

A STUDY OF HISTONE KINASE ACTIVITY AND HISTONE  
PHOSPHORYLATION IN PINUS PINEA COTYLEDONS  
FOLLOWING X-IRRADIATION

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## ABSTRACT

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### A STUDY OF HISTONE KINASE ACTIVITY AND HISTONE PHOSPHORYLATION IN PINUS PINEA COTYLEDONS FOLLOWING X-IRRADIATION

A cyclic-AMP-independant histone kinase was partially purified from developing cotyledons of Pinus pinea. Purification on DEAE-Sephadex A-50 yielded one major and one minor enzymatically active fractions (35-fold purification). The enzyme was magnesium-dependant and optimal enzyme activity occurred at pH 7.8 in 5  $\mu$ M Tris-HCl buffer. Both iodoacetate and p-chloromercuribenzoate inhibited enzyme activity and thus suggested the presence of essential sulphydryl group(s). 2-Mercaptoethanol reversed p-chloromercuribenzoate inhibition.  $K_m$  for P. pinea histone was 41.0  $\mu$ g/ml protein and  $V_{max}$  was 0.243 pMol  $^{32}$ P transferred/minute. The order of substrate specificity was: P. pinea histone > calf thymus histone > protamine > casein.

Histone isolated from day-14 nuclei showed nine major peaks after gel electrophoresis and were tentatively identified as: very lysine-rich F1 (X, 1, 2, 3 and 4), arginine-rich F3 (5), arginine-rich F2a2 and F2b (6 and 7 respectively) and moderately arginine-rich F2a1 (8).

X-Irradiation induced no electrophoretic change in any particular histone class except for the very lysine-rich fraction X. In vivo, low-level X-irradiation did not alter the UV absorption profiles of chromatin isolated from day-14 cotyledons, but the thermal denaturation profiles showed complete transition from the unirradiated "two-step" profile to a "one-step" profile. Cotyledons irradiated with 1.0 krad exhibited selective inhibition of  $^{32}\text{P}$  (inorganic) uptake in the arginine-rich fractions. Irradiation of day-13 cotyledons with 3.0 krads resulted in 68% loss of enzyme activity whereas in vitro irradiation of isolated enzyme resulted in as much as 21% loss of activity. This data suggests the possibility of direct radiation-induced damage to the enzyme. Irradiation of day-13 cotyledons with 3.0 krads yielded a histone isolated from nuclei with 42% loss of substrate specificity as compared with the controls. In vitro irradiation of isolated substrate, however, showed no such alteration in the ability of P. pinea histone to serve as substrate as compared with control values. Irradiation of day-13 seedlings with 0.5 and 1.0 krad resulted in mitotic arrest within six hours, however, both groups returned to near-normal control values within 22 hours.

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## INTRODUCTION

There is an extensive literature on the subject of histones as gene repressors. For more than twenty-five years, research has been based on the original proposals by Stedman and Stedman which suggested that histones exhibit tissue-specificity and in some way modulate or regulate tissue-specific transcription<sup>137</sup>. As a result of these studies, much is known about the chemistry, structure and metabolism related to histone and nucleohistone. Histones comprise a rather unique class of small, basic proteins<sup>39</sup>. It is generally accepted that histone may be found associated with the DNA of all eukaryotes with the possible exception of the fungi<sup>84</sup>. To date, only a few histone types have been isolated and identified on the basis of their unique column, or electrophoretic mobilities. They are classed into four major categories according to their lysine/arginine composition ratios. Table 1 illustrates this classification for the four histone classes in calf thymus.

Direct experimental evidence for possible in vivo function of a histone-DNA association was examined by comparing template activities of whole purified chromatin and deproteinized or purified DNA<sup>98,16</sup>. The result of these findings, which indicated lowered RNA synthetic rates in whole

Table 1 Physico-chemical characteristics  
of calf thymus histone.

Class	lys/arg ratio	mol. wt.	no. subfractions
I (F1) (F2a2)	22	21,000 15,000	3-8
II (F2b)	2.5	13,770	2-3
III (F3)	0.8	14,900	1
IV (F4)	0.7	11,300	1

(after Elgin et al., 1971)<sup>39</sup>

chromatin, prompted either of two explanations. Either an overall lowered rate of RNA transcription was occurring or only a specified portion of the DNA in chromatin was available for transcription. Direct evidence is available which indeed suggests this latter possibility<sup>47,99</sup>. Experiments were also devised to confirm that the mechanisms responsible for template restriction which operated, in vitro were the same as those operating in vivo<sup>111</sup>. By using a cell-free RNA synthesizing system, deproteinized chromatin showed greater rates of hybridization with RNA transcribed in vivo than did RNA transcribed from whole chromatin. The data further signified that in vitro synthesized RNA was similar to the natural RNA produced in vivo. The significance of these

results helps to suggest further the functional involvement of histone in vivo and to establish that its in vitro function is not merely artifactual.

The functional involvement of histones does not end with the inhibition of template activity. At about the same time the template inhibition studies were initiated, evidence was given for a possible role for histones in regulating DNA synthesis<sup>15</sup>. Table 2 presents data which shows the effects of histones, either whole or fractionated, on a DNA-synthesizing preparation. The results assigned some specificity of action to some histone fractions (i.e., lysine-rich F1 and arginine-rich F3 fractions). A mechanism for the inhibition of DNA synthesis involves the aggregation and precipitation of DNA as DNP (deoxynucleo-protein) by the association of histone to DNA.

Table 2 The effects of histones on an in vitro DNA-synthesizing system.

histone ( $\mu$ g)	Calf thymus histone fractions added				
	none	F1	F2a	F2b	F3
0	0.900*	-	-	-	-
1	-	0.685	0.785	0.830	0.819
10	-	0.391	0.074	0.592	0.000
40	-	0.010	0.043	0.007	0.032

\*  $\mu$ Mol nucleotide incorporated/10 minutes (after Billin and Hnilica, 1964.)<sup>15</sup>

Much attention has been given in recent years to the study of histone modification by means of acetylation, methylation and phosphorylation. These modifications may be involved in conferring tissue-specificity or gene-specificity to histone inhibition. It does not seem at this time that this is the sole factor contributing to histone specificity. It is felt that post-synthetic histone modification may play a considerable role in the regulation of DNA and RNA synthetic activity during defined periods of replication and gene activation<sup>122</sup>.

There is strong evidence that the enzymatic phosphorylation of histone exerts at least one level of transcriptional control<sup>81</sup>. This is supported by existing information which states that a cyclic-AMP-dependant histone kinase does exist and is widely distributed. The fact that protein kinase is modulated by c-AMP implies possible hormonal involvement. Work by T.A. Langan clearly suggests that the enzymatic phosphorylation of histone reduces the template restriction exerted by histone and allows for the induction of RNA transcription<sup>81</sup>. The activity of protein kinase is determined by c-AMP levels. Direct evidence which favors a c-AMP-dependant histone kinase is shown in Table 3.

Table 3 Stimulation of histone phosphorylation by c-AMP.

substrate	<sup>32</sup> P transferred (nMol)		stimulation
	+ c-AMP	control	(% control)
histone F1	124	23	530
histone F2b	364	92	400
protamine	62	57	111
none	5	8	---

(after Langan, 1969.)<sup>81</sup>

Because the involvement of c-AMP in promoting the action of histone kinase was so direct, the possibility of hormonal induction was examined. The study indicated that possible activation of an adenyl cyclase may occur via hormonal induction. An active adenyl cyclase may in turn activate a specific histone kinase. This would then act in the presence of ATP to phosphorylate DNA-bound histone. The negatively charged phosphate groups will bind with the positively charged regions of histone. This histone-DNA complex would subsequently dissociate or be altered in some way so as to allow transcription or replication.

In recent years, another aspect of histone metabolism has been studied focusing on the action of radiation on

chromatin and in particular histone irradiation and growth inhibition. For many decades, it has been known that ionizing radiations (i.e., soft or hard X-irradiations,  $\gamma$ -irradiations,  $\beta$ -irradiations) may induce profound biological effects on living tissues. The most intensely studied topic concerning these effects have been the effects that ionizing radiations have on nucleic acids in which structural change and impairment of nuclear function occur. This impairment may manifest itself as mutations, chromosomal aberrations and/or cell death.

Intense studies on the nature of radiation injury have long since disclosed that DNA is the primary target molecule of the cell. In general, X-irradiation results in strand breakages of the DNA super-helix or depolymerization and disruption of hydrogen bonding<sup>58</sup>. Such damage is caused primarily by two distinct methods, that is, either by a "direct hit" by the X-ray photon or  $\beta$ -particle or by radical formation in the cell water (i.e., peroxide formation). In an in vivo situation, the latter is probably the predominate mode of damage. Damage to proteins by either the direct or indirect mechanism may alter a protein's physico-chemical properties and thus alter its functional capacity as an enzyme. At high radiation doses,

denaturation readily occurs and is primarily due to the disruption of secondary bonds (i.e., hydrogen or disulphide bonds)<sup>3</sup>.

Because of these secondary effects, the physiological effects of radiation damage are usually not immediately displayed in the organism as a whole. Structural alterations of DNA and template will eventually lead to altered or diminished pools of mRNA which result in decreased enzyme and protein production. However, for a more or less defined lag period, a certain amount of mRNA translation will occur utilizing the endogenous supply of pre-irradiated mRNA. Cell division and DNA synthesis also come to a halt, but again, physiological and morphological manifestations are not immediately apparent.

In spite of this, several important biochemical criteria have been used to immediately assess the extent of radiation-induced cellular and biochemical damage. These include physical analysis of chromatin and nucleohistone by UV absorption, viscosity measurements and marked hyperchromicity due to thermal denaturation<sup>60</sup>.

The role that histones play in affecting the radiosensitivity of the DNA molecule is unclear. Comparative



viscosity measurements of the irradiated nucleohistone and DNA indicate that nucleohistone is more radiosensitive<sup>14</sup>.

However, nucleoproteins do provide protection to DNA base alteration<sup>40</sup>. In one study, in vivo, X-irradiation of

rat thymus nuclei resulted in 20% loss of both DNA and histone. Gel electrophoresis of the isolated histones revealed marked changes in their electrophoretic patterns<sup>8</sup>.

One may conclude from these studies that there is an obvious correlation between histone alteration and DNA depolymerization as a result of the radiation-injury processes.

It is well known that the DNA replicative processes in eukaryotic organisms is a segment in a series of chronological events occurring in the cell cycle<sup>5,141,96,140,104</sup>. In general, for mammalian cells, DNA synthesis (S) follows the period of time necessary for the preparation for DNA synthesis (G<sub>1</sub>) and precedes a premitotic growth stage (G<sub>2</sub>) which is a period of preparation for mitotic division (M). There is a profound inhibitory effect by ionizing radiations on the progress of cells through division and on the rates of division and nucleic acid and protein synthesis and promotes chromosomal aberrations<sup>108,100,28,11,32</sup>. This delay in DNA synthesis is variable and depends on X-ray dose and is tissue-specific and as well, irradiation-

induced mitotic block may be reversible<sup>93</sup>. It is known that radiosensitivity of the cell may differ at various phases of the cell cycle, although this may differ markedly from tissue to tissue and species to species. Data by Mak and Till<sup>96</sup> clearly showed that there is increased radiosensitivity in the S-phase and nearly all cells tested and irradiated in the S-phase showed mitotic block. However, cells irradiated in the G<sub>1</sub>-phase will initiate DNA synthesis with only a delay of the onset of DNA synthesis. Delay of DNA synthesis is characterized by a reversible block which occurs in G<sub>2</sub> as irradiated cells move from DNA synthesis (S) and G<sub>1</sub><sup>93</sup>. This G<sub>2</sub> mitotic block is of variable duration and depends on the time of irradiation in the cell cycle. For example, cells irradiated in G<sub>1</sub> show lesser delay than cells irradiated in G<sub>2</sub><sup>48,140, 148</sup>.

In Pinus pinea, each of the twelve cotyledons in the dry seed consists entirely of G<sub>1</sub> cells with a 2C DNA content<sup>25</sup>. Each cell possesses a large nucleus of 10-25  $\mu$ m and even moderate X-ray doses of less than 15 kR will induce radiation-damage symptoms. Roy et al.<sup>121</sup> showed that 15 kR exposure caused up to 25% DNA synthesis reduction, mitotic delay, chromosomal aberration and decreased cotyledon length.

There were decreased levels of  $^3\text{H}$ -uridine into RNA and  $^{14}\text{C}$ -leucine into protein after 14 days of germination.  $^{14}\text{C}$ -thymidine incorporation into DNA and  $^3\text{H}$ -lysine incorporation into protein was significantly reduced on days 9-11 even though this was the period of maximal DNA synthesis. Such overall evidence attempts to provide a greater understanding of the mechanism(s) involved in the inhibition of DNA synthesis. One such mechanism may involve the unimpaired synthesis and metabolism of the lysine-rich histones and is viewed as a prerequisite step for DNA synthesis and cell replication. The inhibition of histone synthesis may be closely related to radiation-induced mitotic block<sup>4, 123, 150</sup>.

Until early 1975, there existed little published information concerning the characterization of histone and nucleohistone obtained from gymnosperm sources. A single report on Cycad, a primitive gymnosperm, was available<sup>22</sup>. It was emphasized that there was striking similarity between the amino acid composition and electrophoretic mobilities of Cycad F3 histone and bovine F3 histone. An earlier report described a photometric study of histone, DNA, RNA and cytoplasmic proteins in developing apices of white spruce (Picea glauca) which method utilized alkaline fast green as a staining reagent specific for histone. That report

indicated that during the yearly three-phase growth cycle of this species, the rates of synthesis and levels of DNA and histone were synchronous during early development but that later in the growing season the DNA ratio exceeded histone. The appearance of new needle primordia occurred at the same time that there was noted a loss of histone and a rise in RNA production and increased levels of cytoplasmic proteins<sup>26</sup>. Berkofsky, 1975<sup>13</sup> reported that Pinus pinea nucleohistone possessed similar chemical and physical characteristics to nucleohistone obtained from other plant sources at a comparable stage of growth. He identified by means of polyacrylamide gel electrophoresis eight histone subfractions and identified each fraction on the basis of their electrophoretic mobilities. He also showed that X-irradiation of isolated nucleohistone or histone did not alter electrophoretic mobility nor did physico-chemical characteristics change in nucleohistone isolated from whole irradiated nuclei given 1.0 and 5.0 kR X-ray doses. The 1.0 kR exposure to day-14 seedlings induced immediate mitotic arrest by disrupting histone deposition into nucleohistone and subsequently delayed tissue differentiation. In addition, histone modification by acetylation, methylation, and phosphorylation was slight in the isolated whole nuclei.

X-irradiation at 1.0 and 5.0 kR doses did not affect histone modification in the isolated nuclei. 5.0 kR irradiation did, however, decrease histone F1 (I) 1, F1 (I) 2, F1 (I) 3, F2a2 (IIb1) 6, and F2b (IIb2) 7 phosphorylation.

In view of this previous work on a pine species and evidence given for the strong interest in histone metabolism and the enzymatic modification of histone and its role in cellular metabolism, this thesis will study the extent of histone phosphorylation in developing cotyledons of Pinus pinea following exposure to X-irradiation-induced modification of the physical characteristics of nucleoprotein isolated from irradiated cotyledons and controls. An attempt will be made to phosphorylate histone in vivo. In addition, the study will be extended to include the isolation and partial purification of a protein kinase which will enzymatically phosphorylate Pinus pinea histone in vitro. A comparison of histone kinase activity between irradiated and non-irradiated tissues will evaluate the capacity of irradiated and non-irradiated enzyme to phosphorylate both irradiated and non-irradiated histone substrates. The involvement of cyclic AMP and hormonal modification (kinetin) will also be investigated.

### LITERATURE REVIEW

The control and regulation of protein biosynthesis and DNA replication have been studied for many years and coincided almost at the same time with the discovery of DNA as being the hereditary material. The most significant advance made concerning the control and regulation of protein synthesis in the prokaryotic organisms has been the proposal of an enzyme induction system in E. coli by Jacob and Monod<sup>68</sup>. However, the DNA of higher organisms and its structure and function is considerably more complex and is subject to much more intricate and refined controls. These controls operate at three levels of cellular hierarchy in the eukaryotic organism: 1) transcriptional (DNA), 2) translational (RNA) and 3) epigenetic control (developmental control due to protein modification by means of intrinsic factors; i.e., the inherent three-dimensional conformational characteristics common to proteins which permit the existence of an enzyme in different functional states)<sup>97</sup>. These controls are imposed upon the cell and account for the regulated synthesis of structural and functional proteins in terms of qualitative, quantitative and temporal distribution.

One type of control mechanism which operates at the level of transcription of mRNA has been implicated by the close association of nuclear proteins with DNA. Watson and Crick had also discovered in their analysis of the molecular structure of the nucleic acids that a metal used in the isolation procedures had complexed tightly to the DNA and neutralized the phosphate groups of the double helix. These phosphate groups are responsible for giving the native DNA its strongly acidic properties<sup>147</sup>. DNA isolated as chromatin is rarely complexed with metal but is found in close association with several classes of nuclear proteins which together constitute nucleoprotein. Chromatin, in addition to DNA, consists of a small amount of RNA, as well as basic proteins (including histones) and a lesser amount of many acidic polypeptide species (non-histones). Preliminary studies on histone-DNA complexes dates back as far as the mid-nineteenth century. Frederick Meisher discovered a certain species of basic protein which strongly complexed with isolated DNA obtained from salmon sperm nuclei. Later analysis on this species of basic protein had shown that it was chemically different from histone found in somatic cells<sup>1</sup>. Following the course of development of sperm, it was found that chemical changes take place in the basic histones. In fully developed sperm, it was seen

that the histones were replaced by an even more basic species termed protamine and that these events were paralleled by a significant decrease in the non-histone components. Such studies were among the first to implicate the developmental significance of the nucleohistone complex. The possible role of histone in gene regulation and evidence given for it has been previously discussed in the introduction. In summary, however, it was found in those early reports that DNA template restriction would increase with the amount of histone complexed. These experiments were later severely criticized, however, because of the limited solubility of nucleohistone in the buffer system chosen<sup>113</sup>. The early excitement caused by Huang and Bonner's work soon subsided when it was realized that histones, albeit did suppress transcription in vitro, could not be thought of as a specific gene inhibitor because of their stringent chemical and structural uniformity from cell to cell, tissue to tissue and even species to species. Much attention was then focused on the possibility that the non-histone chromosomal proteins (NHCP) are indeed the protein species involved in specific gene recognition required for the synthesis of particular proteins. The first such experiments implicating this role for the NHC proteins were those of Paul and Gilmore<sup>54,112,53</sup>. Using



DNA-RNA hybridization techniques, they firmly established by analysis of the transcription products, that NHC proteins do indeed interact with DNA to produce only specific proteins. Other factors which tend to further implicate the role of the NHC proteins are reports which indicate that NHC proteins tend to accumulate in active tissue whereas histone levels remain relatively constant in all tissues<sup>36,46,47,92</sup>. In addition, analyses of the NHC proteins show them to be highly heterogenous and capable of displaying tissue and species specificity<sup>149, 146,125,139</sup>.

As shown in those early experiments which implicated histone as a regulator of the onset of DNA synthesis<sup>15</sup> attempts have been made to assign a similar role to the NHC proteins. If this assumption is correct, then one would expect to witness a variation in the rate and level of NHC protein synthesis and its turnover. One would also expect variation in specific fractions of the NHC proteins in response to different stages of the cell cycle. Such changes could be analyzed by gel electrophoresis. Experiments have already indicated such expected changes in the gel profiles of specific NHC proteins immediately during G<sub>1</sub> of the cell cycle<sup>144,6</sup>. Analysis of <sup>3</sup>H- and <sup>14</sup>C- labelled amino acid incorporation into protein has

shown the selective and active synthesis of particular classes of NHC proteins which coincides with particular phases of the cell cycle<sup>135</sup>. Turnover rates were reported to be highest during replication and were lowest during S phase<sup>134,21</sup>. It has also been implicated that the modification of NHC proteins by phosphorylation is selective and these are phosphorylated at specific periods of the cell cycle<sup>116</sup>. There is also much evidence given for the intermediate action of steroid hormones acting at the transcriptional level by interacting with specific cytoplasmic hormone receptor sites<sup>59</sup>. These hormones include estrogen, progesterone, aldosterone, hydrocortisone and androgens<sup>136</sup>. Both histone and NHC protein have been shown to be involved with binding of steroid hormone<sup>127,44,126,142</sup>. A model has since been proposed to explain how a simple inductive signal like hormone recognition could effect the onset of a large number of non-contiguous genes (i.e., the entire process of replication) and do so in an integrated manner<sup>23</sup>. In theory it resembles very much the lac operon enzyme induction system as proposed by Jacob and Monod in which a specific inducing agent binds to a particular "sensor" gene which in turn stimulates an "integrator" gene. The product of this gene is an "activator RNA" which activates certain receptor genes which allows the

transcription of a specified set of structural genes. It is realized that if several receptor or integrator genes are activated by the intervention of a single controlling element, (i.e., hormones), then a series of integrated and varied functions can occur.

The phosphorylation of the NHC proteins has been extensively studied. The highest concentration of intracellular-bound phosphate is located in the nucleus. Of this fraction, up to 90% is bound to NHC protein. Most of it is present as o-phosphoserine and may be as high as 50% of all the amino acid residues<sup>80</sup>. NHC proteins become rapidly phosphorylated, using ATP as the energy source in conjunction with a protein kinase and occurs independantly of protein synthesis. They are subject to rapid turnover. The proteins are phosphorylated and de-phosphorylated by separate enzymes<sup>136</sup>. That NHC protein phosphorylation does indeed induce gene activation is suggested by correlation between NHCP phosphorylation and the onset of gene activity in a number of tissues<sup>124</sup>. Polyacrylamide gel analysis of <sup>32</sup>P- labelled NHC proteins have shown them to be tissue and species-specific and stage-specific. NHC protein phosphorylation was shown to effect cell-free RNA synthesis. The regulation of NHC protein

phosphorylation is thought to occur via "second-mediation" by cyclic AMP and its action on varied cAMP-dependant protein kinase fractions<sup>75</sup>. The mechanism of gene activation or more properly, gene de-repression, was originally proposed by Kleinsmith and Allfrey, 1966<sup>76</sup>. It was postulated that positively-charged histones become displaced from the histone-DNA complex when in the presence of the negatively charged phosphate groups of phosphorylated NHC proteins. Once histone was removed, RNA synthesis could occur. This hypothesis was subsequently refined by the evolution of a newer model<sup>70</sup>. This model envisages the induction of phosphorylation of NHC proteins by histone interaction. This modification results in increased negativity on the NHC protein which in turn induces ionic bonding between the NHC protein and the positively charged histones. It is thought that this force of attraction is severe enough to displace histone and thus allow transcription to occur. Thus, this model sees the NHC proteins playing a more active role in gene regulation than do the histones, which nevertheless are an integral part of this complex system. The problems which remain the same are those which still ask why and how acidic proteins could bind specifically to only certain histone/DNA regions.

## MATERIALS AND METHODS

### A. Histone Isolation and Characterization

#### (1) Plant materials and growth conditions

Growth conditions were standardized for each experiment. Typically, enough Pinus pinea seeds were collected to fill a 600 ml beaker (i.e. about 480 seeds) for nucleohistone extraction procedures and about 200 ml volume of seeds (i.e. about 175 seeds) were used for the crude enzyme preparation. The seeds were vigorously washed in running tap water to remove the fine dust-like sediment which occurs on the surface of the seed. Small, discoloured or misshapen seeds were discarded. The seeds were then surface-sterilized in a plastic pan containing 1% javel water for 1 hour after which they were thoroughly rinsed in running tap water. They were allowed to stand in ordinary water overnight at 21°C.

It must be noted that in determining the age of the seedlings, day 0 begins with the sterilization step. The cotyledons are removed from the flats either on day 13 or 14, depending on the experiment. Nucleohistone extraction procedures were always performed on day 14, the period of maximal cell division.

After surface sterilization, the seeds were germinated in moist sand in 50 cm x 30 cm x 7 cm trays. The tray bottoms were punctured to allow for proper drainage and a length of paper towelling was placed over the bottom to prevent the loss of sand. The sand was first prepared by sifting to remove small stones and foreign objects, then sterilized by autoclaving at 121°C for 15 minutes and was air-dried. The flats were filled to a depth of about 5.0 cm. The seeds were planted in regularly spaced rows about 1.5 cm deep. It must be noted that seeds of Pinus pinea show polarity and must be planted with the broad-based area, characterized by two dark spots on the seed coat, facing upwards. The flats are maintained in a controlled-environment chamber with 400 ft-c illumination, 16 hour photoperiod and 45% relative humidity at 21°C. Temperature is critical and was monitored daily. The flats were watered with tap water every other day.

(ii) Isolation of plant nuclei

After exactly 14 days germination, each seedling was carefully removed from its bed of nearly dry sand. Care was taken to preserve the root structure. The seed coat and endosperm were carefully removed and both were discarded. The exposed seedling usually revealed twelve well-developed

cotyledons. Seedlings were selected in which overall length from apical tip to root tip ranged between 20 to 30 mm in length, eliminating discrepancies in developmental stage. In practice, about 30% of the seedlings were discarded at this point, the majority of which were less than 20 mm or have altogether failed to germinate. The seedlings were washed with distilled water to remove any traces of sand, dried between layers of absorbant towels and were loosely placed in a thin guage plastic bag which was subsequently sealed. Typically, between 75-100 g fresh weight of cotyledons were used for each nuclear preparation. All procedures were carried out between 0° - 5°C by means of refrigerated centrifuges, walk-in cold rooms and portable ice-buckets. All glassware and reagents were pre-chilled to 5°C. The following procedures for the isolation of nuclei are outlined in Appendix I.

Immediately after placing the cotyledons into the plastic bags, they were frozen between blocks of dry ice. Between 10 - 20 frozen cotyledons were ground at a time in a frozen mortar and pestle which contained a small piece of dry ice. As soon as the powder was prepared, it was placed into the grinding medium. The procedure was repeated until all the cotyledons were disrupted. This procedure helped initiate penetration of the grinding medium.

The tissue was allowed to stand in a Waring blender with 5 volumes of grinding medium. The grinding medium consisted of 0.4 M sucrose, 0.01 M  $MgCl_2$ , 0.01 M Tris, 0.025 M  $NaHSO_3$  in double distilled water adjusted to pH 8.0 with NaOH. This medium was kept as a 5x stock concentration with the exception that  $NaHSO_3$ , being unstable, is added just prior to use. Sodium bisulphite is used to prevent proteolysis<sup>109</sup>. The relatively high sucrose concentration ensures the integrity of the nuclei by maintaining a hypertonic environment and thus eliminates rupture due to osmotic shock. Magnesium ions stabilize the nuclear envelope.

After this 60-minute incubation period, the cells are disrupted by 90 seconds homogenization at full speed. The homogenate was then filtered through four layers of "J-cloth". The residue was then combed from the "J-cloth", resuspended in grinding medium and re-homogenized and re-filtered in "J-cloth" to ensure a larger nuclear yield. The entire filtrate is then filtered through a series of Nitex (Tober, Ernst and Traber, Inc., Elmsford, N.Y.) nylon mesh filters of decreasing pore size (375 $\mu$ , 100 $\mu$ , 25 $\mu$ , 10 $\mu$ ). The smaller pore sizes necessitated the use of a tap aspirator and Buchner funnel. This simple procedure



was highly successful in removing whole cells and cell fragments larger than  $10\mu$ . The filtrate was then centrifuged at  $500 \times g$  at  $5^{\circ}\text{C}$  for 10 minutes. This procedure effectively removed any whole cell or large particle which escaped the  $10\mu$  screening. The pellets were then recombined and resuspended in a wash medium consisting of 0.25 M sucrose, 0.10 M  $\text{MgCl}_2$ , 0.01 M Tris, 0.025 M  $\text{NaHSO}_3$ , 1% (v/v) Triton X-100 in double glass distilled water at pH 8.0 (NaOH). As in the case of the grinding medium, this wash medium was kept as a five-fold concentrated solution, adjusting the pH and adding the bisulphite just before use. This wash, by the detergent action of Triton X-100, removed the chloroplasts from the pellet. The resuspended pellet was then re-centrifuged at  $500 \times g$  at  $5^{\circ}\text{C}$  for 10 minutes. This procedure was performed twice and effectively removed all chloroplast contamination.

After this second centrifugation, nuclei, starch grains and some cell wall fragments remained in the pellet. The starch grains by themselves are no serious source of contamination but the cell wall fragments are certain to contain attached ribosomes which could be a source of basic protein contamination. This purification step required

ultracentrifugation through a sucrose density gradient.

The pellet was prepared for ultracentrifugation by first homogenizing the combined pellets in 2.3 M sucrose in a Potter-Elvehjem glass and teflon homogenizer which was

kept on ice. A low motor speed was used at five second

intervals, in an effort to dissipate heat build-up. The

homogenate which resulted was now less than 2.3 M sucrose

due to dilution. This suspension was then layered

carefully by Pasteur pipet over the sucrose density

gradient medium in the ultracentrifuge tubes. The sucrose

density gradient medium was prepared fresh with 2.3 M

ribonuclease-free sucrose (Sigma Chemical Co.), 0.01 M

MgCl<sub>2</sub>, 0.01 M Tris, 0.025 M NaHSO<sub>3</sub>, 1% (v/v) Triton X-100

and double distilled water. The medium was adjusted to

pH 8.0 with NaOH.

The interface between the two gradients was slightly disturbed to remove the interfering surface tension.

Centrifugation proceeded in an MSE Superspeed-75 ultra-

centrifuge equipped with a 3 x 23 ml swing-out bucket

rotor at 5°C for 2½ hours at 60,000 x g. After centri-

fugation, the pellets consisted of dark, sticky nuclei

over a tight layer of starch grains. Cell membranes and

ribosomes were discarded in the supernatant. The overlying

nuclei could now be physically separated from the layering starch grains. The combined nuclear pellets were then saved for the preparation of chromatin, nucleohistone and histones.

(iii) Chromatin, nucleohistone and histone  
preparation

Chromatin preparation methods are outlined in Appendix II. Once the nuclear pellet was physically separated from the starch grains, the nuclei were ruptured by homogenization and osmotic shock. Three or four strokes in a Dounce homogenizer were used with about 5 volumes of cold hypotonic medium consisting of 0.025 M  $\text{NaHSO}_3$  with 0.020 M EDTA adjusted to pH 8.0. The nuclei were then centrifuged at  $20,000 \times g$  at  $5^\circ\text{C}$  for 10 minutes. The supernatant was removed and the pellet was resuspended in the same medium and recentrifuged. The pellets at this point became solidified and sticky. The pellets were then gently resuspended in cold, double glass distilled water, and centrifuged at  $20,000 \times g$  at  $5^\circ\text{C}$  for 10 minutes. This procedure was performed twice. With each centrifugation, the pellet swelled due to hydration and became easily separable from any remaining starch pellet contamination.

The gel was allowed to swell further in double distilled water overnight at 5°C. The chromatin was then sheared for 90 seconds in a semi-micro head of a Waring blender at maximum speed at 5°C. This action solubilizes the nucleohistone. The sheared chromatin was further centrifuged at 20,000 x g at 5°C for 10 minutes. The pellet now contained residual starch grains, some nuclear membrane material and some DNA and was discarded.

#### (iv) Histone extraction

The extraction of histone from soluble nucleohistone is outlined in Appendix III. Acidic proteins were first extracted by stirring the prepared chromatin in 0.4 N  $\text{H}_2\text{SO}_4$  for 3 hours at 5°C. At this high acid concentration, the non-histone chromosomal proteins become dissociated from the DNA and were precipitated out of solution. They were removed by centrifugation at 20,000 x g for 10 minutes at 5°C. The resulting histone sulphates left in solution were dialyzed against cold 95% ethanol overnight. The precipitated histone sulphates were collected the next day by centrifugation at 20,000 x g for 10 minutes. The pellet was washed twice with acetone and stored at about -20°C. This extraction procedure gave between 5 - 8 mg histone from 75 - 100 g cotyledons.

(v) Physical analysis of chromatin

a. UV-spectral analysis, peak ratios and  
purity criteria

The UV-spectrum was determined from 350 nm to 230 nm using the Unicam SP 8000 recording spectrophotometer. Typical spectra are illustrated in Figures 4, 5 and 6. The spectra were used to analyze the response of chromatin to X-irradiation and as a criteria for purity by analyzing the UV peak ratios 260/240, 260/280 and 320/260. Criteria for purity were established by Bonner et al.<sup>19,20</sup>.

b. Thermal denaturation

Bonner et al.<sup>19</sup> described a method for the measurement of thermal denaturation in chromatin. Thermal denaturation can be used as an assay procedure for the analysis of the extent of radiation injury on chromatin<sup>60</sup>. Direct or indirect interaction of ionizing radiations results in the rupture of hydrogen bonds and depolymerization. These structural changes can be evaluated by noting decreased viscosity or hyperchromicity.

The sample of chromatin was first prepared by dilution to a known absorbance standard (typically 0.50 O.D. at 260 nm). Analyses were made using a Bausch and Lomb Spectronic 700.

spectrophotometer or Unicam SP 8000 recording spectrophotometer at a fixed wavelength of 260 nm. The sample was then sealed in a glass container and was gradually heated in an open water bath. After reaching the desired temperature ( $\pm 1^{\circ}\text{C}$ ) and allowing the sample to remain at that temperature for 5 minutes, absorbance was read at 260 nm immediately after this period without allowing the sample to cool. Readings were taken at  $5^{\circ}\text{C}$  intervals. Values for  $T_m$  were calculated by taking the midpoint between the O.D. value at  $20^{\circ}\text{C}$  and the highest O.D. value at the highest temperature. This method was modified for two-step  $T_m$  curves where noted. No attempts were made to subtract absorbance increases due to turbidity changes at higher temperatures.

(vi) Protein determination

Histone sulphates were dissolved in 15% sucrose (w/v) in 0.9 N acetic acid. Protein determinations were routinely made by the method of Lowry. Standard curves were made with commercially prepared bovine serum albumin (Fraction V, Sigma Chemical Co.). In some cases, histone concentrations were determined by the turbidity method. Two ml samples of diluted histone solutions were precipitated by an equal volume of cold 2.2 M TCA. The solutions were kept

on ice for 30 minutes and the optical density was measured at 400 nm with the Bausch and Lomb SP 700 spectrophotometer. Optical density increases linearly with increasing turbidity from 0.00 to 0.15 O.D.<sup>19</sup>. Commercially prepared calf thymus histone was sometimes used to prepare the standards. (Whole histone, Nutritional Biochemical Corp.) and was diluted and assayed in the same manner as plant histone.

(vii) Polyacrylamide gel electrophoresis of histone

a. Preparation of stock solutions


The fractionation of Pinus pinea histone into its subcomponents was done by polyacrylamide gel electrophoresis as described by Spiker<sup>129</sup>. No important changes were introduced from the procedures outlined, however, there were some slight modifications. The preparation of the polyacrylamide stock solutions and reagents can be made several weeks in advance if kept at 5°C in air-tight, darkened glass bottles. The acrylamide (Eastman Organic Chemicals, electrophoresis grade) was prepared by making a 70% (w/v) solution by dissolving 70 g acrylamide in 100 ml double-glass distilled water. The solution was stirred and brought to 45°C. The acrylamide was purified by the addition of activated animal bone charcoal. The resulting slurry was allowed to settle for several minutes and was filtered under

suction using two layers of Whatman #1 qualitative filter paper and a Buchner funnel. The solution was made 60% acrylamide (w/v) and 0.4% (w/v) N,N'-methylene BIS-acrylamide (Eastman Organic Chemicals). N,N,N',N'-tetramethylethylenediamine (TEMED) was also purchased from Eastman Organic Chemicals and was used to speed up the polymerization step. A 4% (w/v) TEMED stock solution was prepared in 43.2% acetic acid and stored in a dark bottle at 5°C. A 4.0 M stock urea solution was also prepared using double glass distilled water. Ammonium persulphate was used as a catalyst and was added to the required amount of urea solution just prior to use (see "Preparation of gels" for required amount).

b. Preparation of the electrophoresis tubes

Twelve polyacrylamide electrophoresis tubes of 6 mm internal diameter and 10 cm length were used for each analysis. After washing the tubes, they were rinsed several times with tap water. They were then acid washed, allowed to soak in several changes of distilled water and were followed by an acetone rinse, prior to air-drying at 85°C.

After allowing the tubes to cool, they were coated with a 1% solution (v/v) of dichlorodimethylsilane in benzene. This coating allows for greater ease of removal of the gels





from their tubes. One end of each tube was doubly wrapped with "Parafilm M". The tubes were then supported in a commercially designed stand.

c. Preparation of gels

Sixteen ml of a 15% acrylamide, 0.1% BIS-acrylamide gel solution was prepared by adding 4.0 ml acrylamide stock, 2.0 ml TEMED stock, 10.0 ml urea stock and 0.02 g ammonium persulphate into a 50-ml Erlenmyer flask with sidearm. The solution was thoroughly degassed using suction. One ml of the resulting solution was then added to each tube taking care not to cause any bubble formation. About 0.5 ml of cold 0.9 N acetic acid was then carefully layered above the liquid acrylamide to establish a flat surface on the gel. This step ensures that protein banding throughout the gel remained straight. The gel usually polymerized within 30-45 minutes when placed near a fluorescent light source. The gels thus prepared were easily handled but were somewhat brittle. The degree of molecular sieving afforded by the 15% gel was ideal for plant histone analysis.

The entire electrophoretic chamber was kept cool by a running, cold water jacket. Ammonium persulphate and TEMED were subsequently removed by electrophoresis in 0.9 N

acetic acid by applying 130 volts negative polarity across the tubes for 1½ hours. The voltage was supplied by a Buchler Instruments model 3-1014A power supply. Care was taken to ensure proper polarity, that is the cathode was in the lower chamber and the anode was in the upper chamber of the electrophoresis unit. Completion of this pre-electrophoresis step was marked by the migration of a marker dye, benzene-azo- $\alpha$ -naphthylene (Matheson, Coleman and Bell) in 15% sucrose solution. The dye migrates the distance of the tube in 90 minutes. The event is also marked by a current drop from 8 ma to 3-4 ma per tube. The voltage was subsequently adjusted to bring the current back to 8 ma per tube. The gels were allowed to cool for 30 minutes after pre-electrophoresis by maintaining the cold water flow inside the cooling jacket.

d. Application of histone to the gels.

Histone sulphates were dissolved in 0.9 N acetic acid containing 15% sucrose (w/v). Usually, 30  $\mu$ g of histone solution (1 mg/ml) were applied to each tube and was again electrophoresed at 130 volts, ensuring proper polarity and constant current for 90 minutes. The completion of this time period coincides with the complete migration of the marker dye, which was used in only 2 of the available 12

tubes. At the end of electrophoresis, the gels were carefully removed from the glass tubes by gently reaming the inner wall with a blunted 20 gauge hypodermic needle with slight water pressure applied. The gels were then placed in 0.1% (w/v) amido black solution containing 7% (w/v) acetic acid and 20% ethanol and were left overnight. The addition of 20% ethanol prevents hydration of the gel by the aqueous component of the dye. Such swelling can be considerable and would prevent the re-entry of the gel into the destaining tubes. Ethanol alone does not immobilize the protein, rather it is precipitated by the amido black. The next day, unbound dye was removed using 0.9 N acetic acid as electrophoresis buffer and by applying 130 volts, constant current across the tubes.

#### e. Gel analysis

The destained gels were maintained in their destaining tubes and were analyzed using an ISCO gel scanner model 659 with an ISCO type 5 optical unit with a 579 nm filter. The optical unit was connected to an ISCO model UA-4 absorbance monitor set to record a full scale deflection of 0.00 - 1.00 O.D. A Bausch and Lomb VOM 5 chart recorder was used to record the absorbance traces. Areas under the curve were assumed to be directly proportional with histone

concentration up to 10  $\mu\text{g}$  protein per band<sup>43</sup>. Areas under the curve were computed with a Keuffel and Esser compensating polar planimeter model 58815.

(viii) X-irradiation and dosimetry

Several experiments required either whole plant irradiation or irradiation of isolated plant histone or partially purified histone kinase. For both histone and histone kinase isolation procedures, irradiation was always performed on day-13, allowing for a full 24 hours before extraction procedures began. Seedlings were removed carefully from the germination flats, washed to remove traces of sand and were dried between layers of absorbant towels. Designated batches of seedlings were placed in a thin plastic bag and were labelled and sealed.

The seedlings were then placed directly under the beam path of a Müller MG 300 X-ray unit at a distance of 70 cm from the tube. No attempt was made to reduce the backscatter and 1.0 mm aluminium filtration was used to remove the harmful soft X-rays. Treated seedlings received either 0.5, 1.0 or 3.0 kilorads of 260  $\text{kV}_p$  X-rays at 7.0 ma at 35 R/minute. After receiving the desired amount of X-irradiation, incubation procedures were initiated for in vivo phosphorylation of plant histone using inorganic

radiophosphate. Control groups or samples to be used for kinase isolation procedures were maintained at  $21^{\circ}\text{C}$  between moist paper towelling inside loose, plastic bags and were subjected to conditions previously described under "Plant materials and growth conditions". Irradiation of partially purified kinase or isolated histone involved similar procedures. Both crude enzyme extract and histone were irradiated in small tightly capped glass vials. The samples were maintained on crushed ice throughout the irradiation procedures. X-ray exposure dose was determined using a Victoreen condenser R-meter with a 250 R probe or by ferrous ammonium sulphate dosimetry. A suitable method described by Law<sup>82</sup> modifies the standard Fricke ( $\text{FeSO}_4$ ) dosimeter to accurately monitor doses below 1.0 krad. The dosimeter was composed of 0.8 N  $\text{H}_2\text{SO}_4$ ,  $10^{-3}$  M  $\text{Fe}(\text{NH}_4)_2 \cdot 6\text{H}_2\text{O}$ ,  $10^{-3}$  M NaCl made in double glass distilled water. The solution was made fresh each time used and was protected from the light.

- (ix) Radioactive materials, monitoring and labelling procedures for the in vivo incorporation of inorganic  $^{32}\text{P}$  into histone subfractions.

Labelling procedures used in the in vitro enzymatic phosphorylation of histones by organic phosphate

will be described in the appropriate section. 1.0 mCi amounts of  $\text{NaH}_2^{32}\text{PO}_4$  were purchased from New England Nuclear. It was obtained as an aqueous solution at a concentration of 2.0 mCi/ml. The specific activity on assay date was 100 mCi/mM. The isotope was first prepared by dilution to 5.0 ml with double distilled water and the vial gently shaken. A 125  $\mu\text{Ci}$  aliquot of radiophosphate was added to 250 ml distilled water which was contained in a 500-ml glass beaker. The incubation medium was maintained on a water bath at  $21^\circ\text{C}$  and was gently aerated and illuminated. Immediately after irradiating the seedlings intended for the in vivo histone phosphorylation studies, they were submersed into the incubation medium for exactly sixteen hours. This period of time was previously determined to be necessary for sufficient  $^{32}\text{P}$  accumulation into all histone fractions under controlled conditions. The next day (day 14), the seedlings were removed from the incubation medium and washed several times with distilled water to remove surface contamination. The roots were then carefully excised and discarded. The remaining tissue was blotted dry with paper towelling. The material was then ready for nuclear extraction procedures as previously described.

x. Solubilization of acrylamide gel slices.

With the aid of a light source and dissecting microscope, protein bands were hand sliced and collected from 4 acrylamide gels. Thus 4 slices were obtained and combined for each fraction. The purpose of this procedure was to ensure count rates significantly above background even for the smaller protein bands. The gel slices were dissolved in 0.4 ml 30%  $H_2O_2$  and 0.2 ml 60% perchloric acid and were incubated for 1 hour at 60°C. After the vials had cooled, 10 ml Aquasol (New England Nuclear) was added to each and activity was monitored in an Ansitron liquid scintillation monitor.

## B. Histone Kinase Isolation and Purification

### 1. Isolation and purification procedures

There is relatively little information concerning the isolation and purification of plant histone kinases. Rather, much information on various tissues and phyla of the animal kingdom have been reported. Such reports give evidence for both cyclic AMP-dependant and independant protein kinases which enzymatically phosphorylate casein, protamine and/or histone. This enzyme was initially isolated in muscle<sup>145</sup>, rat liver<sup>151</sup>, pig brain<sup>105</sup>, hypothalamus<sup>95</sup>, rabbit reticulocytes<sup>86</sup>, pancreas<sup>87</sup> and hepatoma cells<sup>30</sup>. Kuo and Greengard<sup>79</sup> reported in a significant study that

there is widespread occurrence of cyclic-AMP-dependant protein kinases in many tissues of many members of the animal phyla and includes several invertebrate representatives.

It is interesting to note that in this early report, Kuo and Greengard reported the absence of c-AMP-dependant protein kinase in plant tissues but commented further by saying that this evidence was not at all conclusive and indeed the assay methods exclusively employed to study animal protein kinase were not suitable to the plant study.

Keates<sup>72</sup> successfully isolated a cyclic-nucleotide-independent protein kinase from pea shoots which enzymatically phosphorylated casein or phosvitin but not histone. Cyclic nucleotides did not affect the reaction rate. More recent reports show the presence of protein kinase in Chinese cabbage and tobacco<sup>119</sup> and a cyclic-AMP-binding protein in Jerusalem artichoke<sup>51</sup>.

A method utilized for the isolation of kinase from carrot root culture<sup>103</sup> was modified for Pinus pinea cotyledons and is outlined in Appendix IV. Methods for the partial purification of the crude enzyme are outlined in Appendix V. All operations were carried out at 0 - 5°C. Twenty-five grams (fresh weight) of day 14 cotyledons were harvested and combined with 10 volumes of cold 0.05 M Tris-HCl buffer with



0.05 M NaCl and 6 mM 2-mercaptoethanol adjusted to pH 7.8.

2-Mercaptoethanol was added to stabilize the enzyme. The tissue was ground in a cold, semi-micro head of a Waring blender for 90 seconds at maximum speed. The brei was then filtered through 4 layers of "J-cloth", then through a series of Nitex nylon mesh filters as previously described (375, 100, 25, 10  $\mu$ ). This step removed large, unbroken cell fragments and yielded a filtrate which consisted essentially of nuclei, chloroplasts, starch grains, cell wall fragments less than 10  $\mu$  in diameter and the cytoplasmic fraction. The filtrate was centrifuged at 500 x g for 10 minutes at 5°C and the supernatant was carefully withdrawn by Pasteur pipet. A thin, floating lipid layer was first removed with the aid of a plastic spatula. The supernatant was then combined with 15 g Polyclar AT (Serva Biochemicals, Heidelberg) in an effort to remove phenolic and lignin contaminants and was immediately centrifuged at 500 x g for 10 minutes at 5°C to remove the Polyclar AT. The supernatant was transferred to the ultracentrifuge and was spun at 105,000 x g for 60 minutes at 5°C. Protein was analyzed here and at all purification steps using the Lowry method.

After withdrawing the samples from the ultracentrifuge

tubes, the protein solution was treated with Dowex-IX2 for 20 minutes ( $\text{Cl}^-$  form, 1:10 w/v) which had been previously equilibrated with 0.05 M Tris-HCL, 0.05 M NaCl and 6 mM 2-mercaptoethanol overnight. The protein was then removed from the resin by suction filtration on a Buchner funnel using #4 Whatman filter paper. The protein was then extracted from solution by  $(\text{NH}_4)_2 \text{SO}_4$  precipitation and the 35% - 65% fraction was collected by centrifugation at 500 x g at  $5^\circ\text{C}$  for 10 minutes. This precipitate was resuspended in 4.0 ml of 0.05 M Tris-HCl buffer adjusted to pH 8.5 and contained 0.05 M NaCl and 6 mM 2-mercaptoethanol. This solution was dialyzed in the same buffer overnight. The precipitate which had formed by the next day was separated by centrifugation at 500 x g for 10 minutes at  $5^\circ\text{C}$  and was discarded.

Approximately 150 mg protein in a 5.0 ml volume of solution was applied to a Sephadex G-25 column to desalt the protein. The 2.5 cm x 35 cm column was equilibrated overnight with 0.05 M Tris-HCl in 0.05 M NaCl and 6 mM 2-mercaptoethanol adjusted to pH 8.5. The flow rate was adjusted to 2.3 ml/minute and 5.0 ml fractions were obtained dropwise in a fractionator. Absorbance was monitored at 280 nm using an ISCO model UA-4 absorbance monitor. A 300 ml

eluant was used and the fraction which showed the greatest kinase activity was collected and pooled for further purification and was usually that fraction collected between 230 ml to 250 ml (fraction numbers 46-50).

This fraction was then applied (usually 4.0 - 4.5 mg/ml protein) to a DEAE-Sephadex A-50 column (1.0 x 15.0 cm) which had been previously equilibrated with 0.05 M Tris-HCl buffer, pH 8.5 and contained 0.05 M NaCl with 6 mM 2-mercaptoethanol. The column was washed with 75 ml of the same buffer at a flow rate of 0.6 ml/minute. After stabilization, a linear concentration gradient maker was prepared to apply 100 ml of a salt gradient from 0.05 M to 0.60 M NaCl using the same buffer at the same flow rate as previously described. The fractions were collected dropwise in 3.0-ml samples and were immediately assayed. That fraction collected between 0.18 M to 0.22 M NaCl contained the highest kinase activity. Absorbance was monitored at 280 nm. Because the specific activity of the enzyme was sufficiently high to carry out further experiments, the enzyme was not further purified. Only on one occasion were those fractions which showed the highest kinase activity purified further on Sephadex G-200 (fine) using a 0.9 cm x 20 cm column. The 200-ml eluant previously described

for Sephadex G-25 was used at a flow rate of 0.6 ml/minute. Six peaks (not shown) were obtained with the highest enzyme activity occurring in the first peak (highest activity collected at 24 ml - 36 ml eluant). Further purification of the enzyme was not attempted, owing to the small amount of protein available after Sephadex G-200 gel filtration.

ii. Assay procedure for histone kinase

The assay procedure used throughout this work was standardized and was based on the method of Yamamura et al.<sup>151</sup>. Enzyme activity was assayed by using a Millipore glass filter (type AP-200) to collect acid-precipitated protein which had been labelled with adenosine 5'-tri-phosphate, tetra (triethylammonium) salt,  $\gamma$ -<sup>32</sup>P purchased from New England Nuclear. The specific activity on assay date was 27.0 Ci/mM at a concentration of 0.0370  $\mu$ M/mCi. The standard reaction mixture contained 50  $\mu$ g/ml calf thymus histone, 5  $\mu$ g/ml kinase, 2.5  $\mu$ M  $\gamma$ -<sup>32</sup>P ATP at a specific activity of 0.2  $\mu$ Ci, 3  $\mu$ M 2-mercaptoethanol, 5 mM  $Mg^{2+}$ -acetate and 5  $\mu$ M Tris-HCl buffer adjusted to pH 7.5. The total reaction volume was 0.25 ml and was incubated at 30°C for 5 minutes, on an open water bath. The reaction was immediately halted at the end of the incubation period by the addition of ice cold 5% TCA with 0.25% sodium tungstate

(pH 2.0) and by removal of the reaction vessels to 0°C.

The precipitated protein was collected on Millipore glass filters under suction and were washed extensively with 5% TCA, 0.25% sodium tungstate, acetone, ethanol and finally ether. All wash solutions were maintained at 0 - 5°C.

Radioactivity was assayed using an Ansitron liquid scintillation spectrophotometer and a scintillation cocktail containing 0.4% PPO, 0.01% POPOP in 1.0 l toluene. Twenty-three-ml glass scintillation vials were used and were tightly capped. Fifteen-ml volumes of cocktail were used for each filter. Window settings were adjusted to 50-990 volts and no less than 4 minute counts were taken for each sample.

### iii. pH dependance and buffer types

There is evidence from the literature that protein kinase can operate in vitro under a fairly broad physiological range of pH. Such examples are carrot root culture, 37°C, pH 8.6 in 5  $\mu$ M Tris-HCl buffer<sup>103</sup>, rat liver, 37°C, pH 7.5 in 5  $\mu$ M potassium phosphate buffer<sup>151</sup> and pea shoots, 30°C, pH 6.5 in 0.05 M sodium  $\beta$ -glycerophosphate buffer<sup>72</sup>. In this study, attention was paid to proper buffer types in order to determine the pH optimum. The buffers used were 5  $\mu$ M Tris-HCl from pH 6.5 - 8.5, 10  $\mu$ M

potassium phosphate, pH 7.0 - 9.0 and 10  $\mu$ M glycine, pH 8.5 - 9.5. Standard assay conditions were used, which included 5  $\mu$ g enzyme preparation in the assay mixture and 5 minutes incubation at 30°C.

iv.  $Mg^{2+}$  dependance

Standard assay conditions were used. Magnesium acetate concentrations were prepared from 0 - 10 mM. Optimum pH was determined for phosphate buffer as described above.

v. c-AMP and kinetin dependance

Standard assay conditions were used. Adenosine 3', 5'-cyclic-phosphate was purchased from Calbiochem and was used in the assay at a concentration range of 0.0 - 10.0  $\mu$ M. Kinetin (6-furfurylaminopurine) was purchased from the Nutritional Biochemical Corporation and was also assayed under standard assay conditions and was tested at a concentration range of 0.0 - 10.0  $\mu$ M.

vi. Thermal lability and stability

Thermal lability of the enzyme was assayed as described by Nakaya et al.<sup>103</sup>. Using standard assay conditions, each reaction mixture was incubated for 5 minutes at the appropriate temperature which ranged between 20 - 95°C. The stability

of the enzyme was determined by immediate assay after recovery from DEAE-Sephadex A-50 and by assay of the same sample for periods up to 6 weeks when stored at  $-20^{\circ}\text{C}$  temperatures.

#### vii. Enzyme kinetics

Standard assay conditions were employed for the analysis of enzyme kinetics. The Michaelis-Menton constants ( $K_m$ ) were calculated for ATP, Pinus pinea histone and calf thymus histone by the method of Lineweaver and Burk.

#### viii. Inhibition studies

Standard assay conditions were used to conduct tests for the presence of sulphydryl groups and for the reversibility of inhibition by the action of 2-mercaptoethanol. p-Chloromercuribenzoate (p-chloromercuribenzene sulphonic acid) and iodoacetic acid (free acid, anhydrous) were purchased from the Sigma Chemical Corporation. 2-Mercaptoethanol was used to test for the reversibility of inhibition. EDTA (ethylenediamine tetraacetate,  $\text{Na}^+$  salt) was used to demonstrate  $\text{Mg}^{2+}$  dependence by chelating the divalent cation.

#### ix. Substrate specificity

Standard assay conditions were used to test for

substrate specificity. The specific activities were assayed using both 10 and 20  $\mu$ g additions of substrate. Both calf thymus histone and Pinus pinea histone were used. In addition, casein, and protamine sulphate obtained from salmon sperm were analyzed and were purchased from the Sigma Chemical Corporation.

#### x. X-irradiation procedures

Either whole tissue irradiation as previously described or histone and/or histone kinase were irradiated at 0.5, 1.0 and 3.0 krad doses using the Müller MG 300 X-ray unit. Dose rate and distances used were identical as for irradiated cotyledons used for nuclei extractions. Irradiation of both isolated histone and histone kinase was carried out in aqueous solution maintained at 0 - 5°C.

#### xi. Post-irradiation mitotic recovery and Feulgen staining

Growing cotyledons were irradiated at various doses using procedures previously described. Irradiation was performed on day-13 and recovery was monitored for the next 48 hours. The cotyledons from different seedlings were randomly chosen at the prescribed time period from each of the three irradiated groups (0.5, 1.0, 3.0 krad) and control (0.0 krad). The cotyledons were fixed in acetic



acid:ethanol (1:3) overnight at 5°C. The next day, the cotyledons were rinsed in 70% ethanol and were later stored in 70% ethanol at 5°C. Feulgen staining of the nuclei proceeded by hydrolyzing the cotyledons in 1.0 N HCl at 60°C for exactly 7 minutes. The stained tissue was subsequently cooled on ice. The cotyledons were rinsed in distilled water and were stained with fresh Feulgen reagent overnight. The following day, the cotyledons were rinsed in distilled water and the growing apical tips were excised and placed on a microscope slide, containing a drop of 45% acetic acid. The fibers were thoroughly macerated with the aid of two dissecting needles. A cover slip was placed on top and considerable pressure was applied to make a squash preparation and temporary slide of the tissue. At least 2,500 cells from three different apical tips from each seedling were scored for each irradiated group. Thus some 10,000 cells were scored for 4 individual plants for each given dose and for each given time period. Mitotic indices were scored as % mitosis and standard errors were calculated.

## RESULTS AND DISCUSSION

### A. Physical Analysis of Chromatin

#### 1. U.V. absorption - peak ratios and purity criteria.

Sheared chromatin was diluted 1:10 in cold, double-distilled water and adjusted to neutral pH. U.V. analysis gave typical absorption peaks at 260 nm and 220 nm (Figures 1,2,3). Purity criteria for isolated Pinus pinea chromatin is shown in Table 4. The data shows that based on Bonner's criteria,<sup>19,20</sup> the chromatin isolated after exposure to 0.0, 0.5 and 1.0 kilorad irradiation and incubation with 125  $\mu$ Ci inorganic radiophosphate is reasonably free of chromosomal aggregates or non-chromosomal proteins.

Table 4. Criteria for purity of isolated chromatin.

X-ray exposure (krads)	Absorbance (260 nm)	Absorbance (320 nm)	320/260
0.0	0.69	0.04	0.06
0.5	0.66	0.05	0.06
1.0	0.61	0.05	0.08

The U.V. peak ratios were also determined and values are displayed in Table 5. The 260/240 ratios fell between 1.08 and 1.21 and 280/260 ratios were observed from 1.55 to

Figure 1: UV-absorption spectrum of P. pinea  
chromatin isolated from unirradiated  
cotyledons.

*Handwritten scribble*

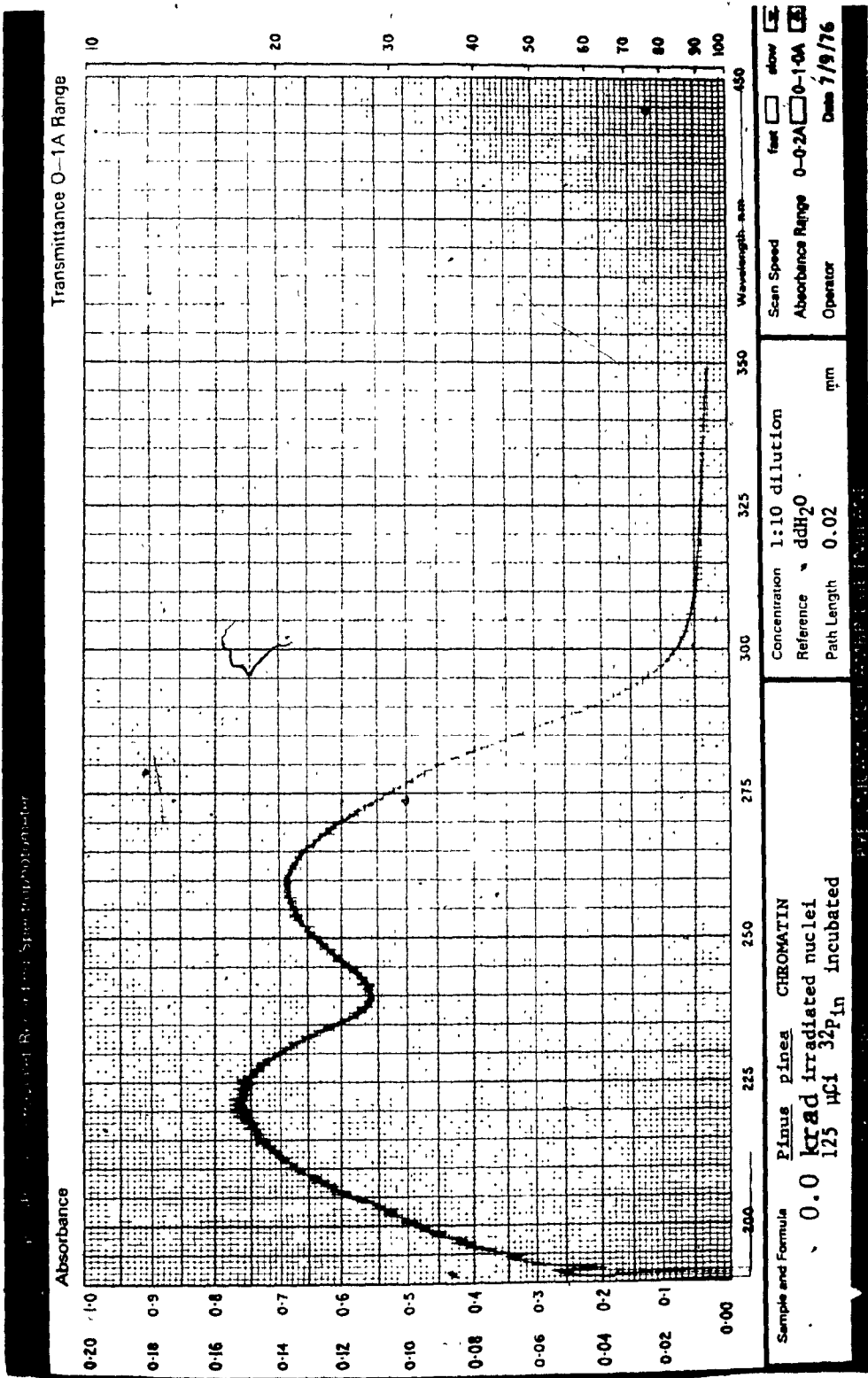
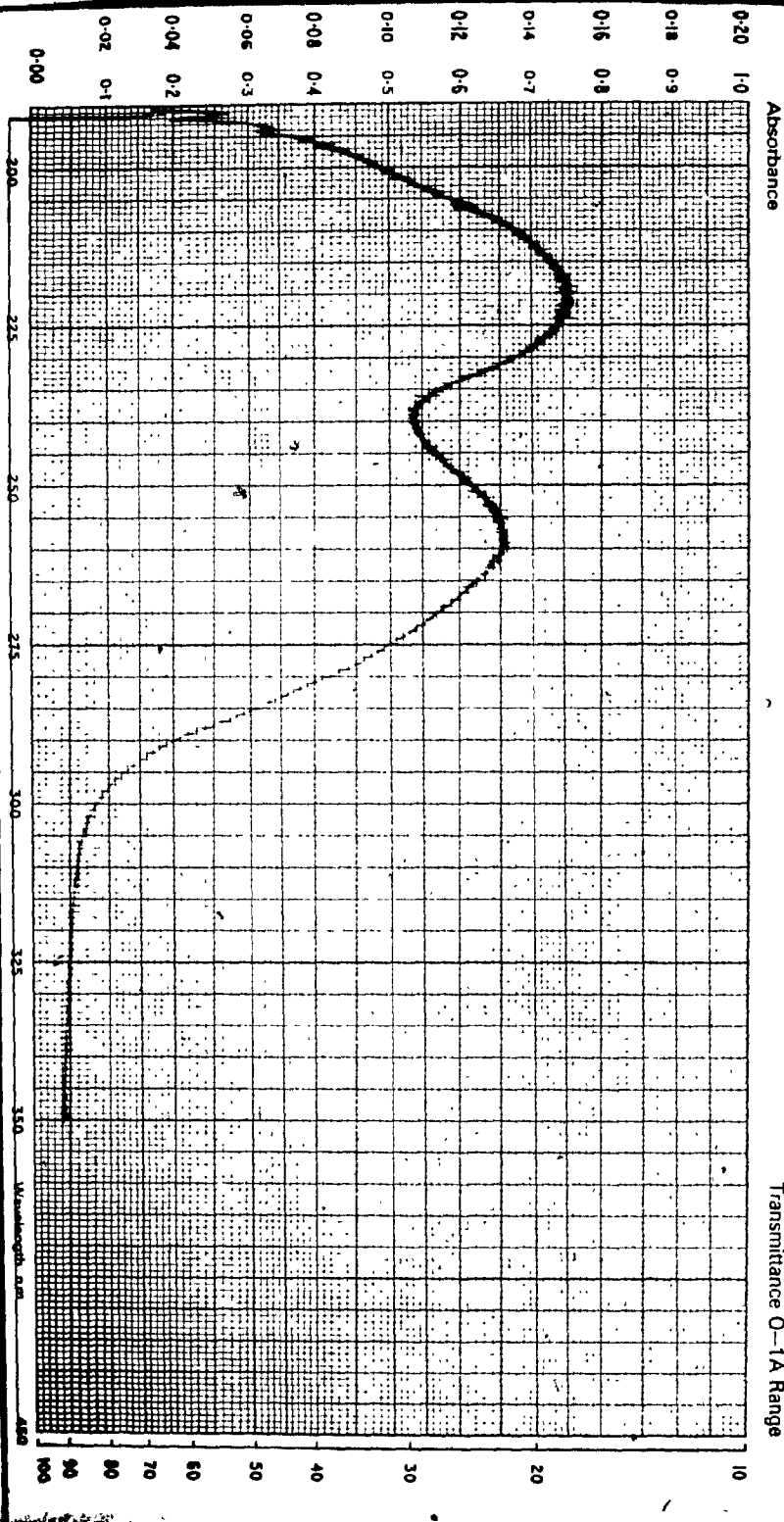


Figure 2: UV-absorption spectrum of P. pinea  
chromatin isolated from 0.5 krad  
irradiated cotyledons.

Transmittance 0-1A Range

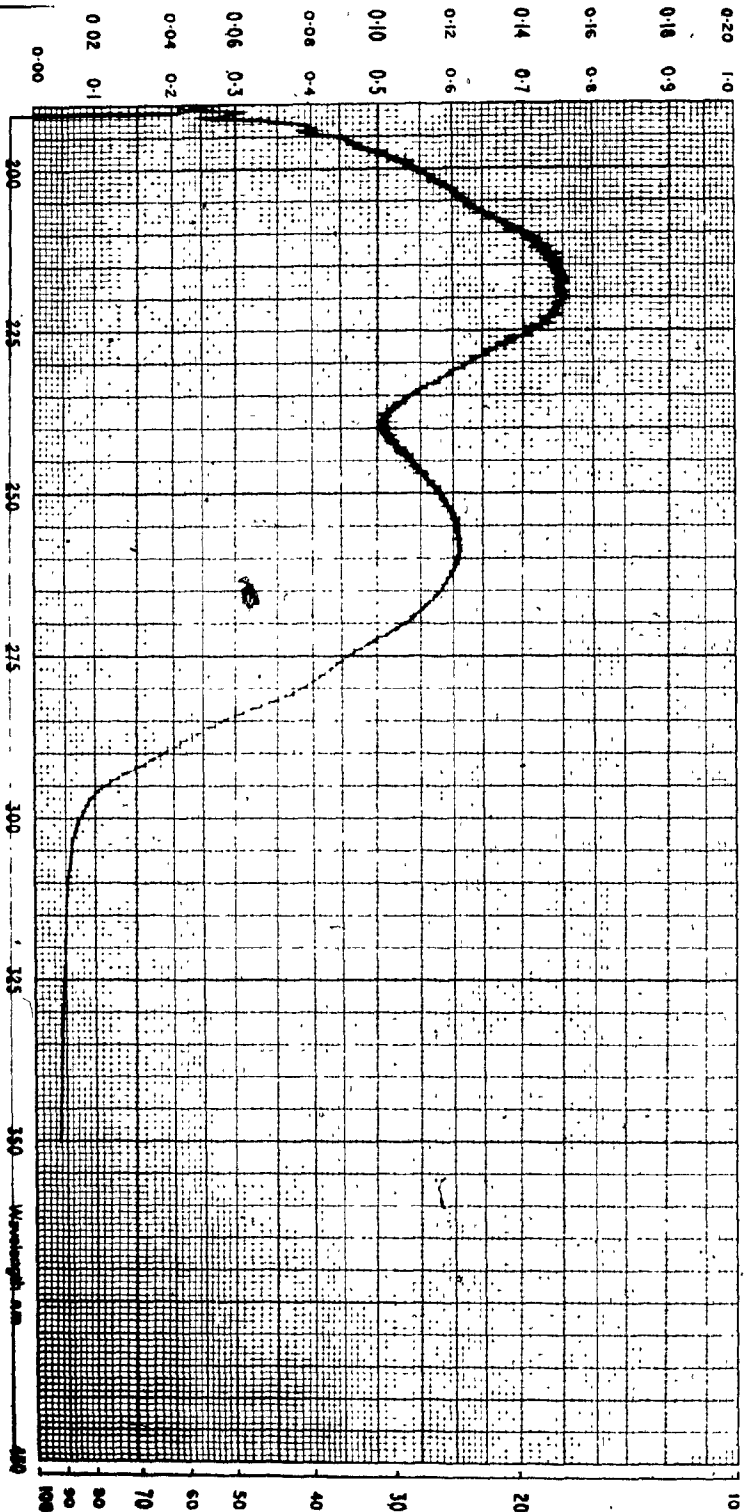


Sample and Formula	Pinus pinus CHROMATIN	Concentration	1:10 dilution	Scan Speed	fast <input type="checkbox"/> slow <input checked="" type="checkbox"/>
0.5 Krad irradiated nuclei		Reference	ddH <sub>2</sub> O	Absorbance Range	0-0.2A <input type="checkbox"/> 0-1.0A <input checked="" type="checkbox"/>
125 KCl 32P <sub>4n</sub> incubated		Path Length	0.02 mm	Operator	Due 7/31/76

Figure 3: UV-spectra of P. pinea chromatin  
isolated from 1.0 krad irradiated  
cotyledons.

Absorbance

Transmittance 0-1A Range



Sample and Formula  
 1.0  $\mu$ g/ml CHROMATIN  
 125  $\mu$ Cl 32P in incubated

Concentration  
 Reference 1:10 dilution  
 Path Length 0.02 mm

Scan Speed ☐ fast ☒ slow  
 Absorbance Range 0-0.24 ☐ 0-1.0A ☒ 0-1.0A  
 Operator Date 7/24/75



2.22. The data suggests no discernable difference of the 260/240 ratios between control and irradiated groups; however, there seems to be evident a sharp decline in the 280/260 ratio from control to 1.0 krad exposure, perhaps implying loss of nuclear protein.

Table 5. Peak ratio analysis in response to X-irradiation

X-ray exposure (krads)	O.D. ratios	
	260/240	280/260
0.0	$1.21 \pm 0.09$	$2.22 \pm 0.15$
0.5	$1.20 \pm 0.10$	$1.61 \pm 0.09$
1.0	$1.20 \pm 0.06$	$1.55 \pm 0.13$

The primary task for the in vitro analysis of P. pinea histone structure, metabolism and function is the necessity for the purification of chromatin or nucleohistone. Chromosomes as found in the intact nucleus are collectively termed chromatin and are the site of DNA biosynthesis and DNA-dependant RNA biosynthesis. In only the last fifteen years, research has led to the development of modern chromatin isolation techniques which allows for a more detailed and comprehensive analysis of its structure and function.

Chromatin, once isolated, is found to be composed of protein complexed with DNA and more or less defined amounts of RNA. In the in vivo situation, only selected portions of the DNA or genome are found to be associated with a basic protein or histone. Such genomes are genetically "masked" whereas other portions are available or accessible for RNA transcription<sup>47,98,99,111,128,133</sup>. Histones are defined as a complex arrangement of small yet unique basic proteins which are at times associated with DNA in the cell nucleus. The complex between these basic proteins and DNA is defined as nucleohistone or deoxyribonucleoprotein or DNP<sup>128</sup>. However, it is absolutely essential for the study of histone chemistry that purified chromatin be used to isolate and further purify histone. Without such technical interaction, other nuclear components will contaminate the preparation. Both preparative acid extraction of whole tissue or the crude isolation of nuclei will include other basic, ribosomal proteins, residual nuclear proteins and non-histone chromosomal proteins (acidic proteins). These non-histone components are often present in far greater amounts than the histones themselves<sup>20</sup>. The residual nuclear proteins have been reported to represent by themselves over 50% of the nuclear protein

content<sup>153,154</sup>. It has been suggested that a portion of this fraction comes from the cytoplasmic membranes and it has been suggested that these proteins are denatured acidic nuclear proteins<sup>64</sup>. In summary then, there has been assembled five classes of nuclear proteins: 1) nuclear globulins which are soluble in 0.14 M NaCl, 2) Tris-extractible nuclear ribosomal proteins, 3) acid soluble basic nuclear proteins which includes histones and protamines, 4) acid nuclear proteins (non-histone chromosomal proteins) and 5) residual nuclear proteins<sup>64</sup>.

There are in general, certain basic considerations which must be given prior to the preparation of nucleohistone. The first major consideration must be given to the action of proteolytic or degradative enzymes. Careful attention must be given to both time and temperature of the isolation procedures, especially at the early preparative stages<sup>128</sup>. Hnilica (1967) comments on the advisability of isolating nuclei as the preliminary step, disregarding early wash and differential centrifugation stages<sup>64</sup>. However, some authors advise that the degree of difficulty in isolating a relatively pure nuclear fraction depends on the tissue used and that starting material characteristics may preclude high yield and speed as secondary considerations

114,132. These time-consuming and enzyme-degradative processes may be eliminated for those certain tissue types.

Early published reports used calf thymus as starting material which was considered an excellent source of nucleohistone because of the relatively low proportion of cytoplasm. This in itself made prior nuclear extraction unnecessary. One such method, the Chargaff procedure, involved tissue disruption by homogenization in suitable buffers (i.e., 0.10 M NaCl, 0.05 M sodium citrate, pH 7.0, 0 - 4°C), low-speed selective sedimentation of nuclei and washing to remove exogenous electrolyte with buffered distilled water<sup>27,29,101,49,133</sup>. The Zubay-Doty procedure<sup>37,155</sup> represented several important refinements to the preliminary Chargaff procedures and included defined speed control of the Waring blender and the addition of capryl alcohol blended with saline-versene solvent adjusted to pH 8.0 with NaOH. This greatly improved suppression of surface denaturation. The inclusion of EDTA in the solvent ensured deoxyribonuclease I inhibition by the chelating action of divalent cations. The maintenance of a relatively higher pH inhibits deoxyribonuclease II and inhibits histone denaturation by the action of cathepsins.

The basic methods utilized for the isolation of pure nuclei from plant sources could not be used without modification due to the fact that plant cells are surrounded by a cell wall. Two early extraction procedures described the direct extraction of nucleohistone from plant homogenates without prior nuclei isolation<sup>66,17,10</sup>. Early attempts by Berkofsky<sup>13</sup> in an effort to isolate nucleohistone from Pinus pinea by the Johns and Butler<sup>69</sup> procedure failed. This method attempted a direct chemical extraction from whole cells which was similar to the Zubay-Doty procedure but resulted in low chromatin yield and high basic protein contamination. The methods of Fambrough and Bonner<sup>42</sup> and Tautvydas<sup>138</sup> required the isolation of pure nuclei but both resulted in unacceptable levels of contamination. Finally, the modification of the method used by Spiker<sup>129</sup> led to acceptable levels of nuclear purity and thus a suitable material for histone extraction was obtained.

Bonner et al.<sup>19,20</sup> states that properly prepared chromatin is a clear gelatinous pellet which exhibits very little or non-existent absorption at 320 nm and must be less than 0.10 of the absorbance at 260 nm. In addition, pure chromatin is characterized by a protein/DNA (280/260) ratio of 1.3:1.0 - 2.0:1.0 and RNA/DNA ratio of less than

0.2. Pure chromatin migrates as a single band in zone electrophoresis and high melting profiles attest to DNA stabilization by protein.

Properly prepared calf thymus nucleohistone is characterized by stability for several days at 5°C. The extinction coefficient,  $E_{259 \text{ m}\mu}^{1\%}$ , is  $106 \pm 5$  with a DNA content of  $47 \pm 2\%$  and a nitrogen/phosphorous ratio of  $3.7 \pm 0.3$ <sup>128</sup>. In addition, properly prepared calf thymus DNA and DNP have the following physico-chemical characteristics as outlined in Table 6.

Evidence for enzymatic degradation is marked by decreased rigidity of the molecule which would result in a decrease in viscosity. An increase in viscosity, on the other hand, marks DNP aggregation which is initiated by high salt content or the presence of multivalent cations<sup>128</sup>.

It should be noted that in this study, absorption spectra analyses were made 24 hours after in vivo, whole-tissue irradiation, whereas Berkofsky<sup>13</sup> quotes data for the irradiation of isolated nuclei. On this basis, the data suggests an observed irradiation effect on the level of protein synthesis (i.e., loss of nucleoprotein or decreased amounts produced) as well as a certain amount of decrease in

the DNA levels. Such loss of chromosomal protein may be the result of radiation injury to both portions of the nucleoprotein complex. It is well documented that X-irradiation causes weakening of the DNA/histone association<sup>8,9,94</sup>.

Table 6 Physico-chemical properties of nucleohistone/DNA

Reference	Molecular Weight	$[\eta]$ , dl/g	$S_{20, w}$
		(intrinsic viscosity)	(sedimentation coefficient)
<u>DNP</u>			
Zubay and Doty <sup>155</sup>	$19 \times 10^6$	35	50
Bayley, Preston Peacocke <sup>10</sup>	$20.5 \times 10^6$	30	---
Lloyd and Peacocke <sup>94</sup>	--	--	50 for 70% of the material
<u>DNA</u>			
Zubay and Doty <sup>155</sup>	$8 \times 10^6$	70	22

(After B.P. Sonnenberg and G. Zubay, 1966)<sup>128</sup>

#### ii. Thermal denaturation

The  $T_m$  values and values of hyperchromicity for P. pinea control and irradiated chromatin are displayed in Table 7. The  $T_m$  profiles are presented in Figures 4, 5, 6.

The results indicate that  $T_m$  decreases as an X-ray exposure increases. Also, there is a very definite transition from a two-step  $T_m$  profile for the unirradiated chromatin to a one step  $T_m$  profile for chromatin irradiated at 1.0 krad which closely resembles the  $T_m$  profile for deproteinized chromatin or nucleohistone rather than whole chromatin. The data strongly suggests that irradiated chromatin is structurally damaged or modified to resemble nucleohistone perhaps as a result of loss or alteration of the chromosomal nuclear proteins. ( $T_m$  is defined as thermal denaturation).

Table 7 Thermal denaturation and hyperchromicity of control and irradiated chromatin. (Values are measured at 260 nm, without buffer system, using ambient temperature determinations)

X-ray exposure ( krad )	$T_m$ 1 °C	$T_m$ 2 °C	Hyperchromicity (%)
0.0	61.3 $\pm$ 0.9	82.5 $\pm$ 1.1	28
0.5	57.5 $\pm$ 0.7	87.5 $\pm$ 0.8	24
1.0	---	75.0 $\pm$ 1.2	62

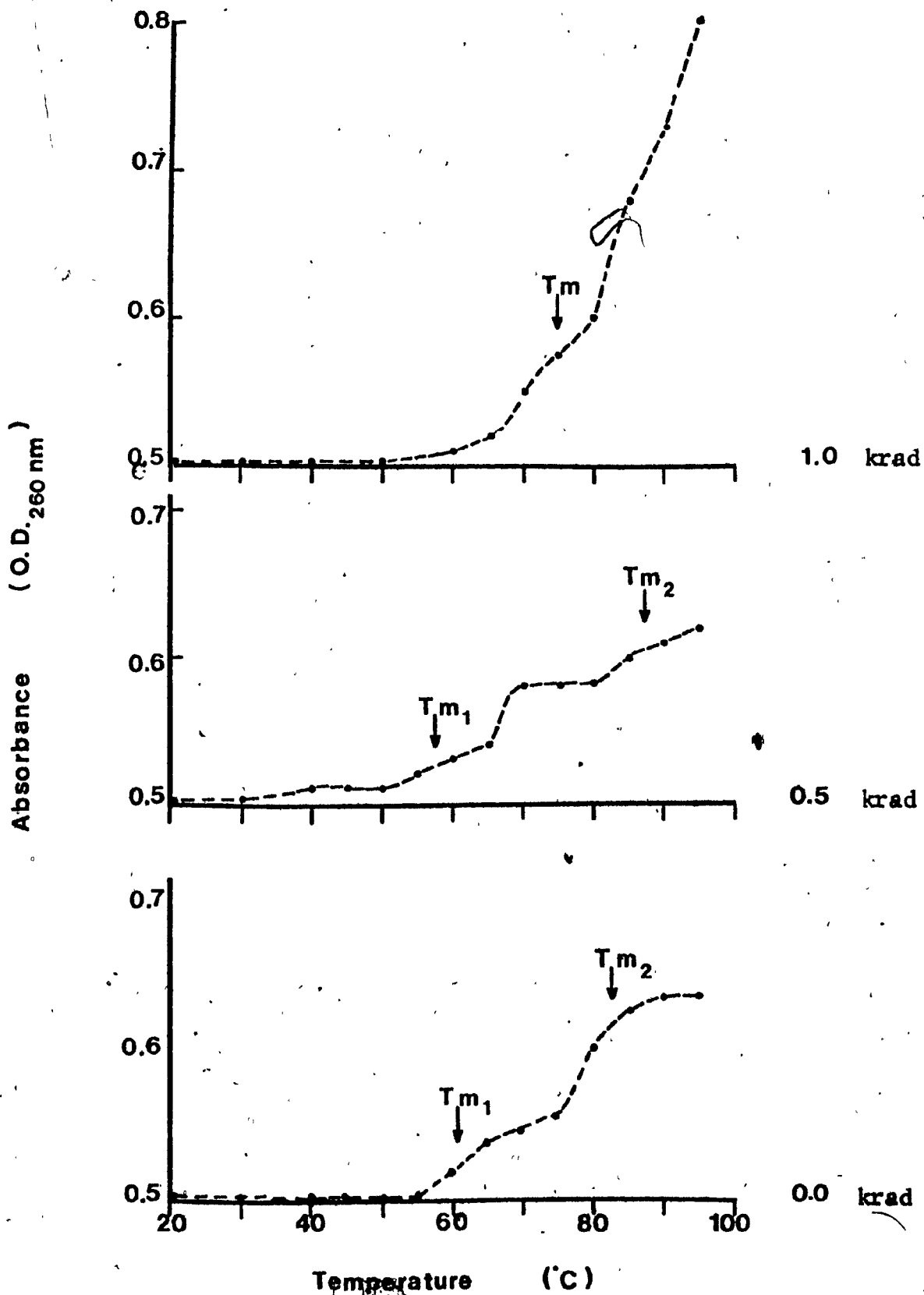
Thermal denaturation of double-stranded DNA results in the dissolution of hydrogen bonding between complementary base pairs and subsequently allows the double helix to unwind and separate with resulting physical and chemical



Figure 4: Thermal denaturation profile of 1.0 krad irradiated chromatin isolated from P. pinea nuclei. (Values were recorded at 260 nm, using an unbuffered aqueous system and ambient temperature determinations.

Figure 5: Thermal denaturation profile of 0.5 krad irradiated chromatin isolated from P. pinea nuclei.

Figure 6: Thermal denaturation profile of 0.0 krad unirradiated chromatin isolated from P. pinea nuclei.



changes. This change has been referred to as the "helix  $\rightarrow$  coil" transition<sup>31</sup>. Polynucleotide absorption at 260 nm is due to its base components; however, absorption is reduced or suppressed when polynucleotide bases are rigidly held in stacked formation as is the case with double stranded DNA. Equimolar amounts of free bases or even nucleotides show far greater absorbancies for this reason. This absorbance suppression is lifted as the DNA undergoes helix  $\rightarrow$  coil transition and an increase in 260 absorption or hyperchromicity is observed. The helix  $\rightarrow$  coil transition will occur at specified  $T_m$ 's regulated by several factors. The complexity of the DNA molecule will affect helix  $\rightarrow$  coil transition, (i.e., "homogenous" DNA as found in prokaryotic organisms (viruses, bacteria) will melt over a narrow temperature range as compared with "heterogenous" DNA of the eukaryotes). Owing to the fact that (G+C) base pairs are intrinsically more stable than (A+T) base pairs, then it is easy to understand that (G+C) rich DNA regions (i.e., bacteriophage lambda) will melt at higher temperatures than (A+T) rich regions. Also, the type, ionic strength and  $pH$  of the solvent will affect base-pairing interaction and will subsequently alter or modify the transition<sup>31</sup>:

There are two generally accepted methods described for

the measurement of  $T_m$ 's. The first method<sup>38</sup> measures the absorption quickly after the DNA solution has reached the desired temperature and is then read at that temperature. The second method<sup>50</sup> measures the absorbance of DNA which has been quickly cooled after heating to produce a condition of irreversible denaturation. The first method is preferred because it relates to base stability of the DNA in the original state<sup>19</sup>. The  $T_m$  measurements for this study were made by the preferred ambient temperature method and whole chromatin, not nucleohistone was chosen for this study and this represents an important difference. Native, untreated chromatin displays a typical, two-phase  $T_m$  curve (Figures 7 and 8). Bonner and Huang<sup>18</sup> noted that pea DNA, when complexed with histone, exhibited a  $T_m$  14°C higher than deproteinized DNA. When nucleohistone is associated with RNA and acidic protein, as in whole chromatin, there must exist a different structural form to allow for a two-step  $T_m$  profile. Bonner and Huang (1964), also stated that the 2nd  $T_m$  step ( $T_{m2}$ ) corresponds to the  $T_m$  of native nucleohistone whereas the 1st step ( $T_{m1}$ ) corresponds to the  $T_m$  of native DNA.

The basic model for two-step (or multi-step) thermal denaturation profiles rests on the premise that DNA base pairs

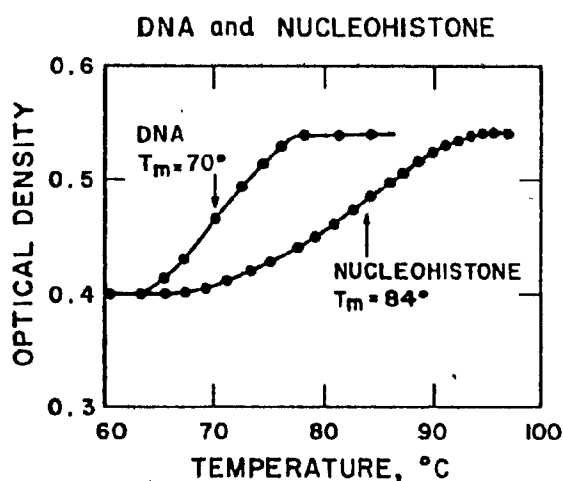


Fig. 2a.—Deproteinized DNA and native nucleohistone.

Figure 7

Pea embryo DNA melting profiles

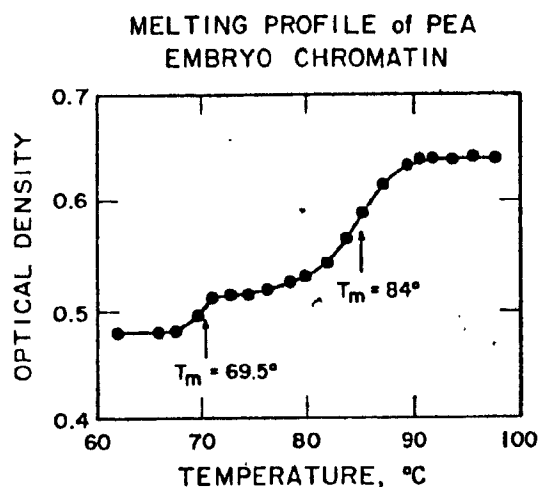


Fig. 2b.—Chromatin.

Figure 8

(J. Bonner and R-c.C.Huang, 1964)<sup>18</sup>

in the chromatin or nucleohistone complex can be identified as two groups; one a protein-free and the other, a protein-bound DNA genome. It is this difference which confers different degrees of protection which allows for multi-step denaturation profiles<sup>89</sup>. Several experiments report on artificial polylysine or polyarginine - DNA complexes which show biphasic melting curves as a result of the polypeptide association<sup>143,85,106,107,71</sup>. Early reports giving the denaturation profiles for pea bud chromatin and selectively-removed histone could not be reconciled with the above model for biphasic denaturation<sup>88,89</sup>. Biphasic denaturation for these cases was interpreted to be closely linked with the

physical properties of the individual histone molecules and that two melting bands, one at 66°C and the other at 81°C were due to DNA interaction with a less-basic portion and the more-basic portion of the histone molecule respectively. However, selective removal of lysine-rich histone caused reduced amplitudes of both melting bands and suggested that DNA stabilization is unlikely due to any one class of histone. Instead, the Li - Bonner model attributed different melting bands to different degrees of stabilization by different histone "ends". The ends are defined by their lysine/arginine ratios. The more basic end confers greater electrostatic protection and thus a more stabilized DNA is established which exhibits a higher melting temperature. It would be difficult to assign which model would account for biphasic  $T_m$ 's in P. pinea chromatin but it is possible that perhaps both mechanisms could operate. It is equally possible that any form of degradation of either stabilizing systems (i.e., loss of chromosomal protein or loss of basic histone) would account for altered  $T_m$  profiles. For example, chromatin autolysis and trypsin digestion of chromosomal proteins which resulted in exposed and altered DNA stabilization resulted in changes in the  $T_m$  profile<sup>2</sup>. Similarly, one can interpret

these results as being analogous to the radiation-induced effect. Several experiments have indicated altered DNA and/or histone content following whole cell or nuclei irradiation<sup>8,9,83,7</sup>. Bauer et al.<sup>8</sup> has demonstrated lowered  $T_m$ 's for both DNP and reconstituted DNP (deoxyribonucleo-protein) as a result of the radiation effect and further suggested that this resulted from damage or alteration of the histones.

B. Histone Analysis and Characterization and the Effect of X-Irradiation on Relative Mobilities of the Histone Subfractions

Electrophoregrams of calf thymus histone  $^{32}\text{P}$  phosphorylated P. pinea histones are illustrated in Figures 9, 10, 11, 12. Because of the stated constant electrophoretic mobility of the arginine-rich F2a1 (IV) histone subfractions<sup>73,110,130,131</sup> of histone samples obtained from various plant and animal sources, band 8 (see Figure 10) was used to align the P. pinea electrophoregrams with calf thymus histone F2a1 (IV) in an attempt to further characterize the other fractions. In all cases, 30  $\mu\text{g}$  of histone were applied. In all cases, at least eight or sometimes nine fractions could be separated from P. pinea histone. That fraction having the highest mobility was band 8 and was

labelled as the arginine-rich F2a1 histone. The major peak nearer the cathodic end was labelled as bands 6-7. Histones of this control group were electrophoresed several times and consistently gave reproducible results. In Figure 11, partial resolution of peak 6-7 is achieved. Berkofsky (1975) claims that this apparent failure in separating these peaks is due to the amount of histone applied and the degree of chemical modification suffered but nevertheless this peak does represent histones F2a2 (6) and F2b (7)<sup>13</sup>.

Because of the relatively low electrophoretic mobilities of the first four numbered fractions for P. pinea histone, this group was arbitrarily labelled as the lysine-rich F1 fraction and showed comparable mobility with calf thymus F1 histone. In one group, i. e. ,unirradiated P. pinea histone, the persistent occurrence of a fraction with less electrophoretic mobility than fraction 1 is seen (Figure 10). This fraction completely disappears in the irradiated groups. This fraction is presumably lysine-rich and was tentatively labelled as fraction X. Certain unirradiated histone fractions aligned perfectly with the calf thymus histone fractions. These are histones 3 (F1), 6-7 (F2a2, F2b) and 8 (F2a1). Histone 5 is of variable



nature and seems to correspond with calf thymus histone F3 (III). Variability in the areas under the curve, that is, the amount of protein constituting each fraction, is greatest for fractions 5, 6-7 and 8; however, fractions 1-4 were more constant. Except for band X, there seems to be little evidence of an X-ray-induced effect on any one particular fraction of P. pinea histone after 24 hours post-irradiation (Table 9). This is perhaps due to the fact that proteins in solution are relatively radio-insensitive at low exposures and only de novo synthesized histones may be altered at the time of irradiation being subject to template mutation and/or alteration. Thus from this data and the data obtained from UV spectral analysis (Table 5), it seems that the lowered 280/260 ratios obtained for irradiated chromatin is not due to loss of any one particular histone fraction but rather to loss of histone and perhaps other chromosomal protein in general.

A discussion of the analysis and characterization of the histones must include at least an awareness of the preponderance of symbols and differing nomenclatures which have been assigned to describe the same histone fraction. Murray (1968) similarly commented on this state of affairs<sup>102</sup>. He pointed out that originally Stedman and Stedman<sup>137</sup> labelled

Figure 9: Electrophoregram of commercially prepared calf thymus histone (fraction IV). Direction of migration as illustrated is from left to right (i.e., from anode to cathode). 30  $\mu$ g applied to each tube.

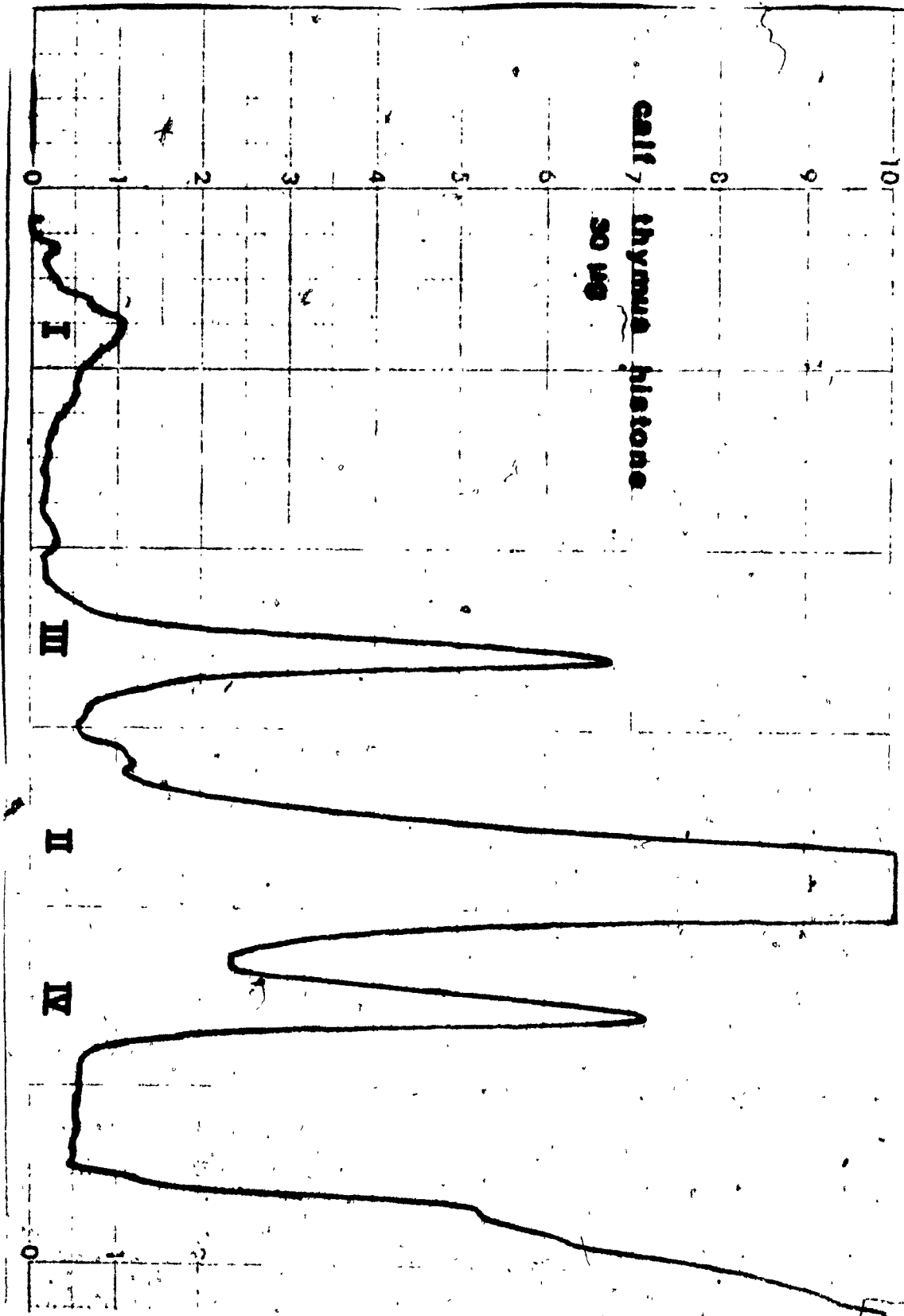


Figure 10: Electrophoregram of histone obtained from unirradiated (control) Pinus pinea cotyledons following 16 hours incubation in aqueous inorganic radiophosphate (125  $\mu\text{Ci}$ ). Direction of migration as illustrated is from left to right (i.e., from anode to cathode). 30  $\mu\text{g}$  histone applied to each tube.

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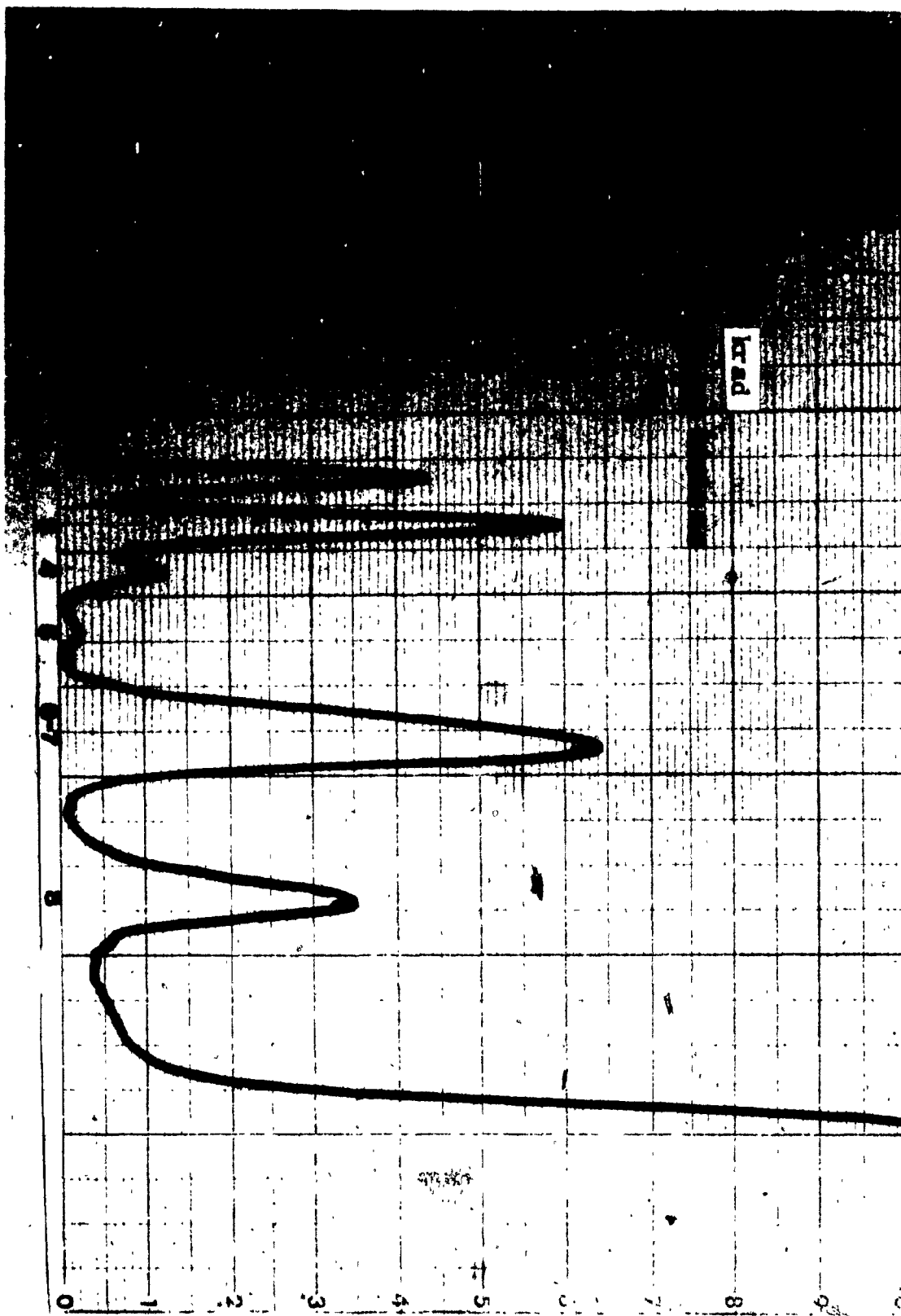


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**Figure 11:** Electrophoregram of histone obtained from 0.5krad irradiated Pinus pinea cotyledons following 16 hours incubation in aqueous inorganic radio-phosphate (125  $\mu$ Ci). Direction of migration as illustrated is from left to right (i.e., from anode to cathode). 30  $\mu$ g histone applied to each tube.

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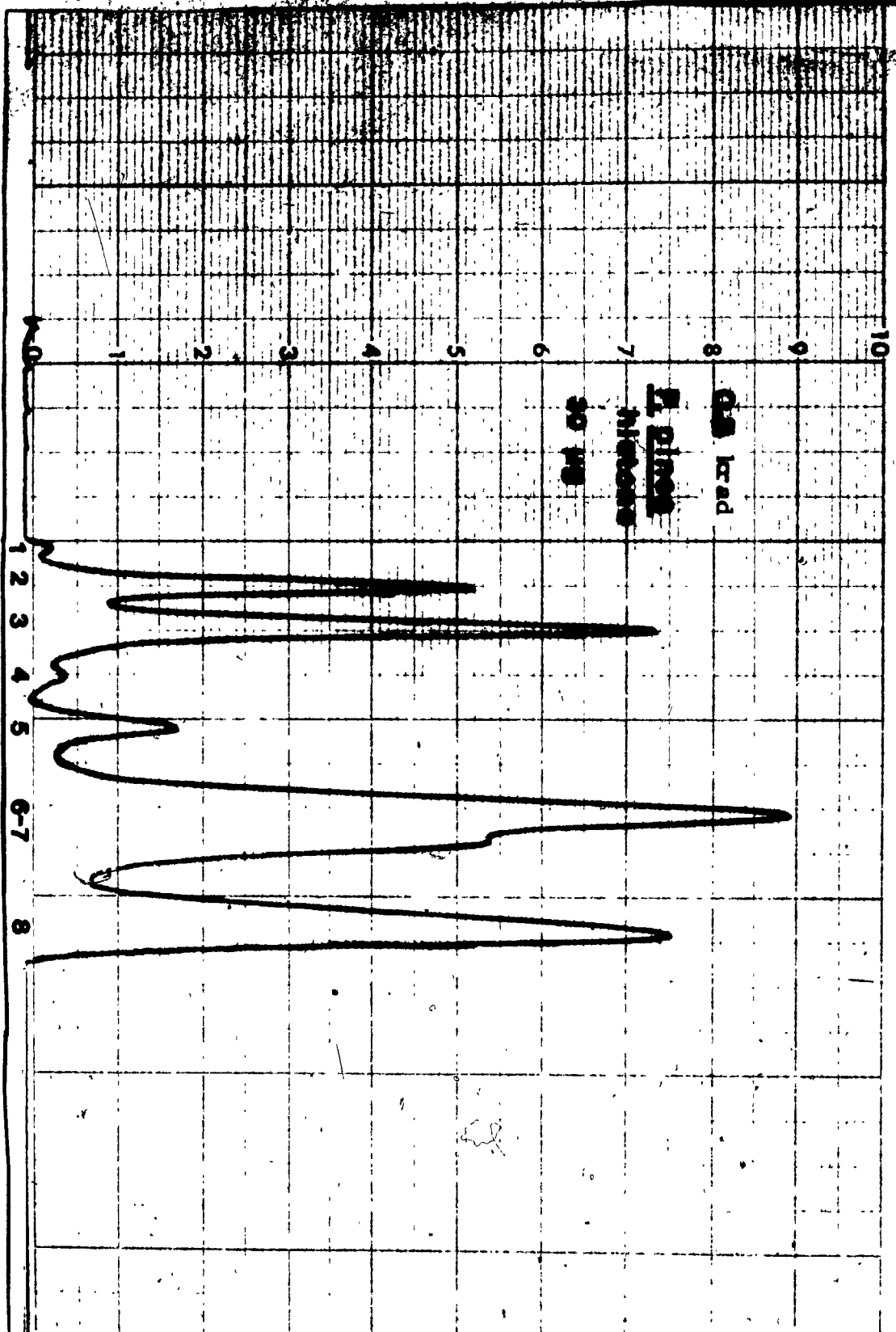
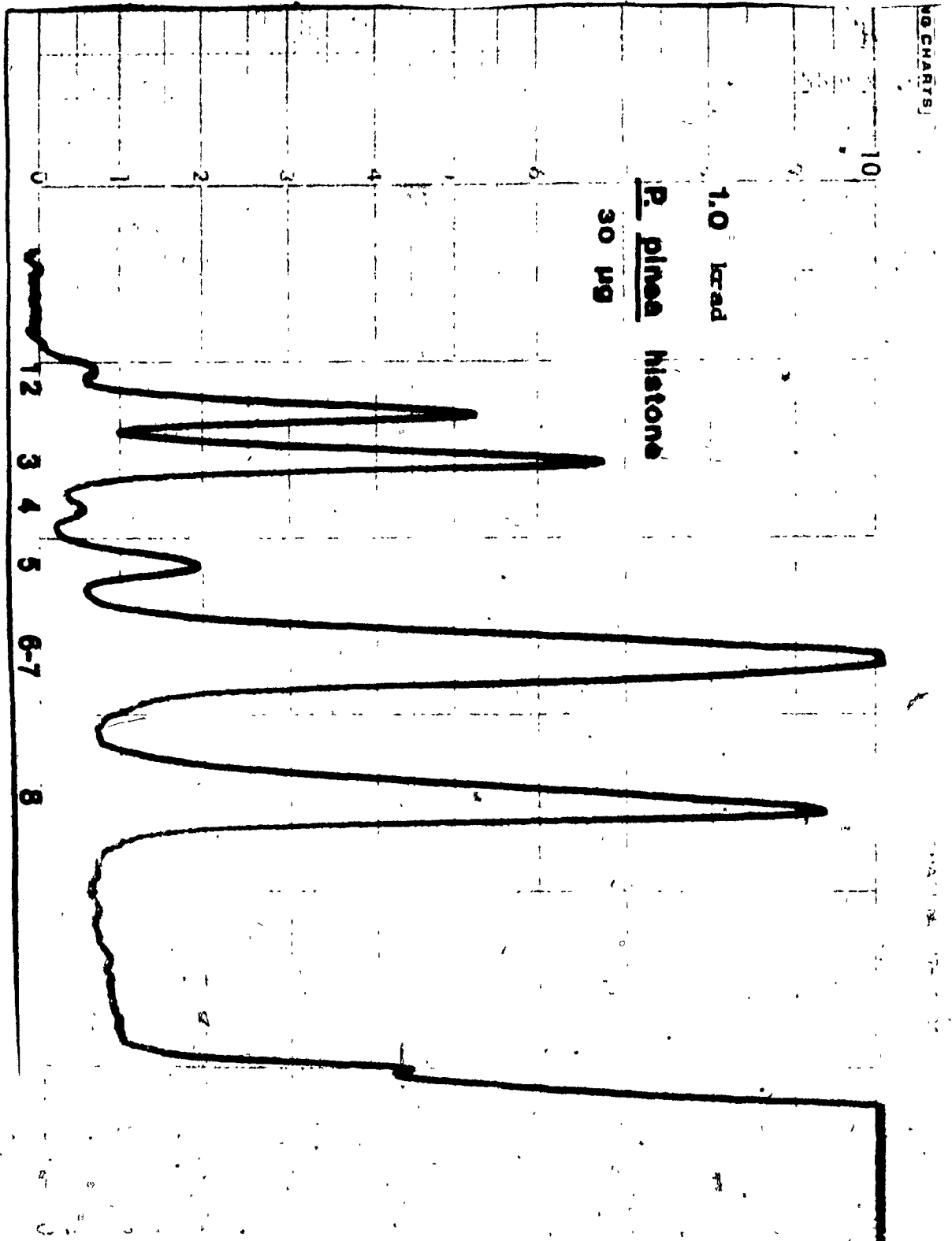


Figure 12: Electrophoregram of histone obtained from 1.0 kradirradiated Pinus pinea cotyledons following 16 hours incubation in aqueous inorganic radio-phosphate (125  $\mu$ Ci). Direction of migration as illustrated is from left to right ( i.e., from anode to cathode). 30  $\mu$ g histone applied to each tube.



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the histones on the basis of their electrophoretic mobilities and assigned the symbols  $\alpha$ ,  $\beta$ , and  $\gamma$  to them. Since that time there has been no less than four new nomenclature systems which are summarized in Table 8 and have been conspicuously tabulated in the preface of a recent text on chromatin structure<sup>90</sup>.

Table 8. Nomenclature for histone subfractions

Lysine-rich histones	H1	I	F1
	H5	V	F2c
Slightly-lysine rich histones	H2a	IIb1	F2a2
	H2b	IIb2	F2b
Arginine-rich histones	H3	III	F3
	H4	IV	F2a1

(after H.J. Li and R.A. Eckhardt, eds., 1977)<sup>90</sup>

Until such time that major technical difficulties could be resolved in the preparation of chromatin and isolated pure, unaltered histone, there were conflicting reports on the similarity or dissimilarity of histones from different tissues, organs or species. Until nucleoprotein preparations could be made without basic protein contamination or degradation by proteolysis reports would indicate histone homogeneity from species to species<sup>62,63</sup> or that histones

display individual species and tissue specificity<sup>73,115,74</sup>.

It is now generally accepted that there are five major classes of histones which can be identified on the basis of their electrophoretic mobilities. Most are heterogenous species with only the lysine-rich fraction showing some tissue-specific microheterogeneity<sup>73,74</sup>. In addition, a study on the comparison of vertebrate histone based on their electrophoretic mobilities by Panyim et al, 1970<sup>110</sup>, conclusively showed that over a wide range of vertebrate types, two histone fractions, F3 and F2a1, have been closely conserved throughout evolution and indeed there is evidence for only minor changes in their mobilities among the various phyla. Only histone F1 shows any indication of tissue-specificity. The primary amino acid structures for the four groups, F2a1, F2b, F3 and F2a2 have been analysed<sup>74,67,33,152</sup> and partial sequencing for histone F1 has been attempted<sup>118</sup>. A comparison between calf thymus and pea bud histones F2a1 revealed an extremely conservative alteration of merely two amino acid changes and has shown that there has been no divergence between this fraction for nearly 1.5 billion years<sup>34</sup>.

### C. Inorganic $^{32}\text{P}$ Incorporation and X-Irradiation

Data for the recovery of inorganic  $^{32}\text{P}$  from gel slices of the histone fractions as well as corresponding areas under the curves obtained from the electrophoregrams are listed in Tables 9A-C. The data show that for all subfractions,  $^{32}\text{P}$  incorporation is markedly reduced as the radiation exposure increases. It is interesting to note that three fractions obtained from unirradiated histone, fractions 1, 4 and 5 show relatively high specific activities. This is not unusual and is in accordance with the findings of Langan<sup>81</sup> who demonstrated and purified a phosphokinase and phosphatase specific for rat histone fraction F1. Phosphorous was found to be preferentially associated with the lysine-rich histones and is bound to the serine residues as o - phospho L-serine.

The effect of X-irradiation on the relative rate of incorporation of  $^{32}\text{P}$  into histone is represented in Figure 13. The results indicate that the lysine-rich subfractions of P. pinea histone tend to show less radiation effect than do the more arginine-rich histones. The increase in the percent inhibition of  $^{32}\text{P}$  incorporation for both irradiated groups is linear with decreasing lysine-content. For example, 1,000 rads ionizing radiation induced only 58%

inhibition of  $^{32}\text{P}$  i uptake in fraction number 1 as compared with control data. The same radiation dose induced up to 98.5% inhibition of phosphate uptake in the arginine-rich fraction 8.

It is known that in whole-body irradiated rats there is a general and immediate decrease in the histone/DNA ratios of isolated nuclei<sup>41</sup>. Bauer et al.<sup>9</sup>, showed that for irradiated rat thymus nuclei there were no perceptible differences in either the electrophoretic mobilities of the major bands of the fractionated histones following irradiation or the relative amounts of histone. There was, however, significant decrease (up to 20%) in the amounts of DNA and histone 24 hours after in vivo lethal irradiation and as well, the loss of DNA is both gradual and non-selective. However, differential turnover rates of synthesis of the histone fractions are not uncommon following X-irradiation and the arginine-rich histones of thymus are particularly depressed<sup>77,78</sup>. Thus, if these reports are correct, the data now seems to imply that there is no X-ray-induced  $^{32}\text{P}$  inhibition of incorporation per se, but rather there are maintained levels of phosphorylated F1 histones due to the rapid and selective turnover of the F1 histones. One could imagine that if the turnover rates for F1 lie

Table 9A The in vivo uptake of  $^{32}\text{P}_1$  (as  $\text{NaH}_2^{32}\text{PO}_4$ ) into P. pinea histone.  
Table 9A, unirradiated seedlings.

Fraction Number	Tentative Classification	Net Count Rate/ 4 gel slices	Amount protein	Area under the curve	Specific activity	$^{32}\text{P}$ incorporation
		(cpm)	( $\mu\text{g}$ )	( $\text{cm}^2$ )	(cpm/ $\mu\text{g}$ protein)	% inhibition
X	F1	1676	1.64	1.0	255.5	0.0
1	F1	992	0.49	0.3	506.1	0.0
2	F1	1218	5.25	3.2	58.0	0.0
3	F1	1128	5.57	3.4	50.6	0.0
4	F1	978	1.15	0.7	212.6	0.0
5	F3	454	0.33	0.2	343.9	0.0
6-7	F2a2, F2b	2478	11.15	6.8	55.6	0.0
8	F2a1	1516	4.43	2.7	85.6	0.0
TOTALS		10440	30.00	18.3	---	---

Table 9B The in vivo uptake of  $^{32}\text{P}_1$  (as  $\text{NaH}_2^{32}\text{PO}_4$ ) into P. pinea histone.  
 Table 9B, 0.5krad irradiated seedlings.

Fraction Number	Tentative Classification	Net Count Rate/ 4 gel slices	Amount protein	Area under the curve	Specific activity	$^{32}\text{P}$ incorporation
		(cpm)	( $\mu\text{g}$ )	( $\text{cm}^2$ )	(cpm/ $\mu\text{g}$ protein)	% inhibition
X	F1	---	---	---	---	---
1	F1	369	0.24	0.2	384.4	24.0
2	F1	290	1.90	1.6	38.2	34.1
3	F1	479	4.05	3.4	29.6	41.6
4	F1	269	1.55	1.3	43.4	79.6
5	F3	302	3.57	3.0	21.2	93.8
6-7	F2a2, F2b	373	11.90	10.0	7.8	86.0
8	F2a1	167	6.79	5.7	6.2	92.8
TOTALS		2249	30.00	25.2	---	---

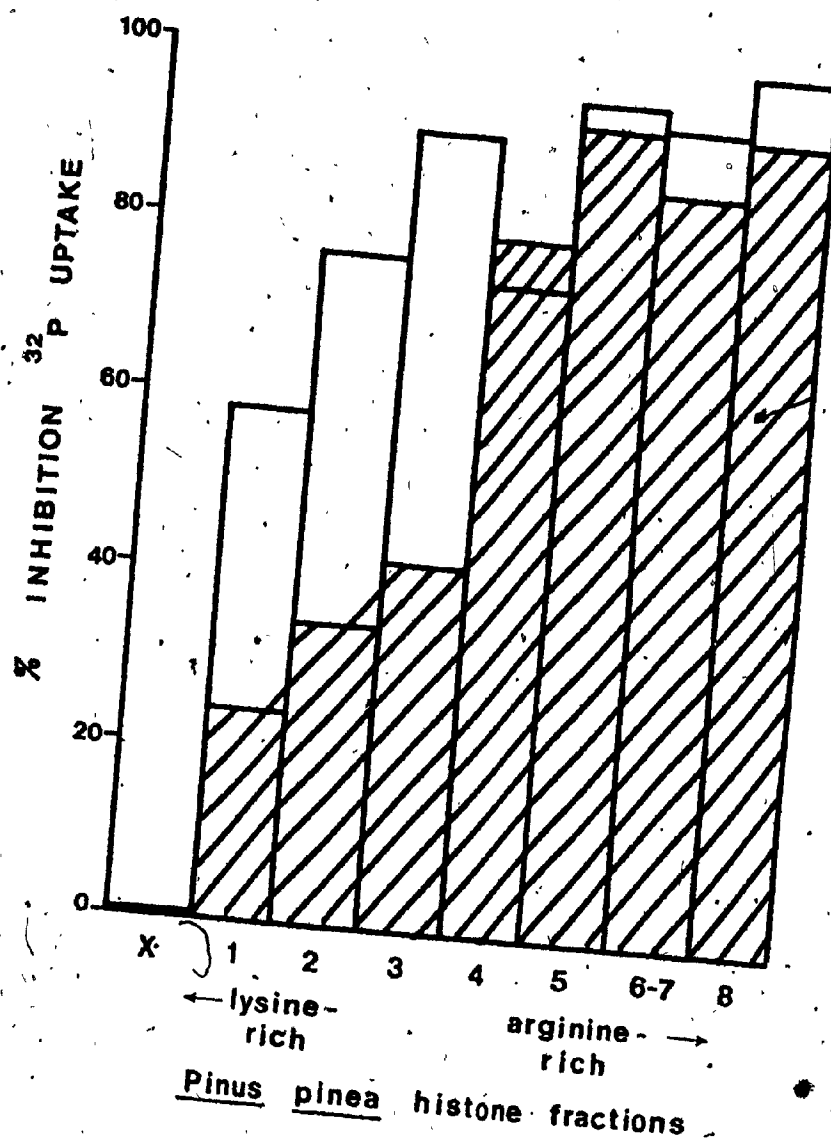
Table 9C The in vivo uptake of  $^{32}\text{P}_4$  (as  $\text{NaH}_2^{32}\text{PO}_4$ ) into P. pinus histone.  
Table 9C, 10krad irradiated seedlings.

Fraction Number	Tentative Classification	Net Count Rate/ 4 gel slices	Amount protein	Area under the curve	Specific Activity	$^{32}\text{P}$ incorporation
		(cpm)	( $\mu\text{g}$ )	( $\text{cm}^2$ )	(cpm/ $\mu\text{g}$ protein)	% inhibition
X	F1	---	---	---	---	---
1	F1	536	0.63	0.5	212.7	58.0
2	F1	172	3.14	2.5	13.7	76.4
3	F1	76	4.02	3.2	4.8	90.5
4	F1	91	0.38	0.3	59.9	71.8
5	F3	83	1.26	1.0	16.5	95.2
6-7	F2a2, F2b	163	12.18	9.7	3.4	93.9
8	F2a1	45	8.41	6.7	1.3	98.5
TOTALS		1166	30.00	23.9	---	---



Figure 13: % Inhibition of inorganic  $^{32}\text{P}$  incorporation into P. pinea histone following X-irradiation.

For all cases, the control, unirradiated histone remained at an arbitrary 0% value for X-irradiation induced inhibition of  $^{32}\text{P}_i$  incorporation. Values shown previously for the specific activities were used to calculate % inhibition for the irradiated groups. Cross-hatched bars represent 0.5 krad irradiated tissue and the clear bars, represent 1.0 krad irradiated tissue. For all cases, irradiation caused significant inhibition of the in vivo phosphorylation of P. pinea histone. In only one case, fraction 4, did the 0.5 irradiated group show greater X-ray induced effect than 1.0 krad irradiation.



within the 16 hour incubation period which immediately followed X-irradiation then, F1 histones lost during and immediately after irradiation could be replaced and re-phosphorylated sometime during this period.

Gurley and Walters (1971) reported the rapid response of histone turnover and concomitant phosphorylation in response to X-irradiation in CHO\* cells<sup>57</sup>. It was previously known that histone F1 turnover in CHO cells stopped for a short time in response to X-irradiation<sup>55,56</sup>. The study showed up to 50% decrease in the level of F1 phosphorylation one hour after irradiation (800 rads); however, by three hours post-irradiation, the F1 phosphorylated histone level returned to 86% of its normal value.

Further work must be done on the in vivo phosphorylation of P pinea histone. A similar study, like that of Gurley and Walters, (1971) which investigated the temporal relationship of histone F1 turnover and phosphorylation in response to X-irradiation must be initiated to better understand the data presented of Tables 9A - 9C. The study must also include the analysis of the relationship between F1 histone phosphorylation and the onset of recovery following G<sub>1</sub> or G<sub>2</sub> mitotic block.

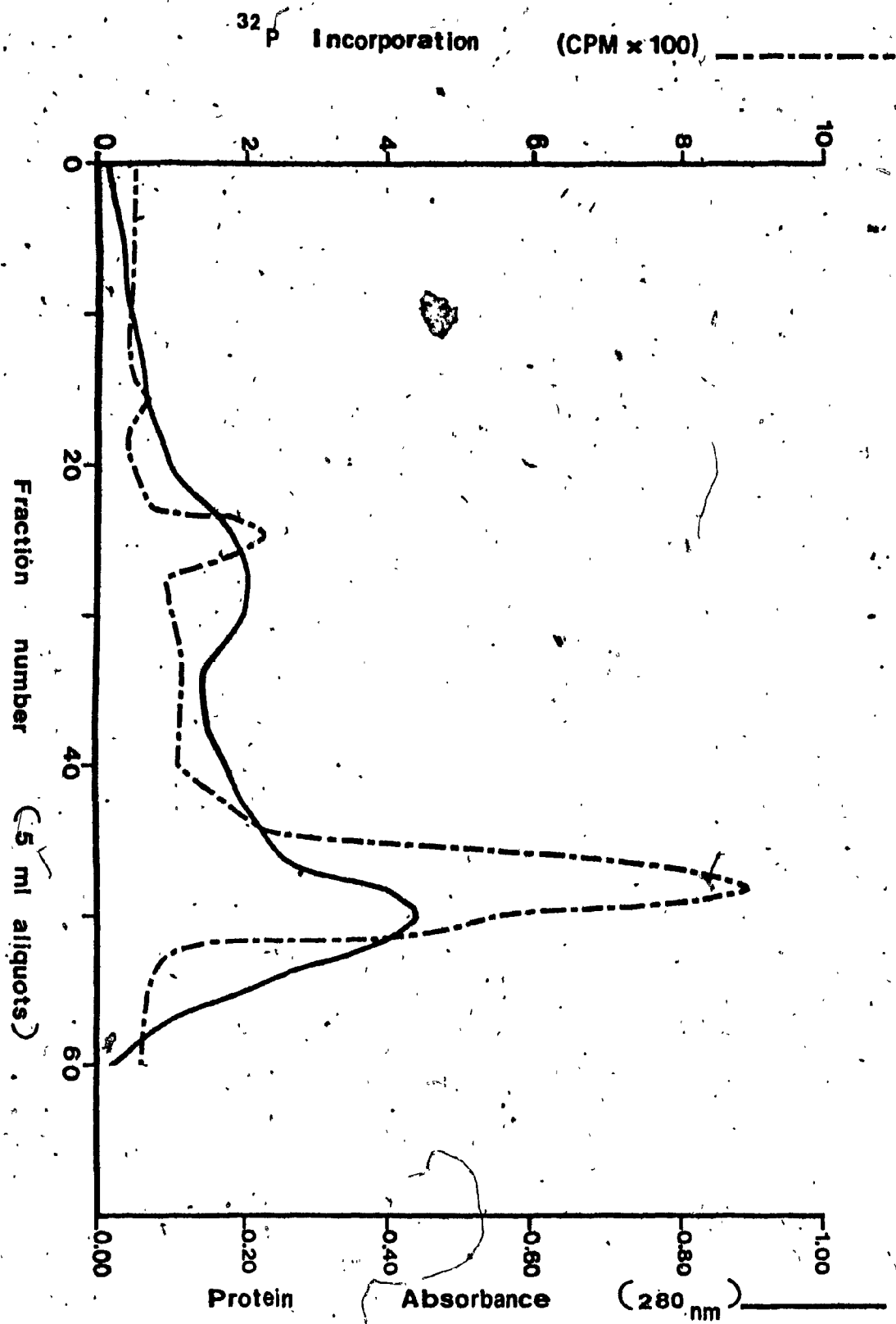
\*CHO - Chinese hamster ovary cells

D. The physical properties of Pinus pinea histone kinase

1. Purification of the protein kinase.

The chromatographic elution peaks and  $^{32}\text{P}$  activity peaks for both Sephadex G-25 and DEAE-Sephadex A-50 are shown in Figures 14 and 15 respectively, Figure 14 represents the gel filtration profile of Sephadex G-25 on the crude enzyme preparation. Figure 14 reveals the presence of two major peaks. The first peak represents a fraction of high molecular weight proteins and is broad and undefined. There is evidence of some radioactivity in this broad fraction and was collected in fraction numbers 23-26. However, its peak activity was only 22.4% of the radioactive peak of the second lower molecular weight fraction. Because the activity contained within this second peak was minimal, it was neither purified further nor assayed. Only that protein fraction eluted between 230 ml and 250 ml was pooled and further purified on an ion-exchange resin. It is not understood why any enzymatically active fraction could be found at all in this higher molecular weight protein pool. The large pore size of Sephadex G-25 could not effectively separate two different enzyme forms with identical function. Multiple kinase forms have been reported and are fairly common

Figure 14: Chromatography of the crude enzyme preparation on a Sephadex G-25 (five) column. The protein was eluted with 300 ml of 0.05 M Tris-HCl buffer pH 8.5 containing 0.05 M NaCl and 6 mM 2-mercaptoethanol. The 5.0-ml fractions were collected and assayed as described in the methods. The solid line represents changes in optical density at 280 nm (arbitrary units). The enzyme was most concentrated between 230 and 250 ml eluant. These fractions were pooled and further purified.

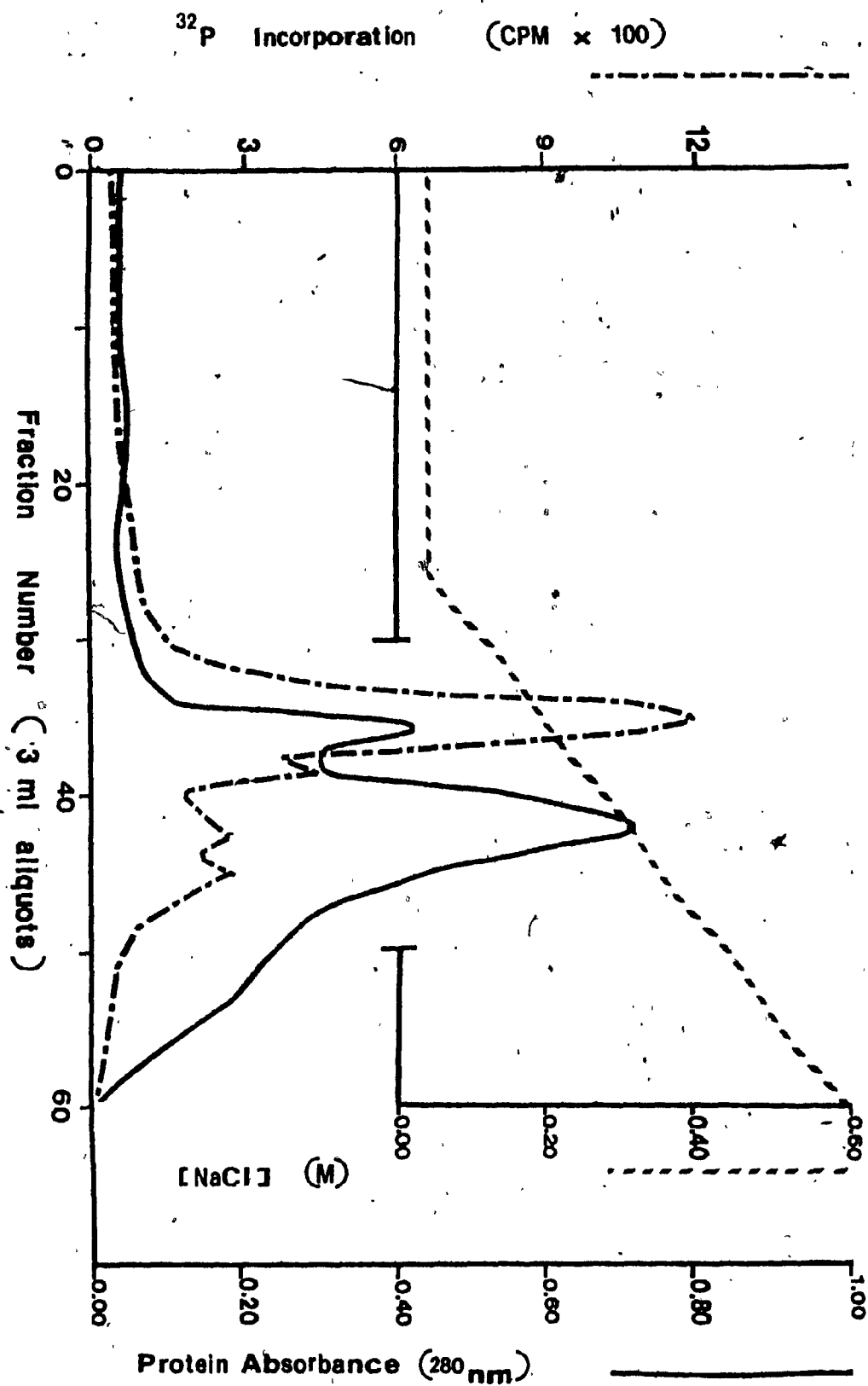


(rat liver<sup>151</sup>; pea shoots<sup>72</sup>; silkworm<sup>61</sup>) and one kinase has been described as being a holoenzyme which can be dissociated<sup>65</sup>. In all cases, however, the appearance of a multiple-form kinase was seen only after more refined purification steps were utilized (i.e., CL-Sepharose 6B gel filtration<sup>65</sup>; Sephadex G-150<sup>61</sup>; DEAE-cellulose D-52<sup>72</sup>; hydroxyapatite<sup>151</sup>.) The only explanation which can be given without further analysis of this fraction is that it probably contains soluble cytoplasmic proteins capable of being enzymatically phosphorylated by the protein kinase or other kinases or non-enzymatically phosphorylated in the presence of  $AT^{32}P$  alone. These proteins may be low molecular weight globulins or even perhaps the cytoplasmic pool of nascent P. pinea histone.

The major peak obtained from Sephadex G-25 chromatography was further purified on DEAE Sephadex A-50 ion exchange resin and the results are displayed in Figure 15. The results show a large, single major peak which has been eluted at a salt concentration gradient from 0.18 M to 0.22 M NaCl. This fraction yielded the highest enzymatic activity. A second broader peak of radioactivity is also seen for the fraction eluted at 0.32 M to 0.36 M NaCl. The radioactivity of this peak represents about 38.8%

Figure 15: Elution of P. pinea histone kinase from DEAE-Sephadex A-50 column using a linear NaCl gradient between 0.05 and 0.60 M NaCl, in 0.05 M Tris-HCl buffer,  $pH$  8.5 and containing 6 mM 2-mercaptoethanol. The solid line represents protein concentration and is the change in optical density at 280 nm (arbitrary units).





of the combined radioactivity of the first peak and is contained in twice the volume of eluant. Further assays which involved the kinetics, substrate specificity,  $Mg^{2+}$  ion concentration, c-AMP and  $pH$  dependancies of the enzyme were made only for the enzyme collected in Peak 1.

Table 10 shows the degree of purification attributed to each of the various purification steps. It was realized that the enzyme contained within the 35% to 65%  $NH_4SO_4$  fraction may be of sufficient specific activity to carry out certain preparative experiments.

ii.  $pH$  dependance.

Data for the effect of  $pH$  on enzyme activity is shown in Figure 16. The data shows a  $pH$  optimum in Tris-HCl buffer of 7.8 with rapid decline for  $pH$  values below or above this figure. Phosphate buffer indicates a  $pH$  optimum of 7.9. Glycine as indicated is an unsuitable buffer for this application. Table 11 below gives surveys of the  $pH$  optima obtained for histone kinases obtained from different sources. The data for the combined sources indicate that the average  $pH$  optimum for a variety of histone kinases obtained from plant sources is about  $pH$  7.9 and agrees with the value obtained for P. pinea kinase.

Table 10 Purification data for the isolation of Pinus pinea histone kinase.

Fraction	Total activity (net CPM)	Protein concentration (mg/ml)	Specific activity (pMol $^{32}$ P /min)	Purification factor (x crude)	yield (%)
Crude extract	1142	28.7	0.0007	1.00	100.0
*Dowex-IX2	---	---	---	---	---
105, 000 x g pellet	8406	21.8	0.0040	2.12	76.0
35% - 65% $\text{NH}_4\text{SO}_4$	16467	12.5	0.0780	7.41	43.6
Dialysis	17660	9.2	0.0840	11.68	32.1
Sephadex G-25	28951	7.2	0.1380	17.37	25.1
DEAE Sephadex A-50	39407	3.4	0.1880	34.50	8.4

\*Data not obtained for Dowex-IX2.

Figure 16:

• pH dependance of histone  
kinase activity.

5  $\mu$ M Tris-HCl  
10  $\mu$ M phosphate  
10  $\mu$ M glycine

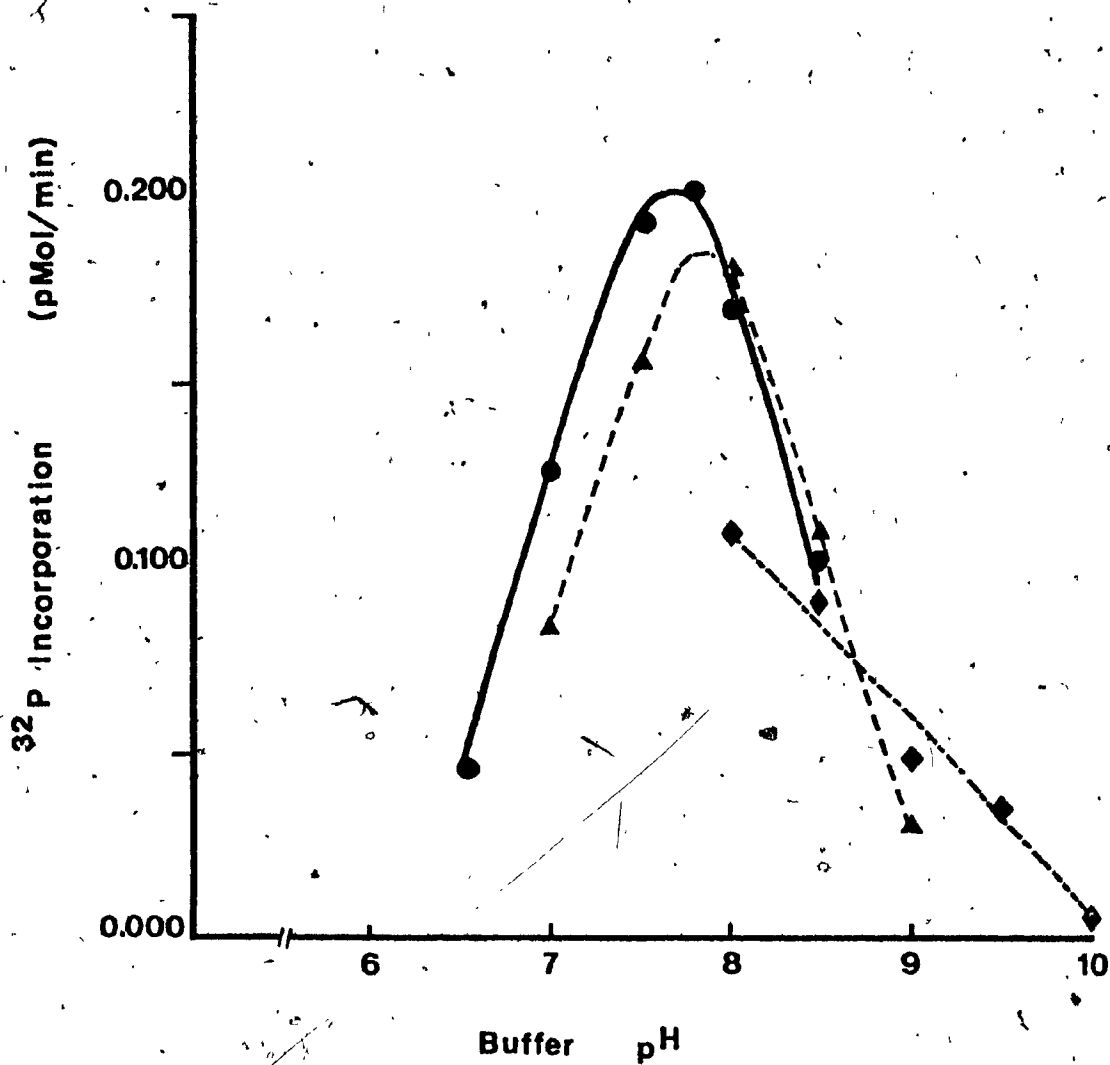
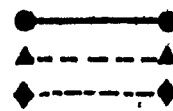


Table 11 - Histone kinase  $p^H$  optima obtained from various sources.

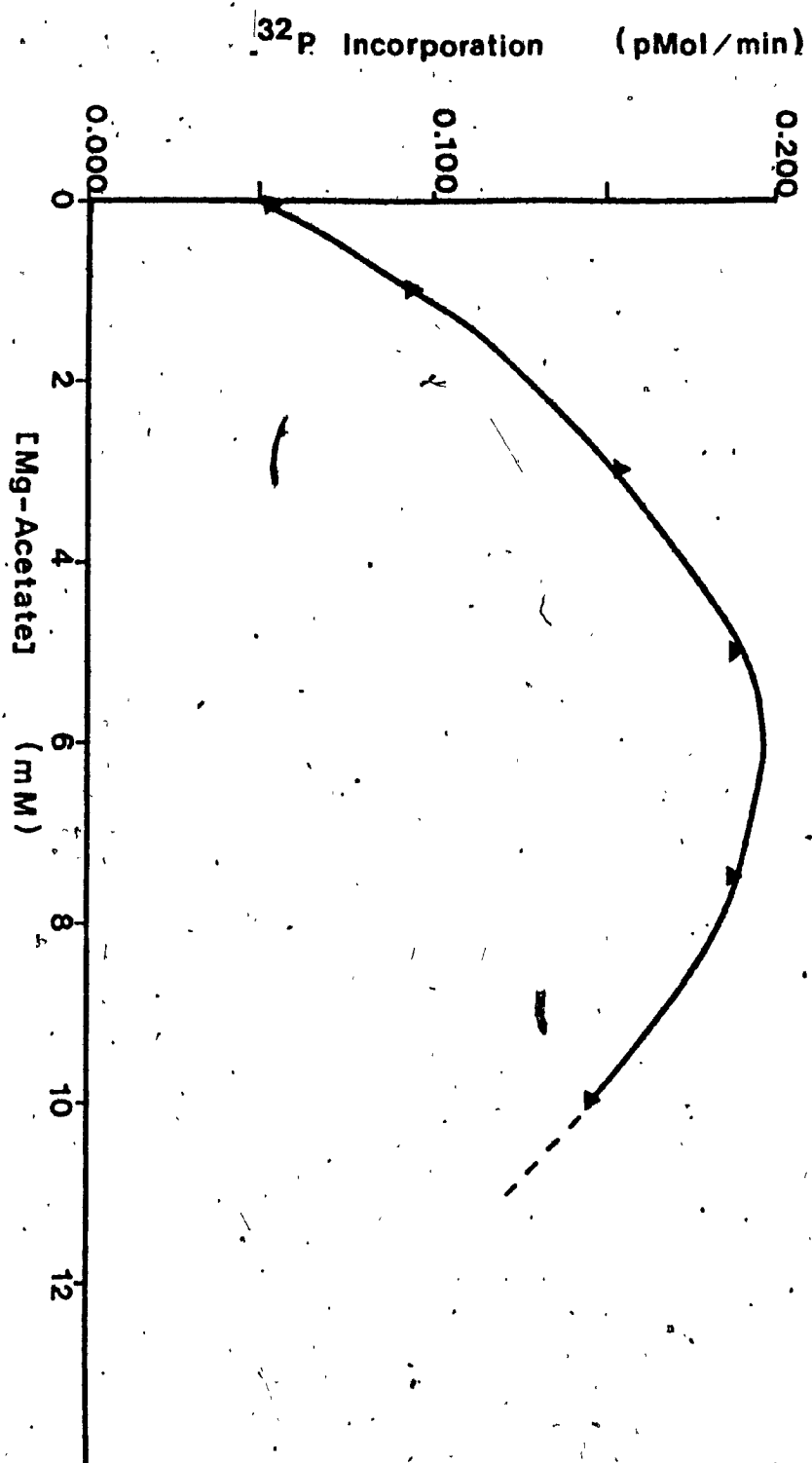
*Author	Source	Buffer	$p^H$ range
Nakaya <u>et al.</u> , 1975 <sup>103</sup>	carrot cell culture	0.05 M Tris-HCl	7.5 - 9.0
Keates, 1973 <sup>72</sup>	pea shoots	0.05 M $\beta$ -glycero-phosphate	6.5
		0.05 M Tris-(hydroxymethyl)methyl 2-amino methane sulphonic acid	8.5
Lin <u>et al.</u> , 1976 <sup>91</sup>	soybean	20 mM Tris-HCl	8.0 - 9.0
Yamamura <u>et al.</u> , 1970 <sup>151</sup>	rat liver	5 $\mu$ M phosphate	7.5
McKelvey, 1975 <sup>95</sup>	hypothalamus	5 $\mu$ M phosphate	7.0

iii.  $Mg^{2+}$  dependance.

The effect of a divalent cation,  $Mg^{2+}$  on the activity of P. pinea histone kinase is represented in Figure 17. The data shows almost linear increase in the specific activity of the enzyme up to 3.0 mM concentration. The optimum concentration of magnesium for this assay lies between 5 mM and 7.5 mM. Concentrations higher than 7.5 mM become inhibitory. At the zero concentration, there still

7

Figure 17: The effect of various  $Mg^{2+}$  concentrations  
on the enzymatic activity of histone  
kinase. (Standard assay conditions are  
used).





remains as much as 27% of the maximal activity, regardless of its absence in the reaction mixture. Very similar evidence is given for the effect of  $Mg^{2+}$  ion concentration on kinase activity in cultured carrot cells (Nakaya et al., 1975). Maximal kinase activities were reached at 0.5 mM but fell rapidly at concentrations above 1.0 mM. About 26% activity of the maximal value was obtained at the zero magnesium concentration. Optimal magnesium concentrations for plant kinases have been reported to be 0.5 mM for carrot root culture<sup>103</sup> and 5.0 mM for soybean<sup>91</sup>.

Certain other divalent metal cations enhance the enzymatic phosphorylation of kinase. These are  $Mn^{2+}$ , and  $Co^{2+}$ <sup>91</sup>. Millimolar amounts of either  $Ca^{2+}$ ,  $Zn^{2+}$  or  $Hg^{2+}$  added to the incubation medium can result in up to 50% inhibition of the primary activity.

#### iv. Effect of kinetin and cAMP.

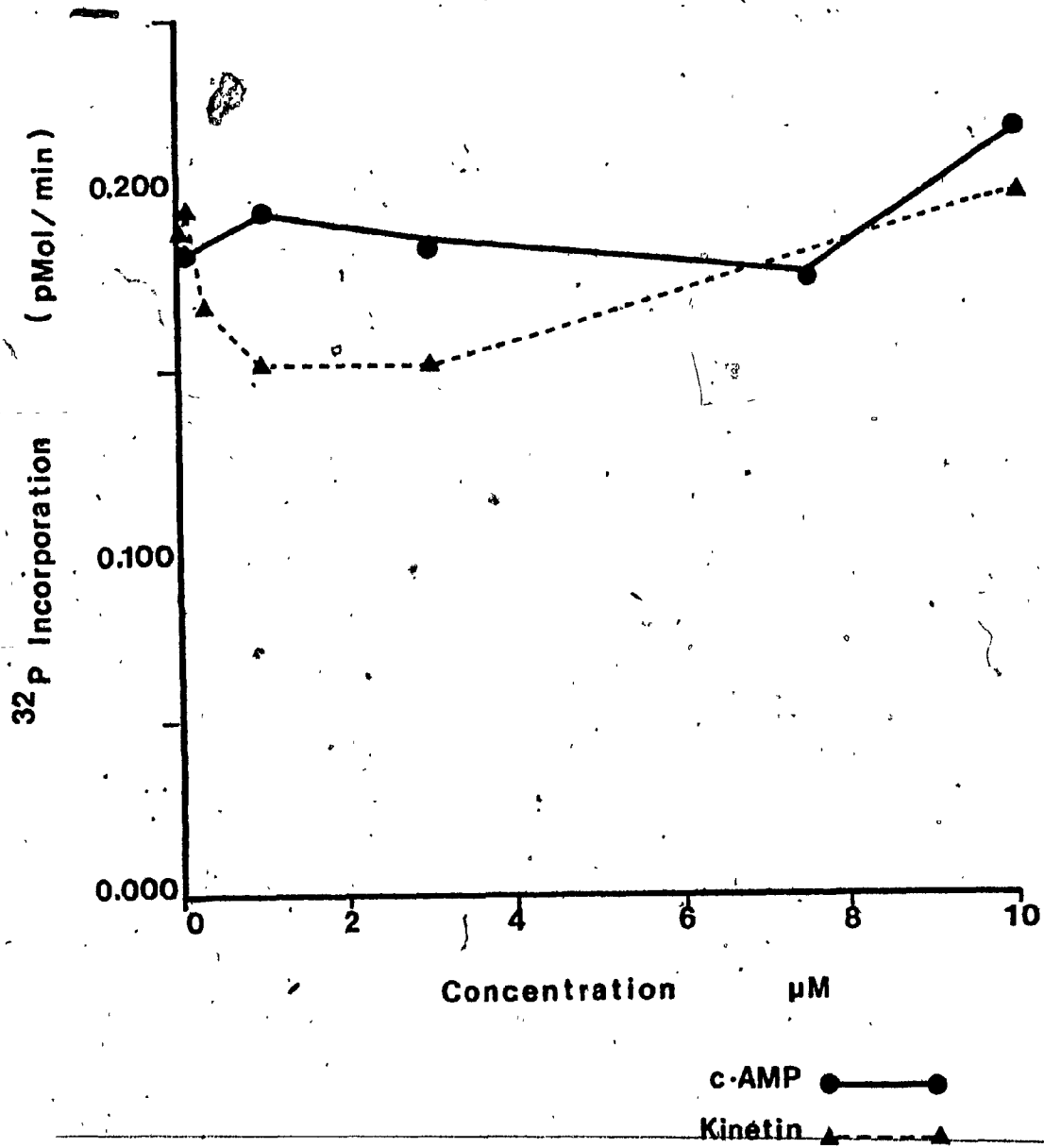
Figure 18 shows the results obtained for the effect of the inclusion of cAMP or kinetin into the reaction medium and the effects produced on the reaction rate. The results indicated the absence of any dependence shown by P. pinea histone kinase for these two substances. The results indicate this effect for only the in vitro situation.

No assumptions from the results can be made concerning their individual roles in vivo in P. pinea. It should be noted that there are few reported cases of c·AMP-dependant protein kinases in the plant phylae<sup>51,120</sup> although the c·AMP-dependant forms are by far the more common. Other cyclic nucleotides and their analogues were not tested.

This situation differs from that of kinase isolated from animal tissues in which a unifying theory for the mechanism of action of c·AMP has been postulated<sup>79</sup>. Ralph et al., 1972 postulated that the cytokinins play the same role in plant tissues as does cyclic·AMP in their animal counterpart<sup>119</sup>. For this reason, the influence of kinetin (6-furfurylaminopurine) on the reaction rate was studied. This data is also shown in Figure 18. The results give no clear evidence of kinase stimulation as a result of kinetin addition. There is instead, a slight degree of inhibition from 0.3 to 3.0  $\mu$ M kinetin. Evidence for kinetin inhibition is not uncommon<sup>119</sup>, although the reverse situation is more frequently observed. The mechanism of kinetin inhibition is not well understood.

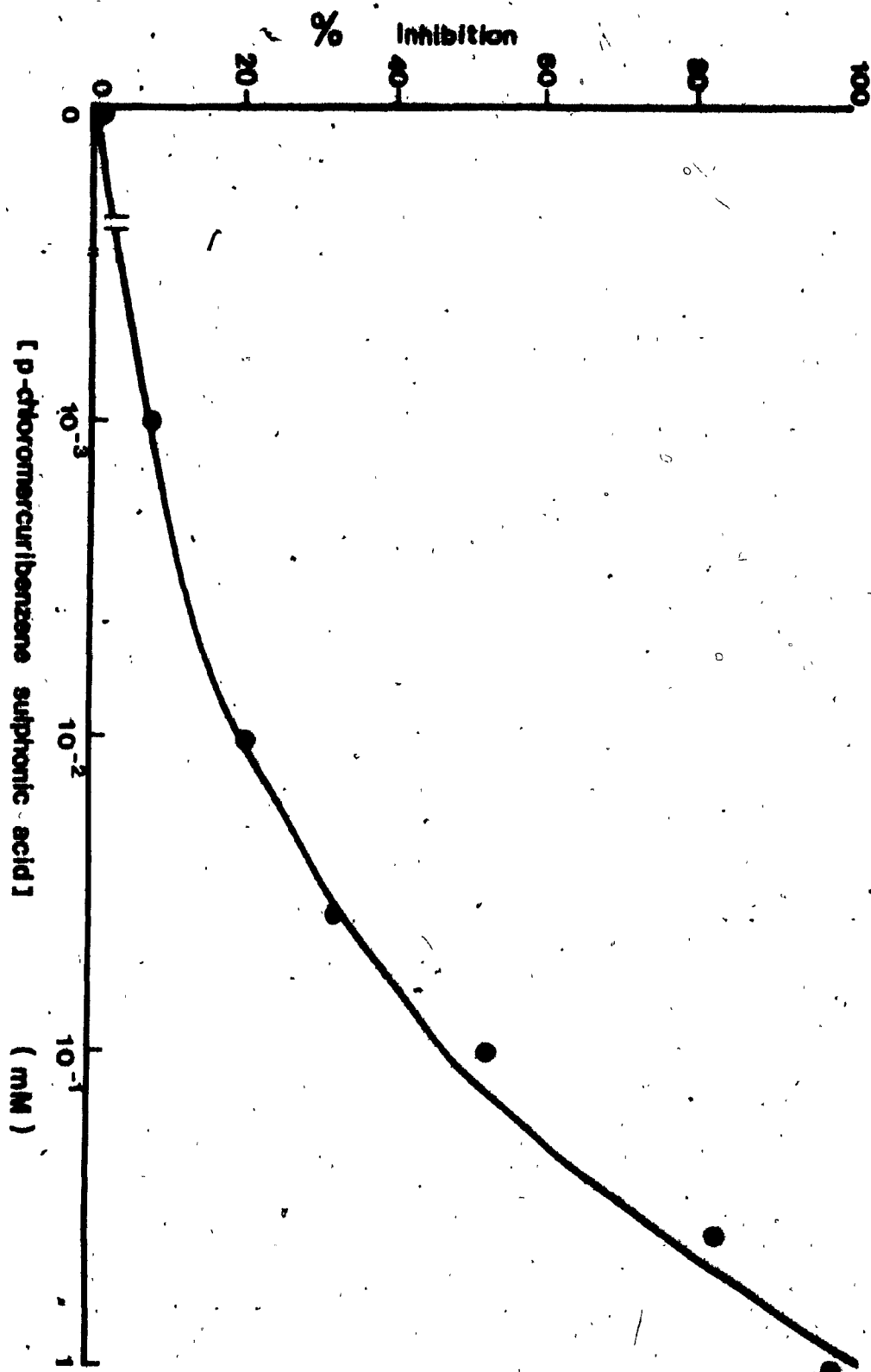
#### v. Inhibitor studies.

Figure 18: The action of 6-furfurylaminopurine  
(kinetin) and cyclic adenosine 3', 5'-  
monophosphate on histone phosphorylation.  
Standard assay conditions as described in the  
text were used.



To test for the presence of sulphydryl groups in the enzyme and to determine how essential these groups are for the activity of the enzyme, p-chloromercuribenzoate and iodoacetate were added to the reaction mixtures in individual tests. The addition of 2-mercaptoethanol was also assayed and its necessity in the reaction mixture and its ability to overcome -SH group inhibition was determined. The effect of p-chloromercuribenzenesulphonic acid on kinase activity in the absence of a mercaptan is displayed in Figure 19. The data presented shows a high degree of correlation between the amount of inhibitor and the degree of kinase inhibition associated. The addition of up to 1.0 mM p-chloromercuribenzoate results in almost complete inhibition. The mode of action of enzyme inhibition by p-chloromercuribenzoate is thought to be due to these facts; 1) p-chloromercuribenzoate is a powerful and selective inhibitor of -SH groups and attacks both exposed and concealed groups<sup>24</sup>, and 2) it is well known that -SH groups confer important tertiary structural characteristics to the enzyme which is necessary for its complete function<sup>45</sup>. Any reduction in the number of these sulphydryl bonds (i.e., -S-S-) can result in exaggerated conformational and structural changes which can result in loss or altered activity.

Figure 19: The effect of p-chloromercuribenzene sulphonic acid on the inhibition of kinase activity in the absence of 2-mercaptoethanol. Standard assay conditions were used.



It is quite common to include in all preparative steps and in the assay mixture a suitable reducing agent (i.e., 2-mercaptoethanol, cysteine, -CN). They function by protecting the exposed -SH groups which are prone to oxidation. Data given in Table 12, Section B and Figure 20 show results obtained for the addition of increasing amounts of 2-mercaptoethanol at a fixed concentration of p-chloromercuribenzoate (0.5 mM). The data show almost complete reversal of inhibition by the 0.3 mM concentration of the mercaptan. Excess mercaptoethanol became inhibitory to the system. This type of data further suggests that inhibition of enzymatic activity by p-chloromercuribenzoate is due to inhibition of the suphydryl groups. Data in Table 12, Section A, shows up to 14% inhibition of activity when 2-mercaptoethanol is excluded from the reaction medium. There is an obvious requirement for the inclusion of this reagent into the buffer system at all phases of isolation and handling.

Data for Section C of Table 12 and Figure 21 show the effect of increasing concentrations of monoiodoacetic acid on enzyme activity. Iodoacetate is known to affect -SH groups in much the same way as p-chloromercuribenzoate<sup>117</sup>, and was used to further demonstrate the presence of this



Table 12 The modification of histone kinase activity by  
sulphydryl reagent and a chelating reagent  
under standard assay conditions.

System	Formation of acid insoluble $^{32}\text{P}$	% inhibition	
	Net CPM	Specific activity (pMol/min)	
A			
Complete	40423	0.192	0.0
-Histone	907	0.004	97.8
-2-mercaptoethanol	34609	0.165	14.2
B			
0.5 mM p-chloromercuribenzoate 0.0 $\mu\text{M}$ 2-mercaptoethanol	7128	0.034	82.3
0.5 mM p-chloromercuribenzoate 3.0 $\mu\text{M}$ 2-mercaptoethanol	27401	0.130	31.2
0.5 mM p-chloromercuribenzoate 30.0 $\mu\text{M}$ 2-mercaptoethanol	37983	0.181	5.9
0.5 mM p-chloromercuribenzoate 0.3 mM 2-mercaptoethanol	40219	0.192	0.3
0.5 mM p-chloromercuribenzoate 3.0 mM 2-mercaptoethanol	17392	0.083	56.9
C			
0.0 $\mu\text{M}$ monoiodoacetic acid	40423	0.192	0.0
20.0 $\mu\text{M}$ monoiodoacetic acid	39551	0.188	2.0
0.2 mM monoiodoacetic acid	36261	0.172	10.4
2.0 mM monoiodoacetic acid	18081	0.086	55.2
10.0 mM monoiodoacetic acid	10904	0.052	72.9
D			
0.0 $\mu\text{M}$ EDTA	40422	0.192	0.0
20.0 $\mu\text{M}$ EDTA	33772	0.161	16.1
0.2 mM EDTA	19213	0.091	52.6
2.0 mM EDTA	13877	0.066	65.6
20.0 mM EDTA*	10524	0.050	74.0

\* This value is probably not valid since EDTA becomes  
somewhat insoluble at this concentration and pH.

Figure 20: The reversal of inhibition of p-chloromercuribenzoate (0.5 mM) by 2-mercaptoethanol.

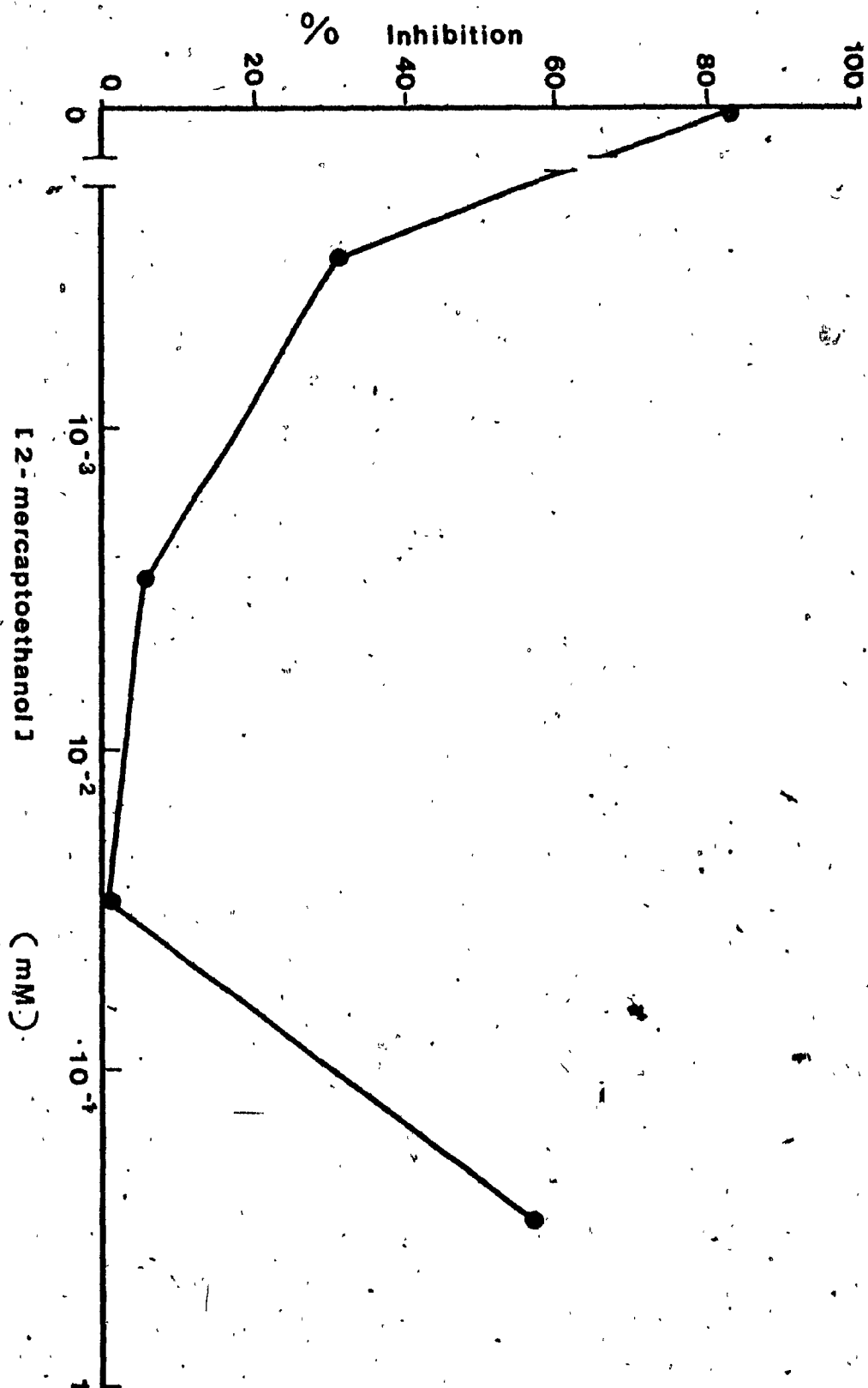


Figure 21: The inhibition of histone kinase  
activity by monoiodoacetic acid.

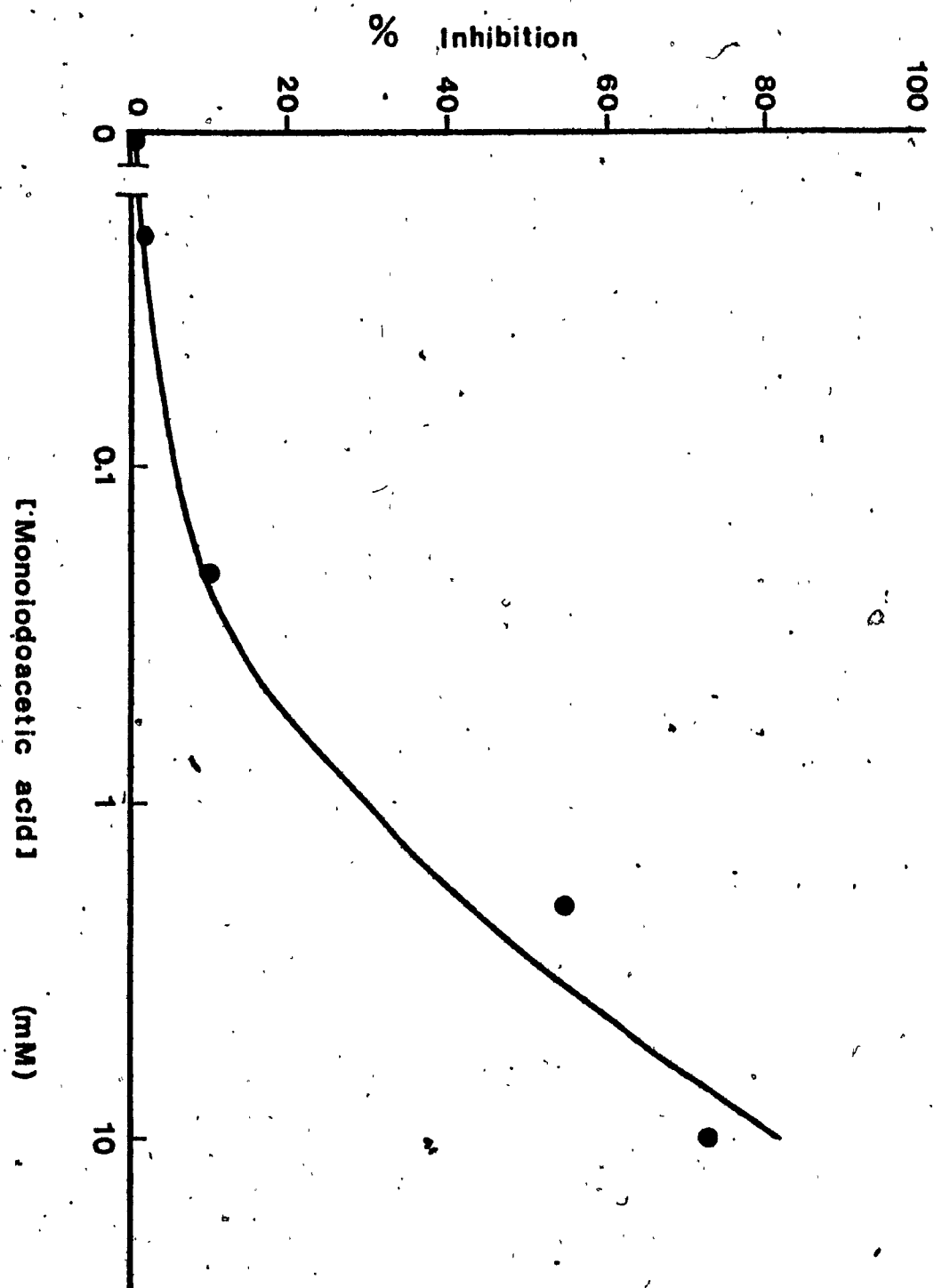
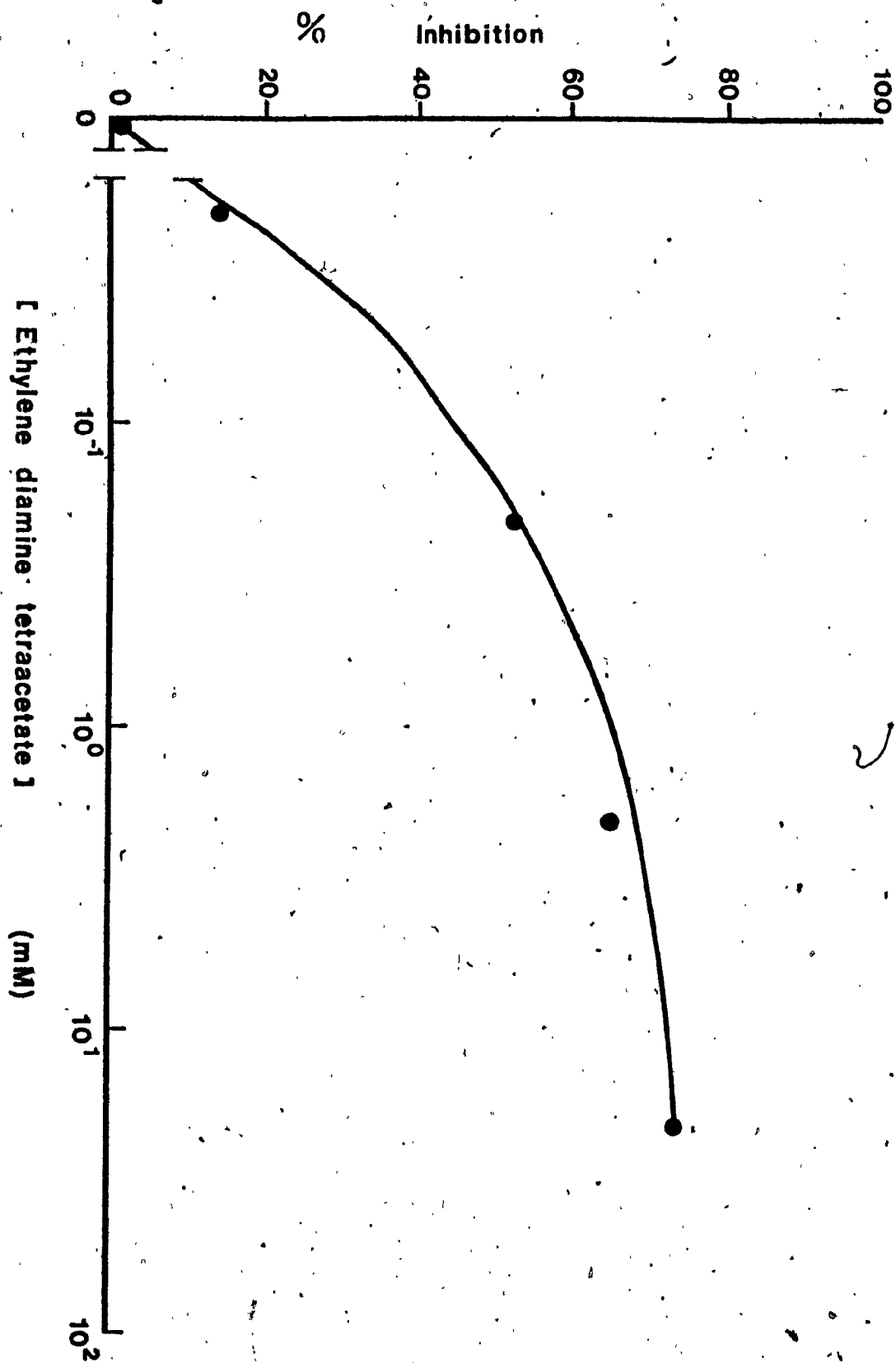


Figure 22: The inhibition of histone kinase  
activity by ethylenediaminetetraacetate  
(EDTA).



essential group. Iodoacetic acid does not have the same degree of specificity as does p-chloromercuribenzoate in inhibiting -SH groups. This may reflect on the lesser degree of inhibition by iodoacetate when compared with p-chloromercuribenzoate. For example, 10.0 mM p-chloromercuribenzoate induced about 99% inhibition whereas under the same conditions, iodoacetate at the same equimolar concentration caused only about 73% inhibition.

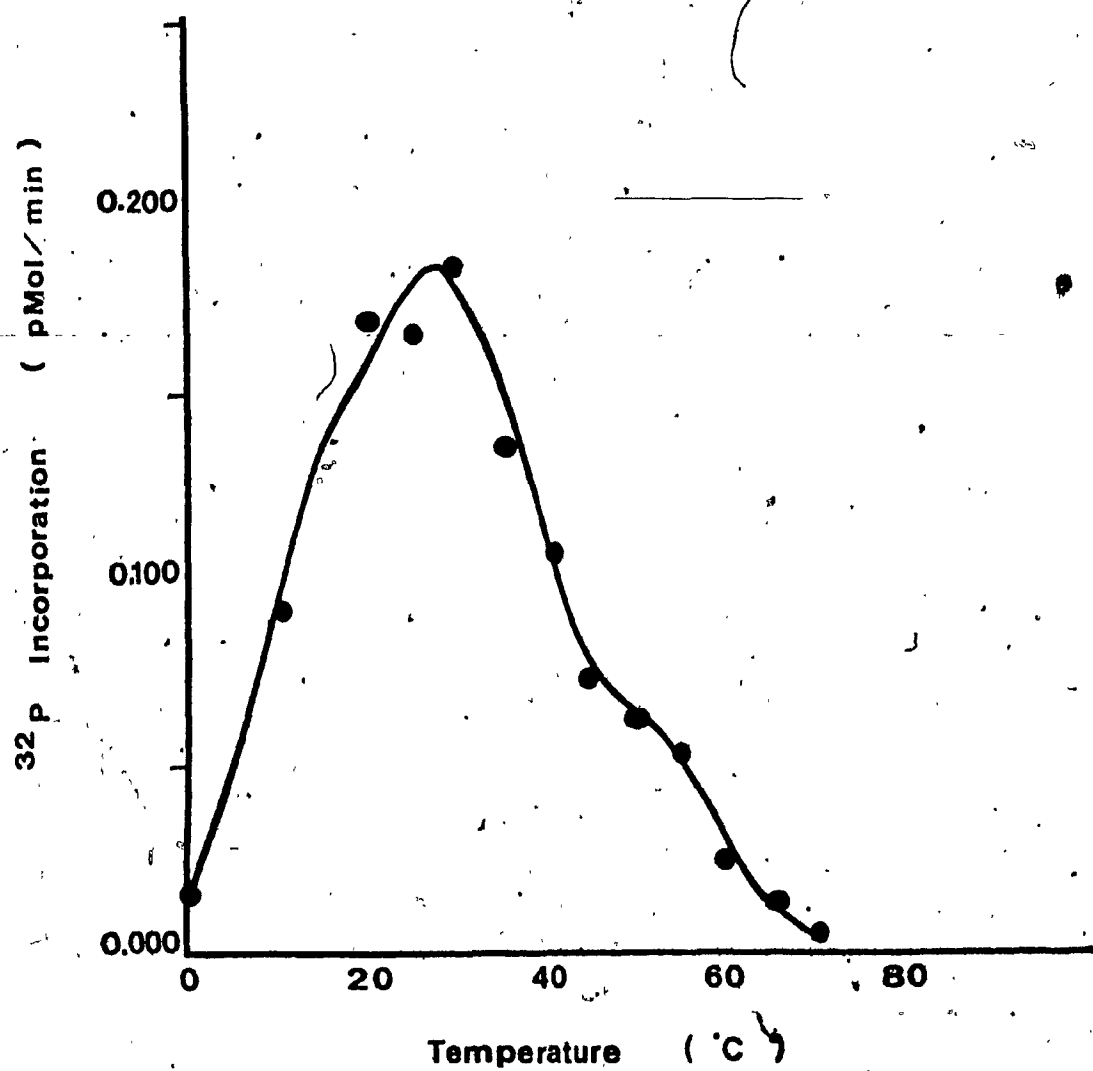
Section D of Table 12 and Figure 22 shows data for the inhibition of enzyme activity by a chelating agent, ethylene diamine tetraacetate. The data shows almost concentration-proportional decline in activity up to 0.2 mM EDTA. Maximum inhibition (73%) occurred at the highest concentration of EDTA (20 mM). In view of the data presented in Figure 17 for  $Mg^{2+}$  dependence for this enzyme, it is clear that any typical chelating agent for magnesium such as EDTA will indeed effectively "remove" the necessary component from the reaction mixture. The data in Table 12, Section D, re-confirms the earlier statement which defined  $Mg^{2+}$  ion dependence.

vi. Thermal lability and half-life determination.

Figure 23 shows data obtained for the temperature optimum and thermal lability of histone kinase. The data



Figure 23: Thermal lability of histone kinase.



shows a temperature optimum which falls within a narrow range ( $26^{\circ}\text{C}$  -  $29^{\circ}\text{C}$ ) and declines rapidly as the temperature increases above  $30^{\circ}\text{C}$ . There remains half the optimum activity at a temperature of between  $40^{\circ}\text{C}$  to  $45^{\circ}\text{C}$ .

Activity is completely lost at  $70^{\circ}\text{C}$ . The enzyme has a relatively long half-life of about three months if stored in Tris-HCl buffer with 2-mercaptoethanol, at  $-20^{\circ}\text{C}$  (data not illustrated). The half life is reduced to 48 hours if maintained under similar conditions but at  $0^{\circ}$  -  $5^{\circ}\text{C}$ .

#### E. Substrate and enzyme kinetics

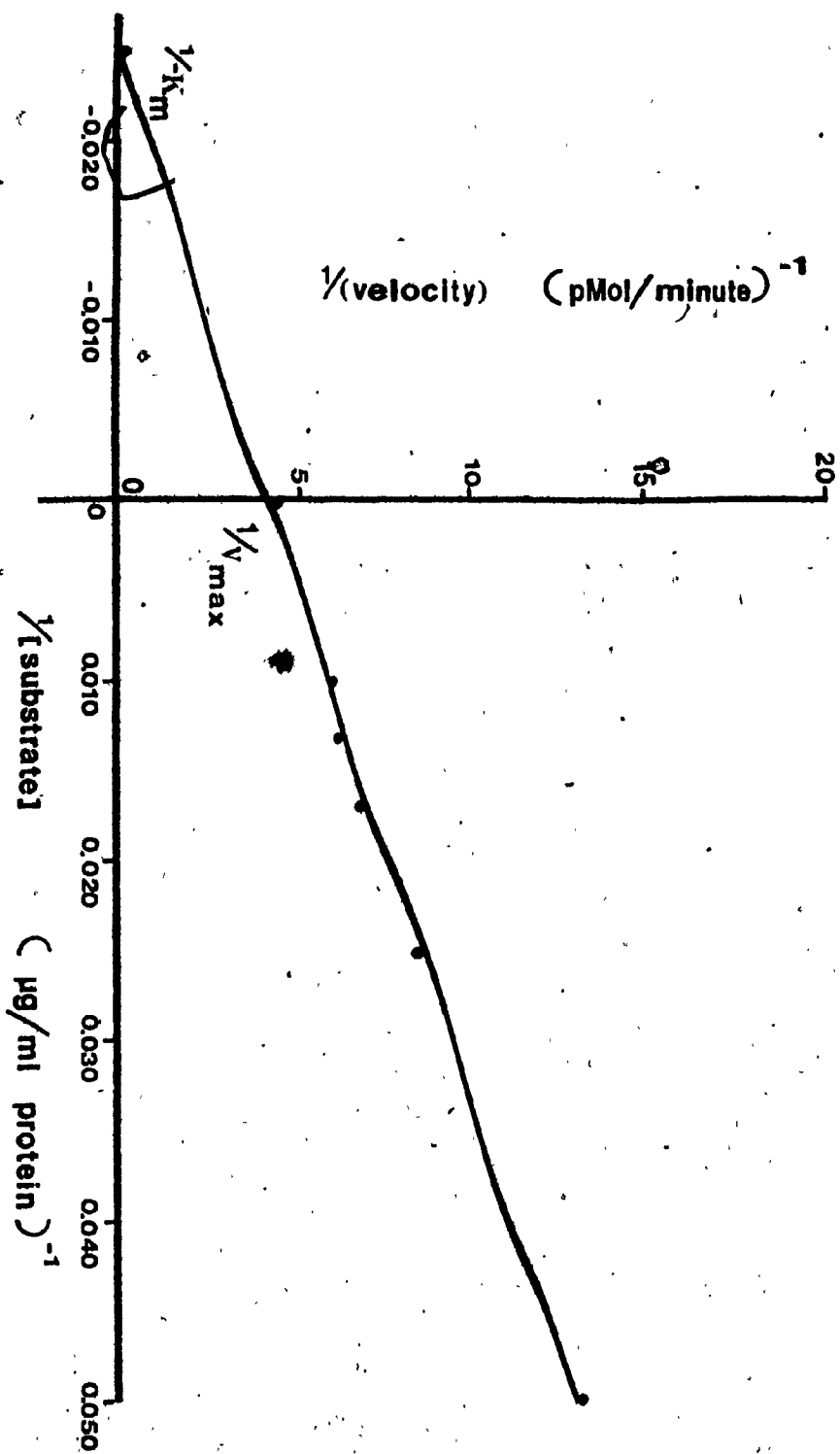
##### i. Pinus pinea and calf thymus histone substrate kinetics.

Data obtained for the substrate kinetics for P. pinea histone are shown in Figure 24. Substrate kinetics for calf thymus histone are outlined in Figure 25. In all cases, standard assay conditions were used for these determinations. Data for both substrates represents pooled averages for two different assays. The apparent values for  $K_m$  and  $V_{max}$  were determined graphically by the Lineweaver-Burk double reciprocal plot.  $V_{max}$  for Pinus pinea histone was  $0.243 \text{ pMol/min.}$  with a resultant  $K_m$  value of  $41 \text{ } \mu\text{g/ml}$  protein.  $V_{max}$  for calf thymus histone was somewhat lower,  $0.220 \text{ pMol/min.}$  and the  $K_m$  calculated was  $59.5 \text{ } \mu\text{g/ml}$  protein.

Figure 24: The double reciprocal Lineweaver-Burk plot  
( $1/v$  vs  $1/[S]$ ) for Pinus pinea histone  
substrate kinetics. The  $[S]$  range was  
arbitrarily chosen for optimal  
determination of  $K_m$  and  $V_{max}$ .

$$V_{max} = 0.243 \text{ pMol/min.}$$

$$K_m = 41 \text{ } \mu\text{g/ml protein.}$$



There is no difference in the magnitude of these values and probably there is no real difference in substrate specificity. Because the substrate concentrations chosen for these experiments were limited, it is impossible to comment further on any possible substrate inhibition as a result of the higher substrate concentrations. For comparative purposes, the histone kinase partially purified 10-fold from carrot root cell culture<sup>103</sup> had a  $K_m$  value for calf thymus histone of 50  $\mu\text{g/ml}$  protein,

ii. Pinus pinea histone kinase kinetics.

Results for the effect of various enzyme concentrations on the reaction rate using standard assay conditions are shown in Figure 26. The substrate used was freshly prepared calf thymus histone and was used in the reaction mixture at a concentration of 20  $\mu\text{g/ml}$  protein rather than the usual 50  $\mu\text{g/ml}$  protein as described under the standard assay conditions. The results show maximum activity reached at a concentration of 35  $\mu\text{g/ml}$ , and that the enzyme is fully saturated at this and even higher concentrations at the given substrate concentration. The reaction is linear up to 12  $\mu\text{g/ml}$  protein.

iii. Determination of optimal reaction time.

Figure 25: The double reciprocal Lineweaver-Burk plot ( $1/v$  vs  $1/[S]$ ) for calf thymus histone substrate kinetics. The  $[S]$  range was arbitrarily chosen for optimal determination of  $K_m$  and  $V_{max}$ .

$$V_{max} = 0.220 \text{ pMol/min.}$$

$$K_m = 59.5 \text{ } \mu\text{g/ml protein}$$

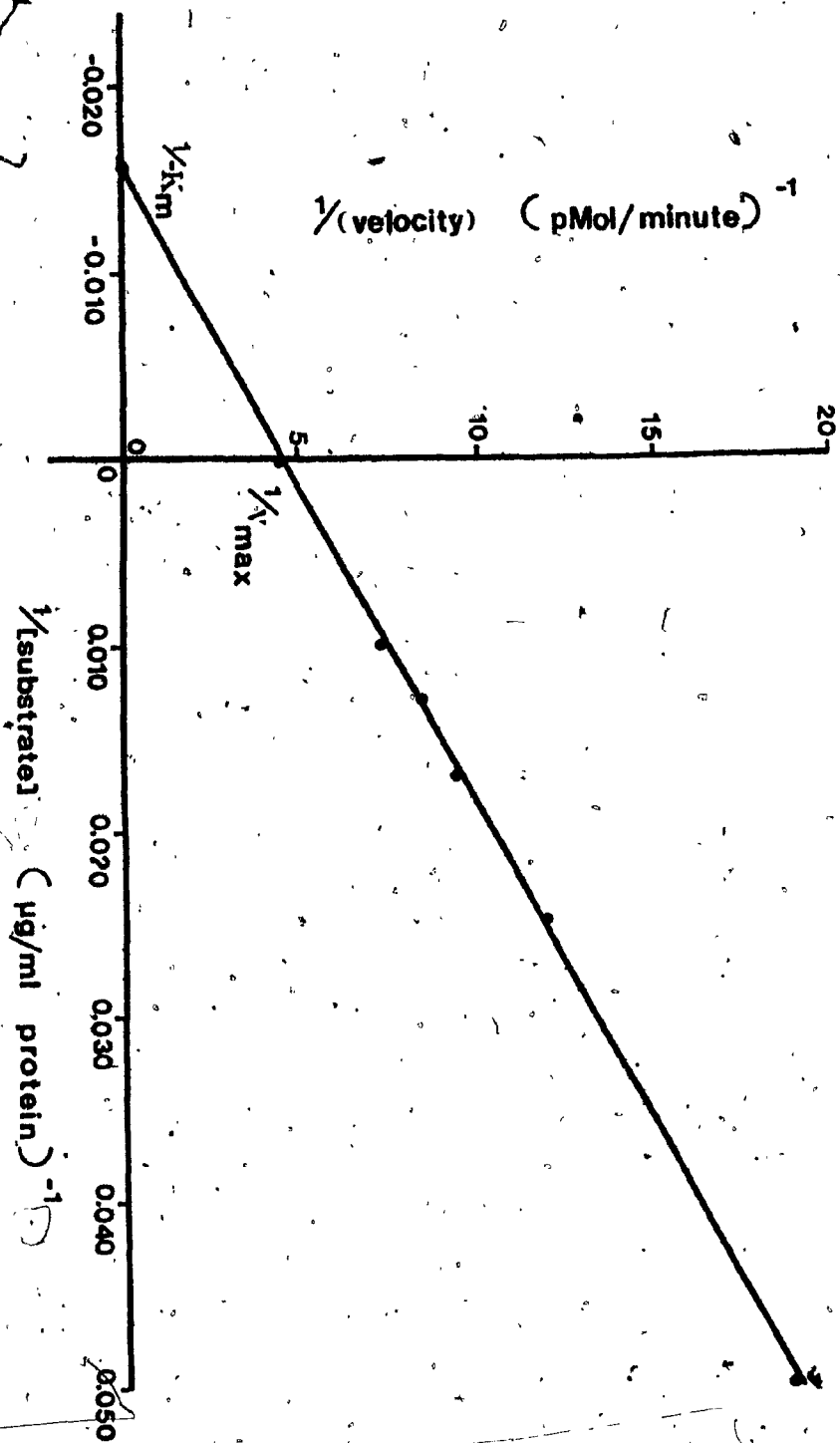
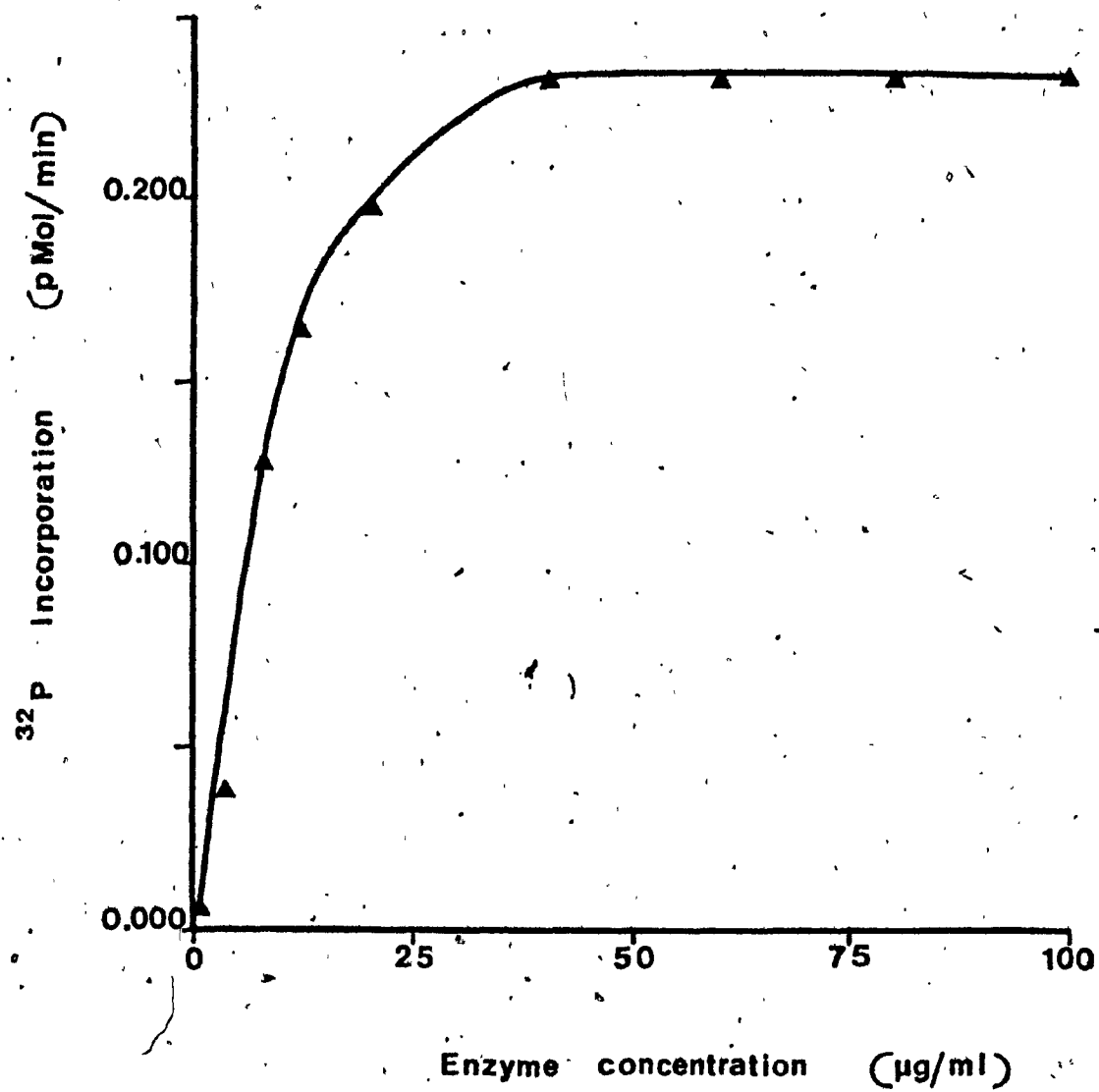




Figure 26: The kinetics of histone kinase obtained from cotyledons of Pinus pinea (20.0  $\mu\text{g/ml}$  calf thymus histone). The effect of enzyme concentration on reaction rate is represented.



Data for the effect of time on the reaction rate for P. pinea histone kinase is illustrated in Figure 27. The data shows clearly that the rate of reaction is quite linear with respect to time up to 5 minutes. Close to 90% of the reaction is complete at this time. Maximal reaction is reached in 10 minutes. Incubation periods longer than 10 minutes result in noticeable decline in the reaction rate. Close to 20% inhibition is incurred at 30 minutes incubation time. This is perhaps due to proteolysis, oxidation or simply thermal denaturation.

#### iv. Substrate specificity.

Data is presented in Table 13 for the specificity of different substrates for the enzyme. Two substrate concentration ranges were used (40  $\mu\text{g/ml}$  and 80  $\mu\text{g/ml}$ ) to ensure that at least one assay was performed at a less than saturating level. For both concentration groups, P. pinea histone showed maximum activity for the enzyme, although the difference between this value and the value obtained for calf thymus is probably only mathematically significant. Protamine served well as substrate for kinase at the 80  $\mu\text{g/ml}$  concentration. Its activity was greater than the value obtained for calf thymus histone at this concentration. It did not serve equally well at the lower

Figure 27: The enzymatic phosphorylation of calf  
thymus histone (50  $\mu$ g) by histone  
kinase obtained from Pinus pinea.  
The effect of incubation duration  
on reaction rate is represented.

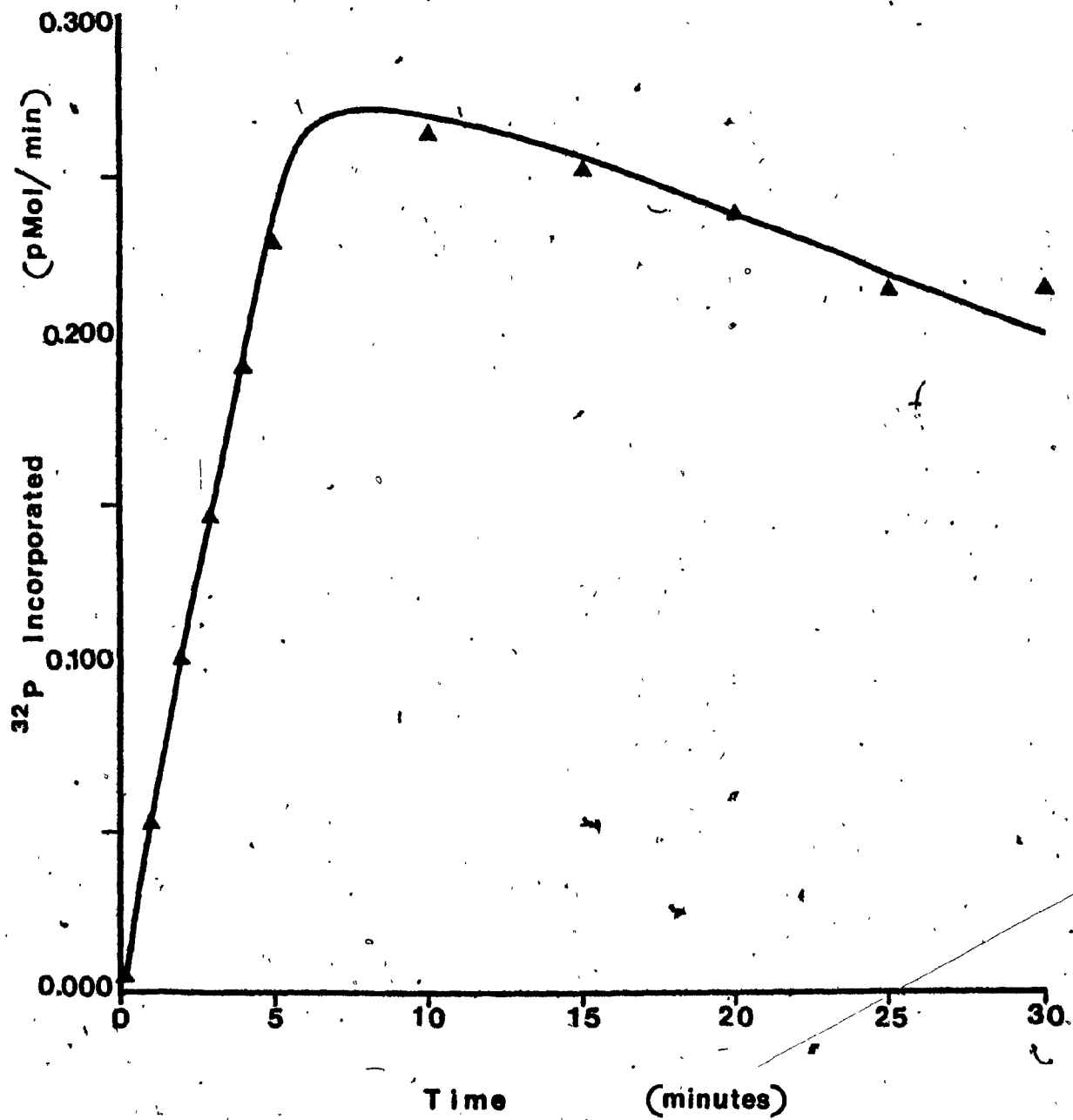


Table 13 The specificity of histone kinase for  
different substrates

Substrate concentration	Calf thymus histone	<u>Pinus pinea</u> histone	Casein	Protamine
40 µg/ml (net CPM)	19531	24073	2520	12658
80 µg/ml (net CPM)	40694	49875	5040	46704
40 µg/ml specific activity (pMol/min)	0.093	0.115	0.012	0.060
80 µg/ml specific activity (pMol/min)	0.194	0.238	0.024	0.222

Pinus pinea histone > calf thymus histone > protamine > casein

concentration. Casein remained consistently poor as substrate at both concentrations. By averaging the specific activities obtained for both concentration groups, the order of specificity of substrate for the enzyme becomes: P. pinea histone > calf thymus histone > protamine > casein.

F. Radiation-induced inhibition of enzymatic activity.

Data in Table 14 and Figure 28 represent the results obtained for the effect on the in vitro reaction rate of histone kinase isolated from 16-hour post-irradiated P. pinea cotyledons. These experiments were designed to further analyze the results shown in Tables 9A - 9C which

Figure 28: The effect of X-irradiation on both the activity of in vivo irradiated P. pinea histone kinase and the ability of in vivo irradiated P. pinea histone to serve as substrate.

Calf thymus histone { ● — ● 0.0 krad  
                           ○ — ○ 0.0 krad  
P. pinea histone { ▲ — ▲ 0.5 krad  
                           △ — △ 1.0 krad  
                           ■ — ■ 3.0 krad

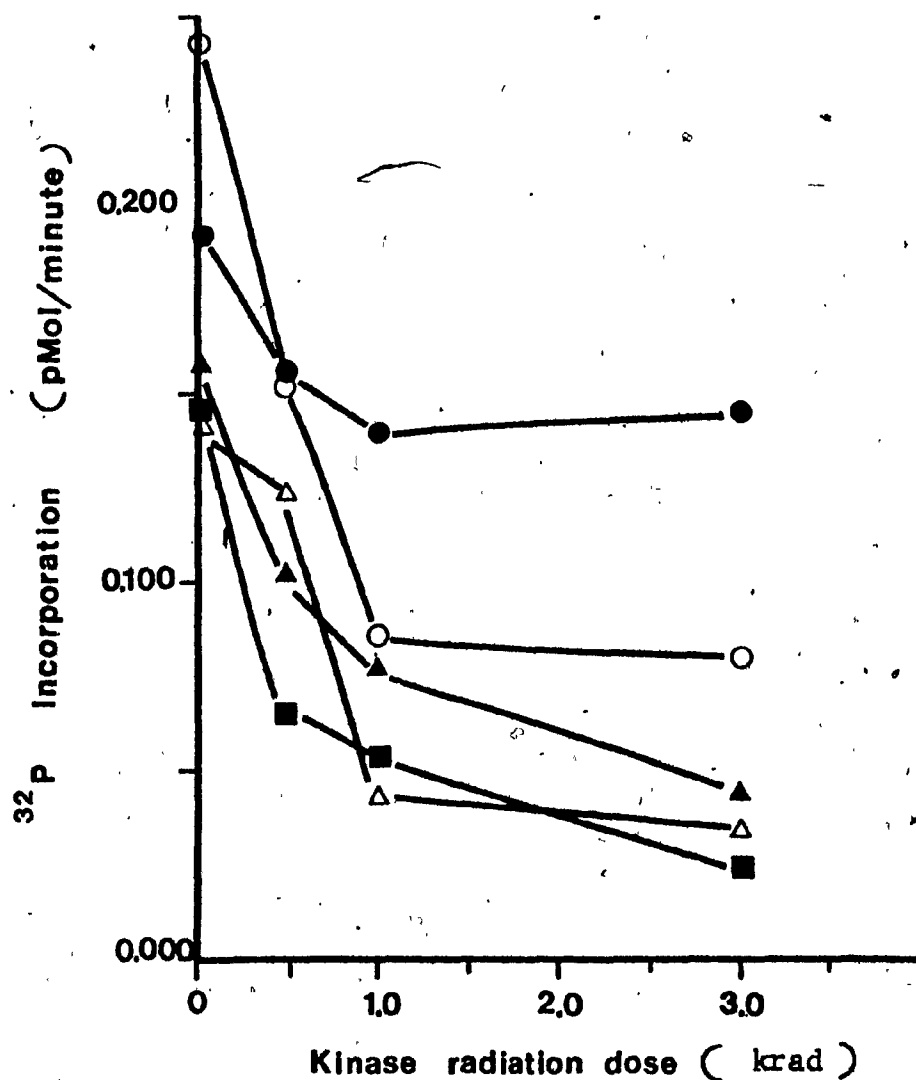




Table 14 The effect of X-irradiation on both the enzymatic activity of histone kinase and the ability of Pinus pinea histone (and calf thymus histone, non-irradiated) to serve as substrate.

	Substrate	Substrate exposure	Enzyme exposure	Formation of acid insoluble $^{32}\text{P}$		% Inhibition of control
		(krads)	(krads)	Net CPM	Specific activity (pMol/min)	
A	CTH*	0.0	0.0	40261	0.192	0.0
	CTH	0.0	0.5	32813	0.156	18.5
	CTH	0.0	1.0	29350	0.140	27.1
	CTH	0.0	3.0	30558	0.146	24.1
B	PPH**	0.0	0.0	50924	0.243	0.0
	PPH	0.0	0.5	38655	0.152	37.3
	PPH	0.0	1.0	17774	0.085	65.1
	PPH	0.0	3.0	16348	0.078	67.9
C	PPH	0.5	0.0	32798	0.156	35.6
	PPH	0.5	0.5	21186	0.101	58.4
	PPH	0.5	1.0	15839	0.076	68.9
	PPH	0.5	3.0	9167	0.044	82.0
D	PPH	1.0	0.0	29386	0.140	42.3
	PPH	1.0	0.5	25465	0.122	50.0
	PPH	1.0	1.0	9269	0.044	81.8
	PPH	1.0	3.0	6824	0.033	86.6
E	PPH	3.0	0.0	17668	0.143	41.1
	PPH	3.0	0.5	13598	0.065	73.3
	PPH	3.0	1.0	11408	0.054	77.6
	PPH	3.0	3.0	5297	0.025	89.6

CTH\* - Calf thymus histone

PPH\*\* - Pinus pinea histone

gave data for the in vivo, rather than the in vitro, incorporation of inorganic radiophosphate into the various histone subfractions and the resulting inhibition of phosphorylation induced by irradiation. It will be recalled from the data in Tables 9A-9C that even the moderately low dose of 0.5 krads was sufficient to induce from about 80 - 95% inhibition of in vivo  $^{32}\text{P}$  uptake in the arginine-rich fractions; however, lesser degrees of inhibition were caused in the lysine-rich fractions. What must be asked as a result of this data is to what extent does irradiation exert its effect on substrate and enzyme alone? Reduced levels of phosphorylated histones may be the result of altered histone structure or direct damage to the enzyme which may be the result of either enzyme inactivation (despite the sub-lethal level of irradiation applied) due to direct interaction of the ionizing radiation or indirectly by peroxide or radiation-product formation. Also, reduced levels of phosphorylated histones may be the result of a decrease in the total pool of mRNA or its availability as a result of template or ribosomal damage. Evidence for this latter possibility would be marked by a decrease in activity when the substrate is enzymatically phosphorylated in vivo but not in vitro.

The effect on the reaction rate using in vivo irradiated P. pinea histone and unirradiated enzyme can be studied by analyzing the values obtained for all 0.0 krad exposed enzyme (Sections B,C,D,E in Table 14 and also Figure 28). The data shows more than 40% inhibition of phosphorylation using in vivo irradiated histone and unirradiated kinase in the reaction mixture. However, data for the effect on reaction rate using in vitro irradiated histone (Table 15 and Figure 29) shows no significant radiation-induced inhibition of  $^{32}\text{P}$  uptake at all even at the 3.0 krad exposure. The obvious difference between these two sets of data may be the result of at least two factors. The histone irradiated in vivo is subject to more extensive damage due to the nature of its environment, (i.e., the intact biochemical machinery of the cell). It is subject to more complex and damaging peroxides and radiation products than would be the case for histone in aqueous solution alone. Also, it must be taken into account that the in vivo irradiated histone has had at least 16 hours recovery time (i.e., necessary period of time for significant levels of inorganic radiophosphate incorporation under in vivo conditions) following X-ray exposure whereas the histone isolated for the in vitro experiment was isolated from unirradiated plants.

Table 15 The in vitro irradiation of isolated histone kinase and Pinus pinea histone.

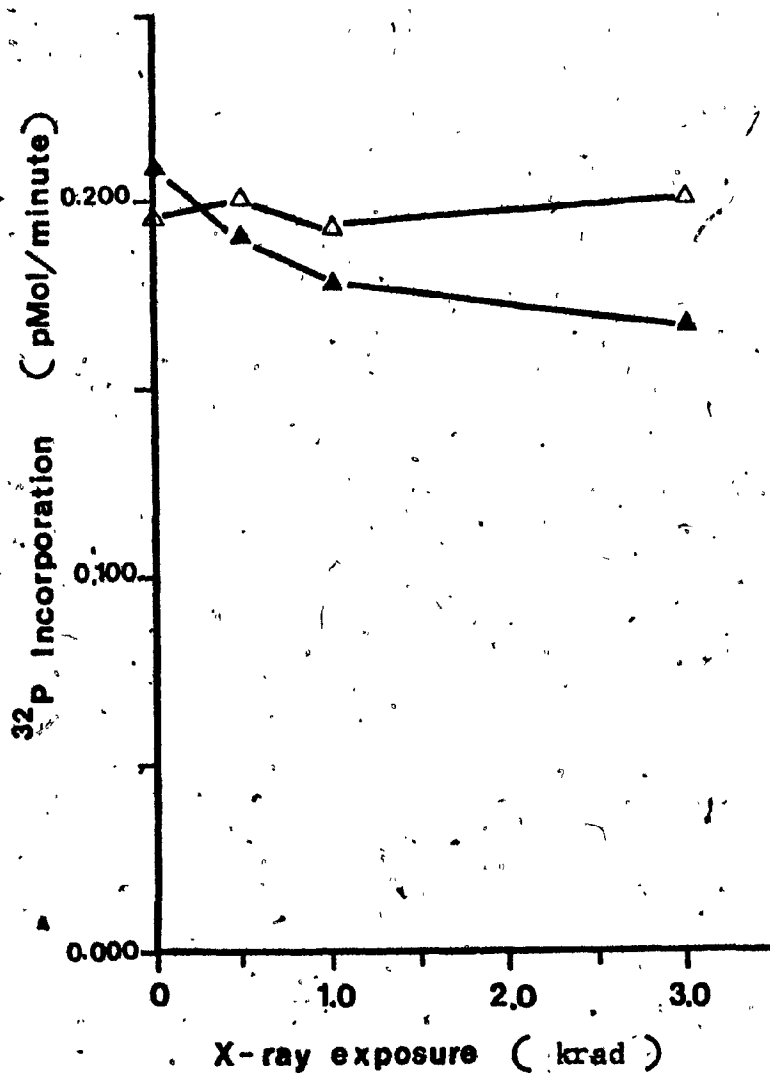
Substrate exposure	Enzyme exposure	Formation of acid insoluble $^{32}\text{P}$		% Inhibition of control
(krads)	(krads)	Net CPM	Specific activity (pMol/min)	
0.0	0.0	11123	0.209	0.00
0.0	0.5	10121	0.190	9.09
0.0	1.0	9473	0.178	14.83
0.0	3.0	8834	0.166	20.57
0.0	0.0	10378	0.195	0.00
0.5	0.0	10697	0.201	----
1.0	0.0	10218	0.192	1.54
3.0	0.0	10750	0.202	----

On the basis of the results obtained in Tables 9A-9C, it was implied that F1 histone (at least) showed a rapid turnover in response to irradiation and that theoretically, there should exist two classes of F1 histone, "old" and "new", sometime within the 16 hour post-irradiation recovery period. Also, in results to be discussed later (Figure 30), data is presented which shows almost complete mitotic arrest induced by the 0.5 and 1.0 krad exposures as early as 6 hours post-irradiation. These two factors may result in the production of a histone with fewer available phosphorylation sites depending on whether it was isolated prior to irradiation ("old" histone) or after irradiation ("new" histone). Figure 30

Figure 29: The in vitro X-irradiation of isolated  
histone kinase and histone from P.  
pinea.

▲—▲ P. pinea kinase

△—△ P. pinea histone



shows that at 14 hours recovery time, 34.4% of the original mitotic activity is recovered at 0.5 krad and 46.0% is recovered for 1.0 krad. This also implies that histone isolated at 16 hours post-irradiation may contain different proportions of lysine-rich and arginine-rich histone. The significance of these results lies in the implication at least that damage to the substrate as caused by the action of ionizing radiation is probably of an indirect nature as is evidenced by the lack of radiosensitivity shown for the in vitro irradiated substrate. This damage may be the result of mitotic block and preferential and rapid turnover of the F1 histones (as indicated by indirect evidence) and the possibility of interaction of the substrate with a greater variety of ionization products when located in situ.

Data in Table 14 and Figure 28 also show the effect on the reaction rate of in vivo irradiated kinase and unirradiated substrate on the reaction rate. There is an increased radiosensitivity of the enzyme as compared with the reaction rates using in vivo irradiated substrate and unirradiated enzyme. Almost 68% of the control value is lost at 3.0 krad exposures (Section B). However, what is especially interesting is the data presented in Table 15 and

Figure 29 which show almost 21% inhibition of reaction rate as a result of the same 3.0 krad exposure dose, using in vitro irradiated enzyme under the exact same conditions. This data implies the possibility of direct radiation damage to the enzyme and may involve the inactivation or denaturation of an essential group or active site of the enzyme. A suphydryl group or groups may be involved and its presence was tested and accounted for in the inhibitor studies.

Sections C,D and E of Table 14 (see also Figure 28) show the interaction between different levels of in vivo irradiated enzyme and substrate. It is not surprising, based on the data given above, that 3.0 krad, in vivo irradiated, P. pinea histone and 3.0 krad, in vivo irradiated kinase showed almost 90% inhibition of  $^{32}\text{P}$  uptake, as compared with the control value (Section B). It is also interesting to note that only 0.5 krad is sufficient radiation to induce significant inhibition of the reaction rate for both enzyme and substrate and further reflects the relatively high radiosensitivity of this system.

Table 14 and Figure 28 also shows data for the effect on the reaction rate of in vivo irradiated P. pinea histone kinase using calf thymus histone as substrate. The data in



Section A shows up to 27% inhibition of phosphorylation at 1000 rads. Section B shows data for the same situation, but using P. pinea histone as substrate. As much as 68% inhibition is induced as a result of 3000 rads irradiation. This data has two implications: 1) in vivo irradiated enzyme does indeed show radiation effect and 2) there seems to be greater specificity for P. pinea histone over calf thymus histone because of the increased radiosensitivity as noted. This may serve to imply damage to a selective area of the enzyme which also confers greater specificity for the plant substrate. None of this data differentiates between enzyme loss nor inactivation.

The significance of these experiments is the implication that at least part of the radiation-induced damage to the enzyme is direct and results in its inactivation; however, there is no conclusive evidence implying that it is merely present in reduced amounts in the cell following irradiation. Further experiments should include analysis of its  $K_m$  values during the recovery period and an attempt should be made to quantify the amount of enzyme present per cell during and after irradiation. Further, it is realized that the X-ray-induced inhibition of in vivo phosphorylation of whole histone in situ may be the result of indirect damage or

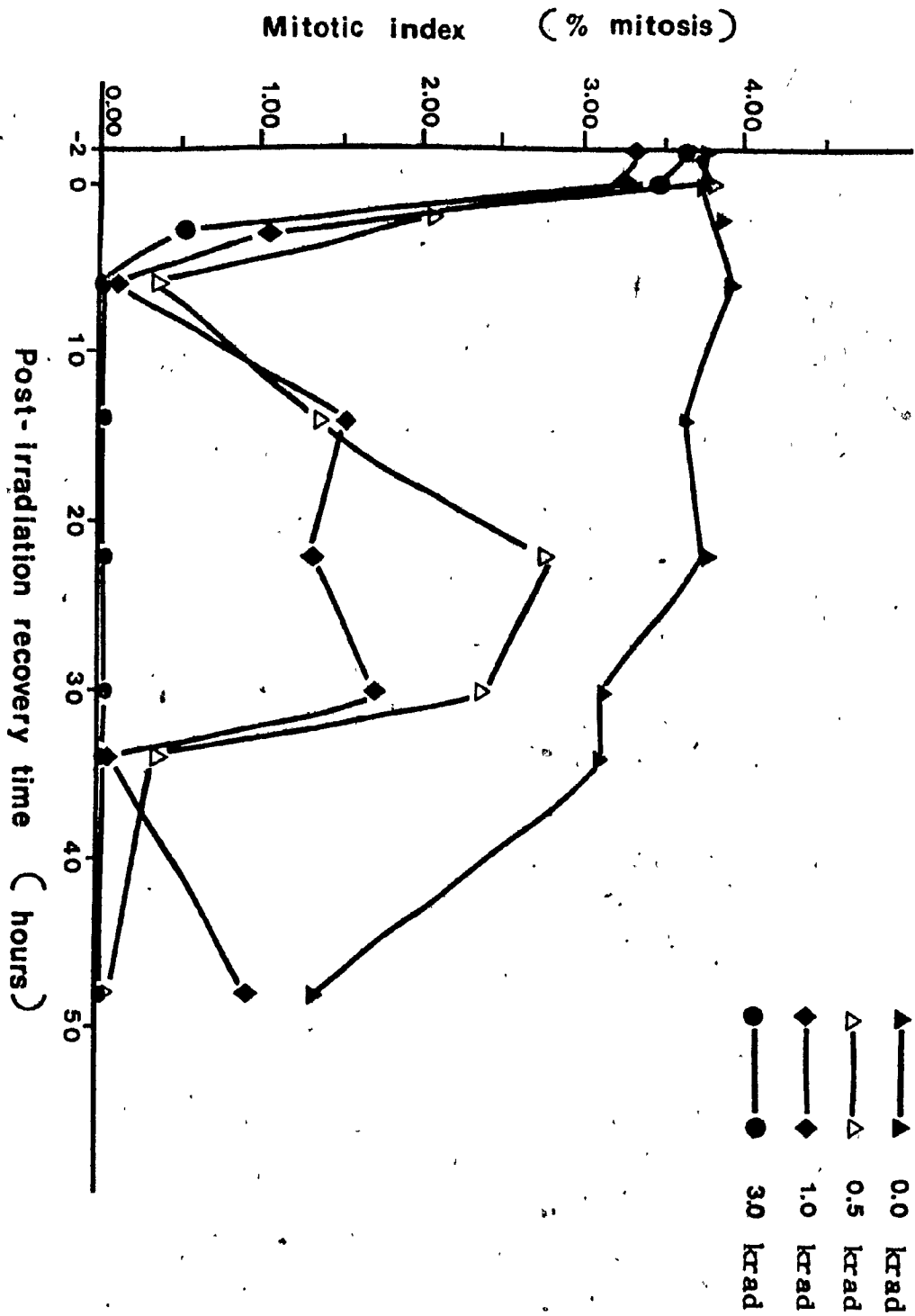
alteration to the substrate or direct damage to the enzyme (possibly at the -HS - SH- groups) or perhaps due to a mechanism which limits the amount and availability of kinase.

#### G. Mitotic arrest in response to X-irradiation

The effect of X-irradiation and 48 hours recovery on the mitotic activity of actively growing day-13 P. pinea cotyledons is seen in Figure 30. Three low-level radiation doses were used in this assay, 0.5, 1.0 and 3.0 krad and an unirradiated batch served as control. As seen in the control data, cell division is maximal up to day-14 and drops markedly in the next 24 hours (day-15). No data was collected for periods prior to day-13 nor after day 15. This information is available Roy et al., 1972<sup>121</sup>. In their study, mitotic activity was initiated in the lower segments of the cotyledon by day 5 in the controls, and the highest value was seen by day-7 where mitoses were uniform throughout the cotyledon. There was also rapid decline in mitotic activity beginning at day-14 through day-17.

Data in Figure 30 shows that irradiated cotyledons show marked arrest at all radiation levels six hours after exposure. During the next 16 hours (22 hours post-irradiation) there

Figure 30: The inhibition of mitotic activity  
and its recovery following X-  
irradiation.



is immediate and rapid return to near-normal mitotic indices for the 0.5 and 1.0 krad groups. 3.0 krad exposure showed no evidence of recovery within the 48-hour period. Once these two exposure groups (0.5 and 1.0 krad) had shown maximal recovery, there was again the expected decline in mitotic activity after day 14.

There are several significant points as a result of this study. It further confirms the relatively high radiosensitivity of the gymnosperms as shown by the onset of mitotic block even at 0.5 krad exposure. Also, an interesting correlation is made between the time period seen for the recovery from mitotic block (between 6 and 22 hours) and the period of time postulated previously from indirect results which implied rapid turnover and re-phosphorylation of the F1 histone. Since it is already known that F1 histone is a much more effective template inhibitor than are the arginine-rich histones<sup>12</sup> and it is also known that histone phosphorylation (especially F1 phosphorylation) plays an important role in DNA replication<sup>4,123,148</sup>, it is possible to envisage a system whereby any damage (i.e., radiation) to either histone kinase or histone or both will inhibit F1 phosphorylation. This in turn will inhibit DNA replication and subsequent mitosis. Because

of the uniqueness of the F1 histone it is selectively re-synthesized and preferentially re-phosphorylated. In becoming phosphorylated it reduces its association with the DNA template. This action ultimately alters the DNA replication complex and so allows recovery from mitotic block to occur. The question which obviously arises from such a proposed system is what mechanism is available for the selective and rapid turnover of the F1 histone? The loss and recovery of F1 phosphorylated histone has been closely linked with the loss and recovery of  $^3\text{H}$ -thymidine incorporation into DNA in CHO cells<sup>57</sup> but the mechanism and function of F1 turnover is unknown. Further work must be done with Pinus pinea to ensure that F1 turnover does indeed occur within the 16 hour period after complete mitotic block in order to support fully the above statements.

### SUMMARY AND CONCLUSIONS

This is the first known purification of Pinus pinea cyclic-AMP-independant histone kinase. The enzyme was partially purified (35 fold) on DEAE-Sephadex A-50 ion exchange resin and yielded one major enzymatically active peak and one lesser one. The kinase showed a  $pH$  optimum of 7.8 in 5  $\mu M$  Tris-HCl buffer and  $pH$  8.0 in 10  $\mu M$  phosphate buffer. The enzyme was magnesium-dependant and showed maximal activity between 5.0 and 7.5 mM  $Mg^{2+}$ . The addition of up to 10.0  $\mu M$  kinetin and 10.0  $\mu M$  c-AMP showed no stimulatory effect. The presence of active sulphydryl groups were tested for by the addition of p-chloromercuribenzene sulphonic acid and iodoacetate. In the absence of 2-mercaptoethanol, 10.0 mM p-chloromercuribenzene sulphonic acid induced almost total inhibition of  $AT^{32}P$  uptake into calf thymus histone under the prescribed assay conditions. The inhibitory effects of iodoacetate were less pronounced. The inhibition induced by 0.5 mM p-chloromercuribenzene was found to be reversible by the addition of 0.3 mM 2-mercaptoethanol. The enzyme showed maximal activity at  $30^{\circ}C$  and was stable for up to three months at  $-20^{\circ}C$ .  $V_{max}$  for P. pinea histone was 0.243 pMol/min and the  $K_m$  calculated was 41.0  $\mu g/ml$  protein.  $V_{max}$  for calf thymus

histone was 0.220 pMol/min and the  $K_m$  value was 59.5  $\mu$ g/ml protein. The enzyme became fully saturated at 35  $\mu$ g/ml protein using standard assay conditions. Maximal reaction was reached in 10 minutes and was linear but longer incubation periods resulted in loss of activity. The histone kinase purified showed an order of substrate specificity as follows: P. pinea histone > calf thymus histone > protamine > casein. Casein was not a suitable substrate for the reaction and represented only 10% of the maximal activity obtained with P. pinea histone.

Histone and nucleohistone were also isolated from P. pinea cotyledons. Low-level radiation doses (0.5 and 1.0 krad) did not alter the UV absorption profile of the isolated chromatin to any marked extent. However, thermal denaturation profiles and changes in hyperchromicity were markedly affected by these same sub-lethal exposures suggesting the possibility of an altered histone/DNA complex. The  $T_m$  for native, unirradiated chromatin was biphasic and gave two values, 61.3°C and 82.5°C. The 0.5 krad irradiation dose caused an altered profile resulting in altered  $T_m$  values, 57.5°C and 87.5°C, but nevertheless remained biphasic. The 1.0 krad radiation exposure induced a complete shift from a biphasic curve to a single-step melting profile with a calculated



$T_m$  of 75.0°C. This  $T_m$  profile strongly suggested again altered histone/DNA association and resembled the melting profiles usually attained for deproteinized DNA alone.

Pinus pinea histone isolated from chromatin was characterized by polyacrylamide gel electrophoresis and revealed the presence of up to nine major electrophoretic peaks. Band 8 showed the highest mobility (Figure 10) and was arbitrarily aligned with the arginine-rich F2a1 histone fraction of calf thymus histone. Bands 6-7 could be only partially resolved and were identified as the F2a2 and F2b fractions respectively on the basis of their relative mobilities with respect to calf thymus histone. Band 5 was of a variable nature and was identified as histone F3. The remaining bands (X,1,2,3,4) were thought to be the lysine-rich histones due to their low relative mobilities and showed comparative mobility with calf thymus histone F1. That fraction showing the least electrophoretic mobility (X) disappeared completely in the 0.5 and 1.0 krad irradiated groups. There was no evidence of an X-ray-induced effect on any other particular P. pinea histone fraction.

X-irradiated whole cotyledons incubated for 16 hours in aqueous inorganic radiophosphate solution gave evidence of  $^{32}\text{P}$  uptake inhibition in only certain fractions (i.e., over

90% inhibition of uptake in the arginine-rich fraction at 0.5 krad irradiation). The lysine-rich fractions seemingly were not as affected (i.e., the F1 fractions were inhibited only 24% - 42% as compared with the control values). It was suggested that instead of the seeming radioinsensitivity as indicated by the results, this fraction displayed marked and rapid turnover of de novo synthesized F1 histone in response to X-irradiation and thus accounted for the low inhibition values.

The effect of in vivo irradiated enzyme on the reaction rate shows that at 3.0 krad irradiation, there is a resulting 68% loss of activity when incubated with unirradiated substrate as compared with the control value. The effect of 3.0 krad in vitro irradiated enzyme under the same conditions caused up to 21% inhibition of the control reaction rate. This data serves to imply direct damage to the enzyme, probably as a result of damage to an essential group or active site of the enzyme (i.e., sulphydryl group). The effect of 3.0 krad in vivo irradiated P. pinea histone showed up to 42% inhibition of  $AT^{32}P$  uptake as compared with the control value and using unirradiated histone kinase in the reaction medium. The 3.0 krad irradiated substrate, under exactly the same conditions, showed no radiation-induced inhibition of the

reaction rate. This indicated that the in vivo irradiated substrate is subject to more extensive radiation damage when situated in the cell and may be prone to peroxide and other radiation product formation of the cell water. The discrepancies observed in the values for the reaction rates between the in vivo and in vitro irradiated histone may also be due to the possibility based on previous reports that F1 histone, in certain tissues at least, shows selective and rapid turnover in response to X-irradiation. Therefore, at the time of chromatin isolation (i.e., 16 hours post-irradiation) there may exist two classes of F1 histone, one fraction in some way inactivated by irradiation and the other, a newer, de novo synthesized fraction capable of being phosphorylated in vivo. It must be rigidly stressed that no direct observation exists in this report that Pinus pinea F1 histone did indeed show rapid and selective turnover. This finding remains to be experimentally determined although literature has been cited which shows that it does so in other tissues.

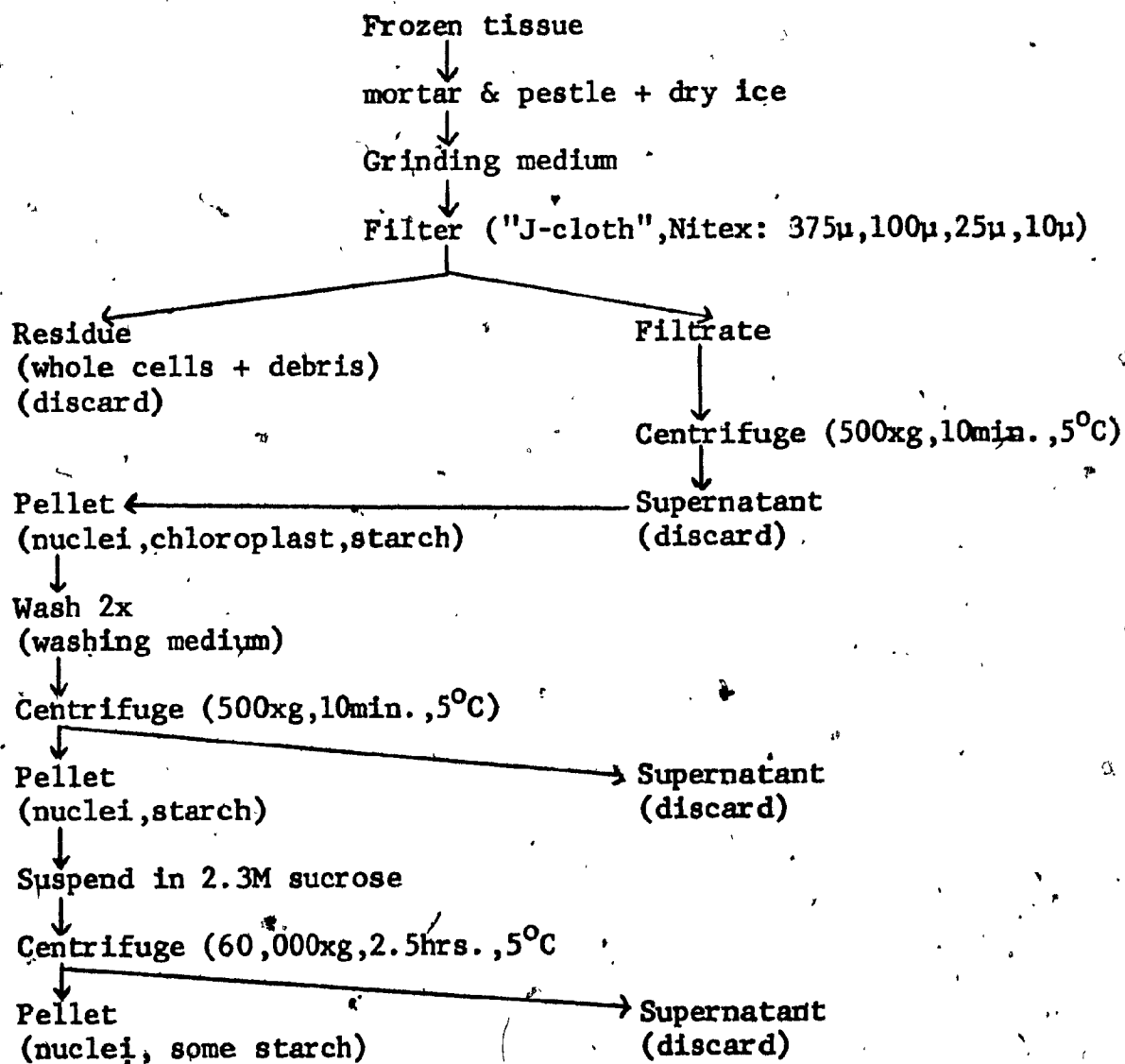
The analysis of mitotic arrest and recovery in Pinus pinea over a period of 48 hours post-irradiation beginning on day-13 showed marked arrest 6 hours post-irradiation at all exposures given (0.5 krad, 1.0 krad, 3.0 krad). However,

within 22 hours post-irradiation, there was immediate and rapid return to near normal control values. There was no recovery noted for the 3.0 krad exposure group. This information confirms the high radiosensitivity reported for the gymnosperms. The data also implies the existence of a mechanism for the recovery of radiation-induced mitotic block. Given that lysine-rich F1 histone is a more effective template inhibitor than the arginine-rich histones, then a mechanism may exist whereby any radiation-induced damage to histone kinase or histone or both which ultimately inhibits F1 phosphorylation will in turn inhibit DNA replication and subsequent mitoses. However, it is postulated that F1 histone becomes selectively re-synthesized in response to irradiation or mitotic block and becomes re-phosphorylated under the experimental conditions prescribed. Once phosphorylated, it reduces its association with DNA due to interaction with the highly charged phosphate groups of DNA and thus allows derepression of the particular gene segments involved with the subsequent production of the necessary enzymes and replication regions to allow division to occur. Such an analysis is admittedly highly speculative but not improbable and remains to be experimentally determined. What must now be done under laboratory situations are the determinations

necessary to insure that rapid F1 turnover and phosphorylation does indeed occur in P. pinea cotyledons within the defined 16 hour recovery/incubation period.

Appendix I

Isolation of plant nuclei

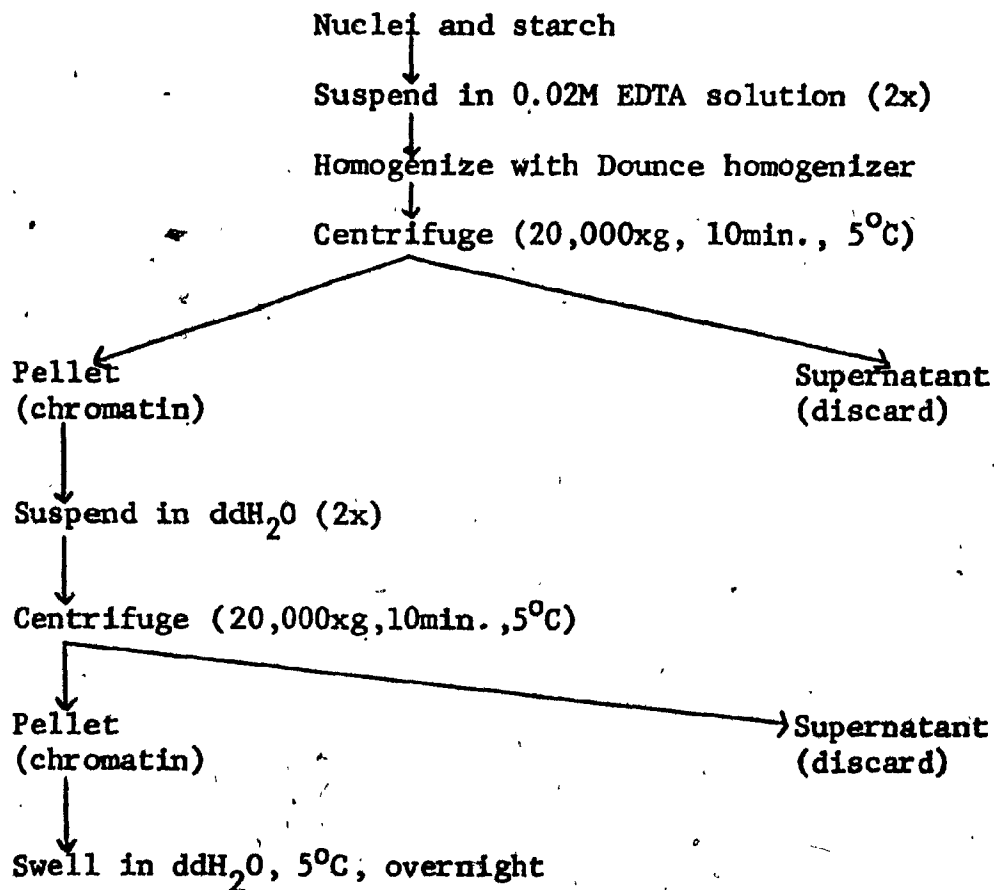


(all steps at 5°C)

(after Berkofsky, 1975<sup>13</sup>)

Appendix II

Isolation of chromatin

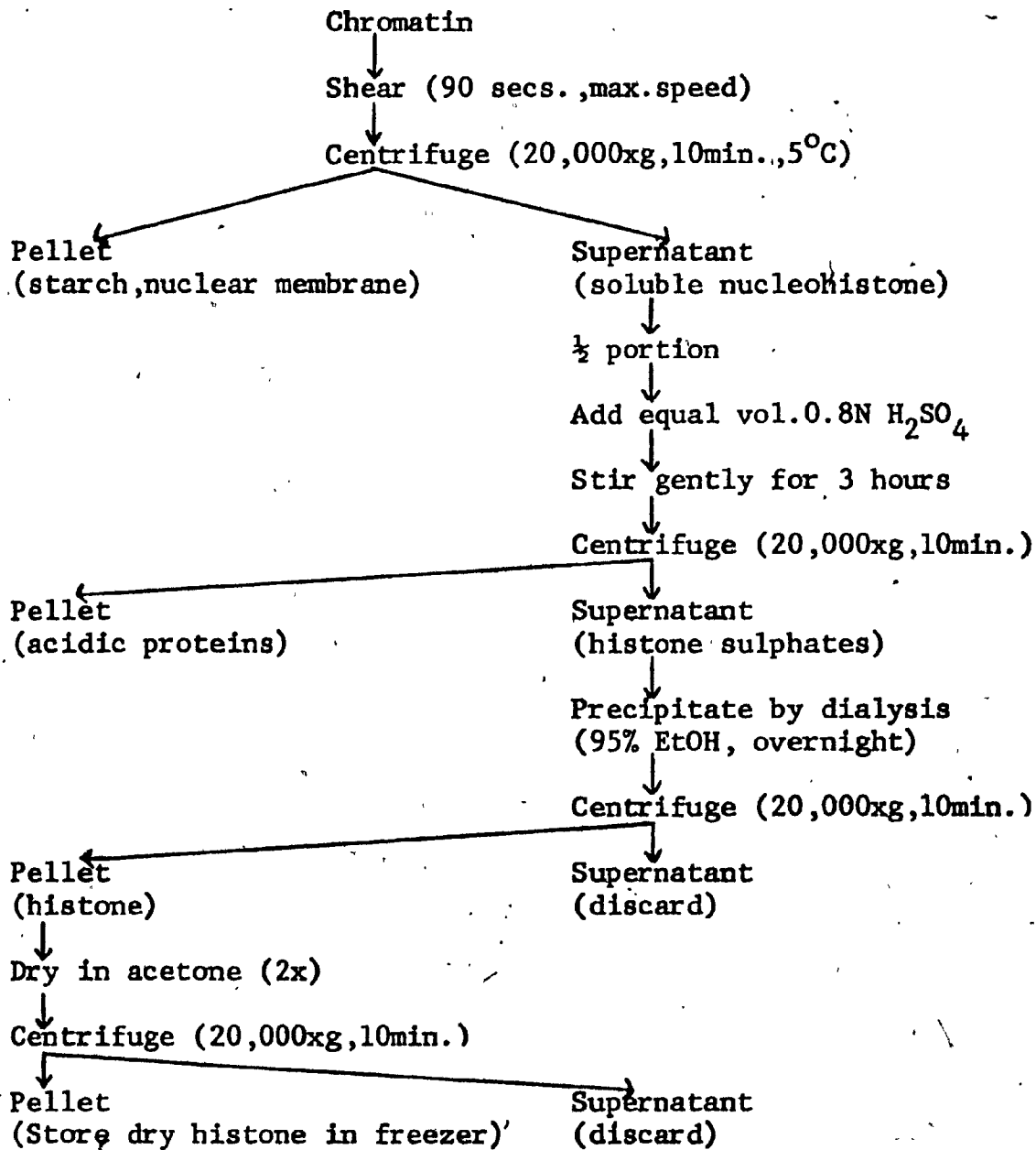


(all steps at 5°C)

(after Berkofsky, 1975<sup>13</sup>)

Appendix III

Histone isolation



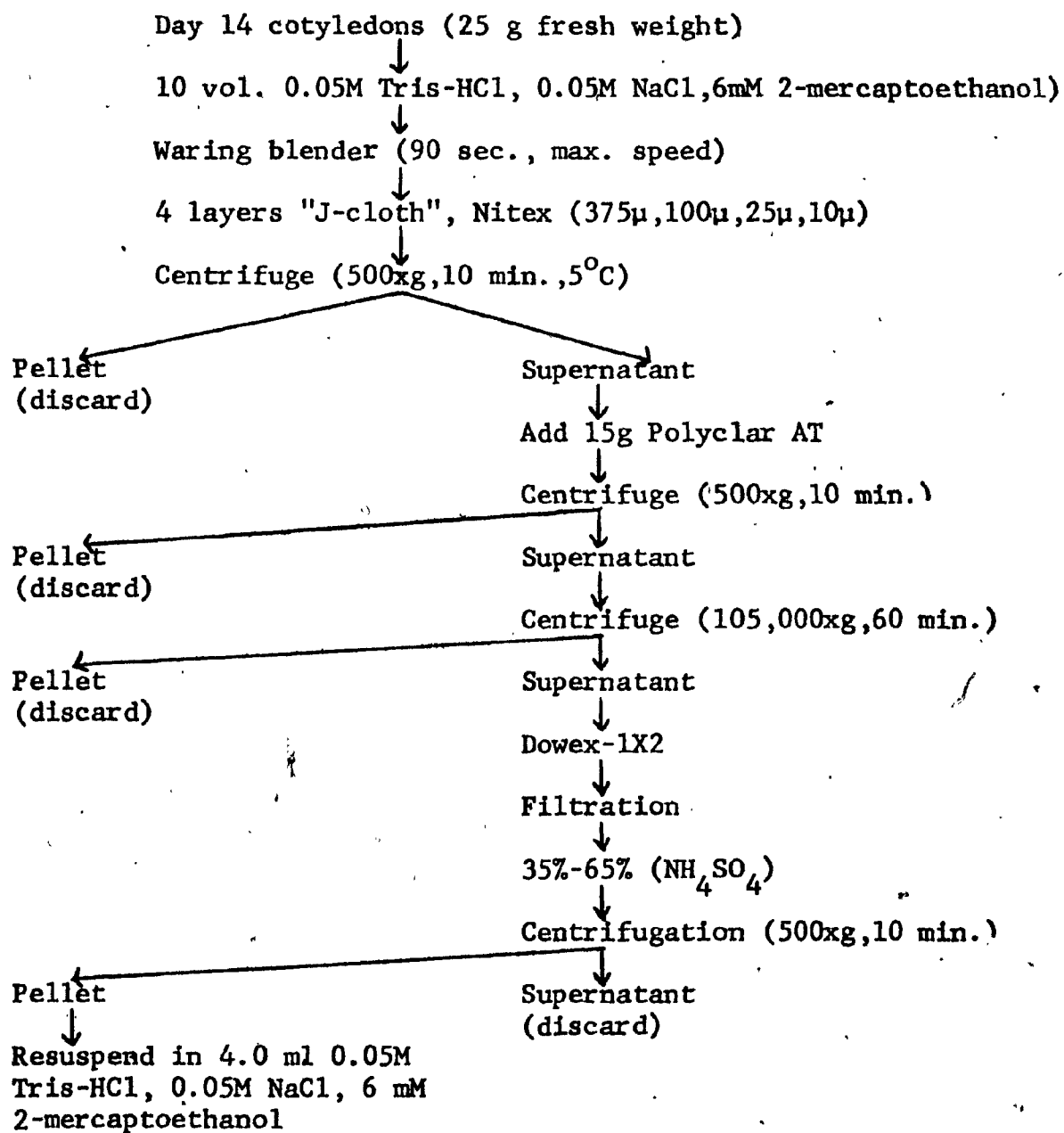
(all steps at 5°C)

(after Berkofsky, 1975<sup>13</sup>)



Appendix IV

Preparation of crude enzyme



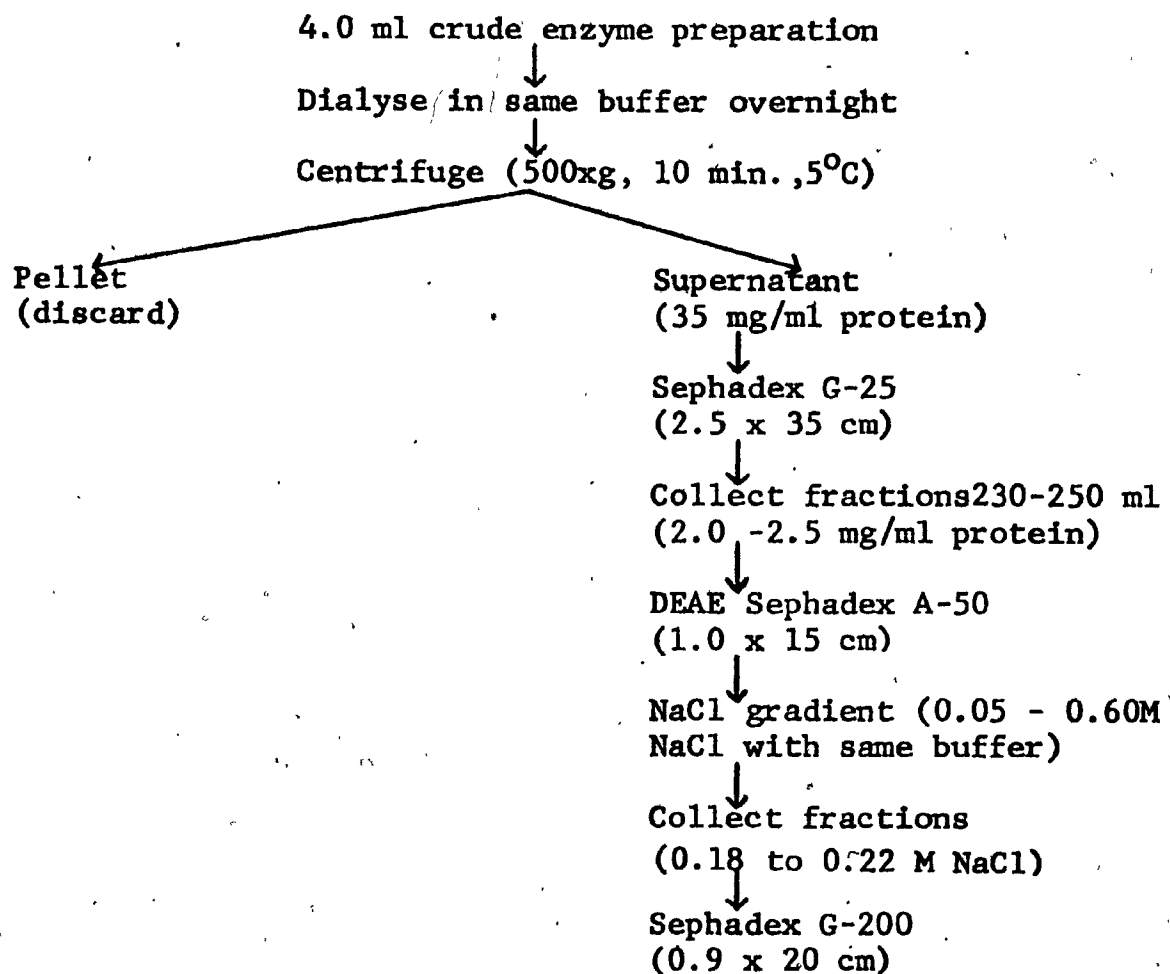
(all steps at 5°C)

(after Nakaya et al, 1975<sup>103</sup>)

Appendix V

Partial purification of crude enzyme

preparation



(after Nakaya et al, 1975<sup>103</sup>)

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