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Title EFFECTS OF WASHING AND AGING ON THE METABOLISM OF RED BEET ROOT SLICES.

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The rate of the $C^{14}O_2$ release from glucose, specifically labeled with C^{14} at 1, 2, 3+4, and 6 positions, was used to study the relative participation of pathways of glucose catabolism in red beet slices. Evidence was obtained which indicated that in mature red beets glucose was catabolized mainly via glycolysis-tricarboxylic acid cycle (EMP-TCAC). Approximately 15-22 percent of the total glucose catabolized in fresh slices appeared to be mediated via the pentose phosphate pathway (PPP). Older beet roots were found to have more PPP activity than young beet root slices.

When red beet slices were washed in either demineralized distilled water or 0.01M KH_2PO_4 solution by shaking in a gyratory shaker, the relative participation of the PPP increased with the duration of washing. Increase in the relative participation of the PPP was

more in the slices washed in KH_2PO_4 solution than in water alone. However, changes in the relative participation of pathways were less marked with red beet slices than with other storage tissues as reported by other workers. The TCAC was active in both fresh and washed beet slices. C^{14} release from specifically labeled glucose by fresh beet slices was very strongly inhibited by 0.05M malonate, whereas much less inhibition was found in washed slices. This strongly suggests TCAC participation in the fresh slices. Enzyme assays with crude extracts prepared from red beet slices indicated that the following enzyme activity changes occurred during a 24-hour washing period. Glucose-6-phosphate dehydrogenase and isocitrate dehydrogenase (TPN) slightly increased in activity; TPN reduction and malic dehydrogenase (TPN) activities slightly decreased; TPNH oxidizing power increased significantly; 6-phosphogluconate dehydrogenase activity increased 3-5 fold. The increase in TPNH oxidizing power may play a role in increasing the relative participation of PPP, but the most marked increase was found in 6-phosphogluconate dehydrogenase activity and the rate of gluconate utilization. Washing periods longer than two days caused a marked decrease in glucose-6-phosphate dehydrogenase and triose phosphate dehydrogenase activities. Experiments with glucose specifically labeled with H^3 indicated that the TPNH produced by the PPP was utilized for biosynthetic purposes rather than as

a respiratory substrate.

Metabolic changes of slices aged under moist conditions in petri dishes were also examined. The rate of oxygen uptake increased 3-5 times in 20-24 hours. O_2 uptake by fresh slices was inhibited 60-70 percent by 8×10^{-5}M HCN. The sensitivity of respiration to HCN decreased rather rapidly during aging and resulted in a cyanide stimulation (about 30 percent) of respiration in about 10-12 hours. After about 36 hours of aging, cyanide sensitivity began to reappear.

The rates of C^{14} release from specifically labeled glucose by fresh and aged red beet slices showed that both the PPP and the EMP-TCAC were stimulated by aging. Preferential increase in PPP participation was indicated to be very small, if any. Release of $C^{14}O_2$ from specifically labeled acetate, succinate, aspartate and glutonate showed TCAC activity in both fresh and aged slices. Glucose-6-phosphate dehydrogenase activity doubled and 6-phosphogluconate dehydrogenase activity increased by about 300-400 percent as a result of aging for 36 hours. The rate of gluconate utilization increased 10-20 times by aging over that of fresh slices. The rate of uptake and utilization of organic acids, amino acids and glucose increased several fold due to 24-hours of aging. Biosynthetic activities such as protein synthesis of these tissues were also indicated to be increased during aging.

From these studies, it does not seem justifiable to explain age-induced respiration as being due to a release of inhibition on TCAC activity or as preferential increase in PPP activity, but it seems to be a stimulation of the activity of the total metabolic machinery probably caused by removal of a metabolic block which is still not understood.

EFFECTS OF WASHING AND AGING ON THE METABOLISM OF RED BEET ROOT SLICES

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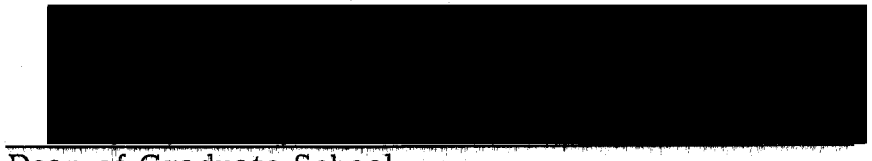

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TO MY WIFE

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I. INTRODUCTION

The rate of respiration of an organism, although it encompasses a wide variety of complex series of reactions, very often can give valuable information about the overall picture of the physiological state of the organism. A large amount of data is available in this regard, particularly because of the convenience involved in measuring respiratory rates. Although plants in general respire at a slower rate compared to animals, the respiratory rate of certain plant organs such as skunk cabbage flowers (the arid spadix) exceeds that of several animals if not most of them. Numerous physiological, non-physiological and pathological conditions are known to alter the rate of respiration of plant tissue. A wide variety of infections, fungal, viral and bacterial, are known to increase the rate of respiration of plants. Accumulation of solutes against concentration gradients is well known to be accompanied by a respiratory increase (109, p. 1-3; 125, p. 253-478; 108, p. 63-78). The metabolically controlled absorption of water into plant tissues (63, 0. 183-188) results in a concomitant respiratory increase. Hormonally induced growth is frequently accompanied by increased respiration (24, p. 429-436; 36, p. 230-247). A variety of substances, such as toxic materials, metabolic inhibitors, uncouplers, etc., are also known to alter respiratory rates. Physiological phenomena, such as dormancy, climacteric and senescence, also alter respiratory rates in plants (83, p. 215-299). Mechanical injuries, such as wounding, bruising, etc., cause dramatic increase in rate of respiration in plants.

Bulky storage tissues, such as potato, respire at a very low rate when they are intact. Any injury seems to increase the rate of respiration. Slicing or even peeling for example increases the respiration rate several fold. This increased respiration is known as "wound respiration." Development of wound respiration is very rapid and indications are that it is only a quantitative change. If these freshly prepared slices are maintained aerobically the rate of respiration increases further and reaches a maximum after a period of time. This relatively slow increase in respiration has sometimes been taken as part of wound respiration, probably without justification. The increase in the rate of respiration and other metabolic changes associated with it depend a great deal on the conditions under which slices are maintained from the time of slicing, and since different laboratories maintain slices under different conditions, results from different laboratories often are hard to compare. For example, slices have been aged by washing in water or different salt solutions or by keeping in moist atmosphere. Since there are good reasons to believe that the different conditions under which slices are maintained may have different effects on the slices, it is misleading to designate such different changes by one term. However, in general, at least qualitatively, they resemble each other enough to warrant a common term with which they can be designated temporarily. Even when the possible role of the minerals in the washing medium and the mechanical disturbances such as shaking that have been used in aging experiments, are eliminated by maintaining the slices in a moist atmosphere, dramatic metabolic changes happen and these are designated as "aging" effects on storage tissue slices.

It has been suggested that fresh slices of potato catabolize glucose mainly via pentose cycle and that the EMP-TCA pathway becomes active as a result of aging by washing in 10^{-4}M $CaSO_4$ solution (110, p. 20-29). It has also be reported that fresh potato slices and carrot slices metabolize glucose mainly via the EMP-TCA pathway and that the PPP becomes increasingly important in the slices with washing in 0.02M KH_2PO_4 solution for 24 hours (6, p. 839-847). In view of such contradicting reports, it would be interesting to study the effect of washing and aging on pathways of glucose catabolism in storage tissue slices.

Since PPP contribution has been suggested to be much larger to the induced respiration in potatoes and carrots, it would be of value to know whether the same is true for other storage tissues. Since conditions under which the slices are maintained from the time of slicing are indicated to have effects on the respiratory increase, it would be of interest to know the effects of these conditions on pathways of glucose catabolism. The work described herein was designed to study the pathways of glucose catabolism in fresh red beet slices and the effects of washing and aging on the relative participation of pathways. Attempts were also directed to study the effect of washing and aging on the metabolism of related substrates such as organic acids and amino acids. Red beet was chosen as the experimental material for several reasons. Red beet slices can be maintained under different conditions for long periods of time without loss of turgor and pigmentation. Fresh slices of this storage tissue take up and utilize externally administered substrates more rapidly than some other storage tissues such as potatoes.

II. LITERATURE REVIEW

About three quarters of a century ago Boehm observed (21, p. 671-675; 22, p. 681-692) tremendous enhancement of CO_2 production when potato tuber was cut. This stimulation was attributed by Boehm to wounding per se (20, p. 200-202). A few years later H. M. Richards while confirming an older observation of C. Stich (127, p. 1-57), showed (105, p. 531-582) that the enhancement of respiration could be prevented by reuniting cut portions of the tuber and closing the wound with clay. The reopening of the wound was invariably associated with a rather sudden increase in CO_2 production. These early observations were followed by similar observations in other storage tissues also. Dramatic enhancement in rate of respiration was observed in potatoes when they were quartered (68, p. 75-88; 72, p. 339-340), peeled (4, p. 265-294; 77, p. 679-690), sliced (126, p. 576-611), bruised (95, p. 429-451), or ventilated (115). To avoid complications from respiratory changes that might occur during the cutting and measuring time interval, Schade et al (113, p. 85-96) and Sharpensteen (117) measured the rate of respiration immediately after slicing potato tuber and found that specific respiration rate of slices (0.5-0.75 mm thick) was ten times that of the whole tuber. J. S. Turner (133, p. 274-306) and Turner and Hanly (134, p. 149-171) reported four to five fold stimulation of respiration in carrots as a

result of slicing (1.0 mm thick). When the respiration rate of whole tissue, reported by Choudhury (35, p. 238-257), is compared to respiration rate of slices of red beets and other storage tissues, it becomes clear that the enhancement of respiration rate due to slicing is a phenomenon common to all bulky tissues.

Ever since Stiles and Jorgenson (129, p. 349-367) used storage tissue slices for the investigation of permeability and salt absorption, tissue slices have become a popular experimental material of biochemists and physiologists. On using these tissue slices, various investigators began to notice certain pronounced changes in the physiology of the slices as they aged. Thus, the metabolic changes that occur in storage tissue slices were the subject of study for more than half a century. Although the basic reason for such dramatic changes remains unknown even today, several aspects of this interesting puzzle have been well studied.

Immediately after slicing, the respiration rate of plant tissue slices is about five to seven times that of the intact tissue. As the slices age, their respiration rate increases gradually. This gradual increase of respiration has been observed in a variety of storage tissues. The respiratory rise which occurs when potato slices are incubated in water has been studied by Steward et al (126, p. 576-611) Stiles and Dent (128, p. 1-34), Schade and Levy (114, p. 211-219),

Thimann et al (132, p. 239-257), Sharpensteen (117), Hackett (58, p. xl) and others. Similar studies were made on carrots, red beets, mangold roots, sugar beets, swede, etc., by Stiles and Dent (128, p. 1-34), Robertson et al (109, p. 1-8), Bennet-Clark and Bexon (13, p. 65-92), MacDonald and DeKock (89, p. 429-448) and others. Although there are disagreements and discrepancies among the various investigators, the general qualitative pattern is well agreed upon by all workers. From the time of slicing a gradual increase in respiration rate is observed in all tissues examined thus far. The rate of respiration reaches a maximum (3 to 5 times that of fresh slices) after a period of time depending on the tissue, its physiological state, the conditions under which the slices are maintained after slicing, etc. After maintaining this maximum rate of respiration for a period of time, again depending on the factors mentioned above, the rate of respiration begins to decrease.

In spite of the agreement in the qualitative pattern of respiratory increase, there are a large number of discrepancies in the literature. For example, Bennet-Clark and Bexon (13, p. 65-92) prepared red beet slices from dormant beets and washed them in running aerated water at 12-16°C and found a maximum of 130μl/hr/gm, about four times that of fresh slices (30μl/hr/gm), after 300 hours. This higher respiration rate remained "roughly constant" for many

days. Robertson et al (109, p. 1-8) found it difficult to reconcile their results with those of Bennet-Clark and Bexon, because they found that slices prepared from dormant beets when aerated in distilled water reached a maximum respiration rate of two and one-half times that of fresh slices in about 30-70 hours after slicing. After the initial 30-70 hour interval, the rate subsequently fell and decreased slowly for 300 hours. During a second 300-hour interval, the respiration rate decreased even more slowly than during the first 300-hour interval. Stiles and Dent (128, p. 1-34) maintained red beet discs in running aerated tap water at 12°C and observed the maximum respiration rate after about nine days and then the respiration rate gradually decreased. More recently MacDonald and DeKock (89, p. 429-448) studied the effect of temperature of aging on the respiration rate of a variety of storage tissues and attributed the discrepancies mentioned above to the differences in aging temperature.

Effect of temperature on aging has been overlooked by some investigators. Bennet-Clark and Bexon (13, p. 65-92) and Stiles and Dent (128, p. 1-34) washed red beet discs at 12-16°C and under these conditions the maximum rate of respiration was obtained only after a week. As MacDonald and DeKock point out (89, p. 429-448), Robertson et al (109, p. 1-8) overlooked the effect of temperature

when the latter workers found it hard to reconcile their results with those of Bennet-Clark and Bexon and Stiles and Dent. MacDonald and DeKock found maximum respiration rate in beet discs after 50 hours when slices were maintained at 25°C in aerated running tap water, whereas it took a week when the temperature was 12°C. These authors demonstrated the effect of temperature on the change in respiration and the change in sensitivity to respiratory inhibitors in a variety of storage tissues (89, p. 429-448). The increase in respiration due to aging is reported to be completely inhibited by maintaining the slices at very low temperature 0-20°C (110, p. 20-29; 117). This together with the high temperature coefficient supports the view that aging effect requires synthetic activity of the tissue. If the potato discs were preincubated at 25°C for two hours before being placed in the cold, the induced respiration was fully developed by an additional 40 hours of incubation in the cold.

The medium in which slices are maintained play a very significant role in controlling the respiratory changes. Bennet-Clark and Bexon (13, p. 65-92), Stiles and Dent (128, p. 1-34), MacDonald and DeKock and others used aerated tap water or distilled water. Robertson et al (109, p. 1-8) used distilled water as aging medium and observed pronounced increments in the rate of respiration due to addition of salts into the medium. Bennet-Clark and Bexon

(13, p. 65-92) observed that 0.05N KCl or 0.05N $CaCl_2$ in the washing medium of red beet discs gave a higher rate of respiration as compared to controls in water. Ca^{++} was less efficient than K^+. While Robertson (107, p. 265-277) reported similar results in carrot root discs, Steward and Preston (123, p. 85-97) found that in potato discs Ca^{++} had a retarding effect on respiration (77 percent) while K^+ had an enhancing effect (175 percent). More recently Laties reported (79, p. 378-391) that 0.05M Li^+ in the washing medium prevented increase in respiration, that normally accompanies aging in chicory root slices. Aging effects are found to be quite different if aging is done in air. For example, two maxima have been observed in the respiration-time curve of potato slices respiring in air (68, p. 75-88; 87, p. 429-455). Steward et al (126, p. 576-611) reported that unwashed potato discs respiring in air gave greater carbon dioxide output than similar discs immersed in dilute salt solution, while discs washed for a few hours and then exposed to air gave intermediate values. Stiles and Dent (128, p. 1-34) extended such observations to other storage tissues and concluded that respiratory activity of thin slices of storage tissue, when exposed to air with little preliminary washing, increased rapidly to a maximum in about a day and then fell slowly while similar slices, when immersed in aerated water, took several days to reach a maximum respiration rate.

Thimann et al (132, p. 239-257) have reported that four days under aerated water was necessary to bring about changes in sensitivity to respiratory inhibitors which are comparable to changes in sensitivity of potato tissue aged on nets over water in a day. Other biochemical changes also have been reported (62, p. 553-560; 122, p. 436-453; 126, p. 576-611) to be more intense or faster in the discs aged in air when compared to those aging in aerated water or salt solution.

Effect of thickness of discs on aging of storage tissue has been studied by various investigators. Richards (105, p. 531-582) found that observable effects of injury on potato tissues was confined to a more or less restricted zone of activity as suggested by the ever-increasing rate of respiration which followed successively severe injuries. Zaleski (145, p. 331-339) also noticed more pronounced effects with smaller pieces of tissue and concurrent larger surface area. Steward et al (126, p. 576-611) found that the specific rate of respiration decreased with thickness of the discs in potatoes. From such experiments they concluded that the surface cells contributed to the total respiration to a much greater amount, relative to their mass, than the inner ones. Laties more recently (77, p. 679-690; 83, p. 215-299) studied this problem in great detail and found that the respiration rate of freshly cut potato discs was independent of

thickness. The specific respiration rate of potato core cut in cylindrical sections, 12mm in diameter and 18mm in length, was found to be the same as that of fresh discs which were 0.5mm thick. He reported that whole tubers, when peeled, achieved a comparable respiration rate rather quickly. However, when the discs were aged for 24 hours, the respiration rate became dependent on thickness. Thus, according to his results, it is only the outermost millimeter thick mantel of tissue that achieves the several fold increase in respiration. He attributed the effects of thickness reported in the older literature to aging effects, as the measurements reported were all done after washing for relatively long periods of time.

Starch disappearance below the cut surface in potato tissue has long been known (101, p. 96-155). Hopkins (68, p. 75-88) observed starch disappearance and a corresponding increase in reducing sugars in potato slices. He correlated this to respiratory increments and from it concluded that the increase in reducing sugar was a cause for the respiratory increment. Steward et al (126, p. 576-611) reported results obtained by microscopic examination of potato slices. They observed that starch disappearance proceeded to a depth of 0.8mm (6 to 7 cells) in unwashed discs respiring in moist air, and to only 0.22-0.24mm in discs respiring under water. The

suberization and cambial development that are usually observed in the slices exposed to air occurred only to either a limited extent or not at all in water. Steward (122, p. 436-453) calculated the depth of tissue in which active metabolism occurs and found it to be four cells (0.47mm) in water, eight to nine cells (0.92mm) with tissues in air which had been given a short washing in water and 12 cells (1.44mm) deep in air after a superficial rinsing. Later, Hackett and Thimann (62, p. 553-560) made similar observations in potato slices. More recently MacDonald and DeKock (89, p. 429-448) examined a variety of storage tissues and found that corresponding to the increase in respiration, there was an increase in reducing sugar content of all storage tissue. These authors pointed out that sugar beet, red beet and potato, in which sugar content commenced at a level below 1 mg/gm fresh weight required about three or four days to reach their respiratory maximum, whereas similarly treated discs of swede and carrots containing substantial amounts of glucose when cut, reached their respiratory maximum within 24 hours. This good correlation between reducing sugar increase and increase in the rate of respiration, obtained by these workers, agrees very well with Hopkin's earlier observations (68, p. 75-88). However, it is doubtful whether the attempts of these authors to explain the increment in respiration on the basis of this striking correlation of sugar

increase to respiratory increments are justifiable in view of the fact that substrate is usually not the rate limiting factor of respiration in storage tissues. More recently gradual increments in reducing sugar content has been reported in carrot slices during washing in water (103).

As might be anticipated, nitrogenous substances within the cell also undergo striking transformations. Zaleski (145, p. 331-339) and Hettlinger (66, p. 248-250) seem to have been the first to show that in onion and potato the total protein nitrogen markedly increased in three to four days after slicing. The relationship between the high respiration following injury and protein synthesis became more clear when Zaleski could not observe the increase in protein nitrogen when slices were kept in hydrogen. Kovchoff (76, p. 449-462) and Smirnoff (121, p. 26-38) observed increments of protein nitrogen in injured onion when maintained aerobically. Considerable amount of this increase in protein nitrogen was in protein not digestible by gastric juice--the so-called nuclein. They ascribed the increased respiration to enhanced content of insoluble nucleoprotein. Friedrich (48, p. 330-348) observed that increase of protein nitrogen was associated with decrease in soluble nitrogen and starch in injured potato exposed to air. Steward and Preston (123, p. 85-101) after studying the protein and soluble nitrogen levels of

potato discs in great detail, found that protein level increase was accompanied by corresponding decrease in soluble nitrogen. Potato discs aged for 63 hours in 0.075N KBr solution almost doubled in protein content. Calo et al (31, p. 1142) observed eight fold increase in alanine incorporation due to aging of potato slices. They attempted to inhibit protein synthesis in aging potato slices by incubating potato discs for 24 hours in the presence of chloramphenicol and found that it could prevent normal aging effects on respiration. Although Click and Hackett (38, p. 243-250) recently raised the possibility of a general toxic effect of the high concentration (1 mg/ml) of chloramphenicol used in these experiments, Calo et al found that the presence chloramphenicol during the measurement of of oxygen uptake had little effect on the respiration of fresh or aged slices. However, they did not report any evidence that the inhibitor was actually blocking protein synthesis. Jacoby and Sutcliffe (70, p. 377-383) have recently reported chloramphenicol inhibition of aminoacid incorporation into protein in carrot slices. They also reported that chloramphenicol inhibited net protein synthesis in carrot root slices suspended in aerated solutions. Recently, Click and Hackett (38, p. 243-250) used puromycin to inhibit protein synthesis and leucine-1-C^{14} incorporation to measure the rate of protein synthesis. They found that the respiratory increase associated with

aging depended on protein synthesis. It is interesting to note that Jacoby and J. F. Sutcliffe (70, p. 377-383) observed chloramphenicol insensitive incorporation of aminoacids into protein in carrot slices and Click and Hackett (38, p. 243-250) observed puromycin insensitive incorporation of leucine into protein in potato slices. Since this inhibitor-insensitive incorporation of aminoacids into protein seems to be quantitatively small, the conclusions drawn from such experiments are not significantly affected by it.

Click and Hackett (38, p. 243-250) also used uracil-2-C^{14} incorporation to measure the rate of RNA synthesis and actinomycin D to inhibit RNA synthesis in aging potato slices. From such experiments, they concluded that the development of respiration depends on protein synthesis which must be preceeded by RNA synthesis. It is interesting to note that they could partially block the development of respiration by delayed addition of the inhibitors. It is remarkable that Kovchoff (76, p. 449-462) and Smernoff (121, p. 26-38), more than 60 years ago, drew similar conclusions from relatively primitive experiments. Thus, it seems clear that, at least in the potato, aging effects depend on protein and RNA synthesis. Such observations have not been extended to other tissues to any extent.

Phosphate metabolism has attracted attention of many investigators primarily due to the central role inorganic and organic phosphates play in respiratory metabolism. Calo et al (31, p. 1142) measured the phosphorylative capacity of potato discs by determining the extent of incorporation of P^{32} into organic phosphates. From such experiments they concluded that the increment of respiratory capacity is coupled to phosphorylation, thus eliminating uncoupling as a possible reason for increased respiration. Loughman (84, p. 418-424) reported a 100 fold increase in phosphate uptake and 20 fold increase in the ability of potato slices to esterify the accumulated phosphate, during a 24-hour aging period at 25°C. However, at high concentrations of phosphate, fresh and aged discs showed very little difference in their ability to accumulate and esterify phosphate. For example, only about five percent of the phosphate absorbed in ten minutes from a 10^{-5}M solution is esterified, while up to 70 percent may be esterified at 10^{-2}M. However, phosphate is not a rate limiting factor in the respiration of fresh slices, because immersing in 10^{-2}M phosphate solution does not bring about any change in rate of respiration of fresh slices. Hackett et al (61, p. 8-19) also observed similar increases in P^{32} uptake in aging potato discs and found that respiration in aged tissue is as efficient as in fresh tissue as far as phosphate esterification is concerned. More recently, Bieleski and

Laties (15, p. 580-594) studied in great detail the incorporation of P^{32} into various organic phosphates in aged and fresh potato slices. They confirmed the results of Loughman (84, p. 418-424) and Hackett et al (61, p. 8-19). There was more RNA synthesized in the aged tissue, but only small differences could be observed in soluble organic phosphates. Phosphate esters became labeled in the same order in aged and fresh slices, but a faster rate could be observed in aged slices. The authors estimated in vivo P/O ratios using data on the concentrations and turn over rates of the esters and rate of respiration. Such estimations indicated a P/O ratio of about 0.25 for fresh tissue and 0.6 for aged tissue, thus indicating that respiration is more efficient in aged tissue than fresh tissue. If such estimations give a reliable indication of the true in vivo P/O ratio, then the P/O ratios obtained from isolated mitochondria (61, p. 8-19) do not give us an indication of the actual changes in the tissue. There does not seem to be any similar information available on other storage tissues.

Enhanced utilization of inorganic ions by aged storage tissues is not confined to phosphate. Recently Ellis (44, p. 129-136) reported that sulphate uptake by red beet discs increased with the number of days of washing up to eight days. Slices washed for 8 days took up $S^{35}O_4^{=}$ about nine times as fast as fresh discs. However, the maximum incorporation of S^{35} into cysteine was observed in two day

washed beet discs, S^{35} incorporation into cysteine in two-day washed tissue was about fifty times that of fresh and 75 times that of fresh in presence of added L-serine.

Increase in uptake of minerals other than phosphate and sulphate due to aging has been reported (125, p. 253-478). Recently MacDonald and Laties (90, p. 38-44) have reported kinetic studies on ion absorption by fresh and aged potato discs.

Enhanced uptake by aged storage tissue is not confined to inorganic solutes. Grant (103) studied sugar uptake by plant cells in great detail and found a dramatic increase in uptake due to aging of carrot root discs. ApRees and Beevers (6, p. 839-347) observed about a five-fold increase of glucose uptake in carrot discs washed in 0.02MKH_2PO_4 solution for a day and two-fold increase in potato discs washed in a similar manner. Romberger and Norton observed a similar increase in glucose uptake in aged potato discs (110, p. 20-29). Similar increases in uptake of amino acids also have been observed by Ellis (44, p. 129-136) and Bert and Hird (18, p. 277-286) in both red beet and carrot discs. It is well known that respiration of storage tissues is mediated primarily through a cytochrome system. However, in the past, several workers concluded that polyphenol oxidase was involved in the respiration of some storage tissues (40, p. 454). Such conclusions were based upon the observation that the

respiration of potato tissue could be stimulated by phenol-like substances. But according to Schade et al (113, p. 85-96), catechol is an unsatisfactory substrate for the elucidation of the enzymatic systems involved in the endogenous respiration of the potato tuber. Barron et al (9, p. 377-398), observed that respiration could be slightly inhibited by 8-hydroxy quinoline and from this they concluded that polyphenol oxidase could account for part of the respiration. In spite of such conclusions, evidence for cytochrome systems has been overwhelming. Levy et al (85, p. 273-286) showed that the respiration of freshly cut potato slices was inhibited by cyanide. They also observed that inhibition of respiration by carbon monoxide was reversible by light. Similar observations were made in a variety of storage tissues by various investigators (88, p. 241-256; 130, p. 469-475; 132, p. 239-257). In addition to such indirect evidence, direct evidence such as isolation and characterization of enzymes such as cytochrome oxidase (51, p. 41-47) succinic oxidase (93, p. 123-132), spectroscopic data on the oxidation and the reduction of cytochrome in potato slices (14, p. 112-120), spectroscopic evidence for presence and participation of cytochromes in red beets (142, p. 109-122), studies on isolated mitochondria from storage tissues like potato and red beets (55, p. xxv; 58, p. xl; 59, p. vii; 142, p. 109-122), etc., seems to have confirmed the role of cytochrome oxidase in the

terminal electron transport system of these tissues. Qualitative changes in respiratory pathways have been observed in aging storage tissue discs. Change in sensitivity to respiratory inhibitors gave the first indication of such changes. Schade et al (114, p. 211-219) used cyanide and carbon monoxide as diagnostic inhibitors in fresh and washed potato discs. Their results were interpreted to mean that there were two oxidases participating in potato respiration, one cytochrome oxidase which mediates respiration of intact tubers, the other a pO_2 sensitive oxidase which participates more in the respiration of washed discs. Although many of such experiments have been criticized by various investigators (26, p. 521-543; 71, p. 245-260), Thimann et al (132, p. 239-257) under conditions which eliminated such criticisms showed that respiration of freshly cut potato slices were inhibited by carbon monoxide and that this inhibition was fully photo-reversible. Moreover they showed that washing the potato slices for four days or keeping them at 25°C for 24 hours eliminated their sensitivity of respiration toward CO to a large extent. Instead, carbon monoxide stimulated the respiration of such discs. Carbon monoxide oxidation was eliminated as a possible explanation for their observations. They demonstrated that potato slices insensitive to carbon monoxide were also insensitive to cyanide. From pO_2 sensitivity studies it was concluded that the respiration of aged slices was

mediated by a new enzyme which was either a cytochrome system insensitive to carbon monoxide and cyanide or a flavoprotein of high oxygen affinity. Changes in sensitivity to cyanide has been observed at a subcellular level also. For example, Hackett and Hass (59, p. vii) reported that a concentration of cyanide which inhibited oxygen uptake of fresh tissue mitochondria 80-90 percent did not affect the oxygen uptake by aged tissue mitochondria although phosphorylative capacity of aged mitochondria was inhibited about 50 percent. Hackett et al (61, p. 8-19) showed that potato slices became insensitive to antimycin A as they aged. That aged potato discs became less sensitive to respiratory inhibitors like cyanide has been well documented by several other investigators (7, p. xx; 32, p. xlvii; 54, p. xlvi; ii, p. 241-256) although observations such as those of Schade et al are in disagreement (114, p. 211-219) in the fact that they observed the same amount of inhibition by 1×10^{-3}M KCN in fresh as well as four-day washed potato. Such changes in sensitivity to respiratory inhibitors is a general characteristic of aging storage tissue slices. Robertson et al (128, p. 1-34) observed that red beet discs, when washed in water for long periods of time, became relatively insensitive to 1×10^{-3}M CN^-. However, they observed this only in two experiments. In the majority of their experiments, cyanide reduced the rate of respiration in water to a constant CN^- insensitive level which they

observed even after washing for 360 hours. They noticed changes in cyanide sensitivity in washed carrots also. Middletone (92, p. 422-434) suggested that in beet discs the basal cyanide resistant system is of limited activity and any respiratory increase induced by wounding, salts, etc., would be carried by the cyanide sensitive system. Such clear-cut distinctions are unjustifiable according to MacDonald (88, p. 241-256) who observed a small increase in cyanide sensitivity during the first four days of washing, followed by rather quick decrease in sensitivity of respiration. Then, after a period of cyanide stimulation, inhibition developed. He also studied changes in sensitivity to inhibition in a variety of other tissues such as swede, sugar beet, red beet, carrots and potato. In swede he observed a rapid decrease in sensitivity to cyanide during aging, which resulted in a cyanide-stimulation of about 40 percent in about three days. After eight days a gradual increase in inhibition could be observed. Even when the discs were pretreated at 7°C a rapid decrease (and later increase) in sensitivity to cyanide could be observed. In sugar beet discs aged at 25°C the sensitivity pattern was similar to red beets. An interesting feature of the pattern was that when slices were aged at 15°C a rapid decrease in sensitivity was observed rather than an initial increase in sensitivity that was seen in slices aged at 25°C. Surprisingly, fresh carrot discs were completely insensitive to cyanide at 12°C (or even stimulated), but they developed sensitivity to cyanide as a result of aging. After three or four days of aging at 12°C cyanide sensitivity decreased slightly. At 25°C, sensitivity to respiration rapidly decreased and cyanide then caused a slight stimulation after four or five days.

After about ten days, inhibition began to develop. In potatoes aged at 25°C, a rapid decrease in cyanide sensitivity was observed and in two days small stimulation became observable. Slices aged at 12°C took longer to undergo the same changes. It should be pointed out that the extent of inhibition and the sensitivity patterns depend on concentration of cyanide used. For example, MacDonald (88, p. 241-256) found that the respiration of potato discs aged for four days at 25°C was stimulated about 25 percent by 1×10^{-4}M KCN while 5×10^{-4}M KCN inhibited the respiration of similar slices about 35 percent. Although quantitatively dissimilar, qualitatively similar observations were made by Thimann et al (132, p. 239-257). MacDonald (88, p. 241-256) also found similar changes in sensitivity to azide and carbon monoxide in sugar beet discs.

Malonate sensitivity has been used by Laties and others (15, p. 580-594; 77, p. 679-690; 79, p. 378-391; 110, p. 20-29) to characterize the aging process in storage tissue slices. In chicory root slices initial respiration was shown to be completely insensitive to malonate inhibition, while the respiratory increment was entirely repressed by malonate. Moreover, the addition of malate to fresh tissue either in the presence or absence of malonate was completely without effect whereas the addition of malate to aged tissue, previously inhibited by incubation with malonate, restored the rate of respiration to the non-inhibited level. From such observations, Laties seems to conclude (83, p. 215-299) that freshly prepared discs were unable to oxidize malate while aged discs were able to oxidize it very well. Mitochondrial preparations from fresh tissue oxidized malate

as rapidly as mitochondrial preparations from aged tissue. Thus, it was postulated that either some cofactor of oxidation or some ancillary enzyme system concerned with malate oxidation other than by electron transfer must develop in the presence of air at room temperature during aging. However, if malate was not a rate limiting factor in the respiration of fresh tissue, malate addition would not increase the rate of its respiration, and normally malate concentration is very unlikely to be the rate determining factor. Malate, succinate and citrate (0.05N) have been reported (13, p. 65-92) to have stimulatory effects on the respiration of red beet slices. This suggests (103) the presence of an inactive pool and a rather small active pool. Thus, the added acids enter the metabolically active pool causing the observed effects. Such compartmentation of organic acids seems to be generally present in plant tissues (91). In the aged tissue, malate almost restored malonate inhibited respiration (79, p. 378-391). In this case, the tricarboxylic acid cycle is hindered at the succinic dehydrogenase level resulting in the accumulation of succinate. This reduces the concentration of fumarate and the next few members of TCA cycle. Such a block may be restored, although not always completely, by administering fumarate or the next few members of TCA cycle, and thus, malate restores respiration under such conditions. Conclusions drawn by Laties from such observations do not seem to be justifiable. Laties is of the opinion that aging in chicory root slices is associated with the onset of vigorous TCA cycle activity. This is in agreement with the development of malonate sensitivity which is also associated with the aging process of storage tissues as described by Laties. Malonate

inhibition has been studied in great detail in potato slices also by Laties (77, p. 679-690). He observed that malonate inhibition was a characteristic of aged slices and this inhibition was not observed when aging was done in high pCO_2 or in the presence of bicarbonate. This interesting observation may be explained on the basis of increased organic acid concentration caused by CO_2 fixation. Bieleski and Laties (15, p. 580-594) studied the effect of malonate on P^{32} incorporation into the organic phosphates of potato slices. They found that malonate altered P^{32} distribution in the phosphate esters in the aged tissues only. If malonate resistant respiration in the aged tissue represents the entire respiration of the fresh tissue, their P^{32} patterns should be similar. With such a rationale in mind, the P^{32} patterns were examined and serious differences were observed. It is clear that aged discs in the presence of malonate are not biochemically equivalent to fresh discs. All the biochemical changes that occurred during aging were not stopped by addition of malonate.

Malonate has also been used by Romberger and Norton (110, p. 20-29) to study the qualitative changes in glucose metabolism that occur during aging. It was found that 5×10^{-2} malonate did not inhibit $C^{14}O_2$ release from radioactive glucose by fresh tissue whereas severe inhibition was observed in aged tissue. Glucose uptake was also not inhibited in fresh slices whereas severe inhibition was observed in aged slices. From such observations and others they came to the conclusion that malonate insensitive respiration in fresh tissue was mediated via the pentose cycle and the malonate sensitive respiration which developed during aging was mainly TCAC activity. In view of

of the fact that malonate inhibition depends very much on the concentration of malonate in the reaction zone, the conclusions drawn from a comparison of malonate inhibition in fresh and aged slices seem to require re-examination, especially because uptake of solutes are known to be enhanced tremendously by aging (6, p. 839-847; 18, p. 277-286; 90, p. 38-44; 103; 110, p. 20-29). If malonate uptake is very sluggish in fresh tissue and relatively rapid in aged tissue, then the increased sensitivity of aged tissue to malonate may be explained simply as due to increased concentration of malonate in the reaction zone rather than as a qualitative change in metabolism. A detailed study of malonate uptake by fresh and aged tissue would help clarify this problem.

Dinitrophenol, a popular uncoupling agent was shown to increase the respiration rate of freshly cut slices of storage tissues (6, p. 839-847; 12, p. 91-96; 79, p. 378-391; 117). Sharpensteen (117) observed that the immediate stimulation of respiration by dinitrophenol in fresh potato slices progressively diminished as the aging effect on respiration increased. Laties (79, p. 378-391) found that DNP increased the rate of respiration of freshly cut chicory root slices, to a level which is usually observed in one-day aged slices. Furthermore, DNP did not increase the rate of respiration of aged slices. ApRees and Beevers reported similar results in carrot slices (6, p. 839-847). The observation of Laties (79, p. 378-391) that, in contrast to the basal respiration, both DNP-induced and age-induced respiratory increments were malonate sensitive, led him to suggest that malonate sensitive respiration was associated with oxidative phosphorylation and that

phosphorylative processes were limiting in fresh tissue. However, the increase in respiration due to aging is not a simple reflection of the removal of restraint on the phosphorylative metabolism. Qualitative and quantitative similarity of age-induced and DNP-induced respiration does not mean that they are of the same origin. In fact, there is enough evidence (15, p. 580-594; 61, p. 8-19) to postulate that age-induced respiration is as efficient or more so than the respiration of fresh tissue. Moreover, DNP-induced respiration and age-induced respiration differ in the fact that DNP-stimulated respiration of fresh tissue is sensitive to cyanide and other respiratory inhibitors (51, p. 8-19). So, the similarity in malonate sensitivity may be misleading unless caution is taken to examine other evidences. It may be reasonable to suppose that the higher rates of synthetic activities, already demonstrated in aging tissues, probably utilize more energy and thus ATP turnover becomes faster and consequently maintains a high rate of respiration. However, the mechanism which triggers this process is not known. DNP in the aging medium prevented respiratory increment that is normally associated with aging. This indicates that the respiratory increment requires energy conservation in the tissue. Such a conclusion is supported by a variety of observations. If the tissue is maintained at low temperature, in presence of HCN, malonate, under anaerobic conditions, etc., a normal aging effect cannot be seen. These observations clearly indicate that the aging process requires a cyanide and malonate sensitive respiration. In view of the fact that

synthetic activities (31, p. 1142; 38, p. 243-450; 48, p. 330-348; 131, p. 274-279) are associated with aging, a requirement for respiratory energy is only natural.

The dramatic increments in respiration and the changes in sensitivity to respiratory inhibitors such as cyanide, carbon monoxide, azide and antimycin A, accompanying aging processes prompted Hackett and co-workers to look for an electron transport system that is insensitive to classical cytochrome oxidase inhibitors (55, p. xxv; 58, p. xl; 59, p. vii; 61, p. 8-19). On a subcellular level, mitochondria prepared from aged tissue were found to be less sensitive to cyanide than similar preparations from fresh tissue. However, at a mitochondrial level, there was no increase in the oxidative ability associated with aging. Aging for one day brought about several fold increases in DPNH oxidase, diaphorase and DPNH-cytochrome c reductase activities. However, significant increases in cytochrome c oxidase and succinic-cytochrome c reductase could not be observed. Although such increases in enzyme activity levels were encouraging, the rapid DPNH oxidase remained sensitive to cyanide, azide and antimycin. So, the actual basis for inhibitor insensitivity still remains unexplained. Even though the author did not seem to think microsomes were respiratory centers, he could demonstrate a marked increase in the microsmal DPNH-cytochrome c reductase. The

soluble, cyanide-sensitive DPNH oxidase system (117) was not considered, apparently because such a system is not probably involved in tissue respiration to any extent. One of the possible explanations put forward (34, p. 33-49) is the so-called "excess" hypothesis or "cushioning effect." According to this hypothesis the net electron transfer rate will not be inhibited by very large changes in the steady state oxidation level of the oxidase when this component is not limiting. If the inhibition of respiration by inhibitors (cyanide and azide) is taken to mean that cytochrome oxidase is a rate limiting factor in fresh tissue, then the insensitivity developed by aging should be accompanied by a large increase in the cytochrome oxidase system. However, Hackett and co-workers failed to observe a significant increase in the measured amount of activity of cytochrome c oxidase. Thus, the "excess" hypothesis does not seem to explain all the observations. The other possibility is a bypass pathway in which an autooxidizable cytochrome b_7 plays an important role. Spectroscopic examination (61, p. 8-19) showed flavoprotein, cytochrome a, a_3, b_7, and c components in mitochondria from fresh and aged tissue. A study of difference spectra revealed a striking increase in b-type cytochromes relative to the a-a_3 components, due to aging. According to calculations made with data from difference spectra, b_7 concentration increased at least two-fold. This observation together with the increase

in activity of the region between DPNH and cytochrome c, may be taken as an evidence for b_7 oxidase hypothesis (bypass). However, the high P/O ratio observed (15, p. 580-594; 61, p. 8-19) in aged tissue would seem to require participation of a cytochrome oxidase system unless a b_7 to O_2 pathway, coupled to phosphorylation, exists. The inhibition of phosphate uptake by carbon monoxide without blocking respiration seems to suggest that this pathway is not coupled to phosphorylation. The inhibition of phosphorylation by cyanide also would agree with a non-phosphorylative bypass. Thus, it seems likely that such a bypass is operative in presence of cyanide but not in the absence of inhibitor. It is interesting to note that tissues having a vigorous cyanide insensitive respiration possess strong b_7 component and the respiration of aged slices is reminiscent of the well-known skunk cabbage respiration (34, p. 33-49; 60, p. 27-32). It must be pointed out that the problem of cyanide-insensitive respiration is far from clear.

Qualitative changes in the metabolism of aging storage tissue discs are well documented by the changes in sensitivity to various inhibitors. It has been reported that R. Q. remains unity during the aging process (6, p. 839-847; 13, p. 65-92; 128, p. 1-34; 133, p. 274-306) except in tissue washed for very long periods (128, p. 1-34)

or stored for a long time in presence of salts like nitrate (133, p. 274-306) or in presence of 0.05N malate, succinate or citrate (13, p. 65-92). Thus, carbohydrate is indicated to be the primary respiratory substrate. Romberger and Norton (111, p. xii) indicated that fresh potato slices metabolize glucose via pentose cycle, tricarboxylic acid cycle and possibly other pathways. In carrot slices, participation of PPP and glycolytic pathway was demonstrated by ApRees and Beevers (5, p. 830-838). These authors also studied the carbohydrate metabolism in aged slices of carrots and potatoes (6, p. 839-847). Using glucose specifically labeled with C^{14}, they observed that the relative rates of release of the individual carbons of glucose changed during washing. Thus, in aged tissue, carbon one of glucose was released faster than carbons 3, 4, whereas in fresh tissue, carbon 3, 4 was released faster than carbon one. This was interpreted to mean that the PPP contribution to the induced respiration was much greater than its contribution to the basal respiration. This was true for both carrots and potatoes. They suggested increased TPNH oxidation and increased reductive events in aged tissue as two possible explanations for their observed results.

Alberghina and Marré (2, p. 1771) presented evidence for increased pentose cycle participation in aged storage tissue slices. They reported two to eight-fold increase in glucose-6-phosphate

dehydrogenase activity due to aging of potato slices for 24 hours. Honda and Oda (67, p. 24-29) also observed an increase in TPN linked dehydrogenase activity upon aging. Such results also support the contention of ApRees and Beevers that pentose cycle participation is much greater in aged tissue than in fresh tissue.

A very high participation of pentose cycle does not explain the repeated claims of increase in malonate sensitivity (111, p. xii). Romberger and Norton (110, p. 20-29) used malonate inhibition and the ratio of release of glucose carbon one to carbon six to study the relative participation of pathways in fresh and aged potato discs. They observed that 0.05M malonate at pH5 in the medium did not inhibit glucose uptake or $C^{14}O_2$ production from uniformly labeled glucose, whereas in aged tissue under identical conditions $C^{14}O_2$ production and uptake was severly inhibited by malonate. In fresh tissue they observed a sharp drop in C_1/C_6 ratio during the first two hours, whereas, the C_1/C_6 drop in presence of malonate was soon reversed. Such observations were interpreted to mean that onset of TCA cycle is the primary change that happens during aging. Their conclusions agree with those of Laties (80, p. 382; 81, p. 364-377) who contends that the onset of TCA cycle is the major change that happens during aging. However, the conclusions drawn from $C^{14}O_2$ production during extremely short initial time interval may be questionable, and no

measurements were reported to indicate the concentration of malonate actually present in the tissue. In view of the tremendous increase in uptake of solutes, usually associated with the aging process, the concentration of malonate in the cytoplasm of the aged tissue is very likely to be much higher than in the fresh tissue. Moreover, these workers reported that out of the 12,000,000 counts administered to fresh tissue as glucose-C^{14} (110, p. 20-29), only a few hundred counts were released as $C^{14}O_2$, whereas aged discs released considerable fraction of the total administered counts. The reliability of the conclusions drawn from such data are questionable. The observation of Laties (81, p. 364-377) that malate had no effect upon the respiration of fresh tissue which was DNP-treated and malonate inhibited, whereas malate restored the respiration of aged chicory slices which were inhibited by malonate to the normal level suggested to Hawker and Laties (65, p. 498-500) that respiration of fresh tissue might be limited by endogenous pyridine nucleotide levels. So they determined the levels of DPN and TPN in fresh and aged potato slices. During a 24-hour incubation period, the concentration of DPN increased about 1.6 times, whereas TPN concentration increased 2.7 times. This increase was rather small when compared to the increase in respiration. Moreover, fresh tissue mitochondria did not respond to addition of DPN. These observations led the authors to

conclude that the increase in pyridine nucleotides is not enough to support the contention that they might limit the rate of respiration of fresh tissue. According to the authors the increase of TPN may no more cause the increase in the PPP with aging of potato slices than the rise in DPN causes the onset of citric acid cycle activity. However, TPN/DPN ratio might be significant enough to change the relative participation of pathway of glucose catabolism in favor of pentose cycle. In view of the disagreement regarding the changes in relative participation if pathways, a further examination of the problem became desirable and such a study is described in the following sections.

Even though our knowledge about the biochemical changes that accompany the aging process has been greatly improved as a result of the recent investigations, the underlying mechanisms which control the process are not well understood. Boehm originally attributed wound-respiration to the wounding itself (20, p. 200-202). However, it soon became agreed somewhat generally that increased oxygen availability was the basic reason for the observed respiratory changes (72, p. 339-340; 105, p. 531-582; 126, p. 576-611; 127, p. 1-57). This interpretation was subsequently questioned (29, p. 121-134; 35, p. 238-257; 41, p. 419-442; 52, p. 207-232; 83, p. 215-299) on the grounds that the storage tissues have sufficient oxygen available inside the tissue for the various oxidases to function and they do not

respond to external changes in pO_2 contrary to the increased oxygen availability theory. Rather recently, Woolley (144, p. 793-798) after a detailed study of the problem of oxygen availability and ventilation in potato tissue, concluded that oxygen supply would not be limiting except in pieces of tuber whose minimum diameter approached 10 cm. Laties presented (77, p. 679-690) additional evidence to support such a view. A rather old observation (19, p. 273-283) that respiration of intact potato tubers in air could be increased five-fold almost immediately by gamma radiation, seems to argue very strongly against an oxygen limitation theory. In view of such strong evidences, the increased oxygen availability theory is no longer tenable. Such arguments hold especially true in the case of aging effects, as the effects are confined to a very thin mantle of tissue.

Laties has proposed (77, p. 679-690; 78, p. 129-155; 83, p. 215-299) without much direct evidence that regulation of respiratory development represents a so-called negative feed back process in which control is effected by a volatile respiratory product. It has been reported (9, p. 377-398) that pyruvate may either be decarboxylated to acetaldehyde in potato or oxidized and directly channeled into TCA cycle, and that aging is accompanied by the onset of vigorous glycolysis (110, p. 20-29). Out of the total volatile carbon released by the potato respiration, over 7 percent is reported to be some

product other than CO_2 (124, p. 409-447). Acetaldehyde and a variety of aldehydes inhibit the respiratory increments normally associated with aging of the discs (78, p. 129-155; 99, p. xxxii). Amyl alcohol, which is produced by potatoes, also inhibits the respiratory increment but only at a narrowly limited, non-physiologically high, range (0.05-0.025M), thus making it a less likely candidate for a physiological regulator. On such grounds, Laties chose acetaldehyde as a model for the volatile endogenous metabolite. Thus, according to his theory, diffusion of acetaldehyde (or a similar metabolite) regulates the respiration. B. Payes and G. G. Laties (99, p. xxxii) have attributed a regulatory role to glyoxylate whose concentration is reported to be sharply reduced with aging. They isolated a condensation product of glyoxylate and oxaloacetate and found that α-keto β-hydroxy glutarate was a potent inhibitor of citrate and α-keto glutarate oxidation by potato mitochondria. However, the theory of Laties does not explain DNP-induced respiration. Moreover, no direct evidence is available to support his theory. Hence, the mechanism of aging process is considered unknown at present.

III MATERIALS AND METHODS

Plant Tissues

For preliminary experiments, red beets (Beta vulgaris L) were purchased from a local wholesale firm. For the majority of the experiments, however, beets freshly dug each morning from a local farm were used. For the first series of experiments, cylinders were cut from well washed red beets using a cork borer 12 mm in diameter. These cylinders were placed immediately in 0.01M KH_2PO_4 solution, pH 5.0. The cylinders were then sliced into 1mm thick slices with a slicing device. The slicing device was double-edged razor blades spaced with washers and held tightly together by two threaded rods. Slicing was done within a 30-minute period and the slices used immediately are designated as fresh slices. Slices were well randomized before using and comparisons of results were made only between slices of a single batch. This was necessary because both the age and type of storage conditions influenced the behavior of the slices. For washing purposes, slices were placed in 250ml erlenmeyer flasks containing 125ml of 0.01M KH_2PO_4 solution, pH5.0, and shaken in a temperature-controlled gyratory shaker at about 300rpm at 26°-27°C. The medium was replaced every 30 minutes during the first two hours and later at longer intervals. During the first

24-hour washing period the medium was changed 8-10 times and after that about four times daily. During the initial phase of the investigation, visual symptoms such as exudation of pigment, loss of turgidity and cloudiness of the medium were used as an indication of bacterial contamination. However, since this method was found to be unsatisfactory, aseptic techniques were employed in further experiments. Freshly dug beets, which were chosen for uniformity in size (medium) and age, were well washed in water and immersed in 25 percent Chlorox solution (1·3% NaOCl) for 15-20 minutes. These surface-sterilized beets were then well washed in sterile water and placed in a glove box under ultraviolet light. All the glassware and cutting apparatus were sterilized before each experiment. Slices were cut as described before and these aseptic slices were transferred into an erlenmeyer flask which was connected to a reservoir containing sterile 0.01M KH_2PO_4 solution. By this means, the washing medium could be replaced at frequent intervals without opening the system, thus avoiding possible bacterial contamination. To check for bacterial contamination, slices were taken at the start of each experiment and ground in sterile water in a mortar under sterile conditions. This extract was plated onto complete nutrient agar using a serial dilution technique. The agar plates were then incubated for several days, and bacterial colonies were counted. At the end of 24 hours washing, the

maximum contamination observed was a few hundred bacterial counts per 15 slices. However, it was extremely difficult to wash the slices for several days without contamination of the order of 10^7-10^8 counts per 15 slices.

For all the aging experiments, slices (9mm in diameter) were prepared under aseptic conditions as described above but using demineralized distilled water containing dihydrostreptomycin sulphate (50μg/ml) in place of 0.01M KH_2PO_4. Higher concentrations of dihydrostreptomycin sulphate with penicillin were found to injure the tissue. Fifty beet slices were placed on a filter paper, supported by glass beads in a petri dish containing 15ml of sterile demineralized distilled water. Dihydrostreptomycin sulphate was added (50μgm/ml) to prevent possible contamination. The slices were placed in such a way that the upper half of each slice was exposed to air and the lower half to water. The petri dishes were incubated in an incubator at 25°C in the dark. Throughout the aging process, sterile conditions were preserved and the slices used were checked for contamination by plating on agar plates as described before. When contamination was detected results were discarded. Carrots and potatoes were purchased from a local grocery store and slices were prepared as described before.

Rate of Respiration

The rate of oxygen uptake of tissue slices was measured with standard Warburg respirometers at 25°C. Eight slices were immersed in the appropriate medium (0.01M KH_2PO_4 solution or demineralized distilled water) in the flasks, with alkali in the center well, keeping the total liquid volume at 3ml. Since washing and aging increased the fresh weight and decreased the dry weight, an equal number of slices was used in each flask for comparison. The effect of glucose, at the concentration used in the radiorespirometry experiments, on the rate of respiration was determined by tilting in glucose solution from the side arm at the desired time.

To determine the effect of hydrogen cyanide on the rate of oxygen uptake, standard Warburg techniques were used with KCN-KOH mixture in the center well according to the method of Robie (106, p. 307). With 0.5ml of 0.5M KOH plus 4.8M KCN solution in the center well, after equilibration the medium contained 8×10^{-5}M HCN as determined colorimetrically, according to a modification of the method reported by Aldridge (3, p. 474). Oxygen uptake was measured after an equilibration time of one hour. Four readings at 30-minute intervals were subsequently taken during which time oxygen uptake was found to be constant.

To study the effect of peeling on the rate of respiration, eight freshly dug red beets (1.25Kg) were well washed and placed in a three liter container. Dry and CO_2-free air was circulated through the container and air flowing out of the container at a rate of 83ml/min was analyzed continuously for CO_2 using a recording infrared gas analyzer. A few hours after the whole beets showed a steady rate of CO_2 output, the beets were quickly removed, peeled and placed back in the chamber. This process took less than ten minutes. After recording the rate of CO_2 output of the peeled beets for a few hours the peeled beets were again quickly removed, quartered and placed in the chamber and the CO_2 analysis was continued for a few more hours. The percent CO_2 in the exhaust air was used as the rate of CO_2 output since the air flow was kept constant using a flow meter.

Enzyme Activity Level Measurements

Of all the extraction methods attempted, a liquid nitrogen freeze-powdering method gave the most satisfactory results as far as enzyme activity and reproducibility are concerned. Slices were counted on two folds of filter paper and the surfaces were wiped dry. These slices were then dropped into liquid nitrogen contained in a precooled mortar. As the liquid nitrogen evaporated the frozen

slices were quickly ground, using a precooled pestle, into a fine powder. To this powder was added a measured volume of 0.05M tris-HCl buffer pH 7.6 containing 0.005M 2-mercaptoethanol and the frozen slurry was allowed to thaw in an ice bath. When thawed, the slurry was well ground with or without sand and passed through several layers of cheese cloth. The extract was then centrifuged for 20 minutes at 14500xg in a refrigerated centrifuge. The supernatant was decanted and used as the source of the enzyme. Protein determinations were carried out using a micro Kjeldahl method described by Umbreit et al (135, p. 274).

Determination of Rate of Gluconate Utilization

Fifteen fresh slices were placed in a respirometer funnel which contained 4ml of demineralized distilled water and 2.78 moles of potassium gluconate-1-C^{14}. The slices were continuously aerated by a stream of air (43 ml/min). The CO_2-trapping solution was changed at hourly intervals and the experiment was stopped at the end of two hours. The cumulative recoveries during the two-hour period was determined and the averages of duplicate experiments were used. This experiment was repeated at regular intervals using slices aged for varying lengths of time.

Experiments With Tritiated Glucose

Glucose-1-H^3 and glucose-6-H^3 obtained in an alcohol solution, were equilibrated with water and lyophilized. Aliquots of the lyophilizate were counted as described elsewhere. The labile or exchangeable tritium was found to be less than 0.05 percent of the total tritium present. In the respirometer, described elsewhere, 15 beet slices were placed in 4ml 0.01 KH_2PO_4 solution, pH 5.0, containing 2.78 μmoles of glucose-1-H^3 or glucose-6-H^3, and aeration was kept steady at the rate of 43ml/min. The H^3_2O vapor escaping with the sweeping air was trapped in a series of traps containing ethylene diamine solution and counted. This radioactivity was added to the total radioactivity of H^3_2O. Three identical pairs of samples were set up and at the desired intervals of time, aeration was stopped and medium was withdrawn from one pair of samples. The slices were well washed and the medium, washwater and slices were lyophilized separately. The lyophilizate was condensed in a trap immersed in a dry ice-acetone bath. Aliquots of the water fraction were counted in a liquid scintillation counter as described elsewhere.

Radiorespirometry Experiments

Fifteen slices of fresh or appropriately treated slices were placed in a long narrow funnel of 30ml capacity with sintered glass

bottom, containing 4ml of medium. As soon as the slices were dropped into this medium, containing appropriate amounts of the substrate, the respirometer funnel was swept continuously by a stream of air (43-45ml/min) saturated with water vapor, through the sintered glass bottom. The CO_2 in the respiratory gas was trapped by bubbling through a trapping funnel containing 0.5ml ethylenediamine, 0.5ml ethylene glycol and 4ml 2-methoxy ethanol. At hourly intervals the trapping solution was drained directly into a counting vial and the trap was washed with 2ml of 2-methoxy ethanol into the same vial. This $C^{14}O_2$ solution was counted in a liquid scintillation counter after adding 10ml of toluene containing 6 gm of PPO (2, 5 diphenyl oxazole) per liter. At the end of experimental time the medium was drained off and the slices kept frozen until further analysis. An aliquot of the medium was counted to determine the C^{14} remaining in the medium. To determine the rate of uptake, at two-hour intervals 0.05ml of the medium was withdrawn using a Hamilton microliter syringe, and counted in the liquid scintillation counter.

Chemicals

Glucose-1-C^{14}, glucose-6-C^{14}, glucose-2-C^{14}, acetate-2-C^{14}, acetate-1-C^{14}, succinate-1, 4-C^{14}, succinate-2, 3-C^{14}, glucose-1-H^3 and glucose-6-H^3 were purchased from New England Nuclear

Corporation. L-aspartate-4-C^{14}, L-glutamate-1-C^{14} and L-alanine-1-C^{14} were purchased from the Cal Biochem Corporation. Gluconate-1-C^{14} was purchased from the Nuclear Chicago Corporation. Glucose-3, 4-C^{14} was prepared according to the method of Wood et al (143, p. 475-489) and gluconate-6-C^{14} and gluconate-2-C^{14} were prepared according to the method of Moore et al (94, p. 293-311). TPNH, TPN, DPN, glucose-6-phosphate, 6-phosphogluconate and d, l-isocitrate were purchased from the Sigma Chemical Company.

Radioactivity Measurements

$C^{14}O_2$ was trapped in an organic base solution and counted directly in a liquid scintillation counter as described before. Aliquots of all aqueous solutions and $H^3{}_2O$ were mixed with a scintillation solution of the same composition used in $C^{14}O_2$ counting and counted in a Packard liquid scintillation counter. Internal standards of naphthalene-C^{14} and toluene-H^3 or $H^3{}_2O$ were used to determine counting efficiency. C^{14} was counted with a counting efficiency of 30 percent and H^3 was counted at about 12 percent efficiency. Tissue residues were counted as $C^{14}O_2$ after combustion in an oxygen atmosphere according to the method of Kelly et al (75, p. 267-273). Radioactivity measurements were carried out to a standard deviation of not greater than 2 percent.

Experiments With Sorghum Seedlings

Sorghum seedlings var. Rox orange were germinated from seeds obtained from Mr. Paul Long, Alexandria, Nebraska. Germination was in an inorganic nutrient solution according to the method of Reed (104, p. xxxii). After three to four days of growth, 30 seedlings, detached from seeds (dry weight 92± 3mg), were placed in each respirometer funnel, containing 4ml of nutrient solution and 0.5mg of the radioactive glucose. The samples were aerated at the rate of 43ml/min. After one hour the medium was removed and the seedlings were washed well. The washing medium and seedlings were separately lyophilized and the radioactivity in the water fraction was determined as described before. The dry seedlings were extracted overnight with ether in a soxhlet extraction apparatus. The ether was evaporated off under reduced pressure. The lipid material was dissolved directly in the toluene scintillation solution and counted in the liquid scintillation counter.

In each experiment, duplicate samples were used and all the experiments described in this thesis were repeated at least once.

RESULTS AND DISCUSSION

Part A.

Experiments With Washed Red Beet Slices

1. Effects of Washing Medium on Relative Participation of Pathways of Glucose Catabolism

In view of the apparent disagreements regarding the changes in relative participation of pathways of carbohydrate metabolism for washed storage tissue slices, preliminary experiments were designed to get information about the influence of the washing medium on glucose catabolism. Washing was with either demineralized distilled water, 0.01 M KH_2PO_4 pH 5, or a nutrient solution which consisted of a mixture of four salts (104, p. xxxii). The relative recoveries of $C^{14}O_2$ were compared using glucose-1-C^{14}, glucose-3, 4-C^{14} and glucose-6-C^{14} as exogenously administered substrates. These preliminary experiments indicated that red beet slices washed in KH_2PO_4 solution for one day absorbed and utilized glucose most rapidly and the slices washed in distilled water took up and utilized glucose, the least rapidly, while the slices washed in nutrient solution were intermediate, but similar to the phosphate-washed slices. Moreover, judging from relative rates of release of C^{14} from specifically labeled glucose, washing in KH_2PO_4 solution

seemed to have enhanced C_1 release more than washing in any other medium.

When a glucose molecule undergoes catabolism via the glycolysis- tricarboxylic acid cycle (EMP-TCAC) pathway carbon one (C_1) and carbon six (C_6) have an equal chance of appearing in the respiratory CO_2, whereas the pentose phosphate pathway (PPP) causes a preferential release of C_1 during the early phase of the pathway. Based on this rationale, C_1/C_6 ratio has been used to study the relative participation of the two pathways. However, this method is for several reasons quite inadequate when used to draw conclusions about quantitative aspects of pathway participation (5, p. 830-838; 69, p. 580-582; 110, p. 20-29). C_1/C_6 ratios tend to approach unity irrespective of the pathways if the tissue is allowed to catabolize the administered glucose for very long periods of time, however, due to drainage of intermediates for biosynthetic purposes C_1/C_6 usually is greater than unity. Release of C_1 from glucose-6-phosphate via PPP takes only three reactions, whereas C_6 begins to be released by the TCA cycle only after more than 30 reactions. This time factor involved in the relative rates of release of C_1 and C_6 might result in misleading C_1/C_6 ratios, especially when the ratio is calculated from extremely short experiments. In a tissue which has a considerable amount of direct oxidative cleavage of C_6 or triose exchange by

reversal of the aldolase reaction, misleading C_1/C_6 ratios may be obtained. Such difficulties may be at least partially overcome by using glucose-3, 4-C^{14} according to the method of Barbour et al (8, p. 396-400). It must be pointed out that the accuracy of this method depends on the validity of the assumptions upon which the calculations are based. The assumption that pentose formed via the PPP is not further catabolized extensively seems to be the most objectionable assumption (5, p. 830-838; 43, p. 751-756). Assuming that administered gluconate can be readily converted to 6-phosphogluconate which joins the stream of 6-phosphogluconate of PPP, Wang et al (139, p. 1-7) corrected G_6 and $G_{3,4}$ terms for the contribution of C_6 and $C_{3,4}$ to the respiratory CO_2 via PPP. Due to the extremely slow utilization of gluconate by the fresh slices and the drastic changes in gluconate utilization brought about by washing and aging, the corrections of Wang et al (139, p. 1-7) were not attempted in this investigation.

Although usage of C_1/C_6 ratio may be quite misleading as an absolute measure of participation of pathways, changes in this ratio may be used as an indication of qualitative changes in pathway participation and the C_1/C_6 ratio is used in this investigation only to serve this purpose. Estimations based on the equations of Barbour et al (8, p. 396-400) are used here only as a semi-quantitative measure of

changes in relative participation of pathways.

The effect of the washing medium on the relative participation of pathways was further examined by comparing the relative rates of C^{14} release from glucose-1-C^{14}, glucose-3, 4-C^{14} and glucose-6-C^{14}. In these experiments rather mature beets obtained from a local farmer's garden were used. The relative rates of C^{14} release and the yield of C^{14} from slices washed in 0.01M KH_2PO_4 solution (pH 5) and deionized distilled water, metabolizing specifically labeled glucose are summarized in Table I. At the end of experimental time, 94 ± 4 percent of the total administered glucose was taken up by the slices in all cases. The percentage was based on the C^{14} uptake by the end of the experiments. In fresh tissue the rate of release of C^{14} as $C^{14}O_2$ from glucose-3, 4-C^{14} was much faster than from glucose-1-C^{14}. This indicates that the glycolytic pathway is the dominant pathway in fresh red beet slices. However, the relative rates of C^{14} release from glucose-1-C^{14} and glucose-6-C^{14} were not the same. A C_1/C_6 ratio value of 1.56 was obtained when calculated on the basis of C^{14} recovery during the first two hours. This indicates participation of the PPP in addition to glycolysis in the catabolism of glucose in the fresh red beet slices. From the percent recoveries summarized in Table I, using the equations of Barbour et al (8, p. 396-400), it is estimated that about 20 percent of the total glucose

Table I.

The effects of washing in water and potassium phosphate solution on the kinetics of $C^{14}O_2$ release from specifically labeled glucose by red beet slices.

PERCENT RECOVERY

	Fresh			Washed for one day						Washed for four days					
				In KH_2PO_4			In H_2O			In KH_2PO_4			In H_2O		
Hours:	2	4	6	2	4	6	2	4	6	2	4	6	2	4	6
Glucose-1-C^{14}	3	11	19	23	41	45	16	34	38	36	70	75	31	49	53
Glucose-3,4-C^{14}	6	16	23	26	37	40	18	33	36	25	37	41	23	39	43
Glucose-6-C^{14}	2	7	12	8	16	18	7	16	20	12	27	31	13	23	27

Each respirometer flask contained 15 beet slices, (2.25g fresh weight) 4ml nutrient solution and 2.78μ M of C^{14} labeled glucose. Air flow through each respirometer flask was 43ml/minute.

catabolized by the fresh red beet slices proceeded via the PPP. Washing either in KH_2PO_4 solution or deionized distilled water increased $C^{14}O_2$ production from externally administered glucose-1-C^{14}, glucose-3, 4-C^{14} and glucose-6-C^{14} several fold in the initial stages of glucose utilization. Since toward the end of the experimental time $C^{14}O_2$ production would be limited by the concentration of radioactive substrate, the initial stages are more meaningful. Washing for a day in KH_2PO_4 solution increased $C^{14}O_2$ production by red beet slices from glucose-1-C^{14} more than eight-fold during the first two hours, whereas $C^{14}O_2$ production from the other positions of glucose ($C_{3,4}$ and C_6) increased only more than four-fold. Such large differences are not readily apparent if the cumulative C^{14} recoveries at the end of the experiment are compared. However, it is evident from Tables I and II that the increase in the release of C^{14} from glucose-1-C^{14} was considerably greater than from 3, 4 or 6. This indicates a higher PPP participation in the washed slices as compared to fresh slices. Washing in water increased C_1 release almost six-fold, whereas release of C_6 and $C_{3,4}$ increased three to four-fold. Thus, washing in water also increased the relative participation of PPP.

Table II summarizes the cumulative C^{14} recoveries at the end of the experimental time (nine hours). C_1/C_6 ratios are also shown as calculated on the basis of C^{14} recoveries during the first two

Table II

The effects of washing in water and potassium phosphate solution on the cumulative recovery of C^{14} in CO_2 from red beet slices.

PERCENT CUMULATIVE RECOVERY

Substrates	Fresh slices	Duration of washing					
		24 hours		4 days		8 days	
		Washing medium		Washing medium		Washing medium	
		KH_2PO_4	Demineralized water	KH_2PO_4	Demineralized water	KH_2PO_4	Demineralized water
Glucose-1-C^{14}	25	48	42	77	56	72	71
Glucose-3,4-C^{14}	30	42	39	43	44	44	48
Glucose-6-C^{14}	16	21	22	33	28	27	37
C_1/C_6 Ratio	1.5	2.9	2.3	3	2.4	5.9	3.6
Percent PPP Participation	20	40	35	54	40	-	-

hours, along with estimated values for the PPP. The C_1/C_6 ratio increased markedly with the duration of washing. If changes in this ratio are taken as an indication of qualitative changes in relative participation of PPP, the ratios in Table II indicate that PPP participation is increased by washing in either KH_2PO_4 solution or water. A comparison of the values for the slices washed in water and those washed in KH_2PO_4 solution suggests that the increase in PPP participation is probably more pronounced when slices are washed in 0.01M KH_2PO_4 solution than in water. Table I also shows the rate of release of $C^{14}O_2$ from glucose specifically labeled at 1, 3, 4 and 6 positions, by slices washed for four days. Here again C_1/C_6 ratios are indicative of an increased participation of PPP due to increased duration of washing. Estimated values for the percent participation of PPP agree with this. Increase in the rate of $C^{14}O_2$ production from $C_{3,4}$ and C_6 positions due to one-day washing is significant enough to indicate that probably an increase in glycolytic pathway also contributes to the increased catabolism of administered glucose. However, the corresponding changes due to long term washings (four or eight days) were small, or even negative, as shown in Tables I and II. This indicates that washing periods longer than a day or so may damage the EMP-TCA pathway in red beet slices. Such long washing periods are known to change their respiratory quotient

(128, p. 1-34).

2. Malonate Experiments

Results of a radiorespirometry experiment in the presence of 5×10^{-2} M malonate are shown in Table III. Recoveries of C^{14} in CO_2 are recorded as the percentage of radioactivity that disappeared from the medium during 8 hours. Uptake of substrate in the presence of malonate by the beet slices was about one-third of that by the control slices in the case of the fresh tissue and about one-half in the case of the washed tissue. However, the changes in uptake do not enter into the figures presented because recoveries are calculated on the basis of C^{14} taken up. Inhibition of C^{14} release due to the presence of malonate in the medium was not observable during the first hour in fresh tissue. From the second hour onward C^{14} release by fresh tissue was severely inhibited. Thus, $C^{14}O_2$ recovery as percent of control decreased from 100 percent in the first hour to 35 to 43 percent and 15 to 21 percent during the first two hours and the first four hours, respectively. In the washed tissue also there was essentially no inhibition when first-hour C^{14} recoveries from glucose-1-C^{14} and glucose -3, 4-C^{14} were compared, whereas C^{14} recovery from glucose-6-C^{14} was already inhibited about 20 percent. During the next nine hours of the experiment, more inhibition became visible.

Table III

Effect of malonate on $C^{14}O_2$ release from specifically labeled glucose by red beet slices.

	F	FM	Percent of control	WP	WPM	Percent of control	WW	WWM	Percent of control
	PERCENT C^{14} RECOVERY AS $C^{14}O_2$ DURING FIRST TWO HOURS.								
Glucose-1-C^{14}	2.8	1.2	43	36	30	83	31	21	68
Glucose-3,4-C^{14}	5.9	2.8	48	25	23	94	23	21	90
Glucose-6-C^{14}	1.8	0.6	35	12	6.5	52	13	4.4	34
	PERCENT C^{14} RECOVERY AS $C^{14}O_2$ DURING FIRST FOUR HOURS.								
Glucose-1-C^{14}	11	1.6	15	70	49	70	49	34	70
Glucose-3,4-C^{14}	16	3.3	21	37	38	102	39	35	90
Glucose-6-C^{14}	7.2	1.1	15	27	13	47	23	10	42

Each respirometer flask contained 15 slices (2.25g fresh weight) in 4ml nutrient solution containing 5×10^{-2}M malonate and 2.78 μM C^{14} labeled glucose. The pH was adjusted to 5 with KOH. Recoveries are expressed as percent of uptake during 8 hours experimental time. Air flow through each respirometer flask was 43ml/minute. F=fresh slices, FM=fresh slices+malonate, WP=slices washed in KH_2PO_4 solution, WPM=slices washed in KH_2PO_4 solution+malonate in the respirometer, WW=slices washed in demineralized distilled water, WWM=slices washed in demineralized distilled water+malonate in the respirometer.

Thus, when cumulative recoveries during the first two hours and four hours were compared, very little or no inhibition was found in the case of glucose-3, 4-C^{14}, whereas 17 to 30 percent inhibition in the case of glucose-1-C^{14} and 48 to 53 percent inhibition in the case of glucose-6-C^{14} were found in the slices washed in phosphate buffer. When slices were washed in distilled water a qualitatively similar inhibition pattern was obtained.

Malonate is well known to be a competitive inhibitor of succinic dehydrogenase. Thus, the terminal stages of glucose catabolism via TCA cycle are probably hindered in the malonate treated tissue. Carbon-6 of glucose which traverses through glycolysis is equivalent to the methyl carbon of acetate. This carbon can be first released only in the third turn of the TCA cycle. Release of C_3 and C_4 of glucose, being equivalent to carboxyl carbon of pyruvate, cannot be inhibited at the succinic dehydrogenase step, unless the decarboxylation of pyruvate is indirectly prevented by the hindrance in the TCA cycle caused by malonate. Thus, release of C_1 and C_6 would be expected to be inhibited very severely by malonate with less inhibition in the release of $C_{3,4}$. The results of the malonate experiments are in general agreement with such an effect. In fresh tissue there was severe inhibition by malonate after the first hour. This delay in the appearance of inhibition in the fresh tissue might well have been due

to slow uptake of malonate during the initial phase of the experiment. As soon as enough malonate gets into the tissue, severe inhibition sets in. A similar increase in inhibition, although quantitatively much less, was often visible in the case of aged tissue also. Thus, if recoveries at the end of four hour time are considered more meaningful than the early inhibition, then the above results indicate that the respiration of fresh slices is highly sensitive to malonate. This observation, together with the almost equal inhibition of C^{14} release from C_1 and C_6 substantiates the contention that fresh slices metabolize glucose mainly by EMP-TCAC. Similarly, the comparatively poor inhibition of C^{14} release, and the very significant difference in malonate sensitivity between C_1 and C_6 in the washed tissue, agree well with the contention that the contribution of PPP to the total glucose catabolism of beet slices is increased significantly by washing.

These results do not agree with those of Romberger and Norton (110, p. 20-29) who observed no malonate inhibition of C^{14} release by fresh potato slices from randomly labeled glucose. According to their results during the three-hour experimental time, malonate (0.05M) inhibited glucose uptake by fresh slices about 11 percent, whereas uptake by aged tissue was inhibited about 75 percent. Laties also reported that respiration of fresh chicory tissue was not malonate sensitive, whereas aged tissue was highly sensitive to malonate

(77, p. 679-690; 79, p. 378-391). However, in none of the above reports was the actual concentration of malonate in the tissue reported. As pointed out in the literature review, the concentration of malonate in the tissue is very likely to be different in fresh and aged tissue. According to Romberger and Norton (110, p. 20-29) oxygen uptake of fresh potato tissue was inhibited about 15 percent by 0.05M malonate at pH 5.0, whereas oxygen uptake of aged tissue was inhibited about 50 percent. This is only a 3.3-fold increase in sensitivity which might be at least to a large extent due to concentration differences in the tissues, in view of the increase in uptake of organic acids brought about by aging. Moreover, malonate inhibition is not a very reliable tool to evaluate pathways of carbohydrate metabolism or respiratory pathways. Although low concentrations of malonate may have specific inhibitory effects on succinic dehydrogenase, higher concentrations may inhibit other enzymes such as glycolic acid oxidase, pyruvic kinase, etc. (10, p. 183-191; 11, p. 725-735; 37, p. 977-987; 39, p. 1124-1130; 46, p. 438-445; 53, p. 2095-2110; 100, p. 314-324; 141, p. 299-312). Hackett suggested (57, p. 113-146) that TCA cycle activity may not be rate limiting in the respiration of certain tissues like fresh chicory slices and potato slices. This is true in certain leaf tissues (45, p. 346-355), where organic acid concentrations are probably high enough to successfully compete

with low concentrations of malonate. Such a reversal of malonate inhibition by organic acids has been reported (23, p. 311-326; 25, p. 497-518; 82, p. 8-15). In potato slices aged in a high pCO_2 no malonate sensitivity was observed (77, p. 679-690). Here again the organic acid concentration was probably raised by CO_2 fixation, thus enabling the tissue to successfully compete with the low malonate concentration. In support of the contention that TCA cycle activity is probably not a rate limiting factor of respiration in fresh storage tissue slices, it may be pointed out that 2, 4-dinitrophenol (DNP) stimulates the respiration of fresh storage tissue discs (6, p. 839-847; 79, p. 378-391; 83, p. 215-299). Moreover, malonate seems to be generally present in plant tissues, and most plant tissues can metabolize administered malonate (50, p. 498-499; 116, p. 683; 136, p. 629-640). These facts also must be taken into consideration when malonate inhibition is used as a diagnostic tool.

3. Further Studies on the Effects of Washing in KH_2PO_4 Solution on the Glucose Catabolism of Red Beet Slices

Since washing in 0.01M KH_2PO_4 solution was indicated to enhance PPP participation, changes in relative participation of pathways were further examined in another series of experiments. Rather young beets from a local wholesale store were used in this series of experiments. Using the same procedures as before, the kinetics of

C^{14} release from 1, 2, 3+4, and 6 positions of glucose was determined in fresh slices as well as in slices washed in 0.01M KH_2PO_4 solution for varying lengths of time. The interval and cumulative percentage yields from fresh and washed slices are shown in Figures I, II, and III.

Figure I shows that the rate of release of C^{14} from 3, 4 positions by fresh red beet slices was much faster than the other positions of glucose as indicated by the yield of $C^{14}O_2$. C_1 of glucose was released by the fresh tissue faster than C_6. The C_1/C_6 ratio of fresh tissue during the first two hours was 1.4, and approximate estimation according to the equations of Barbour et al (8, p. 396-400) gave a value of about 15 percent for PPP participation. The changes in C_1/C_6 ratio, and estimated percentage participation of PPP, along with radiochemical yield of $C^{14}O_2$ at the end of experimental time, are given in Table IV.

In fresh tissue, the relative rates of release of the individual carbons of glucose indicate a major role for the EMP-TCAC pathway. Participation of the PPP is also suggested by the non-identical yields of $C^{14}O_2$ from C_1 and C_6 as well as from the approximate estimations. It is to be pointed out that C_2 release was slower than C_6 release in fresh slices. A simple glycolytic sequence would release C_2 and C_5 faster than C_6 since the former are equivalent to C_1 of acetate

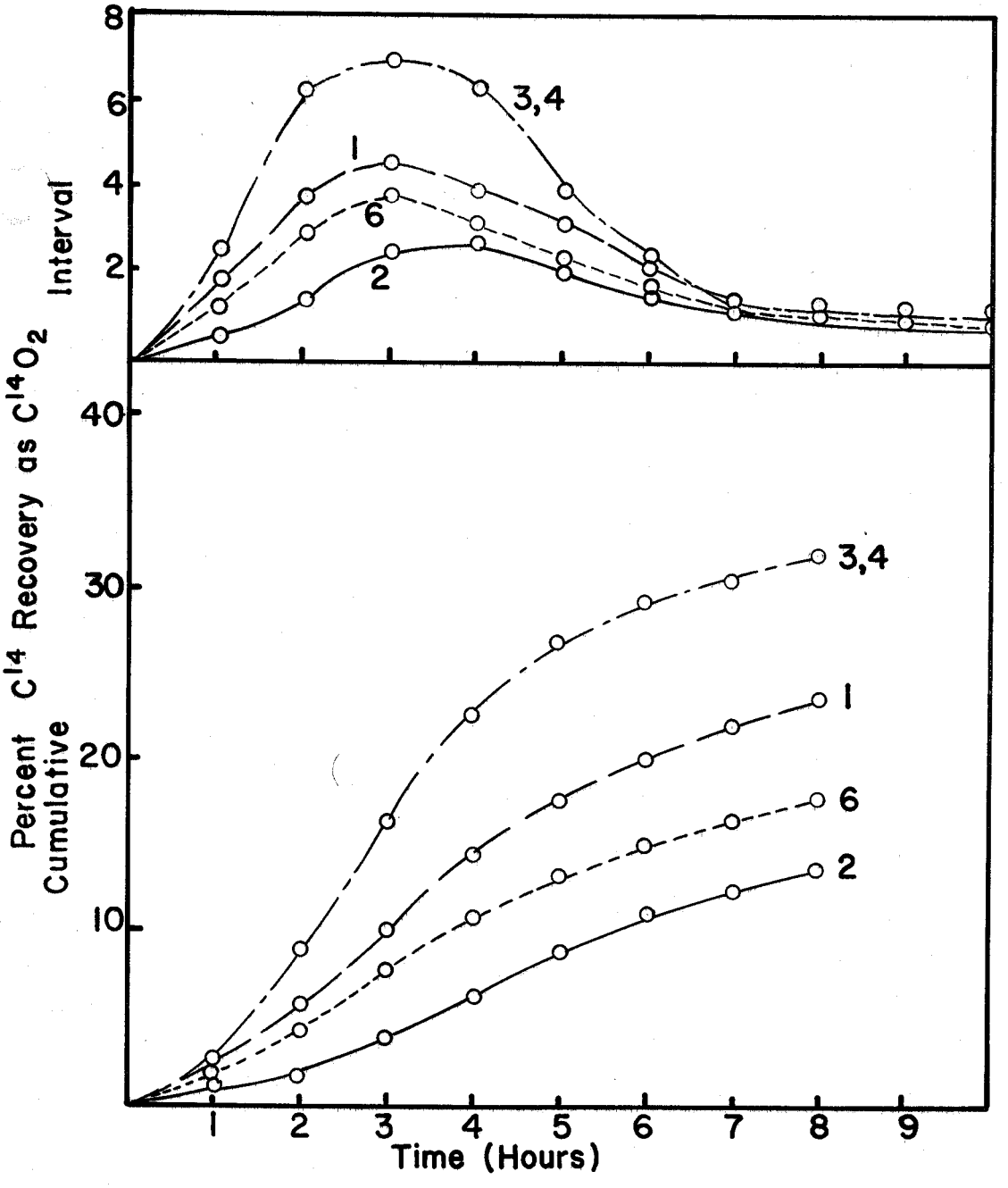


Figure I. Time Course Plots of Percent Interval and Cumulative Respiratory $C^{14}O_2$ Recoveries From C^{14} Specifically Labeled Glucose Metabolized by Fresh Beet Slices.
1 = glucose-1-C^{14}; 2 = glucose-2-C^{14}; 3, 4 = glucose-3, 4-C^{14}; and 6 = glucose-6-C^{14}.
Experimental conditions were the same as those shown under Table I.

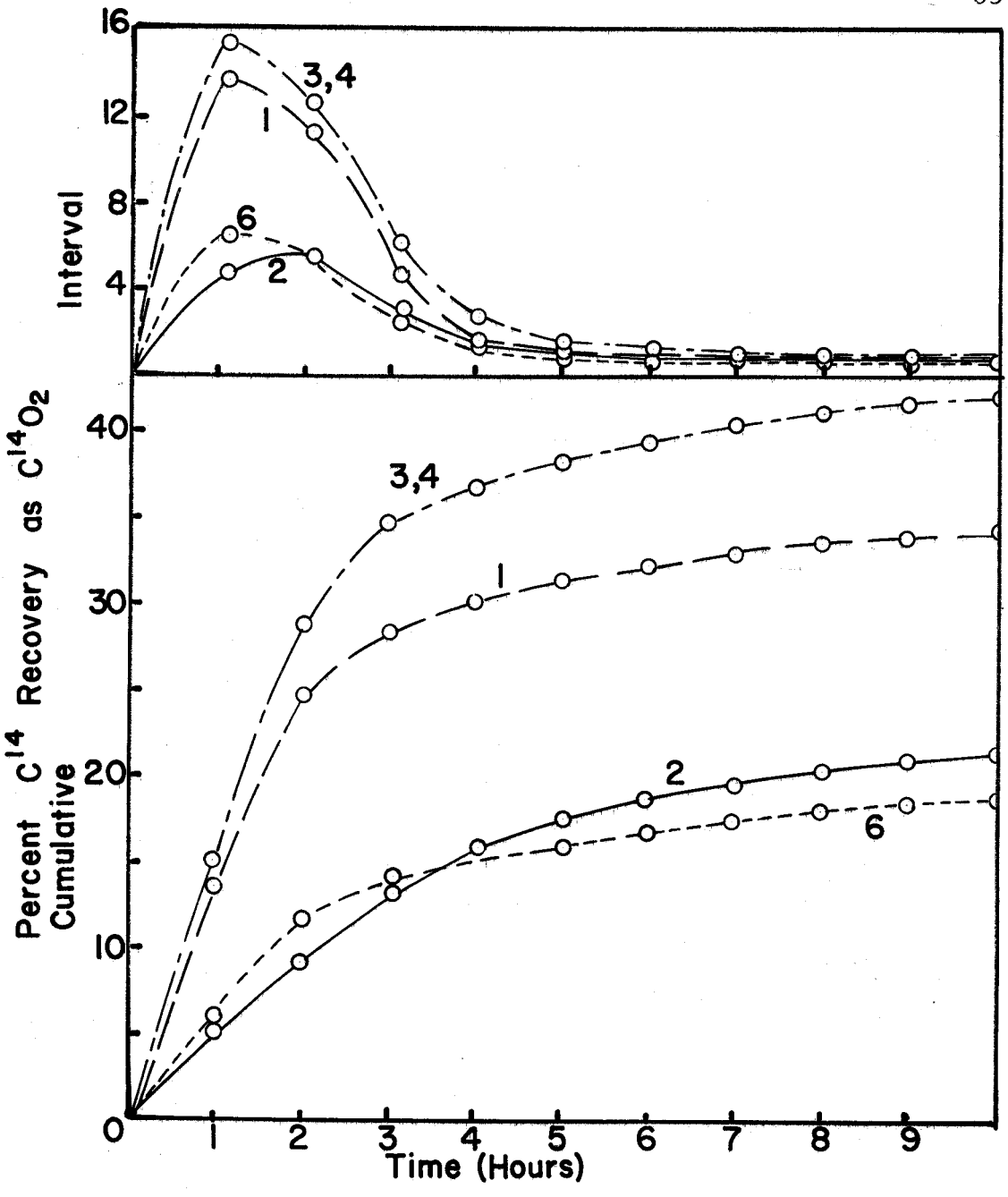


Figure II. Time Course Plots of Percent Interval and Cumulative Respiratory $C^{14}O_2$ Recoveries From C^{14} Specifically Labeled Glucose Metabolized by Beet Slices Washed in 0.01M KH_2PO_4 Solution for 24 Hours. See Figure I for Explanation of Legend. Experimental conditions were the same as those shown under Table I.

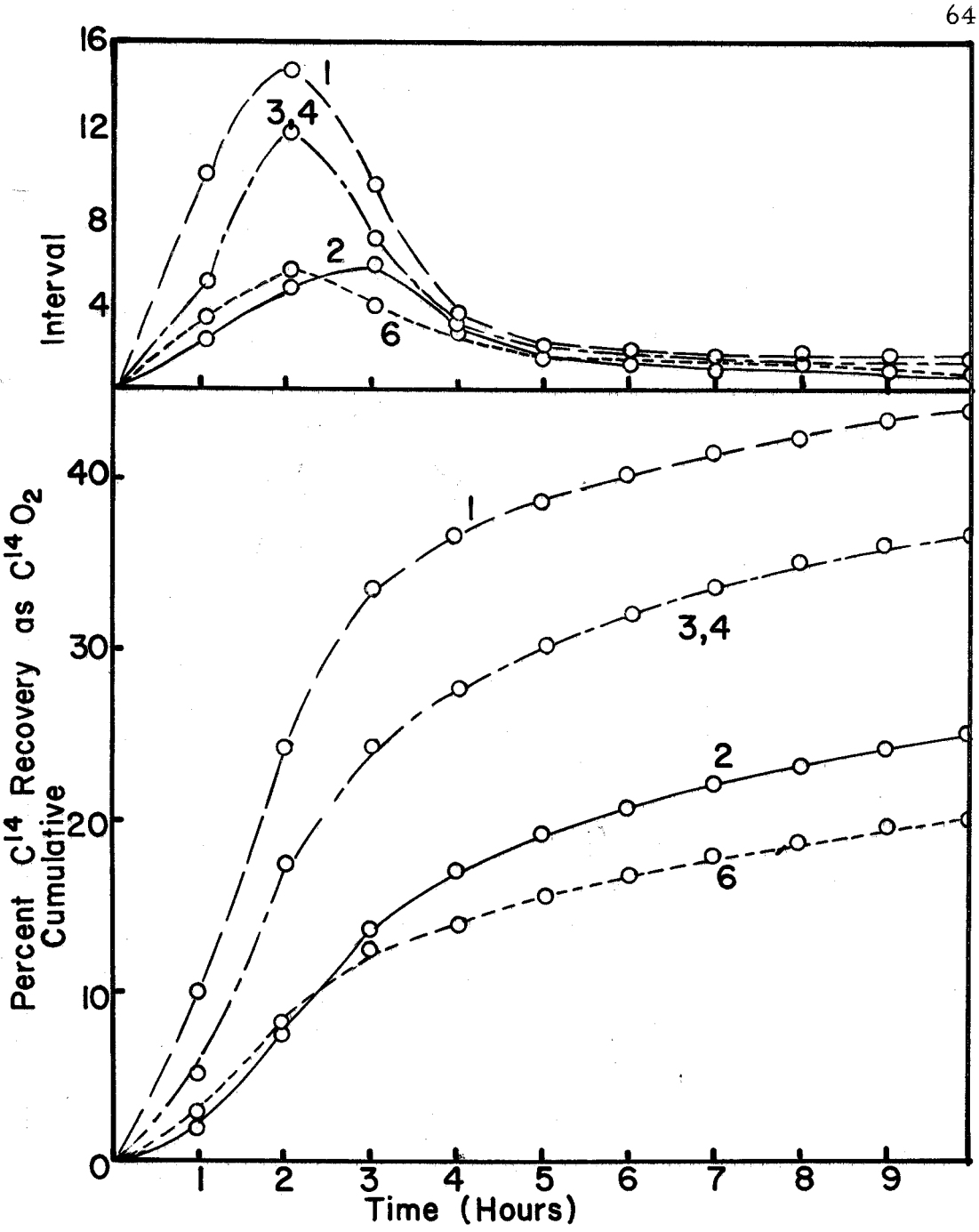


Figure III. Time Course Plots of Percent Interval and Cumulative Respiratory $C^{14}O_2$ Recoveries From C^{14} Specifically Labeled Glucose Metabolized by Red Beet Slices Washed in 0.01M KH_2PO_4 Solution for Four Days. See Figure I for Explanation of Legend. Experimental conditions were the same as those shown under Table I.

Table IV.

Effect of washing in potassium phosphate solution on the cumulative recovery of C^{14} in CO_2 from red beet slices,*

Substrate	Duration of washing			
	Fresh	24-hour	4 days	8 days
Glucose-1-C^{14}	27	35	44	61
Glucose-2-C^{14}	15	22	24	38
Glucose-3, 4-C^{14}	33	42	37	42
Glucose-6-C^{14}	19	19	20	32
C_1/C_6 ratio	1	2.1	2.9	3.4
Percent participation of PPP	15	31	48	51

*Duration of the experiment was 10 hours.

and the latter equivalent to C_2 of acetate. Specifically labeled acetate is shown in a later section to release C_1 faster than C_2, as expected with the function of the TCA cycle. The PPP activity also would cause the release of C_2 much faster than C_6 because C_2 of the original glucose molecules becomes C_1 of the reformed hexose. However, reverse aldolase activity would cause exchange of triose units (112, p. 10-15) and this would result in conversion of C_6 into C_1 and the subsequent release of it via PPP. Moreover, the oxidative release of carbon 6 of hexose is presumably functioning in most plant tissues (97, p. 187-193; 120, p. 146-151). Thus, a C_6/C_2 ratio greater than one is not surprising, since such a ratio seems to be of quite common occurrence. For example, Barbour et al (8, p. 396-400) obtained C_6/C_2 ratios greater than one in tomato fruits. Humphrey and Dugger (69, p. 580-582) and Butt and Beevers (30, p. 21-27) obtained C_6/C_2 ratios of about two with corn roots. Using liver slices from rats fasted for 48 hours, Agranoff et al (1, p. 773-779) also obtained a C_6/C_2 ratio considerably higher than one. Corn root tips, corn coleoptile, wheat and beet were recently reported to release more of C_6 than of C_2 (91).

A comparison of the $C^{14}O_2$ patterns obtained from fresh (Fig. I) and washed red beet slices (Figs. II and III) indicates several interesting metabolic changes upon washing. Washed slices utilized

administered glucose much faster than fresh slices. Conversion carbons 1, 2, and 3+4 of glucose to respiratory CO_2 has been enhanced by washing the slices for 24 hours.

The relative increase in the rate of release of $C_{3,4}$ and increased final yield of $C^{14}O_2$ (ten hour) from the $C_{3,4}$ position of glucose indicates an increased ability of the washed slices to utilize exogenous glucose via the glycolytic pathway. A significant increase in the release of C_6 also is in agreement with such a contention. However, the relative increases in C^{14} release from C_1 and C_2 were significantly higher than the increase in $C_{3,4}$ or C_6. This indicates that participation of the PPP is higher in the washed slices than the fresh slices. Another noticeable change in the pattern was the increase in the release of C_2 as compared to C_6. In the tissue washed for 24 hours, release of C_2 overtook that of C_6 and the final yield of C_2 was clearly more than the yield of C_6. This change would be expected since recycling of the reformed hexoses via PPP would favor the release of C_2. The increase in C_1/C_6 ratio, as shown in Table IV, also suggests that washing has increased relative participation of PPP. Moreover, the approximate estimation of the percent participation of PPP made according to the equations of Barbour et al (8, p. 396-400) shows that percent participation of PPP has doubled as a result of washing for 24 hours.

In Figure III and Table IV are shown changes that occurred during further washing. The $C^{14}O_2$ pattern of slices washed for four days (Fig. III) shows that C_1 release has become the fastest followed by $C_{3,4}$, C_2 and C_6 in that order. The most significant aspect seems to be the dramatic change in the relative rates of C^{14} release from C_1 and $C_{3,4}$ positions. As shown in Table IV, C_1 release gradually increased as the duration of washing increased. A similar increase was noticeable in the case of C_2 also. However, after the increase in $C_{3,4}$ that happened during the first 24-hour washing period, there was no more increase but instead a decrease was noticeable. The rate of release of C_6 did not change very much during continued washing even up to four days. Such a pattern indicated that in the slices washed for four days there was a much stronger participation of PPP than in the fresh or 24-hour washed slices. By the same reasoning, eight-day washed slices had still higher percent participation of PPP. In agreement with this contention is the estimated percent participation of PPP as is shown in Table IV. It must be pointed out that the percent recoveries of C^{14} from externally administered glucose as is used here is not intended to give any indication of the amount of glucose that is catabolized, but only relative importance of participation of pathways.

4. Utilization of TCA Cycle Intermediates by Fresh and Washed Red Beet Slices

Acetate, although not found to any extent as a normal metabolite in plant tissues, can be metabolized by a variety of plant tissues presumably via the TCA cycle. Thus, changes in acetate utilization due to the washing of red beet slices are of interest. Acetate-2-C^{14} was used as a tracer and the cumulative C^{14} recoveries are shown in Figure IV. Fresh slices utilize acetate rather slowly and by the end of experimental time (ten hours) about 25 percent of C^{14} appeared in respiratory CO_2. However, washing of the beet slices resulted in much faster utilization of acetate by them. In the first two hours fresh slices released only a little more than 3 percent of C^{14}, whereas slices washed for 24 hours released about 15 percent and those washed for four days about 25 percent. Washing of the slices for still longer periods increased the C^{14} release from acetate as shown in the case of slices washed for eight days, which released about 33 percent of the administered C^{14}. Uptake evidently could play a part in this increased C^{14} release, but as shown in Table V, even fresh slices took up 75 percent of the administered substrate in the first two hours. Although the washed slices were capable of faster acetate uptake, this difference does not affect the conclusions since the C^{14} release is based on the uptake and slight differences in

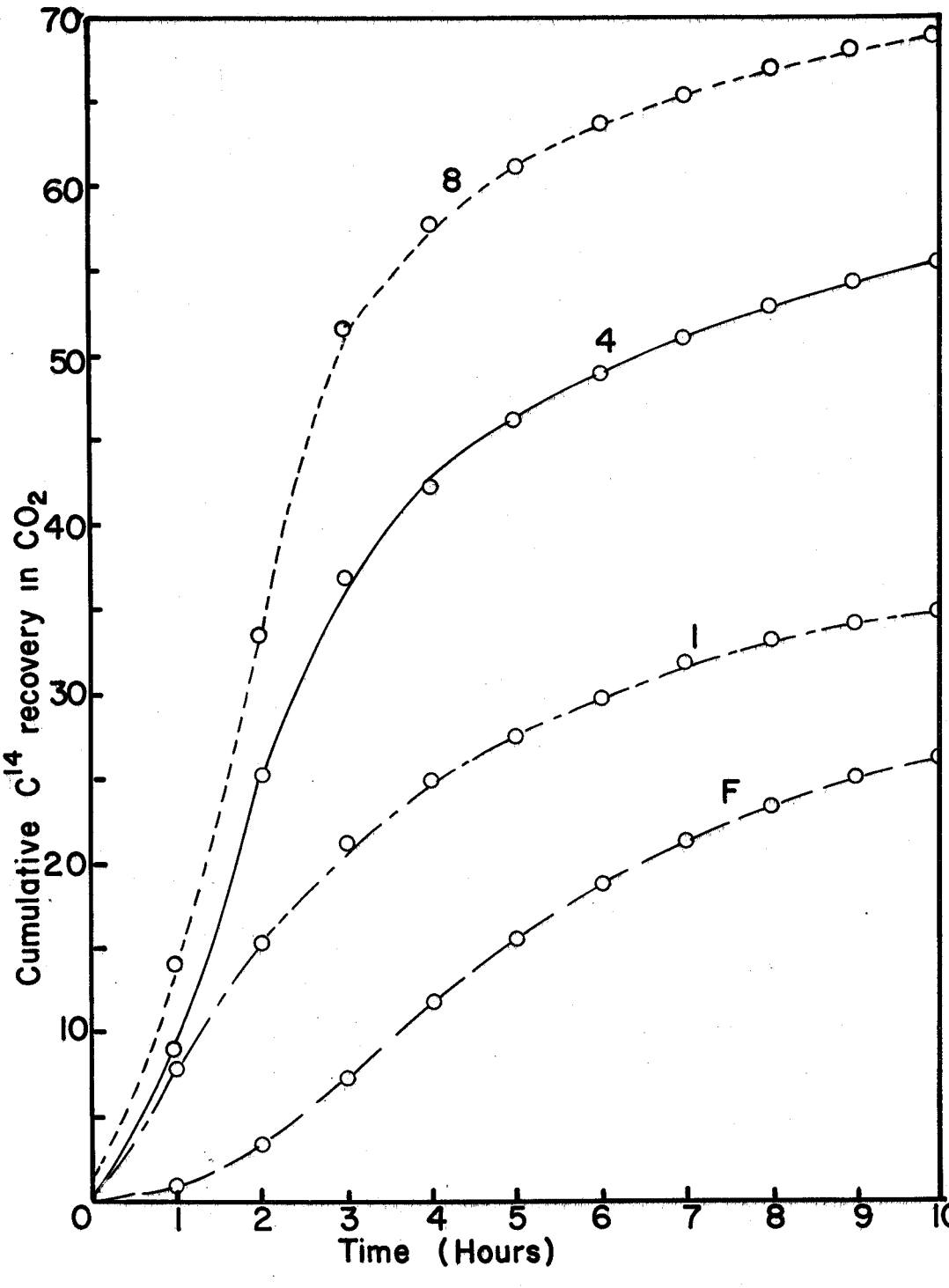


Figure IV. Time Course Plots of Percent Cumulative Respiratory $C^{14}O_2$ Recoveries from Acetate-2-C^{14} Metabolized by Fresh and Washed Red Beet Slices.

F = Fresh 1 = One-day washed

4 = Four-day washed 8 = Eight-day washed

Experimental conditions were the same as those shown under Table I.

uptake is not enough to explain the several fold increase in the rate of C^{14} release. A decrease in pool size of TCA cycle intermediates caused by long term washing is probably a major factor in the increased C^{14} release from acetate by four-day and eight-day washed slices. This view is supported by the fact that the R. Q. for storage tissue has been reported to be changed (128, p. 1-34) and amino acid pools have been found to decrease by long washing periods (44, p. 129-136; 122, p. 436-453).

Table V. Effect of Washing on Substrate Uptake by Red Beet Slices.

Substrate	Percent C^{14} left in the medium					
	Fresh			Washed		
	2hr	4hr	6hr	2hr	4hr	6hr
Glucose	51	25	11	8	3	-
Acetate	25	3.5	-	2	1	-
Succinate	65	35	-	5.5	1	-

Acetate-2-C^{14}, while traversing through the TCA cycle, becomes succinate-2, 3C^{14} and thus C^{14} begins to be released from it in the third turn of the TCA cycle. C^{14} from succinate-1-4-C^{14} would be released faster than C^{14} from succinate-2, 3-C^{14} or acetate-2-C^{14}, as C^{14} is released from it in the first turn of the TCA cycle. So, succinate-1, 4-C^{14} was administered to the fresh slices and

slices washed for different lengths of time and the cumulative C^{14} recoveries are shown in Figure V. Fresh slices released C^{14} at a comparatively slow rate releasing about 27 percent of C^{14} in three hours, whereas slices washed for 24 hours released C^{14} very rapidly and about 70 percent of C^{14} was released in three hours. Any further increase due to longer washing periods would not be clearly indicated because most of the C^{14} was already released. So effects of further washing were not indicated by such experiments. Fresh slices took up about one-third of the administered C^{14} in two hours, whereas washed slices took up essentially all the administered C^{14} (Table V). These results agree well with the contention that the TCA cycle is operative in the fresh and washed tissues. Since changes in pool sizes were not determined, it is not possible to say whether or not TCA cycle activity increased by washing, although washing increased the ability to utilize exogenous organic acids. However, the results would agree with the view that TCA cycle activity is increased by washing.

5. Changes in the Activity Levels of Glucose-6-Phosphate Dehydrogenase Due to Washing

Since washing increased participation of the PPP in the catabolism of glucose, the activity levels of the enzymes of the PPP might

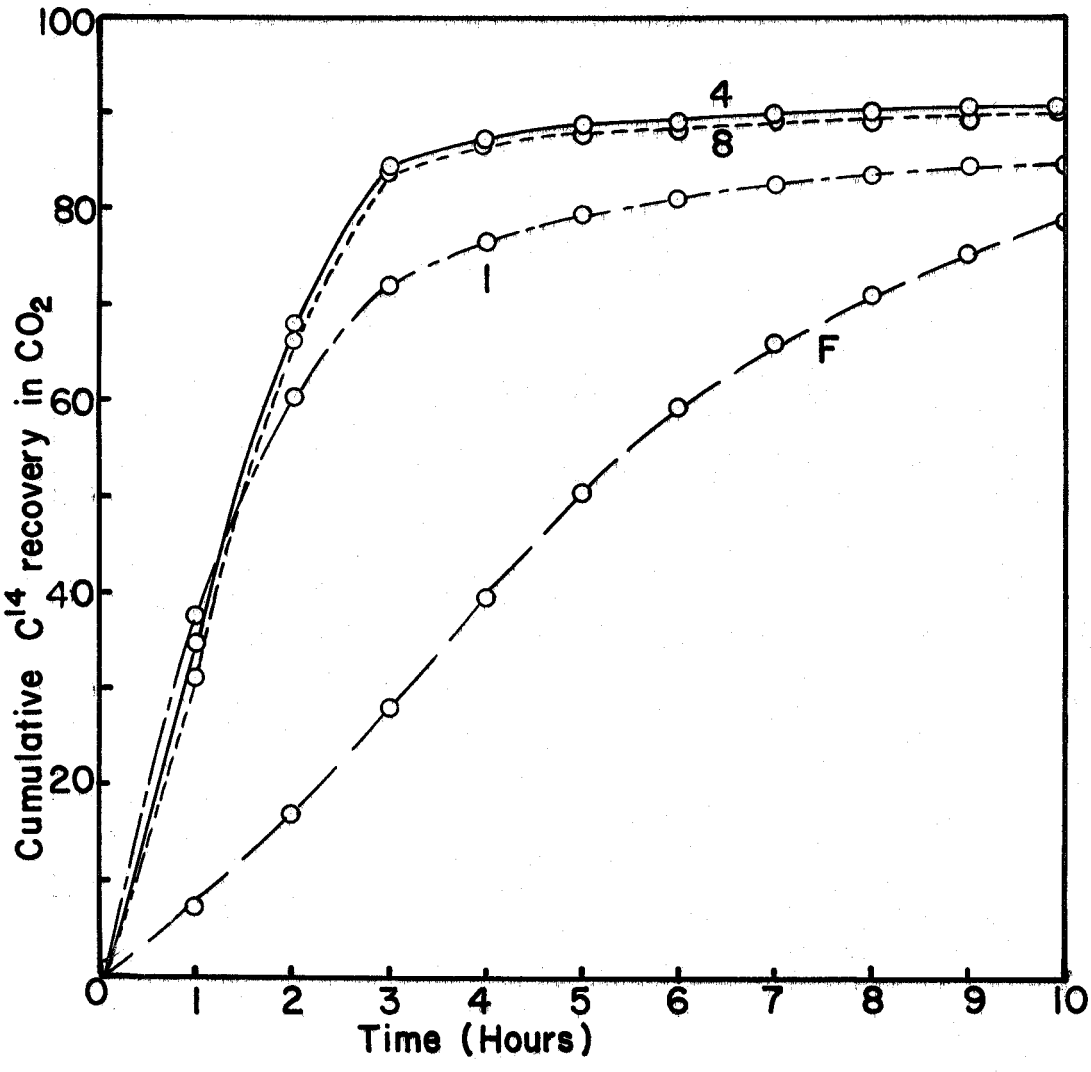


Figure V. Time Course Plots of Percent Cumulative Respiratory $C^{14}O_2$ Recoveries from Succinate-1, 4-C^{14} Metabolized by Fresh and Washed Red Beet Slices.

F = Fresh
1 = One-day washed
4 = Four-day washed
8 = Eight-day washed

Experimental conditions were the same as those shown under Table I.

have undergone considerable changes during the washing period. To examine this aspect, the activity levels of glucose-6-phosphate dehydrogenase was determined in the cell-free extracts of fresh and washed red beet slices. Changes in activity levels of this enzyme during the washing period are shown in Figure VI. Activities are based on unit volume of extracts prepared under identical conditions from an equal number of slices. During the first 24 hours of washing, enzyme activity levels increased by 20 to 30 percent of the original activity. Since this increase was not measured in shorter intervals of the washing period, a more detailed pattern of increase is not known. Washing for two days was accompanied generally by a decrease, resulting in about 90 to 100 percent of the original activity. Continued washing for longer periods of time was accompanied by a rather rapid decrease in activity of the enzyme until about 20 to 30 percent of the original activity was observable after about five to six days, after which time the decrease in activity was extremely slow. Although the level of the enzyme alone is not an indication of the extent of participation of pathways, the results suggest that during the long washing periods, the tissue was probably under severe starvation, and so proteins may be catabolized. In support of this view, it may be pointed out that a decrease in R. Q. (128, p. 1-34) and lowering of S^{35} incorporation by long term washings of red beet slices

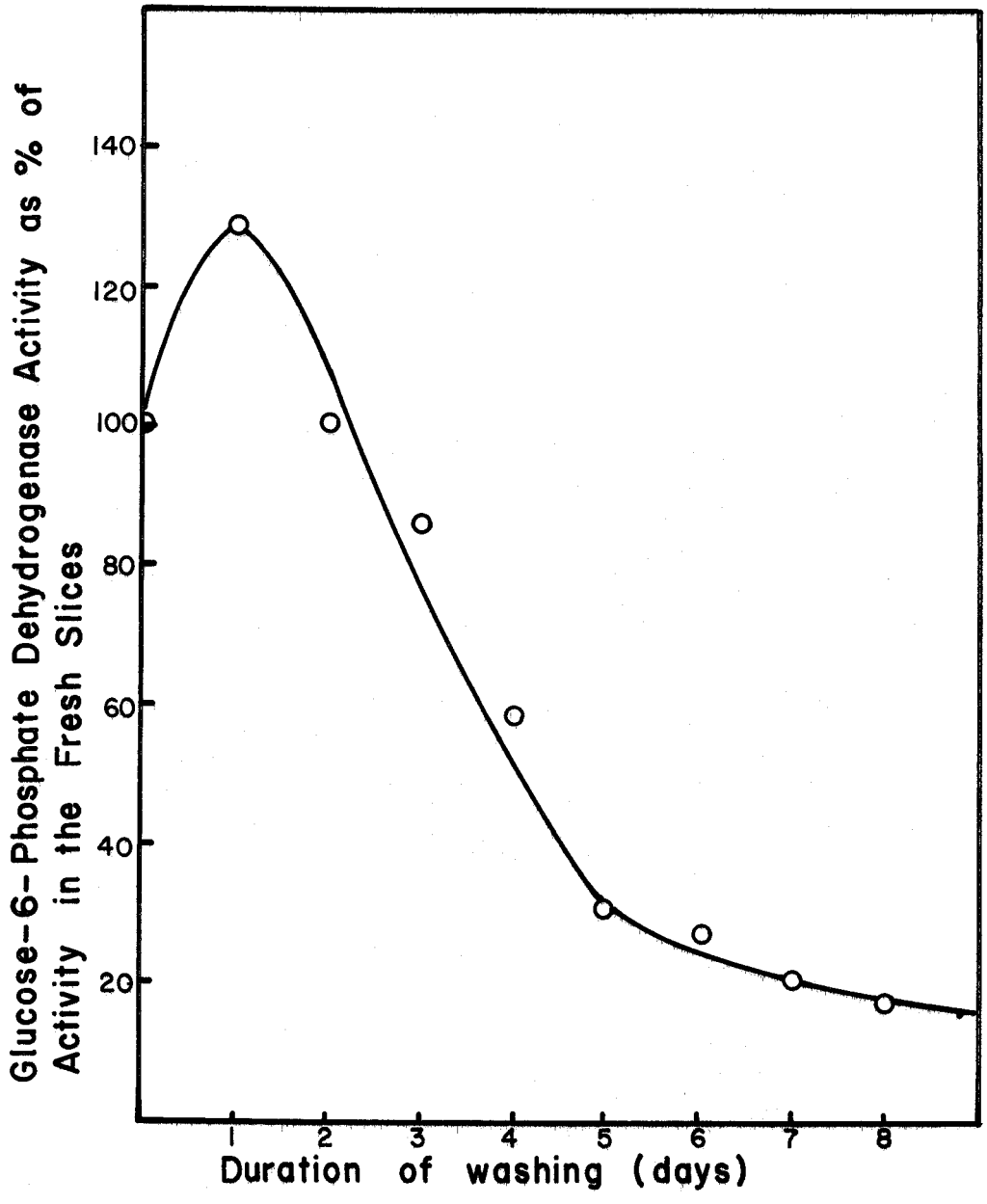


Figure VI. Changes in Glucose-6-phosphate Dehydrogenase Activity in Beet Slices due to Washing in 0.01M KH_2PO_4 Solution. Fifty Slices Were Used to Prepare Extracts. Assay Reaction Mixture Consisted of 0.3 ml Extract, 60 μ moles of $MgCl_2$, 1.2 μ moles TPN, 10 μ moles of Glucose-6-phosphate and 2.4 ml 0.05M Tris buffer (pH 7.6) Containing 0.005M 2-mercaptoethanol in a Total Volume of 3.0 ml. The Reference Blank was Identical to the Reaction Mixture Except for Glucose-6-phosphate. Absorbance Change at 340mμ was Measured in a Bekman DB Spectrophotometer with Recorder.

(44, p. 129-136) have been reported and the respiration rate of such washed tissue is known to be very low (128, p. 1-34). Triosphosphate dehydrogenase was found to be greatly decreased by a washing period of even four days. These results indicate that the latter part of the washing time represents a dying period of the tissue and thus the results obtained with such slices may not be related to the metabolic changes that are usually associated with the aging of storage tissue slices.

In the experiments discussed thus far, slices were not prepared and washed aseptically. The slices did not show symptoms of serious bacterial infection as judged by a lack of exudation of pigment, absence of loss of turgidity, and absence of turbidity in the medium. However, frequent changes of the washing medium could have been the reason for not observing any turbidity. Such criterion have been used as indications of bacterial contamination (13, p. 65-92). However, a closer examination of the slices by grinding the slices in a mortar and plating this suspension in complete nutrient agar using a serial dilution technique under aseptic conditions, showed that the slices when washed for about 4 days contained 10^7-10^8 bacteria per 15 slices. Consequently, for further experiments, aseptic techniques described in the experimental part were used.

PART B.

Experiments With Sterile Red Beet Slices Washed in Potassium Phosphate Solution

1. Changes in the Rate of Respiration

Slices from beets freshly dug from a local farm were used in a series of experiments. Respiration rates as determined by standard Warburg techniques are shown in Table VI. Fresh slices respired at a rather steady level for about three to four hours. Reproducibility of rates of respiration depended upon the previous history of the beet used. For example, beets obtained from a local market usually showed a higher respiration rate as compared to freshly dug beets. The respiration rate was based on an equal number of slices rather than equal weight. Such a procedure became necessary because usually slices when washed increased in fresh weight by about 20 percent and at the same time decreased in dry weight by about 15 percent. Slices washed for 24 hours started with a respiration rate much faster than fresh slices. However, this high rate of respiration fell rather fast and ended up at a respiration rate slightly lower than fresh slices.

The respiration rate of storage tissue slices is known to rise to a maximum and then decrease when the slices are washed

Table VI

Effect of washing on oxygen uptake by red beed slices.

Time	μl O_2/0.5 Hour	
Hours	Fresh	24 Hour washed
0.5	35	55
1.0	34	44
1.5	30	41
2.0	32	33
2.5	33	32
3.0	36	30
3.5	35	27
4.0	39	26

Flask contents: Eight beet slices (9mm in diameter, 1mm thick) fresh weight 0.5 gm

2.3 ml 0.01 phosphate buffer pH 4.9 - 5.0

0.2 ml 10% KOH in center well

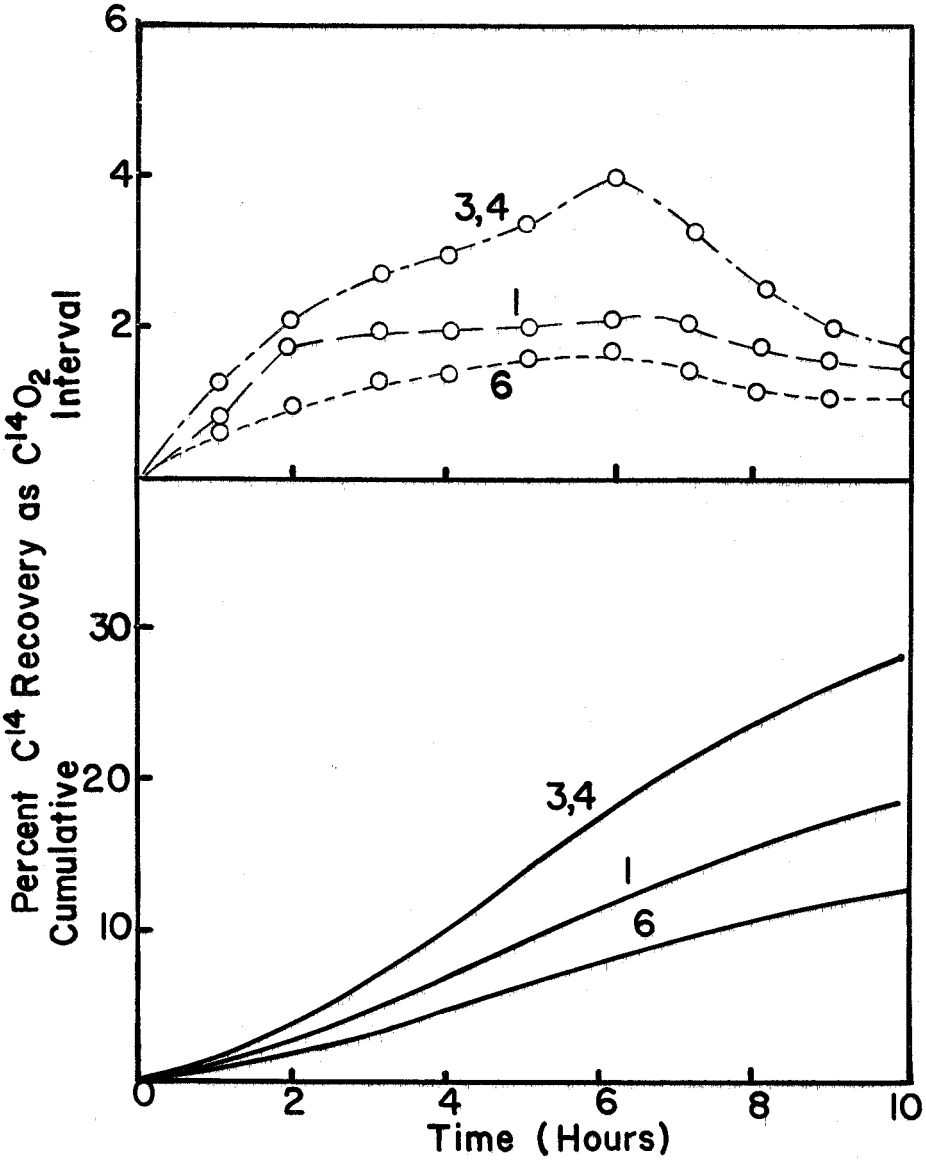
Temperature - 30°C

end of the 24-hour washing period.

2. Radiorespirometry Experiments With Sterile Slices of Red Beets.

A rather steady C_1/C_6 ratio of about 1·5-1·6 was observed during the initial phase of the experiment, whereas the ratio gradually increased to about two during the last few hours. When substrate depletion during the last few hours of the experiment causes such a change in the ratio, it tends to approach unity, whereas in these experiments the ratio was increasing as shown in Table VII. Such a change is indicative of a slight enhancement in PPP during the last few hours of aeration in the respirometry chamber. ApRees and Beevers (6, p. 839-847) reported similar changes in carrots and potatoes.

In Figure VII is shown the interval and cumulative recovery of C^{14} from slices metabolizing glucose-1-C^{14}, glucose-3, 4-C^{14} and glucose-6-C^{14}. Exogenous glucose was slowly metabolized by slices freshly cut from freshly dug beets. Rate of release of the 3, 4 positions of glucose was much faster than any other position, yielding, as $C^{14}O_2$, 27 percent of the total C^{14} that was taken up in ten hours. This is indicative of a major role played by glycolysis in glucose catabolism of these tissues. Carbon one of glucose was as usual released faster than carbon six, yielding 18 and 12 percent of C^{14},



FigureVII. Time Course Plots of Percent Interval and Cumulative Respiratory $C^{14}O_2$ Recoveries from C^{14} Specifically Labeled Glucose Metabolized by Sterile Fresh Beet Slices.

1 = glucose-1-C^{14}; 3, 4 = glucose-3, 4-C^{14}; 6 = glucose-6-C^{14}.

Experimental conditions were the same as those under Table I.

Table VII. Interval C_1/C_6 Ratio of Fresh Red Beet Slices.

Hourly interval	1	2	3	4	5	6	7	8	9	10
C_1/C_6 ratio	1.5	1.6	1.5	1.5	1.5	1.4	1.6	1.8	1.8	2.0

(89, p. 429-448; 109, p. 1-8; 128, p. 1-34). The time required for the respiration rate to reach the maximum has been shown to depend on the temperature (89, p. 429-448). At room temperature, the respiratory maximum in red beet slices has been reported to occur between 30 and 50 hours of washing (109, p. 1-8). In the light of these facts, it may be reasonably assumed that the higher temperature used in our experiments (26-27°C) caused the respiratory maximum to appear much sooner than 30 hours. Moreover, the physical disturbances caused by the shaking that was employed in our experiments, also probably caused the respiratory maximum to occur sooner than expected from the higher temperature alone, especially in view of the fact that mechanical disturbances have been shown to stimulate the respiration of storage tissues (128, p. 1-34). So, it may be reasonably assumed that in our experiments the respiratory maximum occurred sometime before the 24-hour washing was over and hence the slices were in the decreasing side of their respiratory-drift-pattern when the respiratory measurements were made at the

respectively, from one and six positions. This is indicative of the participation of the PPP in the metabolism of glucose in the fresh slices. This is substantiated also by the fact that an approximate estimation using the equation of Barbour et al (8, p. 396-400) gave a value of 22 percent for participation of the PPP.

Slices washed aseptically for 24 hours utilized exogenous glucose much faster than fresh slices as shown in Figure VIII. Just as in fresh slices, $C_{3,4}$ release was much faster than the release of the other positions of glucose. Substrate concentration became limiting after two to three hours and then the rate of recovery of C^{14} remained very low. At the end of ten hours about 33 percent of C_1 was released as CO_2, whereas about 41 percent of $C_{3,4}$ and 20 percent of C_6 was released. The initial C_1/C_6 ratio was about 2.3-2.4, which was higher than the initial ratio of fresh slices which was about 1.5-1.6. Such a change in the C_1/C_6 ratio supports the contention that participation of PPP in the glucose catabolism of washed slices is somewhat higher than that of fresh slices. This is further supported by the estimated 28 percent participation of PPP. Uptake of glucose by the slices freshly cut from freshly dug beets was very low as shown in Table VIII, whereas slices washed for 24 hours took up glucose very rapidly. For example, during the first two hours about 26 percent of the administered glucose was taken up by fresh slices,

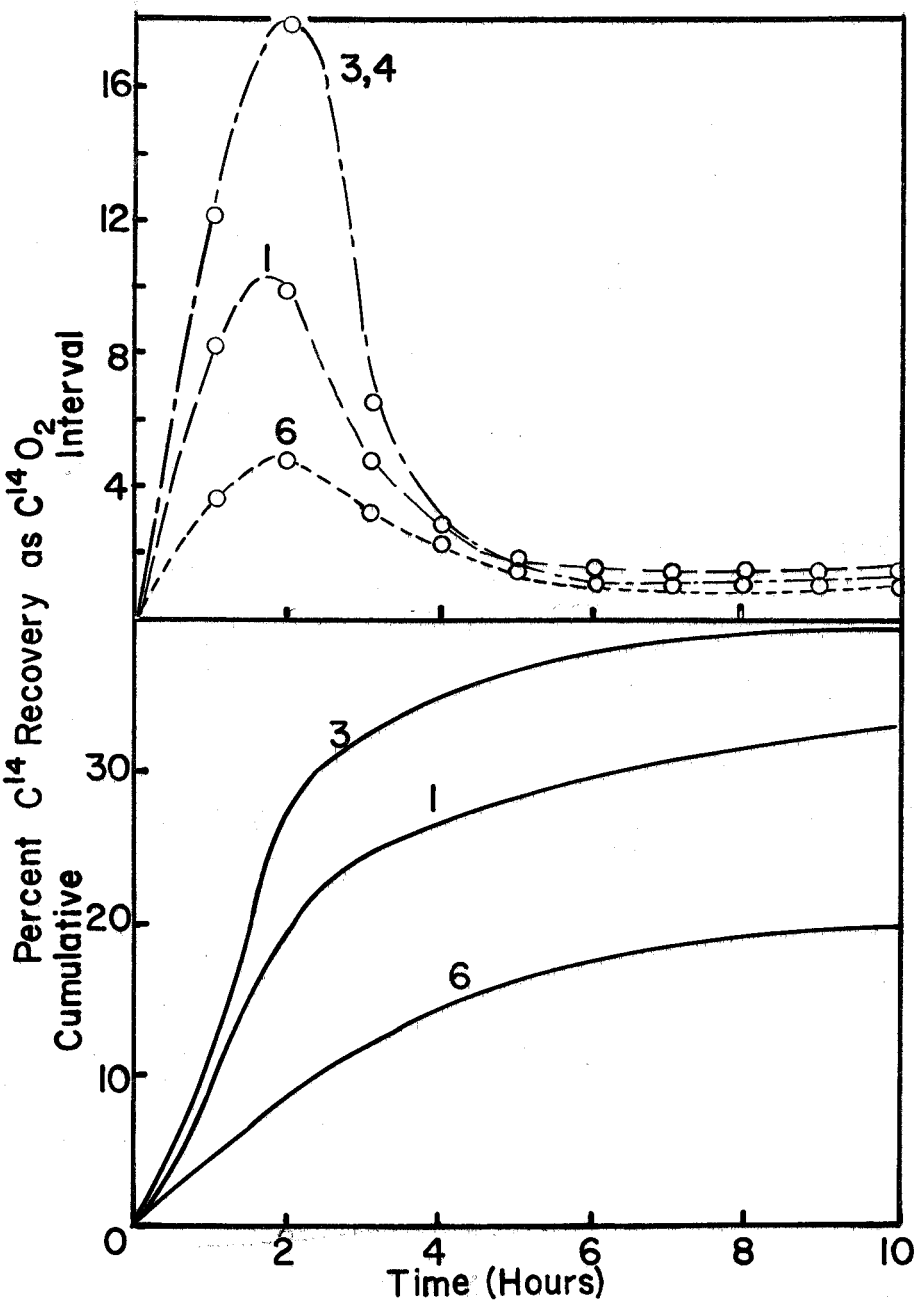


Figure VIII. Time Course Plots of Percent Interval and Cumulative Respiratory $C^{14}O_2$ Recoveries from C^{14} Specifically Labeled Glucose Metabolized by Sterile Fresh Beet Slices.

1 = glucose-1-C^{14}; 3, 4 = glucose-3, 4-C^{14}; and 6 = glucose-6-C^{14}.

Experimental conditions were the same as those under Table I.

whereas the same number of washed slices under identical conditions took up about 95 percent. However, since substrate in the medium became a limiting factor, the 95 percent uptake cannot be compared to that of fresh slices to draw conclusions about quantitative changes in uptake.

Table VIII. Effect of Washing on Glucose Uptake by Red Beet Slices.

Time (hr):	2	4	6	8
	(Percent of administered)			
Fresh.....	26	48	62	74
Washed.....	95	96	97	--

3. Changes in Activity of Levels of Certain Enzymes.

In view of the changes in the relative participation of the PPP in washed beet tissues, as suggested by tracer studies, it would be interesting to know whether the activity levels of certain enzymes participating in carbohydrate metabolism undergo any changes during the washing period. Glucose-6-phosphate dehydrogenase activity of the slices prepared nonaseptically was indicated before to be slightly increased by washing for a day. When aseptically prepared slices were used, similar results were obtained as shown by the typical experimental results in Table IX. Although the increment was not very large, the activity level of this enzyme in washed slices was consistently higher than that in the fresh slices. The increase in activity level of this enzyme, is not believed to cause the change in participation of pathways due to its relatively small increase. Activity of 6-phosphogluconate dehydrogenase was assayed in the crude extracts prepared from fresh and washed slices of red beets and the

Table IX

Effect of washing on enzyme activity levels in red beet slices.

Enzyme Activity	Δ O.D. 340/min* Fresh	Δ O.D. 340/min* 24 Hr. Washed	Percent Change
Endogenous TPN Reduction	0.1	0.07	- 33
Endogenous TPNH Oxidation	0.07	0.16	+ 134
Glucose-6-PO_4 Dehydrogenase	0.37	0.43	+ 16
6-Phosphogluconate Dehydrogenase	0.05	0.20	+ 300
Isocitrate Dehydrogenase (TPN)	0.3	0.53	+ 77
Malic Dehydrogenase (TPN)	0.25	0.16	- 36

*Activity based upon mg of non-dialyzable Kjeldahl Protein.

Assay reactio mixtures: 6-phosphogluconate dehydrogenase - 10 μmoles of 6-phosphogluconate (Na salt), 1.2 μmoles of TPN 100 μmoles $MgCl_2$, 0.3 ml beet extract; Malic dehydrogenase: 10 μmoles oxalo acetate, 0.5 mg TPNH, 0.3 ml beet extract; Isocitrate dehydrogenase: 20 μmoles d,l-isocitrate, 100 μmoles $MgCl_2$, 1.2 μmoles TPN 0.3 ml beet extract. All reaction mixtures were in a total volume of 3 ml of 0.05M Tris-HCl buffer, PH 7.6, containing 0.005M 2-mercaptoethanol. O.D. change at 340 mμ was measured in a Beckman DB spectrophotometer with recorder. Path length 1cm.

results of a typical experiment are shown in Table IX. Comparison of activities of equal volume of identically prepared extracts from an equal number of slices, fresh and washed, showed that washing of the slices for 24 hours increased 6-phosphogluconate dehydrogenase activity level three to five fold. Protein determinations were done on the extracts after dialysis against distilled water for 24 hours. Extracts from washed slices were found to contain 20 to 40 percent less protein than the extracts prepared from fresh slices. It is very interesting to note that in fresh beets the activity of 6-phosphogluconate dehydrogenase was very low and that this activity was quickly developed by washing. Attempts to detect an inhibitor of 6-phosphogluconate dehydrogenase in the fresh beets were not successful.

Rate of reduction of added TPN was measured in the extracts made from fresh and washed slices. As shown in Table IX, a slight decrease in the rate was observed. However, the significance of this observation is hard to assess since the concentration of various TPN linked substrates and activities of TPN linked enzymes are not known in the crude extracts used in these experiments. However, the increased TPNH oxidizing power of the extracts prepared from washed slices, as shown in Table IX, may be significant. This change might indicate a faster turnover of TPNH which in turn may contribute to an increased PPP. Butt and Beevers (30, p. 21-27)

after studying the effect of various artificial electron acceptors on participation of pathways, suggested that the rate of reoxidation of TPNH might be the controlling factor.

Certain other dehydrogenases were also studied in the crude extract. Activity levels of TPN specific isocitrate dehydrogenase were determined in extracts prepared from fresh and 24-hour washed slices and some typical results are shown in Table IX. The activity of this enzyme was consistently found to be increased by 50 to 80 percent, due to washing. Since the function of this enzyme is not very well known, the significance of this observation is not easy to assess, but it may be suggested that the increased activity is serving to meet an increased need for TPNH in increased biosynthetic reductions. Activity of malic dehydrogenase as measured by TPN linked oxaloacetate reduction is shown in Table IX; however, the significance of such slight changes is uncertain. In view of the fact that activity levels were determined in crude extracts, of all the changes in activities observed, the change in 6-phosphogluconate dehydrogenase seem to be the most significant, and it is tempting to postulate a regulatory role for this enzyme. As pointed out before, increase in the rate of oxidation of TPNH might also be significant.

In view of the dramatic increase in 6-phosphogluconate dehydrogenase which occurs during the 24-hour period of washing, the

increase in activity of this enzyme with the duration of washing would be of interest. For this purpose, an equal number of slices were withdrawn at different intervals from a single batch of slices that was undergoing washing, and extracts were made and activities determined under identical conditions. The results of a typical experiment are shown in Figure IX. It appears that rather quick changes in activity levels of the enzyme are noticeable. After about six hours the activity was found to be about doubled.

4. Gluconate Metabolism by Fresh and Washed Red Beet Slices.

Gluconate has been generally assumed to be metabolized via PPP after phosphorylation, and thus its catabolism involves 6-phosphogluconate dehydrogenase. Since the fresh slices have very weak activity of this enzyme, such slices would be expected to metabolize gluconate very slowly. Further, if the increased activity of 6-phosphogluconate dehydrogenase observed in the crude extracts has a functional significance, washed slices would be expected to metabolize gluconate quite well. Table X summarizes results of an experiment designed to test this possibility. Specifically labeled gluconate was used as exogenous substrate to study the catabolism of gluconate in fresh and washed slices. Fresh slices released C^{14} from gluconate-1-C^{14} extremely slowly while carbon 2 and carbon 6 were

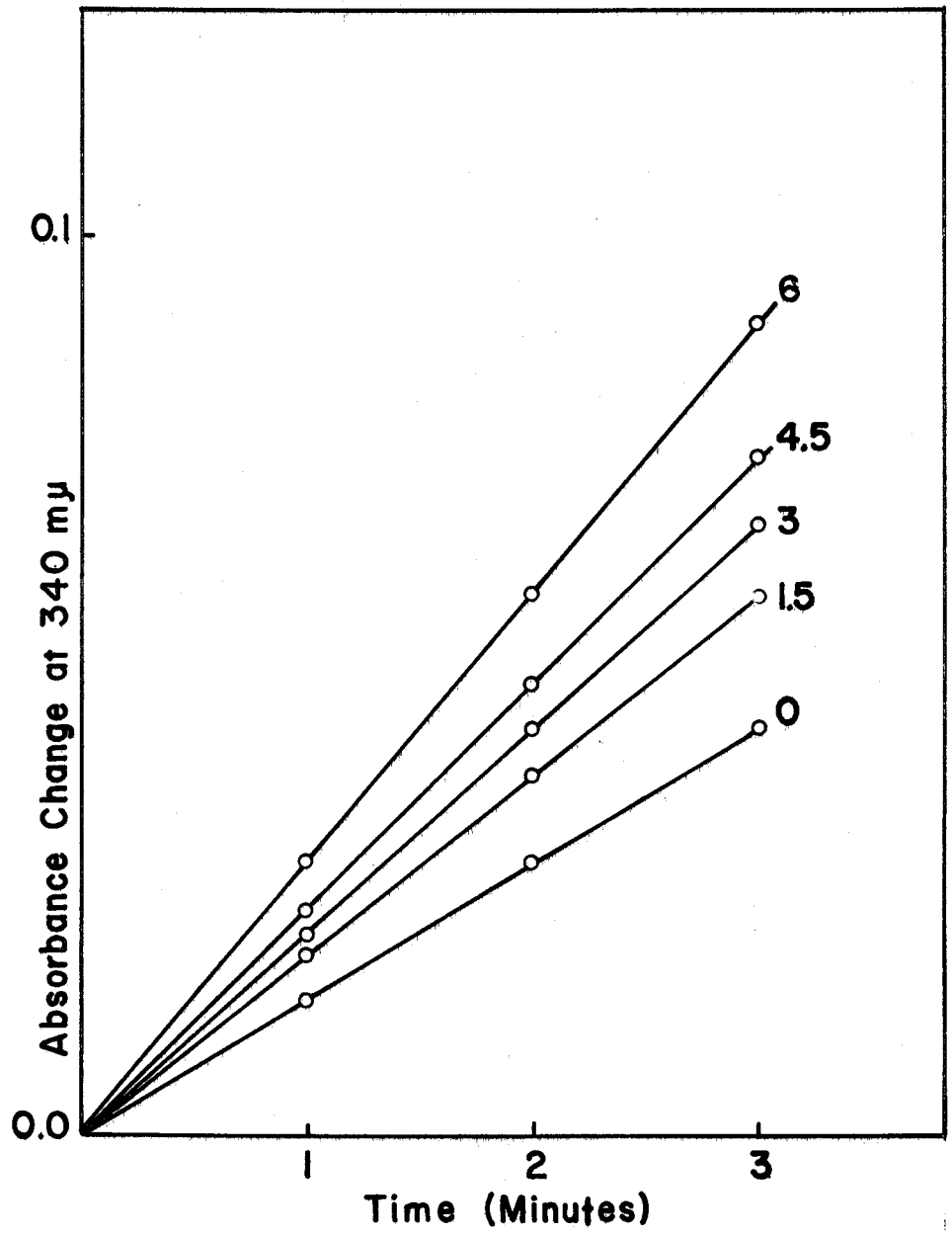


Figure IX. Time Course of Increase in 6-Phosphogluconate Dehydrogenase Activity, due to Washing in 0.01M KH_2PO_4 Solution. Assay Conditions are Identical to Those in Table IX. The Number on Each Line Denotes the Number of Hours of Washing.

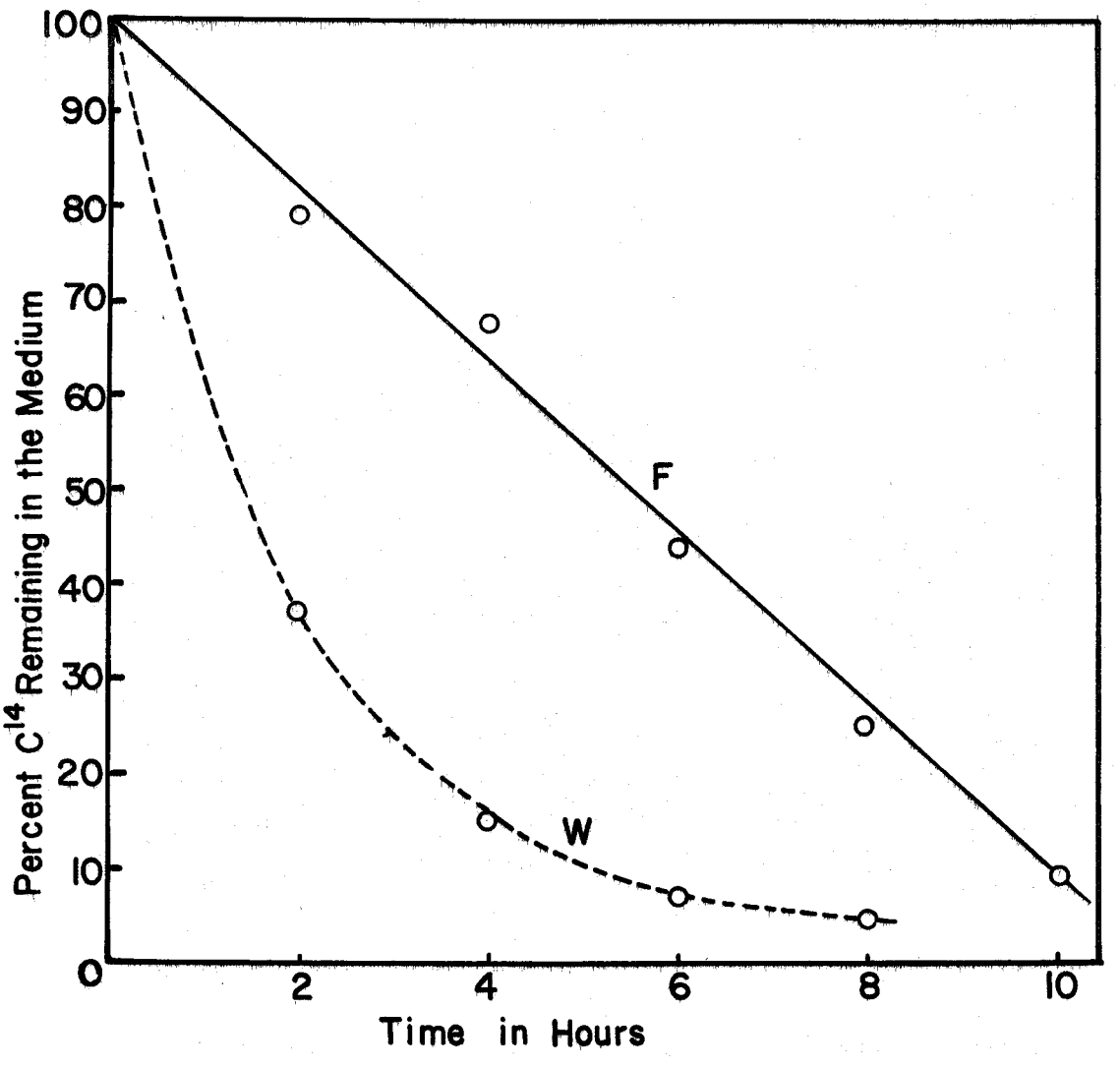


Figure X. Time Course of Gluconate Uptake by Fresh and Washed Red Beet Slices. 0.05ml of the Medium was Withdrawn and Assayed for C^{14} Every Two Hours During the Experiment Summarized in Table X.

F = Fresh; W = Washed.

released so slowly that C^{14} was virtually undetectable in the respiratory CO_2. However, slices washed for 24 hours released C^{14} quite rapidly, releasing about 38 percent of the C^{14} from gluconate-1-C^{14} during the first four hours. Considerable amounts of other carbons were also released as shown by the release of 18 percent of carbon 2 and nine percent of carbon 6 in four hours. If gluconate were to be metabolized, after phosphorylation, via the PPP, carbon 1 would be expected to be released much faster than any other carbon, followed by carbon 2, and carbon 6 would be the slowest to be released. The pattern of C^{14} release by both fresh and aged slices agree with such a scheme. Since uptake of other organic solutes such as glucose, acetate, succinate, etc., was found to be enhanced by washing, changes would be expected in gluconate uptake also. To test this possibility, small aliquots of the media were withdrawn at regular intervals and the disappearance of radioactivity from the medium was used as a measure of gluconate uptake. As shown in Figure X, gluconate uptake by fresh slices was fairly linear but rather slow, whereas washed slices took up gluconate much faster, and hence the substrate in the medium became the limiting factor. In the first two hours, about 20 percent of the gluconate administered was taken up by the fresh slices. On the basis of the activity that was taken up by the slices, about 1.5 percent of the C^{14} was released from gluconate-1-C^{14}

Table X

Effect of washing on gluconate metabolism in red beet slices.

	C^{14} recovery in CO_2 Percent of uptake			
	Fresh		24 Hour washed	
Substrate	2 hours	4 hours	2 hours	4 hours
Gluconate-1-C^{14}	0.30	2.0	20	38
Gluconate-2-C^{14}	0.06	0.26	8	18
Gluconate-6-C^{14}	0.004	0.2	3	9

Respiratory $C^{14}O_2$ output by fresh and washed beet slices metabolising specifically C^{14} labeled gluconate. Experimental conditions were the same as those in table I but 2.78 μmoles of C^{14} gluconate (K salt) was used as substrate.

by fresh slices, whereas the comparable value for washed slices was about 28 percent. Although it is hard to make meaningful quantitative comparisons between enzyme activity levels in a crude extract and the rate of utilization of externally administered substrates, it seems very evident that 6-phosphogluconate dehydrogenase activity was very weak in the fresh slices which utilized externally administered gluconate extremely slowly. Washing brought about a very rapid increase in the activity of this enzyme along with a similar increase in the ability to utilize externally administered gluconate.

5. Experiments With Tritiated Glucose

Increased participation of PPP would contribute to an enhanced production of TPNH which could either be oxidized by some oxidase or utilized in biosynthetic reductions. If the TPNH produced by increased PPP participation were to be oxidized by any of the terminal electron transport systems, more of the hydrogen from carbon 1 of glucose would end up in water than of the hydrogen of carbon 6 of glucose in the initial stages of the experiment. If the TPNH were to be used for biosynthetic reductions, an equal amount of hydrogen from carbon 1 and carbon 6 would be in the tissue water. To test these possibilities, glucose-1-H^3 and glucose-6-H^3 were used as exogenous substrates, and the percent of H^3 that went into water was determined

at varying time intervals. The results are shown in Table XI. In washed slices, the rate of conversion of H^3 from both 1 and 6 positions into H^3_2O was twice that in fresh slices. However, no significant differences between the rate of transfer of H^3 from carbon 1 to water and H^3 from carbon 6 to water was clearly visible under these conditions. In view of the fact that invariably C_1 was released faster than C_6 and the C_1/C_6 ratio was slightly higher in washed slices, it seemed quite likely that the TPNH produced by the enhanced PPP participation was utilized in biosynthetic reductions rather than as a respiratory substrate. The function of the PPP has been suggested to be providing of the TPNH necessary for biosynthetic reductions. Tissues which synthesize cell components more actively are known to have more PPP activity (73, p. 1289-1294; 74, p. 64-68). Foster and Bloom (47, p. 2548-2551) after comparing the ability of glucose-1-H^3 and glycerol-2-H^3 to donate H^3 to fatty acids, cholesterol and water, came to the conclusion that TPNH was utilized in biosynthetic reductions while DPNH produced energy by being a respiratory substrate. They also used glucose-6-H^3 to examine the amount of H^3 from glucose-1-H^3 that might go through the glycolytic sequence and thus be incorporated into cellular components, such as fat and water, without the mediation of TPNH. They found that glucose-6-H^3 contributed more than glucose-1-H^3 to water, whereas glucose-1-H^3

Table XI

Rate of H^3_2O production from specifically H^3 labeled glucose by beet slices and sorghum seedlings.

		FRESH			WASHED		
		Percent of uptake			Percent of uptake		
Substrate	Hours:	1	2	4	1	2	4
Glucose-1-H^3		13.0	20.7	-	25.6	37.9	45.6
Glucose-6-H^3		12.0	18.2	28.3	25.8	40.2	46

In etiolated sorghum seedlings:

	Water	Fat		Fat
Glucose-1-H^3	63	0.67	Glucose-1-C^{14}	0.62
Glucose-6-H^3	79	0.17	Glucose-6-C^{14}	0.81

Each respirometer flask contained 15 slices (fresh weight 2.25g) in 4ml of nutrient solution containing 2.78 μmoles of specifically H^3 labeled glucose. In the experiments with sorghum seedlings each respirometer flask contained 30 seedlings (92 ± 3mg dry weight) and 4ml nutrient solution containing 2.78 μmoles of radioactive glucose. Aeration rate 43ml/minute. Incorporation time was one hours.

contributed more H^3 than glucose-1-H^3 to fat. Although similar differences in the ability of glucose-1-H^3 and glucose-6-H^3 to contribute H^3 to water were not observed in red beets, in sorghum seedlings germinated under conditions known to be conducive for the PPP (104, p. xxxii), the behavior of tritiated glucose was similar to rat liver, as shown by typical experimental results given in Table XI. Glucose-6-H^3 contributed a significantly higher percentage of H^3 to water than glucose-1-H^3, whereas glucose-1-H^3 contributed about four times H^3 to fat as compared to glucose-6-H^3. If all glucose molecules were to traverse through EMP-TCAC, an equal percentage of H^3 from C_1 and C_6 would have gone to water. The difference, 16 percent, probably represents the amount of H^3 that went through TPNH via the PPP. Part of this H^3 must have been incorporated into the lipid constituents of the seedlings as was shown by the higher H^3 incorporation into the lipids from glucose-1-H^3. Such a reasoning is supported by the fact that the proportion of C_1 and C_6 that was incorporated into fat was the same as the proportion of 1-H^3 and 6-H^3 that was found in the tissue water. It must be pointed out that according to Lowenstein (86, p. 1213-1216), glucose-6-phosphate dehydrogenase and hence the PPP is not the major source of TPNH in the livers of animals kept on a normal diet. However, as Foster and Bloom (47, p. 2548-2551) pointed out, direct comparisons of the metabolism of DPNH and

TPNH based on radiochemical yields from different substrates may be misleading because of differences in the rates of utilization of the substrates, intracellular dilution and other factors. To minimize such complications, Foster and Bloom compared all their values relative to water. Similar attempts were made by Ragland and Hackett (102, p. xlvii) to study the comparative aspects of TPNH and DPNH metabolism in pea stems. According to their results with pea stem sections, a higher percentage of H^3 from position one of glucose was found in tissue water as compared to position six, whereas C_1/C_6 ratios indicated a substantial PPP participation in glucose catabolism by pea stem sections. Such findings seem to suggest that hydrogen from C_1 of glucose could be incorporated into water presumably via glucose-6-phosphate dehydrogenase through the mediation of TPNH. A comparison of the results from pea stem sections, rat liver, red beets and sorghum seedlings indicate that the extent to which TPNH incorporates its hydrogen into water or other cell components, such as fat, depends entirely on the particular organism in question, its physiological state, and the balance between various TPNH linked oxidases and biosynthetic reductions. However, if extreme caution is taken in interpreting the results, tritiated substrates could be used to study the problem of TPNH and DPNH metabolism and its correlation to carbohydrate metabolism. For example,

isocitric acid labeled with H^3 on the carbon 2 may be used to gather information on the role of TPN specific isocitrate dehydrogenase, which seems to be present in the cytoplasm of almost all organisms. Thus, it may be possible to compare the ability of this enzyme to that of the pentose cycle to provide TPNH for biosynthetic reductions that are very essential for the cell. Here it may be pointed out that only half of the TPNH molecules produced by the PPP would be labeled with H^3 when glucose-1-H^3 is used as the substrate. The other half of the H^3 generated in the 6-phosphogluconate dehydrogenase step would be unlabeled. In tissues, in which considerable recycling of the reformed hexose occurs, much less than half of the TPNH produced by PPP would be labeled with H^3 when glucose-1-H^3 is used as the substrate. Unless such factors are taken into consideration, the ability of the PPP to provide TPNH for biosynthetic reductions are likely to be underestimated.

6. Comparison of the Effects of Washing by Gyratory Shaker and Aging in Petri Dishes on Potato Slices

Potato slices and carrot slices were subjected to the same treatment as beet slices and the pattern of C^{14} release from specifically labeled glucose determined. The carrot slices showed a marked increase in the participation of the PPP as judged by

relative rates of release of C^{14} from glucose labeled at 1, 3, 4 and 6 positions. The patterns obtained with carrot slices agreed quite well with those reported by ApRees and Beevers (6, p. 839-847). However, potato slices clearly showed signs of damage, such as loss of turgidity, and failed to release C^{14} from labeled glucose at measurable rates. Therefore, activity levels of some enzymes in extracts prepared from freshly cut and washed potato slices were determined and the results are shown in Table XII. Added Mg^{++} ions (0.02M) were necessary for maximum activity of glucose-6-phosphate dehydrogenase enzyme, whereas excess Mg^{++} ions (0.033M) were found to be inhibitory. Six-phosphogluconate dehydrogenase activity was stimulated several fold by the addition of 0.02M Mg^{++} and 0.033M Mg^{++} did not inhibit the activity of this enzyme, unlike glucose-6-phosphate dehydrogenase. Extracts prepared from slices washed for 24 hours on the gyratory shaker were found to be practically inactive as far as glucose-6-phosphate dehydrogenase and TPN reduction activities were concerned. A very weak 6-phosphogluconate dehydrogenase activity was detectable. Protein content was found to be decreased very much by the rather vigorous shaking of the gyratory shaker. However, the extracts prepared from slices aged in petri dishes showed a significant increase in activity of TPN reduction, glucose-6-phosphate dehydrogenase and 6-phosphogluconate

Table XII

A comparison of the effects of washing and aging on certain enzyme activity levels in potato slices.

		Aged in petri dishes		Washed in gyratory shaker
Enzyme Assay	Fresh	24-hour	48-hour	24-hour
End. TPN Reduction.....	0.038	0.0575	0.06	0.0
Glucose-6-PD (no added Mg)	0.095	0.125	--	0.0
Glucose-6-PD +0.02M Mg++	0.113	0.140	0.130	0.0
Glucose-6-PD +0.033M Mg++	0.09	0.135	--	0.0
Glucose-6-PGAD (no added Mg)	0.012	0.025	--	0.0
Glucose-6-PGAD +0.02M Mg++	0.035	0.053	--	0.003
Glucose-6-PGAD +0.033M Mg++	0.035	0.055	0.06	--

TPN reducing activity and 6-phosphogluconate dehydrogenase activity were assayed as described under Table IX. Glucose-6-phosphate dehydrogenase activity was assayed as described under Figure VI.

Glucose-6-PD = 6-phosphogluconate dehydrogenase.
Glucose-6-PGAD = glucose-6-phosphate dehydrogenase.

dehydrogenase. Protein content was found to have increased by about 50 percent also. Thus, the washing treatment on the shaker was found to be too harsh for potatoes. Steward (122, p. 436-453) mentioned that potatoes were more susceptable than other storage tissues to mechanical disturbances. Beet slices when washed for 24 hours on a shaker and then kept in a moist atmosphere in an erlenmyer flask for an additional 24 hours, were found to have about one-third more glucose-6-phosphate dehydrogenase activity than similar slices washed on the shaker for 48 hours. This, together with the significant decrease in protein that was associated with the washing on the shaker and the rather rapid decrease in respiration rate, observed in the washed slices, suggests that the washing treatment was probably too severe on the beet slices also, although the slices appeared perfectly normal on microscopic examination, even after long washing periods. Therefore, a new series of experiments were done in which such a harsh treatment was avoided.

PART C.

Experiments on Aged Red Beet Slices

1. Changes in Rate of Respiration Due to Aging

A series of respiration experiments was done in which the effects of shaking and minerals in the medium, were eliminated. Red beet tissues were incubated on wet filter paper supported on the surface of demineralized distilled water by glass beads in petri dishes in an incubator at 25°C. Rate of respiration of red beet slices aged this way, measured at 25°C, is shown in Figure XI and XII. Fresh slices showed a low level of respiration which gradually increased and reached a maximum in about 18 to 24 hours, during which time the respiration rate became about four-fold greater than fresh slices. Bennet-Clark and Bexen (13, p. 65-92) reported approximately a four-fold increase in the respiration rate in red beet slices due to washing in aerated tapwater for 300 hours at 12-16°C. In view of the reported (13, p. 65-92; 126, p. 576-611) difference between the aging patterns in air and under water and the effect of temperature on aging effects (88, p. 241-256; 89, p. 429-448), the aging method used in this work seems to have caused the aging effects to appear more rapidly than those reported by others (13, p. 65-92;

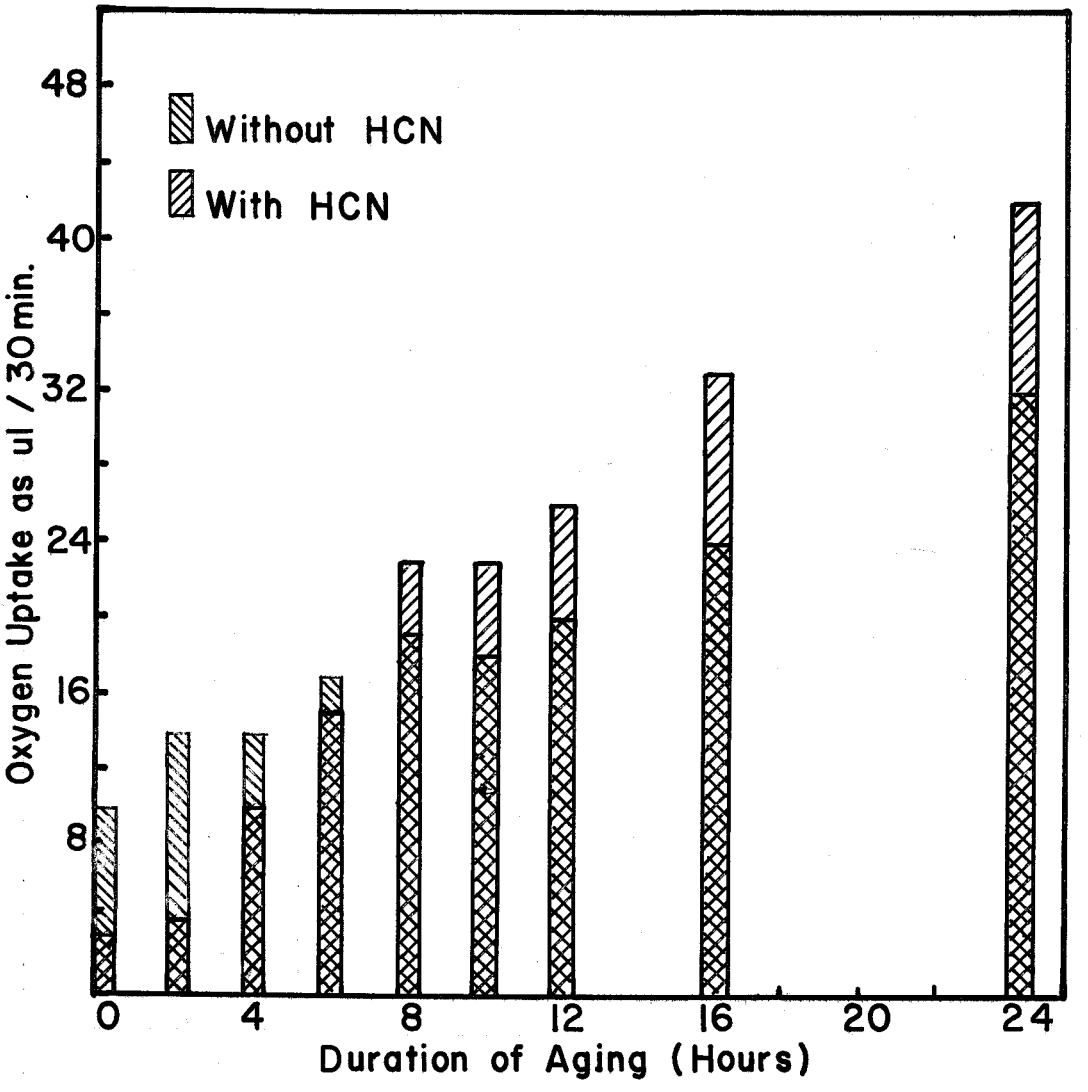


Figure XI. Rate of Oxygen Uptake by Red Beet Slices During Aging and the Effect of Cyanide on the Oxygen Uptake. Each Warburg Flask Contained Eight Slices (0.5 g Fresh Weight) and 2 ml Demineralized Distilled Water; The Center Well Contained 0.5 ml 5N NaOH or 0.5 ml 0.5M KOH+4.8M KCN. After Equilibration Medium Contained 8×10^{-5}M HCN. Temperature Was 25°C.

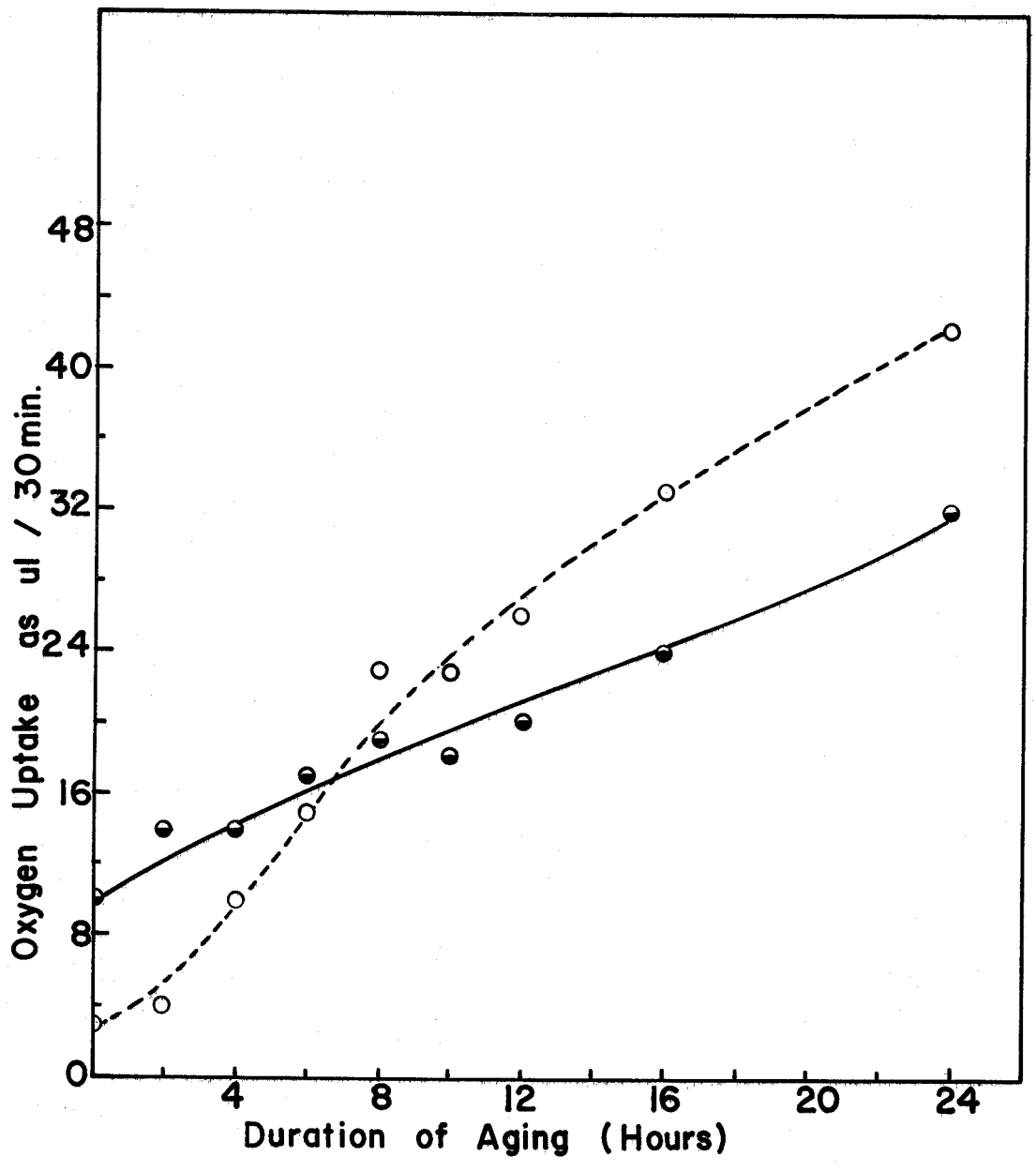


Figure XII. Change in Cyanide Sensitivity During the Aging of Red Beet Slices. Experimental Conditions Were the Same as Those in Figure XI.

---- With HCN ——— Without HCN

88, p. 241-256; 89, p. 429-448; 109, p. 1-8, 126, p. 576-611). Moreover, similar observations on other storage tissues have been reported (38, p. 243-250; 132, p. 239-257).

2. Wound Respiration.

Figure XIII shows the results of an experiment designed to study the effect of peeling on the rate of respiration of whole red beets. At a constant rate of flow of air, the percent CO_2 in the exhaust air represents the rate of respiration. The slow steady rate of respiration of whole beet roots suddenly increased by about 150 percent within 30 to 60 minutes after peeling with a knife. The next phase of the respiratory increase was rather slow but became faster on quartering the peeled whole beets. Similar observations have been reported in other storage tissues. For example, slicing of the storage tissues has been reported to be associated with rather quick changes in the rate of respiration (113, p. 85-96; 117; 134, p. 149-171). Mere peeling of whole potato tuber also caused rapid changes in respiration (77, p. 679-690). Since evidence available at present argues strongly against higher pO_2 as a possible reason for the observed respiration increments (see Literature Review), the explanation might lie in the presence of a volatile inhibitor of the type proposed by Laties and co-workers (77, p. 679-690; 78, p. 129-155;

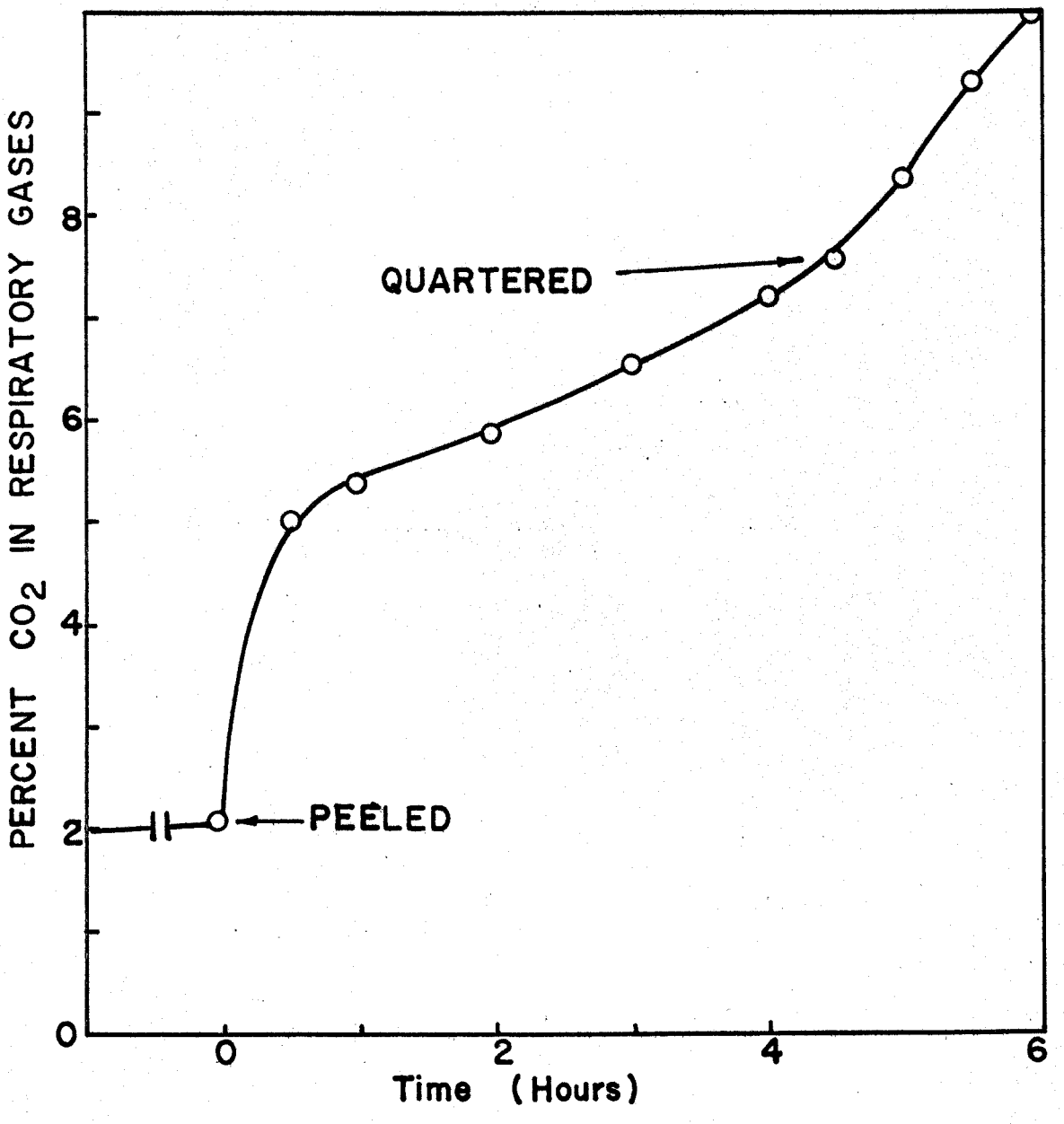


Figure XIII. Time Course of CO_2 production by Whole Beets on Peeling and Slicing. Eight beets (fresh wt. 1, 250 gms) were placed in a three-liter chamber and dry CO_2-free air was passed through the chamber at 85 ml/min. Total time lapse for peeling or quartering = 10 min.

83, p. 215-299). However, no direct evidence along this line is available. It must be pointed out that the kinetic nature of increments in respiration observed when whole beet was peeled was quite different from the respiratory increments observed in the aging slices. The sudden burst of respiration observed immediately after peeling may be identified as classical wound respiration, whereas the respiratory increase due to aging is rather gradual. However, it is quite difficult to separate the two experimentally. Similar observations have been reported by Laties (77, p. 679-690; 83, p. 215-299) in the case of potatoes.

3. Changes in Sensitivity to Cyanide

Since changes in sensitivity to respiratory inhibitors have been reported to be a common phenomenon accompanying aging in storage tissues, beet slices aged in petri dishes were exposed to HCN fumes (8×10^{-5}M). The effect of this low concentration of HCN on the respiration of aging beet slices is shown in Figures XI, XII, and XIV. Histograms in Figure XI show the rate of respiration, in the presence and absence of HCN, at different intervals of time after slicing. Respiration of freshly cut slices was inhibited 60 to 70 percent by 8×10^{-5}M HCN. As the slices aged, the sensitivity of the respiration to this concentration of HCN gradually decreased. As shown in

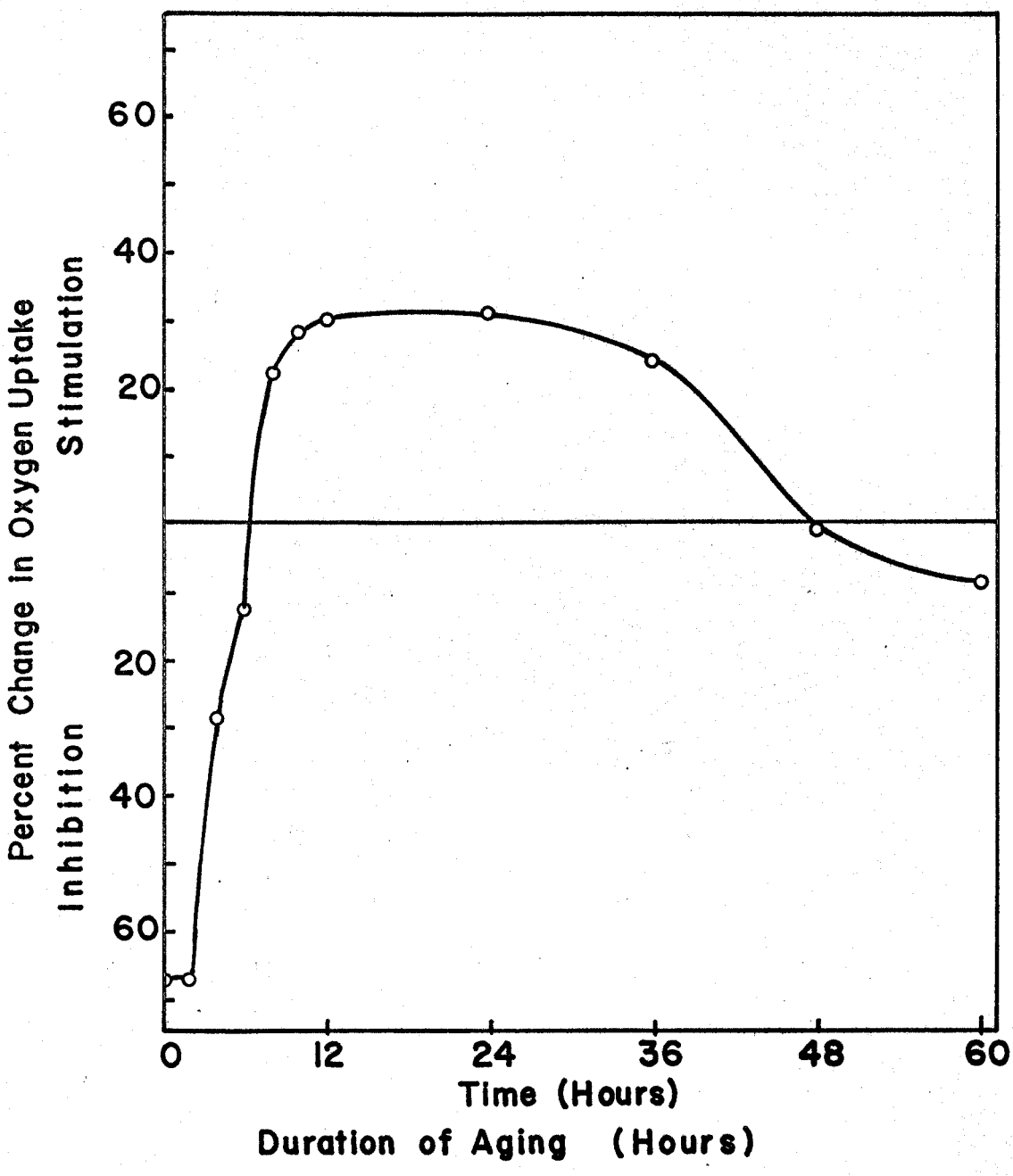


Figure XIV. Disappearance and Reappearance of Cyanide Sensitivity of Respiration During the Aging of Red Beet Slices.

Experimental conditions were identical to those in Figure XI.

Figure XII, after six to eight hours of incubation, the respiration became completely insensitive to cyanide and during the next several hours respiration was stimulated about 30 percent. In Figure XIV, the transition from a respiration strongly sensitive to cyanide to a cyanide stimulated respiration and the reappearance of sensitivity are shown. MacDonald (88, p. 241-256) was unable to detect any change in permeability to cyanide due to aging. Moreover, the stimulation is an indication of the presence of cyanide in the reaction zone.

These results are in qualitative agreement with those of MacDonald (88, p. 241-256) who observed similar patterns of cyanide sensitivity during a washing period of about 16 days or more. The difference may be explained on the basis of the conditions used for aging. MacDonald washed the slices in running tap water. Under these conditions aging effects are known to appear much more slowly than in similar slices aged in moist air (13, p. 65-92; 132, p. 239-257). The slight increase in inhibition during the early phase of aging observed by MacDonald (88, p. 241-256) was not clearly visible in this investigation, presumably because of the rapidity of the aging process or the difference in the physiological state of the tissue. However, Robertson et al (109, p. 1-8) used a higher concentration of cyanide and failed to observe cyanide insensitivity in the majority

of their experiments. In spite of the fact that cyanide insensitivity has been repeatedly reported to be associated with aging of other storage tissue slices (88, p. 241-256; 132, p. 239-257) Schade et al (114, p. 211-219) failed to observe any change in sensitivity of respiration of potato slices to 10^{-3} M KCN. Except for such disagreements, it is now well established that storage tissue slices become less sensitive to respiratory inhibitors, such as cyanide, CO and azide when they are aged. Tissues possessing cyanide insensitive respiration seem to have a strong b_7 component in their cytochrome system (34, p. 33-49; 60, p. 27-32; 61, p. 8-19). Although a b_7 oxidase-bypass hypothesis does not explain all aspects of cyanide insensitive respiration, a significant increase in the b_7 region was observed to be associated with the aging of potato slices (61, p. 8-19). Such detailed studies have not been made in red beets, although spectroscopic studies (142, p. 109-122) and other evidences show that a normal cytochrome oxidase system is the major terminal oxidase that functions in the respiration of beets. A study of the concentration changes of the cytochrome components and their steady state redox conditions in the presence and absence of cyanide might help to better understand the mechanism of the change in sensitivity of respiration to cyanide.

4. Radiorespirometry Experiments on Aging Red Beet Slices.

After having established that aging brings about a four-fold increase in the respiration rate of beet slices, and that this respiration may be qualitatively different from the respiration of fresh slices, information about glucose catabolism by aged slices would be of interest, especially in view of the changes in relative contribution of pathways for glucose catabolism suggested by experiments with washed slices. A series of experiments was done in which the amount of substrate was kept high so that the substrate would not be limiting. Under such conditions, the release of C^{14} attained a rather steady rate within two to three hours with fresh slices and within one to two hours from the aged slices as shown in Figures XV, XVI, and XVII. Under these conditions, the relative rates of release may be used to draw conclusions about qualitative changes in pathways. The rate of release of $C_{3,4}$ was consistently and significantly larger than the rate of release of the other carbons of glucose in both fresh and aged slices. This would be expected if the major part of the glucose was catabolized via EMP and the pyruvate produced was mainly decarboxylated. Thus, the C^{14} release patterns shown in Figures XV, XVI, and XVII show that glucose is catabolized mainly via EMP in both fresh and aged red beet slices. However, in both fresh and aged

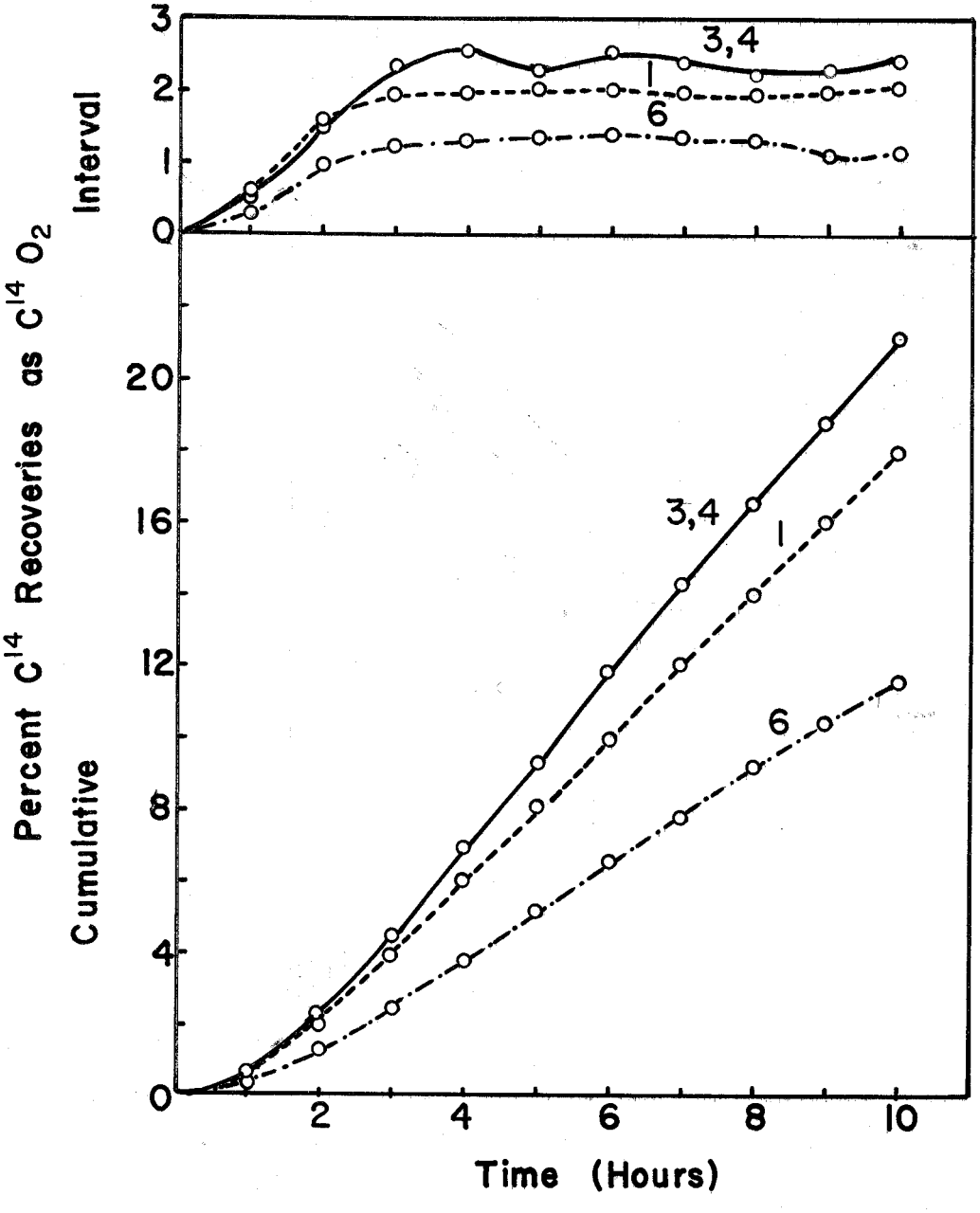


Figure XV. Time Course Plots of Percent Interval and Cumulative Respiratory $C^{14}O_2$ Recoveries from C^{14} Specifically Labeled Glucose Metabolized by Fresh Red Beet Slices. Each respirometer flast contained 15 beet slices (9mm thick, 1mm in diameter, 1.1g fresh weight) in 4ml demineralized distilled water containing 2.78 moles of C^{14} specifically labeled glucose. Air flow 43 ml/min. Percent recoveries were based upon the substrate absorbed by the end of the experiment.

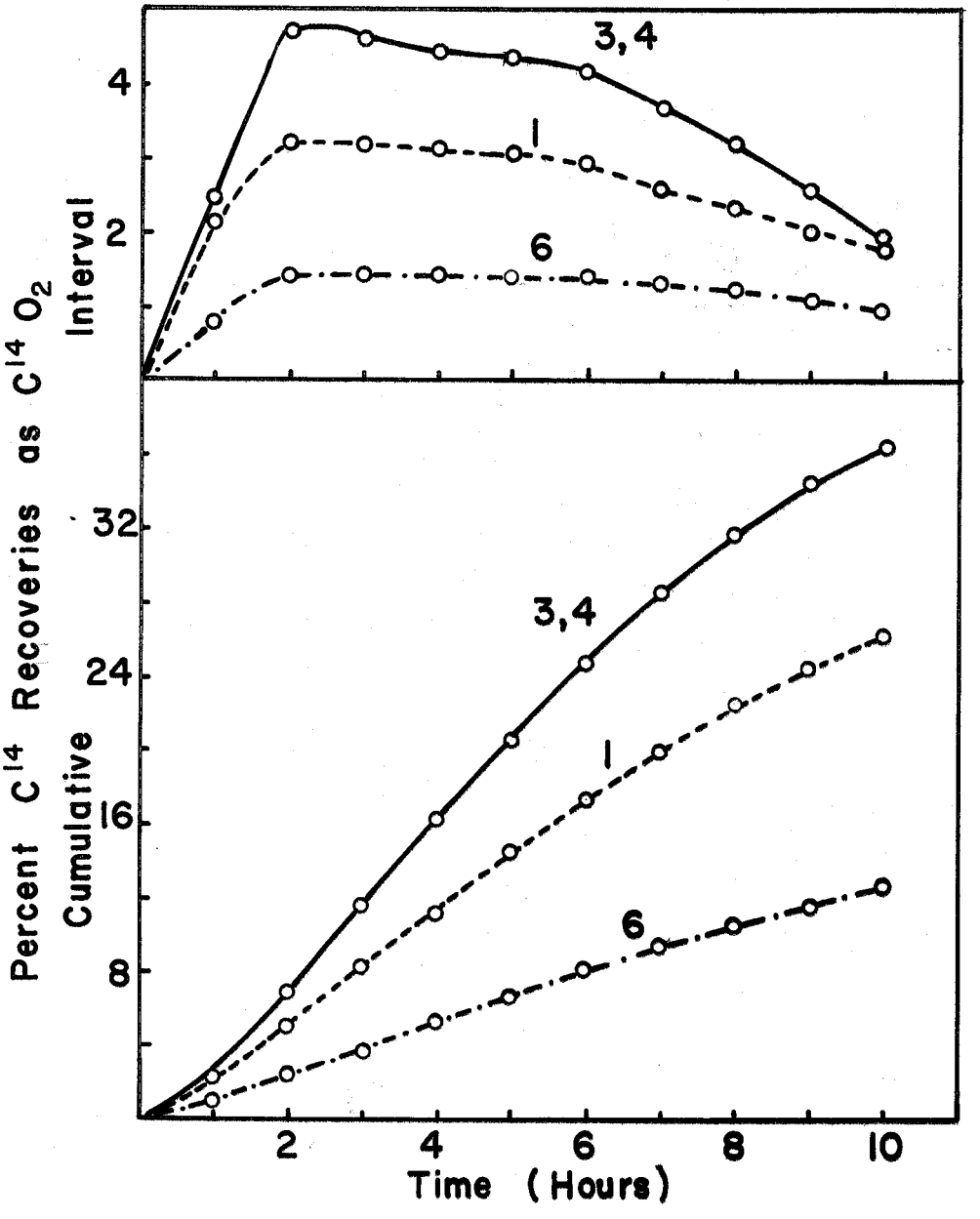


Figure XVI. Time Course Plots of Percent Interval and Cumulative Respiratory $C^{14}O_2$ Recoveries from C^{14} Specifically Labeled Glucose Metabolized by 24-hour Aged Red Beet Slices.

See Figure XV for experimental conditions and legend.

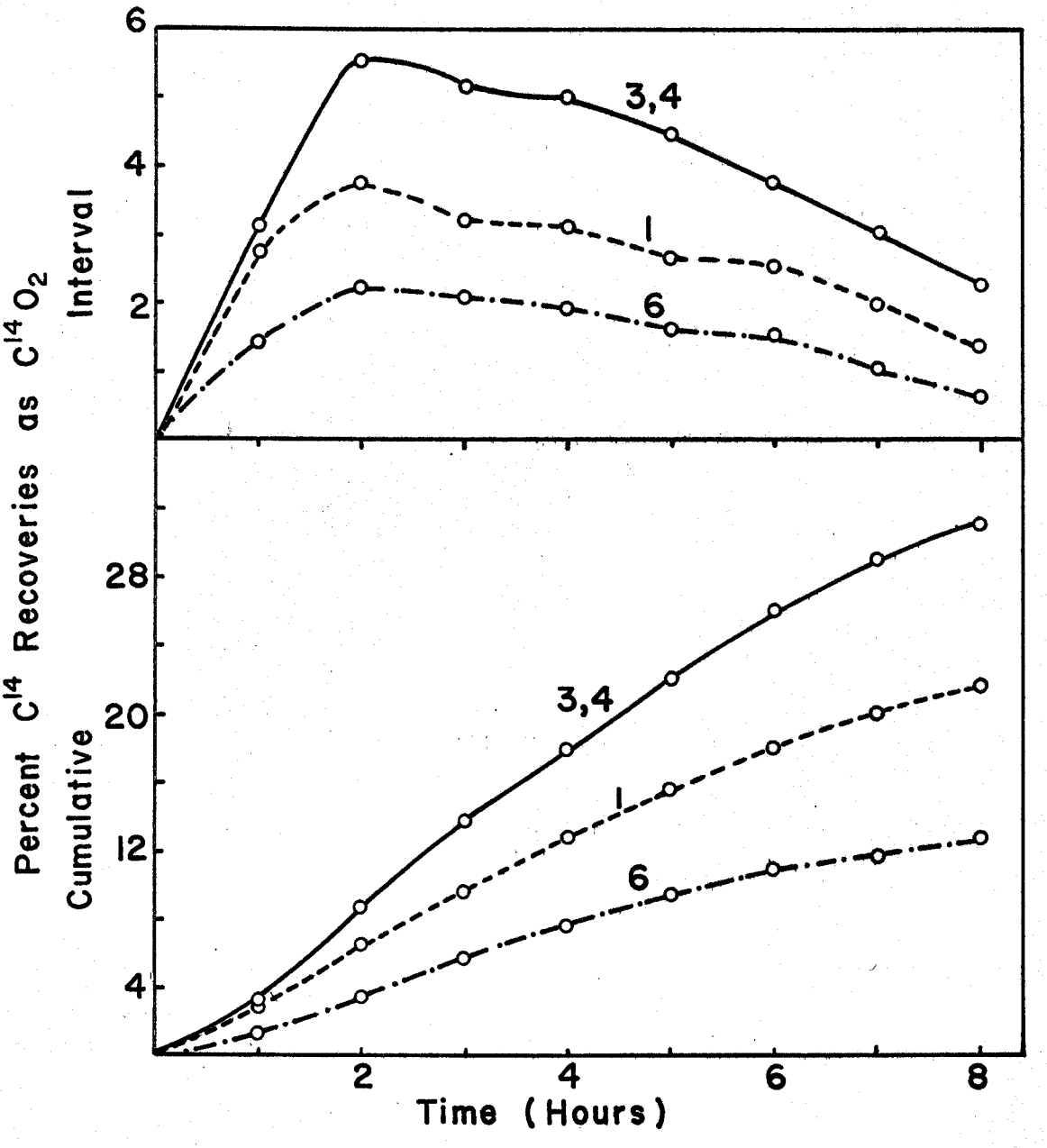


Figure XVII. Time Course Plots of Percent Interval and Cumulative Respiratory $C^{14}O_2$ Recoveries from C^{14} Specifically Labeled Glucose Metabolized by 40-hour aged Red Beet Slices.

See Figure XV for experimental conditions and legend. Since the beet slices used in this experiment were from a different batch, this pattern cannot be directly compared to those in Figures XV and XVI.

slices, C_1 release was significantly faster than C_6 release. This indicates that a pathway, presumably the PPP, which preferentially releases C_1 is active in both fresh and aged slices. The increase in C_1 and $C_{3,4}$ recoveries were substantially higher than the increase in C_6 recovery. Comparing the rates of release of C_1 and $C_{3,4}$, it is very hard to tell whether there is preferential increase in participation of the PPP. However, a comparison of rates of release of C_1 and C_6 shows that increase in C_1 release was greater than C_6 release. Under the experimental conditions employed a change in C_1/C_6 ratio may give a meaningful indication of changes in relative participation of pathways. The relatively steady C_1/C_6 ratio of about 1.5-1.6 changed to about 2.0-2.2 when the slices were aged. An estimation of participation of the PPP using the equation of Barbour et al (8, p. 396-400) showed that in fresh slices about 23 percent of glucose was catabolized via the PPP, whereas the comparable value for aged (24-hour) slices was about 28 percent. This evidence suggests that there may be a slight preferential increase of the PPP. However, in view of the assumptions involved in such estimations, the quantitative change in relative participation of pathways indicated by such estimations may not be very reliable. This is especially true because the present evidence indicates that there may be large differences in the biosynthetic capacities of the fresh and

aged slices. For example, as shown later in this section, retention of C_6 for biosynthetic purposes was much larger in aged tissue than in fresh tissue. Protein and RNA synthesis have been reported to be increased by aging (38, p. 243-250) in potato slices, and available evidence suggests that a similar increase takes place in red beets also. Increased drainage of intermediates for such increased biosynthetic activities decreases the reliability of the estimation. In any case, it is clear from the above results that the age-induced respiration is mediated primarily through EMP and not the PPP, although aging probably caused some preferential increase in the PPP participation. If the increase in the concentration of reducing sugar, reported by other workers (89, p. 429-448; 103) represents an increase in the actual metabolically active pool, and if the externally administered glucose joins this pool, we would expect a larger dilution of the administered glucose in the aged slices, although an increase in uptake caused by aging complicates the problem. Thus, due to the greater intracellular dilution of the administered glucose increase in C^{14} release by the aged slices does not represent the true quantitative increase in the glucose catabolized. In any case, administered glucose constituted only a small fraction of the total respiratory output of carbon by beet slices.

5. Metabolism of TCA Cycle Intermediates and Related Compounds

It has been suggested that the TCA cycle is not very active in fresh slices of storage tissues such as potato and chicory and that it becomes increasingly important as the slices are incubated (79, p. 378-391; 81, p. 364-377; 110, p. 20-29). ApRees and Beevers have stressed that their data indicated that participation of the TCA cycle in the respiration of carrot tissue was unaltered by the development of the induced respiration (6, p. 839-847). In view of such observations in other storage tissues, it became of interest to study the utilization of TCA cycle intermediates by fresh and aged red beet slices. Since glutamic and aspartic acids have been suggested (91) to represent ancillary TCA cycle pools which can readily exchange carbon skeletons with the intermediates of the TCA cycle, utilization of these acids by fresh and aged red beet slices is also of interest.

a. Metabolism of Alanine. L-Alanine is well known to generate pyruvate in vivo in most organisms. Moreover, it is more stable and more easily absorbed by most organisms than pyruvate. Due to these reasons, L-alanine was used to get some information on the metabolism of pyruvate by fresh and aged red beet slices.

L-alanine-1-C^{14}, DL-alanine-1-C^{14}, DL-alanine-2-C^{14} and DL-alanine-3-C^{14} were administered to fresh and aged red beet

slices and the rate of release of C^{14} as $C^{14}O_2$ measured at hourly intervals. The results are shown in Figures XVIII and XIX. Fresh slices released C^{14} rather slowly during the first two to three hours. This probably indicates a lag-period in the uptake. However, the rate of uptake was not measured in these experiments. After the initial lag-period, carbon 1 was released very rapidly, yielding about 65 to 70 percent of the total C^{14} that was taken up by the slices in the experimental time. Carbon 2 was released much more slowly than carbon 1 and amounted to about 12 percent of the total uptake. Carbon 3 was the slowest with a yield of about three percent at the end of the experimental time. This pattern of C^{14} release indicates that alanine is converted into pyruvate. Pyruvate is well known to be decarboxylated rapidly by plant tissues, including carrot and potato discs (6, p. 839-847; 96, p. 409-416). Carbons 2 and 3, after being converted to acetyl CoA, would traverse through the TCA cycle releasing C_2 during the second turn of the cycle, and C_3 begins to be released during the third turn. Tracer experiments on other plant tissues support such a scheme. The incorporation of C_2 of pyruvate into the 1, 4 positions of malate and 1, 5 positions of citrate has been shown in tomato fruits (28, p. 192-195). The correspondence of pyruvate carbons 2 and 3 to acetate-carbons 1 and 2 was clearly indicated in wheat leaves and castor bean endosperm(33, p. xv).

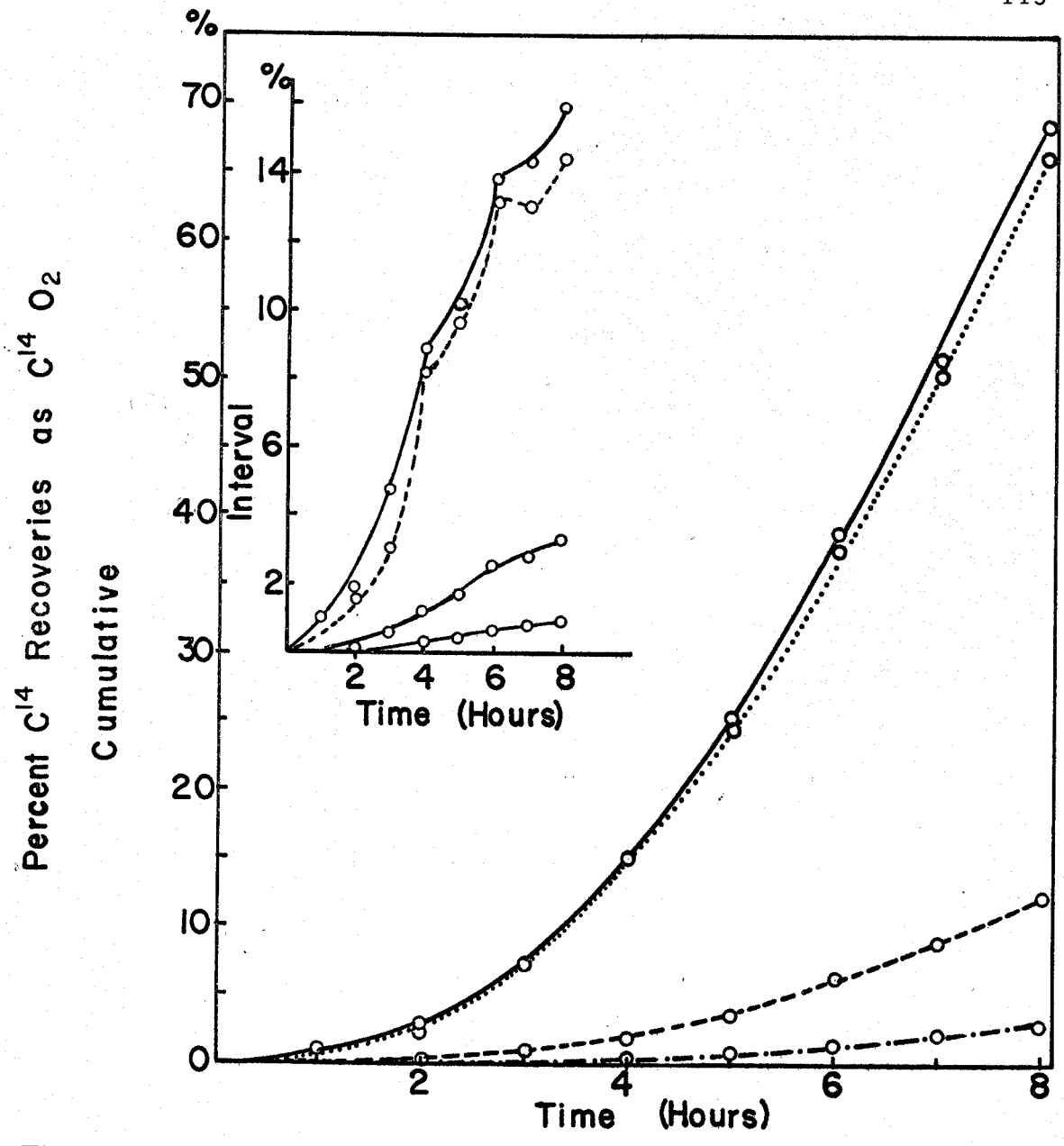


Figure XVIII. Time Course Plots of Percent Interval and Cumulative C^{14} Recoveries in Respiratory $C^{14}O_2$ from Fresh Red Beet Slices Metabolizing C^{14} Specifically Labeled Alanine. Experimental conditions are identical to Figure XV.

—— L-alanine-1-C^{14} (2.78 μ moles)
..... D, L-alanine-1-C^{14} (5.56 μ moles)
----- D, L-alanine-2-C^{14} (5.56 μ moles)
-.-.-. D, L-alanine-3-C^{14} (5.56 μ moles)

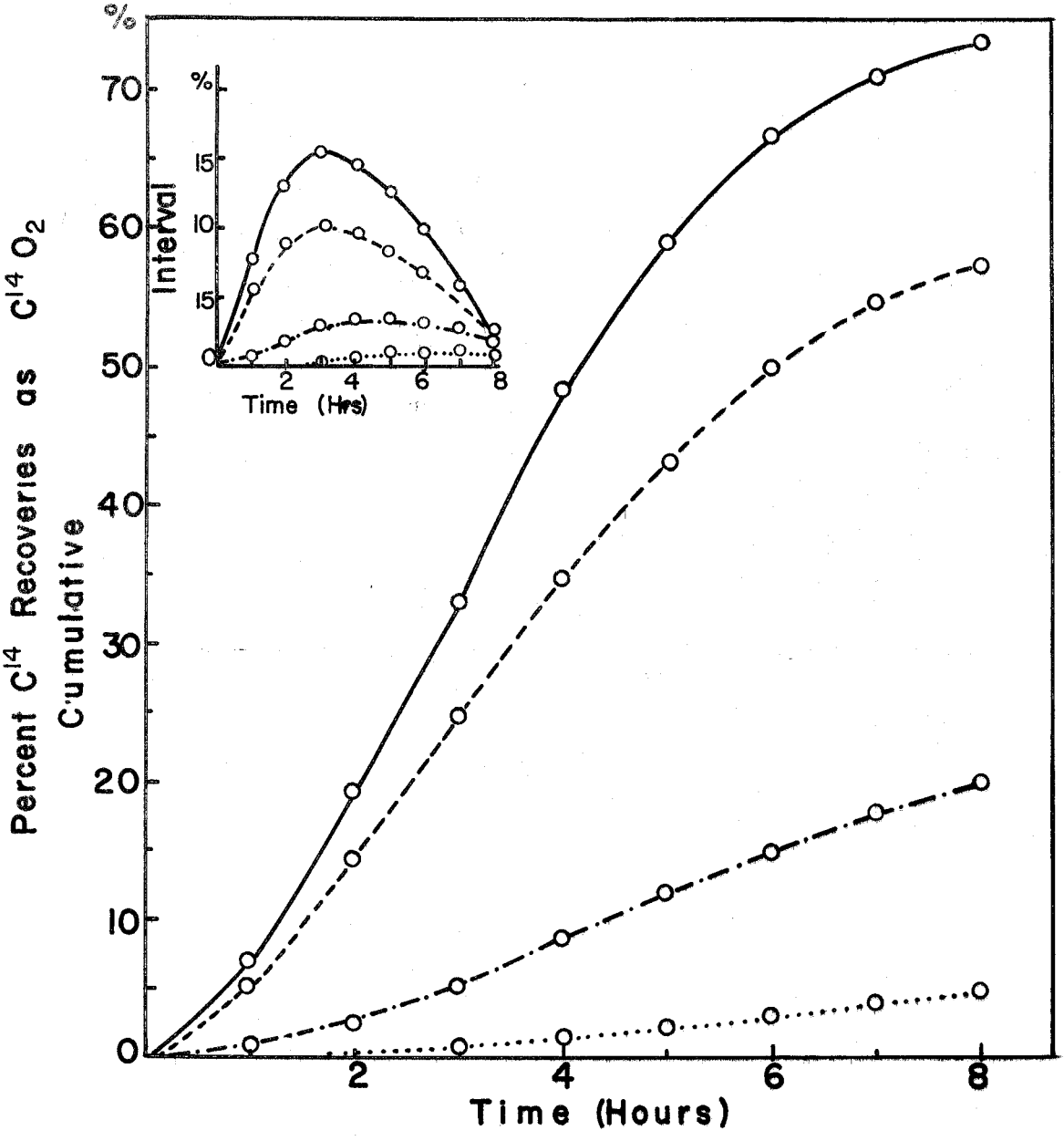


Figure XIX. Time Course Plots of Percent Interval and Cumulative C^{14} Recoveries in Respiratory $C^{14}O_2$ from 24-hour Aged Beet Slices Metabolizing C^{14} Specifically Labeled Alanine.

See Figure XV for experimental conditions, and Figure XVIII for the legend. 2.78 moles of L-alanine and 5.56 moles D, L-alanine were used as substrates.

Aged slices released carbon 1 of alanine extremely rapidly to yield as $C^{14}O_2$ about 73 percent of the total C^{14} that was taken up by the slices. Carbon 2 was released rather slowly giving about a 19 percent yield during the experimental time. Carbon 3 was released extremely slowly, yielding about five percent of the total C^{14} taken up during the experimental time. This pattern is qualitatively identical to the pattern observed in fresh tissue, hence indicating identical metabolic pathways.

The most significant point shown by these experiments is the extremely slow release of the C_2 and C_3 of alanine. This presumably represents utilization of acetyl CoA for biosynthetic purposes. The very high amounts of C_2 and C_3 that were retained in the tissue might be, either incorporated into organic acid pools, which are usually found to be of considerable size in plant tissues (91) or utilized for other biosynthetic purposes. Extraction of the slices after the radiorespirometry experiments showed that 95 percent of the C_3 that was retained could be found in the alcohol soluble fraction of the fresh tissue, whereas only about 70 percent of the C_3 retained by the aged tissue could be found in this fraction. Approximately five percent of C_3 was incorporated into the alcohol insoluble residue of fresh tissue, whereas about 29 percent of C_3 was incorporated into the alcohol insoluble residue in the aged slices. Presumably, the activity that was

incorporated into the insoluble residue was in the protein fraction. In support of this, it may be pointed out that only a negligible fraction of the activity could be found in the neutral fraction of both fresh and aged slices indicating also negligible incorporation of C_3 into the carbohydrate fraction. Eight fold higher incorporation of alanine into protein has been reported in aged potato slices (31, p. 1142; 38, p. 243-250). The high retention of C_2 and C_3 of alanine in the aged tissue is in agreement with high retention of C_6 of glucose observed in these slices.

A comparison of the final yields of $C^{14}O_2$ from carbon 1 of alanine may be misleading because of the very high yields. No reliable conclusions can be drawn from such experiments, regarding the in vivo rates of alanine or pyruvate metabolism in view of the unknown pool sizes that might dilute the administered substrates.

In fresh slices DL-alanine-1-C^{14} released $C^{14}O_2$ almost as fast as L-alanine-1-C^{14} and the final yields of C^{14} were extremely close. Assuming that D-alanine would act as a metabolically inert compound, whenever DL-alanine was used as a substrate, its level was made double that of L-alanine. The fresh slices took up only half as much radioactivity from DL-alanine as L-alanine C^{14}. This evidence suggests that probably only L-alanine was taken up by the fresh slices. D-Aspartic acid and D-glutamic acid have been reported

to be taken up with extreme difficulty (118, p. 360-364; 119, p. 639-643). However, aged slices took up almost all of the DL-alanine administered, and thus out of the total C^{14} that was taken up by the aged slices, only half was the metabolically active L form. Therefore, when the C^{14} release is expressed as a percent of uptake, the yield from DL-alanine-1-C^{14} (55 percent) is much less than the L-alanine (73 percent) in the case of aged tissue. Thus, the yield of $C^{14}O_2$ from DL-alanine-1-C^{14} indicates that D-alanine is probably not very readily metabolized by the red beet slices. If none of the D-alanine was metabolized, then the 29 percent of C_3 incorporated into the insoluble residue could not have been L-alanine units, because all the D-alanine units taken up has been decarboxylated, according to C^{14} yield in respiratory CO_2. However, it seems likely that some alanine was incorporated into protein. This difficulty can be resolved by assuming that some D-alanine was also utilized by aged slices, but much more slowly than L-alanine. C^{14} from C_3 of alanine probably got incorporated at least in part via glutamate and aspartate.

b. Acetate Metabolism. Although free acetate is not usually found in most plant tissues to any great extent, externally administered acetate is well known to be metabolized via the TCA cycle (16, p. 357-363; 42, p. 483-490; 43, p. 751-756; 64, p. 117-123; 137, p. 443-454; 138, p. 741-745; 146, p. 240-250). So, acetate-1-C^{14} and

acetate-2-C^{14} were used as tracers to obtain some information on the TCA cycle activity in fresh and aged red beet slices.

The percent cumulative and interval recoveries are shown in Figures XX and XXI. Acetate was quite slowly taken up from the medium by fresh slices, and the release of C^{14} from it was very slow. Washing activated acetate uptake, but not as much as the uptake of some of the other organic acids. It is clear from the C^{14} yield from C_1 and C_2 of acetate that washing stimulated utilization of acetate, presumably via the TCA cycle. C^{14} from acetate-1-C^{14} as it traverses through the TCA cycle, becomes succinate-1, 4-C^{14} and is decarboxylated in the second turn of the TCA cycle. Thus, the rate of release and the yield of C_1 would be expected to be much larger than C_2 and the results shown in Figures XX and XXI clearly show that this was true in both fresh and aged tissue. The C_2 release would be expected to have a longer lag period than C_1 release (64, p. 117-123). However, such lag-periods were not clear in these experiments, because the intervals used were too large to show them clearly. Nevertheless, the relative yields of C_1 and C_2 are in agreement with the contention that the TCA cycle is active in both fresh and aged tissues. Tracer evidence is so overwhelming that there is no more doubt about TCA cycle activity in almost all kinds of plant tissues. Higher yield of C_1 in the respiratory CO_2 than in C_2 (42, p. 483-490; 43, p. 751-

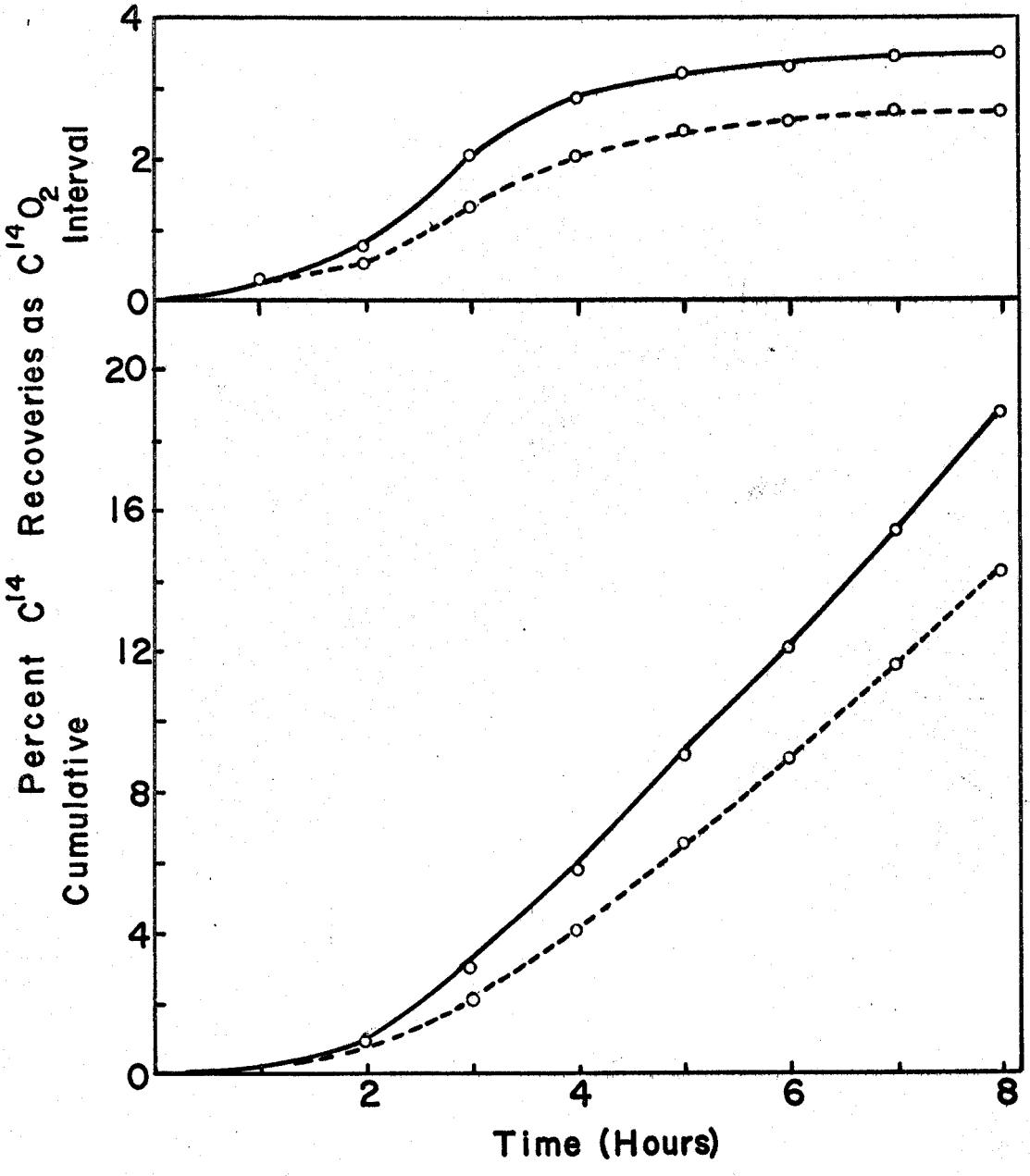


Figure XX. Time Course Plots of Percent Interval and Cumulative Recoveries of Respiratory $C^{14}O_2$ from Fresh Beet Slices Metabolizing C^{14} Specifically Labeled Acetate. 1 = acetate-1-C^{14} and 2 = acetate-2-C^{14}. Experimental conditions were identical to thos in Figure XV. Sodium acetate (2.78 μmoles) was used as the substrate.

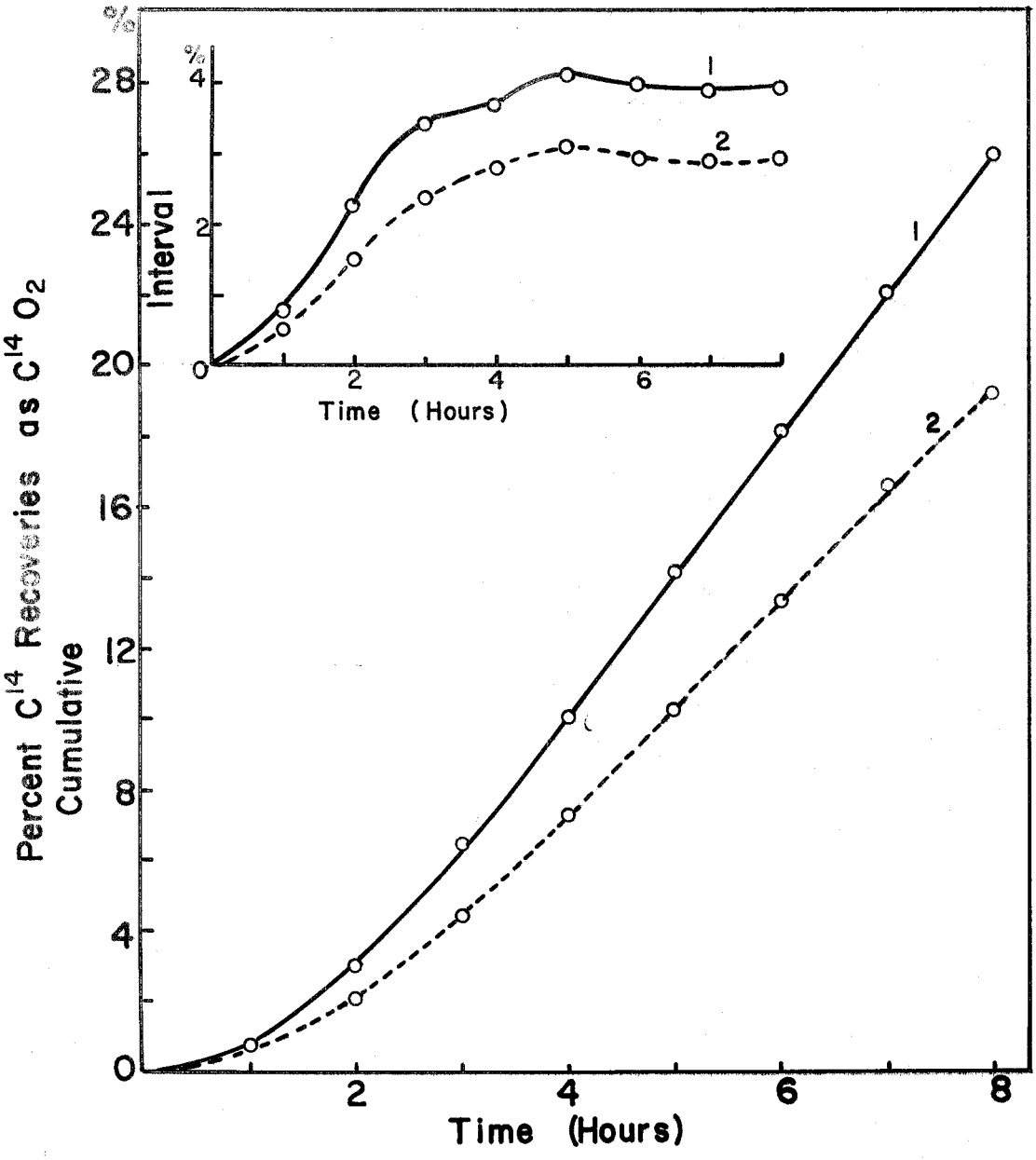


Figure XXI. Time Course Plots of Percent Interval and Cumulative Recoveries of Respiratory $C^{14}O_2$ from 24-hour Aged Beet Slices Metabolizing C^{14} Specificately Labeled Acetate.

See Figure XV for experimental conditions, and Figure XX for the legend. Substrate was 2.78μ moles of sodium acetate.

756; 137, p. 443-454), appearance of acetate C^{14} in other TCA cycle intermediates (64, p. 117-123; 138, p. 741-745; 146, p. 240-250) and related amino acids (16, p. 357-363), etc., have been used to establish presence of TCA cycle in plant tissues. According to the results shown in Figures XX and XXI, the ratio C_1/C_2 is about 1.4, which is quite low when compared to the reported values of 3 to 5 in plant tissues (42, p. 483-490; 43, p. 751-756; 91; 137, p. 443-454). If the administered substrate continuously feeds in C^{14} labeled acetate, at conditions approaching steady state, the ratio could theoretically approach 1, and low ratios of about 1.4 have been reported for Verticillium albo-atrum (27, p. 50-53) and Penicillum digitatum (98, p. 469-476) both having TCA cycle activity. It may be pointed out that in the experiments with acetate, the uptake was very slow and consequently, the radioactive substrate was being fed into the tissue continuously, and under these conditions, the situation approached steady state giving a low C_1/C_2 ratio.

c. Succinate Metabolism. Succinate, being an intermediate of the TCA cycle, would give some information on the TCA cycle activity on the fresh and aged red beet slices. So succinate-1, 4-C^{14} and succinate-2, 3-C^{14} were used as tracers to study succinate metabolism in red beet slices. Succinate uptake by fresh slices was

extremely slow, thus, a reliable measurement of the uptake was not possible by determining the disappearance of radioactivity from the medium. However, the succinate molecules that got into the cells seemed to be metabolized quite rapidly as shown by the $C^{14}O_2$ recoveries shown in Table XIII. Aged slices took up succinate several fold faster and metabolized succinate quite well. As mentioned before, a comparison of the rate of metabolism of endogenous succinate in fresh and aged slices can hardly be based on such results. However, comparison of the rates of turnover of different carbons of the same substrate is quite justifiable. In both fresh and aged slices, C_1 and C_4 of succinate were released several fold faster than C_2 and C_3. This is only natural if TCA cycle is active, because of the fate of succinate carbons in this pathway as discussed in previous sections.

d. Metabolism of L-Aspartate and L-Glutamate. The deamination products of aspartate and glutamate are intermediates of TCA cycle and so the rate of utilization of these amino acids would give some information on TCA cycle activity. Hence, L-aspartate-4-C^{14} and L-glutamate-1-C^{14} were used as tracers to study their metabolism in fresh and aged beet slices. The C^{14} release by fresh and aged slices metabolizing externally administered L-aspartic-4-C^{14} and L-glutamic-1-C^{14} are shown in Table XIV. Fresh slices took up

Table XIII

Effects of aging on succinate utilization by red beet slices.

	Recovery of C^{14} in CO_2 Percent of administered							
	Fresh				Aged			
Hours:	2	4	6	8	2	4	6	8
Substrate								
Succinate-1, 4-C^{14}	1.4	3.4	5.3	7.3	3.2	7.3	10.6	13.6
Succinate-2, 3-C^{14}	0.13	0.6	1.1	1.7	0.22	1.0	2.0	3.0

Experimental conditions were the same as those described under Figure XV, but 2.78 umoles of C^{14} succinate was used as the substrate.

Table XIV

Effects of aging on the utilization of L-aspartate and L-glutamate by red beet slices.

	CUMULATIVE RECOVERY OF C^{14} IN CO_2 PERCENT OF ADMINISTERED C^{14}							
	Fresh				24 Hour Aged			
Substrate Hours:	2	4	6	8	2	4	6	8
L-Aspartate-4-C^{14}	0.6	0.8	1.2	2.2	5.0	10	15	19.5
L-Glutamate-1-C^{14}	0.9	1.5	2.1	2.6	7.5	15	22	28

Experimental conditions were same as those shown under figure 15 but 2.78μmoles of L-aspartate or L-glutamate was used as the substrate. Duration of the experiment was 8 hours.

these dicarboxylic amino acids at an extremely slow rate; so reliable measurement of uptake by disappearance of C^{14} from the medium was not possible. However, once the amino acids entered the fresh tissue, they were metabolized quite readily as shown by the release of C^{14}. Aged slices took up these amino acids several fold faster and readily converted into oxaloacetate and α-keto glutarate, which were then metabolized via the TCA cycle. A major portion of the total L-aspartate and L-glutamate that was taken up seems to have undergone extensive catabolism releasing $C^{14}O_2$. It may be pointed out that positions of the label are such that once the corresponding keto-acids are produced, the labeled carbon can be released very readily. Considerable breakdown of amino acids, such as L-glutamate, L-aspartate, L-alanine, etc., which on deamination give rise to keto-acids which are easily broken down by the TCA cycle, has been reported in carrot discs (18, p. 277-286). Almost one-half of the radioactivity taken up as aspartate-4-C^{14} and less than one-third of the activity taken up as glutamate-1-C^{14} were retained by the aged tissue. Although the location of this activity was not determined, it is very likely that a considerable portion of this activity was incorporated into protein. Aspartic acid incorporation into the protein of carrots has been reported (119, p. 639-643). Similarly, glutamic acid is also known to be incorporated into proteins of higher plants (140, p. 382-385). When the interval recovery of C^{14} was examined on hourly

intervals, aspartic-4-C^{14} gave an interesting pattern. The C^{14} recovery during the first hour was about six times as much as the second hour recovery. After the second hour, as is normally found with other substrates, a gradual increase in recovery was observed. This initial anomalous high recovery was not an artifact of that particular experiment but a very reproducable observation. This pattern was observed even when the slices were well washed in distilled water for fifteen minutes to leach out the cytoplasm from cut cells and surface of the slices. No further attempt was made to investigate this problem, other than speculating that a wound-activated aspartic decarboxylase might be temporarily active.

6. Changes in Activity Levels of Certain Enzymes During Aging.

Crude extracts were prepared as described in section 11 and the rates of TPN reduction were measured with glucose-6-phosphate and 6-phosphogluconate as substrates. Comparisons were made on the basis of an equal number of slices. Addition of a low concentration of 2-mercaptoethanol (0.005M) to the extracting medium resulted in increased activity of both enzymes. Prolonged dialysis of the extract against distilled water destroyed the enzymatic activity of glucose-6-phosphate dehydrogenase. Low concentrations of 2-mercaptoethanol (0.005M) was found to partially protect the enzyme activity

(25 percent). Dialysis for six hours against buffer containing 0.005M mercaptoethanol was found to prevent any loss of enzyme activity. Crude extracts containing 0.005M mercaptoethanol were used routinely for assays. From a single batch of aging slices, slice aliquots were taken and enzyme activity levels were determined. Changes in the rate of TPN reduction with glucose-6-phosphate as substrate are shown in Figure XXII. Significant changes in activity levels began to appear after about four to eight hours of aging. The increase in activity was found to continue up to 36 hours beyond which measurements were not made. In Figure XXIII are shown the changes in the rate of TPN reduction with 6-phosphogluconate as substrate. Again, about four to eight hours were required before significant changes in activity levels could be observed. The rate of increase in activity of this enzyme was faster than that of glucose-6-phosphate dehydrogenase and the increase continued until at least 36 hours beyond which measurements were not made. In Figure XXIV are summarized the changes in activity levels of glucose-6-phosphate dehydrogenase, 6-phosphate gluconate dehydrogenase, and endogenous TPN reducing power. Protein analysis of the extracts showed that significant increases in protein synthesis became noticeable only after four to eight hours of aging. From this, one can imply that the enhanced enzyme activity levels may be the result of an increased rate of synthesis of these enzymes. Changes in the rate of respiration also became significant after about four hours of aging. It is interesting to note that oxygen uptake became completely insensitive to 8×10^{-5}M HCN within six to eight hours of aging. However, the changes in respiration, qualitative and quantitative, became noticeable before

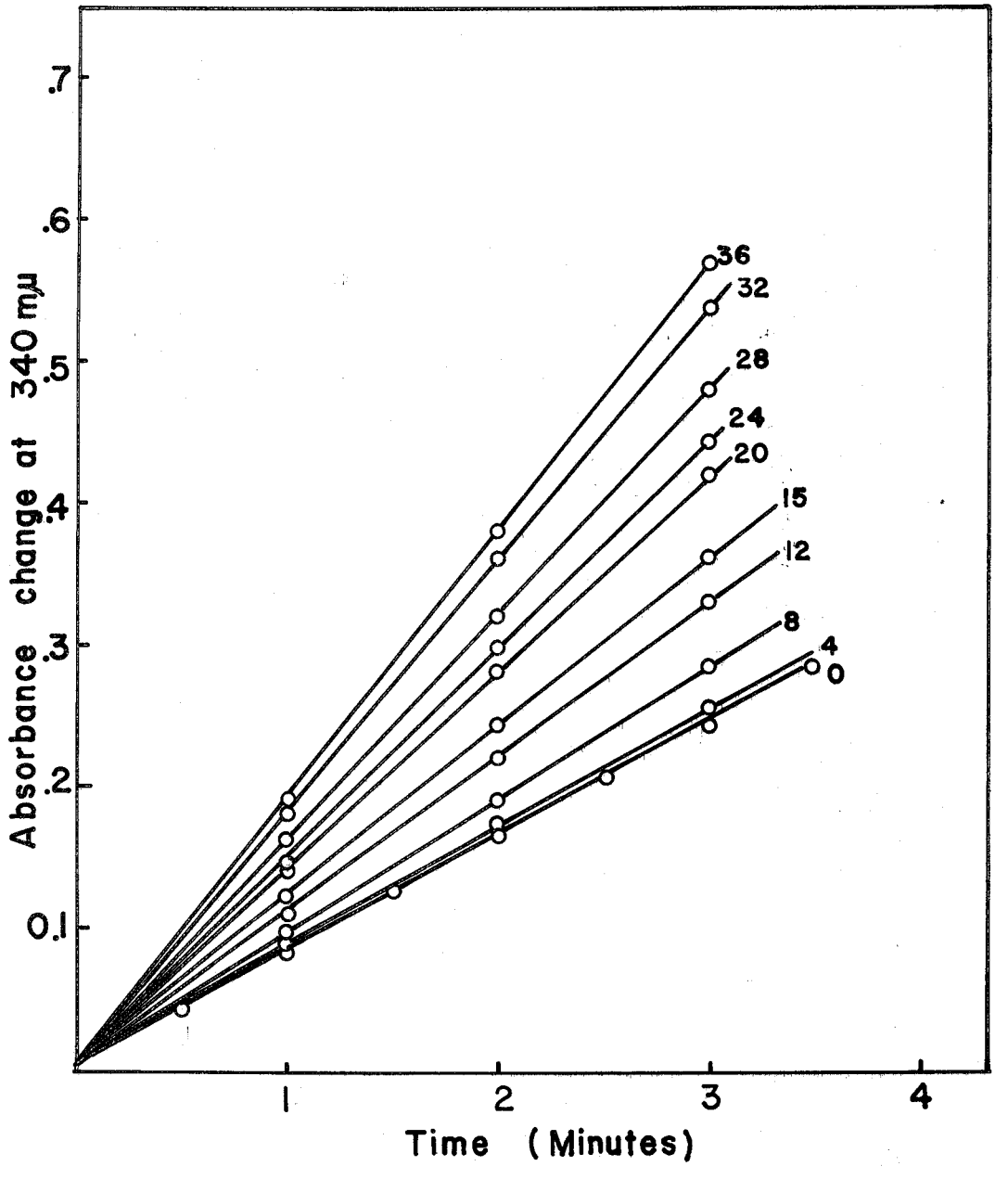


Figure XXII. Changes in Glucose-6-Phosphate Dehydrogenase Activity in Beet Slices Due to Aging.

The number on each line denotes the duration of aging (in hours). The assay conditions were the same as those described under Figure VI.

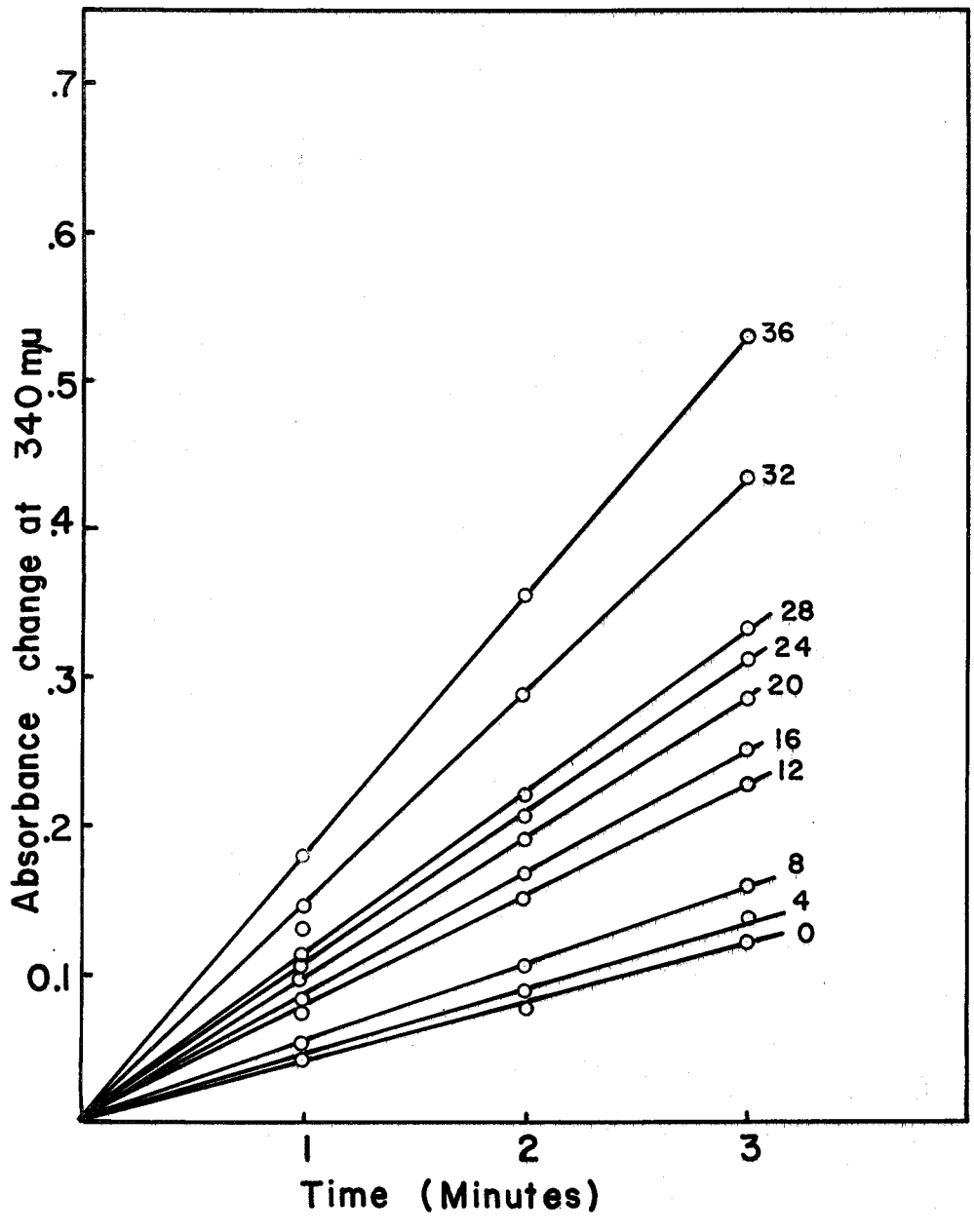


Figure XXIII. Changes in 6-Phosphogluconate Dehydrogenase Activity in Beet Slices Due to Aging. The number on each line denotes the duration of aging (in hours. The assay conditions were identical to those described under Table IX.

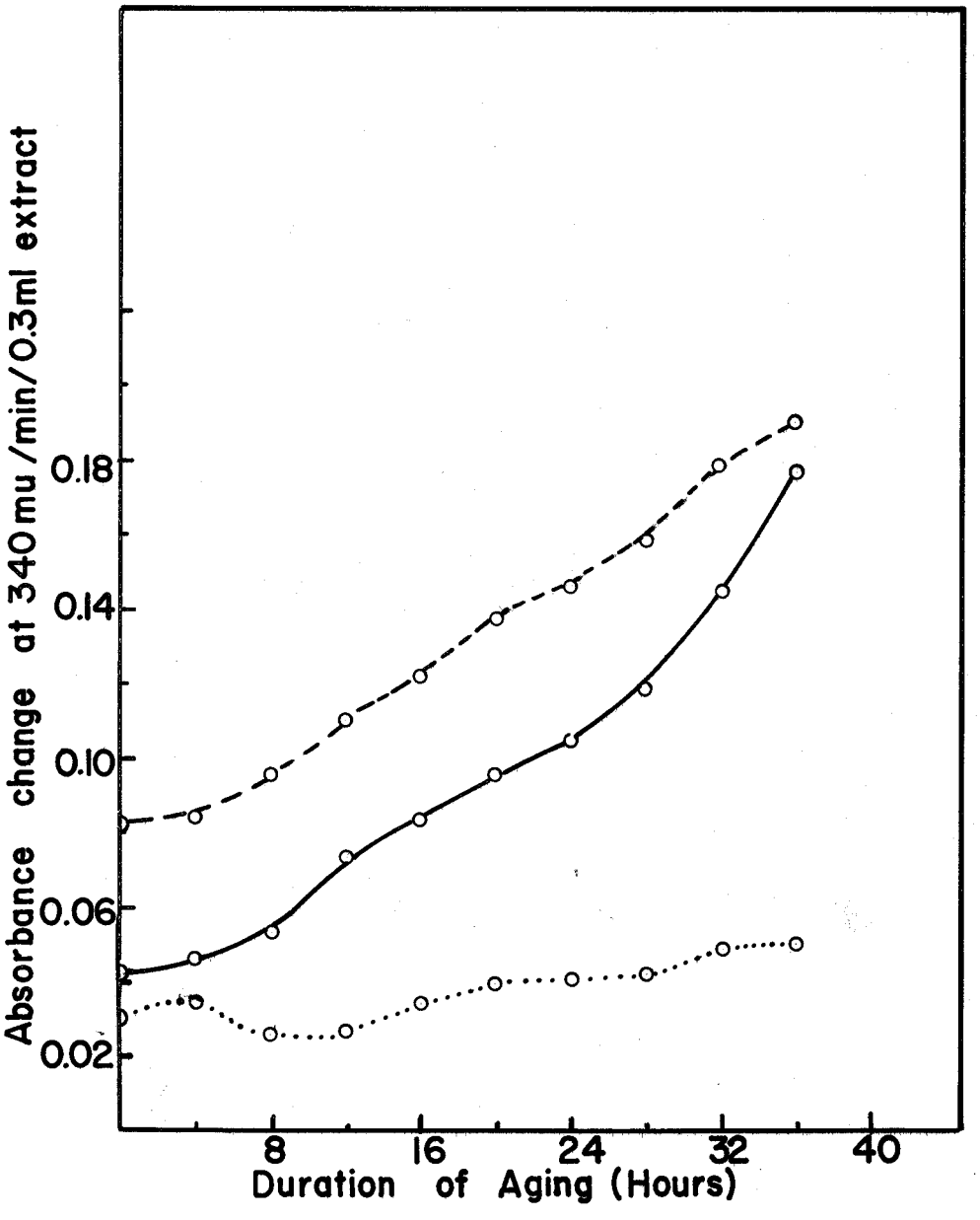


Figure XXIV. Changes in Enzyme Activities due to Aging In Beet Slices. Assay Conditions Were Identical to Those Described Under Figure VI and Table IX.

..... TPN reducting power.

——— 6-phosphogluconate dehydrogenase.

----- glucose-6-phosphate dehydrogenase.

changes in the protein level and activity levels of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, became significant. If a volatile respiratory inhibitor similar to that proposed by Laties (77, p. 679-690; 78; p. 129-155; 83, p. 215-299) is assumed to control the rate of respiration, a change in respiration rate would be the first change to be observable. Click and Hackett (38, p. 243-250) recently reported similar results in aging potato slices in which the respiration rate rose faster and reached a maximum sooner than the rise in observable protein and RNA synthesis. However, other biochemical changes could have taken place before changes in respiration rate, RNA synthesis and protein synthesis became measurable.

7. Gluconate Metabolism of Aging Beet Slices.

It is interesting to note that when compared to glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase activity was very weak in fresh tissue. However, the rate of increase of 6-phosphogluconate dehydrogenase was much larger than that of glucose-6-phosphate dehydrogenase, and thus, towards the end of the aging period of 36 hours, the two enzymes became comparable in activity levels. It may be recalled that when slices were washed, the 6-phosphogluconate dehydrogenase activity level was the one that

increased most markedly. In view of such a dramatic increase in the activity of this enzyme, C^{14} release from gluconate-1-C^{14} is of interest. In an attempt to determine the changes in the rate of release of C_1 of gluconate, at regular intervals, samples of aging slices were transferred from the petri dishes into radioactive potassium gluconate solutions. The $C^{14}O_2$ released during a two-hour interval was collected and counted as described in the experimental section. The results are shown in Figure XXV, along with a plot of the changes in 6-phosphogluconate dehydrogenase activity levels. Fresh slices released C^{14} from gluconate-1-C^{14} extremely slowly. After about four hours of aging, a dramatic increase in C^{14} release became visible. As much as a 15-25 fold increase in C^{14} release could be observed after 20 hours of aging. This increase was in part due to an increase in uptake, but uptake alone could not account for the large increase in C^{14} release. The large increase in gluconate utilization may in part reflect increased utilization of pentose for nucleic acid synthesis. Although it is not possible to evaluate the role of the large increase in 6-phosphogluconate dehydrogenase, it is very interesting to note that aging is accompanied by a marked increase in the ability to utilize gluconate and a concomitant increase in the activity of 6-phosphogluconate dehydrogenase.

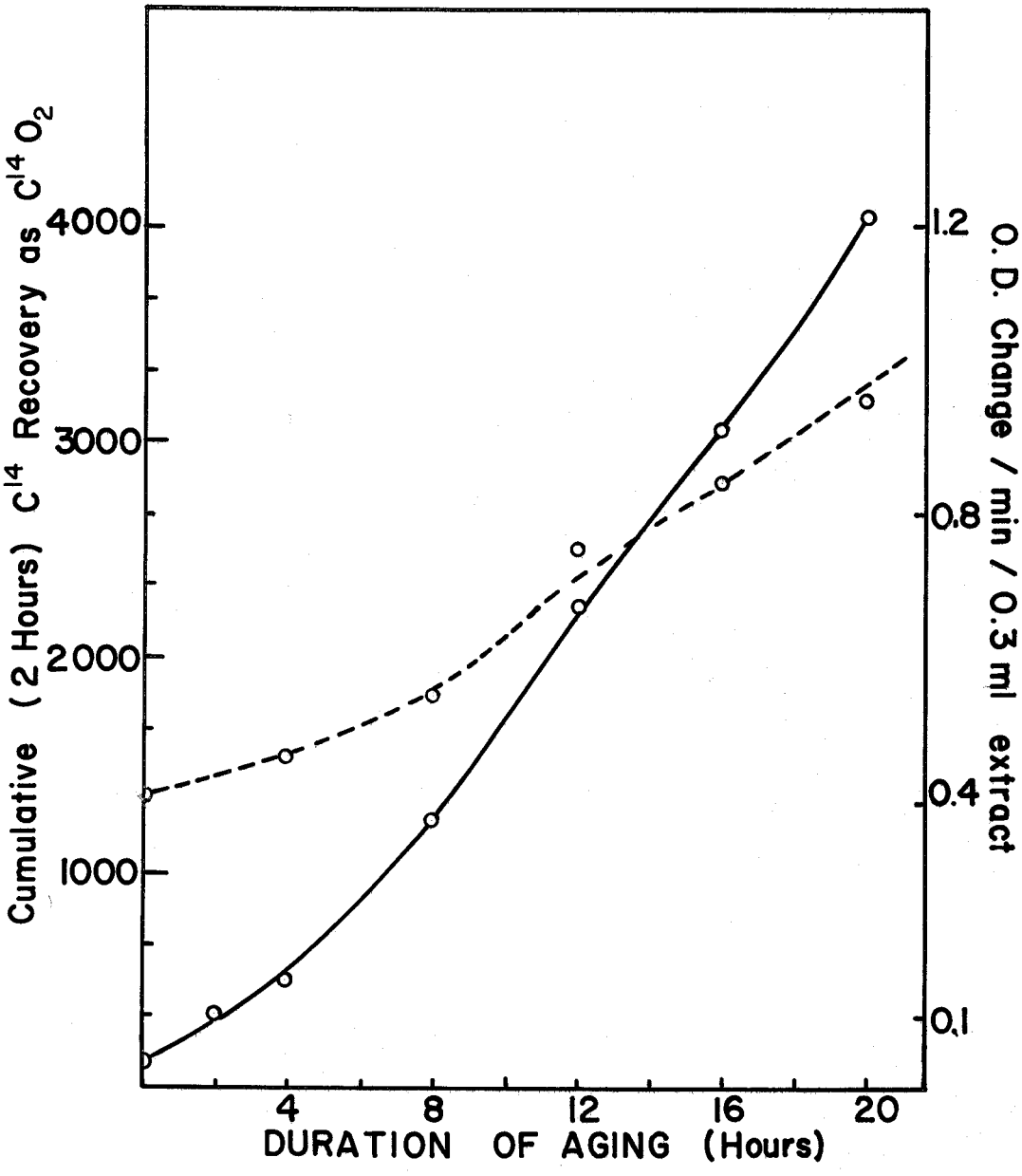


Figure XXV. Changes in 6-Phosphogluconate Dehydrogenase Activity and Yield of C^{14} in CO_2 from Gluconate-1-C^{14} due to Aging. 6-Phosphogluconate Dehydrogenase was assayed as Shown Under Table IX. Conditions of Gluconate Metabolism Experiments are Described in the Experimental Section.

8. Uptake of Substrates.

The rate of uptake of all the substrates used in this investigation was found to be increased by aging the slices as shown in Table XV. Both glucose and L-alanine uptake was increased at least two-fold and acetate uptake was almost doubled by a 24-hour aging period of the beet slices. Uptake of L-aspartate, L-glutamate and succinate by fresh slices was extremely slow, whereas aged slices took up these substrates several fold faster. It must be pointed out that since the primary purpose of these experiments was not uptake rate changes, the experiments were not designed to show the absolute increase in uptake. This is especially true in the case of the substrates such as glucose and L-alanine in which cases substrate levels limited the uptake by aged slices. Increases in uptake of glucose (6, p. 839-847; 103; 111, p. xii), phosphate (15, p. 580-594; 84, p. 418-424), sulphate (44, p. 129-136) and other solutes (125, p. 253-478) have been reported to be associated with washing and aging of slices from several different storage tissues. Indications are that organic solutes, as well as minerals, are probably taken up through the mediation of specific protein molecules, known as "permease," "translocase," etc., which are associated with membrane structures. Since increased protein synthesis seems to be generally associated

Table XV. Effects of aging on substrate uptake by red beet slices.

Substrate	Level in moles	Percent of administered	
		Fresh	24 hr. Aged
Glucose	2.78	43-47	86-92
Acetate	2.78	23-27	40-43
L-Alanine	2.78	26-30	92-94
DL-Alanine	5.56	45-49	95-98
Succinate	2.78	<10	36-40
L-Aspartate	2.78	<10	33-38
L-Glutamate	2.78	<10	36-40

with the aging phenomenon, part of this increase may be due to the increased synthesis of the protein molecules with the specialized function of solute uptake. Moreover, increased respiration provides additional energy which can be utilized for energy requiring processes of the cells, such as solute uptake. Working with carrot slices, Grant suggested an increased synthesis of protein of the permease type as a possible cause for the increased sugar uptake by the washed carrot root slices (103). However, they could not demonstrate an effect of puromycin on sugar uptake by carrot slices.

9. Experiments With Old, Dormant Red Beets

It has been suggested that in plants PPP participation increases with increasing age (49, p. 343-347). Bennet-Clark and Bexon (13, p. 65-92) pointed out that aging effects might depend on whether the tissue is in the growing phase, dormant phase or sprouting phase. Even though in the experiments described thus far beets in the growing phase were used, effects of age were often noticeable. For example, older beets gave a C_1/C_6 ratio of 1.6, whereas younger beets gave a value of 1.4. Older beets were found to have higher activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. In view of this evidence, aging effects on old, dormant beets would be of interest. Beets which were left undug

(until February) but looked healthy were used in a series of experiments. These beets were apparently dormant.

a. Glucose Catabolism in Fresh and Aged Slices of Old, Dormant Red Beets. Slices freshly prepared aseptically from the old dormant beets were used to determine the release of $C^{14}O_2$ from specifically labeled glucose, and the results are shown in Figure XXVI. Unlike growing beets, these dormant beet slices released C_1 and $C_{3,4}$ at about the same rate, indicating a much higher participation of PPP in this tissue than the growing beets used in previous experiments. It may be noted that unlike growing beets, dormant beet slices released C_6 and C_2 at about the same rate. If reformed hexoses recycle through PPP, C_2 would have a better chance to be released than other carbons. This might contribute to the higher C_2 recovery in the old and dormant beets than in growing beets. Fresh slices from growing beets showed an initial C_1/C_6 ratio of about 1.5-1.6, whereas the corresponding value in the old and dormant beet slices was about three. This strongly supports the contention that PPP participation is much greater in old and dormant beets than the rather mature growing beets used in previous experiments. It is further substantiated by an approximate estimation of PPP participation according to the equation of Barbour et al (8, p. 396-400), which

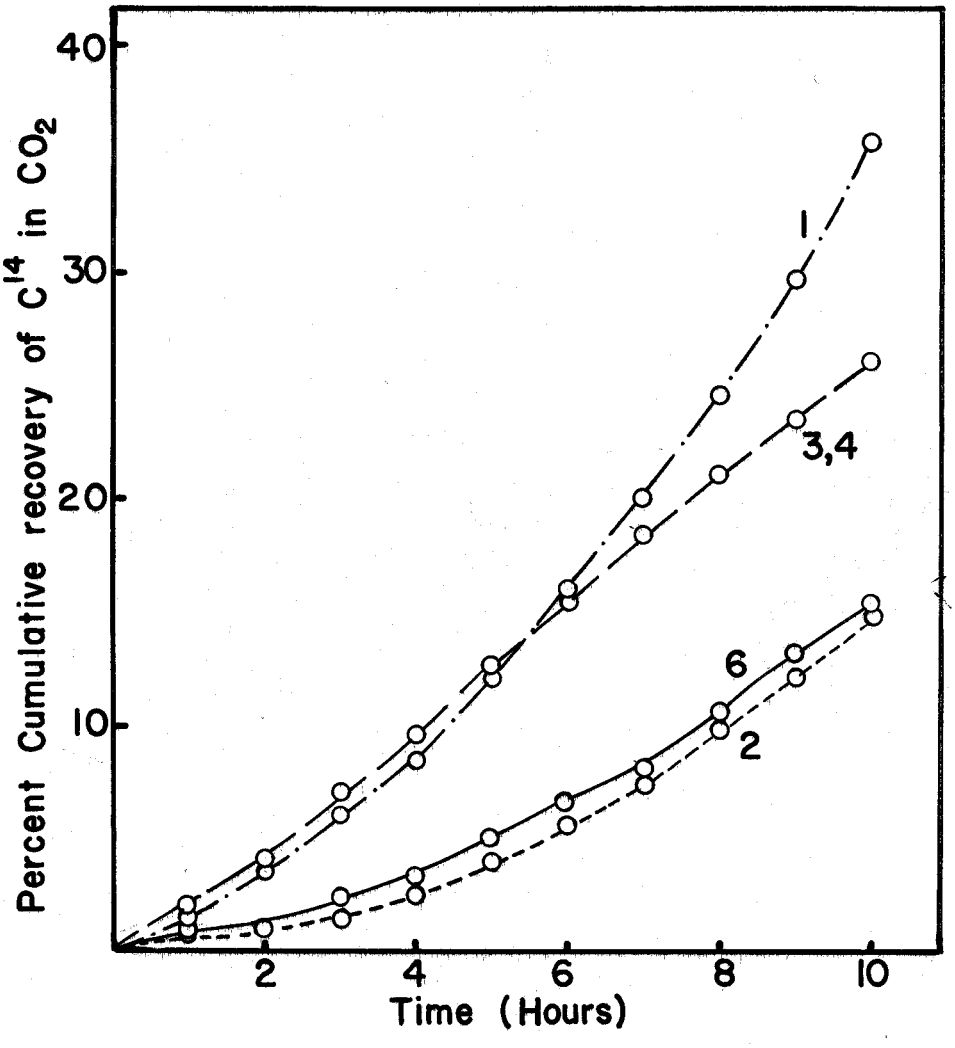


Figure XXVI. Time Course Plots of Percent Cumulative Recoveries of Respiratory $C^{14}O_2$ from Fresh Slices of Old, Dormant Beets Metabolizing C^{14} Specifically Labeled Glucose.
1 = glucose-1-C^{14}; 2 = glucose-2-C^{14}; 3, 4 = glucose-3, 4-C^{14}; 6 = glucose-6-C^{14}.
Experimental conditions were identical to those in Figure XV.

gave a value of 37 percent for PPP participation in old, dormant beet slices, whereas the corresponding value for growing beets was about 20 percent. This result is in line with the belief that older plant tissues have more PPP than younger tissues (49, p. 343-347).

C^{14} release patterns of dormant beet slices aged for 24 hours are shown in Figure XXVII . Release of C_1 was clearly faster than the $C_{3,4}$. C_2 release was evidently faster than the release of C_6. C_1/C_6 ratio in the first two hours was four. This evidence indicates that participation of PPP has increased during the 24-hour aging period. This conclusion is further supported by an approximate estimation of the PPP participation, which gave a value of about 47 percent. It must be pointed out that glucose uptake seemed to have decreased by aging of this tissue. For example, fresh slices took up about 76 percent of the administered glucose in ten hours, whereas 24-hour aged slices took up only about 61 percent of the administered glucose in ten hours.

b. Gluconate Utilization by Fresh and Aged Slices of Old, Dormant Red Beets. Ability of these slices to utilize gluconate was examined by administering 0.5mg gluconate-1-C^{14} to fresh and aged slices. Fresh slices from dormant beets took up gluconate better (40 percent of the administered) than the aged slices (30 percent). In contrast, when the percent cumulative recoveries are calculated on

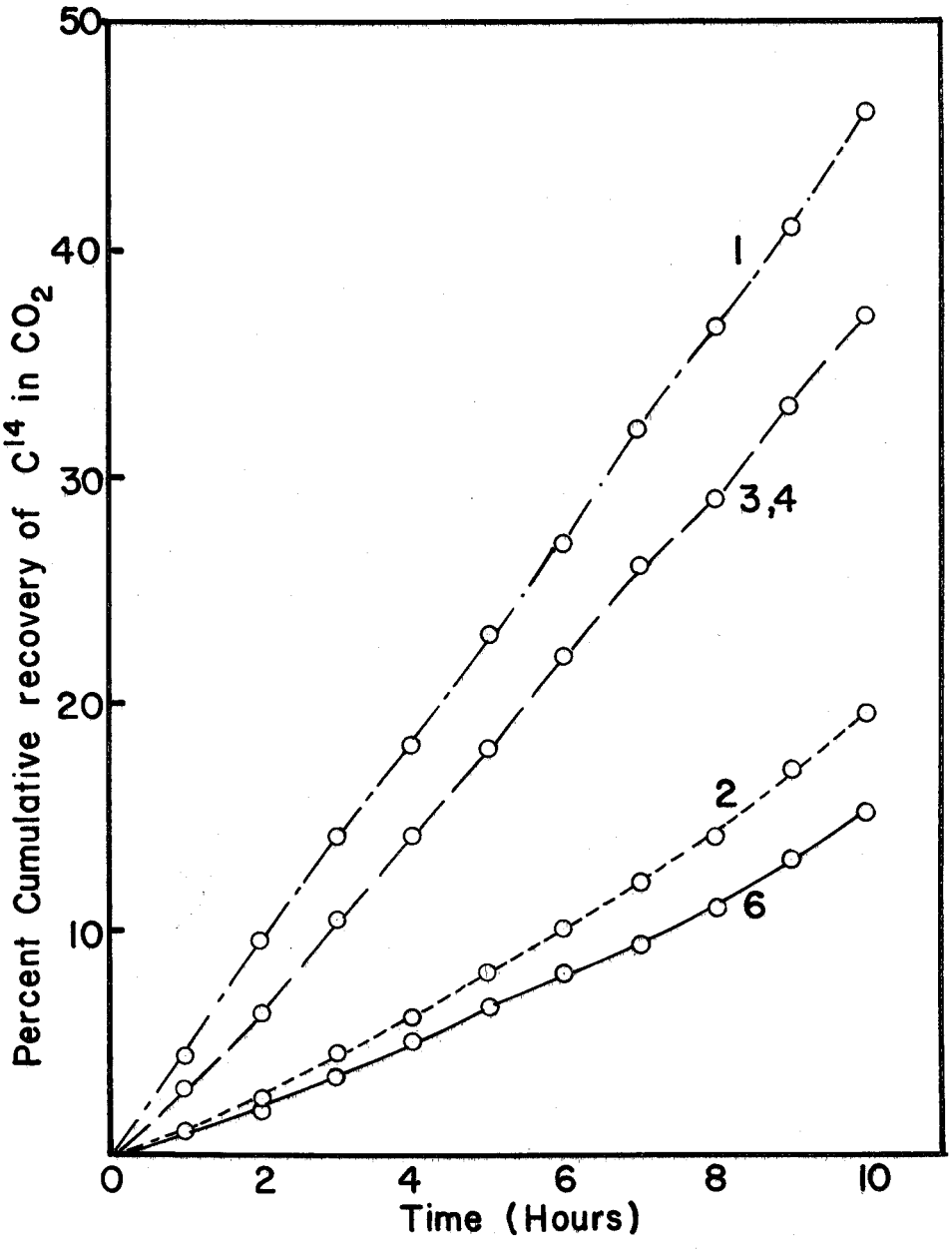


Figure XXVII. Time Course Plots of Percent Cumulative Recoveries of Respiratory $C^{14}O_2$ from 24-Hour Aged Slices of Old, Dormant Beets Metabolizing C^{14} Specifically Labeled Glucose.
1 = glucose-1-C^{14}; 2 = glucose-2-C^{14}; 3, 4 = glucose-3, 4-C^{14}; and 6 = glucose-6-C^{14}.
Experimental conditions were identical to those in Figure XV.

the basis of uptake, aged slices utilized gluconate faster than fresh slices, as shown in Table XVI. In the initial stages, 24-hour aged

Table XVI. Effect of Aging on the C^{14} Release from Gluconate-1-C^{14} by Old, Dormant Beet Slices.

Time (hr):	1	2	3	4	5	6	7	8	9	10
Tissue	Percent C^{14} Recovery from Gluconate-1-C^{14}									
Fresh...	1.1	1.9	3.2	5.2	8.3	13	19	26	35	47
Aged....	1.9	4.2	7.0	10.0	14.0	19	24	30	38	48

slices released C_1 of exogenous gluconate about twice as fast as fresh slices. However, toward the end of the experiment, fresh slices began to release C^{14} as fast or faster than aged slices, and thus, the final yield of C^{14} was about the same in both tissues. This may be due to the fact that the fresh slices were aged during the 10-hour experiment.

c. Leucine Metabolism in Fresh and Aged Slices of Old, Dormant Red Beets. L-leucine utilization by old, dormant beet slices was determined by administering L-leucine-U-C^{14} to the fresh and aged slices. Both tissues utilized L-leucine quite well. Fresh tissue took up L-leucine (78 percent in eight hours) probably slightly better than aged tissue (76 percent in nine hours). Fresh tissue incorporated about 34 percent of the C^{14} into alcohol insoluble residue in eight hours, whereas aged tissue incorporated 41 percent in nine

hours. Leucine incorporation into the protein fraction of potato slices has been reported (38, p. 243-250). The release of C^{14} from L-leucine-U-C^{14} was extremely slow in both tissues, as shown in Table XVII. Just as with aspartic-4-C^{14}, leucine-U-C^{14} also released C^{14} rather quickly during the first hour and then only slowly. This phenomenon was present only in freshly cut slices. This may be a part of wound respiration, which is the sudden increase in the rate of respiration observed immediately after peeling or slicing.

Table XVII. Effect of Aging on the C^{14} Release from L-leucine-U-C^{14} by Old, Dormant Beet Slices

Time (hr):	1	2	3	4	5	6	7	8
	Percent C^{14} Recovery from L-leucine-U-C^{14}							
Tissue								
Fresh...	0.92	1.33	1.6	1.8	2.0	2.3	2.6	3.1
Aged....	0.08	0.47	0.79	1.1	1.4	1.7	2.0	2.3

As a result of aging, slices prepared from old dormant beets seem to undergo changes in participation of pathways of glucose catabolism while no significant increase in leucine incorporation could be observed. Increase in gluconate utilization also was not very significant when compared to the growing tissue. The old and dormant beet tissues could utilize gluconate quite well, even when they were fresh. Thus, the fresh slices of the old dormant beets appeared to be already at the stage which growing beets reach when their slices are aged. The respiration rate of the slices from old and dormant

was not measured and so respiratory changes in this tissue are unknown. In any case, this is a tissue in which participation of PPP seems to have increased, but no significant increase in protein synthesis could be detected. A study of the metabolic changes in aging slices prepared from beets in growing, dormant, and sprouting phases may give more information about the aging process itself.

CONCLUSIONS AND SUMMARY

Experiments described in the preceeding sections were designed to study the metabolic changes that accompany aging in red beet slices. The main effort was directed toward changes in pathways of glucose catabolism.

Time course patterns of $C^{14}O_2$-release from C^{14} specifically labeled glucose by fresh red beet slices indicate that fresh slices catabolize glucose mainly via EMP-TCAC pathway, but that the PPP contribution to glucose catabolism is significant in this tissue. About 15-22 percent of the total glucose catabolism is estimated to be routed via the PPP. In fresh slices, C_6 of glucose was released faster than C_2, indicating either a triose exchange via reverse aldolase and subsequent recycling of the reformed hexose through PPP or a direct decarboxylation of C_6.

When the slices were washed in 0.01M KH_2PO_4 solution for 24 hours, the rate of uptake and utilization of externally administered glucose increased several fold. The $C^{14}O_2$ release patterns of the washed red beet slices, metabolizing specifically C^{14} labeled glucose indicated that the relative participation of the PPP contribution in the glucose catabolism increased due to washing. In agreement with this conclusion was the marked increase in the release of C_2 of glucose,

presumably due to increased recycling of the reformed hexose. Washing for longer periods of time increased the relative participation of PPP as indicated by the $C^{14}O_2$ release patterns of four and eight day washed slices metabolizing specifically labeled glucose. The results indicate that the EMP-TCAC pathway is damaged more than the PPP in the red beet slices washed for four to eight days.

The fresh slices of red beets utilized acetate and succinate quite well indicating that TCAC was quite active in fresh slices. The uptake and utilization of acetate and succinate increased markedly by washing. However, due to the unknown intracellular dilution of these substrates, the actual rate of endogenous acetate and succinate metabolism cannot be determined from these results, although the increase of acetate and succinate utilization caused by 24-hour washing strongly suggests increased TCAC activity.

Slices washed for long periods of time (four days or more) were found to be contaminated with bacterial growth, although visual symptoms of bacterial contamination were absent. The contribution of the bacteria, if any, to the $C^{14}O_2$ release patterns of the slices washed for four and eight days was not determined. Due to the uncertainties caused by bacterial contamination, aseptic conditions were used for further experiments. A comparison of the results of the experiments with sterile beet slices and those with non-sterile

beet slices shows that bacterial contamination did not become sufficient to influence the results, at least as long as the washing period was kept as short as 24 hours. It was found also that exogenous glucose utilization was slower with the slices prepared from freshly dug beets as compared to those from beets obtained from local markets.

Determinations of certain enzyme activities in the extracts prepared from fresh and washed slices indicated certain interesting changes in the activities of some enzymes related to carbohydrate metabolism. Glucose-6-phosphate dehydrogenase showed a small but consistent increase due to a 24-hour washing period. Malic dehydrogenase showed a small decrease. Isocitrate dehydrogenase showed a considerable increase. A small decrease in the ability of the extracts to reduce added TPN was observed. These rather small changes in enzyme activity levels are not believed to be of great significance. On the other hand, the rate of TPNH oxidation was found to be significantly increased by washing for 24 hours. This is believed to represent increased turnover of TPNH in the washed tissue which could contribute to the enhancement of the PPP as was observed in washed slices. The most marked change was found with 6-phosphogluconate dehydrogenase activity, which increased three to five fold during a 24-hour washing period. This increase in activity was

quite rapid; for example, within six hours of washing, 6-phosphogluconate dehydrogenase activity doubled. It is interesting that this change could be correlated to the very marked increase in gluconate utilization associated with the washing.

Experiments with tritiated glucose suggest that the TPNH produced via the PPP is utilized for biosynthetic reductions rather than as a respiratory substrate in red beet slices.

When fresh red beet slices were aged by maintaining slices at the surface of demineralized distilled water under sterile conditions, the rate of oxygen uptake gradually increased and reached a maximum in about 18-24 hours and then remained rather steady for at least 20 hours before it began to decline. The oxygen uptake of fresh slices was strongly inhibited (60 to 70 percent) by 8×10^{-5}M HCN; but the cyanide sensitivity decreased rather rapidly with increasing duration of aging. After six to eight hours of aging, the respiration of red beet slices became completely insensitive to cyanide and further aging was accompanied by a cyanide-stimulation of respiration by about 30 percent. After about 48 hours of aging, the inhibitory effect of cyanide began to reappear. No attempt was made to examine in detail the mechanism of these changes in cyanide sensitivity.

A study of the relative participation of pathways of glucose catabolism in the fresh and aged slices using radiorespirometric techniques

shows that glucose is catabolized by both fresh and aged slices primarily via EMP-TCAC. The $C^{14}O_2$ release patterns also indicate that the relative participation of PPP is slightly increased by aging. For example, in fresh tissue slices, about 22 percent of glucose was catabolized via the PPP, whereas in the aged counterpart, about 28 percent of glucose was found to traverse through PPP. The increase in the relative participation of PPP caused by aging was much smaller than that caused by washing. Thus, it appears that both the EMP-TCAC and the PPP are stimulated by aging; but there is a slight preferential increase in the PPP pathway. However, this small change in the relative participation of pathways is probably not directly related to the basic mechanism of age-induced respiration.

The $C^{14}O_2$ release patterns obtained using labeled acetate, succinate and alanine strongly support the view that the EMP-TCAC pathway is operative in fresh red beet root slices. Although the dicarboxylic amino acids (aspartic and glutamic) were very slowly absorbed by fresh red beet slices, once they entered the tissue, they were rather readily oxidized, again indicating TCAC activity in the fresh slices. Malonate inhibition of C^{14} release from labeled glucose by fresh red beet slices also supports this view. The $C^{14}O_2$ release patterns of aged slices metabolizing specifically labeled acetate, alanine, succinate, aspartate, and glutamate clearly indicated very active TCA cycle in the aged

slices.

Glucose-6-phosphate dehydrogenase activity and 6-phosphogluconate dehydrogenase activity were found to be markedly increased by aging. The increase in the former activity was less than the increase in the latter. In fact, only the increase in 6-phosphogluconate dehydrogenase was comparable to the increase in respiration. It is very likely that glucose-6-phosphate dehydrogenase is present in fresh tissue far in excess as far as PPP participation is concerned. However, 6-phosphogluconate-dehydrogenase activity was much less than glucose-6-phosphate dehydrogenase activity in the fresh slices. In view of these observations, one could suggest a regulatory role for 6-phosphogluconate dehydrogenase in this tissue. The marked increase in 6-phosphogluconate dehydrogenase was accompanied by a dramatic increase in the ability of beet tissue to utilize gluconate.

The extent of PPP participation was found to depend on the age of the tissue; the relative participation of the PPP was found to be more in older beets. For example, in a rather young beet sample, about 15 percent of the total glucose catabolism was mediated by PPP, whereas, in a sample of old, dormant beets, as much as 37 percent of the total glucose catabolism was indicated to be mediated via the PPP.

The rate of uptake of all substrates used in this investigation was found to be increased by aging. Fresh slices absorbed glucose and alanine quite readily; acetate and gluconate were less readily absorbed

but succinate, aspartate and glutamate were extremely slowly absorbed. The increase in the rate of uptake could result from increased synthesis of protein molecules of the permease type, increased availability of energy that can be expended for solute uptake or increased rate of catabolism of the substrates.

Aging was found to increase the biosynthetic capacity of the red beet slices. Increased retention of C_6 of glucose and carbons C_2 and C_3 of alanine appears to result from increased biosynthetic activities of the aged tissue. Moreover, larger amounts (29 percent) of C_3 of alanine was incorporated into the alcohol insoluble residue of aged tissue as compared to the fresh tissue (5 percent). It should also be pointed out that the protein content of the tissue was found to be increased by about 50 percent due to aging for 24 hours.

In the light of the results of this investigation and those reported in the literature on the biochemical changes that accompany the aging process in storage tissue slices, the following scheme represents a possible explanation for the aging effects observed in storage tissue slices.

In fresh storage tissues, the biosynthetic activities are kept inhibited at some level. This inhibition is released in some unknown fashion upon aging, thus triggering the biosynthetic activities, which in turn cause a rather rapid turnover of ATP. This increases the availability of ADP, which was limiting the respiration rate in fresh slices. The same effect may be brought about by DNP which is known to increase the availability of ADP. The increased availability of ADP increases the rate of DPNH oxidation via the cytochrome oxidase system and thus brings about the increase in respiration rate. The increased biosynthetic reactions consume TPNH for biosynthetic reductions and thus cause an increased participation of PPP. The extent to which the terminal oxidases might be consuming the TPNH is unknown. In any case, aging does not bring about very marked increase in the relative participation of PPP in red beet slices. It is not known how or at what level the biosynthetic activities of the tissue are kept inhibited in the fresh slices or what triggers the onset of increased biosynthetic activities.

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