Measurement of the Activity of the Hexose Monophosphate Pathway of Glucose Metabolism with the Use of [^{18}O]Glucose. Variations in Its Activity in *Escherichia coli* with Growth Conditions*

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ABSTRACT: A method for measuring the hexose monophosphate pathway (HMP) with the use of [1-^{18}O]-glucose is described. With this method we find that about 25% of the glucose metabolized by *Escherichia coli* during active growth in minimal medium passes through the HMP, and that this value remains constant for a range of glucose concentrations. The HMP becomes significantly less active as the cells enter the stationary phase, or if growth is interrupted by exhaustion of the nitrogenous source, or by removal of methionine (in the case of a methionine-requiring mutant) or by addition of colicine K. Anaerobic growth also leads to lower HMP participation in glucose catabolism. The HMP is less active when minimal medium is enriched with casein hydrolysate or yeast extract. Growth in nutrient broth–glucose results in enhanced HMP activity; the addition of 6.5×10^{-2} M sodium phosphate or 0.8% NaCl abolishes this enhancement and results in a level of HMP activity which is the same as that in the enriched minimal medium. Levels of HMP activity in strain Br 15 which was first adapted to growth on ribose are the same in the presence and absence of ribose. It is suggested that the activity of the HMP in *E. coli* is regulated by the availability of oxidized nicotinamide–adenine dinucleotide phosphate (NADP$^+$).

The hexose monophosphate pathway (HMP)[1] of glucose catabolism is widely distributed (Hollman, 1964) and its products, NADPH, and ribose 5-phosphate are essential to growth. Results from a number of laboratories (Cohen, 1951; Wang *et al.*, 1958; Allen and Powelson, 1958; Jones, 1961; Rittenberg and Ponticorvo, 1962; Scott and Cohen, 1953) show that the HMP accounts for a variable fraction of the glucose utilized by *Escherichia coli*. We have investigated this variation with the use of a new method for measuring the HMP which combines the specific yield technique systematized by Wang *et al.* (1958) and the use of [^{18}O]glucose introduced by Rittenberg and Ponticorvo (1962).

When [1-^{18}O]glucose is metabolized by the Embden–Meyerhof pathway, the ^{18}O will be lost as water in the dehydration of 2-phosphoglyceric acid. For each mole of this substrate decarboxylated by way of the HMP, however, a mole of $C^{18}O_2$ will be formed. To evaluate the HMP, the CO_2 produced by the cells from [1-^{18}O]glucose is collected quantitatively, and the glucose consumed during the collection period is measured. After correction is made for the exchange of the oxygens of CO_2 with those of water (Mills and Urey, 1940), the ratio of isotope recovered in the CO_2 to that in the [1-^{18}O]glucose consumed (the corrected specific yield)[2] represents the fraction of the substrate metabolized by the HMP.

This procedure measures only the passage of [1-^{18}O]-glucose through the HMP. Nonisotopic glucose or glucose 6-phosphate arising from gluconeogenesis or from the reconversion of pentose phosphates to hexose phosphate will not contribute to either term of the ratio described above, nor, therefore, to the HMP estimate.

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[1] Abbreviations used: HMP, hexose monophosphate pathway; NADPH and NADP$^+$, reduced and oxidized nicotinamide–adenine dinucleotide phosphate; TCA, trichloroacetic acid; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

[2] The term *specific yield* is defined as (gram-atoms of ^{18}O recovered in CO_2/gram-atoms of ^{18}O in substrate consumed) $\times$ 100 and corrected specific yield is defined as the specific yield adjusted for the exchange with water. The magnitude of the HMP is further defined as equal to the per cent of substrate glucose decarboxylated by the action of 6-phosphogluconate dehydrogenase. This definition of HMP is in accord with that used by Cohen (1951), Dawes and Holmes (1958), and Wang *et al.* (1958, 1962) at times, as well as by many other workers.

Materials and Methods

Bacterial Strains. E. coli strains WT, W113-3 (M^-), and K12 y 87 ($M^-B^-\lambda^+$) were supplied by Dr. Ernest Borek. Strain Br 15 was made available to us through the courtesy of Dr. S. S. Cohen. A colicine K sensitive variety of strain B and colicine K, prepared according to the method of Goebel and Barry (1958), was furnished by Dr. John Foulds. All strains were maintained on Penassay agar slants.

Media. The salts' medium C of Roberts *et al.* (1957) was used. For some anaerobic experiments, 6 g/l. of ammonium nitrate was substituted for the usual 2 g/l. of ammonium chloride. Enriched media were made either with Bacto nutrient broth, or were composed of Roberts' C medium to which had been added the suggested concentration of nutrient broth, 0.2% Bacto casein hydrolysate, 0.2% Bacto yeast extract, or 0.2% of both. All media were adjusted to pH 7.0 $\pm$ 0.05 immediately before use.

Growth of Cells. Cells were inoculated from slants into minimal medium and grown with vigorous shaking on limiting glucose. A small inoculum from such a culture was then grown up again in C medium on limiting glucose. This latter culture was used as the stock culture, and was kept in a refrigerator for not more than 1 week. Fresh overnight cultures, again grown on limiting glucose, were prepared for each experiment from a small inoculum of the stock cells. For anaerobic experiments, the inoculum was grown under an atmosphere of prepurified nitrogen which had been passed through a solution of vanadous sulfate (Meites and Meites, 1948) to remove traces of oxygen. All incubations were at 37°.

Growth was followed in a Klett colorimeter with a 660 filter. In some of the early experiments, viable counts were also made. In the range of importance to these experiments, the Klett readings were approximately linear with cell number. Thirty Klett units correspond to approximately 10^8 cells/ml; 100 Klett units to about 7×10^8 cells/ml. No significant contamination was ever observed at the end of the experiments.

Labeled Substrates. [1-^{14}C]Gluconate and [1-^{14}C]-ribose were gifts of Dr. D. B. Sprinson. [1-^{18}O]Glucose and [6-^{18}O]glucose were prepared as described previously (Rittenberg *et al.*, 1961).

[1-^{18}O]Gluconate was prepared from the δ-lactone by exchange with $H_2{}^{18}O$. Recrystallized δ-lactone (5 g) was suspended in 5 ml of [^{18}O]H_2O (7.34% excess). After 5 days at room temperature, concentrated [^{18}O]NaOH was added to neutrality. The water was removed *in vacuo* and the residue was taken up in 10 ml of hot water, filtered, cooled, and 75 ml of ethanol was added. An oil formed which slowly crystallized. The crystals were filtered off, washed with ethanol–water (7:1), ethanol, ether, dried in air overnight, and finally dried *in vacuo* at 60° over P_2O_5. This sodium gluconate contained 1.80 atom % excess ^{18}O. The analysis was performed by conversion to CO_2 by a modification of the $HgCl_2$–$Hg(CN)_2$ method of Anbar and Guttman (1959).

The aldobenzimidazole of this gluconate was prepared by the method of Moore and Link (1940), and contained no ^{18}O. This is consistent with the view that all of the ^{18}O was in the carboxyl group of the gluconate. The carboxyl oxygen atoms, therefore, contained 6.3% ^{18}O. No back-exchange to normal water occurred in 24 hr at 37°.

Method of Collecting and Measuring the CO_2 Produced by the Organisms

1. Experiments in Which only the ^{18}O Concentration in the CO_2 Was Measured. Air under pressure was passed in sequence through a flow meter, a tube filled with solid $CaSO_4$, two tubes packed with ascarite, then through water to rehydrate it, and into the incubation flask. The gas stream from the incubation flask was passed through a trap immersed in Dry Ice into the collecting trap cooled in liquid nitrogen. The incubation vessel (see Figure 1A) for these experiments consisted of a large test tube to which had been attached a smaller test tube which would fit a Klett colorimeter.

The collecting trap (see Figure 1B) consisted of two long, narrow, U tubes joined by a stopcock. The purpose of the second U tube was to trap any CO_2 that might diffuse in against the prevailing flow direction. At the end of a collection period, both stopcocks were closed, and the trap, still in liquid nitrogen, was transferred to the inlet manifold of a 180° mass spectrometer. Air was pumped out, the liquid nitrogen bath was replaced by Dry Ice, and the frozen CO_2 expanded into the inlet system of the spectrometer.

2. Experiments in Which the Specific Yield of ^{18}O Was Determined. The system was the same with the following exceptions. (1) The air was drawn through the system with a water pump aspirator. The gas outlet was through a water-cooled condensor to minimize loss of water by evaporation. (2) The collecting trap (see Figure 1C) consisted of a 140-cm length of 4-mm i.d. tubing which had been bent into a rectangular coil, and fitted with a stopcock at either end as well as a 10/32 male joint at one end. A small plug of glass wool was inserted near the exit end of the trap, but well below the liquid nitrogen level. This served to prevent the loss of small particles of solid CO_2. This trap was followed by one of the double U tubes described above (1B). The first half of the double U served as a control, to make sure that the collection was indeed quantitative; the second half safeguarded against atmospheric CO_2.

The pressure in the collecting trap was held at about 100 mm by a vacuum controller to avoid condensation of liquid oxygen in the trap. The rate of flow through the system was controlled by a stopcock at the entrance to the collecting trap, so that the pressure in the incubation vessel was close to atmospheric.

Between the Dry-Ice trap and the collecting trap, and also between the collecting trap and the double U tube, three-way stopcocks were inserted so that the collecting traps could be changed without interrupting the gas flow. At the conclusion of a collection period,

the trap was replaced by another, and then connected to a vacuum line (see Figure 1C). Air was pumped out, the liquid nitrogen was removed, and the solid CO_2 was transferred quantitatively to a measured volume. From the pressure of CO_2 developed, the amount of CO_2 formed was calculated. The gas was then transferred to the inlet system of the mass spectrometer and the ^{18}O concentration was measured.

TABLE 1: Specific Yield of ^{18}O and ^{14}C in the CO_2 Formed by *E. coli* WT Growing on [1-^{18}O]Gluconate and [1-^{14}C]Gluconate.[a]

Condition	^{18}O Sp Yield (%)	^{14}C Sp Yield (%)
Normal		
Expt 1	25	—
Expt 2	26	—
Expt 3 (no ascarite)[b]	25	—
Expt 4	24	84
Expt 5	—	86
Nitrogen-free medium	33	
Cells in stationary phase	23	

[a] Cells were grown as described in the materials and methods section, but the inoculum for each experiment was grown on gluconate rather than glucose as a carbon source. The experimental cultures received 0.5 or 1 mg/ml of sodium gluconate either as [1-^{18}O]gluconate, 1.80 atom % excess, or with 0.1 μc of calcium [1-^{14}C]-gluconate. The specific yield was calculated from the cumulative recovery of the isotope after growth had ceased and the evolution of isotopic CO_2 had declined to negligible levels (see Figure 2) and assumed total consumption of substrate. The stationary phase culture was grown overnight on 10 mg/ml of sodium gluconate, harvested, and resuspended in the same volume. The sweep rate for all experiments was 250 cc of air/min. The CO_2 from the ^{14}C experiments was handled in the same way as that from the ^{18}O experiments, except that instead of condensing the measured gas in a bulb, it was condensed in a small flask over frozen 1 N Hyamine–OH (diisobutylcresoxyethoxyethyldimethylbenzylammonium hydroxide) in methanol. The flask was closed off, allowed to warm and to stand overnight with stirring. The mixture was then rinsed into a volumetric flask with methanol, to give a final concentration of 0.25 N Hyamine. A 5-ml aliquot of this solution was added to 10 ml of a PPO–POPOP–toluene scintillating mixture. An aliquot of the starting [1-^{14}C]gluconate was counted in the same solvent mixture used for the $^{14}CO_2$. After these counts had been recorded, a second aliquot of the starting material was added to the vial containing the $^{14}CO_2$ to serve as an internal standard. At the end of the experiment a portion of the culture (medium and cells) was suspended and counted in Bray's (1960) solution as was an aliquot of the medium freed of cells. Total ^{14}C recoveries were 96% for expt 4 and 98% for expt 5.
[b] Atmospheric CO_2 not removed from entering air.

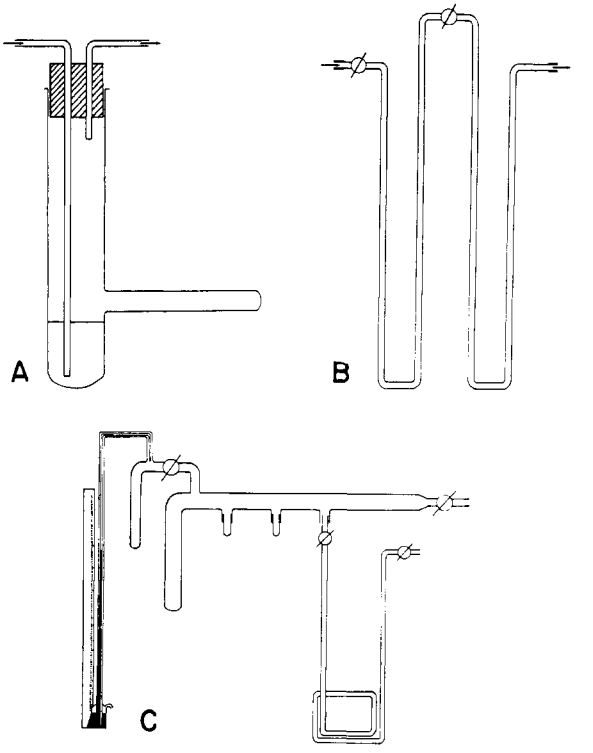

FIGURE 1: Apparatus. (A) Incubation vessel. (B) Double U trap. (C) Vacuum manifold, with coiled trap attached.

Glucose Measurements

Glucose consumption was measured by the glucose oxidase method of Huggett and Nixon (1957), slightly modified. An appropriate aliquot of the culture was filtered through a Millipore type HA filter, and the filtrate was frozen pending analysis. The reaction mixture usually consisted of 0.2 ml of sample, 1 ml of H_2O, and 5 ml of reagent. When greater sensitivity was required, the sample size was increased and the amount of water was decreased. Absorbancy readings at 400 mμ were made in a Beckman DU spectrophotometer. Glucose oxidase, horseradish peroxidase, and *o*-dianisidine were purchased from Worthington Biochemical Co.

Results

Measurement of the Loss of ^{18}O from [^{18}O]CO_2 to Water. About 85% of the ^{14}C present at the C_1 of gluconate was recovered in the CO_2 formed by actively growing *E. coli* (see Table I and also Cohen (1951) and Jones (1961)). The corresponding recovery of ^{18}O from [1-^{18}O]gluconate is only 25% (see Table I and Figure 2). Horecker and Smyrniotis (1953) and Brodie and Lipmann (1955) have shown that at physiological pH, 6-phosphogluconate is not directly reduced to

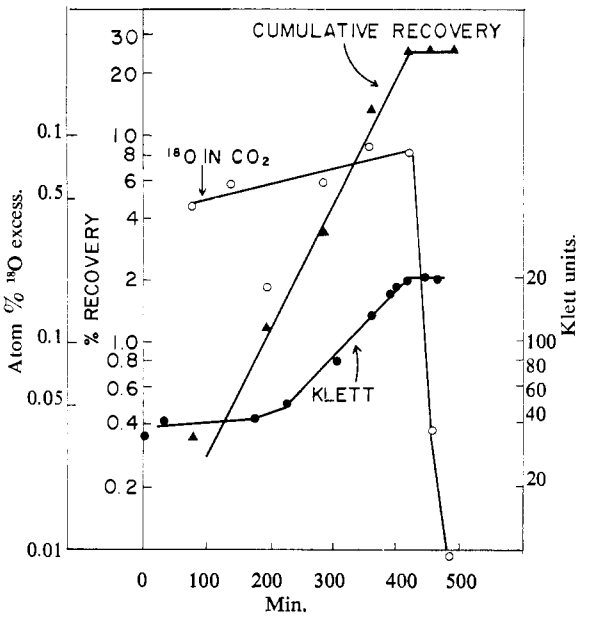

FIGURE 2: Cumulative recovery and concentration of ^{18}O in the CO_2 formed from [1-^{18}O]gluconate by *E. coli* WT. Details of this experiment are given in Table I, expt 2.

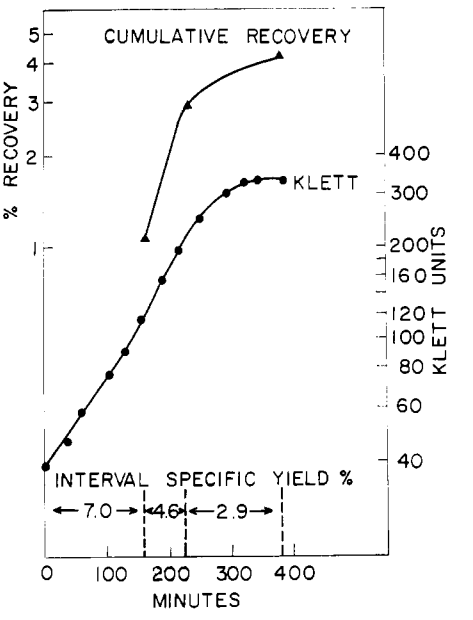

FIGURE 3: Specific yield and cumulative recovery of ^{18}O in the CO_2 formed by *E. coli* WT growing in C medium on [1-^{18}O]glucose (10.5 atom % excess). The initial glucose concentration was 3 mg/ml. The sweep rate was 250 cc of air/min.

glucose 6-phosphate. The metabolism of gluconate must, therefore, occur by either the HMP, or by the Entner–Doudoroff pathway. The former involves an obligatory conversion of the C_1 of gluconate to CO_2; the latter results in the formation of pyruvate, in which the carboxyl carbon is derived from carbon one of gluconate. Both of these routes for the formation CO_2 from carbon one of gluconate should lead to the formation of $^{14}CO_2$ from [1-^{14}C]gluconate and $C^{18}O_2$ from [1-^{18}O]gluconate, in equal yields, if there were no exchange of $C^{18}O_2$ with water. A comparison of ^{14}C with ^{18}O specific yields should, therefore, serve as a measure of the amount of exchange with water that does take place. Table I shows that such an exchange takes place, and that it is of considerable magnitude. The data of a typical experiment is shown in Figure 2.

The ratio of the specific yields of $^{14}CO_2$ from [1-^{14}C]gluconate to $C^{18}O_2$ from [1-^{18}O]gluconate is 3.5 (86:25). This indicates that 71% [100(1 − $1/3.5$)] of the ^{18}O in the CO_2 formed by the cells is lost to the medium by exchange. When cells are deprived of a nitrogenous source, more labeled CO_2 is released from the carboxyl of gluconate than when ammonia is present (see Table I). This has been noted by Cohen (1951), and by Jones (1961) also.

Participation of the Entner–Doudoroff (1952) pathway in gluconate metabolism would lead to the formation of pyruvic acid with isotope at C_1. The metabolic parallel between [1-^{14}C]- and [1-^{18}O]gluconate would still be conserved, however, since decarboxylation of pyruvate derived from either source would lead to isotopic CO_2.

Measurement of the HMP During Growth in a Salts Medium. Once the factor (3.5) which measures the loss of ^{18}O from CO_2 has been established, the specific yield can be corrected to give the size of the HMP pathway. Figure 3 shows a measurement of interval specific yields of ^{18}O during three phases of growth on glucose. The specific yield declines as the culture becomes more dense, this being particularly marked during the last period, in which growth is no longer exponential. These three specific yields correspond to HMP participations of 25, 16, and 10% (specific yield × 3.5).

It is difficult to measure interval specific yields over short time periods. For this reason we measured the concentration of ^{18}O in the CO_2 when it seemed desirable to learn something of the rate at which the HMP activity changes.

To relate changes in the ^{18}O concentration to changes in the activity of the HMP requires, however, the assumption that CO_2 production per cell from other sources remains constant. This assumption is approximately valid since the results agree with the experiments in which specific yields were determined. The ^{18}O concentration in the CO_2 formed from [1-^{18}O]-glucose rises rapidly as the cells pass into the phase of exponential growth (see Figure 4), remains high during the growth period, and then rapidly declines as the system goes into stationary phase.

We have calculated from the rate of exchange of [1-^{18}O]glucose with water (Rittenberg and Graff, 1958) that the ^{18}O concentration of the glucose would decline by about 2.8%/hr, a rate which would very

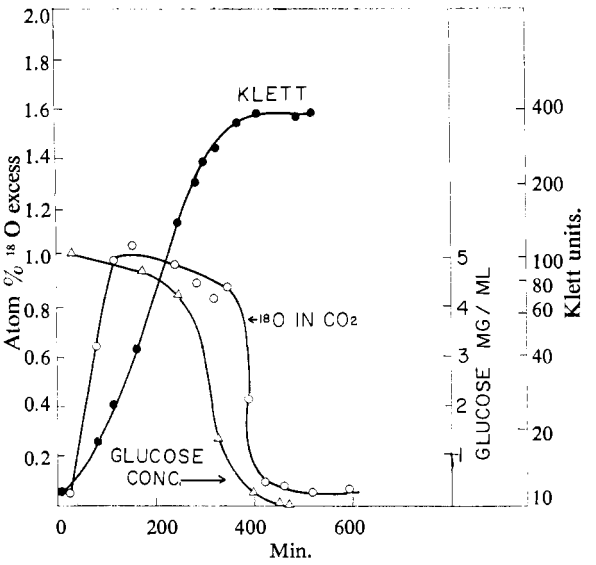

FIGURE 4: ^{18}O concentration of the CO_2 formed by *E. coli* WT from (9.94 atom % excess) [1-^{18}O]glucose in C medium. Cells were grown as described in the Experimental Section, except that the last transfer was in medium containing [1-^{18}O]glucose as carbon source. The sweep rate was 800 cc of air/min.

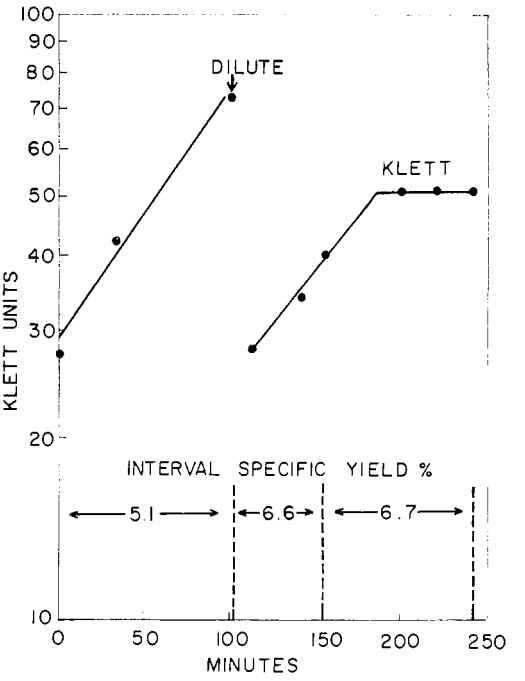

FIGURE 5: Specific yield of ^{18}O in the CO_2 formed by *E. coli* WT growing in C medium on [1-^{18}O]glucose (10.52 atom % excess) under conditions of declining glucose concentrations and limited cell density. The initial volume was 6 ml, the initial glucose concentration, 1.1 mg/ml. At 103 min 20 ml of fresh medium was added, and the glucose concentration was then 0.16 mg/ml. At 155 min the glucose concentration was 0.046 mg/ml. The sweep rate was 250 cc of air/min.

nearly account for the slope of the central portion of the ^{18}O concentration curve in Figure 4.

Since it appeared that the HMP activity declines as cultures approach stationary phase, we allowed cultures of *E. coli* to grow, on [1-^{18}O]glucose, from a density having an absorbancy of about 30 Klett units to a density not exceeding 90 Klett units. These cultures were then diluted with more medium, which in some instances contained additional [1-^{18}O]glucose, and in other instances did not. Growth continued. No significant differences were found in the specific yield before and after dilution, whether fresh [1-^{18}O]-glucose was added or not. Figure 5 gives the results of an experiment in which no glucose was added, and in which the dilution of the original medium was considerable. We conclude from these experiments that the decline in HMP with continued growth is a direct consequence of increases in cell density, and is not attributable to "cellular age" or declining glucose concentrations.

We have measured the HMP during exponential growth. The average of the specific yield of ^{18}O during 18 intervals from 12 experiments was 6.8 ± 0.24% (mean standard deviation) corresponding to an HMP of 24% (6.8 × 3.5). In all the measurements the cell density was between 30 and 90 Klett units, the medium was Roberts' C, and the aeration rate was 250 cc/min. Strains W, B, and K of *E. coli* were used.

In order to confirm that the decline in HMP at high cell densities was not the consequence of the accumulation of a repressing substance, we incubated cells which had been allowed to reach stationary phase in fresh medium with [1-^{18}O]glucose without reducing the cell density. The results (see Table II) show that the HMP continues low under these conditions. The fraction of glucose passing through the HMP in *E. coli* as measured by various methods is tabulated in Table III.

TABLE II: Specific Yield of ^{18}O in the CO_2 Formed from [1-^{18}O]Glucose by *E. coli* WT in Stationary Phase.[a]

Expt	Sp Yield (%)	HMP (%)	Optical Density Klett Units: Beginning	Optical Density Klett Units: End
1	2.9	10	254	295
2	1.6	5	228	252

[a] Cells were grown as described in the Experimental Section except that the overnight culture just prior to the experiment was made with 0.4% glucose. The cells were harvested, resuspended in an equal volume of C medium with 0.5 mg/ml of [1-^{18}O]glucose. The sweep rate was 250 cc of air/min.

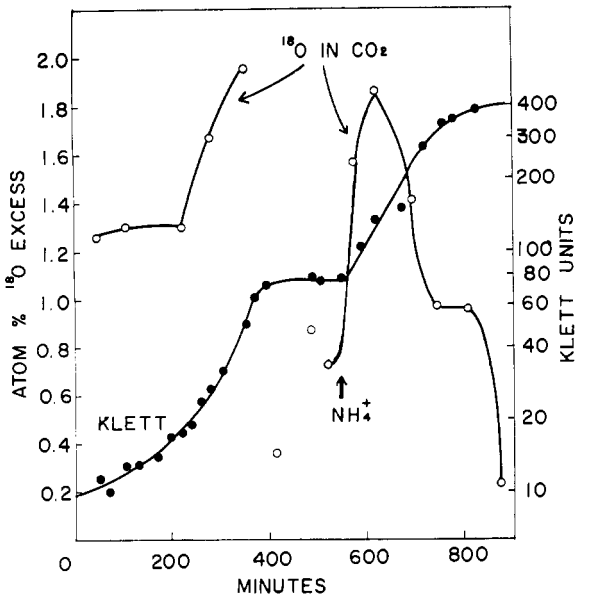

FIGURE 6: Concentration of ^{18}O in the CO_2 formed by *E. coli* WT growing in Roberts' N medium on [1-^{18}O]-glucose (9.96 atom % excess) before, during, and after a period of nitrogen deprivation. The innoculum was grown as described in the Experimental Section, but the last culture was grown on [1-^{18}O]glucose. The medium initially contained 1.5 μmoles/ml of NH_4^+ and 1 mg/ml of glucose. At 325 min an additional 4 mg/ml of [1-^{18}O]glucose was added. At 547 min an amount of NH_4^+ was added which served to bring the medium to the concentration of NH_4^+ usual for C medium. At 778 min there remained 0.94 mg/ml of glucose in the medium. The sweep rate was about 800 cc of air/min.

The low results of Jones (1961), whose methods were very similar to those used by Wang *et al.* (1958), were obtained from cells grown in salts medium to which yeast extract had been added. We will show that the HMP is depressed by yeast extract.

Specific Yield of $C^{18}O_2$ from [6-^{18}O]Glucose. [6-^{18}O]-Glucose was used to test the prediction that the oxygen at C_3 of triose phosphate will not be incorporated into $C^{18}O_2$, and to measure the extent to which resynthesis of glucose from triose results in an interchange of ^{18}O from position 6 to 1 of glucose. The specific yield of ^{18}O in the CO_2 formed from [6-^{18}O]glucose by a growing culture of *E. coli* WT was 0.22%. The conditions used for this experiment, which was performed twice, were the same as those used when the specific yield of ^{18}O from [1-^{18}O]glucose was measured. This low specific yield shows that there is little or no production of $C^{18}O_2$ from [3-^{18}O]triose, and that there was, at most, a transfer of 2.8% of the label originally at C_6 to C_1.[3] Our results here are consonant with the observations of Szynkiewicz *et al.* (1961) who noted that carbon 5 of glucose isolated from the glycogen of glucose-adapted *E. coli* grown on [2-^{14}C]glucose had a radioisotope content equal to 3.8% of that of carbon 2. Sable and Cassisi (1962) observed the same in glucose-adapted resting *E. coli.*

TABLE III: Tabulation of Glucose Passage through HMP.

Type of Measurement	HMP (%)	Ref
^{14}C recoveries	14–37	Cohen, 1951
^{14}C specific yields	28	Wang *et al.*, 1958
^{14}C specific yields	16 or 22	Jones, 1961
^{18}O concentration	10–30	Rittenberg and Ponticorvo, 1961
Enzyme assay	40 max	Scott and Cohen, 1953
^{18}O specific yields	24	Present work

An incidental result of this experiment is the conclusion that no more than 1% (0.2 × 3.5)/0.7 of the substrate glucose passed through the glucuronic acid pathway which has been described by Ashwell *et al.* (1958), since every mole of glucose that goes through this pathway should give rise ultimately to about 0.7 mole of CO_2 derived from carbon 6. The factor 0.7 represents the conversion of the C_1 of pyruvate to CO_2 (Jones, 1961).

Effect of Nitrogen or Methionine Starvation or Addition of Colicine K. Our observation that the HMP declines as cells reach the stationary phase, together with Cohen's report (1951) that it is not active during nitrogen deprivation, suggested that the HMP decline was a general phenomenon associated with cessation of growth. We examined the behavior of cells which had been starved of a nitrogenous source, deprived of an essential amino acid (methionine), or treated with colicine K.

When the cells exhaust a limited nitrogen source in the presence of an adequate supply of glucose, the ^{18}O concentration of the CO_2 and the specific yield fall (see Figure 6 and Table IV). As the cells enter the phase of rapid growth the ^{18}O concentration of the CO_2 rises (see Figure 6) and on cessation of growth drops rapidly. Addition of nitrogen initiates further growth and an increase of ^{18}O in the CO_2. The data on specific yields in a period of nitrogen deprivation (see Table IV) demonstrates that the above results are due to a decrease in HMP activity.

[3] The specific yield of ^{18}O in the CO_2 formed from [1-^{18}O]-glucose was about 7%, and in the CO_2 formed from [6-^{18}O]glucose it was 0.2%. Since the growth conditions for these two experiments were similar, the activity of the HMP should have been the same in each of the two cases. This would mean that the ratio of the specific yields would reflect the ratios in the ^{18}O concentrations at C_1 of [1-^{18}O]glucose and [6-^{18}O]glucose. Thus, in the C_1 of glucose 6-phosphate formed from [6-^{18}O]glucose there will have been (0.2/7.0) × 100% = 2.8 of the ^{18}O present at C_6.

TABLE IV: Specific Yield of ^{18}O in the CO_2 Formed from [1-^{18}O]Glucose by *E. coli* WT before, during, and after a Period of Nitrogen Deprivation.[a]

	Sp Yield (%)	HMP (%)
Before N deprivation (0–116 min)	8.1	28
During N deprivation (116–230 min)	3.6	13
After N deprivation		
Period 1 (230–302 min)	5.3	19
Period 2 (302–440 min)	5.1	18

[a] The innoculum was grown as described in the Experimental Section. The cells were initially suspended in 10 ml of N medium containing, in addition, 1.5 μmoles/ml of NH_4^+ and 1.1 mg/ml of [$1^{18}O$]glucose (9.52 atom % excess). At 116 min, after growth had stopped and had reached 97 Klett units, 10 ml of N medium containing 1.09 mg/ml of [1-^{18}O]glucose was added. At 230 min an amount of NH_4^+ was added which sufficed to bring the medium to the concentration of NH_4^+ usual for C medium. At 422 min 20 ml of C medium were added. The sweep rate was 250 cc/min.

TABLE V: Specific Yield of ^{18}O in the CO_2 Formed by *E. coli* W 113-3 from [1-^{18}O]Glucose before, during, and after Methionine Starvation.[a]

	Before	During	After
Experiment 1			
Specific yield (%)	5.4	2.8	5.0
HMP (%)	19	9.8	17
Atom % excess ^{18}O in CO_2	1.2	0.24	0.82
Experiment 2			
Specific yield (%)	6.2	1.5	5.3
HMP (%)	22	5.2	18
Atom % excess ^{18}O in CO_2	1.3	0.27	0.57

[a] A culture of strain W 113-3M$^-$ was grown as described in the Experimental Section, on C medium supplemented with 30 mg/ml of DL-methionine. The overnight culture was allowed to double on unlabeled glucose prior to the start of the experiment. The starting experimental culture was suspended in 10 ml of C medium containing 30 μg/ml of DL-methionine and 1 mg/ml of [1-^{18}O]glucose (10.52 atoms % excess). When absorbancy was 90 Klett units, they were removed, centrifuged, washed twice, and resuspended in 20 ml of C medium containing 1 mg/ml of [1-^{18}O]glucose but no methionine. After 150-min incubation enough DL-methionine was added to bring the concentration to 30 μg/ml. The sweep rate was 250 cc/min.

The effect of the absence of growth due to a deficiency of an essential amino acid was studied in two methionineless mutants of *E. coli*. The first, W 113-3, in common with the majority of amino acid requiring mutants, promptly ceases RNA synthesis on removal of methionine from the medium. The second, K12 Y87 (identical in all respects with K12 W6) has been shown by Borek *et al.* (1955) to accumulate RNA during methionine starvation.

When W 113-3 was deprived of methionine (see Table V) the participation of the HMP in glucose catabolism was reduced. However, it continues to utilize glucose and to produce CO_2 at an appreciable rate. Figure 7 shows the kinetics of the effect of the removal and subsequent addition of methionine to a culture of W 113-3. Almost identical results were obtained with the "relaxed" strain K12 Y87.

A decline in HMP catabolism of glucose is not incompatible with continued RNA synthesis by strain K12 Y87. Roberts *et al.* (1957) have shown that about 5% of the ^{14}C from uniformly labeled [^{14}C]glucose is incorporated into nucleic acids (hot TCA-soluble portion of the cell). About 60% of this, or 3%, should be in the ribose and deoxyribose moiety. By this calculation, pentose synthesis by the HMP normally exceeds cellular requirements for ribose. An alternative route to ribose exists, moreover, in the transaldolase–transketolase pathway.

The addition of colicine K to a growing culture of a sensitive strain of *E. coli* B rapidly stops growth and lowers the ^{18}O concentration of the CO_2 (see Figure 8). The decline in ^{18}O concentration is limited and remains at its depressed but nonzero level for some time. Colicine K treated cells are not killed, since its effects can be reversed by the addition of trypsin to the medium (Nomura and Nakamura, 1962).

Two almost exactly similar experiments in which the specific yields rather than ^{18}O concentrations were measured gave the following results: specific yield before addition of colicine K 5.5 and 6.8%; after addition of colicine K 3.3 and 3.7%. These figures correspond to a HMP participation of 19 and 24% (5.5 or 6.8 × 3.5) before addition of the colicine and 11 and 13% (3.3 or 3.7 × 3.5) after. This again demonstrates that the changes in ^{18}O concentration were due to changes in HMP activity.

Effect of Ribose. E. coli Br 15 (Cohen and Raff, 1951) are capable of using ribose as a sole carbon source. A culture previously adapted to ribose was grown in a medium containing ribose and [1-^{18}O]glucose. The activity of the HMP in these cells is not diminished by the presence of ribose in the medium (see Table VI). In a control experiment, ribose-adapted cells were grown in the presence of [1-^{14}C]ribose (2.57 mg/ml, 0.2 μc/ml) together with glucose (1.22 mg/ml). Measurement of the radioactivity of the washed cells after 2.5 doublings demonstrated that 3% of the ^{14}C activity of the added ribose was incorporated. None of this

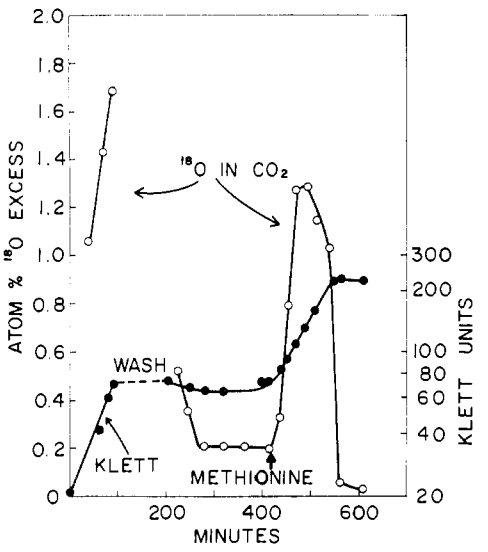

FIGURE 7: Concentration of ^{18}O in the CO_2 formed by *E. coli* W 113-3 before, during, and after a period of methionine deprivation. A culture of W 113-3 was grown as described in the Experimental Section except that the C medium was supplemented with 15 μg/ml of L-methionine, and that the carbon source for the last transfer was [1-^{18}O]glucose rather than glucose. The innoculum was placed in 25 ml of C medium to which had been added 15 μg/ml of L-methionine and 1 mg/ml of [1-^{18}O]glucose (10.52 atom % excess). After 92 min the culture was removed, centrifuged, and washed twice in C medium containing neither glucose nor methionine. It was then resuspended in 25 ml of fresh medium, and the culture was returned to the incubation vessel, which was placed in an ice bath and CO_2-free air was bubbled through it for a further 30 min. At the end of this time the culture was restored to the 37° bath, and received 2 mg/ml of [1-^{18}O]-glucose. At 416 min L-methionine was added to return the concentration to 15 μg/ml. The sweep rate was 800 cc of air/min.

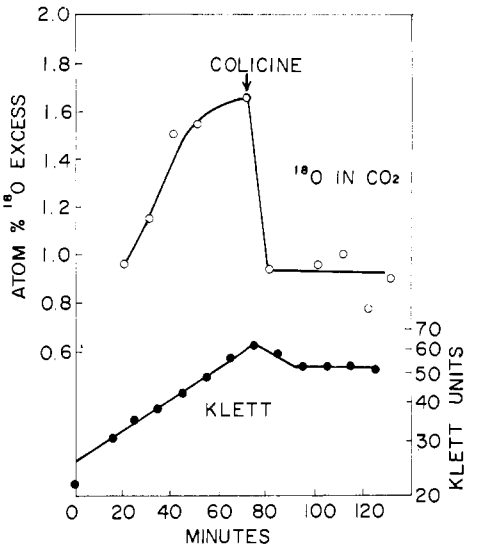

FIGURE 8: ^{18}O concentration in the CO_2 formed by a sensitive strain of *E. coli* B before and after the addition of colicine K to the medium. The strain was grown from a slant in nutrient broth, transferred to C medium containing 2 mg/ml of glucose, and allowed to grow overnight. Before the experiment this culture was again transferred and allowed to grow in C medium containing 2 mg/ml of glucose until it was certain that the cells were growing actively. After two washings the innoculum was suspended in 20 ml of C medium containing 2 mg/ml of [1-^{18}O]glucose (10.52 atom % excess). At 71 min 900 units/ml of colicine K was added. Sweep rate was 250 cc of air/min.

radioactivity could be removed with cold TCA (5%, 0°). We conclude that ribose exerts no inhibitory effect on the HMP, and by extension, that the fall in HMP activity associated with a cessation of growth is most probably not due to an accumulation of ribose 5-phosphate.

Enriched Media. Wang and Krackov's (1962) observation that the HMP is less active in *Bacillus subtilis* when these organisms are grown on complex medium than when they are grown in minimal medium, together with Scott and Chu's (1959) report of lessened glucose 6-phosphate dehydrogenase activity in nutrient broth grown *E. coli* led us to study the effects of more complex media on the HMP catabolism by *E. coli.* The addition of nutrient broth, yeast extract, or casein hydrolysate to minimal (C) medium results in a decreased HMP participation in glucose catabolism (see Table VII). This effect is probably attributable to the addition of amino acids to the medium.

Cells cultured in nutrient broth–glucose metabolize considerably more glucose by the HMP than when they are grown in minimal medium. Addition of either phosphate or NaCl abolishes this enhancement (see Table VII). It is probable that the increase in HMP activity when *E. coli* grow in nutrient broth–glucose is associated with the low ionic strength of this weakly buffered medium rather than to variations of the pH.

Anaerobiosis. Under anaerobiosis the HMP operates at a low level (see Table VIII). When nitrate is added to the medium, the specific yield of ^{18}O rises, but remains lower than when the cells are grown aerobically (see Table VIII). Anaerobically, *E. coli* grow more slowly than they do aerobically, and are less efficient at converting glucose to cellular materials. The addition of nitrate, which serves as a terminal electron acceptor from the cytochrome system (Egami *et al.*, 1959), does not, at the concentrations we used, accelerate growth, nor does it increase the yield of cells from glucose. Nitrate appears to stimulate the HMP, and it markedly raises the ^{18}O concentration in the CO_2.

TABLE VI: Specific Yield of ^{18}O in the CO_2 Formed by *E. coli* Br 15 from [1-^{18}O]Glucose in the Presence and Absence of D-Ribose.[a]

Substrates	Sp Yield (%)	HMP (%)
[1-^{18}O]Glucose + ribose		
Period 1	9.0	31
Period 2	8.2	29
[1-^{18}O]Glucose only		
Period 1	7.4	26
Period 2	8.0	28

[a] Strain Br 15 was grown as described in the Experimental Section except that all cultures but the first transfer from the slant contained ribose as the carbon source. The overnight culture was centrifuged and resuspended in C medium containing 4 mg/ml of ribose. A 10-ml aliquot of this suspension was used as the innoculum, and to it was added 1.2 mg/ml of [1-^{18}O]glucose (10.52 atom% excess). At 107 min 10 ml of fresh C medium containing 4 mg/ml of ribose was added, the CO_2 trap was changed, and an aliquot was removed for glucose determination. At 160 min the culture was removed, 10 ml of it was discarded, the remainder was centrifuged, the pellet was washed once and then resuspended, centrifuged again, and then resuspended in 10 ml of fresh C medium containing no ribose but 1.1 mg/ml of [1-^{18}O]glucose. At 260 min 10 ml of C medium was added, the trap was changed, and an aliquot was removed for glucose determination. The sweeping rate was 250 cc/min of air.

TABLE VII: Specific Yield of ^{18}O in the CO_2 Formed by *E. coli* WT Growing on [1-^{18}O]Glucose in a Variety of Media.[a]

Medium	No. of Expt	Mean Sp Yield (%)	Std Dev of Mean	HMP (sp yield × 3.5), %
C, glucose, pH 7	18	6.7	0.24	24
C, glucose, pH 6	1	5.4	—	19
C, glucose, yeast extract, casein hydrolysate	2	3.9	0.6	14
C, glucose, casein hydrolysate	3	4.7	0.7	16
C, glucose, yeast extract	1	4.1	—	14
C, glucose, nutrient broth	2	3.7	0.5	13
Nutrient broth, glucose	4	9.4	0.5	32
Nutrient broth, glucose, 6.5×10^{-2} M phosphate	1	4.7	—	16
Nutrient broth, glucose, 0.8% NaCl	1	4.9	—	17

[a] Overnight cultures were allowed to grow for two doublings in the appropriate medium. The cells were washed, and a portion was resuspended in 10 ml of the same medium, at a concentration which had an absorbancy of 30–40 Klett units. About 1 mg/ml of [1-^{18}O]-glucose was then added. When the cells had doubled, an equal volume of fresh medium was added, the CO_2 trap was changed, the glucose concentration was measured, and the cells were allowed to continue growing. When the cells had again doubled, the CO_2 trap was changed, the glucose concentration was measured, but no fresh medium was added. The experiment was terminated when there was no glucose left in the medium and when the concentration of ^{18}O in the CO_2 had fallen to a low level. For cells growing in minimal medium, only periods in which the cell density was held to a level equivalent to an absorbancy of fewer than 90 Klett units are considered. For cells growing in enriched media, the over-all specific yield is reported, since in these cases there was no decline in specific yield with increasing cell density. The initial pH of all media except that of expt 2 was 7 ± 0.1. For expt 2 it was 6.02.

Discussion

Methodology. We obtain a measurement of the HMP from a reaction which occurs very early in the catabolic scheme, and which is observed directly and not *via* secondary reactions. Consequently, many reactions on the triose level or below which affect the results of methods based on the use of ^{14}C substrates have no effect on this method.

Besides exogenous glucose, there are other sources of glucose 6-phosphate. In particular, the pentose phosphate formed by the operation of the HMP may be converted to fructose 6-phosphate, and thence to glucose 6-phosphate, which, since it is unlabeled, will dilute the [1-^{18}O]glucose 6-phosphate formed directly from glucose. Such dilution of the glucose 6-phosphate pool will affect the concentration but not the *yield* of $C^{18}O_2$. In effect, the unlabeled glucose 6-phosphate will serve as an additional carrier for the labeled molecules, and provided there is no isotope discrimination, the fraction of unlabeled molecules metabolized by the HMP should be the same as the fraction of labeled molecules, that is to say, as the corrected specific yield. If then, it is assumed that the first step in the metabolism of glucose is its phosphorylation to glucose 6-phosphate, that glucose 6-phosphate formed from exogenous glucose mixes with that from other sources, and that [1-^{18}O]glucose is not discriminated against, the specific yield measures both the fraction of exogenous glucose metabolized by the HMP, and the fraction

TABLE VIII: Specific Yield of ^{18}O in the CO_2 Formed by *E. coli* WT Growing on [1-^{18}O]Glucose Anaerobically in Minimal Medium with and without Added Nitrate.

Expt	Nitrate[a]	Sp Yield to Termination of Growth (%)	HMP (%)	^{18}O Concn in CO_2
1	0	2.5	8.8	0.64
2	0	1.5	5.1	0.63
3	+	3.8	13	1.44
4	+	3.8	13	1.8

[a] Cells were grown as described in the Experimental Section, but were subsequently allowed to grow up overnight under nitrogen on 1 mg/ml of glucose. The innocula for the two experiments without nitrate were prepared by diluting a portion of an overnight culture with an equal volume of fresh medium containing [1-^{18}O]-glucose (10.52 atom % excess) to give a final concentration of 1 mg/ml. The cultures containing nitrate were prepared from a growing culture by sucking an appropriate aliquot into a syringe, expelling the medium but not the cells through a Millipore filter, and then sucking in fresh medium through the same Millipore. The innoculum for the second nitrate experiment was derived from the cells used in the first, immediately after they had stopped growing. The media were deoxygenated first by evacuation and then by bubbling N_2 through for 20 min. The sweep rate was 250 cc of N_2/min.

of glucose 6-phosphate which passes through this pathway.

Cleavage of hexose diphosphate by aldolase, followed by resynthesis, or a sequence of reactions involving transaldolase and transketolase, could catalyze the rearrangement of the carbon atoms of hexose phosphate. Sable and co-workers (Sable and Cassisi, 1962; Szynkiewicz *et al.*, 1961) have studied the distribution of ^{14}C in glucose isolated from the glycogen of *E. coli* which had utilized [2-^{14}C]glucose as their carbon source. They found that so long as the cells had been previously adapted to glucose there was very little redistribution of the isotope. These results, together with our observations on the metabolism of [6-^{18}O]glucose, suggest that randomization of the hexose carbon skeleton is not an important problem.

The Entner–Doudoroff pathway would also serve as a source of $C^{18}O_2$ from [1-^{18}O]glucose. The enzymes of this pathway have been demonstrated in some strains of gluconate-grown *E. coli* (Kovachevich and Wood, 1955), but Jones (1961) and Scott (1956b) have shown that this pathway is not active in *E. coli* grown on glucose.

Fixation of CO_2 into cell constituents will lead to a decrease in the specific yield of isotope, and to an underestimate of the participation of the HMP. Wang *et al.* (1958) and Jones (1961) have reported recoveries ranging between 80 and 90% of the $^{14}CO_2$ from [3,4-^{14}C]glucose metabolized by these cells during their assimilatory phase.

Included in the fraction of isotope which was not recovered is ^{14}C in glucose incorporated into anabolic products, into triose used for the synthesis of amino acids and glycerol, and isotope incorporated into the pentose and deoxypentose of nucleic acids. Only a fraction, perhaps 5–10%, of the unrecovered isotope can, therefore, be fixed as $^{14}CO_2$.

We have calculated from the data of Rittenberg and Graff (1958) that at 37° and pH 7, 2.8%/hr of the ^{18}O in [1-^{18}O]glucose will be lost to water by exchange. Few of the experiments on which our estimate of the HMP during exponential growth are based took longer than three hours, and most were somewhat shorter. It is possible to construct a function with which the specific yields may be corrected to reflect loss of isotope from [1-^{18}O]glucose to the medium (Model, 1965). The application of this formula shows that in a three-hour period of growth on minimal medium, 5% of the ^{18}O is lost to the medium, and that, therefore, our estimate of the HMP is too low by this amount. Intra-experimental comparisons of the specific yield, and therefore HMP, during nitrogen starvation, methionine starvation, etc., result in differences which are much greater than can possibly be accounted for by the exchange of [1-^{18}O]glucose.

Effect of Growth on HMP. Cessation of growth induced by the onset of the stationary phase, by nitrogen deprivation, methionine deprivation, or colicine K treatment leads to a diminution of the fraction of glucose which is metabolized by the HMP. A somewhat smaller fall in the HMP is induced by the addition of nutrient broth, yeast extract, or casein hydrolysate to a salts medium. Growth in nutrient broth enhances the HMP, an effect which is attributed to the low ionic strength of nutrient broth.

Scott has shown that interruption of growth by exhaustion of nitrogen (Scott and Cohen, 1953), anaerobiosis (Scott, 1956a), or the presence of casein hydrolysate together with glucose (Scott and Chu, 1959), does not lead to a decline in the activity of the extractable HMP enzymes.

Since in *E. coli* ammonia is incorporated into amino acids *via* a NADPH-linked system (Adler *et al.*, 1938), and the synthesis of fatty acids requires NADPH, the cessation of growth which accompanies nitrogen or methionine deprivation, or colicine K treatment, may be expected to lead to a lower rate of NADPH oxidation.

In the stationary phase there is reduced synthesis of cellular materials, and a lower oxygen tension, both of which would be consistent with a smaller supply of $NADP^+$. In anaerobiosis the rate of

biosynthetic activity, measured against it either time or glucose consumed is low, and at the same time the need for generalized reducing power must also be small. When preformed amino acids are supplied to the cells the need for NADPH for the reductive amination of α-ketoglutarate is diminished. Consequently, the supply of $NADP^+$ may be reduced, and the HMP, for which $NADP^+$ is a required cofactor, may be less active. Further, there is evidence that NADPH is an inhibitor of the glucose 6-phosphate dehydrogenase of yeast (Glaser and Brown, 1955).

Our results, therefore, are consistent with the hypothesis that the regulation of HMP activity is through the availability of $NADP^+$, or through the $NADP^+$: NADPH ratio. There is evidence that in the tissues of higher organisms, *e.g.*, adipose tissue (Flatt and Ball, 1964), brain (Hoskins and Von Eschen, 1963), erythrocytes (Brin and Yonemoto, 1958), mammary gland (McLean, 1962), and thyroid (Field *et al.*, 1961; Dumont, 1961), there is a coupling between $NADP^+$ availability and activity of the HMP. Eagon (1963) has studied the rate of oxidation of NADH and NADPH, and of NADPH in the presence of NAD^+, by crude extracts from a number of microorganisms. In general he found that extracts from organisms which metabolize glucose mainly by the Embden–Meyerhof pathway had little NADPH oxidase or NADPH: NAD^+ transhydrogenase activity, while extracts from those in which there is extensive glucose metabolism by the Entner–Doudoroff or HMP pathway had a good deal of NADPH oxidase and NADPH:NAD^+ transhydrogenase activity. If the relative activities of these extracts correctly mirror the *in vivo* situation, this would support the hypothesis of a close coupling between biosynthesis and HMP activity, since his data shows that *E. coli* extracts are able to oxidize NADPH only 2% as fast as they can oxidize NADH.

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Photosynthetic Reactions with Pyridine Nucleotide Analogs. III. *N*-Methylpyridinium Iodides*

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ABSTRACT: *N*-Methylpyridinium iodides with certain substituent groups on the pyridine ring actively catalyze photophosphorylation with spinach chloroplast fragments. During photophosphorylation neither reduction nor tritium incorporation could be detected. The reduced forms of the pyridinium salts and the free bases were inactive as catalysts. Photophosphorylation supported by the pyridinium salts was insensitive to anaerobic conditions but was sensitive to *o*-phenanthroline and substituted ureas. Desaspidin influenced photophosphorylation supported by the pyridinium salts in a manner comparable to that supported by triphosphopyridine nucleotide (TPN) plus spinach ferredoxin.

The reaction was not affected by the addition of soluble chloroplast enzymes such as ferredoxin. We suggest (in connection with previous findings with pyridine nucleotide analogs) that electron flow during photophosphorylation is mediated by a radical form of these compounds which does not accumulate but is rapidly quenched, presumably by some component of the chloroplast system or oxygen.

In previous papers (Böger *et al.*, 1966a,b) we reported that chloroplast fragments utilized as a cofactor of photophosphorylation not only TPN[1] but also a number of analogs of DPN and TPN. These studies demonstrated that the quaternary pyridinium portion of the molecule is necessary for photophosphorylation activity. The following observations support this conclusion. Certain pyridine nucleotide analogs could be reduced enzymatically and were unable to support photophosphorylation. The phosphoadenosine portion of the oxidized analogs could be removed with phosphodiesterase (dinucleotide nucleotidohydrolase, EC 3.6.1.9) without substantial loss in activity. The phosphoribose portion could be replaced by a methyl group and the N-methylated pyridinium iodides could serve as model compounds for the pyridine nucleotide analogs. Under appropriate experimental conditions, the rates of photophosphorylation were approximately the same.

Therefore, a number of substituted quaternary pyridinium iodides were synthesized and tested as catalysts for photophosphorylation. This approach to the study of chloroplast reactions is particularly useful since it is not possible to obtain all pyridine nucleotide analogs because DPNase (NAD glycohydrolase, EC 3.2.2.5) does not catalyze an exchange reaction with all substituted pyridines (*e.g.*, Lamborg *et al.*, 1958).

N-Alkylated pyridinium compounds have been studied intensively as model substances for DPN (Karrer *et al.*, 1938; Rafter and Colowick, 1954; van Eys and Kaplan, 1957; Wallenfels and Gellrich, 1959; Wallenfels and Schüly, 1959a,b) but were found not to function as reversible hydrogen carriers in enzyme reactions. Most of these investigations, however, were confined to the 3-carbamylpyridinium salt. The experiments described in this paper suggest that *N*-methylpyridinium salts, with certain substituent groups on the pyridine ring, function as redox carriers in the multienzyme system present in chloroplast material.

Methods

Preparation of Pyridinium Iodides. The *N*-methylpyridinium iodides were synthesized in the following general manner. The pyridine compound with the

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[1] For abbreviations of the *N*-methylpyridinium iodides see Table I. Other abbreviations are: CMU, 3-(*p*-chlorophenyl)-1,1-dimethylurea; PMS, *N*-methylphenazinium methyl sulfate; Chl, chlorophyll; SCE, saturated calomel electrode; TPN and DPN, tri- and diphosphopyridine nucleotides; NAD, nicotinamide–adenine dinucleotide; ATP, adenosine triphosphate; NADP, nicotinamide–adenine dinucleotide phosphate.