ADDITIONAL EVIDENCE ON THE EFFECT OF X-RAY AND ULTRAVIOLET RADIATION ON MUTATION IN MAIZE[1]

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IT is well known that MULLER working with Drosophila (1927) and STADLER working with barley (1928) reported that X-rays cause a striking increase in the frequency of heritable changes, both lethal and viable in the former, and affecting both chlorophyll characters and plant types in the latter. These unsorted heritable changes were hopefully looked upon as the building blocks of hereditary variability and of evolutionary trends. The deleterious nature of the changes that X-rays produced, however, led STADLER to suspect them of being different from the changes that occur in nature, and led him to embark on experiments which showed that the induced heritable changes may be either changes within the gene (gene mutations) or losses or relocation of the gene (extragenic changes). PATTERSON and MULLER (1930) presented extensive data showing that X-rays cause gene mutations. One criterion they used for distinguishing a true gene mutation was whether or not it could revert to the original nonmutant form. Their data demonstrated this type of change.

With the advent of superior cytological techniques such as PAINTER'S salivary gland method in Drosophila and MCCLINTOCK'S pachytene technique in maize and their use in the analysis of the various X-ray induced cases, it became quite clear that the majority of the X-ray induced alterations were actually chromosomal alterations or extragenic changes. Taking a skeptical approach, STADLER began a rather extensive study of individual X-ray induced changes at particular loci in maize to determine whether or not they were actually discrete changes of the gene itself. Such criteria as association with a visible chromosomal aberration, transmission through male and female germ cells, viability of the homozygote, and ability to revert to the original nonmutant form were used in this determination. STADLER (1954) could find no evidence that X-rays cause gene mutation. He suggested that in view of the data a re-examination of our definition of the gene might be necessary.

As the number of radiation experiments multiplied, two important aspects of the problem arose. The first was the discovery of a type of position effect in Drosophila in which an X-ray induced chromosomal aberration that removed a locus, normally residing in euchromatin, to a new position next to heterochromatin could cause that locus to express itself as a mutable allele. The new form generally showed a mosaic of dominant and recessive phenotypes. If this locus was moved away from the heterochromatin it reverted to the normal wild type allele. This behavior satisfied

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the criterion of reversability even though the gene had not undergone a true mutational change.

The second aspect of this problem was the accumulation of a vast amount of data from experiments in which investigators had treated various organisms with X-rays and looked for heritable changes but had not examined the mutants in detail. These cases were reported as gene mutations without going any further into the matter. The very weight of this evidence and the rather loose definition of the word mutation has tended to make investigators feel that X-rays have a strong mutagenic effect, using the narrower sense of the word. However, a careful examination of specific cases usually revealed that a deficiency or some other chromosomal aberration was the basis for the so-called gene mutation. Furthermore, many of the changes which were not associated with chromosomal aberrations exhibited pleiotropic effects suggesting that they involved more than one locus.

A major point in the hypothesis that X-rays cause gene mutation is that ionizing radiations cause a series or a gradation of deficiencies ranging from large cytologically visible cases down to those which are invisible and which grade into a type indistinguishable from spontaneous gene changes. The only way to test this hypothesis is to find some means of defining the limits or the discreteness of the changes that X-rays can produce. Such is the purpose of the experiment outlined in this report.

MATERIALS AND METHODS

Previous experiments designed to determine the mutagenic properties of X-rays and ultraviolet radiation on maize had been limited by the unavoidable crudeness of the genetic indicators that were used. In treatments using single loci or widely spaced markers, it was quite difficult to define the limits within which the changes occurred. The discovery of the sh_2 locus by MAINS (1949) and of the compound nature of the A^b allele at the A_1 locus by LAUGHNAN (1949), permitted the combination of three marked loci within very narrow limits. The two components of the A^b locus, α and β, and the sh_2 locus are located on the long arm of chromosome 3 covering a map distance of less than 0.3 crossover units. Cytologically they are included within the haplo-viable (a-$x1$) deficiency which has been reported as invisible at pachytene with standard cytological technique. They all affect the endosperm (α pale aleurone, β purple aleurone, *sh* shrunken endosperm) and in addition α and β are expressed in the plant. Finally, most of the combinations of these three units can be easily recognized.

If pollen from plants possessing the dominant forms of these three loci is treated with X-rays and used on a female stock having their recessive counterparts, one can predict the types of progeny produced as the result of induced chromosome breaks in different regions in and surrounding the α β *Sh* segment. Losses of the whole α β *Sh* segment will be quite frequent, as they will be included in the larger losses of major portions of the chromosome arm. Losses of β *Sh* together or of *Sh* alone will be considerably less frequent since they require, for the first type, a break between α and β and for the second a break between β and *Sh* as well as a second break somewhere else in the distal portion of the chromosome arm. The second break must be assumed to satisfy the observed fact that in maize losses produced in mature pollen are usually

intercalary. Losses of $\alpha\ \beta$ and of α should be even more rare as they require a break between β and *Sh* in the first case and α and β in the second with a coincident break between α and the centromere. Mutational changes of any of the three loci involved may also occur, but those involving α or *Sh* alone will not be recognized as such because they can not be distinguished from small deficiencies involving the same loci. Losses of the action of β alone without the coincident loss of either α or *Sh* can arise in three ways: (1) coincident breaks between α and β and between β and *Sh* with the subsequent loss of the small β segment; (2) point destruction of the β locus without the alteration of α or *Sh* and (3) a mutation of the β locus to the null level. At present the only criterion for distinguishing between these three possibilities is pairing homology. A deficiency would lack the element necessary to pair with its homologue in the *A* locus while a true gene mutation would not. Precedent for such behavior at the *R* locus in maize has already been established by STADLER and EMMERLING (1956).

Changes of β to its recessive form occur spontaneously at the rate of about 3.85×10^{-4} (LAUGHNAN 1955). However, it has been observed that spontaneous mutations occur early enough in the development of the microspore that they affect both the endosperm and the embryo, while induced cases are produced after the second microspore division and therefore affect only one or the other. This is significant from another standpoint in that it suggests that spontaneous mutation of β is associated with the meiotic cycle.

The $\alpha\ \beta$ *Sh* combination has the disadvantage that the changed form of β is a null form which would not be distinguishable from a point destruction of β. The ideal combination to overcome this difficulty would be a gene which has an extremely low mutation rate but which is known to produce mutants of a type that can be distinguished from point deficiencies. These conditions are met by another A_1 allele, *A:D2*. This allele arose as a mutant from the recessive allele *a* through the action of the gene *Dt*. It has been found that *A:D2* mutates to recessive *a* at a high rate in female gametes (3.4×10^{-4}) and in somatic cells when the *Dt* gene is present (STADLER 1951; NUFFER 1955). The responsibility of *Dt* for these mutational changes can be seen by comparison of the data in lines one and two, table 3. The active unit (hereafter designated *A*) of the *A:D2* allele appears to be a single unit whose phenotypic expression resembles β and which resides at the same general location as β. There is at present no proof, however, that *A* and β are homologous. For this experiment such proof is not essential. In any case, *A* is a hereditary unit which can be extremely stable under certain conditions and yet which can mutate to revertible mutants under certain other conditions. A critical test of the mutagenicity of any external agent would be whether or not it can cause *A* in a stable environment (absence of *Dt*) to change to the revertible recessive form. The close marker (*Sh*) helps identify gross deficiencies and chromosome rearrangements. The *A:D2* stock used had the disadvantage of lacking a marker to the left of *A*. This was found to be relatively unimportant since losses of chromosome segments between *Sh* and the centromere with one break point between β and *Sh* occur at a very low frequency.

Two experiments were undertaken using the above described combinations. In the first, treated and untreated pollen of homozygous $\alpha\ \beta$ *Sh et, Dt* plants was used

on silks of plants that were homozygous a^m *sh Et*, *Dt*. The a^m allele (a highly mutable allele that responds to *Dt* action) was used as female instead of a null α β segment so as to permit easy recognition of changes of β. The expression of α without β is a pale seed which is sometimes hard to distinguish from other types of dilution unless there are full colored *A* dots superimposed on the pale aleurone to demonstrate that the paleness is due to α. The *Dt* gene was added so that a^m would mutate to give the necessary dots. There is no evidence to indicate that either α or β respond to the mutagenic action of *Dt*. In the second experiment, treated pollen from homozygous *A:D2-Sh*, *dt* plants was used on silks of plants that were homozygous *a sh*, *dt*. The use of *dt* stocks here was essential since *A* responds readily to *Dt* action.

In each experiment approximately the same number of ears were pollinated by pollen which had been subjected to ultraviolet radiation, to X-rays, or to no treatment. The ultraviolet treatment consisted of shaking the pollen from freshly opened anthers on a $3'' \times 3'' \times \frac{1}{16}''$ quartz slide, placing the slide between two steri-lamp tubes (which were held four inches apart in a specially constructed box) for 30 seconds, dumping the pollen into a small porcelain combustion boat and finally scattering it evenly on receptive silks. The whole process is completed in less than three minutes.

The X-ray treatment consisted of taking tassel branches containing the mature unopened anthers, placing them on a damp blotter in a petri dish, treating with 1200r of unfiltered X-rays, shaking the pollen into a porcelain boat as soon as the anthers were opened, and then pollinating as before. In this treatment the anthers often had to stand 20 minutes or more after treatment before they opened enough to release the pollen. The pollen from the control was handled exactly like the X-ray treated pollen except that it received no treatment.

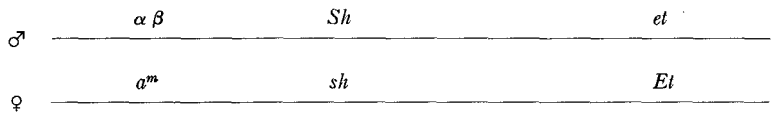
The ears produced in both experiments were examined carefully for seeds of two types, those with the whole seed affected, and those with fractional seed sectors. Mature maize pollen has three nuclei; a vegetative nucleus and two sperm nuclei, one of which may eventually fertilize the female proembryo and the other the female proendosperm. An event occurring as the result of treatment at maturity can affect any one independent of the other two. Therefore, the whole-seed cases represent changes in the male proendosperm nucleus alone and should be accompanied except in rare instances by a normal embryo. Although only the embryo can be used for progeny tests the simultaneous study of endosperm and embryo can be used advantageously to identify contaminants and spontaneous mutations, both of which affect both endosperm and embryo. This is especially important in the first experiment, because spontaneous germinal changes of β in the α β *Sh* stock occur at quite a high frequency.

The second group (partially affected seeds) represent changes that occurred following fertilization even though they must have been initiated earlier. The size of the changed sector depends on the number of cells present in the endosperm at the time the final event occurred, and on the developmental pattern of the progeny of the two daughter cells, one changed and the other apparently normal, arising from the affected but as yet unchanged parental cell. Thus if an event occurred at the two-cell stage and growth rate were uniform and equal for the progeny of the two daughter

cells, one would expect to find a half seed sector; if at the four-cell stage, a quarter seed sector, etc. If, however, there is unequal development, one would expect various sizes of sectors to arise from the event that became effective at the two-cell stage. The very fact that three fourth seed and larger changed sectors occurred in preliminary experiments indicates that the development of the maize endosperm follows an irregular pattern. The sectors recorded were those that covered one eighth of the seed surface or more. Smaller sectors were not easily distinguished from other unclassified effects and therefore could not be used for accurate counts.

EXPERIMENTAL RESULTS

Assuming that $\alpha\ \beta\ Sh$ accurately represents the condition of the tested segment of the third chromosome in the male stock used for treatment as indicated in the following diagram:



and that the female stock was $-\ a^m\ sh$ (or $a^m - sh$ depending on the homology of a^m with α or β) in the same region, one could reasonably expect to find the following unusual types:

(1) — — —	colorless shrunken	deficiency for $\alpha\ \beta\ Sh$, proximal break between centromere and α
(2) α — —	dilute shrunken	deficiency for $\beta\ Sh$, proximal break between α and β
(3) $\alpha\ \beta$ —	colored shrunken	deficiency for Sh or a mutation of Sh to sh
(4) — — Sh	colorless normal	deficiency for $\alpha\ \beta$; two breaks essential, one between centromere and α, and other between β and Sh
(5) — $\beta\ Sh$	colored normal	indistinguishable from $\alpha\ \beta\ Sh$
(6) α — Sh	dilute normal	(a) deficiency for β, either point destruction or two breaks, one on either side of β but between α and Sh (b) mutation of β to null level

Whole seed cases

Actually four of the six expected types were found. Of the two that were not seen, — $\beta\ Sh$ (colored nonshrunken) is phenotypically indistinguishable from the normal sibs, while the other — — Sh (colorless nonshrunken) would have been seen had it occurred, but no cases were found. Of the remaining four, several α — — (dilute shrunken) cases occurred, but they were so difficult to recognize that to avoid possible inaccuracies they were not included in the table of data. The other three types, — — — (colorless shrunken) $\alpha\ \beta$ — (colored shrunken) and α — Sh (dilute, nonshrunken) occurred at various frequencies depending on the type of treatment as indicated in tables 1 and 2.

A comparison of the whole endosperm cases from the two treatments and the control appears in table 1. Gross deficiencies represented as losses of the $\alpha\ \beta\ Sh$ segment occur in the control at a low rate (7×10^{-4}) which roughly corresponds to that

TABLE 1

Whole endosperm losses per 10,000 seeds

a^m *sh, Dt* × A^b *Sh, Dt*					
Treatment	Population	$\alpha\ \beta$ *Sh*	*Sh**	$\alpha\ \beta$	β
Control	9,228	7	0	0	0
UV 30 sec	8,888	267	10	0	11
XR 1200r	8,739	611	7	0	0

* Not entirely reliable as losses of Su_1 occasionally may be mistaken for losses of Sh_2. The female stock included some *Su su* plants.

TABLE 2

Fractional losses, one eighth seed or more, per 10,000 seeds

a^m *sh, Dt* × A^b *Sh, Dt*					
Treatment	Population	$\alpha\ \beta$ *Sh*	*Sh**	$\alpha\ \beta$	β
Control	9,228	17	1	0	0
UV 30 sec	8,888	397	91	0	12
XR 1200r	8,739	90	8	0	0

* Not entirely reliable as losses of Su_1 occasionally may be mistaken for losses of Sh_2. The female stock included some *Su su* plants.

found earlier (STADLER 1939) for loss of the *A* locus. In the ultraviolet treatment the rate was 39 times as high while in the X-ray treatment it rose to 87 times the control frequency. The results are in agreement with findings by other investigators and clearly demonstrate that both treatments are extremely effective in causing chromosome breakage.

If we now look for those deficiencies which include only two of the markers (they require one break in a very narrowly defined region between α and β for the β *Sh* losses and between β and *Sh* for the losses of $\alpha\ \beta$, as well as a second break elsewhere), we are confronted by a complete absence of the one class (losses of $\alpha\ \beta$). This could be attributed to a low frequency of coincident breaks, one between β and *Sh* and the other between the centromere and α, or to the resistance to breakage or perhaps frequent restitution after breakage of this region. The second class (losses of β *Sh*) occurred at a low frequency in both the X-ray and the ultraviolet treatment, but they were so difficult to recognize that their frequency could not be considered as valid data.

Alterations of the *Sh* locus occurred at an increased frequency in both treatments as compared to the control with no significant difference between the two treatments. These cases are of relatively little use here as they represent two types of occurrence: (1) a mutational or deficiency change of the *Sh* locus itself and (2) those gross losses which include the *Sh* locus and any portion of the chromosome distal to it. It is not possible to distinguish between these two in this experiment.

The really diagnostic cases in this experiment are the losses or changes of β alone since they represent the residue of cases that could include possible gene mutations.

Significantly the X-ray treatment as well as the control failed to produce a single valid change of β while a comparable population from the ultraviolet treatment yielded ten (shown as 11×10^{-4} in the table) good clear cases.

Fractional seed cases

Comparison of partial seed losses from the three treatments in table 2 reveals several differences as well as agreements with the whole endosperm data. First of all, the total number of gross deficiencies (α β *Sh* losses) produced by both X-rays and ultraviolet light as compared to the control was increased, though not as much as in the whole seed group. However, the relative frequency from the X-ray and ultraviolet treatment was reversed. The X-ray treatment which had given 87 times as many whole seed cases as the control gave only five times as many partial seed cases while the ultraviolet treatment which gave 39 times as many whole seed cases gave 23 times as many partial seed cases. Taking into account the differences in dosage these data are in rather close agreement with those reported for loss of *A* alone by STADLER (1939).

The fractional losses of *Sh* also differed from those where the whole seed was involved in that the ultraviolet produced many more partially affected seeds. A second agreement occurs in the changes of β. As was the case in the whole seed class, the control and X-ray treatment of the partial seed class failed to include any cases at all, while the ultraviolet treatment yielded 11 (12×10^{-4}).

Reversibility of mutants

In the second experiment, which was designed primarily to discover the reversible type of mutant and not to identify the region affected, the same general results were obtained. As can be seen from the data in tables 3 and 4, gross deficiencies of *A Sh* and deficiencies of *Sh* alone were obtained in roughly the same proportions for both the whole seed and fractional cases. Changes of *A*, which in the light of the failure of X-rays or ultraviolet to produce large interstitial deficiencies of material immediately to the left of *Sh* in the first experiment, can be considered mutations or point destructions of the *A* allele, occur only in the *Dt* and the ultraviolet treatments.

The changes at *A* that arise through the action of *Dt* have long been considered examples of true gene mutation because they occur in somatic cells, they are reversible, they have normal transmission through both male and female germ cells, and

TABLE 3

Whole endosperm losses per 10,000 seeds

a sh × *A-D2 Sh*				
Treatment	Population	*A Sh*	*A*	*Sh*
Control (*dt* × *dt*)	35,733	0.9	0	0.3
Dt (*Dt* × *dt*)	17,506	9*	3	2
UV 30 sec (*dt* × *dt*)	7,457	232	4	11
XR 1200r (*dt* × *dt*)	16,873	321	0	2

* Confirmation tests were not made on these cases.

TABLE 4

Fractional losses, one eighth seed or more, per 10,000 seeds

a sh × *A-D2 Sh*				
Treatment	Population	*A Sh*	*A*	*Sh*
Control (*dt* × *dt*)	35,733	52	0.3	0.6
Dt (*Dt* × *dt*)	17,506	63	8	2
UV 30 sec (*dt* × *dt*)	7,457	679	11	43
XR 1200r (*dt* × *dt*)	16,873	108	0	0

they are not associated with chromosome breakage. All of these qualifications except for the reversibility can be satisfied by a point deficiency of the *A* locus. The question now arises whether the ultraviolet induced mutations which give every indication of filling all the qualifications but the reversibility, will indeed prove to be reversible under proper tests. Since it is impossible to get a progeny test from the endosperm, it is necessary to produce changes of *A* in the embryo which can be grown and progeny tested. STADLER (unpublished) tested three good ultraviolet induced mutations for their reversibility and obtained essentially negative results. However, the *A* allele which he used for treatment had no history of producing reversible recessive *a* mutants under any conditions. Since that time a fourth mutant produced from the same *A* allele as used in the present experiment was produced and tested in collaboration with STADLER and EMMERLING and found to be nonreversible.

The seeds used to obtain the already listed endosperm data were planted in sand benches and grown to discover such embryos. From the small population examined not a single valid case was obtained. Therefore the settling of this point must await the accumulation of more data.

DISCUSSION

The prevailing opinion of the majority of radiation biologists, as amply discussed by MULLER (1954, 1955), is that X-rays cause various heritable changes ranging from gross chromosomal rearrangements, such as translocations, inversions and segmental losses, down through small segmental losses to point deficiencies and finally changes within the gene which can be termed true gene mutations. The gross changes are produced as the result of the passage of an electron track through the chromosome causing breaks followed by rearrangement, rejoining, or complete loss; or are produced as the result of breaks caused by the ion clusters produced at the end of the electron track. The more discrete changes—that is, the point mutations and the true gene mutations if they occur—are produced by single ionizations, molecular activations, or other secondary effects. The data obtained in this investigation have a distinct bearing on this interpretation. They show that for a particular locus in maize, the effects of X-rays are quite gross and include none of the more discrete changes that have been reported in Drosophila and other organisms.

Why is it that no point mutations or true mutations of the gene β occurred as the result of X-ray treatment in this material? One possibility is MULLER'S suggestion that the maize chromosomes in the pollen grain are so condensed as to make it im-

possible for a single quantum of X-rays to affect one gene without affecting its neighbors. It seems unlikely, however, that even in the most condensed chromosomes the genes are this close together, particularly considering the limited sphere of effectiveness expected of a single ionization or molecular activation. Furthermore, treatment of maize at other stages of development has failed to produce point mutations of the kind described by MULLER.

An experiment was conducted at this laboratory by HAHN (unpublished) in which stocks that were homozygous R^r were treated pre-meiotically with 1000 and 1500r of X-rays and the male and female gametes tested for mutation of R^r to r^r. In the female series the rate from treatment was 7.8×10^{-4} in 57,500 gametes compared to a spontaneous rate of 10×10^{-4}. In the male series the treatment frequency was 11×10^{-4} in 150,000 gametes as compared to the spontaneous rate that was known to be 14×10^{-4}.

A second possibility is that different gene loci differ in their response to the effects of X-rays. Perhaps the structure of β is unique among genes and of such a nature that no amount of ionization will alter it without completely destroying it and its neighbors or irreversibly breaking the chromosome in that region. This is not supported by the data from the ultraviolet treatment which show that β is not necessarily stubborn in its response to other types of radiation. One can of course postulate a gene which is selectively susceptible to certain types of outside action and therefore satisfy this possibility.

A third possibility is that X-rays cannot produce discrete changes in the presumed molecular structure of the gene but instead cause only such things as destruction and deficiency by loss of small chromosomal segments. If one holds to the latter hypothesis, one must conclude either that this fact was not recognized in experiments with other organisms because the criteria were not available to point out the difference between true gene mutations and deficiencies or destructions, or that maize is a unique organism in this respect. However, in the face of the vast preponderance of evidence in favor of X-rays causing gene mutations in other organisms, one would hesitate to say on the basis of the data from a single locus in a plant like maize that X-rays do not cause gene mutations.

In comparison the results of ultraviolet radiation are quite different. The data indicate that ultraviolet radiation does cause discrete changes of the β locus. However, the cases obtained still cannot be distinguished as to whether they are deficiencies of β or changes of β to the null level. Until better criteria are developed, this question must remain unanswered. It is clear, however, that ultraviolet can cause changes which are within the limits of our present definition of the gene.

One might argue at this point that the data presented in this paper are not extensive enough to demonstrate that X-rays do not cause gene mutations at the locus tested. It is true that if greater numbers had been involved, some cases of true mutation would perhaps have been found. It should be pointed out, however, that for all practical purposes X-rays do not cause the desired effect at the A locus in maize. It would be of little value to find, for instance, that X-rays merely double a control rate of 30 mutations in one million, while ultraviolet increases the rate 37-fold or more and certain mutator genes increase the rate at least 1,000 times. One should not

conclude, however, that because X-rays cannot cause gene change they are of no value to the geneticist and the plant breeder in altering the hereditary material. In fact, to the breeder it makes little difference whether heritable change is mutation or deficiency. It is possible to visualize rather drastic destructional changes that would be beneficial under certain conditions.

SUMMARY

1. Maize gametes carrying the α β *Sh* segment were treated with X-rays and ultraviolet radiation to determine whether either treatment could cause changes of the middle unit without affecting its neighbors.

2. No changes of β alone were found in the X-ray progeny or the control, while in the ultraviolet progeny a significant number were found.

3. A high frequency of loss of the whole α β *Sh* segment was observed from both treatments but not in the control.

4. A test of the possibility of X-ray or ultraviolet treatments producing reversible mutants from a gene which is known to produce reversible mutations spontaneously was also conducted. The results were inconclusive.

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