ISOLATION AND CHARACTERIZATION OF A GERMINATION INHIBITOR FROM SUGAR BEET FRUIT

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Major professor
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Date October 31, 1966

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF A GERMINATION INHIBITOR FROM SUGAR BEET FRUIT

by Earl D. Mitchell, Jr.

A seed germination inhibitor, cis 4-cyclohexene-1,2-dicarboximide, has been isolated from sugar beet fruit. The isolation procedure involved ether extraction, silicic acid chromatography, cellulose chromatography and crystallization after elution from a sephadex G-10 gel filtration column. Physical data was obtained by infrared, ultraviolet, nuclear magnetic resonance and mass spectral analyses. Identity of the compound was confirmed by synthesis and comparison of physical and biological data. Other inhibitors were present in the sugar beet fruit, but they were not identified. The same procedures were used to investigate inhibitors from wheat chaff, which appears to contain similar compounds.

Fifty percent inhibition of lettuce seed germination was effected by 5×10^{-4}M cis 4-cyclohexene-1,2-dicarboximide. Inhibition was not reversed by gibberellins, but the inhibitor could be removed by washing and then the seeds would germinate. Stimulated respiration of seeds after imbibing water did not occur in the presence of the inhibitor.

The structure of cis 4-cyclohexene-1,2-dicarboximide is unusual for naturally occurring compounds. However, a similar structure occurrs in the synthetic N-ethyl maleimides which are herbicides and in the agricultural fungicide cycloheximide, which is a protein synthesis inhibitor.

ISOLATION AND CHARACTERIZATION OF A GERMINATION INHIBITOR FROM SUGAR BEET FRUIT

By

Earl D. Mitchell, Jr.

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INTRODUCTION

INHIBITION OF GERMINATION

In recent years evidence has been accumulating that dormancy in plants is regulated by many endogenous natural occurring inhibitors and growth substances (1). A seed may be viewed as a resting bud in which biological activity is the result of a marvelous interplay of several hormones that trigger different enzymes. Dormancy is a phase of development. In seeds, one finds a balance between inhibitors and promoters (2). This balance can be broken by removal or alteration of the inhibitors. Thus, dormancy of hard winter wheat can be broken after a heavy rain before harvesting, when the water washes out the inhibitor(s).

A variety of substances can inhibit germination. Inhibitors of germination have been found in grain (3), fruit (4), tubers (5) and buds (6). The many compounds which are toxic to living systems will also, at the toxic concentrations, prevent germination simply by killing the seed. In the latter cases the metabolic processes are irreversibly inhibited.

Inhibition by toxic compounds is often the result of inhibition of certain metabolic pathways. Thus all compounds which interfere with normal metabolic pathways are likely to inhibit germination. Various respiratory inhibitors such as cyanide, dinitrophenol, azide, fluoride and others, all inhibit germination at concentrations similar to those inhibiting metabolic processes. These substances act on germination as a result of their effect on metabolism and not necessarily that of inducing dormancy.

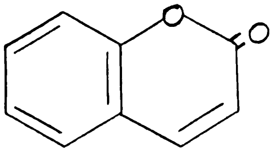
The most interesting substances are those which prevent germination only when they are present but without effecting the seed viability. Of this type, phenolic compounds of various kinds will inhibit germination. Mayer and Evenari (7) found 50% inhibition of lettuce seed by such compounds as $10^{-4}\underline{M}$ coumarin, $10^{-3}\underline{M}$ ferulic acid, $10^{-3}\underline{M}$ salicyclic acid, 10^{-3} resorcinol and 10^{-2} catechol. Because of the widespread occurrance of phenolic compounds in plants, van Sumere (8) has suggested that these substances might act as natural occurring germination inhibitors.

It is also possible to prevent germination of seed by placing them in a solution of high osmotic pressure. When the seeds are removed from such an enviroment and placed in water they can germinate. Some of the compounds responsible for high osmotic pressures may be sugars and inorganic salts such as sodium chloride. One of the more commonly used laboratory compounds is mannitol which gives results somewhat different than sodium chloride (9). Uhvits attributes this difference to the ionic toxicity of the salt.

In the literature there are a number of reports of germination inhibitors being present in seeds (3) and fruits (4). The use of chromatographic technique has resulted in detection of many of these unknown substances but few have been thoroughly characterized. Coumarin and hydroxycinnamic acid and their derivatives have been shown to occur in barley husks. These same types of compounds have been identified in clusters of sugar beet seeds, and Massart (4) suggested that these substances act as germination inhibitors. However, as in the case of most of the inhibiting substance in sugar beet (4), the evidence is very uncertain and no definite conclusion can yet be drawn as to the nature of many of these inhibitory compounds.

Herbicides also inhibit germination to a greater or lesser extent. Many of the commonly used herbicides, such as 2,4-D, affect germination at comparatively low concentrations. Sometimes herbicides are applied in order to kill the weed seedling immediately after germination and before the main crops germinate, and in this case they are not being used as germination inhibitors.

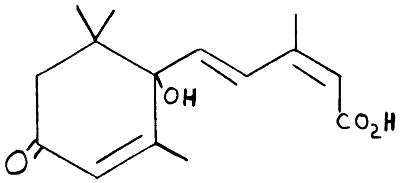
Coumarin, as shown below, has been extensively studied as a germination inhibitor and it is fairly widespread in nature. It is characterized by an aromatic ring and an unsaturated lactone ring. There is no effect of changes in structure of the coumarin molecule on its germination inhibition activity. Therefore there is no single essential group on the molecule which is the cause of its inhibitory activity. The mechanism of coumarin's activity on the germination seed is not clear. Coumarin has been studied with fat metabolism, lipid metabolism, nitrogen metabolism and respiration (10). An additional interaction is that between coumarin and gibberellic acid; the latter is able to reverse the inhibitory action of coumarin in germination. The effect of coumarin on metabolism of the germinating seed has been studied to great depths, because this substance has rather specific effects in the germinating seed. Many of the reported investigations serve to indicate some of the problems and achievements in metabolic studies during germination even though they do not solve them unequivocally.



Coumarin

One natural occuring inhibitor, far more active than coumarin, was first isolated by Addicott from young cotton fruits as a factor involved in abscission of the leaf petiole (11). This inhibitor was called Abscisin. It is identical with Dormin which was isolated about the same time from dormant sycamore buds by Wareing (13). Dormin causes a vegetative bud to change to a winter bud. This inhibitor is a sesquiterpene which contains a hydroxyl group, a carboxylic acid residue and a conjugated ketone of the following structure. Abscisin II was later synthesized by Cornforth (13).

Dormin and Abscisin II may be involved in complicated interactions with auxins and gibberellins as re-



Abscisin II

ported by Wareing (14). It was suggested that 'dormin' appears to inhibit α-amylase production in the gibberelic acid treated barley endosperm. The inhibitor reduces the growth of normal plants and is reversed by gibberellic acid. Dormin was also found to inhibit auxin induced growth which was later restored when gibberellic acid was applied. Coleoptiles did not respond to gibberellic acid in the absence of dormin.

Another Abscisin II like inhibitor is the "β complex" (15) from the potato tuber which showed marked inhibition of wheat coleoptile elongation and of sprouting potato eyes. This inhibition was reversed by gibberellic acid. However, this inhibitor β complex, when stored for 6 months at a -10°, lost the ability to inhibit both coleoptiles and potato buds. Rappaport (15) has also shown that Abscisin II (dormin) when applied at very low dosages almost completely inhibited sprouting of potato buds. Dormin or Abscisin II may be the inhibiting component of the β complex, since Conforth (16) has recently shown that Abscisin II (dormin) is very widespread in being present in buds, leaves, tuber, seeds and fruit.

While the search for the chemical identity of natural occurring inhibitors continued, several synthetic retardants have discovered through serendiptic research. Among these were amo-1618 (17) and cycocel (18, 19). These growth inhibitors seem to inhibit the biosynthesis of gibberellic acid (20) and not the activity of gibberellic acid once it has been formed. Therefore, growth retardants, or inhibitors, are thought not to enter into the processes initiating germination but are operative only when growth has been started by light or gibberellins.

Whether or not any of the natural occurring inhibitors are hormones is a question of semantics. One might define a plant hormone as a chemical which evoked a response, possibly at the DNA and RNA level, which has physiological significance.

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GERMINATION:

A cereal seed has two major components; the embryo (germ) and the endosperm (the food reserve). The cells of the embryo are alive and contain the necessary hormones to initiate cell division and cell enlargement. The most obvious change associated with germination is the rapid uptake of water and oxygen. During the first four days of maize germination most of the water is held by the embryo (21). This water uptake activates the embryo, out of which the entire plant will develop. The storage tissue of the cereal seed endosperm is considered to be nonviable but it contains starch for the growth and respiration of the embryo.

In the course of normal germination, the starch of the endosperm is hydrolysed by α-amylase. Simultaneously Yomo (22) and Paleg (23) discovered that gibberellic acid is the chemical or hormone, which is secreted by the embryo, in order to activate the aleurone layer to secrete the hydrolytic enzyme α-amylase. Recently Varner (26) has demonstrated that gibberellic acid induces the de novo synthesis of α-amylase in the aleurone cells. Thus gibberellic acid probably works at the RNA level by controlling the synthesis of specific RNAs, which in turn controls the synthesis of specific enzymes. In addition to inducing the de novo synthesis of α-amylase, gibberellic acid activates the enzymes that promote the degradation of the cell wall (25).

The germination of lettuce seeds in response to light is associated with a pigment system. Red light promotes the germination of these seeds (26). However, the seeds are sensitive to the illumination only after they have absorbed enough moisture. After imbibition of water, a one minute exposure to 60 foot-candles or red light is sufficient to give 100% germination. The light absorbing pigment is called phytochrome, a bluish colored protein (27). This light absorption may set in motion a mechanism by which the hormones in the embryo are formed or activated. Since gibberellic acid triggers the synthesis of α-amylase, then it is conceivable that the light may trigger the formation of gibberellic acid. The hydrolytic enzymes may weaken the seed coat and thus allow the young root to break through (26). On the other hand, gibberellic acid stimulates the germination of <u>Lactuca sativa</u> in the dark as shown by Kahn et. al (28). In this case gibberellic acid and far-red light with high temperature as done by Evenari (29) have shown that it is not always possible to effectively reverse the gibberellic induced stimulation by the use of far-red light. These experiments have led to the conclusion that gibberellic acid and red light act only partially in the same way and that their mode of action is not identical.

DORMANCY FACTORS:

This investigation has been involved with the isolation of inhibitors of seed germination from sugar beet fruit and wheat seed or chaff. At least 9 organic substances which inhibit seed germination have been isolated from sugar beet fruit (30, 4, 31, 32, 33, 34) but none from wheat. The chemical structures of many of the mentioned compounds have not been determined. Also, only very limited data on the quantity of inhibitors in sugar beet fruits are presently available.

Duym et.al.,(30) have concluded that the aqueous extracts were inhibitory because of the osmotic effect provided by the high concentration of salts present. Fröschel (33) observed that demineralized extracts of sugar beet fruits inhibited the germination of Lepidium and other seeds and that the extracts also emitted volatile substances which caused inhibition. He indicated the presence of specific organic substances as the cause of inhibition. Dekok et al.,(32) indicated that the ether extracts of sugar beet fruits contained inhibitors of germination and that these inhibitors were a mixture of organic substances having a synergistic effect on inhibition of germination. The sugar beet seed inhibitors have also been investigated by Massart (4) who chromatographed extracted material and described these inhibitors as hydroxy organic acids. According to Mankind and Mayamoto (33, 34), water soluble oxalate, which exceed two percent in sugar beet fruits of some sugar beet varieties, may be casually related to germination performance. Snyder et al., (35) showed a significant correlation between water soluble oxalate in the fruit and spud on germination of sugar beet seeds. They also had evidence that at least other inhibitors besides oxalate were present and depressed the speed of germination of sugar beet seeds.

However, as stated earlier, the inhibitory substances in sugar beet seeds have not been fully characterized and evidence for one or more inhibitors is very uncertain. No definite conclusion can be drawn, from the literature, as to the nature of these inhibitory compounds. It has been the purpose of this investigation to look for additional inhibitory substances in seeds which may be very specific, active and unique in respect to other known germination inhibitors.

Masheov (36) was the first to study and report germination inhibitors in wheat kernels. He studied the influence of water extracts of wheat seeds upon their germination and growth. These inhibitors in wheat were found in certain varieties. Everson and Hart (37) reported on the varietal variation of the presence of inhibitors in mature wheat. All wheat varieties with white kernels readily germinated. Those wheat kernels of red color were more resistant to sprouting. There is also a morphological difference between the red and white seed coats (38). The seed coat of the red wheats tightly cover the embryo whereas the seed coat of the white wheats are often separated from the embryo. The morphological differences between the two types of wheat seed coats suggest that water may enter the embryo of the white wheat more easily than that of the red wheat.

Previous Research On the Dormancy Factors At Michigan State University:

This thesis has been a part of a long term continuum of research on dormancy at this University. The orgin of our interest in wheat seed dormancy and attempts to alleviate it through crop breeding had been carried out in Professor Everson's group in the Crop Science Department (37). In the Biochemistry Department, Professor Tolbert's group undertook the task of isolating and characterizing chemical compounds which might be responsible for dormancy. If these compounds were known, it is possible that, in the future, plant breeders may be able to expedite their program by rapid assays for the compounds and by selecting plants with desired amounts of inhibitory material.

Miyamota et al., (39) obtained a small amount of crystalline inhibitor from wheat seed which, however, was not characterized. The latter steps of their isolation procedure differed from the ones reported in this thesis. Gibberellic acid could reverse the effect of this inhibitor on the wheat embryo, but gibberellic acid had no effect on lettuce seed germination in the presence of the inhibitor. Miyamoto et al., (39) decided that postharvest dormancy in wheat was caused by inhibitors found in the seed coat of red wheat. Dormancy was not caused by restricted water or oxygen uptake, a mechanically tough seed coat or an immature embryo.

A. A. Khan, N. E. Tolbert, W. J. Bruin and E. Everson (in manuscript) investigated the biological distribution of the wheat dormancy factors in field grown wheat crops during the 1963 and 1964 season. They found that, although there was some dormancy factor in the seed coat, that a far greater amount was present in the chaff. For that reason wheat chaff and the fruit of sugar beet seed was used in the present investigations. Khan et al., further deliniated the seasonal variation in amounts of inhibitors in chaff and seeds. Units of inhibitors accumulated during maturation until the seeds were nearly mature, after which the amount dropped preceptibly during a rain storm. The urgency of properly harvested material was emphasized. Khan et al., found that inhibitory material was stable if the slightly green chaff were stored at -18°. In the period from 1962 to 1965, A. A. Khan, W. J. Bruin and E. D. Mitchell worked with N. E. Tolbert on the isolation procedures for the dormancy factors from wheat chaff and developed the procedure essentially as outlined in Table 5. Because of limited supply of wheat chaff, F. W. Snyder suggested the use of sugar beet fruit which could be obtained in nearly unlimited amounts. The isolation procedure developed for wheat chaff was found to be applicable to the sugar beet fruit. It has been the purpose of the present investigation to complete the purification and crystallization of one of the dormancy factors so that its identity could be determined.

MATERIALS

All chemicals used in this work were standard reagent grade unless otherwise stated. Anhydrous purified diethyl ether and petroleum ether (30° - 60°) were obtained from J. T. Baker Chemical Co. (Phillipsburg, New Jersey). The silicic acid powder was purchased from Mallinckrodt (St. Louis, Missouri) and the cellulose powder from W. T. R. Balstor (England). Sephadex G-10 was purchased from Pharmacia (Uppsala, Sweden). Gibberellic acid was obtained as Gibrel from Merk and Company (Rahway, New Jersey). Cis 4-cyclohexene-1,2-dicarboxylic acid anhydride was obtained from Aldrich Chemicals.

Infrared spectra was obtained from a Beckman IR - 5 Spectrophotometer. Ultraviolet spectra were obtained from a Beckman DB Spectrophotometer. Nuclear magnetic resonance spectra were received from a Varian HA-60 Nuclear Magnetic Resonance Spectrophotometer using the CAT 1054 computer. Elemental analysis was determined by Spang Micro Analytical Lab. (Ann Arbor, Michigan).

A Soxhlet extractor of 5000 ml. capacity with a 145 mm id., female joint 145/60 was borrowed from Dr. H. M. Sell of this department. Crystalline barley α-amylase was a gift from Dr. J. E. Varner of this department.

The sugar beet fruit was the corky material from lot number (SL 121 x 133) ms x 5822-0 variety of Beta vulgaris L. seed was a gift from the Farmers and Manufactures Beet Sugar Association (Saginaw, Michigan). It was obtained as a very fine powder formed during milling of the sugar beet seed to remove part of the seed coat. I am grateful to Dr. F. W. Snyder for his help in obtaining this material. Upon receiving the material in large bags it was stored at -18^o until used which was generally 1 to 10 months. The White paris lettuce seeds were obtained yearly from the Ferry-Morse Seed Company (Fulton, Kentucky).

Ferric chloride: 2,2'bipyridyl reagent was used to detect weak reducing compounds. 1 ml solution of 0.2% ferric chloride in ethanol and a 1 ml solution of .2% 2,2'bipyridyl (freshly prepared) in ethanol were added to 1.0 ml of the test solution. The ferric chloride solution was added first in order that any Fe++ formed would then immediately be trapped by the addition of the 2,2'bipyridyl. A red color appears immediately or after setting for 10 - 15 minutes which can subsequently be measured spectrophotometrically at 520 mμ after diluting to 25 ml.

For detection of phenolic and other hydroxyl compounds a ceric nitrate reagent was used. A 200 g quantity of ceric ammonium nitrate was dissolved in 500 ml of 2 N nitric acid. Bromine - water and $KMnO_4$ were reagents used for detecting unsaturation.

BIOASSAY

In all studies the inhibitory materials were detected by inhibition of germination of White paris lettuce seeds. A quantity of 25 lettuce seeds were germinated on a 5 cm Whatman No. I filter paper discs moistened with 2 ml of test solution in a petri dish. They were held at room temperature and light and percent germination was recorded after 20 hours in a 2.0 ml medium. A unit of activity was defined as the amount of material that would completely inhibit the germination of 25 lettuce seeds. Specific activity was in units of activity per mg. of dry material. In all fractions to be assayed, it was necessary to remove organic solvent completely or else solvent inhibition of germination would occur. Either the residue after evaporation of the organic solvent was dissolved in water or else an aliquot of the fraction in organic solvent was placed directly on the filter paper, evaporated to dryness at room temperature and then 2 ml of water was added to moisten the paper during germination.

CIS 4-CYCLOHEXENE-1,2-DICARBOXIMIDE:

Twenty-five milliliters of absolute ethanol in a pyrex tube was saturated with ammonia gas at 0^{o} and then 4.1g of cis 4-cyclohexene-1,2-dicarboxylic acid anhydride was added to the cold solution. The tube was sealed and placed in an autoclave with 20 lbs. pressure at 125° for 90 minutes. After being allowed to cool the contents were dissolved in benzene and the insoluble material was obtained by decanting. The benzene solution was evaporated to dryness leaving a crystalline material, mp. 134° - 136° . The imide was recrystallized from water and a total of 0.62 g (15% yield) of crystalline material was obtained, mp. 138° - 139° (lit. (40) 136.5 - 137.3).

The imide was also prepared in the following manner. A 500 ml round bottom flask equipped with an air condenser was charged with 44.4 ml (0.66 moles) of 28% aqueous ammonium hydroxide. A 51.6 g (0.34 moles) quantity of cis-4-cyclohexene-1,2-dicarboxylic acid anhydride was added to the aqueous solution. The reaction mixture was heated with a flame for 2 hours at a temperature of about 300° C to remove water. An oily residue was obtained which solidfied after cooling. The solid was recrystallized from benzene leaving 35.4 g of white needle like crystals (yield 70%). After recrystallizing from water the melting point was 138° - 139° C.

α-AMYLASE BARLEY ENDOSPERM ASSAY:

This assay, was developed by Varner (41), and was used with minor modifications. The barley seed Hardeum vulgare, variety Himalaya, were cut in half and the embryos were discarded. The emdosperm halves were soaked in 1% sodium hypochlorite for 15 minutes, rinsed in sterile deionized distilled water until the odor of sodium hypochlorite was no longer present and then aseptically transferred to sterile moist sand in a petri dish. After three days at room temperature (preincubation period), 10 half seeds were transferred to a sterile Warburg flask containing 1 ml of a solution of 10^{-2}M sodium acetate buffer (pH 4.8), with 10^{-2}M $CaCl_2$ and 0.5 ml of appropriate dilutions of inhibitory substances. Each flask was treated with 50 ug/ml chloramphenicol to prevent the growth of micro organisms. The flasks were shaken on a Gilson differential respirometer for 20 hours at 25° for respiration readings. The gibberellic acid concentration, when used, was 10^{-5} M. After incubation the medium was poured off and saved for α-amylase assay. The half seeds were rinsed with 3.0 ml of 0.01 M acetate buffer (pH 4.8) and then ground for one minute in a mortar with sand and 2.0 ml of 0.2 M NaCl. The mortar with sand was rinsed with 3.0 ml of 0.01 M acetate buffer (pH 4.8) and the combined solutions were centrifuged with a clinical centrifuge and designated as extract. The medium and extract were assayed separately by a modification of the Schuster and Gifford method (42). To 1.0 ml of enzyme solution a 1.0 quantity of soluble starch (1.20 mg/ml) was added for a one minute incubation. The enzyme reaction was killed by adding a 1.0 ml solution containing 3.5 mM I_2 and 2.5 mM KI in 0.05 M HCL. The mixture was diluted to 8.0 ml and a spectrophotometric reading was obtained at 620 mμ . Units of α-amylase activity were calculated according to Varner's method as follows:

$$\text{units} = \frac{\Delta A620 \text{ x vol}}{\text{aliquot x minutes}}$$

α-AMYLASE INHIBITION ASSAY

A 100 g quantity of crystalline α-amylase was incubated with a 16.8 units of inhibitor for a period of one hour in an acetate buffer (pH 4.8). The volume of the incubation medium was 1 ml. A 0.1 ml quantity was removed and diluted to 1 ml. This quantity was assayed as above and units were calculated as previously shown.

α-AMYLASE WHOLE SEED ASSAY

This assay was nearly identical to the half seed assay except that barley seeds were used from which only the tip of the endosperm had been removed to facilitate the imbibition. Ten seeds were placed in a petri dish and covered with 3 ml of deionized distilled water. The seeds were then allowed to stand at room temperature for

30 hours. The medium was saved for assay and the seeds were washed with 2 ml of .01 M acetate buffer (pH 4.8) containing .01 M $CaCl_2$. Preparation of the seed extract by grinding was performed as described for the half seeds.

LETTUCE SEED RESPIRATION

50 lettuce seeds (White paris) were placed in a Warburg flask with suspended trough and incubated with a 1.5 ml solution containing 0.29 mg/ml of the inhibitor and 1 drop of a 0.5 mg/ml penicillin and mysteclin solution to prevent growth of micro-organisms and fungi. Duplicates of the controls and treated flask were set up and manometric readings were obtained each hour on the respirometer.

AVENA STRAIGHT GROWTH

This procedure was obtained from K. Schlender and H. M. Sell of this department. In a buffer solution containing 10^{-2} M citrate, 10^{-2} M phosphate (pH 5), 2% sucrose and 0.1% Tween 80 was dissolved IAA or the inhibitor. The IAA concentration was 10^{-5} M and inhibitor concentration was 10^{-4} M. A control of the buffer solution was used.

Oat seeds (variety Torch) were soaked in the dark for two hours in tap water. The seeds were then planted under green light, on Vermiculite in germinating dishes and germinated under dim red light. After 24 hours, the seeds were covered with Vermiculite and placed in the dark for the rest of the germination period. All subsequent operations were carried out under green light or in the dark. Three days after the seeds were planted, 5 mm sections of the coleoptiles were cut, two to three mm below the tip. These sections were floated for two hours on a distilled water solution containing 1 mg $MnSO_4$-H_2O per liter.

Six inch test tubes containing 1 ml of the assay medium and 10 coleoptiles were placed in a revolving drum, (one revolution per minute). The drum was placed in a dark incubator at 26° and the coleoptiles were allowed to elongate for 22 hours and then removed and placed in a photographic enlarger. The projected shadows were enlarged 5x and measured in millimeters. The experiments were repeated for each solution at least three times. The mean length values were expressed as percent of values obtained by controls with or without IAA.

RESULTS AND DISCUSSION

ISOLATION PROCEDURE FOR SUGAR BEET FRUIT:

A summary of the isolation procedure is given in Table I. A 1.4 kg quantity of sugar beet fruit was extracted in the soxhlet with 3.5 L of ether for 8 hours. After 6 subsequent extractions (a total of 8.4 kg of material extracted) the ether was removed by evaporation and the extracted material weighed about 65 g.

The total material was placed on top of a silicic acid packing in a column of 45 x 5 cm dimensions. The organic substances were eluted with petroleum ether: ether (9:1 to 1:9 v/v) and eventually with ether, as shown in Table II. The silicic acid column was eluted with solvent under 3-6 lbs. air pressure. One liter fractions were collected until a total volumn of 13,000 ml of eluent was obtained. Aliquots of each fraction were bioassayed. The strongest inhibitory fractions (80 - 90% of the total) always appeared between fractions 8 - 11 and usually fraction numbers 9 and 10 were combined and evaporated to near dryness for futher isolation.

The residual material in fractions 9 and 10 was then mixed with powdered cellulose and placed on top a dry cellulose packing in a column of 25 x 5 cm dimensions. Substances were eluted with water and the first 250 ml fraction contained 90% of the inhibitory ma-

Table I

Isolation Procedure for 8.4 Kg of Sugar Beet Fruit

	Residue Wt. in g	Total Units	Specific activity units/mg
Ether extraction	65.0	32,500	.5
Silicic acid column	3.36	4,000	1.19
Cellulose column	1.33	3,800	2.86
Sephadex G-10 column	0.096	319	3.33

Table 2

Elution Pattern for Silicic Acid Columns

Fraction	Volume	Eluent		units/ml
1	1000	Pet.Et: Ether	(9:1)	0.0
2	1000	" "	(8:2)	0.0
3	1000	" "	(7:3)	0.0
4	1000	" "	(6:4)	0.0
5	1000	" "	(5:5)	0.0
6	1000	" "	(4:6)	0.0
7	1000	" "	(3:7)	0.0
8	1000	" "	(2:8)	1.0
9	1000	" "	(1:9)	8.0
10	1000	Ether		2.0
11	1000	Ether		1.0
12	1000	Ether		0.25
13	1000	Ether		0.0

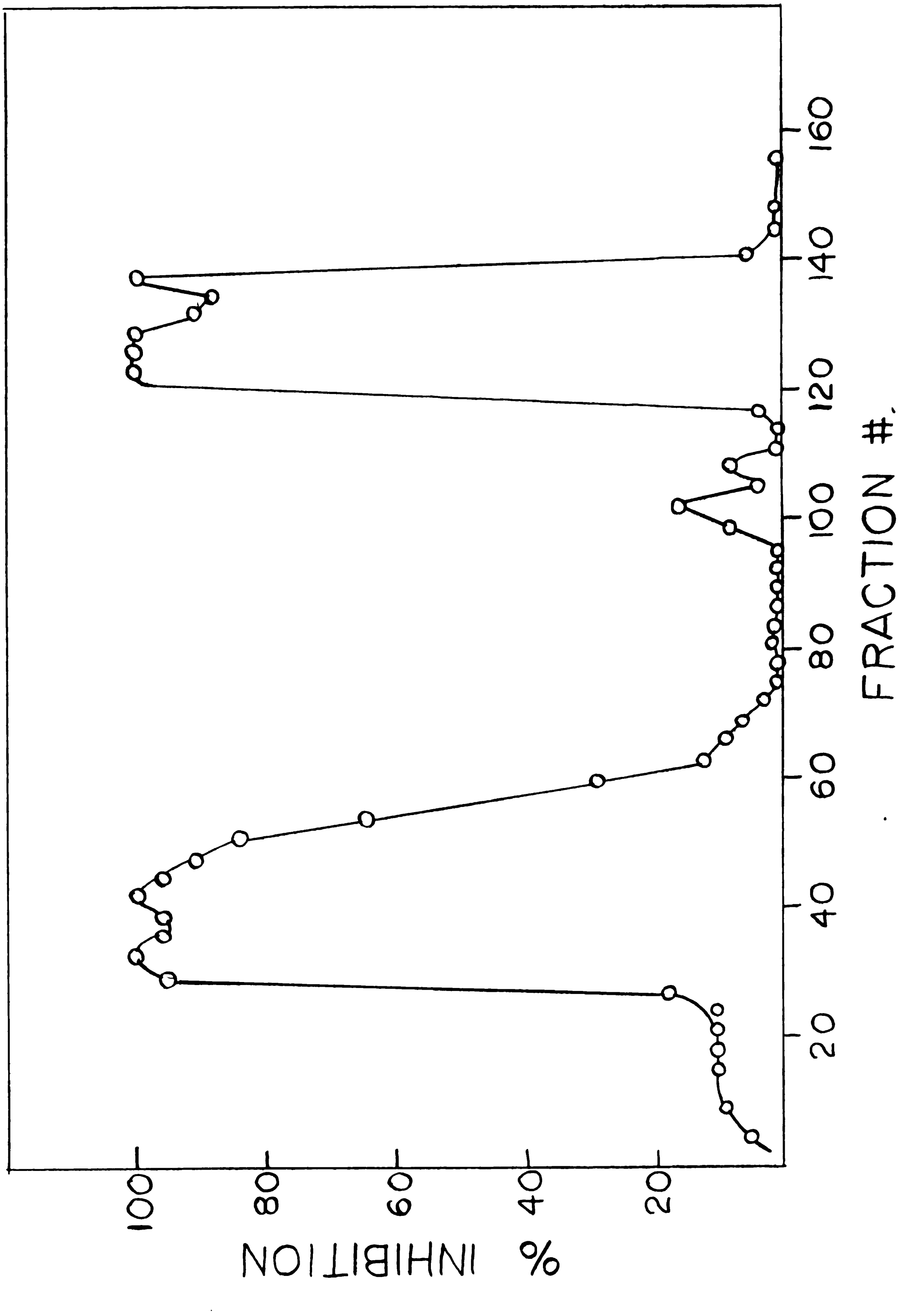
terials. Eluting with water removed only water soluble material and eliminated much of the organic soluble material which was not inhibitory. The pH of the eluent was 4.5. This procedure also removed much acidic material.

The first 250 ml aqueous fraction containing the inhibitory substances from the cellulose column was evaporated to a 3 to 5 ml volume. It was placed on top a sephadex G-10 column of 95 x 2.5 cm dimensions which had been packed and equilibrated with water. Sephadex G-10 is a gel filtration dextran with a molecular weight exclusion of 0 to 700. The eluting solvent was water and flow rate was adjusted between 7 - 8 ml per hour. Fractions were collected every thirty minutes. Inhibitory substances were detected in two bands by the lettuce assay (Figure 1). The inhibitory material in the first fraction after evaporation was an oily residue and it has not been further investigated extensively. The substance in the second band, fractions 121 - 132, upon combination and evaporation, yielded 96 mg of white solid residue (mp 105 - 110). The germination inhibitor in this preparation was crystallized from cold water to constant melting point. The yield was 9.9 mg. The vacumn dried (15 mm Hg at 25° for 12 hours) crystalline preparation melted at 138° - 139° .

Figure 1. Elution pattern of the Sugar Beet inhibitory material from a Sephadex G-10 Column

Flow rate 7 - 8 ml/hr

Eluting solvent - water


% INHIBITION
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60
40
20
FRACTION #
20
40
60
80
100
120
140
160

OTHER ISOLATION PROCEDURES ATTEMPTED:

Acid-base extraction was repeatedly tried with eluent from the silicic acid column. This aqueous extract contained much material including most of the units of inhibition which could be extracted into ether at acidic pH and which was soluble in water at a basic pH above 8 or 9. However, the biological activity was partially lost upon treatment with dilute alkali. Therefore, the principle of organic and aqueous extractions at different pH values was not employed with sugar beet fruit as a method of isolation.

Thin layer chromatography (TLC) with silica gel G and GF was used for qualitative studies and H and HF for preparative work. 2537 A^o and 3660 A^o U.V. light were used for detection of components. Silica gel HF was preferred, since it could be used to detect non-fluorescent materials also. Of the many solvent systems tested, $CHCl_3$: MeOH (9:1) was found to give the best separation without moving the inhibitory material to the solvent front. Areas of the TLC were scraped off the plate and collected on a sintered glass suction funnel. The silica gel fractions were extracted with ether and acetone repeatedly and the extract evaporated to yield the active material. In all this work it was necessary to run controls to ascertain that the absorbent material and solvents did not contain traces of impurities which would be inhibitory in the biological assay. In a typical experiment 480 mg (about 2500 units) in a ml of ether was spotted in 10 TLC plates (200 mm x 200 mm) coated with silica gel HF (0.5 mm). After development 8 components ranging from Rf 0.05 to 0.85 could be observed under U.V. lights. Each component was collected separately. Upon bioassay, only fractions designated as number 3 and 4 were found to contain inhibitory activity against lettuce seed germination. Fraction 3, Rf of 0.6, was pale yellow in color, did not fluoresce in 3660 A^{o} U.V., but absorbed 2537 A^{o} U.V. In about 35 mg of material there was 625 units. Fraction 4, Rf of 0.5, was pale yellow in color, showed fluorescence in 3660 A^{o} U.V., and absorbed 2537 A^{o} U.V. In 27 mg of material there was 830 units.

When the TLC procedure was repeated with either fraction 3 or 4, about 8 components were again observed on the chromatogram and over half of the activity was lost. From many such disasters we conclude that most of the inhibitors were unstable to the TLC procedure with break down into several inactive components. The most likely reason for this loss of activity was either destruction of the compounds by U.V. or oxidative degeneration on the TLC.

For gas chromatography fraction 3 and 4 from TLC was injected onto a SE-30 (3% silicon rubber on Diatoport WAW, 60-80 mesh) gas chromatography column of an Aerograph 661 Gas chromatograph, using a flow rate of 2.6, a pressure of 40 lbs. per in.^{2}, and a temperature of 225°. The three major peaks were detected corresponding to a retention time of 0.7, 1.0 and 8.3 minutes respectively. The last peak generally contained the bulk of the material. The column temperature was 225°. A collection tube was used to collect the peak at a retention time of 8.3 minutes. Inside the tube a crystalline material along with liquid was obtained. The tube was washed with ether and the solution was assayed for units in the usual manner. The weight of the sample collected was obtained by weighing the collection tube before and after washing with ether. There were 480 units obtained in which the weight was 18 mg. TLC of this material in chloroform: Methanol (9:1) on silica gel HF showed non-fluorescent material with an Rf 0.60. The infrared spectra (KBr pellet) showed carbonyl absorbtion at 1775 cm^{-1} and 1725 cm^{-1} along with carbon-hydrogen absorbtion at 2950 cm^{-1}, 2925 cm^{-1} and 2845 cm^{-1}. Collecting the material from gas chromatography was very difficult to duplicate because the crude sample when injected on the column left a residue which eventually eluted with the sample. So gas chromatography was abandoned for the sake of larger preparative methods.

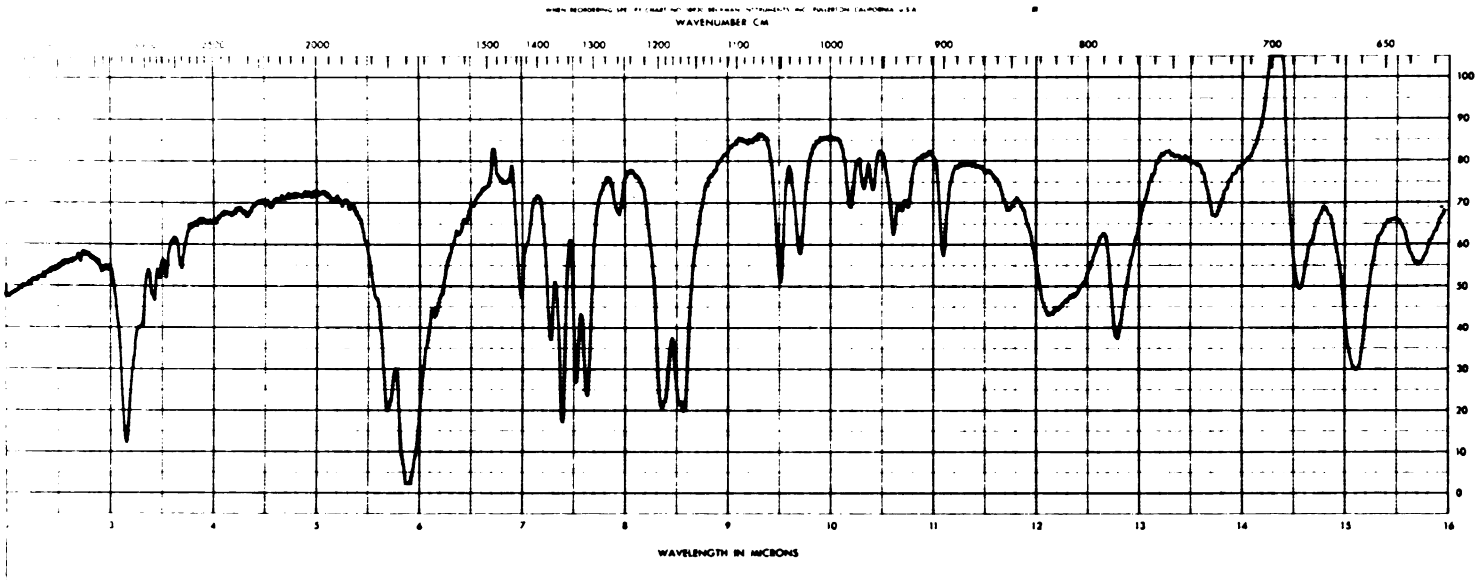
PROOF OF STRUCTURE:

The inhibitory material in the second peak from the G-10 column was recrystallized to a constant melting point of 138° - 139°. From elemental analysis the empirical formula was calculated to be $C_8H_9NO_2$. The i.r. spectrum (KBr pellet), figure (2), showed N-H next to a strong electro-negative atom (3215 cm^{-1}); unsaturated CH stretch (3058 cm^{-1}, 2976 cm^{-1}); methylene C-H (2924 cm^{-1}, 2865 cm^{-1}); carbonyl of a cyclic imide (1767 cm^{-1} and 1704 cm^{-1}); methylene bending frequencies (1425 cm^{-1}) and carbon-carbon double band (1637 cm^{-1}). An absence of absorption at 1600 cm^{-1} (1580) and 1500 cm^{-1} (1450) indicated that the compound did not have an aromatic phenyl nucleus. The strongest indication of a functional group was the double carbonyl absorption at 1767 cm^{-1} and 1704 cm^{-1} in which the lower band was stronger. This is characteristic of a 5 member cyclic imide ring (43). The conclusion from i.r. is that the compound is non-aromatic, unsaturated and a cyclic imide.

The unknown inhibitor gave a positive test with both $KMnO_4$ reagent and with Br_2/H_2O reagent (Table 3). This indicated the presence of an olefinic double bond. With the ferric chloride 2,2'bipyridyl reagent a red coloration indicated a reducing group. The ceric nitrate reagent is a test for phenolic and alcoholic hydroxyl groups.

Figure 2. Infrared spectrum of Unknown Inhibitor

KBr pellet 1mg/400mg of KBr



SPECTRUM NO. 36
DATE 31 [illegible]
SAMPLE [illegible]
[illegible]
SOURCE [illegible]
STRUCTURE $C_8H_9NO_2$
[illegible]
[illegible]
[illegible]
PATH —
SOLVENT [illegible]
CONCENTRATION 1mg 400mg [illegible]
PHASE
COMMENTS [illegible]
[illegible]
ANALYST [illegible]

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Table 3

Chemical Test on the Unknown Inhibitor

	$2,2^1$bipyridyl	$FeCl_3$	Nitrate	Br_2/H_2O	$KMnO_4$
Inhibitor	Yellow	Yellow	Yellow	Clear	Brown
Dithiothreitol	Red	Red	Yellow	red	brown
Abscisin II	Yellow	Yellow	Yellow	clear	brown

For alcohol the reagent changes from a yellow to a red coloration. Phenols change from yellow to brown or green-brown precipitate in aqueous solutions and a deep red to brown coloration is formed in dioxane. These series of qualitative test eliminated alcohols, hydroxy acids and phenolic compounds and confirmed the infrared spectra which showed unsaturation.

Figure 3 shows the mass spectrum. An empirical formula of $C_8H_9NO_2$ corresponds very well with a molecular weight or parent peak at 151. Fragments of C=0 and 0=C-NH are present at 28 and 44 respectively which corresponds with carbonyl character of cyclic imides found in the i.r. spectrum.

The uv spectrum shown in figure 4 had a maximum absorbancy at 242 mμ when measured at a concentration of 1 mg/ml. At the concentration of 6.62×10^{-3}, the extinction coefficient E equals 128 or log E = 2.107. An extinction coefficient (E) of 128 is low for a conjugated carbonyl compounds in which a carbonyl conjugated with an ethylenic linkage should display a strong K-Band (German konjugierte) in the near ultraviolet region of 215-250mμ and have a E max of 10,000 - 20,000 (44). Since the unknown compound showed a low molar absorptivity (E max of 128) then the transition at 242 mμ is not due to K-bands which result from $\pi - \pi^*$ transitions

Figure 3. Mass Spectrum of the unknown Inhibitor
From a Bendix Time-of-Flight

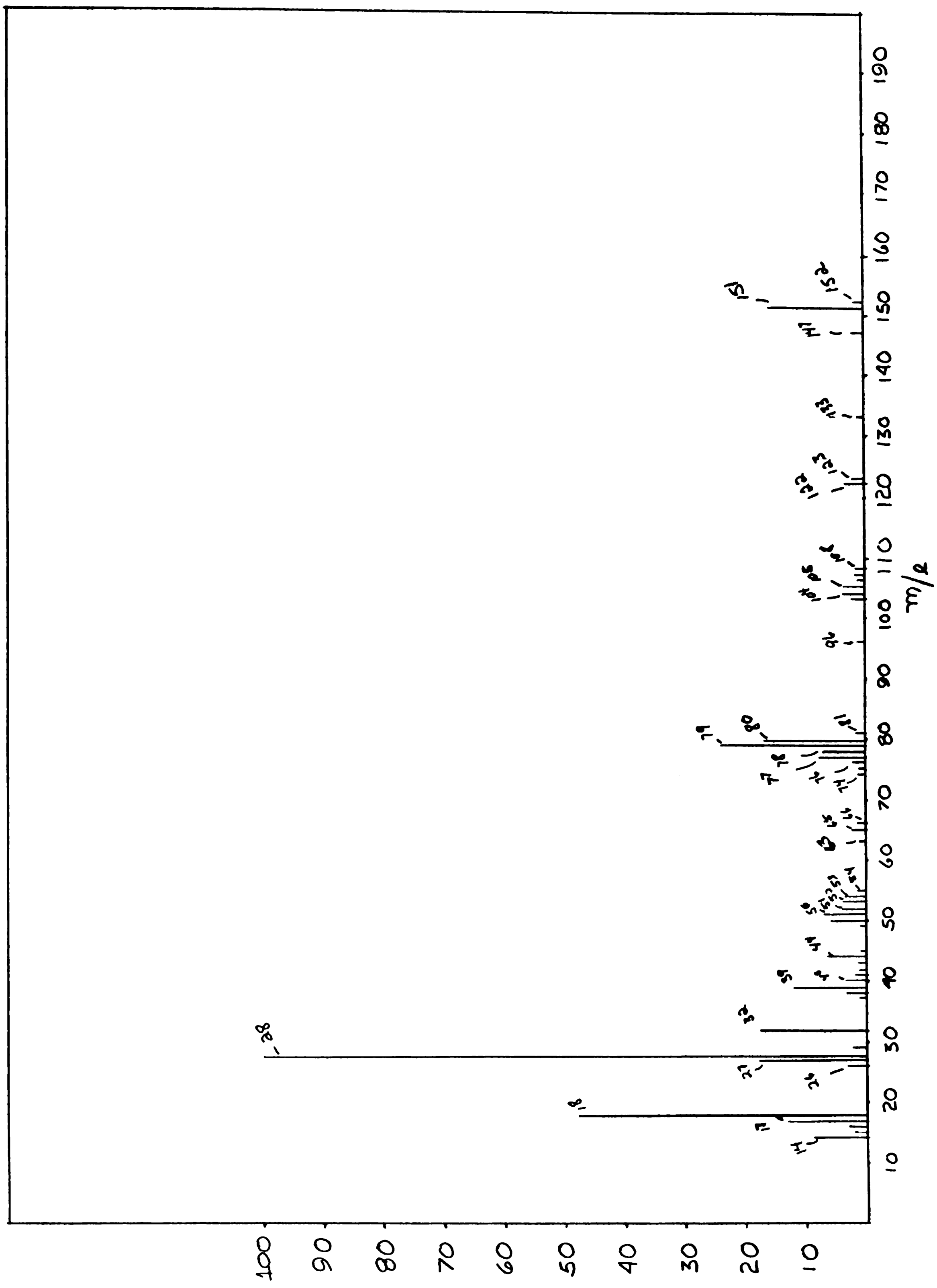

m/e
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
0
10
20
30
40
50
60
70
80
90
100
14
17
18
26
27
28
32
39
50
63
65
74
76
77
78
79
80
81
96
104
106
108
122
123
133
147
151
152

Figure 4. Ultraviolet Spectrum of the unknown Inhibitor

λ_{max} = 242mμ ϵ = 128

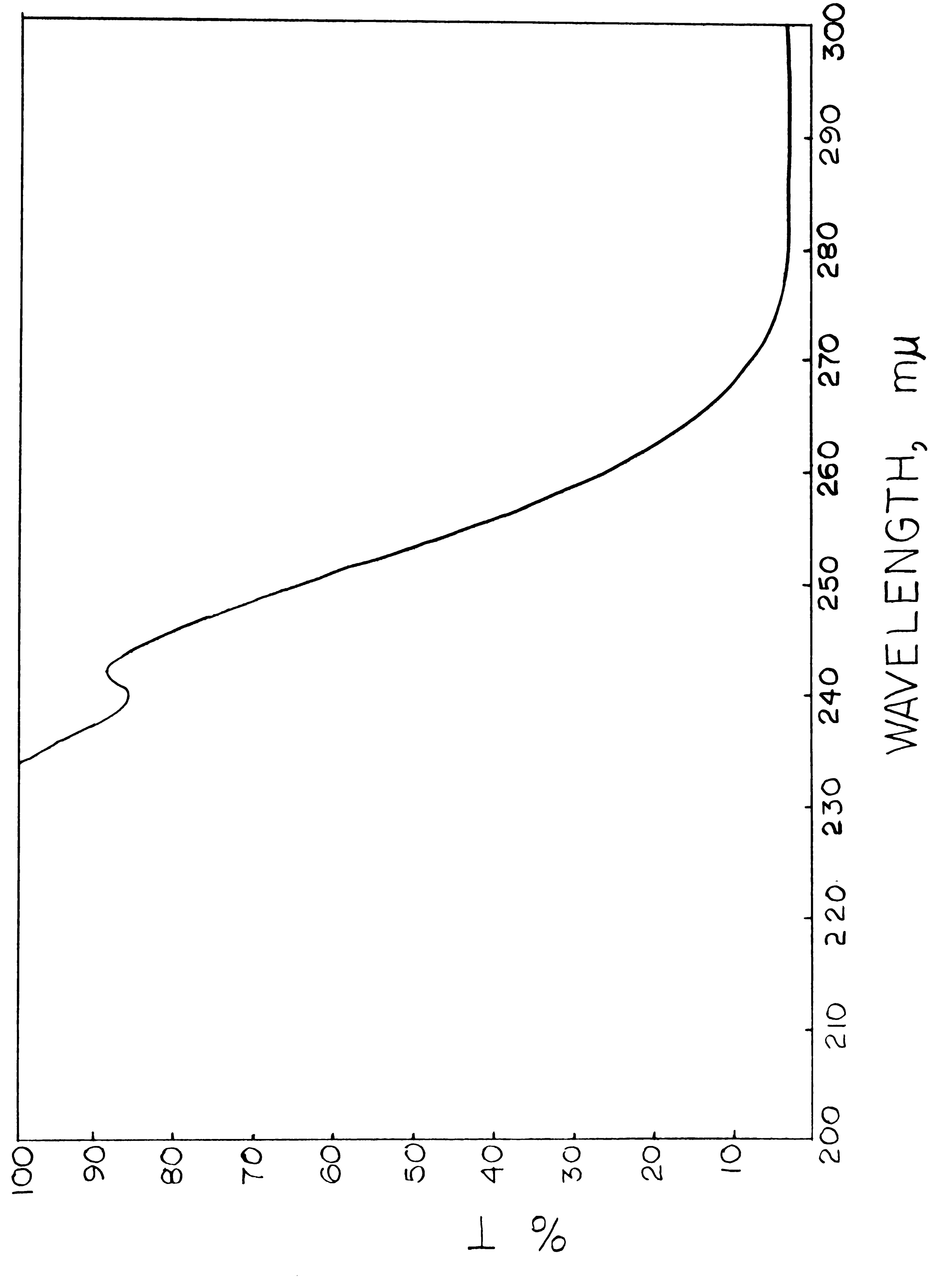
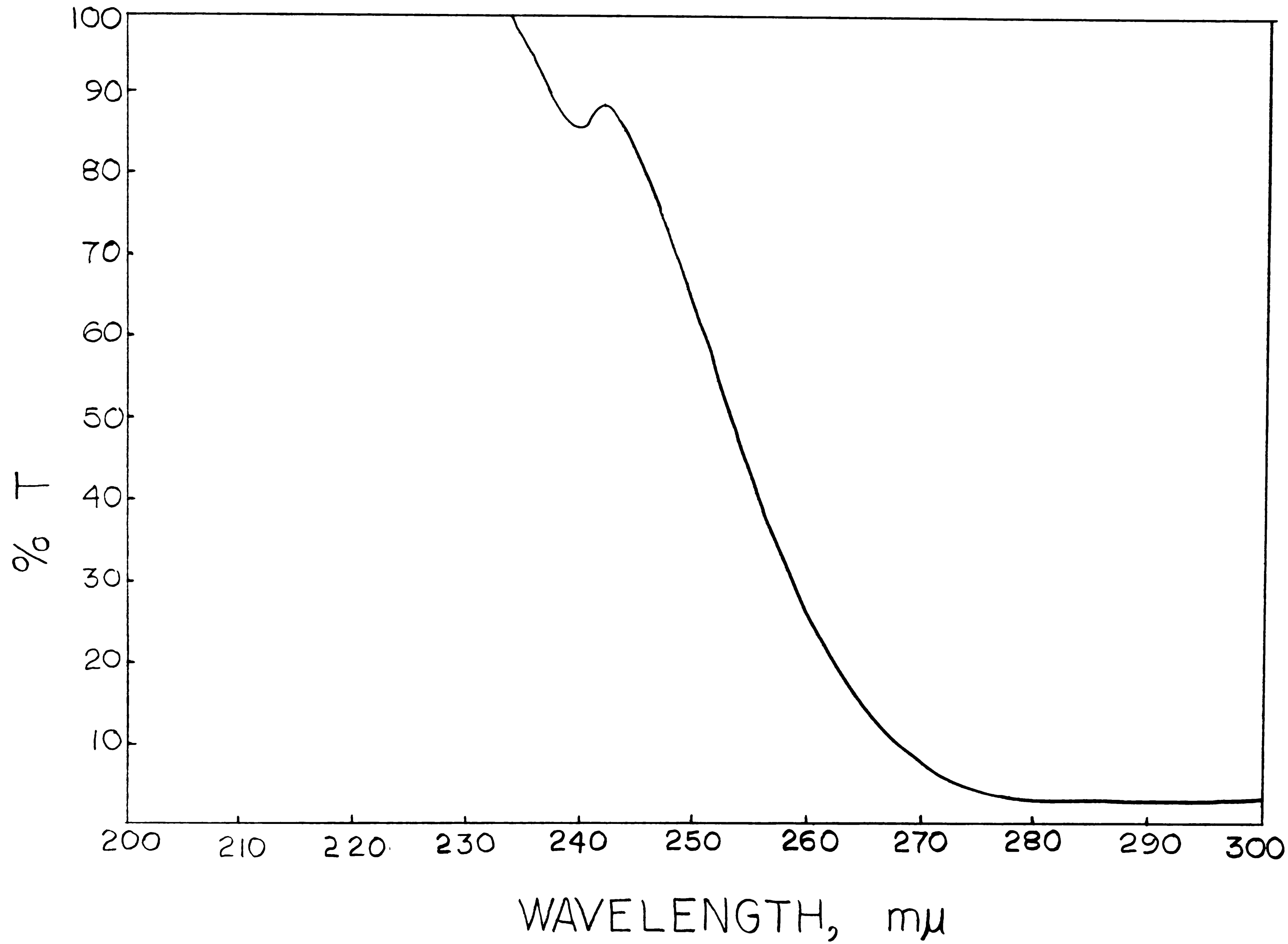

% T
100
90
80
70
60
50
40
30
20
10
200
210
220
230
240
250
260
270
280
290
300
WAVELENGTH, mμ

Figure 4. Ultraviolet Spectrum of the unknown Inhibitor

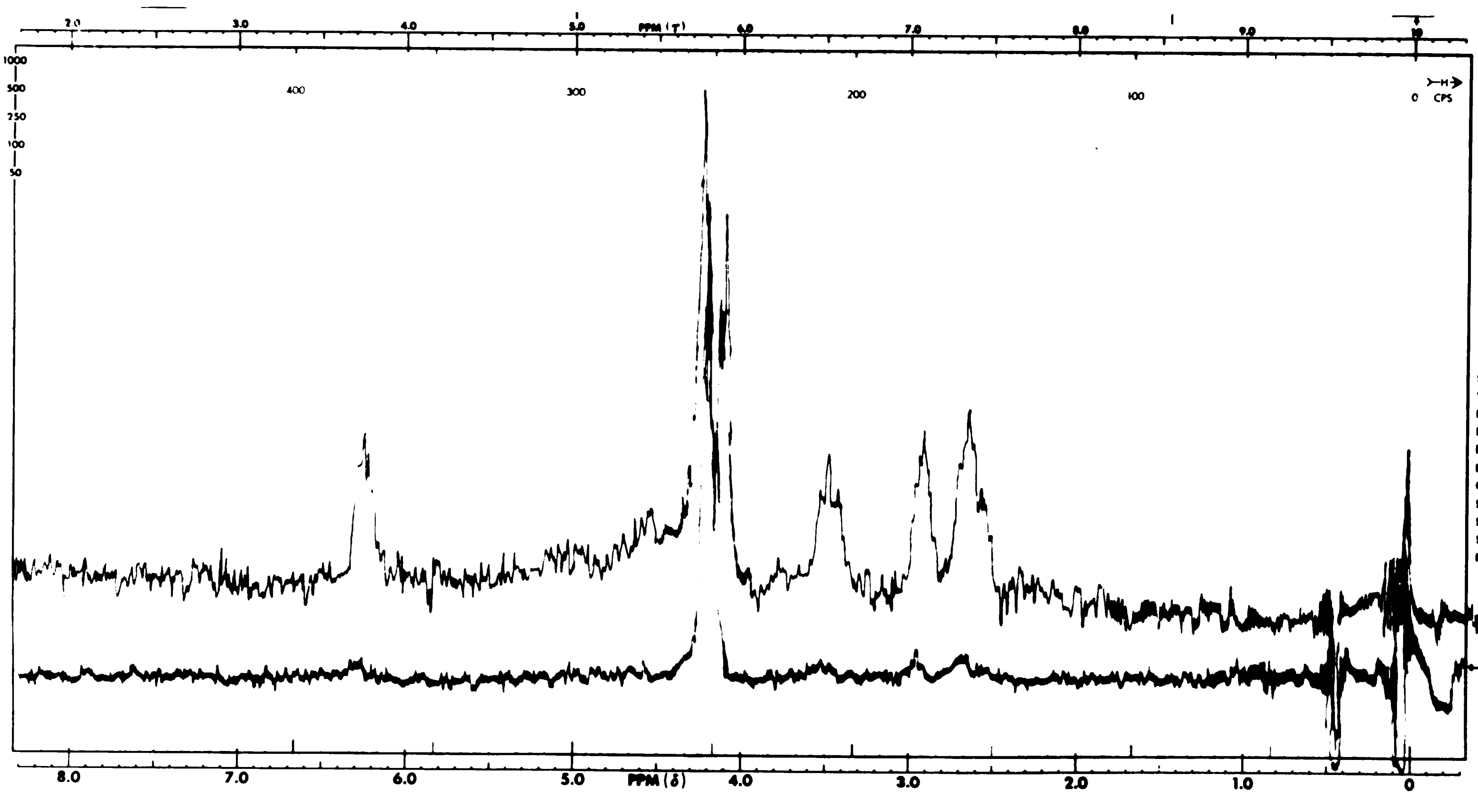
λ_{max} = 242mμ ϵ = 128


100
90
80
70
60
50
40
30
20
10
% T
200
210
220
230
240
250
260
270
280
290
300
WAVELENGTH, mμ

and are characterized by high molar absorptivity. Since the molar absorptivity was 128 then the transition must be from a R-band (German radikalartig) whose orgin is in the $n-\pi^*$ transition of a single non-conjugated chromophoric group. From all of the information thus far presented, it was concluded that the compound contained a cyclic 5-membered imide, a non conjugated carbonyl and a non conjugated double bond. There was no aromatic character (from u.v and i.r) and there was probable an alicyclic ring to satisfy the atomic structure.

From the n.m.r. spectrum in figure 5 was obtained additional information as to the nature of the protons. With only a small sample, there was difficulty in getting a good n.m.r spectrum. The n.m.r spectrum showed no aromatic protons which agreed with u.v and i.r data. There was only one set of methylene on a ring which would stand out as a sharp singlet peak. Two vinyl protons (in the same environment) should show some splitting although only a broad peak was observed. From the n.m.r spectrum in figure 5 it was very difficult to measure spin-spin coupling constants (J values). The absorption peak at 6.86τ appeared as a triplet ($J = 3.0$ cps). A J value close to 6.86τ could be due to tertiary protons next to a methylene group to give a triplet. However, the n.m.r spectrum was not satisfactory for abso-

Figure 5. n.m.r. Spectrum of the unknown Inhibitor
in Deuterated DMSO



HA-60/DA-60 SPECTRUM

60 MC NMR SPECTRUM NO. 3 B

OPERATOR: B Wangel DATE 8/11/66

SAMPLE: Germination Inhibitor

locked on external capillary c̄ TMS

SOLVENT:	D₂O				
TEMPERATURE	[illegible]				°C
R.F. FIELD ATTEN.	-40				db
RECEIVER GAIN	[illegible]				
FREQ. RESPONSE	2.0				cps
INPUT LEVEL	100				
OUTPUT LEVEL	C1F0				
SWEEP TIME	250				sec
SWEEP WIDTH	500				cps
SWEEP OFFSET	000				cps

REMARKS:

m.o. Freq 1500 + 000
m.o. Fld .01 R F
s. Freq Fld .01 R F,
Sp Amp 7RN'

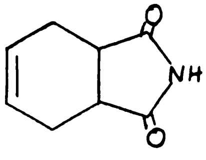
C 1054 after [illegible] sweeps

origin

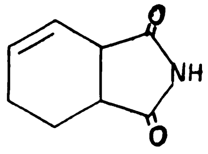
VARIAN associates CHART 560-E

lute proof of structure because the concentration of the solution was less than 1%.

The evidence so far presented pointed to a compound whose structure contains a double bond, a 5-membered cyclic imide (dicarboximide) and no aromatic ring. Two of the likely structures are as follows.



I



II

The above structures would fit the physical data and the empirical formula, u.v., mass and i.r. spectra. Excluding the proton on the nitrogen, structure II shows 6 different protons where as structure I shows 3 different kinds of protons. Structure I and II both have <u>cis</u> and <u>trans</u> dicarboximides. Structure II can be eliminated since it does not agree with the observed n.m.r. spectrum which shows 4 different kinds of protons.

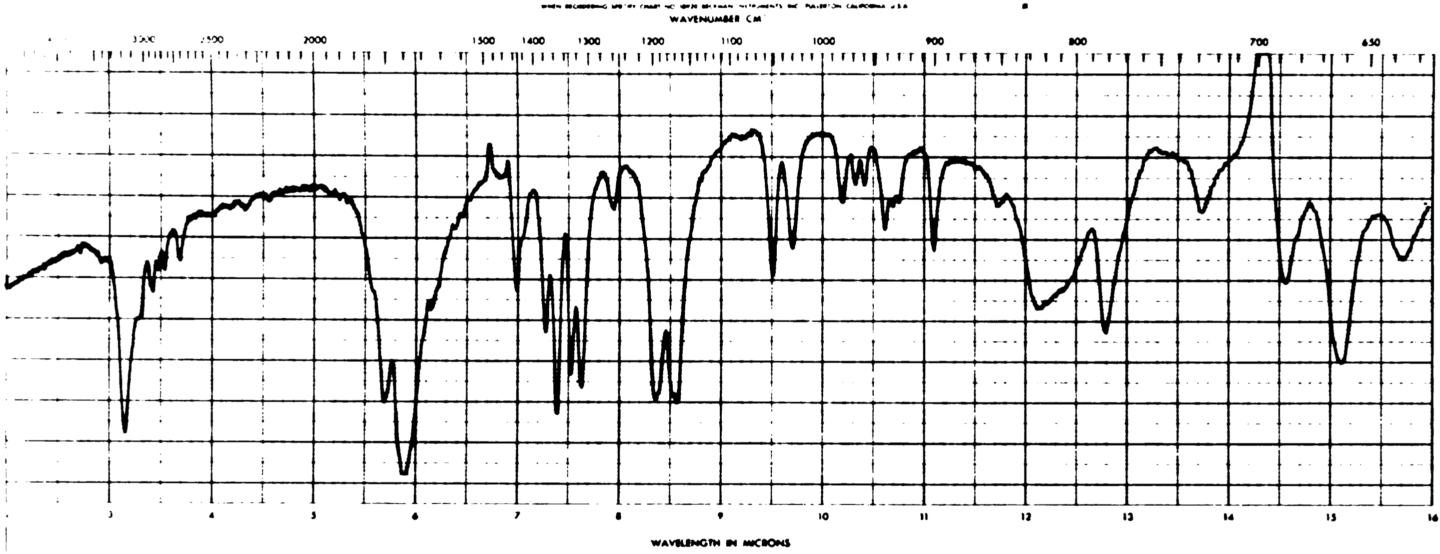
Structure I, cis 4-cyclohexene 1,2-dicarboximide, appeared to be the unknown dormancy factor from sugar beet fruit. It has been synthesized by Snyder and Poos (40). They reported a melting point of 134°- 136° after recrystallizing from benzene which did not agree with the material isolated.

Starting with cis 4-cyclohexene 1,2-dicarboxylic acid anhydride, the dicarboximide was synthesized and recrystallized from benzene (mp 134° - 136°). The melting point corresponded to the value reported in the literature (45). The isolated germination inhibitor had a melting point of 138° - 139°. However, when the synthetic compound was recrystallized from water, the melting point was 138° - 139°. Perhaps the crystallization from benzene carried some solvent with the crystals and thus lowered the melting point even though the crystals were dried in a vacuum desiccator for 15 hours. The infrared spectrum (figure 6) of the synthetic cis 4-cyclohexene 1,2-dicarboximide is identical with the spectra obtained with the isolated germination inhibitor. A mixed melting point with the synthesized compound and the isolated inhibitor showed no depression. The biological activity of the synthetic compound was essentially identical to the isolated material (table 4). There, the structure of the inhibitor was cis 4-cyclohexene 1,2-dicarboximide (as shown) which will be referred to as CCD.

Figure 6. Infrared Spectra of Synthetic and unknown Inhibitor

Top: Isolated inhibitor

Bottom: Cis 4-cyclohexene 1,2-dicarboximide
(Synthetic)



SPECTRUM NO. 36^0
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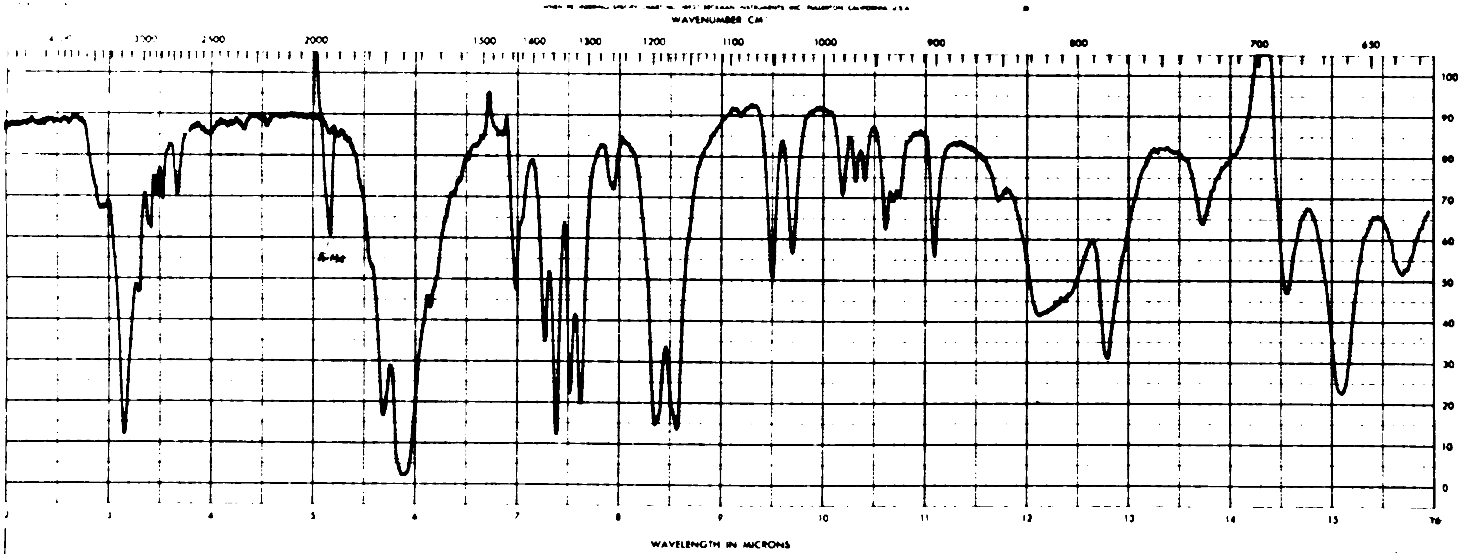
SAMPLE [illegible]
[illegible]

SOURCE [illegible]
STRUCTURE $C_9H_9NO_2$
[illegible]

PATH —
SOLVENT [illegible]
CONCENTRATION [illegible] 400 [illegible]
PHASE
COMMENTS [illegible]
[illegible]

ANALYST [illegible]

Beckman
INFRARED
SPECTROPHOTOMETER



SPECTRUM NO. 36^1
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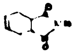
SAMPLE [illegible]
[illegible]
[illegible]
SOURCE [illegible]
STRUCTURE



PATH —
SOLVENT KBr [illegible]
CONCENTRATION [illegible]
PHASE
COMMENTS [illegible]
[illegible]
[illegible]

ANALYST [illegible]

Beckman
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SPECTROPHOTOMETER

Table 4

Comparison of Activity of Isolated and Synthetic Inhibitor*

Isolated Inhibitor			Cis-4 cyclohexene-1,2-dicarboximide		
Conc.		% Inhibition	Conc.		% Inhibition
Molarity X10^3	mg/ml		Molarity X10^3	mg/ml	
3.3	.50	100	3.3	.50	100
1.6	.25	100	1.6	.25	100
.8	.13	83	.8	.13	100
.4	.16	52	.4	.06	79
.2	.03	16	.2	.03	48

*White paris lettuce seeds germinated at 25° in 20 hours

ISOLATION PROCEDURE FROM WHEAT CHAFF:

The summary of the isolation procedure is exhibited in Table 5. The bioassay was the complete inhibition of germination of White paris lettuce seeds and serial dilutions were run on all samples. Hulls of red coat wheat which were harvested in July, 1964, and stored at -10° to -18°were used in 1964 through 1966. An 8.0 Kg quantity of wheat hulls (Chaff) were extracted with 141 liters of purified anhydrous ethyl ether for 12 hours. The hulls were removed by filtration through cheese cloth and the ether was evaporated in a continous flask evaporator under vacuum. The concentrate was mixed with minimum amount of silicic acid powder to form a paste and air dried at room temperature. The mixture was further dried overnight in a vacuum dessicator. It was then placed on top a silicic acid column of 39 x 5 cm dimensions. Final column height was 44 cm. The column was eluted as follows and aliquots were collected and assayed:

(a) Fraction number 1: 1000 ml petroleum ether (30°- 60°), no inhibitor was eluted.

(b) Fraction number 2: 1000 ml 75:25 mixture of petroleum ether: ethyl ether; no inhibitor eluted.

(c) Fraction number 3: 3000 ml of 50:50 mixture

Table 5

Units of activity Recovered in Isolation Procedure from 8 Kg of Wheat Hulls

	Units
Ether Extraction	11,000
<u>Silicic Acid Chromotography</u>	
(a) 50:50 Pet. Et: Ether	3,000
(b) 25:75 Methanol:Ether	6,000
<u>Cellulose Column</u>	5,000
Ether-water Partition (pH 4.0)	4,000
Ether-water Partition (pH 8.5)	3,000
Ether-water Partition (pH 3.0)	2,500

of petroleum ether:ethyl ether: The first 300 to 1000 ml of elute contained considerable inhibitory activity. This fraction was stored in the cold for further investigation. It was unstable at room temperature over prolonged periods of time.

(d) Fraction number 4: 1000 ml of 25:75 methanol: ethyl ether. The addition of a polar solvent immediately eluted a major inhibitory fraction which was used in subsequent steps.

The inhibitory fraction was evaporated to a small volume and the concentrate was mixed with cellulose powder and dried in a vacuum desiccator. This mixture was subsequently applied on top of a cellulose packing which had been packed dry. The material was eluted with water. All of the inhibiting activity was eluted with 100 ml of water and subsequent fractions were inactive.

The pH of the aqueous solution was adjusted to 3.5 and extracted three times with equal volumes of ether. The inhibitor was found in the ether layer, thus leaving some impurities in the aqueous layer. The ether extracts were then extracted with aqueous sodium bicarbonate. The aqueous layer turned yellow in color and all of the inhibitory material was found in the aqueous layer. The aqueous layer was neutralized with Dow -

50-H+ and again extracted with ether in order to transfer the material to an organic solvent.

A small sample has been run through a sephadex G-10 column as described for the isolation procedure for sugar beet fruit (Figure 7). Inhibitory activity was eluted from the column at about the same position as for the cis 4-cyclohexene-1,2-dicarboximide compound from sugar beet. However the amount of material was too small for any analysis except biological activity.

FORMATION OF INHIBITIORS IN WHEAT CHAFF:

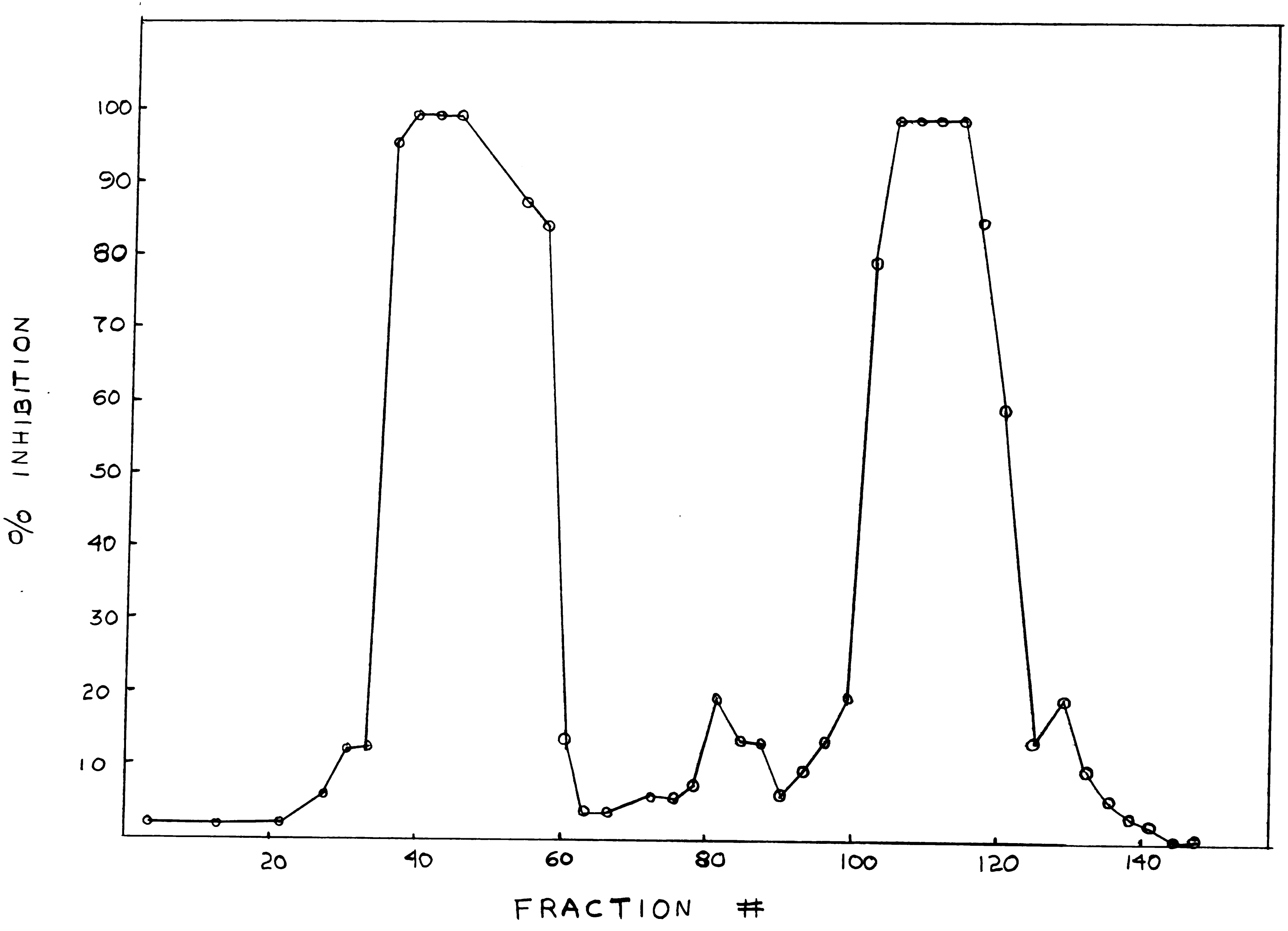
The results in this section were collected by A. A. Khan and N. E. Tolbert and have not been published. Since their results were pertinent to the isolation of inhibitors from wheat, their findings are summarized herein with their permission.

During the summer of 1963 and 1964 field tests were made on the amount of inhibitor in ripening grain. From these studies it was acertained that wheat hulls were a rich source of the dormancy factors whereas the seeds contained much less inhibitor. The amount of inhibitor increased to maximum values about 26 days after anthesis when the grain was fully formed. The amount of inhibitor in the more dormant Red Coat wheat was greater than in the Genesee wheat which is often not dormant at harvest.

Figure 7. Elution pattern of the Wheat Inhibitory Material from a Sephadex G-10 column

Flow rate = 7-10ml/hour

Eluent = water


% INHIBITION
100
90
80
70
60
50
40
30
20
10
20
40
60
80
100
120
140
FRACTION #

The amount of inhibitor in the Genesee wheat hulls decreased more rapidly after 26 days than that in the hulls of the Red Coat wheat. A two day rain occured 41 days after anthesis and only 50% of the inhibitory material remained, probably, because the rain had leached or washed out the inhibitor. It is known that germination will occur in the head after such rain storms.

Consequently, all of the research was done on the Red Coat Wheat. Furthermore, the hulls were a more desirable starting material because ether extractions removed less bulk from hulls than from the seeds from which ether extracted much fat and lipoidal material.

It was necessary to harvest the the hulls 35 days after anthesis which is before normal harvest in order to obtain them with maximum amounts of inhibitory material per gram of weight. The heads were harvested with a stripper and a fan was used to separate the grain from the hulls. The hulls have been stored for over ten months at -10° C but with an appreciable loss in the inhibitory material which can be removed by ether extraction.

PROPERTIES OF THE INHIBITOR FROM WHEAT CHAFF:

Some of the chemical properties are as follows:

(a) Inhibitor is a small molecular weight organic compound.

(b) It is a weakly acid or neutral compound since it is not absorbed appreciably on an ion exchange resin.

(c) It is soluble in polar solvents such as, water, ethanol, methanol, ethyl acetate, ether; but insoluble in carbon tetrachloride and petroleum ether (30° - 60°).

(d) On paper chromatography the inhibitory material has an Rf of 0.60 - 0.90 with a variety of polar solvent systems used. Using thin layer chromatography with the same solvent systems the Rf is from 0.50-0.85. Using chloroform:methanol (9:1) the Rf of the strongest inhibitory band is 0.55.

(e) The inhibitory material forms a brown coloration on paper chromatograms with 1% permanganate, a red coloration with Rhodamine 6 G, and a yellow color with bromo cresol purple.

(f) A solution of the inhibitory material decolorized bromine-water, decolorized permanganate, and did not react with ferric chloride. These results suggested oelifinic unsaturation.

(g) Biological activity was destroyed by reduction with sodium borohydride.

(h) I.r and u.v characteristics can not be discussed in absolute certanity but the i.r always showed a strong carbonyl absorbtion along with carbon-hydrogen (possible aliphatic) absorption. However, there were other components (impurities) with the inhibitor.

BIOLOGICAL STUDIES ON CIS 4-CYCLOXENENE-1,2-DICARBOXIMIDE (CCD):

LIGHT, GIBBERELLINS AND α-AMYLASE SYNTHESIS:

It is known that dormant seeds at harvest do not germinate regardless of the light treatment. Thus there is no evidence at present on any relationship between dormancy and the phytochrome system. So far no interaction between CCD inhibition of seed germination and light treatment has been observed by us in the laboratory. Rather CCD seems to supress the phytochrome effect on germination until the CCD is removed. However further research on the effect of CCD on various phytochrome responses should be explored.

Lettuce seed treated with CCD will not germinate if 1 x 10^{-5} M gibberellin A_3 is also added. As far as these test have gone, gibberellins do not appear to reverse the inhibition of germination induced by CCD.

Since gibberellins regulate α-amylase synthesis, another way to test this conclusion was to examine α-amylase production in germinating seeds. Gibberellins are known to induce the de-novo synthesis of α-amylase in barley endosperm (24), and this was confirmed by my data in table 6. The CCD germination inhibitor at 10^{-3} M did not alter α-amylase production or release by barley endosperms as brought about by 10^{-5} M gibberellin A_3.

As a further test, the effect of CCD was studied on α-amylase synthesis in the whole seed. The purpose of this experiment was to measure the synthesis of α-amylase using endogenously synthesized gibberellins from the embryo. About 70% as much α-amylase enzyme was synthesized with the CCD treated whole seeds (Table 7). Thus CCD did not inhibit severely α-amylase synthesis by the whole seed. There was a lower amount of α-amylase in the medium which suggests that less enzyme was synthesized in the aleurone layer but that no CCD effect exists upon release of the enzyme from the aleurone layers. In the previous experiments with the barley endosperm, there was no effect upon α-amylase synthesis in gibberellic acid treated half

Table 6

α-amylase synthesis in the barley endosperm

Treatment	Medium	Extract	Total
	units	units	units
$-GA_3$	15	7.6	22.6
$+10^{-5}M\ GA_3$	29.3	12.6	41.9
10^{-3} CCD + $10^{-5}M$ GA,	28.5	12.2	40.7

Table 7

α-amylase Whole Seed Assay

Treatment	Medium	Extract	Total
	units	units	units
None	15.3	16.9	34.2
+CCD 10^{-4}M	6.3	17.1	23.4

seeds. The smaller amount of enzyme produced in the whole seeds could mean that the CCD inhibitor possibly inhibited part of gibberellin synthesis in the embryo. However, there were still some gibberellin produced or there was another factor produced by the embryo which is involved in the formation of α-amylase. The latter has been suggested by MacLeod (45).

At present we conclude there appears to be no relationship between gibberellin linked α-amylase synthesis and CCD. Thus CCD is not an "anti-gibberellin" and has little effect on gibberellic induced synthesis of α-amylase. CCD activity as a germination inhibitor must reside elsewhere. In this respect, CCD is different from Abscisin II which prevents the formation of α-amylase without inhibiting respiration, phosphorylation, protein synthesis or RNA synthesis (46). The mechanism of action of Abscisin II seems to be rather specific and clear, whereas CCD has no effect in this system at the concentrations used.

RESPIRATION:

The Q_{O2} (1 O_2 hour $^{-1}$) for 10 barley half seeds measured at 25^{o} was 31 1. For the half seeds treated with gibberellic acid the value was 31 l and for those treated with gibberellic and CCD inhibitor the value was 32 . It appears that respiration of the barley endosperms is not effected by inhibitor (CCD).

Germination of White paris lettuce seeds was inhibited at 10^{-3} to 10^{-4} M concentration of the inhibitor (CCD). In figures 8 and 9 the expression of this phenonmenon is shown as inhibition of respiration of the lettuce seeds. The seeds will begin to germinate at 16 hours after inbibition. The physical evidence for this in the protrusion of the radical which can be visually observed after 16 hours. However, respiration increases after 10 to 12 hours and the rate of respiration (figure 9) reaches a new higher level after 16 hours. The seeds treated with CCD did not germinate nor change their respiration rate after 12 hours of inbibition. Rather CCD treated seeds continued to respire at approximately the same slow initial rate for more than 20 hours . It appears that the inhibitor CCD prevents germination without effecting the initial respiration rate. Thus it does not inhibit germination by killing the seed. In fact after treatment of seeds with a 10^{-4} M CCD for 24 hours, the seeds may be washed and left for an additional 20 hours with fresh water when they will then germinate. This remarkable experiment shows the reversibility of

Figure 8. Total Oxygen Uptake of *White paris* lettuce seeds

open circles	without Inhibitor
closed circles	10^{-3} - 10^{-4} M Inhibitor

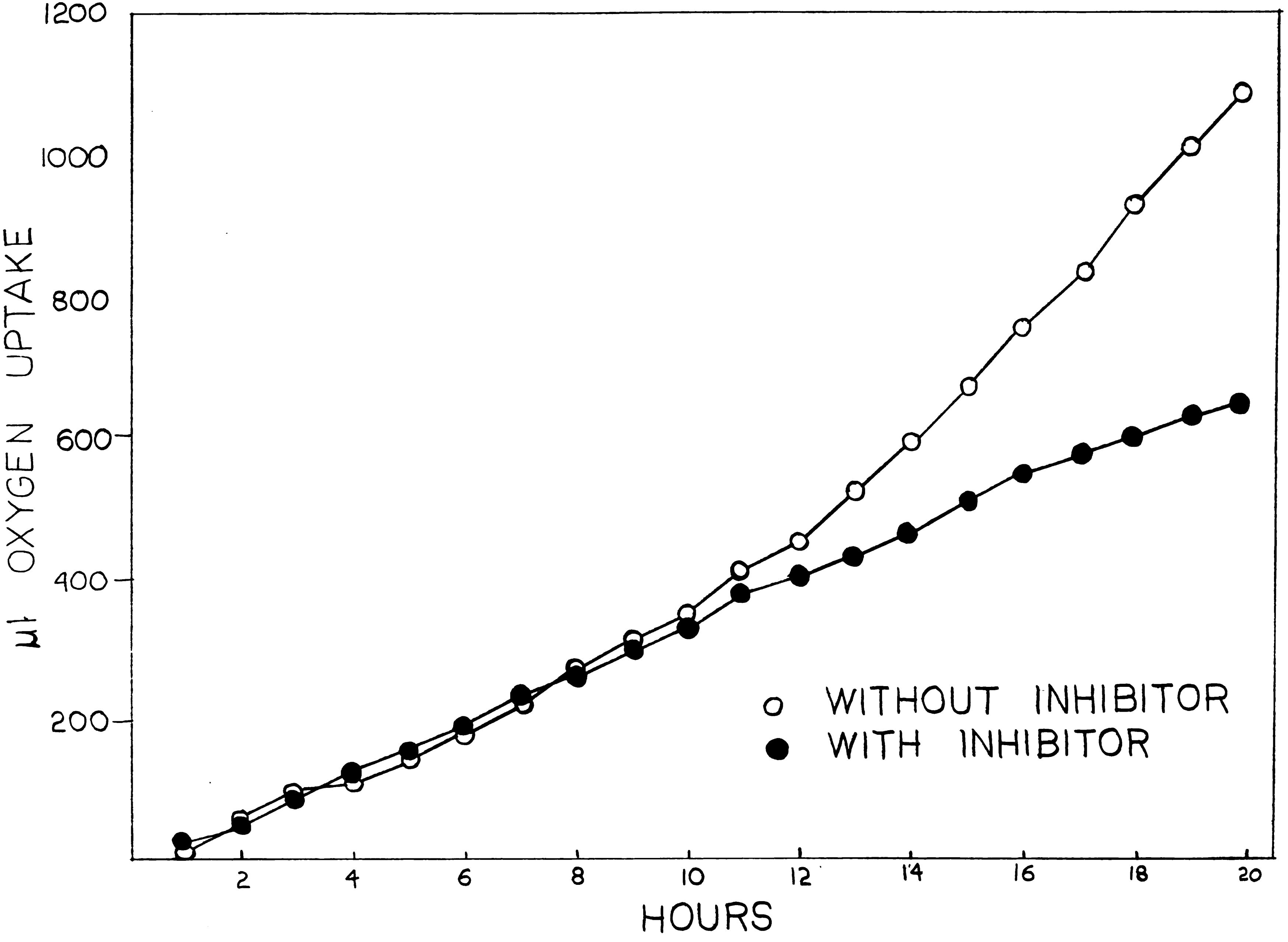
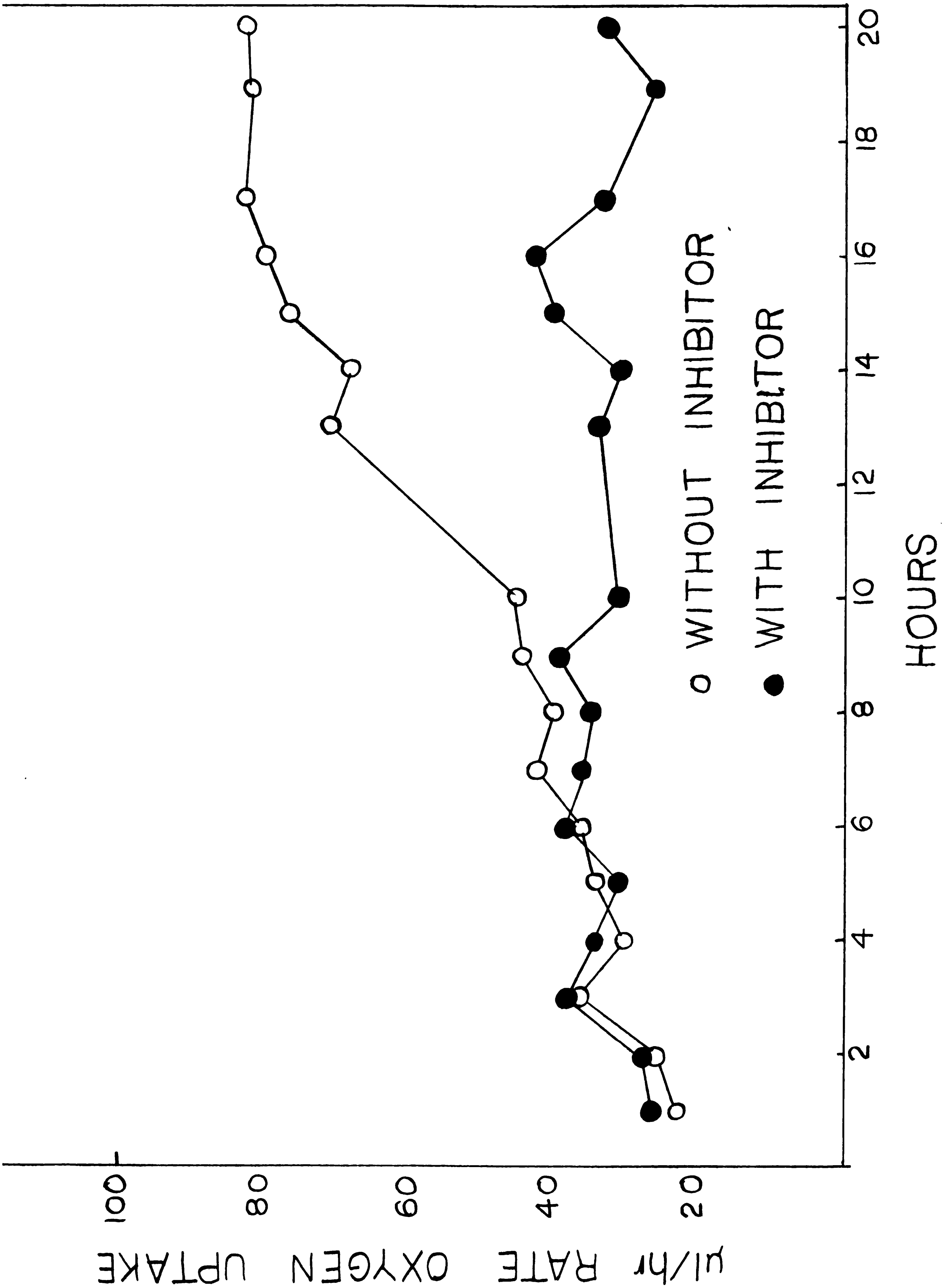

1200
1000
800
600
400
200
µl OXYGEN UPTAKE
2
4
6
8
10
12
14
16
18
20
HOURS
○ WITHOUT INHIBITOR
● WITH INHIBITOR

Figure 9. Rate of oxygen uptake of White paris lettuce seeds

open circles	without inhibitor
closed circles	10^{-3} - 10^{-4} M Inhibitor


µl/hr RATE OXYGEN UPTAKE
100
80
60
40
20
HOURS
2
4
6
8
10
12
14
16
18
20
○ WITHOUT INHIBITOR
● WITH INHIBITOR

the effect of this inhibitor.

AVENA STRAIGHT GROWTH ASSAY:

The results of the *Avena* straight growth assay are summarized in table 8. The IAA + CCD treated coleoptiles showed quite a variation in lengths; however, the mean value was somewhat less than IAA treated coleoptiles. This suggests that the CCD inhibitor at 10^{-3} M may act only as a weak inhibitor of auxin induced growth. The mean value length of CCD treated coleoptiles were the same as the control (buffer only). These length measurements were very consistent and did not vary as those with IAA + CCD.

That all the above physiological experiments were negative indicated the uniqueness of the CCD structure on inhibition seed germination. It suggest that this inhibitor (CCD) does not prevent germination by killing the seed, affecting the seed viability or interacting with other known plant hormones such as IAA and GA. Further studies or mechanism of action should be done at the molecular level for inhibition of RNA synthesis.

Table 8

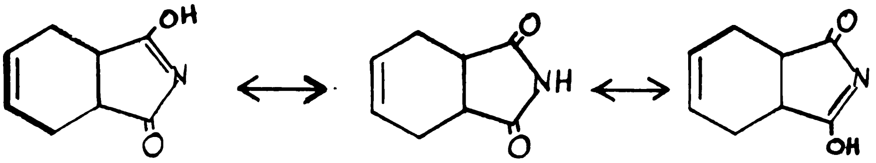
Effect of CCD on Avena Straight Growth Test

Treatment	Length at 0 hours	Length at 24 hours	% control growth
Control	4.9mm	6.8mm	100
IAA	4.9mm	10.0mm	147
IAA + CCD	4.9mm	9.2mm	135
CCD	4.9mm	6.8mm	100

STRUCTURE OF CIS 4-CYCLOHEXENE-1,2-DICARBOXIMIDE AND ITS BIOLOGICAL ACTIVITY:

This structure is unusual for a naturally occuring compound. It is not an alkaloid nor does it belong to the family of terpenoid compounds which contain the isoprenoid unit. The structure fits no known biosynthetic family of compounds.

In the structure of CCD, the dicarboximide functional group seems to be most important by comparison with other similar compounds to be described below. However the single double bond in the hexene ring is undoubtedly of importance. In aqueous solution, the dicarboximide ring must be in equilibrium with two other resonance forms which are identical:

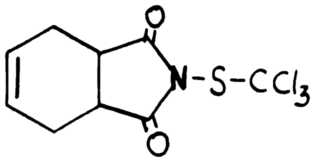


CCD is a weak acid and its PK is about 6.5. Consequently CCD is soluble in both polar and non polar solvents, and this solubility is effected by pH shifts of importance in biological tissue. It can be readily solubilized by water from seeds and other tissue, yet it could penetrate lipoidal layers and non-polar solvents.

CCD is a stable compound. It is not destroyed by autoclaving in aqueous solutions. The loss of inhibitory units experienced in the isolation procedure during thin layer chromatography and acid-base treatments must be attributed to destruction of compounds other than CCD.

The amount of CCD per weight of sugar beet fruit has not been determined. Its effective concentration for complete inhibition of lettuce seed germination was 1×10^{-4} M. CCD is not the only seed germination inhibitor in sugar beet fruit, for in fact there are many inhibitors in the sugar beet fruit such as coumarin, organic acids and phenolic compounds. The nature of many of these other compounds, which are inhibitors, remains unknown. The first peak off the Sephadex G-10 column in the last step of the isolation procedure would be the next most logical inhibitor to identify. This unknown must be relatively pure and may have some of CCD properties, since it has been isolated by the same procedure. This unknown is not Abscisin, since it showed no cotton effect at 247 mu (16).

The basic structure of CCD is very similar to the agricultural fungicide, Captan, which is shown below.

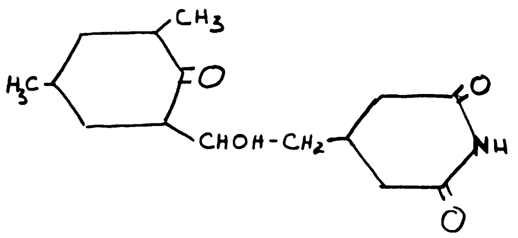


Captan

Captan is used as a agricultural fungicide. It was discovered by Kettelson (49) who also made other derivatives of similar cyclic imide structures. Captan has more effect as an agricultural fungicide, and it is now commonly used as in agricultural practices. Its possible use on the sugar beet fruit before we received the material raises the serious question as to whether CCD is naturally occurring or whether CCD arose as a breakdown product from some chemical treatment of the seed. In a private communication, the West Coast Sugar Beet Seed Company from whom the seeds were obtained, stated that the seeds had not been treated with Captan. Further similar inhibitory material is present in wheat hulls which were grown here by us and which we also know were not treated with Captan. Captan is a very stable compound and is not easily decomposed. All these points suggest that CCD is a naturally occurring material, but further careful investigation will be necessary to establish this point.

If CCD were a decomposition product or metabolite of Captan, then CCD might be the active component of Captan. This is the universal question about active structures of inhibitors such as herbicides, fungicides, pesticides and insecticides. In this case with Captan, is Captan the active structure, or is a decomposition produce or metabolite such as CCD the active structure. For the present, the assumption is made that Captan itself is active and that CCD is a similar naturally occurring compound. Since Captan inhibits fungi growth by inhibiting the germination of spores, both compounds may have similar mechanism of action.

Another interesting compound which has a similar functional group to CCD is Cycloheximide, (shown below), which is produced by Upjohn Company. Cycloheximide has a cyclic imide structure and has been used as an inhibitor of protein synthesis in mammalian systems (48).

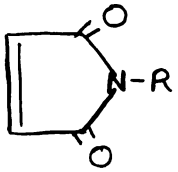


CYCLOHEXIMIDE

Cycloheximide has no effect upon protein synthesis in extracts of Echerichia coli. It is also used as an agricultural fungicide but the mechanism of action in systems other than mammalian has not been thoroughly investigated. However, cycloheximide has been studied in higher plants (24, 51, 52) and at 10 mg liter gives inhibition to germination of Phacelia tanacetifolia seed

seed (49). It inhibits germination completely in darkness and in the light with gibberellic acid. Thus the mechanism of action of CCD and cycloheximide may be identical and may involve inhibition of protein synthesis. Varner (24) has shown that cycloheximide inhibits de novo synthesis of α-amylase by inhibiting protein synthesis.

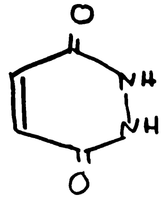
A class of biological inhibitors may be evident from the above structural observations of compounds similar to CCD. They all contain the cyclic imide ring. In this group might go other biological inhibiting compounds such as the maleimides.



R = H, alkyl or aryl

Of the maleimides, the N-ethyl maleimides has been the most widely used as an enzyme inhibitor. N-ethyl maleimides can react with amino groups in proteins under the same conditions which are used to alkylate sulfhydryl groups. Recently, Sharpless and Flavin (50) have studied the reactivity of maleimides with protein amino groups and reported that the amino group adds to the double bond of the maleimide. Thus the significance of the double bond in CCD is emphasized.

Another similar compound is maleic hydrazide (shown below) which acts as a metabolic poison.



Maleic Hydrazide

The above mentioned compounds have differend effect upon biological systems, but they do have similar functional groups. Namely, the cyclic imide. Perhaps this functional group is necessary for biological activity though each has a different function in similar systems. The spectficity or selectivity may be a result of the other differences in structures.

SUMMARY

A compound has been isolated and characterized from sugar beet fruit which is a germination inhibitor. The use of Sephadex G-10 column chromatography was very beneficial in separating low molecular weight organic compounds. This compound's activity is not just due to destroying the seed but is due to action at a site other than some isolated metabolic process. It seems to be very specific in only inhibiting germination. However, more experiments are needed to determine the site of action of this compound. There was no effect upon respiration, gibberellic acid induced synthesis of α-amylase, the activity of the α-amylase enzyme and a questionable effect upon avena straight growth.

The compound is characterized by a 5-membered cyclic imide which is somewhat unusual as a natural occurring biological compound. The closest similar structure is a derivative CAPTAN which is used as an agricultural fungicide. Its action is inhibition of germination of fungi spores. The cyclic imide perhaps may be a particular class of biologically active compounds along with glutarimides and N- substituted maleimides. The future of research with this unique compound will depend upon universival distribution and activity in which the importance rests upon the latter.

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