Final Report for AEC Contract AT(11-1)-332

Genetic and Biochemical Investigations of Barley Chloroplast Mutants

Major emphasis in the research conducted under this contract during the period 1965-70, the period during which the present senior investigator was associated with the contract, was in the following areas:

1. Genetic analysis of induced mutants acting to suppress the effects of a primary virido-xantha mutant on chloroplast structure and content of the various chloroplast pigments. The results of the analysis were summarized in previous reports and have been published in detail in two papers (Tuleen et al., 1968a, 1968b). The major findings from the investigation of 44 induced suppressors, and subsequent partial analysis of 10 additional suppressor mutants, have been: (a) suppression of the original x-ray-induced chloroplast mutant is extremely complex, with not less than 19 loci having been identified by the occurrence of suppressor mutants; (b) no dominant suppressor mutants have been found to result from treatment with ionizing radiations (x-rays or thermal neutrons), whereas approximately one-third of the mutants induced with both monofunctional (ethylenimine) and polyfunctional (triethylene melamine) alkylating chemicals were found to be dominant; (c) evidence for locus specificity in action of the mutagenic agents has been suggested by the occurrence of 4 neutron-induced, 6 x-ray-induced, and 1 TEM-induced mutants at a single locus on chromosome 6 - at which no mutants were found to be induced with the monofunctional chemical.

2. Quantitative investigation of the chloroplast pigments produced in the virido-xantha mutant, normal Himalaya barley, and two of the suppressed strains at both 15°C and 21°C. Copies of a manuscript reporting the results of this work have previously been submitted under the number C00-332-5, and reprints of the paper (Warner et al., 1969) are being submitted with this report. The principal findings from this work were: (a) both chlorophyll content and the ability to produce protochlorophyllide from exogenously supplied 6-aminolevulinic acid are sharply reduced in the mutant at both temperatures in all stages of seedling development up to 21 days; (b) although total carotenoid content is also reduced in mutant seedlings, the 5 carotenoid pigments assayed are not reduced coordinately; (c) antherxanthin was found to be accumulated in mutant seedlings, and zeaxanthin was found to be present in significant quantities in such seedlings although it could not be detected in normal seedlings; (d) the presence of recessive suppressors of the vx mutant resulted in increased contents of the chlorophyll pigments and a partial reversal of the indicated changes in carotenoid pigments noted; (e) a pronounced cumulative effect in restoring both chlorophyll and carotene levels toward normal values was found when the two suppressors studied were combined into the same genotype.

3. Analyses of the ultrastructure of chloroplasts from normal Himalaya barley, the vx mutant, several different suppressed-vx strains, and a limited number of other pigment-deficient mutants were initiated during the last two years of the contract. The major findings from this work, some representative electron photomicrographs of which were...
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submitted with the Technical Progress Report (COO-332-6) for 1968-69, have been: (a) observation of a distinct prolamellar body in early differentiation of chloroplasts in each of the pigment-deficient mutants, and the absence of such bodies in differentiation of normal chloroplasts; (b) the differentiation of internal plastid structure appears to be both delayed and variously aberrant in each of the mutants that has been examined; (c) aberrations in internal structure are consistently more pronounced in chloroplasts from mutant seedlings grown at 15°C than in chloroplasts from seedlings of comparable genotype grown at 22-24°C.

During the 1969-70 contract year a seed increase was obtained from greenhouse grown plants of most of the primary plastid mutants, the suppressors of the vx mutant, and the translocations and various other genetic stocks which have been used in these studies. Primary interest in the continuing genetic investigations consisted of growing additional seedling progenies of ethyl methanesulfonate-, x-ray-, and thermal neutron-treated material of the vx mutant. A large number of additional suppressor mutants, having phenotypes similar to those characteristic of mutants at the su-1 locus on chromosome 6, have been selected and increased. These materials are expected to provide a critical test of the indicated specificity of action of ionizing radiations and polyfunctional alkylating agents at the su-1 locus (at which 10 suppressor mutants induced with x-rays or thermal neutrons and 1 mutant induced with trifunctional triethylene melamine were found previously) or the su-2 locus on the same chromosome (at which single mutants induced with x-rays and thermal neutrons have been found). Previous results, which will be extended as part of this investigation, failed to detect EMS-induced suppressor mutants at either of these loci among a sample of 45 suppressors induced with this agent and selected for analysis on the basis of phenotype. Twenty-five of the most recently obtained suppressors were crossed this spring with mutant alleles representing both the su-1 and su-2 loci, as the first step in determining allelism. Analysis of these and the considerable number of new radiation-induced suppressor mutants that have not yet been crossed will yield information also relating to the unusual and not yet explained phenomenon that all of the radiation-induced suppressors that were examined in the first phase of the genetic studies were found to behave as recessives. It is anticipated that this genetic study relating primarily to specificity of action of the different classes of mutagenic agents will be carried to completion, with timing depending on the availability of funding, facilities and time available.

A large number of additional mutants apparently affecting the chloroplast carotenoids and their precursors was selected from among progenies of EMS- and x-ray-treated normal Himalaya barley during the past year. Anticipated use of these mutants, and of previously obtained mutants, is in: (a) a test of the hypothesis that the stepwise conversion of phytoene into lycopene in barley occurs by way of the intermediate compounds phytofluene, zeta-carotene, and neurosporene; and (b) the investigation of these and later mutant blocks in carotenoid biosynthesis in relation to the associated formation of protochlorophyllide, chlorophyll, and internal structural differentiation of the chloroplasts. Approximately 25 additional mutants resulting in a "paper-white" phenotype have been obtained. Three of these clearly accumulate phytoene, one
appears to accumulate phytofluene, five clearly accumulate zeta-carotene, and I appears to accumulate neurosporene. The pigment characteristics of all of these and the additional mutants will require more detailed study. The consistent ease with which single seedlings, grown in full sunlight in greenhouse sand benches, can be assayed for the accumulation of zeta-carotene is interesting in view of the susceptibility of this pigment to photodestruction found by Anderson and Robertson (1961, Proc. 3rd Int. Cong. Photobiol., p. 477) from work with a corn mutant. The barley mutant which appears to accumulate neurosporene is of particular interest, inasmuch as it seems increasingly likely that this compound is the one in which ring closure occurs and is the most likely precursor of both alpha- and beta-carotene. It is anticipated that this phase of the work will be advanced to the point of publication as rapidly as time and space permit. The other mutants affecting carotenoid biosynthesis are now of greatest interest to us in relation to their possible effects on differentiation of the internal chloroplast structure.

A phenomenon that has been of continuing interest to us is the rather high incidence of mutants blocked in the biosynthesis of chlorophyll b in progenies of normal barley treated with both ionizing radiations and alkylating chemicals. We now have 23 such mutants of independent origin. Twelve of these have now been intercrossed and found to be allelic with one another and with a mutant previously reported by Highkin (1950, Plant Physiol. 25, 294).

Considerable effort during the past year has been expended on refining the procedures for preparation of quantities of chloroplasts from normal and vx mutant barley seedlings. We now feel confident in our ability to obtain workable quantities of clean and apparently undamaged (as checked by electron microscope examination) chloroplasts from both of these genotypes, and to obtain good yields of transfer-RNAs from such chloroplasts. Mrs. Vivian Hiatt has arranged to spend the entire summer in the laboratory of Dr. M.P. Stulberg at Oak Ridge National Laboratory to become proficient in reverse-phase chromatography and related analytical techniques for the study of tRNA populations. The aim of this work is to establish the pattern of tRNA species in barley chloroplasts and to examine several vx suppressor mutants for changes in this pattern. The suppressors of immediate interest are those which behave genetically as dominants, since such behavior is expected of a nonsense suppressor.

The major effort during the last year was directed toward completion of the ultrastructural comparison of chloroplast development in normal and vx mutant barley seedlings. Lamellar development in plastids from seedlings of both of these genotypes grown at 15°C is observed to begin similarly with a few scattered membranous structures. The double membrane structure of these lamellae is clearly defined by the second day after seedling growth being initiated but their spatial orientation differs between normal and mutant. In vx chloroplasts the lamellae are waxy and irregular, and appear to be interrupted at some points along their length. In normal plastids there also appear to be some discontinuities along the length of the lamellae but the general orientation is more strongly linear. Some lamellae appear doubled for part of their length in the mutant but do not resemble paired thylakoids in grana since there is no inter-thylakoid space and the doubled regions-are
highly variable in length. The lamellae in normal plastids pair by short overlappings of ends of lamellae or the association of a short with a longer lamellar structure. These paired structures of similar in length and have the characteristic inter-thylakoid space. The course of differentiation or development differs markedly at this point between plastids of normal and the \textit{vx} mutant. By the third day of seedling growth there are numerous grana containing up to 7 thylakoids per stack in normal plastids. Rows of grana are interconnected by lamellae and these rows are more or less parallel along the length of the plastid. In the mutant plastid, prolamellar body formation is obvious by the third day and is highly developed by the fourth day. These structures are not as regularly crystalline in form as those seen in typical dark-grown plant material; however, there is an intricate lamellar network which encloses numerous osmiophilic globules and often adjoins a starch grain. Lamellar membranes not included in the prolamellar body are usually short and often open up into vesicles. By the fifth day the prolamellar body has broken down, leaving many short lamellae which are beginning to orient in parallel rows and pair as in grana formation in normal plastids. In normal \textit{chi*}oplasts, the grana system is well-developed after the fifth day. The dimensions of the thylakoids are highly regular and the parallel orientation of rows of grana connected by their inter-thylakoid lamellae is retained through the fifteenth day and undoubtedly much longer. The development of grana in the \textit{vx} mutant plastids appears to have been completed by the ninth day, but the average number of thylakoids per granum is less than in normal. The system of inter-thylakoid lamellae remains poorly developed through the fifteenth day so that grana appear randomly distributed in the \textit{vx} chloroplasts with inter-thylakoid lamellae trailing away and often having no obvious connections with other grana. Thus, the major differences in development between normal and \textit{vx} mutant chloroplasts are both in a much delayed development of the mutant plastid structure and also in the extent and orientation of the lamellar structure that eventually takes place.

When normal barley seedlings are grown at 22°C the plastids are found to have complete grana systems by the third day, a day earlier than when growth is at 15°C. Chloroplasts of the \textit{vx} mutant contain grana structures similar to those in mature normal chloroplasts by the seventh day at 22°C. The inter-thylakoid lamellae, which are distinctly abnormal in \textit{vx} chloroplasts which have developed for nine days or longer at 15°C, tend to be well formed and resemble those of normal plastids when growth of the seedlings is at the higher temperature. A prolamellar body is typically observed in \textit{vx} mutant plastids at the fourth day of development at 22°C, and such bodies are often found in chloroplasts with lamellae oriented in parallel rows typical of normal plastid at the second day of development at this temperature. The tendency of the mutant plastids to have a lamellar structure less well formed and oriented than in normal plastids is not entirely overcome by growth at the higher temperature.

Osmiophilic globules of variable size have been seen in virtually all of the chloroplast preparations examined. Von Wettstein (1958) Brookhaven Symp. Biol. 11, 138) found a correspondence between the accumulation of globuli and an unusually high content of chlorophylls and carotenoids in a barley xantha mutant. Breakdown of the globuli was observed to be accompanied by bleaching of the pigments, from which it was concluded that the globuli contain pigments
and represent a chromolipid. On the other hand, Bailey et al. (1963, Biochim. Biophys. Acta 78, 163) found plasto-quinone, a trace of chlorophyll, but no beta-carotene in isolated globuli from spinach and beet chloroplasts. Greenwood et al. (1963, Biochim. Biophys. Acta 78, 148) did a similar isolation of globuli from Vicia faba chloroplasts. These latter workers found neither beta-carotene nor chlorophyll, but rather plasto-quinone and galactolipids from which they suggest that such globules are a general deposit of insoluble lipids. In the normal plastids which we have observed, the pattern of globule accumulation is similar at 15°C and 22°C. The number increases until shortly after grana formation and then does not change perceptibly.

The globules are randomly dispersed throughout the stroma between the interthylakoid lamellae. Beginning at the ninth day at 15°C and at the third day at 22°C, globules are found occasionally in the cytoplasm bordering the chloroplasts. Chloroplasts of the vx mutant also accumulate osmiophilic globules, the increase in number being much more abrupt than in normal plastids and coinciding with the appearance of the prolamellar body. Most of the globules are found within the interstices of the prolamellar body. After the formation of grana, many of the globules are dispersed throughout the stroma while the remainder remain aggregated. Globules can be seen in the cytoplasm by the fifteenth day at 15°C and by the twelfth day at 22°C. At 15°C there are approximately 50% more globules present in mature vx mutant chloroplasts than at 22°C or in normal chloroplasts from seedlings grown at either temperature.

As might be anticipated, the several suppressors of the vx mutant that have been examined tend to shift both the timing, extent and regularity of chloroplast structure toward that in normal plastids. The combination of two suppressors in the same genotype results in only a slightly delayed formation of what is not discernibly different from normal structure.

Although a very limited amount of additional work needs to be done, it is expected that this phase of the work will continue to progress rapidly to satisfactory completion. Unfortunately, time has not permitted examination of some very interesting peripheral questions: e.g., what effect does a single gene block in the biosynthesis of chlorophyll b have on the formation of chloroplast structure.

Graduate students trained and post-doctoral tenures completed:

The following graduate students have been associated with the research program:

- Miss Louise Heine. M.S., 1967. Now Assistant Professor, Metropolitan Junior College, Minneapolis.
- Dr. Fa-ten Kao. Ph.D., 1965. Now Assistant Professor, University of Colorado Medical Center.

The following post-doctoral tenures have been completed in association with the research program:

- Dr. Victoria Bergbusch, 1964-66. Now Associate Professor, Department of Genetics, McGill University.
Dr. Mishrilal Jain, 1964-66. Now Associate Professor, Department of Biology, St. Louis University.

Dr. Neal Tuleen, 1966-68. Now Assistant Professor, Department of Soil and Crop Science, Texas A & M University.

Dr. William Bell, 1968-69.

Bibliography of publications from work done under this contract since 1964:


