival percentages (exponential regression) for the NTG doses (Table 3, $\chi^2 = 2.18$, 3 df, N.S.).

The total numbers of habituated clones at each dose are distributed as the numbers of plated surviving cells (homogeneity $\chi^2 = 7.47$, 3 df, N.S.); combining the data results in a calculation of the frequency of 3/0 cells in a 3/.3 culture of 4.6×10^{-7} (more frequent than the spontaneous mutation rate, above. No wonder the majority of biochemical variants are epigenetic variants). At 4.6×10^{-7}, the 34.2 ml V_{30} in the control flasks would contain 6 3/0 cells and 1.3×10^{-7} 3/.3 cells. A medium to low plating efficiency [typical for plant cell culture (*5*)] combined with NTG-mediated coincident killing of those 6 cells could easily produce cultures without habituants at the frequencies observed (Table 3).

NTG, then, is not causing epigenetic events at least at a rate that can be distinguished from stochastic processes acting on a small number of habituated cells already present. But mutagenesis of such mixed cultures results in some purely 3/.3 cultures. In these cultures the spontaneous rate of habituation can be measured—rather than the equilibrium frequency of habituants—and effects on this rate by mutagens and "epigens" will be able to be distinguished.

Discussion

In Vitro Experiments

During the courses of our experiments we have become aware of (or begun to suspect) many different parameters that do or might affect the results of mutagenesis/epigenesis experiments. Of prime importance in our minds are the following.

Our cell lines, 3/.3 or 3/0, were not true polyhaploids; indeed why use a polyhaploid at all, responsible perhaps for the leakiness of mutants recovered

Table 3. Numbers and distribution of habituant clones in 3/.3 cultures after exposure to NTG for 1 hr.

Dose, μg/ml	V_{30}, ml	Survival	Cultures	Habituants: Numbers: Total	Habituants: Numbers: Variance	Habituants: Cultures with: Obs	Habituants: Cultures with: Exp[a]
0	34.2	0.91	6	15	2.70	6	6.00
10	30.7	0.77	7	10	11.29	2	4.88
20	35.8	0.74	7	25	23.62	4	5.43
50	34.0	0.63	4	2	0.33	2	2.51
						$\chi^2 = 2.18$, 3 df., N.S.	

[a] Expected calculated as $(V_{30,X}/V_{30,0})(S_X/S_0)N_X$, where $V_{30,X}$ is the average V_{30} at dose X, S_X, the predicted survival at those dose from the exponential regression, and N_X is the number of cultures.

(*7*) when the monohaploid, *Nicotiana sylvestris*, gives extremely vigorous cultures and regenerates extremely readily. Our mutation rate with *N. tabacum* "polyhaploid" cells is the same as (or lower than) those rates reported for diploid *N. tabacum* (*3, 8, 16*). Mutagenesis of diploid *N. sylvestris* has been reported (*17*); comparisons of rates and spectra of mutations between haploid and diploid could, for example, confirm some speculations (*5*) about effective selection for certain types of mutant enzyme activity.

Habituation experiments will require monitoring of temperature (*13*) as well as an accurate determination of the time course of kinetin starvation. Our "several days" is not precise enough, especially if rescue of numbers of habituants at or below the minimum inoculation density is desired. Similarly, the afterculture media in mutagenesis experiments needs to be investigated. We found 3/0 cells did better (recovered quicker, had better color) when washed and grown subsequent to mutagenesis in 3/.3 media. Yet kinetin is supposed to suppress the growth of habituants (*14*). Does exposure to mutagen transiently revert epigenetically modified cells with high efficiency? Meins (*14*) and Kandra (*18*) differ in their interpretation of the effect of BUDR on habituation; is NTG also a specific block of differentiation in the right system?

Our putative mutant callus is extremely slow growing—on 3/0, 3/.3 or a supplemented 3/.3 media; Malmberg reports the same for *ts* mutants grown at permissive temperatures. Now, over a year after mutagenesis, our variant clones have increased enough to begin retesting for resistance and regeneration, and to test transmission of the trait. Surely there are post-selective cultural conditions that give greater growth rates for variants.

In Vivo Experiments

Whole leaves of tobacco plants can be considered as biological equivalents of dishes densely covered with cells. For example, green islands in otherwise virus-infected leaves, rescued through tissue culture, are an efficient (50%) way of obtaining virus-free plants (*19*). Very recently (*20*), whole leaves of haploid Wisconsin 38 tobacco plants have been used to select herbicide resistant mutants. Briefly, young polyhaploid plants ($n = 2X = 24$) were irradiated and, when more mature, sprayed with lethal herbicides; green putative-resistant clones are excised, rescued as whole plants *via* tissue culture, and assayed for retention of the resistant phenotype and the transmission genetics of the trait. Plants were regenerated from green sectors; 18% retained the resistance as whole plants. After diploidization, herbicide resistance was recovered in the F_2 of 45% (8% of rescued green sectors) of these; the F_1s were all sensitive. Interestingly, this 8% is similar to the recovery of meiotically transmissible mutations from variants selected after mutagenesis of cell suspension cultures (*7*) or protoplasts (*8*); plant cells, *in vivo* or *in vitro*, it seems, react to agents with a spectra of genetic and epigenetic events.

This whole plant system simultaneously assays "point"-mutagenic capacity as well as two epigenetic capacities of a particular agent. The identity and independence of mutant events is evidenced by the spatial relationship of mutant tissue in the assayed leaves. Test crosses would assign a particular mutation to one of several alleles giving resistance to the selective condition, allowing later comparison of the distribution of mutants produced by several mutagens. Nonresistant plants regenerated from resistant clones and resistance not meiotically transmissible are two distinguishable epigenetic phenomena.

Rescue of whole plant sectors via tissue culture into whole plants themselves can be used in studies of somatic mutation in nonhaploid plants—soybean, tobacco, cotton, *Tradescantia*. Homologous exchange, homeologous exchange in other than diploids, deletion, nondisjunction, gene conversion, point mutation all can produce variant clones in leaves or petals. Rescue, regeneration, and genetic analysis of the mutant sectors produced after exposures to various agents will characterize each agent (and each level of an agent) in a particularly meaningful way.

The challenge in the use of higher plants, haploid or diploid, lies not in the replacement of bacterial bioassays, but in the precision that their higher levels of organization lend to dissection and analysis of the component processes of "mutation." Whether we choose a suspension cell culture to assay biochemical point mutations, or epigenetic events, a haploid leaf system to distinguish two levels of epigenetic modification as well as gene mutation, or a diploid leaf, petal or stamen hair with its host of possible mechanisms to be distinguished, we are approaching an assay, quantitative and qualitative, of the real total biological effect of an agent on a eukaryotic system.

Conclusions

The importance of the distinctions between mutation and mutant, between rates of the former and population frequencies of the latter, can not be overemphasized. We calculate a maximum spontaneous mutation rate for resistance to 5-MT, SAEC, or ETH of 3×10^{-8} per cell plated; calcula-

tion of the mutation rate per cell exposed is preferable, but not possible from our data.

Habituant cells, we find, exist in nonhabituated cultures at a frequency of 5×10^{-7}. Determination of the rate of habituation events was not possible in these cultures; those cultures, devoid of habituant cells, which arise after mutagen treatment can be used to determine the rate of the epigenetic events that cause habituation.

When exposed to mutagenic agents, plant cells, *in vivo* or *in vitro*, give rise to genetically and epigenetically modified cell lineages. In many cases, the epigenetic effects (carcinogenesis, teratogenesis) of an agent may be of greater immediate importance. The higher levels of genetic organization in plant cells as compared to prokaryotes lend themselves to the dissection and analysis of the component processes of "mutation." Higher plants are admirably suited for use in assays, both quantitative and qualitative, of the real, total biological effect of an agent on a eukaryote.

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