THE CATABOLISM OF GLUCONATE IN *ESCHERICHIA COLI*

by

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INTRODUCTION

The field of microbial metabolism offers many intriguing areas for the study of the basic biochemical processes occurring in the cell. Recent efforts by numerous investigators have helped to elucidate the complex nature of the various pathways of carbohydrate catabolism. Their findings as well as the results of future investigations should provide valuable information concerning the life processes since many of the metabolic relationships which have been shown to exist in bacteria have later been identified in higher forms of life.

That bacteria have become widely accepted as an ideal system with which to study carbohydrate metabolism is explained, in part, by the short generation time of the cell. This, coupled with the fact that bacteria supply large statistical populations of cells, permits the tracing of the complete metabolic life cycle of the cell with the added assurance of reproducibility of results.

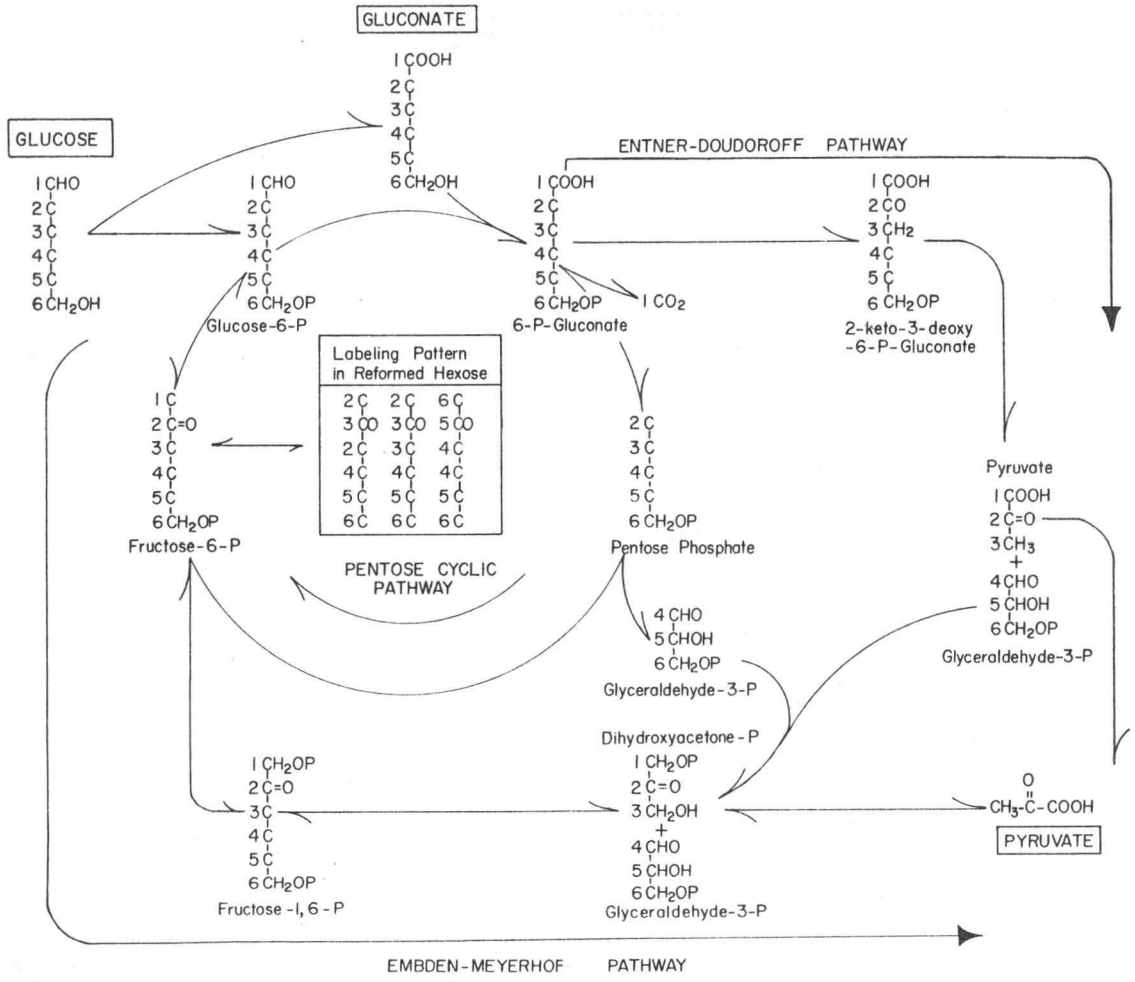
Recent advances in radioactive tracer methodology as well as related technical advances have accelerated the study of the primary respiratory processes.

Pathways of Glucose Catabolism

Although the earliest scientific interests in microbial metabolism may have stemmed from the vast research efforts of Pasteur and his colleagues, the present knowledge of pathways of carbohydrate metabolism arises from the later efforts of Buchner, who in 1897 first demonstrated that cell free yeast juice was able to ferment sugar. Since that time, numerous investigations have revealed that there exist two major pathways of glucose utilization in microorganisms, plants, and animal cells. These are recognized today as the Embden-Meyerhof-Parnas pathway (EMP) and the hexose monophosphate pathway (HMP). Recently a third pathway has been shown to occur to a limited extent in microorganisms, namely, the Entner-Doudoroff pathway (ED) (31, p. 853-862). The nature of these pathways has been dealt with in several recent reviews (10, p. 295-346; 30, p. 145-202; 35, p. 79-128; 50, p. 703-713; 88, p. 245-284). A description of these pathways is summarized in Figure 1.

Both the EMP and the ED pathways are responsible for the degradation of glucose to two molecules of pyruvate, but the mechanism for each case is entirely different. Whereas the carboxyl groups of pyruvate, formed via the EMP pathway, are derived from C-3 and C-4 of glucose, the

FIGURE 1


GLUCONATE
GLUCOSE
ENTNER-DOUDOROFF PATHWAY
Glucose-6-P
6-P-Gluconate
2-keto-3-deoxy
-6-P-Gluconate
Labeling Pattern
in Reformed Hexose
Fructose-6-P
Pentose Phosphate
Pyruvate
PENTOSE CYCLIC
PATHWAY
Glyceraldehyde-3-P
Dihydroxyacetone-P
Glyceraldehyde-3-P
Fructose-1,6-P
PYRUVATE
EMBDEN-MEYERHOF PATHWAY

carboxyl groups of pyruvate, formed via the ED pathway, derive their carbon from C-1 and C-4 of glucose. A key step in the catabolism of glucose by either pathway is the aldol cleavage of a C_6 intermediate to yield two C_3 fragments. In the EMP pathway, fructose-1,6-diphosphate is cleaved to yield dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. In the ED pathway, 2-keto-3-deoxy-6-phosphogluconate is cleaved to yield pyruvate and glyceraldehyde-3-phosphate. These C_3 intermediates for each pathway are then converted to pyruvate.

The HMP pathway involves an oxidation of glucose-6-phosphate to form 6-phosphogluconate, which may undergo phosphogluconate decarboxylation (PGD) to yield pentose phosphate or be further catabolized via the ED pathway. The pentose phosphate, resulting from PGD, may be catabolized by pentose cleavage reactions or be metabolized via the reactions of the pentose cyclic pathway (PC). If the enzymes of the pentose cycle are present, the pentose phosphate, formed from phosphogluconate decarboxylation, becomes essentially a mixture of xyulose phosphate and ribose phosphate. These pentose moieties are formed through the action of an epimerase and an isomerase enzyme upon ribulose phosphate, which is the first intermediate of phosphogluconate decarboxylation. Through a series of enzymatic reactions, involving the enzymes transketolase

and transaldolase, the pentose phosphate is converted to fructose-6-phosphate and triose phosphate. Triose phosphate, by the action of aldolase, may also in turn be converted to fructose-6-phosphate. In either case, the fructose-6-phosphate thus formed may be further catabolized via the reactions of the pentose cycle or those of the EMP pathway. The relative positions of carbon atoms in fructose-6-phosphate, derived from gluconate catabolized via the PC pathway, have been determined by Beevers (9, p. 339-347). These relationships are illustrated in Figure 1. The first reaction of this pathway, the direct oxidation of glucose-6-phosphate, was discovered by Warburg, Christian and Griese in 1935 (96, p. 157-205). Later efforts by such investigators as Dickens (27, p. 1626-1644), Lipmann (65, p. 588-589), Dische (28, p. 252-253) and more recently by Scott and Cohen (81, p. 686-689), Horecker (37, p. 393-403) and Racker (74, p. 141-178) have helped to elucidate the complex scheme of intermediary stages which result in the conversion of pentose phosphate to hexose.

Pathways of Terminal Respiration

The present form of the tricarboxylic acid cycle (TCA), as illustrated in Figure 2, is remarkably similar to that proposed by Krebs in 1937 (60, p. 148-156). The

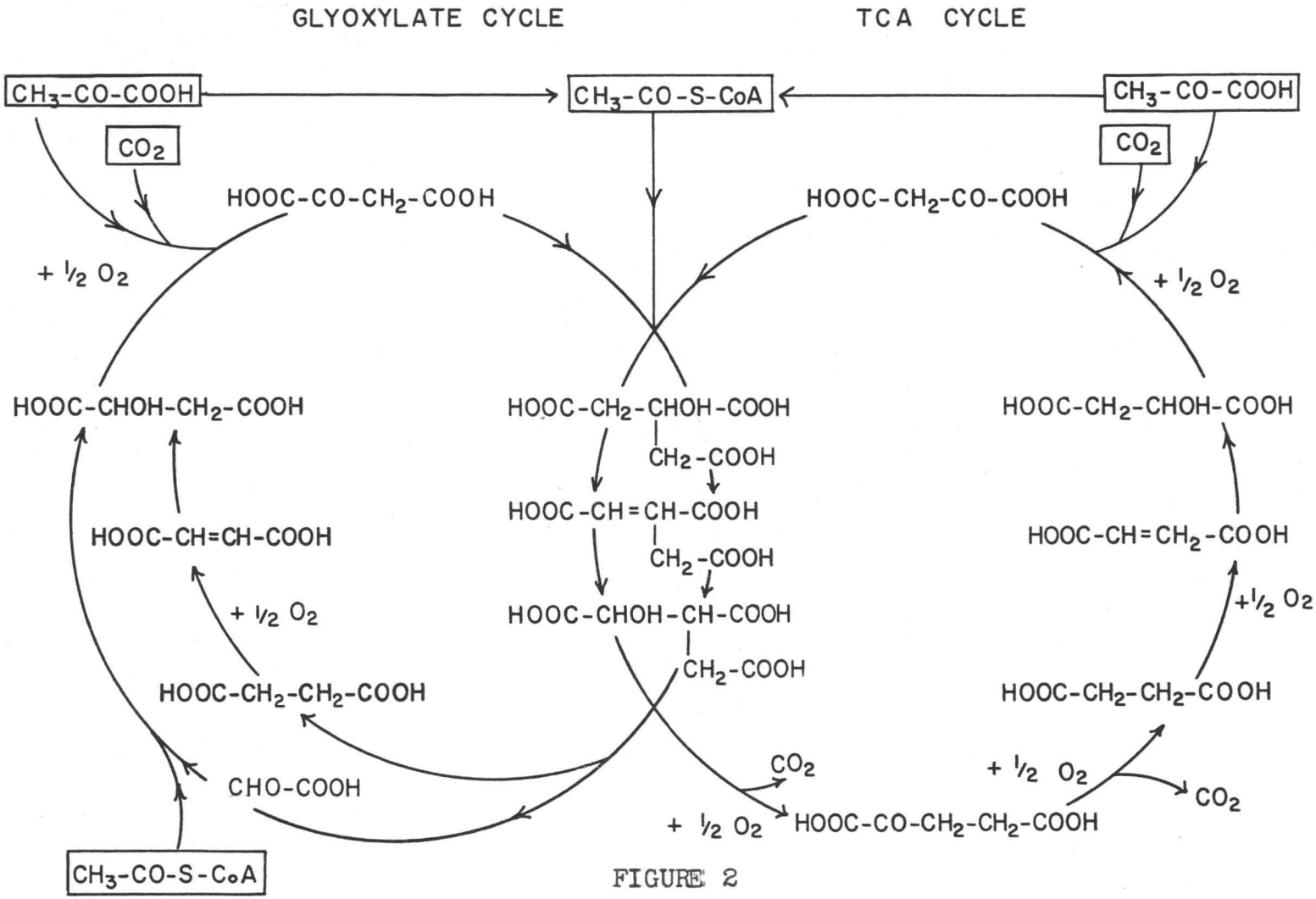

GLYOXYLATE CYCLE
TCA CYCLE
CH3-CO-COOH
CH3-CO-S-CoA
CH3-CO-COOH
CO2
CO2
HOOC-CO-CH2-COOH
HOOC-CH2-CO-COOH
+ 1/2 O2
+ 1/2 O2
HOOC-CHOH-CH2-COOH
HOOC-CH2-CHOH-COOH
HOOC-CH2-CHOH-COOH
CH2-COOH
HOOC-CH=CH-COOH
HOOC-CH=CH-COOH
CH2-COOH
+ 1/2 O2
+1/2 O2
HOOC-CHOH-CH-COOH
CH2-COOH
HOOC-CH2-CH2-COOH
HOOC-CH2-CH2-COOH
CO2
+ 1/2 O2
CO2
CHO-COOH
+ 1/2 O2
HOOC-CO-CH2-CH2-COOH
CH3-CO-S-CoA

FIGURE 2

formation of citrate through the condensation of acetyl CoA with oxaloacetate is the initial step in the sequence of reactions by which acetate is combusted to carbon dioxide and water to produce energy needed for biosynthesis. In addition to its role as a mechanism for energy supply, the TCA cycle may serve to furnish carbon skeletons for several biosynthetic schemes (89, p. 683-688; 69, p. 469-476; 59, p. 614-628; 84, p. 601-611; 61, p. 212-298; 77, p. 218-246). It is obvious that drainage of one or more components will result in the interruption of the TCA cycle. Therefore, it is necessary for the self reliance of the bacterial cell to provide alternate routes for the synthesis of one or more intermediates of the cycle.

Several mechanisms have been proposed to account for the net synthesis of C_4 or C_5 intermediates of the TCA cycle. These are represented by reactions of the type $C_3 + C_1$ to form C_4 in which pyruvate reacts with CO_2 to form oxaloacetate (102, p. 377-388) or malate (70, p. 979-1000). In addition, two mechanisms have been proposed by which $C_2 + C_2$ yields C_4. These are represented by the Thunberg condensation (87, p. 1-91) of 2 molecules of acetate to yield one molecule of succinate, and the malate synthetase reaction in which acetate reacts with glyoxylate to give malate. The latter reaction sequence was established upon discovery of two enzymes, which

catalyze respectively the cleavage of isocitric acid to succinate and glyoxylate, and the formation of malate from glyoxylate and acetyl CoA. These enzymes are termed isocitratase (71, p. 695-696) and malate synthetase (99, p. 3230-3231) respectively and form an important complement to the glyoxylate bypass of the TCA cycle. Reactions of the type $C_3 + C_2$ to yield C_5 have been proposed by Koepsell et al (49, p. 5142-5144) and Katagiri (41, p. 143-153) to explain the formation of α ketoglutarate.

Methodology of Pathway Analysis

In the concurrent operation of two or more metabolic pathways for a given substrate, it is essential to know the relative contribution of each of the pathways so that the function of the respective pathways can be fully evaluated. Several approaches have been employed in the detection of specific pathways of carbohydrate catabolism in microbes. Isolation of a particular enzyme or enzyme system responsible for the specific conversion of a given pathway intermediate has formed the basis for most of the earlier work in this connection. Another method, which has been successfully applied in many studies, is the kinetic relationship between supplied precursors and product formation. One limiting factor of these methods,

however, is that they are usually of qualitative significance and provide little information regarding total glucose metabolism in the intact organism. Several methods have been devised for estimating the relative contribution of individual pathways to the overall catabolism of glucose (2, p. 31-38; 3, p. 773-779; 12, p. 5446; 13, p. 555-563; 14, p. 681-694; 15, p. 6093-6097; 23, p. 551-552; 45, p. 853-868; 47, p. 70-76; 63, p. 273-286; 64, p. 252; 82, p. 389-407; 90, p. 1869-1874).

The radiorespirometric method of Wang et al (91, p. 207-216) is basically a study of the kinetics of concurrent pathways. This method has been tested in fungi, yeast, bacteria, insects, plants and fruit (91, p. 207-216; 11, p. 1-95; 29, p. 751-756; 83, p. 102-104). In applying the radiorespirometric method to the study of pathways of carbohydrate catabolism in microorganisms, important information is gained with regard to the quantitative importance of concurrent catabolic pathways through an analysis of the relative rates of combustion to carbon dioxide of specifically C^{14} labeled carbohydrates or organic acid intermediates. One advantage of this method is that quantitative results regarding pathway distributions are obtained by utilizing intact systems where the organization of enzymes remains undisturbed. The nature of this

method permits one to trace the primary and secondary routes of carbohydrate catabolism by an analysis of the rates and extents of respiratory carbon dioxide evolved during successive time intervals.

Pathways of Carbohydrate Metabolism in Escherichia coli

In recent years considerable efforts have been made to elucidate the pathways by which carbohydrates or their catabolic intermediates are metabolized in E. coli. The work of Roberts and coworkers, utilizing the principles of isotopic competition to examine pathways, is illustrative in this regard (77, p. 1-521). Cohen examined the participation of the EMP pathway and the alternate pathway involving phosphogluconate decarboxylation (PGD) in E. coli (21, p. 746-747). Using isotopically labeled glucose and gluconate, he concluded that the majority of glucose is metabolized by way of the EMP pathway while the extent of glucose oxidation via the gluconate route ranged from a minimum of 14% to a maximum of about 37%. Scott and Cohen have further shown that the first isolable product of gluconate decarboxylation was ribulose phosphate, a key intermediate of the pentose cycle (81, p. 686-689). Wang and coworkers have reported the results of radiorespirometric studies of glucose catabolism in this organism and

indicated that glucose is catabolized to the extent of 72% EMP and 28% via either phosphogluconate decarboxylation or some route giving rise to preferential release of C-1 of glucose as CO_2 compared with glucose C-6 (91, p. 207-216).

The pentose resulting from phosphogluconate decarboxylation may follow many diverse routes of metabolism. One of the major functions of the PGD pathway in E. coli is to supply the pentose moieties of RNA and DNA (62, p. 193-199; 8, p. 1369-1374). Racker (74, p. 141-178) demonstrated that an enzyme in E. coli may catalyze the reversible formation of 2-deoxyribose from acetaldehyde and glyceraldehyde-3-phosphate. A similar enzyme has been recently isolated from Lactobacillus plantarum by Price and Horecker (73, p. 1292-1298). In addition to such degradative cleavage routes, pentose from gluconate decarboxylation may enter the pentose cycle to eventually reform hexose, which may then be metabolized via either the EMP or PGD pathways.

The operation of the Entner-Doudoroff pathway has been demonstrated in several organisms, particularly in the Pseudomonas species (38, p. 853-862; 95, p. 489-492). However, there is little information on the quantitative significance of this route in E. coli. Kovachevich and Wood, working with the dried cells of E. coli, have demonstrated a cleavage of

2-keto-3-deoxy-6-phosphogluconate to pyruvate indicating the presence of the aldolase enzyme of the ED pathway (57, p. 757-767).

The Tricarboxylic Acid Cycle in Escherichia coli

The TCA cycle in E. coli has been the subject of many investigations (4, p. 474-485; 1, p. 1020-1026; 22, p. 353-371; 66, p. 81-95; 78, p. 1013-1019; 86, p. 426-434; 98, p. 317-339). Roberts (77, p. 218-246) has indicated that one of the functions of the cycle in E. coli is to supply carbon skeletons for the biosynthesis of amino acids.

In order for this cycle to play a role in biosynthesis, it is necessary to feed in preformed C_4 acids or other intermediates of the cycle. At present considerable effort is being devoted to this problem. One mechanism involves the fixation of CO_2 with pyruvate which may be of the Wood-Werkman variety (102, p. 377-388) or malic enzyme type (70, p. 979-1000). Roberts has shown that this mechanism of entry may be an important route of C_4 acid biosynthesis in E. coli (77, p. 95-112). Another mode of entry of C_4 acid into the TCA cycle involves the reactions of the glyoxylic acid bypass. The operation of this cycle in E. coli has been the subject of many investigations (54, p. 651-653; 99, p. 3230-3231;

100, p. 970-971). It has recently been indicated that the glyoxylate bypass mechanism of the TCA cycle plays an important role in C_4 acid synthesis when E. coli is grown on acetate as the sole carbon source (56, 438-445; 76, p. 341-345). The operation of this cycle in cells of E. coli, grown on glucose or gluconate as carbon sources, has not been evaluated, but it is known that the content of malate synthetase and isocitratase is considerably lessened when glucose, as well as several other substrates other than acetate are the carbon sources (76, p. 341-345).

There is evidence that the glyoxylate cycle may not function in all strains of E. coli. Glasky and Rafelson (34, p. 2118-2122), utilizing short term time course experiments with isotopically labeled acetate in acetate adapted cells of E. coli (Crookes Strain) showed that the first products of acetate incorporation were succinic acid, glutamic acid and a compound tentatively identified as S-acetyl glutathione. The incorporation of acetate-2-C^{14} into glutamic acid in these experiments resulted in a labeling pattern for glutamic acid not consistent with any known biosynthetic scheme. Furthermore, the presence of isocitratase in these cells could not be detected. Malate synthetase was present, however, as evidenced by a rapid incorporation of acetate label into malic acid when

exogenous glyoxylate was supplied. This latter observation would seem to discount the possibility of the direct conversion of acetate to glyoxylate as a significant pathway in E. coli, although Bolcato, using a trapping technique, has detected glyoxylate as an early oxidation product of acetate in E. coli (16, p. 179-182).

More recently Kornberg (56, p. 438-445) has performed kinetic studies of acetate incorporation into E. coli cells of the Crookes strain. He was able to conclude that the glyoxylate bypass is an important route to C_4 acid synthesis in this strain of E. coli since isocitratase was present and malate became rapidly labeled. In addition to the role in C_4 acid synthesis, glyoxylate is known to also undergo decomposition to active formate and CO_2. The active formate may be metabolized by several routes in addition to further reaction with another molecule of glyoxylate to produce hydroxypyruvic acid which is an intermediate in several biosynthetic schemes (52, p. 1791-1795; 58, p. 593-594).

In addition to the glyoxylate cycle and the $C_3 + C_1$ reactions as supplementary routes to the TCA cycle in growing cells, the possibility exists that at least one other mechanism may play an important role, namely, a $C_3 + C_2$ condensation to form α ketoglutarate or some precursor of α ketoglutarate. Evidence for the operation

of such a pathway is far from conclusive although Dekker, working with rat liver tissue, has recently described an enzyme which is capable of degrading glutamic acid to alanine and glyoxylic acid (24, p. 174-175).

Considerable interest has developed in recent years in the study of the commercial production of αketoglutarate and glutamic acid in bacterial fermentations. At present, attention is focused upon the study of the mechanisms of formation of these acids. Koepsell et al (49, p. 5142-5144) showed that Pseudomonas fluorescens produces 2-ketogluconate from glucose, which later is converted to ketoglutarate. They proposed a $C_3 + C_2$ condensation to account for the large yields of αketoglutarate appearing in the incubation medium. More recently Weimberg (97, p. 727-732) has shown that P. sacchrophila is capable of converting L-arabinose to αketoglutarate without a cleavage of the carbon chain. Thus, a direct C_5 entry into the TCA cycle may be an important additional route for cellular biosynthesis and respiration.

Katagiri has exhaustively studied the formation of αketoglutarate from such substrates as pyruvate, acetate and glucose in E. coli (40, p. 210-214; 41, p. 143-153; 42, p. 188-196). The results of these investigations indicated, that under conditions of limited supply of nitrogen source and enhanced aerobic conditions, E. coli

cells excrete large quantities of this acid into the fermentation medium where its accumulation may account for as much as 50% of the carbon atoms of the administered substrate. On the basis that pyruvate (C_3) plus acetate (C_2) was more effective in leading to large yields of αketoglutarate than was pyruvate or acetate as sole substrate, Katagiri has postulated that a $C_3 + C_2$ condensation occurs between pyruvate and acetate to yield αketoglutarate (C_5).

A renewed interest has developed in the extent of participation of concurrently operative catabolic pathways for the total metabolism of glucose. Recently, Katz and Wood (46, p. 2165-2177) have ascribed theoretical significance to the possibility of total oxidation by the pentose cycle mechanism, for those molecules of glucose-6-phosphate engaged in this pathway. Dawes and Holms, however, have considered the case of equilibration of fructose-6-phosphate formed from glucose via the pentose cycle reactions with that fructose-6-phosphate formed via the Embden-Meyerhof-Parnas pathway (23, p. 551-552). These authors suggest that fructose-6-phosphate formed in either pathway participates in the EMP and HMP routes to the same degree as substrate glucose. Wang and Krackov (93), on the basis of radiorespirometric studies of glucose and gluconate catabolism in Bacillus

subtilis indicate that fructose-6-phosphate arising from glucose catabolism via the pentose cycle mechanism is further catabolized according to the pathways distributions alloted to substrate glucose.

The present study is designed to elucidate the catabolic fate of pentose resulting from phosphogluconate decarboxylation in *E. coli*. This has been accomplished, in part, through radiorespirometric studies of glucose and gluconate catabolism in this organism. Further evidence for the catabolic pathway of gluconate was obtained from a study of the isotopic distribution patterns of selected amino acids which had incorporated carbon atoms from gluconate-1-C^{14}, gluconate-2-C^{14} and gluconate-6-C^{14}. Insight into the mechanism of entry into the TCA cycle of pyruvate deriving its labeling from gluconate-1-C^{14}, gluconate-2-C^{14} and gluconate-6-C^{14} was obtained by an analysis of the isotopic distribution pattern of alanine and α ketoglutarate.

EXPERIMENTAL METHODS

Cultural Conditions

The organism used in this series of studies was Escherichia coli Strain B ATCC 11303 from the S. E. Luria collection, University of Illinois. The culture was stored under sterile mineral oil. Appropriate subcultures were made onto agar having the composition: agar, 2 g.; yeast extract, .5 g.; tryptone, .5 g.; K_2HPO_4, .1 g.; glucose, .1 g.; H_2O, 100 ml. These subcultures were also stored under mineral oil. Working slants were subcultured on ordinary agar slants of the above composition. Transfers were made at intervals of one month.

For the radiorespirometric studies of carbohydrate catabolism by growing cells of E. coli, a medium was used having the following composition: NH_4Cl, 2 g.; Na_2HPO_4, 6 g.; KH_2PO_4, 3g.; $MgCl_2$, 0.010 g.; $NaSO_4$, 0.026 g. and H_2O, one liter (77, p. 5). Yeast extract was also added to the extent of 1 gram per liter of medium. The pH of the medium was adjusted to 6.8 with dilute HCl. Glucose was sterilized separately and added to the extent of 250 mg. per 100 ml. of medium. One hundred milliliter portions of the medium, contained in 250 ml. Erlenmeyer flasks, were innoculated with 0.5 ml. of an active culture

of E. coli cells previously grown on the same medium, and incubated at 37° C. on a rotary shaker. The cells were routinely harvested in the logarithmic growth phase. For radiorespirometric studies with growing cells, the medium was identical to that used for obtaining the initial cell crop with the exception that C^{14} labeled substrates were used to replace the non-isotopic glucose carbon source.

In resting cells experiments where α ketoglutarate production was studied, the medium used for obtaining the cell crop consisted of the following components: glucose, 20 g.; $(NH_4)_2HPO_4$, 1 g.; $(NH_4)_2SO_4$, 1 g.; K_2HPO_4, .5 g.; $MgSO_4 \cdot 7\ H_2O$, 0.5 g.; NaCl, 2 g.; H_2O, 1 liter (41, p. 143-153). The pH of the solution was adjusted to 6.8 with dilute HCl. One hundred milliliters of the medium was dispensed into a 250 ml. Erlenmeyer flask, and the flask was stoppered with a cotton plug before sterilization in the autoclave for 15 min. at 15 pounds per square inch. One hundred fifty milligrams of $CaCO_3$ suspension, previously sterilized, was added aseptically to each flask after autoclaving. These flasks were then innoculated with an actively growing culture of E. coli cells, obtained by inserting 1 or 2 mg. of moist cells from an agar slant into 50 ml. of growth medium which had been enriched by the addition of 1% yeast extract and 1% tryptone. The flasks were then incubated at 30° C. for 6 to 9 hours without

shaking.

Five tenths of a milliliter of the innoculum was pipetted aseptically into 100 ml. of the growth medium. The flasks were then incubated at 30° C. on a rotary shaker set at 180 cycles per minute. The course of growth of the cells was followed by periodic determination of the optical density of an aliquot of cells suspension to which 0.1 ml. of concentrated HCl was added in order to remove excess $CaCO_3$. The maximum growth of the cells, as judged by the lack of change in the optical density, was obtained after 11 to 14 hours of incubation. The cells were then harvested by centrifugation, washed once with phosphate buffer, and resuspended in phosphate buffer having the following composition: K_2HPO_4, 17 g.; $MgSO_4 \cdot 7\ H_2O$, 680 mg.; H_2O, 1 liter. The pH of the buffer was adjusted to 6.2 by the addition of dilute HCl, and the concentration of cells in the phosphate buffer solution was adjusted to a prescribed concentration, usually 0.67 mg./ml., as indicated by the optical density of the suspension.

Radiorespirometric Experiments

Radiorespirometric studies on the utilization of carbohydrates were carried out according to the method of Wang et al (91, p. 207-216). The system was modified

slightly to include two identical CO_2 traps connected by a 3 way stop cock, as illustrated in the M. S. thesis of Bjerre (11, p. 33). This inclusion eliminated the necessity of interrupting aeration of the culture during the incubation experiment.

In a typical experiment, the labeled substrate was diluted with the prescribed amount of non-isotopic carrier and added to the side arm of the incubation vessel. The cell crop, rinsed with carbohydrate free medium, was diluted to a prescribed concentration as indicated by the optical density of the suspension. A defined volume of cell suspension was introduced to the main compartment of each flask. The flasks were attached to the holder and incubated at the desired temperature and shaking rate. The aeration rate was adjusted to a prescribed value, and the cells were allowed to deplete for one-half hour. The substrates were then tipped into the cells suspension at the end of this period. The radiochemical recovery of the substrate activity in respiratory CO_2 from cells metabolizing specifically labeled substrates was examined by collecting CO_2 samples at one hour intervals.

The trapping reagent for CO_2 employed in these experiments was 0.25 N ethanolic hyamine solution. The efficiency of collection was ascertained to be satisfactory in preliminary experiments. For the

radiorespirometric studies performed with growing cells, air which had been passed through a cotton plug was used to sweep respiratory CO_2 through the incubation medium to the CO_2 trap as well as serving as an oxygen source for the cells. In the resting cells experiments, pure oxygen which had been passed through a cotton plug was used as the sweeping gas and oxygen source. Incubations were generally carried out over a period of five to eight hours. The cells were then separated by centrifugation from the incubation medium, after chilling in an ice bath, and both cells and medium were processed for radioactivity determination.

C^{14} Labeled Substrates

Glucose-1, -2, -3 and 6-C^{14} were obtained from Dr. H. S. Isbell, National Bureau of Standards. Glucose-3,4-C^{14} was prepared from rat liver glycogen according to the method of Wood *et al* (104, p. 475-489). Potassium-D-gluconate -2, -3, -3,4 and 6-C^{14} were prepared in this laboratory from the correspondingly labeled glucose compounds, according to the method of Moore and Link (68, p. 293-311). Sodium gluconate-1-C^{14} was obtained from Nuclear Chicago Corporation. Pyruvate-1, -2 and 3-C^{14} were obtained from Merck, Inc. Canada. Acetate-1 and -2-C^{14} were obtained from Tracerlab, Inc. Glyoxylate-1

and -2-C^{14} were prepared in this laboratory by a modification of the procedures of Milas and Terry (67, p. 1412-1418) and Radin (75, p. 60-62).

Incorporation of Glyoxylate-1-C^{14} and Glyoxylate-2-C^{14} into Cellular Amino Acids

Glyoxylate-1-C^{14} and glyoxylate-2-C^{14} were administered to E. coli cells suspended in medium prescribed for growth. The course of utilization of this substrate was followed by the radiorespirometric method. When the rate of $C^{14}O_2$ recovery from glyoxylate-1-C^{14} had passed the initial peak, the experiment was terminated by the addition of 1.0 ml. of concentrated HCl to the cells suspension. The cells were separated from the medium by centrifugation, and the radioactivity in a portion of each was determined by a persulphate combustion. The remaining cells were added to a pyrex tube containing 2.0 ml. of 6 N HCl. The tube was sealed and subsequently autoclaved for 8 hours at 15 pounds per square inch. The cell hydrolysate was filtered to remove humin, and the filtrate repeatedly evaporated to dryness in a vacuum dessicator over P_2O_5 and KOH in order to remove excess HCl.

The amino acids from the cell hydrolysate were separated from each other using a paper chromatographic technique. A portion of the cells hydrolysate was

applied, in a narrow band near the origin, to each of two separate strips of Whatman 3 MM filter paper, having dimensions of 1.5" x 24". One strip was subsequently subjected to chromatography, using 80% liquid phenol in water as the solvent system, while the other strip was chromatographed using 2-butanol:NH_3 as the developing solvent (79, p. 502-505). The positions of the amino acids on paper as well as their identity was established by simultaneous chromatography of appropriate amino acid standards. The bands corresponding to the amino acids were sectioned from the paper, placed in a specially designed vial for counting of paper strips to which toluene, containing phosphor, had been added, and counted according to an established procedure (92, p. 203-205). The counting efficiency was determined as 55% using a standard paper strip to which a known amount of glutamic acid-2-C^{14} had been applied.

Incorporation of C^{14} Specifically Labeled Gluconate into αKetoglutarate and Cellular Amino Acids

Gluconate-1-C^{14}, -2-C^{14} and -6-C^{14} were incorporated into αketoglutarate and cellular amino acids under the experimental conditions previously described. The experiment was terminated after 8.5 hours when the $C^{14}O_2$ recovery from gluconate-1-C^{14} declined to a low value.

The cells were separated from the incubation medium by centrifugation, and further processed for radioactivity determination in the standard manner. The cell free medium was diluted to 50 ml. and appropriate aliquots of the medium taken for radioactivity determination. The balance of the medium was concentrated to a small volume for the continuous ether extraction of α ketoglutarate. The ether was subsequently removed by evaporation under vacuum. The resulting residue of α ketoglutarate was taken up in a small volume of water, applied to sheets of heavy filter paper (S and S 470-A), and chromatographed using butanol: formic acid: water (95:5:21 v/v) as the developing solvent.

The band corresponding to α ketoglutarate was cut from the paper and the α ketoglutarate eluted with water. The resulting solution was concentrated to a volume of 50 ml. The concentration of α ketoglutarate in this solution was determined by colorimetric analysis of the 2,4 dinitrophenylhydrazone derivative (32, p. 415-442). The radioactivity of an aliquot of the solution was determined by diluting 1:10 with ethanolic hyamine hydroxide. One milliliter of the resulting solution was added to a liquid scintillation counting vial containing 4 ml. of 0.25 N hyamine hydroxide and 10.0 ml. of toluene, containing 30 mg. of terphenyl and 0.3 mg. of POPOP. The resulting

solution was counted by liquid scintillation counting at a photomultiplier voltage of 1370 volts and discriminator settings of 10-100 volts and 10- α volts. The counting efficiency for each sample tested was determined as 58% by addition of 0.1 ml. of standard benzoic acid-7-C^{14} in toluene to each of the samples after their initial counting rate had been determined. The net counting rate due to benzoic acid-7-C^{14} was determined by substracting the counting rate due to isotopic α ketoglutarate as well as the background counting rate from the total sample counting rate.

The cellular amino acids which became labeled during the course of the experiment were isolated and subjected to degradation reactions as described in the subsequent section.

Isolation of Amino Acids

Cellular amino acids which became labeled during the course of incorporation experiments, employing specifically labeled gluconates, were isolated as follows. Twenty milligrams of cells from each experiment were transferred to a small pyrex test tube to which 2.0 ml. of 20% HCl was added. The tubes were sealed and autoclaved for 8 hours. The hydrolysate was filtered to remove humin and concentrated to dryness over P_2O_5. Water was then

added and the solution was again evaporated to dryness. This process was repeated three times to remove excess HCl.

Alanine, aspartic acid, and glutamic acid were separated from the other amino acids in the hydrolysate by repeated chromatography using heavy filter paper (S and S 470-A). The amino acid mixture was applied in narrow bands across the origin of 25 x 75 cm. strips of paper and chromatographed using 80% liquid phenol as the developing solvent. Bands, corresponding to aspartic acid, glutamic acid and alanine, were cut from the dried paper, and the acids eluted with water. After concentration to a small volume, the resulting partially purified amino acids were rechromatographed on heavy paper using 2-butanol:NH_3 as the developing solvent. Final purification of the amino acids was obtained by eluting the spots from the paper with water as before and rechromatographing the amino acids in butanol: acetic acid: water (4:1:5 v/v). The amino acids were eluted from the dried paper, filtered through a fine sintered glass filter funnel to remove particulate matter, and concentrated to a volume of 25 ml.

Determination of Specific Activity of Amino Acids

The concentration of the individual amino acids in the eluate was determined by colorimetric analysis using

the ninhydrin procedure (19, p. xii). The radioactivity associated with each isolated amino acid was determined in the following manner: a 1.0 ml. portion of each amino acid solution was concentrated to dryness over P_2O_5 in a vial used for scintillation counting. One tenth milliliter of water was added to this and the contents thoroughly mixed. Five milliliters of 0.25 N ethanolic hyamine was added and the contents again thoroughly mixed. Ten milliliters of toluene, containing 30 mg. of terphenyl and 0.3 mg. POPOP, was then added and the contents mixed before liquid scintillation counting. The counting efficiency was determined by addition of 0.1 ml. of standard benzoic acid-7-C^{14} to 5 ml. of 0.25 N ethanolic hyamine hydroxide in 10 ml. of toluene-phosphor solution. In addition, 0.1 ml. of standard benzoic acid was added directly to the amino acid solution which had been previously counted, and the counting rate due to benzoic acid determined. The data from the foregoing determinations made it possible to calculate the specific activity of each amino acid.

Degradation Study of Alanine

Alanine was decarboxylated, using a modified ninhydrin procedure, to obtain the activity in the C-1 carbon atom (17, p. 260-261). Five tenths of a millimole

of alanine was transferred to a 150 ml. 3-neck flask equipped with a condenser, a CO_2 free air inlet and a standard taper pistol shaped pyrex side arm, containing 300 mg. of ninhydrin. One hundred milligrams of pH 2.5 citrate buffer, dissolved in 20 ml. of water, was added to the alanine. The system was swept with CO_2 free air for 5 minutes, the ninhydrin powder was tipped in and the flask was placed in a boiling water bath for 20 minutes. Acetaldehyde, resulting from the decarboxylation of alanine, was swept from the system and trapped in 1% $NaHSO_3$. CO_2 from C-1 was swept through the bisulphite solution and through an acid permanganate scrubber to remove any SO_2 and finally was trapped in 0.5 N NaOH. The Na_2CO_3 was precipitated as $BaCO_3$ and processed as described in the radioactivity measurements section. The bisulphite trap containing acetaldehyde was transferred to a 100 ml. flask fitted for steam distillation. Six grams of K_2HPO_4 were added to liberate the acetaldehyde which was steam distilled into a receiver placed in an ice bath. The recovered acetaldehyde was degraded by the iodoform reaction to yield iodoform (C-3) and formate (C-2) of the original alanine. A prescribed amount of ice cold 10% KI_3 was added to the chilled acetaldehyde solution which was set aside for several hours until all of the iodoform had precipitated. The iodoform was

filtered off and recrystallized from methanol:H_2O. The filtrate was freed of excess iodine by thiosulphate titration, concentrated to a small volume, acidified with H_2SO_4 and steam distilled. The formic acid recovered in the distillate was combusted to CO_2 by a HgO oxidation (6, p. 137). Iodoform was assayed directly by liquid scintillation counting in the following manner. From 12 to 50 mg. of recrystallized iodoform was added to a liquid scintillation counting vial containing 17 ml. of cold toluene-phosphor solution, prepared by dissolving 1 g. of PPO and 30 mg. of POPOP in 100 ml. of toluene. The contents of the vial were mixed immediately and counted, using a photomultiplier voltage lf 1210 volts and discriminator settings of 10-100 volts and 10-∞ volts. The sample counting rate observed in the 10-100 volt region was used for further calculations.

The counting efficiency for a given weight of iodoform in 17.0 ml. of toluene-phosphor solution had been previously determined by observing the change in counting efficiency of a series of samples, containing equal amounts of benzoic acid-7-C^{14} dissolved in 17 ml. of the toluene-phosphor solution, to which increasing amounts of non-isotopic recrystallized iodoform had been added. Alternatively, a sample, which had been previously

counted, was treated with 0.1 ml. of standard benzoic acid-7-C^{14} in toluene. The net counting rate due to benzoic acid-7-C^{14} was determined by difference. All counting data were corrected for variations in counting efficiency as well as background counting rate.

Degradation Study of Aspartic Acid

Aspartic acid was decarboxylated with ninhydrin to yield the total activity in the C-1 and C-4 carboxyl carbon atoms (17, p. 260-261). The resulting CO_2 was trapped in 0.5 N NaOH which was processed for radioactivity determination as indicated in the radiochemical assay methods section. The activity in C-3 of aspartic acid was determined by the hypoiodite oxidation of aspartic acid. The iodoform (C-3 of aspartic acid) resulting from this degradation was processed for radioactivity determination as indicated under alanine degradation.

Degradation Study of Glutamic Acid

Glutamic acid was degraded by the ninhydrin method (17, p. 260-261) for activity on the C-1 carboxyl. Six tenths millimole of glutamic acid was mixed with 100 mg. of citrate buffer and 300 mg. of ninhydrin and heated for

20 minutes at 100° C. The CO_2 produced was trapped in 0.5 N NaOH and further processed as indicated in the radiochemical assay methods section.

Degradation Study of α Ketoglutarate

Portions of the isolated α ketoglutarate were diluted with carrier α ketoglutarate for degradation studies. The mixture was taken up in water, evaporated to dryness, and redissolved in acetone. Dry benzene was added to the acetone to effect crystallization. The purified acid was degraded by several means. Activity in C-1 was determined by a permanganate decarboxylation (6, p. 143). The resulting succinic acid was isolated by a continuous ether extraction for 24 hours. The ether was evaporated and the acid taken up in a few milliliters of water. Five milliliters of 5% $AgNO_3$ were added and the pH of the solution adjusted to 7.0 with dilute NH_4OH. The precipitated silver succinate was resuspended in water and silver sulfide was removed by filtration and the filtrate containing succinic acid was evaporated to dryness. The melting point of the resulting crystals corresponded to pure succinic acid (M.P. 189° C.). Succinic acid was decarboxylated by means of a Schmidt reaction (43, p. 155-156), yielding CO_2 from C-1 and C-4 of succinate which

corresponds to C-2 and C-5 of the original α ketoglutarate. The yield of CO_2 corresponded to 70% decarboxylation of succinic acid.

In order to determine the activity in the carboxyl group at C-5 of α ketoglutarate, it was necessary to convert α ketoglutarate to glutamic acid. A portion of the above recrystallized sample of α ketoglutarate was converted to glutamic acid by a reductive amination procedure (6, p. 144). The reaction mixture, containing the disodium salt of glutamic acid and $PdCl_2$ catalyst suspended on charcoal, was filtered and the filtrate evaporated to dryness. The residue was dissolved in 2.0 ml. of 0.5 N acetate acid and chromatographed by passage over Dowex-1 (8x) (36, p. 6063-6065). The purity of the amino acid was established by chromatography in three solvent systems: 80% liquid phenol in water; n-propanol-NH_3 (7:1); and ether, acetic acid and water (13:3:1). These solvent systems were designed to test for contamination by other amino acids, non volatile organic acids, and volatile organic acids respectively. Only glutamic acid was ascertained to be present as judged by reactions with indicator sprays containing either ninhydrin or bromcresol green.

The isolated glutamic acid was decarboxylated with ninhydrin to determine activity on C-1 (17, p. 260-261),

while another portion of the acid was decarboxylated with hydrazoic acid to yield CO_2 from C-5 (89, p. 683-688). Total activity in succinic acid, α ketoglutaric acid or glutamic acid prepared from α ketoglutaric acid was determined by a persulphate combustion (18, p. 1225-1226). The activity of C-2 of α ketoglutarate was determined by difference.

Radiochemical Assay Methods

The hyamine hydroxide, used for the trapping of respiratory CO_2 as well as that used as a solvent and diluent component of liquid scintillation counting mixtures, was prepared according to a prescribed procedure (94).

1. Substrate Calibration.

Radioactive substrates were standardized by liquid scintillation counting. Five tenths of a milliliter of a solution of the carrier free substrate, which had previously been dissolved in a prescribed volume of water (usually 25 ml.), was added to a 10 ml. volumetric flask. An additional 0.5 ml. of water was added to this, and the solution diluted to 10.0 ml. with 0.25 N hyamine hydroxide. One milliliter of this solution was added to a scintillation counting vial containing 4.0 ml. of the hyamine hydroxide solution and 10 ml. of a phosphor

solution, consisting of 30 mg. of terphenyl and 0.3 mg. of POPOP in toluene. After a preliminary cooling period of at least 10 minutes, the sample was counted at a photomultiplier voltage of 1370 volts and discriminator settings of 10-100 volts for the lower channel and 10-α volts for the upper channel. Calibration of the counter was accomplished by counting a standard amount of hyamine ($C^{14}O_2$) carbonate, dissolved in the toluene-phosphor solution. The counting efficiency was determined to be 55% for samples counted in the 10-α volt region.

2. Liquid Scintillation Counting of Respiratory $C^{14}O_2$.

The radioactivity of the respiratory CO_2 in the nature of hyamine carbonate in toluene was determined by liquid scintillation counting. The trap solution, consisting of ethanolic hyamine hydroxide solution, was rinsed into a 15 ml. volumetric container and diluted to 15 ml. with absolute ethanol. Five milliliters of the latter solution were transferred to a counting vial containing 10 ml. of a phosphor solution, consisting of .3 mg. of POPOP and 30 mg. of terphenyl in 10 ml. toluene. The sample was then counted at a photomultiplier voltage of 1370 volts and discriminator settings of 10-100 and 10-α volts.

The efficiency of the liquid scintillation counting process used for substrate calibration as well as $C^{14}O_2$ counting was determined by counting a standard solution, prepared by mixing 1.0 ml. of a standardized hyamine carbonate solution with 4 ml. of 0.25 N hyamine hydroxide and 10 ml. of the toluene-phosphor solution. The standard hyamine carbonate solution had previously been prepared by liberating a known amount of $C^{14}O_2$ from $BaCO_3$ with perchloric acid and subsequently trapping the $C^{14}O_2$ in 0.25 N hyamine hydroxide which was then diluted to a standard volume with additional 0.25 N hyamine.

3. Scintillation Counting of Gel Suspensions.

Bacterial cells and fermentation products which had become radioactive during the course of an experiment were processed for the determination of their respective radioactivities in the following manner.

The harvested E. coli cells were suspended in 10 ml. of carbon free incubation medium. One milliliter of this solution was added to 14.0 ml. of a gel scintillator suspension which had been prepared by mixing 18.75 g. of a thixotrophic gel powder; 6 ml. of a 1:9 mixture of tween -80 and span 80; 7.5 ml. of glycerol; 25 mg. of POPOP, 3 g. of terphenyl and 750 ml. of toluene in a Waring blendor. Each sample was shaken vigorously 50 times before counting

in the liquid scintillation counter. A photomultiplier voltage of 1370 volts and discriminator settings of 10-100 volts and 10- $\propto$ volts were used. The counting efficiency for gel was determined by diluting 1.0 ml. of a previously calibrated substrate to 10.0 ml. with non isotopic cell suspension or non isotopic medium, and counting a 1 ml. aliquot in 14 ml. of gel using counting procedures described above.

4. Liquid Scintillation Counting of $C^{14}O_2$ Derived from $BaCO_3$.

For solid $BaCO_3$ samples of very low activity, such as those resulting from the degradation studies of amino acids, a procedure was developed for the liquid scintillation counting of $C^{14}O_2$ derived from these $BaCO_3$ samples.

Accordingly, the $BaCO_3$ was filtered through pre-weighed sintered glass funnels, rinsed with water and 70% ethanol, and dried at 120° C. The dried precipitate was weighed and transferred to the center compartment of a standard taper flask equipped with a side arm containing excess 20% perchloric acid. The flask was attached to an assembly which consisted of a CO_2 free air inlet and an exit leading to a trap containing 5 ml. of 0.5 N hyamine. The perchloric acid was tipped into the center compartment

of the flask and evolved $C^{14}O_2$ swept into the hyamine trap. The trap contents were rinsed into a scintillation vial with 5.0 ml. of toluene containing 15 mg. of terphenyl and 0.15 mg. of POPOP. An additional 5.0 ml. of toluene containing the phosphor was added to the vial and the radioactivity assayed by liquid scintillation counting. The efficiency of the CO_2 trapping process was determined to be 98 to 100% based on liberation of standard $Na_2C^{14}O_3$ to which carrier had been added.

5. Scintillation Counting of Radioactivity on Paper Chromatograms.

The purity of radioactive substrates as well as the determination of radioactivity of fermentation products and cellular amino acids was occasionally examined by paper chromatography combined with liquid scintillation counting of paper strips cut from the chromatograms (92, p. 203-205).

Measurements of radioactivity by liquid scintillation counting techniques were performed in a Packard Tricarb Liquid Scintillation Spectrometer to a standard deviation of no greater than 1%. Counting data were corrected for counting efficiency variations and background in the conventional manner.

6. Geiger-Muller Counting of $BaCO_3$.

$C^{14}O_2$ samples obtained in the degradation studies

of α ketoglutarate were collected in 0.5 N NaOH and subsequently converted to $BaCO_3$ by the addition of $BaCl_2$-NH_4Cl solution. The barium carbonate samples were mounted on aluminum planchets by the centrifugation technique, dried and counted in a thin mylar window gas flow Geiger-Muller counter. The counting efficiency was determined by counting a $BaCO_3$ planchet which had been prepared by precipitation of a standard amount of $NaC^{14}O_2$ as $BaC^{14}O_3$. The countings were carried out to a standard deviation of not more than 3% and counting data were corrected for background and self absorption in the usual manner.

EXPERIMENTAL RESULTS AND DISCUSSION

The catabolism of complex carbohydrates in microorganisms leads to the formation of smaller fragments which are in turn combusted to CO_2 by way of terminal respiratory mechanisms. It is through terminal oxidation that the cell gains energy necessary for biosynthesis. The rates and extents of the CO_2 production process, therefore, provide an important clue to the mechanism of carbohydrate breakdown. The radiorespirometric method of Wang *et al* (91, p. 207-216) is based on the kinetic study of $C^{14}O_2$ production from C^{14} specifically labeled substrates in microorganisms. The radiorespirometric patterns from experiments with such intermediates as acetate or pyruvate as test substrates are compared with those patterns obtained employing C^{14} specifically labeled saccharides. The conclusion drawn from the comparison provides important information concerning the role that a specific substrate carbon atom plays in the overall mechanism. It is from such comparisons that pathways of carbohydrate utilization can be understood.

The present study is designed to determine the basic radiorespirometric patterns for pyruvate, acetate and glyoxylate since there exists considerable confusion in the literature with regard to the catabolic pathways

leading to CO_2 production for each of these substrates.

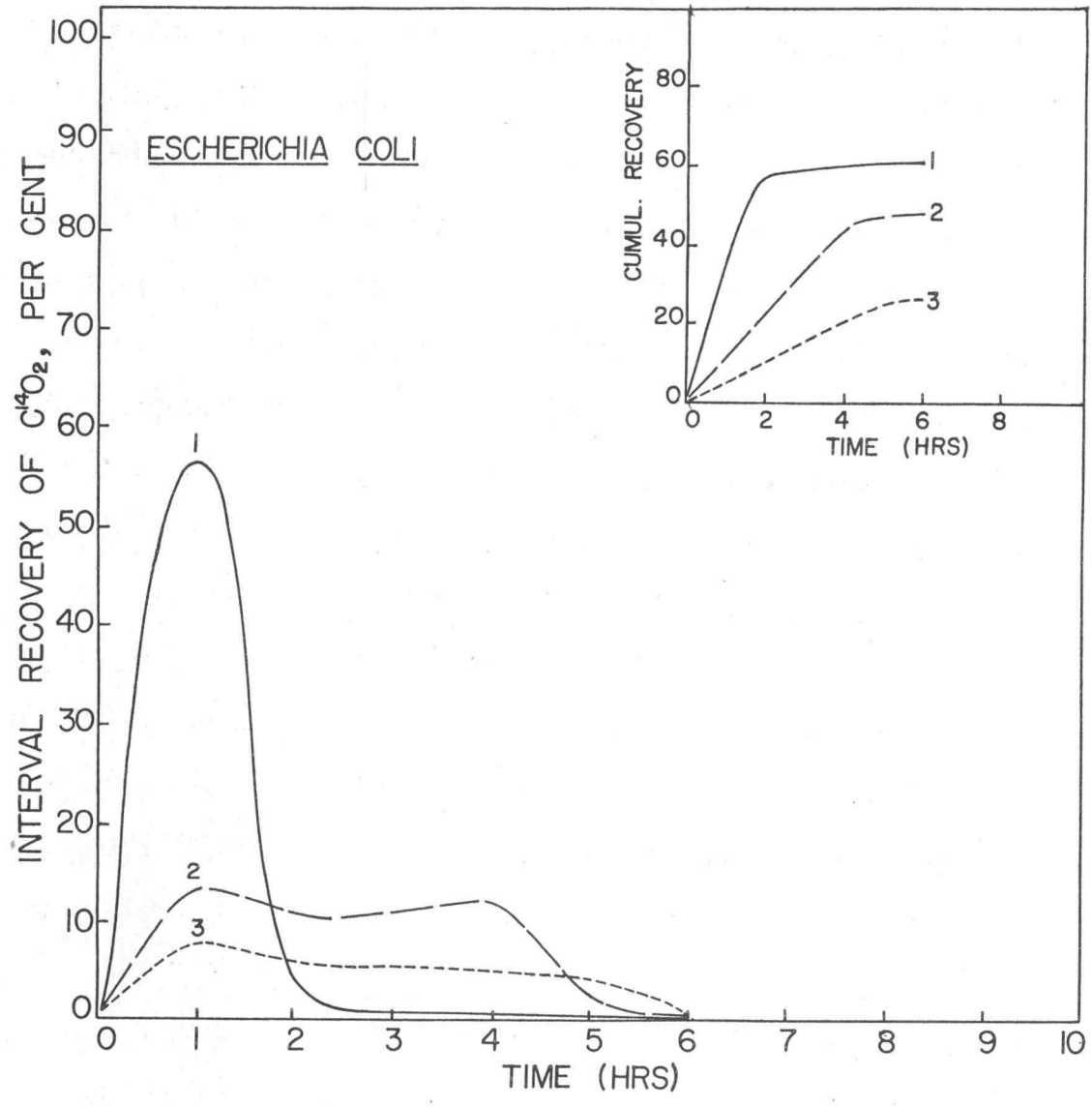
The radiorespirometric patterns to be presented here have been plotted on the basis of time in hours against percent interval recovery of substrate activity in respiratory $C^{14}O_2$. The cumulative $C^{14}O_2$ recoveries represent total $C^{14}O_2$ recovery plotted against time in hours. The radiorespirometric curves shown represent the average results of several experiments.

Radiorespirometry of Pyruvate Catabolism

Pyruvate is a key intermediate of glucose or gluconate catabolism. A radiorespirometric study of this substrate should provide valuable evidence for the major catabolic routes of this essential compound. Roberts *et al* have indicated that in *E. coli* substrate pyruvate behaves like acetate rather than like pyruvate derived from glucose catabolism (77, p. 242).

Interesting information concerning the role of pyruvate in the respiratory function of *E. coli* is obtained by an analysis of the relative rates and extents of $C^{14}O_2$ recovery from each of the labeled pyruvate substrates tested. The radiorespirometric patterns for *E. coli* cells metabolizing pyruvate-1-C^{14}, pyruvate-2-C^{14} and pyruvate-3-C^{14} are shown in Figure 3. The rapid and extensive $C^{14}O_2$ recovery from the carboxyl (C-1) carbon

FIGURE 3



Radiorespirometric Pattern. Escherichia coli metabolizing specifically labeled pyruvate.

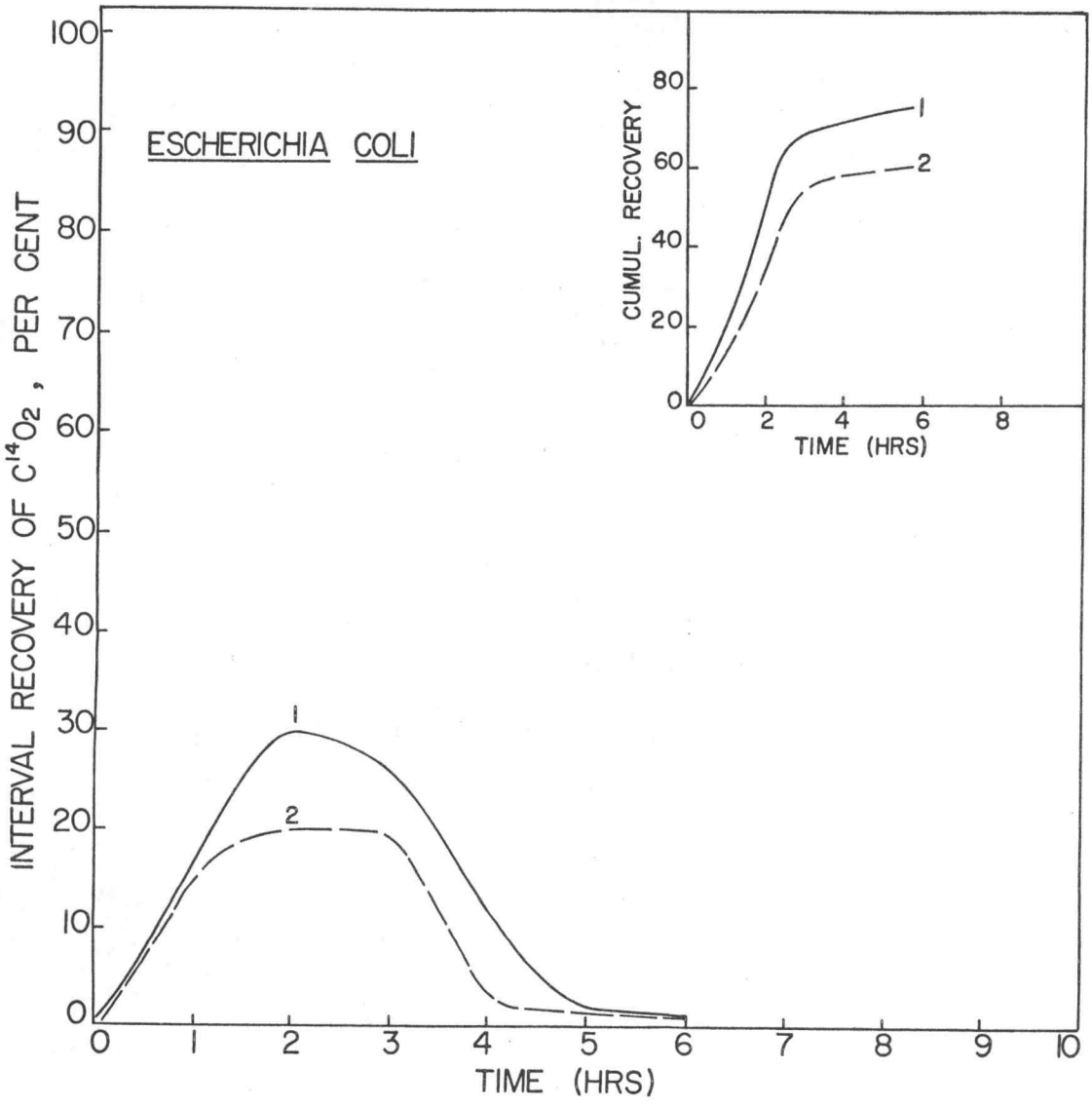
Pyruvate-1-C^{14} ———, -2-C^{14} — — —, -3-C^{14} - - - .

atom of pyruvate is representative of oxidative decarboxylation of pyruvate giving rise to acetate. The relative rates of CO_2 production from C-2 and C-3 of pyruvate suggest that the carbonyl (C-2) and methyl (C-3) carbon atoms become respectively the carboxyl and methyl groups of acetate. The ratio of cumulative $C^{14}O_2$ yields for pyruvate C-2/C-3, taken at the end of the experiment, is of interest in examining the role played by the TCA cycle in biosynthesis and respiration. A value of 1 for pyruvate C-2/C-3 would be expected for the case where no drainage of TCA cycle intermediates occurs. A value approaching 2 would be expected where intermediates of the cycle enter extensively into biosynthetic pathways. In the present case, the value of 1.8 was obtained which indicates that acetate, resulting from pyruvate decarboxylation, is utilized for both biosynthetic and energy producing processes.

Radiorespirometry of Acetate Catabolism

In order to gain insight into the operation of the TCA cycle in E. coli as well as to explore the possibility of alternate oxidative mechanisms, a radiorespirometric experiment was performed using acetate-1-C^{14} and acetate-2-C^{14} as substrates for E. coli cells previously grown on glucose. The results of this study are presented in

FIGURE 4



Radiorespirometric Pattern. Escherichia coli metabolizing specifically labeled acetate.

Acetate-1-C^{14} ——, -2-C^{14} — — — .

Figure 4, where it is observed that the carboxyl carbon (C-1) of acetate is recovered as CO_2 to a greater extent than the methyl carbon (C-2). These results are comparable with those observed with C-2 and C-3 of pyruvate. In the case of acetate, the ratio of cumulative $C^{14}O_2$ yields for acetate (C-1/C-2) has a value of 1.3 which indicates that acetate, as sole carbon source for glucose grown cells of E. coli, serves primarily in the respiratory function.

Radiorespirometry of Glyoxylate Catabolism

The results of many investigations have shown that glyoxylate is an active participant in the formation of malate from acetate in E. coli by way of the glyoxylate bypass pathway (51, p. 49-78; 53, p. 988-991; 55, p. 549-557). Upon entry into the TCA cycle by this mechanism, Figure 2, glyoxylate and acetate become essentially equivalent with respect to the recycling process in the nature of the TCA cycle. This suggests that any observed differences between the CO_2 production behavior of glyoxylate and acetate could be indicative of the occurrence of alternate oxidative pathways or differences in the relative contributions to biosynthetic functions from these two substrates. The literature presents many conflicting reports with regard to the origin and

catabolic routes of glyoxylate catabolism.

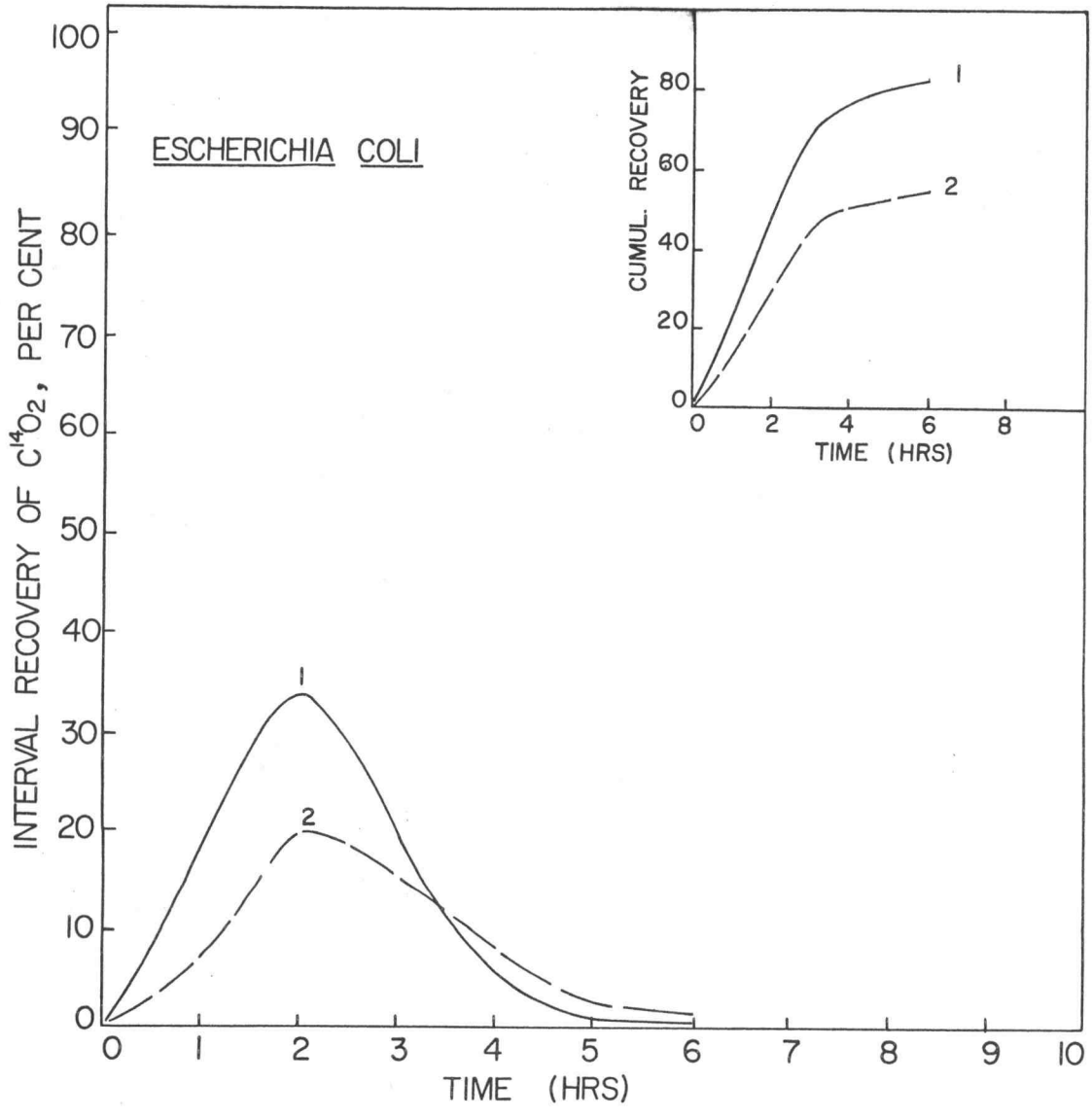
In Figure 5 radiorespirometric patterns for E. coli cells, metabolizing glyoxylate-1-C^{14} and glyoxylate-2-C^{14}, are presented. The patterns are very similar to those obtained with acetate. The ratio of cumulative $C^{14}O_2$ recovery for carbon atoms C-1/C-2 of glyoxylate is 1.4. This value is similar to that obtained with acetate.

The radiochemical recovery of substrate radioactivity in respiratory $C^{14}O_2$, cells and medium, observed in the pyruvate, acetate and glyoxylate experiments are given in Table I along with the experimental conditions employed. The results indicate a reasonable correlation between the overall inventories for the acetyl moiety of pyruvate (C-2 and C-3) and carbon atoms C-1 and C-2 of acetate and glyoxylate. However, the medium values are considerably higher for pyruvate.

Incorporation of Glyoxylate-1-C^{14} and Glyoxylate-2-C^{14}

In order to gain insight into the metabolic pathway of glyoxylate during the course of utilization in E. coli, the incorporation of C^{14} specifically labeled glyoxylate carbon atoms into cellular constituents was examined. The course of the incorporation experiment was followed by the conventional radiorespirometric techniques. The time course of $C^{14}O_2$ production as well as the

FIGURE 5



Radiorespirometric Pattern. Escherichia coli metabolizing specifically labeled glyoxylate.

Glyoxylate-1-C^{14} ——, -2-C^{14} — — — .

TABLE I

DISSIMILATION OF PYRUVATE, ACETATE AND GLYOXYLATE IN ESCHERICHIA COLI

Substrate	Level		Radiochemical Recovery of Substrates, Percent			
	uc	mg	CO_2	Cells	Medium	Total
Pyruvate-1-C^{14}	0.25	10	62	5	33	100
Pyruvate-2-C^{14}	0.24	10	47	13	32	92
Pyruvate-3-C^{14}	0.20	10	26	21	41	88*
Acetate-1-C^{14}	0.22	10	77	18	6	101
Acetate-2-C^{14}	0.21	10	61	21	7	89*
Glyoxylate-1-C^{14}	0.10	10	80	7	9	96
Glyoxylate-2-C^{14}	0.13	10	57	27	11	95

* Low value due to incomplete recovery of cells or medium

Experimental Conditions - incubation temperature, 37° C.; cell age, 12 hours; acidity of growth medium, initial pH 6.8, final pH 5.2; acidity of medium for radiorespirometry, pH 6.8; cell suspension, 15 mg. (dry weight) in 10 ml. of medium; aeration rate, 61 ml. per min.

radiochemical inventory of substrate activity in respiratory CO_2 cells and medium and the experimental conditions employed are shown in Table II. The radioactivity residing in the amino acid fraction of these cells in shown in Table III.

As expected, heavy labeling in glycine, serine, and alanine was observed. The labeling in glycine can be explained on the basis of well known glyoxylate transamination reactions. It is also known that glyoxylate undergoes decomposition reactions in many biological systems to form CO_2 (C-1) and active formate (C-2). The active formate may react with another molecule of glyoxylate to form hydroxypyruvate which could be a precursor of both alanine and serine (52, p. 1791-1795; 58, p. 593-594). Alternatively, formate resulting from glyoxylate decomposition may react with glycine to form serine. Doyle and Wang recently have indicated this latter reaction may account for the heavy labeling of alanine and serine in glyoxylate incorporation studies with tomato fruit (29, p. 751-756).

Radiorespirometry of Glucose Catabolism

In recent years considerable effort has been devoted by biochemists to the development of methods for the

TABLE II

CUMULATIVE $C^{14}O_2$ RECOVERIES AND SUBSTRATE RADIOACTIVITY INVENTORY FOR *E. COLI* CATABOLIZING GLYOXYLATE-1-C^{14} AND GLYOXYLATE-2-C^{14}

Substrate	Cumulative $C^{14}O_2$ Recovery, Percent			
	1 hr.	2 hr.	3 hr.	4 hr.
Glyoxylate-1-C^{14}	18	42	72	80
Glyoxylate-2-C^{14}	7	20	36	48

SUBSTRATE RADIOACTIVITY INVENTORY

Substrate	Substrate Level		Radiochemical Recovery of Substrates, Percent			
	uc	mg	CO_2	Cells	Medium	Total
Glyoxylate-1-C^{14}	.60	15	80	9	11	100
Glyoxylate-2-C^{14}	.67	15	48	40	13	101

Experimental Conditions - incubation temperature, 37° C.; cell age, 11 hours; pH of growth medium, initial pH 6.8, final pH 5.3; pH of medium for radiorespirometry, 6.8; cell suspension for radiorespirometry, 15 mg. in 10 ml. of medium; aeration rate, 61 ml. per min.

TABLE III

ISOTOPIC CONTENT OF CELLULAR AMINO ACIDS DERIVED FROM GLYOXYLATE-C^{14} AND GLYOXYLATE-2-C^{14}

Amino Acid	Carbon Content*	Radioactivity Counts/min.		Relative Specific Activity**	
		C-1	C-2	C-1	C-2
Aspartic	25	53	384	2	16
Glutamic	33	59	260	2	8
Serine	11	102	568	9	50
Glycine	10	143	270	14	27
Threonine	12	42	276	4	24
Alanine	24	146	502	6	21
Lysine	26	***	274		11
		50		1	
Arginine	20		219		11

* The carbon content is reported as milligrams of amino acid carbon per gram of cells.
** The relative specific activity is expressed as cpm/mg of amino acid carbon per gram of cells (dry weight).
*** Lysine and arginine did not separate on the chromatogram. The values represent the total activity in both molecules.

detection and estimation of multiple catabolic pathways in glucose metabolism. Recent interest has been focused upon the quantitative significance of concurrent pathways in the total catabolism of glucose.

Katz and Wood (46, p. 2165-2177) have recently examined the effect of extensive recycling of glucose, in the nature of the pentose cyclic mechanism on the estimation of catabolic pathways by tracer methods. Their analysis was made on the basis that the glucose molecules entering the pentose cyclic mechanism are extensively catabolized through repeated cycling of intermediates. Dawes and Holms, however, have indicated that in Sarcina lutea, where both the EMP and HMP pathways are of major significance, fructose-6-phosphate arising from glucose catabolism via the HMP route is apparently further catabolized via the combined operations of these two pathways (23, p. 551-552). Wang and Krackov (93), on the basis of radiorespirometric studies of glucose and gluconate catabolism in Bacillus subtilis, reported that hexose phosphate formed through the operation of the pentose cycle is catabolized in the same manner as substrate glucose.

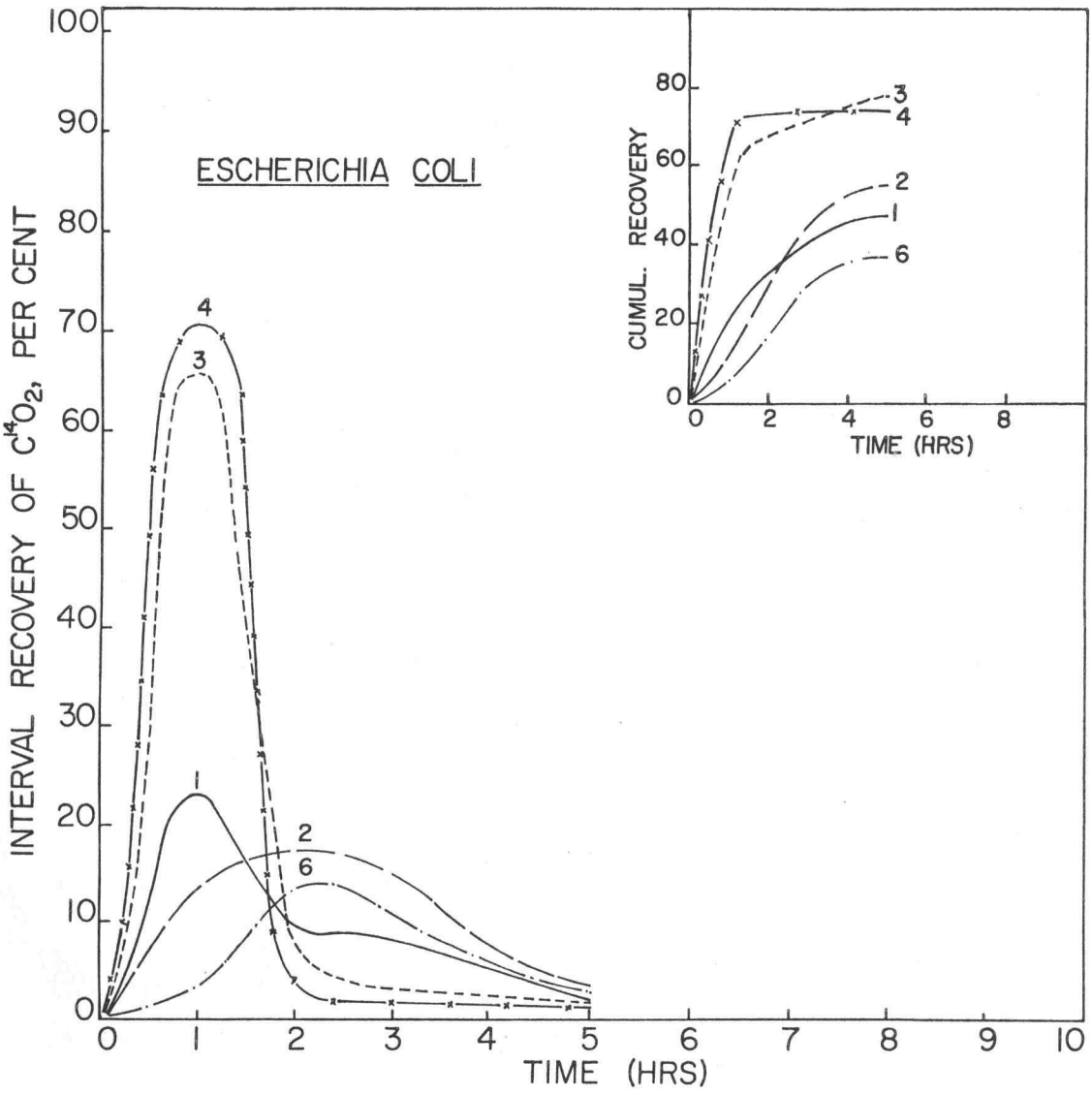
With the basic radiorespirometric patterns for the catabolism of pyruvate, acetate and glyoxylate in hand, it is then possible to direct attention to the study of

glucose catabolism with particular interest focused on the catabolic fate of pentose, arising during the course of glucose oxidation. Wang et al (91, p. 207-216) have reported the results of radiorespirometric experiments of glucose catabolism in E. coli and have indicated that the pathways participation for this substrate is 72 percent via the EMP pathway and 28 percent via a pathway presumably involving phosphogluconate decarboxylation.

A renewed interest in concurrent pathway operation had led to the development in this laboratory of radiorespirometric experiments designed to test for pentose catabolism in E. coli. Recently glucose-3-C^{14} has been made available as a test substrate for radiorespirometric studies. The use of this substrate and glucose-3(4)-C^{14} in radiorespirometric experiments makes possible the calculation of the radiorespirometric pattern for glucose-4-C^{14} by difference. Any observed difference in the rates of $C^{14}O_2$ recovery from C-3 and C-4 of glucose can be interpreted as reflecting the nature of pentose catabolism, since known routes of pentose catabolism call for an unequal rate of $C^{14}O_2$ recovery from the original C-3 and C-4 carbon atoms of glucose.

The radiorespirometric patterns of glucose catabolism in E. coli metabolizing glucose-1-C^{14}, -2-C^{14}, -3-C^{14}, -3(4)-C^{14} and -6-C^{14} are presented in Figure 6. The

FIGURE 6



Radiorespirometric Pattern. Escherichia coli metabolizing specifically labeled glucose.

Glucose-1-C^{14} ———, -2-C^{14} — — —, -3-C^{14} - - - ,

-4-C^{14} — X — X —, -6-C^{14} — . — . — .

radiochemical yield of substrate activity in CO_2, cells and medium as well as the experimental conditions employed are shown in Table IV.

The overall picture of glucose catabolism in this organism, as revealed in Figure 6, is essentially the same as that presented earlier (91, p. 207-216), with the exception that radiorespirometric data on carbon atoms C-3 and C-4 are included in the present work. That there is a significant difference in the rates of $C^{14}O_2$ recovery from glucose C-3 and C-4 is interpreted as indicative that pentose phosphate, formed during the course of glucose oxidation via the pentose cycle, can be further catabolized.

Radiorespirometry of Gluconate Catabolism

With the information that pentose phosphate, formed during the course of glucose utilization by E. coli, can be further metabolized, it was desirable to study in more detail the catabolic pathway for the utilization of this substrate. The pentose phosphate pathway in E. coli was extensively examined in the present study through the use of labeled gluconate as the substrate for radiorespirometric experiments. The employment of gluconate, as the test substrate in radiorespirometric studies as well as the incorporation studies to be presented later, is based

TABLE IV

DISSIMILATION OF GLUCOSE IN
ESCHERICHIA COLI

Substrate	Level		Radiochemical Recovery of Substrates, Percent			
	uc	mg	CO_2	Cells	Medium	Total
Glucose-1-C^{14}	0.25	18	47	40	10	97
Glucose-2-C^{14}	0.24	18	53	36	10	99
Glucose-3-C^{14}	0.03	18	78	26	5	109
Glucose-4-C^{14}*	-	-	76	12	7	95
Glucose-3,4-C^{14}	0.06	18	77	19	6	102
Glucose-6-C^{14}	0.26	18	35	49	12	96

* Calculated values

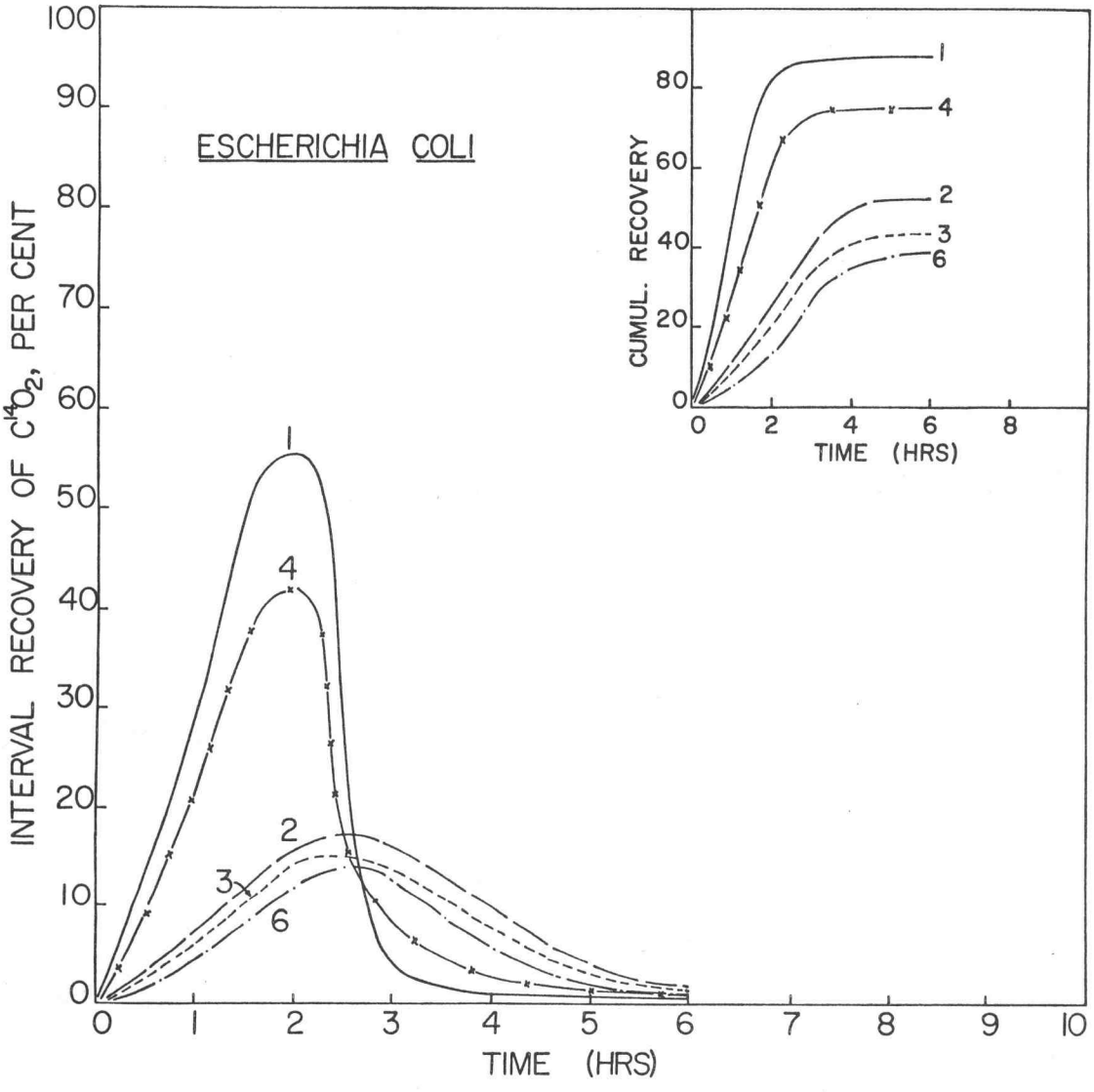
Experimental Conditions - incubation temperature, 37° C.; cell age, 12 hours; acidity of growth medium, initial pH 6.8, final pH 5.4; acidity of medium for radiorespirometry, pH 6.8; cell suspension, 15 mg. (dry weight) in 10 ml. of medium; aeration rate, 61 ml. per min.

upon the following considerations. (1) Gluconate supports the growth of E. coli. (2) Gluconate is a key intermediate of the pentose cycle reactions. (3) The reverse reaction leading from gluconate to glucose is not likely to occur. These facts make it possible to study both the phosphogluconate decarboxylation pathway as well as the Entner-Doudoroff pathway possibly operative in this organism.

The radiorespirometric patterns in E. coli cells metabolizing gluconate-1-C^{14}, -2-C^{14}, -3-C^{14}, -3(4)-C^{14} and -6-C^{14} are presented in Figure 7. The radiochemical yield of substrate activity in respiratory CO_2, cells and medium and the conditions under which the experiment was performed are shown in Table V.

The results of the radiorespirometric studies suggest the following sequence of events. (1) In E. coli, gluconate is rapidly decarboxylated to pentose phosphate presumably after prior phosphorylation, as reflected by the extensive radiochemical yield of $C^{14}O_2$ from carbon atom C-1. (2) The concurrent rapid rate of $C^{14}O_2$ recovery from C-4 of gluconate, followed by the less extensive rates of $C^{14}O_2$ recovery from carbon atoms C-2, C-3 and C-6 of gluconate can be explained on the basis of the combined operations of the pentose cycle and the EMP pathway as concurrent primary catabolic routes with the

FIGURE 7



Radiorespirometric Pattern. Escherichia coli metabolizing specifically labeled gluconate.

Gluconate-1-C^{14} ———, -2-C^{14} — — —, -3-C^{14} - - -,

-4-C^{14} — x — x —, -6-C^{14} — . — . —.

TABLE V

DISSIMILATION OF GLUCONATE IN
ESCHERICHIA COLI

Substrate	Level		Radiochemical Recovery of Substrates, Percent			
	uc	mg	CO_2	Cells	Medium	Total
Gluconate-1-C^{14}	0.23	23.5	86	6	8	100
Gluconate-2-C^{14}	0.20	23.5	52	33	8	93
Gluconate-3-C^{14}	0.02	23.5	45	44	11	100
Gluconate-4-C^{14}*	-	-	73	18	11	102
Gluconate-3,4-C^{14}	0.08	23.5	59	31	11	101
Gluconate-6-C^{14}	0.20	23.5	38	45	12	95

* Calculated values

Experimental Conditions - incubation temperature, 37° C.; cell age, 12 hours; acidity of growth medium, initial pH 6.8, final pH 5.4; acidity of medium for radiorespirometry, pH 6.8; cell suspension, 15 mg. (dry weight) in 10 ml. of medium; aeration rate, 61 ml. per min.

TCA cycle serving as the terminal oxidation mechanism for the EMP pathway. That the Entner-Doudoroff pathway is not operative in this organism is evidenced by the findings in the incorporation experiment to be presented later in the discussion. However, it should be pointed out, that the observed radiorespirometric patterns may be interpreted on the basis of the concurrent operation of the pentose cycle and the Entner-Doudoroff pathway.

The conclusion, that the concurrent operation of the pentose cycle reactions and the EMP-TCA pathway is in accordence with the present radiorespirometric results, is drawn from the studies of Wang and Krackov (93). These investigators have shown that it is possible to predict the total $C^{14}O_2$ yields of individual carbon atoms of gluconate providing the pathways distribution is operative. The necessary considerations for making these predictions are as follows: (1) The reversal of the pentose cycle operations from gluconate to glucose via gluconolactone is not likely to occur. (2) The isotopic distribution pattern in fructose-6-phosphate deriving its carbon atoms from C^{14} specifically labeled gluconate can be predicted (9, p. 339-347). (3) Substrate gluconate behaves metabolically identical to gluconate formed through glucose metabolism. (4) The Entner-Doudoroff pathway is not operative. (5) The fraction of gluconate

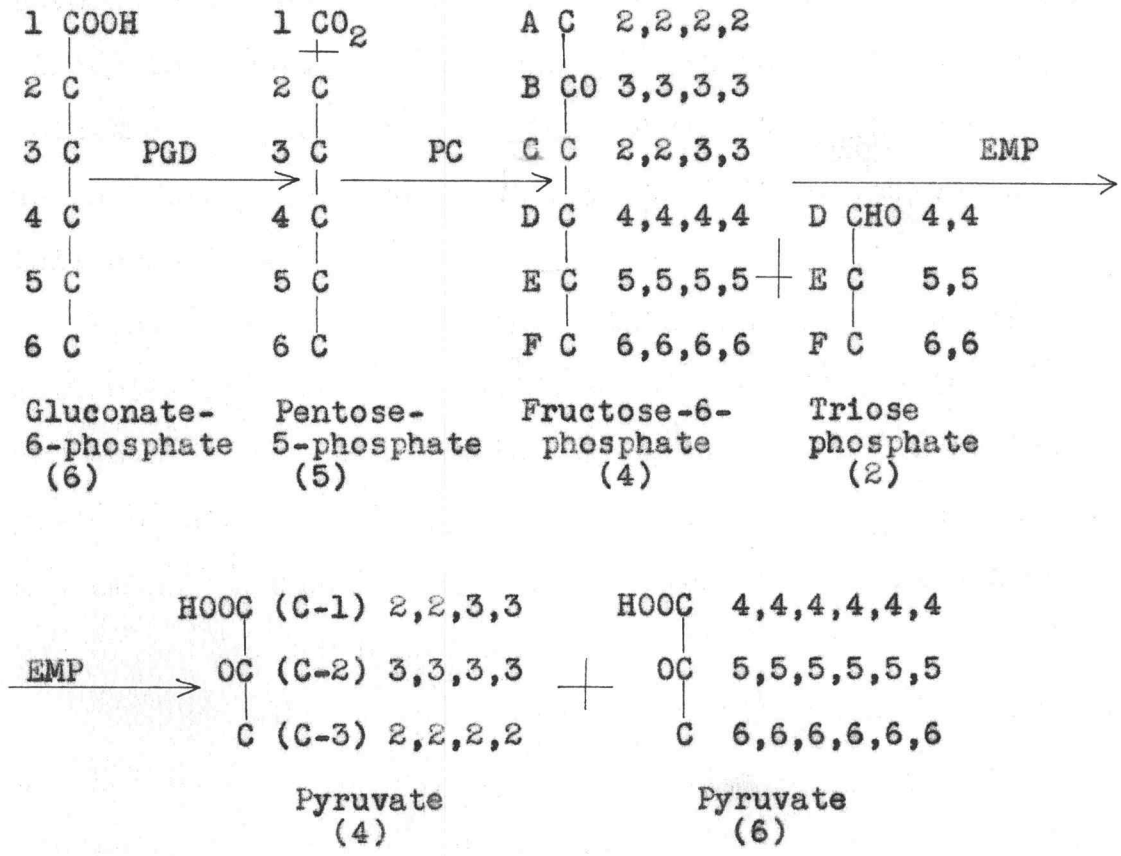
converted to pentose phosphate is represented directly by the percentage recovery of C-1 of gluconate in CO_2. This is true in as much as one mole of pentose phosphate can be derived from one mole of substrate gluconate.

Thus the further catabolism of these fructose-6-phosphate molecules may be predicted as follows: As an example, the total $C^{14}O_2$ recovery from C-2 of gluconate can be calculated from data given in Tables IV and V where the total $C^{14}O_2$ yields from individual carbon atoms of glucose and gluconate are found. According to the pattern of transposed gluconate carbon atoms in re-formed fructose-6-phosphate, as illustrated in Figure 8, the original C-2 of gluconate will be found in fructose carbon atoms A and C. The percentage is expressed as fraction of unity. .67 of this will be found in carbon A and .33 in carbon atom C.

If we now assume that the re-formed fructose-6-phosphate behaves catabolically like administered glucose, the total recovery for carbon atoms A and C will be directly proportional to the recoveries observed for carbon atoms C-1 and C-3 of glucose (Table IV). The proportionality factor, .86, is represented by the fraction of gluconate which is transformed to fructose-6-phosphate as indicated by the $C^{14}O_2$ recovery of C-1 of gluconate (Table V). In this case, C-1 of the re-formed hexose

FIGURE 8

POSITION AND LABELING INTENSITY* OF TRANSPOSED GLUCONATE CARBON ATOMS IN RE-FORMED FRUCTOSE-6-PHOSPHATE



* The labeling intensity represents the fraction of radioactivity of a transposed gluconate carbon atom residing in fructose-6-phosphate carbon atom(s). i.e. for gluconate C-2, the labeling intensity is 0.67 in fructose A and 0.33 in fructose C.

yields 47 percent as $C^{14}O_2$ and C-3 of the re-formed hexose yields 78 percent as $C^{14}O_2$. For purposes of calculation, these may be expressed as fractions of unity. Calculation of the theoretical $C^{14}O_2$ yield for C-2 of gluconate may then be carried out as follows:

From fructose C-1 .86 x .67 x .47 = .27 and

From fructose C-3 .86 x .33 x .78 = .22

Total = 0.49 or

49 percent of substrate activity. The value so calculated represents the sum of the total $C^{14}O_2$ contribution for fructose-6-phosphate bearing labeling from C-2 of gluconate. It is obvious that the contributions of both the indirect EMP pathway and the pentose cycle operation in the total metabolism of gluconate are automatically included in these calculations since the total $C^{14}O_2$ recovery values for glucose carbon atoms C-1 and C-3 reflect total glucose catabolism in both of these concurrently operative systems.

The calculated value of 49 percent agrees well with the observed value of 52 percent for C-2 of gluconate (Table V). The calculated and observed total $C^{14}O_2$ recovery values for C^{14} specifically labeled gluconate carbon atoms are shown in Table VI. The values so recorded are in reasonably good agreement with the observed values. Thus experimental verification is

TABLE VI

OBSERVED AND CALCULATED CUMULATIVE RECOVERIES FOR SPECIFICALLY C^{14} LABELED GLUCONATE CARBON ATOMS

Gluconate Carbon Atom	Fraction of Gluconate Decarboxylated	Fraction of Transposed Gluconate in Fructose-C	Recovery of Glucose-C in CO_2 at 6 hours	Calculated $C^{14}O_2$ Recovery (Fraction)	Calculated $C^{14}O_2$ Recovery Total (Percent)	Observed $C^{14}O_2$ Recovery (Percent)
	A	B	C	AxBxC		
C-2	0.86	0.67 in C-A	0.47	0.27		
	0.86	0.33 in C-C	0.78	0.22	49	52
C-3	0.86	0.67 in C-B	0.53	0.30		
	0.86	0.33 in C-C	0.78	0.22	52	45
C-4*	0.86	1 in C-D	0.76	0.65	65	73
C-4**	0.86	0.83 in C-D	0.87	0.62		
	0.86	0.17 in C-C	0.78	0.11	73	73
C-6*	0.86	1 in C-F	0.35	0.30	30	38
C-6**	0.86	0.83 in C-F	0.35	0.25		
		0.17 in C-A	0.47	0.07	32	38

* Assuming no triose recombination
** Assuming triose recombination

provided for the foregoing described sequence for the catabolism of gluconate.

Quantitative Estimation of Concurrent Pathways Participation

The information gained in the catabolism of glucose and gluconate also permits one to reevaluate the estimation of pathway participation with respect to basic hexose catabolism in this organism. Previously an estimation has been carried out by Wang *et al* using a set of equations derived under a set of assumptions (90, p. 1869-1874). One of the assumptions states that pentose phosphate derived from glucose via the PGD pathway is not catabolized further with respect to the production of respiratory CO_2. The evidence presented in the present work, particularly with respect to the fate of pentose phosphate in its further catabolic function, makes it necessary to revise the original equations. In as much as the participation of pentose phosphate in the respiratory function does not affect in any way the rates and extents relative to the recovery of C-1 of glucose in CO_2, the refinement is, therefore, focused on the term G_6, which represents the $C^{14}O_2$ yield from C-6 of glucose. By definition, the term G_6 represents the overall yield from C-6 of glucose from any pathway. With concurrent

operation of the EMP and the HMP pathways, the contributions of C-6 of glucose toward the production of respiratory CO_2 by the HMP pathway should, therefore, be deducted from the term G_6. By applying this correction, the original equation

$$G_p = \frac{G_1 - G_6}{G_T - G'_T} \qquad (1)$$

is, therefore, revised to read:

$$G_p = \frac{G_1 - (G_6 - A_6 G_p)}{G_T - G'_T} \qquad (2)$$

When $G_T = 1$ and the magnitude of G'_T approaches zero then equation (2) becomes:

$$G_p = \frac{G_1 - G_6}{1 - A_6} \qquad (3)$$

The extent of participation of the EMP pathway remains

$$G_e = 1 - G_p \qquad (4)$$

The derivation of the necessary equations for estimation of pathway participation in the glucose metabolism by E. coli follows.

Let G_1, $G_{3,4}$ and G_6 = percent $C^{14}O_2$ yields from E. coli metabolizing equal amounts of glucose labeled with C^{14} at C-1, C-3 (or 4) or C-6 respectively, taken at 1 RTU (relative time unit, at which the primary catabolic

processes with respect to the administered substrate have been completed) expressed as fractions of unity.

A_1, $A_{3,4}$, A_6 = percent $C^{14}O_2$ yields from E. coli metabolizing equal amounts of gluconate labeled with C^{14} at C-1, C-3 (or 4) or C-6 respectively, taken at 1 RTU, expressed as fractions of unity.

G_T = total activity of each labeled substrate administered, expressed on the percentage basis as unity.

G'_T = fraction of the labeled substrate administered that was not engaged in metabolic processes, expressed as fractions of unity.

G_p = fraction of the administered glucose catabolized via the pentose cyclic pathway, expressed as fractions of unity.

G_e = fraction of the administered glucose catabolized via the glycolytic pathway, expressed as fraction of unity.

The revised equations are based on the following assumptions.

1. Glucose is catabolized in E. coli via two sets of concurrent metabolic sequences, namely, the EMP pathway and the pentose cyclic pathway.
2. The preferential conversion of C-1 of glucose to CO_2 via the pentose cyclic pathway is a rapid and essentially irreversible process.

3. The C_3 unit formed in the glycolytic pathway, best represented as pyruvate, is decarboxylated promptly. It is understood that a small fraction of pyruvate may participate in CO_2 fixation processes; however, the magnitude of such a process is likely to be insignificant.
4. The C_3 units formed in the glycolytic pathway are virtually equivalent to one another at the pyruvate stage with respect to further metabolic reactions.
5. The formation of hexose by way of a recombination of triose is virtually insignificant in magnitude.
6. The randomization of hexose skeleton via the transaldolase exchange reaction or similar processes does not occur to any significant extent.
7. The substrate gluconate is utilized in E. coli in a manner virtually identical to the phosphogluconate derived from glucose in vivo.

A comparison of the values obtained using the former and newly corrected equation is pertinent:

G_1 = 0.30 at 1 RTU (2 hours) from Figure 6

G_6 = 0.14 at 1 RTU (2 hours)

A_6 = 0.27 at 1 RTU (3 hours) from Figure 7

Using equation (1) when $G_T - G'_T = 1$:

$$G_p = G_1 - G_6$$
$$= .30 - .14$$
$$= .16 \text{ or } 16\%$$

Using equation (3):

$$G_p = \frac{G_1 - G_6}{1 - A_6}$$
$$= \frac{.30 - .14}{1 - .27}$$
$$= .22 \text{ or } 22\%$$

The former value for EMP

$$G_e = 1 - G_p$$
$$= 1 - .16$$
$$= .84 \text{ or } 84\%$$

now becomes with corrected value for HMP

$$G_e = 1 - G_p$$
$$= 1 - .22$$
$$= .78 \text{ or } 78\%$$

It is of interest to compare the present set of values for E. coli with those obtained in the earlier experiments of Wang et al (91, p. 207-216) where values of 28 percent HMP and 72 percent EMP were reported. The apparent discrepancy may be ascribed to slight modifications of the cultural conditions and individual technique. In addition, a newer stock culture (ATCC 11303B)

was used in the present studies.

Paege and Gibbs (72, p. 107-110) have recently studied glucose catabolism in E. coli cells under anaerobic resting conditions and concluded that at pH 5 the pentose cycle may be operative to the extent of 10 percent. Cohen had earlier reported a range of values for the direct oxidative pathway which indicated a minimum value of 14 percent and a maximum value of 37 percent for the direct oxidative pathway in E. coli (21, p. 746-747). There are, however, conflicting reports in the literature regarding the extent of glucose oxidation via the HMP pathway (5, p. 184-189).

Incorporation Studies

In order to gain further insight into the function of the pentose cycle pathway in E. coli, particularly with respect to the fate of pentose phosphate, the incorporation of labeled gluconate carbon atoms into the cellular constituents was also studied.

Usually, experiments of this type are carried out with growing cells and use is made of the isotopic distribution of labeling in the cellular amino acids for purposes of pathway analysis. This is necessary since in microorganisms, accumulation of pathway intermediates drained off from the operation of the TCA cycle, cannot be

realized when cells are cultured under growing conditions. The accumulation of pathway intermediates does not usually occur unless an inhibitor is used (39, p. 59-90).

Katagiri has reported the experimental conditions under which resting E. coli cells, metabolizing glucose, pyruvate or acetate, excrete large quantities of α ketoglutarate into the incubation medium (41, p. 143-153). This finding suggested that αketoglutarate, formed from C^{14} specifically labeled substrates, should be relatively free from the effects of randomization thus providing an excellent opportunity for analysis of a key TCA cycle intermediate with respect to the incorporation of substrate carbon atoms. It should be added that there exists no information for the comparison of the isotopic distribution pattern of cellular amino acids with that of the alleged precursor. Such a comparison is of great interest in the elucidation of the overall biosynthetic functions of catabolic pathways. Despite the fact that resting cells have to be used to effect accumulation of α ketoglutarate, labeling of cellular amino acids from C^{14} labeled substrate is expected in view of the study by DeMoss and Swim with resting yeast cells (26, p. 445-451). They conclude that when resting yeast cells are innoculated with C^{14} labeled acetate, the cells

incorporated substrate activity into several amino acids.

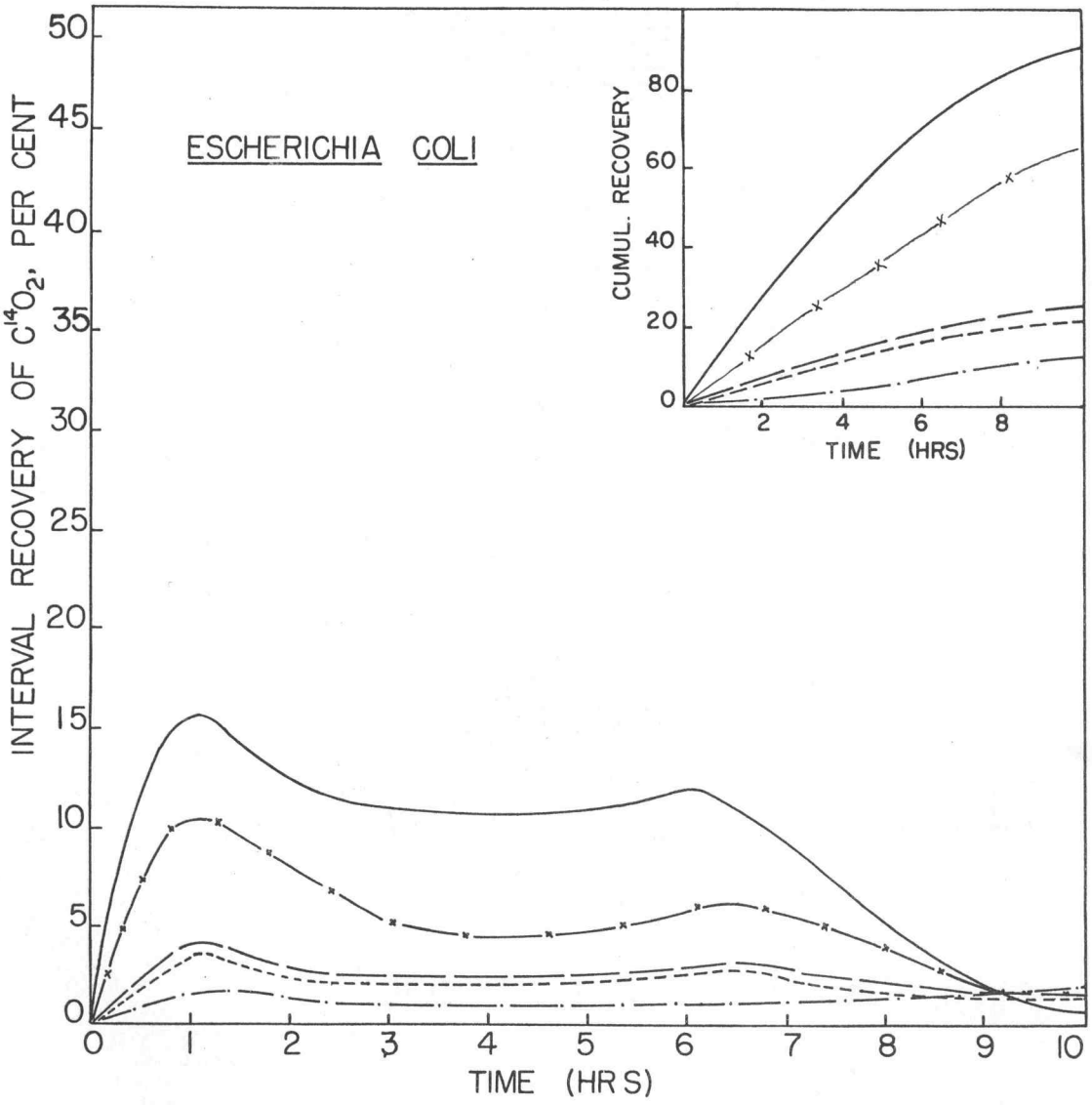
It is of interest to note that on the basis of earlier work, Katagiri has suggested that α ketoglutarate may have been derived from pyruvate by way of a $C_3 + C_2$ condensation with pyruvate and acetate as condensing components. The study of gluconate carbon atoms incorporated into ketoglutarate should, therefore, provide experimental evaluation of the occurrance of the proposed mechanism.

Radiorespirometry of Gluconate Catabolism in Resting Cells of Escherichia coli

Prior to the proposed incorporation studies, it is essential to first understand the catabolism of gluconate in resting E. coli cells with respect to the respiratory functions. For this reason, highly aerobic conditions (pure oxygen) were used. The latter is necessary since it is known that the production of α ketoglutarate is greatly enhanced when an atmosphere of high oxygen content is employed (42, p. 188-196).

The radiorespirometric patterns from resting cells of E. coli, metabolizing gluconate-1-C^{14}, gluconate-2-C^{14}, gluconate-3-C^{14}, gluconate-3(4)-C^{14} and gluconate-6-C^{14} may be examined by an inspection of Figure 9. Here we find that the overall rate of gluconate catabolism is

FIGURE 9



Radiorespirometric Pattern. Resting cells of Escherichia coli metabolizing specifically labeled gluconate.

Gluconate-1-C^{14} ———, -2-C^{14} — — —, -3-C^{14} - - -,

-4-C^{14} — x — x— , -6-C^{14} — · — · —.

TABLE VII

DISSIMILATION OF GLUCONATE BY RESTING CELLS OF ESCHERICHIA COLI

Substrate	Level		Radiochemical Recovery of Substrates, Percent			
	uc	mg	CO_2	Cells	Medium	Total**
Gluconate-1-C^{14}	0.25	23.5	90	0.49	5	95
Gluconate-2-C^{14}	0.22	23.5	27	17	43	87
Gluconate-3-C^{14}	0.02	23.5	23	14	45	82
Gluconate-4-C^{14}*	-	-	62	9	17	88
Gluconate-3,4-C^{14}	0.08	23.5	42	11	31	84
Gluconate-6-C^{14}	0.25	23.5	14	14	60	88

* Calculated values
** Low values due to error in determining the activity in the medium

Experimental Conditions - incubation temperature, 30° C.; cell age, 12 hours; acidity of growth medium, initial pH 6.8, final pH 5.5; acidity of medium for radiorespirometry, pH 6.2; cell suspension, 20 mg. (dry weight) in 30 ml. of phosphate buffer; aeration rate, 84 ml. per min. pure oxygen atmosphere.

considerably reduced as in comparison with Figure 7 where growing cells were used in a similar experiment. This is presumably due to the resting conditions employed. It is also observed that the cumulative $C^{14}O_2$ recoveries from gluconate carbon atoms C-2, C-3 and C-6 are lowered. This fact presumably reflects the drainage of αketoglutarate from the TCA cycle into the incubation medium. The radiochemical yields of substrate activity in respiratory $C^{14}O_2$, cells and medium are shown in Table VII. As expected, the radioactivity in the cells is lowered and that in the medium is increased when compared with the case of growing cells.

Incorporation of Gluconate-1-C^{14}, Gluconate-2-C^{14} and Gluconate-6-C^{14}

An incorporation experiment, using gluconate-1-C^{14}, -2-C^{14} and -6-C^{14}, was performed in the manner indicated for the previous radiorespirometric experiment. The cumulative $C^{14}O_2$ recoveries at selected time intervals, as well as the radioactivity inventory for CO_2, cells and medium along with the experimental conditions employed, are shown in Table VIII.

The amino acids and αketoglutarate which became labeled during the course of the experiment were isolated and the isotopic distribution patterns of alanine,

TABLE VIII

CUMULATIVE $C^{14}O_2$ RECOVERIES AND RADIOACTIVITY INVENTORY OF RESTING CELLS OF *E. COLI* CATABOLIZING SPECIFICALLY LABELED GLUCONATE-1-C^{14}, -2-C^{14} and -6-C^{14}

Substrate	Cumulative $C^{14}O_2$ Recovery, Percent		
	3 hr.	6 hr.	9 hr.
Gluconate-1-C^{14}	37	70	86
Gluconate-2-C^{14}	6	14	20
Gluconate-6-C^{14}	4	8	11

RADIOACTIVITY INVENTORY

Substrate	Level		Radiochemical Recovery of Substrates, Percent			
	uc	mg	CO_2	Cells	Medium	Total
Gluconate-1-C^{14}	10.6	23.5	86	0.7	19	10
Gluconate-2-C^{14}	3.9	23.5	20	8	60	88
Gluconate-6-C^{14}	2.2	23.5	11	6	79	96
Gluconate-U-C^{14}	0.15	23.5	38	13	48	99

Experimental Conditions - incubation temperature, 30° C.; cell age, 12 hours; pH of growth medium, initial pH 6.8, final pH 5.5; pH of phosphate buffer for radiorespirometry, 6.2; cell suspension for radiorespirometry, 20 mg. in 30 ml. of phosphate buffer; aeration rate, 84 ml. per min. pure oxygen atmosphere.

aspartic and glutamic acids and αketoglutarate were determined as indicated in the experimental methods section.

Incorporation of Gluconate-1-C^{14}, Gluconate-2-C^{14} and Gluconate-6-C^{14} into Alanine, Aspartic and Glutamic Acids

Information which helps to elucidate the fate of gluconate in the primary catabolic process may be gained from an examination of the isotopic distribution patterns in alanine, aspartic and glutamic acids deriving their labeling from gluconate-1-C^{14}, -2-C^{14} and -6-C^{14} as shown in Table IX.

An analysis of the labeling patterns in alanine reveals these significant facts: (1) exclusive carboxyl carbon labeling from C-1 of gluconate with nevertheless low specific activity; (2) alanine labeled from C-2 of gluconate is of considerably higher specific activity with labeling in the methyl (C-3) and carboxyl (C-1) carbon atoms in the ratio of 2 for C-3/C-1; (3) alanine from C-6 of gluconate has the highest specific activity and essentially all labeling is in the methyl (C-3) carbon atom.

This information may be readily interpreted on the basis of the previous discussion concerning the proposed catabolic pathway for gluconate catabolism in E. coli. In Figure 8, the positions of transposed gluconate carbon

TABLE IX

ISOTOPIC DISTRIBUTION OF ALANINE, ASPARTIC AND GLUTAMIC ACIDS DERIVED FROM GLUCONATE-1-C^{14}, GLUCONATE-2-C^{14} AND GLUCONATE-6-C^{14}

Amino Acid	Gluconate-1-C^{14}		Gluconate-2-C^{14}		Gluconate-6-C^{14}	
	Corr. S.A. uc/mM	% Dist.	Corr. S.A. uc/mM	% Dist.	Corr. S.A. uc/mM	% Dist.
Alanine						
Whole Molecule	.031	100	.5	100	.6	100
HOOC (C-1)	.03	95	.15	30	.03	5
H_2NC (C-2)	0	0	.02	3	.02	3
H_3C (C-3)	0	0	.30	60	.51	85
Aspartic Acid						
Whole Molecule	.07	100	.4	100	.6	100
HOOC (C-1)	-	-	-	-	-	-
H_2NC (C-2)*	0	0	.12	30	.12	20
C (C-3)	0	0	.16	40	.36	60
HOOC (C-4)	-	-	-	-	-	-
(C-1+ C-4)	.06	81	.12	30	.12	20
Glutamic Acid						
Whole Molecule	.06	100	.6	100	.1	100
HOOC (C-1)**	.05	90	.06	11	.07	7

* Calculated by difference

** Only the alpha carboxyl group analyzed

atoms in fructose-6-phosphate have been shown. In addition, further catabolism of the fructose-6-phosphate via the EMP pathway was shown to lead to the following incorporation patterns in pyruvate:

HOOC(C-1) 2,3,4,4,4
OC(C-2) 3,3,5,5,5
C(C-3) 2,2,6,6,6

Analysis of the labeling patterns in pyruvate derived from 3 molecules of C^{14} specifically labeled gluconate catabolized via the proposed sequential pathway reveals the following information: (1) no labeling occurs from C-1 of gluconate; (2) the ratio of labeling of the methyl carbon atom (C-3) to the carboxyl (C-1) carbon atom (C-3/C-1) for pyruvate labeled from C-2 of gluconate is 2/1; (3) C-6 of gluconate is incorporated exclusively into the C-3 methyl carbon atom of pyruvate.

A close correlation exists between the observed isotopic labeling patterns for alanine with those predicted for pyruvate produced via the pentose cycle pathway, keeping in mind that alanine reflects the labeling pattern of pyruvate. This observation confirms the contention that gluconate catabolism occurs via a reaction involving one-turn of the pentose cycle to the fructose-6-phosphate stage with subsequent catabolism of fructose-6-phosphate via the EMP pathway. The small amount of radioactivity

in C-1 of alanine derived from gluconate carbon atom C-1 can be explained on the basis of the current knowledge of $C^{14}O_2$ fixation and randomization reactions as explained by Wood et al (104, p. 475-489). That the Entner-Doudoroff pathway is not operative to explain the present results is indicated by the lack of intensive labeling in C-1 or C-2 of alanine respectively from C-1 or C-2 of gluconate.

The isotopic distribution patterns for aspartic acid may indicate that this acid is formed via transamination with oxaloacetate which is formed through the operation of the glyoxylate cycle. The fraction of activity in C-1 of glutamate is nearly equal to that indicated for α ketoglutarate, indicating that glutamate is probably derived through transamination with ketoglutarate.

Incorporation of Gluconate-1-C^{14}, Gluconate-2-C^{14} and Gluconate-6-C^{14} into α Ketoglutarate

In the course of catabolism of C^{14} specifically labeled gluconate in E. coli, an important intermediate is formed, presumably in the nature of pyruvate. An insight into the probable mechanism of pyruvate catabolism may be gained by analysis of the isotopic distribution pattern for α ketoglutarate, a key TCA cycle intermediate.

The isotopic distribution patterns for α ketoglutarate, formed during the course of metabolism of gluconate-1-C^{14},

-2-C^{14} and -6-C^{14} in E. coli are shown in Table X.

It is not surprising to find that C-1 of gluconate is incorporated mainly into the αcarboxyl carbon atom of α ketoglutarate inasmuch as incorporation can only be realized through CO_2 fixation. The mechanism which adequately explains these results involves CO_2 fixation of pyruvate by either the Wood-Werkman or malic enzyme reaction to give beta carboxyl (C-4) labeled oxaloacetate or malate. Subsequent metabolism of these C_4 acids via the conventional TCA cycle, involving condensation of oxaloacetate and acetate, yields citrate which upon further metabolism gives rise eventually to α keto-glutarate-1-C^{14}. This type of mechanism has recently been indicated to occur in Kluyera citrophila, an organism which produces α ketoglutarate (7, p. 170-182). The high incorporation of carbon atoms of α ketoglutarate can only be explained on the basis of the extensive operation of the glyoxylate bypass mechanism of the TCA cycle, since alternate mechanisms do not explain the results.

A comparison of the possible mechanisms by which α ketoglutarate may become labeled from C^{14} specifically labeled gluconate carbon atoms is offered as evidence for reaching the preceding conclusions. Examination of Figure 10 reveals that two mechanisms for entry of pyruvate into the TCA cycle are under consideration:

TABLE X

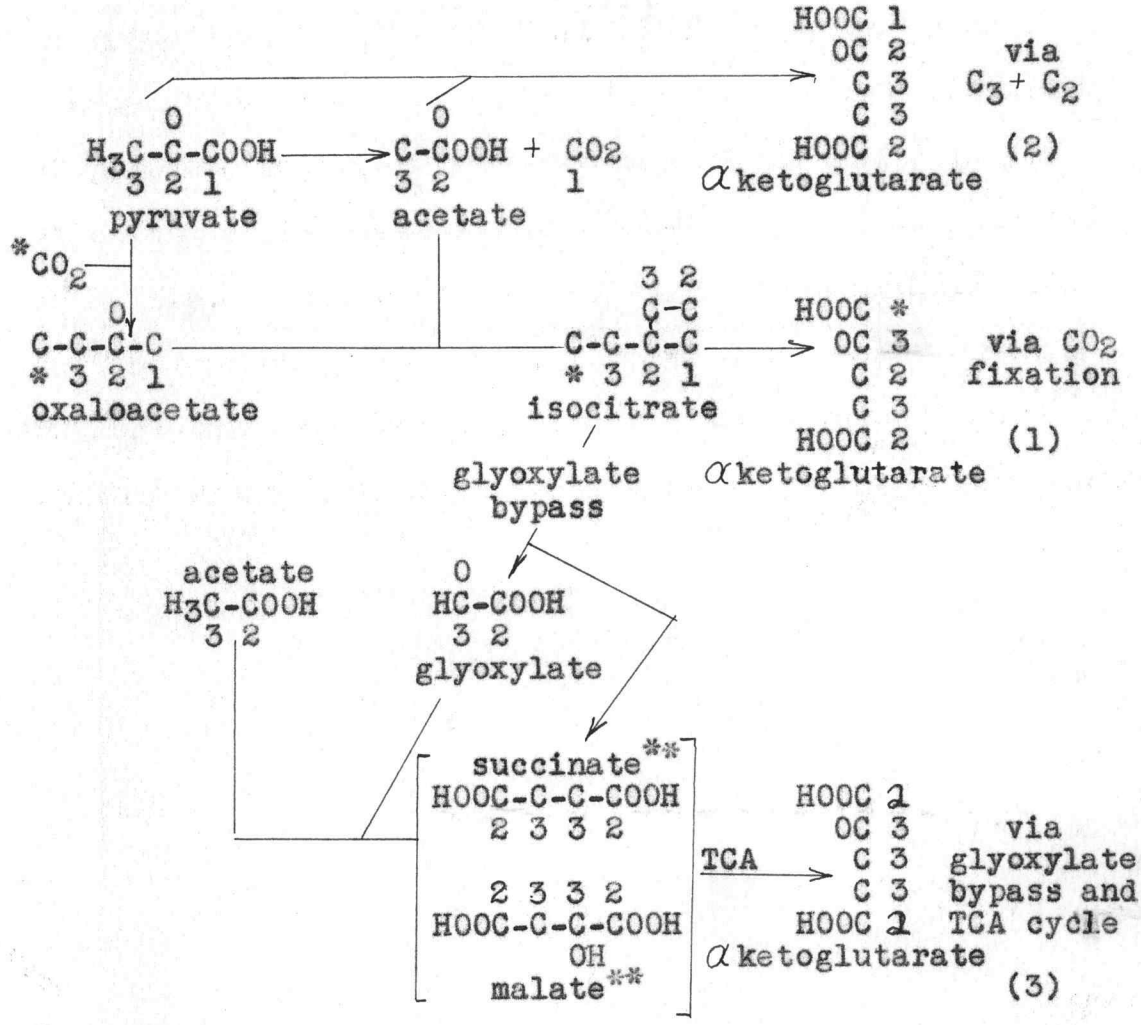
LABELING PATTERNS AND PROPOSED BIOSYNTHETIC MECHANISMS FOR αKETOGLUTARATE DERIVED FROM GLUCONATE-1-C^{14}, GLUCONATE-2-C^{14} AND GLUCONATE-6-C^{14}

Substrate	Isotopic Dist. of αKetoglutarate	Corr. S.A. uc/mM	% Dist. of Label	Origin of Carbon			
				Obs.	via Glyox. bypass	via CO_2 Fixation	via $C_3 + C_2$
Gluconate -1-C^{14}	Whole Molecule:	1.00	100				
	HOOC (C-1)	0.9	90	A_1**	-	A_1	-
	OC (C-2)	0	0	-	-	-	-
	C (C-3)	0	0	-	-	-	-
	C (C-4)	0	0	-	-	-	-
	HOOC (C-5)	0.1	10	A_1**	-	-	-
Gluconate -2-C^{14}	Whole Molecule:	6.0	100				
	HOOC (C-1)	0.9	14.5	A_2**	-	A_2	A_2
	OC (C-2)	1.4	24	A_2	A_2	A_2	-
	C (C-3)	3.5*	59	$2A_2$	A_2	-	A_2
	C (C-4)				A_2	A_2	A_2
	HOOC (C-5)	0.2	3.5	-	-	-	-
Gluconate -6-C^{14}	Whole Molecule:	9.6	100				
	HOOC (C-1)	1.0	10.1	A_6**	-	A_6	-
	OC (C-2)	2.2	23	A_6	A_6	A_6	-
	C (C-3)	6.2*	65	$2A_6$	A_6	-	A_6
	C (C-4)				A_6	A_6	A_6
	HOOC (C-5)	0.2	2.2	-	-	-	-

* Value represents sum of C-3 and C-4 as determined by difference
** Labeling due to CO_2 fixation and/or limited recycling mechanisms

FIGURE 10

POSSIBLE MECHANISMS OF
α KETOGLUTARATE BIOSYNTHESIS



* Position of fixed CO_2
** Equilibrium labeling patterns due to extensive recycling in the glyoxylate cycle.

(1) a $C_3 + C_1$ condensation to give C_4, involving CO_2 fixation into pyruvate; (2) a $C_3 + C_2$ condensation to give C_5, involving pyruvate and acetate as proposed by Katagiri. The glyoxylate cyclic mechanism (3) is under consideration for the complementary entry of acetate into the TCA cycle. One should point out that the mechanisms under consideration lead to the net synthesis of C_4 or C_5 intermediates of the TCA cycle, a condition which is necessary in the present case where α ketoglutarate is drained from the TCA cycle.

An understanding of the labeling patterns of gluconate carbon atoms in α ketoglutarate for each of the possible biosynthetic mechanisms, as shown in Table XI, is aided by an understanding of the position of gluconate carbon atoms in pyruvate. From the isotopic distribution pattern in alanine, it is concluded that pyruvate has the following labeling patterns from carbon atoms C-2 and C-6 of gluconate.

HOOC(C-1)	A_2	where A is either C-2 or C-6 from gluconate
OC(C-2)	-	
H_3C(C-3)	$A_{2,2,6,6,6}$	

It is to be observed that pyruvate labeled from C-2 of gluconate should behave like pyruvate-1,(3)-C^{14} with twice as much label in carbon atom C-3 as C-1.

TABLE XI

POSSIBLE BIOSYNTHETIC MECHANISMS OF αKETOGLUTARATE FORMATION FROM PYRUVATE

α Ketoglutarate	Position of Gluconate Carbon Atoms in α Ketoglutarate Derived from Pyruvate		
	via $C_3 + C_2$	via CO_2 Fixation	via Glyoxylate bypass
HOOC (C-1)	$A_{2,3,4,4,4}$	CO_2 CO_2	$A_{3,3,5,5,5}$
OC (C-2)	$A_{3,3,5,5,5}$	$A_{2,2,6,6,6}$	$A_{2,2,6,6,6}$
C (C-3)	$A_{2,2,6,6,6}$	$A_{3,3,5,5,5}$	$A_{2,2,6,6,6}$
C (C-4)	$A_{2,2,6,6,6}$	$A_{2,2,6,6,6}$	$A_{2,2,6,6,6}$
HOOC (C-5)	$A_{3,3,5,5,5}$	$A_{3,3,5,5,5}$	$A_{3,3,5,5,5}$

An evaluation of the possible biosynthetic mechanisms for α ketoglutarate, as shown in Table XI, is aided by the diagramatic reaction sequences leading to α ketoglutarate, as shown in Figure 10, and information concerning the relative positions and labeling patterns of gluconate carbon atoms in pyruvate as previously indicated. A comparison of the observed labeling patterns in α ketoglutarate (Table X) with those predicted by each of the possible mechanisms leads to the conclusion that operation of the glyoxylate bypass mechanism is important in the net synthesis of C_4 acid intermediates of the TCA cycle and hence in the biosynthesis of α ketoglutarate. This finding has two important consequences, namely, that the glyoxylate cycle, as proposed by Kornberg (53, p. 988-991), is operative in glucose grown cells of E. coli. Furthermore, the $C_3 + C_2$ condensation between pyruvate and acetate, as described by Katagiri (41, p. 143-153), does not appear to be operative on the basis of the results of the present work.

Thus the present experimental findings lead to the conclusion that gluconate and glucose are catabolized via the sequential concurrent operations of the pentose cycle mechanisms and the Embden-Meyerhof glycolytic pathways. Furthermore, pyruvate enters the TCA cycle through

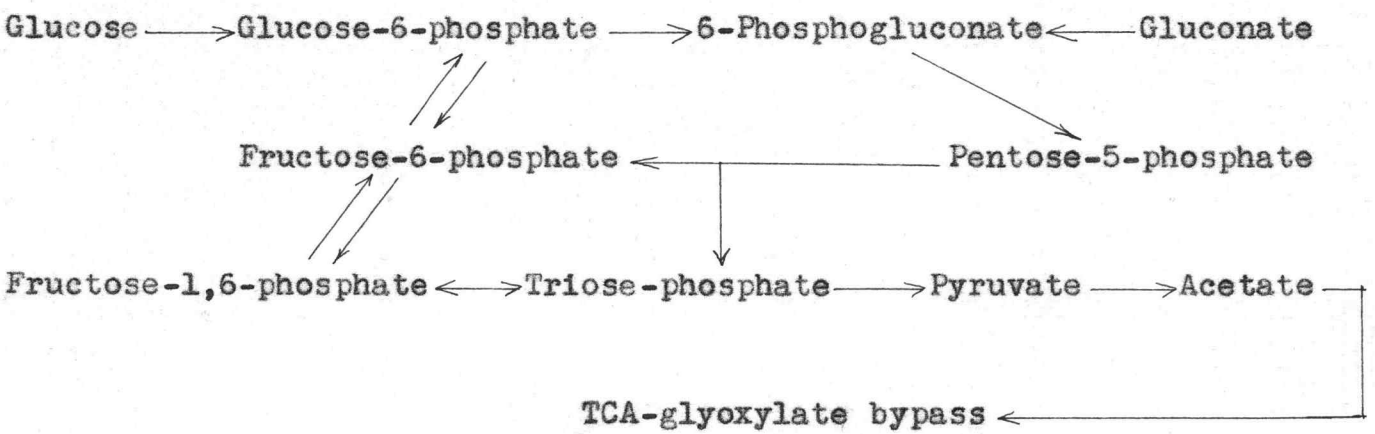
limited CO_2 fixation processes, but primarily after conversion to acetate via oxidative decarboxylation. The resulting acetate enters the TCA cycle through condensation with oxaloacetate to form citrate, and presumably through condensation with glyoxylate derived from isocitrate to form malate.

Glucose and gluconate catabolism in growing or resting cells of E. coli under aerobic conditions, apparently then proceeds according to the scheme shown in Figure 11.

Recently Gibbs and Paege (33, p. 6-9) have shown that pentose catabolism, in resting cells of E. coli under anaerobic conditions, proceeds via the combined pentose phosphate and EMP pathway. In addition, they have shown that at pH 5 under anaerobic resting conditions glucose catabolism in E. coli proceeds primarily by way of the EMP pathway and to a lesser extent through the pentose phosphate pathway (72, p. 107-110).

FIGURE 11

CATABOLIC PATHWAYS OF GLUCOSE AND GLUCONATE IN *ESCHERICHIA COLI*



SUMMARY

Recently it has become possible to evaluate quantitatively the extent of participation of two or more concurrently operative catabolic pathways for glucose utilization in microorganisms (93). The present study is designed to elucidate the gluconate metabolic pathways in E. coli with particular attention paid to the further catabolic fate of pentose phosphate arising in the course of gluconate catabolism. The results of radiorespirometric studies employing C^{14} specifically labeled pyruvate, acetate and glyoxylate indicated the following significant facts:

(1) Pyruvate is catabolized through oxidative decarboxylation to acetate.

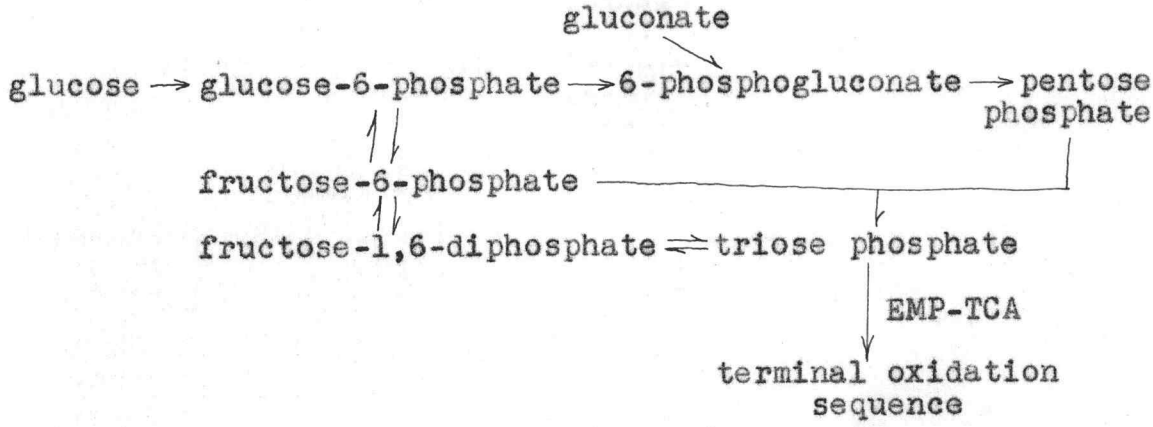
(2) Acetate and glyoxylate appear to be combusted biologically via the TCA cyclic mechanism. Glyoxylate is presumably routed into the TCA cycle by way of the glyoxylate bypass mechanism.

The results of radiorespirometric studies of glucose and gluconate catabolism and incorporation studies of C^{14} specifically labeled gluconates provide the following information:

(1) Gluconate is not catabolized via the ED pathway.

(2) Gluconate, and presumably gluconate arising from glucose oxidation in the hexose monophosphate pathway is

catabolized according to the following sequential steps:



(3) Gluconate and glucose catabolism is recognized to proceed to the extent of 78 percent EMP pathway and 22 percent pentose phosphate pathway.

(4) Triose formed in the catabolic sequence of gluconate is converted to α ketoglutarate by the Krebs cycle mechanism. The glyoxylate cycle pathway which involves isocitratase and malate synthetase as key enzymes appears to be the main route for the net synthesis of C_4 acids. The latter is in turn fed into the Krebs cycle leading to the net synthesis of α ketoglutarate.

(5) The findings indicate that the speculative mechanism, proposed by Katagiri (41, p. 143-153), for the biosynthesis of α ketoglutarate, namely, the condensation of pyruvate and acetate, is not a probable mechanism for the production of α ketoglutarate.

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