Radiation-induced *alcohol dehydrogenase* mutants in maize following allyl alcohol selection of pollen

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SUMMARY

The *alcohol dehydrogenase-1* gene in maize presents advantages for mutational analysis. Foremost among these is the ability to chemically select ADH-negative and ADH-low gametophytes owing to their resistance to allyl alcohol vapour. Immature tassels were irradiated with either 220 kV X-rays or 400 MeV/amu accelerated neon-ions; spontaneous mutants were also selected and recovered. RBE for neon-20 was about 5. A total of 70 presumptive mutants were placed into one of four classes on the basis of allozyme profiles following electrophoresis and ADH staining: (A) dysfunction, (B) underproducer, (C) overproducer, and (D) up-*Adh2* gene. Mutants have been recovered and confirmed in the first three classes. These include two male-transmissible deletion-type lesions induced by X-rays, five underproducer transpositions and one overproducer transposition induced by neon-20. Certain of the neon-induced alleles are unstable in their expression. All 70 mutants are chromosomal aberrations; no intragenic lesions were recovered although our experimental design would have preferentially recovered them if they had occurred.

The Discussion considers the mutagenic action of ionizing radiation, and especially the well-documented differences between maize and Drosophila data. In particular, the effect of these chromosome derangements on the 'programmable' component(s) of the *Adh1* cistron is discussed.

1. INTRODUCTION

The nature of the gene in higher organisms is not understood. The phenomena of determination (or presetting), competence and somatic heredity must surely have correlates at the level of the specific gene. We call this correlate a 'programmable' *cis*-acting gene component(s) whose function is to affect transcription of its adjacent structural gene component(s). A 'program' mutant would alter the rate, timing, balance, cell specificity or gene circuitry of transcription without altering the primary structure of the message. *Induced* mutants affecting programmable components of genes or altering cell-specific gene circuits are very rare (possible examples: McClintock (1951) for gene instabilities generated by breaks in chromosomes; Mottinger (1973) for unstable *Bronze-1*; and cf. Baker, 1968 review of position effect variegation in Drosophila). All of the above cases appear to involve

chromosomal breakpoints near a target gene, and none have proven biochemically accessible. Naturally occurring variants have proven more encouraging. Several presumptive 'programming' *variants* of specific genes have been identified (examples include: Schwartz (1962) for maize esterases and Schwartz (1971) for *alcohol dehydrogenase-1*; Swank, Paigen & Ganschow (1973) for murine β-glucuronidase; Dickinson (1975) for Drosophila *aldehyde oxidase*; Freeling (1975) for both maize *alcohol dehydrogenase* genes; Chovnick *et al.* (1976) for Drosophila *rosy*; cf. Brink, Styles & Axtell (1968) for maize *R*; and cf. McClintock (1956) for transposable elements). Unfortunately, naturally occurring variants are especially difficult to interpret because their evolutionary origins are obscure. The overall aim of these initial studies is to artificially induce and recover mutants in 'programmable' components of the *alcohol dehydrogenase-1* (*Adh1*; ADH enzyme, EC 1.1.1.1.) cistron in maize.
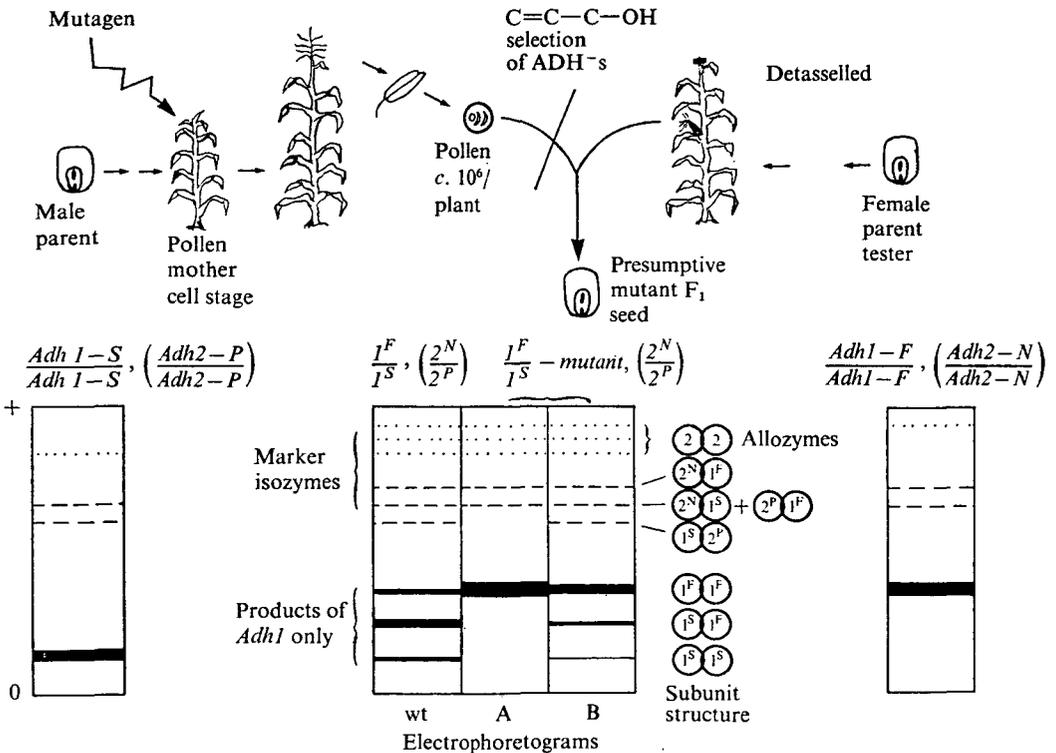
Text-fig. 1. Chemical selection, recovery and classification of *Adh1-mutants*. The paternal or target *Adh1* allele *Adh1-S*, is abbreviated *1ˢ*, etc. The electrophoretograms depict semi-quantitative, banding patterns and intensities after native starch gel electrophoresis. ○ denotes origin; + denotes the anode. The ADH activities reflected in these diagrams are from the wild-type F$_1$ (wt), *Adh1-negative* heterozygote (A) and *Adh1-underproducer* heterozygote (B) extracts obtained from F$_1$ scutellar slivers. The subunit composition(s) of each band is indicated (Freeling & Schwartz, 1973) where subunit 1ˢ is specified by *Adh1-S*, etc. The dashed and dotted bands in the marker isozyme region of gel denote low-activity and usually-not-present, respectively.

The *Adh1* complementation group (i.e. cistron) in maize offers many advantages for mutational analysis. First, ADH catalyses dispensable functions under aerobic conditions (Schwartz, 1969). Secondly, much is known about the maize ADH allozymes and isozymes themselves and the rules underlying their organ-specific expression (Schwartz, 1966, 1971; Freeling, 1975). Thirdly, our laboratory has developed recently an *in situ* ADH staining procedure for maize pollen permitting quantification of intragenic recombination and reversion frequencies

Table 1. *Eight steps for initial mutant characterizations*

I. F_1 scutellar allozyme/isozyme profiles and check for paternal marker. (Class A, B, C, or D.)
II. Viability of F_1 seed.
III. F_1 plant morphology and behaviour.
IV. F_1 chromosomal counts from root tips.
V. Allozyme profile from pollen shed from F_1 plants (F_2 gametophytic population). Especially check for hybrid allozyme indicating hyperploidy for *Adh1* region of 1L.
VI. Pattern of pollen abortion and ADH staining (F_2 gametophytes).
VII. Transmissibility of mutant allele through male and/or female from allozyme analyses of F_2 or backcross progeny scutella.
VIII. Instability or variegation after transmission?

below 10^{-7} (Freeling, 1976). Fourthly, recent results (Schwartz & Osterman, 1976; Cheng & Freeling, 1976) demonstrated that allyl alcohol ($C = C-C-OH$) selectively kills ADH$^+$ gametophytes allowing for preferential fertilization by rare, mutant ADH$^-$ pollen. For these reasons, maize *Adh1* is among the few specific genes in higher organisms which bring together high genetic resolution, a mutation selection scheme and biochemical accessibility. Text-fig. 1 diagrams the first steps in *Adh* mutant induction, selection, recovery and confirmation by allozyme analyses. Table 1 lists further tests to which each single mutant heterozygote (F_1) is subjected by way of preliminary characterization.

Intuitively, accelerated heavy ions (high Z) should generate a unique class of multi-hit, relatively small (0·1–100 kilobases) chromosomal aberrations with single-hit kinetics. Given idealized conditions, Chatterjee *et al.* (1971) and Christensen, Tobias & Taylor (1972) have used LET, energy per amu and the angular dependence of secondary electrons (δ rays) to estimate the length of such single-hit deletions. What actually occurs within the cell depends on repair/degradation processes, the shape of the gene and the like. We employ neon-20 accelerated to 400 MeV/amu at the Bevatron, Lawrence Berkeley Laboratory, as well as X-rays as our ionizing radiations.

Previous attempts to generate intragenic forward mutants in maize and barley using ionizing radiation have failed (Stadler, 1928; Stadler & Roman, 1948; Amano, 1968, and especially results and further citations of Mottinger, 1970). Possible exceptions to this generality are some of the C-series *waxy* mutants (cf. Nelson, 1968), and our *Adh1-FunkFγ25* (unpublished 'point' mutant), both of which were recovered following gamma irradiation of maize seeds. Other organisms do not give this result (Muller, 1955 for review). Certain mutants in *Drosophila*

melanogaster revert with X-rays (Patterson & Muller, 1930; cf. Green, 1961) although the nature of the original mutants (inserts?) remain unknown. The genetic fine structural analysis of XDH structural gene component of *rosy* by Chovnick and co-workers (Gelbart *et al.* 1973; see Chovnick *et al.* 1976) indicated clearly that X-ray induced mutants often behave recombinationally as points, but not necessarily base-substitutions. In microbes, ionizing radiation may induce intragenic lesions including base-substitutions as well as breakage-type lesions (e.g. Bridges, Dennis & Munson, 1967 in *E. coli*; Weber & deSerres, 1965; Malling & de Serres, 1967 in *Neurospora*). As a partial explanation for the discrepancy in data between plants and all other tested organisms, we will advance the notion that two-strand breaks may be ripped-out by exonucleases before reunion. Such chromosomes would not transmit through the gametophytes. We call this suggestion 'chromosome suicide'.

Our system has been designed carefully in order to diminish the preponderance of gross chromosomal aberrations recovered by previous maize geneticists. (1) Pollen mother cells were irradiated prior to the first microspore mitosis (however, tassels contain some germ cells at all stages from premeiotic to mature pollen: a distinct disadvantage of our system). (2) A fertile pollen grain must be ADH⁻ in tube nucleus in order to survive allyl alcohol vapour selection, and must also transmit an *Adh-altered* gene in order to be identified as a mutant in the F_1 allozyme screen. (3) An electrophoretically detectable marker, *Adh2-P* on chromosome 4 was used to rule out contamination and gynogenetic haploids. Given these three considerations, we should have filtered out most macrolesions and chromosomal derangements because they abort at the male gametophyte, or pass through with difficulty. However, a reciprocal translocation resulting from the union of clean breaks, one of which bisects *Adh1*, should pass through our screen via alternate segregation and be recovered.

There are two unlinked *Adh* genes in maize (Schwartz, 1966; Freeling & Schwartz, 1973): *Adh1* (within 2 mu from *Kn* and *Lw* on chromosome 1L; Schwartz, unpublished) and *Adh2* (on chromosome 4S; 20 mu from *Su1*; Dlouhy and Freeling, unpublished collaboration). In total, there are three isozymes of ADH: Set I (ADH1·ADH1 dimer), Set II (ADH1·ADH2 heterodimer) and Set III (ADH2·ADH2 dimer). The scutellum of the quiescent embryo and mature pollen expresses ADH1 subunits in great preponderance. Figure 1 includes subunit compositions of our F_1 allozyme/isozyme profile.

This report gives our first data on mutant recovery via pollen selection, and must necessarily cover our methods and their controls in detail. Initial characterization of our first 69 radiation-induced *Adh* lesions is presented, along with cursory information on three spontaneous point mutants. Relative biological effectiveness (RBE) for neon-ion compared to X-rays is estimated. Among these mutants, and particularly among the neon-induced underproducer class, we have evidence for alterations of *Adh1* programming and stability of expression. None of our 69 radiation-induced mutants are confined to the structural gene component of the *Adh1* cistron.

2. METHODS AND MATERIALS

(i) *Inbred lines,* Adh1 *alleles and nomenclature*

The single cross hybrid, *Funk-G4343*, generously supplied by Funk Seeds International, was used consistently as the detasselled, female tester. This vigorous line carries *Adh1* alleles which specify ADH1 subunits conferring a relatively fast electrophoretic migration rate to dimers containing these subunits. These alleles are called *Adh1-F*. At one point, the allele designation *Adh1-F(Std)* is used; this 'fast' allele is not the same as the *Adh1-F* alleles in *Funk-G4343*. The mutational target alleles were *Adh1-S, Adh2-P* in a line inbred for four generations. The *Adh1-S* allele specifies an electrophoretically slower product than *Adh1-F*, and *Adh2-P* specifies an electrophoretically slower product than the wild-type *Adh2-N* (Freeling & Schwartz, 1973). In one section of results, the naturally occurring *Adh1* alleles *Adh1-C^t* and *Adh1-$\overline{FC^m}$* are used. These alleles have been described previously (Schwartz, 1966).

The nomenclature we use is changed slightly from that used by Schwartz. For example, one of our underproducer mutants is designated *Adh1-S1945*. The '*Adh1*' denotes the gene involved, the '*S*' indicates the wild-type mutant progenitor allele and '*1945*' is the arbitrarily chosen family number of the F_1 presumptive mutant seed. This allele would be abbreviated *1945*. No component of this revised nomenclature reflects the type of mutational lesion or the mutagen. This nomenclature differs from that used by Schwartz in one important respect: the arabic allelic designation is used by Schwartz to denote the electrophoretic behaviour of the mutant's product rather than the product of the progenitor allele of the mutant.

(ii) *Treatment with X-rays and accelerated neon, and dosimetry*

Plants to be irradiated were grown in 10 in. pots. When the immature tassel was at or just before the stage usually chosen for collecting germ cells in pachytene – the 'sporocyting' stage – the potted plant was moved to the radiation source. An immature maize tassel at the stage of irradiation is not composed of a single type of germ cell; premeiotic, meiotic and, in some cases, postmeiotic gametophytic stages were present as mutational targets.

The X-ray source was a Philips 250 kV. We used a 220 kV, 15 mA beam with 0·25 mm Cu and 1·0 mm Al filters. A 20 cm length of stem was irradiated, including the entire immature tassel. The typical dose rate was about 58 rads/min as measured by a Victoreen Condensor 250 kV R meter after the beam had passed through the plant.

400 MeV/amu neon 20 was accelerated at the Bevatron, Lawrence Berkeley Laboratory, on 1 September 1976. Dose rate and beam level is constantly monitored using an on-line parallel-plate ionization chamber. Our target area was a 7 cm diameter circle; certain immature tassels were too large, and the tip of the main tassel stalk was not irradiated. The Bragg peak was placed just past the

target such that plateau LET values prevailed. Mean dose rates were about 50 rads/min.

(iii) *Pollen collection, storage, allyl alcohol treatment and the test-cross*

After mutagenic treatment, potted plants were grown outdoors during the summer months. Shortly before anthesis, plants were transported to a greenhouse isolated three miles away from any *Adh1-deficient* pollen; contamination by mutants already in our collection was eliminated. On days 2 and 3 of shedding, the heaviest days, Lawson 400 pollination bags were closed at 9 a.m. and opened again between 10 and 12 noon for pollen collection; 5×10^5 grains is a reasonable sample since these plants have relatively small tassels. Using Millipore pollen counting techniques (Freeling, 1976), 1 mg of *Adh1-S* pollen was found to reflect about 2×10^3 grains. Collected pollen was separated from anthers using a folded glassine bag and stored at 4 °C for 0·5–4 h. Control experiments (D.S.K.C., unpublished) show that this cold treatment increases germination *in vitro* by about 10%. After cold treatment, pollen was dispensed in about 500 mg lots (10^6 grains) onto glassine paper for allyl alcohol vapour treatment. A 500 ml Mason jar with sealed lid containing 20 ml granular $CaSO_4$ desiccant was used; unless specified, we used between 1 and 6 μl allyl alcohol/500 ml chamber for a total treatment time of 20–30 min; 1 μl allyl alcohol vapour/500 ml equals 75 μM. After treatment, the seal is broken, the jar is cooled in an ice bucket and transported to the field of detasselled plants (*Adh1-F*, *Adh2-N*) whose silks had been cut back the day before. Tester ears were pollinated with about $5·8 \times 10^4$ pollen grains/ear using a camel hair brush; there are about 400 silks, each leading to an ovum. Each ear was then covered until harvest about 2 months later.

(iv) *David's Bread Loaf*

The *in vitro* pollen germination medium and conditions developed by Cook & Walden (1965) have been revised. Our overall procedure is called 'David's Bread Loaf' because the most important revision involves gametophyte germination on a newly cut solid agar surface. The medium is 17% w/v sucrose, 300 mg/l $CaCl_2 \cdot H_2O$, 100 mg/l H_3BO_3 and 0·7% w/v Difco Bactoagar, adjusted to pH 6·4 after the addition of agar but before heating to 120 °C for 8 min. The medium was solidified in a Griffin beaker of the desired diameter and stored at 4 °C until use. Slices, 2 mm thick, were placed onto opaque grids; pollen was dusted onto the newly cut surface using a camel's hair brush. Consistent percentage germinations of 75–95% are obtained, and there is little or no density dependence and minimal differences in the performance of pollen from various inbred and exotic lines (unpublished).

After germination, gametophytes may be cytochemically stained for the presence of ADH enzyme activity. These methods are detailed in Freeling (1977).

(v) *Electrophoretic analyses of ADHs in scutellum and pollen*

The scutellum is a diploid nutritive organ which encloses the embryonic axis: the scutellum plus axis comprise the 'embryo'. The removal of a small ($4 \times 4 \times 1$ mm) sliver of scutellum does not impede germination of the seed but does allow for electrophoretic analyses of ADH enzyme constitution. Slivers were macerated in $100 \mu l$ of 50 mM Trisma-HCl, pH 8·0 containing 10 mM dithiothreitol (Calbiochem). If many seeds were involved in a genotype, their order was maintained by writing numbers directly on the seed and by taping seeds to file cards. The extract was absorbed into Whatman No. 3 filter paper rectangles and subjected to electrophoresis in starch gel according to the methods of Schwartz & Endo (1966); cutting and staining of gels specifically for ADH enzyme activity was according to the modifications of Freeling (1974).

Pollen was extracted and analysed as above, with the following exceptions. A mortar and pestle were used to grind the pollen in extraction buffer, and a small piece of Miracloth (Calbiochem) intervened between the slury and the filter paper rectangles; the latter procedure presumably removes some lipids. All procedures were conducted at 4 °C using precooled equipment.

Our choice of native starch gel electrophoresis over any of various polyacrylamide gel procedures was made after careful comparisons. Even after pre-running with running buffer (Tris-glycine, Tris//cistrate, or borate), native slabs of polyacrylamide separating gel are not as sensitive to ADH activity as starch gel (NBT_{red} band intensity $\times$ time^{-1} $\times$ ADH units loaded^{-1}). The polyacrylamide system leads to the inactivation of various mutant-specified allozymes which remain active under our conditions of starch gel electrophoresis (M. Sachs, this laboratory). In addition, we routinely analyse 288 scutellar slivers in one 8 h manday using a total of 1 l. of gelled starch matrix. Polyacrylamide, while offering superior resolution of protein bands, is inferior to starch in staining sensitivity, gentleness to active ADH enzymes and application to large numbers of samples.

(vi) *Chromosomal counts from root tips*

Plants to be assessed for mitotic chromosome number were grown in Jiffy Pots until several roots pierced the peat bottom. About five root tips (1 cm) were removed, covered with about 2 cm of 0·2 % (w/v) 8-hydroxyquinoline for 3 h at 18 °C or overnight at 6 °C. Roots were then fixed for over 0·5 h in Carnoy's, hydrolysed, and stained by Feulgin procedures (basic fuchsin from Gurr, lot No. 22397). At least three root tips were prepared, and at least five unambiguous cells per root were counted. Our data are presented as a modal chromosome number unless dramatic deviations from the mode occurred. It should be noted that root tip ploidy may sometimes not reflect the ploidy of the apical meristem, especially when chromosomally unstable situations were generated *via* radiation-induced derangements.

(vii) *Plant morphology and vigour*

Whole plant morphology was photographically recorded on 35 mm plus X film after 3 weeks of growth in Jiffy Pots just after transplantation to 10 in. pots. These photographs show early growth rate, leaf shape, symmetry of leaf blades, yellow/ white striping, blotching, tillering and the like. Careful observations were recorded in writing at the time of flowering, especially concerning sterility, haploid syndrome (Chase, 1952) and timing of male and female flower maturity.

(viii) *Transmission of Adh-mutants through pollen and egg*

A few milligrams of pollen were collected as shed from mutant F_1 plants, dispersed onto the surface of isotonic phosphate buffer, frozen, thawed and stained for the presence or absence of ADH enzyme activity as described previously (Freeling, 1976). We made two important modifications: (1) the surface of the ice block was not washed because aborted (shrivelled, hollow) pollen grains tend to float, and (2) the dialysate was not decanted since aborted grains would be preferentially removed. Instead, the dialysate was fortified with an equal volume of $2 \times$ concentrated stain. After 2–3 h of staining at 25 °C, at least four types of gametophytes resulted: plump and blue (wild-type, ADH$^+$), plump and yellow, shrivelled and blue (aborted, ADH$^+$) and shrivelled and yellow. For example, a pollen sample which was 50 % aborted, but of the plump grains, 50 % were ADH$^+$ and 50 % were ADH$^-$, would fit the interpretation that the mutant F_1 was a reciprocal translocation heterozygote where one of the breakpoints inactivated *Adh1-S* function. Clearly, allozyme analysis of pollen adds much to these sorts of preliminary interpretations. In reality, many more than these four classes of pollen often occur, including huge grains, gradations in ADH activity content and small rather than aborted grains.

F_1 mutant plants were self-pollinated when possible, and often backcrossed to one parental line (Funk-G4363 containing an *Adh1-F* and *Adh2-N* alleles) or the other (Standard S, containing *Adh1-S* allele with or without the *Adh2-P* allele). The resulting seeds were genotyped using scutellar slivers and electrophoretic procedures. In addition, F_1 ears which did develop were assessed for seed abortion.

3. RESULTS AND CONCLUSIONS

(i) *Controls on the reliability of allyl alcohol selection and pollen viability*

Pollen was collected as described from an *Adh1-deficient* mutant heterozygote – *Adh1-(Std)F/Adh1-(Funk)Fγ25* where *γ25* is a CRM$^-$ intragenic mutant. This pollen was cold-treated, subjected to allyl alcohol treatment, germinated on fresh slices of David's Bread Loaf solid medium and then stained for the presence of ADH; these methods have been detailed. There are four possible phenotypes: blue grain with tube, blue grain without tube, yellow grain with tube, and yellow grain without tube. In the absence of chemical treatment, pollen viability is about 80 % with half of the gametophytes staining ADH$^+$ (blue) and half ADH$^-$. Plate I shows

this result. Allyl aclohol preferentially kills ADH+ gametophytes, presumably because the relatively innocuous alcohol is oxidized to deadly acrolien in a reaction catalysed by ADH1 activity (Megnet, 1967). Table 2 shows a typical set of comparative kill data for sibling ADH+ and ADH− gametophytes assayed after germination *in vitro*. It is clear that concentrations of allyl alcohol and treatment durations exist which should permit mutant selection with few or no ADH+ escapers.

Table 2. *Allyl alcohol selection of ADH− pollen quantified using germination and cytochemical staining*

Allyl alcohol (mM)	Mean relative percentage pollen germination*	
	ADH+ (blue)	ADH− (yellow)
0	100 (78·5%)	100 (80·7%)
0·07	40	98
0·15	9	99
0·29	Zero	86
0·74	Zero	78
1·47	Zero	56
2·21	Zero	37
2·94	Zero	31
3·68	Zero	8

* The results of four independent experiments are averaged for each data point; mean absolute control was 79%, as indicated parenthetically; allyl alcohol was diluted with methanol such that 50 μl of foreign vapour were present; treated for 40 min. Otherwise, as detailed in Methods and Materials.

According to the data in Table 2, treatment under these conditions with about 0·3 mM allyl alcohol vapour should let just a few ADH+ gametophytes germinate. Does a positive *in vitro* germination assay indicate ability to fertilize an ovum successfully? In order to approximate an answer to this question, progeny tests were done on a pollen sample divided into three Mason jars and treated with 0·3, 0·22, and 0·0 mM allyl alcohol, respectively. Pollen treatment conditions are given under Table 2. After treatment, the pollen was brushed onto silks which had been cut back the previous day. The female testers were homozygous for an electrophoretically unique *Adh1* allele so that test-crossed seeds resulting from fertilization by *Adh1+* or *Adh1−* pollen could be distinguished easily from scutellar allozyme profiles. All seed sets were 99–100%. The 0·3 mM treatment gave zero *Adh1+* alleles transmitted/142 tries, 0·22 mM gave 3 transmissions/65 and the control was 42/80; the latter fraction fits the Mendelian expectation. We conclude that 0·22 mM allyl alcohol vapour is just borderline-selective both in the *in vitro* germination test and in progeny tests. However, the small sample sizes underlying these data can only give an approximate direct correlation between ability to germinate and ability to fertilize an ovum.

(ii) *Spontaneous mutant recovery: a control*

The best test of any mutant recovery system is its ability to deliver heritable mutants. Spontaneous forward mutant frequency in $ADH^+ \to ADH^-$/viable gametophyte for the *Adh1-S* and *Adh1-F* (Standard) isoalleles is below 2×10^{-7} (Freeling, 1977). It was therefore not surprising that we recovered only one spontaneous *Adh1-S* mutant out of an estimated 2.6×10^7 viable pollen grains (Table 3, column 1). This mutant, *1561*, will be described further in subsequent sections. Other naturally occurring *Adh1* isoalleles – *Adh1-C^t* and *Adh-$\overline{FC^m}$* – have yielded spontaneous mutants. Two independent *Adh1-deficient* alleles have been recovered and confirmed out of a total of 1.4×10^6 viable pollen grains; both may be intragenic lesions because transmission through pollen is not affected (mutants *Adh1-C^t1522* and *Adh1-$\overline{FC^m}$1543*; unpublished). Our methods work.

(iii) *Four classes of presumptive* Adh1-S *or* Adh-system *mutants identified by aberrant* F_1 *scutellar allozyme profiles*

With the exceptions of the spontaneous mutants mentioned briefly in the preceding Results and Conclusions section, the *Adh1-S* allele was the mutational target. *Adh1-S* specifies polypeptide subunits which migrate to the anode (+) relatively slowly during native gel electrophoresis. Figure 1 diagrammed the standard test-cross *Adh1-F/Adh1-F* $\times$ *Adh1-S/Adh1-S* ($\male$). Since maize ADHs are dimers, the routine F_1 allozyme profile contains three bands of enzyme activity: $1^S \cdot 1^S$, $1^S \cdot 1^F$ and $1^F \cdot 1^F$ using the subunit abbreviations of Fig. 1. In addition, the male (target) plants carried an electrophoretically distinguishable *Adh2* allele, *Adh2-P*, which marks chromosome 4 (see Fig. 1 and Methods and Materials).

Plants to be used as males were irradiated with X-rays or accelerated neon-20 at the sporocyte stage, as described in Methods and Materials. Pollen shed from these plants were tested for abortion, ability to germinate *in vitro*, treated with just enough allyl alcohol to kill not quite all ADH$^+$ gametophytes (74–296 μM for 20–30 min) and then crossed onto tester ears at a mean of 57 800 pollen grains per ear. Borderline concentrations of allyl alcohol were used in hopes of recovering mutants via pollen with lowered levels of ADH but not total absence. The infrequent F_1 seeds were removed from the ear and subjected to a scutellar biopsy which in no way affected their ability to germinate, and were stored for future use. The scutellar slivers were extracted, and their pattern of ADH allozymes and isozymes were assessed, as described, using starch gel electrophoresis. Our choice of starch over polyacrylamide gel for this particular assay is deliberate and is discussed in Methods and Materials. Plate II shows our nonmutant profile and our classes of aberrant profiles.

Each small slab of starch shown in Plate II analyses three profiles: two nonmutant (marked 'wt') and one presumptive mutant (A, B, C or D). These classes are (A) *Adh1-S* dysfunction, (B) underproducer, which is balanced toward *Adh1-F* product, (C) overproducer, which is balanced toward *Adh1-S* product and (D) *up-Adh2*, where the *Adh2* gene is switched on in scutellum. At least super-
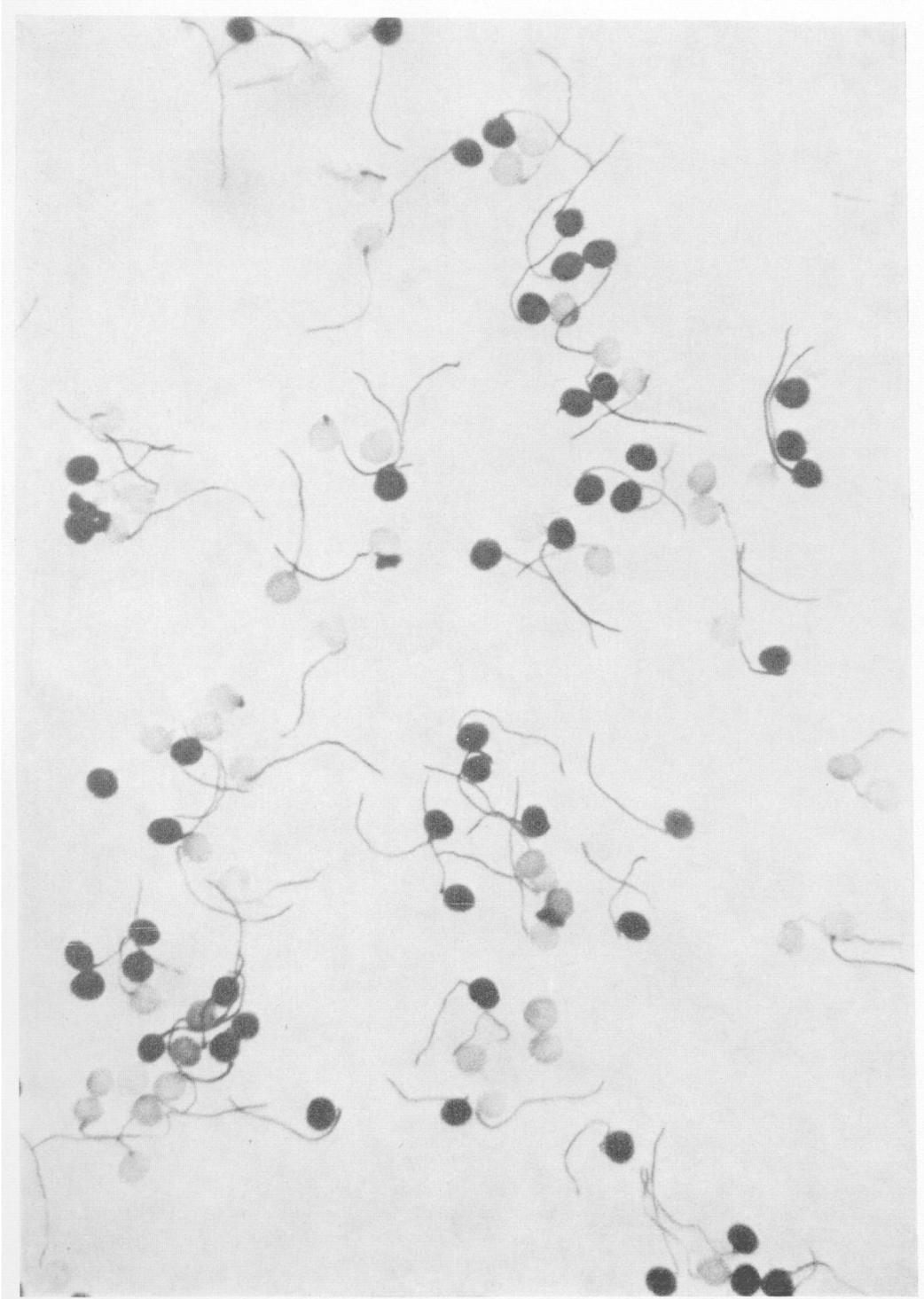
Pollen shed from an *Adh1+/Adh1⁻* plant after *in vitro* germination and *in situ* staining for ADH activity. The dark (ADH+) grains are actually blue and opaque; the light (ADH⁻) grains are actually yellow and translucent. Our kill-curves were obtained by quantifying deviations from controls such as this.
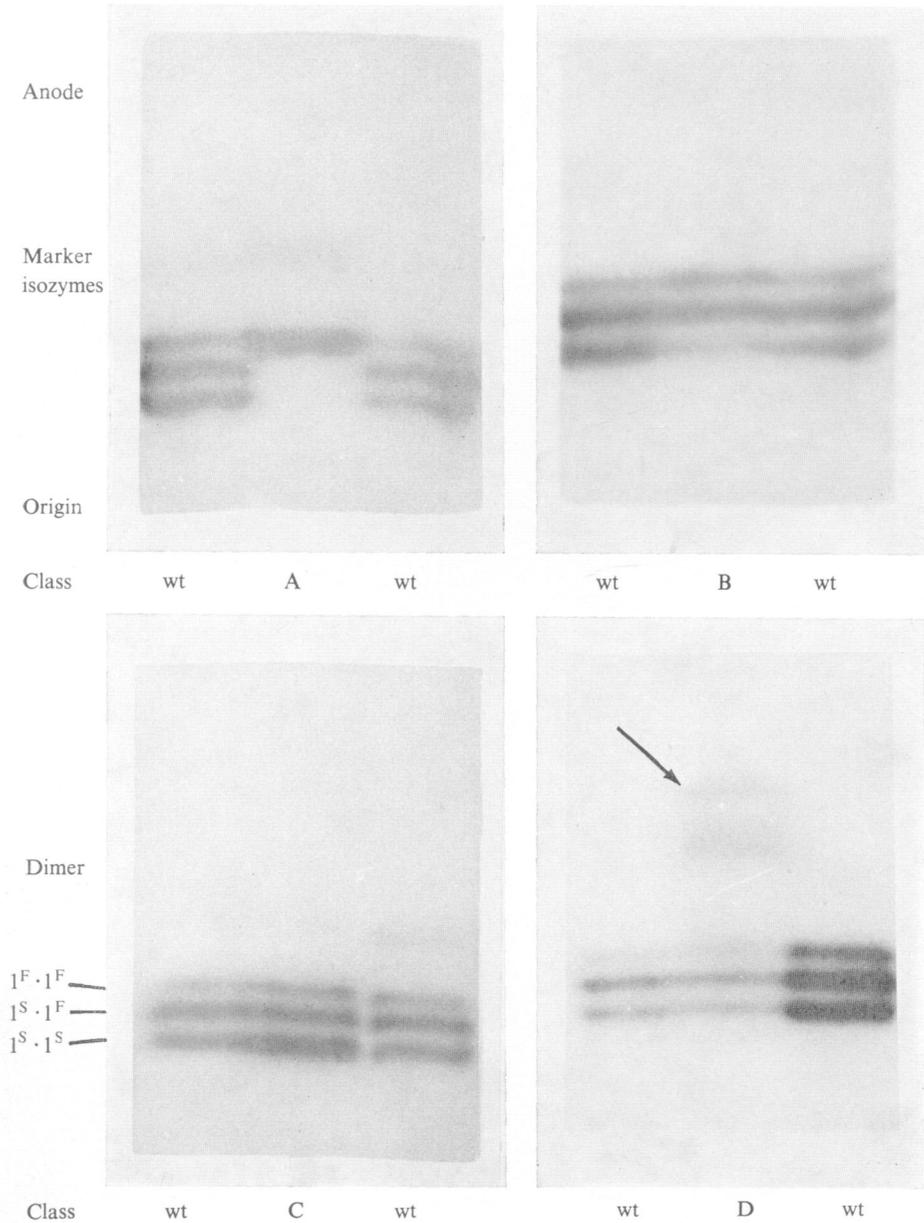
Four classes of *Adh* mutants detected in F_1 seeds by abnormal allozyme ratios. Each electro-phoretogram analyses three extracts, each reflecting a single scutellar sliver. The nonmutant profile is designated 'wt'. Class A is a total *Adh1-S* dysfunction; Class B is an allozyme balance skewed toward *Adh1-F* product; Class C is an allozyme ratio skewed toward *Adh1-S* product; and Class D includes apparent elevation of *Adh2* gene products. The arrow marks the fuzzy ADH2.ADH2 isozyme, which is actually composed of three bands (see Text-fig. 1).

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ficially, Classes B, C, and D fit expectations for regulatory mutants not involving the *Adh1-S* structural gene and Classes C and D are difficult to explain in any other way. The weakly staining bands which move anodally are the result of intergenic hybrid dimers between *Adh1* and *Adh2* gene product; the subunit composition for each band is diagrammed in Fig. 1. Our point in identifying these faint isozymes is that all of our presumptive mutants carried the chromosome 4 marker, *Adh2-P*; therefore, we were not misled by gynogenetic haploids or contaminants, although they did occur infrequently (unpublished).

In the absence of our molecular level assay system, where allelic expression could be easily compared and quantified, the most interesting Classes B, C and D would have gone unnoticed.

(iv) *Recovery and RBE data*

Table 3 condenses our F_1 recovery data for the first 69 *Adh* mutants arising after treatment with ionizing radiation as compared with the single spontaneous mutant. Note that we are measuring recovery frequency and not mutant frequency or

Table 3. *Presumptive* Adh *mutant recovery data with neon-20 RBE to X-ray approximations*

Mutagen:	None	X-ray	Ne[10+]
Mean dose in rads at *c.* 50/rads/min., weighted†	—	327	367
A. Dysfunction	0	13	35
B. Underproducer	1	1	6
C. Overproducer	0	2	2
D. Up-Adh2	0	2	8
Total No. A + B + C + D	1	18	51
% ears with at least one seed*	17·2	28·9	31·1
No. ears with seeds analysed electrophoretically	153	92	33
Total no. of ears analysed	890	318	106
Est. no. pollen grains at $5·8 \times 10^4$/ear ($\times 10^6$)	51·6	18·4	6·1
Est. % pollen viability‡	50	45	60
No. viable pollen ($\times 10^6$)	25·8	8·3	3·7
Mutants/viable pollen ($\times 10^{-8}$)§	3·9	212	1378
Mutants/viable pollen/rad ($\times 10^{-8}$)	—	0·7	3·8

* Allyl alcohol concentrations were about equivalent for all mutagenic treatments. X-ray data includes all ears from all 42 tassels irradiated with 300–500 rads; neon-20 data from two irradiated tassels only.

† Each ear given the rad value of its irradiated pollen parent.

‡ Pollen viability determined empirically. Aborted pollen was estimated to weigh 10 % of a plumb grain. The unmutagenized pollen has such a low viability because it, unlike other pollen samples, was field collected.

§ Note that these are mutant *recovery* frequencies, not mutation frequencies.

mutation rate. The overall relative biological effectiveness (RBE) of 400 MeV/amu plateau Ne[10+] to 220 kV X-rays (in mutants recovered × viable gametophyte[−1] × rad[−1]) is about 5. This RBE is a mere approximation since there is much variation in the exact premeiotic, meiotic or postmeiotic stage when the target germ cell was

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Table 4. *Summary Adh-mutant characterizations in F₁ plants and F₂ gametophytes: initial data*

Treatment (No. F₁ mutants)	Mutant class (No. F₁ mutants)	Fraction surviving	'Mutant' designation	Sporophyte characters	Root tip ploidy	Pollen characters Pollen (allozymes)*	Fraction transmission†	Comments
Control	—	—	*Adh1-S*	Normal	20	2% abort; 98% ADH-blue; size constant = normal	1/2	Non-mutant F₁
X-ray (18)	A (12)	8/10	8 mutants	Short, abnormal; ♂ sterile	N.D.	Usually 50% abort; usually constant size	0, < 1/18 or low/no seed set	Severe chromosomal aberrations with little chance of transmission
		1/1	*1999*	Short, thin, small tassels, anther ear, tillers	N.D.	15% abort; 50% ADH-pink; > 30% ADH-blue	7/64 BC through ♂	Small semi-lethal deletion or equivalent
		1/1	*2008*	Short, normal	N.D.	35% abort, 18% ADH-pink, 50% plump, ADH-blue (mutant allele expressed)	0/36 SP 4/9 BC	As for *1999* above, except the structural gene may be intact; organ-specific differences
	B (1)	1/1	1 mutant	Abnormal, nectotic	N.D.	No pollen	0/5 BC through ♀	Severe chromosomal aberration
	C (2)	1/2	1 mutant	Normal	20	37% abort; size and ADH-colour variable	0/50 BC through ♂; 0/4 SP	Apparently *Adh1-S* lost
	D (3)	3/3	3 mutants	Normal	20	Normal	Normal	Normal; *Adh2-up* mutants not confirmed
	A (35)	20/35	20 mutants	Usually very short; usually sterile	20 when tested	Usually 50% abort	0, < 1/18 or low/no seed set	Severe aberrations; a few may transmit based on pollen characterizations, but none have

Table 4. (*cont.*)

Treatment (No. F_1 mutants)	Mutant class (No. F_1 mutants)	Fraction surviving	'Mutant' designation	Sporophyte characters	Root tip ploidy	Pollen characters*	Fraction transmission†	Comments
Neon ion (51)	B (6)	5/6	1945	Normal	20–30	14% abort; 18% ADH-pink; ~60% ADH-blue; size not variable; allozymes prove hyperploidy	3/20 SP 31/181 BC through ♂	8 of the 31 transmitted Adh1-S alleles were clearly underproduced. *Adh1-S* is transposed to a new centromere; hyperploid gametes result. *Adh1-S* is unstable in its expression
			1946	Normal, late	10–30	Approximately as above (also hyperploid pollen)	11/36 SP 41/123 BC through ♂ and ♀	7 of the 52 transmitted *Adh1-S* alleles were clearly under-produced; 3 over-produced. As above, but more unstable
			1948	Normal, barren tassel	10–18	40% abort, ADH⁺; 40% abort, ADH⁻; 10% large, ADH⁻; 10% large, ADH⁻ (no allozyme data)	13/52 BC through ♀; male sterile	No low-producers confirmed of the 13 *Adh1-S* alleles transmitted. Probably as above
			1951	Normal	N.D.	7% abort; 50% ADH⁺, ~40% var. ADH- colour (also hyperploid pollen)	75/138 BC through ♂ or ♀	Only one of the 75 transmitted Adh1-S alleles were low-balance. Otherwise, as above
			1952	Normal, late	11–30	15% abort; var. ADH- colour and size (also hyperploid pollen)	15/23 BC through ♂ and ♀	Two of the 15 are confirmed low-balances. As above

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Table 4. (cont.)

Treatment (No. F₁ mutants)	Mutant class (No. F₁ mutants)	Fraction surviving	'Mutant' designation	Sporophyte characters	Root tip ploidy	Pollen characters*	Fraction transmission†	Comments
	C (2)	2/2	1950	Normal	20	40% abort; 60% ADH-blue; no var. size	0/92 BC through ♂ or SP	Overproducer probably lost, but Adh1-S was over-expressed in pollen grains. Not confirmed
			1955	Normal, late	20	50% abort; var. ADH⁻ colour and size	18/32 SP	Only one of the 18 Adh1-S alleles transmitted clearly over-produced its product. Confirmed, but unstable
	D (8)	5/8	5 'mutants'	Usually normal	N.D.	Normal	Normal when tested	Adh2-up mutants not confirmed. Epigenetic? Artifact?
Spontaneous (1)	B (1)	1/1	1561	Short, normal	N.D.	Normal; but, all are Adh1-F-specified allozyme	10/22 SP and 1^S expressed	Data seems internally inconsistent; tests are being repeated

N.D. denotes 'no data'.

* The neon ion-induced class B mutants are distinctive in displaying some interallelic hybrid allozyme in pollen extracts, a condition indicating Adh1-S/Adh1-F hyperploid gametophytes; see text.

† This fraction is in the following units: Adh1-S alleles detected in single scutellar extracts/total Adh1 alleles transmitted from the F₁ plant. SP indicates transmission data from self-pollinated ears. BC denotes a backcross where the mutant F₁ was used as either ♂ ♀ for any single cross.

irradiated. The 18 X-ray induced mutants represent 9 different tassels; the 51 mutants generated by heavy-ions represent only 2 irradiated tassels. Given the general lack of clustering of mutants from the same class, the late stage of irradiation, and the unique behaviour of most mutants (Table 4), mutant duplication due to somatic cloning (jackpot effect) is not likely, but might have happened in a few cases. From these data, only Class B mutants appear preferentially with Ne^{10+}, and even this correlation is not statistically significant based on these quantitative data alone. None of our four classes are induced exclusively by either X-rays or neon-ions.

(v) *Three control experiments for the mutant recovery results*

Proper interpretation of the previous data on radiation-induced allyl alcohol selected mutant recovery require three controls: (1) unirradiated and unselected; (2) unirradiated and selected; and (3) irradiated and unselected.

Control 1: $1^S \cdot 1^S$: $1^S \cdot 1^F$: $1^F \cdot 1^F$ dimer (Set I) allozyme ratios from 4000 *Adh1-F/Adh1-S* individuals were electrophoretically analysed as described, as a part of another project in this laboratory. There was no mutagen or allyl alcohol selection involved. Zero mutant electrophoretic profiles were found; the allozyme ratio is remarkably constant. On the other hand, the 69 presumptive induced mutants reported in Table 3 reflect the analysis of only 1722 seeds. The allozyme ratio character appears to be free of spontaneously generated noise.

Control 2: The first column of Table 3 gives the spontaneous mutant recovery data where the allyl alcohol selection system was used. All 69 or the radiation-induced mutants are probably the result of radiation, as would be expected on the basis of previous determinations (Freeling, 1977) of spontaneous mutation rate for *Adh1-S*.

Control 3: The irradiated but unselected control is the most crucial because the irradiated immature tassels might well have contained some proportion of binucleate or trinucleate postmeiotic gametophytes. Since we intentionally chose our allyl alcohol concentrations at borderline concentrations to permit the transmission of ADH-low pollen, the majority (*ca.* 95 %) of our surviving F_1s proved to carry an electrophoretically normal *Adh1-S* allele. If, for example, a sperm nucleus in a mature pollen grain were to lose the entire long arm of chromosome 1, including *Adh1-S*, it might well show up among our presumptive mutants as an unselected volunteer. To test for this possibility, 1094 F_1 seeds were analysed where the pollen mother cells had been irradiated with various doses of X-rays (300–600 rads; 440 rads weighted mean) during the sporocyting stage and crossed to testers without allyl alcohol treatment. We found 8 mutants, all of which belonged to Class A, the *Adh1-S* dysfunction class. Although 6 of these mutants came from one pollen sample, we did not correct these data. When the appropriate normalization per rad is done, the unselected volunteer frequency in mutants $\times$ seed^{-1} $\times$ rad^{-1} for X-rays is $1 \cdot 7 \times 10^{-7}$. The frequency for selected presumptive mutant recovery (mutants $\times$ seed^{-1} $\times$ rad^{-1}) was $9 \cdot 8 \times 10^{-5}$. Note that these recovery units are not those used in Table 3. On the basis of these data, we estimate that about

17% of our presumptive mutants recovered in Table 3 would have occurred without allyl alcohol selection. Since unselected volunteers should rarely transmit through the gametophytes, and since all unselected mutants were Class A (dysfunction), we expected considerable nontransmission (50% abortion) from Class A lesions. Data on unselected neon-20-induced mutants are not available.

(vi) *Mutant characterization and preliminary interpretations*

Each of the 70 mutants recovered in F_1 seeds and classified in Table 3 were subjected to preliminary characterization (see Table 1). Since we irradiated immature germ cells and employed a gametophytic selection scheme, several transmissible lesions in or affecting *Adh1* occurred. These data are condensed and interpreted in Table 4, and are summarized as follows:

(*a*) At least one mutant in each of Class A (dysfunction), Class B (underproducer), and Class C (overproducer) transmitted through self-pollinations (SP) and/or backcrosses (BC) of the original F_1 mutant; Class D (*up-Adh2*) 'mutants' did not transmit and the F_1 plants appeared normal in phenotype.

(*b*) Of the surviving Class A (dysfunction) mutant F_1s, 2 of 9 from X-ray and 0 of 20 from neon proved transmissible deletion-type chromosomal aberrations (Table 4, items 3 and 4). For mutants *1999* and *2008*, we tentatively conclude that at least one breakpoint lies outside the *Adh1* cistron (as delineated by ethyl methanesulfonate-induced point mutants, cf. Freeling, 1976) since (1) pollen abortion is associated with both *1999* (*ca* 15%) and *2008* (*ca.* 35%); (2) pollen abortion is linked to ADH-negativity in pollen from these two mutants stained *in situ* for ADH activity; and (3) both transmissible mutants displayed dominant sporophytic phenotypes characteristic of small chromosomal derangements in maize, but not of whole-arm aneuploidy (as with a 1L hypoploid generated by a Tb-1a translocation). Furthermore, pollen shed from the *Adh1-F/Adh1-S2008* plant contained electrophoretically detectable amounts of ADH1-S subunits. This result suggests that the *Adh1-S* structural gene component remained intact, but was turned off in the scutellum. In the absence of genetic fine structure or pachytene analysis data, these deletion-type lesions could be local inversions, deletions, or insertions. Transpositions involving centromeres other than that of chromosome one are not likely, given the low levels of pollen abortion.

(*c*) Class B mutants (underproducers) were so classified because the F_1 scutellum expressed an abnormally low $1^S \cdot 1^S : 1^S \cdot 1^F : 1^F \cdot 1^F$ allozyme dimer ratio. That is, the binomial distribution of allozyme activity was shifted toward *Adh1-F* product. The single X-ray induced Class B mutant was a severe aberration (Table 4, item 5). However, each of the five surviving neon ion Class B's (Table 4, items 9–13) displayed characteristics in common with one another: variable root-tip ploidy, less than 50% pollen abortion, some fraction of hyperploid pollen containing functional *Adh1-S* and *Adh1-F* alleles with both types of haploid and hyperploid genomes passing through male and female gametophytes. In addition, *1945*, *1946* and perhaps all five of these mutant lesions (Table 4, items 9–13) confer instability onto *Adh1-S* expression in the scutellum. 'Instability' is deduced from an

extremely variable $1^S \cdot 1^S : 1^S \cdot 1^F : 1^F \cdot 1^F$ scutellar allozyme ratio among different *Adh1-S*//*mutant*/*Adh1-F* siblings of the back-cross *Adh1-F*/*Adh1-F* × *Adh1-F*/ *Adh1-S*//*mutant*. Similar cases of breakpoint-associated instability have been reported by Mottinger (1973) for *Bz2* in the aleurone. The last section of Discussion treats this interesting class of neon-20-induced mutants in greater depth.

(*d*) Only one of the Class C mutants (overproducers) clearly transmits: *1955* induced by neon-20 (Table 4, item 15). As with the neon-induced underproducers, the transmission of mutant *Adh1-S* behaviour is infrequent as compared with transmission of apparently normal *Adh1-S* alleles. This seems to be another case of breakpoint-associated instability of gene expression; definitive experiments are in progress.

(*e*) The most mysterious mutant characterized is the single spontaneous Class B (underproducer): *1561* (Table 4, last item). Pollen extracts contained only *Adh1-F* product, but *Adh1-S* was transmitted normally through the male, and was expressed normally in backcross scutella. Further, the pollen from the F_1 plant was apparently plump and ADH$^+$. These data do not appear internally consistent. We have not yet completed a repetition of these data.

(*f*) Except for the deletion-type mutants *1999* and *2008*, all other transmissible mutants could conceivably map away from *Adh1*. Therefore, we use the full designation (e.g. *Adh1-S1999*) for a chromosomal aberration which includes at least a part of the *Adh1* cistron. For other mutants, like *1561*, we leave the mutant number temporarily unattached to a particular gene pending continued investigation.

4. DISCUSSION

(i) *Chemical selection of* Adh *mutants in pollen*

There have been previous reports of allyl alcohol selection of ADH$^-$ gametophytes in maize (Schwartz & Osterman, 1976; Cheng & Freeling, 1976; Freeling, 1977). This report shows that confirmed mutants may be selected via a surviving gametophyte and recovered in F_1 seeds. Our detailed methods of pollen collection, storage, germination on slices from David's Bread Loaf and *in situ* staining of germinated pollen for ADH enzyme activity permitted accurate and reproducible allyl alcohol dose-response data. On the basis of these kill-curves, we were able to choose borderline concentrations and treatment conditions which permitted the escape of a few ADH$^+$ gametophytes. Thus low ADH as well as ADH-negative gametophytes survive our treatment. Since we chose allyl alcohol concentrations which were not 100 % restrictive to ADH$^+$ gametophytes, and since the immature tassel does contain some germ cells past the microspore stage, it was possible that certain of our mutants were unselected volunteers whose occurrence was independent of allyl alcohol treatment. We have performed the appropriate control for our X-ray recovery data (control experiment No. 3, Results and Conclusions): selected recovery frequency was $9 \cdot 8 \times 10^{-5}$ mutants × seed^{-1} × rad^{-1} while unselected volunteers occurred at $1 \cdot 7 \times 10^{-7}$ mutants × seed^{-1} × rad^{-1}. Therefore, approximately 83 % of our 17 X-ray induced mutants were allyl alcohol selected.

A similar control experiment for our neon-ion data has not yet been done. In any case, these early mutant recovery data prove that allyl alcohol can be used to select mutants on the basis of gametophytic survival. Our detailed methods should largely extrapolate to other genes in other plants.

(ii) *Use of allozyme ratios for mutant confirmation and classification*

Most specific gene systems in Drosophila, maize and mice which have been used routinely as mutational targets for ionizing radiations share one unfortunate attribute: they involve the controlled read-out of a hypothetical polypeptide which is unknown or very difficult to assay. *Rosy* in *Drosophila* is an exception. Similarly, maize ADHs are well characterized at the allozyme/isozyme level owing largely to the work of Drew Schwartz and associates. Our choice of using electrophoretically distinguishable *Adh1* alleles for the treated male and tester female was influenced by Schwartz's (e.g. 1969) general scheme for recognizing ethyl-methanesulphonate-induced *Adh1* structural gene mutants. As a result of our nondestructive assaying of each presumptive mutant F_1 for an altered ADH allozyme profile, we recognized classes of mutants in addition to *Adh1-dysfunction* (Plate II). Our classes B, C and D, recognized on the basis of allozyme/isozyme ratio, probably occurred but were undetected by previous investigators working with biochemically inaccessible genes.

We have recovered and transmitted mutational lesions from all classes but D, the *up-Adh2*. Abnormally high ADH2 activity level in the scutellum is often associated with vivipary or defective seeds, although the Class D 'mutant' kernels we included in Tables 3 and 4 appeared normal. We do not as yet have a satisfactory explanation for our failure to confirm Class D mutants; perhaps the endosperm's genotype affects *Adh1-Adh2* balance via the physiological milieu.

We did not find any mutants which specify alterations in the electrophoretic mobility of ADH1-S. As will be discussed, none of our mutants appear to be confined within the *Adh1* gene. Here, the *Adh1* 'gene' is taken to be a complementation group composed of an uninterrupted stretch of dispensable DNA: the *Adh1* structural gene plus adjacent *cis*-acting components. Of course, it is conceivable that lethal or semi-lethal stretches of DNA also act in *cis* to regulate the expression of other less dispensable functions as well as *Adh1*.

(iii) *Recovery frequencies and RBE data: this system's weakness*

A major weakness in irradiating immature germ cells is the extreme heterogeneity of the target cells. Our RBE data on 400 MeV/amu plateau neon-ion vs. 220 kV X-rays for the recovery of *Adh* mutants is certainly accurate, but not complete or rigorously controlled; see Results and Conclusions.

Our experimental design should be modified if further RBE data were desired at the *Adh1* system: (1) reversions could be assayed as rare blue-staining pollen (Freeling, 1976) or (2) forward mutations could be identified by rare allyl alcohol resistant, ADH⁻ gametophytes germinating on an artificial medium (Freeling, 1977).

However, quantifying the *ratio* of mutants among Classes A, B, C and D may be accomplished reliably. Our high-LET radiation generated a particular class (B; underproducers) of mutants originally identified in the F_1 by lowered expression of irradiated *Adh1-S* as compared to the tester allele, *Adh1-F*. All five surviving members of this class showed some but less than 50% pollen abortion, some *Adh1-S/Adh1-F* hyperploid (partially disomic for the *Adh1* region of 1L) pollen, unstable ploidy in root tips, lowered male transmission frequencies, and instability of *Adh1-S* after expression. The presence of such hyperploid pollen suggests that *Adh1-S* has been relocated to a new centromere. Although these five surviving members of this class did come from the same irradiated, immature tassel, each F_1 showed phenotypes which suggest independent mutational origins. Class B transpositions appear specific to the high-LET treatment. The instability of these *Adh1-S* alleles may be particularly important, and is evaluated further in the last section of this Discussion.

(iv) *On the absence of intragenic mutants in maize*

There can be no doubt that most nonplant organisms are capable of confining the damage caused by a chromosomal break to within the complementation group. Thus, ionizing radiations have been shown to generate lesions ranging from multi-break chromosomal abnormalities to base-substitutions. The Introduction cites studies in Drosophila, Neurospora, mice and *E. coli* which substantiate this 'a little bit of everything' conclusion. The fact remains: there are no cases of dominant 'point' mutants or intragenic lesions in forward mutation tests in plants, although scores of small derangement lesions which do transmit through the sensitive male gametophyte were recovered (see Introduction and especially Stadler, 1941; and Mottinger, 1970). The intergenic nature of these mutants was established in a most straightforward way: lowered transmission frequencies through male, lethal phenotype of homozygous plant where spontaneous mutants at the gene delineated a fully dispensable function, and often lowered recombination between markers flanking the region. Our X-ray mutants *1999* and *2008* may now be added to the long list of small deletion-type specific gene events induced in male germ cells by sparsely ionizing radiation. All of our other transmissible mutants are blatantly associated with breakpoints even though our allyl alcohol screen and meiotic irradiation were designed to augment for intragenic mutants which behave recombinationally as points.

Stadler (1944) said of the totality of maize data: The (radiation-)induced mutations observed may be merely gene losses tolerated by the gametophyte. Based on a body of evidence indicating clearly that intragenic lesions do happen in Drosophila, Muller (cf. 1955) dismissed the maize data as reflecting special repair properties of the pollen grain and advanced his argument that, being intragenic, X-rays produced 'progressive' mutants: '... we must admit that at bottom evolution has been built up out of intragenic mutations, together with some super-structure of intergenic structural changes that worked only by utilization of

the gene differences brought about by the intragenic mutations' (Muller, 1955, p. 134). Muller concerned himself with the unitary gene (or structural gene) and progressive evolution. In contrast, Stadler stressed the experimental fact of his system: X-rays induce chromosomal aberrations. It seems important to ask whether or not Drosophila's intragenic lesions are merely microscopic relatives of maize's intergenic aberrations; unfortunately the data are equivocal. All of the radiation-induced reversion studies cited in the Introduction gave ambiguous results because the molecular nature of the original mutant lesions was not under stood. Green (cf. 1977) has demonstrated that insertional-type aberrations (mutants) both should and do revert as a response to chromosome breakage. Chovnick and coworkers (Chovnick *et al.* 1964; Gelbart, McCarron & Chovnick, 1976) tested 41 radiation-induced xanthine dehydrogenase-negative (ry^-) for their ability to complement known complementing alleles: three of 41 tested positive. XDH is a dimer. 6 of 24 EMS-induced ry^- mutants complemented in some *inter se* combination. Unfortunately, complementation might be accomplished even by a polypeptide which is missing amino acids. Indeed, it is now clear that two grossly truncated polypeptides can, in some cases, complement (Morrison, Zipser & Goldschmidt, 1971). Rigorous analyses of the complementing proteins is needed; there is no clear evidence for a base substitution being induced in higher organisms by ionizing radiations. It seems likely that the intragenic mutants Muller used to explain progressive evolution were composed largely of short (point) intragenic aberrations. If such aberrations fell within the structural gene component of the cistron, they would be the least likely candidates for contributing to selectively meaningful change. Ironically, a chromosomal rearrangement (deletion, insertion, inversion, transposition) in a local, *cis*-acting, regulatory component of a cistron might well be progressive (cf. McClintock, 1956; Mottinger, 1973; Wallace, 1975). Perhaps Stadler's emphasis on the negative aspects of chromosome derangement resulting from DNA ionizations was overly pessimistic. After all, only drastic phenotypes could be recognized as mutants.

(v) *Explaining the differences between the maize and Drosophila data*

It must be that the male-transmissible small X-ray-induced deletion-type mutants in maize are caused by a single spur. In addition, it is not realistic to suppose that essentially all single-hit events which result in a two-strand break are repaired with absolute efficiency. Given the above conditions, we envisage two reasonable explanations for the lack of intragenic deletions in plant male germ cells. (1) Two-strand breaks may be excised by exonucleases to a considerable extent (1–100 kilobases) before reunion may occur. The result is 'chromosome suicide' where two-strand breaks are generally filtered out through gametophytes. The degraded region would include lethal or semi-lethal adjacent genes or non-dispensable regulatory components used by the target structural gene. This chromosome suicide alternative may demand clustering of lethal cistrons on the chromosome in order to account for the numerous balanced chromosomal aberrations where the breakpoints themselves are not apparently associated with

gametophytic inviability. Evolutionary explanations for chromosome suicide would be interesting, but certainly academic at this point. (2) A second alternative would seem bizarre except for the distinguished history of transposable elements in maize. A two-strand break may attract an insertional sequence. The inserted DNA may then permit reunions. In addition, the insert need also adversely affect gametophytic viability. In terms of recombination and chromosomal cytology, this insert would be difficult to differentiate from a deletion. In short, this second alternative suggests that intragenic inserts may behave as expected of intergenic chromosomal rearrangements. A third explanation would include both of the above. It should also be clear that neither of these explanations should be called 'repair' unless a repaired chromosome is defined as practically anything with two telomeres on either side of a centromere. While we now feel that at least one of these alternatives must be true to account for the lack of intragenic radiation-induced lesions in maize, we nevertheless admit that ionizing radiations probably cause base-substitutions as well, but at far lower frequencies. Indeed, any source of energy should exacerbate the infidelities underlying spontaneous mutation, a process which, for $Adh1$-S, happens at a frequency below 2×10^{-7} (Freeling, 1977). In addition, it may be that our explanations apply only to plant pollen or pollen mother cells and that data on 'somatic' cells will be canonical.

In the light of the above discussion, we are presently reevaluating our rationale (Introduction, paragraph 3) for using high-Z particles on male germ cells. For example, there is no gain from inducing multiple lesions along 200 nucleotide pairs in a *cis*-acting regulatory component if exonucleases were to cut out several kilobase-pairs on either side of each break.

(vi) *On obtaining mutants in programmable components of the gene*

As stated in the Introduction, the overall aim of these studies was to induce regulatory mutants near, but not in, the *Adh1* structural gene. Definite proof of such mutants must await continued analyses. Mutant *Adh1-S2008* appears to be allelic, entirely outside of the structural gene, and to act differentially in scutellar cells versus pollen. Our Class B (underproducers) and Class C (overproducers) also look promising. These mutants seem to involve the level of gene expression in the scutellum as influenced by breakpoints which are probably close to *Adh1-S*. Further, when these breakpoint-associated *Adh1-S* alleles are transmitted through backcrosses to *Adh1-F* testers, the allozyme ratios in backcross scutella vary from almost zero *Adh1-S* expression to an overabundance of *Adh1-S* expression relative to *Adh1-F*, as is the case for *1946*. Mottinger (1973) reported on two unstable *bronze-1* mutants induced by pre-meiotic X-ray treatment to the male germ cells. These mutants, designated *bz-x3*, *bz-x4* and *bz-x5*, showed instability in the aleurone cells of the endosperm and also in leaf tissue. At *bronze-1*, the instability involves timing rather than level of gene action in development. Mottinger concluded that he had either added a component (like a transposable element) or altered regulatory components affecting *bronze-1*. The fact that our allozyme assay system detected ionizing-radiation-induced balance mutants (Classes B, C and D)

at high frequency (16/69 total mutants is 23%), immediately suggests that the 'programmable component' of *Adh1* is mutable. We trust that mutants in regulatory gene components will prove valuable in understanding development and progressive evolution.

Note added in proof

Data from the progeny of one subsequent round of crosses confirmed these initial results except that *1999* and *2008* also appear to be unstable. The NE[10+]-underproducers are transpositions cytologically; a stable derivative of *1951* is tightly linked but probably outside of the structural gene.

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