CHAPTER I

I N T R O D U C T I O N

Citrus fruits are grown in tropical and sub-tropical regions throughout the world and rank probably third among the sub-tropical fruits of the world.

The origin and history of citrus fruits are not fully known. The most that can be said with confidence is that they are natives of Southern Asia (Bartholomew and Sinclair, 1951). Chinese literature written as early as 2200 B.C. refers to the cultivation of some species of citrus.

The lemon which belongs to the citrus species is commonly believed to be indigenous to India. The most common species grown in India has been identified as C.medica Var-acida (Watt, 1908). These fruits are used for preparing beverages, pickles etc. The juice is also used for flavouring soups, curries, fish etc., since it imparts a pleasant taste and flavour. It is also used in domestic medicine.

The curative value of lemon juice in scurvy led to the identification of citrus fruits as important sources of ascorbic acid. However, citric acid is a major constituent in these fruits.

The origin of citric acid and other constituents present in the fruits has been a subject of great interest.

The main question is whether they are formed in the fruit tissue itself or whether they are formed in the leaf and transferred to the fruit. In the former case question also arises regarding the mechanisms involved in their formation and accumulation in fruit tissue. The latter belief is referred to as the translocation hypothesis.

The translocation hypothesis received the support of Nitsch (1953) according to whom the acids are translocated from the leaves to the fruits which merely act as storage organs. Earlier, Gatet (1939) and Ballard, Magness and Hawkins (1922) found that the acidity in grapes and apples is higher at the centre than at the periphery of the fruit and that a reduction in leaf area decreases the acid content of the fruit. But the results must be considered equivocal as obviously such reduction would affect the supply of carbohydrate necessary for cellular synthesis and would affect organic acid content whether it is formed locally from carbohydrate or supplied by the leaves. Similarly, regional differences in the concentration of enzymes could well account for the greater concentration of organic acid at the centre.

The alternate hypothesis that the presence of organic acids in fruits is due to conversion of carbohydrate into organic acids in the fruit vesicles and not due to translocation seems more plausible in the light of recent studies.

As early as 1933 Ricevuto suggested the formation in lemons of citric acid from reducing sugar and pentosans by enzymic action. In their studies on valencia oranges, Sinclair and Eny (1947) found the distribution of citric, malic and oxalic acids in leaves, juice and peel to vary considerably. The highest concentration of total malic and oxalic acids was found in leaves, whereas the juice contained the highest concentration of citric acid, and the peel, the lowest concentration of citric acid as well as total acids. These findings led them to suggest that the organic acids are synthesized in the vesicles from carbohydrates.

This hypothesis received substantial support from the ingenious studies carried out by Erickson (1957) who grafted a sweet lemon on a sour lemon plant and a sour lemon on a sweet lemon plant. Analyses of the fruits showed that the acidity of sweet lemons remained low (0.44 per cent) and that of sour lemons remained higher (5.2 per cent) irrespective of the leaves by which they were nourished. The sweet lemon also had a much higher reducing sugar concen-

tration (5.17 per cent) than the sour lemon (1.45 per cent). These findings certainly do not support the translocation hypothesis and point to local synthesis. This suggestion has been amply supported by enzyme studies which show that the fruit tissue possesses the enzyme machinery necessary for the synthesis of citric acid in the case of lemon (Ramakrishnan and Varma, 1959) and garcinia (Deshpande and Ramakrishnan, 1961).

Nada (1954) 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 polysaccharides during the initial stages of growth and the reverse phenomenon during the later stages. A similar observation was made by Deshpande and Ramakrishnan (1961) in fruits of Garcinia (Xanthochymus guttiferae). Studies carried out by Ramakrishnan and Varma (1959) showed an association between increase in citric acid content and decrease in carbohydrate during the development of the lemon fruit (Citrus acida). These authors also found that the contents of sugars, protein and acidity of young and old leaves and of stems bearing young fruits and those bearing mature fruits do not differ when considered in terms of percentage dry weight. But, in the case of fruits the sugar

and protein contents decrease, and the acidity increases, with growth. The pattern of organic acids is the same in young and old leaves and stems bearing young fruits and those bearing mature fruits. Citric, malic succinic, fumaric and oxalic acids were present in both categories of leaves and stems. But in the case of fruits the pattern of organic acids varies with development. Young fruits are found to contain only malic, fumaric and oxalic acids. When the fruits attain a size of 1.5 cm diameter citric acid begins to accumulate, fumaric acid disappears, and oxalic acid is still present in small amounts. As they reach maturity, citric acid is the major constituent, malic acid being present in small amounts.

It is found that both the lemon and the garcinia fruit contain the enzymes necessary for the formation of citric acid and that the mature fruit is characterised by a high activity of citrate synthase and the disappearance of aconitase (Ramakrishnan and Varma, 1959; Deshpande and Ramakrishnan, 1961).

The evidence cited above seems to have been ignored by Lioret and Moyse (1963) who favour the hypothesis of translocation. They report the absence of citrate synthase

in fruits accumulating citric acid. This is surprising since the enzyme has been found in lemon (Ramakrishnan and Varma, 1959) and garcinia (Deshpande and Ramakrishnan, 1961). It is possible that the enzyme preparation in their experiments was inactivated due to a sudden release of excess acid during the preparation of the homogenate.

Recent studies suggest that even the carbohydrate necessary for organic acid synthesis may be derived from the outer skin of the fruit which is shown to have photosynthetic capacity. Bean and Todd (1960) while studying the $C^{14}O_2$ uptake by young oranges in light and dark found that the sugars are highly labeled in the photosynthesizing flavedo. Sucrose is found to be labeled to an appreciable extent in the albedo of the intact fruit but only a very small activity is found in sucrose in the illuminated isolated albedo. This suggested that the major activity in the albedo of the intact photosynthesizing fruit is due to translocation from flavedo. A very slight amount of activity is found in illuminated, isolated vesicles. On the other hand, acid fractionation has shown that the peel has a low concentration of citric acid whereas it is a major constituent in the vesicles.

A number of factors indicate that products such as citric acid formed during dark fixation must be formed within each tissue rather than by extensive translocation from one tissue to another. In the intact fruit photosynthesis results in the increase and redistribution of the activity in flavedo but has no effect on that found in the vesicles. Even in the albedo of the intact fruit the changes due to photosynthesis appear to be restricted to carbohydrate components. In contrast the vesicles accumulate a large amount of activity during dark fixation.

However, the fixation of carbon dioxide also occurs in the dark and appears to be a general reaction for plants (Krotkov et al, 1958; Kunitake et al, 1959). In some succulent plants a net uptake of carbon dioxide may occur in the dark to give rise to an increase in organic acid (Gregory et al, 1954; Thomas, 1949; Thomas and Beevers, 1949; Thomas and Ranson, 1954).

The uptake of CO_2 during dark fixation appears to depend upon utilization of sugars to form acceptor units. Sugars are depleted while the acids increase. The reaction of phosphoenol pyruvate with CO_2 (Tchen and Vennesland, 1955) appears to be one of the reactions responsible for the dark fixation

in succulents and other plants (Saltman et al, 1956; Walker, 1962). In citrus vesicles a high activity of phosphoenolpyruvate carboxylase has been demonstrated by Huffaker and Wallace (1959). Davies (1956) has demonstrated the association of phosphoenolpyruvate carboxykinase with pea mitochondria and such systems have been more extensively studied in other tissues by Mazelis and Vennesland (1957) and Benedict and Beevers (1961). A number of enzymes which catalyse the fixation of carbon dioxide into acids in plants have been reported (Vennesland and Conn, 1952; Vennesland, 1960; Vishniac, Horecker and Ochoa, 1957; Walker, 1962).

Reports on the feasibility of cultivating the lemon fruit in vitro in a nutrient medium containing mineral salts and sucrose (Schroeder, 1960; 1961; Kordan, 1962; 1963a, b, c; 1964; 1965b) and the demonstration of starch synthesis in vitro in lemon fruit (Kordan,1965a) indicate the independence of fruit tissue as a metabolic entity capable of synthesizing its own cellular constituents.

The mechanism by which glucose and other compounds are utilised for energy production has naturally been a subject of great interest since their oxidation in living tissues is quite different from their combustion outside the body.

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 carbohydrate (Dickens, 1951; Nord and Weiss, 1951; Green, 1954). The widespread operation of this pathway in other animal tissues (Novikoff et al, 1948; DuBois et al, 1948; Wu and Chang, 1948; de Vincenti, 1947), plants (James et al, 1941; Giri and Ramasarma, 1956), bacteria (Utter and Werkman, 1941; LePage and Umbreit, 1943) and molds (Bernhauer and Iglauer, 1936a; b; Clutterbuck, 1936; Butkevich and Gaevskaya, 1935; Johnson et al, 1937; Bentley and Thiessen, 1957; Butkevich and Fedorov, 1929a; b; 1930; b; Foster and Waksman, 1939a; b; Foster et al, 1949; Foster and Carson 1950) suggest that this is a major pathway for the conversion of carbohydrate to pyruvate in most living tissues, although evidence is now available that glucose may also be metabolised through reactions that bypass anaerobic glycolysis down to glyceraldehyde-3-phosphate by enzyme systems present in yeast, bone marrow and other sources (Warburg et al, 1935; Dickens, 1938a; b; Seegmiller and Horecker, 1952) through a sequence known as the hexose monophosphate shunt.

Several studies were carried out by Thunberg (1920), Knoop (1923), Szent - Gyorgyi (1935, 1936) and Krebs and Johnson (1937) to understand the formation of organic acids during the oxidation of carbohydrates and finally Krebs and Johnson proposed the citric acid cycle, otherwise known as the tricarboxylic acid cycle or the Krebs cycle, to explain the formation of organic acids as intermediates in the oxidation of pyruvate. The operation of the tricarboxylic acid cycle has been widely demonstrated in animal tissues (e.g. Green et al, 1948; Elliott and Kalnitsky, 1950) as well as microorganisms including bacteria and molds (e.g. Gilvarg and Davis, 1956; King et al, 1956; Atkinson 1956; Stone and Wilson, 1952; Beck and Lindstrom, 1955; Wiame and Bourgeois, 1953; Altenbern and Housewright, 1952; Swim and Krampitz, 1954; Blackley, 1952; Englesberg and Levy, 1955; Delwiche and Carson, 1953; Campbell and Stokes, 1951; Barrett and Kallio, 1953; Kogut and Podoski, 1953; Crook and Lindstrom, 1956; Stedman and Kravitz, 1955; Mickelson and Schuler, 1953; Ramakrishnan, 1954; Lewis, 1948; Strauss, 1955; Goldschmidt et al, 1956; Hockenhull et al, 1954; Schatz et al, 1955; Moses, 1955).

These studies show that carbohydrates can be utilized in animal tissues and micro-organisms by a major pathway shown in Fig. 1.

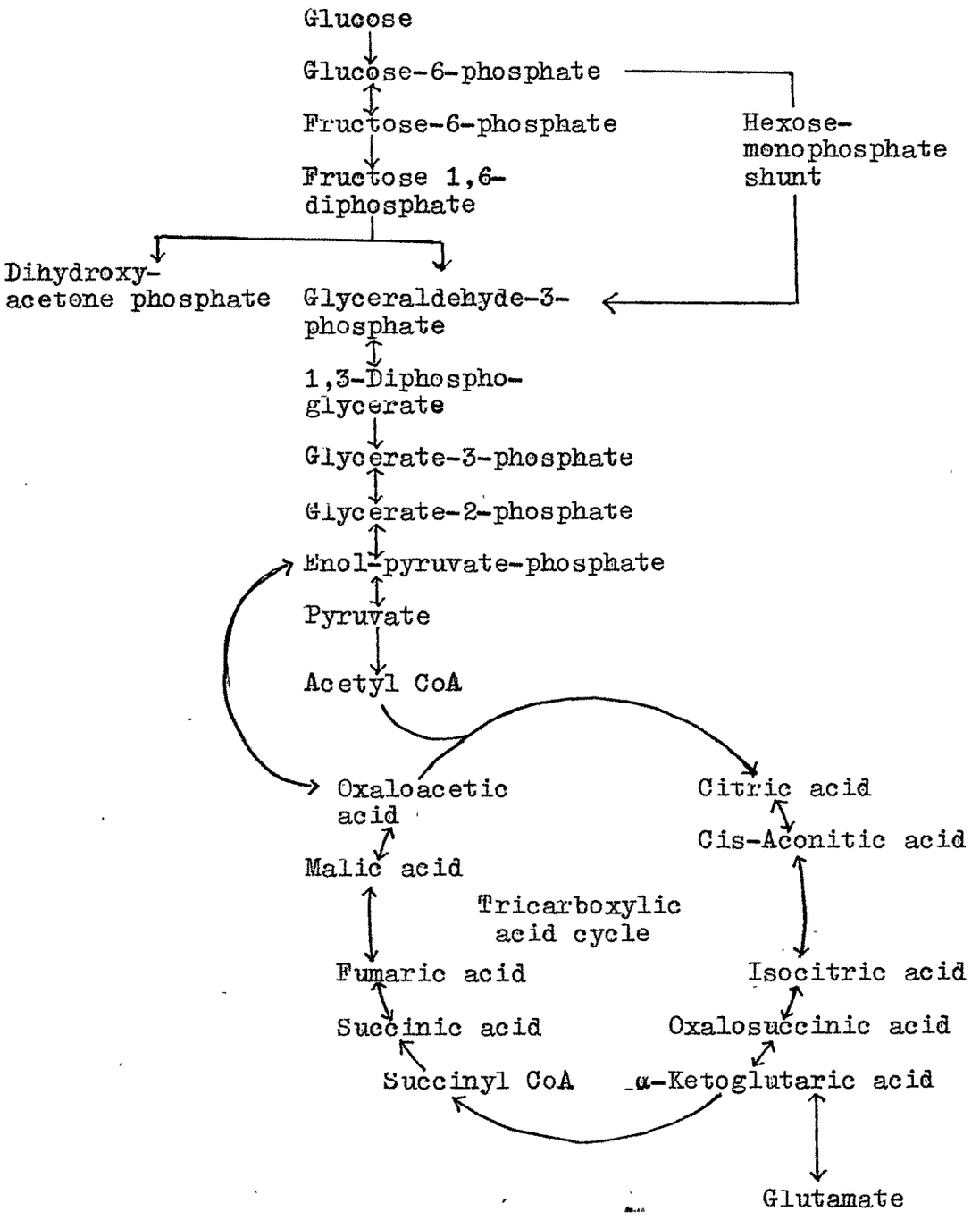

Fig.1:- Carbohydrate metabolism in living cells.

In the case of plant tissues, the studies of Tanko (1936) and Hanes (1940a) on the transformation of starch into phosphorylated sugars by pea meal extract, and those of James et al (1941) on the conversion of sugars into pyruvic acid by barley sap have led to the belief that the mechanism for the conversion of carbohydrates into pyruvate in plants may be similar to that in animals and a yeasts. A considerable amount of evidence has accumulated during the past few years for the operation of the glycolytic cycle in plant tissues. Individual enzymes of the glycolytic cycle have been detected in several plant tissues (Tanko, 1936; Hanes, 1940a; b; Bliss and Naylor, 1946; Porter, 1950; Kursanov and Pavlinova, 1948; Edelman et al, 1955; Saltman, 1953; Gibbs, 1955; Hageman and Arnon, 1955a; b; Cardini, 1951; Ramasarma et al, 1954; Stumpf, 1948; Tewfik and Stumpf, 1949; Giri and Ramasarma, 1956; Axelrod and Bandurski, 1953; Stumpf, 1950). More crucial is the demonstration that all the enzymes of the glycolytic cycle are present in green gram (Phaseolus radiatus) (Giri and Ramasarma, 1956).

Several investigations have similarly been made on the operation of the hexose monophosphate shunt in plant tissues. Conn and Vennesland (1951) demonstrated the presence of glucose-6-phosphate dehydrogenase in several

plant extracts. Benson (1951) was able to identify ribulose in spinach leaf preparation fed with ribose-5-phosphate. Ginsburg and Hassid (1956) found labeled sucrose in canna plant and wheat seedling following the administration of labeled C^{14} pentoses. The detection in plant tissues of the individual enzymes of the hexosemonophosphate shunt such as glucose-6-phosphate dehydrogenase (Gibbs, 1952), 6-phosphogluconic dehydrogenase (Axelrod and Bandurski, 1952), phosphoriboisomerase (Axelrod and Jang, 1954) and transketolase (Horecker et al, 1953) and the work of Clayton (1959) on the pentose cycle activity in cell-free extracts of tobacco leaves and seedlings have added substantially to the evidence for the operation of pathways other than glycolysis in plant tissues.

It is thus, evident that plant tissues have the enzymes required for the operation of both the Embden-Meyerhof pathway and the hexose monophosphate shunt. To determine the relative strengths of these two pathways Beevers and Gibbs (1954) 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 Embden-Meyerhof pathway is the more dominant.

Regarding the further oxidation of pyruvate in plant tissues, the hypothesis that a cycle similar to the tricarboxylic acid cycle in animal tissues might operate in plant tissues was first advanced by Chibnall (1939).

A number of *in vitro* experiments on a variety of plant materials such as segments of *Avena* coleoptiles (Bonner, 1948) and barley root (Laties, 1949), spinach leaves (Bonner and Wildman, 1946) and slices of potato tubers (Barron *et al*, 1950) demonstrate the utilization of various di and tricarboxylic acids such as succinic, fumaric, α-ketoglutaric and citric acids by respiring plant tissue cells as evident from the increased rate of oxidation in these cells on addition of these acids.

The oxidation of the intermediates of the tricarboxylic acid cycle have also been shown in a large number of other plant tissues like *Avena* (Tager, 1954), castor been endosperm (Beevers and Walker, 1956; Walker and Beevers, 1956), cauliflower (Laties, 1953a; b), sweet potato (Akazawa and Uritani,

1954; Lieberman and Biale, 1956), spinach leaves (Ohmura, 1955), developing pepper (Howard and Yamaguchi, 1957) and double beans (Kalimi, 1968).

Millerd et al, (1951) found active mitochondria from mung bean (Phaseolus aureus) hypocotyls to be capable of oxidizing some of the tricarboxylic acid cycle intermediates. Similarly active mitochondrial fractions were isolated from cauliflower (Brassica oleracea Var botrytis) by Laties (1953a; b; c) and from peas (Pisium sativum) by Davies (1953). Davies (1953) demonstrated the occurrence of the essential steps of the tricarboxylic acid cycle in pea mitochondria. Similarly mitochondrial particles isolated from roots, seeds, hypocotyls, floral parts, fruits, petioles and tubers of different plants have been found to utilize tricarboxylic acid cycle intermediates for respiration (Freebairn and Remmert, 1956). Brummond and Burris (1953) showed the net production of citrate from pyruvate and malate in lupine mitochonadria. They incubated the same with pyruvate labeled in the carbonyl carbon using malate as a sparking acid. In addition other individual acids of the cycle were added as traps for any labeled acid of the same type which might be produced. After one hour the reaction was stopped and the acids were separated chromatographically. The carbonyl-C of pyruvate

appeared in each of the acids of the tricarboxylic acid cycle. Similarly Freebairn and Remmert (1957) showed that carbon from succinate-2-C^{14} was incorporated in malate, pyruvate, citrate and glutamate when it was oxidized by a preparation from cabbage.

The operation of the tricarboxylic acid cycle in plant tissues was further strengthened by the study on the individual enzymes involved. Davies (1953, 1954) showed the presence of NAD and NADP specific isocitrate dehydrogenases, fumarate hydratase and aconitate hydratase in the supernatant obtained by treating washed pea mitochondria in a Mickle shaker.

Several attempts have been made to show similar oxidation in fruit tissues. Pearson and Robertson (1954) tried to isolate mitochondria from apples which can oxidise the tricarboxylic acid cycle intermediates. But their preparations showed only a limited activity with succinate, malate and citrate. Neal and Hulme (1958) also demonstrated the oxidation of two intermediates, viz., succinate and malate by the particles of apples in presence of added protocatechuate. Biale and his associates could isolate metabolically active cytoplasmic particles from avocado (Abramsky and Biale, 1957a; b; Biale et al, 1957) 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 (1957a) concluded that the tricarboxylic acid cycle is a major pathway of oxidation of carbohydrates in a variety of tissues in higher plants.

Ramakrishnan and Varma (1959) studied the oxidation of the intermediates of tricarboxylic acid cycle by the homogenates of young and old fruit tissue of Citrus acida. The young fruit tissue in which no citric acid accumulates is able to effect the oxidation of all the intermediates of the tricarboxylic acid cycle. The mature fruit tissue in which citric acid accumulates is not able to oxidise citric acid suggesting a partial block of the tricarboxylic acid cycle at this level. This supports the view expressed by Bonner (1950), namely, that the accumulation of particular cycle acid in a plant tissue might mean that one of the reactions of the cycle was blocked or especially slow. While this may account for the accumulation of citric acid the question arises regarding respiration in these tissues.

The above studies show that carbohydrates are utilized for the formation of citric acid in the fruit tissues due to the operation of glycolytic pathway and the

tricarboxylic acid cycle although the operation of the former has yet to be demonstrated. Further, the question regarding the mode of respiration of the tissue when citric acid accumulates in mature fruit tissue has to be solved. The decrease in the protein content of the tissue in mature fruits (Sinclair and Eny, 1947; Bain, 1958; Varma 1959) raises the question whether protein is utilized for respiration.

In higher plants protein breakdown is known to occur under different conditions. The early experiments of Vickery et al, (1940) with isotopic nitrogen indicate the dyanamic state of all proteins in plants. It is known that the breakdown of tissue protein occurs in starving or senescent organs of the plants (Steward and Street, 1947). Chibnall (1954) found a decrease of protein nitrogen and an increase in non-protein nitrogen in the detached leaves of runner bean (Phaseolus vulgaris). Leaves about 8 weeks old and of similar size were detached from the plant and kept in beakers containing ordinary tap water so that the lower inch of the main petiole was immersed. They were so kept for 6 days at room temperature in a room exposed during the day to strong diffused day light. The leaves which were thus kept in the absence of exogenous sugar supply were analysed for total and protein nitrogen. Similar studies were carried.

out by Krotkov (1939) and Ranjan and Laloraya (1965). The latter workers showed a massive increase in aspartic acid and glutamic acid in tobacco leaves which were detached from the plant and kept for 72 hrs in water. During this period starch was being quickly depleted for energy requirement. However, towards the end of this period rapid breakdown of protein was also evident. From 70 hours onwards hexoses and sucrose along with free amino acids especially glutamic acid and aspartic acid were used for respiration.

Changes in the contents of protein and amino nitrogen have also been observed in plants starved of nutrients other than carbohydrates. Leaves of barley plants were grown under different conditions to be specified below and analysed for sugar, protein nitrogen and amino nitrogen (Gregory and Sen, 1937).

(a) With optimal level of potassium and decreasing levels of nitrogen

(b) With optimal level of nitrogen and decreasing levels of potassium

(c) With minimum levels of potassium and nitrogen

(d) With optimal levels of potassium and nitrogen.

In the leaves of plants subjected to potassium deficiency a very rapid (compared to the control) disappearance of protein was associated with a marked increase in amino nitrogen.

Studies have been made of the effects of starvation induced by preventing photosynthesis on the nitrogen metabolism in etiolated seedlings. Barley seeds were allowed to germinate in the dark and determinations made on different days of respiratory rate and the contents of carbohydrate and nitrogen both in endosperm and embryo (Barnell, 1937; James and James, 1940; and Forward, 1951). During the first 6 days of germination the reserves of the embryo, namely, sucrose, raffinose and small amount of fat were rapidly broken down. The respiratory quotient (R.Q.) during this time was maintained at a value close to unity indicating that carbohydrates furnished most of the respiratory substrate. After this period there was a complete exhaustion of soluble carbohydrates and the respiratory quotient began to fall to a value of about 0.8 indicating that proteins contributed to the respiratory substrates. This fall in R.Q. could be prevented by subjecting the germinating seedling to periodic illumination (Folkes et al, 1952).

The extent to which amino acids and proteins contribute as main respiratory substrates in young seedling is at present uncertain. It is, however, evident from the work of Folkes and Yemm (1958) that the germination of seedlings is characterised by an extensive breakdown of protein of the endosperm and resynthesis of new protein in the growing embryo. Experiments to support this statement were done by growing the seeds in pots of sand supplied with mineral nutrient solution. Samples of the seedlings were taken at 2 days intervals up to a period of 10 days. The seedlings were then dissected into embryo and endosperm and both endosperm and embryo investigated for their protein and non-protein constituent. The protein content of the endosperm changed very little during the first two days followed by a rapid breakdown during next four days. By the sixth day the protein reserves were largely exhausted and relatively little change occurred after the eighth day. Hordein, the chief storage protein, was utilized first. In all cases the protein breakdown was associated with a rise in non-protein nitrogen which reached a maximum on the fourth day. However, this increase did not correspond to the loss in protein nitrogen indicating the early translocation of simple peptides and amino acids into the growing

embryo which showed a marked increase in protoplasmic protein after the second day. Studies on amino acid composition of the protein of the endosperm and the embryo show wide differences in composition between chief storage proteins of endosperm and the protoplasmic proteins of the embryo suggesting that an extensive interconversion of the amino acids must occur during germination (Folkes, 1957). Similar results have been obtained on pea seedlings (Beevers and Quernsey, 1966). Yemm and Folkes, (1958) suggested that the carbon skeleton of these amino acids may be in part drawn into the respiratory metabolism.

Another factor which brings about the breakdown of protein and accumulation of amino acids in plant tissues is water stress. Considerable amount of work has been done on the effect of water status on the amino acid composition of leaves. The rapid loss of protein from the leaves of tobacco plant in the process of curing have been considered important from the point of view of tobacco industry. Vickery and Meiss (1953) noted that when tobacco leaves were hung for a period of two months rapid chemical changes occurred during the first 8 to 12 days of curing. About 75 per cent of initial water evaported and the loss of water was accompanied

by the decomposition of 5 per cent of the protein with the production of soluble nitrogenous substances.

Similar observations have been made by Thompson et al (1966) who prepared wilted leaves of turnips by drying the leaves so that 50 per cent of water content was lost. The partially dehydrated leaves were then incubated for 0,2,5, 10,20,45,70,101 and 164 hours. Protein content decreased at a much faster rate in the wilted leaves. This was associated with a rapid disappearance of sugar followed by a rapid rise in ammonia indicating that deamination of amino acids may provide oxidizable substrates for respiration with the progressive depletion of sugar.

The diurnal variations on the protein metabolism of the runner bean has been studied by Chibnall (1922). Analysis for nitrogenous contituents were carried out on leaves picked about half an hour before sunset when they had been exposed to 14 hours of sunlight and half an hour before sunrise when they had been in the dark for about six hours. A decrease in protein nitrogen and increase in amino nitrogen were found to occur during the dark interval suggesting protein breakdown at night.

Such breakdown of tissue protein has also been found to be characteristics of maturity and senescence in a wide variety of plant tissues such as the shoot, apical meristems and young and old leaves (Steward et al, 1954). The concentration of amino acids is found to be more in older plant tissues. A similar observation has been made with regard to Valencia oranges by Bain (1958) who found that protein nitrogen expressed as a percentage of either fresh or dry weight decreases during growth.

The changes in nitrogen metabolism associated with the development and ripening of the fruits have been worked out to some extent. Most of the studies have been concentrated on climateric fruits such as apples and banana which can ripen both on and off the tree. Such fruits are characterised by a sharp rise in respiration (climateric rise) during ripening and a fall in respiration in the over ripe fruit. In apples the climateric rise in the respiration was found to be associated with a net protein synthesis followed by a steady fall in protein content and an increase in amino nitrogen and asparagine (Hulme, 1948).

Studies of physiological and biochemical changes in the banana fruit at different stages namely, initiation, development, maturation and harvest were made by Steward

et al (1960). During the rapid growth of the fruit its initially high content of soluble nitrogen was depleted suggesting its utilization for anabolic processes. At the stage of harvest there was an increase in asparagine and glutamine.

It would appear from the above studies that protein breakdown is a common feature of conditions such as senescence, light deprivation, starvation and mobilization of the reserves in endosperm for germination.

As mentioned earlier it has been suggested that in germinating seedlings the carbon of amino acids may be used for respiratory purposes. This hypothesis has been sought to be tested by using the respiratory quotient or R.Q. as the criterion. In animal tissues the same is found to be close to unity when carbohydrate is used as fuel and less than one when fat (0.7) and protein (0.8) are used as fuel with intermediate values when a mixture of these substances serves as fuel. In plants the use of fat as fuel source is practically ruled out because of its low concentration in the tissues. In several species of plants studied by James (1953) the R.Q. is found to be close to unity in leaves of normal plants with adequate

reserves of carbohydrates. With light deprivation there is a decrease in carbohydrate content. As mentioned earlier there is also an increase in amino nitrogen. It is interesting that amino acids formed by the breakdown of protein may account for most of the carbon dioxide produced in respiration.

The oxidative deamination of glutamate for respiratory purposes is suggested by Das and Roy (1962) on seedling of Vigna sinesis in which carbon dioxide and ammonia formation were formed at the expense of glutamic acid in mitochondria studied for oxygen uptake in the Warburg apparatus.

Glutamic acid can be reversibly converted to α-keto-glutaric acid by the enzyme glutamate dehydrogenase which is widely present in plant tissues. (Adler et al, 1938; Damondaran and Nair, 1938; Euler et al, 1939 and Berger and Avery, 1944).

The role of hormones in regulating protein breakdown is suggested by the studies of Chibnall (1954) who found that treatment with indoleacetic acid retarded protein breakdown in the blade of mature leaf of the runner bean detached from the plant and kept with the cut and in water. Such treatment was also found to result in the formation of adventitious roots at the petiole. Similar findings have been

made by others using detached leaves of Xanthium (Richmond and Lang, 1957) and wheat (Pearson et al, 1957). Later, Mothes et al (1959) elegantly demonstrated that when solution of kinetin was applied locally to the surface of the mature tobacco leaves the original level of protein was maintained in the areas treated. Similar observations have been made by Osborne (1960) in leaves (Prunus serrulata) treated with auxin. Fully grown leaves were removed from trees and an ethanolic solution of the n-butyl ester of 2,4-dichlorophenoxy acetic acid was applied to the upper surface of the lamina on each side of the main vain to give a total doze of 25 ug/spot. Control leaves were treated with ethanol. At appropriate intervals the levels of the different nitrogen fractions were determined. While nearly 50 per cent of the protein was degraded in control, the protein level was maintained in the tissue treated with 2,4-dichlorophenoxy acetic acid. Further estimations had shown that there was an increase in α-amino acids corresponding to the decrease in protein in the control.

The catabolism of protein is accompanied by an accumulation of the amides-asparagine and glutamine in barley leaves (Yemm, 1937). Tobacco leaves cultured in water in the

dark were found to accumulate asparagine but very little glutamine (McKee, 1949). The two amide fractions show consistent differences in relation to catabolism of proteins and respiration of barley leaves. Glutamine accumulates rapidly at first in the freshly detached leaves while asparagine is formed only at later phases of starvation (Yemm, 1937).

The accumulation of amides in barley leaves may be decisively influenced by a variety of factors such as available carbohydrates, exposure to light, oxygen tension in the atmosphere, stage of development of the plant and age of the leaves. When starvation associated with a reduction in carbohydrate reserves was induced by detaching the leaf from the plant an increase occured in asparagine content. But when this was associated with exposure to light, the increase was in glutamine rather than asparagine. The nature of the amide formed thus varied with availability of carbohydrates and light. The formation of asparagine in corn seedlings germinated for 70-hours in the dark is consistent with these findings (Oakes, 1966).

These studies show that when protein is utilized for respiration asparagine formation takes place in order to trap the ammonia formed during the utilization of amino acids formed by protein breakdown.

Although the catabolism of protein in leaves is always characterised by an accumulation of amide, it is important to decide whether the amides could arise simply by hydrolytic changes of proteins or whether more extensive catabolism and interconversion of amino acids was implied. Data were obtained on the loss of protein nitrogen at different sugar levels and the amide nitrogen formed (Yemm, 1949) and also the amide nitrogen present in the protein isolated from barley leaves. An estimate could be made of the maximum amount of glutamine and asparagine that could be formed simply by hydrolysis of the protein. The examination of the data on the amide nitrogen present in the protein and formed in detached leaves has shown that the increase in amide nitrogen was two to three times greater than that could be accounted for by hydrolysis of protein. This has given an indication that both glutamine and asparagine arise by secondary changes in detached leaves.

Regarding the mechanism of formation of amides it has been shown in plants (Elliott, 1953; Webster, 1953) and animals (Speek, 1949) that glutamine is formed according to the reaction.

$$\text{Glutamate} + NH_3 + \text{ATP} \rightarrow \text{Glutamine} + \text{ADP} + \text{Pi}$$

Regarding the formation of asparagine several mechanisms have been suggested. Evidences for the formation of asparagine by a mechanism similar to glutamine have been given by Webster and Varner (1955). Their study shows the presence in lupine seedlings of an enzyme which can form radioactive asparagine from ^{14}C aspartate and ammonia in presence of adenosine triphosphate.

Pathways other than direct amidation of aspartic acid has been suggested for the biosynthesis of asparagine. Blumenthal-Goldschmidt et al (1963) have noted that hydrocyanic acid was incorporated into asparagine by seedlings of flax, sorghum and white clover that contain relatively large amounts of cyanoglucosides. The extracts of these seedlings are found to incorporate carbon of labeled hydrocyanic acid into amide carbon of asparagine.

The accumulation of citric acid in the mature lemon has been attributed to a block in the TCA cycle at the

aconitate level. As respiration continues at this stage, this raises a question regarding its mechanism. The possibility suggests itself that as the fruit matures protein may be broken down into amino acids which are utilized for respiratory purposes. If this is the case we should expect changes in the contents of protein and amino nitrogen and in the activities of enzymes concerned with carbohydrate and amino acid metabolism. The present studies were designed to test this hypothesis. Fruit tissues of Citrus acida were investigated at two stages of development for:

(a) enzymes and intermediates of glycolytic cycle
(b) protein, amino nitrogen and free amino acids
(c) activities of enzymes concerned with glutamate metabolism.

These studies are detailed in this thesis.