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ABSTRACT

It has been shown that the leaves of Encelia farinosa when applied to tomato and other plants in sand cultures cause a striking growth inhibition. Water and ether extracts of the leaves when fed to tomato seedlings in solution culture cause the death of the plants within one day. Fractionation of the leaf extracts yielded a crystalline compound which was isolated in pure form and is toxic to tomato seedlings in solution culture.

The structure determination and synthesis of this new compound, 3-acetyl-6-methoxybenzaldehyde (AMB), has been successfully worked out and is given in detail. The inhibitory activity of related compounds on the growth of tomato seedlings is demonstrated.

Another crystalline toxic compound has been isolated from leaves of Encelia farinosa gathered in a different geographical location. Its toxic action is even more pronounced than that of AMB. This compound has been partly characterized and shown to be an aliphatic unsaturated lactone containing one hydroxyl group, three double bonds, and having a molecular formula of $C_{16}H_{20}O_4$.

The presence of these growth inhibitors found in the leaves of Encelia farinosa is offered as an explanation of why so few desert annuals are found growing in close relationship with the Encelia shrub on the desert.

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INTRODUCTION

Inhibitory Effects of One Plant on Another

The detrimental effect of one plant on another growing in close relationship has been known from the earliest times. This detrimental effect was first thought to be due to the competition of the plants for raw materials from the soil and air or for the supply of sunlight. De Candolle (1) in 1832 was the first to suggest that the apparent antagonisms between certain neighboring plants might be due to toxic substances exuded from the roots of some plants. He believed that these substances exerted a detrimental effect when absorbed from the soil by specific plants belonging to the same natural order as the plants from which the substances originated, but that their excretions would be harmless to plants of a different order. As examples, he cited the antagonisms between Euphorbias and flax, tares (darnel) and wheat, and thistles and oats. De Candolle used this theory to explain the benefits of crop-rotation while others, and especially Liebig (2), preferred to explain the benefits of crop-rotation by the removal of different ratios of mineral elements from the soil by different species.

Renewed interest in the problem of the detrimental effects of one plant on another occurred in the early 1900's when Pickering (3,4,5) and Pickering and Bedford (6,7) presented sound experimental evidence that toxic substances produced by one plant are toxic or inhibitory to the growth of other plants. The harmful effects of certain grasses on apple trees was attributed to the toxic products derived from the grass; all other factors such as food, water oxygen, carbon dioxide, and sod packing were eliminated. By growing two plants in two pots, one above the other, one receiving the drainage from the other, they obtained reduction of growth from 6-97 percent. These inhibitory effects were exerted on various plants by apple seedlings, mustard, tobacco, tomatoes, clover, and sixteen varieties of grasses.

Hedrick (8) showed that young peach trees were hindered in their development when grown in the same pot cultures with oats, potatoes, tomatoes, mustard, and rape, even when optimum conditions of moisture, aeration, and food supply were maintained.

Dandeno (9) found that the growth of buckwheat was considerably inhibited by Canada thistles when the

two plants were grown together. He also observed that poplars and oaks were injurious to the growth of corn. Since the injurious effects extended to some distance beyond the trees, moisture and shade were not the limiting factors.

It has long been observed, Jones and Morse (10), Cook (11), Massey (12) and Schneiderhan (13), that certain plant associations grown near black walnut trees are severely injured. Walnut trees prevent the growth of cinquefoil within a large area surrounding them and cause the wilting, stunting and frequently the death of corn, tomatoes, potatoes, alfalfa, and apple trees when their roots come in close proximity to those of the walnut. Davis (14) has sought to identify the toxic principle with juglone which he extracted from the hulls and roots of the black walnut tree and showed to be very toxic when injected into the stems of alfalfa and tomato plants.

Other plants reported as injurious to their neighbors are corn, rye, buckwheat, squash, turnips, sesame, rutabages, mangles, and potatoes,(15,16,17,18).

It is now widely accepted that the presence of certain organic compounds in the soil inhibit plant growth. Livingston (19,20) showed that toxic organic substances are present in water expressed from saturated

bog soils. Toxic compounds believed to be of plant origin have been isolated from soils by Schreiner and co-workers (21-27). Among the compounds isolated, identified, and found to be injurious were coumarin, vanillin, dihydroxystearic acid, alanine, glycocoll, neurine, quinone, picolinic acid, and tyrosine. Adsorbing agents and fertilizers greatly reduced the toxicity of many of these compounds.

Sewell (28) showed that kafir leachings had a retarding effect on the growth of wheat. Breazeale (29,30) observed that kafir stubble gives rise to toxic decomposition products which later decompose further becoming nontoxic. The detrimental effect of sorghum roots and stalks on the crops that follow is a well known observation. Hawkins (31) found that the detrimental effects of sorghum disappear in a few months after the crop has been harvested. Conrad (32) suggested that the high content of sugar in sorghum roots stimulates growth of micro-organisms which then compete for nitrogen with the plants. The sugar also would retard the process of nitrification.

Collison (33) found that water extracts of cereal straws, timothy residues, and alfalfa were toxic to plants grown in them.

Another example of a suppressing effect on the

vegetation of other plants in their neighborhood is shown in some Robinia pseudacacia parks in which almost no vegetation can be found. Wake (34) found that extracts of the leaves of Robinia pseudacacia, Wisteria chinensis, Genista tinctoria, Cytisus laburnum and Tilia planifolia have a hindering effect on the growth of barley plants. Robinia leaf extracts gave a much greater effect than the extracts of the other plants; extracts of the bark also hindered growth of barley.

Proebsting and Gilmore (35) showed by a field survey that a high percentage of replanted peach orchards failed to make normal growth. They showed that exhaustion of plant nutrients or diseases carried over from the last orchard could not account for the failure of growth. Peach roots and their alcohol extracts inhibited the growth of peach seedlings when added to sand cultures.

Benedict (36) demonstrated that dried roots of bromegrass are inhibitory to bromegrass grown in sand cultures, and he suggests that the inhibitory substance in the roots may be responsible for the "sod-bound" condition of bromegrass where thick stands thin out after a few years. Myers and Anderson (37) showed that the addition of ammonium sulfate only

partially overcomes this effect.

Bonner and Galston (38) and Bonner (39) found that water or nutrient solution in contact with roots of growing guayule plants accumulate substances which are toxic to the growth of guayule seedlings. Trans-cinnamic acid was isolated in crystalline form from the toxic liquor and was shown to cause 50 percent inhibition of growth at a concentration of 30 milligrams per liter.

Bode (40) and later Funke (41) demonstrated the injurious effect of Artemisia absinthium on eighteen species of plants which were sown near a hedge of Artemisia. Within a distance of 100 centimeters the plants were severely injured and in one case killed. They believed the toxicity to be mainly due to the compound absinthiin which is excreted by the glandular hairs of Artemisia and washed on to surrounding plants by rain.

Thus, we see that there are a number of cases showing that growth inhibitory substances may arise from certain plants. In the experiments on dead roots and root extracts, there is no good evidence that toxic substances are given off by the living plant, or that the material causing the growth inhibition acts as such under natural conditions. However, there are two cases

cited above in which the deleterious effect of one plant on another has been shown to be due to the emanation of definite chemical substances by the living plant. The work of Bode (40) and Funke (41) on Artemisia absinthium is one of the very few if not the only case in which the chemical action of a noxious plant on others has been demonstrated in its natural surroundings. The compound absinthiin, excreted by Artemisia absinthium and causing this species to be harmful to surrounding plants, has not been characterized. The influence of emanations of plants on other species has often been studied under bell jars (42,43,44) but this can hardly explain their ecological significance.

In the other case of Bonner and Galston (38) and Bonner (39) it has been shown that certain substances unfavorable to the growth of guayule plants, emanated from the roots of actively growing plants of the same species, under certain limited environmental conditions (plants grown in gravel cultures). In this case one of the toxic compounds, trans cinnamic acid was actually isolated and identified. However it was not possible to show that these toxic substances of guayule plants accumulate in the soil under field conditions.

The present work on Encelia farinosa gives further indication that chemical compounds arising form one

plant may have a detrimental effect on other plants and thus be an important ecological factor.

The Problem with Encelia

In the deserts of the Southwest it is common for many species of annual plants to grow primarily in and around individuals of shrubby species which appear to provide favorable growing conditions for these annuals. Certain shrubby species, however, do not harbor desert annuals or harbor only a few as compared to shrubs in general. This is true of the composite Encelia farinosa as has been observed by Went (45). Franseria, a shrub of comparable size and density, harbors many more annuals than Encelia, so that the unfavorable effect of the Encelia cannot be due to microclimatic conditions. Observations made by the writer near 29 Palms and in the Castle Dome mountains of Arizona confirm the findings of Went that Encelia harbors few if any desert annuals. This suggests at once that Encelia may give rise to chemical substances that may be detrimental to the growth of surrounding plants. The present work is concerned with the problem of whether or not growth inhibitors do, in fact, arise from the Encelia plant.

Encelia farinosa as described by Beal (46) is a composite shrub found on the deserts of the Southwest.

It has been given many different names such as Incense Bush, Golden Hills, Brittle Bush, and White Brittle Bush. One of the common names used by botanists is Incienso, which came from Mexico, where the resinous gum was burned as incense, giving off a strong fragrance. From Mexico also came the name Yerba del vaso, from its use as a pain reliever. The gum was heated and smeared on the body, especially on the chest and side. The resin also served as a primitive chewing gum and when melted made a good varnish.

The domain of Encelia farinosa extends from Death Valley in California down through the Mojave and Colorado Deserts into Mexico, and eastward into Southern Nevada and Arizona.

It has a pale gray leaf and stem that often appears quite white. It is a somewhat rounded and compact bush 2 to 4 feet or more high. The flowers which crown the naked flower stems consist of broad orange disks encircled by wide golden yellow rays, 3 toothed and somewhat fluted. The felt like ovate leaves 3/4 to 2 inches or more long, are borne in clusters at the end of branchlets. They are silvery with soft short hairs that form a matted covering. Many woody branches extend from the trunk like base; the branches exude drops of amber resin.

EXPERIMENTAL

PHYSIOLOGICAL PROPERTIES OF ENCELIA

Effect of Encelia Roots

Preliminary experiments were carried out to determine if an inhibitory substance is given off by Encelia roots. Barley, tomatoes, and guayule were planted in sand cultures containing Encelia plants in the greenhouse and the growth of these plants in the Encelia culture was compared with that of controls lacking Encelia. After 3 weeks the plants were harvested, dried, and weighed. The results given in table 1 show that little difference was found between the growth of plants in Encelia cultures as compared with the controls.

Table 1.

Growth of guayule, barley, and tomato plants in sand culture with or without associated Encelia plants.

	Total number of plants	Av. dry wt..Gms. per plants Gms.
Guayule with Encelia	36	.32
Guayule alone	35	.36
Barley with Encelia	88	.24
Barley alone	95	.19
Tomatoes with Encelia	18	2.76
Tomatoes alone	17	3.00

Effect of Encelia leaves: Since the roots did not appear to give off inhibitory substances, attention was turned to the leaves. Leaves were gathered from Encelia shrubs in their natural habitat on the Colorado desert of California. The leaves were dried at 70°C. for 48 hours and aliquots were then placed on top of the sand in pots containing recently transplanted tomato plants (24 days old). In the first experiment three grams of Encelia leaves were applied to each of ten pots. Tomato leaves were dried and applied to ten other pots for comparison. A set of control plants received no leaves. The plants were given water and nutrient solution each day, the solutions being poured over the leaves. The heights of the plants were measured periodically. The results obtained are shown in Figure 1. It may be seen that addition of even 3 grams of Encelia leaves per tomato plant causes considerable inhibition of growth. The same amount of tomato leaves causes some retardation of growth as compared to the control, but the effect is small as compared to Encelia.

Another experiment was carried out as described above to determine the effect of different amounts of Encelia leaves. Another sample of leaves was used and in this case the dried leaves were chopped into small pieces and applied to the surface of sand in pots containing

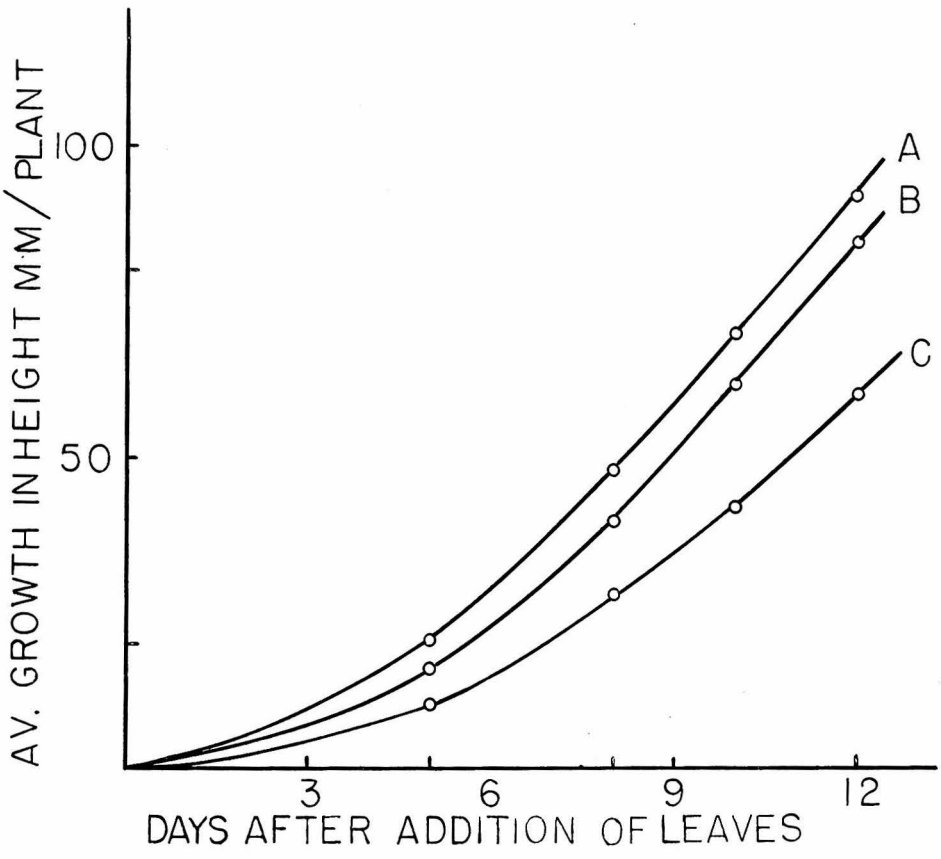


Fig. 1. Effect of tomato or Encelia leaves on the growth of tomato plants in sand culture. A. Control plants, no leaves added. B. Three grams tomato leaves per pot. C. Three grams Encelia leaves per pot.

tomato plants. A portion of the leaves were also extracted with ether and the effect of these extracted leaves compared with that of the non-extracted. The results obtained are given in Figure 2. It can be seen that with increasing concentration of leaves, the inhibitory effect increases, and in fact, a portion of the plants died when 20 grams were applied to each pot. The ether extracted leaves are much less inhibitory than the non-extracted leaves. This indicates that other soluble organic substances may be responsible for a portion of the inhibitory activity. In other experiments in which the Encelia leaves were mixed with the sand in pots, the inhibition was even greater than when the leaves were applied on top of the sand. Less striking growth inhibition was obtained in experiments in which rich garden soil rather than sand was used as the medium for the tomato plants.

An effect of Encelia leaves on plants other than tomato was noted in further experiments. Encelia leaves were applied to small plants of barley, oats, peppers, corn, Encelia plants themselves, sunflowers, and Pea in sand cultures. Encelia leaves caused marked inhibition of growth with peppers and corn, had but little effect on Encelia plants, and exerted no noticeable effect on barley, oats, sunflowers, or Pea.

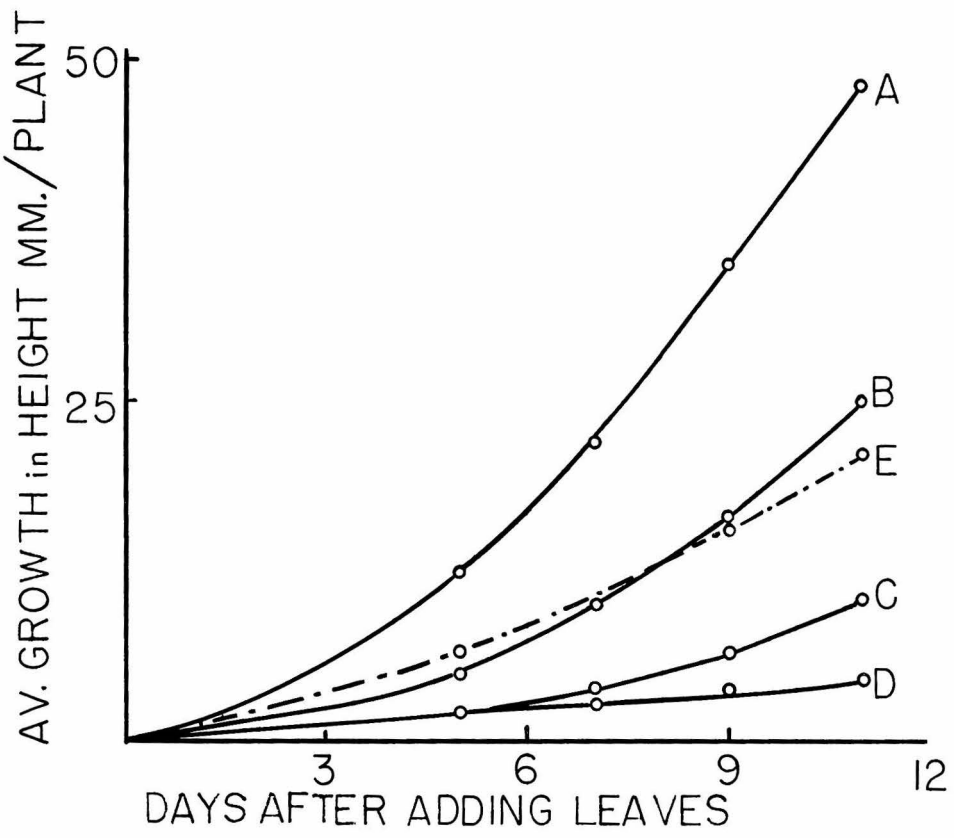


Fig. 2. Effect of varying concentrations of Encelia leaves on the growth of tomato plants in sand culture. A. Control plants, no leaves added. B. Five grams Encelia leaves per pot. C. Ten grams Encelia leaves per pot. D. Twenty grams Encelia leaves per pot. E. Twenty grams ether extracted Encelia leaves per pot.

The Test for Inhibitory Activity

For the isolation of the active principle of Encelia leaves it was essential to have a reliable and convenient assay method for inhibitory activity. Such a test has been described by Bonner and Galston (38) in connection with the growth inhibitors of guayule roots. The following assay for the inhibitory principle of Encelia leaves is based on the earlier assay.

Tomato seedlings were used as the test plant, although other species may also be used. Uniform plants were obtained by planting the seeds about 2 inches apart in sand contained in flats. The plants were used when they had reached a specified size, in general just after the appearance of the first leaves which usually was 12 days from the time of planting. The seedlings were transplanted into shell vials of 16 cc. capacity containing the test solution. This solution consisted of Hoagland's nutrient mixed with an equal volume of distilled water containing the test material. The plants were held in the vials in slits made in cork stoppers and held firmly in place with a small wad of cotton. A small hole in the center of the cork served as a place for supplying distilled water to replace that lost in transpiration. The distilled water was added by means of a small jet from

a wash bottle every few days; this provided a small amount of aeration. Wooden boxes were used to hold the vials. The vials fitted closely into a hole in the top of the box so that the roots were kept in darkness and the stem protruded from the top of the box. Each box contained twenty vials. The plants were measured at the time of transplanting and again after growing in the greenhouse at constant temperature of 60°F. for one week. The average growth for one week was about 20 mm. per plant for the controls given no inhibitory material. In the work reported below the extracts were frequently so toxic that it was possible to use as a criterion of activity the death or survival of the test plants rather than inhibition of growth.

Encelia Leaf Extracts

Water extracts of Encelia leaves are exceedingly toxic to tomato seedlings as shown by the following experiment. Fifty grams of whole green Encelia leaves obtained from plants grown in the greenhouse were soaked in 200 cc. of distilled water for 12 hours. The leaves were filtered off and the filtrate tested as described above. A chopped green leaf extract was prepared in the same manner and also tested. The chopped leaf extract caused death of 3/4 of the plants within 24 hours, while the whole leaf extract caused death of 3/4 of the plants within 3 days. This effect exhibits a certain

specificity since as shown in table 2, similar extracts of tomato or of tangerine leaves are much less toxic.

Table 2.

Effect of aqueous extracts of Encelia leaves on survival of seedling tomato plants.

	Days required to kill 3/4 of plants	
	Whole leaf ext.	Chopped leaf ext.
Encelia leaves	3	1
Tomato leaves	5	4
Tangerine leaves	7	4

After 3-4 days decomposition of the copious organic matter of these extracts by microorganisms occurred which may explain the delayed toxicity of the tomato and tangerine extracts but in any case the extracts of Encelia leaves were much more toxic than the others.

The toxic principle is readily soluble in ether as shown by the following experiment. Fifty grams of the green leaves were dried at 70°C. for 24 hours, chopped, and extracted with purified ether for 18 hours in a Soxhlet extraction apparatus. The ether was evaporated under reduced pressure on a water bath. The green sticky mass was extracted with 200 cc. of hot water, cooled, and filtered to give a clear solution. This solution was

mixed with an equal volume of nutrient solution and fed to tomato seedlings in the standard assay. All plants died within one day. On the other hand the water extract of leaf residue from the ether extraction showed greatly reduced toxicity and took four days to kill the test plants. The clear solutions from the ether extract showed no signs of bacterial decomposition even after the plants had grown in them for ten days. The relation of concentration of the ether extract to toxicity in the standard assay is given in Figure 3.

The ether extract of tomato leaves in contrast to that of Encelia leaves possesses only a small toxic effect as shown by the following experiment. Two hundred grams of green tomato leaves were dried at 70°C. for 48 hours yielding 17.4 grams of dry leaves. Two hundred grams of green Encelia leaves from the desert under the same treatment yielded 92 grams of dry leaves. The leaves were compared on the dry basis using 17.4 grams of dry tomato leaves and 17.4 grams of dry Encelia leaves. Each sample was extracted with an equal volume of purified ether, the ether completely removed, and the residue taken up in equal volumes of water (140cc.). The solutions were boiled, cooled, filtered and tested in the standard tomato assay. The results are shown in table 3.

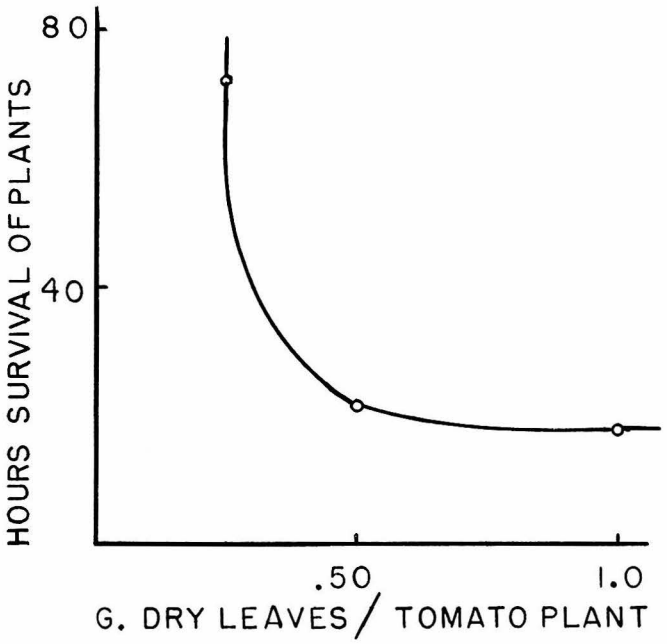


Fig. 3. Survival of tomato seedlings in solution culture in the presence of varying concentrations of the ether extract of Encelia leaves.

Table 3.

Survival of young tomato plants in ether extracts of tomato and Ancelia leaves.

	Dilution	Mgs. original dry leaf per plant	No. of plants dead after	
			1 day	7 days
Ether extract of tomato leaves	1/2	94	0	2
	1/4	47	0	0
	1/8	24	0	0
Ether extract of Ancelia leaves	1/2	94	10	10
	1/4	47	6	10
	1/8	24	0	4

A steam distillation of the green Ancelia leaves was carried out to see if the toxicity might be due to essential oils. The steam distillate containing oil particles gave avery little inhibition, whereas an extract of the residue from the steam distillation caused complete death of all the plants in 2 days. Other observations showed also that drying of the leaves at elevated temperatures (70°C.) did not destroy the inhibitory activity. Exudation of resin is found on the stems of the Ancelia shrub. Some of this resin drops off onto the ground even under natural conditions. The resin was tested, but did not exhibit any inhibitory activity.

The results presented above show conclusively that there is a powerful inhibitor of growth of tomato seedlings contained in Encelia leaves. The isolation of the inhibitor was therefore undertaken.

In addition to ether, other solvents including benzene, petroleum, ether, methyl alcohol, ethyl alcohol, and acetone were also found to extract the inhibitor from Encelia leaves. Benzene and ether were preferred over the other solvents, because the water extracts of the residues from these solvents were clear and practically colorless.

That the toxic component is a neutral compound rather than an acid or base is shown by the following experiment. Equal portions of the ether extract were shaken in separatory funnels with aqeous solutions containing sodium barbonate and sulfuric acid. The greater part of the toxic material was extracted by both the aqueous alkaline phase and the aqueous acid phase, although the ether phases also retained a considerable part. It might be mentioned also that the toxicity of the Encelia extract was independent of pH between pH's 5 and 7.

The water extract of the ether soluble residue was evaporated to dryness without loss of activity. The syrupy residue was dissolved in alcohol and subjected to lead acetate precipitation. After removing the lead from

the precipitate and the filtrate with hydrogen sulfide and boiling off the H_2S, the two fractions were tested. The toxic material was found in the filtrate, e.g. the fraction not precipitated by lead acetate. The syrupy residue of the aqueous extract of the ether soluble material was also taken up in benzene and subjected to chromatographic adsorption on a column of calcium hydroxide and calcium carbonate. It was found that the toxic fraction passed through the column with little purification. The syrupy material was distilled at reduced pressure, but it had to be heated to so high a temperature that decomposition took place. Although the distillate was active, the products obtained might not have been present as such in the original leaves.

Isolation of a Growth Inhibitor

It was possible to obtain an active crystalline inhibitor from Encelia leaves by the following procedure. Dry Encelia leaves were extracted with ether or benzene and the solvent evaporated at reduced pressure leaving a green sticky residue. It was found that by extracting this residue four times with hot water all of the toxicity could be removed. The aqueous solution was then cooled, and the insoluble material filtered off. On extracting the water layer with benzene the bulk of the toxicity passed into the benzene layer. The benzene

was evaporated at reduced pressure leaving a clear yellow syrup. By extracting this residue with hot petroleum ether (60-70°C.) and removing the solvent, a semi-crystalline material was obtained. Further crystallisations from ether yielded an active crystalline compound. In a typical isolation 507 grams of dry leaves yielded 143 milligrams of the crystalline compound. This represents but a portion of the crystalline toxic material in the plant since further crystals separated from the mother liquors and a considerable amount of similar material remained dissolved in the syrupy residue.

The outline of the isolation procedure together with the amounts and inhibitory activity of each fraction is given in table 4. The data of table 4 is based on a representative experiment in which 100 grams of dry leaves were used as the starting material.

Table 4.

Isolation of Inhibitor from Encelia Leaves.

Fraction	Weight (Grams)	Conc. for 50% inhibition of tomato seedlings (mg./L.)
1. Dry leaves	100	3750
2. Extraction with benzene		
A. Benzene soluble	10.5	337
B. Benzene insoluble	89.5	5750
3. Hot water extract of 2A after evaporating benzene		
A. Water soluble	2.34	75
B. Water insoluble	8.15	7000
4. Benzene extract of 3A(liquid-liquid)		
A. Benzene layer	1.33	45
B. Water layer	1.02	200
5. Hot petroleum ether extract of 4A after evaporating benzene		
A. Petroleum ether soluble	0.847	25
B. Petroleum ether insoluble	0.448	110
6. Ether crystallization of 5A		
A. Crystalline compound	0.0449	115
B. Non-crystalline residue (Contains much 5A)	0.791	145

The insoluble fractions 2B, 3B, and 4B showed practically no inhibitory activity and were discarded. A portion of the inhibitory activity was lost in the petroleum ether insoluble fraction 5B. The petroleum ether

soluble fraction 5A was highly toxic. This fraction was separated into two components, a toxic crystalline material and a less toxic non-crystalline, although neither fraction was as toxic as the material before separation. Recombination of the two fractions did not appear to restore the original activity. The total amount of the crystalline toxic compound present in the plant cannot be concluded from the amount isolated in pure form since the final residue 6B appeared to contain much of the same material in a condition from which it can be crystallized with difficulty.

Activity of the Crystalline Compound

The crystalline material was recrystallized three times from ether to yield a compound with a sharp melting point of 144°C. The pure compound was tested for inhibitory activity at different concentrations on tomato seedlings in solution culture and the results are given in Figure 4. Essentially complete inhibition of growth was given by 250 mg./l. of the material while substantial inhibition was given by concentrations as low as 50 mg./l.

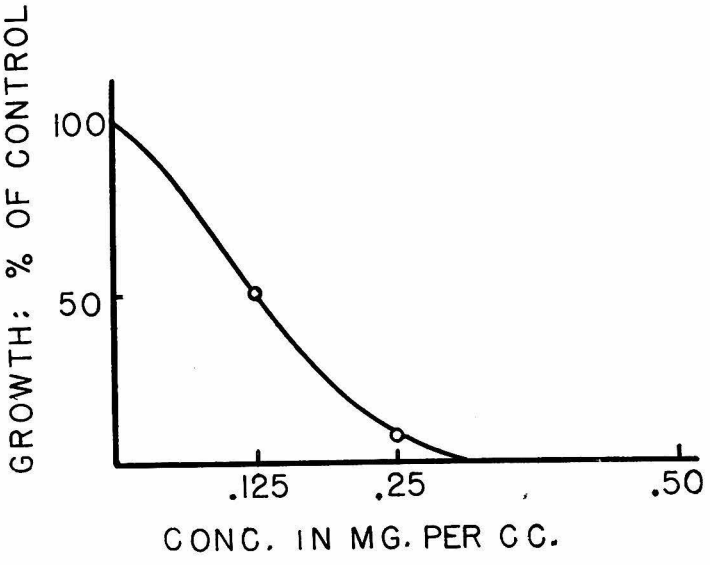


Fig. 4. Growth of tomato seedlings in solution culture in the presence of varying concentrations of the crystalline inhibitory compound isolated from Encelia leaves.

Effect of Encelia Leaves on the Germination of Seeds

Experiments were carried out to determine the effect of Encelia leaves on the germination of seeds. The tests were carried out in petri dishes, each containing a filter paper moistened with 4 cc. of the solution tested. Fifty tomato seeds were placed in each dish and 2 dishes were used for each concentration. The cover was replaced and the dishes were kept in the dark at room temperature. The number of seeds that germinated were counted after 3 days and after 7 days. The results are shown in the following table.

Table 5.

Effect of Encelia leaf extracts on germination of tomato seeds.

Solution tested	% Germination after 3 days	7 days
Water (control)	82	91
Water extract of Encelia leaves (2g. lvs./20cc. water)	0	3
Same, diluted 1/2	0	66
Same, diluted 1/4	16	94
Water extract of ether extracted Enc. leaves (2 g. leaves/20 cc. water)	1	51
Same, diluted 1/2	10	88
Same, diluted 1/4	28	90
Water saturated with volatile oils from steam distillation of leaves.		92
Second hot water extract of the ether extract of Encelia leaves.	0	0
Same, diluted 1/2	0	2

The table shows that the water extract of the Encelia leaves contains substances which markedly inhibit germination. The leaves after being extracted with ether show much less effect which means that the ether extracts some of the substances which are detrimental to germination of tomato seeds. The ether extract completely prevented germination.

The crystalline compound isolated from the Encelia leaves did not show such an inhibitory effect on the germination of tomato seeds as did the ether and water extracts, as shown by the following experiment. The tests were carried out as described above using different concentrations of the pure crystalline compound. The results are shown in the following table.

Table 6

Effect of crystalline compound isolated from Encelia leaves on the germination of tomato seeds.

Conc. of cmpd. mg./cc.	% Germination after 3 days	6 days
0 (Water control)	90	92
0.50	0	24
0.25	4	81
0.125	69	95
0.050	72	92

Thus, it is seen that the toxic compound delays germination somewhat, but it does not prevent germination of tomato seeds at the concentrations tested. At the highest concentration (0.50 mg./cc.), however, it does markedly inhibit germination. Therefore, it seems that the toxic compound isolated from the California Encelia leaves has more of an inhibitory effect on the growing plant than on germination of the seeds.

Structure Determination

The present chapter is concerned with the identification of the toxic compound which was isolated from the Knoellia leaves. The tests were all carried out on a micro scale, since only about 300 milligrams of the pure crystalline compound was available.

Physical and Chemical Properties

The toxic material crystallizes in colorless needles from ether or alcohol. It starts to sublime at 115°C. and melts sharply at 144°C. Sublimation causes no change in melting point. The crystals show weak double refraction when examined under the polarizing microscope. The compound has no odor at room temperature, but when the crystals are heated a pleasant perfume-like odor is detected. It burns with a smoky flame leaving no residue after ignition.

An elementary analysis using the sodium fusion method was carried out using a few milligrams of the unknown compound. Tests for nitrogen, sulfur, and halogens were all negative; evidently the compound is composed only of the elements carbon, hydrogen and oxygen.

Solubility tests showed the toxic compound to be slightly soluble in ether and very slightly soluble in hot water. It dissolves readily in alcohol, acetone, benzene, and chloroform. It is insoluble in cold water, 5 percent hydrochloric acid, 5 percent sodium hydroxide,

petroleum ether, and carbon tetrachloride. The compound dissolves in concentrated sulfuric, hydrochloric, and hydrobromic acids producing orange colored solutions.

Classification tests (47,48) were carried out on the toxic compound to determine the functional groups present. A red color is produced when the compound reacts with anhydrous aluminum chloride and chloroform according to the Friedel-Crafts reaction. This gives some indication that the compound may be a homologue of benzene. It reacts slowly with bromine in chloroform giving off hydrogen bromide, indicating a substitution reaction rather than an addition reaction. It decolorized cold aqueous potassium permanganate solution showing that the compound is either unsaturated or easily oxidized. A precipitate is obtained by treating the compound with 2,4-dinitrophenyl-hydrazine reagent in alcohol, showing that the compound may be an aldehyde or a ketone. It gives a green colored solution with Fehling's solution showing some reduction of the reagent. It also reduces ammoniacal silver nitrate solution (Tollen's reagent) giving a black precipitate of silver. With fuchsin-aldehyde reagent (Schiff's reagent) a good violet color is obtained when the crystals are dissolved in alcohol. Therefore an aldehyde group is definitely present. The ferric chloride test for phenols was negative as was also the hydroxamic acid (49) and saponification tests for esters. A positive test for methyl

ketones is obtained with both the sodium nitroprusside reagent (50) and the ortho-nitrobenzaldehyde test (50).

The classification tests show that an aldehyde group and a methyl ketone (acetyl) group may be present in the toxic compound which may also contain the benzene nucleus.

2,4-Dinitrophenylhydrazine Derivative. A cold saturated solution of 2,4-dinitrophenylhydrazine in 95% ethyl alcohol was added to 5 mg. of the compound dissolved in 1 cc. of alcohol. After adding a drop of concentrated hydrochloric acid, an orange colored precipitate formed immediately. The derivative was filtered off, washed twice with alcohol and dried, m.p. 258-261°C. The derivative was insoluble in hot alcohol, ethyl acetate, and most organic solvents, and could be recrystallized only from nitrobenzene.

Quantitative Analysis. A micro analysis* of the pure compound gave results agreeing closely with the empirical formula $C_{10}H_{10}O_3$.

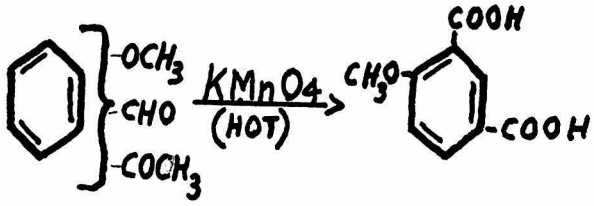
	Found for toxic cmpd.	Calculated For $C_{10}H_{10}O_3$
% C	67.14	67.41
% H	5.62	5.62
% O	27.04	26.97
Mol. Wt. (Rast)	192	178
% OCH_3	17.98	17.40

* Micro analyses done by Dr. G. Oppenheimer and G. Swinehart

A calculation of the molecular weight assuming one methoxyl group per molecule gives 172. A quantitative precipitation with 2,4-dinitrophenylhydrazine using the method described above shows that two moles of the reagent combine with one mole of the toxic compound giving 184 for the molecular weight. A Zerewitinoff determination of groups reactive to Grignard reagent showed the absence of active hydrogen.

The results indicate that the toxic compound is a benzene derivative containing an aldehyde, methyl ketone, and a methoxyl group, and having a molecular weight in the range 172-192. Considering the molecular formula $C_{10}H_{10}O_3$ to be correct, no other groups could be present on the benzene ring. A search in the chemical literature revealed no known compound satisfying these requirements, therefore, degradation to simpler known compounds was undertaken (1).

Degradation



(1)

I II

Oxidation with Hot Permanganate. A 20 mg. sample of the toxic compound was mixed with 2 cc. of saturated potassium permanganate solution in a small reflux tube. The tube was sealed and heated on a steam bath until the color of the permanganate had disappeared; this took 2 1/2 hours. The tube was cooled, opened, and the contents emptied into a centrifuge tube. After centrifuging, the clear supernatant was drawn off. Upon acidifying with dilute hydrochloric acid, a white precipitate formed. The precipitate was filtered off, recrystallized from hot water, dried, and sublimed; m.p. 264-267°C.

Examination of the carboxylic acid derivatives of anisole found in the literature gave the following possibilities for the oxidation product of the toxic compound:

5-methoxyisophthalic acid, m.p. 265(51),270(52).

4-methoxyisophthalic acid, m.p. 245(53),261(54,55), 275(56)

2-methoxyterophthalic acid, m.p. 274-275(51), 276-279(58) 281(59).

The melting points of these acids vary considerably as determined by different investigators. Therefore, the acids were synthesized in order that mixed melting points with the oxidative product of the toxic compound could be taken.

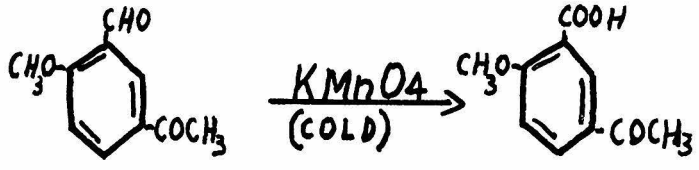
5-Methoxyisophthalic acid. This compound was prepared by methylating 5-hydroxy-1,3-dimethylbenzene with dimethyl sulfate and oxidizing the side chains with permanganate as described above. After recrystallizing from hot water and subliming, it melted at 269-270°C. A mixed melting point with the oxidation product of the toxic compound gave a considerable depression.

4-Methoxyisophthalic acid(II) (a) Prepared from p-Cresol. --An aldehyde group was substituted in the position ortho to the phenolic group of p-cresol using chloroform and alkali (Reimer-Tiemann Reaction). The resulting 3-methyl-6-hydroxybenzaldehyde was methylated with dimethyl sulfate and then the side chains were oxidized to acids with permanganate. The product was recrystallized from hot water, dried and sublimed, m.p. 264-267°. A mixed melting point determination with the oxidation product of the natural toxic compound gave no depression in melting point.

(b) Prepared from 2,4-Dimethyl phenol. --By methylating 5 g. of this compound with dimethyl sulfate in strong alkali, the methyl ether was obtained, b.p. 192°C. To 2.72 g. of the ether in a round-bottomed flask fitted with a reflux condenser was added 200 cc. of water containing 12.64 g. of potassium permanganate. The mixture was heated on a steam bath until the color

of the permanganate had all disappeared (eight hours.). The manganese dioxide was filtered off and the filtrate acidified with hydrochloric acid. The white crystals which separated were filtered off, recrystallized from hot water, and dried in an oven, m.p. 273-275°, yield 1 g., 25% of theoretical. After subliming the melting point was lowered to 264-267°.

The above reaction tentatively establishes the identity of the oxidative product of the toxic compound with 4-methoxy isophthalic acid.(II) This indicates that the toxic compound which contains an aldehyde and a methyl ketone group must be either 3-acetyl-6-methoxy-benzaldehyde, or 3-acetyl-4-methoxybenzaldehyde. The question as to which isomer is the correct one was answered by a selective oxidation of the aldehyde group with cold potassium permanganate solution.(2)



(2)

III IV

Selective Oxidation of the Aldehyde Group.--A sample of 30 mg. of the toxic compound was mixed in a small test tube with 1 cc. of saturated potassium permanganate solution. The mixture was shaken for 45 minutes, centrifuged, and the supernatant drawn off. A few drops of ethyl alcohol were added to reduce the excess permanganate. A drop of 5 N sodium hydroxide solution hastened the reaction. After the violet color had disappeared the solution was heated to 60°C on a water bath and centrifuged. The clear supernatant was drawn off and concentrated to 1/3 cc. by bubbling air through the solution while heating on a water bath. The solution was acidified with dilute hydrochloric acid. After standing a few minutes, crystals began to separate and after two hours the crystals were filtered off and re-crystallized from hot water. The yield was approx. 10 mg, m.p., 150-151°C. This product gave a positive test for methyl ketones but gave a negative test for aldehydes.

The identity of this cold permanganate oxidative product was established by its synthesis. The literature reports 152°C. as the melting point of 3-acetyl-6-methoxybenzoic acid. This compound was synthesized and a mixed melting point determination made with the cold permanganate oxidative product of the toxic compound.

3-Acetyl-6-methoxybenzoic acid.-- The corresponding phenol was prepared according to the directions of Bialobrzeski and Menki (60). Eighty grams of Salicylic acid was

dissolved in 100 g. of acetyl chloride contained in a 500 cc. flask. One hundred grams of anhydrous ferric chloride was then added in small portions with vigorous stirring. With the addition of the ferric chloride, a lively reaction set in and the solid acetyl ester of salicylic acid was produced. Upon further addition of ferric chloride the material again became liquid and dark in color. Hydrogen chloride was evolved and when nearly all of the ferric chloride had been added, the flask was heated cautiously over a flame until the temperature reached 110°C. Fifteen minutes after the addition of all the ferric chloride, the melt was cooled, triturated with cold water, filtered by suction, and washed several times with cold water. The red colored mass was then taken up in several portions of boiling water (several liters of hot water were necessary) and the hot solution filtered. A little hydrochloric acid was added until the color of the solution changed from violet to yellow. On cooling, the 3-acetyl-6-hydroxybenzoic acid crystallized in orange needles. The acid was purified by recrystallization from hot water and sublimation, giving colorless needles that melted at 209-210°C.; yield 42g., 40.3% of theoretical.

The methyl ether of the phenol was prepared as described by Kranmichfeldt (61); 25 g. of 3-acetyl-6-hydroxybenzoic acid were dissolved in 130 cc. of 10 percent sodium hydroxide solution. To this solution, 52.5 g. of dimethyl

sulfate was added. After a few minutes, the same amount of alkali and dimethyl sulfate were again added. After shaking 20 minutes, the flask was heated under reflux to boiling. An oily layer of the ester separated. Solid sodium hydroxide was added while heating the flask until the ester was completely saponified. A few grams of calcium hydroxide was added and the flask and contents heated for 1 hour. The insoluble calcium salt of the unchanged 3-acetyl-6-hydroxybenzoic acid precipitated and was filtered off. The filtrate was acidified with dilute hydrochloric acid, and on cooling the 3-acetyl-6-methoxybenzoic acid crystallized from the solution. It was purified by recrystallization from hot water and after drying, melted at 150-151°C; yield, 15 grams.

A mixed melting point determination with the cold permanganate oxidative product of the toxic compound showed no depression in the melting point. This determination shows that the aldehyde group is ortho to the methoxyl group and that the methyl ketone group must be in the para position in the toxic compound. Therefore, the structure of the toxic compound isolated from the Encelia leaves is established as 3-acetyl-6-methoxybenzaldehyde(III). This compound has not been reported in the chemical literature.

SYNTHESIS

Since more of the toxic compound was needed for physiological testing, and since it had not been prepared before, synthesis of the compound was undertaken. A number of methods for the synthesis of aromatic aldehydes and ketones were tried without any success. It might be well to mention some of the reactions that were tried in an attempt to synthesize the toxic compound, 3-acetyl-6-methoxy-benzaldehyde.

Reimer-Tiemann Reaction.-- p-Hydroxy acetophenone was prepared from phenol and acetic anhydride using the Fries rearrangement. The phenyl acetate first formed was subjected to rearrangement with anhydrous aluminum chloride producing a mixture of ortho and para-hydroxyacetophenone. The ortho compound was removed from the para by steam distillation. The p-hydroxyacetophenone in the residue was purified by recrystallization from benzene and treated with sodium hydroxide and chloroform according to the Reimer-Tiemann Reaction. A polymerized product was obtained instead of the aldehyde. In other reactions the carbonyl group was protected by forming the semicarbazone and also the acetal, but no aldehyde could be isolated from the reaction mixture in either case after reacting with chloroform in alcoholic sodium hydroxide solution.

Gattermann-Koch Reaction.--Starting with p-methoxyacetophenone, it was attempted to substitute an aldehyde group into the benzene ring using zinc cyanide and anhydrous hydrogen chloride according to the method as described by Adams and Montgomery(62). However, no aldehyde could be detected in the reaction mixture, most of the p-methoxyacetophenone being recovered unaltered.

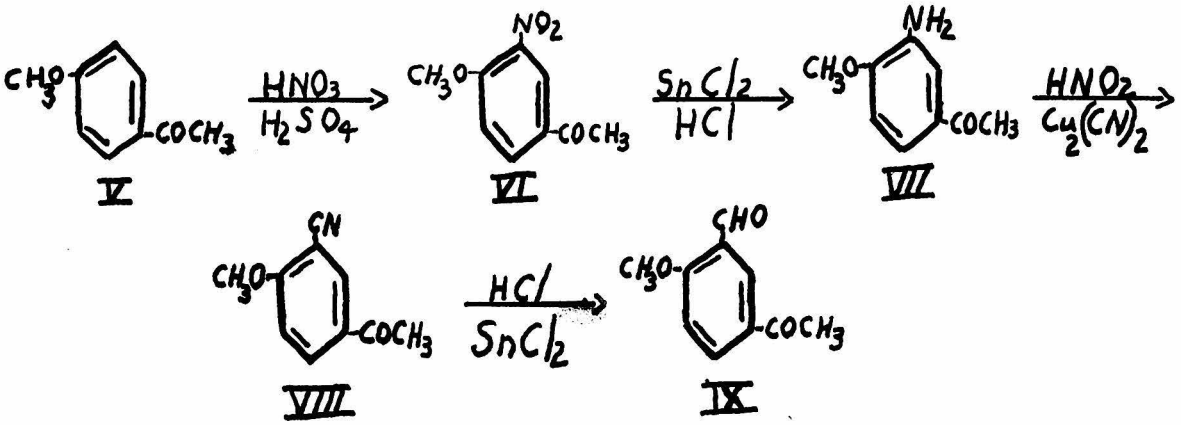
Rosenmund Reduction.--The acid, 3-acetyl-6-methoxybenzoic acid was prepared as described above according to the directions of Bialobrzeski and Mencki(61). It was undertaken to prepare the acid chloride of this acid by reacting with phosphorous pentachloride or thionyl chloride using various precautions, and then reducing the acid chloride by the Rosenmund-Reduction using a poisoned palladium catalyst. The reaction with thionyl chloride was difficult to regulate and only tarry products were obtained. As a result the acid chloride was not isolated and upon hydrogenation no aldehyde could be detected.

Fries Rearrangement.-- The acetyl derivative of salicylaldehyde was prepared and subjected to rearrangement with anhydrous aluminum chloride. A product containing a methyl ketone group could not be found in the reaction mixture.

The reaction of o-methoxybenzaldehyde with acetic anhydride and aluminum chloride according to the method of Noller and Adams(63) was also unsuccessful.

Many other syntheses were tried but none of them yielded the desired compound. Some of the reactions seemed to work, but the conditions were not mild enough to prevent decomposition or polymerization.

Finally a method was found by which the compound could be synthesized, starting with p-methoxyacetophenone according to the following scheme.



4-Methoxy-3-nitroacetophenone (VI).-- The preparation of this compound was accomplished by nitration of p-methoxyacetophenone according to the procedure of Bogert and Curtin (64). Twenty five grams of p-methoxyacetophenone were dissolved in 100 cc. of concentrated sulfuric acid at room temperature. The solution was then cooled to 0°C. and a mixture of 12.3 cc. of concentrated nitric acid and 12.3 cc. of concentrated sulfuric acid was added dropwise over a period of one-half hour while stirring rapidly. The temperature was kept between 0-5°C. and after stirring 15 minutes longer, the solution was poured into 500 cc. of ice water. After standing an hour the precipitate was filtered off, washed several times with cold water and recrystallized from alcohol. The yield was 30.8 g., 95 percent of the theoretical; m.p. 96-98°C.

3-Amino-4-methoxyacetophenone (VII).-- The preparation of the amino compound from the corresponding nitro compound using tin and hydrochloric acid has been mentioned by Bogert and Curtin (64). Directions were not given. In the present work poor yields and a colored product were obtained using tin and hydrochloric acid. It was found that stannous chloride in hydrochloric acid gave better yields and a colorless product.

Powdered stannous chloride dihydrate (180g.) was dissolved in 180 cc. of concentrated hydrochloric acid contained in a 1-liter Erlenmeyer flask. The solution was cooled to 0°C. in an ice salt bath and 40 g. of 4-methoxy-3-nitroacetophenone was added all at once with stirring. The ice bath was removed, and the nitro compound dissolved as the reaction proceeded. When the heat of the reaction had raised the temperature to 95°C., the flask and contents were cooled slightly to 85°C. in the ice bath and then the ice bath was removed. Light yellow crystals of the double salt of the hydrochloride of the amine and stannic chloride started to separate immediately. The mixture was stirred and allowed to cool slowly to room temperature. The mixture was cooled in the ice bath and the double salt was filtered off. The amine was released by adding 50 cc. of water to the salt and then adding an excess of 40% sodium hydroxide solution. The amine was filtered off by suction, washed with cold water,

and recrystallized from alcohol. Small colorless platelets were obtained which melted at 100-101°C. Yield, 76% of theoretical.

3-Acetyl-6-methoxybenzonitrile.-- The nitrile was also prepared by Bogert and Curtin (64) using the Sandmeyer method, but no details were given. The original Sandmeyer method with some modification was found to give a reasonably good yield.

To 20 g. of cuprous chloride suspended in 80 cc. of water was added a solution containing 30 g. of sodium cyanide dissolved in 50 cc. of water. Heat was evolved and after cooling, the solution was filtered. An additional 160 cc. of water was added and the solution cooled to 0°C. in an ice salt bath. Meanwhile, 18 g. of 3-amino-4-methoxyacetophenone was mixed with 100 cc. of water. To this mixture was added 20 cc. of concentrated hydrochloric acid. The amine dissolved and 18 cc. more of conc. hydrochloric acid was added. The solution was cooled to 0°C. in an ice salt bath with stirring. Fine crystals of the amine hydrochloride separated. The cold solution was diazotized by adding from a dropping funnel a cold solution of 11 g. of sodium nitrite dissolved in 50 cc. of water, until an excess was indicated by starch iodide paper. The temperature was kept at 0°C. during the reaction. The diazonium solution was poured slowly with vigorous shaking into the cold cuprous cyanide solution contained in

a 2-liter Erlenmeyer flask. A yellow-brown precipitate formed. Both solutions were kept at 0°C. until the addition was complete. The mixture was stirred two hours longer while allowing the contents to reach room temperature. The mixture was then heated on a water bath for 30 minutes and finally heated to boiling over a burner. The reaction was carried out under a hood, as hydrogen cyanide was given off. The mixture was cooled, filtered, and the residue dried in the room. The residue was extracted with hot benzene. A white copper salt remained behind. From the hot benzene solution light yellow needles of 3-acetyl-6-methoxybenzonitrile crystallized, m.p. 157-158°C. Yield, 68.6% of theoretical.

3-Acetyl-6-methoxybenzaldehyde.-- This compound has not been reported previously. The aldehyde was prepared from the corresponding nitrile using Stephen's reaction. After a number of experiments it was found that the following conditions gave the best yield.

To 30 grams of anhydrous stannous chloride (prepared by the action of acetic anhydride on stannous chloride dihydrate) contained in a 3-necked flask fitted with a reflux condenser, a mercury sealed stirrer, and an inlet tube reaching nearly to the bottom of the flask, was added 200 cc. of absolute ether. Dry hydrogen chloride was passed in through the inlet tube while stirring the mixture. After four hours a clear

lower layer separated, and the inlet tube was replaced by a dropping funnel. A solution of 12.7 grams of 3-acetyl-6-methoxybenzonitrile dissolved in 80 cc. of warm chloroform was added in a small stream from the dropping funnel while stirring vigorously. After a few minutes crystals of the aldimine-stannichloride began to separate. Dry hydrogen chloride was again passed into the solution for 1-1/2 hours. Stirring was continued 1/2 hour longer and the flask and contents were allowed to stand overnight. The yellow precipitate of the aldimine-stannichloride was filtered off and washed with 50 cc. of dry ether. The salt was heated with 100 cc. of water and filtered. The residue and filtrate were both extracted with benzene. The aldehyde was removed from the benzene by extracting with 20% sodium bisulfite solution. The aldehyde was released upon acidifying and heating the bisulfite solution. After cooling, the 3-acetyl-6-methoxybenzaldehyde was filtered off and dried. It was recrystallized from alcohol, m.p. 141-143°C. Yield, 39% of theoretical. After recrystallizing from ether the melting point was 143-144°C. This compound gave an aldehyde test with Schiff's reagent and a positive test for methyl ketones with sodium nitroprusside reagent. A mixed melting point with the natural toxic compound isolated from Encelia leaves showed no depression in melting point.

Thus the identity of the toxic compound has been confirmed by its synthesis. This method provides a way by which other aromatic compounds containing an aldehyde and a ketone group on the same ring may be synthesized.

INHIBITORY EFFECT OF SYNTHETIC AND NATURAL COMPOUND AND ACTIVITY OF RELATED COMPOUNDS.

The inhibitory effect of the synthetic compound was compared to that of the natural compound. Both compounds were tested in the same concentrations using the toxic assay test described earlier, in which the compounds to be tested are supplied to young tomato seedlings in solution cultures. The results are shown in Figure 5. It may be seen that the inhibitory effect of the synthetic aldehyde is essentially the same as that of the natural aldehyde. Concentrations of 250 mg. per liter caused death of most of the plants and concentrations of about 125 mg. per liter caused 50% inhibition of growth of the tomato seedlings in both cases.

Substances related to the toxic compound which was isolated from Encelia leaves were tested to determine which group or combination of groups is responsible for the toxic effect. The pure compounds were weighed out and dissolved in a known volume of distilled water, the difficultly soluble solid compounds being taken up in hot water. These solutions were mixed with an equal volume of Hoagland's nutrient solution and fed to tomato seedling contained in vials. The plants were measured at the time of transplanting and again after growing in the greenhouse at a constant temperature of 80°F. for one week. Four or five dilutions of each substance (1000, 500, 250, 125, and 0 mg. per liter) were tested using

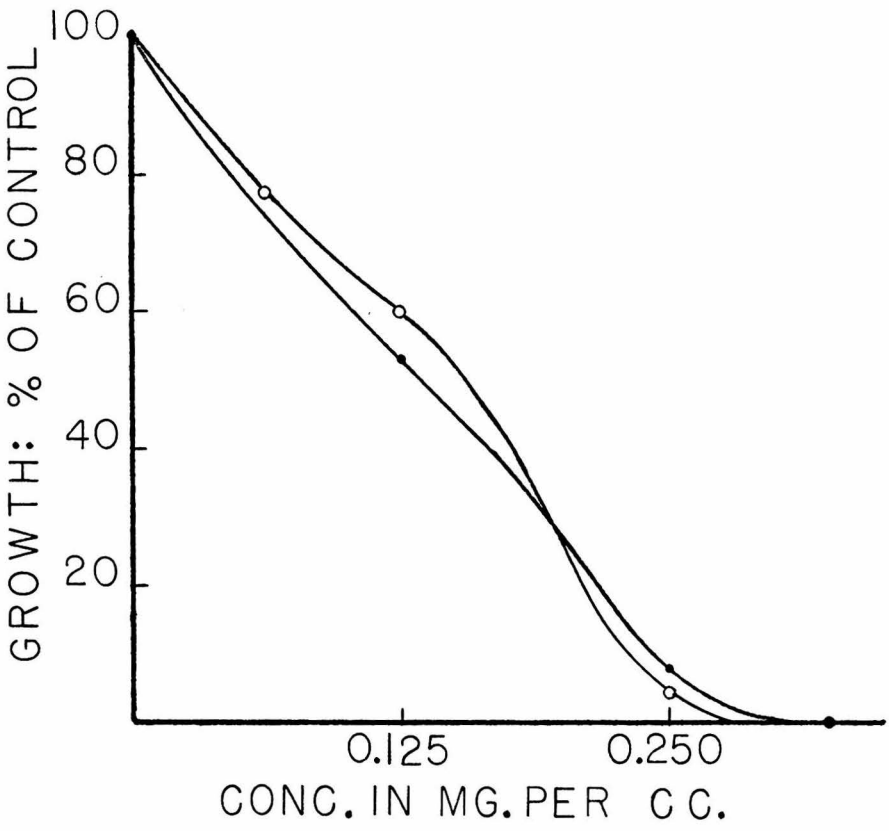


Fig. 5. Effect of varying concentrations of synthetic and natural 3-acetyl-6-methoxybenzaldehyde on the growth of tomato seedlings in solution culture.

o: Synthetic compound; •: Natural compound.

ten plants for each dilution. Concentration of inhibitor was plotted against growth in height as percent of control for each substance as in Figure 5, and the values for 50% inhibition determined by interpolation. The results are shown in Table 7.

Table 7

INHIBITORY ACTIVITY OF PURE COMPOUNDS ON GROWTH OF TOMATO SEEDLINGS

Substance	Conc. needed for 50% inhibition in height growth (mg./L.)	No. of plants dead after 1 week	
		500 mg/L.	250 mg/L.
Benzene	1000	0	0
Anisole	1000	0	0
Benzaldehyde	165	7	3
Acetophenone	365	1	0
p-Methoxyacetophenone	145	10	0
o-Methoxybenzaldehyde	170	6	4
m-Acetylbenzaldehyde	140	7	4
3-Acetyl-6-methoxybenzaldehyde	127	10	8
3-Acetyl-6-methoxybenzonitrile	90	10	10
3-Amino-4-methoxyacetophenone	40	10	10
4-Methoxy-3-nitroacetophenone	45	10	10
3-Acetyl-6-methoxybenzoic acid	237	7	2
4-Methoxyisophtahalic acid	270	1	0
2-Methoxy-5-methylbenzaldehyde	125	10	5
Phenol	225	2	0
Salicylaldehyde	125	9	0
p-Hydroxyacetophenone	130	10	3
o-Hydroxyacetophenone	325	0	0
o-Methoxyacetophenone	175	8	0
3-Acetyl-4-hydroxyacetophenone	60	10	0
Aniline	155	0	0
Nitrobenzene	100	0	0
Benzoic acid	150	10	3

It may be seen from the table that benzene itself is not toxic and that substitution of a methyl ether group in the ring does not increase the toxicity. Introduction of an aldehyde group into the benzene ring greatly increased the inhibitory activity, whereas a methyl ketone (acetyl) group is much less inhibitory than the aldehyde group. Combination of a methyl ketone group with a methoxyl group is more inhibitory than the combination of an aldehyde and methoxyl group. The toxic compound isolated (3-acetyl-6-methoxybenzaldehyde) which contains all three groups is more toxic than combinations of any two of the other groups. The substitution of a cyano, nitro, or amino group for the aldehyde group causes increased inhibition, the amino group having the most toxic effect. The last two columns in the table show that most of these compounds do not cause death of the plants even though they may cause more inhibition in height growth than the naturally occurring inhibitor. Only the nitro, cyano, and amino substituted analogs brought about as high a mortality as the natural substance itself.

TOXIC COMPOUND FROM ARIZONA ENCELIA LEAVES

Isolation and Inhibitory Activity

Encelia leaves gathered near the Castle Dome Mountains in Arizona yielded a different toxic compound than the Encelia leaves gathered in California, although they were both of the same species, Encelia farinosa. The green leaves were dried in a forced draft oven at 70°C. These Arizona leaves gave inhibitory effects similar to the others described earlier when added to tomato plants in sand cultures. The leaves were extracted in the same manner as the California Encelia leaves, and the toxic activity also followed the same fractions.

Isolation.-- Fourteen hundred grams of dry chopped Encelia leaves (Arizona variety) were added to 4 liters of benzene in a large flask. The flask was warmed in a warm water bath at 60°C. for an hour and the leaves were allowed to soak in the benzene overnight at room temperature. Nearly all of the benzene was removed from the leaves by means of a press. The solution was filtered and the benzene removed by heating in a water bath at reduced pressure, leaving a green, sticky mass. All of the toxic activity was removed from this residue with four extractions with 500 cc. of hot water each. The water containing the toxic material was cooled and filtered, and then extracted with an equal volume of benzene with two extractions in a separatory funnel. On evaporation

of the benzene at reduced pressure, crystals separated. The crystalline material was recrystallized twice from hot benzene to give a white crystalline compound melting sharply at 194.5-195°C. The yield of the crystalline material was 1.8 grams which corresponds to 0.13% of the dry weight of the leaves. This compound from the Arizona Encelia leaves was later shown to be a lactone.

Inhibitory Activity.-- The pure compound was tested for inhibitory activity on tomato seedlings growing in solution culture using the method described earlier. The crystals of the lactone were dissolved in hot water at different concentrations and then mixed with equal volumes of Hoagland's solution. The tomato plants were allowed to grow in the vials containing the test solution for one week at a constant temperature of 80°F. Ten plants were used for each dilution. The height of the plants was measured before and after a weeks growth, and the growth was compared with the controls which received only nutrient solution. The results are shown in Figure 6. At concentrations of 250 and 125 mg. per liter all of the plants died. It can be seen from the curve that very small amounts of the compound caused considerable inhibition of growth; 62.5 mg. per liter caused 90% inhibition of height growth, and 50% inhibition corresponds to a concentration of 12.5 mg. per liter.

Thus we see that the lactone has greater inhibitory activity than the first compound, 3-acetyl-6-methoxybenzaldehyde,

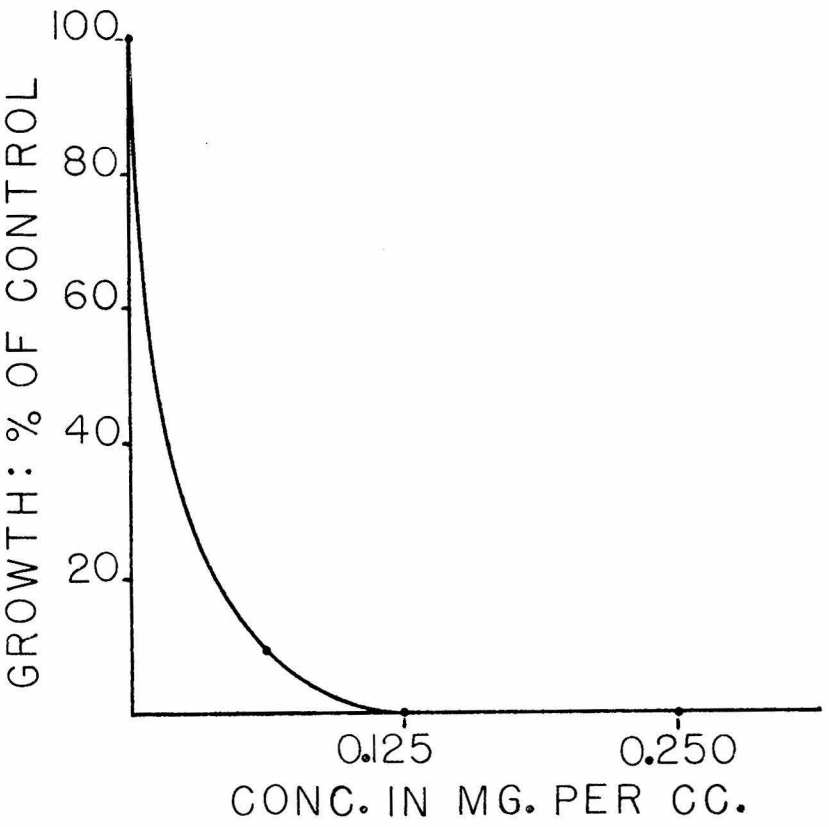


Fig. 6. Growth of tomato seedlings in solution culture in the presence of varying concentrations of the crystalline lactone isolated from Arizona Encelia leaves.

which was isolated from the California Encelia leaves.

The inhibitory activity of the lactone was tested on Encelia seedlings themselves which were obtained by germinating some Encelia seeds which were gathered on the desert. The plants were 20 to 30 mm. high and contained 6 to 8 leaves per plant when they were transplanted from the sand flats to the vials. The tests were carried out in the same manner as with the tomato seedlings. Only 5 plants were used for each concentration. The measurements were made after one weeks growth. The results are shown in the following table.

Table 7

Effect of lactone isolated from Encelia leaves on growth of Encelia seedlings in solution culture.

Conc. of lactone	Condition of plants after 1 week
1.0 mg./cc.	all 5 dead
0.375	4 dead, 1 survived, no growth
0.187	4 dead, 1 survived, no growth
0.0625	3 dead, 2 survived, no growth
0 (control)	0 dead, 5 survived, average growth 1.6 mm/plant

It is shown in the control that the Encelia seedlings were able to survive in nutrient solution, although they did not grow very rapidly. Since all other concentrations of the lactone tested caused most of the plants to die, we may conclude that the lactone isolated from the Encelia plants seems to be quite toxic to Encelia plants themselves when grown in solution culture.

Germination tests.-- The effect of the lactone on germination of tomato seeds was not so striking as the effect on the tomato plants. The tests were carried out as described earlier, different concentrations of the lactone being used in each case. Fifty seeds per petri dish were used and two petri dishes were used for each dilution of the lactone. The seeds were placed in the dark at room temperature and the number of seeds that germinated were counted after 3 days, and after 6 days. The results are shown in table 8.

Table 8

Effect of lactone on the germination of tomato seeds.

Conc. of lactone	% Germination after 3 days	6 days
water (control)	90	92
0.50 mg./cc.	2	37
0.25	5	76
0.125	22	75
0.062	46	86

It is seen from the table that although the lactone delays germination, it does not prevent germination in the same concentrations that killed the plants.

CHARACTERIZATION OF THE TOXIC COMPOUND FROM ARIZONA ENCELIA LEAVES

Physical and Chemical Properties

The toxic compound from the Arizona Encelia leaves crystallizes in colorless needles from benzene forming colorless needles that show strong double refraction when examined under the polarizing microscope. The compound melts sharply at 194.5-195°C. It can be crystallized from hot water giving fine needles with the same melting point. The compound has little odor but often causes a person to sneeze. It has a bitter taste; it burns with a smoky flame leaving no residue after ignition.

Elementary analysis using the sodium fusion method showed the absence of nitrogen, sulfur, and the halogens.

Solubility tests show the compound to be insoluble in cold water, 5% hydrochloric acid, and 5% sodium bicarbonate solution; it is slightly soluble in ether, cold benzene, and hot water, and very soluble in 5% sodium hydroxide, alcohol, and hot benzene.

Classification Tests(47, 48).-- The unknown compound gives no color reaction with anhydrous aluminum chloride in chloroform which shows that the benzene ring may be absent. Although it dissolves in 5% sodium hydroxide, the ferric chloride test for phenols is negative. Generic tests for acids were negative since it is not acidic enough to be titrated with 0.02 N sodium hydroxide in alcoholic solution. The compound shows no reaction with sodium even when dissolved in benzene. However, the Grignard determination shows the presence of an active hydrogen atom. Therefore, there must be an alcoholic hydroxyl group present. The compound also reacts with acetic anhydride. This compound does not give a test for aldehydes with the fuchsin-aldehyde reagent, nor does it give a positive test for methyl ketones with the sodium nitroprusside reagent (50). The iodoform test was negative. The compound does form a precipitate with 2,4-dinitrophenylhydrazine reagent which shows that a ketone group may be present. However in trying to form a derivative with 2,4-dinitrophenylhydrazine in alcoholic solution, no crystalline material could be obtained.

A qualitative test for aromatic methylene ethers (65), which consisted of adding a drop of 5% gallic acid in alcohol to a solution of the compound in concentrated sulfuric acid, was negative. A sample of piperonal gave a good positive test (emerald green color) with the reagent. Another quantitative test for methylene ethers (65) using phloroglucinol in sulfuric acid solution gave a red precipitate in excess of the theoretical amount, and since the reagent is not specific, the results of this test were later disregarded.

The toxic compound decolorizes 2% cold aqueous permanganate solution very readily showing that the compound may be unsaturated. The compound also decolorizes a 5% bromine in carbon tetrachloride solution when the compound is dissolved in chloroform. Therefore, the compound is undoubtedly unsaturated.

The compound gives a good positive test (violet color) with the hydroxamic acid test (49) for esters, acid anhydrides, and lactones. In the saponification test for esters no other products of saponification could be detected, and on acidification the compound was recovered unaltered. This is characteristic of lactones. The compound cannot be classified as an acid, since it is not acidic enough to be titrated by alkali. The classification tests show that the toxic compound from the Arizona Encelia leaves is an unsaturated lactone possibly containing a hydroxyl group.

Quantitative Analysis.-- Micro analysis * of the pure compound gave the following results.

	Found		Atomic ratio		Calc'd for
	Sample 1	Sample 2	Sample 1	Sample 2	$C_{16}H_{20}O_4$
Carbon	69.18%	69.77%	3.95	4.06	69.56
Hydrogen	7.45	7.25	5.1	5.08	7.25
Oxygen	23.37	22.95	1.0	1.0	23.19
Mol. Wt.	300	279			276

The atomic ratio of the elements as shown in columns 3 and 4 of the above table corresponds to an empirical formula of C_4H_5O. A molecular formula of $C_{16}H_{20}O_4$ of molecular weight 276 is obtained by multiplying by a factor of 4. This corresponds nicely to the molecular weights obtained by analysis.

The saponification equivalent was determined using potassium hydroxide in diethylene glycol (47). A sample of 77 mg. of the lactone was heated to 130°C. with 1.04 grams of 1 N. potassium hydroxide -- diethylene glycol solution in a stoppered weighing bottle. The excess base was titrated with 0.02 N acid using phenolphthalein as indicator. The saponification equivalent was found to be 285 in one trial and 288 in the second trial. This agrees closely with the molecular weight 276 and molecular formula $C_{16}H_{20}O_4$. Therefore the compound must contain only one saponifiable group per molecule.

The number of unsaturated bonds was determined by catalytic hydrogenation. The hydrogenation was carried out

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* Most of the micro analyses were done by Dr. G. Oppenheimer and G. Swinehart. Some were done by the author.

in glacial acetic acid at atmospheric pressure using platinum oxide as catalyst. Using 36.53 mg. of the lactone, and 13.42 mg. of the catalyst, 8.48 cc. of hydrogen was taken up at S.T.P. This corresponds to 2.94 moles of hydrogen being taken up per mole of lactone. In another determination 3.1 moles of hydrogen were taken up per mole of lactone, assuming the molecular weight of 276. This means that the unknown compound contains three double bonds per molecule.

A determination of the number of double bonds was also done by titration with bromine in chloroform. To 85 mg. of the lactone dissolved in chloroform was added an excess of .2 N bromine in chloroform solution. After shaking in a glass stoppered flask a few minutes and then standing in the dark for 15 minutes, the excess bromine was determined by adding potassium iodide and titrating the liberated iodine with .1 N sodium thiosulfate solution. This determination showed that only three atoms of bromine were taken up per mole of the lactone under these conditions.

A determination of the groups reactive to Grignard reagent was made. A sample of 13.208 mg. of the lactone yielded .97 cc. of methane which corresponds to one active hydrogen per mole. Two moles of the reagent reacted with the unknown compound without the liberation of methane. The active hydrogen must be present as an alcoholic hydroxyl group as no phenols or acids are present.

Optical Rotation.-- The optical rotation of the lactone was measured using a concentration of 5 mg. of the lactone per cc. of 96% ethyl alcohol. The specific rotation at 25°C. with the sodium D line was calculated from the observed rotation (-0.59°) and found to be -118°.

Ultra-Violet Absorption Spectra.-- The ultra-violet absorption spectra was determined to see if it would give some indication as to whether or not the double bonds in the compound are isolated or conjugated. A sample of 25 mg. of the pure lactone was weighed out on the microbalance and dissolved in 5 cc. of 96% alcohol in a volumetric flask. The solution had to be diluted with alcohol to a concentration of 0.025 mg. per cc. in order to obtain good readings on the spectrophotometer. The absorption spectra was also run in 5% sodium hydroxide solution at the same concentration. The density (log I/I) is plotted against the wavelength in millimicrons in Figure 7. It can be seen that the absorption maximum occurs at 241 millimicrons which indicates that the double bonds may be conjugated. The alkaline solution shows a shift in the absorption spectrum which indicates that the lactone group may be in conjugation with the double bonds.

Derivatives

Acetyl Derivative.-- Fifty milligrams of the pure lactone was placed in a 50 cc. distilling flask, 0.4 cc. of acetic anhydride and 25 mg. of fused sodium acetate powder were added

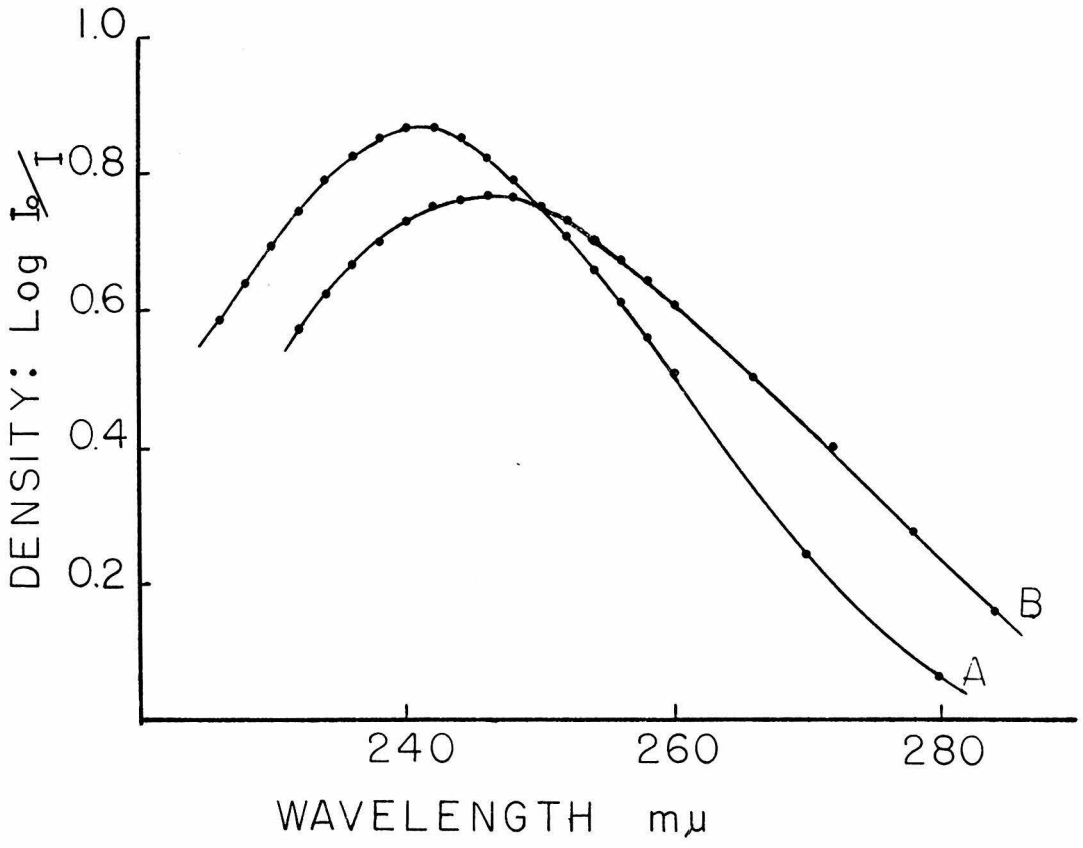

Fig. 7. Ultra-violet absorption spectrum of toxic lactone isolated from Arizona Encelia leaves.

A. 0.025 mg. lactone per cc. of 96 % ethyl alcohol.

B. 0.025 mg. lactone per cc. of 5 % aqueous sodium hydroxide solution.

and the mixture heated 2 hours on a steam bath. The acetic anhydride was removed under reduced pressure and the residue washed with 5 cc. of cold water. The flask was again dried under reduced pressure. The residue was extracted with 2 cc. of hot benzene. Most of the benzene was evaporated in a small beaker on a hot plate. On cooling, white needles formed. The crystals were washed with ether, filtered, and recrystallized from warm ether yielding 20-30 mg. of fine white needles melting at 184.5-185°C. A micro analysis of this product gave the following results:
67.43% C, 6.97% H, and 25.6% O. If the hydrogen atom of the hydroxyl group of the original compound ($C_{16}H_{20}O_4$) were replaced by the acetyl group (CH_3CO-) on acetylation, this would give a compound having the molecular formula ($C_{18}H_{22}O_5$). This acetyl derivative would contain 67.92% C, 6.93% H, and 25.15% O which agrees closely with that actually found.

A saponification equivalent was run on the acetyl derivative using a 12 mg. sample and the potassium hydroxide-diethylene glycol saponification reagent. The saponification equivalent was found to be equal to 160 which corresponds to two saponifiable groups per molecule and a molecular weight of 320. This agrees closely with the formula $C_{18}H_{22}O_5$ having a molecular weight of 318. The results of this analysis add evidence as to the correctness of the proposed molecular formula ($C_{16}H_{20}O_4$) for the toxic lactone.

2,4-Dinitrophenylhydrazine Derivative.-- A 25 mg. sample of the lactone was dissolved in 2 cc. of alcohol and a saturated solution of 2,4-dinitrophenylhydrazine in alcohol and a drop of hydrochloric acid were added. No precipitate formed, even after adding water. After standing for some time a few crystals formed that were filtered off and dried; m.p. 250-253°C. By adding a hot saturated solution of an alcoholic solution of 2,4-dinitrophenylhydrazine to a concentrated solution of the lactone in alcohol and a drop of hydrochloric acid, a jell-like precipitate formed. This material was not crystalline and did not behave as a true hydrazone.

Hydrogenation Product.-- The hydrogenation was carried out as described above under Quantitative Analysis. The catalyst was filtered off and the acetic acid was removed under reduced pressure while heating on a water bath. The residue was recrystallized from benzene giving a compound melting at 221-223°C. This hydrogenation product gave a positive test for esters and lactones with the hydroxamic acid test.

Action of Alkali.-- The unknown compound could be dissolved in 5% sodium hydroxide and on acidification the original compound of m.p. 195°C. was obtained. The lactone was saponified as described earlier with potassium hydroxide-ethylene glycol (1 N) and acidified. The solution was too dilute for the compound to precipitate, so it was extracted with ether. The ether was evaporated and the residue

recrystallized from benzene giving a compound melting at 187-190°C. The melting point of the starting material was 192-193°C. A mixed melting point determination of the two materials gave no depression, so it was assumed that the saponification product was the same as the starting material. The product also gave a good positive test for lactones with hydroxamic acid and ferric chloride.

By boiling in strong alkali (25% sodium hydroxide) a few minutes, the lactone is converted into a new compound. After acidifying the solution, a precipitate was obtained which melted at 283-285°C., after being washed and dried. This product gave no test for lactones with the hydroxamic acid test. It was insoluble in hot benzene and was not acidic enough to give the generic tests for acids.

Under different conditions, the lactone on treatment with alkali behaves in a different manner. If the lactone is dissolved in 20% sodium hydroxide and then a few pellets of solid sodium hydroxide added, a syrupy material separates. This material dissolves on dilution with water. On acidification, very little material separated so the solution was extracted with ether. On evaporation of the ether a syrupy material was obtained. Attempts to crystallize the material were unsuccessful. The syrupy material gave positive tests for esters or lactones.

Alkali fusion of the lactone with potassium hydroxide in a nickel crucible, first causes the oily material to separate

which then remains insoluble and becomes charred on further heating. On dilution of the product, and acidification, some brown material separated. This material gave no test for phenols with ferric chloride. Coumarin under the same treatment yields a nice crystalline phenolic compound. This experiment suggests that the lactone is not an aromatic lactone such as coumarin and its derivatives.

Oxidation with Potassium permanganate.-- Twenty milligrams of the lactone was oxidized with 3.5 cc. of saturated potassium permanganate solution in a sealed tube. After 1 hour of heating on the steam bath, all of the permanganate color had disappeared. The manganese dioxide was centrifuged off and the filtrate concentrated to .5 cc. on a water bath by bubbling air through the solution in a test tube. On acidification no precipitate formed, and extraction with ether gave no solid material. Evidently the oxidation breaks the compound down into small fragments.

Less vigorous oxidation with less permanganate, and in a solution of acetone gave no recognisable products.

Methylation and Oxydation.-- A sample of 100 mg. of the lactone was treated with .5 cc. of methyl alcohol, and .8 cc. of dimethyl sulfate. After cooling to -5°C., 1 cc. of 43% potassium hydroxide solution was added. The mixture was heated under reflux for 1/2 hour and the same amount of dimethyl sulfate and potassium hydroxide again added. An oil separated and several pellets of solid KOH were added to saponify any

ester formed. On cooling and acidifying, an acid precipitated. This acid was oxidized with permanganate as described above, but gave no crystalline product. Aromatic lactones give methoxy benzoic acids under the same treatment (66). This experiment indicates further that the compound is an aliphatic lactone rather than an aromatic lactone.

About all that can be concluded concerning the structure of the toxic compound from the Arizona Encelia leaves is that it is unsaturated, possibly containing three conjugated double bonds, and that it is an aliphatic lactone containing one lactone ring and one alcoholic hydroxyl group, and that it has a molecular formula of $C_{16}H_{20}O_4$. The other oxygen atom which is unaccounted for is almost limited to being present as a cyclic or fairly long chained ether.

DISCUSSION

It has been shown that the leaves of Encelia farinosa when applied to tomato and other plants in sand cultures exert a marked growth inhibition. Water or ether extracts of the leaves are also inhibitory to the germination of seeds and may even cause death of tomato seedlings grown in solution culture. Leaves of other species tested did not appear to be toxic or inhibitory to the degree in which Encelia shows this property. By fractionation of the toxic leaf extract a pure crystalline compound has been isolated which is toxic to tomato seedlings in solution culture.

The presence of the growth inhibitor in the leaves of Encelia may have ecological importance in relation to the fact that only few specimens of desert annuals are to be found growing in close relationship with the Encelia shrub on the desert. It is known that the organic matter from most shrubs provides a medium favorable for the growth of shrub-associated annuals such as Malacothrix, Emmonanthe, and Rafinesquia (Went, (45)). The dry Encelia leaves either on the plant or after they have fallen upon the ground retain their toxic activity for a period of many months. It is possible that rain may leach the toxic material from the fallen leaves and cause inhibition of germination and growth of such seeds as come to rest under the Encelia plant.

It should be noted however that the toxic effect of Encelia leaves was found to be less striking on tomato plants

grown in rich garden soil than on similar plants grown in sand culture. It may be that the toxic material of Encelia like that of the guayule is destroyed by the microflora of the soil as was shown by Bonner (39). This fact is in any case of uncertain importance in the ecological relations of Encelia, a plant which in general grows in sandy well-drained locations.

After determining the structure of the toxic compound isolated from the California Encelia leaves and finding it to be 3-acetyl-6-methoxybenzaldehyde, it was desired to determine which part of the molecule was responsible for the toxic effect. A number of pure compounds were tested on the growth of tomato seedlings in solution culture. Since neither benzene nor anisole showed any inhibitory action even at relatively high concentrations, the benzene ring and the methyl ether group are certainly not effective alone. The aldehyde group seems to be the most toxic constituent of the molecule, since benzaldehyde was much more inhibitory than acetophenone. However the presence of the three groups (aldehyde, methyl ketone, and methyl ether) together in the same compound has somewhat of an additive effect, as the toxic compound was more toxic than combinations of any two of the three groups. This compound 3-acetyl-6-methoxybenzaldehyde (AMB) is quite closely related to the toxic compound, vanillin (4-hydroxy-3-methoxybenzaldehyde), which was isolated from

certain soils by Schreiner and co-workers (21-27). Both compounds have an aldehyde and a methoxy group.

The method of synthesis of the toxic compound (3-acetyl-6-methoxybenzaldehyde) which was successfully worked out may be used for the synthesis of other difficult-to-make benzene derivatives containing an aldehyde and a ketone group on the same ring.

It is of interest that although the ecological relationships of Encelia farinosa appear to be similar over a wide geographical range, the active growth inhibitory principle may differ with different geographical races of the species. Leaves of Encelia farinosa collected in the Castle Dome mountains of southern Arizona yielded a crystalline toxic material different from the one found in the California leaves. This new compound from the Arizona leaves has been shown to be an unsaturated aliphatic lactone, containing one hydroxyl group and having a molecular formula $C_{16}H_{30}O_4$. The lactone was found to be more toxic than the AMB. Concentrations of the lactone as low as 62.5 mg. per liter caused 90% inhibition of growth in tomato seedlings in solution culture. Other unsaturated lactones such as coumarin, which was isolated from soils by Schreiner and co-workers (21-27), are known to be injurious to plant growth. In other instances (67) several unsaturated lactones have been reported to be growth inhibiting which may be due to their antagonistic effect to plant growth hormones.

In conclusion, it may be said that the observations of Encelia plants in their natural habitat on the desert, which show few plant associations with Encelia and the finding of toxic growth inhibitors in the leaves of Encelia farinosa, strengthens the idea that specific chemical substances arising from plants may play an important part in plant ecology and sociology.

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