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METABOLISM OF PHENOLIC SUBSTANCES BY THE CHLOROPLASTS—III.*

PHENOLASE AS AN ENZYME CONCERNING THE FORMATION OF ESCULETIN

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Abstract—The chloroplasts of *Saxifraga stolonifera* are able to convert *cis*-caffeic acid to esculetin. This reaction is enzymic and the enzyme responsible is phenolase (*ortho*-diphenol: O_2 oxidoreductase, EC.1.10.3.1). An *ortho*-quinone produced from *cis*-caffeic acid by the enzyme may convert spontaneously to esculetin. The results present a new explanation for the first step in the formation of the lactone ring of esculetin.

INTRODUCTION

ESCULETIN (6,7-dihydroxycoumarin) was first found in the leaves and bark of horse-chestnut (*Aesculus hippocastanum*) as a glucoside,[1] and several plant tissues were later shown to contain esculetin or its glucoside.[2]

It has been shown that esculetin can be produced from *cis*-caffeic acid by a non-enzymic mechanism,[3,4] and it has been suggested that when esculetin is found in a plant extract which also contains caffeic acid, it might be formed from the *cis*-isomer of the latter during paper chromatographic procedures.[3]

During the course of the study on the metabolism of phenolic substances in *Saxifraga stolonifera*,[5,6] however, the present author observed that the chloroplasts of this plant converted *cis*-caffeic acid enzymically to esculetin.

It seems now to be clear that the first step in the biogenesis of natural coumarins is the introduction of a hydroxyl group *ortho* to the established side-chain of *trans*-cinnamic acids, as shown in studies on coumarin,[7–10] herniarin,[9,11] and umbelliferone.[11–13] Although no studies have been carried out on the biosynthesis of esculetin, it is believed that this coumarin is no exception and is formed from caffeic acid by the same step as for other coumarins.[14]

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The results of the present experiments, however, show that *cis*-caffeic acid is first oxidized to its *ortho*-quinone, which then spontaneously gives rise to esculetin, and that this is controlled by an enzyme of the phenolase type present in the chloroplasts.

RESULTS

When a *trans–cis* mixture of radioactive caffeic acid was incubated with an acetone powder of the leaves of *Saxifraga stolonifera*, a radioactive substance could be observed to arise, the

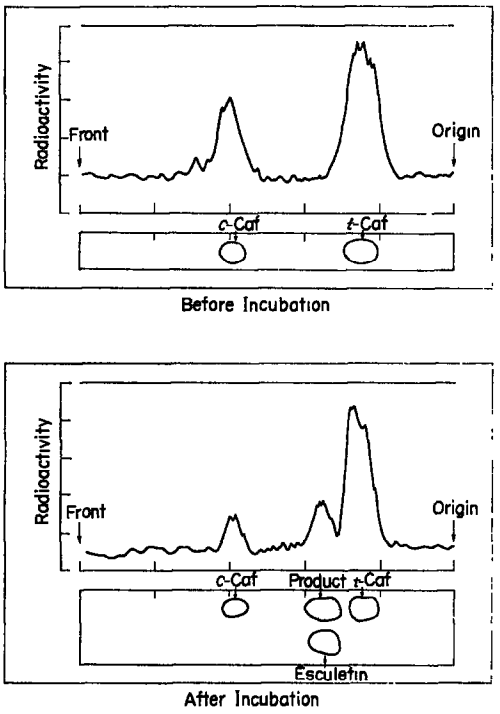

FIG. 1. SUBSTANCE FORMED WHEN *trans–cis* MIXTURE OF CAFFEIC ACID WAS INCUBATED WITH ACETONE POWDER OF THE LEAVES OF *S. stolonifera*.

1 ml of 0·1 M phosphate buffer (pH 6·8) containing 1·5 mg of *trans–cis* mixture of radioactive caffeic acid (see Experimental) and 1 ml of suspension of acetone powder (10 mg) were mixed and the mixture was incubated for 30 min at 30°. An aliquot of the reaction mixture (10 μl) was chromatographed on Whatman No. 1 paper using 5% acetic acid. Substances on the chromatogram were detected by u.v. lamp (maximal output at 360 nm) and by an automatic paper-chromatoscanner (Aloka 4π). Upper, immediately after mixing; lower, after 30 min incubation.

fluorescence and chromatographic behaviour of which were similar to esculetin (Fig. 1). The substance was isolated and some of its properties are shown in Table 1, showing it is apparently esculetin. The u.v. spectra of the product is identical with that of an authentic sample. A mixture of a small amount of the radioactive sample obtained by paper chromatography (amorph; *ca.* 2 mg) and unlabelled esculetin (15 mg) showed an approximately constant specific radioactivity (35·5, 38·2 and 39·9 mμc/mg, respectively) during three successive crystallizations.

Since esculetin can be formed non-enzymically from *trans*-caffeic acid via the *cis*-isomer,[3] it was doubtful whether the formation of esculetin was enzyme catalysed. In order to clarify

this point, esculetin-forming activity of an untreated powder was compared with that of a heat-treated one, and the result was shown in Fig. 2. Little esculetin was formed in the reaction mixture containing the heat-treated powder, while the production of esculetin by the unboiled

TABLE 1. SOME PROPERTIES OF THE PRODUCT

Solvent	R_f values: Product	R_f values: Esculetin
(*a*) 5% Acetic acid	0·34	0·34
(*b*) *n*-Butanol:acetic acid:water (4:1:2)	0·89	0·89
(*c*) Water-saturated butanol	0·84	0·84
(*d*) Ethanol:conc. ammonia:water (20:1:3)	0·34	0·34
Reagent	**Colour**	
(*a*) Alc. 1% ferric chloride	Green	Green
(*b*) 1% Phosphomolybdic acid	Yellow	Yellow
(*c*) Höpfner's reagent	Orange	Orange
(*d*) Diazotized *p*-nitroaniline	Orange	Orange
then treated with alkali	Brown	Brown
Fluorescence in u.v. light (360 nm)	White blue	White blue

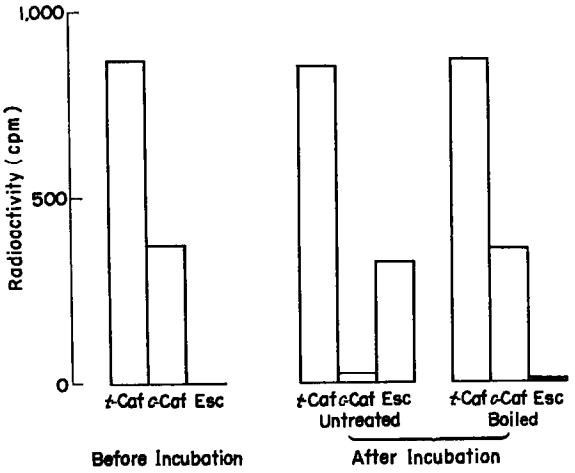

FIG. 2. EFFECT OF HEAT-TREATMENT ON THE FORMATION OF ESCULETIN.

The reaction mixture (see Fig. 1) was incubated for 3 hr at 30°. Boiled sample was obtained by heating a suspension of acetone powder at 100° for 10 min before mixing. Activity was expressed as cpm detected in 10 μl of the reaction mixture.

powder was fairly significant. A proportionality was observed between the amount of the powder used and the esculetin formed (Fig. 3), and this also indicates that esculetin formation is the enzyme catalysed.

It was clear that esculetin was derived from the *cis*-, not from the *trans*-isomer of caffeic acid. As can be seen in Figs. 1 and 2, the production of esculetin was accompanied by the

consumption of *cis*-caffeic acid, while the decrease of *trans*-isomer was not significant (a slight decrease of the latter isomer could be attributed to the action of phenolase; the activity of *trans–cis* isomerase which was found in *Melilotus alba*[15] could not be detected when a reaction mixture of *trans*-isomer and powder was incubated for 3 hr in the dark). A time-

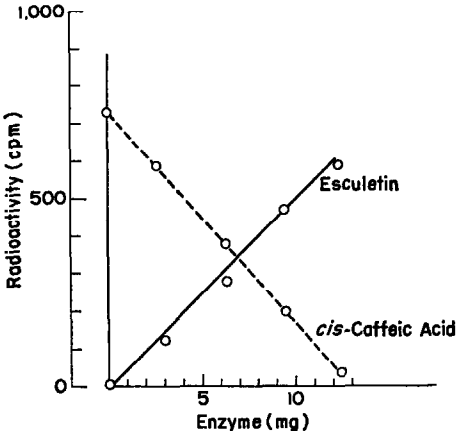

FIG. 3. THE RATE OF ESCULETIN FORMATION AND *cis*-CAFFEIC ACID UTILIZATION AS A FUNCTION OF ENZYME CONCENTRATION.

1 ml of substrate solution (1·5 mg of *trans-cis* mixture of caffeic acid in 0·1 M phosphate buffer, pH 6·8) and 1 ml of suspension containing various amount of acetone powder were mixed and the mixture incubated for 30 min at 30°. Activity is expressed as cpm detected in 20 μl of the reaction mixture.

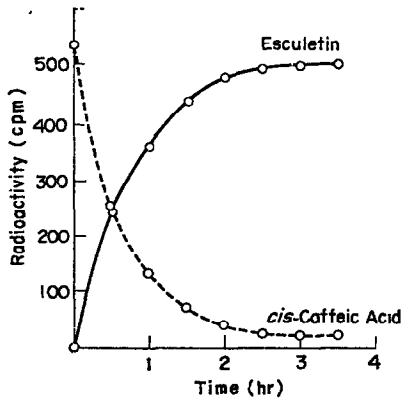

FIG. 4. THE TIME COURSE OF ESCULETIN FORMATION AND *cis*-CAFFEIC ACID UTILIZATION.

2 ml of substrate solution (3 mg *trans–cis* mixture of caffeic acid in 0·1 M phosphate buffer, pH 6·8) and 2 ml of suspension of acetone powder (10 mg) were mixed, and the reaction was run at 30°. Every 30 min, an aliquot (10 μl) was chromatographed and the radioactivity of both substances were measured.

course curve showed that esculetin production completely paralleled *cis*-caffeic acid consumption (Fig. 4).

The enzyme seems to be localized in the chloroplasts (Table 2). The protein fraction obtained from the cytoplasm of the leaves was not able to convert *cis*-caffeic acid to esculetin.

[15] J. R. STOCKER, *Biochem. Biophys. Res. Commun.* **14**, 17 (1964).

TABLE 2. CELLULAR DISTRIBUTION OF THE ESCULETIN-FORMING ENZYME

Fraction	Chlorophyll (μg from 1 g leaf)	Activity (cpm/20 μl)	Specific activity (cpm/μg chl.)
150 *g* for 2 min	61	187	3·1
1100 *g* for 5 min	192	610	3·2
15,000 *g* for 20 min	17	52	3·1

Reaction system consisted of 0·5 ml of substrate solution (1·5 mg *trans-cis* mixture of caffeic acid in 0·1 M phosphate buffer, pH 6·8) and 0·5 ml of suspension (corresponding to 1 g fresh leaves), and the reaction was run for 30 min at 30°. Activity was expressed as cpm detected in 20 μl of the reaction mixture.

To characterize the enzyme involved in this reaction, experiments were carried out using inhibitors. Of the metal-chelating reagents tested, 8-hydroxyquinoline, diethyldithiocarbamate and cyanide strongly inhibited the reaction. Substances which combine more or less specifically with ferrous ion (α,α′-dipyridyl and *o*-phenanthroline), however, did not cause any inhibition. The enzyme did not appear to have a SH group in the active site because *p*-chloromercuribenzoate and iodoacetate were not greatly inhibitory (Table 3).

TABLE 3. EFFECT OF INHIBITORS ON ENZYME ACTIVITY

Inhibitor (Final conc. $6·7 \times 10^{-4}$ M)	Esculetin formed (cpm, $\times 10^{-3}$)	Inhibition (%)
None (control)	1·79	—
Cyanide	0·10	95
8-Hydroxyquinoline	0·34	81
Sodium diethyldithiocarbamate	0·10	95
Thiourea	1·48	17
Ethylenediamine tetraacetic acid	1·41	21
α,α′-Dipyridyl	1·79	0
o-Phenanthroline	1·79	0
p-Chloromercuribenzoate	1·52	15
Monoiodoacetic acid	1·53	14

Reaction system consisted of 0·5 ml of 2×10^{-3} M inhibitor or water (none), 0·5 ml of substrate solution (1·5 mg *trans-cis*-mixture of caffeic acid in 0·1 M phosphate buffer pH 6·8) and 0·5 ml of suspension of acetone powder (5 mg). Reaction was run for 40 min at 30°. Esculetin was expressed as cpm detected in 20 μl of reaction mixture.

The reaction was completely suppressed in nitrogen, and it thus seems that molecular oxygen is indispensable for esculetin formation.

These results suggest that a copper enzyme, which could be presumed to be phenolase present in the chloroplasts of this plant,[5,6] might participate in the reaction. An addition of a small amount of ascorbic acid brought about a complete cessation of esculetin formation (Fig. 5).

The chloroplast fraction from *S. stolonifera* was blended in water, sodium cholate or Triton X-100, and the solubilized protein precipitated by acetone and collected by centrifugation. Its activities in both esculetin formation and as a catecholase were compared. As

shown in Table 4, the esculetin-forming enzyme could be solubilized by Triton X-100, and this fraction also contained a high catecholase activity.

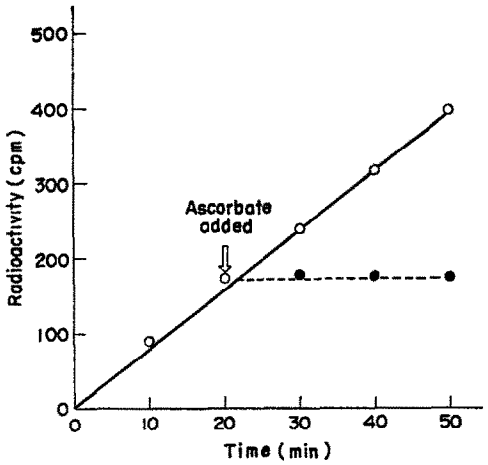

FIG. 5. EFFECT OF ASCORBIC ACID ON THE ESCULETIN FORMATION.

Reaction system consisted of 2 ml of substrate solution (3 mg of *trans-cis* mixture of caffeic acid in 0·1 M phosphate buffer, pH 6·8) and 2 ml of suspension of acetone powder (10 mg), and reaction was run at 30°. After 20 min, one part (1·5 ml) of the reaction mixture was transferred to another tube containing 1 mg ascorbic acid. Every 10 min an aliquot (10 μl) was taken for estimation. ○—without; ●—with ascorbic acid.

TABLE 4. SOLUBILIZATION OF THE ENZYME FROM THE CHLOROPLASTS

Fraction		Activity			
		Esculetin formation*	Catecholase†		
			Homocatechol	Chlorogenic acid	Caffeic acid
Whole chloroplasts		182	63	6	7
Water (10 min)	Extract	—	—	—	—
	Residue	240	74	9	8
0·5% Sodium cholate (10 min)	Extract	6	8	—	—
	Residue	226	74	8	8
0·5% Triton X-100 (10 min)	Extract	33	12	3	2
	Residue	197	59	8	9
1·0% Triton X-100 (30 min)	Extract	197	59	7	7
	Residue	68	11	6	1

* Reaction system consisted of 0·5 ml of substrate solution (1·5 mg *trans-cis* mixture of caffeic acid in 0·1 M phosphate buffer, pH 6·8) and 0·4 ml of enzyme (corresponding to 8 mg chloroplasts dry wt.), and the reaction mixture was incubated for 20 min at 30°. Activity was expressed as cpm in 10 μl from the reaction mixture.

† Activity was expressed as μl O_2 for 10 min. Conditions of the reaction, see Experimental.

Figure in parentheses, time for blending; —, activity negligible.

The presumption that phenolase takes part in esculetin formation was further supported by the fact that in several ammonium sulphate saturated fractions, phenolase (tyrosinase) prepared from the common mushroom, the activity of esculetin formation appeared to parallel catecholase activity (Table 5).

TABLE 5. ESCULETIN FORMATION BY MUSHROOM PHENOLASE

Fraction (fractionation at $(NH_4)_2SO_4$ saturation)	Activity			
	Esculetin formation†	Catecholase*		
		Homocatechol	Chlorogenic acid	Caffeic acid
–0·36	24	36	18	14
0·36–0·41	31	58	33	26
0·41–0·46	79	103	58	65
0·46–0·52	108	130	61	81
0·52–0·60	28	66	25	12
0·60–0·66	20	60	20	10

* A mixture of 0·5 mg *trans-cis* caffeic acid in 0·5 ml of 0·1 M phosphate buffer, pH 6·8, and 0·2 ml of enzyme solution (100 times dilution of the original solution) was incubated for 10 min at 30°. Activity was expressed as cpm in 10 μl of the reaction mixture.

† Reaction system consisted of 0·5 ml of 0·01 M substrate, 0·5 ml of 0·1 M phosphate buffer, pH 6·8, and 0·5 ml of enzyme solution (20 times dilution of the original solution). Activity was expressed as μl O_2 uptaken for 10 min.

The ability of the saxifrage enzyme to form coumarins from *cis*-isomers of other cinnamic acids was examined and it was found that none of the cinnamic acids tested (cinnamic acid, *p*-coumaric acid and ferulic acid) gave rise to the corresponding coumarins. It must be remarked that the leaves of *S. stolonifera* contain neither esculetin nor free caffeic acid, although chlorogenic acid is present.

DISCUSSION

From the results shown above, it is clear that the chloroplasts of *Saxifraga stolonifera* are able to convert *cis*-caffeic acid to esculetin.

Of the several theories proposed on the mechanism of lactone ring formation of natural coumarins,[7–13,16–18] the following seems now to be plausible. *Ortho*-hydroxylation and glucosylation take place in *trans*-cinnamic acid or its derivatives to give a product, which is isomerized, probably by sunlight, then hydrolysed by a specific glucosidase and finally lactonizes by spontaneous dehydration. This scheme appears to be more or less well established in the biogenesis of such coumarins as coumarin itself,[7–10] herniarin[9,11] and umbelliferone.[11–13] Although there have been no reports on the biogenesis of esculetin, it has been believed that this coumarin can arise by steps analogous to those shown above.[14] In this connexion, it is of interest that Butler and Siegelman[3] showed a non-enzymic mechanism of esculetin formation from *trans*-caffeic acid via its *cis*-isomer. They assumed that the formation of esculetin from *cis*-caffeic acid could go through an *ortho*-hydroxylated compound or through an *ortho*-quinone and suggested that the former was more likely (Fig. 6).

The present study, however, shows that the first step in esculetin formation is not the production of an *ortho*-hydroxylated substance but an *ortho*-quinone of the *cis*-caffeic acid. Esculetin formation was completely blocked in the presence of ascorbic acid which can reduce *ortho*-quinones to the corresponding *ortho*-diphenols. Furthermore when heavy metal ions

[16] R. D. HOWORTH, *J. Chem. Soc.* 448 (1942).
[17] H. GRISEBACH and W. D. OLLIS, *Experientia* **17**, 4 (1961).
[18] C. A. BUNTON, G. W. KENNER, M. J. T. ROBINSON and B. R. WEBSTER, *Tetrahedron* **19**, 1001 (1963).

are present, ascorbic acid can also promote the production of *ortho*-hydroxylated substances.[19–20] The fact that the *cis*-isomers of cinnamic acids other than caffeic acid (cinnamic, *p*-coumaric and ferulic acids) were not converted to its corresponding coumarin by the enzyme showed that the presence of an *ortho*-dihydroxyl group is necessary for esculetin formation.

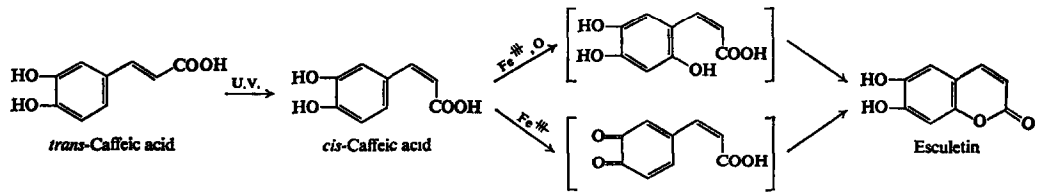

FIG. 6. PROPOSED SCHEME OF NON-ENZYMIC TRANSCONVERSION OF *trans*-CAFFEIC ACID TO ESCULETIN (BY BUTLER AND SIEGELMAN[3]).

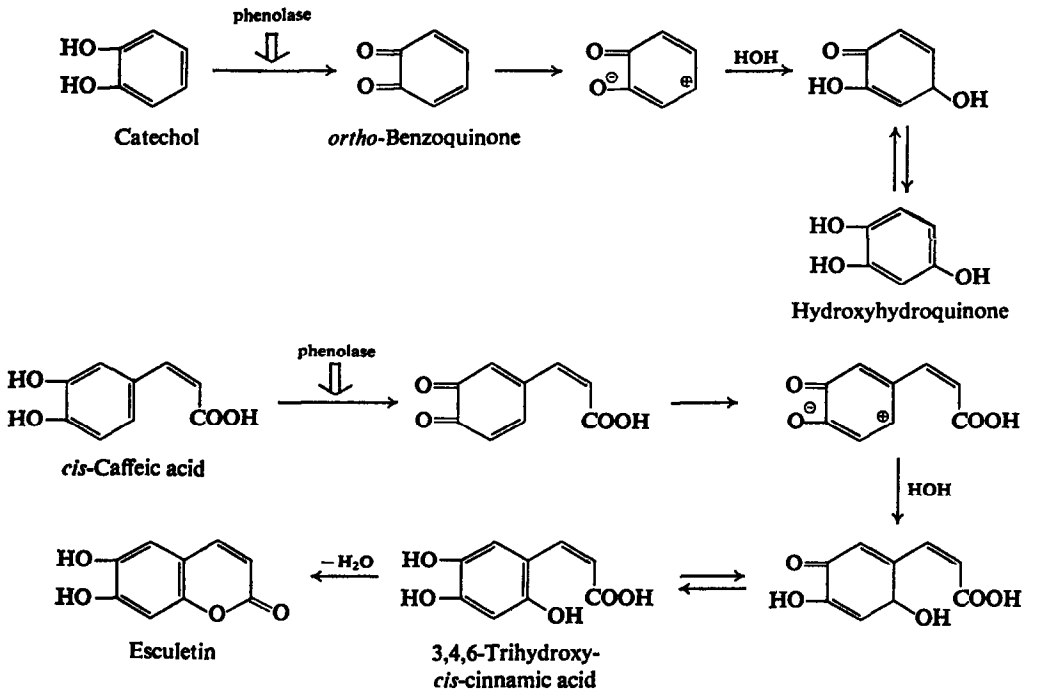

FIG. 7. PROPOSED SCHEME OF FURTHER CHANGE OF *ortho*-BENZOQUINONE (BY NELSON AND DAWSON), AND SCHEME OF TRANSCONVERSION OF *cis*-CAFFEIC ACID INTO ESCULETIN (BELOW).

The above facts, together with the information obtained from the inhibitor experiments, strongly suggest that the reaction was controlled by an enzyme of the phenolase type. The enzyme could be dissociated effectively from chloroplast preparations by Triton X-100, and in this fraction a high catecholase activity was also found. Chloroplast phenolase has been shown to be not easily solubilized, and Triton X-100 has been used for its solubilization.[21–22]

[19] R. O. C. NORMAN and G. K. RADDA, *Proc. Chem. Soc.* 138 (1962).
[20] J. H. GREEN, B. J. RALPH and P. J. SCHOFIELD, *Nature* **198**, 754 (1963).
[21] E. HARREL, A. M. MAYER and Y. SHAIN, *Phytochem.* **4**, 783 (1965).
[22] U. R. L. WALKER and A. C. HULME, *Phytochem.* **5**, 259 (1966).

The presumption that phenolase would catalyse esculetin formation was supported by the fact that phenolase (tyrosinase) prepared from common mushroom did produce esculetin from *cis*-caffeic acid, and in several ammonium sulphate saturated fractions esculetin formation paralleled catecholase activity.

It is clear that the first step in the formation of esculetin from *cis*-caffeic acid is the formation of the *o*-quinone catalyzed by an enzyme of the phenolase type. Two possible ways may be envisaged for the subsequent change of this intermediate; one, as pointed out by Butler and Siegelman, is a Michael addition β to the carbonyl group followed by lactonization (this was considered by these authors to be less likely, because Michael additions do not generally occur under mild condition); two, involves the formation of a hydroxyhydroquinone. It has been suggested that by the action of phenolase, phenols of the catechol type are first oxidized to *ortho*-benzoquinones and the latter then converted to hydroxyhydroquinones (Fig. 7).[23–24]

The phenolase of the chloroplasts of this plant, however, could not have any physiological significance in coumarin biosynthesis so far as this plant is concerned, because it does not contain esculetin or its possible precursor, caffeic acid.

MATERIAL AND METHODS

Acetone Powder

Fresh leaves of *Saxifraga stolonifera* (harvested from August till October; no activity could be detected in the acetone powder of the leaves harvested before July) were previously frozen in cold acetone (−30°) and blended in a mixer. The homogenate was filtered and the residue rehomogenized in acetone and filtered several times to obtain finally a finely ground powder. It was dried in the air and further in a desiccator. The yield from 100 g fresh leaves was 5·4 g. The esculetin-forming activity could be detected in the powder stored in a freezer (−20°) for 6 months or so.

Cellular Fractionation

Leaves (20 g) were blended in a cold medium (150 ml) containing 0·4 M sucrose, 0·05 M Tris-HCl buffer (pH 7·8) and 0·01 M NaCl. The homogenate was filtered through silk, and the filtrate centrifuged at 12,000 *g* for 20 min. The pellet was suspended in the same buffer (100 ml) and the suspension successively centrifuged for 2 min at 150 *g*, for 10 min at 1100 *g* and for 20 min at 12,000 *g*. The cytoplasmic protein fraction was prepared as described previously.[5]

Solubilization of the Enzyme from the Chloroplast Fraction

The pellet (130 mg in dry; corresponded to 10 g fresh leaves) obtained from the 1100 *g* centrifugation was suspended in 10 ml of water, 0·5% sodium cholate, 0·5% or 1% Triton X-100 (in 0·05 M phosphate buffer, pH 6·8) and the suspension was gently blended in a homoblender at 2° for 10 min or 30 min. The homogenate was centrifuged for 30 min at 15,000 *g*, giving the supernatant (extract) and residue fractions. The extract was dialysed against two changes of water (2 l.) for 48 hr. To the dialysate was added 3 vol. of cold acetone (−25°) and after standing for 30 min the protein was collected by centrifugation. It was dissolved in 2 ml of cold water and dialysed for 24 hr. The residual fraction of the above centrifugation was washed with water twice. Each fraction was made up in 5 ml of water.

Preparation of Mushroom Tyrosinase (Phenolase)

This was prepared from 1 kg of common mushroom according to steps 1–5 of the method of Bouchilloux *et al.*[25] Protein fractions obtained by step 5 (ammonium sulphate fractionation) were dissolved in 5 ml of water.

Trans–cis *Mixture of Caffeic Acid*

Since *trans*- and *cis*-caffeic acids are readily interconverted under ordinary experimental conditions (especially in the light[26]), a mixture of both isomers was prepared as substrate for semi-quantitative assay of

[23] J. R. NELSON and C. R. DAWSON, *Advan. Enzymol.* **4**, 99 (1944).
[24] H. DRESSLER and C. R. DAWSON, *Biochim. Biophys. Acta* **45**, 515 (1960).
[25] S. BOUCHILLOUX, P. MCMAHILL and H. S. MASON, *J. Biol. Chem.* **238**, 1701 (1963).
[26] J. S. CHALLICE and A. H. WILLIAMS, *J. Chromatog.* **21**, 357 (1966).

the enzyme activity. *Trans*-caffeic acid-2-^{14}C (specific radioactivity 0·27 μc/mg) was dissolved in ethanol (1 mg in 1·5 ml) and the solution irradiated under 20 W u.v. lamp (maximal output at 360 nm) from a distance of 10 cm for 30 min. The resulting solution was dried and the residue dissolved in an appropriate volume (in general 1·5 mg in 1 ml) of 0·1 M phosphate buffer (pH 6·8; a preliminary experiment showed the activity was highest in the pH range from 6·5 to 7·0). The solution contained *ca.* 35% of *cis*-caffeic acid.

Conditions of the Reaction

To the substrate solution prepared as above (1·5 mg/ml), an appropriate volume of enzyme or suspension of acetone powder (5 mg/ml) (see figures or tables) was added and the mixture was shaken at 30°.

Measurement of Radioactivity

After the reaction was over, the mixture was boiled for 5 min and when powder, chloroplasts or chloroplast fragments were used the mixture was allowed to stand for 10 min before an aliquot (10 or 20 μl) was taken and applied to Whatman No. 1 paper as a band and immediately chromatographed with 5% acetic acid. The substances were located under the u.v., and pieces of the corresponding bands were placed in planchets, and the radioactivity directly measured with a counter (Aloka low-background β counter). Values (average of three estimations) were expressed as cpm detected in 10 μl (or 20 μl) taken from the reaction mixture.

Radiochromatogram

Activity was measured directly on chromatograms with an Aloka 4π chromatoscanner.

Isolation of the Product

100 mg *trans–cis* mixture of caffeic acid were taken in 80 ml of 0·1 M phosphate buffer (pH 6·8) and 200 mg acetone powder from the leaves of *S. stolonifera* were added. The mixture was incubated for 3 hr at 30°. The mixture was boiled for 5 min, filtered, and the filtrate acidified with HCl to pH 2·0. The phenolic compounds were extracted with 100 ml of ethyl acetate. The extract was concentrated to dryness, and the residue dissolved in 100 ml of water. The pH was brought to 8·0 with dil. NaOH, and extraction with an equal volume of ether was repeated five times. The combined ether extracts were evaporated to dryness and the residue dissolved in dilute ethanol (charcoal) on a water bath. After standing overnight, a crude crystalline solid was obtained. Although further purification was unsuccessful because of the shortage of material, the sample gave a single spot on paper chromatograms which corresponded to that of authentic esculetin.

Chemicals

Radioactive *trans*-caffeic acid-2-^{14}C was synthesized from radioactive malonic acid-2-^{14}C and 3,4-dihydroxybenzaldehyde;[27] specific activity 0·27 μc/mg (48·6 μc/m mol). Esculetin was prepared by the method of Pechmann for the synthesis of umbelliferone.[28]

Paper Chromatography

Whatman No. 1 filter paper was used with 5% acetic acid. Other solvents were also used for the identification of the product: (*a*) *n*-butanol:acetic acid:water (4:1:2, by volume); (*b*) water-saturated *n*-butanol; (*c*) ethanol:conc. ammonia:water (20:1:3, by volume). Detecting reagents were (*a*) alcoholic 1% ferric chloride; (*b*) 1% phosphomolybdic acid; (*c*) Höpfner's reagent;[29] (*d*) diazotized *p*-nitroaniline.[30]

Measurement of Catecholase Activity

This was carried out by the manometric method. 0·5 ml each of enzyme solution, 0·1 M phosphate buffer (pH 6·8) and 0·01 M substrate were incubated at 30°.

Estimation of Chlorophyll

This was done according to the literature.[31]

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Examination of the Phenolic Substances in the Leaves of S. stolonifera

10 g of fresh leaves of this plant were extracted three times with 100 ml of 80% isopropanol on a boiling water bath. The combined extracts were concentrated to dryness, and water-soluble substances in the residue were taken in 20 ml of hot water. The water solution was further concentrated to about 1 ml, and an aliquot was chromatographed with 5% acetic acid in the first direction and with *n*-butanol:acetic acid:water (4:1:2) in the second. The chromatogram was examined with the reagent (*d*).

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