AN ABSTRACT OF THE THESIS OF

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Title: GLUCOSE CATABOLISM IN NORMAL AND FLUORIDE INJURED MUNG BEAN (PHASEOLUS AUREUS) SEEDLINGS

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Studies in other laboratories have established the presence of the Embden-Meyerhof-Parnas (EMP) pathway and the pentose phosphate (PP) pathway in plant tissue. Research in this laboratory with mung bean (Phaseolus aureus) seedlings has confirmed the presence of these two pathways as well as the glucuronic acid (GA) pathway. However, no reliable method for estimating the relative participation of these concurrent pathways in plants has existed.

In this study the primary pathways of glucose catabolism in ten-day old mung bean seedlings were identified. A new approach, designated as the catabolic rate method, was developed to estimate the relative participation of three concurrent catabolic pathways of glucose. Then the effect of fluoride on glucose catabolism was examined using the catabolic behavior data.

A continuous substrate feeding technique was developed in connection with the catabolic rate method thereby permitting the collection of radiorespirometric data on mung bean seedlings respiring at a

metabolic steady state. These data in turn provided reliable information on the rate of evolution of respiratory $^{14}CO_2$ evolved from intact mung bean seedlings catabolizing ^{14}C specifically labeled glucose substrates in the dark. Comparative examination of the rate data on $^{14}CO_2$ formation made it possible to calculate the extent of participation of individual catabolic pathways of glucose.

It was found that in ten-day old mung bean seedlings glucose was catabolized via the EMP pathway, the PP pathway, and the GA pathway to the extent of 74%, 17%, and 9% respectively.

Fluoride ion at a concentration of 20 ppm in the intact plant was found to inflict a severe inhibitory effect on the EMP-pyruvate decarboxylation pathway. It was of even greater interest to observe that in the presence of fluoride the PP pathway and the GA pathway were playing more important roles in over-all glucose catabolism of intact mung bean seedlings.

Glucose Catabolism in Normal and Fluoride Injured Mung Bean (*Phaseolus aureus*) Seedlings

by

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GLUCOSE CATABOLISM IN NORMAL AND FLUORIDE INJURED MUNG BEAN (PHASEOLUS AUREUS) SEEDLINGS

I. INTRODUCTION

Glucose catabolism in plant tissues has been studied in several laboratories (3, 8, 15, 16, 20, 33). Previous workers have been primarily concerned with demonstrating the concurrent operation of two catabolic pathways, the Embden-Meyerhof-Parnas (EMP) pathway and the pentose phosphate (PP) pathway, and the estimation of their relative participation in over-all glucose catabolism. In addition the glucuronic acid (GA) pathway has recently been demonstrated by Ikeda (16) to be operative in mung bean (Phaseolus aureus) seedlings. The presence of the tricarboxylic acid cycle (TCA) pathway in higher plants has also been well established (9, 11).

Existing methods for the estimation of glucose pathways in biological systems involve the administration of ^{14}C specifically labeled glucose substrates in single doses into either tissue preparations or intact biological systems. Glucose pathway information may then be obtained by examining either the relative specific activity (22, 36) or the yield (21, 43) of glucose derivatives, such as lactate or $^{14}CO_2$, from ^{14}C specifically labeled glucose substrate. The attention of most pathway workers has been placed primarily on the EMP pathway and PP or pentose cycle (PC) pathway in plants and animals. This is due to the nearly universal presence of these two mechanisms in glucose catabolism. Other pathways such as the GA pathway (16) have been investigated but there does not presently exist

a useful method for the estimation of three concurrent pathways for glucose catabolism in plants.

It should also be noted that existing methods for pathway estimations are concerned with the direct assessment of the PP (or PC) pathway contribution with the EMP pathway being determined by the difference between the PP pathway and the total glucose metabolized. This method assumes, however, that all administered glucose participates in either the PP pathway or EMP pathway to the exclusion of any anabolic pathways or other catabolic pathways.

The initial fate of substrate glucose in the catabolic pathways has been of primary concern to Wang and his co-workers (41, 43) and hence their method is devised to estimate the contribution of the PP pathway but not the PC pathway in over-all glucose catabolism. The initial fate of substrate glucose is visualized by these authors to be as follows:

via the EMP pathway

G_e = fraction of glucose catabolized via the EMP pathway

$$3 \text{ glucose} \xrightarrow{G_e} 3 \text{ glucose-6-P} \rightarrow 3 \text{ fructose-1,6-diP} \rightarrow 6 \text{ triose-3-P} \rightarrow 6 \text{ pyruvate}$$

via the PP pathway

G_p = fraction of glucose catabolized via the PP pathway

$$3 \text{ glucose} \xrightarrow{G_p} 3 \text{ glucose-6-P} \rightarrow \begin{cases} \rightarrow 3\ CO_2 \\ \rightarrow 3 \text{ pentose-P} \rightarrow 2 \text{ fructose-6-P} + 1 \text{ glyceraldehyde-3-P} \end{cases}$$

Wang and his co-workers recognize that fructose-6-phosphate, derived from the PP pathway, can be catabolized either by the PC pathway or the EMP pathway.

On the other hand, Katz and Wood (22, 47) have focused the objective of their method on the assessment of the net participation of the PC pathway. They have defined the PC pathway as:

via the PC pathway

PC = fraction of glucose catabolized via the PC pathway

3 glucose-6-P $\rightarrow$ 3 CO_2 + 1 glyceraldehyde-3-P + 2 fructose-6-P

2 fructose-6-P $\rightarrow$ 2 glucose-6-P

net reaction becomes

1 glucose $\xrightarrow{PC}$ 1 glucose-6-P $\rightarrow$ 3 CO_2 + 1 glyceraldehyde-3-P

It should be noted that the contribution of the pentose cycle pathway, designated as PC, and the participation of the PP pathway in over-all glucose catabolism, designated G_p by Wang and co-workers, can readily be related to each other (21, 43) through the expression

$$G_p = \frac{3\ PC}{1 + 2\ PC}$$

Useful information on the relative participation of the two major pathways operating concurrently in biological systems has been obtained by existing methods, but the findings have been subject to the validity of several key assumptions. Katz and co-workers in their work on the PC pathway assumed that glucose is primarily engaged in triose phosphate pathways. They also assumed that any pentose phosphate derived from the PP pathway is not engaged in any biosynthetic processes (47). These assumptions cannot be readily verified.

The fact that existing methods do not provide a direct estimation of the participation of the EMP pathway is of prime importance. Estimation of the EMP pathway by difference assumes that glucose is not engaged in any anabolic processes and that the PP pathway and the EMP pathway are the only catabolic routes. These assumptions can be questioned on the basis that it is known that glucose can be incorporated into such substances as starch or glycogen and that the GA pathway is operative in such biological systems as higher plants (16).

With plants the experimental approach of existing methods of pathway studies has primarily utilized plant tissue preparations. Findings from tissue preparations of plants may not reflect the catabolic behavior of intact plants. In those few studies utilizing intact plants (2, 16) it may be observed that substrate glucose was administered via vacuum infiltration in a single dose. The administration of substrate by this single dose method causes a transient increase of glucose in the plant. This method does not provide the steady state conditions necessary for meaningful kinetic information that can lead to a correct understanding of the catabolic fate of glucose in the plant.

Establishing the effect of the fluoride ion on glucose catabolism of intact plants is of much interest. Previous work in this regard has centered primarily on the use of plant tissue rather than the intact plant. There are reports indicating that fluoride is a potent inhibitor on several plant enzyme systems in vitro (10, 17). Early work on the effect of fluoride on plant tissue was carried out by Caldwell and Meiklejohn (7) when they observed a 90% decrease in

uptake of oxygen by isolated tomato plant tissue when placed in 0.033 M NaF solution. Subsequent work by James and his co-workers (19) found pyruvate formation in young barley leaves was markedly inhibited by 0.025 M NaF. James and Beevers (18) found a decrease in uptake of oxygen by Arum spadix at fluoride concentrations as low as 0.004 M. Bonner and Wildman (5) observed sodium fluoride inhibited respiration in spinach leaf tissue at a concentration of 0.0024 M. Bonner and Thimann (6) found marked inhibition of Avena coleoptile respiration by fluoride at concentrations above 0.004 M.

In work with extract from pea seeds, Hatch and Turner (12) reported the inhibition of glycolysis by fluoride ion. Laties (23) in his studies found the respiration of excised barley roots was inhibited by fluoride at 0.005 M. He also found that addition of pyruvate to the fluoride inhibited barley roots caused respiration to approach the normal rate indicating that the action of the fluoride was on the enzyme enolase. The plant Chenopodium murale was found by Miller (29) to be sensitive to fluoride concentrations as low as 0.4 ppb in air. He attributed the observed inhibition of plant tissue respiration to the fluoride ion, which he believed formed a magnesium-fluorophosphate complex that inactivated the enzyme enolase. Miller also believed the complex ion thus formed occupied the activating site on the enzyme that was normally occupied by the magnesium ion. This belief is in agreement with that of Warburg and Christian (45), who arrived at a similar conclusion in their work in which enolase was obtained as an extract of yeast. The presence of

the enzyme enolase in higher plants has since been established in work by Stumpf (37) and Tewfik and Stumpf (38).

Certain workers, however, have found no significant effect of fluoride on plant respiration or have found stimulation of respiration by fluoride. In work on the effect of fluoride on pinto beans grown in a hydrogen fluoride atmosphere McCune and co-workers (25) found that plant leaf discs containing up to 500 µg of fluoride per gram dry weight of leaf tissue did not exhibit any apparent effect in the evolution pattern of $^{14}CO_2$ when the leaf tissue was incubated in glucose-1-, -2-, -3(4)- or -6-^{14}C substrate solutions. In similar work on corn they found an increase in the evolution of $^{14}CO_2$ when plants accumulated up to 250 µg of fluoride per gram dry weight of tissue. McCune and co-workers (26) on another occasion reported decreased levels of pyruvate in tendergreen bean plants exposed to an atmosphere containing 1.7 to 7.6 $\mu g/m^3$ of fluoride. They also reported similar results with Milo maize plants. Presumably a decrease in pyruvate levels would lead to a decrease in the rate of decarboxylation of C-3 and C-4 of glucose as reflected by a reduced rate of CO_2 evolution. Work reported by Pack and Wilson (31) found no significant effect by fluoride on inhibition of enzymes that catalyze reactions of phosphorylated compounds. This included the phosphoglycerates in tendergreen bean seedlings which accumulated 275 ppm of the fluoride upon exposure of the intact plant to hydrogen fluoride in a phytotron.

Ross (32) and Ross, Wiebe and Miller (33, 34) in work with nearly mature plants of Polygonium orientale found that excised leaves when administered 0.005 M KF through the petioles for two days

exhibited a significant increase in O_2 uptake over the controls. Similarly, increased yields of $^{14}CO_2$ from glucose-1-^{14}C and glucose-6-^{14}C were observed in the fluoride treated plants when compared to the controls. In comparing the increase in yield of $^{14}CO_2$ for these two labels of glucose substrate Ross, Wiebe and Miller arrived at the conclusion that the PP pathway and glycolysis were stimulated with the PP pathway being stimulated to the greater extent. They concluded that even though glycolysis might be inhibited by fluoride, injury to the cells by the fluoride caused an increase in permeability of the cell to glucose, leading to a net increase in yield of $^{14}CO_2$ from the administered glucose-6-^{14}C. In work with young plants of Chenopodium murale they obtained $^{14}CO_2$ yield data allowing conclusions similar to those arrived at in their P. orientale work. They left unexplained, however, why P. orientale exhibited an increase in O_2 uptake and C. murale a decrease in O_2 uptake in fluoride injured plants.

The belief that fluoride injury may be the cause of increased respiration rate has also been suggested by Hill and co-workers (13) who found that the amount of fluoride in leaf tissue did not appear to be a significant factor in the respiration rate but rather was contingent on injury to some of the adjacent plant tissue. Applegate and co-workers (2) in working with three to five day old intact bush bean seedlings, vacuum infiltrated with sodium fluoride solution, found that at fluoride levels up to 10 ppm, oxygen uptake was accelerated whereas at concentrations greater than 100 ppm oxygen uptake was inhibited. The effect occurred over a relatively short period of

time; thus it was believed that tissue damage was not a contributing factor as has been suggested by Hill and Ross, Wiebe and Miller.

The effects of fluoride on plants may be divided into two kinds, chronic and acute. Studies concerned with the chronic effect of fluoride on plant tissue have typically utilized two general methods of providing fluoride to the plant. The fluoride has been provided over a period of several days either as hydrogen fluoride and introduced into the growth chamber of the plants (1, 24, 25, 26, 27, 46, 48) or added at very low fluoride levels as a soluble salt to the nutrient solution of the plant (24, 28, 39). Acute fluoride effect studies have been carried out by adding fluoride to the plant tissue culture medium (2, 5, 20, 33, 35, 40) and then observing the effect on respiration over a period of several hours. With the exception of Applegate and co-workers (2), who used intact bean seedlings to observe oxygen uptake in determining the respiration rate, all studies on the acute fluoride effect on plant respiration have utilized plant tissue sections.

In view of the fact that previous workers have not observed the effect of fluoride on the relative participation of concurrent catabolic pathways of glucose in intact plants it appeared to be desirable to make such a study. Additionally, the use of intact plants would provide a condition similar to the normal physiological environment of the plant, thereby allowing for the collection of more meaningful information.

It was of primary importance to the objectives of this study that a method be developed which would provide for the direct

estimation of three concurrent pathways of glucose catabolism. Such a method would overcome limitations of existing methods and provide a means by which work much broader in scope could be carried out on carbohydrate catabolism in higher plants.

In the present work glucose catabolism in mung bean seedlings was studied by means of the radiorespirometric method. The ^{14}C specifically labeled glucose substrates were administered to the intact plants by a novel continuous feeding method. This approach provided data on the rate of evolution of $^{14}CO_2$ from the mung bean seedlings utilizing labeled glucose under metabolic steady state conditions. The data were then used to calculate the individual catabolic rates of three concurrent pathways: the EMP pathway, the PP pathway, and the GA pathway.

II. MATERIALS

Plants

The mung bean (Phaseolus aureus) seedlings were grown from seed obtained through a Corvallis seed store from the Beal Seed Company of Ontario, Oregon.

Radiochemicals

Glucose-1-, -2-, -3-, -3(4)- and -6-^{14}C were purchased from New England Nuclear Corporation, Boston, Massachusetts. The toluene-^{14}C used as a primary standard in liquid scintillation counting was purchased from Packard Instrument Company, Downers Grove, Illinois.

Miscellaneous

Chemicals used for preparation of nutrient solutions, unlabeled substrate glucose solutions, and sodium fluoride solutions were reagent grade.

III. METHODS

Plants

The mung bean seeds were uniformly scattered on top of one inch of vermiculite in aluminum trays with a spacing of about 0.5 inches. The seeds were covered with approximately 0.3 inches of vermiculite and moistened with a sufficient amount of water to saturate the vermiculite. A second aluminum tray was then inverted over the planted flat and the covered flat left undisturbed four days in a greenhouse. The greenhouse temperature was regulated to 24 ± 4°C. At the end of four days the cover tray was removed and water added to saturate the vermiculite. On the sixth and eighth days post planting the vermiculite was saturated with a nutrient solution (14, p. 31) containing 0.14 g KH_2PO_4, 0.50 g KNO_3, 0.82 g $Ca(NO_3)_2$, 0.24 g $MgSO_4$, 0.0029 g H_3BO_3, 0.0018 g $MnCl_2 \cdot 4H_2O$, 0.00022 g $ZnSO_4 \cdot 7H_2O$, 0.00008 g $CuSO_4 \cdot 5H_2O$, 0.00009 g $H_2MoO_4 \cdot H_2O$, and 0.005 g $FeC_4H_4O_6$ per liter of solution. The vermiculite was saturated with water on all other days during the ten-day period. A sixteen hour daylight exposure for the plants was maintained by supplementing natural light with artificial light.

The cotyledons dropped off by the ninth day and the plants were then ready for experimentation. All plants used were ten days old and on the average had a stem length of 13 cm, a leaf area of 7 cm^2, and a plant weight of 0.4 g each. The plants for each experiment were visually selected on the basis of uniformity of leaf area and stem length.

The selected plants were gently pulled from the vermiculite and placed in sufficient water to cover the roots. A razor blade was used to sever the roots from the plant at a point just above the uppermost secondary root while the roots were immersed in water to prevent the introduction of air into the vascular system.

Four experiments were carried out simultaneously, which required the preparation of four sets of plants consisting of ten plants each. These plants were selected at random from the detached mung bean shoots; thus any effect due to time of cutting was randomized. The weight for ten plants was 3.2 ± 0.1 g for each experiment.

Substrate

The ^{14}C specifically labeled glucose substrate solutions were administered by the continuous feeding method. With this method ten detached mung bean seedlings were placed in 2 ml of substrate solution in a two dram shell vial. This arrangement allowed the plants to take up substrate solution of known composition at a uniform rate over an extended period of time.

The substrate glucose solutions at the prescribed chemical and radioactivity levels were prepared from the following stock solutions: glucose at a known chemical level, normally 1 mg/ml; ^{14}C specifically labeled glucose solution at a known chemical level (negligible in amount); and nutrient solution.

Experiments designed to study the effect of fluoride on glucose catabolism required the addition of substrate solution at a prescribed time during the experiment. The administered substrate solution

contained the same glucose and radioactivity levels as the original substrate solution, plus sodium fluoride at a concentration which upon dilution by the substrate solution already in the vial would provide the desired fluoride level. The additional substrate solution was administered to the plants through a polyethylene feed tube with a 1 ml disposable syringe. The feed tube was then purged of all administered substrate solution by rinsing with 0.2 ml of distilled water, followed by 100 ml of air which created a mixing action in the substrate vial.

Radiorespirometer

Plant Chamber. The plant chamber of the radiorespirometer (42, p. 326) is shown in Figure 1. The chamber was constructed to provide ready removal of the substrate vial and feed tube. The feed tube, which extended to the bottom of the vial, was equipped with an attached hypodermic needle (18 gauge x 1.5 inch) to permit administration of additional solution via a disposable syringe.

Air-flow Scheme. The schematic diagram for the air-flow pattern of the radiorespirometer is given in Figure 2. Respired $^{14}CO_2$ was swept from the plant chamber with a stream of air at a regulated flow rate of 500 ml per minute. From the outlet of the chamber the air passed through a drying column (#26668-007 Scientific Supplies Company, Portland, Oregon) containing eight mesh Drierite and then entered a one liter ion-chamber. From the ion-chamber the air flow passed through a flowmeter (#FM1044B Manostat Corp.) connected to a needle valve (Model B-4MA, Nuclear Products Company, Cleveland, Ohio)

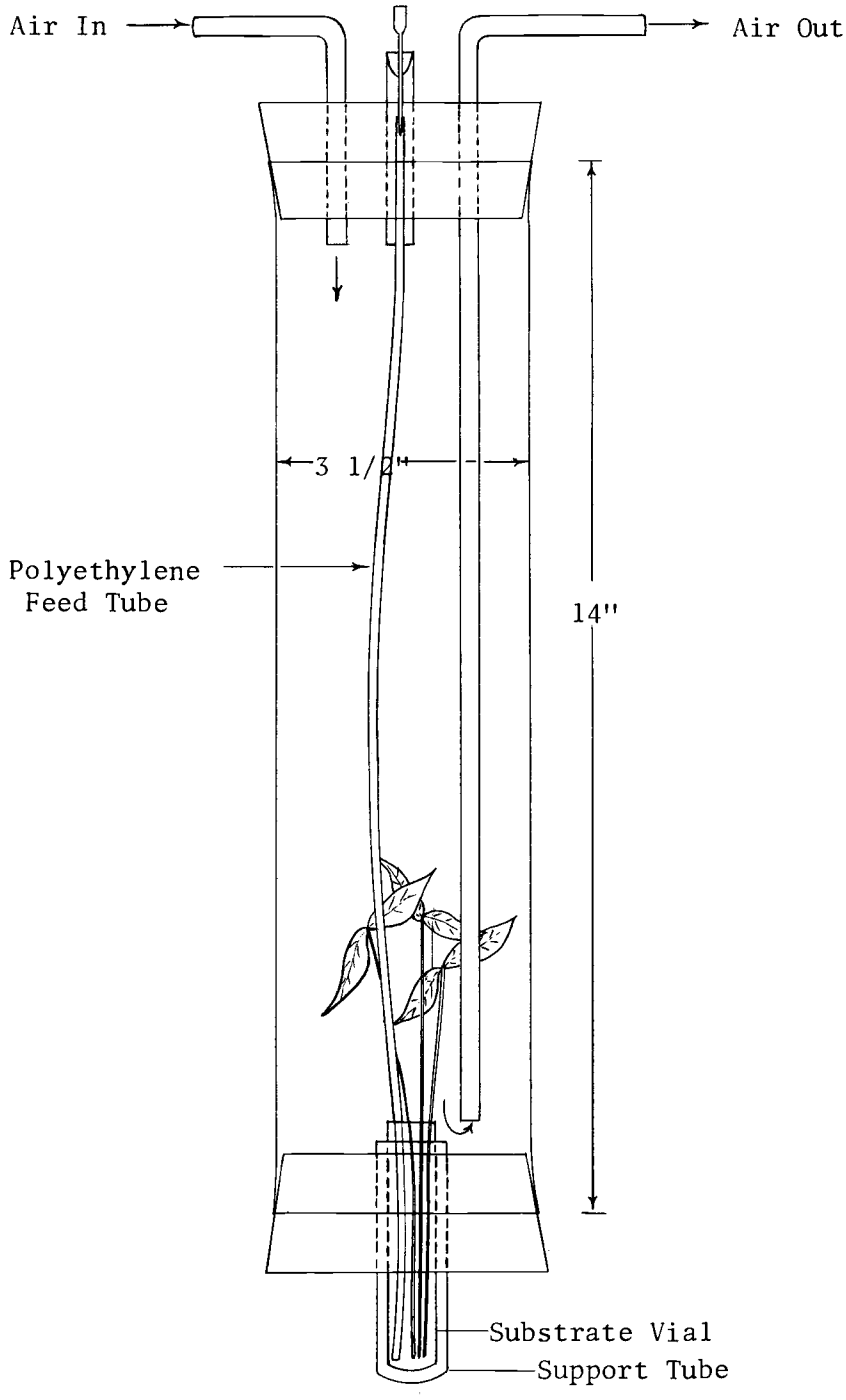

Figure 1. Plant chamber for radiorespirometric studies.

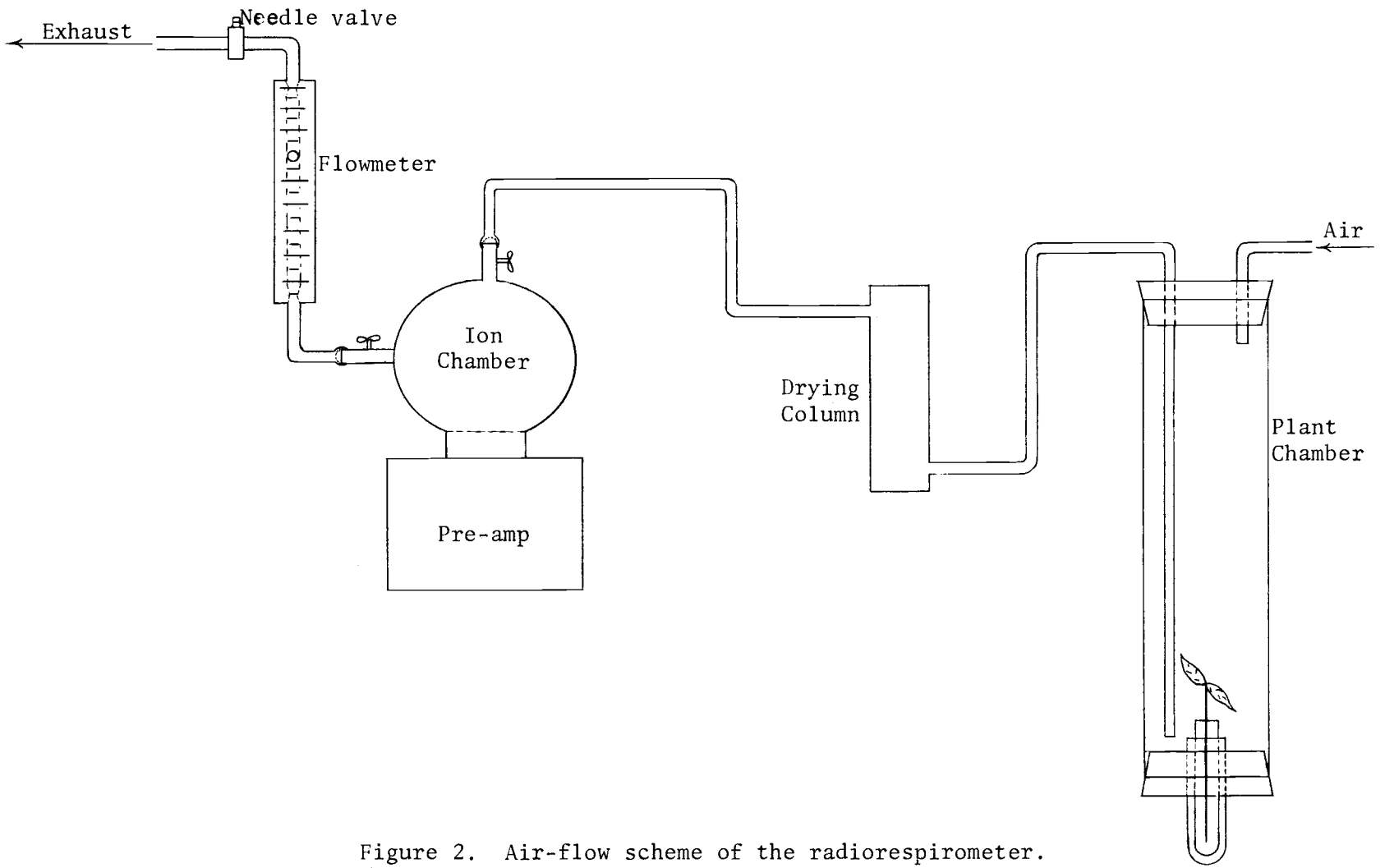

Figure 2. Air-flow scheme of the radiorespirometer.

for flow regulation. The air was then exhausted through a Gast pump (Model V-70572, Brentwood Company, Brentwood, Missouri) which maintained the necessary negative pressure that provided for the constant rate of air flow throughout the system.

Determination of Respiratory $^{14}CO_2$. The assembly of a four channel system is illustrated in the block diagram given in Figure 3. The flowing air containing the respiratory $^{14}CO_2$ passed into a one liter ion-chamber (Applied Physics Corporation, Monrovia, California). The ion-chamber was mounted on a base housing a preamplifier which was in turn connected to a vibrating reed electrometer (Cary Model 31, Applied Physics Corporation). The electrometer provided an output signal ranging from 0 to 30 V to the V-F converter (Dymec Model DY-2210-R, Hewlett-Packard Corporation, Palo Alto, California). The Dymec V-F converter in turn supplied the digital signal to a six-decade scaler (RIDL Model 49-43, Radiation Instrument and Development Laboratory, Melrose Park, Illinois) for recording. The scaler was also connected to a programmer (RIDL Model 52-44) which, coupled with an electronic timer (RIDL Model 5408), provided a pre-scheduled time interval for data print-out by means of a printer (Hewlett-Packard Model H44-562A). Operational settings for the system were the following: electrometer input voltage was set for 100 mv range; V-F converter input voltage was set at 10 volts full scale (corresponding to a frequency of 10,000 cycles per second full scale); ion-chamber polarizing potential was set at 90 volts; timer for data printout was set at 10-minute intervals for routine experimentation and 30-minute intervals for recording of background radiation.

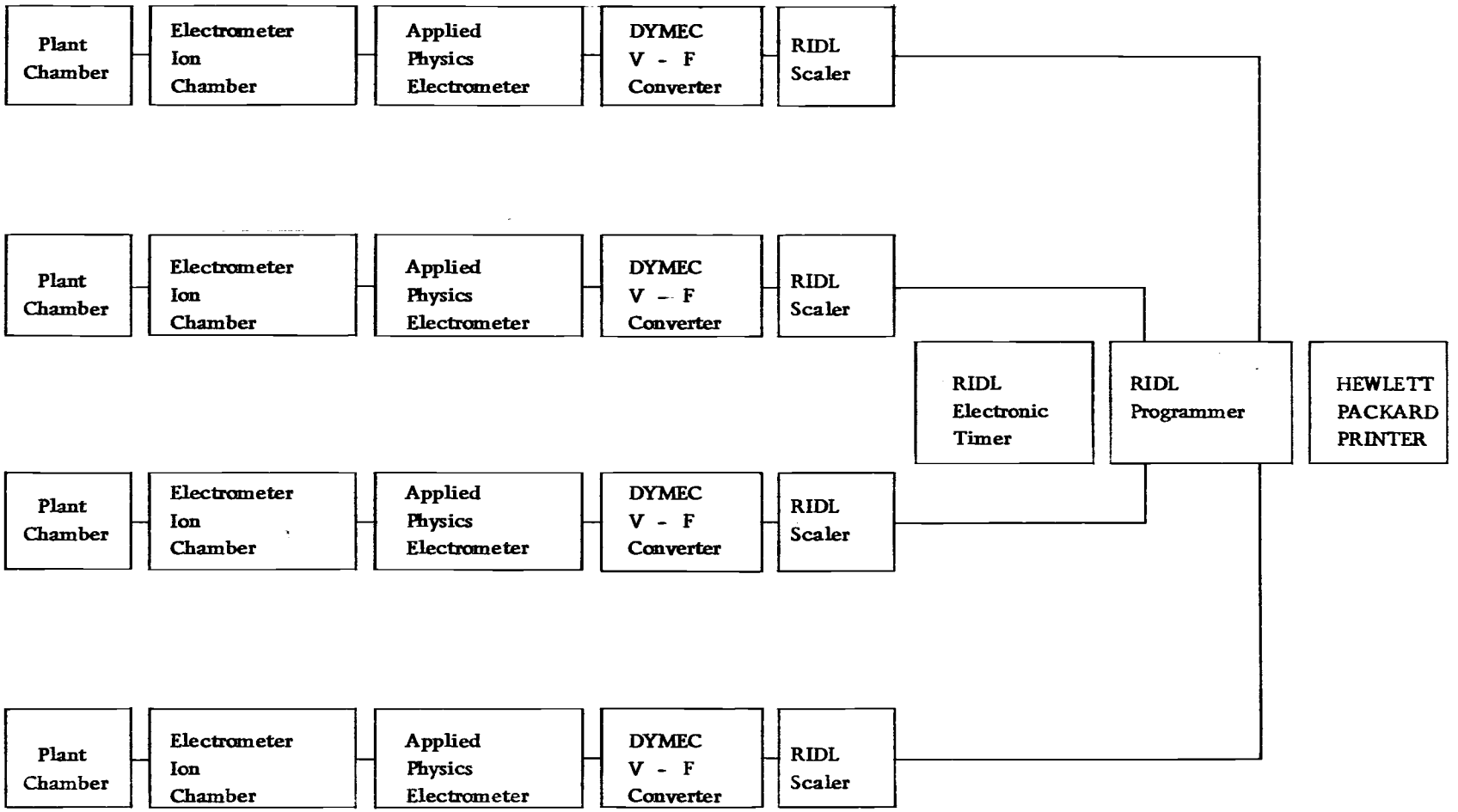

Figure 3. Block diagram of the radiorespirometer.

The beta radiation resulting from radioactive decay of ^{14}C in $^{14}CO_2$ produced ionization of the air in the ion-chamber. Electrons resulting from this ionization were collected at the anode producing a current proportional to the amount of $^{14}CO_2$ present in the chamber. This current was converted by the system into a digital readout on the printer. Thus the digital value was a quantitative expression of the amount of $^{14}CO_2$ passing through the ion-chamber during the pre-selected time interval.

Calibration. The radiorespirometer was calibrated by passing a stream of air containing a prescribed amount of $^{14}CO_2$ through the system from a storage tank. The air flow was maintained at a uniform rate of 500 ml per minute and exhausted through a solution of absolute ethanol-ethanolamine (1:1 volume ratio) to trap the $^{14}CO_2$ contained therein. An aliquot of the resulting ethanolamine carbonate solution was then counted by the liquid scintillation counting technique over a prescribed time interval. The ratio of the digital reading registered on the scaler to the amount of $^{14}CO_2$ passing through the ion-chamber, as determined by liquid scintillation counting, over a prescribed time interval provided the efficiency for the detection of $^{14}CO_2$ by the ion-chamber electrometer radiorespirometer system.

Substrate Calibration

The substrate was calibrated by taking 50 µl of the ^{14}C specifically labeled glucose substrate and quantitatively diluting it with distilled water to give an activity of about 3×10^4 disintegrations per minute per ml. A 0.1 ml aliquot of this diluted substrate

was then added to a counting vial which contained 5 ml of absolute ethanol-ethanolamine solution (1:1 volume ratio) and 10 ml of a toluene scintillation solution which was 0.3% p-terphenyl and 0.003% POPOP. The radioactivity of the preparation was then determined by means of a liquid scintillation counter (Packard Tricarb Model 314EX2). Counting efficiency of the counter was determined by the use of toluene-^{14}C as an internal standard (44, p. 131).

Transpiration Rate

The rate of uptake of substrate glucose solution by detached mung bean seedlings was determined under normal experimental conditions. In six duplicate experiments ten detached mung bean seedlings weighing 3.2 ± 0.1 g had an average uptake rate of 0.14 ± 0.02 ml per hour as determined over a ten-hour period.

IV. RESULTS AND DISCUSSION

Glucose catabolism in plants has been studied by many workers during the past two decades. The majority of these studies have been carried out using various types of tissue preparations. The radiotracer technique has revealed that the EMP pathway plays a predominant role in plant catabolism, although the PP pathway and the GA pathway are also known to be present.

No reliable method has existed, however, to estimate the concurrent operation of the three identified pathways in plants. Also, previous radiotracer experiments on glucose catabolism with plant tissue preparations have not permitted study of the catabolic behavior of plants under metabolic steady states.

With mung bean seedlings, Ikeda (16) has studied glucose catabolism making use of ^{14}C specifically labeled glucose substrate administered as one single dose. Whereas considerable information has been collected in his study, his findings on relative participation of glucose pathways remain to be verified.

In the present work methods were developed permitting administration of ^{14}C specifically labeled glucose substrates to detached mung bean seedlings by continuous feeding. This approach permitted collection of reliable information on the production of $^{14}CO_2$ from intact plants metabolizing ^{14}C specifically labeled glucose substrates under essentially a metabolic steady state. This approach also permitted study of the effect of external factors, such as fluoride ions, on glucose catabolism in plants. The above procedure coupled

with the use of the fast response ion-chamber electrometer system permitted continuous determination of the radioactivity of the respiratory $^{14}CO_2$ with precision, thereby providing good kinetic information on $^{14}CO_2$ formation.

Route of Substrate Administration

The objective of the present study was to gain kinetic information on the production of respiratory $^{14}CO_2$ from intact plants utilizing ^{14}C specifically labeled glucose. To this end the substrate glucose had to be administered to the plant at a rate comparable to the rate of glucose utilization. Since the uptake of glucose solution by roots or leaves of intact mung bean seedlings is a slow process, it appeared to be desirable to use detached mung bean seedlings, thus allowing administration of substrate in solution form by infiltration through the severed stem. Findings in a set of preliminary experiments revealed that when detached seedlings are immersed in 2 ml of substrate solution the rate of transpiration is constant at 0.14 ± 0.02 ml per hour. This transpiration rate made it possible to carry out experiments over a ten-hour period without replenishing the substrate solution.

Substrate Glucose Level

One of the most important facets in designing a radiotracer experiment on glucose catabolism in intact plants was the realization that the substrate level, in this case glucose level, had to be within an optimum range. An excessive amount of substrate administered in a

given time period might have overwhelmed the catabolic mechanism of the plant, thereby creating a physiologically undesirable environment. On the other hand, if the level of administered glucose was too low the findings based on production of respired $^{14}CO_2$ might have reflected a distorted catabolic picture. This is true because several catabolic mechanisms are known to operate in plant tissue, each having a set of defined catabolic rates. Inasmuch as these concurrent catabolic mechanisms are competing with each other for the glucose substrate, an insufficient level of substrate glucose may create an artificial preference for one or more of the catabolic mechanisms.

Consequently, a series of experiments was carried out directed at the determination of the optimum glucose substrate level for the present work. Consideration had to be given first to the level of endogenous hexose in mung bean seedlings. It was reasonable to believe that the hexose level in Phaseolus aureus is similar to that of Phaseolus vulgaris reported by Onslow (30, p. 52). It was also known that the roots of mung bean seedlings are enriched with glucose (4). It was inferred, therefore, that detached mung bean seedlings contain approximately 0.07% free glucose. Calculation then determined that 3.2 g of detached mung bean seedlings, as used in the present work for each of the experiments, contained approximately 2 mg of free glucose. The optimum level of administered substrate glucose in an experiment of five to ten hours duration needed to be compatible with the level of endogenous glucose.

The set of experiments designed to determine the optimum substrate level made use of glucose-3(4)-^{14}C as the test substrate.

This choice of substrate was reasonable since the EMP pathway is known to be the predominant route for glucose utilization (16), and C-3 and C-4 of glucose are promptly converted to CO_2 via the EMP pathway. The substrate levels observed ranged from 0.17 mg/ml to 3.0 mg/ml, equivalent to a net uptake ranging from 0.024 mg/hr to 0.42 mg/hr when the observed transpiration rate is 0.14 ml/hr.

From data shown in Figure 4 one can conclude that when the substrate level is 0.17 mg/ml a steady state with respect to $^{14}CO_2$ production cannot be realized throughout the duration of the experiment. At higher substrate levels the rate of evolution of $^{14}CO_2$ from mung bean seedlings utilizing glucose-^{14}C enters a plateau phase three hours after initiation of the respective experiments. Inasmuch as total radioactivity at each substrate level was constant, the areas under the respective rate curves reflect the relative yields of $^{14}CO_2$ from the administered substrate at given substrate levels. One can therefore conclude that when the level of substrate increases, proportionally less of the administered substrate has been engaged in catabolic functions. For this reason the substrate level at 0.33 mg/ml, equivalent to a net uptake of 0.046 mg/hr, is considered to be optimum. At this substrate level the rate of $^{14}CO_2$ production assumes a constant level three hours after substrate administration, indicating that the administered glucose is utilized by the plant under an approximate metabolic steady state. Moreover, it appears that at this substrate level the bulk of the administered glucose has been engaged in catabolic functions.

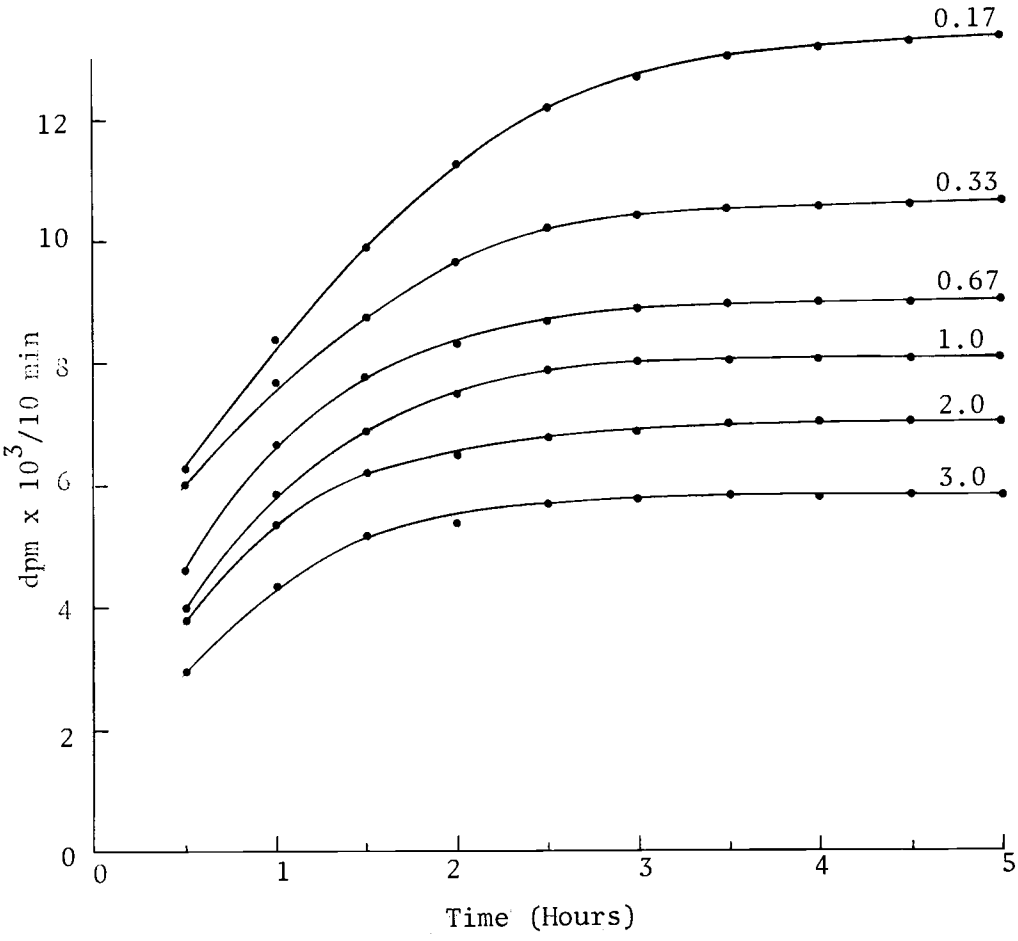

Figure 4. The radiorespirometric pattern of $^{14}CO_2$ production from mung bean seedlings metabolizing glucose-3(4)-^{14}C at various substrate levels. (Numerals refer to level of administered substrate in mg/ml.)

Glucose Catabolism in Normal Mung Bean Seedlings

Once the optimum glucose level was determined, a series of radiorespirometric experiments using various ^{14}C specifically labeled glucose substrates was carried out. The findings, representing the average of three experiments, are given in Figure 5. The rate for conversion of C-4 of glucose was calculated by difference from the rate of evolution of respiratory $^{14}CO_2$ observed in glucose-3-^{14}C and glucose-3(4)-^{14}C experiments. The deviation of the $^{14}CO_2$ data with each experiment was approximately 7%.

Much information on the catabolic behavior of mung bean seedlings can be determined from these data. The relatively more extensive conversion of C-3 and C-4 of glucose to respiratory CO_2 reflects the predominant role played by the EMP-pyruvate decarboxylation pathway in this plant. The fact that the rate of $^{14}CO_2$ production from C-4 of glucose is slightly greater than that from C-3 is indicative of the presence of other catabolic mechanisms for glucose utilization. For example, it is known that C-4 of glucose is preferentially converted to respiratory CO_2 in comparison to C-3 when glucose is catabolized via the PP pathway, giving rise to pentose phosphate and then re-formed fructose-6-P which in turn can be catabolized by either the PC pathway or the EMP pathway.

The rate for $^{14}CO_2$ production from C-1 of glucose was considerably greater than that from either C-2 or C-6, a fact indicative of the operation of the PP pathway for glucose catabolism. The rate for $^{14}CO_2$ production from C-1 of glucose assumed a mild ascending slope

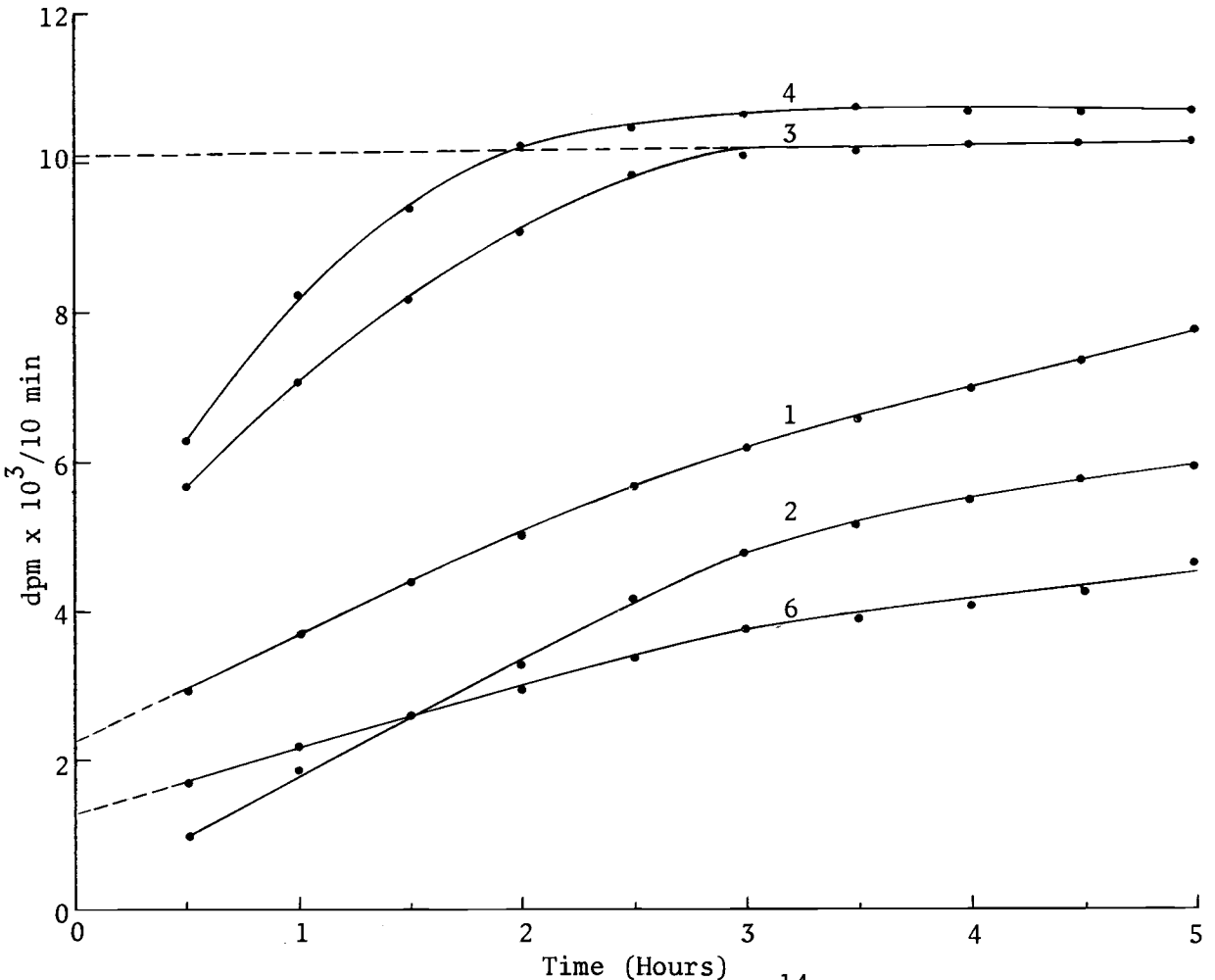

Figure 5. The radiorespirometric pattern of $^{14}CO_2$ production from mung bean seedlings metabolizing ^{14}C specifically labeled glucose substrates at the 0.33 mg/ml level. (Numerals refer to the labeled position of glucose.)

throughout the experiment. This is understandable since C-1 of glucose can be converted to respiratory CO_2 via the EMP-TCA pathway and the PP pathway with the latter a much more direct process. Hence, the initial rate of $^{14}CO_2$ production in the glucose-1-^{14}C experiment is believed to reflect directly the extent of the operation of the PP pathway.

Similar analyses can also be applied to the glucose-6-^{14}C experiments since C-6 of glucose can be converted to respiratory CO_2 either by the EMP-TCA route or the more prompt GA pathway. The initial rate for $^{14}CO_2$ production observed in the glucose-6-^{14}C experiment is therefore a direct index for the conversion of C-6 of glucose to CO_2 via the GA pathway.

With glucose-2-^{14}C as the test substrate the observed rate for $^{14}CO_2$ production appears to reflect primarily the EMP-TCA pathway. This is evidenced by the fact that the extrapolated CO_2 rate curve, observed in the glucose-2-^{14}C experiment, intersects with the origin. If the PC pathway or the GA pathway is involved in converting C-2 of glucose to respiratory CO_2, one would expect to find a steeper slope of the rate curve reflecting the more prompt processes.

From the findings in these radiorespirometric experiments one can conclude that qualitatively mung bean seedlings catabolize glucose predominantly via the EMP pathway, with the PP pathway and the GA pathway playing minor roles. The over-all catabolic behavior is summarized in Figure 6.

Use can be made of the radiorespirometric data given in Figure 5 to estimate the relative participation of each of the three

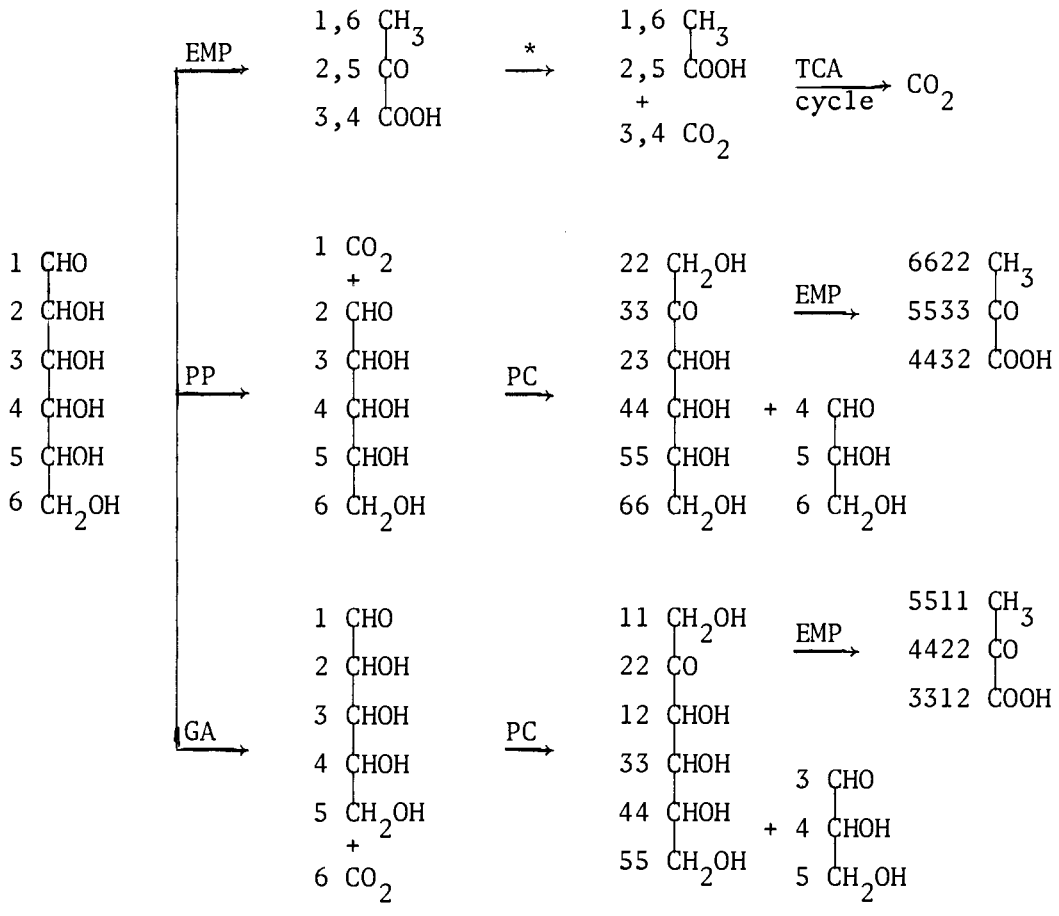

Figure 6. Behavior of catabolic pathways of glucose. Three moles of pentose are used to show the over-all reaction scheme.

* Pyruvate decarboxylation

recognized catabolic sequences. The method for pathway estimation designated as the "catabolic rate method" was devised with the following conceptual understandings:

(1) At the metabolic steady state, the constant rate of CO_2 production from C-3 of glucose reflects primarily the catabolic rate of the EMP-pyruvate decarboxylation pathway. This understanding is based on the assumption that pyruvate derived from glucose via the EMP pathway is promptly converted to acetyl CoA and respiratory CO_2 and the operation of other minor pathways contributes little to the production of CO_2 from C-3 of glucose. When the rate curve is extrapolated to the ordinate axis, the rate information should be that of the EMP-pyruvate decarboxylation pathway exclusively without interference of other complicating factors such as randomization of glucose labeling via the PP pathway.

(2) The rate of CO_2 production observed in the glucose-1-^{14}C experiment consists of essentially two components: the contribution of the EMP-TCA pathway, a slow process; and the contribution of the PP pathway, a prompt process. Consequently, if one extrapolates the initial slope of the curve representing CO_2 production from C-1 of glucose to the ordinate, the intersecting point represents exclusively the rate of conversion of C-1 of glucose via the PP pathway.

(3) The rate of $^{14}CO_2$ production observed in the glucose-6-^{14}C experiment consists of essentially two components: the contribution of the EMP-TCA pathway, a slow process; and the contribution of the GA pathway, a prompt process. Consequently if one extrapolates the initial slope of the curve representing CO_2 production from C-6 of glucose to the ordinate, the intersecting point represents exclusively the rate of conversion of C-6 of glucose via the GA pathway.

The extrapolated rates of CO_2 production, expressed in dpm/min, for C-1, C-3, and C-6 of glucose, can therefore be conveniently used to assess the relative participation of the three recognized catabolic pathways operating concurrently in mung bean seedlings. This is true since for each mole of glucose catabolized via the PP, the EMP, and the GA pathways one would expect to recover, respectively, one mole of respiratory CO_2 from C-1, C-3, and C-6 of glucose.

Let G_{rt} = rate of glucose metabolism, which is equal to the rate of glucose administration, expressed as µg/min.

G_{rp} = extrapolated rate of glucose catabolism via the PP pathway at the time of substrate administration, expressed as µg/min.

G_{re} = extrapolated rate of glucose catabolism via the EMP pathway at the time of substrate administration, expressed as µg/min.

G_{rn} = extrapolated rate of glucose catabolism via the GA pathway at the time of substrate administration, expressed as μg/min.

G_{ra} = rate of glucose anabolism via the anabolic pathways at the time of substrate administration, expressed as μg/min.

G_{r1} = extrapolated rate of $^{14}CO_2$ production from glucose-1-^{14}C at the time of substrate administration, expressed in dpm/min.

G_{r3} = extrapolated rate of $^{14}CO_2$ production from glucose-3-^{14}C at the time of substrate administration, expressed in dpm/min.

G_{r6} = extrapolated rate of $^{14}CO_2$ production from glucose-6-^{14}C at the time of substrate administration, expressed in dpm/min.

A = specific activity of the administered glucose-1-, -3-, and -6-^{14}C respectively, expressed as dpm/μg.

G_T = administered glucose that has engaged in metabolic processes, i.e. $G_T = 1$.

G_p = relative participation of the PP pathway in glucose metabolism, expressed as percent of the administered glucose.

G_e = relative participation of the EMP pathway in glucose metabolism, expressed as percent of the administered glucose.

G_n = relative participation of the GA pathway in glucose metabolism, expressed as percent of the administered glucose.

G_a = relative participation of anabolic pathways in glucose metabolism, expressed as percent of the administered glucose.

It follows that

$$G_{rt} = G_{rp} + G_{re} + G_{rn} + G_{ra} \quad (1)$$

and

$$G_T = G_p + G_e + G_n + G_a \quad (2)$$

The value of G_{rt} is calculated as 0.77 µg/min, since the glucose solution, having a concentration of 330 µg/ml, is taken up by the seedlings at a rate of 0.0023 ml/min. It was observed, in a separate experiment, that upon termination of glucose feeding the rate of production of respiratory $^{14}CO_2$ declined drastically within a few minutes. This fact indicates that the substrate glucose, once administered to the plant, is engaged immediately in metabolic functions.

The relationship between rate of $^{14}CO_2$ production and rate of glucose catabolism via a given pathway can be expressed as:

$$G_{rp} = \frac{G_{r1}}{A} \quad (3)$$

$$G_{re} = \frac{G_{r3}}{A} \quad (4)$$

$$G_{rn} = \frac{G_{r6}}{A} \quad (5)$$

Relative participation of individual metabolic pathways in the over-all glucose metabolism can therefore be expressed as:

$$G_p = \frac{G_{rp} \times 100}{G_{rt}} \tag{6}$$

$$G_e = \frac{G_{re} \times 100}{G_{rt}} \tag{7}$$

$$G_n = \frac{G_{rn} \times 100}{G_{rt}} \tag{8}$$

$$G_a = \frac{[G_{rt} - (G_{rp} + G_{re} + G_{rn})] \times 100}{G_{rt}} \tag{9}$$

Making use of the preceding equations, the relative participation of various pathways in the over-all metabolism of the administered glucose is tabulated in Table 1.

As observed from the data given in Table 1, about 47% of the administered glucose is engaged in anabolic processes. Presumably it is either incorporated into the polysaccharide fraction or enters the free glucose pool. Insofar as the catabolic mechanism is concerned the EMP pathway plays the predominant role, with the PP pathway and the GA pathway participating to a less significant extent. The findings are in accordance with those reported earlier by Ikeda (16), whose results were obtained in radiotracer experiments based on cumulative yield which utilized the administration of substrate glucose by the single dose method.

Table 1. Estimation of relative participation of glucose pathways in mung bean seedlings.

Experimental Findings		Relative Participation of Pathways	
Glucose uptake rate G_{rt} (μg/min)	0.771	Metabolic Pathways of glucose	Per cent of metabolized glucose
Sp. act. of glucose A (dpm/μg)	33.8×10^2	Total metabolism $G_T = G_p + G_e + G_n + G_a$	100
Rate of CO_2 production for C-1: $^2G_{r1}$ (dpm/min)	2.3×10^2	Catabolic pathways	
for C-3: G_{r3} (dpm/min)	10.1×10^2	Participation of the:	
for C-6: G_{r6} (dpm/min)	1.3×10^2	PP pathway $G_p = \frac{G_{rp} \times 100}{G_{rt}}$	9
Rate of catabolic pathways			
PP pathway $G_{rp} = G_{r1}/A$ (μg/min)	0.068	EMP pathway $G_e = \frac{G_{re} \times 100}{G_{rt}}$	39
EMP pathway $G_{re} = G_{r3}/A$ (μg/min)	0.298	GA pathway $G_n = \frac{G_{rn} \times 100}{G_{rt}}$	5
GA pathway $G_{rn} = G_{r6}/A$ (μg/min)	0.038		
		Anabolic pathways $G_a = \frac{[G_{rt} - (G_{rp} + G_{re} + G_{rn})][100]}{G_{rt}}$	47

Glucose Catabolism in Mung Bean Seedlings Administered with Fluoride

Once the catabolic behavior of glucose pathways functioning in normal mung bean seedlings was obtained the way was paved to undertake the study of the effect of fluoride ions on glucose catabolism in these plants. Efforts in the present work focused on the study of the acute effect when a solution containing fluoride ions was applied to the plants by infiltration via the stems. It was believed that any such acute fluoride effect on glucose catabolism in mung bean seedlings might provide a clue to the cause of the chronic effect observed by many other workers.

An important parameter in designing an experiment aimed at the above stated objective was to determine the necessary concentration of fluoride solutions that would bring out immediate change, if any, in the catabolic behaviors of the plant. Previous studies with plant tissue preparations carried out by numerous workers reported that plant respiration can be readily inhibited at fluoride concentrations ranging from 10^{-3} to 10^{-1} M in culture media (2, 5, 7, 19, 20, 23, 32). On this basis a series of experiments was carried out in which the rate of $^{14}CO_2$ production from mung bean seedlings utilizing glucose-3(4)-^{14}C in the presence of the fluoride ion at various concentrations was observed. Glucose-3(4)-^{14}C was chosen as the test substrate because the two labeled carbon atoms are known to be converted to CO_2 promptly and extensively via the EMP pathway, the predominant respiratory pathway functioning in mung bean seedlings. Any effect of fluoride on respiratory mechanisms in mung bean

seedlings would be reflected in the observed yield of $^{14}CO_2$ from these two labeled carbon atoms of glucose. Experimental procedures in this fluoride work were essentially the same as those previously described. The seedlings were fed with glucose solution during the first three hours of the experiment at which time additional glucose solution containing a prescribed amount of sodium fluoride was introduced into the substrate vial and the radiorespirometric experiment was allowed to proceed for an additional five hours.

In order to assess whether there was any effect on glucose catabolism due to the presence of a non-toxic salt in the substrate solution, separate experiments were carried out in which substrate glucose solution containing sodium chloride at concentrations as high as 0.1 M were administered to the seedlings.

The findings in this series of experiments are given in Figure 7. The data given for the control experiment reveal that the administration of additional substrate solutions to that already in the substrate vial results in a slight reduction, with subsequent recovery, in the rate of $^{14}CO_2$ production from labeled substrate. In the case of the sodium chloride experiment the presence of the salt in the substrate solution creates a small effect on glucose catabolism as indicated by the slightly reduced rate of $^{14}CO_2$ production from ^{14}C specifically labeled glucose substrate.

The radiorespirometric data obtained in a series of glucose-3(4)-^{14}C experiments utilizing the addition of sodium fluoride into the substrate solution at various concentrations are given in Figure 8. It may be seen that at fluoride concentrations of

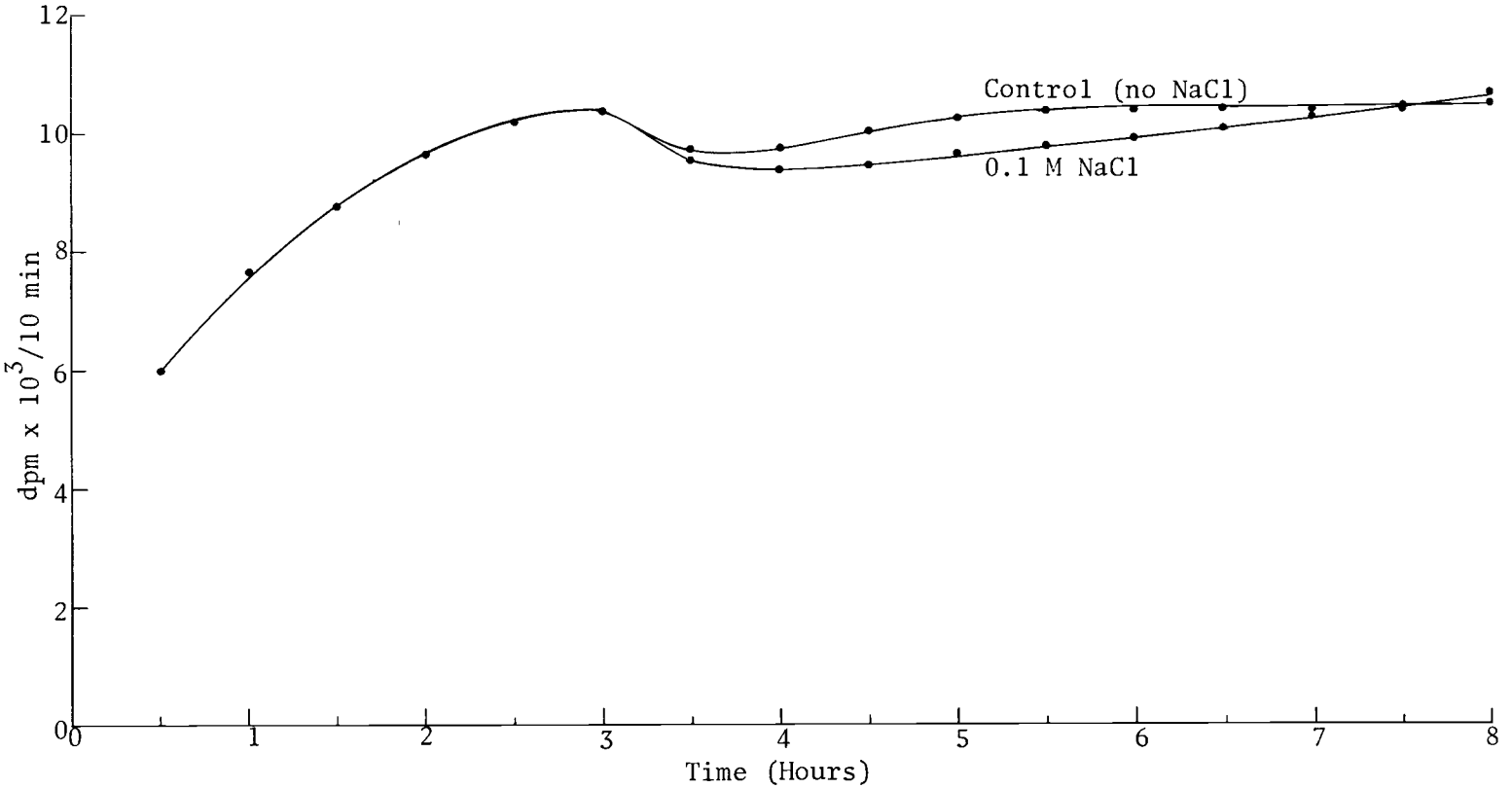

Figure 7. The radiorespirometric pattern of $^{14}CO_2$ production from mung bean seedlings metabolizing glucose-3(4)-^{14}C at the 0.33 mg/ml level in the presence of 0.1 M NaCl as compared to control.

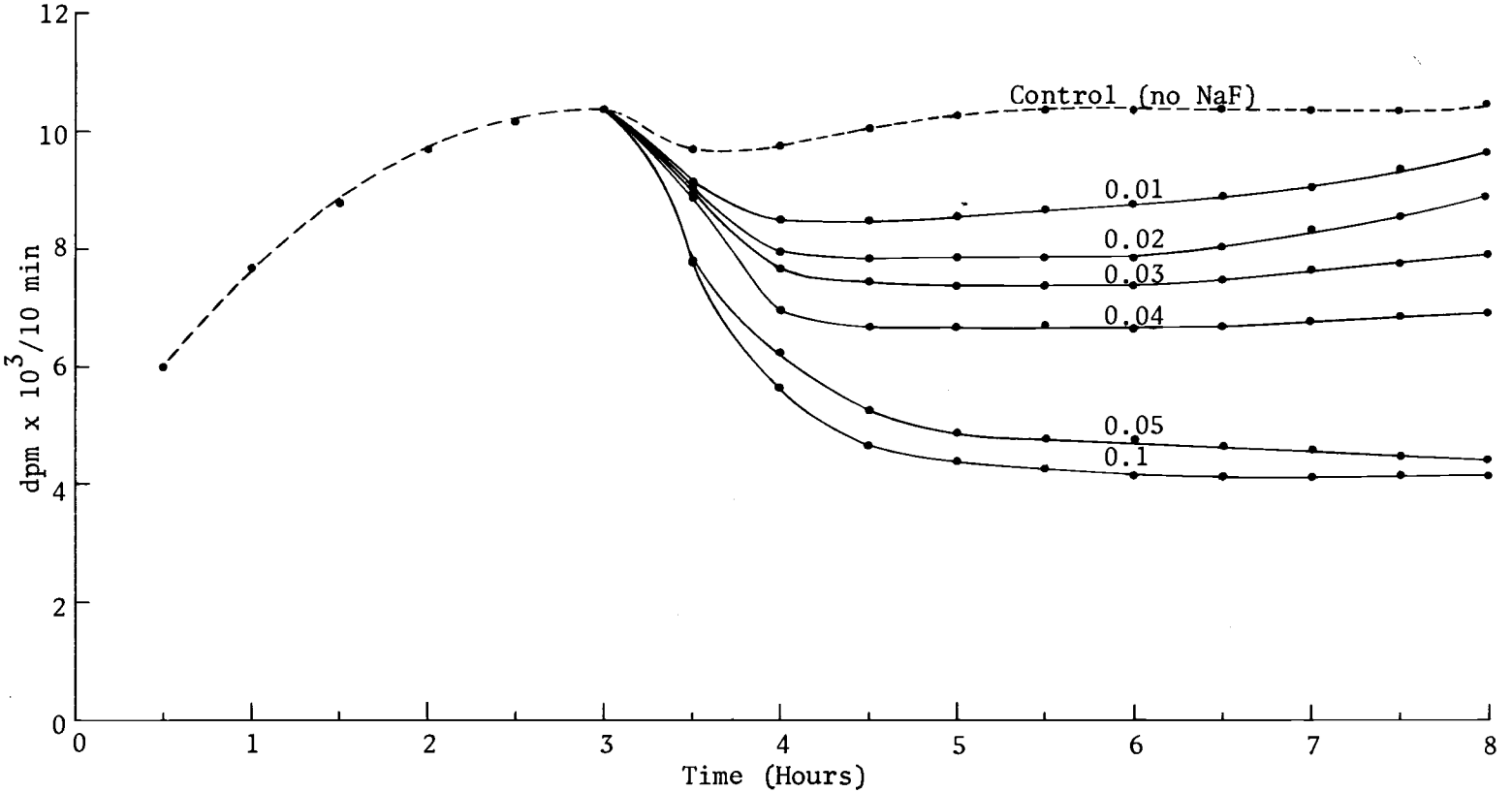

Figure 8. The radiorespirometric patterns of $^{14}CO_2$ production from mung bean seedlings metabolizing glucose-3(4)-^{14}C at the 0.33 mg/ml level in the presence of NaF. (Numerals refer to molarity of substrate in NaF.)

0.01 M to 0.03 M in the substrate solution a significant effect on glucose catabolism is reflected by the noticeable reduction in rate of $^{14}CO_2$ production from mung bean seedlings utilizing glucose-3(4)-^{14}C. The effect appears to be temporary in nature, particularly in the experiment with a fluoride concentration of 0.01 M in the substrate solution. The magnitude of the effect appears to be proportional to the fluoride concentration. When the fluoride concentration in the substrate solution is increased to 0.04 M a sustaining effect is observed. At concentrations above 0.04 M, such as 0.05 M and 1.0 M, the effect is much more severe and may reflect extreme injury to the respiratory system of the plant. In view of these findings it appears to be desirable to examine the effect of fluoride on rates of conversion of individual carbon atoms of glucose substrate to CO_2 at a fluoride concentration in the substrate solution of 0.04 M.

The radiorespirometric data obtained in a series of experiments designed to study the effect of fluoride on catabolic pathways of glucose in mung bean seedlings at substrate solution concentrations of 0.04 M sodium fluoride are given in Figure 9. It may be noted that in these experiments the effect of fluoride was definitely observable within thirty minutes after administration of fluoride to the substrate solution. After this elapsed time period of thirty minutes 3.2 g of plants had taken up approximately 0.05 mg of fluoride ion (approximately 20 ppm based on a wet weight of 3.2 g for 10 mung bean seedlings). The drastic reduction in rates of conversion of C-3 or C-4 of glucose to CO_2 by fluoride ions points to an inhibitory effect on the EMP-pyruvate decarboxylation sequence. Previously

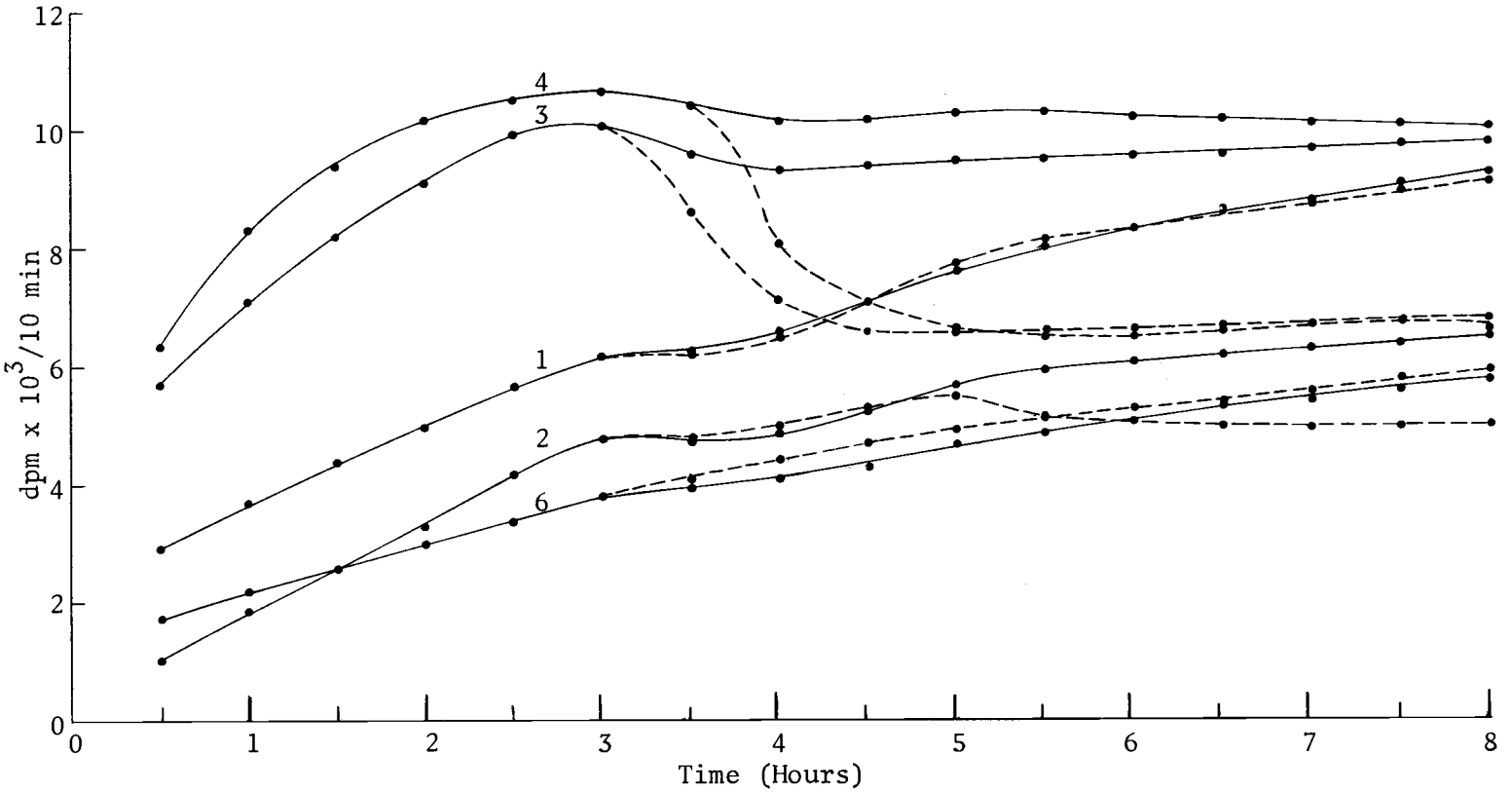

Figure 9. The radiorespirometric patterns of $^{14}CO_2$ production from mung bean seedlings metabolizing ^{14}C specifically labeled glucose substrates at the 0.33 mg/ml level in the presence of 0.04 M NaF. (Numerals refer to the labeled position of glucose. Solid lines represent control for respective fluoride curves which are the broken lines.)

Warburg and Christian (45) have observed inhibition by fluoride ion on the enzymic reaction catalyzed by enolase. Subsequently Bonner and Wildman (5) were able to restore oxygen consumption to fluoride inhibited respiration of spinach leaves with the addition of pyruvate but not with the addition of glucose. This observation led them to believe that the inhibition involved the enzyme enolase. Investigations by Miller (29) on properties of enolase in extracts from pea seeds found fluoride inhibition of the enzymic activity of enolase. However, the findings given in the present work represent, for the first time, fluoride inhibition on the EMP-pyruvate decarboxylation pathway in intact plants.

The observed inhibitory effect of the fluoride ion on the EMP-pyruvate decarboxylation pathway implies that the amount of pyruvate that can be converted to acetyl CoA is proportionally reduced. It is therefore not surprising to find that the rate of conversion of C-2 of glucose to CO_2 is significantly reduced in plants to which fluoride solution has been administered. This is true since, as stated previously, C-2 of glucose is converted to respiratory CO_2 primarily via the EMP-TCA pathway.

Of even greater interest is the finding in the glucose-1-^{14}C and glucose-6-^{14}C experiments. The rate of conversion to CO_2 of either the C-1 or the C-6 carbon atoms is not affected by the presence of fluoride ion to any noticeable extent. It is recognized that C-1 of glucose is converted to CO_2 by the concurrent operation of the EMP-TCA pathway and the PP pathway. The demonstrated inhibition on the EMP-pyruvate decarboxylation pathway would reduce the availability

of acetyl CoA, the entry intermediate to the TCA pathway, and should be reflected in a proportional reduction in the rate of conversion of C-1 of glucose to CO_2, provided the participation of the PP pathway remains at a constant level. The observation that the rate of conversion of C-1 of glucose to CO_2 is not noticeably affected by fluoride provides, consequently, an indication that the PP pathway is participating to a greater extent in the over-all catabolism of glucose when the fluoride ion is present.

Similarly it is recognized that C-6 of glucose is converted to CO_2 by the concurrent operation of the EMP-TCA pathway and the GA pathway. The demonstrated inhibition of the EMP-pyruvate decarboxylation pathway should also result in a reduction of the rate of conversion of C-6 of glucose to CO_2, provided the participation of the GA pathway remains at a constant level. Therefore the observation that the rate of conversion of C-6 of glucose to CO_2 is not affected by fluoride indicates that the GA pathway is participating to a greater extent in the over-all catabolism of glucose in the presence of the fluoride ion.

Previously Ross (32) and Ross, Wiebe and Miller (33) have studied the effect of fluoride on glucose catabolism in plant samples using glucose-1-, -2-, and -6-^{14}C as tracing substrate. They observed that the yields of $^{14}CO_2$ from glucose-1-^{14}C and glucose-6-^{14}C were considerably higher with fluoride treated plant samples and concluded that glycolysis was stimulated. By examining the ratio of CO_2 derived from C-6 of glucose with CO_2 derived from C-1 of glucose, these authors stated that glycolysis may have been inhibited despite the

apparent stimulatory action of fluoride. They attributed this apparent stimulatory effect to an increased permeability which results in an increased penetration of substrate glucose into the cells of injured tissue. They further stated that the decreased ratios may also indicate that the PP pathway is somewhat stimulated. It should be noted, though, that glucose-3-^{14}C and glucose-3(4)-^{14}C were not used as test substrates by these authors to permit them to draw a more defined conclusion. Of more importance is the fact that these authors did not recognize the role that can be played by the GA pathway.

It is believed that results presented in this work provide much more insight regarding the effect of fluoride on glucose catabolism in plants. Data obtained in this work indicate without any doubt that the inhibitory effect of fluoride on glycolysis is prompt and severe. In addition, findings in this work clearly demonstrate the stimulatory effect of fluoride on the PP pathway and for the first time establish the stimulatory effect of fluoride on the GA pathway. Further work is needed to elucidate the mechanism underlying the observed effect.

V. SUMMARY

A new method for the estimation of relative pathway participation in the catabolism of glucose in intact plants has been developed. This method utilized the radiorespirometer to provide the necessary kinetic data on the formation of respiratory $^{14}CO_2$ from ^{14}C specifically labeled glucose substrate which allowed the estimation of the catabolic rate of the pentose phosphate (PP) pathway, the Embden-Meyerhof-Parnas (EMP) pathway, and the glucuronic acid (GA) pathway in mung bean (Phaseolus aureus) seedlings. It was found that the relative catabolic participation of each of these glucose pathways was 17% for the PP pathway, 74% for the EMP pathway and 9% for the GA pathway.

The basic information gained on the catabolic behavior of mung bean seedlings was used to study the effect of fluoride on individual pathways. A noticeable effect on the participation of the EMP-pyruvate decarboxylation pathway was observed at fluoride ion concentrations in plants as low as 5 ppm. Data analysis revealed that at the fluoride ion concentration in plants of 20 ppm the EMP-pyruvate decarboxylation pathway was severely inhibited. Of more interest was the finding that in the presence of substrate solution which was 0.04 M in fluoride, the PP pathway and the GA pathway were playing a more important role in over-all glucose catabolism in mung bean seedlings. Evidently when the predominant pathway in mung bean seedlings was inhibited alternate routes were called into play to fulfill the need of respiratory functions. Such a phenomenon in plants is reported here for the first time.

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