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THE FUNCTION OF THE PENTOSE PHOSPHATE PATHWAY IN *PHASEOLUS MUNGO* HYPOCOTYLS

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Key Word Index—*Phaseolus mungo*; Leguminosae; black gram; pentose phosphate pathway; gluconate metabolism; glucose catabolism; differentiation.

Abstract—The metabolism of D-gluconate-[1-^{14}C] and -[6-^{14}C] by segments from etiolated hypocotyls of *Phaseolus mungo* has been studied. The release of $^{14}CO_2$ from gluconate-[1-^{14}C] was greater than that from gluconate-[6-^{14}C] in all parts of hypocotyls examined. Incorporation of the radioactivity from gluconate-[6-^{14}C] into RNA, lignin and aromatic amino acid fractions was greater in the upper (younger) part of the hypocotyls. Incorporation into sugars was greater in the lower (more mature) parts.

INTRODUCTION

As part of our studies [1,2] on the significance of the pentose phosphate (PP) pathway in etiolated *Phaseolus mungo* seedlings, we have investigated the metabolism of D-gluconate-[1-^{14}C] and -[6-^{14}C] in three parts of the hypocotyls, representing different stages of differentiation and ageing. Results are compared with those obtained when either D-glucose-[1-^{14}C] or -[6-^{14}C] was fed to younger parts of the hypocotyls.

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RESULTS AND DISCUSSION

Table 1 shows the incorporation of ^{14}C into various metabolites after Part I (see Experimental) of the hypocotyls had been incubated for 2 hr with glucose-[1-^{14}C] or -[6-^{14}C]. Although

Table 1. Incorporation of ^{14}C from glucose-[1-^{14}C] and -[6-^{14}C] into various fractions in Part I of the hypocotyls of *Phaseolus mungo* seedlings

Fraction	Radioactivity recovered (× 10^3 dpm) [1-^{14}C]		[6-^{14}C]	
CO_2	68	(14·4*)	36	(10·1*)
EtOH-soluble fraction	228	(48·2)	175	(49·0)
sugar phosphates	21	(4·4)	24	(6·7)
glucose	47	(9·9)	37	(10·0)
sucrose	41	(8·7)	22	(6·2)
citrate	6	(1·3)	4	(1·1)
malate	42	(8·9)	37	(10·0)
lactate, succinate	5	(1·1)	6	(1·7)
aspartate	7	(1·5)	7	(1·9)
glutamate	14	(3·0)	15	(4·2)
alanine	10	(2·1)	9	(2·5)
threonine	11	(2·3)	5	(1·4)
tyrosine, valine	8	(1·7)	8	(2·2)
leucine, isoleucine, phenylalanine	4	(0·8)	5	(1·4)
glutamine	3	(0·6)	trace	(–)
EtOH-insoluble fraction	177	(37·4)	146	(40·9)
carbohydrates	127	(26·8)	107	(30·0)
uronic acids	47	(9·9)	36	(10·1)
lignin	3	(0·6)	3	(0·8)

* Percentage of the radioactivity found in the fractions or compounds to the total radioactive substrates absorbed.

release of $^{14}CO_2$ from glucose-[1-^{14}C] was appreciably greater than that from glucose-[6-^{14}C], differences between the distribution of radioactivity into sugar phosphates, amino and organic acids and ethanol-insoluble carbohydrates from glucose-[1-^{14}C] and that from glucose-[6-^{14}C] were not obvious.

In an attempt to determine more clearly the function of the PP pathway in different parts of hypocotyls, gluconate-[1-^{14}C] or -[6-^{14}C] was fed. If most of the gluconate-[^{14}C] supplied was metabolized via the PP pathway, the distribution of radioactivity should afford more direct evidence for the function of the pathway than that obtained from the experiments with glucose. The incorporation of radioactivity from gluconate-[1-^{14}C] into $^{14}CO_2$ was extremely high. When gluconate-[6-^{14}C] was used as a substrate, most of the ^{14}C was recovered in cellular metabolites (Table 2). This suggests that the supplied gluconate

Table 2. Incorporation of ^{14}C from gluconate-[1-^{14}C] and -[6-^{14}C] into various compounds in Parts I, III and V of the hypocotyls of *Phaseolus mungo* seedlings

Compound	Part I [1-^{14}C]		Part I [6-^{14}C]		Part III [1-^{14}C]		Part III [6-^{14}C]		Part V [1-^{14}C]		Part V [6-^{14}C]	
Experiment I	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
CO_2	685	(76·3)	137	(17·1)	349	(60·6)	46	(8·9)	337	(57·8)	53	(9·1)
Sugars	4	(0·4)	19	(2·4)	19	(0·2)	59	(11·5)	2	(0·3)	87	(14·9)
glucose	†		3		†		4		†		18	
fructose	†		4		†		4		†		3	
sucrose	†		1		†		44		†		53	
Organic acids**	179	(19·9)	209	(26·1)	207	(35·9)	253	(49·1)	228	(39·1)	259	(44·4)
Amino acids	8	(0·9)	81	(10·1)	7	(1·2)	24	(4·7)	4	(0·7)	45	(7·7)
aspartic acid, threonine, serine, asparagine,	2		6		2		3		1		7	
glutamine	3		40		4		10		3		27	
glutamic acid	3		5		*		3		*		1	
proline	—		2		—		*		—		1	
glycine, alanine	—		21		1		4		*		5	
methionine	—		3		—		2		—		2	
phenylalanine	—		4		—		2		—		2	
Insoluble carbohydrates	3	(0·3)	86	(10·7)	1	(0·2)	10	(2·0)	3	(0·5)	31	(5·4)
hexoses	—		28		—		5		—		10	
pentoses	—		8		—		1		*		2	
uronic acids	3		26		1		2		3		12	
Protein amino acids	1	(0·1)	52	(6·4)	*	(—)	5	(1·0)	1	(0·2)	9	(1·5)
aspartic acid	*		3		—		1		*		2	
serine, glutamic acid	—		22		—		3		—		5	
proline	—		6		—		—		—		—	
glycine, alanine	—		6		—		2		—		3	
leucine, isoleucine	—		16		—		—		—		—	
Lignin	7	(0·8)	118	(14·7)	2	(0·3)	52	(10·0)	1	(0·2)	34	(5·8)
Experiment II												
Nucleotides	6	(0·7)	8	(1·0)	3	(0·5)	6	(1·2)	3	(0·5)	7	(1·2)
RNA	2	(0·2)	61	(7·6)	2	(0·2)	20	(3·9)	2	(0·3)	15	(2·6)
Lipids	3	(0·3)	30	(3·7)	4	(0·7)	40	(7·8)	3	(0·5)	43	(7·4)

The rates of incorporation of the radioactivity are expressed as $\times 10^3$ dpm per g fr. wt (a), and as the percentage of total radioactivity absorbed (b). See Table 1.

* trace; —, not detected; †, not determined.

** The main constituent of the organic acid fraction was unmetabolized gluconate.

was converted to 6-phosphogluconate which was then decarboxylated (releasing the carbon atom at the '1' position as CO_2). This assumption is supported by other similar studies using higher plants [3–5]. The large release of $^{14}CO_2$ from gluconate-[1-^{14}C] suggests that refixation by phosphoenolpyruvate carboxylase is slight.

The distribution of radioactivity in fractions from the extracts of segments incubated with gluconate-[1-^{14}C] and -[6-^{14}C] for 4 hr is also shown in Table 2. In Part I (a hook of the hypocotyl), a large amount of label from gluconate-[6-^{14}C] was incorporated into lignin, RNA, amino acids (both free and protein constituents) and insoluble carbohydrates. The results suggest that the PP pathway supplies precursors for the biosynthesis of RNA and lignin in this part of the hypocotyl. As high activity of phosphoribosylpyrophosphate synthetase was observed in this part [2], a higher rate of incorporation from gluconate-[6-^{14}C] into RNA is reasonable. However, the results also indicate that gluconate may be metabolized through a variety of other metabolic pathways; e.g., by way of fructose-6-phosphate and glyceraldehyde-3-phosphate to the glycolytic pathway, to gluconeogenetic pathways, and into amino acids and insoluble carbohydrates. In this part, amino acids formed from gluconate were used for the active biosynthesis of protein. In Part III (an intermediary part of the hypocotyl) and Part V (a more aged and differentiated part of the hypocotyl), the patterns of incorporation of ^{14}C from gluconate-[1-^{14}C] and -[6-^{14}C] were similar. At the same time, the amount of ^{14}C into RNA, lignin, protein amino acid and insoluble-carbohydrate fractions was less than that observed with Part I.

In the previous paper [1], it was shown that the PP pathway operates most actively in Part I. The results obtained in the present work suggest that the PP pathway may play a more important role as the supplier of the building blocks for biosynthesis in the younger parts of the hypocotyls of *Phaseolus mungo* seedlings than in the aged parts.

EXPERIMENTAL

Plant material. Hypocotyls of 4-day-old etiolated *Phaseolus mungo* (black gram) seedlings grown under aseptic conditions were divided as described previously [1]. Part I (the apical 5 mm of a hypocotyl including a hook), Part III (the segment 15–25 mm from the tip) and Part V (the segment 45–55 mm from the tip) were used.

Administration of glucose-[^{14}C] and gluconate-[^{14}C]. 15 segments of hypocotyls (Part I, *ca* 0·3 g; Part III, *ca* 0·75 g; Part V, *ca* 1 g) were incubated in 2 ml of K_2HPO_4–KH_2PO_4 buffer (10 mM, pH 6) containing 0·3% glucose and 1 ml of 5 μCi gluconate-[^{14}C] or glucose-[^{14}C] solns in a flask with a centre well (containing 0·2 ml of 20% KOH). D-Glucose-[1-^{14}C] (sp. act. 33·8 mCi/mmol), D-glucose-[6-^{14}C] (sp. act, 10·0 mCi/mmol) (Daiichi Kagaku Yakuhin Co., Tokyo). Na salts of D-gluconic acid-[1-^{14}C] (sp. act. 3·0 mCi/mmol) and D-gluconic acid-[6-^{14}C] (sp. act. 3·4 mCi/mmol) (Radiochemical Centre, Amersham) were used.

Separation of various fractions and isolation of sugars, amino acids, uronic acids and lignin. Samples were killed and extracted with EtOH as described in Ref. 6. The extract was concentrated *in vacuo* at 35°, and separated into basic, acidic and neutral fractions using Amberlite IR-120B (H^+ form) and Dowex 1 × 4 ($HCOO^-$ form). In the experiments using glucose-[^{14}C], the EtOH–soluble fraction was resolved using two dimensional PC in PhOH–H_2O (72 : 28, w/w) plus 1 mM EDTA, then equal parts of *n*-BuOH–H_2O (15:1), and HOPr–H_2O (31:40). EtOH–insoluble materials were dried and powdered. Samples for analysis of protein amino acids were hydrolyzed at 130° with 6 M HCl for 24 hr [7]. The hydrolysates were passed through a column of Amberlite IR-120B (H^+ form) from which amino acids were eluted. Other samples were treated with 72% H_2SO_4 for 2 hr at room temp. and the resultant suspensions diluted with H_2O to 1% H_2SO_4 and heated in sealed ampoules at 100° for 12 hr [8]. The hydrolyzates were filtered. The residue constituted the lignin fraction. The filtrates were neutralized and fractionated further using columns of Amberlite IR-120B (H^+ form) and Dowex 1 × 4 ($HCOO^-$ form). The effluent contained neutral sugars derived from the EtOH–insoluble carbohydrates. The eluate from Dowex 1 × 4 column contained uronic acids. Sugars were separated by PC using BuOH–HOAc–water (4:1:2), or using MeEtCO–Me_2CO–H_2O–C5H5N (150:30:10:1). Amino acids were separated using an automatic amino acid analyzer.

Isolation of lipids, nucleotides and RNA. Segments of the hypocotyls were frozen with liquid N_2, and ground finely with a chilled pestle and mortar. The frozen powder was extracted successively with 5% $HClO_4$ at 2° for 30 min (2 ×), an EtOH–Et_2O mixture (1:1, v/v) at 50° for 15 min (2 ×), and 0·3 M KOH at 37° for 18 hr. The $HClO_4$–soluble fraction was neutralized with K_2CO_3. After removal of precipitated perchlorate, the fraction was subjected to ion-exchange chromatography as described in Ref. 9 for nucleotide analysis. The EtOH–Et_2O extract was considered to be a lipid fraction. The 0·3 M KOH–soluble fraction was neutralized and nucleotides derived from RNA were separated by ion-exchange chromatography.

Measurement of radioactivity. All radioactive samples, except $^{14}CO_2$, were assayed using a liquid scintillation spectrometer. In the case of insoluble materials, radioactivity was measured following combustion in a sample oxidizer. Quench corrections were made using an external standard technique. Respiratory $^{14}CO_2$ was measured with a windowless gas flow counter after conversion to $Ba^{14}CO_3$. Results were corrected for self-absorption by the carbonate and converted to dpm by counting standard samples using both the windowless gas flow counter and the liquid scintillation spectrometer.

REFERENCES

1. Ashihara, H., Komamine, A. and Shimokoriyama, M. (1974) *Bot. Mag.* Tokyo **87,** 121.
2. Ashihara, H. and Komamine, A. (1974) *Plant Sci. Letters* **2,** 331.
3. Wang, C. H., Doyle, W. P. and Ramsey, J. C. (1962) *Plant Physiol.* **37,** 1.
4. Beevers, H. (1956) *Plant Physiol.* **31,** 339.
5. Humphreys, T. E. and Dugger, W. M. Jr. (1959) *Plant Physiol.* **34,** 580.
6. Bassham, J. A. and Calvin, M. (1957) *The Path of Carbon in Photosynthesis,* pp. 16, Prentice-Hall, Englewood Cliffs, New Jersey.
7. Dustin, J. D., Czajkowska, C., Moore, S. and Bigwood, E. J. (1953) *Anal. Chim. Acta* **9,** 256.
8. Jermyn, M. A. (1955) *Modern Methods of Plant Analysis* Vol. 2, pp. 197, Springer, Berlin.
9. Fowler, M. W. and ap Rees, T. (1970) *Biochim. Biophys. Acta* **201,** 33.