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GLYCOLYSIS DURING GLUCONEOGENESIS IN COTYLEDONS OF *CUCURBITA PEPO*

S. M. THOMAS and T. AP REES

Botany School, University of Cambridge

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Key Word Index—*Cucurbita pepo*; Cucurbitaceae; marrow; glycolysis; germination; cotyledons; phosphofructokinase; pyruvate kinase.

Abstract—The aim of this work was to investigate the extent of glycolysis during gluconeogenesis in the germination of marrow (*Cucurbita pepo* L. var. *medullosa* Alef.). The activities of phosphofructokinase (E.C. 2.7.1.11) in extracts of cotyledons, of seeds, and seedlings grown in the dark for 2, 5, and 8 days were 3·5, 4·8, 9·4, and 11·8 nmol substrate consumed per cotyledon per min, respectively. The comparable figures for pyruvate kinase (E.C. 2.7.1.41) were 16·3, 72·3, 974, and 1485. The patterns of $^{14}CO_2$ production from [1-^{14}C], [2-^{14}C], [3,4-^{14}C], and [6-^{14}C]glucose indicated that at all the above stages of germination glycolysis was appreciable and predominated over the pentose phosphate pathway. These patterns, and the distribution of label from [1-^{14}C], and [3-^{14}C]pyruvate supplied to 5-day-old cotyledons, indicated that the pyruvate formed in glycolysis was converted to acetyl units that were used primarily in biosyntheses. It is concluded that glycolysis occurred at all the stages of germination examined and was particularly active during gluconeogenesis. It is suggested that the significance of this glycolysis is the provision of intermediates for biosyntheses, a need that may not be met by corresponding gluconeogenic intermediates as these may be retained within organelles.

INTRODUCTION

WE HAVE stressed the importance of studying the relationship between glycolysis and gluconeogenesis during the germination of fatty seeds.[1] In the same paper we showed that during germination of marrow in the dark, gluconeogenesis is slight for the first 2 days but develops rapidly in the next 3 days to a substantial level that persists at least until 8 days from the start of germination. The present work was undertaken in order to determine the extent of glycolysis during gluconeogenesis, and thus to increase our understanding of the relationship between the two processes. Our approach was to compare, at different stages of germination, the activities of characteristic glycolytic enzymes with the manner in which the cotyledons metabolized specifically labelled glycolytic substrates. In order to achieve as close a comparison as possible with our data for gluconeogenesis, the present work was carried out at stages of germination that were identical to those used previously. The substrates chosen were specifically labelled [^{14}C]glucose and [^{14}C]pyruvate because the extent and pattern of their metabolism indicate the activity of glycolysis. We chose phosphofructokinase (E.C. 2.7.1.11) and pyruvate kinase (E.C. 2.7.1.41) because they are characteristic of glycolysis but not of gluconeogenesis, and because they catalyse steps at which the flow of carbon is likely to be regulated.

[1] S. M. THOMAS and T. AP REES, *Phytochem.* **11**, 2177 (1972).

RESULTS

Enzyme Studies

We have described the general features of the differentiation of marrow cotyledons.[1] This data and that in Table 1 make it possible to relate the present study of glycolysis to carbon dioxide production and oxygen uptake, and to cell number and protein content during germination. We emphasize that little cell division occurred in the cotyledons during germination and that the changes that we observed represent differentiation.

The activities of phosphofructokinase and pyruvate kinase rose during germination (Table 2). Pyruvate kinase activity was greater, and rose more extensively, than that of phosphofructokinase. We checked that the above results were not affected by inhibition of the enzymes during the preparation of the extracts. We determined the effects of extracts from cotyledons at all the stages of germination on the activities of samples of purified phosphofructokinase and pyruvate kinase. No inhibition of either enzyme was detected with any of the extracts. We investigated whether we were using optimum conditions for the assays by determining the effects of varying, independently, the concentration of each component of the assay mixtures on the activities in extracts of seeds and of 5-day-old cotyledons. The results showed that the assay mixtures used to obtain the data in Table 2 gave the highest activities.

TABLE 1. RESPIRATION OF MARROW COTYLEDONS DURING GERMINATION

	Gas exchange (μl/cotyledon/hr)*	
Days germinated	Oxygen uptake	CO_2 production
0	0·7	1·3
2	8·3	6·6
5	248	104
8	163	64

* Values are means of six samples.

Metabolism of [^{14}C]*glucose and* [^{14}C]*pyruvate*

The rates at which the individual carbons of [^{14}C]glucose were released as $^{14}CO_2$ by cotyledons of different ages are given in Table 3. In these experiments we estimated the uptake of [^{14}C]glucose by comparing the radioactivity of the incubation medium at the beginning and end of the incubation. In the 6-hr incubation reported in Table 3 estimates of uptake of [3,4-^{14}C]glucose were 10, 40, 38, and 29% of the added activity for seeds, and 2-, 5-, and 8-day-old cotyledons, respectively. The patterns of $^{14}CO_2$ production were basically similar for all ages of cotyledons. We emphasize three aspects of this pattern. First, the yield of $^{14}CO_2$ from C-3 and C-4 was much higher than that from any other carbon. At all the intervals, with every age of cotyledon, the yield from C-3 and C-4 was at least twice that of the carbon with the next highest yield. Second, at all intervals and with every age of cotyledon, the yield from C-1 was significantly greater than that from C-6. Third, the yields from C-2 and C-6 were generally very similar. The only major change in the above pattern that occurred during germination was that the extent to which C-3 and C-4 were released in excess of the other carbons increased dramatically between days 2 and 5 and then declined. It should be noted that this increase was not accompanied by an increase in the amount of [^{14}C]glucose absorbed by the cotyledons.

TABLE 2. ACTIVITIES OF PHOSPHOFRUCTOKINASE AND PYRUVATE KINASE IN EXTRACTS OF MARROW COTYLEDONS DURING GERMINATION

Days germinated	Activity* (nmol substrate consumed/cotyledon/min)	
	Phosphofructokinase	Pyruvate kinase
0	3·45 ± 0·4	16·3 ± 1·2
2	4·75 ± 0·7	72·3 ± 12
5	9·43 ± 2·9	974 ± 124
8	11·80 ± 1·3	1485 ± 225
Comparison	Fisher's $\tilde{P}$ values†	
0 vs. 2	N.S.	<0·002
2 vs. 5	N.S.	<0·001
5 vs. 8	N.S.	N.S.
0 vs. 5	<0·05	<0·001
0 vs. 8	<0·001	<0·001
2 vs. 8	<0·001	<0·001

* Values are means ± S.E. of activities of 6 different extracts.
† Fisher's $\tilde{P}$ values are given for comparison of extracts from cotyledons of different ages. Values of 0·05 or less are considered significant. Values greater than 0·05 are given as N.S. (not significant).

A characteristic pattern of $^{14}CO_2$ production was obtained with [1-^{14}C] and [3-^{14}C] pyruvate (Table 4). This pattern was the same for seeds and 5-day-old cotyledons and was characterized by a yield from C-3 that was extremely low relative to that from C-1. Note that the rates of $^{14}CO_2$ production from both carbons were linear. Clearly, an unusually high proportion of the acetyl unit derived from the [^{14}C]pyruvate was retained in the tissue.

TABLE 3. RELEASE OF $^{14}CO_2$ FROM SPECIFICALLY LABELLED GLUCOSE BY MARROW COTYLEDONS DURING GERMINATION

Days germinated	Position of ^{14}C in [^{14}C]glucose	% of added [^{14}C]glucose recovered as $^{14}CO_2$ in:				
		1	2	3	4	6 hr*
0	1	0·025	0·072	0·159	0·309	0·659
	2	0·024	0·063	0·122	0·197	0·437
	3,4	0·085	0·315	0·635	1·025	2·045
	6	0·009	0·041	0·106	0·216	0·466
2	1	0·050	0·340	0·830	1·260	2·200
	2	0·014	0·090	0·260	0·470	1·100
	3,4	0·100	0·700	2·130	3·580	6·830
	6	0·019	0·118	0·308	0·538	1·112
5	1	0·080	0·320	0·640	1·050	1·860
	2	0·052	0·202	0·442	0·772	1·512
	3,4	0·360	2·520	5·530	8·780	15·79
	6	0·049	0·199	0·389	0·599	1·059
8	1	0·077	0·327	0·667	1·017	2·107
	2	0·025	0·145	0·345	0·605	1·295
	3,4	0·120	1·120	2·710	4·680	8·910
	6	0·037	0·167	0·397	0·647	1·137

* Hours from addition of [^{14}C]glucose to samples. All values are means of triplicate samples.

TABLE 4. RELEASE OF $^{14}CO_2$ FROM SPECIFICALLY LABELLED PYRUVATE BY MARROW COTYLEDONS DURING GERMINATION

Days germinated	Position of ^{14}C in [^{14}C]pyruvate	% of added [^{14}C]pyruvate recovered as $^{14}CO_2$ in:				
		1	2	3	4	5 hr*
0	1	2·0	3·70	5·40	7·10	8·90
	3	0·05	0·11	0·18	0·26	0·36
5	1	3·0	6·30	8·90	11·10	13·10
	3	0·09	0·37	0·57	0·76	0·96

* Hours from addition of [^{14}C]pyruvate to samples. All values are means of triplicate samples.

We investigated the fate of this acetyl unit by determining the labelling of sugars, lipids, and the fraction that contained the amino acids, by [1-^{14}C] and [3-^{14}C]pyruvate supplied to 5-day-old cotyledons (Table 5). About 70% of the ^{14}C supplied as either [1-^{14}C] or [3-^{14}C]pyruvate was recovered in the fractions that contained the amino acids. However, nearly all of this radioactivity was found in the incubation medium as opposed to the tissue itself. Labelling of the lipid and neutral fractions was slight by comparison but was twice as heavy from [3-^{14}C]pyruvate as from [1-^{14}C]pyruvate. Chromatography of the neutral fractions labelled by [3-^{14}C]pyruvate showed that at least 90% of the ^{14}C in these fractions was present in sugars with a distribution similar to that achieved by [2-^{14}C]acetate.[1]

TABLE 5. DISTRIBUTION OF ^{14}C FROM [1-^{14}C] AND [3-^{14}C]PYRUVATE SUPPLIED TO COTYLEDONS OF 5-day-old MARROW SEEDLINGS

Fraction	% of supplied ^{14}C recovered per fraction*	
	[1-^{14}C]Pyruvate	[3-^{14}C]Pyruvate
CO_2	29·0	1·0
Tissue		
Lipid	1·7	3·8
Neutral components of aqueous extract	1·3	2·6
Basic components of aqueous extract	5·6	4·7
Medium at end of incubation		
Neutral components	0·3	0·6
Basic components	63·0	68·0

* Replicate samples were incubated in [^{14}C]pyruvate for 5 hr.

The distribution of label from [^{14}C]pyruvate suggested that much of it was converted to alanine that was then lost to the medium. This could have reflected a situation in the intact seedling where alanine was formed from glucose via pyruvate and then immediately translocated to the remainder of the seedling as a source of amino nitrogen. If this were so, then $^{14}CO_2$ production from [^{14}C]glucose and [^{14}C]pyruvate could be misleading as it could have been due primarily to the uptake and metabolism of [^{14}C]alanine that had accumulated in the medium because its normal sink, the rest of the seedling, had been severed from the cotyledons. We investigated this possibility in two ways. First, we analysed the medium from the experiment reported in Table 3 in which [3,4-^{14}C]glucose

was supplied to 5-day-old cotyledons for 6 hr. At the end of this incubation the basic fraction of the medium contained only 0·8% of the added radioactivity. Second, we determined the effect of adding an excess of non-radioactive alanine to the incubation medium on $^{14}CO_2$ production from [^{14}C]pyruvate by 5-day-old cotyledons. In a 5-hr incubation the percentages of label, added as [1-^{14}C] and as [3-^{14}C]pyruvate, that were recovered as $^{14}CO_2$ were 3·65 and 0·24. The corresponding figures for replicate samples incubated in media that contained 5 mM alanine as well as the [^{14}C]pyruvate were 2·9 and 0·19.

DISCUSSION

Our measurements of enzyme activities do not appear to have been affected by failure to assay the enzymes under optimum conditions or to inhibition of the enzymes during extraction. We conclude that our measurements reflect the catalytic activities of the cotyledons. At each stage of germination the activity of phosphofructokinase was appreciable and was comparable to that of non-gluconeogenic tissues of plants.[2,3] Our feeding experiments indicate that little of the acetyl-CoA, formed from the pyruvate produced in glycolysis, was oxidized to CO_2. Thus about two molecules of CO_2 would be produced per molecule of hexose entering glycolysis. On this basis, phosphofructokinase activity in seeds and in 2-, 5-, and 8-day-old cotyledons could support CO_2 production at 25° at 10, 14, 28, and 35 μl per hr per cotyledon, respectively. These activities permit glycolysis to account for all of the CO_2 produced during the first 2 days of germination and to make significant contributions thereafter. The activity of pyruvate kinase exceeded that of phosphofructokinase at all the stages of germination examined. This excess became more marked during germination as the activity of pyruvate kinase rose much more than that of phosphofructokinase. These data, together with those reported previously[1] for glyceraldehyde-3-phosphate dehydrogenase, leave little doubt that marrow cotyledons possess considerable capacity for glycolysis during the first 8 days of germination. The results also indicate that this capacity increased during germination and that the greatest increase occurred during the development of gluconeogenesis.

The feeding experiments indicate the extent to which the above glycolytic capacity was used *in vivo*. For all ages studied, the pattern of $^{14}CO_2$ production from [^{14}C]glucose strongly suggests that the glucose was oxidized predominantly via glycolysis and that the pyruvate so produced was converted to acetyl-CoA that was used primarily for biosyntheses rather than in oxidation to CO_2. This explains the contrast between the high yield of CO_2 from C-3 and C-4 and the low yield from the remaining carbons of glucose. It also explains the similarity of the yields from C-2 and C-6. This hypothesis is strongly supported by the fact that the carboxyl group of [^{14}C]pyruvate was released as CO_2 in very much greater amounts than the methyl group (Tables 4 and 5). Further support is provided by the low yield of $^{14}CO_2$ from [2-^{14}C]acetate.[1]

The relative yields of $^{14}CO_2$ from C-1 and C-6 of glucose indicate activity of the pentose phosphate pathway at all the ages examined. Some indication of the relative activities of this pathway and glycolysis can be obtained by comparing $^{14}CO_2$ production from C-1 with that from C-3 and C-4 of glucose. In these particular experiments the yield from C-1 is a guide to the maximum activity of the pentose phosphate pathway and that from C-3 and C-4 indicates the activity of glycolysis. The pentose phosphate pathway can release C-3 and

[2] M. W. Fowler and T. ap Rees, *Biochim. Biophys. Acta* **201**, 33 (1970).
[3] C. P. P. Ricardo and T. ap Rees, *Phytochem.* **11**, 623 (1972).

C-4 as CO_2 during recycling.[4] However, entry into this pathway entails the loss of C-1 as CO_2. Thus it is probable that the maximum contribution of the pathway to CO_2 production from C-3 and C-4 is given by the yield from C-1. At all intervals in all our experiments with all ages of cotyledons the yield from C-3 and C-4 greatly exceeded that from C-1. We suggest that this excess represents the minimum contribution of glycolysis. Consequently we propose that the activity of the pentose phosphate pathway was small relative to that of glycolysis throughout the first 8 days of germination. This proposal is consistent with our previous suggestion[1] that $NADH_2$, not $NADPH_2$, is the reducing power used in gluconeogenesis.

We think that the only significant difference between the patterns of $^{14}CO_2$ production during germination was that in 5-day-old cotyledons the yield from C-3 and C-4 of glucose as a percentage of [^{14}C]glucose uptake, was about double that at the other ages examined. There was no corresponding rise in the yields from the other carbons of glucose. These observations indicate that the activity of glycolysis rose between days 2 and 5 and fell thereafter and that this increase in glycolysis was accompanied by an increase in the proportion of acetyl units retained in the tissue.

The close agreement between the above different types of experiment leads us to conclude that glycolysis operated in the cotyledons for at least the first 8 days of germination. The increases in the activities of the glycolytic enzymes and the relative increase in the yield of CO_2 from C-3 and C-4 of glucose suggest strongly that glycolysis increased during germination and was particularly active at the time of maximum gluconeogenesis.[1] This situation differs from that generally found in animals where glycolysis appears to be relatively low during gluconeogenesis.[5,6] We note that there is also evidence for glycolysis during gluconeogenesis in the endosperm of castor bean.[7] The simultaneous operation of glycolysis and gluconeogenesis in the same tissue poses two problems in particular. Firstly, what is the significance of glycolysis in a tissue in which fat breakdown and gluconeogenesis involve the formation of intermediates and high energy compounds normally provided by glycolysis? Secondly, what arrangements allow the two opposing pathways to operate simultaneously in the same tissue?

We hoped to answer the first question by feeding [^{14}C]pyruvate. However, the complex manner in which this compound was metabolized precludes any definitive answer. About 70% of the label added as [1-^{14}C] and as [3-^{14}C]pyruvate was recovered in the basic fraction and most of it was in the medium. This indicates that much of the added pyruvate was converted, without cleavage, to a basic compound that rapidly leaked into the medium. The simplest explanation is that of conversion to alanine, particularly since alanine was heavily labelled by [1-^{14}C]pyruvate in castor bean endosperm.[8] As feeding [^{14}C]glucose did not produce an equivalent labelling of the basic fraction of the medium, we suggest that the pyruvate produced from [^{14}C]glucose in glycolysis was separate from the site at which the exogenous pyruvate was metabolized to the basic compound. This means that the fate of pyruvate formed in glycolysis cannot be traced unequivocally by feeding [^{14}C]pyruvate. Thus, although [^{14}C]pyruvate labelled sugars during gluconeogenesis, we may not conclude that pyruvate formed in glycolysis is used in gluconeogenesis. The [^{14}C]pyruvate could have been converted to precursors of sugar at a site separate from that concerned with the

[4] T. AP REES, E. BLANCH, D. GRAHAM and D. D. DAVIES, *Plant Physiol.* **40,** 910 (1965).
[5] H. A. KREBS, *Proc. R. Soc.* **159B,** 545 (1964).
[6] M. C. SCRUTTON and M. F. UTTER, *Ann. Rev. Biochem.* **37,** 249 (1968).
[7] M. J. KOBR and H. BEEVERS, *Plant Physiol.* **47,** 48 (1971).
[8] G. E. NEAL and H. BEEVERS, *Biochem. J.* **74,** 409 (1960).

product of glycolysis. A similar difficulty may arise in considering the labelling of sugars by [^{14}C]pyruvate in castor bean endosperm.[7,8] Indeed, we find it difficult to attach any significance to glycolysis that merely produced pyruvate for consumption in gluconeogenesis.

The view that the function of glycolysis is the production of pyruvate for the synthesis of alanine for export is ruled out by the fact that [^{14}C]glucose did not label, significantly, the basic fraction of the medium. This result, and the failure of alanine to cause appreciable reduction in $^{14}CO_2$ production from [^{14}C]pyruvate make it unlikely that $^{14}CO_2$ production from glucose and pyruvate was an artifact arising from the conditions of incubation.

We suggest that the significance of glycolysis in gluconeogenic tissues like marrow cotyledons may be as follows. In such tissues the very effective conversion of fat to sugar may be achieved at the expense of a compartmentation of the cell that isolates the intermediates from the rest of the cell.[9] Thus reactions outside the gluconeogenic compartments that require intermediates or products of glycolysis would be unable to make direct use of gluconeogenic intermediates. This would then lead to a need for glycolytic intermediates and products for biosynthesis that was comparable to that in non-gluconeogenic tissues. This hypothesis is supported by the fact that [2-^{14}C]acetate labelled the fractions that contained amino acids, nucleotides and lipids in 5-day-old cotyledons,[1] and by the distribution of label from [^{14}C]pyruvate. A greater proportion of the added ^{14}C moved from [3-^{14}C]pyruvate than from [1-^{14}C]pyruvate into the fractions that contained lipids and amino acids. This difference could represent the acetyl unit derived from [3-^{14}C]pyruvate that labelled the pyruvate produced in glycolysis.

Our results do not explain how glycolysis and gluconeogenesis operate in the same tissue. The most likely explanations are inter- or intra-cellular compartmentation. We favour the latter as most of the cells in the cotyledons are of one type, and because Kobr and Beevers[7] have reported that proplastids from castor bean contain some of the enzymes required for the synthesis of sugar from phosphoenolpyruvate.

EXPERIMENTAL

Material. [3,4-^{14}C]Glucose was obtained from New England Nuclear Corporation, Boston, Mass. All other [^{14}C]labelled compounds were obtained from the Radiochemical Centre, Amersham, Bucks. Seeds of vegetable marrow (*Cucurbita pepo* L. var. *medullosa* Alef.) of the variety Long Green Bush were grown and harvested as described previously.[1]

Measurement of gas exchange. Gas exchange was measured manometrically at 25° in the dark by the direct method. The numbers of cotyledons per sample were 20, 14, 15, and 15 for seeds, 2-, 5-, and 8-day-old cotyledons, respectively. In order to eliminate diffusion problems, each sample was placed, in the Warburg flask, on filter paper that had been moistened with 0·25 ml 0·02 M KH_2PO_4 (pH 5·2).

Assay of enzymes. Extracts of the cotyledons were prepared as described previously.[1] Phosphofructokinase was assayed according to Scott, Craigie and Smillie[10] except that the buffer was 40 mM glycylglycine (pH 8·0) and the final concentration of ATP was 1 mM. Pyruvate kinase was assayed according to Bucher and Pfleiderer[11] except that the buffer was 50 mM glycylglycine (pH 7·5) and the final concentrations of phosphoenolpyruvate and KCl were 3·9 and 150 mM, respectively.

Metabolism of labelled substrates. Samples were prepared as described previously[1] and were incubated in 100 ml Erlenmeyer flasks in the dark at 25°. The methods used for the collection of $^{14}CO_2$ have also been described.[12] For the experiments with [^{14}C]glucose, replicate samples were incubated in 5·0 ml 0·02 M KH_2PO_4 (pH 5·2) that contained [^{14}C]glucose at 0·3 mM. The ^{14}C added to each sample was 0·5 μCi for [1-^{14}C] and [6-^{14}C]glucose, and 0·2 μCi for [2-^{14}C] and [3,4-^{14}C]glucose. For measurement of $^{14}CO_2$ production from [^{14}C]pyruvate (Table 4) the samples were incubated in 5·0 ml 0·02 M KH_2PO_4 (pH 5·2) that was 5·0 μM with respect to pyruvate and contained either 0·5 μCi [1-^{14}C]pyruvate or 0·375 μCi [3-^{14}C]pyruvate.

[9] H. Beevers, *Ann. N.Y. Acad. Sci.* **168,** 313 (1969).
[10] K. J. Scott, J. S. Craigie and R. M. Smillie, *Plant Physiol.* **39,** 323 (1964).
[11] T. Bucher and G. Pfleiderer, in *Methods in Enzymology* (edited by S. P. Colowick and N. O. Kaplan), Vol. 1, p. 435, Academic Press, New York (1955).
[12] T. ap Rees, E. Blanch and D. D. Davies, *Plant Physiol.* **40,** 748 (1965).

All measurements of $^{14}CO_2$ production are the means of triplicate samples. We emphasize that $^{14}CO_2$ production from different carbons of a particular substrate were compared only within the same batch of replicate samples. For the analysis of the fate of [^{14}C]pyruvate, samples were incubated in 5·0 ml 0·02 M KH_2PO_4 (pH 5·2) that was 7·9 μM with respect to pyruvate and contained 0·6 μCi [^{14}C]pyruvate. At the end of the incubation, the medium was removed, the tissue was washed twice with 2·5 ml portions of 0·02 M KH_2PO_4 (pH 5·2) and the washings were added to the medium. The tissue was killed with boiling 80% aqueous ethanol and then extracted in a Soxhlet apparatus in the same solution for 12 hr. This ethanolic extract was then reduced to dryness *in vacuo* at 30° and the residue was extracted with chloroform. This chloroform extract is the lipid fraction. The material that was insoluble in chloroform was extracted with water. The water extracts and the media were fractionated by ion-exchange and paper chromatography as described previously.[1] ^{14}C was assayed in a Tracerlab Coru/Matic II liquid scintillation spectrometer as described previously.[1]

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