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Title RADIOTRACER STUDIES OF CARBOHYDRATE CATABOLISM IN TOMATO FRUIT

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Glucose-1, -2 and -6-\textsuperscript{14}C substrates were incorporated into individual tomato fruits in order to study the catabolic fate of substrate glucose, especially with regard to the possible occurrence of triose recombination, as well as to investigate the pathways leading to the biosynthesis of C\textsubscript{4} acids in the fruit. After metabolizing the administered glucose-\textsuperscript{14}C substrates for a period of 12 hours, the fruits were individually processed for the separation of organic acids and carbohydrates. Subsequently, both glucose and malic acid isolated from the tomatoes were degraded in order to determine their isotopic distribution pattern.

Data obtained from the carbohydrate fractions reflected the presence of a large endogenous pool of carbohydrates in the fruit, and a comparison of the specific activities of fruit glucose and fructose indicated that glucose-6-phosphate and fructose-6-phosphate probably are not in isotopic equilibrium in this organism. The isotopic distribution pattern in fruit glucose clearly established the occurrence of triose recombination via both the
Embden-Meyerhof-Parnas (EMP) and the pentose phosphate (PP) pathways. A theoretical analysis of the impact of triose recombination on the equations derived for the estimation of pathway participation indicated that recombination processes do not significantly alter the previously calculated values.

The examination of organic acids isolated from the fruits indicated that the EMP pathway, in conjunction with the tricarboxylic acid cycle, is the primary source of carbon skeletons for the biosynthesis of fruit acids. The isotopic distribution pattern of malic acid demonstrated that a CO₂-fixation reaction of the C₃ + C₁ type is largely responsible for the net biosynthesis of C₄ acids in tomato fruit.
RADIOTRACER STUDIES OF CARBOHYDRATE CATABOLISM IN TOMATO FRUIT

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This Thesis is Dedicated
To my Parents
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RADIOTRACER STUDIES OF CARBOHYDRATE CATABOLISM IN TOMATO FRUIT

INTRODUCTION

The relative participation of the Embden-Meyerhof-Parnas (EMP) and the pentose phosphate (PP) pathways, as well as the complex metabolic role played by these sequences, has been of increasing interest in recent years (2, p. 267-298, 3, 4, 5, 7, 13, 24). More precise information of their physiological function as well as the interplay between these two catabolic mechanisms is essential to an understanding of the overall metabolism of glucose in plant systems.

The oxidation of glucose carbon atoms to CO₂ and the attendant production of respiratory energy in the form of adenosine triphosphate is generally recognized as the primary function common to both of these pathways. The tricarboxylic acid (TCA) cycle, operating in conjunction with the EMP and PP sequences, may serve as the major mechanism to accomplish the extensive oxidation of glucose to CO₂. On the other hand, the PP sequence, via successive pentose cycle reactions, can bring about the oxidation of the top three carbon atoms of glucose to CO₂.

Also, metabolic intermediates derived from glucose by the operation of either of these pathways may be utilized as a source of carbon skeletons for biosynthetic purposes. Thus, C₅ units arising via the PP reactions may be incorporated into such plant constituents as nucleic acids and pentosans (34, 36), and C₇ units
can serve as precursors for aromatic acids (11). Glucose carbon atoms traversing the EMP-TCA sequence may be drained off for the biosynthesis of compounds such as amino acids or lipids. Inasmuch as an additional source of carbon skeletons such as C₄ acids will have to be provided before TCA cycle reactions can be used for biosynthetic purposes, derivatives of glucose in the EMP sequence may also be mobilized for the synthesis of C₄ acids or allied compounds.

The production of reduced triphosphopyridine nucleotide (TPNH) has also been regarded as an important function of the PP catabolic sequence (13). This coenzyme, arising from the oxidation of glucose-6-phosphate to CO₂ and pentose phosphate in the PP pathway, may be utilized in biological reduction reactions.

As is the case with many plant systems, it has been established with tomato fruit that glucose is catabolized via both the EMP and the PP pathways (5, 21). In order to understand the complete fate of glucose in a biological system such as fruit, it is of primary importance to know the relative participation of each of these two concurrent catabolic pathways of glucose.

Numerous methods have been reported for the estimation of pathway participation in biological systems. Since its introduction by Bloom and Stetten (10) in 1953, the use of the ratios of the yields of C¹⁴O₂ from glucose-1-C¹⁴ and glucose-6-C¹⁴ to trace the distribution of substrate glucose between the EMP and PP pathways has seen extensive application (7, 35, 41). Other investigators have employed similar radiotracer methods for pathway estimation.
based on $^{14}\text{C}O_2$ yields, or on other products derived from substrate glucose (14, 15, 17, 18, 19, 28). These methods, based on the catabolic equivalence of C-1 and C-6 of glucose via the EMP-TCA sequence and the preferential oxidation of C-1 of glucose to $CO_2$ via the PP sequence, have been shown to be subject to serious limitations, particularly with respect to the validation of some of the assumptions and the interpretation of data from radiotracer experiments.

As pointed out by several authors, in the PP sequence, further metabolism of the pentose phosphates formed by the decarboxylation of labeled 6-phosphogluconate will lead to the reconstruction of hexose phosphates with randomized labeling patterns (6, 28, 53). Mixing of these reconstructed hexose-6-phosphates with substrate glucose will result in dilution of the specific activity of the substrate glucose, particularly at C-1, C-2 and C-3 (28, 52). This fact makes it difficult to interpret data obtained with experiments carried out under steady state conditions, i.e., where there is a continual balance between inflowing glucose and its outflowing metabolic products. These complications have been examined by Katz and Wood (28) and by Wood and Katz (53), resulting in the development of theoretical treatments for data analysis. It should be noted that one of the basic assumptions underlying their derivations (28) required the complete isotopic equilibration of glucose-6-phosphate and fructose-6-phosphate.

Doyle and Wang (19) and Barbour, Buhler and Wang (5) in 1958 estimated the relative participation of two concurrent catabolic
pathways of glucose in tomato fruit. The complete time course for the utilization of the administered labeled glucose was followed. Use was made of cumulative radiochemical yields of respiratory C\textsuperscript{14}O\textsubscript{2} observed at the end of the time course for glucose utilization. Equations were derived (5) to estimate relative pathway participation on the basis of that portion of administered glucose substrate actually engaged in catabolic functions.

This type of treatment allows an estimation of pathway participation to be made which is independent of a knowledge of the isotopic equilibration of the hexose-6-phosphates. Moreover, the derivation is not affected by the complications derived from the dilution of substrate glucose with reconstructed hexose phosphates arising via the PP pathway. This is true since, with a one-dose administration of labeled glucose, all the glucose carbon atoms destined to be catabolized by one pathway or the other will have done so by the end of the time course of glucose utilization. Thereby, the necessity is eliminated to correct for differences in metabolic rates along the various catabolic sequences, or for the dilution of substrate glucose by metabolites in endogenous pools.

The use of C\textsuperscript{14}O\textsubscript{2} yields, with the introduction of a new term, "specific yield", for pathway estimation has also been recently reported by Landau et al. (31, 32) as being applicable to steady state systems. However, it should be noted that under steady state conditions, one is not able to follow the complete time course for utilization of a given amount of administered glucose and, hence, the interpretation of data are subject to all the limitations.
previously described by Katz and Wood (28, 52, 53).

The basic method for pathway estimation as reported by Wang, et al. in 1958 (5, 19) did not take into account the catabolic fate of pentose phosphates derived from substrate glucose in the PP sequence. Subsequently, in 1962, Wang, Doyle and Ramsey (48) reported a revised method for pathway estimation which included the tracing of the catabolic fate of glucose in the PP pathway. The irreversibility of the glucose-6-phosphate to 6-phosphogluconate conversion (26) allowed the utilization of specifically labeled gluconate-$C^{14}$ substrates in correcting for $C^{14}O_2$ produced from C-3, 4 and C-6 of glucose via the PP sequence.

The results of experiments carried out by Wang et al. (5, 21) indicated that the EMP and PP pathways are the important routes for glucose catabolism in tomato fruit. Approximately 73 percent of the catabolized glucose substrate appears to be routed through the EMP-TCA sequence (48). Subsequently it was also found that there is a definite change in pathway participation during the ripening process in tomato fruits (40). Experiments with gluconate-$C^{14}$ substrates also indicated that recycling activity in the nature of the pentose cycle processes is limited, but that recombination of triose units derived from the PP pathway may be taking place to a considerable extent (48).

Of equal interest is the fate of triose phosphate, or pyruvate, as derived from the PP or EMP pathways, in further metabolic reactions. It is generally recognized that the conversion of pyruvate to $CO_2$ and acetyl coenzyme A may result in the oxidation of
the acetate carbon atoms to CO₂ via the TCA cycle reactions, and the operation of such a pathway has been demonstrated in tomato fruit (12, 46).

As indicated previously, the TCA cycle alone cannot function as a biosynthetic pathway. The net synthesis of C₄ acids requires that other mechanisms provide a source of TCA intermediates so that metabolites of the TCA cycle may be utilized for biosynthetic purposes. One such anabolic pathway is CO₂-fixation of the C₃ + C₁ type (37), which is known to function in various plant tissues (1, 8, 45) and has been demonstrated in tomato fruit (12). Another mechanism capable of providing for the net synthesis of C₄ acids is the malate synthetase reaction of a C₂ + C₂ type (29, 30, 51, 54). This type of reaction has also been demonstrated to be operative in tomatoes (20). It should be noted that previous experimental findings did not provide any quantitative evaluation of the relative importance of these two pathways for the net synthesis of C₄ acids (12, 20).

The present work is designed to elucidate more extensively the fate of glucose traversing through primary catabolic pathways leading to the formation of pyruvate, and through secondary pathways leading to the biosynthesis of C₄ acids in tomato fruit. Particular emphasis has been placed on the effect and significance of the recombination of triose phosphates via the reversed aldolase reaction to form hexose phosphates, and on the relative quantitative participation of the C₃ + C₁ versus the C₂ + C₂ mechanisms with respect to the net synthesis of C₄ acids.
EXPERIMENTAL AND RESULTS

Materials

The tomatoes used in this investigation were mature green fruits of the Michigan State Forcing variety, obtained from the Field and Bagley greenhouses in Salem, Oregon. The fruits were selected on the basis of uniformity in size, shape and color, and were used for experimentation within four hours after harvest.

C-14 labeled substrates

The specifically labeled glucose-C\(^{14}\) substrates used in the experiments were obtained from commercial sources. Glucose-1-C\(^{14}\) was purchased from the Volk Radiochemical Company, while glucose-2-C\(^{14}\) and glucose-6-C\(^{14}\) were obtained from the National Bureau of Standards.

Measurement of radioactivity

In those experiments in which the specific activity of C\(^{14}\)O\(_2\) was desired, Geiger counting of BaC\(^{14}\)O\(_3\) was used. Carbon dioxide was collected in 1 N NaOH and precipitated as BaCO\(_3\) by 0.5 M BaCl\(_2\). The BaCO\(_3\) was then mounted on aluminum planchets by centrifugation and counted in a Nuclear Chicago thin-window low-background flow counter to a standard error of less than three percent. After correcting for background and self absorption, the observed count rate was compared with that of BaC\(^{14}\)O\(_3\) planchets prepared from standard NaHC\(^{14}\)O\(_3\) (obtained from the National
Bureau of Standards) to determine the absolute counting efficiency of the instrument. All counting data thus obtained were converted to disintegrations per minute per millimole of carbon.

Liquid scintillation counting was used to assay for radioactivity of water-soluble biological compounds such as organic acids and amino acids. In these instances, 0.2 M ethanolic hyamine hydroxide was used to assure solubility of the C\(^{14}\)-containing compounds in the scintillator solution (toluene containing 0.3 percent p-terphenyl and 0.003 percent POPOP as primary and secondary scintillators, respectively). The liquid samples were then counted in a Tri-Carb Series 314E liquid scintillation spectrometer at a high voltage setting of 1120 V and a discriminator setting of 1000-1000 divisions. The use of an internal standard of toluene-C\(^{14}\) (obtained from the National Bureau of Standards) enabled one to determine the counting efficiency of the individual samples.

Radioactivity in the effluent resulting from ion exchange column operation was qualitatively detected by the use of a Tri-Carb flow monitor. For this purpose, the column outlet was directed through an anthracene-packed cartridge which was optically coupled to two photomultiplier tubes operating in coincidence at an applied voltage of 1100 V. The output of the photomultiplier tubes was monitored by a ratemeter connected to a Varian strip-chart recorder.
Incorporation of glucose-1, -2, and -6-C$^{14}$

In order to determine the fate of the isotopic carbon within metabolic intermediates of tomato fruit utilizing specifically labeled glucose substrates, glucose-1-C$^{14}$, glucose-2-C$^{14}$ and glucose-6-C$^{14}$ were administered to individual tomatoes by the vacuum infiltration technique (5). The fruits were allowed to metabolize the administered substrates for a period of 12 hours, at which time the radioactivity in respired C$^{14}$O$_2$ had subsided to a steady low level. The cumulative radiochemical yields of respiratory C$^{14}$O$_2$ observed in these incorporation experiments are given in Table I.

**TABLE I.** The Incorporation of Specifically Labeled Glucose-C$^{14}$ Substrates into Tomato Fruit.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Administered Radioactivity (µc)</th>
<th>Substrate Level (mg)</th>
<th>Weight of Fruit (gm)</th>
<th>Percent Recovery of C$^{14}$ in C$^{14}$O$_2$ at 12 hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-1-C$^{14}$</td>
<td>9.3</td>
<td>2.0</td>
<td>65</td>
<td>11.3</td>
</tr>
<tr>
<td>Glucose-2-C$^{14}$</td>
<td>2.9</td>
<td>2.0</td>
<td>66</td>
<td>6.9</td>
</tr>
<tr>
<td>Glucose-6-C$^{14}$</td>
<td>8.3</td>
<td>2.0</td>
<td>65</td>
<td>4.7</td>
</tr>
</tbody>
</table>

Upon termination of the experiments, the fruits were immediately removed from the respiration chambers, individually homogenized in hot 80 percent ethanol, then subjected to 80 percent ethanol extraction in a Soxhlet apparatus for an additional 12 hours. The alcohol extract was then evaporated to near-dryness in a rotary flash evaporator, and the residue was taken up in water and evaporated once again. Several successive operations of this type
removed all the ethanol. The aqueous solution was then filtered, and subjected to liquid-liquid extraction with petroleum ether to remove any remaining traces of fatty substances.

The aqueous portion from the above mentioned ether extraction was then concentrated to a small volume, and further processed for the separation of amino acids, organic acids, and carbohydrates.

Separation of amino acids

The aqueous solution obtained by the above procedure was passed through a cation exchange resin column (Dowex 50 W-X8, 200-400 mesh, hydrogen form) to remove the amino acids. After rinsing the column with water to wash through the acidic and neutral substances, the amino acids were eluted from the column with 4 N HCl. Repeated evaporation of the eluent to near dryness removed the mineral acid, and the resulting amino acid fractions were assayed for radioactivity by liquid scintillation counting. The radioactivity contained in these amino acid fractions is shown in Table II.

TABLE II. Radioactivity Incorporated into Water-Soluble Fractions Isolated from Tomato Fruit Metabolizing Specifically Labeled Glucose-C14 Substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Percent of Administered Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amino Acids</td>
</tr>
<tr>
<td>Glucose-1-C14</td>
<td>3.5</td>
</tr>
<tr>
<td>Glucose-2-C14</td>
<td>3.5</td>
</tr>
<tr>
<td>Glucose-6-C14</td>
<td>1.5</td>
</tr>
</tbody>
</table>
Separation of organic acids

The effluent from the above operation, containing acidic and neutral substances, was passed through an anion exchange resin column (Dowex 1-X8, formate form) to remove the organic acids. When radioactivity contained in the neutral fraction was completely rinsed through the column, as indicated by the Tri-Carb flow monitor, the organic acids retained on the column were selectively eluted from the exchange resin by gradient elution with formic acid according to the method of Palmer (38). Peaks of radioactivity in the eluent were observed to appear when the eluting acid concentration was between 2.0 and 3.4 N.

Malic, succinic, and citric acids accounted for the bulk of the radioactivity present. The appropriate fractions were combined, and the eluting acid was removed by repeated evaporations to near-dryness. The total radioactivity contained in the organic acids is given in Table II.

The purity of the malic, succinic and citric acid samples isolated was established by means of chromatography on Whatman No. 1 paper with iso-propyl alcohol:t-butyl alcohol:benzyl alcohol:water:formic acid (1: 1: 3: 1: 0.1, v/v) (44), and n-butanol: acetic acid: water (25: 6: 25, v/v) as solvents. The amount of each acid present was determined by titration with 0.02 N NaOH, and their radioactivity was assayed by liquid scintillation counting. The specific activity of these acids is shown in Table III.
TABLE III. Normalized Specific Activity of Individual Organic Acids Isolated from Tomato Fruit Metabolizing Specifically Labeled Glucose-C\textsuperscript{14} Substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific Activity(\times 10^{-5})</th>
<th>Malic Acid</th>
<th>Succinic Acid</th>
<th>Citric Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-1-C\textsuperscript{14}</td>
<td>14.3</td>
<td>9.6</td>
<td>6.4</td>
<td></td>
</tr>
<tr>
<td>Glucose-2-C\textsuperscript{14}</td>
<td>16.8</td>
<td>9.8</td>
<td>5.6</td>
<td></td>
</tr>
<tr>
<td>Glucose-6-C\textsuperscript{14}</td>
<td>12.5</td>
<td>7.3</td>
<td>4.7</td>
<td></td>
</tr>
</tbody>
</table>

* Specific activity is normalized to equal amounts of radioactivity in the individual glucose substrates.

Degradation of malic acid

Paper chromatography revealed a contaminant present in the malic acid fractions isolated from the column operation. Consequently, the acid fractions were further purified by streaking the solutions on S&S 470-A chromatography paper, then developing the paper in n-butanol: acetic acid: water (25: 6: 25, v/v). The malic acid band was cut out and eluted with water, and was subsequently shown to be chromatographically pure.

The malic acid thus obtained was diluted with carrier and a sample of it was subjected to persulfate combustion to determine the molar specific activity.

The specific activity of the carboxyl carbon atoms of malic acid was determined from the CO\textsubscript{2} obtained by a permanganate decarboxylation according to the method of Friedeman and Kendahl (23, p. 25-28). The specific activity in C-1 of malic acid was determined by subjecting the malic acid to the action of 100 percent
sulfuric acid at 50°C (39, p. 26-30), and the resulting CO was then oxidized to CO₂ by CuO at 400°C. The specific activity of C-4 of malic acid was then determined by difference.

The acetaldehyde formed in the permanganate reaction (representing C-2 and C-3 of malic acid) was trapped in NaHSO₃, then released from its bisulfite addition compound by the addition of K₂HPO₄, and purified by distillation. The acetaldehyde was then further degraded by the action of NaOIr in the cold (16, p. 567-568). The iodoform thus obtained from C-3 of malic acid was separated from the reaction mixture by filtration, oxidized to CO by the action of AgNO₃ (43), and further oxidized to CO₂ by passing it over CuO at 400°C.

Formic acid resulting from the iodoform reaction was recovered from the reaction mixture by steam distillation and oxidized directly to CO₂ with mercuric acetate (16, p. 568), thus providing a means of determining the specific activity of C-2 of malic acid. The distribution of activity within the individual carbon atoms of malic acid is given in Table IV.

Separation of carbohydrates

The neutral effluent obtained from the operation of the Dowex 1 (formate) column was concentrated to a small volume, and its radioactivity was assayed by liquid scintillation counting. The total radioactivity contained in the carbohydrate fractions is given in Table II.
TABLE IV. Distribution of Radioactivity Within the Individual Carbon Atoms of Malic Acid Isolated from Tomato Fruit Metabolizing Specifically Labeled Glucose-\(\text{C}^{14}\) Substrates.

<table>
<thead>
<tr>
<th>Carbon Atom of Malate</th>
<th>Glucose-1-C(^{14}) (% of total)</th>
<th>Glucose-2-C(^{14}) (% of total)</th>
<th>Glucose-6-C(^{14}) (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-1</td>
<td>12</td>
<td>33</td>
<td>8</td>
</tr>
<tr>
<td>C-2</td>
<td>33</td>
<td>21</td>
<td>38</td>
</tr>
<tr>
<td>C-3</td>
<td>38</td>
<td>19</td>
<td>40</td>
</tr>
<tr>
<td>C-4</td>
<td>17</td>
<td>27</td>
<td>14</td>
</tr>
</tbody>
</table>

Separation of the carbohydrates was then carried out on a preparative scale by the use of paper chromatography with Whatman 3 MM paper (previously washed in oxalic acid and water). After streaking the carbohydrate solutions on the paper, the chromatograms were developed by the ascending technique in 80 percent phenol, dried, then developed again in the same solvent. The carbohydrate bands were then detected with a periodic acid-benzenidine reagent (25), and the bands corresponding to glucose and fructose were cut out and eluted with water. The identity and purity of these carbohydrates was confirmed by paper chromatography on Whatman No. 4 paper in n-butanol: acetic acid: water (25: 6: 25, v/v). The carbohydrates were also co-chromatographed with authentic samples of glucose and fructose in iso-propyl alcohol: pyridine: acetic acid: water (8: 8: 1: 4) (25). The amount and specific activity of glucose and fructose were then determined by the isotopic double-dilution technique, the results
of which are given in Table V.

TABLE V. Normalized Specific Activity of Individual Carbohydrates Isolated from Tomato Fruit Metabolizing Specifically Labeled Glucose-C\textsuperscript{14} Substrates.

| Substrate          | Glucose | | | Fructose | | |
|-------------------|---------|---------|---------|----------|---------|
|                   | μmole   | specific activity | μmole   | specific activity |
| Glucose-1-C\textsuperscript{14} | 40      | 2.9        | 38      | 0.4       |
| Glucose-2-C\textsuperscript{14} | 42      | 5.1        | 42      | 1.5       |
| Glucose-6-C\textsuperscript{14} | 45      | 5.7        | 34      | 2.4       |

* μmole/gm fruit is calculated on a fresh weight basis.
** Specific activity is expressed in (dpm/μmole of hexose) x 10\textsuperscript{-4}, and is normalized to equal amounts of radioactivity in the individual glucose substrates.

Degradation of glucose

In order to determine the isotopic distribution pattern within the glucose samples isolated above, bacterial degradation by the action of Leuconostoc mesenteroides was utilized. This fermentation process results in the production of CO\textsubscript{2} from C-1 of glucose, ethanol from C-2 and C-3, and lactic acid from C-4, C-5 and C-6 (16, p. 571-580, 42, p. 40-41). The lactic acid and ethanol may then be further degraded to ascertain the radioactivity contained in each carbon atom in the glucose molecule.

Lyophilized cultures of Leuconostoc mesenteroides were obtained from the American Type Culture collection (ATCC No. 12291), and grown on the medium described by Bernstein et al. (9, p. 140-141). Actively fermenting cells were harvested by centrifugation at
3000 x g, and stored by freezing in the glycerol-buffer solution described by Lesley (33). The cells were then thawed and activated at 37°C for 30 minutes before being used to degrade the glucose-C\textsuperscript{14} samples.

The fermentations were carried out on the labeled samples after dilution with carrier glucose. Carbon dioxide arising from C-1 of glucose during the fermentation was trapped in 1 N NaOH, after which the cells were removed from the fermentation medium by centrifugation.

The medium was then made alkaline to the phenol red end point (pH 8.4) with NaOH. Ethanol formed in the fermentation process was then removed from the medium by distillation and oxidized to acetic acid by the action of acid dichromate (16, p. 575). Acetic acid was recovered from the reaction mixture by steam distillation, and converted to its sodium salt with NaOH. After being evaporated to a small volume, the sodium acetate was oven-dried at 110°C.

The sodium acetate thus obtained was subjected to the Schmidt degradation with hydrazoic acid (27), resulting in the formation of CO\textsubscript{2} from the carboxyl carbon atom of acetate, derived from C-3 of glucose.

The methyl amine arising from the methyl carbon atom of acetate via the Schmidt reaction was removed from the reaction mixture by distillation. Methyl amine was then oxidized with alkaline permanganate (27) to CO\textsubscript{2} derived from C-2 of glucose.

Lactic acid formed during the fermentation of the glucose-C\textsuperscript{14} samples was contained in the residue of the ethanol
distillation in the form of sodium lactate. After adjusting the pH of the residue to 3.0 with sulfuric acid, the solution was subjected to continuous liquid-liquid ether extraction by the method described by Sakami (42, p. 42-45). The lactic acid thus obtained was subjected to steam distillation to remove any steam-volatile impurities carried over by the ether extraction.

The lactic acid remaining in the residue of the steam distillation was converted to its sodium salt by the addition of NaOH, and the solution concentrated to a small volume. The sodium lactate was then subjected to permanganate decarboxylation (27) to yield CO₂ from the carboxyl carbon atom of lactate, derived from C-4 of glucose.

The acetic acid (representing C-5 and C-6 of glucose) resulting from the decarboxylation of lactate was removed from the reaction mixture by steam distillation, concentrated, and further degraded as described previously. Thus, C-5 of glucose was obtained as CO₂ from the carboxyl carbon atom of acetate, and C-6 of glucose was obtained as CO₂ from the methyl carbon atom of acetate.

The isotopic distribution pattern in fruit glucose obtained by the above degradation is given in Table VI.
TABLE VI. Distribution of Radioactivity Within the Individual Carbon Atoms of Glucose Isolated from Tomato Fruit Metabolizing Specifically Labeled Glucose-C\textsubscript{14} Substrates.

<table>
<thead>
<tr>
<th>Carbon Atom of Glucose</th>
<th>Glucose-1-C\textsubscript{14} (%) of total</th>
<th>Glucose-2-C\textsubscript{14} (%) of total</th>
<th>Glucose-6-C\textsubscript{14} (%) of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-1</td>
<td>84</td>
<td>6</td>
<td>31</td>
</tr>
<tr>
<td>C-2</td>
<td>1</td>
<td>70</td>
<td>3</td>
</tr>
<tr>
<td>C-3</td>
<td>2</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>C-4</td>
<td>2</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>C-5</td>
<td>-</td>
<td>14</td>
<td>-</td>
</tr>
<tr>
<td>C-6</td>
<td>11</td>
<td>2</td>
<td>60</td>
</tr>
</tbody>
</table>
DISCUSSION

As shown in Table I, the overall glucose metabolism of the tomato fruits used in these experiments is essentially the same as that observed previously with respect to the production of respiratory C\textsuperscript{14}O\textsubscript{2} (5). This fact implies that the conclusions reached in the previous work are applicable in the interpretation of data obtained in the present experiments.

The general fate of substrate glucose-C\textsuperscript{14} in tomato fruit is indicated to some extent by the data given in Table II. The extensive incorporation of C\textsuperscript{14} into the carbohydrate fractions as compared to that in the organic acid and amino acid fractions may reflect the large endogenous pool of carbohydrates present in tomatoes, which is serving as a trapping mechanism for the administered substrates. Doyle and Wang (21) reported earlier that substrate glucose administered to tomato fruit was diluted with approximately 17 mg of fruit hexose. This is to be compared with the amount of labeled glucose administered to the fruit in the present experiments, i.e., 2 mg.

It is noted that the glucose and fructose content in tomato fruits (given in Table V) is actually much higher than the pool size of fruit glucose actively engaged in catabolism reported by Doyle and Wang (21). This is understandable since the labeled glucose administered by the vacuum infiltration technique is distributed almost exclusively in the vascular tissue, and thereby it is diluted only by the amount of glucose in that defined area.
Of great interest is the finding that the label of glucose-1-C\textsuperscript{14} detected in fruit carbohydrates was significantly less than that found in either the glucose-2-C\textsuperscript{14} or the glucose-6-C\textsuperscript{14} experiments. This is also substantiated by the specific activity of the fruit glucose or fructose as shown in Table V. A possible interpretation of this finding can be made on the basis that the loss of C-1 of glucose via the PP pathway is a rapid process relative to the rate of mixing of the administered substrate with the endogenous pool. Hence, a significant amount of the radioactivity contained in the glucose-1-C\textsuperscript{14} substrate would be expected to be lost as C\textsuperscript{14}O\textsubscript{2} via the PP pathway before extensive dilution by endogenous hexoses has occurred.

A key assumption made by several workers in evaluating pathway participation has been that glucose-6-phosphate and fructose-6-phosphate were in complete isotopic equilibrium after the administration of glucose-C\textsuperscript{14} to a given biological system (28, 53). Such an assumption appears to be not applicable in the case of tomato fruit. As shown in Table V, the amounts of glucose and fructose present in the fruit appear to be comparable, yet the specific activity of fructose is considerably less than that of glucose in all three of the labeled glucose-C\textsuperscript{14} experiments.

Previously, by the use of specifically labeled gluconate-C\textsuperscript{14} substrates (48), it was possible to trace the fate of triose phosphates derived from glucose via the PP pathway. A significant portion of the triose phosphate so formed was found to engage in recombination processes via a reverse aldolase reaction. However, it was not
known whether the triose phosphates derived from glucose via the EMP pathway would display a similar behavior. As shown in Table VI, the labeling pattern observed in the fruit glucose isolated from the three glucose-C\textsuperscript{14} experiments provides definite information that triose recombination is an important process in tomato fruit, regardless of the route of formation. Thus, 11 percent of the radioactivity contained in fruit glucose derived from glucose-1-C\textsuperscript{14} was found to be relocated to the C-6 position. This fact indicates that a sizable amount of the triose phosphate derived via the EMP pathway has engaged in isomerization and recombination reactions. This conclusion is confirmed by similar findings in the glucose-2-C\textsuperscript{14} and glucose-6-C\textsuperscript{14} experiments. The relocation of radioactivity from C-6 to C-1 of fruit glucose derived from glucose-6-C\textsuperscript{14} is more extensive (i.e., 31 percent) than that observed with glucose-1-C\textsuperscript{14}. This is understandable since, in this case, the relocation of C-6 to C-1 actually represents recombination of triose units derived from both the PP and EMP pathways.

In view of the triose recombination observed in the present experiments, it is of interest to examine the validity of the derivations underlying the use of C\textsuperscript{14}O\textsubscript{2} yields for the estimation of pathway participation. A brief description of the derivation concept (5, 47, 48) is described here in order to facilitate the discussion.

The estimation of pathway participation is based on the immediate catabolic fate of substrate glucose in entering either the EMP or the PP pathway. Consequently, the EMP pathway is defined as:
I Glucose-6-P → fructose-1,6-di-P → 
  x triose-3-P → y pyruvate

in which x and y are actual yields of the respective compounds, with a limiting magnitude of two. The PP pathway is defined as:

\[
\begin{align*}
3 \text{ Glucose-6-P} & \rightarrow 3 \text{ CO}_2 \\
 & \rightarrow 3 \text{ pentose-P} \rightarrow z \text{ fructose-6-P} + \\
 & \quad q \text{ glyceraldehyde-3-P}
\end{align*}
\]

in which z and q are actual yields of the respective compounds, and as an upper limit, z = 2 and q = 1.

The derivation of equations for estimating the participation of these two pathways in biological systems are based on the following assumptions.

1. The EMP and PP sequences are the only pathways accounting for the primary breakdown of glucose.

2. The conversion of C-1 of glucose to CO₂ via the PP pathway is a rapid and irreversible process.

3. Triose phosphates formed in the EMP sequence are equivalent to each other with respect to their further metabolism.

4. Pyruvate derived from triose phosphate is oxidatively decarboxylated, giving rise to CO₂ and acetate.

5. The recombination of triose units to form hexoses does not occur to a significant extent.

6. Substrate gluconate is catabolized in the biological system in a manner identical to that of 6-phosphogluconate formed via the PP pathway.
The derivation of equations for estimating pathway participation then follows from the foregoing assumptions. Let \( G_1, G_3,4 \) and \( G_6 \) = cumulative radiochemical yields of respiratory \( \text{C}^{14}\text{O}_2 \) from equal amounts of respectively labeled glucose substrates. Let \( A_1, A_3,4 \) and \( A_6 \) = cumulative radiochemical yields of respiratory \( \text{C}^{14}\text{O}_2 \) from equal amounts of respectively labeled gluconate substrates. All the above yields are expressed as a fraction of unity.

\[
G_T = \text{Total radioactivity of each labeled glucose administered.}
\]

\[
G_T' = \text{Fraction of the administered labeled glucose that was not catabolized.}
\]

\[
G_t = \text{Fraction of the administered labeled glucose that was catabolized. Therefore, } G_t = G_T - G_T'.
\]

\[
G_p = \text{Fraction of } G_t \text{ catabolized via the PP pathway.}
\]

\[
G_e = \text{Fraction of } G_t \text{ catabolized via the EMP pathway.}
\]

All the above values are expressed as fractions of unity.

Thus, \( G_T \) is always equal to one.

The basic expression for pathway estimation (3) is then

\[
G_p = \frac{(G_1 - G_6)}{G_t}
\]  \( \text{(I)} \)

And, since the PP and EMP pathways are the only primary catabolic routes for glucose dissimilation,

\[
G_e = 1 - G_p
\]  \( \text{(II)} \)

The total glucose catabolized is estimated from the equation
The production of CO$_2$ from C-3, 4 and C-6 of glucose via the PP pathway necessitates a correction term equal to the magnitude of the C$^{14}$O$_2$ yield from the labeled gluconate substrate times that fraction of the catabolized glucose routed through the PP pathway. Thus, equation I becomes

\[
G_p = \left[ G_1 - (G_6 - A_6G_p) \right] / G_t
\]

and equation III becomes

\[
G_t = G_1 - (G_6 - A_6G_p) + (G_{3, 4} - A_{3, 4}G_p)
\]

\[
= G_1 - G_6 + G_{3, 4} + G_p(A_6 - A_{3, 4})
\]

The occurrence of triose recombination reactions demonstrated in the present work consequently invalidates one of the key assumptions underlying the derivation of equation IV. The term G$_1$ will not actually represent the yield of respiratory CO$_2$ derived from the PP and the EMP pathways. This is true since the amount of C-1 relocated to C-6 of glucose will not be preferentially decarboxylated by the PP pathway. On the other hand, the relocation of C-6 of glucose to the C-1 position will not affect the validity of the use of the term G$_6$ in the pathway estimation equation. This is true since via the EMP-TCA pathway the fate of C-6 of glucose will not be affected by recombination process, and the corrective term
applied to \( G_6 \) by the use of gluconate-6-\( \text{C}^{14} \) will include a correction for any interference from triose recombination via the PP pathway.

Therefore, the impact of triose recombination upon the derivation concept of equations III and IV is focused on the fact that the true yield of respiratory \( \text{CO}_2 \) from C-1 of glucose should be greater than the observed \( \text{C}^{14} \text{O}_2 \) yield from glucose-1-\( \text{C}^{14} \). The discrepancy is naturally dependent on the extent of triose recombination.

If the term \( R_1 \) is defined as that fraction of radioactivity located in C-1 of fruit glucose derived from glucose-1-\( \text{C}^{14} \), then the quantity \((1 - R_1)\) is a measure of the extent to which radioactivity has been removed from C-1 of glucose. Therefore, as an approximation, the term \( G_p(1 - R_1) \) should represent the maximum extent to which the yield of respiratory \( \text{CO}_2 \) from C-1 of glucose via the PP pathway has been affected by the relocation of radioactivity from C-1 to C-6 of glucose. \( G_1' \) is defined as the yield of \( \text{C}^{14} \text{O}_2 \) from glucose-1-\( \text{C}^{14} \) corrected for triose recombination, and is defined as

\[
G_1' = G_1 + G_1 \left[ G_p(1 - R_1) \right] = G_1 + G_1(G_p - G_p R_1) \quad \text{VI}
\]

It should be substituted for the term \( G_1 \) in equations IV and V which then become respectively

\[
G_p = \frac{(G_1' - G_6 + A_6 G_p)}{G_t}
\]

\[
= \frac{\left[ G_1 - G_6 + G_p(G_1 - G_1 R_1 + A_6) \right]}{G_t} \quad \text{VII}
\]
\[ G_t = G_1' - (G_6 - A_6 G_p) + (G_{3,4} - A_{3,4} G_p) \]

\[ = G_1 - G_6 + G_{3,4} + G_p (G_1 - G_1 R_1 + A_6 - A_{3,4}) \] VIII

Wang et al. has determined that \( G_p \) is equal to approximately 0.27, or 27 percent, in tomato fruit (48). And in the present work, Table I shows that \( G_1 = 11.3 \) percent and Table VI shows that \( R_1 = 0.84 \). By the use of these values and equation VI, one finds that the \( G_1 \) term is increased from 11.3 to 11.8 percent by correcting for the occurrence of triose recombination in tomato fruit.

It can, therefore, be concluded that the occurrence of triose recombination introduces a complicating factor in pathway estimation to all the reported methods. A number of uncertain parameters prevents one from devising an equation to calculate the exact extent of triose recombination occurring. The method recently reported by White and Wang (49, 50) in their work with \( A. xylinum \) cannot be applied directly in the present case, since the presence of the large endogenous pool of unlabeled glucose invalidates one of their key assumptions. It should be noted that the estimation given above by the use of equation VI indicates that the extent of triose recombination observed in the present work will not significantly change the pathway participation reported previously by Wang et al. (48) for glucose catabolism in tomato fruit.

With the information on glucose metabolism, as discussed in the preceding section, on hand, it is possible to examine the biosynthesis of fruit acids, particularly the very important \( C_4 \) acids,
from glucose derivatives. From Table II it can be seen that the radioactivity from glucose-\(^{14}\)C substrates was incorporated into the fruit acids to approximately the same extent regardless of the labeling position in the glucose substrate. This fact, in contrast to the findings on the transfer of substrate radioactivity into the carbohydrate fractions, strongly suggests that the source of carbon skeletons for the synthesis of fruit acids is derived exclusively from intermediates along the EMP pathway. This is true since any significant role played by the PP pathway will show preferential incorporation of C-6 and C-2 of glucose over that of C-1.

Similar conclusions can also be drawn from the data on the specific activity of fruit acids given in Table III. It is noted in the case of malate that the specific activity of malate derived from glucose-1-\(^{14}\)C or glucose-2-\(^{14}\)C is slightly higher than that of malate derived from glucose-6-\(^{14}\)C. This observation presumably reflects the role played by CO\(_2\)-fixation in the biosynthesis of this acid. This is true since the radioactivity observed in respiratory C\(^{14}\)O\(_2\) from the glucose-1-\(^{14}\)C and glucose-2-\(^{14}\)C experiments was higher than that observed with glucose-6-\(^{14}\)C.

The magnitudes of the specific activities of malate, succinate and citrate are not directly comparable since they are dependent on the pool size and mechanism of formation of each of the acids. Thus, if a C\(_3\) + C\(_1\) type of reaction is responsible for the formation of C\(_4\) acids, malate will exhibit a higher specific activity than succinate.

A considerable amount of information on the nature of the biosynthetic mechanism for C\(_4\) acid formation can be obtained by
analyzing the labeling distribution pattern of malate observed in the three glucose-\textsuperscript{14}C experiments, which is given in Table IV.

It will be helpful in this regard to first review the possible biosynthetic pathways leading to the formation of malate in tomato fruit. Previous investigations (12, 20) have demonstrated, in tomato fruit, the occurrence of a CO\textsubscript{2}-fixation reaction and the malate synthetase reaction, both of which can give rise to the net synthesis of malate.

The fixation of CO\textsubscript{2} to pyruvate derived from substrate glucose will give rise to malate with a labeling pattern as shown.

\[
\begin{array}{ccccc}
\text{O} & \text{OH} \\
\text{CO}_2 + \text{CH}_3 - \text{C} - \text{COOH} & \rightarrow & \text{HOOC} - \text{CH}_2 - \text{CH} - \text{COOH} \\
1 & 2 & 3 & 1 & 2 & 3 \\
6 & 5 & 4 & 6 & 5 & 4 \\
\end{array}
\]

The condensation of glyoxylate, derived from glucose via acetyl-coenzyme A and isocitrate, with acetyl-coenzyme A will give rise to malate with a labeling pattern as shown.

\[
\begin{array}{ccccc}
\text{O} & \text{O} \\
\text{CH}_3 - \text{C} - \text{COOH} & \rightarrow & \text{CH}_3 - \text{C} \sim \text{SCoA} \\
1 & 2 & 3 & 1 & 2 \\
6 & 5 & 4 & 6 & 5 \\
\end{array}
\]

\[
\begin{array}{ccccc}
\text{O} & \text{O} & \text{OH} \\
\text{HOOC} - \text{CH} + \text{CH}_3 - \text{C} \sim \text{SCoA} & \rightarrow & \text{HOOC} - \text{CH} - \text{CH}_2 - \text{COOH} \\
2 & 1 & 1 & 2 & 2 & 1 & 2 \\
5 & 6 & 6 & 5 & 5 & 6 & 6 & 5 \\
\end{array}
\]

However, the labeling pattern of malate, regardless of its mode of formation, can be readily randomized if the malate is
subjected to TCA cycle reactions. This randomization can be re-
presented as follows for one turn of the TCA cycle.

\[
\begin{align*}
\text{HOOC} & \quad - \quad \text{CH}_2 & \quad - \quad \text{CH} & \quad - \quad \text{COOH} & \quad \overset{\Delta}{\longrightarrow} & \quad \text{HOOC} & \quad - \quad \text{CH}_2 & \quad - \quad \text{CH} & \quad - \quad \text{COOH} \\
\text{O} & \quad + & \quad \bullet & \quad + & \quad \bullet & \quad \bullet & \quad \bullet & \quad +
\end{align*}
\]

The malate labeling pattern may also be randomized by
participating in a reversible fumarase reaction. In this mechanism,
fumarate, by virtue of being a symmetrical compound, will lead to
randomization between C-1 and C-4 and between C-2 and C-3 of
malate. Table IV shows a nearly equal distribution of radioactivity
between C-1 and C-4 and between C-2 and C-3 of malate, which
must reflect extensive randomization of fruit malate via a symmetri-
cal intermediate such as fumarate. The anticipated labeling pattern
of malate derived from C-1, C-2 or C-6 of glucose, before and
after randomization through a symmetrical intermediate, is
summarized in comparison with the observed labeling pattern in
Table VII.

It is evident that the labeling pattern of malate observed
in the glucose-2-C\textsuperscript{14} experiment is by far the most informative one,
since in this case, C\textsuperscript{14} can be incorporated into the middle carbon
atoms of malate only via a CO\textsubscript{2}-fixation process. Thus, with 40
percent of the activity of malate from glucose-2-C\textsuperscript{14} residing in
the middle two carbon atoms, it can be concluded that the minimal
amount of malate formed via a CO\textsubscript{2}-fixation reaction in tomato
fruit is 40 percent.
TABLE VII. Isotopic Distribution Patterns of Malate for Various Pathways Before and After Randomization via the Fumarase Reaction.

<table>
<thead>
<tr>
<th>Pathway for Malate Synthesis</th>
<th>Expected and Observed Isotopic Distribution in Malate (percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HOOC</td>
</tr>
<tr>
<td>With glucose-1-C¹⁴:</td>
<td></td>
</tr>
<tr>
<td>via CO₂-fixation (initial)</td>
<td>0</td>
</tr>
<tr>
<td>via CO₂-fixation (randomized)</td>
<td>0</td>
</tr>
<tr>
<td>via Malate Synthetase (initial)</td>
<td>0</td>
</tr>
<tr>
<td>via Malate Synthetase (randomized)</td>
<td>0</td>
</tr>
<tr>
<td>observed</td>
<td>17</td>
</tr>
<tr>
<td>With glucose-2-C¹⁴:</td>
<td></td>
</tr>
<tr>
<td>via CO₂-fixation (initial)</td>
<td>0</td>
</tr>
<tr>
<td>via CO₂-fixation (randomized)</td>
<td>0</td>
</tr>
<tr>
<td>via Malate Synthetase (initial)</td>
<td>50</td>
</tr>
<tr>
<td>via Malate Synthetase (randomized)</td>
<td>50</td>
</tr>
<tr>
<td>observed</td>
<td>27</td>
</tr>
<tr>
<td>With glucose-6-C¹⁴:</td>
<td></td>
</tr>
<tr>
<td>via CO₂-fixation (initial)</td>
<td>0</td>
</tr>
<tr>
<td>via CO₂-fixation (randomized)</td>
<td>0</td>
</tr>
<tr>
<td>via Malate Synthetase (initial)</td>
<td>0</td>
</tr>
<tr>
<td>via Malate Synthetase (randomized)</td>
<td>0</td>
</tr>
<tr>
<td>observed</td>
<td>14</td>
</tr>
</tbody>
</table>

However, in reality, the contribution of CO₂-fixation reactions to malate biosynthesis may be considerably greater than 40 percent. On the surface, the detected labeling at the carboxyl carbon atoms of malate in the glucose-2-C¹⁴ experiment may reflect the operation of the malate synthetase reaction. However, it should be noted that the label of C-2 of glucose can occur at the carboxyl carbon atoms of malate even though the latter is synthesized via the CO₂-fixation process. Firstly, any recycling of
malate via the TCA cycle pathway will transfer the label from the middle carbon atoms of malate to the carboxyl carbon atoms. The occurrence of this mechanism is evidenced by the finding of label of C-6 of glucose in the carboxyl carbon atoms of malate. In fact, the observed label distribution pattern of malate in the glucose-6-C\textsuperscript{14} experiment provides a direct indication to the extent of occurrence of this randomization mechanism. And another complicating factor is the fact that when malate is converted to oxalacetate and condensed with acetyl-coenzyme A derived from glucose (the labeling pattern is \( \text{O} \ \text{CH}_3 - \text{C} \sim \text{S} \text{CoA} \)) the resulting citrate, upon conversion to malate, will have the label of C-2 of glucose located at the carboxyl carbon atoms.

By taking into consideration the foregoing mechanisms for label randomization, it can be readily concluded that the direct incorporation of label from C-2 of glucose occurred primarily in the middle carbon atoms of malate. This contention implies that the CO\textsubscript{2}-fixation process is by far the most important mechanism for the biosynthesis of malate in tomato fruit.
SUMMARY

Glucose-1-C\textsuperscript{14}, glucose-2-C\textsuperscript{14} and glucose-6-C\textsuperscript{14} substrates were incorporated into tomato fruit, with subsequent isolation and degradation of labeled metabolic intermediates. The data thus obtained indicated the existence of a large endogenous pool of carbohydrates in tomato fruit, and a comparison of the specific activities of fruit glucose and fructose has indicated that glucose-6-phosphate and fructose-6-phosphate may not be in isotopic equilibrium in this organism. The distribution of radioactivity in fruit glucose also established the fact that triose recombination is occurring via both the EMP and the PP pathways. The impact of this process on the derivation of equations for the estimation of pathway participation was examined, and it was concluded that the previous estimation of pathway participation in tomato fruit would not be significantly altered by the occurrence of triose recombination.

Examination of the organic acids isolated from the fruits indicated that the EMP-TCA sequence is the primary source of carbon skeletons for the biosynthesis of fruit acids. The labeling distribution pattern of malate demonstrated that a CO\textsubscript{2}-fixation reaction of the C\textsubscript{3} + C\textsubscript{1} type is primarily responsible for the net biosynthesis of C\textsubscript{4} acids in tomato fruit.
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