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STUDY OF A PROPOSED METABOLIC RELATIONSHIP BETWEEN WATERCORE AND INTERNAL BREAKDOWN IN DELICIOUS APPLES

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INTRODUCTION

"Watercore" is a physiological disorder that occurs in a number of apple varieties, including Delicious. Watercore manifests itself as a watery, translucent area which begins development near the vascular bundles of the fruit and spreads outward toward the skin as severity increases. These symptoms suggest movement of sap into the vascular area from adjacent areas and thus a flooding of the intercellular spaces. Though not often objectionable in itself, watercore has been reported to be a contributing factor in the development of "internal breakdown" (3, 19), a deterioration of the tissue resulting in a brown discoloration and a bitter taste that destroys the value of the fruit. The relationship between watercore and internal breakdown may be one of initiation of anerobiosis during watercore as the intercellular spaces become flooded and oxygen is excluded. A build-up of toxic substances may result, thus causing disorganization of protoplasm and breakdown of the tissues which is manifested as the physiological disorder internal breakdown.

The experiments described herein will test the hypothesis that the presence of watercore is initiating anaerobiosis and thereby producing a toxic substance(s) such as acetaldehyde or ethanol that is poisoning the tissues and causing breakdown. This study has been conducted to determine, specifically, if watercore: (1) initiates anaerobiosis in Delicious apples; (2) causes acetaldehyde, ethanol, or some similar substance to

accumulate in the affected tissues; and (3) affects mito-
chondrial behavior.

LITERATURE REVIEW

The causes of watercore (Figure 1) are not clearly understood, but two theories have been presented to explain the disorder. In 1931, Fisher, Harley, and Brooks (10) reported that a rapid conversion of starch to sugar preceded initiation of watercore. From their data they concluded that watercore was a result of premature and non-uniform starch conversion, and that this may be induced by the effect of comparatively high temperatures on diastatic activity. High soluble sugar concentrations resulting from the hydrolysis apparently set up abnormal osmotic relations, resulting in the guttation of the affected tissues with water.

Brown's (4) observations on Illinois apples in 1943, however, did not agree with this hypothesis. He found that the amount of starch in watercored areas appeared to be equal to or even greater than that in non-watercored tissue. In addition, he found that the normal conversion of starch as the fruit matured was not associated with the incidence of watercore, and he observed watercore in some fruit long before there was any evidence of a decrease of starch in the cortex. As the fruit matured, regions around the vascular tissue, where watercore first appeared, were among the last to lose starch. Watercored tissue was not consistently higher and was often lower in soluble solids than the non-watercored tissue of the same apple.

Figure 1. Various stages of development of watercore in Delicious apples. 1) none; 2-3) slight; 4-5) moderate; 6-7) severe..

Williams (30) in 1966 also found no correlation between total sugar levels and the degree of watercore in Delicious and Winesap apples. He observed an exudation of liquid both from the stem and cluster base when watercored Winesap apples were removed from the spur, and from the watercored vascular areas when these fruit were cut open. Analysis of this exudate and of expressed juice showed that the sap from watercored tissue contained less sugar than that from non-watercored tissue. However, sap from watercored tissue contained large quantities of sorbitol, in contrast with only small amounts of sorbitol in the sap of non-watercored tissue.

Webb and Burley (29) have investigated translocatory carbohydrates in Golden Delicious apples and found that sorbitol, the polyhydric sugar alcohol of glucose, is a major constituent of the carbohydrates moving through the Golden Delicious apple stem. Williams (30) found a relationship between the loss of sorbitol from leaves and its accumulation in fruit, and proposed that the capacity of fruit tissue to metabolize translocated photosynthates may be an important factor in the development of watercore. As a fruit approaches maturity, the growth rate and ability to deal with incoming materials decreases, possibly due to a change in enzyme activity and a build-up of end products. This condition, coupled with an influx of metabolites due to a high leaf-to-fruit ratio or to senescence, could result in an increase in solutes in the intercellular spaces. Since the intercellular spaces are usually small, a slight change in the osmotic concentrations of the intercellular sap would result in sufficient water-holding capacity to produce the

the translucent appearance associated with watercore. This explanation of watercore appears to be much more plausible than the earlier explanation by Fisher et al. (10).

A sound explanation for the occurrence of internal breakdown following watercore has not been proposed, however. Indeed little work has been done to even disclose the nature of this disorder. "Breakdown" has been described as a physiological decay accompanied by internal browning which terminates the life of the fruit (24). The browning may be internal, having a layer of uncolored sound flesh immediately beneath the skin of the apple, or may occur toward the outer surface of the apple and show a distinct skin discoloration. There are various forms of breakdown, arising apparently from different causes, and the terminology employed to describe these disorders is not clearly defined. Eaves and Hill (8), for example, have described numerous types of breakdown and have classified them on the basis of causation into: (1) low temperature breakdown (soft scald, soggy breakdown, and internal browning); (2) breakdown associated with senility (Jonathan breakdown, mealy breakdown, core flush or core browning, cortical flush, and vascular breakdown); (3) breakdown associated with storage atmosphere (gas storage injury, superficial scald, and scald breakdown); (4) breakdown associated with orchard conditions (Bitterpit, watercore breakdown, and cork or corky breakdown).

Merely describing the form of internal breakdown being observed so as to distinguish it from similar-appearing disorders but ones arising from entirely different causes is

quite difficult. Thus it is virtually impossible to correlate literature reports dealing with "internal breakdown", and it is not surprising that hardly any studies of the physiological nature of these disorders have been attempted.

That one form of internal breakdown is associated with watercore has, however, long been known. In 1926 (3), watercored apples were reported to have a high degree of susceptibility to internal breakdown. Kemp and Beare (16), and more recently Lord and Damon (19), have shown this relationship by visually scoring samples at harvest and at intervals during storage. A form of internal breakdown is definitely associated with the presence of watercore in the apples at harvest. This form of breakdown was placed by Eaves and Hill (8) in their class 4 (breakdown associated with orchard conditions). Internal breakdown that follows watercore may develop in and around the water-soaked areas, the cells becoming brown and the flesh soft and spongy. Typical examples of this disorder are shown in Figure 2.

In the earlier studies of the relationship between watercore and internal breakdown mentioned above, it was necessary to cut open each fruit and examine it for the presence of these disorders. Hence the fruit was destroyed and the actual development of the disorders and changes in the fruit could not be followed. The recent development of spectrophotometric, non-destructive methods of detection of both watercore and internal breakdown (1) has made it possible to examine the relationship between these disorders within individual fruits.

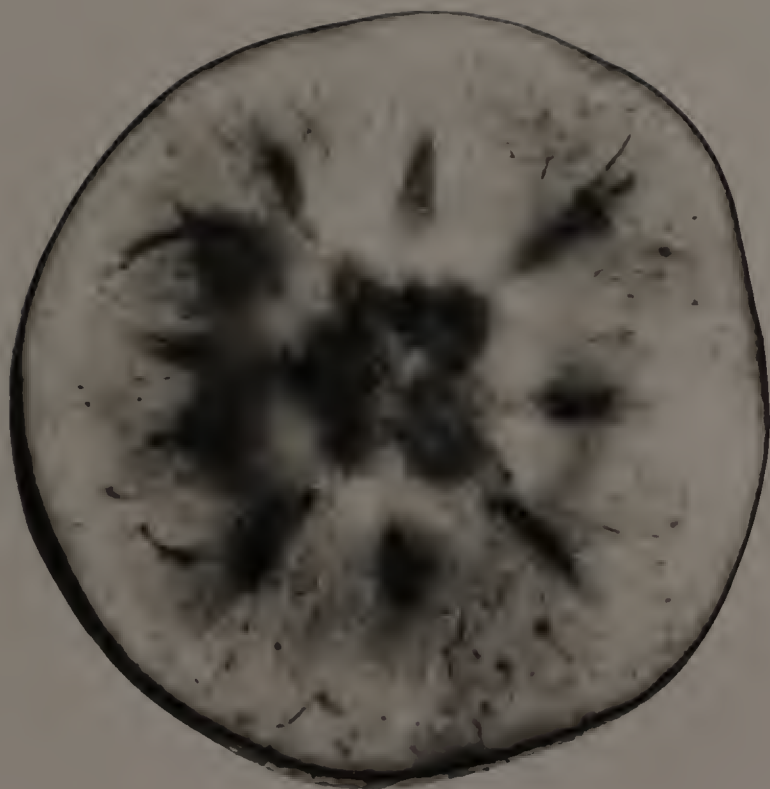


Figure 2. Typical examples of internal breakdown following watercore in Delicious apples.

Using these techniques, Bramlage and Shipway (2) found that watercore disappeared rapidly from fruit during cold storage. However, the fruit with watercore at harvest had greater susceptibility to internal breakdown than those fruits free of watercore at harvest, and the greater the initial intensity of watercore, the more likely was the fruit to develop internal breakdown.

Early work by Kemp and Beare (16) and later by Lord and Southwick (20) implied that both disorders were found in the fruit at the same time, but the light transmittance studies (2) showed that watercore often completely disappeared before internal breakdown developed. A simultaneous occurrence of watercore and internal breakdown was sometimes noted, but each was located in different areas of the fruit. The great majority of fruit completely lost watercore before internal breakdown appeared.

Length of persistence of watercore in the fruit may be an important factor in this relationship. The more severe the watercore was at harvest, the longer it has been found to persist in the fruit (2). Thus, the greater susceptibility to internal breakdown with greater watercore intensity at harvest may be due more to longer watercore persistence than to actual watercore intensity. Bramlage and Shipway (2) did find that an earlier dissipation of watercore during one season was followed by a reduced incidence of internal breakdown.

No prior attempt to find the physiological or biochemical link between watercore and internal breakdown has been reported. Nevertheless, considering the nature of watercore, a relationship between it and internal breakdown may be envisioned as follows. Since watercore is characterized by a water-soaked condition of the tissues, it is likely that a partially anaerobic environment is induced within these tissues. This supposition is supported by the report of Fisher <u>et al</u>. (10) that watercored tissue contains high concentrations (up to 0.8% fresh weight) of ethanol. Apple tissues can survive extended periods of fermentation (9) but during this process acetaldehyde and other secondary fermentation products, as well as ethanol, may accumulate.

Thomas (27) studied the effects of different atmospheres on respiration of apple tissue. His data indicated that a high CO_2 environment, in the presence of some O_2, could create a condition in the apple tissue similar to a completely anaerobic environment. In other words, "zymasic cleavage" of carbohydrates, leading to the production of higher than normal amounts of acetaldehyde and ethanol, can occur even though varying amounts of O_2 are present in the tissue if the CO_2 level is high. However, there is a marked distinction between anaerobic and CO_2 zymasis; he found that the relative concentrations of acetaldehyde and ethanol produced by and accumulating in the tissues are quite different. In CO_2 zymasis, associated with the presence of O_2, there is a greater accumulation of acetaldehyde and a lesser accumulation of ethanol,

the ratio of ethyl alcohol/acetaldehyde being 2/1. In striking contrast, anaerobic zymasis produced an ethanol/acetaldehyde ratio of 50/1. Thus in CO_2 zymasis, acetaldehyde is a major accumulating product. The concentration of acetaldehyde found in normal healthy tissue is relatively low, approximately 0.005%, whereas concentrations of 0.018%-0.040% acetaldehyde accumulates in tissue undergoing CO_2 zymasis. Ethanol increased from 0.006% in normal tissue to approximately 0.08% as a result of CO_2-zymasis.

Progressive injury, tissue browning, and disorganization of protoplasm occurred more rapidly under CO_2-zymasis than under anaerobic fermentation, which Thomas believed was probably due to the greater accumulation of acetaldehyde (Kidd and West, (cited by Thomas)). That not all fruits broke down simultaneously in the same environment was attributed to varying tolerance to specific quantities of acetaldehyde by different fruits.

Thomas postulated that CO_2 zymasis might be due to CO_2 inactivating the oxidation system concerned in normal respiration without influencing zymase splitting (fermentation). He refers to the work of Willslatter and Stol (31), who indicated that the activity of peroxidase systems was reduced 50 percent in water saturated with CO_2, though no destruction of the enzyme occurred.

Thomas and Fidler (28) later studied fermentation in apples at different O_2 concentrations, finding that rising O_2 concentration progressively retarded fermentation. The

concentration of O_2 at which no alcohol was produced was termed the "Extinction point of nitrogen respiration". The extinction point of Newton Wonder and Bramley's Seedling apples was between 1- 3% O_2 early in the storage season and shifted to a higher concentration of O_2 later in storage. It was suggested, though not demonstrated, that in older apples alcohol may accumulate even in 100% O_2. Thus oxygen stress may become more injurious as the storage season progresses and the fruit becomes more senescent.

Since watercore consists of an accumulation of liquid in intercellular spaces, it seems likely that the watercore condition imposed on Delicious apple tissue would impair the movement of gases both in and out of the affected areas of the fruit. The lessened availability of oxygen to these tissues and also the accumulation of CO_2 remaining in the tissue well might affect the nature of respiration of the tissue, perhaps inducing the CO_2 zymasis described by Thomas. Consequently, high concentrations of acetaldehyde as well as ethanol might accumulate in the watercored tissues.

Clijsters (6, 7) has recently found that ethanol and acetaldehyde accumulation preceded the development of internal breakdown in Jonathan apples, and he proposed that acetaldehyde was causing internal breakdown by poisoning the tissues. An anaerobiosis induced by watercore might, therefore, be causing internal breakdown by producing substances (perhaps acetaldehyde) that later poison the tissue.

If in fact a partially anaerobic environment is created by the imposed watercore condition, this would result in a change in the overall respiratory pattern of the tissues. Decarboxylation of pyruvic acid would result in a rise of the Respiratory Quotient (R.Q.), which is the ratio of CO_2 evolved to O_2 consumed. The R.Q. of a tissue can readily be determined by measuring O_2 consumption and CO_2 evolution. However, a high R.Q. is only an indication of fermentation processes, as other factors can also cause a change in the normal R.Q. value. Respiratory Quotient measurements need support from actual measurements of the accumulation of fermentation products for a valid assessment of fermentation. With the development in gas chromatographic analyses, methods are now at hand to determine the accumulation of such materials and determine if they are accumulating to levels which might be toxic.

Since the living cell is a complex structure composed of many discrete organelles, if a substance such as acetaldehyde were indeed poisoning the cell, there are many potential sites for such poisoning. Cellular respiration occurs within the organelle called the mitochondrion. If this energy-producing entity were poisoned or in any way injured by such an accumulating substance, the effect could be catastrophic for the cell and ultimately for the tissues and the entire fruit. If the imposed condition irreversibly damaged these organelles, it could be initially manifested as an abnormal respiratory capacity, either a greater than normal oxidation of a given substrate if uncoupling of oxidative phosphorylation has occurred, or as a lower rate of oxidation if the organelles

were extremely damaged. In either case, reduction in the generation of energy by these organelles could result in disruption of other cellular functions and lead to protoplasmic disorganization and eventual collapse of the tissues, symptomatic of internal breakdown. Thus, the mitochondria are prime sites for disruptive effects to act, and it is well known that many poisons do act upon these organelles (18, 25).

The early work of Pearson and Robertson (23) showed that particulate fractions containing many enzymes of the Krebs Cycle could be isolated from the tissue of mature apple fruits. Since then many workers have obtained mitochondrial preparations having considerably higher activity than the early reports. These preparations have been found to contain cytochromes a, a_3, b, and c, and to have the capacity for oxidative phosphorylation. Obstacles to the isolation of enzymes and intact mitochondria from apple fruit include the high acidity of the tissue and the phenolic compounds (leucoanthocyanidins, catechins, quercetin, and cyanidin glycosides) present in the fruit (26). The reduction in activity by phenolic compounds appears to be brought about in two ways (13); firstly by direct inhibition, and secondly by "co-precipitation" with the mitochondria of inactive protein complexed with phenolic material of various stages of oxidation and polymerization. The use of buffers during the extraction and the presence in the extraction medium of substances such as Polyvinylpyrrolidone (P.V.P.) and Polyethylene Glycol (P.E.G.), to remove polyphenols have been instrumental in preparing mitochondria

exhibiting high activity. In addition, Jones, et al. (15) show an almost complete lack of phosphorylation by mitochondria prepared without the incorporation of P.V.P. into the extraction medium.

Casein has also been employed successfully as an inert protein to compete with tannin-enzyme reactions as mitochondria are released from tissue into an extraction medium (12). In addition to precipitating or complexing polyphenols, reducing agents, such as ascorbic acid and L-cysteine hydrochloride have been used to keep the polyphenols in a reduced and inactive state. Using such extraction methods, it is possible to extract active and coupled mitochondria from apples. The functioning of the organelles can then be assessed.

MATERIALS AND METHODS

Apple fruit used: Richared Delicious apples grown at the Massachusetts Agricultural Experiment Station in Amherst were harvested on October 26, 1966, by which time watercore had become intense. The harvested fruits were separated with a Biospect[1/] spectrophotometer into three classes of watercore intensity: severe, moderate and none. Since fruit size influences Biospect readings (1), only fruits of 2½ to 3-inch diameter were used. All apples were held at room temperature before and during determination of internal characteristics and measured while in a vertical position, with the light beam passed parallel to the core. The optical density difference ΔOD (740 nm - 800 nm) was used to measure watercore intensity and ΔOD (840 nm - 740 nm) was used as a measure of internal breakdown. Watercore during the 1966 growing season was uncharacteristically located throughout the core and not outward from the vascular bundles, so a relationship between Biospect readings and watercore intensity different from that previously used (2) had to be established. The fruits used for the experiments were those that were differentiated into three distinct groups according to optical density difference; (1) ΔOD less than 19; (2) ΔOD, 23-26; (3) ΔOD greater than 30. These groupings represented what were designated visually as, respectively: (1) severe watercore, (2) moderate watercore;

1/ Manufactured by Agricultural Specialties Co., 11313 Frederick Ave., Beltsvile, Md.

and (3) no watercore. This grouping minimized the chance of overlap among fruits within different groups.

A fruit was judged to be free of internal breakdown if the optical density difference (840nm - 740nm) was positive, and to have internal breakdown if the optical density difference was negative (11). Only fruits free of internal breakdown were used for the subsequent experiments.

All fruits were then stored in air at 32°F and drawn upon for experiment I, to determine the respiratory pattern of tissue affected with watercore, and for experiment IV to determine the respiratory capacity of mitochondria extracted from severely watercored tissue.

A second series of samples of severely, moderately and non-watercored fruit was separated at harvest in the above manner. At 2-week intervals, all of these fruits were withdrawn from storage and measured for watercore and internal breakdown, using the Biospect. In this manner, a record of the watercore history was compiled for each fruit. These fruits were used in experiments II and III to determine the respiratory pattern and volatile constituents in fruits having lost watercore during the storage period.

Delicious apples grown in a commercial orchard in Massachusetts and stored in controlled atmosphere were used as a source of severely watercored fruit for volatile extractions. Richared Delicious grown at the Massachusetts Agricultural Experiment Station, harvested on October 14, 1966 (before any significant watercore had developed) and stored in air at 32°F,

served as a source of non-watercored fruit for extraction of volatiles.

Experiment I. Respiratory study of watercored tissue:

To determine the effect of watercore on the overall respiratory pattern of Delicious apple tissue, the O_2 consumption and CO_2 evolution of tissue cylinders was measured in a Gilson Differential Respirometer, model GRP 20. At 2-week intervals from November 14, 1966 to December 26, 1966, fruits from the three watercore classes were sampled and treated as follows. The top and bottom portions of the fruit were removed with a knife and the watercore condition confirmed visually. A No. 4 (9mm.) stainless steel cork borer was used to sample tissue in the area of the vascular bundles, and the top 2 cm of this cylinder was taken as the sample for analysis. Samples of severely watercored tissue were completely translucent and exhibited a totally water-soaked condition, moderately watercored samples were only partially water-soaked containing seemingly non-watercored areas, and the no watercore samples were completely free of any trace of water-soaked tissue. Six cylinders were removed from every apple, each was put in a Gilson flask containing 2.0 ml of a potassium phosphate buffer of pH 4.0, which was found by experimentation to be optimum (Appendix Table 1). Three flasks were used to measure O_2 consumption by including filter paper and 0.2 ml 20% KOH in the center well; the center wells of the remaining three flasks were kept empty, and so in these vessels CO_2 evolution minus O_2 consumption was measured. Carbon dioxide evolution

was determined by adding to the values of CO_2 - O_2 those obtained for O_2 consumption. Average values were obtained from the 3 flasks for each fruit. Measurement was made at 25°C, using a shaker speed of 125 strokes per minute and allowing 15 minutes for temperature equilibration before readings were begun. Gaseous exchange was measured in microliters at 15 minute intervals over a 2-hour period. The cylinders were then carefully removed and dried at 60°C overnight and their dry weight obtained. The values obtained were used to express respiration on a dry weight basis. One fruit of each class was used for each replication and three replications were made at each of the four sampling dates.

<u>Experiment II. Respiratory pattern of tissue following recovery from watercore</u>:

To determine if there is a residual effect on tissue after watercore disappears, a respiratory study similar to that in Experiment I was conducted. Samples were selected according to their history of Biospect readings: those never having had watercore; those initially having had moderate watercore; and those initially having had severe watercore. All fruits chosen were by then free of watercore according to Biospect readings (ΔOD 740 nm - 800 nm > 33) and this was confirmed visually as the tissue was sampled. The samples were handled in the same manner as in experiment I and O_2 uptake and CO_2 production were determined.

Experiment III. Extraction, identification and comparison of volatile materials in normal tissue and tissue affected by watercore:

To determine the occurrence of materials similar to ethanol and acetaldehyde in the apple tissues, highly volatile constituents of the tissue were extracted and measured.

Preliminary work involved lyophilizing frozen macerated tissue for several hours and capturing the evolved materials in three traps immersed in acetone-dry ice baths. This procedure attempted to stop enzymatic changes of tissue constituents by keeping the tissue sample in a frozen state as the volatile materials were extracted. When the trapping efficiency of this apparatus was tested by extracting acetaldehyde and ethanol from a solution containing 1% of each substance, it was found to be inadequate. Replacing the acetone-dry ice baths with liquid nitrogen baths greatly increased the trapping efficiency; however, to use liquid-N baths for such a long period of time seemed economically unreasonable. An Aerograph 90 -P chromatograph equipped with a thermal conductivity detector, was used during this phase of work and this lacked the sensitivity to detect the amounts of acetaldehyde trapped in the above manner.

A new approach was taken which would concentrate the amount of acetaldehyde in the final sample and accurately measure it. This involved inactivating the enzymes by exposing them to trichloroacetic acid, trapping the volatiles in a more efficient system, and using a chromatograph equipped

with a flame ionization detector. Specifically, the extraction and trapping method finally employed was as follows. The sliced pulp of three apples was frozen in liquid nitrogen and ground in a Waring blendor to a snowy consistency. Thirty grams of the frozen powdered tissue was placed in a 200 ml - capacity mason jar containing 150 ml of a 15% trichloroacetic acid solution to inactivate enzymes and prevent conversion of materials at the time of sampling. This slurry was mixed in an Osterizer for 10 seconds, transferred to a 1000 ml flask, and attached to the trapping apparatus. A Duo-seal vacuum pump was used to draw a vacuum for 15 minutes and the volatiles removed from the solution were trapped in two liquid-N traps.

The trapping system (Figure 3) was designed by R. A. Southwick, Department of Plant and Soil Sciences, University of Massachusetts, and has three important features. (1) Entry into each trap is through a large aperture enabling large amounts of water to be trapped without stopping the flow through the system. (2) Entry into the trap is below the joint, so all connections are exposed to ambient temperature. This allows immediate uncoupling of the ground glass joints when the vacuum is released. (3) The location of the inlet to each trap results in a circular flow around the stem of the male portion of the joint before going through the stem to the next trap. Plugs of glass wool were also placed between the 1000 ml flask and the first trap to interrupt the rapid flow of the highly volatile materials coming from the sample.

At the end of the evacuation period, the pump was stopped, the vacuum was gradually released, and the stopcock above trap

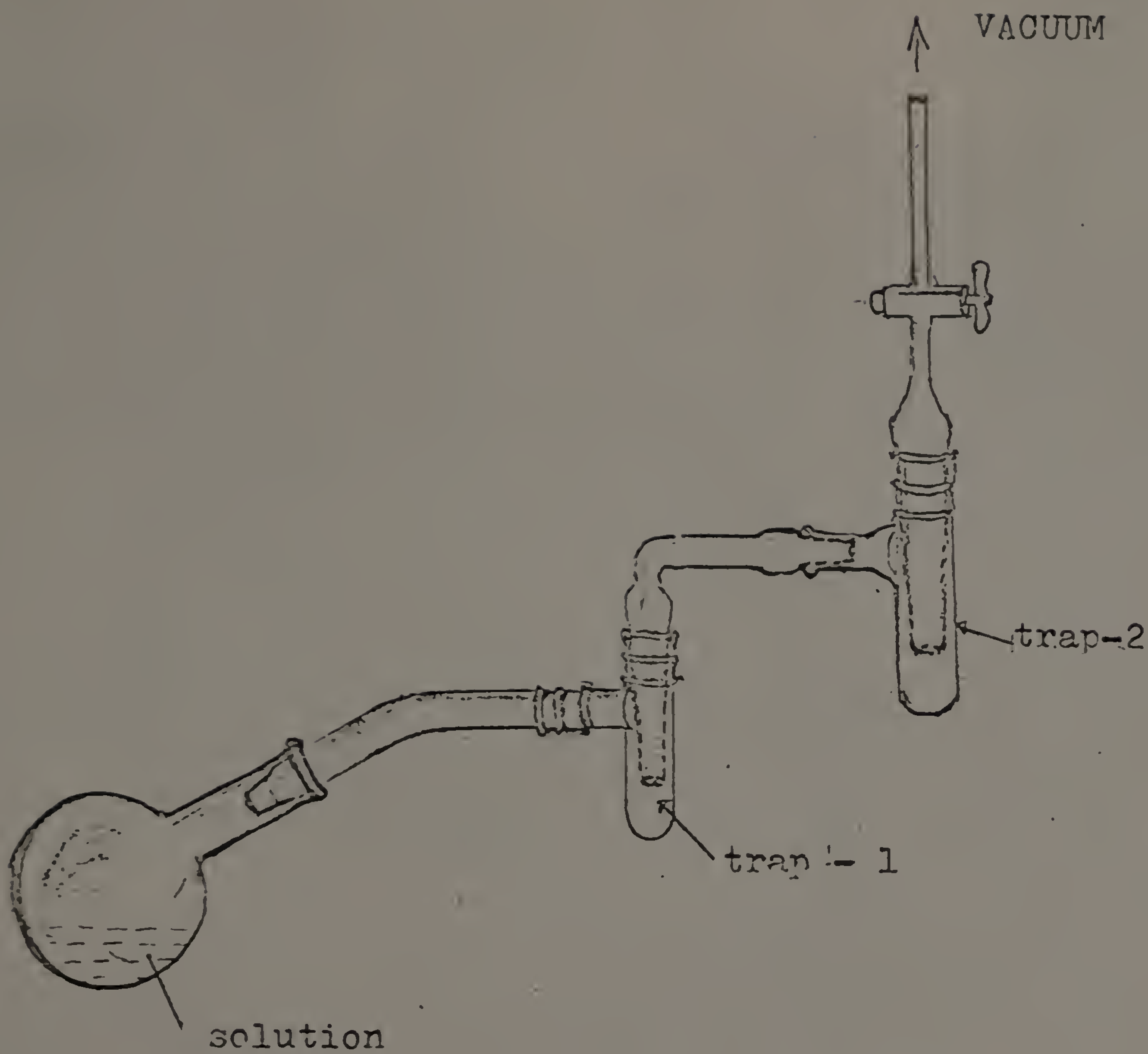


Figure 3. Graphical representation of the trapping apparatus employed to trap materials evolved from a cold slurry of apple tissue under vacuum.

II was closed. The elbow and 1000 ml sample flask were removed from trap I and a balloon was placed over the opening. Before trap I was lifted out of the liquid-N bath, it was disconnected from trap II and balloons were placed over these openings. Balloons rather than rubber caps were used for the following reasons. As the traps were removed from their liquid-N baths, a pool of liquid was noticed at their base. This quickly volatilized and tightly fitting rubber caps popped off during preliminary experiments. Part of this pool was probably liquified nitrogen since a similar pool of liquid was produced when a sealed trap was immersed in liquid N. Rubber balloons can expand and release this pressure very slowly and eliminate the possibility of an outrush of gas taking acetaldehyde or alcohol with it. Chromatographic analysis of head space during the melting procedure indicated that no acetaldehyde or ethanol was being lost through the balloon.

The frozen material in trap I was allowed to melt at room temperature and then used to pick up the adhering small droplets on the inside of the trap. The frozen volatiles in trap II were melted in the same manner. Since trap I contained the most liquid, its contents were transferred to trap II and used to take up the condensate in this trap. The combined volatiles of trap I and trap II constituted the sample that was analyzed. The sample volumes varied slightly among extractions varying from 2.0 ml to 2.7 ml, partly due to the fact that some droplets were unavoidably left in the traps.

It is assumed that the droplets left behind contained the same relative concentrations as the sample and their loss should not alter the results. This assumption is supported by good duplication between extraction of the same frozen tissue powder.

Extractions using solutions of known acetaldehyde and ethanol concentrations indicated that a 15 minute vacuum period was best for our purposes. After 15 minutes, more water relative to acetaldehyde and ethanol was extracted, causing a decrease in concentration of these two substances in the final sample. The concentration of volatiles in the sample is not the actual concentration in the tissue; however, if two tissue samples are handled in the same manner, any differences in the concentration of volatiles in the tissues should be reflected in the extracted sample. The effectiveness of the trapping system was demonstrated by extracting from duplicate samples of the tissue powder, whereby excellent duplication was consistently obtained. Samples were stored in a room maintained at -40° and were kept in a cold water bath of 15°C during the analysis period. Gas chromatographic analysis of the trapped volatiles was made with an Aerograph Hy-fi instrument (flame-ionization detector) equipped with an Esterline Angus Speed Servo recorder. A 6-foot long, 1/8-inch diameter copper column packed with 20% Carbowax on Chromosorb P was used in the Chromatograph. Helium served as the carrier gas, flowing through the column at a rate of 13 ml per minute. The peaks were identified by their retention

times at 94°C and 70°C and by addition of known substances to the samples.

Volatile extracts from severely watercored tissue, tissue which had recovered from watercore and tissue exhibiting internal breakdown were made and compared to extracts from normal tissue. In addition, extracts were made from severely watercored tissue of Jacob Sweet apples, to determine if the watercore of another variety produced the same effect as it did in Delicious.

Experiment IV. Respiratory capacity of mitochondria:

Preliminary experiments were conducted to determine optimum extraction and assay procedure for mitochondria, and these are described in Appendix II. These led to the adoption of the following procedures. For extraction, 100 grams of appropriate apple pulp were frozen in liquid nitrogen, macerated in a chilled Waring blendor, and transferred to 400 ml of extraction medium. This medium was composed of: 0.4M sucrose; 0.2M citrate (Na); 0.01M KH_2PO_4; 0.01M ethylene diaminetetraacetic acid (EDTA); 0.75% (w/v) polyvinyl-pyrrolidone (PVP) (13); and 0.01M cysteine hydrochloride. This medium was maintained at pH 8.0 with concentrated H_3PO_4 and with 20% KOH as the frozen macerated tissue was slowly added and blended into the solution. A Beckman Model G pH meter was used during this procedure. The cold slurry which resulted was centrifuged at 3,500 RPM (GSA rotor) for 10 minutes in a refrigerated Sorvall centrifuge to remove heavy cellular material and starch, and the supernatant fluid was strained through fine cloth. This supernatant fluid was further

centrifuged in a Spinco Model L ultracentrifuge at 28,000 RPM (30 rotor) for 30 minutes, and the green mitochondrial pellet and starch fraction obtained was washed, using a glass homogemizer, in 50 ml of a solution containing 0.4M sucrose, 0.1M EDTA, and 0.01M Tris (made to pH 7.5). The homogenate was spun for 10 minutes at 3000 RPM (30 rotor) to remove starch still remaining with the mitochondrial fraction. The pellet which resulted from centrifugation at 28,000 RPM (30 rotor) for 30 minutes was suspended in 5.0 ml of 0.2M sucrose (pH 7.5) using the homogenizer, and was used as the mitochondrial preparation.

Succinoxidase activity was determined in a Gilson Differential Respirometer, model GRP 20. The Gilson flasks contained 400 umole sucrose, 37.5 umole KH_2PO_4, 10 umole $MgSO_4$, 3 mg crystallized bovine plasma albumin, 2 mg yeast concentrate, 40 umole succinate, 0.56 umole cytochrome-c, 10 umole ADP (when used), 0.25 to 1.0 ml of mitochondrial preparation, and water to make a total of 3 ml. Yeast concentrate was used as a source of co-factors such as ATP, NAD, thiamine pyrophosphate and CoA (13). The reaction mixture was adjusted to pH 7.5 and the mitochondrial preparation and reaction mixture temperature equilibrated separately before combining. Oxygen uptake was measured at 25°C, using a shaker speed of 125 strokes per minute and allowing 15 minutes for temperature equilibration before mitochondria were added to the medium and readings were begun. The succinoxidase activity is expressed as ul O_2/mg protein-hr. on the basis of the first 15 minutes of activity.

Protein content of the mitochondrial preparation was used as an indication of the amount of mitochondria present. The amount of protein extracted per preparation ranged from 4.5 mg to 11.0 mg, averaging 1.7 mg/ ml prep. The Lowry method (21) of protein determination, slightly modified (Apendix III), was found to be consistent and convenient and was employed in all experiments dealing with mitochondria.

To compare the respiratory capacity of mitochondria isolated from normal and severely watercored tissue, five mitochondrial preparations of each type of tissue were made periodically between December 13, 1966 and February 1, 1967. Succinoxidase activity was used as a measure of their respiratory capacity. The respiratory control of representative preparations was also measured by the addition of ADP to the reaction medium.

RESULTS

Experiment I. Respiratory pattern of watercored tissue:

Both O_2 uptake and CO_2 evolution were depressed in Delicious apple tissue exhibiting watercore, as is illustrated in Table 1. Considerable variability did exist among replications, probably because one apple was used for each replication and variation in respiration rates between individual apples can be expected; nevertheless, when the replications for each sampling date were averaged (Table 1); there existed a marked depression of O_2 uptake in severely watercored tissue and a lesser depression of O_2 uptake in moderately watercored tissue. A similar pattern was recorded for CO_2 evolution, but the magnitude of depression was considerably less. The R.Q. (CO_2/O_2) values were consequently higher for both moderately and severely watercored tissue than for that free of watercore. An average of 12 assays (Table 2) shows a 26% decrease in O_2 consumption in severely watercored tissue, significant at the 2.5% probability level, and a decrease in CO_2 evolution of 14%, significant at the 10% probability level. For tissue exhibiting severe watercore, the R.Q. rose 12%, from 1.51 in normal tissue to 1.71 in the affected tissue and this was significant at the 2.5% probability level. These data indicate a disruption of the normal respiration pattern due to the presence of watercore, suggesting that a partially anaerobic environment may have been created by the watersoaked condition of the tissue.

TABLE 1. Effect of watercore on O_2 uptake, CO_2 evolution and Respiratory Quotient (R.Q.) of Delicious apple tissue at four sampling dates.

Watercore intensity	Sampling date			
	11/14	11/28	12/11	12/26
	O_2 uptake (ul O_2/gm (dw) - 2 hours)			
None	580[1/]	553	660	796
Moderate	527	635	510	476
Severe	453	466	476	518
	CO_2 evolution (ul CO_2/gm (dw) -2 hours)			
None	822[1/]	887	871	1135
Moderate	759	994	862	990
Severe	697	782	796	957
	R. Q. (CO_2/O_2)			
None	1.42[1/]	1.64	1.32	1.42
Moderate	1.44	1.56	1.69	1.65
Severe	1.53	1.67	1.67	1.67

1/ Each value is an average of 3 replications.

TABLE 2. Summation of effects of watercore on respiration of Delicious apple tissue.

Watercore intensity	ul O_2 uptake per gm (dw) - 2 hr.	ul CO_2 evolved per gm (dw) - 2 hr.	R.Q. (CO_2/O_2)
None	647 1/	929	1.51
Moderate	569	901	1.60
Severe	478 2/	808 3/	1.71 4/

1/ Each figure is an average of 12 assays (3 replications of 4 periods).

2/ Significant at 1% probability level.

3/ Significant at 10% probability level.

4/ Significant at 2.5% probability level.

Some preliminary work done with watercored Yellow Newtown apple tissues also suggested the presence of a partially anaerobic condition in affected tissues. Table 3 shows that the effect of severe watercore on the respiratory pattern of this tissue was rather similar to the effect on Delicious tissue. Oxygen consumption was depressed 30 - 35% by the disorder. Carbon dioxide evolution was greatly increased over that of the control, unlike the small but consistent decrease in CO_2 evolution from watercored Delicious tissue. Consequently, the R.Q. was increased two-fold by the watercored condition in Yellow Netown apple, an increase much greater than was found for Delicious.

<u>Experiment II. Respiration of tissue following recovery from watercore</u>:

The storage behavior of fruits selected for use in this experiment was in many ways similar to that of previous years (2). Fruits exhibiting watercore at harvest were more susceptible to internal breakdown during storage than those free of watercore (Table 4). By January 7, about 2½ months after harvest, none of the fruit without watercore had developed internal breakdown, while those containing moderate and severe watercore developed 22% and 89% breakdown, respectively. No additional breakdown occurred after this time in fruit classed as having had moderate watercore, and only 3% more internal breakdown appeared in the severe watercore class, this occurring by February 11. A great number of the fruit, especially those severely watercored, developed internal breakdown while watercore was present. Only one severely watercored fruit

TABLE 3. Respiratory pattern of severely watercored Yellow Newton apple tissue[1].

Watercore intensity	ul O_2/gm (fw) -2hr.		ul CO_2/gm(fw) -2 hr		R.Q. (CO_2/O_2)	
	Oct. 6	Oct. 15	Oct. 6	Oct. 15	Oct. 6	Oct.15
None	57.0[2]	60.1	69.2	65.8	1.21	1.09
Severe	37.0	43.1	88.0	106.0	2.38	2.45

1/ Each Gilson flask contained a 1-gram tissue cylinder, taken with a No. 4 (9 mm) stainless steel cork borer, and 2 ml of pH 4.0 potassium phosphate buffer.

2/ Each value represents an average of 2 duplicate flasks.

TABLE 4. Storage behavior of fruits stored at 32°F and used in Experiments I and II.

Initial watercore intensity	Date								
	11/11	11/25	12/9	1/7	1/26	2/11	2/25	3/30	5/11
	Percent of fruits free of both watercore and internal breakdown								
None (25)[1]	100	100	100	100	100	100	100	100	100
Moderate(32)	0	13	34	56	72	75	75	75	78
Severe (112)	0	0	1	4	5	5	5	5	5
	Percent of fruits with internal breakdown								
None (25)	0	0	0	0	0	0	0	0	0
Moderate (32)	2	6	13	22	22	22	22	22	22
Severe (112)	4	17	46	89	91	92	92	92	92

1/ Numbers in parentheses refer to fruits in each watercore class.

devloped internal breakdown after watercore disappeared; all others developed internal breakdown while some watercore was present. The simultaneous occurrence of both disorders has often been observed (16, 20). However, this season's storage behavior is in striking contrast to the behavior during the 1965-1966 season (2), when about 94% of the fruit lost watercore before internal breakdown developed. This illustrates the seasonal variation of watercore behavior. It can be seen in Table 4 that not all fruit containing watercore at harvest developed internal breakdown; many moderately water-cored and a few severely watercored fruit recovered from this disorder without developing internal breakdown. During this study none of the fruit initially determined to be free of watercore developed internal breakdown in storage, although in previous studies (2) limited amounts of breakdown did occur in fruit never having had detected watercore.

Experiment II was designed to determine the existence of any residual effect of watercore on the respiration of Delicious apple tissue. The respiration rate of tissue having moderate and severe watercore at harvest but now free of disorder is compared in Table 5 to the respiration rate of tissue never having had watercore. An average of 6 replications indicates that there was little difference in O_2 consumption, CO_2 evolution, or R.Q. among the tissues sampled. In comparing tissue never having had watercore with tissue initially having had severe watercore but now free of it, the latter possessed a 7% greater O_2 uptake and a 2% lower CO_2 evolution. The average respira-

TABLE 5. Respiratory pattern of Delicious apple tissues following recovery from watercore.

Initial watercore intensity	ul O_2 uptake/gm (dw) - 2hrs.	ul CO_2 evolved/gm (dw) - 2 hrs.	R.Q. (CO_2/O_2)
None	685 ± 161	1086 ± 288	1.6 ± 0.11
Moderate	790 ± 157	1078 ± 243	1.4 ± 0.07
Severe	738 ± 94	1070 ± 170	1.5 ± 0.13

1/ Each value is an average of 6 replications, with the calculated standard deviations indicated.

tory quotient for severely watercored tissue was consequently 6% lower than for "normal" tissue. The existence of only these small and inconsistent differences seem to indicate that once watercore disappears the pattern of respiration in the tissues returns to essentially that of fruit never having had the disorder. Thus there appeared to be no permanent effect of watercore on the respiration of apple tissue.

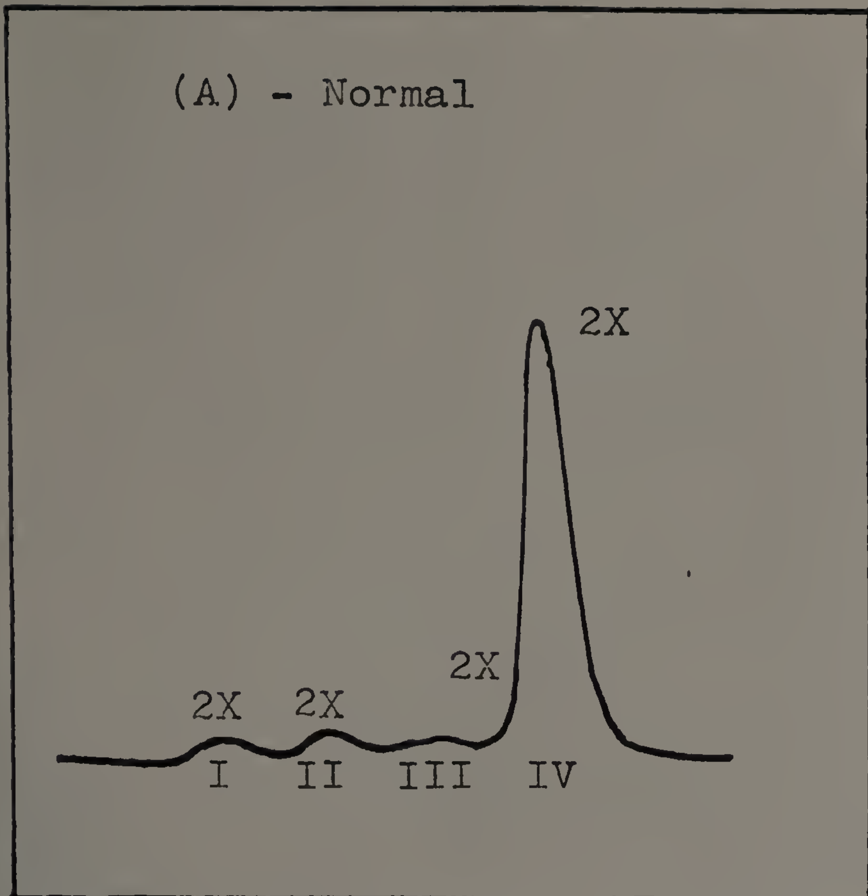
In a supplementary study, the respiratory pattern of some tissue exhibiting internal breakdown was determined in the same manner as in Experiments I and II. The results of two replications are given in Table 6. The tissue exhibiting internal breakdown consumed less O_2 and evolved equal or greater amounts of CO_2 than normal tissues. Hence, the R.Q. values were considerably higher for the internal breakdown tissue than for the normal tissue.

Experiment III. Extraction, identification, and comparison of some volatile materials in normal tissue and tissue affected by watercore:

Typical chromatograms of extracts from normal and severely watercored tissue are illustrated in Figure 4. The major chromatogram peaks were tentatively identified, in the order of increasing retention times, as acetaldehyde, acetone, ethyl acetate, and ethyl alcohol by comparing their retention times to those of known substances at 94°C and at 70°C. A symmetrical increase in peak size after addition of small amounts of a known substance to the sample further verified their identification. The data obtained were relatively consistent among

TABLE 6. The effect of internal breakdown (IB) on the respiratory pattern of Delicious apple tissue.

Measurement	August 24		August 27	
	Tissue condition			
	Normal	IB	Normal	IB
ul CO_2/gm (fw) - 30 min.	20.1	19.3	20.3	27.6
ul O_2/gm (fw) - 30 min.	19.1	11.2	18.0	11.6
R.Q. (CO_2/O_2)	1.1	1.7	1.3	2.4



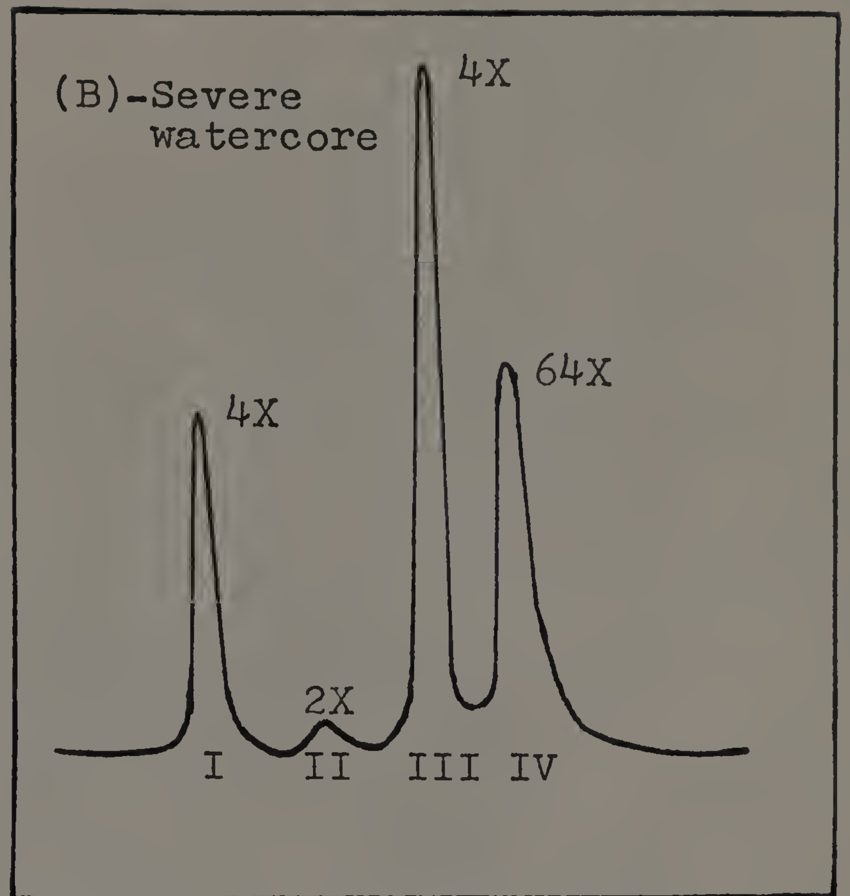


Figure 4. Tracings of typical chromatograms of volatile extracts from normal (A) and severely watercored (B) Delicious apple tissue.[1] Peaks were identified as I, acetaldehyde; II, acetone; III, ethyl acetate; IV, ethanol

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1/ 2.0 ul samples of extracts were analyzed chromatographically at 70° as described in "Materials and Methods", Experiment III. The symbol x refers to attenuation, 2x means ½ maximum sensitivity, 4x means ¼ maximum sensitivity, and so forth.

the apples samples and between consecutive extractions of same apple tissue (Appendix, Table 5).

Analysis of the volatiles extracted (Table 17) reveals the presence of greater than normal amounts of acetaldehyde and ethanol in severely watercored tissue. The area under the acetaldehyde peaks recorded for severe watercore samples averaged 5.3 times greater than the area under those peaks recorded for normal tissue. The acetone peaks remained relatively constant among all extracts and therefore the areas were not measured. The area under the ethanol peaks was 12.2 times greater for severe watercore samples than for extractions from normal tissue. A third component in the tissue extracts which was consistently greater in watercored tissue was identified as the ethyl ester of acetic acid. The area under these peaks was on the average 10.7 times greater than normal in extracts of severely watercored tissue.

After watercore disappeared, the concentrations of acetaldehyde, ethyl acetate, and ethanol decreased, with ethanol decreasing the most. The concentrations appeared to be even less in tissues exhibiting internal breakdown, yet were still much greater than the concentrations found in normal apple tissue.

The build-up of acetaldehyde and ethanol in severely watercored tissue indicates a shift from the normal metabolic pathways of aerobic respiration to those involved in fermentation, since these materials are typically fermentation products.

TABLE 7. Comparison of three volatiles extracted from tissue representing the normally observed pattern of water-core (WC) behavior during cold storage of Delicious apples.

Volatile components	Condition of tissue			
	Normal[1/]	Severe WC[1/]	Recovered from severe WC[2/]	Internal breakdown[3/]
Acetaldehyde	5.7×10^{-3} [4/]	30.2×10^{-3}	24.7×10^{-3}	11.0×10^{-3}
Ethyl acetate	55.7×10^{-3}	61.3×10^{-3}	34.8×10^{-3}	26.5×10^{-3}
Ethanol	1.1×10^{-1}	13.4×10^{-1}	3.1×10^{-1}	2.3×10^{-1}

1/ Values for normal and severe WC tissues are the averages of 6 extracts, 3 replications with duplicates of each.

2/ Values for tissue having recovered from severe WC are the averages of 5 extracts, 3 replications with duplicates for only 2.

3/ Values for internal breakdown tissue are the averages of 4 extracts, 2 replications with duplicates of each.

4/ All values represent areas under peaks recorded when 2 ul samples were analyzed chromatographically at 90 °C as described in "Materials and Methods", Experiment III. Areas were measured with a planimeter and are expressed as square centimeters.

In a supplementary test, some severely watercored Jacob Sweet apples were extracted in the same manner as Delicious tissue. However, no "normal" tissue of this variety was available, so comparison can be made only with "normal" Delicious tissue. These extracts from severely watercored tissue of Jacob Sweet apples contained large amounts of acetaldehyde, ethanol and ethyl acetate (Table 8): twice the amount of acetaldehyde, 10 times the amount of ethyl acetate, and 4-5 times the amount of ethanol as in non-watercored Delicious. These data support the premise that watercore results in the accumulation of fermentation products in apple tissues.

In a brief study, meant to confirm the very early reports of acetaldehyde toxicity, Delicious apple were determined to be free of internal breakdown, using the Biospect, and were subjected to an atmosphere high in acetaldehyde, ethyl acetate, or ethanol. This was done by placing seven fruit in a 9-liter capacity desiccator containing 2 ml of acetaldehyde, ethyl acetate, or ethanol. Superficial browning occurred on only one of seven fruits exposed to the ethyl acetate over a three-week period, whereas all seven of the fruit subjected to the acetaldehyde exhibited superficial tissue browning in 60 hours (Figure 5), and browning to the core in three weeks (Figure 6). No effect of the atmosphere high in ethanol was observed over the three-week period.

As a follow-up to this study, 5 apples were exposed to a greater amount of ethanol (10 ml/9 L air) and again there was no observable response.after a period of three weeks.

TABLE 8. Measurement of some volatile materials extracted from severely watercored Jacob Sweet apple tissue.

Replication	Acetaldehyde	Ethyl acetate	Ethanol
1	14.2×10^{-3} [1/]	54.1×10^{-3}	4.9×10^{-1}
2	12.9×10^{-3}	55.9×10^{-3}	4.1×10^{-1}

1/ Values represent areas under peaks recorded when 2 ul samples were analyzed chromatographically as described in "Materials and Methods", Experiment III. Areas were measured with a planimeter, and are expressed as square centimeters of area.

Figure 5. Internal condition of Delicious apples exposed to 2 ml of acetaldehyde or ethyl acetate in 9 L air for 60 hours at 85°F. Note only one of seven fruit was affected by the ethyl acetate atmosphere.

Figure 6. Internal condition of 'Delicious apples exposed to 2 ml of acetaldehyde or ethyl acetate in 9 L air for 19 days at 85°F.

Experiment IV. Respiratory capacity of mitochondria:

Any damage occurring to the mitochondria in cells would be expected to produce recognizable injury in the fruit tissues. Thus, tissue breakdown might result from injury to these organelles while cells are subjected to a watercored condition. Mitochondria can be extracted from cells and deviations from their "normal functioning" can be determined experimentally.

Normal activity has been described in three ways (14): 1) as a constant rate of oxidation of a given substrate by the mitochondria; 2) as a doubling of this oxidation rate when twice the amount of mitochondria (within certain limits) are assayed; and 3) as a positive response to addition of ADP, in terms of increased substrate oxidation by the mitochondria. Extracted mitochondria which demonstrated these capacities were considered to be functioning normally. Chance and Williams (5) have described a variety of metabolic states of mitochondria and the associated oxidation-reduction levels of the respiratory enzymes. Two of these states are of particular relevance to our experiment. "State 4" was described as a "resting" state, characterized by a low respiration rate even though substrate is present. Definite conditions are required for its establishment; mitochondria must be carefully prepared and supplied with substrate, but without phosphate acceptor (ADP). When adequate substrate and phosphate acceptor are present, an "active" state of rapid respiration and phosphorylation is established, and this was termed "state 3".

In accordance with Lance, et al. (17), the state 3 rate of activity was taken as the most reliable criterion of respiratory capacity. A summary of the results for the measurements of state 3 succinoxidase activity of the mitochondria is given in Table 9. It can be seen that no consistent difference in succinoxidase activity existed between the types of tissue from which mitochondria were extracted. Furthermore, duration of watercore in the tissues had no effect on mitochondrial activity. Thus, the presence of watercore in the apple tissue appears to have had no effect on the succinoxidase activity of extracted mitochondria. Furthermore, it can be seen in Table 9 that the amount of protein extracted during each mitochondrial preparation was relatively uniform, and that no consistent change occurred with time or with condition of the tissue. This indicates that extraction was equally efficient for both types of tissue.

In Figure 7, typical state 3 succinoxidase activity for mitochondria extracted from both normal and severely watercored tissue are illustrated. For both mitochondrial extracts, there was linearity of response and there was a doubling of the oxidation rate when twice the amount of mitochondrial preparation was used. While these data represent only one assay, they are typical of the results obtained in this experiment. Hence, mitochondria from both types of tissue appeared to be functioning normally.

TABLE 9. Comparison of total protein extracted, and of "state 3" succinoxidase activity of mitochondria isolated from the pulp of normal and severely watercored (WC) Delicious apple after different intervals of storage.

Tissue condition	Date	ul O_2 uptake/mg protein -hr[1]	Total mg of protein extracted
Normal	Dec. 13	122	10.0
Normal	Dec. 28	176	4.5
Normal	Jan. 10	107	10.5
Normal	Jan. 19	73	11.0
Normal	Jan. 31	93	9.5
Average		114	9.1
Severe WC	Dec. 14	124	7.0
Severe WC	Dec. 29	120	6.5
Severe WC	Jan. 11	79	11.0
Severe WC	Jan. 20	104	8.0
Severe WC	Feb. 1	103	8.5
Average		103	8.2

1/ Based on first 15 minutes of activity using 0.5 ml of mitochondria preparation.

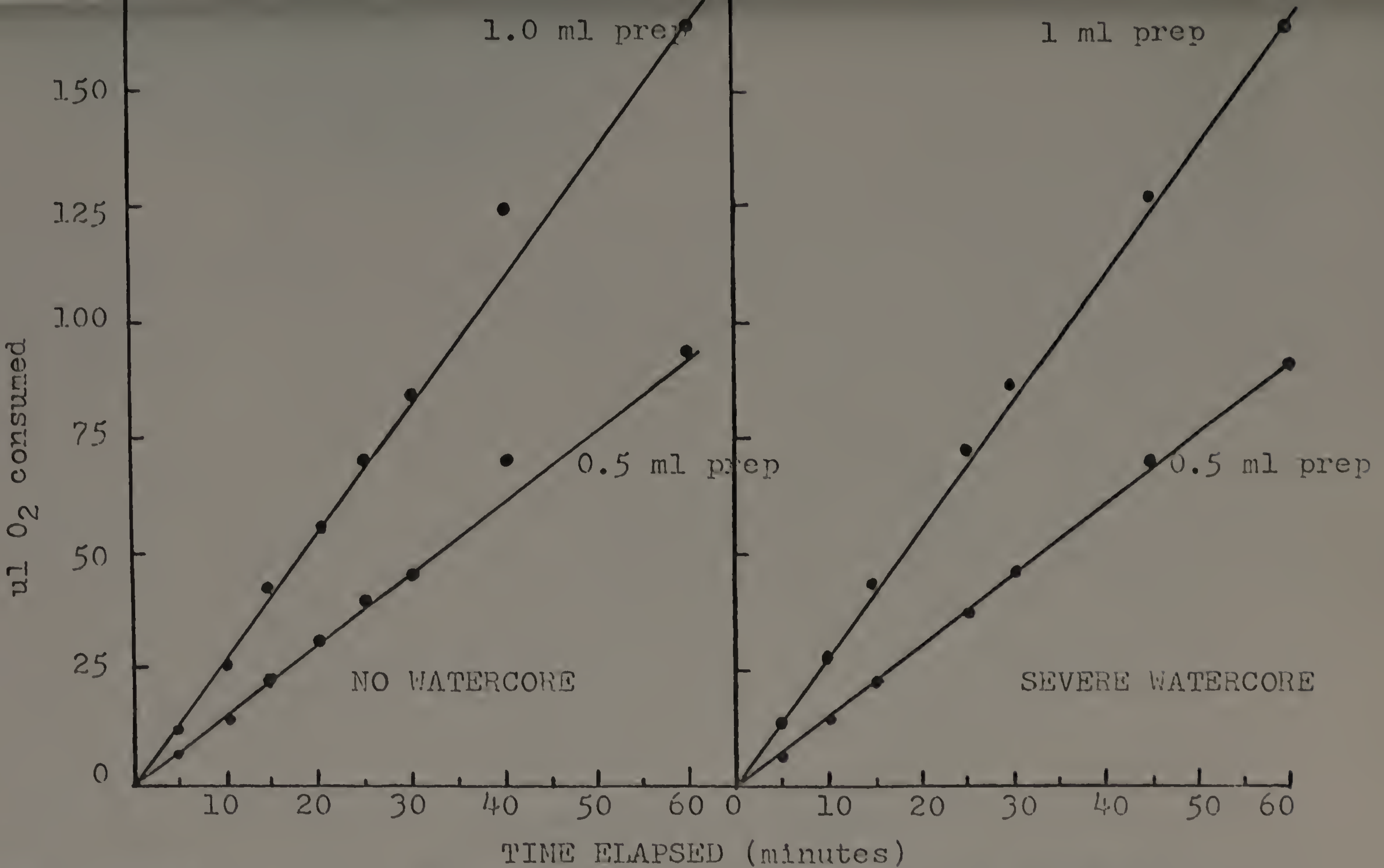

Figure 7. "State 3" succinoxidase acitivty of mitochondrial preparations from normal and severely watercored Delicious apple tissue[1/].

1/ Oxygen consumption is expressed in terms of the amount of mitochondrial preparation used. Both preparations contained 2.2 mg protein per ml prep.

The third criterion for "normal" behavior of mitochondria is a positive response to the addition of ADP, that is, an increased substrate oxidation by the mitochondria. This phenomenon is referred to as "Respiratory Control" and following the terminology of Chance and Williams (5), it is a change of state 4 respiration to state 3. In Table 10, the response of the apple mitochondria to the addition of ADP can be seen. The mitochondria from both the non-watercored and the watercored tissue did exhibit the positive response to ADP (i.e., respiratory control) for as ADP was added to the reaction mixture, an increased rate of O_2 consumption occurred.

To compare the magnitude of respiratory control (R.C.) in mitochondria from the normal and watercored tissue, I calculated R.C. values in the following manner. The O_2 uptake during the 30-minute period after the addition of ADP was divided by 30 minutes of O_2 uptake in the absence of ADP. Representative respiratory control values are shown in Table 10. Values of about 2.2 were common for mitochondria isolated from both normal and severely watercored tissue. These data show that mitochondria from both types of tissue responded very similarly to added ADP and it can consequently be concluded that both mitochondrial extracts possessed the same degree of "coupling" of oxidative phosphorylation.

In experiment IV, it was demonstrated that mitochondria from non-watercored and watercored tissues were functioning normally and very similarly, and therefore watercore seems to have had no permanent effect on mitochondrial behavior.

TABLE 10. Respiratory Control (R.C.) values for mitochondria isolated from normal and severely watercored tissue.[1]

Tissue used	ul O_2 uptake/0.5 ml prep.- 30 min.		R.C.	Mg protein/ml prep.
	+ ADP	-ADP		
Normal	46.0[2]	23.2	2.0	1.5
Severe WC	38.6	18.6	2.1	1.4

1/ Mitochondria were isolated as described in "Materials and Methods", Experiment IV. The assay medium contained; 400 umole sucrose, 37.5 umole KH_2PO_4, 10 umole $MgSO_4$, 3 mg Bovine serum albumin, 2 mg yeast concentrate, 40.0 umole succinate, 0.56 umole cytochrome-c, and 10 umole ADP (when used) brought to a final volume of 2.5 ml with H_2O. The reaction was started by the addition of 0.5 ml mitochondrial preparation.

2/ Each value represents an average of duplicate flasks. In determining the R.C. value for the "normal" prep., ADP was added at zero time, whereas ADP was added 2 hours after zero time for the severe watercore prep.

DISCUSSION

Some relationship between watercore and internal breakdown has long been known to exist, yet little research has been done to clarify its nature. Experiments were initiated to determine some of the effects of watercore on Delicious apple tissue in hopes of finding an explanation for the development of internal breakdown.

The results of respiratory studies of tissue cylinders showed that both O_2 consumption and CO_2 evolution were depressed and the R.Q. was increased by the presence of watercore in Delicious apples. Changes in the R.Q. can result from decarboxylations unrelated to respiration proper as well as from reductive events such as sulfate and nitrate reduction or oxidative events such as those that occur in the phenolase system. However, the accumulation of ethanol and acetaldehyde, which are typical products of fermentation, strongly suggests that the increased R.Q. was at least largely due to induction of partial anaerobiosis in the watercored tissues.

Although the concentrations of O_2 and CO_2 in affected tissue have not been measured, it seems likely that the watercore condition, which includes infiltration of intercellular spaces with liquid, would reduce O_2 availability and retain, to a certain extent, respired CO_2 in the tissue. Thomas (27) showed that normal aerobic respiration can be altered in a high CO_2 environment, even though varying amounts of O_2 are

present, and this can result in the formation of higher than normal amounts of acetaldehyde and ethanol just as if O_2 were absent. In this fermentation associated with some O_2, the ratio of ethyl alcohol/acetaldehyde that accumulated in the tissues was 2/1, contrasting strikingly with the 50/1 ratio obtained in anaerobic fermentation. He believed that a high CO_2 environment in the presence of O_2 may be even more injurious to apples than anaerobic conditions, owing to the greater accumulation of acetaldehyde and its apparent toxicity to apple tissue, for partial anaerobiosis was accompanied by protoplasmic disorganization and browning of superficial cells. This cellular disorganization and tissue browning following CO_2 -zymasis seems to be similar to the condition which occurs in Delicious apple tissue as they develop "breakdown", except that here the condition is internal whereas in Thomas' work it was external.

I have presented evidence indicating that acetaldehyde and ethanol do accumulate in watercored tissue, and that their concentrations decreased but were still relatively high after watercore disappeared and right up to the development of internal breakdown, but I have not determined the actual concentrations that were present. Hence, direct comparison of acetaldehyde and ethanol concentrations can not be made between tissue exhibiting watercore and tissue in which Thomas induced browning and protoplasmic disorganization. However, a comparison of the relative increase of both acetaldehyde and ethanol in these two types of tissues can be made. From the

data in Table 7, the ratio of the area under the ethanol to that under the acetaldehyde peak for extracts from normal tissue was calculated ($1.10 \times 10^{-1}/0.06 \times 10^{-1} = 19.3/1$). This was compared to the same ratio of areas under peaks of ethanol and acetaldehyde representing extracts from water-cored tissue ($13.4 \times 10^{-1}/0.30 \times 10^{-1} = 40.1/1$). The ratio 19.3/1 obtained for normal tissue should not be taken to mean that the ethanol concentration was 19.3 times greater than the acetaldehyde concentration. Equal amounts of ethanol and acetaldehyde, analyzed chromatographically with a flame ionization detector, will not be recorded as peaks of equal area because each ionizes to a different extent. Nevertheless, the degree of ionization of a relatively pure substance is constant and detection of twice the amount of this substance will result in a peak having twice the area. Therefore, an increase in the area representing a substance, or in our case an increase in the ratio of areas of two substances, can be taken as reliable evidence of the relative increase in the concentrations. The ratio of the area under ethanol peak/acetaldehyde peak rose from 19.3/1 in volatile extracts from normal tissue to 40.1/1 in extracts from severely watercored tissue; this indicates that ethanol increased 2.1 times as much as acetaldehyde.

When Thomas' data (27, p 940) are recalculated and represented in the same manner(the ratio of ethanol concentration/acetaldehyde concentration in normal tissue divided by the ratio of ethanol concentration/acetaldehyde concentration

in affected tissue), ethanol is shown to increase 2.2 times as much as acetaldehyde in tissue exhibiting CO_2-zymasis. That increases in ethanol relative to increases in acetaldehyde are similar in both types of tissue seems to indicate that only a partially anaerobic enrivonment was created in watercored tissue.

A third volatile, identified as the ethyl ester of acetic acid, was also found to accumulate in watercored tissue. On chromatograms of normal tissue, the peaks were usually smaller than acetaldehyde peaks and the areas under them were difficult to measure. The area under ethyl acetate peaks on chromatograms representing severely watercored tissue were on the average 10.7 times greater than for normal tissue. The accumulation of ethyl acetate in watercored tissue was twice that of acetaldehyde. It has been shown that ethyl acetate can produce injury to apple tissues (24). Subjection of Jonathan apples to an atmosphere reportedly saturated with ethyl acetate produced superficial scald, a killing of the epidermal and hypodermal cells of the fruit.

When I exposed Delicious apples to an atmosphere high in ethyl acetate (2 ml in 9 l air), superficial tissue browning occurred on only one of seven fruits. On the other hand, all seven of the fruits subjected to an atmosphere containing the same concentration of acetaldehyde exhibited superficial tissue browning in 60 hours, and browning to the core in three weeks. The browning resulting from the acetaldehyde environment was associated with a more intense vascular browning and with a spongy texture, which are characteristics of Delicious

apple tissue exhibiting internal breakdown following watercore. An atmosphere containing 2 ml of ethanol per 9 l of air had no apparent effect on Delicious apple tissue over a three-week period. These observations suggest that of the three volatiles found accumulating in watercored tissue, acetaldehyde is the most toxic. However, it remains to be determined the actual concentrations of these substances that accumulate in a watercored area and whether or not these levels are toxic to the tissue.

Mitochondria extracted from severely watercored tissue and assayed in a medium containing adequate substrate and co-factors exhibited respiratory capacity in terms of succinoxidase activity, and respiratory control similar to mitochondria extracted from normal tissue. These results suggest that the watercore environment was not altering the capacity of mitochondria to oxidize succinic acid (although the possibility that utilization of other respiratory intermediates might be irreversibly affected can not be ignored). The *in vitro* demonstration of normal respiratory activity of mitochondria does not mean, of course, that the mitochondria were functioning normally in a watercore environment. In fact, respiratory studies of intact watercored tissue cylinders indicated that they were not functioning at full capacity in such environment, for O_2 consumption was suppressed. But once watercore disappeared from the tissues, their respiratory pattern was no longer distinguishable from that of tissue that never possessed the disorder. These findings

support the contention that watercore merely imposes a temporary environmental control over respiration and that this environment can be removed and its effect reversed. The mitochondria seem to be free of any actual injury from watercore.

SUMMARY

A series of experiments were designed and conducted to determine if the presence of watercore initiated anaerobiosis in apple tissue. The overall respiratory pattern of intact tissue cylinders was studied and the accumulation of volatile materials such as ethanol was determined. The physiological condition of the mitochondria of affected tissue was studied by extraction and assay of these subcellular particles.

These studies showed that:

1. Severe watercore caused a highly significant decrease (26%) in O_2 consumption by Delicious apple tissue. CO_2 evolution was decreased 14% in severely watercored tissue and this was significant only at the 10% level. Consequently, the R.Q. rose from 1.51 in normal tissue to 1.71 in severely watercored tissue, significant at the 2.5% level.

2. When Delicious apple tissue recovered from severe watercore, the respiratory pattern, in terms of O_2 consumption, CO_2 evolution, and R.Q. differed very little from tissue never having had watercore.

3. Three volatile materials, tentatively identified as acetaldehyde, ethyl acetate and ethanol, --- were shown to accumulate in watercored Delicious apple tissue. Since the actual concentrations of these volatiles in this tissue were

not determined, their increase is expressed in terms of area under the recorded peaks. The areas under the acetaldehyde, ethyl acetate and ethanol peaks recorded for severe watercore samples averaged 5.3, 10.7 and 12.2 times greater, respectively, than the areas under those peaks recorded for normal tissue.

4. After tissue recovered from severe watercore, the concentrations of acetaldehyde, ethyl acetate, and ethanol decreased, but still remained relatively high right up to the time that the tissues developed internal breakdown. In breakdown tissue, the areas under the acetaldehyde, ethyl acetate, and ethanol peaks averaged 2.1, 4.6 and 1.9 times greater, respectively, than normal tissue.

5. The respiratory capacity, in terms of both succinoxidase activity and the respiratory control value, was similar for mitochondria extracted from normal and from severely watercored Delicious apple tissue.

CONCLUSION

Watercore appears to be imposing an environmental effect on Delicious apple tissue, by creating a partially anaerobic condition in the affected areas. The fermentation products which accumulate in these water-soaked tissues remain in relatively high concentration after watercore disappears, even though the respiratory pattern seems to return to "normal". In watercored areas, the effect on mitochondria in cells does not appear to be a permanent one, as removal to an "ideal" environment shows no obvious damage in terms of succinoxidase activity and respiratory control.

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APPENDIX

I. Selection of pH:

To insure that all tissue plugs were in a similar environment, they were immersed in 2 ml of buffered solution. Neal and Hulme (22) reported use of a pH 4.0 buffer in determining respiration of apple peel discs but without an explanation for his choice of pH. A preliminary study was conducted to determine the effect of pH on the respiratory pattern of tissue plugs. The data presented in Appendix Table 1 show that greatest O_2 uptake and CO_2 evolution was obtained when a phosphate buffer at or near pH 4.0 was used. This, coupled with the fact that an apple tissue macerate has a pH around 4.0, led to the use of a pH 4.0 buffer in our experiments.

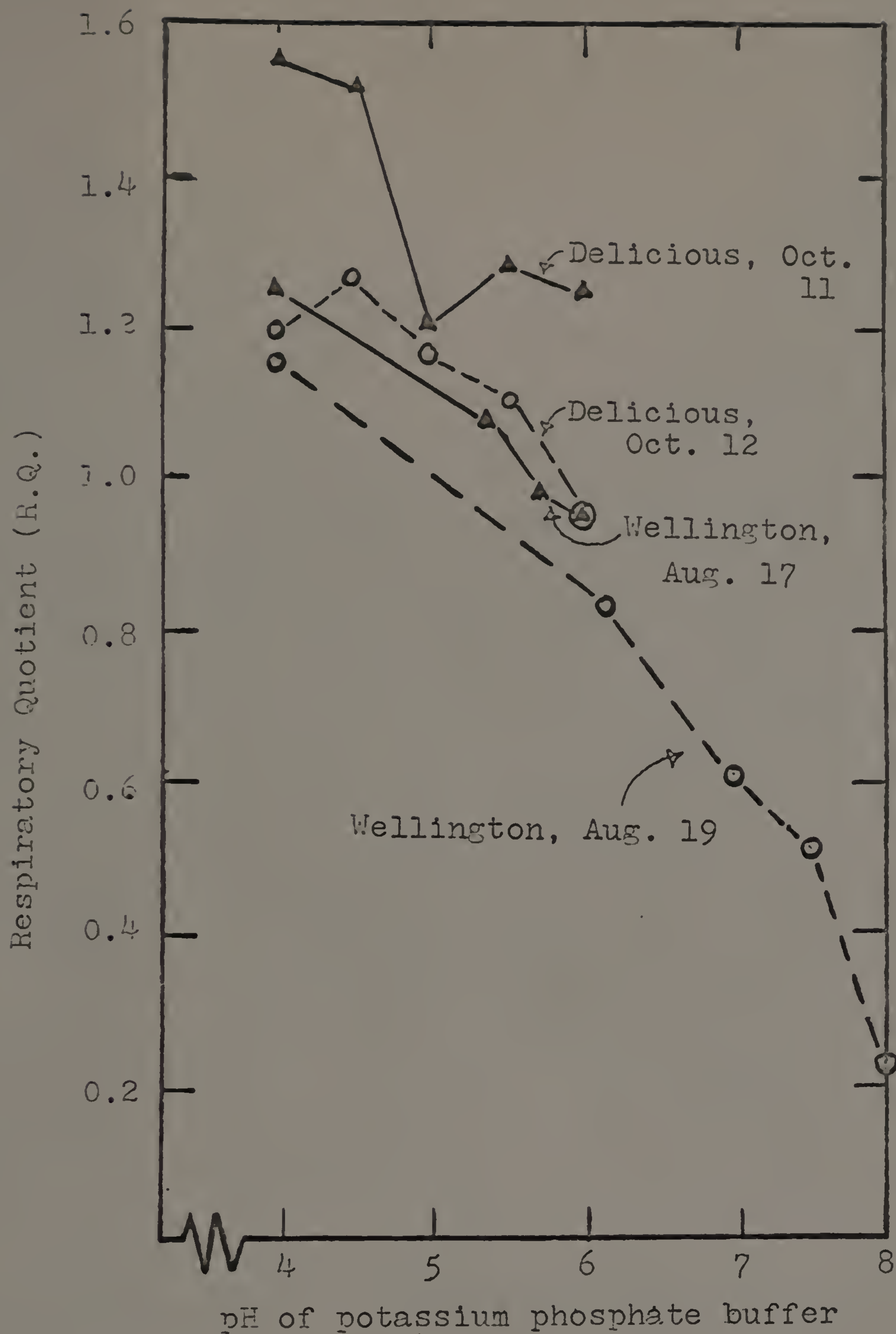
Appendix Figure 1 shows the effect of pH on the respiratory quotient (R.Q.). As the pH increased from 4.0 to 8.0, the R.Q. decreased. An explanation for this phenomena may be a difference in solubility of CO_2 at various pH values; more CO_2 dissolves in a buffer solution of higher pH values.

II. Preliminary approaches to mitochondrial extraction and assay.

Preliminary work was conducted using early apple varieties, such as Early McIntosh, June Wealthy and Wellington, and the following extraction medium and wash solution, which proved to be very successful for Haard (12)

Appendix Table 1. Effect of potassium phosphate buffer pH on O_2 uptake, CO_2 evolution and Respiratory Quotient (R.Q.) of Wellington and Delicious apple tissue.

Tissue used	Buffer pH	ul O_2 uptake per gm(fw)-2K		CO_2 Evolution per gm (fw)-2K		R.Q.	
		Oct.11	Oct.12	Oct.11	Oct.12	Oct.11	Oct.12
Delicious	4.0	42.6	66.9	65.8	77.5	1.55	1.16
	4.5	36.4	67.8	55.3	85.1	1.52	1.26
	5.0	43.1	66.9	51.8	76.9	1.20	1.15
	5.5	41.0	68.9	52.8	76.5	1.29	1.10
	6.0	27.2	74.9	34.1	71.2	1.25	0.95
		Aug.17	Aug.19	Aug.17	Aug.19	Aug.17	Aug.19
Wellington	4.0	109.9	92.6	135.3	118.8	1.23	1.21
	5.4	88.6	-	95.5	-	1.08	-
	5.7	75.2	-	73.3	-	0.98	-
	6.1	76.8	65.3	72.7	54.9	0.95	0.84
	7.0	-	68.2	-	42.4	-	0.62
	7.5	-	78.2	-	36.6	-	0.53
	8.0	-	79.4	-	19.4	-	0.24



Appendix Figure 1. Effect of potassium phosphate buffer pH on Respiratory Quotient (R.Q.) of Wellington and Delicious apple tissue cylinders1/

1/ Oxygen uptake and CO_2 evolution were measured as described in "Materials and Methods", Experiment I.

in extracting intact mitochondria from banana fruit, were used. The Haard extraction medium contained 0.4M sucrose, 5 mMolar EDTA, and 5 mMolar MgCl. To 400 ml of this solution, 50 ml of dialyzed casein and 0.2 gm of cysteine hydrochloride were added. The frozen macerated apple tissue was slowly added to this solution and the slurry which resulted was subjected to the following centrifugation: Spin I (Sorval Centrifuge - GSA Rotor) -3,500 RPM for 10 minutes (supernatant liquid strained through cheese cloth); Spin II - (Model L ultracentrifuge - 30 rotor) 28,000 RPM for 30 minutes (pellet taken up and washed in 50 ml of wash solution); Spin III - (30 rotor) - 3000 RPM for 10 minutes (pellet again washed in 50 ml of wash solution); Spin IV - (30 rotor) - 28,000 RPM for 30 minutes (pellet taken up in 5 ml of wash solution and used as the mitochondrial suspension). The wash and suspension solution contained 0.4 M sucrose, .01 M Tris and 2 mg/ml of BSA, ATP and MgCl. The respiratory capacity of the extracted mitochondria was determined in a Gilson Differential Respirometer Model GRP-20 using 40 umoles of succinic acid as a substrate. In addition, the assay medium contained 3 x 10^{-5} mMolar cytochrome - c, 40 uM potassium phosphate, and water to 3 ml. Oxygen uptake was measured at 25°C using a shaker speed of 125 strokes per minute and a period of 15 minutes was allowed for temperature equilibration before readings were begun.

Mitochondria were also extracted using the extraction and the wash solution described by Hulme (13). This extraction medium (maintained at pH 7.5) was composed of the following:

0.4 M sucrose; 0.2M citrate; 0.01 M KH_2PO_4; 0.01 M ethylene diminetetraacetic acid (EDTA); and 0.7% (w/v) polyvinylpyrrolidone (PVP). Later, 0.01 M cysteine Hydrochloride was also added to the extraction medium. The wash solutoon contained 0.04 M sucrose, 0.01 M EDTA and 0.01 M Tris (made to pH 7.5). The assay medium contained 400 umole sucrose, 37.5 umole KH_2PO_4, 10 umole $MgSO_4$, 3 mg crystallized bovine plasma albumin, 2 mg yeast concentrate, 40 umole succinate and 0.56 umole cytochrome-c.

Appendix Table 2 shows the response of mitochondria extracted from Wellington apples using the extraction medium, wash solution and reaction mixture of Haard, and Hulme's extraction medium (with and without cysteine-HCl), wash solution and assay medium.

These data indicate that the extraction medium described by Hulme (13) was better suited for our work. Addition of cysteine Hydrochloride to Hulme's basic extraction medium also resulted in greater succinoxidase activity.

Data in Appendix Table 3 suggested that some constituent of the reaction medium was limiting. In addition, doubling the amount of mitochondrial preparation did not result in the expected doubling of the O_2 consumption (Appendix Figure 2). Consequently, cytochrome-c, ADP and NADH were added to the reaction medium. Their effect is seen in Appendix Table 4.

The addition of 10 umoles of ADP increased the respiratory capacity markedly; however, cytochrome-c and NADH at concentrations of 0.5 umole each did not improve the rate of

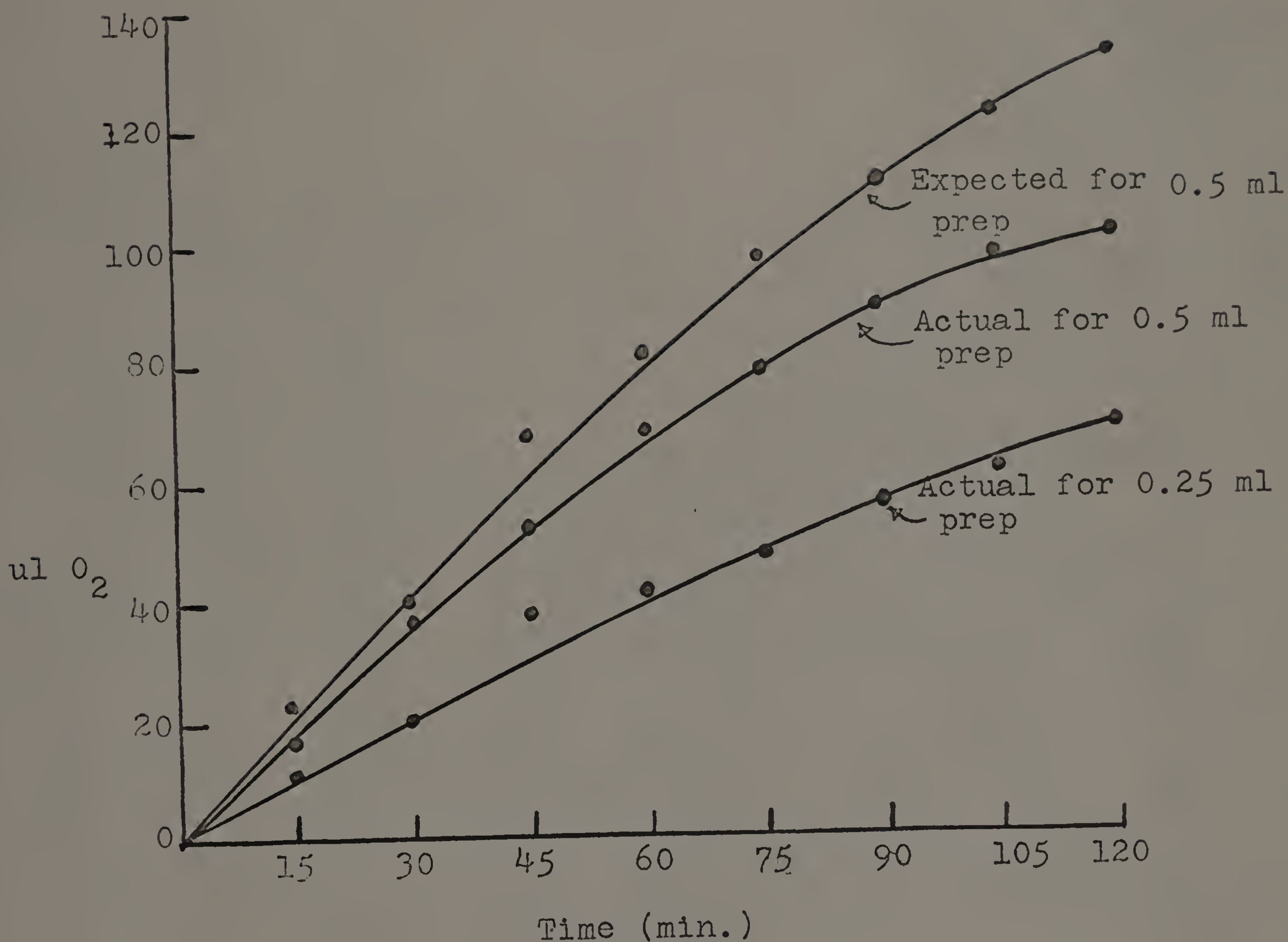
Appendix Table 2. Succinoxidase activities of mitochondria isolated from Wellington apple tissue using three types of extraction media and two types of wash solutions and assay media.

Extraction medium	Wash solution	Assay medium	ul O_2 uptake/ mg protein-hr.
Haard (casein - cystein-HCl)	Haard	Haard	18.9[1/]
Hulme (PVP)	Hulme	Hulme	42.3
Hulme (PVP - cystein-HCl)	Hulme	Hulme	56.4

1/ All values are an average of 2 preparations. One-half ml of preparation was assayed in 1.0 ml of assay medium.

Appendix Table 3. Succinoxidase activity of mitochondria isolated and assayed using the extraction medium, wash solution and assay medium of Hulme.

Apple tissue	ul O_2/hr./mg protein	
	0.25 ml mito./ml react. mixture	0.50 ml mito./2 ml react. mixture
Wellington	60.3	67.1
Wellington	62.5	66.3
Wellington	71.1	80.4
Delicious	70.8	80.9
Delicious	54.6	66.7



Appendix Figure 2. Succinoxidase activity of mitochondria isolated from Wellington apple tissue and assayed in 1 ml of Hulme's basic assay medium.1/

1/ Mitochondria were isolated as described in "Materials and Methods", Experiment IV.

Appendix Table 4. Effect of additional ADP, cytochrome-c and NADH on succinoxidase activity of mitochondria isolated from Delicious apple tissue.[1]

Reaction mixture	ADP (10 uM)	Cyt.-c(0.5 uM)	NADH (0.5 uM)	ul O_2/0.5ml prep.-hr.
1 ml				44.5[2]
1 ml	x			83.6
1 ml		x		47.9
1 ml	x	x	x	87.0

1/ The extraction procedure used is that described in "Materials and Methods", Experiment IV.

2/ Each value is an average of two flasks.

oxidation. When mitochondria were assayed in 1 ml of reaction mixture plus 10 uM ADP and 0.5 uM cytochrome - c, the expected doubling of activity with twice the amount of mitochondria was observed and this is illustrated in Appendix Figure 3.

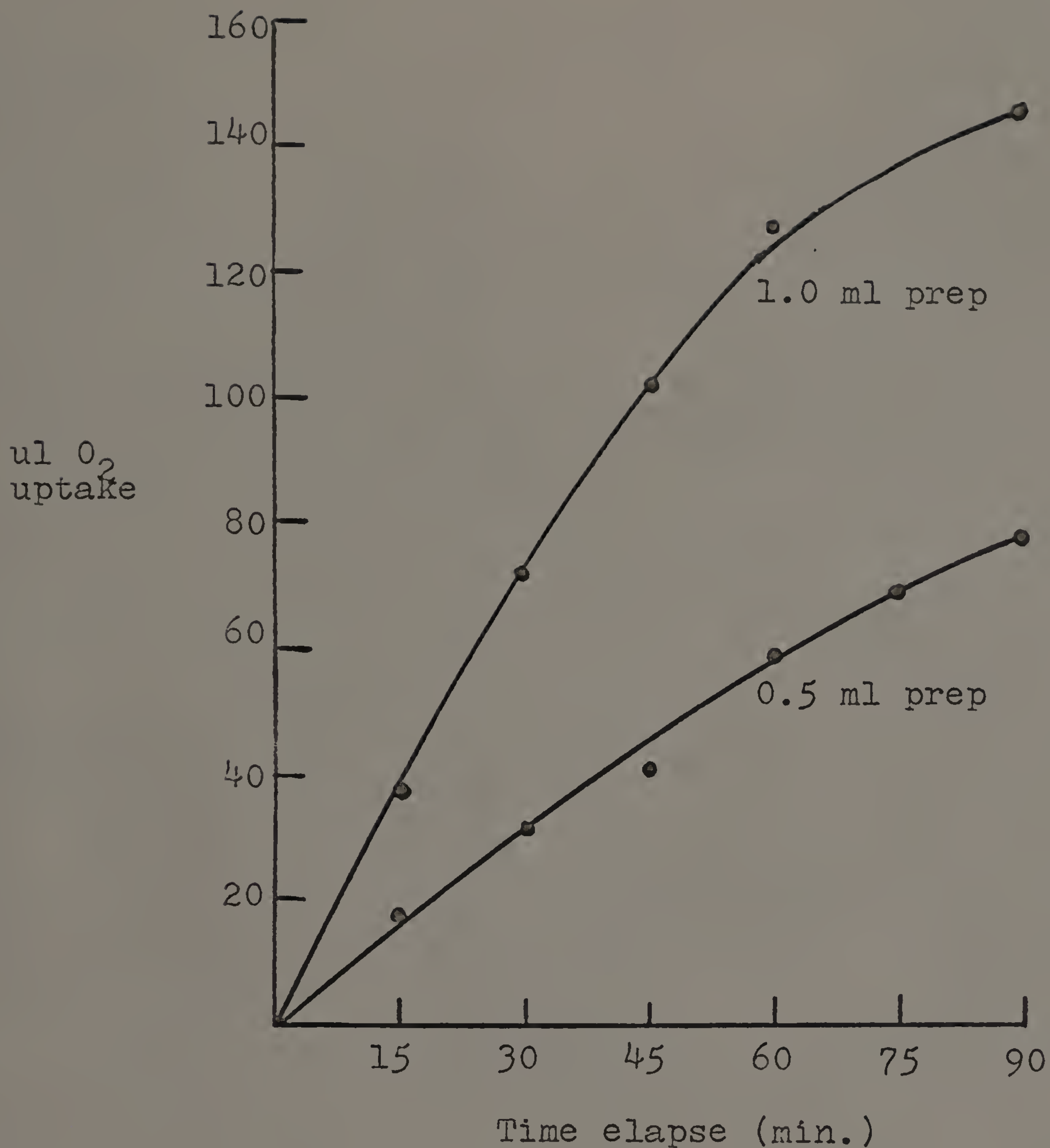
III. Determination of Protein in the mitochondria preparation.

The protein concentration of the mitochondrial prepatation was determined by the Folin method:

Reagent 1. - mis 1:1 $CuSO_4$ (1%) and Na K Tartrate (2%)

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

1. Samples of protein (generally 0.02 ml of preparation) were pipetted into tubes of equal inside diameter and the volume was brought to 0.6 ml with distilled water. A 0.6 ml sample of water was used as a blank.
2. A 3 ml aliquot of reagent 2 was added to each tube, and tubes were mixed and allowed to stand at room temperature for 10 minutes.
3. A 0.3 ml aliquot of Folin reagent (diluted 1:1 with 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. The protein concentration was calculated from a standard curve made with Bovine serum albumin as the protein.



Appendix Figure 3. Succinoxidase activity of mitochondria isolated from Delicious apple tissue and assayed in Hulme's basic assay medium plus 10 uM ADp and 0.5 uM cytochrome-c.1/

1/ Mitochondria were isolated as described in "Materials and Methods", Experiment IV.

IV. Composited data from volatile extractions.

Appendix Table 5. Area under chromatograph peaks recorded for extracts representing the normally observed pattern of watercore (WC) behavior during cold storage of Delicious apples.

Volatile component	Extract	Condition of tissue			
		Normal	Severe WC	Recovered from WC	Internal breakdown
Acetaldehyde	1 a[1]	5.7[2]	22.7	20.6	12.9
	b	5.2	24.3	25.8	13.5
	2 a	4.4	13.2	25.8	13.5
	b	4.6	25.8	20.6	7.7
	3 a	7.7	47.2	29.2	-
	b	6.7	42.8	-	-
Ethyl acetate	1 a	5.7	57.1	43.9	27.7
	b	5.7	82.6	44.4	18.1
	2 a	5.7	33.4	20.3	29.7
	b	5.7	55.2	18.1	31.0
	3 a	7.7	72.3	47.2	-
	b	5.8	67.1	-	-
Ethanol	1 a	67.1	929.0	329.8	203.9
	b	87.7	980.6	289.7	283.9
	2 a	89.0	683.9	260.1	187.1
	b	90.3	949.7	368.5	268.4
	3 a	134.2	1403.9	306.6	-
	b	123.9	1622.7	-	-

1/ Letters a and b represent duplicate extractions of the same powdered tissue sample.

2/ All values represent areas under peaks recorded when two microliter samples were analyzed chromatographically at 90°C as described in "Materials and Methods", Experiment III. Areas were measured with a planimeter and are expressed as square centimeters x 10^{-3}.

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Study of a Proposed Metabolic Relationship Between Watercore and Internal Breakdown in Delicious Apples

A Master Thesis

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