1-1-1971

Effects of supraoptimal carbon dioxide concentrations on mitochondrial activity of 'Richared Delicious' apples.

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EFFECTS OF SUPRAOPTIMAL CARBON DIOXIDE CONCENTRATIONS ON MITOCHONDRIAL ACTIVITY OF 'RICHARED DELICIOUS' APPLES.

A Dissertation Presented

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B.Sc., University of London, England (1965)

Submitted to the Graduate School of the
University of Massachusetts in
partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

September 1971

Major Subject: Plant Science

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

"CO_2 injury" is a physiological disorder of apples that develops during storage under high levels of CO_2. The concentration likely to produce injury depends on the specific cultivar and duration of storage period under consideration. Many cultivars such as 'Delicious' are highly susceptible to injury and will tolerate no more than 4%CO_2 when exposed to the gas for a long duration (2). On susceptible cultivars higher levels produce damage at a much faster rate than occurs at 4%CO_2.

The disorder was first observed in England in 1911 in shipments of apples from Australia. Injury appeared as a brown area in and around the core region and because of its location was initially referred to as "brown heart". However, it was not until 1923 that Kidd and West (41) reproduced the disorder experimentally and demonstrated that it was caused by excessive amounts of CO_2 in the storage atmosphere.

There are numerous reports (34, 40, 56) showing that CO_2 has a retarding influence on respiration, but little is known of its role in this process.

Hulme (27) reported that apples injured by storage in high CO_2 atmospheres accumulated succinic acid and from this he concluded that the succinoxidase system of mitochondria was being inhibited. Such a restraint could shift intermediate metabolism in the direction of fermentation. Not

only would this change reduce energy production within the mitochondrion but it could also give rise to formation of acetaldehyde and ethanol, both potentially toxic compounds capable of further suppression of activity (70, 74).

It was the purpose of this investigation to determine the effects of increased levels of CO_2 on the mitochondrial metabolism of apples. Of primary importance was to test whether CO_2 specifically affected succinate oxidation or whether it had an effect on the oxidation of other citric acid cycle acids. In addition, it was intended to follow changes in mitochondrial oxidation rates and in oxidative phosphorylation during the development of "CO_2 injury" in stored fruit.

L I T E R A T U R E R E V I E W

Since the time of de Saussure's "Recherches chimiques sur la vegetation" published in 1804 (68) it has been known that plants become injured when placed in an atmosphere containing high concentrations of CO_2. However, the exact nature of the injury has still not been clearly defined. The inhibitory effect of CO_2 on respiration was first recorded by Kidd (40) who showed that high levels of CO_2 had a depressing effect on both the CO_2 output and the O_2 uptake of germinating white mustard seeds. Further work has shown the rather diverse effects of CO_2 on certain vital cellular processes. In various plant species it has been shown that by increasing the CO_2-bicarbonate levels in the rooting medium, there is a depressing effect on growth (73), respiration (34, 40, 56), mineral absorption (33), nutrient translocation within the plant (64) and the rates of certain enzyme reactions, such as cytochrome-c oxidase (54). However, in the present study we are not concerned with the effects of CO_2 on the whole plant but rather, on the detached fruit of the apple (Malus domestica. Bork). Hence, a thorough review of the voluminous literature on whole plant effects will not be attempted.

Although "CO_2 injury" of apples is a storage disorder the incidence and severity will be influenced by certain preharvest factors. During storage the intensity will depend on the CO_2 concentration, temperature and O_2 concentration. The effects of these storage factors will be discussed separately

under the section headed "Post-harvest Factors".

1. Factors Affecting Development of "CO2 Injury".

A. Pre-harvest Factors.

Susceptibility to "CO_2 injury" is very much dependent on the cultivars of apple being stored. Many, such as 'Delicious', tolerate less than 4%CO_2 when stored for long periods of time. However, others such as 'Bramley's Seedling' may be stored in concentrations as high as 10%CO_2 without damage for extended periods of time.

Large fruits are more prone to damage than smaller ones (11, 37). Kerrawala showed that 'Jonathan' apples stored in 18%CO_2 and 3%O_2 exhibited damage in only 9% of the fruits weighing 121-125 grams, but this increased to 100% in fruits weighing more than 160 grams.

Advanced maturity (11), low N (16) and the presence of water core (11) have all been shown to increase the incidence of the disorder.

B. Post-harvest Factors.

There is controversy in the literature as to whether high or low temperatures increase "CO_2 injury". Wilkinson (82) cites the evidence of Kidd and West (41, 42) who reported that apples are more susceptible to "brown heart" at lower than at higher temperatures. Hulme (27) showed that fruit stored in 20%CO_2 for 11 days at 3°C developed injury, but fruit stored at 10°C for 90 days showed none.

However, there are also reports which show that damage is more pronounced at higher than lower temperatures (13). Furthermore, to add to the confusion Blanpied and Smock (7) found opposite results in 2 successive seasons with 'McIntosh' apples.

The level of O_2 in the storage atmosphere also has an effect on the development of "CO_2 injury". High concentrations increase the susceptibility to CO_2 (37). Anderson (2) found such an effect beginning at 3%O_2. However, evidence from Hansen and Mellenthin (22) suggests that the reverse is true for pears.

Although high levels of CO_2 produce damage during storage, certain low levels in combination with low O_2 can produce beneficial effects. The early work of Kidd and West (42) showed that by increasing the CO_2 level and reducing the O_2 level, not only was respiration reduced but also the storage life of the fruit was increased. These initial discoveries eventually led to the world-wide application of controlled atmosphere storage to apples and pears.

2. "CO2 Injury" of Apples.

In the apple high concentrations of CO_2 can produce distinct injury. This manifests itself as a browning in and around the core region. In its initial stages this is nothing more than a slight discoloration; however, as the disorder progresses the browning increases in intensity. At the same time the affected tissue readily loses water to the

adjacent tissues so that cavities are eventually formed (18). Similarly, when the apple is cut the brown tissue dries more rapidly than the unaffected tissue. Typical symptoms of moderate "CO_2 injury" are shown in Figure 1.

A. Effect of CO2 on Apples.

There have been several attempts to relate the development of "CO_2 injury" to the accumulation of certain metabolites during storage. Thomas (74, 75) discovered that exposure of apples to aerobic atmospheres containing high CO_2 concentrations resulted in the accumulation of acetaldehyde and ethanol and he termed this induction of fermentation products by CO_2, "CO_2 zymasis". He inferred that some oxidative process was inhibited. Furthermore, he found that the relative concentrations of acetaldehyde and ethanol produced were different under "CO_2 zymasis" from those under anaerobic conditions. Under "CO_2 zymasis" there was greater accumulation of acetaldehyde and a lesser accumulation of ethanol. The ratio of ethanol to acetaldehyde was 2:1, whereas tissues exposed to anaerobic conditions gave a ratio of 50:1. Clearly, under the conditions of "CO_2 zymasis" the conversion of acetaldehyde to ethanol was inhibited. Furthermore, Harley and Fisher (25) found that acetaldehyde accumulated in tissues showing breakdown in pears. Later, Harley (24) measured the amount of CO_2 in the intercellular spaces of pears prior to the appearance of damage and found that the maximum amount of CO_2 occurred a short time before the disorder appeared; thus, the accumulation of acetaldehyde might

Figure 1. Typical symptoms of moderate internal "CO2 injury". Note the browning in and around the core region of the fruit.

have been an expression of the effect of high CO_2 in the tissues. Although "CO_2 zymasis" was induced in many tissues by CO_2 in concentrations above 50% it has not been reported in concentrations much below this value (72).

Further evidence that high concentrations of CO_2 induce a change in metabolism has been produced by Hulme (27). He showed that apples injured by storage in 20% CO_2 at 3°C were higher in succinic acid than uninjured apples stored in air. A small but measurable increase in succinic acid was also observed with 10% CO_2, whilst only trace amounts of the acid were found in healthy tissue. He therefore implied that the accumulation of succinic acid under high CO_2 was due to an inhibitory effect on the succinoxidase system.

This system is an important constituent of the citric acid cycle and it has been suggested by Faust et al. (18) that accumulation of succinic acid may be due to the inhibition of this cycle. Under these conditions the regeneration of oxalacetate (OAA) is diminished, and the oxidation of pyruvate may not keep pace with its production. Accumulating pyruvate is likely to be diverted through alternative pathways, one of which involves the conversion of pyruvate to acetaldehyde and ethanol. This would explain Thomas's (74, 75) observation that acetaldehyde and ethanol do accumulate under "CO_2 zymasis".

Apart from these results, the work concerning the effects of high CO_2 on apples is very limited. Nevertheless,

work in other areas has provided some evidence regarding the modifying effects of CO_2 on metabolism.

Bown et al. (10) working with Iris rhizomes showed an increase in the main acids except malate during storage in 12%CO_2 and 10%O_2. Ranson (61) stored carrot, oat, and Kalanchoe tissues in different CO_2 levels and found that the results common to all were an accumulation of succinic acid and a decrease in malic acid content in the tissues. Similar effects of high CO_2 were observed in pears by Williams and Patterson (84) who, in addition, found an accumulation of citric acid. There seemed, however, to be a modifying effect of CO_2 on the depletion of malic acid, as fruits stored in air were depleted at a slightly faster rate. Kollas (43) confirmed this effect of CO_2 on the rate of depletion of malic acid during storage. Working with 'McIntosh' apples stored in 5%CO_2 - 3%O_2 and in air, he showed that the total acid content was much higher in the CO_2-stored fruit than that stored in air. Malic acid accounted for most of the difference in total acid between the 2 treatments.

In interpreting such findings, it is important to distinguish whether the change in acid content is due to a difference in the production rate, depletion rate, or both. In the case of malic acid there is evidence that the higher content in apples stored in 5%CO_2 may have resulted from CO_2 fixation (1), which will be considered more thoroughly later. However, the mechanism of succinic and citric acid

accumulation might best be interpreted as a result of changes in the citric acid cycle, which operates within the mitochondria of a cell.

B. Effects of CO_2 on Citric Acid Cycle.

Before proceeding with this topic a brief review of terminology is considered necessary.

Mitochondria contain the enzymes necessary for the oxidation of a variety of acids. These oxidations are accomplished through the removal of electrons and protons from specific organic acid molecules, an act performed by specific dehydrogenases and acceptors consisting of pyridine nucleotides and flavoprotein. The electrons and protons then pass through a system of carriers to a carrier that reacts with oxygen. The passage of each pair of electrons from substrate to oxygen is coupled to the phosphorylation of ADP to ATP in a process known as oxidative phosphorylation. The number of molecules of ATP formed in this process per atom of oxygen uptake has been defined as the P:O ratio.

In "tightly-coupled" systems oxygen consumption is dependent on ADP concentration, and this dependence is termed "respiratory control". Furthermore, the "respiratory control ratio" (RCR) is the rate of respiration in the presence of ADP divided by the rate in its absence. The significance of these characteristics in relation to mitochondrial integrity will be considered later.

There is no direct evidence showing the effects of various CO_2 concentrations on the metabolism of apple fruit mitochondria. Nevertheless, some work of this type has been carried out with the mitochondria isolated from the endosperm of germinating castor bean seeds. Bonner (8) and Bendall et al. (4), working under controlled pH conditions, observed that the succinate-oxidase system was sensitive to CO_2-bicarbonate mixtures above 10%CO_2. Further work by Bendall et al. (5) with various components of the respiratory chain suggested that it was the succinate-cytochrome-c reductase component that was most sensitive to CO_2-bicarbonate. The inhibition was competitive with respect to succinate concentration and for this and other reasons they suggested that the CO_2 - sensitive component of the reductase was succinic dehydrogenase. In addition, higher concentrations of CO_2-bicarbonate mixtures, especially above 40%CO_2, also inhibited reduced diphosphopyridine nucleotide-cytochrome-c reductase, and cytochrome-c oxidase. Similarly, Miller and Evans (54), studying the effect of bicarbonate on the cytochrome-c oxidase activity of extracts from several plant species, showed that bicarbonate inhibited this enzyme. These and other effects of CO_2-bicarbonate have been reviewed by Miller (53). Furthermore, Ranson et al. (62, 63) also showed that citrate synthase, (earlier called the "condensing enzyme") could be included in the category of enzymes affected by CO_2-bicarbonate mixtures.

Miller and Hsu (55) showed more far-reaching effects of CO_2-bicarbonate mixtures than had been previously shown. They reported that concentrations of 15%CO_2 inhibited oxidation rates of mitochondria prepared from cauliflower. Inhibition occurred with citrate, isocitrate, malate, succinate, and NADH as substrates. Phosphorylation was also inhibited.

Kasbekar (36), using mitochondria extracted from rat liver, showed that 5% and 15% CO_2 inhibited respiration rates of a different group of substrates, namely, citrate, alpha keto-glutarate, malate, pyruvate, succinate, and beta-hydroxy butyrate. The ADP/O ratios were not markedly affected although a slight increase was observed with increasing CO_2 concentrations. He showed that the oxidation of succinate was inhibited to a considerably greater degree than the NADH-linked substrates, suggesting that the CO_2-bicarbonate mixtures inhibited the succinate-cytochrome-c reductase component of the system more than the NADH-cytochrome-c reductase or cytochrome-c oxidase component.

Bown et al. (10), working with mitochondria isolated from Iris rhizomes, showed that the succinoxidase system was inhibited by CO_2 concentrations as low as 6%. Measurements were not made on oxidation rates in this study but rather, directly on the cytochrome-c reductase component of the system.

Fanestil et al. (17), working with rat liver mitochondria, showed that in the presence of CO_2 the uptake of Pi decreased and the uptake of substrate increased, suggesting an uncoupling

effect of CO_2 on oxidation and phosphorylation. However, in this case there was no direct measurement of oxygen uptake.

All the evidence to date supports the view that succinate accumulation is due to the inhibition of the succin-oxidase system. There is no evidence to suggest that it is produced from malate by a reversal of the citric acid cycle. This is supported by Allentoff et al. (1), whose experiments with labelled CO_2 in apples showed that the label was incorporated into and remained in malate and was absent from succinate.

C. Origin of CO2 Within the Fruit.

Carbon dioxide, as one of the end products of respiration, is continually being produced within the fruit during storage. Therefore, external CO_2 would not have to penetrate the fruit in order for accumulation to occur. The amount accumulating would reflect the equilibrium established between the internal and external environment. It is therefore reasonable to expect that fruit held in an atmosphere high in CO_2 will eventually achieve a high level internally. Certain morphological factors may have a major effect on the free exchange of gases between the internal and external atmospheres and hence, the equilibrium that is established. The large volume of tissue and thick waxy cuticle common to apple fruits could possibly impede the diffusion process, thereby producing an atmosphere substantially different inside the fruit compared with outside. Evidence from Hardy (23), showed that the greatest

resistance to diffusion came from the skin; resistance from the flesh was negligible in comparison. Furthermore, Williams and Patterson (83) showed that the internal atmospheres of pears removed from controlled atmosphere storage were never more than 2 to 3 percentage points higher in CO_2 than the level in the storage atmosphere. Smith (71) obtained similar results with apples; in air no more than 2 percentage points separated internal and external atmospheres, and in external atmospheres of 5% and 10%CO_2, the internal atmospheres were no more than 1 percentage point different from the external ones. There was, however, a gradient in concentration across the fruit, but this was small. CO_2 concentration increased with depth in the fruit and O_2 concentration decreased. Fruits stored for 10 days at 12°C in 5%CO_2 and 2.2%O_2 showed a CO_2 concentration of 5.5% near the surface increasing to 6.0% at the core. Thus, in the apple there is apparently only minor resistance to movement of O_2 and CO_2 into or out of the fruit during cold storage.

Within the fruit, CO_2 diffuses freely among the cells. Krogh (46) showed that CO_2 differs markedly from carbonic acid in that it can penetrate cell membranes more easily than any other known sustance, including water. In contrast, Rabinowitch (60) concluded that the cell membrane is almost impermeable to bicarbonate ions.

D. CO2 Concentrations Within the Fruit.

The internal CO_2 concentrations recorded for fruits of

apple and pear are variable and depend on many factors, especially temperature and respiration rate. Although high temperatures increase respiration, low temperatures increase the solubility of CO_2 in the tissues. Fidler (19) states that the solubility of CO_2 in apple tissues at 0°C is 43% greater than at 12°C. Nevertheless, despite the problems involved, Williams and Patterson (83) were able to show that in pears the internal atmosphere varied with respiratory activity during ripening. When the respiration was low, there was a low concentration of CO_2 and a high concentration of O_2 in the core area; with increased respiration intercellular CO_2 increased and O_2 decreased.

Magness (52) showed that concentrations of 5-10% CO_2 within a fruit in air are common and in some cases levels as high as 30%CO_2 were recorded. Harley (24) reported from 15% to 30%CO_2 in tissues of 'Bartlett' pears sometime before core breakdown. However, Williams and Patterson (83) never reported levels above 10%CO_2 in pears stored in air.

Any difference in values may be real or they may be an artifact of the technique used to analyze the atmosphere. Smith (71) suggested that the high levels for CO_2 obtained by Magness (52) and by Harley (24) were due to the fact that they used tissue plugs and that damaging the tissue may have increased the concentration of CO_2 within the tissue.

E. Changes in pH Due to CO2.

There is the possibility that the observed depressing

influence of CO_2-bicarbonate on certain cellular reactions may be in part attributable to pH changes, since the CO_2-bicarbonate ratio cannot be changed without a simultaneous change in pH.

When CO_2 gas is dissolved in pure water H^+ ions are generated and the pH is reduced (80). Thornton (79) observed this same effect when CO_2 was dissolved in the sap extracted from plant tissues. However, he further reported that when living tissues were exposed to CO_2, the pH increased. In the potato the effect was dependent on the presence of O_2. Apart from saying that this increase in pH was due to an "indirect effect" he offered no explanation.

From the equation:

$$CO_2 \text{ (gas)} \rightleftharpoons CO_2 \text{ (diss.)} \; H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

it can be seen that any change resulting in an increase in pH would have to be a result of the reduction of CO_2 within the system. The removal of CO_2 would bring about a dissociation of H_2CO_3 to restore the equilibrium. Furthermore, there would have to be an association of H^+ and HCO_3^- to replace the H_2CO_3. This removal of H^+ ions from the system would cause an increase in pH. The reduction of CO_2 within the system could be brought about by a depression in respiration resulting in less CO_2 production, or removal of CO_2 from the system by fixation reactions. The effects of CO_2 on respiration have already been discussed. Evidence for "CO_2 fixation" will be discussed presently.

It is questionable, however, whether such observations on the overall pH of tissues throws any light on the direct effect of CO_2 on the pH of the cytoplasm or on the pH within the various sub-cellular organelles such as mitochondria.

F. CO2 Fixation.

A pertinent aspect of CO_2 metabolism that has been mentioned previously is that of "CO_2 fixation". Certain intermediate acids accumulate in plant cells, presumably in the vacuole. Of these acids malate and citrate most commonly occur, with the former being the predominant acid in apples. However, the accumulation of these acids cannot be explained purely in terms of their production via the citric acid cycle. When acetyl Co A combines with OAA the 2 carbons gained by OAA are balanced by the 2 lost as CO_2 in the regeneration of OAA. Thus, the accumulation of any acid intermediate would immediately arrest the cycle. There are, nevertheless, 3 known systems whereby intermediate acids can be produced, other than via the citric acid cycle.

1) Phosphoenolpyruvate (PEP) carboxylase.

$$PEP + CO_2 + H_2O \xrightarrow{Mg^{++}} OAA + Pi$$

2) Malic enzyme.

$$Pyruvate + CO_2 + NADPH_2 \overset{Mn^{++}}{\rightleftharpoons} Malate + NADP$$

3) PEP carboxykinase.

$$PEP + CO_2 + ADP \overset{Mn^{++}}{\rightleftharpoons} OAA + ATP$$

According to Hulme and Rhodes (29), only PEP carboxykinase is a possible mitochondrial enzyme and for this enzyme to fix CO_2 would require permeability of the mitochondrial membranes to PEP.

Malic enzyme is known to exist in apples (29) but there is little evidence to show that it works in the direction of malate formation. Nevertheless, Allentoff et al. (1) did show that labelled CO_2 could be fixed into malic acid and they suggested the possibility that it was being incorporated via pyruvate and malic enzyme. Dilley (14) specifically assayed for malic enzyme activity in 'Delicious' apples and observed that prolonged exposure to 5%CO_2 in storage resulted in a decrease in the activity of the enzyme. Further, a recovery of the enzyme activity in the fruit occurred after CO_2 treatment was terminated, suggesting that malate formation via this enzyme is not a major pathway in apples under high CO_2.

Walker (81) suggests that reaction 1) is most likely to function in tissues as a source of C_4 acids since the equilibria of the other 2 favor decarboxylation of these acids.

G. Changes in Mitochondrial Structure.

Koncalova (44,45) studied the effect of CO_2 on the shape of wheat, barley, onion and pea mitochondria. Sensitivity to CO_2 was manifested in a change in shape of the mitochondria, but this change differed with plant species studied. Pea and wheat, which are more adapted to growth in poorly aerated

soils, contained mitochondria which were more resistant to structural change than those of barley or onion. Furthermore, the mitochondria in the aerial parts were more susceptible to damage than those connected with the root system, presumably due to the adaption of the roots to higher levels of CO_2 in the soil medium. When the mitochondria of barley and onion were exposed to 5%CO_2 there was elongation and eventually fragmentation. Prolonged periods of exposure proved to be lethal.

Frenkel (20), working with pears, showed that the mitochondria from high CO_2-stored fruit were reduced in size, were present in larger numbers and were characterized by the deformation of the outer membrane and cristae system.

Whether all, or indeed any, of these changes are related to damage to the mitochondria, or are just changes in conformation under different environments is unclear.

3. Isolation of Mitochondria.

Many procedures have been used to extract and isolate mitochondria from plant tissues. Although the techniques used to produce tightly-coupled mitochondria are basically very similar, the finer details often differ. The methods in general involve gentle disruption of the cells in a suitable extraction medium. The homogenate is then passed through a coarse filter, and the supernatant is separated into the various sub-cellular fractions by differential centrifugation. The mitochondrial pellet produced is washed in a suitable

medium and resedimented at high speed.

The greatest difficulties are encountered when isolating active mitochondria from plant tissues that contain large quantities of organic acids and/or phenolic compounds. Apple fruits contain large quantities of both substances (28).

The pioneering work concerning the isolation of mitochondria from apple fruits was done by Pearson and Robertson (58). They obtained particulate fractions which contained many of the enzymes of the Krebs cycle. Since then, Hulme (28) and Wiskich (85, 86) have produced highly active preparations and Romani (65, 66), working with pears, has been able to obtain tightly-coupled mitochondria.

Organic acids and phenolic compounds (Leucoanthocynanidins, catechins, quercetin and cyanidin glycosides) present in the fruit (69) are both capable of inactivating enzyme systems when they come into contact with the mitochondria during isolation (28). The phenolic compounds also reduce activity of mitochondria by "co-precipitation" of inactive protein complexed with phenolic materials in various stages of oxidation and polymerization (28).

A. The Extraction Medium.

The use of buffers in the extraction medium, together with close monitoring of the pH, is generally successful in counteracting the effect of high acidity. The buffer employed in the medium is usually either phosphate (67), tris-HCl (47,48), or both (15). Buffer concentration is also

important since high concentrations can cause deleterious effects on mitochondrial activity (47).

The pH of the isolation medium also varies considerably within the range 6.8-7.8 (31). A pH higher than 8.0 has been shown to be detrimental.

To prevent the harmful effects of polyphenol oxidase on phenolic compounds, polyvinypyrrolidone (PVP), cysteine-HCl or casein can be added to the extraction medium. PVP acts by complexing with the polyphenols thus reducing the "co-precipitation" of tannins with mitochondrial preparations. The concentration most widely used is 0.75-1.00% (w/v). Cysteine-HCl acts as a reducing agent and keeps the polyphenols in the reduced and inactive state. However, since cysteine-HCl is itself auto-oxidized and the product harmful (28) the concentration should be kept to a minimum (below 5 mM) and the chemical deleted from the wash and suspension medium. Casein acts as an inert protein to compete with tannin-enzyme reactions (21).

Bovine serum albumin (BSA) is a protective agent used to obviate the deleterious action of fatty acids and other compounds released during cell rupture. It is used in the isolation medium by some but not all workers.

EDTA is used in the isolation medium to prevent any detrimental action of cations such as Mg^{++} or Ca^{++}.

Although the most commonly used osmoticum for the isolation of higher plant mitochondria is either sucrose or

mannitol (31), workers extracting apple or pear mitochondria seem to prefer sucrose.

Another important point concerning the isolation medium seems to be the ratio of the quantity of medium to the quantity of plant material being used. Most workers seem to prefer a 3:1 or 2:1 ratio (v/w) of medium to plant material (9, 32, 48, 66).

B. The Extraction Procedure.

Grating (47, 48, 67), grinding (9, 15, 67), blending (87), milling (28), and liquid nitrogen with blending (21) have all been used to disrupt the fruit tissue during mitochondrial extraction. These can be used singly or in combination (86). The principle of tissue disruption is to break the tissues gently enough to release intact mitochondria into the isolation medium. Any harsh method will disrupt not only the cell walls but also the mitochondria. Severe blending or the use of a mortar and pestle have been shown to be extremely damaging to the resulting mitochondrial fraction (31). By using gentle disruptive techniques it is possible to obtain highly active, tightly-coupled mitochondria. However, yield of mitochondria is low under these conditions, since many mitochondria are not removed from the cellular debris. Another disadvantage of the technique is the need for large quantities of isolation medium necessitating rather long extraction procedures. Palmer (57) and Sarkissian and Srivastava (67) have shown that the activity and respiratory

control of the isolated mitochondria are diminished as the time in the isolating medium increases. This is partly due to "aging" and partly because the mitochondria are subjected to the inhibitors, released during the disruption of the cell, for longer periods. As mitochondria "age" they tend to lose activity and respiratory control. Damaged mitochondria "age" much faster than intact ones and the ability to retain activity and coupling over a period of many hours is used as a means of assessing mitochondrial integrity (31, 32, 65). Palmer (57) and Sarkissian and Srivastava (67) therefore set out to develop a procedure whereby the extraction time could be markedly reduced. This was successfully achieved using nylon fabric in the initial stages of the extraction procedure and by using high speed centrifugation. Firstly, the use of nylon fabric makes the initial slow speed centrifugation step unnecessary and secondly, the incorporation of high speed centrifugation for shorter periods markedly reduces the extraction time. These did not appear to damage the mitochondria.

'Delicious' apples contain large quantities of starch, especially in the earlier stages of maturity. This is a problem, not only because starch has been shown to affect the activity and coupling of the mitochondria (67), but also because mitochondria become trapped in it during sedimentation. Nylon fabric, used in the early stages of isolation, effectively removes large quantities of starch from the extraction

medium.

Differential centrifugation throughout the rest of the extraction procedure should aim at minimizing contamination by non-mitochondrial particles.

The wash and suspending media usually contain fewer chemicals than the isolation medium. Important ingredients are osmoticum, buffer to keep the pH above 7.2 and sometimes a stabilizing agent such as BSA.

C. The Assay Medium.

The major constituents in the assay medium are an osmoticum, phosphate, magnesium, and substrate. The osmoticum concentration in the assay medium is often lowered to an isomolar level from the hyperosmotic level in the isolation medium. The concentration of phosphate is generally 10mM, the magnesium 5mM and the optimal pH of assay medium 7.2.

D. Properties of Isolated Mitochondria.

Studies made with isolated mitochondria must always be interpreted with caution, because the mitochondria may have been changed or damaged in some way during isolation procedure. In addition, the preparation may have been contaminated with non-mitochondrial material. However, it has now become possible to define precisely which properties isolated mitochondria must possess if it is to be assumed that they are reasonably intact and have maintained their morphological integrity.

Lehninger (49) has shown that high respiratory control

and phosphorylation are good measures of mitochondrial intactness. However, he further suggests that a high respiratory control should be maintained for some time after isolation.

Furthermore, highly active tightly-coupled mitochondria are associated with low ATPase activity. Inferior preparations, or those showing a high degree of damage, show high ATPase activity and poor respiratory control (6, 26). In addition, disruption of mitochondrial integrity by sonication produces preparations with high rates of endogenous ATPase activity.

Bonner (9), working with plant mitochondria, and Hedman (26), working with animal mitochondria, furthermore reported that damaged mitochondria lose cytochrome-c and that this is manifested in cytochrome-c stimulation of oxidation of various substrates.

Although most studies indicate that in terms of fundamental structure and function plant and animal mitochondria are alike, some apparent differences have been shown to exist. Peter and Lee (59), working with chicken muscle mitochondria, suggested that the inability to respire added NADH most likely indicated a degree of membrane integrity or purity of mitochondria. However, Ikuma and Bonner (32), working with higher plant mitochondria, state that their ability to respire added NADH is one of the major differences between plant and animal preparations. Another difference between plant and animal mitochondria is that plant mitochondria seem to lose activity

and respiratory control much faster than those from animals. This loss is commonly referred to as "aging".

M A T E R I A L S A N D M E T H O D S

1. Fruit Handling Procedures.

Pre-climacteric 'Richared Delicious' apples grown at the University of Massachusetts orchard in Amherst were harvested in early October, 1970, before any significant water-core had developed, and were immediately placed into storage in air at $0^{o}C$. These fruits were used for the studies involving the effects of CO_2-bicarbonate mixtures on mitochondrial activity.

Other samples of fruit were placed in various controlled atmospheres containing potentially injurious levels of CO_2. The 3 atmospheres tested were: $4\%CO_2$ and $13\%O_2$; $6\%CO_2$ and $3\%O_2$; and $12\%CO_2$ and $3\%O_2$. The remainder of each atmosphere consisted of nitrogen. Some fruits were stored in air as a control. These fruits were stored for 4 months at $0^{o}C$ and 90% R.H. with samples removed periodically to assess changes associated with the development of "CO_2 injury".

An external generating system was connected to the CA rooms and the required atmospheres were maintained automatically through a central control monitor. This equipment was developed by the Atlantic Research Corporation, Alexandria, Virginia and is still in the experimental stages. The atmospheres within each room were analyzed every 20 minutes and were generally maintained within 0.5% of the pre-set values. A continuous record was maintained through a teletype connected to the control module.

At monthly intervals fruits were removed from the various CA treatments and examined both immediately and after a 5-day holding period in air at 23°C. On each occasion separate 30-fruit samples were assessed for external and internal appearance and any evidence of "CO_2 injury" was recorded. Firmness was measured on a 20-fruit sample with a Magness-Taylor pressure tester using a 7/16" plunger. Total soluble solids were assessed using a Bausch and Lomb hand optical refractometer using a 10-fruit sample. The development of scald, a physiological disorder causing browning on parts of the skin of the fruit, was followed throughout the storage period together with any distinct changes in flavor.

At the end of the storage period two 50-fruit samples of apples, one measuring less than 2 3/4" in diameter and the other greater than 2 3/4", were taken from each treatment in order to assess the effect of fruit size on the development of "CO_2 injury". Fruits were classified as follows. "None" signified fruit with no internal damage. "Slight" included fruit with faint signs of browning between the locules of the central core region. "Moderate" described fruit with more intense browning, but still restricted to the central core region. "Severe" contained fruit with intense browning extending beyond the core region into the cortex.

2. Determinations Using Apple Mitochondria.

A. Preparation of Mitochondrial Fraction.

Mitochondria were isolated from 'Delicious' apples

stored under the conditions described earlier. Five 2 3/4" diameter fruits were selected, peeled, and grated into 750ml of extraction medium using the apparatus shown in Figure 2. With this procedure approximately 350-400 grams of tissue were grated into the extraction medium, thus maintaining a medium: tissue ratio of 2:1. The extraction medium was composed of: Sucrose, 0.4M; citrate, 0.2M; KH_2PO_4, 0.01M; polyvinyl-pyrrolidone (PVP)-pharmaceutical grade, 0.75% (w/v); ethylenediamine tetra-acetic acid (EDTA), 0.01M; and cysteine hydrochloride, 0.01M, added immediately prior to extraction. This medium was maintained at pH 7.8 throughout the extraction procedure by dropwise addition of 20% KOH and using a Beckman Model G pH meter. The medium was continuously stirred using a magnetic stirrer. All operations were carried out between 0 and 4°C and all apparatus was pre-chilled. During the grating process downward pressure was exerted upon the tissue as it was passed over the grating surface, thereby rupturing the cells of the tissue. Care was taken to insure that the grating surface was maintained below the surface of the extraction medium so that once the cells were disrupted the contents were immediately bathed in buffered extraction medium. The resulting extract was strained through Ca 50 u mesh nylon fabric (Size D. General Biological Co. 8200 S. Hayne Ave., Chicago, Ill) to remove the major portion of starch present within the extract. This procedure circumvented the need for low-speed centrifugation at this stage.

Figure 2. Apparatus used in the extraction of apple mitochondria.

a) Beckman Model G pH meter.
b) Plastic grater in a porcelain dish containing 750 ml of extraction medium.
c) Magnetic stirrer.

The filtrate was then centrifuged for 10 min at 37,000 x g (SS-34 rotor) in a refrigerated Sorvall RC2B centrifuge. The green mitochondrial pellet obtained, together with some adhered starch, was washed using a Thomas homogenizer (glass-teflon size C) in 30 ml of wash medium containing sucrose, 0.4M, EDTA, 0.01M, and trishydroxymethylaminomethane (Tris), 0.1M. This medium was adjusted to pH 7.5. The homogenate was centrifuged for 10 min at 1,000 x g to remove any adhering starch from the mitochondrial fraction. The pellet which resulted from the centrifugation at 37,000 x g for 10 min was suspended in 2 ml of sucrose, 0.2M(pH 7.2) using a Thomas homogenizer (glass-teflon 5 ml size). This gave a 3 ml mitochondrial preparation. Total extraction time varied between $1^1/4$ and $1^1/2$ hours. The extraction procedure is schematically illustrated in Figure 3.

B. Conditions for RCR and Oxidation Rates.

All determinations of oxidation rates and respiratory control ratios were measured polarographically at 28°C using a recording oxygen cathode (Oxygraph Model KM, Gilson Medical Electronics.) fitted with a Clarke-type oxygen electrode (Yellow Springs Instrument Co.) and teflon (0.001") membrane in a 1.5 ml cuvette. Polarizing voltage was maintained at 0.8v throughout. The teflon membrane and half-saturated KCl electrolyte were renewed at the beginning of each experiment. Materials were introduced into the chamber through a movable well with a small opening, and the reaction mixture was stirred

by means of a small magnetic stirrer. The temperature of the reaction medium was maintained using a thermostatically controlled pressurized water jacket. Reaction rates were calculated from a recorder trace on the basis of 0.23 umoles O2/ml in an air-saturated medium. (See Appendix Figure 1 for sample calculation). Oxygen consumption was expressed as mug atoms O2/min-mg protein. The reaction medium contained: Sucrose, 0.25M; $MgCl_2$, 5mM; KH_2PO_4-K_2HPO_4 buffer, 10mM; Tris-HCl buffer, 10mM, pH 7.2; substrates alpha keto-glutarate, citrate, fumarate, malate, pyruvate, succinate, all 16mM; and NADH, 1mM. Also bovine serum albumin, 3mg/ml, and yeast extract, 2mg/ml, were added immediately prior to assay. The latter was used as a source of co-factors such as ATP, NAD, thiamine pyrophosphate and Co A (28). The reaction mixture was maintained at a pH of 7.2 and the temperature at 28°C throughout, and the reaction times were held to a maximum of 5 min in order to reduce errors arising from the diffusion of O_2 from the air into the reaction medium as respiration progressed.

C. Measurement of Respiratory Control Ratios.

The reaction medium was placed in the cuvette followed by substrate and a base-line established. Between 1 and 2 mg of mitochondrial protein was added to start the reaction. Once the initial rate was established 0.38 umoles of cytochrome-c and 0.3 umoles of ADP were added at intervals and the respiratory control ratio was calculated as shown in

TISSUE GRATED INTO ISOLATION MEDIUM,
STRAINED THROUGH 4 LAYERS OF CHEESECLOTH

DISCARD PULP

FILTRATE
Strained through nylon fabric
(Ca 50u mesh)

DISCARD PRECIPITATE

FILTRATE
Centrifuge: 37,000g for 10 min

DISCARD SUPERNATANT

Suspend pellet in wash medium
Centrifuge: 1,000g for 10 min

DISCARD PELLET

SUPERNATANT
Centrifuge: 37,000g for 10 min

DISCARD SUPERNATANT

MITOCHONDRIAL PELLET
Suspend in 0.2M sucrose

Figure 3. Flow-chart for the isolation of mitochondria from apple tissue.

Appendix Figure 2. Respiratory control ratios were established at the start, half-way through and at the termination of the experiment.

D. Measurement of Oxidation Rates.

This procedure was the same as described previously except that 0.38 umoles cytochrome-c and 0.3 umoles ADP were added initially instead of at intervals during the reaction. The reaction was allowed to continue until 0.10 umoles of O_2 had been used up. Reaction time was always less than 5 min. This was especially important when CO_2-bicarbonate mixtures were being used in the incubation medium, because keeping the reaction time to a minimum reduced the possibility of error due to the escape of CO_2 from the cuvette to the atmosphere.

For the experiments involving the effects of CO_2-bicarbonate mixtures on the oxidation rates of mitochondrial extracts, control rates were first established using reaction medium and substrate alone. Separate traces were then made using various concentrations of CO_2-bicarbonate (Na-salt) complemented by traces replacing the $NaHCO_3$ with the equivalent amount of NaCl to give equimolar concentrations of Na^+ ions. Bendall et al. (4, 5) and Bown et al. (10) had shown that there was also a non-specific "salt effect" on the mitochondria. This was manifested as a slight stimulation at low concentrations of NaCl and an inhibition at higher concentrations.

E. Regulation of CO2 Concentrations.

Aqueous solutions (25 ml) of $NaHCO_3$ were prepared at molar concentrations calculated on the basis of the Henderson-Hasselbach equation to adjust to pH 7.20 at 28°C when in equilibrium with 3%, 6%, 12%, and 18% CO_2 (80). This is presented graphically in Appendix Figure 3 and sample calculations are shown in Appendix Table 1. CO_2 gas was slowly bubbled through the solutions for approximately 10 min until the pH was maintained at 7.20 on a Corning Model 10 expanded-scale pH meter. Once the solutions had reached equilibrium they were immediately transferred to 10 ml volumetric flasks which were filled to overflowing and the ground glass stoppers replaced. These mixtures were then pipetted into the cuvette of the Oxygraph and the assay run to completion. This method gave highly reproducible results between duplicate assays and also between replicates. Changes in pH due to the loss of CO_2 from the medium were insignificant (less than 0.05 pH units at the 18% CO_2 level).

F. Organic Chemicals Used in These Studies.

Compound	Grade	Source
Citrate	-	Fischer Scientific Co.
Phenol reagent 2N	-	" " "
Yeast extract	Bio.Cert.	" " "
Sucrose	-	Baker
EDTA	-	"
Tris	-	Nutritional Biochem. Corp.
Alpha keto-glutarate	-	" " "
ADP (Na dihydrate)	-	" " "
L-malate	-	" " "
Oxalacetate	-	" " "
Succinate	-	Sigma

Compound	Grade	Source
PVP-40	pharmaceutical MW 40,000	Sigma
NADH di-sodium salt	B from yeast grade III	"
Cytochrome-c	horse-heart type III	"
Serum albumin (bovine)	fatty acid poor	Mann
Fumarate	-	Schwarz/Mann
L-cysteine HCl	-	" "

All inorganic chemicals were of analytical reagent grade and solutions were prepared in distilled-deionized water. The pH of all substrates was adjusted to 7.2 before use and the co-factors to near 7.0. All chemical reagents were prepared gravimetrically.

3. Assay of ATPase Activity.

Mitochondria were extracted from 'Delicious' apples and suspended in 10 ml sucrose, 0.2M, pH 7.5. One ml of mitochondrial preparation was incubated for 0, 2, or 5 min in the following reaction medium at 37°C: ATP, 50mM; NAD, 2mM; Nicotinamide, 40mM; $KHCO_3$, 75mM; $MgCl_2$, 30mM; and Tris-HCl, 40mM. Samples were continuously shaken in a Dubnoff-metabolic shaking incubator. Reaction was stopped with 0.5ml of 0.25M silicotungstic acid. A zero-time blank was treated the same way with mitochondria added immediately after the addition of silicotungstic acid. The precipitated protein was removed by centrifugation for 10 min in an IEC clinical centrifuge. A 1 ml aliquot of the silicotungstic acid extract

was then introduced into an extraction tube and to this was added 0.5 ml of 0.05M silicotungstic acid followed by 3.0 ml isobutanol-benzene (v/v) and 0.5 ml volumes of 0.28M H_2SO_4 and 1% ammonium molybdate. Tubes were then capped and mixed for 20 seconds on a vortex mixer, and the phases allowed to separate. 2 ml of the isobutanol-benzene layer was then removed and diluted to 5 ml with 3.2% (v/v) sulphuric acid in absolute ethanol. 0.5 ml of 0.5% $SnCl_2$ in 1.5M H_2SO_4 was then added and the absorbance read at 700 mu in a 1.0-cm cuvette.

4. Assay for Cytochrome-b1.

In order to confirm that the protein extracted was mainly of mitochondrial origin, an assay for cytochrome-b_1 was obtained. Mitochondria were extracted over a period of 2 weeks and frozen until the assay was initiated. Aliquots of 0.2 ml from the total extracts (3 ml) were pipetted into tubes of equal internal diameter. To each tube in turn was added 1mM NADH, 1.5 ug antimycin A and water to give a final volume of 4 ml. NADH served as substrate and antimycin A served to block the electron transport chain between cytochrome-b and cytochrome-c. The tubes were kept under oxidizing conditions and the absorbance measured in a Spectronic 20 spectrophotometer at 557.5 mu against a distilled water blank.

5. Protein Determination.

The determination of the protein content of the mitochondrial extract was used as an indication of the amount of mitochondria present. The Lowry method (51) of protein determination, slightly modified (Appendix Table 2) was found to be reliable and consistent in the determination of mitochondrial protein.

6. Comparison of Protein Concentration with Oxidation Rate.

Appendix Table 3 shows that generally the amount of protein present coincided closely with the rate of respiration obtained. However, in some cases a very high protein reading gave a very low reading for the respiration rate. Furthermore, total protein extracted increased with storage, probably due to the softening of the fruit and increased ease of extraction of the mitochondria. Also a reduction in starch content of the apples during storage probably reduced the amount of mitochondria trapped in it during sedimentation. Because of these anomalies the relative rates of respiration were recorded in 3 different manners. These are shown in Appendix Table 4. It can be seen from these values that there is a close relationship between the rates however determined.

R E S U L T S

In reporting the results of these experiments, various treatments will usually be referred to by the CO_2 concentration alone. Thus, $4\%CO_2$-$13\%O_2$ becomes $4\%CO_2$; $6\%CO_2$-$3\%O_2$ becomes $6\%CO_2$; and $12\%CO_2$-$3\%O_2$ becomes $12\%CO_2$. However, whenever any interaction seems involved the various treatments will be presented in full.

1. "CO2 Injury" to Apples.

During this investigation, the various concentrations of CO_2 and O_2 in the atmosphere had some marked effects on the stored apples. These effects consisted of the development of external and internal lesions, changes in fruit firmness, and changes in soluble solids content of the fruit. Less distinct effects included flavor changes and scald development. These atmospheric effects will be considered here separately and in detail.

A. Internal Lesion Development.

The classical symptom of "CO_2 injury" in apples shown in Figure 1 was readily observed in this experiment. It first became apparent in fruit from the $12\%CO_2$ treatment after 12 weeks storage (Table 1). Although only 10% of the fruits showed the disorder on removal from storage, after a subsequent 5-day holding period in air at 23°C, 60% of fruits were affected. In the majority of the cases the disorder at this time was just a mild browning around the core region. No lesions were observed in fruit from the other 2 treatments.

Table 1. Occurrence of internal "CO_2 injury" 1/ in apples.

Weeks storage at 0°C	Percentage of fruits 2/ with internal injury			
	Storage atmosphere			
	Air	4%CO2 13%O2	6%CO2 3%O2	12%CO2 3%O2
12	0 (0)3/	0 (0)	0 (0)	10 (60)
16	0 (0)	0 (0)	0 (0)	11 (95)
20 4/	0 (0)	0 (0)	85 (100)	90 (100)

1/ Typical appearance shown in Figure 1.

2/ Each sample consisted of 30 apples.

3/ Figures in parentheses are the percent occurrence after 5 days in 23°C air following removal from storage.

4/ Sixteen weeks in indicated atmosphere plus 4 weeks in air.

This disorder was initiated by the storage atmosphere but clearly it had the capacity to develop in ambient air.

After 16 weeks of storage results similar to the above were obtained, except that the 12%CO_2 atmosphere now produced injury in 95% of fruits after the 5-day holding period. However, after 16 weeks the damage was much more pronounced, with an intense browning around the core region (Figure 4) and in more severe cases, the first signs of tissue collapse (Figure 5).

After 20 weeks of storage (16 weeks of treatment followed by 4 weeks in air), 90% of the fruit from the 12%CO_2 atmosphere were affected immediately out of storage and this increased to 100% after the holding period. In addition, the majority of the fruits showed severe symptoms of the disorder (Figure 6). More importantly, lesions were now present in nearly all fruits that had been stored in 6%CO_2, although the damage was much less intense (Figure 7). There was, however, still no damage apparent in the fruit from the 4%CO_2 atmosphere.

The incidence and intensity of this internal form of the injury seemed to be directly related to the concentration of CO_2 in the atmosphere, and in the atmospheres tested this injury was not affected by O_2 concentration.

B. External Lesion Development.

Another manifestation of "CO_2 injury" was the formation of external lesions. The injury appeared as sunken necrotic

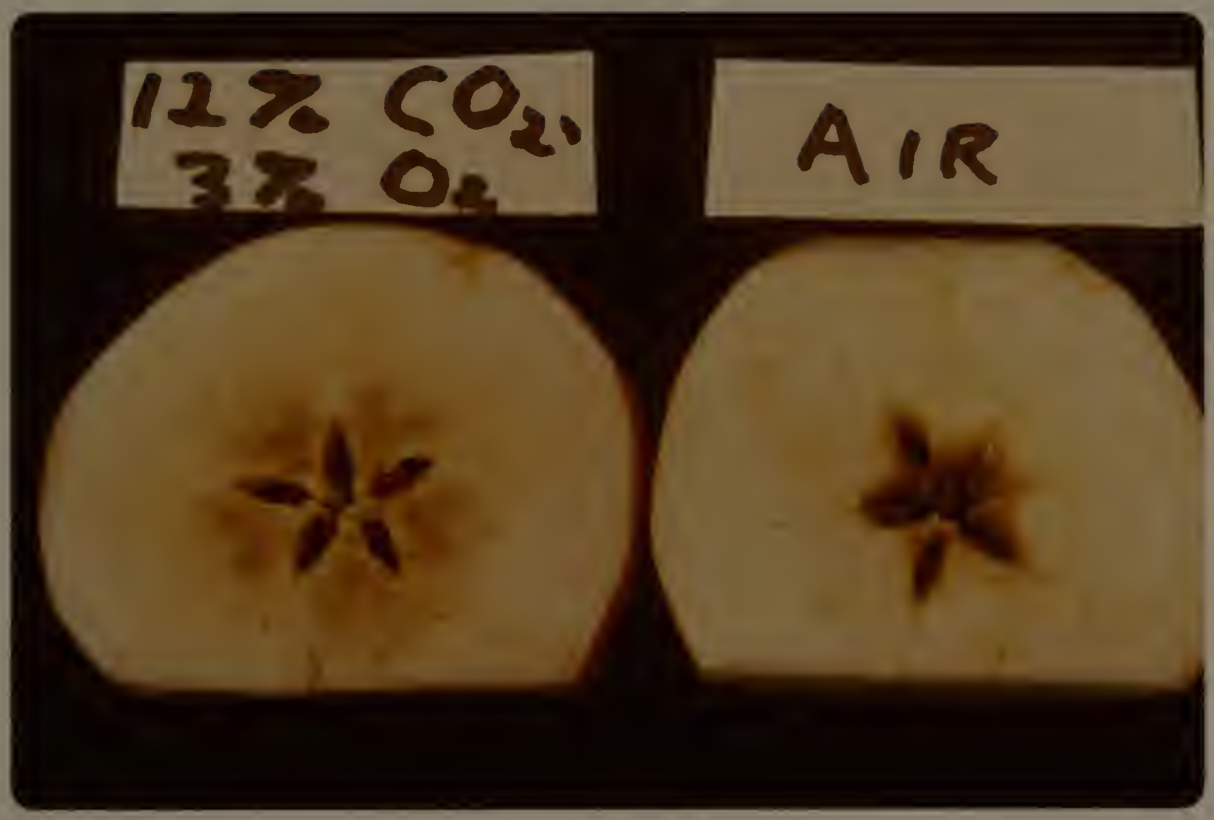

Figure 4. Characteristic symptoms of internal "CO2 injury" in apples stored in 12%CO2-3%O2 for 16 weeks then held for 5 days in 23°C air, compared with apples stored in air for the same period. Note intensity of browning in the core region.

Figure 5. Apple, stored in 12%CO2-3%O2 for 16 weeks then held for 5 days in 23°C air, showing the first signs of tissue collapse and cavity formation.

Figure 6. Apple stored in 12%CO2-3%O2 for 16 weeks followed by 4 weeks in 0°C air and 5 days in 23°C air. Fruit shows severe internal "CO2 injury" extending out from the central core region into outer cortex of the fruit.

Figure 7. Apples stored in the atmospheres shown for 16 weeks plus 4 weeks in air at 0°C and 5 days in 23°C air. The 12%CO2-3%O2 fruit shows severe "CO2 injury", and the 6%CO2-3%O2 fruit shows very slight injury.

areas around the lenticels in the skin of the fruit (Figure 8). Unlike the internal lesions, which have frequently been reported in the literature, these external symptoms have not been previously related to "CO_2 injury" on apples.

This disorder was first observed in the 12%CO_2-3%O_2 fruits at the end of 6 weeks storage and it was present on nearly all the fruits. Fruits from the other CO_2 atmospheres were not examined at this time. However, after 12 weeks of storage, all the treatments were assessed and it was found that the 4%CO_2-13%O_2 fruits were more severely affected than those from the 12%CO_2-3%O_2 atmosphere (Figures 9, 10, 11). Furthermore, the majority of the 4%CO_2-13%O_2 treated fruits showed a splitting of the skin around the stalk (pedicel) and calyx regions of the fruit (Figure 12). At this time, no injury of any type was apparent on fruit from the 6%CO_2-3%O_2 atmosphere. Those external lesions already present did not progress during the 5-day holding period, but the 6%CO_2-3%O_2 fruits showed faint signs of necrosis around the lenticels at the calyx end of the fruit after 5 days at 23°C, indicating formation of new lesions after transfer to air. However, these were not sunken as in the other 2 treatments.

During the rest of the storage period there was no further increase in the intensity of the symptoms except on the 6%CO_2-3%O_2 fruits, where the necrotic areas eventually showed some signs of a sunken appearance (Figures 13, 14). Scald, which had developed during storage, tended to mask the

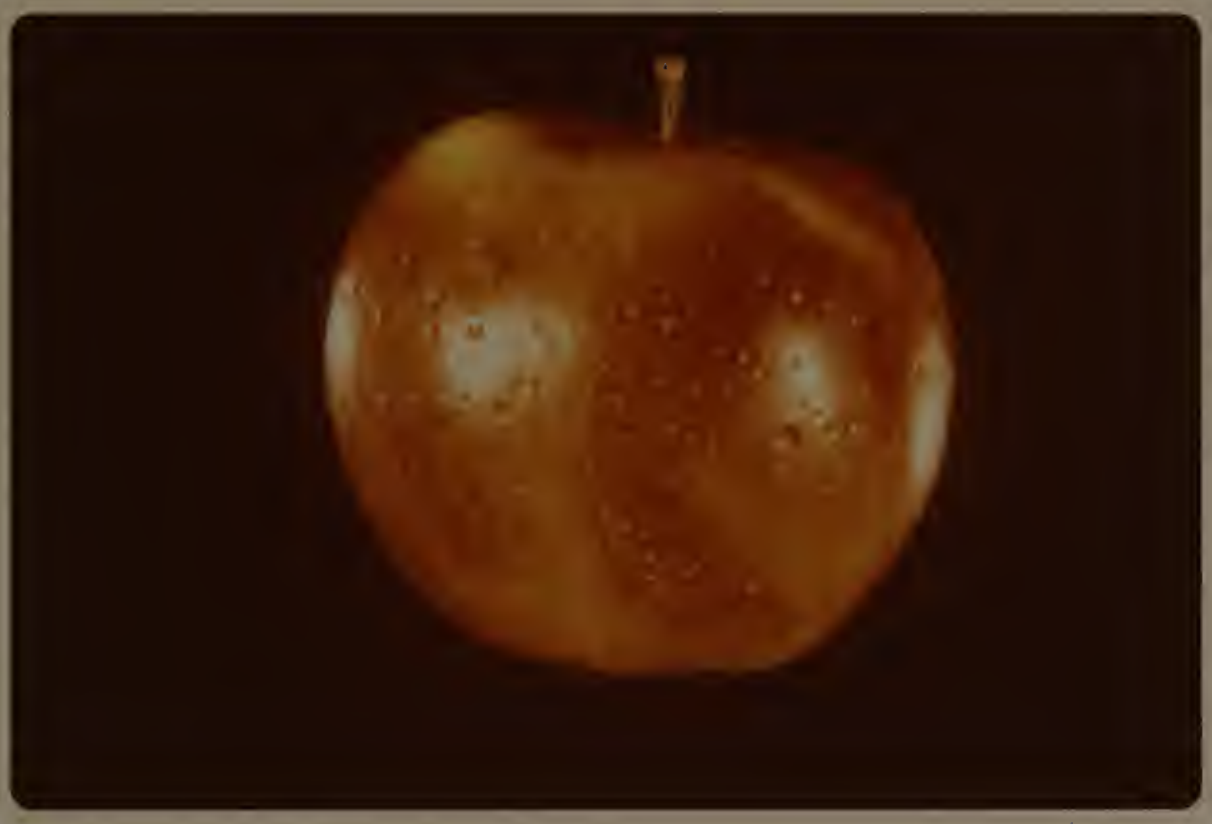

Figure 8. Severe symptoms of external "CO2 injury". Note the sunken necrotic areas around the lenticels in the skin of the fruit.

Figure 9. Apples stored for 12 weeks in the various atmospheres shown. Fruits show the intensity of external "CO2 injury" at this stage. The 4%CO2-13%O2 fruit shows the most severe damage followed by the 12%CO2 apple. No damage was apparent from the other 2 atmospheres.

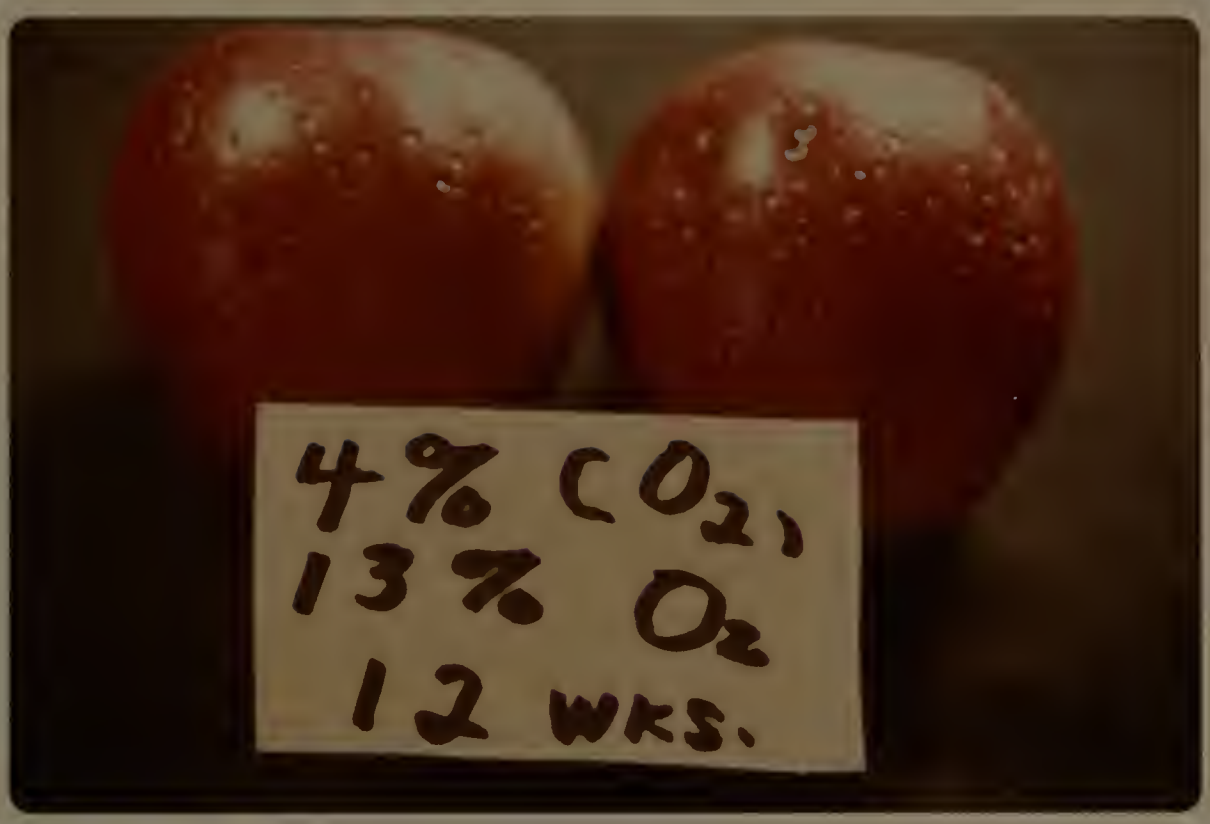

Figure 10. Apples showing the intensity of the external symptoms of "CO2 injury" after storage in 4%CO2-13%O2 for 12 weeks at 0°C. Compare with Figure 11.

Figure 11. Apple showing the intensity of the external symptoms of "CO2 injury" after storage in 12%CO2-3%O2 for 12 weeks at 0°C. Compare with Figure 10.

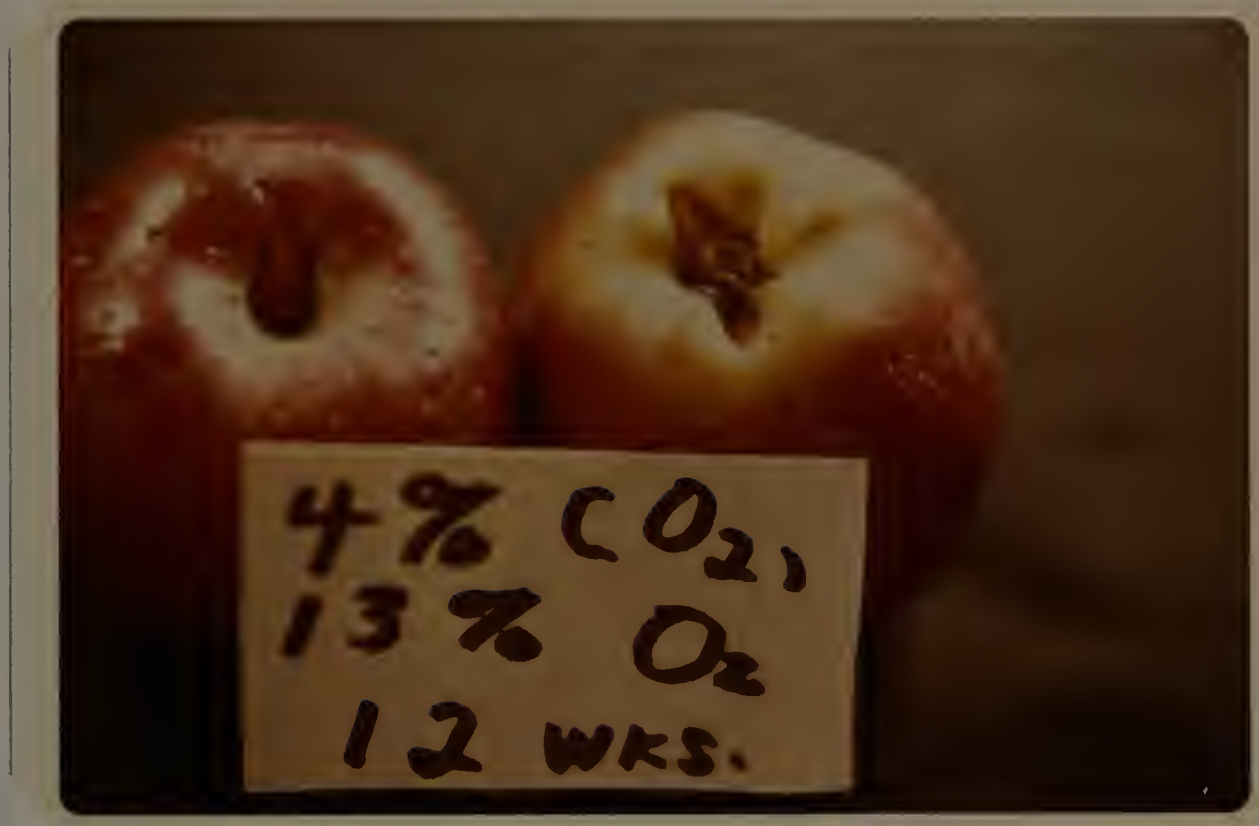

Figure 12. Apples showing splitting of the skin around the calyx and stalk cavities of fruit stored in 4%CO2-13%O2 for 12 weeks at 0°C.

external effects of "CO_2 injury" during the later stages of the experiment (Figure 13).

Not only did the $4\%CO_2$-$13\%O_2$ fruits show more external damage, but they were also much more susceptible to secondary fungal infection through this injury, especially around the cracks in the calyx and stalk regions.

C. Effect of Fruit Size on the Development of Internal "CO2 Injury".

There is evidence that fruit size affects the incidence and intensity of internal "CO_2 injury" (11). Observations early in this experiment indicated that fruit size was a factor influencing the findings. This was verified when, at the end of the experiment, fruits were separated by size and examined for internal "CO_2 injury" (Table 2). The larger fruits not only had higher incidence of injury, but also were more severely affected. Air-stored fruits were free of the lesions, as were the smaller fruits from the $4\%CO_2$ atmosphere. However, some of the larger fruits from $4\%CO_2$ showed signs of injury, showing that even $4\%CO_2$ was a damaging concentration under some conditions.

The effect of size was, nevertheless, most striking in the higher CO_2 treatments. In the $6\%CO_2$ apples twice as many of the larger fruits were affected with "CO_2 injury" as the smaller ones, and at the same time more fruits appeared with moderate and severe injury. In the $12\%CO_2$ apples a similar effect was observed. However, since most of the fruits were

Figure 13. Apples stored in the atmospheres shown for 16 weeks at 0°C. Note the first signs of "CO2 injury" in the 6%CO2-3%O2 stored fruits. Furthermore, note the masking effect of scald on the symptoms of "CO2 injury".

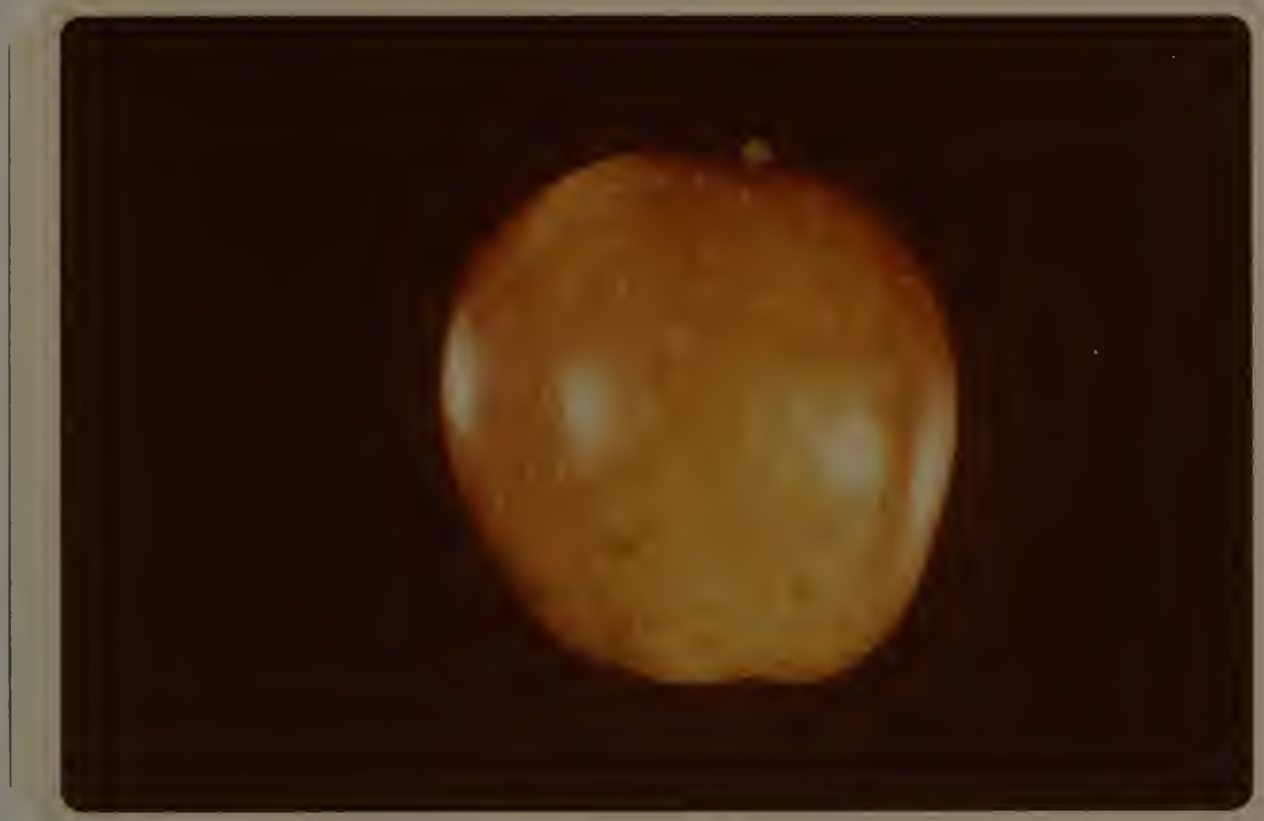

Figure 14. Apple stored in 6%CO2-3%O2 for 16 weeks plus 4 weeks in air at 0°C. Note signs of sunken necrotic areas around the lenticels at the calyx end of the fruit.

Table 2. Effect of fruit size on occurrence of internal "CO2 injury" in apples.

Storage 1/ atmosphere	Fruit diameter (inches)	Percentage of fruits with "CO2 injury", rated on intensity of disorder			
		None	Slt.2/	Mod.	Sev.
Air	< 2 3/4	100	0	0	0
Air	> 2 3/4	100	0	0	0
4%CO_2-13%O_2	< 2 3/4	100	0	0	0
4%CO_2-13%O_2	> 2 3/4	92	5	2	1
6%CO_2-3%O_2	< 2 3/4	61	26	11	2
6%CO_2-3%O_2	> 2 3/4	21	33	31	15
12%CO_2-3%O_2	< 2 3/4	14	30	27	29
12%CO_2-3%O_2	> 2 3/4	1	9	28	62

1/ Stored for 16 weeks at 0°C in indicated treatment plus 4 weeks in 0°C air plus 5 days in 23°C air.

2/ Subjective categorization of intensity, described in Materials and Methods.

affected in both size categories, it was mainly an effect on intensity of occurrence. Whereas in the smaller fruits the numbers of apples with various degrees of injury were evenly spaced over the 3 categories, in the larger fruits the majority were judged as having moderate and severe damage.

It is also important to note that at this stage a large number of the fruits in the severe category had developed a form of internal breakdown. Although it was not possible to completely distinguish between severe "CO_2 injury" and breakdown, there is no doubt that breakdown occurrence was directly related to CO_2 levels since breakdown did not occur in fruits from the air treatment.

D. Effect on Fruit Flesh Firmness.

During storage the 4%CO_2-13%O_2 fruits softened at a faster rate than those in the other atmospheres (Table 3). After 20 weeks they were, on average, 1 lb softer than those held in air, 6%CO_2, or 12%CO_2, among which there was no difference.

During the 5-day holding period in 23°C air the difference between those stored in 4%CO_2 and those stored in air increased markedly, with a 3 lb difference being recorded after 16 and 20 weeks of storage. However, dramatic rates of softening also occurred during this time in fruits that had been stored in 12%CO_2-3%O_2, so that these fruits were eventually as soft as the ones from the 4%CO_2-13%O_2 treatment. There appeared to be little or no effect of 6%CO_2-3%O_2 on softening during or

Table 3. Firmness (lbs pressure) of apples following storage in different atmospheres.

Weeks storage at 0°C	Storage atmosphere			
	Air	4%CO2 13%O2	6%CO2 3%O2	12%CO2 3%O2
12	12.3 (12.4)[1/]	12.7 (11.7)	14.0 (12.7)	13.5 (9.4)
16	13.7 (12.9)	11.3 (9.9)	13.2 (13.2)	14.0 (11.0)
20[2/]	12.7 (12.9)	11.8 (9.9)	12.9 (11.6)	12.8 (10.1)

1/ Figures in parentheses are the values for fruit held 5 days in 23°C air following removal from storage.

2/ Sixteen weeks in designated atmosphere plus 4 weeks in air.

following storage.

The data suggest that high CO_2 concentrations accelerated softening, but also that an interaction between O_2 and CO_2 was involved in this effect, since 4%CO_2 plus 13%O_2 was at least as effective as 12%CO_2 plus 3%O_2 in producing this response.

E. Effect on Total Soluble Solids.

The measurement of total soluble solids is essentially an assessment of the amount of sugars present within the fruit. The results of soluble solids determinations during the experiment (Table 4) showed that with increasing levels of CO_2, sugars were depleted at an increasingly faster rate than in air. After 20 weeks in storage the 4%CO_2, 6%CO_2, and 12%CO_2 treatments showed a reduction in sugars of 5%, 9% and 20%, respectively, in comparison with air-stored fruit. During the 5-day holding period there was a further depletion in these levels but the differences were essentially the same, namely, 2.5%, 8%, and 21% of the air values. The atmospheric effect seen here appeared attributable solely to CO_2 concentrations, since no O_2 interaction was evident.

F. Effects on Scald Development and Flavor.

Scald, although varying in intensity, developed on fruit from all atmospheres during and following storage, with the highest levels being observed on fruit from the air and 12%CO_2 atmospheres (Table 5). Fruits from the 4%CO_2 and 6%CO_2 treatments seemed to be much less affected by the disorder and, in

Table 4. Percentage soluble solids of apples following storage in different atmospheres.

Weeks storage at 0°C	Storage atmosphere			
	Air	4%CO2 13%O2	6%CO2 3%O2	12%CO2 3%O2
12	14.0 (14.0)[1/]	13.5 (12.9)	14.0 (13.7)	13.0 (12.1)
16	13.0 (12.8)	12.7 (11.9)	12.2 (11.8)	12.0 (11.7)
20[2/]	13.3 (12.5)	12.6 (12.2)	12.1 (11.5)	10.3 (9.9)

1/ Figures in parentheses are the values for fruit held 5 days in 23°C air following removal from storage.

2/ Sixteen weeks in designated atmosphere plus 4 weeks in air.

addition, were affected later in the storage period. There was some indication that 12%CO_2 accelerated the rate of development of this disorder.

Flavor of fruits was assessed at all examination times, and never was there an indication of "off-flavor" that might be associated with fermentation. Conversely, neither was there a definite suppression of flavor by any of the storage atmospheres, although in fact even the air-stored fruits lacked a pleasing flavor probably due to the harvesting at an early stage of maturity.

Table 5. Percentage of fruits exhibiting scald following storage of apples at 0°C.

Storage atmosphere	Weeks in storage		
	12	16	20[1]/
	Percentage scald		
Air	21[2]/	24	68
4%CO_2-13%O_2	0	26	39
6%CO_2-3%O_2	0	26	40
12%CO_2-3%O_2	24	59	74

1/ Stored for 16 weeks in indicated atmosphere plus 4 weeks in air.

2/ Before assessment all fruits were held for 5 days at 23°C following removal from storage.

2. Activity of Isolated Mitochondria.

The degree of mitochondrial integrity was assessed using the parameters outlined previously. Following the terminology of Chance and Williams (12) the ADP-stimulated rate of respiration will be referred to as state 3 oxidation and the rate of respiration in the absence of ADP will be referred to as state 4 oxidation. Under this interpretation the respiratory control ratio is defined as the state 3 rate divided by the state 4 rate.

A. Effect of Increasing Amount of Mitochondrial Suspension.

Figure 15 shows the increase in state 3 rate of oxygen utilization with increasing amounts of mitochondrial suspension. The rates are approximately doubled and trebled with similar increments in the amount of mitochondria added. This demonstrates the homogeneity of the mitochondrial preparation. Because of the high rate of activity for the 0.09 ml of preparation, and the linearity of response for the utilization of the first 0.10 umoles of O_2, this volume of extract was used in all succeeding assays. This amount of suspension contained between 1-2 mg protein.

It is noteworthy that the oxidation rate of the mitochondria was unaffected by the O_2 concentration to almost zero O_2 in the reaction mixture, as seen in Figure 15.

B. Effect of Added Cytochrome-c, ADP and DNP.

Under state 4 conditions the addition of 0.38 umoles cytochrome-c produced a negligible increase in the utilization of

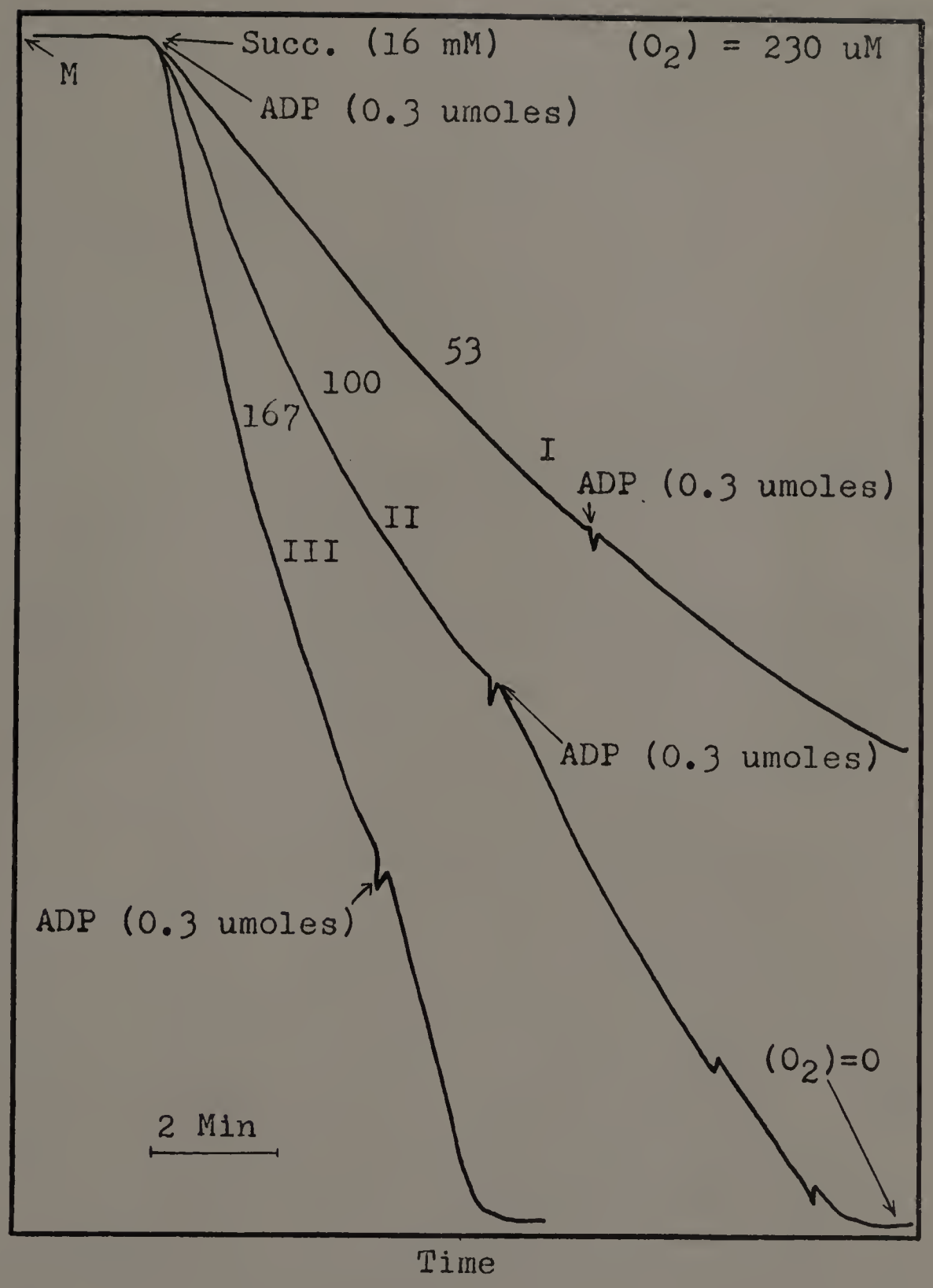

Figure 15. Polarograph traces showing the influence of increased amounts of mitochondrial suspension (M) on the rate of oxygen utilization, succinate as substrate. Assayed in basic reaction medium described in Materials and Methods with repeated additions of 0.3 umoles ADP as indicated. The numbers on the traces represent the decrease in mug atoms O2/ min. Roman numerals indicate: I, 0.03 ml of suspension; II, 0.06 ml suspension, and III, 0.09 ml suspension.

oxygen (Figure 16). With the addition of ADP to the reaction medium a state 4-to-state 3 transition was obtained, thus demonstrating respiratory control, which provided an indication of the structural integrity of the mitochondria. Furthermore, the addition of 2, 4, dinitro-phenol (DNP) brought a stimulation of oxygen utilization indicating an uncoupling effect on oxidative phosphorylation (Figure 17).

Preliminary work in developing the mitochondrial extraction procedure showed that with the procedure utilized, high rates of succinate oxidation and high respiratory control values were obtained with mitochondria obtained from several apple cultivars other than 'Richared Delicious' (Appendix Table 5).

C. "Aging" Property of the Mitochondria.

It was extremely important for the mitochondria to maintain the level of respiratory activity and respiratory control over the whole period of the assay. Any marked increase or decrease in these levels during this period would prevent any meaningful conclusions from being drawn. Furthermore, any rapid decline in activity or respiratory control would signify probable damage to the mitochondria.

Throughout the assay period the mitochondria were maintained in an ice bath and were occasionally shaken gently. The results presented in Figures 18 and 19 show that there were no marked changes in either oxidation or respiratory control ratios during the 3-hour standing period.

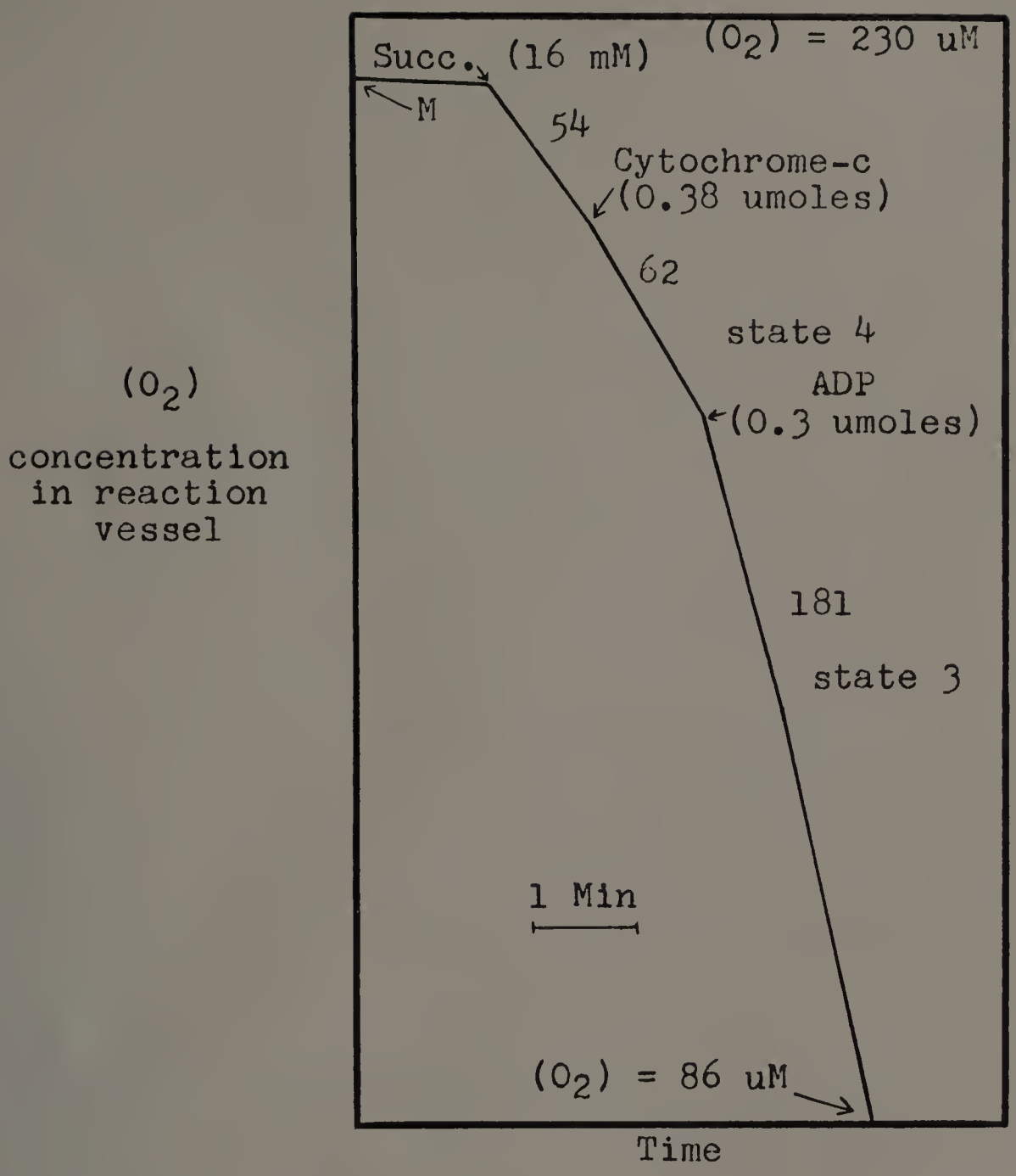

Figure 16. Polarograph trace showing the influence of adding 0.38 umoles cytochrome-c and 0.3 umoles ADP on the rate of oxygen utilization, succinate as substrate. Assayed in basic reaction medium described in Materials and Methods. The numbers on the traces represent the decrease in mug atoms O2/min; 0.09 ml of mitochondrial suspension (M).

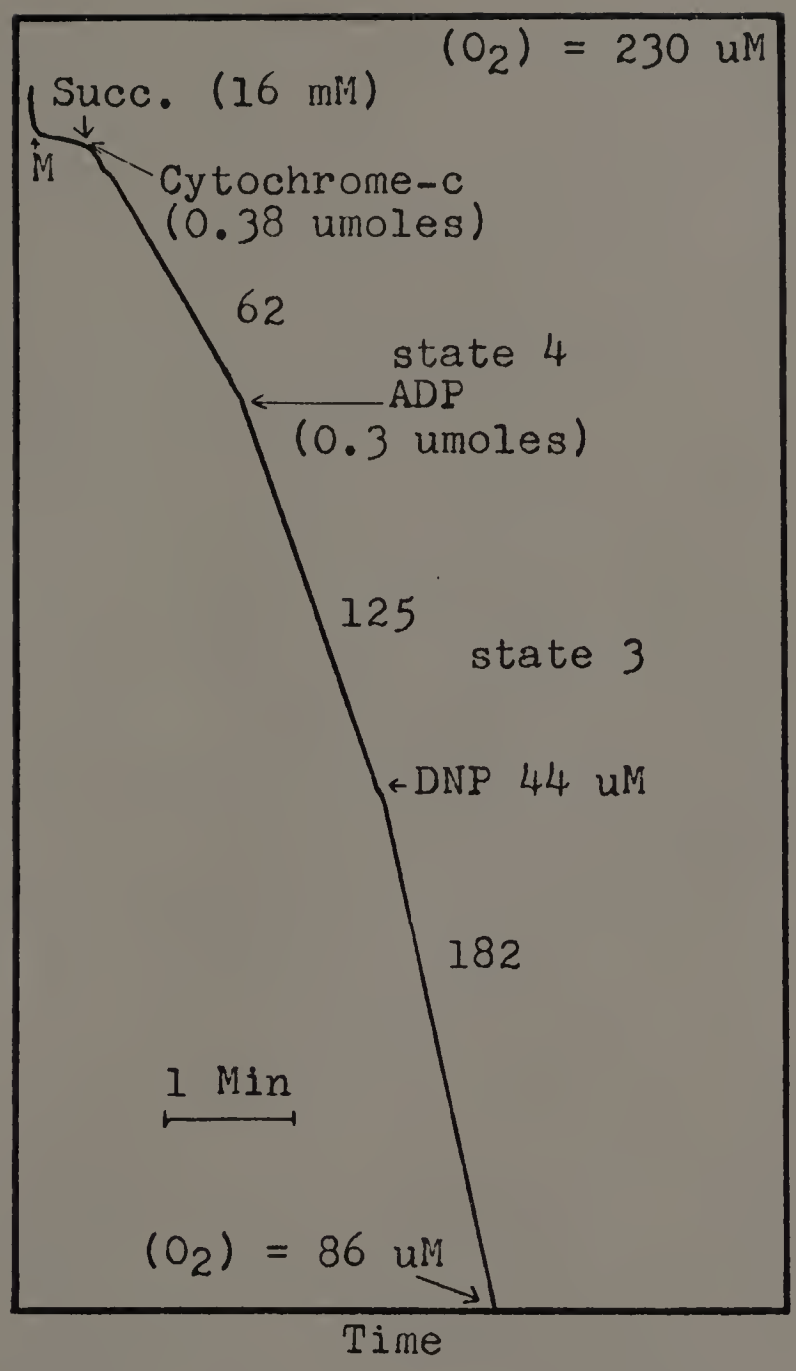

Figure 17. Polarograph trace showing the influence of adding 44 uM DNP on the rate of oxygen utilization, succinate as substrate. Assayed in basic reaction medium described in Materials and Methods. The numbers on the traces represent the decrease in mug atoms O2/min-0.09 ml of mitochondrial preparation (M).

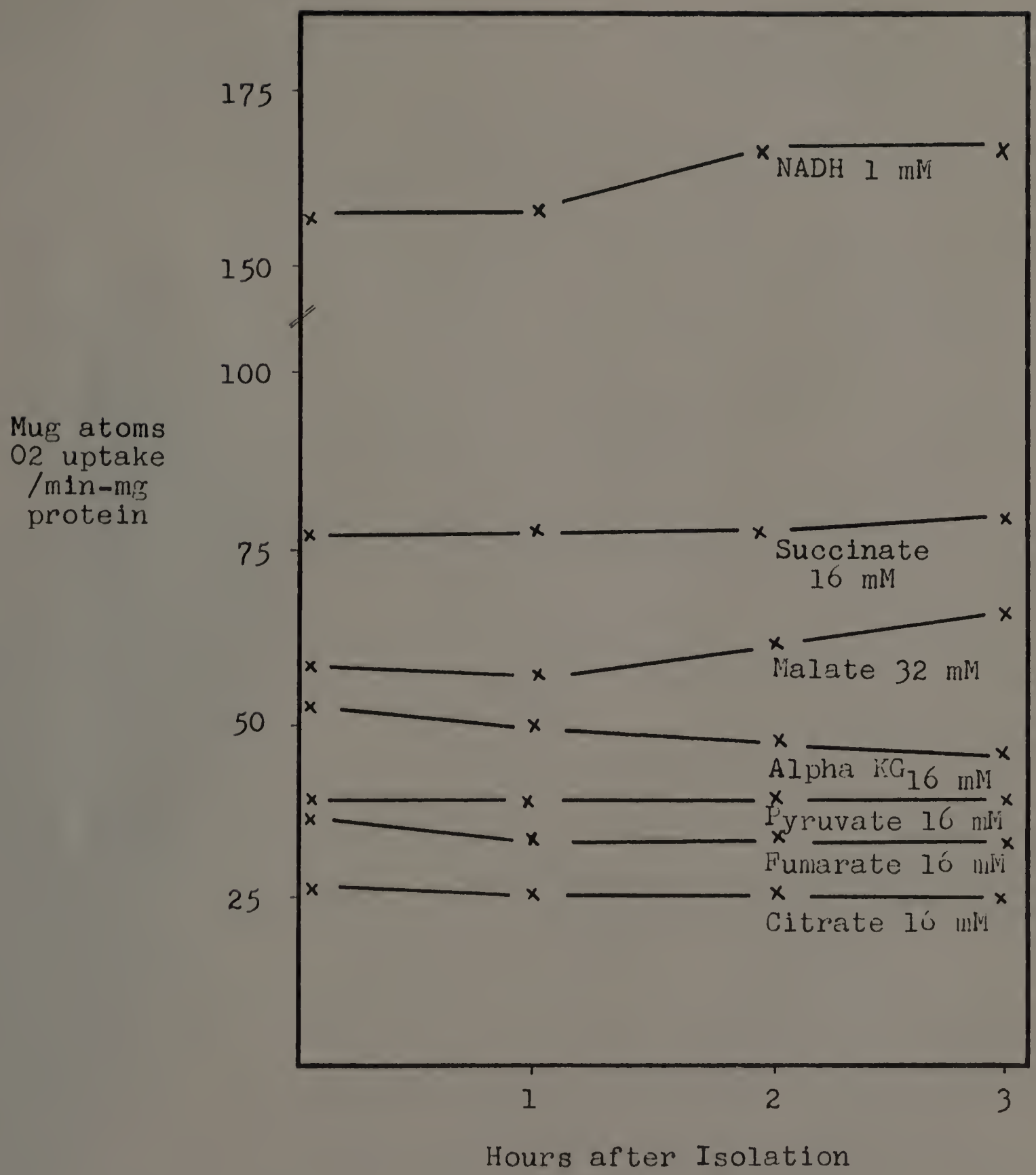

Figure 18. Effect of time on activity of mitochondria assayed in basic reaction medium described in Materials and Methods, plus 0.3 umoles ADP and 0.38 umoles cytochrome-c.

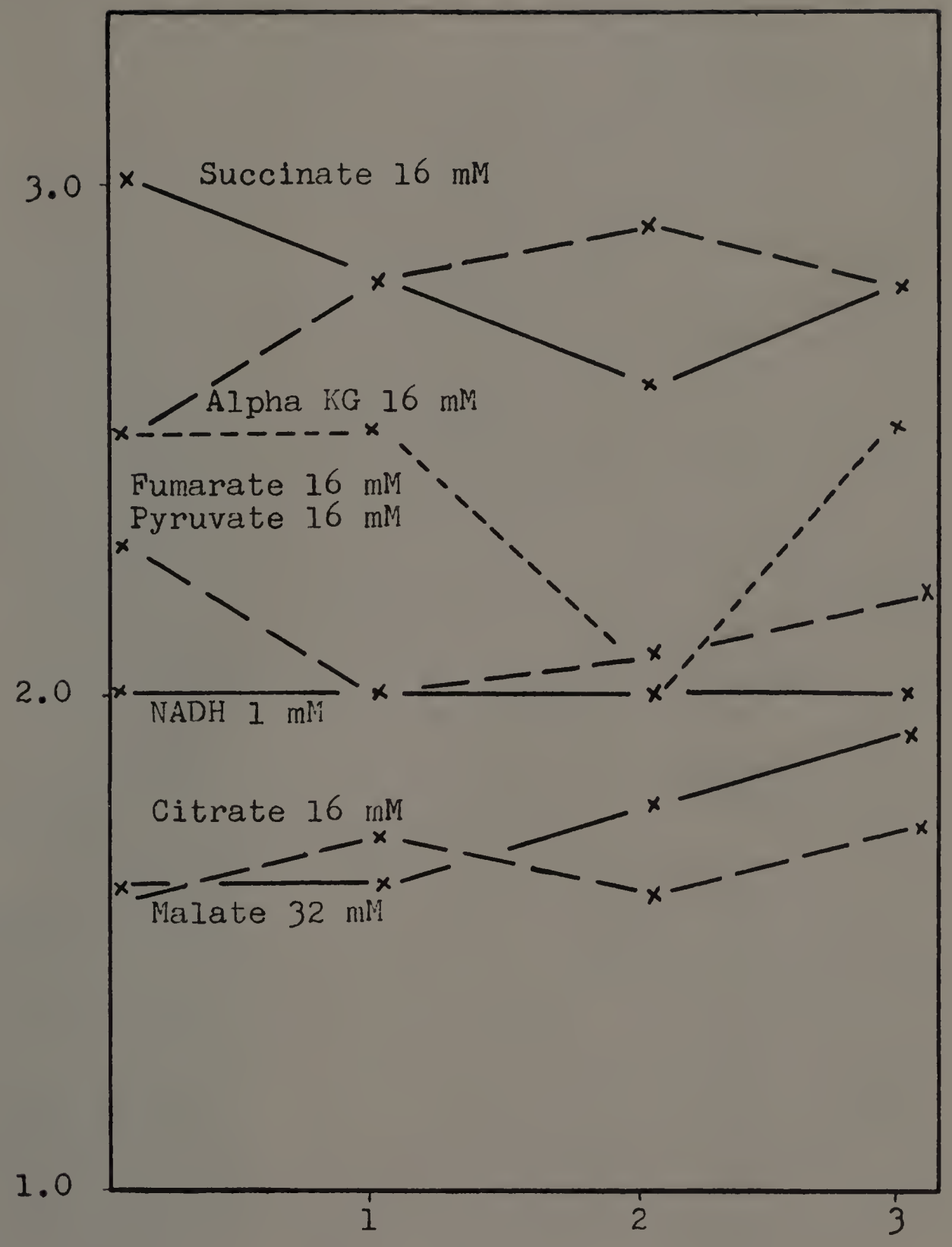

Figure 19. Effect of time on RCR of mitochondria assayed in basic reaction medium described in Materials and Methods, plus 0.3 umoles ADP and 0.38 umoles cytochrome-c.

D. ATPase Activity of the Mitochondria.

The ATPase activity of the mitochondria is presented in Figure 20 and Table 6. This assay was carried out at the end of the storage season when the mitochondria had lost a considerable proportion of their activity and respiratory control. This relatively low ATPase activity of the mitochondrial preparations is additional indication of extraction of relatively intact mitochondria.

E. Effect of pH of Assay Medium.

To determine the optimal pH for the assay medium when using succinate as substrate assays were carried out between pH 6.6 and 7.4. Figure 21 shows that there was a broad pH optimum over the range 6.8 to 7.2 for state 3 respiratory activity.

F. Rates of Oxygen Utilization and Respiratory Control.

The mitochondria obtained were capable of oxidizing all the citric acid cycle acids tested and NADH to a greater or lesser degree. The state 3 oxidation rates shown in Table 7 were highly reproducible, as can be seen from the small standard errors shown after the various rates. Consistency was also apparent with the respiratory control ratios. Furthermore, in the majority of cases state 4 respiration was at least doubled, with the addition of ADP, suggesting a reasonably high degree of coupling. Although these ratios are not as high as those reported for some other plant tissues (32, 67, 87) they are nevertheless equal to, or in the case of

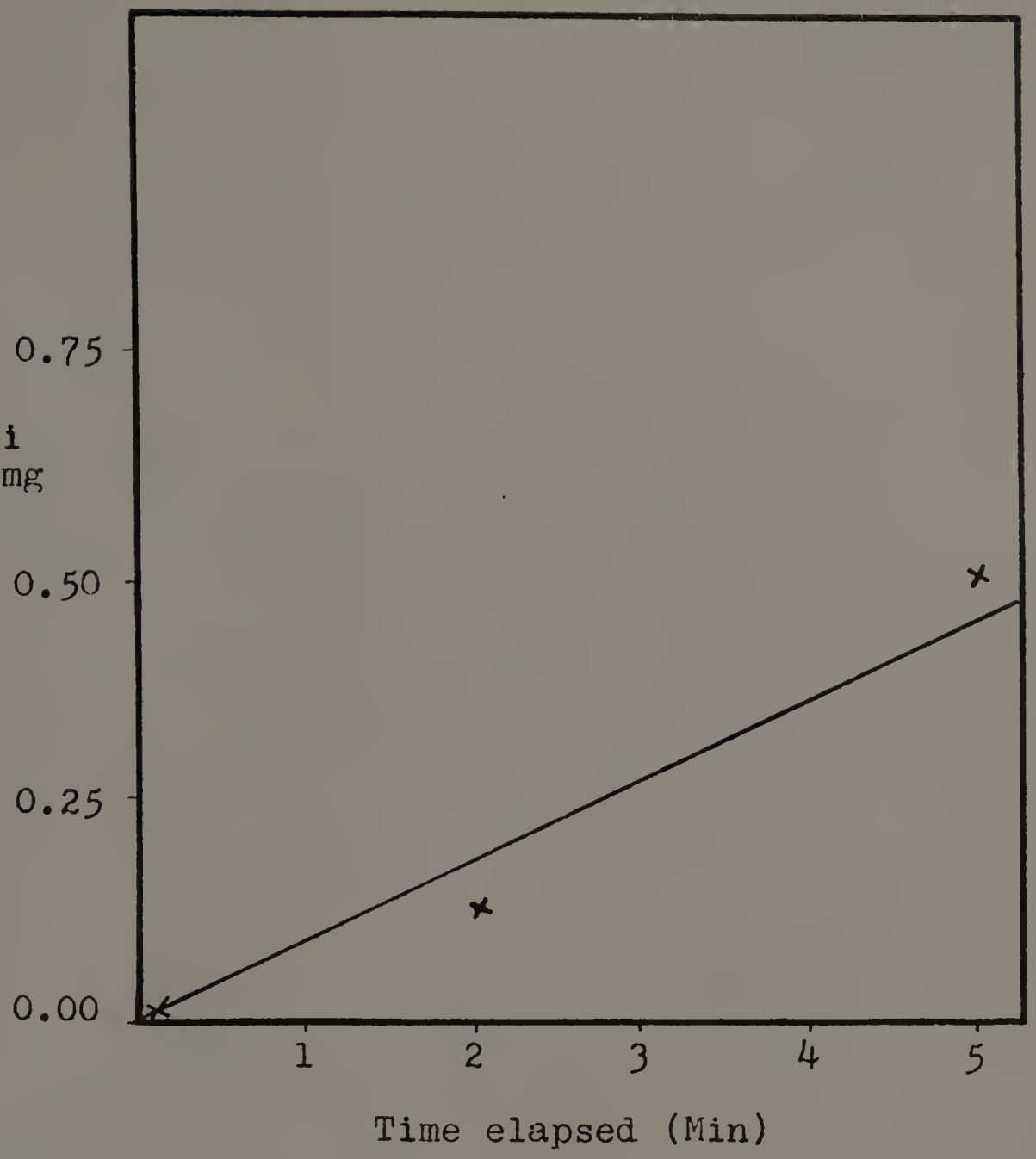

Figure 20. ATPase activity of mitochondria isolated from 'Delicious' apples, expressed as umoles Pi/mg protein. Assay was carried out by the procedure outlined in Materials and Methods.

Table 6. ATPase activity of mitochondria isolated from 'Delicious' apples and assayed by the procedure outlined in Materials and Methods.

Time (min)	Experiment	Activity[1]	Specific Activity[2]
0	1 2	0	0
2	1	1.20	0.15
	2	1.45	0.18
5	1	4.40	0.55
	2	3.45	0.45

1/ Activity = umoles Pi released/ml mitochondrial suspension.

2/ Specific activity = umoles Pi released/mg protein during time stated.

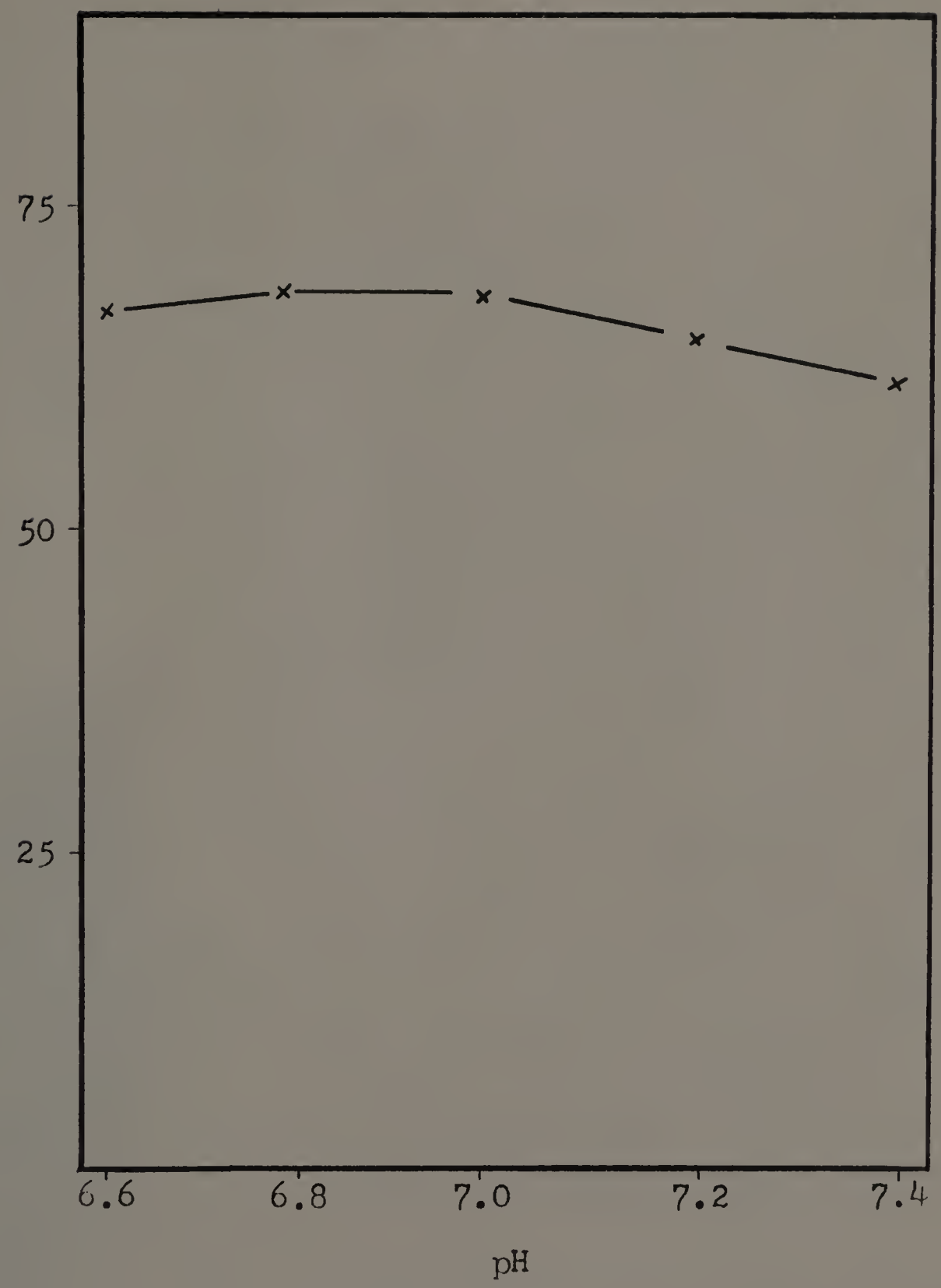

Figure 21. Effect of pH of assay medium on the rate of oxygen utilization, succinate as substrate, for mitochondria isolated from 'Delicious' apple tissue and assayed in basic reaction medium described in Materials and Methods plus 0.3 umoles ADP and 0.38 umoles cytochrome-c.

Table 7. Oxidative phosphorylation of mitochondria isolated from 'Delicious' apples. Assayed in basic reaction medium plus 0.3 umoles ADP and 0.38 umoles cytochrome-c, for the various substrates shown.

Substrate	Rate of O2 uptake (mug atoms /min-mg protein)	Respiratory control ratio
Citrate	25 (±3)	1.7 (±0.0)
Fumarate	35 (±1)	2.4 (±0.3)
Pyruvate	40 (±6)	2.2 (±0.1)
Alpha keto-glutarate	48 (±3)	2.8 (±0.3)
Malate	59 (±4)	1.7 (±0.1)
Succinate	77 (±0)	2.8 (±0.2)
NADH	162 (±15)	2.0 (±0.0)

succinate higher than, those recorded to date for apple fruits (30,85).

When testing the capacity of the extracted mitochondria to respire the various citric acid cycle acids, oxalacetate was used as a substrate but because of its rather rapid decarboxylation to pyruvate, this substrate was omitted from the final assays. Possible evidence that decarboxylation was occurring is shown in Appendix Table 6.

3. Effects of CO2 on Mitochondrial Metabolism.

A. Effect on the Oxidation of Various Substrates.

The first objective was to determine the effect of increasing levels of CO2 on the mitochondrial oxidation of various citric acid cycle acids. Only fruits that had been stored in air were used for these experiments. The effects of various CO_2-bicarbonate mixtures were determined polarographically using the technique described earlier. These CO_2 effects are presented as a percentage of control respiration after allowing for any "salt effect", which was approximately a 10% inhibition at the 18%CO_2-bicarbonate level and 0% inhibition at the 3%CO_2-bicarbonate level.

Figure 22 shows the effect of increasing levels of CO_2 on the oxidations of malate, citrate, alpha keto-glutarate and NADH. Two distinct responses were observed. The oxidation of malate was significantly stimulated by 6%, 12% and 18%CO_2. The stimulation varied between 5% and 10% but there was no significant difference among these 3 concentrations of CO_2,

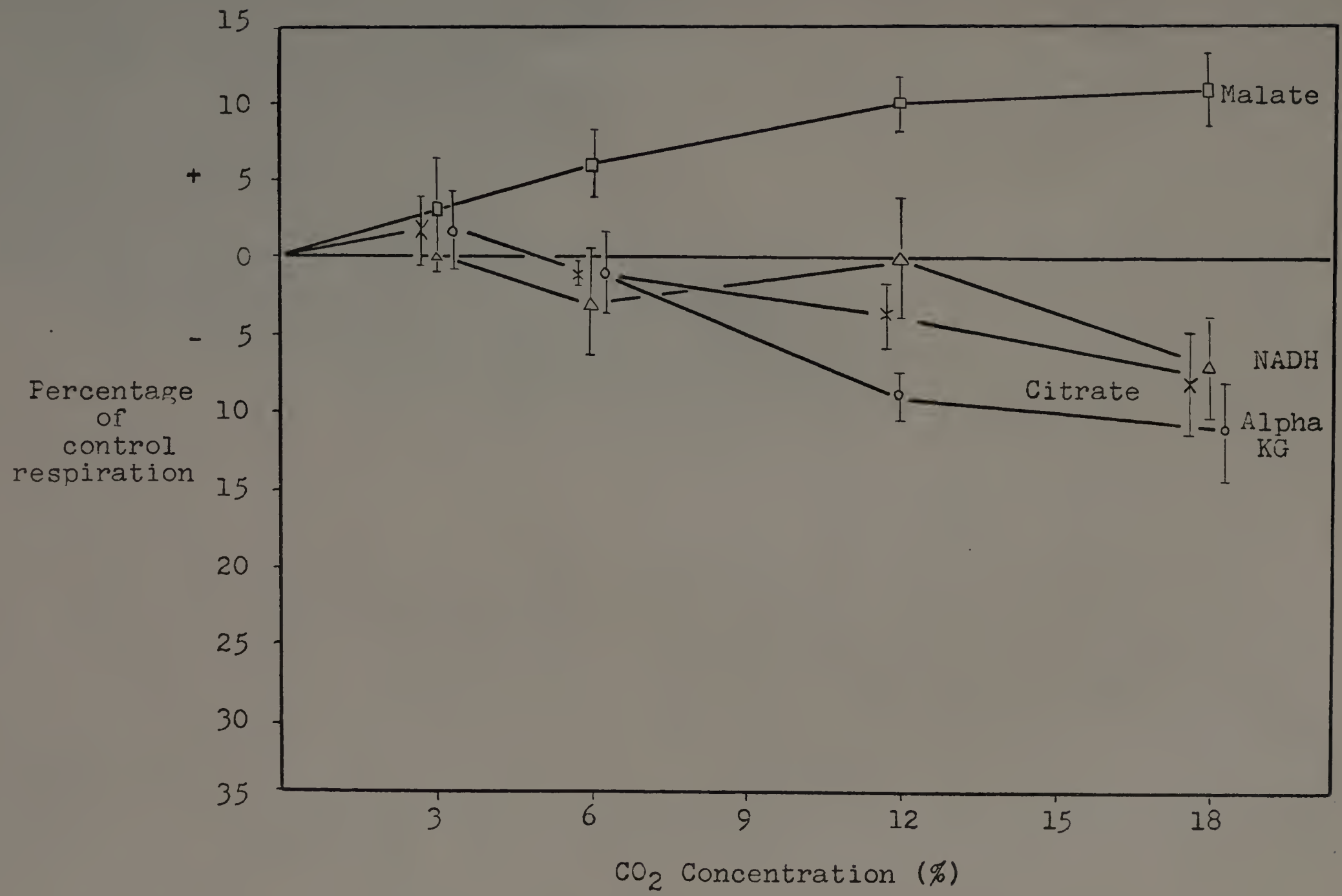

Figure 22. Effect of CO2-HCO3$^-$ mixtures on O2 uptake of mitochondria extracted from apples stored in 0°C air and assayed in basic reaction medium plus 0.3 umoles ADP. Substrate concentration: malate 32 mM: citrate and alpha KG 16 mM and NADH 1 mM.

suggesting a saturation of this response at a very low CO_2 concentration.

In contrast to the effect of CO_2 on malate oxidation, the oxidations of alpha keto-glutarate, citrate, and NADH showed a significant inhibition at the 18%CO_2 level approximating 5% to 10%. Furthermore, alpha keto-glutarate and citrate were about equally inhibited by both 12% and 18% CO_2.

Figure 23 shows a third type of response of acid oxidation to increasing CO_2 concentrations. The oxidations of succinate, fumarate, and pyruvate were markedly suppressed by increasing levels of CO_2. An almost linear response between 3%CO_2 and 18%CO_2 was observed, increasing to about a 30% inhibition at the 18%CO_2 level for all substrates. This inhibition was statistically significant at all levels above 6%CO_2 and for all 3 substrates. Furthermore, the extreme sensitivity of succinate oxidation to CO_2 was shown by the significant inhibition even at the 3%CO_2 level.

B. Changes in Succinate and NADH Oxidation during Storage.

The second objective was to determine whether the capacity of the mitochondria to respire added succinate and NADH changed during storage, and furthermore whether this was affected in any way by increased levels of CO_2 in the storage atmosphere. For these assessments fruit was stored for up to 20 weeks in the various atmospheres and in air as a control. Succinate and NADH were chosen as substrates because of their relatively high initial rates of oxidation. Table 8 shows

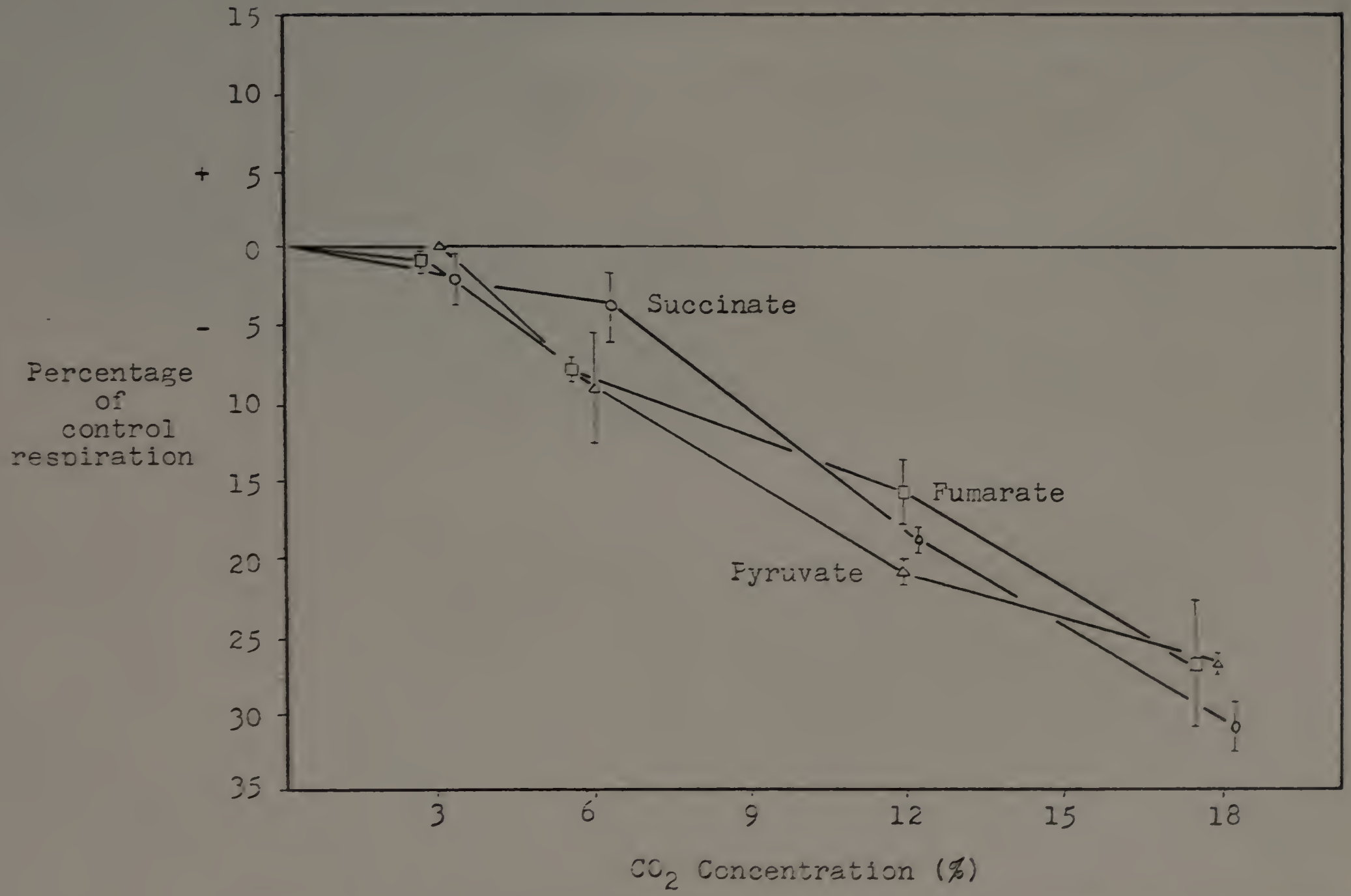

Figure 23. Effect of CO2-HCO3- mixtures on O2 uptake of mitochondria extracted from apples stored in 0°C air and assayed in basic reaction medium plus 0.3 umoles ADP. Substrate concentrations all 16 mM.

Table 8. Oxidation of succinate by apple mitochondria[1/] extracted after increasing lengths of storage of fruit in different atmospheres.

Weeks storage at 0°C	Storage atmosphere: Air	4%CO2-13%O2	6%CO2-3%O2	12%CO2-3%O2
	Rates of O2 uptake (mug atoms/min-mg Protein)			
0	77(±0)[2/]	77(±0)	77(±0)	77(±0)
4	142(±5)	-	-	110(±13)
6	146(±8)	-	-	101(±10)
8	106(±30)	-	-	98(± 2)
12	113(±8)	154(±50)	145(±15)	84(±15)
16	74(±3)	87(±3)	72(±9)	58(±4)
20[3/]	50(±1)	44(±1)	39(±8)	24(±1)

1/ Assayed in basic reaction medium described in Materials and Methods.

2/ All values are for state 3 rates of oxidation.

3/ Sixteen weeks in indicated atmosphere plus 4 weeks in air.

the changes in the capacity of mitochondria to oxidize succinate. This capacity was shown to rise to a peak after 4 to 6 weeks and then to decline steadily over the rest of the storage period. After 16 weeks storage the oxidation rates for succinate were significantly lower in the 12%CO_2-3%O_2 treatment than in air. However, after a further 4 weeks storage in air there was a dramatic decline in the capacity of the mitochondria to oxidize succinate with increasing levels of CO_2.

This type of change in respiration rate previously reported by Jones et al. (35) resembles the occurrence of the respiratory climacteric in apples and probably corresponds to its development in the fruit. However, mitochondria extracted from fruit which had been stored in 12%CO_2-3%O_2 showed a marked suppression and shortening of this rise compared with that seen with the mitochondria from the air-stored fruit. This can be seen more clearly when the data are presented graphically (Figure 24). Furthermore, the data presented in Figure 25 show that this was not a readily reversible effect since removal to air cold storage for 1 week did not markedly change the rate of respiration.

Table 9 shows the effect of storage and CO_2 concentrations on the capacity of the mitochondria to respire NADH. Although there are no data available for the 4 to 6 week period it is again evident that the mitochondria extracted from the fruit stored in 12%CO_2-3%O_2 lost the capacity to respire NADH at a much faster rate than those stored in air.

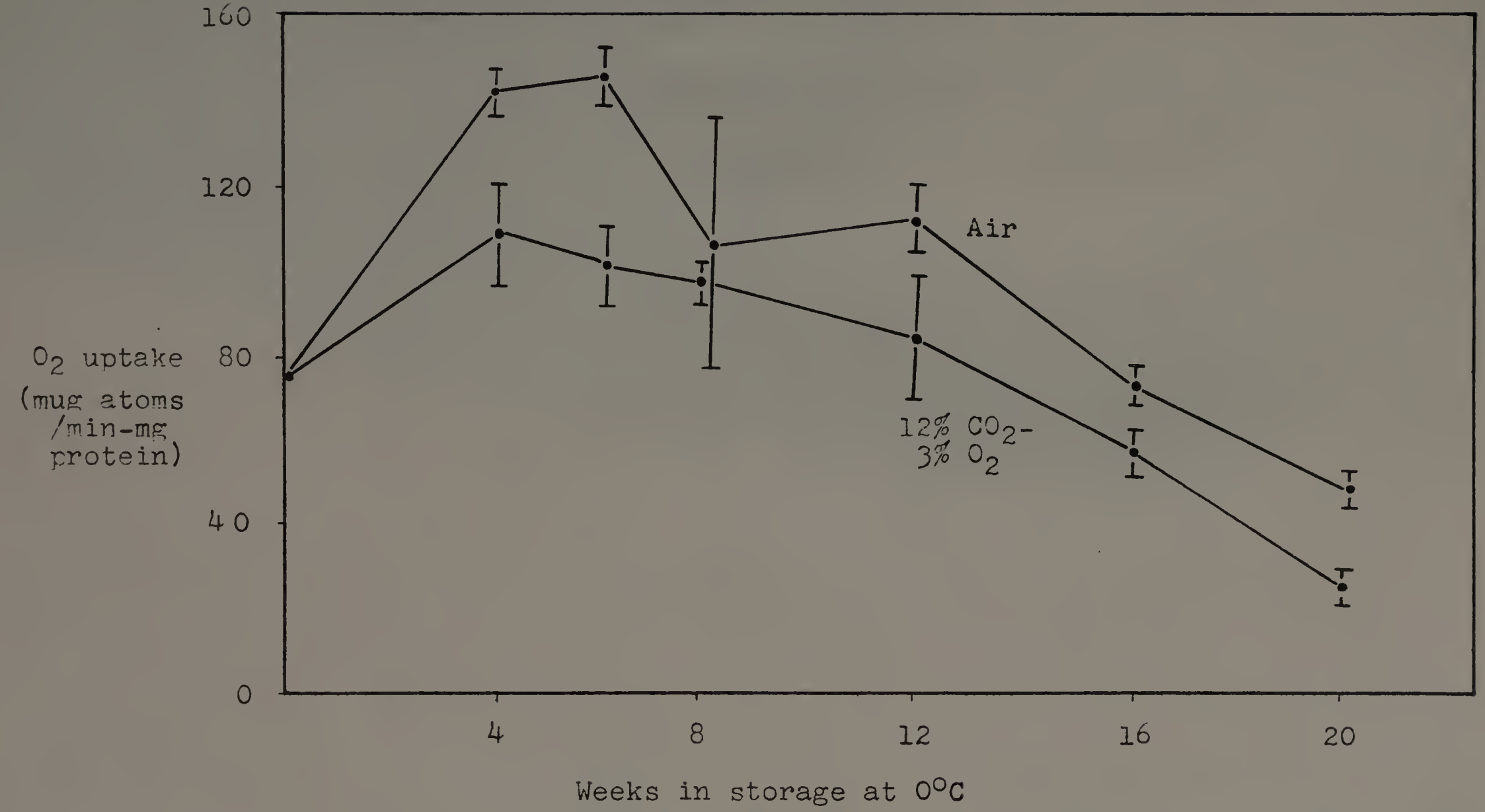

Figure 24. Oxidation of succinate by mitochondria extracted after increasing lengths of storage of fruit in air and 12%CO_2-3%O_2.

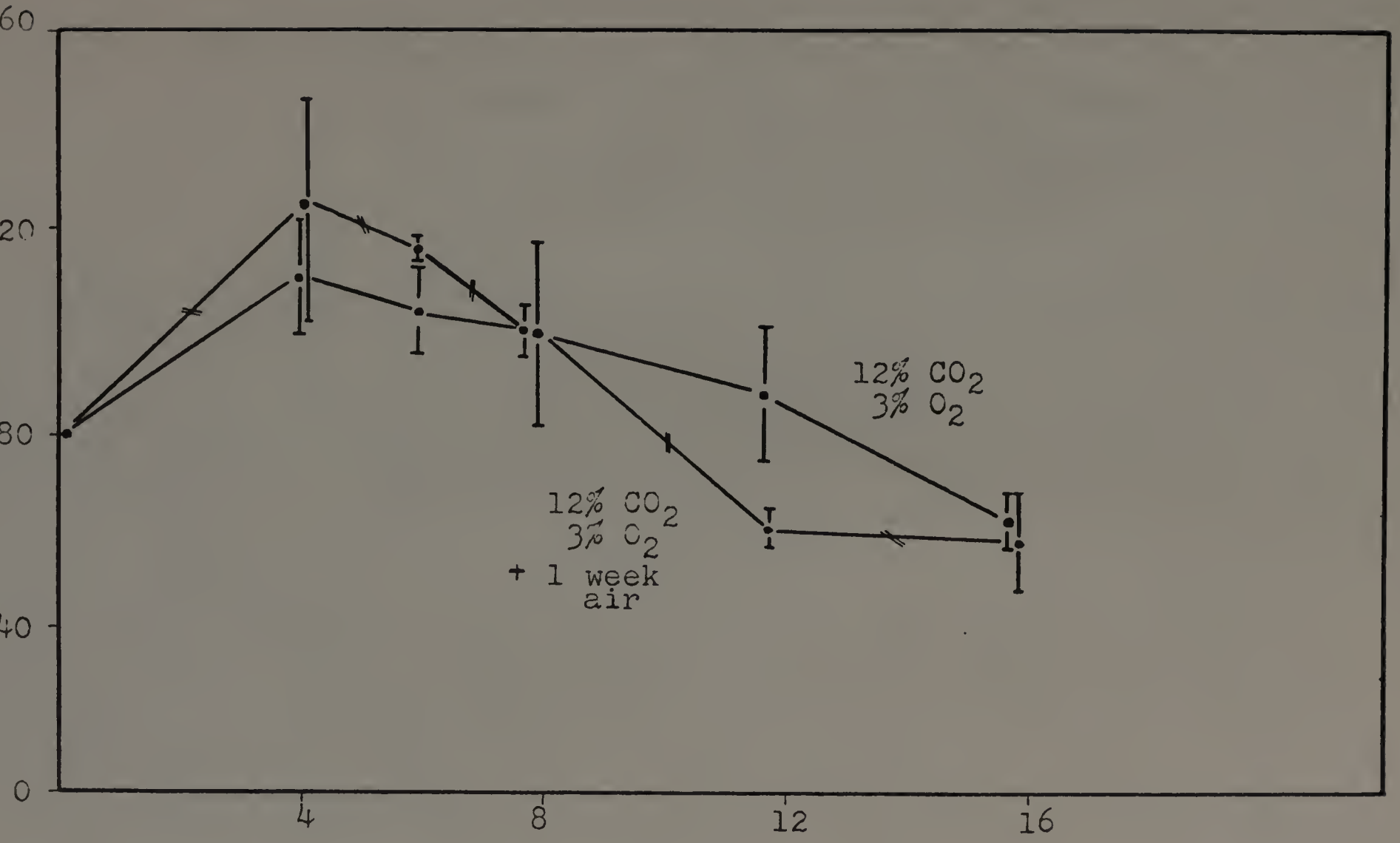

Figure 25. Oxidation of succinate by mitochondria extracted after increasing lengths of storage of fruit in 12% CO_2-3% O_2 and 12% CO_2-3% O_2 plus 1 week in 0°C air.

Table 9. Oxidation of NADH by apple mitochondria [1/] extracted after increasing lengths of storage of fruit in different atmospheres.

Weeks storage at 0°C	Storage atmosphere			
	Air	4%CO2-13%O2	6%CO2-3%O2	12%CO2-3%O2
	Rates of O2 uptake (mug atoms/min-mg Protein)			
0	162(±15) [2/]	162(±15)	162(±15)	162(±15)
8	166(±36)	-	-	132(±10)
12	169(±17)	159(±41)	159(±6)	116(±9)
16	125(±5)	94(±3)	84(±10)	89(±3)
20 [3/]	102(±2)	70(±1)	60(±20)	37(±1)

1/ Assayed in basic reaction medium described in Materials and Methods.

2/ All values are for state 3 rates of oxidation.

3/ Sixteen weeks in indicated atmosphere plus 4 weeks in air.

Furthermore, the dramatic decline in the respiration rate with increasing levels of CO_2 was again evident after 20 weeks of storage.

C. Changes in Respiratory Control Ratios during Storage.

The third objective was to follow changes in respiratory control during the storage period. These determinations were made to test whether CC_2 was acting as an uncoupler of oxidative phosphorylation.

Table 10 shows the changes in respiratory control of apple mitochondria during storage in air at 0°C for 18 weeks. For all the substrates shown there is a decline in respiratory control during the storage period. Yet, the mitochondria clearly retained respiratory control even at the end of the experiment.

Table 11 shows the effect of 12%CO_2-3%O_2 storage on the respiratory control of apple mitochondria oxidizing succinate. During the 6 to 12 week storage period the mitochondria from the 12%CO_2 fruit were less tightly-coupled than those from air-stored fruit. Furthermore, storage for 1 week in air seemed to accentuate this difference. However, after 16 weeks there was no longer any significant difference between fruits from air and 12%CO_2, and the respiratory control values were still relatively high for apple mitochondria.

D. Changes in Sensitivity to CO2.

The final objective was to determine whether long-term storage of fruit in high CO_2 would saturate the sensitivity

Table 10. Respiratory control with various substrates by apple mitochondria 1/ extracted after increasing lengths of storage of fruit in air.

Weeks storage at 0°C	Substrate			
	Succinate	NADH	Alpha keto-glutarate	Fumarate
0	2.8(±0.2)	2.0(±0.0)	2.8(±0.3)	2.4(±0.3)
8	2.3(±0.1)	1.8(±0.0)	-	-
16	1.9(±0.1)	1.5(±0.0)	1.3(±0.0)	1.4(±0.1)
18	2.0(±0.0)	1.6(±0.0)	1.6(±0.2)	1.7(±0.3)

1/ Assayed in basic reaction medium plus 0.38 umoles cytochrome-c followed, after an interval by 0.3 umoles ADP as described in Materials and Methods.

Table 11. Respiratory control with succinate by apple mitochondria 1/ extracted after increasing lengths of storage of fruit in different atmospheres.

Weeks storage at 0°C	Storage atmosphere		
	Air	12%CO2 3%O2	12%CO2-3%O2 plus 1 week in air
0	2.8(±0.2)	2.8(±0.2)	2.8(±0.2)
4	2.4(±0.0)	2.5(±0.3)	2.3(±0.3)
6	2.6(±0.0)	2.1(±0.0)	1.8(±0.2)
8	2.3(±0.1)	2.0(±0.1)	1.7(±0.1)
12	2.7(±0.1)	2.2(±0.1)	2.2(±0.1)
14	1.9(±0.1)	-	-
16	1.9(±0.1)	2.0(±0.1)	2.1(±0.2)

1/ Assayed in basic reaction medium plus 0.38 umoles cytochrome-c followed, after an interval by 0.3 umoles ADP as described in Materials and Methods.

of extracted mitochondria to CO_2. Again succinate oxidation was used because of its high rate and high sensitivity to CO_2.

Table 12 shows that mitochondria from air-stored fruit lost at least 50% of their sensitivity to CO_2 by the end of the storage period. This was evident at both the 18%CO_2 and 12%CO_2 levels. The mitochondria from the 12%CO_2 and 3%O_2 treatment also lost about 50% of their sensitivity during this period, but the change occurred at a much slower rate. In the first 12 weeks of storage the 12%CO_2-stored fruits were always significantly more sensitive to CO_2 than the air-stored fruits.

E. Reversibility of CO2 Effect.

Whenever samples of fruit were removed from the various CO_2 treatments during the storage period a second sample was transferred to air cold storage for 1 week to determine the reversibility of the CO_2 effects.

Table 13 again shows that the capacity of isolated mitochondria to respire added succinate rose and then fell in all treatments as the storage period progressed. Placing the fruit in air cold storage for 1 week did not reverse or slow the effect. After extended storage the 12%CO_2 treatment produced a marked reduction in capacity of mitochondria to respire added substrate compared with those from air-stored fruit, even after the fruit were held in air for 1 week. This suggests that possible irreversible damage occurred to the mitochondria during storage of the fruit in 12%CO_2. However, there was no

Table 12. Effects of 18% and 12% CO2-HCO3- mixtures on oxidation of succinate by mitochondria 1/ extracted after increasing lengths of storage of fruit in air and 12%CO2-3%O2.

Weeks storage at 0°C	Storage atmosphere: Air		12%CO2-3%O2	
	18%CO2	12%CO2	18%CO2	12%CO2
	Percentage of Control			
0	-31%(±2)	-19%(±1)	-31%(±2)	-19%(±1)
4	-22%(±1)	-14%(±1)	-35%(±1)	-20%(±3)
6	-19%(±1)	-13%(±2)	-26%(±1)	-20%(±3)
8	-18%(±1)	- 7%(±1)	-26%(±2)	-13%(±3)
12	-19%(±0)	- 7%(±3)	-23%(±2)	-14%(±2)
14	-15%(±1)	- 7%(±1)	-	-
16	-12%(±1)	-11%(±2)	-16%(±2)	-12%(±4)
18	-14%(±1)	- 8%(±2)	-	-

1/ Assayed in basic reaction medium plus 0.38 umoles cytochrome-c and 0.3 umoles ADP plus the appropriate concentration of NaHCO3 as described in Materials and Methods.

Table 13. Effects of removing fruit from CO_2 storage atmospheres, and of placing them in air cold storage for 1 week, on the oxidation rate of extracted mitochondria, with succinate and NADH as substrate.

Weeks storage at 0°C	Storage atmosphere						
	Air	4%CO2 13%O2	Plus 1 week 0°C air	6%CO2 3%O2	Plus 1 week 0°C air	12%CO2 3%O2	Plus 1 week 0°C air
	Succinate as substrate						
0	77(± 0)[1/]	77(± 0)	77(± 0)	77(± 0)	77(± 0)	77(± 0)	77(± 0)
12	113(± 8)	154(±50)	97(± 4)	145(±15)	130(± 3)	84(±15)	38(± 1)
16	74(± 3)	87(± 3)	65(± 2)	72(± 9)	83(±13)	58(± 4)	57(±11)
	NADH as substrate						
0	162(±15)	162(±15)	162(±15)	162(±15)	162(±15)	162(±15)	162(±15)
12	166(±36)	159(±41)	86(±31)	159(± 6)	179(±15)	116(± 9)	59(± 4)
16	125(± 5)	94(± 3)	90(± 3)	84(±10)	113(±19)	89(± 3)	71(± 8)

1/ All values are for state 3 rates of oxidation and assayed in basic reaction medium described earlier in Materials and Methods.

clear effect of 6%CO_2 or 4%CO_2 on the capacity of extracted mitochondria to oxidize succinate. Table 13 shows a similar effect with NADH as substrate. Storage in 4%CO_2 or 6%CO_2 had no clear effect on oxidation, but storage in 12%CO_2 markedly suppressed oxidative capacity, and the suppression was not relieved by subsequent storage of the fruit in air for 1 week.

Table 14 shows the effect of 1 week of storage in 0°C air following 12 and 16 weeks in the various atmospheres on the respiratory control of the extracted mitochondria. After 12 weeks of storage, mitochondria from fruit stored in 6% and 12%CO_2 exhibited significantly lower respiratory control, and this effect was not overcome by subsequent storage in air.

Table 15 shows the effect of 1 week of storage in air on the sensitivity of the extracted mitochondria to CO_2. Again the decline in sensitivity to CO_2 with aging of the fruit is shown, and it can be seen that after holding CO_2-stored fruits in air for 1 week, sensitivity of mitochondria to CO_2 was generally equivalent to that of mitochondria from air-stored fruit.

Table 14. Effects of removing fruit from CO2 storage atmospheres, and of placing them in air cold storage for 1 week, on respiratory control ratios, with succinate and NADH as substrates.

Weeks storage at 0°C	Storage atmosphere						
	Air	4%CO2 13%O2	Plus 1 week 0°C air	6%CO2 3%O2	Plus 1 week 0°C air	12%CO2 3%O2	Plus 1 week 0°C air
	Succinate as substrate						
0	2.8(±0.2) [1/]	2.8(±0.2)	2.8(±0.2)	2.8(±0.2)	2.8(±0.2)	2.8(±0.2)	2.8(±0.2)
12	2.7(±0.1)	2.6(±0.1)	1.9(±0.1)	2.3(±0.2)	2.4(±0.1)	2.2(±0.1)	2.2(±0.1)
16	1.9(±0.1)	1.8(±0.2)	2.1(±0.2)	1.7(±0.1)	2.0(±0.0)	2.0(±0.1)	2.1(±0.2)
	NADH as substrate						
0	2.0(±0.0)	2.0(±0.0)	2.0(±0.0)	2.0(±0.0)	2.0(±0.0)	2.0(±0.0)	2.0(±0.0)
12	2.2(±0.0)	2.1(±0.1)	1.5(±0.2)	1.8(±0.2)	1.7(±0.0)	1.6(±0.2)	1.5(±0.1)
16	1.5(±0.0)	1.6(±0.1)	1.8(±0.1)	1.4(±0.1)	1.9(±0.1)	1.4(±0.0)	1.6(±0.2)

1/ Assayed in basic reaction medium described earlier in Materials and Methods.

Table 15. Effects of removing fruit from CO2 storage atmospheres, and of placing them in air cold storage for 1 week, on sensitivity to CO2-bicarbonate mixtures at the 18% and 12% levels with succinate as substrate.

Weeks storage at 0°C	Storage atmosphere						
	Air	4%CO2 13%O2	Plus 1 week 0°C air	6%CO2 3%O2	Plus 1 week 0°C air	12%CO2 3%O2	Plus 1 week 0°C air
				18%CO2			
0	-31(±2)[1/]	-31(±2)	-31(±2)	-31(±2)	-31(±2)	-31(±2)	-31(±2)
12	-19(±1)	-20(±2)	-17(±5)	-21(±1)	-23(±3)	-23(±2)	-11(±1)
16	-12(±1)	-18(±1)	-15(±1)	-16(±3)	-12(±1)	-16(±2)	-16(±0)
				12%CO2			
0	-19(±1)	-19(±1)	-19(±1)	-19(±1)	-19(±1)	-19(±1)	-19(±1)
12	- 7(±3)	-11(±1)	-11(±1)	-12(±2)	-10(±3)	-14(±0)	-13(±1)
16	-11(±2)	-11(±1)	- 8(±1)	- 9(±3)	- 9(±3)	-12(±4)	-14(±1)

1/ Assayed in basic reaction medium described earlier in Materials and Methods. Values presented are percentage of control.

D I S C U S S I O N

Apples subjected to high concentrations of CO_2 for an extended period of storage have been shown to develop "CO_2 injury". Yet, a close study of the disorder during its early development has not previously been undertaken. It was therefore the object of these experiments to follow the development of the disorder and to try and find a physiological or biochemical link to its appearance.

The browning reaction occurring around the core region of the fruit is clear evidence of "CO_2 injury". However, it gives little indication of the role that CO_2 has played in the development of this disorder. Such a reaction, occasioned by high concentrations of CO_2, is probably a result of increased action by phenolases in response to the leakage of abnormally large quantities of phenolic compounds from the vacuole into the cytoplasm. Under normal conditions phenols and organic acids presumably accumulate in the vacuole (29). Such compartmentalization would serve as a barrier between substrate and enzyme. There is, however, little information available concerning the control of the movement of these compounds from the vacuole through the tonoplast to the site of metabolism. Nevertheless, any such leakage of phenols from the vacuole into the cytoplasm would suggest a change in membrane permeability. This change may be a result of storage in high CO_2 concentrations, and if so it is probably a secondary

response to the atmospheric composition.

There are several ways in which CO_2 might be causing such an effect to occur. Firstly, this change could be the result of a direct effect of CO_2 on the membranes themselves. Secondly, it could be the result of a reduction in the energy level within the cell leading to changes in membrane permeability. Or thirdly, it may be due to the action of increased levels of certain metabolites which have accumulated under high CO_2 concentrations.

Direct effects of CO_2-bicarbonate mixtures were observed on mitochondria extracted from fruits which had been stored in air, although the effects were not necessarily attributable to membrane changes. The results showed clearly that CO_2-bicarbonate mixtures significantly decreased, without any lag period, the capacity of these mitochondria to respire added citric acid cycle acids and NADH (Figures 22 and 23). Malate oxidation was the only exception to this suppression and it showed slight stimulation by CO_2. From these results, the accumulation of succinate but not malate under high CO_2, noted in earlier research (10, 27, 84) is quite understandable. Although studies with isolated mitochondria must always be interpreted with caution, it is clear that if similar effects on mitochondrial activity were produced within fruit under high CC_2 conditions, a marked reduction in oxidative phosphorylation would occur within the fruit. Clearly, the effect of CO_2 on mitochondrial metabolism was not restricted to one specific

enzyme such as succinic dehydrogenase, as has been suggested earlier (4, 5), but was rather a broad effect, perhaps upon the mitochondrion itself. One can only speculate on what effect CO_2 may be having, but there are 2 possibilities that might be considered.

1. A disruption of the structural integrity of the mitochondrion, or less severely a conformational change, inflicted by CO_2 could account for the pronounced inhibition of the mitochondrial enzymes. For example, structural damage to the mitochondrion during the isolation procedure drastically affects not only its ability to respire the citric acid cycle acids but also the coupling of oxidation to ATP formation (9, 26, 49). Furthermore, damaged mitochondria have been shown to readily lose cytochrome-c, a vital component of the electron transport chain (9). Indeed, the chemical, chemiosmotic, and conformational coupling hypotheses for the mechanism of energy conservation during electron transport all invoke a dependence on the intactness of the mitochondrial structures for efficient energy generation (50). Clearly, even slight conformational changes could consequently affect oxidative phosphorylation.

2. The inhibition noted with CO_2-bicarbonate mixtures may have been attributable to pH changes. Although the pH of the assay medium was maintained at a constant point for all levels of CO_2, it has been shown that CO_2 flows freely through membranes (46). Diffusion of CO_2 across the mitochondrial

membrane could change the internal pH and affect enzyme activities and hence, oxidation rates.

Regardless of the means by which CO_2 suppressed acid oxidation by the mitochondria, the suppression was effected. Within the cell, a result would be a reduced utilization of pyruvate by the citric acid cycle system, probably causing its accumulation. Pyruvate would then likely be diverted through alternative pathways to OAA, malate, ethanol and acetaldehyde and other substances, e.g., the amino acids alanine, lysine and valine. However, with high levels of CO_2 the routes to OAA and malate should become saturated, and acetaldehyde and ethanol should begin to accumulate, perhaps to potentially toxic levels. Accumulation of acetaldehyde and ethanol has been found in earlier work (25, 74) to be associated with "CO_2 injury" in fruit.

Any change (77) from a predominantly aerobic to partially anaerobic system would also have an effect on energy production. Acetaldehyde and ethanol are still both in a highly reduced state containing the majority of the energy originally present in glucose. To produce the same amount of energy under anaerobic conditions, a much greater amount of glucose would have to be oxidized. Such a change in metabolism is likely to lead to a "wasteful" consumption of carbohydrate reserves in the cell. Furthermore, volatilization of these compounds could lead to additional depletion rather than conservation of carbon within the system. However, despite their volatility, under high CO_2

concentrations these compounds are likely to accumulate at a faster rate than they can be dispersed.

The greater utilization of sugars under high CO_2 than in air (Table 4) suggests that, in fact, a change from predominantly aerobic to partially anaerobic metabolism did occur within the fruit. There was approximately a 25% greater consumption of sugars under 12%CO_2-3%O_2 than in air. With lower CO_2 concentrations, this stimulation also occurred, but to a lesser extent.

Atkinson (3) has discussed the possible regulatory mechanisms whereby a drop in the level of energy within the cell would stimulate sugar utilization. Several enzymes involved in either glycolysis or the citric acid cycle have been shown to be regulated by the relative concentrations of AMP, ADP, and ATP *in vitro*. There is, therefore, the possibility that *in vivo* the course of energy metabolism may be controlled by the intracellular concentrations of AMP, ADP, and ATP. Phosphofructokinase, isocitrate dehydrogenase, and citrate synthase have been shown to be regulated by the energy balance within the cell. With a slowing down of aerobic respiration, energy production would be reduced leading to a lowering of the ATP/AMP ratio. Under normal conditions there would be a stimulation of the activity of citrate synthase and isocitric dehydrogenase in response to such a low energy balance. However, if the citric acid cycle is being inhibited by CO_2, as our results showed, then this controlling influence would no longer be effective.

However, under these circumstances the stimulation of phosphofructokinase by low levels of ATP is a distinct possibility. This would lead to the increased utilization of sugars under high CO_2. If the increased utilization of sugars to produce energy still could not keep pace with the energy needed to maintain membrane integrity and function, then with reduced energy levels membrane permeability might change and have an effect on cellular compartmentalization. This, in turn, may give rise to an increased rate of leakage of organic acids and phenols from the vacuole into the cytoplasm. Enzymatic oxidation of the liberated phenols would account for this browning reaction.

Another way in which energy production can be reduced within the cell is the actual poisoning of the mitochondria by either acetaldehyde and ethanol or by organic acids and phenols. Damage to the mitochondria could drastically effect energy production within the cell. Such damage if irreversible could lead to the disruption of energy production. Indeed, even if the effect were reversible it could only be sustained for a short period before the energy balance would be affected. Damage to the mitochondria should manifest itself as an abnormality of respiration. Faster rates of oxidation are generally symptomatic of uncoupling, if associated with a reduced uptake of Pi. Slower rates of oxidation would be observed if the mitochondria were severely damaged. Either of these effects would likely lead to a reduction in the amount of energy being

produced by oxidative phosphorylation.

From the results presented here it is clear that CO_2 was not acting as a strong uncoupler of oxidative phosphorylation, although there were signs of mild uncoupling by the 12%CO_2-3%O_2 atmosphere midway during storage (Table 11). However, there was evidence of some other form of damage to the mitochondria by 12%CO_2-3%O_2 after all investigated intervals of storage. The increasing capacity of the mitochondria to oxidize added succinate with time of storage in air was suppressed by storage in 12%CO_2-3%O_2 (Figure 24). Furthermore, after 16 weeks of storage both the 6%CO_2-3%O_2 and 12%CO_2-3%O_2 had imposed an effect on the mitochondria which reduced their capacity to respire added succinate (Table 8). With NADH as substrate this effect was extended to the 4%CO_2-13%O_2 atmosphere (Table 9). This effect was not reversed by storage in 0°C air for 4 weeks. Indeed, this reduction in capacity to respire both substrates, imposed by storage in CO_2, was enhanced by later storage in air, which suggests that this modifying effect was representing damage that was irreparable.

Although the mitochondria extracted from apples subjected to supraoptimal CO_2 concentrations showed distinct indications of injury, they were still functional at the termination of the experiment. These mitochondria were still capable of at least half the rate of succinate oxidation exhibited by mitochondria from air-stored fruits (Table 8), and mitochondria from all the atmospheres had equal respiratory control ratios (Table 11).

While there was a direct effect of CO_2 on mitochondria and there was probably some reduction in energy level of the cells due to CO_2, "CO_2 injury" might also represent damage to the fruit from accumulation of certain metabolic products.

Acetaldehyde and ethanol are capable of producing browning types of injury in 'Richared Delicious' apples when they are injected into the atmosphere surrounding fruits (70). The mechanism of their action is unresolved, but it is a possibility that they are causing damage to the mitochondrial and vacuolar membranes, perhaps by reacting with the lipid fraction of the membrane.

It is interesting that holding fruit at 23°C for 5 days in air following high CO_2-low O_2 increased the incidence and severity of internal "CO_2 injury" (Table 1). Under these circumstances the increase in temperature would increase metabolic activity, and the increase in oxygen concentration might stimulate the activity of oxygen-requiring enzymes. An increase in metabolic activity would not only increase the cells' requirements for energy but if mitochondrial damage had occurred, it may increase the possibility of pyruvate being diverted to acetaldehyde and ethanol and causing further damage.

A suggestion that "CO_2 injury" may be caused by something other than CO_2 itself may be implied by the greater susceptibility of larger fruit to damage (Table 2) and the fact that injury begins in the center of the fruit. For larger fruits gases must diffuse a greater distance for exchange to

occur between the external environment and the cells in the center of the fruit. Evidence by Smith (71) and Williams and Patterson (83) has shown little difference in CO_2 concentration between the center of the fruit and the exterior, so the effect of size should not be attributable to differential CO_2 concentrations in the fruit. However, while only small and presumably insignificant differences in the concentrations of CO_2 and O_2 may exist between the interior and exterior of an apple, the same may not be true of acetaldehyde and ethanol. Significant gradations of acetaldehyde and ethanol might exist across a fruit and be sufficient to cause more damage in the larger fruits and initiate damage at their center. Thus, acetaldehyde and ethanol could be the causative agents of the injury rather than CO_2 itself.

From our results it is possible to put forward a hypothesis to explain both the beneficial and damaging effects of CO_2 on the storage life of fruit: At low concentrations the partial inhibition of the respiratory enzymes by CO_2 retards respiration, conserves carbon within the system, and prolongs storage life by slowing the degradative actions of aging. However, at too-high CO_2 concentrations excessive inhibition of the respiratory enzymes diverts pyruvate from aerobic respiration to fermentation. Such an effect, if prolonged, leads to the accumulation of acetaldehyde and ethanol to levels toxic to the fruit. This in turn may lead to mitochondrial damage producing further loss of energy within the cell,

and resulting in cellular disorganization and eventual tissue collapse that manifests itself as "CO_2 injury".

S U M M A R Y

A study was conducted to determine whether the development of "CO_2 injury" in 'Richared Delicious' apples could be related to any changes in mitochondrial metabolism. Apples were stored in atmospheres containing 6%CO_2-3%O_2; 12%CO_2-3%O_2; and 4%CO_2-13%CO_2 at 0°C for 4 months then for 1 month in air. Changes in appearance, firmness, and soluble solids were recorded during the storage period. Mitochondria were isolated from air-stored fruits and assayed for the effects of 3%, 6%, 12%, and 18%CO_2 concentrations on metabolism. Furthermore, mitochondria were isolated at periodic intervals from fruit stored in the various atmospheres and were assayed for changes in rates of oxygen uptake, respiratory control, and sensitivity to CO_2, using succinate as substrate.

The results are summarized as follows :

1). Characteristic symptoms of internal "CO_2 injury" were observed in all storage atmospheres by the end of the experiment. The severity increased with increasing CO_2 concentration and duration of exposure, and was greater in larger than in smaller fruits.

2). An external symptom not previously related to "CO_2 injury" was also observed in all atmospheres but was most pronounced in the 4%CO_2-13%O_2 fruits.

3). High CO_2 concentrations accelerated fruit softening, but there was also an interaction between O_2 and CO_2 since 4%CO_2 plus 13%O_2 was at least as effective as 12%CO_2 plus

3%O_2 in producing this response.

4). Soluble solids determinations during the experiment showed that with increasing levels of CO_2, sugars were depleted at an increasingly faster rate than in air.

5). Extracted mitochondria were capable of oxidizing to a greater or lesser degree all 6 citric acid cycle acids tested and NADH. Consistently high rates of oxidation and respiratory control ratios were obtained for apple mitochondria. These together with low ATPase activity and slow "aging" were suggestive of reasonably intact mitochondria.

6). Using various citric acid cycle acids as substrates increased levels of CO_2 were found to have a broad effect on mitochondrial oxidations. Malate oxidation was stimulated approximately 10% at the highest levels of CO_2. Alpha ketoglutarate, citrate, and NADH were inhibited up to 10% with increasing levels of CO_2. The greatest effect was observed with fumarate, pyruvate, and succinate, which showed inhibition as high as 32% at the highest CO_2 concentration.

7). Mitochondria extracted from air-stored fruit showed an increased capacity to respire succinate early in the storage period and this was followed by a decline. High CO_2 markedly suppressed this rise.

8). CO_2 did not act as a strong uncoupler of oxidative phosphorylation, although there was evidence of mild uncoupling midway through the storage period.

9). There was a greater decline in capacity of mitochondria to respire added succinate and NADH after storage in high

CO_2 than in air-stored fruits.

10). At most sampling intervals CO_2-stored fruits were more sensitive to CO_2-bicarbonate mixtures than air-stored fruits.

11). The effects of CO_2 on mitochondrial activity were not reversed by placing fruits in 0^oC air for 1 or 4 weeks.

12). These results suggest that too-high CO_2 concentrations produce excessive inhibition of the respiratory enzymes, diverting pyruvate from aerobic respiration to fermentation. Such an effect, if prolonged, could lead to the accumulation of acetaldehyde and ethanol to levels toxic to the fruit. This in turn may lead to mitochondrial damage, producing further loss of energy within the cell and resulting in cellular disorganization and eventual tissue collapse that manifests itself as "CO_2 injury".

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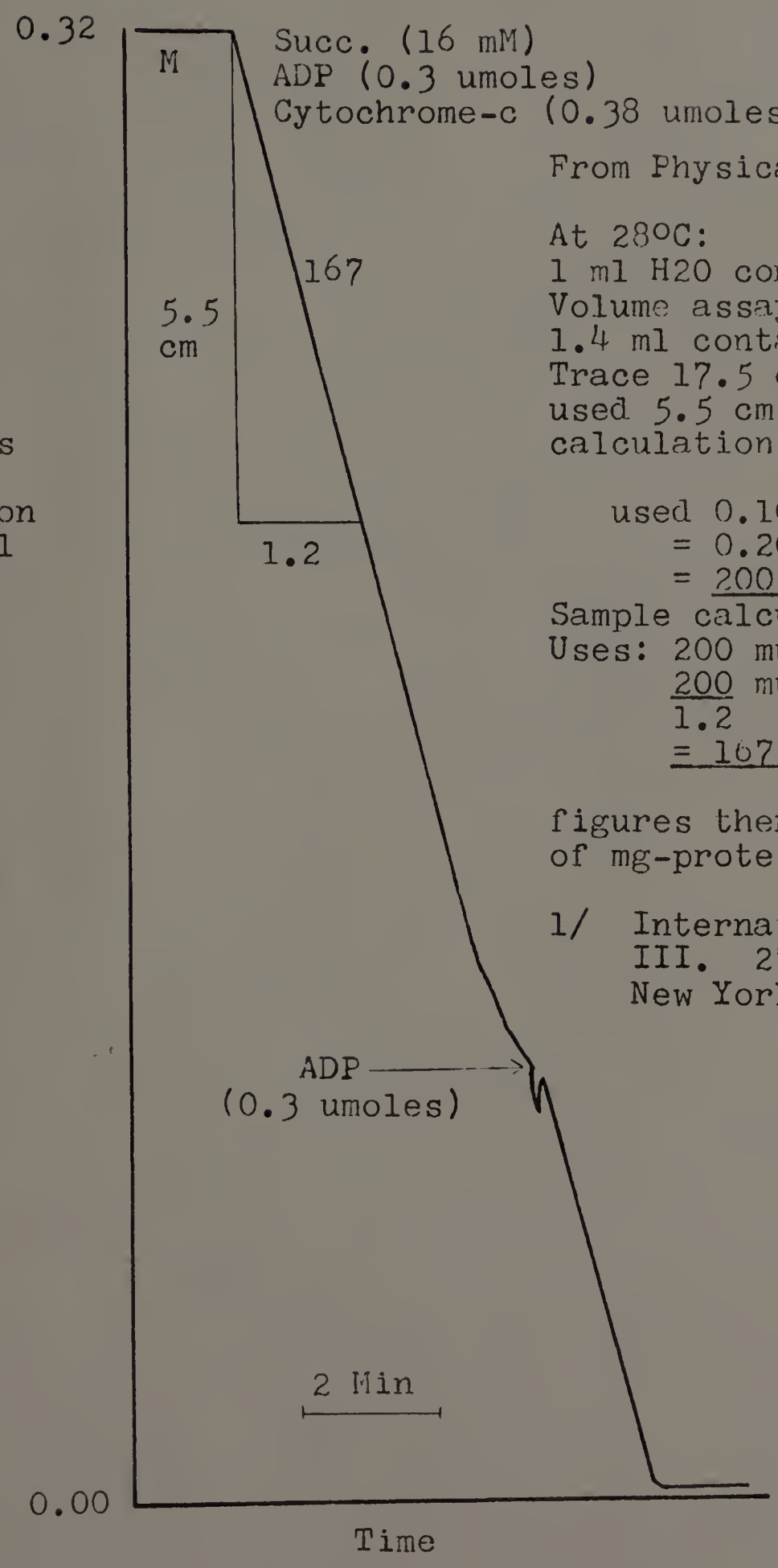
A P P E N D I X



From Physical Tables:1/

At 28°C:
1 ml H20 contains 0.23 umoles 02
Volume assay medium = 1.4 ml
1.4 ml contains 0.32 umoles 02
Trace 17.5 cm = 0.32 umoles 02
used 5.5 cm of the trace in calculation

used 0.10 umoles 02
= 0.20 ug atoms 02
= 200 mug atoms 02

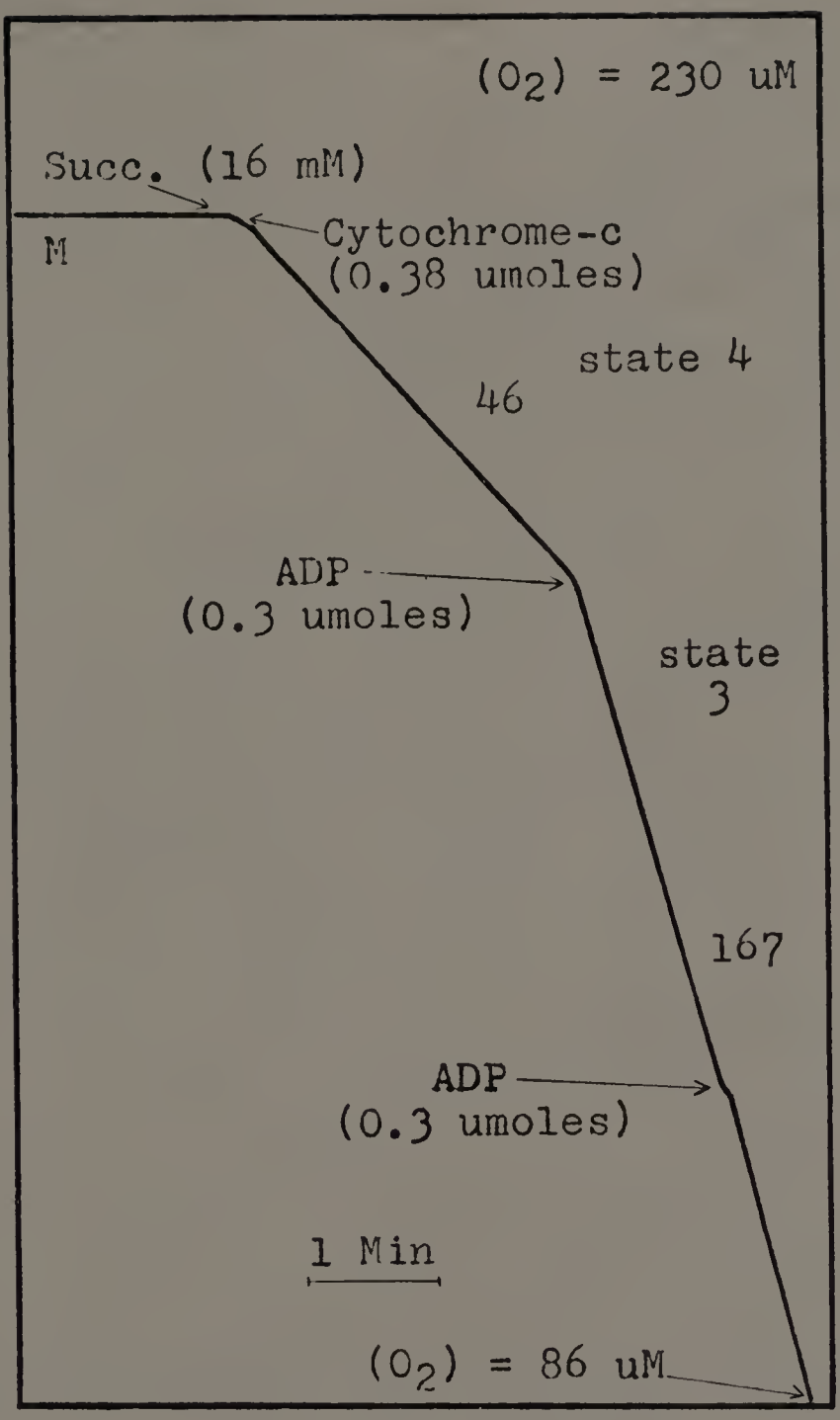
Sample calculation.
Uses: 200 mug atoms 0.2 in 1.2 min

$$\frac{200}{1.2} = 167$$ mug atoms 02/min

figures then expressed on basis of mg-protein.

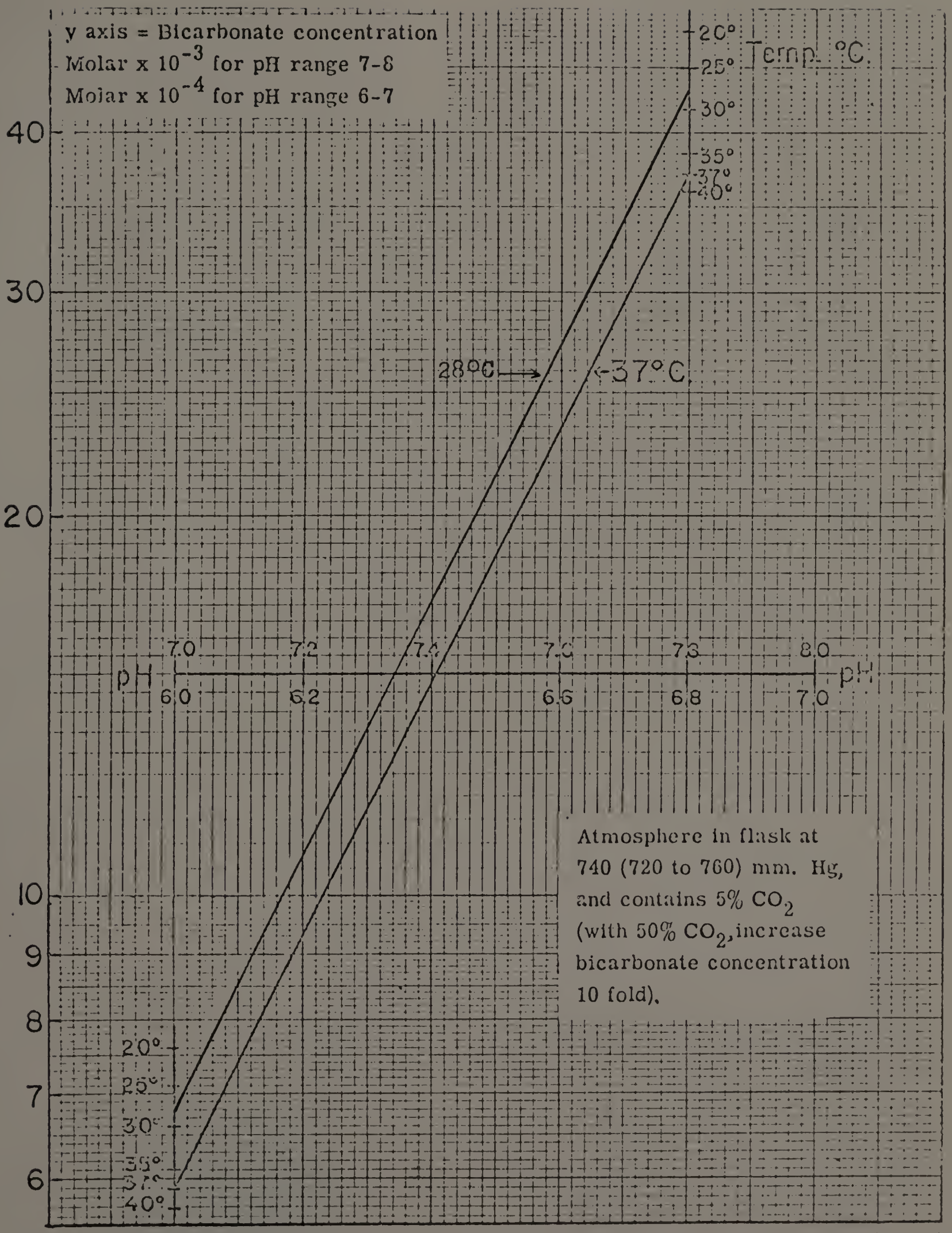
1/ International Critical Tables III. 271-272. McGraw-Hill. New York.

Appendix Figure 1. Method of calculating oxidation rates from polarograph.



Appendix Figure 2. Polarograph trace showing the influence of 0.38 umoles cytochrome-c and 0.3 umoles ADP on the rate of oxygen utilization, succinate as substrate. Assayed in basic reaction medium described in Materials and Methods. The numbers on the traces represent the decrease in mug atoms 02/min-0.09 ml of mitochondrial suspension.
Method for calculating respiratory control ratio (RCR).

$$RCR = \frac{\text{State 3}}{\text{State 4}} = \frac{167}{46} = 3.6$$



Appendix Figure 3. Chart for determining the proper bicarbonate concentration to use at a given pH, pCO2 and temperature.
From: Manometric Techniques. Umbreit, W. W., R. H. Burris and J. F. Stauffer.

Appendix Table 1. Calculation of CO2-bicarbonate concentration.

The quantity of sodium bicarbonate necessary to produce the required CO_2 concentration within the medium was calculated from graph shown in Appendix Figure 3.

Carbon dioxide forms carbonic acid which dissociates to form H^+ and HCO_3^- in accordance with the following equation.

$$\underset{(gas)}{CO_2} \rightleftharpoons \underset{(Diss)}{CO_2} + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

However over 99% of carbon dioxide in solution is in the form of dissolved carbon dioxide. Less than 1% exists as H_2CO_3, H^+, or HCO_3^-.

From Table.

Temperature : 28°C
pH required : 7.2
Concentration of CO_2 : 3%, 6%, 12%, 18%.

For above conditions and 5%CO_2 requires 10.8×10^{-3}M $NaHCO_3$

" " 18%CO_2 requires 10.8×10^{-3} " $\times \frac{18}{5}$

= 0.0389M $NaHCO_3$

" " 12%CO_2 = 0.0259M $NaHCO_3$

" " 6%CO_2 = 0.0129M $NaHCO_3$

" " 3%CO_2 = 0.0065M $NaHCO_3$

Appendix Table 2. Determination of protein in mitochondrial preparation.

The protein concentration of the mitochondrial preparation was estimated using the Folin-Ciocalteu reagent. The Lowry method (51), slightly modified, was used.

Reagent 1. Mix 1:1 $CuSO_4$ 1% with NaKTartrate 2% to give final concentration 0.5% $CuSO_4$ in 1% NaKTartrate.

Reagent 2. Mix 1 ml of reagent 1 with 49 ml of 2% Na_2CO_3 in 0.1N NaOH.

Procedure:-

1. Samples of protein (generally 0.01 ml) were pipetted into test-tubes and the volume brought to 0.6 ml with distilled water. A 0.6 ml sample of distilled water was used as a blank.

2. A 3 ml aliquot of reagent 2 was added to each tube and the contents were mixed and allowed to stand at room temperature for 10 mins.

3. A 0.3 ml aliquot of Folin reagent (normally 2N, but diluted to 1N by an equal volume of water) was added and the solution mixed immediately.

4. Samples were allowed to stand at room temperature for 30 minutes.

5. The optical density of this solution was read at 500 mu against the blank with a Spectronic 20 spectrophotometer and the protein concentration calculated from a standard curve made with bovine serum albumin as the protein.

Comments:-

a. 500 mu used instead of 750 mu because of the relatively high protein content of the sample.

b. Lowry method chosen for sensitivity; less liable to inaccuracies due to turbidity.

Appendix Table 3. Comparison of total protein extracted, and of 'state 3' succinoxidase activity 1/ of mitochondria isolated from pulp of 'Delicious' apples. 2/

Substrate	Total mg of protein extracted 3/			Mug atoms O2 uptake /min-mg protein		
	1	2	3	1	2	3
Succinate	49.5 4/	49.5	45.0	76.5	76.5	76.4
Malate	39.0	36.0	30.0	58.3	61.1	74.0
Alpha keto-glutarate	46.5	43.5	42.0	44.8	45.0	53.6
Fumarate	46.5	45.0	45.0	34.1	33.7	37.8
Citrate	56.4	78.0	56.4	27.8	18.6	28.6
Pyruvate	63.0	45.0	40.5	28.4	42.9	47.1
NADH	39.0	49.5	33.0	177.5	133.3	176.0

1/ Assayed in basic assay medium plus 0.3 umoles ADP and 0.38 umoles cytochrome-c.

2/ Extractions carried out between Oct 25-Nov 25 1970.

3/ Results of 3 different extractions are presented individually.

4/ Means of 4 separate traces.

Appendix Table 4. A comparison of different methods of expressing succinoxidase activity of apple mitochondria.

Storage atmosphere[1/]	Rates of O2 uptake (mug atoms/min)[2/] Per mg protein	Per 100gm F.W.	Per umole cytochrome-b1
Air	74(1.4)[3/]	30(1.2)	2350(1.3)
4%CO2-13%O2	87(1.6)	34(1.3)	2330(1.3)
6%CO2-3%O2	72(1.4)	30(1.2)	1997(1.1)
12%CO2-3%O2	53(1.0)	25(1.0)	1801(1.0)

1/ Stored for 16 weeks at 0°C.

2/ Mitochondria were isolated from 'Delicious' apple tissue and assayed in basic assay medium plus 0.3 umoles ADP and 0.38 umoles cytochrome-c.

3/ Figures in parentheses signify ratios of respective rates.

Appendix Table 5. Oxidative phosphorylation of various apple cultivars.

Cultivars	Date	Rate of O2 uptake succinate as substrate (mug atoms/min-mg protein at 25°C.) Succinate concentration 8mM	16mM	Respiratory control ratio
'E. McIntosh'	Sept 2-14	51(±4)	-	-
'Niagara'	Sept 15-18	112(-)	-	4.5(±0.2)
'Delicious' (Bisbee)	Sept 22-23	-	60(±8)	3.9(±0.7)
'Delicious' (Richared)	Oct 25-Nov 25	-	77(±0) 1/	2.8(±0.2)

1/ Assayed at 28°C.

Appendix Table 6. Evidence for the decarboxylation of oxalacetic acid to pyruvic acid either non-enzymatically or by enzymic catalysis. Note that after 4 days OAA oxidation rates are close to those of pyruvic acid suggesting conversion of OAA to pyruvic acid.

$HOOCCOCH_2COOH$ → $CH_3COCOOH + CO_2$

Oxalacetic acid → Pyruvic acid

Substrate	Rates of O_2 uptake (mug atoms/min-mg protein)		
	1 [1/]	2	3
OAA (freshly prepared)	3.1	-	3.4
OAA (4 days old)	-	6.4	7.6
Pyruvic acid	5.8	8.4	8.8

1/ Separate extracts.

A C K N O W L E D G M E N T

Numerous people have extended help, advice and guidance during the course of this work as well as in the preparation of the manuscript. Special thanks are due to Dr. W. J. Bramlage for his excellent advice, guidance, and personal encouragement.

My sincere thanks also to Drs. H. O. Hultin, and P. H. Jennings who served on my Dissertation Committee and who provided helpful suggestions throughout the work and during the preparation of the manuscript. I am further indebted to Dr. Hultin for the use of some of his facilities and equipment.

Special appreciation for my wife Margaret's understanding and encouragement throughout this study, and for her patience in the tracing of the various figures.

To her sister Susan I am indebted for her excellent accomplishment in typing this manuscript.

EFFECTS OF SUPRAOPTIMAL CARBON DIOXIDE CONCENTRATIONS ON MITOCHONDRIAL ACTIVITY OF 'RICHARED DELICIOUS' APPLES.

A Dissertation

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