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COMPARATIVE STUDY OF *IN VIVO* STIGMASTEROL BIOSYNTHESIS IN *NICOTIANA TABACUM* AND *HORDEUM VULGARE*

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; *Hordeum vulgare*; Gramineae; barley; phytosterols; stigmasterol; sitosterol.

Abstract—Six-day-old tobacco (*Nicotiana tabacum*) and barley (*Hordeum vulgare*) seedlings rapidly incorporated and metabolized exogenously supplied [4-^{14}C]sitosterol but neither plant was able to convert it into stigmasterol. However, a sterol metabolite was isolated from both species and the acetate derivative was slightly more polar, on $AgNO_3$–silica gel TLC, than stigmasteryl acetate. A similar metabolite was also obtained with [4-^{14}C]cholesterol, indicating a general metabolic reaction of plants to exogenous sterols. Both species incorporated [2-^{14}C]mevalonic acid into sitosterol and stigmasterol. We suggest that in vascular plants, whether monocotyledons or dicotyledons, the pathway of stigmasterol biosynthesis is not *via* sitosterol but through a common precursor which is derived from mevalonic acid.

INTRODUCTION

In most vascular plants the two major sterols are sitosterol (stigmasta-5-en-3β-ol) and stigmasterol (stigmasta-5,22-diene-3β-ol) and because of their structural similarity, substrate-to-product relationship has become generally accepted [1]. However, this hypothesis is based largely on indirect evidence with [2-^{14}C]mevalonic acid (MVA) [2–4] and [3-^{3}H]α-spinasteryl acetate [5] as substrates. In a recent communication, Navari-Izzo and Izzo [6] presented data that barley (*Hordeum vulgare*) apparently converts sitosterol directly into stigmasterol. This conversion, however, could not be confirmed with *Nicotiana tabacum* seedlings [7], although tobacco readily incorporated and metabolized [4-^{14}C]sitosterol. A similar failure to show direct conversion of sitosterol to stigmasterol had previously been reported by Waters and Johnson [8] with *Glycine max* and by Bennett and Heftmann [9] with *Digitalis lanata*. Based on the limited amount of available information, it has been proposed [7] that dicotyledons cannot convert sitosterol to stigmasterol, while monocotyledons apparently can. This suggestion implies that monocotyledons and dicotyledons have different pathways to synthesize stigmasterol: monocotyledons *via* sitosterol and dicotyledons through an independent pathway without going through sitosterol. The present comparative investigation with barley and tobacco seedlings was undertaken to test the above hypothesis.

RESULTS AND DISCUSSION

The hypothesis that sitosterol in vascular plants is the immediate precursor of stigmasterol was originally based on indirect evidence [2–4]. Recently, however, it was reported that barley seedlings were able to convert sitosterol directly to stigmasterol [6], but this conversion could not be confirmed with tobacco seedlings [7]. As tobacco is a dicotyledon and barley a monocotyledon, it was suggested that monocotyledons and dicotyledons might have different pathways to synthesize stigmasterol [7].

Both tobacco and barley, when continuously incubated with [4-^{14}C]sitosterol, showed rapid substrate incorporation and metabolism. After 24 hr of incubation, 55–60% of the incorporated radioactive sitosterol was metabolized and essentially no difference in the metabolic rate could be established between the two species. However, in pulse labelling studies (3 hr pulse), barley seedlings showed a somewhat greater rate of sitosterol metabolism. For example, after 20 hr 65% of the incorporated sitosterol was metabolized by barley vs 50% by tobacco. In the present study the metabolism of sitosterol by tobacco was slightly higher than previously reported [7] but, more importantly, sitosterol metabolism by barley, even though higher than by tobacco, was significantly lower than previously observed [6]. Navari-Izzo and Izzo [6] recovered, after a 2.5 hr pulse of [4-^{14}C]sitosterol, 5.11% of the ^{14}C activity in the sterols and after an additional 24 hr of incubation they found only 4.42%. No reason can be given for the observed difference in the rate of sitosterol metabolism by barley by the two laboratories, except to point out that light has a pronounced effect on sterol synthesis [4]. The present studies were carried out under continuous illumination (34 J/m^{2}sec) but it is not clear what light conditions were used by Navari-Izzo and Izzo [6].

Sitosterol and stigmasterol are the major 4-demethylsterols of 6-day-old tobacco and barley seedlings (Table 1). In barley, sitosterol accounted for over half of the total free sterols and stigmasterol for another 29%. Tobacco had 31% sitosterol and 38% stigmasterol. Both species, following a 3 hr pulse with [2-^{14}C]mevalonic acid (MVA), rapidly incorporated ^{14}C activity into their sterols. With time, the percentage of radioactivity in the Δ^5-sterols (sitosterol, campesterol and cholesterol) decreased while that of $\Delta^{5,22}$-sterol (stigmasterol) increased

Table 1. Free sterol composition of 6-day-old tobacco and barley seedlings

	Tobacco		Barley	
	(μg/g fr. wt)	(%)	(μg/g fr. wt)	(%)
Sitosterol	87	31.0	109	52.8
Stigmasterol	108	38.4	60	29.1
Campesterol	66	23.5	36	17.5
Cholesterol	20	7.1	1	0.5

(Table 2); therefore, the ratio of radioactivity of the $\Delta^{5,22}$- to Δ^5-sterols increased with longer incubation. Tobacco seedlings, when compared to barley, showed a significantly larger increase in the radioactivity ratio (Table 2), probably reflecting the higher stigmasterol level of tobacco (Table 1). The initial, more rapid, incorporation of [2-^{14}C]MVA into sitosterol and its later but higher incorporation into stigmasterol has been cited as indirect evidence that these two sterols have a substrate-to-product relationship [2–4].

When [4-^{14}C]sitosterol, however, was used as substrate, the digitonin-precipitated sterols of tobacco and barley, after acetylation and argentation chromatography, showed essentially no ^{14}C label in the zone corresponding to stigmasterol, even after an incubation period of 24 hr (Table 3). Both species, however, had a major metabolite at R_f 0.22 and a minor radioactive component at R_f 0.75. The R_f values for stigmasteryl acetate and sitosteryl acetate were 0.35 and 0.55, respectively, and resolution between the R_f 0.22 metabolite and stigmasteryl acetate was complete. With incubation, the R_f 0.22 metabolite accumulated significant amounts of radioactivity; in tobacco the increase was from 7.9% of total sterols after 2 hr to 13.8% after 24 hr, and in barley seedlings the increase was from 5.6% after 2 hr to 14.2% after 24 hr (Table 3). The occurrence of the R_f 0.22 metabolite had previously been reported for tobacco [7].

When [4-^{14}C]cholesterol was substituted for [4-^{14}C]sitosterol both barley and tobacco produced a chromatographically quite similar metabolite (Table 4). As with sitosterol, after 24 hr about 12–14% of the free sterol radioactivity was recovered in the R_f 0.22 TLC zone. The R_f 0.22 metabolite was not obtained when radioactive MVA was used as substrate, and it appears that the formation of the metabolite was a general response and an artifact to exogenous sterol. Based on TLC analysis this component was quite different from the product isolated from potato (*Solanum tuberosum*) leaves incubated in [4-^{14}C]cholesterol [10].

Our results do not support the previously reported observation that barley converts sitosterol to stigmasterol [6]. Navari-Izzo and Izzo [6] found that when seedlings were incubated with [4-^{14}C]sitosterol, the stigmasterol fraction, as separated by TLC argentation chromatography, showed significant radioactivity and that the ^{14}C activity increased from 8% immediately after a 2.5 hr pulse to 26.1% after 24 hr of incubation. In our experiments the stigmasterol zone from TLC did not become radioactive. However, a metabolite at R_f 0.22 accumulated radioactivity and it increased with incubation (Table 3). Navari-Izzo and Izzo [6] did not observe this metabolite or any other radioactive product, except the one corresponding to stigmasterol. These differences in results are possibly explained by the difficulties in separating the various steryl acetates. As previously discussed [7], resolution of the metabolite from stigmasterol is difficult

Table 2. Incorporation of radioactivity into Δ^5 and $\Delta^{5,22}$ sterols by tobacco and barley seedlings following a 3 hr [2-^{14}C]MVA pulse. The free sterols were acetylated and separated by TLC on $AgNO_3$–silica gel containing 0.5% CMC with $CHCl_3$ as solvent

	Δ^5-Sterols		$\Delta^{5,22}$-Sterols		$\Delta^{5,22}/\Delta^5$-^{14}C Ratio	
Time (hr)	Barley	Tobacco	Barley	Tobacco	Barley	Tobacco
	(dpm/g fr. tissue)					
0	4005	4295	243	196	0.061	0.046
3	4212	5772	444	1157	0.105	0.201
20	6730	5716	1830	3640	0.219	0.637

Table 3. [4-^{14}C]Sitosterol metabolism [2-^{14}C] by tobacco and barley seedlings over a 24 hr incubation period following a 3 hr pulse. The digitonin-precipitated sterols were acetylated and separated by TLC on $AgNO_3$–silica gel containing 0.5% CMC with $CHCl_3$ as solvent

	Tobacco			Barley		
	2 hr	4 hr	24 hr	2 hr	4 hr	24 hr
Radioactivity/plate, dpm × 10^{-3}	6.9	11.2	26.7	8.3	21.9	79.8
Sitosteryl acetate, %	82.4	78.8	73.9	74.2	73.9	74.1
Stigmasteryl acetate, %	2.8	3.0	1.5	2.6	1.5	2.4
R_f 0.22, metabolite, %	7.9	8.4	13.8	5.6	11.3	14.2
Rest of plate, %	6.9	9.8	10.8	17.6	13.3	9.3

Table 4. Metabolism of exogenously supplied [4-^{14}C]cholesterol by tobacco and barley seedlings after 24 hr of continuous incubation. The digitonin-precipitated sterols were acetylated and separated by TLC on $AgNO_3$–silica gel containing 0.5% CMC with $CHCl_3$ as solvent

	Tobacco	Barley
Radioactivity/plate, dpm × 10^{-4}	15.1	11.9
Cholesterol, %	79.3	77.3
Stigmasterol, %	1.4	1.9
R_f 0.22, metabolite, %	11.6	13.8
Rest of plate, %	7.7	7.0

even by argentation chromatography. We found that the addition of 0.5% of carboxymethyl cellulose (CMC) to 12.5% $AgNO_3$–silica gel was beneficial in resolving the various acetate components.

Navari-Izzo and Izzo [6] observed that the stigmasterol fraction isolated from barley seedlings that were incubated with [4-^{14}C]sitosterol in combination with [22,23-^{3}H]sitosterol had a lower ^{3}H:^{14}C ratio than the substrate, indicative of the functioning of a 22,23-dehydrogenase. They found, however, that the tritiated substrate was more rapidly metabolized than [4-^{14}C]sitosterol and thus the ^{3}H:^{14}C ratio of the stigmasterol fraction decreased with incubation. To verify their observation they examined strawberry (*Fragaria vesca*), a vascular plant that has only trace amounts of stigmasterol [11]. They found that the isolated sterols from strawberry leaves incubated in dual-labelled sitosterol showed no decrease in ^{3}H:^{14}C ratio. We also used strawberry (*F.* × *ananassa*) and found that, although [4-^{14}C]sitosterol was readily incorporated and metabolized, no metabolite with an R_f value lower than sitosterol could be recovered. For example, after 24 hr of continuous incubation with [4-^{14}C]sitosterol, 81% of the recovered sterol radioactivity was in the sitosterol fraction, 1.7% in the stigmasterol zone, 1.9% in the fraction corresponding to the metabolite at R_f 0.22, 6.8% in a metabolite at R_f 0.75 and 8.6% from the rest of the plate. The metabolite at R_f 0.75 also appeared in tobacco and barley but at much lower levels. The sterol composition of strawberry differs from that of tobacco and barley in that sitosterol accounts for 95% of the sterols and stigmasterol occurs in only trace amounts [6, 11], and it appears that metabolism of exogenously administered sterols is species specific.

The data lead us to conclude that (*a*) both barley and tobacco metabolize exogenously supplied sitosterol and cholesterol in a similar manner, (*b*) stigmasterol is not derived from sitosterol *via* a 22,23-dehydrogenation and (*c*) biosynthesis of stigmasterol and sitosterol is by two independent routes. The conclusion that stigmasterol synthesis by monocotyledons and dicotyledons is not through sitosterol is consistent with the hypothesis for poriferasterol biosynthesis in algae such as *Ochromonas malhamensis* [12] and *Chlorella ellsoidea* [13] in which a 22,24(28)-diene intermediate is postulated.

EXPERIMENTAL

Plant material. Tobacco (*Nicotiana tabacum* L. var. Burley 21) and barley (*Hordeum vulgare* L. var. Himalaya) seeds were germinated for 6 days on Whatman No.1 filter paper in germination chambers under continuous light, 34 J/m^{-2}/sec, at 22°. The seedlings and filter paper were then placed in Petri dishes with 10 ml of medium under the same condition for incubation. To administer the desired radioactive substrate, 2 μCi of either [2-^{14}C]MVA (36.5 mCi/mmol) or [4-^{14}C]sitosterol (58 mCi/mmol) were dissolved in 0.1 ml MeOH and taken up in H_2O containing 0.1% Tween-20. Incubation was either continuous or with a 3 hr pulse. For pulse studies, the seedlings without removal from the filter paper, were washed 3 × with 20 ml H_2O and transferred to a clean Petri dish with 10 ml H_2O. The 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 700 ml H_2O, dried, weighed, ground in Me_2CO, extracted by Soxhlet for 14 hr and filtered. All sterol fractions were purified by digitonin pptn after 1 mg sitosterol and 1mg stigmasterol were added as carriers.

Sterol analysis. The acetylated sterols (pyridine–Ac_2O, 1:1) were separated on $AgNO_3$–silica gel G (1:8) by developing the plates with freshly distilled $CHCl_3$. For best resolution of steryl acetates, 0.5% carboxymethyl cellulose (cellulose gum type 7HP, Hercules Chemical Co.) was mixed with the silica gel. The plates were activated at 80° for 2 days and dried between runs at 40°. Sterols were visualized with berberine, then removed and extracted with MeOH. The steryl acetates were extracted from the MeOH with hexane (3 ×) and assayed by liquid scintillation counting. Sterol quantitation was by GC using a 5% OV-101 column and He as carrier gas [4].

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