AN ABSTRACT OF THE THESIS OF

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Little is known regarding the catabolic mechanisms involved in the breakdown of glucose in mung bean (Phaseolus aureus) seedlings. Studies in other laboratories have shown that the TCA cycle pathway is operative in mung bean leaves, and have demonstrated the presence of enzymes for the oxidation of glucose to glucuronic acid in mung bean seedlings. In the present study, the primary pathways responsible for the catabolism of glucose in detached 10-13 day old mung bean shoots were identified and the relative participation of each of these pathways was estimated.

The radiorespirometric method was employed for these experiments, using an ion chamber-electrometer system to monitor the respiratory $^{14}CO_2$ evolved from detached mung bean shoots catabolizing ^{14}C-labeled glucose substrates in the dark. Substrates were administered in two different ways, i.e., by means of a one-dose method and a continuous feeding method. The one-dose

experiments gave rise to respiratory $^{14}CO_2$ yield data which were used to estimate the relative pathway contributions. With the continuous feeding experiments, a metabolic steady state with regard to glucose utilization was realized, and the data on $^{14}CO_2$ production enabled one to calculate the catabolic rates of the individual glucose pathways. Comparison of the relative rates also provided one with information on the relative participation of glucose pathways.

By use of both the yield method and the catabolic rate method, it was concluded that in mung bean (Phaseolus aureus) seedlings, glucose is catabolized mainly by way of the Embden-Meyerhof-Parnas pathway, and that the pentose phosphate pathway and the glucuronic acid pathway played minor roles in the overall catabolism of glucose.

Glucose Catabolism in Mung Bean (*Phaseolus aureus*) Seedlings

by

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INTRODUCTION

The catabolic mechanisms involved in the breakdown of glucose in plant tissues have been studied in many laboratories. They have, in the main, centered on demonstrating the operation and estimating the relative participation of the Embden-Meyerhof-Parnas (EMP) and the pentose phosphate (PP) pathways and their interrelationships. In 1958, Loewus and coworkers suggested that glucose may also be catabolized in plant tissues by way of the glucuronic acid pathway (46), a metabolic route demonstrated by Eisenberg et al. to be operative in rats (20). The occurrence of the tricarboxylic acid cycle (TCA cycle) in higher plants for the catabolism of glucose intermediates has been well established; the state of knowledge of this has been reviewed by Hackett (29). However, more precise information on the relative participation and interplay among the primary catabolic mechanisms for glucose catabolism including the role of the TCA cycle pathway in the catabolism of glucose intermediates would be important if one desires to understand the overall metabolism of glucose in plant systems.

One of the primary functions of these glucose pathways is the production of energy through the oxidation of glucose carbon atoms to

CO_2, giving rise to reduced pyridine nucleotides and adenosine triphosphate. The primary catabolic mechanisms for the breakdown of glucose, i.e., the EMP, PP, and glucuronic acid pathways, result in the formation of pyruvate which is in turn catabolized by the TCA cycle pathway and the electron transport chain, resulting in the formation of energy.

Metabolic intermediates formed by the operation of any of the three primary pathways or the TCA cycle pathway may be used as a source of carbon skeletons for biosynthetic purposes. Glucose carbon atoms, while undergoing degradation via the EMP-TCA scheme, may be drained off as carbon skeletons for lipids and amino acids. The pentose units arising by way of the PP reactions may be incorporated into nucleic acids, arabans and xylans; the C_3 and C_4 fragments may be used for the synthesis of aromatic compounds via the intermediate, shikimic acid (50). In addition, the glucuronic acid pathway serves in the synthesis of ascorbic acid, pectin and hemicelluloses, as well as another source of non-cellulosic polysaccharides as pentosans (45, 47).

Numerous plant tissues have been used for the study of glucose metabolism: wheat shoots (51), wheat leaves (57), Avena coleoptile (25), corn coleopile (59), plum leaves (1, 2), strawberry leaves (10), Kalanchoë leaves (12, 13), tobacco leaf discs (49), tobacco tissue culture (22), leaf tissue from castor bean, coffee, pea, and

sunflower (25), stem tissue of Bryophyllum, Coleus, pea, sunflower, tomato (25), roots of castor bean (25), roots of corn (14, 25, 37). Glucose metabolism in storage tissues of various types has been investigated: carrots (3, 4, 5, 6, 48), turnips (5, 6), pumpkin mesocarp (6), potatoes (4, 36, 43, 44, 56), and beets (9, 42, 55, 65). Various types of fruit have been used for this purpose: tomato (18, 66), pepper (17), orange, lime and cucumber (8), parenchyma of pear (30).

Glucose metabolism has been studied in various intact algae. Syrett and coworkers have studied glucose catabolism in Chlorella (15, 60, 61) as has Devlin (16). Bidwell and Ghosh have studied glucose catabolism in brown algae (11), and Wildon and ap Rees have investigated glucose catabolism in Anabaena cylindrica (69). In addition, glucose metabolism in various intact plants has been studied: wheat seedlings (19), barley seedlings (58), etiolated sorghum seedlings (54), pea seeds (63, 70).

The present state of knowledge in this regard may be summarized as follows: Glucose is thought to be catabolized mainly by EMP-TCA cycle pathways and to a lesser extent (up to 30%) by the PP pathway, as estimated by use of C-1/C-6 ratios (6). Certain workers have used intermediates such as fructose, gluconate, pentoses, pyruvate and acetate as substrates in plants to confirm the operation of the two primary catabolic pathways and the secondary

TCA cycle pathway.

Work with plant tissues and slices do serve a purpose in that they reveal the presence of certain enzymes and metabolic schemes operative in the region being investigated, but such approaches do have disadvantages. First, these systems are usually decaying systems, that is, the systems are not capable of rejuvenation. Secondly, the systems are quite susceptible to attack by bacteria and molds, which could give erroneous results on catabolic pathways operative in the tissue.

For studying glucose catabolism in plants under physiological conditions, it would be highly desirable to use an intact plant as the test system inasmuch as it would reflect more accurately the mechanisms of glucose catabolism which operate in the undisturbed state in nature. However, the use of intact plants for study of glucose catabolism is difficult because of the limited permeability of the roots to substrate solutions. The limited permeability of substrate into the plant via the root system would make it nearly impossible to have a meaningful study of glucose catabolism by the addition of exogenous glucose. It is therefore more desirable to carry out experiments by removing the roots to facilitate transport of substrate to the stem and leaves. This is justifiable since the functions of the roots are to absorb water and minerals, to anchor the plant, to store food, and to conduct dissolved substances and food.

The roots do not generally take an active part in the catabolism of carbohydrates or respiration; most of the respiratory reactions are known to take place in the leaves and to a limited extent, in the stems of the plant.

The relative participation of pathways of glucose catabolism in mung bean seedlings is not known. Work with plant organs and tissues have shown that the EMP and PP pathways as well as the TCA cycle pathway are operative in plant systems. In addition, mung bean seedlings are known to contain enzyme systems for the oxidation of glucose to glucuronic acid (23), and glucuronic and galacturonic acid metabolism has been studied by Hassid's group in very young mung bean seedlings (41). Graham and Walker have shown that the TCA cycle pathway is operative in mung bean leaves by the use of ^{14}C-labeled TCA cycle intermediates (28).

The present study is designed, using detached Phaseolus aureus shoots, to detect the operation of individual pathways for the catabolism of glucose, and then to estimate more precisely the relative participation of each of these pathways.

MATERIALS AND METHODS

Plant Material

Mung bean (Phaseolus aureus) seeds were obtained from a local seed store. For germination, they were soaked for one day in water, then planted in a tray of moist vermiculite. The vermiculite was kept moist for the first five days covered with an inverted tray. During this period the plant developed a substantial root system and small leaves appeared. The cover was removed on the fifth day and the plants were allowed to grow in the greenhouse under long-day (15 hours) conditions for another five to seven days. Hoagland's nutrient solution #1 (33, p. 31), diluted 1:1 with water, was given on the seventh and ninth days, counting from the day the seeds had been planted; water was supplied once daily on the other days. On the tenth or eleventh day after planting, the cotyledons dropped off, and the plants were then ready for use. Experiments were performed with plants which were ten to thirteen days old, counting from the date of planting. Plants generally had at this stage of growth a stem length of about 14 centimeters and a total leaf area of about 7 (6 to 8) square centimeters; they weighed on the average, 0.4 gram each.

The plants were selected on a basis of comparable size, with

regard to both stem length and leaf area. After removal of the individual plants, the roots were cut off immediately above the uppermost secondary root. The detached shoots were divided into groups of ten, each group weighing 3 ± 0.1 grams. The cut ends of the detached shoots were then put into distilled water for about 45 minutes.

Administration of Substrate

For experiments involving one-dose substrate administration, each group of shoots was placed in a two-dram shell vial into which had been placed 0.2 ml of substrate solution. The cut ends of the stems were carefully arranged so as to be in contact with the solution at the bottom of the vial, the assembly placed in a vacuum desiccator, and a slight vacuum (to give 550 mm Hg) was pulled for a few seconds. The vacuum of the system was slowly removed. As the system approached atmospheric pressure, the substrate solution was drawn up into the stems of the plants. A "rinse" of 0.2 ml distilled water was quickly put into the vial, and the vacuum infiltration process was repeated, this time using only a very slight negative pressure to prevent drawing out the substrate solution. The whole process of infiltration was performed in less than three minutes. Two hours after placing in the plant chamber of the respirometer (Figure 1), the shoots were supplied with 2 1/2 ml of water which were placed

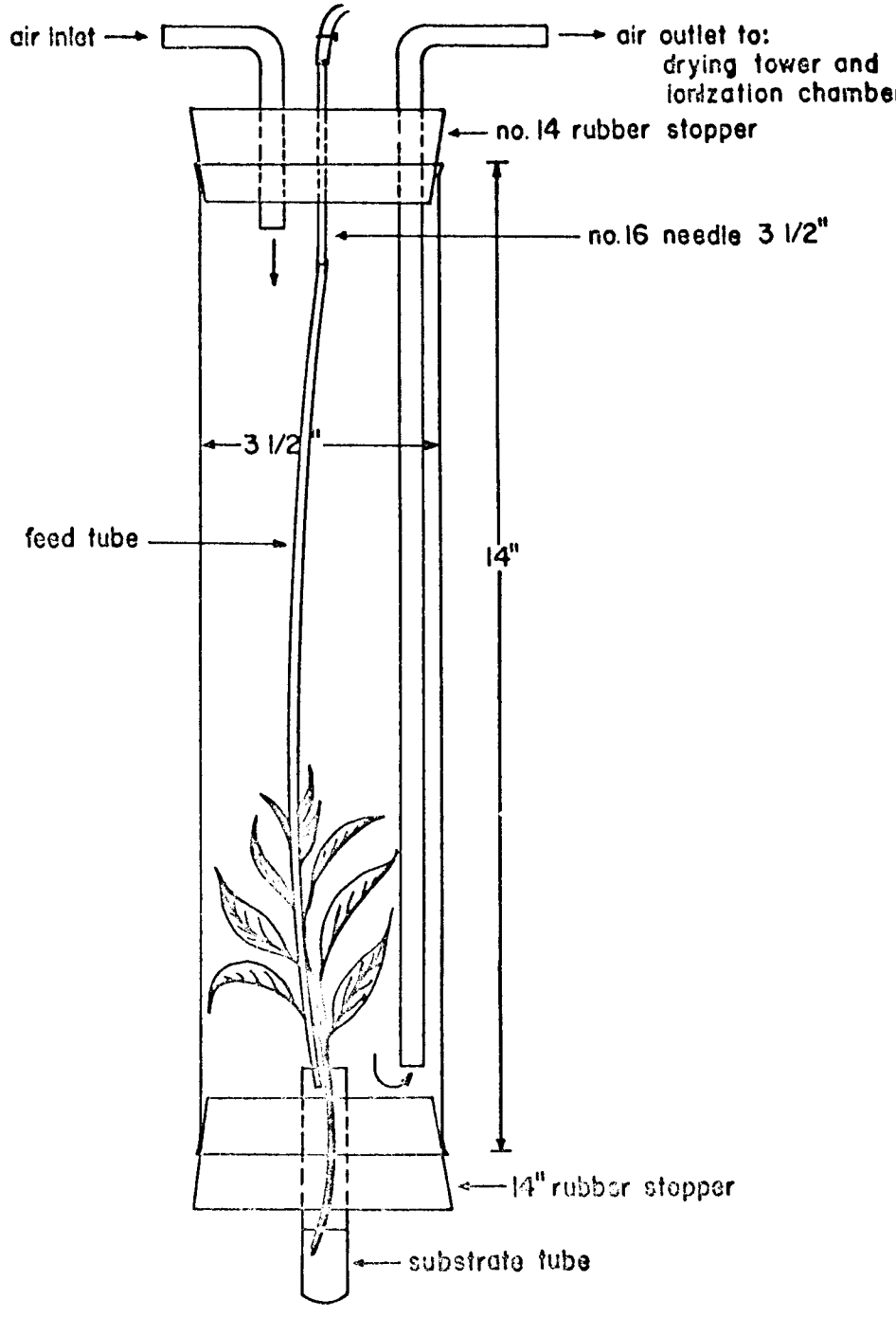


Figure 1. Respiration chamber for radiorespirometric experiments using plants.

in the vial via the feed tube.

For the experiments involving continuous feeding of substrate, the plants were prepared as described earlier, and the vial was loaded with 2.0 ml of substrate solution, ten times the amount used for the one-dose experiments. The vial and shoot assembly was placed in a vacuum desiccator as above, and the vacuum infiltration procedure was applied only once. The shoots were then placed in the respirometer chamber as before, and readings were taken at set intervals. Flow rate of sweeping air was 500 ml per minute; the plants were allowed to respire in the dark as before. It took 12 1/2 hours for 2.0 ml of substrate solution to be absorbed by the shoots.

That the vacuum infiltration method for substrate administration results in a rapid, homogeneous distribution of substrate in the leaves was shown by radioautography of pressed plants after substrate administration. For such an experiment, plants were derooted and put into shell vials with 0.2 ml of substrate solution as for the one-dose experiments, the assemblies were placed in the desiccator, and vacuum was pulled and slowly removed. Timing was started from the moment the substrate was drawn into the stem. After a fifteen minute period, the detached shoots were put between two sheets of Schleicher and Schuell 470-A filter paper and were "steam pressed" by placing the papers between two hotplates heated

to approximately 300°C, upon which surfaces were placed an asbestos sheet and a piece of wet Schleicher and Schuell 470-A filter paper. The plants were sandwiched between the two hotplates for one minute, and this resulted in a pressed plant which had been instantly killed. The wet paper served to prevent scorching of the plants. The pressed plants were allowed to dry, then placed on Eastman blue-sensitive x-ray film for ten days. It was found that fifteen minutes after substrate administration, the substrate was distributed homogeneously throughout the leaves.

Transpiration Rate in Detached Shoots

The transpiration rate was determined for mung bean seedlings under experimental conditions. Plants which were of suitable size for experimentation were derooted and put in a measured volume of water, then placed in the plant chambers. Air was drawn over the plants at a rate of 500 ml per minute. After a ten-hour period, the volume of water absorbed by the plants was noted. The average rate observed with several determinations was 0.16 ml per hour.

Determination of Respired $^{14}CO_2$

For the determination of respired $^{14}CO_2$, the plants were placed in a plant chamber of the respirometer described by Wang (64). A block diagram of this system is shown in Figure 2.

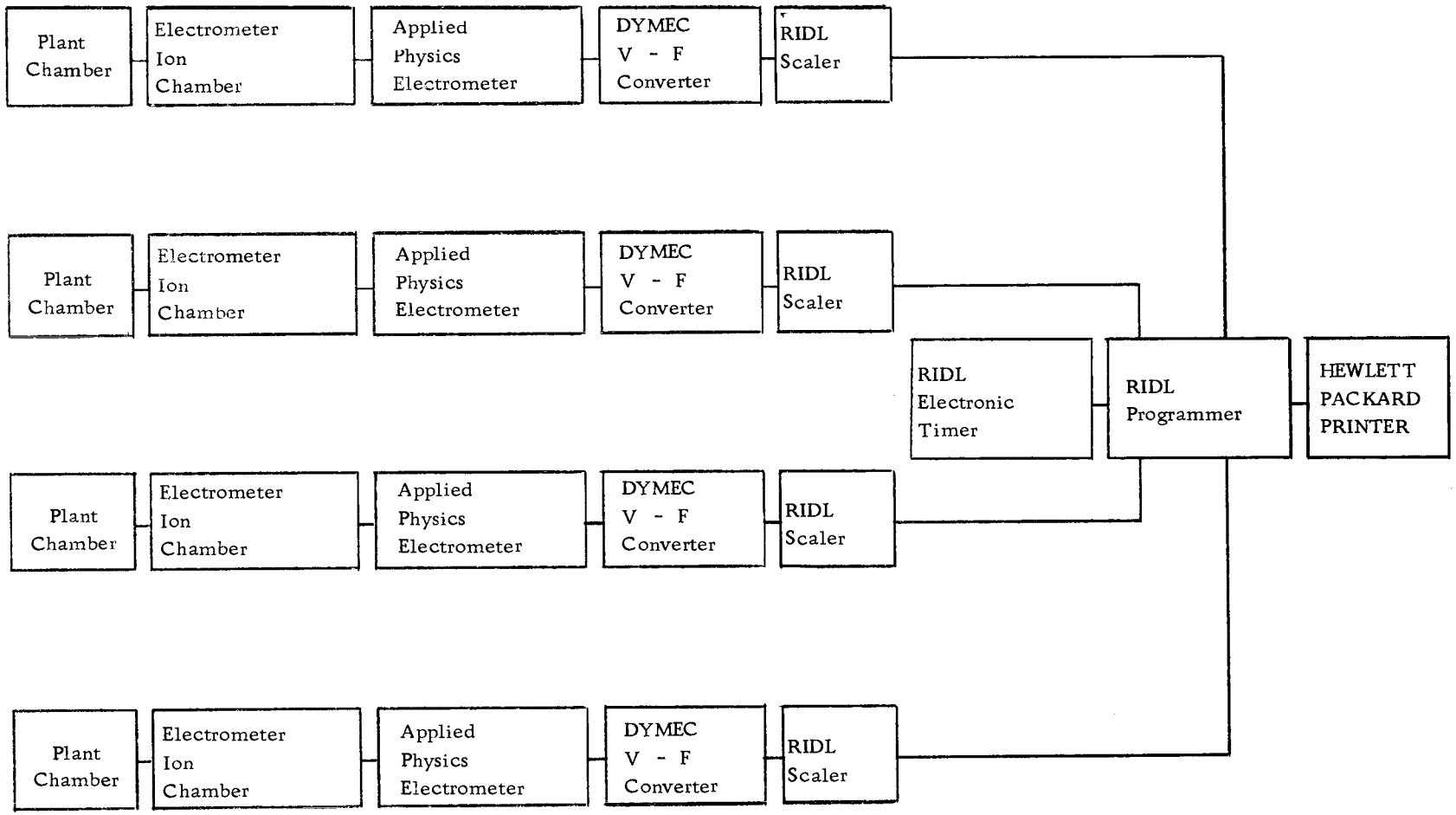


Figure 2. Block Diagram of Radiorespirometer with Printout System

Respiratory $^{14}CO_2$ from four concurrent experiments was measured continuously by means of an ion-chamber and vibrating reed electrometer; readout of data was accomplished in digital form. Flow rate of air sweeping through the plant chamber was adjusted to a defined rate. The air from the plant chamber was then passed through a Drierite gas drying unit to remove moisture from the air stream; this dried air was then passed through the ion-chamber and exhausted via a Gast pump.

The beta radiation from the $^{14}CO_2$ which flows through the ion-chamber produces electrons which are collected on the anode of the ion-chamber. A current is produced by these electrons; this current is directly proportional to the radioactivity present in the chamber. This d-c signal is converted to an a-c signal in the pre-amplifier unit of the electrometer; this a-c signal is then amplified by the amplifier of the electrometer, is demodulated, and used as the input to a voltage-to-frequency converter (VFC). The VFC produces an output frequency that is proportional to the input voltage; it serves to convert the analog information provided by the electrometer to the digital form. The digital output of the VFC is displayed by a six decade transistorized scaler. The scaler of each experiment, a timer, and printer are connected to a programmer. When the preset time is reached on the timer, the information from each scaler is printed on tape by the printer.

Procedure for Typical Experiment

In a typical experiment, after administration of substrate, the plants and their container were placed in the plant chamber of the respirometer. Air was swept through the chamber at a rate of 500 ml per minute, and the respiratory $^{14}CO_2$ was measured by the system described above. The chambers containing the plants were covered with a layer of black taffeta, and the room was kept dark to permit the plants to respire in the dark. The plants were left in the dark until the respiratory $^{14}CO_2$ dropped to a negligible level, usually 16 to 20 hours.

At the end of this period, the plants were removed from the chambers and killed by immersing in 50 ml of boiling 80% ethanol.

Procedure for Substrate Inventory

For the experiments requiring complete substrate inventories, the plants were homogenized in a Waring blendor, using a 360 ml Eberbach monel vessel, and the homogenate was filtered through Whatman No. 41 filter paper. The residue was then extracted with 80% ethanol in a Soxhlet extractor for 16 hours. The extracts and filtrate were combined, an aliquot removed for liquid scintillation counting, and the remaining ethanol extract was evaporated to near dryness in a rotary evaporator. A small amount of water was added

to the flask, and the evaporation process was repeated. This was done several times to ensure complete removal of ethanol. The ethanol extract was dissolved in water and subjected to liquid-liquid extraction with petroleum ether for 24 hours to remove any fatty substances. The petroleum ether and aqueous fractions were separated and assayed for radioactivity.

Isolation of Amino Acids: The aqueous fraction obtained by the above procedure was passed through a 1 x 15 cm column of Dowex 50 W x 8, 100-200 mesh (in H^+ form), to remove amino acids. The column was rinsed with water to remove all the acidic and neutral substances; the retained amino acids were then eluted from the column with 4 N HCl. The HCl was removed by rotary evaporation; the residue was dissolved in water and assayed for radioactivity; this was designated as the "amino acid fraction."

Isolation of Organic Acids: The effluent from the Dowex 50 column, which contained neutral and acidic substances, was passed through a 1 x 15 cm column of Dowex 1 x 8, 100-200 mesh (in formate form), to remove the organic acids. The column was rinsed with water to wash through the neutral substances, and the organic acids retained by the resin were eluted with 4 N formic acid. The effluents were assayed for radioactivity by liquid scintillation counting. The fraction which was washed through the formate column with water was designated as "simple carbohydrates," and

the effluent with formic acid was designated as "organic acids."

Determination of Starch: The residue from the Soxhlet extraction was treated with perchloric acid for extraction of starch according to the method of Hassid (31). After extraction with perchloric acid, an aliquot of the starch extract was taken and precipitated with iodine-potassium iodide reagent. The use of 25 mg of soluble starch as a carrier improved the recovery of starch, since mung bean seedlings contain little starch. The precipitated starch-iodine complex was centrifuged, washed twice with "ethanolic sodium chloride" (31), and the complex was dissociated with 0.25 N ethanolic sodium hydroxide. After centrifugation of the liberated starch, the starch was hydrolyzed according to the method of Pucher (53), and the solution of the liberated glucose was assayed for radioactivity by liquid scintillation counting.

Substrates

Glucose-1-^{14}C, glucose-2-^{14}C, glucose-3,4-^{14}C, and glucose-6-^{14}C were purchased from New England Nuclear Corporation, Boston, Massachusetts; Na gluconate-1-^{14}C was obtained from Nuclear Chicago Corporation, Des Plaines, Illinois. Na glucuronate-1-^{14}C and Na glucuronate-6-^{14}C were obtained from Nuclear Research Chemicals, Inc., Orlando, Florida, and glucose-U-^{14}C from Calbiochem, Los Angeles, California. Glucuronic-2-^{14}C was

prepared in the laboratory from glucose-2-^{14}C according to the method of Finkle (21). Substrates were calibrated for radioactivity by liquid scintillation counting of an aliquot of a diluted sample in a Packard Tri-Carb 314-EX2 liquid scintillation spectrometer.

Liquid Scintillation Counting of Radioactivity

Liquid scintillation counting was used to assay for radioactivity of water-soluble biological compounds. For the calibration of substrate solutions, 0.1 ml of a diluted (1:100) sample was put into 5 ml of 1:1 ethanolamine-absolute ethanol solution, and to this was added 10 ml of a toluene scintillator solution containing 0.3 percent p-terphenyl and 0.003 percent POPOP as primary and secondary scintillators, respectively. The samples were then counted in a Packard Tri-Carb 314-EX2 liquid scintillation spectrometer at a high voltage setting of 1020 v for the photomultiplier and a discriminator setting of 100-1000 divisions. For assay of radioactivity of the various plant extracts and column effluents, a 0.5 ml sample was dissolved in 15 ml of "diotol" scintillator (toluene-dioxane-methanol 350:350:210 by volume, containing 73 g naphthalene, 4.6 g 2,5 diphenyl oxazole, and 0.08 g POPOP per liter) (32). The absolute counting efficiency for each sample was determined by recounting following addition of an internal standard of toluene-^{14}C (New England Nuclear Corporation).

Calibration of the Electrometer System

Each ion-chamber electrometer system was calibrated, using a tank of compressed gas, 0.51% CO_2 in air containing $1.96 \pm 0.01 \times 10^4$ dpm of ^{14}C per liter. The ^{14}C radioactivity in the gas was determined beforehand by trapping the $^{14}CO_2$ in ethanolamine-ethanol (1:1) and counting the resulting ethanolamine carbonate in a liquid scintillation counter. By calibration of the system, the total number of digits accumulated by the scaler in a given period of time can be converted to the amount of radioactivity passing through the ion-chamber during that same period of time.

RESULTS AND DISCUSSION

The objective of the present study is to identify and estimate the relative participation of the various pathways responsible for the catabolism of glucose in mung bean seedlings. A method for the estimation of three concurrent pathways of glucose catabolism i.e., the EMP, PP, and glucuronic acid pathways, has not yet been developed. Even the estimation of two concurrent pathways presents problems in that the "specific yields," i.e., yields expressed in terms of the net amount of utilized glucose (8, 40, 66), are difficult to determine, since the amount of substrate which is engaged in anabolic processes cannot be readily determined with certainty.

For the identification of pathways, information from a one-dose substrate administration experiment is most useful. By the use of specifically ^{14}C-labeled glucose substrates, the interval yields of respiratory $^{14}CO_2$ may be plotted versus time and the curves so obtained (i.e. radiorespirometric patterns) reflect the mechanisms and associated kinetics by which glucose was catabolized to CO_2 in the system. The cumulative yields of $^{14}CO_2$ at the end of the time course serve as a basis for the estimation of the relative participation of pathways of glucose catabolism by employing equations devised by Wang (8, 66, 68).

In Figure 3 are shown the radiorespirometric patterns obtained

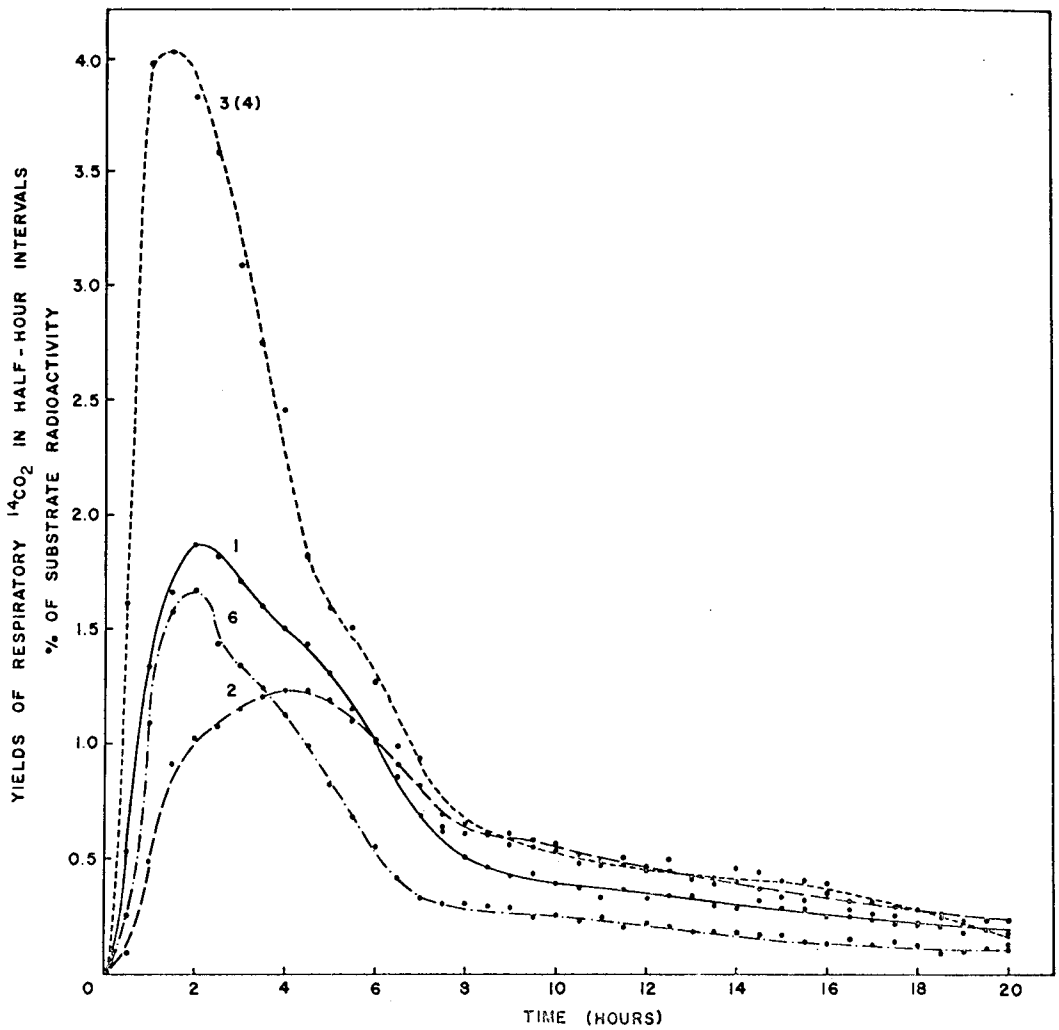


Figure 3. The Radiorespirometric Pattern for the Utilization of ^{14}C Specifically-Labeled Glucose by Ten (3.0 g fresh weight) Detached Mung Bean Shoots.

Glucose-1-^{14}C _____ Glucose-2-^{14}C _ _ _ _ _

Glucose-3, 4-^{14}C ----- Glucose-6-^{14}C _._._._.

Substrate Level: 300 μg in 0.2 ml administered by means of one-dose feeding.

Average of results from three experiments.

by the catabolism of glucose labeled with ^{14}C in various positions. The substrate level for these experiments was 300 μg. As stated by Wang (64), the slopes of the curves in this type of experiment provide the following information: 1) The ascending slope of each curve is thought to represent the following processes: a) the uptake of the substrate, which involves transport and absorption processes, b) rates and relative participation of sequential reactions for the production of respiratory CO_2 from the substrate. 2) The descending slope of the curve is thought to represent the following events: a) exhaustion of the labeled substrate in the system, b) dilution of the labeled substrate by endogenous compounds.

The extensive and rapid recovery of C-3 and C-4 of glucose as $^{14}CO_2$, shown in data given in Figure 3, is indicative of the operation of the EMP-TCA cycle pathways. The higher recovery of $^{14}CO_2$ from C-1 than those from C-6 or C-2 indicates that the PP pathway is operative. The operation of the EMP-TCA cycle pathways alone would result in the conversion of C-2 of glucose to CO_2 being more extensive and rapid than that from C-1 or C-6 of glucose, since C-2 and C-5 of glucose correspond to the carboxyl group of acetyl CoA, and C-1 and C-6 to the methyl group of acetyl CoA derived from glucose. The fact that conversion of C-6 of glucose to CO_2 is rapid and occurs in higher yield than conversion of C-2 of glucose to CO_2 in the early phase of the experiment indicates that C-6 is oxidatively

decarboxylated via the glucuronic acid pathway at a rather rapid pace. The appearance of a very early peak in the $^{14}CO_2$ yield data with glucose-6-^{14}C, presumably via the glucuronic acid pathway, is understandable since the decarboxylation of C-6 of glucose via this pathway requires only a few enzymatic steps, whereas via the EMP-TCA cycle pathway, the oxidation of C-6 of glucose to $^{14}CO_2$ occurs through extensive metabolism of acetyl CoA, derived from the substrate glucose, via the TCA cycle pathway.

The $^{14}CO_2$ yield curve obtained in the experiment using glucose-1-^{14}C is consistent with the operation of the PP pathway; here again, the preferential oxidative decarboxylation of C-1 of glucose requires only a few enzymatic steps. Carbon atom 2 of glucose is thought to be metabolized to $^{14}CO_2$ via two possible routes: via the EMP-TCA cycle pathways, where C-2 of glucose corresponds to the carboxyl group of acetyl CoA, and via the PP pathway, which results in re-formed fructose-6-P. The re-formed hexose is then mainly catabolized by the EMP-TCA cycle pathways; only a very few percent of the re-formed hexose appears to be catabolized via the pentose cycle (PC) pathway.

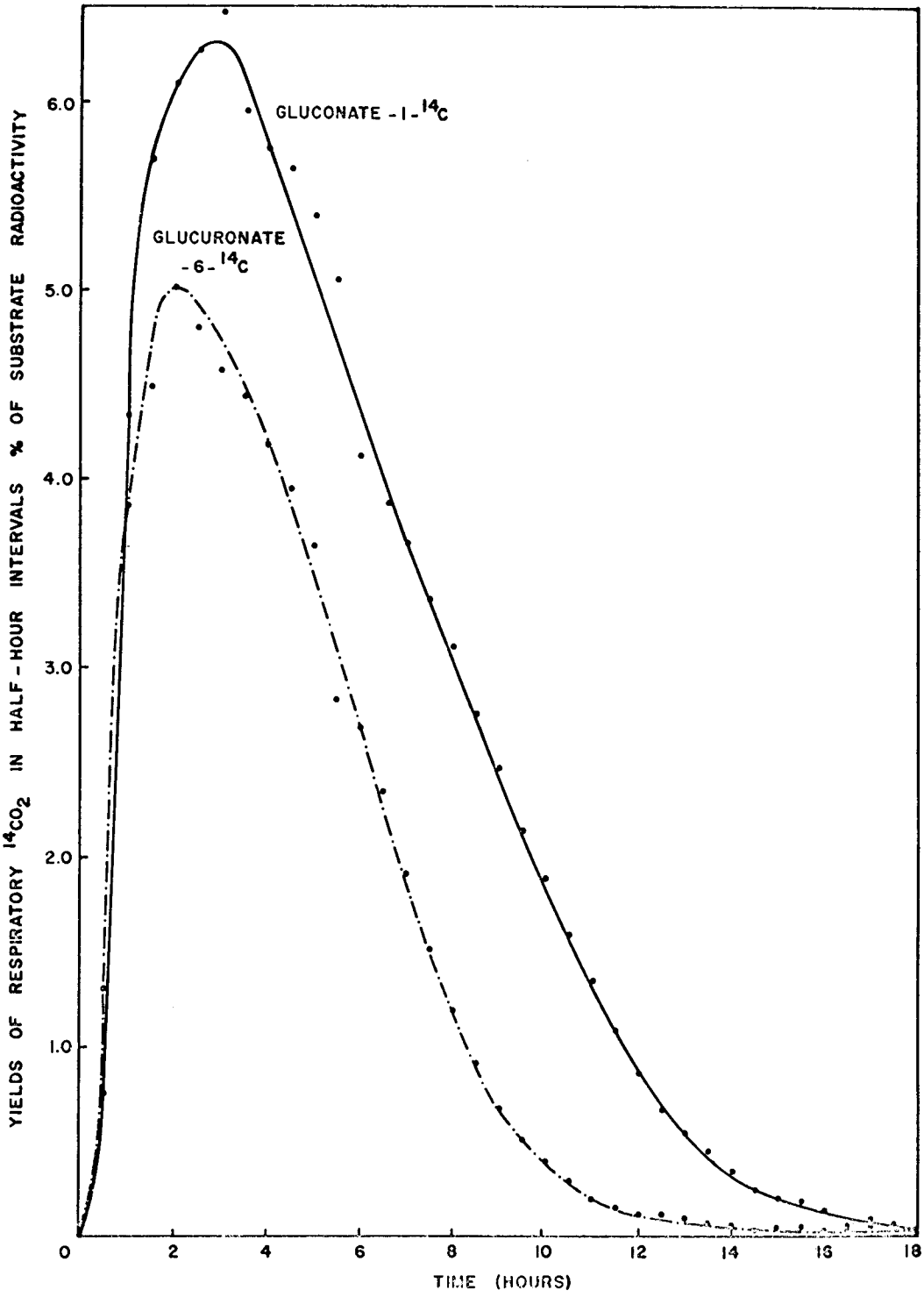
Data on $^{14}CO_2$ yields obtained from the experiments with Na gluconate-1-^{14}C at 200 μg level and Na glucuronate-6-^{14}C at 100 μg level (Figure 4) show that both of these substances were rapidly and extensively catabolized by mung bean seedlings; the cumulative

Figure 4. The Radiorespirometric Pattern for the Utilization of ^{14}C Specifically-Labeled Gluconate and Glucuronate by Ten (3.0 g fresh weight) Detached Mung Bean Shoots.

Gluconate-1-^{14}C _____ Glucuronate-6-^{14}C _._._._.

Substrate Level: Gluconate at 200 μg in 0.2 ml
Glucuronate at 100 μg in 0.2 ml
Both administered by means of one-dose feeding.

Average of results from three experiments.


YIELDS OF RESPIRATORY $^{14}CO_2$ IN HALF-HOUR INTERVALS % OF SUBSTRATE RADIOACTIVITY
6.0
5.0
4.0
3.0
2.0
1.0
0
2
4
6
8
10
12
14
16
18
TIME (HOURS)
GLUCONATE -1-^{14}C
GLUCURONATE -6-^{14}C

$^{14}CO_2$ yields at 20 hours were 92 and 56 percent, respectively. These experiments confirm the operation of both the PP pathway and glucuronic acid pathway in this plant. In separate experiments using Na glucuronate-1-^{14}C and -2-^{14}C as substrates, at a substrate level of 100 μg per 3 grams fresh weight of detached mung bean shoots, it was found that the labeled carbon atoms were converted to respiratory CO_2 at a very slow pace and gives rise to cumulative $^{14}CO_2$ yields of only 11% and 8%, respectively, at the end of the utilization time course. These findings indicate that the primary degradation products of the glucuronic acid pathway, i.e., pentoses (20) derived from glucose via the glucuronic acid pathway, are not further catabolized to any great extent.

The present state of knowledge indicates that there are three possible routes of glucose catabolism in plants. These routes are namely, the EMP pathway, the PP pathway, and the glucuronic acid pathway. The pentose phosphate derived from either the PP pathway or the glucuronic acid pathway can be further catabolized via the oxidative pentose cycle pathway. Despite the fact that the reductive pentose cycle is the mechanism for photosynthesis in plants, information so far in the literature leads one to believe that the pentose cycle pathway does not play an important role for glucose catabolism in plants in the dark (66). In fact, Gibson and Wang reported that in the photosynthetic organism Rhodospirillum rubrum,

glucose cannot be utilized at all by the organism (27).

The catabolic rate of each of these routes is controlled by the concentration of enzyme systems involved and kinetics of the enzymatic reactions. Hence, when substrate glucose is administered to the plant, it is subjected to competition from each of these metabolic routes, and if the quantity of substrate is limited, the catabolic rates of the individual pathways would then be the determining factor for the fate of the substrate. Thus in a typical radiorespirometric experiment, when the level of substrate glucose is low, the administered glucose would be routed preferentially to the pathway that is equipped with enzyme systems capable of a rapid turnover of substrate glucose. The radiorespirometric pattern so obtained would then represent only a distorted picture, not the normal catabolic mechanisms, for glucose utilization in the plant. Consequently, use cannot be made of the results of this type of experiment for the estimation of relative pathway participations. As indicated previously, the conversion of C-6 of glucose to CO_2 via the glucuronic acid pathway and the conversion of C-1 of glucose to CO_2 via the PP pathway involve only a few steps of enzymatic reactions. The respective decarboxylation processes are rapid, as evidenced by the data given in Figure 3. It is therefore necessary to maintain a sizable reservoir of substrate glucose so that the effect, in the nature of an artifact, derived from the variation in

catabolic rates of pathways, can be minimized or eliminated.

It is therefore evident that, for the purpose of estimating the relative participation of different catabolic pathways of glucose, it is of paramount importance that an optimal substrate level be used in the radiorespirometric experiments. The substrate must be administered at a level which will not constitute an overdose, i.e., at a level such that the available enzyme systems will be taxed or unable to handle such a quantity of substrate. In fact, with glucose catabolism, the Crabtree effect (34, 35, 38, 62) has been observed to be called into play when a massive dose of glucose is administered to biological systems such as mammalian tissues, tumors, and yeasts. Such an effect will give a distorted picture of glucose catabolism in the normal state of the system. On the other hand, a substrate level too low in magnitude is equally undesirable as discussed in the preceding section. An additional criterion for the selection of an optimal substrate level is that the administered substrate be utilized by the test plants within a period of 10-12 hours, generally considered to be the length of the normal dark period for plants.

According to Onslow (52, p. 57), it is calculated that 4.0 grams fresh weight of intact plants such as Phaseolus vulgaris (equivalent to 3.0 grams of detached shoots), contain 3.7 mg hexoses. Assuming that Phaseolus aureus seedlings are similar to

Phaseolus vulgaris plants in content of constituents, it can be inferred that total free glucose content is about 3 mg in 10 intact mung bean seedlings (4.0 g fresh weight). Of this, perhaps half would be found in the roots (7), leaving 1.5 mg contained in the detached shoot. Consequently the level of substrate glucose would have to be approximately equal in magnitude to this amount.

From results given in Table 1, it is seen that small amounts of glucose (50-400 μg) are rapidly utilized by detached mung bean shoots, as reflected by the observed short time course of utilization. As the level of substrate is increased, a more defined pattern depicting the participation of various glucose pathways is observed. This fact implies that the distortion of catabolic patterns, which resulted from variations in catabolic rates via different pathways, has been either minimized or eliminated. It is noted that when the substrate levels were within the range of 800 μg to 2,000 μg, the cumulative yield of $^{14}CO_2$ from the administered glucose-U-^{14}C was maximal and constant. At a higher substrate level, the yield of $^{14}CO_2$ declined, presumably as a result of overloading of the plants with external glucose. It can therefore be concluded that the optimal substrate level for the radiorespirometric experiments should be set at between 1,000 to 1,500 μg of glucose. The experiments designed for the estimation of glucose pathways were those using a substrate level of 1,000 μg.

Table 1. Cumulative Yields of Respiratory $^{14}CO_2$ from Detached Mung Bean Shoots Metabolizing Glucose-U-^{14}C at Various Substrate Levels

Substrate Level, μg	Time Elapsed After One-dose Substrate Administration, in Hours								
	1	2	3	4	5	6	8	10	20
50	2.4	8.4	12	15	17	18	21	24	32
100	1.7	5.4	8.5	11	13	15	19	22	31
300	1.1	4.5	8.4	11	13	16	19	22	31
400	1.5	5.0	8.3	11	13	15	18	22	32
800	0.7	3.2	6.3	8.5	11	14	18	22	33
1,000	0.7	3.1	6.1	8.3	11	13	18	22	33
1,500	0.5	2.5	5.1	7.0	9.3	12	16	20	33
2,000	1.1	3.6	6.4	8.7	11	13	16	20	34
5,000	0.5	1.9	3.6	5.6	7.7	9.8	14	17	30
10,000	0.6	2.1	3.9	6.1	8.3	10	14	18	31
20,000	0.3	1.2	2.3	3.8	5.5	7.3	11	15	28
50,000	0.2	0.5	1.0	1.5	2.1	2.8	4.5	6.5	20

As shown in results (given in Figure 5) obtained in experiments using ^{14}C specifically-labeled glucose samples as substrate, the rapid and abundant yield of $^{14}CO_2$ from C-3 and C-4 of glucose indicates the important role played by the EMP-TCA cycle pathways. The fact that the interval $^{14}CO_2$ yields from C-1 of glucose is greater than those from C-2 or C-6 of glucose is indicative of the operation of the PP pathway. The small but significant maximum observed in the early phase of the radiorespirometric data of glucose-2-^{14}C experiments presumably reflects the limited conversion of C-2 of glucose via the pentose cycle pathway. The second maximum appears to reflect the conversion of C-2 of glucose to CO_2 via the EMP-TCA cycle pathway. Similarly, a small but noticeable maximum observed in the radiorespirometric experiment with glucose-6-^{14}C as substrate provides evidence that the glucuronic acid pathway is operative but does not contribute much to the overall catabolism of glucose. This is in sharp contrast to the radiorespirometric findings in the experiments with 300 μg of substrate glucose (Figure 3), showing that the glucuronic acid pathway was an important pathway in the total catabolism of glucose. Evidently, the experiments with the low substrate level presented a distorted picture due to reasons that have been explained previously. The second maximum observed in the radiorespirometric pattern on the utilization of glucose-6-^{14}C at 1 mg substrate level can be explained

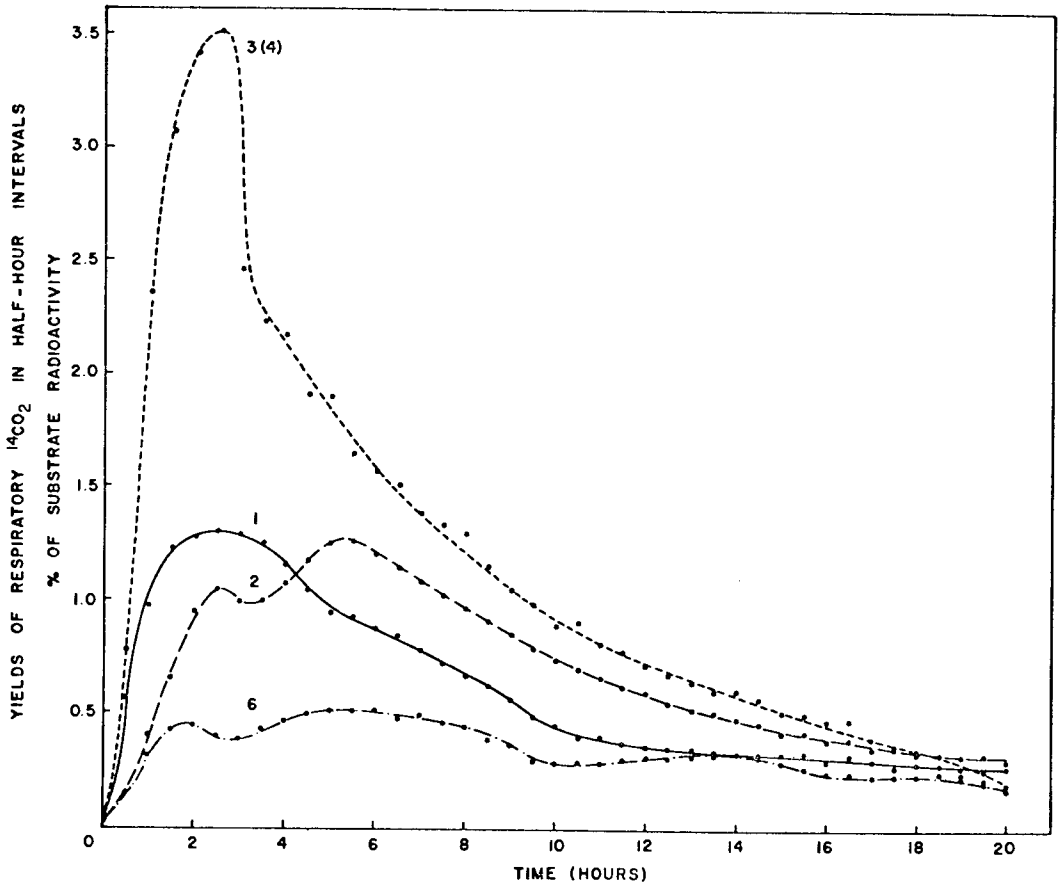


Figure 5. The Radiorespirometric Pattern for the Utilization of ^{14}C Specifically-Labeled Glucose by Ten (3.0 g fresh weight) Detached Mung Bean Shoots.

Glucose-1-^{14}C _____ Glucose-2-^{14}C _ _ _ _ _

Glucose-3, 4-^{14}C ----- Glucose-6-^{14}C _._._._.

Substrate Level: 1 mg in 0.2 ml administered by means of one-dose feeding.

Average of results from three experiments.

on the basis of the conversion of C-6 of glucose to CO_2 via primarily the EMP-TCA cycle pathways.

One finds in the literature that there exists no method for the estimation of relative participation of three concurrent glucose pathways (the EMP, PP, and glucuronic acid pathways) in biological systems. This is due to the fact that the present methods for the estimation of glucose pathways relies on the estimation of the contribution of the PP pathway by direct measurement of the preferential conversion of C-1 of glucose to CO_2. The contribution of the EMP-TCA cycle pathways is estimated by difference (66, 68). The conversion of C-6 of glucose to CO_2 via the glucuronic acid pathway prevents one, first, to determine the exact extent of the preferential conversion of C-1 of glucose to CO_2, since the latter determination relies on the assumption that complete metabolic equivalence is realized between C-1 and C-6 of glucose in pathways other than the PP pathway. Such metabolic equivalence is indeed the case with the EMP-TCA sequence. However, the glucuronic acid pathway involves a preferential conversion of C-6 of glucose to CO_2. Secondly, the occurrence of three concurrent pathways prevents one from estimating the contribution of the EMP-TCA cycle pathways by difference unless the contribution of the glucuronic acid pathway in the overall glucose catabolism can be determined directly.

In the present study, the radiorespirometric findings do not

provide direct quantitative information on the participation of the glucuronic acid pathway in the overall catabolism of glucose. However, the kinetic information observed in these experiments does present one with a means to estimate, crudely, the extent of operation of the glucuronic acid pathway. If one visualizes that the descending slope of the first maximum of the radiorespirometric pattern observed in the glucose-6-^{14}C experiment represents the decline of the contribution of the glucuronic acid pathway in overall glucose catabolism, extrapolation of the slope downward to the abscissa would then provide one with information on the conversion of C-6 of glucose to CO_2 via the glucuronic acid pathway (Figure 6). If one subtracts the interval yield data of the extrapolated curve from the overall yield data observed in the glucose-6-^{14}C experiment during the entire time course, one would obtain the corrected yield curve, designated as "6'," for the conversion of C-6 of glucose via exclusively EMP-TCA cycle pathway (Figure 6). Thus the shaded area "A" represents the contribution of the glucuronic acid pathway to the overall conversion of C-6 of glucose to CO_2.

Such an analysis of the radiorespirometric data leads to information on the net contribution of the glucuronic acid pathway to the overall catabolism of the administered glucose. This is represented by the cumulative yield of respiratory $^{14}CO_2$ from C-6 of glucose under the shaded area shown in Figure 6. This is true

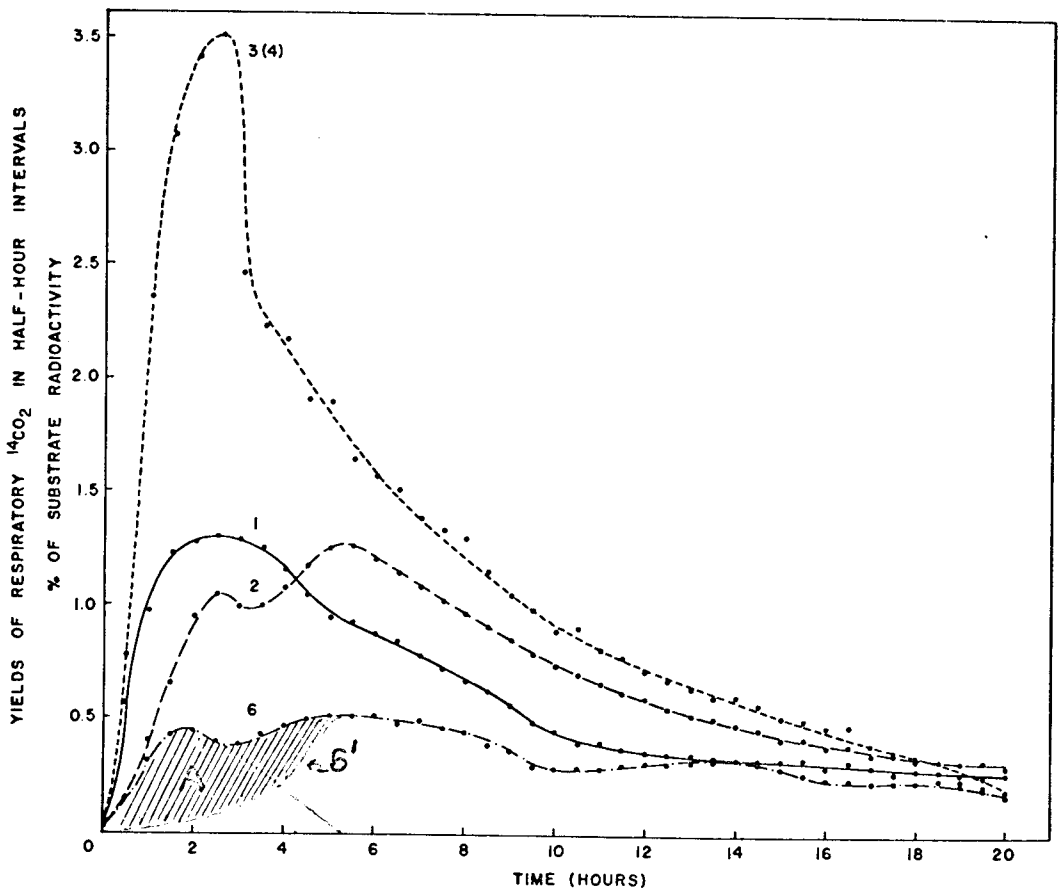


Figure 6. The Radiorespirometric Pattern for the Utilization of ^{14}C Specifically-Labeled Glucose by Ten (3.0 g fresh weight) Detached Mung Bean Shoots, Showing the Extrapolation Technique used to Determine the Contribution of the Glucuronic Acid Pathway to Glucose Catabolism.

Glucose-1-^{14}C _____ Glucose-2-^{14}C – – – – –

Glucose-3,4-^{14}C ---- Glucose-6-^{14}C _·_·_·_·

Shaded Area represents the Approximate Contribution to Glucose Catabolism by the Glucuronic Acid Pathway.

Substrate Level: 1 mg in 0.2 ml administered by means of one-dose feeding.

since, for each mole of glucuronic acid undergoing decarboxylation via the glucuronic acid pathway, one mole of respiratory CO_2 would have been produced. It is understood that the estimation so made does not cover the portion of the glucose that has converted to glucuronic acid, then to ascorbic acid or any other intact 6-carbon skeletons. From the data obtained from the glucuronic acid-6-^{14}C experiment, it is noted that approximately 56% of the administered glucuronic acid was decarboxylated. Bearing this information in mind, the data shown in Figure 6 reveal that the shaded area is equivalent to approximately 2.5% of the administered glucose. And, if one considers that 44% of the glucuronic acid derived from glucose was not decarboxylated, the total amount of administered glucose that has been routed into the glucuronic acid pathway is equivalent to approximately 5%, i.e., 2.5%/0.56 = 5%.

With the information on the relative participation of the glucuronic acid pathway in hand, use can then be made of the respiratory $^{14}CO_2$ yields from experiments with mung bean seedlings utilizing variously ^{14}C-labeled glucose to estimate the relative participation of other pathways. It should be emphasized that these yields, however, are calculated on the basis of the total amount of substrate administered, and hence they are not specific yields.

In order to convert the yield data to those expressed as specific yields, previously, Wang and coworkers (8, 66) have

devised equations for such a conversion. However, the validity of the equations devised by these authors was subject to a number of assumptions, of which several cannot be readily verified. In the present work, the amount of administered glucose engaged in anabolic processes have been estimated directly by determining the amount of substrate radioactivity in the carbohydrate fractions. Thus, the results given in Table 2 indicate that labeled glucose substrates were incorporated into the starch fraction to the extent of 0.4 to 0.6 percent and into the simple carbohydrates fraction to the extent of 5 to 16 percent. Inasmuch as C-1, C-2, and C-6 of glucose can be incorporated into the simple carbohydrates fraction via pathways involving degradation products of substrate glucose, it deems to be desired to rely on the data in this regard observed in the glucose-3,4-^{14}C experiments.

The amount of substrate glucose that has engaged in catabolic processes can be calculated as follows:

$$G_t = G_T - G_{T'} \qquad (1)$$

where G_t = the fraction of administered glucose engaged in catabolic functions;

G_T = the fraction of substrate glucose administered, which is by definition, unity;

Table 2. Distribution of ^{14}C Activity From Administered Glucose in Constituents Isolated From Mung Bean Shoots*

Constituent	Percent of Administered Radioactivity			
	Glucose-1-^{14}C	Glucose-2-^{14}C	Glucose-3,4-^{14}C	Glucose-6-^{14}C
Amino Acids	7.0	4.0	2.2	9.0
Organic Acids	8.4	8.0	3.4	15
Simple Carbohydrates	14	11	5.3	16
Starch	0.6	0.4	0.5	0.5
Fat	0.6	0.2	0.2	2.4
$^{14}CO_2$	25	24	44	18

*Experimental Conditions: Substrate Level: 1 mg by one-dose substrate administration
Plants: 3.0 grams (fresh weight) of detached mung bean shoots
Experimental environment: Dark
Experimental duration: 20 hours

$G_{T'}$ = the fraction of the labeled glucose administered which was engaged in anabolic processes.

From the results given in Table 2 for the glucose-3,4-^{14}C experiment, one finds that the fraction $G_T = 0.053$ (simple carbohydrates) + 0.005 (starch) = 0.06. Hence, the fraction $G_t = 1.00 - 0.06 = 0.94$, that is, 94% of the administered glucose has been engaged in catabolic processes.

Once the fraction of administered glucose that has engaged in catabolic processes is known, the observed cumulative yield of respiratory $^{14}CO_2$ derived from the individual ^{14}C specifically-labeled glucose substrates can be used directly as basic data for the estimation of glucose pathways.

The basic equation for the estimation of three concurrent pathways by means of the yield method can be readily derived as follows: The concept as well as the necessary assumptions underlying the derivation of the equation is much the same as that described previously by Wang (66, 68), except in the present case, consideration is given to the contribution of the glucuronic acid pathway to the overall glucose catabolism. Hence,

$$1 = G_p + G_e + G_{gu} \qquad (2)$$

where G_p = fraction of glucose catabolized by the PP pathway;

G_e = fraction of glucose catabolized by the EMP pathway;

G_{gu} = fraction of glucose catabolized by the glucuronic acid pathway.

$$G_p = \frac{G_1 - (G_6 - (G_p G_6 + G_{gu}))}{G_t} \qquad (3)$$

where G_1, G_6 = cumulative yield of respiratory $^{14}CO_2$ derived from mung bean shoots metabolizing equal amounts of the respectively ^{14}C-labeled glucose. Expressed as a fraction of the administered radioactivity.

Upon simplification of equation (3) and solving for G_p, one obtains

$$G_p = \frac{G_1 - (G_6 - G_{gu})}{G_t - G_6} \qquad (4)$$

In the foregoing equations, the fate of C-6 of glucose in the PP pathway is represented by the term $G_p G_6$ instead of $G_p A_6$, where A_6 designates the yield (in fractions) of C-6 of gluconate in respiratory CO_2. This is justifiable since previously it has been observed that there exists little difference between the yield of C-6 of glucose in respiratory CO_2 as compared to that of C-6 of gluconate (66).

Using the yields of $^{14}CO_2$ given in Table 2 and substituting $G_{gu} = 0.045$ and $G_t = 0.94$, equation (4) becomes

$$G_p = \frac{0.25 - (.18 - .045)}{0.94 - .18} = 0.16 \text{ or } 16\%; \quad G_e = 1 - G_p - G_{gu}$$

$$= 1 - 0.16 - 0.05 = 0.79 \text{ or } 79\%$$

In other words, of the glucose administered to mung bean shoots, 6% was routed into anabolic pathways. For the portion of glucose engaged in catabolic processes, 79% was catabolized via the EMP pathway; 16% was catabolized via the PP pathway; and 5% via the glucuronic acid pathway.

The estimation of relative participation of pathways can also be made by a new method devised by Wang (39, 67). With this method, the catabolic rate of an individual glucose pathway is determined while the biological system is metabolizing substrate glucose under metabolic steady state conditions. Such a condition, using mung bean shoots as a test system, can be realized when substrate glucose is administered by means of the continuous feeding technique. Comparisons of the relative catabolic rates via various glucose pathways provide one directly with information on the relative participations of pathways in the overall glucose catabolism. Catabolic rate of the EMP-pyruvate decarboxylation sequence is revealed by the rate of production of respiratory $^{14}CO_2$ from glucose-3,4-^{14}C. The rate of the PP pathway is revealed by examining the rate of preferential conversion of C-1 of glucose to respiratory CO_2.

As shown in Figure 7, with continuous feeding of substrate glucose, the rate of production of respiratory $^{14}CO_2$ in the glucose-3,4-^{14}C experiment reached a plateau stage approximately 180 minutes after implementation of the experiment and was maintained at that stage for a period of at least 600 minutes. This fact indicates that a metabolic steady state was indeed realized with respect to the utilization of substrate glucose. Similarly, one finds the rate for the preferential conversion of C-1 of glucose to CO_2 obtained by subtracting the interval yield of respiratory $^{14}CO_2$ in the glucose-6-^{14}C experiment from that observed in the glucose-1-^{14}C experiment (designated by 1 (PP)), reached a plateau stage as early as 60 minutes after substrate administration. It is noted that the curve representing the preferential conversion of C-1 of glucose to CO_2 remained at the plateau level for a period of only 250 minutes followed by a sharp decline. The latter phenomenon presumably reflects the increased yield of respiratory CO_2 from C-6 of glucose due to extensive catabolism of this carbon atom via the PP pathway. It should be also noted that inasmuch as the glucuronic acid pathway does not play an important role in glucose catabolism in mung bean seedlings, no correction has been made for the $^{14}CO_2$ yield data of the glucose-6-^{14}C experiment to account for the contribution of the glucuronic acid pathway.

Catabolic rates for the EMP-pyruvate decarboxylation pathway

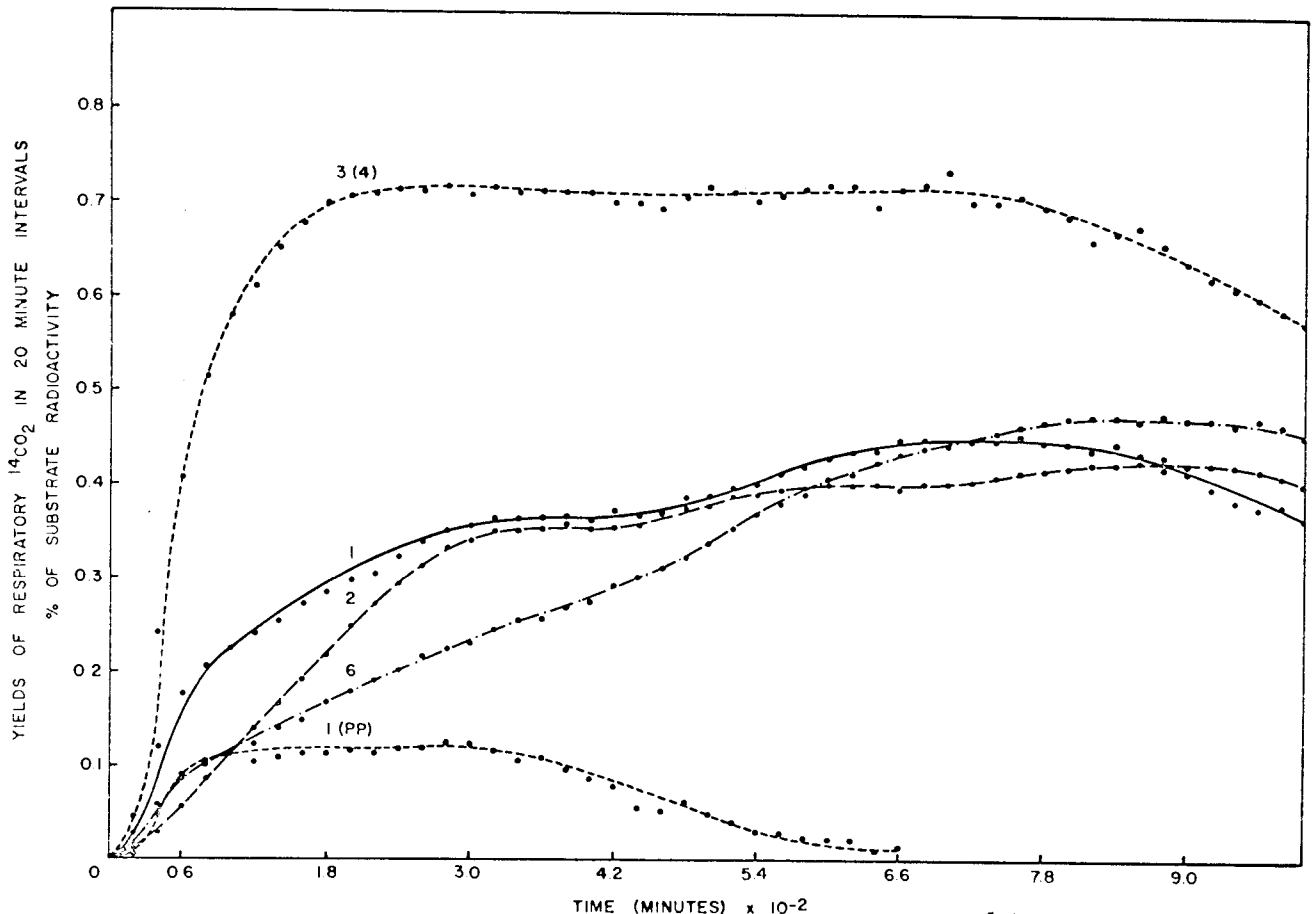


Figure 7. The Radiorespirometric Pattern for the Utilization of ^{14}C Specifically-Labeled Glucose by Ten (3.0 g fresh weight) Detached Mung Bean Shoots.
Glucose-1-^{14}C _____ Glucose-2-^{14}C _ _ _ _ _ _ _
Glucose-3,4-^{14}C ------ Glucose-6-^{14}C _._._._._._
Substrate Level: 10 mg in 2 ml, administered by means of continuous feeding.
Average of results from two experiments.

and the PP pathway were calculated, making use of data observed between 240 and 260 minutes (Figure 7). It is noted that during this time period, metabolic steady states were realized with regard to both of these pathways.

The determination of catabolic rates of glucose utilization via the EMP-pyruvate decarboxylation pathway relies on the understanding that for each mole of glucose catabolized by this sequence, 1 mole of CO_2 will be produced from either C-3 or C-4 of glucose. Similarly, for the catabolism of glucose via the PP pathway, 1 mole of respiratory CO_2 will be derived from C-1 of 1 mole of glucose. From the results given in Figure 7, one notes that the yield of respiratory $^{14}CO_2$ derived from glucose-3,4-^{14}C is 0.724% during the period of 20 minutes, which is equivalent to 0.0362% per minute. During the same period, the yield of $^{14}CO_2$ derived from C-1 of glucose produced via the PP pathway is 0.118%, or 0.0059% per minute. It follows that during this defined period of one minute that the fraction of substrate glucose catabolized via EMP-pyruvate decarboxylation pathway and PP pathway is equivalent to $0.000362 + 0.000059 = 0.000421$, or 0.0421%. One can then calculate that relative participation of the EMP-pyruvate decarboxylation pathway for the catabolism of said fraction of glucose is:

$G_e = \frac{0.000362}{0.000362 + 0.000059} \times 100 = 86\%$. Similarly,

$G_p = \frac{0.000059}{0.000362 + 0.000059} \times 100 = 14\%$. The relative participation of EMP-pyruvate decarboxylation pathway and the PP pathways as calculated by the method of catabolic rates are very close to that calculated by the yield method. It should be emphasized, however, that with the catabolic rate method, the values for G_p and G_e were determined directly, whereas with the yield method, the value of G_e is obtained by difference. However, in the present case the catabolic rate method does not take into consideration the operation of the glucuronic acid pathway, since the latter pathway is operative only to a limited extent in mung bean seedlings.

The close proximity of the values obtained for the relative participation of glucose pathways by the yield method and the catabolic rate method leads one to believe that the findings represent a reliable assessment of the contribution of each of the pathways to the overall glucose catabolism in mung bean seedlings. It can be therefore stated with reasonable certainty that in mung bean seedlings, glucose is catabolized predominantly by the EMP pathway, with the PP pathway and the glucuronic acid pathway playing relatively minor roles in overall glucose metabolism.

SUMMARY

The operation of the various pathways of glucose catabolism in detached mung bean (Phaseolus aureus) shoots was investigated by the radiorespirometric method using an ion chamber-electrometer system. It was found that glucose is catabolized by way of the Embden-Meyerhof-Parnas (EMP), pentose phosphate (PP), and glucuronic acid pathways. Discussion was given regarding the use of an optimum substrate level in glucose catabolism studies. Using this optimum substrate level, the contributions of the three primary pathways to the total glucose catabolism in mung bean shoots were estimated by use of respiratory $^{14}CO_2$ yield data and substrate inventory data.

The catabolic rate method was employed in the study of glucose catabolism by detached mung bean shoots in order to determine the contributions of the EMP and PP pathways to glucose catabolism. Reasonably close agreement was obtained between this method and the $^{14}CO_2$ yield method.

It was concluded that in detached shoots of Phaseolus aureus, glucose is catabolized predominantly by way of the EMP pathway, with the PP pathway and the glucuronic acid pathway playing relatively minor roles in the overall catabolism of glucose.

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