THE EFFECT OF IONIZING RADIATION ON GERMINATION AND SEEDLING GROWTH OF GRAND RAPIDS LETTUCE

LYNN PETRICK YEALY

THE EFFECT OF IONIZING RADIATION ON GERMINATION AND SEEDLING GROWTH OF GRAND RAPIDS LETTUCE

An Abstract

Presented to

the Graduate Council of

Austin Peay State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

Lynn Petrick Yealy

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ABSTRACT

Ionizing radiation is responsible for inactivating many cellular processes which prevent or delay germination. Seedling growth and subsequent plant development are also retarded by exposure of plant tissue to ionizing radiation. The objectives in this study are to determine the effects of ^{60}Co gamma-irradiation on germination and hypocotyl elongation of Grand Rapids lettuce seeds; to demonstrate the interactions of gibberellic acid and 5-fluorouracil on these processes; and to estimate the effects of ionizing radiation on ribosomal ribonucleic acid (RNA) in association with 5-fluorouracil.

Germination of Grand Rapids lettuce seeds was effectively delayed by exposure to 100 kilo roentgens (kR) gamma-irradiation when incubated for 48 hours in water. Both gibberellic acid and kinetin were able to slightly overcome this delay in germination imposed by ionizing radiation. Incubation in 5-fluorouracil significantly depressed germination in irradiated seeds while it did not affect unirradiated seeds.

A dose of 100 kR ionizing radiation was effective in decreasing incorporation of ^{3}H-uridine into high molecular

weight RNA but did not decrease incorporation into transfer RNA. Incubation in the presence of 5-fluorouracil drastically reduced incorporation of labeled precursor into both RNA components, while gibberellic acid stimulated the synthesis of ribosomal and transfer RNA.

Gamma-irradiation retarded hypocotyl length of seedlings grown from irradiated seed more severely in darkness than in continuous light. Hypocotyl elongation was inhibited by 5-fluorouracil which indicated that ribosomal RNA synthesis was necessary for hypocotyl cell elongation to occur. Gibberellic acid was effective in significantly overcoming the inhibitory effects of gamma-irradiation on hypocotyl elongation. In 5-day old seedlings the synthesis of ribosomal RNA was selectively depressed by 5-fluorouracil while gibberellic acid stimulated incorporation of ^{3}H-uridine into ribosomal and transfer RNA in seedlings grown from unirradiated seed.

Similar growth retardation was imposed by ionizing radiation on 21-day old seedlings grown from irradiated seed. In this case gibberellic acid was ineffective in reversing the radiation-induced inhibition of seedling height.

The results indicated that ribosomal RNA synthesis was not necessary for germination to occur as 5-fluorouracil did not prevent germination in unirradiated seeds. However,

5-fluorouracil did delay germination after exposure to ionizing radiation indicating that ribosomal RNA synthesis may be involved in radiation damage and subsequent repair processes. From these data it appears that ionizing radiation delays germination of Grand Rapids lettuce seeds through an inhibition of ribosomal RNA synthesis.

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To the Graduate Council:

I am submitting herewith a Thesis written by Lynn Petrick Yealy entitled "The effect of Ionizing Radiation on Germination and Seedling Growth of Grand Rapids Lettuce." I recommend that it be accepted in partial fulfillment of the requirements for the degree Master of Science, with a major in Biology.

_____

Major Professor

We have read this thesis and recommend its acceptance:

_____

Second Committee Member

_____

Third Committee Member

Accepted for the Council:

_____

Dean of the Graduate School

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I am submitting herewith a Thesis written by Lynn Petrick Yealy entitled "The effect of Ionizing Radiation on Germination and Seedling Growth of Grand Rapids Lettuce." I recommend that it be accepted in partial fulfillment of the requirements for the degree Master of Science, with a major in Biology.

Benj. P. Stone

Major Professor

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CHAPTER I

INTRODUCTION

The effect of ionizing radiation on plant tissues is initiated at the submolecular level of organization. This gives rise to physiological changes which are expressed as minor morphological alterations or ultimately death. In this study an attempt is made to describe the possible sequence of molecular events preceding visual modifications.

Ionizing radiation has been shown to decrease the germination response of seeds from several plant families, the depression being correlated with dose. According to Haber and Luippold (1959) gamma-irradiation induced a physiological state of dormancy in New York variety lettuce seeds. Germination of Grand Rapids lettuce seeds was delayed with increasing doses of ionizing radiation (Stone, 1968). The application of gibberellic acid or kinetin to the incubation medium was effective in overcoming the delaying action of ionizing radiation on the germination response of Grand Rapids lettuce seeds.

It has been indicated that chromosomes may be the primary site of damage in seeds exposed to ionizing radiation (Sparrow et al., 1961). This was apparent at the metabolic

level before it could affect morphology or growth. Due to chromosome aberrations and nuclear damage, growth occurring in irradiated tissue was primarily expressed as cell elongation (Haber and Luippold, 1960). Seedling height was reduced by ionizing radiation and was indicative of the degree of damage suffered by the tissue as a result of irradiation exposure. Other cellular processes inhibited by high energy radiation include respiration, nucleic acid and protein syntheses (Casarett, 1968).

The objectives in this study are: 1) to determine the effects of ^{60}Co gamma-rays on germination and hypocotyl elongation of Grand Rapids lettuce seeds; 2) to investigate the effects of plant hormones and 5-fluorouracil on germination and hypocotyl elongation of seeds exposed to gamma-irradiation; 3) to study RNA synthesis in lettuce seeds and seedlings after 5-fluorouracil and gibberellic acid treatment; 4) to observe the morphological variations and influence of gibberellic acid on lettuce seedlings grown from seeds exposed to ionizing radiation.

CHAPTER II

REVIEW OF LITERATURE

The long term effects of ionizing radiation on plant tissue resulted in severe morphological deformity, growth inhibition, or death (Sparrow et al., 1961). According to Sparrow and Woodwell (1962) the principle site of damage to plants by ionizing radiation was the cell nucleus. These authors stated that measurement of size or weight could be used as the criterion of sensitivity to chronic gamma-irradiation. Variations in sensitivities between different species of higher plants approached 500-fold; these variations were correlated with chromosome number and nuclear volume. Evidence was provided indicating that any environmental factor which influenced the rate of growth by influencing the rate of cell division would affect radiosensitivity.

Ionizing radiation altered the sequence of bases of the DNA molecule which resulted in a series of chromosomal aberrations (Casarett, 1968). Pfahler (1970) has stated that chromosome structural changes, which led to bridges at division and genetic imbalance after mitosis, were the main causes of lethal effects of irradiation. This author suggested that low irradiation doses produced chromosomal

aberrations while high doses were required to inactivate enzymes and damage membranes.

As a result of ionizations induced by radiation exposure, biological molecules underwent a variety of structural changed which resulted in altered function. Radiation-induced chemical changes in purines and pyrimidines led to deamination, ring opening, and the formation of organic hydroperoxides (Weiss, 1964). This author also reported that liberation of inorganic phosphates and breaking of glycosyl linkages occurred in nucleotides exposed to ionizing radiation. This consequently resulted in degradation of the sugar-phosthate backbone and therefore a nonfunctional nucleic acid.

Mergen and Cummings (1965) have reported that the response to irradiation depends on type and rate of exposure, metabolic conditions during irradiation, and environmental conditions prior to, during and after irradiation. Oxygen was considered by Conger et al. (1966) to be a major agent in the initiation of much damage induced in seeds exposed to ionizing radiation. This author concluded that water was an important secondary factor which regulated the degree which oxygen participated with radiation in the induction of biological effects. An inverse relationship between water content (up to 15%) and radiosensitivity was

indicated by Caldecott (1954). Osborne et al. (1963) discussed the mechanism of this phenomenon by proposing that the presence of water in the medium during irradiation and storage initiated radical-water and radical-radical interactions. Thus, less radicals were available to attack biologically important molecules. Above 15 percent hydration tissue was again radiosensitive. These data indicate that a maximum stage of radioresistance regarding optimum humidity exists for each plant system. Conger et al. (1966) have demonstrated that damage to biological systems as a result of gamma-rays was most evident followed by dry storage at room temperature. This was attributed to the persistence of free radicals in the medium. Mergen and Cummings (1965) have stated that the presence of water, radical scavengers of hydrogen donors, the absence of oxygen and storage at low temperatures diminished or prevented the postirradiation effect.

Looper and Aboul-ela (1971) in studies of Fenugreek beans have demonstrated that increasing temperatures and light, speed recovery after exposure to gamma-rays due to increased metabolism and photosynthesis in damaged tissue. Haber and Luippold (1959) found that 250 kR gamma-irradiation administered to dry lettuce seeds prevented about half of the population of originally viable seeds from

germinating. From this study it was conceiveable that gamma-irradiation induced or maintained a physiological state of dormancy in lettuce seeds. Mitosis was also prevented in lettuce seeds by gamma-irradiation (1.3 Mrad ^{60}Co) and consequently germination did not occur after 5 days incubation in water (Haber et al., 1969).

Exposure of peanut seeds to 250 to 500 X-rays reduced germination by 30 to 50 percent (Van Huystee and Cherry, 1967). Only those seedlings grown from seeds irradiated with less than 50 kR were able to grow into plants. These authors concluded that irradiation prevented cell division in meristematic tissues; therefore, germination of these seeds was due to cell elongation.

In studies of gamma-irradiated wheat germination proceeded without detectable mitotic figures (Haber and Foard, 1964). These nuclei did not synthesize deoxyribonucleic acid (DNA); however, they were capable of synthesizing RNA. Therefore, the irradiation that prevented DNA synthesis and mitosis had not completely inactivated all nuclear functions. This study demonstrated that gamma-irradiation prevented initiation of immature vessels at the apical meristem, but did not prevent maturation of vessels detectable prior to irradiation. These gamma plantlets were growing only by processes of cell elongation.

Mergen and Cummings (1965) in studies of Pinus rigida have shown that germination was decreased by low doses of gamma-irradiation with a slower recovery period for higher levels of radiation. These studies demonstrated that stratification under cold moist conditions increased total germination, but did not affect the rate of seedling development. When seeds were stored under these conditions for 3 weeks, an increase in germination occurred at all dose levels.

Mujeeb and Greig (1972) reported that the growth phenomenon of Phaseolus vulgaris seedlings after exposure to 7.5 kR ^{60}Co gamma-rays decreased with ascending dosage. Total chlorophyll content and leaf spotting were observed to progress with increasing dose of gamma-irradiation. In Summer Bibb lettuce (exposed 26 days postsowing) more damage was suffered by higher experimental levels of ionizing radiation or low levels of long duration (Bottino and Sparrow, 1971).

After 5.2 kR ionizing radiation the meristem of tobacco seedlings became completely inactive with few linear bud primordia apparent (Venketeswaren and Partanen, 1966). Lower doses of ionizing radiation produced initial morphological effects of stunting leaf growth and injury to cells of the apical meristem. However, after 80 days in culture the early morphological effects disappeared and a stimulation of growth of seedlings was evident at low doses.

Venketeswaren and Partanen (1966) indicated a highly significant difference in radiosensitivity between callus tissue of higher plants and organized plants in the form of young seedlings. These authors stated that the apical meristem of the organized plant was of great morphological significance, of high mitotic activity and also dependent on the total organism for nurture and functioning. Therefore, it was highly radiosensitive as compared to the cell suspension where there was little interdependence. In studies of seeds, seedlings and callus tissue of Phaseolus vulgaris Bajaj et al. (1970) also reported a great difference in radiosensitivity when each tissue was exposed to ^{60}Co gamma-rays. Callus tissue was more radioresistant, perhaps due to its undifferentiated state and structural simplicity. Germinated seeds of barley were shown to be 30 times more sensitive to ^{60}Co gamma-rays than dormant seeds (Conger et al., 1973).

Gibberellic acid was reported to promote germination of New York lettuce seeds exposed to gamma-irradiation (Haber et al., 1969). This germination process was described as involving cell expansion only of those cells lacking DNA synthesis, which implied that nuclear DNA synthesis was not necessary for the action of gibberellic acid on lettuce seed germination. These results are consistent with data reported

by Rose and Adamson (1969) who have shown that gamma-irradiated wheat (500 kR) produced coleoptiles growing without cell division. Elongation was promoted by gibberellic acid and kinetin in a manner similar to normal coleoptiles. In lentil epicotyls, however, evidence was provided that continuous DNA synthesis was essential for cell elongation (Nitsan and Lang, 1966). The data obtained suggested that exogenous gibberellic acid enhanced elongation and DNA synthesis which, in turn, stimulated ribosomal RNA synthesis. According to Glasziou (1969) DNA synthesis is essential for elongation of certain plant cells and their elongation response to gibberellic acid.

Haber and Luippold (1960) have demonstrated that gibberellic acid did not have restorative effects on the irradiation-induced damage to lettuce seeds. Gibberellic acid was ineffective in reversing the ionizing radiation effects on oxygen consumption and chromosome aberrations. The authors have proposed that gibberellic acid may increase cell elongation by stimulating RNA synthesis.

Gamma-irradiation significantly inhibited incorporation of ^{3}H-leucine and ^{3}H-uridine into protein and nucleic acids in washed sugar beet root tissue (Dunham et al., 1971). In this study ionizing radiation was also demonstrated to decrease RNA polymerase activity and inhibit DNA template

availibility. When RNA polymerase was irradiated in vitro, transcriptional error was increased when an undamaged template was employed.

Ananthaswamy et al. (1971) have shown that seedling growth of gamma-irradiated wheat was impaired by ^{60}Co gamma-rays at 20 to 200 kR dose levels. Presoaking of the control and irradiated grains in gibberellic acid for 16 hours resulted in stimulation of seedling growth at lower dose levels. These authors also indicated that gibberellic acid stimulated amylase and ribonuclease syntheses in control seeds and reversed the adverse effects of irradiation at low doses. The effect was less pronounced at higher doses. The authors postulated that gamma-irradiation might impair a possible endogenous gibberellin-synthesizing system in wheat, and that this could be counterbalanced by exogenous gibberellic acid in seeds irradiated at low doses. Gorden (1957) however has suggested that auxin synthesis was one of the main possible loci of biochemical damage in the inhibition of growth by ionizing radiation.

Chang and Mericle (1964) have demonstrated that the total nucleic acid content of irradiated barley embryos was depressed by 30 to 36 percent mostly due to the effect upon RNA. The inhibition of DNA synthesis was inversely correlated with embryonic stage irradiated with younger embryos

receiving greater damage. However, depression of RNA content was not affected by embryonic stage or postirradiation time.

In studies of nucleic acid and protein syntheses of irradiated barley seedlings, Ledoux et al. (1962) concluded that X-irradiation inhibited an increase in RNA, DNA, protein and soluble nitrogen content. Ribonuclease activity, however, seemed to be unaffected even at higher dose levels. Quite different results regarding irradiation effects on nucleic acid were reported by Tano (1971). In dry barley seeds exposed to 20 kR gamma-rays, the synthesis of DNA and RNA was compared to that of nonirradiated seeds. No distinct differences were observed in RNA preparations from irradiated and unirradiated seeds as determined by polyacrylamide gel electrophoresis patterns. However, the label was included in the medium for only 1 hour which may not have been long enough to result in any differences.

Stimulation of plant tissues by lower doses of ionizing radiation has been reported to occur. According to Cherry (1962) X-irradiation (250 kR) greatly enhanced the incorporation of uracil into RNA in excised corn root tissue. In addition growth of root tips from irradiated seed had an initial ribonuclease activity 2- to 4-fold greater than normal tissue. Van Huystee and Cherry (1967) have shown that nucleic acid synthesis in cotyledons of peanut seeds

was stimulated by X-rays if seeds were germinated immediately after exposure. However, storage for 2 to 4 weeks reduced X-ray enhanced nucleic acid synthesis; therefore, all doses inhibited nucleic acid synthesis after 4 weeks.

Van Huystee et al. (1968) have demonstrated that exposure of peanut seeds to 500 kR X-rays followed by weeks of storage before planting, resulted in a severe reduction of ^{14}C-amino acid incorporation into protein by the ribosomal material. These authors stated that polyribosomes were nearly absent from ribosomal material obtained from irradiated tissue and synthesis of RNA and protein was inhibited. Since monoribosomes and transfer RNA from irradiated tissue supported a normal level of protein synthesis directed by exogenous messenger RNA, it was concluded that X-irradiation of seeds inhibited the synthesis of functional messenger RNA.

DNA biosynthesis as well as RNA synthesis was inhibited by 5-fluorouracil without any major inhibition of total protein synthesis in bacteria (Heidelberger, 1965). Paranjothy and Raghavan (1970) proposed that 5-fluorouracil inhibited DNA synthesis through interference of thymidylate synthetase activity. This is in agreement with data reported by Cherry and Van Huystee (1965). Aronson (1961) reported that 5-fluorouracil prevented the synthesis of 70s

ribosomal RNA particles in magnesium starved bacteria, Echerichia coli.

Elongation of intact soybean roots was not inhibited by 5-fluorouracil; however, ribosomal and transfer RNA syntheses were inhibited (Lin and Key, 1968). Protein synthesis was not affected and it appeared that ribosomal and transfer RNA syntheses were not essential for cell elongation to occur. This is consistent with data reported by Nooden (1968) in the artichoke tuber, where 5-fluorouracil suppressed ribosomal and transfer RNA syntheses. It was further stated that incorporation of 5-fluorouracil into RNA did not necessarily reduce its ability to function as a template for protein synthesis. In studies of the effects of 5-fluorouracil on RNA synthesis in buds of Xanthium, Cherry and Van Huystee (1965) concluded that it was unlikely that cell activity was influenced by production of nonfunctional messenger RNA since normal protein was coded for properly. In this study 5-fluorouracil enhanced messenger RNA synthesis while ribosomal RNA synthesis was inhibited.

In studies of lettuce seeds, variety Attractie, 5-fluorouracil inhibited germination of intact seeds (Smith and Frankland, 1966). However, germination was not inhibited by 5-fluorouracil in Grand Rapids lettuce seeds (Khan, 1966). Since nucleic acid precursors were unable to reverse the

depression of lettuce seedling growth imposed by 5-fluorouracil, Khan (1966) reasoned that other processes were being inhibited. Gibberellic acid-induced growth of hypocotyls was markedly inhibited by uracil analogs in light (Khan, 1966) and in darkness (Khan, 1967). This indicated the possibility that 5-fluorouracil was depressing a process that gibberellic acid was stimulating in its enhancement of cell elongation.

In soybean hypocotyls Key (1966) has demonstrated that 5-fluorouracil selectively inhibited ribosomal and transfer RNA syntheses without affecting the synthesis of D-RNA. Ribosomal RNA synthesis was almost completely inhibited by 5-fluorouracil within 1 hour after inclusion in the incubation medium. The manner in which ribosomal RNA synthesis was depressed could be blockage of ribosomal RNA precursor or synthesized ribosomal RNA could be degraded because of lack of ribosomal protein synthesis. Key (1966) presented evidence favoring the former condition. The author concluded that ribosomal RNA synthesis, per se, was inhibited because 5-fluorouracil markedly inhibited the incorporation of ^{32}P into ribosomal RNA precursors.

CHAPTER II

METHODS AND MATERIALS

Seed Germination

Seeds of Latuca sativa variety 'Grand Rapids' were purchased from Joseph Harris Seed Company, Rochester, New York, and used throughout the study. The seeds were exposed to a ^{60}Co source delivering an intensity of 2.9 kR per minute at doses from 100 to 800 kR at Oak Ridge National Laboratory, Oak Ridge, Tennessee. After irradiation seeds were stored at 0° to minimize postirradiation effects.

To determine the germination response of irradiated seeds, 4 milliliters (ml) of a hormone or inhibitor solution were added to 9-centimeter (cm) petri dishes lined with filter paper. Seeds of each irradiation dose were placed in dishes that contained either gibberellic acid (2.8×10^{-4} molar), kinetin (10 ppm), gibberellic acid and kinetin, 5-fluorouracil (250 µgrams/ml), 5-fluorouracil and gibberellic acid, or distilled water. A 10^{-4} molar (M) concentration of penicillin and streptomycin was used to inhibit microbial growth. Duplicates of approximately 100 seeds were used per treatment. All incubations were carried out in a Percival 135L incubator at $22^{\circ} \pm 2^{\circ}$ under cool white fluorescent

lamps. Seeds were allowed to germinate for 24, 48 and 72 hours in 16 hours white light and 8 hours darkness. Protrudence of the radicle was used as the criterion for germination.

For statistical evaluations of germination, percent data was converted by the arc sine transformation (Sokal and Rohlf, 1969), and Duncan's new multiple range test (Steele and Torrie, 1960) was employed in determining significance. The chemicals used were obtained from the following sources: gibberellic acid (GA_3) from Eastman Organic Chemicals, Rochester, New York; 5-fluorouracil from Hoffman-La Roche Incorporated, Nutley, New Jersey.

Hypocotyl Elongation

Hypocotyl Elongation in seedlings was studied by selecting uniformly germinated seeds previously exposed to ionizing radiation. This population of germinated seeds was obtained by first germinating seeds in distilled water. Seeds receiving a greater dose of ionizing radiation required a longer incubation period in water to germinate. The following incubation schedule was necessary to achieve maximal germination for each dose: 800 and 600 kR, 4 days; 400 and 300 kR, 3 days; 200 kR, 2 days; 100 and 0 kR, 1 day. Twenty uniformly germinated seeds of each irradiation dose

were transferred to petri dishes containing experimental solutions as indicated in the results. All experiments were run in duplicate and all solutions contained a 10^{-4} M concentration of penicillin and streptomycin. Germinated seeds were allowed to grow for 5 days at 22° in continuous light or darkness. Hypocotyl length was measured to the nearest millimeter (mm).

Morphological Effects

Seeds of each irradiation dose were germinated according to the previously described schedule for hypocotyl elongation. Forty germinated seeds of each dose (100 to 400 kR) were planted in water-soaked peat cubes purchased from Fertl Incorporated, South Norwalk, Connecticut. Incubation was at 22° on a 16-8 daylight-darkness schedule with all seedlings treated equally regarding regular waterings by subirrigation

After 10 days growth six seedlings from each dose were removed and the length of roots, hypocotyls and primary leaves were measured. Fresh and dry weight were also determined for each irradiation dose.

The remaining seedlings were separated into two groups per dose (17 each). Spraying with either gibberellic acid or distilled water was initiated and continued for 14 days being applied every 2 days. When seedlings were 21 days

old the experiment was terminated and all seedlings evaluations were recorded as indicated in the results.

Incorporation of Labeled Precursor into Lettuce RNA

Uridine-5-H-3 (^{3}H-uridine) specific activity 21.7 curies per millimole was obtained from New England Nuclear Corporation, Boston, Massachusetts. For incorporation of ^{3}H-uridine into RNA, 1 gram of lettuce seed previously exposed to 100 kR ionizing radiation was incubated in a filter-sterilized solution of gibberellic acid, 5-fluorouracil, gibberellic acid and 5-fluorouracil, or water. One gram of unirradiated seed incubated in water was used as a control. A 10^{-4} M concentration of penicillin and streptomycin was applied to each dish. The seeds were incubated for 48 hours at 24° in continuous white light. Ten microcuries (μc) per dish of ^{3}H-uridine was added to the seeds the last 12 hours of the 48-hour incubation period.

The extraction method employed for lettuce seed RNA was modified from Summers (1970) as follows. After 48-hour incubation, the seeds were placed in a Gooch crucible attached to a suction flask and rinsed with distilled water to remove any precursor adhering to the seed coat. The seeds were homogenized on ice in a VirTis homogenizer with 25 ml of Tris homogenizing buffer (0.01 M Tris, pH 7.6, 0.06 M KCl,

0.01 M $MgCl_2$), 4.5 ml of 11 percent Duponol (sodium lauryl sulfate) and 0.9 ml diethyl pyrocarbonate. Homogenization was carried out for a duration of 1 minute high, 30 seconds medium and 30 seconds high speed followed by rinsing the homogenizing flask with 2 ml of 0.01 M Tris buffer.

Soluble label precursor pool within the tissue was estimated by precipitating 1 ml of the homogenate with 10 percent cold trichloroacetic acid (TCA) overnight. The amount of RNA in the supernatent represented an estimation of soluble precursor pools. After centrifugation at 10,000 X g for 10 minutes, a 50 µliter aliquot was removed, placed on filter paper and dried under an infrared lamp. The filter was transferred to a liquid scintillation vial containing 10 to 15 ml of scintillation fluid (4 grams of 2,5-diphenyl-oxazole and 50 milligrams of 1,4-bis-2-(5-phenyloxazole) benzene per liter of toluene) and counted in a Nuclear Chicago Unilux II liquid scintillation counter for 10 minutes.

The remaining homogenate was centrifuged at 10,000 X g for 10 minutes, then the supernatent was removed and incubated at 37° for 5 minutes and chilled. A half volume of saturated NaCl was then added which precipitated the protein. The solution was recentrifuged and the nucleic acid supernatent removed. The RNA was precipitated with three volumes

of cold 95 percent ethanol overnight at 5 degrees. The RNA precipitate was collected by centrifugation at 15,000 X g for 15 minutes and dissolved in 1 ml of 3E buffer (0.12 M Tris, 0.06 M sodium acetate, 0.003 M sodium ethylene diamine tetraacetic acid) at a dilution of 1:10. The dissolved RNA was stored at 0° until use.

In RNA extraction from lettuce seedlings, 1 gram of unirradiated seeds was germinated in water for 24 hours at described conditions. The water was poured off and the filter-sterilized experimental solutions added. Tritiated uridine was applied the last 14 hours of a 48-hour incubation period. The RNA extraction method employed with lettuce seedlings differed from the method employed with lettuce seeds. The extraction method of RNA used in lettuce seedlings was a modification of the following procedure as described by Cherry et al. (1965).

In extraction of RNA from seedlings the homogenizing solution consisted of 30 ml Tris homogenizing buffer, 1.5 ml bentonite, 4.5 ml Duponol, 0.3 ml diethyl pyrocarbonate and 15 ml Tris saturated phenol. The seedlings were ground in a VirTis homogenizer at 1 minute low, 1 minute medium and 45 seconds high speed. After rinsing the homogenizing flask with 1 ml 0.01 M Tris buffer, 0.5 ml of the homogenate was removed and precipitated with 5 percent cold TCA for

soluble precursor pool determination. The remaining homogenate was filtered through Miracloth (Chicopee Mills Incorporated, New York) at 0° and the cellular debris discarded.

The aqueous layer was removed after centrifugation at 10,000 X g for 10 minutes followed by addition of 0.5 ml bentonite, 1 ml Duponol, and one half volume (15 ml) phenol. The solution was stirred on ice for 15 minutes and centrifuged. The phenol extraction was repeated without the addition of bentonite and Duponol. After the final centrifugation at 10,000 X g for 10 minutes, the aqueous layer was removed and made 0.2 M with potassium acetate. Three volumes of cold 95 percent ethanol were added to precipitate the RNA overnight at 5 degrees. The nucleic acid precipitate was collected after centrifugation at 15,000 X g for 15 minutes and dissolved in 1 ml of E buffer (3E diluted 1:3).

A 25 μliter aliquot of the dissolved RNA was diluted to 1.5 ml with 3E or E buffer. The absorbancy was read at 260 and 280 nanometers (nm) in a Gilford Spectrophotometer. To determine the purity of RNA a ratio of the absorbance 280/260 was used.

The dissolved RNA extract from lettuce seedlings was dialyzed using 45 mm Spectrapor dialysis membrane with a molecular weight range of 12,000 to 14,000. The dialysis

tubing was placed in a 0.01 M Tris buffer solution which was changed three times during the approximate 24-hour period. The RNA was precipitated with ethanol as previously described and stored at 0 degrees.

Lettuce seedling and lettuce seed RNA were fractionated on a Sephadex G-100 column (900 X 15 mm) previously washed with 0.05 M ammonium acetate, pH 5.1. The total extracted RNA from lettuce seeds or one half of the RNA extracted from lettuce seedlings was separated by Sephadex gel filtration. All RNA samples were applied to the surface of the Sephadex gel. After the RNA sample had been absorbed into the gel bed, the column walls were washed with 2 ml of the elutant and a reservoir of 0.05 M ammonium acetate, pH 5.1 was connected to the column. Thirty 3-ml fractions were collected at a flow rate of 3 ml per 8 minutes or 18 minutes for lettuce seeds and seedlings respectively. The first 15 ml of the effluent was discarded. Lettuce seedling RNA was fractionated at 5° and lettuce seed RNA was fractionated at room temperature.

The absorbancy of each effluent fraction was read at 260 nm on the spectrophotometer. To estimate purity the 280/260 ratio for peak fractions from the G-100 Sephadex effluent profile was calculated. The fractions were precipitated for 1 hour at 5° after addition of two drops of

carrier RNA and 10 ml of cold 5 percent TCA. The RNA precipitate was collected on Reeve Angel glass fiber filter papers (GF/A) then dried for 30 minutes. To determine the radioactivity, each dried filter paper was placed in a scintillation counter programmed for counting single labeled tritium samples.

CHAPTER IV

RESULTS

Effects of Ionizing Radiation, Plant Growth Substances and an Antimetabolite on Lettuce Seed Germination

According to Haber and Luippold (1959) varying exposures of gamma-irradiation produced a delay in germination of New York variety lettuce seeds. A dose of 250 kR ^{60}Co gamma-irradiation prevented half the population of originally viable seeds from germinating when incubated in water. Haber (1959) concluded that ionizing radiation was effective in inducing or maintaining a physiological state of dormancy in lettuce seeds. The germination response of lettuce seeds exposed to ionizing radiation was stimulated by application of gibberellic acid. It has been known for some time that gibberellic acid is effective in overcoming other inhibitory mechanisms such as darkness, supra-optimum temperatures, dark osmotic inhibition and far red light on germination of Grand Rapids lettuce seeds (Khan, 1960).

An experiment was conducted to determine the interactions between the inhibitory effects of ionizing radiation on germination and the growth substances, gibberellic acid and kinetin. Figure 1 illustrates the results of lettuce

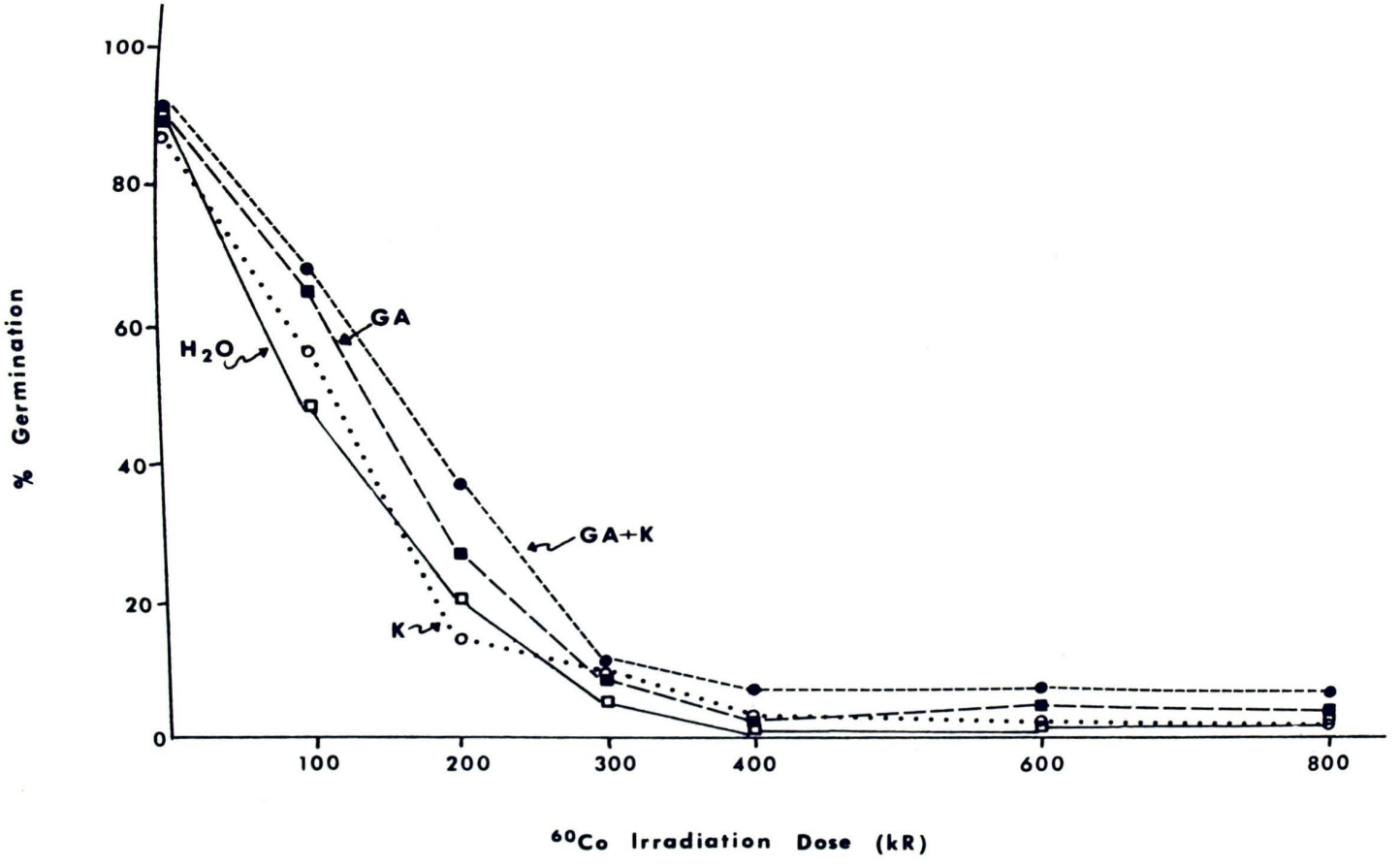

Figure 1. Effect of gamma-irradiation on Grand Rapids lettuce seed germination after 48 hours incubation in plant growth substances.

Seeds were germinated at 12 days postirradiation. Gibberellic acid (GA), 2.8×10^{-4} M; Kinetin (K), 250 µgm/ml.

seed germination as a function of ^{60}Co irradiation dose. After 48 hours incubation on a 16-hour photoperiod, the germination of Grand Rapids lettuce seeds in water decreased with increasing doses of gamma-irradiation. Exposure of seeds to 400 kR represented a threshold dose for maximum inhibition of 48-hour germination. Both gibberellic acid and kinetin alleviated slightly the delaying action imposed by ionizing radiation on the germination response. When applied together, germination was stimulated to a greater degree than when either hormone was applied separately. A synergistic effect resulted when both growth substances were present in the incubation medium.

The same germination pattern was observed after 72-hour germination (Figure 2). Seeds exposed to all dose levels exhibited increased germination with increased incubation time (Figure 3). This indicates that ionizing radiation imposes a delay on the germination response and suggests the ability of lettuce seeds to recover from damage by ionizing radiation. However, exposure of seeds to 600 and 800 kR was still effective in inhibiting germination even in the presence of hormones after 5 days incubation. Stone (1968) reported that complete germination occurred in all seeds (100 to 800 kR) after 7 days.

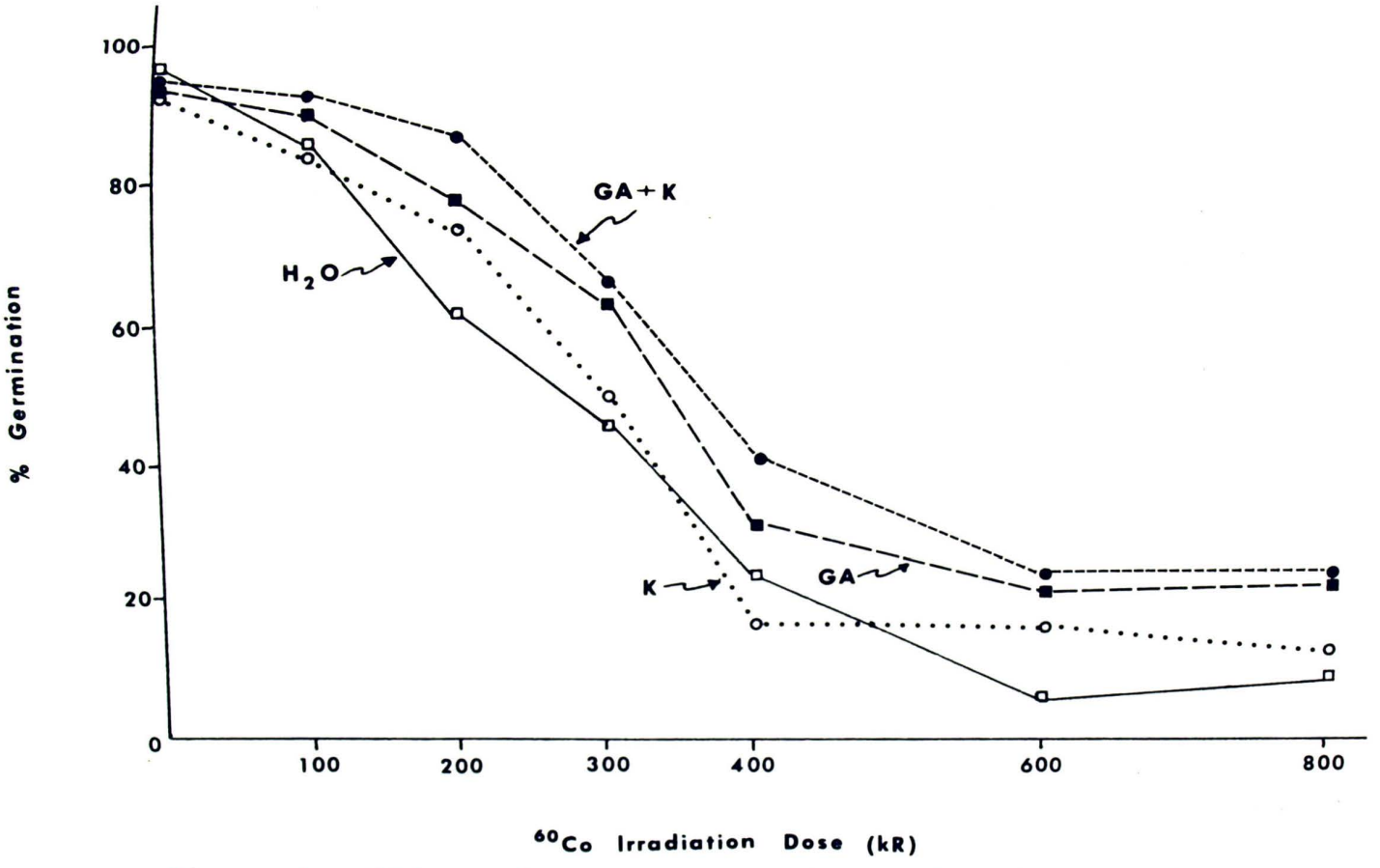

Figure 2. Effect of gamma-irradiation on Grand Rapids lettuce seed germination after 72 hours incubation in plant growth substances.

Seeds were germinated at 12 days postirradiation. GA, 2.8×10^{-4} M; K, 250 µgm/ml.

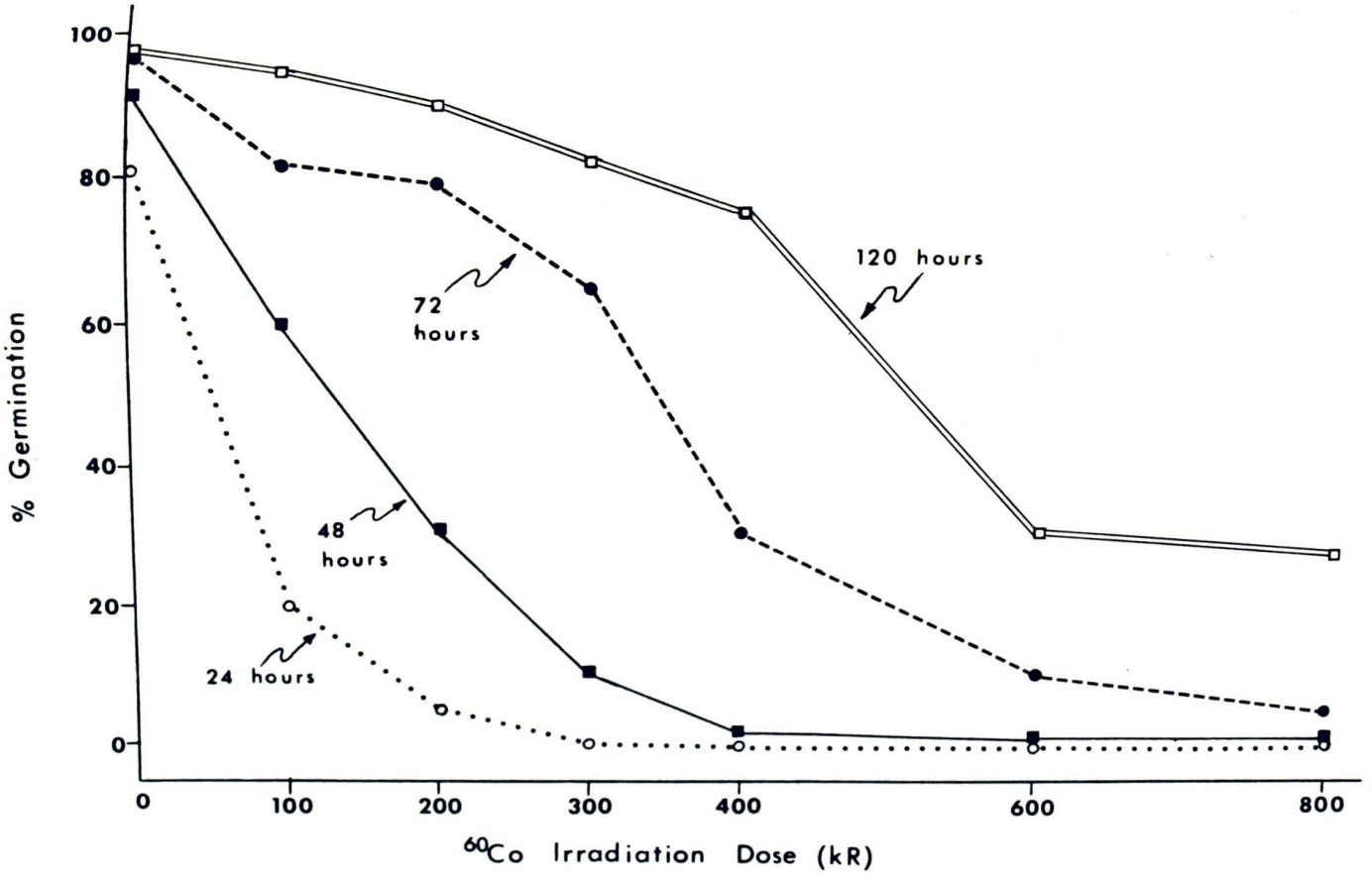

Figure 3. Effect of gamma-irradiation on Grand Rapids lettuce seed germination.

All seeds were germinated in distilled water.

The antimetabolite, 5-fluorouracil, was reported to be an effective inhibitor of DNA and RNA syntheses in bacterial and plant systems (Heidelberger, 1965). In studies of Grand Rapids lettuce seeds, 5-fluorouracil did not inhibit germination after 24 hours incubation in light (Khan, 1966) nor did it reverse the stimulation of gibberellic acid on lettuce seeds incubated in darkness (Khan, 1967). Smith and Frankland (1966) however reported complete inhibition of lettuce seed germination (variety Attractie) after 20 hours incubation in 5-fluorouracil.

An experiment was conducted to observe the effects of 5-fluorouracil on the germination response of Grand Rapids lettuce seeds in conjunction with ionizing radiation. After 48 hours incubation in the solutions indicated in Figure 4, germination percentages were determined for each irradiation dose level. The results indicated that 5-fluorouracil inhibited lettuce seed germination at all irradiation doses while it did not have an effect on unirradiated seeds. The lack of response of unirradiated seeds to 5-fluorouracil is consistent with data reported by Khan (1966). Using Duncan's new multiple range test (DMR) as described by Steele and Torrie (1960), the difference between the means of unirradiated seeds was not found to be significant at the 0.05 protection level. This level was employed in all

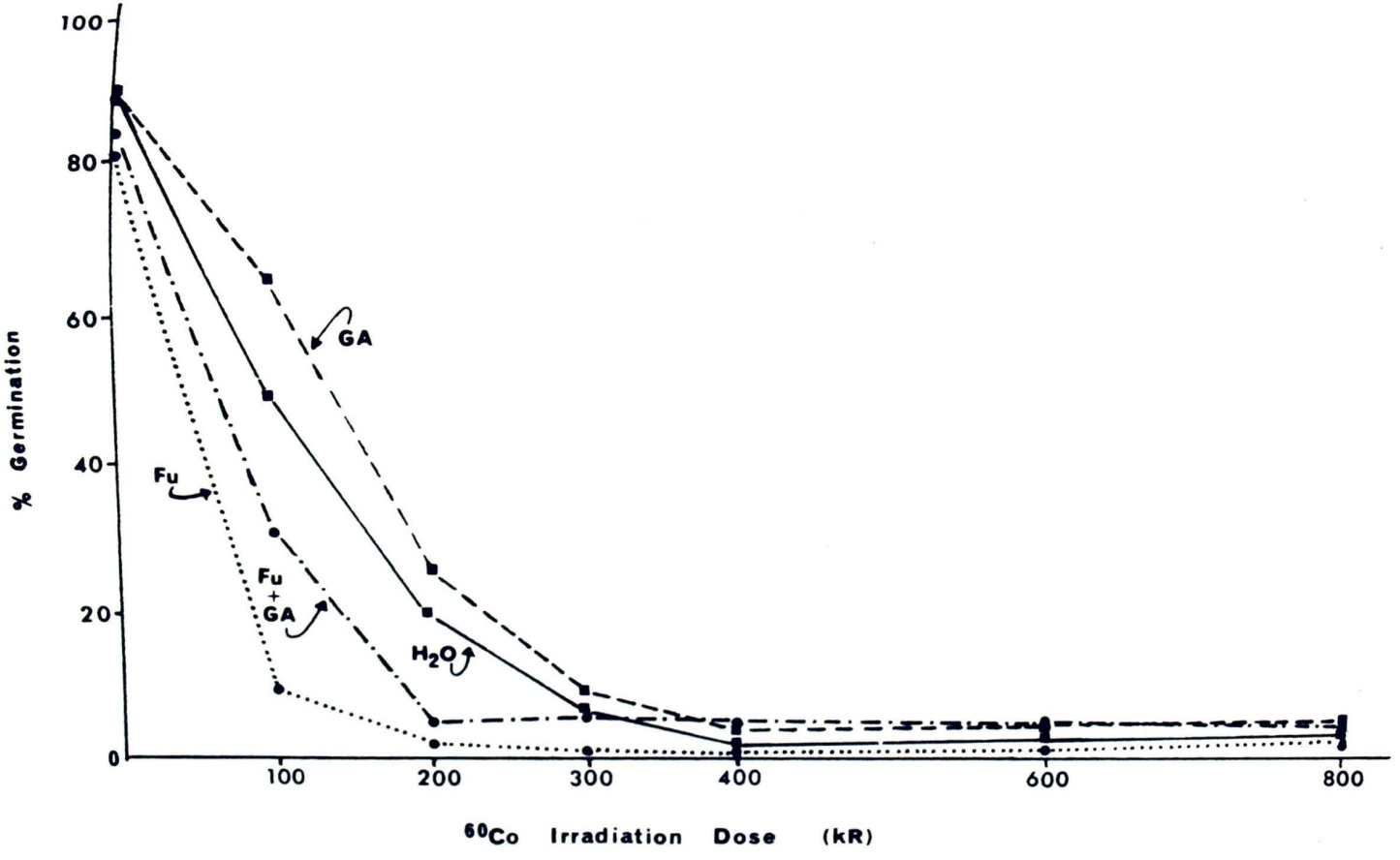

Figure 4. Effect of gamma-irradiation on Grand Rapids lettuce seed germination after 48 hours incubation in 5-fluorouracil or gibberellic acid.

Seeds were germinated at 12 days postirradiation. Fu, 5-fluorouracil.

statistical analyses. However, 5-fluorouracil did significantly inhibit germination at doses of 100 to 300 kR. Seeds exposed to 100 kR ionizing radiation and incubated in 5-fluorouracil resulted in only 10 percent germination, while those incubated in water and gibberellic acid resulted in 50 and 62 percent germination respectively. Gibberellic acid was effective in stimulating the germination response of lettuce seeds previously exposed to gamma-irradiation. The gibberellic acid stimulatory effect upon germination in seeds exposed to ionizing radiation at all dose levels was effectively reversed by 5-fluorouracil.

After a germination period of 72 hours the same germination response was observed for all dose levels, with 5-fluorouracil displaying a significant inhibition of germination (Figure 5).

This inhibitory effect of 5-fluorouracil, in conjunction with ionizing radiation, was evident in seeds that had germinated for a time period of 5 days. Seeds exposed to 300 kR and above and germinated in the presence of 5-fluorouracil germinated very poorly (less than 20 percent). However, those seeds germinating in the presence of gibberellic acid, gibberellic acid and 5-fluorouracil, or water exhibited a maximal germination response (greater than 80 percent). This indicates an inability of lettuce seeds

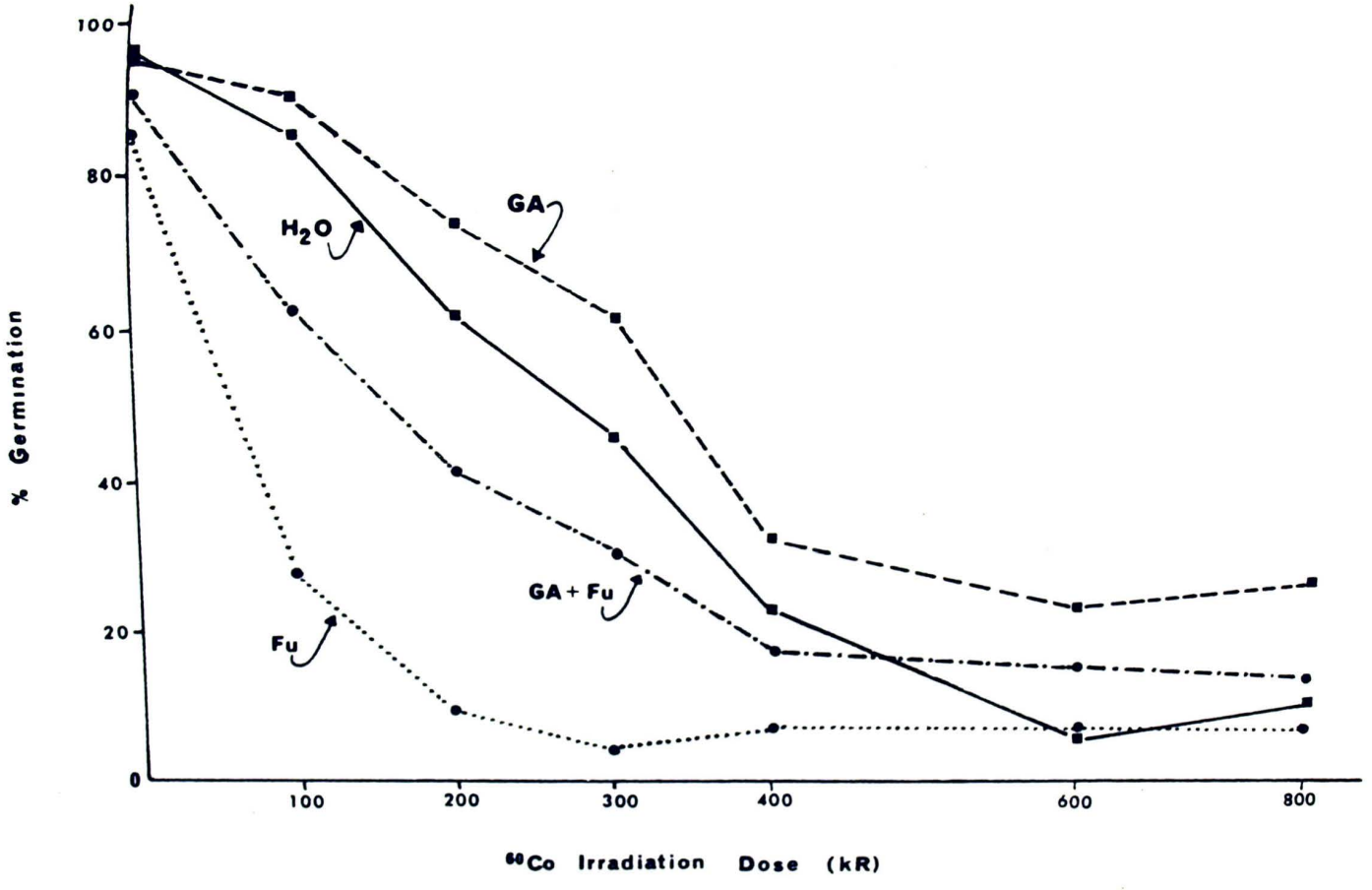

Figure 5. Effect of gamma-irradiation on Grand Rapids lettuce seed germination after 72 hours incubation in 5-fluorouracil or gibberellic acid.
Seeds were germinated at 12 days postirradiation.

to recover from 5-fluorouracil inhibition in association with ionizing radiation.

Germination studies of Grand Rapids lettuce seeds after exposure to ionizing radiation were conducted with incubation in continuous light by Stone (1968). In the present study germinating seeds were exposed to only 16 hours white fluorescent light. All other incubation conditions were identical. Table I illustrates the results of irradiated seeds germinated under continuous light or 16 hours white light and 8 hours darkness. Unirradiated seeds were not affected by variations in lighting time; however, seeds receiving 100 kR ionizing radiation resulted in decreased germination under the shorter photoperiod for all treatments. The same pattern of germination was still present with 5-fluorouracil resulting in the lowest germination percentages.

According to Conger et al. (1966) the environmental conditions during exposure and subsequent germination alter the effectiveness of a given dose of ionizing radiation. Lettuce seeds in this study were irradiated dry and stored at 0° in normal atmospheric conditions. The water content of seeds was not evaluated but since seeds were air dry medium degrees of moisture content were assumed. Storage of seeds might result in a decrease in damage provided the

TABLE I

GERMINATION RESPONSE OF IRRADIATED LETTUCE SEEDS INCUBATED IN CONTINUOUS OR 16 HOURS WHITE LIGHT

Treatment	Incubation Time and Lighting Duration					
	24 Hours		48 Hours		72 Hours	
	Cont	16 Hours	Cont	16 Hours	Cont	16 Hours
GA	75	9	96	58	98	80
Fu	30	3	55	22	63	33
GA + Fu	38	9	79	41	94	56
H_2O	72	10	96	64	97	83
H_2O - 0 kR	93	89	97	96	98	98

Germination was measured after exposure of seeds to 100 kR ionizing radiation. Seeds receiving continuous light were in the 30th day of postirradiation and seeds on 16 hours light were germinated at 34 days postirradiation. Approximately 100 seeds were contained per dish with each treatment run in duplicate. GA, 2.8×10^{-4} M; Fu, 250 µgm/ml.

water content was maintained at 8 to 11 percent under anerobic conditions (Casarett, 1968). This was attributed to recombination of free radicals and a decreased opportunity for the formation of biologically damaging peroxyl radicals.

Only slight postirradiation effects were observed for lettuce seeds germinated at 5, 12, 26 and 30 days after exposure. Germination for each treatment after storage decreased predominately in seeds exposed to doses greater than 300 kR. However, in a second batch of lettuce seeds in which germination was scored at 2 and 8 days postirradiation more pronounced storage effects were observed. A decrease in germination occurred in seeds exposed to 100 and 200 kR when germination was measured after 24-hour incubation. Postirradiation effects upon the germination response in seeds exposed to all dose levels were most dramatic in those seeds allowed to germinate in the presence of 5-fluorouracil.

Incorporation of ^{3}H-Uridine into Lettuce Seed RNA

It has been suggested that gibberellic acid affects the transcriptional process and breaks dormancy by gene derepression mechanism (Jarvis et al., 1968a). Nucleic acid synthesis was inhibited by 5-fluorouracil in Xanthium where DNA and ribosomal RNA syntheses were impaired, but transfer RNA and messenger RNA syntheses were not affected (Cherry

and Van Huystee, 1965). In soybean hypocotyls DNA, transfer and ribosomal RNA syntheses were inhibited as a result of 5-fluorouracil application (Key, 1966).

The effects of ionizing radiation on RNA synthesis in various plant systems have been studied. Particularly relevant were studies by Stone (1968) on Grand Rapids lettuce seeds where this author reported that a delay of accumulation of leucine-^{14}C and uracil-^{14}C into protein and RNA occurred with increasing exposure to gamma-irradiation.

A study was conducted to observe the interactions and possible associations of gibberellic acid, 5-fluorouracil and ionizing radiation on RNA synthesis in lettuce seeds. RNA synthesis was measured by inclusion of ^{3}H-uridine in the germinating medium. The nucleic acid was extracted as described in Methods and Materials and separated on a Sephadex G-100 column. The absorbancy of each fraction was determined on a spectrophotometer. A typical effluent profile of RNA is illustrated in Figure 6. A peak of high molecular weight RNA appears in fractions seven to 10 and a transfer RNA peak in fractions 13 to 15.

The incorporation of ^{3}H-uridine into lettuce seed RNA after exposure to 100 kR gamma-irradiation is shown in Table II as counts per minute per absorbance at 260 nm

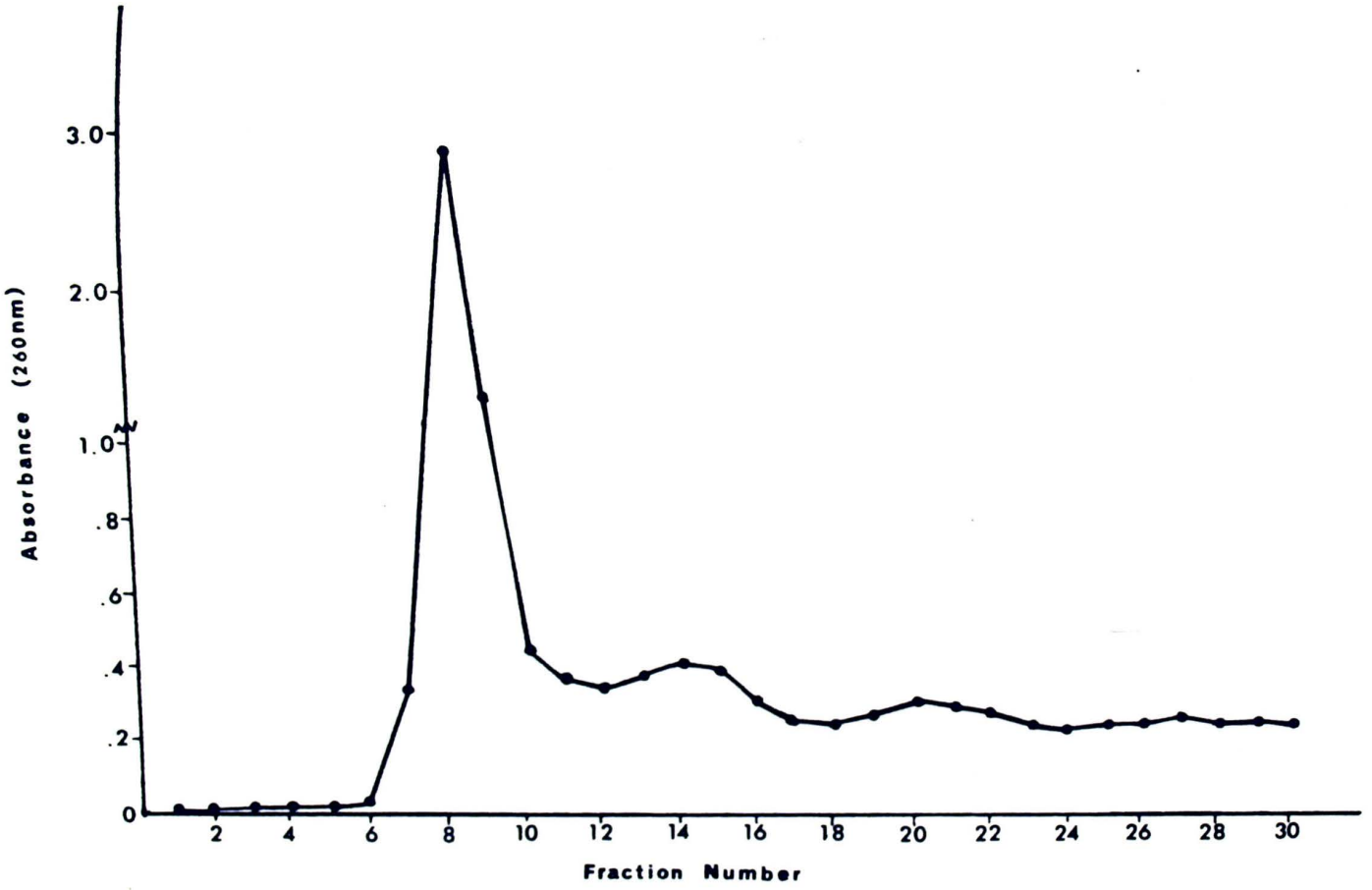

Figure 6. Sephadex G-100 effluent profile of RNA.

TABLE II

INCORPORATION OF ^{3}H-URIDINE INTO LETTUCE SEEDS EXPOSED TO 100 kR ^{60}Co GAMMA RAYS

Treatment	CPM/A_{260}	
	High Molecular Weight RNA	Transfer RNA
GA	14228	25683
Fu	1174	1863
GA + Fu	1661	1487
H_2O	2385	9906
H_2O - 0 kR	3765	7347

The CPM/A_{260} represents summation of peak fractions of a Sephadex G-100 effluent profile. GA, 2.8 X 10^{-4} M; Fu, 250 µgm/ml.

(CPM/A_{260}) of peak fractions. Gibberellic acid greatly stimulated incorporation of ^{3}H-uridine into RNA yielding a 6-fold increase in high molecular weight RNA and almost a 3-fold increase in transfer RNA. Gibberellic acid is known to effectively stimulate RNA synthesis in other plant systems; however, such tissues have not been exposed to ionizing radiation. A definite inhibition of high molecular weight RNA synthesis was observed in seeds incubated in 5-fluorouracil. Gibberellic acid was ineffective in overcoming 5-fluorouracil inhibition. Transfer RNA synthesis was similarly impaired by treatments with 5-fluorouracil. Ionizing radiation inhibited incorporation of labeled precursor into high molecular weight RNA by 40 percent as compared with unirradiated tissue. These results agree with previously reported data regarding RNA inhibition by gamma-irradiation in sugar beet tissue (Dunham et al., 1971) and total RNA reduction after exposure to X-rays in corn (Cherry et al., 1962). However, in the present study gamma-irradiation stimulated incorporation of ^{3}H-uridine into transfer RNA by 35 percent (Table II). The delaying action of ionizing radiation on germination appears to be associated with inhibition of ribosomal RNA synthesis. This concept is further substantiated by the effects of 5-fluorouracil on

RNA synthesis. Thus, ionizing radiation appears to selectively inhibit ribosomal RNA synthesis in lettuce seeds.

Effects of Ionizing Radiation on Hypocotyl Elongation

The promotion of hypocotyl elongation by gibberellic acid in Grand Rapids lettuce is well known (Frankland and Wareing, 1960). The rate of elongation was proportional to the concentration of gibberellic acid employed (Ikuma and Thimann, 1960). Khan (1966) stated that the length of lettuce hypocotyls was inhibited by incubation in 5-fluorouracil which was not relieved by any RNA or DNA precursors. Studies involving ionizing radiation effects of hypocotyl elongation in wheat indicated that seedling height decreased with increasing doses (Haber and Luippold, 1960).

A study was conducted to determine the effects of ionizing radiation on hypocotyl elongation in continuous light relative to gibberellic acid and 5-fluorouracil treatments. The results are presented in Figure 7. Hypocotyl elongation decreased with increasing doses of ionizing radiation regardless of treatment. Lettuce seedlings grown from seeds previously exposed to gamma-rays showed slight inhibition of hypocotyl elongation when germinated in water or 5-fluorouracil at each irradiation dose level. The presence of 5-fluorouracil significantly reversed gibberellic acid

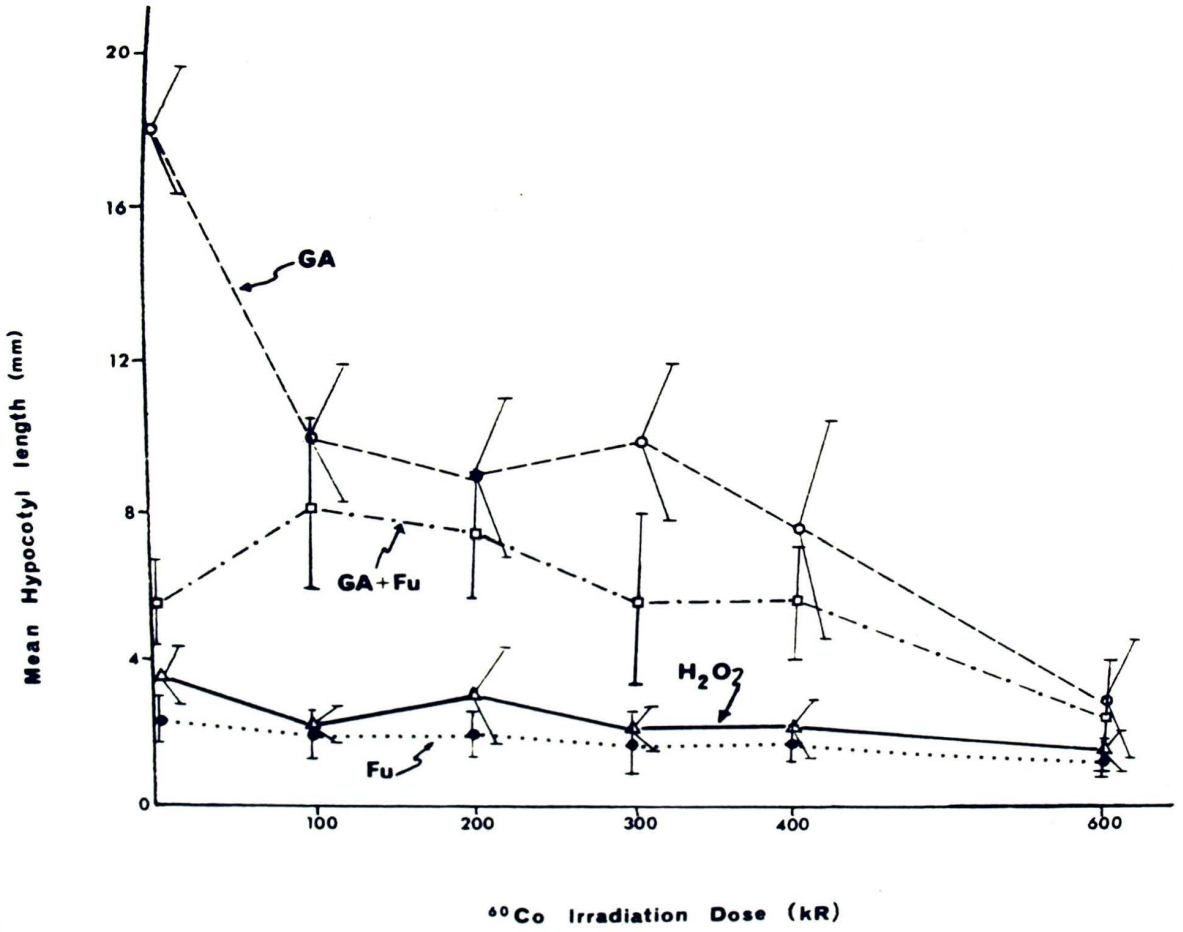

Figure 7. Lettuce hypocotyl elongation response after exposure of seeds to γ-rays and incubation in continuous light.

The experiment was conducted at 34 days postirradiation.

stimulation of lettuce hypocotyl elongation. Ananthaswamy et al. (1971) in studying germination changes in gamma-irradiated wheat suggested that at higher irradiation dose levels gibberellic acid promoted less growth than at lower doses. This is also apparent in the present study of lettuce hypocotyls.

The same procedure was performed in darkness with similar results (Figure 8); however, 5-fluorouracil showed a greater inhibition of growth as compared to those seeds incubated in water. The results of treatments with unirradiated seeds are in agreement with studies by Khan (1966) on gibberellic acid-induced dark hypocotyl elongation studies. Hypocotyl length of unirradiated seeds incubated in water averaged 15.7 mm in darkness, but in continuous light averaged 3.5 mm. This illustrates the inhibitory effects of light on hypocotyl elongation. It is interesting to note that low doses of ionizing radiation significantly stimulated hypocotyl elongation (61 percent) as compared to unirradiated seedlings treated with 5-fluorouracil.

During this experiment data were collected to determine the effects of ionizing radiation on root elongation. Table III illustrates the effects of 100 kR ionizing radiation upon root length in seedlings grown from irradiated seed. Ionizing radiation drastically reduced root length in seeds

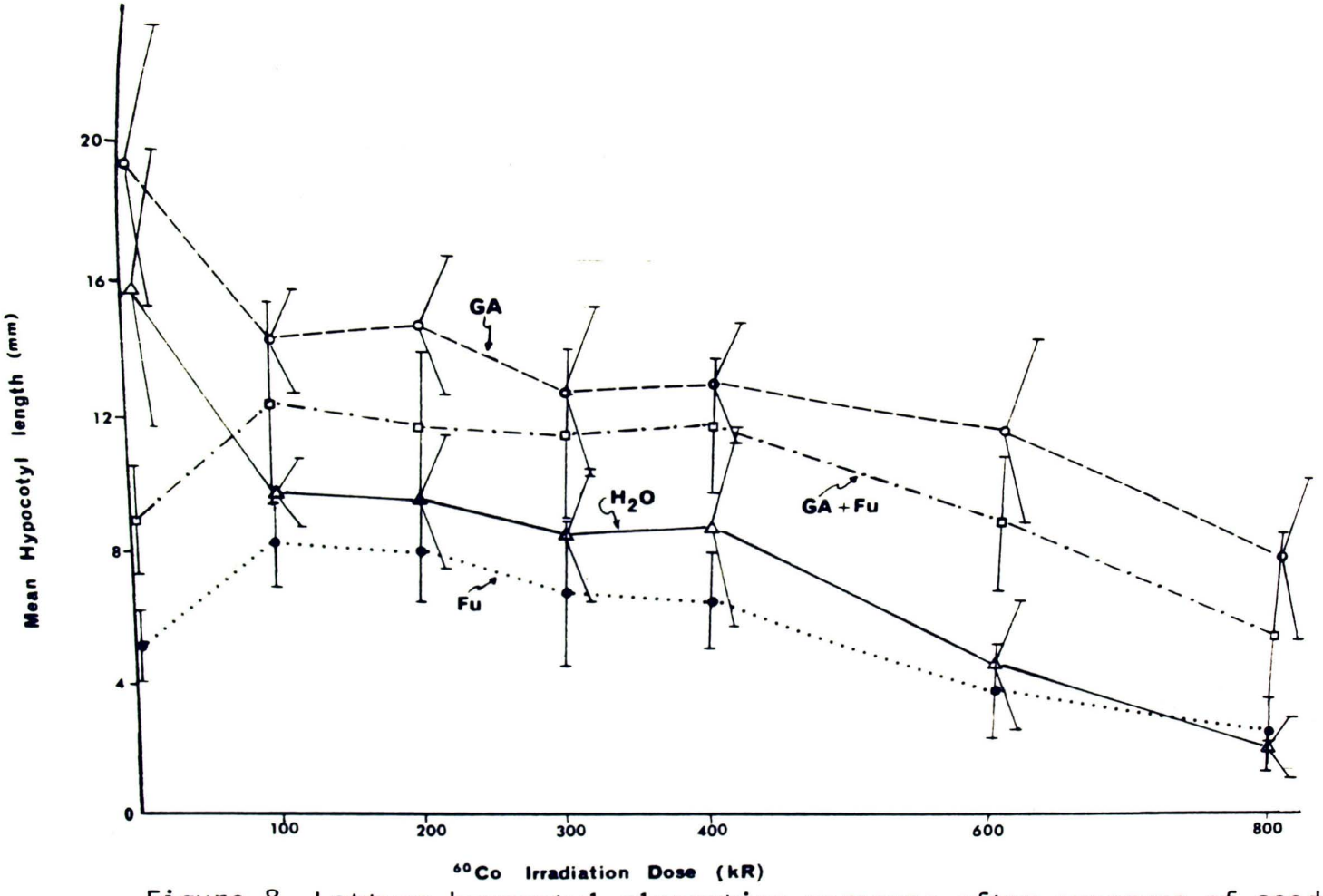

Figure 8. Lettuce hypocotyl elongation response after exposure of seeds to γ-rays and incubation in darkness. Time: 42 days postirradiation.

TABLE III

AVERAGE ROOT LENGTH OF GRAND RAPIDS LETTUCE SEEDLINGS AFTER 5 DAYS DARK INCUBATION

Treatment	Irradiation Dose (kR) 0 $\bar{X}$	0 S.D.	100 $\bar{X}$	100 S.D.
GA	25.12	±11.35	3.54	± 0.81
Fu	4.05	± 1.10	2.47	± 0.56
GA + Fu	3.63	± 1.19	2.37	± 0.49
H_2O	23.94	±12.91	2.86	± 0.85

Each mean represents the average of 40 roots. Incubation was at 22° in GA, 2.8 X 10^{-4} M or Fu, 250 µgm/ml. A 10^{-4} M concentration of penicillin and streptomycin was included in all experimental solutions.

incubated in water (12 percent of the control); however, hypocotyl elongation was less severely depressed (61 percent of the control) after the same treatment (Figure 7). Johnson and Klepinger (1967) reported that roots were inhibited to a greater degree than shoots in seedlings grown from irradiated Yucca seeds. Gibberellic acid was ineffective in reversing the inhibitory effects of ionizing radiation on root length (Table III) as indicated by the severe decrease in elongation after exposure to 100 kR.

Incorporation of ^{3}H-Uridine into Lettuce Seedling RNA

To determine what effects 5-fluorouracil might have on RNA synthesis in lettuce seedlings, the incorporation of ^{3}H-uridine into RNA was assayed. Table IV represents the results of labeled precursor incorporation into seedlings incubated in continuous light. Gibberellic acid was effective in stimulating both high molecular weight and transfer RNA syntheses in lettuce seedlings. Synthesis of high molecular weight RNA was selectively depressed by 5-fluorouracil, but it stimulated incorporation of ^{3}H-uridine into transfer RNA.

Morphological Response of Lettuce Seedlings

Several authors have reported a direct correlation of

TABLE IV

INCORPORATION OF ^{3}H-URIDINE INTO RNA OF LETTUCE SEEDLING HYPOCOTYLS

Treatment	CPM/A_{260}	
	High Molecular Weight RNA	Transfer RNA
GA	6330	8081
Fu	1909	4669
GA + Fu	2442	6089
H_2O	2963	2589

The CPM/A_{260} represents summations of peak fractions of a Sephadex G-100 effluent profile. GA, 2.8 X 10^{-4} M; Fu, 250 µgm/ml.

seedling height and irradiation dose characterized by reduced growth at higher dose levels. According to Conger and Stevenson (1969), seedling height of an individual seedling is a true and valid indication of how much irradiation damage that seed has undergone. The relationship holds true irrespective of dose, treatment or degree of damage. Chromosome aberrations corresponded closely to a decrease in seedling height. The following experiment was directed to observe the damaging effects of ionizing radiation on seedling height and possible gibberellic acid interaction.

Germinated seed exposed to 100 and 400 kR gamma-irradiation were grown in peat cubes on a 16-hour photoperiod and measured after 21 days growth. Exposure to ionizing radiation at all dose levels decreased growth of hypocotyls and primary leaves (Figure 9). Gibberellic acid was ineffective in overcoming the inhibitory effects of ionizing radiation on hypocotyl length as it did in 5-day old seedlings (Figures 7 and 8). However, in unirradiated seedlings gibberellic acid significantly stimulated hypocotyl elongation (Figure 9). A slight but mathematically significant stimulation of cotyledon growth resulted after gibberellic acid application in irradiated seedlings. Cotyledon growth of unirradiated seedlings was not affected by gibberellic acid; however, length of subsequent leaves was

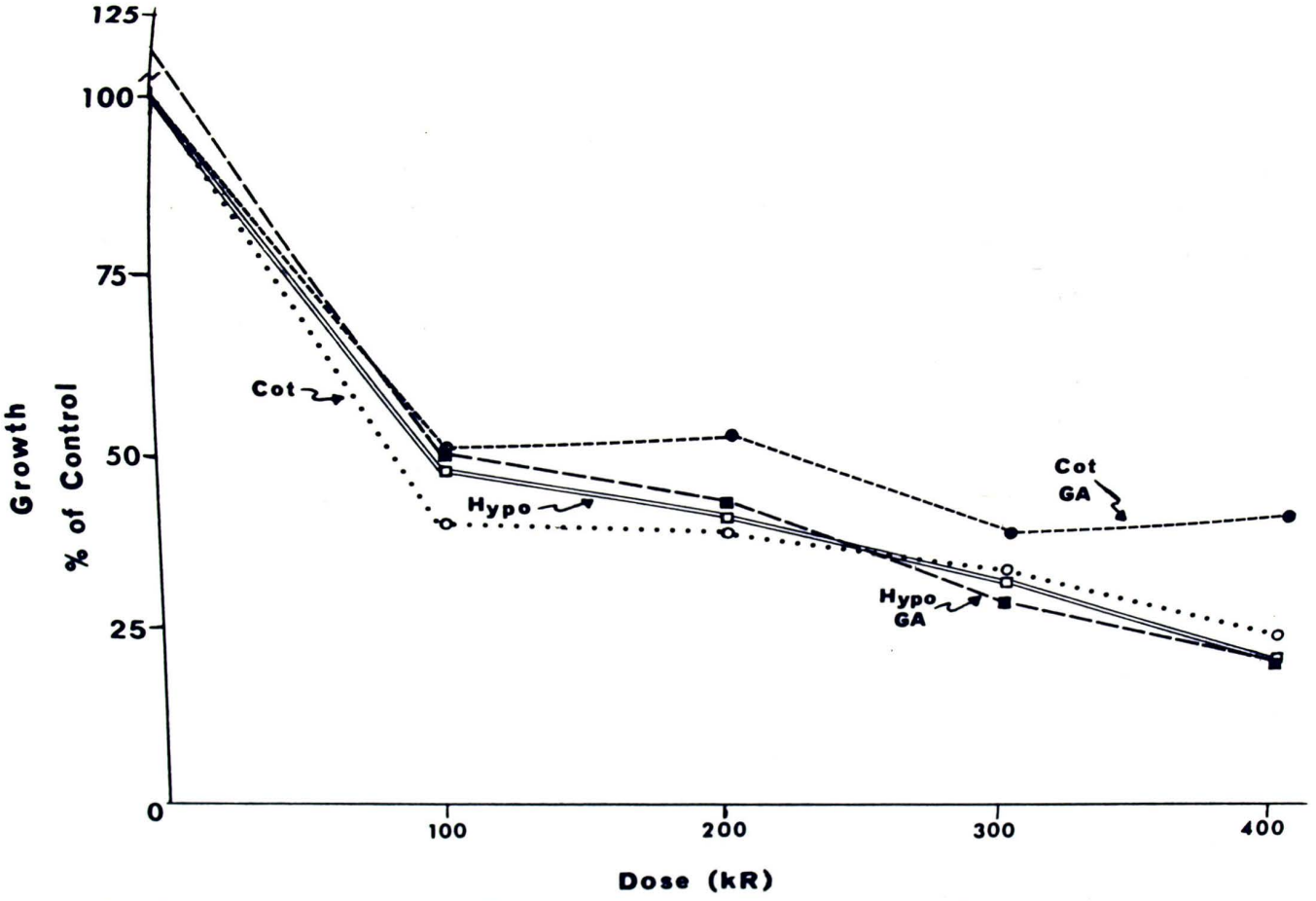

Figure 9. Growth of lettuce seedlings grown from seed exposed to γ-rays. Each entry represents the mean of 17 seedlings. Seedlings were sprayed every 2 days with GA (2.8×10^{-4}M). Measurements were conducted at 21 days growth. Hypo, hypocotyl length; Cot, longitudinal cotyledon length.

stimulated. The first and second true leaves increased in length by 114 and 126 percent respectively. Growth of true leaves did not occur in seedlings grown from irradiated seed.

Figure 10 illustrates the response of fresh and dry weight of seedlings grown from irradiated seed. A dose of 100 kR was effective in reducing fresh and dry weight to 5 and 22 percent of the control. Gibberellic acid effectively stimulated a slight increase in both fresh and dry weight after application at all doses.

Seedlings were examined after 7 days growth for morphological abnormalities. The results are presented in Table V. As the dose of ionizing radiation increased the percentage of germinated seeds breaking through the soil surface decreased. However, at termination all seeds did emerge despite some abnormalities.

The apical meristem of each seedling was cut longitudinally and examined at 30X magnification. In seedlings grown from unirradiated seed the meristem appeared organized with leaf bud primordia present in a typical bilateral fashion. No visible organized meristem was apparent in seedlings grown from seeds irradiated with doses of 100 to 400 kR. The tissue was discolored and orderly meristem organization was absent.

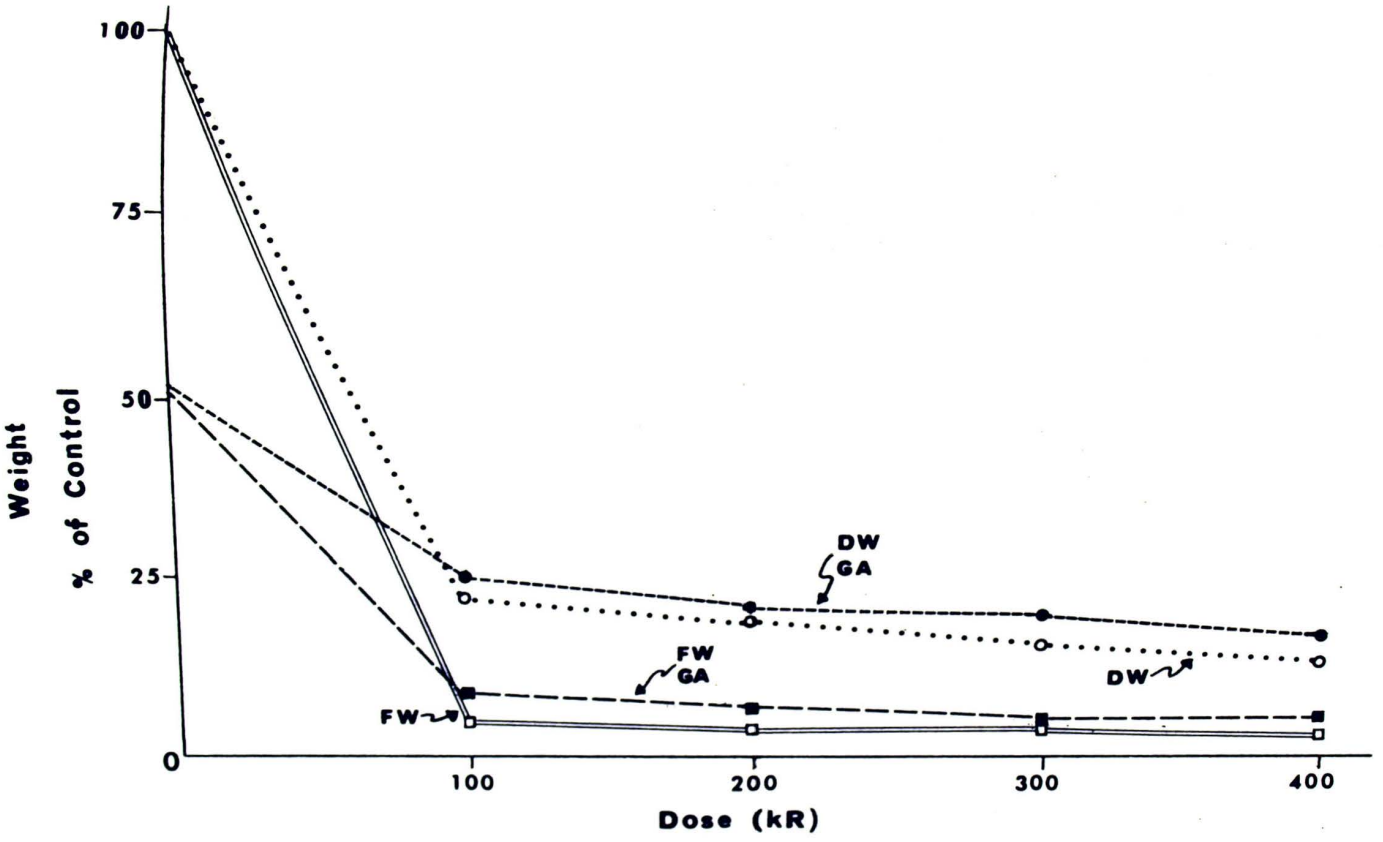

Figure 10. Fresh and dry weight of lettuce seedlings grown from seeds exposed to ionizing radiation.

DW refers to dry weight and FW refers to fresh weight. Experimental conditions were as described in Figure 8.

TABLE V

GROWTH CHARACTERISTICS OF 7-DAY OLD LETTUCE SEEDLINGS GROWN FROM IRRADIATED SEED

Dose (kR)	Percent Above Soil	Number Bud Primordia	Percent Seedlings Distorted	Average Root Length	Average 1st Leaf Length
0	100	2	None	30.0	10.7
100	95	None	None	3.8	None
200	97	None	None	3.0	None
300	86	None	14	2.5	None
400	75	None	20	2.0	None

The data are based on 40 germinated seeds per dose.

Seedlings at the 300 and 400 kR dosage level exhibited an unusual phemonenon regarding emergence from the soil substrate. A small percentage of the seeds protruded from the medium with the radicle first followed by the hypocotyl. The cotyledons were partially beneath the soil surface and only shed the seed coat near the concluding days of the experiment; thus, displaying contorted and abnormal growth.

Gamma-irradiation greatly inhibited root growth as compared to unirradiated seedlings. Gibberellic acid was ineffective in stimulating root growth of 7-day old seedlings grown from irradiated seed. This was also apparent in 5-day old seedlings (Table III).

CHAPTER V

DISCUSSION

Figures 1 and 2 illustrate that ionizing radiation delays germination of Grand Rapids lettuce seeds with increasing doses when incubation proceeds in water. This phenomenon has been reported in various plant systems; however, the mechanism of inhibition still remains unclear. The most obvious result of exposure to high energy radiation is destruction and alteration of chromosomes and subsequent mitotic retardation (Weiss, 1946). However, gamma-irradiation effects on germination of New York variety lettuce seeds could not be explained by interference with mitotis since the lack of mitotic activity could only be correlated with failure to germinate (Haber and Luippold, 1959). High doses of ionizing radiation are known to prevent mitosis in seeds without directly killing the embryo (Haber, 1968) or altering other nuclear functions such as RNA synthesis (Haber and Foard, 1964).

It has been stated that germination in seeds exposed to ionizing radiation is a result of cell elongation only with little mitotic activity (Casarett, 1968). In the present study exposure of seeds to 800 kR ionizing radiation permitted 34 percent germination after 5 days incubation in

water. It is doubtful that any DNA synthesis occurred after administration of this dose which implies that germination can be initiated without DNA replication. This indicates that germination was a result of elongation of those cells existing in the embryo prior to irradiation. If chromosome damage due to exposure to high energy radiation did not prevent germination in lettuce seeds, then it can be assumed that other essential processes are being inhibited.

Since red light increased respiratory activity in Grand Rapids lettuce seeds and far red light caused respiration to occur at a slower rate, Toole et al. (1965) concluded that energy was required for the onset of germination. Increased enzyme synthesis was also proposed to occur during incubation prior to cell elongation. If metabolic energy and enzyme synthesis are necessary for germination to proceed, suppression of these processes would delay germination. Ionizing radiation was shown to decrease respiratory rates in New York lettuce seeds during germination (Haber and Luippold, 1959). It is doubtful that a lack of metabolic energy is the primary effect of gamma-irradiation in depressing germination of Grand Rapids lettuce seeds (Stone, 1968). However, this radiation-induced energy reduction might be partially responsible for the delay in germination produced by ionizing radiation.

Figures 1 and 2 also indicate the stimulatory effects of gibberellic acid and kinetin in overcoming the delaying action of ionizing radiation on germination. The manner in which gibberellic acid breaks dormancy in unirradiated seeds involves its ability to overcome yet undescribed processes. Breaking of dormancy was reported to involve changes in the level of nucleic acid synthesis (Jarvis et al., 1968b) which was measured by increased incorporation of $^{32}PO_4$ into total RNA of hazel seeds. This study proposed that gibberellic acid first controlled the available DNA template sites and thereby stimulated transcription, secondly caused an increase in RNA polymerase activity, and finally increased RNA synthesis in vivo. In studies of barley seeds it has been postulated that gibberellic acid controls the synthesis of α-amylase in the aleurone layer by causing production of specific messenger RNA's (Varner and Chandra, 1964).

Further substantiation of gibberellic acid stimulation of nucleic acid synthesis is demonstrated in the present study. Gibberellic acid is shown to effectively increase ribosomal and transfer RNA syntheses in Grand Rapids lettuce seeds (Table II) and lettuce seedlings (Table IV) after 48 hours incubation. Seeds exposed to 100 kR ionizing radiation and germinated in the presence of gibberellic acid resulted in increased germination after 48 hours incubation.

Therefore, an increase in RNA synthesis accompanied increased percent germination when seeds were incubated in gibberellic acid. It has been suggested that nucleic acid and protein syntheses are essential processes in imbibing seeds and precede germination (Stone, 1968). If ionizing radiation prevents these necessary cellular functions or decreases their efficiency, then it would also delay the germination process. Since gibberellic acid effectively stimulates RNA synthesis, this may be its mode of action in overcoming the delaying effects imposed by ionizing radiation in lettuce seed germination.

Other detrimental effects of gamma-irradiation on lettuce seeds such as chromosomal aberrations, decreased respiratory activity and reduced seedling height were not overcome by gibberellic acid (Haber and Luippold, 1959). These authors also stated that DNA synthesis was not necessary for the action of gibberellic acid on lettuce seed germination because an enhancement of germination occurred after gamma-irradiation had negated mitosis. Since germination in seeds exposed to ionizing radiation is assumed to be primarily a process of cell elongation, it is not surprising that gibberellic acid still functions in promoting germination. Lockhart (1960) has proposed that gibberellic acid is effective in increasing the plasticity of the cell wall

followed by a greater uptake of water.

Germination can occur with increased time after exposure to ionizing radiation (Figure 3). Thus, it is likely that damaged cellular substances are being synthesized. The damage caused by ionizing radiation may be the destruction of RNA. Therefore when RNA synthesis is stimulated by gibberellic acid, germination is able to occur.

In Escherichia coli 5-fluorouracil was incorporated into bacterial RNA replacing 50 percent of the uracil (Heidelberger, 1965). This substitution had an inhibitory effect on enzyme systems and cellular reactions as a consequence of translational error. In RNA the presence of 5-fluorouracil resulted in accumulation of abnormal ribosomes that could not be converted into larger particles (Aronson, 1961). In contrast Key (1966) has shown that 5-fluorouracil markedly inhibited incorporation of ^{32}P into ribosomal RNA precursor of soybean hypocotyls. This author concluded that 5-fluorouracil inhibited ribosomal RNA synthesis, per se, rather than prevent accumulation into larger particles. Other reports indicated that 5-fluorouracil prevented mature ribosomal formation by inhibiting synthesis of protein components of ribosomes (Osawa, 1965). Although the precise mechanism of 5-fluorouracil inhibition is at present undescribed, most authors agree that ribosomal

RNA synthesis is selectively depressed. DNA biosynthesis was also impaired by 5-fluorouracil presumably due to conversion of the latter to 5-fluorodeoxyuracil (Cherry and Van Huystee, 1965); however, this action is not relevant in the present study since DNA synthesis is not occurring in the cells studied.

It may be hypothesized that ionizing radiation causes a destruction of ribosomes in lettuce seeds and that these organelles must be intact for germination. It is then reasonable to infer that during the delaying time period priod to the germination response, there is a requirement for synthesis of ribosomal RNA in those seeds previously exposed to ionizing radiation. The presence of an inhibitor of ribosomal RNA synthesis, 5-fluorouracil, in the medium would prevent repair processes and subsequent germination. The distinct inhibition of ribosomal RNA synthesis in association with ionizing radiation is illustrated by decreased germination (Figures 4 and 5) and decreased incorporation of ^{3}H-uridine into RNA (Table II). Since 5-fluorouracil did not suppress germination in unirradiated seeds, it is probable that the necessary ribosomes and ribosomal RNA had already been synthesized in vivo and that further synthesis was not a requirement for germination. From the available data it is conceivable that ionizing radiation

decreased germination of Grand Rapids lettuce seeds through an inhibition of ribosomal RNA synthesis. Damage to polyribosomes as a consequence of X-rays has been demonstrated by Cherry (1962) in corn tissue. Transfer RNA synthesis was also impaired by 5-fluorouracil application, but since ionizing radiation enhanced incorporation of ^{3}H-uridine into this RNA component (Table II) it was very unlikely that germination was delayed by decreased transfer RNA synthesis.

The lack of germination (less than 20 percent) of irradiated seeds incubated for 5 days in 5-fluorouracil illustrated the severity of inhibiting ribosomal RNA synthesis. It is possible that protein synthesis was also inhibited due to lack of functional ribosomes. Since 5-fluorouracil effectively reversed the promotion of germination and RNA synthesis by gibberellic acid, it would be reasonable to infer that ribosomal RNA synthesis was necessary for the actions of gibberellic acid on lettuce seeds.

It has been demonstrated that one of the consequences of exposure to gamma-rays in lettuce seeds was a reduction of ribosomal RNA based on reduced incorporation of ^{3}H-uridine into RNA. However, ionizing radiation might also alter several other biological reactions. Gamma-irradiation is characterized by a low LET (linear energy transfer) resulting

in a diffuse distribution of ionization over a large volume (Casarett, 1968). It would seem unlikely that only a few cellular constituents would be damaged due to irradiation exposure. Any entity escaping damage by direct contact of an ionized particle might later be affected in storage due to free radical formation.

Damage to ribosomes would be expected to alter protein synthesis. Any enzymes necessary for respiration or seed coat digestion might thus be present in a nonfunctional capacity. Stone (1968) has suggested that the delay in germination of irradiated seeds may be due to inactivation of an enzyme functioning in digestion of the endosperm layer. Ionizing radiation may cause enzyme inactivation indirectly through ribosomal RNA inhibition. Degradation of the sugar-phosphate backbone of nucleic acids is known to occur after exposure to ionizing radiation (Weiss, 1964). This action on ribosomal RNA could easily render ribosomes inactive and incapable of performing in protein synthesis.

In washed sugar beet tissue studies (Dunham et al., 1971) gamma-irradiation decreased RNA polymerase activity and increased transcriptional errors. Cherry (1962) reported that X-irradiation in corn root tips increased ribonuclease activity 2- to 4-fold. Even if ionizing radiation did not destroy ribosomal RNA directly, it might have

prevented accumulation through decreased RNA polymerase and increased ribonuclease activities. From these studies it becomes apparent that ionizing radiation adversely affects many biological processes as a result of primary and secondary ionizations and the persistence of free radicals. Respiration, RNA, DNA and protein syntheses may be inhibited by ionizing radiation. The depression of any of these mechanisms would prevent or delay plant growth.

It is apparent that light influences the germination of irradiated seeds (Table I). However, unirradiated seeds did not differ in germination response in the two photoperiods studied. Stone (1968) reported that the photoreversible phytochrome system was still functional in seeds exposed to ionizing radiation. It appears that the processes involved (ribosomal RNA synthesis) in recovery of radiation damage require exposure to continuous light for repair mechanisms to proceed maximally. This seems to imply that the process of ribosomal RNA synthesis is photosensitive. Doolittle (1973) has demonstrated in studies of Anacystis nidulans that postmaturational cleavage of 23s ribosomal RNA was stimulated by light. This process was mechanistically related to prematurational cleavage in mammalian cells which preceded and was required for ribosomal function. If prematurational cleavage occurs in

lettuce ribosomal RNA, it too may be photosensitive. The increase in germination of Grand Rapids lettuce seeds under continuous light would be explained by this postulate.

The inhibitory effects of ionizing radiation on hypocotyl elongation may be similar in action to those on germination since both are essentially processes of cell elongation. Ionizing radiation decreased hypocotyl length with increasing dose as was evident in continuous light (Figure 7) and darkness (Figure 8). These results are consistent with data regarding decreased seedling growth of New York lettuce (Haber and Luippold, 1959), impaired growth of wheat hypocotyls (Ananthaswamy et al., 1971) and retarded seedling height of Phaseolus vulgaris (Mujeeb and Greig, 1972). In each system decreased growth response was correlated with increased dose of high energy radiation.

In Figure 7 gibberellic acid effectively overcame the inhibition in hypocotyl elongation caused by ionizing radiation although at higher dose levels it was less effective. Gibberellic acid stimulated hypocotyl elongation during the same time period when it increased incorporation of ^{3}H-uridine into ribosomal and transfer RNA (Table IV). It may be reasonable to infer that gibberellic acid increased cell elongation in those seedlings exposed to ionizing radiation by stimulation of RNA synthesis. Hypocotyl

elongation was effectively inhibited by 5-fluorouracil in the presence of gibberellic acid, further indicating the necessity of ribosomal RNA synthesis in gibberellic acid mediated action.

In darkness a more distinct inhibition of hypocotyl elongation occurred in the presence of 5-fluorouracil in association with ionizing radiation (Figure 8). Since light itself was inhibitory to lettuce hypocotyl elongation, there was a somewhat masked effect of 5-fluorouracil in continuous light. This antimetabolite decreased incorporation of ^{3}H-uridine into ribosomal RNA (Table IV). Assuming RNA synthesis was necessary for cell elongation (Key, 1964) the inhibition of elongation may have been due to decreased ribosomal RNA synthesis induced by ionizing radiation.

Transfer RNA synthesis was not affected by 5-fluorouracil and it is unclear whether transfer RNA is essential for cell elongation. In soybean hypocotyls 5-fluorouracil appreciably inhibited ribosomal and transfer RNA syntheses but did not influence growth (Key, 1966). The author concluded that only the synthesis of D-RNA was necessary to support continued protein synthesis. In lettuce hypocotyls ribosomal RNA was required for cell elongation to occur since the presence of 5-fluorouracil significantly inhibited growth (Figure 8) in unirradiated and irradiated

seedlings.

The stimulation of hypocotyl elongation by low doses of ionizing radiation was apparent in those seedlings treated with 5-fluorouracil (Figure 8). Ionizing radiation is known to stimulate invertase production in sugar beet tissue which was correlated with an increased capacity of the tissue to methylate transfer RNA (Stone and Cherry, 1972). Nucleic acid synthesis in cotyledons was reported to be stimulated by X-rays if seeds were germinated immediately after exposure (Van Huystee, 1967). It is conceivable that ionizing radiation in association with 5-fluorouracil may have stimulated cell elongation by arresting an inhibitory process which required ribosomal RNA synthesis. Since this effect was more pronounced in darkness light may have initiated the inhibitory process. Therefore, the data imply that a process occurred in continuous light which decreased hypocotyl elongation and was dependent on ribosomal RNA synthesis. This provides further information indicating that the accumulation of RNA precursor components might indeed be photosensitive.

Ionizing radiation was effective in severely depressing root growth in 5-day old seedlings grown from irradiated seeds (Table IV). Gibberellic acid was ineffective in overcoming root inhibition as a result of ionizing radiation.

Roots have been shown to be more radiosensitive (Johnson and Klepinger, 1967) as smaller doses of ionizing radiation produced a greater growth inhibition. Gamma-irradiation (100 kR) resulted in much more damage in roots than shoots. Therefore, stimulation of RNA synthesis by gibberellic acid was ineffective in mediating repairs as the damage to cellular constituents was too great.

Irradiation of seeds drastically decreased height of plant seedlings (Casarett, 1968). This fact was demonstrated in the present study as doses of greater than 100 kR severely reduced seedling growth (Figure 9). A dose of 10 to 12 kR gamma-irradiation retarded growth of Phaseolus vulgaris and also delayed flowering and maturity (Bajaj et al., 1970). Surrey (1956) reported that X-irradiation inhibited growth of sunflower seeds with increasing doses.

Hypocotyl and cotyledon length were reduced by 50 and 60 percent as a result of exposure to gamma-rays (Figure 9). Gibberellic acid was ineffective in overcoming the inhibitory effects of ionizing radiation on 21-day old seedlings; however, hypocotyl growth was stimulated by gibberellic acid after 5 days growth (Figures 7 and 8). Since gibberellic acid treatment was not begun until seedlings were 7 days old maximum cell elongation may have already occurred. Apparently these cells were not capable of further stimula-

tion as gibberellic acid application did not result in cell elongation. It is also possible that gamma-irradiation may have rendered the tissue less responsive to a given amount of gibberellic acid or reduced water movement through the tissue. Thus, the time period prior to gibberellic acid treatment might have been responsible for increased damage and a reduction in gibberellic acid responsiveness in 21-day old seedlings. Cotyledon length, however, was significantly stimulated (DMR) by gibberellic acid indicating a longer duration of sensitivity to exogenously applied gibberellic acid or perhaps the cotyledons suffered less irradiation damage. Variation in radiosensitivity of plant parts has been noted by Looper and Aboul-ela (1971).

Fresh and dry weight of Grand Rapids lettuce seedlings were greatly inhibited by all doses of gamma-irradiation (Figure 10). Looper and Aboul-ela (1971) attributed a decrease in fresh and dry weight of Fenugreek beans exposed to 30 kR gamma-rays to decreased metabolism and genetic damage. In addition to inhibiting these processes gamma-irradiation may have also inhibited cell elongation by decreasing RNA synthesis in lettuce seedlings. Gibberellic acid slightly stimulated an increase in fresh and dry weight of lettuce seedlings grown from irradiated seed. The stimulation of fresh weight implies increased water absorption.

Gibberellic acid is known to influence cell elongation by increasing the concentration of osmotically active materials inside the cell or overcoming the resistance of the cell wall to stretching (Overbeek, 1966). Both processes result in a greater absorption of water by the cell; thus, increasing fresh weight. It is not clear why gibberellic acid increased dry weight of seedlings exposed to ionizing radiation. Possibly gibberellic acid influenced protein synthesis through nucleic acid stimulation which caused an increase in dry weight.

Doses of 100 kR and above of gamma-irradiation completely prevented growth of the apical meristem in lettuce seedlings (Table V). Subsequent leaf development did not occur in seedlings grown from irradiated seed as all bud primordia were destroyed. This phenomenon can be correlated with a radiation-induced lack of mitotic activity since cell division is a primary function of the meristem. Root meristems were likewise injured by ionizing radiation. The observed response indicated that growth of these seedlings was primarily due to cell elongation which was less severely inhibited by ionizing radiation than cell division. The data obtained are consistent with the idea that ionizing radiation virtually eliminates cell division in the meristematic zone of growing seedlings without affecting cell

expansion.

As a result of these studies on the germination response and hypocotyl elongation of Grand Rapids lettuce seeds, the principle effect of ionizing radiation appears to be an inhibition of ribosomal RNA synthesis. This process is essential for both germination and cell elongation phenomenon.

CHAPTER VI

SUMMARY

Ionizing radiation was effective in delaying the germination response of Grand Rapids lettuce seeds. Maximal germination occurred at dose levels from 100 to 400 kR after 5 days incubation in water. Thus, with increasing incubation time germination did occur. Both gibberellic acid and kinetin were effective in overcoming this delay in germination imposed by ionizing radiation. The presence of 5-fluorouracil, a selective inhibitor of ribosomal RNA synthesis, severely reduced germination at all irradiation dose levels. However, 5-fluorouracil did not prevent germination in unirradiated lettuce seeds. Those seeds previously exposed to ionizing radiation and germinated in the presence of 5-fluorouracil resulted in a low germination response even after 5 days incubation.

The incorporation of ^{3}H-uridine into high molecular weight RNA was effectively inhibited by 100 kR ionizing radiation; however, transfer RNA synthesis was not impaired. Gibberellic acid stimulated synthesis of ribosomal RNA by 6-fold and transfer RNA by 3-fold. Incorporation of labeled precursor into both RNA components was severely inhibited by 5-fluorouracil.

From these data it is reasonable to infer that ribosomal RNA synthesis was not necessary for germination to occur in unirradiated seeds, as 5-fluorouracil did not prevent the germination response. However, 5-fluorouracil did delay germination in seeds previously exposed to ionizing radiation which indicated that ribosomal RNA synthesis was essential for germination in those seeds. It is proposed that ionizing radiation is effective in the destruction of ribosomes and that these organelles must be repaired prior to germination. The subsequent delay in germination may be the time required for repair processes involving ribosomal RNA synthesis. The presence of 5-fluorouracil prevents the onset of repair processes.

Hypocotyl length of 5-day old lettuce seedlings was retarded by exposure of seeds to gamma-irradiation in continuous light and darkness. This inhibition was more pronounced in darkness as light was inhibitory to lettuce hypocotyl elongation. Since 5-fluorouracil was effective in reducing hypocotyl length, it is likely that ribosomal RNA synthesis was necessary for cell elongation. Gibberellic acid significantly stimulated hypocotyl elongation in irradiated amd unirradiated seedlings. When 5-fluorouracil and gibberellic acid were included in the incubation medium 5-fluorouracil significantly reversed the stimulation caused

by gibberellic acid alone.

In unirradiated lettuce seedlings the synthesis of ribosomal RNA was selectively depressed by 5-fluorouracil while gibberellic acid stimulated incorporation of ^{3}H-uridine into ribosomal and transfer RNA. The observed response implies that the synthesis of ribosomal RNA is correlated with lettuce hypocotyl elongation.

In 21-day old seedlings growth was similarly retarded after exposure of seeds to gamma-irradiation. Fresh weight, dry weight, hypocotyl length and cotyledon length were all inhibited as a result of irradiation treatment. Gibberellic acid was ineffective in reversing the radiation-induced depression of seedling height. However, gibberellic acid slightly stimulated fresh and dry weight in lettuce seedlings exposed to ionizing radiation. Cell division did not occur in the apical meristem of seedlings exposed to gamma-irradiation nor did secondary leaves develop. These results reflect that growth of irradiated seedlings was primarily due to cell elongation.

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