Acetate Utilization by Maize Roots [1, 2]

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The aim of this work was to study the entry of acetate-C^{14} into the metabolic pathways of the root tips of maize. Particular attention was paid to the kinetics of its incorporation into the various acids of the tricarboxylic acid (TCA) cycle and the appearance of each of the carbons in the respired CO_2. The results lead to conclusions about the operation of the TCA cycle in this tissue. A preliminary report has appeared previously (3).

Materials & Methods

Maize grains (var. Wf 9 × 38-11 obtained from Agricultural Alumni Seed Association, Lafayette, Ind.) were soaked in tap water for 24 hours and germinated for 24 hours at 30 C between sheets of moist filter paper. The apical 1 cm of their radicles were excised, washed, blotted, and weighed. Samples of 1 g fresh weight were used for tracer experiments and samples of up to 10 g were employed to estimate the quantities of acids in the tissue. Samples of 20 roots (0.2 g) were used in Warburg respirometers.

Roots were allowed to absorb acetate-1-C^{14} or acetate-2-C^{14} (specific activity 2.5-14.3 × 10^4 cpm/micromole) from solutions containing 2.5 micromoles of acetate per 10 ml 0.025 M potassium phosphate at pH 5.0. During uptake the roots were agitated by a stream of air bubbles in a sintered glass aeration tube. For short uptake periods the roots were placed in a perforated Gooch crucible which was shaken manually in the acetate medium.

After the appointed time of exposure the roots were killed by adding boiling 80 % ethanol and boiled over a steam bath for 2½ hours. The ethanol was decanted and the roots were ground in a glass homogenizer. The residue after filtering was extracted successively with hot 80 % ethanol, hot water, and hot 80 % ethanol. The solutions were combined (ethanol-water extract) and taken to dryness at 40 C under reduced pressure. Lipid soluble material was removed from the residue by two washings with 10 ml of dry ether. The residue was then dissolved in water and made to volume (water soluble material).

The water soluble material was fractionated using Dowex resins in 1 cm × 6 cm columns (2). The extract was passed sequentially through Dowex 50 × 8 (H^+) and Dowex 1 × 10 (formate). The basic fraction was eluted from Dowex 50 with 1 N NH_4OH and the acidic fraction was eluted from Dowex 1 with 8 N formic acid.

The basic or amino acid fraction was further fractionated (4) by passage through Dowex 1 × 10 (acetate). A dicarboxylic amino acid fraction containing free glutamic and aspartic acids was obtained by elution with 2 N acetic acid. The effluent, containing neutral and basic amino acids was treated with 2 N H_2SO_4 to hydrolyze the amides, neutralized, and again passed through a Dowex 1 acetate column. The eluate was mainly glutamic and aspartic acids derived from glutamine and asparagine.

The organic acid fraction was separated by gradient elution from 1 × 11 cm columns of Dowex 1 (formate) using 8 N formic acid in the acid reservoir (2, 6). Sequentially eluted samples of 2 to 3 ml (according to the experiment) were collected, taken to dryness in an air stream at 35 to 40 C and made up to 3 ml with water before titrating or counting.

Succinic, malic, citric, and aconitic acids were identified by co-chromatography with known acids on the column and on paper. The glucose-6-phosphate peak was shown to contain glucose and phosphate after hydrolysis. Fumaric acid was detected in trace amounts on paper chromatograms and spectrophotometric measurements at 260 mμ (7) showed absorption in the area in which known fumaric acid was eluted.

With extracts of large samples of roots, the quantities of various acids were determined by titration in CO_2-free conditions against standard base. Closely similar values for each acid were observed in replicate extracts of large samples (10 g). These values were used as a basis for calculating specific activities of acids in experiments in which acetate-1-C^{14} was added to smaller samples (1 g).

In the experiments with labeled acetate the sample size was 1 g and titration values were small. In order to identify the individual radioactive acids in the samples eluted from the column, authentic acids were routinely added *after* killing and extraction. One-third of each of the samples from the gradient elution was dried on a nickel planchet and counted and the remainder titrated to determine the position

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of the acid eluted. Although the elution pattern was sufficiently reproducible for the radioactive peaks to be assigned to particular acids without the titration data, this procedure was valuable in the shortest experiments when the C^{14} content was low.

In experiments in which total counts in each acid were determined, the samples comprising each radioactive peak were pooled, and combusted by the wet persulphate method (5). The $C^{14}O_2$ was precipitated as $BaC^{14}O_3$, plated on microporous porcelain planchets, and counted.

The evolution of $C^{14}O_2$ from samples treated with acetate-C^{14} was estimated over short intervals of time by use of the apparatus shown in figure 1. The sample of roots was contained in A, a funnel with a sintered diaphragm at its base. This was connected below to a reservoir, B, containing acetate in buffer, and above with a two-way tap, C, narrow bore tubing and sintered glass bubblers to two identical reservoirs of KOH, D, and D'. When tap E was opened, air was blown through the apparatus and acetate was applied to the roots in A. The CO_2 evolved was collected in D for a determined time and then the tap C was turned and CO_2 collected in D'. D was meanwhile replaced by a new tube of KOH.

The CO_2 collected in the KOH was precipitated as $BaCO_3$ by adding $BaCl_2$ and the carbonate spun down and plated. By placing $BaC^{14}O_3$ in A and HCl in B it was determined that $C^{14}O_2$ was swept into D in less than 1 minute and the recovery was satisfactory. Respiratory measurements were also made using standard Warburg procedures and when acetate-C^{14} was present in the flasks $C^{14}O_2$ was collected in the center well in KOH. This was recovered, the carbonate precipitated as $BaCO_3$, plated, and its radioactivity determined.

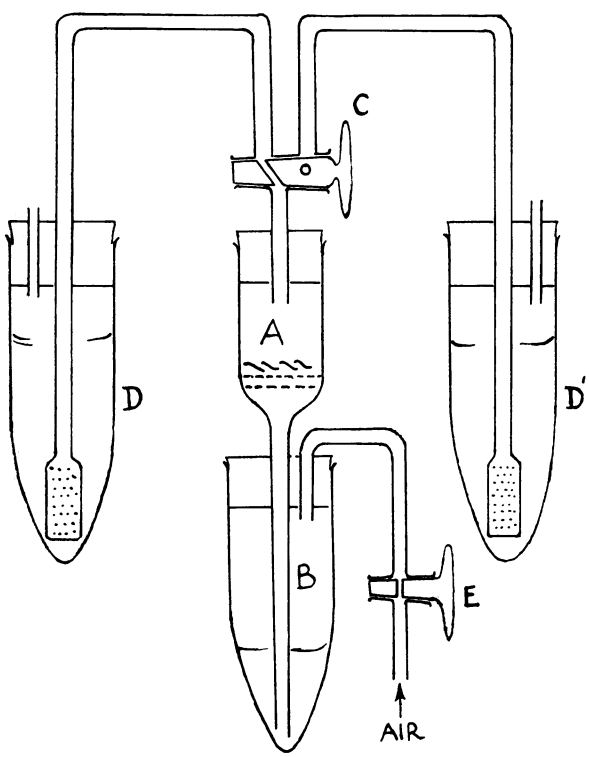

Fig. 1. Apparatus used for collecting respired $C^{14}O_2$ (see Methods).

Results

General Features of Acetate Utilization: Corn roots rapidly absorbed acetate under our experimental conditions. Figure 2 includes a curve showing results obtained when 1 g roots were aerated in 5 ml solution containing 1.25 micromoles acetate. During the first 30 minutes, 70 % of the added acetate was absorbed and at 90 minutes less than 10 % remained in the solution.

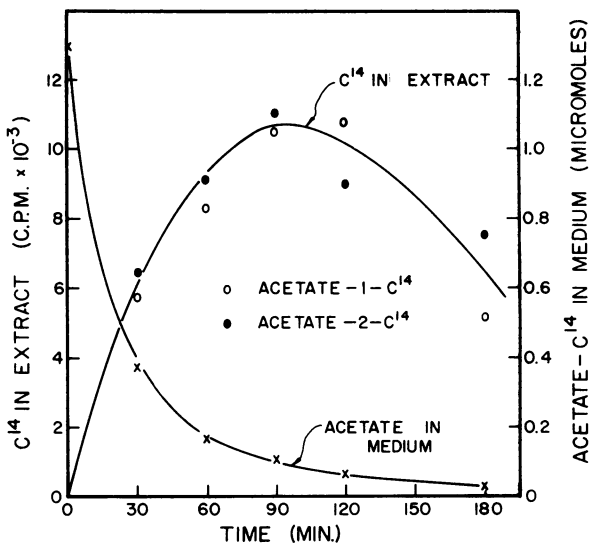

Fig. 2. Uptake of acetate-C^{14} by corn root tips and appearance of C^{14} in the ethanol-water extract.

The absorbed acetate was converted partly to soluble cell constituents, partly, and more especially in the later stages, to insoluble material (cell wall & protein), and partly to CO_2. In addition, it was presumed to be present in the tissue as free acetate and other volatile compounds which were lost during the extraction procedures. Figure 3 shows the progress of incorporation into components other than CO_2 during the first 15 minutes. Clearly, the major destination was water soluble compounds and it is on these and on the respired CO_2 that we concentrated our attention. Acetate carbon continued to accumulate in soluble components until about 90 minutes when the added acetate C^{14} was almost exhausted (fig 2). The subsequent fall in the C^{14} content of the soluble fraction was then due to continued utilization of the labeled components and progressive dilution with endogenous carbon.

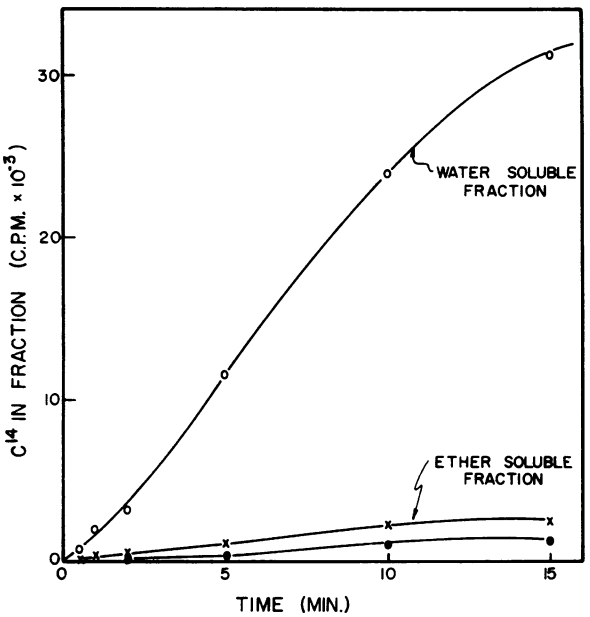

Fig. 3. Incorporation of C^{14} from acetate-2-C^{14} into various components of corn roots. The lowest curve shows the C^{14} in the insoluble residue after ether and water extraction.

Separation of the soluble compounds into basic (amino acid) acid (organic acid) and neutral (sugar) fractions yielded the information shown in figure 4. The curve for percentage incorporation into the acid fraction shows clearly that conversion to non-volatile organic acids was the initial fate of the added acetate. Of the C^{14} in the soluble fraction, 98 % was present in this component at 30 seconds.

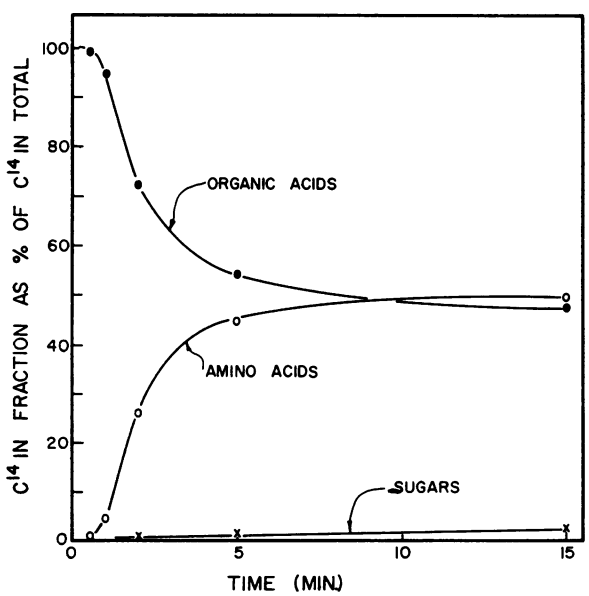

Fig. 4. Progress of incorporation of carbon from acetate-2-C^{14} into components of the water soluble fraction.

The amino acid curve rose sharply from zero while that for the organic acids was declining. This shows that the amino acids acquired C^{14} in a secondary process and that their precursors were components of the organic acid fraction.

Chromatography on paper and radioautographs showed that glutamic acid contained the bulk of the C^{14} in the amino acid fraction. Glutamine was also labeled and aspartic acid and asparagine contained less C^{14}. When the amino acids from roots which had been exposed to acetate-2-C^{14} for 30 minutes was further fractionated (see Methods) glutamic and aspartic acids together were found to contain 80 % of the C^{14}, and their amides a further 12 %. Thus less than 10 % of the C^{14} in the amino acid fraction was present in components other than glutamic and aspartic acids and their amides.

Organic Acids: Figure 5 shows the titratable acidity of successive 2 ml samples obtained by gradient elution of the acid fraction obtained from 9.8 g freshly cut corn roots. The identity of the known peaks is shown and the total amounts of acid in each, in milliequivalents per 10 g. The acids eluted from the column before succinate have not been certainly identified and the second peak has since been resolved on paper into at least seven components including glycolic and lactic acids.

The large peak at tubes 145 to 165 appeared in the same region as authentic fumaric acid. However, almost all of the titratable acid in this region in the eluate from corn roots is inorganic phosphate. Spectrophotometric measurements (see Methods) showed that the amount of fumarate did not exceed 3-4 microequivalents per 10 g of roots. α-Ketoglutarate was detected only in traces in tubes 201 to 205.

These results showed that easily measurable and separable amounts of four of the acids of the TCA cycle were present in samples of 10 g. These amounts do not change appreciably during experimental periods of 180 minutes (MacLennan, Beevers, & Harley, in preparation). The fact that only very small amounts of α-ketoglutarate and fumarate (& isocitrate) were present does not rule out their participation in a cyclic catalytic sequence. We might speculate, rather, that only a fraction of the total pools of those acids present in large amounts was undergoing metabolic turnover.

Incorporation of Acetate Into Organic Acids: The progress of incorporation of label from acetate-2-C^{14} into components of the acid fraction is shown in figure 6A. At the earliest times C^{14} was found only in citric acid and in those acids (not members of the TCA cycle) which are eluted prior to succinate. It is emphasized that in all of the acid samples obtained between 2 and 15 minutes, 20 to 25 % of the C^{14} eluted from the acid column was found in these unidentified components. It was of great interest to find that succinate became appreciably labeled only after 2 minutes and malate even later. At all times the sum of the C^{14} recovered from the fumar-

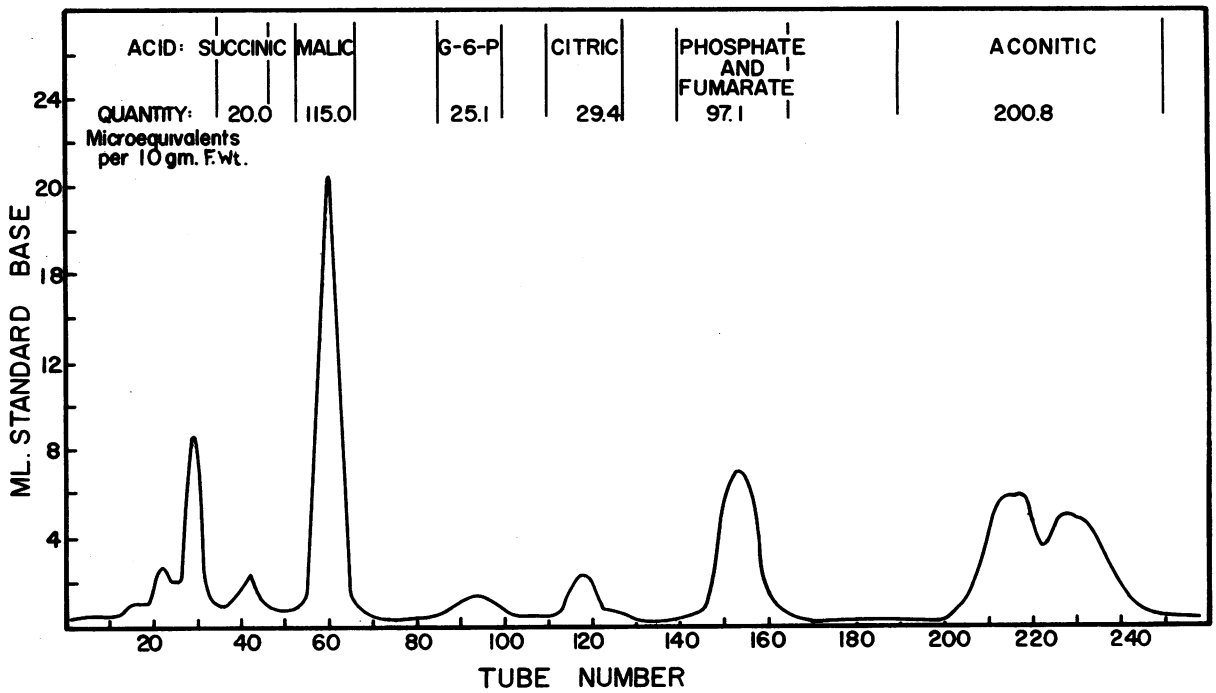

Fig. 5. Titratable acids in corn root tips. The tube numbers refer to samples obtained by gradient elution from Dowex-1 resin. The identified acids and the amounts present are indicated above the respective peaks.

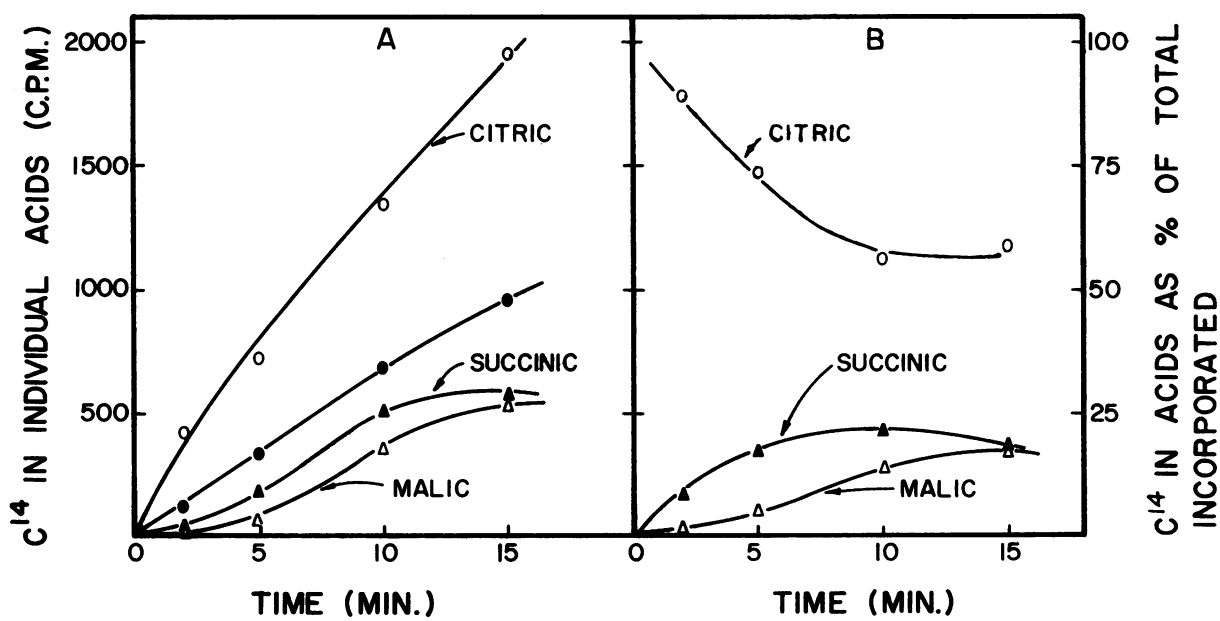

Fig. 6. (A) Progress of C^{14} incorporation from acetate-2-C^{14} into individual acids. The unlabeled curve shows the C^{14} content of the acids (unidentified but not members of the TCA cycle) appearing from the column before succinate. B, C^{14} in citric, malic, and succinic acids as percentage of the total C^{14} in these acids.

ate and α-ketoglutarate areas was less than 1 % of the total recovered. In no experiment with acetate C^{14} did glucose-6-phosphate become appreciably labeled.

The relationships between the TCA cycle acids (citrate, malate, & succinate) are brought out in figure 6B, where the incorporation of C^{14} into each acid is expressed as a percentage of the total in these acids. Citrate was clearly the first of the acids to become labeled and the curve extrapolates to 100 % at time O. The C^{14} content of the succinate pool rose strikingly as the percentage C^{14} in citrate declined and the malate curve showed a clear lag before it too became appreciably labeled. It should be emphasized that the order of appearance of C^{14} in these acids is not a function of the pool sizes. The data on specific activities (cpm per micromole) shown in table I lead to the same conclusion, namely that carbon from acetate appears in the acids of the TCA cycle in the predicted sequence, and there is no evidence for the direct incorporation of acetate by ancillary reactions into malate or succinate. The fact that a significant proportion of the acetate carbon finds its way to other acids, not members of the TCA cycle, does not affect this conclusion. We may note that if the turnover pools of the acids in the TCA cycle comprised only small fractions of the total (measured) acids and these were not in ready equilibrium with the bulk of the individual acid in remote pools, the measured specific activities of the individual acids would rise quickly to constant levels. The fact that the specific activities continued to increase during the 15 minute experimental period (table I) shows at once that an appreciable part of the total pool must be equilibrating rapidly with the turnover pools. In addition to the citrate, succinate, and malate pools, that of aconitate (not measured in this experiment) must also be considered as a point where the acetate carbon traversing the cycle might suffer dilution. And the extensive production of glutamate—presumably in equilibrium with the minute α-ketoglutarate pool—may be regarded as imposing a further potentially large pool into the cycle.

Certainly these considerations would be expected to have repercussions on the timing of the appearance of C^{14} from acetate as $C^{14}O_2$ and on its subsequent specific activity. Insofar as carbon from C-1 of acetate is released from the TCA cycle during the so-called second turn and that from C-2 of acetate begins only after isocitrate in the third turn, the effects of the pools on the release of C-2 would be expected to be more aggravated.

Table I

Specific Activities (cpm/micromole) of Acids of Corn Roots During 15 Minute Exposure to Acetate-2-C^{14}

Time (min)	1	2	5	10	15
Citrate	59	539	921	1,724	2,525
Succinate	13	29	170	509	533
Malate	0	2	10	63	96

Table II

Respiration of Corn Root Tips in 0.02 M Phosphate*

Experiment	Time (min)	O_2 Uptake (microliters/hr/g fr wt)	CO_2 Output (microliters/hr/g fr wt)	R.Q.
I	0–105	720	700	0.98
	105–195	625	507	0.92
II	0–60	722	707	0.98
	60–120	688	—	—

* pH 5.0 at 22 C.

Production of $C^{14}O_2$ From Acetate-C^{14}: The roots used in these experiments respired at a uniform rate for more than one hour, after which the rate fell slowly with time. The RQ was at first very close to unity, after which it, too, fell slightly. The results of separate Warburg experiments are shown in table II.

The release of $C^{14}O_2$ from specifically labeled acetates was determined over short time periods using the apparatus described. Typical results are shown in figure 7. The curves have essentially the same form and consist of a lag phase before detectable $C^{14}O_2$ output, followed by a phase where the total $C^{14}O_2$ release was proportional to some power of time and finally a phase, clearer with acetate-1-C^{14}, where $C^{14}O_2$ release tended to become proportional to time. The lag before detectable CO_2 emission is presumably due to the following components.

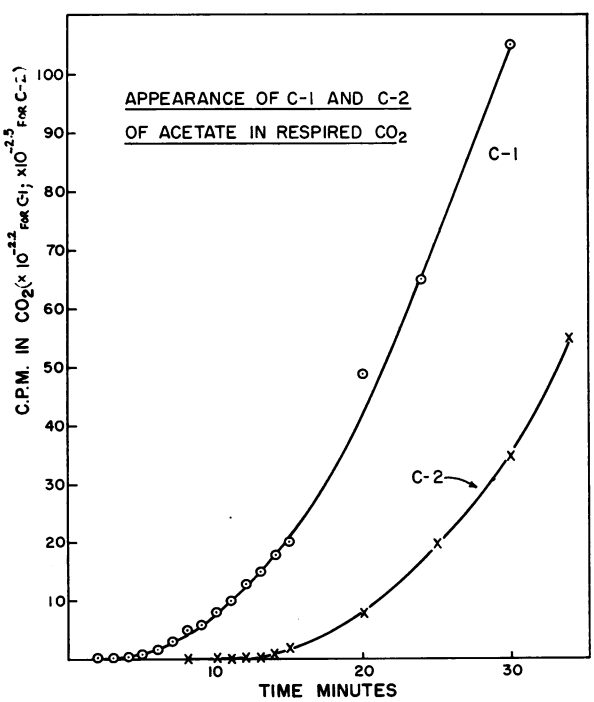

Fig. 7. The initial phase of production of $C^{14}O_2$ from acetate-1-C^{14} and acetate-2-C^{14}. Note that the curves are plotted on different scales.

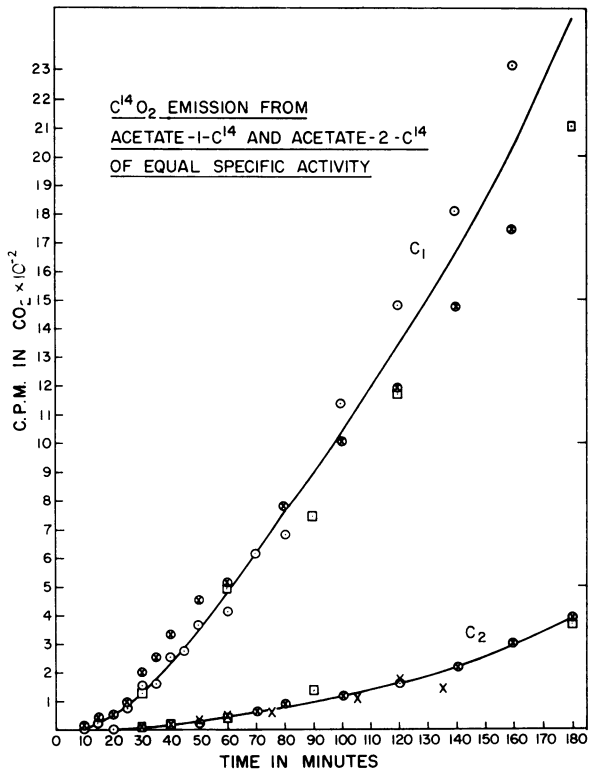

Fig. 8. Progress of $C^{14}O_2$ production from specifically labeled acetates. The results were obtained from four separate experiments with acetate-1-C^{14} and acetate-2-C^{14} of equal specific activities.

I. That dependent on the penetration of acetate-C^{14} to the sites of utilization. This would be the same for acetate-1-C^{14} and acetate-2-C^{14}.

II. The delay of the apparatus in sweeping $C^{14}O_2$ into the KOH. This was shown to be small experimentally and would in any event be equal for the two forms of acetate.

III. The delay in the appearance of C^{14} in the carboxyl groups of malic acid—for from these the CO_2 released in the next turn of the cycle is derived. This delay would be expected to be greater for C-2 than for C-1 (see above). The observed delay periods for the appearance of C^{14} in CO_2 in a series of experiments were about 5 minutes for C-1 and 14 minutes for C-2. It is clear, from a comparison with figure 6 that the appearance of $C^{14}O_2$ from acetate-1-C^{14} occurred only after the malic acid had become labeled, and this at a time when the total C^{14} in the TCA cycle acids, principally citrate, was already quite impressive.

A further important point which emerges from figure 8 is that the time taken for the production of a particular amount of $C^{14}O_2$ from acetate-2-C^{14} is roughly three times that from acetate-1-C^{14} of the same specific activity. Such a figure is expected on theoretical grounds.

Discussion

There is already a considerable amount of evidence from inhibitor and feeding experiments that the TCA cycle is of major importance in plant respiration (for a review see reference 1). The present results provide convincing new evidence for its operation in maize radicle. In particular, the following are weighty arguments in favor: A, the demonstration that citrate is the first acid of the cycle to become labeled from acetate-C^{14} and that the other acids of the TCA cycle acquire C^{14} in the predicted order with time: B, the increases in specific activity of the individual acids with time is related to their position in the cycle: C, $C^{14}O_2$ release from acetate-C^{14} does not occur until after C^{14} has appeared in malate, and D, the amino acids, principally glutamate, acquire C^{14} in secondary reactions related to the cycle. The greater delay in the appearance of $C^{14}O_2$ from acetate-2-C^{14} as compared with acetate-1-C^{14} is likewise in agreement with expectation. On theoretical grounds it would be expected that all the carbons of the acids would become labeled with acetate-2-C^{14} but only the carboxyl carbons with acetate-1-C^{14}. Hence, it would be expected that the CO_2 from the former would take between two and three times as long as the latter to become equally labeled and this was observed.

The lag in appearance of $C^{14}O_2$, the delay in its attainment of a constant specific activity, and the relatively slow increase in specific activity of malate during acetate-1-C^{14} utilization are here interpreted as indicating partial equilibration of the main pools of cycle acids with the operative metabolic pools, rather than as evidence for a sluggish operation of the TCA cycle (8). Glutamate, which accounted for almost half of the C^{14} in the soluble fraction after 15 minutes, was presumably in equilibrium with α-ketoglutarate and this would impose a further point of dilution of acetate carbon traversing the cycle.

Information on the question of what portion of the total pools of individual acids does undergo metabolic turnover during acetate utilization can be obtained by comparing specific activities of CO_2 and acids from tissues respiring acetate-1-C^{14}. Experiments of this kind with a variety of tissues will be described in a subsequent paper (MacLennan, Beevers, & Harley, in preparation).

Summary

Acetate-1-C^{14} or acetate-2-C^{14} was supplied to root tips excised from 2-day-old corn seedlings. The progress of $C^{14}O_2$ evolution was followed at intervals of 1 minute and the progressive contribution of C^{14} from the acetate to various metabolites was determined.

After 30 seconds 98 % of the alcohol soluble C^{14} was present in organic acids (other than acetate) and the percentage declined to about 50 at 30 minutes as the percentage of C^{14} in amino acids (prin-

cipally glutamic & aspartic acids & their amides) rose from 0 to 50 during the same periods. No incorporation into the neutral (sugar) faction occurred. Kinetic experiments showed clearly that citrate was the first of the acids of the tricarboxylic acid (TCA) cycle to become labeled; C^{14} appeared subsequently in succinate and later in malate. Parallel experiments showed that $C^{14}O_2$ began to appear from roots provided with acetate-1-C^{14} only after a lag corresponding to the time for the production of malate-C^{14}, and that from roots provided with acetate-2-C^{14} showed a considerably greater lag. The results provide new and definitive evidence for the view that the TCA cycle is the all important pathway in the oxidation of acetate to CO_2 in this tissue.

Acknowledgment

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