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Pyruvate Utilization in Castor-Bean Endosperm and Other Tissues

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Although pyruvate occupies a pivotal position in metabolism, its utilization by intact higher plant tissues has not been intensively studied. There is, however, ample evidence for its production by soluble glycolytic systems, for its oxidation by mitochondria and for its participation in other reactions such as transamination, reduction and decarboxylation by isolated enzyme systems. The fact that it is usually found in only small amounts in plant tissues attests to the activity of the various consuming reactions *in vivo*; if for any reason the internal pyruvate concentration increases to a level above that which can be accommodated by the oxidation systems, ethanol and carbon dioxide or, occasionally, lactate accumulate. It is for this reason that when substrate levels of pyruvate are

supplied to plant tissues the usual response is a large increase in the respiratory quotient, although stimulations in uptake of oxygen have been observed (e.g. Laties, 1949a, b).

The availability of labelled pyruvate has made possible experiments in which its metabolism can be followed under conditions where gross gas exchanges are unaffected, and specifically where internal levels are not increased to the point where ethanol is formed.

Pyruvate samples specifically labelled with ^{14}C in individual carbon atoms were used in the present experiments. The fates of the carbon atoms were determined by measuring the respiratory release of $^{14}\text{CO}_2$ and analysing samples of tissue at suitable intervals. In most of the experiments virtually all of the added pyruvate was accounted for as carbon dioxide, organic acids, amino acids or carbohydrates.

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A major objective of the experiments was to measure pyruvate utilization in the castor-bean endosperm, which converts fat into carbohydrate, conceivably by way of pyruvate. Pyruvate metabolism in this specialized tissue was compared with that which occurs in three other types of material. These were (a) petiole of fully mature castor-bean leaf, which has ceased active growth and in which synthetic reactions are probably at a minimum, (b) mesocotyls from young corn seedlings, which grew appreciably in length during the experiments, and (c) leaf of *Bryophyllum calycinum*, which is known to be especially rich in organic acids.

MATERIALS AND METHODS

Plant materials. (a) Castor beans were germinated as described previously (Beevers & Walker, 1956). Endosperms were removed from 5-day-old seedlings and sections about 0.5 mm. thick were cut by hand. They were washed in two changes of water before use.

(b) Sections from the petioles of mature castor-bean leaves were prepared in the same way.

(c) Corn (maize) seeds of variety Wf9/38-11 (Agricultural Alumni Seed Improvement Association, Lafayette, Ind., U.S.A.) were grown in vermiculite in the dark at 29°. After 4-5 days, when the mesocotyls were 2-3 cm. long, they were removed, cut into 1 cm. lengths and rinsed in water before use.

(d) Plants of *Bryophyllum calycinum* were placed in bright light for 24 hr. before use. Disks (1 cm.) were then removed from the laminae with a sharp cork borer.

Equal samples of the individual materials were weighed out and placed in Warburg vessels containing 2 ml. of 0.05 M-potassium phosphate, pH 5.0. The centre wells contained 0.2 ml. of carbonate-free 20% sodium hydroxide. The temperature for all experiments was 25°.

Analytical methods. At the end of the chosen time interval, the alkali was removed and boiling 80% ethanol was added to the flasks. Extraction was completed with successive 25 ml. portions of 80% ethanol or by continuous treatment with approximately 180 ml. of 80% ethanol in a Soxhlet apparatus. The extracts were evaporated under reduced pressure to a volume of 2-5 ml. and made up to 25 or 50 ml. with water. The colouring matter was removed from the leaf and petiole extracts with a few millilitres of hexane or benzene. Fatty materials were removed from the endosperm and mesocotyl extracts by shaking briefly with a small quantity of ether.

The extracts were then passed through columns containing 3 g. of Dowex 50 ($\times 8$) cation-exchange resin in the hydrogen form (regenerated with 2N-hydrochloric acid). The adsorbed amino acids were subsequently eluted with 100 ml. of N-aq. ammonia soln. The extracts were next passed through a column containing 3 g. of Dowex 1 ($\times 10$) anion-exchange resin in the acetate form and the organic acids were subsequently eluted with 50 ml. of 6N-formic acid. The final solutions, containing neutral materials (mainly the sugar fraction), and the eluted acids and bases, were evaporated under reduced pressure almost to dryness and then made up to a known volume (10 or 25 ml.) in water. The components of the various fractions

were separated chromatographically on Whatman no. 1 paper by means of the following solvents.

Organic acids: the upper phase of water-butanol-formic acid (5:4:1, by vol.).

Amino acids: phenol-water (4:1, v/v), plus a few drops of 1% ethylenediaminetetra-acetic acid, followed by the organic phase of water-butanol-formic acid (5:4:1, by vol.) in the second direction.

Sugars: the upper phase of ethyl acetate-water-acetic acid (3:2:1, by vol.).

The ethanol-insoluble residues were dried and weighed. A 30 mg. sample was placed in 2-5 ml. of 72% sulphuric acid and the vessel was shaken to aid solution. Corn mesocotyl tissue did not dissolve completely during this process, but the balance sheet indicated that the fibrous material which remained did not contain appreciable amounts of carbon from the added pyruvate. The solution was made to a volume of 180 ml. with water and boiled under reflux for 18 hr. It was then neutralized with solid barium carbonate. The slurry was filtered under vacuum through Whatman no. 42 paper and the precipitate was thoroughly washed with hot water.

The filtrate and washings were evaporated under reduced pressure, filtered, the filtrate was made up to 25 ml. with water and then separated into organic acid, amino acid and sugar fractions by the methods described above.

Determination of radioactivity. Specifically labelled sodium pyruvate (The Radiochemical Centre, Amersham, Bucks.) was diluted with unlabelled sodium pyruvate and made up with dilute phosphoric acid so that 0.1 ml. of solution contained 1 μ mole of pyruvate, pH 5.0. Stock solutions were maintained at -15°. The radioactivities of the solutions were determined by wet combustion in a Stutz & Burris (1951) apparatus. Losses of carbon were found to occur when pyruvate solutions were evaporated to dryness. The solutions for combustion were therefore frozen in the combustion flasks and the complete oxidation mixture was added from the side arm. The CO₂ was converted into barium carbonate, which was filtered off on to sintered-porcelain disks and counted in a windowless gas-flow counter. All figures are corrected for background and self-absorption.

[1-¹⁴C]-, [2-¹⁴C]- or [3-¹⁴C]-Pyruvate (2 μ moles) containing a known amount of radioactivity (50 000-60 000 counts/min.) were added as required to Warburg flasks. The ¹⁴C content of the respired CO₂ was determined by transferring the sodium hydroxide from the centre wells of the Warburg flasks, with washings, into 8 ml. of a 10% barium chloride-1% ammonium chloride solution. The precipitated carbonate was plated and counted as above. The radioactivity of the various fractions obtained from the columns was usually determined by evaporating a measured fraction to dryness on a nickel-plated planchet (after adding a few drops of 0.1N-sulphuric acid to ensure complete removal of any residual pyruvate) and then counting directly. In order to make these values directly comparable with the figures for total combustion and respired CO₂, so that balance sheets could be prepared, it was necessary to convert them into the equivalent 'barium carbonate counts'. A factor was therefore empirically determined by measuring the radioactivity of comparable samples which had been dried on metal planchets or converted into barium carbonate before counting. This factor, metal-planchet count/2.8 = barium carbonate count, was used

throughout, although it is now known (T. apRees & H. Beevers, unpublished work) that slightly different factors should be used in converting values from the different fractions. The error involved is apparently small so that the results are not substantially altered; in some experiments the radioactivities of all fractions were determined by combustion to CO_2 and the results confirmed others obtained by the method usually employed. The ^{14}C content of the insoluble residues was always determined by total combustion of a weighed amount. Radioautographs were prepared from chromatograms by the use of Kodak No-Screen X-Ray film. The distribution of activity on the chromatograms was also estimated either by direct counting on the paper, or by counting an evaporated eluate of a radioactive area cut from the paper.

Degradation of glucose was carried out by the *Leuconostoc mesenteroides* procedure of Gunsalus & Gibbs (1952).

RESULTS

In preliminary experiments it was shown that (a) the spontaneous breakdown of the pyruvate solutions under the experimental conditions was negligible; the maximum amount of $^{14}\text{CO}_2$ released in a 24 hr. run was from $[1-^{14}\text{C}]$ pyruvate and amounted to 0.35 % of that added; (b) for each of the tissues, the amount of pyruvate which was added (not more than $2\text{ }\mu\text{moles}/2\text{ ml.}$) was well below the level which brought about increase in the rate of CO_2 output.

Respiration of specifically labelled pyruvate by the four tissues. The aim in these experiments was to follow the release of the individual carbon atoms of pyruvate into the respired CO_2 and to relate these figures to the amount of carbon supplied. It should be emphasized that each of three equal samples of tissues received equal amounts of pyruvate and respired at the same rates throughout. In each experiment the treatments were in duplicate; the agreement between these was good (Fig. 2b). In addition, the various experiments have been repeated at least once. The results which are presented are representative ones.

The respiration results from experiments with castor-bean petiole, *Bryophyllum* leaf and castor-bean endosperm are collected in Figs. 1 and 2. In a similar experiment with corn mesocotyl, 90 % of C-1 of pyruvate had appeared in the respired CO_2 after 20 hr., when less than 20 % of C-3 had been converted into CO_2 . The first point to be emphasized is that in every case it is the C-1 of pyruvate which appears first and in greatest abundance in the respired CO_2 . By contrast, at each state the respired CO_2 is considerably less rich in C-2 of pyruvate and C-3 makes a yet smaller contribution. In three of the tissues, the amount of C-1 finally recovered in the CO_2 was close to 100 % of that added, showing that all of the added pyruvate has undergone at least one reaction. At any point on the time scale, the percentage release of C-1 gives

a minimal figure for the amount of pyruvate that has undergone conversion into a two-carbon-atom unit (presumably acetyl coenzyme A) from C-2 and C-3. The extent to which C-2 and C-3 are released as CO_2 , and the order of their appearance, provide information about the subsequent utilization of the two-carbon-atom unit.

In the castor-bean petiole (Fig. 1) the release of C-2 does not lag far behind that of C-1, and it, too, reaches 100 % by the end of the experiment, whereas in *Bryophyllum* leaf (Fig. 2a) and endo-

Fig. 1. Progress of $^{14}\text{CO}_2$ production from equal portions ($1\text{ }\mu\text{mole}$) of specifically labelled pyruvate. ●, $[1-^{14}\text{C}]$; ○, $[2-^{14}\text{C}]$; △, $[3-^{14}\text{C}]$. Material: 500 mg. samples of castor-bean petiole.

Fig. 2. Progress of $^{14}\text{CO}_2$ production from equal portions ($2\text{ }\mu\text{moles}$) of specifically labelled pyruvate. ●, $[1-^{14}\text{C}]$; ○, $[2-^{14}\text{C}]$; △, $[3-^{14}\text{C}]$. Material: (a) 750 mg. samples of *Bryophyllum* leaf; (b) 500 mg. samples of castor-bean endosperm.

sperm (Fig. 2b) the release of C-2 lags strikingly behind that of C-1. In endosperm, over 60% of C-2 had appeared as CO₂ by the end of the experiment. From the leaf, the final yield from C-2 was less than one-third of that from C-1.

The disparity between the appearance of C-1 and C-3 (methyl carbon atom of pyruvate) is even

Table 1. *Utilization of [3-¹⁴C]pyruvate by castor-bean petiole*

Tissue (500 mg.) was incubated with 1 μ mole of pyruvate.

Fraction	Percentage of added radioactivity incorporated	
	In 6 hr.	In 28 hr.
CO ₂	11.6	73.0
Amino acids*	11.8	5.0
Organic acids†	13.6	3.7
Sugars	0	0.4
Ethanol-insoluble residue	1	5.0

* Comprised: 79% of glutamic acid, 7% of aspartic acid, 8% of γ -aminobutyric acid, 6% of alanine and trace of glutamine.

† Malate only.

Table 2. *Utilization of [3-¹⁴C]pyruvate by corn mesocotyl*

Tissue (1 g.) was incubated with 2 μ moles of [3-¹⁴C]-pyruvate.

Fraction	Percentage of added radioactivity incorporated	
	In 10 hr.	In 28 hr.
CO ₂	11.2	28.1
Amino acids*	37.8	40.7
Organic acids†	24.8	9.2
Sugars	1.1	1.3
Ethanol-insoluble residue‡	11.6	17.1

* Alanine > glutamic acid = aspartic acid > asparagine = γ -aminobutyric acid.

† Malate > citrate or isocitrate.

‡ 28 hr. hydrolysate: of the amount of radioactivity recovered, the fractions contained: amino acids 86%, organic acids 11%, sugars 3%.

Table 3. *Utilization of [3-¹⁴C]pyruvate by Bryophyllum leaf disks*

Tissue (750 mg.) was incubated with 2 μ moles of [3-¹⁴C]-pyruvate for 24 hr.

Fraction	Percentage of added radioactivity incorporated
CO ₂	6.7
Amino acids	9.3
Organic acids*	35.0
Sugars†	9.7

* Comprised: 37% of malate, 56% of citrate and isocitrate, 2% of fumarate and 5% of succinate.

† Comprised: 46% of sucrose, 27% of glucose and 27% of fructose.

greater. However, in the petiole a steady release of C-3 does occur and 70% had been converted into CO₂ by the end of the experiment. Thus of the total pyruvate carbon supplied to this tissue more than 90% was recovered as CO₂. In each of the other three tissues, however, the final yield of C-3 in the respired CO₂ was less than one-third of that which appeared from C-1. Sizeable portions of the products of pyruvate decarboxylation were retained even after 24 hr. in these tissues.

Analysis of tissues which received [3-¹⁴C]pyruvate.

In attempting to explain this retention, the fate of C-3 is particularly interesting, and a series of experiments was carried out in which the disposition of ¹⁴C from [3-¹⁴C]pyruvate was determined.

(a) Petiole (Table 1). At the 6 hr. stage, 38% of the added pyruvate had been metabolized, and of this amount about two-thirds was present as organic and amino acids; less than one-third had appeared as CO₂. After 28 hr., however, when 87% of the pyruvate had been used, less than 10% was in the organic and amino acids; the great bulk of the added C-3 had by then appeared as CO₂. There had been a small incorporation of C-3 into ethanol-insoluble material and virtually none into the sugar portion.

(b) Corn mesocotyl (Table 2). At the earlier stage, incorporation into organic and particularly amino acids is striking; more than 40% of the C-3 which had undergone reaction was found in a variety of amino acids. By 28 hr., when all of the pyruvate had been used, 10% of C-3 was in the organic acids and the amino acid component was still high. Another striking difference from the utilization by other tissues is shown by the incorporation into the ethanol-insoluble portion. At 10 hr. almost 12% of the added C-3 had appeared in this fraction and this increased during the subsequent 18 hr. The high content of ¹⁴C in the amino acids in the hydrolysate of this portion should be especially noted.

(c) *Bryophyllum* leaf (Table 3). The striking feature here is that, although pyruvate utilization was far from complete, a greater proportion of the added C-3 was present in the organic acid fraction, even at the end of 24 hr., than was ever found in any of the other tissues. Almost 60% of the C-3 recovered from the tissue was in the form of citric (isocitric) acid and malic acid. In contrast with the findings for mesocotyl and petiole, a significant amount of radioactivity was found in the sugar portion. Although the flasks were darkened during the experiment, the possibility cannot be excluded that some of this arose during the unavoidable exposure to light during manipulations for sampling the CO₂.

(d) Castor-bean endosperm (Table 4). The very low yield of CO₂ from C-3 at a time when virtually

Table 4. Utilization of [3-¹⁴C]pyruvate by castor-bean endosperm

Endosperm slices (500 mg.) were incubated with 2 μ -moles of pyruvate for 10 hr.

Fraction	Percentage of added radioactivity incorporated
CO ₂	5.6
Amino acids*	35.3
Organic acids†	13.1
Sugars‡	31.1
Ethanol-insoluble residue	5.7

* Comprised: 98% of alanine, 2% of glutamate and a trace of glutamine.

† Principally malate, some citrate and succinate.

‡ Sucrose, traces of glucose and fructose.

Fig. 3. Progress of incorporation of ¹⁴C from 2 μ moles of specifically labelled pyruvate into CO₂, amino acids, organic acids and sugar. ●, [1-¹⁴C]Pyruvate; ○, [2-¹⁴C]pyruvate; △, [3-¹⁴C]pyruvate. Material: 500 mg. samples of castor-bean endosperm tissue.

Table 5. Incorporation of pyruvate carbon atoms into endosperm constituents

Samples (500 mg.) of tissue were incubated with 2 μ moles of the particular labelled pyruvate for 4 hr.

Labelled pyruvate	Percentage of added radioactivity in component		
	[1- ¹⁴ C]	[2- ¹⁴ C]	[3- ¹⁴ C]
CO ₂	49.3	23.8	2.9
Sugars	7.5	15.1	22.8
Organic acids	1.1	8.0	8.5
Amino acids	33.2	29.5	33.2

all of the pyruvate had been used should be especially noted. The high content of C-3 in the amino acid portion is dealt with separately below. The biggest contrast with the rest of the tissues is seen in the sugar fraction, which acquired more than 30% of the added C-3 of pyruvate.

Further experiments with endosperm tissue. Each of the labelled pyruvates was supplied to 500 mg. of endosperm tissue and, at intervals during a 4 hr. period, flasks were removed and the incorporation of radioactivity into the various fractions was determined. Fig. 3 shows the results of these determinations; the incorporation of ¹⁴C from the three pyruvates into each fraction are grouped together. The figures for percentage incorporation into the various fractions at the end of 4 hr. are given in Table 5.

(a) CO₂ (cf. also Fig. 2b). The very prompt appearance of C-1 is striking; the release of C-2 occurs at a much lower rate, with a detectable lag, and that of C-3 is very slow indeed. It seems that shortly after adding the pyruvate, there would be a time when although C-1 release had begun there would be as yet no carbon atoms from C-2 and C-3 in the CO₂.

(b) Amino acids. The values in Fig. 3 show that from each of the labelled pyruvates there was a rapid appearance of ¹⁴C in the amino acids. The striking feature is that the rates of this incorporation were virtually identical from C-1, C-2 and C-3; the single curve accommodates all of the points. The amino acids were separated chromatographically and the following were revealed by ninhydrin: phenylalanine, isoleucine, valine, tryptophan, γ -aminobutyrate, proline, alanine, threonine, glutamate, glutamine, aspartate, asparagine, serine and glycine. It was found that, of these, alanine was the only radioactive component when [1-¹⁴C]-pyruvate was supplied, and contained all but a minute fraction of the total radioactivity in the amino acids when [2-¹⁴C]pyruvate and [3-¹⁴C]-pyruvate were the substrates. The virtual absence of ¹⁴C in the amino acids related to α -oxoglutarate is noteworthy. Since alanine can arise from pyruvate without cleavage, the equal incorporation of each carbon atom from pyruvate into the amino acid fraction is thus accounted for. For incorporation into amino acids, we can now write incorporation into alanine.

It will be recalled that the conversion of C-1 of pyruvate into CO₂ is virtually complete after 16 hr. This would presumably require that any C-1 which is present at an earlier time in any other component is subsequently converted into CO₂. The results in Table 6 show that for alanine, the only component which gains more than a few per cent of radioactivity in the first 5 hr., this is indeed so; at the end of the experiment alanine contained only 2% of the supplied C-1.

(b) Organic acids. The curves in Fig. 3 show that at no time does the organic acid fraction contain more than a few per cent of the carbon atoms from pyruvate. However, significantly more of C-2 and C-3 than C-1 enter the organic acid fraction and the

forms of the three curves are quite different from each other. At 30 min., when a sizeable amount of C-1 had appeared in CO_2 and virtually none is present in the organic acids, precisely the reverse is true for C-3. Both CO_2 and organic acids have appeared from C-2 by this time. This is strong evidence for the view that the two-carbon-atom fragments left after removal of C-1 enter the organic acid fraction, from which C-2 is also subsequently lost. A further consideration here is that, whereas from C-1 malate was the only organic acid to become labelled (see Discussion) citrate (isocitrate) and succinate were also radioactive when $[2\text{-}^{14}\text{C}]\text{pyruvate}$ and $[3\text{-}^{14}\text{C}]\text{pyruvate}$ were supplied.

(c) Sugars. Particular interest attaches to the large-scale conversion of pyruvate carbon atoms into sugar (principally sucrose). C-3 is a more effective precursor of sucrose carbon atoms than C-2, and C-1 is the least efficient. The incorporation of C-3 continues at a steady rate only for a few more hours than the period shown in Fig. 3; the maximum incorporation which was observed was 35% of that added in an 8 hr. experiment. By this time most of the added pyruvate had been utilized, and the subsequent changes are therefore less important. During this time, however, some of the radioactivity previously incorporated into the sucrose is lost from this fraction. The complete recovery of C-1 as CO_2 at the end of the longer experiments illustrates this point and in a 24 hr. experiment with $[3\text{-}^{14}\text{C}]\text{pyruvate}$ it was found that only 4–5% of the supplied ^{14}C was still present in the soluble sugars. However, at the same time there was a noticeable increase in the amount of radioactivity in the ethanol-insoluble fraction;

19.2% of the added C-3 was recovered in one experiment in this form, and at least 27% of this was in polysaccharide.

Sucrose samples isolated from 4 hr. experiment with $[1\text{-}^{14}\text{C}]\text{pyruvate}$ and $[3\text{-}^{14}\text{C}]\text{pyruvate}$ were hydrolysed and the glucose moieties separated chromatographically. Degradation of the glucose samples yielded the results shown in Table 7. Even after 4 hr. most of the C-1 of the pyruvate is confined to C-3 and C-4 of the hexose, and, by contrast, C-3 of pyruvate does not appear in these positions to any extent.

DISCUSSION

The results show that pyruvate is readily utilized by all of the plant tissues in ways other than by simple decarboxylation. The following features of the results with petiole, leaf and mesocotyl are in accord with the view that oxidative decarboxylation occurs, followed by entry of the resultant two-carbon-atom unit into the tricarboxylic acid cycle: (a) the early preponderance of C-1 of the pyruvate in the respired carbon dioxide; (b) the appearance of C-3 (and C-2) in organic acids of the tricarboxylic acid cycle and related amino acids; (c) subsequent appearance of C-2 and C-3 of pyruvate in order, in the respired carbon dioxide.

Several factors may be expected to contribute to the delay in the appearance of C-2 and C-3 as carbon dioxide. The first of these is the time required for the two-carbon-atom units to undergo the necessary circuits of the cycle which lead to release of C-2 and C-3 of pyruvate. Secondly, if some of the intervening pool sizes are large there will be further delays in the complete appearance, as carbon dioxide, of the entering two-carbon-atom unit. Thirdly, to the extent that drainage of intermediates of the tricarboxylic acid cycle to form amino acids and hence proteins occurs, a smaller or larger fraction of the two-carbon-atom unit may never appear in the carbon dioxide even during a prolonged experiment. Any other reaction which utilizes either the two-carbon-atom unit itself, or a product which results from its introduction to a metabolic sequence, would lead to retention of C-2 and C-3 of pyruvate. Transfer to pools in which turnover is not occurring should also be included as a likely possibility in some tissues.

The analyses in Tables 1–4 show that the tissues which were used provide examples of some of the above possibilities. In only one tissue, the petiole, was the pyruvate oxidized fairly completely during the experimental period. All of C-1 and most of C-2 of the pyruvate had appeared as carbon dioxide and the yield from C-3 was 70% and still rising. This tissue was one in which growth had been completed and was not rich in organic acids. Reactions

Table 6. *Temporary appearance of C-1 of pyruvate in alanine*

Samples of endosperm slices (500 mg.) were incubated with 2 μmoles of $[1\text{-}^{14}\text{C}]\text{pyruvate}$.

	Time after adding pyruvate (hr.)	
	5	24
Percentage of added radioactivity in CO_2	58	99
Percentage of added radioactivity in alanine	30	2

Table 7. *Distribution of ^{14}C from specifically labelled pyruvate in sugar produced by endosperm slices*

Substrate	Percentage of ^{14}C in carbons of glucose	
	C-1, C-2, C-5, C-6	C-3, C-4
$[1\text{-}^{14}\text{C}]\text{Pyruvate}$	25	75
$[3\text{-}^{14}\text{C}]\text{Pyruvate}$	91	9

leading to permanent retention of parts of the two-carbon-atom unit were at a minimum.

The *Bryophyllum* leaf (Fig. 2a, Table 3), although mature, is known to have a high content of acids. Although there is evidence that much of this may not be in 'metabolic' pools (Stutz & Burris, 1951), it is clear from Table 3 that the major fate of C-3 of pyruvate is in fact incorporation into organic acids, principally malic acid and citric acid, and that this incorporation slowed down, by dilution, the conversion of this carbon into carbon dioxide.

Production of amino acids is a major drain on the two-carbon-atom unit in mesocotyl tissue, which was in an actively elongating condition during the experiments. There is evidence for the passage of the two-carbon-atom unit through pools of organic acids into amino acids; the amount of C-3 from pyruvate in the organic acid showed an early rise and subsequently declined whilst the ^{14}C in the amino acid fraction was still rising. In addition to the considerable incorporation of C-3 into amino acids, there was also a deposition into ethanol-insoluble material yielding radioactive amino acids on hydrolysis. This active production and utilization of amino acids accounted for the observed results on carbon dioxide released; the respired carbon dioxide was derived largely from C-1 of the pyruvate.

The results from endosperm tissue show firstly that it can convert pyruvate into sucrose with about 20% efficiency. However, not all of the pyruvate carbon atoms contribute equally to this synthesis; the incorporation of C-1, C-2 and C-3 respectively is roughly in the ratio 1:2:3 (Table 5). It is therefore quite clear that production of hexose by a complete reversal of glycolysis is not the major route of conversion.

The rapid production of carbon dioxide from C-1, and the sharp increase to a steady level in organic acids from C-3 preceding the sustained incorporation of C-3 into the sugar (Fig. 3), lead to the conclusion that it is the two-carbon-atom unit derived from C-2 and C-3 of pyruvate which is the important precursor of sucrose. It has already been established (Beevers, 1957) that acetate itself is rapidly converted into sucrose in this tissue and the utilization of C-2 and C-3 of pyruvate resembles closely that of C-1 and C-2 of acetate. It will be recalled that acetate utilization by this tissue most probably occurs through the reactions of the glyoxylate cycle (Kornberg & Beevers, 1957), and a similar utilization of an acetyl unit derived from pyruvate would go far towards accounting for the results observed here.

The relatively small but highly significant incorporation of C-1 of pyruvate into sucrose requires separate consideration. Clearly, if the only way in which pyruvate could contribute to sucrose syn-

thesis was by producing an acetyl unit, C-1 of the pyruvate would not be expected to appear in sucrose. It is therefore possible that, to the extent that C-1 is incorporated, the pyruvate skeleton is converted intact into sugar, and only the excess of C-2 and C-3 which is found in sucrose would have to be accounted for by the glyoxylate cycle. However, this view is rendered unlikely by the finding (Stiller, Neal & Beevers, 1958) that during conversion of fat into carbohydrate in this tissue there is a remarkably active fixation of carbon dioxide, comparable with that described by Bradbeer (1958) in sunflower seedlings. The carbon dioxide which is fixed appears initially in the carboxyl groups of malate, and in the hexose unit it is confined almost entirely to C-3 and C-4. It is therefore to be expected that, in the $[1-^{14}\text{C}]$ pyruvate experiments, in which a rapid release of $^{14}\text{CO}_2$ occurred, some of this $^{14}\text{CO}_2$ would be incorporated by reactions unrelated to the actual utilization of pyruvate itself. Support for this contention comes from an experiment in which the incorporation of radioactivity from $[1-^{14}\text{C}]$ pyruvate into sucrose was increased when sodium hydroxide was not included in the centre well of the Warburg vessel. This view of the incorporation of C-1 would minimize still further the possibility of direct pyruvate incorporation, though such a conversion does occur, at what by comparison are feeble rates, in diaphragm tissue (Hiatt, Goldstein, Lareau & Horecker, 1958). Certainly the present results rule out pyruvate itself as an intermediate in the synthesis of carbohydrate from acetate, and in ascending from the C-4 acid the entry into the glycolytic sequence must indeed be at the phosphoenol-pyruvate level (Krebs, 1954). It should be emphasized that the tissue slices used in these experiments were actively converting fat into carbohydrate, and the 'reducing power' required in the formation of hexose comes presumably from the oxidation of the fatty acids.

James & Slater (1959) have described some experiments on pyruvate utilization in barley roots and apple tissue. In neither of these tissues was pyruvate converted into sugars. We can conclude that 'oxidative re-synthesis' of hexose from pyruvate is not a general feature of plant respiration.

SUMMARY

1. Micromolar amounts of pyruvate specifically labelled with ^{14}C in the 1, 2, and 3 positions were supplied to four different plant tissues (castor-bean petiole, corn mesocotyl, *Bryophyllum* leaf and castor-bean endosperm) in order to determine the extent of utilization and the fates of individual carbons.

2. The release of C-1 of pyruvate in the respired carbon dioxide occurred promptly and regularly

from all four tissues and from all except *Bryophyllum* the eventual recovery was virtually quantitative.

3. Carbon atoms 2 and 3 appeared subsequently in sequence in the respired carbon dioxide, but in such a way as to indicate that the two-carbon-atom unit was undergoing very different fates in the four tissues.

4. In castor-bean petiole, a fully mature tissue, all of C-2 and 70% of C-3 was recovered as carbon dioxide.

5. In corn mesocotyl, which grew noticeably during the experiment, the bulk of C-2 and C-3 were retained in the tissue as amino acids and protein.

6. In *Bryophyllum* leaf, a tissue rich in acids, more than half of C-3 was retained in acids of the tricarboxylic acid cycle.

7. In castor-bean endosperm, where carbohydrates were being produced from fat, an active conversion of pyruvate into sucrose occurred. Results are provided to support the conclusion that pyruvate was acting as a sucrose precursor only in so far as it was converted into acetyl units which entered the glyoxylate cycle.

8. Direct reversal of glycolysis from pyruvate plays, at best, a minor role in the production of

sucrose from pyruvate; the relatively small incorporation of C-1 of pyruvate is ascribed to the (dark) fixation of carbon dioxide into sucrose by endosperm slices.

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