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THE INCORPORATION OF MEVALONATE-[2-^{14}C] INTO THE STEROL FRACTIONS OF *CALENDULA OFFICINALIS*

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Key Word Index—*Calendula officinalis*; Compositae; incorporation of mevalonate-[2-^{14}C] into phytosterols; steryl esters; steryl glucosides; acylated steryl glucosides; water-soluble sterol complexes.

Abstract—The incorporation of mevalonate-[2-^{14}C] into the free sterols, steryl esters, steryl glucosides, acylated steryl glucosides and water-soluble complexes was investigated and the sterols of each fraction were separated into stanols, Δ^7 sterols, Δ^5 sterols, stigmasterol, clerosterol and methylene-cholesterol. The stanols and Δ^7 sterols were more strongly labelled in the steryl esters than in the free sterols. The Δ^5 sterols and stigmasterol were more intensively labelled in the free sterols than in the steryl esters. All sterol types were more labelled in the steryl glycosides than in the acylated steryl glucosides. Stanols were probably formed from Δ^7 or Δ^5 precursors.

INTRODUCTION

The previous paper described the qualitative and quantitative determinations of the sterols present as free compounds, steryl esters, steryl glucosides, acylated steryl glucosides and water-soluble complexes in the seedlings and leaves of *Calendula officinalis* [1].

The present work deals with the dynamics of labelling, with mevalonate-[2-^{14}C], of the sterols present in these fractions in the seedlings of *C. officinalis*.

RESULTS AND DISCUSSION

The sodium salt of mevalonic acid-[2-^{14}C] was fed to the 3-day-old *C. officinalis* seedlings for 16 hr. The utilization of the precursor absorbed by the plant was rather slow, even after 2 days the mevalonate-[2-^{14}C] could be detected by TLC. The seedlings were processed after different times from 16 hr to 18 days in the manner described in the earlier paper [1]. For each fraction the radioactivity was measured in the different types of sterols which could be separated by means of $AgNO_3$–silica gel TLC, namely the stanols, Δ^7 sterols, Δ^5 sterols, stigmasterol, clerosterol and methylenecholesterol.

Table 1 and Figs. 1–5 present the results of these experiments. In Table 1 is presented the total radioactivity incorporated into each fraction by the embryo axes and the cotyledons of 100 seedlings at each sampling time. Nearly equal quantities of radioactivity were incorporated into both the embryo axes and the cotyledons. From the first to the eighteenth day the greatest amount of radioactivity was incorporated into the free sterols (70–80% both in the embryo axes and the cotyledons) then into esters (*ca* 20%), glucosides (*ca* 3%) and acylated glucosides (*ca* 1%). In the water-soluble complexes traces of radioactivity were detected only in Δ^5 sterols and stigmasterol. The proportion of radioactivity incorporated into each fraction by the embryo axes and the cotyledons of seedlings resembled to some extent the quantitative relationships among the sterols of the different fractions in the 3-day-old seedlings. However, the percentage of the radioactivity incorporated into the steryl glucosides and the acylated steryl glucosides was less than expected from the concentrations of these fractions [1].

After 16 hr only the free sterols and steryl esters were labelled and there was more radioactivity in the free sterols. The free sterols exhibited maximal radioactivity at the fourteenth day and the steryl esters at the fifth day for the embryo axes and at the seventh day in the cotyledons. The labelling of the steryl glucosides could be detected after the

Table 1. Radioactivity incorporated into sterols (A) by the embryo axes and (B) by the cotyledons of 100 seedlings of *Calendula officinalis*

	Time (days)	Free sterols cpm	%	Steryl esters cpm	%	Steryl glucosides cpm	%	Acylated glucosides cpm	%	Total cpm
	16 hr	490	80	110	20	0		0		600
	1	730	57	390	31	90	7	50	4	1260
	2	5100	68	1990	26	220	3	190	3	7500
(A)	3	17110	76	4670	21	450	2	150	1	22380
	5	22850	77	5990	20	690	2	240	1	29770
	7	24990	73	8260	24	610	2	240	1	34100
	10	24750	79	5460	17	750	3	280	1	31240
	14	25390	79	5300	17	950	3	400	1	32040
	18	23300	77	5500	18	990	3	440	2	30230
	16 hr	360	60	240	40	0		0		600
	1	970	66	440	30	0		60	5	1470
	2	2510	72	640	18	260	7	100	3	3510
(B)	3	8970	78	1970	17	420	4	160	1	11520
	5	18090	61	10460	35	1024	3	400	1	29970
	7	23490	71	8460	25	900	3	340	1	33990
	10	21730	74	6190	21	1158	4	450	1	29530
	14	26910	78	5550	16	1290	4	640	2	34260
	18	18260	69	5610	22	1430	6	690	3	25990

first day and the radioactivity incorporated into these fractions increased continually and was always higher in the steryl glucosides than in the acylated glucosides. These results indicate that in *C. officinalis* the following biosynthetic sequences may operate: (a) free sterols → steryl esters and (b) free sterols → steryl glucosides → acylated steryl glucosides [2,3]. The total steryl esters reached a peak of radioactivity earlier than the free sterols which points to a faster metabolism of the former fraction. A rapid turnover of some steryl esters was also suggested in *Nicotiana tabacum* [4].

At all times more than 95% of the radioactivity was incorporated into the Δ^5 sterols and stigmasterol with only 4% in the stanols, Δ^7 sterols, clerosterol and methylenecholesterol (about 1% in each group).

Figure 1 shows the labelling of the Δ^7 sterols in the embryo axes (A) and in the cotyledons (B). At the early times there was more radioactivity in the free Δ^7 sterols than in Δ^7 steryl esters but between 3 and 10 days in the embryo axes and between 5 and 14 days in the cotyledons they were labelled more intensively. In the embryo axes the Δ^7 steryl

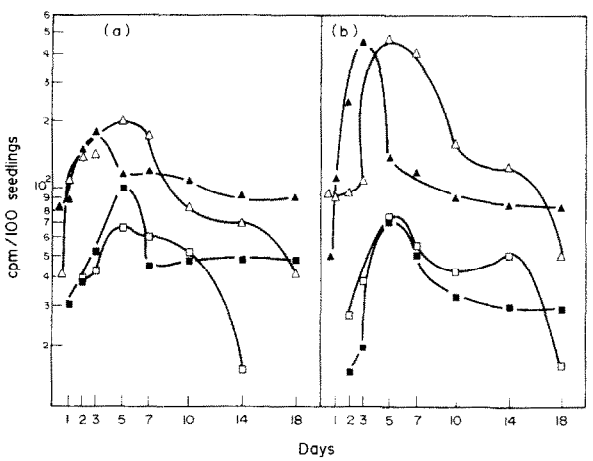

Fig. 1. Radioactivity incorporated into Δ^7 sterols (a) by the embryo axes and (b) by the cotyledons of seedlings of *Calendula officinalis*. ▲—Free sterols; △—steryl esters; ■—steryl glucosides; □—acylated steryl glucosides.

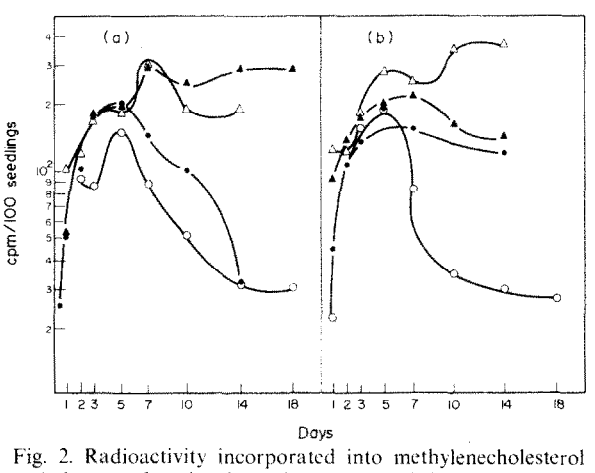

Fig. 2. Radioactivity incorporated into methylenecholesterol and clerosterol (a) by the embryo axes and (b) by the cotyledons of seedlings of *Calendula officinalis*. ●—Free methylenecholesterol; ○—methylenecholesterol esters; ▲—free clerosterol; △—clerosterol esters.

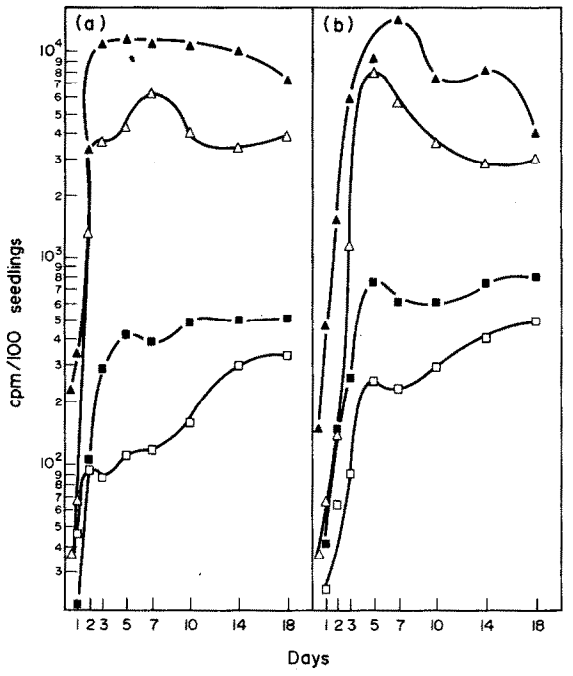

Fig. 3. Radioactivity incorporated into Δ^5 sterols (a) by the embryo axes and (b) by the cotyledons of seedlings of *Calendula officinalis*. Designations as in Fig. 1.

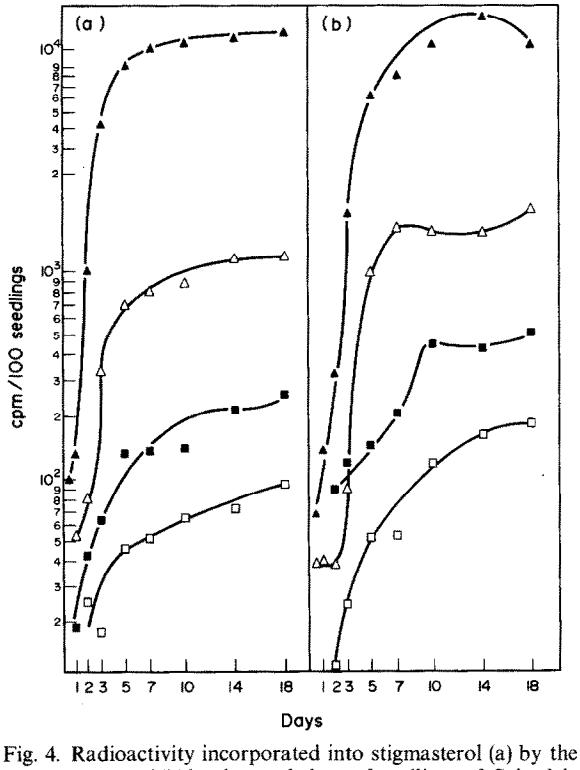

Fig. 4. Radioactivity incorporated into stigmasterol (a) by the embryo axes and (b) by the cotyledons of seedlings of *Calendula officinalis*. Designations as in Fig. 1.

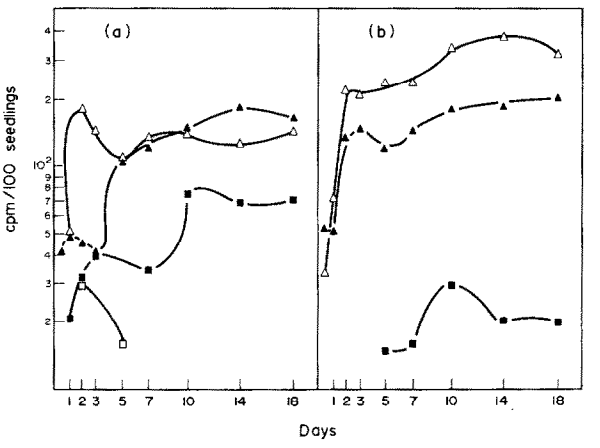

Fig. 5. Radioactivity incorporated into stanols (a) and by the embryo axes and (b) by the cotyledons of seedlings of *Calendula officinalis*. Designations as in Fig. 1.

glucosides were more strongly labelled than the Δ^7 steryl acylated glucosides but in the cotyledons the labelling of both these fractions was nearly equal.

In all fractions the Δ^7 sterols exhibited distinct maximums of radioactive incorporation. The peaks of radioactivity in the free Δ^7 sterols appeared at the 3rd day in the embryo axes and the cotyledons and at the 5th day in the bound steryl fractions which indicates that the free Δ^7 sterols are transformed into bound sterol forms. Distinct decreases of radioactivity after passing the peaks were observed for the Δ^7 steryl esters and the acylated glucosides which shows that these sterol fractions are actively metabolised in both the embryo axes and the cotyledons. The radioactivity in the other sterol forms stabilised at a fairly even level after passing the maxima.

The labelling of methylenecholesterol (Fig. 2) was determined only for the free and esterified sterols. The free methylenecholesterol was more strongly labelled than its esters in both tissues. The peaks of radioactivity appeared at the fifth day in both fractions followed by a distinct decrease of radioactivity especially in the ester fraction. These results show that the metabolism of methylenecholesterol in the free and esterified sterols is rather fast and similar to that of the Δ^7 sterols.

With the Δ^5 sterols (Fig. 3) and stigmasterol (Fig. 4) more radioactivity was incorporated into the free sterols than into steryl esters, also more radioactivity was in the steryl glucosides than in acylated steryl glucosides. The labelling of the Δ^5 sterols gave no sharp maxima for either the free

sterols or the steryl esters in the embryo axes but more distinct peaks were observed for both fractions in the cotyledons. After the fourteenth day a decrease in radioactivity was observed in the free sterols and there was a stabilization in the esters with both the embryo axes and the cotyledons. In the Δ^5 steryl glucosides and acylated glucosides a continuous increase of radioactivity was observed for both embryo axes and cotyledons.

In stigmasterol the radioactivity increased very rapidly in all forms in both tissues until the fifth day. In the free stigmasterol of the cotyledons a small decrease of radioactivity was observed after the fourteenth day.

For clerosterol (Fig. 2) determinations were only for the free and ester fractions. The free sterol was more strongly labelled than the clerosterol ester fraction in the embryo axes but the reverse was observed in the cotyledons. The radioactivity in these forms increased very rapidly to the seventh day in both tissues and then did not change in embryo axes for the free clerosterol but decreased in the clerosteryl esters. In the cotyledons the radioactivity of the free clerosterol decreased while that of the clerosterol esters increased to the end of the experiment.

The stanyl esters (Fig. 5) were more strongly labelled than the free stanols from the first to seventh day in the embryo axes and during the whole experimental period in the cotyledons. Stanyl glucosides were more strongly labelled than the acylated glucosides in the embryo axes. Stanols exhibited a peak of radioactivity even earlier than did the Δ^7 sterols. After the initial rapid incorporation of radioactivity into the free stanol and stanyl esters there was a small drop followed again by an increase to the 10th day. This may suggest that stanols can be formed from precursors earlier than Δ^7 sterols as well as from other precursors with double bonds at the Δ^7 and Δ^5 positions.

It should be kept in mind that the stanols, Δ^7 sterols and Δ^5 sterols were mixtures of C_{27}, C_{28} and C_{29} compounds. The individual components of these mixtures may have exhibited peaks at slightly different times and so caused the small deformations in the radioactivity curves of these groups of sterols.

The results obtained indicate that in most sterols there was more radioactivity incorporated into the free sterols than into their esters. This was true for Δ^5 sterols, stigmasterol and methylenecholesterol in the embryo axes and cotyledons and for clerosterol in the former tissue. The contrary was observed for the labelling of Δ^7 sterols and stanols in the embryo axes and cotyledons and for clerosterol in the cotyledons. Differences in the labelling of the different free sterols and steryl esters have been observed in *Nicotiana tabacum* [4].

The earlier appearance of the radioactive peaks in the sterols of the embryo axes indicates that in this tissue the metabolism of all forms of sterols is faster than in the cotyledons. The graphs illustrating the dynamics of sterol labelling represent the sum of the different transformations of these compounds. For example Δ^7 sterols, after being synthesized, are partly esterified and glycosylated, partly transformed to Δ^5 sterols and also most probably to stanols. The transformation of the free and bound sterols proceeding at the same time in various cellular organelles, with some delay due to intracellular transportation processes, cannot be excluded. The quantitative evaluation of each of these processes is impossible at present.

EXPERIMENTAL

Material. The seedlings of *Calendula officinalis* var. Radio were cultivated under the conditions described previously [1].

Administration of radioactive precursors. The seed coats were removed from 3-day-old seedlings and 900 were fed with 90 μC of DL-sodium mevalonate-[2-^{14}C] for 16 hr. Subsequently the seedlings were washed and cultivated on non-radioactive medium. One hundred seedlings were taken for analysis after 16 hr, 1, 2, 3, 5, 7, 10, 14 and 18 days of cultivation. The embryo axes and cotyledons were analysed separately.

Fractionation of the material. The free sterols, steryl esters, steryl glucosides, acylated steryl glucosides and water-soluble complexes were isolated, hydrolysed and fractionated by the methods described earlier [1]. Stanols, Δ^7 sterols, Δ^5 sterols, stigmasterol, campestrol and methylenecholesterol from the different sterol fractions were obtained by silica gel TLC as described previously [1]. The separation steps were monitored by autoradiography. The radioactivity was measured by scintillation counting at a counting efficiency of 80% with 0·03% POPOP and 0·3% PPO in toluene as the scintillator soln.

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