ISOTOPIC TRACER STUDIES OF
CARBOHYDRATE METABOLISM
IN FRUITS

by

DONALD RAYMOND BUHLER

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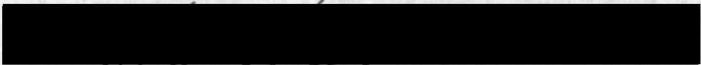
Associate Professor of Chemistry

In Charge of Major

██████████████████
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Chairman of Department of Chemistry

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Chairman of Graduate School Committee

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Dean of Graduate School
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Date thesis is presented   June 1956

Typed by ____Mary S. Fellman____

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INTRODUCTION

Considerable attention has been focused on the fascinating variformed group of plant organs known as fruit, ever since an apple reputedly inspired a seventeenth century physicist to formulate the laws of gravitation. In spite of a multitude of studies carried out on all varieties of fruits which were primarily aimed at practical solutions to problems of preservation and usage, today many pertinent questions concerning the metabolism of fruit remain unanswered, particularly the factors which govern the chemical and physiological transformations occurring during the ripening process.

The availability of radioactive isotopes and the subsequent development of tracer methodology in combination with the new analytical techniques such as chromatography and radioautography have become powerful and effective tools in attacking many difficult biological problems. Thus, in the past few years tracer technology has met with remarkable success, elucidating, for example, the path of carbon in photosynthesis and bringing to light a number of new and largely unsuspected pathways of glucose metabolism.

The present study deals with the application of tracer techniques toward the solution of some of the more baffling problems concerning the metabolism of fruits.

Carbohydrates and fruit respiration. Sugars, constituting the major portion of the carbohydrate materials in fruits, are manufactured in the leaves of higher plants by the process of photosynthesis and are translocated into the fruits. The free sugars of fruits consist mainly of D-fructose, D-glucose and sucrose. Starch is the common form of sugar storage in plants, and is formed in the actively growing fruit during the time when carbohydrate material is transported into this organ in quantity. As growth slows down and maturation occurs, starch rapidly disappears.

Williams and Bevenue (115, pp. 472-474) have examined the specific carbohydrate composition of ripe tomatoes and found that the free sugars, comprising over 60 per cent of the solids in tomatoes, were: D-fructose and D-glucose in a ratio of 1.2 to 1, a small amount of sucrose and a trace of ketopentose. Polysaccharides made up only 19 per cent of the total tomato solids and consisted of: 22 per cent pectic substances, 17 per cent α-cellulose, 17 per cent protein material, 13-23 per cent of material yielding D-xylose + D-glucose on hydrolysis and 21 per cent araban-galactan material showing L-arabinose, D-galactose and glucuronic acid upon hydrolysis. They were unable to find starch in tangible amounts.

Fruits oxidize organic materials such as sugars, organic acids, and fats first to certain intermediate compounds and ultimately to carbon dioxide and water. Respiration in fruits serves two chief

functions, as it does ubiquitously in all living cells: (a) to pro-
vide energy for endergonic cellular process and (b) to oxidize
materials such as carbohydrates into various organic acids etc. which
are needed by the fruit for synthetic purposes. The overall equation
for the complete oxidation of glucose may be represented as:

$$C_6H_{12}O + 6O_2 \longrightarrow 6CO_2 + 6H_2O + energy \qquad (1)$$
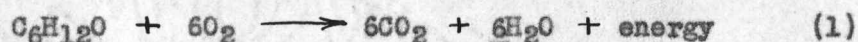
The respiration rate of a fruit is not constant, but changes with
maturation of the organ. If one examines the life history of each
fruit, the following four periods become apparent, namely: (a) a
growth period of high respiration rate with extensive cell division,
characteristic of young fruits; (b) a period of much lower respiration
rate during which cells enlarge enormously; (c) a maturation period,
of essentially constant respiration rate, terminated by a sudden rise
in respiration, which has been termed the "climacteric" by Kidd
(57, p. 327), marking the onset of fruit ripening; and (d) a decrease
in respiration during the senescence and breakdown of tissues.

The marked rise in oxygen uptake and carbon dioxide evolution,
the climacteric, is a characteristic phenomenon of the ripening
process, transitions from greeness to ripeness taking place in
certain fruits during or immediately following the climacteric
(16, p. 190). In contrast to this, ripening lemons and oranges do
not show a climacteric rise of CO_2 production in air but do so under
concentrations of oxygen exceeding 33 per cent (15, pp. 301-309).

Generally speaking, external factors which increase the respiration rate of fruits also hasten the onset of the climacteric and therefore, ripening, while factors which decrease respiration correspondingly delay ripening. These factors include: concentration of oxygen and carbon dioxide in the external atmosphere, temperature, nutrition of the fruit and the effect of stimulating chemicals such as ethylene.

It is not surprising that the partial pressure of oxygen plays and important role in the respiration and growth of plant organs since it is one of the reactants in the process of respiration. Kidd and West (58, pp. 467-504) reported that oxygen concentration influenced the time of the onset of climacteric in apples and Claypool and Allen (28, pp. 103-113) observed lower respiration rates of apricots, plums, peaches, pears and grapes at oxygen levels lower than air. If the oxygen concentration of the atmosphere surrounding the fruits is reduced below the critical value of 0.5 to 5 per cent oxygen at 15° C., anaerobic respiration would result, as indicated by the increased rate of carbon dioxide evolution (18, pp. 412-445). On the other hand when oxygen concentration is increased above 21 per cent, the respiration rate of fruits is considerably increased (58, pp. 467-504).

In general, the respiration rate of fruits decrease with increasing concentration of CO_2 in the surrounding atmosphere (100, pp. 371-402 and 102, pp. 453-458). The mechanism for this phenomenon is yet uncertain although it might be conveniently

explained by the law of mass action. It has also been suggested that
the presence of CO_2 displays an effect on the pH of fruit tissue which
might also effect respiration (100, pp. 371-402). In controlled atmos-
phere storage of fruits, use is made of the foregoing observations by
employing high concentrations of CO_2 and low concentrations of oxygen
to slow respiration and delay ripening.

It has long been recognized that the respiration rate per unit
weight of sections of plant organs (carrot root, beet root and potato
tuber) is 3-6 times higher than that of the intact organs. Scott
(91, pp. 1-150) showed that this phenomenon is not due to oxygen dif-
fusion but rather is the result of relatively higher internal CO_2
pressure inside the plant organs. Denny (31, pp. 383-396) has demon-
strated that large amounts of CO_2 are retained by plant tissues
especially at higher temperatures.

The temperature of the fruit markedly affects the concentration
of oxygen and CO_2 in the intercellular spaces of fruits. At higher
temperatures when the cells of the fruit are actively respiring,
oxygen cannot diffuse into the fruit as fast as it is being consumed
and similarly, CO_2 cannot diffuse out of the fruit as rapidly as it
is being produced. Magness (67, pp. 308-316) has found that in
apples at 30° C. the internal oxygen concentration is only 3 per cent
while the internal CO_2 concentration has risen to 21 per cent.

The respiration rate of fruits is generally proportional to the
temperature, increasing with an increase in temperature. A measure of

this increase in respiration rate for any ten degree change in temperature, is the temperature coefficient (Q_{10}). The Q_{10} for respiration of fruits varies with variety, age of the fruit, temperature, composition of the atmosphere etc. In avocado (16, p. 192) the value of Q_{10} in the range of 5-15° C. changed from approximately 3.5 in the preclimacteric phase to 7.0 at maximum respiration. In general, however, the Q_{10} for most green fruit is in the range of 2-3 but this value may increase markedly with ripening.

The volume of carbon dioxide produced divided by the volume of oxygen consumed in a living system is defined as the respiratory quotient (R.Q.) and it may be seen from equation (1) that in the complete oxidation of glucose, the R.Q. is unity. The R.Q. reflects the proportion of oxygen as compared with carbon in the material being oxidized, having values of less than 1 for oxygen "poor" materials such as fats and values of greater than 1 for oxygen "rich" organic acids.

Not only does the R.Q. vary with the age of the fruit (increasing with age) but it also varies considerably with temperature. Thus, Gerber (37, pp. 1-279) has obtained the following data for grapes: between 0° and 20° C., the R.Q. is 0.7; around 20° it becomes 1.0; at 30° it is 1.2; and at 35° C. it reaches 1.6. These facts have been interpreted by Gerber to indicate that under 20° C. sugars are incompletely respired and malic acid is formed, at 20° C. sugars are

completely metabolized, at 30° C. malic acid is chiefly oxidized and at 35° C. tartaric acid also is burned.

Until recently, it was thought that hexose was converted to pyruvic acid in higher plants only via the classical Embden-Meyerhof-Parnas glycolytic pathway (EMP) and that pyruvic acid is further oxidized via the Krebs tricarboxylic acid (TCA) cycle through the intermediary of the common organic plant acids to CO_2 and H_2O (21, pp. 167-218).

However, early studies by Warburg (111, pp. 157-205 and 112, pp. 287-295), Lipmann (65, pp. 588-589) and Dickens (32, p. 1057) indicated that there was a mechanism in yeast for the oxidation of glucose to carbon dioxide which did not involve the EMP-TCA pathway. After the lapse of many years, ribose-5-phosphate was identified as an oxidation product of this reaction (90, pp. 509-530) and ribulose-5-phosphate was established as the primary decarboxylation product (49, pp. 383-396). Isolation and identification of hexose monophosphate and diphosphate (34, pp. 252-253), heptulose monophosphate and diphosphate and ribulose diphosphate (14, pp. 703-716) occurred in rapid sequence in both plant and animal systems. The cyclic nature of this pathway, variously called the "hexose monophosphate shunt", the "direct oxidative pathway", the "pentose cycle", the "Warburg-Lipmann-Dickens pathway" or more precisely "phosphogluconate oxidation pathway", has been established (51, pp. 214-219 and 84, pp. 141-182). Enzymes of this pathway have also been isolated and characterized in higher

plant tissue (8, pp. 619-634; 11, pp. 115-122; 12, pp. 322-324; and 38, pp. 34-39).

Horecker et al., in recent investigations with rat liver preparations (51, pp. 393-403) and with pea roots and leaves (41, pp. 812-820), have determined the isotope pattern in the hexose monophosphate formed from pentose-C^{14} and found the distribution to be in agreement with the proposed pathway.

In the EMP-TCA cycle both C-1 and C-6 positions of glucose are converted to the methyl groups of pyruvate and thereafter metabolized identically. Thus the order of appearance of the glucose carbons in the respiratory CO_2 from the operation of the EMP-TCA pathway is

$$glucose \longrightarrow CO_2(3,4) + CO_2(2,5) + CO_2(1,6) \qquad (2)$$
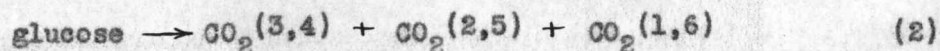
However, in the phosphogluconate oxidative pathway as well as other alternate pathways of glucose catabolism (35, pp. 853-862; 39, pp. 689-694; and 43, pp. 871-875), C-1 of glucose is preferentially converted to CO_2 and consequently C-1 and C-6 are metabolized at different rates; the fate of the carbon skeleton of glucose is illustrated in Figure 1. Because of this difference, the most important contributions to an evaluation of alternate pathways has come from isotope studies. Using glucose labeled with carbon[14] at various positions on the molecule, a number of investigators have studied the pathways of glucose metabolism and found evidence for operation of one or more alternative pathways in: yeast (13, p. 640; and 109), Penicillium digitatum (74, pp. 14-58), mammalian tumors (61, pp. 70-76), mammalian liver (19, pp. 555-563; and

Figure 1

55, pp. 853-868), corneal epithelium (60, pp. 72-73) and higher plants (41, pp. 343-347). These experiments are based upon the consideration that the mechanism of glucose breakdown will determine the relative rates of conversion of radioactive glucose carbon into respiratory CO_2; thus, if the EMP-TCA pathway alone is operating, the per cent radiochemical yield of $C^{14}O_2$ from glucose-6-C^{14} divided by the per cent radiochemical yield of $C^{14}O_2$ from glucose-1-C^{14} (G-6/G-1) will be unity. However, if the phosphogluconate oxidative pathway or other alternative pathways are in operation, the C-6/C-1 ratio will be less than one. The smaller this ratio, the greater the contribution from pathways other than the classical EMP-TCA.

Calculations which have been devised to assess the relative importance of the alternative pathways to the overall catabolism of glucose, have met with varied success (1, pp. 31-38; 2, pp. 773-779; 19, pp. 555-563; 20, pp. 6093-6097; 55, pp. 853-868; 61, pp. 70-76; 64, pp. 273-286; and 109). These calculations are based upon a number of assumptions which, in general, appear to oversimplify the extremely complex interrelationship between the various metabolic pathways. However, certain of the proposed methods of calculation seem to offer a promise of circumventing this objection.

Organic acids are intermediates in the oxidation of glucose to CO_2 and water in plants, and thus occupy a central position in the metabolism of fruits (26, pp. 91-114). It is well known that in growing fruits the general pattern is one of progressively increasing

organic acid content followed by a decrease during the ripening process. Furthermore, the organic acid content varies also with external factors, such as climate and temperature. For example, Peynaud (80, pp. 177-180) has demonstrated a correlation between climate and tartaric acid content of grapes, the acid increasing in rainy periods and decreasing in dry sunny periods. The effect of temperature on organic acid metabolism has been demonstrated in leaves of Bryophyllum (83, pp. 123-132). The relatively lower night-time temperatures stimulate production of organic acids, whereas temperatures higher than 30° C. cause acid levels to decrease.

Di- and tricarboxylic acids are very abundant in fruit tissue, reaching as much as 7 per cent in peeled lemons (94, p. 146). The principle acids of fruit are: malic (pome fruit), citric (citrus fruit), tartaric (grapes) and isocitric (blackberries). Using column chromatography, Bulen, Varner and Burrell (25, pp. 187-190) found the acid content of ripe tomato fruit expressed in milliequiv-alents $x\ 10^2/10$g. fresh weight to be: acetic 2.1, formic 0.9, lactic 1.0, trans-aconitic 3.9, malic 4.2 and citric 66.

The biosynthesis of these acids has recently been investigated in fruits by means of radioisotopes. Wang et al. (108, pp. 683-688) have shown that, following injection of sodium acetate-1-C^{14} into mature green tomatoes, radioactivity appears in the respiratory CO_2, protein, organic acid and certain other fractions isolated from the homogenized fruits. Degradation of citric and malic acids isolated therefrom,

indicated the formation of citric acid and the active operation of the tricarboxylic acid cycle as the major pathway of acetate metabolism and the biosynthesis of citric acid in tomato fruit. However, these authors point out that this cycle alone could not account for any net synthesis of plant acid and therefore, some other mechanism is available for synthesizing the required C_4 acids. Such synthesis could occur by the Wood-Werkman reaction (116, pp. 377-388), by the "malic fixation" scheme (76, pp. 979-1000), or by a C_2-C_2 condensation of the Thunberg type (101, pp. 1-91); these possibilities would all lead to exclusive carboxyl carbon labeling in malic acid, as Wang and coworkers have observed.

Allentoff et al. (5, pp. 231-234 and 6, pp. 234-238) studied the fixation of $C^{14}O_2$ into mature, stored McIntosh apples kept in the dark and found that approximately 64 per cent of the incorporated radioactivity appeared in malic acid. Upon degradation of this acid, 99 per cent of the activity was found to be equally distributed between the carboxyl carbons of the isolated malic acid. The hypothesis was advanced that CO_2 fixation into malic acid represents a significant contribution to the accumulation of acid in the ripening fruits. The rate of malic acid production was also found to be proportional to the internal CO_2 concentration.

Fruit ripening and ethylene. The ripening of fruits is accompanied by a group of characteristic physiological and chemical changes which are similar for different species of fruits. Typical symptoms

of the ripening process are: decrease in firmness due to pectic transformations, disappearance of pigments such as chlorophyll and the appearance of new secondary pigments, a decrease in acidity, conversion of polysaccharides to simpler sugars, and a change in the respiration rate. Sando (89, pp. 1-38) found that, in general, throughout the ripening period in tomatoes there is an increase in moisture and sugars and a decrease in solids, acids, total nitrogen, starch, pentosans, crude fiber and ash. The most striking change is that undergone by carbohydrates; sugars increase from 25.7 per cent (dry basis) in fruits 14 days old to 48.3 per cent in ripe fruits. Starch decreases in the same interval from 15.8 per cent to 2.65 per cent (dry basis). During the early stages of the ripening process the sucrose content is low but increases sharply during the final stages.

Application of ethylene gas in concentrations as low as 1 part per million accelerates the onset of the climacteric in fruits if it is applied while the fruits are in the pre-climacteric stage (16, p. 195). In citrus fruits, which do not display a climacteric rise in air, the respiration rate is stimulated by ethylene (29, pp. 322-329). On the other hand in fruits with a climacteric rise, ethylene does not modify the normal respiratory pattern except to shift the time axis (16, p. 192). Ethylene exerts a stimulating influence on subsequent ripening of tomatoes, within the limits of concentration from 1:1000 to 1:30,000, the optimum concentration being 1:8000

(88, pp. 315-322). Ethylene also accelerates ripening in Bartlett Pears (105, pp. 257-264) and in apples (34, pp. 637-643).

Hansen (45, pp. 145-161) has investigated the effects of ethylene on chemical changes in pears and observed an increase in the rate of starch hydrolysis, higher sugar content and accelerated transfer of protopectin to pectin. Ethylene is also known to reduce the acid content in apples (3, pp. 381-441) and hasten lycopene formation as well as chlorophyll decomposition in tomatoes (104, pp. 929-955). All of these changes occur during normal ripening and, in fact, Biale (16, p. 192) states that nearly all the changes brought about by ethylene treatment were changes that would occur during the regular course of ripening.

Ethylene has been identified as a prominent constituent among the volatile emanations from many plants. Hansen and Christensen (46, pp. 403-409) using a quantitative microbromination procedure, determined the amounts of ethylene produced by several varieties of apples and pears during ripening and found it to be within a range of 0.001 to 0.28 ml./kg. fruit/hour. Ethylene is also produced by bananas (71, pp. 357-361), turnip and rutabaga roots (30, pp. 431-438), detached leaves (81, pp. 16-18) and the fungus _Penicillium digitatum_ (117, pp. 304-310). It is interesting to note that not all fruits produce ethylene in ripening; Biale _et al_. (17, pp. 168-174) have confirmed previous observations that oranges and lemons, fruit which display no climacteric rise in air, do not produce ethylene.

The biosynthetic pathway leading to the production of ethylene
is rather obscure. Hall (44, pp. 55-65) has studied the effect of
different substrates upon the production of ethylene from crude
extracts of apple juice and Penicillium digitatum and postulated that
ethylene arises by enzymatic degradation of a number of active
substrates during respiration. Maximum stimulation of ethylene produc-
tion was observed by the addition of arabinose, ethanol, pectin, pectic
acid, pyruvic acid, fructose or galactose to the test system. However,
this work has been seriously criticized because of a lack of specific-
ity in the method used to detect ethylene (17, p. 172).

At a certain stage of maturity, fruits by their own metabolism
produce ethylene but it remains to be clarified, whether ethylene
actually induces the climacteric rise in a type of autostimulation or
is merely a by-product of the ripening process. Recently Biale et al.
(17, pp. 168-174) studied the relation of ethylene production to the
climacteric rise in 14 species of fruits. In general, they found no
definite evidence whether ethylene production precedes or follows the
onset of the respiratory rise and advanced the hypothesis that ethylene
is a product of the ripening process rather than a causal agent.
Hansen (47, pp. 543-548) showed that ethylene production was not
directly related to respiration in pears. With an increase in temper-
ature beyond 20° C., respiration continued to rise and ethylene produc-
tion dropped until at 40° C. no significant emanation could be
detected.

It has been suggested that concentration of phosphate acceptors and not ethylene may be the controlling factor of the climacteric. Thus, Robertson and Turner (87, pp. 92-107) proposed that the respiration rate in plant tissues may often be limited by the low concentration of phosphate acceptors available when the phosphorylations from respiration are more rapid than the dephosphorylations of synthesis. They suggest that the climacteric is brought about by the increased production of some phosphate acceptor resulting in a more rapid utilization of energy-rich phosphate. Millard et al. (70, pp. 521-531) on the other hand suggest that the climacteric results from the uncoupling of oxidation from phosphorylation due to the production of a natural uncoupling agent. Pearson and Robertson (79, pp. 1-17), using small discs of apple tissue, observed that 2,4 dinitrophenol (DNP) and adenosine triphosphate (ATP) increased the oxygen uptake of preclimacteric fruits and that neither compound had an effect on postclimacteric fruits. Millard et al. (70, pp 521-531) verified these observations using mitochondrial particles from preclimacteric and climacteric avocados and observed that addition of a phosphate acceptor, adenylate, increased the respiration of the particles from immature but not from mature fruits.

In view of the confusion or lack of information concerning the various pathways of carbohydrate metabolism in fruits, the present investigation has been carried out in respect to the following scheme (a) the primary breakdown patterns of glucose, qualitative and

quantitative studies; (b) the fate of the glucose breakdown products; (c) the influence of external factors on (a) and (b); and (d) the origin and mode of action of ethylene.

EXPERIMENTAL AND RESULTS

Green tomato fruits were used in the major part of this work as the test system, however, in some instances, other fruits have been employed for comparative studies on certain aspects of carbohydrate metabolism. The tomatoes used in early studies were greenhouse grown and picked at the mature green state; field grown tomato fruits were also used in later experiments. The green cucumbers and ripe oranges and limes used in other experiments were purchased on the local market.

A critical requirement for studies of the present type is the uniformity of the conditions under which results are obtained and compared. In actual practice, it has been a difficult task in obtaining fruits which are comparable in respect to their stage of maturity and physiological condition. Although efforts were made to minimize these variations, fruits of approximately equal weight, size and shape picked at the same state of maturity being used in each set of experiments, nevertheless, one must realize certain limitations are inherent to the sampling problem in experiments involving biological systems.

Other methods of circumventing the limitations imposed by sample variations have been reported. Thus, aliquots of a homogenate prepared from a single fruit may be employed as test system in a given set of experiments. Aside from the difficulties involved in the preparation of an active plant homogenate, however, no feasible method could yet

be devised to keep such preparations free from microbial contamination during the long experimental periods required for the present studies. The same limitations also apply to the application of tissue slice techniques.

A. Detection of Carbohydrate Breakdown Patterns:

For these studies, small amounts of various specifically carbon^{14}-labeled glucose samples were introduced into intact, excised fruits and the respiratory CO_2 collected. By comparison of the rates of utilization of the different glucose substrates in an air atmosphere as indicated by the rate of $C^{14}O_2$ evolution, it was possible to detect and estimate quantitatively the pathways of glucose metabolism normally occurring in fruits.

Due to the nature of the development of techniques in the present study, the earlier versions of these procedures suffered from various shortcomings. The results obtained, nevertheless, provide qualitative information which led to the perfection of the experimental method and are, therefore, included here.

1. Introduction of labeled substrates:

Glucose-1-C^{14}, glucose-2-C^{14} and glucose-6-C^{14} were purchased from the National Bureau of Standards; glucose-U-C^{14} was purchased from Tracerlab, Inc., all on allocation from the Atomic Energy Commission. For most experiments, labeled glucose was diluted with a calculated

amount of nonradioactive glucose so that all glucose substrates had identical specific activities.

In preliminary experiments, the labeled glucose, in solution, was injected into the fruits by means of a sterile needle and syringe (104, pp. 741-745), however, this technique has proved unwieldy and subject to error. Consequently, a technique has been developed which enabled the operator to rapidly introduce solutions of labeled substrate with a high degree of accuracy and at the same time the diffusion of labeled material into the fruits was greatly improved, as indicated by trials with colored dyes.

In practice, the region of the fruit selected for treatment was first disinfected with 95 per cent ethanol (if this precaution was not taken, extensive mold growth occurred in the vicinity of the drilling site within 48 hours). A sterile cork borer was then used to drill into the center of the fruit leaving, after removal of the plug of flesh, a cylindrical well approximately one inch in depth. The desired amount of radioactive solution was next pipetted into this cavity by means of a sterile drawn-out pipette and the opening finally sealed from the air with paraffin wax.

With these precautions, no noticeable mold growth or rotting has been observed over a period as long as 169 hours following introduction of labeled material.

To further ensure maximum possible penetration of the labeled substrate, the treated fruits were placed in an evacuated container for

5 minutes prior to the actual start of the experiments.

2. <u>Collection of respiratory</u> CO_2:

a). <u>Closed system with internal</u> CO_2 <u>collection</u>:

The fruits, after introduction of labeled substrate, was placed in a 1500 ml. wide-mouthed dark brown bottle. A 50 ml. beaker containing 20 ml. of 50 per cent KOH for collection of respiratory CO_2 was also placed into the bottle and the latter was then sealed with a large rubber stopper into which had been inserted a short piece of glass tubing equipped with a rubber vial seal. The apparatus is illustrated in Figure 2. Usually a battery of 2 or 3 bottles was employed in each experiment, each bottle containing a fruit into which had been introduced a different glucose substrate.

The bottles were evacuated by means of a hypodermic needle inserted through the vial seals and then were connected to manometers to test for leakage, after which, pressure inside the bottles was restored to normal by venting to air.

At the end of the experimental period, the vessels were opened, the CO_2 trapping solutions taken out and the respiratory CO_2 from the respective fruits recovered as $BaCO_3$ by the addition of 1 N $BaCl_2$ plus 1 N NH_4Cl solution, followed by filtration.

In a trial experiment using this equipment, an orange fruit was placed in the respiration chamber for 84 hours and, at the end of this period, the residual CO_2 was swept from the bottle with

Figure 2 Static System

CO_2-free air and trapped in a conventional sintered glass scrubber with CO_2-free NaOH. Approximately 1 per cent of the metabolic CO_2 was found in this external trap, the remaining 99 per cent being recovered in the internal KOH trap. This suggests a very high trapping efficiency for an internal trap of this sort, hence respiratory CO_2 could not have accumulated to any great extent in the system.

However, this procedure still suffered from several serious disadvantages. In this system, there was a constant decrease in the oxygen partial pressure inside the experimental flask since no attempt was made during the experiment to replenish the oxygen taken up by the fruit (CO_2 was being continuously absorbed by the trap solution, hence a partial vacuum developed). Of a more serious nature was the accumulation of volatile fruit emanations such as ethylene which might have serious effects on overall fruit metabolism.

In an effort to overcome or at least reduce the effect of these factors, the internal KOH trap solutions were periodically replaced with fresh solutions at approximately 10 hour intervals, the atmosphere inside the bottles simultaneously being replaced. Furthermore, a second beaker containing a solution of mercuric perchlorate (118, pp. 551-555) was introduced into each bottle in order to trap any ethylene gas produced by the fruits and thus prevent it from accumulating in the system.

In a typical experiment employing all of the above

modifications, two mature green tomato fruits of approximately equal weight (weights 118 and 121 g.), color and diameter were selected; and into the first was introduced 0.05 ml. of a solution of glucose-1-C^{14} (specific activity 3.55 x 10^7 c.p.m./mmole) containing 79,500 c.p.m. using the technique described in Section 1. An identical amount of glucose-6-C^{14} having the same specific activity was then added to the second fruit. Each fruit was placed in a bottle of the type described above and allowed to stand at room temperature (20-25 $^\circ$ C.), the KOH trapping solution being replaced at 9, 16.5, 24, 35 and 45 hours, respectively.

The $BaCO_3$ samples recovered from the trapping solution in the glucose-1-C^{14} and glucose-6-C^{14} experiments were mounted on standard flat planchets using special Atomlab-Exstein demountable centrifuge tubes (The Atomic Center, New York) and counted to a standard error of 5 per cent under a shielded thin window GM tube (Tracerlab, Inc., Model TGC-2) in the conventional manner with corrections for background and self-absorption properly applied.

The results from this experiment are tabulated in Table I along with the results of similar studies carried out on green cucumbers.

b). Intermittent sweeping and trapping of metabolic CO_2:

Table I

Oxidation of Glucose-1-C^{14} or Glucose-6-C^{14} to $C^{14}O_2$

by Fruits in Closed Systems

Exp. No.	Exp. Duration (hours)	Type of Fruit	Glucose[a] Label	Level Used (cpm)	Activity per mmole BaCO_3 (cpm x 10^2)	Total Activity (cpm)	$\frac{G-6}{G-1}$[b]
1	48	Cucumber	G-1	49,800	13.5	10960 (22%)	
"	"	"	G-6	"	8.69	6550 (13%)	0.60
2	45	Tomato	G-1	75,000	14.8	6128 (7.6%)	
"	"	"	G-6	"	9.57	4455 (5.6%)	0.73

a % radiochemical yield
   Specific activity of glucose-1-C^{14} and glucose-6-C^{14} was 3.55 x 10^7 c.p.m./mmole glucose.
b G-6/G-1 is the % radiochemical yield from glucose-6-C^{14}/% radiochemical yield from glucose-1-C^{14}

Subsequently, a modified procedure was developed which permitted qualitative comparative studies to be made simultaneously on several different species of fruits under varying experimental conditions. The test fruits, after introduction of labeled substrates, were placed in a battery of 1500 ml. wide-mouthed brown bottles equipped with rubber stoppers and two stopcocks each. The bottles were next evacuated to test for leakage in the manner outlined previously and meanwhile permitting better diffusion of the administered labeled glucose throughout the fruits. After a period of 5 minutes, the bottles were restored to normal pressure and allowed to stand at room temperature during the test period. Following a suitable time interval (usually three hours), the bottles were connected to a NaOH scrubbing tower and flushed with CO_2-free air and the respiratory CO_2 trapped as Na_2CO_3. The bottles were swept as rapidly as practicable (200 ml. air/minute) for a standard period of 15 minutes, and upon completion of the sweeping period, the stopcocks were closed again until the next sweeping.

After examination of the radioactivity of the $BaCO_3$ samples recovered after each sweeping, it was possible to compare in a qualitative manner the internal accumulated radioactivity in the respiratory CO_2 from the respective C^{14}-labeled glucose substrates, throughout the experimental period.

Since both CO_2 and fruit emanations such as ethylene were allowed to accumulate in the bottle by this procedure (the CO_2 in

even greater amounts than in the closed system) one could not expect
to obtain the completely normal picture for the oxidation of the
labeled glucoses. However, it is nevertheless possible to amass
valuable data of a qualitative nature by means of this technique.
The results from a number of experiments of this type are given in
Table II.

c). Continuous sweeping and absorption of respiratory CO_2:

The experimental technique was further improved by the in-
novation of continuous sweeping of the respiratory CO_2. In this pro-
cedure, flasks of the type described previously were attached to NaOH
traps and a stream of CO_2-free air (100 ml. air/minute) was led
through each bottle to flush the metabolic CO_2 of the fruits and trap
it in CO_2-free NaOH solution; a photograph of the experimental appara-
tus is shown in Figure 3. At suitable intervals, the trapping solution
was replaced and $BaCO_3$ precipitated from the solution in the manner
previously described.

The use of a continuous stream of fresh, CO_2-free air elimi-
nates certain of the difficulties encountered in the earlier experi-
ments. The accumulation of any biologically active fruit emanations
(ethylene) and of CO_2 which might alter the pattern of carbohydrate
metabolism was circumvented. Also, the production of $C^{14}O_2$ could be
followed more closely by changing the NaOH trapping solutions at short
time intervals, hence minimizing any possible time lag between the

TABLE II

OXIDATION OF GLUCOSE-1-C^{14}[a], GLUCOSE-6-C^{14} OR GLUCOSE-U-C^{14} TO C^{14}O$_2$
BY FRUITS WITH INTERMITTENT SWEEPING

Exp. No.	Type of Fruit	Level Used (c.p.m.)	30 hour % Radiochemical Yield			G-6/G-1 [b]	45 hour % Radiochemical Yield			G-6/G-1
			G-1 (%)	G-6 (%)	G-U (%)		G-1 (%)	G-6 (%)	G-U (%)	
3	Lime	83,000	12.4	4.4	-	0.35	-	-	-	-
4	Orange	195,500	-	-	10.6	-	-	-	17.8	-
5	"	78,200	10.9	4.2	-	0.39	17.3	7.7	-	0.45
6	"	94,500	7.2	5.3	-	0.74	14.0	9.5	-	0.68
7	"	47,500	9.8	6.5	-	0.66	14.4	9.1	-	0.63
8	"	163,000	3.2	1.7	-	0.52	6.8	2.3	-	0.34
9	Cucumber	83,000	21.1	9.2	-	0.44	-	-	-	-
10	Tomato	156,000	6.3	2.8	-	0.44	9.0	4.5	-	0.50

[a] Specific activity of glucose-1C^{14} and glucose-6-C^{14} was 3.55 x 10^7 c.p.m./mmole glucose; specific activity of glucose-U-C^{14} was 1.39 x 10^7 c.p.m./mmole glucose.
[b] G-6/G-1 is the per cent radiochemical yield from glucose-6-C^{14}/ per cent radiochemical yield from glucose-1-C^{14}
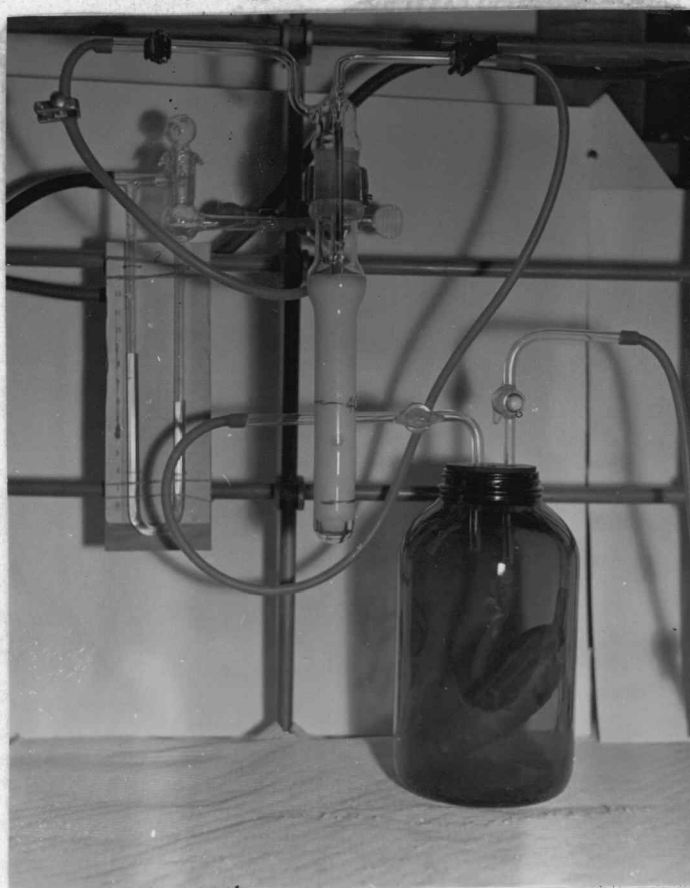
Figure 3 Continuous Sweeping System

actual time of $C^{14}O_2$ production and the time of trapping. The closed

system described under (a) and to a lesser extent, the intermittent

sweeping system (b) both yield $BaC^{14}O_3$ from respiratory CO_2 which has

been released by the fruits over a fairly long time interval. Thus

there is a "totalizing" effect in which $C^{14}O_2$ of relatively higher

specific activity, released during the early hours of the experimental

period, is mixed with that of lower specific activity produced at a

later time, the net result being a reduction in the over-all specific

activity, which does not represent the true picture of the distribution

of specific activity. In the continuous sweeping system there is no

such problem since the specific activity of the respiratory CO_2 being

produced in _any_ given time period is known with a fair degree of

accuracy.

In Table III are summarized the results from three separate

sets of experiments carried out on green cucumber fruits using the

three different experimental procedures. It is apparent from these

data that the relative conversion of labeled glucose to CO_2 is affected

by the experimental procedure employed and that glucose-6-C^{14} is the

most sensitive to these changes. Table IV gives the data from a

number of separate experiments on fruits utilizing glucose-1-C^{14},

glucose-2-C^{14}, glucose-6-C^{14} and glucose-U-C^{14}. The interval radio-

active recovery and cumulative radioactive recovery in the respiratory

CO_2 from two experiments of the continuous sweeping type with green

tomato fruits, are presented in Figures 4,5 and 6 respectively. In

TABLE III

OXIDATION OF GLUCOSE-1-C^{14} OR GLUCOSE-6-C^{14} TO $C^{14}O_2$ BY CUCUMBER FRUIT

Exp. No.	Type of Procedure	Exp. Duration (hours)	Position Labeled	Level Used (c.p.m.)	Activity per mmole $BaCO_3$ (c.p.m. x 10^{-2})	Total Activity (c.p.m.)	% Radiochemical Yield (%)	$\frac{G-6}{G-1}$ [b]
1	Closed System	48	G-1	49,800	13.0	10,960	22.0	
								0.60
"	"	"	G-6	"	8.0	6,550	13.2	
9	Intermittent Sweeping	30	G-1	83,000	23.1	17,500	21.1	
								0.44
"	"	"	G-6	"	13.3	7,600	9.2	
12	Continuous Sweeping	30	G-1	83,000	19.5	13,900	16.8	
								0.32
"	"	"	G-6	"	7.7	4,500	5.5	

[a]Specific activity of glucose-1-C^{14} and glucose-6-C^{14} was 3.55 x 10^7 c.p.m./mmole glucose.

[b]G-6/G-1 is the per cent radiochemical yield from glucose-6-C^{14}/per cent radiochemical yield from glucose-1-C^{14}.

TABLE IV

OXIDATION OF GLUCOSE-1-C^{14}, GLUCOSE-2-C^{14}, GLUCOSE-6-C^{14} OR GLUCOSE-U-C^{14} TO $C^{14}O_2$
BY FRUITS USING CONTINUOUS SWEEPING PROCEDURE

| Exp. No. | Type of Fruit | Level Used (c.p.m.) | 30 hour % Radiochemical Yield | | | | $\frac{G-6}{G-1}$ [d] | $\frac{G-2}{G-6}$ [e] | $G-3$ [f] |
			G-1 (%)	G-2 (%)	G-6 (%)	G-U (%)			(%)
11	Lime	80,000 [a]	6.6	-	3.2	-	0.49	-	-
12	Cucumber	83,000 [a]	16.8	-	5.5	-	0.32	-	-
13	Tomato	80,000 [a]	9.8	7.3	4.4	-	0.45	1.66	-
14	"	80,000 [b]	9.1	6.8	5.2	-	0.58	1.32	-
15	"	80,000 [b]	8.7	6.8	5.0	-	0.58	1.39	-
16 [h]	"	105,000 [c]	7.5	4.4	4.4	9.8 (10.9) [g]	0.57	1.00	19.5

---

[a] Specific activity of glucose-1-C^{14}, glucose-2-C^{14} and glucose-6-C^{14} 3.55 x 10^7 c.p.m./mmole glucose.
[b] Specific activity of glucose-1-C^{14}, glucose-2-C^{14} and glucose-6-C^{14} 3.28 x 10^7 c.p.m./mmole glucose.
[c] Specific activity of glucose-1-C^{14}, glucose-2-C^{14}, glucose-6-C^{14} and glucose-U-C^{14} 1.85 x 10^7 c.p.m./mmole glucose.
[d] G-6/G-1 is the per cent radiochemical yield from glucose-6-C^{14}/per cent radiochemical yield from glucose-1-C^{14}.
[e] G-2/G-6 is the per cent radiochemical yield from glucose-2-C^{14}/per cent radiochemical yield from glucose-6-C^{14}.
[f] G-3 is the 30 hour per cent radiochemical yield calculated for glucose-3-C^{14} by Equation 3.
[g] In a parallel experiment, 6 times as much glucose-U-C^{14} (6.3 x 10^5 c.p.m.) was introduced into this fruit.
[h] Field grown tomato fruits were used in this experiment.

Figure 4

Experiment 15
INTERVAL RADIOCHEMICAL RECOVERY IN
RESPIRATORY CO_2 OF TOMATO FRUIT
UTILIZING LABELED GLUCOSE

Figure 5

CUMULATIVE RADIOCHEMICAL RECOVERY
IN RESPIRATORY CO_2 OF TOMATO FRUIT
UTILIZING LABELED GLUCOSE
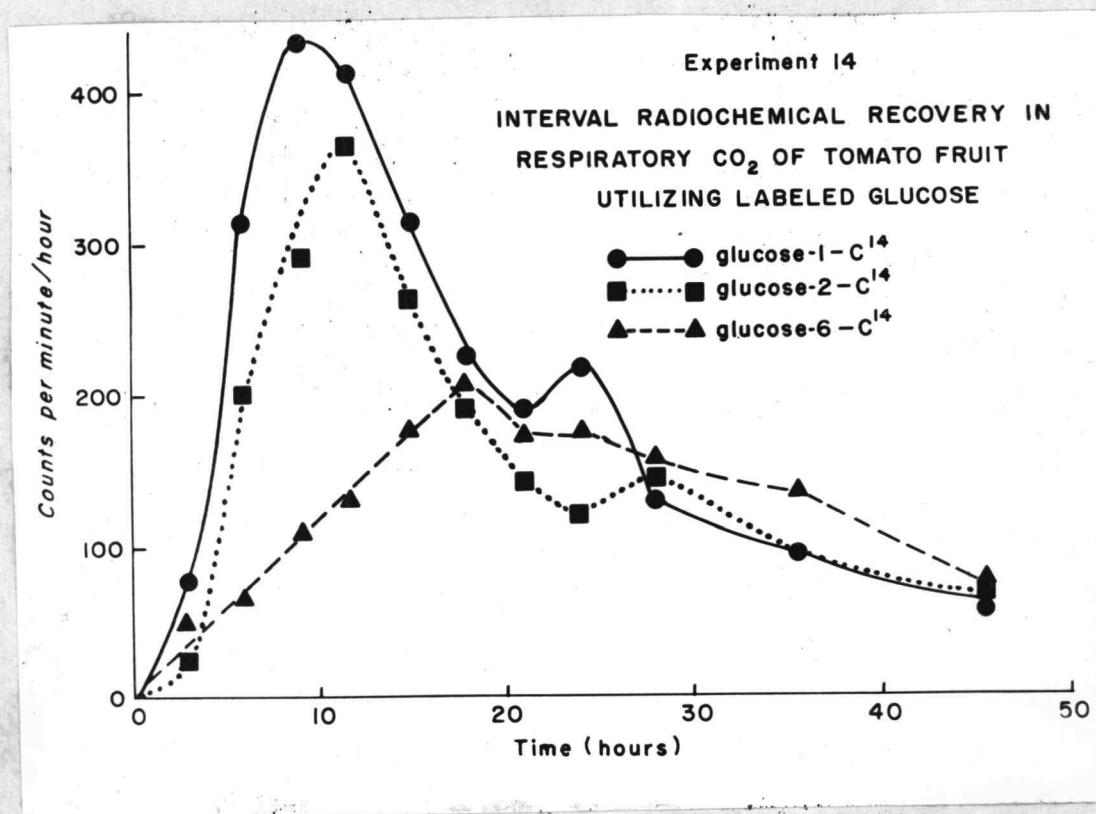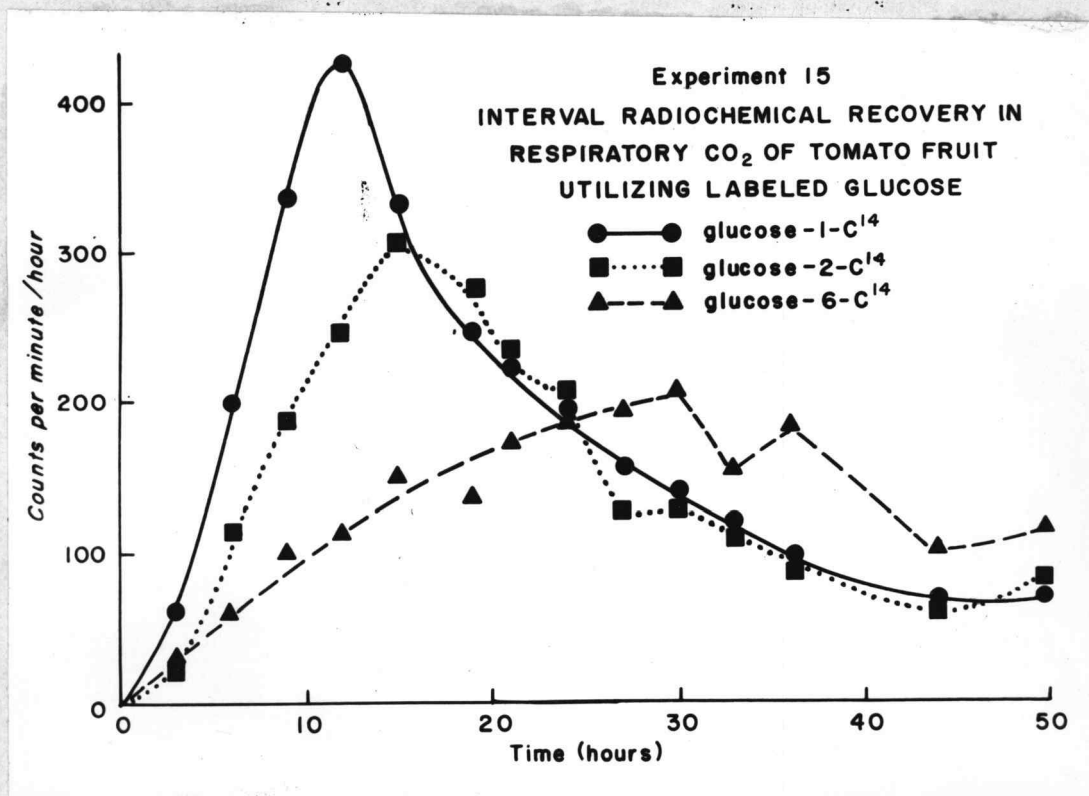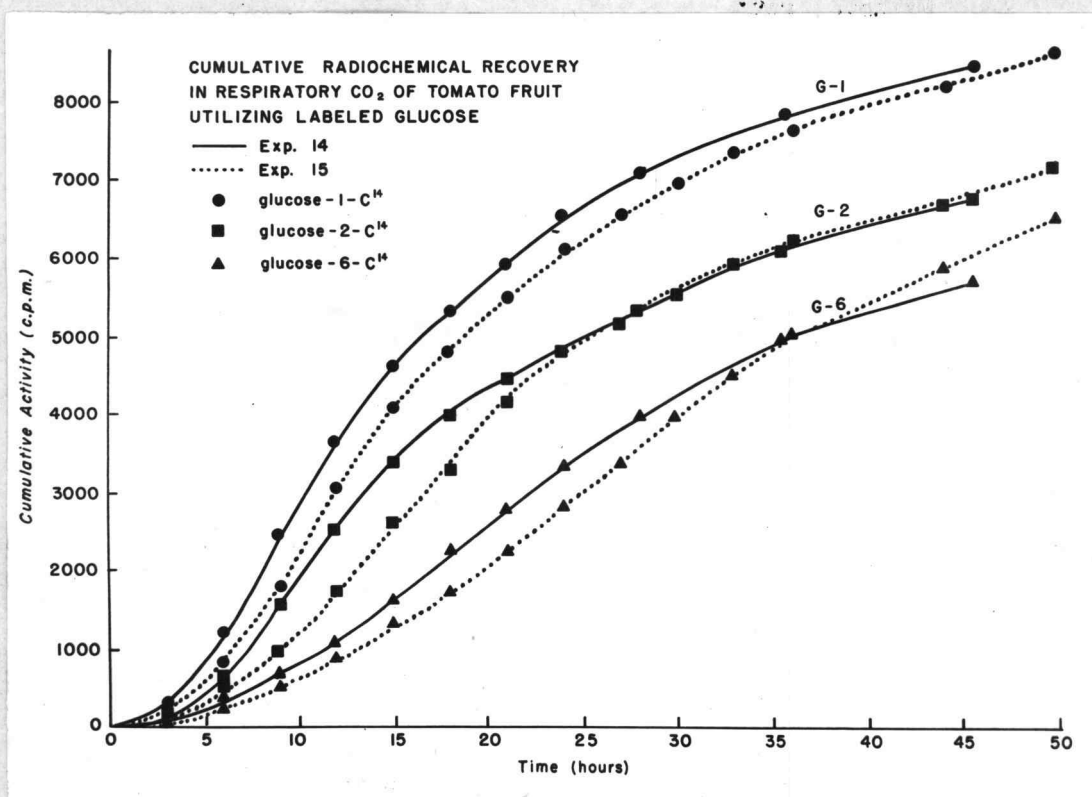
Figure 6

Table V are listed the radiochemical recovery in the respiratory CO_2 with respect to time for a representative tomato experiment.

3. Incorporation of glucose activity into fruit constituents:

Upon completion of one of the tomato respiration experiments described above, an attempt was also made to examine the distribution of the glucose radioactivity incorporated into the fruits, particularly that into the organic acid fraction. The fruits were sliced and homogenized with equal weights of distilled water in a Waring blendor for approximately 3 minutes. After centrifugation and filtration, aliquots of the aqueous extracts from the respective fruits were plated on stainless steel planchets by the "direct plating" technique and their radioactivity measured in the conventional manner. The remaining solutions were acidified with sulfuric acid and subjected to exhaustive ether extraction after which the organic acids were recovered from the ether by alkali extraction. Aliquots of the respective acid solutions were assayed for radioactivity as described above.

The distribution of radioactivity derived from glucose among various fractions of the tomato fruits is given in Table VI.

B. Fate of Glucose Breakdown Products:

Pyruvic acid occupies a central position in the metabolism of carbohydrate and is thus a compound of unusual biological interest. Pyruvate may arise from glucose via either the Embden-Meyerhof-Parnas

Table V

Oxidation of Glucose-1-C^{14a}, Glucose-2-C^{14}

and Glucose-6-C^{14} to C^{14}O$_2$ by Tomato

Fruits Under Continuous Sweeping

Experiment 14

Time (hours)	G-1[b] (%)	G-2 (%)	G-6 (%)	G-6[c] / G-1	G-2[d] / G-6
3	0.29	0.08	0.19	0.67	0.42
6	1.5	0.82	0.43	0.30	1.91
9	3.1	1.9	0.85	0.27	2.27
12	4.5	3.2	1.3	0.29	2.44
15	5.8	4.2	2.0	0.35	2.10
18	6.6	5.0	2.8	0.42	1.77
21	7.3	5.5	3.5	0.47	1.59
24	8.1	6.0	4.1	0.51	1.45
28	8.8	6.7	4.9	0.56	1.36
35.5	9.7	7.6	6.2	0.64	1.23
45.5	10.5	8.4	7.0	0.68	1.19

a Specific Activity of glucose-1-C^{14}, glucose-2-C^{14} and glucose-6-C^{14} was 3.28×10^7 c.p.m/ mmole glucose; 80,000 c.p.m. was the level used.

b Per cent radiochemical yield

c G-6/G-1 is the % radiochemical yield from glucose-6-C^{14}/% radiochemical yield from glucose-1-C^{14}.

d G-2/G-6 is the % radiochemical yield from glucose 2-C^{14}/% radiochemical yield from glucose-1-C^{14}.

TABLE VI

DISTRIBUTION OF RADIOACTIVITY IN TOMATO FRUITS UTILIZING

GLUCOSE-1-C^{14}[a] OR GLUCOSE-6-C^{14}

Experiment 13

Exp. Duration (hours)	Position Labeled	Respiratory CO$_2$[b] (%)	Aqueous Extract (%)	Organic Acids (%)
95	G-1	17.1	46.2	3.6
"	G-6	11.0	48.0	3.5

[a] Specific activity of glucose was 3.28 x 10^7 c.p.m./mmole glucose; 80,000 c.p.m. total activity used in this experiment.

[b] % is the per cent radiochemical yield.

glycolytic pathway or the pentose cycle. Pyruvate may be converted to alanine, to fats, to acetate, to acetaldehyde, to malate, to oxalacetate or oxidized completely to CO_2 through the tricarboxylic acid cycle.

In order to follow the products of glucose metabolism, the metabolically related substrates $CH_3C^{14}OCOOH$, $C^{14}O_2$ and $CH_3C^{14}OONa$ were employed in studies on tomato fruits. Whenever necessary, isolation and degradation of fruit constituents has been carried out (24).

1. Introduction of labeled compounds:

a). Carbon[14] dioxide:

Mature green tomato fruits (6 fruits, total weight 657 g.) were placed in a desiccator under slightly reduced pressure and exposed to 1.4×10^8 c.p.m. of $C^{14}O_2$ which was generated internally by injecting 20 per cent perchloric acid into a small vial containing $BaC^{14}O_3$ (obtained from Oak Ridge National Laboratory; specific activity 9.27×10^8 c.p.m./mmole). The tomatoes were kept at room temperature for 48 hours in the dark (a period determined by preliminary experiment to give optimum incorporation), at which time the system was swept with CO_2-free air and any residual $C^{14}O_2$ was trapped in CO_2-free NaOH.

b). Pyruvate-2-C^{14}:

A solution of $CH_3C^{14}OCOOH$ (obtained from Tracerlab, Inc.;

specific activity 1.02×10^8 c.p.m./mmole) containing 0.134 mmole

pyruvate dissolved in 3.5 ml. of water was administered into the

locules of 5 mature green tomato fruits (total weight 987 g.) by

means of an injection technique (108, pp. 741-745). The fruits

were placed in a large vacuum desiccator, protected from the light

and allowed to stand at room temperature. The respiratory CO_2 was

swept continuously from the system with CO_2-free air and collected

in CO_2-free NaOH solution. The trap solution was replaced periodically

with fresh NaOH and carbonate recovered as $BaCO_3$ for radioactive assay.

The specific activity of the respiratory CO_2 reached a max-

imum in approximately 15 hours (Figure 7). In order to ensure a

steady state with respect to the incorporation of radioactivity into

various components of the fruits, the experiment was terminated after

46 hours at which time the cumulative activity curve of the metabolic

CO_2 had leveled off in the manner illustrated in Figure 7.

c). Sodium acetate-1-C[14]:

Since incorporation of acetate into tomatoes has been

carried out previously (108, pp. 741-745), the present work was

devoted to the time course study of the utilization of acetate for

the formation of organic acids and amino acids.

Two freshly picked mature green tomato fruits were sliced

in such a manner as to give 8 segments having approximately equal

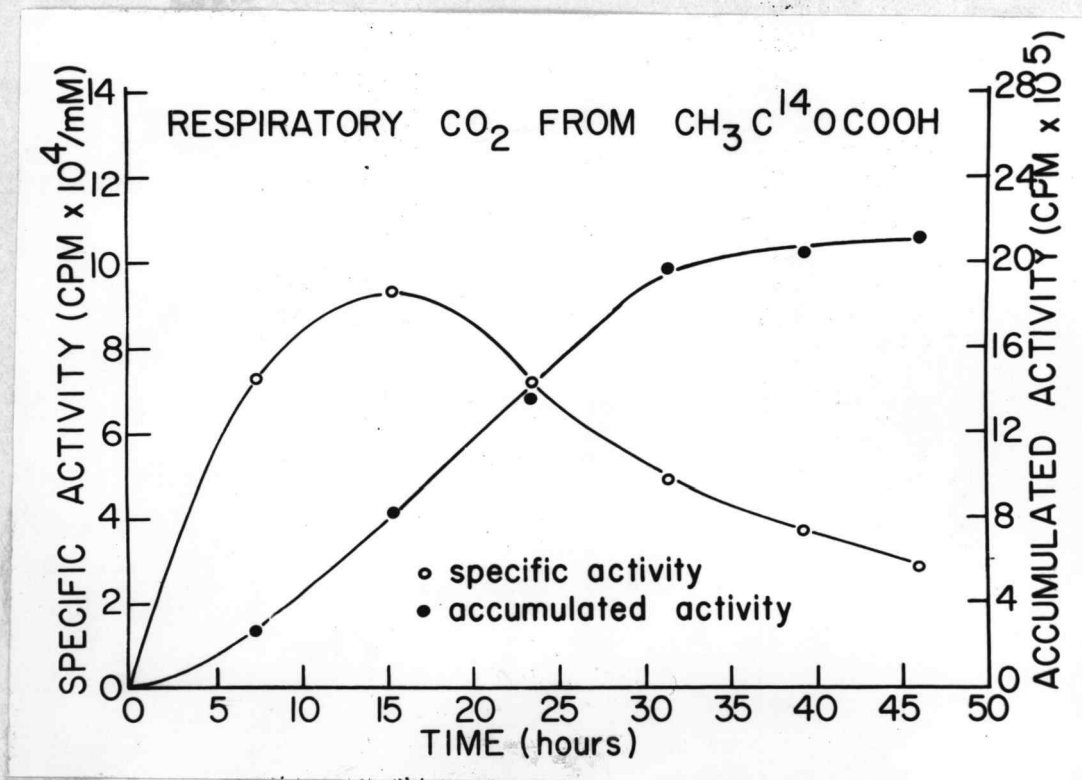weights (12g.). Onto the freshly cut surface of each slice was

RESPIRATORY CO$_2$ FROM CH$_3$C^{14}OCOOH

Figure 7

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pipetted 0.05 ml. of a solution of $CH_3C^{14}OONa$ (prepared in our laboratory from $BaCO_3$; specific activity 5.15×10^8 c.p.m./mmole) containing 2.6×10^5 c.p.m. The slices were then allowed to stand in air at room temperature.

2. <u>Examination of the incorporation of labeled substrates into</u>

<u>fruit constituents</u>:

a). <u>Carbon dioxide fixation experiment</u>:

At the termination of this experiment, the fruits were quickly sliced and homogenized in a Waring blendor. The juice was next separated by centrifugation and the pulp washed three times with water, followed by ethanol and then ethyl ether, dried and stored. The combined juice and aqueous washings were rapidly passed through an Amberlite IR-120 ion exchange column to remove amino acids; the effluent was next concentrated and passed through an Amberlite IRA-400 anion exchange column. The organic acids were recovered from this resin by means of HCl elution. The eluate, after concentration and removal of HCl, was acidified with sulfuric acid and subjected to exhaustive ether extraction to recover the organic acids. After removal of ether, these acids were redissolved in water. Aliquots of the various fractions were assayed for radioactivity by direct plating techniques and the distribution is presented in Table VII.

b). <u>Pyruvate utilization experiment</u>:

Table VII

Distribution of C^{14} Activity in Fractions

Isolated from Tomato Fruit

Fraction	CO_2 Experiment 17		Pyruvate Experiment 18	
	sp. act. (cpm/mmole)	total act. (cpm.)	sp. act. (cpm/mole)	total act. (cpm.)
Activity administered	9.27×10^8	1.4×10^8	1.02×10^8	1.37×10^7
Respiratory CO_2	4.26×10^6	3.17×10^7	6.32×10^4	2.11×10^6
Organic Acids	-	8.95×10^6	-	1.92×10^6
Amino Acids	-	1.1×10^6	-	0.40×10^6
Sugars	-	0.01×10^6	-	0.26×10^6
Fat	-	0	-	-

The procedure for isolation of the fruit acids was modified from that used above due to the detection of interaction of fruit sugars with the strongly basic ion exchange resin IRA-400 resulting in production of large amounts of lactic acid (23, pp. 481-482). Consequently, the combined juice and aqueous washings from the homogenized fruits was passed through an Amberlite IR-120 exchange column, the effulent concentrated, acidified with sulfuric acid and subjected to exhaustive ether extraction. The ether was removed from the combined extracts and the residual mixed organic acids made up to volume with water.

The distribution of radioactivity among the various components is given in Table VII.

c). Time course study in the utilization of acetate:

Following the administration of the labeled substrate, slices were dropped at intervals into boiling absolute ethanol after 15, 30, 45, 60, 90, 120, 150 and 180 minutes, respectively. After homogenization in a Waring blendor, each mixture was reheated to boiling and held at this temperature for 5 minutes. Upon filtration of the ethanol extracts, aliquots were taken for radioactivity measurements (acetic acid first removed by steam distillation) and ethanol was next removed from the remaining solution under vacuum in the cold and the residue dried completely over P_2O_5 in vacuo.

The extracts from the 30, 60, 120 and 180 minute samples,

after redissolving in water, were each passed through Amberlite
IR-120 ion exchange columns in order to remove amino acids. The
effluents from these columns were concentrated, acidified and sub-
jected to exhaustive ether extraction. The ether extracts were
evaporated to dryness _in vacuo_ to remove the solvent and any residual
acetic acid; organic acids were then extracted with NaOH. Amino acids
were eluted from the columns with HCl, the eluates concentrated and
evaporated to dryness over P_2O_5 and KOH pellets _in vacuo_. The recovery
of radioactivity in the various samples is summarized in Table VIII.

Aliquots of the amino acid fraction were chromatographed on
paper with 80 per cent phenol:water (114, pp. 238-243) and n-butanol:
acetic acid:water (4:1:5) (95, pp. 696-697). The organic acids were
separated on paper using n-butanol:acetic acid:water (4:1:5) (66, pp.
98-111) and isopropyl alcohol:tert-butyl alcohol:benzyl alcohol:
formic acid:water (99, pp. 413-415) as solvents. The compounds were
identified by the usual spraying techniques and the radioactivity
estimated by radioautography with Eastman "No-Screen" X-ray film.
The results from these studies are given in Table IX.

3. Isolation and degradation studies on fruit acids:

a). Isolation procedure:

A portion of the aqueous solutions of mixed organic acids
from the CO_2-fixation and pyruvate experiments were taken to dryness

Table VIII

Time Course Utilization of $CH_3C^{14}OONa$[a]

by Tomato Slices

Experiment 19

Elapsed Time (minutes)	Ethanol Extract (Acetic acid removed) (%)[b]	Amino Acids (%)	Organic Acids (%)	Neutral Fraction (%)
15	2.4	-	-	-
30	4.0	0.7	2.5	1.8
45	7.0	-	-	-
60	10.8	1.4	3.7	5.5
90	15.2	-	-	-
120	20.1	3.0	7.9	9.2
150	26.4	-	-	-
180	29.9	9.5	12.6	11.6

a 2.6×10^5 c.p.m applied to each slice; specific activity 5.15×10^8 c.p.m./mmole

b % is per cent radiochemical yield.

Table IX

Relative Distribution of Radioactivity in Tomato Slices

Utilizing $CH_3C^{14}OONa$ [a]

Experiment 19

	Time in minutes					
	30	60	90	120	150	180
Aspartic acid	-	-	-	-	*	**
Glutamic acid	-	-	*	*	**	***
Glycine-Serine	-	-	-	-	*	**
Histidine	-	-	-	-	-	*
Phenylalanine	-	-	-	-	-	*
Tryptophane	-	-	-	-	-	*
Tyrosine	-	-	-	-	-	*
Lactic acid	-	-	*	*	*	*
Trans-aconitic acid	*	**	**	**	**	***
Citric acid	**	***	****	****	****	****
Malic acid	-	*	**	**	**	***

a 2.6×10^5 c.p.m. applied to each slice; specific activity 5.15×10^8 c.p.m./mmole; **** very heavy labeling; *** heavy labeling; ** moderate labeling; * detectable.

over P_2O_5 _in vacuo_, acidified with a little sulfuric acid and both subjected to silica gel column chromatography using n-butanol and chloroform as solvents according to the method of Bulen, Varner and Burell (25, pp. 187-190). The location and concentration of the component acids were determined by titration of the acid content in the individual fractions with 0.02 N NaOH. The identity and purity of all fractions was established by paper chromatography using ethyl ether:acetic acid:water (54, pp. 394-396), 1-pentanol:5 M aqueous formic acid (22, pp. 489-491) or n-butanol:acetic acid:water (4:1:5) (66, pp. 98-111) and radioautography on Eastman No-Screen X-ray film. The content of citric and malic acids in the original mixed acids fraction and pure acid fractions obtained in the chromatographic separation were estimated colorimetrically using the method of Pucher et al. (82, pp. 288-291) for malic acid and that of Speck et al. (98, pp. 119-144) for citric acid.

Malic acid was purified through its lead salt with subsequent removal of lead ion with hydrogen sulfide. Inert carrier malic acid was then added and the diluted malic acid was further purified by recrystallization from a solution of dry benzene and acetone.

Citric acid was isolated as its calcium salt after appropriate dilution with inert carrier acid.

b). Degradation procedures:

The total activity of malic acid was determined by dry

combustion to carbon dioxide, since wet combustion techniques gave consistently low recoveries of CO_2. A portion of the acid was decarbonated with concentrated sulfuric acid according to the method of Racusen and Aronoff (85, pp. 25-40); the CO, after conversion to CO_2 by passage over hot copper oxide, giving the specific activity of carbon 1. A third sample of malic acid was oxidized with potassium permanganate according to the procedure of Wood et al. (116, pp. 377-388) yielding CO_2 from carbons 1,4 and acetaldehyde corresponding to carbons 2,3. The aldehyde was converted to iodoform, giving the specific activity of carbon 3 directly and carbon 2 by difference.

The molar specific activity of the citric acid was obtained by either dry combustion or persulfate oxidation. Other samples of citric acid were treated with concentrated sulfuric acid according to the Martin and Wilson modification of the method of Weinhouse, Medes and Floyd (113, pp. 691-703) to yield CO, CO_2 from carbon 6 and carbons 1,5 respectively. The acetone obtained in the same reaction was oxidized further by the wet combustion method of Van Slyke and Folch (103, pp. 507-541) giving the specific activity of carbons 2,3,4.

Acetone was also obtained from carbons 2,3,4 as the mercury-acetone complex by the dichromate oxidation method of Lewis and Weinhouse (63, pp. 2500-2503). The mercury-acetone complex was converted to iodoform to give the specific activity of carbons 2,4 directly and that of carbon 3 by difference; a portion of the Deniges complex was also oxidized to carbon dioxide for the total activity of carbons 2,

3,4. As a further verification of the degradation results, citric acid was degraded by the method of Martin, Wilson and Burris (68, pp. 103-111) to give the total activity in carbons 1,5,6. The penta-bromoacetone from the three central carbon atoms was converted to CO_2, giving the sum of carbons 2,3,4.

All samples were counted as barium carbonate in the conventional manner to a standard error of 2 per cent and corrected for background and self-absorption.

The distribution of the incorporated activity into malic and citric acids is given in Table X.

C. Effect of External Factors Upon the Utilization of C^{14}-labeled Glucose, Sodium Acetate-1-C^{14}, Sodium Acetate-2-C^{14} and Sodium Phosphate-P^{32} in Fruits:

Experiments have been carried out to test the effect of ethylene, nitrogen, oxygen, dinitrophenol or temperature on the utilization of various substrates by fruits. In these studies, either the respiratory CO_2 was collected or else the radioactive distribution was determined among several fruit constituents.

1. Effect of ethylene upon the utilization of $CH_3C^{14}OONa$ by mature orange fruit:

One ripe Valencia orange (222 g.) obtained from the local market was placed in a large closed desiccator along with a 100 ml.

Table X

Isotopic Distribution Pattern in Malic and Citric Acids in Tomatoes Utilizing $CH_3C^{14}OCOOH$ and $C^{14}O_2$

Carbon Atom	$C^{14}O_2$ Experiment 17		$CH_3C^{14}OCOOH$ Experiment 18	
	Sp. activity (cpm x 10^4/ mmole)	% total	Sp. activity (cpm x 10^4/ mmole)	% total
Malic Acid				
C-1	24.9	35	23.7	40
C-2	0	0	4.5	7.7
C-3	0	0	2.0	3.4
C-4	40.1	57	26.8	46
Whole molecule	70.5	100	58.7	100
Citric Acid				
C-1 + C-5	12.2	43	6.97	87
C-2	0	0	0.13	1.6
C-3	0	0	0.13	1.6
C-4	0	0	0*	0
C-6	15.3	54	1.32	16.4
Whole molecule	28.4	100	8.04	100

* based on theoretical consideration

beaker containing 60 ml. of 50 per cent KOH. A second fruit (weight 230 g.) was placed in a desiccator similarly equipped, which was then evacuated and air containing ethylene gas at a concentration of 1 in 1000 parts was introduced. The two fruits were allowed to stand in their respective atmospheres at room temperature for 84 hours.

At the end of the experimental period, both fruits were removed and a solution of $CH_3C^{14}OONa$ (synthesized in our laboratory from $BaC^{14}O_3$; specific activity 5.14 x 10^8 c.p.m./mmole) containing 1.82 x 10^7 c.p.m was introduced into each fruit by the technique previously described. The control fruit was returned to its desiccator with its air atmosphere and the KOH trapping solution replaced with fresh solution. Concurrently, the ethylene treated fruit was replaced into its desiccator, the KOH trap replenished and the ethylene atmosphere renewed (1 to 1000 in air). The two desiccators were again allowed to stand at room temperature for an additional 71 hours.

The fruits were then removed, peeled and a simplified fractionation of constituents of the peel and pulp of each fruit was carried out as follows. The peelings were homogenized with 80 per cent ethanol in a Waring blendor, the resulting mixture was heated and filtered. After removal of the ethanol, the aqueous extract was acidified and subjected to exhaustive ether extraction. The ether was then extracted with NaOH solution to recover organic acids. The fruit pulp was treated in a manner similar to that of the peelings.

The various fractions were assayed for radioactivity in the usual manner. The distribution of radioactivity in the two fruits is given in Table XI.

Paper chromatograms and radioautographs by the techniques outlined earlier showed no detectible differences in distribution of radioactivity among the organic acids from the two fruits.

2. **Effect of ethylene upon the utilization of $C^{14}H_3COONa$ by tomato fruits:**

A solution containing 1.61×10^6 c.p.m of sodium acetate-2-C^{14} (purchased from Tracerlab, Inc.; specific activity 1.4×10^8 c.p.m./mmole) was introduced into two green tomato fruits of equal weight (97.5 g.) by the method described in Section A, and the fruits were then placed in large dark bottles. One bottle, designated as the control, was swept continuously with a stream of CO_2-free air, the respiratory CO_2 being trapped in CO_2-free sodium hydroxide. The second bottle was swept with a mixture consisting of 1 part oxygen and 4 parts nitrogen containing ethylene gas in a concentration of 1 part per 1400 parts of nitrogen and the metabolic CO_2 was trapped as above. The sweeping rate was maintained at 125 ml/minute for both samples and sweeping continued for 49.75 and 50.5 hours, respectively. During the experimental period, the NaOH solution in the traps was periodically changed and the carbonate precipitated as barium carbonate,

Table XI

Distribution of Radioactivity from $CH_3C^{14}OONa$[a] in Normal and Ethylene[b] Treated Orange Fruits

Experiment 20[c]

Fraction	Control Radioactivity (c.p.m. x 10^5)	% Radiochemical Yield (%)	Ethylene Treated Radioactivity (c.p.m. x 10^5)	% Radiochemical Yield (%)
Peel				
Ethanol extract	6.7	3.7	2.2	1.2
Ether extract	4.0	2.2	0.69	0.4
Organic acids	1.4	0.8	0.65	0.4
Ether soluble neutrals	2.3	1.3	0.22	0.1
Pulp				
Ethanol extract	14.5	8.0	17.3	9.5
Ether extract	10.2	5.6	7.7	4.2
Organic acids	5.1	2.8	4.5	2.5
Ether soluble neutrals	0.75	0.4	0.74	0.4

a Specific activity 5.14 x 10^8 c.p.m./mmole; applied activity 1.82 x 10^7 c.p.m.; b Ethylene in air 1:1000; c Experiment duration 71 hrs
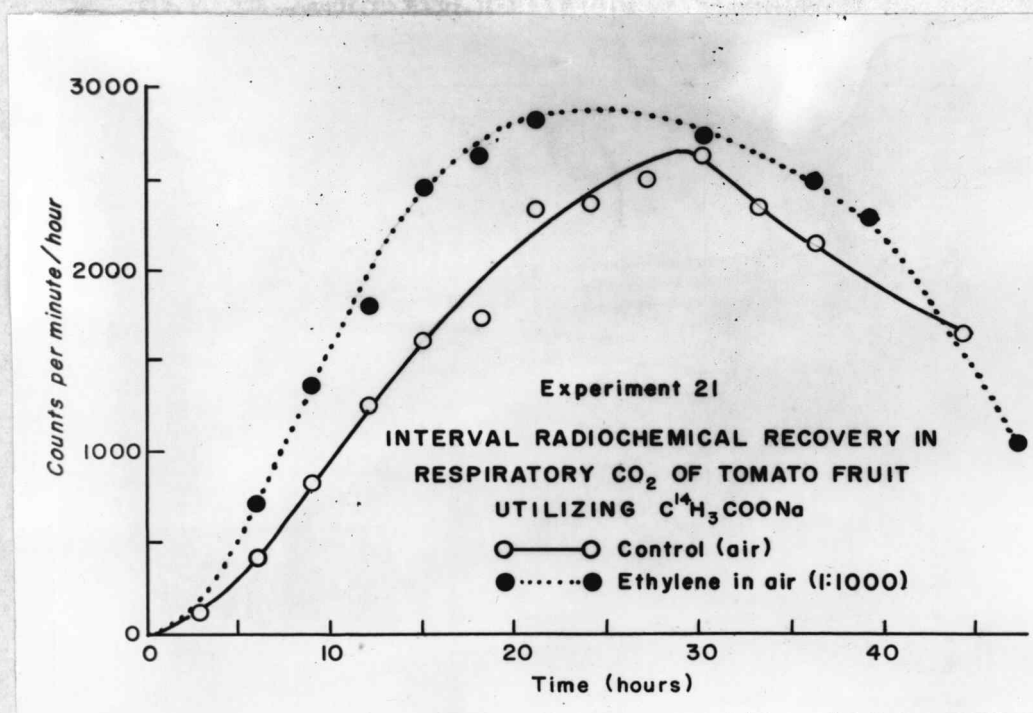
Figure 8

then plated and counted.

As may be seen in Figure 8, the interval radiochemical recovery of the respiratory CO_2 reached a maximum at the end of 21 hours in the case of the ethylene treated fruit and at 30 hours for the control.

After termination of the experiment, the fruits were homogenized in a Waring blendor, the juice separated from the pulp by centrifugation and the pulp washed with water. The combined juice and washings from each fruit were acidified and subjected to exhaustive ether extraction, the organic acids being recovered from the ether by extraction with NaOH. The distribution of radioactivity is shown in Table XII.

Scanning of paper chromatograms prepared from the two acid samples using a Geiger-Mueller counting tube showed no appreciable difference in the distribution of radioactivity among various fruit constituents.

3. Effect of ethylene upon the utilization of labeled glucose
by fruit:

Experiments of the type described under section A were carried out on green tomatoes and cucumbers and ripe oranges and limes. However, in place of an air atmosphere in the experimental bottles, a mixture of ethylene in air was introduced. In the static system (Section A2a) and intermittent sweep system (Section A2b) this

TABLE XII

DISTRIBUTION OF RADIOACTIVITY FROM $C^{14}H_3COONa$[a] IN NORMAL

AND ETHYLENE[b] TREATED TOMATO FRUITS

Experiment 21[c]

CONTROL

	Radioactivity (c.p.m. x 10^5)	Per cent Radiochemical Yield (%)	Specific Activity (c.p.m./mg. $BaCO_3$)	Weight (mg. $BaCO_3$)
Respiratory CO_2	0.86	5.3	84.1	1018
Aqueous extract	5.5	34.0	-	-
Organic acids	3.0	18.7	-	-

ETHYLENE TREATED

Respiratory CO_2	0.96	6.0	76.5	1258
Aqueous extract	4.7	29.2	-	-
Organic acids	1.5	9.3	-	-

[a] Specific activity 1.4 x 10^8 c.p.m./mmole; total activity 1.61 x 10^6 c.p.m.

[b] Ethylene in air 1:1000.

[c] Experiment duration 50 hours.

was accomplished by reducing the pressure and refilling with pure
ethylene to the desired pressure. Two techniques were employed in
the continuous sweep (Section A2c). First, when a mixture of ethylene
in nitrogen (1:1400) became available, studies were carried out in
which the respective fruit were swept continuously with 100 ml/minute
of the ethylene-nitrogen mixture and 25 ml/minute of oxygen, flow
being rigidly controlled by means of low pressure reduction valves
and flow meters. More recent experiments employed continuous sweeping
with a specially prepared ethylene-air mixture (1:1000).

The specific activity and radiochemical yield in the respir-
atory CO_2 from these experiments are presented in Table XIII.

4. <u>Effect of nitrogen upon the utilization of labeled glucose</u>
<div align="right"><u>by fruit</u>:</div>

The experimental technique is the same as that described
previously under Section A. However, nitrogen gas was used instead
of air in providing the atmosphere of the closed system and as the
continuous sweeping gas. The results from these studies on green
cucumber and tomato fruits are listed in Table XIV along with the
results from concurrent control experiments.

5. <u>Effect of oxygen upon the utilization of labeled glucose</u>
<div align="right"><u>by fruit</u>:</div>

TABLE XIII

OXIDATION OF GLUCOSE-1-C^{14}, GLUCOSE-2-C^{14} OR GLUCOSE-6-C^{14} TO C^{14}O$_2$
BY FRUITS EXPOSED TO ETHYLENE

Exp. No.	Type of Fruit	Level Used (c.p.m.)	Gas Used	45 hour % Radiochemical Yield			$\frac{G-6}{G-1}$ [b]	45 hour Accumulated Specific Activity		
				G-1 (%)	G-2 (%)	G-6 (%)		G-1	G-2 (c.p.m./mg. BaCO$_3$)	G-6
7[c]	Orange	47,500	air	14.4	-	9.1	0.62	6.6	-	3.5
"	"	"	ethylene	13.9	-	7.0	0.49	4.8	-	2.2
8[c]	"	163,000	air	6.8	-	2.3	0.34	15.5	-	5.6
"	"	"	ethylene	5.7	-	1.8	0.31	15.1	-	3.9
13[d]	Tomato	80,000	air	12.1	9.9	6.8	0.57	8.9	8.2	5.0
"	"	"	air[e]	11.3	-	6.9	0.61	8.3	-	4.9
14[d]	"	"	air	10.5	8.3	7.0	0.68	9.2	8.9	5.6
"	"	"	ethylene	11.0	6.8	6.8	0.62	9.2	3.9	5.0
15[d]	"	"	air	10.3	8.3	7.5	0.73	8.6	6.8	4.8
"	"	"	ethylene	10.8	8.1	4.8	0.43	7.9	5.0	2.8

[a] Ethylene in air (1:1000). [b] G-6/G-1 is the per cent radiochemical yield from glucose-6-C^{14}/per cent radiochemical yield from glucose-1-C^{14}. [c] Intermittent sweeping. [d] Continuous sweeping. [e] Fruits exposed to ethylene-air mixture (1:1000) for 10 hours and then swept continuously with CO$_2$-free air.

TABLE XIV

OXIDATION OF GLUCOSE-1-C^{14} OR GLUCOSE-6-C^{14} TO C^{14}O$_2$ BY TOMATO AND CUCUMBER
FRUITS EXPOSED TO NITROGEN OR OXYGEN GASES

Exp. No.	Type of Fruit	Level Used (c.p.m.)	Gas Used	45 hour % Radiochemical Yield		$\frac{G-6}{G-1}$ [c]	45 hour Accumulative Specific Activity	
				G-1 (%)	G-6 (%)		G-1 (c.p.m./mg. BaCO$_3$)	G-6
1 [a]	Cucumber	49,800	air	22.0	13.2	0.60	6.6	4.1
"	"	"	nitrogen	16.8	3.1	0.18	8.8	1.9
"	"	"	oxygen	22.0	16.5	0.75	6.7	5.3
13 [b]	Tomato	80,000	air	12.0	7.4	0.57	8.9	5.0
"	"	"	nitrogen	9.6	7.4	0.78	12.6	10.3
"	"	"	oxygen	16.0	10.0	0.63	10.2	8.0

[a] Static system; specific activity of glucose used 3.55 x 10^7 c.p.m./mmole.

[b] Continuous sweeping system; specific activity of glucose used 3.28 x 10^7 c.p.m./mmole

[c] G-6/G-1 is the per cent radiochemical yield from glucose-6-C^{14}/per cent radiochemical yield from glucose-1-C^{14}.

The method employed was the same as described in Section A with the exception that oxygen gas replaced air in providing the atmosphere in the system. The data from these studies are also compiled in Table XIV.

6. Effect of temperature on the utilization of labeled glucose by tomato fruit, with and without ethylene gas:

a). Control experiment (without ethylene):

In order to evaluate the effect of temperature upon the utilization of differentially carbon[14]-labeled glucose substrates by green tomato fruit, continuous sweeping type experiments were carried out at different temperatures using the continuous sweeping technique previously described in Section A2c.

The experimental bottles, containing the respective glucose tagged fruits, were placed in a thermostatically controlled water bath maintained at 32° C. throughout the experimental period, while CO_2-free air was passed through the bottles at a rate of 125 ml/minute. Periodically the NaOH trap solutions were replaced and $BaCO_3$ recovered for radioactive assay.

A plot of the interval radioactive recovery against time is presented in Figure 9 and the data are presented in Table XV.
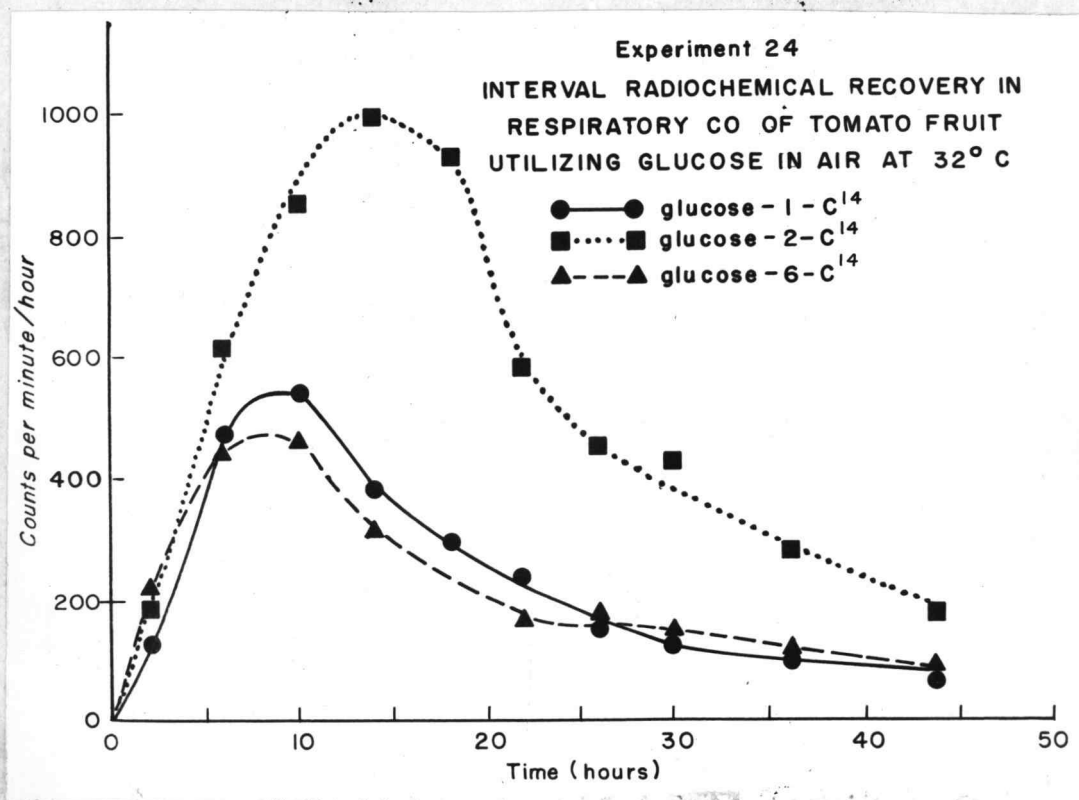
b). Ethylene experiments:

Figure 9

Table XV

Effect of Temperature[a] and Ethylene Upon the Oxidation of

Glucose-1-C^{14}[b], Glucose-2-C^{14} Glucose-6-C^{14} or

Glucose-U-C^{14} to C^{14}O$_2$ by Tomato Fruits

Experiment 24

Gas Used	18 hour % Radiochemical Yield (%)	Accumulated Specific Activity (c.p.m./mg. BaCO$_3$)
Air		
G-1	6.7	9.8
G-2	13.3	23.0
G-6	6.2	8.2
G-U	14.8	24.6
G-3[c]	25.0	-
G-6/G-1	0.93[d]	-
G-2/G-6	2.14[e]	-
Ethylene (in air 1:1000)		
G-1	7.4	8.9
G-2	4.8	6.4
G-6	2.3	3.0
G-U	9.7	19.8
G-3	19.5	-
G-6/G-1	0.30	-
G-2/G-6	2.14	-

a. Temperature 32 °C.   b. Glucose specific activity 1.85 x 10^7 c.p.m./ mmole glucose; level used 105,000 c.p.m.   c. G-3 is the 18 hour % radiochemical yield calculated for glucose-3-C^{14} using Equation 3.   d. G-6/G-1 is the % radiochemical yield from glucose-6-C^{14}/ % radiochemical yield from glucose-1-C^{14}.   e. G-2/G-6 is the % radiochemical yield from glucose-2-C^{14}/% radiochemical yield from glucose-6-C^{14}.

The experimental technique is the same as described in Section A2c. A mixture of ethylene in air (1:1000) was passed through the respiration chambers maintained at 32° C. in order to sweep the metabolic carbon dioxide from the respective fruits. The interval radioactive recovery with respect to time is plotted in Figure 10. For comparison purposes, in Figure 11, the cumulative radioactive recovery curves of the control counterparts are compared with the cumulative radioactive recovery curves from the ethylene treated set. The experimental data are listed in Table XV.

7. Effect of ethylene and dinitrophenol on the utilization of sodium phosphate-P^{32} by tomato fruits:

By means of the technique described in Section A, 0.1 ml. of a solution of sodium phosphate-P^{32}, pH 7.0, containing 1.82×10^7 c.p.m. was introduced into each of 8 mature green tomato fruits. Four of the fruits were then placed in separate dark brown bottles and flushed with a mixture of ethylene in air (1:1000) and then allowed to stand at room temperature. The remaining 4 fruits were allowed to metabolize in air at room temperature. At intervals of 1, 4, 6.5 and 23 hours, respectively, one fruit from each set was removed for radioactive assay. In a similar experiment 1.5 mg. of 2,4 dinitro-phenol (approximately 10^{-4} M/fruit) was administered to each of two fruit along with sodium phosphate-P^{32} containing 1.82×10^7 c.p.m. These tomatoes were allowed to stand in air and examined after 6.5 and
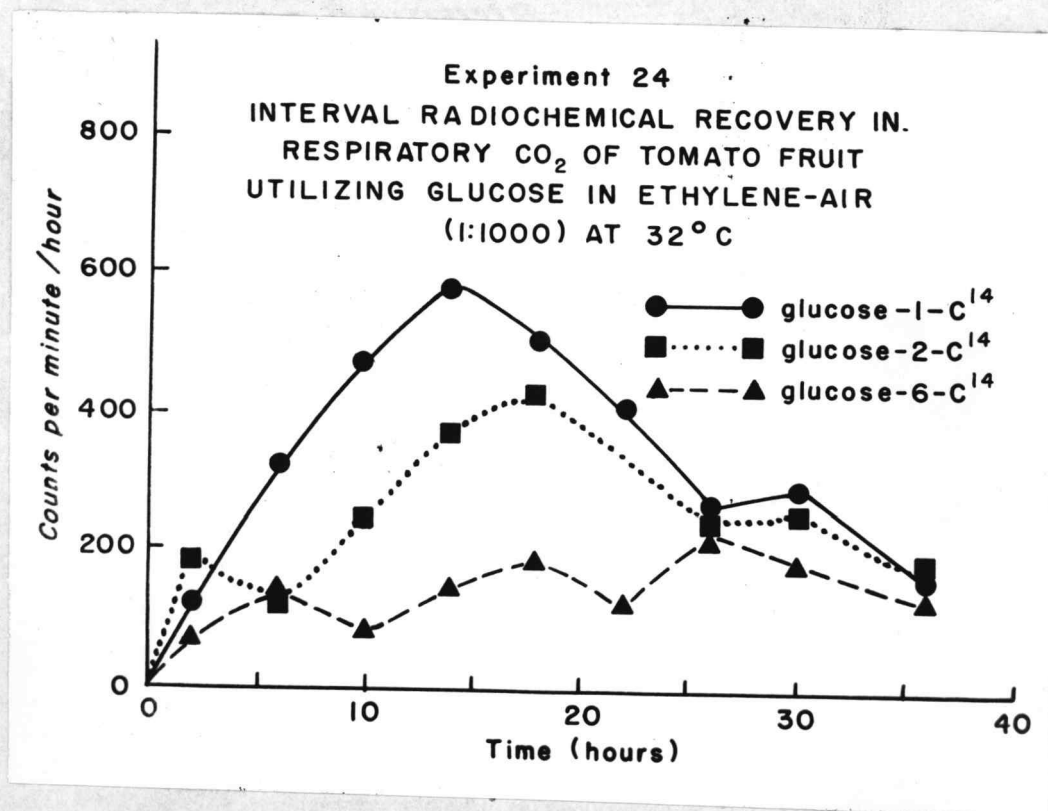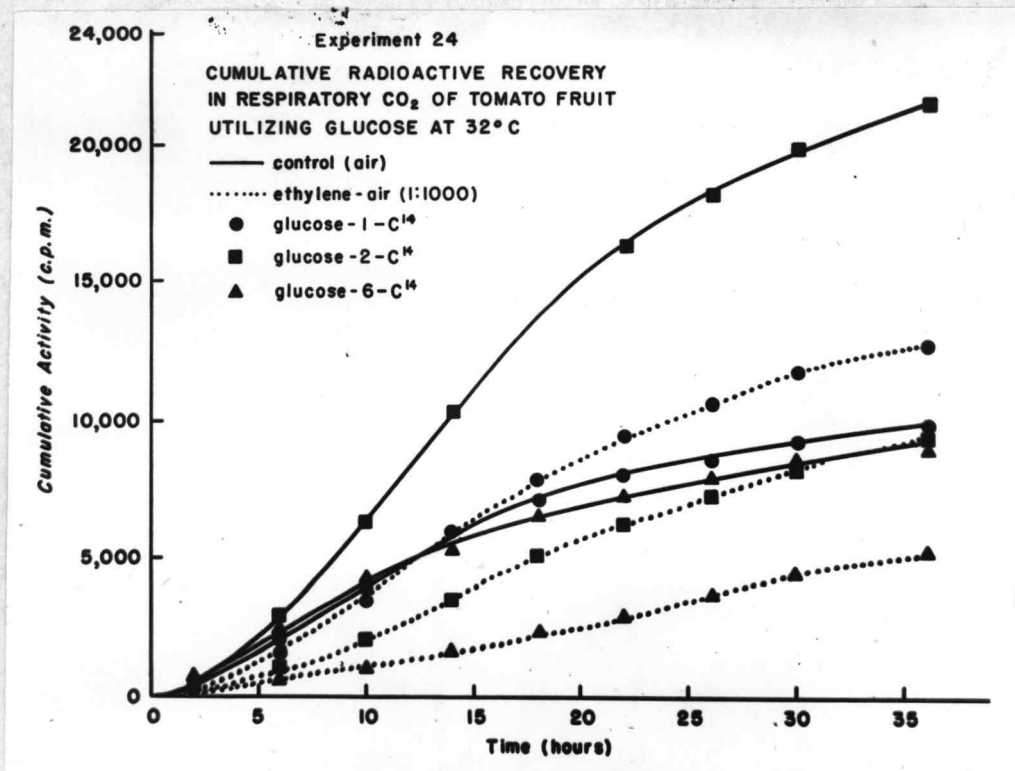
Figure 10

Figure 11

23 hours, respectively.

Each fruit was homogenized in a Waring blendor with an equal weight of water after which the aqueous extract was removed by centrifugation. A 2 ml. aliquot of the filtered extract was taken for separation of inorganic phosphate from soluble organic phosphate according to the procedure of Nielsen and Lehninger (72, pp. 555-570). This procedure relies on an isopropanol-benzene extraction of the orthophosphate in the form of phosphomolybdic acid from the aqueous layer containing the organic phosphate.

The separation procedure was carried out for each fruit and aliquots of the original juice and of the separated organic phosphate were counted in a GM counter in the manner previously described. The distribution of radioactivity between organic and inorganic phosphate is given in Table XVI.

D. Incorporation of Ethylene-1,2-C^{14} into Fruits:

Since it has been demonstrated that ethylene indeed has an effect upon the pathways of glucose utilization in fruit, it was of great interest to investigate the mechanism of its physiological effect and the mode of ethylene production in different fruits. Consequently, experiments were carried out with C^{14}-labeled ethylene in an attempt to study the possible incorporation of ethylene into fruit constituents by way of an exchange reaction or reversible process which might reflect the nature of the ethylene precursor or the site of its action. It was

TABLE XVI

EFFECT OF ETHYLENE[a] AND DINITROPHENOL[b] UPON THE UTILIZATION

OF SODIUM PHOSPHATE[32] BY TOMATO FRUITS

|	Control		Dinitrophenol Treated		Ethylene Treated	
Elapsed Time (hours)	Organic[c] Phosphorous (%)	Inorganic Phosphorous (%)	Organic Phosphorous (%)	Inorganic Phosphorous (%)	Organic Phosphorous (%)	Inorganic Phosphorous (%)
1	6	94	-	-	4	96
4	15	85	-	-	12	88
6.5	36	64	39	61	24	76
23	52	48	49	51	48	52

Experiment 25[d]

a Ethylene in air (1:1000)
b Dinitrophenol 1.5 mg/fruit
c Per cent of total water soluble phosphorous activity
d Level used 1.82×10^7 c.p.m.

reasoned that if the ethylene gas evolved by fruit is an end product and is in equilibrium with some unknown precursor (EtP), carbon[14]-labeled ethylene in the surrounding atmosphere might be incorporated into the precursor (or active site) if the equilibrium constant of the reverse reaction (k_2) were of reasonably large value:

$$EtP \underset{k_1}{\overset{k_2}{\rightleftarrows}} CH_2 = CH_2 + P$$
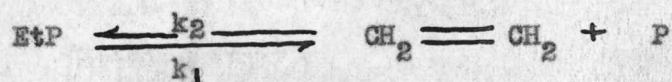
1. Introduction of labeled substrate:

The fruits were exposed in large desiccators to portions (2.8×10^7 c.p.m.) of 1,2-C^{14}-labeled ethylene gas (obtained from Tracerlab, Inc.; specific activity 1.4×10^8 c.p.m./mmole) in concentrations of 1 part of ethylene to 1000 parts of air at normal pressure. Each desiccator was equipped with a beaker of KOH solution to absorb the respiratory carbon dioxide. Periodically reduction of the oxygen tension within the system, as indicated by an attached manometer, was restored with oxygen gas from a cylinder.

2. Examination of the fruit constituents for incorporated radioactivity:

Experiments of the above type were carried out on green apples, pears, tomatoes, grapes, ripe oranges, avocados, papayas and limes. A small amount of ethylene radioactivity was found to be

incorporated into avocados and pears, mainly into the organic acid fraction, and a slight amount was incorporated into papayas. Subsequent experiments carried out in an atmosphere of oxygen or nitrogen failed to improve the extent of incorporation.

In a typical experiment, two ripe avocado fruits (total weight 604 g.) were exposed at room temperature to ethylene 1,2-C^{14} in the manner outlined above, for a period of 10 days. At the end of the experimental period, the radioactive ethylene was swept from the desiccator and trapped in mercuric perchlorate solution (118, pp. 551-555). The respiratory CO_2 was recovered from the KOH trap solution as $BaCO_3$, plated and counted in the conventional manner. It was found to be not labeled.

The fruits were mechanically separated into three portions (seed, pulp, and peel), and each section was treated in the manner previously described (Section B2) in order to ascertain the distribution of radioactivity which is compiled in Table XVII.

The results from a similar study on green pears are also listed in Table XVII.

3. <u>Isolation</u> <u>and</u> <u>degradation</u> <u>of</u> <u>fruit</u> <u>acids</u>:

After exhaustive ether extraction of the combined pulp organic acids, they were concentrated and chromatographed on silica gel column using increasing concentrations of n-butanol in chloroform (25, pp. 187-190). The fractions representing the acid peaks were

TABLE XVII

INCORPORATION OF RADIOACTIVITY FROM ETHYLENE 1,2-C^{14}[a,b]

INTO PEAR AND AVOCADO FRUIT

AVOCADO: Experiment 26

    Seeds
        Ethanol extract   1700 c.p.m.
        D-persitol   200 c.p.m.

    Peelings
        Aqueous extract   3000 c.p.m.
        Organic acids   2740 c.p.m.

    Pulp
        Aqueous extract   9000 c.p.m.
        Organic acids   6850 c.p.m.

    Respiratory CO_2 0

PEAR: Experiment 27

    Peelings
        Ethanol extract   5000 c.p.m.
        Organic acids   3520 c.p.m.

    Pulp
        Aqueous extract   8000 c.p.m.
        Organic acids   3520 c.p.m.
        Amino acids   0
        Sugars and other neutrals   540 c.p.m.

    Respiratory CO_2 0

---

[a] Applied radioactivity 2.8×10^7 c.p.m.

[b] Ethylene in air 1:1000.

combined and concentrated. Fumaric and succinic acids were isolated therefrom in the pure state. The purity and identity of all acid fractions was established by paper chromatography in 1-pentanol:5 M aqueous formic acid (22, pp. 489-491) or n-butanol:acetic acid:water (4:1:5) (66, pp. 98-111).

The specific activity of fumaric acid was determined by wet combustion. A portion of the acid was degraded according to the method of Allen and Ruben (4, pp. 948-950) and Foster et al. (36, pp. 663-672), in which fumaric acid was oxidized with acid permanganate at low temperature resulting in conversion of all of the carboxyl carbon and half of the middle carbon atoms to CO_2, leaving the remaining half as formic acid. The latter was steam distilled from the reaction mixture and oxidized to CO_2 by the procedure of Horowitz and King (52, pp. 125-128).

The activity of the entire succinic acid molecule was determined by wet combustion. The acid was degraded by high temperature pyrolysis, according to Kushner and Weinhouse (62, p. 3558), yielding $BaCO_3$ from the carboxyl carbons. The activity of C-2,3 was obtained by difference.

All samples were counted as $BaCO_3$, using a Tracerlab, Inc. Model SC-16C Windowless Flow Counter, to a statistical accuracy of 2 per cent. Results from the degradation studies are presented in Table XVIII.

TABLE XVIII

DISTRIBUTION OF RADIOACTIVITY IN FUMARIC AND SUCCINIC ACIDS

ISOLATED FROM AVOCADO FRUIT TREATED WITH

ETHYLENE 1, $2C^{14}$[a],[b]

	Specific Activity c.p.m./ mmole	Per cent of total
Fumaric Acid		
COOH	89.7	38.4
CH	27.0	11.6
CH	27.0	11.6
COOH	89.7	38.4
whole molecule	234.0	100.0
Succinic Acid		
COOH	40.1	38.4
CH_2	12.0	11.5
CH_2	12.0	11.5
COOH	40.1	38.4
whole molecule	104.0	100.0

a Activity supplied 2.8×10^{7} cpm
b Ethylene in air 1:1000

DISCUSSION

It can be seen from the experiments described above that efforts have been made in elucidating the metabolic pathways of carbohydrates and their related intermediates in fruits with the aid of radioactive tracer techniques.

A. Carbohydrate Catabolism in Fruits:

Using uniformly labeled glucose as a reference substrate, it appears that only 10 per cent of the applied radioactivity is found in the respiratory CO_2 of tomato fruits after 30 hours (Table IV) and only 11 per cent in the CO_2 of orange fruits after the same time period (Table II). This low recovery of glucose activity is due to either (1) continuous dilution of the labeled glucose by unlabled glucose (or related compounds) newly formed from polysaccharides; or (2) utilization of the applied glucose for biosynthetic purposes.

Metabolic pathwaywise, it is readily apparent from the results of the studies carried out on fruits using specifically carbon[14]-labeled glucose samples (Tables I, II, III, or IV), that fruits are capable of metabolizing glucose by some route other than the classical pathway consisting of the Embden-Meyerhof-Parnas glycolysis (EMP) and tricarboxylic acid cycle (TCA). With fruits the G-6/G-1 ratio (per cent radiochemical recovery in CO_2 from glucose-6-C^{14}/per cent radiochemical recovery in CO_2 from glucose-1-C^{14}) which is customarily used as an indication of the relative contribution of glycolysis and alternate pathway participation in higher plants

(41, p. 345), varies from 0.32 to 0.74 (Tables I, II, III and IV) indicating considerable contribution from an alternative pathway toward overall glucose catabolism in fruits. Of the fruits tested, cucumbers oxidized the greatest amount of labeled glucose substrate to $C^{14}O_2$ which may reflect a lower glucose content or higher enzymatic activity (7, pp. 675-684).

The G-6/G-1 ratio of the metabolic $C^{14}O_2$ changes as the experiments progress, thus, as given in Table V, the observed ratio changed from a value of 0.27 to a value of 0.68 during the course of the experiment. It is well established that the rate of conversion of the individual carbon atoms of glucose to metabolic CO_2 varies according to the metabolic pathway involved. Degradation of glucose according to the alternate pathway probably involves 2 intermediate compounds before glucose C-1 becomes CO_2, on the other hand, the same conversion following the EMP-TCA cycle pathway would require 20 intermediate steps. It is therefore essential to select a time at which the cumulative radiochemical recovery curves from the various labeled glucose substrates have leveled off, only at which time the G-6/G-1 ratio reflects a true comparison. With this concept in mind, the results from the present studies are generally compared after periods of 30 or 45 hours.

The time course plots of interval radiochemical recoveries in respiratory CO_2 from Experiments 14 and 15 (Figures 4 and 5) furnish additional evidence for the operation of an alternate pathway of

glucose catabolism in fruits. If glucose were metabolized solely via the EMP-TCA pathway, one would expect the highest peak to be that from glucose carbon number 2 (C-2) while those from glucose C-1 and C-6 would be smaller and of equal size. Moreover, glucose C-2,5 should have been converted to respiratory CO_2 earlier than that from glucose C-1,6.

It is evident that this was not the case in the fruits tested. Appreciable amounts of glucose C-1 activity rapidly appeared in the respiratory CO_2 along with lesser activity from glucose C-2 and least from glucose C-6. Furthermore, the order of appearance of the interval radiochemical recovery peaks of $C^{14}O_2$ differed from that expected if the EMP-TCA cycle alone was operating (thus, in Figure 4 peaks in the interval recovery of $C^{14}O_2$ from glucose C-1, C-2 and C-6 was observed after 12, 15 and 30 hours, respectively).

The rapid appearance of glucose C-1 in the respiratory CO_2 could be the result of the participation of the pentose cycle, the Entner-Doudoroff pathway (35, pp. 853-862) or some other alternate pathway of glucose transformation. Judging by the well demonstrated function of pentose cycle type metabolism in the vegetable kingdom in combination with the fact that the Entner-Doudoroff pathway has been detected only in a few microorganisms, it is likely that the alternate pathway of glucose metabolism in fruits probably belongs to the pentose cycle type.

The pathway which is responsible for conversion of C-2 of glucose

to CO_2 cannot be definitely established in the present experiments.
Carbon atom 2 of glucose can be converted to CO_2 by way of either
glycolysis-TCA cycle route or the pentose cyclic pathway; the latter
pathway would give rise to CO_2 from the glucose carbons in the order
C-1, C-2 and C-3 (Figure 1). Since operation of the TCA cycle has
been established as one of the major pathways in respiratory function
and hydroxy acid biosynthesis in fruit, it is reasonable to assume
the bulk, if not all, of the $C^{14}O_2$ from glucose-2-C^{14} is probably
the result of EMP-TCA cycle metabolism.

This is further evidenced by the findings on the ratio between
the $C^{14}O_2$ derived from glucose-2-C^{14} and that from glucose-6-C^{14}
(G-2/G-6 ratio) in the present studies. In conventional glycolysis
the key intermediate, pyruvate, would be labeled in the methyl carbon
from glucose C-1,6; the carbonyl carbon from glucose C-2,5; and the
carboxyl carbon from glucose C-3,4, respectively. Similarly, the
methyl carbon of acetate would be derived from glucose C-1,6 and the
carboxyl of acetate from glucose C-2,5. Combustion of acetate accord-
ing to the tricarboxylic acid cycle pathway would then result in the
immediate conversion of acetate carboxyl carbon (glucose C-2,5) to
metabolic CO_2 followed somewhat later by the appearance of acetate
methyl carbon (glucose C-1,6). If one were to examine the G-2/G-6
ratio in the metabolic CO_2 (analogous to the comparison of the carboxyl
carbon atom to methyl carbon atom in acetate), one could then expect
to find a variation in this ratio with time, in the following sequence:

(1) during the early stages of the experiment, acetate carboxyl carbon (glucose C-2) is a chief source of respiratory CO_2 hence the G-2/G-6 ratio would be high; (2) with extensive TCA cycling, acetate methyl carbon is gradually converted to respiratory CO_2 and results in a lower G-2/G-6 ratio; (3) subsequently, acetate methyl and carboxyl activity will contribute equally to the metabolic CO_2 and the ratio will then be unity.

As may be seen from Table V, the G-2/G-6 ratio for tomato fruits increases during the first 12 hours to a maximum of 2.44 and thence decreases to 1.19 after a period of 45.5 hours, in the manner predicted above. With a change in this ratio with time, it is again important to allow sufficient time for the various pathways to reach an equilibrium state before the G-2/G-6 ratio attains qualitative significance.

In the foregoing discussion it is assumed that the C-6 of glucose is converted to CO_2 only by way of the EMP-TCA cycle. However, it is entirely possible that the recombination of two moles of triose by way of the aldolase reaction would give rise to glucose molecules with the original carbon atom 6 now relocated at the C-1 position. The operation of the direct oxidative pathway of glucose catabolism will then result in the preferential conversion of C-6 of glucose to CO_2. Although the contribution of this possible route can not be defined in the present study, it is unlikely to be extensive as indicated by the rapid utilization of pyruvate by tomato fruits (Experiment 18) and the negligible incorporation of pyruvate into glucose (Table VII) in the

$CH_3C^{14}OCOOH$ experiment.

Wang and coworkers (109) have recently presented a method for estimating the contribution of individual pathways in overall glucose metabolism of microorganisms, based on the relative contribution of the individual glucose carbons as indicated by the radiochemical yield from glucose-1-C^{14}, glucose-2-C^{14}, glucose-6-C^{14} and glucose-U-C^{14} in the respiratory CO_2. On the basis of a few basic assumptions, it is possible to calculate the contribution of C-3 of glucose to metabolic CO_2 according to the following equation:

$$G_3 = \frac{6G_U - (G_1 + 2G_2 + G_6)}{2} \qquad (3)$$

where G_U, G_1, G_2, G_3 and G_6 represent the radiochemical yield in the respiratory CO^2 from glucose-U-C^{14}, glucose-1-C^{14}, glucose-2-C^{14}, glucose-3-C^{14} (calculated value) and glucose-6-C^{14}, respectively. One may expect that the exhaustion of the administered labeled glucose in the cells would be directly reflected by the disappearance of C-3 of glucose from the respiratory CO_2 which will be indicated by the leveling-off on the time-course curve of the cumulative radiochemical yields in $C^{14}O_2$ from glucose C-3.

In a system such as fruit, the continuous supply of nonlabeled glucose from polysaccharides dilutes the administered labeled glucose gradually to the extent beyond one's means of detection. Under these

circumstances, it is only practical to estimate the total labeled glucose metabolized by the following equation:

$$T = G_3 - G_6 + G_1 \qquad\qquad (4)$$

It is evident that these equations are based on the following major assumptions: (1) the administered glucose is metabolized only via phosphogluconate decarboxylation and glycolysis in conjunction with the TCA cycle (these pathways are the only ones so far detected in higher plants which carry out this function); (2) the pentose resulting from phosphogluconate decarboxylation is not further metabolized extensively by way of the pentose cycle or other pathways; (3) the removal of C-1 from glucose via phosphogluconate decarboxylation and the oxidative decarboxylation of pyruvate (loss of glucose C-3,4) formed in the glycolysis both occur promptly (this assumption is supported by the rapid appearance of glucose-1-C^{14} and glucose-U-C^{14} activity in the respiratory CO_2); and (4) very little hexose is formed from triose (as substantiated by the $CH_3C^{14}OCOOH$ experiment).

Although these assumptions may not be entirely valid in fruit systems and hence the significance of the subsequent calculations based on these assumptions may have only limited value, nevertheless, they represent the marginal limits of the estimations of these pathways.

The calculations for the estimation of individual metabolic pathways can be made according to the following equations:

(1) Fraction of glucose metabolized by phosphogluconate

decarboxylation (G_p):

$$G_p = \frac{G_1 - G_6}{T} \qquad (5)$$

(2) Fraction of glucose metabolized by the glycolytic pathway (G_e):

$$G_e = 1 - G_p \qquad (6)$$

(3) Fraction of acetate carboxyl converted to CO_2 (R_c):

$$R_c = \frac{G_2}{G_3} \qquad (7)$$

(4) Fraction of acetate carboxyl utilized in biosynthesis (S_c):

$$S_c = 1 - R_c \qquad (8)$$

(5) Fraction of acetate methyl converted to CO_2 (R_m):

$$R_m = \frac{G_6}{G_3} \qquad (9)$$

(6) Fraction of acetate methyl utilized in biosynthesis: (S_m):

$$S_m = 1 - R_m \qquad (10)$$

In Table XIX are listed the results from such calculations per-
formed with the experimental data from Experiment 16, a time course
study on the utilization of glucose by green tomato fruits. It
appears that the contribution from phosphogluconate decarboxylation
reaches an early maximum of 27 per cent after 6 hours and subsequently

TABLE XIX

AN ESTIMATION OF THE RELATIVE CONTRIBUTION OF THE PATHWAYS

OF GLUCOSE METABOLISM IN TOMATO FRUITS IN AIR

Experiment 16

Time (hours)	G_p[a] (%)	G_e (%)	R_c (%)	S_c (%)	R_m (%)	S_m (%)
6	27	73	-	-	-	-
12	18	82	-	-	-	-
18	15	85	-	-	-	-
24	16	84	23	77	18	82
33	14	86	-	-	-	-

[a] G_p is the fraction of glucose metabolized by phosphogluconate decarboxylation; G_e is the fraction of glucose metabolized by the glycolytic pathway; R_c is the fraction of acetate carboxyl converted to CO_2; S_c is the fraction of acetate carboxyl utilized for biosynthesis; R_m is the fraction of acetate methyl converted to CO_2; and S_m is the fraction of acetate methyl utilized for biosynthesis.

declines to 14 per cent after 33 hours. This observed trend may stem
from the previously mentioned differences in reaction rates between
glycolysis and the phosphogluconate decarboxylation. Nevertheless,
it is clear that the direct oxidative pathway does play an essential
role in the catabolism of glucose in tomato fruits.

The calculated values of R_c, S_c, R_m and S_m are given only for
24 hours in Table XIX, at which time the radioactivity recovered in
CO_2 from glucose C-3 had leveled off. The relatively higher contri-
bution of acetate carboxyl carbon to total respiratory CO_2 and the
relatively higher contribution of acetate methyl carbon in bio-
synthesis are in good agreement with what has been reported in yeast
(109) and Streptomyces griseus (110) as a result of the operation of
TCA cyclic processes in these organisms.

In Table VI may be seen the distribution of radioactivity into
various fractions isolated from tomato fruits used in Experiment 13.
There is no noticeable difference in the distribution of activity
from glucose-1-C^{14} and glucose-6-C^{14} among various fruit constituents.

B. The Nature of the Metabolic Pathways of Glucose Breakdown Products:

The fate of some of the major glucose breakdown products in
tomatoes are demonstrated in the experiments on the incorporation of
$CH_3C^{14}OCOOH$, $C^{14}O_2$ and $CH_3C^{14}OONa$.

The distribution of incorporated activity into various fractions
of tomatoes as derived from $CH_3C^{14}OCOOH$ and $C^{14}O_2$ is given in Table

VII. The incorporated C^{14} activity from either substrate was found to occur to the greatest extent in the organic acids with smaller amounts present in the amino acids and sugar components. Wang et al. (108, pp. 741-745) found that approximately 45 per cent of the $CH_3C^{14}OOH$ activity appeared in the metabolic CO_2 at the time of saturated utilization in contrast to 15 per cent recovered in the present pyruvate-2-C^{14} experiment at an equivalent time. This suggests that pyruvate is not oxidized to the same extent as acetate but instead, is utilized more extensively by the fruits for biosynthetic purposes. Nevertheless, the activity incorporated in the organic acids in the present pyruvate experiment and that of the acetate study by Wang and coworkers are more or less identical (14 and 10 per cent, respectively).

The isotopic distribution patterns of malic and citric acids isolated from the CO_2 and pyruvate experiments are given in Table X. In view of the difference in the quantity of acids present in the fruits, the labeling level of various carbon atoms was corrected for the pool size of each acid in the fruit used in the present experiments. This corrected specific activities as well as the pool size of malic and citric acids are given in Table XX.

From $C^{14}O_2$, as expected, one finds the activity located exclusively in the carboxyl groups of the fruit acids. The considerably heavier labeling in C-4 of malic acid as compared to that of C-1, indicates that the mechanism of CO_2 fixation in tomatoes belongs to the Wood-Werkman (116, pp. 377-388) or malic enzyme (76, pp. 979-

Table XX

Distribution of Radioactivity in Citric and Malic Acids from Tomato Fruits utilizing $CH_3C^{14}OCOOH$ and $C^{14}O_2$ Corrected for Pool Size

Carbon Atom	$C^{14}O_2$ Experiment Sp. activity cpm x 10^5/m mole	$CH_3C^{14}OCOOH$ Experiment Sp. activity cpm x 10^5/m mole
Malic Acid found in the fruit	6.5 m mole	2.3 m mole
C-1	1.6	0.55
C-2	0	0.1
C-3	0	0.05
C-4	2.6	0.7
Whole molecule	4.6	1.4
Citric Acid found in the fruit	15.3 m mole	15.2 m mole
C-1 + C-5	1.9	1.1
C-2	0	0.02
C-3	0	0.02
C-4	0	0*
C-6	2.3	0.2

* assumed to be unlabeled

1000) type. It is interesting to note that the partial randomization of the malic acid carboxyl groups observed here (probably as a result of equilibration with fumarate) is in contrast to the recent report on CO_2 fixation in apples (5, pp. 231-234 and 6, pp. 234-238) where complete randomization was realized. In citric acid, the tertiary carboxyl carbon was significantly more heavily labeled than the primary carboxyl carbon atoms. By assuming that (a) C-5 of citric acid is unlabeled as shown by the absence of $C^{14}O_2$ activity in the fat fraction (Table VII), hence the "acetate" unit; and that (b) the C_4 condensing component in the citric acid synthesis is symmetrical as indicated by the equal labeling of C-2 and C-3 of citric acid in the pyruvate experiment, one could then assign the activity of primary carboxyl carbon atoms to C-1 alone and expect to find equal labeling in C-1 and C-6 of citric acid. The observed excessive labeling in the tertiary carboxyl carbon atom consequently reflects the occurrence of some other CO_2 incorporation mechanism such as the formation of isocitrate from alpha-ketoglutarate and CO_2 (10, pp. 68-71; 42, pp. 461-465 and 75, pp. 133-157).

Since the demonstration of the occurrence of $C_1 + C_3$ condensation (with CO_2) in tomatoes does not necessarily imply the quantitative significance of this reaction in the overall acid biosynthesis, the pyruvate experiment was therefore carried out. Pyruvate-2-C^{14}, a key intermediate in the glycolytic scheme, is capable of giving rise to C_4 acid with labeling mainly in C-2 and C-3 by way of CO_2 fixation. On

the other hand, this compound can also be converted to acetate which is in turn used to synthesize a C_4 acid through Thunberg-type condensation (101, pp. 1-91) with labeling confined to C-1 and C-4. Comparison of the specific activity of the C-2 and C-3 with that of C-1 and C-4 will then provide a direct indication of the extent of the operation of the $C_3 + C_1$ reaction. Thus, in yeast which was utilizing pyruvate-2-C^{14} as sole carbon source, Wang et al. found 62 per cent of the activity in C-2 and C-3 of aspartic acid and concluded that the $C_3 + C_1$ type reaction is responsible for the metabolism of as much as 70 per cent of the available pyruvate in the yeast.

In the present experiment, the detection of labeling in the non-carboxyl carbon atoms of malic acid and citric acid substantiates the fact that CO_2 fixation occurs in this fruit. However, the presence of only 11 per cent of the C^{14} activity in the C-2,3 of malic acid indicates that CO_2 fixation with pyruvate is probably not a major pathway in the biosynthesis of malic acid in tomatoes under these conditions. Judging from the specific activity curve of the respiratory CO_2, (Figure 7) it is reasonable to believe that a steady state in labeling had been reached at the time the pyruvate experiment was terminated; yet the specific activity of malic acid was higher than that of the malic acid skeleton in citric acid even after pool size is taken into consideration (Table XX). This fact implies that malic acid was synthesized from pyruvate prior to citric acid through a pathway unrelated to the citric acid cycle. Although the nature of this pathway

cannot be defined in the present work, it is possible that conden-
sation of two C_2 units of the Thunberg type might play an important
role in the overall acid biosynthesis in this fruit. In Aspergillus
niger, as much as 40 per cent of the citric acid was reported to
originate through the C_2-C_2 condensation pathway (93, pp. 68-80).
Seaman and Naschke (92, pp. 1-12) have recently described a reversible
cleavage of succinate to acetyl coenzyme A in Tetrahymena which is
DPN, CoA and ATP dependent, and also report its presence in mammalian
and other microbial systems.

The isotopic distribution pattern of citric acid in the pyruvate
experiment is in good agreement with that of malic acid. Thus, if one
assumes that C-1, 2, 3, 6 in citric acid are derived from the carbon
skeleton of malic acid by way of the conventional citric acid bio-
synthesis (108, pp. 741-745), the activity of C-1, 6 and of C-2,3
will then be 9 per cent of and 91 per cent respectively of the four-
carbon fragment in citric acid. These figures compare favorably with
11 per cent and 86 per cent respectively observed in malic acid.

It is also interesting to note that C-2 and C-3 of citric acid
are approximately equal in activity. Since these two carbon atoms are
equivalent to C-2 and C-3 of the C_4 condensing component in the classi-
cal citric acid biosynthesis, it is therefore indicated that the C_4
compound must be symmetrical in respect to its labeling pattern. In
view of the fact that malic acid isolated from the same fruit is not
symmetrically labeled it is unlikely that oxalacetate could have been
the condensing partner. This is true since randomization of the latter

by equilibration with a symmetrical C_4 acid (such as fumaric acid) would have involved malic acid as an intermediate. It thus appears that a symmetrical C_4 acid such as fumaric or succinic acid is probably participating in the citric acid formation in tomato fruits.

In the time course study of acetate incorporation into tomato slices, it is apparent that the administered acetate is rapidly metabolized since approximately 30 per cent of the applied radioactivity appears in the acetic acid-free ethanol extract within a period of 3 hours (Table VIII), the incorporated activity being detected in the organic acid and amino acid fractions. However, it is interesting to note that appreciable amount of activity from the acetate carboxyl carbon was also incorporated into the neutral fraction. The nature of this fraction is yet undetermined.

Among the organic acids, C^{14} activity was detected in citric and trans-aconitic acids after 30 minutes, at which time no activity was yet detectable in malic acid (Table IX). This is very interesting especially in view of the fact that in tomatoes, the normal citric acid content is 3-7 times greater than that of malic acid (25, pp. 187-190). After 60 minutes, activity was detected in malic acid and at 90 minutes activity had appeared in lactic acid. These results imply that acetate condenses with oxalacetate to form labeled citrate which is then subsequently converted to labeled trans-aconitate and finally malate by the operation of the TCA cycle. This is also evidenced by the fact that the relative activity in citrate was higher than that of

malate throughout the experimental period. Incorporation of radio-
activity into lactate probably occurred by decarboxylation of oxal-
acetate to pyruvate with subsequent reduction of the resulting
pyruvate.

The distribution of activity among the various amino acids is
also in line with the foregoing discussion, thus, activity appeared
first in glutamic acid and subsequently in aspartic acid. At the end
of the experimental period (3 hours) detectable radioactivity finally
appeared in glycine-serine, histidine, phenylalanine, tryptophane and
tyrosine.

It is understood that the incorporation pattern observed here is
primarily the result of labeling by way of exchange reactions which
does not necessarily reflect biosynthetic pathways and consequently
does not rule out the occurrence of a C_2-C_2 condensation in C_4 acid
biosynthesis. However, the rate of labeling in these acids does
reveal interesting information concerning the rate of reactions in
the Krebs cycle sequence in tomato fruits.

C. Effect of External Factors on Metabolic Pathways in Fruit:

Studies were also made in testing the effect of ethylene, nitro-
gen, oxygen, dinitrophenol (DNP) or temperature upon the utilization
of glucose-1-C^{14}, glucose-2-C^{14}, glucose-6-C^{14}, glucose-U-C^{14}, sodium
acetate-2-C^{14} or sodium phosphate-P^{32} by fruits in an effort to collect
additional information for the elucidation of the nature of the meta-
bolic pathways of fruits.

1. <u>Effect</u> <u>of</u> <u>temperature</u> <u>upon</u> <u>glucose</u> <u>metabolism</u> <u>in</u> <u>tomatoes</u>:

In the early studies on fruits utilizing differentially C^{14}-labeled glucose substrates discussed previously, no attempts were made to control the temperature and consequently during the course of a given experiment, the temperature varied from 20-25° C. Therefore, in an effort to ascertain the effects of temperature, if any, upon the assimilation of labeled glucose by fruits, a study was performed on green tomatoes at controlled temperature (Experiment 24, 32° C.).

The glucose assimilation pattern of tomatoes was found to be extensively altered at elevated temperatures (Figure 9). Thus, in respect to the specific activity and radiochemical recovery of respiratory $C^{14}O_2$, much higher values were observed in the $C^{14}O_2$ derived from glucose-2-C^{14} at 32° C. than that obtained at room temperature (Tables IV and XV). Since the CO_2 production of the fruits was also increased (approximately double that of experiments at room temperature), it appears that the pronounced increase in contribution of C-2 of glucose toward the respiratory CO_2 is probably the result of increased TCA cycle activity.

It is interesting to note that the increase in CO_2 production did <u>not</u> lead to an appreciable increase in the radiochemical recovery from glucose-1-C^{14} in the metabolic CO_2 as compared to similar experiments conducted at lower temperatures (in the high temperature Experiment 24, the radiochemical yield from glucose C-1 in respiratory CO_2

at 30 hours was 8.7 per cent as compared to 9.8, 9.1 and 8.7 per cent in Experiments 13, 14 and 15). However, the recovery from glucose-6-C^{14} was substantially increased (8.0 per cent at 30 hours in Experiment 24 as compared to 4.4, 5.2 and 5.0, in Experiments 13, 14 and 15, respectively), resulting in a marked increase in G-6/G-1 ratio: after 18 hours the G-6/G-1 ratio in Experiment 14 was 0.42 (Table V) in contrast to a value of 0.93 in high temperature Experiment 24 (Table XV).

The contribution of individual catabolic pathways of glucose in tomatoes at 32 °C. has also been calculated in the manner described previously (Equations 3, 4, 5, 6, 7, 8, 9 and 10). The results indicate that 98 per cent of the glucose is catabolized in tomatoes at 32 °C. by way of glycolysis in conjunction with the TCA cycle (Table XXI).

2. <u>The effect of ethylene upon fruit metabolism at room temperature</u>:

In Table XI are presented the results from the experiments with oranges in which the effect of ethylene upon the utilization of sodium acetate-1-C^{14} was examined. Although there was no appreciable difference in the fractions isolated from the pulps of the control and ethylene-treated fruits, considerable difference was observed in the quantities of radioactivity incorporated into <u>all</u> fractions of the peelings. Paper chromatographic examination of various fractions did not reveal any qualitative difference in labeling patterns.

TABLE XXI

AN ESTIMATION OF THE RELATIVE CONTRIBUTION OF THE PATHWAYS

OF GLUCOSE METABOLISM AT HIGH TEMPERATURE[a] AND UNDER

THE INFLUENCE OF ETHYLENE

Experiment 24[b]

ATMOSPHERE	G_p[c]	G_e	R_c	S_c	R_m	S_m
	(%)	(%)	(%)	(%)	(%)	(%)
air	2	98	54	46	25	75
ethylene (in air 1:1000)	21	79	25	75	12	88

[a] Temperature 32° C.

[b] Values calculated from 18 hour data (Table XV).

[c] G_p is the fraction of glucose metabolized by phosphogluconate decarboxylation; G_e is the fraction of glucose metabolized by the glycolytic pathway; R_c is the fraction of acetate carboxyl converted to CO_2; S_c is the fraction of acetate carboxyl utilized for biosynthesis; R_m is the fraction of acetate methyl converted to CO_2; and S_m is the fraction of acetate methyl utilized for biosynthesis.

It appears that ethylene reduced the incorporation of C^{14}-activity into peeling constituents. This observation can be explained on the basis of: (1) the enhanced production of acetate or its related compounds from fruit carbohydrates, thus greatly diluting the administered labeled acetate; or (2) possible effect of ethylene upon the enzymatic processes in fruit biosynthesis. It is noteworthy that much less contrast is observed in the pulp fractions examined in this experiment, which is possibly the result of the greatly reduced ethylene concentration inside the fruit.

In tomatoes (Experiment 27), a similar effect was also observed in the reduced amount of incorporation of acetate activity in the organic acid fraction (Table XII). It is interesting to note that although the interval radiochemical yield in the respiratory CO_2 is higher in the ethylene treated tomato fruits (Figure 8), the specific activity of the respiratory CO_2 is much greater in the control fruit (Table XII). The latter finding is in line with the view that ethylene stimulates the degradation of polysaccharides in fruits.

Preliminary studies on the effect of ethylene upon glucose metabolism in oranges and tomatoes were carried out with the aid of specifically labeled glucose, using the intermittent sweeping (Section A2b) and continuous sweeping (Section A2c) techniques. It appears that ethylene definitely displayed an effect upon the metabolic pathways of glucose in these fruits (Table XIII). In general, the radiochemical recoveries in the $C^{14}O_2$ from C-1 of glucose is only slightly affected.

On the other hand, that from C-2 and C-6 are noticeably suppressed. It is also interesting to note that in Experiment 13, where tomato fruits were exposed to an ethylene-air mixture for 10 hours prior to introduction of labeled glucose substrate and then swept continuously with a normal atmosphere, ethylene does not display any noticeable "persisting" effect, as indicated by the specific activity and radio-chemical recovery data in Table XIII.

3. The effect of ethylene upon the metabolism of glucose by tomato fruits at 32° C.:

It was found that superior reproductability could be ascertained with experiments on glucose metabolism in fruits when carried out at controlled temperature. After the metabolic pattern of glucose in tomatoes at the controlled temperature under investigation has been established, it is then possible to investigate the effect of ethylene on the assimilation of glucose in these fruits.

At elevated temperature (32° C.), the presence of ethylene in the atmosphere displays a much more pronounced effect on fruit metabolism than that obtained previously at lower temperatures. The interval radiochemical recovery patterns in $C^{14}O_2$ derived from labeled glucose becomes completely altered upon exposure of fruit to ethylene (see Figure 9 and Figure 10).

In Table XV are given the specific activities and radio-chemical recoveries of C-1, C-2, C-6 and the calculated C-3 (Equation

3) of glucose in the respiratory CO_2 of tomato Experiment 24 after 18 hours. The reason for the presentation of results at 18 hours, stems from the fact that a pronounced leveling off of the $C^{14}O_2$ calculated C-3 recovery curve was observed at this time.

It may be readily seen that upon exposure to ethylene the radiochemical recovery in $C^{14}O_2$ from glucose-1-C^{14} is increased by 11 per cent, while that from glucose-2-C^{14} is drastically reduced by 64 per cent, that from glucose-6-C^{14} is also reduced 63 per cent, that from glucose-U-C^{14} is reduced by 34 per cent and that from glucose-3-C^{14} (calculated) is reduced by 22 per cent. These changes are also reflected in the marked alteration in the G-6/G-1 ratio from 0.93 in the control fruits to 0.30 in the ethylene-treated fruits. No change was observed in the G-2/G-6 ratio (Table XV).

Quantitative estimation of the contributions of various pathways according to the method given previously (Equations 3,4,5,6, 7,8,9 and 10) were also carried out on the results obtained from ethylene-treated tomatoes at 32° C. and are presented in Table XXI. The values given in this table are calculated from the accumulated radiochemical recoveries in $C^{14}O_2$ of glucose carbon atoms after 18 hours (Table XV) at which time the "available labeled glucose" was exhausted from the fruits as indicated by the leveling of the calculated cumulative radiochemical yield of glucose C-3 in the respiratory CO_2.

Examination of these values as well as the cumulative

recoveries and specific activities of various carbon atoms of glucose in $C^{14}O_2$ (Table XV) revealed the following:

(1) It is evident that ethylene did not affect appreciably the phosphogluconate pathway as indicated by the data on $C^{14}O_2$ recoveries from glucose-1-C^{14} (Table XV). The findings are in good agreement, nevertheless, more pronounced, with the results obtained in preliminary experiments at lower temperatures (Table XIII).

(2) The radiochemical recoveries of glucose C-2, C-6 and C-3 (calculated) in $C^{14}O_2$ are greatly suppressed. This is in line with the finding in the acetate experiments (Experiments 20 and 21) that the rate of degradation of polysaccharides in fruits is stimulated by the presence of ethylene in the surrounding atmosphere resulting in the dilution of labeled breakdown products of glucose.

(3) It is evident that the degradation product of polysaccharides could not have been glucose-1-phosphate, a direct product from the hydrolysis of polysaccharides, as indicated by the absence of dilution effect on the glucose C-1 recovery in $C^{14}O_2$.

(4) Calculated values of the efficiency of the conversion of the acetate carbon atoms to CO_2 or fruit constituents (values R_c, S_c, R_m and S_m given in Table XXI) renders further evidence to the dilution effect of the breakdown products of labeled glucose from fruit polysaccharides.

Consequently, one tends to speculate that the presence of ethylene in the atmosphere stimulates the degradation of polysaccharides in fruits. This is in line with the well established fact that

during the ripening process of fruits, polysaccharides are known to decrease in content while the monosaccharide content gradually increases (73, pp. 199-236 and 89, pp. 1-38)

In order to ascertain the true nature of this process, it is first necessary to examine the transformation of sugars in plants somewhat more thoroughly. In Diagram I are summarized certain relationships of hexoses in plants.

If ethylene stimulates the breakdown of starch to glucose-1-phosphate or glucose in fruits, one would expect the specific activity of glucose-6-phosphate (derived from labeled glucose in the present experiments) to be markedly reduced by the influx of unlabeled glucose-6-phosphate (Diagram I). This would result in a reduction in the radiochemical yield of $C^{14}O_2$ derived from all of the carbon atoms of glucose. However, the present studies have demonstrated that the ethylene effect is selective in that ethylene reduces the amount of labeled glucose metabolized via the glycolytic and TCA cycles but does not affect thatundergoing phosphogluconate decarboxylation. Consequently, it appears that, either (a) starch degradation was not accelerated in fruits exposed to ethylene in the present experiments or (b) more likely, fruits exposed to ethylene degrade starch by some pathway in which glucose-6-phosphate is not an intermediate, for example, the nonphosphorylated pathway of glucose oxidation present in certain microorganisms (84, p. 176).

1.

starch

4.

glucose-1-PO$_4$ +   fructose $\rightleftharpoons$ sucrose

2.   5.   6.

glucose $\longrightarrow$ glucose-6-PO$_4$ $\longrightarrow$ 6-phosphogluconate

7.   9.

fructose-6-PO$_4$

pentose cycle

8.

fructose-1,6-di-PO$_4$

glyceraldehyde-3-PO$_4$
etc.

TCA cycle

1. Amylase and maltase
2. Hexokinase
3. Sucrose phosphorylase
4. Phosphorylase
5. Phosphoglucomutase
6. Glucose-6-phosphate
   dehydrogenase
7. Phosphohexoisomerase
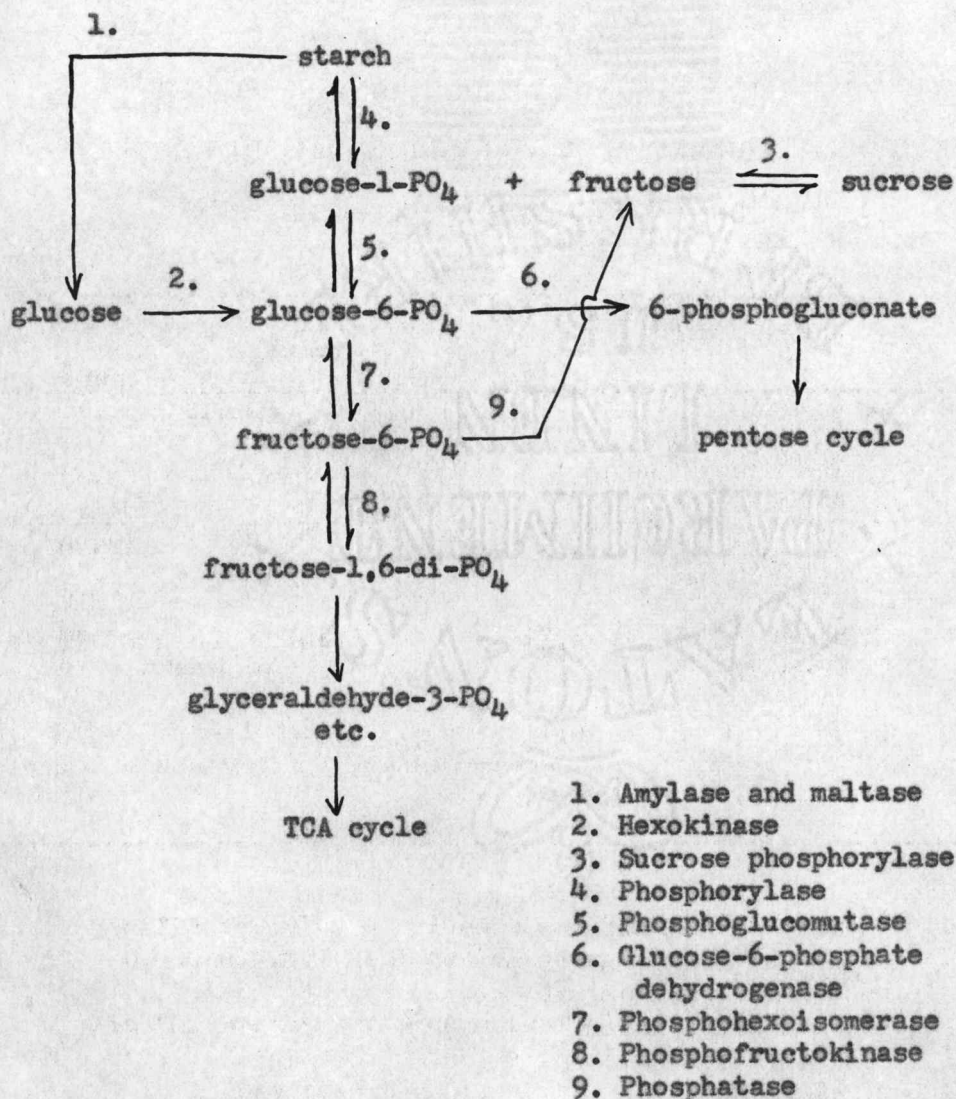8. Phosphofructokinase
9. Phosphatase

DIAGRAM I

REACTIONS LEADING TO THE PENTOSE CYCLE AND TRICARBOXYLIC

ACID CYCLE IN PLANT TISSUES

The concentrations of other polysaccharides change during the ripening of fruits. Thus, of the chemical changes associated with ripening, perhaps the most characteristic involves the pectins. As fruit ripening sets in, water-insoluble protopectin is rapidly converted first to pectin, then pectic acid and ultimately galacturonic acid. The metabolic fate of the galacturonic acid thus produced is somewhat obscure, however, it probably is eventually oxidized to CO_2 by way of the tricarboxylic acid cycle. Acceleration of the breakdown of fruit pectins to galacturonic acid by ethylene might well explain the results of the present study since galacturonic acid would be further metabolized via some non-glycolytic pathway in conjunction with the TCA cycle. The net result would be a dilution of labeled Kreb's cycle intermediates (derived from labeled glucose via classical glycolysis) by an influx of non-labeled pectic degradation products. Phosphogluconate decarboxylation would not be affected and, therefore, the radiochemical recovery in CO_2 from glucose C-1 would be unchanged. This possibility would be in line with the observed stimulation of pectic transformations in fruits exposed to ethylene (45, pp. 145-161).

During the ripening of fruits, the starch content decreases markedly and that of sucrose increases in an inverse manner (89, pp. 1-38). Sucrose is probably formed from glucose-1-phosphate and fructose by the action of an enzyme such as sucrose phosphorylase, thus:

$$glucose\text{-}1\text{-}phosphate + fructose \rightleftharpoons sucrose + P_i$$
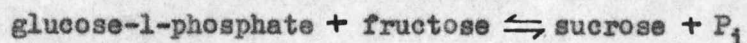
Glucose-1-phosphate undoubtedly is derived from starch by the action of phosphorylase (Diagram I), however, it is of great interest to examine the source of the fructose used in sucrose formation. The fructose content of fruits is somewhat lower than that of glucose (115, pp. 472-474) indicating that the large quantities of fructose utilized by fruits during the ripening process for sucrose synthesis must be formed at that time by active biosynthesis. One may speculate that fructose also is ultimately derived from starch and is probably formed by the action of a phosphatase upon fructose-6-phosphate formed glyco-lytically from starch (Diagram I).

This "siphoning off" of fructose-6-phosphate for the pro-duction of sucrose, would very adequately explain the observed effect of ethylene on the utilization of labeled glucose by fruits. There would, in effect, be a competition for fructose-6-phosphate between this reaction and the remaining portion of the EMP- TCA cycle pathway. This competition should have no effect on hexose oxidized via the pen-tose cycle (since the pentose cycle and EMP-TCA cycle have a common precursor, glucose-6-phosphate). Thus, upon exposure of fruits to ethylene, one would find that the radiochemical recoveries from glucose C-2, C-3 and C-6 would be decreased (more C^{14}-activity being shunted off for sucrose formation) while that from glucose C-1 would be unaffected. This hypothesis is in line with the observation that starch breakdown and sucrose formation are accelerated in fruits ex-posed to ethylene (45, pp. 145-161).

The results obtained in the present study are not necessarily due to the action of ethylene on a single key site but instead may be the combination of a number of separate responses. Moreover, the physiological effect of ethylene is not necessarily restricted to polysaccharides alone, but could involve any one of a number of respirable substrates. Thus, in addition to the possibilities already mentioned, the reduced radiochemical recoveries of glucose C-2, C-3 and C-6 in respiratory CO_2 (with no appreciable effect on glucose C-1) could be caused by an influx of TCA cycle intermediates as the result of ethylene stimulation of: (a) the breakdown of fats to acetyl CoA; (b) breakdown of proteins to certain amino acids which may be converted to TCA intermediates via transamination; or (c) conversion of pentose to α-ketoglutarate in a manner analogous to that observed in Pseudomonas saccharophila (119, pp. 607-624).

4. Utilization of labeled glucose by fruits under nitrogen
atmosphere:

To further investigate the possible effects of environmental conditions on the pathways of glucose catabolism in fruits, experiments were carried out under anaerobic conditions with the ordinary atmosphere surrounding the fruits replaced with nitrogen.

An examination of the mechanism of the classical glycolytic process shows that the anaerobic breakdown of glucose is essentially

a self-perpetuating process, DPNH formed in the oxidation of glyceraldehyde-3-phosphate is utilized for the reduction of (a) pyruvic acid to lactic acid or (b) acetaldehyde (produced from pyruvate by decarboxylation) to ethanol. When oxygen is introduced, it becomes a preferred hydrogen acceptor and the production of alcohol or lactic acid is retarded, or entirely suppressed at above about 5 per cent oxygen concentration.

On the other hand, in the conversion of glucose-6-phosphate to ribulose-5-phosphate, by way of 6-phosphogluconate decarboxylation, two moles of TPN are reduced per mole of glucose. Under aerobic conditions, TPNH is reoxidized by molecular oxygen by way of the cytochrome system and the flavoprotein, TPN-cytochrome reductase. The effect of anaerobisis on the operation of the pentose cycle in fruits is, therefore, of great interest.

In a study of the glucose metabolism in yeast, Beevers and Gibbs (13, pp. 640-641) have investigated the participation of various catabolic pathways of glucose with the aid of labeled glucose and found that unaerated cultures showed a decrease in the per cent recovery in CO_2 from glucose-1-C^{14} amounting to 27 per cent as compared to a decrease in radiochemical recovery of 72 per cent for glucose-6-C^{14}. The G-6/G-1 ratio thus changed from a value of 0.50 in the aerated controls, to 0.19 in the unaerated samples. Blumenthal et al. (20, pp. 6093-6097) have also observed a marked reduction in the relative specific activity of the respiratory CO_2 derived from glucose-1-C^{14} and glucose-6-C^{14} in Saccharomyces cerevisiae and Torulopsis

utilis anaerobically, the conversion of glucose C-6 to $C^{14}O_2$ being

most drastically curtailed. Calculations, according to the method

devised by these workers, revealed that glucose was metabolized al-

most exclusively by way of the EMP process, with a minute occurrence

of the pentose cycle under these conditions. Wang (110) has found

that in S. cerevisiae, the radiochemical recovery of glucose-6-C^{14}

in the respiratory CO_2 was reduced during the early phases of an-

aerobisis on a growing culture but that the radiochemical recovery

from glucose-1-C^{14} was not significantly different from that of the

control. This results in a decrease in the G-6/G-1 ratio during the

early phases of the experiment.

In the present fruit studies under nitrogen atmosphere,

cucumbers displayed a metabolic pattern remarkably similar to that

obtained by Beevers and Gibbs in yeast (13, pp. 640-641). Under

nitrogen, the glucose C-1 radiochemical recovery in respiratory CO_2

decreased by 24 per cent after 45 hours in comparison to the control

while the glucose C-6 recovery decreased by 76 per cent (Table XIV).

The specific activity data from the same experiment (Experiment 1),

however, reveal some rather interesting findings. As expected, the

specific activity of the $C^{14}O_2$ derived from glucose-6-C^{14} is decreased

under the influence of nitrogen but that derived from glucose -1-C^{14}

actually increases anaerobically. Concurrently, the total CO_2 pro-

duction of the nitrogen treated cucumbers was only one-half that of

the control fruits. With reference to the specific activity and

radiochemical recovery data in Table XIV, it appears that (a) while nitrogen reduces the radiochemical recovery in CO_2 from glucose-1-C^{14}, it also induces a more pronounced reduction in endogenous CO_2 production, thus resulting in an actual increase in the specific activity of the $C^{14}O_2$ derived from glucose C-1, and (b) nitrogen inhibits the conversion of glucose-6-C^{14} to $C^{14}O_2$ more completely than it does endogenous CO_2 production, resulting in a decrease in the specific activity of $C^{14}O_2$ derived from glucose C-6. It appears, therefore, that absence of oxygen markedly curtailed operation of the tricarboxylic acid cycle. Of perhaps greater significance is the fact that phosphogluconate decarboxylation apparently occurs under anaerobic conditions and indicates that cucumbers are equipped with some suitable hydrogen acceptor which, in the absence of oxygen, regenerates TPN.

In contrast to the above, are the results obtained with tomatoes (Experiment 13) under nitrogen atmosphere. Thus, after 45 hours, the glucose C-1 recovery in the metabolic CO_2 was reduced some 20 per cent below that of the control but the recovery from glucose C-6 was not affected, hence the G-6/G-1 ratio increased (Table XIV). However, after 95 hours both glucose C-1 and C-6 recoveries in $C^{14}O_2$ were reduced by similar amounts (21 and 23 per cent of control values, respectively) and consequently, the G-6/G-1 ratio was identical to that of the control fruits. It is interesting to note that with tomatoes, the specific activity of $C^{14}O_2$ derived from glucose-1-C^{14} and glucose-6-C^{14} both increased in the absence of oxygen (Table XIV).

As observed with cucumbers, the endogenous CO_2 production also de-creased, in nitrogen, to about one-half of that obtained from control fruits.

These results substantiate the previous findings with cucumbers and indicate that tomatoes are also capable of carrying out decarboxylation of phosphogluconate under anaerobic conditions. However, the response of tomato fruits to oxygen depletion differs somewhat from that of cucumbers in that the conversion of glucose-6-C^{14} to $C^{14}O_2$ in tomatoes is not readily affected in changing from aerobic to anaerobic conditions. Since the observed reduction in endogenous CO_2 production under anaerobic conditions is probably the result of inhibition of the TCA cycle, the lack of response in the con-version of glucose C-6 to $C^{14}O_2$ upon removal of oxygen suggests that tomatoes may be able to oxidize the number 6 carbon atom of glucose to CO_2 via some pathway not involving the TCA cycle.

5. <u>Utilization of labeled glucose by fruits under oxygen</u>

<u>atmosphere</u>:

Experiments in which pure oxygen replaced air in the atmos-phere surrounding the fruits revealed that oxygen had little effect upon the relative contribution of the pentose and EMP-TCA pathways as evidenced by the slight increase in the G-6/G-1 ratios in comparison to that of the control experiments (Table XIV). The specific activities of $C^{14}O_2$ derived from glucose C-1 and C-6 were increased somewhat over

control values in both cucumbers and tomatoes. The radiochemical recovery from glucose-6-C^{14} in the respiratory CO_2 is increased to a somewhat greater extent than that from glucose-1-C^{14} in cucumbers and tomatoes as compared to the respective controls. (Table XIV). The specific activities of $C^{14}O_2$ derived from glucose C-1 and C-6 were increased over control values. These data support the view that the increased oxygen tension enhances operation of the TCA cycle thus resulting in the observed increase in CO_2 production and radiochemical recoveries from glucose C-1 and C-6. It is interesting to note that phosphogluconate decarboxylation was not significantly favored over glycolysis at high oxygen tensions.

6. Effect of ethylene and dinitrophenol upon the utilization of
sodium phosphate-P^{32} by tomato fruits:

Millerd et al. (70, pp. 521-531) have investigated the factor which control respiration rate and hence the climacteric in fruit and found that oxidation of substrate by avocados is coupled to the oxidative production of ATP. With the aid of the uncoupling agent dinitrophenol (DNP), they found that phosphorylation may be uncoupled from oxidation in the preclimacteric fruit but not in the climacteric fruit.

In an effort to establish DNP-uncoupling in fruits, an investigation was carried out on tomatoes using sodium phosphate-P^{32} (Experiment 25). However, this effect could not be demonstrated in

pre-climacteric tomato fruits since there was no detectable difference in the incorporation of P^{32} into the organic phosphate fractions of control and DNP-treated fruits (Table XVI). The apparent lack of effect of DNP on oxidative phosphorylation in tomatoes, however, may be merely due to the inability of dinitrophenol to penetrate the cell walls (Millerd and coworkers used mitochondrial preparations in their studies).

On the other hand, experiments of a similar nature indicated that the incorporation of P^{32} into organic phosphate was markedly re-duced in fruits which had been exposed to ethylene (Table XVI). The reduced incorporation of P^{32}-activity into organic phosphate is most pronounced during the early phases of the experiment and, after 23 hours, little difference exists between the control and ethylene-treated fruits.

It is difficult to elucidate the exact nature of this effect from the present experiment since the observed reduction of P^{32}-incor-poration into organic phosphate in ethylene-treated fruits could be the result of several factors, namely: (a) increased utilization of high energy phosphate for certain endoergonic processes, (b) reduced production of high energy phosphate due to the slowing down of certain metabolic processes or (c) uncoupling of oxidation from phosphorylation.

It is interesting to note that, if ethylene accelerates the conversion of starch to sucrose in fruits, one would expect a more rapid turnover of phosphorous. Thus, in the formation of one mole of sucrose from glucose-1-phosphate and fructose-6-phosphate, two moles

of inorganic phosphate are formed (from glucose-1-phosphate by sucrose phosphorylase and from fructose-6-phosphate by a phosphorylase). High energy phosphate would not be utilized, in this transformation. Thus the observed reduction of P^{32} incorporation into organic phosphate in the presence of ethylene might actually reflect a reduction in hexose phosphate content.

D. The Biosynthesis of Ethylene in Fruits:

The mechanism responsible for the physiological effect of ethylene on fruits and the biosynthetic pathways leading to its production are problems of major interest to plant biochemists. In an effort to gain some insight into the mode of formation of ethylene, studies have been carried out on the incorporation of ethylene-1,2-C^{14} into fruits.

In the present study, it is impossible to distinguish clearly between (a) the incorporation of ethylene into its active physiological site or (b) the incorporation of ethylene into its immediate biosynthetic precursors. However, in spite of the very low incorporation of ethylene activity into the fruits tested, approximately 0.1-0.2 micromoles of ethylene must have been taken up by the fruits (derived from the activity data in Table XVII). This is a fairly large quantity of ethylene on a molecular basis, and suggests that since ethylene is physiologically active such minute quantities, the observed incorporation of ethylene activity in the present experiment is probably the result of an exchange reaction with its immediate biosynthetic precursor.
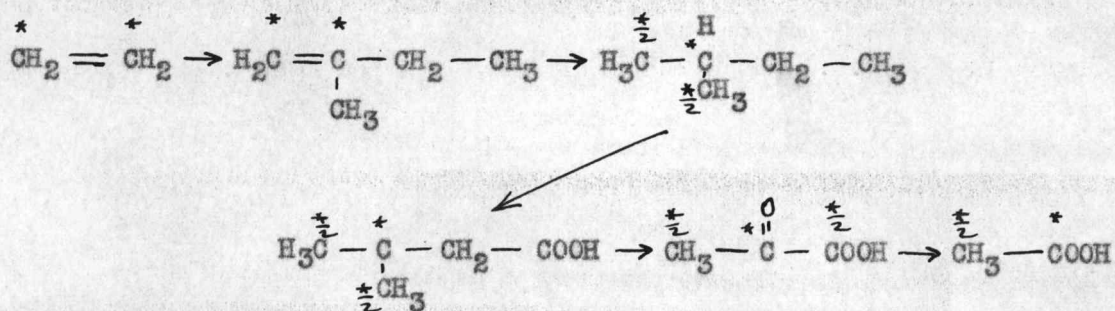
As may be seen from Table XVII, the majority of the incorporated activity is concentrated in the organic acid fractions of the fruit peelings and pulp, suggesting that ethylene is normally produced by a route which is closely connected to organic acid metabolism.

The fumaric acid isolated from Experiment 26 on avocado fruits was approximately double the specific activity of the succinic acid isolated from the same experiment. Interestingly enough, fumaric acid had a higher specific activity than succinic acid even after correction for pool size. This might indicate that fumaric acid is more closely related to the precursor of ethylene than succinic acid. In light of this close relationship between ethylene and the tri-carboxylic acid cycle, it is interesting to note than Hansen (47, pp. 543-558) has found that the ethylene production of fruits is markedly retarded in the absence of oxygen. Moreover, Hall(44, pp. 55-65) has reported that ethylene production in apple juice or Penicillium digitatum is stimulated slightly by addition of citrate, succinate, malate or fumarate and is significantly increased by addition of pyruvate. These observations are further evidence for the close connection between oxidative processes (i.e. the TCA cycle) and the production of ethylene.

Degradation of the isolated fumaric and succinic acids revealed a very interesting labeling pattern (Table XVIII). Approximately three times as much radioactivity was located in the carboxyl carbons of these acids as was found in the middle carbon atoms. It is difficult
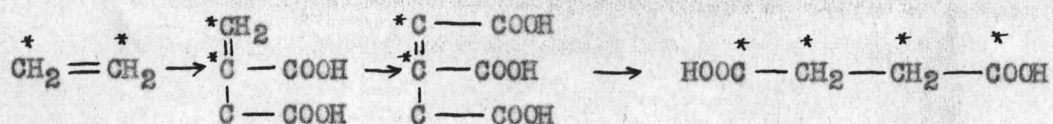
to conceive of a mechanism involving the TCA cycle which makes use of a symmetrically labeled C_2 unit (derived from ethylene directly) which could account for the observed pattern. It may be easily demonstrated that, through the operation of the TCA cycle, acetate carboxyl carbon activity appears only in the carboxyl carbons of the C_4 acids while acetate methyl carbon activity appears in all carbons of the C_4 acids, with more activity appearing in the middle carbon atoms than in the carboxyl carbons. The observed labeling pattern, in succinate and fumarate, therefore, could not arise from symmetrically labeled acetate.

Consequently, one might speculate that the symmetrically labeled carbon to carbon bond stemming directly from ethylene, must either have been ruptured and the fragments metabolized separately, or alternatively, one of the labeled carbons has been "randomized" to a greater extent than the other. For example, in plants the immediate precursor of ethylene may be isoprene (or a related compound) and with the incorporation of the ethylene unit, one might find the following reactions occurring:

$$\overset{*}{C}H_2 = \overset{*}{C}H_2 \rightarrow H_2\overset{*}{C} = \underset{CH_3}{\overset{*}{C}} - \overset{*}{C}H_2 - CH_3 \rightarrow H_3C - \underset{\overset{*}{\pm}CH_3}{\overset{\overset{\pm}{\frac{1}{2}} H}{\overset{*}{C}}} - CH_2 - CH_3$$

$$\underset{\overset{*}{\pm}CH_3}{H_3\overset{\overset{\pm}{\frac{1}{2}}}{C}} - \overset{*}{C} - CH_2 - COOH \rightarrow \overset{\overset{\pm}{\frac{1}{2}}}{C}H_3 - \overset{\overset{O}{\overset{*}{\parallel}}}{C} - \overset{\overset{\pm}{\frac{1}{2}}}{C}OOH \rightarrow \overset{\overset{\pm}{\frac{1}{2}}}{C}H_3 - \overset{*}{C}OOH$$

Thus, it is apparent that, if the two carbon atoms derived from
ethylene are incorporated into a molecule in which one of the two
atoms may be randomized, it is then possible to obtain labeled
acetate in which the majority of the activity is in the carboxyl
carbon.

Other intriguing possibilities exist. Itaconic acid, which
occurs in plants and microorganisms, is a very interesting compound
having no known biological function. Itaconic is readily formed from
cis-aconitic acid (or citric acid) by mild heating. If labeled
ethylene were incorporated into itaconic acid (it would therefore be
the normal ethylene precursor) one might expect to obtain symmetrically
labeled fumarate or succinate in the following manner:

$$
CH_2 = CH_2 \rightarrow
\begin{array}{l} *CH_2 \\ *C - COOH \\ C - COOH \end{array}
\rightarrow
\begin{array}{l} *C - COOH \\ *C - COOH \\ C - COOH \end{array}
\rightarrow
HOOC - CH_2 - CH_2 - COOH
$$

The observed labeling pattern in fumaric and succinic acids could arise
via operation of a pathway of this type in conjunction with a second
pathway leading to carboxyl labeled acetate. It is noteworthy in this
respect that the degradation of succinic and fumaric acids is subject
to certain errors and, consequently, the ratio of activity in the car-
boxyl carbons to that in the middle carbons may be less than the ob-
served 3 to 1 ratio.

However, the greater activity in fumaric acid (even after cor-
rection for pool size) suggests that activity was also incorporated

into the organic acid fraction by some route other than via the condensation of acetate with oxalacetate.

Hall (44, pp. 55-65) was unable to demonstrate increased ethylene production in his test systems when glucose was supplied as the substrate, but found a marked increase in production occurred when arabinose, galactose, fructose, pectic acid or pectin were supplied. The mechanism for the observed stimulation of ethylene production by pectins and certain sugars (but _not_ glucose) is yet unknown, but may be related to galacturonic acid metabolism.

The present study indicates that labeled ethylene may be incorporated to a small extent in fruits, mainly into the organic acid fraction. The unique labeling pattern observed in the isolated C_4 acids suggest that labeled ethylene is _not_ metabolized as an intact C_2 unit derived from ethylene directly, but instead is partially randomized, then converted to acetate and ultimately the C_4 acids.

SUMMARY

Carbohydrate metabolism has been studied in fruits with the aid of glucose-1-C^{14}, glucose-2-C^{14}, glucose-6-C^{14}, glucose-U-C^{14}, $C^{14}O_2$, $CH_3-C^{14}OCOOH$, $C^{14}H_3COONa$, $CH_3C^{14}OONa$ and sodium phosphate-P^{32}.

1. Fruits are capable of metabolizing glucose via conventional glycolysis or via the direct oxidative pathway. Quantitative estimations suggest that in green tomato fruits, approximately 16 per cent of the glucose is oxidized via phosphogluconate decarboxylation and the remaining 84 per cent is glycolyzed.

2. Evidence has been obtained that CO_2 fixation is not a major pathway for organic acid synthesis in excised tomato fruit in the dark. The incorporation of $C^{14}O_2$ activity into the tertiary carboxyl carbon atom of citric acid has also been observed.

3. Further evidence for the occurrence of the tricarboxylic acid cycle in fruit has been obtained.

4. At 32° C. approximately 98 per cent of the glucose is metabolized glycolytically, indicating that the relative contribution of glucose catabolic pathway in fruits varies with the temperature.

5. Exposure of fruit to ethylene reduces the amount of labeled glucose oxidized via the EMP-TCA pathway but does not affect that under going phosphogluconate decarboxylation. The theory is advanced that ethylene stimulation of sucrose synthesis creates a demand which competes with the tricarboxylic acid cycle for labeled glucose carbons,

and results in the observed reduction in labeled glucose metabolized via the EMP-TCA pathway.

6. Experiments revealed that fruits are capable of metabolizing glucose via phosphogluconate decarboxylation even under anaerobic conditions, suggesting that some mechanisms for the regeneration of TPN are present under these circumstances.

7. Experiments on fruits with ethylene $1,2-C^{14}$ demonstrated that ethylene activity may be incorporated to a slight extent, mainly in the organic acid fraction. Evidence is presented which suggests that ethylene is produced from some compound closely related to the tricarboxylic acid cycle.

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