C H A P T E R I.

INTRODUCTION

A normal fruit is essentially a matured ovary of a flower, including its seed or seeds and any part of the flower that may be closely associated with the matured ovary. The fruit may be formed either from an enlarged ovary wall, or from the tissue supporting the ovaries, or from the fusion of floral parts with the ovarian tissue. Despite this diversity in structure and differing climactic adaptabilities, patterns of ripening behaviour are few and somewhat similar.

Although of relatively low caloric value, fruits are an important element of human nutrition. Fruits can supply abundant vitamins, mineral salts and carbohydrates. Jaffee et al[1] have shown that the banana supplies the people of Venezuela with 7% of their calories, 26% of their vitamin C and 48% of their vitamin A (in the form of carotene).

The commercial importance and nutritive value of fruits have made their study not only of academic but also of applied interest. Moreover, the study of fruits avoid complexities resulting from the inter-play of processes like photosynthesis and respiration in leaves. Knowledge of the biochemical and physiological events that occur in fruits can contribute enormously to the understanding of the process of development and aging in many other organisms.

The life history of a fruit can be divided into three distinct phases: (a) cell division, enlargement and maturation during which growth occurs at the expense of assimilation derived from the parent plant; (b) ripening, during this period the fruit becomes edible, often with change in colour and (c) senescence, when autolysis sets in and tissue breakdown occurs.

Several excellent reviews have appeared on the biochemistry and physiology of fruits including those of Biale,[2-5] Biale and Young,[6] Hulme,[7] Porrit,[8] Miller,[10,11] Burg,[9] Ulrich,[12] Smock,[13] Nitsch.[14] Most of the information on changes during storage and ripening is available on bananas,[15] although reviews on apples, pears, citrus, cherries, mango and papaya have also appeared.[3,7,10,13,16-18]

Biale[4] made a tentative classification of fruits as "climacteric" and "nonclimacteric" types. In the climacteric fruits ripening is associated with a rapid increase in respiration, whereas, in the nonclimacteric variety there appears to be a simple gradual decline in respiration throughout maturation and during senescence; the changes characteristic of ripening often occur at a constant slow rate. In the former class he listed cherimyo, mango, papaya, passionfruit, papaw, peach, pear, plum, sapote, apple, apricot and banana, and in the latter class he included cherry, cucumber, fig, grape, orange, grapefruit, lemon, melon, pineapple and strawberry.

The substances that are known and suspected to play a role in the ripening process are proteins,[11,12] carbohydrates,[4] fats,[19] acids,[4,5,7,12] minerals,[7] vitamins and pigments,[7,12,20] and certain cell wall constituents,[5,7,21,22] such as, hemicellulose and pectin.

Carbohydrates, which might be expected to provide the main sources of energy for respiration in fruits, do show marked changes qualitatively as well as quantitatively, so much so that Czyhrinciw[23] classified tropical fruits on the basis of their starch content. According to him fruits can be divided into two groups, those containing reserve polysaccharide, especially starch in the unripe mature stage like banana and mango, and those which contain no reserve polysaccharide in the unripe, mature stage like guvava, pineapple and papaya. Among the polysaccharides starch has gained a major factor of interest, since its disappearance has been used as one of the parameters to find the optimal time of harvest for pomefruits[7] and maturity of Florida mango.[293]

Starch is generally deposited in plant cells as granular particles. Different plant species have their own specific starch granules varying in size, shape and the location of the hilum. Apple[7] and mango[23] starch have been shown to have a similar structure like the cereal and tuber starch granules, with the difference that the molecular weight of its components were found to be smaller.

Kidd et al[25,26] have provided evidence that on the removal of starchy fruits from the tree starch synthesis stops and its degradation starts.

The chemical composition of the edible portion varies considerably among fleshy fruits at harvest time.[2,27] The dry matter content may be as high as 80% of the total weight for some varieties of date, though in most fruits it is between 10 and 15%. Carbohydrate content (fresh weight basis) in the majority of species is 10 to 20%, but there are notable exceptions, such as 5% or less for the avocado and about 75% for the date.[2] Fruits, low in carbohydrate, are frequently high in fat content. Protein content varies from values as low as 0.4% of fresh weight for pineapple to a highest of 1.7% for avocado. Total acid content is also highly variable ranging from about 5% for lemon to 0.1% for persimmon.[2]

The major portion of the dry matter of most fruits is made up of carbohydrate material. Krishnamurthy et al[28] have studied the relationship between sugar and flavour in mango fruit. Generally glucose, fructose and sucrose are found to be present at all stages of ripening and there is a trend towards their enhanced synthesis during ripening. The exact proportion of each sugar depends upon the species, maturity and to some extent upon variety. In apples and pears[29] fructose and glucose form the principal sugars, whereas sucrose is the major sugar in peaches.[30] The amount and

proportion of these sugars also vary in different parts of the fruit. Harding[31] has found that fructose is the main sugar in the flesh and dextrose in the skin of pears.

In pears an increase in the total soluble sugars during ripening at 15° C has been shown.[32] In bananas about 60% of the loss of dry matter is due to the disappearance of the total carbohydrates.[15] Starch disappearance is most marked lasting about nine days at temperatures of 70-85° F. As a result of starch hydrolysis, reducing sugars are formed in much greater quantities than sucrose.

Handerson et al[33] discovered a trisaccharide in the course of ripening of Cavendish bananas. The oligosaccharide found appeared to be identical with the one formed by the action of banana invertase on sucrose and also with a component of the reaction of yeast invertase with sucrose indicating that banana invertase has the capacity of transfructosylation. The compound on crystallisation from banana was tentatively identified as fructosyl-sucrose.

Church[34] found that in avocado, after harvest, sugars were consumed in the early but not in the later stages; Bean[35] noted that ketosugars were preferentially used during ripening. Biale[36] compared sugar consumption and carbon dioxide production and concluded that changes in hexoses were insufficient to account for the observed

respiratory activity at climacteric. He further reported that the changes in carbohydrates during ripening of tropical and sub-tropical fruits were of a similar nature.

Nada,[37] who analysed fruits of Vitis vinifera at different stages of development for their sucrose and total acid contents, observed an increase in acidity and a decrease in sucrose and polysaccharide contents during initial stages of growth and the reverse phenomenon during the later stages. Onslow et al[38] found that sucrose level never falls to zero in pears, but that there is a critical residual amount, the basal sucrose content, which never enters into the respiratory process. They associated a high level of this basal sucrose content with bad keeping quality of the fruit.

Ash and Reynolds[39] have detected (in small amounts) at least two keto-oligosaccharides in several varieties of pears. On hydrolysis, xylose, glucose and fructose were detected from one of these oligosaccharides. They suggest, that as in other plant tissues transfructosylation might be occurring in fruits.

From available data, it is pertinent to view an association between precise chemical changes and normal course of ripening. Many of the transformations are of a general nature and occur in a variety of fruits, due to enzymic actions. A number of reports on enzymic changes

accompanying the ripening of fruits are available.[40-53] McCready and McComb[54,55] have demonstrated a high polygalacturonase and pectin-methyl-esterase activities in ripe pears, whereas in unripe pears very little activity was observed. The failure of earlier attempts to demonstrate pectinase activity in fruits, has been shown to be due to the presence of two inhibitors of this enzyme.[56,57] One of these was found to be thermolabile, and has been isolated from the pear sap by acetone precipitation and is non proteolytic in nature.

Hall[58] showed that a cellulolytic enzyme, that reduces the viscosity of carboxy methyl cellulose was found in the extracts of ripe tomatoes but not in the unripe fruit. The increase in malic enzyme and pyruvate carboxylase in William pears during ripening has also been shown.[59,60]

Due to the enormous literature available on sugars in fruits, there was a belief that sugars, including starch form the lone substrates for metabolic processes yielding energy in fruits. The energy level of the fruit might then have been expected to determine the length of its life on detachment from the tree. Theories based on the data accumulated have not stood the test of time. The discovery of Tricarboxylic acid (TCA) cycle and the energy released in the phosphorylating mechanism involved have emphasized the importance

of organic acids in the respiration processes of plants.

A relation between carbohydrate and acid metabolism of fruits has been suggested [6,9,61] but biochemical evidence on this point is lacking. In general the organic acids vary with external factors such as climate and nutrition of the plant. It is well known that fruits grown in cold and rainy climates are sour.

The bulk of acids present in fruits are of the same nature as those operating as intermediates in the TCA cycle. Di and tri carboxylic acids are very abundant in fruit tissues. While they amount to 1-2% of fresh weight in apples and pears, their concentration may reach as high as 4% in black currants[62] and 7% in lemons.[63]

The polycarboxylic acids that are found in fruits are principally malic acid (pome fruit and mangoes), citric acid (citrus fruits), tartaric acid (grapes), and isocitric acid (black currants). Instead of one particular acid predominating, many fruits contain a mixture of these acids.[64] The general pattern is one of progressively increasing organic acid content during fruit growth and its decrease during ripening.[22,62,65-69] Hulme and his group[44,70] have proved beyond doubt that in apples malate decarboxylation and the enzymes associated with this process increase considerably during ripening and suggested that this may be one of the reasons for the upsurge of respiration at the climacteric.

Respiration in a rigorous sense, is restricted to the reactions requiring oxygen, whereas fermentation, also known as glycolysis or Embden - Meyerhof - Parnas (EMP) pathway is characteristic of biological oxidation in an oxygen free environment. Fermentation may also take place in the presence of oxygen. A distinction is therefore made between "aerobic" and "anaerobic" glycolysis, on the basis of the conditions to which the fermenting material is exposed. The following reactions illustrate the two processes:

Fermentation:-

$$C_6H_{12}O_6 \longrightarrow \underset{\text{Pyruvic acid}}{2CH_3CO.COOH} + 4(H) \quad ....(1)$$

Respiration:-

$$CH_3.CO.COOH + 2\tfrac{1}{2}\ O_2 \longrightarrow 3CO_2 + 2H_2O \quad ....(2)$$

The breakdown of sugars to pyruvic acid (1) consists of the phosphorylation of glucose or fructose to a hexose-di-phosphate, the splitting of the six carbon sugar phosphate into two triose units, isomerisation, oxidation of phosphoglyceraldehyde to phosphoglyceric acid and the successive transformation of the latter into pyruvic acid. The coupled oxidation reduction results in the formation of adenosine-tri-phosphate (ATP) from adenosine-di-phosphate (ADP) and inorganic phosphate (Pi).

Once the fruit is detached from the parent plant it lives an independent life by utilising substances accumulated during growth and maturation. Biological oxidation, with its respiratory and fermentative features

assumes the dominant role, and the chemical changes then taking place are directly or indirectly related to it.

In the case of animal tissues and micro-organisms, studies carried out by Hopkins, Meyerhof, Parnas, Lohmann, Embden, Harden, Neuberg, Warburg, Cori and Cori and others helped to postulate the glycolytic pathway for the initial breakdown of carbohydrates.[71-73]

The widespread operation of this pathway in other animal tissues,[74-77] plants,[78,79] bacteria[80,81] and moulds,[82-91] suggests that this is a major pathway for the conversion of carbohydrates to pyruvic acid in most living tissues.

During respiration, pyruvic acid, the cleavage product of glycolysis, is completely oxidised to carbon dioxide and water as indicated in reaction (2). The metabolic pathway responsible for this oxidation is known as "Kreb's cycle" or the TCA cycle.[92] The essential features of of this cycle are the condensation of a two carbon fragment derived from pyruvate with a four carbon acid oxaloacetate, to form citrate and the successive transformation of the latter into aconitate, isocitrate, oxalosuccinate, α-ketoglutarate, succinate, fumarate, malate and oxaloacetate. The operation of the TCA cycle has been widely demonstrated in animal tissues,[93,94] as well as in micro-organisms including bacteria and mould.[95-104] The complete cycle of carbon through fermentation and respiration is given in Fig. 1.

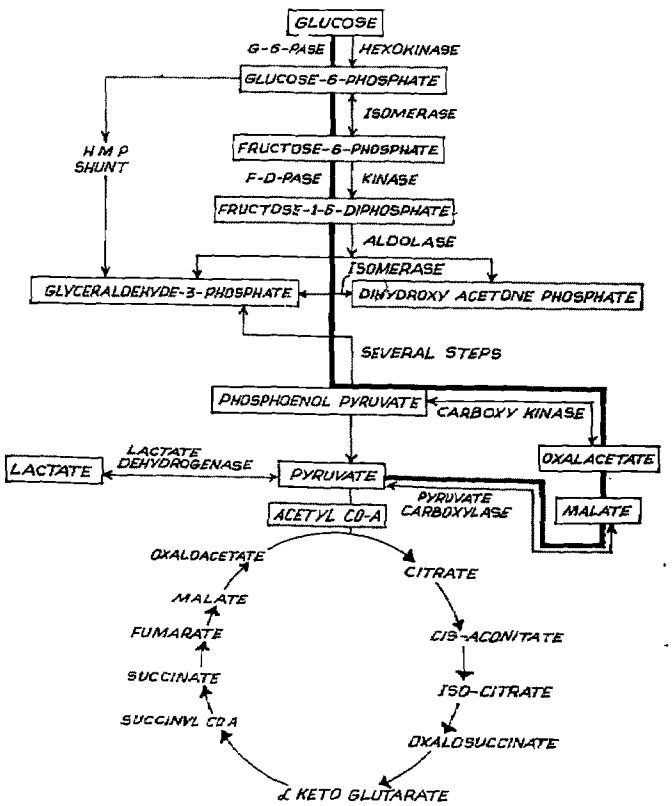

FIG. 1 CARBOHYDRATE METABOLISM IN CELLS

→ GLYCOLYSIS VIA TCA CYCLE
→ GLUCONEOGENESIS

A modification of this cycle for some plant tissues has been suggested by Beevers and Kornberg.[105] According to this, succinate is bypassed, and glyoxalate formed from isocitrate, condenses with acetate to form malate Fig 2.

It should be added that glycolysis, TCA cycle and glyoxalate shuttle are not the only metabolic cycles that account for carbohydrate oxidation. The pentose phosphate pathway[106-109] also known as the hexose monophosphate (HMP) shunt in which glucose may be metabolised through reactions that bypass glycolysis, has been reviewed by Horecker and Mehlar,[110] Axelrod and Beevers[111] also explains the complete degradation of hexose and pentose.

It is thus evident that plant tissues have the enzymes required for the operation of both the EMP-pathway and the HMP-shunt. To determine the relative strengths of these two pathways Beevers and Gibbs,[112] and Hartman[113] carried out isotopic studies on a variety of plant tissues. They introduced equal amounts of glucose-1-C^{14} and glucose-6-C^{14} into several plant tissues and compared the initial yields of $C^{14}O_2$. They found that in most of these tissues some of the glucose was broken down through a reaction sequence in which C-1 was split off at an earlier stage than C-6. They concluded, therefore, that the EMP pathway is more dominant.

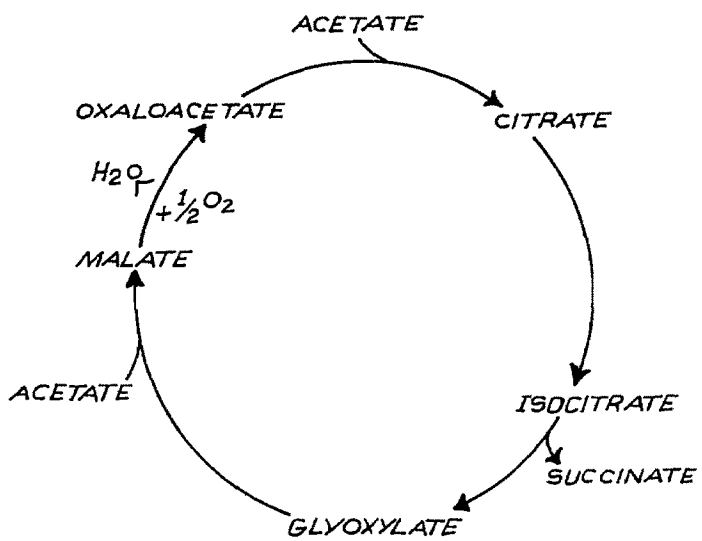

FIG. 2 GLYOXYLATE BYPASS

Regarding the further oxidation of pyruvate in plant tissues, the hypothesis that a cycle similar to the TCA cycle might operate was first advanced by Chibnall.[114] A number of *in vitro* experiments on a variety of plant materials such as segments of *Avena* coleoptiles,[115] barley root,[116] spinach leaves[117] and slices of potato tuber[118] have shown the utilisation of various dicarboxylic acids such as succinic, fumaric, α-keto glutaric and citric acid. Oxidation of the intermediates of TCA cycle have also been shown in a large number of other plant tissues like castor bean endosperm,[119,120] *Avena*,[121] cauliflower,[122,123] sweet potato,[124,125] spinach leaves,[126] and developing pepper.[127,128]

Millerd *et al*[129] isolated active mitochondria from mung bean hypocotyls (*Phaseolus aureus*) to be capable of oxidising some of the TCA cycle intermediates. Similarly active mitochondrial fractions were isolated from cauliflower[122,123] and pea.[130] Davis[130] demonstrated the occurence of the essential steps of TCA cycle in pea mitochondria. Similarly mitochondrial particles isolated from roots, seeds, hypocotyls, floral parts, fruits, petioles and tubers of different plants have been shown to utilise TCA cycle intermediates for respiration.[131]

The operation of the TCA cycle in plant tissues was further strengthened by the studies on the individual enzymes. Davis[130,132] showed the presence of

nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) specific isocitrate dehydrogenase, fumarase and aconitase in the supernatant obtained by treating washed pea mitochondria.

Considerable information is available on the catabolism of glucose in various plants,[78,79,110-132] but there is a paucity of information on the overall catabolism of glucose in fruits. The operation of the TCA cycle has been demonstrated in tomato,[133] pepper[127] and apple.[44] Glucose oxidation involving a C1-C5 cleavage has been reported in cucumber, lime and orange.[134] Barbour et al[46] have shown that in tomatoes 84% of the fruit glucose is catabolised via the EMP pathway and the TCA cycle and 16% via the HMP shunt.

Tager and Biale[135] reported that a shift in the metabolic pathways in banana occurs with ripening. Tager[136] obtained some evidence for the operation of pentose cycle in the unripe banana which shifted to the glycolytic pathway as the fruit ripened. Rakitin[137] reported an increase in carboxylase activity in persimmon during ripening. Tager's results[135,136] on the increase in aldolase activity were reinvestigated by Young[138] who found no difference in aldolase activity in the supernatant fractions of cell homogenates obtained from green and ripe bananas.

Pearson and Robertson[43] attempted to isolate active mitochondria from apples which can oxidise the

TCA cycle intermediates. But their preparation showed only limited activity with succinate, malate and citrate. Neal and Hulme[44] also demonstrated the oxidation of the two intermediates, viz, succinate and malate by the particles of apple.

Patwardan[139] was able to isolate an actively respiring mitochondrial fraction from mango. He found that it contained no endogenous oxygen uptake and that the succinate oxidation values were comparable with the values of the mitochondrial fraction from apples.

Biale and associates[140] were able to isolate metabolically active cytoplasmic particles from avocado, which were found to carry on the oxidation of all the intermediates of the cycle as well as phosphorylation. From these and other related studies Abramsky and Biale[141,142] concluded that the TCA cycle is a major pathway of oxidation of carbohydrates in a variety of tissues in higher plants.

Work on apples, by Hulme and his group,[160,257] has revealed that the level of oxaloacetate (OAA) plays an important role during ripening. OAA was found to inhibit succinate and malate oxidation and the removal of this inhibition by the development of a mechanism e.g. a special enzyme system for metabolising OAA, has been suggested to lead to the increase in the rise in respiration at the climacteric.

Fruit ripening is considered as just one manifestation of aging in a living tissue. The formation of substances that play a key role in the maturation and ripening of fruits is interwoven with metabolic patterns. Till a clear insight into the dynamics of metabolism in the fruit is achieved the study of ripening will be superficial and limited.

Mango is one of the major fruits cultivated in India. The well marked phases of ripening and its physico-chemical characteristics make it an ideal tool for investigating the mechanism of ripening in this fruit.

This fruit is one of the oldest fruits. It having originated in the Indo-Burma-Malaya region of south eastern Asia, was cultivated and developed for centuries before spreading to other parts of the tropical world. The tree enters into Hindu religious observances and mythology, and its importance as a food producing tree must have been established at least 4000 years ago. Almost all the varieties of this fruit, both cultivated and wild, grow in India. Commercially, however, only a few varieties are important, and Alfanso is considered to be one of the best among them.

According to Banerjee et al[145] the Alfanso mango ripens on the fifth day after plucking and putrefaction sets in after ten days. Wardlow and Leonard[146] showed a rapid decline in acidity during ripening of West Indian

mangoes. They also observed a marked gradient of acidity from skin to stone. This was, however, found to decrease with ripening.

Cheema et al[147] studied the chemical composition of the green and ripe fruit of different varieties of mango. Their work correlated the acidity and storage of the fruit, the storage being short in the case of low acidic fruits and long for highly acidic fruits. According to Leley et al[148] during the growth of Alfanso mango the main activity of fruits is the accumulation of starch and during ripening this reserve starch gets completely hydrolysed with the formation of sugars.

While studying the biochemical changes that take place during ripening of mango, Modi and Reddy[65] reported that the rise in pH during ripening favour the synthetic reactions like the formation of carotene, lipid and sugar. Further they have shown that catabolism of malate preceded that to citrate and the catabolism of acids would serve for the generation of high energy phosphate (ATP) and reduced NADP, which are required for the synthetic reactions. These results were substantiated by the finding that an increase in malic enzyme, glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase favoured the production of NADPH during ripening. Reddy[149] also reported that inspite of an increase in the activities of the enzymes glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase an increase in

sugars (sucrose, fructose, glucose and pentoses) occured four fold. Among these sugars sucrose was found to constitute the major part in the pulp.

Mattoo[153] has found that mangoes at 20° C contained one half or 50% as much free sugar as compared to those kept at 35° C the temperature at which fruits are generally ripened in India. Further he observed that fruits stored below 5° C developed chilling injury. Studying the changes in the cellular constituents of the chilled fruits it was found that chilled injured tissue contained less sucrose, but there was an unusual invertase active at 0° C.[152]

The results cited above indicate that the metabolism of carbohydrates is undoubtedly one of the important features during storage and ripening of mango fruit. In order to gain more knowledge about the role and metabolism of carbohydrates in this fruit, the present investigation was undertaken.

An attempt has been made to study the changes in the hydrolytic enzymes cellulase, amylase and pectinesterase and the glycolytic breakdown of sugars during ripening.

Sucrose is the most abundant and widespread disaccharide in nature. The efforts of Leloir and his group[150,151] have proved that the synthesis of sucrose is

not merely the reversal of its breakdown. An active invertase[152] has been reported in the mango fruit although considerable increase in the levels of sucrose has been observed,[65] suggesting thereby that the fruit may have high capacity to synthesise sucrose and to regulate its breakdown. In the present enquiry, it was therefore of interest to study sucrose biosynthesis by mango cell free extracts.

Modi and Reddy[65] observed an increase in the reducing power at the climacteric in mango which was associated with an increase in the enzymic activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase and malic enzyme. The regulation of these enzymes by β carotene and fatty acids, (fruit constituents which increase during ripening) are reported here.

Mattoo[153] observed that the ripening of mango is associated with a high respiratory rate, which is accompanied by a considerable increase in the enzymic activities viz catalase, peroxidase, amylase, invertase, phosphoglucoisomerase and aldolase. Further Mattoo and Modi[153,154] reported that the increase in some of these enzymes at ripening was due to the inactivation of proteinic inhibitors present in the unripe mango. The proteinic inhibitors were found to inhibit catalase, peroxidase and amylase. The investigation with reference to the isolation, partial purification and characterisation of the inhibitor with respect to invertase forms a part of this thesis.

Earlier successful storage[291] of unripe mature mangoes has been achieved by wax coating and subsequent storage at 5° C for a period of 45 days. However, prolongation of storage for 60 days was encountered with a loss of 40 to 50% due to the development of microbial infection mainly of the fungal type. Studies carried out to minimise the fungal spoilage by using antifungal antibiotic aureofungin are also reported in this investigation.