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THE PATHWAYS OF AMINO ACID OXIDATION DURING GERMINATION OF MUSTARD SEEDS (*SINAPIS ALBA*)

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Key Word Index—*Sinapis alba*; Cruciferae; white mustard; amino acids; oxidation; germination.

Abstract—During the germination of *Sinapis alba* seed, alanine and some other amino acids were oxidized via the tricarboxylic acid cycle (TCA), although the pentose phosphate pathway (PPP) was also operative and may account for *ca* 50% of glucose oxidation. The relative operation of the PPP and the TCA cycle was influenced by changes in the concentrations of glutamic acid and glycine.

INTRODUCTION

The metabolism of a number of amino acids was investigated during early germination by applying L-[U-^{14}C]-labelled amino acid solutions to imbibing seeds, when it was found that the carbon dioxide evolved became labelled [1]. It is generally assumed that amino acids are degraded and the carbon skeletons eventually oxidized to CO_2 via the TCA cycle [2, 3], following pathways similar to those found in mammals and micro-organisms [4, 5]. However, in some seeds the PPP is operative during germination [6–8] and may provide the predominant oxidative pathway. Indeed, Roberts and Smith [8] suggest that high PPP activity is essential for germination to proceed. The oxidation of some amino acids may therefore proceed via the PPP in addition to, or in preference to, oxidation via the TCA cycle. To investigate this possibility, the pathways leading to the evolution of labelled carbon dioxide from some exogenously applied amino acids were examined in germinating *Sinapis alba*.

RESULTS AND DISCUSSION

Incorporation of ^{14}C from labelled substrates into CO_2 during germination

About 30% of the carbon from [U-^{14}C]-labelled alanine was evolved as $^{14}CO_2$ (Table 1). This was the largest percentage of CO_2 evolved from any of the amino acids added. Pyruvate was oxidized to a similar extent and, during early germination, much of this evolved CO_2 originated from the C-1 (i.e. carboxyl) position. The high yield of CO_2 from alanine may indicate pyruvate dehydrogenase (EC 1.2.4.1) activity during this period. Later, however, [U-^{14}C]pyruvate contributed more label to CO_2 than [1-^{14}C]pyruvate, indicating that [U-^{14}C]pyruvate and its products had undergone more than one decarboxylation reaction, possibly in the TCA cycle. Those amino acids which yielded between 5 and 10% of the imbibed label as $^{14}CO_2$ are shown in Table 2. All other protein amino acids yielded less than 3.5% as $^{14}CO_2$, with

Table 1. Oxidation of alanine and pyruvate during germination of mustard seeds

Compound	$^{14}CO_2$ released (as % ^{14}C uptake) at time: 0.5 hr	3.0 hr	6.0 hr	48.0 hr
L-[U-^{14}C]alanine (d)*	0.2	5.9	13.7	28.3
L-[U-^{14}C]alanine (l)	4.2	6.5	15.9	27.0
[U-^{14}C]pyruvate (d)	0.9	3.5	5.5	25.5
[U-^{14}C]pyruvate (l)	0.9	3.6	5.9	29.7
[1-^{14}C]pyruvate (d)	1.3	4.0	3.8	17.7
[1-^{14}C]pyruvate (l)	1.3	4.8	6.4	17.6

*Average of two experiments; (d) seeds germinated in dark conditions; (l) seeds germinated in white light.

1 g seed was germinated on filter paper in 9 cm Petri dishes under white light or in the dark, in 1.5 ml water containing *ca* 1 μCi radioactive label. Carbon dioxide was collected on filter papers impregnated with 10% KOH, which were changed at intervals, and the radioactivity of the evolved $^{14}CO_2$ was measured.

Table 2. Oxidation of some amino acids during germination of mustard seeds

Compound	$^{14}CO_2$ released (as % ^{14}C uptake) at time: 0.5 hr	3.0 hr	6.0 hr	48.0 hr
Aspartic acid (d)*	0.1	1.5	3.6	9.6
Glutamic acid (d)	0.4	1.2	3.2	9.2
Glutamic acid (l)	0.1	0.9	2.0	7.6
γ-Aminobutyric acid (d)	0.3	0.6	0.9	9.3
γ-Aminobutyric acid (l)	0.1	0.6	0.8	7.5
Arginine (d)	0.1	2.3	4.0	7.1
Arginine (l)	0.2	1.8	4.7	8.7
Glycine (d)	0.1	2.8	3.6	5.6

*Average of two experiments; (d) seeds germinated in dark conditions; (l) seeds germinated in white light.

Other details as in Table 1.

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methionine, isoleucine, lysine, valine, tryptophan and phenylalanine evolving less than 1% of the imbibed label as $^{14}CO_2$ [1].

It appears then that the carbon dioxide derived from some of the amino acids reflected the degree to which they were utilized as substrates in the TCA cycle.

Oxidation of amino acids via the PPP and the TCA cycle

The relative operation of the PPP and glycolysis was first determined by comparing the oxidation of D-[1-^{14}C]glucose with D-[6-^{14}C]glucose during germination [9]. The results (Table 3) indicated that about half of the glucose was oxidized via glycolysis and half via PPP under both light and dark conditions. Whilst a number of workers [6–8, 10–12] have used the Bloom and Stetten [9] technique, the determination of the relative activity of PPP and glycolysis by this method has been subject to criticism [13–16]. The main problems are the recycling of labelled compounds, randomization of label, and the operation of other oxidative pathways (particularly pentan synthesis) in addition to the PPP and glycolysis. Other techniques for measuring the activities of the two pathways are available [16, 17], but most of these techniques are subject to criticisms similar to the C_6/C_1 ratio method, or they may be inaccurate due to technical difficulties. In this investigation, the initial incubation time was short, which may help to minimize the effects of randomization and recycling. Indeed, results shown here (Table 3) indicate that increasing the incubation time leads to an apparent increase in glycolysis and so the results of 8–10 hr could be artefacts due to recycling, randomization or pentan synthesis; if labelled glucose was added to seeds germinated for 20 hr without label, the amount oxidized via PPP returned to *ca* 50%. Furthermore, in this investigation, the technique was not used to gain absolute values for the operation of the two pathways but to provide, at least, qualitative data regarding carbohydrate oxidation so that the subsequent effects of amino acids on this process could be investigated.

Table 3. Glycolytic activity during germination of mustard seed

	Germination conditions			
	White light		Darkness	
Time (hr)	Glucose oxidized via glycolysis (%)	± s	Glucose oxidized via glycolysis (%)	± s
0.5	48.3		51.3	
1.0	43.2	10.8	51.6	5.8
2.0	44.8	8.2	38.5	4.2
3.0	52.6	8.1	45.3	6.9
4.0	53.0	6.9	51.3	8.2
5.0	59.4	7.7	78.0	8 1
6.0	66.0	6.1	61.3	5 2
8.0	72 0		80.4	
10.0	81.8		83 0	
21.0	41.0		42.1	
22.0	58 5		55.5	
23.0	53.3		59.3	
24.0	67 0	10.4	65 0	9.4
25.0	80 2	10.7	98 6	10.2
26.0	83.0	8.0	93 3	2 3
27.0	90.0	9.6	91 4	10.3
29.0	80 0		114 6	

Results are expressed as percentage glucose oxidized via glycolysis, derived from the expression:

$$\frac{CO_2 \text{ yield from } [6\text{-}^{14}C]\text{glucose}}{CO_2 \text{ yield from } [1\text{-}^{14}C]\text{glucose}} \times 100 \quad [9]$$

Standard deviation (± s) is given where seven or more readings were obtained. Other figures represent averages of more than three experiments. 1 g seed samples were germinated on filter paper in 9 cm Petri dishes containing 1 4 ml water. 0.1 ml water containing 1 μCi of either [1-^{14}C]- or [6-^{14}C]glucose was added at 0 hr or 20 hr. The evolved $^{14}CO_2$ was collected over 1 hr periods on filter paper soaked with 10% KOH and the radioactivity was measured

The relative amount of oxidation of amino acid substrates via the TCA cycle or PPP was compared by competition studies. Unlabelled glycine and glutamic acid, at different concentrations, competed with the specifically labelled glucose for oxidation in pathways leading to the production of $^{14}CO_2$. The results showed (Tables 4 and 5) that different effects on the oxidation of glucose were produced. For instance, early (up to 6 hr) during imbibition and germination, the oxidation of [1-^{14}C]glucose was enhanced in the presence of glycine (Table 4), whilst later during germination, glycine competed with the oxidation of both [1-^{14}C]- and [6-^{14}C]glucose. Glutamate also tended to increase the oxidation of [1-^{14}C]glucose during early germination whilst the oxidation of [6-^{14}C]glucose fluctuated (Table 5). Later during germination, glutamate enhanced both [1-^{14}C]- and [6-^{14}C]glucose oxidation.

A smaller evolution of CO_2 than that of the control suggested that the unlabelled amino acid was competing with the specifically labelled glucose for oxidation. This may occur by competitive inhibition between the two substrates (or intermediates derived from them) or by inhibition of an allosteric enzyme or enzymes in the reaction sequence. An alternative explanation may be that the amino acids that cause a decrease in CO_2 evolution do so by providing the necessary conditions and perhaps substrates for the retention of labelled compounds as in, for example, carboxylation reactions (e.g. the production of phosphoenol pyruvate from pyruvate during gluconeogenesis).

Enhanced CO_2 evolution is more difficult to explain. One possibility is that normally carbon from glucose can contribute to the amino acid pool and on addition of a high concentration of exogenous amino acid, the glucose is no longer required for the synthesis of that amino acid and so the excess is oxidized via the PPP or the TCA cycle. However, it was found that only *ca* 7% of the glucose was incorporated into the amino acid skeletons [1]. This corroborated previous findings in bean [17] and in pea [18]. Therefore it seems unlikely that the increase in $^{14}CO_2$ evolution in the presence of amino acids is entirely due to the diversion of glucose from amino acid biosynthesis. It is usually assumed that the PPP and glycolytic pathway are operating simultaneously and that they share common intermediates. Thus the addition of amino acids may cause an imbalance of some intermediate(s), which may in turn alter the relative activities of the two pathways. This may occur by modification of the activity of an enzyme, either directly concerned with carbohydrate

Table 4. Effect of glycine on glucose oxidation during germination of mustard seeds

Time (hr)	$\frac{[1\text{-}^{14}C]\text{Glucose} + \text{glycine}}{[1\text{-}^{14}C]\text{Glucose}} \times 100$		$\frac{[6\text{-}^{14}C]\text{Glucose} + \text{glycine}}{[6\text{-}^{14}C]\text{Glucose}} \times 100$	
	5×10^{-4} M glycine	5×10^{-3} M glycine	5×10^{-4} M glycine	5×10^{-3} M glycine
1	91	38	119	155
2	107	39	153	65
3	84	79	83	94
4	201	154	99	82
5	156	127	74	101
6	152	137	118	86
23	51	30	36	24
24	59	59	61	56
25	52	40	43	39
26	60	39	49	53
27	66	37	41	44
28	70	55	44	45

Average of four experiments. 1 g seed samples were germinated in 1.4 ml glycine solution. 0.1 ml of a similar solution containing 1 μCi of [1-^{14}C]- or [6-^{14}C]glucose was added at 0 hr or 22 hr and the $^{14}CO_2$ evolved was collected. Results were compared with $^{14}CO_2$ evolved from seeds germinated in water to which 0 1 ml water containing 1 μCi specifically labelled glucose was added at 0 hr or 22 hr.

Table 5. Effect of glutamate on glucose oxidation during germination of mustard seeds

Time (hr)	$\frac{[1\text{-}^{14}C]\text{Glucose} + \text{glutamate}}{[1\text{-}^{14}C]\text{Glucose}} \times 100$		$\frac{[6\text{-}^{14}C]\text{Glucose} + \text{glutamate}}{[6\text{-}^{14}C]\text{Glucose}} \times 100$	
	5×10^{-4} M glutamate	5×10^{-3} M glutamate	5×10^{-4} M glutamate	5×10^{-3} M glutamate
1	181	44	117	49
2	136	72	89	92
3	139	141	69	87
4	124	124	132	138
5	102	232	92	119
6	112	179	109	113
23	394	406	168	687
24	252	223	227	250
25	133	128	139	183
26	138	143	120	144
27	88	71	100	120
28	72	59	75	74

Average of two experiments. Other details as in Table 4.

oxidation, or indirectly, by affecting enzymes of other pathways. For instance, amino acids may provide nitrogen for nucleic acid synthesis [19, 20], which utilizes pentoses from the PPP and therefore increases the activity of the PPP.

It would appear that a number of amino acids may be oxidized according to their ease of entry into the TCA cycle, and that this is their main oxidative pathway. Nevertheless, some amino acids compete with glucose for oxidation via the PPP and furthermore, changes in the relative operation of the TCA cycle and the PPP can be initiated by changes in the amino acid concentration.

EXPERIMENTAL

Germination of seeds. 1 g of seed was surface-sterilized with 2% Chloros, then washed in 250 ml 0.01 M HCl prior to rinsing with 250 ml sterile H_2O. Seeds were then transferred to 9 cm diameter

Petri dishes and germinated on a layer of Whatman No. 1 filter paper in 1.5 ml H_2O or amino acid soln (1 μCi) for 48 hr at 23 $\pm 2°$. The white light source was 2×60 W Atlas 'Growlux' fluorescent tubes situated 0.5 m above the seeds. Dark conditions were ensured by wrapping the dishes in two layers of aluminium foil.

Addition of labelled glucose. This was added to the seeds in 0.1 ml (1 μCi) after the H_2O or unlabelled amino acid soln. Using a microsyringe, *ca* 0.5 μl was added to the film of H_2O covering each seed. The Petri dishes were then sealed with Vaseline.

$^{14}CO_2$ *measurement.* $^{14}CO_2$ evolved from the seeds was absorbed into 0.2 ml 10% KOH on a strip of filter paper in a plastic vial enclosed within the sealed Petri dishes. After different times (1–3 hr), the paper was removed, dried and placed in a counting vial to which 8 ml of scintillation fluid was added. Radioactivity was measured in a scintillation counter using a channels ratio method of quench correction. Results are presented as the percentage of label taken up by the seeds. Total uptake was calculated as the difference in cpm between the added label and the cpm that could be washed from the filter paper supporting the seeds and the Petri dish.

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