ACCUMULATION OF CITRIC ACID IN FRUIT

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This is to certify that the

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ACCUMULATION OF CITRIC ACID IN FRUIT

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ABSTRACT

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ACID IN FRUIT

by Moshe Tishel

The acetone powders of the orange, lemon, tangerine, grapefruit, strawberry, and tomato fruits (all citric acid accumulators), the apple and sweet lime fruits (malic acid accumulators), the orange flavedo (an oxalate accumulator) and the Bartlett pear (a fruit with high malic and citric acid content) were assayed for the activity of enzymes associated with citrate metabolism.

Aconitase, isocitric dehydrogenase, and malic dehydrogenase showed activities which did not differ considerably from one fruit to another. Citric synthase varied markedly from fruit to fruit, but no correlation between citric acid content and this activity was apparent. The activities of isocitric dehydrogenase (DPN dependent) and isocitritase were not detected in these tissues.

Acetate 2-^{14}C was injected to detached green tomatoes and apples. Half an hour after injection the incorporated ^{14}C was associated with citric acid in both fruits.

After twenty-four hours citric acid remained the most labeled acid in the tomato while malic acid was most highly labeled in the apple.

Glucose-U.L. ^{14}C, was injected into detached green tomatoes and apples. In the apple, citric acid became highly labeled ~~acid~~ one hour after injection but malic acid became the dominantly labeled acid after twenty-four hours. In the tomato an unknown compound incorporated a major portion of the ^{14}C from glucose within thirty minutes after injection and declined with time with a concurrent increase in labeled citric and malic acids and after twenty-four hours most of the label was in the citric acid fraction and none in the unknown.

A double label experiment with ^{14}C and ^{32}P indicated that the unknown acid must be a phosphorylated compound. Homogenate studies established the dependence of this unknown on ATP for its biosynthesis. The label of glucose U.L.-^{14}C as well as glucose 1-^{14}C and glucose 6-^{14}C was found in this compound, eliminating decarboxylation as a step in its biosynthesis. In addition acetate 2-^{14}C did not serve as a precursor.

Acetate 2-^{14}C was fed to apples and tomatoes, the

distribution of radioactivity between particles precipitated at 22,000 x g and the supernatant fraction was compared. In the apple, 33% of the organic acid radioactivity was found in the particles compared with only 5% in the tomato. Citric acid was the only labeled acid in the apple particles.

The organic acid profile in the fruit tissues examined does not correlate with the enzyme activity profile. Labeled precursor experiments corroborate the enzyme survey finding. Finally the intracellular distribution of newly synthesized organic acids indicates the possibility of compartmentation as a factor in acid accumulation in fruits.

ACCUMULATION OF CITRIC ACID IN FRUIT

by

Moshe Tishel

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I. INTRODUCTION

Organic acids are important constituents of food. They contribute to the flavor of foods and act as food preservatives. On the other hand, acids take part in undesirable changes in food processing. Chlorophyll degradation, off-flavors, and browning may be caused by acids during processing and storage.

Citric acid is an important food additive. It is produced commercially either by microbial fermentation or obtained from fruits such as lemon fruits which contains about six percent citric acid. The biochemical and physiological process by which citric acid accumulates in plants is therefore interesting both from the academic and practical standpoints.

Plant tissues, in contrast to animal tissues, tend to accumulate one or more organic acids. Citric acid is the major acid in the juice of citrus fruit, tomato, and strawberry fruits. Malic acid is the main acid of apples, cherries, and quinces.

The accumulation of a certain acid is not a property

characteristic of a family or a genus or even of a given species. Anjou pear accumulates malic acid, whereas the Barlett variety contains about equal amounts of both citric and malic acids. Even in a given fruit such as the naval orange, the flavedo accumulates oxalate while citric is the main acid in the juice. There are now indications that different acids may be distributed unequally within the same cell.

This study was conducted to elucidate the process of citric acid accumulation in fruits. A dual approach was employed: a) a comparative study of the enzymatic activity of tissues of high and low citric acid content, and b) a comparative study of the incorporation and metabolism of organic acid precursors in the two types of tissues.

II. LITERATURE REVIEW

Acid Accumulation

Malic and citric acids are most frequently the major components of the organic acid fraction in fleshy fruits. Citric predominates in orange, lemon, strawberry, gooseberry, and tomato; whereas malic is accumulated in apples, plums, and cherries (Ranson, 1965). Other acids are also found in significant amounts in plant tissues. Roberts and Martin (1954) reported that aconitic acid is the major acid component in sugar cane. It is the most plentiful acid in young shoots of wheat and rye as well as in maize roots and coleoptile (Ranson, 1965). MacLennan and Beevers (1964) have shown that about 95% of the aconitate which accumulates in maize roots supplied with acetate was of the trans-configuration type.

Grapes accumulate tartaric acid in addition to malic (Peynaud and Maurie, 1953) and *Phaseolus coccineus* accumulates malonic acid as well as malic and citric acids (Bently, 1952). The fruits of blackberry, and the leaves of *Bryophyllum* and *Kalanchoe* accumulate isocitric acid (Pucher *et*

al., 1949; Whiting, 1958). A recent review of the distribution of organic acids in higher plants has been published by the U.S.D.A. (Buch, 1960).

An interesting aspect of acid metabolism is the Crassulacean acid metabolism which has been reviewed by Ranson and Thomas (1960). In the dark, malate is produced in the cytoplasm and accumulates in the vacuole. In the light, the vacuole is depleted of malic acid. Citrate generally varies in phase with malate, but with markedly smaller amplitude. Isocitric acid, often the acid present in highest concentration, shows very little diurnal change (Vickery, 1952).

Although the types and the quantities of organic acids are supposedly determined genetically, Kenworthy and Harris (1964) noted that the geographic location determines the acid profile of apples. This observation is supported by Rasmussen and Smith (1960) who noted that oxalate level in orange leaves responds to changes in Ca and K while levels of citric acid remain unchanged.

Acid Translocation

It is often stated that the acids which accumulate in fruits are largely synthesized in the leaves and trans-

located into the developing organs. Pucher et al. (1937, 1949) observed that during germination of Lupinus angustifolius the acid content decreased in the cotyledons and increased in the seedling axis. This tendency was shown for each main acid component, malic, citric, phosphoric and an unknown.

Allsop, according to Ranson (1965), found that oxalic, malic, and citric acids decreased in the rhizomes of sprouting rhubarb and increased in the shoots developing from them. Each of these instances supplied indirect evidence that acids were translocated unchanged from one organ to another. Kursanov (1963), however, found that malic and citric were not translocated from assimilating cells. He pointed out that in the phloem sap sugars were the main soluble component, although aliphatic acids, amino acids and other compounds were found. Webb and Barley (1964) stated that stachyose and sucrose were the compounds which were mostly translocated in various plants.

CO_2 Fixation

Wood and Werkman (1938) reported that dark fixation of CO_2 occurs in bacteria. Bonner and his co-workers (1948)

postulated and later confirmed CO_2 fixation into malate which accumulated in the leaves of Crassulaceae. This was confirmed by Wood (1952), Gregory et al. (1954), and Moyse (1955).

Allentoff, Phillips and Johnston (1954) reported that most of the CO_2 which was fixed by apple fruit was incorporated into malic acid. Ninety-nine percent of the label was incorporated in the carboxyl groups.

Bean and Todd (1960) reported on light and dark CO_2 fixation in the young orange fruit. The flavedo exhibited high photosynthetic activity in contrast to the juice vesicles. In the dark, however, the juice vesicles were far more active in dark fixation than any other tissue. Malic and citric acids were almost equally labeled and they were the major labeled compounds.

CO_2 fixation in citrus fruits was confirmed by Bogin and Wallace (1966) and Bogin (1966). Phosphoenolpyruvate (PEP) carboxylase, malic enzyme and isocitric dehydrogenase were considered as the enzymes which were responsible for this fixation.

Clark et al. (1961), while examining CO_2 fixation in orange and avocado, showed that either phosphoenolpyruvate

or ribulose-5-phosphate can serve as the CO_2 acceptors. They demonstrated also the presence of a polyphenolic inhibitor of CO_2 fixation in avocado leaves.

Several enzymes were postulated as responsible for dark CO_2 fixation:

1. Malic enzyme (Ochoa et al., 1947).
   Pyruvate + CO_2 + $TPNH_2$ $\rightleftharpoons$ Malate + TPN. This enzyme is widely distributed in plant tissues (Vennesland and Conn, 1952).
2. PEP carboxylase catalyses the following reaction:
   PEP + CO_2 $\rightleftharpoons$ Oxalacetate + P_1. This enzyme was first described by Bandurski and Greiner (1953) and is very widely distributed in plant tissue (Mazelis and Vennesland, 1957). According to the latter authors malic dehydrogenase which catalyses the reduction of oxalacetate to malate is also widespread in plants. Together these two enzymes may account for the fixation of CO_2 into malate.
3. Mazelis and Vennesland (1957) also reported that another enzyme, PEP carboxykinase, was widely distributed in plants.

PEP + CO_2 + ADP $\rightleftharpoons$ oxalacetate + ATP.

4. Isocitric dehydrogenase is found in many plant tissues (Vennesland and Conn, 1959).

α- Ketoglutarate + CO_2 + $TPNH_2$ $\rightleftharpoons$ isocitric + TPN

Moyse and Jolchime (1956) suggested that isocitrate which is accumulated may be synthesized by carboxylation of α-ketoglutarate.

Walker (1957) argued that the equilibrium of malic enzyme is unfavorable toward malic acid synthesis while synthesis of oxalacetate by PEP carboxylase is virtually irreversible. Walker and Brown (1957) pointed out that PEP carboxylase has a high affinity to CO_2. It will reach optimum activity at 0.5% CO_2 while carboxylation of pyruvate by the malic enzyme reaches a maximum at 30% CO_2.

Buhler et al. (1956)evaluated the physiological importance of CO_2 fixation in tomato. They injected 2-^{14}C pyruvate into tomato and determined the radioactivity in each of the carbons in malic and citric acids. They concluded that although CO_2 fixation on pyruvate does exist it is of little physiological importance. This supports Walker's data but does not shed light on the role of CO_2 fixation via PEP or α-ketoglutarate.

Clark and Wallace (1963) attempted to correlate CO_2 fixation to the amount of total acidity in citrus fruits. They found that sweet lime had the highest CO_2 incorporation, while lemon had the lowest, and orange was intermediate. The relative acid content was found to be 1:11:37 for sweet lime, orange and lemon, respectively, which is in the reverse order to the CO_2 fixation capacity. The authors concluded that CO_2 fixation may not be the controlling mechanism for acid accumulation.

The Tricarboxylic Acid (TCA) Cycle

There is little doubt today that the TCA cycle is present in plant cells. There is ample evidence to justify the view that the TCA cycle is the major route of pyruvate utilization in plant, as in animal cells. (Beevers, 1961). Evidence has accumulated that the TCA cycle may play a role in acid accumulation.

Markakis and Embs (1964) demonstrated that labeled sugars may be converted to radioactive organic acids in detached strawberry fruits.

Deshpande and Ramakrishnan (1961) suggested that the TCA cycle enzymes may be involved in regulating acid

accumulation in fruit. In their work on Garcinia fruit they demonstrated that citric acid production was concurrent with a ten-fold increase in citric synthase activity and a simultaneous decrease in aconitase activity. On ripening, when the amount of citric acid decreased, citric synthase activity diminished and citric desmolase appeared.

Jangaard, et al., demonstrated that ATP caused a 20-fold increase in the apparent Km of citric synthase toward acetyl CoA; therefore, ATP may act as a regulator of acid accumulation.

Compartmentation

Plant cells have a large vacuole which frequently accounts for 90% of their volume. Stiller (1959) calculated that if malic acid in Bryophyllum leaves were confined to the cytoplasm, its concentration would be 7-8 M; but if it were in the vacuole also, its average concentration would be 0.25 M, a more credible value. Her experimental results also indicate compartmentation of malate, since the malate formed from $^{14}CO_2$ remained asymmetrically labeled, but asymmetrically labeled malate provided to the tissue from outside became randomized. The vacuole need not be consid-

ered as the only storage reservoir of metabolites, however, since a degree of compartmentation within the cytoplasm itself has been invoked as a rational explanation for results of labeling patterns (Porter and May, 1955).

McLennan et al. (1963) designed experiments to determine the existence and extent of compartmentation. They argued that if a labeled compound isolated from a biological tissue had a higher specific activity than its labeled precursor, then only a fraction of the precursor pool is involved in the interconversion. Using a 1-^{14}C acetate, they demonstrated that when the specific activity of the carbon dioxide respired by maize root tips became constant, indicating that equilibrium had been reached in the turnover pools of the acids of the Krebs cycle, there were great differences in the specific activities of the individual acids. The differences were ascribed to the existence of pools of acids not in ready equilibrium with turnover pools, but which mixed with them during extraction. Evidence from maize root shows that, as vacuolation occurs, the relative amount of acid not in turnover pools increased markedly.

Some workers examined the ability of isolated mito-

chondria to incorporate metabolites. They found that the mitochondria were impermeable to externally added citrate (Schneider, Striebich and Hogeboom, 1956; Bartley and Davies, 1953; Amoore, 1958). It was also demonstrated that various chemicals can influence the accumulation and oxidation of citrate by isolated mitochondria (Chappel, 1964; Williams, 1965; Gamble, 1965; Meyer and Tager, 1966). L-malate in the absence of inorganic phosphate increases the permeability of mitochondria towards citrate. When inorganic phosphate is added the nitochondria become impermeable to citrate. Dinitrophenol and ADP + Pi block the entrance of citrate into the mitochondria. Max and Purvis (1965) found that succinate enhances the entry and exit of citrate into mitochondria.

Temperatures close to 0^{o} will retard metabolite incorporation by mitochondria and decrease metabolic activities of mitochondria. Accumulation of citrate by mitochondria is energy linked (Max and Purvis, 1965) which explains some of the effects of various compounds on citrate accumulation.

Cerijo-Santalo (1966) examined the conditions which cause mitochondrial swelling and found that isotonic solu-

tion and neutral pH favor retention of mitochondrial structure. Swelling is promoted by hypotonic solutions with subsequent leakage of substances.

III. MATERIAL AND METHODS

Preparation of Enzymes for Assay

The enzymes were prepared as acetone powder according to Nason (1955). This method has the advantage of giving a chlorophyll-free preparation with a minimum contamination of gum and resin-like material. At least in one case (aconitase activity in apple) the enzyme could be demonstrated in the acetone powder, but not in the apple homogenate, due probably to a polyphenol inhibitor (Hulme et al., 1964). Extraction of the enzymes was accomplished by suspending 300 mg powder in 10 ml phosphate buffer pH 7.1. After 15 minutes the powder was filtered and the clear filtrate was assayed.

Assay of Enzymes

All enzyme assays were performed at room temperature. The specific reaction condition are given in legend to appropriate figures.

Aconitase

This enzyme catalyses the following reaction:

citric acid ⇌ isocitric acid

cis-aconitic acid

Aconitase was assayed by the method of Anfinsen (1955). This method is based on the conversion of citrate and isocitrate to aconitate, and the spectrophotometric determination of the latter at 240 mμ.

Isocitric dehydrogenase (TPN dependent)

The following reaction is being catalysed by this enzyme:

Isocitrate + TPN + $\rightleftharpoons$ α-ketoglutarate + CO_2 + $TPNH_2$

Kornbergs' method (1955) was used for the assay of TPN dependent isocitric dehydrogenase. The reduction of TPN in the presence of isocitrate was followed at 340 mμ.

Isocitric dehydrogenase (DPN dependent)

The following reaction is catalysed by this enzyme:

Isocitrate + DPN + $\rightleftharpoons$ α- ketoglutarate + CO_2 + $TPNH_2$

Two methods were employed. In both, reduction of DPN in presence of isocitrate was followed at 340 mμ. While Kornberg (1955) uses a neutral pH, Davies (1953) uses pH 9.

Malic dehydrogenase

Malic + DPN $\rightleftharpoons$ oxalacetate + $DPNH_2$

Here the oxidation of DPNH in the presence of oxalacetate is followed at 340 mμ. According to Ochoa (1955) the equilibrium lies in the direction of malate formation.

Citric synthase

This enzyme catalyses the following reaction:

Oxalacetate+acetyl CoA ⇌ citrate + CoA

Ochoa's method (1955) for assaying citric synthase activity is based on the coupling of citric synthase with malic dehydrogenase. DPNH formation in the presence of malate and acetyl CoA is followed at 340 mμ.

Isoctritase

Isocitritase catalyses the following reaction:

Isocitrate ⇌ sccinate + glyoxylate

Two methods were employed, both were based on the formation of glyoxylate from isocitrate. According to Carpenter and Beevers (1959) the reaction is run at pH 7.6 and the color of the 2.4 dinitrophenylhydrazones of glyoxylate is read at 445 mμ. According to Olson (1959) the reaction mixture is buffered to pH 6.0 and the semicarbazones of glyoxylate are followed at 252 mμ.

Controls

All enzyme assays were run with two controls: 1) the reaction mixture with one reagent missing and 2) the reaction mixture with a boiled enzyme.

Acid Extraction and Determination

The fruit acids were extracted with acidified boiling water, and depectinized with ethanol. The chlorophyll was removed by petroleum ether extraction. The partially purified mixture of the acids was then passed through an anion exhanger (Dowex-1X8). The column was washed by ca 60 ml deionized H_2O to remove neutral compounds. Subsequently the acids were separated by gradient elution using acetic and formic acids. The fractions were dried and titrated with NaOH solution (Markakis et al., 1963).

Administration of Radioactive Material

Two techniques of administering radioactive precursors to the fruit were employed.

1. Radioactive compounds were injected by syringe into single tomatoes or apples. The compounds were: uniformly labeled glucose -^{14}C, glucose -1-^{14}C, glucose -6-^{14}C, 2-^{14}C acetate, and ^{32}P-phosphoric acid. About 1 μC per fruit was administered. At appropriate time intervals, which are specified in "Results and Discussions," the whole fruit was macerrated in boiling water.

The extraction of the acids was executed as described in the previous section.

2. For the compartmentation study experiments the following technique was used:

The needle of a syringe was inserted into the center of the fruit (tomato and apple) and the cylinder was fitted through the rubber stopper of a vacuum dessicator. The radioactive solution was placed in the syringe and the dessicator was evacuated through a second hole in the stopper (Figure 1).

The radioactive solution was made from 1 μC of 2-C acetate dissolved in 5 ml of 0.35 M mannitol.

The solution diffused into the flesh of the fruit by displacement of the intercellular air during evacuation. Approximately 1 ml of solution per 15 gram of fruit was administered (Dilley, 1967). At appropriate time intervals single whole fruits were homogenized and fractionated as is described in the previous section.

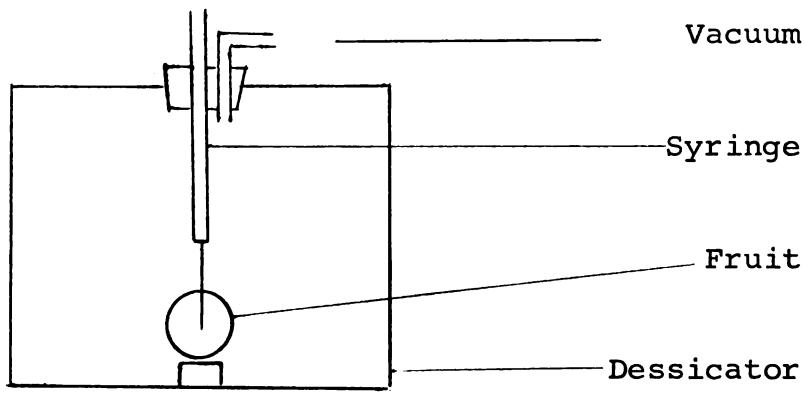

Figure 1. The injection of fruit under vaccuum.

Determination of Radioactive Material

Aliquotes of the fractionated organic acids were dried on disposable aluminum planchets as an infinitely thin layer and counted by a scaler, automatic changer, and time printing instrument. (Nuclear Chicago Company)

A liquid scintillation spectrometer was employed for simultaneous counting of ^{32}P and ^{14}C. For this a Packard tri-carb liquid scintillation spectrometer Model 3003 was used with a window setting of 50-250 for carbon and 350-∞ for phosphorus. The scintillation liquid was prepared as follows:

160 gm Naphthalene
9 gm BBOT 2,5 [2- (5 tertbutylbenzoxozolyl) Thiophane]
75 gm Cab-O-Sil

770 ml P- dioxane
770 ml Xylene
462 ml Ethanol

Fifteen ml of the scintillation liquid was employed with 0.5 ml of aqueous samples.

The Vanguard automatic chromatogram scanner, Model 880 was used for counting paper chromatograms.

Paper Chromatography

The compound was run ascendingly along side with the markers on a Whatman No. I paper. The solvent system was ethyl- methylketone: water saturated butanol:propanol:formic acid 45:25: 5:20(v/v) (Gerlach et al., 1955).

After the solvent front traveled the entire length, the paper was dried at room temperature and irrigated a second time with the same solvent. The following spray was used: 5 ml of 60% W/W $HClO_4$, 10 ml of 1 N HCl, 25 ml of 4% W/V $(NH_4)_2MoO_4$, made up with water to 100 ml (Block et al., 1958).

Isolation of Sub-cellular Particles

All steps in the preparation of mitochondria were conducted at 0-2°C. Since the pH of the ground tomato suspension was about 4, it was necessary to neutralize the acid

during grinding. This was achieved by grinding the tomatoes with 1.5 X their weight 0.1 M phosphate buffer pH 7.5, 0.4 M sucrose, which brought the slurry to pH 7.0-7.2. Ten grams of tomatoes were ground and immediately diluted to 50 ml with 0.1 M phosphate buffer pH 7.0, 0.4 M sucrose. In a similar manner the apple tissues acids were neutralized by adding an equal weight of 0.4 M phosphate buffer pH 7.5, 0.25 M sucrose. The suspension was diluted to 50 ml with 0.05 M phosphate buffer pH 7.0, 0.4 M sucrose. The homogenate was filtered through cheese cloth and milk filter paper and centrifuged at 22,000 x g for 10 minutes.

IV. RESULTS AND DISCUSSION

Theories

It is well known that the Krebs cycle operates in all living organisms. While the intermediate acids of the cycle can be found in catalytic amounts in animal cells, one or more di- or tri-carboxylic acids may be accumulated in high concentration in plant cells.

The amount of acids in plants and the acid profile are probably genetically determined, since they are unique to any given plant tissue.

Two theories have been advanced to explain this phenomenon:

1. The enzymic imbalance theory according to which the acid profile of the fruit is determined by a corresponding enzymic profile.
2. Preferential compartmentation. According to this theory the characteristic acid profile results from a specific mechanism involving removal of a certain acid from its biosynthetic site to a storage site,

and a consequent shifting of equilibrium toward further synthesis of this acid.

While the first theory emphasizes the role of the enzymes, the second stresses the migration of the acids as cause for acid accumulation. These two possibilities are not mutually exclusive, however.

Enzymic Imbalance

It may be assumed that the enzyme activity of the tricarboxylic acid cycle (TCA) in animal cells is carefully balanced and controled and the accumulation of acids is consequently avoided. In plants, however, it is conceivable that enzyme activity is unbalanced which may lead to high acid concentrations. Hence citric acid may be accumulated either as a result of a high activity of citric acid forming enzymes or because of a low activity of citric acid degrading enzymes. Citric synthase which catalyses the formation of citric acid may have a high activity, whereas enzymes like aconitase, isocitric dehydrogenase, malic dehydrogenase which catalyse the degradation of citric acid may demonstrate a low activity.

When attempting to correlate metabolite accumulation with an in vitro estimation of enzyme activity it is assumed

that the enzyme is the limiting factor and an increase or a decrease in its activity will affect the concentration of the metabolite. This assumption is not always valid. Laties (1964), for example, demonstrated that Krebs cycle enzymes were present in young potato tubers in a latent state and that the cycle began to operate upon the aging of the tuber. Standardized procedures for the extraction of the enzyme and their assay is another inherent difficulty in such a survey. They do not ensure optimal activity for the various possible enzymic multiple forms. The various cofactors, inhibitors, activators do not necessarily respond equally well to the standardized treatment, and may result in deviation from the optimum. The activity of the enzyme under physiological conditions may differ from that under assay conditions. Although these reservations are not uncommon to other in vitro experiments, the validity of the conclusions of the *in vitro* studies were checked with *in-vivo* experiments in this study.

Comparison of Enzyme Activity in Various Fruits

Two groups of fruits were assayed for their enzyme activity.

1. Citric accumulators included the fruits of

tomato, strawberry, Bartlett pear, and a few species from the citrus family like orange, tangerine, grapefruit, and lemon fruits.

2. Non-citric accumulators included the fruit of apples and sweet lime which are malic accumulators and Naval orange flavedo which is an oxalate accumulator.

Five enzymes of the Krebs cycle were assayed: aconitase, isocitric dehydrogenase (TPN dependent), isocitric dehydrogenase (DPN dependent), malic dehydrogenase, and citric synthase. From the glyoxylate cycle isocitritase was assayed. All the results are averages of three experiments, and the maximum deviation is ± 15%.

Aconitase

Apples and sweet limes (non-citric accumulators) and oranges and strawberries (citric accumulators) were assayed for their aconitase activity. The results are summarized in Figure 2 and Table I and indicate no difference between citric and malic accumulating fruits.

Isocitric dehydrogenase (TPN dependent)

Apples (malic accumulator) and oranges and straw-

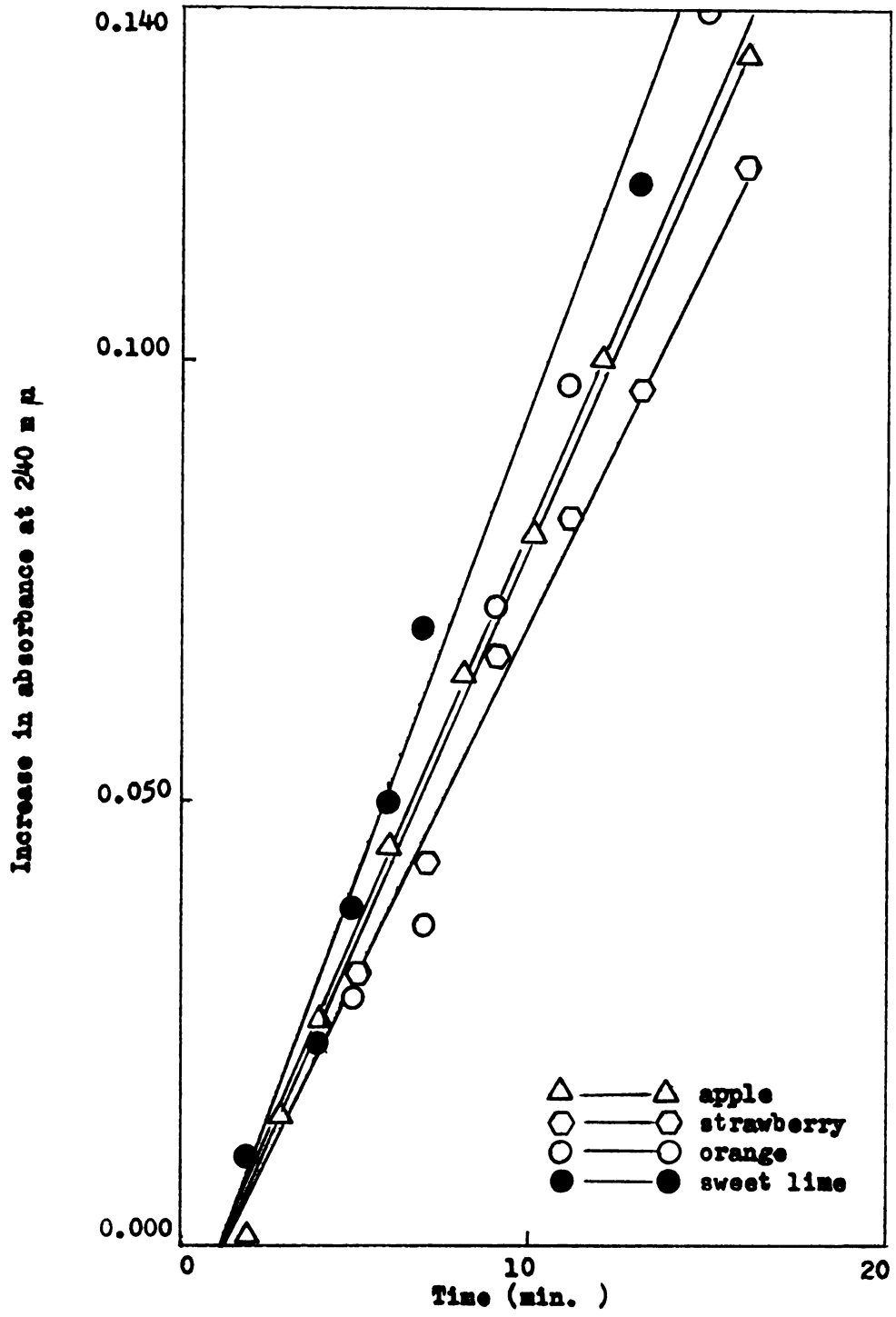

Figure 2. Aconitase activity in some fruits.

Reaction mixture: 0.1 ml enzyme, 225 μ mole potassium phosphate pH 7.4, 75 μ mole citrate pH 7.4.
Tot. vol. 3 ml.

TABLE I

ACONITASE SPECIFIC ACTIVITY

Fruit	Specific activity*
Apple	0.070
Strawberry	0.050
Orange	0.058
Sweet line	0.060

*Specific activity is defined as: increase in absorbance at 240 mμ per gram fresh tissue per minute.

TABLE II

ISOCITRIC DEHYDROGENASE (TPN DEPENDENT) SPECIFIC ACTIVITY

Fruit	Specific activity*
Apple	0.14
Strawberry	0.09
Orange	0.12

*Specific activity is defined as: increase in absorbance at 340 mμ per gram fresh tissue per minute.

berries (citric accumulators) were assayed for isocitric dehydrogenase activity and the results are summarized in Figure 3 and Table II. The activity of TPN dependent isocitric dehydrogenase is similar in citric and malic accumulators.

Isocitric dehydrogenase (DPN dependent)

Apples, strawberries, and oranges were assayed for the activity of DPN dependent isocitric dehydrogenase. No activity was detected, even though the assay was performed at two pH levels (7 and 9). This is supported by Bogin (1966) who found only TPN dependent isocitric dehydrogenase activity in lemon and sweet lime. The reaction mixture was: 20 μmole isocitric acid, 100 μmole potassium phosphate pH 7.0 or 9.0, 10 μmole magnesium nitrate, 0.2 ml enzyme. Total volume 3.0 ml.

Malic dehydrogenase

Apples, oranges, sweet lines, and lemons were assayed for malic dehydrogenase activity. Again no difference in activity could be demonstrated between the two groups (Figure 4). It is noteworthy that in terms of TPN reduction malic dehydrogenase activity is at least ten times higher than that of any other dehydrogenase tested in this work.

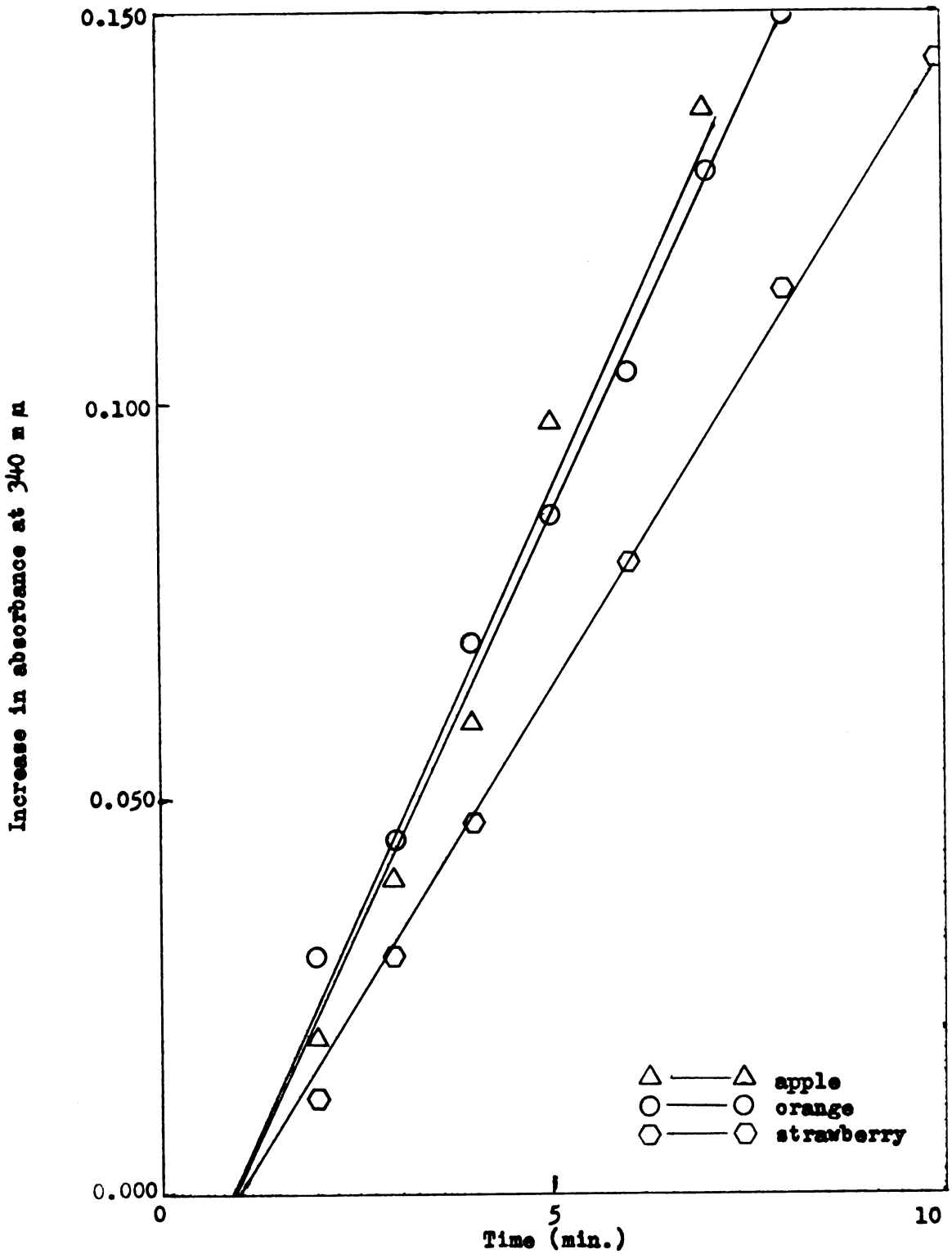

Figure 3. Isocitric dehydrogenase (TPN dependent) activity of some fruits.
Reaction mixture: 100 μmole potassium phosphate pH 7.0, 30 μmole isocitrate, 10 μmole magnesium nitrate, 0.5 μmole TPN, 0.2 ml enzyme. Tot. vol. 3.0 ml.

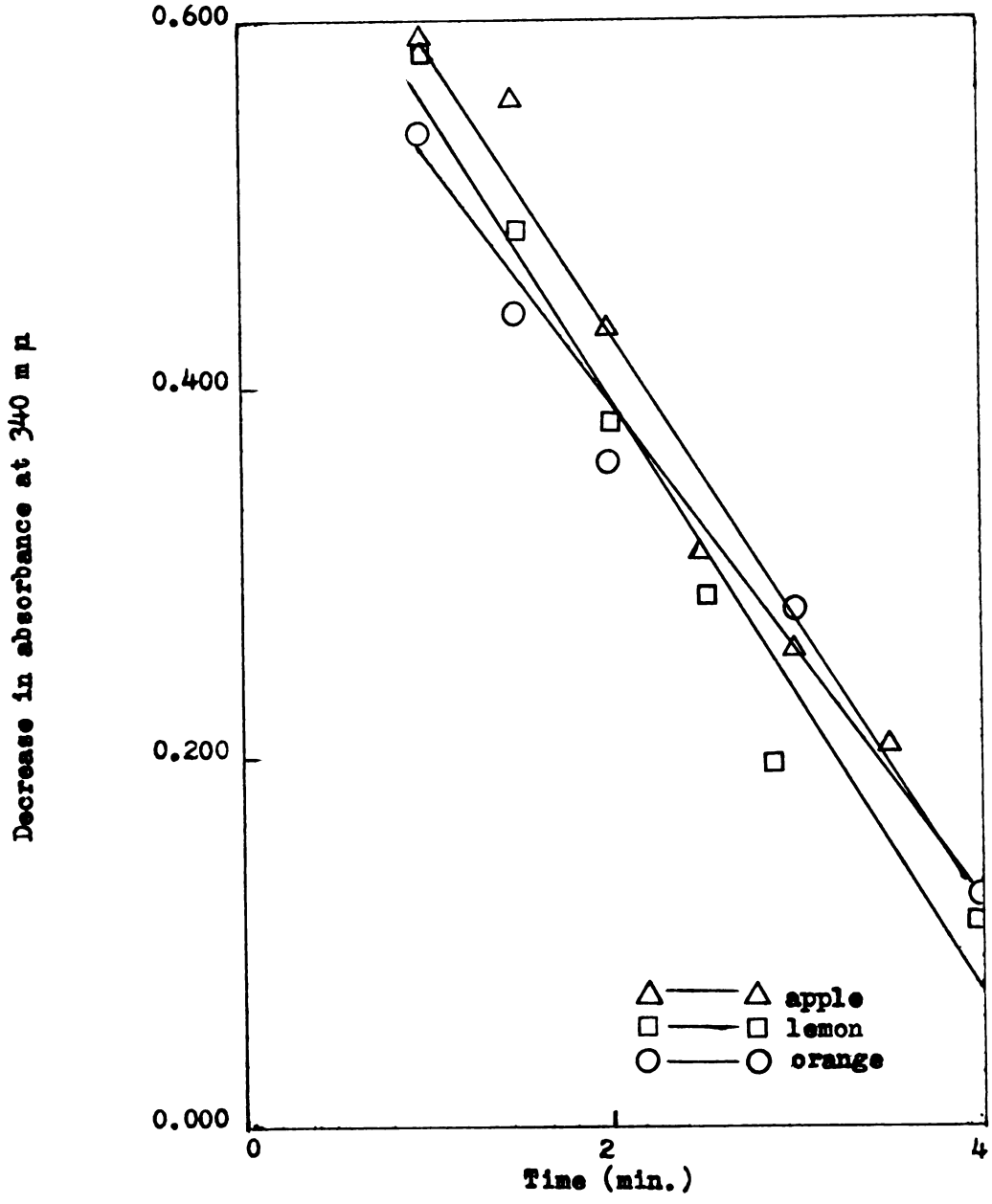

Figure 4. Malic dehydrogenase activity of some fruits.

Reaction mixture: 1 μmole oxalacetate pH 7.4, 0.1 μmole DPNH, 100 μmole potassium phosphate pH 7.4, 0.02 ml enzyme. Tot. vol. 3.0 ml.

Citric synthase

Preliminary experiments indicated that citric synthase activity unlike the rest of the enzymes in this survey is not identical in all fruits. Since this enzyme may be a key factor in citric acid biosynthesis, a rather extensive survey, involving ten tissues was conducted in order to see whether a correlation existed between citric synthase activity and citric acid accumulation. From the group of citric accumulators the following fruits were examined: green tomatoes, red and green strawberries, Bartlett pears (which accumulate equally well citric and malic acids) and from the citrus family the juice and juice vesicles of orange, lemon, and tangerine. The other group of fruits included the malic accumulators, apples and sweet limes and an oxalate accumulator - the flavedo of the naval orange. A broad spectrum of activities could be demonstrated both on the basis of miligram acetone powder and gram fresh tissue (Figure 5, Table III).

All the fruits of the citrus family exhibited a high citric synthase activity, regardless of the acid which they accumulated. Thus the orange, grapefruit, tangerine, and lemon (all citric accumulators), the sweet lime (a malic

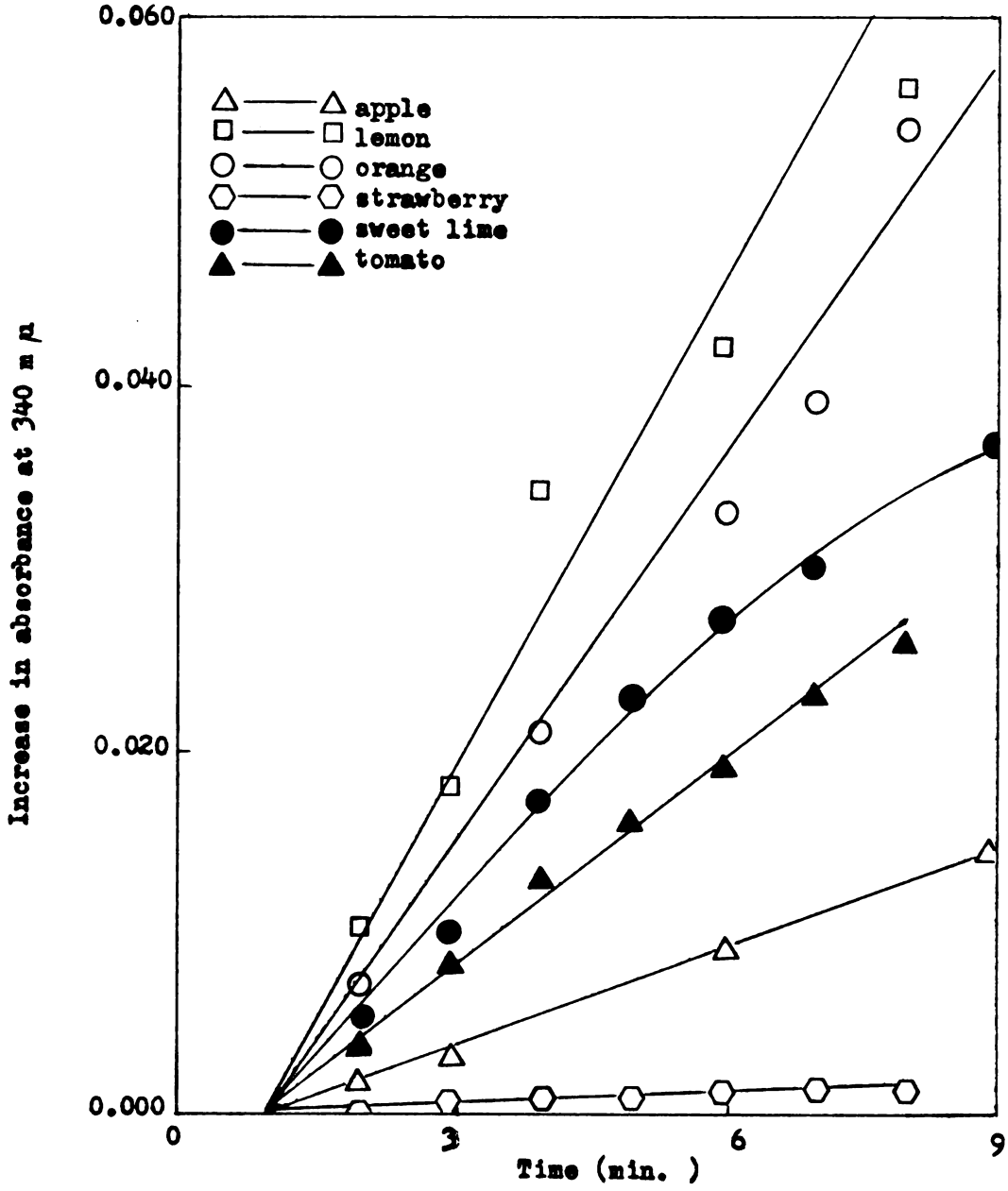

Figure 5. Citric synthase activity of some fruits.

Reaction mixture: malic acid 10 μmole, DPN 0.3 μmole, potassium phosphate pH 8.0 75 μmole, acetyl CoA 0.2 μmole, 0.1 ml enzyme. Tot. vol. 1.0 ml.

TABLE III

CITRIC SYNTHASE SPECIFIC ACTIVITY

Fruit	Specific activity*
Tangerine	0.090
Orange	0.070
Lemon	0.070
Grapefruit	0.060
Tomato	0.060
Strawberry	0.004
Bartlett pear	0.050
Orange flavedo	0.240
Sweet lime	0.055
Apple	0.020

*Specific activity is defined as increase in absorbance at 340 mμ per gram fresh tissue per minute.

accumulator), and the orange flavedo (an oxalate accumulator) had a high activity. Tomato (a citric accumulator) and apple (a malic accumulator) demonstrated an intermediate activity. The Bartlett pear (malic and citric accumulator) had a medium activity, whereas the strawberry which is a citric accumulator exhibited a very low activity regardless of the degree of ripeness.

Dialysis

To remove all possible low molecular weight activators or inhibitors the enzyme extracts from oranges and apples were dialyzed and retested. As can be seen in Figure 6 dialysis reduced the citric synthase activity in both oranges and apples, but the ratio of activities remained unchanged, 3.6 and 3.8 for undialyzed orange and apple and for dialyzed orange and apple, respectively. Apparently, no dialyzable activator or inhibitor is associated with high citrate synthase activity of the orange over that of the apple.

Isocitritase

Two different reaction mixtures and two pH values as well as different reagents were tried in the assay for isocitritase activity. Isocitritase is the key enzyme of the glyoxylate pathway but no activity was observed in the tissue

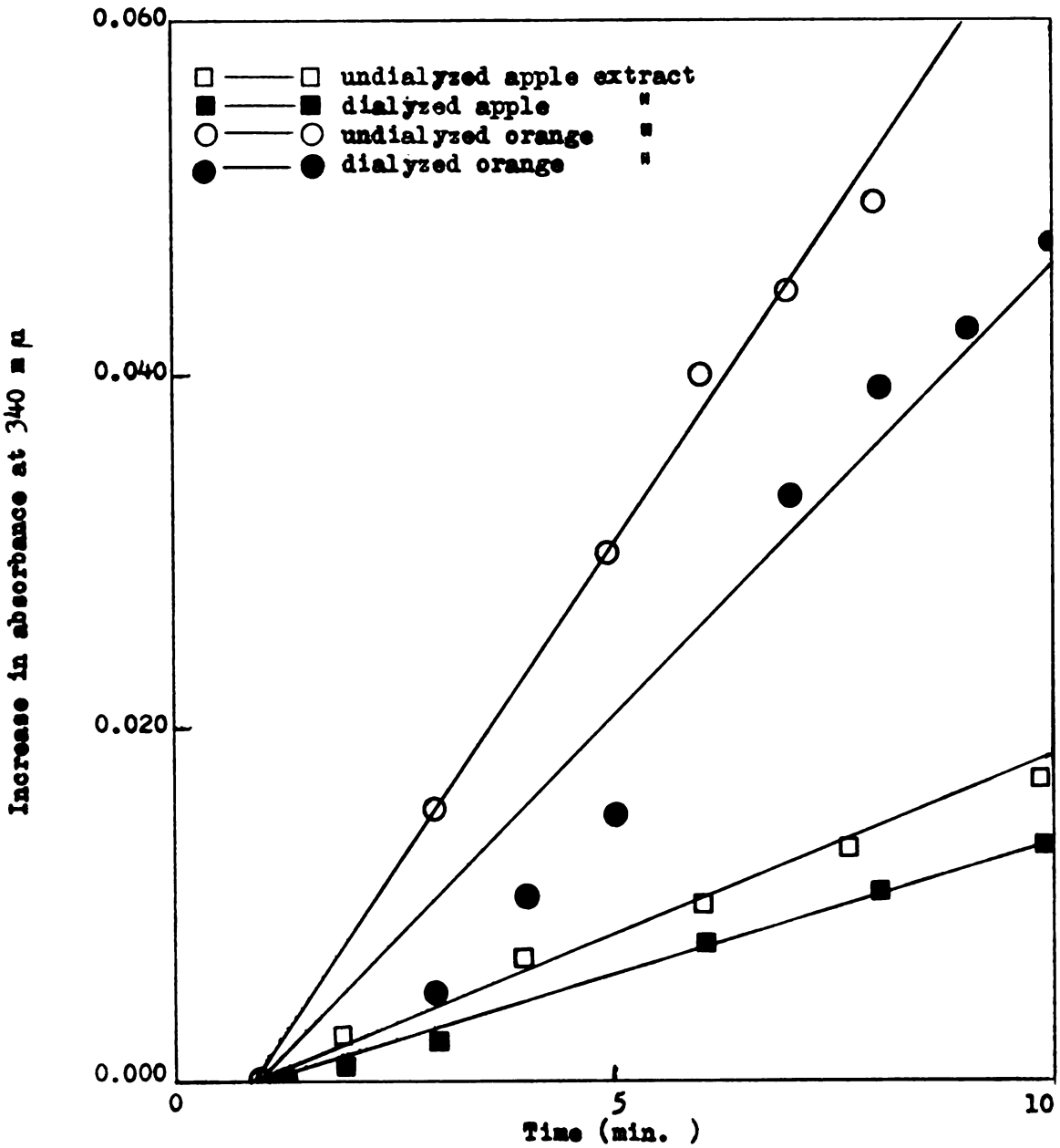

Figure 6. Effect of dialysis on citric synthase activity of apples and oranges.

Reaction mixture: malic acid 10 μmole, DPN 0.3 μmole, potassium phosphate pH 8.0 75 μmole, acetyl CoA 0.2 μmole, 0.1 ml enzyme. Tot. vol. 1.0 ml.

examined. This is in line with other observations (Carpenter and Beevers, 1959). The two reaction mixtures were as follows: 1) 200 μmoles potassium phosphate pH 7.6, 15 μmoles $MgSO_4$, 6 μmoles cysteine HCl, 24 μmoles isocitrate, water to 2.8 ml and 0.2 ml enzyme. The reaction was terminated by the addition of trichloroacetic acid. 2.4 dinitrophenylhydrazine was the color reagent. 2) Twenty μmoles cysteine HCl, 180 μmoles semicarbazide, 50 μmoles $MgSO_4$, 600 μmoles, 20 μmoles sodium isocitrate, 200 μmoles potassium phosphate pH 6.0, water added to 2.8 ml and 0.2 ml enzyme. The semicrbazones were followed at 252 mμ.

In conclusion, the comparison of enzymatic activities in various fruits which are citric or malic accumulators did not substantiate the theory that enzymic imbalance in the TCA cycle is the cause for citric acid accumulation. The activity of aconitase, isocitric dehydrogenase (DPN and TPN dependent) and malic dehydrogenaseis virtually identical in malic and citric accumulators. From the standpoint of citric acid accumulation all these enzymes can be considered degradative enzymes, that are leading to the consumption of citric acid.

Citric synthase on the other hand exhibited a wide

range of activities, but no correlation could be found between the activity of this enzyme, and acid accumulation in fruit.

If any of the accumulated citric acid originates in the TCA cycle, a source of replenishing the cycle intermediates biosynthetically related to citric acid must exist. There are metabolic pathways capable of feeding the cycle with intermediate compounds. Kornberg (1957) who worked on the glyoxylate pathway in bacteria, and showed that it can lead to a net synthesis of malic acid, hypothesized that this may lead to the replenishment of malate needed for citric acid accumulation. But a survey conducted by Carpenter and Beevers (1959) failed to show isocitritase activity in any plant except for certain germinating seeds. The present study with isocitritase confirmed Carpenter and Beevers' observation that fruit tissues are devoid of isocitritase activity.

Reversal of the isocitric dehydrogenase reaction could replenish isocitric acid into the cycle. The incorporation of CO_2 to isocitric acid was reported by Vennesland and Conn (1955). By using the oxidation of TPNH as the sole criterion of CO_2 addition to α-ketoglutarate by orange acetone powder; no formation of isocitric acid could be demonstrated.

The decarboxylation of isocitric acid has been established equally well in all fruits which were examined. α-keto-glutaric acid is a very unlikely source for citric acid accumulation since citric acid is the only known source for its formation.

Radioactive Isotope Experiments

Since enzyme activity was assayed *in vitro* it was of interest to correlate it with *in vivo* experiments. Injection of radioactive precursors seemed to answer this requirement. In injecting small quantities of radioactive compounds with high specific activity, it was assumed that the tagged compound would mix with the same compound in the fruit and cause a minimal interference with the metabolism of the fruit. This method has inherent difficulties. A radioactive precursor administered from outside the cell may reach the internal pools and biosynthetic sites at a different rate than the native precursor. This is especially true when intermediates are being injected. *In vivo*, enzyme complexes may catalyse a successive transformation of a compound, without allowing any leakage of intermediates to the existing pool. It is quite conceivable that when an intermediate is administered from outside the cell it may participate in other

reactions before reaching the active site. The side reactions may mask the main reaction in which this intermediate participates *in vivo*.

Incorporation of Acetate 2-^{14}C

Labeled acetate 2-^{14}C was injected into tomatoes and apples (Tables IV and V). During the first three hours the label was mainly incorporated into citric acid in both fruits. However, 24 hours after the injection, citrate remained in the tomato as the main labeled acid, while malic acid was the main labeled acid in the apple.

It may be concluded that the initial rate of citric acid synthesis is very similar in tomato and apple regardless of the acid which is accumulated. After longer incubation time, the ^{14}C distribution shows similarity to the acid pattern in the given fruit. In the apple the ratio of ^{14}C in the citric acid to the ^{14}C in the other acids shows a decline from 1:1.1 half an hour after injection to 1:23 twenty-four hours after injection. The inverse happens to malic acid which increases from a ratio of 1:30 half an hour after injection to 1:3.5 twenty-four hours after injection (Table V). There are at least two possible explanations for this observation. One is the conversion of citrate to malate

TABLE IV

INCORPORATION OF ^{14}C OF ACETATE 2-^{14}C INTO THE ORGANIC ACIDS OF DETACHED GREEN TOMATO FRUITS*

Time after injection	Counts per minute per fruit			Citric /rest	Malic /rest
	Malic	Citric	Total		
20 min	700	5650	11700	1:1	1:16
40 min	700	5400	9350	1:0.7	1:12
180 min	550	7000	11000	1:0.6	1:19
18 hr	1500	17000	20000	1:0.2	1:12
24 hr	11650	38550	57900	1:0.5	1:4

*Each time interval represents one fruit.

TABLE V

INCORPORATION OF ^{14}C OF ACETATE 2-^{14}C INTO THE ORGANIC ACIDS OF DETACHED PRECLIMACTERIC NORTHERN SPY APPLE FRUITS*

Time after injection (hours)	Counts per minute per fruit			Citric /rest	Malic /rest
	Malic	Citric	Total		
0.5**	300	4300	9300	1:1.1	1:30
1.0	650	7700	14100	1:0.8	1:20
2.5	3400	4300	19200	1:3.5	1:4.5
24.0**	6800	1300	31000	1:23	1:3.5

* Each time interval represents one fruit.

** Data represent two single fruit experiments.

through the TCA cycle; in the tomato only part of the citrate would be further processed in the cycle, while the rest would be removed from it; in the apple practically all of the citrate would be converted to malate. A second speculation would involve the slow and steady condensation of malate (Thunberg reaction) while citrate is accumulated (tomato) or degraded (apple).

These results support the observation from the enzyme activity study in that the Krebs cycle enzymes, although possibly participating in the synthesis of citric acid and malic acid, do not control their accumulation and do not determine the acid profile.

Another theory which has been advanced to explain citric acid accumulation is the existence of an inhibitor which blocks one of the citric degrading enzymes in the TCA cycle, and thereby causes citric acid accumulation.

The accumulation of radioactive malic acid in tomato from labeled acetate refutes this theory indirectly. If an inhibitor had been present there would not have been any accumulation of labeled malic acid.

Incorporation of Uniformly Labeled Glucose - ^{14}C

Uniformly labeled glucose - ^{14}C was the second precur-

sor that was injected to both apples and tomatoes. The results appear in Table VI. While the results do generally support our observation with acetate 2-^{14}C there are also marked differences.

In the apple, during the first 2 1/2 hours, citric acid accounts for 65% pf the total acid radioactivity and only later, at 24 hours, does malic acid take over (Table VI). In the tomato, however, the picture becomes more complicated by the appearance of an unknown radioactive peak which is the major peak during the first few hours after infiltration (Figure 7). It later declines in activity while the activity of citric acid and malic acid goes up (Table VII). In accordance with the previous results, 24 hours after the glucose injection in the tomato the highest radioactive count is found in the citric acid fraction. By injecting simultaneously glucose-UL - ^{14}C and phosphate - ^{32}P it was shown (Figure 8) that this unknown compound is phosphorylated.Incorporation of ^{14}C in the unknown compound appearing early after injection and then declining with a concurrent increase in radioactivity in citric and malic acids, suggest that this unknown compound is a precursor of citric acid.

An attempt was made to test whether the phosphorylated

TABLE VI

INCORPORATION OF ^{14}C OF GLUCOSE U.L. ^{14}C INTO ORGANIC ACIDS OF DETACHED APPLE FRUITS

Time (hours)	Counts per minute per fruit			Citric /rest	Malic /rest
	Malic	Citric	Total		
0.5	0	2300	3500	1:0.5	-
1.0	50	2800	5250	1:0.9	1:100
2.5	-	600	1500	1:1.5	-
29	1800	1200	4350	1:2.5	1:1.4

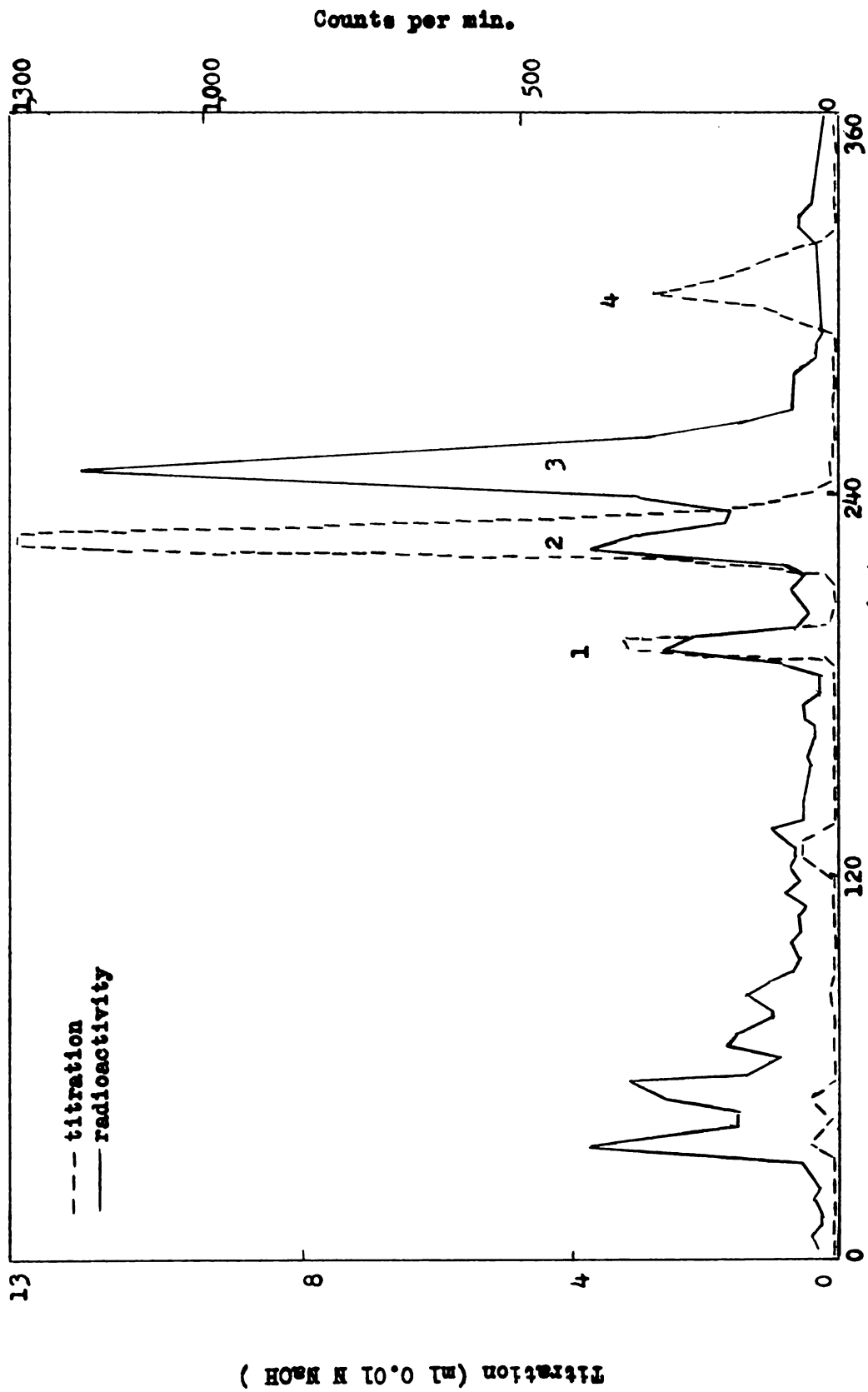

Figure 7. Incorporation of the label of glucose- UL - ^{14}C into the organic acids of detached tomato fruits, 1 hour after injection.

Peaks: (1) malic acid, (2) citric acid, (3) unknown, (4) phosphoric acid.

TABLE VII

INCORPORATION OF THE LABEL OF GLUCOSE UL-^{14}C INTO ORGANIC ACIDS IN DETACHED GREEN TOMATO FRUITS

Time after injection	Counts per minute per fruit				Malic /rest	Citric /rest	Unknown /rest
	Malic	Citric	Unknown	Total			
25 min	800	650	330	8250	1:90	1:11	1:1.5
60 min	1100	2800	7400	19200	1:16	1:6	1:1.6
**60 min	680	1600	6000	11600	1:18	1:6	1:1
120 min	3600	4800	5400	17600	1:4	1:2.5	1:2.2
180 min	5200	6100	5700	25000	1:4	1:3	1:3.3
24 hr	12000	12600*	0-0-0	36000	1:2	1:2	
**24 hr	9400	14400	0-0-0	28600	1:2	1:1	
36 hr	5100	14100	0-0-0	26000	1:4	1:0.8	
4 days	2700	13400	0-0-0	22800	1:7.5	1:0.7	

* A test tube was broken, count would probably reach 18000 counts per minute.

** Fruit extract passed through Dowex-50 before column chromatography on Dowex-1.

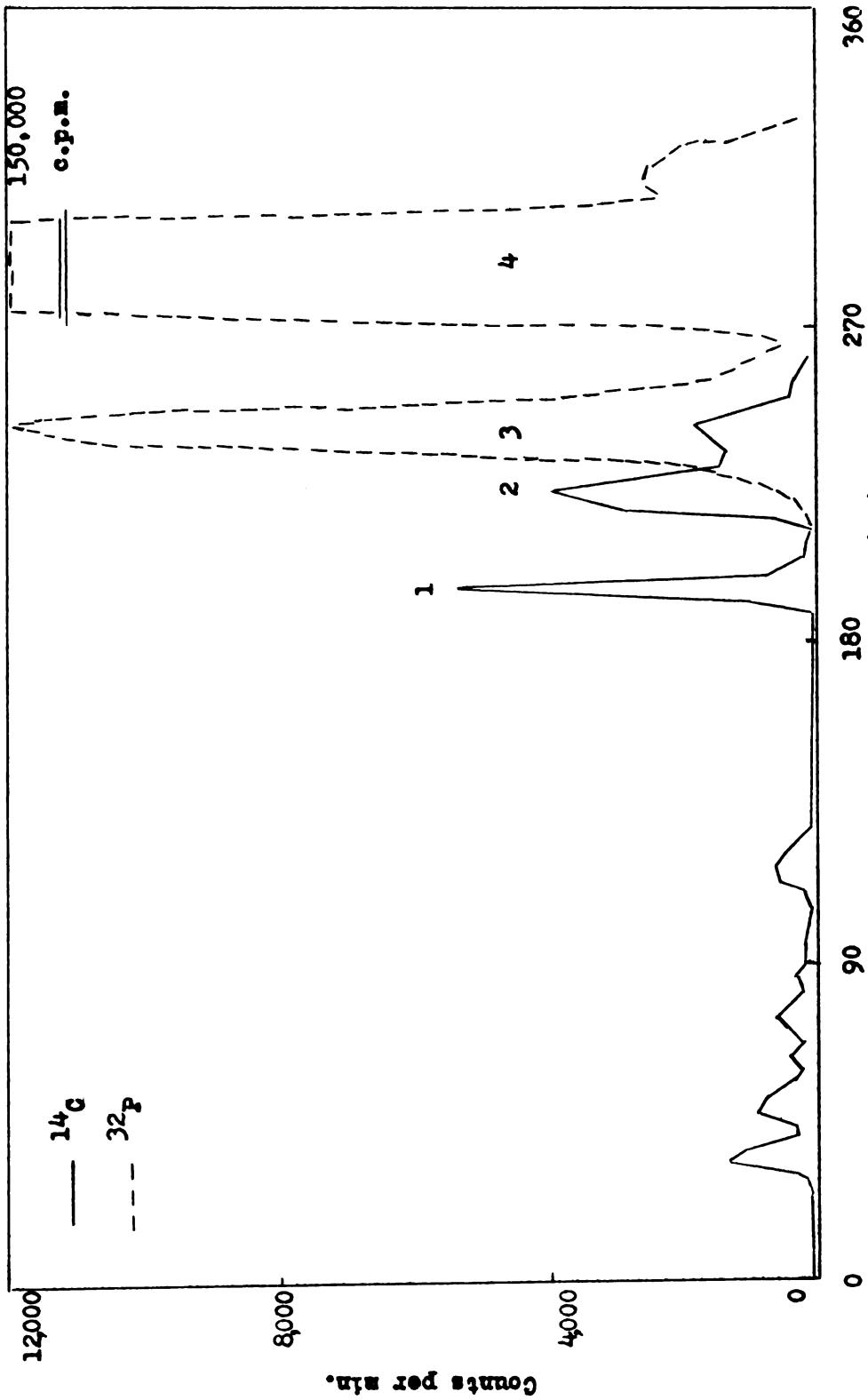

Figure 8. Incorporation of the labels of glucose - UL - ^{14}C and phosphate - ^{32}P into the organic acids of detached tomato fruits, 2.5 hours after injection.

Peaks: (1) malic acid, (2) citric acid, (3) unknown, (4) phosphoric acid.

compound might be a precursor of citric acid. Double-labeled unknown ^{14}C and ^{32}P which was isolated by column chromatography from tomato fruit was injected into another tomato. After an hour the analysis failed to show any radioactivity in the citric acid, but some radioactivity appeared in the phosphoric acid fraction. These results suggest that this compound was probably hydrolysed in the cells of the tomato. Hence, the role of this unknown compound is still obscure. It has not been shown that it is a precursor of citric acid, but because of its hydrolysis it can not definitely be ruled out as a precursor. This compound was not found in apple.

Bakowski et al. (1964), and Markakis and Embs (1964) working on snap beans and strawberries respectively, demonstrated an acidic compound that appeared in the elution pattern on the shoulder of the citric acid peak. This peak coincides with the unknown in the present study.

Markakis and Embs (1964), however, demonstrated that their unknown compound contained radioactivity after 24 hours when radioactive fructose was administered to strawberries while in the present investigation no traces of radioactivity were found in the unknown after 24 hours. This discrepancy may be due to different tissues employed.

Homogenate

In order to learn more about this unknown compound and its relationship to citric acid synthesis, uniformly labeled glucose -^{14}C as well as glucose -1-^{14}C and glucose -6-^{14}C were added to tomato homogenate. When ATP, DPN, and Mg were added, the unknown became labeled. All attempts to label the citric acid fraction failed. (Table VIII). The formation of the unknown was repressed by addition of boiled tomato extract. On the basis of the present knowledge it is difficult to speculate on the nature of this repression. Addition of $NaHCO_3$, in hopes that CO_2 fixation may result in formation of citric acid, failed to change the ^{14}C distribution in the acid profile. Addition of sucrose or its withdrawal from the homogenized media did not have an influence on the ^{14}C distribution and did not affect appreciably the rate of incorporation. It may be concluded that the degree of integrity of the mitochondria is of no importance in the biosynthesis of the unknown. The fact that glucose -1-^{14}C and -6-^{14}C served as precursors rules out the pentose shunt as a possible source of this unknown and almost rules out decarboxylation as a step for its formation.

It must be emphasized that these experiments also

TABLE VIII

INCORPORATION OF ^{14}C FROM GLUCOSE-^{14}C INTO THE ORGANIC ACIDS BY TOMATO HOMOGENATE

Homogenate with the following additives	Glucose	Counts per minute per three gram tomato Malic	Citric	Unknown
*Sucrose	UL-^{14}C	0	0	200
*Suc., ATP, DPN, TPN, Mg	UL-^{14}C	0	0	4500
*Suc., ATP, DPN, TPN, Mg	6-^{14}C	0	0	3500
*ATP, DPN, TPN, Mg	1-^{14}C	0	0	3000
*Suc., ATP, DPN, TPN, Mg	1-^{14}C	0	0	4500
*Suc., ATP, DPN, Mg	1-^{14}C	0	0	4500
**Suc., ATP, DPN, TPN, Mg	UL-^{14}C	0	0	4300
**Suc., ATP, DPN, TPN, Mg, boiled tomato	UL-^{14}C	0	0	1400
**Suc., ATP, DPN, TPN, Mg, $NaHCO_3$	UL-^{14}C	0	0	6000
**Suc., ATP, DPN, TPN, Mg, $NaHCO_3$, boiled tomato	UL-^{14}C	0	0	1400

Homogenate plus additives incubated for three hours.

Homogenate is composed of 3 g tomato, 300 μmole potassium phosphate pH 7.5 homogenized for 20 seconds, the final pH is 7.1.

*Final volume 10 ml.

**Final volume 20 ml.

Boiled tomato consists of 5 g tomato homogenized in 250 μmole potassium phosphate pH 7.5, boiled for 5 minutes. Total volume 10 ml.

Glucose	1 μC
ATP	40 μmole
TPN	5 μmole
DPN	5 μmole
$Mg(NO_3)_2$	10 μmole
$NaHCO_3$	100 μmole

failed to clarify the role of the unknown as a precursor of citric acid. This is especially so since attempts to achieve citric acid biosynthesis by tomato cell-free extract from glucose failed.

Attempts to Identify the Unknown

Double Label Experiments

Glucose -1-^{14}C and phosphoric acid -^{32}P were injected simultaneously into the tomato. The carbon peak and the phosphate peak coincided indicating that this compound contains both ^{14}C and ^{32}P.

Column Chromatography

The unknown compound is an acid. It is not adsorbed by Dowex-50, a cation exchange and is adsorbed by Dowex-1 which is an anion exchange. Its position on the elution pattern between citric and phosphoric acid indicates the relatively strong anionic nature of this acid.

Paper Chromatography

The unknown compound was subjected to paper chromatography with known compounds (Table X). The Rf of the unknown differed from all others, thus eliminating few possibilities but yet leaving open the question of its identity.

TABLE IX

INCORPORATION OF ^{14}C OF GLUCOSE U.L. - ^{14}C AND PO_4 - ^{32}P INTO ORGANIC ACIDS OF DETACHED GREEN TOMATO FRUITS

Time after injection	Counts per minute per fruit									
	Malic		Citric		Unknown		Phosphoric		Total	
	C	P	C	P	C	P	C	P	C	P
2.5 hr	7000	Ω	11700	5200	6200	47500	Ω	500000	35800	552000
4 hr	15000	Ω	20200	24500	0	92500	Ω	34500	52500	484700

TABLE X

Rf VALUES OF SOME PHOSPHORYLATED COMPOUNDS

Compound	R_f
Glyceraldehyde phosphate	0.23
3-phospho glyceric acid	0.19
Phospho enol pyruvic acid	0.30
Adenosine 5' mono phosphate	0.03
Adenosine 5' diphophate	0.02
Inosine 5' mono phosphate	0.04
Fructose -1- phosphate	0.05
Glucose -1 phosphate	0.05
Unknown	0.13

A twice ascending chromatography was employed. The paper used was Whatman No. 1, and the solvent was ethyl-methyl-ketone: water saturated butanol: propanol: formic acid 45:25: 5:20 (V/V)

Compartmentation

The enzymic survey and the precursor studies did not support the theory that overproduction of citrate is the cause of citrate accumulation. The data in Tables V and VI, however, are compatible with, although not a proof for, the theory of compartmentation. According to this assumption, citric acid in apple, although biosynthesized both from glucose and acetate in the first few hours, does not accumulate but is metabolized directly into malic acid which accumulates in stable pools. The other possibility is that citric acid is metabolized to CO_2 and H_2O and malic acid arises via a different mechanism. This assumption seems to explain the slowness in the appearance of malic acid in apples when glucose is the precursor.

According to the compartmentation theory the accumulation of an acid is a result of active transport from the site for synthesis into a passive pool. This rather than excess of enzyme prompts further synthesis of this acid.

Bennet-Clark and Bexon (1943) have experimental evidence that individual acids may be constrained inside the vacuole so that an apparent diffusion gradient is maintained

between the vacuole and the cytoplasm. Compartmentation of cytoplasm has been invoked by many authors (Porter and May, 1955; Steward, Bidwell and Yemm, 1958; Cowie and McClure, 1959). Beevers and his collaborators (Harley and Beevers, 1963; MacLennan et al., 1963; Lips and Beevers, 1966 a, b; Lips, Steer and Beevers, 1966) demonstrated compartmentation in some plant tissues.

Actual compartmentation of acids has been shown by Schneider et al., (1956). They showed that in fluoroacetate poisoning, which causes citrate accumulation, most of the citric acid is found in the mitochondria of rat liver.

A serious drawback in compartmentation studies is that the most important compartment, namely the vacuole, is mixed with the cytoplasm, during the isolation of particles. It is also true that all possible and hypothetical compartments in the cytoplasm will be mixed. Nevertheless, since particles, especially the mitochondria, seem to be the location for the synthesis and degradation of organic acids, this examination seemed to be of merit.

Studies were made to measure the ^{14}C incorporation into particles and supernatant of both the tomato and apple at several intervals (Table X) after injecting them with

2-^{14}C acetate. The isolation of mitochondria was done at 0^{o}-2^{o}C to reduce leaking and further metabolism of the acid by the mitochondria stem.

While 5% of the radioactivity was confined in particles of tomatoes, 30% was found in the particles of the apple, after the administration of the 2-^{14}C acetate. When the incubation time was extended, particles were depleted from radioactivity in both fruit. Citric acid was the only labeled acid found in the particles from apples.

This may suggest that although citric acid is the first acid to be biosynthesized by apple from acetate, it is retained in the particles and metabolized there. It may be either converted to malic acid and then released to the cytoplasm or completely oxidized to H_2O and CO_2 and the malic acid which is accumulated by the apple may be synthesized by a different biochemical route.

These results may point to another factor in acid accumulation, namely, membrane permeability and compartmentation.

TABLE XI

INCORPORATION OF ^{14}C FROM ACETATE -2-^{14}C INTO THE ORGANIC ACIDS OF PARTICULATE AND SUPERNATANT FRACTIONS OF APPLE AND TOMATO FRUITS

Fruit	Time after injection (hours)	Counts per minute		Ratio particles /super.
		Particles	Supernatant	
Apple	0.5	500	1000	1:2
	4.0	120	700	1:7
	15.0	50	600	1:12
	24.0	30	530	1:17
Tomato	1.0	270	5000	1:18
	9.0	150	4000	1:29
	24.0	60	5100	1:85

V. SUMMARY AND CONCLUSIONS

1. The acetone powders of the orange, lemon, tangerine, grapefruit, strawberry, and tomato fruits (all citric acid accumulators), the apple and sweet lime fruits (malic acid accumulators), the orange flavedo (an oxalate accumulator) and the Bartlett pear (a fruit with high malic and citric acid content) were assayed for the activities of the following enzymes: aconitase, isocitric dehydrogenase (TPN dependent), isocitric dehydrogenase (DPN dependent), malic dehydrogenase, citric synthase, and isocititase.

2. The activity of aconitase, isocitric dehydrogenase, malic dehydrogenase, and citric synthase were observed in all of the tissues studied. The activities of isocitric dehydrogenase (DPN dependent) and isocitritase were not detected in these tissues. Aconitase, isocitric dehydrogenase, and malic dehydrogenase showed activities which did not differ considerably from one fruit to another. Citric synthase varied markedly from fruit to fruit, but no correlation between citric acid content and this activity was apparent.

3. Acetate 2-^{14}C was injected to detached green tomatoes and apples. Half an hour after injection the incorporated ^{14}C was associated with citric acid in both fruits.

After 24 hours citric acid remained the most labeled acid in the tomato while malic acid was most highly labeled in the apple.

4. Glucose-U.L.-^{14}C, was injected into detached green tomatoes and apples. In the apple, citrate was highly labeled ~~acid~~ one hour after injection but malic acid became the dominant labeled acid after 24 hours. In the tomato an unknown compound incorporated a major portion of the ^{14}C from glucose within 30 minutes after injection and declined with time with a concurrent increase in labeled citric and malic acids and after 24 hours most of the label was in the citric acid fraction and none in the unknown.

5. A double label experiment with ^{14}C and ^{32}P indicated that the unknown acid must be a phosphorylated compound. Paper chromatography revealed that the unknown was not one of the following compounds: glyceraldehyde-phosphate, 3-phosphoglyceric acid, phosphoenolpyruvic acid, adenosine monophosphate, adenosine diphosphate, inosinemonophosphate, fructose-1-phosphate, glucose -1 phosphate and glucose - 6

phosphate. Homogenate studies established the dependence of this unknown on ATP for its biosynthesis. The label of glucose-U.L.-^{14}C as well as glucose-1-^{14}C and glucose-6-^{14}C was found in this compound, eliminating decarboxylation as a step in its biosynthesis. In addition acetate-2-^{14}C did not serve as a precursor.

6. Acetate 2-^{14}C was fed to apples and tomatoes, and the distribution of radioactivity between particles precipitated at 22,000 x g and the supernatant fraction was compared. In the apple, 33% of the organic acid radioactivity was found in the particles compared with only 5% in the tomato. Citric acid was the only labeled acid in the apple particles.

7. It is concluded that the organic acid profile in the fruit tissues examined does not correlate with the enzyme activity profile. Labeled precursor experiments corroborate the enzyme survey finding. Finally the intracellular distribution of newly synthesized organic acids indicates the possibility of compartmentation as a factor in acid accumulation in fruits.

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