# Isolation from Sugar Beet Fruit and Characterization of *cis*-4-Cyclohexene-1,2-dicarboximide as a Germination Inhibitor*

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ABSTRACT: The isolation of *cis*-4-cyclohexene-1,2-dicarboximide as a germination inhibitor from the fruit of sugar beet seeds has been followed by its inhibition of lettuce seed germination. A similar compound was present in wheat chaff or dormant wheat seed. The isolation procedure involved ether extraction, silicic acid chromatography, cellulose chromatography, Sephadex G-10, and crystallization. Identity was confirmed by synthesis and comparison of physical and biological properties. Derivatives were synthesized, and the most active structure was the *N*-butyl analog which inhibited lettuce seed germination at $10^{-5}$ M. Inhibition of germination was measured by failure of the embryo to grow, by the absence of increased respiration, and by low rates of leucine-$^{14}$C incorporation into protein. Germination inhibition by the compounds was not reversed by gibberellin, indole acetic acid, or light treatments.

Seeds inhibited by the compounds would germinate when washed with water to remove the inhibitors. *cis*-4-Cyclohexene-1,2-dicarboximide is a naturally occurring biological compound which is structurally comparable to other dicarboximide inhibitors such as cycloheximide, Captan, and maleimides.

Dormancy in plants and seeds may be regulated by many endogenous inhibitors and growth substances (van Overbeek, 1966). Inhibitors of seed germination have been recognized in grain (Miyamoto *et al.*, 1961), fruit (Massart, 1957), tubers (Hemberg, 1949), and buds (Robinson *et al.*, 1963). Chemical characterization of most of these substances has not been accomplished. However, Abscisin II has been identified as a dormancy factor in buds (Ohkuma *et al.*, 1965; Cornforth *et al.*, 1965). The presence of inhibitors in sugar beet seeds has been studied repeatedly (Duym *et al.*, 1947; DeKock *et al.*, 1956) and attributed partially to hydroxy organic acids (Massart, 1957) or to oxalate (Miyamoto, 1957; Snyder *et al.*, 1965). In this investigation, one of several inhibitory substances from sugar beet seeds has been identified as *cis*-4-cyclohexene-1,2-dicarboximide. This type of structure seems to be characteristic of several other compounds which are inhibitors of biological growth.

## Materials and Methods

*cis*-4-Cyclohexene-1,2-dicarboxylic acid anhydride was obtained from Aldrich Chemicals. Gibberellic acid was obtained as Gibrel from Merck & Co.

Sugar beet fruit was the corky material removed from around dried seeds of *Beta vulgaris* L. variety (SL 129 X 133) ms X 5822–0. The sugar beet material was obtained as a fine powder formed during milling to remove the outer part of the fruit prior to planting the seed. It was donated by Farmers and Manufacturers Beet Sugar Association, Saginaw, Mich. Bags of the material were stored at −18° for 1–10 months before use. Red Coat wheat was grown locally. White Paris lettuce seeds were obtained yearly from Perry–Morse Seed Co.

Infrared spectra were run with a Beckman IR-5 and ultraviolet spectra with a Beckman DB spectrophotometer. Nuclear magnetic resonance spectra were run with a Varian HA-60 using a CAT 1024 computer. Elemental analysis was determined by Spang Microanalytical Laboratory, Ann Arbor, Mich.

*Bioassay.* All fractions and materials were assayed by inhibition of germination of White Paris lettuce seeds. A quantity of 25 lettuce seeds was germinated on a 5-cm Whatman No. 1 filter paper disk moistened with 2 ml of test solution in a small petri dish. The per cent germination at room temperature (22°) and light was recorded after about 20 hr. A unit of activity was defined as the amount of material that would completely inhibit germination of the seeds. Specific activity was expressed in units of activity per milligram of dry material. Organic solvents had to be completely removed for the germination assay. Therefore, fractions were either evaporated to dryness and redissolved in water or placed directly on the filter paper for the germination assay, dried at room temperature, and then 2 ml of water was added for the germination assay.

*α-Amylase Assays.* The barley endosperm assay for α-amylase as reported by Varner *et al.* (1965) was used. In each assay 100 dry seeds were cut in half, sterilized, and the endosperm half of the seeds was preincubated for 3 days on moist sand. The half seeds

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were then incubated for 20 hr at 25° by shaking in a buffered solution with appropriated dilutions of *cis*-4-cyclohexene-1,2-dicarboximide and with or without $10^{-5}$ M gibberellic acid. Amylase activity was measured (Schuster and Gifford, 1964) in the incubation solution and also in extracts of the half seeds as obtained by homogenization in acetate buffer at pH 4.8. α-Amylase production was also followed for whole seeds rather than half seeds by a nearly identical procedure. In each assay, ten seeds were used with the tip of the endosperm removed to facilitate imbibition.

*Lettuce Seed Respiration.* Fifty lettuce seeds were placed in a 15-ml Warburg flask and incubated with 1.5 ml of solution containing 0.29 mg/ml of the inhibitor and about 25 μg of penicillin and 25 μg of mysteclin to prevent growth of microorganisms and fungi. Conventional $O_2$-uptake measurements were made with KOH in the center well.

*Avena Straight Growth.* This procedure with oat seeds (variety, Torch) has been developed for measuring the effectiveness of various IAA[1] analogs (Schlender *et al.*, 1966). We used IAA concentrations of $10^{-5}$ M and *cis*-4-cyclohexene-1,2-dicarboximide at $10^{-4}$ M. The increase in mean length was expressed as a per cent of the values obtained with controls, both with or without IAA.

*Synthesis of cis-4-Cyclohexene-1,2-dicarboximide.* To 25 ml of absolute ethanol in a Pyrex tube saturated with $NH_3$ gas at 0° was added 4.1 g of *cis*-4-cyclohexene-1,2-dicarboxylic acid anhydride. The tube was sealed and autoclaved with 20 psi at 125° for 90 min. The contents were dissolved in benzene, the benzene was evaporated to dryness, and the imide was recrystallized from water with 15% yield: mp 138–139°, lit. (Snyder and Poos, 1950) mp 136.5–137.3°.

The imide was also prepared by heating $NH_4OH$ and *cis*-4-cyclohexene-1,2-dicarboxylic acid anhydride. In a 500-ml round-bottom flask equipped with an air condenser, 44.4 ml (0.66 mole) of 28% aqueous $NH_4OH$ and 51.6 g (0.34 mole) of the anhydride were heated for 2 hr at about 300° to remove water. The imide formed was recrystallized from benzene and then water: yield 70%, mp 138–139°.

*Synthesis of N-Alkyl-cis-4-cylohexene-1,2-dicarboximide.* The boiling point and melting point of the N-substituted derivatives which were synthesized are listed in Table I. The preparation of *N*-methyl-*cis*-4-cyclohexene-1,2-dicarboximide exemplifies these syntheses. Into 30.5 g (0.54 mole) of KOH in 120 ml of 75% ethanol was poured 800 ml of absolute ethanol containing 80 g (0.53 mole) of *cis*-4-cyclohexene-1,2-dicarboximide. The alcoholic solution was evaporated to dryness, and the salt was used without further purification. The *N*-potassium *cis*-4-cyclohexene-1,2-dicarboximide was refluxed with excess $CH_3I$ for 12 hr. KI was removed by filtration and excess $CH_3I$ by vacuum. The *N*-methyl derivative was recrystallized from water with an over-all yield of 72%.

*endo-cis-Bicyclo[2.1.1]-5-heptene-2,3-dicarboximide.*

TABLE I: *N*-Alkyl-*cis*-4-cyclohexene-1,2-dicarboximide Derivatives.

| Alkyl | Pressure (mm) | Bp (°C) Lit.[a] | Bp (°C) Obsd |
|---|---|---|---|
| H | 760 | 136–137[b] | 138–139[b] |
| Methyl | 760 | 72.5–73[b] | 70–72[b] |
| Ethyl | 0.3 | 86–90 | 86–90 |
| *n*-Propyl | 0.2 | 86–91 | 85–92 |
| Allyl | 3.0 | 127–129 | 120–127 |
| *n*-Butyl | 0.7 | 128–130 | 125–129 |
| *n*-Amyl | 0.2 | 96–101 | 95–100 |
| *n*-Hexyl | 0.4 | 117–122 | 115–123 |
| *n*-Heptyl | 0.3 | 127–130 | 127–135 |

[a] Rice *et al.* (1954); Culberson and Wilder (1960). [b] Melting point in degrees centigrade.

This compound was prepared by heating for 10–15 min 8.8 ml of 28% $NH_4OH$ with 11 g (0.068 mole) of *endo-cis*-bicyclo[2.1.1]-5-heptene-2,3-dicarboxylic acid anhydride which was a gift from Velsicol Chemical Corporation. The imide was recrystallized from hot water: mp 188–189°, lit. (Culberson and Wilder, 1960) mp 186–187°.

*Cyclohexane-1,2-dicarboximide.* This compound was prepared by reduction of *cis*-4-cyclohexene-1,2-dicarboximide with one atmosphere of $H_2$ as catalyzed by 0.2 g of platinum oxide. The theoretical volume of $H_2$ uptake was observed in 5 min. The compound was recrystallized from water: yield 86%, mp 136–137°, lit. (Snyder and Poos, 1950) mp 137°.

*Leucine-$^{14}$C Incorporation.* Lettuce seeds (100) were surface sterilized with 80% ethanol for 3–5 min and then washed with water. The seeds were incubated for 10 hr at room temperature with 1 ml of $10^{-3}$ M *cis*-4-cyclohexene-1,2-dicarboximide containing 0.5 μCi of L-leucine-$^{14}$C (uniformly labeled). Then the medium was decanted and the seeds were washed four times at 5-min intervals at 0° with $10^{-3}$ M leucine-$^{14}$C. They were ground in a mortar at 0° for two 5-min periods with 2 ml of 0.2 M NaCl each time. Aliquots were counted for total $^{14}$C. Other aliquots were centrifuged, and then the protein was precipitated by 7.5% TCA, washed with ethanol–ether (50:50) and with ether, and resuspended. Aliquots of the TCA-insoluble protein were counted for $^{14}$C and assayed for total protein by the method of Lowry *et al.* (1951).

## Results

*Isolation Procedure from Sugar Beet Fruit.* Each fraction from each step of the procedure was bioassayed, and a summary of the steps and results of the isolation is given in Table II. Separate 1.4-kg quantities of the corky material from sugar beet fruit were extracted in a 5-l. soxhlet extractor with 3.5 l. of ether for 8 hr.

[1] Abbreviations used: IAA, indole-3-acetic acid; TCA, trichloroacetic acid; $GA_3$, gibberellin $A_3$.

TABLE II: Summary of Isolation Results from 8.4 kg of Sugar Beet Corky Material.

| | Residue Wt (g) | Total Units | Sp Act. (units/mg)[a] |
|---|---|---|---|
| Ether extraction | 65.0 | 32,500 | 0.5 |
| Silicic acid column | 3.36 | 4,000 | 1.19 |
| Cellulose colunm | 1.33 | 3,800 | 2.86 |
| Sephadex G-10 column | 0.096 | 319 | 3.33 |

[a] One unit of activity was obtained from 0.25 mg of synthetic *cis*-4-cyclohexide-1,2-dicarboximide which yields a calculated specific activity of 3.95 for the pure compound.

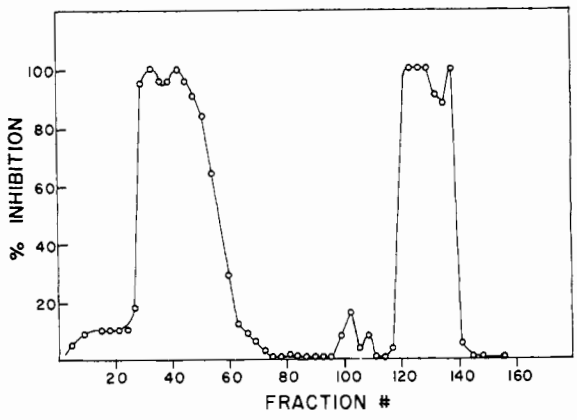



FIGURE 1: Elution pattern of the sugar beet inhibitory material from a Sephadex G-10 column: flow rate, 7–8 ml/hr; eluting solvent was water.

After six different extractions, the ether was removed from the combined fractions by evaporation to yield 65 g of residue containing 32,500 units of activity for inhibiting lettuce seed germination. The total material was placed on top of a silicic acid packing of 45 × 5 cm dimensions. The material was eluted from the column with petroleum ether (bp 30–60°)–ether (9:1 to 1:9, v/v) under 3–6 psi and eventually with ether, as described in Table III. Fractions (1 l.) were collected and each was bioassayed. About 80% of the total units of activity was not recovered from this column, since we selected for only one group of inhibitors. Oxalic acid and phenolic acids, known inhibitors in sugar beet fruit, were excluded by the collected fractions. Of the inhibitory material which was eluted from the column with petroleum ether–ether, about 80% always appeared in fractions 8–11. Usually fractions 9 and 10 were combined and evaporated almost to dryness. This residual material was mixed with powdered cellulose and placed on top of a 25 × 5 cm dry cellulose packing. The cellulose column was eluted with water, and the first 250-ml fraction contained 90% of the inhibitory material. This eluate, containing ether-soluble and water-soluble constituents, had a pH of 4.5.

TABLE III: Elution Pattern for Silicic Acid Column.

| Fraction[a] | Eluent: Petroleum Ether–Ether | Units/ml |
|---|---|---|
| 7 | 3:7 | 0.0 |
| 8 | 2:8 | 1.0 |
| 9 | 1:9 | 8.0 |
| 10 | Ether | 2.0 |
| 11 | Ether | 1.0 |
| 12 | Ether | 0.25 |
| 13 | Ether | 0.0 |

[a] Each 1000-ml fraction was eluted and assayed. Fraction 1 had a petroleum ether–ether ratio of 9:1, fraction 2 had an 8:2 ratio, etc., to fraction 7 as shown. No activity was found in the first seven fractions.

The first 250-ml aqueous fraction from the cellulose column was evaporated to between 3 and 5 ml. It was placed on a Sephadex G-10 column of 95 × 2.5 cm dimensions which had been equilibrated with water. The column was developed with 7–8 ml of water/hr, and fractions were collected every 30 min. Inhibitory substances were detected in two bands by the lettuce seed assay (Figure 1). The material in the first fraction was an oily residue after evaporation and has not been extensively investigated. It was not Abscisin, since it showed no Cotton effect at 247 mμ. The substances in the second band, fractions 121–132, upon combination and evaporation, yielded 96 mg of residue. A material in this fraction was recrystallized from cold water to a constant melting point. The yield was 9.9 mg, and the vacuum-dried material melted at 138–139°.

*Identification.* The inhibitory material from sugar beet fruit isolated in the second peak from the Sephadex G-10 column had an empirical formula of $C_8H_9NO_2$ from elemental analysis and a mass spectral parent peak representing a molecular weight of 151. The infrared spectrum in KBr pellet (top of Figure 2) showed NH next to a strong electronegative atom (3215 $cm^{-1}$), unsaturated CH stretch (3058 and 2976 $cm^{-1}$), methylene CH (2924 and 2865 $cm^{-1}$), carbonyl of a cyclic imide (1767 and 1704 $cm^{-1}$), methylene bending frequencies (1425 $cm^{-1}$), and carbon–carbon double bond (1637 $cm^{-1}$). The strong double carbonyl absorption at 1767 and 1704 $cm^{-1}$, in which the lower band was stronger, is characteristic of a five-member cyclic imide ring (Nakanishi, 1964).

The unknown inhibitor gave a positive test with both $KMNO_4$ and $Br_2$–$H_2O$ reagents to indicate an olefinic double bond. There were no reducing groups strong enough to react with ferric chloride–2,2′-bipyridyl reagent and no phenolic or hydroxy group reactions with ceric nitrate.

The ultraviolet spectrum had a maximum absorbance

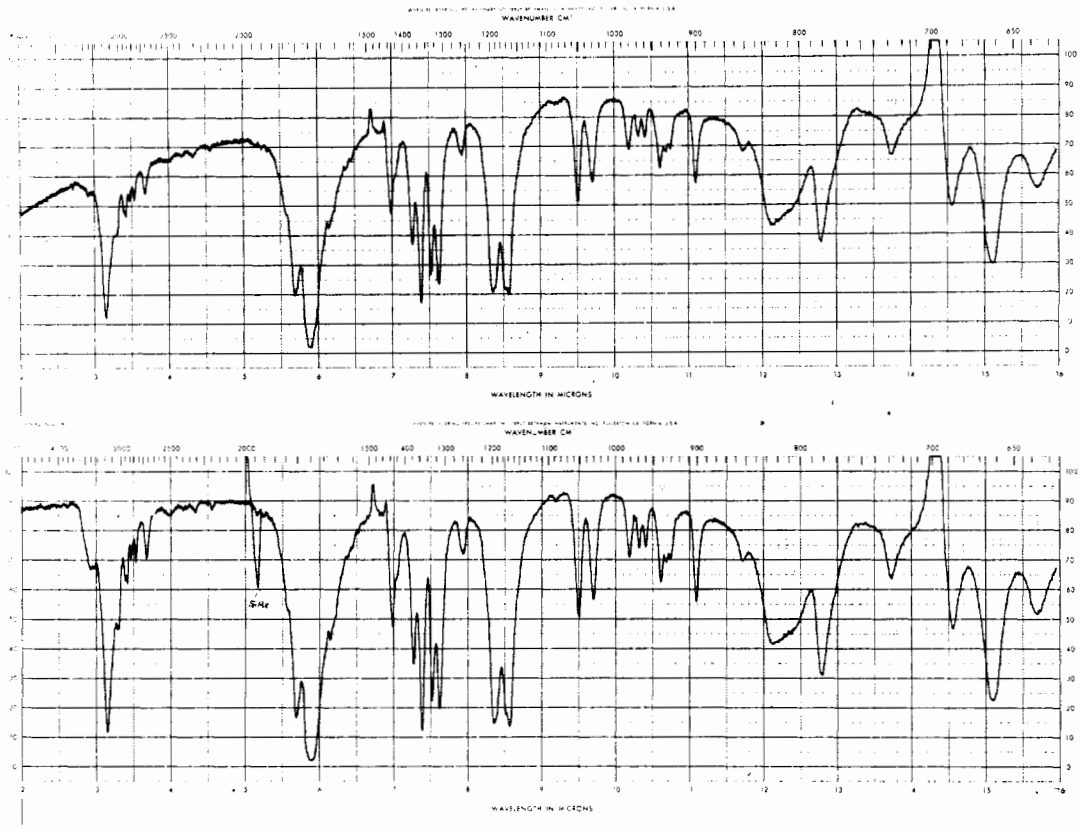
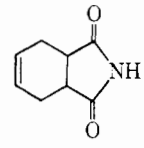


FIGURE 2: Infrared spectra of synthetic and unknown inhibitor. Top: isolated inhibitor. Bottom: synthesized *cis*-4-cyclohexene-1,2-dicarboximide.

at 242 mμ when measured at a concentration of 1 mg/ml, but the extinction coefficient ($E$) was only 128 at 242 mμ. This value was too low for a carbonyl conjugated with an ethylenic group which should have an $E_{max}$ of 10,000–20,000 (Silverstein and Bassler, 1963). The low $E_{max}$ of 128 at 242 mμ could not be due to K bands which result from $n$–$\pi^*$ transitions but rather from an R band whose origin is in the $n$–$\pi^*$ transition of a single nonconjugated chromophoric group.

From the above data, the unknown inhibitor appeared to contain a five-membered cyclic imide ring, a nonconjugated carbonyl, a nonconjugated double bond, and an alicyclic ring to satisfy the elemental composition. The structure of *cis*-4-cyclohexene-1,2-dicarboximide satisfied these requirements as well as the empirical formula and the ultraviolet and infrared




spectra. The location of the double bond was further established by nuclear magnetic resonance analysis which showed four different kinds of protons at $\tau$ 3.72, 6.51, 7.12, and 7.35. If the double bond were located so that the unknown were a 3-cyclohexene-1,2-dicarboximide, then seven protons of different environment would have been observed.

*cis*-4-Cyclohexene-1,2-dicarboximide has been synthesized (Snyder and Poos, 1950) with a reported melting point of 134–136° after recrystallizing from benzene. This value did not agree with the melting point of the isolated inhibitor. Upon synthesis of the compound from *cis*-4-cyclohexene-1,2-dicarboxylic acid anhydride and recrystallization from benzene, we, too, observed a melting point of 134–136°. However, when the synthetic dicarboximide was recrystallized from water, as had been done with the unknown, the melting point was 138–139° and the mixture melting point was unchanged. The infrared spectrum (Figure 2) of the synthetic *cis*-4-cyclohexene-1,2-dicarboximide was essentially identical with the spectrum of the isolated germination inhibitor. The biological activity for inhibition of lettuce seed germination by the isolated material was similar within the limits of the biological assay to that with the synthetic material (Table IV).

*Partial Isolation of Inhibitors from Wheat Chaff.* The isolation procedure for the dicarboximide was developed in part with wheat chaff or wheat seeds which contain materials inhibiting seed germination (Miyamoto *et al.*, 1961). In earlier experiments, the production and distribution of the inhibitor in wheat had been ascertained (A. A. Kahn, N. E. Tolbert, and E. H. Everson, unpublished data). Chaff from dormant Red Coat wheat was harvested 35 days after anthesis

TABLE IV: Comparison of Activity of Isolated and Synthetic Inhibitor.[a]

| Concentration | | Inhibition (%) | |
|---|---|---|---|
| M × $10^{-3}$ | mg/ml | Isolated Inhibitor | *cis*-4-Cyclohexene-1,2-dicarboximide |
| 3.3 | 0.50 | 100 | 100 |
| 1.6 | 0.25 | 100 | 100 |
| 0.8 | 0.13 | 83 | 100 |
| 0.4 | 0.06 | 52 | 79 |
| 0.2 | 0.03 | 16 | 48 |

[a] White Paris lettuce seeds were incubated at 22° for 20 hr.

and stored at −10° for up to 10 months. The same isolation procedure as in Table II for inhibitory material was followed by the lettuce seed germination assay. In the last step, a similar elution pattern from the Sephadex G-10 column was obtained, but owing to other impurities in the second peak and insufficient final material, crystallization of a product from wheat chaff was not achieved. However, the material showed the same characteristic ultraviolet and infrared spectra for strong carbonyl absorption without an aromatic ring, and it tested for olefinic unsaturation. Similarities in isolation procedure and physical properties suggest that wheat chaff contained a compound similar to *cis*-4-cyclohexene-1,2-dicarboximide as one component responsible for dormancy of wheat seeds.

*Biological Tests.* It is known that dormant seeds at harvest do not germinate regardless of the light treatment for alteration of the phytochrome system. Viable lettuce seeds, when treated with *cis*-4-cyclohexene-1,2-dicarboximide, also do not germinate, regardless of light treatment. Thus, for lettuce seed germination, the inhibitor seems to induce a dormancy which could be the suppression of the phytochrome system.

The White Paris lettuce seeds used in our bioassay showed an increased rate of respiration after imbiding water for 10–12 hr (Figure 3), and protrusion of the radicle was first evident by about 16 hr. When treated with $10^{-3}$ or $10^{-4}$ M *cis*-4-cyclohexene-1,2-dicarboximide, the stimulated respiration rate, which precedes visible growth of the embryo after 10 hr, did not occur. The inhibition of respiration seems to be an expression of inhibition of germination. If the seeds were incubated with the inhibitor for 24 hr or longer, they could be washed with running water to remove the inhibitor, after which they germinated in an additional 10 hr. The reversibility of the action of the inhibitor is emphasized by its removal and subsequent release of dormancy.

Lettuce seed treated with inhibitory amounts of *cis*-4-cyclohexene-1,2-dicarboximide did not germinate when treated with a concentration ($10^{-5}$ M) of gibberellin $A_3$ which normally stimulates germination. Thus, gibberellin did not reverse the inhibition of germination induced by the dicarboximide. Gibberellins are also known to induce the *de novo* synthesis of α-amylase in barley endosperm (Varner *et al.*, 1965) (Table V). The production or release of α-amylase by barley endosperms, as induced by the addition of $10^{-5}$ M gibberellin $A_3$, was not altered by subsequent addition of $10^{-3}$ M *cis*-4-cyclohexene-1,2-dicarboximide (Table V). However, even $10^{-4}$ M inhibitor did reduce α-amylase synthesis by the whole seed in the absence of added gibberellin. In this case, 5 × $10^{-3}$ M inhibitor would have been required to completely prevent germination of the barley seeds. Thus, it would appear that the dicarboximide inhibitor affects a germination process before the gibberellin-controlled step in α-amylase synthesis, but that the inhibitor has no direct effect upon gibberellin action.

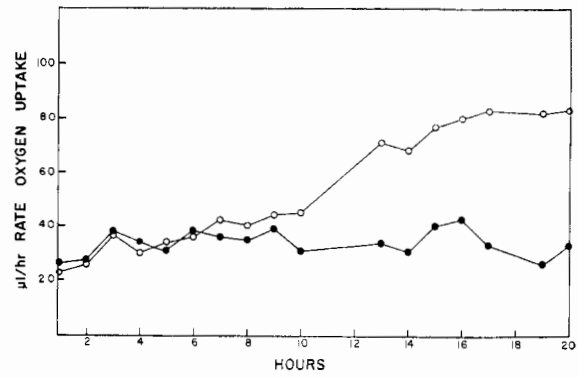



FIGURE 3: Rate of oxygen uptake by White Paris lettuce seeds. Open circles: without inhibitor. Closed circles: $10^{-3}$–$10^{-4}$ M *cis*-4-cyclohexene-1,2-dicarboximide.

In the *Avena* straight growth assay, the mean value increase in the growth of the *Avena* coleoptiles in 24 hr was not affected by 1 × $10^{-3}$ M *cis*-4-cyclohexene-1,2-dicarboximide. Further, a 47% increase in growth rate induced by 1 × $10^{-5}$ M IAA was only slightly reduced in the presence of the dicarboximide inhibitor.

During the first 10-hr period of incubation, $10^{-3}$ M *cis*-4-cyclohexene-1,2-dicarboximide or the *N*-*n*-propyl

TABLE V: α-Amylase Synthesis in Barley.

| Treatment | Medium (units) | Extract (units) | Total (units) |
|---|---|---|---|
| In 10 Barley Endosperms | | | |
| None | 15 | 7.6 | 22.6 |
| 1 × $10^{-5}$ M $GA_3$ | 29.3 | 12.6 | 41.9 |
| 1 × $10^{-3}$ *cis*-4-cyclohexene-1,2-dicarboximide plus 1 × $10^{-5}$ M $GA_3$ | 28.5 | 12.2 | 40.7 |
| In 10 Whole Seeds | | | |
| None | 15.3 | 16.9 | 32.2 |
| 1 × $10^{-4}$ M *cis*-4-cyclohexene-1,2-dicarboximide | 6.3 | 17.1 | 23.4 |

TABLE VI: Effect on Leucine-$^{14}$C Incorporation.

| Treatment | Total Uptake (cpm/g fresh wt) | cpm in Protein/ g Fresh Wt | cpm/mg of Protein |
|---|---|---|---|
| Water | 119,000 | 40,500 | 296 |
| $10^{-3}$ M *cis*-4-cyclohexene-1,2-dicarboximide | 36,360 | 5,680 | 56 |
| $10^{-3}$ M *N-n*-propyl-*cis*-4-cyclohexene-1,2-dicarboximide | 35,000 | 9,700 | 75 |

TABLE VII: Inhibition of Lettuce Seed Germination by Various Analogs.

| Compound | % Inhibition of Germination | | | | |
|---|---|---|---|---|---|
| | $1 \times 10^{-6}$ | $1 \times 10^{-5}$ | $1 \times 10^{-4}$ | $5 \times 10^{-4}$ | $1 \times 10^{-3}$ |
| *cis*-4-Cyclohexene-1,2-dicarboximide | 0 | 4 | 48 | 79 | 100 |
| Cyclohexane-1,2-dicarboximide | 4 | 4 | 50 | 100 | 100 |
| Phthalimide[a] | 0 | 0 | 0 | 0 | 0 |
| Cycloheximide | 0 | 62 | 100 | 100 | 100 |
| *N*-Methyl-*cis*-4-cyclohexene-1,2-dicarboximide | 0 | 8 | 100 | 100 | 100 |
| *N*-Ethyl-*cis*-4-cyclohexene-1,2-dicarboximide | 8 | 13 | 60 | 100 | 100 |
| *N*-Propyl-*cis*-4-cyclohexene-1,2-dicarboximide | 8 | 40 | 68 | 100 | 100 |
| *N-n*-Butyl-*cis*-4-cyclohexene-1,2-dicarboximide | 16 | 52 | 100 | 100 | 100 |
| *N*-Allyl-*cis*-4-cyclohexene-1,2-dicarboximide | 8 | 35 | 94 | 100 | 100 |
| *N-n*-Amyl-*cis*-4-cyclohexene-1,2-dicarboximide | 0 | 60 | 88 | 100 | 100 |
| *N-n*-Hexyl-*cis*-4-cyclohexene-1,2-dicarboximide | 0 | 9 | 88 | 100 | 100 |
| *N-n*-Heptyl-*cis*-4-cyclohexene-1,2-dicarboximide | 0 | 17 | 60 | 100 | 100 |
| *endo-cis*-Bicyclo[2.2.1]-5-heptene-2,3-dicarboximide | 0 | 4 | 4 | 60 | 92 |

[a] Phthalimide showed no inhibition at $10^{-2}$ M.

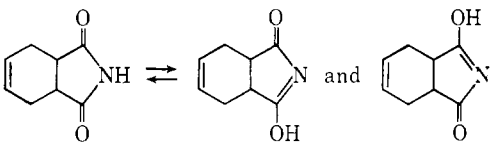
derivative markedly inhibited leucine-$^{14}$C incorporation into protein (Table VI). Proteins formed at this stage are thought to be mostly hydrolytic enzymes. Total leucine-$^{14}$C uptake was also reduced. This result is also another expression of the inhibition of germination by the dicarboximide derivatives.

From the above biological experiments, it appears that dormancy induced by the naturally occurring compound, *cis*-4-cyclohexene-1,2-dicarboximide, is not reversed by gibberellin or IAA, nor does the dicarboximide inhibit the action of gibberellin or IAA. Dormancy induced by the compound has been expressed by inhibition of embryo growth, by the prevention of the normal stimulation of respiration prior to germination, and by the blocking of leucine-$^{14}$C incorporation into protein. Although the mechanism of action is unknown, the dormancy may be reversed by simply washing away the compound.

*Analogs.* Some analogs of *cis*-4-cyclohexene-1,2-dicarboximide were synthesized (Methods and Table I) and tested for biological activity by the lettuce seed bioassay (Table VII). The cyclohexane derivative was as active as the naturally occurring compound, but the fully unsaturated aromatic derivative, phthalimide, showed no inhibitory activity. Aliphatic N substitution increased activity. *N-n*-Butyl- and *N-n*-amyl-4-cyclohexene-1,2-dicarboximides were as active at $10^{-5}$ M as the parent compound, 4-cyclohexene-1,2-dicarboximide, at $10^{-4}$ M. With an increase in size of the alkyl group beyond C-4 or C-5 activity decreased to some extent.

## Discussion

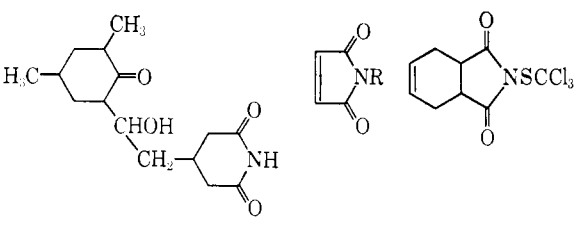
The structure of *cis*-4-cyclohexene-1,2-dicarboximide is unusual for a naturally occurring compound. It is not an alkaloid, nor does it belong to the family of terpenoid compounds which contain the isoprenoid unit. In the structure, the dicarboximide functional group seems to be the most important. In aqueous solution, the dicarboximide ring must be in equilibrium with two other resonance forms which are tautomers.



It is soluble in both polar and nonpolar solvents, and this solubility is affected by pH shifts in the biological range, since its $pK_a$ is about 6.5. The dicarboximide is a stable compound, not destroyed by autoclaving in aqueous solutions.

The amount of *cis*-4-cyclohexene-1,2-dicarboximide per unit weight of sugar beet fruit or per seed was not determined. Many other inhibitors such as coumaric acid, phenolic compounds, and other organic acids are also present in such seed coats. Thus, the relative importance of the dicarboximide on sugar beet seed dormancy in relationship to these other inhibitors of germination is not known. That the dicarboximide inhibitor may occur in other seeds was suggested by the isolation of similar material from wheat chaff.

The primary mechanism of action of the dicarboximides in maintaining dormancy or inhibiting seed germination has not been elucidated. A class of biological inhibitors may be evident from a group of compounds which have in common the cyclic imide ring structure found in *cis*-4-cyclohexene-1,2-dicarboximide. Cycloheximide, a naturally occurring fungicide, inhibits germination of seed in light or with gibberellins (Chen and Thimann, 1966) (Table VII), and it has been used as an inhibitor of protein synthesis in plant and mammalian systems (Chrispeels and Varner, 1967). Thus, the biological action of *cis*-4-cyclohexene-1,2-dicarboximide and cycloheximide may be very similar. A wide variety of synthetic dicarboximides are inhibitors of biological growth. A *N*-thio-substituted dicarboximide, called Captan, has been used as an agricultural fungicide; It is unlikely that Captan contamination or Captan decomposition could account for the *cis*-4-cyclohexene-1,2-dicarboximide in sugar beet fruit and wheat chaff. The West Coast Sugar Beet Seed Co., from whom the sugar beet seeds were obtained, stated that the seeds had not been treated with Captan, and the wheat hulls from our own Experiment Station were not treated with Captan. Captan is very slightly soluble in water, and a water-saturated solution of Captan was not inhibitory in our lettuce seed assay. N substitution by phosphothionates have produced fungicides (Tolkmith *et al.*, 1967). Another group of cyclic imides are the maleimides, of which *N*-ethylmaleimide has been widely used as an enzyme inhibitor. The metabolic poison, maleic hydrazide, also has a somewhat similar structure.

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