
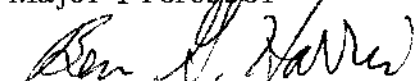


EFFECTS OF 4-<sup>2</sup>-CHLOROGLUTARANILIC ACID ON GROWTH  
AND DEVELOPMENT OF SUNFLOWER SEEDLINGS

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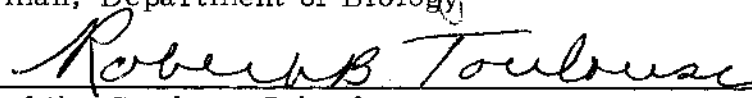
  
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Larsen, Stephen P., Effects of 4'-chloroglutaranilic Acid on Growth and Development of Sunflower Seedlings, Doctor of Philosophy (Cellular Biology), August, 1972, 113 pp., 13 tables, bibliography, 172 titles.

The potential growth-regulating compound 4'-chloroglutaranilic acid (CGA) was tested in whole-plant bioassay systems which utilized sunflower seedlings (Helianthus annuus, L.). Test systems included the growth of plants in soil, solid inert (Vermiculite) substrate, and hydroponic (Seed-Pak) pouches.

Concentration studies involving the foliar application of CGA to soil-grown plants indicated that an increase in growth inhibition is associated with an increase in concentrations. No additional inhibition or phytotoxic effects occurred using solutions of  $10^{-1}$  M or higher concentrations. Foliar applications of CGA produced growth inhibition which was detectable within twenty-four hours following initial treatment, and which persisted for at least thirty days. Application of CGA to roots of plants grown in a solid-inert substrate produced a growth inhibition which was 15 per cent greater than values reported for a foliar application of the same concentration. Foliar or root application of CGA at concentrations of  $10^{-5}$  M or greater produced

gross leaf morphology changes in all growth occurring subsequent to treatment. Total plant-height inhibition produced by CGA treatment was found to be principally associated with elongation inhibition of the stem area located between primary leaves and the shoot meristem. Concentration studies involving root application of CGA to hydroponic-grown (Seed-Pak) plants revealed that  $10^{-8}$  M solutions produced optimal growth enhancement and that  $10^{-3}$  M solutions are lethal. CGA was found to be 32 per cent less active than IAA as a growth promoting compound but was 76 per cent more active as a growth inhibitor at equivalent concentrations. The growth-enhancement capacity of CGA was equal to that of 2,4-D, but was a ten-fold less active inhibitor than 2,4-D. Comparative, quantitative plant-organ studies using the hydroponic (Seed-Pak) root application assay and  $10^{-4}$  M CGA solution revealed that the most severely affected organ was the leaf and that the least affected organ was the root system. Dark-grown plants cultivated in hydroponic (Seed-Pak) pouches, which received root application of  $10^{-8}$  M and  $10^{-4}$  M concentrations of CGA, exhibited both the enhancement and inhibition activity seen in light-grown plants. Growth inhibition produced by root treatment of  $10^{-4}$  M CGA is augmented by the addition of equimolar concentrations of glucose, sucrose, and 6-BAP and is blocked by GA and is not affected by IAA. Growth inhibition and enhancement produced by  $10^{-8}$  M and  $10^{-4}$  M

CGA applied to roots of Seed-Pak-grown plants is not related to the pH of test solutions. Leaves from soil-grown plants treated with a  $10^{-3}$  M foliar application of CGA possessed several abnormalities. These included an increase in thickness and dry weight, a reduction in chloroplast starch vacuoles, extractable starch, soluble hexoses, and soluble proteins.

Modification of the 4'-chloroglutaranilic acid molecule by reduction of the alkylcarboxylic acid carbon number from five to zero resulted in activity changes. When applied to roots at  $10^{-3}$  M concentration, all homologues except 4'-chloroaniline were lethal. At  $10^{-4}$  M concentrations, a decrease in the growth-inhibiting ability of the compounds paralleled a reduction in side-chain carbon number. At  $10^{-8}$  M concentrations, the odd-carbon-number compounds and 4'-chloroaniline possessed growth-augmentation activity; whereas even numbered side-chain acids demonstrated minimal activity.

EFFECTS OF 4'-CHLOROGLUTARANILIC ACID ON GROWTH  
AND DEVELOPMENT OF SUNFLOWER SEEDLINGS

DISSERTATION

Presented to the Graduate Council of the  
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By

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## CHAPTER I

### INTRODUCTION

Within the past few years there has been an obvious emphasis placed on the need for increased food production on a world-wide basis. A fundamental requirement necessary to accomplish the task of providing for increasing population nutritional needs is that of accumulating information in the areas of agronomy and plant physiology. Hopefully, from research efforts in the areas of basic and applied plant physiology will come answers that can provide man with the capabilities for increasing both the quantity and quality of plant food yields. It is recognized that green leaves are the world's largest source of protein and research is being conducted to remove economically the presence of indigestible fiber, undesirable flavor, and color (85).

A prerequisite to increasing the yield and quality of a plant tissue to be used as a source of nutrition is additional knowledge concerning those substances which alter the growth of plants.

A wide variety of both inorganic and organic substances have been found to evoke measurable or observable plant growth responses which can be considered both normal and abnormal in nature.

The quest for individual inorganic plant requirements and proper concentration mixtures which can produce physiological changes has an extensive past history. This period extends from Woodward's observations in 1699 (164) that plants grow better in muddy water than in rain water, to recent plant growth studies which indicate that growth modifying factors are present in extra-terrestrial materials of lunar origin (156, 154, 157).

From the time that Darwin's (35) experiments with grass coleoptile provoked thought in the minds of scientists such as Paal (118) about the possibility of a substance being produced in one part of the plant which could cause a response in another part of the same plant, the "race" was on. Since then, ambitious workers have been isolating, synthesizing, testing for, and accidentally discovering a number of organic compounds which can affect plant growth in various ways.

It soon became evident that a large number of organic compounds possessed biological activity in plant systems, and the phrase "plant growth regulator" became a popular expression for these compounds. Obviously high concentrations of strong organic acids would have an inhibitory effect on plant growth by direct tissue destruction and could be classified as plant growth regulators. However, the phrase "plant growth regulator" became more precisely

descriptive in nature when a definition was proposed by the American Society of Plant Physiology to include only those organic compounds which, in small amounts, inhibit, or in other ways modify, physiological processes in plants. Along with this statement, further attempts were made to standardize the nomenclature, including placing active compounds into 1 of 9 categories based on the type of plant response produced (147). Thoughtful efforts have since been made to establish a single acceptable term to describe organic compounds which affect or modify plant growth. A recent contribution by Steward (127) recognizes that many organic chemical agents act to produce observable or measurable changes in plant morphology and physiology and do so through complex metabolic schemes. He, therefore, proposed to name collectively such substances by employing the Latin noun moderamen, which denotes a means of moderating the management and control of direction. Because of the difficulty in ascribing a single physiologic activity to any one growth regulating compound, only a limited organization or categorizing of the substances has been accomplished. The following scheme is offered as an attempt to bring about the necessary organization to a current discussion of growth regulating compounds.

### Growth Regulating Compounds

Organic compounds which in small amounts ( $10^{-10}$ - $10^{-1}$  M) perceptibly modify physiological processes in plants

- A. **Phytohormones** - All naturally occurring organic compounds which have been isolated from viable plant tissue and produce some characteristic or series of characteristic physiologic responses based on established bioassays (23). The following compounds are included in this group.
1. **Auxins** - Substances which enhance the rate of cell elongation in plant tissue.
    - a. (indole-3)-acetic acid (84)
    - b. p-(indole-3)-propionic acid, (indole-3)-glyoxylic acid, and (indole-3)-glycolic acid (45)
    - c.  $\gamma$ -(indole-3)-butyric acid (24)
    - d. (indole-3)-pyruvic acid (160)
    - e. (indole-3)-acetonitrile (77)
    - f. (indole-3)-acetaldehyde (89)
  2. **Gibberellins** (88) - Identified with the ability to cause elongation of genetically dwarfed plants. Thirty-four currently recognized analogs, which are derivatives of the gibbane skeleton.

3. Cytokinins - Induce cell division. Are substituted adenine derivatives.
    - a. Zeatin (93)
    - b. Zeatin riboside (93)
    - c. Isopentyl adenosine (94)
  4. Inhibitors - Compounds which are associated with senescent organs and quiescent cells.
    - a. Abscisin II (Dormin) (1)
    - b. Tannins and related compounds (3,4,5-trimethoxybenzoic acid, chlorogenic acid, caffeic acid, ellagic acid, ferulic acid, transcinnamic acid, coumarin, and 2',3',4',3,4-pentahydroxychalcone) (31)
    - c. "Inhibitor B" (18)
    - d. New non-aromatic inhibitor (34)
  5. Ethylene - Induced in tissues by auxin application, and may alter the way tissues grow in presence of auxin (53).
- B. Synthetic Growth Regulators - Synthetic organic compounds which exert influence on plant growth and development. Following the identification of Indole acetic acid as a natural auxin, laboratories began to synthesize compounds which were similar to naturally occurring phytohormones but which possessed minor



modifications. These compounds were tested in plant bioassay systems to determine what similarities and differences in plant growth regulation might occur as a result of the molecular alterations.

Some of the most commonly known compounds which are highly active in modifying plant growth and to some degree mimic the qualitative response of one of the naturally occurring growth regulators are listed below.

1. 2,4-dichlorophenoxyacetic acid (17)
2. 2,4,5-trichlorophenoxyacetic acid (61)
3. 1-naphthalene acetic acid (NAA) (171)
4. 2-naphthoxyacetic acid (70)
5. 4-chloro-2-methylphenoxy acetic acid (MCPA) (113)
6. phenylacetic acid (172)
7. 2,3,6-trichlorobenzoic acid (2,3,6 TBA) (169)
8. N,N-D, methyl dithiocarbanylacetic acid (79)
9. Morphactins - Derivatives of fluorene-9-carboxylic acid (56)

C. Synthetic Growth Retardants - Synthetic organic compounds which retard stem elongation and increase green color of leaves without causing malformations of the plant. The most widely reported examples of this group include the following substances.

1. 2,4-dichlorobenzyl-tributyl phosphonium chloride  
(Phosphon) (121)
2. 4-hydroxyl-5-isopropyl-2-methylphenyl trimethyl ammonium  
chloride, 1-piperidine carboxylate (AMO-1618) (161)
3. (2-chloroethyl) trimethyl ammonium chloride (CCC) (145)

The above groups of synthetic compounds are representative of only a few of the organic substances that have been tested for phyto-biological activity. Literally thousands of compounds have been synthesized in the search for synthetic growth regulating substances. An example of this type of research is reflected in the work of Thompson et al. (144) in which 1,060 new chemical compounds were synthesized expressly for the purpose of use in plant growth regulation activity tests. Attempts to quantitate the growth-regulating activity of compounds were initiated in 1928 by Went in his design of the *Avena* curvature test. This test is based on the curvature induced in decapitated oat coleoptiles when the substance absorbed in agar blocks is placed asymmetrically on the cut surface. Since that time, many tests have been devised which detect and assess the physiological responses that occur when organs of plants or sections of plant tissue are exposed to growth regulators. These tests are the oat (*Avena*, sp.) or wheat (*Triticum*, sp.) straight growth test,

pea curvative test, tomato leaf epinasty test, tomato parthenocarpy test, root cutting elongation tests, dwarf corn and pea tests, tissue protein and chlorophyll tests, and seen germination tests (151). Two of the most recently proposed assays involve the hyponastic response of bean plant leaf blades (96), and a rapid, triple-test regime which includes a Chlorella and sorghum, cucumber, oat bioassay (86). Because of the differences in morphological characteristics and enzyme complements in different species of plants, there may be qualitative and quantitative responses in different plants to a single compound. Therefore, information regarding the effect of a growth regulating compound on a specific plant type is best obtained by an intact, growing plant assay (115).

Organic chemistry became a useful tool to the plant physiologist in that it could provide for the synthesis of a series of compounds that differed slightly from a biologically active parent compound. These compounds could then be tested for plant growth activity and a systematic analysis made to determine what molecular substituents are necessary for physiological activity. Results from these systematic studies strongly suggest that compounds which demonstrate a high degree of growth regulating capability usually include the following molecular substituents or configurations.

1. A carboxyl group. Although a compound may exhibit activity in the absence of carboxyl group in the molecule, it appears to be a necessary constituent for a compound to possess a high degree of growth regulating activity (148).

2. A ring system containing at least one double bond. This hypothesis has received support from experiments such as those conducted by Went (158) which involved the testing of compounds which were similar in structure with the exception of presence or absence of one double bond. The active substance had one double bond adjacent to the side chain.

3. Presence of a hydrogen atom on the carbon which is alpha to the carboxyl group. Studies which include isotope and halogen substitution of alpha hydrogen atoms indicate that this generalized rule for structural activity is a valid one (134, 58).

As part of a study designed to add more information to the growing input of data concerning molecular structure versus plant growth activity, Sargent (125) synthesized a series of organic, isoteric compounds which conformed to the previously described criteria for plant growth activity.

These compounds, O-[N-(p-chlorophenyl) carbamoyl]-3-hydroxy-propionic acid,<sup>1</sup> N-[N-(p-chlorophenyl) carbamoyl]-β-

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<sup>1</sup>The abbreviation CCH will be used for future reference to this compound.

alanine,<sup>2</sup> and 4'-chloroglutaranilic acid<sup>3</sup> (Figure 1), had not been tested for growth regulating activity in higher plants. Preliminary studies indicated that these compounds possessed some activity (90) and it was decided that additional experiments should be conducted to characterize further the effects of these substances in a whole plant assay.

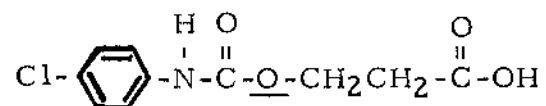
Selection of the plant species used for this research was based in part on the commercial importance and food source potential of sunflower plants (Helianthus annuus, L.). Sunflowers are fifth in world production of seed oils and it is probable that certain species will become useful as staples in plant proteins (128). Current studies are underway to establish sunflower seeds and leaves as an economical and nutritious source of fatty acids and protein for livestock (106).

Two basic approaches are being employed in an attempt to improve the nutritional characteristics of sunflowers. These include the treatment of seeds and whole plants with chemical compounds which might alter growth characteristics in the parent strain and

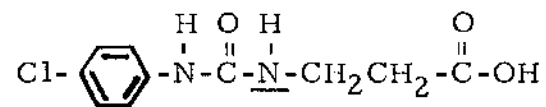
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<sup>2</sup>The abbreviation CCA will be used for future reference to this compound.

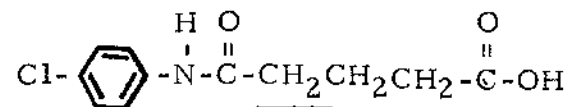
<sup>3</sup>The abbreviation CGA will be used for future reference to this compound.



O - N-(p-chlorophenyl)carbamoyl -3-hydroxypropionic acid (CCH)



N - N-(p-chlorophenyl)carbamoyl -β-alanine (CCA)



4'-chloroglutaranilic acid (CGA)

Fig. 1--Substituted isosteric compounds. These compounds are similar in construction, with the exception of oxy, amine, and methylene substituents occurring at the underscored positions.

subsequent generations, and which will result in plants of the M<sub>3</sub> generation acquiring some agriculturally valuable properties such as short stems, bigger diameter of their calathide, higher seed productivity, and higher husk and oil contents (135, 133). The other procedure is by conducting breeding experiments which would result in a more nutritionally desirable lipid and amino acid composition (81). Because of the increasing importance of sunflower plants as previously cited, and the availability of a new series of potential growth regulating compounds the following experiments were conducted and are reported herein.

The basic purpose of this research effort is to add a significant piece of data to the body of knowledge regarding plant growth regulation.

## CHAPTER II

### MATERIALS AND METHODS

#### Bioassay Method Employing Soil Grown Plants

Untreated seeds (Turtox 70VII) from sunflower plants (Helianthus annuus, L.) were planted in 23 x 26 x 8 cm wooden flats filled with composted soil which consisted of one part of peat moss and three parts dirt. Boxes were positioned so that soil surface received approximately 800 ft-c of light from fixtures located 15 cm vertically above the flats which contained four each, 40 w, F40D Westinghouse fluorescent lamps. Continuous illumination was provided throughout the growing period with light fixtures being raised to maintain an approximately constant distance of 15 cm between plant tops and light source. To insure a relatively equivalent illumination to all plants, boxes were rotated 180 degrees every 12 hours with respect to long axis of light tube source. Air temperature was  $26 \pm 2^{\circ}$  C. Five hundred ml of tap water was added to each flat at 12 hr. intervals. Ten days after seed implantation, plants were culled leaving a population of 20 plants per box which were allocated as 10 test and 10 control plants.



To quantitate the effect of test compounds as growth regulators, plant measurements were taken at 3 day intervals following treatment with a mm ruler using soil surface and stem meristem apex as reference points. Measurement precision was  $\pm 3$  mm. Values for the test and control plants were averaged and reported as average total plant height in cm or as per cent of control height values.

#### Bioassay Method Employing Seed-Pak Cultivated Plants

For all growth studies utilizing the diSPo<sup>1</sup> No. B1220 Seed-Pak growth pouch, the following procedure was followed: Untreated seed (Turtox 70VII) from sunflower plants (Helianthus annuus, L.) were planted and 20 ml de-ionized water were added. Three days following planting, pouches were examined for presence of ungerminated seeds which were removed. This procedure eliminated the necessity of pre-washing seeds with an antimycotic agent. Illumination method was the same as that described for soil grown plants with the exception that light distance to plant tops was maintained at 12 cm instead of 15 cm. De-ionized water was added to maintain an approximate volume of 20 ml per pouch. Seven days following

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<sup>1</sup>diSPo is a trademark of Scientific Products Division of Amer. Hosp. Supp. Corp.

planting, all pouches were drained and 30 ml of the test solution added to each pouch. Five days after treatment, 20 ml of a nutrient solution (107) were added to the existing pouch contents. Control plants received water and nutrient solutions only. Ten days following application of test solutions, plant measurements were taken using the top edge of the pouch seed trough and the stem meristem apex as reference points. Measurement precision was  $\pm 2$  mm. Values for plants were averaged and reported as average total plant height in cm or per cent of control height values.

#### Bioassay Method Employing an Inert-Solid Substrate

For growth studies involving Vermiculite substrate, conditions were the same as those described for soil grown bioassays with the exception of soil replacement with Vermiculite and nutrient solution being added 3 days following germination of seedlings and at 6 day intervals thereafter till completion of the experiment.

#### Preparation of Test Compounds and Solutions

The test compounds, CCH, CCA, CGA (Fig. 1), were prepared as described by Sargent (125). 4'-chlorosuccinanic acid, 4'-chloromalonanilic acid, 4'-chlorooxanilic acid, and 4'-chloroaniline (Fig. 13) were prepared by the methods of Barufinni (14), Barufinni (12), Barufinni (13), and Thompson (144) respectively. Test

solutions were prepared by dissolving sufficient quantity of a compound in de-ionized water to produce the desired concentration. All test solutions used for foliar application contained 0.1 per cent Tween 20 (polyoxyethylene (20) sorbitan monolaurate) to facilitate absorption. Compounds were applied by spraying leaves to point of wetness with a Kontes 40 ml atomizer.

#### Dark Growth Experiments

Non-treated sunflower seeds were allowed to germinate in a non-illuminated Sargent incubator at  $27^{\circ}\text{C} \pm 0.5^{\circ}$ , and at 4 days of age received test solutions. Two days later total plant height measurements were taken. In an effort to reduce the visible light energy spectrum to a minimum, all necessary plant manipulations were accomplished in the presence of a 50 watt tungsten bulb filtered through 3 layers of duPont 300 MSC red cellophane. Following the dark growth experimental period, plants were exposed to light conditions as previously described for Seed-pak growth studies, and observations made to detect the rate of etiolated plant greening.

#### Supplementation Studies

In the case of supplementation studies, Seed-Pak grown sunflower seedlings were drained of the aqueous solution at 7 days following seed germination and an inhibitory concentration of CGA

( $10^{-4}$  M) was added to the pouches. In addition, an equimolar concentration of either 6-BAP, GA, or IAA was added to the nutrient solution. Two per cent solutions of glucose and sucrose were also tested. Plant growth response was measured in terms of total plant height, and the results summarized in Table 7. If a supplemental test compound potentiated the inhibitory effect of CGA, it was reported as positive augmentation. If the test compound reduced or prevented the inhibitory effect of CGA, it was reported as positive blockage.

#### Physical Measurement and Visual Examination of Leaf Tissue

Physical measurements and examination of leaf tissue were accomplished by staining  $1\ \mu$  sections with Paragon metachromatic stain and taking measurements with a Nikon microscope fitted with a Unitron Ke 10X ocular micrometer and 20X Nikon objective.

#### Tissue Ultrastructure Examination

Electron micrograph studies of chloroplast ultrastructure were carried out in the following manner. Ten-day-old soil grown plants received foliar application of  $10^{-3}$  M CGA and 10 days following treatment, 1 cm leaf discs were punched from areas adjacent to the midrib. Tissues were fixed in a 5 per cent buffered solution of gluteraldehyde, impregnated with osmium tetroxide, dehydrated with ethanol, treated with propylene oxide and embedded in Epon 812 resin

(98). Sections were cut on a Porter-Blum MT-2 Ultramicrotome, mounted on 300 mesh grids, and stained with uranyl acetate and lead citrate. Specimens were examined with an RCA-36 electron microscope fitted with 250- $\mu$  condenser and 50- $\mu$  objective apertures. Two electron micrograph sections were prepared from each leaf sample making a total of 18 control and 20 experimental specimens examined.

#### Photography

Photographs appearing in Figures 3 and 4 were taken with a Yashica, J-5, 35 mm camera fitted with a 1:1.8 lens and a No. 2 extension tube. Film used was Kodachrome II, K135-20 and settings employed were f/4 at 1/125 sec.

#### Leaf Chemistry--Soluble Sugars

Ten-day-old soil grown plants received foliar application of a  $10^{-3}$  M concentration of CGA, and 10 days after treatment, 12 mm discs were punched from affected secondary leaves of treated and control plants. Immediately following cutting of leaf discs to be used in sugar analysis, samples were placed in a Boekel Model 1078 hot-air oven at 55<sup>o</sup> C to prevent enzymatic autolysis of tissue compounds. Weighings were taken at various intervals on drying leaf samples to determine time required to give constant dry weights. Precision error in weight of dry leaf samples was  $\pm$  0.05 mg. Dried leaf

samples were homogenized in 5 ml of distilled water with a Potter-Elvehjem Teflon grinder for 5 min at 3000 rpm and filtered through Whatman No. 5 paper. Microscopic examination of homogenate concentrates revealed no intact cells with debris consisting of cell wall fragments, trachied spiral elements and green spheroplasts. Filtrate concentrates examined showed amorphous debris of approximately  $7 \mu$  or less in size. Leaf sample homogenates were quantitatively tested for hydrolyzable reducing sugars by the microcolorimetric phenol-sulfuric acid method (38). One ml of homogenate solution was added to 1 ml of 5 per cent reagent grade phenol prepared with glass distilled water. To this mixture was added 5 ml of concentrated sulfuric acid. The mixture was incubated at  $27^{\circ}\text{C}$  for 20 min and color read on a Bausch and Lomb Spectronic 20 at  $490 \text{ m}\mu$ , which is the maximum absorption reported for hexoses and their methylated derivatives. To determine the precision of the colorimetric technique employed, seven 50 microgram glucose knowns were analyzed which yielded an average optical density of  $0.428 \pm 0.015$ . The Student's "t" test statistical model was applied to data using combined sample degrees of freedom ( $N_1 + N_2 - 1$ ) and probability level of 0.05. A test for the specificity of sugar assay employed was prepared by adding known amounts of glucose standard to solutions of control leaf tissue homogenates for which sugar values had been obtained. Total

sugar content in each mixture was calculated from known molar extinction coefficients, and compared to experimental data obtained.

#### Leaf Chemistry--Extractable Starch

Leaf tissue to be analyzed for starch content was obtained and homogenate samples prepared as described for the soluble sugar procedure. Starch separation from tissue homogenates was accomplished using a modified method as described by McCready (101). Five ml of 80 per cent EtOH was added to 5 ml quantities of homogenate and incubated at 70° C for 5 min. The sample was centrifuged at 2500 rpm for 5 min. Supernatant solutions were discarded and the residue was extracted by adding 10 ml hot (70° C) 80 per cent EtOH for 5 min. Samples were centrifuged again and the supernatant solution discarded. This extraction procedure was repeated 3 times to insure removal of soluble sugars from residue. The residue was treated with 3.5 ml 52 per cent perchloric acid to solubilize starch, incubated at room temperature for 15 minutes, and centrifuged at 2500 rpm for 5 min. The supernatant solution containing the digested starch extract was collected in a 25 ml volumetric flask and the step repeated using a 30 min incubation time. De-ionized water was added to the volumetric flask to bring the final solution up to 25 ml. One ml of this extraction solution was recovered following filtration through Whatman

No. 1 paper and exposed to the phenol-sulfuric acid colorimetric procedure previously described.

#### Leaf Chemistry--Starch Content vs. Growth Inhibition

Ten-day-old soil grown plants received foliar application of the test compound and leaf discs were punched at daily intervals from affected secondary leaves and analyzed for extractable starch. Daily total plant height measurements were also taken and recorded along with starch values in Figure 13. Data represents average of at least 6 treated and control individual starch analysis values for each daily interval and plant height measurements represent averages of triplicate experiments consisting of 10 plants each.

#### Leaf Chemistry--Soluble Proteins

Much reported information regarding plant proteins is based on measurement of the nitrogen content of protein; however, as nitrogen is found in cellular constituents other than proteins such as soluble amino acids and nucleic acids, it is necessary in nitrogen determinations to effect separation of the proteins from other nitrogen containing compounds. Also, it is reported that there is found to be a direct correlation between the amount of nitrogen which can be measured and the difficulty of the technique (74).



Several methods are reported as being "rapid" or "micro-techniques" but were rejected because they either require large amounts of material (in the range of 0.5 to 1 gm) or require protein extraction steps (15, 100, 114, 120, 122). After careful examination of the data reported by Lowry (97) on the Folin-phenol method of protein determination it seemed that this technique, by virtue of its specificity and lack of interference by various non-protein compounds, would be an acceptable procedure, if carefully validated.

Because of the problems related to certain biochemical tests in plant tissue as previously noted, it was necessary to validate the effectiveness of the Lowry test for protein by determining if the crude homogenate from plant leaves absorbed at the spectral frequency of 750 m $\mu$  which is used in the testing procedure. Leaf tissue samples obtained as previously discussed for soluble sugars were homogenized in 3 ml of distilled water at room temperature with a Potter-Elvehjem Teflon grinder for 5 min at 2500 rpm. Crude homogenate was then centrifuged at 3000 rpm for 15 min in an International Equipment Company Model CL clinical centrifuge. One ml aliquots were analyzed in a Coleman Model 124 Hitachi Double-Beam Grating Spectrophotometer fitted with a Model 165 Strip Chart Recorder. Conditions for the scan were chart speed 20 mm/min,

input 5 mv, and scan 800 to 220  $m\mu$  at a rate of 240  $m\mu$ /min. Results indicated that negligible absorbance occurred at the 750  $m\mu$  region.

A protein standard curve was established using Calbiochem crystalline, bovine, plasma albumin, grade A, Lot 802136, electrophoretically pure with a pH of 5.1 and moisture content of less than 5 per cent, and an ash and carbohydrate content of 0.05 per cent. Fifty, 75, 100, 250, and 500  $\mu\text{g}$  samples were prepared by dissolving an appropriate amount of crystalline albumin in distilled water. One ml aliquots of each standard were transferred to 15 x 120 mm tubes to which was added 1 ml of Lowry Reagent A which consisted of 1 ml 1% aqueous  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , w/v, 1 ml 2% Sodium Tartrate, w/v, and 100 ml 2%  $\text{Na}_2\text{CO}_3$ , w/v. After 10 min period, 5 ml of 1 N Folin-Ciocalteu reagent was added, allowed to incubate at 27 C for 45 min, and then per cent T recorded at a wavelength of 750  $m\mu$ .

One ml samples of control and test tissue homogenates were treated as described above and per cent T values obtained. Final values were expressed as  $\mu\text{g}$  of Bovine Plasma Albumin (BPA) protein equivalents per mg of leaf tissue dry weight. Precise optical density ( $L = 2 - \log 6$ ) against galvanometric readings (% T). Using the average molar extinction coefficient (E) calculated from standard curve data and the formula concentration equals optical density divided by the extinction coefficient E, concentration of

protein per 1 ml of tissue homogenate was obtained and multiplied times three to obtain the total amount of soluble protein resulting from homogenization of each sample leaf disc in 3 ml of distilled water. This value, representing the total amount of soluble protein, was then divided by dry weight of leaf samples to obtain the final reported value of  $\mu\text{g}$  of soluble protein BPA equivalents/mg of leaf tissue dry weight ( $\mu\text{g}$  BPA protein/mg dry weight).

To test for the specificity of the Lowry test for protein in crude leaf tissue homogenate an "addition" test was performed. This procedure consisted of testing a suitable number of leaf tissue specimens to obtain protein values and then adding a known amount of BPA protein to aliquot portions of leaf tissue homogenate sample. Theoretical values calculated from the test for protein in homogenate aliquots plus the amount of BPA protein added were recorded and compared to values obtained by testing addition samples. A comparison of calculated versus experimental values from tests reveals an average range of  $\pm 2.5 \mu\text{g}$  of differences. These data revealed that if a known amount of BPA protein ( $50 \mu\text{g}$ ) is added to a crude leaf tissue homogenate for which the soluble protein value has been determined ( $40 \mu\text{g}$ ), the resultant value of the "addition" mixture determined by the Lowry method will be  $90 \mu\text{g} \pm 2.3 \mu\text{g}$ . This information suggests that substances other than proteins in crude sunflower leaf

tissue homogenate do not greatly affect the specificity of the Lowry protein test. Analysis of three aliquot portions from an experiment consisting of six individual tissue homogenate samples gives good reproductibility of soluble protein values. The average range was  $\pm 2.8 \mu\text{g}$  of BPA protein equivalents.

#### Leaf Chemistry--Extractable Fatty Acids

CGA treated and control tissues were obtained as described for soluble hexose studies. Fresh tissue was weighed and ground in 5 ml of chloroform-methanol (2:1, v/v) using Potter-Elvehjem Teflon grinder for 5 min at 5000 rpm. The homogenate was centrifuged at 3000 rpm for 15 min and the supernatant solution collected. Resuspension of the sediment was carried out using an additional 5 ml of chloroform-methanol and the supernatant solutions pooled. Five ml of the extraction solution received 1/2 ml of a C<sub>17</sub> internal standard and was evaporated to dryness with nitrogen using a 40° C water bath. The C<sub>17</sub> standard consisted of 1 mg/ml of heptadecanoic acid (Applied Science Laboratories) in methanol. Two ml of 0.5 N NaOH in methanol was added to the residue and the closed tube placed in boiling water for 5 min. After cooling to room temperature, three ml of BCl<sub>3</sub> (10%)-CH<sub>3</sub>OH (Applied Science Laboratories) was added, and the closed tube was heated for 2 min at 100° C

(104). The tube was cooled and the contents transferred to a 30 ml separatory funnel. The screw-cap tube was washed with 10 ml of chloroform which was then added to the separatory funnel, followed by 3 ml of de-ionized water. The chloroform phase was evaporated with nitrogen and 0.5 ml of chloroform was added to the residue.

Two microliters of the sample were injected into an Aero-graph gas chromatograph (Varian Associates, Palo Alto, California; Model 204-1C) with a flame ionization detector. Columns (5 ft. x 1/8 inch) containing 15 per cent diethylene glycol succinate polyester on Chromosorb W (60/80 mesh) were operated at 180°C. Detector and injector temperatures were 225°C and the nitrogen flow was 25 ml/min respectively. Range was  $10^{-10}$  and attenuation was 16. Areas of peaks were calculated by multiplication of peak height by peak width at 1/2 height (69).

## CHAPTER III

### RESULTS AND DISCUSSION

#### Effects of Three New Isosteric Organic Compounds on Sunflower Seedling Growth

To determine the relative activity of CCH, CCA, and CGA as growth-regulating compounds, ten-day-old, soil-grown sunflower seedlings received a  $10^{-4}$  M foliar application of each test solution and total height measurements were taken at 3-day intervals. Three days following application of the compounds, measureable decreases in height had occurred in all test plants. 2,4-D treated plants served as a reference for relative potency of compounds tested. The least active compound, in terms of total height reduction as compared to controls, was CCH, which achieved 89 per cent of control plant values at 18 days following treatment (Fig. 2). CCH, CGA, and 2,4-D plants had reached approximately equivalent values of 75 per cent of controls by 18 days. However, at 3, 6, 9, 12, and 15 days, CGA-treated plant values were lower than all other compounds tested. At 12 days post-treatment, CGA-treated plants were maximally inhibited, having achieved only 60 per cent of control plant height values. Also noted at 12 days following treatment were observable

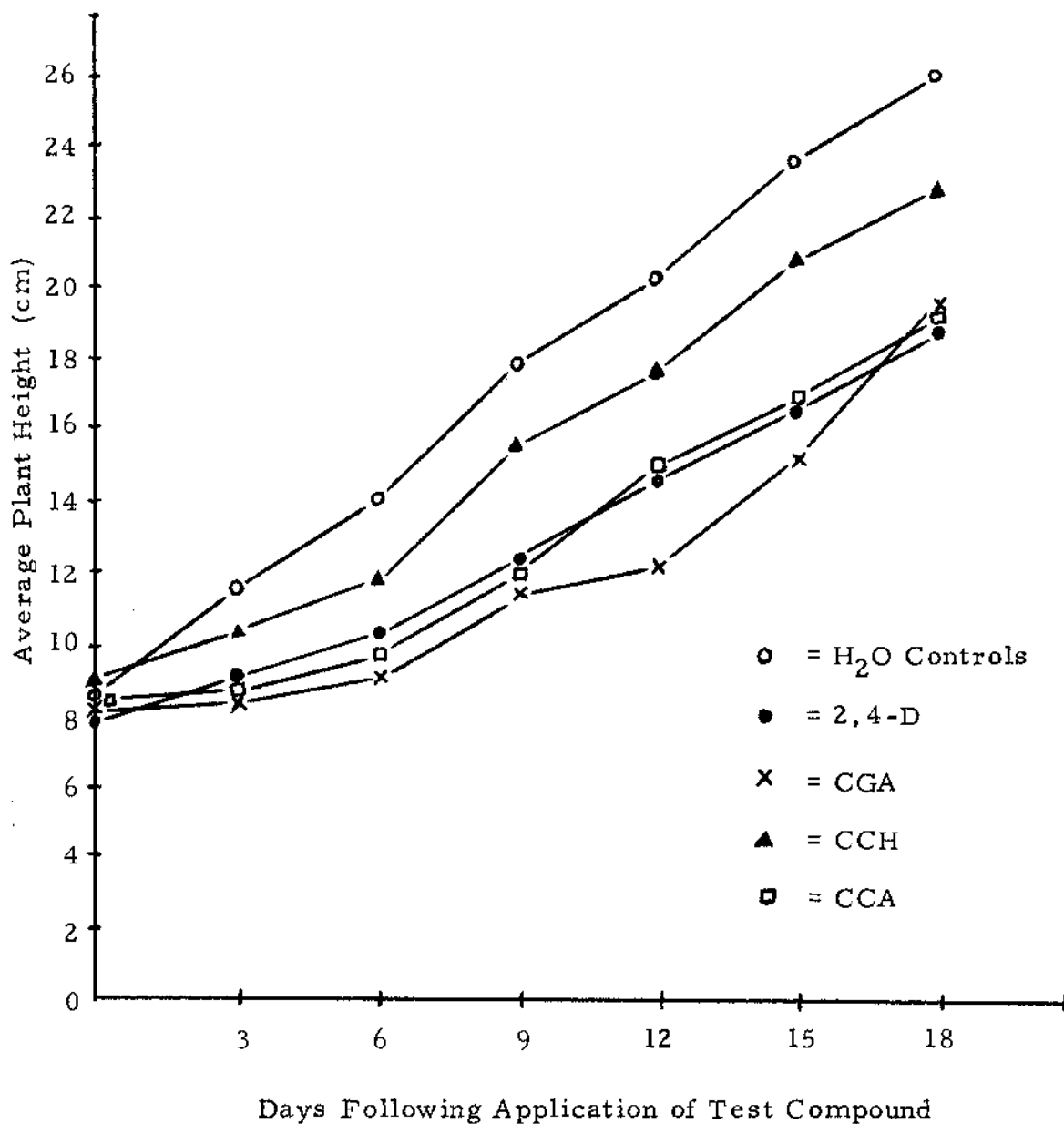


Fig. 2--Comparison of growth activity of three substituted isosteric compounds. Ten-day-old soil grown sunflower seedlings received a  $10^{-4}$  M foliar application of each test solution and total plant height measurements were taken at three-day intervals. A 2,4-D application was included as a standard reference for relative potency. Data represent average values for a minimum of 8 plants per compound tested.

changes in leaves of all CGA-treated plants. These changes were found to be almost exclusively associated with new foliar growth that occurred after the time of CGA application. Primary leaves which received foliar application were relatively unaffected. These leaf alterations include upward and inward curling of margins toward the midrib (hyponasty), hyperchromicity, and surface convolutions (Fig. 3). None of these characteristics were observed in control plant leaves (Fig. 4). A description of leaf surface changes which may be similar to those observed in CGA treated plants has been reported by Watson (155). The changes described by this author were noted in plants treated with auxin-type herbicides, and include reduction in size, and "crinkling" of leaves. "Crinkling" is described as an abnormal leaf growth which occurs when vein growth is more retarded than mesophyll growth and the latter tissue bulges out between the veins giving leaves a convoluted surface.

Epinastic leaf movements (curling down of leaf margins) has been frequently reported as having been observed when growth-regulating compounds have been applied to either the upper or lower leaf surfaces (7). This phenomenon is attributed to the more rapid expansion of cells on the upper leaf surface. Auxin-induced hyponasty has been observed and noted by several authors to have occurred in tomato, cotton, and spinach and bean plants (68, 95, 108, 124, 168).





Fig. 3--Treated sunflower seedlings. Affected secondary leaves display upward curled margins, hyperchromicity and surface convolutions. Primary leaves which received test compound are relatively unaffected. Ten-day-old soil-grown plants received foliar application of  $10^{-3}$  M 4'-chloroglutaranilic acid and photographs were taken 10 days following treatment.



Fig. 4--Control sunflower seedlings. Primary and secondary leaves of 20-day-old plants are shown.

Recently, Lippincott and Lippincott (96) completed a more detailed study of auxin-induced hyponasty on the bean leaf blade. They found that leaves are most sensitive to auxin shortly after they first unfold, and leaves which have grown to approximately 60 per cent or more of their ultimate surface area no longer give a hyponastic response. The activity was specific for auxin and was inhibited by anti-auxins such as trans-cinnamic acid and p-chlorophenoxyisobutyric acid. Ethylene and ethylene-generating compounds failed to induce hyponasty. The amount of leaf edge curvature was found to be roughly proportional to a concentration range of  $10^{-6}$  to  $10^{-3}$  M for indoleacetic acid. These findings are of interest because of the similarities of response in sunflower leaves treated with  $10^{-4}$  M CGA.

2,4-D-treated leaves developed abnormally and in a manner that was different from CGA-treated leaves. About 12 days following application with  $10^{-4}$  M 2,4-D, primary leaves which received the spray application developed scattered chlorotic spots, twisting of the leaf on the longitudinal (midrib) axis and downward curling of leaf margins (epinasty).

Under these experimental conditions, it appears that CGA is producing a morphological response in sunflower leaves which is similar to that produced by the naturally-occurring auxin

indoleacetic acid in bean leaves, and dissimilar to those produced by 2,4-D in sunflowers.

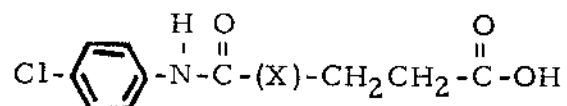
No perceptible leaf changes occurred in plants treated with CCH or CCA. None of the compounds tested at the  $10^{-4}$  M concentration produced growth augmentation or caused plants to exceed control height values. Further screening tests employing hydroponic-Seed-Pak-grown plants were conducted to determine if selective activity occurred following treatment with the isosteric substituted compounds CCA, CCH, and CGA. It was discovered that for the growth conditions described, CCA produced leaf changes similar to those associated with CGA treatment. However, noticeable changes occurred later than those produced by CGA. CCA was approximately 50 per cent as effective a plant height inhibitor. CCH did not alter leaf growth and had negligible effect on plant height inhibition. However, unlike CCA and CGA, both of which caused no observable changes in roots, CCH treated plants showed roots with a brown discoloration which began peripherally and extended acropetaly. This change was first noticeable at day 4, post-treatment, and progressed to obvious necrosis of root tips by day 10.

CGA produced characteristic leaf changes within 24 hours and was the most potent plant height inhibitor. These qualitative differences are summarized in Table I. The selective toxicity for

TABLE I

SELECTIVE ACTIVITY OF ISOSTERIC COMPOUNDS ON  
SUNFLOWER SEEDLINGS

A  $10^{-4}$  M concentration of each isosteric compound was applied to roots of 7-day-old, hydroponic-pouch grown plants. Observation of changes in leaves, roots, and total height values were recorded 10 days following application of compounds. The basic molecule is as follows, with substitutions being made at point in structure marked "(X)":



Substituent (X)	Activity		
	Leaves	Roots	Height
$-\text{NH}-^1$	+	-	+
$-\text{O}-^2$	-	+	-
$-\text{CH}_2-^3$	+	-	+

$^1 = \text{CCA}$

$^2 = \text{CCH}$

$^3 = \text{CGA}$

root tissue demonstrated by CCH may be due to the presence of oxygen in the substituted alkyl portion of the molecule. It has been reported that when the growth-regulating activity of ten methylchlorophenyl glycines was compared with that shown by the corresponding methylchlorophenoxyacetic acids, the active analogs with the -O- substituent were all superior to those with the -NH- group (28). A direct comparison in this case would be difficult because the alkyl substitution is adjacent to the ring which is not the case in the CCA, CCH, and CGA isosteres. Bioassays employed in this evaluation were the wheat cylinder, pea segment, and pea curvature tests. Oat coleoptile elongation tests employing CCA, CCH, and CGA at concentrations ranging from  $10^{-5}$  to  $10^{-4}$  M, consistently indicated that CCH was the most active and CCA the least active compounds (125).

#### Concentration Study--Foliar Application--Soil Grown Plants

As CGA demonstrated the greatest degree of qualitative and quantitative growth-regulating activity when subjected to the tests previously described, subsequent work was restricted to this compound. Experiments were conducted to further characterize the effects of CGA on sunflower seedlings. Concentration studies were performed on soil-grown, ten-day-old plants to determine the effects of increased concentrations applied via foliar application.

To facilitate leaf absorption (47) and to assist in the preparation of CGA solutions at high concentrations, the surfactant Tween 20 was added to all test solutions.

Evidence has accumulated which indicates that surface-active agents such as Tween 20 can interfere with bioassays, and in some cases, demonstrate growth-regulating activity (49, 72, 71, 109). It was felt that an experiment should be performed to determine if Tween 20 alone would augment or inhibit growth under the conditions employed for growth-regulation bioassays.

The concentration of Tween 20 experimentally employed was a ten-fold higher concentration than the 0.1 per cent which is recommended for aqueous solutions of test compounds (141). Results indicate that one per cent Tween 20 applied to leaves of sunflower seedlings grown under the conditions described does not produce significant changes in total plant height (Fig. 5). No observable changes in the aerial plant body were noticed. It was then assumed that at the 0.1 per cent concentration level used, Tween 20 would not be directly responsible for growth inhibitory activity observed in test plants.

Interpretation of the data for various concentrations of CGA (Fig. 5) leads to the conclusion that foliar application of the compound in increasing concentrations from  $10^{-3}$  M to  $10^{-1}$  M are

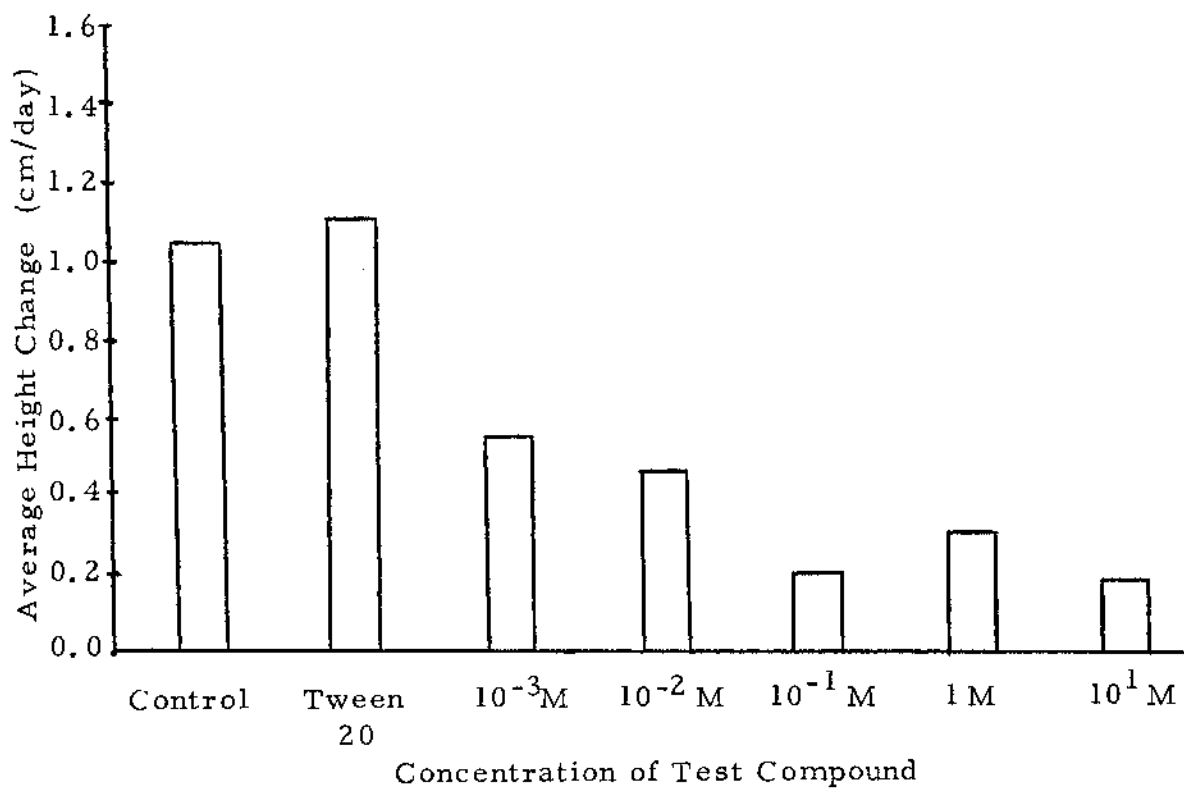


Fig. 5--Average change in height rates for sunflower seedlings treated with various concentrations of 4'-chloroglutaranilic acid and a 1 per cent concentration of Tween 20. Ten-day-old, soil-grown plants received foliar application of approximately 0.4 ml per plant of test solutions and daily total plant height measurements were recorded. Data represent average values for a minimum of 12 plants for each test.

accompanied by an increased inhibition of daily plant height growth. At the  $10^{-1}$  to  $10^1$  M concentration range no further increase in growth inhibition occurred. Also, no additional qualitative changes in the aerial plant body were observed, with the exception of a slight increase in leaf hyponasty, and leaf-surface convolutions as the test solution concentration was increased.

Failure of additional phytotoxic response at concentrations of  $10^{-1}$  to  $10^1$  M is thought to be due to decreased solubility of CGA at these concentrations in the formulation employed for these experiments. CGA is crystalizing as evidenced by white residue remaining on leaves upon drying, and in a crystalline form is apparently not capable of penetration of the outer leaf surface (33).

These concentration studies indicate that when an aqueous solution of CGA, containing 0.1 per cent Tween 20 is applied to leaves of sunflower seedlings, no additional growth response is obtained using concentrations of greater than  $10^{-1}$  M.

#### Growth Study--Foliar Application--Soil-Grown Plants

Experimental results which are depicted in Figure 6 establish two facts. These facts are, that within the limits of the experimental system employed, inhibition of growth is detectable as early as 24 hours following foliar application of CGA and that growth



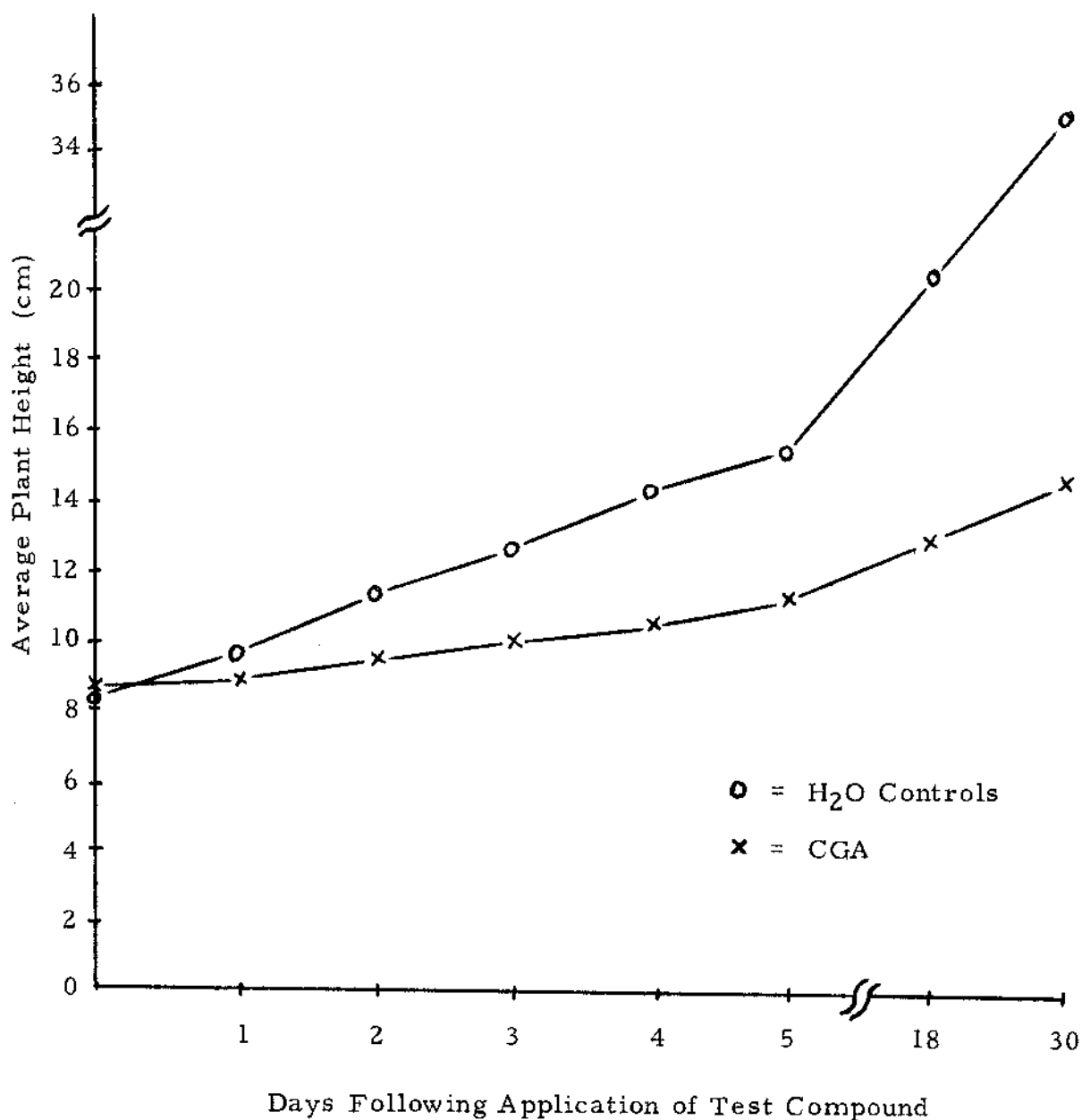


Fig. 6--Average change in total plant heights of sunflower seedlings treated with a foliar application of approximately 0.4 ml per plant of  $10^{-3}$  M 4-chloroglutaranilic acid. Ten-day-old soil-grown plants were treated and total plant height measurements taken at daily intervals up to 5 days and again at 18 and 30 days. Values were obtained from average figures for a total of 96 control and 89 treated plants.

inhibition continues to occur for a period of at least 30 days following treatment of CGA. At 30 days post-treatment test plants have achieved only 40 per cent of control plant values. This information suggests that CGA is capable of penetrating the leaf cuticular barrier and that the compound or its metabolite is translocated to sites of new growth, where it produces growth changes which are detectable within 24 hours.

Two factors appear to be of importance regarding the nature of a foliar penetrant. The importance of optimum balance between lipophilic and hydrophilic groupings has been implied (117, 62). This molecular "balance" is important in achievement of maximum leaf tissue penetration. Bennett (19) in reviewing the foliar absorptive behavior of a series of phytotoxic compounds noted an inverse relationship between the rate of absorbance and molecular size. It would appear that CGA possesses those molecular qualities necessary for relatively rapid foliar penetration in sunflower seedlings.

The persistence of CGA activity could be due to conjugation of the compound with plant constituents such as glucose, aspartic acid, polypeptides, proteins, or pectic acid, as has been reported for several phenoxyalkanoic acids in a variety of plants (143, 131, 6, 102).

Also, the sunflower may lack an enzyme complement that would be necessary for degradation of CGA as a detoxication mechanism.

#### Growth Study--Root Application--Vermiculite-Grown Plants

This study was performed to determine if CGA is absorbed through the root system, and if it would cause the occurrence of previously described growth changes that were observed for foliar application of the compound.

The data recorded in Figure 7 indicate that CGA was absorbed by the root system, and that the compound or its metabolite was translocated to the growing tissues of the aerial plant body and exerted a characteristic response similar to that described for foliar applications of the compound. This finding was not surprising in light of extensive reports on the absorption of organic compounds which show that many substances, including growth-regulating compounds, are readily absorbed by higher plant roots (46). However, this experimental information was of value in indicating that not only was root absorption occurring, but that the compound or its metabolite was acropetally translocated.

A diversity of mobility patterns occur in a variety of growth-regulating compounds. Literature reports indicate that certain

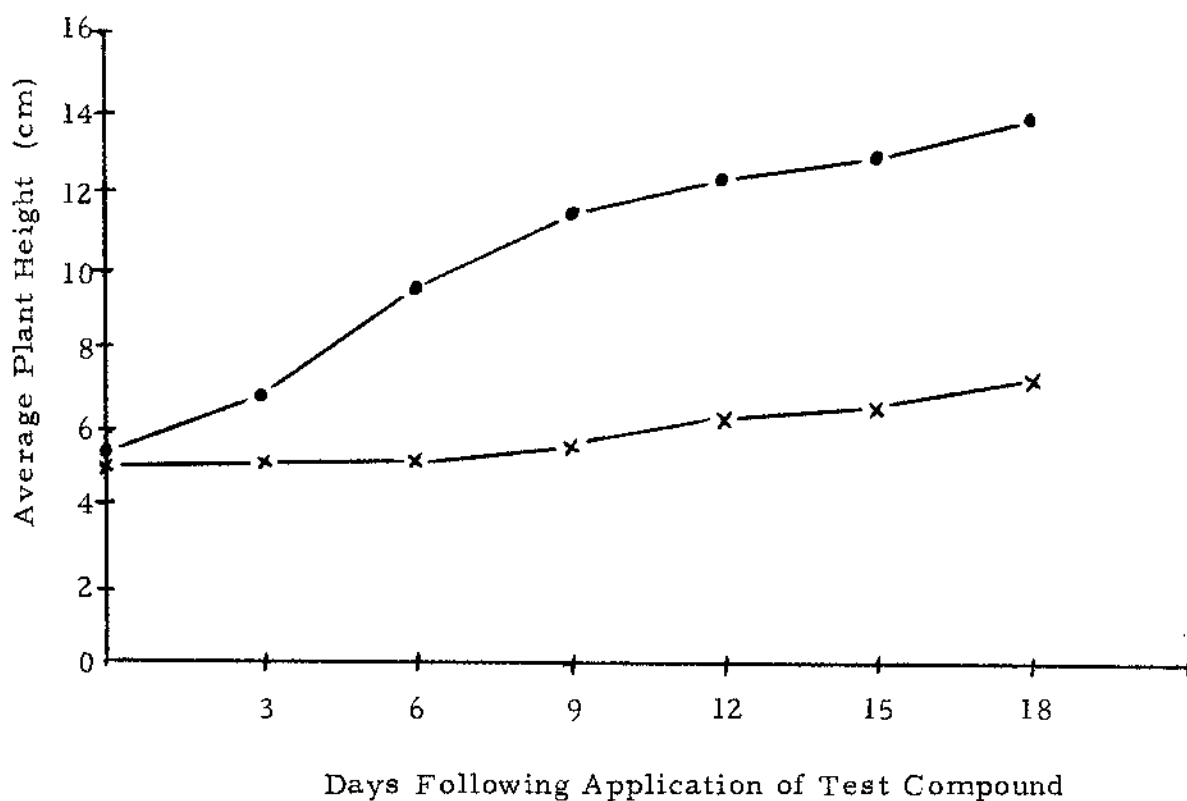


Fig. 7--Average change in total plant heights of sunflower seedlings treated via roots with approximately 20 ml per plant of  $10^{-3}$  M 4'-chloroglutaranilic acid. Ten-day-old plants maintained in a chemically inert (Vermiculite) substrate were exposed to the test solution by application to substrate, and measurements were obtained. Data represent average values for a minimum of 5 plants.

organic compounds tend to be accumulated very strongly in the metabolically active regions of the plant. Other substances are less mobile and tend to accumulate in the root portion of the plant body. Translocation studies have been conducted which include the use of  $C^{14}$ -labeled growth-regulating compounds (32). Those substances tested included 2,4-D, Amitrole, maleic hydrazide, urea, monuron, dalapon, simazine, and IAA.

It was discovered that all chemicals were highly absorbed by roots; 2,4-D was moved to plant tops in the least amount, urea was next, and the other seven were moved in larger quantities. Supportive evidence of CGA movement in the intact plant when applied via the root organ will have to come in the form of  $C^{14}$ -label experiments.

In addition, there was a 15 per cent greater inhibition than was observed for foliar applied CGA at an equivalent concentration.

#### Shoot Elongation Studies--Foliar Application--Soil-Grown Plants

To further characterize the action of CGA as a growth-regulator, experiments were conducted to determine if a specific portion of the plant longitudinal axis was being affected in the height-inhibitor response. Table II data established that while some elongation inhibition in the soil to primary-leaf-stem area occurs, the

TABLE II

SHOOT ELONGATION MEASUREMENTS ON HELIANTHUS ANNUUS,  
L. FIVE DAYS FOLLOWING FOLIAR APPLICATION OF  
 $10^{-3}$  M CGA<sup>1</sup> TO TEN-DAY-OLD SOIL-GROWN  
PLANTS

Measurement Method	Values <sup>2</sup>		
	Control (cm)	Treated (cm)	Differences in Treated vs. Control Plants (%)
Soil Level to Shoot Meristem	19	12.5	34 Decrease
Soil Level to Primary Leaves	10	9	10 Decrease
Primary Leaves to Shoot Meristem	9	3.5	61 Decrease

<sup>1</sup>4-chloroglutaranilic acid.

<sup>2</sup>Values were obtained from average figures for a total of 84 control and 78 treated plants. Three independent replicate experiments were performed.

principle shoot portion which fails to elongate is that region as measured from the primary leaves to shoot meristem (Fig. 8). This failure of stem elongation in the shoot apical meristem region results in a rosette pattern as subsequent leaf development occurs (Fig. 3).

It is well established that stem elongation or plant body extension is a function of collective shoot cellular enlargement (5). This process is initiated by the release from cells of an unidentified wall-loosening factor. This release apparently requires the presence of unstable transport protein (or proteins), a supply of energy and in some tissues, auxin must be present (29).

It can therefore be postulated that CGA is directly or indirectly acting to inhibit the action of cell wall-loosening factor(s), to interfere with the production or function of transport proteins, to reduce or to uncouple available endogenous energy supplies, or to behave as an anti-auxin which blocks the physiologic activity that is produced by necessary auxins. CGA may cause a reduction in total plant height by producing a lesion which affects one or a combination of these processes.

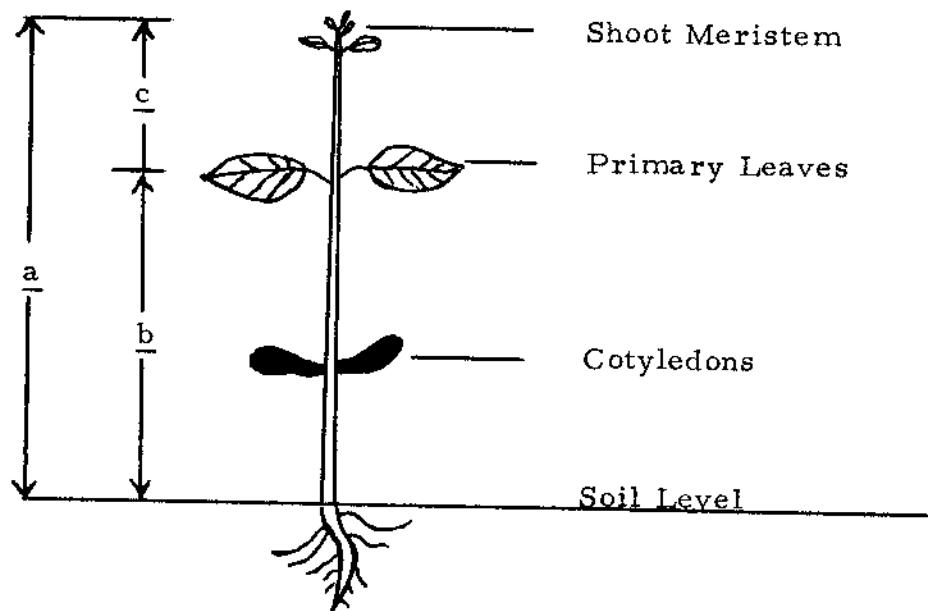


Fig. 8--Method of elongation measurements used to determine region of shoot affected by application of 4'-chloroglutar-anilic acid. Region a represents soil level to shoot meristem. Region b represents soil level to primary leaves. Region c represents primary leaves to shoot meristem.



CGA, 2,4-D, and IAA Concentration Studies--Root  
Application--Seed-Pak Grown Plants

The use of Seed-Pak growth pouches to hydroponically grow test plants has several advantages. These include closer control of concentrations applied to the intact plant and ready observation of root growth patterns. Also, augmentation or inhibition of root growth can be more readily quantitated. Therefore, a concentration study was undertaken to determine the effect of high and low concentrations of CGA on intact plant growth. The results presented in Figure 9 show a two-phase growth response curve for CGA and IAA. Concentrations of  $10^{-9}$ ,  $10^{-8}$ ,  $10^{-7}$ , and  $10^{-6}$  M CGA produced an increase in total plant height values as compared to controls of 15, 22, 18, and 10 per cent respectively. Concentrations of  $10^{-5}$ ,  $10^{-4}$ , and  $10^{-3}$  M CGA produced growth inhibition of 11 per cent and 36 per cent for  $10^{-5}$  and  $10^{-4}$  values, with the  $10^{-3}$  M solution being a quite active inhibitory concentration resulting in death for 50 per cent of the test plants within 3 days following initial treatment. No additional growth occurred in the balance of the plants up through termination of the experiment at 10 days.

IAA-treated plants showed marked increases in total height growth with values of 46, 54, 63, 58, and 23 per cent reported for  $10^{-9}$ ,  $10^{-8}$ ,  $10^{-7}$ ,  $10^{-5}$ , and  $10^{-4}$  M concentrations respectively. A

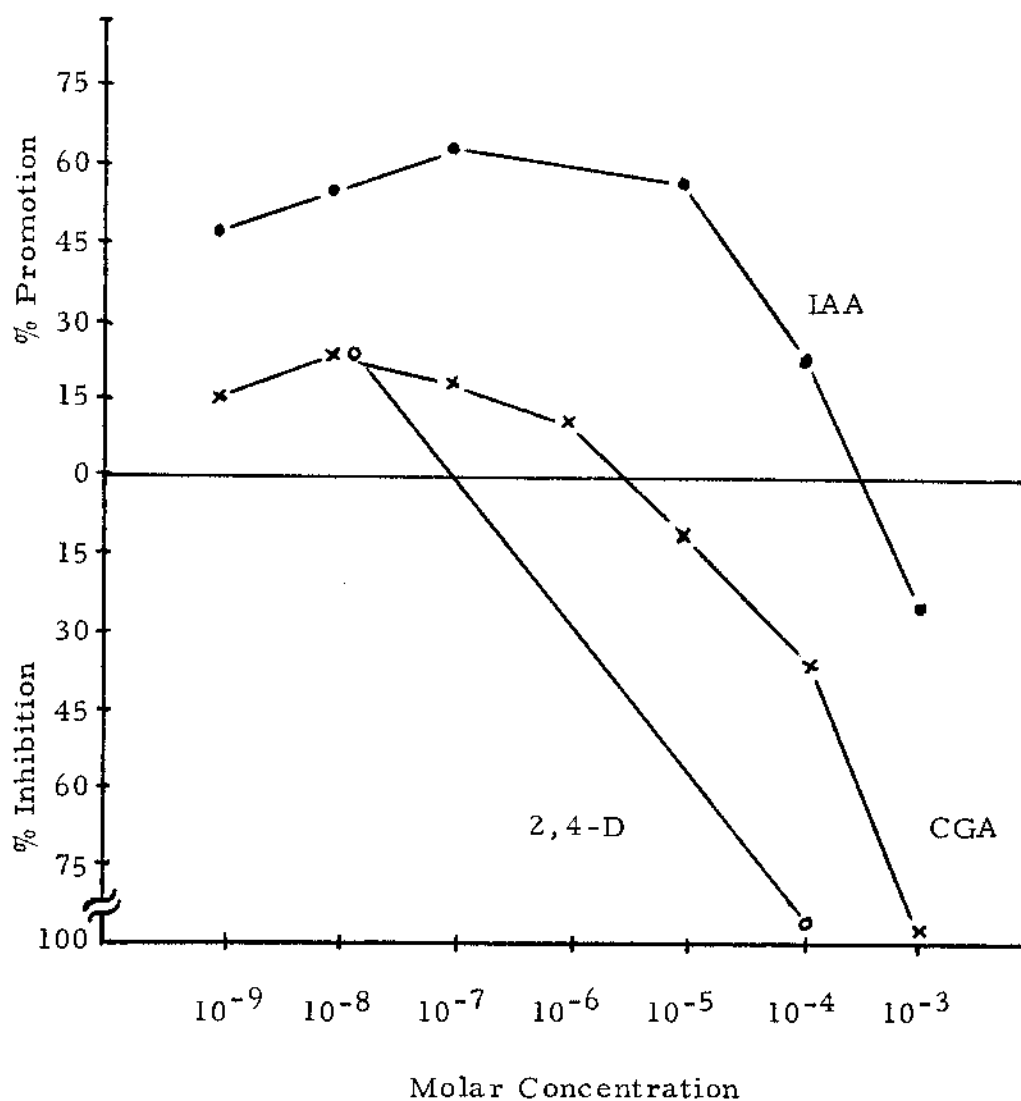


Fig. 9--Sunflower seedling growth response curves for various concentrations of 4'-chloroglutaranilic acid (CGA), indoleacetic acid (IAA), and 2,4-dichlorophenoxyacetic acid (2,4-D). Roots of 7-day-old hydroponic pouch-grown test plants were exposed to different concentrations of experimental solutions and total plant height recorded 10 days following treatment. All individual concentrations of test compounds were added to separate pouches containing 5 plants in equivalent volumes of 30 ml per pouch.

growth inhibition value of 24 per cent was recorded for a  $10^{-3}$  M solution of IAA.

A  $10^{-8}$  M concentration of 2,4-D produced an increase in growth values of 22 per cent, with the  $10^{-4}$  M solution proving to be lethal for 50 per cent of the test plants within 2 days following initial treatment.

This evidence showed that CGA is 32 per cent less active as a growth-promoting compound than is IAA at equivalent concentrations under the conditions tested, but the compound was 76 per cent more active than IAA as a growth inhibitor. The CGA concentration for optimum growth is about ten-fold less than the IAA value for optimum growth. Compared to 2,4-D, CGA demonstrated an equivalent growth-enhancing capacity and was a ten-fold less active growth inhibitor.

This two-phase growth response has been reported for roots, buds, and stems of peas (Pisum, Sp.) to a range of auxin concentrations with each organ having a promotive and an inhibitory range. Roots show the lowest optimum concentration ( $10^{-10}$  M), buds an intermediate value ( $10^{-8}$  M), and stems the highest concentration requirement of  $10^{-5}$  M (142). It is interesting to note that the optimum growth-promoting value for IAA applied to intact sunflower seedlings is  $10^{-7}$  M, which is an approximate intermediate

value between the concentrations necessary for optimum root and stem growth in some other plants.

#### Plant Organ Study--Seed-Pak Grown Plants

Having acquired the experience necessary for successful cultivation of sunflower seedlings in Seed-Pak growth pouches, a quantitative evaluation of growth and development of the major plant organs was undertaken using the optimum growth promotion and inhibitory concentrations of CGA determined by concentration studies (Fig. 9). Concentrations of CGA employed were those which had been shown to produce maximum growth promotion ( $10^{-8}$  M) and inhibition ( $10^{-4}$  M) without killing the test plants.

Table III data, which record total shoot length, are the result of a number of experiments and show that at  $10^{-8}$  M sunflower seedlings achieved a height of 17 per cent greater than that of the controls. At a  $10^{-4}$  M concentration, the average decrease in plant height was 33 per cent of the control plant values. This experiment also yielded information regarding the consistency of test plant responses. An average difference in values between the six replicate experiments was  $\pm 4$  per cent. This precision was not obtained with any isosteric compounds tested. Zimmerman (170) has found this information to be of potential value in the stunting of

TABLE III

SHOOT ELONGATION MEASUREMENTS OF SUNFLOWER SEEDLINGS  
TREATED WITH  $10^{-4}$  M 4'-CHLOROGLUTARANILIC ACID

Test compounds were added to the roots of 7-day-old sunflower seedlings grown in hydroponic pouches. Ten days following application of the compounds, total shoot length measurements were taken.

Concentration of Test Compound	Shoot Length <sup>1</sup> (cm)	Differences in Treated vs. Control Plants (%)
H <sub>2</sub> O Controls	5.7	-
$10^{-8}$ M	6.7	17 Increase
$10^{-4}$ M	3.8	33 Decrease

<sup>1</sup>Data for each test is an average of values from 6 replicates consisting of 10 plants per replicate.

sunflowers, and is in possession of a quantity of CGA for further studies to examine its effect on plants that have grown to maturity and also to study mutagenic effects of the compound.

Table IV, which presents data regarding inhibition on elongation of roots, shows that the concentration which produces shoot growth enhancement does not significantly increase intact root lengths. However, the concentration which is inhibitory for shoot elongation is also somewhat inhibitory for root elongation.

Previous studies suggested that the CGA shoot growth enhancement and inhibition had also altered leaf size. Data reported in Table V reveal that the concentration of CGA which produces shoot growth enhancement also produces a 51 per cent increase in approximated leaf surface area values. This finding had practical implications in that if a very weak concentration of CGA could produce a significant increase in leaf size, the food potential of sunflowers might be enhanced by commercial application of CGA.

The plant organ which is most severely affected by non-lethal doses of CGA is the leaf. A 60 per cent decrease in approximated leaf surface area results from treatment with a  $10^{-4}$  concentration of the test compound (Table V).

TABLE IV

ROOT ELONGATION MEASUREMENTS OF SUNFLOWER  
SEEDLINGS TREATED WITH  $10^{-4}$  M  
4'-CHLOROGLUTARANILIC ACID

Test compounds were added to the roots of 7-day-old sunflower seedlings grown in hydroponic pouches. Ten days following application of the compounds, plants were removed from growth pouches, the roots extended and measurements taken.

Concentration of Test Compound	Root Length <sup>1</sup> (cm)	Difference in Treated vs. Control Plants (%)
H <sub>2</sub> O Controls	19.6	-
$10^{-8}$ M	20.0	2 Increase
$10^{-4}$ M	17.3	13 Decrease

<sup>1</sup>Data for each test present an average of values obtained from 10 plants.

TABLE V

LEAF SIZE MEASUREMENTS OF SUNFLOWER SEEDLINGS  
TREATED WITH  $10^{-4}$  M 4'-CHLOROGLUTARANILIC ACID

Test compounds were added to the roots of 7-day-old sunflower seedlings grown in hydroponic pouches. Ten days following application of the compounds, primary leaves were removed, measured across the length (L) and width (W) and an approximation of leaf surface area calculated (L x W).

Concentration of Test Compound	Approximation of Leaf Surface Area ( $\text{cm}^2$ )	Differences in Treated vs. Control Plants (%)
H <sub>2</sub> O Controls	3.5	-
$10^{-8}$ M	5.3	51 Increase
$10^{-4}$ M	1.4	60 Decrease

<sup>1</sup>Data for each test present an average of values obtained from 10 plants.



### Dark Growth Studies --Seed-Pak Grown Plants

Reports indicate that a variety of growth-regulating compounds require the presence of light to exert their inhibitory or growth promoting activity. These compounds include kaurenoic acid, 1-naphtylacetic acid, 2,4-D, abscisic acid, indoleacetic acid, kinetin, haloxydine, paraquat, atrazine, amitrole, sirmate, simazine, pyridine analogs (82), and pyrazon (48).

An experiment was conducted to determine if the inhibitory and enhancing effects of CGA on shoot elongation would occur in dark-grown sunflower seedlings. Concentrations employed were the same as those which produced optimal changes in Seed-Pak cultivated plants grown in the light. Data summarized in Table VI show that a  $10^{-8}$  M concentration produces a 10 per cent growth promotion as compared to 17 per cent seen in light-grown plants. It is also of interest to note that a  $10^{-4}$  M concentration of CGA produces the same amount of inhibition (30 per cent) as compared to 33 per cent for light-grown plants.

Dark-grown sunflower seedlings treated with a growth-inhibitory concentration of CGA ( $10^{-4}$  M) were returned to the same light source used for previous growth studies. Observations were made at 2-hour intervals up to 48 hours to determine if the rate of development of green coloration of cotyledons, primary leaves, and

TABLE VI

DARK-GROWN SUNFLOWER SEEDLING RESPONSE TO VARIOUS  
CONCENTRATIONS OF 4'-CHLOROGLUTARANILIC ACID

Four-day-old hydroponic pouch grown seedlings, germinated in an unlighted growth chamber, received root application of test solutions, and total plant height measurements were recorded 2 days following application of compounds.

Concentration of Test Compound	Shoot Length <sup>1</sup> (cm)	Differences in Treated vs. Control Plants (%)
H <sub>2</sub> O Controls	6.1	-
10 <sup>-8</sup> M	6.7	10 Increase
10 <sup>-4</sup> M	4.3	30 Decrease

<sup>1</sup>Data represent values for an average of 15 plants for each concentration studied.

shoots was inhibited as compared to control plants. The rate of green color development was determined to be approximately equivalent in treated and control plants.

The greening of dark-grown, etiolated tobacco tissue has been correlated with plastid replication and growth, chlorophyll synthesis, accumulation of soluble proteins, synthesis of lipids, and development of internal membranes (25, 59).

These findings indicate that the accelerated and inhibited development of shoot tissue produced by CGA in sunflower seedlings is not a light-dependent reaction. Further, it is proposed that at the concentration levels studied, CGA does not markedly interfere with plastid development and chlorophyll synthesis.

Auxins have been reported to exert their principal growth-regulating activity of cell elongation in bioassay systems which exclude light (51, 52). Therefore, the dark-growth studies conducted on CGA suggest an auxin-like activity for this compound.

#### Supplementation Studies -- Root Application CGA -- Seed-Pak Grown Plants

A procedure which has been employed in experiments designed to characterize new growth-regulating substances is that of bioassay supplementation with known phytoactive compounds (36, 103, 91). Pouch-grown sunflower seedlings were exposed to an inhibitory

concentration ( $10^{-4}$  M) of CGA. In addition, equimolar concentrations of glucose, sucrose, 6-BAP, GA, and IAA were added to the test solution. The results are summarized in Table VII.

When only 2 per cent glucose or sucrose was added to pouch-grown sunflower seedlings, growth changes were negligible. However, when a 2 per cent glucose or sucrose solution was used to make up a  $10^{-4}$  M CGA test solution, significant inhibition was recorded at 48 hours following treatment and at 8 days all plants were dead. It was anticipated that if the CGA growth inhibition process was acting by some mechanism which disturbed the availability of hexose nutrients, an exogenous source of carbohydrate such as sucrose or glucose would alleviate the response. This was not the case; in fact, augmentation of the inhibitory processes occurred. The failure of sucrose or glucose to reverse or block the CGA inhibitory effect may indicate that the stunting process is not related to a reduced tissue hexose content. The augmentation phenomenon demonstrated by the presence of glucose and sucrose finds some support in work reported by Farr (42) and Moore (110) who found that 2 per cent solutions of these hexoses potentiated the effects of IAA on oat coleoptile segments and suppressed rooting and senescence in radish (Raphanus sativus, L.).

TABLE VII

EFFECT OF EQUIMOLAR CONCENTRATIONS OF VARIOUS  
COMPOUNDS ON 4'-CHLOROGLUTARANILIC ACID  
GROWTH INHIBITION

A mixture consisting of the test compound and 4'-chloroglutaranilic acid at  $10^{-4}$  M concentrations was added to roots of 7-day-old hydroponic pouch-grown sunflower seedlings. Observations were made 10 days following application of compounds.

Compound	Response <sup>1</sup>		
	Augmentation	Blockage	No Effect
Glucose	+		
Sucrose	+		
6 BAP <sup>2</sup>	+		
GA		+	
IAA			+

<sup>1</sup>Response = reduction of total plant height values.

<sup>2</sup>6-Benzylaminopurine.

6-BAP is a synthetic cytokinin which mimics the activity of naturally occurring compounds (129). While the principal role of cytokinins is one of stimulating cell division and growth, it has been established that various ratios of kinetin to auxin produces a variety of morphogenic phenomena (130). In typical cell elongation bioassay systems, kinetin tends to inhibit auxin-stimulated longitudinal growth (105). Plants treated with  $10^{-4}$  M 6-BAP showed a 40 per cent reduction in total height, whereas the same concentration of CGA produced a 33 per cent decrease in height values. A test mixture of equimolar concentrations of 6-BAP and CGA applied to test plants resulted in negligible growth and death of all plants at 5 days. The simultaneous application of 6-BAP and CGA results in an additive effect of the stem elongation and cytotoxic potential of each compound at the concentrations studied.

Gibberellin produces its most dramatic growth-regulating effect on stem elongation in intact plants, and this activity is not restricted to genetically dwarfed plants (119). A  $10^{-4}$  M concentration of GA tested resulted in a 48 per cent increase in plant heights. Supplementation of the growth-inhibiting CGA test solution with an equimolar concentration of GA resulted in plants which attained heights that were equivalent to control plant values. This blockage of the inhibiting properties of CGA by gibberillic acid tempts one to

assign the role of an anti-gibberillin to CGA. However, this proposed property of CGA is probably not the only activity of this compound as evidenced by the occurrence of the characteristic CGA leaf changes seen in GA supplemented test solutions. Most active growth-regulating compounds produce more than one physiological response in bioassay systems. The stem elongation inhibition and leaf morphology changes produced by CGA are an example of this generalization.

The possible anti-auxin properties of CGA were challenged by supplementation of test solutions with IAA. Plants treated with IAA alone grew to heights that were increased 23 per cent above control plants. Supplementation of test solutions with an equimolar concentration of IAA did not affect the demonstrated inhibitory effect of CGA and produced plants which had an average height reduction of 28 per cent as compared to controls. IAA did not protect against CGA induced leaf changes as was also the case with hexoses, 6-BAP, and GA. These findings which show a failure of IAA to block the effects of CGA in intact plants suggests that CGA is not an anti-auxin-like compound. However, oat coleoptile elongation studies revealed that in this test system, IAA could produce a 90 per cent reversal of CGA-induced inhibition of elongation (125).

## Leaf Studies -- Physical Measurements

Leaves taken from sunflower plants which had been treated with a  $10^{-3}$  M foliar application of CGA were physically examined for an explanation to the morphological changes previously described from gross observations. These tests included thickness measurements, weighing of leaf discs, and examination of cross sections by light microscopy. Data on weight and thickness measurements are summarized in Table VIII. The thickness of treated leaf tissue taken from two different locations was increased by a value of 45 microns as compared to controls. An average increase in wet weight of 10 mg accompanied the increased thickness of treated tissue. Microscopic examination of treated tissue preparations showed no major disruptions in tissue architecture, nor was any cellular pleomorphism evident. Even though no obvious accumulations of extracellular fluid could be detected by microscopic examination, one gets the impression from low power (10X) examination that an increased succulence accompanies treatment of leaves with inhibitory levels of CGA. It has been reported that when sublethal doses of the growth-regulating compounds simazine and diuron are applied to the soil of pot-grown sunflower seedlings, a measurable increase in leaf water content occurs (55). Also, studies conducted on the growth of pine (Pinus rosenosa, L.) seedlings subjected to sublethal concentrations of



TABLE VII

PHYSICAL MEASUREMENTS OF LEAF SAMPLES FROM  
SUNFLOWER SEEDLINGS TAKEN TEN DAYS  
FOLLOWING APPLICATION OF  $10^{-3}$  M  
4'-CHLOROGLUTARANILIC ACID

Ten-day-old soil-grown plants received foliar application of the test compound and 12 mm leaf discs were punched from two different locations of secondary leaves for analysis

Tissue Tested	Thickness ( $\mu$ )	Wet Weight (mg)
Control - midrib	158	21.2
Control - peripheral vein	155	20.1
Treated - midrib	201	32.3
Treated - peripheral vein	200	30.3

2,4-D developed abnormal division and expansion of cotyledon and leaf mesophyll cells (165). It is possible that the increase in weight and thickness of CGA treated leaf disc samples is due to the abnormal accumulation of water. However, when a quantity of 1 cm treated and control leaf discs were dried in a hot air oven to constant weight, treated discs were from 28 to 40 per cent heavier than controls. This finding indicates that the increased weight is due to an increase in dry weight matter in treated leaf samples. This increase in dry weight could reflect the presence of additional mesophyll tissue present in convoluted leaf tissue samples.

#### Leaf Studies--Chloroplast Ultrastructure

It has been reported that some of the most active plant growth inhibitors act by disruption of the Hill reaction. These compounds are substituted amides which have the general formula

$$R_1-N-\overset{H}{\underset{O}{\parallel}}-C-R_2,$$

where  $R_1$  is an aromatic ring dichlorinated in positions 3 and 4, and  $R_2$  is an alkyl amino or alkyl group (112). As the compound 4'-chloroglutaranilic acid possesses this general formula, electron micrographs were made of treated leaf tissue to determine if a corollary existed between growth inhibition and observable disruption of the chloroplast ultrastructure. Treated tissue chloroplasts showed normal biconcave shapes, orientation parallel to cell walls,

presence of grana oriented parallel to long axis of chloroplasts, intact grana, periodicity of thylakoid membranes, and intact thylakoid and intergranal membranes (Fig. 10). These characteristics are all considered normal for chloroplasts found in leaves of higher plants (92). A major observable difference between control tissue and CGA treated leaf chloroplasts was an absence or decrease in the number of starch vacuoles (Figs. 11 and 12). It was felt that this observation should be quantitated and a number of specimens were examined for the presence or absence of starch vacuoles. The average per cent occurrence of starch granules in CGA treated leaf chloroplasts was 11 per cent as compared to a 60 per cent value for control tissue (Table IX).

Relatively little information has been assembled which describes ultrastructural tissue changes associated with growth inhibition caused by application of growth-regulating compounds. It has been reported that 3-amino-s-triazole (amitrole) blocks the development and structural differentiation of proplastids in wheat (Triticum aestivum, L.) (9). Examination of leaves from 3,4-dichlorobenzylmethylcarbamate (dichlormate) treated wheat showed no granafret membranes or ribosomes in their chloroplasts (10). Klein (83) described alterations in chloroplast fine structure that occur following growth inhibition caused by 3-(p-chlorophenyl)-1,1-



Fig. 10--Chloroplast from treated sunflower leaf mesophyll tissue indicating apparently intact internal fine structures. X 8,500. Ten-day-old plants received foliar application of  $10^{-3}$  M 4-chloroglutaranic acid and leaf samples were taken for electron micrograph study 10 days following treatment.



Fig. 11--Chloroplasts from control sunflower leaf mesophyll tissue showing presence of multiple starch vacuoles in 20-day-old plants. X 6,000.

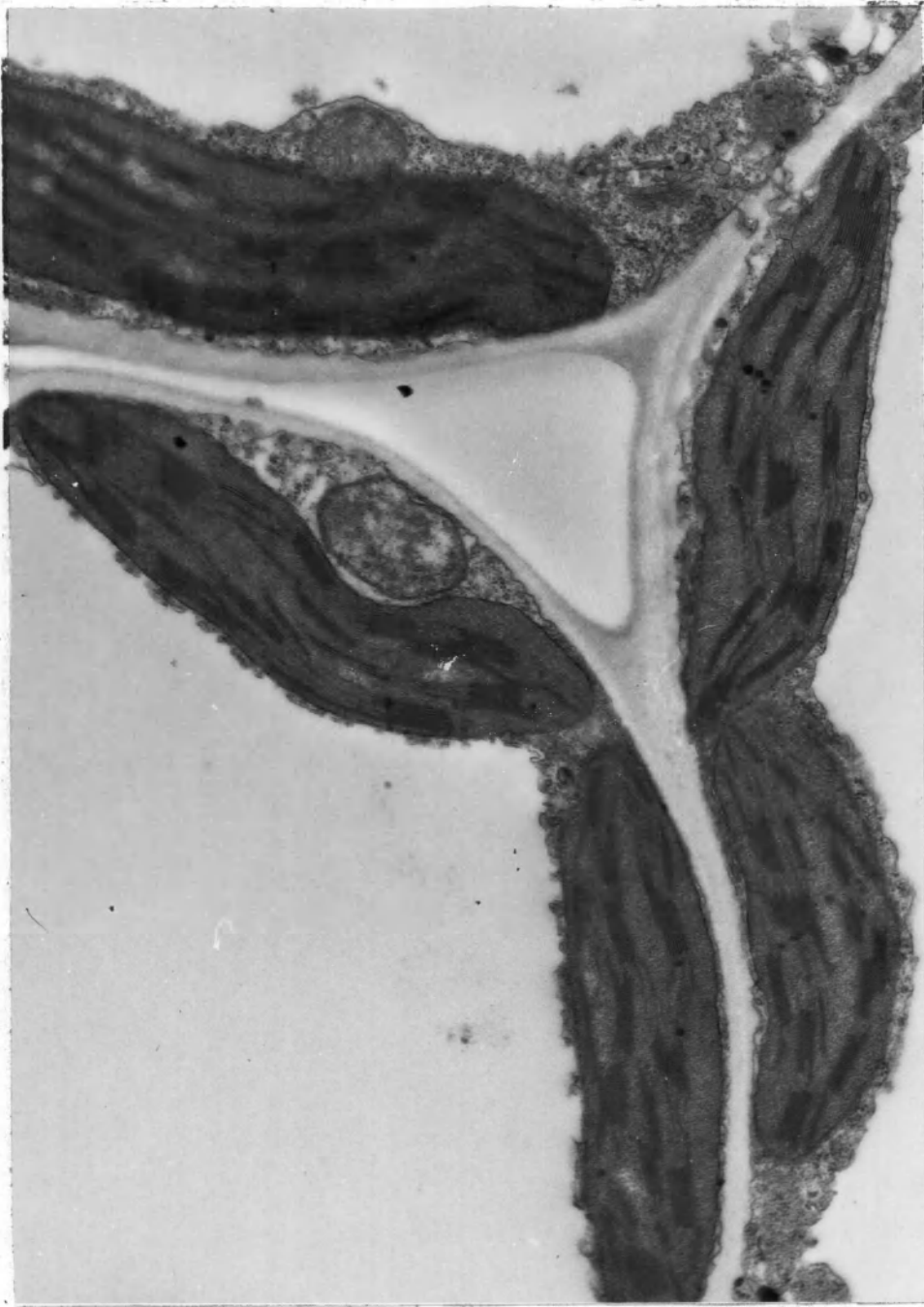


Fig. 12--Chloroplasts from treated sunflower leaf mesophyll tissue showing absence of multiple starch vacuoles. X 6,000. Ten-day-old plants received foliar application of  $10^{-3}$  M 4-chloroglutaranilic acid and leaf samples were taken for electron micrograph study 10 days following treatment.

TABLE IX

PER CENT OCCURRENCE OF STARCH GRANULES IN CHLOROPLASTS OF SUNFLOWER SEEDLINGS TREATED WITH  $10^{-3}$  M 4'-CHLOROGLUTARANILIC ACID

Ten-day-old soil-grown plants received foliar application of compound and leaf samples were taken for electron micrograph study 10 days following treatment. The following is an example of how quantitation procedures were accomplished:

<u>Microscopic Sample No.</u>	<u>Chloroplasts Counted</u>	<u>Starch Granules Counted</u>	<u>% Occurrence of Starch Granules</u>
1	108	67	62

Tissue Examined	Number of Leaf Samples <sup>1</sup>	Average % Occurrence of Starch Granules
Control	9	60
Treated	10	11

<sup>1</sup>Two electron micrograph sections were prepared from each leaf sample taken, making a total of 18 control and 20 treated specimens examined.

dimethylurea (monuron). These changes included reduced number of grana per chloroplast in treated bean (Phaseolus vulgaris, L.) leaves and enlargement of individual grana.

The effects of 2-chloro-4-(Ethylamino)-6-(isopropyl-amino)-s-triazine (atrazine) on bean leaf tissue as reported by Ashton (3) include a spherical rather than the normal shaped chloroplasts, absence of starch granules in the lamellar system, destruction of fretwork, and swollen or ruptured granal compartments. Hill et al. (66) reported similar atrazine effects on barnyard grass (Echinochola crusgall, L.) chloroplasts. The effects of 5-amino-4-chloro-2-phenyl-3(2H)-pyridazinone (pyrazon) on bean leaf chloroplast ultrastructure observed by Anderson and Schaelling (2) were spherical and swollen chloroplasts, clumping and lack of orientation of chloroplasts, inhibition of grana formation, swollen and perforated thylakoids, absence of starch vacuoles, and breakage of outer chloroplast membranes. Results of chloroplast ultrastructural changes similar to those described for atrazine and pyrazon have been reported for 2,4-dichloro-phenoxyacetic acid (60) and analogues of pyrimidine such as 2-thiouracil (64) and bromouracil (67, 4).

Reports by Bartels and Pegelow (11) indicate that 3,4-dichlorobenzylmethylcarbamate (sirmate) causes breakdown of chloroplasts and disappearance of the 70S chloroplast ribosomes in bean seedling leaves.



Mature leaves of barley (Hordeum vulgare, L.), radish (Raphanus sativus, L.), and marigold (Chrysanthemum segetum, L.) from plants treated with 3,5-dichloro-2,6-difluoro-4-haloxypyridine (Haloxydine) possessed chloroplasts which varied in structure from slightly swollen to completely destroyed (37).

The reported effects of 1,1-dimethyl-4,4-bi-pyridylium-2A (paraquat) on mesquite (Prosopis juliflora, L.) mesophyll cell chloroplasts include, rupture of chloroplast membranes, loss of chloroplast turgor, and reduction in the number of starch vacuoles present per chloroplast (16).

In all of the previously cited observations, the growth-regulating compound to be tested was applied in concentrations sufficient to produce observable plant growth inhibition as measured by reduction of total plant height as compared to controls.

Several proposals have been made to explain the chloroplast abnormalities described. These are inhibition of the light reaction and, hence, photosynthesis, by inhibiting thylakoid structural protein formation which depends upon the synthesis of ATP, either directly or indirectly, and thus, disrupting existing thylakoid structure, direct lipid solvent effect on membranes to bring about dissolution of the organelle structure, interference with nucleic acid metabolism,

blockage of protein synthesis, and reduction in carotenoid pigment synthesis thereby exposing the chlorophylls to photo-oxidation.

The lack of ultrastructure changes in leaf chloroplasts, other than a reduction in starch vacuoles, from plants which have been treated with a growth-stunting concentration ( $10^{-3}$  M) of 4'-chloroglutaranilic acid suggests that the inhibiting effect is not caused primarily by observable disruption of chloroplast ultrastructure as appears the case for the compounds previously described.

A decrease or absence of starch vacuoles in electron micrograph visualizations of leaf chloroplasts has been reported for plants treated with growth-regulating compounds. These findings were previously mentioned in cases where plants were treated with atrazine, pyrazon, 2,4-dichloro-phenoxyacetic acid, and paraquat. It would thus appear that a decrease in the number of observable starch vacuoles is not unique to the compound 4'-chloroglutaranilic acid. Possible explanations for the decrease in starch granules are presented in a discussion regarding chemical analysis performed on treated leaf tissue samples.

#### Leaf Chemistry--Soluble Hexoses and Extractable Starch

A survey of the major chemical leaf compounds seemed in order in an effort to pinpoint major areas of interference of leaf

organ metabolism by inhibitory levels of CGA. Quantitative analyses were performed to determine the presence of soluble hexoses, extractable starch, soluble proteins, and total extractable fatty acids (Table X).

Several methods of soluble leaf sugar quantitation were tried and rejected for lack of precision. The good precision and apparent specificity of the phenol sulfuric acid sugar determination suggests this to be a valid microcolorimetric test for leaf tissue crude homogenate sugars. Excluding that portion of leaf homogenate which was removed by filtration it can be assumed that all polysaccharides that are hydrolyzable to hexose monomers, under the conditions described, were being tested colorimetrically. The statistical "t" values calculated for experimental sugar tests were greater than values obtained from significance limit tables and it can be stated that for the conditions defined, the difference in data values is a real change and similar changes can be expected in identically treated future samples. The reduced quantity of soluble hexoses in treated leaves is felt to be due to the activity of CGA. Experimental tissues were exposed to a starch extraction procedure and the phenol sulfuric acid method was employed to quantitate extraction solutions. The low value reported (68 per cent of controls) confirms the leaf chloroplast ultrastructure studies which indicated a reduction in the

TABLE X

CHEMICAL ANALYSIS OF SUNFLOWER SEEDLING LEAVES  
TREATED WITH  $10^{-3}$  M 4'-CHLOROGLUTARANILIC  
ACID

Ten-day-old soil-grown plants received foliar application of the test compound and 10 days after treatment, 12 mm discs were punched from affected secondary leaves of treated and control plants. Analysis was performed on extracts of leaf discs.

Components	Control <sup>1</sup> $\mu\text{g}/\text{mg}$ dry wt	Test <sup>1</sup> $\mu\text{g}/\text{mg}$ dry wt	% of Control
Soluble Hexoses	97.5	74.4	76.3
Extractable Starch	233.0	159.0	68.3
Soluble Proteins	127.0	101.0	79.5
Total Extractable Fatty Acids	73.0	75.0	102.7

<sup>1</sup>Data are averages of values obtained from 10 plants.

amount of leaf storage starch in CGA treated plants. Alterations in carbohydrate metabolism produced by growth-regulating substances are known to occur. Hayashi reported that gibberellins affect  $\alpha$ -amylase production in wheat grains as early as 1940 (63). Since that time, numerous experiments have been conducted which implicate the activity of growth-regulating compounds with plant carbohydrate metabolism. It has been shown that  $\alpha$ -amylase shows a two-fold increase in activity in leaves when the plant receives treatment with cytokinen and GA (39). A series of growth-regulators tested in barley (Hordeum vulgare, L.) seeds were shown to almost completely inhibit the activity of  $\alpha$ -amylase induced by exogenous gibberellic acid. These compounds were applied at  $10^{-4}$  M concentrations and included 2,3,6-trichlorophenyl acetic acid (fenac), 3,5-dibromo-4-hydroxybenzotrile (bromoxynil), 2,3-dicarboxylic acid (endothal), and 1,1'-dimethyl-4,4'-bipyridinium (paraquat) (76). Cytokinen has been reported to reverse the abscisic acid inhibition of growth and  $\alpha$ -amylase production with a concomitant decrease in starch occurring in barley seeds (80). Galsky and Lippincott (50) reported that substrate levels of glutamate and aspartate induced  $\alpha$ -amylase production in barley seeds.

The effects of kinetin on starch and sugar levels on floated Chinese cabbage (Brassica pekinensis, L.) leaf discs were investigated

by Berridge and Ralph (22), and revealed that kinetin caused gross starch degradation. Neutral sugars were depressed by 30 to 40 per cent in leaf tissue treated with kinetin. S-triazines applied to leaves of pea (Pisum sativum, L.) and sweet corn (Zea mays, L.) at sublethal doses caused an increase in the activity of  $\alpha$ -amylase and a decrease in starch and soluble sugars (166).

One can propose several possible modes of involvement whereby CGA might affect starch metabolism. These possibilities are listed below.

1. Inhibition of the Hill-Bendall reaction with its system of photophosphorylation and production of NADPH reducing equivalents.
2. Disruption of the Calvin cycle which reduces CO<sub>2</sub> to carbohydrate precursors of starch.
3. Inhibition of a step or steps in the intermediary pathway that results in synthesis of starch from glucose.
4. Uncoupling of an energy utilization system which would result secondarily in depletion of starch reserves.
5. Cause stimulation or inhibition of endogenous levels of growth-regulating substances which controls starch synthesis or degradation.

Selection of one of these proposed mechanisms is quite speculative, but some previously acquired data may allow the discarding of the first scheme. Sargent (125) tested the effects of inhibitory levels of CGA on oat coleoptiles under dark-growth conditions, and found a reduction in hydrolyzable sugars. If the hydrolyzable sugars reflect starch content then it would appear that CGA can exert its effect on starch metabolism in the absence of light. This information plus the references cited regarding the influence of growth-regulating compounds on  $\alpha$ -amylase makes proposal number 5 the most attractive hypothesis.

If CGA were able to exert its effect on starch metabolism in plants other than sunflower, it may be of practical value to the sugar producing industry. Following a report to the scientific community regarding CGA influence on carbohydrate metabolism, a communication was received from Wise<sup>1</sup> of the U.S. Department of Agriculture which stated in part:

A compound which would increase the efficiency of sucrose storage at the expense of starch in the sugar beet would be a significant contribution to the beet industry (162).

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<sup>1</sup>Personal Communication, Wise, U.S.D.A., Agricultural Research Service Plant Science Research Division, Texas Research Laboratory, Logan, Utah:

### Leaf-Chemistry--Starch Content vs. Growth Inhibition

It has been determined that a  $10^{-3}$  M concentration of CGA applied to leaves of soil-grown sunflower seedlings produced a 33 per cent average reduction in total plant height. Also, data showed that a 32 per cent decrease of leaf starch occurred 10 days following CGA treatment. It seemed that a time-course study would be in order to possibly determine if a physiological relationship existed between these two observations. It can be seen from the graphed data presented (Fig. 13), that a decrease in total plant height is detectable at some point between zero time and 24 hours following CGA treatment. This inhibition in stem elongation continues to occur at a relatively steady rate at least up to the 10th day, at which time a 33 per cent average height reduction is expressed. Under the experimental conditions employed, a decrease in the level of leaf starch is not detectable for the first 24 hours. After 24 hours, the level is reported to drop relatively rapidly up through the 4th day and then continues to decrease but at a much slower rate up to day 10.

These findings imply that CGA may exert its physiological effects of stem growth inhibition and storage starch depletion independently of each other. It would seem, at least, that inhibition of stem elongation is not dependent on, or is a secondary result of, leaf starch depletion.



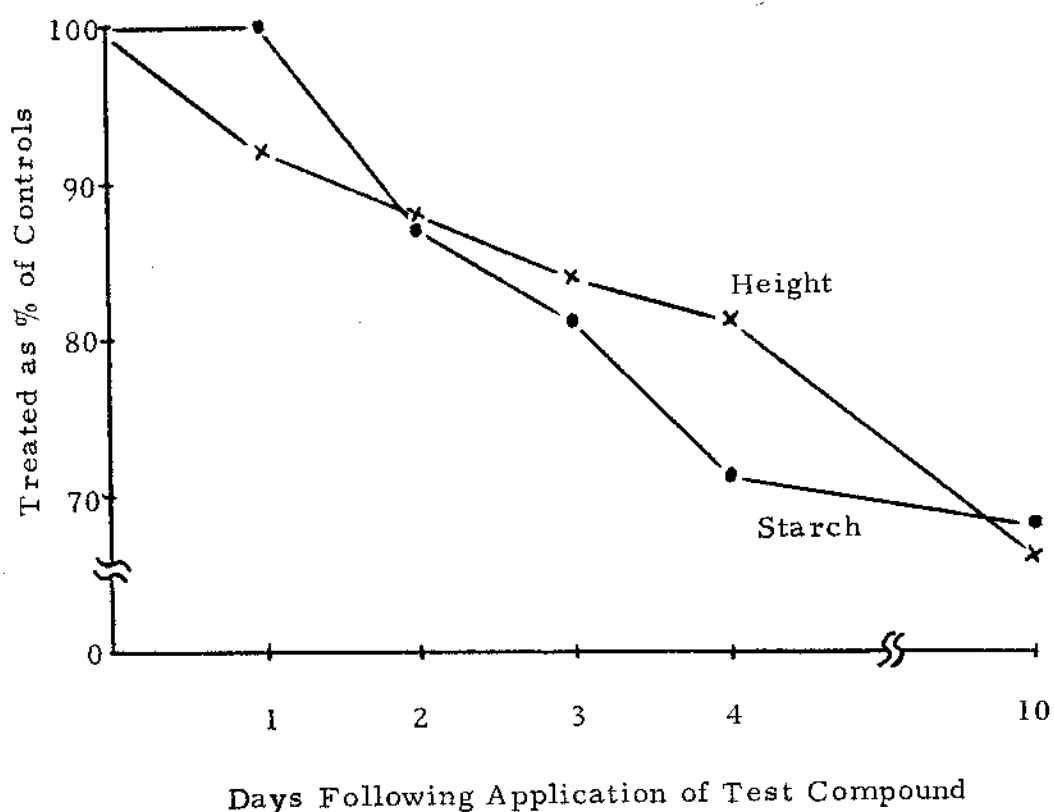


Fig. 13--Leaf starch content of sunflower seedlings treated with  $10^{-3}$  M 4-chloroglutaranic acid versus change in total plant height. Ten-day-old soil-grown plants received foliar application of the test compound and 12 mm leaf discs were punched at daily intervals from affected secondary leaves of plants and analyzed for extractable starch. Data represent averages of at least 6 treated and control individual starch analysis values for each daily interval and plant height measurements represent averages of triplicate experiments consisting of 10 plants each.

### Leaf Chemistry--Soluble Proteins

It has been reported that the Lowry technique can be used to estimate buffer soluble plant leaf proteins provided they are first precipitated with trichloroacetic acid, and that if proteins are not precipitated shortly after extraction, interfering substances contribute to large errors in the determinations (36). Information obtained from known protein addition tests and triplicate analysis data indicate that if leaf tissue homogenates are prepared in distilled water as a solvent instead of a buffer solution the lack of precision associated with the Lowry test is not a problem and that precipitation of soluble proteins is not a necessity in obtaining valid data. On the basis of information presented it appears that if the conditions for preparation of leaf tissue homogenates are followed as described, the Lowry method is a useful technique for comparing relative amounts of soluble proteins found in leaf tissues of 20-day-old sunflower (Helianthus annuus, L.) seedlings. The average value of 127  $\mu$ g BPA protein equivalents per mg dry weight (Table X) represents 12.7 per cent soluble protein per mg dry tissue weight which compares favorably with reported soluble protein values of 9 per cent for Folin reagent analysis on crude homogenate of barley (Hordeum vulgare, L.) leaves (75), 20 per cent for Biuret analysis of 15-day-old pea (Pisum sativum, L.) leaves (132) and 13.4 - 27.5 per

cent for three species of edible plants (Solanum melongena, L. ; Solanum modification, L. ; and Veronia amygdalina, L.) (116)

The 20 per cent soluble protein reduction in leaves of 4'-chloroglutaranilic acid treated sunflower seedlings (Table X) cannot be explained on the basis of experimental information acquired to date. Some explanations, however, can be provided by way of speculation. The cellular proteins that are most likely to be soluble in an aqueous solution would be enzymes, nucleic acid bound basic proteins, and structural proteins would tend to be insoluble in distilled water and would require more vigorous extraction procedures and specific ionic strength and pH solutions for solubilization. Available evidence suggests an involvement of plant growth regulators in protein synthesis. Whitmore (159) presented data showing that indole acetic acid at a  $10^{-4}$  M concentration stops formation of peroxidase isoenzymes in both soluble and bound enzyme fractions. Vorob'ev has reported that 2,4-dichlorophenoxyacetic acid selectively inhibits plant protein synthesis as evidenced by strongly depressed rates of  $S^{32}$  incorporation into leaf proteins (149). Studies on a large number of herbicides demonstrated that in vivo RNA and protein biosynthesis was inhibited in treated plants (111). The ability of plant-growth regulators such as indole acetic acid, gibberillic acid, 2,4-dichlorophenoxyacetic acid, and 2,4,5-

trichlorophenoxyacetic acid, at concentrations of  $10^{-4}$  M, to cause decreases in the thermal melting of highly polymerized DNA is additional evidence of the possible indirect control of protein synthesis by plant growth regulators (8). If 4'-chloroglutaranilic acid is exerting a physiological effect on plants similar to that of currently studied plant growth regulators, then a possible role in the control of protein synthesis can be assigned to this compound.

#### Leaf Chemistry--Extractable Fatty Acids

Leaf tissue samples obtained from soil-grown sunflower seedlings, which had received foliar application of  $10^{-3}$  M 4'-chloroglutaranilic acid, were analyzed for total extractable fatty acids (Table X). The principal fatty acids detected were hexadecanoic (Palmitic) 23 per cent, octadecadienoic (Linoleic) 18 per cent, and octadecatrienoic (Linolenic) 58 per cent (Table XI). Data from the literature regarding type and quantity of fatty acids most commonly found in higher plants, indicated that the major components are C16:0, C18:2, and C18:3 (65), which were also the predominant fatty acids identified in this study.

C18:3 usually predominates in leaf lipids along with C16:0 and both have been found in these respective quantities in leaves of the kapok tree (Ceiba yentandra, L.), Panama bush (Sterculia

TABLE XI

FATTY ACID ANALYSIS OF SUNFLOWER SEEDLING LEAVES  
TREATED WITH  $10^{-3}$  M 4<sup>2</sup>-CHLOROGLUTARANILIC ACID

Ten-day-old, soil-grown plants received foliar application of the test compound and 10 days after treatment 12 mm discs were punched from affected secondary leaves of treated and control plants. Analysis was performed on extracts of leaf discs.

Fatty Acid	Percentage of Total Fatty Acid	
	Control <sup>1</sup> (%)	Treated <sup>1</sup> (%)
16:0	24	23
18:2	16	18
18:3	59	58

<sup>1</sup>Data presented are averages of values from 5 leaf disc samples.

foetida, L.), and choolmoyric tree (Hydnoearpus wightiane, L.) (78). The major fatty acids reported for leaves of rye (Secale cercale, L.), English rye grass (Lolium perenne, L.), and wheat (Triticum estivum, L.) were C16:0, C18:2, and C18:3, with C18:3 occurring in the highest quantities and C18:2 in the smallest amounts for these 3 fatty acids (87).

Most fatty acids of young, green leaves of higher plants appear to be incorporated into galactolipids, sulfolipids, and phospholipids (126, 127, 20, 163), and these compounds are associated primarily with chloroplast and mitochondrial membranes (21). A photosynthetic function was initially proposed for linolenic acid (cis-9, 12, 15-octadecatrienoic acid) in organelle lamellar lipids of plant leaves (40). Recent evidence suggests that an integral relationship occurs between the normal lipid metabolism of intact isolated chloroplast membranes of spinach, bean, and pea leaves and photosynthetic competence (153, 73, 146, 27). It has also been shown that intact spinach leaf chloroplasts (57) and developing castor bean proplastids (167) are capable of fatty acid synthesis. It follows, therefore, that if a growth-inhibitory level of CGA is acting directly or indirectly to disrupt lipid metabolism in leaf cell organelles, that this irregularity might be reflected in quantitative and qualitative fatty acid changes.

The fatty acid values reported in Table XI indicate that under the experimental conditions employed, no significant quantitative or qualitative fatty acid changes were detectable in sunflower seedling leaf samples treated with growth-inhibitory levels of CGA.

It is possible, however, that alterations in lipid metabolism could have occurred that would not be detectable by the method employed. Shifts in fatty acid content within a lipid class could be occurring that would not be detectable by total fatty acid analysis.

Additional evidence which may indicate a lack of interference by CGA in sunflower plant lipid metabolism, at the levels tested, would be the apparently intact ultrastructure of leaf chloroplasts as shown in Figure 11.

It would seem that if lipid metabolism was being grossly disrupted by CGA, either by interference with the lipid synthesis-degradation process or by acting as a surfactant, that evidence of this activity would be reflected in structural changes in chloroplast membrane integrity. A noteworthy observation is found in the work reported by Mann (99). Thirty synthetic growth-regulating compounds were tested for possible effects upon the incorporation of radioactivity from malonic acid-2-<sup>14</sup>C into lipids by excised hypocotyls of hemp (Sesbania exaltata, L.). At the concentration levels tested, which were equivalent to those used to produce growth inhibition by CGA,

only 7 of the 30 compounds examined caused more than 25 per cent alteration in lipid biosynthesis. Inhibition of lipid synthesis was caused by 3-nitro-2,5-dichlorobenzoic acid (dinoben), 2-chloro-N,N-diallylacetamide (CDAA), 2-chloro-allyl diethyldithiocarbamate (CDEC), 7-oxabicyclo(2.2.1)heptane-2,3-dicarboxylic acid (endothall), 2,6-dichlorobenzonitrile (dichlobenil), 3,5-diiodo-4-hydroxybenzonitrile (ioxynil), and pentachlorophenol (PCP).

#### CGA Homologous Compound Studies

Synherholm and Zimmerman (139) tested a long series of *w*-phenoxyalkanecarboxylic acids for phytochemical activity. Compounds with an increasing length of the side chain from the 2,4-D acetic acid to the octanoic acid were tested. These investigators found that, when tested by the tomato epinasty test, those compounds having an even number of carbon atoms in the acid chain were active whereas those having an odd number were not. This phenomenon was explained by suggesting that the test plant must possess a  $\beta$ -oxidizing enzyme system capable of degrading the various chains down to the 2-carbon acetic acid active compound. It was felt that the odd chains were degraded to phenols that are not active as growth regulators. A great number of even carbon number phenoxy acid compounds have been tested in wheat sections and were found to be








active but were inactive when tested in pea sections. However, pea sections placed in solutions containing wheat sections and even numbered phenoxy acid compounds would exhibit a growth response. It was concluded that some plants possess a  $\beta$ -oxidation mechanism which is capable of degrading the even-numbered compounds of the series, while other plants did not possess this enzyme complement (152). Additional testing has proved this assumption to be true (43, 150, 140). Compounds which have been tested in a homologous series include amides and nitriles, W-(indole-3)alkanecarboxylic acids (44), and W-(N,N-dimethyldithiocarbamoyl)alkanecarboxylic acids (54). Stumpf (138) also found that in the peanut plant (Arachis hypogea, L.) all members below the C<sub>14</sub> homologue fatty acid behaved in a manner which was consistent with stepwise degradation by  $\beta$ -oxidation involving the loss of two carbon fragments at each stage. As no information could be found which dealt with a homologous series study on alkyl carboxylic acid substituted 4'-chloroaniline compounds, a series was synthesized to complete a group extending from the 5-carbon 4'-chloro-blutaranilic acid to 4'-chloroaniline. These compounds were subjected to growth-regulation studies in sunflower seedlings and the results summarized in Table XII.

At  $10^{-3}$  M concentrations all compounds exhibited a pronounced phytotoxicity with the exception of 4'-chloroaniline which caused a 61 per cent decrease in average height values. The  $10^{-4}$  M

TABLE XII

## EFFECT OF HOMOLOGOUS COMPOUNDS ON SUNFLOWER SEEDLINGS

Various concentrations of the test compounds were added to the roots of 7-day-old plants grown in hydroponic pouches and total plant height measurements recorded 10 days following treatment.

Compounds	Concentrations		
	$10^{-8}$ M	$10^{-4}$ M	$10^{-3}$ M
(a) Cl-  - $\overset{\text{H}}{\underset{ }{\text{N}}}-\overset{\text{O}}{\parallel}{\text{C}}-\text{CH}_2\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}{\text{C}}-\text{OH}$	19% increase <sup>1</sup>	36% decrease <sup>1</sup>	100% decrease <sup>1</sup> (death)
(b) Cl-  - $\overset{\text{H}}{\underset{ }{\text{H}}}-\overset{\text{O}}{\parallel}{\text{C}}-\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}{\text{C}}-\text{OH}$	5% decrease	20% decrease	100% decrease (death)
(c) Cl-  - $\overset{\text{H}}{\underset{ }{\text{N}}}-\overset{\text{O}}{\parallel}{\text{C}}-\text{CH}_2-\overset{\text{O}}{\parallel}{\text{C}}-\text{OH}$	21% increase	19% decrease	100% decrease (death)
(d) Cl-  - $\overset{\text{H}}{\underset{ }{\text{N}}}-\overset{\text{O}}{\parallel}{\text{C}}-\overset{\text{O}}{\parallel}{\text{C}}-\text{OH}$	6% increase	25% decrease	100% decrease (death)
(e) Cl-  - $\text{NH}_2$	39% increase	10% increase	61% decrease

<sup>1</sup>Total plant height values expressed as per cent of control values.

- (a) 4'-chloroglutaranilic acid
- (b) 4'-chlorosuccinanilic acid
- (c) 4'-chloromalonanilic acid
- (d) 4'-chlorooxanilic acid
- (e) 4'-chloroaniline

concentration tests revealed that 5-carbon 4'-chloroglutaranilic acid was the most active inhibitor of growth, and a general decrease in growth inhibiting capacity was associated with a reduction in carbon chain length. At this concentration it was noted that the even carbon compounds 4'-chlorosuccinanic acid and 4'-chlorooxanilic acid produced a similar cytotoxicity pattern by causing a distinct leaf tip necrosis. The odd carbon compounds did not produce this effect. Solutions containing a  $10^{-8}$  M concentration produced some varied results which may find a corrolary in carbon chain numbers. The most active compound at this concentration was 4'-chloroaniline which produced a 39 per cent increase in average plant heights. The least active compounds were the odd carbon compounds 4'-chlorosuccinanic acid and 4'-chlorooxanilic acid, with the next highest degree of activity occurring in the odd carbon compounds 4'-chloroglutaranilic acid and 4'-chloromalonanilic acid.

#### pH Effect on Growth Response

In 1934, Bonner (26) reported that the growth of oat coleoptile sections was 8 times greater at pH 4.1 than at 7.2. Evans (41) reported that hydrogen ions had a stimulatory effect on cell enlargement both in the presence and absence of IAA. Rayle and Cleland (123) recently found that rapid cell elongation and wall loosening

occurred in Avena coleoptile, and it was shown that the optimal pH for growth is about 3.0 and that both the maximal growth rate and wall extensibility are similar to that produced by the presence of optimal amounts of auxin. Studies conducted on the influence of pH on phytotoxicity of growth-regulating compounds revealed that phytotoxicity increased as the soil pH increased and reached a maximum at pH 6.5 for the weak aromatic acids 3,6-dichloro-o-anisic acid (dicamba) and 2,4-D. Soil pH levels between 4.3 and 7.5 had no effect on the phytotoxicity of the weak aromatic acids 3-amino-2,5-dichlorobenzoic acid (chloramben) and 4-amino-3,5,6-trichloropicolinic acid (picloram (30)). Awareness of the above information, which indicates that pH can affect plant growth, led to some concern as to whether the augmentation and inhibitory growth effects produced by CGA could be related to pH of the test solutions. Although CGA is a weak aromatic acid, calculations employing an estimated  $pK_a$  for weak acids revealed that a  $10^{-4}$  molar aqueous solution would have a pH low enough to possibly alter growth in the sunflower seedling test plants. An experiment was conducted in an effort to dismiss the possibility that pH of the test solution might be causing the growth alteration effects ascribed to CGA.

The pH values for test solutions of  $10^{-8}$  and  $10^{-4}$  M concentrations of CGA were found to be approximately 6.8 and 4.0

respectively. An inorganic acid solution (HCl) was prepared and adjusted to an approximate pH 3.0. Data presented in Table XIII show that Seed-Pak-grown plants exposed to the pH 3.0 acid solution resulted in a growth enhancement of 27 per cent. Sunflower seedling bioassays receiving  $10^{-8}$  M and  $10^{-4}$  M concentrations of CGA responded by producing a 19 per cent increase and 35 per cent decrease in growth values respectively. Although the acidity of  $10^{-4}$  M CGA is within 1 pH unit of the inorganic acid test solution pH of 3, CGA is producing growth inhibition while the acid test solution is producing growth enhancement. The  $10^{-8}$  M CGA solution produces a growth enhancement but the pH value approaches that of neutrality and therefore, the growth response would not appear to be due to a low pH effect. These findings tend to rule out the pH of test solutions as being responsible for the growth responses produced by  $10^{-8}$  M and  $10^{-4}$  M concentrations of CGA, or the homologous series of CGA compounds studied.

TABLE XIII  
pH EFFECT ON GROWTH RESPONSE OF SUNFLOWER  
SEEDLINGS

A non-buffered, acid-water solution was prepared, adjusted to pH of 3.0 and the pH of 4'-chloroglutaranic acid test solutions were recorded. Test compounds were added to the roots of 7-day-old sunflower seedlings grown in hydroponic pouches. Ten days following application of the compounds, total shoot length measurements were taken.

Test Compound	Concentration (Molarity)	Acidity (pH)	Growth Response <sup>1</sup> (% of Controls)
4'-chloroglutar- anilic acid	10 <sup>-8</sup>	6.8	19 Increase
4'-chloroglutar- anilic acid	10 <sup>-4</sup>	4.0	35 Decrease
Acid H <sub>2</sub> O (HCl)	10 <sup>-3</sup>	3.0	27 Increase

<sup>1</sup>Data for each test are averages of values from 2 replicates, consisting of 10 plants per replicate.

## CHAPTER IV

### SUMMARY AND CONCLUSIONS

Three new potential growth-regulating isosteric compounds were tested at a  $10^{-4}$  M concentration by foliar application to 10-day-old sunflower soil-grown seedlings. None of the three compounds produced growth augmentation at this concentration. The most active compound as determined by reduction in total plant height values was CGA. This alteration in growth was accompanied by a characteristic change in leaf morphology. A hydroponic assay utilizing Seed-Pak growth pouches and solutions of  $10^{-4}$  M concentration revealed that a selective activity was associated with the isosteric compounds tested. The substituted amine affected plant leaves but did not affect roots and had a low level of growth inhibition activity. The oxy substituted compound did not affect leaves, did produce root necrosis and had a negligible effect on plant height. Methylene substitution resulted in a positive effect on leaves, no observable effect on roots, and inhibition of plant height.

Concentration studies indicated that CGA applied to leaves of 10-day-old sunflower seedlings produces an increase in plant height

inhibition up to  $10^{-1}$  M concentration and that no additional inhibition or phytotoxic effects occur at higher concentration applications.

CGA applied to leaves of 10-day-old sunflower seedlings produced growth inhibition which was detectable by 24 hours following initial treatment, and the height inhibition persisted for at least 30 days.

Application of CGA at a  $10^{-3}$  M concentration to roots of 10-day-old sunflower seedlings grown in an inert substrate produced an inhibition 18 days following treatment which was 50 per cent of control values. This was a 15 per cent greater inhibition than that observed for foliar applied CGA of an equivalent concentration. Root application also produced the characteristic leaf changes associated with foliar application.

Total plant height inhibition in sunflower seedlings produced by CGA treatment was principally associated with an elongation inhibition of the stem area located between primary leaves and the shoot meristem.

Concentration studies revealed that  $10^{-8}$  M solutions of root applied CGA produced optimal enhancement of growth and that  $10^{-3}$  M concentrations are lethal. CGA was 32 per cent less active than IAA as a growth-promoting compound but was 76 per cent more active as a growth inhibitor at equivalent concentrations in sunflower



seedlings. Growth enhancement capacity of CGA was equal to that of 2,4-D but was a ten-fold less active inhibitor.

Comparative quantitative plant organ studies in Seed-Pak-grown sunflower seedlings revealed that the organ most severely affected by  $10^{-4}$  M CGA was the approximated area of the leaf surface and that the roots were least affected.

Dark-grown Seed-Pak cultivated sunflower seedlings exposed to  $10^{-8}$  M and  $10^{-4}$  M concentrations of CGA showed both the growth enhancement and inhibition activity produced in light-grown plants.

CGA growth inhibition produced in Seed-Pak-grown sunflower plants was augmented by the addition of glucose, sucrose, and 6-BAP, was blocked by gibberillic acid, and was not affected by IAA.

A 29 per cent increase in the thickness of leaves occurred in  $10^{-4}$  M CGA treated soil-grown plants, and leaves also developed a 28 to 40 per cent increase in dry weight.

Leaves of soil-grown plants treated with a  $10^{-3}$  M foliar application of CGA showed a 49 per cent reduction in the chloroplast starch granules and no other observable chloroplast ultrastructure damage occurred.

Chemistry studies showed that tissue from leaves of plants treated with  $10^{-3}$  M CGA developed a 24 per cent reduction in soluble hexoses, a 32 per cent reduction in extractable starch, a 20 per cent

reduction in soluble proteins and that no significant changes occurred in the total extractable fatty acid content.

Modification of the 4'-chloroglutaranilic acid molecule by varying the alkylcarboxylic acid carbon chain number from 5 to 0 revealed that when applied to roots of Sed-Pak-grown sunflower seedlings at  $10^{-3}$  M concentration levels, all homologues except 4'-chloroaniline were lethal. At  $10^{-4}$  M concentrations a decrease in the growth inhibiting ability of the compounds paralleled a reduction in side chain carbon number. At  $10^{-8}$  M concentrations, the odd carbon number side chain compounds and 4'-chloroaniline possessed a growth augmentation activity while even numbered side chain acids demonstrated minimal activity.

Studies concerned with the pH of test solutions revealed that the growth inhibition and enhancement produced by  $10^{-8}$  M and  $10^{-4}$  M CGA applied to roots of Seed-Pak grown sunflower plants was not related to the pH of test solutions. The same conclusion applied to test solutions used in the CGA homologous series experiments.

It can be concluded that CGA possesses chemical properties which resulted in modification of plant growth in sunflower seedlings. The amounts of CGA required to produce growth changes are in the concentration range of endogenous, naturally-occurring plant growth

altering compounds and it is felt that CGA can be referred to as a synthetic growth-regulating compound in sunflower seedling bioassays.

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