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BIOSYNTHESIS OF FLAVAN-3-OLS BY LEAF EXTRACTS OF *ONOBRYCHIS VICIIFOLIA**

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Key Word Index—*Onobrychis viciifolia*; Leguminosae; sainfoin; biosynthesis; reductases; condensing step; dihydroflavanols; flavan-3-ols; proanthocyanidins; condensed tannin.

Abstract—A subcellular enzyme fraction ($M_r > 20 \times 10^6$) from immature sainfoin leaves catalysed the two-step NADPH-dependent reduction of (+)-dihydromyricetin to (+)-gallocatechin. No enzyme-mediated (−)-epigallocatechin was formed under the conditions employed, and only traces of 2*R*,3*S*-*trans*-3*S*,4*S*-*cis*-leucodelphinidin were observed. The two-step reductase activity mirrored proanthocyanidin content, rising to a maximum before leaflet unfolding, then declining during leaf expansion. When [^{14}C](+)-dihydromyricetin and [C_4-^{3}H]2*R*,3*S*-*trans*-3*S*,4*S*-*cis*-leucodelphinidin were supplied to the same fraction, preferential utilization of (+)-dihydromyricetin was observed. Enzymic formation of proanthocyanidin dimers could not be detected using these preparations under a variety of conditions. However, a small portion of radioactivity was bound to the subcellular fraction after addition of labelled (+)-catechin. In addition, *in vivo* incorporation of (+)-dihydromyricetin into proanthocyanidins was observed. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Proanthocyanidin polymers (condensed tannins) are widespread plant natural products composed of linear or branched C_4–C_8 or C_4–C_6 linked chains of flavan-3-ol units [1, 2]. They exhibit considerable diversity in their molecular weight distribution and hydroxylation pattern. Current understanding of the biosynthesis of proanthocyanidins is based on *in vivo* radiolabelling data [3–5], cell-free enzymology in Douglas fir cell cultures, barley testa/pericarp and sainfoin (*Onobrychis viciifolia* Scop.) leaves [4, 6, 7], chemical synthesis [8–10] and analysis of proanthocyanidin polymer structure [3, 11]. Enzymological results have shown that 2*R*,3*S*-*trans*-flavan-3-ols are derived from (+)-dihydroflavanols by the sequential action of two classes of NADPH-dependent reductases (Fig. 1). The first class, comprised of dihydroflavanol reductases, is common to both anthocyanin and proanthocyanidin biosynthesis. Representative dihydroquercetin reductase proteins and genes have been isolated and characterized both biochemically and genetically from several sources [12–15]. The second, comprising leucoanthocyanidin reductases [6, 16, 17], is unique to 2*R*,3*S*-*trans*-flavan-3-ol biosynthesis; however, full enzyme purification, biochemical characterization and gene isolation have not been reported.

It has been suggested that 2*R*,3*R*-*cis*-flavan-3-ols are derived *in vivo* from reduction of the corresponding 2*R*,3*S*-*cis*-dihydroflavonols [18, 19] and that they may arise because of symmetric flav-3-ene-3-ol intermediates [3] or the action of epimerases [18]. However, *in vitro* biosynthesis of 2*R*,3*R*-*cis* flavanols has not been demonstrated, leaving the mechanism of their formation unclear.

The activity of a condensing step enzyme responsible for proanthocyanidin polymer formation has also not been demonstrated. It has been proposed that the condensing step occurs by the sequential addition of C_4 carbocation or quinone methide intermediates to a flavan-3-ol monomer such as (+)-catechin or a pre-existing flavan-3-ol polymer [16, 18]. This reaction can occur spontaneously [9, 20], leading one to question whether condensation occurs enzymically *in vivo*. However, the discovery of a unique barley seed mutant allele, *ant 26*, in which the seedcoat synthesizes anthocyanins and (+)-catechin and only trace amounts of proanthocyanidin [16], combined with data which illustrate that sainfoin leaves synthesize polymers of different composition as a function of leaf development [21], strongly suggests that condensation is under some type of enzymic control.

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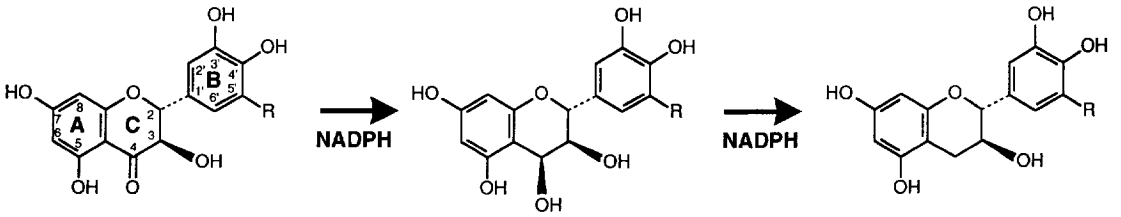

Fig. 1. Proposed pathway for the biosynthesis of flavan-3-ols from dihydroflavonols by the sequential action of two NADPH-dependent reductase enzymes.

In this paper, we report *in vitro* studies using subcellular enzyme fractions from immature leaves of sainfoin. These studies were undertaken to clarify the biosynthetic pathway leading to the immediate precursors of proanthocyanidins in this plant. We also report on experiments to determine whether condensation is enzymically driven.

RESULTS

In vitro *formation of flavan-3-ols during leaf development*

Following incubation of a stage 2 leaflet fraction [21] with [^{14}C](+)-dihydromyricetin (**1**) (DHM) and NADPH for 120 min, radiochemical HPLC analysis, two-dimensional TLC and autoradiography of the EtOAc-soluble products indicated that the major enzyme-mediated product was (+)-gallocatechin (**3**; (+)-GC) (Fig. 2). The small amount of the intermediate 2*R*,3*S*-*trans*-3*S*,4*S*-*cis*-leucodephinidin (**2**; *cis*-LD) produced after 45 min incubation (data not shown) was reduced to trace quantities by 2 hr (Fig. 2). Occasional traces of 2*R*,3*S*-*trans*-3*S*,4*R*-*trans*-leucodelphinidin (*trans*-LD, R_t = 17.5 min; Fig. 2) were also observed (data not shown) and have been attributed to the breakdown of *cis*-LD on the C18 column or during substrate handling, a phenomenon which also has been observed when handling 2*R*,3*S*-*trans*-3*S*,4*S*-*cis*-leucocyanidin (**5**; *cis*-LC) (data not shown). The radioactivity in peaks at 33 and 47 min was attributed to nonenzymic products of (+)-DHM, because they were present in equivalent amounts in reactions with boiled and fresh enzyme (Fig. 2) but not present in HPLC traces of purified (+)-DHM substrate. The absence of enzymically produced (−)-epigallocatechin ((−)-EGC) was always confirmed by two-dimensional TLC, because this compound was not well resolved by HPLC from traces of (+)-catechin ((+)-C) **6** present as an impurity in some of the (+)-DHM preparations. The peaks at 43 and 47 min did not give phloroglucinol adducts [11], suggesting that these compounds were not

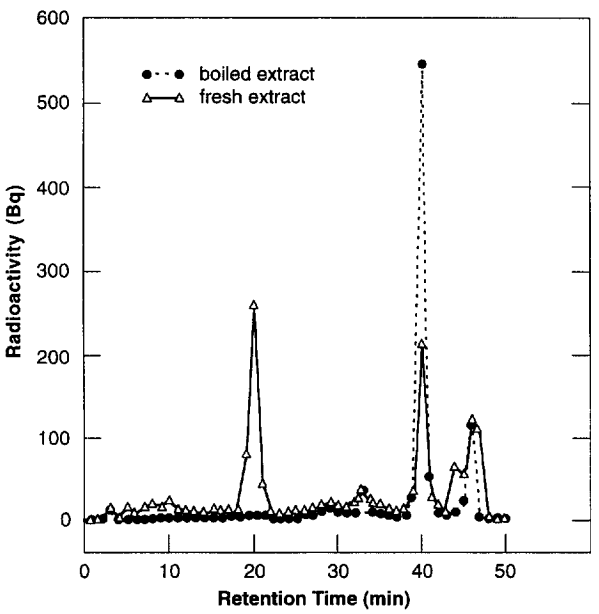

Fig. 2. HPLC trace of radioactive ethyl acetate soluble products from the two-stage NADPH-dependent enzymic reduction of [^{14}C](+)-DHM. *cis*-LD, R_t = 8 min; (+)-GC, R_t = 20 min; (+)-DHM, R_t = 39.5 min.

polymeric. Reductase activity was never observed with boiled plant extracts or in the absence of NADPH in any of the experiments in this study.

The same sainfoin leaf extract also catalysed the double-step reduction of [^{14}C](+)-dihydroquercetin (**4**; (+)-DHQ) to (+)-C **6** (data not shown). The course of this reaction was similar to the reduction of (+)-DHM, in that *cis*-LC (**5**) was only observed early in the reaction and in small quantities.

When the DHM reductase activity and proanthocyanidin content were analysed as a function of development (Fig. 3), enzyme activity rose during leaflet unfolding, then declined after stage 2 as the opening leaflets expanded. This profile (Fig. 3) directly mirrors total polymer content, which changed in a similar fashion as a function of leaflet development when expressed either on a fresh weight or per leaf basis [21]. It also mirrors the accumulation of (+)-GC polymer subunits which rise to a maximum by stage 2 [21]. It is interesting to note here that leucocyanidin reductase activity is higher at stage 1 than at later stages of development (B. Skadhauge, unpublished data), a profile which differs from our two-stage reductase activity. This reflects the earlier accumulation of maximum levels of (+)-C polymer subunits compared with (+)-GC subunits in developing leaves (B. Skadhauge, pers. comm.) [21].

Our data, indicating that proanthocyanidin biosynthesis and accumulation of polymers in sainfoin leaflets occurs primarily during the early stages of leaf development, is supported by ultrastructural evidence suggesting that proanthocyanidins accumulate during sainfoin leaflet development sequentially in two unique cell types; first in a network of cells which begin to develop on the abaxial surface before leaf emergence, followed later by their appearance in large sac-like cells on the adaxial surface [22]. The reduction of total proanthocyanidin content is further supported by the observation that the cellular vacuoles were depleted of proanthocyanidins during leaf expansion without a concurrent increase in electron density in the cell wall [22]. This suggests that proanthocyanidins may be recycled in sainfoin leaves rather than oxidized to form insoluble complexes, such as are found in seed coats [23]. Although this has not been tested, the differential expression of flavanol reductases observed during leaflet development in our study may not only result in the differential accumulation of unique proanthocyanidin isomers, but each of the enzyme activities may actually be compartmentalized into the different cell types observed microscopically [22].

Sequential formation of flavanols in vitro

Reduction of between 10 and 50% of the administered (+)-DHM was routinely observed in these reductase assays depending on the preparation and stage of leaflet development, but in all cases enzyme-mediated production of (−)-EGC was not detected in the reaction mixtures. The absence of any incorporation of (+)-DHM into (−)-EGC indicates that there was neither enzymic epimerization of the dihydroflavonol nor production of a symmetrical intermediate—situations that would permit formation of flavanol products with appropriate stereochemistry. This suggests that (−)-EGC subunits, which are one of the major components of sainfoin leaf proanthocyanidin polymers [11], may be formed by enzymic reduction of a 2*R*,3*S*-*cis*-dihydroflavanol. However, one cannot eliminate the possibility that they are formed spontaneously as a part of a condensing step, that the enzyme was inactivated, or that a low molecular weight component was missing from the extracts.

The reduction of [^{14}C](+)-DHM to a flavan-3-ol with only trace formation of the intermediate *cis*-LD contrasts with the *in vitro* reduction of unlabelled (+)-DHM observed in a previous study with *Ginkgo biloba* and Douglas fir cultures in which large amounts of *cis*-LD compared with (+)-GC were observed [17]. It suggests the possibility that there may be preferential utilization of enzyme-bound substrates in sainfoin leaves, a phenomenon known as 'substrate channelling' [24, 25]. These results are also consistent with the model proposed by Stafford [23] in which the

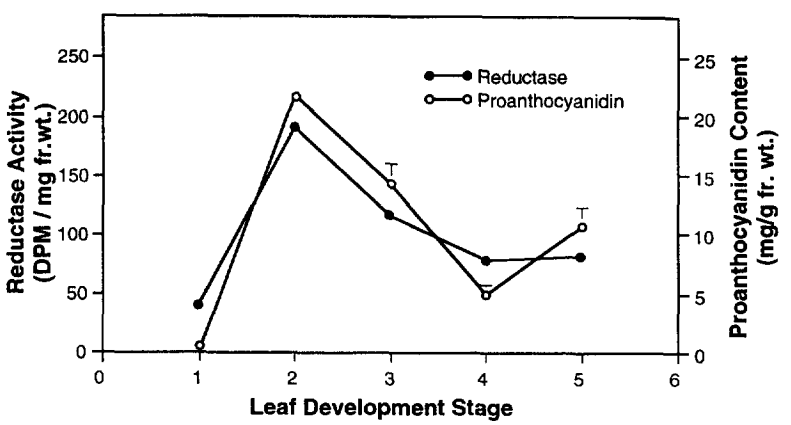

Fig. 3. NADPH-dependent reductase activity and proanthocyanidin content as a function of leaflet development. S.E. ($n=4$), where not indicated, fall within the symbol.

dihydroflavanol and leucoanthocyanidin reductases are situated in close proximity to each other in a membrane-bound complex. This hypothesis was tested by simultaneous administration of both [^{14}C](+)-DHM and [C_4-^{3}H]*cis*-LD to the enzyme preparation and examination of the proportion of each radioisotope in the (+)-GC product (channelling ratio = ^{14}C/^{3}H). Incomplete resolution of the broadly tailing breakdown product, [C_4-^{3}H] 2*R*,3*S*-*trans*-3*S*,4*R*-*trans*-leucodelphinidin (R_t = 17.5 min), from (+)-GC (R_t = 20 min) under these HPLC conditions led to difficulties in assessing the channelling ratio directly at the (+)-GC peak maximum, but radioisotope ratios could be calculated from peak trailing fractions. Estimates of the molar incorporation ratios indicated preferential incorporation of (+)-DHM rather than *cis*-LD into (+)-GC with a peak channelling ratio of 5.5±0.8 (Fig. 4). Channelling ratios of up to 10 have been reported previously for the conversion of phenylalanine to *p*-coumaric acid by buckwheat preparations [26], but channelling by enzymes in the flavonoid pathway has not been observed to date.

In vitro *condensation reactions*

In vitro enzymic production of oligomeric proanthocyanidin was investigated by incubating [^{14}C]-(+)-C, NADPH and either unlabelled (+)-DHQ or (+)-DHM with enzyme preparations at pH 7.2. However, no significant quantities of oligomeric material could be detected in either of these types of experiments. Because the conditions for these assays were essentially those of the reductase assays and because condensation could involve additional mechanisms, we sought conditions that might prove optimal in the demonstration of enzymic conversion of monomeric flavanols to oligomeric forms (Table 1).

No reductase or condensing step activity was associated with microsomal fractions prepared by centrifugation of the crude extract at 100 000 *g* for 40 min before assaying with (+)-DHM for activity, although radioactive products identical to those using the CL4B void volume extract were produced with the 100 000 *g* supernatant. The addition of Mg^{2+}, Mn^{2+}, Ca^{2+} and Zn^{2+}, and also substitution of the Tris buffer with MES, borate or phosphate had no effect on the ratio of *cis*-LD and (+)-GC formed by the 100 000 *g* supernatant or CL4B void volume.

Table 1. Conditions and cofactors used to test a subcellular fraction for reductase and condensing step enzyme activity

Temp. (°)	5–55
Cofactors/additives	NADPH, NADH, H_2O_2, PVPP
Ions	Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}
pH	4–11
Buffer fractions	Tris, MES, phosphate, borate
100 000 *g* fractions	Pellet, supernatant
Inhibitors	EDTA, KCN, DEDC*, DTT

*Diethyldithiocarbamate.

Addition of the Cu-chelating agent, diethyldithiocarbamate (DEDC, 2 mM), to the reaction mixture containing (+)-DHM inhibited the amount of *cis*-LD and (+)-GC, compared with the standard reaction, by 50% but did not change relative product proportions. EDTA was only mildly inhibitory and KCN had no effect. Substitution of NADPH with NADH reduced the amount of *cis*-LD and (+)-GC formed to 15% (1 mM NADH) and 42% (5 mM NADH), but the relative proportion of products remained similar to the NADPH-dependent assay. Removal of the dithiothreitol (DTT) from the reaction mixture increased the formation of *cis*-LD two-fold relative to that formed in the standard assay. This indicates that either disulfide bridging is important for maximum activity of the first reductase or that the second reductase activity is dependent on free sulfhydryl groups. No polymeric products were observed with any of the aforementioned biochemical additions.

Optimal activity for both reductase enzymes was observed at 30° and between pH 6.4 and 7.4 when the temperature was varied according to Table 1. No dimeric products were observed under these conditions. Activity was lower than the optimum, but completely

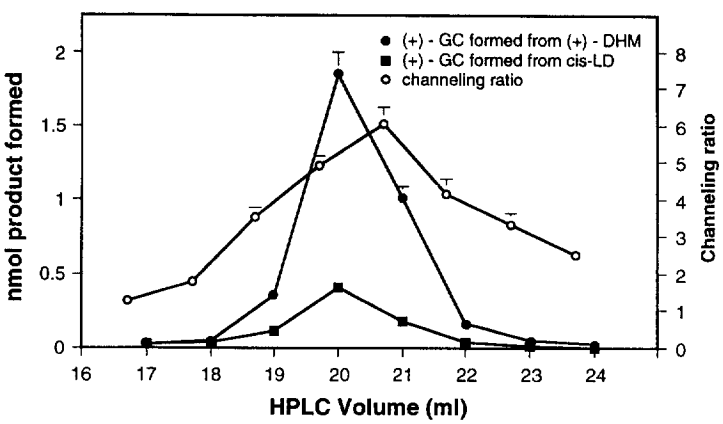

Fig. 4. Apportionment of radioisotope in (+)-GC after simultaneous enzymic reduction of [^{14}C](+)-DHM and [C_4-^{3}H]*cis*-LD. Channelling ratio = ^{14}C/^{3}H. S.E. (n = 4), where not indicated, fall within the symbol.

stable for 36 hr at 5°. The broad pH optimum for our two-step reductase reaction is similar to that observed for single-step leucocyanidin reductase activity in desalted 20 000 *g* supernatants made from young sainfoin leaves or barley kernels [6]. There was no evidence of a peroxidase-mediated reaction, since addition of H_2O_2 produced a ratio of products similar to the reductase assay. When the plant extract was pretreated with PVPP to remove monomeric phenolics and condensed tannins which might inhibit the reaction, no changes were observed in the ratio of products compared with the base two-step reductase assay.

Finally, to test the hypothesis that proanthocyanidins are formed as a result of transport of flavanols into the vacuole, we examined whether there was any evidence of (+)-C uptake or association with membrane vesicles. When stage 2 subcellular enzyme preparations were incubated with [^{14}C](+)-C and Mg^{2+}, approximately 2% of the applied radioactivity was consistently associated with the enzyme preparation as measured by exclusion of radiolabel together with the turbid, chlorophyll-containing fractions eluting in the void volume of an analytical CL4B column (Fig. 5). However, the low levels of radioactivity in these experiments prevented conclusive localization of isotope to a specific membrane compartment after sucrose gradient centrifugation. In addition, the association of [^{14}C](+)-catechin with the enzyme preparation was not increased by addition of ATP (data not shown). In a subsequent experiment in which a 100 000 *g* washed pellet fraction replaced the usual enzyme preparation, preliminary results indicate that 16% of the radiolabel co-chromatographed with the void volume.

Further studies with larger amounts of labelled catechin will be necessary to determine whether these results reflect specific or non-specific sequestration and whether the catechin was actually membrane-bound.

In vivo *synthesis of proanthocyanidins*

Because neither flavanol epimerase nor condensing activity could be demonstrated *in vitro*, we examined the ability of stage 2 sainfoin leaflets to synthesize proanthocyanidins from [^{14}C](+)-DHM *in vivo*. Of the original radiolabel used, 0.4% was observed in the (+)-GC subunit of the polymeric fraction, with only traces of radiolabel in (−)-EGC, (+)-C and (−)-EC (data not shown). The low incorporation of radiolabel into condensed tannins is likely to be a function of the efficiency of uptake of the dihydroflavanol into sainfoin leaves. However, the unbalanced incorporation into (+)-GC at the expense of (−)-EGC supports the idea that the epi-isomers are synthesized via a different route in the flavonoid pathway, since at this stage of leaf development the sainfoin leaf polymers were composed of roughly 50% (−)-EGC and only 30% (+)-GC [21].

DISCUSSION

Previous studies of the enzymic reduction of dihydroflavonols have employed isolation techniques and media that favour dissociation of enzymes from weakly stable complexes or membranes [5, 17] and in several plants the reductase activity has proved quite unstable [6] (M. Y. Gruber *et al.*, unpublished data). The enzyme fractions used in the present study were prepared by homogenizing leaflets under mild conditions, followed by centrifugation and gel filtration on Sepharose CL4B to yield a subcellular fraction substantially free of low molecular weight soluble protein and cofactors. By using these mild conditions we were able to measure and characterize the activity of two sequentially acting, closely associated reductase enzymes as a function of tissue development in sainfoin leaflets. Either the first or both reductases appear to require Cu^{2+} for full activity and are affected by a sulfhydryl reducing agent.

We were unable to demonstrate a mechanism by which 2*R*,3*R*-*cis*-flavanols such as (−)-EGC are formed, and we were also unable to demonstrate enzymic conversion of flavanols to proanthocyanidins *in vitro*. These situations may be a result of enzyme inactivation or a missing low M_r component. However, our experiments do not rule out the possibility that

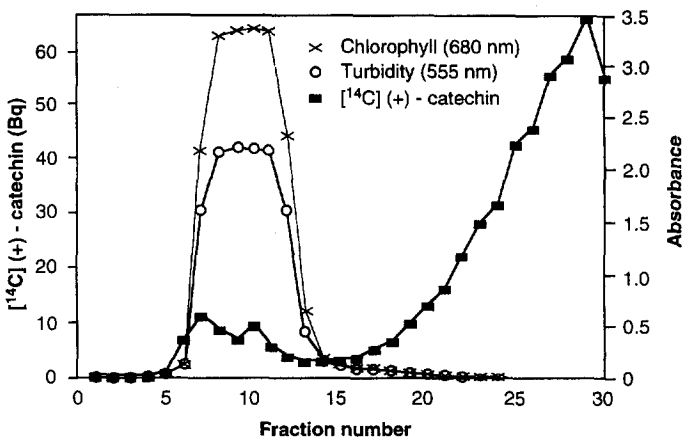

Fig. 5. Representative CL4B gel permeation chromatogram of stage 2 leaflet extract after incubation with [^{14}C](+)-catechin.

(−)-epi-isomers are formed earlier than the reductase steps via a different set of enzymes.

The demonstration of enzymically driven condensation *in vitro* probably requires several sequential reactions in order to mimic *in vivo* subcellular conditions. The first reaction would be the production of flavan-3-ols by the second reductase under the same type of reducing conditions used in our reactions. This reaction does not appear to require intact membranes or the enzyme activity is easily solubilized and, as such, has been characterized in other plants [6] (M. Y. Gruber *et al.*, unpublished data). The second reaction would be the formation of stable C_4 carbocations. This may well require addition of a cofactor to form a good leaving group, although our experiments with radiolabelled catechin do not indicate that ATP is involved. These first two reactions would be followed by a third lyase-type reaction to form oligomers in which the C_8 or C_6 of the flavan-3-ols is first rendered highly nucleophilic. The formation of the proanthocyanidin oligomer from two different metabolic pools has been inferred from evidence of disproportionate labelling of monomeric flavanol structures by cinnamate precursors [27].

Conditions that would mimic the microenvironment of the active sites for such condensing step enzymes would necessarily be different from the reductase conditions—hence the need for several steps. The second and third steps could be mediated by unique active sites on different moieties or different subunits of the same integral vacuolar membrane protein or by different proteins. The demonstration of these latter steps *in vitro* may depend on the addition of an intact vacuole to the reaction to form oligomeric proanthocyanidins, as well as to prevent oligomers from inactivating protein. The presence of more than one unique barley *ant* mutant, post-reductase, supports a requirement for multiple gene products in this reaction [16]. In addition, the relative concentration of flavan-3-ol to carbocation might be important to drive the reaction forward. Hence, a reaction *in vitro* might well be more successfully demonstrated if organized into separate stages, first in which cytosolic and loosely-bound vacuolar proteins, the required cofactors were present and the reaction was allowed to generate different concentrations of flavan-3-ols (i.e. with a >100 000 *g* supernatant). Purified flavanols and cofactors could replace this reaction. The condensation stage would likely follow after addition of whole purified vacuoles and non-protein cytosolic factors. The condensing steps may be more stable and more easily detected *in vitro* if tested in a weak organic solvent [28]. It may also be beneficial to determine whether organic free radicals are part of the reaction mechanism.

EXPERIMENTAL

General. Plants of sainfoin (*O.* Scop. cv. Melrose) were maintained under a 16/8hr photoperiod with an 18°/14° day/night temp., and developing leaflets harvested at five stages of development: 1, youngest leaves (leaflets not separated); 2, leaflets separated but folded; stage 3, leaflets partially unfolded; stage 4, leaflets fully unfolded; and stage 5, older mature leaves [21]. HPLC was performed on a Lichrocart RP-C18 (5 μ) column (250×4 mm) (1 ml/min). Radiolabelled flavonoids were detected using a radiochemical HPLC detector or by liquid scintillation counting of HPLC or TLC frs, and also by their UV spectral characteristics. Rotary evapn was performed at 30°. 2*R*,3*S*-*trans*-3*S*,4*S*-*cis*-LC and its 3*S*,4*R*-*trans* isomer were chemically synthesized as described in ref. [6]. Flavanol isomers were identifed by co-chromatography with authentic standards and comparison of UV spectra. Protein was determined by the Bradford assay [29]. Proanthocyanidin content was measured by a modification of the tannin–PVPP binding assay [30]. Acid-washed PVPP powder (0.1 g) was added to 0.1g of ground plant material and the mixt. was vortexed and reground with MeOH (5 ml). Samples were allowed to stand for 12 hr with intermittent vortexing, centrifuged (2000 *g*) and the supernatant discarded. The pellet was washed twice with fresh MeOH (10 ml). Total extractable (bound to PVPP) and unextractable proanthocyanidins (remaining in the plant residue) were measured together as released anthocyanidins by spectrophotometry at 550 nm after acid hydrolysis of the washed pellet at 70° (5.0 ml of *n*-BuOH–HCl (70:30) and 0.08 ml of 2% $NH_4Fe(SO_4)_2 \cdot 12H_2O$) [31].

Labelled substrates. [^{14}C](+)-DHM was prepd *in vivo* by incorporation of radiolabelled phenylalanine into *Leptarrhena pyrrofolia* [17, 32]. Leaf discs (14–20 g, 1 cm diameter) were harvested from 20–25 plants, surface-sterilized and vacuum-infiltrated for 20 sec with 1 MBq L-[U-^{14}C]-phenylalanine (17.4 GBq mmol^{-1}, Dupont-NEN) (270 μl) diluted to 10 ml with 1/2 strength sterile MS salts. Discs were incubated in the solution in sealed Petri dishes for 1–3 days at 25° under a 16/8 hr light/dark photoperiod. Alternatively, discs were incubated for 5 days without vacuum infiltration on sterile solid medium (0.8% agar) spread with a similar quantity of radioactive phenylalanine. Discs were rinsed twice in distilled H_2O, ground in cold MeOH–H_2O (3:1), centrifuged 5 min (1000 *g*) and the pellet re-extracted until the supernatant was colourless. The combined MeOH frs were concd to the aq. phase and extracted with EtOAc (×3). The combined EtOAc extracts were subjected to prep. TLC cellulose (1 mm) (MN300NR) developed in H_2O. [^{14}C](+)-DHM (R_f 0.2, dark blue fluorescence under UV; specific activity 14 MBq mmol^{-1}) was eluted with MeOH, reduced to dryness, and stored frozen at −80°. Purity was confirmed by HPLC and by comparison of the UV spectrum with an authentic standard. Unlabelled (+)-DHM was isolated from leaves of *L. pyrofolia* in a similar fashion.

[^{14}C](+)-Catechin was prepd by incubating 4.5 g of *Taxus brevifolia* callus cultures (G. H. N. Towers, unpublished data) in 50 ml SH medium with hormones containing 367 kBq L-[U-^{14}C]phenylalanine (17.4 GBq

mmol^{-1}) with shaking for 5 days at 25° using a 16/8 hr light/dark photoperiod. Calli were recovered by filtration and extracted as for *Leptarrhena*. [^{14}C](+)-catechin was recovered from the EtOAc fr. by descending prep. PC on 3MM paper (*n*-BuOH–HOAc–H_2O (4:1:5, upper layer); R_f 0.8, dark under UV). The [^{14}C](+)-catechin (specific activity 3.2 MBq mmol^{-1}) was eluted with MeOH, the purity confirmed by HPLC (1% HOAc–MeOH (80:20); R_t 10 min; HPLC system 1) and by comparison of the UV spectrum with an authentic standard, freeze dried and stored at 4°.

[C_4-^{3}H]-2*R*,3*S*-*trans*-3*S*,4*S*-*cis*-leucodelphinidin (*cis*-LD) was synthesized in two stages, first by reduction of 3.2 mg (+)-DHM in dry EtOH with 185 MBq solid NaB^3H_4 (18.1 GBq mmol^{-1}) (20°, 2 hr). [C_4-^{3}H]-2*R*,3*S*-*trans*-3*S*,4*R*-*trans* leucodelphinidin (*trans*-LD) produced by this procedure was then epimerized to the 3*R*,4*S*-*cis* isomer by reaction with 0.1% (v/v) HOAc (50°, 3.5 hr). These reactions were based on those used to synthesize [C_4 - ^{3}H] - 2*R*,3*S* - *trans* - 3*S*,4*S* - *cis* - leucocyanidin [5, 6, 33] and unlabelled *cis*-LD [17]. The products were monitored by HPLC (5% HOAc, isocratic, HPLC System 2; 3,4-*cis* epimer, R_t 4 min; 3,4-*trans* epimer, R_t 7.2 min) to avoid acid-catalysed synthesis of proanthocyanidin dimers. Labelled *cis*-LD and *trans*-LD were purified by HPLC on a phenyl-silica column (250×4 mm) eluted with H_2O (1 ml min^{-1}). These reactions yielded 4.9 MBq *cis*-LD (96% pure, 21 GBq/mmol, by HPLC analysis). Radiolabelled *cis*-LD was characterized by comparison of its behaviour on TLC, and on phenyl and C18 HPLC columns with reported values [6, 17], by UV spectrum (λ_{max} 268 nm) and by acid conversion to the anthocyanidin and co-chromatography on TLC plates with authentic delphinidin. [C_4-^{3}H]-2*R*,3*S*-*trans*-3*S*,4*S*-*cis*-leucocyanidin and the 3*S*,4*R* isomer were also synthesized [5, 6] and a ^{1}H NMR spectrum matched with the published spectrum [5]. Flavan-3,4-diols were freeze-dried and stored at −80°.

Enzyme preparation. All manipulations were performed at 4°. Leaflets (2–3 g fr. wt) were sorted into specific stages of development [21], washed in distilled H_2O, chilled on ice and ground without abrasive in ice-cold N_2-purged lysis buffer (4 ml g^{-1} fresh wt tissue) containing 25 mM Tris pH 7.3, 250 mM sucrose, 10 mM KCl, 5 mM DTT, 1.5% PVP-40, 1mM PMSF, and 0.2% BSA (buffer A). Lysates were also made without DTT. The homogenate was filtered through two layers of Miracloth™ and centrifuged at 10 000 *g* for 5 min at 4°. The supernatant was passed through a Sepharose CL4B gel filtration column (20 ml bed vol) previously equilibrated with 2 bed volumes of the lysis buffer but without PMSF, BSA, and PVP (buffer B). After supernatant application, the column was eluted with buffer B; then the turbid fraction eluting at the void volume was used as an enzyme source. The 10 000 *g* pellet was found to contain no enzyme activity and was discarded.

In vitro *assay procedures.* Standard reductase assays contained 1 or 2 kBq of [U-^{14}C](+)-DHM, NADPH (1mM), G-6-P (3 mM), G-6-P dehydrogenase (1U ml^{-1}, Sigma) and stage 2 leaflet CL4B column void fraction in 2.5 ml, and were incubated at 30° for 120 min. Microassays were scaled down proportionally to 250–500 μl final assay volume. Following incubation at 30° for 45–120 min, HOAc (150 μl) was added and the reaction mixt. extracted 3× with 3 ml H_2O-saturated EtOAc. EtOAc extracts were combined and concentrated *in vacuo* at 30° to *ca* 1 ml; then 1 ml of H_2O was added. The remaining EtOAc was removed *in vacuo* and the aq. residue was frozen in liquid N_2 and freeze-dried. The residue was redissolved in 50 μl MeOH–H_2O (7:3), and analysed by 2D-TLC (cellulose; *t*-BuOH–HOAc–H_2O (3:1:1) (TBA) and 6% HOAc), and gradient HPLC. The HPLC gradient conditions were 1% HOAc (A) and MeOH (B): 5% B, t=0–20 min; 5–20% B, t=20–25 min; 20% B, t=25–40 min; 20–70% B, t=40–45 min; 70% B, t=45–50 min; 70–95% B, 50–55 min (HPLC system 3). Flavans and 3,4-diols were detected after spraying with 4% vanillin (w/v) in MeOH–0.1% HCl, by direct autoradiography or by liquid scintillation counting of HPLC or TLC frs.

Proanthocyanidin content and reductase activity were assayed as a function of leaf development to determine the stage of maximum enzyme activity [21]. Enzyme specific activities were calculated as DPM mg^{-1} fresh wt, based on the radioactivity detected in the (+)-GC peak. The activity of enzyme preparations from stage 2 leaflets from different plant samples were standardized by dilution for all subsequent experiments.

For studies to determine substrate channelling, 250 kBq of [C_4-^{3}H]-2*R*,3*S*-*trans*-3*S*,4*S*-*cis*-leucodephinidin and 2 kBq of [^{14}C]-(+)-DHM were added to 1.5 ml aliquots of the stage 2 enzyme prepn with cofactor concs as described above. Substrate channelling was calculated as a ratio of the concentration of the differently labelled (+)-GC radioisomers formed (^{14}C/^{3}H), using substrate radiospecific activity to determine flavanol concentration in nmoles. In all cases, control assays contained boiled plant extracts (10 min) or no NADPH.

To investigate *in vitro* formation of PA dimers, 800 μl aliquots of stage 2 plant extract was incubated in base assays with 1 mM (+)-DHQ or (+)-DHM, 5 kBq of [^{14}C](+)-catechin and the NADPH-generating system under the conditions described for the reductase assays. Assay conditions were then varied using combinations of the conditions listed in Table 1. Control assays contained boiled enzyme (10 min). The EtOAc-soluble products were analysed using HPLC system 1.

To determine whether (+)-catechin became associated with subcellular organelles, aliquots of stage II enzyme fr. (1 ml) were incubated for 30 min (30°) with 2.5–5 kBq of [U-^{14}C](+)-catechin, $MgSO_4$ (5 mM) and Na_2ATP (5 mM). Control assays did not contain ATP. After incubation, the entire sample was subjected to analytical gel filtration on CL4B column (10×1 cm) equilibrated with buffer B (0.7 ml min^{-1}), and 0.7 ml frs were collected and analysed by liquid scintillation

counting. In one experiment, a 100 000 *g* washed pellet fraction replaced the standard enzyme preparation.

In vivo *labelling of condensed tannins*. Stage 2 leaflets were incubated in 1/2-strength MS salts containing 3.3 kBq [^{14}C](+)-DHM for 24 hr by flotation. Alternatively, radioisotope was incorporated into leaflets by absorption through the petiole of whole leaves. Proanthocyanidin polymers were purified and their individual flavan-3-ol subunits were fractionated on 2D-TLC cellulose [11]. This experiment was repeated 10 times using different leaf harvests.

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