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REVISION OF THE PATHWAY OF D-PINITOL FORMATION IN LEGUMINOSAE

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Key Word Index—*Medicago sativa*; *Ononis spinosa*; *Trifolium incarnatum*; Leguminosae; cyclitols; biosynthesis; D-pinitol.

Abstract—Tracer studies with labelled inositols demonstrate that in contrast to earlier reports the formation of D-pinitol in *Medicago sativa*, *Ononis spinosa* and *Trifolium incarnatum* does not proceed by epimerization of sequoyitol but via D-ononitol. Hence, the novel pathway detected in *Simmondsia chinensis* occurs also in the Leguminous family and thus represents a major biosynthetic pathway for D-pinitol.

INTRODUCTION

Until recently, the unchallenged view of D-pinitol (1D-3-*O*-methyl-*chiro*-inositol) biosynthesis in higher plants assumed the sequence *myo*-inositol → sequoyitol (5-*O*-methyl-*myo*-inositol) → D-pinitol; this had been shown particularly in Gymnosperms [1] and Leguminosae [2]. However, investigations of plant constituents of 'Jojoba' (*Simmondsia chinensis*) revealed the presence of D-pinitol in leaves without the required occurrence of the precursor sequoyitol; instead, D-ononitol (1D-4-*O*-methyl-*myo*-inositol) was detected, which proved to function as a D-pinitol precursor in this plant [3]. Since D-pinitol also occurs together with D-ononitol in leguminous plants, the speculation arose that possibly two pathways of D-pinitol formation may exist in this particular plant family: one via sequoyitol and the other via D-ononitol. However, our investigation revealed that only the epimerization of D-ononitol represents the operating pathway, while earlier reports in the literature are misleading because of the use of an inappropriate substrate in enzymatic studies.

RESULTS AND DISCUSSION

The main issue of the present investigation was to elucidate whether D-ononitol can function as a precursor of D-pinitol in leguminous plants, which have been described to use only sequoyitol [2]. The classical approach to this question would be to run a $^{14}CO_2$-pulse-chase experiment, using photosynthesis to incorporate label into all inositols present in the plant and to evaluate the sequence of turnover of the individual compounds during the chase period. Fortunately, such kinetic studies with *Medicago sativa*, *Ononis spinosa* and *Trifolium incarnatum* formerly prepared for other purposes were at hand for analysis. The inspection of the paper chromatograms revealed that sequoyitol had been formed in neither plant. In contrast, appreciable levels of D-ononitol, whose label was decreasing with time in favour of D-pinitol, could be detected. The nature of D-ononitol had to be corroborated separately using high-voltage electrophoresis in borate buffer, since the chromatographic properties of sequoyitol and D-ononitol are quite similar and sometimes indistinguishable.

This preliminary indication of a biogenetic relationship between D-ononitol and D-pinitol as well as the lack of sequoyitol was conclusively probed by feeding [U-^{14}C]-labelled precursors, *myo*-inositol, sequoyitol and D-ononitol, via the transpiration stream to leaves of *Medicago sativa*, *Ononis spinosa* and *Trifolium incarnatum*. Leaf samples were taken at intervals and subjected to paper chromatographic analysis and confirmative high-voltage electrophoresis. While feeding of sequoyitol to all three plants led only to a minor degree of demethylation to *myo*-inositol (0.2%), the corresponding application of D-ononitol resulted in the formation of D-pinitol. The data given in Table 1 are selected from the representative experiment with *Trifolium incarnatum*, a plant, which formerly was the subject of enzymatic studies with sequoyitol [4]. The conversion rate of D-ononitol to D-pinitol yielded 80.9% after three days, a rate that convincingly points to the validity of the novel pathway, while sequoyitol epimerization could not be achieved at all. This

Table 1. Metabolites of two feeding experiments with young leaves of *Trifolium incarnatum* using labelled precursors

	Feeding time (hr)					
	myo-Inositol				D-Ononitol	
Metabolites	3	24	48	72	24	72
myo-Inositol	78.3	38.8	25.8	19.7	0	0
D-Bornesitol	0	0.5	0.7	0.7	0	0
D-Ononitol	12.2	32.0	26.1	21.0	44.1	17.6
D-Pinitol	1.3	22.2	41.0	48.9	54.6	80.9
D-*chiro*-Inositol	0.2	0.4	0.4	0.5	1.3	1.5
Pectic compounds	8.0	6.1	6.0	9.2	0	0

Data given in % of total radioactivity recovered from the leaf sample. Compounds fed were uniformly labelled with ^{14}C.

basic scheme was further supported by the results of the feeding experiment with *myo*-inositol. The data included in Table 1 first of all show the lack of sequoyitol formation; instead, D-ononitol is being synthesized by methylation of *myo*-inositol. Furthermore, trace amounts of a likewise monomethylated *myo*-inositol, namely D-bornesitol, could be detected. While the latter shows only very low levels of ^{14}C-incorporation and does not gain any importance, D-ononitol exhibits the characteristics of a kinetic precursor: increase of incorporated label, maximum incorporation point and finally a decrease of labelling. In contrast to Gymnosperms, which are able to convert D-pinitol further to methyl-*muco*-inositol [1], the sequence of epimerizations in the three leguminous plants tested stops at D-pinitol; eventually D-*chiro*-inositol can be detected, but this compound is a well known product of demethylation of D-pinitol.

The clear relationship beteeen D-ononitol and D-pinitol, which we have demonstrated earlier in *Simmondsia chinensis* [3] and presently in three leguminous plants provokes the question why an erroneous pathway via sequoyitol had been described to function in the latter plant family [4]. One of the numerous publications on cyclitols in Leguminosae [5] indeed describes that prior to D-pinitol formation D-ononitol has to be synthesized in *Ononis spinosa*. However, since trace amounts of sequoyitol were thought to occur in *Ononis* and since the biosynthesis of D-pinitol in Gymnosperms indeed was known to proceed via sequoyitol, no further questions had been asked. Such a conclusion appears the more understandable as D-ononitol does not look—at least not at the first glance—like a possible precursor. Only the turning of the molecular space model of D-pinitol reveals the biogenetic relationship between both cyclitols [3]. The report of an enzymatic study with *Trifolium incarnatum* apparently demonstrating the conversion of sequoyitol to D-pinitol can be explained on the basis that the precursor was isolated from the very plant species used in the study and D-ononitol easily can be mistaken for sequoyitol without proper controls [4].

The initially raised question, whether two pathways of D-pinitol formation exist in leguminous plants can be answered so far as only the newly detected pathway via D-ononitol operates in this plant family and that the previously held view of sequoyitol epimerization can be attributed to experimental error.

EXPERIMENTAL

PC, radioautography and high-voltage electrophoresis for analysis and compound identification were carried out as previously described [6].

Feeding experiment. A short shoot section of *Medicago sativa*, *Trifolium incarnatum* and *Ononis spinosa* with a young leaf at the tip was placed in a small test-tube containing the aq. soln of either 10 μCi myo-[U-^{14}C] inositol, 2 μCi D-[U-^{14}C] ononitol or 2 μCi [U-^{14}C] sequoyitol. When the soln had been consumed, further distilled H_2O was added. Leaf sections were cut out at intervals and analysed for labelled products.

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