FURTHER EVIDENCE FOR A CHEMICAL REACTION BETWEEN PLANT GROWTH-REGULATORS AND A PLANT SUBSTRATE

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Introduction

The substitution of atoms or groups in both ortho positions of phenoxyacetic, phenylacetic, phenylbutyric and indoleacetic acids causes the compounds to be ineffective in promoting the elongation of sections of Avena coleoptiles (**9, 15**). The obvious interpretation is that these atoms or groups in the ortho position block a chemical reaction taking place at that point. Considerable evidence exists that electronegative atoms or groups attached to the benzene ring in phenoxyacetic and phenylacetic acids enhance the activity of these compounds, and this feature suggests that the substrate reacting at the ortho position is nucleophilic in nature (**15**). BENTLEY (**2**), however, has reported that 2,3,6-trichlorobenzoic acid is very effective in promoting the elongation of coleoptile sections, although both ortho positions are occupied by chlorine atoms. Investigations of the activities of a number of benzoic acid derivatives have shown that the active compounds have one or both ortho positions occupied by electronegative atoms or groups (**9, 26**), and it has been suggested that with these compounds the reaction takes place by a displacement of the electronegative atom or group by the nucleophilic substrate (**9**).

One of the active derivatives of benzoic acid is 2,6-dichlorobenzoic acid. If such a substance does react at an ortho position by nucleophilic displacement of a chloride ion, then chemical analysis of the tissue and the solution in which growth has taken place should detect an increase in the amount of chloride ion. Such analyses were made and an increase in Cl^- was detected.

Materials and methods

Avena plants were grown and coleoptile sections were harvested as reported previously (**16**) except that the sand in which the plants were grown was freed of chloride by heating at 150° F overnight with concentrated sulphuric and nitric acids. The sand was then washed with hot, doubly distilled water until the washings gave a negative sulphate test with barium chloride. All apparatus used in the experiment was cleaned scrupulously and then washed with doubly distilled water. Best results were obtained when all procedures were conducted in a small, carefully scrubbed, dark room in which only the investigator worked.

One lot of sections was placed in 25 ml. of the solution of the chemical, and a second lot was placed in 25 ml. water to serve as a control. The sections were allowed to grow for eight hours before analysis. This period of time for growth was used to avoid bacterial activity. After eight hours, the sections were removed; and the liquids were evaporated to dryness under a heat lamp. The chloride content of the residues was determined along with that of the sections. The sections were ground thoroughly in a mortar and dried until crisp under a heat lamp to insure rupture of all cells. The dried sections were ground again and extracted six times with hot water, the water being decanted into a centrifuge tube after each extraction. The combined washings were centrifuged to remove suspended material and evaporated to dryness under the heat lamp.

The analysis for chloride was made by the procedure developed by KIRK and coworkers (**12**) for the determination of chloride in the presence of protoplasmic material. Actually very little such material was present in the solutions analyzed since the protein material was rendered insoluble by the heat treatment and removed by centrifugation. Titrations were made with 0.01 *N* $AgNO_3$, and the volume of the solution at the beginning of the titration was 0.8 ml.

Experimental results and discussion

The validity of the technique employed was established by several preliminary experiments. Comparable data were obtained from five experiments and the data from two such experiments are summarized in table I. The data show that the amount of chloride ion does increase when growth takes place in the presence of 2,6-dichlorobenzoic acid or 2,4-dichlorobenzoic acid. The possibility that the increase of chloride ion is due to hydrolysis of the compounds under the conditions of the experiment was examined by evaporation of their solutions under the heat lamp and also by boiling

TABLE I

CHLORIDE CONTENT OF COLEOPTILE SECTIONS AND GROWTH MEDIUM AFTER GROWTH PERIOD OF EIGHT HOURS

Exp. no.	Compound	Molar conc.	No. of sections	Length of sections	Fresh wt. of sections	Micrograms of chloride	
						Sections and solution	Sections and water control
				mm.	*g.*		
1.	2,6-dichloro-benzoic acid	5×10^{-4}	50	5.5	0.35	46	38
2.	2,6-dichloro-benzoic acid	1×10^{-4}	162	3.0	0.62	75	50
	2,4-dichloro-benzoic acid	1×10^{-4}	162	3.0	0.62	62	50

the compounds in aqueous solution. No ionic chloride was obtained in either treatment of 2,6-dichlorobenzoic acid. The evaporation of 25 ml. of 1×10^{-4} M 2,4-dichlorobenzoic acid solution under the heat lamp resulted in the detection of 2.5 micrograms of ionic chloride. This observation indicates that Cl^- is more easily removed from the 2,4-acid. However, during the growth of the sections much more chloride is released from the 2,6-acid. The 2,4-acid does not promote elongation of the sections, and the chloride released during the growth period (12 micrograms) may be the result of hydrolysis. The possibility also exists that the chloride may be removed from the 4-position rather than the 2-position.

Quantitative data on the ease of removal of Cl from the benzoic acids are not available in the chemical literature; however, such data are available for the analogous chloronitrobenzenes. Nucleophilic groups remove chlorine 144 times faster from the 2,4-dichloronitrobenzene than from the 2,6-dichloronitrobenzene and both chlorine atoms are removed (**1**). Presumably the greater stability of the 2,6-dichloronitrobenzene toward nucleophilic displacement is due to the steric inhibition of resonance (**1**) which

TABLE II

PERCENTAGE ELONGATION OF SECTIONS OF COLEOPTILES IN SOLUTIONS OF INDOLEACETIC ACIDS AS COMPARED WITH PERCENTAGE ELONGATION IN WATER

	Molar concentrations $\times 10^{-4}$							
	2.0	1.0	0.5	0.1	0.05	0.01	0.005	0.001
4-Chloroindoleacetic acid	+7	+20	+20	+19	+28	+21	+17	+13
Indoleacetic acid				+24		+20		+7

results from the substitution of the two ortho positions forcing the nitro group out of the plane of the ring. The same steric hindrance of resonance has been shown (**17**) to occur with 2,6-dichlorobenzoic acid.

The larger amount of chloride released from 2,6-dichlorobenzoic acid in experiment 2 was correlated with greater elongation of the sections in the lower concentration of the chemical. At concentrations greater than 2×10^{-4} M the promoting effect of the compound on elongation decreases (**16**). If one assumes that every chloride ion displaced represents a molecule of 2,6-dichlorobenzoic acid reacting, the data of experiment 2 show that 1.13×10^{-6} mole of the compound reacts per gram of coleoptile tissue and only 28% of the molecules present actually are involved in the reaction.

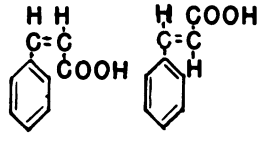
These data are additional evidence for the hypothesis that compounds which promote elongation of the cells do so by a chemical reaction at the ortho position involving a displacement of hydrogen (phenoxyacetic, phenylacetic, and phenylbutyric acids) or electronegative atoms or groups (benzoic acids). Evidence exists that the ortho effect is operative for indoleacetic acids (**9, 16**). Substitution at the 2 position diminishes the activity of in-

doleacetic acid; substitution at the 2 and 4 positions essentially nullifies the activity. It was of interest therefore to determine the activity of a compound substituted only at the 4 position, and to this end the synthesis of 4-chloroindoleacetic acid was carried out and its activity is shown in table II.

It is apparent from the data in table II that the substituted acid has an effect on elongation which is equal to or greater than the effect of indoleacetic acid. This observation may be explained by the aromatic nature of the five-membered ring (**14**) and the favorable electronic effect of substitution reactions in this ring brought about by the chlorine at position 4.

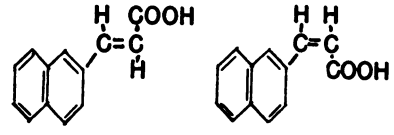
VELDSTRA (**23**) has presented results of investigations on the growth activity of cis and trans acids such as cinnamic acid and the phenylcyclopropane carboxylic acids which do not seem to be accounted for by the hypothesis of the ortho effect. In the following acids, he reports the cis isomers are active and the trans isomers are not (evidence for the structures of the naphthylacrylic acids and the tetra hydronaphthylideneacetic acids has been obtained by HAVINGA and NIVARD, **10**):

Cinnamic acids



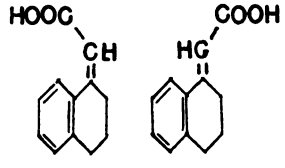
Cis Trans

B-Naphthyl-1-acrylic acids



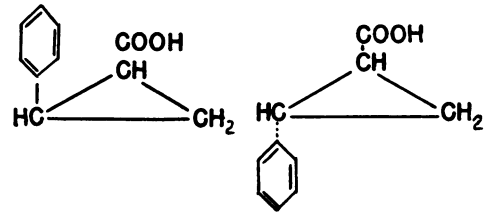
Trans Cis

1,2,3,4-Tetrahydronaphthylidene acetic acids



Cis Trans

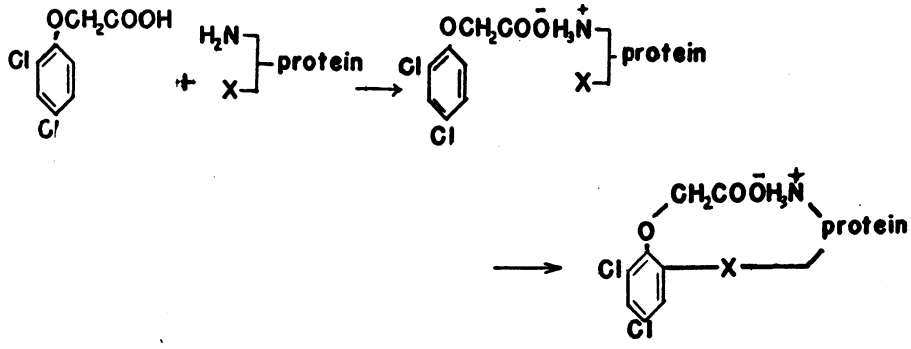
1-Phenylcyclopropane carboxylic acids



Cis Trans

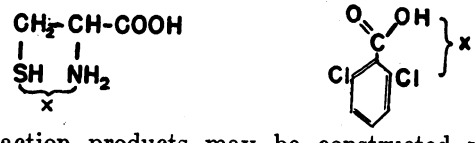
The activity of the cis isomer is explained by Veldstra by postulating that in these isomers the carboxyl groups are out of the plane of the ring while in the trans isomers the carboxyl groups are in the plane of the ring, and the carboxyl group must be out of the plane of the ring for activity (**4, 24**). However, with certain assumptions the activity of the cis isomers can be interpreted in terms of a reaction at the ortho position. Since a carboxyl group or a group convertible to a carboxyl group is essential for growth activity, a reasonable assumption is that the function of this group is to

attach the molecule to a protein by reaction with a basic group such as an amine. Such a reaction would be fast compared to the nucleophilic substitution at the ortho position. The first step would be salt formation. Following this attachment, reaction at the ortho position with another point on the protein would lead to a cyclic reaction product according to the following equation:



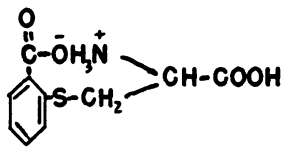
The ring could form with a minimum of strain in the cis form of the acids. In the tetrahydronaphthylideneacetic acid the carboxyl group is cis to a ring in each form; however, the reaction could take place only through the unsaturated ring (**13**). In the trans forms of the acids much greater strain (presumably prohibitive) would be involved in the formation of such a ring. Using Hirschfelder models, a ring can be formed from cis cinnamic acid and cysteine by having an amide linkage between the cinnamic acid carboxyl group and the cysteine amine group with the sulphur of the cysteine attached at the ortho position on the benzene ring. It is not possible to form such a structure using the trans form of cinnamic acid. Using a salt linkage instead of an amide linkage would involve less strain however. Strains of the type described will prevent the formation of certain rings in organic molecules (**7**).

Cysteine or a cysteinyl unit of a protein is regarded as the most likely substrate reacting with the growth regulator because of the distince, X, between the basic group, —NH_2, and the nucleophilic group, —SH. The minimum distance separating the groups would be that in the benzoic acid structure since some derivatives of the acid have considerable effect in causing elongation. This minimum distance is equal to X as is shown in the following formulae:

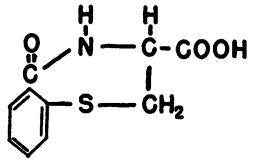


The cyclic reaction products may be constructed with Hirschfelder models for the combination of cysteine and benzoic or phenoxyacetic acids using either an amide or a salt linkage as shown below:

Benzoic acids

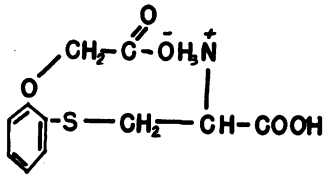


Salt linkage

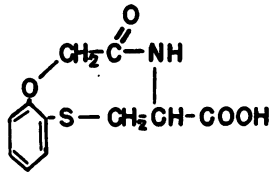


Amide linkage

Phenoxyacetic acids



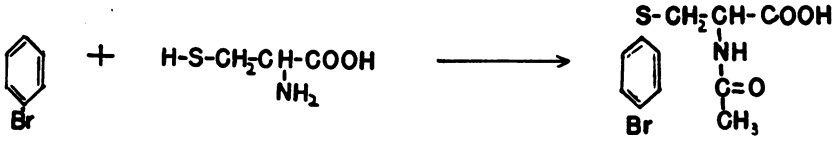
Salt linkage

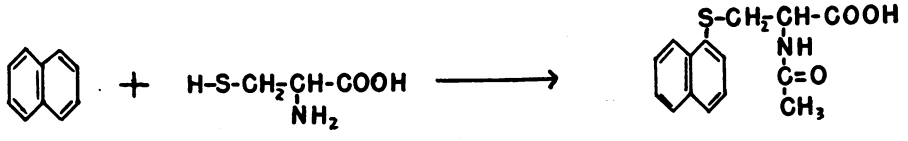


Amide linkage

The rings would have a minimum of seven members in the case of the amide linkage of the benzoic acids, and as many as 11 members in the case of the γ-phenylbutyric acid with the salt linkage.

The nucleophilic substitution of an aromatic nucleus is not unreasonable from either the chemical or biological point of view. A cysteine unit is introduced into aromatic molecules in various animals (**5, 11, 18**) in the following manner:



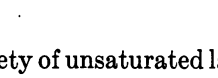


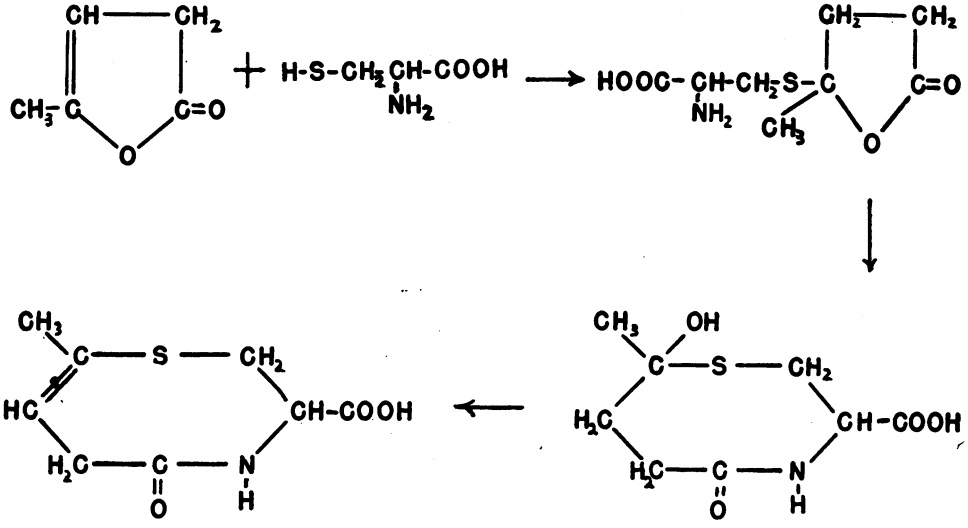
It is interesting to note that the amino group is acetylated by the animal which would correspond to the amide formation as postulated for the growth regulators. STEKOL (**19**) has shown that naphthalene or bromobenzene stop growth in animals, but the effect of these two compounds can be reversed by feeding L-cysteine or DL-methionine. These observations indicate the importance of the —S—S— $\rightleftharpoons$ —SH equilibrium in animal growth.

The observation of THIMANN and BONNER (**21, 22**) that iodoacetate, arsenite and *p*-chloromercuribenzoate (which react with —SH groups) inhibit the elongation of Avena and Pisum sections may be interpreted as indicating a combination of the growth regulators with sulfhydryl enzymes.

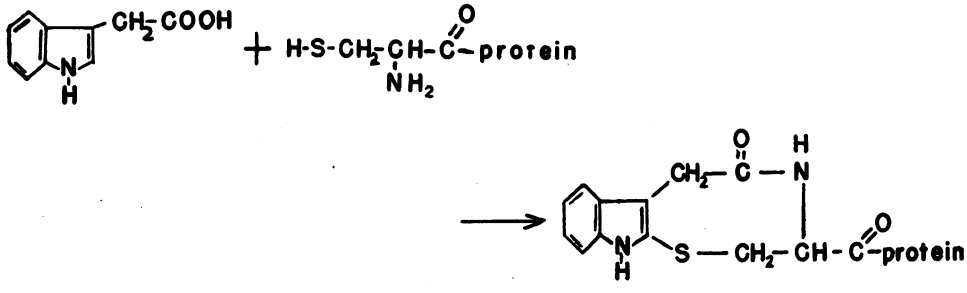
The inhibition of growth could not be reversed with 2,3-dithioglycerol, butane-dithiol-2,3 or thioglycolate (**22**). If the growth regulators react with —SH groups and the inhibitors react more rapidly with the —SH groups or an excess of sulfhydryl compounds is added, a reversal of inhibition would not be possible.

BONNER (**3**) has shown that L-cysteine inhibits the elongation of Avena coleoptile sections, and this may be explained by the reaction of the cysteine with the growth hormone thus preventing the hormone from reacting with a cysteinyl unit of a protein molecule.

A variety of unsaturated lactones of the general structure  inhibit growth of plants (**8, 25**). CAVALLITO and HASKELL (**6**) have shown that these lactones react with cysteine according to the following mechanism:



Thus the inhibiting effect of the lactones on growth could be due to their reaction with the cysteinyl unit of a protein which prevents the growth regulator from reacting at that point. If the growth regulators activate an enzyme system by reaction with a cysteinyl part of a protein molecule as follows:



then an unsaturated lactone, iodoacetate, arsenite or any substance which would effectively compete with the growth regulator for the cysteinyl enzyme unit would inhibit growth provided the cyclic reaction product would not operate in the next step of the growth reaction.

Another interesting aspect of the relationship of chemical structure and physiological activity in the growth regulators which may be explained on the basis of the ortho reaction is the difference in the activity of D and L optical isomers. The data on the isomers has been summarized recently by THIMANN (**20**). As an example, the D isomer of 2,4-dichloro-α-naphthoxypropionic acid is twice as active as the racemic mixture. If optically active growth regulators react with an optically active amino acid such as cysteine in a protein enzyme, then the resulting diastereoisomers differ greatly in their chemical and physical properties. Thus, the two enzymes resulting from the reaction of the D and L forms of the growth regulator with the protein would have different properties and one might be more effective in the growth reaction than the other.

Summary

Increased elongation of Avena coleoptile sections brought about by 2,6-dichlorobenzoic acid is accompanied by the release of chloride ion. This observation substantiates the interpretation of the reaction of the growth regulator as a displacement of the electronegative atom by a nucleophilic substrate.

The reaction of the growth regulator through the carboxyl group and the ortho position requires a basic group and a nucleophilic group on the substrate which are separated by a minimum distance corresponding to that between the carboxyl group and the ortho position of benzoic acid. The cysteinyl unit of a protein appears to be the most likely such substrate in the plant cell. Cyclic reaction products between cysteine and the benzoic and phenoxyacetic acids are possible on chemical grounds.

The different activities of D and L optical isomers and cis and trans isomers, and the inhibitory effects of unsaturated lactones, arsenite, iodoacetate, *p*-chloromercuribenzoate, and cysteine may be explained by the ortho reaction hypothesis for plant growth-regulators.

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