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SUMMARY

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Significance: To help elucidate mechanisms of mutation in a eucaryotic system, the biochemical and morphogenetic consequences of mutation in the system, and the nature and effects of induced suppressor mutations.

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IN NUCLEAR SCIENCE ABSTRACTS

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PROGRESS REPORT

The investigations conducted under this contract during the present year have continued to be concerned primarily with the genetic analysis of radiation and chemically-induced reversions of a yellow-virescent (yv) chlorophyll mutant in barley, and with comparisons of the mutagenic effects of ethylmethanesulfonate and triethylene melamine on normal Himalaya barley. The closely related investigations concerned with the cytological, morphogenetic, and biochemical definition of the phenotypes of the yv mutant and revertants in relation to the progenitor wildtype barley have of necessity been somewhat curtailed because of resignations of personnel associated with the research.

The yv mutant originated in the X_2 progeny from an x-radiated plant of normal Himalaya barley and behaves as a simple recessive in crosses with the parental variety and other genetically normal barley lines. Location of the mutant locus on chromosome 7 was accomplished by crosses with a number of translocation stocks in which the lengths of the chromosome segments interchanged are sufficiently unequal to permit recognition of the translocated chromosomes at the metaphase stage in root-tip squashes. The yv mutant was found to be closely linked, i.e., less than 3% recombination, with the breakpoints in translocations 1-7c, 1-7f, 3-7d, 6-7a, and 6-7d. A small percentage of the progeny from a barley plant heterozygous for a translocation are trisomic. These trisomics owe their origin to 3:1 disjunction from the ring of four chromosomes present at anaphase I in meiosis. Chromosomes 6 and 7 are satellited and the breakpoints in T 6-7d are in the short (satellited) arm of chromosome 6 and near the centromere in chromosome 7. In the F_2 generation from the cross of T 6-7d with the yv mutant three of the yv progeny were trisomic. All three of these plants were tertiary trisomics in which the extra chromosome was the translocated 7; i.e., the diploid complement had the normal chromosomes and the extra chromosome had a satellite on each arm. Since the entire short arm of chromosome 7 is included in this translocated chromosome, these plants should have had normal phenotypes if the yv locus was located in this arm. Therefore, the yv locus appears to be in the long arm of chromosome 7, and recombination between the locus and the breakpoints of a selected group of translocations places it close to the centromere.

An initial group of revertants of the yv mutant phenotype was obtained in 1959 following treatment of seeds with thermal neutrons at a flux of approximately $7.0 \times 10^8/\text{cm}^2/\text{sec}$ for one, two, three, and four hours, or with 3500r or 7000r of x-rays followed by hydration in the presence of either oxygen or nitrogen. These treatments resulted in 104 independent detected revertants from the 8,573 surviving X_1 plants. Part of this work was repeated in 1960, using 5000r of x-rays followed by hydration in the presence of oxygen, since most of the revertants detected in the previous year were lost as the result of virus infection. During the two years a total of 176 independent cases of reversion toward the normal green phenotype were obtained from the 12,792 surviving X_1 plants that were tested.

A second group of revertants has subsequently been obtained from seed treatment with two alkylating chemicals, monofunctional ethyl methanesulfonate (EMS) and polyfunctional triethylene melamine (TEM). The rationale for the selection of the two chemicals came from the demonstration by Heslot et al. (1959) that EMS greatly enhanced the mutation

rates of genes concerned with chlorophyll synthesis in barley but was not an effective chromosome-breaking agent, and from the finding that TEM was both non-toxic and highly efficient in the induction of point mutations and chromosome breaks in mature sperm of *Drosophila* (Snyder, 1963). The treatment procedure with the chemicals consisted of soaking previously dried seeds in buffered aqueous solutions of the alkylating agents for six hours at 30°C in a water bath shaker, followed by twenty minutes of washing in tap water. In most of the work with EMS, a concentration of 0.01M in 0.05M TRIS buffer adjusted to a pH of 6.5 was used. This concentration was found to be less than optimal for mutation frequency, but higher concentrations were found to result in substantially increased sterility in the treated plants and their progeny. The concentration indicated yielded 252 independent reversions from the 1,976 C₁ plant progenies tested. Based on evidence from irradiation work that there are approximately five culm primordia present in the embryos of selected large seeds, up to five heads from each C₁ plant have been screened for mutants in all work of this type. TEM has been used at concentrations of 3.03, 4.5, and 6.5 x 10⁻⁴M in distilled water at pH 6.5; concentrations above 6.5 x 10⁻⁴M have been found to result in a marked reduction in vigor and survival under field conditions. In excess of 8,000 C₁ plant progenies have been tested, but only nine independent revertants have been obtained from the TEM treatments.

The scoring for reversions of the yv mutant phenotype has been done by planting head progenies from treated plants in clean sand in greenhouses maintained at approximately 15°C. Seedlings selected as probable revertants have been transplanted and grown to maturity, and their progenies grown and compared with seedlings of the yv mutant in a lighted 15°C growth room. When grown under these conditions the degree to which chlorophyll pigmentation has been restored in the different revertants ranges from only slightly greener than the yv mutant through yellow-green and light-green to one revertant that does not differ perceptibly from the normal green progenitor. The revertants induced by each of the mutagenic agents also show a wide range of variation in fertility, growth habit, vigor, and maturity.

Genetic analysis of revertants: Twenty-five revertants obtained from treatment with ionizing radiations, 5 produced with neutrons and 20 from x-irradiation, are being investigated. Each of these revertants has been crossed with the yv mutant, and in each case the resulting F₁ plants have the normal green phenotype and a segregation of approximately three yv to one revertant has been observed in the F₂. All of the revertants have now also been crossed with normal green barley, and the segregation of seedlings with yv phenotypes in the F₂ generations from these crosses indicate that the revertants represent recessive suppressor mutations. Data from intercrosses among the 25 revertants indicate that 10 of the suppressors are allelic; 4 of the 10 were induced by neutron treatment and 6 by x-radiation. The mutants in this group of alleles have now been crossed in all possible combinations and the F₁ hybrids show a consistent pattern of non-complementarity; i.e., all F₁'s have the revertant phenotype. The 10 allelic suppressors have very similar phenotypes, and represent the maximum restoration of chlorophyll development associated with any of the radiation-induced suppressors. Data that are now available for 93 of the possible 105 diallel crosses among the remaining 15 suppressors indicate that a minimum of 10 additional loci

involved, with two of these loci being represented by 2 alleles each.

Fifteen of the 20 EMS-induced revertants originally selected for analysis are now under investigation. The remaining 5 were discarded because of their high sterility and the difficulty encountered in maintaining the homozygous lines. Each of the revertants has been crossed with the yv mutant and based on hybrid phenotypes and the segregation of yv and revertant phenotypes in the F₂ generation indicate that 9 of the mutants are recessive and the remaining 6 are incompletely dominant. The F₁ hybrids between each of the latter mutants and the yv mutant are perceptibly lighter in seedling chlorophyll pigmentation than are the respective homozygous revertant mutants. The homozygous revertant mutants in this group of incompletely dominant revertants have phenotypes which range from a light yellow-green to a close approximation of the normal green. All 15 of the EMS-induced revertants have been crossed with the normal green progenitor, and segregation for the yv phenotype observed in all but one of the F₂ progenies indicate that 14 of the revertants represent suppressor mutations. The F₂ from the remaining cross, involving one of the darker green dominant revertants, segregated 562 normal green seedlings to 171 with the revertant phenotype; i.e., this reversion involves either a suppressor mutation at a closely linked locus or a mutation at the same locus as the yv mutant.

Data obtained during the present year from intercrosses among the EMS-induced suppressors indicate that a minimum of 10 loci are involved, with one of the loci being represented by two recessive alleles. In considering the analysis of allelism among these suppressors, it was anticipated that linkage could represent a considerable impediment to the analysis in crosses in which one or both of the suppressor mutants was a dominant. The F₁ hybrids from these crosses are phenotypically revertant (suppressed yv) due to the dominance of the suppressor mutant from one or both of the parents, and the demonstration of non-allelism of the suppressor mutants depends on the appearance of seedlings with the yv mutant phenotype in the F₂ generation. On the other hand, failure of the yv mutant phenotype to appear in the F₂ can be due either to close linkage of the suppressor mutants or to their allelism. The power of the test for non-allelism in such cases depends on linkage intensity and the size of the F₂ population grown.

The F₂ progenies from the 15 possible intercrosses among the 6 dominant suppressors have been grown and establish that there are no cases of allelism among these mutants. Thirty-eight crosses of a dominant with a recessive suppressor have been made, and in all but one of these the non-allelism of the mutants involved has been established by the appearance of segregants with the yv phenotype in the F₂ generation. In the one exceptional cross in this group, none of the 129 F₂ seedlings had the yv phenotype. Additional F₁ seeds of this dominant x recessive suppressor cross have been obtained for the production of a larger number of F₂ progeny.

Only four of the TEM-induced revertants are being analyzed genetically at present. Crosses of each of these revertants with the yv mutant stock indicate that two of them are dominant and the other two are recessive. Data from the F₂ generations from crosses of the recessive revertants with normal Himalaya indicate that both of them are attributable to suppressor mutations. The darker green of the two recessives has been found to be allelic to the group of 10 recessive suppressor alleles produced by treatment with ionizing radiations.

When homozygous, one of the dominant revertants induced by TEM results in a phenotype that is indistinguishable from that of normal Himalaya barley, although in hybrids with the yv mutant and in resulting F₂ segregants the phenotypes of plants heterozygous for the revertant mutant are detectably lighter than normal. This is not found in comparable crosses between the yv mutant and normal Himalaya. No seedlings having the yv mutant phenotype have been found among approximately 2,000 F₂ segregants from crosses between this dominant revertant and normal barley. The second dominant revertant has a yellow-green phenotype, and the F₂ generation of crosses between this revertant and normal Himalaya have segregated 456 normal green to 84 revertant seedlings. These data indicate that the two dominant revertants involve mutation either at the same locus as the yv mutant or at relatively closely linked loci.

Location of suppressor mutants: The location of the yv mutant near the centromere of chromosome 7 led to the use of a set of six translocation tester stocks having this chromosome in common, i.e., T 1-7f, T 2-7d, T 3-7d, T 4-7b, T 5-7a, and T 6-7a, in an attempt to relate the loci responsible for the revertant phenotypes to specific chromosomes. It was subsequently found that the T 5-7a stock that was being used in crosses with the various suppressors did not carry the translocation. Information on the possible location of suppressor loci on chromosome 5 is being obtained from crosses with translocations 1-5a and 4-5e which are homozygous for the yv mutant and from crosses with the primary trisomic for chromosome 5.

Barley is a species in which there is a considerable excess of alternate over adjacent chromosome segregation at meiosis in plants that are heterozygous for a translocation. If a single crossover in the interstitial segment, i.e., the region between the translocation breakpoint and the centromere, is followed by alternate segregation, the crossover chromatids end up in spores that are genetically deficient and will abort. This results in a reduction in genetic recombination for regions included in the interstitial segment. If a gene is located at the centromere, the region between the gene and any translocation breakpoint will be an interstitial segment, and all translocations involving that chromosome would be expected to show close linkage with the gene. Since the yv mutant locus is located near the centromere of chromosome 7, it should show close linkage with the breakpoint of any translocation involving this chromosome.

The expected segregation ratio in the F₂ generation from a cross of a translocation with a dominant or a recessive suppressor of yv is 12 normal green : 3 suppressed yv : 1 yv, or 12 normal green : 1 suppressed yv : 3 yv, respectively, if the suppressor is segregating independently of the translocation. Linkage of either a dominant or a recessive suppressor with a translocation is indicated by a low ratio of yv to suppressed yv phenotypes in the F₂ seedling progeny. Table 1 gives the minimum number of F₂ plants required to detect linkage between a mutant, such as yv, and a suppressor mutant from the cross of a translocation tester stock with a suppressed mutant stock. The table, as noted, is based on a probability of .05 of concluding independence if linkage of the indicated intensities actually exists between the mutant and either a recessive or a dominant suppressor. These calculations are based also on the assumption of equal viability of gametes and zygotes and that p is normally distributed with a variance of 1/information.

Table 1. Minimum number of F₂ progeny required to detect linkage between a mutant and a suppressor from the cross of a translocation tester stock with the suppressed mutant so that the probability of concluding independence is .05 if the true recombination value is p .

Type of suppressor	True recombination value (p)						
	.45	.40	.35	.30	.25	.20	.15
recessive	3133	752	318	169	102	65	43
dominant	3350	861	391	225	146	103	77

Linkage data from crosses of the translocations involved in the tester set with genetic markers on chromosome 7 and cytological data on the positions of the translocation breakpoints from root-tip squashes indicate that any locus on chromosome 7 that is linked with the breakpoint of one of these translocations should be linked with the breakpoint of at least one of the other four translocations in the set. Therefore, any suppressor that shows linkage with two or more of the translocations is located on chromosome 7, while linkage with only one of the translocations indicates that the suppressor mutant is located not on chromosome 7 but on the other chromosome involved in the translocation. As an example of this, the F₂ segregation data from crosses of two of the recessive suppressors of *yv* with translocations in the tester set are given in Table 2. The data indicate that *suX30-yv*, one of the group of 10 allelic suppressors induced with ionizing radiation, is located on chromosome 6. In this case, for which linkage data with the 2-7 and 5-7 translocations are not available, the chromosome 7 breakpoints of the translocations that were used is important. The breakpoint of T 6-7a is in the long arm very close to the centromere, as also is the chromosome 7 breakpoint of T 6-7d. The breakpoint of T 1-7f is also in the long arm of chromosome 7, about two recombination units from the centromere, and is thus very close to that of the two 6-7 translocations. The chromosome 7 breakpoint of T 4-7b is in the satellite on the short arm, and for both T 2-7d and T 3-7d it is near the middle of the short arm. It follows that the close linkage of *suX30-yv* and T 6-7a places the mutant near the breakpoint in one of these chromosomes and the absence of linkage of the suppressor, particularly with T 1-7f, eliminates the latter chromosome. Similarly, the linkage of *suT2-yv*, one of the TEM-induced suppressors, with T 3-7d but not with T 2-7d or any of the other translocations indicates that this mutant is located on chromosome 3.

By the use of translocations and intercrosses among the suppressor mutants it has been possible to identify the following additional loci at which suppressor mutations have been induced: (a) a second locus on chromosome 6 with two alleles, one induced by thermal neutrons and the other by x-rays; (b) single loci on chromosomes 1, 4, 5, and 7, represented in each case by single alleles induced by x-radiation; and (c) an additional locus on chromosome 3, a single locus on chromosome 1, and

Table 2. Data on F₂ segregations from crosses of two recessive suppressors of yv with translocations in the tester set.

Suppressor	Translocation	Phenotype of F ₂ progeny		
		normal	<u>yv</u>	<u>su-yv</u>
<u>suX30-yv</u>	T 1-7f	47	13	3
	T 3-7d	38	7	2
	T 4-7b	72	16	4
	T 6-7a	1974	46 ♂	693
<u>suT2-yv</u>	T 1-7f	90	28	10
	T 2-7d	114	31	9
	T 3-7d	310	2	78
	T 4-7b	86	25	7
	T 6-7a	372	105	40

♂ 39 of these yv segregants were trisomics.

3 separate loci on chromosome 4, represented in each case by single EMS-induced suppressor alleles. The radiation-induced suppressor mutation on chromosome 4 is not allelic with any of the three EMS-induced suppressors on this chromosome, nor are the EMS- and TEM-induced suppressors on chromosome 3 allelic. One of the suppressor mutants induced by EMS which it has not been possible to locate as to chromosome from limited data from crosses with the translocations may represent a fifth locus on chromosome 4 on the basis of non-allelism with the other mutants on this chromosome but apparent linkage with one of these mutants induced also by EMS. Yet another EMS-induced suppressor appears on the basis of limited data with the translocation tester stocks to be located on chromosome 3 but is not allelic with the mutants at either of the other two loci known to be on this chromosome. It is not known whether the two suppressors located on chromosome 1, one of which was induced by x-rays and the other by EMS, are allelic. It is also not known whether any allelism exists among the four suppressors located near the centromere of chromosome 7 and yv locus, but one of these mutants, a recessive induced by x-rays, is non-allelic with the yv mutant.

Of the 25 radiation-induced suppressors, there are 9, 2 of which are allelic, that have not yet been located as to chromosome. Seven of the 15 suppressors induced by EMS, again including 2 that are allelic, also have not been located as to chromosome. In all of these cases a considerable amount of data has been obtained from crosses with some or all of the translocation testers, but without conclusive results. In addition, 4 of the x-ray-induced suppressors, one representing each of the two known loci on chromosome 6, the one known to be located on chromosome 4, and one of the pair of alleles of unknown location, have been crossed with each of the 7 suppressors of unknown location that were induced by EMS. No evidence of allelism was obtained in the progenies from these crosses. All of the suppressors of unknown location have been crossed with most or all of the 6 primary trisomic stocks homozygous for the yv mutant. As noted in the previous progress report, the trisomic for

chromosome 1 has not been included in these stocks because of its very low fertility. Data from the crosses with the trisomics should make it possible to assign unambiguously any of the suppressor mutants to a specific chromosome.

Although not all of the suppressor mutants have been located, and some of the ones induced by ionizing radiation have not been tested for allelism with some of those induced by EMS treatment, the data summarized above indicate that a minimum of 14 different loci are represented by the 44 suppressors of yv that are being investigated. The frequency with which allelism has been detected, with the notable exception of the group of 11 alleles on chromosome 6, indicates that at least several additional loci are represented by the suppressors that have not yet been genetically located.

Implications of the genetic data: It is evident from the above data that treatment of the yv mutant with either physical or chemical agents results in a high frequency of reversions toward the normal green phenotype, that most, if not all, of these reversions are due to suppressor mutations, and that these may occur at a considerable number of loci. The point should be made that no meaningful comparison between the physical and chemical mutagens regarding their efficacy in induction of the reversions is readily possible: (1) the treated materials and their progenies were grown and tested under a variety of conditions over a period of years, and by different workers, and (2) the various treatments were not standardized on any basis readily admitting to such comparison. However, certain comparisons between the mutagenic agents with respect to frequencies and spectra of the induced reversions appear to be warranted.

All of the 25 reversions induced by ionizing radiations that were selected for investigation have been shown to represent suppressor mutations at loci readily separable from that of the yv mutant, and 10 of these are alleles at a locus on chromosome 6. The 25 suppressors do not represent a random sample of the revertants induced by ionizing radiations since their selection was biased in favor of mutants with darker green phenotypes. The 10 allelic suppressors represent the maximum restoration of chlorophyll development found in any of the radiation-induced reversions. The only surviving plants following neutron treatment for the induction of reversions of the yv mutant came from the 1-hour treatment at the flux given above. Five reversions were selected on the basis of phenotype from among the 12 reversions of independent origin obtained when 966 N₁ plant progenies were tested. Four of the 5 proved to be alleles at the locus on chromosome 6. The remaining 6 alleles at this locus originated independently among the progenies of 6,914 X₁ plants grown in two different years following treatment with 3,500 or 7,000r of x-rays and subsequent hydration of the treated seeds in the presence of O₂ or N₂. No mutants at the locus of the 10 radiation-induced allelic suppressors have been found among the EMS-induced reversions. The 20 investigated reversions in this group were also selected with a bias in favor of those mutants resulting in darker green phenotypes, and a suppressor at the chromosome 6 locus in question undoubtedly would have been included if it resulted in a phenotype at all comparable to those of the radiation-induced alleles. All 20 of the EMS-induced reversions have been crossed with one or another of the latter alleles, and no cases of allelism have been found. To obtain additional data on this point, 6 reversions with phenotypes as dark or almost as

dark as those of the radiation-induced suppressor alleles were selected from progenies of 1,500 EMS-treated plants grown this year. These reversions were crossed with one of the radiation-induced alleles, and again gave no evidence of allelism. Thus, no suppressor mutations at the chromosome 6 locus, at which such mutations are induced at relatively high frequencies by ionizing radiations, were obtained in a large sample of EMS-induced reversions of yv. On the other hand, 1 of the 4 TEM-induced reversions under investigation involves a suppressor mutant at the locus in question.

With the exception of the 11 allelic suppressors at the locus on chromosome 6, relatively few cases of allelism have been found among the mutants represented in the selected sample of revertants. Two alleles, one induced by neutron treatment and the second by x-radiation, occur at a second locus on chromosome 6; two alleles, both induced by x-radiation, at a locus not yet located as to chromosome; and two EMS-induced alleles at yet another locus that has not been located. Additional allelism may be found among the 4 mutants, 1 induced by x-rays, 2 by TEM, and the fourth by EMS, that have been found to be located near the locus of yv on chromosome 7. No allelism has been found between any of the mutations induced with ionizing radiations and those induced by EMS treatment. The absence of allelism between mutations belonging to the two groups is not of great significance in view of the limited allelism that has been found overall. An additional implication of the limited allelism among all of the selected yv suppressors under investigation is that a number of other loci at which such suppressors could occur have not yet been detected.

Another comparison that appears to be valid concerns the dominance of the suppressor mutations induced by the two alkylating chemicals and by the ionizing radiations. None of the suppressor mutations induced by ionizing radiations has been found to be expressed as a dominant in the usual sense. A few of them are not completely recessive since reasonably effective selection can be made, usually in later stages of seedling development, for heterozygous segregants in the F_2 progeny from crosses of these revertants with the yv mutant. In all cases, however, the heterozygotes resemble the yv mutant more closely than they do the homozygous suppressed-yv. This is true also for a few of the EMS-induced suppressors that have been classified as recessives. The 6 EMS-induced suppressors classified as dominants are not completely dominant, but the F_1 hybrids between each of these mutants and the yv mutant resemble the homozygous suppressed-yv more closely than they do the yv mutant with respect to chlorophyll development. One of the two TEM-induced dominant suppressors is very similar to this latter group, whereas the second one results in the most nearly normal phenotype of any of the revertants both when homozygous and in F_1 hybrids with the yv mutant.

One limitation of the present investigation has been the inability to obtain other mutations allelic with the original yv, with the result that possible allele-specificity of any of the suppressor mutations cannot be determined by a direct approach. Recent work by Brody and Yanofsky (1965), Capecchi and Gussin (1965), Carbon et al. (1966), and others, provides compelling evidence that allele-specific suppressors, which classify logically as either missense or nonsense suppressors, act at the level of the adapter tRNA molecules. The mechanism of action has not yet been specified in any of these cases, e.g., whether through change in the amino acid activating enzyme or change in a minor tRNA

species which causes it to accept an atypical amino acid or to recognize an atypical codon, but the effect is restoration in a small proportion of cases of a functional primary structure in the polypeptide specified by the gene that was altered in the primary mutation. As in the case of suppression of amber mutations in bacteriophages, such suppressors would be expected to reverse (partially) the effect of a specific type of mutational change occurring in any gene. In view of these considerations and the absence of other mutant alleles at the yv locus, 3 of the suppressors of yv which have been separated from yv were crossed in most combinations with 12 phenotypically similar mutants. The necessary F₂ progenies from these crosses are being obtained and will be grown within the next six weeks. Any cases of suppression within this very limited group of combinations would be of particular interest in relation to possible allele specificity of the suppressor mutation(s) involved. On the other hand, the absence of such suppression would provide only limited support to the alternative; that most, if not all, of the suppressors obtained act to bring about some change in metabolism such that the aberrant function resulting from the yv mutation is at least partially by-passed. The latter phenomenon is suggested both by the considerable number of loci at which suppressor mutations can occur and by the diversity in level of suppression that is found.

Biochemical investigations: As noted in previous reports, these investigations were initiated to achieve better definition of the yv mutant and derived revertants in relation to the normal green progenitor and certain other chlorophyll mutant types. The results of much of the work completed through the previous contract year have recently been published (Jain, 1966). This work was concerned primarily with a comparative investigation of chlorophyll content and composition and qualitative and quantitative composition of both the free amino acid pool and the protein amino acids. Data were obtained from different seedling developmental stages and from seedlings grown under different light and temperature conditions using principally the yv mutant, a suppressed-yv involving a recessive suppressor from the group of 10 radiation-induced alleles on chromosome 6, and normal Himalaya barley. Electrophoretic separation of soluble leaf proteins in polyacrylamide gels initially revealed no marked differences among these three genotypes although one protein fraction was found to be consistently intense in electropherograms of extracts from normal Himalaya, only faintly represented in the yv mutant, and intermediate in the suppressed-yv. This band was found to be intense also in preparations from 3 allelic light-green mutants and was absent in protein extracts from etiolated seedlings of all of the genotypes investigated.

Dr. Jain terminated his work on the project in June to accept a position at Louisiana State University. Prior to his departure the major emphasis in the biochemical investigations consisted of continued efforts to resolve the protein fractions in (disc) electropherograms of soluble leaf proteins extracted from the same group of genotypes that provided the materials for the earlier investigations. The use of low pH (4.0) gel, variations in sample size, and a large-pore gel formulation of Canal Industrial Corporation all failed to result in improved resolution of the protein fraction previously observed to be more intense in preparations from the normal than from the yv or suppressed-yv seedlings. Numerous runs were also made with the standard 7.5% gel at a pH of 9.4 during the course of this work, and it was found that two

differences among the three genotypes that had been suggested by earlier work could be demonstrated reliably. A fraction represented by a band of moderate intensity located close to the origin can be shown to be consistently absent in preparations from the yv mutant but present in both the normal and suppressed-yv. A second fraction, represented by a band that is less well-defined and farther from the origin, can be demonstrated with some consistency in preparations from the suppressed-yv seedlings but has never been detected in either of the other genotypes.

Since the early work using acrylamide gel electrophoresis did not provide a satisfactory answer to the question of whether or not qualitative differences in soluble leaf proteins occurred among the genotypes investigated, Dr. Jain started work with immunoelectrophoresis. This work was undertaken with the advice and assistance of Dr. R. K. Lindorfer of the Department of Veterinary Bacteriology. Protein extracts were obtained at low temperatures from 7-day-old seedlings of normal barley and the yv mutant that had been grown at 15°C in a lighted cold room. To obtain antiserum, rabbits were injected subcutaneously twice each week for four weeks with 0.5 ml of the supernatant derived from the protein extraction procedure. The animals were bled 12 days after the last injection and serum was prepared by centrifugation of the coagulated blood at 3500 x g for 30 minutes. A qualitative test against the crude protein extract on a standard agar plate, i.e., the Ouchterlony technique, was used to assess antibody build-up. Basically the same procedure, with antiserum in an elongate central trough on the slide and crude extracts from the yv mutant and normal seedlings in wells located near the middle of the long axis of the slides and on opposite sides of the central trough. The slides used were standard microscope slides coated with 20 ml of a 1.5% solution of Noble agar in 0.02M veronal buffer (pH 8.6), containing 100 ppm merthiolate. Some difficulty was encountered initially in obtaining an appropriate antibody level in the antiserum and subsequently in resolution of the precipitin bands. Work with immunoelectrophoresis was stopped when it was found that differences among the genotypes in major protein fractions could be demonstrated in the acrylamide gel electropherograms.

The biochemical aspects of the work have now been altered in focus, or at least in approach, with the aim of defining the nature of the block imposed by the yv mutant. The work that is in progress is concerned with paper and thin-layer chromatographic procedures for the separation of the photosynthetic pigments and their precursors and with techniques for the introduction of C¹⁴-labeled compounds into intact early seedlings and, if possible, into leaf discs. The compounds that are being used are C¹⁴-glycine and C¹⁴-alpha-amino-levulinic acid. The latter is the last compound before the appearance of ring structures in the biosynthesis of the heme compounds; i.e., two molecules of alpha-amino-levulinic acid condense to give a pyrrole ring and 4 pyrrole rings condense to give the porphyrin ring from which the synthesis of either the chlorophylls or the cytochromes proceeds. Glycine and succinyl-Co A are the precursors of alpha-amino-levulinic acid. The immediate goal of the work that is in progress is to determine whether accumulation of any chlorophyll precursors occurs in the yv mutant. The rationale and procedures have been formulated through consultation with Dr. H. R. Warner, Department of Biochemistry, and the work is being conducted with his participation and direct supervision.

Morphogenetic investigations: A major objective of this work has been the analysis of morphological differences characterizing normal Himalaya barley and the yv mutant and suppressed-yv phenotypes. Previous work on the normal and yv mutant seedlings, using stained sections of the first leaf of seedlings grown for 3-10 days at 15°C, indicated that the most striking differences between the two lines were in the development of chloroplast structure and possibly also in the numbers of chloroplasts. Dr. Bergbusch left the program at the end of June, having accepted a position in the Department of Genetics at McGill University. During the last few months that she was associated with this work, it was decided that she could most profitably focus her attention on the comparative development of the chloroplasts in the normal and yv mutant lines.

Seeds were germinated on blotting paper and grown for varying lengths of time at 15°C with 16 hours of light per day. These conditions were chosen to balance satisfactory seedling development and the greatest effect of the yv allele on chloroplast structure. After stripping the epidermal layer from sections of the first seedling leaf, cells of the spongy mesophyll were teased out into a drop of sucrose or sucrose-TRIS buffer solution (pH 7.0-7.4) on a microscope slide. A considerable proportion of the cells were broken by this treatment and a thick suspension of relatively undamaged chloroplasts in solution were obtained. The chloroplasts were examined under phase contrast.

The concentration of sucrose necessary to prevent excessive swelling of the chloroplasts was found to vary with developmental stage; thus relatively immature chloroplasts required relatively high concentrations of sucrose (1M or higher). To prevent either shrinkage or swelling of mature chloroplasts, the optimum sucrose concentration was about 0.3 to 0.4M. Chloroplasts were in some cases deliberately swollen in order to check on the presence or absence of "lamellae." This proved to be a more reliable test than the appearance of positively identifiable grana; the swelling of mature chloroplasts led to a reduction in the apparent size and density of the grana, followed by the appearance of balloon-like "blebs" at the surface of the chloroplast. At low concentrations of sucrose, the whole chloroplast is found to disintegrate into these "blebs," which float free in solution as intact structures. These have been described by Spencer and Wildman (1962), who considered them to be stroma lamellae "bags," visible under phase contrast at comparatively low magnification (compared to the electron microscope studies of lamella membrane width) by virtue of the light reflected from their margins. In the first leaves of seedlings that are from 3 to 20 days old, these "blebs" appear at much higher concentrations of sucrose than those reported by Spencer and Wildman (e.g., <0.2M); in fact, chloroplast swelling was found to be difficult to control in developing chloroplasts of both the mutant and normal lines.

Chloroplast maturation in the yv mutant is very much retarded compared with that of normal barley, as judged by the criteria of size, general shape, the appearance of identifiable grana, and "bleb" formation on swelling. Not until visible greening appears at the leaf tip do any of the chloroplasts of the mutant line show any sign of structural differentiation under phase contrast. This occurred after about 10[±] days of growth under the conditions used. Until this time, although there was some increase in the size of the mutant chloroplasts, they remained featureless, of very irregular shape, very variable in size, and very plastic, i.e., in intact cells were seen to be not only very irregular in shape, but individual

chloroplasts were subject to considerable shape variation. The "interconnecting strands" between mature chloroplasts of normal cells, reported from phase contrast observations of other workers, were found to be very numerous and easily seen in cells of the yv mutant; the "jackets" of these chloroplasts appeared to be very mobile. It is possible that the segmentation of the chloroplasts observed in the intact cells of the mutant represented genuine chloroplast "division;" however, the conditions of isolation and the toxicity of the surrounding medium may have induced an artificial segmentation. A similar, but very transient, stage was occasionally observed in seedlings of the normal line that were less than three days old. At this stage, however, the cells are small, dense, and crowded with rather large and highly reflective "bubble-like" structures which made observation and identification of chloroplasts in intact cells very difficult. When in solution, these chloroplasts either swelled into featureless invisibility or contracted into very small, smooth-surfaced structures, depending to some extent on the molarity of the surrounding medium. In 5-day-old seedlings of the normal line, the chloroplasts were found to have a very well defined lamella formation.

The development of the chloroplasts in both the normal and yv mutant have been recorded photographically. A brief comparison of chloroplast development in the suppressed-yv line that has been used extensively in the biochemical investigations has shown that it falls between the extremes represented by the yv mutant and normal barley, without incorporating any especially distinctive features. A manuscript reporting the results of the morphogenetic investigations is in preparation.

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COMPREHENSIVE REPORT

The last comprehensive report of the over all research activities on this contract was submitted by Dr. R. S. Caldecott at the beginning of the 1965-66 contract year; the present principal investigator has been associated directly with the contract since the latter part of that year. At the time of the previous report the stated objectives of the research were: (1) determination of the role of pre- and post-irradiation temperature on x-ray-induced injury in seeds; (2) determination of recurrent radiation effectiveness in inducing "diploidization" in polyploid species of Avena and Triticum; (3) determination of the effectiveness of x-irradiation in inducing reverse mutations or suppressor mutations modifying the effects of primary mutations concerned with chlorophyll development in barley; and (4) biochemical determination of the nature of changes in the chloroplast pigments associated with such mutant reversion or suppression.

Research pertinent to the first two of the above objectives was terminated during the 1965-66 contract year; when Dr. Caldecott resigned his research position to become dean of the College of Biological Sciences at the University. The major results of investigations concerned with the effects of recurrent radiation on polyploid series in Avena and Triticum have now been published (Caldecott et al., 1964; Kao and Caldecott, 1966.) Populations of the Avena species were carried through one additional cycle of irradiation in 1965. The results obtained were generally consistent with those obtained in the previous cycles; (1) chlorophyll mutations obtained in the tetraploid and hexaploid species segregated in all cases as single gene differences, whereas occasional cases of segregation indicating complementary genes had been found in the tetraploid and hexaploid species of Triticum; (2) mutants characterized by presence of only the yellow pigments were found to represent between 5 and 10% of the total chlorophyll mutant frequency in diploid, tetraploid, and hexaploid species, whereas they were found, in about the same frequency, in only the diploid Triticum species; and (3) the relative proportions of albino mutants in the diploid, tetraploid, and hexaploid species were found to be approximately 50%, 28%, and 6%, respectively; whereas about 50% of the chlorophyll mutants observed in each of the comparable Triticum species were of this type. An obvious interpretation of these data, consistent also with the low mutation frequency in hexaploid Triticum after a number of cycles of radiation and a large body of evidence concerning the probable progenitors of Triticum polyploid species, is a closer genetic relationship between the progenitors of hexaploid wheat than existed between the progenitors of hexaploid oats.

A major part of the research effort during the last three contract years has been directed toward the last two of the originally stated objectives. Treatment of the yellow-virescent (yv) chlorophyll mutant, on which most of the work has centered, with either physical or chemical agents results in a high frequency of reversions toward the normal green phenotype. Most, if not all, of these reversions are the result of suppressor mutations induced at other loci. The mutants investigated, selected with a bias toward those resulting in maximum suppression of the yv mutant, were found (1) to represent a minimum of 14 different loci, (2) to show only limited allelism interse; with the exception of a group of 11 alleles at a locus on chromosome 6; and (3) to be characterized by wide differences in the degree of suppression of the yv mutant. The genetic analyses of the

suppressor mutations permit two comparisons of interest relating to the effects of the different mutagenic agents used. Ten allelic suppressors at the previously mentioned locus on chromosome 6 were induced by x-radiation or thermal neutrons, and eleventh allele was induced by the polyfunctional alkylating chemical, triethylene melamine (TEM), but no mutants at this locus have been found among a large number induced by the highly mutagenic monofunctional agent, ethyl methanesulfonate (EMS). The second comparison relates to dominance of the induced suppressors: none of the 20 suppressor mutants induced by x-radiation or the 5 that resulted from thermal neutron treatment behaves as a dominant, whereas 6 of the 20 EMS-induced and 2 of the 4 TEM-induced suppressors that have been investigated are dominant.

The results of the work on genetic aspects of suppression of the yv mutant allele, which are given in more comprehensive detail in the accompanying progress report, are being prepared for publication. The genetic information that is now available on this system provides presumptive evidence that at least most of the suppressors obtained are general rather than allele-specific; i.e., that they condition changes, possibly in organization or metabolism, that do not relate directly to restoring a functional primary structure to the primary product of the yv allele. The apparently large number of loci at which suppressor mutations can occur supports this view, as also does the wide variation in the level of suppression associated with different ones of the suppressors (although different mutant alleles at a given locus result in very similar apparent levels of suppression.)

When biochemical investigations of the barley chlorophyll mutants were initiated in 1964, an immediate objective of the work was identification of the specific block in chlorophyll synthesis imposed by the yv mutant allele. The work done by Dr. Jain during the two years that he was associated with the program served to identify some of the characteristics of the mutant and one of its induced suppressors in relation to normal, although some properties of potential interest and importance (e.g., the relative levels of reducing sugars) were not considered. Electrophoretic separation of soluble leaf proteins has been successful to the extent of establishing differences in two protein fractions among the above three genotypes; i.e., a well-defined fraction found in preparations from both the normal and the suppressed mutant is absent in the primary (yv) mutant, and a second protein, represented by a less intense band in the gel electropherograms, is found only in preparations from the suppressed mutant. These proteins represent major fractions in the soluble extracts, and it appears probable that they represent structural proteins. The preparations were from extracts using entire leaves, for reasons of convenience, and there remains a need to know whether the protein differences that have been found relate to the chloroplasts.

Phase contrast observations of chloroplast development in the yv mutant in relation to normal barley have established that very little structural differentiation occurs in the mutant chloroplasts until the seedlings are 10-12 days old (when grown in 15°C). Prior to this the chloroplasts are very irregular in shape and size, and it is a valid assumption, based on the extensive investigation of fine structure of normal and mutant barley chloroplasts by von Wettstein (Brookhaven Symp. Biol. 11, 138, 1959), that these differences from the normal reflect differences in internal structure. Unfortunately, Dr. Bergbusch did not continue her association with the program long enough to conduct investigations on the development of fine structure in chloroplasts of the yv

mutant and the suppressed mutant, although this work was started. If the biochemical investigations establish that the alteration imposed by the yv mutant is at the level of chloroplast structure, i.e., involves a structural protein related to the chloroplasts rather than the regulation of chlorophyll synthesis or one of the enzymatic steps in chlorophyll synthesis, information on fine structure of the chloroplasts will become especially important.

Plans for continuation of present objectives: Work concerned with factors influencing radiation-induced alterations in seeds and with recurrent irradiation of polyploid plant species, has been terminated and there are no plans for the resumption of work in these areas.

Genetic analyses of the several groups of induced suppressors of the yv mutant have been both extensive and productive. There remain several aspects on which additional information should be obtained to complete this phase of the work. One of these, a critical demonstration of non-allelic interaction between at least certain suppressor mutations to produce a phenotype that is darker than that of either of the homozygous suppressors, would provide information and materials potentially very useful in the biochemical investigations.

The biochemical part of the work concerned with the nature of changes induced in the photosynthetic apparatus by forward mutations and ameliorated by either reverse mutations or suppressors, was phrased in terms of alterations in chloroplast pigments in the originally stated objective. This work has focused on the yv mutant and its suppression, a system in which the experiments that have been done indicate that the only significant alteration in the photosynthetic pigments is quantitative. With the minor redefinition that is implied, investigations in this area are no less timely or potentially productive than when the work was undertaken. A major limitation heretofore has been in biochemical insight, but with the interest and active participation of Dr. H. R. Warner in the program this has been remedied. Work on experiments designed to determine the nature of the defect in chlorophyll synthesis is in progress, and the rationale and procedures appropriate to such investigations are included in the accompanying proposed technical program.

Graduate students trained and post-doctoral tenures completed:

The following graduate students have been associated with the program during the period since the last comprehensive report:

Louise Heine, 1964-66. M.S., without thesis, to be conferred 1967.

Vivian Hiatt, 1963-66. PhD. student, now Instructor, Department of Genetics, University of Minnesota.

Fa-ten Kao, 1957-65. PhD., 1965, now Assistant Professor, University of Colorado Medical Center.

Gudrun Willeke Pfendler, 1964-67. M.S. to be conferred 1967.

The following post-doctoral tenures have been completed on the program during the same period:

Victoria Bergbusch, 1964-66. Dr. Bergbusch is now Assistant Professor, Department of Genetics, McGill University.

Mishrilal Jain, 1964-66. Dr. Jain is now Assistant Professor, Department of Biology, Louisiana State University, New Orleans.

Neal Tuleen, 1966-.

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- Hiatt, V. S., D. T. North, N. A. Tuleen, and R. S. Caldecott. 1964. Radiation-induced phenotypic reversions in Hordeum. Radiation Res. 22, 197. (abstract).
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- Tuleen, N. A., R. S. Caldecott, L. A. Snyder, and V. S. Hiatt. In press. In: Research in the Production and Use of Induced Mutations in Plant Breeding. Pergamon Press.

The present state of knowledge in this area of research: For reasons that are readily understandable, in terms of complexity of the systems, investigations concerned with the control of structural and enzymatic functions have been less extensive and substantially less productive in higher organisms, and particularly in higher plants, than in lower forms. Control, in this sense, is intended to refer both to the synthesis and subsequent activity of the enzymes. The rationale for the use in such work of mutants in which a function is totally or partially blocked, and suppressors which act to remove the block, would appear to be sufficiently apparent as to not require elaboration.

The system that is being used in work on this contract, i.e., the photosynthetic apparatus, is a biologically important one, and serves to illustrate the state of knowledge relating to such complex systems generally. Although mutations causing impairment in the chlorophyll apparatus have been obtained in a considerable number of green plants, the question of control of chlorophyll and cytochrome synthesis has not yet been rigorously investigated. Many of the enzymatic steps in the pathways are known or can be inferred (cf. Lascelles, 1965), but relatively little is known of their genetic or biochemical regulation. The latter phenomena are of considerable importance in the understanding of such systems, and loom especially important in relation to the central problem of development.