INTRAGENIC RECOMBINATION IN MAIZE: POLLEN ANALYSIS METHODS AND THE EFFECT OF PARENTAL *Adh1*+ ISOALLELES.[1]

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ABSTRACT

The ability to stain mature pollen grains for the presence of alcohol dehydrogenase (ADH) activity permits the quantitation of ADH+ gametophytes at frequencies below 10^{-6}. This resolution allows reversion and genetic fine structure analyses. The rationale of pollen analysis follows Nelson's prototype studies with *waxy*. As with the *waxy* gene, revertant frequencies for seven *Adh1*-deficient (*Adh1*$^-$) alleles appear to be in excess of microbially derived expectations. Each of the seven *Adh1*$^-$ alleles were derived from one of three naturally occurring isoalleles. Based on SCHWARTZ's protein level characterizations of the mutants' products, it was anticipated that the seven *Adh1*$^-$ alleles should recombine to yield ADH+ cistrons in certain pairwise combinations. This expectation was not met. The parental "wild-type" isoalleles from which the mutants were derived appear to be structurally divergent. The DISCUSSION interprets these data in view of understanding naturally occurring cistronic variation.

INTRAGENIC recombination is potentially a valuable tool with which to explore the nature of the cistron in higher organisms. While there have been numerous demonstrations of intracistronic recombination in higher plants and animals—especially in *Drosophila melanogaster* (reviewed by FRISTROM and YUND 1973)—only rarely has the structure of the cistron itself been approached: notable are the *rosy* cistron in *Drosophila melanogaster* (CHOVNICK *et al.* 1964; GELBART *et al* .1974) and the *waxy* locus in maize (NELSON 1958; 1968). Studies with *rosy* and *waxy* are reviewed extensively in the DISCUSSION. In this report, the *alcohol dehydrogenase-1* (*Adh1* gene; ADH enzyme EC 1.1.1.1.) cistron is genetically analyzed because it is possible to stain specifically for the presence or absence of ADH activity in mature pollen grains.

Genetic fine structure determinations usually employ mutants induced from a common allele. In these studies, *Adh1*-deficient (*Adh1*$^-$) mutants, chosen as representatives of three different "wild-type" isoalleles, were crossed in most of the pairwise combinations. The mutant lines and much data on their protein products were obtained from DREW SCHWARTZ. Five of these mutants have point

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lesions within the structural gene component of the cistron. The pattern of recombination failure implies that the three wild-type *Adh1* isoalleles are all structurally different from one another. Structural and regulatory gene differences between two naturally occurring *Adh1* alleles (*Adh1-S* and*Adh1-F*) have been extensively studied by SCHWARTZ (1971a) and later by FREELING (1975). The DISCUSSION relates these data to the general question: What constitutes a selectively meaningful polymorphism?

Three terms must be defined: "cistron," "recombinant" and "revertant." "Cistron" is used here to denote a simple, single complementation group. For the purposes of this report, a cistron is composed of one structural gene along with a hypothetical *cis*-acting regulatory component(s). *Adh1* intracistronic "recombinants" (used interchangeably with "convertants") are used loosely to signify those ADH$^+$ gametes generated by a heteroallelic pair of *Adh1*$^-$ mutants after those ADH$^+$ gametes arising from the homoallelic pairs ("revertants") have been subtracted. In any given instance, and ADH$^+$ pollen grain might be the result of conversion, site or second-site reversion, suppression by any mechanism, activation of a repressed *Adh*, an overproducer, or the like. However, the most frequent source of ADH$^+$ gametes specific to heteroallelic pairs is intragenic recombination; alternatives will be discussed. A "revertant" is the general term for an ADH$^+$ gametophyte from a homoallelic *Adh1*$^-$ plant. A "reversion" is the mutational event somewhere in the lineage of a revertant pollen grain. These terms do not imply any particular mechanisms.

Two unlinked genes, *Adh1* and *Adh2*, specify ADH enzymes in maize. When both genes are "on", three electrophoretically separable sets of ADH dimers occur: ADH1·AHD1, ADH1·ADH2 and ADH2·ADH2 (SCHWARTZ 1966; FREELING and SCHWARTZ 1973). The balance of expression between these genes and among their naturally-occurring alleles is differentially regulated in different organs. Pollen expresses only *Adh1*, and most of the ADH1 polypeptides in the mature pollen grain are synthesized after meiotic anaphase II (SCHWARTZ 1971a).

MATERIALS AND METHODS

Adh1-*deficient mutants*

The naturally occurring variant, *Adh1-C*^{m}, has been described (SCHWARTZ 1966); ADH-C^m enzyme subunits are about 5% as active as other wild-type subunits under the standard assay conditions (LAUGHNER 1970). The five mutants derived from the naturally occurring *Adh1-S* allele and the mutant derived from an *Adh1-F* wild allele were induced by ethyl methanesulfonate, recovered and partially characterized at the protein level by SCHWARTZ (1971b, 1975 and personal communication). His biochemical characterizations permit the assignment of certain lesions to the structural gene component of the cistron; see SCHWARTZ (1971b) for these methods. Table 1 summarizes the data for these mutants.

Plant rearing and the avoidance of foreign pollen

Seeds of known *Adh1* constitution were germinated between September and February. The resulting plants were reared in greenhouses under an artificial 14 hr day and flowered in about two and one-half months. Greenhouse temperature was constant at 23° ± 2° with rare afternoon temperatures up to 30°. Two days prior to flowering, plants were hosed down to lyse or remove

TABLE 1

Adh1-*deficient mutants from* SCHWARTZ

Adh1-allele designation*	Progenitor wild-type *Adh1*-allele	Protein-level characterization (See SCHWARTZ 1971b)	Structural gene involved?
05657	*S*	Cross reacting material negative (CRM)	perhaps
0664	*S*	CRM-	perhaps
S296	*S*	Probably inactive, faulty dimerizing, CRM+ in heterodimers	yes
F908	*S*	Low activity, altered electrophoretic mobility	yes
S1108	*S*	Temperature-sensitive product	yes
C^m variant	C^m	Very low activity, CRM+, unique electrophoretic mobility	yes
C70-6	*F*	†Zero activity, CRM+ in heterodimers	yes

* All mutants were induced by ethylmethane sulfonate; C^m is naturally occurring.
† These conclusions anticipate data given in RESULTS and SCHWARTZ's (1975) results cited as unpublished data.

pollen and the tassels were protected from foreign pollen by an open Lawson 402 pollination bag. These plants were then moved to one of the two collection greenhouses. The first was reserved for plants homoallelic for *Adh1*-deficient mutants; the second was for the heteroallelic plants. Neither greenhouse contained plants with a wild-type *Adh1* allele.

Staining ADH in pollen

At about 10 a.m. on a collection day, the tassel was shaken to free older pollen grains and the bag already protecting the tassel was then closed. Two to three hrs later, 5×10^5 to 2×10^6 grains were collected and dispersed onto the surface of 50 ml of 0.1 *M* sodium phosphate buffer, pH 7.3 in a petri dish. Allowing 15 mins for healthy pollen to sink, the samples were frozen at −23° for three hrs on an iron plate used as a heat sink. The surface of the ice, containing anthers and other flotsam, was removed with tap water and the sample was defrosted for 45 mins at room temperature on an iron plate rotating at 30 rpm. Endogenous dehydrogenase substrates are presumed to dialyse out through the perforations in the pollen wall and membranes caused by the slow freezing and thawing. The buffer was withdrawn and replaced with an alcohol dehydrogenase specific stain based on the ethanol-dependent cytochemical reduction of p-nitrobluetetrazolium chloride: 86 m*M* sodium phosphate buffer, pH 7.3, 0.3 m*M* p-nitrobluetetrazolium chloride (NBC Co.; stock in 100% methanol), 1.0 m*M* NAD+ (NBC Co.; ethanol free) and 9.5% v/v ethanol. Stain is added at about 15 ml per 10^6 pollen grains, rotated at 30 rpm for 2 to 4 hrs. The reaction was stopped by replacing stain with 100% methanol or by automatically cooling the plates to 2°. Pollen may be stored in methanol, 2° in the dark for at least four months without jeopardizing further procedures. ADH+ gametophytes stain shiny, opaque blue; ADH- grains are yellow-pink and translucent. The RESULTS present data on the reliability of these methods.

Estimation of ADH+ frequencies

Pollen samples in methanol were suspended in 40% technical glycerin in a 250 ml graduate cylinder to a final concentration of $4\text{–}6 \times 10^3$ pollen grains per ml by visual estimation. A 1.0 ml sample of homogeneous suspension was further diluted into 19.0 ml 40% glycerine and resuspended. 10.0 ml of this diluted sample was evenly layered over a gridded Gelman filter (GA-6, 0.45μm, 47 mm diam.) and quickly deposited by evacuation; circular currents were avoided in the layering process. Particles in four radial strips, each containing 5 squares (each square is one percent of the total area of the filter), were counted under incident light at a total magnification of 16X. In the rare instances where these numbers were significantly different from a 1:1:1:1 (by χ^2), the entire sampling process was repeated. For each original pollen sample, two filters

were prepared, counted and preserved for further reference; this gave eight statistically equivalent numbers on which to base our estimates of total pollen grains per ml of original suspension. Known volumes of the original suspension were then dispensed into gridded plastic plates and every grain was screened for the rare ADH+ phenotype. Occasional light blue or sectored pollen grains were counted separately, and not included with the ADH+s.

ADH+ frequencies for a particular family are reported in two forms. A "family" is composed of sibling plants. (Form 1) all plants in the family are pooled, and the raw ADH+ per total grains screened (raw f) is reported. (Form 2) The ADH+ frequency is determined for each plant and the family mean and standard error are calculated ($\bar{f} \pm SD/\sqrt{N'}$) by excluding data points over 2 SDs from the recalculated mean of N′ plants. In one case, f's of zero were excluded because the sample size was so small that one ADH+ grain would have given an f within 2 S.E's of $\bar{f}$. In only two cases do the raw data (Form 1) and the corrected data (Form 2) lead to different interpretations. The biological justifications for these data exclusions are presented with the RESULTS.

ADH extraction and electrophoresis

Extraction of ADH from the dry, quiescent embryo and electrophoretic analyses in starch gel have been described (see FREELING 1973).

RESULTS AND CONCLUSIONS

Reliability and sensitivity of ADH staining

The cytochemical reduction of p-nitrobluetetrazolium chloride is a well-known assay for NAD(P)+-linked dehydrogenases in the primary substrate (oxidized) to primary substrate (reduced) reaction direction. The specificity of the assay lies in the enzyme's specificity for substrate. To use the pollen staining procedures detailed in MATERIALS AND METHODS with confidence, controls were necessary and are detailed as follows.

Pollen grains from an *Adh1*$^+$/*Adh1*$^-$ plant were stained specifically for ADH as described. Figure 1 is a photograph, taken with incident light on a light blue background before treatment with methanol; methanol removes red pigment from ADH$^-$ grains. The ADH$^+$ (shiny, blue and opaque) to ADH$^-$ (yellow and translucent) ratio was consistent with the Mendelian expectation of 1:1, as it was in all four repeats of this experiment. There was no blue pigment in the absence of ethanol.

Since ADH+ gametophyte frequencies were determined for many plants in a family shedding over a three-month period, it was important to confirm that almost all *Adh1*$^+$ genes will express themselves as a shiny, blue and opaque (ADH$^+$) pollen grain. That is, all pollen should be "stainable". In place of ethanol in the stain, sodium lactate (final concentration 100 m*M*) or malic acid·NaOH (pH 7.3; final concentration 100 m*M*) were substituted. In 47 samples, each from a different wild-type plant, no sample had over 3% unstained pollen (equivalent to ADH$^-$); the mode was below 1%. The red monoformazan pigment visible before methanol treatment in ADH$^-$ negative gametophytes requires the presence of diaphorase enzyme activity. Every stained pollen sample was checked for an excessive (greater than 3%) number of non-pink grains; no sample was discarded by this criterion. Possible differences in the condition of shed pollen do not appreciably affect their "stainability".

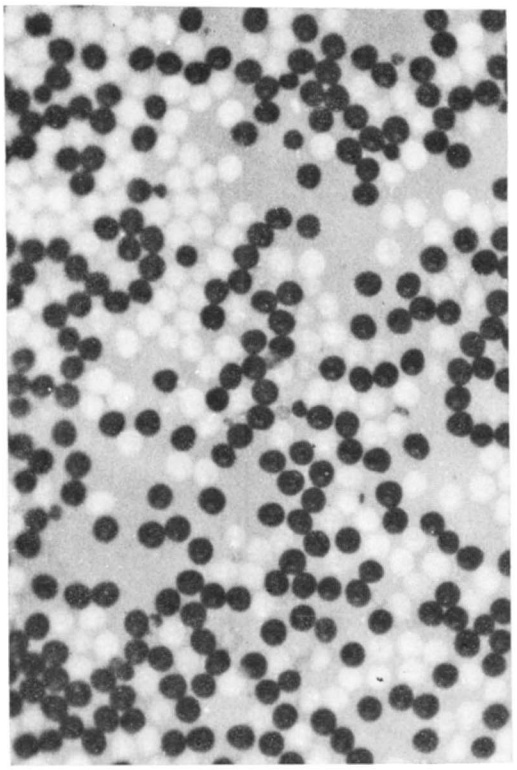

FIGURE 1.—A photomicrograph of pollen segregating *Adh1*+ and *Adh1*- after staining specifically for ADH enzyme activity. The shiny, blue and opaque grains are ADH+.

The naturally occurring variant—*Adh1-C*^{m}—specifies a stable ADH1-C^{m} subunit which has about 5% wild-type (ADH1-S) specific activity (units ADH/subunit under standard assay conditions) (LAUGHNER 1970). After 24 hrs of staining pollen from an *Adh1-C*^{m}/*Adh1–05657* heterozygote, 50% of the pollen was light blue while the other 50% was yellow. The light blue grains, even after 24 hrs of staining, would not have been classified as ADH+. To be scored as an "ADH+" gametophyte requires somewhat greater than 5% of wild-type ADH activity.

The staining methods are specific to ADH, sensitive and reliable.

Homoallelic ADH+ frequencies

In the absence of genetic markers flanking *Adh1* that are scorable in the pollen grain, our criterion for the existence of intragenic recombination is the significant elevation of ADH+ gametophyte frequency in the heteroallelic pair as compared to the ADH+ frequencies characterizing the comparable homoallelic parents. Accurate homoallelic frequencies are essential. ADH+ *frequencies* due to reversion (e.g., site or second site back-mutation, suppression, activation of other genes, etc.) should not be confused with a reversion *rate*. Typically, we screen 10^{6} gametophytes which are the progeny of many mitoses. Table 2 gives

TABLE 2

Frequencies of ADH+ pollen grains for homoallelic Adh1 *families*

Adh1-genotype	Family No.†	N	Total grains screened	No. ADH+ gametophytes	Raw f_{ADH+}	N′	$\bar{f}_{ADH+}$	$\pm 1\ SD/\sqrt{N'}$
05657	74*85	12	38,562,415	58	0.15	11	0.16	± 0.04
0664	74*86	9	31,972,235	194	0.60	9	0.73	± 0.14
S296	74*87	8	33,256,938	115	0.34	7	0.21	± 0.10
F908	74*94	10	35,890,885	264	0.73	10	0.72	± 0.11
S1108	74*88	9	12,822,465	118	0.92	9	1.08	± 0.17
Cm	74*93	8	8,436,490	15	0.17	7	0.12	± 0.03
C70-6	74*92	3	1,557,935	9	0.57	—	—	—

f = frequency ADH+ gametophytes $\times 10^5$.
$\bar{f}$ = mean frequency.
N = number of plants.
N′ = number of plants left after exclusion of plants with f's over 2 SD's from the mean of the corrected distribution.
† all pollen collected in March and April 1975.

the raw ADH+ frequencies for the seven *Adh1*-deficient homoalleles. The last columns of Table 2 give the corrected mean frequencies ± one standard error ($1\ SD/\sqrt{N'}$) when frequencies greater than two SDs from the mean of the new distribution (of N′ plants) are excluded. These exclusions follow the same rules used to correct heteroallelic data and are justified since some of the reversions are expected to happen relatively early in sporocyte development resulting in a clone of ADH+ grains.

The apparently significant differences in revertant frequency among the seven *Adh1*⁻ alleles must be interpreted with great caution. While contamination by ADH+ pollen was avoided, the mutant lines were not isogenic. The median homoallelic ADH+ frequency of 5.7×10^{-6} seems higher than one might expect from simple site or second-site back-mutations. The DISCUSSION further interprets these homoallelic data.

The primary purpose in deriving the homoallelic data was to set a recombinational baseline: *mean heteroallelic ADH+ frequencies which are below 10^{-5} or are less than one standard error above 10^{-5} are taken to be due to reversion alone.*

Heteroallelic ADH+ frequencies among mutants derived from the Adh1-S *allele*

The first 14 lines of Table 3 give the ADH+ frequency data for all ten possible pairwise heteroallelic combinations of the five *Adh1-S* derived mutants (see Table 1), with some duplications. In every case, both raw and corrected mean ADH+ gametophyte frequencies are well above the homoallelic controls. That is, the f (minus one standard error) is well above 10^{-5}. I conclude, following NELSON's (1958, 1968) studies with *waxy*, that these elevated frequencies reflect intracistronic recombination and that all five *Adh1* mutants induced from the same progenitor allele behave as point mutants. The recombinational matrix in Figure 2 consolidates the results of these data. Alternative explanations are entertained in the DISCUSSION.

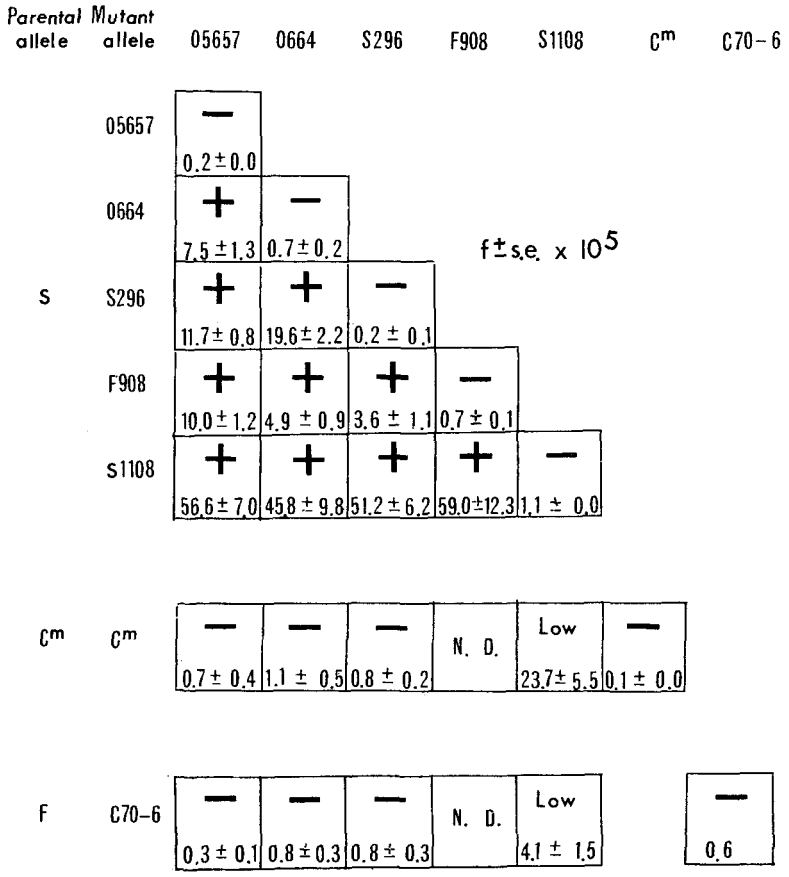

FIGURE 2.—A "recombinational" matrix summarizing the $\bar{f} \pm 1$ standard error data from Tables 2 and 3. All data are from Winter 1974–75. "+" means that the pair of alleles do intragenically recombine to form ADH+ gametophytes; "—" means that they do not; the arbitrary designation "Low" means that there is apparent recombination, but at a rate far below expectations. The $\bar{f}$'s due to reversion were not subtracted from the heteroallelic $\bar{f}$'s. "N.D." means "no data available" and the *Adh1-C^m/Adh1-C70-6* pair was not constructed due to biological problems in the field.

Even if it were theoretically possible to construct a map based on data such as these, when we have analyzed the same heteroallelic pair in different family backgrounds (lines 1, 2; 3, 4, 5; 8, 9 of Table 3), very different ADH+ frequencies resulted; see DISCUSSION.

Of special interest are the high ADH+ frequencies obtained when *Adh1-S1108* was one of the heteroalleles. The *Adh1-S1108* homoallelic controls (Table 2, line 5) gave a raw ADH+ f of 0.92 and a corrected $\bar{f}$ of $1.08 \pm 0.17 \times 10^{-5}$; these frequencies were the highest among the homoalleles. The recombinational frequencies are about an order of magnitude above expectations based on reversion alone. The *S1108* subunit is the least abnormal of the seven *Adh1*⁻ products and is easily complemented by other CRM+ subunits (SCHWARTZ 1975). Interallelic complementation in rare disomic pollen grains is a possible explanation of elevated

TABLE 3

Frequencies of ADH+ pollen grains for heteroallelic Adh1⁻ *families*†

	Adh1⁻ allele	Progenitor alleles	Family No.	Season	N	Total grains screened	No. ADH+	Raw f_{ADH^+}	N′	$\bar{f}_{ADH^+}$	±1 SD/$\sqrt{N'}$
1	05657/0664	S/S	74*40	W74–75	10	5,120,686	345	6.74	10	7.53	± 1.29
2	05657/0664	S/S	74*32	F74	6	8,158,750	224	2.74	6	3.14	± 0.16
3	05657/S296	S/S	74*41	W74–75	10	5,556,619	648	11.66	10	11.68	± 0.82
4	05657/S296	S/S	74*4	W73–74	11	8,243,850	1296	15.72	10	17.30	± 0.25
5	05657/S296	S/S	74*35	F74	17	6,858,070	1429	20.83	15	19.30	± 0.95
6	05657/F908	S/S	74*48	W74–75	8	3,525,065	322	9.14	7	9.95	± 1.17
7	05657/S1108	S/S	74*42	W74–75	9	1,933,550	1002	51.82	9	56.61	± 6.97
8	0664/S296	S/S	74*49	W74–75	8	3,790,560	696	18.36	8	19.63	± 2.17
9	0664/S296	S/S	74*36	F74	7	5,440,625	815	14.97	7	14.70	± 0.38
10	0664/F908	S/S	74*56	W74–75	7	2,568,064	120	4.67	7	4.89	± 0.85
11	0664/1108	S/S	74*50	W74–75	6	2,762,085	1025	37.10	6	45.78	± 9.77
12	S296/F908	S/S	74*63	W74–75	9	6,729,670	177	2.63	7‡	3.64	± 1.09
13	S296/S1108	S/S	74*57	W74–75	8	2,295,530	1088	47.40	8	51.23	± 6.22
14	F908/S1108	S/S	74*69	W74–75	8	1,736,235	945	54.43	8	58.95	± 12.29
15	C^m/05657	C^m/S	74*47	W74–75	10	4,992,908	161	3.22	8	0.66	± 0.36
16	C^m/S664	C^m/S	74*55	W74–75	6	4,491,240	54	1.20	6	1.12	± 0.45

17	C^m/0296	C^m/S	74*62	W74–75	6	4,168,110	34	0.82	6	0.81	± 0.18
18	C^m/F908	C^m/S				No data					
19	C^m/S1108	C^m/S	74*68	W74–75	9	2,898,590	605	20.87	9	23.70	± 5.48
20	C70-6/05657	F/S	74*46	W74–75	6	2,877,780	12	0.41	5	0.26	± 0.13
21	C70-6/0664	F/S	74*54	W74–75	7	5,434,785	72	1.32	6	0.82	± 0.25
22	C70-6/S296	F/S	74*61	W74–75	5	3,512,690	29	0.82	5	0.77	± 0.27
23	C70-6/F908	F/S				No data					
24	C70-6/S1108	F/S	74*67	W74–75	5	3,165,115	111	3.51	5	4.13	± 1.51

† See Table 2 for definitions of symbols.

‡ Two plants with zero ADH+ grains were excluded because samples were so small that one ADH+ would have given an f within 2 S.E.'s of $\bar{f}$.

ADH+ gametophyte frequencies, and may be important in heteroallelic pairs of *S1108* with complementing alleles. Alternatively, the *S1108* lesion may somehow resist participation in coconversions with other mutants. These explanations will be discussed later. In certain crosses involving *S1108*, there were high levels of light blue grains, but these were not shiny, blue, opaque ADH+s; this particular allele is under investigation. It is tentatively concluded that *Adh1-S1108* is particularly recombinogenic.

Heteroallelic ADH+ frequencies involving Adh1-C^m *and an* Adh1-F *progenitor allele*

In addition to the five *Adh1* mutants analyzed in the previous RESULTS section, the *Adh1-C^m* naturally occurring variant and *Adh1-C70–6* mutant (from *Adh1-F*) were also studied. Table 1 describes these alleles; both differ from the *Adh1-S* allele in at least the structural gene component of the cistron; both are CRM+.

The last 10 lines of Table 3 give the ADH+ gametophyte frequency data for the crosses of these two new alleles with the five *Adh1-S*-derived mutants. Of the eight successful crosses, only those involving (the apparently recombinogenic) *Adh1-S1108* yielded frequencies above revertant frequency background, and even these were relatively low. The matrix of Figure 2 summarizes these results. These two new alleles are clearly unlike the five *Adh1-S*-derived mutants.

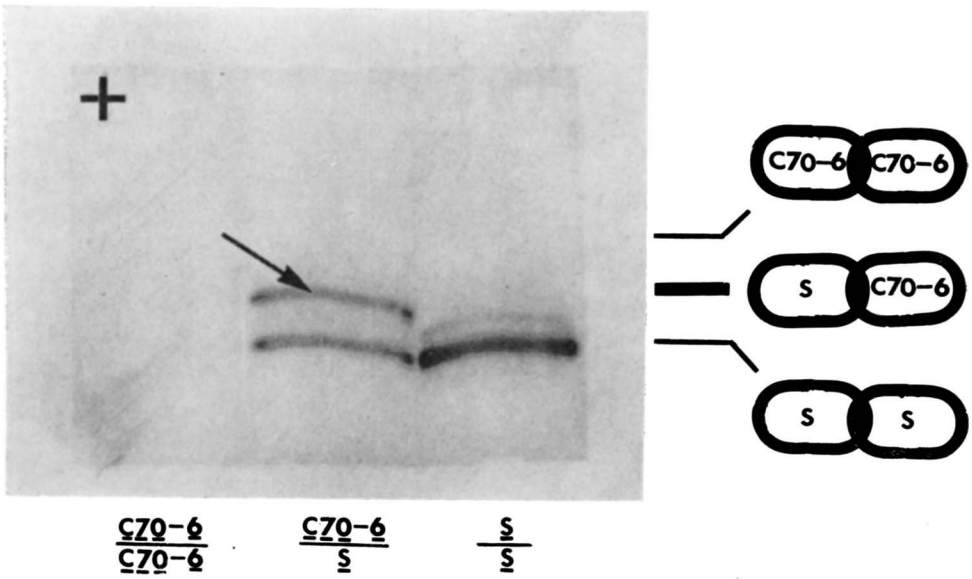

FIGURE 3.—Electrophoretograms showing the ADH1·ADH1 allozyme profiles for the three F_2 segregants of the cross *Adh1-S/Adh1-C70–6* self pollinated. The subunit compositions of each "band" are shown to the right; the arrow denotes the S·C70–6 heterodimer. The position of this heterodimer is coincident with the position of F·F allozyme (the product of the wild-type progenitor of *Adh1-C70–6*). "+" denotes the anode. These data merely confirm SCHWARTZ's (1975) citation of unpublished data.

If the *Adh1-C70–6* allele, derived from the *Adh1-F* allele, were other than a point lesion, these results would not be surprising. SCHWARTZ (1975, citing unpublished data) reported that *Adh1-C70–6* specifies an inactive, electrophoretically altered polypeptide. Since the assignment of *C70–6* to the structural gene component of *Adh1-F* is central to the interpretations, some biochemical data are now presented. The three electrophoretograms in Figure 3 represent the ADH1·ADH1 dimer allozyme profiles for the three F_2 genotypes from the cross *Adh1-S/Adh1-C70–6* self-pollinated. Scutellar ADHs were assayed. The *ADH1-C70–6* subunit, although inactive, is able to form a hybrid dimer with ADH1-S subunits (marked with an arrow in Figure 3), and the mutant subunit confers one additional unit of negative charge leading to an electrophoretic mobility faster than that conferred by the progenitor ADH1-F subunit. Most likely, the mutational lesion is a base-substitution and certainly involves the structural gene component of the *Adh1* cistron.

DISCUSSION

Estimates of recombinational frequency using pollen analysis

The rationale of using the male gametophyte to achieve the resolution necessary to score rare ($10^{-4} - 10^{-7}$) genetic events was developed for the *waxy* locus in maize by NELSON (1958). By staining unshed pollen grains with an I_2-KI solution, NELSON was able to estimate the frequency of intragenic recombination in plants heteroallelic for *wx* mutants; *Wx* recombinant or revertant grains stained blue (starchy); *wx* mutants stained red (waxy). Heteroalleles were said to recombine if the *Wx* frequencies were significantly elevated over the *Wx* frequency predicted by reversion alone, and only recombinants leading to a phenotypically starchy phenotype were quantitated. NELSON (1962) was able to demonstrate that *Wx* frequencies for the heteroalleles *wx-C* and *wx-90* were the same when estimated by pollen analysis, or by more laborious conventional methods involving endosperm tests of back-crossed progeny. Using the latter technique, with endosperm flanking markers, Nelson found that about half (58% of 108) of *Wx* kernels were nonparental for flanking markers. The prototype studies with *waxy* lend credence to these studies with *Adh1*.

As in the *waxy* system, pollen analysis at the *Adh1* cistron uses elevated ADH^+ gametophyte frequencies specific to heteroallelic plants as the sole criterion for intragenic recombination. These studies do not employ flanking markers, hence the correlation of intragenic recombination and conventional chromosomal recombination was not studied. The terms "intragenic recombination" and "gene conversion" are used interchangeably since they probably reflects alternative (reciprocal or non reciprocal) resolutions of a common heteroduplex (FOGEL and MORTIMER 1969). However, since apparent recombinant ADH^+ gametophytes have not yet been recovered and genetically or biochemically characterized in progeny, any *particular* ADH^+ grain could be the result of a conversion, site or second site reversion, a suppression, overproduction of a low-activity ADH, the derepression of another *Adh* gene, etc. The conclusion that heteroalleles often recombine is a statistical inference, but a highly plausible one.

Apart from recombination, there is another mechanism which might routinely give elevated ADH+ frequencies specifically in heteroallelic plants. If the heteroalleles both specied CRM+ polypeptides which showed interallelic complementation via a partially active heterodimer, and if there were appreciable frequencies of nondisjunctions involving chromosome one such that disomic (diploid for the *Adh1* heteroalleles), ADH-complemented pollen grains were produced, then elevated heteroallele-specified ADH+ would result. Since SCHWARTZ has intensively studied each of the mutants at the protein level (Table 1), I was able to rule out this alternative as a general explanation for the heteroallelic data. For example, the CRM- mutants *05657* or *0664* cannot complement, but participate in heteroallelic pairs yielding recombinational-level ADH+ frequencies. On the other hand, the high ADH+ frequencies in certain heterozygotes involving the easily-complemented *S1108* allele might include an increment due to intragenic complementation in hyperploid pollen. BELLING and BLAKESLEE (1924) estimated that 0.33 percent of Datura pollen was n + 1(13) in chromosome number, probably reflecting nondisjunction during meiosis. Since the methods of pollen analysis do not require competitive or viable gametophytes, there is a real possibility that pollen disomic for chromosome one could occur at frequencies above 10^{-5}. Since both C^m and *C70–6* subunits will stablize *S1108* subunits in heterodimers (SCHWARTZ 1975), it is possible that the exceptional frequencies in Table 3, lines 19 and 24 (*S1108/*C^m and *S1108/C70–6*) result from such hypothetical nondisjunctions followed by interallelic complementation in the disomic pollen grain.

Pollen analysis with *Adh1* differs from that with *waxy* in three important respects. (1) the *Adh1* mutants are from known progenitor alleles and are characterized at the protein level. (2) An ADH+ pollen grain clearly contains ADH activity. This direct staining method allows for approximate quantitation of the *Adh1* gene product itself; at least we know that any reversion to less than five percent of wild-type specific activity will go undetected. (3) Pollen grains are scored after shedding. 10^6 pollen grains per plant per day is the routine sample. The ability to resolve frequencies around 10^{-7} has been especially useful in the analysis of revertant frequencies in homoalleles.

ADH+ frequencies in homoallelic families

Since it is relatively easy to screen $3–5 \times 10^7$ pollen grains from a family of 10 plants, accurate revertant frequencies in homoallelic plants were determined (Table 2). Revertant f's ranged from 0.12 to 1.02×10^{-5}, which is consistent with similar data collected for some of the *wx* homoalleles (NELSON 1968: frequencies ranged from 0.1 to 2.7×10^{-5}). Since NELSON used unshed pollen, contamination by foreign *Wx* gametophytes was virtually eliminated. Likewise, the *Adh1* homoallelic samples were taken in an isolated greenhouse using procedures designed to thwart contamination (MATERIALS AND METHODS). As seen from the comparisons of the various ADH+ mean frequencies with their narrow confidence limits, an ADH+ contamination frequency of 10^{-6} is the maximum possible, and even this seems unlikely.

Given the resolving power of pollen analysis for *Adh1*, it seemed reasonable to attempt a comparison among the revertant frequencies for the individual *Adh1*$^-$ mutants. In general, the *Adh1-S*-derived mutants listed in Tables 1 and 2 are ordered from the most "abnormal" polypeptide (CRM$^-$; 05657) to the most normal polypeptide (S1108). Not surprisingly, ADH$^+$ frequencies in Table 2 tend to increase from *05657* to *S1108*. Since the untested influence of genetic background could easily obliterate cistron-specific differences, this correlation must be interpreted with caution. I tentatively conclude that the reversion-frequency differences are generally mutant-specific. If this conclusion proves true, revertants might reflect site or second site back-mutations, suppressions, or overproduction for those mutants specifying partially active product. Excluded by this tentative conclusion is the notion that these high revertant frequencies are accounted for by derepression of other *Adh* genes (such as *Adh2*, FREELING and SCHWARTZ 1973). It is especially important to recover and analyze biochemically the ADH of revertants.

STADLER (1942) estimated the spontaneous mutation frequency (clonally independent mutants/gamete) for *Su*, *C*, *Y*, *Sh*, and *Wx*. Zero *waxy* seeds were found among one and one-half million; the other genes yielded mutants at one or two per million. From studies with microbes, reversion rates should be 10 to 100 times *lower* than comparable forward mutation rates (see DRAKE 1970). Both NELSON (1968, with *wx*) and I obtained median revertant frequencies (revertants/pollen grain) two to six times *higher* than forward mutation frequency expectations. In addition, a pollen grain must have over 5% and perhaps 30% wild-type ADH levels to be scored as a revertant; our revertant frequencies appear surprisingly high. Ideally, one should compare forward with backward mutation *rates* (in specific gene mutations/allele replication). Conversion of mutant frequencies to mutation rates is complex and much depends on the nature of pollen development. Using appropriate calculations and control experiments, it now appears that *Adh1*$^- \rightarrow$ ADH$^+$ revertant frequency exceeds *Adh1*$^+ \rightarrow$ ADH$^-$ mutant frequency as scored in pollen grains (conclusion based on unpublished data; manuscript in preparation).

Recombination among the Adh1-S-*derived mutants*

Figure 2 shows the recombinational matrix for all mutants studied; mean heteroallelic ADH$^+$ frequencies are from Table 3 (W74–75 data) and are not corrected for the negligible contribution from reversion. The five mutants derived from the *Adh1-S* progenitor allele recombine significantly in all ten possible pairwise combinations. Since these mutants are unselected and are the only *Adh1-S*-derived mutants for which any recombinational evidence exists, they should reflect "typical" EMS-induced lesions. From these data and the revertant f data of Table 2, there is no evidence for gross chromosomal deletions, inversions or duplications. These five lesions behave as would be expected of point mutants.

The same two heteroalleles in similar but not isogenic background can give significantly different ADH$^+$ frequencies. Repeats of the same heteroalleles are enclosed by brackets in Table 3. Although the influence of genetic background

has not been estimated, that mean ADH+ frequencies within a family have low standard errors, even though the plants were planted in four cycles at two-week intervals, suggests that fluctuation in greenhouse environment is not the major variable. Perhaps the influence of genetic background accounts for our inability to construct a linear map based on ADH+ frequencies. Perhaps marker effects preclude recombinational frequency mapping (reviewed by STADLER 1973). Recently, Sherman and coworkers (MOORE and SHERMAN 1975; SHERMAN *et al.* 1975) have definitively shown gross noncorrespondence of conversion frequencies and intervening number of base pairs among mutants affecting iso-1-cytochrome c of yeast.

One of the *Adh1-S*-derived mutants—*S1108*—is particularly recombinogenic, yielding ADH+ frequencies about 50×10^{-5} with any other of the four related mutants, as compared with a median ADH+ frequency of about 7×10^{-5} for heteroalleles not including *S1108*. Considerations made in the first section of the discussion exclude nondisjunction followed by intragenic complementation as the primary explanation. Since *S1108* is also the mutant which reverts at the highest frequency (10^{-5}) and specifies the least altered subunit, it is likely that *S1108* is relatively easily corrected by either reversion or conversion. Why high reversion and conversion rates should be correlated is not apparent and may be fortuitous.

The effect of the mutant progenitor allele

A surprising result in these studies is the low frequency of recombination in hybrids involving mutants derived from *Adh1-S* with the other two mutants (*Adh1-C70–6* from *Adh1-F* and variant *Adh1-C^m*). As detailed in Table 3 (lines 15 to 24) and summarized in Figure 2, these "heterologous" crosses gave zero recombination, or—for the cases involving the apparently recombinogenic *Adh1-S1108* mutant—greatly reduced ADH+ frequencies.

If either *Adh1-C70–6* or *Adh1-C^m* were a known deletion, inversion or the like, the results summarized in Figure 2 would be easily explained. On the contrary, biochemical characterizations of the products of *C70–6* and *C^m* (Table 1 and Figure 3) show that both differ from *Adh1-S* in the structural component of the cistron. Both mutants are CRM+ and both are capable of dimerizing with ADH1-S subunits. *A priori* there is no reason to suppose that any of the mutants studied or their parental *Adh1+* alleles differ from one another by gross chromosomal aberrations. Therefore, the effect of progenitor alleles on intracistronic recombination is an unexpected finding.

NELSON (1968) was able to construct a linear map of *wx* mutants only with difficulty. NELSON's *wx* mutants were largely uncharacterized as to progenitor *Wx* allele and nothing was known about the gene product. Based on recombinational frequency data, NELSON found that some pairs of mutants which "should" have recombined, did not, and vice versa. Qualitative (topological) data were less ambiguous. As observed by NELSON (1968): "The [mutant progenitor] *Wx* alleles in these different lines were all capable of supporting amylose synthesis. This does not necessarily imply that all the *Wx* alleles were exactly the same

structurally." The results of these studies on *Adh1* are consistent with NELSON's point.

CHOVNICK and coworkers (review: CHOVNICK *et al.* 1975; GELBART *et al.* 1974) have analyzed five naturally occurring *rosy*$^+$ (*ry*$^+$) variants identified by altered electrophoretic mobilities of xanthine dehydrogenase (XDH), the *rosy* product. Therefore, the five *ry*$^+$ variants differ from one another in at least the structural gene component of the *ry* cistron. (The three *Adh1* variants used in these studies provide the comparable situation in maize.) CHOVNICK and coworkers derived *ry*$^-$ (rosy eye color; no XDH activity) mutants from each of these five variants, and then constructed some of the appropriate heteroalleles for intragenic recombination tests. Employing both flanking markers and a chemical selection system, *ry*$^+$ flies arising by reciprocal or nonreciprocal outcomes of intragenic recombination were recovered. There was no evidence for defective or lowered recombination among mutants induced in different *ry*$^+$ isoalleles (CHOVNICK, personal communication). The failure of variant isoalleles to permit intragenic recombinations to wild-type apparently does not hold for *rosy* in Drosophila, and is probably not a general phenomenon.

We have not yet analyzed a series of mutants from *Adh1-F*, as was done for *Adh1-S*. Therefore, it is possible that *Adh1-F* rarely or never participates in a successful recombination with any other allele without regard to progenitor. In any case, variants *Adh1-S*, *Adh1-F* and *Adh1-C*^{m} all differ when intracistronic recombination is used as an assay. The imagination may pick from a wide field of possible mechanical explanations for these phenomenological data: structural gene divergence, small chromosomal rearrangements, abnormally long heteroduplexes, inability to pair in register (see WALLACE and KASS 1974, for the concept of genic fluidity) and more. Appropriate experiments are underway.

On the maintenance of intracistronic linkage disequilibrium

Knowledge about the mechanism underlying the general failure of *Adh1* variants to recombine (to form ADH$^+$ gametes) is important, but will not alter the deduction that one expects *Adh1-S*, *Adh1-F* and *Adh1-C*^{m} cistrons, or at least the structural gene component, to evolve as units. This permits multiple "mutant" sites on the same intracistronic stretch of DNA to remain in complete linkage disequilibrium within a population. This is a particularly meaningful deduction since a great deal is known about the protein products and differential regulation of *Adh1-S* and *Adh1-F* naturally occurring alleles (SCHWARTZ 1971a). As argued in a previous report (FREELING 1975), evidence indicates that *Adh1-S* and *Adh1-F* differ in at least two parameters: (1) a product surface charge difference implying at least one site difference between the structural gene components of the two cistrons, and (2) organ-specific regulatory behavior suggesting (but not proving) differences in *cis*-acting receptor components of the cistron. The important data of MARSHALL, BROUÉ and PRYOR (1973) on the adaptive significance of the *Adh1-S* and *Adh1-F* polymorphism has been reinterpreted (FREELING 1975) to support the hypothesis that it is the regulatory difference which is selectively meaningful, with the electrophoretically detectable structural gene differ-

ence neutrally maintained due to very close linkage. If *Adh1-S* and *Adh1-F* cistrons do not intragenically recombine (to yield ADH+ gametes), then one expects obligatory linkage disequilibrium for all variant sites within the gene.

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