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Regulatory Effect of Pyruvate on the Glucose Metabolism of *Clostridium thermosaccharolyticum*

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Whole cells and cell-free extracts of *Clostridium thermosaccharolyticum* 3814 grown in media containing 0.5% glucose or 0.6% pyruvate were evaluated for their metabolic activities toward these compounds. Glucose-grown cells rapidly fermented glucose with the production of gases (CO_2 and H_2), acids, and alcohol, but they did not ferment pyruvate well. Pyruvate-grown cells, on the other hand, readily fermented pyruvate, while fermenting glucose at a rate of one-half that of pyruvate. An analysis of the enzyme levels in the two cell culture conditions revealed that pyruvate-grown cells had lower levels of most of the glycolytic enzymes and increased levels of the hexose monophosphate pathway enzymes. Incorporation studies with the use of labeled glucose demonstrated that cells do have a control mechanism(s) whereby they can discriminate between a carbon (glucose) and an energy (pyruvate) source, selectively utilizing glucose in the synthetic pathway while obtaining energy from the phosphoroclastic degradation of pyruvate.

Clostridium thermosaccharolyticum is an obligate thermophilic anaerobe that can utilize a variety of carbohydrates as the carbon and energy source. Sjolander (19) found that the end products of glucose and xylose fermentation by growing cultures were CO_2, H_2, and butyric, acetic, and lactic acids, but he did not elucidate the metabolic pathways involved. Recently, the phosphoroclastic utilization of pyruvate involving ferredoxin was investigated by Wilder et al. (21).

During the course of the studies on the energy metabolism of *C. thermosaccharolyticum* 3814, we observed that fermentation products of glucose in this organism were significantly different from those reported by Sjolander. The organism was unable to ferment pyruvate readily unless grown on pyruvate. Nevertheless, our preliminary work confirmed the presence of both glycolytic and phosphoroclastic systems in this organism. This apparently anomalous phenomenon led us to investigate pyruvate metabolism and the control of the glycolytic pathway by pyruvate.

MATERIALS AND METHODS

Organism and conditions of growth. C. thermosaccharolyticum, National Canners Association strain 3814, was obtained through the courtesy of George York, University of California. Stock cultures of this strain were stored at 3 C in pea broth (8) and transferred monthly into fresh pea broth. The cultures were grown anaerobically at 56 C in a medium which consisted of the following (grams per liter): Trypticase (BBL), 10.0; yeast extract (Difco), 1.0; K_2HPO_4, 1.25; KH_2PO_4, 0.9; sodium thioglycolate, 1.0. "Glucose medium" contained 0.5% glucose, and "pyruvate medium" contained 0.05% glucose plus 0.6% sodium pyruvate. The cells were harvested at the end of exponential growth.

Preparation of cell-free extracts. After being harvested by centrifugation, the cells were washed twice with 0.01 M tris(hydroxymethyl)aminomethane (Tris) chloride buffer (*p*H 7.0), resuspended in the same buffer, and adjusted to a relative absorbancy of 75 at 420 mμ by use of a Bausch & Lomb Spectronic-20 colorimeter with water as the colorimetric blank. This was accomplished by taking a sample of the cell suspension and diluting it 300-fold with water. The suspension was then sonically treated for 60 sec at 0 C with a Branson Sonifier (Heat System Co., Great Neck, N.Y.). Cellular debris was removed by centrifugation at 27,000 × *g* for 20 min, and the clear supernatant fraction was used for the enzyme assays. Protein was determined by the biuret method (13) with crystalline bovine serum albumin as the standard.

Measurement of growth. Growth was measured by following the changes in relative absorbancy of cultures at 600 mμ by use of a Bausch & Lomb Spectronic-20 colorimeter with the uninoculated medium as the colorimetric blank. Dilutions with the blank medium were made when necessary.

Determination of fermentation products. Nongaseous end products of glucose fermentation were analyzed by column chromatography on silicic acid,

essentially as described by Ramsey (16). The compounds were identified by including known standards. The quantity present was determined by measuring the radioactivity. Manometric techniques were those described by Umbreit et al. (20).

Measurement of radioactivity. All samples were counted with the use of a Packard Tri-Carb Liquid Scintillation Counter (Packard Instruments Co., LaGrange, Ill.). Samples (0.5 ml) of column fractions were introducted into vials containing 15 ml of scintillation fluid: 0.5% PPO (2,5-diphenyloxazole) and 0.03% dimethyl POPOP [1,4-bis-2-(4-methyl-5-phenyloxazolyl) benzene] in toluene. Correction for quenching was made by the method of Bruno and Christian (4). For the incorporation studies with labeled substrates, cells were fractionated according to the procedures of Roberts et al. (17). These samples were counted in a scintillation fluid of the following composition: 0.7% PPO, 0.03% dimethyl POPOP, and 10% naphthalene in dioxane.

Enzyme assays. Enzyme activities were determined, whenever possible, spectrophotometrically by coupling to a pyridine nucleotide-dependent system in such a way that the enzyme to be tested was rate-limiting. The total volume in each cuvette was 3 ml. A unit of enzyme activity was defined as that amount of enzyme catalyzing the formation of 1 μmole of product per minute. Specific activity was expressed as units of enzyme per milligram of protein. A molar extinction coefficient of 1.44×10^3 (22) was used for the calculation of phosphoenol pyruvate and 6.22×10^6 (11) for reduced nicotinamide adenine dinucleotide ($NADH_2$). Hexokinase was determined spectrophotometrically by use of the following system: 100 μmoles of Tris buffer (*p*H 7.4), 5 μmoles of $MgCl_2$, 6 μmoles of adenosine-5-triphosphate (ATP), 1 μmole of nicotinamide adenine dinucleotide phosphate (NADP), 4 μg of glucose-6-phosphate dehydrogenase, 10 μmoles of glucose, and the extract. The glucose-phosphate isomerase assay system contained 100 μmoles of Tris buffer (*p*H 7.4), 5 μmoles of $MgCl_2$, 6 μmoles of ATP, 1 μmole of NADP, 4 μg of glucose-6-phosphate dehydrogenase, 5 μg of hexokinase, 10 μmoles of fructose, and extract. Phosphofructokinase was determined in a system containing 100 μmoles of Tris buffer (*p*H 8.0), 5 μmoles of $MgCl_2$, 6 μmoles of ATP, 20 μmoles of cysteine-HCl, 0.3 μmole of $NADH_2$, 25 μg of fructose diphosphate aldolase, 25 μg of glycerolphosphate dehydrogenase, 5 μg of hexokinase, 10 μmoles of fructose, and extract. Fructose diphosphate aldolase was assayed in a system containing 50 μmoles of triethanolamine buffer (*p*H 7.5), 0.3 μmole of reduced NADP ($NADPH_2$), 25 μg of glycerolphosphate dehydrogenase, 5 μmoles of fructose-1,6-diphosphate, and extract. Triosephosphate isomerase (3), glyceraldehydephosphate dehydrogenase (12), phosphoglycerate kinase (5), phosphoglycerate phosphomutase (9), phosphopyruvate hydratase (6), pyruvate kinase (18), and the phosphoroclastic system (14) were determined by current methods, as indicated. Glucose-6-phosphate dehydrogenase was assayed in a system containing 10 μmoles of triethanolamine buffer (*p*H 7.5), 1 μmole of NADP, 20 μmoles of glucose-6-phosphate, and extract. Phosphogluconate dehydrogenase was determined in a system containing 10 μmoles of triethanolamine buffer (*p*H 7.5), 1 μmole of NADP, 4 μmoles of 6-phosphogluconate, and the extract. All spectrophotometric assays were conducted at 30 C by use of a Beckman DU spectrophotometer equipped with a model SR Sargent recorder (E. H. Sargent & Co., Chicago, Ill.).

Chemicals. ^{14}C-glucose (uniformly labeled) and ^{14}C-pyruvic acid (uniformly labeled) were purchased from Calbiochem, Los Angeles, Calif., and Nuclear-Chicago Corp., Des Plaines, Ill., respectively. Enzymes and other biochemicals were obtained from Sigma Chemical Co., St. Louis, Mo., Nutritional Biochemicals Corp., Cleveland, Ohio, Mann Fine Chemicals, Inc., New York, N.Y., and P-L Biochemicals, Milwaukee, Wis.

RESULTS

Fermentation of glucose. Glucose-*U*-^{14}C was fermented by glucose-grown cells in Warburg flasks at 56 C. The soluble end products were separated by silica gel column chromatography and the radioactivity was determined. A typical chromatographic profile of end products (Fig. 1)

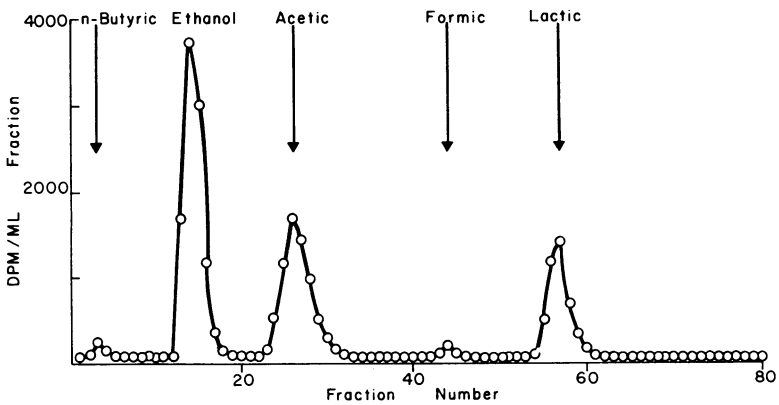

FIG. 1. *Chromatographic profile of end products of glucose fermentation in silica gel column.*

shows five peaks of radioactivity, representing, in order, *n*-butyric acid, ethyl alcohol, and acetic, formic, and lactic acids. The production of only trace amounts of *n*-butyric acid and a large amount of ethyl alcohol was unexpected, and is in contrast to the results of Sjolander (19). Molar quantities of each product are presented in Table 1.

Fermentation of pyruvate. The results of studies on glucose fermentation suggested that glucose-grown cells fermented glucose to the pyruvate level and that the pyruvate was further converted to various end products. The results of the fermentation of glucose and pyruvate by glucose- and pyruvate-grown cells are presented in Fig. 2. Gas production under anaerobic conditions was used as a fermentation index in these experiments. Glucose was fermented at a rapid rate by glucose-grown cells but was fermented at about half this rate by pyruvate-grown cells. On the other hand, pyruvate was actively fermented by pyruvate-grown cells but only very slowly by glucose-grown cells. These observations raised the following questions. (i) Why are glucose-grown cells unable to ferment pyruvate actively? (ii) Why do pyruvate-grown cells have decreased activity for glucose fermentation while fermenting pyruvate actively? To understand the utilization of pyruvate by the organism, it was desirable to determine the organism's growth rate in both the pyruvate and glucose media. As shown in Fig. 3, the culture reached the maximal optical density

TABLE 1. *Fermentation products of glucose by Clostridium thermosaccharolyticum 3814*[a]

Product	Amt
	μmoles
Initial glucose	10
Final glucose	0
Carbon dioxide	12
Hydrogen	11.3
n-Butyric acid	0.2
Ethyl alcohol	10.9
Acetic acid	7.5
Formic acid	0.75
Lactic acid	3.1
Carbon recovered (%)	99.4
Oxidation-reduction index	0.74

[a] Fermentation was carried out at 56 C in the Warburg vessel containing 1 μc of glucose-*U*-^{14}C, 10 mg (dry weight) of glucose-grown cells, 100 μmoles of potassium phosphate buffer, and 0.2 ml of 2 N KOH, in a total volume of 3.0 ml. Fermentation products were analyzed by silica gel column chromatography as described in Materials and Methods.

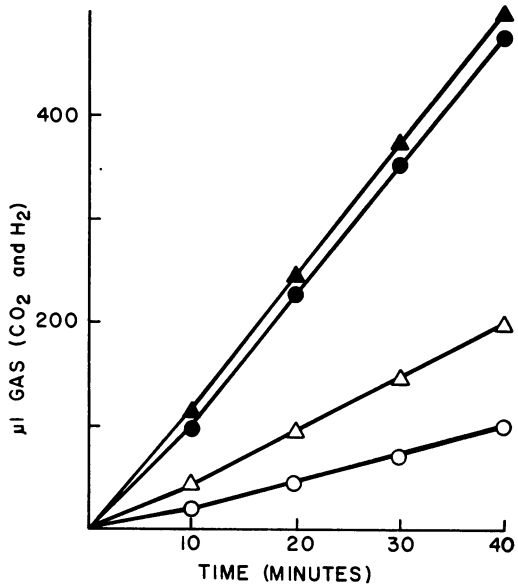

FIG. 2. *Fermentation of glucose and pyruvate by glucose- and pyruvate-grown cells of Clostridium thermosaccharolyticum 3814. Symbols:* ▲, *pyruvate-grown cells on pyruvate;* ●, *glucose-grown cells on glucose;* △, *pyruvate-grown cells on glucose;* ○, *glucose-grown cells on pyruvate.*

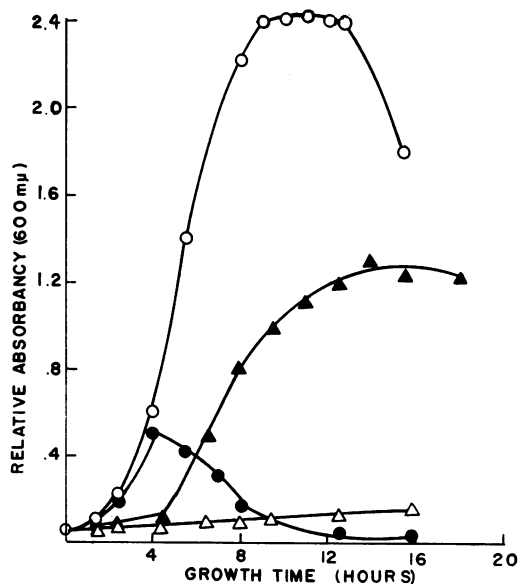

FIG. 3. *Growth response of Clostridium thermosaccharolyticum 3814 in glucose and pyruvate medium as determined spectrophotometrically at 600 mμ. Symbols:* ○, *0.5% of glucose;* ●, *0.05% of glucose;* △, *0.6% of Na pyruvate;* ▲, *0.05% of glucose plus 0.6% of Na pyruvate.*

(OD) in 9 hr in glucose medium. When pyruvate was the sole source of carbon and energy, no growth occurred. Only when a small amount of glucose (0.05%) was added to the pyruvate medium did growth occur and reach a maximum OD of 1.25. The fact that 0.05% glucose alone produced an OD of not more than 0.5 indicated that pyruvate was used in combination with glucose. One of the characteristics of growth in pyruvate media was a relatively long lag phase and the absence of diauxic growth. This initial long lag seemed to indicate that pyruvate molecules prevented the utilization of glucose by the cells. To examine this possibility, washed glucose-grown cells were tested for their metabolic activity with glucose in the presence and absence of pyruvate in Warburg flasks. As shown in Fig. 4, no inhibitory effect of pyruvate on glucose fermentation was observed. The cells fermented both substrates simultaneously and independently. Thus, it appears that factors controlling glucose fermentation in a growing system may be different from those of a nongrowing system.

Studies on enzyme activities. To evaluate the possibility that the low rate of glucose fermentation by pyruvate-grown cells was due to a repression of glucose-utilizing enzymes by pyruvate, cell-free extracts were prepared from cells grown in glucose and pyruvate media and assayed for their enzymatic activity. The extracts were used immediately after preparation. The results (Table 2) indicated that the enzymes may be placed into three groups in terms of their response to the growth substrate. In the first group, in which pyruvate-grown cells exhibited a repressed activity (40 to 80%) were the enzymes hexokinase, phosphofructokinase, fructose diphosphate aldolase, triosephosphate isomerase, glyceraldehyde phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate phosphomutase, and phosphopyruvate hydratase. The second group, which, contrary to the first group, showed an increased level of activity in the extract of pyruvate-grown cells, consisted of glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase, pyruvate kinase, and the phosphoroclastic system. Glucose phosphate isomerase, which was the lone member of the third group, had comparable activity in both preparations. Since our pyruvate media contained 0.05% glucose in addition to pyruvate (0.6%), the changes in level of enzyme activities in pyruvate-grown cells could be due to low concentration of glucose rather than to feedback repression by pyruvate. To clarify this doubt, we examined glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase activities

TABLE 2. *Specific activity[a] of glycolytic enzymes of glucose- and pyruvate-grown cells*

Enzymes	Specific activity	
	Glucose-grown cell extracts	Pyruvate-grown cell extracts
Hexokinase	8.63×10^{-2}	3.85×10^{-2}
Glucose phosphate isomerase	1.34×10^{-1}	1.32×10^{-1}
Glucose-6-phosphate dehydrogenase	2.5×10^{-3}	4.8×10^{-3}
Phosphogluconate dehydrogenase	2.5×10^{-3}	5.2×10^{-3}
Phosphofructokinase	9.6×10^{-2}	5.1×10^{-2}
Fructose diphosphate aldolase	1.84×10^{-2}	1.28×10^{-2}
Triosephosphate isomerase	1.92	1.28
Glyceraldehyde phosphate dehydrogenase	1.6×10^{-2}	1.28×10^{-2}
Phosphoglycerate kinase	2.08×10^{-1}	1.2×10^{-1}
Phosphoglycerate phosphomutase	8.4	4.9
Phosphopyruvate hydratase	560	360
Pyruvate kinase	1.28×10^{-2}	3.36×10^{-2}
Phosphoroclastic system	0.88	1.32

[a] Specific activity = units of enzyme activity per milligram of protein; 1 unit = 1 μmole of product per min.

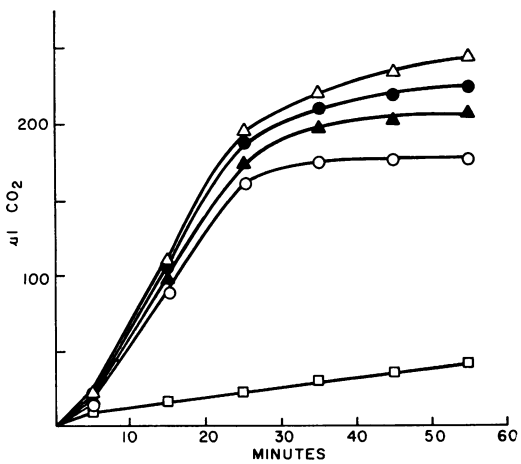

FIG. 4. *Effect of pyruvate on the glucose fermentation by glucose-grown cells of Clostridium thermosaccharolyticum 3814. In each Warburg vessel 10 μmoles of glucose was added except where indicated. The data have been corrected for endogenous activity. Symbols: ○, no pyruvate; ▲, 2 μmoles of pyruvate; ●, 5 μmoles of pyruvate; △, 15 μmoles of pyruvate □, 20 μmoles of pyruvate without glucose.*

of cells obtained from glucose and pyruvate media. The results (Table 3) showed that the levels of dehydrogenase activity were similar in the cells grown with 0.5 and 0.05% glucose, the glucose concentrations in the two growth media studied, but that the pyruvate-grown cells had an increased activity. As experimentally determined, the hexokinase activity was comparable in the cells from the two glucose media, but was reduced in the cells from the pyruvate medium (Table 1). This suggested that it was the presence of the pyruvate rather than the glucose concentration which caused a change in the activity of these enzymes.

Incorporation of radioactive glucose and pyruvate. The results of the studies on the comparative enzymatic activity of pyruvate-grown cells demonstrated that certain oxidative reactions were increased two- to threefold in pyruvate-grown cells. The increased oxidative capacity is believed to favor the flow of glucose metabolism into the hexose monophosphate pathway, thus producing more reduced pyridine nucleotide and also providing more ribose moiety for nucleic acid synthesis. To substantiate this hypothesis, an experiment was designed to follow the incorporation of substrates into the cellular material in both glucose and pyruvate media. At the time of inoculation, 2 μc of glucose-*U*-^{14}C was introduced into 100 ml each of glucose and pyruvate media. When the cultures reached their maximal growth, the cells were harvested, washed, and fractionated (17). The supernatant liquid of the culture media showed a complete exhaustion of glucose. The results are presented in Table 4. On the whole-cell basis, the radioactivity of the pyruvate-grown cells was almost four times higher than that of the glucose-grown cells. This suggested that glucose molecules were mainly used in cells growing in pyruvate for the synthetic reactions, whereas their energy requirements were met by a degradation of pyruvate by the phosphoroclastic reactions. Additional evidence for the inefficiency of pyruvate as a building material was provided by following pyruvic-*U*-^{14}C incorporation into the cells. One-third of the pyruvate still remained in the media at the time of harvest. The total radioactivity of the cells, had the entire pyruvate-*U*-^{14}C been utilized, would have been 13,819 counts/min. This figure corresponds to about one-half that of the ^{14}C-glucose incorporated into the cells in the glucose culture and to about one-seventh of the glucose incorporated into the cells in the pyruvate culture.

TABLE 3. *Specific activity of phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase*

Cells grown in media containing	Phosphogluconate dehydrogenase	Glucose-6-phosphate dehydrogenase
0.5% glucose	2.09×10^{-3}	2.09×10^{-3}
0.05% glucose	1.93×10^{-3}	1.77×10^{-3}
0.6% pyruvate + 0.05% glucose	5.63×10^{-3}	4.34×10^{-3}

TABLE 4. *Distribution of radioactivity among cellular fractions*

Fraction	Culture media		
	Glucose, 0.5%, and 2 μc of glucose-*U*-^{14}C (26,841 counts/min)[a]	Glucose, 0.05%, pyruvate, 0.6%, and 2 μc of glucose-*U*-^{14}C (99, 821 counts/min)	Glucose, 0.05%, pyruvate, 0.6%, and 2 μc of pyruvate-*U*-^{14}C (9,213 counts/min)
	%	%	%
Trichloroacetic acid-soluble	0.22	5.5	5.8
Ethyl alcohol extract	14.6	8.8	13.9
Hot trichloroacetic acid-soluble	38.6	45.8	23.1
Hot trichloroacetic acid-insoluble	46.6	39.9	57.1

[a] Represent the amount of radioactivity in the entire population.

DISCUSSION

The analysis of fermentation products from glucose revealed that *C. thermosaccharolyticum* 3814 produced *n*-butyric, acetic, and lactic acids, and ethyl alcohol, as well as CO_2 and H_2. The low level of *n*-butyric acid and the large amount of ethyl alcohol produced is in contrast to previous findings for this organism. Sjolander (19) observed the production of relatively large amounts of *n*-butyric acid but no ethyl alcohol. These discrepancies might be due to the difference in the fermentation systems employed. Sjolander used a growing system in a malt sprout medium, and our system employed resting cells in a buffer solution.

Growth of the organism in pyruvate medium produced several interesting changes compared with growth in a glucose medium. As demonstrated by the growth curves (Fig. 3), the organism did not grow as rapidly in the pyruvate medium as it did in the glucose medium. The main end product in pyruvate medium was acetic acid (found by the measurement of radioactive acetate in spent medium containing cold glucose- and pyruvate-*U*-^{14}C), indicating the phosphoroclastic utilization of pyruvate. We also demonstrated that glucose and pyruvate were simultaneously utilized during growth. This is in agreement with

the apparent lack of a diauxic phase in the growth curve. Resting cells obtained from the pyruvate medium exhibited a high capacity to ferment pyruvate and possessed an enhanced phosphoroclastic system. The reduced ability of pyruvate-grown cells to ferment glucose could be correlated with the low levels of glycolytic enzymes in cell-free extracts.

A comparison of the enzymes of the phosphoroclastic system indicated that pyruvate-grown cells possessed greater specific activities than did glucose-grown cells. It is postulated that this high activity of the pyruvate-grown cells for pyruvate would partially explain the more active fermentation of pyruvate by such cells, but, as glucose-grown cells also contain the necessary enzymes and apparently utilized metabolically generated pyruvate at a normal rate, the possibility of differences in permeability between the two cell preparations must also be considered. Barrett et al. (2) reported that fumarate grown cells of *Pseudomonas fluorescens* possessed the citrate-oxidizing enzymes to a comparable level with that of citrate grown cells but that fumarate grown cells could not oxidize citrate effectively. Difficulty of permeation of tricarboxylic acid intermediates has also been reported for yeast (1).

The repression and induction of glycolytic enzymes in pyruvate-grown cells are significant. It has been shown by Hommes (10) that the glycolytic enzymes of yeast could be induced by changing the amount of glucose in the culture media. However, it has never been reported, to the best of our knowledge, that these enzymes were repressed by pyruvate. Pyruvate can be regarded as an end product of the glycolytic pathway. Therefore, the repression of enzymes by pyruvate demonstrates the presence of end-product repression in the Embden-Meyerhoff pathway.

The incorporation of a larger amount of radioactive carbon from glucose in pyruvate-grown cells than in glucose-grown cells indicated that the pathway of glucose metabolism in the two systems was different. Cohen (7) pointed out the importance of the hexose monophosphate pathway in growing cells by reporting that, under oxidative conditions, resting cells of *Escherichia coli* metabolize glucose by means of the Embden-Meyerhoff pathway, whereas rapidly growing cells use chiefly the hexose monophosphate (HMP) pathway. Recently, Model and Rittenberg (15) came to a similar conclusion on the participation of the HMP pathway during glucose metabolism in *E. coli*. They found that 25% of the glucose metabolized by *E. coli* during active growth in minimal medium passed through the HMP pathway, although this pathway became significantly less important during the stationary phase or if the growth was interrupted by exhaustion of the nitrogen source. In our present system, it appears that pyruvate-grown cells are able to utilize glucose chiefly via the hexose monophosphate pathway.

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