5-Dehydroshikimate Reductase in the Tea Plant (*Camellia sinensis* L.)

PROPERTIES AND DISTRIBUTION

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1. A method for the extraction of 5-dehydroshikimate reductase (EC 1.1.1.25) from tea plant tissues in an active soluble state has been developed. It is dependent on the use, in the extraction medium, of an insoluble polyphenol adsorbent (Polyclar AT), which prevents the polyphenols present from precipitating all the proteins. 2. The enzyme has the following properties: pH optima at pH 10.1 in glycine-sodium hydroxide buffer and at pH 7.7 in tris-hydrochloric acid buffer; K_m (NADP) $32\mu\text{M}$ and K_m (shikimate) 0.43mM ; and NADP-specificity. It was completely inhibited by 0.33mM -*p*-chloromercuribenzoate and this inhibition was completely reversed by 10mM -cysteine. Iodoacetate and arsenite inhibited the enzyme to a smaller extent. 3. The specific activity of the enzyme was higher in the parts of the actively growing shoot tips (third leaf > stem > second leaf > first leaf > bud) than in the mature leaves. However, the mature leaves had the greatest total activity. 4. The importance of these findings with respect to flavanol biosynthesis in tea plants is discussed.

The shikimic acid pathway (Neish, 1960, 1964) is the sole route by which aromatic compounds are formed in *Escherichia coli* (Davis, 1955). However, in higher plants the importance of this pathway in the biosynthesis of aromatic compounds is not yet clear. Shikimic acid has been found in many plants and it is a good precursor of a wide variety of aromatic compounds in higher plants, but there is also evidence for the operation of other pathways in these organisms (Neish, 1960, 1964). Enzymic studies should help to clarify this point, but very few reports on this subject have appeared (Conn, 1964).

Plant tissues that would presumably contain the highest concentration of the enzymes involved in the biosynthesis of aromatic compounds, namely those containing high concentrations of these compounds, are the most refractory for enzyme studies because of the protein-denaturing properties of such compounds (Sanderson, 1965*a*). However, techniques have recently been developed that enable soluble enzymes to be extracted from such tissues (Loomis & Battaile, 1964; Sanderson, 1964; Badran & Jones, 1965) and these techniques have now been applied to extraction of enzymes of the shikimic acid pathway from tea plants.

Flavonoid compounds usually comprise 20–35% of the dry weight of actively growing shoot tips of the tea plant (*Camellia sinensis* L.) (Vuataz, Brandenberger & Egli, 1959) and they are of great economic importance in these tissues because of their dominant role in the manufacture of black

tea (Roberts, 1962; Sanderson, 1965*b*). The possible importance of shikimic acid in the biosynthesis of flavanols in tea plants has been shown with [^{14}C]shikimic acid (Zaprometov, 1962; Zaprometov & Bukhlaeva, 1963), but enzymic studies would be useful for a fuller understanding of the biosynthesis of these compounds in these plants. In the present paper the occurrence, properties and distribution of 5-dehydroshikimate reductase (EC 1.1.1.25) in tea plants are described.

MATERIALS AND METHODS

Chemicals. NAD, NADP, NADH_2 , NADPH_2 and tris were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Shikimic acid, D-catechin and glycine were purchased from Calbiochem A.-G., Lucerne, Switzerland. Polyclar AT (an insoluble cross-linked polyvinylpyrrolidone) was purchased from Antara Chemicals Division of General Aniline and Film Corp., New York, N.Y., U.S.A. All other chemicals were purchased from British Drug Houses Ltd., Poole, Dorset.

Source of plant material. This was taken from blocks of clonal tea growing in fields adjoining this Laboratory (1500 m. elevation). Rapidly growing shoot tips, comprising the bud, first two leaves and the included stem, from mature plants in plucking (cf. Eden, 1958; Harler, 1963) of clone TRI.777 were used in all experiments except as otherwise noted.

Preparation of enzyme extracts. Fresh shoot tips (5.0 g.) were frozen at -15° for 1 hr. The frozen shoot tips were ground with 3.0 g. of Polyclar AT, about 2.5 g. of acid-

washed sand and 50ml. of 0.1M-sodium phosphate buffer, pH7.0. The homogenate was sieved through muslin cloth and the filtrate was centrifuged at 3000g for 20min. The supernatant solution was used as enzyme preparation. All these operations were carried out in a cold room at 4°.

Assay of 5-dehydroshikimate reductase. The assay was carried out as described by Balinsky & Davies (1961a) by spectrophotometrically following the increase in extinction at 340m μ due to the production of NADH₂ according to the following reaction:

The standard assay was run in silica cuvettes with a 1.0 cm. light-path at room temperature (about 22°). The reaction mixture contained shikimic acid (1.0 mm) and NADP (170 μ M) in 2.5 ml. of 0.1M-glycine-NaOH buffer, pH10.1, 0.1 ml. of enzyme preparation (0.08–0.11 mg. of protein) and 0.4 ml. of water.

Protein determination. Protein was precipitated at 4° with 5% (w/v) trichloroacetic acid overnight. The precipitate was collected, washed with 5% (w/v) trichloroacetic acid, and nitrogen was determined by a micro-Kjeldahl procedure (Bailey, 1962). The factor 6.25 was used to convert nitrogen content into protein content.

Flavanol determination. Flavanols were determined by the diazotized sulphanilic acid method described by Nakagawa & Torii (1964). D-Catechin was used as standard.

Units of enzyme activity and calculations. One unit of activity is defined as that amount of enzyme catalysing the oxidation of 1 μ mole of shikimic acid (or the reduction of 1 μ mole of NADP)/min. Specific activity is defined as units of activity/mg. of protein (*Report of the Commission on Enzymes of I.U.B.*, 1961). A value of 6.22×10^6 (Horecker & Kornberg, 1948) for the millimolar extinction coefficient, ϵ_{mM} , was used to convert E_{340} readings into NADPH₂ concentrations.

RESULTS

Effect of the amount of Polyclar AT on the extraction of soluble 5-dehydroshikimate reductase from tea shoot tips. Shoot tips were homogenized together with various proportions of Polyclar AT to determine the optimum amount for the extraction of soluble enzyme. Maximum activity was obtained with 0.6 g. of Polyclar AT/g. fresh wt. of tissue. This coincided with the amount of Polyclar AT at which maximum protein and minimum flavanols were extracted (Table 1).

All enzyme preparations contained some flavanols (Table 1), although when 0.6 g. or more of Polyclar AT/g. fresh wt. of tissue was used the concentration of flavanols was very low. Balinsky & Davies (1961b) have reported that polyphenolic compounds similar to those occurring in tea shoot tips (Roberts, 1962) inhibit the 5-dehydroshikimate reductase extracted from peas, so it was desirable to determine the effect of removing these residual polyphenols from the preparations. Accordingly, the original extracts were treated with additional amounts of Polyclar AT as explained in Table 1. This second treatment with Polyclar AT was effective in removing residual flavanols without affecting the protein concentration in these extracts. Further, the enzyme activity was increased in the two extracts containing significant amounts of both protein and flavanols, namely the extracts made with 0.2 and 0.4 g. of Polyclar AT/g. fresh wt. of tissue. After removing the enzyme-inhibiting polyphenols the specific activities of all active

Table 1. *Effect of Polyclar AT on the extraction of soluble 5-dehydroshikimate reductase from tea shoot tips*

Extraction of tea shoot tips was carried out as described in the Materials and Methods section except that the amount of Polyclar AT in the extraction medium was varied as shown below. These extracts are the original extracts. Portions of the original extracts were treated with additional Polyclar AT for 30 min. at 4°. After this treatment these latter solutions were centrifuged at 3000g for 20 min. at 4° and the supernatants are denoted as Polyclar-treated extracts. All assays and analyses were carried out as described in the Materials and Methods section.

Amount of Polyclar AT in extraction medium (g./g. fresh wt. of tissue)	Amount of Polyclar AT used to treat original extracts (g./g. fresh wt. of tissue)	Protein (mg./ml.)		Flavanols (μ g./ml.)		Total activity (unit/ml.)		Specific activity (unit/mg. of protein)	
		Original extract	Polyclar-treated extract	Original extract	Polyclar-treated extract	Original extract	Polyclar-treated extract	Original extract	Polyclar-treated extract
0	0.5	0.01	0.01	2270	11	—	0	—	0
0.1	0.4	0.05	0.05	525	0	0	0	0	0
0.2	0.3	0.36	0.36	110	0	0	0.05	0	0.14
0.4	0.1	0.84	0.84	41	0	0.10	0.13	0.12	0.15
0.6	0.1	1.06	1.06	23	0	0.16	0.15	0.15	0.14
0.8	0.1	1.21	1.21	24	0	0.15	0.16	0.12	0.13
1.0	0.1	1.26	1.26	22	0	0.16	0.15	0.13	0.12

preparations were the same except those made with large amounts (more than 0.6 g./g. fresh wt. of tissue) of Polyclar AT. This decrease in specific activity when large amounts of Polyclar AT are used has been noted repeatedly but the cause is not known.

These results generally agree with those obtained previously for Polycaprolaktam powder in the extraction of soluble catechol oxidase from these same tissues (Sanderson, 1964), except that Polyclar AT is about twice as effective on a dry-weight basis as Polycaprolaktam in adsorbing polyphenolic compounds and thereby preventing them from precipitating the proteins.

Effect of time and enzyme concentration. Experiment showed that ΔE_{340} was proportional with time up to at least 7 min. and with enzyme concentration up to at least 65 $\mu\text{g.}$ of protein/ml. of reaction mixture under the conditions of the standard assay described in the Materials and Methods section. There was no reaction when the enzyme preparation was heated in a boiling-water bath for 3 min. before the assay.

Requirement for substrate and cofactors: activity of related enzymes. When shikimate or NADP was omitted from the standard reaction mixture, no change in E_{340} occurred. Also, no change in E_{340} occurred when quinate was substituted for shikimate in the presence of either NAD or NADP. Neither NADH_2 nor NADPH_2 was oxidized by these enzyme preparations when they were substituted for NADP in the standard reaction mixture.

Fig. 1. Effect of pH and buffer composition on 5-dehydroshikimate-reductase activity. Enzyme activity was determined as described in the Materials and Methods section except that the buffer used in the reaction mixture was varied as shown: ●, 0.1M-tris-HCl buffers; ○, 0.1M-glycine-NaOH buffers. The enzyme preparation contained 0.94 mg. of protein/ml. and its specific activity was 0.15 unit/mg. of protein at pH 10.1.

Effect of pH and buffer composition. The standard assay was modified by substituting 0.1M-glycine-sodium hydroxide or 0.1M-tris-hydrochloric acid buffers at various pH values for the 0.1M-glycine-sodium hydroxide buffer, pH 10.1, normally used. A sharp pH optimum occurs at pH 10.1 in 0.1M-glycine-sodium hydroxide buffer (Fig. 1). However, in 0.1M-tris-hydrochloric acid buffer the pH optimum is at pH 7.7.

Effect of changes in concentration of NADP and shikimic acid. The effects of varying separately the concentrations of NADP and shikimic acid in the

Fig. 2. Reciprocal plot of the effect of NADP concentration on 5-dehydroshikimate-reductase activity. Enzyme activity was determined as described in the Materials and Methods section except that the NADP concentration was varied as shown.

Fig. 3. Reciprocal plot of the effect of shikimate concentration on 5-dehydroshikimate-reductase activity. Enzyme activity was determined as described in the Materials and Methods section except that the shikimate concentration was varied as shown.

standard assay system were measured and the results are presented as reciprocal plots (Lineweaver & Burk, 1934) in Figs. 2 and 3. The K_m for NADP was $32\mu\text{M}$ and K_m for shikimic acid was 0.43mM .

Effect of various compounds. The effect of several compounds on 5-dehydroshikimate reductase is shown in Table 2. *p*-Chloromercuribenzoate was an effective inhibitor of the enzyme and this inhibition could be completely reversed by cysteine. These results suggest that the enzyme contains essential thiol groups. Two other reagents for thiol groups, namely arsenite and iodoacetate, inhibited the enzyme to a much smaller extent.

Distribution of 5-dehydroshikimate reductase in the tea plant. Mature tea plants in plucking (Eden, 1958; Harler, 1963) were sampled to determine the distribution of the enzyme. The following samples, which were taken simultaneously, were

collected: shoot tips, which were dismembered into (1) buds, (2) first leaves, (3) second leaves, (4) third leaves and (5) stems; (6) maintenance leaves, which are the mature leaves in whose axils the shoot tips to be plucked arise; (7) 'old' leaves, which are mature leaves found within the tea bush below the plucking table. All samples were extracted as described in the Materials and Methods section by using 0.6 g. of Polyclar AT/g. fresh wt. of tissue.

Table 3 shows that all parts of the plant sampled had appreciable amounts of 5-dehydroshikimate-reductase activity. The specific activity in both samples of mature leaves was low, but because of their relatively large size they still contained more total activity than the parts of the shoot tips.

DISCUSSION

5-Dehydroshikimate reductase has been extracted from *E. coli* (Yaniv & Gilvarg, 1955), etiolated pea epicotyls (Balinsky & Davies, 1961a) and mung-bean seedlings (Nandy & Ganguli, 1961). These enzymes were purified 9.5-, 68- and 6.5-fold, to obtain preparations with specific activities 0.08, 0.23 and 0.13 unit/mg. of protein respectively. The specific activity of the crude enzyme preparations obtained from tea shoot tips compared favourably with specific activities of the most pure of these earlier preparations (Table 4). Table 4 shows that the four enzymes are indeed very similar.

The distribution of 5-dehydroshikimate reductase in the tea plant is of particular interest because of its possible importance in the biosynthesis of flavonoid compounds. Zaprometov (1962) and Zaprometov & Bukhlaeva (1963) have shown that shikimic acid is readily incorporated into the flavanols of tea shoot tips in experiments with [^{14}C]shikimic acid. In addition, Zaprometov (1963)

Table 2. *Effect of various compounds on 5-dehydroshikimate-reductase activity*

Assays were carried out as described in the Materials and Methods section. Compounds to be tested were incubated with the enzyme in the 0.1M-glycine-NaOH buffer, pH10.1, for 5 min. before adding shikimic acid and NADP.

Compound added	Final concn. in reaction mixture (mM)	Activity (% of original)
None	—	100
EDTA	5	115
Potassium cyanide	1	75
Cysteine	10	105
<i>p</i> -Chloromercuribenzoate	0.33	0
<i>p</i> -Chloromercuribenzoate + cysteine	0.33 } 10	100
Arsenite	1	75
Iodoacetate	1	70

Table 3. *Distribution of 5-dehydroshikimate reductase in the tea plant*

All methods used were as outlined in the Materials and Methods section. The samples are described in the text.

Part of plant sampled	Fresh wt. of part (mg.)	Total activity (units/g. fresh wt. of tissue)	Specific activity (unit/mg. of protein)	Total activity/plant part (% of activity in second leaf)
Buds	53	1.15	0.12	21
First leaves	88	1.51	0.15	46
Second leaves	166	1.73	0.18	100
Third leaves	212	1.74	0.27	129
Stems	150	1.04	0.22	54
Maintenance leaves	525	1.09	0.07	199
'Old' leaves	867	0.38	0.05	115

Table 4. Comparison of the properties of 5-dehydroshikimate reductase extracted from *E. coli*, pea epicotyls, mung-bean seedlings and tea shoot tips

Enzyme property	<i>E. coli</i> (Yaniv & Gilvarg, 1955)	Pea epicotyls (Balinsky & Davies, 1961a)	Mung-bean seedlings (Nandy & Ganguli, 1961)	Tea shoot tips (this paper)
Sp. activity in crude extracts	0.008	0.003	0.020	0.15–0.27
Sp. activity in purified preparation	0.078	0.23	0.13	—
pH optimum	8.5	10.0	8.0	10.1
K_m (NADP)	31 μ M	7 μ M	117 μ M	32 μ M
K_m (shikimate)	0.06 mM	0.23 mM	0.09 mM	0.43 mM
NADP-specificity	Yes	Yes	Yes	Yes
Inhibition by <i>p</i> -chloromercuribenzoate	—	92% by 2.5 mM	100% by unspecified concn.	100% by 0.33 mM
Inhibition by iodoacetate	—	16% by 10 mM	—	30% by 1 mM

has shown that photosynthetically assimilated $^{14}\text{CO}_2$ is in large part incorporated into flavanols in the young (first, second and third) leaves but only in small part in the old (fifth and sixth) leaves. The present findings support those of the Russian workers in two ways: (1) an enzyme required for the operation of the shikimic acid pathway in tea plants has been demonstrated; (2) variations in specific activity of 5-dehydroshikimate reductase in tea plants would be expected to produce the pattern of labelling from photosynthetically assimilated $^{14}\text{CO}_2$ that they found.

Now that a method for the extraction of active soluble enzymes from tissues containing high concentrations of protein-precipitating polyphenols has been developed, it should be possible to detect and assay other enzymes in tea plants and so to answer many of the questions about flavanol biosynthesis.

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