Title: QUALITATIVE CHANGES IN CYTOPLASMIC PROTEINS IN PLANTS TREATED WITH PLANT GROWTH REGULATING CHEMICALS

Abstract approved: Redacted for Privacy

This thesis concerns the alteration of the course of selective protein synthesis and morphogenesis in plants treated with growth regulating chemicals. Treatment of 48-hour-old "Alaska" pea seedlings (Pisum sativum L.) with 2, 4-D, IAA, NAA or picloram caused inhibition of epicotyl and primary root elongation and proliferation of massed lateral roots. Squash (Cucurbita maxima L.) and corn (Zea mays L.) seedlings also produced abnormal lateral roots after 2, 4-D treatment.

The lateral roots induced by 2, 4-D in pea seedlings were initiated in the pericycle opposite all xylem poles over the length of the root between 6 and 9 hours after treatment. Lateral roots in control pea seedlings were also initiated in the pericycle but at a later time and were restricted to widely separated centers opposite a single xylem pole at a given point in the root.
The soluble cytoplasmic proteins of plant root sections were fractionated by anionic gel electrophoresis in 10% acrylamide gels. Some proteins were common to all sections of pea roots but others varied quantitatively with position in the root. A few proteins were found only in certain portions of the root and may be associated with root development. Proteins with an Rf of 0.41 (band 0.41) were prominent members of the latter group. Corn and squash seedlings gave similar results including the prominent changes in the intensity of band 0.41. However, the electrophoretic patterns were markedly different among species.

The electrophoretic patterns of proteins from roots of 2, 4-D-treated corn and squash seedlings and from roots of 2, 4-D-, IAA-, NAA- or picloram-treated pea seedlings differed quantitatively and qualitatively from patterns associated with untreated seedlings. Band 0.41 appeared in all sections and increased in intensity with distance from the root tip and time after treatment. In pea seedlings, band 0.41 was visible 12 hours after treatment with 2, 4-D. Pea seedlings preferentially incorporated alanine-C\(^{14}\) into proteins in band 0.41 between 6 and 12 hours after 2, 4-D treatment suggesting that synthesis of these proteins was initiated during this interval. The distribution of band 0.41 and the pattern and timing of lateral root development were clearly associated in primary root sections of control, 2, 6-D- and ethylene-treated pea seedlings.
The close temporal and spatial association of band 0.41 with the initiation of lateral roots suggests a cause and effect relationship between these events. The identity of the proteins in band 0.41 is unknown, and there is no assurance band 0.41 from different root sections and species contains the same proteins. A procedure is described for the isolation of the proteins in band 0.41 by preparative scale gel electrophoresis.
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Date thesis is presented

August 15, 1969

Typed by Opal Grossnicklaus for Logan Allen Norris
I lovingly dedicate this thesis
to my wife, Betty.

She suffered the pain of the failures and
relished the joys of the triumphs with me. Her
unselfish support, patience and understanding
made the completion of this degree program
possible.
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QUALITATIVE CHANGES IN CYTOPLASMIC PROTEINS
IN PLANTS TREATED WITH GROWTH
REGULATING CHEMICALS

INTRODUCTION

This thesis is concerned with plants treated with growth regulating chemicals. The specific hypothesis is: applying growth regulating chemicals to plants results in alteration of the interdependent course of selective protein biosynthesis and morphogenesis.

This hypothesis is based on three assumptions. (1) Each cell has a protein complement which is characteristic of a particular function, stage of development, or environmental condition. (2) An alteration in expression of genetic information may be observed at the tissue level by a change in morphology or function. (3) An alteration in expression of genetic information is manifested at the cellular level by changes in the protein complement.

The scope of these studies is restricted to the chemicals 2, 4-dichlorophenoxyacetic acid (2, 4-D), indole-3-acetic acid, (IAA), α-napthaleneacetic acid (NAA), 4-amino-3, 5, 6-trichloropicolinic acid (picloram), ethylene and 2, 6-dichlorophenoxyacetic acid (2, 6-D). Treatments were applied to peas (Pisum sativum L., var. Alaska), corn (Zea mays L., var. Tendermost), and squash (Cucurbita maxima L., var. warted Hubbard). Morphological observations were made on whole plants; anatomical examinations involved roots of
pea plants. Biochemical observations concerned soluble cytoplasmic proteins from roots of pea, corn, and squash seedlings.

My objectives were to: (1) investigate changes in the protein complement which accompany morphogenesis induced by growth regulating chemicals, and (2) determine whether changes in the protein complement were causally related to morphogenesis. These studies were undertaken to provide some insight into the impact of artificial growth regulating chemicals on the biochemistry of plants.

Biological differentiation is the acquisition of specialized structures or functions in the course of development. We do not now understand the mechanisms which control differentiation at the cellular level.

Differentiation is observed at all levels of organization. The development of chloroplasts from proplastids after exposure to light is differentiation at the subcellular level. Spore formation in Bacillus subtilis is an example of differentiation in a single cell; Guard cell formation in the epidermis of a leaf is differentiation at the tissue level. A better understanding of the mechanisms which control differentiation is important to plant physiologists, to those concerned with the biological impact of xenobiotics and to those studying some metabolic disturbances such as cancer in man.

Studies of differentiation in multicellular organisms concern the questions: (1) How can a single cell, the fertilized egg, give
rise to a mature individual of many cell types organized into diverse tissues each with its own specific functions? and (2) In the face of this diversity, how can the basic biochemistry of these cells remain the same?

The constancy of the basic biochemistry of these cells is understandable based on our knowledge of the genetic control of enzyme synthesis, the classical chromosome theory of heredity and the mechanics of somatic cell division. All somatic cells of a given individual are the same genotype, as illustrated by the phenomenon, totipotency, i.e., the capacity of a single somatic cell to give rise to a complete individual. Zeevaart (1965, p. 145) gives an impressive list of examples of regeneration of complete plants from one or a few somatic cells. A fundamental question is: How is the genotype in a totipotent cell modulated to give a particular phenotype?

In the macroscopic world, the phenotype of an organism has traditionally been considered the result of an interaction between genotype and environment. This relationship is true for the expression of genotype at the cellular level as well. However, the environment of the individual cell is composed of not only the macroscopic environmental factors but also of certain stimuli such as metabolites, hormones, or bioelectric potentials from neighboring cells and possibly foreign stimuli like artificial growth regulating chemicals. A change in the cell environment may result in a change of phenotype.
of that cell just as a change in the organism's environment may result in a change in its phenotype.

Differentiation on the cellular level might be defined as the preferential expression of a portion of the total genome. Haemmerling's (1963) work with the giant unicellular alga *Acetabularia* illustrates both the residence of the genome in the nucleus and the preferential expression of part of this genome leading to a distinct morphological event. This alga consists of a rhizoidal part, a stalk (with the nucleus at the base) and a cap (all part of one cell). The morphology of the cap is species specific. The stalk and cap are regenerated if they are removed from the base. If the nucleus of one species of *Acetabularia* is implanted in the enucleated base of another species, a stalk and cap are regenerated with the morphology of the species from which the nucleus was taken. The interaction of cytoplasm with the implanted nucleus permits expression of that portion of the genome concerned with regeneration of the stalk and cap (but not the rhizoid part). However, the particular genotype expressed is that of the nuclear donor.

An equally impressive example from the laboratory of Gurdon (1968) illustrates selective expression of genetic information. A nucleus from an epithelial cell from the intestine of *Xenopus laevis* (frog) was implanted in an enucleated unfertilized egg of the same species. This pseudofertilized egg was cultured to a completely
normal adult frog. This illustrates totipotency of a type, but more important for our discussion, exposure of the epithelial nucleus to the environment of the cytoplasm of the egg resulted in a new phenotype.

The one cistron-one polypeptide (one gene-one enzyme) theory suggests genetic information is expressed through a synthesis of proteins. But most of the genetic information of a given cell is not expressed at any one time, so most of the genes are not expressed in synthesis of specific proteins. Control of the availability of genetic information for protein synthesis is basic to the control of differentiation.

The appearance of new macromolecules such as specific proteins may be regarded as differentiation at the subcellular level. Such biochemical differentiation may precede cellular differentiation leading to the formation of new structures or acquisition of new functions. How the production of new proteins ultimately results in new forms and functions is not fully understood, but logically this relationship between biochemical differentiation and morphogenesis should exist.

Skoog and Miller (1957) have shown the impact of auxin and cytokinin growth hormones on differentiation. However, attempts to link the action of growth regulating chemicals to changes in the expression of genetic information have been largely unsuccessful.
Really definitive experiments have not yet been designed for three reasons. First, the control mechanisms of protein synthesis are not completely understood. Increasing evidence suggests these mechanisms are considerably more complex in higher organisms than in bacteria. Further, it is difficult to demonstrate an obligatory relationship between the production of specific macromolecules and the evolution of specific forms or functions. Finally, most studies of the impact of growth regulating chemicals have concerned only general trends in nucleic acid and protein biosynthesis. The synthesis of one or more specific macromolecular species associated with differentiation has not been adequately investigated.
Gene Expression and the Control of Protein Synthesis

We understand the mechanisms by which the genetic information of chromosomal DNA is expressed in the formation of specific proteins. Most recent biochemistry texts contain an adequate treatment of the subject so I need only briefly outline the processes here.

The enzyme RNA polymerase catalyzes the synthesis of RNA using portions of the DNA molecule as a template to specify the sequence of ribonucleotides. Three general classes of RNA are produced in this manner, ribosomal RNA (r-RNA), transfer or soluble RNA (s-RNA), and messenger RNA (m-RNA). The r-RNA and s-RNA are synthesized over relatively small portions of the DNA molecule, while m-RNA is synthesized over a much larger portion.

The m-RNA serves as a template for the synthesis of specific proteins whose amino acid sequences are determined by the ribonucleotide sequence of the m-RNA, which in turn reflects the deoxyribonucleotide sequence of the DNA. Protein synthesis occurs on polyribosomes consisting of several ribosomes on an m-RNA molecule. The ribosomes are made of r-RNA and ribosomal protein. Amino acids are carried to the site of peptide bond formation by specific s-RNA species which interact with the m-RNA to insert the
amino acid at the correct point giving the m-RNA-dictated amino acid sequence.

There are only a few species of r-RNA. There are more than 20 s-RNA species since there are one or more distinct s-RNA species for each amino acid. There are many species of m-RNA. Each protein or group of proteins must have its own message; so it is clear why the majority of the DNA codes for m-RNA while relatively small amount of DNA codes for s- and r-RNA (White, Handler, and Smith, 1964, pages 584-587, 598-610).

Not all the genetic information in the genome of a given cell is expressed at any one time. The totipotency previously described, however, clearly illustrates the total genetic complement remains even in specialized cells. How is the genetic information modulated so that only selected portions of the genome reach expression in the synthesis of specific proteins?

Jacob and Monod (1963), in their classical work with bacteria, proposed that the synthesis of specific proteins is determined by the rate of synthesis of the m-RNA which codes for that protein. Control of protein synthesis at this level is called transcriptional control. According to Jacob and Monod, the genetic material (in bacteria) consist of structural genes which specify the primary structure of the protein, regulator genes which determine whether a structural gene is active or not and an operator gene which is linked to the
structural gene. The operator plus one or more structural genes constitute an operon or transcriptional unit.

The regulator gene produces an allosteric protein called the repressor which can exist in two conformations. In an inducible system, the repressor molecule may block the operator gene preventing transcription of the structural gene. However, when another chemical (the effector or inducer) such as a hormone, a metabolite, or a substrate is present the conformation of the repressor changes. It can no longer block the operator gene, and transcription of the structural gene can occur. In a repressible system, on the other hand, the operator gene is not blocked by the repressor alone. Repressor conformation changes in the presence of an effector however, permitting interaction with the operator gene preventing transcription.

This simple and highly specific control mechanism probably operates in bacteria (Jacob and Monod, 1963) and possibly in higher organisms (Varner, 1964). However, control of protein synthesis could occur at the translational level as well as the transcriptional level. Thus, even though a gene might be derepressed and a specific m-RNA synthesized, the message might not be translated and the coded protein not synthesized.

There are several points of possible translational control. For instance, the m-RNA synthesized on the DNA may not be released from the template without interaction with a release factor.
Stent (1964) has shown ribosomes are involved in the synthesis and release of m-RNA from the DNA template. Ribosomes plus a purified preparation of RNA polymerase failed to stimulate in vitro m-RNA synthesis indicating the need for another material, presumably protein (Shin and Moldave, 1966). Revel and Gros (1967) reported that a protein factor required for translation of natural m-RNA markedly stimulated m-RNA synthesis (transcription) in the presence of purified RNA polymerase, ribosomes, and T-4 DNA. Thus, transcription may be under the influence of a protein factor which also modulates translation.

Some specificity of translation is believed to reside in the s-RNA. Recent advances in column chromatography have revealed the existence of more than one s-RNA for some amino acids. As many as six distinct species of s-RNA are reported which accept only leucine (Anderson and Cherry, 1969).

Söll et al. (1966) and Caskey, Beaudet and Nirenberg (1968) reported some specificity in codon recognition among s-RNA species coding for a given amino acid. Vold and Sypherd (1968) observed changes in the ratios of certain amino acid-accepting species of s-RNA at different stages of wheat germination. These reports suggest specific s-RNA species coding for a given amino acid may be specifically required for synthesis of groups of proteins associated with certain stages of development.
Anderson and Cherry (1968) found six leucine accepting species of s-RNA in the hypocotyl and cotyledons of soybeans. But only four of these species in the hypocotyl were charged with leucine due to a lack of tissue specific leucyl-amino-acyl synthetases. It may appear there is not sufficient diversity conferred by few s-RNA species or synthetase enzymes to account for the specificity required for protein synthesis. However, if entire groups of proteins associated with particular stages of development are controlled by a specific s-RNA-codon recognition interaction, diversity need not be great. Furthermore, I am confident many more species of both s-RNA and synthetase enzyme will be resolved as the technology of separation improves.

Another potential point of translational control concerns the availability of the message for translation. Based on patterns of protein synthesis in sea urchin eggs, Spirin (1966) has postulated the existence of masked forms of RNA in the cytoplasm. In the presence of actinomycin-D, which precludes DNA dependent RNA synthesis (Hurwitz et al., 1962), cleavage to the blastula stage is attained implicating protein synthesis on preexisting m-RNA. Other experiments show unfertilized eggs contain m-RNA and the necessary protein synthesizing apparatus but are incapable of supporting protein synthesis unless an artificial template or message is provided (Spirin, 1966). These reports support the hypothesis that some m-RNA is not available for translation except under special
environmental conditions. The specificity inherent in the transcriptional control hypothesis of Jacob and Monod (1963) also resides in a masked m-RNA. The interaction of an effector substance such as a cell metabolite or hormone with a masking agent (probably a protein) could either be direct or involve an intermediate repressor-like molecule. This mechanism offers sufficient variability in structure and conformation to confer a high degree of specificity in the interaction.

The control of macromolecular synthesis in higher organisms obviously is quite complex. Some control of protein synthesis may reside at various levels from transcription to the final release of the polypeptide. But, I stress that whatever the mechanism controlling selective genome expression, the result is the same—synthesis of a specific complement of proteins.

Relation of Protein Synthesis to Morphogenesis

The presence of specific proteins may lead to differentiation at the cellular level and interaction among cells may lead to differentiation at the tissue level and ultimately to changes in structure or function. It seems reasonable that an obligatory relationship exists between cellular biochemistry and morphogenesis, but this concept is difficult to establish experimentally. Paul and Gilmour (1966) and Ursprung et al. (1968) have used DNA-RNA hybridization techniques
to show organ specific genome translation in rabbit and mouse tissues. The implication is that selectivity in partial genome translation may lead to specificity of protein synthesis related to organ structure or function. Some proteins are specifically associated with a particular tissue or cell type, for instance hemoglobin in erythrocytes, myosin in muscle, and tryosinase in melanocytes. Whether these proteins are a result of differentiation or a cause of it is unclear.

The type of experimental data needed to establish an obligatory relationship between biochemical differentiation and morphogenesis must include evidence that:

1. The protein is synthesized before morphogenesis occurs and

2. Deletion of the protein prevents morphogenesis.

Some pertinent evidence comes from studies of the development of the slime mold *Dictyostelium discoideum* by Sussman and associates (summarized by Sussman, 1966). *Dictyostelium* spores germinate into amoebae which feed on bacteria and increase exponentially in number. As the amoebae enter the stationary phase of growth, they form multicellular aggregates from which a finger-like projection called the pseudoplasmodium is organized. The pseudoplasmodium migrates over the substratum of cells eventually developing a fruiting body on the end of a stalk.
Several carbohydrates have been temporally associated with stages of Detyostelium development, but Sussman has concentrated on an acid mucopolysaccharide containing galactose, galactosamine, and galacturonic acid. Galactose is incorporated into this polysaccharide by the enzyme UDP-galactose polysaccharide-transferase. The first enzyme activity is detected in early pseudoplasmodia and the mucopolysaccharide is detected about one hour later.

There are two mutants which do not reach the morphogenetic stage where the polysaccharide normally appears. They exhibit no transferase activity and fail to synthesize the polysaccharide. Two other mutants develop to the migrating pseudoplasmodium stage, but develop no mature fruiting bodies. The enzyme appears in these mutants but is not released or destroyed, events which normally accompany the final stages of fruiting body development in the wild type. These results suggest the enzyme is closely associated with development of the pseudoplasmodium and fruiting body.

The appearance of the enzyme is actinomycin-D sensitive up to four hours before it actually appears. Treatment with actinomycin-D before this time suppressed appearance, suggesting de novo protein synthesis. These results indicate the cistrons coding for the enzyme are transcribed at least four hours before the message is translated and may offer additional evidence for the masked forms of m-RNA Spirin (1966) discussed.
These experiments with Dictyostelium satisfy the requirement that the protein appear before morphogenesis. However, the evidence is not conclusive that the protein leads to morphogenesis. Failure of the protein to appear in certain mutants could result from a lack of morphogenesis, rather than the converse.

The absolute dependence of morphogenesis on the synthesis of a specific protein could be shown by finding a morphologically deficient organism with a related point deletion. That is, the deletion of one cistron, and therefore one polypeptide, would result in deviation from normal morphogenesis.

**Biological Activity of Some Growth Regulating Chemicals**

The growth regulating properties of 2, 4-D, IAA, and NAA have been recognized for some time. Kiermayer (1963) has reviewed the effects of these compounds on cell division, elongation, and differentiation. It is sufficient to note that these chemicals are active in the common bioassays (Avena coleoptile curvature and straight growth tests, pea stem elongation, and pea split stem tests) for auxin activity. Picloram, 2, 6-D, and ethylene however, need additional consideration.

Picloram is particularly active as an herbicide and performs consistently as an auxin. Kefford and Caso (1966) reported it was stimulatory at low concentrations and inhibitory at high concentrations
to the growth of wheat and oat coleoptiles, pea stem internodes, and pea root tips. Picloram was stimulatory at $10^{-9}$ M, had no effect at $10^{-8}$ M and was inhibitory at $10^{-7}$ M in the pea root tip growth test.

Picloram also promoted extension of bean epicotyls at $5 \times 10^{-7}$ M and showed maximum activity in the soybean hypocotyl straight growth test at $10^{-5}$ M to $10^{-4}$ M (Eisenger, Morré, and Hess, 1966). Schranck (1968) noted $10^{-5}$ M to $10^{-4}$ M picloram stimulated elongation of oat coleoptiles but markedly repressed geotropic responses.

Based on the classical auxin bioassays, 2,6-D has little activity in comparison with potent auxins like NAA, IAA, and 2,4-D, but biological activity is relative. Bourke, Butts, and Fang (1962) reported 2,6-D inhibited the uptake of glucose by pea root tips as effectively as 2,4-D. Chkanikov, Kostina, and Gertsuskii (1966) indicated 2,4-D and 2,6-D in the range of $10^{-2}$ M to $10^{-5}$ M behaved exactly the same as inhibitors of cyclic photophosphorylation in beans. Finally, Osborne et al. (1955) showed 2,6-D was active in the Went pea curvature test, although 2,4-D was considerably more active.

Although 2,6-D certainly is not without biological activity, its activity as an auxin is in question. All authors claim the use of highly purified materials but low-level contamination of 2,6-D by a highly active auxin like 2,4-D seems likely.

ethylene has been reported to have a growth modifying effect on several plants. In addition to its association with fruit ripening
and leaf abscission, Burg and Burg (1966) reported ethylene inhibited pea stem elongation but stimulated lateral swelling. They also observed that $1 \times 10^{-6}$ M IAA stimulated ethylene production in pea stem sections and suggested inhibition of stem elongation by IAA results from stimulation of ethylene.

A large number of plants will evolve ethylene in response to treatment with a variety of growth regulating chemicals. For instance, IAA, NAA, or 2,4-D stimulated ethylene evolution from bean, corn, cotton, and sunflower tissues (Abeles, 1966) and from zebrina, coleus, tobacco, and coffee plants (Abeles and Rubenstein, 1964).

It may be tempting to suggest that stimulation of ethylene synthesis is the basis for the action of growth regulating chemicals, but recent research indicates differences in their mechanisms of action. For instance, ethylene inhibition of excised pea root sections was influenced by tissue age and solution composition; these factors had no major effect on IAA inhibition. In addition, conditions which caused a reduction in IAA-stimulated ethylene evolution failed to affect the inhibitory action of IAA on root elongation (Andreae et al., 1968).

Holm and Abeles (1968) felt the herbicidal action of 2,4-D was not ethylene mediated since plants exposed to 10% ethylene for 48 hours recovered in an ethylene free environment. They reported, however, that some of the 2,4-D-induced nucleic acid and protein
effects might be ethylene mediated since exposure of some plants to ethylene leads to increases in synthesis of DNA, RNA, and protein. Holm et al. (1968) reported 2, 4-D and ethylene evoked similar increases in RNA polymerase activity in soybean hypocotyls. These reports indicate ethylene is biologically active and is evolved from tissues treated with growth regulating chemicals. The importance of ethylene in auxin action is in doubt, however.

Mode of Action of Some Growth Regulating Chemicals

Numerous mechanisms of action have been proposed for growth regulating chemicals. These include alteration of membrane permeability, respiration rate, substrate distribution patterns and many others. These aspects of auxin action are reviewed by Van Overbeek (1966) and Galston and Davies (1969).

Penner and Ashton (1966) cite over 300 references dealing specifically with effects of the chlorophenoxy herbicides at the cellular level. They included main chapters dealing with the impact of these herbicides on plant processes involving carbohydrates, lipids, nitrogen metabolism, organic acids, ethylene, alkaloids, steroids, vitamins, pigments, mineral nutrition, water relations, endogenous auxins, nucleic acids, enzymes, respiration, and photosynthesis. Nearly every aspect of plant growth, development, and metabolism is influenced by the chlorophenoxy herbicides suggesting that
compounds like 2, 4-D either have no central mode of action or they affect a process basic to many metabolic and developmental activities.

Patterns of growth regulating chemical-induced nucleic acid and protein synthesis have been intensively investigated in recent years. Silberger and Skoog (1953) first reported auxin-induced nucleic acid increases which preceded increases in fresh weight of plants. In soybean hypocotyl, the maximum concentration of RNA was found just prior to initiation of cell proliferation in mature, normally non-meristematic tissue (Key and Hanson, 1961).

Chrispeels and Hanson (1962) felt that the real effect of 2, 4-D was exerted in the nucleus through DNA-directed RNA synthesis. In detached leaves, 2, 4-D maintained incorporation of leucine-\(^{14}\)C into protein while a marked decline occurred in untreated leaves (Osborne and Hallaway, 1964). IAA and NAA stimulated the incorporation of P\(^{32}\) and \(^{14}\)C-adenine into RNA of nuclei isolated from pea and mung seedlings and mature onion bulb scales (Maheshwari, Guha, and Gupta, 1966). These reports indicate a general effect of growth regulating chemicals on metabolic activities involving nucleic acids and proteins.

Metabolic inhibitors have been used to determine which steps in the sequence of expression of genetic information are important in auxin responses. RNA and protein synthesis are reported to be essential for cell elongation in soybean hypocotyls (Key, 1964) and
in pea stem sections (De Hertogh et al., 1965).

Stimulatory concentrations of 2, 4-D enhanced nucleotide-$\text{C}^{14}$ incorporation into RNA (primarily r-RNA) from elongating regions of excised soybean hypocotyls. Inhibitory concentrations decreased incorporation and Actinomycin-D inhibited auxin-stimulated synthesis of r-RNA (Key and Shannon, 1964). Pretreatment of pea stem tissue with actinomycin-D (two hours before auxin treatment) completely inhibited auxin-induced elongation. Pretreatment with auxin and then actinomycin-D (one hour later) resulted in no inhibition of elongation until two hours after treatment with inhibitor. The two-hour effective life of the actinomycin-D-sensitive material suggests it may be m-RNA (De Hertogh et al., 1965).

A similar dependence of auxin-stimulated extension growth on both RNA and protein synthesis are reported for soybean hypocotyls, maize mesocotyls, and oat coleoptiles (Coartney, Morfe, and Key, 1967) and artichoke tubers (Noodén, 1968). Several studies showed auxin-induced increases in nucleic acid and protein levels resulted from de novo synthesis and not retardation of normal degradative processes (Osborne and Halloway, 1964; Key, 1964; Roychoudhury and Sen, 1964; Key and Shannon, 1964; Noodén and Thimann, 1965, 1966; Trewavas, 1968).

Auxin-mediated increases in RNA and protein levels may seem quite significant in auxin action. However, they may have little
specific connection with processes of differentiation (Evans and Ray, 1969). Would similar results be obtained by varying certain environmental parameters like light, moisture, and nutrients to maximize growth potential? A general increased synthesis of RNA and protein following application of growth regulating chemicals could be a response to an increasing growth rate or metabolic activity. A change in direction of differentiation may require instead, changes in synthesis of specific RNA and proteins. The majority of studies of effects of growth regulating chemicals on nucleic acid and protein synthesis have not demonstrated changes in the complement of specific RNA or protein species.

If transcriptional control of protein synthesis is operative, chemical alteration of growth requires synthesis of specific new species of m-RNA, which cannot be detected directly with the present technology. Differential centrifugation indicated heavy incorporation of radioactive RNA precursors into the ribosomal fraction of soybean hypocotyls following treatment with 2, 4-D (Key and Shannon, 1964; Key and Lin, 1966). This distribution of label could result from incorporation of labeled r-RNA into polyribosomes or to a lesser extent, incorporation of labeled m-RNA and s-RNA into the protein synthesizing unit, or from some combination of the three.

Chromatography of RNA on columns of methylated albumin on kieselguhr (MAK) gives neither satisfactory resolution of RNA species
nor satisfactory nucleic acid recovery (Masuda and Tanimoto, 1967; Vanderhof and Key, 1968). Much of the research using MAK column chromatography needs critical evaluation.

Trewavas (1968) reported IAA-treated pea stem sections preferentially incorporated radioactive precursors into a species of RNA with a slow rate of turnover (probably r-RNA). Hybridization experiments between pea DNA and the labeled RNA from these plants also indicates synthesis of r-RNA with little incorporation of radioactivity into m-RNA (Trewavas, 1968).

Inhibition of auxin-induced growth by actinomycin-D is usually considered to indicate an obligatory requirement for RNA synthesis in the growth response. Roychoudhury, Datta and Sen (1965) reported actinomycin-D inhibited auxin-induced DNA-dependent RNA synthesis in isolated coconut milk nuclei. However, raising the concentration of IAA from $10^{-5}$ M to $10^{-2}$ M partially overcame the inhibitory effect of the actinomycin-D. It appears that the IAA and actinomycin-D were acting competitively, perhaps not directly on DNA-dependent RNA synthesis. Similar results were reported by Datta and Sen (1965) when they investigated auxin-induced incorporation of amino acids into protein in the same system. Cleland (1965) found oat coleptile wall extensibility was not dependent on RNA synthesis, because normal extensibility was observed in the presence of auxin and sufficient actinomycin-D to cause 90% reduction in RNA synthesis.
Evans and Ray (1969) found extension growth of oat coleoptiles commenced 10 minutes after exposure to IAA. This response was actinomycin-D insensitive, and they felt it was too rapid to permit transcription, translation, and protein action.

Trewavas (1968) reported that cell expansion in auxin-treated pea stem sections is maximal at the time RNA and protein changes are just beginning. These reports do not support the hypothesis that protein and nucleic acid synthesis are obligate prerequisites of tissue expansion.

Although all investigators do not agree on the requirement for DNA-dependent RNA synthesis in auxin action, there is general agreement that protein synthesis is necessary (Datta and Sen, 1965; De Hertogh et al., 1965; Key, 1964; Masuda and Tanimoto, 1967; Noodén and Thimann, 1965; Cleland, 1965). Treatment with inhibitors of protein synthesis has invariably resulted in inhibition of auxin effects. Auxin-induced growth would seem to require not protein in general but a particular complement of proteins, that is, specific proteins which are not present in sufficient quantity or activity in the absence of auxin to result in the growth response.

Relation of Protein Complement to Specific Plant Structures or Functions

There are reports of specific proteins associated with certain
plant tissues, or stages of development. Steward, Lyndon, and Barber (1965) studied the electrophoretic properties of certain soluble proteins of pea seedlings in polyacrylamide gels. They found that (1) many proteins were common to all tissues and all stages of growth; (2) some proteins were tissue specific; and (3) a few proteins in a given tissue were found only at certain stages of development. Their results are an illustration of differential expression of genetic information as preferential synthesis of certain proteins.

Mills and Crowden (1967), with acrylamide gel electrophoresis, examined the protein complement of developing pea seedlings. They reported both qualitative and quantitative variation in electrophoretic patterns among organs at different stages of development but pointed out the danger in assuming bands with similar Rf from different samples are the same protein. Examination of peroxidase, esterase, and amylase activity in the gels indicated some bands were composed of more than one protein component.

Sahulka (1967) found only minor electrophoretic differences in soluble proteins from various root sections of *Vicia faba*. However, the general pattern of protein distribution resembled the electrophoretic pattern obtained by Morris (1966) in developing pea seedling roots. Morris (1966) found at least five bands of protein specifically associated with certain stages of root development in 48-hour-old pea seedlings.
McCown, Beck, and Hall (1968) examined the protein complement of leaves and stems from three different Dianthus species by electrophoresis in polyacrylamide gels. Most of the highly reproducible bands of protein appeared in extracts from all three species and many were common to both leaves and stems. A few bands were characteristic of the variety or the tissue.

Other reports have dealt with the influence of growth regulating chemicals on protein complement in plant tissues. Sacher, Hatch, and Glasziou (1963) and Glasziou and Waldron (1964) report IAA stimulation of an invertase in sugar cane stalks. Inhibition by chloramphenicol suggested de novo protein synthesis. Venis (1964) studied the enzyme-catalyzed formation of benzoyl-aspartate in pea stem segments. This reaction was stimulated by treatment with IAA but was inhibited in the presence of actinomycin-D and puromycin suggesting both DNA-dependent RNA synthesis and RNA-dependent protein synthesis were involved.

Sudi (1966) reported that IAA, NAA, and 2,4-D induced an enzyme catalyzing the formation of IAA- or NAA-aspartic acid complexes. Datta, Sen, and Datta (1965) observed a 100% increase in the specific activity of isocitrate lyase in potato tubers treated with $10^{-5}$ M IAA. Inhibition by puromycin and actinomycin-D suggested de novo synthesis of RNA and protein were required.

The disappearance of at least one protein from extracts of IAA-
treated bean hypocotyledonary hooks has been reported (Spelsberg and Sarkissian, 1966, Sarkissian and Spelsberg, 1967a; Sarkissian and Spelsberg, 1967b). Sarkissian and Spelsberg (1967a) also found treatment with IAA at either $10^{-3}$ M or $10^{-6}$ M resulted in the appearance of three new bands of protein after electrophoresis in acrylamide gels. Morris (1966) reported a protein component of pea root sections which was associated with meristematic zones in control roots but appeared in all portions of the roots of seedlings 24 hours after 2, 4-D treatment.

Patterson and Trewavas (1967) incubated sub-apical sections of etiolated peas with C$^{14}$- or H$^{3}$-labeled amino acids. After exposing the C$^{14}$ labeled sections to IAA and the H$^{3}$-labeled sections to buffer, they were recombined, extracted, and analyzed as one sample. The extract was fractionated by differential centrifugation and column chromatography and fractions were assayed simultaneously for C$^{14}$ and H$^{3}$. A constant C$^{14}$-H$^{3}$ ratio implied no change in protein complement with treatment. They found an actinomycin-D sensitive change in the carbon-tritium ratio following treatment with IAA.

Fan and Maclachlan (1967) found a 12- to 16-fold increase in the specific activity of cellulase in extracts of pea epicotyls three days after treatment with IAA. Inhibition of the IAA-induced component by puromycin and actinomycin-D implicated de novo RNA and protein synthesis. Datko and Maclachlan (1968) found pectinase
induction by IAA in pea epicotyl sections but 1, 3-glucanase and pectinesterase activity increased only in proportion to the total protein content.

On the basis of these reports, I feel it is reasonable to expect some relationship between protein complement and specific tissues or functions. It is equally reasonable to expect changes in the protein complement of certain tissues treated with growth regulating chemicals. What is yet to be demonstrated is a cause and effect relationship between the induction of a specific protein and morphogenesis.
MATERIALS AND METHODS

Germination of Plants

Dry pea seeds, Pisum sativum L. var. Alaska were soaked 10 minutes in 95% ethanol, rinsed with distilled water, soaked 10 minutes in 10% Clorox, then rinsed three times in distilled water. The seeds were then soaked in an aerated solution of 0.1 mM MgCl₂ and 3 mM CaCl₂ for 8 hours, drained and planted in vermiculite moistened with 1mM KH₂PO₄. Germination was in the dark at 27°C for 40 hours. Dry corn seeds, Zea mays L., var. Tendermost (Beal Seed Company, Ontario, Oregon) and dry squash seeds, Cucurbita maxima L., var. warted Hubbard were germinated like the peas except the inhibition medium contained 50 µM chloramphenicol and germination proceeded for 52 and 64 hours respectively. The imbibition period started at 10 AM for peas and squash and 10 PM for corn. Thus, all subsequent chemical treatments were applied at the same point in the daily growth cycle.

1 Seeds were a gift from W. Brotherton Seed Co., Moses Lake, Wash.
2 Seeds were a gift from Floyd Ashton, Univ. Calif., Davis.
Purification and Application of Growth Regulating Chemicals

The growth-regulating chemicals were from standard laboratory stock and had been purified by others according to the following methods.

Indole-3-acetic acid (IAA) and 1-naphthaleneacetic acid (NAA) (both Mann Res. Lab., N. Y.) were recrystallized from ethanol-water.

The 2,4-dichlorophenoxyacetic acid (2,4-D)(Mann Res. Lab., N. Y.) was washed with hot hexane and recrystallized first from benzene then from ethanol-water. The 4-amino-3,5,6-trichloropicolinic acid (picloram) (Dow Chemical Co., analytical standard) was recrystallized from ether-benzene.

The 2,6-dichlorophenoxyacetic acid (2,6-D) was prepared by refluxing one mole 2,6-dichlorophenol (Aldrich, D7020) with one mole monochloracetic acid (Eastman 68) in 150 ml water (pH 9) at 100°C for 2 hours. The mixture was adjusted to pH 6 with HCl and steam distilled to remove unreacted phenol. On cooling, the residue was acidified to pH 1. The precipitate was washed with cold water and then hot hexane. The 2,6-D was recrystallized first from benzene and then from ethanol-water.

For treatment, seedlings were rinsed and placed in aerated solutions of 1 mM KH$_2$PO$_4$ containing picloram, 2,4-D or 2,6-D at the appropriate concentration. After exposure they were rinsed
three times with distilled water and replanted in vermiculite.

It was necessary to expose seedlings continuously to IAA or NAA to obtain the desired growth response. For this exposure, seedlings were placed, roots down, on a coarse mesh stainless steel screen. The screen was supported by a plexiglass frame set in a plastic pan which served as a sump. The treatment solution (1mM KH$_2$PO$_4$ containing IAA or NAA) was pumped to a chromatogram sprayer connected to an air line. The liquid and air flows were adjusted to give a spray of about 5 seconds duration every 10 seconds (Figure 1). The treatment solution was completely changed every 12 hours. At the end of the treatment period the seedlings were rinsed three times with distilled water and replanted in vermiculite.

For exposure to ethylene (0.1% ethylene in N$_2$ from Matheson, Newark, Calif.), seedlings were suspended, roots down, on coarse mesh stainless steel screen in a 3-liter glass chamber. A small petri dish containing 5 M NaOH was placed in the bottom to absorb respired CO$_2$. Seedlings were exposed to 50 ppm ethylene in air at 50 ml/min in the dark at 27°C for 48 hours. Control plants were handled in exactly the same manner as treated plants except the treatment solution or gas lacked the growth regulating chemical.

Pea seedlings (48-hours-old) were treated with 5 x 10$^{-5}$ M 2,4-D for 4 hours, rinsed three times with distilled water and the roots lightly blotted with tissue. The apical 10 mm of the roots were
Figure 1. Pump and sprayer for applying IAA, NAA, or alanine-$C^{14}$ to pea seedlings.
marked at 1 mm intervals with India ink under a dissecting microscope. The seedlings were replanted in vermiculite and at intervals the position of the India ink marks were determined on 15 seedlings. The experiment was repeated with control pea seedlings.

**Anatomical Investigations**

Roots 15 mm long were killed and fixed in FAA (Johansen, 1940), dehydrated in a tertiary butyl alcohol series and infiltrated with paraffin wax according to Johansen (1940). Groups of five roots were embedded in Paraplast (melting point 56-57°C).

Fifty 10 micron serial sections were taken 5 mm and 10 mm behind the root tip with a rotary microtome. Sections were stained with safranin and fast green in clove oil (Johansen, 1940, pages 59, 62) and permanently mounted in Harleco Synthetic Resin. Ten roots were sectioned for any given treatment or sampling time.

**Extraction of Soluble Cytoplasmic Proteins**

All operations were conducted in ice in a cold room (2-4°C). Tissue samples were collected with new razor blades. If serial sections were taken, several new blades were bolted together with spacers between each blade to give the desired section size. The tip of the root was placed at right angles to the blades and sectioned most of the way through by gently pressing with a dry microscope slide.
The cuts were completed by rolling a glass rod covered with surgical tubing across the roots, and the desired sections were placed directly into the homogenizer. No more than 50 seedlings were sectioned with a set of cutting edges.

Tissue samples were gently ground in 1 ml Dual glass homogenizers (Ace Glass Co.) with homogenization medium (Solution 1, Appendix) consisting of 0.4 M sucrose, 20 mM Tris-chloride, 3 mM CaCl$_2$ and 0.1 mM 1, 4-dithiothreitol (pH 6.9 at 0°C.). The homogenate was centrifuged at 23,500 x g for 30 minutes (3°C.). The resulting crude supernatant (used in most experiments) was pipetted into a clean tube and kept in ice.

Gentle mechanical homogenization in a near neutral homogenization medium containing 0.4 M sucrose and a divalent cation will maintain the integrity of most cell organelles (Allfrey, 1959; De Duve, 1964). Thus, the above methods result in a supernatant of soluble cytoplasmic proteins with minimum contamination from nuclei, mitochondria, and ribosomes.

**Determination of Protein**

Protein concentration of plant extracts was determined by the method of Lowry et al. (1951). In some cases the protein content of the crude supernatant was measured without prior trichloroacetic acid (TCA) precipitation. In these instances, 0.05 ml sample and 0.15 ml
homogenization medium were added to 3.0 ml of 2.0% Na₂CO₃, 4.0% NaOH, 0.01% CuSO₄ · 5H₂O and 0.02% NaK-Tartrate (Solution 4 - Appendix). After 10 minutes, 0.3 ml of Folin's phenol reagent (solution 5 - Appendix) was added with shaking and the mixture allowed to stand for 30 minutes at room temperature. The optical density at 750 nm was determined with reference to a homogenization medium reagent blank.

A more accurate measurement of protein content was obtained with a TCA precipitation step. An aliquot of sample (less than 0.5 ml) was added to 3.0 ml 50% TCA. After 30 minutes in ice, the sample was centrifuged at 12,000 x g for 10 minutes. The supernatant was carefully decanted and a measured volume of 1.0 M NaOH added. This material was allowed to stand overnight at room temperature. The protein content of the NaOH-soluble fraction was measured as previously described.

The amount of protein in each sample was determined from a bovine serum albumin standard curve which was linear between 10 and 150 micrograms protein per determination. Protein measurements without the TCA precipitation step gave values about 30% higher.

Analytical Scale Polyacrylamide Gel Disc Electrophoresis

The principal analytical tool used in these studies was disc gel
electrophoresis based on methods of Ornstein (1964) and Davis (1964). The gel matrix was composed of polymers of acrylamide (Eastman 5521) cross-linked with N, N'-Methylenebisacrylamide (Bis) (Eastman 8383). The acrylamide and Bis were recrystallized from chloroform and acetone, respectively, and stored in vacuo in the dark at 0°C. Ammonium persulfate and N, N, N', N'-Tetramethylethylene-diamine (TEMED) (Eastman 8178) comprised the catalyst system for the polymerization reaction. The stock solutions (listed in the appendix) were reported by Davis (1964) to have a useful shelf life of several months when stored in the dark in a refrigerator.

The electrophoresis apparatus was constructed of plexiglass by the Oregon State University Physics Shop. The apparatus (Figure 2) is 36 cm long, 9-1/2 cm wide, and 14 cm high. The upper portion of the apparatus fits into the 700 ml capacity lower reservoir which contains the anode. The upper portion of the apparatus contains the cathode in a 1-liter reservoir mounted on top of a box through which cooling liquid can be circulated. The tubes in which the gels are poured are open to the upper reservoir, pass through the cooling chamber and protrude from the bottom. Thus the lower end of the tube is in contact with the lower reservoir buffer.

This electrophoresis apparatus contains 30 tubes 11 cm long and 5 mm inside diameter. A series of marks scribed on the tubes aid in preparing gels of uniform length and to insure uniform
Figure 2. Analytical gel electrophoresis apparatus.
migration of the buffer front.

After the apparatus was thoroughly clean and dry, the bottom end of each tube was sealed with a double layer of parafilm. The analytical gel solution (Appendix) was degassed in vacuo for 1 minute then poured. It was then carefully overlaid with a 1:8 dilution of lower reservoir stock solution (solution 12, Appendix) to obtain a smooth flat upper surface when the gel polymerized. This step was absolutely necessary for adequate resolution. After 30 minutes at room temperature, a line at the top of the gel indicated polymerization was complete and the overlying solution was carefully withdrawn.

The stacking gel solution was degassed and poured on top of the analytical gel. It was overlaid with a 1:8 dilution of upper gel buffer stock solution (solution 9, Appendix) to produce a flat upper surface. Polymerization was complete in about 30 minutes. After rinsing the upper portion of the tube with upper reservoir buffer and removing the parafilm, the gels were ready to use.

Samples (up to 0.45 ml) were put on the gels in 0.4 M sucrose and further stabilized against convection by carefully adding small amounts of dry P-10 polyacrylamide powder (Biogel P-10, 50 to 100 mesh, Bio-Rad Laboratories). The P-10 was carefully stirred with a glass rod until a thin gel resulted. This operation was critical to successful gel electrophoresis. The proper consistency of the P-10 stabilized sample is difficult to describe and is best learned
by experience.

After sample stabilization, the lower ends of the tubes were immersed in lower reservoir buffer and upper reservoir buffer containing tracking dye carefully added to the upper reservoir.

The electrodes were connected to a Spinco Con-stat constant current power supply and electrophoresis at 3 ma/tube continued at 24°C until the tracking dye migrated 55 mm in the 65 mm analytical gel. Each tube was sealed when the dye reached the desired point.

When all samples had migrated 55 mm, the power supply was disconnected and the gels carefully removed by rimming the lower end of the tube with a water-filled hypodermic syringe. Gels were stained in 0.5% amido black in 10% acetic acid for a minimum of 1 hour, then destained in warm 10% acetic acid for 6 to 12 hours to remove unbound dye. Gels were stored in 10% acetic acid.

For long-term storage gels should be stored in the dark in the cold. Storage at room temperature in the light results in reduced stain intensity over a period of months. Narayan, Narayan, and Kummerow (1966) indicate, however, the degree of fading among bands is proportional to the original intensity of staining in a given gel.

The gels were examined by one or more of several methods. One method involved scanning the gel with a Gilford 2410 linear transporter connected to a Gilford 2000 spectrophotometer mounted on a Beckman DU monochromator. In a few instances, photographs were
made of gels against a diffuse light source. The gels were in Klett colorimeter tubes which provided considerable lateral magnification.

**Preparative Scale Polyacrylamide Gel Disc Electrophoresis**

The resolving power of acrylamide gel electrophoresis was used to isolate a particular soluble cytoplasmic protein from a mixture of about 20 prominent components and an unknown number of minor components.

Preparative and analytical scale gel electrophoresis are based on the same principles but differ in that the latter only provide separation of proteins in a gel cylinder, while isolation of protein components are obtained with the preparative scale instrument by continuing electrophoresis until proteins migrate off the anode end of the gel. Two gel systems were used in Canalco Corporation's Model PB-1 preparative instrument. One system has a single gel called a sieving-stacker single gel and was used for preliminary fractionation. Final purification was accomplished with a dual gel system composed of a stacking gel on the top and a separate sieving gel on the bottom. The latter system is called the stacking and sieving dual gel system. It has gel properties approaching those of the analytical gel system. The stock and working solutions for these gel systems are described in the Appendix.

The bottom of the PD-2/230 column was covered with parafilm
before 10 ml of degassed 10% sieving-stacker single gel solution was poured. It was carefully overlaid with 3 ml of 1:10 diluted gel buffer stock solution (solution 19, Appendix). After polymerization (20 min.) the upper part of the column was rinsed, the parafilm removed, and the gel was ready to use.

The PD-2/70 column was used for the sieving and stacking dual gel system. The bottom of the column was covered with parafilm before 2 ml of degassed 15% sieving gel solution was poured. It was overlaid with 1:8 dilution of dual gel elution buffer stock solution (solution 23, Appendix). After polymerization was completed (30 min.), the overlying solution was removed and 2 ml of degassed 3.5 percent stocking gel solution poured. It was overlain with 1:8 dilution of upper gel buffer stock solution (solution 22, Appendix). After polymerization (20 min.) the upper part of the column was rinsed, the parafilm removed, and the gels were ready to use.

Incorporation of Radioactive Alanine Into Soluble Cytoplasmic Proteins

Dry pea seeds were germinated as previously described. At 41 hours of age 225 seedlings were placed, roots down, in the same apparatus used for spray treating with IAA and NAA (Figure 1). For the next 7 hours, these peas were sprayed with 50 µc alanine-U-C\(^{14}\) (Nuclear Chicago, specific activity 162 mc/mm) in 50 ml of 10\(^{-4}\) M
KH$_2$PO$_4$ and 50 μM chloramphenicol. The entire apparatus was enclosed in a large plastic bag in a fume hood.

After a 7-hour spray period the seedlings were lightly rinsed with distilled water and divided into one group of 75 seedlings which was treated with 5 x $10^{-5}$ M 2,4-D in $10^{-3}$ M KH$_2$PO$_4$ for 2 hours and a control group of 150 seedlings which was treated with $10^{-3}$ M KH$_2$PO$_4$ for 2 hours. At the end of this treatment period the seedlings were rinsed and returned to the spray treatment apparatus.

Spraying with radioactive alanine continued for the duration of the experiment. Samples of the treatment solution were taken at the start of spraying, 12 hours later, and again at the end of the experiment to determine the pattern of C$^{14}$ uptake.

Radioactivity was measured with a liquid scintillation counter (Packard Tricarb 314-EX). Samples were counted in a scintillation solution of Triton-toluene-Omnifluor (Appendix) which has excellent counting characteristics for aqueous samples. Samples of control and 2,4-D treated roots were collected and the crude supernatant prepared as previously described. Aliquots were subjected to analytical electrophoresis (in triplicate) in the 10% acrylamide gel matrix.

The gels were stained with amido black, destained and scanned at 500 nm using a Gilford 2410 linear transporter on a Gilford 2000 spectrophotometer. Gels were then sliced laterally into 1.6 mm
sections.

Slices from one gel for each treatment were counted individually. The two remaining gels for each treatment were also sliced but only sections 11 through 20 were counted individually. Sections 1 (origin) through 10 were combined with sections 21 to the buffer front (pooled samples).

The individual slices were placed in separate scintillation vials and allowed to air-dry for 6 hours before 0.4 ml of 30% H$_2$O$_2$ was added. Solubilization of the slice was usually accomplished in 8 to 12 hours in tightly capped vials in a 50°C water bath. When the sample was cool, 0.5 ml of distilled water was added and then 15 ml of scintillation solution. Solubilization of pooled samples required 24 hours in 7.0 ml of 30% H$_2$O$_2$. Sample volume was adjusted to 15.0 ml with distilled water and a 1.0 ml aliquot removed for counting. All the solubilized slices from one gel were spiked with toluene-C$^{14}$ (4630 DPM), the samples recounted, and the counting efficiency calculated. The efficiency varied between 57.5 and 59.6% but was not a function of position in the gel.

This method of gel slice solubilization yielded 85 to 100% C$^{14}$ recovery. The incubation temperature should be carefully controlled around 50°C; since C$^{14}$O$_2$ can be lost at incubation temperatures of 55 and 60°C (Tishler and Epstein, 1968).
Isolation of a Particular Protein

Pea seedlings (48 hours old) were treated for 2 hours with 5 x $10^{-5}$ M 2, 4-D in the usual manner. The lower 15 mm of the roots were excised 48 hours later and extracted with homogenization medium. The homogenate was centrifuged at 23,500 x g for 30 min. (3°C.) and protein content adjusted to 3 mg Lowry protein per ml.

Ammonium sulfate crystals were dissolved in the supernatant to 50% of saturation and the solution allowed to stand in ice for 10 min. before centrifugation at 12,000 x g for 10 min. Additional $(\text{NH}_4)_2\text{SO}_4$ to 90% of saturation was dissolved in the supernatant and after 10 min. in ice, the solution was centrifuged at 12,000 x g for 10 min. The pellet was suspended in a minimum volume of dialysis medium (solution 2, Appendix) and dialyzed for 2 hours against two changes of dialysis medium. Rapid dialysis was accomplished by placing the dialysis bag between two arms of a rotating (30 RPM) glass frame. Rotation completely mixed solutions in and outside the bag maintaining the most favorable concentration gradient for rapid dialysis. After dialysis and centrifugation (23,500 x g for 15 min.) the supernatant was frozen at -70°C.

Samples of this $(\text{NH}_4)_2\text{SO}_4$ purified material were thawed in ice and carefully layered on top of a 10% sieving-stacker single gel system in the PD-2/320 column in the preparative scale.
electrophoresis apparatus. The sample was stabilized against convection with Bio-Gel P-10 and upper reservoir buffer layered over the sample. The electrodes were connected to the Spinco constant current power supply, and protein stacking proceeded at 15 ma (about 20 minutes). The tracking dye required about 60 minutes to move through the sieving-stacker single gel at 7.5 ma. The current was reduced to 2 ma as the tracking dye approached the bottom 5 mm of the gel.

Elution buffer was pumped across the bottom of the sieving-stacker single gel at 2.5 ml/min. with a Buchler peristaltic pump. Absorbance (280 nm) of the "eluate" was monitored with a Vanguard model 1056 U.V. analyzer, and 30 drop fractions were collected with a LKB Ultrarack 7000 fraction collector. Appropriate fractions were combined into dialysis tubing, concentrated in the cold by dehydration with powdered polyethylene glycol 2000 and dialyzed against homogenized medium (solution 1, Appendix). Small aliquots were examined by analytical gel electrophoresis and the remainder stored at -70°C.

Final fractionation was done in a stacking and sieving dual gel electrophoresis system whose gel characteristics approached those of the analytical system. A sieving gel with a relatively high concentration of acrylamide was overlain by a stacking gel which was low in acrylamide and had no sieving properties. Three attempts were
made to achieve separation. In each case the sample, a selected fraction containing six to eight components from the sieving-stacker single gel system, was stabilized over the stacking gel with Bio-Gel P-10 and overlaid with upper reservoir buffer. The first two attempts used sieving gels 25 cm long which were high in acrylamide (20 and 15%). Diffuse bands and slow protein elution resulted in fractions too dilute for further use.

In the third and successful attempt, a 1 cm 15% sieving gel and a 1 cm 3.5% stacking gel were poured in the PD-2/70 column. Electrophoresis proceeded at 7 ma through the sample, at 10 ma for protein stacking in the stacking gel and at 12 ma for dye front migration through the sieving gel. The elution buffer was pumped across the lower end of the sieving gel at 1.5 ml/min. and fractions were collected at 1 minute intervals.

Lowry protein determination of certain fractions indicated "elution" of a protein between 51 and 57 minutes after migration of the tracking dye from the end of the gel. The contents of tubes 51 through 57 (11 ml) were combined and stabilized with Bio-Gel P-10 above a 1 cm 3.5% stacking gel in the PD-2/70 column. The protein was concentrated electrophoretically at 15 ma through the stacking gel (no sieving gel) and into a small dialysis bag fixed over the lower (anode) end of the column. In about 15 min. the tracking dye migrated through the sample and stacking gel and entered the dialysis bag.
Current was maintained for an additional 5 minutes to ensure that all the protein had migrated from the stacking gel.

The bag was removed from the end of the stacking gel and the sample dialyzed for 2 hours at 2-4°C against two changes of homogenization medium. The volume of the dialysate was 0.55 ml. An aliquot was subjected to analytical gel electrophoresis for confirmation of purification.
RESULTS

**Morphology of Plants Treated with Growth Regulating Chemicals**

The response of seedlings to chemical treatment was easily separated into immediate effects (up to 48 hours post treatment) and later effects (between 48 and 120 hours post treatment).

**Pea Seedlings Treated with 2, 4-D**

I measured the root growth of control and 2, 4-D-treated seedlings to determine which portions of the root showed extension growth. In control seedlings the first mm behind the root tip showed little elongation, while zones 1 to 2 mm and 2 to 3 mm showed a five-fold elongation and zones 3 to 4 mm and 4 to 5 mm showed a twofold or less elongation between 48 and 72 hours of age. Zones more than 5 mm behind the root tip did not increase in length (Figure 3). Seedlings treated with 2, 4-D showed no primary root elongation.

The morphology of etiolated Alaska pea seedlings during early development is depicted in Figure 4a. Exposure to 2, 4-D (5 x 10^{-5} M between 48 and 50 hours of age) caused immediate cessation of primary root and epicotyl elongation. Radial swelling occurred in the epicotyl and was particularly pronounced 3 to 5 mm behind the root tip. Rows of bumps along the root axis 48 hours after
Figure 4a, b, c, d, e. Morphogenesis of pea seedlings between 48 and 168 hours of age. Seedlings were grown in vermiculite in the dark at 27°C and were treated with 1 x 10^{-3} M KH₂PO₄ alone (control) or containing 5 x 10^{-5} M 2,4-D between 48 and 50 hours of age, 5 x 10^{-5} M IAA or 1 x 10^{-5} NAA between 48 and 96 hours of age or 1 x 10^{-5} picloram between 48 and 52 hours of age. The number of hours in the figure is total seedling age from the beginning of the imbibition period.
Figure 4a. Control seedlings at 48, 60, and 72 hours of age.
Figure 4a (cont'd). Control seedlings at 96 and 168 hours of age.
Figure 4b. 2,4-D-treated seedlings at 60 and 72 hours of age.
Figure 4b (cont'd). 2, 4-D-treated seedlings and a root tip at 96 hours of age.
Figure 4b (cont'd). 2, 4-D-treated seedlings and a root tip at 168 hours of age.
Figure 4c. NAA-treated seedlings at 60 and 72 hours of age.
Figure 4c (cont'd). NAA-treated seedlings and a root tip at 96 hours of age.
Figure 4c (cont'). NAA-treated seedlings and a root tip at 168 hours of age.
Figure 4d. IAA-treated seedlings at 72 hours of age and seedlings and a root tip at 96 hours of age.
Figure 4d (cont'd). IAA-treated seedlings and a root tip at 168 hours of age.
Figure 4e. Picloram-treated seedlings at 60 and 72 hours of age.
Figure 4e (cont'd). Picloram-treated seedlings and a root tip at 96 hours of age.
Figure 4e (cont'd). Picloram-treated seedlings and a root tip at 168 hours of age.
Figure 5. Morphogenesis of squash seedlings between 72 and 196 hours of age. Seedlings were grown in vermiculite in the dark at 27°C. and were treated with $1 \times 10^{-3}$ M KH$_2$PO$_4$ alone (control) or with $5 \times 10^{-5}$ M 2,4-D between 72 and 78 hours of age. The number of hours in the figure is total seedling age from the beginning of the imbibition period.
Figure 5 (cont'd). Control and 2,4-D-treated squash seedlings at 120 and 196 hours of age.
Figure 6. Morphogenesis of corn seedlings between 60 and 180 hours of age. Seedlings were grown in vermiculite in the dark at 27°C and were treated with 1 x 10^{-3} M KH$_2$PO$_4$ alone (control) or containing 1 x 10^{-3} M 2,4-D between ages 60 and 62 years of age. The number of hours in the figure is total seedling age from the beginning of the imbibition period.
Figure 6 (cont'd). Control and 2, 4-D-treated corn seedlings at 84, 108, and 180 hours of age.
Figure 6 (cont'd). 2, 4-D-treated corn seedlings and a root tip at 180 hours of age.
treatment preceded the proliferation of massed lateral roots which
predominated the later effects (Figure 4b).

Pea Seedlings Treated with IAA, NAA, or Picloram

Continuous exposure for 48 hours to a spray of IAA or NAA
(5 x 10^{-5} M and 1 x 10^{-5} M, respectively, between 48 and 96 hours
of age) gave a 2, 4-D-like response. NAA caused greater radial
swelling and epicotyl elongation but less intense lateral root prolifera-
tion than 2, 4-D (Figure 4c). IAA gave results similar to NAA ex-
cept that epicotyl and lateral root elongation were more inhibited by
IAA (Figure 4d). Seedlings exposed to IAA or NAA at concentrations
up to 1 x 10^{-4} M for 2, 4 or 6 hours showed an immediate 2, 4-D-
like response but primary root and epicotyl elongation resumed with-
in 48 hours after treatment.

Pea seedlings treated with picloram (1 x 10^{-5} M for 4 hours)
showed more pronounced swelling 3 to 5 mm behind the root tips and
greater inhibition of epicotyl elongation than seedlings treated with
2, 4-D (Figure 4e). Figure 4e shows three seedlings which resumed
primary root elongation between 96 and 168 hours of age. About half
of all picloram treated seedlings responded this way and were dis-
carded.
Squash and Corn Seedlings Treated with 2, 4-D

In order to compare the effects of growth regulating chemicals on other species, squash (generally regarded as sensitive) and corn (resistant) were treated with 2, 4-D. Treatment of Hubbard squash with 2, 4-D (5 x 10^{-5} M for 6 hours starting at age 72 hours) caused immediate inhibition of primary root and epicotyl elongation. Proliferation of lateral roots occurred but it was not as extensive as in peas and was restricted to the area just behind the root tip (Figure 5).

Corn treated with 2, 4-D (1 x 10^{-3} M between 60 and 62 hours of age) showed some effects similar to those of 2, 4-D in peas. Coleoptile elongation ceased but resumed later at a slow rate. Primary root elongation was inhibited but some proliferation of lateral roots did occur (Figure 6).

Anatomy of Roots of 2, 4-D-Treated Pea Seedlings

Cross sections of roots from control and 2, 4-D-treated pea seedlings were examined to determine the time and location of cell divisions leading to initiation of lateral roots.

I counted the number of cells per section with prominent nucleoli or condensed chromatin and assumed these cells would soon divide or had recently done so (Swanson, 1957.) (Table 1). Each value represents the mean of 25 observations from five sections of five
different roots. A t-test (Li, 1957) indicated no significant difference in the number of cells per section with prominent nucleoli or condensed chromatin from control and 2, 4-D-treated seedlings either 3 or 6 hours after treatment. Differences between control and treated seedlings were significant 9 and 12 hours after treatment however, indicating cell divisions leading to lateral root proliferation were initiated between 6 and 9 hours after 2, 4-D treatment. Lateral root initiation occurred around all xylem poles at all levels of the root (Figure 7). Cell divisions were absent in the cortex and infrequent in xylem parenchyma. Thus, 2, 4-D-induced cell division were restricted to the pericycle and then only opposite the xylem poles.

Table 1. Number of dividing cells per section.

<table>
<thead>
<tr>
<th>Time after treatment (hours)</th>
<th>Number of cells with condensed chromatin or prominent nucleoli/section*</th>
<th>CONTROL</th>
<th>TREATED</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>155</td>
<td>164</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>170</td>
<td>158</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>155</td>
<td>273**</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>188</td>
<td>325**</td>
<td></td>
</tr>
</tbody>
</table>

*Mean of 25 observations for each point (five sections from each of five roots).

**Difference between control and treated significant at 5% level.
Figure 7a, b, c, d. Cross sections of pea roots collected 10 mm behind the root tips 12, 24, 36, and 48 hours after exposure to $5 \times 10^{-5}$ M 2,4-D between 48 and 50 hours of age. Roots were killed in FAA, sectioned at 10 microns in Paraplast, and sections stained with safinan and fast-green.
Figure 7a. Section collected 12 hours after 2, 4-D treatment.

Figure 7b. Section collected 24 hours after 2, 4-D treatment.
Figure 7c. Section collected 36 hours after 2, 4-D treatment.

Figure 7d. Section collected 48 hours after 2, 4-D treatment.
Figure 8. Cross sections of control pea roots collected 15 mm below the cotyledons at 120 hours of age. Roots were killed in FAA, sectioned at 10 microns in Paraplast, and sections stained with safranin and fast-green.
In control seedlings, lateral roots normally emerged in sections 10 to 20 mm below the cotyledons around 120 hours of age. Cell divisions leading to lateral root initiation were observed at 96 but not at 72 hours of age. These divisions were never found around more than one xylem pole per section and many sections contained no centers of cell division (Figure 8).

**Development of Techniques for Analytical Gel Electrophoresis**

**Extraction and Electrophoresis**

The reproducibility and resolution of protein patterns obtained with Morris' (1966) techniques were not adequate. A fairly intense background stain and the presence of an intensely stained skewed band with an $R_f$ which varied between 0.6 and 0.8 were particularly perplexing.

The interference from the skewed band and the high background intensity were reduced by increasing the level of sucrose in the homogenization medium to 0.4 M and by stabilizing samples against convection over the stacking gel with Bio-Gel P-10. Increasing the level of acrylamide in the analytical gel from 7.5% to 10% decreased $R_f$ values but markedly improved resolution.

I tried several other procedures but they did not improve resolution or decrease the background. Adding 10% sucrose to the
spacer gel to prevent gel deformation or increasing the level of dithiothreitol from 0.1 to 0.2 mM (McCown et al., 1968) did not improve results. Adding polyvinylpyrrolidone (PVP) (Loomis and Battaile, 1966) to remove phenols and quinones from the homogenate did not significantly change the electrophoretic pattern. Precipitation of proteins by ammonium sulfate (90% of saturation) followed by resuspension in and dialysis against homogenization medium eliminated some components with Rf values greater than 0.7 but decreased clarity of the protein patterns.

The optimum amount of protein to apply to an analytical gel varies with the diameter of the gel and the number of proteins in the sample. My preparations from pea roots usually contained about 20 distinct bands over a continuous background of stain which decreased with increasing Rf. I found 150 to 350 µg Lowry protein per gel was a useful range. I usually examined 150 and 300 µg of protein from each extract to be sure of the location of minor components and to identify any interaction between Rf and amount of protein.

To learn more about the skewed band I extracted proteins from sections 0 to 2 mm, 2 to 4 mm and 4 to 6 mm behind the root tips of control pea seedlings. Various amounts of these proteins and of a composite sample containing equal amounts of protein from the 0 to 2 mm and 4 to 6 mm sections were subjected to analytical gel electrophoresis. The Rf of the skewed band was related to the
amount of protein applied to the gel and also to the portion of the root from which it was extracted (Figure 9). Morris (1966), Sahulka (1967) and McCown et al. (1968) all reported the presence of a skewed band or bands which showed variations in Rf with the amount of protein applied to acrylamide gels.

**Protein Staining**

Amido black is a general protein stain (Davis, 1964). The amount of stain bound is linear up to 10 µg protein per band with a standard deviation of 4% (Fambrough, Fujimura and Bonner, 1968). Narayan, Narayan and Kummerow (1966) reported amido black stained bands were reduced in intensity after several months storage. The loss was proportional to the original stain intensity, however, and relative intensity among bands changed very little.

Coomassie blue has also been used as a general protein stain in acrylamide gel electrophoresis (Chrambach et al., 1967; Fazekas et al., 1963). I found gels stained with coomassie blue and amido black had the same patterns and relative band intensities. The coomassie blue was twice as sensitive as amido black but the bright blue background interfered with gel analysis. Occasional gels were stained with coomassie blue as a check but the majority were stained with amido black.
Figure 9. Influence of amount and origin of proteins on the $R_f$ of the "skewed" band in 10% acrylamide gels. Aliquots of proteins from sections 0 to 2 mm, 2 to 4 mm, and 4 to 6 mm behind the root tips of control pea seedlings 48 hours of age and of a composite sample of equal amounts of protein from sections 0 to 2 mm and 4 to 6 mm behind the root tips were subjected to analytical gel electrophoresis.
Analysis of Electrophoretic Patterns

Presenting data from studies of the electrophoretic behavior of complex mixtures of proteins from crude plant extracts is difficult. Spectrophotometric scanning was of little value due to its inherently limited resolution. Photographic methods suffer from a related disadvantage in that critical alignment of the camera lens with a particular portion of the gel is required to observe minor components. In short, the mechanical methods of gel analysis fall far short of the resolving power of the eye.

Consequently, I have relied almost entirely on diagrammatic representations of gels (measured Rf values and estimated band intensity). In order to show the objectivity of this procedure, I have included a comparison of diagrammatic, spectrophotometric and photographic analysis of four gels (Figure 10). The correspondence of my drawings with the major features in photographs of gels is clearly seen along with the limitations of the spectrophotometric method.

Steward et al. (1965) and Morris (1966) used 7.5% acrylamide gels. To facilitate comparison with my results in 10% acrylamide gels I determined the correspondence of bands in 7.5% and 10% gels based on shape, color, intensity and relation to neighboring bands (Table 2).
Figure 10a, b, c, d. Diagrammatic, photographic, and spectrophotometric methods of illustrating the distribution of soluble cytoplasmic proteins in 10% acrylamide gels after analytical gel electrophoresis.
Figure 10a. Proteins from 1 to 2 mm behind the root tips of 48 hour old control pea seedlings.
Figure 10b. Proteins from 4 to 5 mm behind the root tips of 96 hour old pea seedlings treated with $5 \times 10^{-5}$ M 2, 4-D between 48 and 50 hours of age.
Figure 10c. Proteins from 5 to 7 mm behind the root tips of 120 hour old squash seedlings treated with 5 x 10^{-5} M 2, 4-D between 72 and 78 hours of age.
Figure 10d. Proteins from 5 to 7 mm behind the root tips of 108 hour old corn seedlings treated with $1 \times 10^{-3}$ M 2, 4-D between 60 and 62 hours of age.
Table 2. Correspondence of bands in 7.5% and 10% acrylamide gels.

<table>
<thead>
<tr>
<th>Rf in 7.5% gel</th>
<th>Rf in 10% gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corresponds to</td>
<td></td>
</tr>
<tr>
<td>0.30</td>
<td>0.16, 0.18 or 0.20</td>
</tr>
<tr>
<td>0.48</td>
<td>0.31</td>
</tr>
<tr>
<td>0.60</td>
<td>0.38</td>
</tr>
<tr>
<td>0.66</td>
<td>0.41 or 0.44</td>
</tr>
<tr>
<td>0.73, 0.75</td>
<td>0.54, 0.58 or 0.63</td>
</tr>
<tr>
<td>0.91</td>
<td>0.81</td>
</tr>
<tr>
<td>0.96</td>
<td>0.96</td>
</tr>
</tbody>
</table>

Electrophoretic Behavior of Soluble Cytoplasmic Proteins

Proteins from Control Pea Seedlings

The electrophoretic behavior of the protein complement of 48-hour-old control pea seedling root sections was determined. Proteins were extracted from section 0 to 1, 1 to 2, 2 to 3, 3 to 4, 4 to 5, 5 to 7, 7 to 9 and 9 to 11 mm behind the root tips and subjected to electrophoresis in 10% acrylamide gels. As shown in Figure 11, bands of protein with \( R_f \) values of 0.24, 0.28, 0.31, 0.38, 0.48, 0.51, appeared in all sections with constant relative intensity of stain. Bands with \( R_f \) values of 0.12, 0.15, 0.17, 0.36, 0.59 were also present in all gels but varied in intensity between extracts from
Figure 11. Electrophoretic pattern of soluble cytoplasmic proteins from sections 0 to 1, 1 to 2, 2 to 3, 3 to 4, 4 to 5, 5 to 7, 7 to 9, and 9 to 11 mm behind the root tips of control pea seedlings 48 hours of age.
different root sections. Bands with $R_f$ values 0.19, 0.41, 0.71, 0.73, 0.84, and 0.96 also increased or decreased in intensity with distance from the root tip but were not present in all sections. The most prominent member of the latter group was band 0.41.\(^3\)

Some proteins or sets of proteins are common to all sections of the root while others vary quantitatively with position in the root. A few proteins are found in certain portions of the root and may be associated with root development. In order to determine the influence of seedling age, I examined the protein complement of sections 0 to 1, 1 to 2, 2 to 3, 3 to 4, 4 to 5, 5 to 7, 7 to 9 and 9 to 11 mm behind the root tips of seedlings 50, 60, 72, and 96 hours of age. These seedlings were exposed to $1 \times 10^{-3}$ M $\text{KH}_2\text{PO}_4$ between 48 and 50 hours of age (control treatment). The electrophoretic patterns differed little from those in Figure 11 suggesting a similar complement of proteins despite differences in total seedling age. Proteins were also extracted from sections 10 to 20 mm below the cotyledons of control pea seedlings 72, 96 and 120 hours of age. The electrophoretic distribution of these proteins is depicted in Figure 12. Band 0.41 was absent in control sections at 72 hours of age but appeared in increasing quantities at 96 and 120 hours of age. The intensity of many bands was reduced with increasing age in these root sections.

\(^3\)Bands are identified by $R_f$ or the abbreviation: band 0.16 meaning a band with $R_f$ 0.16.
Figure 12. Electrophoretic pattern of soluble cytoplasmic proteins from root sections 10 to 20 mm below the cotyledons of control pea seedlings 72, 96, and 120 hours of age.
Proteins from 2, 4-D-Treated Pea Seedlings

Proteins also were extracted from sections 0 to 1, 1 to 2, 2 to 3, 3 to 4, 4 to 5, 5 to 7, 7 to 9, and 9 to 11 mm behind the root tips of seedlings harvested 12, 24, 36, and 48 hours after treatment with 2, 4-D (5 x 10^{-5} M between 48 and 50 hours of age). The electrophoretic patterns of gels from this experiment are in Figure 13. Diagrams of proteins from sections 0 to 1 mm behind the root tip show most of the bands of protein observed. The remaining diagrams show only those bands which change with treatment or position in the root, or that are necessary for reference.

Bands with Rf values of 0.05, 0.24, 0.27, 0.31, 0.38, 0.44, 0.48 and several others showed little or no changes from control patterns in Figure 11. Bands 0.12, 0.19 and 0.36, increased in intensity with time in all sections following 2, 4-D treatment. Bands 0.19, 0.41 and 0.84 were not present in all sections of control seedlings but were observed in all sections of 2, 4-D-treated seedlings. An increase followed by a decrease in the resolution of bands around Rf 0.56 to 0.58 was observed following 2, 4-D treatment.

Band 0.32 was seen irregularly in extracts of control seedlings. It appeared in all extracts of treated seedlings but the intensity was variable suggesting an artifact. I have included it in some diagrams only to indicate its occasional presence.
Figure 13a, b, c, d. Electrophoretic pattern of soluble cytoplasmic proteins from sections 0 to 1, 1 to 2, 2 to 3, 3 to 4, 4 to 5, 5 to 7, 7 to 9, and 9 to 11 mm behind the root tips of pea seedlings treated with $5 \times 10^{-5}$ M 2, 4-D between 48 and 50 hours of age.
Figure 13a. Electrophoretic pattern of proteins 12 hours after 2, 4-D treatment.
Figure 13b. Electrophoretic pattern of proteins 24 hours after 2, 4-D treatment.
Figure 13c. Electrophoretic pattern of proteins 36 hours after 2, 4-D treatment.
Figure 13d. Electrophoretic pattern of proteins 48 hours after 2, 4-D treatment.
The most prominent difference between electrophoretic patterns of proteins from control and 2, 4-D-treated seedlings concerned band 0.41. In control seedlings it decreased in intensity with distance and was not found in sections 4 to 11 mm behind the root tip (Figure 11). Band 0.41 was present in extracts of all root sections 12 hours after 2, 4-D treatment (Figure 13a). It increased in intensity with time after treatment until it was a moderately prominent component of the electrophoretic pattern 48 hours after treatment (Figure 13d). Band 0.41 was in a congested portion of the gel which prevented adequate spectrophotometric detection, but it was observed in some photographs (Figure 10b).

**Incorporation of Alanine-C$^{14}$ into Proteins**

I measured the uptake of C$^{14}$-labeled alanine into root proteins to define more closely the initial appearance of band 0.41 following 2, 4-D treatment and to determine if it was present in low levels in root tips or sections below the cotyledons of control seedlings. Pea seedlings were exposed to a spray of uniformly labeled alanine-C$^{14}$ between 41 and 48 hours of age and again between 50 and 72 hours of age. About 89% of the radioactivity applied was absorbed between 41 and 53 hours of age and an additional 2% was absorbed during the next 19 hours. Some seedlings were treated with $5 \times 10^{-5}$ M 2, 4-D while others received only $1 \times 10^{-3}$ M $\text{KH}_2\text{PO}_4$ between 48 and 50
hours of age.

Root sections of control and 2,4-D-treated seedlings were collected according to the following schedule:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Seedling Age (hours)</th>
<th>Hours since start of 2,4-D of buffer treatment</th>
<th>Number of seedlings per sample</th>
<th>Portion of root sampled</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Control</td>
<td>48</td>
<td>0</td>
<td>25</td>
<td>3-11 mm behind apex</td>
</tr>
<tr>
<td>2 Control</td>
<td>54</td>
<td>6</td>
<td>25</td>
<td>3-11 mm behind apex</td>
</tr>
<tr>
<td>3 Control</td>
<td>60</td>
<td>12</td>
<td>25</td>
<td>3-11 mm behind apex</td>
</tr>
<tr>
<td>4 Control</td>
<td>60</td>
<td>12</td>
<td>25</td>
<td>10-20 mm below cotyledon</td>
</tr>
<tr>
<td>5 Control</td>
<td>72</td>
<td>24</td>
<td>20</td>
<td>3-11 mm behind apex</td>
</tr>
<tr>
<td>6 Control</td>
<td>72</td>
<td>24</td>
<td>20</td>
<td>10-12 mm below cotyledon</td>
</tr>
<tr>
<td>7 2,4-D</td>
<td>54</td>
<td>6</td>
<td>20</td>
<td>3-11 mm behind apex</td>
</tr>
<tr>
<td>8 2,4-D</td>
<td>60</td>
<td>12</td>
<td>15</td>
<td>3-11 mm behind apex</td>
</tr>
<tr>
<td>9 2,4-D</td>
<td>72</td>
<td>24</td>
<td>15</td>
<td>3-11 mm behind apex</td>
</tr>
</tbody>
</table>

Only 0.09% of the radioactivity applied was incorporated into soluble cytoplasmic proteins. This figure considers only the proteins in a small portion of the plant however, and incorporation into the total protein of the whole seedlings was probably greater than indicated.

Changes in the specific activity (adjusted for differences in alanine-C\(^{14}\) uptake) of proteins subjected to gel electrophoresis showed there was continuing incorporation of alanine into proteins over the course of the experiment (Table 3). The proportion of radioactivity precipitated by 50% TCA from the crude supernatant was larger than anticipated. Alanine of course readily undergoes
Table 3. Specific activity of soluble cytoplasmic protein containing alanine-C$^{14}$ from root sections of control and 2,4-D-treated pea seedlings.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Radioactivity in soluble fraction (DPM/25 seedlings)</th>
<th>Lowry protein in soluble fraction (µg/25 seedlings)</th>
<th>Radioactivity in soluble fraction precipitated by 50% TCA (%)</th>
<th>Radioactivity bound in gel by amido black (% of applied)</th>
<th>Specific Activity DPM/µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$x \times 10^3$</td>
<td>$x \times 10^3$</td>
<td>7.8</td>
<td>6.7</td>
<td>12.9</td>
</tr>
<tr>
<td>2</td>
<td>143</td>
<td>1.41</td>
<td>8.3</td>
<td>7.4</td>
<td>16.9</td>
</tr>
<tr>
<td>3</td>
<td>122</td>
<td>1.15</td>
<td>11.5</td>
<td>10.8</td>
<td>23.4</td>
</tr>
<tr>
<td>4</td>
<td>51</td>
<td>1.02</td>
<td>11.2</td>
<td>10.0</td>
<td>12.4</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>2.04</td>
<td>13.5</td>
<td>12.1</td>
<td>24.2</td>
</tr>
<tr>
<td>6</td>
<td>35</td>
<td>1.31</td>
<td>11.9</td>
<td>11.2</td>
<td>17.0</td>
</tr>
<tr>
<td>7</td>
<td>133</td>
<td>1.64</td>
<td>12.5</td>
<td>13.0</td>
<td>11.6</td>
</tr>
<tr>
<td>8</td>
<td>234</td>
<td>2.52</td>
<td>20.2</td>
<td>16.5</td>
<td>19.6</td>
</tr>
<tr>
<td>9</td>
<td>128</td>
<td>2.40</td>
<td>16.1</td>
<td>14.2</td>
<td>13.0</td>
</tr>
</tbody>
</table>

*Specific activity is expressed as DPM precipitated by 50% TCA per µg Lowry protein adjusted by proportion for differential uptake of alanine-C$^{14}$ among treatments.
transamination and the resulting pyruvate is susceptible to oxidation. Large quantities of $^{14}\text{C}_2\text{O}_2$ may have been lost by this mechanism.

Proteins were subjected to analytical gel electrophoresis. The destained gels were scanned with a spectrophotometer, sectioned and counted. The results are expressed as a histogram of radioactivity (normalized) over $R_f$ superimposed on a tracing of the spectrophotometer scan (Figure 14). The histogram of radioactivity represents single observations between $R_f$ values of 0.0 and 0.28 and between 0.55 and 1.0. Average values and the standard error of the mean from three separate gels are plotted between $R_f$ values of 0.38 and 0.55 (see materials and methods).

There was only a general correspondence between radioactivity and optical density at 500 nm (OD$_{500}$). Discrepancies between peaks of OD$_{500}$ and radioactivity around $R_f$ values of 0.08, 0.15, 0.38, 0.62, and 0.75 show some proteins are synthesized or metabolized more rapidly than others or differ in amino acid composition.

The general level of radioactivity declined with increasing $R_f$ around $R_f$ 0.41 in all gels except those containing proteins from 2, 4-D-treated seedlings (Figure 14). Band 0.41 was not preferentially labeled until 12 and 24 hours after 2, 4-D treatment which means band 0.41 first appeared between 6 and 12 hours after 2, 4-D treatment (Figures 14c, f, and i). Band 0.41 declined in radioactivity between 12 and 24 hours after treatment suggesting a more rapid
The distribution of OD$_{500}$ and C$_{14}$ in 10% acrylamide gels following analytical gel electrophoresis of soluble cytoplasmic proteins from root sections of control and 2, 4-D treated pea seedlings exposed to alanine-C$^{14}$. Seedlings were grown in vermiculite in the dark at 27 deg. C until exposed to alanine-C$^{14}$ between 41 and 48 hours of age. Seedlings were then treated with $1 \times 10^{-3}$ M KH$_2$PO$_4$ containing $5 \times 10^{-5}$ M chloramphenicol alone (control) or with $5 \times 10^{-5}$ M 2, 4-D between 48 and 50 hours of age, seedlings were rinsed and returned to the alanine-C$^{14}$ treatment solution. Proteins were extracted and subjected to analytical gel electrophoresis. Distribution of OD$_{500}$ in stained gels was measured with a Gilford linear transporter on a Gilford spectrophotometer. Gels were sliced and the radioactivity in individual slices measured with a scintillation counter. Distribution of C$^{14}$ is based on single observations between R$_f$ values of 0.0 and 0.28 and between 0.55 and 1.0. Average values and the standard error of the mean from three separate gels are plotted between R$_f$ 0.28 and 0.55.
Figure 14a. Proteins from sections 3 to 11 mm behind the root tips of control seedlings 48 hours of age.
Figure 14b. Proteins from sections 3 to 11 mm behind the root tips of control seedlings 54 hours of age.
Figure 14c. Proteins from sections 3 to 11 mm behind the root tips of seedlings 54 hours of age, 6 hours after treatment with 5 x 10^-5 M 2, 4-D.
Figure 14d. Proteins from sections 3 to 11 behind the root tips of control seedlings 60 hours of age.
Figure 14e. Proteins from sections 10 to 20 mm below the cotyledons of control seedlings 60 hours of age.
Figure 14f. Proteins from sections 3 to 11 mm behind the root tips of seedlings 60 hours of age, 12 hours after treatment with $5 \times 10^{-5}$ M 2,4-D.
Figure 14g. Proteins from sections 3 to 11 mm behind the root tips of control seedlings 72 hours of age.
Figure 14h. Proteins from sections 10 to 20 mm below the cotyledons of control seedlings 72 hours of age.
Figure 14i. Proteins from sections 3 to 11 mm behind the root tips of seedlings 72 hours of age, 24 hours after treatment with $5 \times 10^{-5}$ M 2, 4-D.
metabolism of proteins in this band than in adjacent bands.

Proteins from Pea Seedlings Treated with IAA, NAA or Picloram

Pea seedlings were treated with concentrations of IAA, NAA or picloram sufficient to produce morphological effects similar to those caused by 2,4-D (Figures 4b, c, and d). The soluble proteins were extracted from various root sections 24 and 48 hours after treatment, and their electrophoretic behavior was compared with proteins from 2,4-D-treated seedlings. The electrophoretic patterns of proteins from sections 0 to 1, 1 to 2, 2 to 3, 3 to 4, 4 to 5, 5 to 7, 7 to 9, and 9 to 11 mm behind root tips collected 48 hours after treatment are in Figure 15.

The changes in the electrophoretic patterns following treatment with IAA, NAA or picloram were remarkably similar to those produced by 2,4-D (Figures 13 and 15). Bands 0.12, 0.15, 0.36, 0.41 and 0.84 appeared about the same in extracts from 2,4-D-, IAA-, NAA- or picloram-treated seedlings but differed from control seedlings. Bands 0.24, 0.28, 0.44, 0.48 looked the same in extracts from all treated and control seedlings. When compared to results with 2,4-D-treated seedlings, band 0.31 decreased in intensity and band 0.38 increased in intensity in seedlings treated with IAA, NAA or picloram.

Proteins were analyzed from a section 0 to 10 mm behind the
Figure 15a. Proteins from sections 0 to 1 or 1 to 2 mm behind the root tips.
Figure 15b. Proteins from sections 2 to 3 or 3 to 4 mm behind the root tips.
Figure 15c. Proteins from sections 4 to 5 or 5 to 7 mm behind the root tips.
Figure 15d. Proteins from sections 7 to 9 or 9 to 11 mm behind the root tips.
root tips of IAA-, NAA- and picloram-treated seedlings 24 hours after treatment. The electrophoretic patterns were generally intermediate between control seedlings and 2,4-D-treated seedlings harvested 48 hours after treatment. This same pattern was observed with 2,4-D-treated seedlings 24 and 48 hours after treatment (Figures 13b and d).

Band 0.41 was present in all sections of roots from IAA-, NAA- or picloram-treated seedlings 24 hours after treatment. The intensity of band 0.41 increased with time after treatments, and with the exception of picloram-treated seedlings, was a prominent component of sections more than 3 mm behind the root tip. The general behavior of band 0.41 from 2,4-D- or IAA-, NAA- or picloram-treated seedlings was similar.

Proteins from Pea Seedlings Treated with Ethylene

According to Burg and Burg (1966), the action of some growth regulating chemicals is mediated by the production of ethylene.

Figure 16 shows the response of pea seedlings exposed to 2,4-D (5 x 10^{-5} M for 2 hours) or ethylene (50 ppm for 72 hours). The morphogenesis of ethylene- and 2,4-D-treated seedlings was similar except ethylene-treated seedlings developed a normal complement of lateral roots rather than the massed lateral roots typical of 2,4-D treatment.

The electrophoretic pattern of proteins from a section 0 to 10
Figure 16. Morphogenesis of pea seedlings between 48 and 168 hours of age. Seedlings were grown in vermiculite in the dark at 27° C. and were treated with $1 \times 10^{-3}$ M KH$_2$PO$_4$ alone (control) or with $5 \times 10^{-5}$ M 2, 4-D between 48 and 50 hours of age or 50 ppm ethylene between 48 and 120 hours of age. The numbers of hours in the figure are total seedling age from the beginning of the imbibition period and indicate seedling age at the beginning of the treatment period and when photographed.
mm behind the root tips of ethylene-treated seedlings resembled in part patterns associated with both control and 2, 4-D-treated seedlings (Figure 17). The intensity of band 0.41 increased with time, and after 48 hours of ethylene treatment it appeared the same as a similar band from control root tips or roots extracted 12 hours after 2, 4-D treatment. After 72 hours, the intensity of band 0.41 resembled that from seedlings 48 hours after 2, 4-D treatment.

Proteins from Pea Seedlings Treated with 2, 6-D

Seedlings treated with $5 \times 10^{-5}$ M 2, 6-D showed little effect except for slight stem and root swelling. About 50% of the seedlings exposed to $5 \times 10^{-4}$ M 2, 6-D showed a 2, 4-D-like response but it was less intense than would be expected from exposure to $5 \times 10^{-5}$ M 2, 4-D. The other seedlings continued primary root elongation but had a markedly swollen area where the root tip was located at the time of treatment. Seedlings which did not continue primary root elongation showed massed lateral root proliferation which was particularly intense in the swollen region behind the root tip. Seedlings continuing primary root elongation showed normal lateral root development except in the swollen region which showed 2, 4-D-like lateral root proliferation between 96 and 168 hours of age. The electrophoretic patterns of proteins from a section 0 to 10 mm behind the root tips of seedlings continuing primary root elongation after treatment with $5 \times 10^{-5}$ M or $5 \times 10^{-4}$ M 2, 6-D were the same as patterns from control seedlings (Figures 11 and 18). Extracts from
Figure 17. Electrophoretic pattern of soluble cytoplasmic proteins from section 0 to 10 mm behind the root tips of 96 and 120-hour-old pea seedlings exposed continuously to 50 ppm ethylene from 48 hours of age
Figure 18. Electrophoretic pattern of soluble cytoplasmic proteins from sections 0 to 10 mm behind the root tips of 96-hour-old pea seedlings exposed to $5 \times 10^{-5}$ M 2, 6-D between 48 and 50 hours of age. Protein were extracted from sections 0 to 10 mm behind the root tips of seedlings which continued primary root elongation after exposure to either $5 \times 10^{-5}$ M (A) or $5 \times 10^{-4}$ M (B) 2, 6-D. Proteins were also extracted either from sections 0 to 10 mm behind the root tips of seedlings which failed to continue primary root elongation (C) or from the swollen portion of roots which did continue primary root elongation (D) after exposure to $5 \times 10^{-4}$ M 2, 6-D. The portion of the root which showed swelling after 2, 6-D treatment was located just back of the root tip at the time of treatment.
roots of seedlings which did not elongate or from the swollen area of roots which did elongate after exposure to $5 \times 10^{-4}$ M 2, 6-D showed an electrophoretic pattern similar to those from roots of 2, 4-D-treated seedlings (Figures 13 and 18). It may be significant that band 0.41 was prominent in these latter two cases since 2, 4-D-like proliferation of lateral roots occurred in these same areas.

Proteins from Control and 2, 4-D-treated Squash and Corn Seedlings

Electrophoretic patterns of proteins from roots of pea seedlings which seemed to be associated with stages of development or 2, 4-D treatment prompted me to look for similar relationships in corn and squash seedlings. Distinct differences were observed among electrophoretic patterns.

In control squash seedlings, a band with Rf 0.41 was found only in sections less than 4 mm behind the root tip (Figure 19a). After 2, 4-D treatment however, band 0.41 appeared in all sections and showed a marked increase in intensity with distance from the root tip (Figure 19b). Among several other changes noted, band 0.28 declined sharply from the levels noted in control seedlings. Bands 0.45 and 0.55 declined with distance from the root tip in control seedlings but were maintained in intensity following 2, 4-D treatment.

In control corn seedlings, bands 0.21 and 0.30 decreased and bands 0.25 and 0.28 increased with distance behind the root tip
Figure 19a. Proteins from root sections of control squash seedlings.
Fig. 19b. Proteins from root sections of 2, 4-D treated squash seedlings.
In 2, 4-D-treated seedlings the reverse occurred, bands 0.21 and 0.30 increased and bands 0.25 and 0.28 decreased in intensity with distance behind the root tip (Figure 20b). Bands 0.05 and 0.15 were found in all sections of 2, 4-D-treated seedlings but in only some control root sections. Band 0.41 was of particular interest. It declined in intensity with distance behind the root tip of control seedlings although it never disappeared. In 2, 4-D-treated seedlings band 0.41 was present in high concentrations in all sections and increased in intensity with distance from the root tip.

**Isolation and Stability of Induced Proteins**

The association of band 0.41 with certain meristematic portions of control roots and its regular appearance in all portions of roots of seedlings treated with a variety of growth regulating chemicals is striking. Characterization of the proteins in band 0.41 is desirable because of the possibility of a causal relationship between its appearance and the initiation or emergence of lateral roots. Toward this end, I have developed a procedure of isolating the proteins in band 0.41 which appeared following 2, 4-D treatment of pea seedlings.

Gentle hand homogenization of the roots in glass in the cold immediately followed by centrifugation (23,500 x g for 30 min.) was required to prevent degradation of the proteins in band 0.41. The crude supernatant lost about 50% of the intensity of band 0.41 when
Figure 20a and b. Electrophoretic pattern of soluble cytoplasmic proteins from section 0 to 1, 1 to 2, 2 to 3, 3 to 4, 4 to 5, 5 to 7, 7 to 9, and 9 to 11 mm behind the root tips of 108 hour old corn seedlings exposed to $1 \times 10^{-3}$ M KH$_2$PO$_4$ alone (control) or with $1 \times 10^{-3}$ M 2,4-D between 60 and 62 hours of age.
Figure 20a. Proteins from root sections of control corn seedlings.
Figure 20b. Proteins from root sections of 2, 4-D treated corn seedlings.
stored in ice for 12 hours or at -70°C. for 21 days.

Storing roots or fresh homogenates in ice or frozen at -20°C, -70°C or -196°C. resulted in an immediate loss of band 0.41. Centrifugation of the homogenate markedly repressed this loss. Maceration of tissue in Virtis or Waring homogenizers also resulted in rapid loss of band 0.41 despite immediate centrifugation. These results suggest that freezing or vigorous homogenization releases degradative enzymes which are normally bound to cell particulates.

Ammonium sulfate fractionation was useful for stabilization and purification of proteins on band 0.41. Table 4 shows the pattern of protein precipitation by ammonium sulfate. Analytical gel electrophoresis showed no distinct banding of proteins precipitated by less than 50% of ammonium sulfate saturation. Proteins in band 0.41 which were recovered from the 50 to 60%, 60 to 75% and 75 to 90% ammonium sulfate fractions showed little degradation when stored at -70°C. for 30 days. Ammonium sulfate fractionation is a good means of preparing large quantities of protein mixtures which contain components of band 0.41.

In another test proteins were extracted from the lower 20 mm of control and 2,4-D-treated pea seedlings. They were fractionated into proteins insoluble in 50 to 60%, 60 to 75%, or 75 to 90% of ammonium sulfate saturation and subjected to analytical gel electrophoresis. Similar patterns for both control and treated seedlings
Table 4. Protein precipitation by ammonium sulfate.

<table>
<thead>
<tr>
<th>(NH$_4$)$_2$SO$_4$ (% of saturation)</th>
<th>Crude supernatant</th>
<th>Protein precipitated</th>
<th>(cumulative % of total recovered)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>109.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–20</td>
<td>0.3</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>20–30</td>
<td>0.3</td>
<td>0.4</td>
<td>0.8</td>
</tr>
<tr>
<td>30–40</td>
<td>25.0</td>
<td>31.7</td>
<td>32.5</td>
</tr>
<tr>
<td>40–50</td>
<td>27.1</td>
<td>34.5</td>
<td>67.0</td>
</tr>
<tr>
<td>50–60</td>
<td>14.4</td>
<td>18.3</td>
<td>85.3</td>
</tr>
<tr>
<td>60–75</td>
<td>10.6</td>
<td>13.4</td>
<td>98.7</td>
</tr>
<tr>
<td>75–90</td>
<td>1.1</td>
<td>1.4</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Total Recovered 78.8 mg  % Recovered 72
were observed although the intensities of some bands varied between treatments (Figure 21). Band 0.41 was prominent in extracts of treated seedlings showing that the electrophoretic properties of its component proteins were not influenced by high salt concentrations.

Further purification was accomplished in two steps with a preparative scale gel electrophoresis apparatus (Canalco Corp.). In this instrument electrophoresis continues until the protein moves off the anode end of the gel and is swept away by buffer. Figure 22 shows the results of a typical preparative run where the buffer which swept the anode end of the gel was monitored for absorbance at 280 nm and assayed for Lowry protein. The fractions indicated by the arrows were concentrated and examined by analytical gel electrophoresis.

The order of band "elution" corresponded to the order of migration in analytical gels (Figure 23). Fractions 24 through 32 contained several proteins including those with $R_f$ 0.41. In other tests, fractions from the last half of the peak of $OD_{500}$ near fractions 25 to 35 were combined for further purification.

The final purification step was accomplished with a stacking and sieving dual gel system in the preparative electrophoresis instrument. A small amount of protein moved off the gel in fractions collected between 51 and 57 minutes after "elution" of the tracking dye. These fractions were combined, dialyzed against homogenization
Figure 21. Electrophoretic distribution of ammonium sulfate fractionated soluble cytoplasmic proteins from sections 0 to 20 mm behind the root tips of 96-hour-old pea seedlings exposed to $1 \times 10^{-5}$ M $\text{KH}_2\text{PO}_4$ alone (control) or with $5 \times 10^{-5}$ M 2, 4-D between 48 and 50 hours of age. Proteins were fractionated into groups insoluble in 0 to 50% (not shown), 50 to 60%, 60 to 75%, and 75 to 90% of ammonium sulfate saturation at 0°C.
Figure 22. Elution of OD$_{280}$ and Lowry protein from the anode end of a sieving-stacker single gel in the preparative scale gel electrophoresis apparatus. The sample contained the 50 to 90% of ammonium sulfate saturation-precipitable soluble-cytoplasmic proteins which were extracted from 0 to 20 mm behind the root tips of 2, 4-D-treated pea seedlings 96 hours of age. Electrophoresis was conducted at 24°C. at 7.5 ma until the dye front was within 5 mm of the end of the gel when the current was reduced to 2 ma. Fractions contained 30 drops of eluate and those marked with arrows were concentrated and subjected to analytical gel electrophoresis (Figure 23).
Figure 23. Electrophoretic pattern of proteins from fractions marked by arrows in Figure 22. Fractions were concentrated in dialysis bags by dehydration with polyethylene glycol 2000 and then dialyzed against homogenization medium.
medium and concentrated electrophoretically into a small dialysis bag. Examination of this final preparation by analytical gel electrophoresis showed one major and two minor components (Figure 24). The spectrophotometer scan was analyzed with a DuPont curve resolver which revealed 88% of the area under the three peaks was contributed by the major component which had an $R_f$ value of 0.41 in 10% acrylamide gels. Complete separation of band 0.41 from other components probably could be achieved by using larger quantities of protein or proteins containing radioactive amino acids.
Diagrammatic, photographic, and spectrophotometric record of the distribution of proteins isolated from fractions 25 to 35 (Figure 22 and 23) using the stacking and sieving dual gel preparative scale gel electrophoresis apparatus. Electrophoresis was conducted at 24 deg. C. and 12 ma, and 1.5 ml fractions were collected each minute. A small peak of Lowry protein moved off the gel between 51 and 57 minutes after passage of the tracking dye. These fractions were concentrated electrophoretically and subjected to analytical electrophoresis in 10% acrylamide gels.
DISCUSSION

Morphology of Plants Treated with Growth Regulating Chemicals

Treatment of pea seedlings with 2, 4-D, IAA, NAA, or picloram caused inhibition of epicotyl and primary root elongation followed by emergence of massed lateral roots. Continued exposure rather than single doses of IAA and NAA were required to produce persistent effects, however. Eliasson (1961) and Morris (1966) found similar effects in pea and aspen seedlings, and pea seedlings, respectively.

The response of pea seedlings to 2, 4-D, IAA, NAA, and picloram is understandable in terms of the patterns of metabolism of these chemicals. Andreae (1967) reported 2, 4-D was largely unmetabolized in pea root segments, which accounts for the success of short term exposure to 2, 4-D in producing persistent effects. In contrast, IAA and NAA were extensively metabolized in 6 and 16 hours, respectively, in pea root segments. Thus it is not surprising that long exposure periods were required to produce persistent effects with IAA and NAA. Picloram resists metabolism in peas but is translocated towards the top of the plant and is excreted by the roots\(^4\) which explains why many picloram-treated seedlings resumed

\(^4\)Scott, P. C., Research Associate, Oregon State University, Dept. of Agricultural Chemistry, Personal Communication, Corvallis, Oregon, June 10, 1969.
primary root elongation although the epicotyls remained completely inhibited.

Corn is not sensitive to 2, 4-D for purposes of selective weed control but my results show that sensitivity is relative, for 1 \times 10^{-3} M 2, 4-D produced morphological effects in corn somewhat similar to those found in 2, 4-D-treated pea seedlings. Eames (1949) felt monocots and dicots were affected similarly by phenoxy herbicides and attributed differences in their external appearance following treatment to different growth patterns. Botrill and Hanson (1968) found proliferation of secondary lateral roots in corn seedlings treated with 2, 4-D. Their pictures of treated seedlings closely resemble Figure 6.

The mode of action of a chemical may be defined as the series of steps leading to the overall effect on the organism. Picloram, IAA, NAA, and 2, 4-D may have a common mode of action in pea seedlings. Some similarities in the morphological responses of peas, corn, and squash seedlings to 2, 4-D suggest a common mode of 2, 4-D action among species.

Anatomy of 2, 4-D-Treated Pea Seedlings

I observed an increased number of pericycle cell divisions opposite the xylem poles in pea seedling roots 9 hours after 2, 4-D treatment. These divisions ultimately led to emergence of massed
lateral roots. Scott (1938) and Bond (1948) found the greatest meristematic activity in the pericycle opposite the xylem poles of IAA-treated pea stem segments and 2, 4-D-treated pea roots, respectively. They reported few divisions in the cortex or vascular tissue.

Wilde (1951) and Fisher, Bayer and Weir (1968), working with 2, 4-D- and picloram-treated bean seedlings, respectively, and Key and Lin (1966), working with 2, 4-D-treated soybean hypocotyls, reported cell divisions leading to the proliferation of lateral roots as early as 12 hours after chemical treatment. Colonial bentgrass (Callahan and Engle, 1965) and corn (Botrill and Hanson, 1968) treated with 2, 4-D also produce massed lateral roots from the pericycle. Eames (1949) found 2, 4-D-induced anatomical changes were essentially the same in monocots and dicots. Indolebutyric acid, NAA, and 2, 4-D increased the number of active cell divisions in the pericycle of Pyrus caucasi suggesting eventual lateral root proliferation (Katulska, 1966).

These reports include research with a diverse group of species treated with several different growth regulating chemicals. The similarity of response is striking. It is significant that in every case sublethal concentrations induced cell divisions in or near the pericycle over the length of the primary root causing proliferation of lateral roots opposite xylem poles. In normal seedlings, lateral roots are also initiated in the pericycle opposite the xylem poles.
but these centers of activity are widely separated. In other words, these growth regulating chemicals induced divisions in cells which have meristematic potential but most of which normally remain inactive. Thus the morphological response of massed lateral root proliferation would seem to differ only quantitatively from the norm.

Electrophoretic Behavior of Plant Proteins

Electrophoresis of soluble cytoplasmic proteins from various root sections of pea seedlings showed some proteins common to all sections, other proteins which varied in amount from section to section, and a few proteins which were only found in certain sections. Steward et al. (1965) and Morris (1966) also investigated the electrophoretic behavior of proteins from pea seedlings in acrylamide gels.

The extraction and purification procedures Morris (1966) and I used were nearly the same and from the correspondence of $R_f$ values in Table 2, our results appear quite similar if $R_f$ values greater than 0.6 are ignored. The justification for ignoring these values is the presence of the shifting skewed band which interfered with gel interpretation. Both Morris (1966) and Steward et al. (1965) showed bands of protein which might have changed $R_f$ with increasing distance from the root tip, which is a characteristic of my skewed band (Figure 9).

My results and Morris' (1966) differ from those of Steward et al. (1965) probably because of differences in extraction and
purification procedures. In particular, Steward et al. (1965) did not use sucrose or a sulfhydryl agent in their homogenization medium and centrifugation was too slow to sediment the lighter particulate fractions. Finally, they concentrated their samples by evaporation in dialysis bags in a stream of air at room temperature. These conditions are harsh and almost certainly resulted in degradation or conformational changes sufficient to influence electrophoretic behavior of proteins.

The appearance of band 0.41 was prominent among several changes in the electrophoretic pattern of proteins from 2, 4-D-treated pea seedlings. Band 0.41 was first detected 12 hours after treatment and increased in intensity with time after treatment and distance behind the root tip. It was also associated with root tip sections of control seedlings. Morris (1966) found a band ($R_f 0.66$ in 7.5% gels) in extracts of root tips of pea seedlings which also appeared in all root sections following 2, 4-D treatment. I do not know if the proteins I found in band 0.41 (10% acrylamide gels) are the same ones reported by Morris (1966) ($R_f 0.66$ in 7.5% acrylamide gels) but a comparison of $R_f$ values in Table 2 suggests they may be similar.

Is the apical 10 mm section of roots from 96-hour-old 2, 4-D-treated seedlings equivalent to the apical 10 mm section of roots from 96-hour-old control seedlings? In some respects they are
The epidermal and cortex tissues in this section of control seedlings are younger than in similar sections of 2, 4-D-treated seedlings. In fact, root tips of 2, 4-D-treated seedlings are more nearly the same age as sections 10 to 20 mm below the cotyledons of control seedlings. Further, lateral roots normally emerge from these latter sections at 96 and 120 hours of age, respectively.

However, differences in the electrophoretic patterns of proteins from sections 10 to 20 mm below the cotyledons of control seedlings and from roots of 2,4-D-treated seedlings increased with time. Significantly, band 0.41 was absent in these control sections at 72 hours of age but was present in small quantities and large quantities, respectively, at 96 and 120 hours of age. Many other bands were drastically reduced in intensity at 120 hours of age in extracts from control sections. These differences suggest little biochemical equivalence between root sections 10 to 20 mm below the cotyledons of control seedlings and roots of 2, 4-D-treated seedlings. On the other hand, similarities in the electrophoretic patterns of proteins from the lower portions of control and 2, 4-D-treated roots suggests similar biochemical states.

Incorporation of Radioactive Alanine into Proteins of Band 0.41

Radioactivity in band 0.41 in extracts from 2, 4-D-treated seedlings neither establishes nor excludes the possibility that the
appearance of this band represented de novo protein synthesis. This point can best be established by immunological assay. The results do show, however, that band 0.41 first appeared between 6 and 12 hours after treatment and that preferential incorporation of alanine-C\(^{14}\) into band 0.41 in control root tips or sections 10 to 20 mm below the cotyledons did not occur.

Chloramphenicol in the solution of radioactive alanine prevented the incorporation of radioactivity by microorganisms (Noodén and Thimann, 1965). The absence of radioactivity in band 0.41 in extracts from both kinds of control tissues also suggests microbial activity was not an important source of error.

**Proteins from Pea Seedlings Treated with IAA, NAA, or Picloram**

The electrophoretic patterns of proteins from root sections of seedlings treated with IAA, NAA, or picloram resembled in many respects patterns from 2,4-D-treated seedlings. Of particular interest was the appearance of band 0.41 in extracts of all portions of these roots 24 or 48 hours after treatment.

Spelsberg and Sarkissian (1966) and Sarkissian and Spelsberg (1967b) reported sections of IAA-treated bean hypocotyls contained three new proteins after exposure to \(1 \times 10^{-6}\) M IAA. Exposure to \(1 \times 10^{-3}\) M IAA also led to the appearance of three new proteins but
caused the loss of three other proteins as well. These investigators froze their sections, however, and did not include a sulfhydryl protecting agent in their homogenization medium. I found these conditions gave poor resolution and patterns suggesting degradation of protein. This may account for the fact only nine bands were reported despite application of 400 µg protein per gel. Patterson and Trewavas (1967) used methods other than gel electrophoresis to show IAA alteration of patterns of protein synthesis.

I found no previous reports of the induction of specific proteins by NAA or picloram. Based on their known impact on nucleic acid and protein metabolism, however, it is not surprising to find such induction (Maheshwari et al., 1966; Esnault, 1965; Malhotra and Hanson, 1966).

Proteins from Pea Seedlings Treated
with Ethylene or 2, 6-D

Pea seedlings treated with ethylene showed morphological effects similar to those treated with 2, 4-D except proliferation of the massed lateral roots did not occur. The electrophoretic pattern of proteins from roots of ethylene-treated seedlings did not show band 0.41 until 48 hours after the start of treatment. The electrophoretic pattern resembled in part patterns associated with both control and 2, 4-D-treated seedlings.
Ethylene has been associated with auxin action by some investigators (Burg and Burg, 1966; Abeles and Rubenstein, 1964) while others have found differences in their modes of action (Andreae et al., 1968). The lack of proliferation of massed lateral roots in ethylene-treated seedlings and differences in the electrophoretic patterns of proteins from ethylene- and IAA-treated seedlings are clear separations of ethylene and auxin effects.

Some 2, 6-D-treated seedlings showed a 2, 4-D-like morphological response. Band 0.41 was present in tissues showing the 2, 4-D response and conspicuously absent in tissues which did not show this response. The recurring association of band 0.41 with the emergence of lateral roots is striking.

Using gas chromatography, I assayed the 2, 6-D for 2, 4-D and found less than 5% contamination (minimum detectable level). $5 \times 10^{-4}$ M 2, 6-D would contain $2.5 \times 10^{-5}$ M 2, 4-D which is probably sufficient to produce visible 2, 4-D effects. The swelling of roots and shoots and the appearance of band 0.41 in extracts of certain roots following 2, 6-D treatment may have been due to low level 2, 4-D contamination.

**Band 0.41**

I have placed emphasis on proteins in band 0.41 in 10% acrylamide gels. Does band 0.41 represent a real or an artificial part of
the protein complement? An unequivocal answer is not possible, but the shape, color, and behavior and consistent appearance of band 0.41 in treated tissues and some control tissues suggests it contains proteins which are true in vivo components of the seedlings examined.

Sarkissian and Spelsberg (1967a, b) reported a protein which disappeared from the electrophoretic pattern of IAA-treated bean seedlings and suggested IAA binding to the protein might have altered its electrophoretic mobility. All the growth regulating chemicals used in my experiments could presumably also bind to proteins. Morris (1966) however, reported no binding of 2, 4-D with proteins in 7.5% acrylamide gels following electrophoresis of soluble proteins from 2, 4-D-C^{14}-treated pea seedlings.

Macey (1965) reported the release of bound forms of pectin methylesterase following treatment of artichoke tubers with 1 x 10^{-5} M 2, 4-D. Northen (1942) reported decreases in the structural viscosity of protoplasm following treatment of bean seedlings with IAA and NAA. Both of these mechanisms could conceivably result in the appearance of a "new protein in an electrophoretic pattern.

My data on the incorporation of radioactive alanine into proteins is consistent with the synthesis of new protein in band 0.41 but does not entirely exclude the mechanisms operative in the experiments of Macey (1965) and Northen (1942).

The appearance of band 0.41 following treatment with growth
regulating chemicals may be the result of \textit{de novo} protein synthesis, the release of bound proteins, or alteration of soluble protein conformation and charge. Whatever the mechanism, the appearance of the proteins in band 0.41 is in close temporal and spatial relationship with anatomical and morphological events which are triggered by exposure to certain growth regulating chemicals.

Band 0.41 was found in the root tips of untreated pea and squash seedlings, in the diminishing quantities with increasing distance behind the root tip of corn seedlings, and in all root sections of seedlings treated with 2,4-D, IAA, NAA, or picloram. It was also found in certain sections of pea seedlings treated with ethylene or 2,6-D and in sections below the cotyledons of control pea seedlings.

The significance of the presence of band 0.41 in all these cases is uncertain. There is no assurance band 0.41 contains the same proteins in all cases. Brewbaker \textit{et al.} (1968), Warner and Upadhya (1968), Mäkinen (1968), Mills and Crowden (1968), Alvarez and King (1969), and others, working with a diversity of plant species, plant parts, and extraction procedures have all reported the presence of more than one protein in many single bands following electrophoresis in acrylamide or starch gels. However, the consistent presence of band 0.41 in primary root sections which are actively elongating and in areas from which lateral roots will soon emerge is striking.

The proteins in band 0.41 must comprise a certain minimum
proportion of the total protein content in order for band 0.41 to be visible in acrylamide gels. The protein content was low in root sections near the cotyledons of control pea seedlings; so relatively small amounts of protein in band 0.41 could make it appear as a major component of the electrophoretic pattern. In the root tips of control pea seedlings, however, the protein content was high which means large amounts of protein must have been in band 0.41 in order for it to be visible. The appearance of band 0.41 in all root sections of 2, 4-D-, IAA-, NAA-, and picloram-treated seedlings represented a large increase in the relative amount of the total protein complement in band 0.41. Based on these suppositions, there may be a relationship between the amount of protein which bands with an $R_f$ of 0.41 and the magnitude of lateral root proliferation.

The identity and number of proteins which band with $R_f$ 0.41 is unknown, and the possibilities are numerous. However, the possible relationship between root elongation or lateral root emergence and the appearance of band 0.41 makes the work of Sutcliffe and Sexton (1968) of interest. They found an excellent correlation between the localization of Beta-glycerophosphatase and emergence of lateral roots in developing pea seedlings. Sutcliffe and Sexton (1968) found this enzyme increased to a maximum in sections 2 mm behind the root tip and then declined sharply. The concentration of enzyme increased steadily with distance in sections 10 to 40 mm behind the
root tip of pea seedlings (age 120 hours). The \( \beta \)-glycerophosphatase was localized in the cortical cells of the primary root which bordered the cells of the lateral root. High levels of this enzyme were also found in the surface layers of roots where radial expansion was occurring. Fan and Maclachlan (1967) and Datko and Maclachlan (1968) reported the appearance of large quantities of cellulase and pectinase in IAA-treated pea seedlings. Their data indicate increased levels of RNA synthesis preceded the synthesis of these hydrolytic enzymes. They feel these enzymes are important in the fragmentation of the walls of expanding parenchyma cells.

I do not know whether the proteins in band 0.41 in any of the experiments I have done are the same as the enzymes investigated by Sutcliffe and Sexton (1968), Fan and Maclachlin (1967), or Datko and Maclachlin (1968). However, sufficient quantities of the proteins in band 0.41 may be isolated by the procedure I have developed to permit their characterization.
CONCLUSIONS

The hypothesis of this thesis states, "Applying growth regulating chemicals to plants results in alteration of the interdependent course of selective protein biosynthesis and morphogenesis." The objectives were to: (1) Investigate changes in the protein complement which accompany morphogenesis induced by growth regulating chemicals, and (2) to determine whether changes in the protein complement were causally related to morphogenesis.

The hypothesis is partly true and the objectives have been partly satisfied. Biochemical differentiation was observed using the resolving power of gel electrophoresis. Qualitative and quantitative changes were detected in the complement of soluble cytoplasmic proteins from various root sections of developing pea, squash, and corn seedlings. The proteins with an $R_f$ value of 0.41 in 10% acrylamide gels were of particular interest. Band 0.41 was found only in the first few millimeters behind the root tips of control pea and squash seedlings and in diminishing quantities with distance behind the root tips of control corn seedlings. In pea seedlings, band 0.41 reappeared in root sections near the cotyledons prior to the emergence of normal lateral roots. It seems likely the proteins in band 0.41 are associated with the appearance of normal lateral roots and the enlargement or elongation of normal primary roots.
It may be significant that band 0.41 was found in all root sections of pea seedlings treated with 2, 4-D, IAA, NAA, and picloram and in root sections from corn and squash seedlings treated with 2, 4-D. The proliferation of massed lateral roots was a striking morphological response common to all these treated seedlings.

In terms of the original hypothesis and objectives then, this thesis has shown biochemical differentiation can be observed following application of growth regulating chemicals. It has also shown that, the timing and location of the appearance of proteins in band 0.41 were correlated with the emergence of lateral roots in both control and treated seedlings; however, there is no assurance the two events were causally related.

Several questions have been raised by this research. Does band 0.41 from the extracts of control and treated pea, corn, and squash seedlings contain the same proteins in all cases? How many proteins are in band 0.41? What are the proteins in band 0.41, and how are they related to the morphological responses of pea, corn, and squash seedlings to growth regulating chemicals? Does the appearance of band 0.41 represent de novo protein synthesis? If so, are changes in patterns of nucleic acid metabolism involved? The procedure I have developed for the isolation of proteins in band 0.41 can be used as a starting point for answering some of these questions.
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APPENDIX
APPENDIX

Each stock solution is numbered. Stock solutions used to prepare a particular working solution are identified by name and/or number.

1. Homogenization medium

Sucrose  
13.72 g

Trizma base (Sigma)  
0.242 g

CaCl₂  
0.033 g

1, 4-dithiothreitol (Cal Biochem)  
0.0015 g

HCl 1 N to pH 6.9 @ 0°C. dilute to 100 ml with distilled water

2. Dialysis medium

Sucrose  
512.9

HCl 1 N  
15 ml

CaCl₂  
0.441 g

1, 4-dithiothreitol  
0.046 g

Trizma base to pH 7.5 @ 25°C. dilute to 31 with distilled water.

3. Stock Solutions for Lowry Protein Determination

\[ \text{Na}_2\text{CO}_3 \]  
2.0 g

NaOH  
4.0 g

Dilute to 100 ml with distilled water
4. CuSO₄ . 5 H₂O  
   NaK Tartrate  
   Dilute to 100 ml with distilled water

5. 50 ml of 3 + 1 ml of 4 (prepare fresh)

6. Phenol reagent (Folin and Ciocalteu)  
   (Hartman and Leddon Co.)

Stock Solutions for Analytical Scale Electrophoresis

7. Upper acrylamide stock solution  
   Acrylamide  
   Bis  
   Dilute to 100 ml with distilled water

8. Lower acrylamide stock solution  
   Acrylamide  
   Bis  
   Dilute with water to 100 ml

9. Upper gel buffer  
   HCl 1.0 N  
   TEMED  
   Trizma base to pH 6.9 at 24°C  
   Dilute to 100 ml with distilled water
10. Lower gel buffer

HCl 1.0 N 48 ml
TEMED 0.1 ml
Trizma base to pH 8.9 at 24°C
Dilute to 100 ml with distilled water

11. Upper reservoir buffer stock solution

Glycine 28.8 g
Trizma base to pH 8.3 at 24°C.
Dilute to 1,000 ml with distilled water

12. Lower reservoir buffer stock solution

HCl 1.0 N 480 ml
Trizma base to pH 8.9 at 24°C

13. Ammonium persulfate stock solution

Ammonium persulfate 0.14 g
Dilute to 100 ml with distilled water.
This solution must be made fresh for each use.

Working Solutions for Analytical Gel Electrophoresis

Composition of 7.5% or 10% Analytical Gel

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>7.5% gel volume</th>
<th>10% gel volume</th>
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</thead>
<tbody>
<tr>
<td>Lower acrylamide (8)</td>
<td>2.0 ml</td>
<td>3.3 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.0 ml</td>
<td>1.45 ml</td>
</tr>
</tbody>
</table>
Lower gel buffer (10) 1.0 ml 1.25 ml
Ammonium persulfate (13) 4.0 ml 4.0 ml

Composition of the Stacking Gel

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper acrylamide (7)</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Upper gel buffer (9)</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Ammonium persulfate (13)</td>
<td>4.0 ml</td>
</tr>
</tbody>
</table>

Upper Reservoir buffer

Upper reservoir buffer stock solution (11) 100 ml
0.5% bromophenol blue in 95% Ethanol 1 drop
Dilute to 1,000 ml with distilled water.

Lower Reservoir Buffer

Lower reservoir buffer stock solution (12) 100 ml
Dilute to 800 ml with distilled water

Composition of Scintillation Solution

14. Triton X-100 (Harleco) 1800 ml
Toluene 2560 ml
Vacuum filter through 28-200 mesh Silica gel (Davidson, grade 14--dried overnight at 80°C in vacuo)
15. Omnifluor (New England Nuclear) 10 g
Toluene 100 ml
Mix 50 ml of 15 with 950 ml of 14 to prepare scintillation solution.

**Common Stock Solutions for Preparative Scale Gel Electrophoresis**

16. Acrylamide stock solution
Acrylamide 50 g
Dilute to 100 ml with distilled water.

17. Bis stock solution
Bis 1.2 g
Dilute to 50 ml with distilled water.

18. Lower Reservoir Buffer stock solution
Trizma base 121 g
HCl 10 N to pH 8 at 24°C
Dilute to 1,000 ml with distilled water.

**Special Stock Solutions for the Sieving-Stacker Single Gel System Electrophoresis**

19. Gel buffer stock solution
HCl 1.0 N 48 ml
TEMED 1.2 ml
20. **Upper reservoir buffer stock solution**

Glycine  
Trizma base to pH 8.3 at 24°C  
Dilute to 1,000 ml with distilled water.

21. **Ammonium persulfate stock solution**

Ammonium persulfate  
Dilute to 100 ml with distilled water.

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**Special Stock Solutions for the Stacking and Sieving Dual Gel System Electrophoresis**

22. **Upper gel buffer stock solution**

HCl 1.0 N  
TEMED  
Trizma base to pH 6.9 at 24°C  
Dilute to 100 ml with distilled water

23. **Dual gel elution buffer stock solution**

HCl 1.0 N  
Trizma base to pH 8.9 at 24°C  
Dilute to 1,000 ml with distilled water
24. **Upper reservoir buffer stock solution**

Glycine 144 g

Trizma base to pH 8.3 at 24°C

Dilute to 1,000 ml with distilled water

25. **Ammonium persulfate stock solution**

Ammonium persulfate 0.28 g

Dilute to 100 ml with distilled water.

26. **Composition of 10% sieving-stacker single gel**

**Gel Buffer (1:10 dilution of gel buffer stock solution (19))**

- Acrylamide stock solution (16) 3.4 ml
- Bis stock solution (17) 0.71 ml
- Distilled water 3.4 ml
- Ammonium persulfate (21) 8.0 ml

**Single gel Elution buffer**

- Gel buffer stock solution (19) 10 ml

Dilute to 1,130 ml with distilled water

**Upper reservoir buffer**

- Upper reservoir buffer stock solution (20) 100 ml

0.5% bromo-phenol-blue tracking dye in 95% ethanol 1 drop

Dilute to 1,000 ml with distilled water.
Lower reservoir buffer

Lower reservoir stock solution (18) 100 ml

Dilute to 1,000 ml with distilled water.

Working Solutions for Stacking and Sieving Dual Gel System

Composition of 15% sieving gel (lower gel)

Dual gel elution buffer stock solution (23) 1.0 ml
TEMED (4.6 ml/ml) 0.5 ml
Acrylamide stock solution (16) 2.4 ml
Bis stock solution (17) 0.15 ml
Distilled water 1.95 ml
Ammonium persulfate (25) 2.0 ml

Composition of 3.5% stacking gel (upper gel)

Upper gel buffer (22) 1.0 ml
Acrylamide stock solution (16) 0.56 ml
Bis stock solution (17) 0.2 ml
Distilled water 4.24 ml
Ammonium per sulfate (25) 2.0 ml

Upper Reservoir Buffer

Upper Reservoir buffer stock solution (24) 100 ml
0.5% bromophenol blue in 95% Ethanol 1 drop

Dilute to 1,000 ml with distilled water.
Lower Reservoir Buffer

Lower reservoir buffer stock solution (18)  100 ml
Dilute to 1,000 ml with distilled water.

Elution Buffer

Dual gel elution buffer stock solution (23)  100 ml
Dilute to 800 ml with distilled water.