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Studies of Intermediary Metabolism in Germinating Pea Cotyledons

THE PATHWAY OF ETHANOL METABOLISM AND THE ROLE OF THE TRICARBOXYLIC ACID CYCLE

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1. The pathway of ethanol metabolism in cotyledons of 3-day-old pea seedlings has been examined by incubating tissue slices with [1-^{14}C]ethanol and [2-^{14}C]ethanol for periods up to 1hr. 2. Ethanol was rapidly incorporated into citrate and glutamate but relatively small amounts of ^{14}C were present in the evolved carbon dioxide even after 1hr. of ethanol metabolism. 3. Similar data were obtained from experiments in which [1,2-$^{14}C_2$]acetaldehyde and [^{14}C]acetate were supplied. 4. The results are interpreted as indicating that ethanol is metabolized essentially via the reactions of the tricarboxylic acid cycle with a substantial drain of α-oxoglutarate to support the biosynthesis of glutamate. 5. It is concluded that oxaloacetate, required for the incorporation of ethanol into citrate, arises mainly from the transamination of aspartate and the fixation of carbon dioxide.

Germination of seeds is characterized by intensive metabolic activity, the final result of which is growth of the embryo. These reactions are generally of three main types: first, breakdown of storage materials within the storage organs of the seed; secondly, synthesis of new compounds from these breakdown products; thirdly, transport of these new materials from the storage organ to the developing embryo (Mayer & Poljakoff-Mayber, 1963).

During the earliest stages of germination many seeds experience a brief period of natural anaerobiosis due largely to poor penetration of oxygen through the seed coat (James, 1953). In pea seeds this period of anaerobiosis persists until the seed coat is ruptured by the developing embryo and is accompanied by large accumulations of ethanol and lactic acid within the cotyledons (Cossins & Turner, 1959, 1963; Cossins, 1964). As the embryo develops, the ethanol and lactate contents of the cotyledons are rapidly depleted and experiments with [^{14}C]-ethanol and [^{14}C]lactate (Cossins & Turner, 1963; Cossins & Beevers, 1963; Cossins, 1964) have shown that these compounds are rapidly metabolized. Cossins & Beevers (1963) showed that several different plant tissues were capable of ethanol metabolism and concluded that this proceeded via acetyl-coenzyme A.

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As the amounts of ethanol metabolized by germinating pea seedlings are considerable (Cossins & Turner, 1963) it is possible that this compound plays an important role in intermediary metabolism as the precursor of compounds required for growth of the developing embryo. The present studies have therefore examined the pathway of ethanol and lactate metabolism in pea cotyledons. These studies have indicated utilization of ethanol via an active tricarboxylic acid cycle which functions synthetically for glutamate and glutamine biosynthesis.

MATERIALS AND METHODS

Plant materials. Seeds of pea (*Pisum sativum* L. *var.* Homesteader) were soaked overnight in distilled water for 18hr. at 25°. After this soaking period, the seeds were transferred to moist vermiculite and allowed to germinate for 48hr. at 25° in the dark. At this stage of germination the radicles were approx. 2·0cm. in length.

^{14}C-labelled compounds. The metabolism of a number of different ^{14}C-labelled compounds was investigated. These included $NaH^{14}CO_3$, [1-^{14}C]ethanol, [2-^{14}C]ethanol, [1-^{14}C]-acetate, [2-^{14}C]acetate, [1,2-$^{14}C_2$]acetaldehyde, [5,6-$^{14}C_2$]-isocitrate, α-oxo[5-^{14}C]glutarate and [3,4-$^{14}C_2$]glutamate. In all cases the labelled compounds from the supplier were diluted with carrier to give final specific radioactivities within the range 0·3–5·8 μc/μmole/0·1ml. of solution (see the Results section).

Feeding experiments. In all experiments the cotyledons were removed and sliced as described by Cossins & Turner (1963). After washing in distilled water, the slices were

placed in Warburg flasks containing 0·5ml. of 0·1M-phosphate buffer, pH5·5. Incubations with the labelled substrates were carried out as described by Cossins & Beevers (1963).

Analytical methods. At the end of the experimental period the tissues were rapidly killed by boiling in 10ml. of ethanol followed by grinding in a hand-operated blender. The procedure of extraction and fractionation of the extracts was as described by Cossins & Beevers (1963). This resulted in fractionation of the aqueous-ethanol-soluble materials into four fractions, namely acidic amino acids, neutral and basic amino acids plus amides, organic acids and sugars, by use of ion-exchange resins (Canvin & Beevers, 1961).

Further fractionation of the organic acids was achieved by gradient elution of the acids from Dowex 1 (X10; formate form) resin by the method of Palmer (1955). Identity of the organic acids was established by their position of elution from the resin and by co-chromatography with authentic organic acids on paper. This was accomplished with phenol–water (8:3, v/v) and butan-1-ol–acetic acid–water (4:1:5, by vol.) as solvent systems.

Asparagine and glutamine present in the neutral and basic amino acid fraction were hydrolysed to aspartate and glutamate respectively by boiling with 2N-HCl for 4hr. The hydrolysate was dried *in vacuo* at 40° to remove all traces of HCl, dissolved in 10ml. of distilled water and passed through a column of Dowex 1 (X10; acetate form) to remove aspartic acid and glutamic acid (Hirs, Moore & Stein, 1954).

The components of the neutral and basic amino acid fraction were separated by two-dimensional descending paper chromatography in phenol–water (8:3, v/v) followed by butan-1-ol–acetic acid–water (4:1:5, by vol.) as solvent systems. The acidic amino acids, principally aspartic acid and glutamic acid, were separated by descending paper chromatography with phenol–water (8:3, v/v) as solvent system.

Radioactive areas on the paper chromatograms were located by radioautography with Kodak No-Screen X-ray film and by scanning with a Nuclear–Chicago 4 Pi Actigraph (model no. 4502). Radioactivity of these areas was assayed after elution from the chromatograms with distilled water and plating portions of the eluate (0·2ml.) on to nickel-plated steel planchets and drying under an infrared lamp (Sinha & Cossins, 1964). Radioactivity was assayed in a gas-flow counter with 20% efficiency (Nuclear–Chicago Corp. model no. C110B). The counts were corrected for background.

Carbon dioxide evolved during the feeding experiments was absorbed in carbonate-free NaOH solution (20%, w/v) added to the centre wells of the Warburg flasks at the start of each experiment. The absorbed CO_2 was converted into $BaCO_3$ and assayed for radioactivity as described by Sinha & Cossins (1964). The counts were corrected for background and self-absorption.

Determination of specific radioactivities. Samples of labelled amino acids separated by ion-exchange and paper chromatography as described above were treated with ninhydrin reagent in methylCellosolve (Moore & Stein, 1948). Extinctions of the solutions were determined in a model DB spectrophotometer (Beckman Instruments Inc., Palo Alto, Calif., U.S.A.). Reference curves were constructed with authentic amino acids.

Degradation of labelled glutamate samples. Samples of ^{14}C-labelled glutamate, extracted from the tissues, were mixed with carrier L-glutamic acid (10μmoles) and incubated with excess of glutamate decarboxylase (L-glutamate 1-carboxy-lyase, EC 4.1.1.15) purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Incubations were carried out at 37° in 0·1M-acetate buffer, pH5·0, and terminated after 1hr. by addition of ethanol. Carbon dioxide evolved from C-1 of glutamate was absorbed in 20% (w/v) NaOH solution. The carbonate formed was then assayed for ^{14}C content as described above. For assay of the ^{14}C content of C-5, further samples of labelled glutamate were treated with azide as described by Aronoff (1961).

Determination of organic acid pool sizes. Samples of pea cotyledons (20g.) were killed and extracted as described above. The organic acids were fractionated by gradient elution from columns of Dowex 1 (X10; formate form) with formic acid as described by Palmer (1955). All traces of formic acid in the tubes were removed in a stream of air at 40°. Then 1ml. of CO_2-free distilled water was added to each tube and titration with 6mN-NaOH carried out.

Enzyme studies. For assay of each enzyme, 10g. of 3-day-old cotyledons was ground in 10–20ml. of buffer solution (see below) at 2°. The homogenate was cleared by centrifuging (10000*g* for 20min.) and protein determined spectrophotometrically (Warburg & Christian, 1941).

Alcohol dehydrogenase (alcohol–NAD$^+$ oxidoreductase, EC 1.1.1.1): the tissue was extracted with 0·1M-phosphate buffer, pH8·5; enzyme activity was assayed according to Racker (1950*a*).

Aconitase [citrate (isocitrate) hydro-lyase, EC 4.2.1.3]: the tissue was homogenized in 0·1M-phosphate buffer, pH7·4; aconitase activity was assayed at 240mμ (Racker 1950*b*).

Isocitrate dehydrogenase [D$_s$-isocitrate–NAD$^+$ oxidoreductase (decarboxylating), EC 1.1.1.41]: the enzyme was extracted in 0·1M-tris–HCl buffer, pH7·4, and assayed as described by Davies (1955).

Glutamate dehydrogenase [L-glutamate–NAD$^+$ oxidoreductase (deaminating), EC 1.4.1.2]: the tissue was homogenized in 0·1M-phosphate buffer, pH7·6, and the homogenate dialysed against distilled water for 36hr.; enzyme activity was assayed spectrophotometrically (Olson & Anfinsen, 1952) and by following incorporation of α-oxo[^{14}C]glutarate into glutamate in the presence of NH_4^+ ions.

Glutamate–oxaloacetate transaminase (L-aspartate–α-oxoglutarate aminotransferase, EC 2.6.1.1): the enzyme was extracted in 0·1M-phosphate buffer, pH7·1, and after dialysis against distilled water for 18hr. was assayed by using α-oxo[^{14}C]glutarate and aspartate; the experimental conditions were as described by Cossins & Sinha (1965).

RESULTS

Titratable organic acids in germinating cotyledons. When extracts of germinating pea cotyledons were fractionated and the titratable organic acids determined, six 'peaks' of acidity were detected (Fig. 1). Paper chromatography of these 'peaks' revealed the presence of lactic acid, glycollic acid, succinic acid, malic acid, citric acid, isocitric acid

and sugar phosphates. The quantities of these acids did not vary greatly during the 5-day period of germination examined.

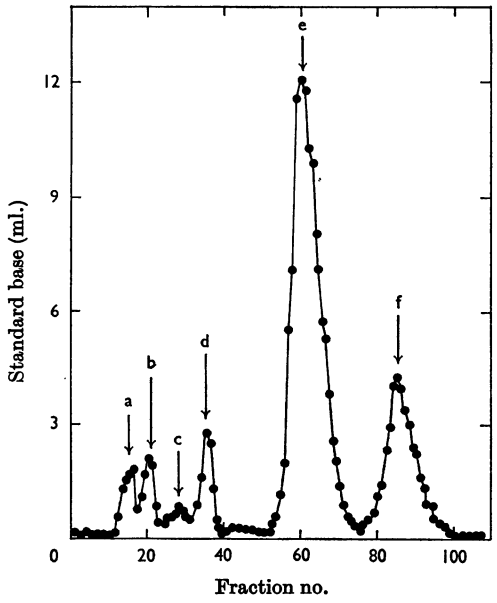

Fig. 1. Titratable organic acids in 3-day-old pea cotyledons. Organic acid fraction from 20g. of cotyledons was chromatographed on Dowex (formate from) resin as described in the Materials and Methods section. a, Lactic acid; b, glycollic acid and two unidentified acids; c, succinic acid; d, malic acid; e, citric acid and isocitric acid; f, sugar phosphates.

Incorporation of labelled ethanol. When samples of 3-day-old pea cotyledons were incubated with [1-^{14}C]ethanol and [2-^{14}C]ethanol for periods up to 1hr. (Table 1), ^{14}C was rapidly incorporated into all of the fractions isolated. The C-1 of ethanol was more rapidly converted into carbon dioxide than the methyl carbon atom. However, only small amounts of ^{14}C were incorporated into carbon dioxide even after 1hr. of ethanol metabolism. In the experiments of only 5min. duration more than 50% of the incorporated ^{14}C was present in the organic acid fraction. Examination of this fraction by ion-exchange chromatography (Fig. 2) and by two-dimensional paper chromatography showed that citrate and isocitrate were the major radioactive compounds present. As the percentage of incorporated ^{14}C in the organic acids decreased with time, the percentage of the total ^{14}C incorporated into the acidic amino acids and amides increased. The principal amide labelled was glutamine and the neutral and basic amino acid fraction contained large amounts of labelled γ-aminobutyrate.

Incorporation of labelled acetaldehyde. In earlier experiments with labelled ethanol, Cossins & Turner (1963) reported a rapid labelling of acetaldehyde followed by labelling of several organic and amino acids. If acetaldehyde is an intermediate in the pathway of ethanol metabolism, [^{14}C]acetaldehyde should be rapidly incorporated into the compounds labelled during ethanol metabolism. This possibility was examined in the experiment summarized in Table 2. Only small amounts of acetaldehyde carbon were incorporated into carbon dioxide. Acetaldehyde was, however, rapidly

Table 1. *Sequence of incorporation of ^{14}C into products of* [1-^{14}C]*ethanol and* [2-^{14}C]*ethanol metabolism*

Slices (0·5g.) of pea cotyledons were incubated at 30° with 50μmoles of phosphate buffer, pH5·5, and 1μmole of ethanol (containing 5μc of ^{14}C) in a total volume of 0·6ml.

Time of incubation (min.) ...	% of incorporated ^{14}C							
	5		15		30		60	
Fraction	[1-^{14}C]-Ethanol	[2-^{14}C]-Ethanol	[1-^{14}C]-Ethanol	[2-^{14}C]-Ethanol	[1-^{14}C]-Ethanol	[2-^{14}C]-Ethanol	[1-^{14}C]-Ethanol	[2-^{14}C]-Ethanol
Lipids	3·2	4·5	3·3	4·4	3·7	5·1	4·4	6·4
Neutral compounds	6·4	5·9	5·8	5·5	3·6	3·9	3·6	3·9
Acidic amino acids								
glutamate	22·8	17·5	36·6	30·4	39·7	34·8	33·2	28·0
aspartate	7·4	5·7	8·3	3·7	9·3	4·6	8·5	5·9
Amides	1·5	1·6	2·2	3·6	4·4	7·6	5·6	8·7
Neutral and basic amino acids	3·3	3·9	3·2	4·0	2·9	2·2	3·4	3·0
Organic acids	52·8	54·3	38·2	45·1	32·2	37·9	32·1	36·7
Carbon dioxide	0·5	0·2	0·9	0·1	1·8	0·1	5·2	0·3
Insoluble residue	2·1	6·4	1·5	3·2	2·4	3·8	4·0	7·1
Total ^{14}C incorporated (counts/min.)	90300	64400	294700	192600	688000	452300	992500	829200

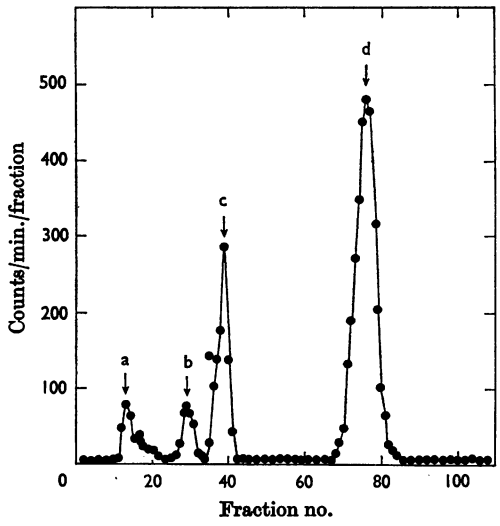

Fig. 2. Organic acids labelled after incubation with [1-^{14}C]-ethanol for 5 min. a, Lactic acid; b, succinic acid; c, malic acid; d, citric acid and isocitric acid.

Table 2. *Sequence of incorporation of* ^{14}C *into products of* [1,2-$^{14}C_2$]*acetaldehyde metabolism*

Slices (0·5 g.) of pea cotyledons were incubated at 30° with 50 μmoles of phosphate buffer, pH 5·5, and 2·5 μmoles of acetaldehyde (containing 2·5 μc of ^{14}C) in a total volume of 0·6 ml.

Time of incubation (min). ... ... Fraction	% of incorporated ^{14}C 5	15	30	60
Lipids	3·7	2·7	2·0	1·6
Neutral compounds	24·4	16·7	12·1	9·4
Acidic amino acids				
glutamate	15·3	19·6	32·4	23·9
aspartate	2·5	2·5	4·5	5·8
Amides	1·2	4·7	6·3	8·9
Neutral and basic amino acids	7·0	7·0	3·4	4·4
Organic acids	39·7	39·3	31·8	35·2
Carbon dioxide	0·8	1·9	2·4	3·6
Insoluble residue	5·4	5·6	5·1	7·2
Total ^{14}C incorporated (counts/min.)	24200	48400	114600	207000

Table 3. *Labelled products isolated after short periods of* [1-^{14}C]*acetate metabolism*

Slices (0·5 g.) of pea cotyledons were incubated at 30° with 50 μmoles of phosphate buffer, pH 5·5, and 1 μmole of sodium [1-^{14}C]acetate (containing 5 μc of ^{14}C) in a total volume of 0·6 ml.

Time of incubation (min.) ... ... Fraction	% of incorporated ^{14}C 1	2	3	5
Lipids	0·9	1·3	2·3	2·3
Neutral compounds	1·7	1·7	1·6	2·4
Glutamate	1·3	1·7	3·0	7·3
Glutamine	0·4	0·5	0·5	0·7
Organic acids				
Citrate + isocitrate	52·7	52·6	54·0	54·2
Succinate	21·0	21·9	22·2	15·6
Malate	6·5	5·6	3·8	5·8
Other organic acids	15·5	14·7	12·6	11·7
Total ^{14}C incorporated (counts/min.)	110600	121900	134300	146100

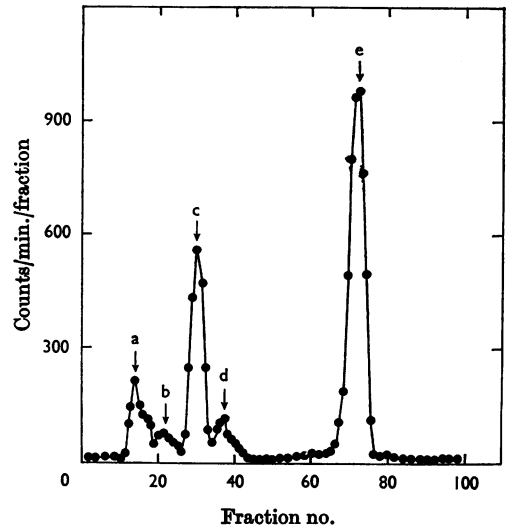

Fig. 3. Organic acids labelled after incubation with [1-^{14}C]-acetate for 1 min. a, Lactic acid; b, glycollic acid; c, succinic acid; d, malic acid; e, citric acid and isocitric acid.

incorporated into the organic acids. Chromatography of this fraction showed that citrate and isocitrate were the most heavily labelled acids after 5 min. As the experimental period was lengthened, succinate and malate also became labelled. In addition to these products, labelling of glutamate was considerable. Paper chromatography of the neutral fraction showed that all of the radioactivity here was associated with one compound. This compound was found to travel with authentic acetoin (3-hydroxybutan-2-one) when subjected to paper chromatography in a variety of different solvent systems.

Incorporation of labelled acetate. Pea cotyledons rapidly incorporated [1-^{14}C]acetate into a number of compounds (Table 3), particularly into citrate, isocitrate and succinate (Fig. 3), which accounted for more than 70% of the ^{14}C recovered in products

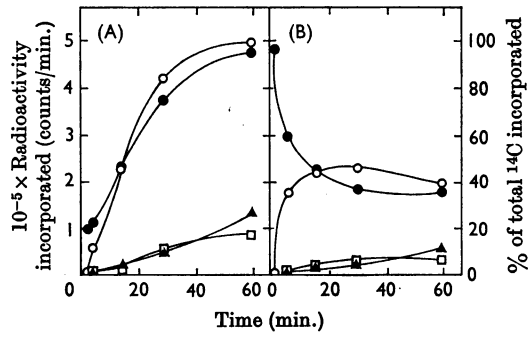

Fig. 4. Incorporation of [1-^{14}C]acetate by pea cotyledons. (A) Incorporation of ^{14}C into individual fractions. (B) Incorporation of ^{14}C expressed as percentage of total recovered in the isolated fractions. ○, Acidic amino acids; ●, organic acids; ▲, amides; □, carbon dioxide.

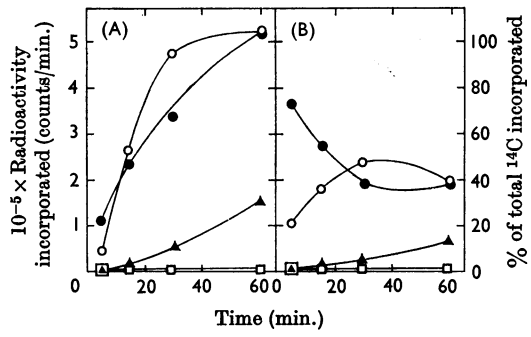

Fig. 5. Incorporation of [2-^{14}C]acetate by pea cotyledons. (A) Incorporation of ^{14}C into individual fractions. (B) Incorporation of ^{14}C expressed as percentage of total recovered in the isolated fractions. ○, Acidic amino acids; ●, organic acids; ▲, amides; □, carbon dioxide.

after 1min. of acetate metabolism. As the experimental period was extended to 5min. the percentage of the total incorporated ^{14}C present in glutamate rose sharply. In experiments of longer duration (Figs. 4 and 5), [1-^{14}C]acetate and [2-^{14}C]acetate were supplied for periods up to 1hr. It is clear that the sequence of the incorporation of the individual carbon atoms of acetate was very similar. The only exception to this was the slightly greater incorporation of C-1 into carbon dioxide. As in the ethanol and acetaldehyde experiments (Tables 1 and 2), acetate carbon labelled initially the organic acid fraction but as the incubation was continued, the acidic amino acids became heavily labelled. When expressed as a percentage of the total ^{14}C incorporated, the line for the organic acids has a pronounced negative slope (Figs. 4B and 5B), whereas the line for the acidic amino acids shows a positive slope.

Incorporation of labelled lactate and acids associated with the tricarboxylic acid cycle. The results of the ethanol- acetaldehyde- and acetate-feeding experiments show that these compounds are all rapidly incorporated into acids of the tricarboxylic acid cycle. However, after incubation for periods up to 1hr. only relatively small amounts of the incorporated ^{14}C were evolved as carbon dioxide and considerable amounts of radioactivity were accumulated in the acidic amino acids. These findings may be interpreted as indicating a relatively slow cycling of carbon through the tricarboxylic acid cycle accompanied by a rapid removal of ^{14}C from the cycle acids and its accumulation in glutamate. Alternatively, ethanol, acetaldehyde and acetate may be extensively oxidized to carbon dioxide, which is then re-fixed and utilized for amino acid biosynthesis.

To examine these possibilities, experiments were carried out to determine the metabolic fates of certain acids associated with the tricarboxylic acid cycle. The position of ^{14}C in these labelled substrates was such that theoretically they would be extensively converted into labelled carbon dioxide (e.g. [1-^{14}C]lactate and [5,6-$^{14}C_2$]isocitrate) or would be incorporated into glutamate without extensive conversion into labelled carbon dioxide (e.g. α-oxo[5-^{14}C]glutarate).

The results of these experiments are summarized in Table 4. In agreement with earlier published work (Cossins, 1964), labelled lactate was incorporated into all of the fractions isolated. In the experiment of 2hr. duration, the major product of this metabolism was carbon dioxide. However, labelling was also detected in the organic acids, including malate and citrate and in glutamate and aspartate. Alanine, which accounted for more than 90% of the ^{14}C present in the neutral and basic amino acid fraction, was found to be entirely labelled in C-1 when treated with ninhydrin (van Slyke, Dillon, MacFadyen & Hamilton, 1941). Clearly the release of C-1 of lactate as carbon dioxide is consistent with operation of lactate dehydrogenase and oxidative decarboxylation of the resulting pyruvate. However, the obvious labelling of malate, citrate, aspartate and glutamate points to some re-fixation of labelled carbon dioxide.

When the products of acetate, isocitrate, α-oxoglutarate and glutamate metabolism are examined (Table 4), it is clear that these compounds were all incorporated into the acidic amino acids and amides. In all cases the organic acid fraction contained appreciable amounts of labelling in citrate, succinate and malate. It is also clear that in these experiments of 6hr. duration the isotopic carbon in isocitrate

Table 4. *Incorporation of ^{14}C-labelled lactate and acids associated with the tricarboxylic acid cycle*

Experimental procedure was as described in Table 1. Cotyledon slices were incubated with ^{14}C-labelled compounds as indicated: lactate, 1·7 μmoles (5·0 μc of ^{14}C); acetate, 1 μmole (2 μc of ^{14}C); isocitrate, 7·4 μmoles (2·0 μc of ^{14}C); α-oxoglutarate, 1 μmole (2·0 μc of ^{14}C); glutamate, 1 μmole (3 μc of ^{14}C). N.D., Not detected.

	% of incorporated ^{14}C					
Time of incubation (min.) ...	30	120	360			
Fraction	[1-^{14}C]Lactate		[2-^{14}C]-Acetate	[5,6-^{14}C$_2$]-Isocitrate	α-Oxo[5-^{14}C]-glutarate	[3,4-^{14}C$_2$]-Glutamate
Lipids	3·6	2·0	5·6	0·2	0·6	0·3
Neutral compounds	43·0	4·1	8·2	2·3	3·1	0·7
Acidic amino acids						
glutamate	2·0	3·6	9·6	11·8	11·1	—
aspartate	1·4	1·9	N.D.	0·5	0·5	1·8
others	N.D.	N.D.	N.D.	N.D.	0·4	4·0
Amides	0·9	0·4	17·3	5·8	14·2	30·7
Neutral and basic amino acids	23·0	36·5	5·2	2·5	5·5	19·4
Organic acids	5·9	4·1	38·7	33·1	29·8	21·1
Carbon dioxide	9·9	36·7	10·8	31·6	32·0	5·3
Insoluble residue	10·3	10·7	4·6	12·2	2·8	16·7
Total ^{14}C incorporated (counts/min.)	189700	389000	289100	335800	1035100	1199100

Table 5. *Sequence of incorporation of ^{14}C into products of [^{14}C]carbonate metabolism*

Experimental procedure was as described in Table 1. n.a., Not radioactive.

	Radioactivity in fractions (counts/min.)				
Time of incubation (min.) ...	2	5	15	30	60
Fraction					
Neutral compounds	n.a.	n.a.	n.a.	n.a.	320
Acidic amino acids	570	1250	4500	8300	15800
Neutral and basic amino acids	1500	4160	6300	12600	21000
Organic acids	3900	7200	14000	27300	40900
Insoluble residue	578	1500	3600	8000	27700
Total ^{14}C incorporated	6548	14110	28400	56200	105720

and α-oxoglutarate was appreciably converted into carbon dioxide.

Incorporation of labelled carbonate. The labelling of malate, citrate and the acidic amino acids after feeding with [1-^{14}C]lactate (Table 4) is most readily explained on the basis of fixation of labelled carbon dioxide. To investigate the ability of these tissues to utilize labelled carbonate and to determine the nature of the products formed, the experiments summarized in Table 5 were carried out. After an experimental period of only 2 min., the label was found in the organic acid fraction (entirely due to [^{14}C]malate) and in the amino acid fractions. As the experimental period was extended, other organic acids became labelled (Fig. 6) and glutamate and glutamine became progressively more radioactive. When the labelling of the acidic amino acids is examined more closely (Table 6), it is clear that the specific and total radioactivity of aspartate rapidly reach maximum values and then decline. In contrast, the glutamate pool increases in total and specific radioactivities throughout the experiment. Further, the specific radioactivities of asparagine and glutamine after 60 min. and 180 min. were higher than the values for their corresponding acidic amino acids. We interpret these results as indicative of a rapid depletion of the supplied carbonate during the experiment and a transfer of ^{14}C from the primary products to the glutamate pool, which gradually increases in radioactivity. Further, aspartate and glutamate must be 'compartmented' within the tissues with a portion of the total content of these amino acids serving as a precursor pool for synthesis of the corresponding amides.

Enzyme studies. A number of the enzymes

Table 6. *Changes in total radioactivities and specific radioactivities of aspartate and glutamate during feeding with* [^{14}C]*carbonate*

Experimental procedure was as in Table 1, with the exception that 1 μmole of [^{14}C]carbonate containing 15 μc of ^{14}C was supplied. After 60 min. the specific radioactivities of asparagine and glutamine were 16580 counts/min./μmole and 6600 counts/min./μmole respectively. After 180 min. the corresponding values were 9363 and 11875 respectively.

	Aspartic acid		Glutamic acid	
Time of incubation (min.)	counts/min.	counts/min./μmole	counts/min.	counts/min./μmole
2	3200	1280	not radioactive	
5	4680	1350	1720	477
15	9000	3000	3820	955
30	16900	8470	5400	1620
60	9900	3313	8500	1840
180	6000	1515	10200	2040

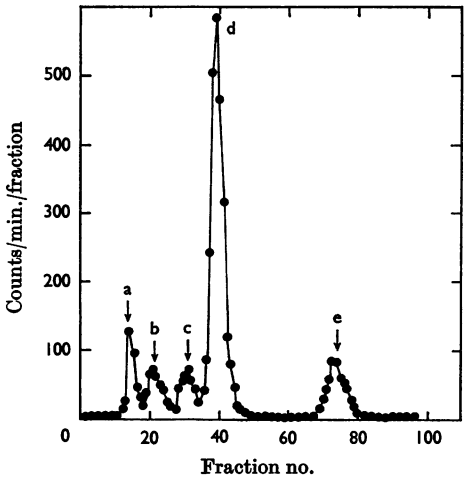

Fig. 6. Organic acids labelled after incubation with [^{14}C] carbonate for 5 min. a, Lactic acid; b, glycollic acid; c, succinic acid; d, malic acid; e, citric acid and isocitric acid.

involved in ethanol metabolism in pea cotyledons were examined in further experiments (Table 7). Pea cotyledons contain relatively large amounts of alcohol dehydrogenase. This enzyme has recently been studied in some detail (E. A. Cossins, unpublished work) and has properties similar to those described for the enzyme from yeast and mammalian sources (Racker, 1950*a*; Theorell & Bonnichsen, 1951; von Wartburg, Bethune & Vallee, 1964). Enzymes catalysing the biosynthesis of glutamate either by reductive amination of α-oxoglutarate or by transamination of this keto acid were also present in large amounts in this tissue. These findings therefore corroborate the data obtained from the ethanol-feeding experiments, namely that the incorporation of ethanol carbon atoms into glutamate proceeds very rapidly and enzymes necessary to catalyse these reactions are demonstrable in tissue extracts.

DISCUSSION

The present studies of the kinetics of ethanol, acetaldehyde and acetate metabolism have shown that these compounds are initially incorporated into the acids of the tricarboxylic acid cycle followed by an accumulation of the label in glutamate. In these studies the individual carbon atoms of ethanol and acetate were incorporated into these products in a similar manner. Perhaps the most striking feature accompanying these incorporations is the very minor conversion of the labelled substrate into carbon dioxide. These experiments indicate that these compounds are metabolized mainly via the reactions of the tricarboxylic acid cycle. Cossins & Beevers (1963) concluded that ethanol was converted into acetylcoenzyme A before entering the organic acids; the present work is in agreement with this conclusion. However, the comparatively minor conversion of these compounds into carbon dioxide in experimental periods of 1 hr. duration may be explained on the basis of: (*a*) cycling of carbon through the tricarboxylic acid cycle is either negligible or extremely sluggish; or (*b*) large storage pools of the cycle acids gradually come into equilibrium with smaller metabolic pools of these acids and therefore trap large quantities of ^{14}C until equilibrium is established; or (*c*) the partial reactions of the cycle function in a synthetic manner whereby oxaloacetate and ethanol carbon atoms are utilized for the biosynthesis of glutamate and glutamine.

The first alternative, namely that cycling of carbon is negligible or sluggish, appears unlikely as citrate, succinate and malate were all rapidly labelled with ^{14}C after only 1 min. of acetate

Table 7. *Specific activities of some enzymes associated with ethanol metabolism in pea cotyledons*

Specific activities are expressed as mμmoles of substrate transformed/min./mg. of protein.

Enzyme	Substrate	Concn. of substrate (μmoles/ml.)	pH	Sp. activity
Alcohol dehydrogenase	Ethanol	50·0	7·4	52·4
Aconitase	Citrate	29·5	7·4	0·9
Isocitrate dehydrogenase	Isocitrate	66·7	7·4	5·6
Glutamate–oxaloacetate transaminase	α-Oxoglutarate	1·1		
	L-Aspartate	0·5	7·1	7·7
Glutamate dehydrogenase dialysed homogenate	α-Oxoglutarate	0·3		
	NH_4^+	3·3	7·2	4·3

metabolism (Table 3). Further, labelled isocitrate and α-oxoglutarate were converted into other acids of the cycle and into carbon dioxide (Table 4).

MacLennan, Beevers & Harley (1963) have made a detailed study of the 'compartmentation' of organic acids in plant tissues. These workers reported that a significant part of the acids present in their experimental tissues were in storage pools, which did not readily equilibrate with smaller acid pools associated with the tricarboxylic acid cycle. If this situation occurs in the cotyledons of peas, labelled citrate from the metabolic pool would tend to exchange with unlabelled citrate in the storage pool until their specific activities approached equality. Thus labelled carbon dioxide would not be produced in large amounts until the storage pools of the cycle acids came into equilibrium with their respective metabolic pools. Titration of the organic acids present in pea cotyledons (Fig. 1) indicated considerable quantities of citrate and isocitrate together with smaller amounts of malate and succinate. After prolonged periods of [^{14}C]-acetate metabolism, the amounts of radioactivity in these acids tended to reflect their individual pool sizes (Cameron, 1965).

Accompanying the heavy labelling of the organic acid pools there was an accumulation of label in glutamate. Fig. 2 shows that as the percentage of the total ^{14}C entering the organic acids decreased that entering glutamate increased sharply. This relationship shows that glutamate was labelled at the expense of the labelled organic acids. Clearly labelling of glutamate represents initially a withdrawal of ^{14}C from the acids of the cycle and therefore also reduces the likelihood of ethanol or acetate carbon atoms being converted into carbon dioxide. However, if the glutamate pool gradually reaches equilibrium with the acids of the tricarboxylic acid cycle, the C-1/C-5 ratio of ^{14}C content should approach 0·5 after extensive cycling of carbon. When samples of [^{14}C]glutamate, recovered from the tissues after feeding with [1-^{14}C]ethanol or [1-^{14}C]acetate, were degraded (see the Materials and Methods section) the distribution of ^{14}C as a percentage of the total radioactivity was: C-1, 5%; C-5, 95%. This distribution was essentially unaltered even after 1 hr. of [1-^{14}C]ethanol or [1-^{14}C]-acetate metabolism. Glutamate samples recovered from tissues after feeding with [2-^{14}C]ethanol or [2-^{14}C]acetate contained negligible amounts of ^{14}C in C-1 and C-5. Thus even after 1 hr. of ethanol or acetate metabolism the labelling of the glutamate pool still reflected a distribution of ^{14}C that would be expected if α-oxoglutarate generated by the reactions of the cycle was withdrawn for glutamate biosynthesis without extensive cycling of ^{14}C. However, when [3,4-$^{14}C_2$]glutamate was supplied for 6 hr. (Table 4), considerable amounts of ^{14}C were incorporated into carbon dioxide and the acids of the cycle. Therefore the supplied glutamate must have entered an endogenous pool of glutamate whereby some exchange with a metabolic pool of α-oxoglutarate was possible.

The present experiments strongly suggest that a large part of the supplied substrate was utilized for the biosynthesis of glutamate and glutamine. Thus ethanol, acetaldehyde and acetate metabolism appears to lead a to net synthesis of this amino acid and amide. Clearly if glutamate arises from α-oxoglutarate (Table 4) and a large part of this glutamate is then rapidly utilized for the synthesis of glutamine (Fig. 4 and Table 4), the overall result of these syntheses will be a continuous removal of carbon atoms from the tricarboxylic acid cycle. As the total acid pool of this tissue was found to remain relatively constant during the early stages of germination, this synthesis does not appear to occur at the expense of the organic acids.

Changes in the amino acid contents of germinating pea seedlings have been studied by Lawrence & Grant (1963) and by Larson & Beevers (1965). These studies have shown that the glutamate and glutamine contents of the cotyledons increase markedly during germination. In contrast the

aspartate and asparagine contents decrease sharply. Although a portion of the aspartate and asparagine decreases may be accounted for by concomitant homoserine biosynthesis, it appears that oxaloacetate, arising from aspartate, is utilized for glutamate and glutamine formation via the partial reactions of the tricarboxylic acid cycle (Larson & Beevers, 1965).

Alternatively oxaloacetate may arise as a result of carbon dioxide fixation in the phosphoenolpyruvate carboxykinase reaction (Walker, 1962). This enzyme has been demonstrated to occur in pea cotyledons by Mazelis & Vennesland (1957) and the present work demonstrates that these tissues can very readily incorporate $^{14}CO_2$ into malate and aspartate (Table 5). As the experimental period was extended, ^{14}C was gradually accumulated in glutamate and glutamine and the specific activities of these compounds rose as those of aspartate and asparagine decreased. Thus carbon dioxide fixation appears to be quantitatively significant in the biosynthesis of glutamate and glutamine in these tissues. The importance of carbon dioxide in supplying oxaloacetate to support the tricarboxylic acid cycle in synthetic reactions has been emphasized by Splittstoesser (1966).

In pea cotyledons intermediary metabolism is therefore centred on an active tricarboxylic acid cycle, which functions synthetically for the production of glutamate and glutamine. In this respect oxaloacetate, required for continuous synthesis of these products, is generated from aspartate or from carboxylation reactions. Ethanol and lactate, produced during the early stages of germination (Cossins & Turner, 1963; Cossins, 1964), are therefore utilized in these synthetic reactions during the later stages of germination.

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REFERENCES

Aronoff, S. (1961). *Techniques of Radiochemistry*, p. 228. Ames, Iowa: Iowa State University Press.

Cameron, D. S. (1965). M.Sc. Thesis: University of Alberta, Edmonton, Canada.

Canvin, D. T. & Beevers, H. (1961). *J. biol. Chem.* **236**, 988.

Cossins, E. A. (1964). *Nature, Lond.*, **203**, 989.

Cossins, E. A. & Beevers, H. (1963). *Plant Physiol.* **38**, 375.

Cossins, E. A. & Sinha, S. K. (1965). *Canad. J. Biochem.* **43**, 495.

Cossins, E. A. & Turner, E. R. (1959). *Nature, Lond.*, **183**, 1599.

Cossins, E. A. & Turner, E. R. (1963). *J. exp. Bot.* **14**, 290.

Davies, D. D. (1955). *J. exp. Bot.* **6**, 212.

Hirs, C. H., Moore, W. S. & Stein, W. H. (1954). *J. Amer. chem. Soc.* **76**, 6063.

James, W. O. (1953). *Plant Respiration*, p. 101. London: Oxford University Press.

Larson, L. A. & Beevers, H. (1965). *Plant Physiol.* **40**. 424.

Lawrence, J. M. & Grant, D. R. (1963). *Plant Physiol.* **38**, 561.

MacLennan, D. H., Beevers, H. & Harley, J. L. (1963). *Biochem. J.* **89**, 316.

Mayer, A. M. & Poljakoff-Mayber, A. (1963). *The Germination of Seeds*, p. 139. New York: The Macmillan Co.

Mazelis, M. & Vennesland, B. (1957). *Plant Physiol.* **32**, 591.

Moore, W. S. & Stein, W. H. (1948). *J. biol. Chem.* **176**, 367.

Olson, J. A. & Anfinsen, C. B. (1952). *J. biol. Chem.* **197**, 67.

Palmer, J. K. (1955). *Bull. Conn. agric. Exp. Sta.* no. 589.

Racker, E. (1950*a*). *J. biol. Chem.* **184**, 313.

Racker, E. (1950*b*). *Biochim. biophys. Acta*, **4**, 211.

Sinha, S. K. & Cossins, E. A. (1964). *Biochem. J.* **93**, 27.

Splittstoesser, W. E. (1966). *Plant Physiol.* **41**, 755.

Theorell, H. & Bonnichsen, R. K. (1951). *Acta chem. scand.* **5**, 1105.

van Slyke, D. D., Dillon, R. T., MacFadyen, D. A. & Hamilton, P. (1941). *J. biol. Chem.* **141**, 627.

von Wartburg, J. P., Bethune, J. L. & Vallee, B. L. (1964). *Biochemistry*, **3**, 1775.

Walker, D. A. (1962). *Biol. Rev.* **37**, 215.

Warburg, O. & Christian, W. (1941). *Biochem. Z.* **310**, 384.