Incorporation of [^{14}C]Phenylalanine and [^{14}C]Cinnamic Acid into Leaf Pieces and Mesophyll Protoplasts from Oat Primary Leaves for Studies on Flavonoid Metabolism at the Tissue and Cell Level

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Z. Naturforsch. **36 c**, 222–233 (1981); received January 14, 1981

Avena sativa, Primary Leaf Tissues, Protoplasts, [^{14}C]Phenylpropanoid Incorporation, C-Glycosylflavones

When the abaxial epidermis was peeled from 5 to 6 day old oat primary leaves, and 3 cm segments were floated on radioactive phenylalanine or cinnamic acid solutions, more than 90 per cent of the radioactivity was incorporated within 3 to 7 h depending on the developmental stage of the leaf. C-glycosylflavones were labelled within 15 min and radioactivity in these compounds increased for several hours. Pulse labelling and pulse chase experiments with either phenylalanine or cinnamic acid, unequivocally demonstrate that oat flavones are stable end products of metabolism. However, this procedure does not distinguish between sequential biosynthesis of various flavones and their interconversion. Cinnamic acid was more efficiently (ca. 20 ×) converted into oat leaf flavones than was phenylalanine, when the precursor was fed to leaf pieces, and flavones recovered from mesophyll protoplasts. Different labelling patterns were obtained with whole leaf segments and protoplasts which apparently reflect differences in tissue specific flavone biosynthesis of mesophyll and epidermis.

Isolated mesophyll protoplasts incubated with [^{14}C]cinnamic acid synthesize ^{14}C-labelled flavones characteristic of the mesophyll, as well as several unidentified phenylpropanoid derivatives not found in the intact tissue. Data suggest that photosynthetically active mesophyll cells are a main site of tissue specific flavone biosynthesis.

Introduction

In earlier experiments on flavonoid metabolism of oat seedlings [1] [^{14}C]phenylalanine was injected into the milky endosperm three days after sowing and efficiently incorporated into the leaf C-glycosylflavones 7-methoxy-vitexin 2″-rhamnoside (F_1) isovitexin 2″-arabinoside (F_2), vitexin 2″-rhamnoside (F_3) and isovitexin (F_5) [2]. As the oat primary leaf develops, the flavones accumulate mainly in the middle and apical section of the leaf [1, 3]. During subsequent leaf development total radioactivity as well as the amount of flavones, remained fairly constant for 14 days. This suggests that there was no turnover, or interconversion, of these compounds. However, there may be interconversion of one flavone to another during leaf differentiation and there are different tissue-specific accumulation patterns of the major flavones F_1, F_2 and F_3 in oat leaves [4, 5]. Flavone profiles obtained from intact mesophyll and from mesophyll protoplasts deviate characteristically from epidermal patterns. Is flavonoid accumulation in leaves, or in various tissue regions, autonomous in each of the tissues or are there regulatory interrelationships between these tissues?

When precursors are injected into the endosperm, they must be transported over considerable distances before they reach sites where flavonoids are synthesized. In the present investigation we have used a more direct method of radioactive precursor application to leaf tissues without interference of long distance movements. Leaf pieces with high flavone biosynthetic activity [3] were prepared from different developmental stages of the primary leaf and floated on ^{14}C precursor solutions with direct contact of the intact mesophyll after peeling off the abaxial epidermis. In addition, pulse labelling as well as pulse chase experiments can be carried out with leaf pieces, followed by protoplast isolation from the mesophyll [6], and subsequently, labelling patterns of protoplasts can be compared with parent intact leaf pieces which consist of mesophyll and complementary epidermis. Furthermore, a direct precursor application to isolated protoplasts could indicate whether this cell type is able to synthesize mesophyll flavones autonomously.

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0341-0382/81/0300-0222 $ 01.00/0

Materials and Methods

1. Plants

Avena sativa L. cv. "Gelbhafer-Flämingskrone" was grown as previously described [7]. Developmental stages of primary leaves are characterized by the seedlings age, 5 days (leaf length 5–6 cm), 5.5 days (6–7 cm) and 6 days (7–8 cm) [3]. Leaves were harvested at the beginning of a 13 h photoperiod.

2. Preparation of leaf pieces

A shallow transverse cut was made with a razor blade, on the adaxial surface of the leaf, about 1 cm below the tip. The tip and the abaxial epidermis of the whole leaf, was peeled away and discarded. The basal part of the peeled leaf was cut off to leave a leaf sample 3 cm in length. Using this method, no microscopically visible part of the mesophyll contaminated the peeled epidermis and the mesophyll, and adaxial epidermis remains intact.

3. Feeding experiments

Leaf pieces were floated in a petri dish on either a L-[U-^{14}C]phenylalanine solution (specific activity 513 mCi/mmol; Amersham-Buchler, Braunschweig) in distilled water or on a 2% ethanolic aqueous [3-^{14}C]cinnamic acid solution (spec. act. 46.3 mCi/mmol; CEA, Gif-Sur-Yvette, France). For 5 leaf pieces either 2.25 ml of the radioactive phenylalanine solution, or 2.05 ml of that of cinnamic acid, was used. To a purified suspension of mesophyll protoplasts of known titer, a definite amount of the [^{14}C]cinnamic acid solution was added. For substrate concentrations see legends of figures. The incubation was performed at 22–25 °C under dim white light (approx. 500 lux) with gentle shaking of the petri dish.

4. Protoplast isolation

Mesophyll protoplasts were isolated from the peeled leaf segments by slight modifications of the procedure previously described in ref. [6]. Tissues were preplasmolyzed on 0.6 M sorbitol in H_2O. Protoplasts were isolated by incubating the plasmolyzed tissues after a preceding vacuum infiltration for 5–10 min in 0.6 M sorbitol, adjusted to pH 5.6–5.8 with KOH, and containing 1.5% Cellulysin grade B (Calbiochem. Co., California, USA) at 22 to 25 ° in dim light (ca 500 lux) for from 2 to 3 h with gentle shaking. Protoplasts were collected by passing the digestion mixture through a stainless steel screen to remove undigested tissues from the protoplast suspension and pelleted by centrifugation for 3 min at $55 \times g$. Protoplasts were washed three times by resuspending the pellets in 0.6 M sorbitol containing 5 mM MES/KOH buffer, pH 5.9. The protoplasts were essentially free from cell debris. Intact protoplasts were counted by a hemocytometer. For details of protoplasts integrity see [6].

5. Extraction of plant material and analytical procedures

Flavones were quantitatively extracted with boiling water, separated and purified after our standard procedures [7, 8]. Total flavone was separated from the aqueous extracts by polyamide column chromatography using 80% aq. methanol after elution of non-flavonoid impurities with water [8]. This MeOH-fraction contains flavones and several cinnamic acid derivatives. Separation of the various flavones F_1, F_2, F_3, F_5 and/or purification to constant specific radioactivity was performed using five different solvent systems in 1- or 2-dimensional cellulose- and polyamide-TLC, according to ref. [1, 8], followed by radioautography. In most instances the first TLC-separation step performed with chloroform, acetic acid 3:2, water saturated (CAW), was sufficient for a 90–100 per cent radiochemical purity of flavones. Aliquots of soluble fractions, and the water insoluble residue were counted by liquid scintillation spectrometry and with a Geiger-Müller counter, respectively [1]. Samples were corrected by external standardization. For quantitative spectrophotometric flavone measurements see [7]. Chlorophyll content of leaf pieces and protoplasts was estimated according to ref. [9]. Radioautography of 2-dimensional TLC-plates was performed with "Industrex Instant 620"-paper, Kodak.

Liquid chromatographic separation was done on a 250 × 4 mm chromatographic column prepacked with LiChrosorb RP-8 (5 µm, Merck, Darmstadt, Germany) on a Spectra-Physics (Santa Clara, Calif., USA) system. Flavones were quantified with a Schoeffel SF UV-VIS spectroflow monitor coupled with an Autolab System I computing integrator (Spectra-Physics). Separation was accomplished by a 15 min gradient elution from 20 to 50% solvent B

in A + B (A, 1.5% *ortho*-phosphoric acid in water; B, 1.5% *ortho*-phosphoric acid, 20% *gl.* acetic acid, and 25% acetonitrile in water) at a flow rate of 1 ml/min.

Results and Discussion

1. Labelling experiments with [^{14}C]phenylalanine

When leaf pieces from 5 day old primary leaves were floated for 3 or 4 h on 4.9 μM, 11 μM or 24.6 μM of [^{14}C]phenylalanine, total radioactivity (TA), sum of water insoluble residue (WR) and water soluble fraction (WS), as recovered from leaf pieces is enhanced only 1.25- and 2.5-fold, respectively, by increasing the precursor concentration to 11 or 24.6 μM (Fig. 1 a). Corresponding relative incorporation values show a general decrease (Fig. 1 b). However, labelling of the flavones F_1-F_3 is not influenced under these conditions (Fig. 1 a, insert).

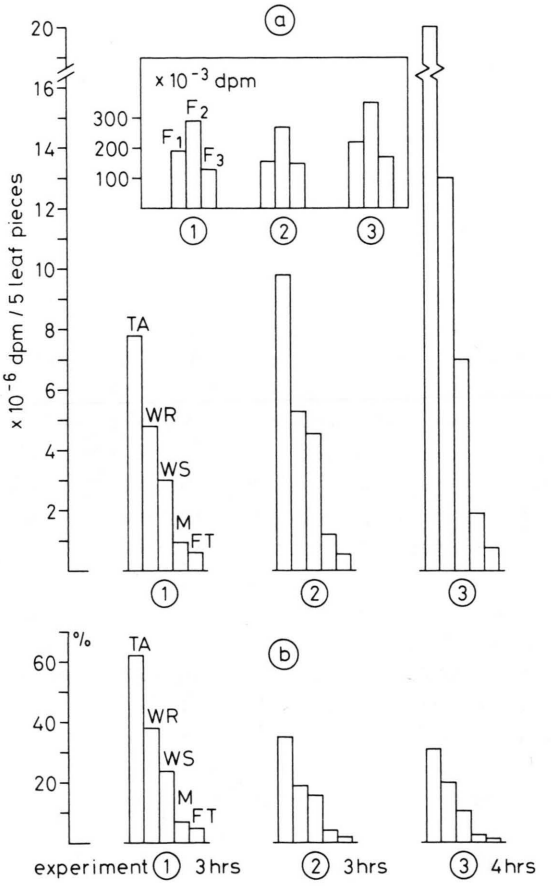


Fig. 1. Incorporation of [^{14}C]phenylalanine into fractions WR (water insoluble residue), WS (water soluble), TA (total leaf radioactivity = WR + WS), MeOH (methanol fraction), FT (total flavone = sum of F_1-F_3) and individual flavones F_1, F_2, F_3 of leaf pieces floated on radioactive substrate solutions increasing in concentration. Age: 5 day old primary leaves. (a) Values in dpm per 5 leaf pieces after 3 or 4 h of incubation. Starting concentration $c°$ of [^{14}C]phenylalanine at time zero in dpm per 2.25 ml distilled water and 5 leaf pieces: experiment 1: 12.5×10^6 dpm (4.9 μM), experiment 2: 28×10^6 dpm (11 μM), experiment 3: 62.5×10^6 dpm (24.6 μM). (b) Relative distribution of radioactivity of leaf pieces in per cent of $c°$ administered = 100%.

When leaf pieces were floated on either 11 μM non-radioactive phenylalanine solution or on distilled water for 5 h, no quantitative changes of flavone compounds were detected; levels are comparable with values obtained from leaf pieces extracted immediately after removing the lower epidermis. Thus no enhancement affected by phenylalanine or by wounding is occurring. In labelling experiments performed with other plants, higher concentrations of phenylalanine were fed; 100 μM to isolated petals of *Petunia hybrida* [10] and even 2.6 mM and 10 mM to strawberry leaf disks and buckwheat cotyledons, respectively [11, 12]. The 100 μM concentration did not have any influence on anthocyanin accumulation in *Petunia* and even the high concentration used with strawberry did not increase flavonol accumulation in comparison to water controls. Only a slight enhancement of 1.5–15% was observed for C-glycosylflavones of buckwheat, incubated with 10 mM phenylalanine. From other authors similar results are reported [13–15].

Fig. 2 demonstrates the time course of phenylalanine incorporation into various fractions of 5 day (a) and 6 day (b) old primary leaves during 7 h of incubation. Within the first 3 h, the rate of incorporation is about three times higher in the 6 day old leaves (TA). Labelling of water-insoluble residues (WR) increases steadily in both cases with time, whereas water soluble (WS) portions differ; an increase for the first 2 h is followed by a continuous decrease in the 6 day old tissues, but an increase up to 7 h in 5 day old tissues. Although only the flavones have been identified, we assume that the decrease in radioactivity of the water soluble portion of 6 day old leaf pieces is caused by polymerisation of soluble precursors into insoluble material of cell walls.

Younger leaf pieces develop a dominant F_2 label, whereas in the older ones flavone F_1 is labelled primarily. This pattern is not surprising since F_2 is the major flavone in 5 day old leaves; in 7 day old leaves grown under similar conditions flavone F_1 is

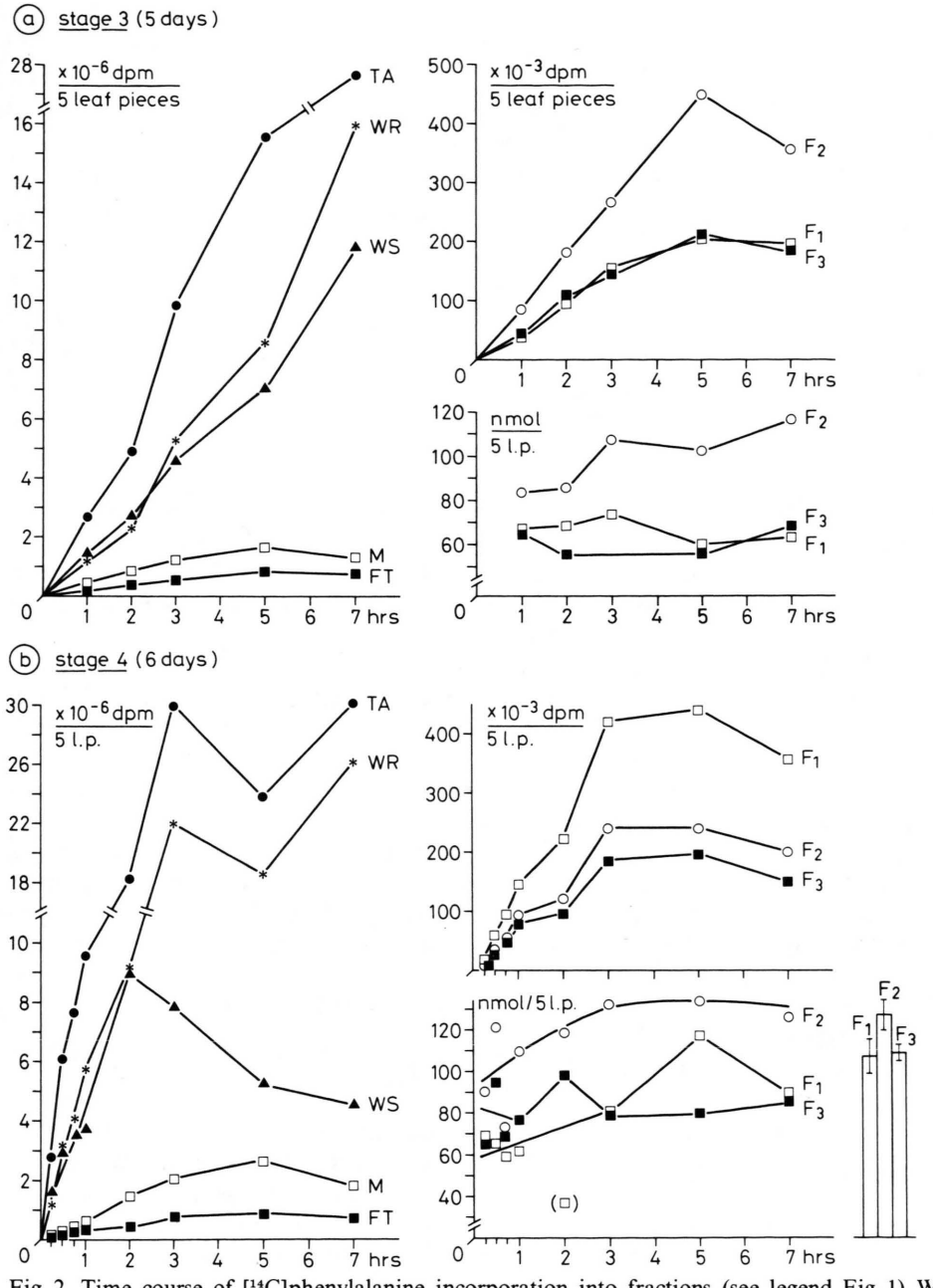
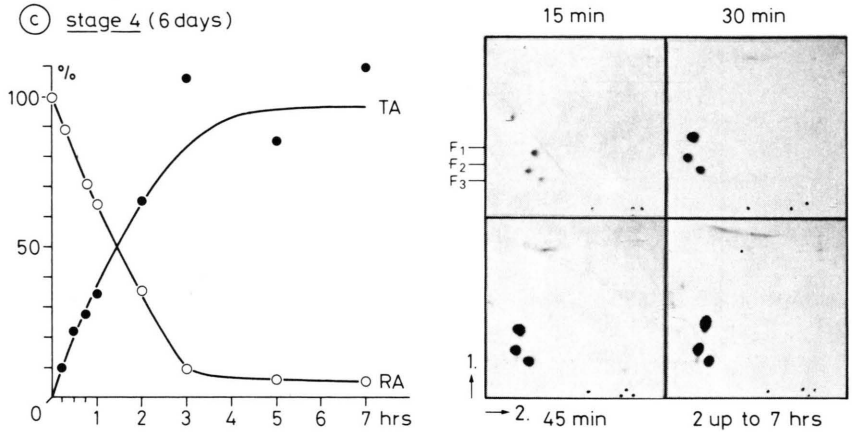


Fig. 2. Time course of [^{14}C]phenylalanine incorporation into fractions (see legend Fig. 1) WR, WS, TA, MeOH, and flavones FT, F_1, F_2, F_3 of leaf pieces of (a) 5 day old and (b) 6 day old primary leaves. Values in dpm and nmol per 5 leaf pieces which were floated on a 11 µM ($c^0 = 28 \times 10^6$ dpm/5 leaf pieces) [^{14}C]phenylalanine solution for several times, removed at time intervals indicated, and analysed. In (b) standard deviations of nmol $F_1 - F_3$ (columns) are indicated by vertical bars and were calculated from 10 independent preparations of 5 leaf pieces each with intact upper and lower epidermis to which no [^{14}C]phenylalanine was administered. Compared to leaf pieces lacking the lower epidermis, an approximately 30% higher $F_1 - F_3$ content is obtained because this percentage is contained in the lower leaf epidermis [5]. (c) (see next page) Time course of [^{14}C]incorporation into 6 day old leaf pieces (TA) in comparison to radioactivity left in the floating solution (RA), 100% = concentration of [^{14}C]phenylalanine c^0, administered at t_0 (left diagram). For radioautography of flavone labelling patterns during time (on the right) identical aliquots of each extract (MeOH fraction) were chromatographed two-dimensionally, solvent CAW (1), solvent 15% HAc (2), and exposed to "Industrex" paper. Flavones $F_1 - F_3$ as indicated, F_5 visible only in trace on the original radioautograms.



the dominant compound [4, 5]. Thus, although flavone F_2 is still a major compound of 6 day old leaf pieces (comp. Fig. 2b, nmoles) [^{14}C]phenylalanine is channeled primarily into F_1, expressing the highest biosynthetic activity for this flavone.

Fig. 2c shows the course of radioactive incorporation into soluble plus insoluble fractions (WS + WR = TA). Almost all of the parent radioactivity is taken up from the medium (residual activity RA) and is incorporated into the various leaf fractions.

Considering dynamics of flavone metabolism of the individual compounds F_1-F_3 and F_5, each major flavone (F_1-F_3) shows an almost linear and continuous increase in dpm per leaf piece. Even in short time experiments (Fig. 2b), the initial increase is almost identical and label is found in all three major flavones to a similar extent within 15 min after feeding [^{14}C]phenylalanine (Fig. 2c). However, no notable radioactivity is detected in flavone F_5, which accumulates in minor or trace amounts [comp. ref. 1]. Its normal fate may be interconversion by O-arabinosylation to compound F_2.

Based on chemical similarities of oat flavone compounds [2, 8] biogenetic relationships are likely to be: F_5 (isovitexin) → F_2 (isovitexin 2″-arabinoside); and isovitexin ⇌ vitexin, followed by O-rhamnosylation to vitexin 2″-rhamnoside (F_3); F_3 → F_1 (7-methoxyvitexin 2″-rhamnoside).

This reaction scheme is supported by reports of an UDP-arabinose: isovitexin-arabinosyltransferase in *Silene dioica* petals [16] and in oat leaves [unpublished results]. The *in vivo* conversion of vitexin to isovitexin, but not the reverse reaction, is reported in *Lemna minor* [17]. Rhamnosyltransfer reactions originating from UDP-Rhamnose to C-glycosylflavone, anthocyanins and flavonols [18–20] as well as methyltransfer reactions from S-adenosylmethionine to several flavonoid compounds are known [21].

As shown in Fig. 2, a maximum or stationary phase of label is reached for the major flavones F_1-F_3 three to five hours after feeding and it is questionable whether the slight decrease in radioactivity during the late phase of the experiment is due to turnover.

To test whether or not a definite pool size of a single flavone is retained with time, pulse chase experiments were performed using [^{14}C]phenylalanine and [^{14}C]cinnamic acid.

2. *Pulse chase experiments*

Comparison of [^{14}C]phenylalanine and [^{14}C]cinnamic acid incorporation

Five and one half day old leaf segments were incubated on either a 4.9 μM [^{14}C]phenylalanine solution or a 49.2 μM [^{14}C]cinnamic acid solution. After a pulse of 3.5 h, [^{14}C] precursors were substituted by corresponding unlabelled solutions of the same concentration at t_0 for additional 20.5 h. Although substrate concentrations differ by a factor of ten, the results were comparable (Fig. 3). In these pulse-chase experiments, radioactivity of the individual flavones F_1-F_3 varies slightly due to experimental error (comp. Fig. 2b, 3c). However, we conclude that no significant decrease of radioactivity of any flavone occurs during the 20.5 h chase period. Thus, no turnover or transformation is taking place

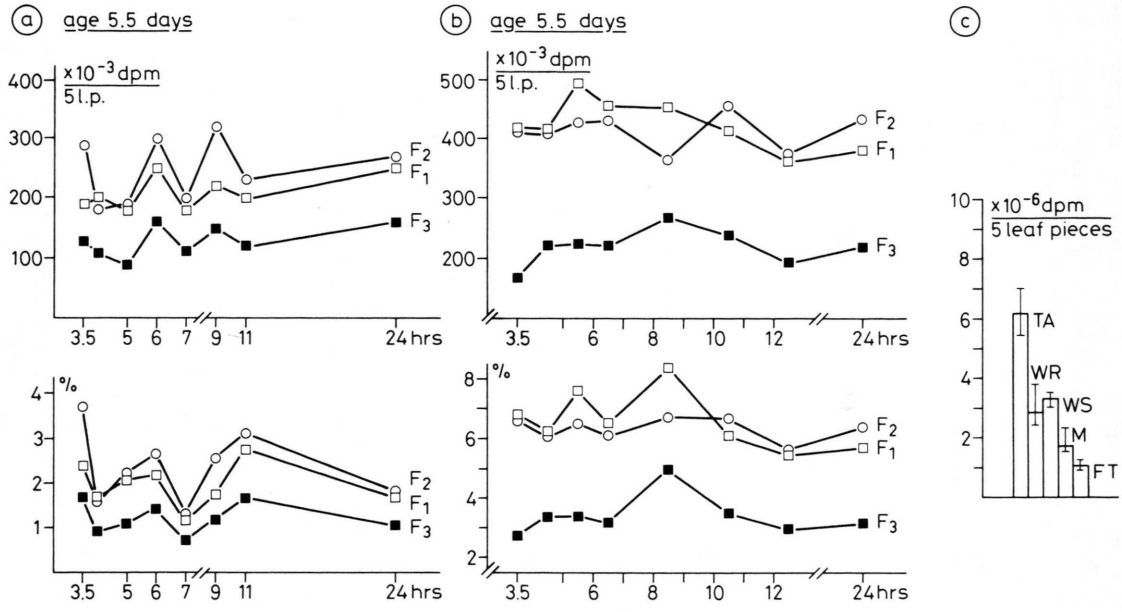


Fig. 3. Pulse-chase experiment with [^{14}]phenylalanine and [^{14}]cinnamic acid. Leaf pieces from 5.5 day old leaves were floated for 3.5 h either (a) on a 4.9 µM [^{14}C]phenylalanine solution ($c^0 = 12.5 \times 10^6$ dpm/2.25 ml $H_2O \times 5$ individuals) or (b) on a 49.2 µM [^{14}C]cinnamic acid solution ($c^0 = 10.4 \times 10^6$ dpm/2.05 ml H_2O, 2% ethanol, × 5 individuals). After pulse labelling, radioactive solutions were substituted by corresponding non labelled substrate solutions identical in concentration (c^0) and leaf pieces were incubated up to 24 h (chase). At time intervals indicated, 5 leaf pieces each were removed and analysed. Flavone radioactivity in dpm per 5 leaf pieces (upper graphs) and relative labelling in per cent (lower graphs), 100% being the total radioactivity (TA) taken up into leaf pieces, (c) incorporation of radioactivity into fractions TA, WR, WS, MeOH and FT (comp. Fig. 1) from [^{14}C]cinnamic acid (values from experiment Fig. 3b). Columns without bars indicate values of that leaf sample which had been harvested 3.5 h after administering cinnamic acid (pulse) whereas bars show absolute variations of samples harvested during the 20.5 h chase period.

and individual oat flavones $F_1 - F_3$, after being synthesized are stable endproducts of metabolism under these experimental conditions.

[^{14}C]cinnamic acid, as commercially available with only about a tenth of the specific radioactivity of [^{14}C]phenylalanine, was used in a ten fold higher concentration to start the pulse labelling experiment with almost identical absolute radioactivity per leaf piece (Fig. 3). Despite this high cinnamic concentration, flavone compounds are labelled only about twice as much with cinnamic acid as with phenylalanine, whereas no substantial differences are observed in total radioactivity (TA) and of water soluble and insoluble activities of leaf fractions (WS and WR; comp. Fig. 1, experiment 1, with Fig. 3c and Fig. 6, experiment 2). Furthermore, the proportion of radioactivity of flavone compounds $F_1 : F_2 : F_3$ does not differ markedly and flavone F_5 is labelled only slightly in both cases.

A separate set of experiments was performed to check whether or not the cinnamic acid concentration used in the pulse-chase experiments influences the flavone content of 5 and 6 day old leaf pieces. Leaf pieces devoid of the abaxial epidermis were floated on a 50 µM unlabelled cinnamic acid solution and a comparison was made with leaf pieces floated on water (Fig. 4). In two additional experiments, after administering cinnamic acid for 3.5 h, leaf pieces were floated afterwards for 3 additional hours either on water or on a 0.6 M sorbitol solution (see experiments with protoplasts, below). In each case, as measured by high performance liquid chromatography (HPLC) no significant differences in flavone content of compounds F_1, F_2, F_3 between "controls" and "samples" were found. Variations as obtained with the controls and/or with samples are in the same order of magnitude and therefore cinnamic acid concentration although ten times of that of phenylalanine does not influence flavone metabolism of oat leaf pieces. Furthermore, no visible turgor change or damage of tissues fed with labelled or unlabelled cinnamic acid was observed.

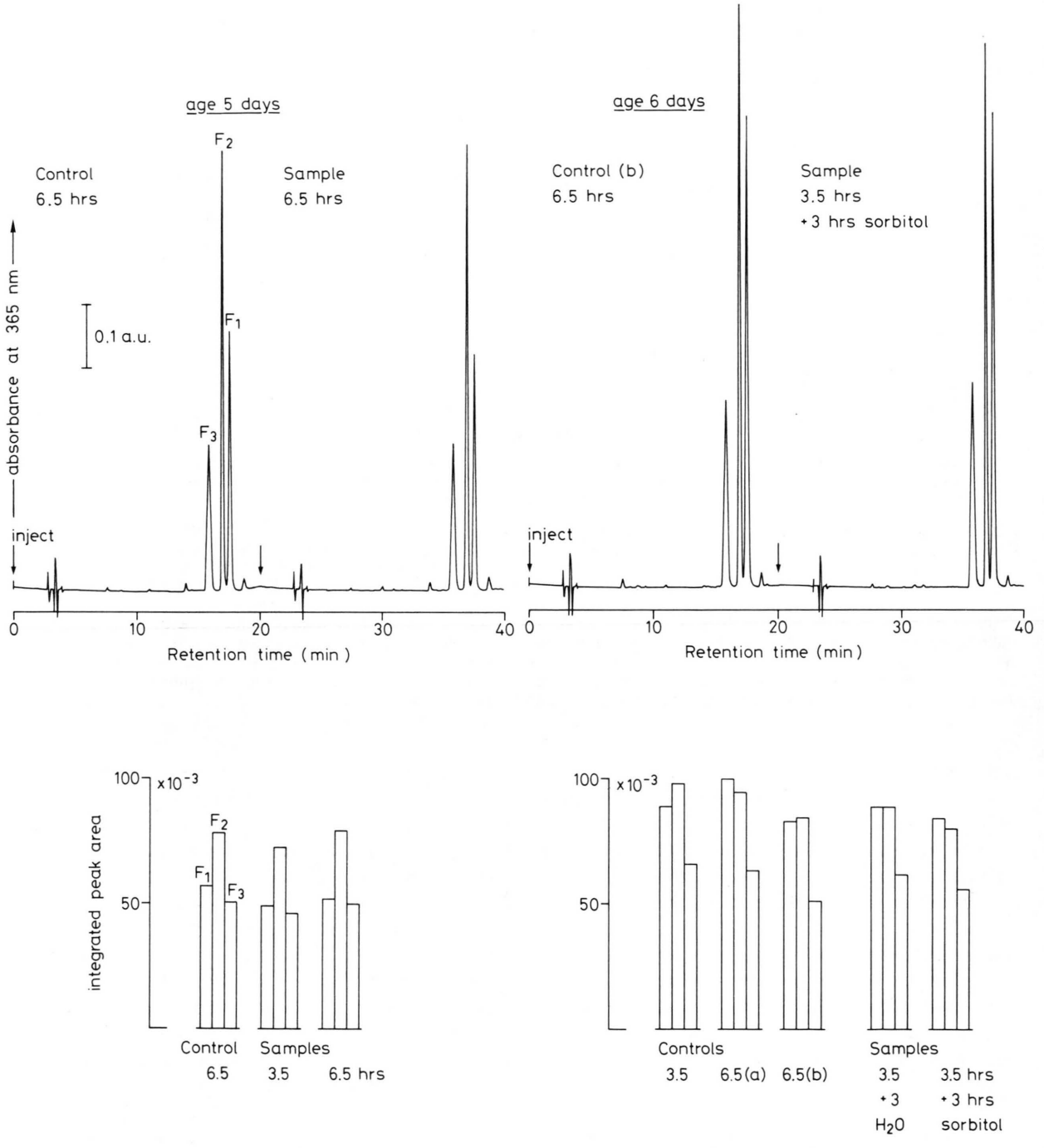


Fig. 4. Flavone content of leaf pieces prepared from 5 day old (left) and 6 day old (right) primary leaves after feeding on a 50 µM non labelled cinnamic acid 2% ethanol. solution (samples) in comparison to water controls. Quantitative data of flavones F_1, F_2, F_3 (integrated peak area) were calculated per 25 individuals (lower diagrams) after HPLC separation of the compounds (see experimental section). Sample size for HPLC was 20 µl out of 2 ml extracts prepared from 20 (5 day old) and 25 (6 day old) leaf pieces (upper graphs). In separate feeding experiments, 20 (5 d) and 25 (6 d) leaf pieces were floated on an aqueous 50 µM cinnamic acid solution (samples) or on dest. water (controls) for 3.5 or 6.5 h. In two additional experiments when the substrate had been fed for 3.5 h, leaf pieces were washed with water and floated either on water for 3 additional hours (3.5 + 3 h H_2O) or on 0.6 M sorbitol solution for 3 h (3.5 + 3 h sorbitol).

Table I. Flavone radioactivity and dilution of label after feeding [^{14}C]phenylalanine and [^{14}C]cinnamic acid to leaf pieces for 3.5 h. Mean values calculated from experiments of Fig. 3 a, b.

Flavone content (nmol/5 leaf pieces)		Total radioactivity (dpm/5 leaf pieces) after feeding	
		[^{14}C]phenylalanine	[^{14}C]cinnamic acid
F_1	65	209×10^3	425×10^3
F_2	100	248×10^3	414×10^3
F_3	70	129×10^3	221×10^3

Flavone	Specific activity		Specific activity	
	(dpm/nmol)	dilution	(dpm/nmol)	dilution
F_1	3215	354	6538	15.7
F_2	2480	459	4140	24.8
F_3	1843	618	3157	32,6

Our results are in agreement with data obtained with *Petunia* petals [10]. When these organs were fed with solutions of 10 μM and 100 μM cinnamic acid, during an incubation period of 25 h, this concentration did not have any significant influence on the rate of anthocyanin accumulation. On the other hand, when high cinnamic acid concentrations, higher than 4–5 mM, are applied to excised buckwheat hypocotyls for 24 h, loss of turgor of the hypocotyl is observed; due apparently to damage of the tissue [15]. However, concentrations up to about 4 mM are tolerated by buckwheat, higher by a factor of 80 than levels fed to oat leaf pieces. Nevertheless, in cell suspension cultures of *Daucus carota* a reduction from 93 to 34% in anthocyanin accumulation was observed when cinnamic concentrations are increased from 0.1 μM up to 100 μM during a 72 h feeding period [22]. Furthermore, the growth rate of carrot cells was inhibited by 100 μM cinnamic acid.

Comparing the data of [^{14}C]phenylalanine and [^{14}C]cinnamic acid incorporation into flavone compounds of oats after 3.5 h of labelling, from the average, dilution factors may be calculated (Table I).

[^{14}C]cinnamic acid is incorporated from 19 to 23 times more efficiently into oat flavones than is [^{14}C]phenylalanine, and no significant differences are found in the relative proportions of dilution values of F_1–F_3. With *Petunia* petals, when similar experiments were performed to compare [^{14}C]phenylalanine and [^{14}C]cinnamic acid incorporation into anthocyanins [10], dilution values were lower by factors of 3.5 and 5 for individual *Petunia* anthocyanins after phenylalanine application compared to cinnamic acid. The differences of the *Petunia* and oat systems may be due to the fact that endogenous pools of the common phenylpropanoid pathway are influenced in different ways by the two precursors which were added exogenously. In addition, differences in uptake, transport within tissues and cells, utilization in other reactions must be considered [*cf.* 15].

3. Incorporation studies with [^{14}C]cinnamic acid into leaf pieces and mesophyll protoplasts

To estimate labelling patterns of several fractions, protoplasts were isolated after feeding [^{14}C]cinnamic acid to leaf pieces (Fig. 6). On the other hand, the labelled precursor was fed directly to isolated protoplasts (Fig. 7) to determine the autonomy of this cell type* to synthesize flavonoids. To check the fate of radioactivity during 3 h of incubation for protoplast isolation, one group of leaves were extracted immediately following 3.5 h on the [^{14}C]cinnamic acid solution and the other was floated for three additional hours on distilled water (Fig. 5).

Radioactivity distribution of several leaf fractions was almost identical in both cases. Accordingly, no significant influence on labelling patterns or change in radioactivity of fractions is expected during protoplast isolation.

Fig. 6 presents labelling data of leaf pieces prepared from 5, 5.5 and 6 day old leaves and corresponding protoplasts which were obtained after feeding the precursor to whole leaf pieces for 3.5 h followed by protoplast isolation (experiments 1–3). For a direct comparison of absolute radioactivity of the several fractions, values are indicated in dpm per 5 leaf pieces (left columns, l.p.) and in dpm per

* At present it is not known whether mesophyll protoplasts consist of a homogenous cell population with respect to flavone content (comp. [23]).

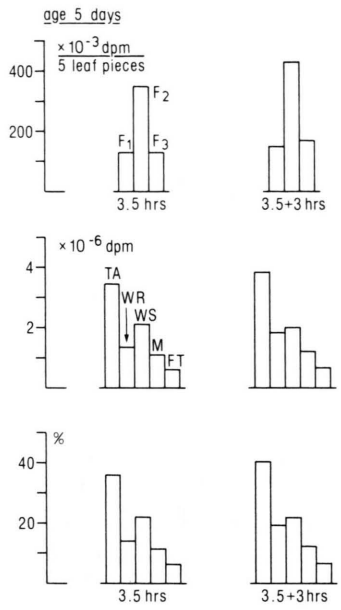


Fig. 5. Distribution of radioacitivity within fractions TA, WR, WS, MeOH, FT and F_1-F_3 (see legend Fig. 1) of 5 day old leaf pieces after 3.5 h of floating on a 46.4 µM [^{14}C]cinnamic acid solution, $c^0 = 9.5 \times 10^6$ dpm/5 leaf pieces (left diagram 3.5 h). The diagram on the right (3.5 + 3 h) demonstrates the same experiment but after the precursor had been administered for 3.5 h, leaf pieces were washed with distilled water to remove the label from the surface and were floated immediately on distilled water for 3 h. Values in dpm/5 leaf pieces and relative incorporation in per cent of $c^0 = 100\%$.

protoplasts corresponding to 15 leaf pieces (right columns, PP) with a protoplast yield of 100% calculated on the basis of chlorophyll recovery. The actual yield of protoplasts isolated from leaf mesophyll were estimated to be 93%, 78% and 76%, respectively, for the individual experiments, 1, 2 and 3. Consequently, a factor of three is to be considered when data of leaf pieces and protoplasts are compared per unit of leaf piece.

Several characteristics are striking. In the young 5 day stage compound F_2 is dominant in both whole leaf pieces and in isolated protoplasts. In older stages characteristic differences become obvious. For protoplasts, F_2 is the major flavone at all stages, whereas flavone labelling patterns of leaf pieces change with age. Six day old leaf pieces show high levels of flavone F_1 and compound F_3 increases markedly with age. For the three stages shown in Fig. 6, flavone content of isolated protoplasts was similar, 1.0 (± 10%) µmol per mg chlorophyll, and compound F_2 amounts to about 60% of the total both of which is in agreement with our earlier findings [6].

Compound F_a, isoorientin 2″-arabinoside, is not accumulated in whole oat primary leaves grown under our standard phytotron conditions [24]. Traces of F_a are detected occasionally in protoplasts (Fig. 6, expts 1 and 3) and flavone F_a is accumulated as a major component of field grown intact leaves, primarily of the mesophyll [5].

In Fig. 6 labelling patterns of several other fractions of leaf pieces and protoplasts are shown and remarkable differences are obvious: Highest label is found in the water insoluble residue (WR) of leaf pieces which amounts to 60% of the total activity. In contrast, protoplasts have a very low radioactivity of only 10% in the WR-fraction but show highest values in the water soluble portion (WS). However relative activity of total flavone (FT), in per cent of WS-fractions, increases with age in both cases, from approx. 30 to 100% for leaves and from 20 to 40% for protoplasts.

When total flavone radioactivity (FT) in dpm of protoplasts isolated from 15 leaf pieces (100% protoplast yield assumed; comp. Fig. 6) is related to that of 15 corresponding whole leaf pieces, the portion of protoplast FT-activity amounts to 39, 23 and 28%, respectively, for the 5, 5.5 and 6 day stage. This activity portion is lower, by about 20%, compared to that of the flavones accumulating in the whole mesophyll. Flavone content of whole mesophyll preparations were found to be in the range of 50% of that in leaf pieces consisting of upper epidermis plus mesophyll [4, 5]. Flavone radioactivity of protoplasts compared to whole mesophyll may be due to flavones, accumulating in other than photosynthetically active cells of the mesophyll or even in cell walls [25], lost during isolation procedures or during protoplast purification.

As oat flavones appear to be stable metabolic end products, one may assume that our results reflect differences in tissue specific flavone biosynthesis, primarily of the mesophyll and epidermis (comp. ref. [4–6]). Accordingly, photosynthetically active mesophyll cells seem to be a main site of flavone biosynthesis of the primary leaf. In a recent paper we have shown that mesophyll protoplasts from oat contain "flavanone synthase" activity, a key enzyme of specific flavonoid biosynthesis [26].

[^{14}C]cinnamic acid was fed to a suspension of intact mesophyll protoplasts (Fig. 7). At time inter-

vals indicated, $t_{30}-t_{180}$ min, identical portions of the homogenous suspension were removed. After washing and resuspending, the number of intact protoplasts was estimated and various fractions were analyzed. As was observed with protoplasts isolated after feeding parent tissues with labelled cinnamic acid (comp. Fig. 6), protoplasts which were directly incubated with the precursor showed highest radioactivity in the water soluble fraction (WS). This activity amounts to approx. 95% of total activity (TA) incorporated after 30 min of incubation. During time, up to 180 min, percentage decreases to 80%, whereas the proportion of insoluble material (WR) increases from 5 to 20%. One may speculate whether this is due to conversion of soluble cinnamic acid derivatives to insoluble polymers such as lignin or lignin-like substances during reconstitution of cell walls [27–29]. When the recovery of intact protoplasts is compared for different time intervals of incubation, up to 120 min evidently no decrease in protoplast number is observed and no visible change in protoplast morphology was stated. Thus, protoplasts remained intact when a 5.1 µM cinnamic acid concentration was used. Nevertheless, after 180 min of incubation the number of intact protoplasts was reduced to 50% of the starting material. However,

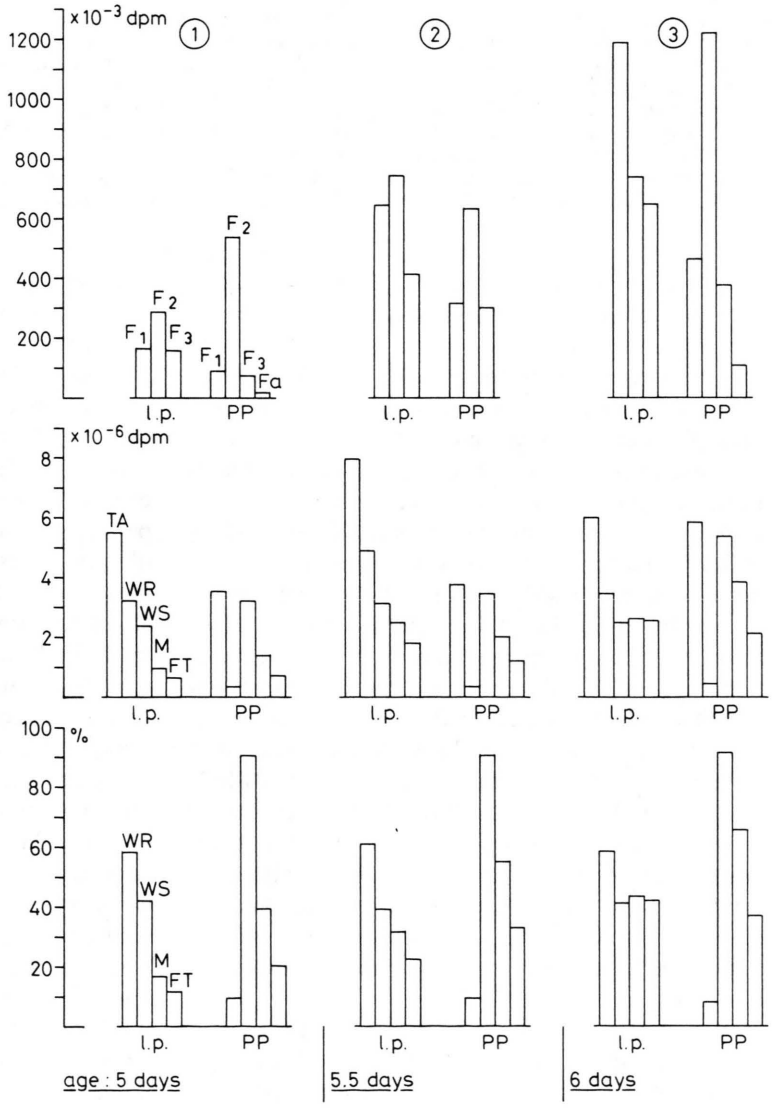


Fig. 6. Comparison of labelling patterns of leaf pieces and isolated protoplasts after feeding [^{14}C]cinnamic acid to leaf pieces followed by protoplast isolation. For each separate experiment, 4 × 5 leaf pieces with peeled epidermis were floated on a 8 ml aqueous [^{14}C]cinnamic acid solution, $c° = 10\ (\pm 0.8) \times 10^6$dpm/5 leaf pieces (48 µM soln) for 3.5 h, leaf age 5 (experiment 1), 5.5 (experiment 2) and 6 days (experiment 3). After the pulse, all leaf pieces were washed with distilled water and one portion of 1 × 5 leaf pieces floated for 3 additional hours on distilled water (left columns l.p.); the other portion of 3 × 5 leaf pieces was treated for protoplast isolation and after 3 h of lysis released protoplasts were washed three times in resuspension medium and collected by our standard techniques (right columns, PP). Values of fractions (see legend Fig. 1) and individual flavones F_1, F_2, F_3, (F_a) are indicated in dpm per 5 leaf pieces (l.p.) and in dpm per protoplasts (PP) isolated from 15 leaf pieces with a yield of 100% which was calculated after estimation of the actual protoplast yield in each experiment 1–3. Relative distribution of activity in various fractions calculated in %, being TA = 100% the radioactivity incorporated into undigested leaf pieces and into protoplasts, respectively.

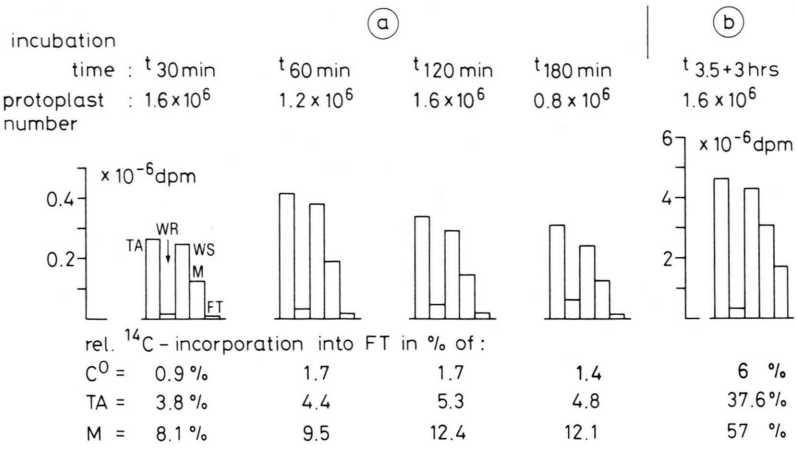


Fig. 7. Time-dependent labelling of various fractions of isolated protoplasts after feeding [^{14}C]cinnamic acid. For abbreviations see legend of Fig. 1. (a) Protoplasts were isolated from leaf pieces, age 6 days, and to a 8 ml suspension containing 6×10^6 intact protoplasts, 20 µl of [^{14}C]cinnamic acid were added, $c^0 = 4.2 \times 10^6$ dpm; resulting conc. 5.1 µM. For each time of incubation ($t_{30}-t_{180}$ min) a 2 ml portion of the homogenous suspension of protoplasts was removed, centrifuged, washed with resuspension medium once (1:10 by vol) and resuspended to 2 ml. After checking the protoplast titer at $t_{30}-t_{180}$ min in comparison to t_0, incorporated radioactivity was analysed. Values in dpm/2 ml of protoplast suspension containing 1.6 to 0.8×10^6 intact protoplasts, as indicated. (b) Distribution of radioactivity of 1.6×10^6 intact protoplasts isolated from leaf pieces fed with a 48 µM [^{14}C]cinnamic acid concentration before protoplast isolation. 6 day stage, 3.5 h of labelling followed by a 3 h lysis and protoplast isolation (comp. Fig. 6).

when *e. g.* the total radioactivity (TA) is calculated per 1.6×10^6 protoplasts and is compared with the value obtained for 30 min, an increase by a factor of 2.4 is stated. Furthermore, 23% to 55% of the precursor activity initially applied is taken up into protoplasts under these conditions.

When single flavones were analyzed, compounds F_1 and F_2 were found to be primarily labelled; radioactivity of F_2 was about two to three fold as much as with F_1. In compound F_3 only a trace of activity was detected which could be due to direct transformation of F_3 to F_1. In addition, various unidentified radioactive compounds moving in the "hydroxycinnamate area" of TL chromatograms [7] were observed which are not detectable on chromatograms prepared from extracts of intact leaf tissues. Thus, isolated protoplasts contain several enzymes which must be required for this cell type to be autonomous in specific flavone biosynthesis. When intact protoplasts were treated for 2–4 sec with ultrasonication, followed by [^{14}C]cinnamic acid feeding, no reaction product was formed and cinnamic acid was left completely, apparently due to destruction of cell compartmentation.

When total flavone radioactivity (FT) is considered, comparative low amounts are found (see below). However, when the relative ^{14}C-incorporation into flavones originating from 5.1 µM cinnamic acid (C° = 100%) is calculated, a distinct increase is observed from 0.9% after 30 min to 1.7% after 60 or 120 min of incubation (Fig. 7). A similar enhancement is found when values are related to total radioactivity (TA) or to that of the methanol fraction (M).

In Fig. 7 dpm-values of the various fractions as expressed per protoplast number (a) may be compared with data obtained for a comparable number of protoplasts (b) which have been isolated after feeding a ca. 10-fold cinnamic acid concentration of 48 µM to parent leaf tissues (comp. Fig. 6, experiment 3). It is evident that protoplasts which were directly fed with the low 5.1 µM cinnamic acid concentration incorporate about a tenth of that of the high precursor concentration. Differences suggest that uptake behavior of isolated protoplasts and intact mesophyll cells is similar related to cinnamic acid. In contrast, flavone radioactivity recovered from isolated protoplasts is markedly lower, by factors of 5 to 8, when either based on absolute (dpm) or relative (per cent of TA, WS or M) values. However, we cannot at present draw conclusions whether the low radioactivity reflects flavone biosynthetic activity of isolated protoplasts; labelled flavone may have been lost from protoplasts during washing procedures.

Summarizing our results, we conclude that under the conditions of a defined feeding experiment, mesophyll protoplasts synthesize flavones usually accumulating in this cell type in the mesophyll. Differences or a decrease in metabolic activity as observed with oat protoplasts compared to the intact tissue, may be due to the fact that metabolic channeling of the precursor or of intermediates usually occurring in the tissue and leading to high concentrations of specific end products, flavones $F_1 - F_3$, is interrupted to a major part when isolated protoplasts are used. It may be mentioned that release of one protoplast necessitates cutting and resealing of $10^3 - 10^4$ protoplasmic connections with neighbouring cells [30] and therefore pool sizes of intermediates are changed and physiological gradients are broken down. Furthermore, plasmolysis may also cause a serious trauma.

On the other hand, similar feeding experiments were performed using [1-^{14}C]acetate with protoplasts isolated from *Nemesia strumosa* petals [23] and it was demonstrated that these protoplasts do contain a whole set of enzymes involved in specific anthocyanin biosynthesis of cyanidin-3-xylosylglucoside. But in contrast to oat, *Nemesia* protoplasts show higher incorporation values for the anthocyanin and specific activity was found to be more than three times higher than that obtained with parent tissues. At present we cannot decide whether differences of the two cell types are due to reduction of "physiological integrity" of oat protoplasts under our conditions or whether they are due to cell- or tissue-specific characteristics of a photosynthetically active and photosynthetically inactive system. Further experiments are necessary to characterise the enzymatic profile of oat protoplasts with respect to phenylpropane and flavone biosynthetic pathways. Comparative studies with epidermal cells or protoplasts from the epidermis are promising in order to determine tissue specific differences of oat primary leaves [31].

Acknowledgements

Financial support by the Deutsche Forschungsgemeinschaft is gratefully acknowledged. We thank J. W. McClure, Oxford, OH, for valuable discussions and suggestions on the manuscript.

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