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Title: THE ESTIMATION OF GLUCOSE PATHWAYS IN SACCHAROMYCES CEREVISIAE BY THE CATABOLIC RATE METHOD

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Existing methods for the estimation of relative participation of glucose catabolic pathways make use of yield data of respiratory $^{14}CO_2$ or intermediary ^{14}C products derived from glucose substrates specifically labeled with ^{14}C. Cumulative yields of respiratory $^{14}CO_2$ observed at the end of time course of complete substrate utilization are analyzed to provide direct information on the participation of the pentose phosphate pathway only. The other glucose catabolic pathways such as glycolysis and the Entner-Doudoroff pathway are obtained by difference.

In the present work a novel method for pathway estimation has been developed which uses data of the catabolic rate of individual glucose pathways. This method relies on newly designed and constructed apparatus which employs an ion-chamber-electrometer assembly to determine the amount of respiratory $^{14}CO_2$ produced by

biological systems metabolizing substrates labeled with ^{14}C. Kinetic information on the production of respiratory $^{14}CO_2$ is obtained with excellent resolution. Hence, the new method has been designated as the catabolic rate method based on high resolution radiorespirometry.

With the high resolution system, the catabolic rate, derived by following the $^{14}CO_2$ yield data over short time intervals (one or two minutes), of not only the PP pathway, but also the glycolytic pathway can be determined directly. Information on the total rate of glucose catabolism permits one to estimate the participation of anabolic pathways if the overall rate of glucose assimilation is known. Thus, insight on the total metabolism of glucose in biological systems is provided by the new method.

Saccharomyces cerevisiae has been employed as the test organism in the development of the catabolic rate method by high resolution radiorespirometry. Since the catabolic rate method uses data collected over a short period of time, it is possible to examine the metabolic mechanisms of glucose metabolism in yeast cells at different growth phases. Such information is of great importance in the understanding of developmental physiology in microorganisms and possibly other biological systems.

The individual catabolic and anabolic rates of glucose metabolism in Saccharomyces cerevisiae have been assessed as a function of growth physiology with the new method. The catabolic rate of the

pentose phosphate pathway decreases as cells develop from middle logarithmic to early stationary phase (0.27 μmole of glucose per minute per 10^9 middle log cells compared to 0.17 μmole of glucose per minute per 10^9 early stationary cells). Since the glucose assimilation rate also decreases during the same growth period, the relative participation of pentose phosphate pathway in overall glucose metabolism remains essentially constant. However, the glycolysis-Krebs cycle pathway assumes a more dominant role in glucose metabolism in yeast growth physiology through the middle log to the early stationary phase. The role played by anabolic pathways resembles the case of the pentose phosphate pathway, i.e., decreasing as growth continues in the cell population.

It appears that the increased resolving power of high resolution radiorespirometry can be successfully employed in the study of other physiological phenomena such as substrate transport and oscillatory mechanisms occurring in metabolic sequences.

The Estimation of Glucose Pathways in Saccharomyces cerevisiae by the Catabolic Rate Method

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

THE ESTIMATION OF GLUCOSE PATHWAYS IN SACCHAROMYCES CEREVISIAE BY THE CATABOLIC RATE METHOD

I. INTRODUCTION

The estimation of the relative participation of concurrent glucose pathways in living organisms has been intensely studied in several laboratories over the past 15 years. The methodological approaches in these studies have been reviewed by several authors (7, 14, 15, 29, 30, 37, 40, 41). Hence, only a few brief statements are herewith given to summarize previous findings.

Early radiotracer methods used to elucidate the mechanisms of carbohydrate catabolism can be classified into two main categories:

(1) Those radiotracer methods involving the determination of the relative specific activity of respiratory $^{14}CO_2$ derived from carbohydrates specifically labeled with ^{14}C (1, 3).

(2) Those methods which compare the specific activity of key metabolic intermediates derived from substrates specifically labeled with ^{14}C (4).

The major disadvantage of methods based upon the information of specific activity is the complication resulting from the uncertainty of the extent of endogenous substrate dilution. Estimating the extent of endogenous dilution in labeled substrate is by no means an easy task.

Katz and Wood (16, 17, 41) have presented extensive theoretical analyses of the estimation of glucose pathways making use of specific activity data of intermediates derived from specifically-labeled ^{14}C glucose substrates. These authors were interested in the net contribution of the "pentose cycle pathway" (PC) to overall glucose metabolism. Their equations rely on several key assumptions, one of which is the rapid and complete isotopic equilibration of glucose-6-phosphate and fructose-6-phosphate through hexose-phosphate isomerase. Another assumption is the rapid and complete isotopic equilibration of triose phosphates through triose-phosphate isomerase. Such assumptions have been verified to a certain extent in the case of hexose-phosphate isomerase (19), however, several recent reports indicate that triose-phosphate isomerization is not a rapid process (15). In contrast to Katz and Wood, whose calculations of PC pathway recycling are based on the net conversion of glucose to end products (41), Dawes and Holms have calculated the contribution of the PC pathway on the basis of hexose-phosphate turnover (10, 11).

The inherent problems of using specific activity data for pathway studies were overcome to a large extent with the development of another method. The new method, generally referred to as the "$^{14}CO_2$ yield method," was first used by Bloom and Stetten (3) in 1953 to measure the relative amount of glucose metabolized by the pentose phosphate and glycolytic pathways. However, the reliability of their

findings is open to question since their yield data pertaining to respiratory $^{14}CO_2$ were collected before substrate had been completely utilized, i.e., incomplete time course of substrate utilization and hence, were subject to differences in rates of individual catabolic sequences.

The methodology based upon the relative radiochemical yield of respiratory $^{14}CO_2$ derived from ^{14}C specifically labeled carbohydrate substrates was extensively developed by Wang and co-workers in this laboratory (30, 31, 34, 35, 37) and has been designated as the radiorespirometric method. The salient feature of this method is the concept that in making use of $^{14}CO_2$ yield data, it is mandatory to collect the cumulative yield data at the end of the time course of complete substrate utilization. This is true since at the end of the time course any complications derived from differences in the rates of individual glucose pathways would have been self compensated. It should also be noted that pathway estimations by the yield method are not affected by the dilution of labeled substrate with endogenous intermediates. It should be further pointed out that the term "pentose phosphate pathway" (PP) used by Wang and co-workers refers to only the initial fate of glucose-6-phosphate, derived from the substrate glucose, as it is metabolized by glucose-6-phosphate dehydrogenase and 6-phosphoglucoate dehydrogenase, the first two enzymes of the pathway.

More recently, Katz, Wood and co-workers (15, 16) have

employed the concept of specific yield for pathway estimation studies. This method relies on ^{14}C yield information of CO_2 and key intermediates expressed as a fraction of utilized substrate. Landau and Katz (18) have applied their theoretical models to experimental studies on rat adipose tissue. They estimated that up to 15 percent of glucose metabolism proceeded via the PC pathway but an estimation of the glycolytic pathway could not be obtained since isotopic equilibration between triose-phosphates was not realized. It should be again emphasized that the pathway designated as the PC pathway by Katz and Wood is the entire fraction of substrate glucose catabolized via the pentose phosphate pathway initially, and, subsequently, the catabolism of the reformed hexose-phosphate via the pentose cycle pathway.

The yield methods of Wang and co-workers (34) and Katz, Wood and co-workers (41) have provided useful information on the relative participation of concurrent glucose pathways in biological systems. However, the usefulness and reliability of the yield methods are limited by several serious complications:

(1) In the case of microorganism studies by means of the radiorespirometric method, it is unlikely that optimal physiological conditions have been maintained during the estimation of glucose pathways. First, cell harvesting and washing procedures followed by resuspension of cells in a fresh medium

perturbs the physiological state of the organism. Second, the single dose treatment of substrate administration forces the organism to adjust rapidly from a substrate-free condition to one in which substrate is relatively plentiful. Moreover, the substrate concentration must be limited if one wishes to have a complete time course of substrate utilization within a few hours. Even during this brief period, the cells may have developed through different growth phases with the possibility of altered pathway participation. Thus, the regulatory mechanisms operative within the organism as it goes through these abrupt environmental changes preclude the study of cells in an unperturbed state.

(2) Aside from the physiological considerations, it would be interesting to study the growth dynamics of cell populations with respect to glucose pathway participation. However, existing methods are technically limited in obtaining rapid kinetic information on pathways of glucose participation and their enzyme activities. Yield methods are unable to distinguish differences in the relative participation of pathways in a given growth phase since the methodology requires a complete time course of substrate utilization, a process generally lasting several hours in a conventional study.

(3) The yield method provides information on the

participation of the PP or PC pathway by direct measurement (34, 41). However, the relative participation of the other concurrent pathways such as glycolysis (34) or Entner-Doudoroff (E-D) (37) pathway is obtained by difference under the assumption that there are only two major catabolic pathways in the biological system.

Considering the limitations of the existing methods for pathway estimations, it is useful to set down the features desirable in a better method, and concentrate particularly on the estimation of glucose pathways in unperturbed microbial systems:

(1) Unperturbed culture conditions for microorganisms are of fundamental importance. This requirement can be accomplished by eliminating the cell harvesting and washing procedures prior to experimentation. In other words, the physiological state of cells during experimentation on pathway estimations should be as close as possible to the actual growth conditions of the microorganism.

(2) In view of the unique advantage inherent in radiotracer methodology, it should be possible to introduce labeled substrate, high in specific activity but low in chemical level relative to substrate concentration, to the culture medium while cells are maintained under unperturbed growth conditions. This would obviate abrupt environmental changes in chemical levels

associated with single dose substrate administration.

(3) In order to assess the relative participation of glucose pathways in cells undergoing growth phase changes, it is necessary to estimate respiratory $^{14}CO_2$ produced over short time intervals, i.e., minutes or seconds. A suitable method could not rely on $^{14}CO_2$ yield data obtained from complete time course of substrate utilization as required in the existing radiorespirometric method. Therefore, the new method should employ a radiorespirometer with greatly improved resolving power for kinetic $^{14}CO_2$ production data.

The primary objective of the present work is, therefore, to develop a new method for the estimation of glucose pathways. It is not only important that the new method overcomes the limitations inherent in the existing methods described above, but also that it provides a much broader scope in application to studies on carbohydrate metabolism, developmental biology and allied areas.

Saccharomyces cerevisiae was selected as the test organism for this study. The early studies of Beevers and Gibbs (1), Blumenthal, Lewis and Weinhouse (4), Chen (8, 9), as well as early studies in this laboratory (32, 33, 36) established that the overall pattern of glucose metabolism in yeast resembled that of mammalian tissue metabolism. Thus, the glycolysis-Krebs cycle pathway is the predominant catabolic sequence for glucose utilization, while the PP

pathway participates to a minor extent (30). More recent studies by Polakis, Bartley and co-workers (22, 23, 24) have followed the changes in the intracellular enzyme activities and gross cell composition as related to various environmental conditions such as carbon source, aerobiosis and anaerobiosis. Other current areas of interest in yeast biochemistry include the biosynthesis of mitochondria (20), oscillatory phenomena (6, 12, 21, 25), and membrane transport (26). While these problems are not the major concern of this work, they are intimately related to the regulatory mechanisms of glucose metabolism in cell development, the area of primary emphasis in this work.

II. MATERIALS

Yeast. The strain of Saccharomyces cerevisiae used in this study was isolated from Fleischmann's bakers' yeast in this laboratory.

Radiochemicals. All of the radiochemicals utilized in this work were obtained from New England Nuclear Corporation. They include glucose-1-^{14}C, glucose-2-^{14}C, glucose-3(4)-^{14}C, glucose-6-^{14}C, glucose-U-^{14}C, sodium carbonate-^{14}C, and toluene-^{14}C (purchased and used as a primary standard for liquid scintillation counting).

Miscellaneous Materials. Reagent grade chemicals were used whenever possible for medium preparation and supplementary procedures. Organic constituents of liquid and solid media were purchased from Difco Laboratories Incorporated. Glucostat reagent (glucose oxidase) for the quantitative determination of glucose was obtained from the Worthington Biochemical Company. Argon (99.999 percent purity) was obtained from the National Cylinder Gas Company.

III. METHODS

Culture Methods

Stock cultures of S. cerevisiae were maintained at 4° C on malt extract-agar slants containing 10 percent (weight per volume) malt extract (Difco Brand), 0.2 percent KH_2PO_4 and 2 percent agar. The pH of the slant was 4.8. New slants were routinely inoculated every month and allowed to grow for 48 hours at 30° C prior to cold storage.

Malt extract slant inoculum was grown in medium containing: 2.5 g $(NH_4)_2SO_4$, 2.0 g NaCl, 2.0 g KH_2PO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.25 g $CaCl_2 \cdot 2H_2O$, 1.0 mg H_3BO_3, 1.0 mg $ZnSO_4$, 1.0 mg $MnSO_4 \cdot 4H_2O$, 1.0 mg $FeCl_3$, 0.1 mg $CuSO_4 \cdot 5H_2O$, 0.1 mg KI, 18 g glucose, and 0.1 g Difco yeast extract all dissolved in 1 liter of distilled water (32). The pH of the medium was adjusted to 4.5 with HCl. This is the standard medium utilized in the major portion of this study. Deviations from the above composition are indicated in individual experimental sections.

Preliminary experiments with the high resolution radiorespirometer employed shake culture cells grown in the following manner: Inoculum from a malt extract-agar slant was transferred to 100 ml of the standard medium in a 250 ml Erlenmeyer flask.

The cells were grown aerobically at 30° C on a gyrotory shaker (New Brunswick Scientific Model G25) operating at 200 cycles per minute. Rate of growth was followed turbidimetrically using a Klett-Summerson colorimeter (filter number 66; 640-700 millimicrons). An optical density versus dry cell weight curve (Klett units versus dry cell weight per ml) was prepared by periodic sampling of the cell suspension, collection of cells on Millipore filters (GSWP 130 00) and determination of cell weight upon drying.

In order to obtain information on glucose pathway participation in cells under optimal physiological conditions, cell harvesting and washing procedures were eliminated in some experiments. Shake culture suspensions, grown as described above, were used directly for experimental purposes.

In addition to shake culture cells, some experiments employed cells which were grown directly in the radiorespirometer flask under gas-purged conditions. These cells were grown in 50 ml micro-Fernbach flasks (see Figure 1) containing 30 ml of standard medium. Aeration was provided from the time of inoculation at a flow rate of 150 ml per minute. Filtered, water-saturated air was employed in order to keep the loss of water from culture medium to a minimum. Rate of cell growth was followed by individual cell counts according to a modified procedure of Townsend and Lindegren (28). Essentially, 0.5 ml of cell suspension was aseptically removed and diluted to 10

Figure 1. Radiorespirometer flasks (modified 25 ml and 50 ml micro-Fernbach flasks) for high resolution radiorespirometry.

ml with a 0.2 M KH_2PO_4 solution containing 0.2 percent methylene blue and 5 percent (volume per volume) absolute ethanol. The cell-stain suspension was then mixed for 30 seconds with the aid of a vortex stirrer. Cell counts were made in a hemacytometer (double Neubauer type; American Optical Company). A growth curve was constructed by plotting cell counts per ml versus time (see Figure 11).

The Radiorespirometer

Cell Incubation

The water bath and shaking mechanism of a Gilson differential respirometer (Model G8) was modified to accomodate the radio-respirometer flasks. All experiments were performed at 30°C with a shaking rate set at 90 cycles per minute. Cell incubations and path-way estimation experiments were carried out in modified 25 ml micro-Fernbach flasks which served as the radiorespirometer flasks. The flask is shown in Figure 1 along with a 50 ml model used as a culture growth flask. Rubber serum stoppers provide a means for substrate entry and an air flow inlet. Figure 2 shows an overall view of a four-flask assembly used in this study.

The Gas-flow System

The schematic diagram illustrating the gas-flow system for a

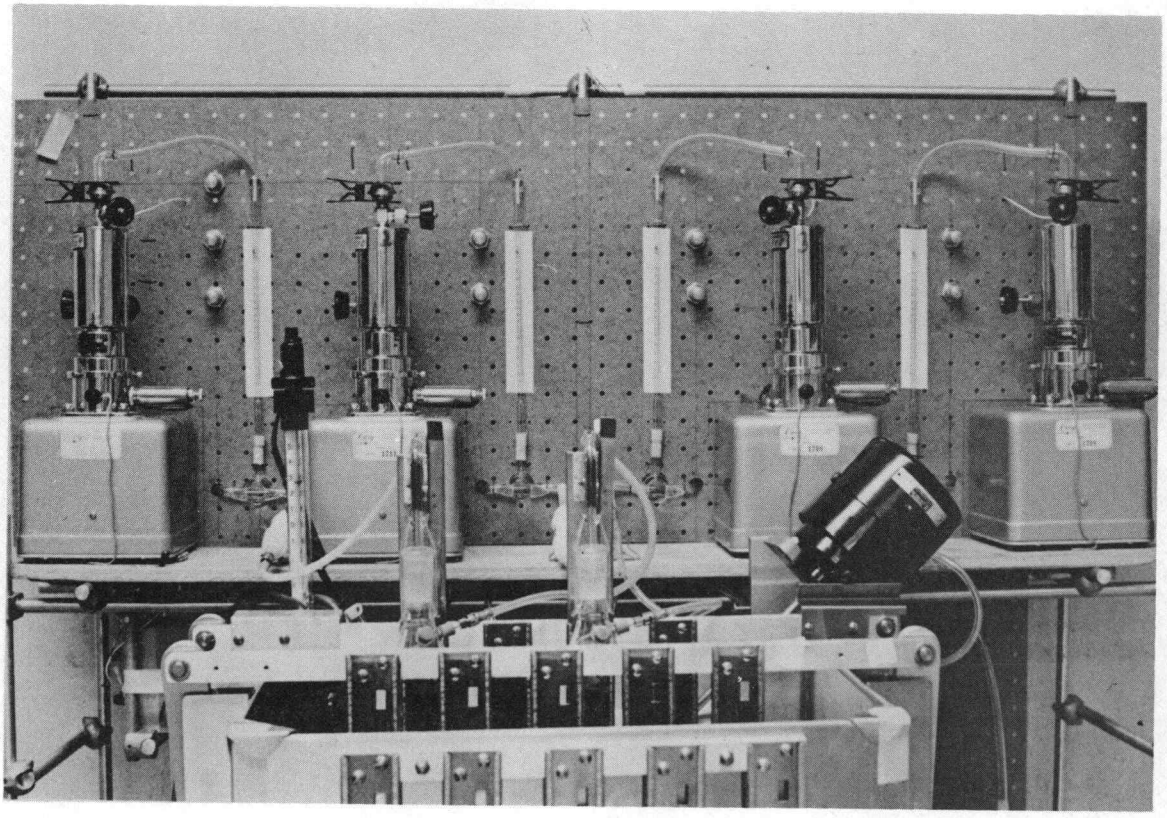

Figure 2. Assembly of a four-flask high resolution radio-respirometer showing the incubator-shaker bath, gas-flow assembly and the ion-chambers mounted on the preamplifiers of the Cary Model 31 Electrometer.

single radiorespirometer flask is presented in Figure 3. The system is carefully designed to minimize dead-volume, i.e., the over all volume of the gas-flow system. Thereby, adequate resolution of $^{14}CO_2$ production is provided.

Respiratory $^{14}CO_2$ is swept from the radiorespirometer flask with filtered air flowing at a rate of 95 ml per minute. Aeration is maintained at this flow-rate during pathway estimation experiments with the use of Nupro metering valves (Nuclear Products Company, Cleveland, Ohio; Type 4MA). Water is removed from the emerging air flow by passing it through a packed 20-mesh anhydrous calcium chloride tube. The dried air containing respiratory $^{14}CO_2$ is mixed with a supplementary argon flow by means of a three-way stopcock (Pyrex #7440; 2 millimeter bore) as shown in the center of Figure 3.

The addition of argon into the gas-flow system is of fundamental importance in the performance of the new radiorespirometer assembly. To obtain kinetic information on respiratory $^{14}CO_2$ with high resolution, there must be a rapid turnover of ion-chamber atmosphere at a rate comparable in magnitude to that of the radiorespirometer flask. The argon flow in conjunction with the air flow through the ion-chamber assures a turnover rate equivalent to that of the respirometer flask. The argon flow rate is maintained by the same type of Nupro metering valve employed on the air-flow system. The combined rate of air-argon flow through the ion-chamber is

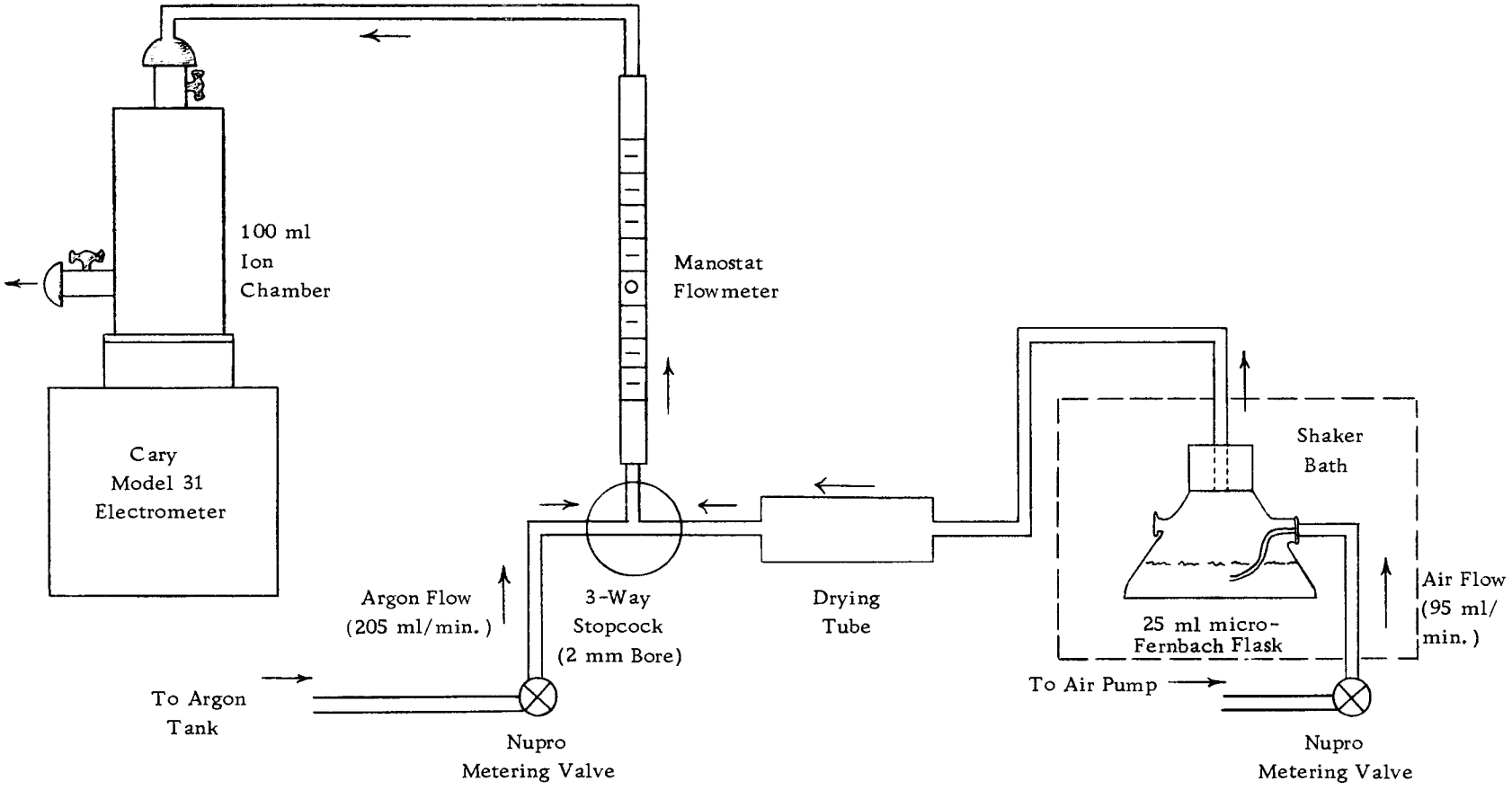

Figure 3. The gas-flow system of the high resolution radiorespirometer.

300 ml per minute. Individual or combined flow rates are measured with a built-in flowmeter (Predictability Flowmeter, 36-541-07; Manostat Corporation). The presence of argon in the ion-chamber is also important from the standpoint of efficiency of radioactivity detection. Since argon has a lower mean energy loss per ion pair formed (compare 26.5 ev per ion pair for argon to 34.0 ev per ion pair for air) under the effects of ionizing radiation, higher sensitivity for ^{14}C detection is achieved.

Respratory $^{14}CO_2$ Determination

The combined air-argon flow carrying respiratory $^{14}CO_2$ passes through the flowmeter directly into a 100 ml ion-chamber (Cary Model 3095-200) as shown in Figure 3. Ion-chambers are mounted on the preamplifiers of the vibrating-reed electrometers (Cary Model 31). A detailed description on the use of ion-chamber-electrometer systems for radioactivity detection in biological systems can be found elsewhere in the literature (31).

A block diagram of the associated measurement and data collection system for the four-flask radiorespirometer is given in Figure 4. The signal of the electrometer, modified to provide 0 to 30 volt output is converted to a proportional audio frequency by a voltage-to-frequency converter (Dymec Model DY-2210). The frequency is scaled on an electronic counter (RIDL Model 49-43) to give

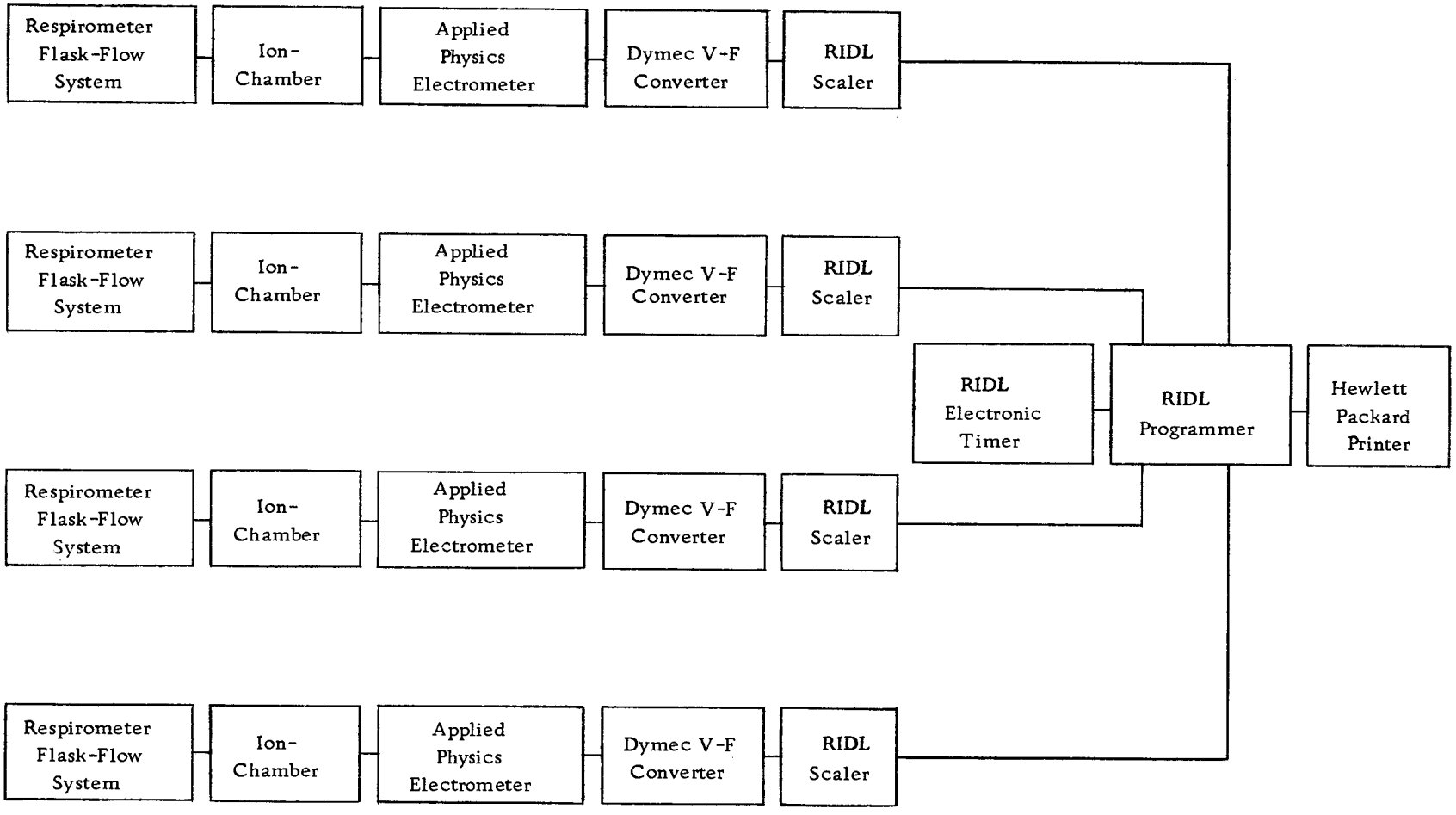

Figure 4. A block diagram of the radiorespirometer including the data printout system.

a direct-reading digital display of the electrometer d-c voltage level. The read-out system for four channels consists of a RIDL scaler-programmer (Model 52-44) and a Hewlett-Packard digital printer (Model H44 562A). The normal operational settings for the electronic equipment are the following: electrometer input voltage range, 100 millivolts; voltage-to-frequency converter input voltage range, 10 volts full scale (corresponding to 10,000 cycles per second at full scale); ion-chamber polarizing potential, 90 volts. An electronic timer establishes the preset time interval which is usually 1 to 2 minutes for most experiments requiring kinetic information and 10 to 30 minutes for background measurements.

Ion-chamber-electrometer Calibration

The calibration of the ion-chamber-electrometer system is accomplished by measuring a known quantity of $^{14}CO_2$ radioactivity liberated when $Na_2{}^{14}CO_3$ solution is injected into dilute sulphuric acid in the radiorespirometer flask. The detailed procedure follows:

Into each radiorespirometer flask is pipetted 10 ml of 2 normal sulphuric acid. This volume is equivalent to the amount of cell suspension that would be present in an actual experiment. The flask containing the acid is positioned on the incubator-shaker bath and is equilibrated with respect to the prevailing air flow rate (95 ml per minute) and temperature (30° C) for 15 minutes. All 4 flasks are

set up in identical fashion. Air and argon flow rates are adjusted to 95 ml per minute and 205 ml per minute respectively. A $Na_2{}^{14}CO_3$ solution is then injected through the entry port of each flask with a 1-ml syringe (B-D Yale Type 1YTL) calibrated in one-hundredths ml subdivisions. The calibration syringes are matched to the individual flasks and later serve as the radiochemical substrate addition syringes to that same flask.

Data on radioactivity released in the form of $^{14}CO_2$ from the $Na_2{}^{14}CO_3$ injection is registered on the digital scaler. Since the digital information is proportional to the amount of $^{14}CO_2$ activity released, a calibration factor can be established for each ion-chamber-electrometer system based on digits registered per μC of radioactivity. Therefore, digital information is collected over a period of time sufficient to account for the complete passage of a known amount of $^{14}CO_2$ through the ion-chamber. Experimentally, data are collected over a 25 minute period. The results of an experimental calibration are given in Table 1.

Information on the lag time of the gas-flow system is radio-activity detection can be obtained by measuring the rate of appearance of $^{14}CO_2$ in the ion-chamber. Experimental results for individual channels are given in Figure 5. Data are accumulated over 6 second intervals and plotted accordingly. The response time of the individual assemblies is remarkably uniform and the average $^{14}CO_2$ activity

Table 1. Calibration data for the determination of $^{14}CO_2$ by means of the ion-chamber-electrometer system.

	Flask Number[a]			
	1	2	3	4
Experiment 1				
Radioactivity of $^{14}CO_2$ (μC)	0.0622	0.0622	0.0622	0.0622
Total Registered Digits[b] (x 10^5)	7.18	7.44	7.41	7.41
Registered Background (x 10^5)	0.28	0.24	0.18	0.21
Net Digits (x 10^5)	6.90	7.20	7.23	7.20
Digits per μC (x 10^7)	1.11	1.16	1.16	1.16
Experiment 2				
Digits per μC (x 10^7)	1.14	1.17	1.17	1.18
Experiment 3				
Digits per μC (x 10^7)	1.14	1.18	1.17	1.19
Averages	1.13 ± .02	1.17 ± .01	1.17 ± .01	1.18 ± .02

[a]Flasks contained 10 ml of 2 N H_2SO_4; 0.80 ml of $Na_2{}^{14}CO_3$ solution containing 0.0622 μC.

[b]Digits registered over a 25 minute period. Air flow rate: 95 ml per minute; Argon flow rate: 205 ml per minute.

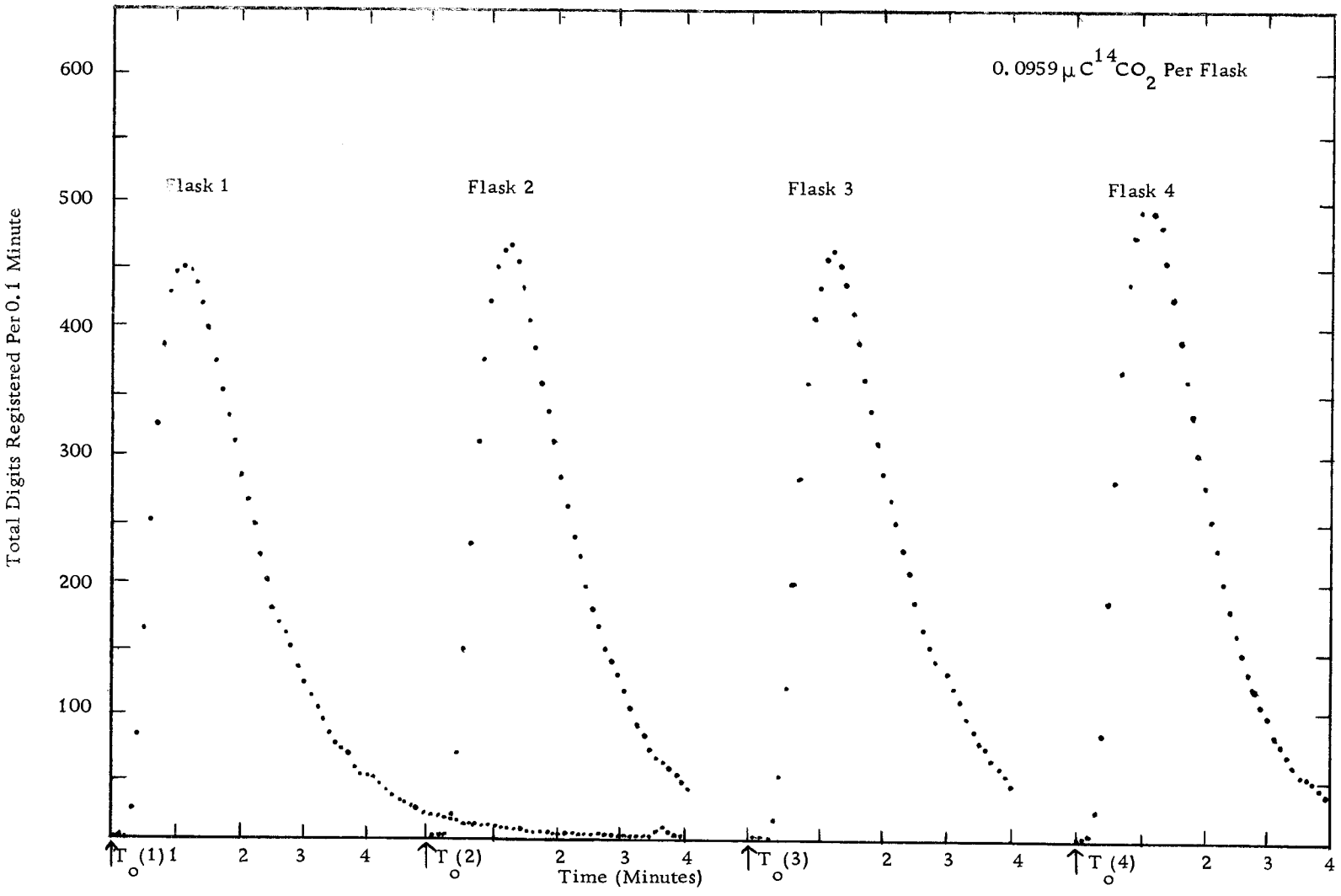

Figure 5. Resolving power of the high resolution radiorespirometer with respect to the rate of $^{14}CO_2$ production.

peaking time is approximately 70 seconds. Since the release of $^{14}CO_2$ from the acid solution is instantaneous upon injection of $Na_2{}^{14}CO_3$, this lag time can be attributed to the dead volume of the system and the gas flow rate involved in the delivery of $^{14}CO_2$ from the flask to the ion-chamber. Two other considerations must be taken into account. First, the ion-chamber-electrometer system has a slow electronic response time of about 10 seconds which contributes to the apparent flow lag time. Second, the extent of gas mixing in the dead space of the flow system can be evaluated from the asymmetry of the $^{14}CO_2$ curve. The profile of the curves shown in Figure 5 indicates that while some mixing of $^{14}CO_2$ has occurred (as indicated by tailing), it does not seem to be a serious drawback and the relatively rapid turnover of gas volumes is realized. The estimated dead volume of the flow system is 30 ml for each assembly. Employing the relationship

$$t_{0.5} = (\text{volume/flow rate}) \log_n 2$$

to estimate turnover time (39), it is calculated that for the gas-flow system the turnover time is 13 seconds and that for the ion-chamber (100 ml) it is 14 seconds.

Pathway Estimation Experiments

Re-evaluation of Single Dose Experiments

A set of experiments were carried out to apply high-resolution radiorespirometry to the existing yield method. In these experiments the procedures were slightly modified from those reported elsewhere (27).

Yeast cells were harvested at the middle of the logarithmic growth phase by centrifugation at room temperature in a Sorvall Model SS-4 (SS-34 head) operating at 3,000 rpm for 5 minutes. After decantation of the supernatant medium, the cells were resuspended and washed with glucose-free standard medium. The cells were again collected by centrifugation and the washing procedure repeated. The final glucose-free suspension of cells in glucose-free medium was pipetted into individual 25 ml radiorespirometer flasks (containing 10 mg of dry cell weight) and incubated on the shaker-bath at 30^{o} C with an air flow of 95 ml per minute. Various incubation periods were employed ranging from 5 to 90 minutes before the addition of substrate. Specifically labeled ^{14}C glucose and carrier glucose were administered simultaneously to the radiorespirometer flask by syringe injection. The flask and its contents were hand shaken for 10 seconds immediately after injection in order to effect rapid mixing of the labeled glucose. Approximately 4.0 μmoles of

glucose per mg dry cell weight were used in the single dose experiments. Rates of production of respiratory $^{14}CO_2$ were obtained by setting the electronic timer to a preset time of 1 or 2 minutes depending on the nature of the experiment.

Upon completion of substrate utilization, the radiorespirometer flasks were cooled to 0° C with ice. The cell suspension was quantitatively washed into pre-cooled centrifuge tubes and the separation of cells and medium was effected in a refrigerated Sorvall centrifuge (Model RC2B) operating at 0° C (5,000 rpm with SS-34 Head). The medium was decanted and the cells were resuspended and washed with glucose-free standard salt solution. The washings were pooled with the original medium after recentrifugation. Cells and medium were then analyzed for ^{14}C content. The single dose experiments required approximately 1 μC of each specifically labeled glucose (specific activity, 0.04 millicurie per millimole) in order to obtain reliable kinetic information.

Continuous Feeding Experiments

In order to obtain more reliable information on pathway estimations, the single dose experiment was replaced by a method in which a shake culture was used directly for radiorespirometric experiments. The intent was to determine the catabolic rate of individual glucose pathways assuming that cellular metabolic

steady-state could be attained by this approach. Cell harvesting and washing procedures were thus eliminated and middle log phase shake culture suspension was pipetted into the radiorespirometric flask. A suspension volume (8 to 10 ml) containing 10 mg dry cell weight was normally employed for pathway estimation experiments. The cell suspension was aerated at 95 ml per minute for various time periods. Tracer level quantities of specifically labeled glucose substrates were added to individual flasks. Since shake culture cells were used directly, the utilization of substrate glucose was never interrupted although the kinetic data on $^{14}CO_2$ production from individual carbon atoms of glucose were obtained only after the addition of the labeled substrate. Immediately prior to tracer addition, an aliquot from a control radiorespirometer flask was removed for cell count and glucose concentration determination. Cell proliferation rate and glucose assimilation rate were determined by periodic sampling of the control flask during the course of the experiment. Kinetic information on respiratory $^{14}CO_2$ production was collected in 1 minute intervals extending over periods up to 200 minutes in some cases. However, most experiments were terminated after 60 minutes. The handling of cells and medium upon termination of the experiment have already been described in the previous section.

A primary concern of the continuous feeding experiments was the concentration of substrate glucose to be employed in the culture

medium. It was realized that high glucose concentrations can produce Crabtree effects (13, 20) in aerobic yeast cultures. Conversely, low glucose concentration can produce decreases in cellular catabolic rates due to the rate-limiting gradient effects derived from low glucose concentration in the medium. Therefore, we needed to know optimal glucose concentration that would allow a cellular metabolic steady-state for the duration of the experiment.

Upon examining the kinetic data of respiratory $^{14}CO_2$ obtained from glucose-3(4)-^{14}C in single dose experiments, it was concluded that the peak rates of $^{14}CO_2$ production might serve as a useful parameter for determining the glucose concentration necessary to achieve a cellular metabolic steady-state. Therefore, a series of experiments were designed using procedures similar to those described for the single dose experiments. Cells were harvested at middle log phase, washed and suspended in glucose-free standard medium. Each flask contained an identical quantity of cells (13.2 mg dry cell weight) which were aerated at 95 ml per minute and incubated at 30° C for 60 minutes prior to the addition of substrate. Glucose-3(4)-^{14}C and carrier glucose, ranging in quantities from 25 to 200 μmoles, were added to the individual flasks. Respiratory $^{14}CO_2$ was measured over 1 minute intervals. The experiment was terminated after the peak rates of $^{14}CO_2$ production for each level of substrate concentration had been clearly established.

From these studies, it was estimated that 0.1 M glucose in the standard medium met concentration requirements not only for continuous feeding experiments, but also for the series of experiments to be described in the next section.

Unperturbed Culture Experiments

Upon analysis of the data obtained in the continuous feeding experiments, it was found that middle-log phase shake culture entered a premature stationary phase upon initiation of aeration in the radiorespirometer flask. While catabolic rate information on individual glucose pathways can be obtained from this perturbed system, it was realized that more reliable data, from a physiological standpoint, could be collected if cell development were not interrupted. Therefore, air-purged cell cultures were grown directly in the radiorespirometer as described in the culture methods section. Pathway estimations with the physiologically unperturbed cells were carried out in the following manner.

At a given growth phase of a typical growth curve, aliquots were pipetted from the 50 ml micro-Fernbach growth flask to the 25 ml micro-Fernbach radiorespirometer flask. The operation required less than 4 minutes for an experiment involving 4 flasks, and it was demonstrated by subsequent cell counting that the growth physiology of the cell suspension in the 25 ml flask was not altered.

Immediately after pipetting, the cells were incubated at 30° C with the air-flow rate set at 95 ml per minute for a 5 minute period. Just prior to radiotracer addition, an aliquot of cell suspension was removed so that a "T_o" or initial level of glucose concentration in the medium and cell population could be determined. Tracer level quantities of specifically labeled glucose substrates were then administered to individual flasks. Kinetic information of respiratory $^{14}CO_2$ production was collected in 1 minute intervals by presetting the electronic timer. After 30 one-minute intervals, the glucose-6-^{14}C flask was removed from the system and cell population and glucose concentration in the medium were determined. Approximately 10 minutes later, or after an elapsed time of 40 minutes, the glucose-1-^{14}C flask was removed and treated similarly. The glucose-3(4)-^{14}C flask was removed after 50 to 60 minutes for the final cell count and glucose level determination. The periodic sampling of cell population and glucose chemical concentration in the medium provided information for the calculation of cell proliferation rate and glucose assimilation rate respectively. Preparation of yeast cells for the determination of incorporated radioactivity has been previously described.

Other Procedures

Radiochemical Substrate Standardization

The radioactivity of individual substrates was determined by

means of liquid scintillation counting in a Packard Tricarb Model 314-EX 2 counter. Radiochemicals were diluted with distilled water to the approximate desired concentration. Aliquots (usually 0.2 ml) of the radiochemical were counted directly or were further diluted to 50 ml from which 1 ml samples were counted in Bray's solution (5). Potential across the photomultiplier tubes was set at 1050 volts. Toluene-^{14}C was used as an internal standard to determine the absolute counting efficiency of the counter.

Determination of ^{14}C Radioactivity in Cells and Medium

Washed yeast cells were suspended in distilled water to approximately 0.2 mg dry cell weight per ml. Aliquots of 0.5 ml cell suspension were mixed with 15 ml of the thixotropic gel suspension (38) and the radioactivity of the preparation was determined by liquid scintillation counting. Glucose-U^{14}C, previously standardized against the primary toluene-^{14}C and counter-checked by combustion to $^{14}CO_2$ for standardization, was used as an internal standard for the gel system.

Radioactivity in the incubation medium was determined by direct liquid scintillation counting of either 1 ml or 0.5 ml aliquots in Bray's solution. Toluene-^{14}C was used as an internal standard for the system.

Determination of Glucose in the Incubation Medium

The Worthington Glucostat method, employing the glucose oxidase reaction coupled to the oxidation of o-dianisidine with H_2O_2 in the presence of horseradish peroxidase, was used to determine the concentration of glucose in medium filtrates. Cell-suspension aliquots taken in pathway estimation experiments were filtered through Millipore filters (Type GSWP 013 33). The filtrate was immediately chilled at 0° C with ice and then frozen for analysis at a later time. For analysis the filtrates were thawed and diluted 100 fold in phosphate buffer at pH 7.0. A glucose standard curve, constructed from 3 concentration ranges (0.1 mg per ml, 0.2 mg per ml, and 0.3 mg per ml in phosphate buffer) was used with each determination. The standards and unknown were read on a Beckman DU-2 spectrophotometer at 410 millimicrons (5.50 sensitivity and approximately 0.03 mm slit width).

IV. RESULTS AND DISCUSSION

Re-evaluation of Single Dose Experiments

Radiorespirometry experiments with harvested cells and single-dose substrate administration were performed in order to evaluate the efficacy of the high resolution apparatus and to compare the findings with those obtained by conventional methods (27, 34). The radiorespirometric data obtained through use of high resolution equipment are presented in Figure 6. Yields for 2 minute intervals, in contrast to hourly intervals employed in previous studies (27), are plotted versus time. For comparison, the results of a study using the conventional methods of the low resolution system are given in Figure 7 (27).

Information on cumulative $^{14}CO_2$ yield, cellular incorporation and residual medium activities are summarized in Table 2 for both studies. When the contribution of the PP catabolic pathway is estimated using the conventional yield method (27, 34) as shown in Table 3, the correction factor for anabolic contribution, i.e., $G_T - G_T'$, representing the net amount of substrate glucose engaged in catabolic pathways (34; Table 3), is applied in order to compare values of G_p calculated from data obtained in both experiments.

The value of G_T', representing the amount of substrate glucose

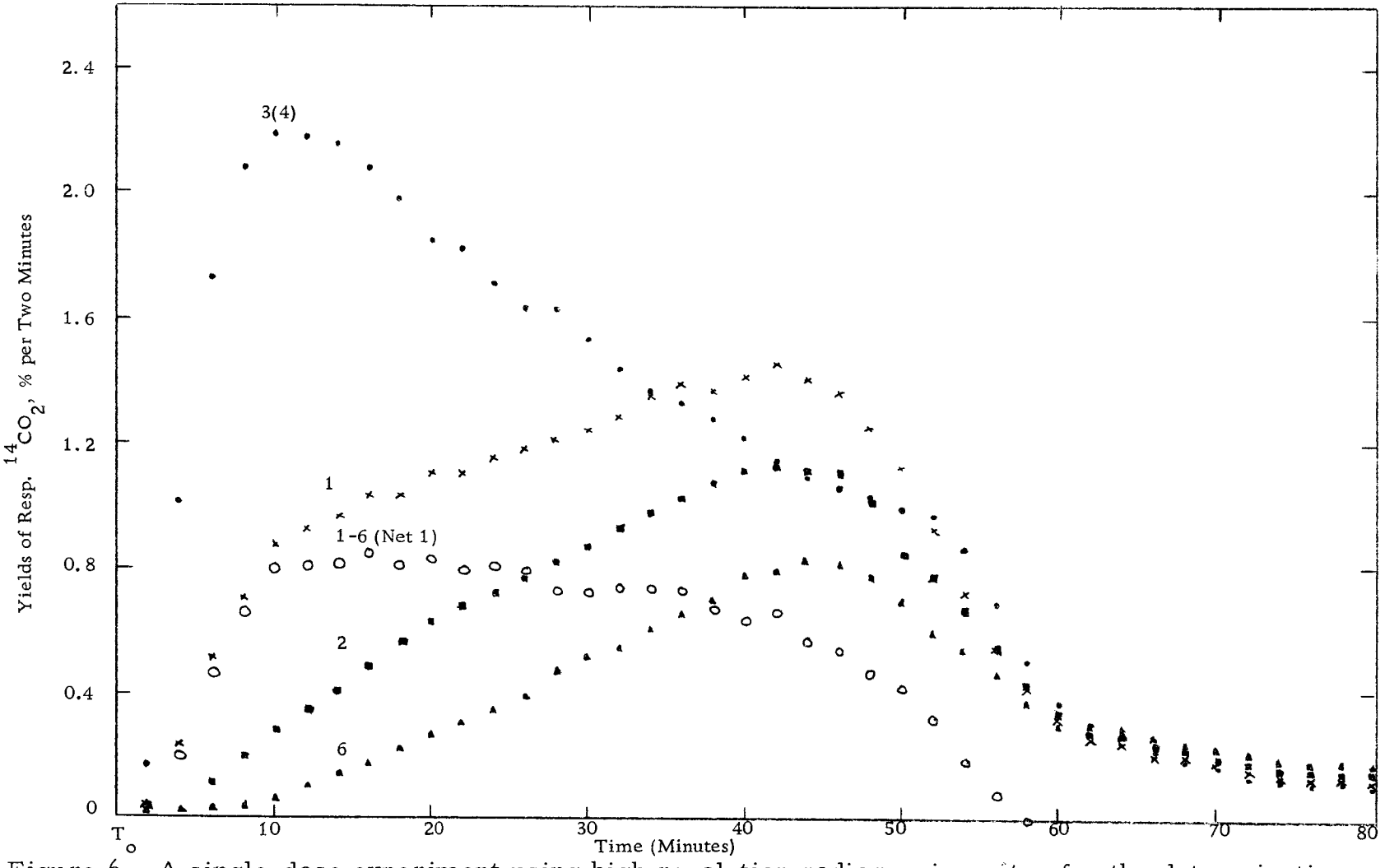

Figure 6. A single dose experiment using high resolution radiorespirometry for the determination of $^{14}CO_2$ production from Saccharomyces cerevisiae metabolizing ^{14}C specifically labeled glucose substrates. (Numerals refer to the labeled position of glucose)

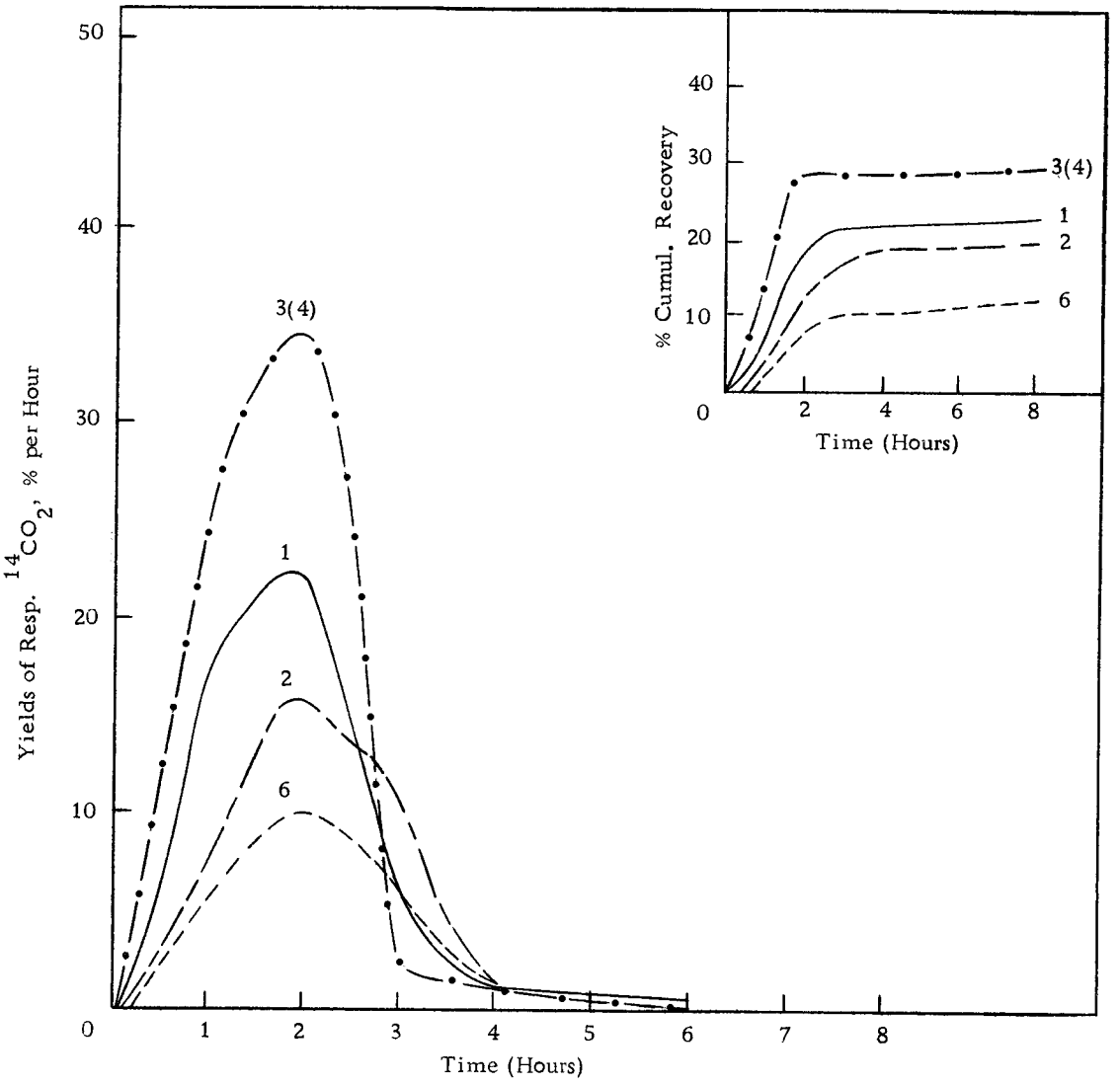

Figure 7. A single dose experiment using low resolution radiorespirometry for the determination of $^{14}CO_2$ production from Saccharomyces cerevisiae metabolising ^{14}C specifically labeled glucose substrates. (Numerals refer to the labeled position of glucose)

Table 2. Utilization of ^{14}C specifically labeled glucose substrates by Saccharomyces cerevisiae as studied by high resolution radiorespirometry and low resolution radiorespirometry.

Method	Substrate	Substrate Level		Radiochemical Yield (%) in			
		μC	μmole	CO_2	Cells	Medium	Total
High Resolution Radiorespirometry (see Figure 6)	Glucose-1-^{14}C	1.00	25	31	58	7	96
	Glucose-2-^{14}C	1.00	25	21	71	10	102
	Glucose-3(4)-^{14}C	1.00	25	42	50	3	95
	Glucose-6-^{14}C	1.00	25	14	72	15	101
Low Resolution Radiorespirometry (see Figure 7)	Glucose-1-^{14}C	0.25	167	46	44	9	99
	Glucose-2-^{14}C	0.25	167	38	51	6	95
	Glucose-3(4)-^{14}C	0.03	167	60	32	4	96
	Glucose-6-^{14}C	0.25	167	24	61	6	91

Table 3. Estimations of the relative participation of glucose pathways in Saccharomyces cerevisiae from yield data obtained with high resolution radiorespirometry and low resolution radiorespirometry.

Method	G_1	G_6	$G_{T'}$	$G_T - G_{T'}$	G_p	G_p*
High Resolution Radiorespirometry	31	14	0.50	0.50	20	47
Low Resolution Radiorespirometry	46	24	0.32	0.68	30	50

Yield and incorporation data are those observed at the completion of substrate utilization; G_1 = respiratory $^{14}CO_2$ yield from glucose-1-^{14}C substrate; G_6 = respiratory $^{14}CO_2$ yield from glucose-6-^{14}C substrate; $G_{T'}$ = fraction of substrate glucose engaged in anabolic pathways as indicated by the amount of radioactivity incorporation into cells in glucose-3(4)-^{14}C experiments; $G_T - G_{T'}$ = fraction of substrate glucose catabolized; G_p = fraction of substrate glucose catabolized via the PP pathway;

$$G_p = \frac{G_1 - G_6}{1 - G_6} \; ; \; G_p{}^* = \frac{G_1 - G_6}{G_T - G_{T'} - G_6}$$

(see ref. 27, 34).

engaged in anabolic pathways, cannot be readily assessed. However, as a crude estimation, the value of G_T' is approximately the same as the amount of C-3(4) of glucose that has been incorporated into the cellular constituents. It is interesting to note that the 2 G_p* values (Table 3), representing the G_p values corrected for anabolic pathways, are in close agreement, despite the six-fold difference in glucose substrate concentration which exists between the two methods.

As a result of the high resolving power of the new apparatus, detailed kinetic information of respiratory $^{14}CO_2$ production becomes available. By examining the plateau region of the $^{14}CO_2$ curve derived from glucose-3(4)-^{14}C in Figure 6, it should be possible to estimate the catabolic rate of the glycolytic pathway, making use of the yield data within this region. Likewise, the catabolic rate of PP pathway can be estimated by subtracting the $^{14}CO_2$ yield curve in the glucose-6-^{14}C experiment from the $^{14}CO_2$ yield curve in the glucose-1-^{14}C experiment. A curve so obtained is representative of the $^{14}CO_2$ yield curve from C-1 of glucose via the PP pathway. The plateau nature of this curve permits the calculation of the catabolic rate of the PP pathway, again making use of the yield data within a short time interval.

Continuous Feeding Experiments

As indicated in the previous section, the use of high resolution

radiorespirometry provides detailed kinetic information on the production of respiratory $^{14}CO_2$ from glucose substrates. Therefore, it is possible to develop a new method for glucose pathway estimation which is independent of cumulative $^{14}CO_2$ yields and relies exclusively on catabolic rate information. Consequently, the high resolution radiorespirometric method, once developed, constitutes a complete change in conceptual approach to the problem of pathway estimation.

In order to develop a method that makes use of the information on catabolic rates of individual glucose pathways, careful consideration must be given to the following points:

(1) The required resolution of kinetic data for the production of $^{14}CO_2$ must be adequately defined. Hence, all pertinent parameters associated with resolution of the kinetic data must be carefully examined.

(2) Cellular metabolic steady-state with respect to substrate utilization must be maintained during the experiment. This requirement can be met using the continuous feeding method for substrate administration.

(3) The physiological state of the cells under which pathway estimations are made must remain unperturbed throughout the course of the experiment.

Consideration of the above points led to a series of experiments which utilized the method of continuous substrate feeding.

Experiments were designed for the direct determination of glucose catabolic rates by high resolution radiorespirometry. The results of a typical study are presented in Figure 8. The curves of $^{14}CO_2$ yield given in Figure 8 can be analyzed by the conditions determining the usefulness of such data in catabolic rate studies. For example, consider the $^{14}CO_2$ yield curve in the glucose-3(4)-^{14}C experiment shown in Figure 8. The rapid transport and utilization of glucose via the glycolytic sequence is revealed by the extensive $^{14}CO_2$ production observed soon after addition of the labeled substrate, i.e., in less than 60 seconds, if one considers that the first 70 seconds represents the lag time of the gas flow system.

Parameters defining the adequacy of the resolving power of $^{14}CO_2$ yield data can be generally classified into two main categories:

(1) Those parameters related to cell physiology such as substrate transport rate, substrate utilization rate and CO_2 or bicarbonate countertransport rate.

(2) Those parameters that are associated with purely physical significance such as CO_2 evolution from the medium, gas-flow rate to the ion-chamber, dead volume turnover rate and response time of the instrumentation for $^{14}CO_2$ detection.

The high resolution radiorespirometer used in this study has sufficient resolving power to permit calculation of catabolic rates from respiratory $^{14}CO_2$ data. However, improvements within the

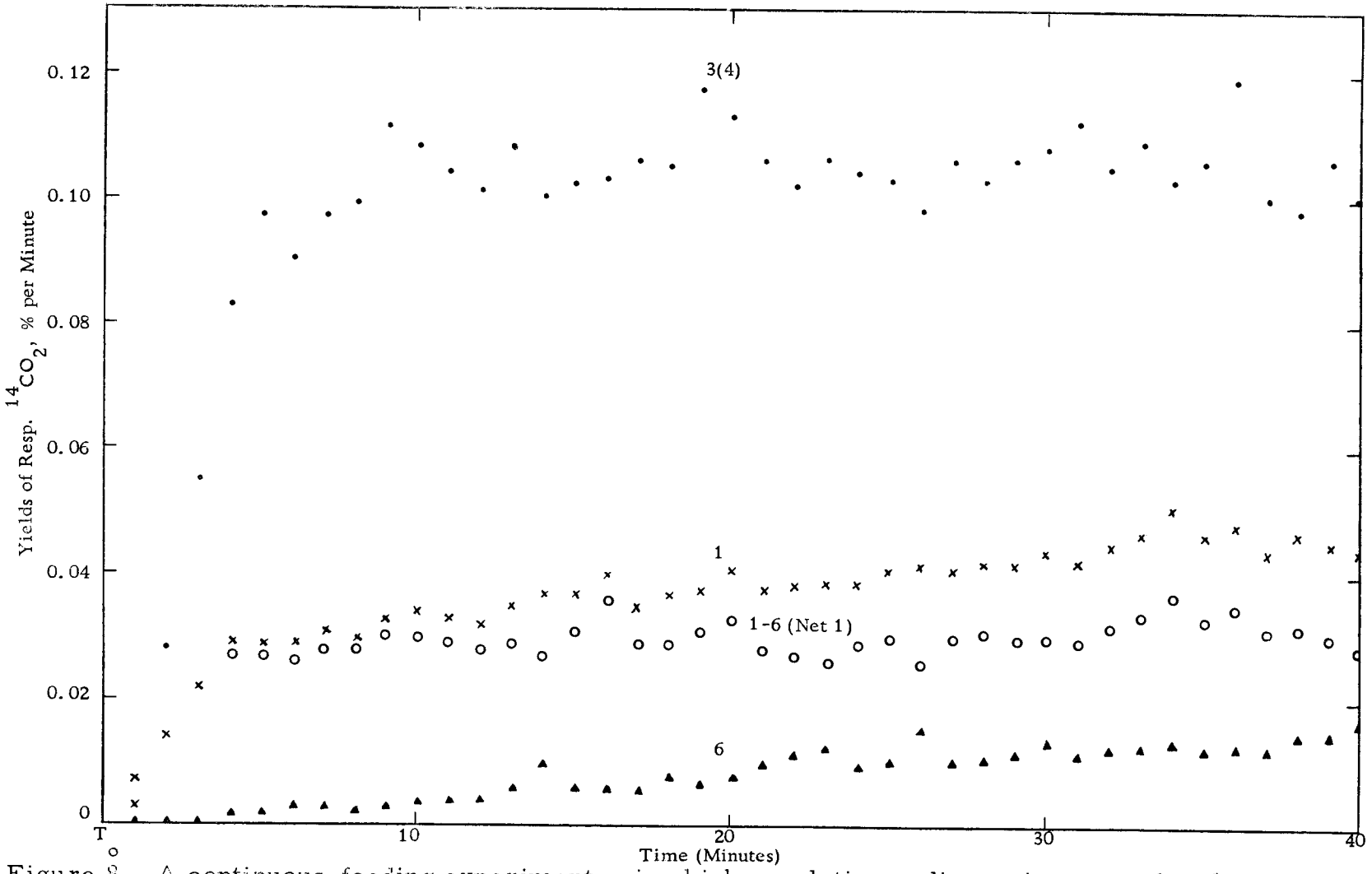

Figure 8. A continuous feeding experiment using high resolution radiorespirometry for the determination of $^{14}CO_2$ production from Saccharomyces cerevisiae metabolizing ^{14}C specifically labeled glucose substrate. (Numerals refer to the labeled position of glucose)

gas-flow system and electronic response time are necessary in order to observe very rapid physiological phenomena related to transport and certain oscillatory phenomena. Evolution of $^{14}CO_2$ from the medium is considered to be rapid at pH 4.5. In addition, the sweeping effect of air flow at 95 ml per minute through 10 ml of cell suspension facilitates the removal of CO_2 as soon as it is released by the cell. It appears that the turnover time of the gas-flow system and that of the ion-chamber (approximately 14 seconds for $t_{0.5}$) in addition to the response time of the electrometer (about 10 seconds) are the limiting factors underlying the resolving power of the apparatus.

The resolving capabilities of the apparatus having been established, consideration was given to the maintenance of metabolic steady-state conditions during pathway estimation studies. The concentration of glucose substrate was critical in the execution of continuous feeding experiments with shake-culture suspensions. The problems associated with undefinable glucose concentrations have been previously mentioned. Examination of glycolytic rates obtained from $^{14}CO_2$ yield data in the single dose experiment suggested that a series of experiments could be designed and executed to provide information on the optimal concentration of glucose necessary to maintain cells at metabolic steady-state.

The data shown in Figure 8 from glucose-3(4)-^{14}C single dose experiments demonstrate that the uptake and utilization of glucose in

the glycolytic sequence occurs with remarkable speed. It appears that the rate of $^{14}CO_2$ production from C-3(4) of glucose, resulting from the overall process of glucose transport → conversion of glucose to pyruvate via glycolysis → decarboxylation of pyruvate to CO_2 and acetate, might serve as a parameter to assess the optimal glucose concentration in the medium. The term "optimal glucose concentration" is used in a kinetic sense, i.e., the transport sites of yeast cells are saturated with glucose so that zero order kinetics can be assumed in data analysis. Also, optimal concentration implies that cells are in metabolic steady-state with respect to substrate turnover.

The rates actually determined in this experiment were the peak $^{14}CO_2$ production rates from glucose-3(4)-^{14}C as a function of glucose concentration in the medium. Single dose experiments employing harvested and washed cells were required so that a defined quantity of glucose could be added to the final glucose-free cell suspension in the radiorespirometer flask. The results of a typical experiment are shown in Figure 9. One minute interval $^{14}CO_2$ recovery is plotted against time for quantities of glucose ranging from 25 to 200 μmoles. The peak in $^{14}CO_2$ production rate is reached in 12 minutes at the 25 μmole level and in 14 minutes at the 200 μmole level. The peak rate is thought to represent the degree of substrate saturation in cells with respect to glucose in the medium. The inserted graph in Figure 10 shows a plot of the peak velocity versus concentration.

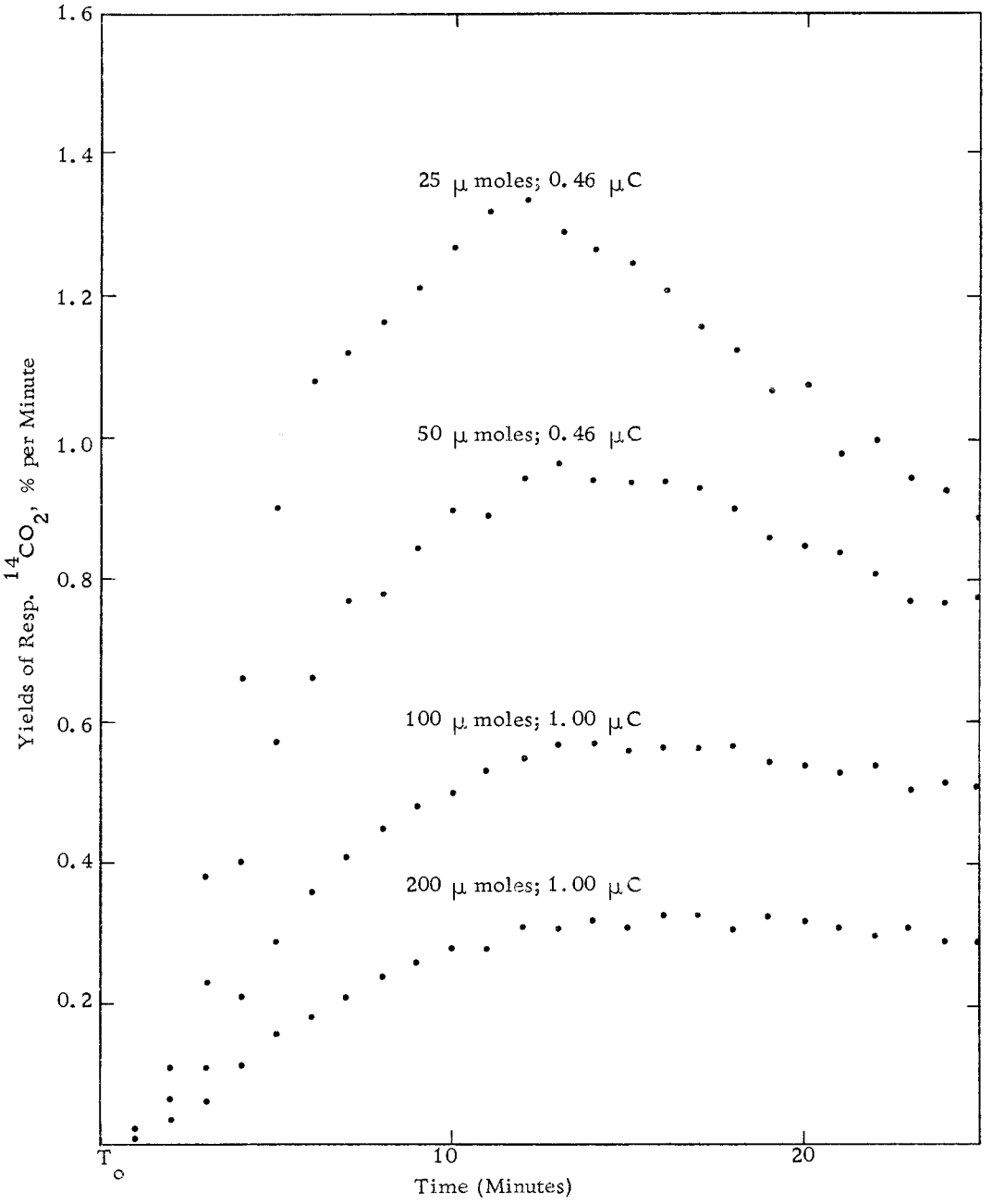

Figure 9. Respiratory $^{14}CO_2$ production from Saccharomyces cerevisiae metabolizing different chemical levels of glucose-3(4)-^{14}C.

From this curve one can assess the apparent saturation level of glucose in the cell. In Figure 10 it is observed that the velocity data, when subjected to the Lineweaver-Burk double reciprocal treatment, appear to obey Michaelis-Menton kinetics. The Km and Vm parameters derived from this plot may reflect the rate limiting step in the overall process of transport, glycolysis and decarboxylation. It is not possible at this time to determine which step is limiting, however, it has been suggested that in yeast, the glucose transport process is the rate limiting step (26).

From these data it was concluded that metabolic steady-state would be maintained over a considerable period if the glucose concentration in the medium exceeded 40 μmole per ml at the end of late log phase.

A glucose concentration of 100 μmoles per ml (0.1 M) was selected as the standard concentration to be employed in continuous feeding experiments. At the end of the late log phase, glucose in the medium (originally 100 μmole per ml) was well above 40 μmole per ml, the minimum thought to be required for substrate saturation in cells.

Therefore, by maintaining yeast cells in a metabolic steady-state with respect to glucose turnover, it is possible to estimate the concurrent glucose pathways by the catabolic rate method. The catabolic rate of the glycolytic pathway can be evaluated from the

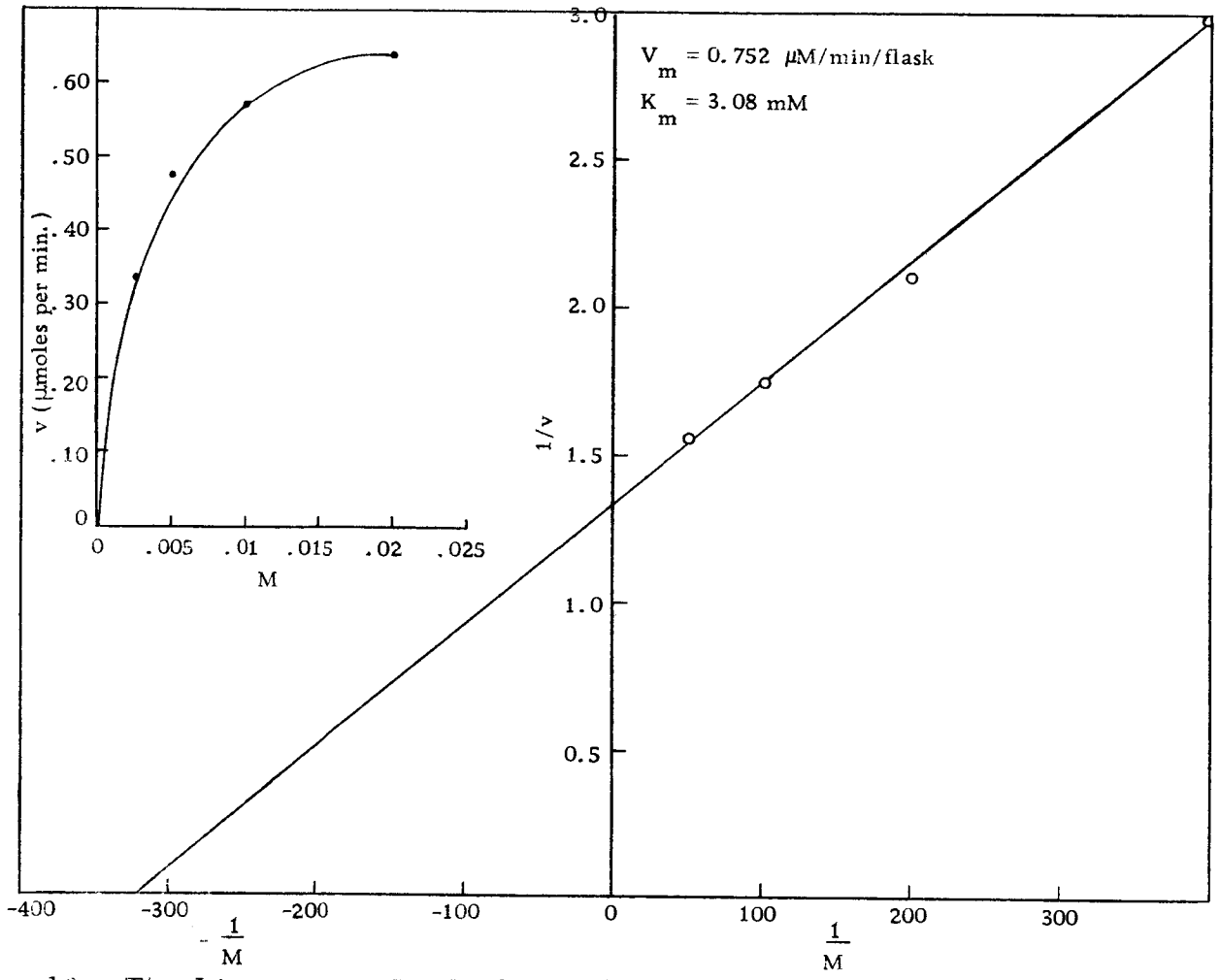

Figure 10. The Lineweaver-Burk plot of the peak velocity of the transport→glycolytic sequence versus substrate concentration. (The inserted figure is a plot of peak velocity versus substrate concentration)

$^{14}CO_2$ yield data in the glucose-3(4)-^{14}C experiment (Figure 8). Assuming that pyruvate formed via glycolysis is predominantly metabolized by pyruvate decarboxylase and pyruvate dehydrogenase (28) and that the drainage of other glycolytic intermediates is minimal, this treatment provides a direct determination of the catabolic rate of the glycolytic pathway. The catabolic rate of the PP pathway can be determined by subtraction of the $^{14}CO_2$ yield data of the glucose-6-^{14}C experiment from the $^{14}CO_2$ yield data of the glucose-1-^{14}C experiment as described for the single dose method.

The studies based on continuous substrate feeding using aerated shake-culture established that cells at metabolic steady-state could provide useful catabolic rate information for pathway estimations. However, upon the initiation of air purging within the radiorespirometer flask, cell growth from middle log phase was prematurely interrupted for a period of time exceeding 100 minutes. The higher degree of oxygenation in the air-purged medium may have perturbed the physiology of the cells, possible by stimulation of mitochondrial synthesis (20).

Consequently efforts were made to design a series of experiments aimed at the preservation of normal cell physiology during high resolution radiorespirometry experiments.

Unperturbed Culture Experiments

In the previous section, it was demonstrated that radiorespirometric data obtained from continuous feedings experiments could be used to estimate the catabolic rates of individual glucose pathways. However, it was recognized that physiological perturbations, resulting from certain culture handling procedures, produced atypical growth patterns, thus negating the possibility of estimating glucose catabolic pathways under optimal physiological conditions.

For this reason a modified culture procedure was employed to establish unperturbed cellular physiology throughout the duration of the pathway estimation experiments. As described in the section on methods, cells were grown in 50 ml micro-Fernbach flasks directly on the shaker-incubation bath of the radiorespirometer and aerated at an air-flow rate of 150 ml per minute. Cell growth was followed by periodic examination of the cell population. A typical growth curve of the cells raised by this culture procedure is shown in Figure 11.

Since the catabolic rate method of pathway estimation is dependent on CO_2 yield data collected within a few minutes, it is possible to use this method to assess pathway participation in the developmental physiology of yeast growth. In the study which follows, pathway estimations are made on middle log phase, late log phase

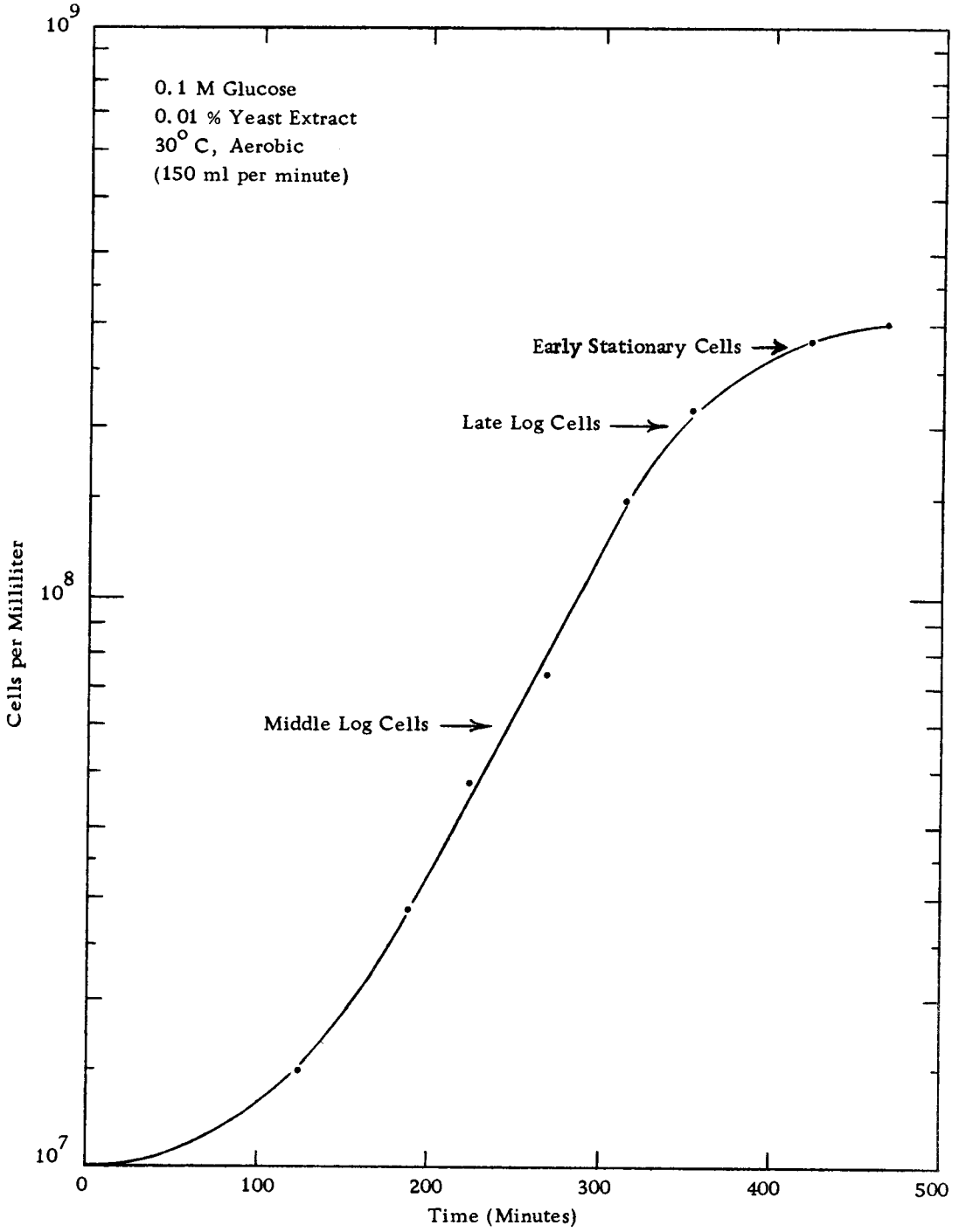

Figure 11. Growth curve of Saccharomyces cerevisiae incubated under gas-purged conditions.

and early stationary phase cells. The results of the experiments using cells obtained from the early stationary phase are presented in Figure 12. Tracer quantities of glucose-3(4)-^{14}C, glucose-1-^{14}C and glucose-6-^{14}C were added to three individual flasks, each containing 1.7×10^9 cells as determined by cell counting immediately before radiotracer addition, at time T_o. Glucose concentration was determined at T_o also. Because the cells had been grown and experimentally employed without perturbation, the kinetic data on respiratory $^{14}CO_2$ production shown in Figure 12 was obtained under optimal physiological conditions.

Pathway estimations from $^{14}CO_2$ yield data given in Figure 12 are derived in the following manner. Since the net amount of glucose utilization is proportional to cell concentration, there should be a steadily increasing rate of $^{14}CO_2$ production as cell concentration increases by cell division and growth. In Figure 12, the cell population in the radiorespirometer flask increased from 1.7×10^9 cells at T_o to 2.5×10^9 cells after 62 minutes. (Cell population data is summarized in Table 4.) Since the increase in cell population can be followed throughout the experiment, it is possible to normalize the $^{14}CO_2$ yield data in Figure 12 to a given cell population, usually that established at T_o. The normalized plot is given in Figure 13. From the normalized $^{14}CO_2$ yield data, it is possible to extrapolate the yield curve to intersect with the ordinate, i.e., at T_o. The

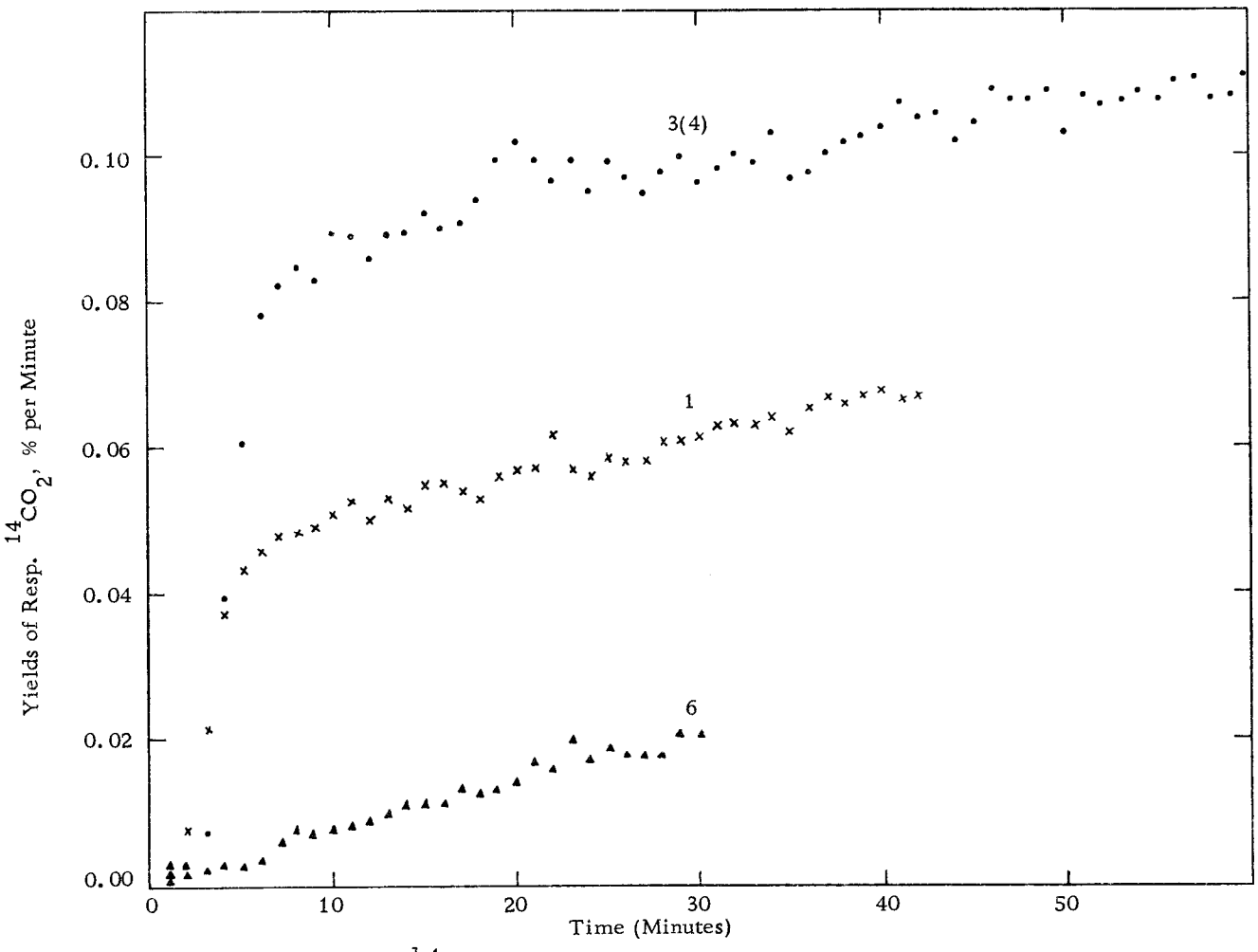

Figure 12. Rate of respiratory $^{14}CO_2$ production from Saccharomyces cerevisiae, in early stationary phase, metabolizing ^{14}C specifically labeled glucose substrates. (Numerals refer to the labeled position of glucose)

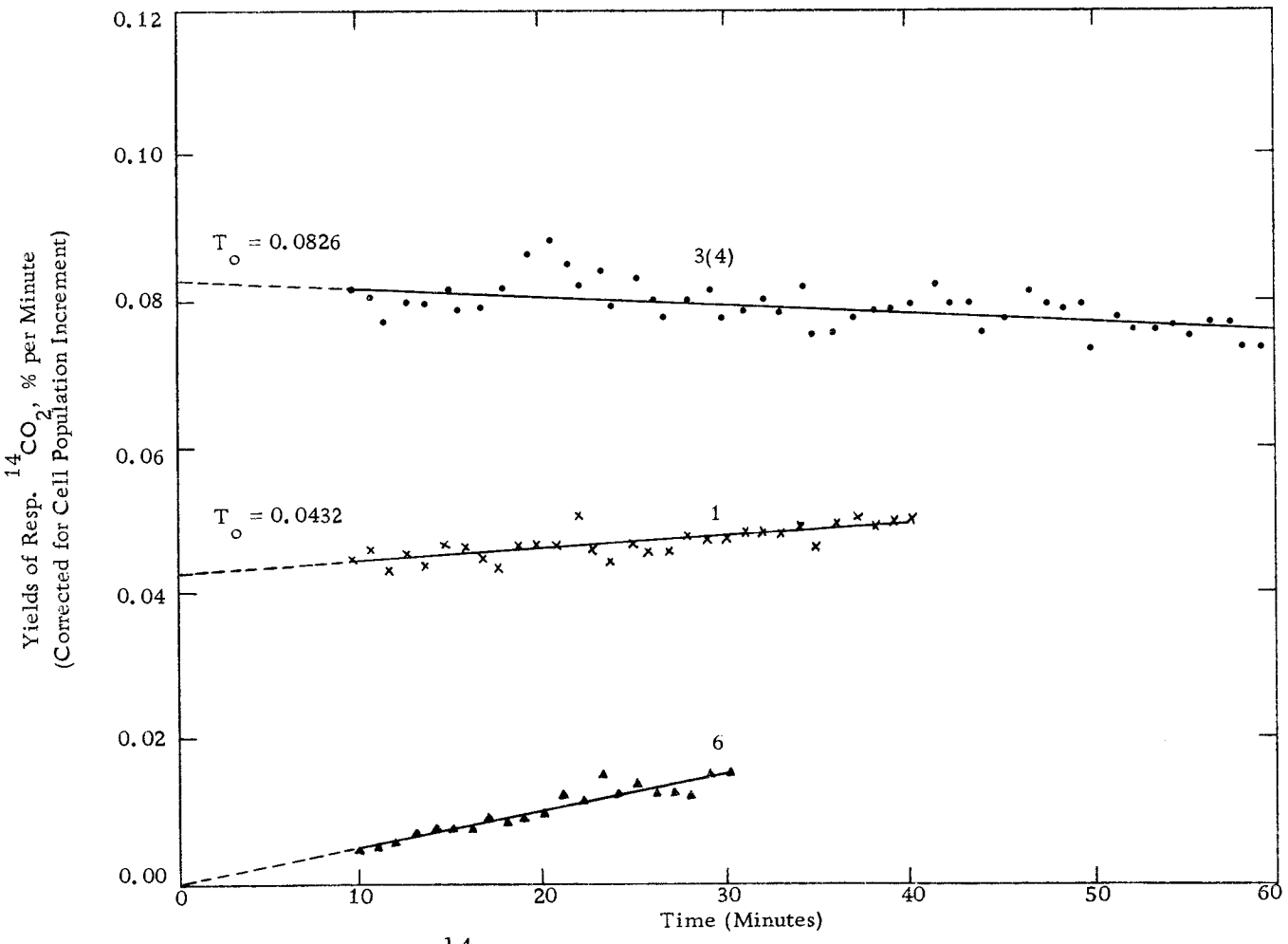

Figure 13. Rate of respiratory $^{14}CO_2$ production from Saccharomyces cerevisiae, in early stationary phase, metabolizing ^{14}C specifically labeled glucose substrates and corrected for cell population increment. (Numerals refer to the labeled position of glucose)

Table 4. Cell populations and substrate levels (chemical and radioactivity) employed in unperturbed culture experiments.

Culture Phase	Time (min)	Cell Counts Per Flask ($\times 10^9$)	Substrate Level Per Flask			
			µmole	Radioactivity (µC)		
				G-1-^{14}C	G-3(4)-^{14}C	G-6-^{14}C
Middle Log	Initial T_o	0.6	774	5.04	3.04	5.69
	60	1.2	746			
Middle Log	T_o	0.5	747	5.04	3.04	4.85
	30	0.8	-			
	52	1.1	719			
Late Log	T_o	1.2	689	5.04	3.04	4.85
	30	1.6	655			
	42	1.6	655			
	56	1.7	641			
Late Log	T_o	1.5	703	4.38	3.04	4.85
	28	1.9	675			
	49	1.9	661			
Early Stationary	T_o	1.7	686	3.76	3.04	4.85
	30	2.0	648			
	42	2.1	635			
	62	2.5	603			

extrapolated yield data can then be used for the calculation of catabolic rates of individual glucose pathways.

The normalized $^{14}CO_2$ yield curve for the glucose-3(4)-^{14}C experiment in Figure 13 displays a slightly negative slope. This decreasing rate of respiratory $^{14}CO_2$ production is attributed to a decrease in the rate of glucose utilization occurring with cells in the early stationary phase. Further evidence supporting this contention will be presented later (Table 5). It must be emphasized that the slightly negative slope of the yield curve in Figure 13 does not cause significant differences in the extrapolated yield data at T_o.

In Figure 13, the $^{14}CO_2$ yield data from the glucose-1-^{14}C experiment and particularly the glucose-6-^{14}C experiment display positive slopes. This observation can be interpreted by considering the extended sequential operation of enzyme reactions associated with the glycolysis-Krebs cycle pathway. In addition to the long sequence of reactions, the pool of the Krebs cycle intermediates may retard the production of $^{14}CO_2$ by a slow turnover. Thus, the production of respiratory $^{14}CO_2$ from glucose-1-^{14}C and glucose-6-^{14}C is delayed and the slope should not reflect the slightly reduced rate of glucose utilization by cells in the early stationary phase.

The extrapolation of $^{14}CO_2$ yield data for the glucose-6-^{14}C experiment leads to 0 as shown in Figure 13. This implies that the extrapolated $^{14}CO_2$ yield data from the glucose-1-^{14}C experiment

represents exclusively the yield data of $^{14}CO_2$ production from C-1 of glucose via the PP pathway. This is not unreasonable since the PP pathway calls for prompt conversion of C-1 of glucose to respiratory $^{14}CO_2$.

The extrapolated yield data originating from C-3(4) and C-1 of glucose through respiratory CO_2 production are not distorted by complicating factors such as hexose reformation and glyceraldehyde-phosphate formation occurring via the PP pathway, triose recombination, and hexose recycling through the PP pathway.

The results of pathway estimation experiments for middle log phase cells are given in Figure 14 which shows uncorrected data and in Figure 15 which shows the normalized results. In Figure 15, the yield of respiratory $^{14}CO_2$ derived from glucose-1-^{14}C and that derived from glucose-3(4)-^{14}C are presented in separate graphs in order to give clearer definition to the normalized curves. The analysis of the data for middle log phase cells is the same as that previously described for early stationary phase cells. However, with cells from middle log phase, the slope of the normalized yield data for the production of $^{14}CO_2$ from C-3(4) of glucose did not display a significant decrease with time. Apparently, cells in this phase of growth are utilizing substrate glucose at a constant rate which is proportional to their concentration for the duration of the experiment. Cells from late log phase produce respiratory $^{14}CO_2$ patterns similar

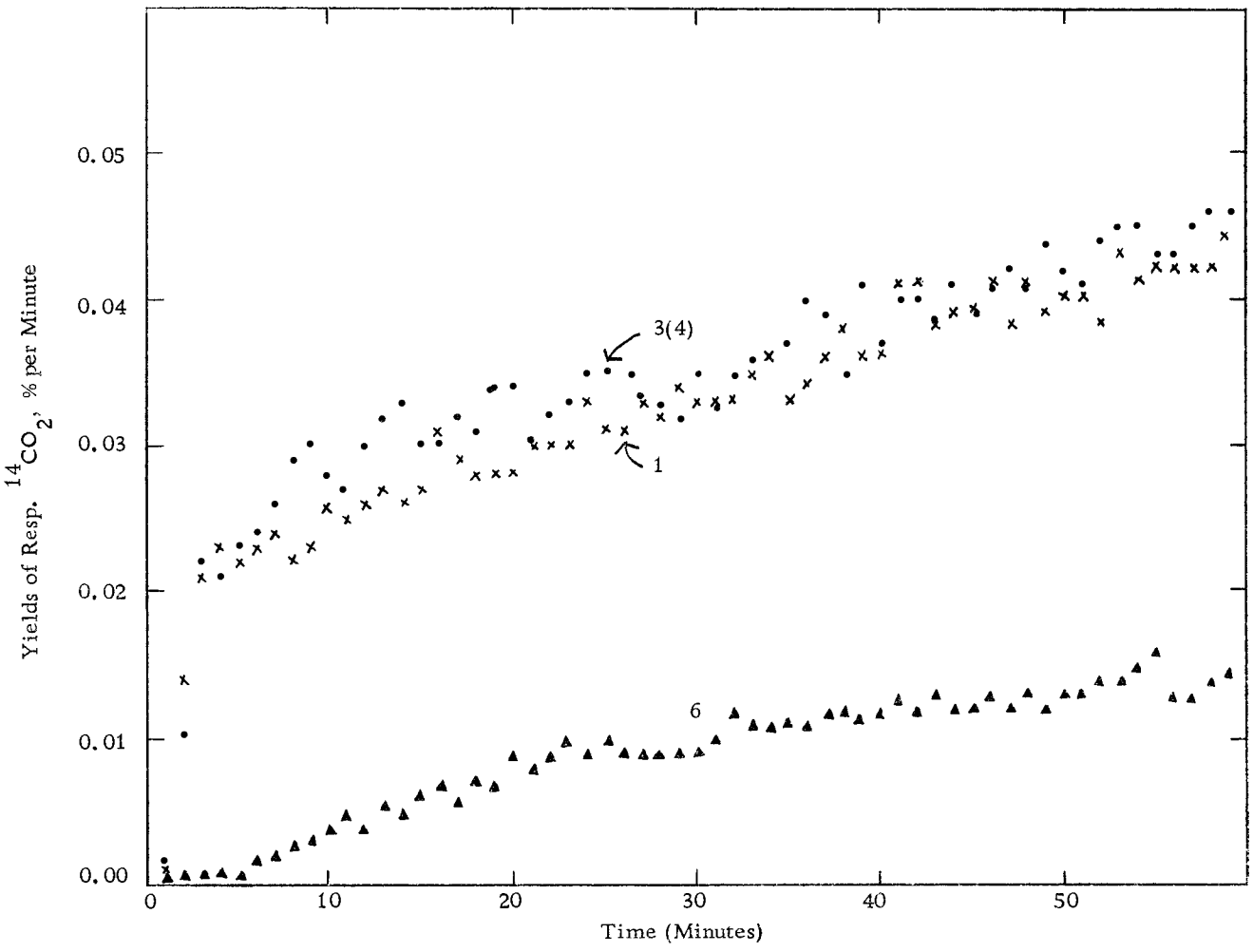

Figure 14. Rate of respiratory $^{14}CO_2$ production from Saccharomyces cerevisiae, in middle log phase, metabolizing ^{14}C specifically labeled glucose substrates. (Numerals refer to the labeled position of glucose)

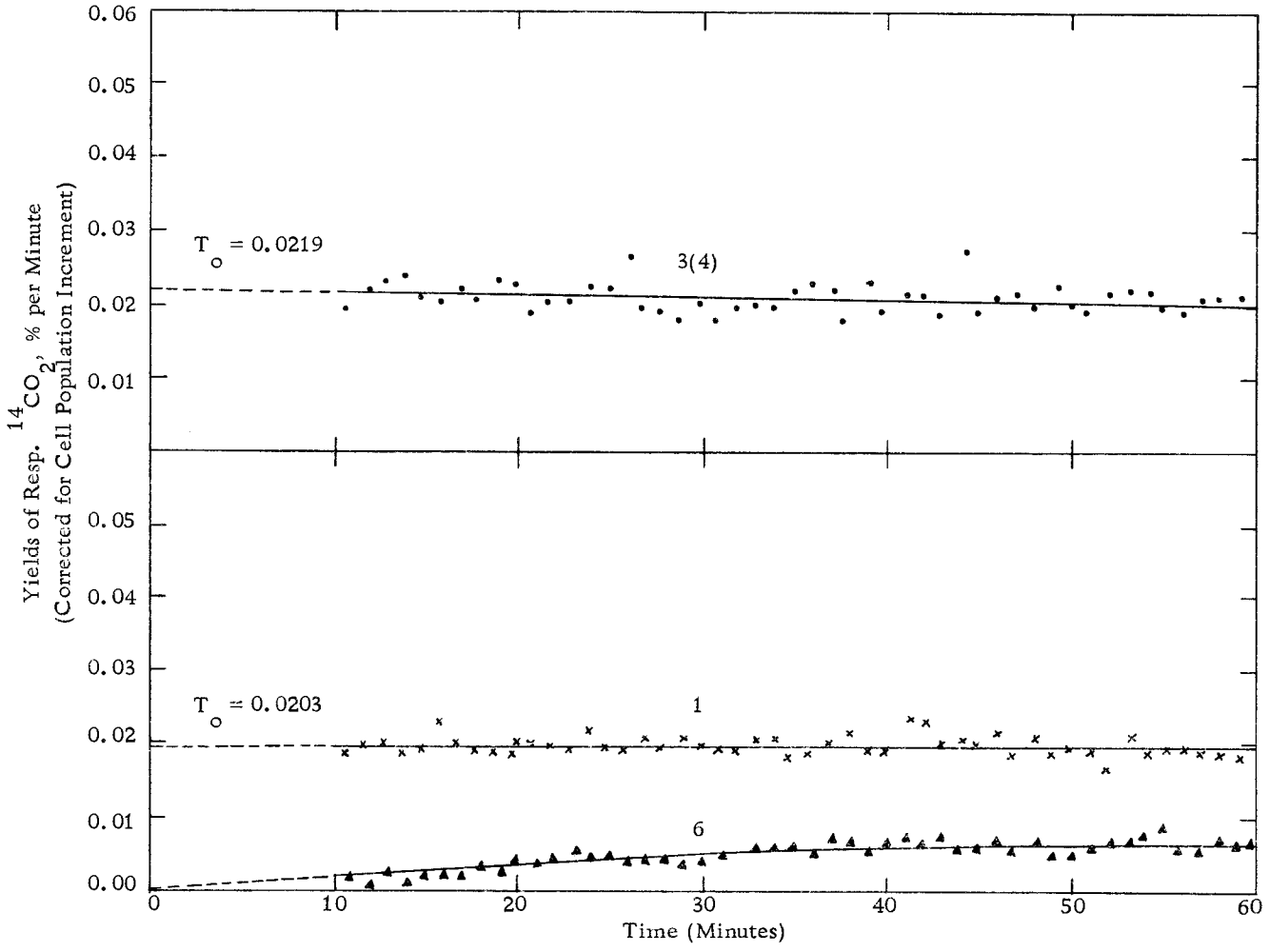

Figure 15. Rate of respiratory $^{14}CO_2$ production from Saccharomyces cerevisiae, in middle log phase, metabolizing ^{14}C specifically labeled glucose substrates and corrected for cell population increment. (Numerals refer to the labeled position of glucose)

to those obtained from cells of the early stationary phase (see Figures 12 and 13).

The extrapolated yield data at T_o can be transformed into an expression of catabolic rate, e.g., yield per minute. The rate data of this type, although related to the yield of respiratory $^{14}CO_2$ over a short period, are not to be confused with the term "cumulative yield" as used by Wang and Krackov (34) nor with the term "specific yield" employed by Katz and Wood (16) in their respective methods for the estimation of glucose pathways. Furthermore, the catabolic rate method allows direct determination of the glycolytic pathway which in older methods was established by difference (34).

All catabolic rate data used in the following calculations are those extrapolated to T_o and normalized for 1×10^9 cells. The glucose disappearance curve, established by periodic sampling of the medium, provides information on the overall glucose assimilation rate of the system. For example, from the data of the early stationary phase experiment shown in Figure 13 and Table 4, the following T_o values are derived:

Cell population = 1.7×10^9 cells per flask containing 9.0 ml.

Total glucose = 686 μmole per flask.

Glucose assimilation rate (G_{ar}) = 0.75 μmole per minute.

Catabolic Rate of the Glycolytic Pathway (G_{er}):

From yield data (Figure 13) of the glucose-3(4)-^{14}C

experiment, radioactivity yield in respiratory $^{14}CO_2$ per minute = 0.083% of total substrate radioactivity or,
0.00083 per minute x 3.04 μC = 0.00252 μC per minute.

Correcting for 10^9 cells,
0.00252 μC per minute ÷ 1.7 x 10^9 cells per flask =
0.00148 μC per minute per 10^9 cells.

The specific activity of substrate glucose is 0.00443 μC per μmole.

Therefore, the catabolic rate of glycolysis is,

$$\frac{0.00148\ \mu C \text{ per minute per } 10^9 \text{ cells}}{0.00443\ \mu C \text{ per } \mu mole} =$$

0.33 μmole per minute per 10^9 cells.

Catabolic Rate of the PP Pathway (G_{pr}):

From yield data (Figure 13) of the glucose-1-^{14}C experiment, radioactivity yield in respiratory $^{14}CO_2$ per minute = 0.043% of total substrate radioactivity,
(same treatment as above)
Therefore, PP pathway catabolic rate =

0.17 μmole per minute per 10^9 cells.

Catabolic Rate of Glucose Metabolism (G_{cat}):

$$G_{cat} = G_{er} + G_{pr} = 0.33 + 0.17 = 0.50 \text{ μmole per minute}$$

Anabolic Rate of Glucose Metabolism (G_{an}):

$$G_{an} = G_{ar} - G_{cat} = 0.75 - 0.50 = 0.25 \text{ μmole per minute}$$

Relative Participation of Glucose Pathways:

$$\text{Glycolytic Pathway } (G_{er}) = \frac{(100)\ (0.33)}{0.75} = 44\%$$

$$\text{PP Pathway } (G_{pr}) = \frac{(100)\ (0.17)}{0.75} = 23\%$$

$$\text{Anabolic Pathway } (G_{an}) = \frac{(100)\ (0.30)}{0.75} = 33\%$$

The calculations presented above have been applied to the yield data of respiratory $^{14}CO_2$ from experiments on middle log, late log and early stationary cultures. The results of these calculations are summarized in Table 5 which presents the individual rates of catabolism, rate of anabolism and the relative participation of glucose metabolic pathways based on glucose assimilation rate.

These data indicate that the rate of glucose utilization (G_{ar}) declines as cells undergo changes in growth physiology from middle log to early stationary phase. Thus, cells in middle log phase utilize glucose at the rate of 0.97 μmole per minute while cells at stationary phase utilize only 0.75 μmole of glucose per minute.

It is of interest to note that the catabolic rate of the PP pathway (G_{pr}) decreases with increasing cell age. Thus, the cells at early stationary phase have a PP catabolic rate of 0.17 μmole per minute which is 36 percent below the catabolic rate of cells in the middle log phase (0.27 μmole per minute). Since the glucose assimilation rate also decreases as cells pass through their growth phases, the participation of PP pathway remains almost constant. It appears that

Table 5. The rates of catabolic and anabolic pathways and the relative participation of pathways for glucose metabolism in unperturbed cultures of Saccharomyces cerevisiae.

Pathway	Rates, μmole per minute			Relative Participation[b]		
	Middle Log[a]	Late Log[a]	Ear. Stat.	Middle Log	Late Log	Ear. Stat.
Glycolytic (G_{er})	0.30	0.31	0.33	31	39	44
PP (G_{pr})	0.27	0.19	0.17	28	24	23
Catabolic (G_{cat})	0.57	0.50	0.50	59	63	67
Anabolic (G_{an})	0.40	0.30	0.25	41	37	33
Glucose Assimilation Rate (G_{ar})[c]	0.97	0.80	0.75	100	100	100

[a] Middle and late log phase values are averages from two experiments; G_{er} and G_{pr} average values agree to within 4%

[b] Relative participation of glucose pathways based on G_{ar}.

[c] G_{ar} average values agree to within 10%

the PP pathway activity in yeast is governed by the overall rate of glucose assimilation which is dependent upon growth physiology.

In contrast, the catabolic rate of the glycolytic pathway remains constant at approximately 0.31 μmole per minute (Table 5) for the three growth phases described. However, since the rate of glucose assimilation decreases as cells transform from middle log to early stationary phase, the glycolytic pathway assumes a more prominent role in relative pathway participation. The anabolic contribution to glucose metabolism decreases from middle log to early stationary phase. The decrease in anabolic rate reflects the reduced need for polysaccharides in cultures whose cell division rate is approaching zero. Thus, there appears to be a change in the direction of increased respiratory activity via the glycolytic-Krebs cycle pathway as cell growth physiology changes from middle log to early stationary phase.

In conclusion, with the use of high resolution radiorespirometry, it is possible to determine directly the catabolic rates of not only the PP pathway, but also the glycolytic pathway. Knowledge of the catabolic rate having been established directly, the anabolic rate of glucose metabolism can be estimated by difference from the glucose assimilation rate. Thus, the direct determination of glucose catabolic pathways under physiologically unperturbed conditions provides more reliable information on the relative participation of

glucose pathways in comparison to conventional methods. Since catabolic rate studies can be conducted within a few minutes, its application to problems in developmental biology and cell growth physiology should provide new information in these fields.

In addition, high resolution radiorespirometry can be applied to physiological studies unrelated to relative glucose pathway participation. For example, oscillatory phenomena (12, 21, 25, 32) can be observed in the respiratory $^{14}CO_2$ curve derived from glucose-3(4)-^{14}C metabolism in yeast (see Figure 16). These phenomena are recognized as important indicators of control mechanisms operative in the glycolytic sequence (6). Similarly, the data obtained in the single dose experiments by means of high resolution radiorespirometry may contain useful information on the transport kinetics of a wide variety of substrates.

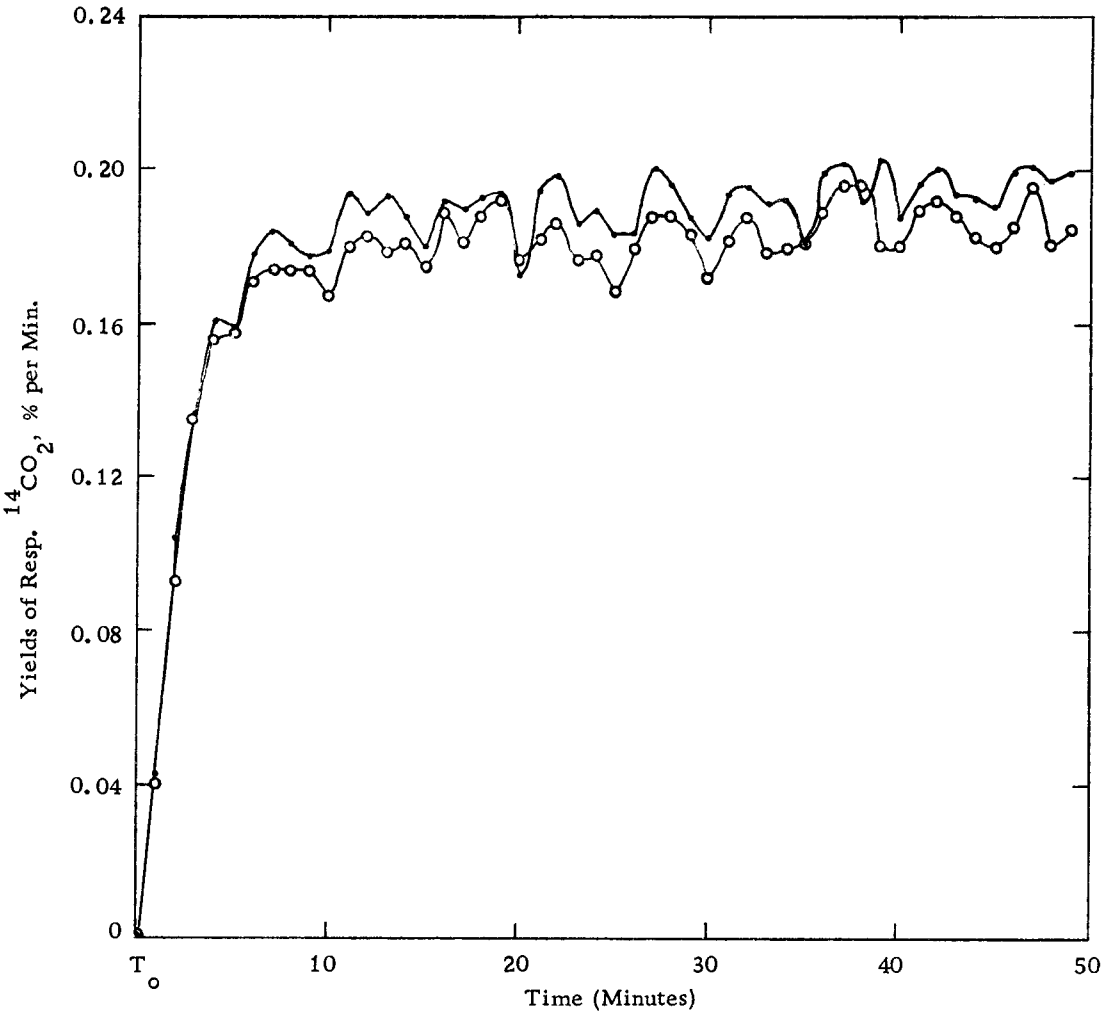

Figure 16. Oscillatory phenomena of respiratory $^{14}CO_2$ from yeast metabolizing glucose-3(4)-^{14}C and revealed by high resolution radiorespirometry.

V. SUMMARY

A unique method for glucose pathway estimation has been developed by the use of high resolution radiorespirometry. With this method physiologically unperturbed yeast cells at metabolic steady-state can be used to determine the catabolic rate of PP and glycolytic pathway directly. The anabolic pathway is determined by difference. The high resolution radiorespirometer can provide new information in the study of transport mechanisms, oscillatory phenomena and developmental biochemistry.

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