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DARK CO_2 FIXATION BY POTATO TUBER TISSUE

C. J. CLEGG and C. P. WHITTINGHAM

The Botany Department, Imperial College, London, S.W.7

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Abstract—Dark fixation of $^{14}CO_2$ has been observed in whole potato tubers and in freshly cut and aged disks of tuber tissue. The immediate products, due to catalysis by phosphenolpyruvate carboxylase, are malate and aspartate, and the further metabolism of these compounds in tuber tissue has been investigated. It has been confirmed that the tricarboxylic acid cycle is partially blocked in fresh disks, and that it is activated during ageing. Bacterial contamination of disks has been determined and shown not to account for the fixation of CO_2.

INTRODUCTION

THE ABILITY to fix CO_2 in the dark occurs widely in plants[1] although it has not so far been reported in potato tuber tissue. The tricarboxylic acid (TCA) cycle is only partially operative in intact tubers and freshly cut slices of tuber tissue; glucose breakdown occurs largely by the pentose phosphate (PP) pathway.[2–4] After disks have been aged for 24 hr, however, the TCA cycle is fully functional.[2]

Experiments reported here establish that potato tuber tissue fixes CO_2 in the dark by the action of phosphenolpyruvate (PEP) carboxylase, recently shown to be present in tuber tissue.[5] The oxaloacetic acid formed is converted into several amino acids and organic acids. The possible significance of this dark CO_2 fixation for tuber metabolism is discussed.

RESULTS

$^{14}CO_2$ Fixation by Whole Tubers

Small whole tubers were enclosed in a desiccator with $^{14}CO_2$ (released from $Na_2\ ^{14}CO_3$ solution), for 3 days. A sample was withdrawn for analysis at the end of $^{14}CO_2$ feeding, and extracted with aqueous ethanol. The radioactivity in the ethanol-soluble fraction, and that remaining insoluble in the tissue was determined (Table 1).

TABLE 1. ACTIVITY RECOVERED FROM WHOLE TUBER TISSUE FED WITH $^{14}CO_2$ FOR 3 days

Fraction	cpm/g fresh wt.	% of total
Aqueous ethanol-soluble	16,080	96·3
Insoluble	620	3·7

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The ethanol-soluble metabolites were separated into acidic, basic and neutral fractions and the compounds in each fraction separated and identified using paper chromatography. The ^{14}C activity of each compound was determined on the paper (Table 2). It can be seen that after 3 days in contact with $^{14}CO_2$, malate and aspartate account for 46 per cent of the total ^{14}C activity in the ethanol-soluble fraction. Glycollate and citrate, together with glutamate and glutamine, also contain substantial amounts of radioactivity.

TABLE 2. ^{14}C ACTIVITY IN ETHANOL-SOLUBLE METABOLITES FROM WHOLE TUBER TISSUE FED $^{14}CO_2$ FOR 3 days

Acidic fraction	cpm/g fresh wt.	% total	Basic fraction	cpm/g fresh wt.	% total
Citrate/isocitrate	1350	8·4	Alanine	100	0·6
Glycollate	1690	10·5	Aspartate	2380	14·8
Malate	5030	31·3	Asparagine	1190	7·4
PEP	820	5·1	Cystine/cysteine	110	0·7
PGA	50	0·3	Glutamate	1900	11·8
Succinate	100	0·6	Glutamine	430	2·9
Sugar phosphates	150	0·9	Glycine/serine	610	3·8
Total	9190	57·1		6720	42·0

No ^{14}C activity was recovered from the neutral fraction (mainly sugars) of the ethanol-soluble metabolites.

3·7 per cent of the total activity recovered was insoluble in aqueous ethanol. The greater part of this insoluble activity was found to be in protein.

$^{14}CO_2$ Fixation by Disks

Fresh and 23-hr-aged disks were incubated with $^{14}CO_2$ for 1 hr, in air containing 0·1 per cent $^{12}CO_2$ and $^{14}CO_2$. Table 3 shows the distribution of recovered activity in the ethanol-soluble and insoluble fractions and Table 4 the activity in individual metabolites of the soluble fraction.

TABLE 3. ACTIVITY RECOVERED FROM FRESH AND AGED DISKS FED $^{14}CO_2$ FOR 1 hr

Fraction	Fresh disks		Aged disks	
	cpm/g fresh wt.	% of total	cpm/g fresh wt.	% of total
Aqueous ethanol soluble	906,850 (± 11,160)	99·8	68,280 (± 3,310)	94·8
Insoluble	1,300 (± 440)	0·2	3,710 (± 570)	5·2

Mean of two replicates.

Both fresh and aged disks fix CO_2, but fresh disks fix at over twelve times the rate. The proportion of radioactive ethanol-insoluble compounds increases greatly with ageing of the disks. The majority of this insoluble activity is in protein. Malate and aspartate contain the greater part of the ^{14}C activity in the soluble fraction in both fresh and aged disks although aspartate is much reduced in the latter.

TABLE 4. ^{14}C ACTIVITY IN ETHANOL-SOLUBLE METABOLITES FROM FRESH AND AGED DISKS FED $^{14}CO_2$ FOR 1 hr

	Fresh disks		Aged disks	
	cpm/g fresh wt.	% total	cpm/g fresh wt.	% total
Acidic fraction				
Citrate/isocitrate	450	0·05	480	0·7
Fumarate	90	0·01	140	0·2
Glycollate	360	0·04	70	0·1
Malate	206,760	22·8	23,630	34·6
PEP	90	0·01	210	0·3
PGA				
Succinate	180	0·02	1,840	2·7
Sugar phosphate	15,420	1·7	210	0·3
Triose phosphate	90	0·01	140	0·2
Total	213,450	25·9	26,700	39·1
Basic fraction				
Alanine	1,810	0·2	550	0·8
Aspartate	543,200	59·9	15,710	22·8
Asparagine	22,670	2·5	1,090	1·6
Cystine/cysteine	0	0	210	0·3
Glutamate	37,180	4·1	10,520	15·4
Glutamine	23,580	2·6	6,280	9·2
Glycine/serine	9,980	1·1	3,620	5·3
Proline	0	0	340	0·5
Threonine	0	0	680	1·0
Tyrosine	0	0	1,370	2·0
Total	638,420	70·4	40,360	59·1

No activity was recovered from the neutral fraction.

The decrease in the percentage activity in the basic fraction with ageing is perhaps associated with incorporation of labelled amino acids into insoluble protein. An enhanced TCA cycle activity in aged disks would be consistent with the fall in ^{14}C activity in both citrate/isocitrate, and in sugar phosphates in aged disks.

The Effect of Sodium Pyruvate on CO_2 Fixation by Disks

Fresh disks were incubated with 10^{-2} M sodium pyruvate-^{12}C during 1 hr feeding with $^{14}CO_2$ (Tables 5 and 6). The retention of the greater part of ^{14}C activity of ethanol-soluble metabolites in the acidic fraction from pyruvate-treated fresh disks was due to accumulation of labelled citrate and isocitrate, relative to the activity in aspartate. The levels of activity in malate in treated and untreated disks is comparable.

Fresh and aged disks were also incubated with sodium pyruvate-U-^{14}C in the presence of 0·1 per cent $^{12}CO_2$ for 1 hr (Tables 7 and 8).

Fresh disks absorb approximately only one-tenth of the quantity of pyruvate-U-^{14}C absorbed by aged disks and, unlike aged disks, evolve the greater part of this activity as $^{14}CO_2$. Of the activity remaining in the ethanol-soluble fraction in fresh disks, most remains as pyruvate or alanine (51 per cent total), but some activity accumulates in succinate (15 per cent) and in citrate/isocitrate (16 per cent).

TABLE 5. ACTIVITY RECOVERED FROM FRESH DISKS FED $^{14}CO_2$ FOR 1 hr, IN THE PRESENCE OF SODIUM PYRUVATE-$^{12}C\mu$

Fraction	Pyruvate-treated disk (10 mM)		Control fresh disks	
	cpm/g fresh wt.	As % of recovered activity	cpm/g fresh wt.	As % of recovered activity
Aqueous ethanol soluble	1,120,820 (± 150,480)	99·7	925,180 (± 162,400)	99·7
Insoluble	3,580 (± 770)	0·3	2,710 (± 320)	0·3

Mean of two replicates.

TABLE 6. ^{14}C ACTIVITY IN ETHANOL-SOLUBLE METABOLITES FROM FRESH DISKS FED $^{14}CO_2$ FOR 1 hr IN THE PRESENCE OF 10^{-2} M SODIUM PYRUVATE

	Pyruvate-treated disks		Control fresh disks	
	cpm/g fresh wt.	% total	cpm/g fresh wt.	% total
Acidic fraction				
Citrate/isocitrate	515,410	46·0	62,910	6·8
Fumarate	3,810	0·3	3,700	0·4
Glycollate	3,810	0·3	4,630	0·5
Malate	360,120	32·1	251,650	27·2
PEP	15,240	1·4	9,250	1·0
PGA			11,100	1·2
Succinate	6,670	0·6	1,850	0·2
Sugar phosphate	32,390	2·9	18,500	2·0
Triose phosphate	1,910	0·2	930	0·1
Total	939,350	83·8	364,520	39·4
Basic fraction				
Alanine	0	0	1,850	0·2
Aspartate	122,380	10·9	455,190	49·2
Asparagine	6,830	0·6	13,880	1·5
Glutamate	3,900	0·3	43,480	4·7
Glutamine	0	0	930	0·1
Glycine/serine	0	0	22,200	2·4
Proline	7,620	0·6	6,480	0·7
Threonine	0	0	3,700	0·4
Total	140,730	12·4	547,710	59·2

No activity was recovered from the neutral fraction.

Aged disks incorporate over 20 per cent of the absorbed activity into the insoluble fraction. More metabolites in the ethanol-soluble fraction become labelled in aged disks than in fresh, but the percentage activity in alanine, pyruvate, succinate and in citrate/isocitrate is much lower. The percentage activity in glutamate is increased greatly in aged disks. Considerable ^{14}C activity also entered the lipids, and some into the neutral fraction.

TABLE 7. ACTIVITY RECOVERED FROM FRESH AND AGED DISKS FED SODIUM PRYUVATE-U-^{14}C FOR 1 hr, IN THE PRESENCE OF $^{12}CO_2$

	Fresh disks		Aged disks	
	cpm/g fresh wt.	% total	cpm/g fresh wt.	% total
$^{14}CO_2$ recovered	298,900 (± 11,100)	53·1	1,809,690 (± 109,060)	33·7
^{14}C activity in ethanol-soluble	262,220 (± 25,880)	46·6	2,628,150 (± 58,510)	50·0
Insoluble ^{14}C activity	1,680 (± 290)	0·3	927,000 (± 45,410)	17·3

Mean of two replicates.
25 μc (1·505 μmoles) of sodium pyruvate-U-^{14}C used in each experiment.

TABLE 8. ^{14}C ACTIVITY IN ETHANOL-SOLUBLE METABOLITES FROM FRESH AND AGED DISKS FED SODIUM PYRUVATE-U-^{14}C FOR 1 hr IN THE PRESENCE OF $^{12}CO_2$

	Fresh disks		Aged disks	
	cpm/g fresh wt.	% total	cpm/g fresh wt.	% total
Acidic fraction				
Citrate/isocitrate	42,210	16·1	49,940	1·9
Fumarate	9,440	3·6	5,260	0·2
Glycollate	15,470	5·9	10,510	0·4
Lactate	0	0	15,770	0·6
Malate	7,870	3·0	176,090	6·7
Oxalate	0	0	10,510	0·4
PEP	1,050	0·4	2,630	0·1
PGA	790	0·3	15,770	0·6
Pyruvate	48,770	18·6	34,170	1·3
Succinate	40,120	15·3	39,420	1·5
Sugar phosphate	0	0	31,540	1·2
Triose phosphate	0	0	15,770	0·6
Total	165,720	63·2	407,380	15·5
Basic fraction				
Alanine	81,810	31.4	105,130	4·0
Aspartate	1,310	0·5	220,770	8·4
Asparagine	0	0	89,360	3·4
Cystine/cysteine	0	0	52,560	2·0
Glutamate	6,030	2·3	651,780	24·8
Glutamine	260	0·1	160,320	6·1
Glycine/serine	0	0	78,840	3·0
Methionine	0	0	31,540	1·2
Proline	0	0	13,140	0·5
Tryptophan	0	0	57,820	2·2
Tryosine	260	0·1	49,940	1·9
Fats	0	0	352,170	13·4
Total	89,670	34·4	1,863,360	70·9
Neutral fraction				
Fructose	0	0	2,630	0·1
Glucose	0	0	94,610	3·6
Sucrose	0	0	7,880	0·3
Totals	0	0	105,120	4·0

Degradation of Malate

Samples of ^{14}C-labelled malate, obtained from fresh and aged disks, were completely oxidized to CO_2 and H_2O by persulphate oxidation. A second equal sample was incubated with malate-adapted *Lactobacillus arabinosus* which specifically decarboxylates C_4 of malate. The CO_2 given off in both cases was trapped as barium carbonate, and counted. The results are given in Table 9.

TABLE 9. DEGRADATION OF MALATE FROM FRESH AND AGED DISKS TO FIND % ^{14}C ACTIVITY AT C_4

Origin of malate sample	Activity in cpm		% ^{14}C activity in C_4
	total	C_4	
Fresh disks fed with $^{14}CO_2$	2792	2470	88·5
	3018	2984	98·9
Aged disks fed with $^{14}CO_2$	2593	2548	98·5
	2584	2654	102·7
Disks fed with pyruvate-U ^{14}C and glucose-U-^{14}C	4958	1596	32·2

The malate formed by fresh and aged disks fed with $^{14}CO_2$ is exclusively labelled in C_4 of malate. This confirms that $^{14}CO_2$ fixation in potato tissue is catalysed by PEP carboxylase.

Malate formed in disks fed with glucose-U-^{14}C and pyruvate-U-^{14}C would be expected to contain approximately 25 per cent of its total ^{14}C activity at C_4. However, if $^{14}CO_2$ produced from these substrates by disks is retained and combined with PEP-^{12}C, the resulting malate obtained will have somewhat more than 25 per cent of the total activity of malate at C_4. This was shown to be the case.

Bacterial Contamination

The number of viable bacteria in potato tuber tissue were determined with an accuracy of ± 5 per cent (Table 10). Fresh disks had a low level of bacterial contamination but the number of bacteria increased sharply when disks were aged for 24 hr. Chloramphenicol at both 25 μg/ml and 50 μg/ml reduced the viable bacteria count to below that in fresh, untreated disks. The counts of viable bacteria in potato tuber tissue are comparable with those reported for carrot root disks by Leaver.[6]

TABLE 10. THE NUMBERS OF VIABLE BACTERIA IN POTATO TUBER DISKS TISSUE

State of disk	Viable bacteria/g fresh wt.
Fresh	5×10^3
Aged 24 hr	$2{\cdot}5 \times 10^7$
Aged with 25 μg/ml chloramphenicol	4×10^3
Aged with 50 μg/ml chloramphenicol	4×10^3
Fresh after 15 min in water at 55°	5×10^3

[6] C. J. LEAVER, Ph.D. Thesis, University of London (1966).

Freshly cut disks of potato tuber tissue were subjected to a pretreatment of 15 min in water at 55°. Bacterial contamination in such disks was determined and found to be comparable with that of freshly cut, untreated disks (Table 10). Since heat-pretreated disks did not fix $^{14}CO_2$, it is concluded that the bacteria in potato tuber tissue are not responsible for the significant fixation of CO_2 observed.

DISCUSSION

The pattern of fixation of $^{14}CO_2$ by whole tubers and disks is consistent with the operation of the enzyme PEP carboxylase. Walker observed[1] that PEP carboxylase constitutes one of the most effective carboxylating mechanisms known. Further, malic enzyme activity has been shown to be absent in freshly cut potato tuber tissue.[7] The oxaloacetate produced by dark CO_2 fixation in tuber tissue is reduced to malate or aminated to aspartate and whilst the major part of these secondary, stable products is stored in the cells, some is metabolized into other intermediates. The further metabolism in whole tubers differs from that in disks in that the former accumulate much more glycollate and citrate/isocitrate. The respiratory metabolism of the intact tuber remains obscure, partly because cutting or even handling of tubers alters their metabolism.[8] The ways in which the initial ^{14}C-labelled products of dark CO_2 fixation are metabolized by the tuber during storage is being investigated and the results will be given in a later paper.

The further metabolism of malate and aspartate by disks as they age is more easily interpreted. Ageing of disks induces two major changes in the ^{14}C-labelling pattern within the cells. First, there is an increase in the percentage of absorbed activity that becomes insoluble int he cells, mostly in protein. Thimann and Loose showed that protein is rapidly synthesized in potato slices during the first 48 hr after cutting, after which this synthesis falls off.[9] It was subsequently shown[10] that the changes in magnitude and mechanism of respiration in potato tuber disks induced by cutting are dependent upon the synthesis of new nucleic acid and proteins. Consequently the conversion of ^{14}C activity from aspartate ^{14}C into protein by 24-hr-aged disks is in agreement with previously reported studies on disk metabolism.

Secondly, the number of amino acids that become labelled increases on ageing. In aged disks cystine/cysteine, threonine, tyrosine and proline become radioactive. All of these amino acids have been previously reported in the soluble, non-protein fractions of potato tuber tissue.[11] 23-hr-aged disks "fed" $^{14}CO_2$ accumulated a higher percentage of radioactivity in succinate, glutamate and glutamine, and a lower percentage activity in aspartate and asparagine. Activity in citrate/isocitrate and glycollate was little changed with ageing.

These observations are in agreement with the previously reported partial block in the TCA cycle in fresh tuber tissue. This block is reported to be removed after 2 hr ageing of tissue at 25°.[2] As the TCA cycle becomes fully operative and α-ketoglutarate production is resumed, further glutamate/glutamine production becomes possible.

Of the two chief products of dark CO_2 fixation in tuber tissue, aspartate is predominantly

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drawn upon as a substrate as TCA cycle activity increases; malate appears to be stored inaccessibly. This hypothesis is confirmed in two ways.

(i) The malate produced in both fresh and aged disks during $^{14}CO_2$ feeding is exclusively labelled at C_4. This confirms that the acid is produced by the actions of PEP carboxylase and malic dehydrogenase. The malate can only slowly equilibrate with fumarate in the TCA cycle, since this would lead to equal ^{14}C activity at C_1 and C_4.

(ii) When $^{14}CO_2$ fixation occurs in the presence of exogenous sodium pyruvate in fresh disks aspartate ^{14}C is depleted and labelled citrate/isocitrate accumulates. The level of malate ^{14}C in pyruvate-treated and untreated fresh disks is comparable.

Lipps and Beevers have shown that in corn roots the malate produced by CO_2 fixation is in an extramitochondrial compartment physically separated from the malate of the TCA cycle.[12]

The accumulation of citrate in fresh disks in the presence of exogenous pyruvate implies that pyruvate was at a low concentration in such disks. When the concentration of this metabolite was augmented from an exogenous source some of the aspartate produced by dark CO_2 fixation was metabolized by the TCA cycle, at least as far as isocitrate. Thus the partial block in the TCA cycle between isocitrate and α-ketoglutarate is further confirmed. If, however, aged disks were fed pyruvate-U-^{14}C, radioactive glutamate accumulated and not citrate/isocitrate.

The existence of dark CO_2 fixation in potato tuber tissue has some important implications for the study of respiratory metabolism in this tissue. Fixation of CO_2 by the intact tuber might be expected to result in accumulation of malate and aspartate or related metabolites during storage. Asparagine has been shown to be the largest constituent by weight of the free amino acids and amides in tuber tissue.[11] Citrate occurs at high concentrations in tuber tissue[13] and has been shown to make up 50 per cent of the total titratable acidity in King Edward VII variety tubers.[14] It remains to be shown whether these metabolites accumulate as a direct result of CO_2 fixation. CO_2 fixation by disks must also result in respiratory products (RQ's) of less than unity in a tissue whose food reserve is exclusively starch. Recent measurements of the RQ of fresh and 24-hr-aged disks cut from King Edward VII variety tubers gave values of 0·73 and 0·78 respectively.[15] Burton reported that although the RQ of stored King Edward VII tubers was at or a little below unity throughout most of storage, at sprouting there was a sharp upward trend in CO_2 output, accompanied by little or no change in oxygen intake, that resulted in RQ's as high at 4·5.[16] Rapid oxidation of the accumulated products of dark CO_2 fixation at sprouting may account for these high RQ values.

EXPERIMENTAL

Tissue

King Edward VII variety potato tubers were obtained from commercial sources locally and were stored in the dark at 3–5° until required. Disks, 1 cm × 1 mm, were cut and washed as described previously.[17]

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Reagents

All chemicals were of the highest purity available and usually Analar grade. Radioactive compounds were obtained from the Radiochemical Centre, except malate $^{14}C_4$, provided by Dr. D. A. Walker, Imperial College. $^{14}CO_2$ was generated from a $Na_2{}^{14}CO_3$ solution of specific activity 56 $\mu c/\mu M$.

$^{14}CO_2$ Feeding of Whole Tubers

Twenty whole tubers (av. wt. 55 g) were fed in a large sealed darkened desiccator at 15°. Air was pumped through the desiccator prior to and after feeding. $Na_2{}^{14}CO_3$ solution (10 mc of CO_2) was injected through a serum cap into 50% lactic acid solution, suspended below the bung in the desiccator lid. The tubers were left in the $^{14}CO_2$ for 3 days.

Ageing and Feeding Disks

Disks were aged, or incubated with substrate or $^{14}CO_2$ during feeding experiments, in the dark, in groups of ten disks (0·75–0·85 g fresh wt.) resting on stretched nylon netting at the surface of 15 ml of medium in a 150 ml Berzelius beaker. The medium was stirred magnetically, and a moist air stream was passed over the tissue and subsequently through a CO_2-absorption tube containing 20 ml of 4 N NaOH solution.

The incubation medium was a 10 mM K phosphate–0·1 mM $CaCO_3$ buffer solution containing 25 μg chloramphenicol per ml. In each experiment the medium was finally adjusted to pH 6·0 using 4 N NaOH. $^{14}CO_2$ (200 μc) was fed for a period of 1 hr.

Analysis of Labelled Metabolites

At the end of feeding period, tissue was killed and extracted with aqueous ethanol. The extract was then separated into basic, acidic and neutral fractions using Dowex exchange resins. The method was that of Neal and Beevers[18] except that during extraction ten disks or an equivalent weight of whole tuber tissue, was boiled under reflux in 80% ethanol (20 ml) for 15 min. This was repeated twice, using fresh ethanol solution, and then the disks maintained in cold 20% ethanol (20 ml) overnight. The ethanolic extracts were combined. Aliquots, plated on aluminium planchets, were counted using a Nuclear Chicago gas-flow counter.

Labelled metabolites in the three fractions were separated by two dimensional paper chromatography using Whatman No. 3 paper, and the solvents phenol/water (2·56:1·0 w/v) and *n*-butanol/glacial acetic acid/water (3·95:2·63:1·0 by volume). Labelled compounds were located and counted using an IDL End Window G.M. tube. Individual compounds were identified by one-directional co-chromatography with marker substances.

Insoluble ^{14}C activity remaining in the tissue was estimated by wet combusting aliquots using the technique of Porter *et al.*[19] The remainder of the tissue was hydrolysed to determine the proportion of insoluble activity in protein.[20]

Labelled malate was degraded by the method of Cockburn[21] to estimate the proportion of activity in C_4.

Estimation of Bacterial Contamination

Bacterial contamination in fresh and aged disks, and in disks treated with chloramphenicol, was estimated by the viable count, dilution plate technique as described by Leaver.[6]

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