

PATHWAYS OF CARBOHYDRATE METABOLISM IN MICROORGANISMS

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Carbohydrates serve as a major source of carbon and energy for the growth of many microorganisms. Thus, in understanding the chemical basis of biological processes, a knowledge of carbohydrates, their chemical properties and transformations, and the pathways of carbon and energy liberation assumes importance, as does control of the reaction routes and formation of biosynthetic precursors.

Several deterrents face the seeker of such knowledge, stemming principally from the complexity of organisms and their processes, limitation in imagination of the investigator, particularly as to the questions asked, in his ability to generalize fruitfully from the existing data and to recognize its limitations in completeness and accuracy. Attempts to collect and to present these data in unified form for use by students and other investigators suffer in addition from limitations of the observer and his judgment in selecting pertinent data and of clearly conceiving and presenting their implications and consequences. Moreover, these concepts are subject to continuous obsolescence as new data and their interpretation reorient understanding and relate seemingly random observations into a conceptual if not complete scheme. The present effort, like many before it, is subject to these shortcomings. Should it prove accurate in re-

cording the data and concepts now current, helpful in planning future experimentation, time saving for instruction, and a helpful guide to information on microbial carbohydrate metabolism, we shall consider the effort expended in assembling, considering and recording the status of knowledge in this area well spent.

The *methods* and *objectives* of studying carbohydrate metabolism vary continuously. In the economic sense, (a) the quantitative transformation of cheap, often by-product carbohydrate to useful products, by fermentation or partial oxidation with growing cultures for industrial or epicurean purposes, has, at least for the former, given way largely to synthetic industrial practice and is replaced by (b) the formation, in more limited amounts, of more complex molecules of medicinal and nutritional importance. In addition, microbial cells enjoy increased use as (c) food or feed adjuncts for the minor (vitamin) components, and as tools for (d) the study of the formation of the intermediates in the glycolytic and oxidative pathways (*i.e.*, phosphate esters) and for the preparation of the cofactor forms of the vitamins as medical curios and more fruitfully as tools for the further elucidation by the chemist and the microbiologist, of the many chemical processes of the living cell still unclassified or undocumented.

In the search for understanding of living processes, the objectives have shifted principally from the quantitative description of the conversion of substrate to product and control of product yields, through identification of fermentation intermediates, description of the reactions they undergo and the catalysts responsible for these reactions, to a consideration of the energy, equilibria and mechanisms of these transformations and now principally to their relationship to cell formation.

Many problems of fermentations studied abundantly at the substrate to product level with growing cultures and with cell suspensions have not been subjected to the enzymatic and isotopic analysis essential to an understanding of the routes involved. Particularly outstanding among these is the propionic fermentation in which isotope labeling amply demonstrates that the Embden-Meyerhof scheme is not the sole pathway, but enzymatic clarification of the steps is lacking.

Although for most microorganisms degrading carbohydrates, the pathway, or pathways, of carbon and energy transformation is becoming clear, this may prove to be only the phase preceding recognition of still further divergences. As examples, the recent observations on the function of ribulose diphosphate in CO₂ incorporation, the route of formation of rhamnose and other cell wall components may be cited.

Also, still unstudied are many problems of induced enzyme formation, the transport of compounds including carbohydrates from the cellular environment to enzymatic sites, the steps in formation of many amino acid and other carbon skeletons and their transport to the sites of synthesis.

I. FERMENTATION *via* HEXOSEDIPHOSPHATE REACTIONS AND PATHWAYS

Glucose, because of its wide distribution and importance in animal metabolism, has served as the initial substrate for most studies of product formation and mechanism of carbohydrate metabolism in microorganisms. Furthermore, glucose is assigned a major role: (a) in reactions leading from polysaccharides, (b) as a substrate for phosphorylation prior to rearrangement and cleavage in fermentation and oxidation, and (c) as a point of departure for the alternate pathways of the hexosediphosphate, or Embden-Meyerhof,

glycolytic scheme and for both the fermentative and oxidative hexosemonophosphate pathways. Thus in the present discussion, the reactions of glucose serve as the first example and main theme. The analogous reactions of aldoses and ketoses of 3 to 7 carbon atoms are discussed with the documentation of glucose reactions. Their import in carbohydrate pathways is discussed in subsequent sections. An attempt to verify the routes followed by carbohydrates in fermentation and oxidation, along with the necessary assignment of sequence to the enzymatic steps, is postponed until after discussion of the specific reaction types.

1. Aldose and Ketose Reactions

a. *Kinases*. Kinases, which transfer phosphate from adenosine triphosphate (ATP) to carbon skeletons, were recognized first for their role in glucose dissimilation. Later, with the separation of kinases from several sources, their substrate specificity and role in fermentation of other carbohydrates were clarified. The variety of carbohydrates fermented by microorganisms has in several instances been traced to ability to form kinases, either constitutively or in response to added inducers, thereby initiating their dissimilation *via* phosphate esters. With increasing study, kinases have been shown to possess specificity for both the position of phosphorylation and the configuration of the acceptor. The known carbohydrate kinases, their source and specificities are summarized in table 1.

Hexokinase, which phosphorylates glucose, fructose and mannose, at relative rates of 100:140:30 (24), has been crystallized from yeast (13, 24, 230) and measured in animal tissue such as brain, muscle, heart, liver and kidney (38, 71, 72, 83, 347, 416).

A *glucokinase* which phosphorylates glucose, but not fructose or mannose, has been reported in muscle and in liver (72, 77). In microorganisms, few studies have utilized purified kinases, but the phosphorylation of glucose and of other carbohydrates entering the Embden-Meyerhof pathway has been shown in a wide variety of fermentative microorganisms. A glucokinase specific for the conversion of glucose to glucose-6-phosphate (G-6-P) has been reported in *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) and in *Escherichia coli* (54) and "hexokinase" activity shown in extracts of *Leuconostoc*

TABLE 1
Kinases: Carbohydrate Phosphorylation

Enzyme	Substrate	Product	Source
1. Aldokinase (hemiacetal bond) Galactokinase	Galactose	GA-1-P	Bacteria (54, 331) Yeast (221, 380) Animal (219)
2. Aldokinases (ester bond) Hexokinase	Glucose Fructose Mannose Glucosamine	G-6-P F-6-P MA-6-P GAM-6-P	Bacteria (125, 331, 350) Yeast (24, 230) Animal (72, 347)
Glucokinase	Glucose	G-6-P	Bacteria (54) Animal (72)
Ribokinase	Ribose	R-5-P	Yeast (333) Bacteria (69, 180)
Phosphoglucokinase	G-1-P	G-1,6-P	Yeast (311) Muscle (311) Plant (55)
3. Ketokinases (ester bond) Fructokinase	Fructose	F-1-P	Animal (80, 163, 244)
6-Phosphofructokinase	F-6-P	F-1,6-P	Yeast (72) Bacteria (393) Animal (319, 321) Plants (9)
1-Phosphofructokinase	F-1-P	F-1,6-P	Muscle (347)
5-Phosphoribulokinase	Ru-5-P	Ru-1,5-P	Plants (418)
4. Aldonic kinases (ester bond) Gluconokinase	Gluconate	6-PG	Bacteria (67, 350) Yeast (332)
2-Ketogluconokinase	2-Ketogluconate	2-KPG	Bacteria (88, 289)

Abbreviations: GA-1-P = galactose-1-phosphate; G-1-P = glucose-1-phosphate; G-6-P = glucose-6-phosphate; F-6-P = fructose-6-phosphate; MA-6-P = mannose-6-phosphate; GAM-6-P = glucosamine-6-phosphate; R-5-P = ribose-5-phosphate; G-1,6-P = glucose-1,6-diphosphate; F-1-P = fructose-1-phosphate; F-1,6-P = fructose-1,6-diphosphate; 6-PG = 6-phosphogluconate; 2-KPG = 2-keto-6-phosphogluconate; Ru-5-P = ribulose-5-phosphate; Ru-1,5-P = ribulose-1,5-phosphate.

mesenteroides (95), *Streptococcus faecalis* (350), *Lactobacillus bulgaricus* (331), and *Clostridium butyricum* (125), as well as in the aerobic (non-glycolyzing), *Pseudomonas aeruginosa* (65) and *Pseudomonas putrefaciens* (206, 207).

Galactokinase, unique among the aldokinases in that the number one carbon is phosphorylated

to form α -galactose-1-phosphate (GA-1-P), has been studied extensively in yeast (221, 380) and to a lesser extent in animal tissue (219) and in galactose fermenting microorganisms, *i.e.*, *E. coli* (54) and *L. bulgaricus* (331). Galactose-1-phosphate is converted to glucose-1-phosphate (G-1-P) by a "galactowaldenase", also found in

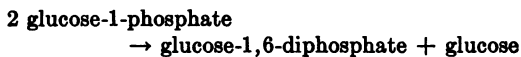
galactose fermenting microorganisms (331). "Galactowaldenase" requiring uridine diphosphohexose as a coenzyme functions in the epimerization of GA-1-P to G-1-P (242). This reaction will be described in detail in section III, but is mentioned here as an example of the route from other carbohydrates to glucose phosphates which are metabolized by a classical pathway bypassing the kinases for glucose.

Fructokinase, which requires magnesium and potassium ions for activity (163, 359), has been demonstrated in animal tissues (80, 244). The phosphorylated product, which accumulated in the presence of fluoride, was identified as fructose-1-phosphate (F-1-P). Fructose phosphorylation has been observed in *Pseudomonas putrefaciens*, but purified enzymes have not been prepared and the product of phosphorylation has not been identified. The high fructose concentration required suggests, however, that the reaction is catalyzed by a hexokinase (207).

Phosphofructokinase, an enzyme peculiar to the Embden-Meyerhof pathway, resembles fructokinase in phosphorylating fructose-6-phosphate (F-6-P) at carbon 1 to yield fructose-1,6-diphosphate (F-1,6-P) (319, 376). Although ATP was initially considered to be the only phosphate donor, uridine triphosphate and inosine triphosphate have recently been shown also to serve as phosphate donors (248). A specific phosphofructokinase has been reported in plants (9), in brain (321, 389) and purified from rabbit muscle (319, 376). Phosphofructokinase is presumed to function in those organisms which accumulate fructose-1,6-diphosphate during hexose fermentation, but little evidence has been published as to its occurrence in microorganisms. An exception is the data of VanDemark and Wood (393) who demonstrated an ATP-dependent conversion of fructose-6-phosphate to triose phosphates in extracts of *Microbacterium lacticum*. A second *phosphofructokinase* which phosphorylates fructose-1-phosphate to fructose-1,6-diphosphate has been purified from rabbit muscle (347) but so far has not been studied in bacteria.

Phosphoglucokinase, which forms glucose-1,6-diphosphate (G-1,6-P) from glucose-1-phosphate, has been found in muscle and yeast (311). An apparently different kinase present in *E. coli*, *Klebsiella pneumoniae* and *Aerobacter aerogenes* forms glucose-1,6-diphosphate from

glucose plus ATP (238). The mechanism was studied with a partially purified preparation and postulated to involve a primary phosphorylation followed by a transphosphorylation from one glucose-1-phosphate molecule to another as follows (238):



Pentokinases, phosphorylating the pentoses at the fifth carbon atom, have been studied in bacteria and yeast but with the exception of ribokinase, purified little if any. The substrate specificity and the identity of the first product of phosphorylation in many cases remain to be clarified. Ribokinase, which phosphorylates ribose to ribose-5-phosphate (R-5-P), has been found in yeast (333) and from purified ribose grown *Aerobacter aerogenes* cells (180). Inducible ribo- and arabinokinases have been demonstrated in *E. coli* (69).

A *phosphoribulokinase*, which converts ribulose-5-phosphate to ribulose-1,5-diphosphate, has been purified from green leaves (418). Ribulose diphosphate has been found in extracts of algae and of green leaves (22, 23), but its occurrence in nonphotosynthetic microorganisms has not been reported. The enzymatic carboxylation of ribulose diphosphate yields 3-phosphoglycerate (3-PGA) (318). Data obtained with crude extracts of lactic acid bacteria suggest the existence of keto kinases for the ketopentoses, xylulose and ribulose (233), but enzymatic reactions have not been reported.

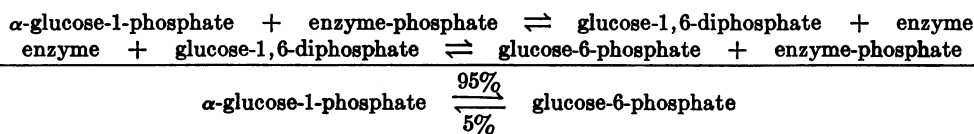
Gluconokinase, which phosphorylates gluconate to 6-phosphogluconate (6-PG), was first demonstrated in gluconate grown *E. coli* cells and shown to be specific for gluconate as the phosphate acceptor (67). Gluconokinase has been demonstrated in gluconate adapted *Streptococcus faecalis*, a fermentative organism (350), baker's yeast (332), and *Aerobacter cloacae* (88) as well as in aerobic bacteria such as *Pseudomonas fluorescens* (289) and *Pseudomonas aeruginosa* (65). The presence of gluconokinase is now considered to be evidence for a nonglycolytic pathway of hexose utilization.

2-Ketogluconokinase, which phosphorylates 2-ketogluconate presumably to 2-keto-6-phosphogluconate (2-KPG), has been found in *Aerobacter cloacae* and *Pseudomonas fluorescens*. This kinase, like gluconokinase, is induced by growth

on the specific substrate. The presence of gluconokinase and 2-ketogluconokinase in the pseudomonads, in which the oxidation of glucose to gluconate and to 2-ketogluconate, without phosphorylation, has been known for some time, indicates that these oxidative processes supply the inducing substrates, which activate kinase formation and further substrate utilization by one of the hexosemonophosphate pathways. 2-Ketogluconate was previously considered to be the endproduct of an incomplete oxidation pathway. These reactions are discussed in detail in section II, 6.

b. *Mutases*. Mutases transfer phosphate from one carbon to another, frequently between hemiacetal and primary hydroxyl, or between primary and secondary hydroxyls. Such transfer reactions occur with hexose- and pentosephosphates and with phosphoglyceric acids.

Phosphoglucomutase, which effects the equilibrium between glucose-1-phosphate and glucose-6-phosphate, has been demonstrated in many tissues and cells including yeast and muscle (75, 76) and has been crystallized from rabbit muscle (287). A coenzyme, glucose-1,6-diphosphate, which mediates in this mutase reaction was isolated from impure fructose-1,6-diphosphate by Leloir and his coworkers (52, 237) and was shown by Capputo *et al.* (49), Sutherland *et al.* (374), Pullman and Najjar (288, 317), and others (186) to function by a mechanism best outlined as follows:



The mechanism indicated is based on recent experiments of Najjar and Pullman (288) with substrate amounts of crystalline phosphoglucomutase in which incubation of the enzyme with glucose-1- or glucose-6-phosphate formed glucose-1,6-diphosphate and "dephosphorylated" enzyme. Upon reincubation with glucose-1,6-diphosphate, the enzyme was rephosphorylated.

Phosphoglucomutase is considered to function in oligosaccharide degradation by converting the glucose-1-phosphate formed by phospholytic cleavage to glucose-6-phosphate. Phosphoglucomutase will also interconvert mannose-1- and mannose-6-phosphates (239) and ribose-1-

and ribose-5-phosphates (147, 208). The enzyme can be activated by the corresponding diphosphates or by glucose-1,6-diphosphate. A mutase specific for *phosphoribose* has been demonstrated in smooth muscle and separated from phosphoglucomutase (147).

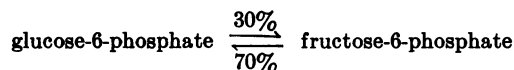
Phosphoglucomutase has not been isolated or studied in detail in bacterial preparations, but its activity has been demonstrated in *Pseudomonas fluorescens* (431), *Lactobacillus bulgaricus* (331), and its presence is assumed in extracts which ferment or oxidize glucose-1-phosphate.

Phosphoglyceromutase interconverts 3-phosphoglyceric acid and 2-phosphoglyceric acid (271, 302). A 2,3-diphosphoglycerate isolated from blood by Greenwald (146) as early as 1925 was shown by Sutherland *et al.* (373) to function by a mechanism analogous to that of glucose-1,6-diphosphate in phosphoglucomutase. Thus, the conversion of 2,3-diphosphoglycerate to pyruvate shown by Lennerstrand (243) was explained. This mutase has not been studied in bacteria.

c. *Isomerases*. Isomerases interconvert aldo and keto groups of carbohydrates. Enzymes specific for hexose-, pentose-, and triosephosphates and for nonphosphorylated pentoses have been observed. The isomerization of phosphate esters, long recognized as important in glycolysis, is now recognized as important also in pentose metabolism. The xylose-xylulose (166, 167, 280) and ribose-ribulose (70) isomerases have been studied. Many more isom-

erases active on nonphosphorylated sugars seem certain to be discovered.

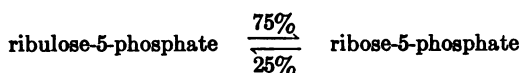
Phosphohexose isomerase which catalyzes the reaction:



was recognized as a key enzyme in the glycolytic pathway soon after the identification of glucose-6-phosphate (258). This isomerase was purified from animal tissues (348), yeast (258), and plants (351) and demonstrated in several bacteria including *Pseudomonas fluorescens* (431) and *Microbacterium lacticum* (393).

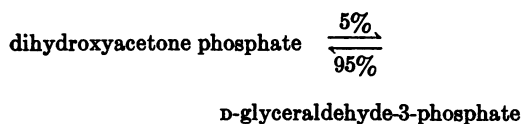
Phosphomannose isomerase (346, 348) which interconverts mannose-6- and fructose-6-phosphates has been partially separated from the isomerase interconverting glucose-6- and fructose-6-phosphates (Lohmann's isomerase). Since fructose-6-phosphate is a product common to both mannose-6- and glucose-6-phosphate isomerization, the term "phosphoglucosomerase" has been suggested as more appropriate for the Lohmann isomerase previously termed phosphohexose isomerase (258).

Pentose phosphate isomerase, discovered in yeast by Horecker *et al.* (170), catalyzes the reaction:

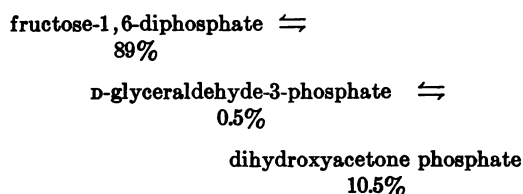


The enzyme was demonstrated in animal tissue (173) and highly purified from plant sources (11). Its presence in a wide variety of bacteria including *Pseudomonas fluorescens* (431), *Acetobacter suboxydans* (161), *Azotobacter vinelandii* (281) and *Microbacterium lacticum* (393) has been inferred from the accumulation of heptulose phosphate with ribose-5-phosphate as substrate, *i.e.*, transketolase action on ribose- and ribulose-5-phosphates—see section II, 3.

Triosephosphate isomerase interconverts dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate as follows:



Muscle (278) and yeast (21) are rich sources of this enzyme which has been purified by Meyerhof and Beck (278) and crystallized from muscle by Meyer-Erendt *et al.* (267a). Before fructose-1,6-diphosphate aldolase and triosephosphate isomerase were separated, Meyerhof (268, 270) had applied the term "zymohexase" to preparations catalyzing both reactions with the following equilibria (277):



Utter and Werkman (384) obtained similar equilibrium data with *E. coli* extracts as a source of "zymohexase".

d. *Aldolases and ketolases.* Aldolases, of which several are now known, catalyze a reversible cleavage of carbon chains adjacent to secondary hydroxyl groups as indicated by the general reaction:



The specific aldolases described and assigned functions in carbohydrate metabolism are listed in table 2.

Fructose-1,6-diphosphate aldolase (table 2, reaction 1), first recognized by Meyerhof (268, 269, 272), was reported to show absolute specificity for dihydroxyacetone phosphate but not for D-glyceraldehyde phosphate, which is replaceable with many aldehydes, including acetaldehyde, to form sugar phosphates of various configurations (272, 273). More recent studies with crystalline aldolase confirmed the substrate specificity patterns suggested by Meyerhof (272). Among the reactions catalyzed is the condensation of dihydroxyacetone phosphate with D- and with L-glyceraldehyde forming, respectively, fructose-1- and sorbose-1-phosphates (382). A condensation with glycolaldehyde phosphate forms xylulose-1,5-diphosphate, and with glycolaldehyde forms xylulose-1-phosphate (42). All of the condensations reported form *trans*-hydroxyls. One cleavage of a *cis*-hydroxyl containing ester, tagatose-1,6-diphosphate, was reported (382).

Aldolase has been separated from triosephosphate isomerase (162) and the equilibrium shown to be 89% toward hexosediphosphate (270). Aldolase has been purified from yeast (415) and crystallized from rabbit muscle (375, 414). In contrast to muscle aldolase, the enzymes from yeast (415), *Aspergillus niger* (187) and *Clostridium perfringens* (17) show divalent metal activation—the yeast and clostridial enzymes are in addition cysteine activated. Among the divalent metals, Zn⁺⁺, Co⁺⁺ and Fe⁺⁺ activate the yeast and *Aspergillus* aldolases; but only Fe⁺⁺ activates the clostridial enzyme. Simplified aldolase assays, especially the colorimetric procedure of Sibley and Lehninger (344), have been used to demonstrate aldolase in microorganisms

TABLE 2
Aldolases for phosphate esters

Enzyme	Substrate	Product	Source
1. F-1,6-P aldolase	DHA-P + G-3-P	F-1,6-P	Yeast (415) Plant (371) Animal (414, 375) Bacteria (17, 261)
2. F-1-P aldolase	DHA-P + glycerol	F-1-P	Liver (244, 245)
3. Phosphoketotetrose aldolase	DHA-P + HCHO	E-1-P	Liver (58)
4. Transaldolase	G-3-P + S-7-P	E-4-P + F-6-P	Animal (176) Yeast (182) Plant (10)
5. DR aldolase	G-3-P + AcAl	DR-5-P	Bacteria (322)
6. KDPG aldolase	KDPG	Pyruvate + G-3-P	Bacteria (223, 263)

Abbreviations: DHA-P = dihydroxyacetone phosphate; E-1-P = erythrulose-1-phosphate; G-3-P = glyceraldehyde-3-phosphate; S-7-P = sedoheptulose-7-phosphate; KDPG = 2-keto-3-deoxy-6-phosphogluconate; DR-5-P = deoxyribose-5-phosphate; glycerol = glyceraldehyde.

including *Clostridium perfringens* (17), *Penicillium notatum* (261), *Aspergillus sp.* (6, 187), *Bacillus subtilis* (124), *Escherichia coli* (361) and *Lactobacillus bifidus* (228). Low activity was detected in *Pseudomonas fluorescens* (431) whereas *Leuconostoc mesenteroides* was devoid of demonstrable aldolase and isomerase activity (93).

Fructose-1-phosphate aldolase, (table 2, reaction 2) which cleaves fructose-1-phosphate to dihydroxyacetone phosphate and D-glyceraldehyde, was obtained from liver (164, 244, 245). This enzyme is inactive toward fructose-1,6-diphosphate and is thus distinct from the Meyerhof-Lohmann, fructose-1,6-diphosphate aldolase (164). The conversion of fructose-1- to fructose-6-phosphate as originally observed (80) has been shown to occur by the combined action of fructose-1-phosphate aldolase, triosephosphate isomerase, fructose-1,6-diphosphate aldolase and fructose-1,6-diphosphate phosphatase (164, 245). Evidence for an additional pathway of fructose degradation via fructose-1-phosphate in muscle has been presented (80).

Phosphoketotetrose aldolase. Liver preparations catalyze an aldolase-type of condensation of dihydroxyacetone phosphate and formaldehyde to erythrulose-1-phosphate (table 2, reaction 3)

by an enzyme distinct from fructose-1,6-diphosphate aldolase (58).

Transaldolase, (table 2, reaction 4) a new type of aldolase active in the transfer of a dihydroxyacetone group from sedoheptulose-7-phosphate to glyceraldehyde-3-phosphate forming fructose-6-phosphate and erythrulose-4-phosphate (182, 183), was first found in yeast (176, 182). This enzyme is present in plant and animal tissues (10, 176) and in aerobic bacteria including *Pseudomonas fluorescens* (431), *Microbacterium lacticum* (393) and *Acetobacter suboxydans* (161). The specificity for dihydroxyacetone is similar to that of fructose-1,6-diphosphate aldolase for dihydroxyacetone phosphate (182, 183). Transaldolase, however, transfers but does not liberate dihydroxyacetone.

Deoxyribose-5-phosphate aldolase (table 2, reaction 5) catalyzes the reversible cleavage of deoxyribose-5-phosphate to acetaldehyde and D-glyceraldehyde-3-phosphate (322). The enzyme is found in *Corynebacterium sp.* and was purified from *E. coli* extracts by Racker (322). The equilibrium favors deoxyribose-5-phosphate formation; no cofactors have been demonstrated for the purified enzyme.

2-Keto-3-deoxy-6-phosphogluconate aldolase (table 2, reaction 6) cleaves 2-keto-3-deoxy-

6-phosphogluconate (KDPG) to pyruvate and D-glyceraldehyde-3-phosphate. This enzyme was discovered in *Pseudomonas saccharophila* (262, 263) and has been purified from this organism and from *Pseudomonas fluorescens* extracts (223). The enzyme converts the KDPG, formed from 6-phosphogluconate by 6-phosphogluconate dehydrase, to pyruvate and D-glyceraldehyde-3-phosphate (223, 263). The KDPG aldolase does not cleave fructose-1,6-diphosphate nor deoxyribose-5-phosphate (223). The function of this reaction in carbohydrate metabolism will be dealt with in greater detail in section II, 3.

Transketolase transfers glycolaldehyde among a number of aldose phosphates (175, 323). Like transaldolase, this is a transfer and not a cleavage enzyme, *i.e.*, does not form free glycolaldehyde. Glycolaldehyde donors among the ketose phosphates include ribulose-5-phosphate, fructose-6-phosphate and sedoheptulose-7-phosphate; the acceptors include ribose-5- and deoxyribose-5-phosphates, glyceraldehyde-3-phosphate, glyceraldehyde and glycolaldehyde and form the corresponding ketoses, as will be shown in figure 5 (section II, 3). Hydroxypyruvate also serves as glycolaldehyde donor producing CO₂. Diphosphothiamine and a divalent metal serve as cofactors presumably as a carbanion acceptor and donor (175, 323). Transketolase has been highly purified from spinach leaves and liver (178) and crystallized from yeast (87). The importance of these transfer reactions in hexose metabolism is discussed in section II, 3.

2. Triosephosphate Reactions

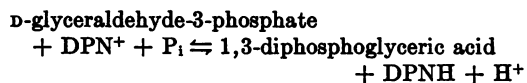
Until recently, the triosephosphates, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate were considered only as obligatory intermediates in glycolysis (Embden-Meyerhof fermentation pathway) in which they arise by the reversible cleavage of fructose-1,6-diphosphate by its specific aldolase, and undergo further reaction by interconversion catalyzed by triosephosphate isomerase and by oxidation of the glyceraldehyde-3-phosphate to 1,3-diphosphoglyceric acid by its specific dehydrogenase. A side reaction of dihydroxyacetone phosphate leading to glycerol *via* α -glycerol phosphate is catalyzed by its specific dehydrogenase.

The many reports of new enzymatic reactions of both triosephosphates have, however, greatly

broadened the concepts of their importance in the diverse pathways of carbohydrate metabolism. Especially noteworthy are the acceptor and transfer reactions of transaldolase and transketolase for 4, 5, 6, and 7 carbon aldose and ketose mono- or diphosphates, and the new aldolase cleavages of 2-keto-3-deoxy-6-phosphogluconate forming glyceraldehyde-3-phosphate. These findings assign to the triosephosphates a role common to several pathways of sugar metabolism (figure 1) similar to the position long enjoyed by pyruvate. Those reactions that are common to triose- and hexosephosphates, *i.e.*, isomerases and the enzymes forming the triosephosphates by cleavage and transfer, have been discussed in the foregoing section dealing with *Aldose and Ketose Reactions*.

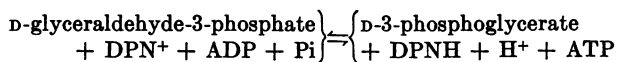
a. *Dehydrogenases*. The role of the triosephosphates in oxidation and reduction reactions to form 1,3-diphosphoglyceric acid from D-glyceraldehyde-3-phosphate and α -glycerol phosphate from dihydroxyacetone phosphate was recognized in the earliest studies of fermentation mechanisms.

Glyceraldehyde-3-phosphate dehydrogenase catalyzes the DPN mediated oxidation of D-glyceraldehyde-3-phosphate with incorporation of inorganic phosphate (292, 411) as follows:



The crystalline dehydrogenase catalyzes the analogous oxidation of acetaldehyde in the presence of phosphate to acetyl phosphate (158). The crystalline dehydrogenase from yeast (411) and rabbit muscle (78) has been used in extensive studies of the reaction mechanism (37, 79, 225, 308, 342, 395). The enzyme contains 2 molecules of bound DPN and is sensitive to sulfhydryl reagents. An enzyme bound —SH group is implicated in the acyl generation mechanism. The complete reaction requires inorganic phosphate which can be replaced with arsenate yielding 3-phosphoglycerate (291, 411)—presumably *via* 3-phosphoglyceryl-1-arsenate, which hydrolyzes spontaneously. Glyceraldehyde-3-phosphate dehydrogenase has been shown in fermentative bacteria, *Leuconostoc mesenteroides* (93), *Clostridium butyricum* (125) and many aerobic bacteria, *Microbacterium lacticum* (393), *Bacillus subtilis* (124), *Pseudomonas fluorescens*

Phosphoglyceric acid transphosphorylase was crystallized from yeast by Bücher (43) and shown to transfer phosphate to ADP but not to adenosine monophosphate, forming ATP. This enzyme with triosephosphate dehydrogenase catalyzes the incorporation of inorganic phosphate into an anhydride (pyrophosphate) linkage as follows (275):



Phosphopyruvate transphosphorylase transfers phosphate from phosphoenolpyruvate to ADP to form ATP. The crystalline enzyme has been prepared from muscle (227), and the reaction shown to be reversible with magnesium (35, 36) and potassium ions (235). These transphosphorylases have not been studied directly in microbial extracts but are presumed to function in the generation of ATP during growth and fermentation.

3. Evidence of an Embden-Meyerhof Pathway in Bacteria

Glycolysis by the Embden-Meyerhof pathway, as found in yeast and muscle (276), was long considered a main pathway of carbohydrate fermentation in microorganisms with the diversity of products attributed to the many pyruvate reactions observed in microbial extracts, cell preparations, or in growing cells (115). Evidence for an Embden-Meyerhof pathway from glucose to pyruvate in microorganisms was based on: (a) production and utilization of postulated intermediates; (b) the presence of enzymes catalyzing reactions of the Embden-Meyerhof pathway; (c) sensitivity of these enzymes to inhibitors effective with muscle and yeast enzymes, *i.e.*, iodoacetate, $m/1,000$, and NaF, $m/50$; and (d) fermentation of C^{14} labeled glucose to products labeled as predicted by Embden-Meyerhof pattern, *i.e.*, glucose-3,4- C^{14} to carboxyl labeled lactate or to labeled CO_2 in the yeast ethanol fermentation (figure 8). The data accumulated with microorganisms were fragmentary, however, since complete evidence of the glycolytic process was not accumulated for any one organism.

Pyruvate as an intermediate in fermentation was partially validated by demonstration of its accumulation in the presence of bisulfite with

living cells of *Escherichia coli* (74), *Propionibacterium arabinosum* (426) and *Clostridium acetobutylicum* (84). Phosphate ester accumulation (hexosemonophosphates and hexosediphosphate) was observed with *Streptococcus faecalis* (309), *Lactobacillus casei* (401), *Staphylococcus aureus* (122), *Brucella suis* (330) and propionic acid bacteria (422).

Phosphoglycerate accumulation in growing cells was shown by addition of $m/50$ sodium fluoride to the culture medium. Colon-aerogenes, lactic acid, propionic acid bacteria, among other organisms (362), accumulated significant amounts of phosphoglycerate. The amount accumulated varied greatly with the organism and conditions of culture, *i.e.*, with the propionic acid bacteria, the formation of a fluoride resistant strain during growth was indicated (420). The accumulation of phosphoglycerate, as indicated by barium precipitable organic phosphate stable to hydrolysis and by optical rotation of the molybdate complex (399), was widely interpreted as evidence of an Embden-Meyerhof glycolysis pattern.

Conversion of phosphate esters such as hexose-diphosphate, phosphoglycerate and glycerol phosphate to fermentation products, or to other glycolytic intermediates, at a rate compatible with the over-all fermentation rate was also considered evidence for this glycolytic scheme in microorganisms. Some limitation to the approach resides in reduced reaction rates in extracts due to limiting levels of hydrogen or phosphate donors (or acceptors) and of coenzymes. A fluoride sensitive system of *E. coli* (116) and of propionic acid bacteria (420) was shown in the presence of acetaldehyde as hydrogen acceptor, to degrade hexosephosphate esters to pyruvate, inorganic phosphate, reducing the aldehyde to ethanol. The phosphoglycerates, which are at the oxidation level of pyruvate rather than glucose, form pyruvate and orthophosphate with *Mycobacterium phlei* (114) and with extracts of *Lactobacillus* sp. (200) and *Leuconostoc* sp. (200).

Glycolytic enzymes which serve in the Embden-Meyerhof pathway have been demonstrated in numerous bacteria; and their presence is cited as evidence for this system. Such evidence,

discussed also in the foregoing portion of this manuscript, is not proof of the scheme or of the order in which the enzymes serve since diverse routes to triosephosphates and pyruvate are now amply documented, as outlined in the section on hexosemonophosphate pathways (see, for example, figure 1). Thus, only phosphohexokinase, aldolase specific for fructose-1,6-diphosphate, and triosephosphate isomerase, enzymes that convert fructose-6-phosphate *via* fructose diphosphate to an equilibrium mixture of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, are unique to the Embden-Meyerhof pathway. All other enzymes of this pathway are also common to other known pathways of both aerobic and anaerobic carbohydrate metabolism.

The fermentation of glucose-C¹⁴ by a variety of lactic acid bacteria (127, 316), clostridia (18, 310, 428), and bacilli (293) has furnished data compatible with the postulates of the Embden-Meyerhof pathway and with the data obtained by its fermentation with muscle enzymes. Thus, glucose-1-C¹⁴ yielded methyl labeled products. Glucose-3,4-C¹⁴ yielded carboxyl labeled lactate in *Lactobacillus casei* (127), but with *Clostridium thermoaceticum* the methyl of acetate was more heavily labeled although both carbons of acetate became labeled (18, 428) in keeping with their equilibrium with C¹⁴O₂ in this organism.

Other data inconsistent with an Embden-Meyerhof pattern were obtained by fermentation of glucose-1-C¹⁴ with cells of *Leuconostoc mesenteroides* (150), *Propionibacterium pentosaceum* (236, 328) and *Pseudomonas saccharophila* (118). These data and the consequent enzyme experiments clarifying the pathways in *Leuconostoc* (95, 97) and *P. saccharophila* (118, 263) (figure 8) have amply repaid the labor invested in checking what at first glance appeared to be adequate data. Similar data for *E. coli* (68, 246) and *Streptomyces* sp. (66) have more recently become available and are included in section II on *Hexosemonophosphate Reactions and Pathways*. In summary, then, Embden-Meyerhof glycolysis occurs in microorganisms as a means, but not the sole means, of energy liberation and carbon transformation.

II. HEXOSEMONOPHOSPHATE REACTIONS AND PATHWAYS

In recent years, exceptions to the Embden-Meyerhof scheme as "the" pathway of carbo-

hydrate breakdown have accumulated until it is clear that this is not the sole pattern of carbohydrate degradation in either fermentative or oxidative microorganisms. The early experiments of Warburg and Christian (405, 406, 407, 408), of Dickens (98, 99, 100) and of Lipmann (249) demonstrated the terminal oxidation of glucose-6-phosphate as far as pentose phosphate. At that time, an oxidative route, termed a hexose monophosphate pathway or "shunt", was visualized as successive terminal oxidations and removal of single carbon atoms bypassing the glycolytic pathway, at least until the triose stage. Recently, it has become clear that this type of stepwise oxidation and decarboxylation is not the main route of aerobic carbohydrate degradation, but rather, after the initial oxidation to aldonic or ketoaldonic acids, cleavage by specific aldolases occurs with further oxidation of the 3, or 2 and 3 carbon fragments. The following discussion deals with the specific enzymes of hexosemonophosphate pathways.

1. Oxidations

a. *Glucose-6-phosphate dehydrogenase*. One of the earliest deviations from the Embden-Meyerhof pathway was reported by Warburg and Christian (405, 406, 407, 408) who found that both yeast preparations and hemolysates of erythrocytes oxidize glucose-6-phosphate to 6-phosphogluconate. The enzyme, glucose-6-phosphate dehydrogenase or "Zwischenferment", was subsequently purified from red cells (408) and from yeast (218, 292a) and shown to reduce specifically a new coenzyme, triphosphopyridine nucleotide (TPN) (408). Thus from its discovery, TPN was associated with oxidative pathways as a counterpart of diphosphopyridine nucleotide (DPN) in the fermentative systems. The product of glucose-6-phosphate oxidation, 6-phosphogluconate, also was isolated by Warburg and Christian (408).

More recently, Cori and Lipmann (81) have shown the enzymatic oxidation of glucose-6-phosphate, in common with the chemical oxidation, to proceed in two steps: (a) oxidation of the pyranose ring of glucose-6-phosphate to 6-phospho- δ -gluconolactone, and (b) hydrolysis of the lactone ring (figure 2). The hydrolysis, which occurs slowly at neutral pH, is catalyzed by a specific delactonizing enzyme. Brodie and Lipmann (40, 41) have shown δ -gluconolactone

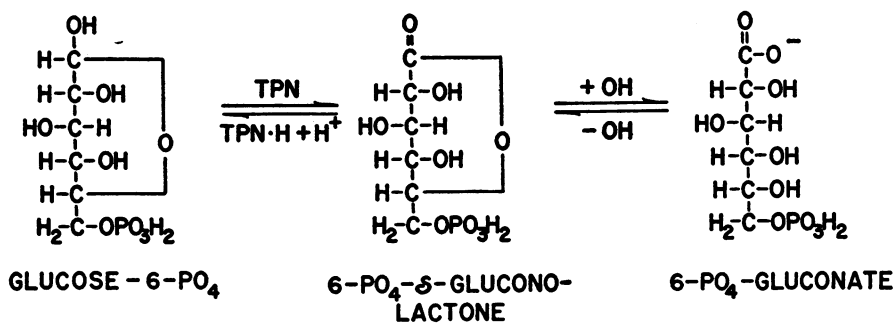


Figure 2. Oxidation of glucose-6-phosphate

to be hydrolyzed by a similar delactonizing enzyme in extracts of *Azotobacter vinelandii* and of yeast. The equilibrium for the over-all reaction for both glucose and glucose-6-phosphate oxidation is far to the right, but the oxidative steps (reaction 1) are readily reversible in the presence of reduced TPN (TPN·H) and phosphogluconolactone (177) or DPN·H and gluconolactone, respectively, (367).

Glucose-6-phosphate dehydrogenase has been found in a variety of animal tissues (101, 136, 137, 138) and in numerous microorganisms including *Escherichia coli* (338), *Pseudomonas fluorescens* (431), *P. saccharophila* (118), *P. lindneri* (94), *Azotobacter vinelandii* (281), *Bacillus subtilis* (85), *Bacillus megaterium* (85), *Leucomostoc mesenteroides* (96), and *Streptococcus faecalis* (350). The *E. coli* enzyme has been purified and shown to require magnesium or calcium ions for activity (338).

The high affinity of glucose-6-phosphate dehydrogenase for glucose-6-phosphate has permitted its use for quantitative determination of hexosemonophosphates, TPN, ATP, and also of several enzymes including hexokinase, phosphohexose isomerase, and phosphoglucomutase. Although TPN is an obligatory coenzyme for the dehydrogenase from animal sources (408), in crude preparations of *Leucomostoc mesenteroides* (96) and *Pseudomonas fluorescens* (431), DPN also serves as a hydrogen acceptor. The enzyme from *L. mesenteroides* reacts with both nucleotides (96) whereas that from *P. fluorescens* reduces DPN via a TPN-specific dehydrogenase coupled with pyridine nucleotide transhydrogenase (73).

b. *6-Phosphogluconate dehydrogenase*. The further degradation of 6-phosphogluconate was first demonstrated in yeast by Warburg and Christian (406, 407, 408) who found TPN to be the coenzyme (410) and obtained evidence for a C₅

product (409, 410). Lipmann (249) characterized the reaction as an oxidative decarboxylation and suggested that arabinose-5-phosphate would be formed. With a partially purified yeast preparation, Dickens (99, 100) obtained evidence for the accumulation of pentose phosphate, which he believed to be ribose-5-phosphate rather than arabinose-5-phosphate, since the former was rapidly metabolized by yeast extracts whereas the latter was not. Thus, ribose-5-phosphate was assumed to be an intermediate in the oxidation of glucose-6-phosphate by yeast.

More than ten years later, Scott and Cohen (337) confirmed the formation of ribose-5-phosphate as a product of phosphogluconate oxidation by yeast preparations, and, subsequently, Horecker and Smyrniotis (168) isolated ribose-5-phosphate as a product of 6-phosphogluconate oxidation with a purified enzyme preparation from yeast. The apparent anomaly in the configuration of the three hydroxyl group of glucose which corresponds to arabinose rather than the ribose was assumed to result from epimerization of carbon 3 of glucose by a hydrogen shift *via* enol or enediol intermediates (101, 337). Horecker, Smyrniotis and Seegmiller (170), however, isolated and identified the product of 6-phosphogluconate oxidation by purified 6-phosphogluconic dehydrogenase (169) as a ketopentose, ribulose-5-phosphate. Ribulose-5-phosphate accumulated first in the reaction mixture and was converted, subsequently, to ribose-5-phosphate (figure 3). The latter reaction is catalyzed by traces of a pentose phosphate isomerase also present in the preparation. At equilibrium, ribose-5-phosphate and ribulose-5-phosphate were present in a three to one ratio. The earlier appearance of ribulose-5-phosphate was attributed to the oxidation of 6-phosphogluconate at the third carbon atom (168), but the suggested

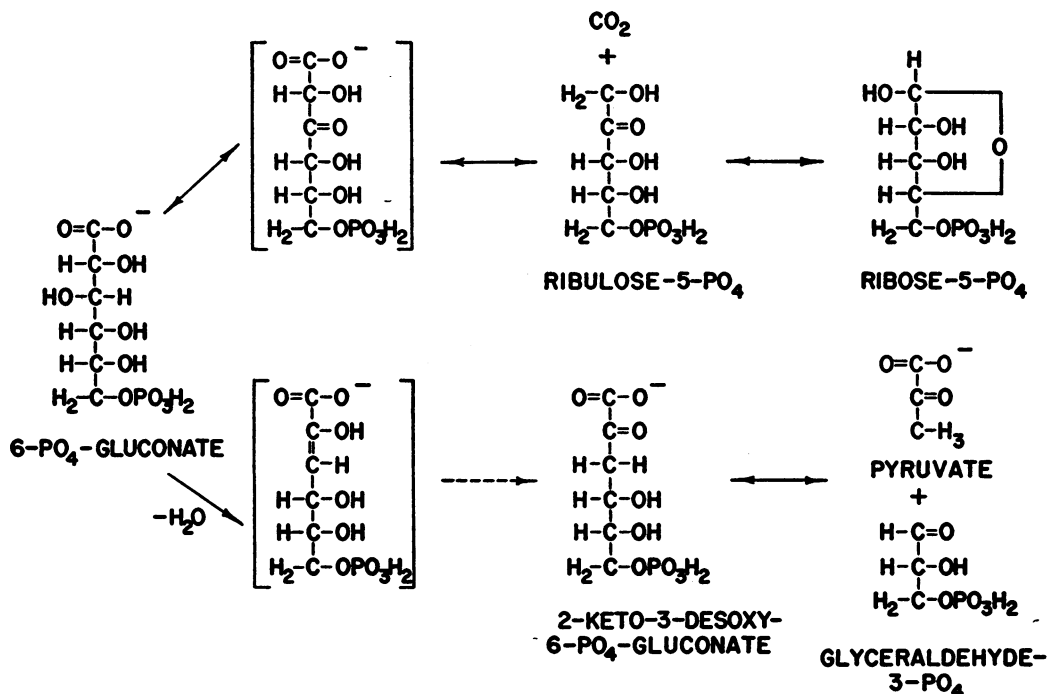


Figure 3. Reactions of 6-phosphogluconate

intermediate, 3-keto-6-phosphogluconate, has not been detected. The lack of evidence of 3-keto-6-phosphogluconate has led to a suggestion that the enzyme possesses two functions, dehydrogenation and decarboxylation, similar to that postulated by Ochoa (306) for the malic enzyme mechanism.

Although the equilibrium of 6-phosphogluconate oxidation favors oxidative decarboxylation, the reaction is reversible as demonstrated by the enzymatic fixation of C^{14}O_2 into carbon atom one of 6-phosphogluconate and by the reductive carboxylation of ribulose-5-phosphate in the presence of TPNH and CO_2 (172). The equilibrium of lactonization (figure 2) is far toward hydrolysis, and hence would appear to limit the rate of 6-phosphogluconolactone formation to a level incompatible with net hexose regeneration *via* this route. Recent evidence demonstrates that photosynthetic carbon dioxide incorporation into hexose occurs *via* ribulose-1,5-diphosphate, which is the primary carbon dioxide acceptor in a carboxylation and cleavage reaction which forms phosphoglycerate (318, 418, 419).

Pentose phosphate formation occurs in bac-

terial extracts, but the details of the mechanism have not been studied. Extracts of *Escherichia coli* (337) oxidize 6-phosphogluconate, but an analysis of the endproducts produced by purified preparations was not reported. Phosphogluconic dehydrogenase has been demonstrated in *Bacillus subtilis*, *B. megaterium* (85), *Azotobacter vinelandii* (281), *Leuconostoc mesenteroides* (97), and *Pseudomonas fluorescens* (431).

The oxidation of phosphogluconate to form keto-6-phosphogluconates has been postulated repeatedly (98, 168, 249, 337, 383), and the production of 2-ketogluconate and 5-ketogluconate from glucose by oxidative organisms lends credence to this possibility. Thus, Uehara (383) proposed an alternate mechanism of phosphogluconate oxidation which does not involve direct oxidative decarboxylation as follows: (a) oxidation of 6-phosphogluconate to 2-keto-6-phosphogluconate; (b) cleavage of 2-keto-6-phosphogluconate to hydroxypyruvate and glyceraldehyde-3-phosphate; (c) transketolase transfer between hydroxypyruvate and glyceraldehyde-3-phosphate to yield ribulose-5-phosphate. The proposed mechanism appears unlikely since the yeast preparations which form ribulose-

5-phosphate from 6-phosphogluconate do not utilize hydroxypyruvate and glyceraldehyde-3-phosphate (181).

A new pathway of phosphate ester degradation involving phosphorylated 2-ketogluconate, presumably 2-keto-6-phosphogluconate, has been found in *Pseudomonas fluorescens* by Narrod and Wood (289) and an *Aerobacter cloacae* by DeLey (88, 89). The intermediate is further degraded with the accumulation of phosphoglycerate with the *A. cloacae* preparation (89) or to two moles of pyruvate with the extract of *P. fluorescens* (290). The evidence for these reactions is detailed in section II, 6c.

2. Isomerization, Phosphorylation and Dehydration

a. *Phosphopentose isomerase*. Pentose phosphate isomerase catalyzes an equilibrium between ribose-5-phosphate and ribulose-5-phosphate, thus clarifying the earlier problem of ribose-5-phosphate formation from phosphogluconate by crude extracts (169). This isomerase is found in plants (175), animals (173), and yeast (168); Axelrod and Jang (11) have purified the enzyme from alfalfa and studied its characteristics. The alfalfa enzyme does not isomerize glucose-6-phosphate or triosephosphates.

b. *Phosphoribulokinase*. This enzyme has been purified from spinach extracts and shown to catalyze the phosphorylation of ribulose-5-phosphate with the formation of a diphosphate ester of ribulose (418). Ribulose diphosphate has been found in extracts of algae and green leaves (22). Its occurrence in nonphotosynthetic microorganisms has not been reported.

c. *6-Phosphogluconate dehydrase*. 6-Phosphogluconate degradation to pyruvate and triosephosphate by enzymes from *Pseudomonas saccharophila* (118) was shown by MacGee and Doudoroff (262, 263) to occur by way of an intermediate which was isolated and characterized as 2-keto-3-deoxy-6-phosphogluconate (figure 3). The enzyme has been purified from extracts of *Pseudomonas fluorescens* by Kovachevich and Wood (222) and shown to require glutathione and ferrous ions for activity. The data are consistent with a mechanism involving dehydration between carbons 2 and 3 followed by a tautomeric shift of hydrogen as postulated by Entner and Doudoroff (118); hence, the enzyme has been termed 6-phospho-

gluconate dehydrase. This enzyme has thus far been reported only in the *Acetobacter* (223), *Pseudomonas* (223) and *Azotobacter* species (281).

3. Cleavage and Transfer

Ribose-5-phosphate degradation, both aerobic and anaerobic, has been observed in red cell hemolysates (103), in extracts of liver and yeast (100, 135, 333) and in microorganisms (25, 26, 266, 320, 403). Although Dickens (99) reported an oxidation of pentose phosphate to phosphopentonic acid and Sable (333) demonstrated a TPN requirement for ribose-5-phosphate oxidation, a pentose phosphate dehydrogenase has not been found. Thus, experiments of Sable (333) and of Glock (135) suggested an oxidation after a primary attack upon ribose-5-phosphate.

Red cell hemolysates (103) and liver extracts were shown to convert adenosine and ribose-5-phosphate to hexosemonophosphate and hexosediphosphate (103, 135, 333). These data were interpreted as indicating a cycle in hexosemonophosphate oxidation. However, with these preparations glucose-6-phosphate was not formed from fructose-1,6-phosphate (102). Seegmiller and Horecker (341) with liver and bone marrow extracts demonstrated the degradation of 6-phosphogluconic acid with a transient accumulation of pentose—presumably a mixture of ribulose- and ribose-5-phosphates—followed by the formation of glucose-6-phosphate. Later it was shown that with these preparations, fructose-1,6-diphosphate did not yield glucose-6-phosphate, thereby, eliminating as a mechanism an aldolase condensation of triosephosphates to fructose-1,6-diphosphate and its conversion to glucose-6-phosphate by the combined action of a 1-phosphofructophosphatase and phosphohexose isomerase.

Pentose phosphate cleavage to triosephosphate was shown in red cells by Dische (102, 103) and in bacteria by Marmur and Schlenk (266). A more detailed study of a similar reaction with the extracts of yeast and of *Escherichia coli* by De la Haba and Racker (86) and with yeast and brain tissue by Sable (333) indicated that several enzyme steps participated in this pathway. Efforts to isolate and identify the remaining two-carbon fragments at the oxidation level of glycolaldehyde were unsuccessful.

a. *Transketolase*. The pentose phosphate cleaving enzyme is found in plant (10, 175), animal

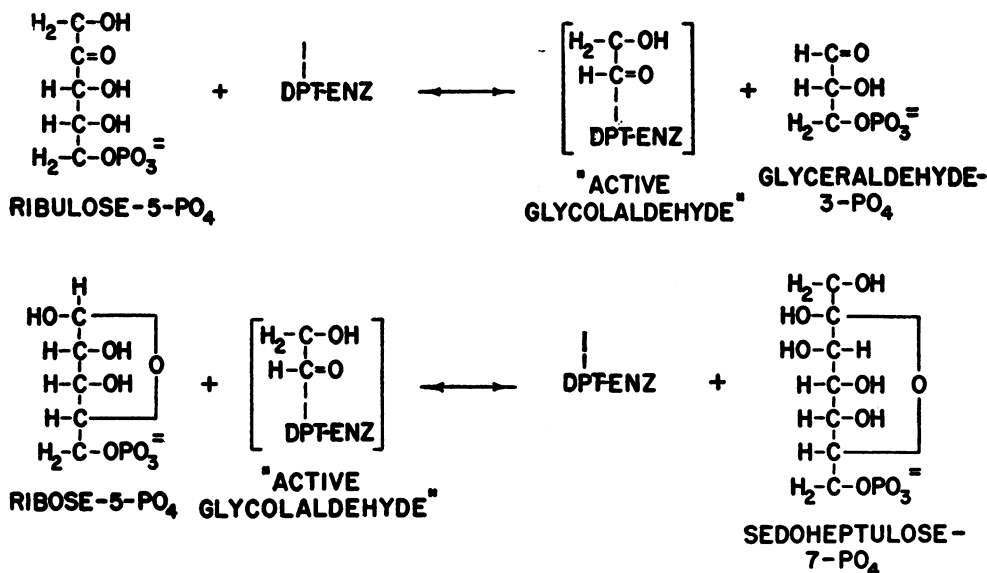


Figure 4. Sedoheptulose-7-phosphate formation (Transketolase)

(174), and bacterial cells (87, 281a, 393, 431) and has been crystallized from yeast by Racker, De la Haba and Leder (323) and highly purified from spinach and from liver by Horecker, Smyrniotis and Klenow (178). This enzyme contains diphosphothiamin (DPT) as a tightly bound prosthetic group (175, 323). The DPT was removed by dialysis against ethylenediamine tetraacetic acid (versene) or by precipitating the enzyme at an acid pH. Ribulose-5-phosphate rather than ribose-5-phosphate is the substrate cleaved, the reaction occurring only if an appropriate acceptor for the two-carbon fragment also is present (323) (see figure 4); thus the transketolase is a transferase. The spinach and liver preparations (178) contain pentose phosphate isomerase which interconverts ribulose- and ribose phosphates, with the latter serving as "active glycolaldehyde" acceptor to yield sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate. Sedoheptulose phosphate has been isolated and identified by chemical means (173, 178). Among the compounds which serve as precursors for "active glycolaldehyde" are hydroxypyruvate (87), L-erythrulose (178), fructose-6-phosphate (324), sedoheptulose-7-phosphate (178) and D-xylulose-5-phosphate (372). Likewise, several aldehydes can serve as "active glycolaldehyde" acceptors including glycolaldehyde, glyceraldehyde, glyceraldehyde-3-phos-

phate (87), ribose-5-phosphate (173) and deoxyribose-5-phosphate (325) (figure 5). In line with these observations both Horecker *et al.* (180) and Racker and coworkers (324) have obtained evidence that transketolase transfers two carbon units between fructose-6-phosphate and glyceraldehyde-3-phosphate, forming ribulose-5-phosphate and a tetrose tentatively identified as D-erythrose-4-phosphate. Purified transketolase lacks a high degree of stereospecificity in that carbohydrates containing both *cis* and *trans* configurations at carbons 3 and 4 are split and formed (325).

Akabori, Uehara and Muramatsu (3) report the formation of pentose phosphate from triose phosphate and dihydroxymaleic acid by a mechanism believed to involve the formation of hydroxypyruvate and its decarboxylation to "active glycolaldehyde".

Free sedoheptulose accumulates in plants (230a) and is found as sedoheptulose-7-phosphate in photosynthesizing algae (23). Heptulose phosphate formation from ribose-5-phosphate, presumably by transketolase, also has been observed in extracts of *Rhodospirillum rubrum* (23), *Pseudomonas fluorescens* (431), *Microbacterium lacticum* (393), and *Azotobacter vinelandii* (281a). Fractionation of growing *Azotobacter vinelandii* (281a) cells also yielded heptulose phosphate, further strengthening the supposition

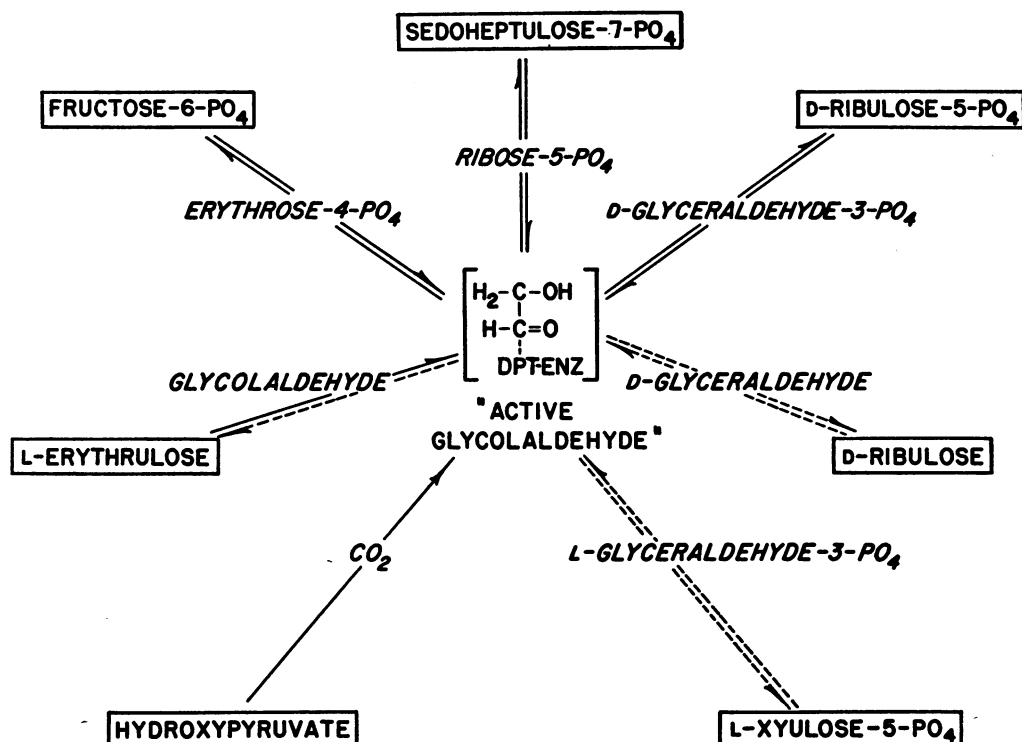


Figure 5. Reactions of transketolase

that it plays an important role in carbohydrate metabolism and is not an artifact of the experimental conditions.

b. *Transaldolase*. Pentose phosphate metabolism by crude liver (171), spinach extracts (10) and pea (131) preparations yields sedoheptulose-7-phosphate which in turn is transformed into glucose- and fructose-6-phosphates (figure 6). Fructose-6-phosphate (135) is formed first, and glucose-6-phosphate appears only if hexose phosphate isomerase is also present (176, 179). The liver and yeast preparations convert sedoheptulose-7-phosphate to hexosephosphates only if triose phosphate is also added (179). Evidence for transaldolase is based on: (a) the formation of glucose-6-phosphate labeled in carbon atoms 4, 5, and 6 with uniformly labeled fructose-1,6-diphosphate (source of triosephosphate) and unlabeled sedoheptulose phosphate as the substrates (182); and (b) free dihydroxyacetone added with labeled sedoheptulose-7-phosphate does not enter the hexosemonophosphate fraction (fructose-6-phosphate + glucose-6-phosphate) as indicated by lack of

dilution of the C¹⁴ furnished by the top 3 carbons of the sedoheptulose-7-phosphate (182).

Transaldolase has been purified about 300-fold from yeast (179, 182). Evidence for the presence of a coenzyme or prosthetic group or for the type of linkage between dihydroxyacetone and the enzyme has not been obtained. No substrates thus far tested other than sedoheptulose-7-phosphate or fructose-6-phosphate have been found to act as a dihydroxyacetone donor, and only glyceraldehyde-3-phosphate and erythrose-4-phosphate will act as acceptors (182).

The second product of sedoheptulose-7-phosphate cleavage by transaldolase is a tetrosephosphate, presumably *D*-erythrose-4-phosphate (179, 183). The evidence for this structure is: (a) a condensation of this product with dihydroxyacetone phosphate, catalyzed by muscle aldolase, forms sedoheptulose-1,7-diphosphate, and (b) a reaction with "active glycolaldehyde" (transketolase) formed fructose-6-phosphate. Sedoheptulose-1,7-diphosphate serves as an efficient precursor of shikimic acid in *E. coli* extracts (195).

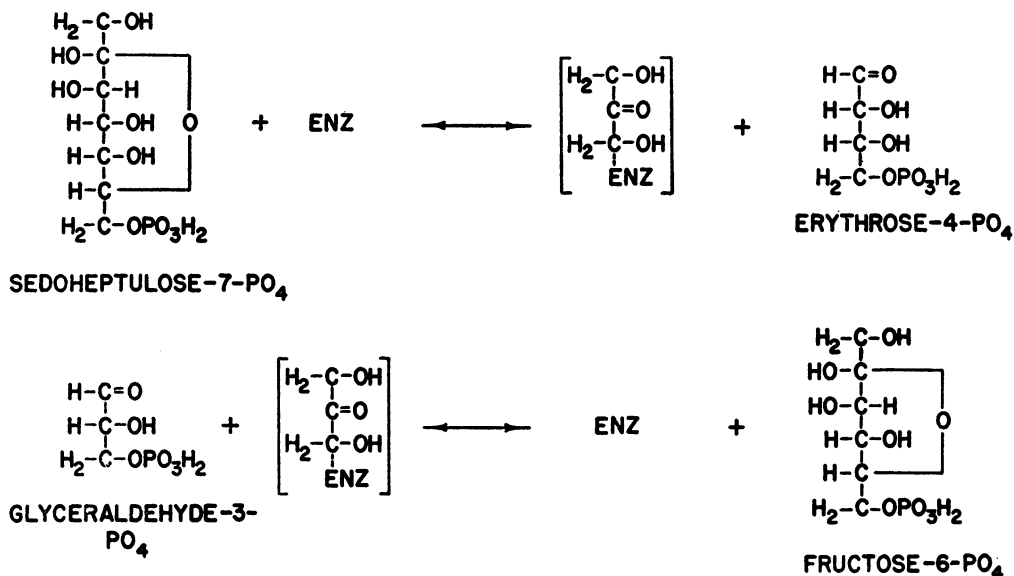


Figure 6. Fructose-6-phosphate formation (transaldolase)

c. *Aldolases*. In addition to fructose diphosphate aldolase which also splits sedoheptulose-1,7-diphosphate (183), specific aldolases which cleave 2-keto-3-deoxy-6-phosphogluconate and deoxyribose-5-phosphate are found in bacteria. The former, postulated by Entner and Doudoroff (118) as part of the mechanism of pyruvate and glyceraldehyde-3-phosphate formation, has been purified from extracts of *P. fluorescens* by Kovachevich and Wood (223) and has been found in several *Pseudomonas* and *Acetobacter* species and in *Escherichia coli* (223). No cofactor requirement has been found. Although accurate determinations have not been made, at equilibrium the ratio of pyruvate to 2-keto-3-deoxy-6-phosphogluconate appears to be about 3:1.

Deoxyribose-5-phosphate aldolase has been purified from *E. coli* extracts by Racker (322) and shown to catalyze the reversible formation of deoxyribose-5-phosphate from acetaldehyde and glyceraldehyde-3-phosphate. Crude extracts of *E. coli* form deoxyribose-5-phosphate from ribose-5-phosphate when acetaldehyde is present.

4. Hexosemonophosphate Oxidation Pathways

The distribution and importance of the several pathways for carbohydrate oxidation are not completely clear. It is evident, however, that more than one pathway exists and that several may contribute to the over-all rate of substrate oxidation. Which pathway is involved and

whether or not a particular intermediate accumulates depends upon the number and amount of enzymes present since these in turn dictate the amount of donors and receptors which are present for transfer reactions.

Evidence as to whether one or two dehydrogenations occur at carbon one of glucose is furnished by measuring the rate of carbon dioxide release from glucose-1-C¹⁴ as compared to that of glucose labeled in other positions. The preferential release of carbon atom one by tissue slices (2, 32, 33, 68) and by *E. coli* (68), *Streptomyces* sp. (66), *Aspergillus niger* (5), and yeast (20, 246) has been demonstrated.

a. *Interconversions of 5, 6 and 7 carbon phosphate esters*. Complete oxidation of phosphate esters to carbon dioxide *via* a hexosemonophosphate pathway can be visualized as shown in figure 7. The cyclic process catalyzed by the combined action of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, pentose phosphate isomerase, transketolase, transaldolase, and hexosephosphate isomerase could yield one mole of carbon dioxide and two moles of reduced pyridine nucleotide, the latter in turn being reoxidized by one mole of oxygen (R.Q. = 1). Only the first two steps of such a cycle are oxidative, the second of these being accompanied by decarboxylation to yield pentose phosphate. Succeeding anaerobic reactions can regenerate fructose-6-phosphate and glucose-6-phosphate by three possible means:

tions by the incorporation and rearrangement of labeled carbon from ribose-5-phosphate-1-C¹⁴ and ribulose-5-phosphate-2,3-C¹⁴, into the hexose molecule. The labeling of the glucose-6-phosphate isolated after enzyme action indicated two mechanisms of fructose-6-phosphate formation; one involving transaldolase and transketolase with sedoheptulose-7-phosphate as a substrate; the other involving transketolase and erythrose-4-phosphate with ribulose-5-phosphate as the 2 carbon donor.

b. *Routes from hexosemonophosphate to pyruvate.*

(1) *Pentose phosphate cleavage.* Degradation of ribose-5-phosphate by *Microbacterium lacticum* extracts (393) has been studied with the following findings: (a) under anaerobic conditions the successive formation of sedoheptulose-7-phosphate and fructose-6-phosphate was observed as reported by Horecker *et al.* (180) and by Axelrod *et al.* (10); (b) aerobically, ribose-5-phosphate was converted largely to pyruvate; hexose and heptulose phosphate esters did not accumulate. It appears that a pentose phosphate cleavage different from the known reactions of transketolase exists and that glyceraldehyde-3-phosphate dehydrogenase competes with transaldolase by oxidizing glyceraldehyde-3-phosphate, the potential three carbon acceptor. These data suggest that the importance of the oxidative cycle is governed by the amount of triosephosphate dehydrogenase and the enzymes which convert phosphoglycerate to pyruvate relative to the amount of transaldolase activity.

(2) *2-Keto-3-deoxy-6-phosphogluconate cleavage.* Doudoroff and his collaborators (118, 263) in studies with *Pseudomonas saccharophila* have expanded the concept of diverse pathways of carbohydrate metabolism by their demonstration of a third pathway of hexosemonophosphate utilization by which glucose yields nearly two moles of pyruvate. The carboxyl group of one pyruvate is derived from carbon atom one of glucose. The mechanism was suggested by the finding that 6-phosphogluconate is converted to pyruvate and triosephosphate under anaerobic conditions (118). Since glyceric acid was not converted to pyruvate, Entner and Doudoroff postulated the formation of a new ester, 2-keto-3-deoxy-6-phosphogluconic acid, as an intermediate and cleavage by an aldolase-type enzyme to form pyruvate and glyceraldehyde-3-phosphate (figure 3). MacGee and Doudoroff (262, 263) have re-

covered the intermediate phosphate ester as a crystalline sodium salt and established a structure as that postulated. This pathway has been demonstrated to be of major importance in *Pseudomonas fluorescens* (431). The enzymes converting 6-phosphogluconate to pyruvate and triosephosphate have been separated and characterized by Kovachevich and Wood (222, 223). The first enzyme, 6-phosphogluconate dehydrase, forms 2-keto-3-deoxy-6-phosphogluconate and requires glutathione or cysteine and ferrous ions for activity. The second enzyme, 2-keto-3-deoxy-6-phosphogluconate aldolase, cleaves this ester to pyruvate and D-glyceraldehyde-3-phosphate. Evidence for this alternative pathway of 6-phosphogluconate degradation has now been found in several members of the genus *Pseudomonas* (223), *Azotobacter* (281a), and in *Escherichia coli* (223).

5. Hexosemonophosphate Fermentation Pathways

Evidence of a hexosemonophosphate pathway in which intermediates derived from the substrate replace oxygen as the hydrogen acceptor has directed attention to the role of this pathway in anaerobic processes. As presently visualized, the fermentative hexosemonophosphate mechanism may involve coupled oxidative reactions to yield 6-phosphogluconate, which is either oxidized to ribulose-5-phosphate or converted anaerobically to 2-keto-3-deoxy-6-phosphogluconate. The pentose phosphate may be cleaved between carbon atoms 2 and 3 as in the aerobic pathways or the 2-keto-3-deoxy-6-phosphogluconate converted to pyruvate and triosephosphate.

a. *Heterolactic (ethanol-lactic).* A fermentative hexosemonophosphate pathway was postulated in *Leuconostoc mesenteroides* by DeMoss, Bard and Gunsalus (93) and Gunsalus and Gibbs (150) upon the following evidence: (a) equimolar quantities of carbon dioxide, ethanol, and lactate were produced, and the ratio of these was constant under varying conditions; (b) aldolase could not be demonstrated, (c) carbon dioxide arises from carbon 1 of glucose, the lactate from carbons 4, 5 and 6 and the methyl carbon of ethanol from carbon 2 of glucose (figure 8). Although the mechanism is still obscure, extracts of the organism were shown to possess glucose-6-phosphate dehydrogenase (TPN- or DPN-linked) (95), and a DPN dependent 6-phosphogluconate dehydrogenase (97). Ribulose-5-phosphate and carbon dioxide were among the endproducts. At present,

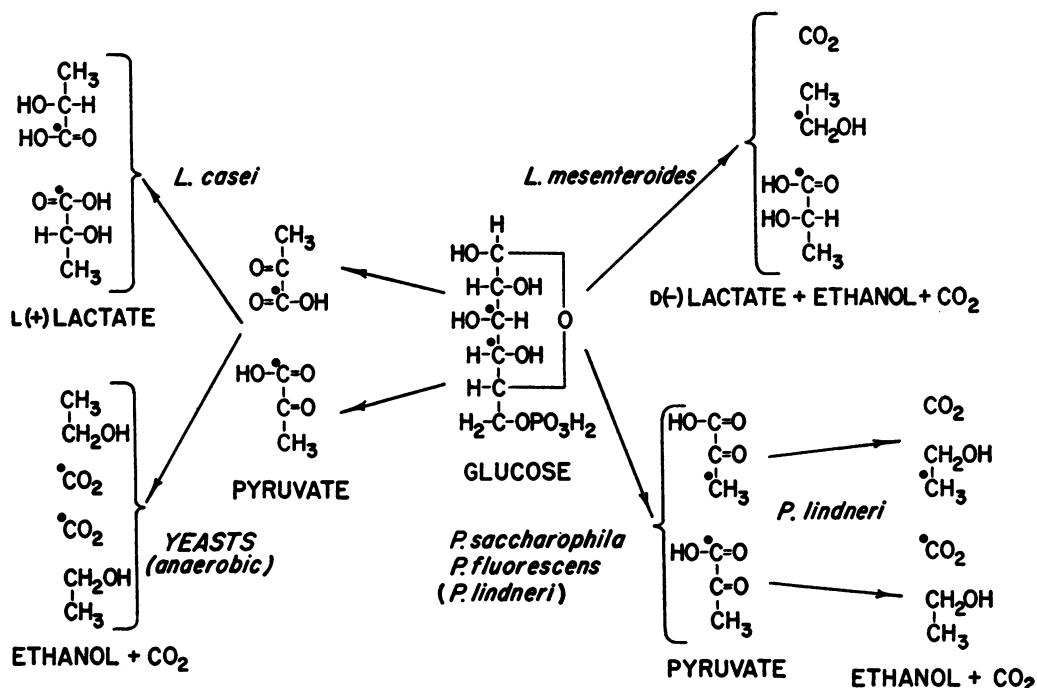


Figure 8. The distribution of glucose carbons in products from various fermentations

key problems are the steps in ethanol formation and the means of forming the three-carbon fragment. If the mechanism involves ribulose-5-phosphate cleavage to "active glycolaldehyde" and glyceraldehyde-3-phosphate, the extracts could convert the latter to lactate (128). Although the route from "active glycolaldehyde" to ethanol is obscure, whole cells of this organism reduce acetate to ethanol. The sedoheptulose phosphate cycle cannot be involved because ethanol derived from glucose-3,4-C¹⁴ contains C¹⁴ only in the carbinol group (150).

b. *Bacterial ethanol*. *Pseudomonas lindneri*, studied by Kluver and Hoppenbrouwers (209), ferments glucose to 1.8 moles of ethanol, 1.8 moles of CO₂ and traces of lactate (128). The pattern resembles that of *Leuconostoc mesenteroides* in that glucose-1-C¹⁴ yields labeled carbon dioxide. In contrast to *L. mesenteroides*, glucose-3,4-C¹⁴ yields 50% of the C¹⁴ of glucose as CO₂; the specific activity of the CO₂ was one-half of that in either carbon 3 or 4 of the glucose (130). Fermentation of 2- and 6-labeled glucose has demonstrated that ethanol arose from carbons 2 and 3 or from 5 and 6 with the carbons 2 and 5 contributing the carbinol carbon. The mechanism has

been clarified by the demonstration of a yeast-type carboxylase (326). This is the distribution of carbon expected from a pathway generating pyruvate by the Entner-Doudoroff cleavage of 6-phosphogluconate (figure 8).

c. *Induced aldonic acid*. *Streptococcus faecalis* can be induced by growth on gluconate or 2-ketogluconate to fermentation by the hexosemonophosphate pathway as indicated by: (a) gluconate but not glucose grown cells ferment gluconate-1-C¹⁴ to yield labeled carbon dioxide and labeled lactate. The adapted cells also ferment 2-ketogluconate and yield one mole each of carbon dioxide and lactate, (b) hexokinase and gluconokinase are present as are dehydrogenases for glucose-6-phosphate and 6-phosphogluconate. The fermentation mechanism is still under study (350).

d. *Pentose*. Pentose fermentation, adaptive in lactic acid bacteria (232), yields labeling patterns in the products from pentose-1-C¹⁴ which implicate a cleavage of ketopentose between carbons 2 and 3 (figure 9). Such a cleavage would be similar to the transketolase reaction except that the 2 carbon fragment would be free rather than transferred to an acceptor. Thus, D-ribose-1-C¹⁴ and D-xylose-1-C¹⁴ fermentation by *Lactobacillus*

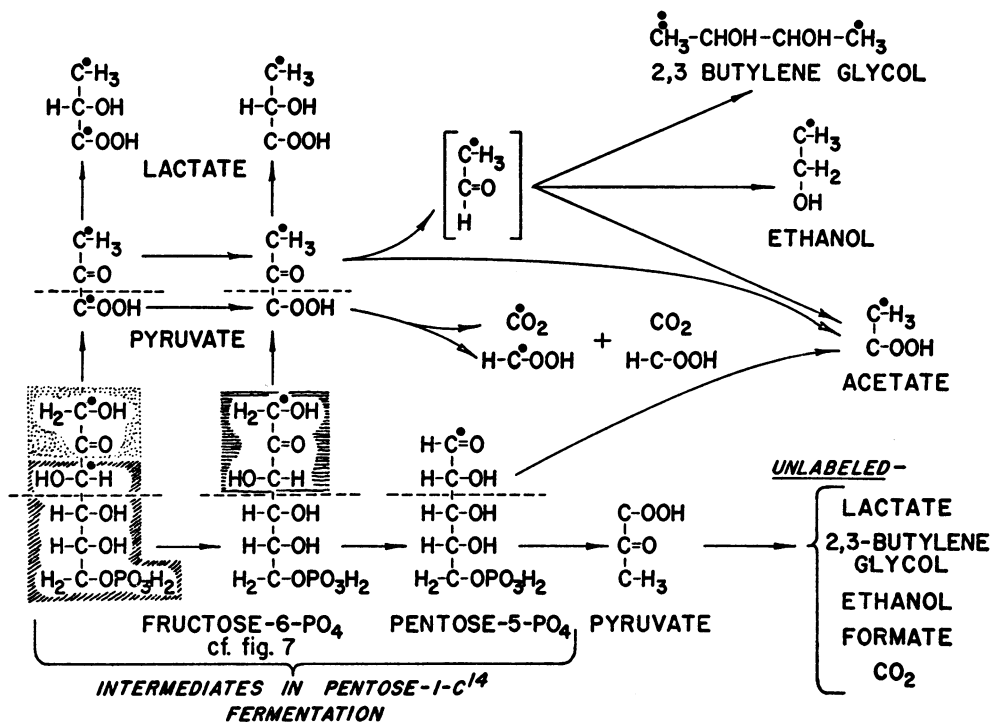


Figure 9. Pathways to pentose fermentation

pentosus (30, 126, 231) and L-arabinose-1-C¹⁴ fermentation by *Lactobacillus pentoaceticus* (327) yielded methyl-labeled acetate; unlabeled lactate was produced by the lactobacilli. D-Xylose-1-C¹⁴ fermentation by *Fusarium lini* (132) yielded ethanol, acetate and CO₂ with the label in the methyl group of acetate.

Although many reactions of pentoses have been reported (34) including isomerization, before or after phosphorylation, the pathways of degradation have not been defined. The phosphorylation of xylose by extracts of xylose adapted *L. pentosus* ultimately yielded ribose-5-phosphate; xylose-5-phosphate was not found (233). Ribulose-5-phosphate was postulated as the precursor of ribose-5-phosphate in this reaction and also as the intermediate cleaved to 2 and 3 carbon units. Xylose also was converted to xylulose by the same extracts (280) and by an enzyme from *Pseudomonas hydrophila* (166, 167). Thus, the reaction sequence was postulated to be xylose → xylulose → xylulose-5-phosphate → ribulose-5-phosphate. This mechanism has been in part confirmed by the isolation of xylulose-5-phosphate (372). Similar isomerizations of D-arabinose to D-ribulose by *E. coli* enzymes have been reported

by Cohen (70), and of L-arabinose to L-ribulose by extracts of *L. pentosus* by Lampen (234). The induced formation of a specific D-arabinokinase and a ribokinase has been reported by Cohen *et al.* (69).

Fermentation of pentose-1-C¹⁴ by *Escherichia coli* (133) and by *Aerobacter aerogenes* (294) indicates the operation of a different pathway in these organisms. With xylose-1-C¹⁴ and D-arabinose-1-C¹⁴, resting cells of *E. coli*, at an acid pH, formed more than one mole of lactate per mole of pentose fermented, as Carson (56a) had already observed with xylose. The methyl groups of lactate and acetate contained the highest radioactivity. C¹⁴ also was present in the lactate carboxyl group and in formate, whereas the β carbon of lactate and the carboxyl of acetate were inactive (133). With D- and L-arabinose-1-C¹⁴, the products of fermentation by *A. aerogenes* were labeled as if derived from methyl and carboxyl-labeled pyruvate (294). The complete conversion of pentose to triosephosphate *via* sedoheptulose-7-phosphate and fructose-6-phosphate was postulated to account for the pattern of labeling in the products (figures 7, 9).

6. Nonphosphorylated Hexose Oxidation

Many organisms of the *Pseudomonas* and *Acetobacter* genera oxidize glucose with the accumulation of gluconate (45, 255) and ketogluconates (28, 29, 210, 229, 370). A similar oxidation of lactose and maltose to their bionic acids (211, 362) and pentoses to the corresponding pentonic acids (256) has been reported. The further degradation of 2-ketogluconate by growing cultures yields α -ketoglutarate (257), pyruvate (213, 404) and acetate (47), whereas the oxidation of 5-ketogluconate yields tartarate, glyoxalate, oxalate, and formate (185). The accumulation of these acids, particularly gluconate and 2-ketogluconate, is dependent upon the cultural condition including the iron (213) and nitrogen content of the medium (213, 214). The pseudomonads are reported to accumulate 2-ketogluconate (255), whereas different acetobacter strains accumulate 2-ketogluconate (28, 29), 5-ketogluconate (29, 370), both 2-ketogluconate and 5-ketogluconate (28, 229), or 2,5-diketogluconate (201).

a. *Glucose and gluconate oxidation.* From studying the mechanism of gluconic and ketogluconic acid formation and oxidation by cell suspensions, Entner and Stanier (117) concluded that in *Pseudomonas fluorescens* the oxidation of glucose to gluconate is affected by constitutive enzymes, whereas the further oxidation of gluconate to 2-ketogluconate is dependent on an inducible system formed in response to gluconate. They also concluded from stoichiometric data that 2-ketogluconate *per se* is not an intermediate in glucose oxidation and suggested that its accumulation might result from the dephosphorylation of a hypothetical 2-keto-6-phosphogluconate.

On the other hand, Stokes and Campbell (363), using dried cells of *Pseudomonas aeruginosa*, and Claridge and Werkman (64), with extracts of the same organism, reported a conversion of both glucose and gluconate to 2-ketogluconate with the uptake of two and one atoms of oxygen per mole of these substrates, respectively. There was no evidence for the further metabolism of 2-ketogluconate or for the participation of phosphate esters in 2-ketogluconate formation. The conclusion that a phosphorylation mechanism was absent was based on the observations that glucose oxidation was insensitive to $M/50$ sodium fluoride, was not stimulated by ATP (363), and that phosphate esters could not be extracted from freshly harvested glucose grown cells (48).

In further studies of glucose oxidation by *Pseudomonas fluorescens*, Wood and Schwerdt, using cell-free extracts, demonstrated in addition to the oxidation of glucose and gluconate to 2-ketogluconate (430) the oxidation at similar rates of glucose-1-phosphate, fructose-6-phosphate, glucose-6-phosphate, 6-phosphogluconate, and ribose-5-phosphate; with each phosphate ester carbon dioxide was evolved (431). The 2-ketogluconate-forming system was separated from phosphate ester-oxidizing system, and in agreement with Campbell's conclusion (363), the oxidation of glucose to 2-ketogluconate was shown to proceed without phosphorylation. A similar and separate pathway for hexosephosphate oxidation also has been demonstrated in *P. aeruginosa* (65).

The main contribution of these studies has been a clarification of the previous findings that living pseudomonad cells oxidize part of the glucose to carbon dioxide, whereas dried cells and extracts oxidize glucose only to the 2-ketogluconate stage. The demonstration of both a hexosemonophosphate pathway and a pathway from glucose to 2-ketogluconate without phosphorylation has widened the concept of carbohydrate oxidation in these organisms. The steps in the further oxidation of the ketogluconates, for which evidence is accumulating (89, 289), still require clarification.

Although the oxidation of glucose and gluconate to 2-ketogluconate does not appear to furnish useful energy at the substrate level, nor to generate intermediates for biosynthesis, a cytochrome system mediates in the hydrogen transport and may well yield high energy phosphate *via* oxidative phosphorylation. Some knowledge of this electron transport system has been derived from studies of cell extracts and particulate preparations of *P. fluorescens* (430). In this organism no evidence for a pyridine nucleotide linked glucose dehydrogenase such as found in the mammalian liver (367) or for a flavoprotein-type glucose oxidase as found in molds (82, 203) has been obtained. So far the complete oxidation system, which exists in particles (334, 430) separable by high speed centrifugation or by ammonium sulfate precipitation, has not been solubilized or the individual components defined. With such preparations, the formation of reduced bands corresponding in wavelength to the cytochrome "b" and cytochrome "c" bands appears on the addition of glucose and disappears on

aeration (430). Since absorption was not observed in the 600 $m\mu$ to 620 $m\mu$ region and the oxidation of glucose and other substrates was not sensitive to 10^{-3} M cyanide, no evidence was obtained for a cytochrome oxidase. These observations demonstrate a linkage of glucose oxidation with cytochromes probably by means of unidentified carriers and enzymes. Until these enzymes can be solubilized, it is not possible to determine if a new pyridine nucleotide component or flavoprotein carrier is involved.

b. *Gluconokinase and 2-ketogluconokinase.* The relationship between phosphorylated and non-phosphorylated carbohydrate oxidation pathways has been clarified recently by the demonstration of gluconokinase and 2-ketogluconokinase in extracts of glucose grown *P. fluorescens* (289) and an inducible 2-ketogluconokinase in *Aerobacter cloacae* (89). With an ammonium sulfate fraction of either *P. fluorescens* (290) or *A. cloacae* (89), the product of 2-ketogluconate phosphorylation accumulates. This acid stable ester has been isolated, purified by cellulose column (290) or paper chromatography (89), and partially characterized as 2-keto-6-phosphogluconate.

The formation of gluconokinase and 2-ketogluconokinase of *A. cloacae* (88) and *P. fluorescens* (290) is induced by growth on the specific substrate. Since growth on 2-ketogluconate does not induce gluconokinase formation, gluconokinase and 2-ketogluconokinase appear to be distinct enzymes. A different enzyme termed "hexonokinase" is found in *A. cloacae* following growth on D-galactonate (400). This kinase also phosphorylates gluconate and 2-ketogluconate.

c. *Routes from gluconate to pyruvate.* In the presence of ATP, crude extracts of *P. fluorescens* anaerobically convert gluconate or 2-ketogluconate to pyruvate (289), whereas 6-phosphogluconate and 2-keto-6-phosphogluconate are converted to approximately 1 and 2 moles of pyruvate, respectively, in the absence of ATP (290). Glyceraldehyde-3-phosphate is not an intermediate in 2-keto-6-phosphogluconate degradation, and hydroxypyruvate does not accumulate. Crude extracts of *A. cloacae* (89) degrade 2-keto-6-phosphogluconate to a mixture of phosphate esters (88) containing 3-phosphoglycerate (89). Although steps in the degradation of 2-keto-6-phosphogluconate remain to be elucidated, dehydration and cleavage reactions similar to those converting 6-phosphogluconate to 2-keto-3-

deoxy-6-phosphogluconate and then to pyruvate and glyceraldehyde-3-phosphate may be involved. In the case of 2-ketophosphogluconate degradation, however, pyruvate and 3-phosphoglycerate would be formed and the latter converted to pyruvate.

Thus, two pathways of glucose oxidation exist in these pseudomonads (figure 10): (a) glucose is oxidized to gluconate and the latter phosphorylated to form 6-phosphogluconate—the 6-phosphogluconate is then converted to pyruvate and triosephosphate by the two enzyme system discovered by Entner and Doudoroff (118) and found in *P. fluorescens* (431), or oxidized via pentose-5-phosphate, presumably involving sedoheptulose-7-phosphate and fructose-6-phosphate as intermediates; (b) glucose is oxidized to 2-ketogluconate before phosphorylation, phosphorylated by 2-ketogluconokinase and then degraded to pyruvate by an unidentified pathway. Glucose is not degraded via Embden-Meyerhof glycolytic system as indicated by: (a) the inability to isolate phosphate esters from glucose grown *P. aeruginosa* (48); (b) the lack of hexokinase and phosphohexokinase in extracts of glucose grown *P. fluorescens* (289); (c) the distribution of C^{14} from position labeled glucose in the pyruvate and acetate isolated (246, 247) and the inability of resting cells of *P. aeruginosa* to ferment glucose or glucose-6-phosphate (65).

III. ROUTES TO KNOWN PATHWAYS

The occurrence in nature of a wide variety of monosaccharides other than glucose and fructose, as well as oligo- and polysaccharides, implies the occurrence of enzymatic pathways for their formation and use. An array of mono- and oligosaccharides has been used as energy and carbon sources to characterize microorganisms. At an enzymatic level, oligo- and polysaccharides are converted by hydrolysis or phosphorolysis to monosaccharides, and the monosaccharides of other configurations are converted to intermediates of glucose or ribose metabolism. Thus far, evidence has not been found for independent routes for the metabolism of other carbohydrates.

1. Monosaccharides

a. *Mannose.* Hexokinase (table 1) converts mannose to mannose-6-phosphate (230); and a specific isomerase, mannose-6-phosphate isomerase, converts this substrate to fructose-6-

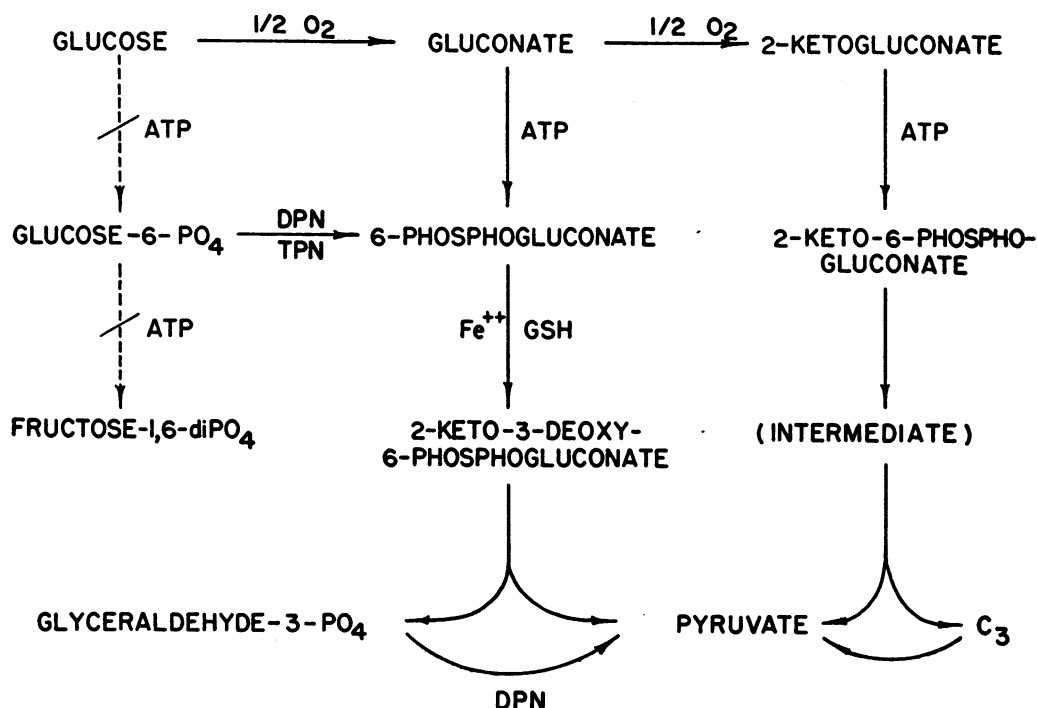


Figure 10. Pathways to pyruvate: *Pseudomonas fluorescens*

phosphate (346, 348)—a known intermediate in glucose metabolism. This isomerase, isolated from liver by Slein (348), is distinct from phosphoglucoisomerase (section I). Phosphoglucomutase interconverts mannose-1- and mannose-6-phosphates (239). The implication of these reactions and of diverse mannose compounds, for example, guanosine diphosphate mannose (46) is not clear. Certain bacteria ferment mannose at approximately 60% the rate of glucose fermentation; their enzymatic patterns have not been studied (123).

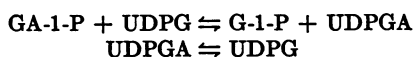
b. *Fructose*. Hexokinase phosphorylates fructose to fructose-6-phosphate, an intermediate in glucose fermentation (table 1). Fructose-6-phosphate is converted in aerobic organisms to glucose-6-phosphate by phosphoglucoisomerase to enter the hexosemonophosphate pathway. Cleavage by transketolase may also occur (180, 324) as discussed fully in section II. Fructose is also metabolized by another route; a specific fructokinase forms fructose-1-phosphate (80, 244) which is cleaved by a specific aldolase, 1-phosphofructoaldolase (section I) to dihydroxyacetone phosphate and free glyceraldehyde (56, 164, 244, 245). The latter is phosphorylated by a specific kinase (164) to glyceraldehyde-3-phosphate.

Among the unresolved problems in fructose metabolism are the routes in *Pseudomonas fluorescens* and *Pseudomonas saccharophila* by way of mannose (113) to accumulate D-mannonic acid (313).

c. *Galactose*. Galactose fermentation was shown by Leloir *et al.* to occur through galactose-1-phosphate (GA-1-P) and its conversion to glucose-1-phosphate by galactowaldenase (50, 51, 53). A new coenzyme, uridine diphosphoglucose (UDPG) and uridine diphosphogalactose (UDPGA), was isolated and shown to mediate the conversion (51, 53).

A specific galactokinase which converts galactose to galactose-1-phosphate (table 1) is of wide occurrence in yeast (221, 380), animal tissues (219) and bacteria (54, 331).

The interconversion of galactose and glucose through hydroxyl inversion of the 4 carbon, as first postulated by Kosterlitz (220), has been studied with preparations of yeast (221) and *L. bulgaricus* (331) and is best summarized as follows:



The two reactions have not been separated. Uridine triphosphate activates crude preparations (285) but with purified enzymes, UDP hexose (157, 196) is specifically required. The equilibrium between glucose-1- and galactose-1-phosphate is about 3:1 as shown with phosphoglucomutase-free preparations of *L. bulgaricus* (156), or with arsenate present to inhibit the mutase (240). Excellent reviews of these studies and of the isolation, chemistry and function of UDPG have been written by Leloir (239, 242) and by Kalckar and Klenow (197). The general availability of the uridine coenzymes should accelerate clarification of these reactions.

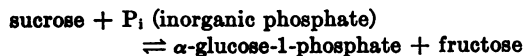
Some evidence was presented for an alternate fermentation route paralleling the Embden-Meyerhof pathway from galactose-6-phosphate *via* tagatose-6-phosphate and tagatose-1,6-diphosphate (379).

Several lactic acid bacteria, which catalyze a homolactic fermentation of glucose, have been reported to form only 1 mole of lactate per galactose (360); the reactions have not been investigated at the enzyme level.

2. Disaccharides and Polysaccharides

a. *Phosphorylases*. Phosphorolysis, an enzymatic transfer of a terminal glycosyl unit to inorganic phosphate, occurs with sucrose, maltose, starch and glycogen to form, respectively, fructose, glucose and polysaccharides plus glucose-1-phosphate. The sucrose phosphorylase cleaves glycosyl-1,2 bonds, whereas the other three cleave glycosyl-1,4 bonds. In both cases, the equilibrium favors the formation of the glycosidic bond, though in the presence of phosphoglucomutase, glucose-6-phosphate accumulates. The sucrose and maltose phosphorylases are of bacterial origin and the glycogen and starch phosphorylases are of general occurrence (160).

Sucrose phosphorylase which catalyzes the reaction—



has been purified from *Pseudomonas saccharophila* (107, 108) and is also found in *P. putrefaciens* (112) and *L. mesenteroides* (194, 417).

Early evidence for an enzyme substrate compound and for a "transglycosidase" reaction was also obtained with the sucrose phosphorylase of

P. saccharophila by demonstrating: (a) an exchange of other monosaccharides for the fructose moiety of sucrose in the absence of glucose-1-phosphate (109, 110, 159); (b) P^{32} incorporation in glucose-1-phosphate in the absence of fructose (159); and (c) an arsenolysis of glucose-1-phosphate (111).

Uridine coenzymes have not been implicated in the sucrose phosphorylase, but sucrose is formed by an independent enzyme found in seeds with UDPG + fructose (241) as substrates.

Maltose phosphorylase differs from sucrose phosphorylase in that (a) β -glucose-1-phosphate is formed, and (b) the P^{32} exchange and arsenolysis reaction with β -glucose-1-phosphate do not occur, thus indicating a different reaction mechanism (121).

b. *Hydrolases*. Enzymes such as invertase, maltase, lactase, amylase and cellulase form glucose from polysaccharides by hydrolysis of the glycosidic linkages. These enzymes have been reviewed elsewhere (140, 286, 301, 315, 394).

c. *Polyhydric alcohols*. Many hexitols and glycerol are fermented or oxidized by microorganisms. Since the hexitols, compared to the hexoses, possess 1 additional pair of hydrogen atoms and glycerol an additional pair as compared to triose, fermentation of these substrates leads to more of the reduced products or occasionally to new products. The known enzymatic steps of metabolism of these substrates are oxidative either with or without phosphorylation.

Mannitol and sorbitol oxidation by *P. fluorescens* cells occurs *via* 2 inducible enzymes, one oxidizing mannitol and sorbitol to fructose, and the other sorbitol to L-sorbose (340). The data do not indicate whether or not phosphorylation is involved. Hexitol phosphate (mannitol phosphate) is formed by a DPN-linked fructose-6-phosphate reductase from *E. coli* (425) with an equilibrium favoring hexitol phosphate formation.

Glycerol is oxidized to dihydroxyacetone by *Acetobacter suboxydans* (402), *Escherichia coli* (7) and *Aerobacter aerogenes* (44). The enzymes from *E. coli* and *A. aerogenes* have been studied in extracts and shown to be DPN-linked. The analogous pathway *via* α -glycerol phosphate has been shown in extracts of *Escherichia freundii* (279), *A. aerogenes* (264), *Streptococcus faecalis* (48) and *Mycobacterium* sp. (184). The *S. fae-*

calis glycerol phosphate dehydrogenase, apparently linked through flavoproteins to oxygen, generates hydrogen peroxide (106, 148), whereas that of *A. aerogenes* involves cytochromes but not DPN and forms H₂O. The occurrence of still another mechanism for glycerol oxidation involving neither α -glycerol phosphate nor DPN has been suggested by data obtained with *Acetobacter suboxydans* (204). Much of the mechanism of polyhydric alcohol formation and use remains unknown, both at the enzymatic and at the fermentation balance level.

IV. PYRUVATE REACTIONS

Pyruvate gained favor as a key intermediate leading to the many products of microbial fermentation in the early 1940's when phosphorylative glycolysis was established as a main route of carbon and energy transformation during anaerobic carbohydrate breakdown. The formation of pyruvate by dephosphorylation of phosphoenolpyruvate concludes the energy yielding phase of fermentation, leaving of interest only problems of product formation, their economic use, the source of biosynthetic intermediates, and of curiosity as to the reaction mechanisms. The pyruvate reactions best understood at the enzyme level were reduction to L(+) lactic acid by DPN-linked lactic dehydrogenases (119, 274, 335) and cleavage to acetaldehyde plus CO₂ (260, 295, 398) by diphosphothiamin mediated carboxylases.

As means for growing microorganisms and for extracting their enzymes developed, evidence for DPN-linked lactic dehydrogenases and "yeast type" carboxylases in bacteria accumulated. In addition, unmistakable evidence also accumulated for other reactions of pyruvate, as well as for other types of lactic dehydrogenases and pyruvate decarboxylases. In fact, more recent data assign to diphosphothiamin (cocarboxylase, DPT) a broader role than decarboxylation coenzyme, and now implicate this cofactor in the transketolase (175, 323), dicarbonyl, and pyruvate cleavages (149, 336, 343), as a carbanion acceptor and transfer agent. The cleavage functions include a variety of α -keto compounds, forming "aldehydes", and an equally wide variety of acceptors for these aldehydes, in some cases with acyl generation (152, 153). An additional DPT-mediated pyruvate reaction of microbiological importance, the "clastic" reaction (198), yielding acetyl plus formate (252, 385), or plus

CO₂ and H₂ (212), differs from previously known DPT-mediated reactions in the apparent direct generation of acyl (as thioester or phosphate anhydride) without aldehyde as an intermediate.

Microbial fermentation studies revealed in addition to reduction and decarboxylation a third pyruvate reaction—carboxylation to 4 carbon dicarboxylic acids. The CO₂ fixation occurs by two routes—(a) reductively to malate (202, 305), and (b) ATP-mediated to oxalacetate (388, 397). An alternate route of carboxylation at a more reduced level, yielding succinate as the C₄ dicarboxylic acid, has also been implicated in microbial (90, 91, 421) and animal (307) enzyme studies of propionyl metabolism. A further diversity of products is assured through condensation of the carbon skeletons at the aldehyde (carbanion) and acyl (carbonium ion) oxidation levels. Only those pyruvate reactions, arising during carbohydrate fermentation and studied at an enzymatic level, will be documented in this section.

1. Reductions

a. *Lactic dehydrogenases.* Four lactic dehydrogenases have been observed in microorganisms (figure 11). The most common DPN-linked forming L(+) lactic acid has been crystallized from tumor, pig heart (227, 365) and rat liver (134). This enzyme is found in homofermentative lactic acid bacteria and in *Escherichia coli* (202, 216, 217). A second lactic dehydrogenase DPN-linked but yielding D(-) lactic acid is present in heterofermentative lactic acid bacteria such as *Leuconostoc mesenteroides* (93) and in *Lactobacillus arabinosus* (202). The latter organism forms DL-lactic acid from glucose and, in extracts, racemizes lactate by linking two stereospecific DPN dependent dehydrogenases (202).

A third lactic dehydrogenase, flavin coenzyme linked, is present in *Lactobacillus delbrueckii* (155, 250). This lactic dehydrogenase, measured by Krebs dismutation reactions or by oxygen utilization, is independent of pyridine nucleotides (155). Such lactic dehydrogenases may be widespread in microorganisms, particularly the lactic acid bacteria. A similar lactic dehydrogenase not requiring a pyridine nucleotide carrier has also been observed in *Proteus vulgaris* (284).

A fourth lactic dehydrogenase (12,188), crystallized from yeast by Appleby and Morton (4), contains both flavinmononucleotide and cytochrome b. A less specific α -hydroxy acid oxidase

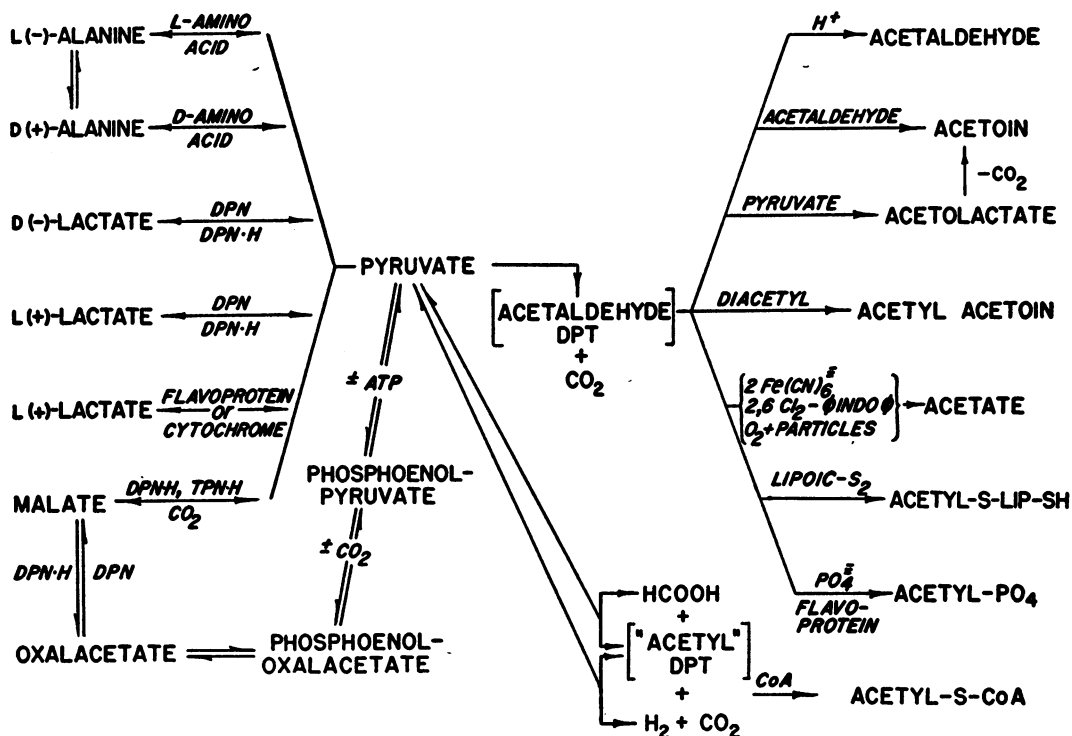


Figure 11. Reactions to pyruvate (See text, IV. 2, for detailed description of the reactions shown at right)

of apparently similar nature is present in rat kidney (31).

b. *Racemase and amination reactions.* Racemization of D- or L-lactic acid by lactate forming organisms has been clarified only with respect to *Lactobacillus arabinosus* through the revelation of two stereospecific dehydrogenases (202). Lactate forming organisms, especially clostridia, and several strains of lactic acid bacteria (63, 205) possess soluble DPN independent racemases. These enzymes, plus a similar soluble racemase for mandelate (151, 358) another α -hydroxy acid, present an interesting area for study since neither their mechanism nor their prosthetic groups have been clarified. Racemization of the analogous α -amino acid, alanine, is pyridoxal phosphate mediated and appears not to involve pyruvate as an intermediate (429) (see figure 11).

Amino acid formation from pyruvate occurs by transamination (267) with several potential amino donors of which glutamate is considered the most important quantitatively (39, 120).

Transamination, from the viewpoint of the keto acid aminated, is a reductive reaction—the amino acids are at the oxidation level of their hydroxy acids rather than of the keto acids.

Amino acid racemase, specifically alanine racemase (429), unlike the DPN-mediated racemization of lactic acid by the coupling of two dehydrogenases seems not to occur through pyruvate. Known pathways for formation of other D-amino acids, however, do involve pyruvate: *i.e.*, amination to L-alanine by transaminase, racemization to D-alanine, and transfer of the amino group from D-alanine to a keto acid forming the D-amino acid in question with regeneration of pyruvate (377). Thus pyruvate would fulfil the role of a coenzyme in the equilibration of D- and L-amino acids other than alanine, which is the real site of shift in configuration.

2. Decarboxylases and Aldehyde Transfer

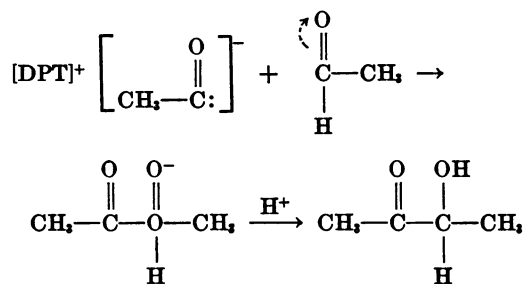
a. *Direct decarboxylases.* Either direct or oxidative cleavage of keto acids, and of other dicar-

bonyl compounds, occurs as a DPT-mediated reaction in most, if not all, microorganisms. The direct and the oxidative decarboxylases differ not in the decarboxylation step but in the subsequent fate of the aldehyde—the specificity for which resides in the enzyme. These reactions are most rationally viewed as forming “aldehyde-DPT” or “aldehyde-DPT-enzyme” compounds, followed by donor reactions for the “aldehyde”, as indicated in figure 11. Although a direct demonstration of an “aldehyde-DPT” is lacking, several evidences favoring this concept can be cited: (a) all DPT-mediated keto-, and dicarbonyl, cleavage systems yield at least traces of free aldehyde—interpreted as a measure of the instability of the “aldehyde-DPT” compound (105, 152, 336); (b) the addition of yeast-type carboxylase to an *E. coli* type pyruvate system decreases the acyl hydroxamate formed without inhibiting carbon dioxide release, suggesting a competition for free “aldehyde-DPT” (61), i.e., diversion of the aldehyde from acyl forming to free acetaldehyde forming reactions. Similar evidence arises from the release of acetaldehyde by *Pseudomonas lindneri* carboxylase during acetyl phosphate reduction with dimercaptioctanoic acid in the presence of *E. coli* fraction A (61). These experiments are far from complete and should be considered indicative rather than proof. Similar attempts to divert the acyloin formation toward acyl generation with *A. aerogenes* and *E. coli* extracts did not yield evidence for such a diversion (369)—thus suggesting caution.

Among the DPT-mediated reactions outlined in figure 11, as occurring through “aldehyde-DPT”, is (a) the yeast type carboxylase, with a proton as acceptor. This reaction may occur slowly in the absence of enzyme, but, if the formulation is correct, it is greatly enhanced in direct decarboxylases yielding free acetaldehyde. The postulated mechanism, like the early formulation of Neuberg *et al.* (296, 297, 298, 299, 300), assigns two sequential steps to the carboxylase reaction: (a) a cleavage of pyruvate forming “acetaldehyde-DPT” with a release of CO₂; (b) transfer of “acetaldehyde” to a proton thereby forming free acetaldehyde. To date, neither the yeast nor bacterial yeast-type carboxylases have been separated into two protein fractions. As indicated above, however, the release of acetal-

dehyde from the oxidative carboxylases—presumed to possess only step (a)—by the bacterial yeast-type carboxylase of *P. lindneri* (326) is presumptive evidence for a two-step reaction. Direct pyruvate decarboxylases have been found in *Acetobacter suboxydans* (326) and *Pseudomonas lindneri* (326) and their properties compared to the yeast and wheat germ enzymes (143, 226, 326, 343). These carboxylases vary slightly in dissociation constant for DPT, pH optimum and in the amount of acetoin formed per unit of carboxylase. They are similar in Mg⁺⁺ activation, sensitivity to sulfhydryl reagents, particularly *p*-chloromercuribenzoate, and to heavy metals. The location of the —SH group, enzyme or DPT, the chemical mechanism of decarboxylation, and the structure of the intermediates will require further study.

b. *Acyloin condensations.* Neuberg and Hirsch's (296) concept of acyloin formation by yeast decarboxylase included; step (a), “carboxylase” now visualized, as indicated, in figure 11, as “acetaldehyde-DPT” formation—; step (b), “carbolicase” transfer of “acetaldehyde-” to free aldehyde forming acyloin. The reaction has been indicated as:



Acetaldehyde as Acceptor. Reaction 2, upper right of figure 11, expresses the Neuberg and Hirsch postulate of acetoin formation by the yeast enzyme (296). The direct yeast and oxidative *E. coli* decarboxylases differ in one respect—the yeast enzyme liberates acetaldehyde to serve as acceptor whereas the *E. coli* oxidative decarboxylase does not form acetoin unless acetaldehyde is added. Acetoin formation relative to CO₂ release increases with aldehyde concentration (192, 193) for both enzymes. The acceptor aldehyde is virtually nonspecific though alkyl aldehydes are more active acceptors than the

more acidic, aryl, or substituted aryl aldehydes (165, 297, 299, 300, 349). The several yeast carboxylases retain during purification a constant ratio of CO₂ release to acetoin formation, but this ratio differs among enzymes—possibly resulting from a partial separation of steps 1 and 2. Further evidence of a 2 step catalysis in acetoin formation was presented by Gibbs and DeMoss (129) who obtained partial removal of acetoin forming activity from the *P. lindneri* decarboxylase, and by Chin (61) who removed by dialysis and adsorption much of the acetoin forming activity of the *E. coli* decarboxylase without destroying decarboxylase activity.

The optical rotation of the acyloins formed depends on the enzyme, e.g., the yeast carboxylase yields primarily (+) acetoin—and is used for the commercial production of mixed acyloins, i.e., biologically active *l*(+) ephedrine (297). The wheat germ carboxylase forms racemic acetoin with a slight excess of (+) isomer [72% (+), 28%(-)] (343).

Pyruvate as Acceptor. The bacterial acetoin forming system from *Aerobacter aerogenes* (27, 189) and *Streptococcus faecalis* (104) among other genera (189) forms acetoin through (+) α -acetylacetyl lactate (189) with pyruvate acting as acceptor (reaction 3, figure 11) (189). A manganese but not DPT-activated β -carboxylase, specific for the (+) isomer of α -acetylacetyl lactate, forms only (-) acetoin (104), through optical specificity of both the “condensing” and “decarboxylating” enzymes. The yeast type pyruvate decarboxylases form acetoin slowly from acetaldehyde alone, thus indicating the partial reversibility of the “aldehyde-DPT” + H⁺ \rightarrow acetaldehyde + DPT reaction (reaction 1, figure 11) (144, 343, 378).

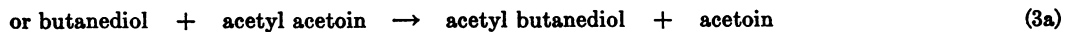
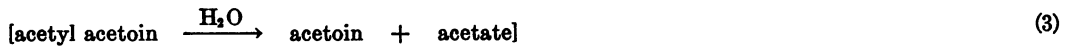
Diacetyl as Acceptor. Diacetyl was shown by Juni and Heym (190, 191) to accept “aldehyde” from “aldehyde-DPT” forming acetyl acetoin—also termed diacetyl methyl carbinol (DAMC). Diacetyl as both aldehyde donor and acceptor forms acetyl acetoin rapidly in an enzyme system studied first in *Aerobacter aerogenes* and in a *Corynebacterium* isolated by enrichment with acetoin as substrate (190, 191). More recently, this system has been found in other aerobic and facultative bacteria—i.e., *Pseudomonas fluorescens*, *Micrococcus lysodeikticus* (192, 193). Diacetyl is also a substrate for the oxidative pyruvate decarboxylases of pig heart (145, 193) and of *Streptococcus faecalis* (105) with some formation

of acetyl acetoin. With pyruvate as substrate, acetyl acetoin is formed if diacetyl is present in sufficient concentration to compete as acceptor for aldehyde (192, 336). The pig heart and *E. coli* systems also form α -acetylacetyl lactate (DL racemate) but lack acetylacetyl lactate decarboxylase and thus do not form acetoin from pyruvate alone (193).

The products of these pyruvate reactions—acetaldehyde, acetoin, acetylacetyl lactate—are reduced in the bacterial fermentations to ethanol, 2,3-butanediol, and acetylbutanediol (190). Complete reduction of acetylbutanediol and of α -acetylacetyl lactate to analogous tri- and dihydroxy alcohols has not been achieved. Reduction of acetoin to 2,3-butanediol is catalyzed by a pyridine nucleotide mediated dehydrogenase (92). The optical isomers of butanediol—2 asymmetric carbons—formed by various microorganisms have been only partially clarified (339, 357).

c. Acetate generation. Acetaldehyde, formed by yeast type decarboxylase in several species of the genus *Acetobacter*, is oxidized to acetate by both DPN- and TPN-specific dehydrogenases (326). This pathway can be considered as an alternate route to acetate formation. Other acetate generating mechanisms include the oxidation of the “aldehyde-DPT” formed by bacterial oxidative pyruvate decarboxylases by: (a) chemical electron acceptors, ferricyanide and 2,6-dichlorophenol indophenol, and (b) oxygen as acceptor *via* bacterial particles (282, 283, 329) (figure 11). These acceptors may or may not involve further enzymatic steps. A decrease in the rate of decarboxylation during purification of both the *E. coli* (153) and pig heart enzymes (336) measured in the presence of these electron acceptors as compared to the dismutation assay and Dolin's (106) recent separation of flavoproteins from *Streptococcus faecalis* with substrate specificity for these dyes indicate that enzymatic steps are involved. The oxidation systems are cytochrome mediated (cyanide sensitive) in both *Proteus vulgaris* and *E. coli* (329), but the nature of the coupling at the “aldehyde-DPT” level is unclear. Both *E. coli* and *P. vulgaris*, however, contain pyruvate decarboxylases—in the lipoic acid mediated fraction A—which do not connect to bacterial particles (329). The diacetyl cleavage system of *Corynebacterium* sp. and *Aerobacter aerogenes* also fails to link to the bacterial particles.

The diacetyl cleavage enzymes described by Juni are acetate generating (192):



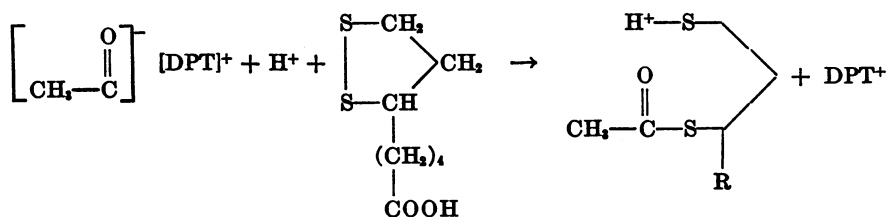
(Sum *via* Reactions
1 to 4 or 1, 2, 3a, 4, 5)

Both the initial cleavage and the subsequent hydrolyses—still tentative—generate acetate and appear to constitute an independent oxidative mechanism for generating acetate (192).

d. *Acetyl generation.* The pyruvate decarboxylases of *Escherichia coli*, *Proteus vulgaris* and *Streptococcus faecalis* have been measured in extracts by following the Krebs' dismutation reaction (217),



Intermediate steps require DPT, presumably forming "aldehyde-DPT" and lipoic acid. The reductive acylation of lipoic acid is suggested to occur by a mechanism similar to the acetoin condensation generating a free mercapto group and a thioester, rather than a hydroxyl and a carbon-carbon bond (152, 153, 345). The reductive acylation step has been visualized as:



If this, or a related formulation is correct, the new disulfide cofactor assumes importance in energy rich bond formation at the substrate level (152, 153, 345). Lipoic acid, through its sensitivity in the reduced state to trivalent arsenicals (314), is implicated in the brain (60), pig heart (329), *E. coli* (149) and *P. vulgaris* oxidative acyl generating pyruvate decarboxylases (329). In *S. faecalis* both trivalent arsenical sensitivity and activation of apopyru-

vate decarboxylase by addition of lipoic acid show its function (149).

The lipoic acid-mediated systems transfer the acyl group from acetyl lipoate to coenzyme A by a lipoic transacetylase (152) and thence to phosphate by phosphotransacetylase. The presence of lipoic transacetylase has been shown in the *E. coli*, *S. faecalis*, *P. vulgaris* and pig heart (152) pyruvate decarboxylase systems and shown to function before coenzyme A by the accumulation of stoichiometric amounts of acetyl lipoate in the absence of CoA (59). Acetyl lipoate is arsenolyzed in the presence of CoA, phosphotransacetylase and lipoic transacetylase. The slow ferricyanide oxidation of "aldehyde-DPT" (figure 11) is not lipoic or CoA dependent, but the more rapid oxidation by *S. faecalis* apopyruvate decarboxylase with ferricyanide as electron acceptor is lipoic dependent presumably by reoxidation of reduced lipoic through DPN (lipoic dehydrogenase) and a flavoprotein DPNH-

oxidase (106). In *S. faecalis*, the ferricyanide reaction rate equals the dismutation rate, whereas in *E. coli* the ferricyanide reaction is only about 1/1,000 as fast (154).

The *Lactobacillus delbrueckii* pyruvate oxidation system generates acyl phosphate (251) by still another mechanism requiring neither lipoic acid, CoA, nor presumably DPN (155). This system does require DPT and flavin adenine dinucleotide and is linked to a flavoprotein lactic

dehydrogenase—see reductive pyruvate reactions, section IV, 1.

The reactions of DPT, indicated at the right side of figure 11, are only part of the reactions in its role as an aldehyde acceptor—the transketolase and dicarboxyl cleavage enzymes, not shown, also involve formation of “aldehyde-DPT” complexes.

3. Clastic Reactions

Lipmann (253) equated three DPT-mediated bacterial pyruvate cleavage reactions through their formation of acetyl phosphate. Two of these were termed phosphoro- “clastic” through the demonstration with bacterial extracts of *Escherichia coli* (198, 386) and *Clostridium butylicum* (212) that phosphate incorporation conserves the bond energy as contrasted to hydrolysis (hydro- “clastic”) liberating approximately 15 kilocalories as heat. Even though knowledge is still fragmentary, and the terminology transient, these two reactions appear to differ from the pyruvate decarboxylases in yielding an “acetyl-” (carbonium ion) rather than an “aldehyde-” (carbanion). In addition, rapid equilibration with the carboxyl cleavage product, formate (387), or $\text{CO}_2 + \text{H}_2$ (424), differs from the slight CO_2 exchange by the decarboxylases (139, 306).

a. *Acetyl plus formate.* The colon-typhoid bacteria possess the formate producing clastic reaction. In extracts of *E. coli*, the forward and formate exchange reactions require inorganic phosphate, CoA, and DPT (57, 198, 366, 368). The acetyl fraction, however, does not equilibrate if added as acetyl phosphate even though it can be demonstrated as the ultimate product. Attempts to separate the enzymes and clarify the mechanism are still in progress (304).

b. *Acetyl plus CO_2 and H_2 .* The clostridia possess a clastic reaction forming CO_2 and hydrogen which provides the source of hydrogen among the fermentation products of this organism. Experiments with extracts of *Clostridium butyricum* (423) have demonstrated a DPT, CoA, inorganic phosphate, and Fe^{++} requirement for the forward reaction, yielding acetyl phosphate, CO_2 and H_2 . For C^{14}O_2 exchange, DPT, CoA, inorganic phosphate and Mn^{++} are required (424). The ferrous iron presumably functions in the hydrogen forming mechanism and is, therefore, not essential for C^{14}O_2 exchange. Substrate amounts of CoA replace the inorganic phosphate

requirement, thus indicating a terminal function for phosphate in acetyl phosphate formation (424).

The enzymes have not been separated, but fractions have been obtained in which the hydrogen liberating mechanism is lost and decarboxylation will occur if artificial electron acceptors, particularly furacin and neotetrazolium, are added. These, however, block the CO_2 exchange reaction, presumably by displacing the electron equilibrium.

4. Carboxylations

As has been already indicated, three carboxylation systems are known in bacteria: (a) *the malic enzyme* first discovered by Ochoa and co-workers in animal tissue (305) and studied in *Lactobacillus arabinosus* as an inducible enzyme (215); (b) *an ATP-activated carboxylation* of phosphoenolpyruvate or decarboxylation of phosphoenoloxalacetate—the Wood-Werkman reaction (figure 11); (c) *the formation and cleavage* of C_4 dicarboxylic acids *via* succinate forming or carboxylating propionyl CoA (307).

The equilibrium between malate plus DPN, and pyruvate, CO_2 plus DPNH favors decarboxylation but can be forced toward synthesis by high concentrations of both reduced pyridine nucleotide and carbon dioxide (305).

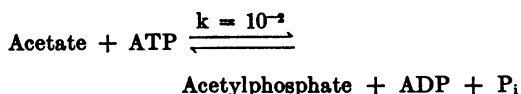
The pyruvate carboxylation *via* phosphoenolpyruvate to phosphoenoloxalacetate has been studied by carbon dioxide exchange reactions in chicken liver and in spinach by Utter *et al.* (390, 391, 392) and by Bandurski *et al.* (14, 15). The decarboxylation by the liver enzyme has been shown to be dependent upon catalytic amounts of ATP or ITP. The formation of phosphoenolpyruvate from pyruvate may occur through reductive carboxylation by malic enzyme, plus oxidation and phosphorylation to phosphoenoloxalacetate and decarboxylation to phosphoenolpyruvate (224).

The third carbon dioxide fixation reaction indicated above is not a reaction of pyruvate but of propionate. This mechanism involves the carboxylation of propionate to succinate and requires substrate amounts of ATP and cofactor amounts of CoA (90, 91, 421). Recent enzyme experiments by Flavin and Ochoa (307) have implicated methyl malonate as an intermediate between propionyl CoA and succinyl CoA.

5. Acyl Condensations and Reductions

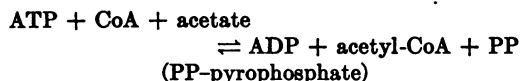
Free fatty acids arising *via* fermentation can form fermentation products of greater chain length and more reduced nature, or enter biosynthetic pathways (62), after activation by one of the three mechanisms described below. Such fatty acids, for example acetate, are formed during fermentation by: (a) oxidation of pyruvate to acetate *via* a so-called bypass mechanism (section IV, 2c); (b) hydrolytic action on thioester or acylphosphate formed by acyl forming pyruvate cleavage (section IV, 2d), *i.e.*, CoA-deacylase (325a) or acetylphosphatase (253); or (c) by transferring an energy rich link to other systems, *i.e.*, thioester linkage *via* CoA-transferases (356), or phosphate anhydride to ATP *via* acetokinase.

a. *Acid activations. Acetokinase* (199, 330a) which catalyzes the reaction



has been purified from *E. coli* and *Streptococcus hemolyticus*, shown to be activated by Mn^{++} or Mg^{++} , not to require CoA and is presumed to proceed by the reaction of both acetate and ATP with the enzyme since the exchange of either acetate- C^{14} or $\text{HP}^{32}\text{O}_4^-$ requires both, plus ADP. This enzyme, forming acetylphosphate, is found only in organisms which contain phosphotransacetylase and appears to furnish acetyl through formation of acetyl-CoA (326).

ATP-CoA-Acetate "Transferase" (254, 325a) catalyzes the reaction

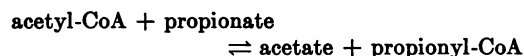


The enzyme has been studied in yeast and animal tissue (254) and is found in those aerobic bacteria devoid of phosphotransacetylase, *i.e.*, species of *Acetobacter* and *Pseudomonas* (326).

An analogous enzyme for succinate forms succinyl-CoA and P_i —though by a somewhat different mechanism (325a). Succinyl-CoA enzymes liberating succinate by deacylase (325a) and generating energy rich phosphate have also been studied (202a). These may play a role in fermentative succinate formation and in the propionic acid fermentations (91).

CoA-transphorase described by Stadtman (356)

equilibrates an acyl with a free acid, *i.e.*, acetyl with propionate, or as shown with acetate- C^{14} , acetyl with acetate as follows:



Delwiche (91) has presented data for a transphorase equilibrating succinyl and propionate in *Propionibacterium*, and Stadtman (356) and others for equilibration of acetyl with acids from propionate to caprylate as well as vinylacetate, among other acids.

Acetyl-CoA generated by these and other reactions can in microbial systems (a) be reduced to ethanol or (b) undergo condensations followed by reduction to form longer chain fatty acids and their alcohols.

b. *Reductions. Ethanol formation from acetyl-CoA* through DPN linked aldehyde dehydrogenase was described in *Clostridium kluveri* (44a). *Ethanol dehydrogenases*, both DPN and TPN linked, are known in a wide variety of bacteria (96a, 326, 335). In the absence of enzymatic data, the reductions of acetate- C^{14} to ethanol during glucose fermentation in species of *Leucomostoc* (150) and *Aerobacter indologenes* (344a) and to butanol in clostridia (427) are presumed to occur by related or similar reactions.

After condensations (see below) acetoacetyl-CoA is reduced *via* β -hydroxybutyryl-, crotonyl- or vinylacetyl-CoA to butyryl-CoA (19, 354). The latter is deacylated, hydrolytically or through CoA transphorase to yield butyrate (356) or is thought to be reduced to butanol by enzymes similar to the bacterial acetyl to ethanol system (352, 355, 427).

c. *Condensations.* Longer chain fatty acids are formed in *C. kluveri* under reducing conditions (excess of ethanol over acetate) (352) and in *C. saccharobutyricum* under reducing but not oxidizing conditions (8). Butyrate formation in both animal tissue (261a, 265) and bacterial preparations (356a) follows condensation *via* thioester catalyzing the reaction



Among the reactions of acetoacetyl-CoA, the reductions are considered under (b) above. Its deacylation forming free acetoacetic acid may precede acetone formation *via* a specific decarboxylase (84a, 341a). This is inferred from the data of Stadtman and others (356).

Summary

The reactions leading to products of carbohydrate metabolism by microorganisms *via* pyruvate or other intermediates of fermentation or oxidation pathways have been discussed insofar as enzymatic reactions for their formation are known or may be deduced with reasonable certainty. Often, the complete scheme even for known pathways is not documented for a single species or even for a single genus. In many more instances, the reaction mechanism is little understood, and pertinent data are fragmentary or unavailable. Reactions one or more steps beyond pyruvate, acetaldehyde, triosephosphates or other intermediates of carbohydrate fermentation or oxidation are, for the most part, omitted from the present discussion; for example, $\text{CO}_2 + \text{H}_2$ generation from formate by the hydrogenlyase of the the colon-aerogenes bacteria. Many further degradation and energy liberating reactions, and their relationship to biosynthetic pathways, remain to be clarified. Although in some instances, the reactants may be formed by the pathways of carbohydrate metabolism, and in still others the mechanisms may be similar to known reactions in glycolysis or other pathways, these are beyond the scope of the present effort.

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