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IN VIVO CONVERSION OF [4-^{14}C]SITOSTEROL AND [22,23-^{3}H]SITOSTEROL TO STIGMASTEROL IN BARLEY SEEDLINGS

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Key Word Index—*Hordeum vulgare*; Gramineae; barley; sitosterol; stigmasterol; phytosterols; free sterols; esterified sterols; glycosidic sterols; acylated glycosidic sterols.

Abstract—Six-day-old barley seedlings were allowed to take up [4-^{14}C]sitosterol and [22,23-^{3}H]sitosterol for 2.5 hr and the incorporation into the sterol fractions was determined after 0, 6, 12 and 24 hr. Sitosterol was readily incorporated into every sterol class. The ^{3}H/^{14}C ratio in the free forms dropped when compared with the ^{3}H/^{14}C ratio of the administered sitosterol. In the free sterol, radioactive stigmasterol, showing a ^{3}H/^{14}C ratio half that of the sitosterol ^{3}H/^{14}C ratio, was isolated and its radiochemical purity established by dilution with carrier material and crystallization to constant specific activity.

INTRODUCTION

The principal sterols in *Hordeum vulgare* occur as free sterols, esters, glycosides and acylated glycosides. It has been reported that in barley shoots sitosterol (24α-ethylcholest-5-en-3β-ol) and stigmasterol (24α-ethylcholesta-5,22-dien-3β-ol) are present in about the same amounts [1]. At present it is not clear if sitosterol is the immediate precursor of stigmasterol or if both sterols arise from a common intermediate. It has been postulated that they are directly related by a hydrogenase–dehydrogenase system and that 22,23-dehydrogenase converts sitosterol to stigmasterol [2].

In *Nicotiana tabacum* [3] and in *Musa sapientium* [4], using [^{14}C]mevalonic acid as precursor, in the first hours of incubation a higher specific radioactivity was found in sitosterol, but with longer incubation time the specific radioactivity of sitosterol decreased whereas that of stigmasterol increased, indicating a precursor–product relationship. The direct conversion of labelled sitosterol into stigmasterol was not observed in five days from administration [5] but it was shown after six weeks incubation [6].

The question of whether stigmasterol arises, at least in part, from sitosterol is quite important; indeed the ratio of sitosterol to stigmasterol changes during seed germination [7], with senescence and plant development [8, 9] and under different sunlight conditions [9, 10]. The experiments described in this paper were designed to follow the *in vivo* incorporation of [4-^{14}C]sitosterol and [22,23-^{3}H]sitosterol into the sterol forms of barley seedlings and to clarify the possible conversion of sitosterol into stigmasterol.

RESULTS AND DISCUSSION

The sterol content and composition of the free, esterified, glycosidic and acylated glycosidic forms of six-day-old barley seedlings incubated with [4-^{14}C]sitosterol and [22,23-^{3}H]sitosterol are reported in Table 1. The distribution of sterol shows that free sterols are the dominant form (79.8%), followed by steryl glycosides (8.9%), steryl esters (7.9%) and acylated steryl glycosides (1.9%). The major sterol in the four fractions was always sitosterol, but in the free form the sitosterol to stigmasterol ratio was nearly 1.0. Comparative values for intact barley seedlings have not been reported in the literature, however Bush *et al.* [1] found a very similar pattern for six-day-old barley shoots.

Radioactive sitosterol was rapidly incorporated into the four sterol fractions. It is interesting to note that over 90%

Table 1. Sterol content and composition of six-day-old barley seedlings 2.5 hr after supplying with [4-^{14}C]sitosterol and [22,23-^{3}H]sitosterol

	Sterol content		Cholesterol		Campesterol		Stigmasterol		Sitosterol	
Sterol fraction	μg/g dry wt	%	μg/g dry wt	%	μg/g dry wt	%	μg/g dry wt	%	μg/g dry wt	%
Free	2059.8	79.8	14.6	0.7	343.7	16.7	746.4	36.2	955.1	46.4
Esters	204.8	7.9	9.9	4.8	29.6	14.5	27.8	13.6	137.5	67.1
Glycosides	230.2	8.9	4.6	2.0	43.6	18.9	55.1	23.9	126.9	55.2
Acylated glycosides	84.9	3.4	1.9	2.2	16.4	19.3	19.5	23.0	47.1	55.5

of the total ^{14}C administered was not recovered in the sterol classes. Indeed after a 2.5 hr pulse, only 5.11% of the ^{14}C activity was recovered in the sterols at zero time and 4.42% at 24 hr. These data appear to indicate the active metabolism of 3β-hydroxysterol in plant tissues suggesting that these compounds could take part in the general metabolic pool. The same phenomenon was observed in *Nicotiana tabacum* after [^{14}C]cholesterol administration [11]. Furthermore it has been suggested that sitosterol in plants may be a precursor of other steroids, namely progesterone and the cardenolides [12].

The results of a time-course experiment with [4-^{14}C]sitosterol and [22,23-^{3}H]sitosterol are shown in Table 2. A 2.5 hr pulse gave 91% of ^{14}C recovered from the tissue as free sterols. The free sterol radioactivity decreased continuously over the experimental period with its lowest value after 24 hr (79%). A high recovery of radioactivity was also observed in the acylated steryl glycosides which showed a faster rate of incorporation of precursor than the steryl glycosides. During the 24 hr of the experiment the first form increased from about 6.8% to 13.4% and the second one only from 1.6% to 4.3% of the total recovered ^{14}C radioactivity.

These data agree with the *in vivo* experiments on *Nicotiana tabacum* [13, 14] and on lettuce leaf discs [15] which support the suggestion that acylation of steryl glycosides is not such an easy and rapid reaction in the *in vivo* system as it is in the *in vitro* system, where the first form to become radioactive is always steryl glycoside [16, 17]. Less than 1% of the total radioactivity was recovered at zero time in the steryl ester and this radioactivity increased three fold in the 24 hr. This low radioactivity is not surprising in view of other experiments [18, 19] where the sitosterol and cholesterol used as substrate were incorporated into steryl ester in low quantities and generally a higher conversion of steryl esters to free sterols was observed in short-term experiments. The same has been shown with mammalian tissue where the steryl esterase enzyme seems to work mostly as a hydrolase [20]. The [22,23-^{3}H]sitosterol gave a very similar pattern of incorporation to that of [^{14}C]sitosterol (Table 2). However, the ^{3}H radioactivity recovered in the free sterol form was lower than the ^{14}C radioactivity recovered.

The esterified sterol form showed the smallest increase in activity while the ^{3}H radioactivity of the acylated steryl glycoside increased at a faster rate than that in the steryl glycosides and reached a higher final level. Our results on intact barley seedlings show that plants can utilize labelled free sterols to synthesize the other forms of labelled sterols. In particular our data suggest that the formation of acylated steryl glycosides is independent of steryl glycosides as a precursor. Although in cell-free systems the sequence: free sterol → steryl glycoside → acylated steryl glycoside seems very likely [16, 17], an alternative route can be considered because acylated sugars have been isolated in micro-organisms [21, 22]. However, it still needs to be ascertained if acylated sugars are also present in plants. Furthermore it is necessary to establish whether the pathways observed in the *in vitro* system are also valid for the *in vivo* system. Grunwald [13, 14] reported in *Nicotiana tabacum* seedlings and in *Hordeum vulgare* roots the presence of an unidentified sterol form, intermediate in the synthesis of acylated steryl glycosides. In our system we found the radioactivity associated only in four sterol classes and we were unable to detect any other sterol form. This difference may be due to tissue difference and/or methods of sterol extraction and quantification.

Although the general incorporation pattern of [^{14}C]- and [^{3}H]sitosterol was very similar, what first surprised us was the faster ^{3}H radioactivity decrease compared with the ^{14}C radioactivity decrease in the free sterol form during the whole experimental period which is not readily explainable by the difference in the labelling of sitosterol.

The genus *Fragaria* is characterized by the presence of a large quantity of sitosterol, lesser amounts of cholesterol and campesterol and trace amounts of stigmasterol [23]. The low quantity of stigmasterol could be interpreted as a failure of this genus to acquire the specific dehydrogenase in the necessary quantity for the dehydrogenation of sitosterol.

A study was made of the ability of *Fragaria vesca* (wild strawberry) seedlings (free sitosterol 1969 μg/g dry wt, free campesterol 69 μg/g, free cholesterol 49 μg/g, free stigmasterol 0.83 μg/g) to incorporate [4-^{14}C]sitosterol and [22,23-^{3}H]sitosterol in a pulse-chase experiment as for the barley seedlings (see Experimental). The $^{3}H/^{14}C$ ratio remained unchanged during the whole experimental period (Table 3).

The drop in $^{3}H/^{14}C$ ratio observed in free sterols of barley seedlings suggested that we investigated if labelled stigmasterol could be found in barley seedlings considering that the observed loss of ^{3}H might be due to the formation of the double bond at C-22/C-23 in the side chain. To date the direct conversion of sitosterol to stigmasterol has been studied only in long term experiments. Bennet and Heftmann [6] demonstrated that sitosterol can serve as a precursor of stigmasterol in *Digitalis lanata* by administering [^{14}C]sitosterol *in vivo* for 6 weeks. On the contrary Waters and Johnson [5] did not observe any conversion in 5 days.

Table 2. Incorporation of [4-^{14}C]sitosterol and [22,23-^{3}H]sitosterol into the sterol fractions of intact barley seedlings, as a function of time after a 2.5 hr pulse

	^{14}C (dpm × 10^{-3})				^{3}H (dpm × 10^{-3})			
Sterol fractions	0 hr	6 hr	12 hr	24 hr	0 hr	6 hr	12 hr	24 hr
Free	263.00	242.00	218.00	198.00	1090.0	747.0	562.0	529.0
Esters	1.73	3.14	5.42	7.75	24.3	44.0	76.0	82.6
Glycosides	4.62	10.28	11.93	10.75	38.5	82.3	95.5	94.7
Acylated glycosides	19.65	29.70	35.81	33.50	129	193.0	233.0	222.0
Total	289.00	285.12	271.16	250.00	1281.8	1066.3	966.5	928.3

Table 3. Incorporation of [4-^{14}C]sitosterol and [22,23-^{3}H]sitosterol into the free sterol of strawberry seedlings as a function of time after a 2.5 hr pulse

Chase period (hr)	Sterol fraction (total activity dpm)		Free sterol fraction (activity dpm)	
	^{14}C ($\times 10^{-5}$)	^{3}H ($\times 10^{-6}$)	^{14}C ($\times 10^{-5}$)	^{3}H ($\times 10^{-5}$)
0	3.12	1.25	2.57	10.30
6	3.07	1.23	2.36	9.44
12	2.91	1.16	2.12	8.48
24	2.68	1.07	1.92	7.68

A portion of the free sterol fraction recovered at each incubation time was acetylated and separated by TLC. Radioactivity was recovered only in the zones corresponding to sitosteryl and stigmasteryl acetates. From Table 4 it emerges that after a 2.5 hr pulse sitosterol is readily converted to stigmasterol during the first 6 hr of metabolism. Afterwards the conversion goes on more slowly. The ^{3}H/^{14}C ratio for the stigmasteryl acetate was about half that of the ^{3}H/^{14}C ratio of the sitosteryl acetate which can be explained by a 22,23-dehydrogenation of sitosterol. The purity of the stigmasteryl acetate (and stigmasterol) was checked by melting point determination of the sample recovered from TLC and purified by solvent crystallization to constant specific activity after diluting with pure stigmasteryl acetate. It is evident from Table 5 that the isolated stigmasterol was radiochemically pure.

Although the present results provided evidence for the biosynthesis of stigmasterol from sitosterol, the possibility cannot be excluded that they can also arise from a common intermediate.

EXPERIMENTAL

Plant material. Barley seeds (*Hordeum vulgare* var. Aramir) were germinated in water on Whatman No 1 filter paper in a germination box under constant light (1.2×10^4 ergs/cm^2 sec at 25°) and controlled moisture (95–99%).

Chemicals. All solvents were reagent grade and redistilled. Cholestane, cholesterol, campesterol, stigmasterol and sitosterol standards were purchased from Applied Science Laboratory, College Park, Pa., [4-^{14}C]sitosterol (58 mCi/mmol) and [22,23-^{3}H]sitosterol (42 Ci/mmol) were from the Radiochemical Centre, Amersham.

In order to be sure that the [4-^{14}C]sitosterol and [22,23-^{3}H]sitosterol were free from stigmasterol they were acetylated and purified by TLC (12.5% w/w $AgNO_3$ impregnated silica gel) developed × 2 with EtOH–free $CHCl_3$. The zone corresponding to sitosteryl acetate was removed, hydrolysed (5% KOH in 95% EtOH), neutralized and extracted × 3 with hexane. Purified [4-^{14}C]sitosterol and [22,23-^{3}H]sitosterol were used in all experiments.

In vivo *experiment.* [4-^{14}C]Sitosterol (2.5 μCi) and [22,23-^{3}H]sitosterol (10 μCi) were dried in Petri dishes. The sterols were dissolved in 1.0 ml of absolute EtOH and 25 ml of sterilized distilled H_2O were added. About 6 g of six-day-old barley seedlings were put in the Petri dishes, and the medium was kept at 25°. After a 2.5 hr pulse, the seedlings were chased with unlabelled sitosterol. Samples from unlabelled sitosterol medium were removed after 0, 6, 12 and 24 hr. At the end of each incubation period, the seedlings were washed with H_2O, then with unlabelled sitosterol and finally with at least 5 l. of H_2O in a Büchner funnel and analysed for the sterols.

Material and methods. Sterol extraction, separation (free, esterified, glycosidic and acylated glycosidic sterols), hydrolysis, precipitation and quantification were as reported elsewhere [8–10].

Each sterol fraction, taken up in EtOAc, was evaporated, dissolved in MeOH and counted in Bray's dioxane scintillation

Table 4. Conversion of sitosterol to stigmasterol in six-day-old barley seedlings as function of time after a 2.5 hr pulse. The data are shown as dpm of ^{14}C and ^{3}H total recovered activity

Time (hr)	Sitosteryl acetate					Stigmasteryl acetate				
	^{14}C	^{3}H	^{3}H/^{14}C	C%	H%	^{14}C	^{3}H	^{3}H/^{14}C	C%	H%
0	18401	73604	4.0	92.0	95.2	1600	3680	2.30	8.0	4.8
6	15948	65387	4.1	80.4	88.1	3887	8799	2.26	19.6	11.9
12	12703	49545	3.9	76.3	84.5	3946	9075	2.29	23.6	15.5
24	10729	42918	4.0	73.9	84.4	3789	7898	2.08	26.1	15.6

Table 5. Specific radioactivities, melting point and ^{3}H/^{14}C ratios of the stigmasterol isolated from barley seedlings after incubation with [4-^{14}C]sitosterol (2.5 μCi) and [22,23-^{3}H]sitosterol (10 μCi)

Compound	Solvent used for crystallization	Specific radioactivity dpm of ^{14}C/mg	Observed ^{3}H/^{14}C ratio	Melting point
Stigmasteryl acetate	Dichloromethane–methanol	516	2.09	
	Methanol	499	2.07	
	Methanol	503	2.08	
	Methanol	496	2.06	144–145°
Stigmasterol	Methanol	498	2.08	
	Methanol	501	2.09	168–170°

fluid. Radioactivity, given in dpm, was corrected for cross-over and efficiency. The TLC techniques for sitosterol and stigmasterol separation were as above. After their cochromatography with standards, radioactivity was determined by liquid scintillation counting of the isolated zones.

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