CHARACTERIZATION OF SOLUBLE NUCLEOHISTONE FROM DEVELOPING COTYLEDONS OF *PINUS PINEA* FOLLOWING X-IRRADIATION

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ABSTRACT

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CHARACTERIZATION OF SOLUBLE NUCLEOHISTONE FROM DEVELOPING COTYLEDONS OF PINUS PINEA FOLLOWING X-IRRADIATION

Soluble nucleohistone and histones have been isolated from cotyledons of Pinus pinea; this is the first such isolation from any gymnosperm tissue. The nucleohistone isolated was similar in chemical composition and physical characteristics to that of chromatin obtained from other plant tissues at comparable stages of growth. Histone, purified by gel electrophoresis, possessed 8 sub-fractions, tentatively identified as follows: very lysine-rich F_1 (I), fractions 1-4; arginine-rich F_3 (III), fraction 5; arginine-rich F_2a2 (IIb1), fraction 6 and F_2b (IIb2), fraction 7; as well as moderately arginine-rich F_2a1 (IV), fraction 8.

Nucleohistone from days 15-22 of germination, a time of cell elongation, possessed greater amounts of histone, acidic protein and RNA compared to day 14 'mitotically-active' nucleohistone. This suggests that the proteins may be acting to modify RNA synthetic activity related to differentiation.

In vitro X-irradiation of either nucleohistone or histone did not induce changes in gel-electrophoretic characteristics. Similarly, the physical characteristics and chemical composition of nucleohistone from nuclei irra-

diated with 1 and 5 kR were not significantly different from un-irradiated controls. However, 1 kR exposure to day 14 seedlings induced immediate mitotic arrest and also reduced histone deposition into nucleohistone by 1 day after irradiation which seemed to recover slightly by 2 days post-irradiation. It was concluded that X-irradiation, by delaying mitosis, had disrupted both synchrony of histone deposition into nucleohistone and delayed subsequent differentiation of the cotyledons.

Histone acetylation, methylation, and phosphorylation (from inorganic phosphate) proceeded at low rates in isolated nuclei and were not affected by 1 kR and 5 kR X-irradiation; however, AT^{32}P phosphorylation occurred to a greater extent. Exposure to 5 kR X-rays significantly decreased histone F_1 (I)1, F_1 (I)2, F_1 (I)3, F_2a2 (IIb1)6 and F_2b (IIb2)7 phosphorylation. In retrospect, the high basicity and low phosphate content of F_1 (I) and F_2b (IIb2) histones in irradiated nuclei may cause changes in the ability of irradiated chromosomes to complete mitosis thus delaying further differentiation of nucleohistone characterized by increases in histone content.

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INTRODUCTION

Biological damage in cells induced by X-irradiation has been traditionally associated with observed effects on the nucleus of the cell. Only recently have the effects of irradiation on cellular metabolism and especially macromolecular synthesis been studied in detail. The most radiosensitive cells of an organism seem to be those which are as yet undifferientiated and are in a state of rapid mitotic activity. For this reason most studies with ionizing radiation in plants have been confined to root and apical meristematic cells.

The cell cycle has been defined as the interval between completion of mitosis in a cell and completion of subsequent mitosis in one or both daughter cells[9]. It is divided into four phases, namely: (1) G_1, the interval between completion of mitosis and onset of DNA synthesis, (2) S, the period during which DNA is replicated, (3) G_2, the period between completion of DNA synthesis and mitosis, and (4) mitosis, in its four stages from prophase to telephase. Much has been learned about the structural, morphological, biochemical and physiological aspects of the different periods. It is known[194] that the nuclear membrane, golgi and nucleolus appear at initiation of G_1 and remain until onset of mitosis. The centioles reproduce during S

and migrate to the poles in G_2. The spindle fibres form at G_2 and breakdown at late telophase. Polysomes form during early G_1 and disaggregate at metaphase. Protein, nuclear RNA, rRNA, and tRNA as well as S-stage proteins are synthesized at beginning of G_1 and finish by end of G_2. DNA, acidic proteins, histones and nucleolar RNA are synthesized at maximum rates during the S period. Mitochondrial DNA, mitotic mRNA and proteins are produced maximally in G_2.

There is adequate evidence indicating that irradiation during the cell cycle results in impairment of DNA synthesis and delay of mitotic division. Sparrow, et al.[157], Pelc and Howard[128] in plants and Lajtha[94] in animal tissue culture demonstrated a correlation between irradiation effect and cell stage. Chromosomal damage and DNA synthesis were observed to be greater when cells were irradiated during the first phase of interphase (G_1) than at the time of DNA replication (S) phase. (For a recent review of the effects of irradiation on DNA synthesis in mammalian tissue culture systems see Sinclair[153]).

The most important reversible block produced by irradiation appears to occur in G_2. Irradiated cells move into G_2 from DNA synthesis (S phase) and G_1, but are delayed from going into mitosis for a variable time depending on dose[104]. The duration of G_2-block appears to depend upon the position of the cell in the cell cycle at

time of irradiation, the delay being greater for cells irradiated in the middle of G_2 and least for those irradiated in G_1[51,171,192].

It has also been suggested that the mechanism of action of radiation delay is through an interference with synthesis of proteins or the nucleohistone synthetic process *per se*[6,99,101]. In 1927 Stadler discovered that X-irradiation induced mutations in barley. Since then much work has been done on radiation effects in plant systems. Several reviews are available describing the various effects produced in plants by radiation which include decreases in germination, reductions in growth, chromosome aberrations, chlorophyll deficiencies, changes in morphology of flowering parts, decreases in respiration rate and CO_2 exchange as well as effects on biosynthetic processes, e.g. nucleic acid synthesis, and IAA synthesis[57,93,115,137,184].

Exposure of dry seeds of corn[33], barley[31,34,98], peanut[182], pea[65], and pine (*Pinus pinea*)[139] to ionizing radiation leads to mitotic arrest, inhibitions of growth and of DNA synthesis in meristems. Rates of RNA and protein synthesis were found to be variously affected. Irradiation at levels sufficient to induce inhibitions of cell division does not inhibit cell elongation[33,65,139]. Cherry *et al.*[33] observed that X-irradiation of corn stimulated ^{32}P-incorporation into nuclei while incorporation of amino acids

into protein material was inhibited. In addition, abnormalities in the synthesis and redistribution of soluble and microsomal RNA were noticed after irradiation.

Peanut cotyledons irradiated with 250 kR of X-rays and stored 4 weeks prior to germination exhibit reductions in synthesis of all RNA fractions[182]. Hybridization of RNA to DNA and examination of protein synthesis on polyribosomes in vitro indicated that X-irradiation, sufficient to prevent cell generation, decreased the capacity of germinating seeds to produce functional messenger RNA (mRNA)[183].

Kovács and Van't Hof[92] demonstrated that exposure of synchronized cultures of excised pea root meristems at the G_1/S boundary to 300 R of γ-radiation does not alter the initiation events for DNA synthesis and replication but does result in prolongation of the G_2 stage. Elevated rates of protein synthesis occurred associated with γ-irradiation-induced mitotic delay. Analysis of ^{14}C-labeled protein hydrolysate incorporation into nascent polypeptides revealed that as the G_1/S irradiated population entered G_2, labeling increases over the monomeric region of the polysome profiles compared to non-irradiated controls. Thus it would appear that 300 R of γ-radiation not only impaired requisite protein synthesis but also disrupted the controlling mechanisms responsible for normal polysome organization and protein synthesis. It can be concluded that protein

synthesis is required for the cell to resume division and is associated with, but not necessarily responsible for recovery from G_2 mitotic arrest.

High radiosensitivity of many gymnosperm species is well documented[23,35,66,141]. In view of the possible release of radioactive fallout from nuclear and thermonuclear detonations or reactor effluents it is possible that large scale destruction of the conifer population could occur. In Canada the loss of this dominant species with its numerous dependent ecosystems could result in economic effects of catastrophic proportions.

Pinus pinea is a particularly suitable material for the study of radiobiological effects. Each of the 12 cotyledons of the dry seed is an embryonic meristem, consisting exclusively of G_1-cells of 2C DNA content[27] with a potential for 8 fold increases in DNA content. Since they posses large nuclei of 10-25μ size, moderate doses of irradiation (15 kR) are sufficient to induce symptoms of radiation damage.

Previous work by Roy et al.[139] found that 15 kR X-ray exposure to dry seeds of Pinus pinea induced chromosomal aberrations, growth inhibition of cotyledons, 25% reductions in DNA synthesis and delay of mitosis. Radiation reduced the incorporation of ^{3}H-uridine into RNA and ^{14}C-leucine into protein at day 14 of germination when DNA synthesis and mitotic activity were declining. Interestingly,

^{14}C-thymidine incorporation into DNA and ^{3}H-lysine into protein was inhibited by X-rays at the time of maximum nucleohistone synthesis (days 9-11). In view of recent reports that synthesis of histones are an obligatory requirement for DNA synthesis and cell replication[7,142,188]. Inhibition of synthesis of the lysine-rich histones may be related to radiation effects on delays of DNA synthesis and mitosis.

Nucleohistone of gymnosperms has not yet been isolated and investigated with respect to its physical and chemical properties. It would appear that among the gymnosperms the most suitable material for this type of investigation would be the cotyledons of germinating seeds of Pinus pinea by virtue of their size. The cotyledon is a discrete meristematic tissue in which all cells will enter a period of rapid cell division during the second week of germination. This provides large quantities of asynchronous, but homogeneous, replicating cells with large nuclei and high DNA content, making it possible to isolate sufficient quantities of chromatin while using only small numbers of seeds.

Gunckel and Sparrow[57], in a review on the effects of ionizing radiation in plants reported that very little work had been done on the effect of ionizing radiation in plant nucleohistone even though preparation of deoxyribonucleoprotein, histones and acidic (residual) proteins had been reported from wheat germ and from several animal

tissues by Mirskey and Pollister[109] as early as 1946. Since that time isolation and characterization of nucleohistone and histones has been described from a variety of plant tissues[46,74,114,160,161,176,177].

As yet there have been no reports concerning the effects of ionizing radiation on nucleohistone or histone protein isolated from any species of pine. In this study nucleohistone was isolated from purified nuclei of cotyledons of Pinus pinea sampled at various times of germination. It will be shown that, the physical characteristics and chemical composition of nucleohistone, as well as histone patterns and nuclear metabolism did not change from irradiated material in particular compared to non-irradiated material. Metabolism of some individual fractions of histones isolated from irradiated nuclei were affected by irradiation. The relationship of these changes to the effects of irradiation on cell differentiation and histone synthesis in germinating seeds of this species will be discussed.

LITERATURE REVIEW

A. Nucleohistone

The functional form of the chromosomal DNA of eukaryotes is associated with other macromolecules such as histones, acidic proteins, lipids or lipoproteins and RNA. This complex is called nucleohistone. Chromosomes are ordinarily obtained from cells during interphase and are therefore in the extended state known as chromatin. Sonnenberg and Zubay[156] have reviewed the various methods that have been used to prepare nucleohistone.

The composition of typical chromatin consists of almost equal proportions of DNA and histones as well as smaller amounts of RNA, acidic proteins and lipids. In contrast to the high reproducibility of histone to DNA ratios, the acidic protein content has been found to be much more variable, the yield of which is somewhat dependent upon the method of extraction[49]. It is now believed that the non-histone content of chromatin reflects its transcriptional activity[71], since it is found to be relatively high in tissues active in RNA synthesis and low in repressed chromatin. Also, the non-histone proteins have been shown to influence the specificity of transcription (see later part of this discussion).

In water, chromatin forms a gel which on dilution swells and ultimately dissolves in a viscous dispersion. The ability of chromatin to form rigid gels is regarded by many investigators as characteristic of enzymatically undegraded chromatin samples[49,42,75,196]. DNA under similar conditions does not gel. Dounce and O'Connel[41] p. 2013 stated that they "...consider gel formation to be an indication of an intact desoxyribonucleoprotein, where the DNA is attached to the residual lipoprotein of the chromosomes, probably through covalent bonds." The formation of the gel-state is most likely a result of the interaction between the very lysine-rich F_1 (I) histones[32,103], and thiol groups of the arginine-rich F_3(III) histone[122,47] forming cross-links and disulphide bonds. Bram and Ris[24] proposed that the nucleoprotein fibre consisted of a single DNA double helix molecule that was irregularly supercoiled or folded with an average pitch of 45 Å and cross-section radius of gyration of 30 Å. Attraction between free basic lysine and arginine amino acid residues and free carboxyl groups of histones leads to a high degree of supracoiling of the nucleohistone. Regions of high histone density seem to be separated by areas of DNA free of histones but bound to acidic proteins[195].

Thermal denaturation of chromatin is a method used to probe the structure of nucleohistone and the ways that histones are associated with DNA. The melting temperature of

the DNA in association with histone decreases with guanine and cytosine content of the DNA, indicating a preferential stabilization of adenine and thymine base pairs by histones[118].

Thermal denaturation studies on chromatin samples containing additional histones show them to have a stabilizing effect by increasing the T_m[19,107,151] (defined as the temperature corresponding to one-half the final increase in relative absorbance determined by comparing absorbance at a standard wavelength, e.g. 260 nm, at the temperature sampled relative to absorbance at 25°C).

One of the earliest events accompanying the activation of previously inactive chromatin is its progressive decondensation and the increased binding capacity of DNA for dyes or Actinomycin D[76,112]. Histones have been strongly implicated in the supracoiling of DNA and the further aggregation of these supercoils into mitotic chromosomes[69,117,119]. Histones provide a structural basis for the functioning of DNA in eukaryotic cells. At prophase the DNA condenses to form chromosomes. Histones by virtue of their proximity to DNA have been implicated as essential to properly orient and anchor the DNA in these superstructures[195]. Changes in the binding affinity of histones to DNA may explain the presence of euchromatic and heterochromatic regions in histological staining. Increases of RNA polymerase activity in <u>in vitro</u>

test system containing nuclei or chromatin has been reported after removal of histones. Replacement of histones leads to repression[4,19-21,53,59,74]. RNA transcribed on nucleohistone templates hybridized with DNA to a lesser extent than RNA synthesized on protein-free material[19,20,126]. The results support the supposition that restriction of RNA polymerase transcription must be due to resultant steric alterations of the nucleohistone template by DNA-protein interactions.

Histones have been proposed to act as specific repressors of DNA-directed RNA synthesis[74,163]. According to this viewpoint, histones should be tissue specific, exhibit heterogeneity, vary qualitatively or quantitively during differentiation and development and exert a differential ability of repression on the process of gene transcription.

Chemical and electrophoretic techniques indicate that there are five major histone fractions in most eukaryotic tissues; and in some specialized cases, several other fractions have been detected in minor quantities, probably as a result of chemical modification of histones, or in highly specialized tissues, e.g. chicken eryocytes, spermatozoa. Despite nearly one hundred years of research on histones there is as yet no unified nomenclature for histones. The two most widely used systems are that of

Johns[78] derived from chemical separation (the Johns nomenclature F_1, F_2a2, F_2b, F_3, F_2a1) and that derived from column chromatography on amberlite GC-50 (the Luck nomenclature, I, IIb1, IIb2, III, and IV). There are three major histone fractions in the lysine-rich group and two in the arginine-rich group. The heterogeneous very lysine-rich F_1 (I) histone contains several subfractions, is hydrophobic in nature, consists of about 212 amino acid residues, and has molecular weights in the order of 19,500 - 22,000 daltons. Besides the very lysine-rich F_1 (I), the moderately lysine-(serine)-rich F_2b (IIb2) and the slightly lysine-(arginine)- rich histone F_2a2 (IIb1) represent the lysine-rich histones in higher animals. The arginine-(glycine-arginine)-rich histone F_2a1 (IV) and the arginine-(alanine-arginine)-rich histone F_3 (III) belong to the arginine-rich histone group.

The definite number of histone fractions in various organisms is unknown and may be quite large. Preliminary reports indicate that histones of some primitive and unicellular organisms do not possess F_1 (I) histones[145]. Such is not the case for chromatin obtained from animals and plants. Extensive analytical work indicates that their chromatin contains only five major histone fractions.

The F_1 (I) histone fraction is heterogeneous, the number of subfractions varying between individual tissues in the same organism, as well as among species. There are four or five F_1 (I) histones in rabbit thymus, four in rabbit

mammary gland, three or four in rat and chicken spleen, four in cat spleen, three in calf spleen, and varying numbers in erythrocytes of trout, pike and carp[29,30,83-85,165,185].

Changes in histone amino acid composition or electrophoretic banding pattern have been observed in different organs of peas[48], and during differentiation of peas[89], wheat[172,173], tobacco[162] and radish[56]. Striking changes in histones were observed during development of sea urchin eggs to the gastrula stage[140,174]. Sheridan and Stern[150] observed the formation of a new pre-mieotic histone which gradually disappeared as development proceeded in anther tissue of lily (Lilium longiflorum) and tulip (Tulipa gesneriana). In lily(Hippeastrum belladona) Pipkin and Larson[130] found two more lysine-rich F_1 (I) histone bands in active anther tissue as compared to quiescent tissue. Recently Nadeau et al.[114] have observed the occurrence of unique plant histones which may be modified forms of F_2a2 (IIbI) and F_2b (IIb2). Meanwhile many authors contend that the differences between histones of plant and animal species are so slight that histones must be acting as general repressors of gene transcription [36,46,27,152,155,161,176,195].

Comparison of the amino acid sequence of histone F_2a1 (IV) from pea, and that from calf thymus, reveals striking similarities in various regions with only three

differences in the 104 amino acids of the two molecules[155]. Comparison of the N-terminal amino acid sequence of histone F_3 (III) of various species revealed similarities except at residue 41[25]. Smith et al.[155] commenting on these similarities, suggested that histones perform identical functions in all creatures. Further, because of the high degree of conservatism of the primary sequence, histones must be resistant to evolutionary change.

The specificity with which individual histone fractions act as repressors is controversial. Bonner and his co-workers as well as Mirsky and co-workers contend that removal of the lysine-rich histone F_1 (I) leads to the greatest amount of derepression[4,21,53,74,110,111], whereas there is evidence supporting the arginine-rich histones F_3 (III) as the most efficient repressors[108,159]. Johns and Hoare[80] examined the repressive action of various histones in an RNA polymerase in vitro test-system. They observed that once the DNA-RNA transcription is inhibited by histone, addition of further histone increased solubility of the DNA-histone complex allowing it to become again a template for RNA synthesis. Further, when DNA is precipitated with an equal or lesser amount of histone, histone F_1 (I) is the best repressor. At higher histone ratios, histone F_3 (III) is the best repressor. The authors suggest that the assay for template activity is simply a very sophisticated method for measuring DNA precipitation and that simple

increases in solubility or accessibility to soluble components might suffice to allow RNA synthesis to proceed.

If this is the case, histone removal may not be a prerequisite for initiation of transcription of chromatin DNA. Rather, alterations in some other component of chromatin in transcribable regions might result in increases in DNA transcription. Paul and Gilmour[126] reported that the acidic proteins may act to prevent histones from restricting RNA synthesis. Addition of acidic proteins to a histone-containing *in vitro* RNA polymerase system resulted in partial restoration of the inhibited RNA synthesis[187] by making the chromatin complex more accessible to RNA polymerase. Acidic proteins added to chromatin *in vitro* show tissue specific augmentation of transcription[91]. In differentiated tissues, not undergoing frequent mitosis, the nuclear non-histone proteins are much more metabolically active than histones. Acidic proteins isolated from various tissues have been found to consist of several heterogeneous fractions. It is believed that through interactions with histones the acidic proteins are able to augment transcription[152,164,195].

Several radiosensitive targets have been proposed as sites of the primary lesion in the cell including DNA, long-lived messenger RNA, various intracellular fine structures and cell membranes. Of these, only for DNA is there

a substantial body of evidence. Microbeam irradiation studies[18] have shown that the nucleus is more radiosensitive than the cytoplasm of cells.

Once it had been clearly established that X-rays greatly increased the mutation rate, caused chromosome breaks and loss of genetic material it remained to study the mechanisms by which these effects were brought about. In an early study, Sparrow and Rosenfeld[158] found that *in vitro* irradiation of calf thymus dexyribonucleoprotein (DNP) led to decreases in relative viscosity and streaming birefringence, while Rosenzendael *et al.*[138], in a similar study, found evidence for release of soluble polynucleotides following irradiation. These results seem to indicate that degradation and partial depolymeration of the DNA-protein complex could be a result of either single or double-strand lesions to DNA.

Kaufman *et al.*[82] reported decreased swelling of isolated calf thymus nuclei in water after exposure to 1,000 R of X-rays. Irradiation produced an acceleration of spontaneous loss of viscosity of DNP gels when left for up to 18 hours in the cold. Losses in viscosity seemed to be the result of fragmentation of the DNA as indicated by the formation of low molecular weight fragments and marked decreases in the DNA component. DNP of mouse spleen

extracted in 0.14M NaCl at various times after 850 R of total body X-irradiation[37,154] resulted in exponential drops in concentration of DNP and increases in soluble polynucleotides with time after irradiation. Cole and Ellis[37] concluded that the X-irradiation induced changes in binding between the DNA and the protein component, i.e. labilization, of the nucleoproteins.

Hagen[67] reported that from 1 to 6 hours after in vivo irradiation of rat thymus, extractability of DNA from irradiated tissue increased in proportion to the radiation dose. The results were interpreted as further evidence of radiation-induced labilization of DNA from the DNA complex. Interestingly, when the solubility characteristics and ease of deproteinization of isolated DNA from radioresistant liver and kidney tissue was studied 24 hours after delivery of a lethal X-ray dose to rats, no effects of irradiation could be demonstrated; however, this was not the case for DNP from radiosensitive thymus[12]. Dexyribonuclease activity in whole homogenates of radiosensitive mouse thymus, spleen[129], and radioresistant regenerating rat liver[55] was found not to be increased significantly by irradiation. Release of free DNA from irradiated spleen and thymus

increased with increasing doses of irradiation indicating a shift of DNA to a more soluble form. Increases in the ease of extractability of newly formed DNA occurred 2 hours before the radiation-induced labilization of the pre-existing DNA-protein complex[55;129]. Therefore it can be concluded that the labilizing effects of radiation on the DNA-protein complex are delayed events and not due to the activity of deoxyribonuclease.

Breakage of the strands of DNA and RNA in aqueous solution is caused by attack of radiation-produced reactive species (free radicals) on the sugar moieties resulting in splitting of the phosphate ester bonds[45]. Rupture of the hydrogen bonds between the purine and pyrimidine bases of DNA leads to hyperchromic effects and denaturation of the molecule. Exposure of DNP to dosages of ionizing radiation in the kilorad range does not result in any measurable damage to the nucleic acid bases of nucleoprotein[73,127,143;189]. In the intact nucleoprotein, the free radicals preferentially attack the protein component which seems to provide 90% protection of the DNA.

Irradiation of DNP and histone solutions <u>in vitro</u> with up to 160 kilorads resulted in destruction of approximately 4% histone protein[90,135,136]. Tyrosine, lysine and

and arginine residues were extensively destroyed and accounted for more than one-half of the amino acids released into solution. Destruction of each amino acid in the histone macromolecule was proportional to its concentration and to its rate of reaction with hydroxyl radicals. In addition, the total yield of damaged amino acids of histone solutions was the same as for DNP solutions. This indicates that all the available hydroxyl radicals were scavenged by the histone component in the dose range investigated. Changes in binding affinity between DNA and histone and losses of low molecular weight fragments of histone protein from the irradiated DNP complex[105,168,178] was considered to be the cause of observed decreases in molecular weight of DNP, increases in density of DNA, changes in electrophoretic mobility and in viscosity of DNP when irradiated *in vitro* or *in vivo*.

Losses of histone from the nucleus to the cytoplasm of rat thymocyte nuclei[191] and decreases in chemical extractibility of histone F_2b (IIb2) from calf lymph nodes[8] have been observed after irradiation. Bauer *et al.*[13] isolated DNP and histone from rat thymus 24 hours after delivery of a 1,000 R total body dose. Decreases in the thermal stability of reconstituted DNP (irradiated histone and native DNA) with time after irradiation and changes in histone electrophoretic pattern were observed.

In a study of various DNA primers extracted from livers 24 hours after irradiation and 48 hours after partial hepatectomy, Lehnert and Okada[99] observed that the ratios of irradiated to control ^{3}H-thymidine incorporated in an *in vitro* DNA polymerase system varied in the following way: 1.0 for naked DNA, 0.4 for DNA complexed with histone or acidic protein and 0.3 for nuclei. Radiation-induced reduction of ^{3}H-thymidine incorporation disappeared when the DNP was freed of proteins. Lehnert and Okada concluded that the X-ray-induced lesion resided in the nucleoprotein complex and was not related to changes in the permeability of irradiated nuclear membranes. Further studies by Lehnert and Okada[100] in the same system indicated that ^{3}H-thymidine incorporation into the DNA of irradiated animals was reduced below that of non-irradiated animals. Incorporation of ^{14}C-lysine into histone was unaffected by irradiation. ^{14}C-alanine incorporation into acidic proteins showed increases in incorporation rates into non-irradiated animals not seen in irradiated animals. Any synchrony normally seen in synthesis of protein and DNA in the nucleus is lost after irradiation. The amount of protein, especially histone, relative to DNA increases following irradiation. But in other studies, after total body irradiation, decreases in histone and DNA of rat thymus[11,14], lymphoid organs and liver[170] were found to parallel one another resulting in constant

histone to DNA ratios. In both cases gel electrophoretic analysis indicated that no preferential loss of a specific histone had occurred. It would seem that decreases in histone to DNA ratios after total body irradiation is not a general phenomenon. Further, these observations strengthen the hypothesis proposed by Lehnert and Okada[99] that changes in the binding of the histone component to DNA is not the sole factor involved in the promotion of the primary lesion resulting from X-irradiation.

After it was found that addition of RNA polymerase to an *in vitro* system which contained ATP, GTP, CTP, UTP (triphosphopurine-pyrimidine nucleotides), DNP, DNA or other templates led to RNA synthesis which could be modified by addition or removal of histones[4,20,74], several authors applied the technique to measure the effects of irradiation on the DNA complex. Lehnert and Okada[100,101] found irradiation induced 75% reduction in RNA synthetic activity (priming ability) in liver nuclei. Three inhibitors of protein synthesis, (actinomycin D, 8-azoguanine, and ethinonine), were found to depress thymidine kinase and DNA polymerase activity as much as X-irradiation alone. Changes in enzyme synthesis but not priming ability was found to parallel changes in DNA synthesis *in vivo*. The degree of inhibition induced by irradiation was of the same magnitude. The authors suggested that radiation both interfered with

biosynthesis of enzymes necessary for entry from G_1 into S and changed the state of the DNP template necessary for DNA synthesis reflected in priming ability after the cells entered S stage. In this sense radiation-induced alterations in enzyme activity would be more directly involved in the regulation of DNA synthesis than priming ability.

Weiss and Wheeler[190] found doses up to 12 kRad resulted in 30% reduction in the priming ability of DNP in an *in vitro* test system. Higher doses (36, 48 and 72 kRad) resulted in increases of DNP priming activity such that at 72 kRad activity was approximately 40% greater than control DNP. The initial drop was probably due to radiation damage to the non-complexed DNA. The results seem to suggest that one of the effects of ionizing radiation at high doses is to cause the protein sheath to become more detached from the DNA opening new sites for RNA synthesis.

In a more detailed study Goddard *et al.*[54] and Hagen *et al.*[68] proposed that the 90% loss of priming activity induced by radiation is a result of single specific lesions in DNA which stopped RNA synthesis along the template. At higher doses of ionizing radiation there was evidence of formation of new binding sites for RNA polymerase which may contribute to observed increases in RNA synthesis. The chain length of RNA synthesized was decreased by irradiation,

but not the number of chains synthesized. Since the priming activity of RNA polymerase decreased in proportion to the molecular weight of DNA used as template, a single break induced by radiation may be enough to stop the process of gene transcription in irradiated tissues.

Paskevich[124] observed that the levels of rat liver DNP template activity assayed in an in vitro test-system using either exogenous E. coli RNA polymerase or endogenous RNA polymerase, increased 24 and 48 hours after irradiation with 400, 600 or 800 R of X-radiation. However, the degree of activity exhibited a distinct dose dependence. After 120 hours, RNA synthesis was reduced below that of non-irradiated animals. RNA synthesis of radiosensitive spleen using exogenous or endogenous RNA polymerase was reduced at all levels of radiation below that of non-irradiated animals. The data indicates that most probably radiation of rat liver but not radiosensitive spleen causes de-repression of the DNA template and also a partial activation of RNA polymerase as measured by the in vitro RNA synthesis test system.

Radiation has been shown to cause destabilization of the secondary structure of DNA. In a study of the interactions of histones with heat-denatured, irradiated and native DNA isolated from rat thymus and liver, Umanskii and

co-workers[180,181] observed reductions of template activity of DNA and chromatin after irradiation. Template activity of rat-liver chromatin in vitro was found to increase up to a dose of 10 KRad and then declined thereafter. Histones caused less precipitation and inhibition of template activity for RNA polymerase with irradiated DNA than with native DNA. This was due to the ability of both denatured and irradiated DNA to bind an excess of histones on account of recharging and redistribution of the total positive charge leading to higher solubilities of the histone-denatured DNA complex. It had been suggested by Johns and Hoare[80] that the repressive action of histones is associated with their ability to precipitate DNA. According to this hypothesis increases in RNA synthesis may be achieved by rendering the complex more soluble or more accessible to RNA polymerase by changing the local state of aggregated DNP. It can therefore be assumed that a change in the ability of irradiated DNA to be precipitated by histones is associated with the appearance of denatured or destabilized portions in the irradiated DNA which result in higher solubility of the irradiated DNA complex.

Recently, the effect of radiation on the activity of endogenous chromatin-RNA polymerase isolated from sugar beet tissue has been reported by Cherry and co-workers[43,44]. 300 KRad of γ-radiation at any time following washing of the

tissue was found to result in a 50% inhibition of endogenous RNA polymerase template activity and 60% reductions in incorporation of ^{3}H-uridine into nucleic acids. Exposure of tissue to 400 KRad severely inhibited (46%) the incorporation of ^{3}H-uridine into polyribosomes. Inhibition of RNA polymerase activity was not due to enhancement of ribonuclease activity in irradiated tissue. Activity of endogenous RNA polymerase of isolated chromatin exposed to increasing dosages of γ-radiation was severely inhibited at doses beyond 100 KRad. This indicated that γ-rays altered the function of the enzyme of other chromatin components, such as the DNA template in RNA synthesis. Soluble RNA polymerase preparations of irradiated chromatin were found to transcribe DNA of control or irradiated tissue less than did control enzyme on irradiated DNA suggesting that irradiation of RNA polymerase alters transcription activity more than does damage to the DNA template.

B. Histone Metabolism

Since histones are essential components of eukaryotic chromatin and most likely regulate both its structure and function acting as genetic repressors, ordered rates of histone biosynthesis closely paralleling replication of other chromatin components would seem to be necessary for replication and maintaining the differentiated state of the cell. Cytoplasmic synthesis of histones has been shown to

occur during spermatogenesis in grasshopper[17], salmon testis cells[102], sea-urchin embryos[81], and HeLa cells grown in tissue culture[134], as well as in isolated cytoplasmic polysomes[26,77]. The histone proteins are assembled on small polysomes found only when cells are actively engaged in DNA synthesis and only during the S phase of the cell cycle. If DNA synthesis was blocked by cytosine arabanoside or 5-fluorodeoxyuridine they disappeared rapidly. Blocking messenger RNA (mRNA) synthesis with actinomycin D did not affect *in vitro* histone biosynthesis for at least 90 min indicating relatively long half-lives for histone mRNA's. In rapidly, proliferating and undifferentiated cells, such as HeLa cells, part of the synthesis of all of the histones is independent of DNA synthesis[142] and continues into G_1. Initiation of DNA synthesis leads to a marked increase of histone biosynthesis even in these cases. Evidence from protein synthesis of protamines from salmon testis[102] and histones of HeLa cells[134] indicates that these proteins are transferred to the nucleus immediately following synthesis.

In Novikoff cells when DNA synthesis was decreased without a concomitant decrease in histone synthesis, pulse-chase experiments indicated that the pool size of free histone in actively growing cells was small and equivalent to less than 2 min of histone synthesis time[120]. The pool is extra-nuclear.

In X-irradiated cells obtained from tissue culture, Gurley et al.[58-61] observed irregularities of biosynthesis and turnover of F_1 (I) histone which suggested that there may be an extra-chromatin pool of histones F_1 (I) and F_3 (III). It appears that under normal conditions biosynthesis and turnover of histones are well co-ordinated with that of DNA but after irradiation, histone synthesis begins to exceed DNA.

From the preceding discussion it is evident that chromosomal DNA must be free of histones in order to function as a template for RNA synthesis. In this sense histones are general repressors of transcription. Several authors have proposed that if histones do act in the variable restriction of DNA then there must be a mechanism that would allow selective removal of histones from DNA. Allfrey and Mirsky[4] discovered that partial acetylation of ε-N-amino groups of lysine decreased the inhibitory effects of histone-like protein on *in vitro* RNA synthesis. This finding stimulated an extensive search for chemical modifications of histones which could occur *in vivo*. It was anticipated that such modifications were of significance in the control of transcriptional activity of chromatin. Since then several modifications of histones *in vivo* have been demonstrated; acetylation, phosphorylation and methylation of certain amino acids being the main ones.

Phytohemagglutin (PHA)-stimulation of lymphocyte cell cultures[131], cortisol administration to adrenalectomized rats[1], estradiol-17β given to rat uteri[5], and partial hepactectomy of rat liver[132] all were found to increase rates of acetylation of arginine-rich histones compared to lysine-rich histones. Rapid acetylation of the arginine-rich histones preceded increases in nuclear RNA synthesis seen in these systems. Since acetylation was not affected by puromycin, an inhibitor of protein synthesis, acetylation of histones must occur after biosynthesis and most probably in situ in the chromatin. The authors concluded that acetylation in gene activated systems leads to changes in basic proteins which are prerequisite to later increases in RNA synthetic activity.

Further studies by Allfey and co-workers[1,2,186] established that acetyl-CoA was the acetate donor, that acetylation was enzyme mediated, reversible and not inhibited by protein inhibitors. Acetate was incorporated into the intact molecule and could occur at multiple sites within the same histone molecule. Acetyl-group transfer seemed to be preferentially directed towards the arginine-rich F_3 (III) and F_2al (IV) histones with 50-60% incorporation into calf thymus histone F_2al (IV) of the lysine at position 16. A high specificity of histone acetylation was indicated since only 1 lysine (position 16) residue out of 11

lysines in the molecule was acetylated.

During the interphase portion of the cell cycle of eukaryotic cells, a part of the chromatin remains persistently condensed (heterochromatin), while the remainder is dispersed as finely distributed threads (euchromatin). By use of cytochemical staining methods high histone contents have been found in the condensed, heterochromatic regions of chromatin while the less dense euchromatic regions seemed to be more active in protein and RNA synthesis[15,16].

If acetylation is correlated with increased RNA transcription then acetylation of diffuse chromatin and nuclear chromatin should be higher than in condensed or nucleolar-associated chromatin. Studies on acetylation in these structures[16,50,70] indicate that histones in these two types of chromatin are acetylated at equal rates. In these cases it would seem that histone acetylation is not directly related to the amount of active template. At present it is safe to say that it is not possible to conclude unequivocally that histone acetylation leads to structural changes in chromatin which would result in the activation of DNA transcription.

Synchronized cultures of mammalian cells incubated with ^{3}H-acetate[148] rapidly acetylated histones F_2a2 (IIb1), F2b (IIb2) and F_3 (III) to a maximum in late S, coincident with the termination of DNA synthesis, and declined rapidly

thereafter. Histone F_1 (I) acetate content rose rapidly in mid-S coincident with maximum DNA synthesis and declined thereafter. Histone F_1 (I) was acetylated to the greatest degree but did not retain its incorporated acetate. This would seem to suggest that histone fraction F_1 (I) is synthesized and acetylated prior to the other fractions, which could be construed as an early event in the alteration of the chromatin.

When these same cultures were X-irradiated[149] the DNA, protein and histone contents of the cell population rose during the division delay period, approximating levels attained by normal cells in the G_2 portion of their life cycle. Accumulation of labelled acetate in histone fractions F_1 (I), F_2b (IIb2) and F_3 (III) of irradiated cells paralled control values, while accumulation of incorporated acetate into histone fraction F_2a2 (IIb1) of irradiated cells was depressed below that of controls.

Methylation of histones occurs in nuclei and is inhibited by inhibitors of RNA synthesis. The reaction is enzymatically catalyzed[3,52]. The immediate methyl donor has been identified as S-adenosylmethionine. The transfer of the methyl group of methionine <u>in vivo</u> occurs preferentially to the lysine residues of the arginine-rich histones. In synchronized HeLa cells[22] and Chinese hamster ovary cells[146] F_1 (I) histones are not methylated. Slight

methylation of histone F_2a2 (IIb1) and extensive methylation of arginine-rich histones F_3 (III) and F_2a1 (IV) occurred, rising during S to a maximum after termination of DNA and histone synthesis, coincident with the beginning of mitosis, and began to fall by mid-M. Methyl content of fraction F_2b (IIb2) rose to a maximum in early S coincident with initiation of DNA synthesis and rapidly declined thereafter. In X-irradiated Chinese hamster ovary cells[149] accumulation of labeled methyl groups as methyl lysine derivatives in histone fractions F_2a2 (IIb1), F_2b (IIb2) and F_3 (III) was depressed below control values 24 hours post-irradiation. No methylation of fraction F_1 (I) occurred in either control or irradiated cells.

Methylation occurs independently of histone synthesis[175]. The peak of methylation occurs when rates of DNA synthesis are declining. Methylation does not result in increases of DNA template activity for RNA synthesis. Methylation of histones may be correlated with structural and functional changes in chromatin known to result in the nucleus prior to mitosis, particularly during the condensation of chromatin and during the ensuing curtailment of nucleic acid synthesis. In support of this theory are the observations of Borun et al.[22] that in synchronized HeLa cells, maximal transfer of methyl groups occurred during the time of maximal condensation of the chromatin, i.e. during G_2,

M, and very early G_1 of the cell cycle.

Chromosomal abberations and increased mutation rates were first observed by Sax in X-irradiated Tradescantia sp. microspores. The net abberation frequency depended not only on the initial production of chromosome breaks but also on the existence of rejoining mechanisms dependent on ATP synthesis[193]. This phenomena may be a contributing factor to accumulated sub-lethal effects of irradiation, especially since Creasy and Stocken[39] have shown that nuclear phosphorylation and ATP synthesis are one of the most sensitive processes to ionizing irradiation depressed by as little as 25 Rads in several cell systems.

The serine groups of F_1(I) histone of calf thymus has been found to be actively phosphorylated resulting in o-phosphoserine[87,166]. Histones other than the very lysine-rich histone F_1 (I), including F_2b (IIb2) and F_3 (III), are phosphorylated but to lesser extents[64,86]. In each case, the phosphoryl group donor was identified as being derived from ATP. Phosphorylation of histones in the cell nucleus could not be inhibited by inhibitors of protein synthesis.

Although the biological significance of histone phosphorylation is not yet known, there are a number of observations which relate the process to increases in gene activity. Histone phosphorylation occurs in higher

proportions in active (diffuse) chromatin and is tissue specific[7,64,167].

Histone phosphorylation in target tissues is affected by hormone administration. Cortisol, which stimulates RNA synthesis in liver, increases the phosphorylation of the lysine- and arginine-rich histones within 30-90 min after administration[113]. The lysine-rich fraction shows the greatest increase. The effect is not due to changes in the ATP pool since the specific activity is not significantly altered. Administration of glucagon to rats[97] at doses which are effective in inducing RNA and enzyme synthesis caused a 15-25 fold increase in phosphorylation of very lysine-rich F_1 (I) histones within the first hour. In the same study insulin also stimulated histone phosphorylation. The phosphorylation of histones is catalyzed by specific histone kinases[96]. Activation of the enzyme by adenosine 3'-5'-monophosphate (cyclic AMP) has been demonstrated *in vitro* and *in vivo*[97] by Langan. Lymphocytes transformed by PHA[40,88,131] show increased phosphorylation of histone F_1 (I) within 15 min. Therefore, phosphorylation, like acetylation, precedes the increase in mRNA synthesis characteristic of this system. In regenerating rat liver a tissue undergoing rapid RNA synthesis as compared to normal liver, the rate of phosphorylation and phosphate content of the F_1 (I) very lysine-rich histones increased by a factor of two within 16 to 24 hours[121,166,167]. Histone F_3 (III) shows a

similar rate of phosphorylation but no change in phosphate content. The higher phosphate content of the F_1 (I) seems to be due to an increase in the number of F_1 (I) molecules phosphorylated. The capacity of histone F_1 (L) to suppress DNA dependent RNA synthesis decreases as the phosphate content increases.

In animals exposed to γ-irradiation, phosphorylation of F_1 (I) histone is decreased to the same extent as DNA synthesis. There is no corresponding change in phosphate uptake into F_3 (III). Similarly, irradiation delays phosphorylation of F_1 (I) histone in radiosensitive regenerating rat liver and kidney[121,122,167]. The effects are not likely to be due to changes in the specific activity of the ATP pool since uptake of ^{32}P into labile phosphate was unaffected by irradiation. The peak of phosphorylation occurred before maximum rates of DNA synthesis were achieved.

Rat liver irradiated with 600 R[125] and then examined after 24, 48 and 120 hours exhibited increases in phosphorylation of all nuclear protein fractions, especially the non-histone proteins. Radiosensitive spleen when irradiated at 400 R exhibited decreases in incorporation of ^{32}P into nuclear proteins. Paskevich and co-workers[124,125] concluded that changes in the content of phosphate groups led to changes in the functional properties of the nuclear proteins. Considering the role assigned to phosphate

groups in control of RNA synthesis, disruptions in the state of the nuclear proteins are certainly among the critical factors of post-irradiation disturbances of cellular metabolism.

Work in cell cultures concerning DNA synthesis and phosphorylation of histones during the cell cycle[62,63,147] indicates that ^{32}P-orthophosphate accumulates in F_2b (IIb2) prior to S, rises to a maximum at mid-S and decreases thereafter, i.e. phosphorylation is largely dependent on newly synthesized histone and in the case of histone F_2b (IIb2) occurs before maximum rates of DNA synthesis. No dephosphorylation occurs during G_1 or M. The ^{32}P accumulation in F_1 (I) protein begins prior to S and continues in linear fashion through the remainder of the cell cycle. This indicates that F_1 (I) phosphorylation has little or no dependence on availability of newly synthesized histone and further the phosphorylated histones are turned over rapidly. Histone F_3 (III) phosphorylation occurred during metaphase and was negligable during G_1, S, or G_2. Histone F_3 (III) and a F_1 (I) subfraction were observed to be rapidly phosphorylated only at the time of cells crossing the G_2/M boundary and when transversing prophase. Histone F_1 (I) phosphorylation is absent during G_1 and commences just before S making it a very early event associated with conversion of non-dividing cells into dividing cells. The evidence suggests that phosphorylation of F_1 (I) histones

and possible F_3 (III) is initiated as a preparation for cell division and may be involved in the condensation of interphase chromatin into mitotic chromosomes.

Newly synthesized histone F_1 (I) from mammalian tissue cultures was phosphorylated after a lag period of 30 min of histone synthesis time. Because of the large delay observed between synthesis and phosphorylation, it is apparent that phosphorylation of the histone F_1 (I) must have taken place while the histone molecule was part of the nucleohistone. Therefore, phosphorylation could not be considered to be a transport device but a mechanism to modify the responses of nucleoproteins to stimuli. Turnover of F_1 (I) following irradiation[60] is stopped for the same period of time as phosphorylation inhibition by irradiation. Resumption of F_1 (I) turnover is coincident with resumption of phosphorylation. However, the dose used, 800 Rads, did not affect the phosphorylation of histone F2a2 (11b1), nor did it decrease the rate of DNA replication indicating that phosphorylation of F_1 (I) histone was not directly involved in DNA replication. Also, since F_1 (I) and F_2a2 (11b1) phosphorylation were uncoupled from each other by radiation, they probably have two different functions <u>in vivo</u>. Gurley and Walter[61] recently observed that F_1 (I) synthesis was uninhibited after X-irradiation of Chinese hamster ovary cells. These observations

indicate that normal phosphorylation rates of F_1 (I) are not necessary for either deposition of newly synthesized F_1 (I) into chromatin or for DNA replication.

MATERIALS AND METHODS

A. Plant Material and Growth Conditions

Pine seeds (Pinus pinea) obtained from Arturo Ansaloni, Bologna, Italy were used as a source of plant material in these investigations. The dry seeds were surface sterilized in dilute Javel water for one hour, then soaked in tap water overnight at room temperature prior to planting in flats of fine sterilized sand. The seedlings were then grown for the desired length of time in a controlled environment chamber at 450 ft-c, 16 hr photoperiod, 45% relative humidity at 21°C before the cotyledons were harvested. In most of the experiments the cotyledons were harvested after fourteen (14) days of germination. At this time the cotyledons are in a period of active cell division. Therefore, it is expected that this tissue should yield adequate amounts of nuclear material.

B. Radiation

In several experiments, after 14 days germination, equal batches of seedlings were removed from the flats and their seed coats removed. The seedlings were then placed between moistened paper towels, and one batch received 1 kR of 260 kVp X-rays (7 ma) at 43 R/min, 0.28 mm aluminum filtration, under maximum backscatter conditions, delivered from a Müller MG 300 X-ray machine. Dose measurements were

performed using a Victoreen condenser R-meter with 200 R probe (barometric pressure and temperature corrected) and by Fricke chemical dosimetry. The other batch was not irradiated and served as a control. Both control and irradiated seedlings were then stored at room temperature between the moistened paper towels prior to extraction of nucleohistone.

Irradiation of purified nuclei was performed in centrifuge tubes on crushed ice at a dose-rate of 270 R/min. Conditions and machine settings were the same as those previously described.

C. Feulgen Staining of Nuclei

Cotyledons from different seedlings were chosen at random from both irradiated and control populations prior to extraction of nucleohistone. The cotyledons were fixed overnight in Carnoy's solution, acetic-acid:absolute ethanol (1:3), and then stored in 60% ethanol. The ethanol was removed and the cotyledons were hydrolyzed in 1N HCl at 60°C for exactly 7 minutes. The vials containing the cotyledons were cooled. The cotyledons were rinsed twice with distilled water and stained with freshly prepared Feulgen reagent. The samples were then stored in the refrigerator overnight. Small amounts of tissue from single cotyledon tips were then squashed in a drop of 45% acetic acid, mounted on slides and examined with a light microscope.

D. Isolation of Plant Nuclei

Nuclei were isolated by the method outlined in Fig. 1. In all cases, nuclei were extracted from cotyledons which had been excised, placed in plastic bags and frozen on dry ice immediately after harvesting. The solidly frozen tissue was then ground in an ice-cold mortar and pestle with addition of some powdered dry ice. This facilitated extraction of nuclei while allowing rapid penetration of the grinding medium into the broken fibres which would otherwise hinder the extraction procedures. All operations from extraction of nuclei to final procurement of histone powder were carried out at zero to four degrees centigrade by use of ice buckets, a walk-in cold room, and refrigerated centrifuges. Approximately 50 to 100 grams fresh weight of tissue were used for the extraction of nuclei, although in some cases, smaller amounts were used. The frozen, ground tissues were placed in a Waring blender along with five volumes of grinding medium which consisted of: 0.4M sucrose, 0.01M $MgCl_2$, 0.01M Tris, 0.025M $NaHSO_3$ in double distilled water and adjusted to pH 8.0 with sodium hydroxide. Stock solutions of grinding medium were kept at concentrations five times that desired in all constituents except $NaHSO_3$. This unstable compound was added just as the stock solution was diluted before use. Sodium bisulphite was added to inhibit proteolysis[123]. Sucrose in the grinding medium served to

provide a slightly hypertonic solution to insure that nuclei would not burst due to osmotic shock. Magnesium ions were included to stabilize the nuclear membrane. The tissue was allowed to stand in the grinding medium for one hour to allow the infusion of the magnesium ions in order to stabilize the nuclei. The tissue was then ground for one and a half minutes at maximum speed in a Waring blender to break the cells and release the nuclei. The brei resulting from the grinding was then filtered through four layers of cheese-cloth, and the residue was ground in the grinding medium once more to increase the yield of nuclei. The combined filtrates were then filtered through a series of Nitex (Tober, Ernst and Traber, Inc., Elmsford, N.Y.) nylon meshes in the following order: 375 μ, 100 μ, 25 μ, and 10 μ. In order to facilitate the filtering of the nuclei through the last two screens, suction was used. This process effectively removed all larger cell fragments and whole cells so that only intact nuclei and smaller material were able to pass through the 10 μ mesh filter. The filtrates were then centrifuged at 500 X g at 4°C for ten min. The pellets, after centrifugation, contained nuclei, starch grains, chloroplasts, cell wall material and membranes, but no whole cells. The pellets were then further purified by resuspension in a washing medium consisting of 0.25M sucrose, 0.01M $MgCl_2$, 0.01M Tris, 0.025M $NaHSO_3$, 1% (v/v) Triton X-100 using double distilled water and adjusted to pH 8.0 with

sodium hydroxide. Stock solutions of washing medium were stored at five times the concentration of all constituents except the sodium bisulphite which was added just before use. Triton X-100 is a detergent used to solubilize the chloroplast membranes. Two washes in this medium was found to be sufficient to remove all of the chloroplasts. The pellets obtained after centrifugation at 500 X g for 10 min were resuspended in the washing medium and centrifuged as before. After the second washing the nuclear pellet was relatively pure, but contained some contamination in the form of starch grains, membrane and cell wall material.

The starch grains are of no consequence as a contaminant but the pellets may contain basic proteins of cell membranes with attached ribosomes which must be separated from the nuclei. This separation was accomplished by centrifuging the nuclei and starch grains through a sucrose solution consisting of: 2.3M sucrose, density gradient grade (ribonuclease-free), 0.01M $MgCl_2$, 0.01M Tris, 0.025M $NaHSO_3$, 1% (v/v) Triton X-100 using double distilled water and adjusted to pH 8.0 with sodium hydroxide.

The nuclear pellet after the second washing was homogenized in a 2.3M sucrose using a Potter-Elvehjem glass and teflon homogenizer. The resulting suspension which was now approximately 2.2M in sucrose was then layered on top of the 2.3M sucrose in centrifuge tubes. The interface between the

two sucrose solutions was disturbed slightly, so that the sedimenting nuclei were not prevented from moving by surface tension. Ultra-centrifugation was performed in a MSE SS-75 ultra-centrifuge using a 3 X 23-ml aluminum swing-out rotor for 2 1/2 hours at 4°C with a maximum centrifugal force of 60,000 X g. After completion of the centrifugation, only nuclei and starch grains were left in the pellet. The membranes and cell membrane material with attached ribosomes were decanted with the supernatant. The nuclei resulting from these procedures were then used to obtain chromatin, nucleohistone and histones.

E. Preparation of Chromatin, Nucleohistone and Histones

Chromatin was obtained from the nuclei as outlined in Fig. 2. The nuclei after sedimentation through sucrose were separated from the lower layer of starch grains. The nuclei were then ruptured with 3-4 strokes of a Dounce loose-fitting ball glass homogenizer in a 0.02M solution of ethylenediaminetetraacetate (EDTA) containing 0.025M $NaHSO_3$ adjusted to pH 8.0 with sodium hydroxide. EDTA chelates magnesium making nuclei more susceptible to rupture through osmotic shock. The suspension of ruptured nuclei was then centrifuged at 20,000 X g at 5°C for ten min. The pellets were then resuspended in the EDTA solution and the centrifugation repeated. During this procedure the pellets became compact and sticky. The pellets were then gently homogenized

in cold doubled glass-distilled water at neutral pH and centrifuged at 20,000 X g for ten min at 5°C. The pellet was then resuspended in distilled water and the centrifugation repeated. This procedure of washing the pellet of ruptured nuclei twice with water reduces the ionic strength and molecular binding forces of the chromosome material. The formerly compact, sticky pellet becomes highly expanded, viscous, and forms a gel. Starch grains were easily separated from the gel at this stage.

The gel was allowed to swell in double distilled water overnight, and the chromatin was then sheared for 1 1/2 min at maximum speed at 4°C in a semi-micro head of a Waring blender. Nuclear membrane material containing protein, attached ribosomal particles, lipids and some DNA were removed by centrifugation. The supernatant resulting after shearing and centrifuging contained the soluble nucleohistone and was saved for further analysis.

The soluble nucleohistone was then divided into equal portions. One half was retained for extraction of histones and acidic proteins. The other half was used for isolation of DNA and RNA.

Histones were extracted from nucleohistone by making the nucleohistone 0.4N in H_2SO_4 as outlined in Fig. 3, with addition of an equal volume of 0.8N H_2SO_4. The resulting solution was stirred for 3 hours at 5°C. At this concentra-

tion of acid the histones are dissociated from the nucleic acids and acidic protein leaving the histones sulphates in solution. The histones were collected by centrifugation at 5°C at 20,000 Xg for 10 min. The supernatant containing the histone sulphates was then dialyzed overnight against 95% ethanol. The next day the histone precipitate was collected by centrifugation at 5°C at 20,000 X g for ten min. The pellets were then dried after centrifugation in acetone 2 times and stored frozen prior to analysis. The precipitated DNA-acidic protein complex was mixed with 5 ml of 0.5N perchloric acid at 90°C for ten min. The DNA was then removed by centrifugation at 20,000 X g for ten min. The precipitate (acidic protein) was then centrifuged twice in acetone, dried, and stored frozen. Protein present in the acidic protein and histone fractions was then determined by the method of Lowry et al.[106].

F. Fractionation of Nucleohistone by the Schmidt-Tannhauser Method into DNA and RNA

The fractionation proceedure to obtain DNA and RNA from the nucleohistone is outlined in Fig. 4. Nucleohistone solution was added to an equal volume of 10% v/v trichloroacetic acid (TCA) and was left to stand 20 min on ice. The macromolecules precipitated by this procedure were then collected by centrifugation for 10 min at 1,800 X g. The pellet was then washed once by centrifugation with

petroleum ether (40-60°C) to remove lipid material. The pellet was then suspended in 2 ml of 0.3N KOH and incubated at 37°C for two hours in order to hydrolyze the RNA. After incubation the sample was then cooled on ice. One ml of 1N PCA was added to acidify the sample. At pH <2.0 the DNA precipitates from solution. RNA was then collected as the supernatant after centrifugation for 10 min at 1,800 X g. The precipitate was then washed with 1 ml 1N PCA by centrifugation, the supernatant being added to the RNA sample. The pellet was then washed twice by centrifugation with 10% TCA to remove impurities from the DNA pellet. The washes were discarded. The RNA sample was then neutralized with 1N KOH and left in the cold overnight to ensure maximum precipitation of the potassium perchlorate salts. The DNA-protein pellet was then suspended in 2 ml 0.5N PCA and hydrolyzed at 90°C for ten min. Soluble polynucleotides form which leave the protein as a residue. After cooling, the supernatant was collected for analysis of DNA. The residue containing the acidic protein was discarded after centrifugation. The supernatant, containing the hydrolyzed DNA was then neutralized with KOH and left to stand overnight in the cold.

G. Physical Analyses

(i) Ultraviolet Absorption Analysis of Nucleohistone

The absorption spectra of soluble nucleohistone was

determined from 325 nm to 230 nm with a Unicam SP 800 recording spectrophotometer. Typical spectra are illustrated in Fig.10 and 11. The ultraviolet (UV)-absorption characteristics of soluble nucleohistone of purified material should possess OD ratios 260/240 and 260/280, of 1.3-1.4 and 1.6-1.7 respectively. In addition, the absorption at 320 nm should be close to zero. If not, then the material is most probably contaminated with ribosomal RNA or proteins or both.

(ii) Thermal Denaturation

Irreversible melting as a measure of the thermal denaturation of nucleohistone described by Bonner et al.[19], was carried out using a Bausch-Lomb 700 Spectrophotometer or a Unicam SP 800 Recording Spectrometer at either fixed wavelength or using fast scan in the UV-absorption region. The sample to be analyzed was first diluted to a standard OD at 260 nm, and then heated in a closed vessel on an open water bath. Temperature was kept constant to within one degree for five minutes, after which the sample was cooled to room temperature and the optical absorbance determined at 260 nm. The sample was then heated at the next temperature, selected at 5 degree intervals, cooled and its absorbance read until a series of readings were obtained throughout the temperature range studied. To calculate the midpoint of the thermal denaturation curve, readings at various temperatures

were divided by the reading at room temperature (25°C). One-half the difference of the value at the highest temperature minus the lowest value yields the midpoint or T_m. In some cases, Table 3 and Fig. 11, optical absorbance increases due to turbidity were corrected by extrapolation of the semi-log plots of the absorption at wavelengths beyond 320 nm, i.e., 340, 350, 360 nm, to 260 nm and subtracting the values obtained from the absorbance measured at 260 nm.

(iii) Viscosity Determinations

Since irradiation is known to affect the viscosity of double-stranded nucleohistone and DNA[82,138,158], viscosity of nucleohistone isolated from irradiated nuclei was measured and compared to that of their own controls. In all experiments, samples of nucleohistone were adjusted to a standard absorbance at 260 nm and determined immediately after shearing. Relative viscosities were determined at 25°C using double distilled water as a reference solvent with an Ostwald viscometer. Four or five measurements of the flow rate were taken from which the average was determined. Relative viscosity is defined as the ratio of the average flow time of nucleohistone solution to the flow time of distilled water.

H. Quantitative Analyses

(i) Determination of Protein

Acidic protein was dissolved in 1N NaOH. Histones were dissolved in a solution of 15% sucrose in 0.9N acetic acid or 1N HCl. Two ml of Lowry reagent was added to 0.4 ml of protein solution in 1N NaOH. Lowry reagent consists of 25 ml of solution A and 0.5 ml of solution B. This mixture is highly unstable and must be used immediately. Solution A was prepared by making up 4 g Na_2CO_3 in 200 ml 0.1N NaOH. Solution B was prepared by addition of 0.25 g $CuSO_4 \cdot 5H_2O$ and 0.50 g sodium potassium tartarate to a final volume of 50 ml. The pH was adjusted to 6 by addition of NaOH. The samples were mixed and allowed to stand at room temperature for 10 min before addition of 0.2 ml of Folin-Ciocalteu reagent. The samples were then mixed well and allowed to react for 30 min at room temperature. Optical density was read at 750 nm using a Bausch-Lomb Spectronic 700 spectrophotometer. Standard curves of bovine serum albumin (fraction 5) were prepared each time and used to calculate amounts of acidic protein present. In some cases standard curves of calf thymus histone were used for the determination of histone protein. Histone protein was also determined by the turbidity method. Two-ml samples were precipitated with an equivalent amount of cold 2.2M TCA, left twenty minutes in the cold (5°C) after which their optical

absorbance was read at 400 nm. Amounts of protein present were calculated from a standard curve prepared from calf thymus histone.

(ii) Determination of Nucleic Acids

DNA and RNA were determined both from UV-absorbance[19] and by colour tests. DNA was measured by the diphenylamine method of Burton[28] and RNA by the orcinol method of Ogur and Rosen[116]. To a one-ml sample of DNA, 2 ml of diphenylamine reagent was added. The samples and standards were then incubated overnight at 37°C and their absorbance measured at 600 nm. Standard curves of DNA were prepared each time and the amounts of DNA present in each sample was read from the standard curve. The diphenylamine reagent was prepared by addition of 0.25 ml solution B to 50 ml solution A. Solution A is 0.75 g of diphenylamine and 0.75 ml H_2SO_4 added to 50 ml glacial acetic acid. Solution B consists of 0.2 ml acetaldehyde in 10 ml double distilled water and must be prepared in the cold using a cold pipette.

To determine RNA, 3 ml aliquots of the RNA solution were combined with 6 ml of acid reagent and 0.4 ml orcinol-alcohol solution. The acid reagent contains 2 ml 10% $FeCl_3 \cdot 6H_2O$ in 400 ml concentrated HCl. The orcinol-alcohol solution is 6% orcinol in 95% ethanol and must be prepared freshly each time. The solution of ribonucleotides and

orcinol reagent was stoppered prior to incubation on a boiling water bath for 20 min. The samples were then cooled and their absorbance determined at 600 nm. The amount of RNA in each sample was calculated by comparison to its own standard curve using yeast RNA samples as reference standards which were treated in the same manner as the samples.

I. Acrylamide Gel Electrophoresis of Histones

(i) Preparation of Glass Tubes

Polyacrylamide gels were polymerized in glass tubes of 6 mm internal diameter and 10 cm in length. Before use, the glass tubes were cleaned with acid, rinsed with tap water, followed by washing in double distilled water and acetone. The tubes were then dried at 75°C. To facilitate the removal of the polyacrylamide gels the glass tubes were coated with dichlorodimethylsilane by dipping then in a 1% (v/v) solution of dichlorodimethylsilane in benzene.

(ii) Preparation of Acrylamide Stock Solutions

Approximately enough acrylamide was dissolved in double distilled water by heating and stirring to yield a 70% (w/v) solution. Yellow impurities in the resulting solution were removed by stirring in activated charcoal. The slurry was allowed to stand for several minutes before

suction filtration on a Buchner filter. The filtrate was then used to prepare an acrylamide stock solution which is 60% (w/v) acrylamide and 0.4% (w/v) bisacrylamide. N,N,N',N'-tetramethylethylenediamine (Temed) was used to speed the polymerization reaction. The Temed stock solution was 4% (v/v) Temed and 43.2% (v/v) acetic acid. The initiator of the polymerization reaction is ammonium persulphate which was added to a 4M urea stock solution just before use at a concentration of 0.2% (w/v).

(iii) Preparation of Polyacrylamide Gels

To make 12 7-cm gels of final concentration 15% acrylamide, 0.1% bisacrylamide and 2.5M urea, the stock solutions described above were added together in proportion of 4 ml acrylamide stock, 2 ml Temed stock, and 10 ml 0.2% ammonium persulphate. The final mixture was then degassed using a tap aspirator. One ml of the mixture was then transferred into the glass tubes which were wrapped with parafilm at one end. A rack was used to maintain the gel tubes in an erect position. After addition of the acrylamide solution, 0.5 ml of cold acetic acid solution was added to the top of each tube. The addition of acetic acid eliminates the meniscus of the acrylamide and ensures a flat top on the gels which is necessary for the attainment of straight bands in the final electrophoregrams. Polymerization was usually

completed 45 min after addition of the acrylamide to the tubes.

The ammonium persulphate and Temed were then removed from the gels by electrophoresis in 0.9N acetic acid. A constant direct current of 130 volts was applied to the gels for 1 1/2 hours from a Buchler Instruments model 3-1014 A power supply. Negative polarity was used, that is, the cathode in the lower chamber and the anode in the upper chamber of the electrophoresis apparatus. Benzene-azo-α-naphthylamine dye was layered on top of one of the gels to ascertain when pre-electrophoresis was finished. At pH 2.4 the dye possesses a single charge and because it is of relatively high molecular weight all of the Temed and persulphate are removed by the time the dye has moved out of the gel. Since the gels heat slightly during the pre-electrophoresis step, the power supply should be turned off for 1/2 hour before actual electrophoresis. If the histones are layered on immediately, curved bands may result. Bubbles which form at the bottom of the gels should also be shaken off in order to ensure straight bands.

(iv) Application of Histone to the Gels and Electrophoresis

Histones were prepared for electrophoresis by dissolving in 15% sucrose in 0.9N acetic acid. The sucrose

is more dense than the acetic acid in the electrophoresis apparatus ensuring that the histones will stay on top of the gel. The histones were layered on top of the gels using a micropipet with an attached rubber tube and bulb. The low pH of the buffer ensures that the histones and not acidic or soluble proteins will migrate to the cathode. Optimal concentrations of histone for electrophoresis were found to be 1 mg/ml of which only 30 µg need be applied for accurate analysis.

After the histone solutions had been applied, electrophoresis was started at once. As before, negative polarity was applied at a constant voltage of 130 volts for 1 1/2 hours. This time period was found to provide an adequate separation of all major histone bands present on the 7-cm gels.

(v) Removal of the Gels from the Tubes, Staining and Destaining

At the termination of electrophoresis, the gels were removed from the glass tubes. The gels were first loosened by sliding a stainless steel wire or a blunt 20 guage syringe needle between the gel and the glass wall. A slight water flow through the needle was applied to ensure an adequate pressure on the gel. The pressure on the gel forces the gel out of the tube. The gels were stained overnight in a

solution consisting of 0.1% (w/v) amido black, 7 % (w/v) acetic acid and 20% ethanol. Amido black binds to the protein and precipitates the complex so that neither the stain nor the protein can be removed by electrophoresis or diffusion. Excess stain was removed from the gels using a Metaloglass destaining apparatus with 7% acetic acid as buffer and maximum amperage applied. The gels were then fed into glass tubes and scanned at visible wavelength.

(vi) Analysis of Electrophoregrams

In order to determine the relative concentrations of histone protein present in each of the bands, the gels were scanned in an Instrumentation Specialities Co. Inc. (ISCO) model 659 gel-scanner with an ISCO type 5 optical unit containing a 579 nm filter. An ISCO model UA-4 Absorbance Monitor connected to a Bausch-Lomb VOM5 chart recorder was used to record the absorbance readings.

As has been shown by Bonner and co-workers[48], the staining of histone by amido black, as measured by absorbance at 600 nm, is directly proportional to the amount of histone up to a concentration of 10 µg per band. Thus the area under the curve of the tracing is directly proportional to the amount of histone in that band.

The area under each curve of the tracing corresponding to individual histone bands was calculated by use of a planimeter and when expressed as per cent of the area under all curves corresponds to the percentage of histone present in each fraction.

J. Radioactive Labelling Procedures

Equal amounts of nuclei were layered on each of three 2.3M sucrose gradients. The nuclei after ultracentrifugation and irradiation were suspended in 5 ml of incubation buffer (see Fig. 5). The media used in each of the various studies are described in Table 1. Equal amounts of radioactive substrates were added and the samples incubated on a shaker at 37°C. After incubation, the nuclei were cooled to 5°C. One-tenth of the nuclear suspension was taken to determine the amount of incorporation of label into nuclei. When radioactive ATP was used as substrate, the aliquots containing the nuclear material were first treated with 1/2 volume of 0.2M sodium pyrophosphate, pH 7.4 to remove adsorbed nucleotides before precipitation by addition of 1/2 original volume 3M PCA. In acetylation, and methylation experiments the nuclear material was precipitated with 10% TCA and hydrolyzed at 90°C for 10 min. The nuclear insoluble material was then collected by centrifugation at 1,800 X g for 10 min. The pellets were then incubated

in 1 ml Protosol 55°C overnight. From the counts in the nuclear insoluble material, total nuclear incorporation was calculated. The rest of the nuclear suspension was centrifuged at 20,000 X g for 20 min. The nuclei were resuspended two more times in the incubation buffer to remove adsorbed label and then centrifuged at 20,000 X g for 10 min. Nuclear suspensions labelled with $AT^{32}P$ were washed twice with 2.5 mM of 'cold' ATP added to the washing medium. The 'cold' ATP was added to ensure the physiological integrity of the nuclei while at the same time removing any loosely bound radioactive ATP. The nuclei were then extracted for nucleohistone by methods already described.

(i) Liquid Scintillation Counting Procedures

Samples to be counted were placed in 15 ml scintillator fluor (4 g PPO and 100 mg POPOP made up to 1 liter with toluene) with 2 ml absolute ethanol added in order to solubilize aqueous samples. The samples were then counted in a refrigerated Nuclear Chicago Unilux 11 scintillation spectrometer at optimal counting settings determined for each isotope. Efficiency was determined by channels ratio and all count rates were converted to disintegrations per minute (dpm).

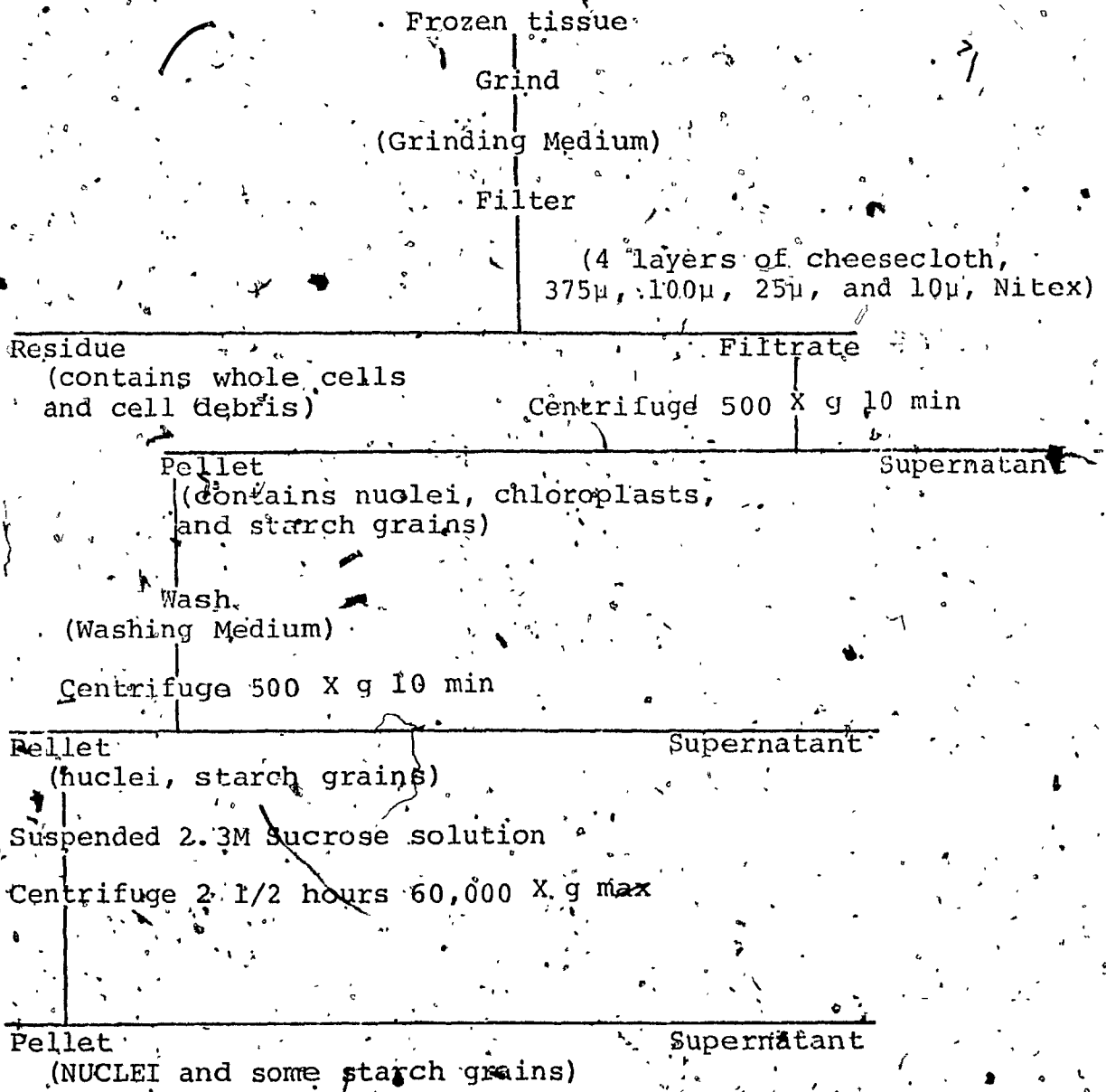
(ii) Gel Counting Procedures

Histones present in the gel after electrophoresis were located by the usual staining procedures. The individual histone bands were cut from the gel with a razor blade into liquid scintillation vials. The fractions were digested at 55°C in a solution of Protosol:toluene:water (9:10:1) for three hours. The fractions were then frozen prior to counting. This operation induced swelling of the acrylamide gel and allows the scintillator to penetrate the gel. Fractions were then counted at optimal settings using standard scintillator fluor without ethanol added. In some cases, internal standardization was performed in order to reduce errors in efficiency determination.

The amounts of individual histone sub-fractions (Table IX) were calculated from the areas under each of the curves of the electrophoregram. The degree of binding of amido black to histone protein is proportional to the amount of protein in the band (Methods I (vi)), allowing expression of the amount of histone present in each band as a percent of the total histone, reported as histone (%) from which the amount of each histone subfraction in µg can be calculated. Count rates from each of the gel slices of individual histones determined the amount of radioactivity present in each histone sub-fraction and is expressed as

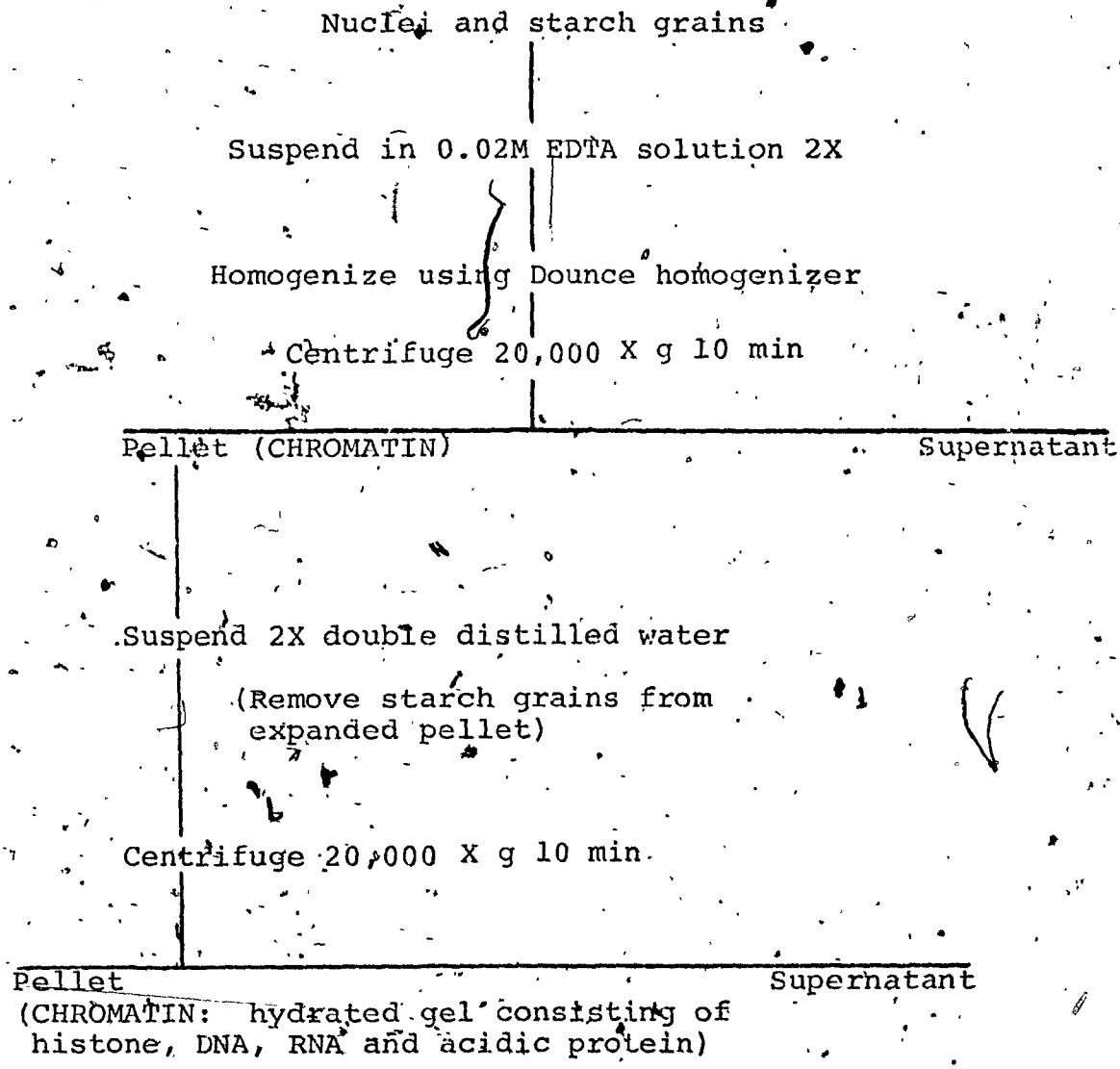
dpm. Total histone count rates were calculated by addition of all sub-fractions. Specific activities were calculated from the dpm's present in each fraction and reported as dpm/mg X 10^{-3}.

Figure 1. Method for the isolation of plant nuclei



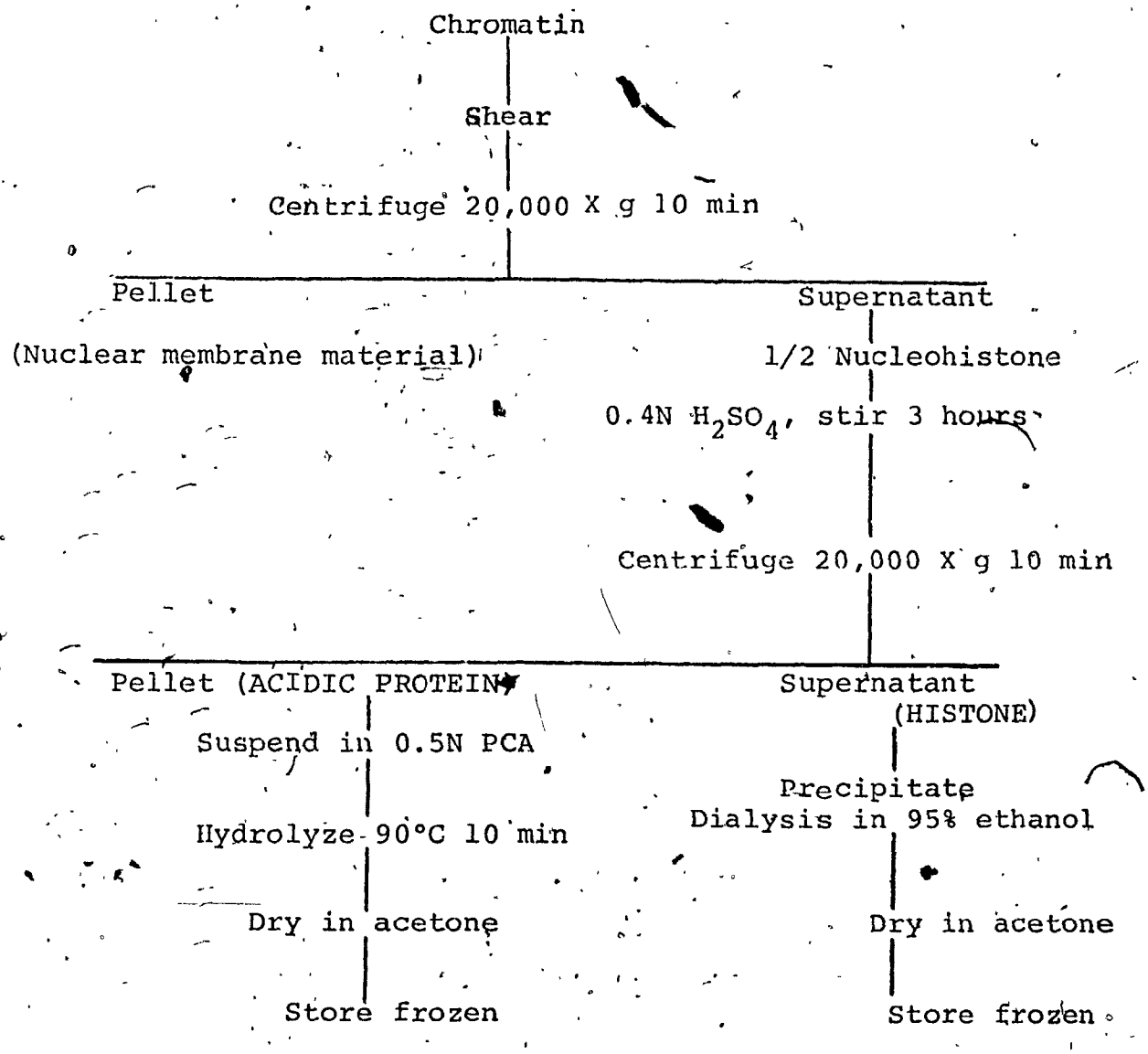
(All operations carried out at 5°C)

Figure 2: Isolation of chromatin from nuclei



(All operations carried out at 5°C)

Figure 3: Isolation of histone from chromatin



(All operations carried out at 5°C)

Figure 4: Extraction of DNA and RNA by the Schmidt-Tannhauser procedure

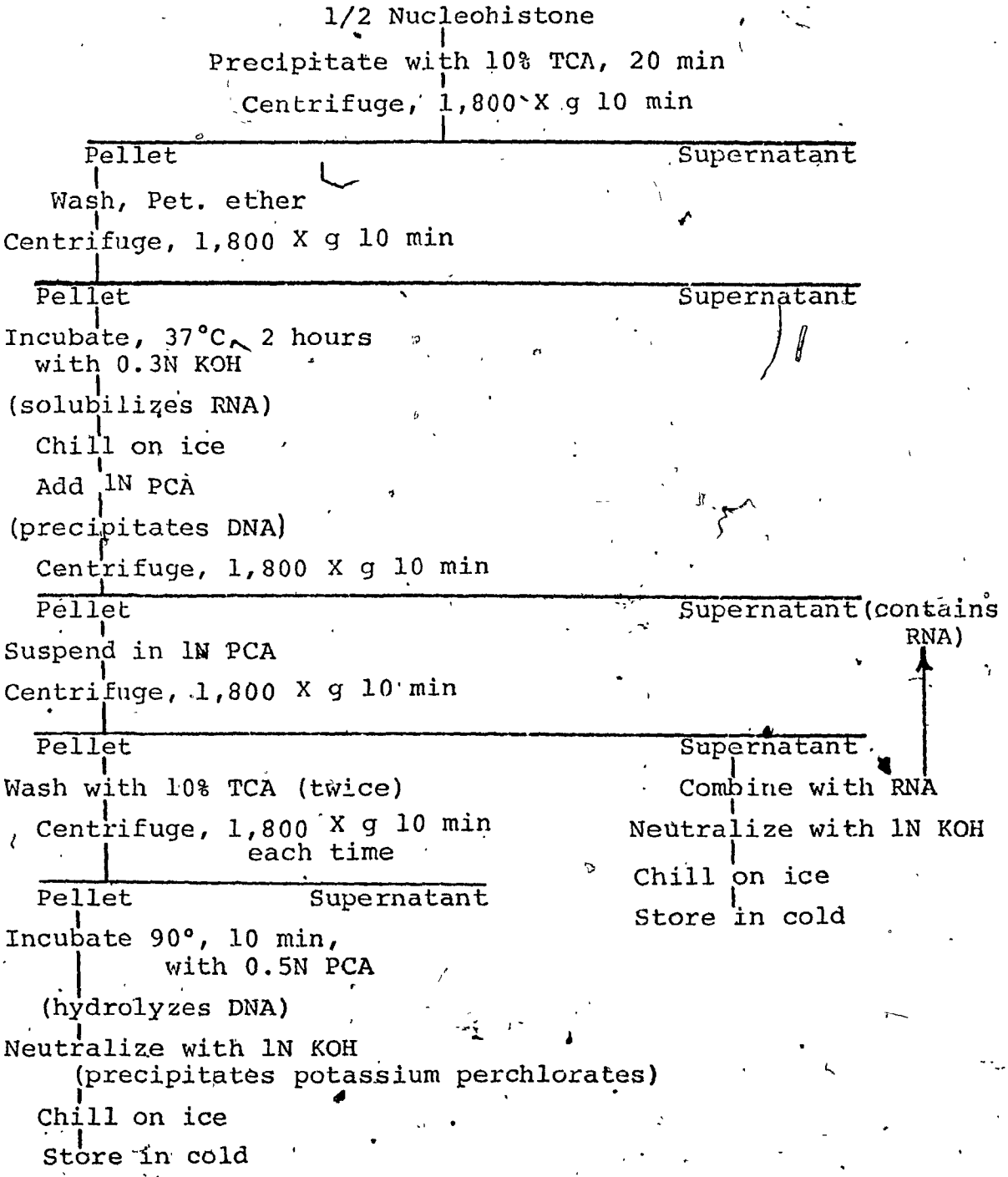
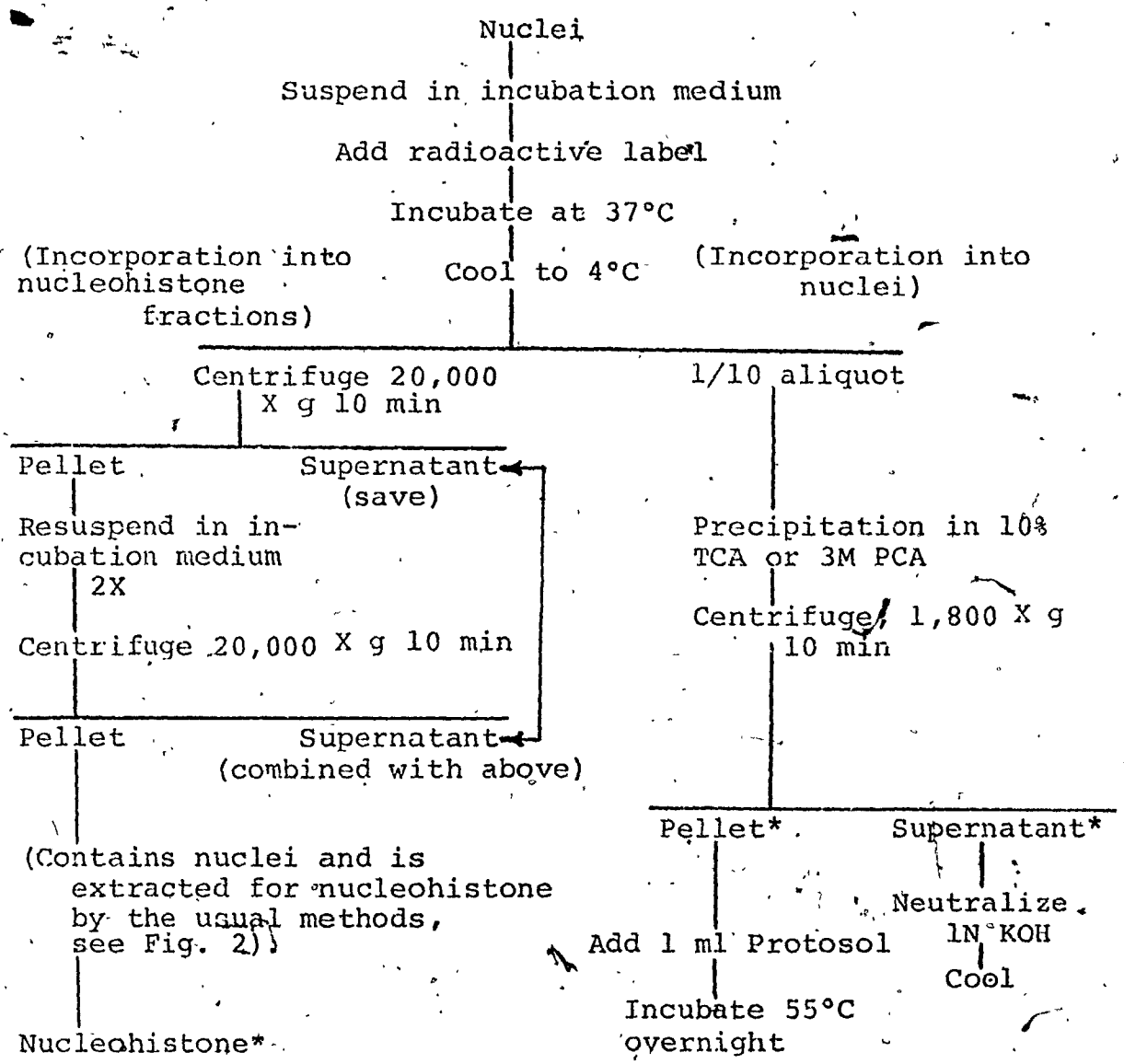


Figure 5: Isotope incubation procedure



*Counted in liquid scintillation counter

Table I: Conditions for Radioactive Labelling of Nuclei

Experiment	Labelled Substrate	Incubation Buffer	Reference
Phosphorylation	Adenosine 5'-triphosphate, tetra (trietylammonium) salt [γ^{32}P] 150 µCi Specific Activity 6 Ci/mM	0.25M Sucrose 0.10M Tris HCl pH 8.0 0.01M $MgCl_2$ 0.025M NaCl	Rickwood et al.[133]
Acetylation and phosphorylation	Sodium Acetate [1-^{14}C] 80 µCi Specific Activity 60 mCi/mM KH_2 $^{32}PO_4$ 150 µCi carrier-free	200 ml 0.1M Sodium phosphate buffer pH 6.0 0.25M Sucrose 160 ml 0.1M Glucose 4 mM NaCl 25 mM $MgCl_2 \cdot 4H_2O$ and 40 ml distilled water	Vidali et al.[186] Kleinsmith and Allfrey[86]
Methylation	S-adensosyl-L-methionine [methyl-^{3}H] 80 µCi Specific Activity 7 mCi/mM	12.5M KCl 12.5M Tris pH 8.0 0.5M $MgCl_2$ 1.0M 2-Mercapto-ethanol	Comb et al.[38]

RESULTS AND DISCUSSION

1. Composition of Chromatin

Plant cells are surrounded by cellulose cell walls and thus plant material does not easily lend itself to nuclear isolation by the methods that have been traditionally applied to animal tissues. Several methods, previously found to be suitable for the isolation of nucleohistone and histones from plant tissues, were employed before a reproducible method was found to extract nucleohistone from the cotyledons of Pinus pinea. Chemical extraction by the method of Johns and Butler[79] using acidified solvent extraction resulted in histone fractions heavily contaminated with cytoplasmic basic protein. The source of the contamination was likely from alkaline-fast green staining basic protein bodies in the cytoplasm. The amounts of basic protein were greatest at early stages of germination decreasing at later stages, indicating that the protein was a form of stored energy reserves. Undoubtably, extraction of whole cells containing such large amounts of cytoplasmic basic protein led to an interference in the ability of the acidified solvents to preferentially extract only the nuclear basic material, i.e., histones. Next, the method of Fambrough and Bonner[46] was tried. Repeated low-speed centrifugation in sucrose-Tris-$MgCl_2$ buffer, of large amounts of blender

homogenates prior to ultracentrifugation through 2 M sucrose, yielded nuclei which were contaminated with large amounts of protein bodies, chloroplasts, cell walls and even whole cells. Nucleohistone prepared from these nuclei possessed inconsistent spectral parameters and was not suitable for further analysis.

Pure nuclei were obtained from 7 to 15 g of cotyledons extracted by the method of Tautvdas[169]. The method was found to be suitable for the isolation of nuclei at very early stages of development. Nuclei were first steeped in a 4.5% solution of gum arabic overnight prior to homogenization in the same medium and sequential filtration through 375, 100, 25, and 10μ nylon mesh (Nitex). The nuclei were then centrifuged at low speed in a discontinuous step-gradient of gum arabic solutions. Nuclei prepared by this method were examined with a phase contrast light microscope and found to be completely free of whole cells, a prerequisite for the isolation of pure nucleohistone from cotyledons of this species. Nucleohistone, of nuclei prepared by gum arabic discontinuous step-centrifugation, was obtained in low yields, with protein contamination as evidenced by the inconsistent absorption spectra and results of analyses of DNA, RNA, acidic protein and histone content. For these reasons, results of experiments which employed this method to isolate the nucleohistone were not presented

in the thesis. These preliminary experiments did point out the necessity of using Nitex screens to isolate pure nuclei, to prevent whole cell contamination and of using protective enzyme inhibitors in the extraction media in order to obtain histone protein fractions without basic protein contamination.

The method described by Spiker[160], modified to include filtration of the homogenates through Nitex filters, yielded nuclei free of whole cells, chloroplasts, or protein contaminants. The method used was found to be rapid, allowing isolation of nuclei from 35 - 75 g of cotyledons, depending on the stage of development when extracted. Typically, 15 mg of nucleohistone from 4,800 cotyledons at day 14 was found to contain 6 mg DNA, 0.4 mg RNA, 1.5 mg acidic protein and 4.5 mg histone. Extraction of nucleohistone from later stages of germination required more washes of the nuclear pellets in order to remove chloroplast-protein contamination from the larger differentiated cells resulting in lower yields of nuclei.

Presence of lipid in chromatin and nucleohistone was not measured but inferred to be present from observations of the persistence of lipid soluble chlorophyll, even after repeated washes with aqueous solvents. Shearing of the chromatin in order to produce nucleohistone resulted in separation of a flocculent material which foamed to the surface of the water. The relative amounts of DNA,

Table II: Composition of Nucleohistone of *Pinus pinea* Isolated at Different Stages of Germination.

Day	DNA	RNA	Acidic Protein	Histone
14	1.00	0.11 ± 0.01	0.30 ± 0.	0.85 ± 0.11
15	1.00	0.17	0.44	1.62
16	1.00	0.14	0.68	1.50
17	1.00	0.12	0.51	1.86
19	1.00	0.54	0.44	1.42
22	1.00	0.35	0.78	1.73
$\bar{X}_{15-22}$		0.26	0.57	1.63
S.E.		0.08	0.07	0.08
t		2.28	3.41	5.45
P		<0.05	<0.01	<0.001

Table III: Composition of Chromatin Isolated From Various Tissues.

Organism	Tissue	DNA	RNA	Acidic Protein	Histone
Pea	Embryonic axis	1.00	0.26	0.29	1.03
Pea	Vegetative bud	1.00	0.11	0.10	1.30
Pea	Growing cotyledon	1.00	0.13	0.36	0.76
Barley	Leaf (7 days)	1.00	0.23	0.56	0.98
Barley	Leaf (25 days)	1.00	0.63	0.39	1.03

Hnilica, L.S. (1972)[71] The Structure and Biological Functions of Histones, p. 100.

RNA, acidic protein and histone present in nucleohistone extracted from cotyledons at various stages of development are presented in Table II. These amounts were found to be characteristic of purified plant chromatin as reported by other authors[19,20,160]. A comparison of the results, Table II, with that of chromatin of other tissues, Table III, shows that the nucleohistone composition of cotyledons at day 14 was very similar to that of growing cotyledons of pea.

The composition of chromatin presented in Table III indicates higher amounts of acidic protein with lower amounts of histone are present in growing pea cotyledon compared to embryonic axis or vegetative bud. The histone of older, more differentiated, barley leaf is present in higher amounts than in less differentiated leaves. The data presented, in combination with reports of higher template activities of nucleohistone of less differentiated tissues[20], lends support to the belief that histones do play a restrictive role during differentiation.

The nucleohistone composition of cotyledons from Pinus pinea were examined at different stages of germination. Values of the nucleohistone content of Table II indicate that the amounts of RNA and protein relative to DNA sampled at day 15 through 22 were substantially greater than those of day 14 cotyledons. This may be due to differences in mitotic activity between the two populations,

since previously[139], it had been observed that day 14 cotyledons are undergoing completion of mitosis, termination of DNA synthesis and that the majority of cells are entering an active stage of cell elongation and biosynthesis.

Work of Bonner and co-workers[20], has indicated that fractionation of chromatin into 'template-active' and 'template-inactive' portions yielded fractions whose histone to DNA ratio showed an inverse correlation with template activity, but did not indicate the extent to which histones were involved in gene expression. The best interpretation of the evidence is that other factors direct or modulate the histone-DNA interaction to obtain tissue specific gene expression. The high basicity of the histones and their probable three dimensional interaction with the other constituents of eukaryotic chromosomes suggests that histones are involved in a complex structure such that the conformation of the protein is rigidly dictated in all regions. Analysis of chromosome structures indicates that at no time are all ionic groups of the protein bound to DNA, or conversely, in loose association, even during DNA replication, are all ionic groups interacting with water. From this information histones are considered to be general and non-specific chromosomal structural proteins which have a capacity to play a reversible role in gene expression.

RNA of chromatin from days 19 to 22 is present in higher amounts than that from day 14 cotyledons and is similar in content to that of well differentiated 25-day old barley leaf and vegetative bud of peas found in Table III. At day 14, RNA and protein synthesis are observed to occur at rapid rates[139], declining at day 17 coincident with termination of cell elongation. High histone contents in the nucleohistone at day 15 and later may be a result of decreased activity of DNA polymerase stimulating differentiation and progression into G_o. Histone deposition into the chromatin at this time may be considered as part of the differentiation process. Considering the active role assigned to histones, the accumulation of histone into chromatin, initiated at a time of cessation of mitosis and DNA synthesis, may be serving to consolidate the structure of the now replicated chromosomes as well as regulating synthesis of macromolecules needed for differentiation. Increases of RNA and acidic protein in chromatin at this time would also seem to reflect tissue specific augmentation of gene activity after day 15 of germination.

Prior work in this species has indicated that RNA synthesis, cell expansion and progress through the cell cycle were delayed following X-irradiation. With this knowledge, this study was undertaken with the aim of finding whether X-irradiation modifes the physical interactions of the

histones to DNA. Acrylamide gel electrophoresis was used to ascertain whether physical-chemical alterations had occurred. In addition, the melting point and viscosity of nucleohistone were determined since X-rays have been shown to affect these parameters. The chemical composition of nucleohistone was examined to see whether X-rays induced the loss of specific components. Various aspects of histone metabolism were studied in response to X-radiation which might be related to inhibition of cell division and altered progression of cells through the cell cycle.

Consequently, histones were isolated from purified nuclei and characterized by acrylamide gel electrophoresis. Since this is the first report of purification of histones from pine or any other gymnosperm, the pattern of histone fractionation will be considered in some detail before effects of irradiation are discussed.

2. Characterization of Histones

For all species so far examined[46,71,114,160], the moderately arginine-rich F_2al (IV) histone has been found to possess a constant mobility upon electrophoresis on 15% acrylamide 2.5M urea gels. Therefore, as indicated in Fig. 6 to 9, the histone F_2al (IV), band 8, was aligned such that each has an identical mobility.

Figure 6: Electrophoregrams of phosphorylated histones. Numbering of sub-fractions is indicated and is the same as that used in the text. The nuclei (day 14) are from experiment 2 (Tables VIII and IX), day 15 is the same as that described in Table VII.

A. Calf thymus standard.

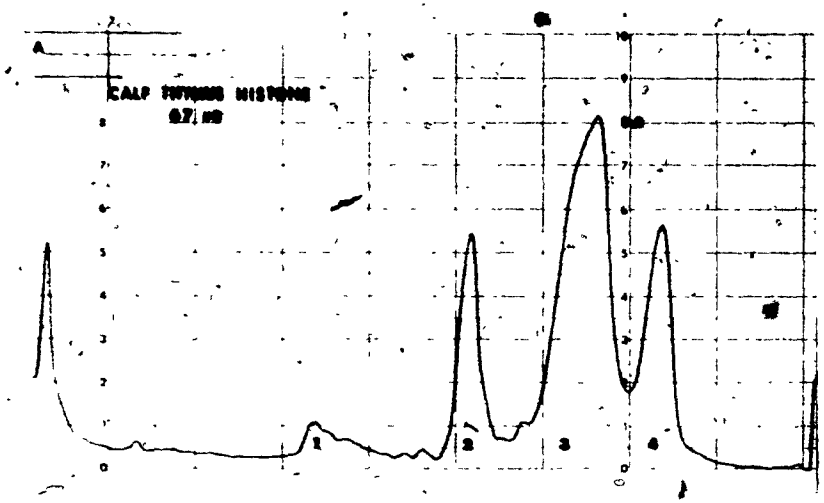
B. Non-irradiated nuclei (day 15) incubated with $KH_2{}^{32}PO_4$.

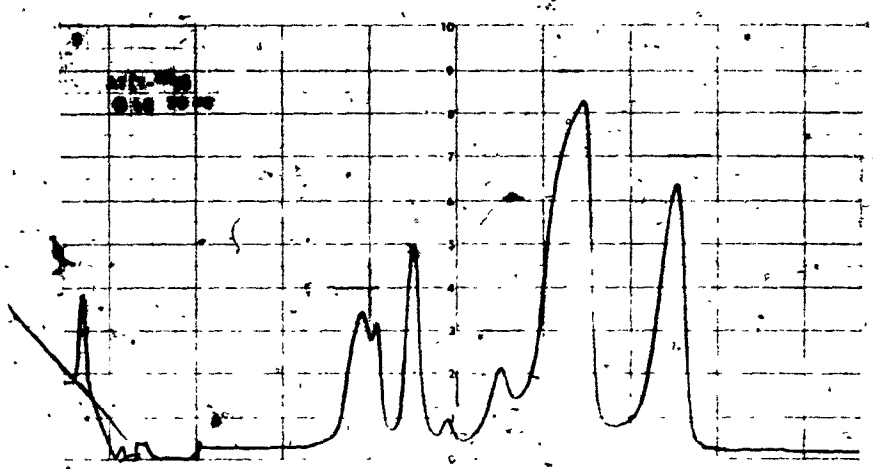
C. Nuclei (day 15) exposed to 1 kR prior to incubation with label.

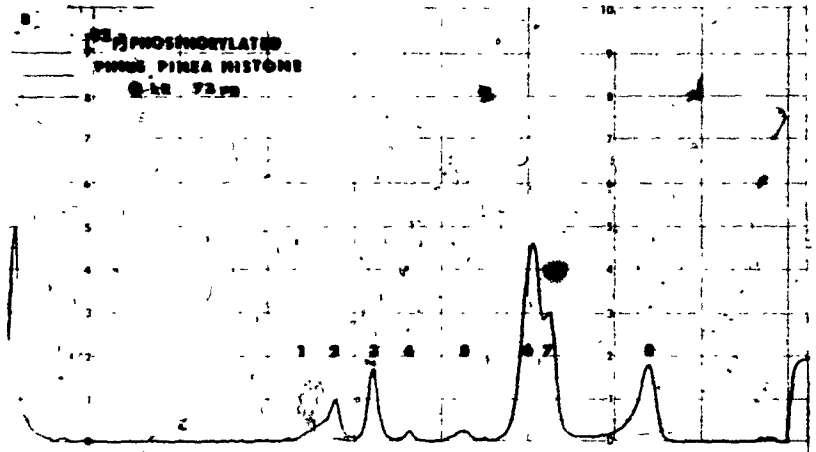
D. Non-irradiated nuclei (day 14) incubated with $AT^{32}P$.

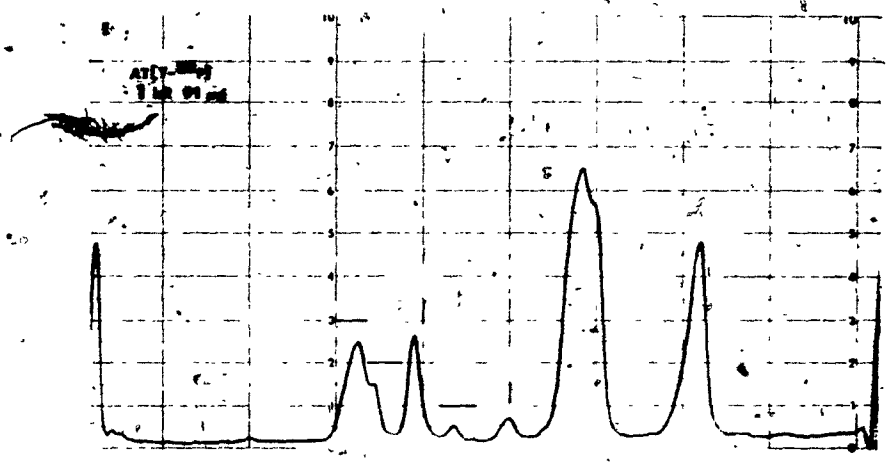
E. Nuclei (day 14) exposed to 1 kR prior to incubation with label.

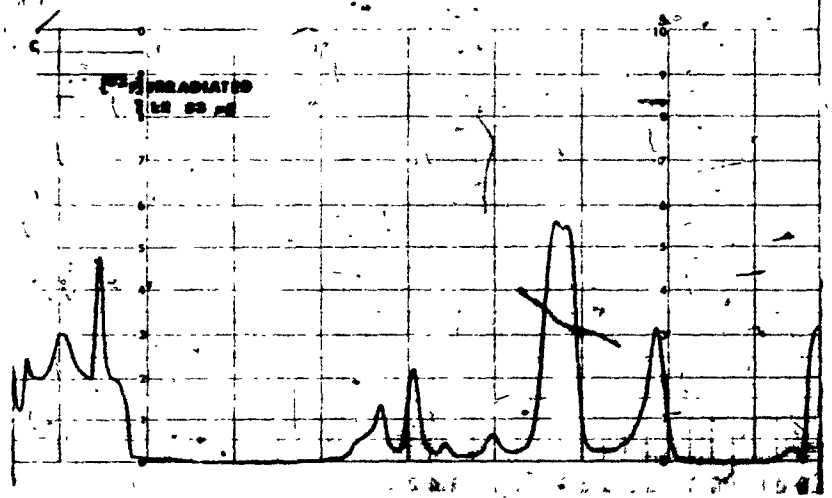
F. Nuclei (day 14) exposed to 5 kR prior to incubation with label.


A
CALF THYMUS HISTONE
0.7 mg




B
PHOSPHORYLATED
PEAS PINEA HISTONE
0.4 mg 72 μg




C
IRRADIATED
1 kR 80 μg

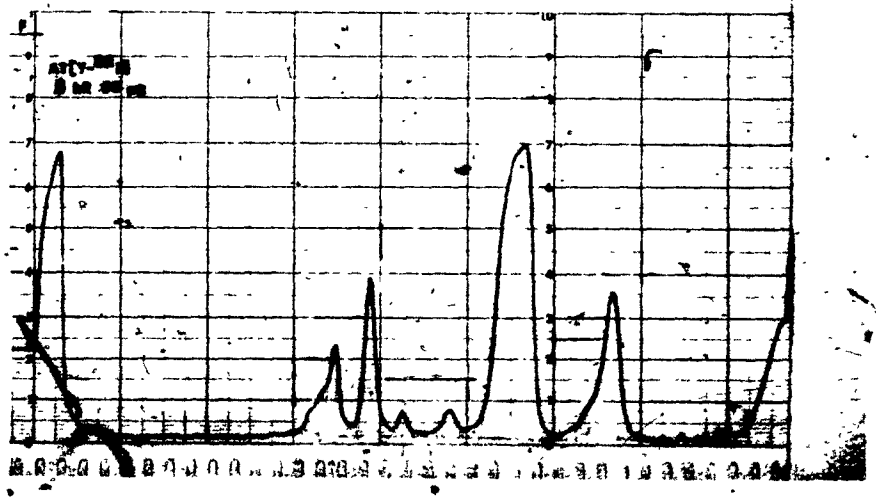


Figure 7: *In vitro* irradiated nucleohistone and histone. The source of irradiated histone and nucleohistones B-F is non-irradiated nucleohistone of Table V.

A. Non-irradiated nucleohistone.

B. Non-irradiated nucleohistone.

C. Nucleohistone irradiated with 5 kR.

D. Histone irradiated with 1 kR.

E. Histone irradiated with 2.5 kR.

F. Histone irradiated with 5 kR.

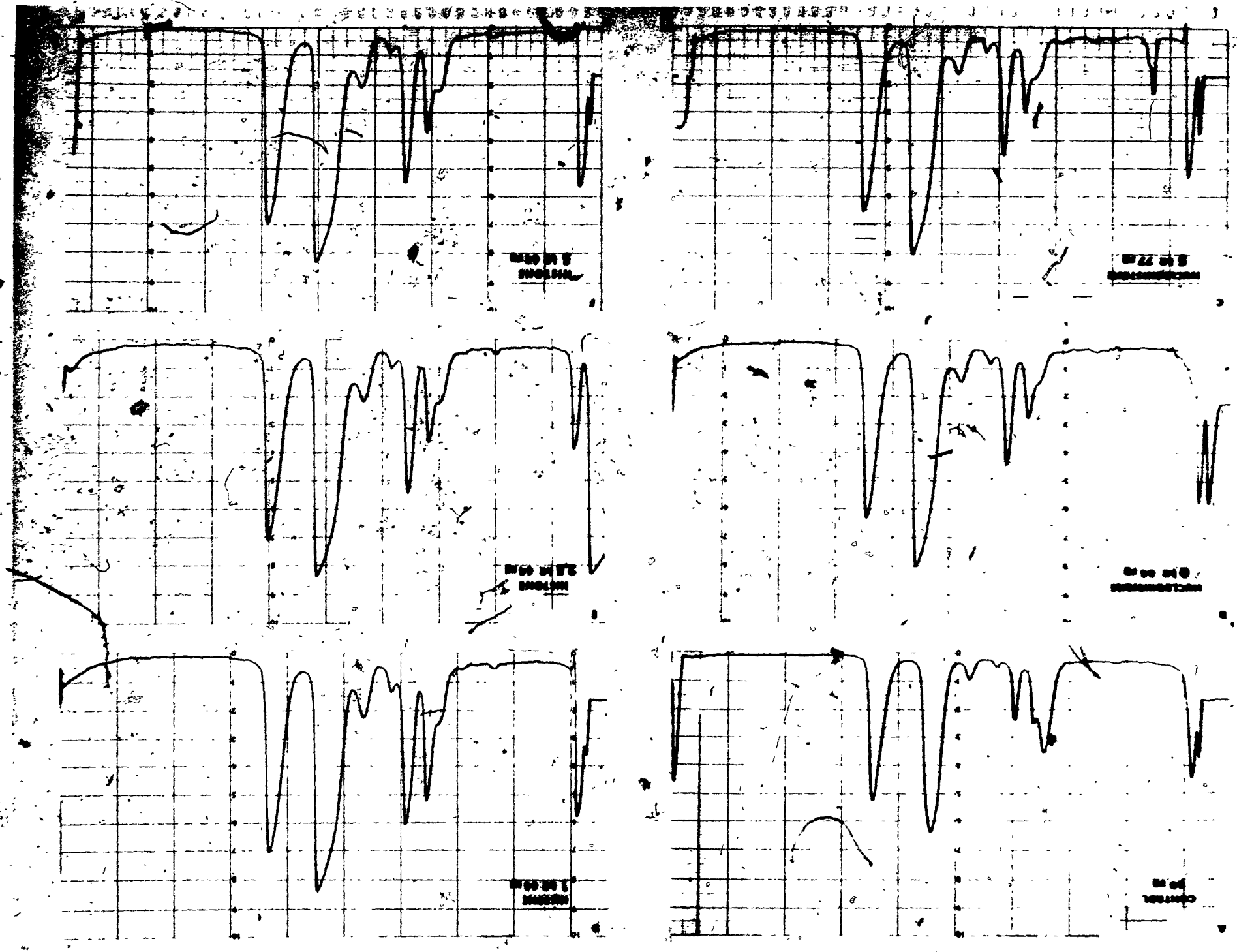


Figure 8: Electrophoregrams of acetylated and methylated histones. The source of acetylated histone (B and C) and methylated histone D-F is the same as described in Table VII.

A. Untreated nuclei.

B. Non-irradiated nuclei incubated with ^{14}C-acetate.

C. Nuclei exposed to 1 kR prior to incubation with label.

D. Non-irradiated nuclei incubated with S-adenosyl-L-methionine (methyl-^{3}H).

E. Nuclei exposed to 1 kR prior to incubation with label.

F. Nuclei exposed to 5 kR prior to incubation with label.

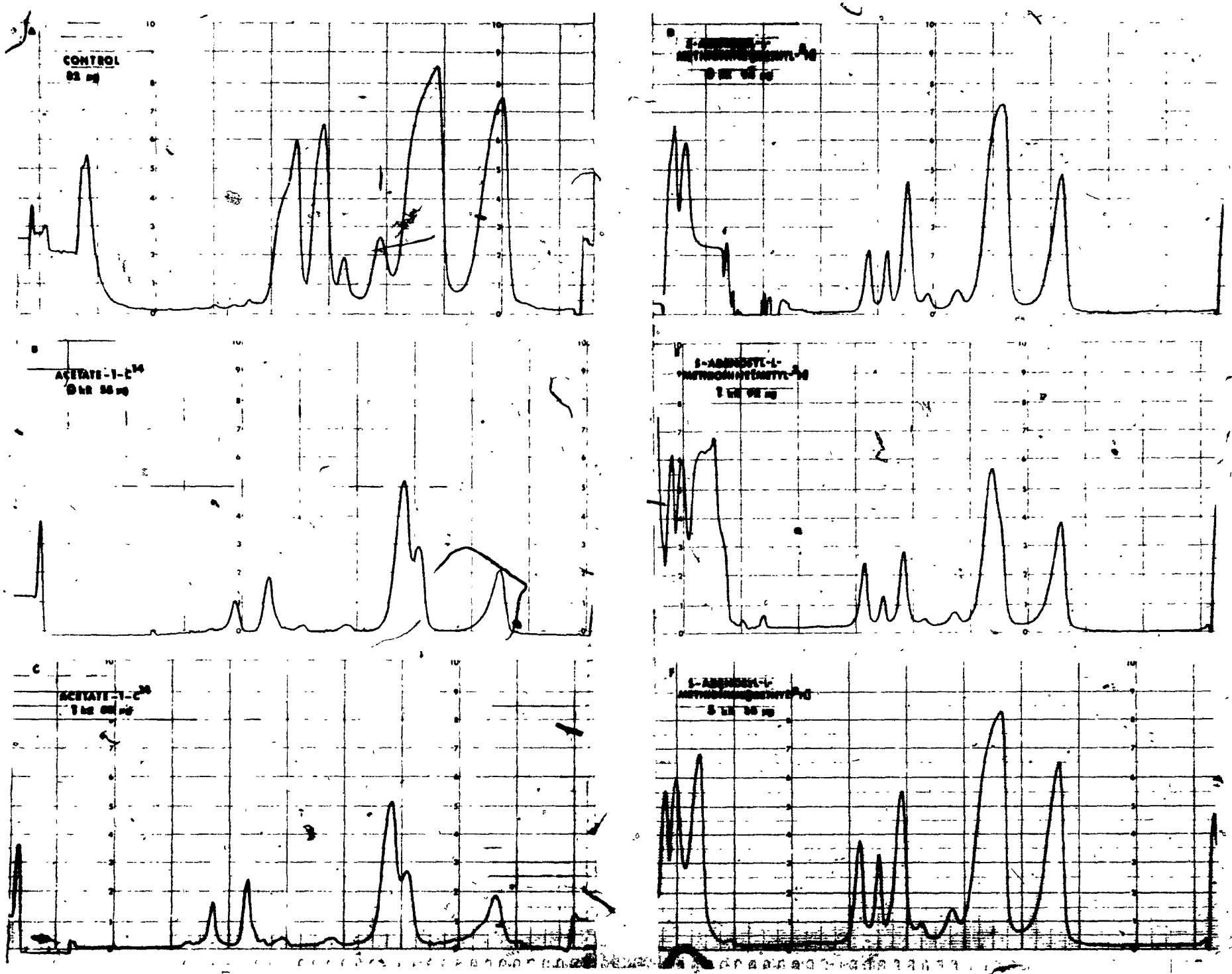

CONTROL
ACETATE-1-C14

Figure 9: Electrophoregrams of phosphorylated histones. The nuclei A-C are from experiment 1 and D-F are from experiment 3 of Tables VIII and IX.

A. Non-irradiated nuclei incubated with AT^{32}P.

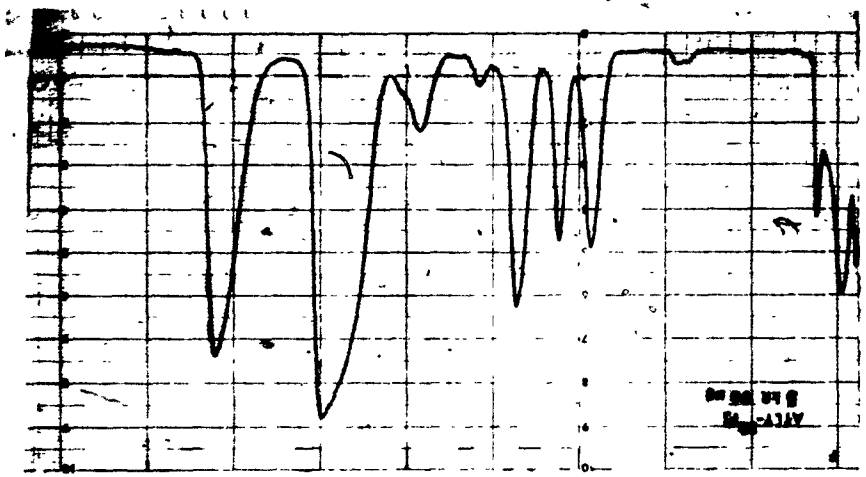
B. Nuclei exposed to 1 kR prior to incubation with label.

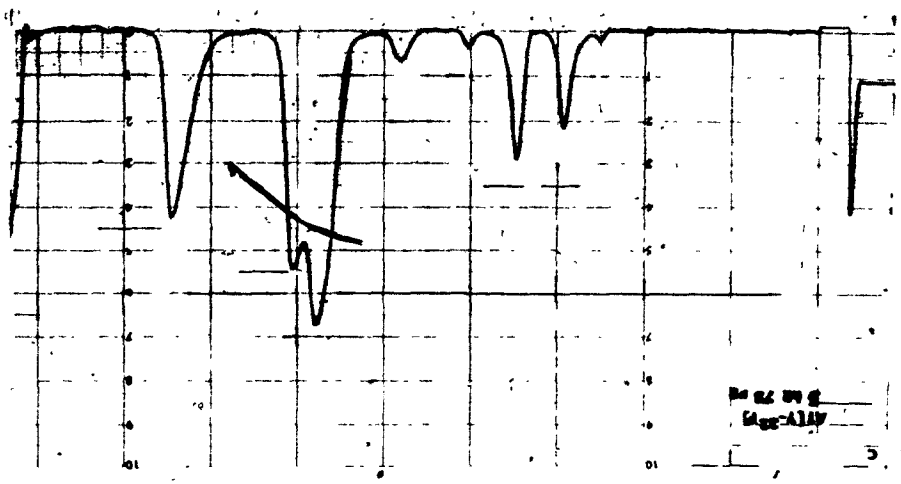
C. Nuclei exposed to 5 kR prior to incubation with label.

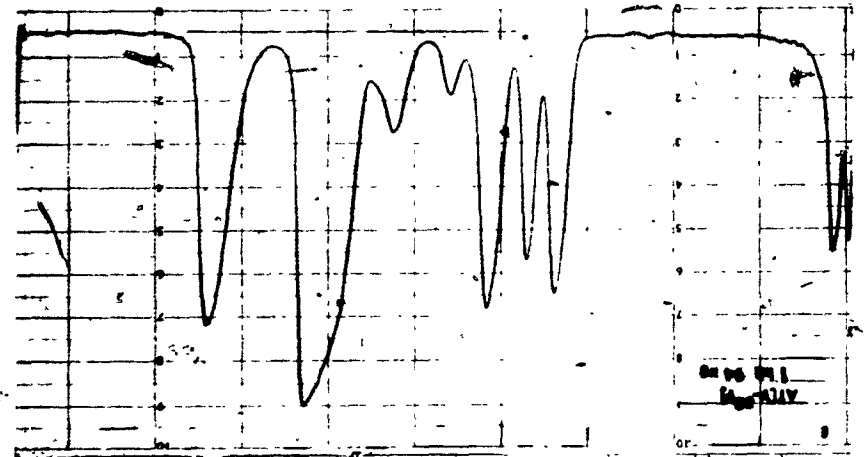
D. Non-irradiated nuclei incubated with AT^{32}P.

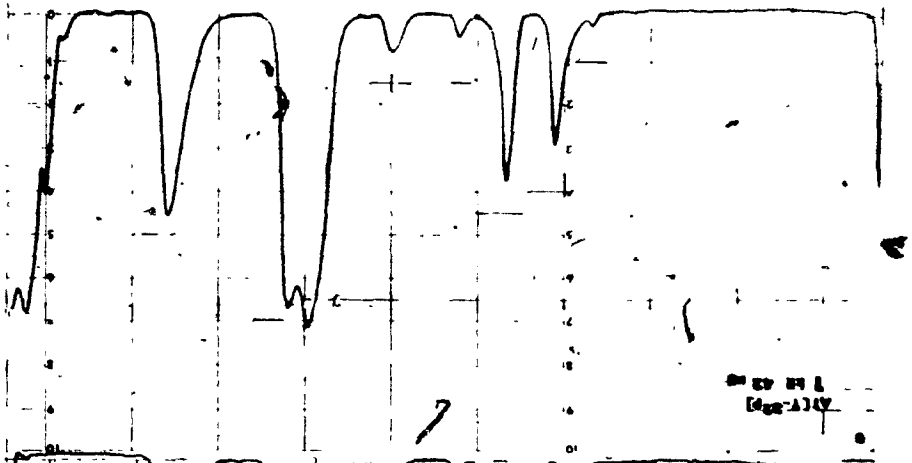
E. Nuclei exposed to 1 kR prior to incubation with label.

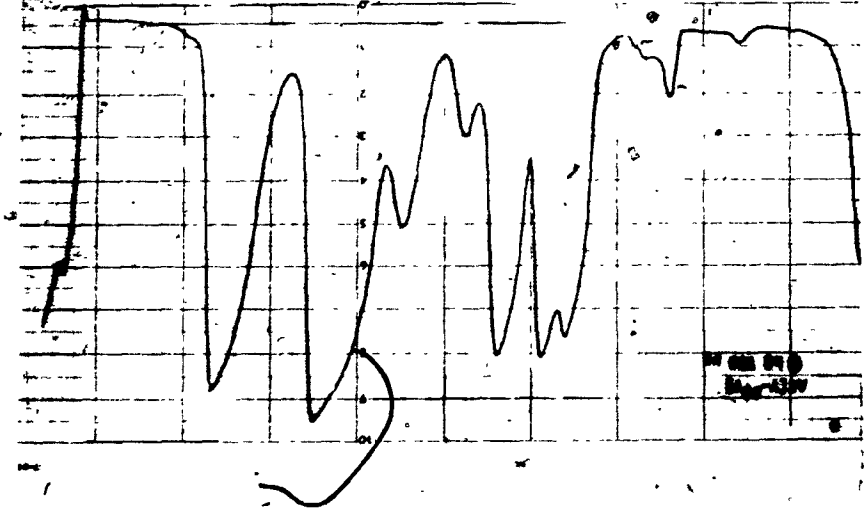
F. Nuclei exposed to 5 kR prior to incubation with label.

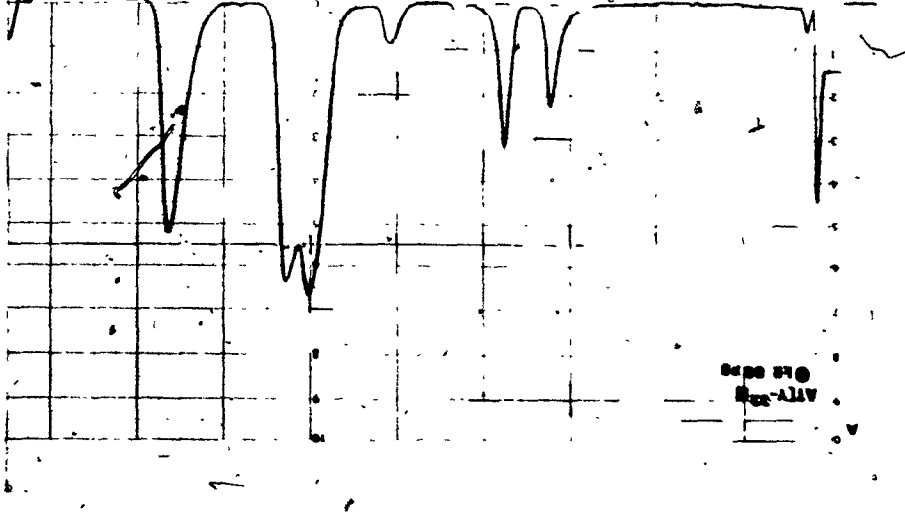












Bands 6 and 7 were separated into one or two bands depending on the load applied to the gel and their degree of chemical modification. Running the histones for longer than 1 1/2 hours, e.g., 2 1/2 hours, did not substantially increase the separation. Based on their relative mobilities, the amount of protein present in these fractions and their degree of response to various labels these two fractions are undoubtably histones F_2a2 (IIb2) and F_2b (IIb1) respectively.

Results of the electrophoregrams, Fig. 6 to 10, indicates that Pinus pinea cotyledons possess eight major histones sub-fractions as compared to four (bands 1 to 4) of calf thymus purified fraction V standard. Band 1 of calf thymus and histone bands 1 to 4 of the cotyledon histone have the least mobility implying that they are identical with lysine-rich histone F_1 (I)[46,160]. Several authors[29, 30,83-85,165,185] have reported that F_1 (I) histones exhibit the greatest heterogeneity amongst species and that even tissues from the same organism can differ in their F_1 (I) histone complement. Rye, barley and wheat have been found to possess three F_1 (I) histones while peas and radish have two[46,144].

To verify that the first four bands were indeed F_1 (I) histones, and were not, for example, oxidized forms of

F_3 (III) histone, a sample of chromatin was stripped of its histone F_1 (I) by the method described by Nadeau et al.[114] The chromatin after dialysis against 0.5 M phosphate buffer with 1 M urea, pH 5.5, overnight was collected by ultracentrifugation and the supernatant F_1 (I) histone collected by precipitation in 5 volumes of absolute ethanol. Subsequent electrophoresis and detection with amido black confirmed that only histone bands 1 to 4 were preferentially extracted by this procedure.

Quantitative differences in the amounts of three 'untreated' controls, (see Fig. 7A, 7B and 8A), each from day 14 chromatin, indicated that the complement of the F_1 (I) histones does vary considerably, even among samples at the same day of germination. Since there is little evidence of proteolytic activity in the samples after electrophoresis, it seems likely that day 14 chromatin can contain different amounts of F_1 (I) histone. Differences in the relative amounts of the F_1 (I) histones may reflect slight differences in the stage of development of the cotyledons induced by the slightest change in humidity or temperature during a crucial period of growth, or, the difference may be an example of differential F_1 (I) histone synthesis and gene regulation amongst cotyledons at the same day of germination. Evidence, not presented, has indicated that there is, to the same degree, variations in the F_1 (I) histone content of

cotyledons at stages of germination later than day 14.

From this it was surmized that if X-irradiation were to effect histones, it would be the F_1 (I) histones which would be affected to the greatest degree. Unfortunately, in vitro irradiation, Fig. 7, indicated that histones are resistant to the effects of ionizing radiation. But, phosphorylated-irradiated F_1 (I) histone, band 1, of Fig. 6E and F, was seemingly affected by irradiation. Discussion reserved for a later time will explain that this was probably due to irradiation-induced reduction in the amount of the phosphorylated histone as compared to more complete extraction of the non-phosphorylated histone F_1 (I).

The fifth band seen in Fig. 6B can be identified as arginine-rich F_3 (III) histone because of its identical mobility to that of the second band of calf thymus, Fig. 6A. When the F_1 (I) histones were removed from the nucleohistone, no histone was evident with the mobility of histones 5 to 8, indicating that none of these fractions could be lysine-rich histone F_1 (I). Electrophoresis of reduced and oxidized samples of peas[160] has indicated that the oxidized form of arginine-rich F_3 (III) has a lesser mobility than that of calf thymus histone F_1 (I) while the reduced form has a mobility greater than that of the F_2b (IIb1) and F_2a2 (IIb2) histones. Nadeau et al.[114], observed that there is a great deal of similarity in the mobilities of

the arginine-rich histone F_3 (III) isolated from calf thymus, rat liver, and three different cereals. It would seem that the electrophoretic mobility of pine histone F_3 (III) is more similar to that of calf thymus and also does not possess a detectable oxidized F_3 (III) arginine-rich fraction. Because of the similarity in the electrophoretic mobility of the histones from pine with those of calf thymus, a similarity in the physico-chemical electrophoretic characteristics is indicated. In addition, both types of histone react similarly on a weight basis to the Lowry test. Therefore, there is some degree of homology between histones of gymnosperms and animals.

It is suspected that examination of histones from earlier stages of germination may also exhibit differences in quantity, and possibly mobility of histones upon electrophoresis, since there is rapid depletion of cytoplasmic basic protein which may possibly represent histone pools that are being used during replication of the genome occurring at early stages. Unfortunately, the methods used were not adequate to isolate histone from cotyledons at early stages of germination suitable for electrophoresis.

3. Respones of Nucleohistone to X-Irradiation

A. Responses of Irradiated Nuclei

In several experiments nuclei were isolated from cotyledons of days 14-17 of germination. Equal portions of nuclei were irradiated and their nucleohistones isolated as previously described. Radiation of nuclei with 1 and 5 kR doses of X-rays did not affect the UV-absorption characteristics, specifically the A260 to 240 and A260 to 280 ratios. The relative viscosities, heats of denaturation, hyperchromicities and the chemical composition of the nucleohistones were also unaffected, see Tables IVa and IVb and Fig. 10 and 11. The values of these parameters were found to vary within the range observed for the un-irradiated controls. Subsequently, the values of these parameters were pooled to yield a mean and a standard error of the mean reported in Tables IVa and IVb.

The finding that irradiation did not alter these characteristics indicates that the parameters so far discussed were not directly affected by radiation. Relative viscosity measurements revealed that 1 kR X-rays induced higher viscosities than those seen in 5 kR irradiated samples or controls, with the exception of one sample which possessed a viscosity equal to that of the control. At low doses increased viscosity (increased flow resistance) is believed

Table IVa: Physical Characteristics of Nucleohistone Isolated from Irradiated and Non-Irradiated Nuclei at Day 14.

UV-Absorption Ratios

	A 260/240			A 260/280		
Dose (kR)	0	1	5	0	1	5
Mean ($\bar{X}$)	1.37	1.40	1.41	1.67	1.70	1.67
S.E.	0.034	.034	.036	.025	.031	.031

Relative Viscosity η/η_o

Dose (kR)	0	1	5
Mean ($\bar{X}$)	1.107	1.220	1.111
η	4	4	4
S.E.	0.030	0.085	0.064

Thermal Denaturation (T_m) °C

Dose (kR)	0	1	5
	78	85	85
	65	62	76
	78	77	79
$\bar{X}$	73.7	74.7	80.0

$\bar{X} = 76.1 \pm 2.6$ °C

Hyperchromicity (%)

Dose (kR)	0	1	5
	18	19	18
	24	25	24
	22	21	28
$\bar{X}$	21.3	21.7	23.3

$\bar{X} = 22.1 \pm 1.2$%

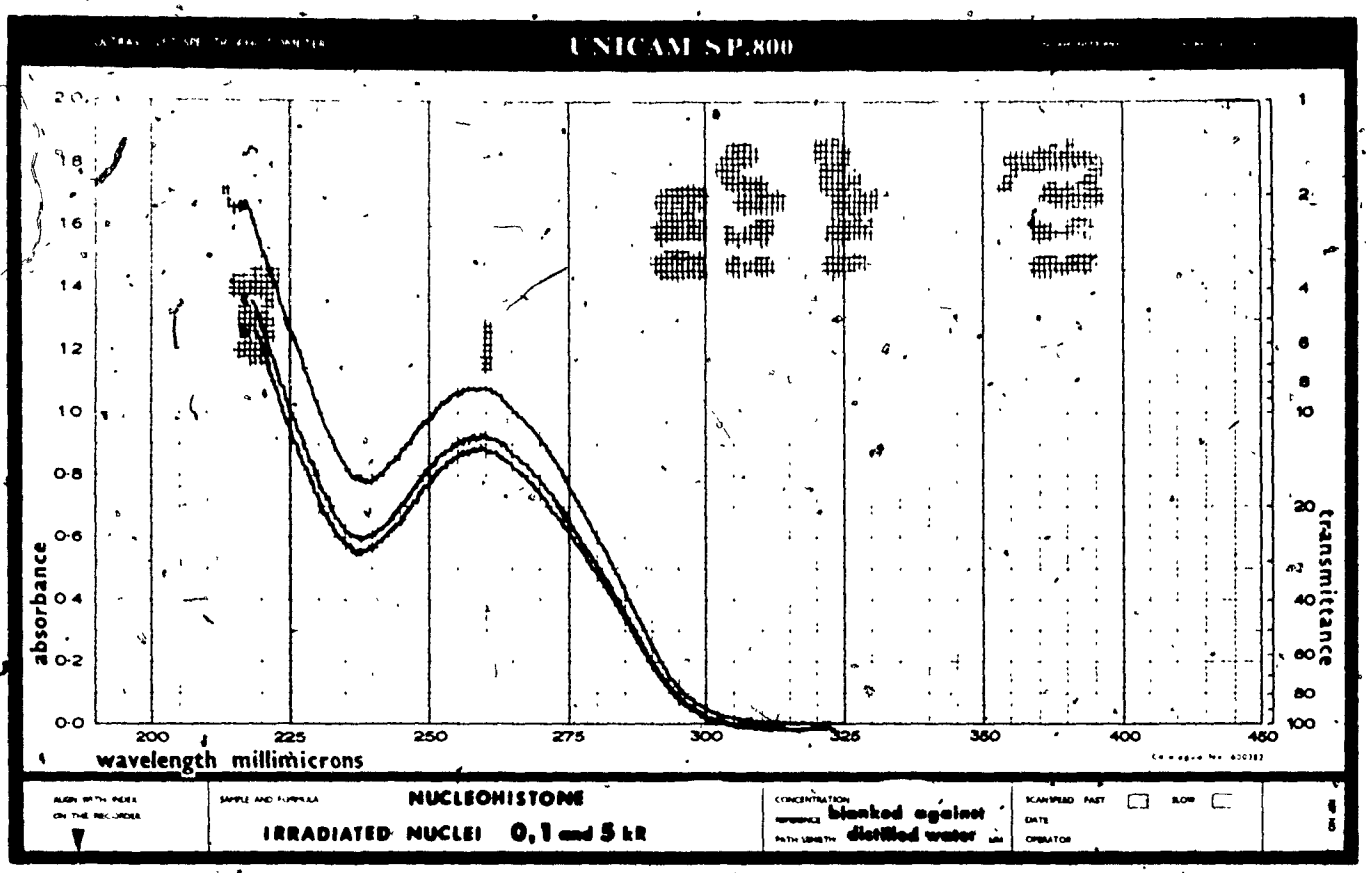
Table IVb. Nucleohistone Composition of Irradiated and Non-Irradiated Nuclei.

Day	Dose (kR)	DNA	RNA	Acidic Protein	Histone
14	0	1.00	0.12 ± 0.02	0.34 ± .10	0.91 ± .12
	1	1.00	0.12 ± 0.02	0.32 ± .08	0.86 ± .19
	5	1.00	0.12 ± 0.01	0.25 ± .07	0.78 ± .28
17	0	1.00	0.12	0.51	1.86
	1	1.00	0.12	0.27	1.09
	5	1.00	0.13	0.30	1.06

Figure 10: Absorption Spectra of Nucleohistone and DNA from Non-Irradiated and Irradiated Nuclei.

Sample	Dose (kR)	UV-Absorption Characteristics A260/240	A260/280
A	0	1.30	1.80
B	1	1.54	1.83
C	5	1.52	1.80

Sample	Dose (kR)	A260/240	A260/280
A	0	1.30	1.32
B	1	1.49	1.48
C	5	1.32	1.40


UNICAM SP.800
absorbance
wavelength millimicrons
transmittance
NUCLEOHISTONE
IRRADIATED NUCLEI 0,1 and 5 kR
blanked against
distilled water

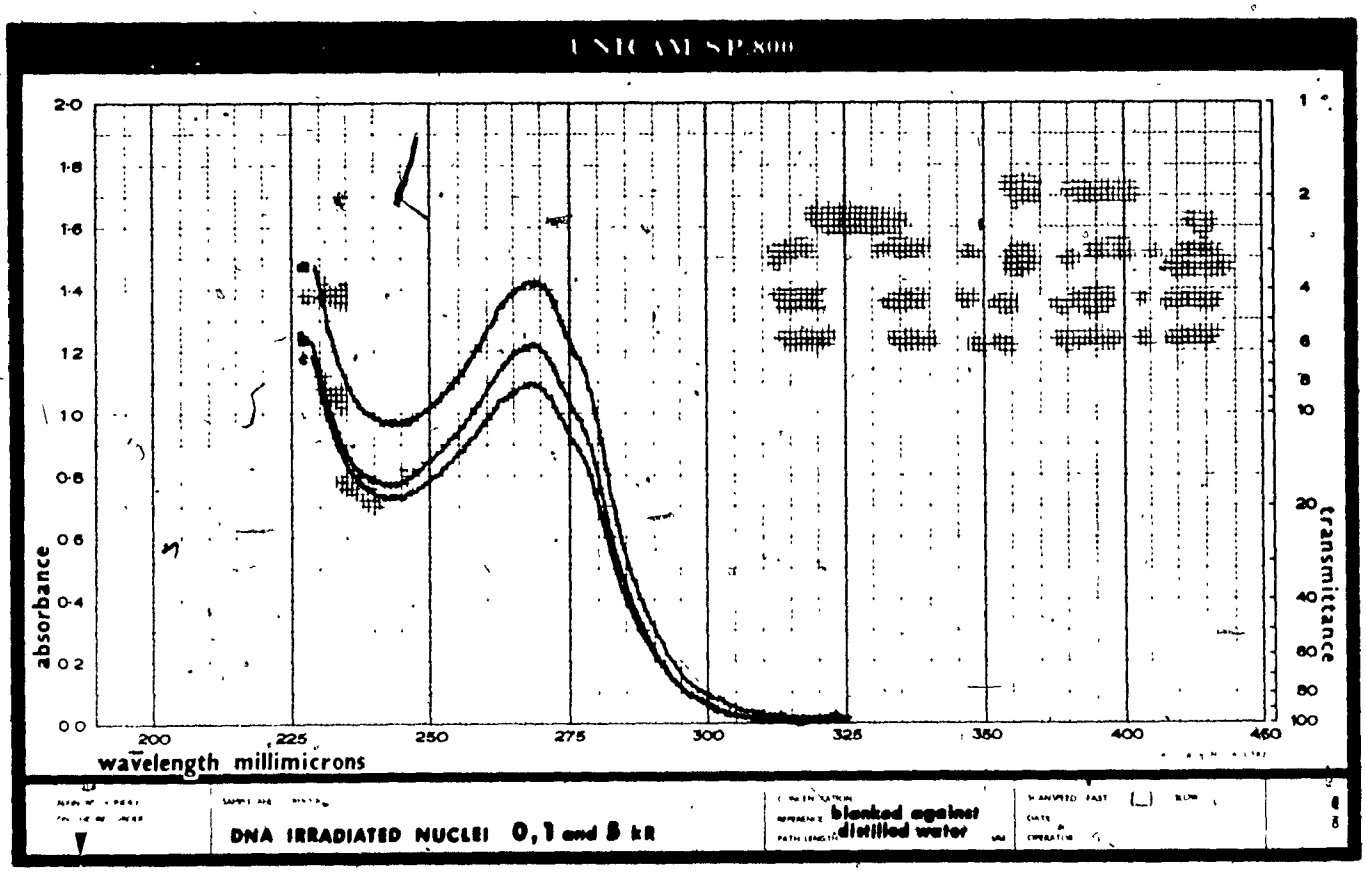
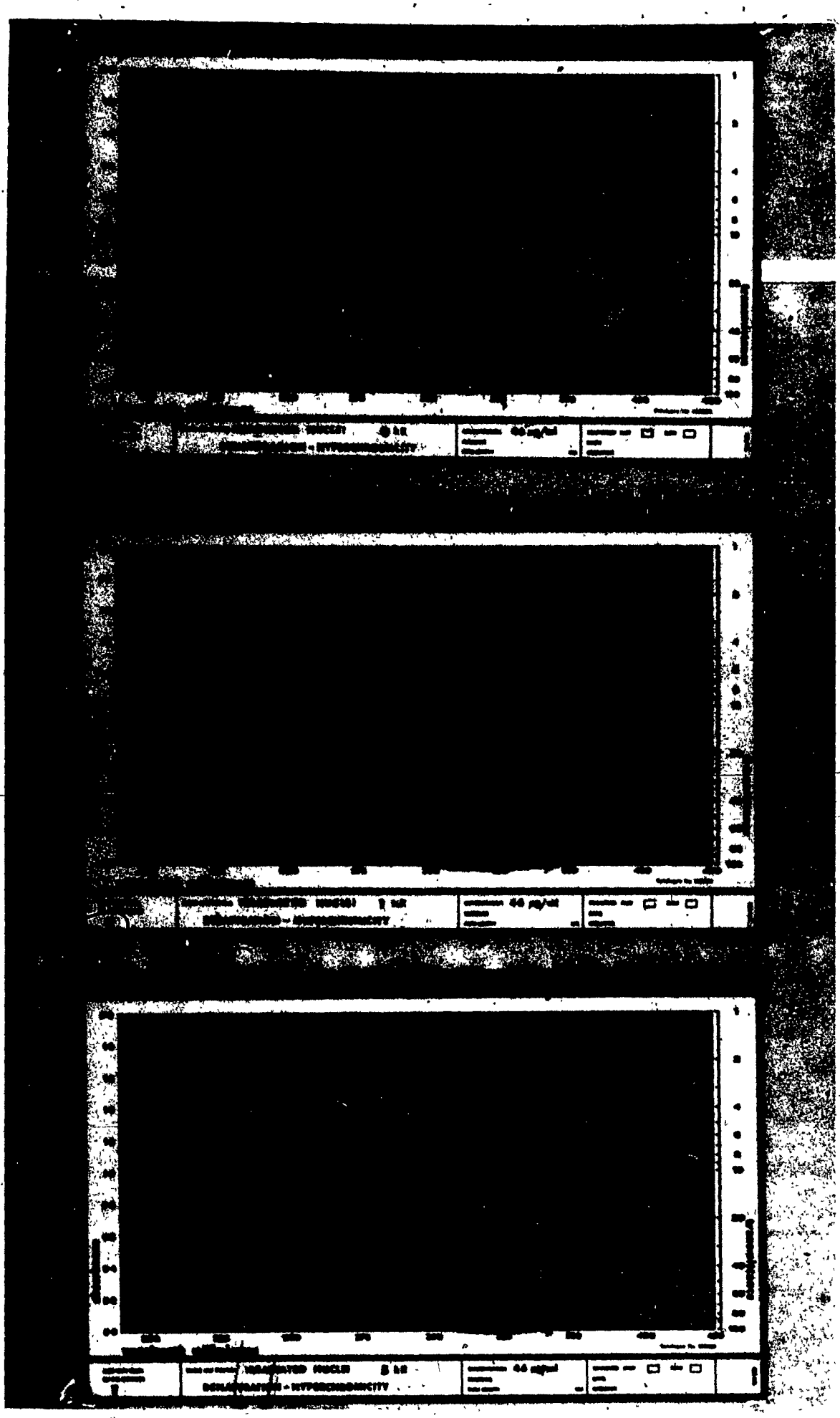

UNICAM SP.800
absorbance
wavelength millimicrons
transmittance
DNA IRRADIATED NUCLEI 0,1 and 5 kR
blanked against
distilled water

Figure 11: Denaturation and Hyperchromicity of Nucleohistone from Irradiated Nuclei. Spectra Determined at 5°C Increments from 60 to 100°C (a-i).

Dose (kR)	Nucleohistone Concentration (µg/ml)	T_m (°C)	Hyperchromicity (%)
0	46	78	22
1	46	77	21
5	46	79	28



to result from unfolding of the nucleohistone followed by release of fragments from the complex[82]. At higher doses aggregation and precipitation presumably occurs masking the effects seen at low doses resulting in decreasing viscosities. This effect of irradiation was not significant (student "t" test t)= 1.77, P = 0.10).

Values of the hyperchromicity and T_m of nucleohistone samples indicated no effect of radiation on these parameters. Consequently, the results were pooled yielding a mean T_m of 76.1 $\pm$ 2.6°C and a mean hyperchromicity of 22.1 $\pm$ 1.2%. The values of the T_m and hyperchromicity are lower than those reported by Bonner et al.[19], of 84°C and 40% for pea nucleohistone because of the use of the method of reversible cooling for measurement of the T_m. Cooling of the nucleohistone after heating is known to result in renaturation and recoiling of the DNA strands thus allowing stabilization of the complex.

The values of the UV-absorption ratios were similar for each of the populations. There is a slight increase with radiation, but the standard error from the mean increases with radiation. The increase is most probably due to variations in histone and acidic protein content caused by irradiation. The variability of the observed effect may be a result, if it is a direct effect, of differences in concentration of the irradiated samples.

The histone and acidic protein contents of nucleohistone (day 14) from nuclei irradiated *in vitro* (Table IVb) was not significantly different than that of controls. The values of RNA remained unchanged after irradiation. Since irradiation had no observable effect, the values obtained for nucleohistone composition, at day 14, were added to Table II and the average of the day 14 nucleohistone composition was compared with those obtained for cotyledons at days 15 to 22 of germination. The older, more differentiated cotyledons were found to possess more histone ($P < 0.001$), greater amounts of acidic protein ($P < 0.01$) and increased levels of RNA ($P < 0.05$) relative to DNA.

B. Response of Nucleohistone and Histones to *In Vitro* Irradiation

Since irradiation of isolated nuclei did not yield observable effects on the nucleohistone, the direct effects of radiation *in vitro* on nucleohistone were investigated. In one experiment, nucleohistone at a concentration of 467 µg/ml, was irradiated with 5 kR X-rays, while an identical sample served as an un-irradiated control. Visible precipitation and turbidity developed immediately following irradiation. The UV-absorption characteristics were significantly different from those of the irradiated specimen, (Table V).

Table V: In vitro Irradiation of Nucleohistone.

Concentration (µg/ml)	Dose (kR)	UV-Absorption Characteristics A260/240	A260/280	Relative Viscosity η/η_o	T_m (°C)	Hyperchromicity (%)
467	0	1.33	1.66	1.58	79	48
467	5	1.16	1.18	0.98		0
46	0	1.40	1.75		78	22
46	1	1.38	1.77		84	17
46	5	1.39	1.76		75	23

Heat denaturation of the samples resulted in hypochromicity of the 5 kR sample, indicating degradation of the nucleohistone indicative of losses of DNA and histone from the complex, probably induced by main-chain scission effects of radiation. Depolymerization, decreases in T_m, decreases in hyperchromicity and viscosity, as well as visible precipitation and losses of histone and DNA from in vitro irradiated nucleohistone or DNP is well documented. In these studies viscosity of the 5 kR sample, reported in Table V, was decreased compared to that of the un-irradiated control, a further indication of precipitation induced by irradiation. In another experiment, nucleohistone at 46 µg/ml concentration did not show any effects upon irradiation with 1 and 5 kR X-rays. A third experiment was carried out subjecting nucleohistone of 50, 100, 150, 200, and 250 µg/ml concentration to 5 kR X-rays, without any observable effects on the T_m, hyperchromicity and with no precipitation.

Histones isolated from the un-irradiated control nucleohistone, and then irradiated in vitro at equal concentrations of 64 µg/ml with 1, 2.5, and 5 kR doses of X-rays did not have different electrophoretic patterns from non-irradiated control, see Fig. 7, E-F, nor exhibit quantitative changes in the amounts of protein present in each of the sub-fractions. Therefore, in accordance with the

literature cited the effects of radiation on histones is slight, suggesting that histones are protecting the DNA from the direct effects of ionizing radiation, rather than being targets themselves.

At best these experiments indicate that the effects of radiation in vitro require a threshold level of targets in order to elicit a response. Radiation-induced changes in DNA depend critically on the exact irradiation conditions. The path of diffusion of an active radical produced from ionization events occurring in water and the distance from the target molecule may both influence the outcome. In the case of irradiated nucleohistone, if the radical does not encounter a target within its zone of diffusion, it has no chance of making an effective collision. If the radiation damage is primarily due to direct effect, as opposed to indirect effect, the number of damaged 'targets' is proportional to concentration at a given dose. When concentration is great, there will be a larger amount of nucleohistone deterioration per Rad absorbed. In addition, a certain minimum number of ionization events must occur in order to penetrate the protein sheath which protects the DNA; such is believed to be the case in these experiments. This is consisteht with a 'single target-multihit' model. Evidence from the literature indicates that most of the effects of ionizing radiation on nucleohistone are due to rupture of

nucleotide-to-protein bonds, or to the disruption of the hydrogen or disulphide bonds of the protein and not the hydrogen bonds of DNA, since recoiling of DNA occurs immediately after irradiation. Further, it has been observed that the phospho-diester bonds of nucleohistone are the most sensitive to the effects of ionizing radiation both in vivo[55,129] and in vitro[45,90,135,136]. Analysis of the electrophoregrams, Fig. 6E of irradiated phosphorylated histones compared to non-irradiated controls suggests that there are two distinct populations of phosphorylated histone F_1 (I) present in band 1; one may have a lower phosphate content than that of the other contributing to different mobilities upon electrophoresis. Quantitative differences exhibited by histone F_1 (I) bands 1 and 2 in these 1 and 5 kR irradiated samples are evidence for the assumption that radiation has altered the solubility of the proteins due to radiation-induced disruption of the hydrogen bonds of the protein. The results depicted in Fig. 6D-F are consistent with the observed changes in solubility of nucleohistone and histone complexes after in vivo irradiation of rats[180,181].

C. Mitotic Delay and Changes in Nucleohistone After X-Irradiation

In three experiments, day 14 seedlings were removed

Table VI: Nucleohistone Characteristics from Irradiation-Arrest Experiments.

Dose (kR)	Hours Post-Irradiation	Day	UV-Absorption Characteristics A260/240	A260/280	Nucleohistone Composition DNA	RNA	Acidic Protein	Histone
0	21	15	1.44	1.63	1.00	0.19	0.62	1.99
1			1.38	1.77	1.00	0.20	0.62	1.09
0	21	15	1.21	1.66	1.00	0.15	0.26	1.15
1			1.13	1.66	1.00	0.16	0.28	0.80
0	40	16	1.37	1.67	1.00	0.14	0.68	1.50
1			1.35	1.61	1.00	0.18	0.73	1.35

from their planters, divided into equal portions, one-half irradiated with 1 kR X-rays, the other half serving as un-irradiated control. In two experiments, the cotyledons were stored for 21 hours, and the other for 40 hours, prior to extraction. Microscopic examination of squash preparations from several Feulgen-stained cotyledons showed that 1 kR of X-irradiation delayed mitosis and produced chromosomal abberations (Appendix 1).

One kR of X-irradiation effectively altered the histone content of nucleohistone (see Table VI) but had no effect on the spectral characteristics. In each of the experiments it is apparent that control nucleohistone possessed more histone at the time of extraction. It has been observed that nucleohistone yields decrease with the stage of development past day 14. Higher yields of nucleohistone were obtained from irradiated specimens indicating that more cells were present which had not yet fully elongated by the time of extraction (day 15 or 16). Decreased histone in irradiated nucleohistone may result from several factors. Messenger RNA and histone synthesis on polyribosomes may have been affected by irradiation[183] or transport of the histones to the nucleus may be occurring at decreased rates, possibly as a result of impairment of a radiosensitive precursor, or possibly because of stimulation of amino acid scavenging enzymes[60,61]; or, histone

synthesis was not affected by irradiation per se but is occurring at a reduced rate in comparison to controls. The latter suggestion is supported by the evidence. It must be remembered that the controls have progressed in age to day 15 and day 16 while the irradiated cells were arrested and have delayed mitosis. Thus, for example, the histone isolated from irradiated day 15 cotyledons is the same essentially as that isolated from day 14 controls (Tables II and IV). For this reason the histone contents of irradiated cotyledons resembles more that of cotyledons still undergoing mitosis rather than cell differentiation.

In conclusion, it would seem that in this system when DNA synthesis and mitosis are stopped by irradiation, increases in the histone content of nucleohistone are also arrested. The effects of radiation on these processes are closely linked. Completion of mitosis leads to differentiation, heterochromatic nucleohistone with low RNA synthetic rates characterized by high histone contents. Irradiation of the cotyledons at a time of rapid growth and synthetic activity results in delays of differentiation and disruption of the normal sequence of events characterized by deposition of histone into nucleohistone by day 15.

Cell division is more sensitive to X-irradiation than is cell elongation. In γ-irradiated plantlets growing without cell division, growth rates were not affected by new

doses of radiation up to 850 k Rad[65]. Chinese hamster ovary (CHO) cells exhibit division delay after irradiation in presence of normal DNA and histone synthetic rates while remaining in a G_2-like state[59,61]. In addition, irradiation of CHO cells at various stages of the cell cycle *in vitro* resulted in decreases of acetylation and methylation of histones F_2a2 (IIb2), F_2b (IIb1), and F_3 (III)[149]. Gurley and co-workers[62,63], have shown that in CHO cells phosphorylation of F_1 (I) histone may be required before cells divide. It was envisioned that phosphorylation of the F_1 (I) molecule is essential for the separation and condensation of sister chromatids prior to and during mitosis. Shepherd *et al.*[149], in a study of the structural modifications to histones after irradiation suggested that G_2 division delay and interphase death may be due to an inability to undergo cytokinesis, rather than effects on DNA or histone synthesis, or histone structural modification.

In cotyledons with radiation-arrested cell division, histone deposition into nucleohistone is also inhibited. The inability of X-irradiated cells to synthesize normal complements of histone may be more related to delays of mitosis rather than effects on histone synthesis.

In order to study the effect of irradiation on the metabolism of nucleohistone, nuclei from day 14 cotyledons

were first irradiated *in vitro* while on crushed ice, and then the degree of acetylation, phosphorylation, and methylation of the histones in the electrophoretic sub-fractions was measured by counting radioactivity from labelled precursors.

Tables VII and VIII show the radioactivity (dpm) incorporated by the TCA-insoluble matter of the nucleus, the nucleohistone and its constituent proteins. Incorporation from nucleohistone into protein and the amount of radioactivity incorporated per mg (specific activity) was included in order to show clearly the effects of radiation on these fractions.

4. Effects of X-Irradiation on Histone Metabolism

When $KH_2{}^{32}PO_4$, ^{14}C-acetate and S-adenosyl-L-methionine [methyl-^{3}H] were used to label histones low count rates were obtained (Table VII). Irradiation did not significantly effect the specific activities (dpm/mg) of nucleohistone, histone or acidic protein. The low ^{14}C-acetate and inorganic ^{32}Phosphate incorporation into nucleohistone and histone indicates a radiation-sensitive high energy dependence of the esterification processes. Since nuclei were able to esterify acetate and phosphate, the nuclei did exhibit normal physiological reactions to some degree.

Table VIIA: Isotope Incorporation into Nuclear Fractions.

Experiment	Dose (kR)	Nuclei dpm X 10^{-6}	Nucleohistone dpm X 10^{-4}	Nucleohistone dpm/mg X 10^{-4}	Acidic Protein dpm X 10^{-4}	Acidic Protein dpm/mg X 10^{-4}	Histone dpm X 10^{-4}	Histone dpm X 10/mg X 10^{-4}
^{14}C-Acetate (1)	0	4.05	1.63	0.167				
	1	8.20	1.21	0.158				
	5	7.38	1.26	0.200				
(2)	0	7.59	1.13	0.796				
	1	8.92	8.97	0.676				
$KH_2{}^{32}PO_4$	0	14.6	10.0	1.85	0.406	15.6	1.55	0.211
	1	5.92	9.11	1.43	0.568	11.4	1.90	0.286
	5	8.91	9.08	1.68	0.776	7.76	1.90	0.178

Table VIIB. Incorporation of ^{3}H-Methyl into Nuclear Fractions

Experiment	Dose (kR)	Nuclei dpm X 10^{-6}	Nucleohistone dpm X 10^{-5}	Nucleohistone dpm/mg X 10^{-4}	Acidic Protein dpm X 10^{-4}	Acidic Protein dpm/mg X 10^{-4}	Histone dpm X 10^{-5}	Histone dpm/mg X 10^{-4}
1	0	2.02	4.30	7.22	3.47	1.03	1.52	7.05
	1	2.52	4.45	6.92	4.74	4.52	1.50	6.49
	5	1.71	3.86	7.10	2.27	2.12	0.915	4.40
2	0	0.617	8.59	20.1	10.3	12.0	.766	1.02
	1	1.74	12.0	24.8	10.0	9.21	1.04	1.42
	5	3.95	16.0	29.0	13.7	8.71	2.09	2.97
3	0	3.13	1.79	7.63	0.213	1.07	0.058	0.821
	1	3.03	1.98	7.65	0.247	1.83	0.090	1.18
	5	2.47	1.46	7.91	0.081	0.623	0.057	1.14

Irradiation of nuclei decreased the orthophosphate specific activity of the acidic protein but not histone. The histones were found to contain insufficient activity such that sub-fractions could not be counted. Irradiation and phosphorylation increased the mobilities of histones (Fig. 6 B and C) when compared to calf thymus standard (Fig. 6A) and non-phosphorylated un-irradiated control histone electrophoresed at the same time (Fig. 8A). Acetylation also decreased the electrophoretic mobilities of histones (Fig. 8 B and C), while radiation with 1 kR caused further decreases compared to the control (Fig. 8A). Further, the ^{14}C-activity of the nucleohistone was too low to allow accurate assessment of the effects of irradiation on individual histone fractions, but there is some indication that X-rays delivered to nuclei or excised cotyledons *in vitro* decreased the specific activities of nucleohistone compared to controls (Table VIIA).

Methylation of irradiated nuclei did not change the electrophoretic mobilities of histones (Fig. 8D to F). Since methylation of F_1 (I) CHO[146,149] histones does not occur to any noticeable degree, no differences in mobility of the F_1 (I) histones was expected. Methylation of the arginine-rich histone F_3 (III) should have induced some changes. Radiation inhibition of methylation of CHO histones has been reported[149]. Methylation of histones

(Table VIIB) was found to be inhibited by radiation in only one of three experiments, whereas stimulation and no effect were observed in the other two cases. The degree of labelling (dpm/mg) of the nucleohistone was moderately high which confirmed previous reports that methylation of histones can occur during mitosis[22,145].

Ord, Stocken and co-workers[121,122,167] and Paskevich and co-workers[124,125], have shown that total body X-irradiation of regenerating rat liver and of hyperplastic kidney but not in non-dividing tissues, decreased histone F_1 (I) phosphate contents which correlated with decreased RNA synthetic activity measured in an in vitro test system. The authors concluded that changes in the content of phosphate groups led to changes in the functional properties of nuclear proteins and that disruptions of the conformation of phosphorylated nuclear proteins as a result of irradiation would become a critical factor in the post-irradiation metabolism of the cell. Gurley and co-workers[59-61] have observed that in CHO cells, blocked during the cell cycle by X-irradiation, F_1 (I) phosphorylation, but not histone synthesis, is decreased while at the same time DNA synthesis is inhibited. It was concluded that normal phosphorylation rates of F_1 (I) were not necessary for either deposition of newly synthesized F_1 (I) into chromatin or for DNA replication but that F_1 (I) phosphorylation may be

related to separation and condensation of sister chromatids prior to and during mitosis[7,63,167]. As of yet there are no reports of an X-irradiation inhibition of histone F_1 (I) phosphorylation in plants, in particular a gymnosperm species such as is Pinus pinea. AT^{32}P incorporation into nucleohistone and histones, isolated from day 14 cotyledons (Tables VIII and IX), was much greater than that observed with day 15 nuclei incubated with inorganic phosphate. This indicated that AT^{32}P was a more direct precursor to histone phosphorylation than was inorganic phosphate. The specific activities of total histone were decreased within 1 hour by irradiation in three of four cases. The activities of the acidic protein and nucleohistone were not consistently affected by irradiation. The inconsistent behaviour of histone specific activity of experiment 1 may be related to the abnormally low nuclear incorporation of the control, the reason for which is unclear.

The degree of phosphorylation of nucleohistone, and histone in particular, was increased after 20 min incubation. The degree of incorporation of label into histone was not affected by the amount of label incorporated into the nuclear insoluble matter, except for the possible anomalous result of experiment 1 where the low count rate may have been a contributing factor. This suggests that

Table VIII: AT ^{32}P Incorporation into Nuclear Fractions.

Experiment (Incubation Time)	Dose (kR)	Nuclei dpm X 10^{-6}	Nucleohistone dpm X 10^{-6}	Nucleohistone dpm/mg X 10^{-5}	Acidic Protein dpm X 10^{-6}	Acidic Protein dpm/mg X 10^{-5}	Histone dpm X 10^{-5}	Histone dpm/mg X 10^{-4}
1 (10 min)	0	9.82	3.62	4.05	2.65	8.27	2.33	2.91
	1	44.1	5.57	5.31	4.30	5.24	2.95	7.02
	5	18.1	2.58	2.62	2.36	5.05	2.55	3.41
2 (15 min)	0	15.4	7.21	8.25	2.38	28.0	4.23	6.07
	1	13.6	9.09	9.87	3.85	89.6	3.31	3.62
	5	34.8	5.39	5.05	2.70	42.9	2.77	3.46
3 (20 min)	0	42.7	10.9	7.61	10.1	66.5	25.6	60.9
	1	34.4	8.60	7.16	5.61	31.5	15.7	41.7
	5	43.6	11.1	9.03	6.09	41.7	15.5	40.5
4 (20 min)	0	23.0	2.48	7.16	0.269	33.6	1.00	3.08
	1	31.9	5.35	16.1	0.512	24.5	1.02	2.21
	5	20.4	1.96	7.16	0.381	47.6	0.650	1.56

Table IX: AT ^{32}P-Phosphorylation of Histones.

Experiment 1 (10 min. incubation)	Histone Fraction							
	Total	1	2	3	4	5	6 and 7	8
0 kR								
Histone (%)	99	0.5	6	8	0.5	3	57	24
Weight (µg)	80	0.40	4.8	6.4	0.40	2.4	46	19
dpm	358	28	26	62	34	44	158	6
dpm/mg X 10^{-3}	4.48	70.0	5.42	9.69	85.0	18.3	3.46	0.31
1 kR								
Histone (%)	100.5	0.5	7	8	1	3	58	23
Weight (µg)	42	0.21	2.9	3.4	0.42	1.3	24	9.7
dpm	504	38	64	108	58	58	166	12
dpm/mg X 10^{-3}	12.0	181	21.8	32.1	138	46.0	6.81	1.24
5 kR								
Histone (%)	99.5	0.5	8	10	1	2	57	21
Weight (µg)	75	0.34	6.0	7.5	0.75	1.5	43	16
dpm	320	6	30	64	38	28	140	14
dpm/mg X 10^{-3}	4.27	17.7	5.00	8.53	50.7	18.7	3.27	0.89

Table IX: AT ^{32}P-Phosphorylation of Histones.

Experiment 2 (15 min incubation)	Histone Fraction							
	Total	1	2	3	4	5	6 and 7	8
0 kR								
Histone (%)	99	9	4	10	1	6	46	23
Weight (µg)	70	6.3	2.8	7.0	0.70	4.2	32	16
dpm	912	194	70	185	68	82	293	20
dpm/mg X 10^{-3}	13.0	30.8	25.0	26.4	97.1	19.5	9.10	1.24
1 kR								
Histone (%)	100	12	4	8	1	2	50	23
Weight (µg)	91	11	3.6	7.3	0.91	1.8	46	21
dpm	491	104	63	88	49	55	123	9
dpm/mg X 10^{-3}	5.4	9.5	17.3	12.1	53.9	30.2	2.70	0.43
5 kR								
Histone (%)	98	3	6	11	2	2	56	18
Weight (µg)	80	2.4	4.8	8.8	1.4	1.8	44	14
dpm	290	39	47	61	31	39	77	0
dpm/mg X 10^{-3}	3.6	16.3	9.79	6.93	22.5	21.5	1.63	0

Table IX: AT^{32}P-Phosphorylation of Histones.

Experiment 3 (20 min incubation)	Histone Fraction						
	Total	1	2	3	4	5	6 and 7	8
0 kR								
Histone (%)	96	11	9	13	3	9	29	22
Weight (µg)	105	12	9.5	14	3.2	9.5	30	23
dpm	8053	1683	700	1022	619	1613	2187	229
dpm/mg X 10^{-3}	76.7	146	74.0	75.0	197	171	72.0	10.0
1 kR								
Histone (%)	100	11	7	14	2	6	39	21
Weight (µg)	94	10	6.6	13	1.9	5.6	37	20
dpm	4085	724	333	468	316	945	1195	104
dpm/mg X 10^{-3}	43.5	70.0	51.0	36.0	168	168	33.0	5.0
5 kR								
Histone (%)	99	9	6	13	1	5	42	23
Weight (µg)	96	8.6	5.8	12	0.96	4.8	40	22
dpm	4053	577	405	524	314	940	1196	96
dpm/mg X 10^{-3}	42.2	67.0	70.0	42.0	327	196	30.0	4.0

Table IX: AT ^{32}P-Phosphorylation of Histones.*

Experiment 4. (20 min incubation)	Histone Fraction							
	Total	1	2	3	4	5	6 and 7	8
0 kR								
Histone (%)	100	13	9	12	2	4	35	25
Weight (µg)	975	127	88	117	20	39	341	244
dpm	7249	1666	631	1102	837	1071	1660	282
dpm/mg X 10^{-3}	7.43	13.1	7.19	9.42	42.9	27.5	4.86	1.16
1 kR								
Histone (%)	100	13	8	12	2	6	32	27
Weight (µg)	1380	179	110	166	28	83	442	373
dpm	9148	1882	1114	1388	966	1363	1946	489
dpm/mg X 10^{-3}	6.63	10.5	10.1	8.38	35.0	16.5	4.41	1.31
5 kR								
Histone (%)	100	12	6	9	2	5	40	27
Weight (µg)	1269	152	76	114	25	64	508	343
dpm	4515	720	517	450	540	917	1149	222
dpm/mg X 10^{-3}	3.56	4.73	6.79	3.94	21.3	14.4	2.26	0.65

*In this experiment the weight of histone indicated was applied to 6 gels from which each of the 8 sub-fractions were combined and counted.

Table X: AT ^{32}P Incorporation Into Histone Fractions. Comparison of specific activities (dpm/mg X 10^{-3}) of 0 and 5 kR. The probability of observing 4 consecutive reductions in 5 kR compared to 0 kR (experiments 1 to 4) is 0.06 and is denoted by an asterisk.

Experiment	Total* (on gel)		F_1 (1)*		F_1 (2)*		F_1 (3)*	
	0	5 kR	0	5 kR	0	5 kR	0	5 kR
1	4.5	4.3 +	70.0	17.7 +	5.4	5.0 +	9.7	8.5 +
2	13.0	3.6 +	30.8	16.3 +	25.0	9.8 +	26.4	6.9 +
3	76.7	42.2 +	146.0	67.0 +	74.0	70.0 +	75.0	42.0 +
4	7.4	3.6 +	13.1	4.7 +	7.2	6.8 +	9.4	3.9 +
	P = 0.06		P = 0.06		P = 0.06		P = 0.06	

	F_1 (4)		F_3 (5)		$F_2a2 + F_2b$ (6&7)*		F_2a1 (8)	
	0	5 kR	0	5 kR	0	5 kR	0	5 kR
1	85.0	51.0 +	18.3	18.7 -	3.5	3.3 +	0.3	0.9 -
2	97.1	22.5 +	19.5	21.5 -	9.1	1.6 +	1.2	0.0 +
3	197.0	327.0 -	171.0	196.0 -	72.0	30.0 +	10.0	4.0 +
4	42.9	21.3 +	27.5	14.4 +	4.9	2.3 +	1.2	0.7 +
	P = 0.13		P = 0.13		P = 0.06		P = 0.13	

AT^{32}P availability was non-limiting and that any differences seen in the metabolism of AT^{32}P by the histone sub-fractions was due to irradiation.

Irradiation was found to decrease phosphorylation of various of the histone sub-fractions. As a group, the very lysine-rich F_1 (I) histones (1-4) were phosphorylated to the greatest degree (Table IX), as can be seen by the lower specific activities of the arginine-rich histones, fractions 6 to 8. In some cases arginine-rich F_3 (III), fraction 5, histone was observed to have the highest specific activity. Irradiation with 5 kR significantly inhibited histone F_1 (1), F_1 (2) and F_1 (3) as well as F_2a2 and F_2b (6 and 7) phosphorylation (dpm/mg X 10^{-3}), P=0.06 (Table X). Arginine-rich histone F_3 (5) was stimulated to a small degree by irradiation with 5 kR X-rays, P=0.13, while phosphorylation of F_1 (4) and arginine-rich F_2a1 (8) was only moderately inhibited by irradiation, P=0.13. The effect of radiation on phosphorylation seems to be specific to the F_1 (I) histones, fraction 1 being the most reduced by irradiation.

Not all of the radioactivity that was present in total histone (Table VIII) was recovered in the histone sub-fractions. The purified histones were found to contain only 10 to 25% of the total radioactivity that was layered

on the gels. Most of the remaining radioactivity, accounting for 40 to 60%, was recovered from that part of the gel nearest the origin. Little activity was found at the bottom of the gel nearest the cathode. This indicates that the specific activities of histone quoted in Table VIII are contaminated by large amounts of adsorbed phosphate; most of which on electrophoresis distributes itself along a diffusion gradient. Therefore, the specific activities of the histones seen in Table IX are more representative of the true activity of the histone preparations since the values were determined on more purified fractions.

Irradiation seemed to have a tendency to increase the percent recovery of radioactivity not present in the histone sub-fractions. Increased amounts of adsorbed phosphate retrieved from electrophoresed phosphorylated-irradiated histones may be due to irradiation-induced reduction of histone phosphorylation resulting in an increased number of net positive charges on the irradiated histones. Therefore, more phosphate was adsorbed. Similarly, altered mobility of irradiated-phosphorylated histones, compared to phosphorylated controls, is also due to an excess of positive charges of irradiated histones.

Because nuclei <u>in vitro</u> do not have a large internal pool of histone or DNA precursors for nucleohistone synthesis, irradiation could not have decreased the

reactions of synthesis. Also $AT^{32}P$ was not limiting. Therefore, reductions in phosphorylation after irradiation could not have been due to inhibition of histone synthesis. Decreased phosphorylation could have been due to decreased activity of histone phosphokinase mediated through the action of cAMP or an increase in nuclear non-specific phosphatase enzymes. Since Shepherd et al.[149] observed that methyl transferase and acetylase activity are not affected by irradiation, decreases in activity of phosphokinase after X-irradiation seems unlikely. Furthermore, the observed selective inhibition of histone phosphorylation would seem to preclude a general effect of radiation on the enzymes that phosphorylate and dephosphorylate histones. Nuclear uptake of $AT^{32}P$ was not affected by radiation. Therefore, it seems likely that radiation has reduced the number of available sites for phosphorylation resulting in lower phosphate content in irradiated histones.

Phosphorylation of F_1 (I) histones may be involved in condensation and decondensation of chromosomes during mitosis[7,63,103]. Phosphorylation of F_1 (I) histone at the time of initiation of DNA synthesis and chromosome replication, mediated through cAMP dependent phosphokinase, is very radiosensitive[63,167]. Now, irradiation affects cell

division to a greater extent than cell growth. The effect is most likely confined to the nucleus, but not necessarily to effects on DNA synthesis per se, since DNA synthesis can occur in cells without division[65]. Histone synthesis and turnover is generally uninhibited by X-rays in mammalian tissue culture systems[10,11,59,61,100]. However, phosphorylation of F_1 (I) histone is substantially reduced by radiation as early as 15 min after dose delivery[39,60,62,121,122,167].

In Pinus pinea cotyledons (day 14) cell division is immediately arrested in interphase as a result of 1 kR dose delivery. Cotyledon growth was not affected (Appendix 1). Analysis of nucleohistone composition isolated 21 hours after irradiation reveals that there are decreases in histone content relative to DNA which seem to recover slightly by 40 hours after irradiation. Cytological examination of 2 day post-irradiated nuclei confirmed that X-irradiated cells complete cell division approximately 2 days after non-irradiated controls and that mitosis is inhibited by 1 hour after dose delivery. Therefore, X-irradiation-induced delay of mitosis is presumably in G_2.

Experiments with radioactive precursors to histone metabolism in nuclei in vitro indicated that X-irradiation altered the structure of nucleohistone but not its chemical composition. It is possible that irradiation-induced reductions of F_1 (I) and F_2a2 (IIb1), F_2b (IIb2) phosphorylation

leading to an excess of positive charge and therefore a difference in physicochemical solubility, may have resulted in delays of mitosis by preventing formation of sister chromatids[163]. Radiation inhibition of histone phosphorylation and consequent failure of chromatids to separate may have caused reversion of the cell to the interphase state, characterized by condensed chromosomes with lower histone contents than controls. X-irradiated cells do not progress through mitosis to G_1 where differentiation occurs in this species, distinguished by histone deposition into nucleohistone. That 40 hours after X-irradiation cotyledons do show mitotic activity and increased histone content lends support to the view that histone deposition into nucleohistone is associated with progression of the cells to G_1 and achievement of the differentiated state.

SUMMARY AND CONCLUSIONS

This is the first reported isolation and study of X-irradiation effects on purified nucleohistone and histones from nuclei of gymnosperms, in particular, a pine species. Nucleohistone was isolated from purified nuclei with demonstrated physiological capacities from growing cotyledons of Pinus pinea. The physical characteristics and chemical composition of isolated nucleohistone were similar to that of chromatin isolated from other plant species at comparable stages of growth. Amounts of histone, relative to DNA, in chromatin from days 15 to 22 of germination was greater than that present in chromatin at day 14. It was suggested that histone deposition into chromatin is acting to repress both DNA replication and also RNA synthetic activity in the later more differentiated cotyledons. Acidic protein and RNA were also found to be significantly increased, but to a lesser extent. Histones isolated at various stages of germination were purified and were found to contain 8 electrophoretic subfractions. Chemical modification by either acetate or phosphate incorporation altered mobilities and increased the number of histone moieties upon electrophoresis.

X-irradiation of nuclei in vitro with 1 and 5 kR X-rays did not significantly alter the physical or chemical

characteristics of nucleohistone but did alter histone mobility upon electrophoresis and reduced F_1 (I) histone phosphorylation to a significant degree. When cotyledons were X-irradiated with 1 kR X-rays, and then stored for approximately one day after radiation, differences in the chemical composition of nucleohistone were observed. Lower histone contents of nucleohistone from cotyledons extracted hours after irradiation were suggested to be related more to X-irradiation-induced delay of mitosis than to direct effects of radiation on the nucleohistone complex, or to synthesis of its components. This view was strengthened by the observation that in vitro X-irradiation of nucleohistone and histones followed by electrophoresis did not induce changes in histones upon electrophoresis. X-irradiation of only high concentrations of nucleohistone in vitro resulted in changes in spectral characteristics and decreases in T_m, hypochromicity and decreases of relative viscosity. The effects were explained to be consistent with a 'single-target-multihit' model of direct radiation effects on macromolecules in aqueous systems.

Histone metabolism of X-irradiated day 14 nuclei revealed that acetylation, methylation, and esterification of histones with $KH_2{}^{32}PO_4$ proceeded at low rates and that none were significantly affected by irradiation. The greater degree of incorporation of $AT^{32}P$ into nucleohistone

and histone allowed counting of the individual histone sub-fractions. X-irradiation with 5 kR significantly decreased phosphorylation of histone F_1 (I) fractions 1, 2 and 3 as well as histone F_2a2 (11b2) and F_2b (11b1) (fraction 6 and 7) which were not always completely separated by electrophoresis. It was suggested that one of the contributing factors for radiation-induced delay of mitosis may be the lack of enough phosphorylated F_1 (I) histones, believed to be important for the condensation and progression of chromosomes through mitosis.

This study has made it obvious that in future, for the purpose of more detailed work on this same topic, synchronized populations of mitotically active cells would have to be used. Determinations of nucleohistone content and degree of phosphorylation of histones would have to be studied at shorter intervals in order to ascertain more exactly the beginning of the effects of X-irradiation on nucleohistone and histone metabolism. In this way the synchrony between histone deposition, phosphorylation of F_1 (I) histone and X-irradiation-induced mitotic delay and their relationship to cotyledon growth and differentiation could be ascertained.

APPENDIX I

Mitotic Activity in Apical Segments of Cotyledons Following Exposure to 1 kR at Day 14.

Stage of Germination (Days)	Non-Irradiated No. of Cotyledons	Cotyledon length (cm)	Mitotic Index (%)	Irradiated No. of Cotyledons	Cotyledon length (cm)	Mitotic Index (%)
14*	8	4.17 ± 0.12	1.29 ± 0.60	5	4.32 ± 0.21	0.0
15	7	3.96 ± 0.36	0.79 ± 0.48	9	3.53 ± 0.22	0.0
16	8	4.56 ± 0.21	0.08	8	4.28 ± 0.22	0.61** ± 0.21
17	4	5.30 ± 0.37	0.0	7	4.67 ± 0.20	1.34*** ± 0.16

* 1 hour post-irradiation
** Chromosomal aberrations observed in 15% of mitoses.
*** Chromosomal aberrations observed in 10% of mitoses.

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