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IN VIVO SYNTHESIS OF STIGMASTEROL IN *NICOTIANA TABACUM*

CLAUS GRUNWALD

Illinois Natural History Survey and Department of Plant Biology, University of Illinois, Champaign, IL 61820, U.S.A.

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; phytosterols; stigmasterol; sitosterol; biosynthesis.

Abstract—Six-day-old tobacco seedlings rapidly incorporated and metabolized exogenously supplied [4-^{14}C]-sitosterol, but none of the radioactivity was recovered from stigmasterol. However, exogenously supplied [2-^{14}C]-mevalonic acid was incorporated into both sitosterol and stigmasterol. Based on these results it is suggested that the biosynthetic pathway of stigmasterol is not via sitosterol but that both sterols have a common precursor.

INTRODUCTION

In most vascular plants, sitosterol (stigmasta-5-en-3β-ol) and stigmasterol (stigmasta-5,22-diene-3β-ol) are the two major 4-demethylsterols. Both sterols have 29 carbon atoms and they differ only in that stigmasterol has a double bond at C-22(23) in its C-17 side chain, whereas sitosterol has a saturated C-17 side chain. Because of the structural similarities of these two sterols, a common biosynthetic pathway, sitosterol → stigmasterol, has been postulated [1, 2]. The existence of a sitosterol-22,23-dehydrogenase finds support in biosynthetic studies following the incorporation of radioactive mevalonic acid (MVA), where the initial specific and total radioactivity of sitosterol was much greater than that of stigmasterol [1, 3, 4], thus supporting the precursor–product relationship. Direct conversion of radioactive sitosterol to stigmasterol, however, has seldom been studied, and the results from the different laboratories do not agree. Walters and Johnson [5] used soybean (*Glycine max*) leaf sections to generate radioactive sitosterol and also to study the conversion of [^{14}C]-sitosterol to stigmasterol. During a 5-day incubation period, using 0.07 μCi per incubation, they found no conversion of sitosterol to stigmasterol. It might be argued that the low level of radioactive sitosterol employed would not allow for an extensive detection of radioactive stigmasterol. Bennett and Heftmann [6] used *Digitalis lanata* to reexamine this question and found that with chemically produced [3-^{14}C]-sitosterol, applying 0.26 μCi twice a week for 5 weeks, 0.41% of the administered sitosterol was recovered as stigmasterol. They interpreted the data as proof that sitosterol can serve as a direct precursor for stigmasterol; however, these low conversion rates of sitosterol over the long incubation period can hardly explain the short term biosynthetic results obtained with MVA [1, 3, 4] which require a much more rapid conversion. In a more recent investigation, Navari-Izzo and Izzo [7] tried to clarify this question. They used barley (*Hordeum vulgare*) seedlings and found very rapid metabolism of incorporated [4-^{14}C]-sitosterol. After a 2.5 hr pulse only 5% of the administered ^{14}C was recovered as sterol, but of this activity, 8% was found as stigmasterol and after 6 hr, 20% of the sterol radioactivity was recovered in the stigmasterol fraction. Furthermore, using [4-^{14}C]-sitosterol in combination with [22,23-^{3}H]-sitosterol, they quite convincingly showed that the radioactive stigmasterol must have been derived from sitosterol.

In vivo experiments with tobacco (*Nicotiana tabacum*) seedlings, which are presented in this communication, do not support the data obtained with barley seedlings [7]. With tobacco seedlings the administered radioactive sitosterol was only slowly metabolized, and only background levels of radioactivity were recovered from stigmasterol even though MVA was readily incorporated into both sitosterol and stigmasterol. The suggestion is put forward that in dicotyledons, such as tobacco, soybean and *Digitalis*, sitosterol does not serve as the direct precursor for stigmasterol.

RESULTS AND DISCUSSION

To study the conversion of sitosterol to stigmasterol, a 3 hr [4-^{14}C]-sitosterol pulse was administered to 6-day-old tobacco seedlings, and after removal of the substrate, the seedlings were incubated in water for various time periods. The tobacco seedlings readily incorporated [4-^{14}C]-sitosterol, and of the radioactivity that was taken up after a 3 hr pulse, 74–82% was, after digitonin precipitation, recovered as 4-demethylsterol. The free sterol fraction had 86–90% of the sterol radioactivity, while the steryl glycosides and the steryl esters had 6–9 and 3–4%, respectively. The amount of radioactivity recovered from the free sterols decreased with incubation time but even after 24 hr, better than 70% was recovered from this fraction. These results differ significantly from those obtained with 6-day-old barley seedlings [7]. As with tobacco, barley rapidly incorporated radioactive sitosterol, but after a 2.5 hr pulse only 5.1% of the accumulated ^{14}C activity was recovered as 4-demethylsterol, and after 24 hr the level had dropped to 4.4%. In barley, 91% of the sterol recovered radioactivity was in the free sterol fraction, while the steryl glycoside and ester fractions had 8.4 and 0.6%, respectively. Barley seedlings were able to metabolize administered radioactive sitosterol 15 times more rapidly than tobacco seedlings. The percent distribution of ^{14}C activity among the various sterol

classes, however, was quite similar. Because 90% of the sterol associated radioactivity in tobacco was in the free fraction, that is those sterols that could be precipitated with digitonin without saponification, only this fraction was further investigated for the possible conversion of sitosterol to stigmasterol.

In a 1975 review, I [8] reported that I had observed a 10% conversion of sitosterol to stigmasterol with tobacco seedlings. These results were not questioned at the time because a substrate to product relationship for sitosterol and stigmasterol was generally assumed [1, 2] on the bases of indirect evidence [1, 3, 4]. However, upon further investigation it became evident that the stigmasterol component, as separated by 12.5% $AgNO_3$–silica gel TLC with chloroform as eluent, contained a highly radioactive contaminate which under proper condition could be resolved. Table 1 shows the radioactive TLC profile obtained from the acetylated digitonin precipitated free sterol fraction. In this experiment the tobacco seedlings were administered a 3 hr [4-^{14}C]-sitosterol pulse followed by a 12 hr incubation. Stigmasteryl acetate (R_f 0.35–0.45) was well resolved from sitosteryl acetate (R_f 0.59–0.76) and from the unknown component of relatively high radioactivity (R_f 0.10–0.18). Clearly stigmasterol did not become radioactive, and the radioactivity of the acetylated stigmasterol zone was that of background as established with [4-^{14}C]-sitosteryl acetate.

In kinetic studies using a 3 hr [4-^{14}C]-sitosterol pulse, the radioactivity of sitosteryl acetate decreased with time, but essentially none of the ^{14}C activity was recovered as stigmasteryl acetate (Fig. 1). The unknown component at R_f 0.10–0.18, however, was labeled immediately after the pulse and it continued to accumulate radioactivity. After 60 hr of incubation the unknown component accounted for 20% of the total digitonin precipitatable radioactivity. The results with tobacco seedlings agree with those obtained with [^{14}C]-sitosterol administered sectioned soybean leaves in which the TLC steryl acetate radiogram had a radioactive zone which corresponded to sitosteryl acetate, but none which corresponded to stigmasteryl acetate [5]. In soybean, a more polar radioactive region which remained near the origin was also observed. It is not clear whether that component is the same as the component observed in tobacco which is more polar than

Table 1. TLC profile of steryl acetates isolated from tobacco seedlings administered a 3 hr [4-^{14}C]-sitosterol pulse and incubated for 12 hr

Zone R_f	Reference compound as acetate	^{14}C-activity (%)
origin–0.10	—	3.2
0.10–0.18	—	11.2
0.18–0.35	—	1.7
0.35–0.45	stigmasterol	1.3
0.45–0.59	—	2.7
0.59–0.76	sitosterol	77.9
0.76–1.00	—	2.0

Free sterols were precipitated with digitonin, acetylated and separated on 12.5% $AgNO_3$–silica gel using $CHCl_3$ as eluant. Total radioactivity of plate was 39 990 dpm.

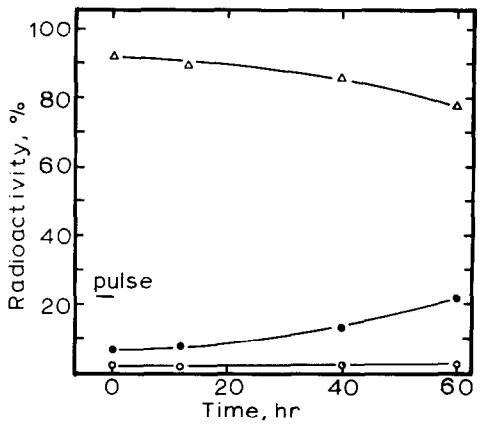

Fig. 1. Distribution of radioactivity of digitonin ppt free sterol fraction isolated from tobacco administered a 3 hr [4-^{14}C]-sitosterol pulse and incubated for various time periods. Sitosteryl acetate (△), stigmasteryl acetate (○), and radioactive component at R_f 0.1–0.2 (●).

stigmasterol (Table 1). Bennett and Heftmann [6], using *Digitalis lanata* plants to administer [3-^{14}C]-sitosterol to leaves over a 6-week period and found that the highest amount of radioactivity, after acetylation of the sterol fraction, was in the sitosteryl acetates, but a minor quantity of ^{14}C activity was recovered from the stigmasteryl acetate zone. Calculations based on the recovery of radioactivity gives a sitosterol to stigmasterol conversion rate of 0.41% over 6 weeks. The authors cite this as proof that stigmasterol was formed from sitosterol; however, it is questionable whether this low rate of conversion can be taken as evidence that sitosterol is the direct substrate for stigmasterol since after 6 weeks of incubation a certain amount of recycling of metabolized [^{14}C]-sitosterol would occur.

The most convincing evidence that sitosterol is the direct substrate for stigmasterol comes from short-term studies with barley seedlings [7]. Even though barley seedlings rapidly metabolized administered sitosterol (95% after a 2.5 hr pulse), Navari-Izzo and Izzo [7] found, using [4-^{14}C]-sitosterol in combination with [22,23-^{3}H]-sitosterol, that the isolated stigmasterol fraction showed the predicted decrease in the ratio of ^{3}H/^{14}C. In addition they observed that the radioactivity of stigmasterol increased from 8% of the sterol radioactivity right after the 2.5 hr pulse, to 26.1% after a 24 hr incubation.

The direct conversion of sitosterol to stigmasterol could not be confirmed with tobacco seedlings (Table 1, Fig. 1). There was the possibility that under present experimental conditions the tobacco seedlings, even though they incorporated sitosterol, were unable to synthesize sterols, particularly stigmasterol. Comparative quantitative sterol analysis of 6- and 7-day-old tobacco seedlings revealed that the free sterols increased by 160 μg/g fresh tissue, or 20%, over 24 hr, and both sitosterol and stigmasterol increased (Table 2).

In a separate experiment, 6-day-old tobacco seedlings were given a 3 hr pulse of [22,23-^{3}H]-sitosterol in combination with [2-^{14}C]-mevalonate and incubated for 12 hr. Of the total MVA incorporated, 6% was recovered from the various sterol fractions of which 67% was in the free, 26% in the ester, and 7% in the glycoside. Clearly tobacco

Table 2. Sterol content of 6 and 7 day old tobacco seedlings

Sterol	Seedling age (days) 6	7	Change
	μg sterol/g seedling		%
Cholesterol	48	40	−17
Campesterol	141	176	25
Sitosterol	384	460	20
Stigmasterol	231	288	25
Total	804	964	20

seedlings were able to synthesize sterols from MVA as substrate. The ^{3}H distribution from sitosterol was 89% in the free, 3% in the ester, and 8% in the glycoside fraction for a total sterol recovery of 86%. Table 3 shows the major radioactive TLC components after acetylation of the digitonin precipitated free sterol fraction. Almost half of the MVA ^{14}C activity remained at the TLC origin; however, the stigmasteryl and sitosteryl acetate regions had significant quantities of ^{14}C activity. These results clearly demonstrate that sitosterol and stigmasterol were synthesized from MVA. Even though ^{3}H-sitosterol was incorporated by tobacco seedlings, only background quantities of ^{3}H were recovered from the stigmasteryl acetate region. Comparing the synthesis of sitosterol and stigmasterol in a kinetic study, the ^{14}C activity from [^{14}C]-MVA increased in stigmasterol and decreased in sitosterol (Fig. 2). This pattern of MVA incorporation has been cited in the part as evidence that sitosterol and stigmasterol have a substrate to product relationship [1, 3, 4]. The level of ^{14}C activity in the sitosteryl acetate region is somewhat overstated since the other Δ^5 4-demethylsterylacetates, particularly campesterol and cholesterol, were also located at this R_f region. The fact, however, remains that MVA was readily incorporated into stigmasterol while exogenously supplied sitosterol was not converted to stigmasterol.

Table 3. TLC profile of steryl acetates isolated from tobacco seedlings administered a 3 hr [2-^{14}C]-MVA and [22,23-^{3}H]-sitosterol pulse with an incubation of 12 hr

Zone R_f	Reference compound as acetate	% Distribution ^{14}C	^{3}H
origin–0.11	—	48.9	4.2
0.11–0.22	—	3.7	14.4
0.22–0.38	—	6.5	3.0
0.38–0.50	stigmasterol	13.9	2.4
0.50–0.64	—	0.9	1.6
0.64–0.72	sitosterol	24.0	72.5
0.72–1.00	—	2.1	1.9

The free sterols were precipitated with digitonin, acetylated, and separated on 12.5% $AgNO_3$–silica gel using $CHCl_3$ as eluant. Total radioactivity of plate was 42066 dpm ^{14}C and 55013 dpm ^{3}H.

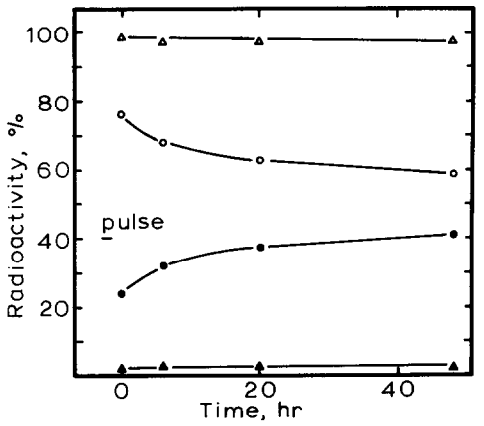

Fig. 2. Distribution of radioactivity of digitonin ppt free sterol fraction isolated from tobacco administered a 3 hr [22,23-^{3}H]-sitosterol and [2-^{14}C]-MVA pulse and incubated for various time periods. Sitosteryl acetate ^{14}C activity (○), stigmasteryl acetate ^{14}C activity (●), sitosteryl acetate ^{3}H activity (△), and stigmasteryl acetate ^{3}H activity (▲).

In summary, while the dehydrogenation of sitosterol to form stigmasterol has been reported in barley [7], the data with tobacco, soybean [5] and *Digitalis* [6] suggest that stigmasterol is not directly derived from sitosterol, and that the introduction of $\Delta^{22(23)}$ of stigmasterol must occur at an earlier point in the sterol biosynthetic pathway. The suggestion that stigmasterol is not directly derived from sitosterol is consistent with the proposed sequence for poriferasterol synthesis in the algae *Ochromonas malhamensis* [9] and *Chlorella ellipsoidea* [10], in which a 22,24(28)-diene intermediate is postulated. The question is still unanswered, "Do all vascular plants synthesize their 29 carbon 4-demethyl sterols via the same pathway?" Goodwin [11], in a recent review, suggested that it may well be that different pathways of desaturation operate in different organisms. Could it be that different vascular plants have different desaturation mechanisms? Tobacco, soybean and *Digitalis* are all dicotyledons, while barley is a monocotyledon.

EXPERIMENTAL

Plant material. Tobacco seeds (*Nicotiana tobacum* L. var. Burley 21) were germinated for 6 days on Whatman # 1 filter paper in germination chambers under a 14 hr photoperiod at 27° as previously described [4].

Incubation. The filter paper with seedlings was cut into 5 cm squares and each square was placed in a separate petri dish with 10 ml of medium. To administer the radioactive pulse, the dried desired substrate, either 5 μCi of [2-^{14}C]-mevalonate (36.5 mCi/mmol) in combination with 9 μCi of [22,23-^{3}H]-sitosterol (60.3 Ci/mmol) or 3 μCi of [4-^{14}C]-sitosterol, was dissolved in 0.1 ml MeOH and taken up in water containing 0.1% Tween-20. If not otherwise indicated the seedlings received a 3 hr pulse. The seedlings, without removal from the filter paper, were washed × 3 with 20 ml distilled water and transferred to a clean petri dish with 10 ml of distilled water. Time at end of pulse was taken as zero time.

Harvest and extraction of sterols. Seedlings were removed from the filter paper, washed in a Buchner funnel × 3 with 500 ml water, dried, weighed, ground in Me_2CO, boiled for 2 hr and filtered. If the sterol classes were separated, the procedure and hydrolysis of the sterol conjugates was as previously described [4]. All sterol fractions were purified by digitonin ppt after 1 mg of sitosterol and stigmasterol were added.

Sterol analysis. The acetylated sterols (pyridine–Ac_2O, 1:1) were separated on $AgNO_3$–silica gel (1:8) by developing the plates × 4 with freshly distilled $CHCl_3$. For best resolution of stigmasteryl and sitosteryl acetate, as well as the other radioactive components, the plates had to be activated at 120° for at least 12 hr, and dried between runs at 40°. Sterols were visualized with berberine, removed and extracted with MeOH. The steryl acetates were extracted from the MeOH × 3 with hexane and assayed by liquid scintillation spectrometry. All data were corrected for quench and in dual labeling experiments for isotope cross-over. Sterol quantitation was by GC using a 5% OV-101 column and He as carrier gas [4].

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