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CAFFEIC ACID, A SUBSTANCE WITH AUXIN ACTIVITY FOUND IN EXTRACTS FROM COLEUS RHENALTIANUS

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In our study on the abscission of debladed petioles carried out with *Coleus rhenaltianus* (VENDRIG, 1960), we detected in extracts from the leaves an auxin that could not be regarded as an indole derivative. It was found that this compound gives a positive response in the *Avena*-curvature test, and that it retards the abscission of debladed petioles of Coleus.

DETTWEILER (1942) had extracted from *Coleus* internodes supplied with an IAA (β-indole acetic acid) preparation an acid stable auxin. The production of this auxin was thought to be enhanced by IAA.

In the present investigation we repeated the experiments of DETTWEILER, and we tried to identify his auxin by means of the chromatographic method, as it seemed to us that this auxin might be identical with the auxin which we ourselves had extracted from the leaves.

In some preliminary experiments, the same chromatographic separation method was used as in our preceding study (VENDRIG, 1960). Besides some spots due to indole derivatives, we especially noticed two spots on the chromatograms which in ultra violet light showed a blue fluorescence, and it appeared that the compounds eluted from these spots gave auxin reaction when used in the *Avena*-coleoptile-section test. The same compounds were also detected in the acid fraction of an extract obtained from untreated plants. The u.v. spectra of both compounds were practically the same as that which was found earlier for the auxin extracted from the leaves. In consequence of this result we decided first to analyse in detail the compounds present in plants that had not been treated with IAA.

METHOD

The plants were cultivated in a glasshouse under long-day conditions. Cuttings were used from the same clone as in our preceding experiments. In each experiment circa 300 g. leaves + stems (fresh weight) were finely ground in a tissue homogenizer with boiling ethanol. After the homogenate had been left for 18 hours at 4° C, it was filtered, and the filtrate concentrated under reduced pressure. The residue was 6 times extracted with about 30 ml. aq. dest. at 40° C. These aqueous extracts were shaken with peroxide-free ether, and each of the ether fractions was 3 times shaken with 20 ml. of an 8 % $NaHCO_3$ solution. The joint bicarbonate fractions were acidified

with 1 N HCl to pH 4.0. The acidified solution was shaken 4 times with ether. After drying over anhydrous Na sulphate, the ether was evaporated.

The residue was dissolved in 2 ml. methanol. This methanol solution was spotted on the start line of a chromatogram (Whatman no. 4, 50 × 50 cm). After equilibration the chromatogram was developed with an isopropanol/water mixture (5:1 v/v) by means of the descending method.

The spots were detected in long wave u.v light (blue fluorescence). They were eluted with ether as well as with ethanol.

The ether eluate was evaporated, and the residue dissolved in a 1 % glucose solution. This solution and some dilutions were tested on auxin activity in the Avena-coleoptile-section test (according to RIETSEMA, 1951); 5 ml. of solution were used for a series of 10–20 sections.

The alcoholic eluates were further purified over aluminium oxide (Merck), and the perfectly clear filtrate was used to determine the u.v. absorption spectrum by means of a *Zeiss* spectrophotometer. After evaporation under reduced pressure the residue was dissolved again in a 1 % glucose solution and tested on auxin activity.

AUXIN ACTIVITY OF THE UNKNOWN COMPOUNDS

On chromatograms developed with isopropanol/water the Rf-values of the two compounds proved to be 0.79 and 0.84. In Table I the

TABLE I

Growth rate of *Avena*-coleoptile sections in a solution of 1 % glucose and the eluates obtained from a chromatogram (spots at Rf 0.79 and 0.84) of the acid fraction from an extract of *Coleus*. Length of the sections 3 mm. Growth recorded at the end of 24 hours. Number of sections between brackets.

Exp. nr.	Increase in length (eye-piece micrometer units)				
	Dilution of the eluate				
	control	1:1	1:10	1:100	1:1000
1 Rf. 0.79	16.8 ± 1.59 (15)	20.8 ± 1.50 (14)	26.2 ± 1.09 (16)	20.9 ± 1.60 (10)	—
2 Rf. 0.79	18.7 ± 1.41 (14)	39.6 ± 2.72 (16)	22.3 ± 1.96 (13)	—	—
3 Rf. 0.79	16.6 ± 1.34 (13)	—	26.2 ± 1.73 (14)	25.7 ± 1.92 (11)	—
4 Rf. 0.79	8.4 ± 0.89 (10)	23.9 ± 1.86 (9)	16.7 ± 2.31 (8)	—	—
1 Rf. 0.84	13.0 ± 0.72 (15)	6.8 ± 0.50 (15)	19.4 ± 0.76 (17)	—	—
2 Rf. 0.84	8.4 ± 0.89 (10)	21.2 ± 2.60 (9)	20.4 ± 1.04 (10)	11.7 ± 1.13 (9)	9.3 ± 1.42 (9)
3 Rf. 0.84	5.2 ± 0.36 (10)	12.4 ± 0.77 (10)	11.2 ± 0.70 (10)	9.4 ± 0.73 (10)	—
4 Rf. 0.84	5.0 ± 0.45 (10)	8.5 ± 0.43 (10)	10.8 ± 0.58 (10)	8.4 ± 0.62 (10)	—

results are recorded of some experiments on the auxin activity of the eluates obtained from these spots. It appears that both compounds enhanced the elongation growth of the coleoptile sections. The diluted solutions too were significantly active.

IDENTIFICATION OF THE COMPOUNDS

In this case too we have tried to identify these physiologically active compounds with indole derivatives. However, all chemical reactions on indole derivatives (LINSER and KIERMAYER, 1957) yielded a negative result.

The two purified alcoholic eluates show (after dilution) the same u.v. absorption spectrum, with a maximum absorption at $\lambda = 2900$ Å and $\lambda = 3250$ Å. Eluates that had not been purified, exhibited a slight shifting of the maximum absorption.

This spectrum resembles some spectra given by flavonoid compounds (GEISSMAN, 1955), and indeed, if we added a solution of neutral lead acetate to the alcoholic eluates, a yellow precipitate was produced. Spraying the chromatograms with this reagent resulted also in the appearance of two yellow spots.

Another useful qualitative test for flavonoid compounds is that of SHINODA (1928), in which the compound is treated with magnesium and concentrated hydrochloric acid. However, with this reagent no colour developed.

Only few of the flavonoids and related compounds listed by GEISSMAN give a blue fluorescence in ultra-violet light and are at the same time colourless in visible light. 3'4' Dihydroxy-flavone satisfies these conditions, but this substance has another absorption spectrum.

The following experiments were carried out to prove that the auxin activity exerted by the eluates is actually due to the compounds which give a yellow precipitate with neutral lead acetate.

The yellow precipitate suspended in ethanol was centrifuged, and the ethanol removed. The precipitate was washed with aq. dest. and centrifuged again. To eliminate the lead, diluted sulfuric acid was added. After filtration, the filtrate was neutralised with K-biphosphate to a pH 2.0, and extracted with ether. After drying and evaporating the ether over anhydrous Na-sulphate, the residue was dissolved in a 1 % glucose solution. Table II shows the results of some experiments with *Avena*-coleoptile sections in which some dilutions of the regenerated compound were tested on their auxin activity. With the compound at Rf 0.84 similar results were obtained.

Other known compounds which give a yellow precipitate with neutral lead acetate and which fluoresce blue in u.v. light, are some phenylpropane derivatives, e.g. chlorogenic acid (PAECH and RUCKENBROD, 1955). The u.v. absorption spectrum of chlorogenic acid given by BJÖRKMAN and HOLMGREN (1960) agrees very closely with the spectra of our unknown compounds. Hoepfner's reagent (1 % $NaNO_2$ in 10 % acetic acid) and $FeCl_3$ give identical colour reactions with chlorogenic acid and with our unknown compounds.

However, commercial chlorogenic acid appeared to have another

TABLE II

Growth rate of *Avena*-coleoptile sections in a solution of 1 % glucose and the regenerated lead precipitate (eluate obtained from the spots in a chromatogram at Rf 0.79). Length of the sections 3 mm. Growth recorded at the end of 24 hours. Number of sections between brackets.

	Increase in length (eye-piece micrometer units)				
	Dilution of the extract				
Exp. nr.	control	1:1	1:10	1:100	1:1000
1	13.4 ± 1.22 (10)	—	—	21.3 ± 1.74 (10)	15.1 ± 1.73 (9)
2	4.3 ± 0.80 (12)	9.1 ± 1.04 (15)	—	7.6 ± 1.22 (12)	8.5 ± 0.57 (13)
3	6.2 ± 0.53 (14)	13.9 ± 0.90 (15)	9.2 ± 0.70 (15)	8.1 ± 0.98 (15)	—

Rf-value (in isopropanol/water: 0.46). Moreover, the elongation growth of Avena coleoptile sections was only slightly enhanced by chlorogenic acid in a 1 % glucose solution. This appears from the experiment recorded in Table III.

TABLE III

Growth rate of *Avena*-coleoptile sections in a solution of 1 % glucose and 0.001–10.0 μg/ml chlorogenic acid. Length of the sections 2 mm. Growth was recorded at the end of 24 hours.

Concentration chlorogenic acid μg/ml	Increase in length (eye-piece micrometer units)	Number of sections
0	9.8 ± 0.72	17
10	12.3 ± 0.74	21
1	12.7 ± 0.70	18
0.1	14.5 ± 0.76	21
0.01	9.2 ± 1.02	18
0.001	8.7 ± 0.89	18

The possibility could not be ruled out that the depside is hydrolysed to quinic acid and caffeic acid. It is known that caffeic acid can act synergistically on IAA and on α-naphthyl acetic acid (REINDERS-GOUWENTAK and SMEETS, 1947; HEMBERG, 1951). According to these authors caffeic acid alone, however, is either slightly inhibitory or inactive.

Caffeic acid too gives a yellow precipitate with neutral lead acetate (due to the two hydroxy groups on the ring). Moreover, we noticed that caffeic acid has the same Rf-value as one of our unknown compounds (0.84 in isopropanol/water). The same correspondence in Rf-value is found when water-saturated ethylacetate is used as a solvent. However, we could detect only one spot in ultra-violet light, if butanol/acetic acid/water (5/2/2 v/v) was used as a solvent. Both caffeic acid and the compounds of our extract had in this case a Rf-value of 0.81.

On chromatograms developed with isopropanol/water only one spot was found, and this proved to have the Rf-value of caffeic acid, when the extract was treated with sulphuric acid.

From leaves of *Solidago virgaurea* BJÖRKMAN and HOLMGREN (1960) extracted besides chlorogenic acid a second compound with the u.v. spectrum of caffeic acid and, if the chromatogram was developed with butanol/acetic acid/water, with the same Rf-value. After hydrolysis

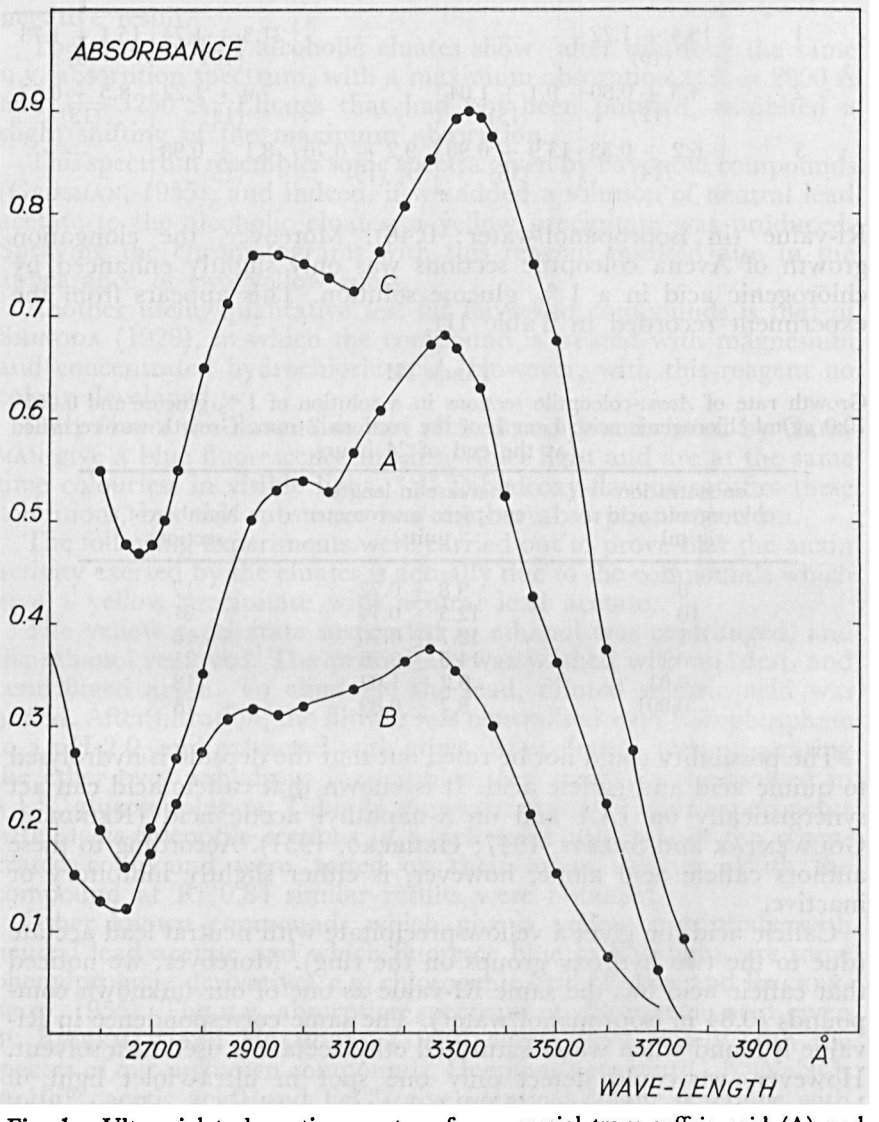

Fig. 1. Ultra-violet absorption spectra of commercial trans-caffeic acid (A) and of the eluates obtained from the spots at Rf 0.79 (B) and 0.84 (C) of a chromatogram developed with isopropanol/water. The substances were dissolved in ethanol.

with hydrochloric acid caffeic acid was obtained as the sole fluorescent substance. According to the authors, their second compound might be identical with iso-chlorogenic acid.

The u.v. absorption spectra of caffeic acid and of our compounds purified from an extract, are reproduced in Fig. 1.

On our chromatograms developed with isopropanol/water, the two fluorescent compounds give the same colour with the following spraying reagents

a) 2 % $FeCl_3$: grey
b) Ammonia vapour: yellow (for a short time)
c) Ammoniacal silver nitrate: black
d) 2.4. Dinitrosalicylic acid in 2 % NaOH: yellow
e) Bariumhydroxyde: yellow.

Auxin activity of caffeic acid

As we now have identified one of the compounds from the acid fraction of a *Coleus* extract with caffeic acid, and the other one as a related substance, it is reasonable to expect that commercial caffeic acid too will show auxin activity in the *Avena* coleoptile section test.

Indeed, caffeic acid, either alone or combined with 0.5 % glucose, greatly enhances the elongation growth of the sections; however, only when applied in low concentrations. Caffeic acid concentrations of 30–100 μg/ml were slightly inhibitory or inactive. Some results are recorded in Table IV. The results of identical experiments with cinnamic acid are recorded in the same table.

Discussion

Bentley (1958) defines auxins as growth regulators which at low concentrations induce cell enlargement. Most of the bioassays that are used to detect auxins in plant extracts are based on this interpretation.

The use of paper and column chromatography has revealed the existence of many unknown substances with auxin activity. Bentley (1958) lists a number of unknown compounds from acid and neutral fractions of ethereal and alcoholic extracts.

The unknown auxin which is present in an ethereal extract from *Coleus* leaves (Vendrig, 1960), is now recognized as consisting of two substances which could be identified by us respectively with caffeic acid and with a related compound.

Caffeic acid has been found in many plants (Tomaszewski, 1960), and is usually considered as a growth inhibitor (Akkerman and Veldstra, 1947). In pedicels of tomatoes caffeic acid is either inactive or exerts but a slight inhibitory effect on cell enlargement. Combined with α-naphthyl acetic acid it has a synergistic effect on the growth hormone (Reinders-Gouwentak and Smeets, 1953). According to these authors caffeic as well as ferulic acid would have some influence on the transport of the natural growth hormone. In tomato the compounds would exert a synergistic effect on applied α-naphthyl

TABLE IV

Growth rate of *Avena*-coleoptile sections in a solution of caffeic resp. cinnamic acid (trans-isomers) in aq. dest. or with 0.5 % glucose. The substances were added 6 hours after the sections had been made and immersed in aq. dest. Length of the sections 2 mm. Growth was recorded at the end of 18 hours. Number of sections between brackets.

			Increase in length (eye-piece micrometer units)			
			Concentration of the acid (μg/ml)			
Exp. nr.	% glucose	Acid	0	3	1	0.5
1	0	caffeic	3.8 ± 0.45 (16)	5.3 ± 0.41 (19)	5.7 ± 0.35 (21)	4.3 ± 0.34 (18)
	0	cinnamic	3.4 ± 0.36 (16)	3.3 ± 0.44 (20)	3.2 ± 0.52 (15)	2.2 ± 0.36 (18)
2	0	caffeic	1.2 ± 0.28 (14)	3.4 ± 0.27 (14)	5.0 ± 0.34 (14)	3.7 ± 0.27 (18)
	0	cinnamic	1.6 ± 0.37 (17)	2.5 ± 0.36 (15)	2.4 ± 0.42 (17)	2.1 ± 0.38 (17)
3	0	caffeic	1.5 ± 0.21 (11)	4.0 ± 0.38 (12)	3.7 ± 0.38 (14)	3.0 ± 0.33 (13)
4	0.5	caffeic	14.2 ± 0.83 (17)	20.0 ± 0.63 (20)	20.1 ± 0.58 (19)	21.8 ± 0.61 (21)
	0.5	cinnamic	14.8 ± 0.57 (24)	13.0 ± 0.84 (14)	12.0 ± 0.92 (12)	13.0 ± 0.80 (12)
5	0.5	caffeic	10.0 ± 0.50 (16)	15.7 ± 0.62 (18)	15.8 ± 0.68 (17)	15.0 ± 0.35 (15)
	0.5	cinnamic	8.3 ± 0.56 (16)	7.8 ± 0.57 (11)	9.5 ± 0.68 (13)	9.8 ± 0.85 (15)

acetic acid, only in those cases where there is no natural growth hormone transport.

HEMBERG (1951) found that caffeic, ferulic and traumatic acid had a synergistic effect on β-indole acetic acid in the *Avena*-curvature test. This author found no effect with caffeic acid alone (at concentrations between 4.4–52 μg/ml), but these concentrations may have been too high to give a positive response. In the *Avena*-coleoptile-section test we found auxin activity only at concentrations between 3 and 0.01 μg/ml.

In peas the content of polyhydroxy cinnamic acids (ferulic, caffeic and chlorogenic acid) proved to be increased after the plants had been sprayed with a solution of gibberellic acid (KÖGL and ELEMA, 1960). According to these authors the polyhydroxy cinnamic acids would act as inhibitors of the IAA-oxydase system, and the "auxin sparing" effect of gibberellic acid would thus be an indirect one.

In our opinion it is not excluded that caffeic acid acts as a true auxin. This can be concluded from our experiments in which the elongation growth of the oat sections proved to be enhanced by caffeic acid while no other auxins were present in the surrounding fluid.

To rule out the possibility that caffeic acid acts synergistically with natural auxin, we will have to carry out experiments with auxin-starved coleoptile sections. In this way NEUMANN (1960) recently could prove that coumarin can act as a stimulator of elongation growth. However, the site of action of this growth regulator in the cells is thought to be different from that of IAA.

VELDSTRA and BOOY (1949) formulated the structural requirements for cell-elongation activity as:

a) a basal ring system (non polar part) with interface activity,
b) a carboxyl group (polar part), or, in general, a group of acidic character, in such spatial position with respect to the ring system that on adsorption of the active molecule to a boundary this functional group will be situated as peripherally as possible.

Introduction of hydrophilic substituents into the lipophilic nucleus would result in inactive or weakly active compounds, as in that way more symmetrically constructed hydrophilic substances are obtained (VELDSTRA, 1953).

Of the stereoisomeric cinnamic acids only *cis*-cinnamic acid is physiologically active (VELDSTRA, 1944).

With these considerations in mind, it is not to be expected that caffeic acid (3'4' dihydroxy cinnamic acid) would surpass cinnamic acid as a growth substance. The introduction of hydrophilic substituents into the lipophilic nucleus results in a more symmetrically constructed compound, and its auxin activity would thus be diminished. At this moment we had at our disposal only the commercially obtainable cinnamic acid and caffeic acid. They proved to be the more stable *trans* isomers. From these two acids only caffeic acid proved to be able to act as an auxin in our experiments. This means that the introduction of hydrophilic substituents (OH-groups) into the lipophilic nucleus results in a more active compound. Moreover, it points out that the *cis* configuration is not necessary for growth activity.

Experiments with the *cis* isomers are now in progress. In future we hope to obtain more details on the steric configuration of the natural caffeic acid and of related compounds, on their occurrence in different plant parts and on their physiological function in plant metabolism.

The use of chromatographic methods in auxin research has revealed the existence of many substances with auxin activity. Now, the question arises to what extent these substances may be identified with cinnamic acid and related compounds. We have found these compounds also in the neutral fraction of the alcoholic extract. This means that dividing an extract in an acid and a neutral fraction is not fully satisfactory.

SUMMARY

In the alcoholic extract prepared from leaves and stems of *Coleus rhenaltianus*, two substances were detected which in the *Avena*-coleoptile-section test showed auxin activity. These compounds fluoresced blue in ultra-violet light. One of the compounds could be identified with *trans*-caffeic acid. The other one is a related compound, giving the same absorption spectrum and colour reactions.

The physiological activity of *trans*-caffeic acid is discussed in connection with the activity of *trans*-cinnamic acid.

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