The effect of gibberellic acid and AMO-1618 on chromatin ribonucleic acid polymerase in soybean hypocotyl tissue.

Gregory Chok-Tsi. Hou

University of Windsor

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THE EFFECT OF GIBBERELLIC ACID AND AMO-1618 ON CHROMATIN RIBONUCLEIC ACID POLYMERASE IN SOYBEAN HYPOCOTYL TISSUE

BY

GREGORY CHO-K-TSI HOU

A THESIS Submitted to the Faculty of Graduate Studies through the Department of Biology in Partial Fulfillment of the Requirements for the Degree of Master of Science at the University of Windsor

WINDSOR, ONTARIO, CANADA 1971
ABSTRACT

Chromatin isolated from soybean (Glycine max) hypocotyls contains a bound RNA polymerase. This enzyme is capable of in vitro RNA synthesis which is dependent on the simultaneous presence of all four ribonucleoside triphosphates and a divalent metal ion (Mg^{++} or Mn^{++}) for activity.

Gibberellic acid, sprayed on intact soybean hypocotyls, seems to enhance the level of chromatin RNA polymerase activity while chromatin isolated from hypocotyls pretreated with AIBO-1618 exhibits a lower polymerase activity relative to control.

Chromatin extracted from treated or untreated seedlings are sensitive to inhibition (in varying degrees) in the presence of actinomycin D, pyrophosphate, or ribonuclease. Preliminary study indicates that the in vivo enhanced (or decreased) RNA-synthesizing capacity of chromatin in response to chemical treatments may probably be due to enhanced (or decreased) synthesis of RNA polymerase.
ACKNOWLEDGEMENTS

I wish to acknowledge my sincere thanks to Dr. D.T.N. Pillay, Department of Biology, University of Windsor, for his guidance and technical instruction during the course of my graduate education and the presentation of this thesis.

For critically reviewing this thesis, I wish to extend my sincere appreciation to Dr. L.B. Sabina, Department of Biology, and Dr. W.P. Aston, Department of Chemistry, both of the University of Windsor.

I am very grateful for the help and encouragement freely given by Dr. A.H. Warner, Dr. W.G. Benedict, Mr. G.S. Soteros and Mr. F.L. Huang.

To my wife, Anita, I wish to express my gratitude for the many hours she spent typing this manuscript and for her unfailing support.

I also wish to gratefully acknowledge the financial assistance provided by the Department of Biology, the University of Windsor, and by Grant No. A-1984 to Dr. Pillay from the National Research Council of Canada.
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ABBREVIATIONS

AMAB  alkyltrimethyl ammonium bromide
AMO  (AMO-1618)  2'-isopropyl-4'-[trimethylammonium chloride]-5'-methylphenyl piperidine carboxylate

\[
\begin{array}{c}
\text{N} \\
\text{O} \\
\text{N}^{+}(\text{CH}_3)_3\text{Cl}^- \\
\text{CH}_3\text{-CH-CH}_3
\end{array}
\]

AMP  adenosine monophosphate
ATP  adenosine triphosphate
B-Nine (or Alar)  N-dimethylaminosuccinamic acid
CCC  2-chloroethyltrimethylammonium chloride
deg Centrifuge
°C  degree Centrifuge
cm  centimeter
cmp  cytidine monophosphate
CTP  cytidine triphosphate
2,4-D  2,4-chlorophenoxyacetic acid
DNA  deoxyribonucleic acid
DNAase  deoxyribonuclease
E. coli  Escherichia coli
s  gravitational force
gibberellic acid A₃

gram

GMP

guanosine monophosphate

GTP

guanosine triphosphate

³H-UMP

uridine-5-³H-5'-monophosphate with radioactive tritium hydrogen

³H-UTP

uridine-5-³H-5'-triphosphate with radioactive tritium hydrogen

IAA

indole-3-acetic acid

i.e.

that is

M

mole

mg

milli-gram

Mg²⁺
magnesium ions

MgCl₂

magnesium chloride

ml

milli-liter

mM

milli-mole

Mn²⁺
manganese ions

MnCl₂

manganese chloride

μµ

milli-micron

NTP

ATP + CTP + GTP

viii
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Phosfon-D</td>
<td>tributyl-2,4-dichlorobenzyl-phosphonium chloride</td>
</tr>
<tr>
<td>PPI</td>
<td>pyrophosphate</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNAase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>r.p.m.</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>Tris-(hydroxymethyl)-aminomethane adjusted to desired pH with hydrochloric acid</td>
</tr>
<tr>
<td>µ</td>
<td>micro</td>
</tr>
<tr>
<td>µC</td>
<td>micro Curie</td>
</tr>
<tr>
<td>µg</td>
<td>micro gram</td>
</tr>
<tr>
<td>UMP</td>
<td>uridine monophosphate</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine triphosphate</td>
</tr>
<tr>
<td>µµ moles</td>
<td>micro micro moles</td>
</tr>
<tr>
<td>w:v</td>
<td>ratio of weight to volume</td>
</tr>
</tbody>
</table>
INTRODUCTION

Previous work in this laboratory (Soteros 1970) was concerned in correlating the actions of the plant growth hormone GA and the growth retardant AMO-1618 at the level of nucleic acid metabolism. Some work (Tu 1970) was also undertaken to investigate the effect of GA and AMO-1618 on the synthesis of cell wall, protein and nucleic acid in addition to growth of pea seedlings.

Current literature on plant growth retardants (AMO-1618, CCC, phosfon-D, B-995 or Alar, AMAB) deals mainly with their morphological and physiological effects especially in their use as a tool in the study of gibberellin physiology in various plant systems (Leng 1968). However, it appears that studies on the effects of growth retardants on nucleic acid metabolism are rare.

Plant and animal hormones are reported to affect nucleic acid metabolism in a wide variety of systems (Key 1969; Tata 1968). Evidence indicates that plant hormones generally enhance RNA synthesis in hormone-responsive tissues. Nevertheless, it is still not clear whether the rate of RNA synthesis or the rate of degradation is affected in different tissues. In order to gain
additional information on the significance of the hormonal effect on RNA metabolism, various studies have been undertaken to investigate the effect of hormones on RNA synthesis by isolated, cell-free, nuclei and chromatin.

Isolated chromatin of higher organisms possess several properties characteristic of the same chromatin in vivo (Bonner et al. 1968). Among the properties are the presence of histone bound to DNA, the state of repression of the genetic material, and the ability to serve as template for RNA synthesis and for the readout of the de-repressed portion of the genome by RNA polymerase.

The present work is an attempt to determine the effect of GA and AMO-1618 on the RNA-synthesizing ability in the nucleus by studying the in vitro chromatin-directed RNA synthesis by isolated chromatin and its bound RNA polymerase of treated and untreated tissues. The chromatin-DNA template availability was also studied. Lastly, it was of interest to understand the relationship between changes induced by these chemicals at the genetic level with particular reference to the morphological and/or physiological changes in the treated plant system.
LITERATURE REVIEW

A growing literature provides evidence that plant and animal hormones strikingly affect nucleic acid metabolism, especially ribonucleic acid synthesis, in a number of systems (Trewavas 1968; Tata 1968; Key 1969). However, little information is available about the mechanisms of hormone-stimulation of nucleic acid synthesis especially in plants. It appears that the hormonal action is involved within the nucleus.

Experimental evidence indicates that hormone-induced changes in RNA synthesis precede the morphological and physiological changes induced by hormones (O'Brien et al. 1968a; McLoughlin et al. 1970; silberger and Skoog 1953). Skoog and his co-workers were the first to show an effect of plant hormones on nucleic acid metabolism. In their experiment on tobacco tissue culture they demonstrated that the auxin-induced increase in nucleic acid occurred prior to the increase in tissue fresh weight and that a proportional increase in RNA and fresh weight took place with increasing time.

The morphological and physiological changes brought about by hormones reflect that hormonal action may be involved in regulation of enzyme activities through nucleic
acids (Trewavas 1968; Key 1969). It is clear that enzyme synthesis depends entirely on nucleic acids and their metabolism; and that DNA, the genetic material in the cell, exercises its regulatory control on protein synthesis by transferring its genetic information to another class of nucleic acids, RNAs, which serve as the protein templates.

Auxins such as IAA and 2,4-D enhance RNA synthesis in intact plants (Rebstock et al. 1954; Key and Hanson 1961; Shannon et al. 1964; O'Brien et al. 1968b), excised tissues (Silberger and Skoog 1953; Key and Ingle 1964), and isolated nuclei (Boychoudhury and Sen 1964; Maheshwari et al. 1966; Cherry 1967). Gibberellins induce increase in RNA synthesis in a number of seedlings (Giles and Myers 1966; Nitsan and Lang 1966), isolated nuclei (Johri and Varner 1968), and excised tissue (Chandra and Varner 1965). The cytokinins also show an effect on synthesis of RNA (Guttman 1957; Osborne 1962). Recently some evidence indicates that ethylene plays some role in RNA metabolism (Jensen et al. 1964; Holm and Abeles 1967; Holm et al. 1968).

That hormone may play its regulatory role through the nucleic acid has been pointed out above. Since most cellular RNA appears to be produced in the nucleus (Prescott 1964), it seems probable that hormone-induced changes in RNA synthesis are originated in the nucleus. Despite
their errors in using crude nuclear preparations with possible bacterial contamination, Roychoudhury et al. (1964) and Maheshwari and co-workers (1966), gave evidence from their experiments on isolated plant nuclei that the site of auxin is in the nucleus and that auxin increased the synthesis of nuclear RNA.

From his experiment on nucleic acid metabolism from peanut cotyledons and soybean hypocotyls, Cherry (1967) also showed that the nucleus is indeed the site where radioactivity is incorporated into RNA. Johri and Varner (1968) found that dwarf pea nuclei showed enhanced RNA synthesis in response to added GA. Matthisse (1968), Matthisse and Phillips (1969) also came to a similar conclusion with their isolated nuclei in response to added auxin.

Johri and Varner (1968) pointed out that purified pea nuclei failed to respond to applied GA because of the loss during isolation of some essential hormone-sensitive factor. Matthisse (1968) and Matthisse and Phillips (1969) also demonstrated that this failure of purified nuclei to respond to applied hormones was probably due to the loss during isolation from the nucleus of some intermediary, hormone-reactive factor which was shown to be a protein (Matthisse and Phillips 1969). For similar reasons, isolated chromatin does not respond to
hormone in vitro in enhancing RNA synthesis (Matthesse 1968; Schwimmer 1968). However, purified pea bud nuclei (Matthesse 1968) and chromatin isolated from hormone-pretreated soybean hypocotyls (O'Brien et al. 1968a, 1968b) exhibited an enhanced RNA synthesis compared to control chromatin.

Investigations in some plant systems have shown that RNA polymerase appears to be bound within the chromatin. Huang and Bonner (1962) reported that in dwarf pea seedlings no soluble RNA polymerase was detected but the enzyme appeared to be bound together with the chromatin. O'Brien et al. (1968a, 1968b) found a similar case for the enzyme in soybean hypocotyls. It should be pointed out that soluble RNA polymerase was also detected in maize (Stout and Mans 1967).

It is obvious that chromatin and its bound RNA polymerase are responsible for carrying out DNA-dependent RNA synthesis; the chromatin-DNA serves as the template for the enzyme RNA polymerase to catalyze the reaction of RNA synthesis.

\[
\begin{align*}
\text{n}_1 \text{ ATP} & + \\
\text{n}_1 \text{ UTP} & + \\
\text{n}_2 \text{ GTP} & + \\
\text{n}_2 \text{ CTP} & \\
\text{Mg}^{++} & \\
\text{AMP} & \\
\text{UMP} & \\
\text{GMP} & \\
\text{CMP} & \\
\text{RNA} & + (2n_1 + 2n_2) \text{ PPI} \\
2n_1 & + 2n_2
\end{align*}
\]
It is found that, when the four ribonucleoside triphosphates (RNA precursors) and a divalent metal ion, like magnesium, are included in vitro in the assay mixture containing chromatin and its associated RNA polymerase, RNA is synthesized; and the overall RNA polymerization reaction (Mahler and Cordes 1968) can be represented as above.
MATERIALS AND METHODS

Plant Material

Soybean seeds (Glycine max L. var. Wayne) soaked in water for 8-10 hours and sown in moist vermiculite, were grown in the dark at 26° C. At the end of three days, seedlings were sprayed by means of an atomizer with GA (10^{-3} M) or/and AMO-1618 (10^{-3} M) and with water to serve as control.

The whole soybean hypocotyls (taken between the cotyledonary hook and the first lateral root) were collected at various times following treatment in a beaker kept on ice.

Chromatin Extraction and Purification

Samples each of 150 gm of chilled hypocotyls from GA and AMO-1618 treated, and control plants were used for chromatin extraction, using the general procedures described by Huang and Bonner (1962) with minor modifications. All steps of extraction were performed either in the cold room or in a container on ice (i.e., temperature maintained at 0-4°C). The tissue was cut into small pieces with a pair of scissors and then homogenized in a Sorvall Omni-mixer in
an equal volume (w:v) of homogenizing medium (sucrose, 0.25 M; Tris-HCl, 0.05 M, pH 8.0; 2-mercaptoethanol, 0.01 M; MgCl₂, 0.001 M). Homogenization was performed for 30 seconds at medium speed followed by 45 seconds at high speed. The homogenate was filtered and squeezed through 4 layers of cheese cloth and finally filtered through a layer of miracloth. The filtrate was centrifuged in a Sorvall RC2-B Centrifuge for 30 minutes at 5,000 x g. The supernatant containing mitochondria and other smaller particles was carefully syringed off. The crude chromatin which formed a brownish, gelatinous layer over the underlying, firm starch layer was carefully scraped with a spatula and suspended in a dounce homogenizer containing 20 ml wash buffer (Tris-HCl, 0.01 M, pH 8.0; sucrose, 0.25 M; 2-mercaptoethanol, 0.01 M). This was again centrifuged for 10 minutes at 10,000 x g. The chromatin layer was again scraped from the pellet and suspended in wash buffer. The chromatin obtained thus far contains approximately 95 per cent of DNA but is contaminated by nonchromosomal protein. To remove the latter, the chromatin suspension was layered over 10 ml of 2 M sucrose (containing Tris-HCl, 0.01 M, pH 8.0; 2-mercaptoethanol, 0.01 M) in a 12.5 ml centrifuge tube and the top portion gently stirred, and centrifuged in a Beckman Ultracentrifuge, model L2-65B for 3 hours at 20,000 r.p.m. The supernatant was carefully
syringed off and the purified chromatin pellet was finally suspended in a dounce tissue homogenizer containing ( Tris-HCl, 0.01 M, pH 8.0; 2-mercaptoethanol, 0.01 M ). The only contaminant at this stage might be some particles of starch but these will not interfere with the chromatin activity during the assays. Aliquots of this chromatin suspension were used in assays for DNA polymerase activity.

Chromatin-bound DNA Polymerase Assay

The assay utilizes the principle that a radioactive ribonucleoside-5'-triphosphate can be incorporated in the presence of other three non-labelled ribonucleoside triphosphates into TCA acid-insoluble product. This can serve as a measure of DNA synthesis. Radioactivity which is not incorporated can be removed by repeated washings with cold TCA. The incorporated radioactivity is then counted in a liquid scintillation spectrometer.

Aliquots of chromatin extract each of 0.1 ml ( equivalent to 15 μg DNA ) were added to 0.3 ml of cold assay reaction mixture containing: Tris-HCl, pH 8.0, 10 μ moles; MgCl₂, 1.0 μ mole; 2-mercaptoethanol, 0.125 μ mole; ATP, 0.2 μ mole; CTP, 0.2 μ mole; GTP, 0.2 μ mole; unlabelled UTP, 0.005 μ mole; labelled ³H-UTP, 10 μCi ( specific activity 13 μCi/micromole ). A zero-time reaction was done by adding 4 ml of cold 10 per cent TCA containing 0.04 ml tetrasodium
pyrophosphate to the reaction mixture immediately after 0.1 ml chromatin had been added. The reaction tubes, in duplicates, were transferred to a water-bath maintained at 37° C. Reaction was started by the addition of chromatin suspension and stopped at the end of 20 minutes (25 minutes in time-course experiments) of incubation by adding 4 ml of cold TCA containing 0.04 M pyrophosphate.

To precipitate the RNA synthesized, the solution was allowed to stand at 0° C for 40 minutes. The resulting precipitate was transferred to a Whatman GF/A glass fiber filter disc and washed 4 times each with 5 ml cold 5 per cent TCA containing 0.02 M pyrophosphate. The filtered discs were then dried under infra-red lamps and put in scintillation liquid (Appendix 1). The radioactivity was counted in a Nuclear-Chicago Liquid Scintillation Spectrometer Mark II. The counting efficiency of the scintillation system was approximately 60 per cent for tritium-UTP.

Determination of DNA Template Availability

The chromatin DNA template availability was determined by using excess RNA polymerase and small amounts of chromatin as template to synthesize RNA. The method of assay and the reaction mixture used are the same as those employed in RNA polymerase activity assay, the only excep-
tion being a reduced amount of chromatin (0.4 - 0.6 μg DNA) and various amounts of *Escherichia coli* RNA polymerase (1 to 10 units) added in excess; units of enzyme are as described by Sigma Co., St. Louis, Mo. The reaction was carried out at 37°C for 20 minutes and stopped by adding 4 ml of cold 10 per cent TCA. The RNA precipitate was processed in the same way as in RNA polymerase activity assay.

**DNA Determination**

Chromatin-DNA was determined according to Giles and Myers (1965) which is a modified method of Burton (1956). The chromatin suspension was first hydrolyzed in 10 per cent perchloric acid at 70°C for 45 minutes. This is followed by the addition of 4 per cent diphenylamine reagent and incubating the DNA solution at 30°C for 18 hours. Optical density at 595 μm and 700 μm in a Beckman DB-G spectrophotometer (see Appendix 2). Calf thymus DNA was used as standard.
RESULTS

Effect of GA and AMO-1618 on Growth of Soybean Hypocotyls

The stimulative properties of gibberellic acid on the growth of many plants are well known and this gives credit to its being recognized as a plant growth hormone. AMO-1618 is commonly known as a plant growth retardant as it can "slow cell division and elongation in shoot tissues and regulate plant height physiologically without formative effects" (Cathey 1964).

In my preliminary experiments, gibberellic acid concentrations below $10^{-3}$ M appeared to promote the elongation of hypocotyls in dark-grown soybean seedlings. The effect of AMO-1618 on the growth of soybean hypocotyls is rather complicated. AMO-1618 at concentrations of $10^{-5}$ M or below appeared to enhance elongation of soybean hypocotyls but caused retardation at $10^{-3}$ M. Since GA ($10^{-3}$ M) enhances elongation of etiolated soybean hypocotyls while AMO-1618 ($10^{-3}$ M) retards, it is possible that these chemicals have similar effect on the fresh weight of the treated tissues. It should be pointed out, that the action of AMO-1618 is highly specific in inhibiting shoot elongation, but it does not affect the number of nodes and weight of leaves (Cathey 1964); therefore
FIGURE 1

Elongation of hypocotyl of intact soybean seedlings.

Each point is average of 15 hypocotyls. Curves labelled with GA, AMO, and H₂O represent seedlings sprayed with gibberellic acid (10⁻³ M), AMO-1618 (10⁻³ M), and water respectively 72 hours after sowing the seeds.
FIGURE 2

Increase in fresh weight of hypocotyls of intact soybean seedlings.

Each point is average of 15 hypocotyls. Curves labelled GA, AMO, and H₂O represent seedlings treated with gibberellic acid (10⁻³M), AMO-1618 (10⁻³M), and water respectively 72 hours after sowing the seeds.
any reduction in weight of treated plants appears to be a primary result of decrease in stem length. Data in Fig. 2 are in agreement with this statement. Previous work in this laboratory provided supporting evidence that AMO-1618 retards growth of soybean hypocotyls (Soteros 1970) and of pea seedlings (Tu 1970).

Fig. 1 indicates the expansion of dark-grown soybean hypocotyls treated with GA \(\times 10^{-3} \text{M}\), AMO-1618 \(\times 10^{-3} \text{M}\) and water respectively. Each point represents the average of 15 hypocotyls from treated and untreated plants. The time zero is taken to be the 72nd hour after sowing seeds. It is obvious that, at the concentrations used, GA caused elongation of hypocotyls over a period 108 hours after treatment while AMO-1618 retarded their growth during the same period of time. Results in Fig. 2 show the effect of GA on increase in fresh weight of GA-treated hypocotyls and decrease in AMO-1618 treated hypocotyls compared with untreated plants.

It is clear that GA promoted growth of etiolated soybean seedlings while AMO-1618 \(\times 10^{-3} \text{M}\) retarded it. The curves level off beyond the 96th hour; that is, when the dark-grown hypocotyls were fully elongated.
TABLE 1

Induction of chromatin-RNA polymerase activity by treatments of GA and AMO-1618.

<table>
<thead>
<tr>
<th>Time in hours</th>
<th>Chromatin from ( H_2O )-treated seedlings</th>
<th>Chromatin from AMO-1618 treated seedlings</th>
<th>Chromatin from GA-treated seedlings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \mu ) moles UMP*</td>
<td>( \mu ) moles UMP*</td>
<td>( \mu ) moles UMP*</td>
</tr>
<tr>
<td>0</td>
<td>15.36</td>
<td>--</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>15.70</td>
<td>14.60</td>
<td>93</td>
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<tr>
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<td>18.56</td>
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<td>14.10</td>
<td>84</td>
</tr>
<tr>
<td>48</td>
<td>13.76</td>
<td>13.62</td>
<td>98</td>
</tr>
</tbody>
</table>

\* per 100 \( \mu \)g DNA
Effect of GA and AMO-1618 on Chromatin-directed RNA Synthesis Over a 48-hour Period

The purpose of these experiments was to determine whether GA and AMO-1618 affect the chromatin-directed RNA synthesis over a period of 48 hours after treatment. Chromatin was isolated at different times following treatment with GA or AMO-1618. Chromatin-RNA polymerase activity was assayed by measuring the incorporation of μμ moles of 3H-UMP per 100 μg DNA. Results presented in Table 1 indicate that RNA polymerase activity was enhanced in GA-treated hypocotyls but reduced in AMO-1618 treated samples. This GA-induced increase in RNA synthesis occurred after 18 hours of treatment but the synthesis declined 36 hours after treatment. Similarly, RNA synthesis brought about by AMO-1618 was reduced during the first 18 hours and therefore increased up to 48 hours.

Reaction Kinetics of Chromatin-RNA Polymerase

McComb et al. (1970) in a recent report indicated that GA markedly enhances the RNA-synthesizing ability of chromatin-RNA polymerase of treated pea seedlings over that of control. They also provided evidence that the maximum time required for the enzyme to complete synthesis of RNA in vitro was about 15 minutes.
FIGURE 3

Reaction kinetics of chromatin–RNA polymerase activity.

The curves represent RNA synthesis as a function of time by chromatin-bound RNA polymerase isolated from AMO-treated (upper curve), from GA-treated (middle curve), and from untreated hypocotyls (lower curve).
FIGURE 3

$\mu$moles UMP incorporated per 100 $\mu$g DNA vs. incubation time (minutes)

- AMO
- GA
- H$_2$O
The results of a time-course experiment in Fig. 3 indicate that maximum incorporation of $^3$H-UMP was achieved at about 20 minutes of incubation. It can be seen that the three curves (GA, AMO-1618, and water) show a similar pattern as they all level off beyond 15 minutes of incubation. Chromatin isolated from GA and AMO-1618 treated soybean seedlings 18 hours after treatment showed a marked increase in RNA synthesis over control. AMO-1618 induced enhancement of RNA polymerase activity in this experiment may be due to insufficient amounts of chemical available to check RNA-synthesizing ability of the hypocotyls in a single spray of 10 ml ($10^{-3}$ M) per tray of size 36 x 30 cm, whereas previous results of Soteros (1970) and Tu (1970) have shown that AMO-1618 treatments caused considerable decrease in RNA synthesis in soybean and pea seedlings. This observation could be due to the fact that plants grown in these experiments were subjected to longer and continuous treatment of retarding chemical.

**Requirements for the Chromatin-RNA Polymerase Reaction**

When assayed separately, the optimal concentration of $Mg^{++}$ ions for the control polymerase activity was found to be 8 mM and that of $Mn^{++}$ ions was about 2 mM. But when
added together in the assay reaction mixture, the optimal concentrations of Mg² and Mn² ions were found, for maximum enzyme activity, to be 2.5 mM and 0.63 mM respectively (Duda and Cherry 1971).

Table 2 shows, that omission of Mn² ions decreased enzyme activity by about 90 per cent in control and 20 per cent in GA-treated hypocotyls. When Mg² ions were absent from the assay mixture, the polymerase activity was decreased by about 44 per cent in control, 68 per cent in AMO-1618 treated and 35 per cent in GA-treated seedlings. The chromatin-ENA polymerase activity was dependent on the presence of four ribonucleoside triphosphates, namely, ATP, GTP, CTP, and UTP. (Table 2). Omission of NTP (that is, ATP, GTP and CTP) decreased the enzyme activity by 12 per cent. 2-mercaptoethanol was included in the reaction mixture. Its complete exclusion from the assay mixture resulted in a 50 per cent loss in enzyme activity. This observation is in agreement with the findings of Chamberlin and Berg (1962) in the E. coli system. It is clear that 2-mercaptoethanol as well as other sulphydryl compounds (Chamberlin and Berg 1962; Cleland 1964) are capable of preventing the inactivation of ENA polymerase in solution and are thus required in extraction media as well as in reaction mixture.
TABLE 2

Requirements for chromatin-directed RNA synthesis. Treatments of GA and AKO-1618 each of $10^{-3} \mu M$ were applied 24 hours prior to isolation of chromatin.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>PERCENT INCORPORATION OF UMP</th>
</tr>
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<tr>
<td></td>
<td>Chromatin from $H_2O$-treated seedlings</td>
</tr>
<tr>
<td>Complete</td>
<td>100</td>
</tr>
<tr>
<td>-Mg</td>
<td>57.77</td>
</tr>
<tr>
<td>-Mn</td>
<td>11.12</td>
</tr>
<tr>
<td>NTP</td>
<td>83.16</td>
</tr>
<tr>
<td>UTP</td>
<td>84.69</td>
</tr>
<tr>
<td>-2-mercapto ethanol</td>
<td>58.02</td>
</tr>
</tbody>
</table>

Incorporations for chromatin from untreated, AKO-1618 and treated seedlings were 17.32, 11.08, and 32.90 $\mu$moles of UMP per 100 $\mu$g DNA respectively.
The optimal pH for the reaction was about 7.9 - 8.0 while the optimal temperature was 35° - 37° C. There was practically no incorporation of ³²H-UMP at 0° C.

Other Properties of Chromatin-RNA Polymerase

The purpose of these experiments was to determine whether or not RNA synthesis directed by chromatin and its bound RNA-polymerase is inhibited in vitro by various additives such as actinomycin D, pyrophosphate, and inorganic phosphate.

A) Effect of Actinomycin D

Actinomycin D is an antibiotic which is generally well recognized as a potent inhibitor of DNA-dependent RNA synthesis. Two different concentrations (0.2 µg/ml and 2.0 µg/ml) of this chemical were added in vitro to the treated and control chromatin preparations in complete assay mixtures. Table 3 indicates that actinomycin D inhibited control chromatin-RNA polymerase activity by 59 per cent and 71 per cent at a higher concentration (2.0 µg/ml) and a lower concentration (0.2 µg/ml) respectively and that chromatin-directed RNA synthesis is minimal. Actinomycin D concentration at
TABLE 3

Effect of various additives on chromatin–RNA polymerase activity. Chromatin was isolated from treated and untreated seedlings 24 hours after treatment.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>PERCENT INCORPORATION OF UMP.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chromatin from H₂O-treated seedlings</td>
</tr>
<tr>
<td>Complete</td>
<td>100</td>
</tr>
<tr>
<td>+ Phosphate</td>
<td></td>
</tr>
<tr>
<td>5μM</td>
<td>95.88</td>
</tr>
<tr>
<td>10μM</td>
<td>89.53</td>
</tr>
<tr>
<td>+ Pyrophosphate</td>
<td></td>
</tr>
<tr>
<td>5μM</td>
<td>15.72</td>
</tr>
<tr>
<td>10μM</td>
<td>11.01</td>
</tr>
<tr>
<td>+ RNAase (100μg)</td>
<td></td>
</tr>
<tr>
<td>17.11</td>
<td>43.76</td>
</tr>
<tr>
<td>+ Actinomycin D</td>
<td></td>
</tr>
<tr>
<td>0.2μg/ml</td>
<td>41.48</td>
</tr>
<tr>
<td>2.0μg/ml</td>
<td>28.95</td>
</tr>
</tbody>
</table>

Incorporations for chromatin from untreated, AMO-1618 and GA-treated seedlings were 17.90, 13.20 and 35.40 μm moles of UMP per 100 μg DNA respectively.
2.0 µg/ml inhibited the AMO-1618 and GA treated chromatin by 78 per cent and 41 per cent respectively.

B) Effect of Pyrophosphate

The end product beside RNAs, of the reaction for the chromatin-directed RNA synthesis is pyrophosphate. The overall reaction is found to be reversible (Mahler and Cordes 1968). Therefore an increase in concentration of pyrophosphate in substrates is expected to shift the direction of reaction in a reverse manner, thus suppressing the formation of RNAs. The data of Table 3 give supporting evidence to show that a concentration of 5 µM and 10 µM of pyrophosphate severely inhibited control enzyme activity by 85 per cent and 89 per cent respectively. The AMO-1618 treated chromatin was also highly inhibited (72 per cent) by pyrophosphate present in assay mixture. It is of interest to note that pyrophosphate appeared to have no specific effect on GA-treated chromatin.

C) Effect of Inorganic Phosphate

At least two enzymes capable of synthesizing
RNA are known, and these are RNA polymerase and polynucleotide phosphorylase. The latter enzyme was discovered much earlier by Grunberg-Manago and Ochoa (1955) who isolated it from extracts of *Azotobacter vinelandii*. This enzyme can catalyze ribonucleoside diphosphates to form RNA and orthophosphate, and the reaction is readily reversible so that in the presence of inorganic phosphate the enzyme will degrade RNA.

A parallel experiment using inorganic phosphate in the assay mixture was run to test the effect of phosphate on the chromatin-bound RNA polymerase activity. It was observed that inorganic phosphate ions had no specific inhibitory or promotive effect on the activity of chromatin-RNA polymerase from treated or untreated plants (Table 3).

D) **Effect of Ribonuclease**

Ribonuclease is specific in degrading RNA into ribonucleotides by hydrolytic reactions. Therefore, inclusion of ribonuclease in the assay mixture can serve as a tool to provide some information whether the product formed during the reaction is RNA or not, when
compared with assay mixture without any ribonuclease. A reduction in incorporation in assay mixture with ribonuclease may indicate that the RNA product being formed was degraded.

Experiments were done in which a concentration of 100 µg of pancreatic ribonuclease was added, in duplicates, to assay mixtures containing chromatin from GA and AMO-1618 treated and control plants. Results in Table 3 indicate that ribonuclease inhibited chromatin-bound RNA polymerase activity by 83 per cent in the control, over 56 per cent and 27 per cent in AMO-1618 and GA treatments respectively.

In vitro Effect of GA and AMO-1618 on Chromatin-bound RNA Polymerase

It was reported that growth retardants, CCC and phosfon-D each stimulated up to 50 per cent in vitro synthesis of polynucleotides by polynucleotide phosphorylase isolated from wheat root (Kessler and Chen 1964). However, the chromatin-bound RNA polymerase activity was reported to be neither enhanced nor inhibited in vitro by plant growth regulators, such as auxin (O'Brien et al. 1968a; Schwimmer 1968; Matthysse and Phillips 1969).
TABLE 4

The *in vitro* effect of GA and AMO-1618 on chromatin and its associated RNA polymerase.

<table>
<thead>
<tr>
<th>Additives</th>
<th>INCORPORATION OF UMP</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>moles UMP per 100μg DNA</td>
<td>percent of control</td>
<td></td>
</tr>
</tbody>
</table>

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>26.89</td>
<td>100</td>
</tr>
<tr>
<td>+ AMO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10⁻⁵M</td>
<td>27.65</td>
<td>102.8</td>
</tr>
<tr>
<td>+ GA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10⁻⁵M</td>
<td>28.96</td>
<td>107.7</td>
</tr>
</tbody>
</table>

GA and AMO-1618 were added *in vitro* to control chromatin in the assay mixture.
gibberellins (McComb et al. 1970; Duda and Cherry 1971).

Experiments were designed to determine whether or not GA and AMO-1618 each have any promotive or inhibitory effect on the in vitro RNA polymerase activity. AMO-1618 (10^{-5} \text{ M}) and GA (10^{-5} \text{ M}) were tested separately, and the results given in Table 4 indicate that these plant regulators had no effect on enzyme activity in vitro.

**Effect of Different Concentrations of GA and AMO-1618 on Chromatin-RNA Polymerase Activity**

Table 5 shows the in vivo effect of various concentrations of AMO-1618 and GA, either applied singly or in combination, on the induction of chromatin-directed RNA synthesis.

When applied singly to soybean seedlings, GA at various concentrations appeared to promote chromatin-RNA polymerase activity; AMO-1618 concentrations at 10^{-5} \text{ M} and above tended to inhibit the enzyme activity but at lower concentration (10^{-7} \text{ M}) enhanced its activity.

When applied in combinations AMO-1618 and GA both of 10^{-5} \text{ M} and 10^{-7} \text{ M} act as though they were AMO-1618 of 10^{-5} \text{ M} and 10^{-7} \text{ M} respectively. However, their combined concentration of 10^{-3} \text{ M} somewhat mimicked the effect of GA at 10^{-3} \text{ M}. It appears that at lower combined concentrations (10^{-5} \text{ M} and 10^{-7} \text{ M}) AMO-1618 rather than GA is the controlling factor whereas at 10^{-3} \text{ M}
TABLE 5

Effect of GA, AMC-1618, and AMC-1618 + GA treatments on chromatin-bound RNA polymerase activity.

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Chromatin-RNA Polymerase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GA</td>
</tr>
<tr>
<td>0</td>
<td>100**</td>
</tr>
<tr>
<td>$10^{-7} M$</td>
<td>393.9</td>
</tr>
<tr>
<td>$10^{-5} M$</td>
<td>195.4</td>
</tr>
<tr>
<td>$10^{-3} M$</td>
<td>198.9</td>
</tr>
</tbody>
</table>

* Seedlings harvested 18 hour after treatment with various concentrations as indicated in the table.

** 100 percent represents incorporation of 18.56 μmole per 100 μg DNA.

*** Equimolar concentrations of AMC-1618 and GA were used.
the reverse is true for GA.

This finding is consistent with the data of Soteros (1970). He also demonstrated that a combined concentrations of AMO-1618 (10^{-4} M) and GA (10^{-4} M) promoted an increased synthesis of nucleic acid in soybean hypocotyls. The high concentration of GA might possibly compete effectively and reduce greatly the inhibitory action of AMO-1618, thus increasing RNA polymerase activity.

**Determination of Chromatin-DNA Template Availability**

To determine the RNA template availability, the chromatin-DNA from treated and untreated hypocotyls were separately saturated at the same levels of exogenous *E. coli* RNA polymerase. The assay method was the same as that for endogenous chromatin-RNA polymerase, except that the amount of chromatin-DNA used per reaction tube was reduced to about 0.4 - 0.6 μg. The results in Fig. 4 indicate neither an increase nor decrease in template availability due to treatments of GA or AMO-1618.

**Relation Between RNA Polymerase Activity Induced by GA and AMO-1618, and Changes in Growth Rate**

As pointed out earlier, it appears that the morphological and physiological changes of hormone-treated plant
FIGURE 4

Saturation of chromatin—DNA from treated and untreated soybean hypocotyls with exogenous *E. coli* RNA polymerase.
tissues occur following hormone-induced changes in RNA synthesis (Silberger and Skoog 1953; O'Brien et al. 1968a; McComb et al. 1970). Fig. 3 shows that chromatin isolated from GA-treated hypocotyls 18 hours after treatment exhibits a marked increase in RNA synthesis over the control. Further, from Fig. 1 it can be seen that the promotive and inhibitory effect of GA and AMO-1618 respectively on elongation of soybean hypocotyls occurs as early as the 6th hour after treatment and reaches a maximum at the 78th hour.

Fig. 5 shows the relation between RNA polymerase activity induced by GA and AMO-1618 and changes in growth rate. This graph was derived from data of Fig. 1 and Table 1. The percent RNA polymerase activity for GA and AMO-1618 was calculated for the respective increase (or decrease) over control at various time intervals (Table 1); percent growth rate was obtained by calculating growth rate (cm/hour) for different points along the curves in Fig. 1 and expressed in terms of control. Thus the chemical-induced RNA polymerase activity and the growth rate can be related. Maximum increase or decrease in RNA synthesis induced by GA or AMO-1618 respectively precedes the maximum increase or decrease in growth rate.

The maximum increase in chromatin-RNA polymerase activity induced by GA treatment occurred 18 hours after
treatment, and gradually decreased with increasing time. This finding is in agreement with the data of various workers (Jarvis et al. 1968; Key 1969). Chrispeels and Varner (1967) related the decrease in RNA synthesis after the initial 24 hour treatment to GA-regulated increase in RNAase activity in intact plants. It seems also logical to assume that one spray of GA solution applied to hypocotyls could only induce a certain amount of RNA synthesis, which was not sufficient enough to maintain RNA synthesis for continued growth.

Fig. 5 indicates that the maximum decrease in RNA polymerase activity brought about by AKO-1618 was also at the 18th hour. The enzyme activity gradually approached the control value after 24 hours of treatment. It may be concluded that one spray of AKO-1618 was not sufficient enough to check RNA synthesis, thus allowing the growth of the hypocotyls.
FIGURE 5

The Comparison Between the Percent Increase in RNA polymerase Activity induced by GA (X--X) and AHO-1618 (+--+) respectively, and Changes in Growth Rate for GA (×---×) and for AHO-1618 (⊕-----⊕).

Data for these curves are obtained from Fig. 1 and Table 1.
DISCUSSION

Assay method employed in the present work involves incorporation of ribonucleoside triphosphates into acid-insoluble products, which demonstrates the existence of RNA polymerase activity in chromatin of intact soybean hypocotyls. Similar enzyme activity was found in the chromatin of 2,4-D treated soybean hypocotyls (O'Brien et al. 1968a). These workers suggested that RNA polymerase appears to be bound in the chromatin of soybean seedlings and that no soluble RNA polymerase could be detected. However, soluble RNA polymerase was found in the corn seedling (Stout and Mans 1967) and in sugar-beet root (Duda and Cherry 1971).

The chromatin-bound RNA polymerase in soybean hypocotyls exhibited certain characteristics commonly shared by partially purified RNA polymerase of Escherichia coli (Chamberlin and Berg 1962), rat liver (Weiss and Gladstone 1959), sugar-beet root (Duda and Cherry 1971) and maize (Stout and Mans 1967). These characteristics include:

(1) that the enzyme is specific for all the four ribonucleoside triphosphate substrates, namely ATP, GTP, CTP, and UTP. Its dependence on
the substrates can be visualized from the
reduction in incorporation brought about by
omission of one or more substrates from the
assay mixture.

(2) that the enzyme requires for optimal activity
a divalent metal ion at low concentrations.

It is demonstrated that magnesium or manganese
ions are required.

At concentrations above $0.5 \times 10^{-3}$ M for
Mn$^{++}$ and above $2.0 \times 10^{-3}$ M for Mg$^{++}$, both
ions are inhibitory. Omission of either ion
caused a reduction in the RNA-polymerase
activity (Table 2).

(3) that the enzyme is temperature-dependent.

Optimal temperature is about $35^\circ - 37^\circ$ C.
Little or practically no enzyme activity is
observed at $0^\circ$ C.

(4) that the reaction is optimal at pH 7.8 - 8.0.

(5) that the activity of chromatin-bound RNA
polymerase is sensitive to inhibitors such
as actinomycin D, pyrophosphate, deoxyribonuclease
and ribonuclease. Presence of ribonuclease in reaction mixture inhibits RNA
synthesis by degrading the RNA product.
Deoxyribonuclease degrades chromatin-DNA, thus
destroying the template and preventing RNA synthesis.

The end products of chromatin-directed RNA synthesis are RNA and pyrophosphate. Inclusion of inorganic pyrophosphate in the assay mixture inhibits RNA synthesis, whereas inorganic phosphate exerts little or practically no effect (Table 3).

Actinomycin D is specific in inhibiting DNA-dependent RNA synthesis. Its binding to DNA is responsible for its interference with DNA function and its selective inhibition of DNA-directed RNA synthesis in both eukaryotic and prokaryotic organisms (Goldberg and Friedman 1971; Hurwitz et al. 1962; Maitra et al. 1967; Hyman and Davidson 1970). The inhibition may be partially restored by adding excess DNA but not adding RNA polymerase (Hurwitz et al. 1962). It is reported that actinomycin D also inhibits DNA synthesis in intact cells (Goldberg and Friedman 1971) or DNA synthesis by isolated chromatin–DNA polymerase (Leffler et al. 1971) but a much higher concentration of the antibiotic is required for this inhibition.
(6) that the product of the chromatin-directed RNA synthesis is a heteropolymer of the four ribonucleoside triphosphate substrates (Duda and Cherry 1971) which can be detected by the radioactive $^3$H-UTP.

Reaction kinetics for chromatin-directed RNA synthesis from treated and untreated soybean hypocotyls exhibits a similar pattern previously described for the same system (O'Brien et al. 1968a), or of other systems (Duda and Cherry 1971; Jarvis et al. 1968). Maximum incorporation of radioactive label ($^3$H-UTP) is completed in 15 minutes following which the curves level off (Fig. 3).

As has been pointed out above, plant growth regulators have a dramatic influence on RNA synthesis in a variety of plant systems (Trewavas 1968; Key 1969). In the present study GA is shown to enhance RNA synthesis by the chromatin-bound RNA polymerase of the soybean hypocotyl, while the plant growth retardant, AMO-1618, inhibits the enzyme activity (Table 1). It should be pointed out that the scarcity of current literature demonstrating effects of plant growth retardants on RNA polymerase activity makes it more difficult to compare experimental results.

While a few reports exist on induction of RNA polymerase activity by plant hormones, little is known about
the mechanisms of hormone-induced stimulation of RNA synthesis in plants. Increase in RNA content in intact plants in response to hormonal action may be due either to increased rate of synthesis or to decreased rate of degradation. In the case of isolated nuclei or chromatin, the increase in rate of RNA synthesis can be ascribed to an induction (or activation) of chromatin-RNA polymerase or to an increase in the sites of available chromatin-DNA templates, or to both.

To solve this problem it is ideal to assay the completely purified RNA polymerase with known amount of an exogenous standard DNA primer, and also in separate experiments, the purified DNA-template with an exogenous RNA polymerase. Thus a direct estimate of RNA polymerase per unit of chromatin-DNA could be possible. It follows that in response to chemical treatment any increase (or decrease) in DNA template availability or in the amount of RNA polymerase might be ascertained. Unfortunately, such a direct estimate cannot be done at present because of unsuccessful attempts to obtain completely purified RNA polymerase from chromatin of plant tissues. Nevertheless, it is possible to solve the problem by an indirect method. Saturation of the chromatin and its associated RNA polymerase with exogenous E. coli RNA polymerase permits some estimation of the chromatin template efficiency.
Preliminary study of DNA template in this chromatin system (Fig. 4) indicates no increase in template availability within 24 hours of chemical treatment. This is in agreement with the findings of O'Brien et al. (1968), Johnson and Purves (1970), and McComb et al. (1970). It is interesting to point out here that Jarvis et al. (1968) observed a drastic increase in template availability in hazel seeds during gibberellin-induced breaking of dormancy prior to any increase in RNA polymerase activity. However, Johnson and Purves (1970) found the reverse situation, i.e., increases in RNA polymerase activity preceding smaller increases in template availability. This and other reasons lead them to suggest that in cucumber system the initial mechanism of hormone-stimulation of RNA synthesis involves RNA polymerase and that increase in RNA template availability in response to hormonal action is probably secondary.

Obviously, information available to date is still not sufficient to answer the question whether plant hormones or retardants cause increase or decrease in RNA polymerase, template availability, or a combination or both. Further work and improvement in techniques is necessary. Holm and Key (1971) point out, "RNA polymerase from control and (hormone)-treated tissue could be isolated separately from the chromatin and assayed with a DNA template", which would then give us some conclusive evidence.
SUMMARY

Gibberellic acid brings about an increase in the RNA content of elongating hypocotyls from dark-grown, intact soybean (Glycine max L. var. Wayne) seedlings. On the other hand, the plant growth retardant, AMO-1618, causes a decrease in the RNA content of the retarded hypocotyls of the same plant system.

At the concentrations tested, GA enhances the chromatin RNA polymerase activity while AMO-1618 inhibits it. This enzyme activity requires a divalent metal ion (Mg\textsuperscript{++} or Mn\textsuperscript{++}) and all four ribonucleoside triphosphates for activity. The \textit{in vitro} RNA synthesis by chromatin RNA polymerase is inhibited by the presence of actinomycin D, or pyrophosphate in the assay mixture. The acid-insoluble product of the assay reaction is degraded by addition of ribonuclease.

Chromatin RNA polymerase from treated or untreated seedlings exhibits no response to either GA or AMO-1618 added \textit{in vitro}. Saturation experiments with \textit{Escherichia coli} RNA polymerase indicate that no observable increase or decrease in DNA template availability is detected within 24 hours of chemical treatments. It may be concluded that the \textit{in vivo} enhanced or decreased chromatin RNA polymerase activity in response to treatment of GA or AMO-1618 is probably due to enhanced or decreased synthesis of RNA polymerase.
APPENDIX 1

The scintillation liquid used for counting dried samples on glass fiber filter discs is composed of toluene, PPO (2,5-diphenyloxazole) and POPOP (1,4-bis[2-(5-phenyloxazolyl)] benzene) in the following composition: 1000 ml, 4 gm, and 50 mg respectively.
APPENDIX 2

Determination of Chromatin-DNA Content

The procedures for estimation of chromatin-DNA content are as follows:

(1) acid hydrolysis of the chromatin-DNA, and

(2) colour reaction of the deoxyribose sugars of hydrolyzed DNA with diphenylamine reagent -- the intensity of the resultant blue colour can be detected by a spectrophotometer.

A blank solution containing no DNA, and standard purified DNA solutions of varying concentrations are required for comparison with test solutions. The blank solution is needed to calibrate the spectrophotometer, serving as the zero readings at 595 µm and 700 µm. The standard DNA solutions serve as standard from which the DNA content of sample solutions can be calculated.

Two diphenylamine methods for estimating DNA content, namely Burton's method (1956) and Giles and Myers' method (1965), were first used. In fact, the latter method is a modified method of the former, which was used in this study. Giles and Myers' method has at least one
improvement over the method of Burton, i.e., increase in sensitivity of the method, being brought about by the reduction in colour of blank solution and use of 4 per cent diphenylamine reagent. This increased sensitivity is very useful in estimating the usually low level of DNA in many plant tissues. During incubation the impurities in DNA extracts precipitated and caused some cloudiness in solution. This may contribute some error in the data obtained from measurement at one wavelength (595 mp), but this error can be eliminated by measuring the 595-700 mp optical density difference. This difference gives a zero blank and a straight line calibration (Giles and Myers 1965).

Because of these advantages, the diphenylamine method of Giles and Myers (1965) was used throughout this thesis.

Method

Chromatin suspension was first incubated in 10 per cent perchloric acid in a water-bath maintained at 70°C for 45 minutes. Duplicates of blank solutions containing 10 per cent perchloric acid and of standard DNA (calf thymus DNA) solution of known concentrations (5 µg/ml, 10 µg/ml, 20 µg/ml, 40 µg/ml) in 10 per cent perchloric acid were incubated simultaneously to serve as standard.
During incubation chromatin-DNA was hydrolyzed to expose deoxyribose sugars which, upon addition of diphenylamine, react to yield a blue colour. At the end of the acid hydrolysis, 2 ml of 4 per cent diphenylamine in glacial acetic acid was added to 2 ml of hydrolyzed DNA solution in 10 per cent perchloric acid. This was followed by the addition of 0.1 ml of aqueous acetaldehyde solution containing 1.6 mg/ml. Same quantities of diphenylamine and acetaldehyde solutions were also added to each blank solution. Finally, they were incubated at 30°C for 18 hours. At the end of incubation, optical density of test and standard solutions were read at 595-700 μ.
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