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THE FATE OF *MYO*-INOSITOL IN *FRAXINUS* TISSUE CULTURES

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Abstract—A callus tissue culture of *Fraxinus pennsylvanica* was grown for 10 weeks in the presence of *myo*-inositol-UL-^{14}C. After the growth period, the specific activities (s.A.) of the free *myo*-inositol extractable from the tissue and from the residual medium, as well as the bound *myo*-inositol in the phosphatidylinositol were identical to the s.A. originally supplied. Thus the tissue is not capable of synthesizing any *myo*-inositol. Seventy-one per cent of the *myo*-inositol metabolized was found in insoluble cell wall material, 21% in phosphatidylinositol. Since the s.A. of the D-galacturonic acids in the cell wall material was less than 0·5% of the s.A. in *myo*-inositol, the *myo*-inositol pathway proposed for pectin formation does not seem to be of major importance.

INTRODUCTION

myo-INOSITOL was one of the first growth factors discovered and chemically characterized.[1,2] It greatly stimulates the growth of many yeast species[3] and it is an absolute requirement for growth of some plant tissue cultures.[4] It was also found to be an essential growth factor for every one of 18 tested tissue cultures of human origin.[5] A function of *myo*-inositol in the growth and survival of cells was indicated by the finding that it occurs in phospholipids;[6,7] thus it seems to be an indispensable membrane component.

More recently, it has been suggested by Loewus and co-workers[8–10] that in plants *myo*-inositol is an important intermediate in the biosynthetic route from D-glucose to the uronic acids and pentose units of cell wall material. However, from the *myo*-inositol feeding data available,[8–10] it has never been possible to estimate quantatively the contribution of the *myo*-inositol pathway to cell wall (e.g. pectin) biosynthesis, since the endogenous synthesis of *myo*-inositol and its pool size were never taken into consideration.

In order to study this question further, the fate of *myo*-inositol-UL-^{14}C was followed when supplied to a tissue culture obligatorily requiring *myo*-inositol as a growth factor. In such

[1] E. WILDIERS, *Cellule* **18,** 313 (1901).
[2] E. V. EASTCOTT, *J. Phys. Chem.* **32,** 1094 (1928).
[3] P. R. BURKHOLDER, I. MCVEIGH and D. MEYER, *J. Bact.* **48,** 385 (1944).
[4] K. E. WOLTER and F. SKOOG, *Am. J. Bot.* **53,** 263 (1966).
[5] H. EAGLE, V. I. OYAMA, M. LEVY and A. E. FREEMAN, *J. Biol. Chem.* **226,** 191 (1957).
[6] R. I. ANDERSON, *J. Am. Chem. Soc.* **52,** 1607 (1930).
[7] E. KLENK and R. SAKAI, *J. Physiol. Chem.* **258,** 33 (1939).
[8] L. F. LOEWUS, S. KELLY and E. F. NEUFELD, *Proc. Natl. Acad. Sci.* **48,** 421 (1962).
[9] R. M. ROBERTS, J. DESHUSSES and F. LOEWUS, *Plant Physiol.* **43,** 979 (1968).
[10] F. LOEWUS, *Ann. N.Y. Acad. Sci.* **165,** 577 (1969).

tissue, no or only very little endogenous synthesis should occur. Tissue cultures of *Fraxinus pennsylvanica* known to possess this property[4] were used in the experiments to be described. The results obtained clearly indicate that less than 0·5% of the total D-galacturonic acid of the cell walls is derived from *myo*-inositol.

RESULTS

Fraxinus pennsylvanica callus tissue was grown for 10 weeks in the presence of 6·6 mg *myo*-inositol-UL-^{14}C (s.A. 0·09 μC/μmol). During this time its dry weight increased from 340 mg to 2·803 g. At the end of this period the tissue contained 73% of the initial radioactivity supplied.

The residual radioactivity in the medium, 21% of the total supplied, was found to be solely in *myo*-inositol. 45% of the total radioactivity of the tissue was extractable with aqueous ethanol and chloroform–methanol (SI) and approximately half of this radioactivity was found in non-metabolized *myo*-inositol (Fig. 1). Twelve per cent of the extractable radioactivity was found in a *myo*-inositol-glycoside, which behaved like galactinol as judged by paper and by sephadex column chromatography. The exact nature of the glycoside could not be established, since the total amount available was too low.

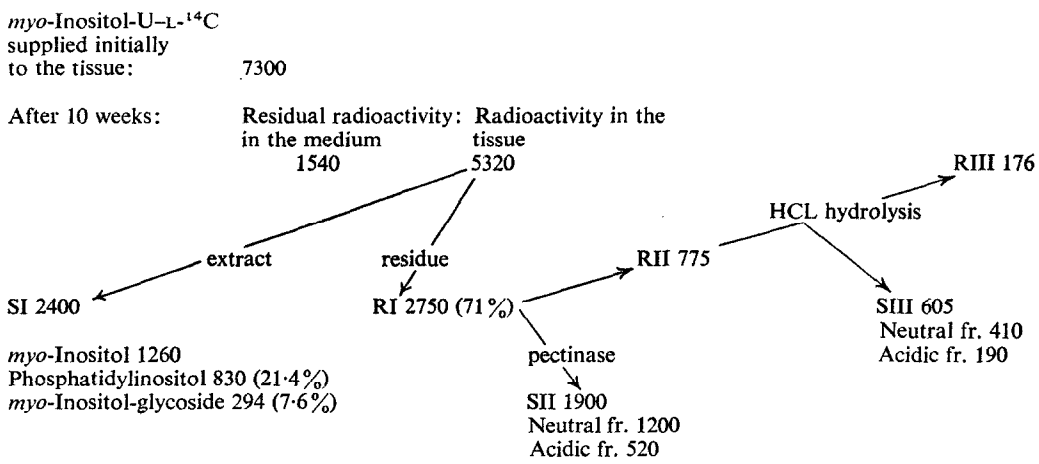

FIG. 1. DISTRIBUTION OF RADIOACTIVITY IN THE TISSUE (dpm $\times$ 10^{-3}).
The percentages given relate to the total amount of *myo*-inositol metabolized as 100%, i.e. the sum of RI and the two compounds of SI.

About 35% of the extractable radioactivity corresponding to 21% of the total *myo*-inositol metabolized was found to be present in the phospholipid fraction. Saponification of this fraction according to Tarlov and Kennedy[11] showed that no radioactivity was present in the fatty acids; the main radioactive component of the water soluble fraction after saponification was chromatographically identical to glyceryl-phosphoryl-*myo*-inositol.[12] Acid hydrolysis of the lipid according to Paulus and Kennedy[13] followed by electrophoresis of the components resulted in 75% of the radioactivity occurring in *myo*-inositol-mono-phosphate and 25% in *myo*-inositol. TLC of the phospholipid-^{14}C following

[11] A. R. TARLOV and E. P. KENNEDY, *J. Biol. Chem.* **240,** 49 (1964).
[12] W. TANNER, *Arch. Mikrobiol.* **64,** 158 (1968).
[13] H. PAULUS and E. P. KENNEDY, *J. Biol. Chem.* **235,** 1303 (1960).

the procedure of Gonzalez-Sastre and Folch-Pi[14] showed that the radioactivity was completely contained in phosphatidylinositol. With this procedure the phosphotidylinositol can be separated from both the phosphatidylinositolphosphates.

The residue (RI) which contained 51·5% of the total radioactivity of the tissue and 71% of the *myo*-inositol metabolized was first treated with EDTA and pectinase according to Roberts *et al.*[9] By this treatment two thirds of the radioactivity was solubilized (SII) (Fig. 1) and further partitioned by Dowex I into a neutral and an acidic fraction. The latter consisted mainly of D-galacturonic acid and acidic oligosaccharides which on acid hydrolysis (2·5 N HCl, 100°, 4 hr) also yielded D-galacturonic acid as the main radioactive product. D-galacturonic acid was identified by paper chromatography; the neutral fraction consisted of mono- and oligosaccharides, which were not further identified.

The insoluble residue remaining after enzymic hydrolysis (RII) was solubilized to 77% (SIII) by acid treatment (2·5 HCl, 100°, 45 min); the composition of the solubilized fraction was qualitatively similar to SII obtained by enzymic hydrolysis (Fig. 1). No radioactive D-glucuronic acid and methylated D-glucuronic acids were detected in these fractions, in contrast to the observations of Roberts *et al.*[9] This is most likely due to the very low specific activities of *myo*-inositol-UL-^{14}C which had to be used.

TABLE 1. SPECIFIC ACTIVITIES OF FREE *myo*-INOSITOL, LIPID-BOUND *myo*-INOSITOL AND D-GALACTURONIC ACID AFTER 10 WEEKS OF TISSUE CULTURE GROWTH

Compound	Specific activity (10^{-3}dpm/μmol) Determined	Corrected for initial tissue
Residual *myo*-inositol in the medium	160	—
	172	—
Extracted free *myo*-inositol from the tissue	170	193
	200	216
myo-Inositol from phosphatidylinositol	177	201
	155	176
D-Galacturonic acid from fraction S II	0·52*	0·59
	0·56*	0·63
	0·37†	0·42
myo-Inositol initially supplied to the tissue	198	

In correcting for the initial tissue it was assumed that the composition of the tissue remained the same during growth.

* Determined from the acidic fraction after separation by Dowex.

† Determined after additional purification by PC.

After extensive purification by paper chromatography the specific activities of the *myo*-inositol in the residual medium as well as of the free *myo*-inositol extracted from the tissue were determined (Table 1). They agree well with the specific activity of the *myo*-insoitol obtained from phosphatidylinositol after acid hydrolysis (5 N HCl, 100°, 3 hr) (Table 1). The specific radioactivity of the D-galacturonic acid obtained from the residue, however, was lower by a factor of more than 300. When the values are corrected for the content of *myo*-inositol and D-galacturonic acid of the tissue explant which was used to start the culture, the data of column 2 are obtained.

[14] F. GONZALEZ-SASTRE and J. FOLCH-PI, *J. Lipid Res.* **2**, 532 (1968).

A duplicate experiment provided very similar results. Again the specific activity of the D-galacturonic acid was found to be less than 1% of that of the free and bound *myo*-inositol.

DISCUSSION

The fact that the specific activity of the free *myo*-inositiol in the medium and in the tissue as well as that of the lipid-bound inositol are identical within experimental error to the initial specific activity of the *myo*-inositol supplied, clearly demonstrates that the tissue does not synthesize any *myo*-inositol. Seventy-one per cent of the total *myo*-inositol metabolized were found in the non-soluble residue and could be solubilized almost completely by pectinase treatment and by acid hydrolysis. Of the solubilized radioactivity 27% was found in an acidic fraction, which consisted almost exclusively of D-galacturonic acid.

These incorporation data agree well with the findings of Loewus *et al.*[8–10] who also observed that most of the *myo*-inositol supplied exogenously to various plant tissues is incorporated into cell wall polysaccharides and also the main individual radioactive compound was D-galacturonic acid. Shantz *et al.*[15] have also reported that, in explants of carrot root phloem tissue, the main metabolic fate of *myo*-inositol is its incorporation into a 'pectic' fraction. These authors observed this, however, mainly for the cell enlargement phase, whereas very little was found to be incorporated during a rapid cell division phase.

To answer the question whether oxidative cleavage of *myo*-inositol[16,17] and the incorporation of the resulting products constitutes a major route for the synthesis of acidic cell wall polysaccharides[10,18] the specific activity of the newly synthesized D-galacturonic acid had to be determined. The very low specific activity found by us indicates that more than 99% of the D-galacturonic acid incorporated into polysaccharides arises by a different pathway, most likely directly via sugar nucleotides.[19]

It therefore seems highly unlikely that this tissue requires exogenous *myo*-inositol to synthesize acidic polysaccharides. Why then does it need this cyclitol? It would be conceivable that the *myo*-inositol-glycoside is absolutely required by the tissue. It has recently been shown that galactinol, L-1-(*O*-a-D-galactopyranosyl)-*myo*-inositol, is the galactosyl-donor in the biosyntheses of the common plant oligosaccharides of the raffinose family.[20–22] However, the *Fraxinus* tissue does not contain any raffinose sugars (unpublished results). Also in yeast cells raffinose sugars do not occur although *myo*-inositol is a growth factor for many species.[2,3] In addition, a *myo*-inositol-mannoside present in *S. cerevisiae* seems only to be a product of phospholipid breakdown, whereas the main fate of *myo*-inositol in yeast is its incorporation into phospholipids.[12]

In contrast to the low specific activity of the pectic fraction, more than 20% of the *myo*-inositol metabolized by the *F. pensylvannica* tissue are incorporated with an unchanged specific activity into phosphatidylinositol. It seems likely therefore that phosphatidylinositol is absolutely essential for survival and growth of the cells. The fact that the lack of *myo*-inositol is so detrimental to these cells indicates that the *myo*-inositol containing phospho-

[15] E. M. SHANTZ, M. SUGII and F. C. STEWARD, *Ann. N.Y. Acad. Sci.* **144,** 335 (1967).
[16] F. C. CHARALAMPOUS, *J. Biol. Chem.* **235,** 1186 (1960).
[17] K. M. GRUHNER and O. HOFFMANN-OSTENHOF, *Hoppe Seyler's Z. Physiol. Chem.* **347,** 278 (1966).
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[22] L. LEHLE, W. TANNER and O. KANDLER, *Hoppe Seyler's Z. Physiol. Chem.* **351,** 1494 (1970).

lipids are not inert building blocks of the membrane, but rather are important for the functioning of the membrane. This is strongly supported by recent observations that transport mechanisms for amino acid uptake into KB cells[23,24] and into *S. cerevisiae*[25] as well as sugar transport into *Neurospora crassa*[26] are greatly severed when these cells are not optimally supplied with *myo*-inositol.

EXPERIMENTAL

Tissue culture and extraction. F. pennsylvanica callus tissue cultures were grown as described previously[4] in a medium containing 10 mg *myo*-inositol/l. After 10 weeks the tissue was freeze-dried and weighed. The tissue was extracted following a procedure previously worked out for *S. cerevisiae*.[12] The insoluble material was further treated following essentially the procedure of Roberts *et al.*[9]

Chromatography and electrophoresis. For paper chromatographic separation, purification and identification of D-galacturonic acid, *myo*-inositol, *myo*-inositol glycoside and of *myo*-inositol-phosphorylglycerol the following solvents (v/v) were used:
EtOAc–*n*–BuOH–HOAc (6:4:3), α-picoline–H_2O–NH_4OH (70:28:2), *iso*-PrOH–H_2O–HOAc (15:3:2) and *iso*-PrOH–NH_4OH (2:1). The chromatograms were sprayed with alkaline $AgNO_3$.[27] *Myo*-inositol-monophosphate was characterized by electrophoresis in 0·2 M ammonium formate pH 3·7 and in NEt_3–HOAc–H_2O (21:6·1:973) pH 8·6. Phospholipids were developed after separation on thin layer[14] using the spray of Vaskovsky and Kostetsky.[28]

Quantitative determination of myo-*inositol and* D-*galacturonic acid. myo*-Inositol was determined enzymically with NAD: *myo*-inositol dehydrogenase prepared from *Aerobacter aerogenes* following the procedure of Weissbach.[29] D-Galacturonic acid was determined according to McComb and McCready.[30]

Determination of radioactivity. The radioactivity of the total tissue and of the insoluble fractions RI, RII and RIII was determined after total combustion according to Kalberer and Rutschmann[31] by scintillation counting. All soluble samples were counted directly in dioxan/PPO; all determinations were corrected for 100% efficiency by internal standardization.

Acknowledgement—We thank Dr. E. Beck for his help in carrying out total combustions of the insoluble fractions.

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[30] E. A. McComb and R. M. McCready, *Analyt. Chem.* **24,** 1630 (1952).
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Key Word Index—Fraxinus pennsylvanica; Oleaceae; fate of *myo*-inositol; biosynthesis; pectic substances.