Phytochemistry, Vol. 24, No. 2, pp. 253–257, 1985.
Printed in Great Britain.

0031–9422/85 $3.00 + 0.00

BIOSYNTHESIS AND DEGRADATION OF α-TOMATINE IN DEVELOPING TOMATO FRUITS

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(*Received* 16 *May* 1984)

Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato; fruit; α-tomatine; biosynthesis; degradation.

Abstract—Fruits of tomato incorporated [2-^{14}C]mevalonic acid lactone into the steroidal glycoalkaloid α-tomatine. Young fruits showed the greatest alkaloid-synthesizing ability but this decreased as the fruits developed. Analysis of sap exuded from fruit stalks and also application of [4-^{14}C]cholesterol to leaves confirmed that tomatine is not transported into fruits from vegetative organs. Accumulation of this alkaloid in fruits thus appears entirely due to synthesis. Excised fruits of all developmental stages degraded injected [^{14}C]tomatine and rates were directly related to fruit age. The pattern of accumulation/decline in fruit tomatine may be explicable on the basis of changing capacity for synthesis/degradation during development. Label from injected [^{14}C]tomatine was present mainly in chlorophylls and carotenoids where it increased with time as that in tomatine decreased. The significance of the relationship between tomatine disappearance and carotenoid development is briefly discussed. The aglycone tomatidine was not detected in green fruits but a Δ^{16}-5α-pregnenolone-like compound was.

INTRODUCTION

In the vegetative tomato plant, the shoot (especially the leaves) and, to a lesser extent, the root are the known major sites of synthesis and accumulation of the steroidal glycoalkaloid α-tomatine [1]. Recently, it was shown [2] that the alkaloid content of fruits increases up to a particular developmental stage and thereafter declines, with the result that young fruits may contain significantly higher tomatine levels than vegetative organs, and mature fruits much less [1]. As yet, however, knowledge of how tomatine accumulates in and subsequently disappears from fruits is meagre. Sander [3] and Ali and Schlösser [4] assumed that leaf-synthesized tomatine is transported to fruits where it is degraded during ripening. Sander [3] found that de-fruited plants accumulate more alkaloid than fruit-bearing plants and also that fruit number and plant tomatine are inversely correlated. The idea of long-distance transport of tomatine has recently been questioned [5] but, nevertheless, the fruit still tends to be viewed principally as an alkaloid-degrading organ, a fact confirmed by injection of 'cold' [3] and radioactive [6] tomatine. The products of tomatine breakdown are not certain. One proposal is that tomatine catabolites are channelled into carotenoid biosynthesis in ripening fruits [7, 8], but 3β-hydroxy-5α-pregn-16-en-20-one has also been implicated [6] in tomatine degradation.

The aim of the present study was to clarify how tomato fruits accumulate tomatine, the extent of this process, and the fate of the degraded steroid nucleus. Such knowledge could have practical value since fruits of related species such as *Solanum khasianum* and *S. mammosum*, the alkaloid of which (solasodine) is being used increasingly for the preparation of medicinal steroids, show a similar pattern of alkaloid accumulation and decline [9, 10].

RESULTS

The ability of fruits to synthesize tomatine was investigated by applying [2-^{14}C]mevalonic acid lactone (MVAL) to fruits ranging from 12 to 63 mm in diameter and from green to pink/red in colour. As shown in Fig. 1, fruits of all developmental stages incorporated MVAL into the alkaloid, the extent of incorporation being inversely related to fruit size. Incorporation into pink/red fruits was 18% of that into large green fruits, which in turn was only 11% of that into small green fruits. Thus, large almost ripe fruits had only 2% of the biosynthetic capacity of small unripe fruits. This pattern of incorporation is closely reflected in the pattern of alkaloid accumulation so that values for the specific activity of tomatine were quite constant. However, the specific activity data are of dubious value due to differential dilution by existing alkaloid and the fact that smaller fruits show a net accumulation of alkaloid during the feeding period while larger fruits show a net loss [2].

The possibility of tomatine transport from vegetative organs to fruits was examined in the first place by analysing sap exuded from fruit stalks following excision of green fruits. A small volume of sap (approximately 7 ml) was obtained from a total of ten fruits over a period of 10 days and analysed by TLC (sensitivity approximately 1 μg) but tomatine was not detected. A radiotracer method was therefore employed involving application of [4-^{14}C]cholesterol to leaves. It was appreciated that some labelled sterol might be transported from leaves to fruits where it could be incorporated into tomatine but this was likely to be very small (< 0.2%) [11]. In the event, there was no significant labelling of fruit tomatine but only of tomatine in treated leaves (Table 1).

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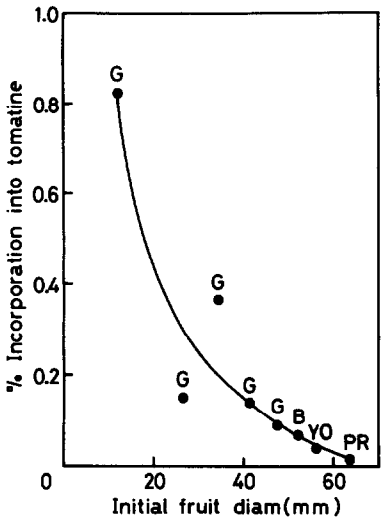

Fig. 1. Incorporation of [^{14}C]MVAL into tomatine in fruits at different stages of development. Aqueous [2-^{14}C]MVAL was applied to the surface of intact tomato fruits daily for 10 days. Letters represent the colouration of fruits at the time of application. G = green, B = breaker, Y/O = yellow/orange, P/R = pink/red. Each point is the mean of five replicates.

Table 1. Distribution of radioactive tomatine in tomato shoots following application of [^{14}C]cholesterol to leaves

Source	Radioactivity (dpm)	Incorporation (%)
Background	20	—
Leaf tomatine	187 812	0.59
Stem tomatine	80	0.00025
Fruit tomatine	28	0.00009

[4-^{14}C]Cholesterol in acetone solution was applied to young leaves three times weekly for 12 weeks. Fruits developed after 9 weeks and material was harvested at week 13. Values are the means of four replicates.

Table 2. Size and appearance of tomato fruits injected with [^{14}C]tomatine

Ref No.*	Diameter range (mm)	Colouration
1	10–19	Green
2	22–30	Green
3	32–38	Green
4	40–50	Breaker
5	54–62	Yellow/orange

*See Figs. 2–5.

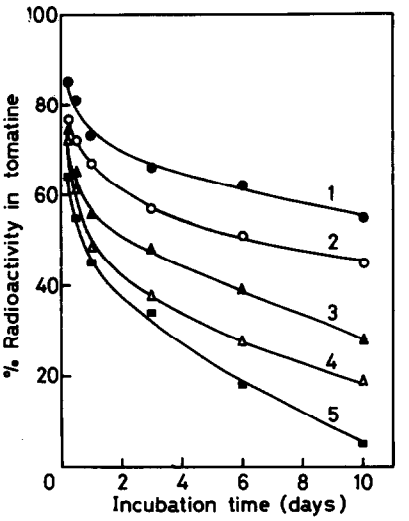

Fig. 2. Radioactivity in tomatine from tomato fruits of different developmental stages following application of [^{14}C]tomatine. Tomatine in PO_4 buffer, pH 5.8, was injected into excised fruits of different sizes. Numbers refer to fruit size and appearance as indicated in Table 2. Tomatine was extracted after varying incubation periods. Each point is the mean of four replicates.

The fate of degraded tomatine was studied by injecting ^{14}C-labelled tomatine into fruits at different stages of development (Table 2) and extracting the label after varying incubation periods. In all fruits, recovery of labelled tomatine decreased progressively with time and according to initial fruit size (Fig. 2). From small green fruits, 85% of the label was recoverable in tomatine after 6 hr but this fell to 56% after 10 days. Corresponding values for the largest fruits were 64% after 6 hr and 5% after 10 days. Fruits between these sizes gave intermediate values.

Of the remainder of the applied radioactivity, 2.5–4% was recovered in an acid fraction which was not identified, 3–4% was not accounted for, and the majority was found in a benzene fraction rich in pigments. In small fruits, only 8% of the label was present in this fraction after 6 hr but this rose to 35% after 10 days. In large fruits, where pigmentation changes were more apparent, initial and final labelling rates in the benzene fraction were 28 and 89%, respectively. TLC of this fraction revealed three major components corresponding to chlorophylls, xanthophylls and carotenes which were separated and assayed. In fruits of all sizes, 7–10% of the label was detectable in chlorophylls after 6 hr. In small fruits, this value increased to 18–24% after 10 days but in large fruits, where carotenoid development occurred, the value increased for *ca* 3 days then declined and at 10 days was close to the initial level (Fig. 3). Incorporation into xanthophylls was initially lower than that into chlorophylls (0.8–8%) but increased in all fruits to a similar level after 10 days (10–25%) (Fig. 4). In small fruits, labelling of carotenes after 6 hr (0.6%) was less than that of other pigments whereas in large fruits it was greater (12%). Labelling increased with time in all fruits and at 10 days carotenes from the smallest fruits (which were still green) had only about half the label found in chlorophylls (7%) while in the large, reddening fruits the value was 57% (Fig. 5).

Green fruits of two sizes (17–25 mm and 40–50 mm diameter) were extracted for the aglycone tomatidine and 3β-hydroxy-5α-pregn-16-en-20-one. Addition of auth-

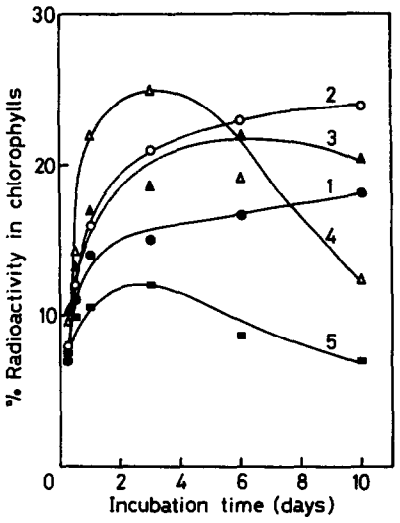

Fig. 3. Radioactivity in chlorophylls from tomato fruits of different developmental stages following application of [^{14}C]tomatine. Tomatine in PO_4 buffer, pH 5.8, was injected into excised fruits of different sizes. Numbers refer to fruit size and appearance as indicated in Table 2. Chlorophylls were extracted after varying incubation periods. Each point is the mean of four replicates.

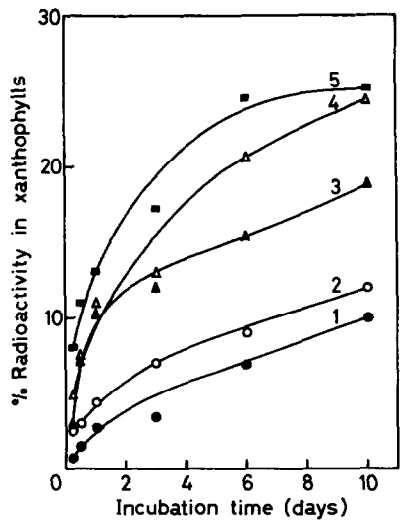

Fig. 4. Radioactivity in xanthophylls from tomato fruits of different developmental stages following application of [^{14}C]tomatine. Tomatine in PO_4 buffer, pH 5.8, was injected into excised fruits of different sizes. Numbers refer to fruit size and appearance as indicated in Table 2. Xanthophylls were extracted after varying incubation periods. Each point is the mean of four replicates.

entic standards to extracts indicated that these compounds could be recovered in 67–70% yields from tomato fruits at a TLC detection limit of 5–10 μg. TLC in up to four solvents failed to reveal either free compound in extracts. From the weight of tissue and volumes used and the calculated detection limits and efficiency of extraction,

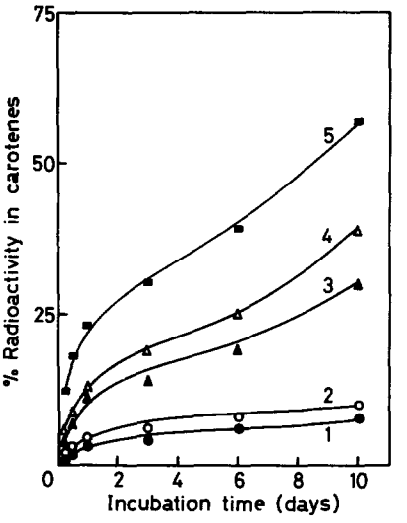

Fig. 5. Radioactivity in carotenes from tomato fruits of different developmental stages following application of [^{14}C]tomatine. Tomatine in PO_4 buffer, pH 5.8, was injected into excised fruits of different sizes. Numbers refer to size and appearance as indicated in Table 2. Carotenes were extracted after varying incubation periods. Each point is the mean of four replicates.

it can be concluded that free tomatidine and Δ^{16}-5α-pregnenolone, if present, must be less than 0.5 μg/g and 0.7 μg/g fruit, respectively. However, when a fruit extract was acid-hydrolysed, a TLC spot corresponding to Δ^{16}-5α-pregnenolone in two solvent systems and in H_2SO_4–chromogen characteristics was apparent in the neutral fraction.

DISCUSSION

Radioactive precursors have earlier been used to demonstrate tomatine synthesis in vegetative organs such as shoots [12] and roots [13]. The radio-labelling data reported here represent the first definitive evidence that synthesis of this alkaloid also occurs in fruits. The inverse relationship between biosynthetic rate and fruit size (and growth) is in keeping with earlier work on vegetative organs which established the interdependence of synthesis and growth [3]. It is interesting that almost mature fruits, in which there has been a net loss of tomatine so that levels are very low, are still capable of alkaloid synthesis. However, the possibility that exogenously supplied precursors might have influenced biosynthetic rates cannot be ruled out at this stage.

Our findings do not support suggestions [3, 4] that tomatine accumulates in fruits as a result of transport from the shoot but are in agreement with recent radiotracer studies [5] which refute transport within or between root and shoot. We are of the opinion that tomatine (and possibly other glycoalkaloids) is not transported either on a large scale or over long distances and, indeed, it is difficult to envisage why such transport should be necessary when all the major organs of the tomato plant are capable of synthesizing the alkaloid. We conclude therefore that accumulation of tomatine during the early stages of fruit development [2] must be due entirely to synthesis.

Although a net decrease in fruit tomatine does not normally occur until fruits are around 28 days old [2], fruits of all developmental stages apparently possess the ability to break down tomatine. This degradative capacity is not therefore unique to ripening fruits as was earlier thought [3], although it does appear to intensify with maturation. Again, caution is necessary when interpreting data derived from introduction of exogenous alkaloid. Development of the tomato fruit would appear to be associated with a decline in its tomatine-synthesizing ability and enhancement of its tomatine-degrading activity. Thus, the biphasic pattern of accumulation of tomatine followed by its disappearance observed in developing fruits [2] is possibly explicable on the basis of changes in the relative rates of synthesis and degradation.

Inverse correlations between tomatine and carotenoids in developing fruits have been reported earlier [7, 8], suggesting utilization of tomatine-degradation products in carotenoid synthesis. Our data provide further evidence in support of this hypothesis. Interestingly, label is also channelled into chlorophylls (particularly in green fruits), although this diminishes as labelling of carotenoids increases. Tomatine catabolites might therefore be utilized for carotenoid biosynthesis either directly, or indirectly via chlorophylls, depending on the developmental stage of the fruit. Such transformations would necessitate metabolite transfer between the vacuole/cytosol [14], chloroplasts and chromoplasts but this has not yet been demonstrated. However, although pigment development and tomatine disappearance may be closely correlated, the relationship is neither obligatory nor causal [2, 15] but may simply reflect the existence of an isoprenoid-scavenging pathway.

Our inability to detect free tomatidine, but the detection of Δ^{16}-5α-pregnenolone in hydrolysed extracts, is consistent with earlier work [3, 6, 16, 17]. The latter compound (or its glycoside) may only be a transient compound in tomatine degradation. This is an area in need of further study.

The biological significance of tomatine degradation is not certain. If, as is widely thought, this alkaloid functions in a protective capacity [18] and carotenoids such as lycopene are primarily attractants of vectors of seed dispersal [19], then the changing (and particularly inverse) accumulation patterns of these compounds become possibly more explicable. Of further interest is that tomatine may be functioning in a dual ecological capacity—initially and directly as a deterrent and subsequently as the source of a key attractant.

EXPERIMENTAL

Plant material. Seed of tomato (*Lycopersicon esculentum* Mill.) cv Sutton's Potentate Best of All was germinated in John Innes No. 1 compost at 20–25°. Seedlings were transferred to No. 2 compost at 10–14 days. Some plants were grown in a greenhouse at 20–25°. Lighting was natural except between October and March when it was supplemented with Hg vapour illumination which also maintained a 16-hr day. These plants were given a liquid feed every 2 weeks. Other plants were grown in a constant environment chamber with a 16-hr 25° day, 15° night, relative humidity 70% and light intensity 1350 μE/m^2 per sec. Following excision, fruits were rinsed with tap H_2O, surface-dried with tissue and their weight, maximum diameter and colouration noted. Where possible, treatment or collection of fruits was randomized between trusses, etc.

Extraction and assay of tomatine. The extraction procedure has been described elsewhere [14]. The assay used for 'cold' tomatine was based on the radioligand method of Heftmann and Schwimmer [20]. Recovery of added authentic tomatine (Sigma) indicated the efficiency of the extraction procedure to be approximately 85%. Radioactive tomatine was first purified by TLC as described below and assayed by a H_2SO_4–chromogen method [21]. The adsorbent used in all TLC analyses was silica gel. For both methods, standard curves were prepared using authentic tomatine.

Application of [^{14}C]MVAL to fruits. [2-^{14}C]MVAL (sp. act. 0.67 GBq/mM; Amersham) as an aq. soln (185 kBq/ml) was applied to the skin of intact fruits ranging from 12 mm diameter (green) to 63 mm diameter (pink/red) daily for 10 days. The total radioactivity administered per fruit was between 85 and 135 kBq. Three days after the last application, fruits were removed and tomatine was extracted. Extracts were subjected to TLC in *i*-PrOH–HCOOH–H_2O (73:3:24), MeOH–EtOAc–NH_4OH (5:4:1), CH_2Cl_2–MeOH (92:8) and CH_2Cl_2–MeOH–H_2O (70:26:4) and tomatine was purified to constant radioactivity. Throughout this work, the TLC-locating reagent used was modified Dragendorff's reagent [21] and radioactivity determinations were made by liquid scintillation spectrometry.

Application of [^{14}C]cholesterol to leaves. [4-^{14}C]Cholesterol (sp. act. 2.16 GBq/mM; Amersham) in Me_2CO soln (37 kBq/ml) was applied to the first leaves of young tomato plants. On each plant, four leaves were treated with 0.1 ml soln 3 × weekly for 12 weeks. Fruits developed 9 weeks after the start of the experiment. One week after the last application, treated leaves, stems and fruits were harvested, washed, dried and extracted. Tomatine was purified by TLC in *i*-PrOH–HCOOH–H_2O (73:3:24), CH_2Cl_2–MeOH (23:2) and CH_2Cl_2–MeOH–H_2O (70:26:4) to constant radioactivity.

Preparation of radioactive tomatine. The method was based on that reported elsewhere [6]. A 0.2 ml aliquot of [4-^{14}C]cholesterol (sp. act. 2.16 GBq/mM; Amersham) in Me_2CO (185 kBq/ml) was applied 3 × weekly for 9 weeks to each of seven leaves of young tomato plants. Five days after the last application, leaves were removed and extracted by methods described previously [16]. Tomatine was purified by TLC in *i*-PrOH–HCOOH–H_2O (73:3:24) and MeOH–EtOAc–NH_4OH (5:4:1) to constant radioactivity. The total amount of tomatine extracted was 140 mg and the total radioactivity recovered in the alkaloid 51 kBq representing a sp. act. of 364 kBq/mM and an incorporation of 0.73%.

Application of [^{14}C]tomatine to fruits. [^{14}C]Tomatine was dissolved in 0.05 M PO_4 buffer, pH 5.8, and injected into excised fruits of five sizes as indicated in Table 2. Within each size, four replicate fruits were used and each received 417 Bq of radioactivity in a vol. ranging from 0.1 ml (small fruits) to 0.4 ml (large fruits). Treated fruits were incubated in a constant environment chamber (22–25°, 16-hr day, 70% relative humidity) for varying periods of time and then extracted as described below.

Recovery of radioactivity from fruits. Fruits were extracted in 96% MeOH containing 2% HOAc (2 ml/g fr. wt), and then Buchner-filtered and extracted in 64% MeOH (× 2). Extracts were combined, reduced to dryness under vacuum at 45°, and taken up in 50 ml 2% HOAc. Repeated partitioning with C_6H_6 removed pigments, after which the HOAc fraction was evapd to dryness and taken up in MeOH. MeOH-insoluble material was re-dissolved in 2% HOAc. Distribution of radioactivity in the three fractions (C_6H_6, MeOH, HOAc) was determined. Only a small proportion of the label (2.5–4%) was present in the HOAc fraction (the components of which were not identified) with most in the C_6H_6 and MeOH fractions. TLC and radiochromatogram scanning revealed that the only radioactive component of the

MeOH fraction was tomatine. This was further purified by TLC in *i*-PrOH–HCOOH–H_2O (73:3:24) and its radioactivity determined. Similar analysis of the C_6H_6 fraction revealed three major bands of radioactivity which coincided with three pigmented bands. TLC in petrol–Me_2CO (9:1) and EtOAc–petrol (3:40) confirmed these to be chlorophylls, xanthophylls and carotenes. These were eluted with C_6H_6 and their radioactivity was determined.

Analysis of fruits for tomatidine. Duplicate batches of fruits (80 g) of two different sizes (17–25 mm and 40–50 mm diameter) were extracted in acidified aq. MeOH as indicated above. Combined extracts were reduced to a small vol. under vacuum at 45°, acidified with 2% HOAc, and ammonified to pH 10. After incubating at 4° overnight, extracts were centrifuged at 27 000 *g* for 30 min at 4°. The supernatant was discarded and the pellet washed 2 × with 1% NH_3. The final pellet was dried in a $CaCl_2$ desiccator and extracted with Et_2O (3 ×) with centrifugation, as above, between each extraction. The Et_2O extracts were combined and evapd to dryness at 30°. Flask contents were washed with C_6H_6 to remove pigments and then re-dissolved in Et_2O. Extracts were subjected to TLC in $CHCl_3$–MeOH (94:6), *i*-PrOH–HCOOH–H_2O (73:3:24), *n*-BuOH–HOAc–H_2O (4:1:5) and CH_2Cl_2–MeOH (92:8). Chromogen formation following spraying with 50% H_2SO_4 and heating to 100° [22] was checked. Recovery of added authentic tomatidine (Sigma) gave an extraction efficiency of approximately 67%.

Analysis of fruits for Δ^{16}-5α-pregnenolone. The method employed was a modification of that described elsewhere [6]. Batches of fruits (100 g) of two different sizes (17–25 mm and 40–50 mm diameter) were used. For each size, two batches were homogenized in H_2O, one of which was subjected to HCl hydrolysis [6]. Non-hydrolysed homogenates were acidified with HOAc. Both acid extracts were partitioned with CH_2Cl_2 (× 3). The aq. fraction was made alkaline with NaOH and extracted with CH_2Cl_2 (× 3) to give a 'basic CH_2Cl_2 fraction'. The acidic CH_2Cl_2 extract was partitioned with an equal vol. of 1 M NaOH leaving a 'neutral CH_2Cl_2 fraction'. The aq. fraction was acidified with HOAc and extracted with CH_2Cl_2 (× 3) to produce an 'acidic CH_2Cl_2 fraction'. The three fractions were dried, reduced to a small vol. under vacuum, and analysed by TLC using *n*-hexane–EtOAc (1:1). A compound corresponding in R_f to authentic 3β-hydroxy-5α-pregn-16-en-20-one (Sigma) was present in the hydrolysed neutral fraction and was eluted with CH_2Cl_2 and subjected to further TLC using EtOAc–MeOH (19:1) followed by C_6H_6–EtOAc (2:1). Chromogen characteristics with 50% H_2SO_4 and heating to 100° were also checked. The efficiency of these procedures as indicated by recovery of authentic pregnenolone was approximately 70%.

Acknowledgements—We thank Mr. J. Kingdon, University of Exeter, for growing the plants. E.A.E. gratefully acknowledges receipt of a Research Scholarship from the University of Khartoum, Sudan.

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