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ALTERNATE PATHWAYS OF GLUCOSE AND FRUCTOSE METABOLISM*

By EFRAIM RACKER, *New Haven, Connecticut*

CONTENTS

I. Introduction

In the course of experimental studies on metabolic pathways investigators occasionally make observations which appear to be in-

* The following abbreviations have been used in the text and particularly in the tables and figures: ATP, ADP, and AMP for adenosine triphosphate, diphosphate, and monophosphate; DPN and TPN for di- and triphosphopyridine nucleotide; Thpp for thiamine pyrophosphate; G-1-p for glucose-1-phosphate; Gal-1-p for galactose-1-phosphate; F-1-p for fructose-1-phosphate, etc.; HDP or F-1,6-p for fructose-1,6-diphosphate and G-1,6-p for glucose-1,6-diphosphate; IAA for iodoacetic acid; DNP for dinitrophenol; P_i for inorganic phosphate. Other abbreviations are indicated in the text or in the tables.

consistent with currently held views. In response to such an event the investigator is likely to follow one of several courses. He may consider himself the discoverer of a new metabolic pathway and present his observations in print in line with this view. On the other hand he may consider the possibility that this reasoning is fallacious and may wish to assemble additional facts to explain his observations. If he is an experienced investigator he may even think of the possibility of an experimental artifact and reattack the problem using other methods and tools. Many examples of such "pathways of short duration" can be quoted. Most errors have resulted from an emphasis on a single approach, *e.g.*, the use of inhibitors, or the demonstration of the presence of an enzyme. For example, numerous papers on nonphosphorylating glycolysis in animal tissues were published during the past two decades in spite of Meyerhof's reiterations that nonphosphorylating glycolysis is nonexistent. Some of the experimental facts which emerged from these studies, such as the inhibitory effect of glyceraldehyde on glycolysis, stimulated further work on this interesting phenomenon. Experiments on intact tissues using P^{32} at first seemed to contradict the concepts derived from studies on fermenting tissue extracts, but later served to reaffirm them and strengthened the view that phosphorylating glycolysis also occurs within the cell structure.

In a frequently quoted paper (1), experiments were reported which showed that in some animal tissues, *e.g.*, muscle, anaerobic glycolysis is inhibited at low iodoacetate (IAA) concentrations while glucose oxidation is not. From this it was concluded that there must be an oxidative pathway which does not proceed through the same enzymic mechanism which degrades glucose to lactic acid under anaerobic conditions. Although not justified on the basis of these experiments the conclusion of an alternate pathway of glucose oxidation in animal tissues was proved correct by subsequent work. Actually, in muscle tissue, where the discrepancy between the aerobic and anaerobic inhibition by IAA is quite pronounced, the alternate pathway of glucose oxidation is not very active (2,169). The assumption that one can prove or disprove the existence of a pathway by the use of inhibitors has lost considerable ground in recent years. Studies of multienzyme systems (3) have given us a deeper insight into the complexities of reactions proceeding in an open system in which any one of many enzymes may be limiting and in which a feed-back mechanism of re-

generated cofactors exists. It has been demonstrated (4), that, in a glycolytic system in which the regeneration of ATP from ADP is the limiting step, and dependent to a large extent on triose phosphate oxidation, a slight inhibition of triose phosphate dehydrogenase may result in a very pronounced inhibition of glucose phosphorylation. Reactions that regenerate ATP (*e.g.*, phosphocreatine and its transphosphorylase) or the addition of triose phosphate dehydrogenase restore glycolysis to such a system. During aerobic oxidations large amounts of ATP are formed and glucose phosphorylation is independent of the minor contribution of triose phosphate dehydrogenation. Under anaerobic conditions, particularly in the presence of ATP-consuming enzymes such as ATPase, the regeneration of ATP is often the limiting step. Then a slight decrease in the rate of its formation may deplete the system of ATP to such an extent that glucose phosphorylation ceases.

In the case of IAA another factor comes into play which helps to explain its more pronounced inhibitory action under anaerobic conditions. It was recently shown (5) that the inhibition of fermentation by IAA can be diminished by exposing the yeast to air prior to the addition of the inhibitor. This fact has been attributed to the somewhat higher concentration of glyceraldehyde-3-phosphate, which can be demonstrated to accumulate within the cells under aerobic conditions. This explanation is supported by the observation made in several laboratories (5,6) that glyceraldehyde-3-phosphate dehydrogenase is markedly protected in the presence of 3-phosphoglyceraldehyde against the inhibitory action of IAA.

It should be apparent from the above considerations that several explanations can be given for the failure of an inhibitor to act as expected, and the existence of an alternate pathway should not be inferred without more direct evidence. Nor does the demonstration of enzymes or of intermediates necessarily prove their participation in a particular metabolic pathway, as demonstrated by the classical example of glyoxalase, which catalyzes the formation of lactic acid from methylglyoxal. Although the enzyme has been detected in a wide variety of tissues and microorganisms, and in spite of the isolation of methylglyoxal which accumulates even in intact cells under certain conditions (7,116), it has been shown conclusively that the glyoxalase reaction does not participate in the formation of lactic acid from glycogen (8).

Studies with the aid of isotopes have been invaluable in the elucida-

tion of enzyme mechanisms as well as in providing new concepts of metabolic pathways. However, difficulties often arise in the interpretation of data based solely on isotope studies, either because of the occurrence of exchange reactions or because of the lack of equilibration. For example, a rapid CO_2 exchange can be demonstrated in the presence of oxalacetate and an enzyme preparation from *Micrococcus lysodeicticus* (*cf.* 9,10), but there can be little doubt that this reaction plays a minor role at best in the actual fixation of CO_2 and in the net synthesis of dicarboxylic acids. Other enzyme mechanisms which fulfill this function will be discussed later.

It is somewhat surprising to find that phosphoglyceric acid is becoming generally accepted as an early intermediate in photosynthesis without an unequivocal demonstration of its net synthesis. It is gratifying to see objections raised by investigators with extensive experience in exchange reactions (10). Nor have these objections been met by kinetic studies (11).

These introductory remarks are not intended to disparage any of the methods used in the study of intermediary metabolism. The important role played by inhibitors such as NaF or IAA in the elucidation of the glycolytic pathway is part of biochemical history. The work with isotopes has undoubtedly revolutionized our biochemical thinking and opened new roads into the wilderness of the metabolic jungle. Nor should one quarrel with the analytical approach of the enzymologist, who wants to become familiar with the fundamental parts before investigating the whole machinery of cellular metabolism. However, emphasis should be laid on a multiple approach and a pooling of the information obtained *in vivo* and with tissue preparations as well as with isolated enzyme systems. The data on the formation and utilization of intermediates, the distribution of the isotope, and the rate of the enzymic reactions should all be assembled to help evaluate the significance of a postulated pathway.

With few exceptions, the work on alternate metabolic pathways has been of a qualitative nature. Most of the attempts to apply a quantitative approach to the relative role of alternate pathways have remained fragmentary and were limited by the scarcity of appropriate methods. The use of inhibitors such as KCN has helped to assess the importance of the cytochrome system in terminal respiration. The lack of specificity of this inhibitor, however, has considerably diminished the significance of the findings. Labeling with isotopes has been

successfully used in a few attempts at quantitative evaluation. Most recently a new approach has been made by Britton Chance in an attempt to throw light through the cloudiness of the intact cell and to measure the metabolic reactions spectrophotometrically (12).

During the past few years a large number of new enzymic reactions have been described in the field of carbohydrate metabolism. In some instances the physiological role of these reactions is apparent, in others it is not. In the following pages the experimental material on these reactions will be presented and subsequently an attempt will be made to integrate these observations with experiments *in vivo* and to evaluate our present state of knowledge on alternate pathways of carbohydrate metabolism.

II. Hexoses (see Table I)

A. PHOSPHORYLATIONS

The enzymes which catalyze the transfer of phosphate from ATP to carbon 1 or 6 of hexoses have been referred to in the literature as

TABLE I

ENZYMES REACTING WITH FREE SUGARS

Enzyme (source)	Cofactor	Substrate	Product	Remarks
1. C-6-Hexokinase (yeast and animal tissues)	ATP Mg^{++}	Glucose, fructose, mannose, glucosamine, desoxyglucose	Hexose-6-p	Acts on fructofuranose
2. C-6-Glucokinase (animal tissues and bacteria)	ATP Mg^{++}	Glucose	G-6-p	Primary product not established in all instances
3. C-1-Fructokinase (liver, muscle)	ATP Mg^{++}	Fructose	F-1-p	Substrate affinity high in liver, low in muscle
4. C-1-Galactokinase (yeast, animal tissues)	ATP Mg^{++}	Galactose	Gal-1-p	Product in intestine not identified
5. Glucose oxidase (molds)	FAD	β-Glucose	δ-gluconolactone	Other sugars oxidized very slowly
6. Glucose dehydrogenase (liver)	DPN TPN	β-Glucose, xylose	δ-Gluconolactone	Other sugars oxidized very slowly
7. Mutarotase (molds)	—	α-Glucose	β-Glucose	
8. Sorbitol dehydrogenase (liver, kidney)	DPN	Fructose, sorbose	Sorbitol, iditol	

hexokinases. In view of the increasing number of different enzymes which fall into this category, a more descriptive nomenclature should be applied when necessary. In the case of enzymes which phosphorylate the free sugars the carbon atom at which this process takes place should be included in the name. In the case of enzymes which act on phosphorylated hexoses it is only necessary to specify the substrate since no phosphorylation reactions other than those concerning carbon 1 or 6 have been described to date.

1. C-6-Hexokinases

Many enzyme preparations which catalyze the phosphate transfer from ATP to carbon 6 of hexoses and which are activated by Mg^{++} or Mn^{++} have been described. The best characterized of these enzymes is that obtained from yeast (13,14) which phosphorylates glucose, mannose, and fructose, as well as glucosamine (15) and 2-desoxyglucose (16). Glucose has a lower K_m value (1.5×10^{-4}) than has fructose (1.5×10^{-3}) and at equal concentrations of the hexoses the competition is such that fructose phosphorylation is nearly completely inhibited (17). An enzyme with a substrate specificity similar to that of yeast is present in animal tissues (17) and tumors (18). There are, however, a number of striking differences between the yeast and the animal enzymes. Of these the most interesting are the susceptibility of some of the animal hexokinases to the inhibitory action of the pituitary factor which is counteracted by insulin (19), and the inhibition of animal hexokinases by glucose-6-phosphate (20,21). Differences between hexokinases from different animal tissues have also been noted. For example, hexokinase from brain and several other tissues was found to be readily sedimented at $18{,}000 \times g$, while the enzyme present in the red blood cells is not (21). The interesting observation that the phosphorylation of glucose by brain hexokinase is markedly stimulated by a non-dialyzable factor from red blood cell hemolyzates (20) requires further elucidation. Several possibilities of explaining this phenomenon have been ruled out by partial purification and by heating the factor for 5 minutes at 80°, thus eliminating phosphohexokinase, hexokinase, myokinase, and triose phosphate dehydrogenase present in crude red blood cell lysates.

An enzyme which acts specifically on glucose has been found in animal tissues (17), in microorganisms (22), and in schistosomes (23).

Homogenates of female schistosomes phosphorylate only glucose while the male attacks fructose and mannose as well. In a mutant of *Pseudomonas putrifaciens* an adaptive and specific glucokinase has been found (24). In most of these cases the glucokinase reaction was tested in rather crude preparations and proof for the direct phosphorylation at carbon 6 was not presented. Since enzymes which catalyze the phosphorylation of hexoses at carbon 1 are known to exist and glucose-1-phosphate is readily transformed into glucose-6-phosphate in most extracts, a demonstration of glucose-6-phosphate formation in the absence of phosphoglucomutase activity should be required to establish the mode of action of these enzymes.

2. C-1-Hexokinases

Enzymes which phosphorylate carbon 1 in the presence of Mg^{++} have been described for fructose and galactose. No enzyme of this type has been demonstrated with glucose as substrate, but it is clear from the above discussion that such an enzyme might be easily overlooked in view of the wide distribution of phosphoglucomutase which would transform 95% of the glucose-1-phosphate into glucose-6-phosphate. The inhibition of the mutase reaction by arsenate (25) should be of considerable value in further studies of this kind.

C-1-Fructokinase (26,27) has been purified from beef liver (28,29). In addition to fructose, which is phosphorylated to fructose-1-phosphate, the enzyme preparation phosphorylates sorbose but the product of this reaction has not been characterized. The K_m of fructose with this enzyme (K_m is 5×10^{-4} M) is considerably lower than with C-6-hexokinase. In contrast to the liver enzyme, the fructokinase from rabbit muscle (17) could not be saturated by high substrate concentrations. Studies on the muscle enzyme were hampered by the presence of a second enzyme which rapidly phosphorylated the product further to fructose-1,6-diphosphate, but recently fructose-1-phosphate was identified as the primary product in the presence of 1 M fructose (30).

C-1-Galactokinase. It has been observed that yeast (31–33), bacteria (22), and animal tissues (34–38) catalyze the phosphorylation of galactose. The enzyme preparation from liver acts also on galactosamine (*cf.* 38). Galactose-1-phosphate has been isolated as the product of liver phosphorylation (39), but has not been established as the product of phosphorylation by intestinal tissue (34,36,37).

The presence of galactose phosphorylation in the intestine is of particular interest in connection with the problem of absorption, since galactose is absorbed faster than any other sugar and is not phosphorylated by C-6-hexokinase. A galactokinase reaction in brain (*cf.* 38) may be of significance in relation to the extensive incorporation of galactose into brain tissue.

B. OXIDATION-REDUCTIONS

The oxidation of glucose to gluconic acid is catalyzed by enzymes of considerable specificity, which have been employed for the quantitative determination of glucose. There are at least two types of enzymes belonging to this class, one a flavoprotein, the other a nucleotide-linked dehydrogenase.

1. Glucose Oxidase (Notatin, Penicillin B)

Glucose oxidase, which is widely distributed in molds, contains FAD as the prosthetic group (*cf.* 40). The enzyme acts specifically on β-D-glucopyranose. The rate with α-glucose, xylose, mannose, 6-methylglucose, and 4,6-dimethylglucose is about 1% of that with β-glucose, other sugars such as galactose being oxidized at a still slower rate. The K_m for β-glucose is about 6×10^{-3} and no inhibition by α-glucose was noted. The mechanism of action of this enzyme was investigated (41) and two reactions were observed, the first leading to an uptake of oxygen, the second to a liberation of an acidic group. This, together with polarimetric observations, indicated the formation of δ-gluconolactone as an intermediate. O_2^{18} was incorporated into the hydrogen peroxide which is formed during the reaction, and no exchange reaction occurred between H_2O^{18} and O_2^{16}. The use of "glucose oxidase" as the name of the enzyme should not imply that oxygen oxidizes the substrate directly, a mechanism clearly ruled out by the experiments with O^{18} (41), but rather should describe the ability of this enzyme to transfer the hydrogen from the substrate to molecular oxygen.

2. Glucose Dehydrogenase

A mechanism similar to that discussed above has been shown to operate in the case of the nucleotide linked enzyme purified from beef liver (42). With glucose as substrate and with either DPN or TPN as cofactor the reaction was shown to proceed as follows:

(1) Glucose + $DPN^+ \rightleftharpoons$ δ-gluconolactone + DPNH + H^+

(2) δ-Gluconolactone + $H_2O \rightleftharpoons$ gluconic acid

While the first step is enzymically catalyzed and readily reversible (K_{eq} = 15 at 30°, pH 6.7), the second step is nonenzymic and not readily reversible. From this equilibrium value the E_0' value for the glucose–gluconate system was calculated to be −0.44 volt. Purified glucose dehydrogenase preparations oxidized xylose one-fourth as fast as glucose. Arabinose and galactose reacted at about 4% the rate of glucose, while other sugars tested were inactive. Since crude enzyme preparations oxidized galactose and arabinose at about 20% of the rate for glucose, the existence of another oxidizing enzyme for these two substrates, or a transformation to a rapidly oxidizable sugar, must be assumed. As in the case of glucose oxidase, β-glucose appears to be the specific substrate since α-glucose is oxidized much more slowly and only after a lag period. At pH 7.6, the K_m for glucose is 0.07 M, and at lower pH values the K_m values tend to increase. The fact that glucose and xylose are the most reactive substrates for this enzyme points to the importance of the stereochemistry of the first four carbons for the combination of the substrate with the enzyme. Since ribose is not oxidized, the stereochemical configuration about carbon 3 seems determinative. A broader survey of the specificity of this dehydrogenase reaction and a comparison with that of glucose oxidase would be of considerable interest for a better understanding of the substrate–enzyme interaction.

Although a slight degree of reversibility of the over-all reaction has been actually demonstrated at pH 4.1 in the presence of high gluconic acid concentrations, it appears unlikely that the direct reduction of an acid to an aldehyde plays an important role under physiological conditions. Gluconate-1-C^{14} and uniform gluconate-C^{14} were found to have been utilized for glucose synthesis, when administered to rats (43,44). Estimates on the relative role of alternate pathways which participate in this reaction revealed that less than 1% of the glucose formed from gluconate could have arisen by direct reduction; about 10% came from the CO_2 derived from C-1 and about 90% involved steps following the loss of carbon 1. These findings are readily explained by the glucose-6-phosphate oxidation cycle, which seems to play a major role in liver metabolism and will be discussed in detail later. Somewhat surprising is the finding that there was no dilution

of the excreted gluconate. This should have occurred if the reaction catalyzed by glucose dehydrogenase proceeds *in vivo* at an appreciable rate and if a miscible pool of gluconic acid exists.

3. Reduction of Hexoses

The oxidation of polyhydric alcohols to keto sugars by bacteria has been known for many years and has been used for preparative purposes as well as for elucidation of the structure of sugars. The formation of polyhydric alcohols in plants and bacteria was also recognized in the last century. In neither case is there any information available on the enzymic mechanism of these reactions.

Sorbitol Dehydrogenase. Liver tissue was found to contain an enzyme which catalyzes the reversible reaction:

$$\text{D-sorbitol} + \text{DPN}^+ \rightleftharpoons \text{D-fructose} + \text{DPNH} + \text{H}^+$$

L-iditol and L-sorbose also were found to be substrates when tested with partially purified enzyme preparations. About 10-fold purification of the enzyme from rat liver was achieved. The K_m for sorbitol is 7×10^{-4} and K_{eq} is 0.24 at 20° and pH 8.0 (45). This study, which represents a considerable advance in our knowledge of the oxidation of polyalcohols, has clarified a number of points in regard to sorbitol metabolism in animal tissues. For the discussion of the older literature and of the antiketogenic effect of sorbitol the original paper should be consulted. The action of the sorbitol dehydrogenase may serve to explain the results obtained with C^{14}-labeled sorbitol and glucose administered to rats. Based on the findings that sorbitol is more extensively oxidized to CO_2 and less effectively incorporated into glycogen than is glucose, Stetten and Stetten (46) make the suggestion that sorbitol enters the glycolytic cycle at some point below glucose-6-phosphate. In view of the existence of sorbitol dehydrogenase in rat liver which forms fructose from sorbitol, a comparative study with labeled fructose would be of interest.

C. MUTAROTATIONS

The first demonstration of an enzyme which catalyzes a mutarotation reaction was made independently in two laboratories (41,47). A highly purified preparation of glucose oxidase was found to oxidize only β-glucose (correcting for spontaneous mutarotation), while other preparations oxidized α-glucose as well. A contaminant was found

in the cruder preparations which accelerates the mutarotation. This heat-labile and nondialyzable factor was called mutarotase. The enzyme is strongly inhibited by excess of glucose and other carbohydrates (while glucose oxidase is not) and is more resistant to alkali than the oxidase, so that a partial separation of the mutarotase from the oxidase could be accomplished.

III. Hexose Monophosphates

A. PHOSPHORYLATIONS AND DEPHOSPHORYLATIONS (see Table II)

1. Fructose-6-phosphate Kinase

This enzyme catalyzes the formation of fructose-1,6-diphosphate from fructose-6-phosphate in the presence of ATP and Mg^{++}. It was purified from rabbit muscle (48,49) and from plants (50) and also was studied in brain tissue (4,51,52). It shows an extreme acid lability and can be inactivated by short exposure at pH 6.0. In some instances the enzyme which is precipitated at that pH can be reactivated with ammonium phosphate or certain other salts (53). The lability of this enzyme might well account for some of the difficulties encountered in its study. On the other hand this rather unusual property can be used for the design of experiments on glycolysis when it is desired to interrupt the Meyerhof-Embden pathway at the step of fructose-6-phosphate phosphorylation. After incubation at pH 6.0 for 10 minutes at 37° a mouse brain homogenate is no longer capable of glycolysis but can be reactivated by the addition of purified

TABLE II

ENZYMES CATALYZING PHOSPHORYLATION OF HEXOSE MONOPHOSPHATE

Enzyme (source)	Co-factor	Sub-strate	Product	Remarks
1. F-6-p kinase (rabbit muscle)	ATP Mg^{++}	F-6-p	F-1,6-p	Unstable at acid pH
2. F-1-p kinase (rabbit muscle)	ATP Mg^{++}	F-1-p	F-1,6-p	
3. G-1-p kinase (muscle, yeast)	ATP Mg^{++}	G-1-p	G-1,6-p	Reaction with other 1-phosphate esters not investigated; muscle enzyme does not act on glucose
4. G-1-p transphosphorylase	—	G-1-p	Glucose + glucose-1,6-p	Possibly due to phosphatase

fructose-6-phosphate kinase (4). Such an approach might lend itself to a study of alternate pathways of hexose monophosphate metabolism.

2. Fructose-1-phosphate Kinase

An enzyme which catalyzes the phosphorylation of fructose-1-phosphate to fructose-1,6-diphosphate has been found in rabbit muscle extracts and is precipitated between 41–50% saturation of ammonium sulfate (17).

3. Glucose-1-phosphate Kinase

An enzyme which in the presence of ATP and Mg^{++} phosphorylates glucose-1-phosphate to glucose-1,6-diphosphate is present in yeast and in animal and plant tissues (54,55). When prepared from rabbit muscle extracts by precipitation at 50% saturation of ammonium sulfate this enzyme is free of C-6-glucokinase activity. It was also purified from yeast and a partial separation from C-6-hexokinase was obtained. No data were reported on the phosphorylation of fructose-1-phosphate by these preparations although this would be of interest in view of the presence of a kinase for this substrate in similar ammonium sulfate fractions of rabbit muscle (17). The specificity of enzymes which phosphorylate hexoses at carbon 1 remains to be investigated.

4. Glucose-1-phosphate Transphosphorylase

An enzyme preparation from *Escherichia coli* has been obtained which forms glucose-1,6-diphosphate from glucose-1-phosphate without the participation of ATP (56). The reaction was formulated as:

$$2 \text{ glucose-1-phosphate} \longrightarrow \text{glucose-1,6-diphosphate} + \text{glucose}$$

A reaction of this type has been proposed by Lipmann (57) on theoretical grounds to explain the high yield of available energy during muscle glycolysis. However, the enzyme preparation from *E. coli* contains phosphatase and attempts to remove this activity have not as yet been successful. Moreover, purified preparations of alkaline phosphatase were also found to catalyze the formation of glucose-1,6-diphosphate from glucose-1-phosphate though with lower yields (58). It is not very likely that a phosphatase-catalyzed reaction could preserve energy during muscle glycolysis unless a complete separation

of the phosphate transfer reaction from ester hydrolysis can be achieved within the cell. A systematic search into the optimal conditions for the phosphate transfer reaction (59) should be rewarding and might permit a functional separation from hydrolysis, similar to that demonstrated for proteolytic enzymes (60). In view of the nonspecificity of these transfer reactions (61), fructose-1,6-diphosphate might be formed by this mechanism and with glycogen as substrate an apparent high energy yield of glycolysis would result.

5. Glucose-6-phosphatase and Fructose-1-phosphatase

A specific enzyme which hydrolyzes glucose-6-phosphate is present in liver and is found to be deficient in liver homogenates of some cases of von Gierke's disease (62). The enzyme plays an important role in the glycogen–glucose shunt in liver in which glycolysis appears to play a minor role. Another phosphatase which catalyzes the hydrolysis of the C-1-ester of fructose-1-phosphate and fructose-1,6-diphosphate (63,64) probably plays a similar role in the diversion of phosphorylated hexoses to the free sugars in liver. Thus a glucose-6-phosphate molecule which has escaped the action of its phosphatase and has been converted to fructose-1,6-diphosphate may still be returned to the glucose pool through the combined action of fructose-1-phosphatase, hexose phosphate isomerase, and glucose-6-phosphatase. The role of the fructose-1-phosphatase in the interconversion of hexose monophosphates will be reviewed later.

B. OXIDATION-REDUCTIONS

1. Glucose-6-phosphate Oxidation Shunt

The early history of the discovery of this pathway has been reviewed often and need not be repeated (65–68). After the pioneer studies of Warburg, Lipmann, Dickens, and Dische (65,69–72) little attention was paid to this pathway for many years. Then, the demonstration of the shunt mechanism in *E. coli* (73), the identification of ribose-5-phosphate as an oxidation product (74), and the recognition of ribulose-5-phosphate as the primary pentose phosphate formed by the oxidative decarboxylation of phosphogluconate (75) followed in rapid succession. At the same time the enzymes which catalyze the breakdown of ribose phosphate and ribulose phosphate were studied. Preparations from *E. coli* (73), yeast and liver (76–79), blood hemoly-

zates (72), and plants (80) were found to form triose phosphate from ribose phosphate, but glycolaldehyde did not accumulate in any of the systems studied and the fate of the two-carbon fragment was not clear. Other products were found to accumulate under specific experimental conditions and were identified as hexose monophosphate and diphosphate (72,81,82), heptulose monophosphate and diphosphate (78,79,83,84), and ribulose diphosphate (84). When pentose phosphate or another source of triose phosphate and free aldehydes were added to crude enzyme preparations, new products were formed.

TABLE III

ENZYMES OF THE GLUCOSE-6-PHOSPHATE OXIDATION SHUNT

Enzyme (source)	Cofactor	Substrate	Product	Remarks
1. G-6-p dehydrogenase (animals, microorganism, plants)	TPN	G-6-p	Gluconate-6-p	DPN-linked bacterial dehydrogenase reported
2. Gluconate-6-phosphate dehydrogenase (animals, plants, microorganisms)	TPN	Gluconate-6-p	Ribulose-5-p + CO_2	DPN-linked dehydrogenase reported with unidentified products
3. Pentosephosphate isomerase (animals, plants, microorganisms)	—	Ribulose-5-p	Ribose-5-p	SH enzyme
4. Transketolase (animals, plants, microorganisms)	Thpp Mg^{++}	Ribulose-5-p + hydroxyaldehyde	Triose-p + keto sugar	Specificity not established
5. Transaldolase (animals microorganisms)	?	Heptulose-p + triose-p	F-6-p + tetrose-p	Specificity not established

Thus, with glycolaldehyde, xylulose-1-phosphate (73,85,86) was shown to accumulate; with acetaldehyde, desoxyribose-5-phosphate (76); with D- and L-glyceraldehyde, fructose-1-phosphate and sorbose-1-phosphate, respectively (87); with erythrose, sedoheptulose-1-phosphate (78,88); and with DL-lactaldehyde, 2-desoxyketohexoses (89). These findings emphasized the complexity of the reactions involved and made a purification and separation of the enzymes imperative. A partial separation has been achieved (90) and one of the enzymes has been crystallized from yeast (91). The main reactions which participate in the metabolism of pentose phosphates have been recognized as transketolizations and transaldolizations

(82,91,92) and have served to explain the negative findings in regard to glycolaldehyde accumulation.

For enzymes of the glucose-6-phosphate oxidation shunt see Table III and the following sections.

Glucose-6-phosphate Dehydrogenase. A useful procedure for the purification of this enzyme has been published (93). A very active preparation can be obtained by this method if a yeast strain with a high dehydrogenase content is used as a starting material (94). The enzyme has become an important reagent on the deep-freeze shelf of the enzymologist. It has been used in many laboratories for the quantitative determination of ATP, ADP, TPN, glucose-6-phosphate, and fructose-6-phosphate, as well as for assays of hexokinase, phosphohexose isomerase, and glutathione reductase. Since some of these enzymes are still found as contaminants of purified glucose-6-phosphate dehydrogenase preparations, it is often essential to remove a particular protein contaminant by further fractionation. This can usually be accomplished by a stepwise isoelectric fractionation of the enzyme in the presence of nucleic acid (95). Glucose-6-phosphate dehydrogenase from yeast (93) and *E. coli* (73,96) react specifically with TPN as coenzyme, while a DPN-linked dehydrogenase has been found in some bacterial species (97,98).

The mechanism of action of glucose-6-phosphate dehydrogenase has been investigated (99). These studies indicate that a phosphogluconolactone is the product of the oxidative step which is followed by hydrolysis of the lactone to phosphogluconic acid. A reduction of the δ-lactone of phosphogluconate with TPNH was recently demonstrated to be catalyzed by the enzyme, which did not react with the γ-lactone (68). Thus it is clear that, as in the case of the free sugar, the glucopyranose form is the substrate which is dehydrogenated to the corresponding lactone. An enzyme which catalyzes the hydrolysis of the δ-lactone of 3,5-diketohexanoic acid has been described (100) and the possibility of an enzyme-catalyzed phosphorolytic cleavage of lactones should be considered. In the case of glucose or its phosphate ester this process would result in the formation of gluconyl phosphate, which might be utilized for the production of ATP. This process would bear some similarity to the oxidation of other aldehydes and keto acids which involve a thiol ester as an intermediate. Such a mechanism would be particularly attractive from the point of view of energy utilization and would also facilitate the

reversibility of the over-all reaction. It may be advanced to join other theories proposed to explain some aspects of carbohydrate synthesis in photosynthesis.

Phosphogluconic Acid Dehydrogenase. This enzyme has been purified from brewers' yeast (101) and from *E. coli* (73,96). The K_m for both coenzyme and substrate is quite low (5×10^{-5}). In yeast and *E. coli* TPN is the coenzyme, while in some bacterial species a DPN-linked reaction has been demonstrated (97,98).

An important contribution to the mechanism of action of this enzyme has been made by Horecker and his collaborators with the identification of ribulose-5-phosphate as the product of the reaction and the demonstration of reversibility (*cf.* 68). The over-all reaction:

$$\text{6-phosphogluconate} + \text{TPN}^+ \rightleftharpoons \text{ribulose-5-phosphate} + CO_2 + \text{TPNH} + H^+$$

proceeds most probably through a 3-ketophosphogluconate as an intermediate. Although no direct evidence for its formation is available several similarities of the reaction with other oxidative decarboxylations of hydroxy acids support this concept. In common with the enzymic decarboxylation of malic and isocitric acids (102), phosphogluconic acid oxidation is activated by Mg^{++} or Mn^{++}, is readily reversible, and forms a keto compound as the end product. In extending the analogy one might expect the proposed keto acid intermediate to be decarboxylated by the purified dehydrogenase and studies on the metabolism of this compound (provided it can be prepared) would be of considerable interest. Horecker's theory of the oxidation of phosphogluconate has been challenged by Uehara (103a), who reverts to the older hypothesis of 2-ketophosphogluconic acid as the intermediate. This compound is suggested to undergo a cleavage to hydroxypyruvate and triose phosphate, followed by a recondensation between the decarboxylated hydroxypyruvate and triose phosphate to form ribulose-5-phosphate (103b). This alternative hypothesis would be quite attractive were it not for the experiments which demonstrated considerable CO_2 fixation into phosphogluconate (68). Decarboxylations of α-keto acids such as hydroxypyruvic or pyruvic acid are not readily reversed and incorporation of $C^{14}O_2$ into the carboxyl group is usually very small and often not detectable. The theory of Uehara can readily be tested experimentally since it includes a condensation between triose phosphate and active aldehyde, a reac-

tion which has been shown to require cocarboxylase (91,92). Removal of this coenzyme from purified phosphogluconic dehydrogenase should permit a decisive test of the proposed mechanism. Although it does not seem likely that this mechanism operates in ribulose-5-phosphate formation the suggested oxidation and cleavage of phosphogluconate may occur in some microorganisms such as *Pseudomonas fluorescens* and *Leuconostoc mesenteroides* (97,98). Data obtained with extracts of these bacteria and with partially purified DPN-linked phosphogluconic dehydrogenase reveal a somewhat different enzyme pattern of the shunt mechanism as compared to that of *E. coli* or yeast. It will be necessary, however, to demonstrate both the formation and cleavage of a ketophosphogluconic acid in these preparations, since the available evidence is still indirect and subject to alternate interpretations.

2-Ketogluconic and 5-ketogluconic acids are known to be glucose oxidation products in bacteria, but 3-phosphogluconic acid has not as yet been detected. It has been suggested (104) that a recently discovered ketogluconic acid (105) may be 3-ketogluconic acid, which would represent a suitable intermediate of kojic acid biosynthesis. However, it appears more likely that the new ketogluconic acid is identical with 2,5-diketogluconic acid (106).

Phosphopentose Isomerase. This enzyme catalyzes the reversible interconversion of ribulose-5-phosphate to ribose-5-phosphate. At equilibrium about 80% of the mixture consists of ribose-5-phosphate (75). The enzyme has been extensively purified from plants (80a) and was found to be very susceptible to inhibitors which react with sulfhydryl groups. The activity of this enzyme can be followed by the appearance of keto sugar, as measured by the carbazole reaction (107), as well as by a convenient spectrophotometric method which was recently developed (108). Since the product formed from phosphogluconate is ribulose-5-phosphate, phosphopentose isomerase is required for the biosynthesis of ribose-5-phosphate, an important constituent of nucleic acid and several nucleotide coenzymes. The enzyme also plays a role in the formation of heptulose phosphate which will be discussed later.

Transketolase. This enzyme has been shown to split ribulose-5-phosphate to triose phosphate and an "active glycolaldehyde." Active aldehyde is also formed by decarboxylation of hydroxypyruvate or from erythrulose, or heptulose phosphate. The active

aldehyde is condensed with an acceptor aldehyde (glycolaldehyde, glyceraldehyde, glyceraldehyde-3-phosphate, ribose-5-phosphate, desoxyribose-5-phosphate) by a ketol condensation to form the corresponding keto sugar:

$$\begin{matrix} R\text{—}CHOH\text{—}CHO \\ + \\ [CHO\text{—}CH_2OH] \end{matrix} \rightleftharpoons RCHOH\text{—}CHOH\text{—}CO\text{—}CH_2OH$$

Transketolase has been crystallized from bakers' yeast (91) and very active preparations were obtained from spinach (92). The enzyme preparations from both sources were shown to require cocarboxylase for activity. In this respect the reaction resembles the acetoin condensation which is catalyzed by purified carboxylase (109,110). In contrast to carboxylase, transketolase does not decarboxylate pyruvic acid, nor does acetaldehyde act as acceptor aldehyde (*cf.* 111). Thus both aldehyde donor as well as the acceptor are different in the case of transketolase, which seems to require the presence of an alcohol group as well as an aldehyde group in its substrates.

Since ribose-5-phosphate can act as acceptor aldehyde it was essential for the elucidation of transketolase action to remove contaminating phosphopentose isomerase. This has been accomplished by extensive purification and by repeated recrystallizations of the enzyme. It was then possible to establish clearly which substrates act as donors and which as acceptor aldehydes. It has been shown that yeast transketolase also catalyzes the decarboxylation of hydroxypyruvate, provided a suitable acceptor aldehyde is present. With glyceraldehyde-3-phosphate as acceptor the formation of a compound which appears to be ribulose-5-phosphate was reported (91). Preparations of transketolase from spinach were shown to form a mixture of pentose phosphates and heptulose phosphates when L-erythrulose and glyceraldehyde-3-phosphate were used as substrates (92). In the light of later findings these observations with the spinach enzyme are most readily explained as being due to a combined action of transketolase and pentosephosphate isomerase.

Provided a source for the formation of active aldehyde is available, transketolase may represent an alternative mechanism for the biosynthesis of pentoses. A potential source for active aldehyde is serine which was shown to undergo transamination reactions in the presence of enzyme preparations from liver (112). Hydroxypyruvate formation from DL-serine has been demonstrated in the kidney (113).

The significance of these findings in relation to studies on the incorporation of labeled acetate (114) and glycine (115) into ribose will be discussed later.

Heptulose Phosphate Synthesis. With ribulose-5-phosphate or hydroxypyruvate as donor and ribose-5-phosphate as acceptor crystalline transketolase catalyzes the formation of a heptulose phosphate (108). Since these experiments were carried out in the absence of pentose phosphate isomerase and other side reactions, this finding establishes the existence of the condensation between a five and a two carbon fragment. A condensation between a three and a four carbon compound is clearly ruled out under these conditions. The reaction is written as:

(1) ribulose-5-phosphate + ribose-5-phosphate $\rightleftharpoons$ heptulose phosphate + glyceraldehyde-3-phosphate

Since transketolase catalyzes the breakdown of ribulose-5-phosphate which has the hydroxyl groups at carbons 3 and 4 in *cis* position, it might be suggested that a similar configuration could be formed when active aldehyde is condensed with ribose-5-phosphate. Alloheptulose, the expected sugar from such a union, has not been found in nature nor has it been synthesized, so that a direct comparison of its properties with sedoheptulose (which has the C-3 and C-4 hydroxyls in *trans* position) or with the product of the transketolase reaction has not been possible. Recent studies (108) on the substrate specificity of crystalline transketolase indicate that in addition to ribulose-5-phosphate and hydroxypyruvate the enzyme can attack sugars that contain hydroxyl groups at carbon 3 and 4 in *trans* position. This lack of specificity leaves open the possibility that compounds such as xylulose-5-phosphate and sedoheptulose-7-phosphate may be synthesized by the enzyme in addition or in preference to the sugars that contain the hydroxyl groups in *cis* position.

The heptulose phosphate which is formed by transketolase has been isolated as a barium salt after elution from a Dowex-1 column. It gave rise to triose phosphate when it was added to a mixture containing transketolase, an acceptor aldehyde (glyceraldehyde) as well as pentosephosphate isomerase. Upon omission of any one of the components no triose phosphate was formed (108). These observations are readily explained by the following sequence of events: Heptulose phosphate is broken down by transketolase (TK) to ribose-

5-phosphate in the presence of an acceptor aldehyde (reaction 2). Ribose-5-phosphate is transformed to ribulose-5-phosphate by the action of the isomerase (reaction 3). Finally ribulose-5-phosphate is cleaved by transketolase to triosephosphate and active aldehyde. The latter condenses either with glyceraldehyde (reaction 4) or with ribose-5-phosphate (reaction 1) to form a keto sugar:

(2) heptulose phosphate + glyceraldehyde $\overset{TK}{\rightleftharpoons}$ ribose-5-phosphate + ribulose

(3) ribose-5-phosphate $\overset{\text{isomerase}}{\rightleftharpoons}$ ribulose-5-phosphate

(4) ribulose-5-phosphate + glyceraldehyde $\overset{TK}{\rightleftharpoons}$ ribulose + glyceraldehyde-3-phosphate

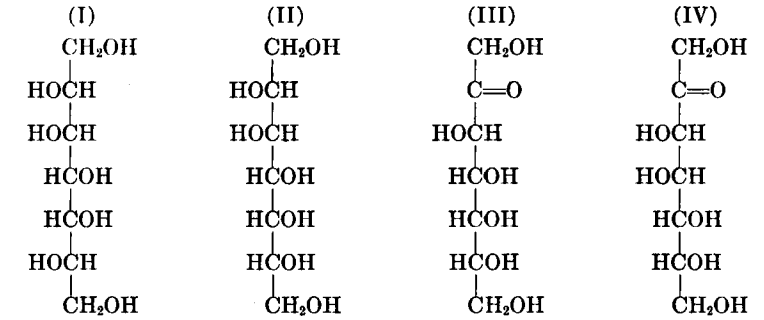
Synthesis of Other Keto Sugars by Transketolase. Purified preparations of transketolase from yeast have been found to react with a number of acceptor aldehydes. Although a final identification of the products has not been completed it may be of interest to discuss some of the reactions. When free glyceraldehyde and ribulose-5-phosphate act as substrates, glyceraldehyde-3-phosphate and ribulose are formed (reaction 4). Upon inspection this reaction may be held to be a phosphate transfer reaction although actually an aldehyde transfer takes place. One might consider such a mechanism in other reactions hitherto held to be phosphate or pyrophosphate transfers. Studies with isotopically labeled compounds will undoubtedly clarify the mechanisms which operate in various transfer reactions.

In analogy with heptulose phosphate formation the condensation of active aldehyde with desoxyribose-5-phosphate should lead to the formation of a 4-desoxyheptulose phosphate. This compound should prove of considerable interest in future studies of the specificity of transaldolase which will be discussed below. A possible relationship to the desoxyketo sugar postulated as an intermediate of phosphogluconate metabolism in *Pseudomonas saccharophila* (116) might be considered. A search for other acceptor aldehydes even in the aromatic series may prove of considerable interest for preparative purposes.

Metabolism of Heptuloses. In view of the increasing interest the seven-carbon sugars have attracted since they were found to become labeled in the presence of $C^{14}O_2$ during the early phases of photosyn-

thesis, a brief review of the biochemistry of these compounds seems desirable.

At the end of the last century compounds have been isolated from nature which were recognized to be polyalcohols containing seven carbons (117,118). They were named perseitol (I) and volemitol (II) after the plants from which they were isolated. The first synthesis of a heptitol (perseitol) from D-mannose by cyanohydrin synthesis was accomplished by Fischer (119), who also obtained the sugar volemose by oxidation of volemitol with hypobromite (120). A few years later Bertrand (121,122) studied the biological oxidation of the heptitols by bacteria (*Acetobacter*) and demonstrated the formation of the ketoheptose perseulose.



The isolation (123,124) of sedoheptulose (III) and mannoheptulose (IV) and the synthesis of a number of heptitols, heptoses, and heptuloses opened new possibilities of an approach to the metabolism of this group of compounds (*cf.* 125,126). Such studies were undertaken and the metabolism of these sugars was studied in animals. It was found (127) that in rabbits mannoheptulose was transformed into a sugar which was most probably glucose, since it was readily fermented by yeast, and its removal from the blood stream was stimulated by insulin. A protection against minimal lethal doses of insulin (in 2 rabbits) and prevention of convulsive seizures was observed after mannoheptulose administration. The aldose D-mannoheptose, on the other hand, was not utilized by rabbits. In rats

(128) neither of the two sugars was used, though some transformation of the heptulose must have occurred since a considerable portion of the reducing substance excreted in the urine did not give a reaction for ketosugar. When the sugars were fed to rats a marked laxative effect was noted, which was explained in subsequent studies by the low rate of absorption of these sugars. In view of the increasing popularity of the avocado fruit, which contains considerable amounts of D-mannoheptulose, the metabolism of this sugar was studied in man (129). It was found that after ingestion of the fruit or of a solution of mannoheptulose a reducing sugar appeared in the urine. The peak of the excretion was reached about 2 to 4 hours after ingestion, but only a portion of the sugar could be accounted for by the excretion. The fate of the remainder was not determined. A point was made of the fact that the sugar excreted after eating avocado fruit is readily differentiated from glucose in a diabetic urine by a positive test given with Benedict's solution in the cold, a characteristic reaction for keto sugars.

In a paper on organic acids in plants, Bennet-Clark (130) includes data on the fluctuation of sedoheptulose in *Sedum prealtum* as influenced by light. An inverse relationship to malic acid which disappears in light and accumulates in the dark was observed. Fluctuations in sedoheptulose concentration were also reported in *Bryophyllum calcynum* (131) and further studies on the effect of light on heptulose formation have been carried out recently (132). A wide distribution of sedoheptulose in plants (133) and the occurrence of heptoses as components of bacterial polysaccharides (134,135) has been reported.

The first indication for the participation of phosphorylated heptuloses in metabolic reactions came with the discovery of a heptulose phosphate ester in a crude preparation of hexose monophosphates obtained from fermenting yeast (136). After oxidation of the aldose sugars with bromine, and removal of the phosphohexonates, the phosphoketo sugar was fractionated and its properties studied. The phosphate ester was found to be hydrolyzed by acid at the same rate as fructose-6-phosphate and its osazone contained phosphate. These observations ruled out the possibility of a heptulose-1-phosphate which should be hydrolyzed more rapidly and should give a phosphate-free osazone. Nevertheless, the suggestion was made that the formation of a heptulose-1-phosphate might have been catalyzed by yeast aldolase with a subsequent transfer of phosphate to a more stable ester. These early findings and speculations are recorded here since they are of interest in the light of later developments.

More than twelve years later a phosphorylated heptulose was suggested to play a role in photosynthesis (84) and sedoheptulose-1-phosphate was isolated from an aldolase catalyzed condensation of erythrose and dihydroxyacetone phosphate (78,88). The findings of the formation of heptulose phosphates from ribose-5-phosphate in preparations from liver (78) and plants (80,92), as well as in hemolyzates of red blood cells (137), demonstrate the wide distribution of enzymes catalyzing this reaction. The formation of heptuloses catalyzed by transketolase, by aldolase, and by transaldolase (which will be discussed shortly), the isolation of sedoheptulose diphosphate (83) and the formation of an unidentified heptulose phosphate from hexose monophosphate (82,108) emphasize the variety of reaction mechanisms which may participate in heptulose metabolism. Finally it should be pointed out that all these enzymic reactions concern phosphorylated intermediates, while in most instances heptuloses found in nature appear to be nonphosphorylated but still participate in metabolic reactions (130,131). It remains to be investigated whether free heptuloses can react also in these transfer systems or must be phosphorylated before they are metabolized.

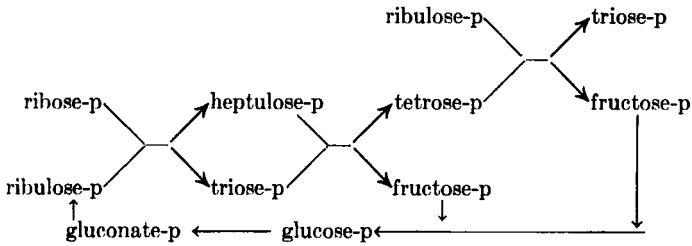
Transaldolase. The formation of hexose phosphates from a pentose derivative was first observed in hemolyzates of red blood cells which metabolized adenosine (72). Glucose-6-phosphate was identified as a product which accumulates when ribose-5-phosphate was added to preparations of liver tissue (81). It was found that hexose monophosphate did not arise from fructose-1,6-diphosphate since the latter was not dephosphorylated to hexose monophosphate in blood hemolyzates (82), nor in partially purified preparations from yeast (77,90) and liver (2,68). No glycolaldehyde could be detected in any of the systems studied and the concept of a transaldolization reaction was therefore introduced (82). The first hexose monophosphate to be formed was reported to be a keto sugar (fructose-6-phosphate) which was converted to glucose-6-phosphate to form an equilibrium mixture of hexose monophosphates. It again became essential to separate the complex enzyme mixture in order to elucidate the mechanism of the participating reactions. A separation of the hexose monophosphate-producing system from transketolase by fractionation of yeast extracts was reported (90) and a transaldolization with heptulose phosphate as donor and triose phosphate as acceptor in a partially purified preparation has recently been described (138). Uni-

formly C^{14}-labeled triose phosphate was incorporated into carbons 4, 5, and 6 of fructose-6-phosphate but not into the first three carbons. This finding is most readily interpreted as a transfer of the dihydroxyacetone portion of the heptulose phosphate to triose phosphate. The tetrose phosphate which would be expected to be formed has not been isolated but, after treatment with phosphatase, a compound corresponding to a tetrose has been spotted on paper chromatograms. Some of the difficulties encountered in attempts to accumulate tetrose phosphate have been explained by the finding that a side reaction takes place in the presence of aldolase, triose phosphate and tetrose phosphate giving rise to the formation of sedoheptulose diphosphate (83).

The mechanism of transaldolase action has not as yet been established. Since in the complete system a transformation of ribose to fructose takes place, an inversion at carbon three must occur. This most likely happens in the course of the condensation reactions which lead to the formation of seven- and six-carbon keto sugars. However, the participation of an enzyme which catalyzes the inversion after condensation must also be considered, particularly in view of the fact that an enzymic "Waldenase reaction" which leads to the inversion of carbon 4 of galactose-1-phosphate has been described (*cf.* 38). The concept of an activated alcohol group which participates in transaldolization (111) should stimulate attempts to explore the active site of transaldolase and the participation of a specific prosthetic group. In reverse, the suggestion of Horecker that an internal transaldolase type of reaction may be responsible for the galactowaldenase activity has been given consideration by Leloir (*cf.* 38). It appears that in neither case of enzymic inversions can a final description of the detailed mechanism be given at the present time.

The Glucose-6-phosphate Oxidation Cycle. The formation of hexose monophosphates has been shown to proceed with a remarkable efficiency in blood hemolyzates (82), yeast (77), and liver (79). Up to 75% of the ribose-5-phosphate added appeared as hexose monophosphate. Our present knowledge of the participating enzymes does not permit us to account for the stoichiometry of this transformation. We can probably assume that the first step in this cycle consists of a reaction between ribulose phosphate and ribose phosphate, to form heptulose phosphate and triosephosphate. This is followed in a second step with the production of 1 mole of fructose-6-

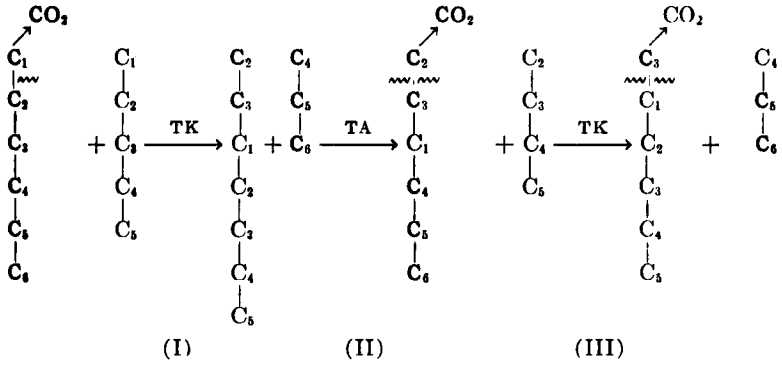
phosphate and 1 mole of tetrose phosphate. Since the actual yield is much greater than 1 hexose per 2 pentoses, and tetrose phosphate has not been shown to accumulate, it becomes apparent that a further transformation of tetrose to hexose must occur. Although an aldolase-catalyzed condensation between a triose and a tetrose has been proposed repeatedly and was actually demonstrated to occur under specific conditions (78,88), the heptuloses formed by these reactions have not as yet been shown to be utilized for hexose formation. If a phosphomutase type reaction converting heptulose-1-phosphate to a C-7 ester exists as suggested many years ago (136), a second cycle of transaldolization may give rise to another mole of fructose-6-phosphate. Attempts to demonstrate this have so far failed. If, on the other hand, transketolase can catalyze condensations in *trans* position, as preliminary experiments (108) indicate, the formation of another fructose-6-phosphate molecule may arise from a direct condensation between tetrose phosphate and active glycolaldehyde. If this proposed series of reactions is then linked to the first two enzymes reactions of the shunt mechanism, namely glucose-6-phosphate dehydrogenase and phosphogluconic acid dehydrogenase, a cycle of glucose oxidation is obtained as outlined in Scheme I. In this scheme



Sum: 3 pentose phosphate ⟶ 2 fructose phosphate + 1 triose phosphate

SCHEME I

a carbon and phosphate balance can be drawn up which accounts fully for the experimental findings. The extent of glucose degradation which can be achieved by such an oxidative pathway may not be apparent on first inspection. If one follows the fate of one glucose-6-phosphate molecule (Scheme II, bold type) in the presence of a ribose-5-phosphate molecule (light type) it becomes evident that only the first three carbons of glucose-6-phosphate are oxidized to CO_2 while the last three become triose phosphate. The ribose-5-



SCHEME II

(I) represents the oxidative decarboxylation of glucose-6-phosphate to ribulose-5-phosphate and condensation with ribose-5-phosphate catalyzed by transketolase (TK) to form heptulose-p and triose-p.

(II) represents transaldolase action on heptulose phosphate and triose phosphate resulting in erythrose-p and fructose-p, the latter being converted to glucose-p and oxidatively decarboxylated to ribulose-p.

(III) represents transketolase action condensing active aldehyde from ribulose-p with erythrose-p to give fructose-p which is again oxidized to ribose-p.

phosphate molecule was added in this representation to emphasize the catalytic role it plays in this series of reactions in a manner quite similar to the carrier role of oxalacetate in the Krebs tricarboxylic acid cycle. One realizes of course that in both cases the "carrier" can undergo side reactions and be oxidized.

2. The Phosphogluconic Fermentation Shunt

In early studies on the hexose monophosphate shunt Warburg, Christian, and Griese (69) stated that phosphogluconic acid is fermented in yeast extracts. This view was corrected by Lipmann (71), who demonstrated the oxidative character of the reaction. This was confirmed thereafter by Warburg and Christian (70) and by Dickens (65).

It now appears that in some bacterial species there actually exists a pathway of phosphogluconate fermentation quite different from the oxidative mechanism. This was discovered by Entner and Doudoroff (116), who have shown that glucose, labeled in carbon 1 with C^{14}, when added to cells of *Pseudomonas saccharophila* gave rise to carboxyl-labeled pyruvate. In cells poisoned with dinitrophenol or

arsenite about 2 moles of pyruvate were formed per mole of glucose used and the specific activity of the pyruvate was close to half of that of glucose on a molar basis. Cell-free extracts of these bacteria exhibited hexokinase and glucose-6-phosphate dehydrogenase activity and catalyzed the anaerobic cleavage of phosphogluconate to 1 mole of pyruvate and 1 mole of glyceraldehyde-3-phosphate, the latter being rapidly converted to free triose by phophatase action. In the intact cells poisoned by DNP glyceraldehyde-3-phosphate was converted to pyruvate, while in the presence of IAA methylglyoxal was formed instead. A direct cleavage of phosphogluconate would be expected to yield triose phosphate and glyceric acid, which on removal of water could yield pyruvic acid. However, free glyceric acid was found to be inactive and the suggestion was therefore made that the removal of water takes place prior to the cleavage, resulting in the formation of a 2-keto-3-desoxy-6-phosphogluconate as an intermediate. This compound could then be split directly to pyruvate and glyceraldehyde-3-phosphate. It was pointed out by the authors that this reaction is quite analogous to the cleavage of desoxyribose-5-phosphate into glyceraldehyde phosphate and acetaldehyde (76). Since the latter reaction has been shown to be readily reversible, a demonstration of the formation of a ketodesoxygluconate would be an important piece of evidence for the proposed mechanism.

3. Reduction of Hexose Monophosphates

When either fructose-6-phosphate or glucose-6-phosphate is added to extracts of *E. coli* a rapid oxidation of DPNH can be observed (95). Glucose-1-phosphate, fructose-1,6-diphosphate, ribose- and ribulose-5-phosphate, heptulose phosphate as well as the free sugars show little or no activity with this enzyme. No evidence for cleavage to triose phosphate could be obtained and the partially purified enzyme actually did not reduce triose phosphate. The specific activity of the fructose-6-phosphate reductase in crude extract was increased about 3-fold by allowing the bacteria to grow in the presence of glucose, and over 8-fold when grown in mannitol. The product of the reaction has not been characterized.

A compound isolated from glucose fermenting *B. casei* was suggested to be a hexitol phosphate on the basis of analysis and absence of reducing power (139). Hexitols are well known to accumulate during some fermentation processes and the observations discussed above

suggest the possibility that the reduction of the hexoses might take place after phosphorylation. However, an enzyme which reduces free hexoses has been described (45) and either one of these two mechanisms may operate in the shunt pathway of polyhydric alcohol formation in plants and bacteria.

C. INTERCONVERSIONS AND CLEAVAGE OF HEXOSE MONOPHOSPHATES

Since the interconversion of hexose monophosphates was covered in an excellent review last year (38) only certain aspects will be discussed. The main enzymic reactions which have been studied are summarized in Table IV.

TABLE IV

ENZYMES CATALYZING INTERCONVERSION OF HEXOSE MONOPHOSPHATES

Enzyme (source)	Cofactor	Substrate	Product	Remarks
1. Hexose-p isomerase (muscle)	—	Glucose-6-p	Fructose-6-p	
2. Mannose-6-p isomerase (muscle)	—	Mannose-6-p	Glucose-6-p or fructose-6-p	Product not established due to presence of hexose-p-isomerase
3. Galactowaldenase (microorganisms, animals)	UDPG	Gal-1-p	G-1-p	
4. Phosphoglucomutase (animal tissues, plants, microorganisms)	G-1,6-p	G-1-p	G-6-p	Acts slowly also on mannose-1-p and ribose-1-p
5. "Cori" fraction (liver)	Mg^{++}	F-1-p	F-6-p or G-6-p	

1. Galactowaldenase

The transformation of galactose-1-phosphate to glucose-1-phosphate is catalyzed by the enzyme galactowaldenase, which is found in microorganisms as well as in animal tissues (140, *cf.* 38) and which has been shown to require a new cofactor identified as uridine diphosphoglucose (UDPG). The enzymically catalyzed reactions (25):

$$\text{UDP-glucose} \rightleftharpoons \text{UDP-galactose}$$

and:

$$\text{glucose-1-phosphate} \rightleftharpoons \text{galactose-1-phosphate}$$

have been shown to reach equilibrium when about 25% of the galactose compound was formed. It was suggested that the first step in the galactose transformation consists of a uridyl transfer from UDP-glucose to galactose-1-phosphate so that UDP-galactose and glucose-1-phosphate are formed. This is then followed by a second step in which UDP-galactose is reconverted to UDP-glucose. Transfer reactions of this type are becoming increasingly recognized (141) and can be very successfully elucidated with the aid of isotopes. In the galactowaldenase reaction it would be of interest to learn whether the label of P^{32}-labeled galactose-1-phosphate is incorporated into UDP-galactose, or alternatively whether P^{32}-labeled UDPG is diluted in the presence of unlabeled galactose-1-phosphate. The mechanism of the second step in which the inversion takes place remains unexplained although a number of possibilities have been excluded (38).

2. Phosphoglucomutase

This enzyme, which has been crystallized from rabbit muscle (142), catalyzes the conversion of glucose-1-phosphate to glucose-6-phosphate and requires glucose-1,6-diphosphate as a cofactor (143). Phosphoglucomutase preparations act on some other phosphorylated sugars such as mannose-1-phosphate (38) and ribose-1-phosphate (144) although considerably more slowly. In all cases the corresponding diester appears to act as cofactor by donating one of its phosphates to the substrate which thus becomes the diester.

3. Fructose-1-phosphate Cleavage and Conversion to Hexose Monophosphate

A very interesting and puzzling interconversion takes place in enzyme preparations from liver which transform fructose-1-phosphate to the glucose-6-phosphate, fructose-6-phosphate equilibrium mixture (64). Several hypotheses have been proposed but none of them seems to satisfy fully the quantitative aspects of the observed phenomenon. The most recent and most plausible explanation centers around the observation that the liver preparations catalyze the cleavage of fructose-1-phosphate to two trioses, one phosphorylated and one nonphosphorylated (145–147), thus reviving an old theory of fermentation. The sequence of events is proposed to proceed as follows: fructose-1-phosphate is cleaved to glyceraldehyde

and dihydroxyacetone phosphate, the latter is being converted to glyceraldehyde-3-phosphate, which condenses with another molecule of dihydroxyacetone phosphate to form fructose-1,6-diphosphate. In the presence of Mg^{++} and specific HDP-phosphatase the diester is split to fructose-6-phosphate. Since the enzymes which catalyze these reactions have been shown to occur in liver and a system has been reconstructed with partially purified enzymes capable of catalyzing the transformation (145,146) it is quite clear that a contribution to fructose-6-phosphate formation from fructose-1-phosphate is made by this mechanism. It should be pointed out, however, that in the absence of an ATP-donating system a maximal yield of 50% of hexose-6-phosphate esters could be obtained and an equivalent of one inorganic phosphate should be liberated. The available data (64) indicate that under some conditions less inorganic phosphate is formed than hexose monophosphate transformed and the yield of hexose-6-phosphates after short incubation periods is well over 50%. After longer periods of incubation some hydrolysis of hexose monophosphates takes place, as indicated by the liberation of free fructose. Thus over 80% of the fructose-1-phosphate which disappears was accounted for either as hexose-6-phosphate or as free hexose. Even neglecting the small contribution made to this value by free glucose (which was not determined by a specific method) it is apparent that the cleavage of fructose-1-phosphate cannot fully explain the findings, although it undoubtedly can contribute to hexose-6-phosphate formation. Although one could speculate on the possible participation of transaldolase or transketolase reactions in the interconversion of hexose monophosphate by liver preparations the fact remains that the phenomenon has been greatly clarified but not completely elucidated.

Perhaps a word of caution should also be added to the interpretation of the cleavage of fructose-1-phosphate as being due to an enzyme other than aldolase (146). Although it is quite possible that there is an enzyme in liver which cleaves fructose-1-phosphate faster than does muscle aldolase it could not shift the equilibrium of the reaction unless it itself is a reagent in the reaction. Since crystalline muscle aldolase catalyzes the condensation between dihydroxyacetone phosphate and free aldehydes it is difficult to see in what respects other than rate and substrate affinity the liver enzyme can differ from muscle aldolase, since the reactants appear to be identical.

IV. From Fructose-1,6-diphosphate to Pyruvic Acid (see Tables V and VI)

Space does not permit us to follow through the metabolic variations of carbohydrate metabolism in all its ramifications. It must suffice to summarize in tabular form the reactions leading to pyruvate and to discuss only some of them which appear to be of special interest.

TABLE V

ENZYMES OF TRIOSE PHOSPHATE METABOLISM

Enzyme	Cofactor	Substrate	Product	Remarks
1. Aldolase	Fe^{++} or Zn or Mn^{++} (in micro-organisms)	Fructose-1,6-di-phosphate	Glyceraldehyde-3-p + dihydroxyace-tone-p	Condenses free aldehydes with dihy-droxyace-tone-p
2. Triosephosphate isomerase	—	Dihydroxyacetone phosphate	Glyceraldehyde-3-phosphate	
3. α-Glycerophos-phate dehydro-genase	DPN	α-Glycerophos-phate	Dihydroxyacetone-p	
4. Glyceraldehyde-3-p-dehydrogen-ase	DPN	Glyceraldehyde-3-p + Pi	1,3-Diphospho-glyceric acid	Slowly oxidises other alde-hydes; TPN linked en-zyme in plants
5. Desoxyribose-5-p aldolase	—	Glyceraldehyde-3-p + acetaldehyde	Desoxyribose-5-phosphate	
6. Transketolase, transaldolase		See Table III		
7. Erythrulose-p aldolase	—	Dihydroxyacetone-p + formaldehyde	Erythrulose-p	Enzyme not identical with HDP aldolase

An oxidation of fructose-1,6-diphosphate in plant preparations has been reported (148). Under the conditions of the test neither hexose monophosphates nor glyceraldehyde-3-phosphate were utilized at comparable rates. In view of the relative inactivity of glyceraldehyde-3-phosphate these findings retain their significance in spite of the demonstration of triosephosphate dehydrogenase in similar extracts (149,150). Perhaps one might consider the possibility of a direct oxidation of the fructose moiety as the initial step; it might be recalled that 5-ketofructose is rapidly fermented by yeast (151).

There are several pathways for the utilization of triose phosphates. The formation of α-glycerophosphate linking them to fat metabolism,

TABLE VI

ENZYMES OF PHOSPHOGLYCERATE AND PHOSPHOPYRUVATE METABOLISM

Enzyme	Cofactor	Substrate	Product
1. Phosphoglyceric transphosphorylase	ATP Mg^{++}	1,3-Diphosphoglyceric acid	3-Phosphoglyceric acid
2. 1,3-Diphosphoglyceric mutase	3-Phosphoglyceric acid	1,3-Diphosphoglyceric acid	2,3-Diphosphoglyceric acid
3. 3-Phosphoglyceric mutase	2,3-Diphosphoglyceric acid	3-Phosphoglyceric acid	2-Phosphoglyceric acid
4. Enolase	Mg^{++}	2-Phosphoglyceric acid	Phosphopyruvic acid
5. Phosphopyruvic transphosphorylase	Mg^{++} ADP	Phosphopyruvic acid	Pyruvic acid
6. Oxalacetate-ATP carboxylase	Mg^{++} ATP	Phosphopyruvic acid + CO_2	Oxalacetate

the formation of desoxyribose phosphate and of ribose phosphate, and the condensation with formaldehyde (152) are but a few examples of the metabolic reactivity of the triose phosphates. Along the main pathway of glycolysis they are oxidized to 1,3-diphosphoglyceric acid, which donates its 1-phosphate to ADP or in some instances transfers it to phosphoglyceric acid to produce 2,3-diphosphoglyceric acid (153). The glyceric acids, phosphorylated in the 2- or 3-positions, have been subjected recently to many graphical alterations in view of their role in photosynthesis. So far no enzymic reaction with these substrates demonstrating an alternate pathway has been found, only negative data on decarboxylation experiments in plant extracts have been reported (154). Hydroxypyruvic acid is readily reduced by DPNH and crystalline lactic dehydrogenase (108,155) but phosphoglyceric acid is not readily oxidized by this enzyme (108) and crude leaf extracts oxidize it only very slowly as compared to lactic acid (148). Phosphoenolpyruvic acid, on the other hand, has been established as an intermediate in CO_2 fixation reactions leading to the net synthesis of oxalacetic acid and the enzyme which catalyzes this reaction has been demonstrated in liver and plants (156, 157). Since this enzyme in combination with malic enzyme and malic dehydrogenase catalyzes the phosphorylation of pyruvate (reactions 1, 2, and 3), one wonders how much this alternate pathway contributes to the formation of phosphopyruvate in the reversal of glycolysis.

which has been shown to be dependent on the presence of potassium ions (*cf.* 158). Curiously enough malic enzyme as well as phosphopyruvate transphosphorylase (159) are stimulated by potassium salts (160):

(1) pyruvate + TPNH + CO_2 $\rightleftharpoons$ malate + TPN
(2) malate + TPN $\rightleftharpoons$ oxalacetate + TPNH
(3) oxalacetate + ATP $\rightleftharpoons$ phosphopyruvate + ADP + CO_2
Sum: pyruvate + ATP $\rightleftharpoons$ phosphopyruvate + ADP

Enzymes of alternate pyruvate utilization will not be discussed because some of them will be covered in another article of this volume. Moreover the field has become so vast that it could not be included in this review.

V. The Pasteur Effect

Although the individual enzymes of the Embden-Meyerhof scheme function aerobically as well as anaerobically, marked quantitative differences in glucose utilization are apparent in the intact cell. The phenomenon, which was first described by Pasteur, consists of a depression of glucose utilization in respiring cells. When air is excluded or certain poisons of respiration or coupled phosphorylation are added, the Pasteur effect is abolished and a rapid rate of glucose utilization sets in. This is the present problem of the Pasteur effect, unconfused by aspects of lactic acid production or carbohydrate resynthesis. There is a multitude of theories (*cf.* 161) which attempt to explain the Pasteur effect but none seems to be fully satisfactory. The most widely quoted ones center around the fact that under aerobic conditions coupled phosphorylation brings about a depletion of inorganic phosphate (162,163). This depletion of inorganic phosphate, even if it could be convincingly demonstrated to occur within the cell, cannot possibly be the direct cause of the phenomenon. Although it could account for an inhibition of lactate formation, which requires both inorganic phosphate and ADP, it cannot account for the inhibition of glucose phosphorylation, which requires neither. Actually, in yeast maceration juice the enzyme pattern is such (*cf.* 164) that the system soon becomes depleted of inorganic phosphate and ADP, resulting in the accumulation of hexose diphosphate (the Harden-Young phenomenon). The Pasteur effect must be of a different nature, since one cannot find large

amounts of hexose phosphates within the cell. Even if these esters would accumulate under aerobic conditions, though the evidence speaks against it (5), such accumulation could not account for a slowing down of the hexokinase reaction, which is essentially irreversible. Inhibition of glucose phosphorylation by glucose-6-phosphate could be suggested to play a role in the Pasteur effect in animal tissues and schistosomes, which have been shown to contain a hexokinase susceptible to product inhibition (20–23). However, purified yeast hexokinase is not susceptible to the inhibitory effect of glucose-6-phosphate (21) nor is a crude yeast extract, as is apparent from the Harden-Young effect. The possibility might be considered that product inhibition does operate within the intact yeast cell. Whatever the cause of the inhibition might be, it seems quite apparent that an inhibitor of glucose utilization must be formed under the conditions of oxidative phosphorylation and is rapidly removed when excessive phosphorylation ceases. It is quite likely that not one, but several different compounds, might function as "Pasteur inhibitors" in various microorganisms. In fact it might be suggested that the formation of such an inhibitor may have permitted an economy of substrate utilization which has helped a struggling microorganism to be selected.

VI. Comparative Biochemistry of Alternate Pathways

Perhaps one is a little bewildered when confronted with so many alternate transformations for each one of the intermediates of carbohydrate metabolism discussed above. What actually happens within the cell? What are the factors that determine which road is taken? What is the probability for a glyceraldehyde-3-phosphate molecule to become a desoxyribose or a glycerol molecule or any other product of transformation? It is quite probable that the structural organization of the cell has some determining influence but there can be little doubt that the properties of the participating enzymes and their intracellular concentration is of importance. This is why several times in the above discussion values of the Michaelis constants for various substrates and their enzymes were included. There has been a recent trend to disparage the significance of these numerical data, stemming from the realization that the K_m value does not necessarily represent the true dissociation constant of the enzyme–substrate complex. This cannot be denied, but one should not

overlook the real significance of the Michaelis constant from a physiological point of view. In the steady state of intracellular metabolism the concentration of the intermediates is usually well below that required to saturate the enzyme. Thus the situation appears to be one of enzymes competing for substrates rather than the reverse. Therefore the relative reactivity of the substrate at low concentrations with various competing enzymes, together with other kinetics aspects, may play an important role in deciding its ultimate fate. Probably the most important factor for determining the pathway is the quantitative distribution of the enzymes in various organisms. There are some microorganisms, *e.g.*, of the *Pseudomonas* and *Leuconostoc* group (97,98), in which the Embden-Meyerhof pathway seems to play a minor role, while there are tissues such as muscle in which it is undoubtedly of primary importance. More difficult to interpret are the events which take place in cells which contain the machinery for several pathways. For example, the enzymes of the shunt mechanism are present in yeast where they were first discovered. Yet, studies with glucose labeled in carbon 1 seem to exclude an active participation of this mechanism in glucose degradation anaerobically or aerobically (165,166). In line with this are the findings of Chance (12), who could not detect in yeast cells a reduction of TPN due to enzymes of the oxidation shunt. Perhaps it should be pointed out that, although yeast has been a favored source for the purification of enzymes of the oxidation shunt, this practice can be attributed largely to the existence of breweries and bakeries which make yeast production a profitable undertaking. Actually the catalysts of the shunt are present in yeast in much lower concentrations than are the classical fermentation enzymes. For example, some bacteria such as *E. coli* contain on a weight basis considerably higher concentration of transketolase than are present in yeast.

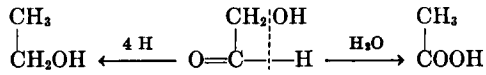
Studies on Alternate Pathways with Isotopically Labeled Glucose. The most important contributions to an evaluation of alternate pathways have come from isotope studies. An investigation of the utilization of glucose-1-C^{14} in *E. coli* (167) revealed a significant contribution of the glucose-6-phosphate oxidation shunt to glucose oxidation. As mentioned previously, in yeast this does not seem to be the case (165,166). Only few data are available for animal tissues. Experiments on the incorporation of glycine (115) and of acetate (114) into the ribose moiety of nucleic acid cannot be accepted as evidence

against a major participation of the shunt in ribose formation (114, 168). Although the condensation of a 3- with a 2-carbon unit to pentose phosphate by transketolase may participate in ribose formation *in vivo*, exchange reactions may also occur with this enzyme and a *quantitative* evaluation of this reaction *in vivo* is therefore not as yet possible. More direct data on the utilization of glucose by the shunt pathway in the rat and in tissue slices have been recently reported (169). While the major part of glucose appears to be utilized via the glycolytic pathway in the intact rat and in the isolated diaphragm, this does not seem to be the case with kidney slices and particularly with liver slices. At least 75% of the CO_2 from glucose is estimated to be derived from the oxidative shunt mechanism in liver. These interesting findings are in line with the quantitative distribution of the enzymes of the shunt pathway in the respective tissues (2).

In the course of studies on the utilization of glucose-1-C^{14} and glucose-3,4-C^{14} for the biosynthesis of kojic acid in molds a preferential oxidation of carbon 1 of glucose was observed (104). The data on $C^{14}O_2$ evolution as well as on the isotope distribution in kojic acid may be readily explained by a glucose-6-phosphate oxidation cycle catalyzed by transketolase and transaldolase as outlined above.

Experiments were performed on the utilization of glucose-1-C^{14} and glucose-3,4-C^{14} by *Leuconostoc mesenteroides* (170). Lactic acid and ethanol, the end products of fermentation, showed a distribution of label which was incompatible with the Embden-Meyerhof scheme of fermentation. Carbon 1 appeared as CO_2 and in experiments with glucose-3,4-C^{14} the label was found only in the carbinol group of ethanol and the carboxyl group of lactic acid. These data, together with studies on cell free extracts (98), suggest that an anaerobic hexose monophosphate shunt must operate in these bacteria. It may be a variant of the known oxidative pathway, but must also include the formation of a hydrogen acceptor to permit a dismutation reaction to occur. This acceptor apparently is acetaldehyde since ethanol is the end product. Thus it remains to be elucidated how acetaldehyde is derived from carbon 2 and 3 of glucose. If hydroxypyruvate is an intermediate as has been suggested (98), the problem becomes quite similar to that emerging from studies of pentose fermentation in lactobacilli (171,172) which was recently discussed by Lampen (173). It

might perhaps be suggested that transketolase participates in the formation of an active glycolaldehyde, which undergoes a dehydration followed by reduction in the case of *Leuconostoc*, or by hydrolysis in the case of lactobacilli as outlined below:



In the case of *Acetobacter acetigenum* free glycolaldehyde is formed from pentoses (174). If another oxidative pathway can be ruled out (*cf.* 173) these data would indicate a pentose split similar to that catalyzed by transketolase, but with a dissociable glycolaldehyde as end product. Such differences in dissociability of the aldehyde formed would be quite similar to those encountered with yeast and animal carboxylase (*cf.* 111). Free glycolaldehyde was shown to accumulate and was first isolated from *Fusaria* by Goepfert and Nord (175).

Recent isotope studies with propionic acid bacteria (177) indicate the participation of a pathway similar to that described by Entner and Doudoroff for *Pseudomonas* (116).

Alternate Pathways and Energy Utilization. The yield of utilizable energy which can be obtained from the aerobic and anaerobic degradation of glucose by the classical pathways is adequately discussed in modern textbooks of biochemistry. Little if anything is said or can be said of the energy which can be gained from glucose degradation by other mechanisms, since no direct quantitative determinations have been reported. It has been experimentally demonstrated (1937) and repeatedly pointed out by Nord and his collaborators that nonphosphorylating glucose utilization occurs in molds (*cf.* 176). Also, in certain species of *Acetobacter* and *Pseudomonas* the nonphosphorylative oxidation of glucose to 2- or 5-keto or 2,5-diketogluconic acid appears to play a major role (*cf.* 105,106,178,179). This, however, does not exclude the possibility of oxidative phosphorylation coupled to hydrogen transport, which could yield as many as 4 to 6 moles of ATP per mole of glucose oxidized to ketogluconic acid. In addition to nonphosphorylating glucose oxidation *Pseudomonas fluorescens* (179) contains a pathway of hexose monophosphate utilization.

Energy data for the glucose-6-phosphate shunt are also lacking. Since the two dehydrogenases are either TPN- or DPN-linked, oxidation of the first three carbons of glucose could result in the formation

of 18 ATP molecules (assuming a P/O ratio of 3 for DPNH oxidation). Thus an efficiency equal to the tricarboxylic acid cycle could be obtained for half of the glucose molecule.

Finally, it should perhaps be reemphasized that the above examples of alternate metabolic reactions probably represent just random samples from the metabolic ocean. The often tiresome attention which is being paid to these reactions is justified only by the ubiquity of their existence in various forms of cellular life.

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