THE PATHWAYS OF GLUCOSE DISSIMILATION

IN PSEUDOMONAS AERUGINOSA

by

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ABSTRACT

The non-phosphorylated oxidative pathway of glucose dissimilation has been established in Pseudomonas aeruginosa and evidence for phosphorylated pathways, other than the Embden-Meyerhof scheme, has been obtained. In the present study the non-phosphorylated and phosphorylated pathways of glucose degradation have been investigated with cell-free extracts of this organism.

Gluconolactone was shown to be an intermediate in the oxidation of glucose to gluconic acid. The enzymatic hydrolysis of the lactone ring has an absolute magnesium ion, or divalent cation requirement. In the presence of phosphate buffer magnesium was chelated and effectively removed from participation in the enzymatic reaction.

As has been reported in the literature, the product of glucose and gluconic acid oxidation was identified as 2-ketogluconate. In the presence of adenosine triphosphate (ATP), glucose and gluconate are phosphorylated and the kinases involved, therefore, link the non-phosphorylated with the phosphorylated pathways.

The demonstration of triphosphopyridine nucleotide (TPN) linked dehydrogenases for glucose-6-phosphate and 6-phosphogluconate, as well as the production of glucose-6-phosphate and 3-phosphoglyceraldehyde from cell-free extracts with gluconate or ribose plus ATP, illustrated the presence of a functional pentose phosphate cycle in this organism. An active 6-phosphogluconate dehydrase and a 2-keto-3-deoxy-6-phosphogluconate aldolase were demonstrated by the production of pyruvic acid from 6-phosphogluconate and indicated the presence of the Entner-Doudoroff pathway.

The oxidation of 3-phosphoglyceraldehyde to 3-phosphoglyceric acid,

initiated by a TPN specific 3-phosphoglyceraldehyde dehydrogenase, and the conversion of phospho-enol-pyruvate to pyruvic acid was shown. It is suggested that the trioses are immediately concerned in the observed CO_2 fixation by this organism. Fructose-1,6-diphosphate aldolase, fructose-1,6-diphosphate phosphatase and phosphohexoisomerase may be involved in the formation of glucose-6-phosphate from triose phosphates.

A direct link between 2-ketogluconate and the phosphorylated pathways could not be shown but the reduction of the phosphate ester of the compound was demonstrated. The feasibility of 2-ketogluconate undergoing a 3:3 split is presented.

No attempt has been made to estimate the relative importance of the various pathways of glucose dissimilation as it is felt that this is determined by the conditions and stages of growth of the organism.

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INTRODUCTION

The non-phosphorylated oxidative pathway of glucose dissimilation through gluconate and 2-ketogluconate has been established as one mechanism of dissimilation in Pseudomonas aeruginosa ATCC 9027 (26), (105), (106). Some of the enzymes of both the pentose phosphate and the Entner-Doudoroff pathways also have been demonstrated to be present in this microorganism (66), (92), (115). A link between the phosphorylated and non-phosphorylated pathways has been suggested by the finding of an adaptive 2-ketogluconate kinase (38).

Wang et al. (124), (138), using C^{14} labeled substrates, have stated that gluconate is oxidized completely via the pentose phosphate pathway whereas 71 per cent of glucose is oxidized by way of the Entner-Doudoroff scheme and the remainder by way of the pentose phosphate pathway. Katz and Wood (85), however, have adequately dealt with the problems involving recycling and incomplete CO_2 yields arising from the use of labeled substrates and the caution that must be exercised in quantitative interpretation of data.

Terminal oxidation in this organism has been shown to involve the conventional tricarboxylic acid (TCA) cycle as well as a glyoxylate by-pass (19), (21), (22).

The occurrence of the pentose phosphate and the Entner-Doudoroff pathways is relatively widespread among microorganisms including the pseudomonads. It has been the intent of this study to determine if these pathways, as well as the non-phosphorylated pathway, function in P. aeruginosa.

LITERATURE REVIEW

The initial step leading to the establishment of the pentose phosphate pathway as an alternative to the Embden-Meyerhof scheme of glucose degradation was put forward by Warburg and Christian in 1931-1933 (63) when they found a dehydrogenase in yeast preparations and in erythrocyte hemolysates that catalyzed the oxidation of glucose-6-phosphate to 6-phosphogluconate in the presence of TPN[1]. Dickens, in 1938 (42), (43), showed that crude yeast enzyme preparations took up 1 µmole O_2/µmole glucose-6-phosphate and $\frac{1}{2}$ µmole O_2/µmole 6-phosphogluconate; with 6-phosphogluconate 1 µmole CO_2/µmole substrate was given off and, further, gluconic acid was not oxidized unless it was phosphorylated. Dickens postulated that from the oxidation of 6-phosphogluconate a ketophosphohexonate was formed and subsequently underwent decarboxylation. The fact that ribose-5-phosphate was oxidized fairly rapidly by yeast extracts led to the proposal that 6-phosphogluconate was oxidized to 2-keto-6-phosphogluconate -- the latter intermediate being decarboxylated to ribose-5-phosphate.

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1. The following abbreviations have been used throughout this presentation:

TPN--triphosphopyridine nucleotide; DPN--diphosphopyridine nucleotide;
TPNH H^+--reduced triphosphopyridine nucleotide;
DPNH H^+--reduced diphosphopyridine nucleotide;
TCA--tricarboxylic acid; ATP--adenosine triphosphate;
P_i--inorganic phosphate; Tris--tris(hydroxymethyl)aminomethane;
DNase--deoxyribonuclease; RNA--ribonucleic acid; DNA--deoxyribonucleic acid

The demonstration of the succeeding steps in the pentose phosphate scheme, leading to a complete pentose phosphate cycle with the production of triose phosphate and with the regeneration of hexose phosphate, was the result of the contributions of a large number of workers. Evidence for a gluconokinase in both bakers' and brewers' yeast was presented (116) and Horecker and Smyrniotis (75) effected a partial purification of 6-phosphogluconate dehydrogenase and determined that the enzyme was stimulated by Mg ions, specific for the substrate and the coenzyme TPN. The reaction product was identified as pentose phosphate. Racker (113) isolated an enzyme from bacterial extracts which produced triose phosphate from ribose-5-phosphate as measured by reduced diphosphopyridine nucleotide (DPNH H^+) oxidation in the presence of excess triose phosphate isomerase and -glycerophosphate dehydrogenase. Cohn and Scott, in 1950, noted that during the incubation of 6-phosphogluconate with yeast enzyme, substrate disappearance was accompanied by the appearance of glyceraldehyde-3-phosphate, a small amount of 2-ketogluconate and a considerable quantity of pentose phosphate -- 25 per cent of which was identified as ribose-5-phosphate.

The occurrence of direct oxidation of glucose-6-phosphate, 6-phosphogluconate and ribose-5-phosphate in a variety of animal tissues, in apparently the same manner as the oxidation in yeast extracts, indicated that the pentose phosphate pathway was not peculiar to microorganisms (45), (73).

Dickens and Glock (45) suggested that the lactone of 2-keto-6-phosphogluconate underwent isomerization to the enolic form and, on the addition of water, the open chain enol of 2-ketogluconate is produced and subjected to concurrent decarboxylation and inversion at carbon atom 3 to yield

ribose-5-phosphate. This is contrary to the postulation of the hypothetical intermediate, 3-keto-6-phosphogluconate (73), which was based on the assumption that oxidation of 6-phosphogluconate occurs at the β carbon resulting in the β keto acid which is decarboxylated to produce ribulose-5-phosphate. The latter hypothesis was perhaps supported in part by the finding of pentose phosphate isomerase which catalyzes the interconversion of ribulose-5-phosphate and ribose-5-phosphate with equilibrium being established at 80 per cent ribose-5-phosphate and 20 per cent ribulose-5-phosphate (76).

Horecker et al. (80) detected sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate as products of transketolase activity with pentose phosphates. The enzyme had an absolute requirement for thiaminepyrophosphate and the suggested mechanism of action was the cleavage of a ketol linkage with the resulting formation of a two carbon intermediate designated as "active glycoaldehyde". After purification of the enzyme, it was found that both ribose-5-phosphate and ribulose-5-phosphate were required for heptulose phosphate and triose phosphate formation (32). With crude extracts, Horecker (74) demonstrated that the disappearance of sedoheptulose-7-phosphate and triose phosphate was followed by the appearance of fructose-6-phosphate which underwent rapid conversion to glucose-6-phosphate. From data based on isotope distribution from labeled pentose phosphates he proposed the following sequence of events:

2 pentose phosphate ⟶ hexose phosphate + tetrose phosphate

pentose phosphate + tetrose phosphate ⟶ hexose phosphate + triose phosphate

2 triose phosphate ⟶ hexose phosphate + inorganic phosphate (P_i)

Transaldolase, which catalyzes the transfer of aldol linkages to a suitable acceptor, was purified by Horecker and Smyrniotis in 1955 (78). The enzyme converted sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate to fructose-6-phosphate and a tetrose phosphate, identified chromatographically as erythrose-4-phosphate (79).

The overall reaction of the pentose phosphate pathway of glucose degradation is as follows:

$$3 \text{ glucose-6-phosphate} + 3\ O_2 + 6 \text{ TPN} \longrightarrow 2 \text{ glucose-6-phosphate}$$
$$\text{glyceraldehyde-3-phosphate} + 3\ CO_2 + 6 \text{ TPNH } H^+$$

and probably serves as an important energy source. The widespread occurrence of this system in animal tissues (15), (16), (45), (122), insects (23), (103) and in plants (4), (58), (80), as well as microorganisms, indicates that it plays more than a minor role in glucose catabolism.

Working with Pseudomonas saccharophilia and using labeled substrates, Entner and Doudoroff (50) found that the carboxyl group of pyruvic acid arose from carbon atom one of glucose as opposed to carbon atoms 3 and 4 as would result from the Embden-Meyerhof pathway. Glucose or gluconate, labeled in the one position with C^{14}, yielded 2 moles of pyruvic acid/mole of substrate with all the C^{14} in the carboxyl group of pyruvate and they, therefore, proposed an oxidative pathway involving a 3:3 split by the cleavage of 6-phosphogluconate, or an isomer of the compound, to provide triose phosphate and pyruvic acid. Alumina ground cells, in the presence of ATP, catalyzed the phosphorylation of glucose; fructose-6-phosphate was also formed establishing the presence, in cell-free extracts, of both glucokinase and phosphohexoisomerase. There was no evidence in the organism

for a pathway other than that involving glucose-6-phosphate oxidation to 6-phosphogluconate followed by degradation to trioses. In 1954, MacGee and Doudoroff (97) found two enzymes in P. saccharophilia, the first catalyzed the dehydration of 6-phosphogluconate to a keto acid and the second, an aldolase, cleaved the keto acid, but not 6-phosphogluconate, into pyruvic acid and 3-phosphoglyceraldehyde. The keto acid was isolated and characterized as 2-keto-3-deoxy-6-phosphogluconate.

The Embden-Meyerhof system of anaerobic triose formation exists in the facultative anaerobe Escherichia coli as well as enzymes required for the function of the pentose phosphate pathway. With the aid of labeled glucose, Cohn (29a), (93) was able to conclude that glucose-grown cells use the oxidative pathway to supply the ribose requirements of the organism. With phage infected cells, however, where ribonucleic acid (RNA) synthesis is eliminated and deoxyribonucleic acid (DNA) synthesis stimulated, CO_2 evolution from carbon atom one of glucose is greatly decreased. The shift to the anaerobic pathway indicating that deoxyribose arises from constituents generated more readily from the Embden-Meyerhof scheme. Extracts of gluconate-grown cells contain the adaptive enzyme gluconokinase (30) and gluconate metabolism proceeds almost completely, if not completely, through the pentose phosphate pathway as the oxidation of glucose-6-phosphate to 6-phosphogluconate is essentially irreversible and, hence, the Embden-Meyerhof scheme is by-passed (119). Isolation and quantitative studies of glucose-6-phosphate and 6-phosphogluconate dehydrogenases from extracts of glucose-grown E. coli led Cohen (120), (121) to conclude that the enzymes of the oxidative pathway account for approximately 40 per cent of the total metabolism of carbohydrate. Anaerobically, C^{14} glucose

dissimilation at pH 8 proceeds entirely by way of the Embden-Meyerhof system whereas at pH 5 $C^{14}O_2$ appears, indicating an anaerobic pentose phosphate pathway (110). High 6-phosphogluconate dehydrase and 2-keto-3-deoxy-6-phosphogluconate activity have been reported to be present in dried cells and extracts of E. coli (92), however, Paege and Gibbs (110), from studies of label distribution in intermediates, have concluded that the Entner-Doudoroff enzymes do not function in this organism.

Dissimilation of glucose by Aerobacter aerogenes and Aerobacter cloacae is similar to that in E. coli as both organisms possess the Embden-Meyerhof system and the pentose phosphate pathway. The latter pathway was shown by the conversion of pentose phosphate by transketolase and transaldolase to triose phosphate and hexose phosphate (1), (35). The nonphosphorylated oxidative pathway from glucose to gluconic acid is also present (9), (35). DeLey (33) has demonstrated the presence of the adaptive specific enzyme, 2-ketogluconate kinase, in A. cloacae as well as the adaptive gluconokinase (35). From its ultra-violet light spectrum, 2-ketogluconate does not contain a free $\geq C = O$ function in acid or neutral solutions but rather exists in its pyranose or furanose form and hence, DeLey (34) has suggested that the open chain form of 2-keto-6-phosphogluconate is in equilibrium with its furanose structure and is consequently cleaved to yield hydroxypyruvate and triose phosphate or the dienol is converted to 2-keto-6-phosphogluconate followed by decarboxylation to ribulose-5-phosphate. Further, it was shown that the oxidation of triose phosphate to 3-phosphoglyceric acid was accompanied by the reduction of 2-keto-6-phosphogluconate to 6-phosphogluconate thus linking the non-phosphorylated oxidative pathway with the pentose phosphate pathway.

Cell-free extracts of Erwinia amylovora (132) have been reported to contain the enzymes for cyclic operation of pentose phosphate pathway in the aerobic metabolism of glucose and the effective operation of the Embden-Meyerhof enzymes in anaerobic dissimilation. Cell-free extracts of Azotobacter vinelandii also possess the enzymes of the pentose phosphate pathway (99), (100).

The anaerobe, Clostridium tetani, which is generally considered to be non-saccharolytic, carries out active dissimilation of glucose initiated by an induced glucokinase. The majority of the enzymes of the Embden-Meyerhof scheme have been demonstrated as well as 6-phosphogluconate dehydrogenase, perhaps indicating a functional pentose phosphate pathway (98).

As with P. aeruginosa, cell-free extracts of Bacillus cereus spores oxidize glucose by the oxidative non-phosphorylated pathway to 2-ketogluconate. Oxidation by the spore extracts, however, involves a DPN linked gluconate dehydrogenase. Hexokinase and gluconokinase have not been detected but a TPN dependent glucose-6-phosphate dehydrogenase and a DPN dependent 6-phosphogluconate dehydrogenase have been demonstrated. Anaerobically, 6-phosphogluconate was converted to pentose phosphate and pentose phosphate yielded fructose-6-phosphate and glucose-6-phosphate, exhibiting a functional pentose phosphate scheme. Two-keto-3-deoxy-6-phosphogluconate aldolase was present and, therefore, indicative of an Entner-Doudoroff pathway but 6-phosphogluconate dehydrase could not be demonstrated. A kinase for ribose was found and, in the presence of ATP, 2-ketogluconate was phosphorylated to form 2-keto-6-phosphogluconate which was subsequently reduced to 6-phosphogluconate by 2-keto-6-phosphogluconate reductase in the presence of reduced DPN (DPNH H^+). Oxidation

of 2-keto-6-phosphogluconate produced pyruvic acid and pyruvic acid production was increased 400 per cent in the presence of DPNH H^+ but 2 to 4 times more pyruvate was formed from 2-keto-6-phosphogluconate than from 6-phosphogluconate. This suggested to Halvorson (46), (65) that an alternative route to pyruvate, other than the pentose phosphate and the Entner-Doudoroff pathways, was present. The difference in rates, however, has been suggested as being a problem of hydrogen transfer rather than the existence of an alternate pathway (24).

In the acetic acid bacterium, Gluconobacter liquefaciens, glucose grown cells and cell-free extracts oxidize glucose, gluconate, 2-ketogluconate and 5-ketogluconate, an intermediate in the oxidation of glucose and gluconate being identified as 2,5-diketogluconate. Hexokinase and gluconokinase were demonstrated by the release of CO_2 from bicarbonate in the presence of substrate and ATP. Reduction of glucose-6-phosphate and 6-phosphogluconate by TPN, together with evidence for transketolase and transaldolase, suggested the pentose phosphate pathway. The presence of reductases for 2 and 5-ketogluconate and 2,5-diketogluconate, the reduction producing gluconate, was supported by relatively poor data and was believed to be followed by phosphorylation. Neither pyruvate formation from 6-phosphogluconate nor the dehydration of 6-phosphogluconate could be detected and this was taken as evidence for the absence of an Entner-Doudoroff scheme. The intermediate 2,5-diketogluconate was found to undergo successive dehydration, oxidation and decarboxylation to yield the aromatic compound rubiginol (127).

The Acetobacter present a similar picture of glucose oxidation in that glucose is oxidized to gluconate and to 2 and 5-ketogluconate (52),

(117) and Katznelson et al. (86) reported Acetobacter melanogenum to produce 2,5-diketogluconic acid from glucose. Acetobacter suboxydans has a TPN specific glucose-6-phosphate dehydrogenase and a DPN specific 6-phosphogluconate dehydrogenase. The result of 6-phosphogluconate oxidation, accompanied by CO_2 evolution, is the production of ribose-5-phosphate and glucose-6-phosphate by way of the pentose phosphate cycle. Kinases for the hexoses glucose, sorbose, mannose, galactose and fructose, the hexonates gluconate, 2-ketogluconate and 5-ketogluconate and the pentoses xylulose and ribose have been noted (53), (54), (67). Fewster (54) believes that 5-ketogluconate is phosphorylated and then decarboxylated to xylulose-5-phosphate establishing a link between the oxidative non-phosphorylated and the phosphorylated pathways. Schramm et al. (117) report the rapid oxidation of glucose to 2-ketogluconate in Acetobacter xylinum with only a minor portion of glucose being phosphorylated directly. Glucose-6-phosphate, therefore, must arise mainly from the pentose phosphate pathway which is operative in whole cells and cell-free extracts. A. xylinum forms acetyl phosphate from pentose phosphate but more rapidly from

fructose-6-phosphate + P_i ⟶ acetyl phosphate + erythrose-4-phosphate (118).

The oxidative characteristics typical of the acetic acid bacteria are located mainly in the cytoplasmic membrane (37).

During the fermentation of glucose-1-C^{14} by Leuconostoc mesenteroides, the C^{14} appeared almost quantitatively in $C^{14}O_2$ and only traces of C^{14} appeared in ethanol and lactic acid and, hence, metabolism differed from the classical Embden-Meyerhof pathway where carbon one of glucose would appear in the methyl carbons of ethanol and lactic acid. The isotope data

indicated that lactate arose from carbons 4,5 and 6 of glucose and ethanol from carbons 2 and 3 (62). This information, together with the demonstration of a glucose-6-phosphate dehydrogenase (41), was strongly suggestive of the pentose phosphate pathway and was supported by the detection of a DPN dependent 6-phosphogluconate dehydrogenase (39). Blakely and Blackwood (5), (7) found that the organism readily adapted to the degradation of gluconate and 2-ketogluconate with labeled substrates yielding CO_2 from carbon 1 and the methyl group of lactic acid from carbon 6, thus being similar to the oxidative pathways. Ciferri et al. (25) subsequently effected a partial purification of a specific, adaptive 2-ketogluconate kinase from 2-ketogluconate grown cells and also demonstrated weak kinases for glucose and gluconate in glucose grown cells. Degradation of 2-ketogluconate in cell-free extracts and resting cells was shown to proceed via phosphorylation to 2-keto-6-phosphogluconate and reduction to 6-phosphogluconate by 2-keto-6-phosphogluconate reductase in the presence of either DPNH H^+ or reduced TPN (TPNH H^+). Concurrent decarboxylation and oxidation of 6-phosphogluconate, by a DPN specific 6-phosphogluconate dehydrogenase, produced CO_2 and ribulose-5-phosphate which was converted to xyulose-5-phosphate by pentose phosphate epimerase (8), (24). Anaerobic dissimilation of glucose differs somewhat from aerobic dissimilation as it appears in the former that electrons are transported to acetyl phosphate or a related metabolite to form ethanol, whereas in the latter, electrons are transferred to oxygen resulting in the production of acetic acid rather than ethanol (6).

An organism isolated from soil and identified as Neisseria winogradskyi (134) oxidized glucose and gluconate at approximately the same rate, with

production of CO_2, but did not oxidize 2-ketogluconate. Evidence was produced for the presence of gluconokinase but not for hexokinase or kinases for 2-ketogluconate or fructose-6-phosphate. In cell-free extracts, TPN was reduced with 6-phosphogluconate or gluconate plus ATP but not with gluconate alone. The TPN reduction was inhibited by iodoacetamide establishing the reduction to be a result of triose phosphate oxidation. Conversion of 2-keto-3-deoxy-6-phosphogluconate to triose phosphate and pyruvic acid by cell-free extracts confirmed the presence of the Entner-Doudoroff pathway. The production of sedoheptulose-7-phosphate from the incubation of extracts with ribose-5-phosphate probably indicates transketolase, phosphoriboisomerase and phosphoribulose epimerase. Enzymes of the Embden-Meyerhof pathway were readily demonstrated but the system was not believed to be functional. As 6-phosphogluconate was apparently not oxidized to pentose phosphate it seemed feasible that fructose-6-phosphate acts as the precursor of pentoses in the organism using the transaldolase and transketolase reaction sequence as described by Dickens (44). Glucose degradation appeared to proceed exclusively via the pathway of Entner and Doudoroff (135).

Hochster and Katznelson (70) have proposed glucose-6-phosphate oxidation in Xanthomonas phaseoli to proceed primarily by way of the Entner-Doudoroff pathway and secondarily by way of the pentose phosphate cycle. Cell-free extracts contain glucose-6-phosphate and 6-phosphogluconate dehydrogenases and convert ribose-5-phosphate to hexose phosphate and triose phosphate by the sequence involving transaldolase and transketolase. Pyruvic acid is produced rapidly from 6-phosphogluconate and a DPN specific 3-phosphoglyceraldehyde dehydrogenase, which is inhibited by iodoacetate,

is also present. Fructose-1,6-diphosphate reduces both DPN and TPN, DPN reduction being sensitive to iodoacetate, thereby indirectly illustrating the enzyme aldolase by measurement of triose phosphate dehydrogenase. Triphosphopyridine nucleotide reduction was insensitive to iodoacetate and, therefore, was essentially a measurement of glucose-6-phosphate dehydrogenase and an indirect measurement of fructose-1,6-diphosphate phosphatase and phosphohexoisomerase.

The Embden-Meyerhof pathway is constitutive in the wild type and mutant strains of Rhodopseudomonas spheroides but it appears to have a relatively limited function because of very low aldolase activity. Phosphogluconate dehydrogenase is either absent or too low in activity to permit utilization of the pentose-phosphate cycle. Glucose and mannose are oxidized to gluconic and mannonic acids and both are converted to 2-keto-3-deoxygluconate by the corresponding specific dehydrases. Cell-free extracts of the wild type contain kinases for glucose and fructose but not gluconate, phosphohexoisomerase, glucose-6-phosphate dehydrogenase but no 6-phosphogluconate dehydrogenase, fructose-1,6-diphosphate aldolase, triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, trace amounts of 2-keto-3-deoxygluconate kinase, an active 2-keto-3-deoxy-6-phospho-gluconate aldolase and phosphatases for 6-phosphogluconate and fructose-1,6-diphosphate. Fructose-6-phosphate or glucose-6-phosphate were produced from ribose-5-phosphate or ribose plus ATP but not from ribose alone, demonstrating the presence of transaldolase, transketolase, ribose kinase and pentose phosphate epimerase. In glucose and gluconate utilizing mutants, but not the wild type, 6-phosphogluconate dehydrase was shown to be present. Aerobic and photosynthetic oxidation of glucose is thought to proceed by

two pathways to the Entner-Doudoroff intermediate 2-keto-3-deoxy-6-phosphogluconate in the wild type. The first involves phosphorylation of glucose to glucose-6-phosphate followed by oxidation to 6-phosphogluconate with subsequent phosphatase and dehydrase activity to yield 2-keto-3-deoxygluconate which is then phosphorylated to 2-keto-3-deoxy-6-phosphogluconate. The second involves oxidation of glucose to gluconate, dehydration of gluconate to 2-keto-3-deoxygluconate followed by phosphorylation to give 2-keto-3-deoxy-6-phosphogluconate. The phosphorylated 2-keto-3-deoxy compound is cleaved by an aldolase to give pyruvate and triose phosphate, two triose phosphates combine to yield fructose-1,6-diphosphate which, after phosphatase activity, isomerizes to produce glucose-6-phosphate which can then recycle (133).

The pathway involving dehydration of 6-phosphogluconate to 2-keto-3-deoxy-6-phosphogluconate followed by a 3:3 split was established by Entner and Doudoroff (50), (97) in P. saccharophilia, as previously mentioned. It was thought by these workers that the established pathway was the only functional one existing in the organism and this was confirmed by Wang et al. (139) using the radiorespirometric technique. Stern et al. (124) concluded that terminal respiration proceeded by way of the conventional TCA cycle.

During aerobic glucose-C^1-C^{14} dissimilation in Pseudomonas lindneri, 99 per cent of the isotope appeared in CO_2 this, together with the detection of glucose-6-phosphate and 6-phosphogluconate dehydrogenases, was taken as evidence for the pentose phosphate pathway (40). Anaerobic degradation of glucose involved a route of ethanol formation differing from either the Embden-Meyerhof or pentose phosphate pathways as CO_2 was evolved from carbons one and four which is indicative of a 3:3 split. From studies with

labeled substrates it was found that carbons 2, 3 and 5, 6 of glucose become ethanol, with carbons 2 and 5 becoming the carbinol group as would result from an Entner-Doudoroff pathway (57).

Anaerobically, cell-free extracts of Pseudomonas hydrophila phosphorylate pentoses which are then converted to heptulose phosphate and finally to hexose phosphate and triose phosphate (71). Under aerobic conditions, TPN specific glucose-6-phosphate and 6-phosphogluconate dehydrogenases and a DPN specific 3-phosphoglyceraldehyde dehydrogenase were demonstrated. Hochster and Stone (126) concluded that insignificant amounts of pyruvate and 3-phosphoglyceraldehyde were generated via the Entner-Doudoroff pathway but they arose essentially from the pentose phosphate pathway.

The finding of rapid oxidation of gluconate to 2-ketogluconate followed by slow oxidation to CO_2 and pyruvate by whole cells of Pseudomonas fluorescens suggested to Koepsell (88) the operation of a functional pentose phosphate cycle. Entner and Stanier (51) blocked adaptive enzyme formation without disturbing enzymatic activities by exposing cell suspensions of the pseudomonad to ultra-violet light. With asparagine grown cells, oxidation of glucose was immediate and oxidation of gluconate and 2-ketogluconate proceeded after a lag. There was negligible oxygen uptake with gluconate and 2-ketogluconate in ultra-violet treated cells, and it was concluded, therefore, that oxidation of both gluconate and 2-ketogluconate was the result of adaptive enzyme formation. As glucose grown cells were found to oxidize glucose and gluconate rapidly and 2-ketogluconate only after a lag, it was felt the sequence of glucose to gluconate was important but 2-ketogluconate was not essential in the main pathway of

glucose oxidation. The presence of 2-ketogluconate after hexose oxidation was explained by the supposition that 2-keto-6-phosphogluconic acid was, in fact, the real intermediate but the action of a phosphatase caused the accumulation of the non-phosphorylated compound or two pathways are present -- the one involving 2-ketogluconate having a partial block.

Cell-free extracts of P. fluorescens oxidized glucose-6-phosphate and 6-phosphogluconate; this oxidation could be inhibited without affecting the oxidation of glucose and gluconate and, therefore, glucose-6-phosphate and 6-phosphogluconate could be eliminated as intermediates in glucose and gluconate oxidation (145). No CO_2 was produced during oxidation of glucose or gluconate but more than one mole/mole of substrate was evolved with the phosphorylated compounds. Both DPN and TPN were reduced during glucose-6-phosphate oxidation whereas only DPN catalyzed 6-phosphogluconate oxidation. Traces of pentose phosphate were formed during 6-phosphogluconate breakdown. Although glucose-6-phosphate and 6-phosphogluconate dehydrogenase were demonstrated, no hexokinase or gluconokinase could be found and hence, it was concluded that the organism was incapable of degrading glucose and gluconate through their phosphate esters and, therefore, the direct non-phosphorylated pathway was used (146), (147). With limiting amounts of TPN and with 6-phosphogluconate as substrate, equal amounts of pyruvate and triose phosphate were isolated accounting for 90 per cent of the substrate, the remaining 10 per cent was converted to pentose phosphate. Under optimal conditions 24 µmoles of 6-phosphogluconate were converted to pentose phosphate and 144 µmoles were cleaved to glyceraldehyde-3-phosphate and pyruvate thus suggesting the sequence involving the 3:3 split to be of major importance (148).

In 1954, Narrod and Wood (101) reported the phosphorylation of gluconate and 2-ketogluconate by dried cells and soluble enzyme preparations of P. fluorescens. At the same time two fractions were obtained from cell-free extracts: the first catalyzed the disappearance of 6-phosphogluconate without pyruvate formation but with the production of a phosphate ester, the second converted the intermediate phosphate ester, but not 6-phosphogluconate, to pyruvate and 3-phosphoglyceraldehyde (90). Subsequently, both 6-phosphogluconate dehydrase and 2-keto-3-deoxy-6-phosphogluconate aldolase were purified from cell-free extracts and their properties described (91), (92).

From isotopic studies, Lewis et al. (94) concluded that the major pathway of glucose catabolism in P. fluorescens was other than the Embden-Meyerhof pathway. The pentose phosphate pathway appeared to be of greater significance accounting for approximately one-half of glucose metabolism and the Entner-Doudoroff pathway accounting for one-third to one-half. Cell-free extracts of glucose grown cells caused more CO_2 evolution from bicarbonate in the presence of gluconate and 2-ketogluconate plus ATP than with ATP alone -- this served as evidence for kinases for the two substrates (102). Wood (144) had previously implicated 2-keto-6-phosphogluconate as an intermediate in glucose dissimilation with pyruvic acid a product of its degradation and later found TPNH H^+ oxidation with 2-keto-6-phosphogluconate as substrate. The reaction was catalyzed by 2-keto-6-phosphogluconate reductase and equimolar amounts of 6-phosphogluconate were formed. As one mole of 2-keto-6-phosphogluconate yields two moles of pyruvate, the pathway of its catabolism was believed to involve 6-phosphogluconate, 2-keto-3-deoxy-6-phosphogluconate, pyruvate and glyceraldehyde-3-phosphate, that is,

the Entner-Doudoroff pathway. The TPNH H^+ specific reduction of 2-keto-6-phosphogluconate was assumed to be coupled with the DPN specific oxidation of glyceraldehyde-3-phosphate by pyridine nucleotide transhydrogenase (56).

Accumulation and subsequent disappearance of both gluconate and 2-ketogluconate has been shown to occur in cultures of P. aeruginosa in a glucose-ammonium phosphate medium. Whole cells oxidized glucose, gluconate and 2-ketogluconate and, from manometric data, it was concluded that gluconate and 2-ketogluconate were intermediates in the oxidation of most, if not all, of the glucose oxidized by the organism (105). Dried cell preparations oxidized glucose with the uptake of one µmole O_2/µmole substrate and gluconate with the uptake of $\frac{1}{2}$ µmole O_2/µmole substrate. The only product of these oxidations detected was 2-ketogluconate, which indicated the absence of phosphate esters (106). Partial purification of gluconic dehydrogenase, which catalyzes the oxidation of gluconic acid without concurrent phosphorylation, was effected and pyocyanine was found to be the most active hydrogen acceptor (114). These data concerning the oxidative non-phosphorylated pathway of glucose to 2-ketogluconate in P. aeruginosa correspond very closely to the previously mentioned results obtained with P. fluorescens, the acetic acid bacteria, the adaptive oxidation of gluconate and 2-ketogluconate by Leuconostoc mesenteroides and the pyridine nucleotide dependent oxidation of glucose by B. cereus spore extracts.

Terminal respiration in P. aeruginosa has been shown to proceed by way of the TCA cycle with all the enzymes for the conventional cycle as well as a glyoxylate bypass (19), (21), (22).

The hydrolysis curves of phosphorylated intermediates of glucose degradation indicated compounds much more resistant to acid hydrolysis than hexose intermediates of the Embden-Meyerhof pathway. Further, under anaerobic conditions, whole cells did not utilize glucose or incorporate inorganic phosphate -- these results established good evidence for the absence of the Embden-Meyerhof pathway (18). Claridge and Werkman (26) found, as did Norris and Campbell (106) with dried cells, that cell-free extracts oxidized glucose and gluconate with no CO_2 evolution and the product isolated in each case was 2-ketogluconate. Hexokinase and gluconokinase were found by observing CO_2 release from bicarbonate in the presence of cell-free extract, ATP and glucose and gluconate. There was no evidence of phosphorylation of 2-ketogluconate. Diphosphopyridine nucleotide and triphosphopyridine nucleotide (27) were reduced in the presence of glucose-6-phosphate and fructose-6-phosphate but not by intermediates of the non-phosphorylated pathway and DPN but not TPN was reduced by ribose-5-phosphate. A phosphatase for fructose-1,6-diphosphate was detected and the compound also reduced TPN and DPN indicating triose phosphate dehydrogenase. These data suggested the possibility of two schemes of glucose degradation to Claridge and Werkman; hexokinase, aldolase and triose phosphate dehydrogenase suggested the Embden-Meyerhof scheme, this was contrary to the previously mentioned conclusions of Campbell et al. (18), and pyridine nucleotide reduction with glucose-6-phosphate suggested the pentose phosphate pathway. Analysis of reaction mixtures of whole cells and labeled 2-ketogluconate revealed labeled amino acids and nucleic acids demonstrating 2-ketogluconate supported oxidative assimilation. Labeled TCA cycle intermediates and glucose-6-phosphate were also found and the latter compound

was believed to have arisen from either a reversal of the Embden-Meyerhof scheme or the reversal of the non-phosphorylated pathway (28).

Campbell et al. (17), (20) showed that almost identical amounts of growth were obtained when limiting equimolar amounts of glucose, gluconate and 2-ketogluconate were used as the sole source of carbon and concluded that P. aeruginosa did not gain energy during the oxidative steps from glucose to 2-ketogluconate. Later, however, Strasdine and Campbell (129) followed P^{32} incorporation into resting cells and found the more oxidized substrates, that is gluconate and 2-ketogluconate, supported considerably less P^{32} incorporation indicating phosphorylation to be coincident with the passage of electrons to oxygen through the electron transport chain.

DeLey and Vandamme (38) found 2-keto-gluconate kinase to be absent in some 2-ketogluconate grown organisms that metabolized the compound, however, when P. aeruginosa was incubated with 2-ketogluconate, a compound identified as 2-keto-6-phosphogluconate, was produced. High 6-phosphogluconate dehydrase and 2-keto-3-deoxy-6-phosphogluconate aldolase activities were exhibited in cell-free extracts establishing good evidence for the Entner-Doudoroff pathway (92).

Data has been presented to indicate that the enzyme systems for dissimilation of glucose are not constitutive in P. aeruginosa (104), however, Eagon et al. (47), (142) believe the enzymes for glucose are constitutive but the enzymes for mannose and fructose dissimilation are adaptive, with mannose being converted to fructose which, in turn, is converted to glucose. Eagon et al. (47), (48) believe that glucose grown cells are impermeable to fructose and adaptation to fructose involves the induction of a transport mechanism located in the cell membrane, as

intact protoplasts of glucose grown cells did not oxidize fructose whereas ruptured protoplasts did. Eagon (142) has concluded that the major pathway of carbohydrate dissimilation is via the pentose phosphate pathway. Phosphoglucoisomerase, phosphomannose isomerase, glucokinase, 6-phosphogluconate dehydrogenase and 2-keto-6-phosphogluconate reductase have been demonstrated in cell-free extracts (115).

Hamilton and Dawes (66) found the dehydrogenases of the non-phosphorylated pathway, kinases for glucose, gluconate and 2-ketogluconate, glucose-6-phosphate and 6-phosphogluconate dehydrogenases and the dehydrase and the aldolase of the Entner-Doudoroff scheme.

Wang et al. from their radiorespirometric data, have concluded that glucose is dissimilated 71 per cent by way of the Entner-Doudoroff pathway and 29 per cent by way of the pentose phosphate pathway, whereas gluconate is utilized exclusively via the Entner-Doudoroff route (138). The interpretation of results from the use of C^{14} labeled substrates has been justly criticized by Katz and Wood (85) who state that the dilution of substrate by recycling and incomplete CO_2 yields must be considered and, even then, no useful quantitative information and only very limited qualitative information on patterns of glucose metabolism can be obtained.

Evidence has been presented in the literature for the absence of active participation of the Embden-Meyerhof scheme in glucose degradation in P. aeruginosa. The presence of 6-phosphogluconate dehydrogenase has indicated the pentose phosphate cycle and 6-phosphogluconate dehydrase and 2-keto-3-deoxy-6-phosphogluconate aldolase have been suggestive of the presence of the Entner-Doudoroff pathway. The oxidative non-phosphorylated pathway has been adequately demonstrated and it is believed to be linked

to the phosphorylated pathways by 2-ketogluconate kinase. Glucose dissimilation in this organism, then, appears to be similar, at least in some respects, to a number of bacteria including the acetic acid bacteria, B. cereus spores, Leuconostoc, Rhodopseudomonas and particularly P. fluorescens.

MATERIALS AND METHODS

I. Organism

Pseudomonas aeruginosa ATCC 9027, the organism used during this study, was maintained in a glucose-ammonium salts medium and stored at 6°C after a twenty-four hour growth period. The culture medium contained the following constituents: 0.1 per cent yeast extract, 0.3 per cent ammonium dihydrogen phosphate, 0.2 per cent dipotassium hydrogen phosphate, 0.2 per cent glucose, 0.5 ppm iron as ferrous sulfate, with pH adjusted to 7.0 and magnesium sulfate heptahydrate added after sterilization to a final concentration of 0.1 per cent. Stock cultures were periodically streaked onto Plate Count agar and examined for both typical colonial and cellular morphology.

Cells required for experimental procedures were obtained by inoculating Roux flasks, containing 100 ml of glucose-ammonium salts medium, with a 1 per cent inoculum from a fresh twenty-four hour stock culture transfer and incubating for twenty hours at 30°C. The cells were harvested by centrifugation at 5,860 x g for 10 minutes at 5°C in a Servall refrigerated centrifuge.

II. Preparation of Cell-free Extracts

1. Hughes' press

The harvested cells were washed twice with distilled water and once with a 0.2 mg per cent aqueous solution of glutathione, with all centrifugation procedures being carried out at 6°C for 10 minutes at 1000 x g. The resulting pellet was packed into open-ended pyrex vials with an inside

diameter of either 11 or 15 mm. The vials were tightly sealed with rubber stoppers and the cells quickly frozen by placing the vials in an ethanol dry-ice bath for 30 minutes. The frozen cells were subsequently stored at -18°C. No loss in activity of the enzymes concerned was observed during storage periods of three months.

As required, cell-free extracts were prepared by crushing the frozen cells in a Hughes' press (82), previously cooled to -22°C, by applying pressure of approximately 12,000 pounds p.s.i. with a Carver hand-operated hydraulic press. The resulting pressate was immediately placed in a teflon Potter homogenizer. A mixture of cold M/1 tris(hydroxymethyl) aminomethane (Tris) buffer pH 7.4 and diluent, in a ratio of 1:6.66, was added to yield an approximate concentration of 200 mg wet weight of cells/ml. The diluent was composed of 0.05 M glycylglycine, 0.05 M Tris and 500 mg per cent egg albumin in distilled water. Prior to homogenization, 0.1 mg of a commercial diphosphopyridine nuclease (DNase) preparation was added to each 10 ml of suspension to decrease the viscosity of the extract. After homogenizing, the suspension was subjected to centrifugation at 6°C for 10 minutes at 1000 x g to remove whole cells. The supernatant fluid was removed and kept in ice. All enzymatic assays were carried out within twelve hours of the preparation of the cell-free extract. Cell-free extracts prepared in this manner will hereafter be referred to as pressates in order to distinguish them from cell-free extracts prepared by the process involving cell lysis.

2. Lysozyme treatment (128a)

Cells harvested from 500 ml of the previously described growth medium were washed once in 0.03 M Tris buffer pH 8.0 and resuspended in the same

buffer to a volume of 2.5 ml. To the suspension was added 2.4 ml of 0.125 M Tris pH 8.0, 3.2 ml of versene pH 8.0 (32 mg/ml) and 1.6 ml of a commercial preparation of lysozyme (4 mg/ml). The mixture was stirred by means of a magnetic stirrer for 30 minutes at room temperature. A 1 mg/ml solution of DNase (0.075 ml) and 0.5 ml of a one molar solution of $MgCl_2$ were added and the mixture was stirred for approximately 5 minutes at room temperature. The suspension was subjected to centrifugation at 5°C for 20 minutes at 5,860 x g, the supernatant fluid removed and the centrifugation procedure repeated. The resulting supernatant fluid was kept in ice until needed.

III. Manometric Studies

1. Oxygen uptake

The oxygen consumption of pressates was followed at 30°C by means of a conventional Warburg respirometer. A typical reaction mixture is represented as follows:

	Endogenous	Test
Pressate (200 mg wet weight of cells/ml)	1.00 ml	1.00 ml
M/1 Tris buffer pH 7.4	0.20 ml	0.20 ml
$MgCl_2$ (100 µM/ml)	0.20 ml	0.20 ml
Substrate (25µM/ml)	--	0.20 ml
Distilled water	1.60 ml	1.40 ml
20 % KOH	0.15 ml	0.15 ml

Variance in procedure, such as the addition of co-factors, was accompanied by an appropriate decrease in the volume of distilled water.

2. Carbon dioxide evolution

The evolution of carbon dioxide during the dissimilation of carbohydrates by pressates was measured in a conventional Warburg respirometer at 30°C by the direct method as described by Umbreit, Burris and Stauffer (137).

3. Carbon dioxide evolution from sodium bicarbonate

The evolution of carbon dioxide from $NaHCO_3$ in the presence of pressate, substrate and ATP would serve as an indication of substrate phosphorylation at pH 7.5 or higher, as the transfer of a phosphate group from ATP liberates an acid equivalent and consequently an equivalent of CO_2 is evolved. The process was measured according to the method of Colowick and Kalkar (29). Incubation temperature was 30°C, the atmosphere was 5 per cent carbon dioxide and 95 per cent nitrogen and the center well contained freshly cut yellow phosphorous. A typical test reaction mixture follows:

Pressate		1.00 ml
$NaHCO_3$ pH 7.5	(63 µM/ml)	1.00 ml
$MgCl_2$	(100 µM/ml)	0.10 ml
ATP	(25 µM/ml)	0.40 ml
Substrate	(25 µM/ml)	0.40 ml
Distilled water		0.25 ml

IV. Spectrophotometric Enzyme Assays

Enzyme assays involving the oxidation or reduction of pyridine nucleotides were followed by measuring changes in optical density at 340 mµ with a Beckman model DU spectrophotometer.

In all assays, substrates were present in excess and, therefore, the reactions were initially zero order -- the rate being dependent on enzyme concentration until the pyridine nucleotide concentration became limiting. All enzyme activities were expressed as change in optical density per unit time or as specific activity which, using Kornberg units (89), is essentially the change in optical density at 25 - 30°C/minute/mg protein of pressate.

1. Glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase activity in pressates was determined by measuring TPN reduction during the conversion of glucose-6-phosphate to 6-phosphogluconate. A modification of the method of Kornberg was used (89) and a typical assay was as follows:

	Pressate control	Substrate control	Test
M/5 Tris buffer pH 7.4	0.20 ml	0.20 ml	0.20 ml
$MgCl_2$ (50 µM/ml)	0.05 ml	0.05 ml	0.05 ml
Glucose-6-phosphate (25 µM/ml)	--	0.10 ml	0.10 ml
Pressate	0.05 ml	--	0.05 ml
Distilled water	0.68 ml	0.63 ml	0.58 ml
TPN .005 M	0.02 ml	0.02 ml	0.02 ml

All constituents, with the exception of TPN, were added to the cuvette and the instrument zeroed on the reaction mixture. Triphosphopyridine nucleotide was added at zero time, the reaction mixture stirred and the increase in optical density recorded.

2. 6-phosphogluconate dehydrogenase

The procedure for 6-phosphogluconate dehydrogenase detection was similar to the glucose-6-phosphate dehydrogenase assay using 6-phospho-

gluconate as substrate.

3. Phosphohexoisomerase and phosphomannose isomerase

The presence of these enzymes in pressates was determined through the conversion of fructose-6-phosphate and mannose-6-phosphate to glucose-6-phosphate followed by oxidation of the latter compound to 6-phosphogluconate with concurrent TPN reduction. The glucose-6-phosphate dehydrogenase assay was used with fructose-6-phosphate and mannose-6-phosphate as substrates.

4. Glucokinase

Glucokinase was measured indirectly by following glucose-6-phosphate dehydrogenase activity in the presence of glucose, ATP and pressate. The rate of TPN reduction, through dehydrogenase activity, was directly dependant on the activity of the kinase. Wood and Schwerdt (147) have stated that dehydrogenase activity is inhibited by ATP and, hence, a commercial source of glucose-6-phosphate dehydrogenase is generally employed for this assay. It was found, however, that a four-fold increase in Mg^{++} concentration alleviated inhibition by ATP and effected an acceptable kinase assay without the addition of an external glucose-6-phosphate dehydrogenase source. The procedure for glucokinase determination was essentially the same as that for glucose-6-phosphate dehydrogenase but with an increase in $MgCl_2$ from 0.05 ml to 0.2 ml and the addition of 0.1 ml ATP (25 µM/ml).

5. Hexokinase

This enzyme was assayed by the procedure for glucokinase using mannose or fructose, instead of glucose, as substrate.

6. Gluconokinase

Gluconokinase was measured indirectly by following 6-phosphogluconate dehydrogenase activity in the presence of gluconate, ATP and pressate.

The procedure for glucokinase was employed but with gluconic acid as the substrate.

7. 2-keto-3-deoxy-6-phosphogluconate aldolase

The products of 2-keto-3-deoxy-6-phosphogluconate aldolase action are 3-phosphoglyceraldehyde and pyruvic acid. The presence of pyruvic acid in reaction mixtures was used as an indication of the existence of the enzyme in pressates. The appearance of pyruvic acid from 2-keto-3-deoxy-6-phosphogluconate[2] incubated with pressates was followed by measuring DPNH H^+ oxidation resulting from the reduction of pyruvic acid to lactic acid in the presence of an excess of a commercial source of lactic dehydrogenase. Iodoacetic acid was used to inhibit diphosphopyridine nucleotide reduction by 3-phosphoglyceraldehyde dehydrogenase. The following reaction mixtures were used.

	Pressate control	Substrate control	Test
M/5 Tris buffer pH 7.4	0.20 ml	0.20 ml	0.20 ml
$MgCl_2$ (50 µM/ml)	0.05 ml	0.05 ml	0.05 ml
2-keto-3-deoxy-6-phosphogluconate (25 µM/ml)	--	0.10 ml	0.10 ml
Iodoacetate (10 mg/ml)	0.10 ml	0.10 ml	0.10 ml
Lactic dehydrogenase (30 µg protein/ml)	0.03 ml	0.03 ml	0.03 ml
DPNH H^+ .005 M	0.02 ml	0.02 ml	0.02 ml
Pressate	0.015 ml	--	0.015 ml
Distilled water	0.585 ml	0.50 ml	0.485 ml

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2. 2-keto-3-deoxy-6-phosphogluconate was kindly supplied by Dr. W.A. Wood, Department of Agricultural Chemistry, Michigan State University.

All the components, with the exception of 2-keto-3-deoxy-6-phosphogluconate, were added to the cuvette and the instrument was "zeroed" at an optical density reading of 0.500. The substrate was added at zero time, the reaction mixture stirred and the decrease in optical density was recorded.

8. 6-phosphogluconate dehydrase

This enzyme was measured indirectly by following the conversion of pyruvic acid to lactic acid accompanied by $DPNH+H^+$ oxidation. The rate of activity was dependant on the initial dehydration of 6-phosphogluconate to 2-keto-3-deoxy-6-phosphogluconate. The assay for 2-keto-3-deoxy-6-phosphogluconate aldolase was used with 0.05 ml pressate and 6-phosphogluconate as substrate.

9. 3-phosphoglyceraldehyde dehydrogenase

As 3-phosphoglyceraldehyde was not available, and, as the pressate was found to contain an active 2-keto-3-deoxy-6-phosphogluconate aldolase, the presence of the dehydrogenase was measured by observing TPN reduction in the presence of 2-keto-3-deoxy-6-phosphogluconate. The procedure was identical to the glucose-6-phosphate dehydrogenase assay.

10. Pyruvic kinase

Pyruvic kinase was assessed through the detection of pyruvic acid in pressates incubated with phospho-enol-pyruvate. The assay for 2-keto-3-deoxy-6-phosphogluconate aldolase was made use of with the following exceptions: iodoacetate was omitted, 0.1 ml KCl (50 µM/ml) was added, the amount of pressate increased to 0.05 ml and phospho-enol-pyruvate was the substrate.

11. 3-phosphoglyceric acid kinase

The measurement of this enzyme involved the determination of the phosphorylation of 3-phosphoglyceric acid in the presence of ATP to yield

1,3-diphosphoglyceric acid followed by reduction of the latter compound to 3-phosphoglyceraldehyde accompanied by concurrent TPNH H^+ oxidation. The glucokinase assay was used with 3-phosphoglyceric acid as the substrate and TPNH H^+ the co-factor. The components of the reaction mixture, except the substrate, were added to the cuvette and the instrument "zeroed" at an optical density reading of 0.500. The substrate was added at zero time and the decrease in optical density was recorded. Appropriate substrate and pressate controls were employed.

12. Enolase and phosphoglyceromutase

These enzymes were assayed by the method for 2-keto-3-deoxy-6-phosphogluconate aldolase. Three-phosphoglyceric acid was the substrate, 5 µM K^+ and 0.02 ml of a 500 µg/ml solution of a commercial preparation of pyruvic kinase were added and the decrease in optical density at 340 mµ was recorded.

13. Fructose-1,6-diphosphate aldolase

As the enzyme converts the substrate into dihydroxyacetone phosphate and 3-phosphoglyceraldehyde, the spectrophotometric assay for 3-phosphoglyceraldehyde dehydrogenase activity, involving TPN reduction, was made use of with fructose-1,6-diphosphate as substrate.

14. Phosphoriboisomerase, phosphopentoepimerase and transketolase

These enzymes are responsible for the production of glyceraldehyde-3-phosphate from ribose-5-phosphate and, therefore, their presence in pressates was assayed for by measuring TPN reduction in the presence of ribose-5-phosphate in the 3-phosphoglyceraldehyde dehydrogenase determination.

15. 2-keto-6-phosphogluconate reductase

The enzyme reduces 2-keto-6-phosphogluconate to 6-phosphogluconate in

the presence of TPNH H^+. The assay, a modification of the method of Reid (115), consisted of measuring the decrease in optical density at 340 mμ. The quantities of constituents were the same as those in the glucose-6-phosphate dehydrogenase assay with 2-keto-6-phosphogluconate[3] as substrate. TPNH H^+ replaced TPN, the substrate was added at zero time and the spectrophotometer set for measuring the oxidation of reduced pyridine nucleotides, as previously described.

16. 2-ketogluconate kinase

Detection of this enzyme in cell-free extracts was attempted by utilizing the 2-keto-6-phosphogluconate reductase assay and measuring TPNH H^+ oxidation. The amounts of constituents in the reaction mixture were the same as those used for glucokinase determination with 2-ketogluconate replacing glucose as substrate.

V. Isolation and Identification of Carbohydrates and their Phosphate Esters

1. Paper electrophoresis

A flat plate, water cooled, enclosed electrophoresis apparatus similar to Resco model E-800-2B was used with a Resco model 1911 power supply. The buffer system was 0.1 M ammonium carbonate ($NH_4HCO_3.NH_2COONH_4$ - Analar) pH 8.6. Standard and unknown solutions were spotted onto Whatman No. 3m.m. paper and, after drying the spotted areas, the paper was dipped in M/10 ammonium carbonate buffer and placed on the flat plate of the electrophoresis apparatus. The current was applied for 1.5 or 2 hours at maximum voltage which varied between 600 and 750 volts depending on the width of the paper used. The paper was subsequently dried in a hot air oven at approximately

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3. 2-keto-6-phosphogluconate was kindly supplied by Dr. F. J. Simpson, National Research Council of Canada, Prairie Regional Laboratory.

75°C prior to development.

2. Developers

a. Alkaline silver nitrate

Because of its marked sensitivity, a modification (2) of the silver nitrate spray of Trevelyn et al. (136) was used to locate carbohydrate spots. After the electrophoretic procedure had been completed, the dried paper was dipped into a silver nitrate - acetone solution which had been prepared by adding 0.1 ml of saturated aqueous $AgNO_3$/20 ml C.P. acetone and dissolving the precipitated $AgNO_3$ by dropwise addition of distilled water. The paper was dried at approximately 90°C and then dipped into a solution of 0.5 N NaOH in 70% ethanol until the spots appeared as dark brown areas against a light brown background. Before drying, the excess Ag_2O in the background was removed by dipping the paper in 5% aqueous solution of $Na_2S_2O_3 \cdot 5H_2O$. This latter treatment resulted in black spots of varying intensity on a white background.

b. Aniline hydrogen phthalate

After electrophoresis, the dried paper was dipped in the following solution and dried in a hot air oven at 90-100°C for approximately 10 minutes.

aniline	0.93 gm
phthalic acid	1.66 gm
water saturated butanol	100 ml (111).

The developer is relatively specific for compounds that readily form furfural derivatives on acid pyrolysis (61).

Standard carbohydrate solutions gave characteristic colors.

glucose, glucose-6-phosphate
fructose, fructose-6-phosphate . . . . . yellow-brown

fructose-1,6-diphosphate	. . . . . pale yellow
pentoses, pentose phosphates 2-ketogluconate 2-keto-6-phosphogluconate 2-keto-3-deoxy-6-phosphogluconate	. . . . pink
gluconate, 6-phosphogluconate	. . . . . no color

This spray served to differentiate compounds that possessed the same rate of mobility under the conditions of electrophoresis employed. For example, glucose and ribose did not move from the origin and, therefore, with the silver nitrate developer both compounds gave black spots which could not be distinguished from one another. With aniline hydrogen phthalate the two carbohydrates were readily differentiated. In a similar manner, 2-ketogluconate or its' phosphate ester could be distinguished from gluconate or its' phosphate ester.

c. Potassium permanganate

Dried paper, previously subjected to electrophoresis, was dipped into a solution containing 1 ml of a 4.8% aqueous solution of potassium permanganate per 29 ml of C.P. acetone and air dried (61). Immediately after dipping, spots appeared as yellow areas gradually fading to white -- depending on both the quantity and reducing ability of the compound in the area involved. The background faded fairly rapidly from a magenta color to a yellow-brown and consequently spots were marked as soon as they appeared. The spray had a low specificity reacting with alcohols, amines and amino acids as well as carbohydrates.

d. Acidic naphthoresorcinol

The developer is specific for fructose and fructose esters (10) and was used for the qualitative detection of fructose-6-phosphate. Test and

standard solutions were spotted on Whatman No. 3 mm paper, dipped into the following solution and heated at 85°C for approximately 10 minutes.

4.75 ml 2% alcoholic naphthoresorcinol

0.50 ml 60% perchloric acid

4.75 ml 2% aqueous CCl_3COOH

Standard solutions produced characteristic colors:

fructose, fructose-6-phosphate fructose-1,6-diphosphate and fructose-6-phosphate plus glucose-6-phosphate	red
ribose-5-phosphate	blue-green
glucose, mannose-6-phosphate	pale blue
glucose-6-phosphate	trace of blue

3. Isolation of carbohydrates and their phosphate esters from reaction mixtures

Reaction mixtures, in a total volume of 15.75 ml, were added to large single side arm Warburg flasks and incubation carried out at 30°C either aerobically or in an atmosphere of 5 per cent carbon dioxide and 95 per cent nitrogen using a conventional Warburg respirometer. The following was a typical reaction mixture.

Pressate (200 mg wet weight of cells/ml)	5.0 ml
M/1 Tris buffer pH 7.4	1.0 ml
ATP (25 µM/ml)	1.25 ml
$MgCl_2$ (100 µM/ml)	1.0 ml
Substrate (25 µM/ml)	1.0 ml
Distilled water	6.5 ml

Warburg flasks, containing reactions to be examined aerobically, did not

contain potassium hydroxide. Anaerobic reactions, however, were performed in the presence of freshly cut yellow phosphorous added to a vessel which was placed in the center of the Warburg flask.

At the termination of the incubation period the reaction mixture in each flask was subjected to the following treatment. Approximately 0.5 ml of wet 200-400 mesh Dowex 50 in the ammonium form was added, the flask shaken and allowed to stand at room temperature for a few minutes. To the reaction mixture was then added 0.75 ml of 60 per cent perchloric acid and, after standing in ice for 20 minutes, the mixture was centrifuged at 1000 x g for 10 minutes at 6°C. All subsequent centrifugations were carried out under the same conditions for the same length of time. The supernatant fluid was removed, the precipitate washed with 2 ml of 12 per cent perchloric acid and allowed to stand in ice for 10 minutes prior to centrifugation. Following centrifugation, the precipitate was discarded and the supernatant fluid combined with the supernatant fluid from the first spinning. The fluid was neutralized with 5 N potassium hydroxide to remove excess perchloric acid and after standing in ice for 10 minutes subjected to centrifugation. The resulting precipitate was discarded and the supernatant fluid made slightly acid with 10 per cent acetic acid followed by the batchwise addition of 340 mg of acid washed charcoal to reduce the nucleotide content. The mixture was allowed to stand 10 minutes at room temperature, subjected to centrifugation, the precipitate discarded and the supernatant fluid was passed through Whatman No. 1 filter paper in a Buchner funnel to remove last traces of charcoal. The filtrate was evaporated to approximately 5-6 ml with a Craig flask evaporator, neutralized with potassium hydroxide, left in ice for 10 minutes and then, sub-

jected to centrifugation. The resulting precipitate was discarded, the supernatant fluid made slightly acid with acetic acid and passed through a 200-400 mesh Dowex 50 H^+ column, 0.7 cm in diameter and approximately 7 cm in height, in an effort to remove cations and amino acids. The column was washed with distilled water equal to approximately four times the volume of the concentrated fluid applied to the ion-exchange resin. The eluate from the column was evaporated to dryness with a Craig flash evaporator, dissolved in 10 ml distilled water and again evaporated to dryness. This procedure was repeated until no odor of acetic acid could be detected. The final dried material was dissolved in 0.6-0.7 ml of distilled water and streaked onto Whatman No. 3 m.m. paper in preparation for preliminary electrophoresis to remove interferring ions.

Electrophoresis of the material proceeded for 2 hours as previously described and, when the paper had been dried, a thin strip was cut from an outer edge and developed with alkaline silver nitrate to locate carbohydrates. The area, or areas, of the filter paper containing carbohydrates were cut out and eluted with distilled water in a manner analogous to descending paper chromatography. The aqueous solution was evaporated to dryness in a flash evaporator and dissolved in 0.4 ml of distilled water in preparation for identification of the compounds by electrophoretic means.

VI. Analytical Procedures

1. Protein determination

The protein concentration of cell-free extracts was measured according to the method of Lowry et al. (96). Cell-free extracts were diluted 1:100, the diluent used in the preparation of pressates was diluted 1:20 and stan-

dard curves were prepared from crystalline egg albumin. The sensitivity of the test is 25-500 µg of protein per tube.

To 0.4 ml of diluted test solution was added 2 ml of a solution containing 0.5 ml of 0.1 per cent $CuSO_4 \cdot 5H_2O$ and 0.5 ml of $NaKC_4H_2O_6 \cdot 4H_2O$ per 50 ml of 2 per cent Na_2CO_3 in 0.1 N NaOH. The tubes were mixed, allowed to stand at room temperature for 10 minutes and 0.2 ml of Folin-Ciocalteau phenol reagent, diluted with distilled water to make 1 N in acid, was added and the tube contents mixed. Optical density readings were taken after 30 minutes at 500 mµ with a Beckman model B spectrophotometer. The optical density readings were applied against the standard curve and appropriate calculations, involving dilution factors and presence of diluent, yielded results in mg of protein/ml of cell-free extract.

2. Inorganic phosphate

Inorganic phosphate determinations on supernatant fluids of Warburg reaction mixtures were performed according to the method of King (87). The reagents used were:

60% C.P. perchloric acid free of inorganic phosphate

5% molybdate -- 5 gm of $(NH_4)_6Mo_7O_2 \cdot 4H_2O$ in 100 ml of distilled water and filtered

Reducing agent -- 0.1 gm 1-amino-2-naphthol-4-sulphonic acid, 6.0 gm anhydrous $NaHSO_3$ and 1.2 gm anhydrous Na_2SO_3 dissolved in 50 ml of distilled water and filtered

To 2.0 ml of test solution, the reagents were added in the following order with shaking after each addition:

2.0 ml perchloric acid

2.5 ml molybdate

1.0 ml reducing agent

A standard curve was prepared from KH_2PO_4 with a range of 10-80 µg inorganic phosphate/tube. After 5 minutes optical density readings were taken at 660 mµ with a Beckman model B spectrophotometer. The readings were applied against the standard curve and calculations converted the readings to µM of inorganic phosphate released/reaction mixture.

3. Qualitative glucose determination

The presence of glucose in test reaction mixtures was determined qualitatively by the method of White and Secor (143).

To two drops of test solution on a spot plate was added 1 drop of a 2 mg/ml solution of a commercial glucose oxidase preparation. After 15 minutes at room temperature, one drop of an aqueous solution of 1 per cent soluble starch and 5 per cent KI was added and immediately followed by the addition of one drop of 5 per cent aqueous KIO_3. A blue color denoted a positive reaction and the test was specific for glucose. Hydrogen peroxide was formed during the enzymatic oxidation of glucose to gluconic acid and the hydrogen peroxide reacted with iodide to liberate iodine which, in turn, reacted with starch to produce a blue color.

4. Qualitative assay for glucose-6-phosphate

Glucose-6-phosphate was detected qualitatively in reaction mixtures by following TPN reduction in the presence of the test solution and a commercial source of glucose-6-phosphate dehydrogenase. The Beckman model DU spectrophotometer was zeroed on the reaction mixture, TPN was added at zero time and the increase in optical density at 340 mµ recorded. The following represents a typical assay.

	Substrate control	Enzyme control	Test
M/5 Tris buffer pH 7.4	0.20 ml	0.20 ml	0.20 ml
Mg^{++} 50 µM/ml	0.05 ml	0.05 ml	0.05 ml
Glucose-6-phosphate 25 µM/ml or	0.10 ml or	--	0.10 ml or
Test solution	0.05 ml	--	0.05 ml
Distilled water	0.63 ml or	0.68 ml	0.63 ml or
	0.68 ml		0.58 ml
TPN .005 M	0.02 ml	0.02 ml	0.02 ml
Glucose-6-phosphate dehydrogenase 10 mg/ml	--	0.05 ml	0.05 ml

Fructose-6-phosphate and mannose-6-phosphate as well as glucose-6-phosphate caused TPN reduction in the presence of the commercial enzyme preparation. The enzyme was obviously contaminated with phosphohexoisomerase and phosphomannose isomerase and, therefore, the test was specific for hexose phosphate.

6. Qualitative determination of deoxy sugars

The assay was carried out according to a modification of the method of Seibert (123).

One gram of diphenylamine was dissolved in 100 ml of glacial acetic acid and 2.75 ml of concentrated sulfuric acid were added. To 0.4 ml of this reagent were added 0.1 ml of test solution and 0.1 ml of distilled water. The solution was heated at 100°C for 10 minutes. The absorption maximum of the color produced was determined with a Beckman model DU spectrophotometer.

Ribose, gluconate and 2-ketogluconate standard solutions produced colorless solutions, deoxyadenosine produced a blue solution with an

absorption maximum at 595 mμ and 2-keto-3-deoxy-6-phosphogluconate produced a purple-blue solution with an absorption maximum at 550 mμ.

Substrates

All inhibitors, co-factors and substrates were neutralized before being added to the test reaction mixtures in the various assay procedures employed. Sodium and potassium salts of substrates were neutralized with KOH or NaOH. Substrates existing as the calcium or barium salt were treated with Dowex 50 in the hydrogen form to remove the divalent cations and then neutralized with KOH or NaOH.

EXPERIMENTAL RESULTS AND DISCUSSION

I. The Non-phosphorylated Oxidative Pathway of Glucose Dissimilation

The oxidation of glucose to 2-ketogluconate, without the involvement of phosphorylation, was reported to occur in Pseudomonas aeruginosa by Stokes and Campbell in 1951 (106). They found that dried cell preparations of the organism oxidized glucose with the uptake of 2 µatoms of oxygen/µmole of glucose, gluconic acid was oxidized with the uptake of 1 µatom of oxygen/µmole of substrate and there was no oxygen uptake with 2-ketogluconate. At the completion of the reaction, 2-ketogluconate was identified by paper chromatography as being the only compound present and it was concluded that 2-ketogluconate was the end product of oxidation of the two substrates. Identical manometric data were obtained with cell-free extracts prepared by sonication (26). Subsequently gluconic acid dehydrogenase was isolated from sonicated cells and it was found to catalyze the oxidation of gluconic acid without concurrent phosphorylation (114). Neither DPN nor TPN stimulated the activity of the enzyme nor were either reduced during substrate oxidation as was indicated by the absence of an increase in optical density at 340 mµ.

Cell-free extracts prepared with a Hughes' press provided the same oxygen uptake data as dried cells and sonicates with glucose and gluconic acid. Gluconolactone also was found to be oxidized with the uptake of 1 µatom of oxygen/µmole substrate (72). This latter observation coincided with the findings of Brodie and Lipmann (13) who found the oxidation of glucose to gluconic acid by sonic cell-free extracts of Azotobacter vinelandii, to proceed by two distinct enzymatic reactions. The first

involved the oxidation of the aldehyde to the lactone and the second involved the enzymatic hydrolysis of the lactone to yield the acid.

Before initiating an investigation of phosphorylated pathways in the organism, it was considered advisable to follow again the sequence of glucose oxidation by pressates in the absence of ATP. Oxygen consumption was measured by the standard aerobic technique with a Warburg respirometer. After endogenous oxygen uptake values had been subtracted, it was found consistently that when 5 µM of glucose were incubated with pressate in the presence of M/2 phosphate buffer pH 7.4 1µatom of oxygen/µM of substrate was taken up. This was contrary to the previously reported results.

When M/1 Tris buffer pH 7.4 was substituted for phosphate buffer in an otherwise identical reaction mixture, oxygen uptake values averaging 1.63 µatom of oxygen/µM of glucose were obtained. The addition of 20 µM of Mg^{++} to reaction mixtures containing either phosphate or Tris buffer resulted in the consumption of 2 µatoms of oxygen/µmole of glucose. Further, if glucose oxidation was followed in phosphate buffer in the absence of Mg^{++} and oxygen uptake was allowed to level off at 1µatom/µM substrate, the addition of 20 µM of Mg^{++} caused the uptake of a second µatom of oxygen. It became obvious, then, that an enzyme involved in the oxidative sequence of glucose to 2-ketogluconate had a Mg^{++} or at least, a divalent cation requirement.

As the organism was grown in a medium containing Mg ions, it is reasonable to suggest that trace amounts of Mg^{++} would be carried over into the pressate. Ammonia has been shown to be an end product of endogenous respiration in this organism and it can be demonstrated in freshly harvested and washed whole cell suspensions (141). Ammonia, therefore, was also

present in pressates probably from both the endogenous source and as a carry-over from the growth medium. It is feasible, then, that in the reaction mixtures containing phosphate buffer an ammonium-magnesium-phosphate complex was formed which effectively removed Mg^{++} from the suspension thus negating its function as an enzyme catalyst. The incomplete oxidation of glucose to 2-ketogluconate in the presence of Tris buffer may be explained on the basis of a deficiency of Mg ions.

The oxidation of glucose to gluconic acid requires one atom of oxygen, the manometric results, therefore, suggested gluconate as being the product of glucose oxidation in the presence of phosphate buffer. However, when gluconic acid was employed as substrate either in the presence or absence of Mg ions, one µatom of oxygen was consumed per µmole of substrate. With gluconolactone as substrate in the absence of Mg^{++}, reaction mixtures containing phosphate buffer showed no net oxygen uptake when endogenous values had been subtracted but reactions with Tris buffer took up 0.65 µatoms of oxygen/µM substrate. The addition of 20 µM of Mg^{++} to reaction mixtures containing either Tris or phosphate buffer resulted in the consumption of one µatom of oxygen/µmole of gluconolactone. Figure 1 illustrates the effect of Mg ions on oxygen uptake with phosphate buffer, pressate and glucose, gluconate or gluconolactone after endogenous oxygen uptake values have been subtracted.

These results demonstrate gluconolactonase, the enzyme which catalyzes the hydrolysis of gluconolactone to gluconic acid, to have a Mg ion requirement. This is in agreement with the observation of Brodie and Lipmann (14) who found the gluconolactonase purified from yeast to have an absolute requirement for divalent cations such as Mg^{++}, Mn^{++} or Co^{++}.

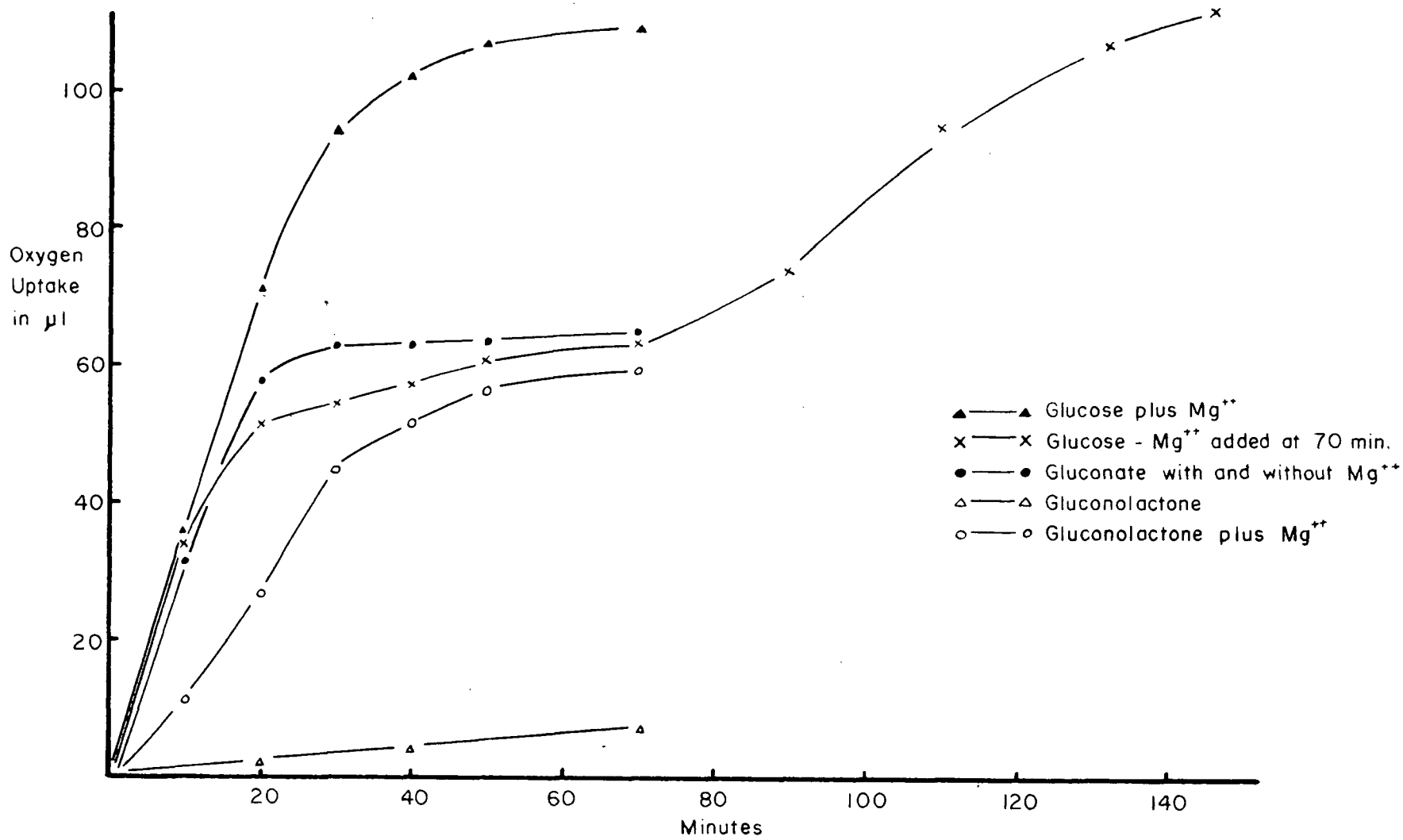

Figure 1. THE EFFECT OF MG IONS ON OXYGEN UPTAKE WITH GLUCOSE, GLUCONATE AND GLUCONOLACTONE

In order to demonstrate that the end product of the non-phosphorylative oxidation of glucose and gluconic acid was, in fact, 2-ketogluconate, separate reaction mixtures containing pressate, M/1 Tris buffer pH 7.4, 100 μM Mg^{++} and 25 μM of glucose, gluconate or 2-ketogluconate were set up in large Warburg flasks as well as an endogenous control. Incubation proceeded at 30°C for 2 hours under aerobic conditions. At the termination of the incubation period, the products of substrate oxidation were isolated as previously described. The compounds were identified by comparing both their electrophoretic mobility and reaction to developers with standard carbohydrate solutions. The alkaline silver nitrate and aniline hydrogen phthalate developers presented the results shown in Table I.

Table I. Electrophoretic identification of the end product of glucose and gluconic acid oxidation

	Silver nitrate	Aniline hydrogen phthalate
Substrate	Location of compound	Location and color of compound
Endogenous Glucose Gluconate 2-ketogluconate	A spot at the origin corresponding to glucose or ribose	A yellow spot at the origin corresponding to glucose
Glucose Gluconate 2-ketogluconate	A spot corresponding in mobility to gluconate or 2-ketogluconate	A pink spot corresponding in mobility to 2-ketogluconate

Glucose and gluconic acid were oxidized to 2-ketogluconate and 2-ketogluconate was not further degraded. These results are identical to those obtained by Stokes and Campbell (106) using dried cell preparations and paper chromatography for identification of the end product. The appearance of a spot at the origin resembling glucose was not unexpected from the

reaction mixture containing glucose as substrate but was totally unexpected from the other reaction mixtures. It appeared that both gluconate and 2-ketogluconate had been reduced to glucose.

Working with the DPN dependent glucose dehydrogenase isolated from beef liver, Strecker and Korkes (130), (131) were able to show the conversion of gluconic acid to glucose in the presence of reduced DPN but the reaction was reversed by the addition of glucose. Stetten and Topper (125) used carbon one labeled gluconate to study the in vivo conversion of gluconate to glucose and were able to conclude that less than 1 per cent of all the glucose carbon derived from gluconate come via the direct reduction without rearrangement of the carbon skeleton. The oxidation of glucose to gluconate in yeast and Azotobacter vinelandii has been reported to be reversible but thermodynamically strongly in favor of the formation of gluconic acid (14). The possibility, then, that the spot in question could be glucose appeared to be very slight. The qualitative but specific glucose oxidase test, which will detect 5 µg of glucose (143), gave negative results with all reaction mixtures and this was taken as conclusive evidence that the compound was not glucose. The unidentified spot at the origin was found to occur in all subsequent analyses of both test and control reaction mixtures and was, therefore, a compound inherent in the pressates rather than a product of substrate degradation. The presence of this compound has been disregarded in succeeding experiments.

In an effort to determine the role of pyridine nucleotides in the oxidative pathway, separate reaction mixtures were set up with glucose, gluconate or 2-ketogluconate as substrate and TPN or DPN as co-factor according to the spectrophotometric assay procedure for the determination

of glucose-6-phosphate dehydrogenase. In no case was there an increase in optical density at 340 mμ to indicate the reduction of pyridine nucleotide. These results concurred with those of Claridge and Werkman (27) and in part with those of Ramakrishnan and Campbell (114) who showed that gluconic acid dehydrogenase did not cause the reduction of either TPN or DPN. As with P. fluorescens (147), the transfer of hydrogen to oxygen during the non-phosphorylative oxidation of glucose and gluconate to 2-ketogluconate is not mediated by pyridine nucleotides.

II. Phosphorylated Pathways of Glucose Dissimilation

1. Hexose phosphorylation and hexose phosphate dehydrogenation

The presence of phosphorylated pathways for degradation of glucose in P. aeruginosa, with the process being initiated by the phosphorylation of hexose, has been implicated by many workers (27), (66), (92). Hogenkamp (72) found consistently that when glucose was incubated with ATP, pressate and NaF 1 μatom of oxygen/μM of substrate was taken up, similarly, with gluconate as substrate 1 μatom of oxygen/μM of gluconate was consumed. These manometric data were later confirmed by Reid (115) who also showed the disappearance of 94 per cent of the glucose during the incubation period. As gluconic acid was apparently not affected by the presence of ATP, it was concluded that an intermediate other than gluconate had accumulated during glucose oxidation in the presence of ATP. The following reaction sequence was postulated by both workers: glucose was oxidized to 2-ketogluconate through gluconic acid and then phosphorylated and reduced to yield 6-phosphogluconate. The two oxidative steps were believed to provide 2 μmoles of a reduced carrier, one of which would be reoxidized during the reduction of 2-keto-6-phosphogluconate to 6-phosphogluconate, resulting in the net

uptake of 1 µatom of oxygen/µmole glucose.

In view of the importance of Mg ion concentration in the conversion of glucose to gluconic acid and the observation of Brodie and Lipmann (14) that inhibition of hydrolysis of gluconolactone to gluconic acid by gluconolactonase was effected in the presence of NaF, it was thought that possibly the conclusions drawn by Hogenkamp and Reid were not valid.

In order to test their hypothesis, standard Warburg reactions were set up containing M/1 Tris buffer pH 7.4, pressate, 5 µM glucose or gluconate, 20 µM Mg^{++} and with and without 6 µM of ATP. Similar test reactions were set up with the Mg^{++} concentration increased to 100 µmoles and the ATP concentration increased to 30 µmoles. Incubation proceeded aerobically for 75 minutes at 30°C and the oxygen consumption values in Table II were obtained after the appropriate endogenous values had been subtracted.

Table II. Oxygen consumption in the presence of substrate and increasing concentrations of ATP.

	µatoms of oxygen taken up/µmole of substrate		
Substrate	No ATP	6 µmoles ATP	30 µmoles ATP
Glucose	1.91	0.95	0.68
Gluconic acid	1.0	0.88	0.99

Increasing concentrations of ATP caused decreasing oxygen consumption with glucose but had no marked effect on oxygen uptake with gluconic acid.

In an effort to identify the products of glucose and gluconic acid oxidation in the presence of ATP, reaction mixtures containing pressate, 100 µM Mg^{++}, 25 µmoles glucose or gluconate, 30 µmoles of ATP and M/1 Tris buffer pH 7.4 were placed in large Warburg flasks. Identical reaction

mixtures, with the Mg ion concentration increased to 500 μmoles and the ATP concentration increased to 125 μmoles, were also prepared. The reactions were carried out aerobically at 30°C for 75 minutes and the products were isolated and identified as shown in Table III.

Table III. End products of glucose and gluconic acid oxidation in the presence of ATP.

Substrate		Silver nitrate Location of Compound	Aniline hydrogen phthalate Location and color of compound
Glucose	30 μM ATP	2 spots: 1. corresponding to gluconate or 2-ketogluconate 2. corresponding to glucose-6-phosphate or fructose-6-phosphate	2 spots: 1. a pink spot corresponding to 2-ketogluconate 2. a yellow-brown spot corresponding to glucose-6-phosphate or fructose-6-phosphate
Glucose	125 μM ATP		
Gluconate	30 μM ATP	1 spot corresponding to gluconate or 2-ketogluconate	A pink spot corresponding to 2-ketogluconate
Gluconate	125 μM ATP	2 spots: 1. corresponding to gluconate or 2-ketogluconate 2. corresponding to glucose-6-phosphate or fructose-6-phosphate	2 spots: 1. a pink spot corresponding to 2-ketogluconate 2. a yellow-brown spot corresponding to glucose-6-phosphate or fructose-6-phosphate

The isolation of 2-ketogluconate from glucose and gluconic acid reaction mixtures indicated that the oxidative non-phosphorylated pathway was active under the conditions employed.

The spectrophotometric enzyme assay for the determination of glucose-6-phosphate was applied to the four test solutions and TPN was reduced in each case confirming the presence of hexose phosphate. The test solutions were

also spotted onto Whatman No. 3 m.m. paper and developed with the acidic naphthoresorcinol spray for fructose esters. A slight red-pink color was produced by all test solutions and this, then, demonstrated the presence of fructose-6-phosphate. In all subsequent results where hexose phosphate has been isolated and identified by paper electrophoresis and the spectrophotometric assay for glucose-6-phosphate, for the sake of convenience, the compound will be referred to as glucose-6-phosphate. As will be shown later, however, the organism contains phosphoglucoisomerase and the "compound" does, in fact, consist of an equilibrium mixture of glucose-6-phosphate and fructose-6-phosphate.

The products of both glucose and gluconic acid degradation suggested the presence of two pathways competing with each other for substrate -- one, the oxidative non-phosphorylated pathway, and the other, a pathway involving substrate phosphorylation. The results strongly indicated the presence of a kinase for glucose which has been previously suggested by Claridge and Werkman (27) and Hamilton and Dawes (66). The appearance of glucose-6-phosphate as a product of gluconic acid degradation in the presence of ATP implied the presence of both a kinase for gluconic acid and the enzymes of the pentose phosphate cycle as the reduction of gluconate to glucose is highly improbable.

The results obtained were not in agreement with those of Hogenkamp and Reid as firstly, the presence of ATP in glucose reaction mixtures did not consistently give oxygen uptake values of one µatom of oxygen/µmole of substrate but rather, the amount of oxygen consumed varied with the amount of ATP added. The ATP concentration apparently influenced the amount of glucose directly phosphorylated. Secondly, no phosphate esters of gluconate

or 2-ketogluconate were found in the reaction mixtures and this was taken as evidence against the phosphorylation of 2-ketogluconate and the reduction of 2-keto-6-phosphogluconate to give 6-phosphogluconate. The results yielding one µatom of oxygen uptake/µmole of glucose are believed to be primarily a function of the inhibition of gluconolactonase by the complexing of Mg ions by sodium fluoride.

To measure substrate phosphorylation, the evolution of carbon dioxide from $NaHCO_3$ was followed in reaction mixtures containing pressate, 10 µmoles ATP, 10 µmoles Mg^{++} and 10 µmoles of glucose, gluconate or 2-ketogluconate. The procedure was carried out under anaerobic conditions as previously described, using the necessary substrate and endogenous controls. After a 2 hour incubation period it was found that CO_2 was liberated in the Warburg flasks containing glucose plus ATP and gluconate plus ATP but not in the flask containing 2-ketogluconate and ATP. It was also observed that all of the endogenous and substrate control reaction mixtures showed a decrease in the volume of gas contained in the flasks. This could not have been the result of oxygen consumption as the phosphorous added to the reaction vessels to remove traces of oxygen showed no evidence of oxidation. It was concluded, therefore, that the decrease in gas volume was a manifestation of carbon dioxide fixation. The manometric data did not allow the calculation of quantitative results but did strongly emphasize the possibility of the presence of kinases for glucose and gluconic acid in pressates of P. aeruginosa.

Further evidence for kinases was obtained by incubating pressate, M/1 Tris buffer pH 7.4, 30 µmoles ATP, 100 µmoles Mg^{++} with 25 µmoles of glucose or gluconic acid in large Warburg flasks and under anaerobic conditions to prevent the oxidation of the substrates to 2-ketogluconate.

After incubation for 2 hours at 30°C, the products were isolated and the results of electrophoretic analysis are shown in Table IV.

Table IV. Products of enzymatic action on glucose and gluconic acid in the presence of ATP under anaerobic conditions

	Silver nitrate	Aniline hydrogen phthalate
Substrate	Location of compound	Location and color of compound
Glucose + ATP	One spot corresponding to glucose-6-phosphate	Yellow-brown spot corresponding to glucose-6-phosphate
Gluconate + ATP	2 spots: 1. corresponding to gluconate or 2-ketogluconate 2. corresponding to glucose-6-phosphate	2 spots: 1. pink spot corresponding to 2-keto-gluconate 2. yellow-brown spot corresponding to glucose-6-phosphate

The presence of hexose phosphate in both test solutions was confirmed by the enzymatic assay for glucose-6-phosphate

The appearance of both glucose-6-phosphate and 2-ketogluconate from gluconic acid and ATP under anaerobic conditions suggested a sequence involving phosphorylation of gluconate followed by reduction to glucose-6-phosphate in the presence of reduced pyridine nucleotide. The oxidized pyridine nucleotide could function in the oxidation of 6-phosphogluconate to 2-keto-6-phosphogluconate and then recycle. The activity of a specific phosphatase for 2-keto-6-phosphogluconate as suggested by Stanier (51) could produce 2-ketogluconate.

To test for the presence of a specific 2-keto-6-phosphogluconate phosphatase, standard aerobic Warburg reaction mixtures were set up with

pressate, M/1 Tris buffer pH 7.4, and 5 µmoles of 6-phosphogluconate or 2-keto-6-phosphogluconate. A flask containing 2-keto-6-phosphogluconate and substrate amounts of DPNH H^+ was also prepared. After a 50 minute incubation period at 30°C, the supernatant fluid from perchloric acid precipitated test reaction mixtures and endogenous controls was examined for inorganic phosphate. When subtracting the amount of inorganic phosphate present in endogenous controls from that in test solutions, it was found that there was no phosphate released from 2-keto-6-phosphogluconate but 0.69 µmole of inorganic phosphate was released from 6-phosphogluconate and 0.22 µmoles were released from 2-keto-6-phosphogluconate plus DPNH H^+. In the absence of pressate, no phosphate was released from 6-phosphogluconate. A phosphatase was present, therefore, but it was specific for 6-phosphogluconate not 2-keto-6-phosphogluconate. The inorganic phosphate released from 2-keto-6-phosphogluconate and DPNH H^+ was concluded to result from the reduction of 2-keto-6-phosphogluconate to 6-phosphogluconate (115).

The compound identified as 2-ketogluconate may actually have been the 2-keto-3-deoxy sugar having arisen from the dehydration of gluconic acid in a manner analogous to the dehydration of 6-phosphogluconate to 2-keto-3-deoxy-6-phosphogluconate. Such a dehydration would eliminate the requirement for an oxidized carrier. The qualitative test for deoxy sugars was applied to both glucose and gluconate test solutions and deoxyadenosine and 2-keto-3-deoxy-6-phosphogluconate were used as standards. The test solutions gave a strongly positive color reaction with an absorption maximum at 595 mµ. The deoxyadenosine standard had an absorption maximum at 595 mµ and the 2-keto-3-deoxy-6-phosphogluconate standard, a maximum at 550 mµ.

The color in the test solutions, therefore, was considered to be due largely to the presence of deoxyribose from DNA of the pressate. The presence of the 2-keto-3-deoxy sugar was not confirmed but, nevetheless, it is suggested as being the most likely compound.

Lipmann (95) and Cori and Lipmann (31) have shown the primary product of chemical and enzymatic oxidation of glucose-6-phosphate to be 6-phosphogluconolactone which is relatively unstable at neutral or alkaline pH. The product of lactone hydrolysis is 6-phosphogluconate. The oxidation of glucose-6-phosphate to 6-phosphogluconate has been reported to be reversible (55), (63), but Gunsalus et al. (63) state that the equilibrium of lactonization is far towards hydrolysis and it would, therefore, appear to limit the rate of 6-phosphogluconolactone formation to such a level that net hexose phosphate regeneration via this route would be negligible. The glucose-6-phosphate in the gluconic acid-ATP reaction mixture must have been derived, not from reduction of 6-phosphogluconate, but from a reaction sequence involving the enzymes of the pentose phosphate cycle.

The spectrophotometric assays for glucokinase and gluconokinase were dependent on the existence of glucose-6-phosphate and 6-phosphogluconate dehydrogenases in the pressate. In order to study the activities of the kinases it was first necessary to establish the presence of the dehydrogenases. Claridge and Werkman (27) observed both TPN and DPN reduction with glucose-6-phosphate and only DPN reduction with 6-phosphogluconate and cell-free extracts of P. aeruginosa. Hamilton and Dawes (66) also demonstrated the presence of both dehydrogenases.

With the previously described assay system for dehydrogenases, both glucose-6-phosphate and 6-phosphogluconate supported TPN reduction as mea-

sured by the increase in optical density at 340 mμ. This illustrated the existence of active glucose-6-phosphate and 6-phosphogluconate dehydrogenases in cell-free extracts prepared with a Hughes' press. Contrary to the findings of Claridge and Werkman, there was more rapid reduction of TPN than DPN with 6-phosphogluconate. Table V shows the relative rates of pyridine nucleotide reduction by glucose-6-phosphate and 6-phosphogluconate dehydrogenases, expressed as specific activity.

Table V. Pyridine nucleotide reduction by glucose-6-phosphate and 6-phosphogluconate dehydrogenases.

Substrate	Co-factor	Specific activity
Glucose-6-phosphate	TPN	0.839
	DPN	0.352
6-phosphogluconate	TPN	0.374
	DPN	0.027

Both enzymes exhibited a considerable decrease in activity when DPN was used as the hydrogen acceptor; the 6-phosphogluconate dehydrogenase, in fact, showed almost negligible activity and it is concluded that TPN is the natural hydrogen acceptor for this enzyme. The activity of glucose-6-phosphate dehydrogenase with both DPN and TPN suggests that either pyridine nucleotide may serve as a hydrogen acceptor or that a situation similar to that in Aspergillus flavus - oryzae exists. Hochster (69) has shown the organism to possess two distinct glucose-6-phosphate dehydrogenases, one specific for TPN and the other specific for DPN.

The glucose-6-phosphate dehydrogenase was apparently more active than the 6-phosphogluconate dehydrogenase but the difference in relative activity

of the two enzymes varied considerably in cell-free extracts prepared with the Hughes' press. The 6-phosphogluconate dehydrogenase of E. coli has been reported to be less stable than the glucose-6-phosphate dehydrogenase (120) and Wood and Schwerdt (147) found considerable variation in the 6-phosphogluconate dehydrogenase content of cell-free extracts of Pseudomonas fluorescens. A comparison of the specific activities of these two enzymes, therefore, is not valid as it appears that at least some of the 6-phosphogluconate dehydrogenase activity may have been destroyed during preparation of the cell-free extract. The possibility of the product of glucose-6-phosphate dehydrogenation contributing to TPN reduction and giving unduly high values for glucose-6-phosphate dehydrogenase activity was examined by Scott and Cohen (121) using purified glucose-6-phosphate and 6-phosphogluconate dehydrogenases from E. coli. The addition of 6-phosphogluconate dehydrogenase to reaction mixtures containing glucose-6-phosphate and glucose-6-phosphate dehydrogenase effected an extremely small increase in TPN reduction and they concluded that the overall contribution was essentially negligible under the conditions of the spectrophotometric assay.

The glucose-6-phosphate dehydrogenase of E. coli is reported to have a requirement for divalent cations such as Ca^{++} or Mg^{++} (120). Glaser and Brown (60), however, state that the glucose-6-phosphate of brewers yeast probably does not have an absolute Mg^{++} requirement as the enzyme retains 70 per cent of its activity in the absence of Mg^{++} and the addition of ethylenediamine tetraacetic acid does not cause a further decrease in activity. To observe the effect of Mg ion on the glucose-6-phosphate and 6-phosphogluconate dehydrogenase of P. aeruginosa TPN reduction was followed in pressates containing glucose-6-phosphate or 6-phosphogluconate in

the presence and absence of 2.5 μM Mg^{++}. The results, expressed as specific activities, are recorded in Table VI.

Table VI. The effect of Mg ions on glucose-6-phosphate and 6-phosphogluconate dehydrogenases.

Substrate		Specific Activity
Glucose-6-phosphate	Mg^{++}	.750
	no Mg^{++}	.917
	5 μM NaF, no Mg^{++}	.738
6-phosphogluconate	Mg^{++}	.468
	no Mg^{++}	.614
	5 μM NaF, no Mg^{++}	.212

The addition of Mg ions promoted some inhibition of both dehydrogenases but the addition of NaF to remove trace amounts of Mg^{++} from the pressate caused a similar inhibitory effect with glucose-6-phosphate dehydrogenase and a more marked inhibitory effect with 6-phosphogluconate dehydrogenase. The 6-phosphogluconate dehydrogenase of brewers yeast is stimulated by low concentrations of Mg^{++} but inhibited by high concentrations (75). It would appear, then, that both dehydrogenases do have a requirement for a divalent cation in trace amounts but 2.5 μM Mg^{++}/10 mg wet weight of cells is sufficiently higher than the required optimal concentration to cause some inhibition. The substitution of 2.5 μM Mn^{++} for Mg^{++} in the reaction mixtures caused a greater decrease in activity with both enzymes than did Mg^{++}.

Spectrophotometrically, phosphorylation of glucose and gluconic acid

was followed by measuring TPN reduction in the presence of pressate, substrate and ATP. The pyridine nucleotide reduction was a result of glucose-6-phosphate or 6-phosphogluconate dehydrogenase activity on the product formed by the action of a kinase. Glucose-6-phosphate dehydrogenase has been found to be inhibited by nucleotides (115), (147) probably because of the formation of a chelate with Mg^{++} (83). The inhibition can be reversed by adding a sufficiently high concentration of Mg ions and this is demonstrated in Table VII. The cuvettes contained M/5 Tris buffer pH 7.4, pressate, 2.5 µM of ATP, 2.5 µM of glucose or gluconic acid, TPN and increasing amounts of Mg^{++}.

Table VII. Reversal of glucose-6-phosphate and 6-phosphogluconate dehydrogenase inhibition.

Substrate	Mg ion concentration	Specific activity
glucose	--	.000
	2.5 µM	.038
	5.0 µM	.101
	10.0 µM	.158
	20.0 µM	.076
gluconate	--	.000
	2.5 µM	.016
	5.0 µM	.022
	10.0 µM	.035

A concentration of 10 µmoles Mg^{++}/1 ml reaction mixture gave the maximum rate of TPN reduction while higher concentrations caused some inhibition

of the activity of the dehydrogenases. The presence of glucokinase and gluconokinase established a definite connection between the oxidative non-phosphorylated pathway and phosphorylated pathways. Further, the presence of gluconokinase indicated that not all of the glucose oxidized to gluconic acid necessarily undergoes further oxidation to 2-ketogluconate.

Phosphohexoisomerase and phosphomannose isomerase were also demonstrated spectrophotometrically by substituting fructose-6-phosphate or mannose-6-phosphate as substrate in the glucose-6-phosphate dehydrogenase assay. Under the test conditions phosphohexoisomerase had a specific activity of 0.062 and phosphomannose isomerase a specific activity of 0.089. These rates of enzymatic activity are relatively low as compared to the glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities as are illustrated in Figure 2. The demonstration of phosphomannose isomerase was contrary to the report of Eagon and Williams (48) who stated that the enzymes for mannose dissimilation in P. aeruginosa are adaptive and extracts of the glucose grown cells do not contain phosphomannose isomerase.

Phosphorylation of either fructose or mannose could not be shown by means of a modified glucokinase spectrophotometric assay. Evidence for phosphorylation of fructose by glucose grown cells has been presented by Eagon and Williams (47).

The presence of kinases for both glucose and gluconic acid and dehydrogenases for glucose-6-phosphate and 6-phosphogluconate was strongly indicative of the importance of phosphorylated pathways in the dissimilation of glucose by this organism.

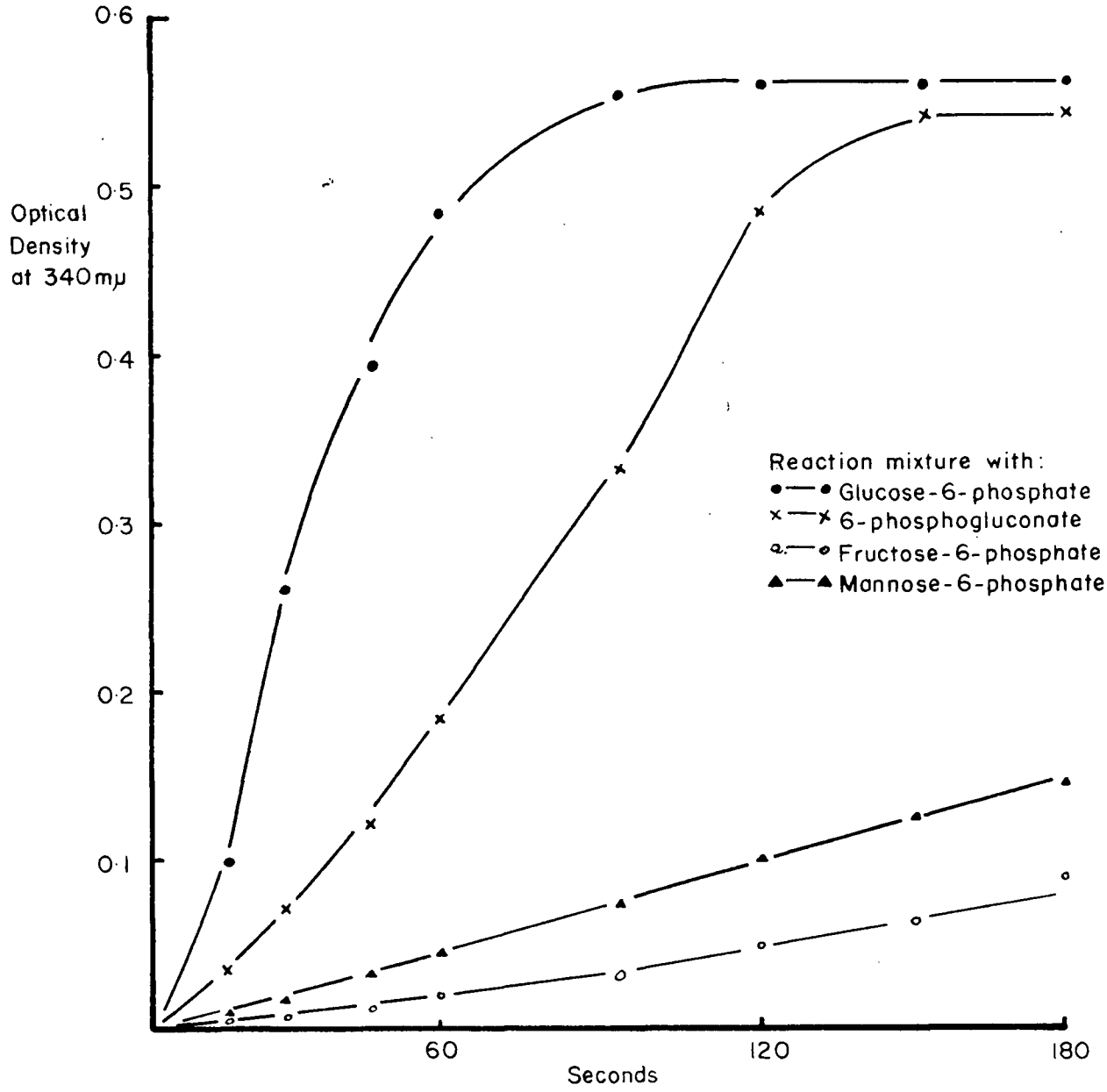

Figure 2. RELATIVE ACTIVITIES OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE, 6-PHOSPHOGLUCONATE DEHYDROGENASE, PHOSPHOHEXOISOMERASE AND PHOSPHOMANNOSE ISOMERASE

2. The pentose phosphate cycle

Claridge and Werkman (27) found DPN, but not TPN, reduction in the presence of ribose-5-phosphate and cell-free extracts of P. aeruginosa and concluded that the extracts contained the enzymes of the pentose phosphate cycle.

The ability of pressates to oxidize ribose-5-phosphate was measured by the conventional aerobic technique with a Warburg respirometer. Pressates were incubated with M/1 Tris buffer pH 7.4, 5 µM of ribose, ribose-5-phosphate or ribose plus 6 µM ATP, and 25 µM Mg^{++} for 90 minutes at 30°C. After the appropriate endogenous oxygen uptake values had been subtracted from the test values, it was found that there was negligible oxygen consumption attributable to substrate oxidation and it appeared that the pressates were unable to dissimilate ribose or ribose-5-phosphate.

The reaction mixtures were prepared again in large Warburg flasks with a five-fold increase in the concentration of the constituents. Incubation proceeded aerobically for 90 minutes at 30°C and, at the termination of the incubation period, the products were isolated and identified by paper electrophoresis as shown in Table VIII. The presence of hexose phosphate in the test solutions was confirmed by the enzymatic assay for glucose-6-phosphate. The reaction mixture containing ribose as substrate gave an appreciably slower rate of TPN reduction than the other reaction mixtures, indicationg a very low concentration of hexose phosphate.

Glucose-6-phosphate could have been generated by way of the pentose phosphate cycle or by CO_2 fixation with pentose phosphate to yield 6-phosphogluconate followed by reduction to glucose-6-phosphate. The feasibility of the reduction of 6-phosphogluconate to glucose-6-phosphate was discussed

previously. Horecker and Smyrniotis (73), (77) investigated the fixation of C^{14}-carbon dioxide into 6-phosphogluconate in yeast extracts incubated with ribulose-5-phosphate. In the presence of reduced TPN, they found a concentration of 6-phosphogluconate equal to 0.35 per cent of the pentose phosphate added. In the absence of TPNH H^+, however, no 6-phosphogluconate was formed from CO_2 and pentose phosphate unless glucose was added to supply the required reduced pyridine nucleotide. The glucose-6-phosphate found in the reaction mixtures as a result of ribose-5-phosphate dissimilation, could only have arisen from the activities of enzymes of the pentose phosphate cycle. These results plus the 6-phosphogluconate dehydrogenase spectrophotometric data confirm the earlier mentioned implication of a pentose phosphate cycle based on the formation of glucose-6-phosphate from pressate incubated with gluconic acid and ATP.

Table VIII. End products of ribose and ribose-5-phosphate dissimilation

	Silver nitrate	Aniline hydrogen phthalate
Substrate	Location of compound	Location and color of compound
Ribose	2 spots: 1. at the origin corresponding to glucose or ribose 2. a faint spot corresponding to glucose-6-phosphate	2 spots: 1. a pink spot corresponding to ribose 2. a faint yellow-brown spot corresponding to glucose-6-phosphate
Ribose + ATP	2 spots: 1. at the origin corresponding to glucose or ribose 2. a spot corresponding to glucose-6-phosphate	2 spots: 1. a pink spot at the origin corresponding to ribose 2. a yellow-brown spot corresponding to glucose-6-phosphate
Ribose-5-phosphate	A spot corresponding to glucose-6-phosphate	A yellow-brown spot corresponding to glucose-6-phosphate

The fact that glucose-6-phosphate was isolated from reaction mixtures containing ribose and ATP demonstrates the presence of ribokinase in the pressates. The production of a small amount of glucose-6-phosphate from ribose reaction mixtures, without ATP, is considered to be due to the phosphorylation of ribose from endogenously produced ATP.

The presence of the pentose phosphate cycle in pressates of Pseudomonas aeruginosa was substantiated by spectrophotometric data. Triphosphopyridine nucleotide reduction was followed in pressates containing M/5 Tris buffer pH 7.4 and 2.5 µM ribose-5-phosphate in the assay for glucose-6-phosphate dehydrogenase. A slow rate of TPN reduction was observed and the reduction could have been the result of the oxidation of either glucose-6-phosphate or 3-phosphoglyceraldehyde. As iodoacetamide inhibits 3-phosphoglyceraldehyde dehydrogenase and does not affect glucose-6-phosphate dehydrogenase (45), 6 µM of the inhibitor were added to reaction mixtures containing glucose-6-phosphate and ribose-5-phosphate and TPN reduction was followed. As can be seen from Figure 3, TPN reduction with glucose-6-phosphate was not affected whereas TPN reduction with ribose-5-phosphate was markedly inhibited. Three-phosphoglyceraldehyde, then, as well as glucose-6-phosphate, was a product of ribose-5-phosphate dissimilation by the pressates.

3. The Entner-Doudoroff pathway

High 6-phosphogluconate dehydrase and 2-keto-3-deoxy-6-phosphogluconate aldolase activity have been shown in dried cells and cell-free extracts of P. aeruginosa (66), (92).

Two-keto-3-deoxy-6-phosphogluconate aldolase was measured by converting a product of its activity - pyruvic acid - to lactic acid in the presence of DPNH H^+ and a commercial source of lactic dehydrogenase. The procedure

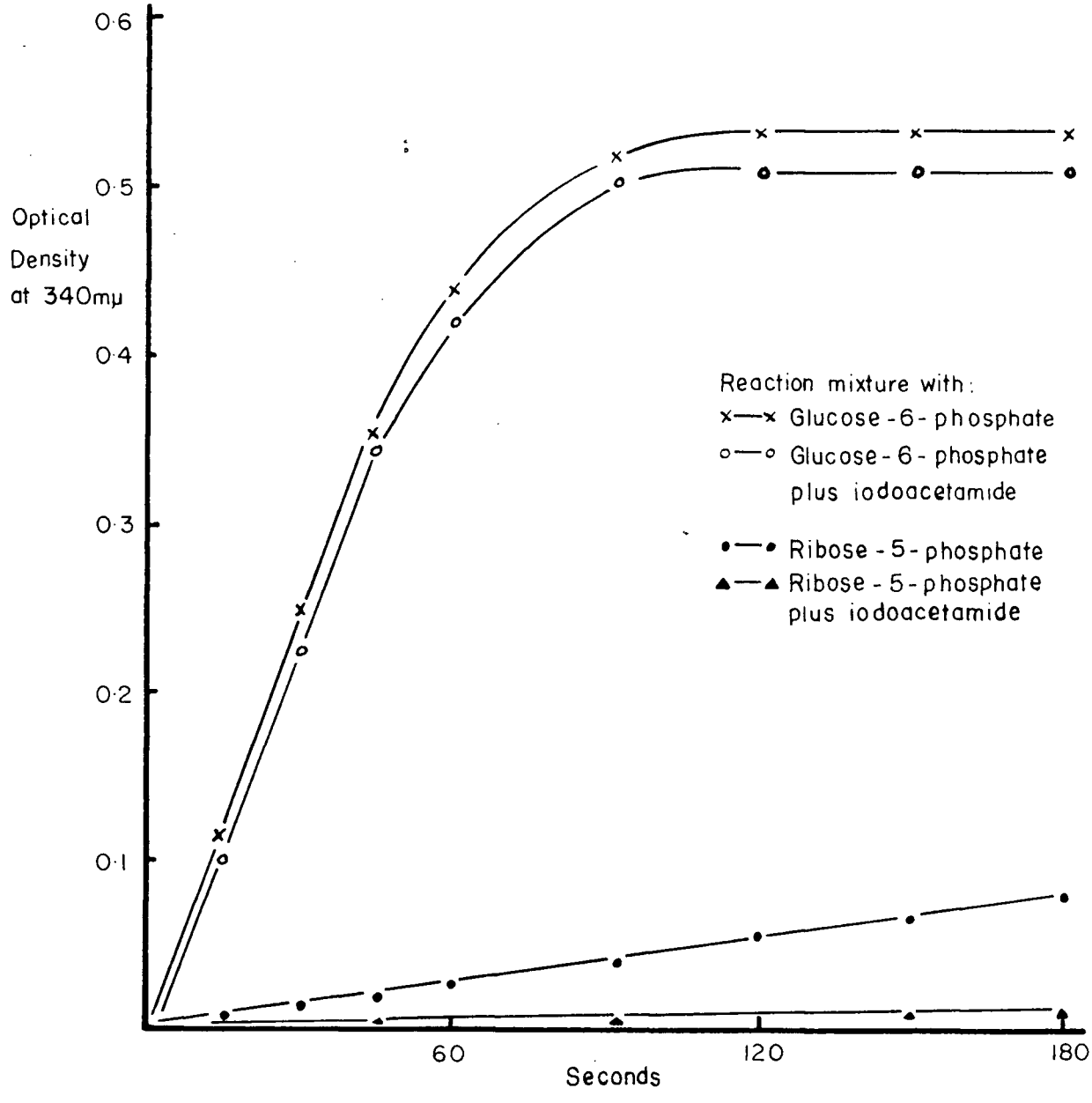

Figure 3. EFFECT OF IODOACETAMIDE ON TPN REDUCTION WITH GLUCOSE-6-PHOSPHATE AND RIBOSE-5-PHOSPHATE

used has been described previously. With 0.05 ml of pressate, the oxidation of DPNH H^+ was completed within 15 seconds and, therefore, a smaller quantity of pressate had to be used. The pressate had an active DPNH H^+ oxidase and corrections for this were utilized in the calculations of the specific activity of the aldolase. The aldolase had a specific activity of 1.968 as compared to a value of 0.980 obtained for glucose-6-phosphate dehydrogenase in the same cell-free extract preparation.

Six-phosphogluconate dehydrase also was measured indirectly by following DPNH H^+ oxidation caused by the reduction of pyruvic acid to lactic acid. Pyruvic acid appeared in the reaction mixtures after 6-phosphogluconate was dehydrated to 2-keto-3-deoxy-6-phosphogluconate and the latter compound split by 2-keto-3-deoxy-6-phosphogluconate aldolase to yield pyruvic acid and 3-phosphoglyceraldehyde. The dehydrase was somewhat less active than the aldolase, therefore, 0.05 ml of pressate was used in the reaction mixtures. After corrections for DPNH H^+ oxidase were made, 6-phosphogluconate dehydrase was found to have a specific activity of 0.549. Figure 4 demonstrates the activity of 2-keto-3-deoxy-6-phosphogluconate aldolase and 6-phosphogluconate dehydrase. The reaction mixture illustrating aldolase activity contained one-fourth of the amount of pressate as that illustrating 6-phosphogluconate dehydrase activity.

4. Triose dissimilation

Three-phosphoglyceraldehyde is a product of glucose dissimilation by way of the pentose phosphate cycle and pyruvic acid and 3-phosphoglyceraldehyde are the products obtained *via* the Entner-Doudoroff pathway.

Campbell *et al.* (19), (20), (140) have established that pyruvic acid is an intermediate in glucose oxidation in *P. aeruginosa* and that the

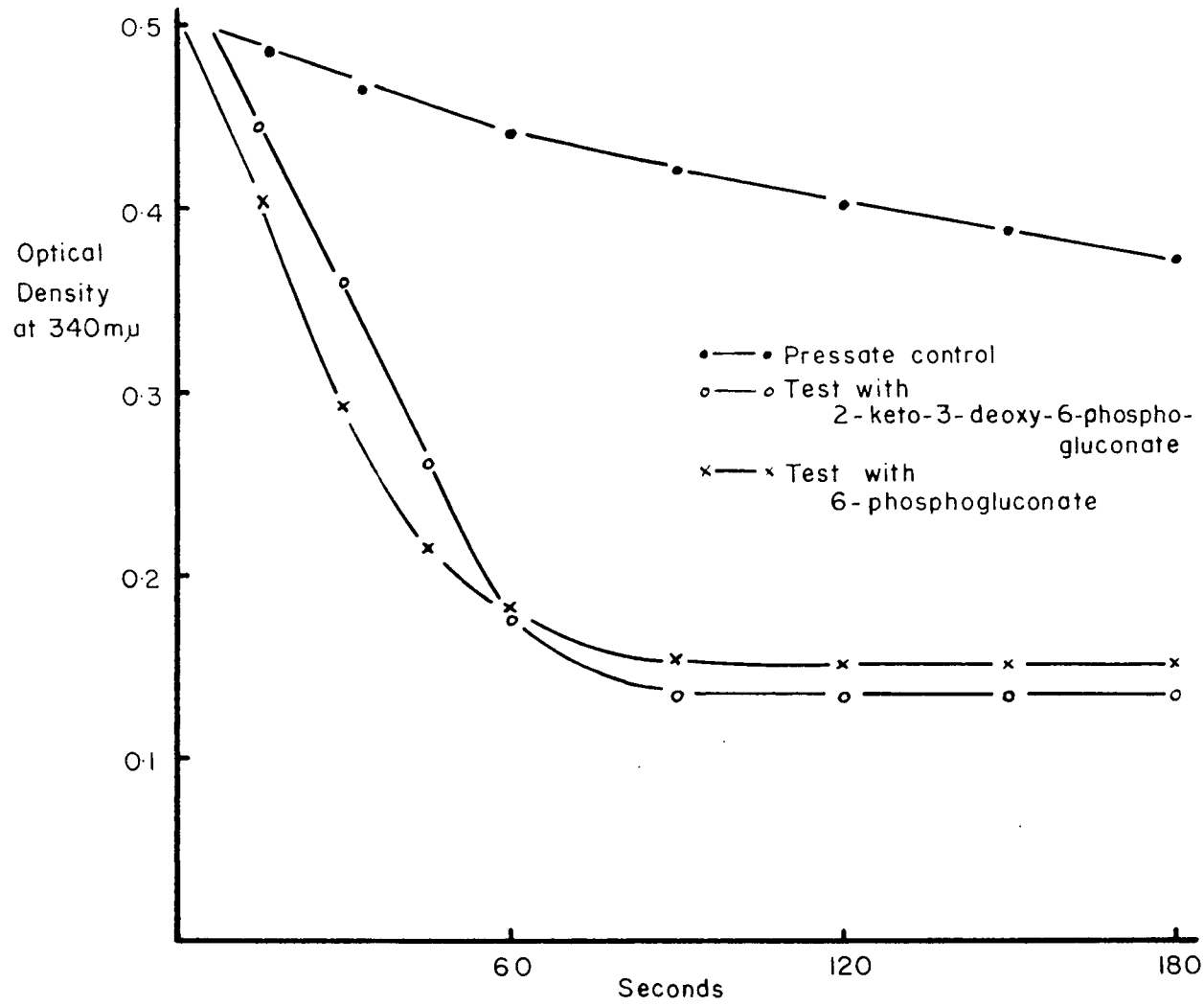

Figure 4. 6-PHOSPHOGLUCONATE DEHYDRASE AND 2-KETO-3-DEOXY-6-PHOSPHOGLUCONATE ALDOLASE ACTIVITY

organism possesses all of the enzymes of the conventional TCA cycle necessary for the oxidation of pyruvic acid as well as a glyoxylate by-pass.

The presence of 3-phosphoglyceraldehyde dehydrogenase was shown earlier by the inhibition of TPN reduction by iodoacetamide in the presence of ribose-5-phosphate. This assay required the action of the enzymes of the pentose phosphate cycle to produce triose phosphate. A more direct spectrophotometric assay, as previously described, was employed with 2-keto-3-deoxy-6-phosphogluconate as substrate, both TPN and DPN were used as coenzymes and the dehydrogenase could be inhibited by 3 µM of iodoacetamide. The specific activity of the enzyme with DPN was 0.028 and with TPN 0.545. Iodoacetamide caused a 90 per cent inhibition of 3-phosphoglyceraldehyde dehydrogenase activity as measured by the marked decrease in the rate of TPN reduction. The activity of the enzyme with DPN, TPN and iodoacetamide are shown in Figure 5.

The 3-phosphoglyceraldehyde dehydrogenase is generally considered to be DPN specific, as has been found in Xanthomonas phaseoli (70), Pseudomonas fluorescens (148), Pseudomonas hydrophilia (126) and others. Claridge and Werkman (27) have reported that DPN and not TPN is reduced with cell-free extracts of P. aeruginosa and ribose-5-phosphate. This is contrary to the findings reported here. However, TPN specific 3-phosphoglyceraldehyde dehydrogenases have been reported to occur in plants (3), Alcaligenes faecalis (11), (12), and Neisseria winogradskyi (135).

Three-phosphoglyceric acid kinase was measured by following TPNH H^+ oxidation in the reversal of 3-phosphoglyceraldehyde dehydrogenase activity. Three-phosphoglyceric acid was incubated with pressate, M/5 Tris buffer pH 7.4, ATP and Mg^{++} as described in the glucokinase assay. In this system

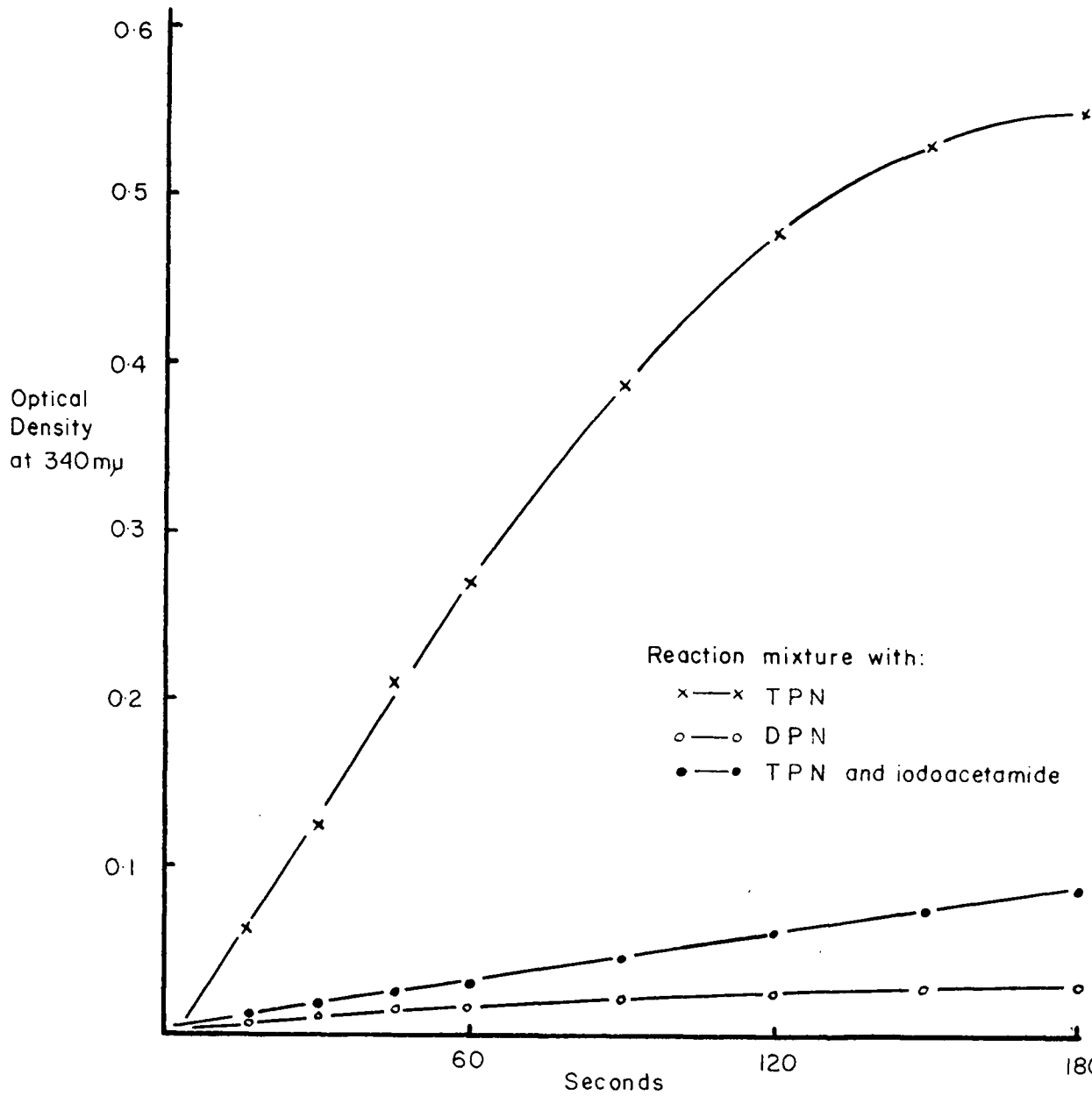

Figure 5. 3-PHOSPHOGLYCERALDEHYDE DEHYDROGENASE ACTIVITY

3-phosphoglyceric acid was phosphorylated by 3-phosphoglyceric acid kinase to give 1,3-diphosphoglyceric acid which, in turn, was dephosphorylated and reduced with TPNH H^+ to 3-phosphoglyceraldehyde by 3-phosphoglyceraldehyde dehydrogenase. There was a slightly more rapid rate of TPNH H^+ oxidation in the test reaction mixture than in the pressate control. The calculated specific activity for the enzyme was 0.037. A comparison of TPNH H^+ oxidation by the test reaction mixture and the substrate and enzyme controls is given in Figure 6.

It was thought that if the 3-phosphoglyceraldehyde arising from the pentose phosphate cycle and the Entner-Doudoroff pathway was converted to pyruvic acid, then phosphoglyceromutase, enolase and pyruvic kinase could be demonstrated by the conversion of 3-phosphoglyceric acid to pyruvic acid as measured by the assay for 2-keto-3-deoxy-6-phosphogluconate aldolase. When 3-phosphoglyceric acid was used as substrate in this assay and 5 µM K^+ added, it was found that there was an insignificant increase in DPNH H^+ oxidation over that found in the pressate control. However, when a commercial source of pyruvic kinase was added to the reaction mixture there was a considerable decrease in optical density at 340 mµ. These results are illustrated in Figure 7. The absence of a pyruvic kinase was implicated by these results.

Reduced DPN oxidation was measured in the previously described assay for pyruvic kinase and it was found that the enzyme was not only present but also fairly active with a specific activity of 0.558. Reduced DPN oxidation in the test reaction mixture and controls is shown in Figure 8. The commercial pyruvic kinase contained a very small percentage of enolase and it is concluded that the oxidation of DPNH H^+ occurring on the addition

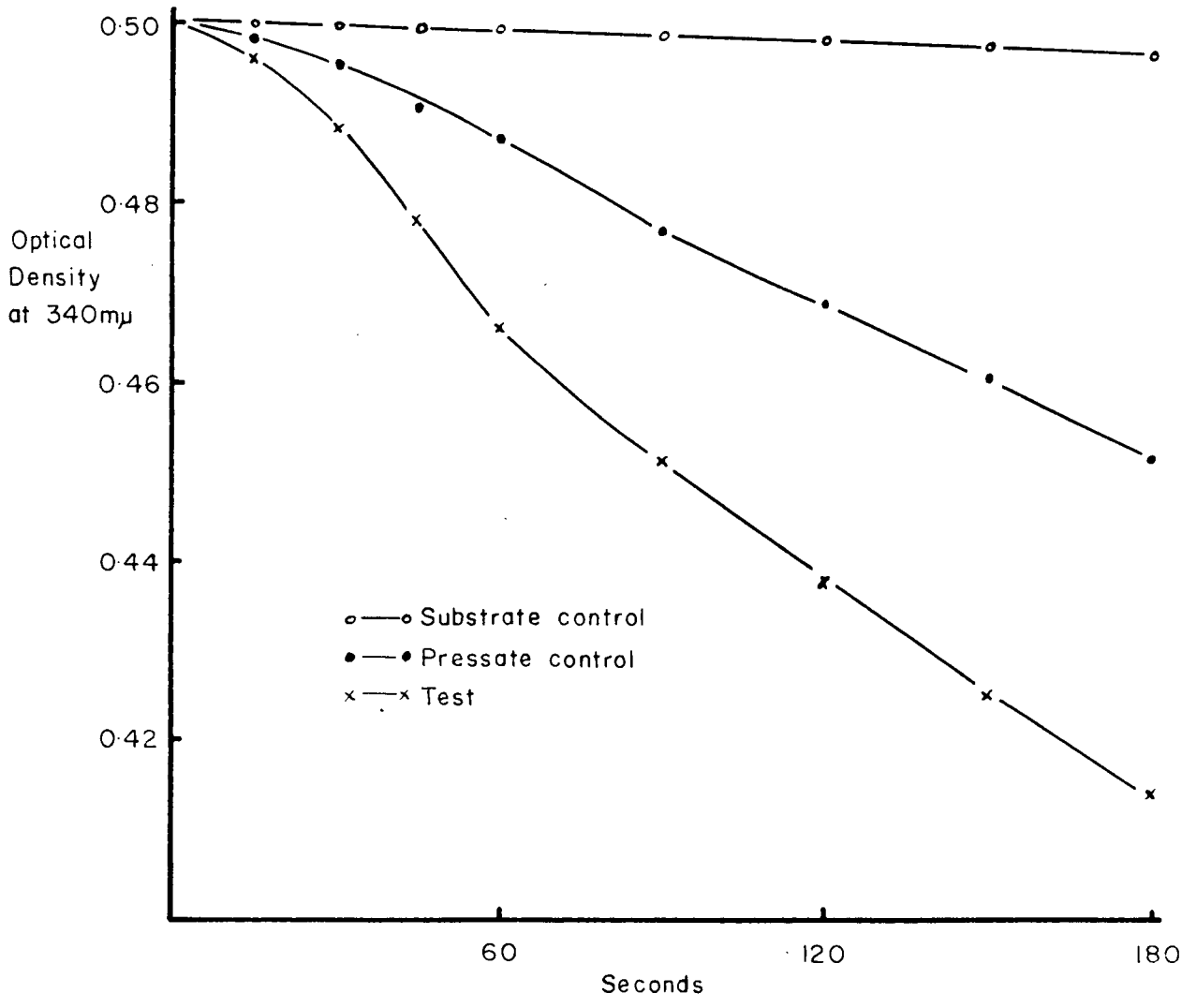

Figure 6. DEMONSTRATION OF 3-PHOSPHOGLYCERIC ACID KINASE ACTIVITY

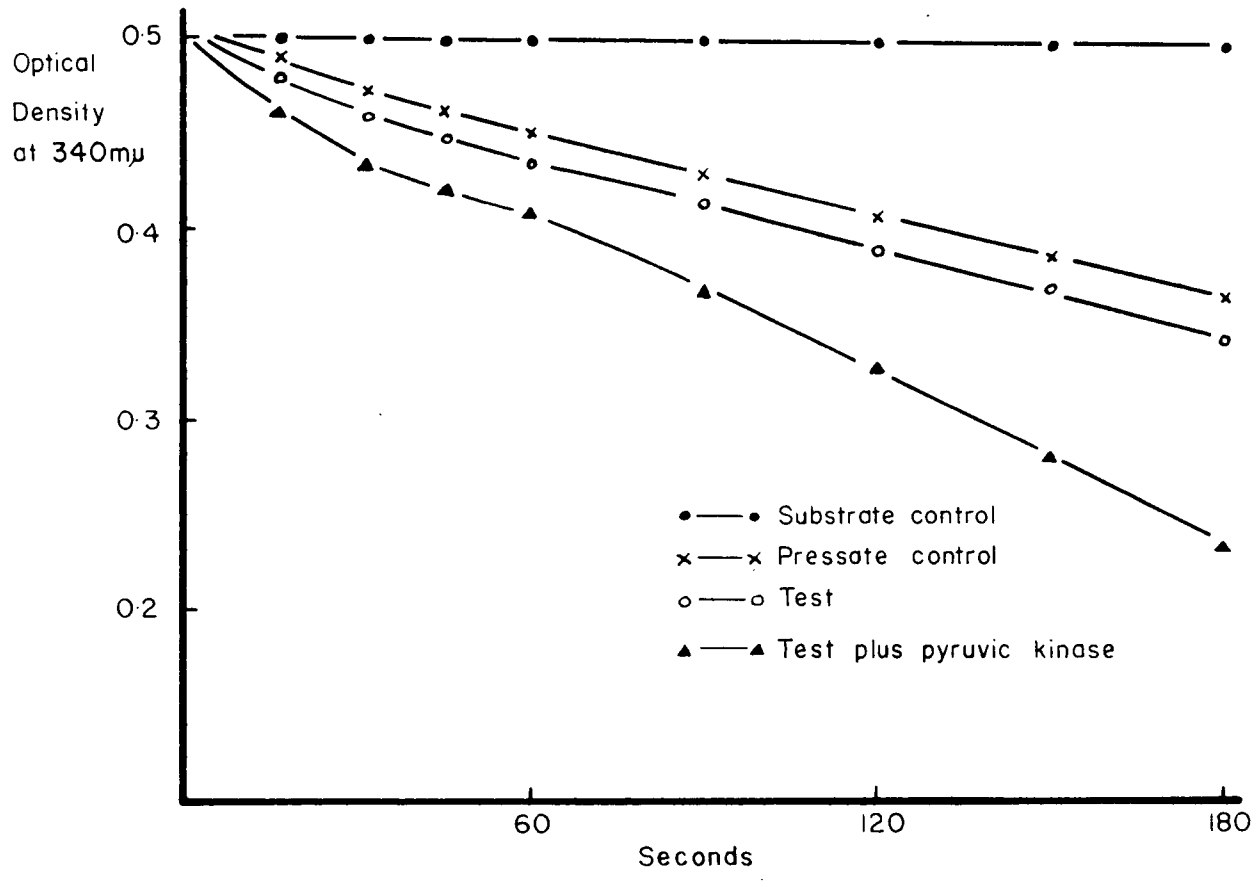

Figure 7. CONVERSION OF 3-PHOSPHOGLYCERIC ACID TO PYRUVIC ACID

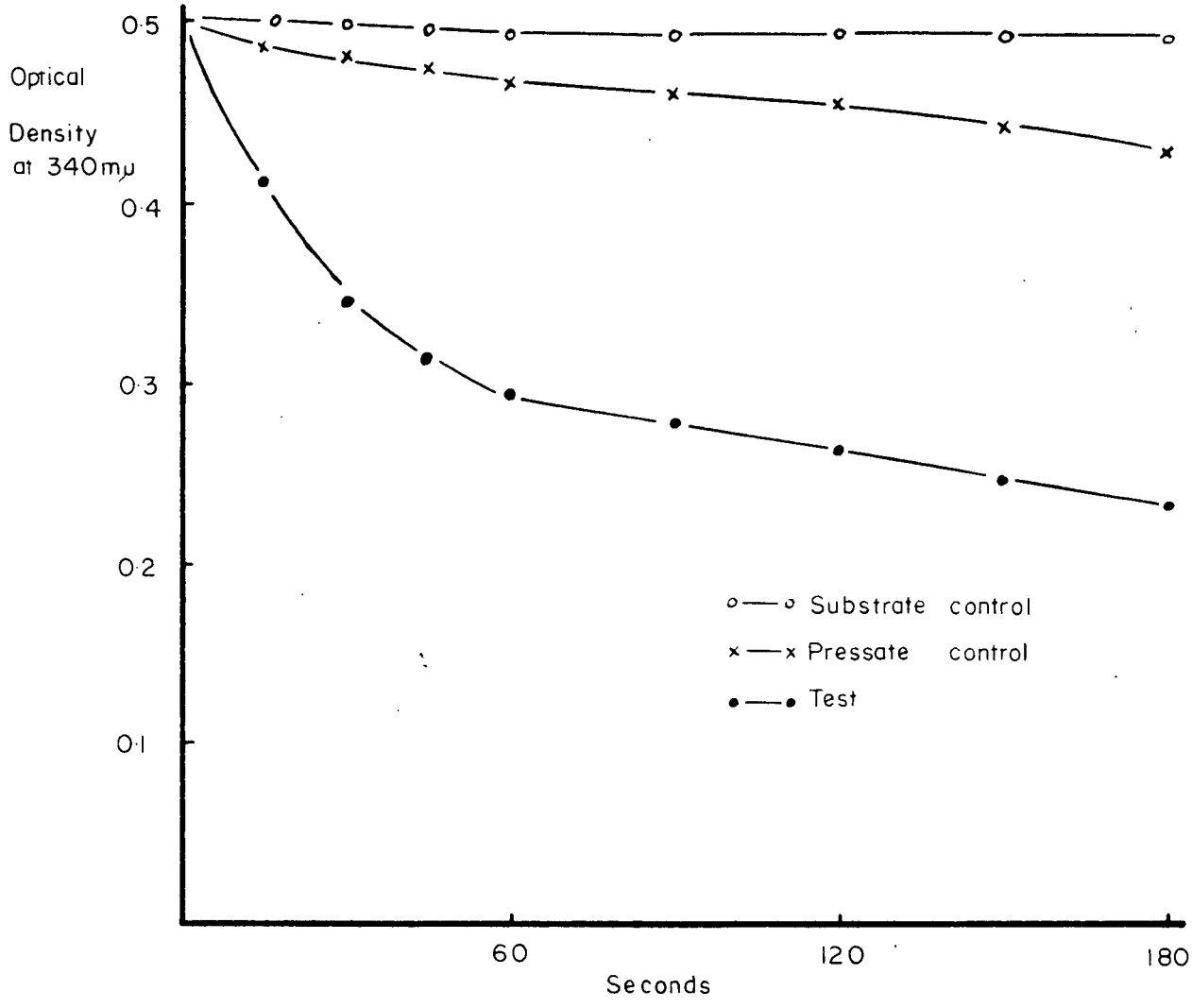

Figure 8. DEMONSTRATION OF PYRUVIC KINASE

of commercial pyruvic kinase to the test solutions containing 3-phosphoglyceric acid, was a result of the activity of the enolase. Under the conditions of assay, then, the pressates did not contain an active enolase, however, this inability to demonstrate enolase activity does not constitute conclusive evidence against its occurrence in the whole cell.

5. Regeneration of hexose phosphate from triose phosphate

Taylor and Juni (135) have demonstrated that glucose oxidation in Neisseria winogradskyi proceeds via a non-phosphorylative oxidation to gluconic acid. Gluconic acid is phosphorylated and then dissimilated 100 per cent by way of the Entner-Doudoroff pathway. The finding of fructose-1,6-diphosphate phosphatase and aldolase and triose phosphate isomerase led them to conclude that two triose phosphates from the Entner-Doudoroff pathway combine by means of the aldolase to produce hexose diphosphate. A phosphate group is cleaved from the one position and the resulting fructose-6-phosphate is utilized for both polysaccharide synthesis and as a precursor of pentoses. This scheme of conversion of triose phosphate to hexose phosphate has been shown to occur in Rhodopseudomonas spheroides (133) and suggested to occur in E. coli (29b), Acetobacter suboxydans (67) and some plant tissues (49).

Fructose-1,6-diphosphate aldolase was assayed for by the procedure for 3-phosphoglyceraldehyde dehydrogenase. Triphosphopyridine nucleotide was reduced in the test system but the reduction was completely inhibited by 6 μM of iodoacetamide. There was only a slight increase in optical density with DPN as coenzyme. As TPN reduction is not inhibited by iodoacetate with glucose-6-phosphate or fructose-6-phosphate, it was concluded that TPN reduction was the result of 3-phosphoglyceraldehyde dehydrogenase activity.

The TPN reduction with these compounds in the presence and absence of inhibitor is illustrated in Figure 9. Wood and Schwerdt (148) have demonstrated a hexose diphosphate aldolase in cell-free extracts of P. fluorescens. Claridge and Werkman (27) found pyridine nucleotide reduction with fructose-1,6-diphosphate and cell-free extracts of P. aeruginosa but there was more rapid reduction with DPN than with TPN.

Strasdine (128) has shown that cell-free extracts of P. aeruginosa contain an active phosphatase for fructose-1,6-diphosphate. The presence of the phosphatase, the aldolase and the earlier demonstrated phosphohexoisomerase may indicate that the organism does regenerate glucose-6-phosphate via the triose phosphate - hexose diphosphate cycle.

6. Metabolism of 2-ketogluconic acid

Evidence for a 2-ketogluconokinase in dried cells and cell-free extracts of P. fluorescens has been presented by Narrod and Wood (101), (102) who found CO_2 evolution from cell preparations in the presence of 2-ketogluconate and ATP. A rapid oxidation of TPNH H^+ occurred in the cell-free extracts incubated with 2-keto-6-phosphogluconate and an equivalent amount of 6-phosphogluconate was formed. The TPNH H^+ specific enzyme involved was 2-keto-6-phosphogluconate reductase (56). The kinase and the reductase could account for 2-ketogluconate oxidation via the pentose phosphate cycle or the Entner-Doudoroff pathway. Stouthamer (127) found a TPNH H^+ specific 2-ketogluconate reductase in cell-free extracts of Gluconobacter liquefaciens. Two-ketogluconate was reduced to gluconic acid before phosphorylation occurred. A specific and adaptive 2-ketogluconokinase was isolated from cells of Leuconostoc mesenteroides grown on 2-ketogluconate (24). The phosphorylated compound was shown to be reduced to 6-phosphogluconate followed by decar-

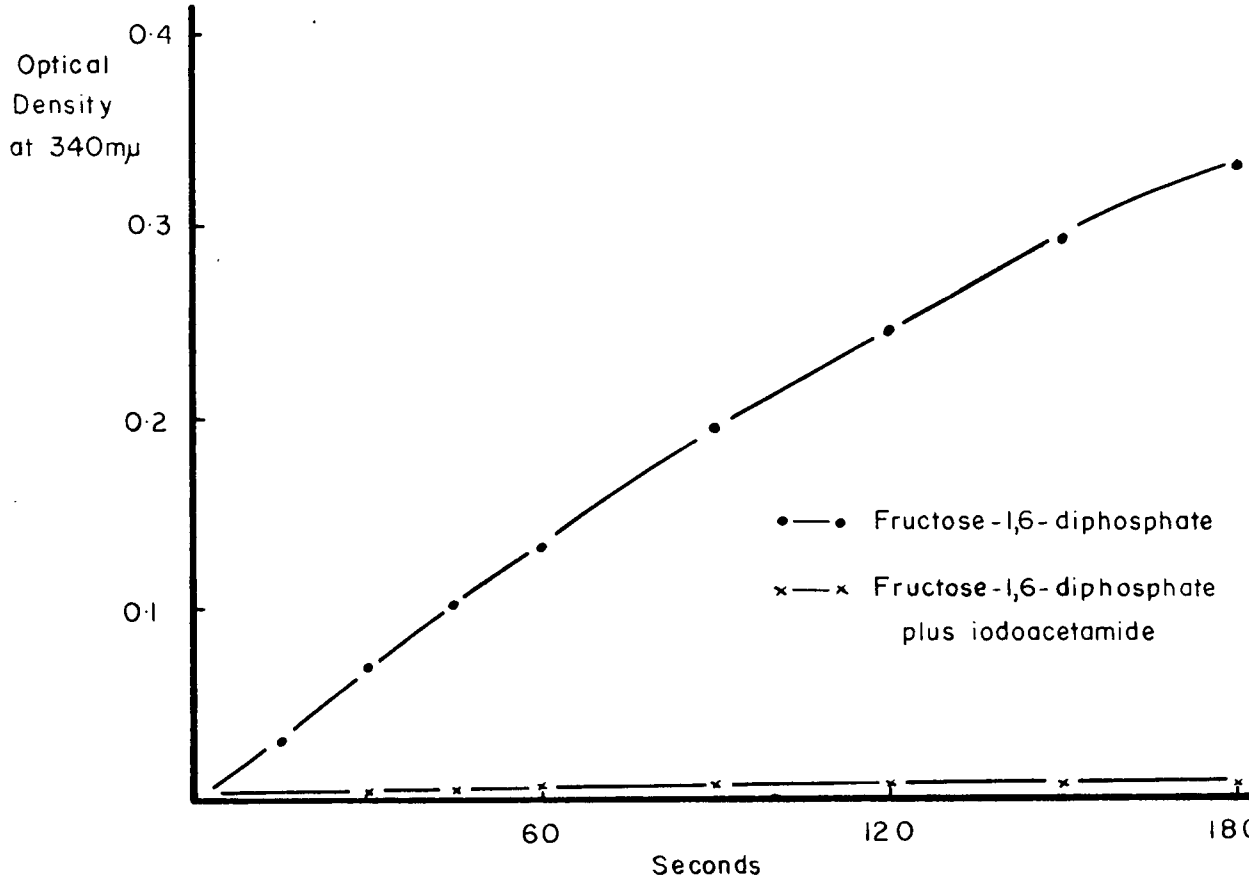

Figure 9. DEMONSTRATION OF FRUCTOSE-1,6-DIPHOSPHATE ALDOLASE

boxylation to pentose phosphate (25). De Ley (35) also found a 2-keto-gluconokinase in extracts of Aerobacter cloacae and concluded that the phosphorylated compound was reduced to 6-phosphogluconate prior to subsequent degradation.

The metabolism of 2-ketogluconate in pressates of P. aeruginosa was expected to proceed either by reduction to gluconic acid followed by phosphorylation or, by phosphorylation to 2-keto-6-phosphogluconate followed by reduction to 6-phosphogluconate.

In a conventional aerobic Warburg run, no oxygen was consumed with pressates incubated with 2-ketogluconate. Neither DPN nor TPN reduction could be detected spectrophotometrically in the presence of 2-keto-gluconate nor DPNH H^+ or TPNH H^+ oxidation and, therefore, it was concluded that 2-ketogluconate was not reduced to gluconic acid by a reduced DPN or TPN specific reductase.

When large Warburg flasks were incubated aerobically or anaerobically at 30°C with M/1 Tris buffer pH 7.4, 25 µmoles of 2-ketogluconate, pressate, 30 µmoles of ATP and 100 µmoles of Mg^{++}, both 2-ketogluconate and glucose-6-phosphate were isolated and identified by electrophoretic means. The presence of hexose phosphate was confirmed by the enzymatic assay for glucose-6-phosphate.

The appearance of glucose-6-phosphate as a product of the reaction suggested that the substrate had been dissimilated by way of the pentose phosphate cycle. In 1938, Dickens (42) postulated the decarboxylation of 2-keto-6-phosphogluconate to produce ribose-5-phosphate. Later, Horecker (73) assumed 6-phosphogluconate to be oxidized to 3-keto-6-phosphogluconate and the latter hypothetical intermediate to be decarboxylated to ribulose-5-phosphate. The direct manometric procedure for CO_2 evolution was employed

in an effort to determine if 2-keto-6-phosphogluconate or 6-phosphogluconate was decarboxylated to pentose phosphate. Reaction mixtures were prepared as described with 2-keto-6-phosphogluconate, 6-phosphogluconate or 2-keto-6-phosphogluconate plus substrate amounts of DPNH H^+. When corrections for oxygen uptake had been considered, it was found that more CO_2 had been evolved from endogenous control flasks than from flasks containing substrates. Obviously no conclusions as to decarboxylation of substrate could be drawn, however, this did serve to emphasize again CO_2 fixation by this organism in the presence of substrate.

The spectrophotometric assay for 2-ketogluconokinase with pressates from glucose grown cells and lysozyme prepared cell-free extracts from 2-ketogluconate grown cells consistently yielded negative results. The manometric technique of measuring substrate phosphorylation by evolution of CO_2 from $NaHCO_3$ also yielded negative results for evidence of a kinase for 2-ketogluconate.

With the previously described method of 2-keto-6-phosphogluconate reductase, it was found that the enzyme was present and active with both TPNH H^+ and DPNH H^+. There was a more rapid oxidation of TPNH H^+ than DPNH H^+ when the pressate controls were subtracted, and the specific activities with these co-factors were .057 and .028, respectively. If the pyridine nucleotide oxidation is, in fact, a measure of 2-keto-6-phosphogluconate reductase, then it is obvious that the enzyme is not very active in the pressates. One would expect the presence of the reductase to be accompanied by the presence of the kinase. It is possible that the kinase is present in the pressates but has an extremely low activity or it may be a very labile enzyme and readily destroyed by the procedures used for

acquiring cell-free extracts. It is also possible that P. aeruginosa does not have a kinase for 2-ketogluconate and the intermediate is metabolized by some other pathway.

Two-ketogluconic acid was incubated anaerobically in large Warburg flasks in the previously mentioned reaction mixture containing ATP, and to one flask a substrate amount of TPN was added. Products of the reaction were isolated and the aniline hydrogen phthalate developer confirmed the presence of 2-ketogluconate. The potassium permanganate developer revealed the presence of two other compounds in the reaction mixture containing substrate and ATP and one additional compound in the reaction mixture containing substrate, ATP and TPN. These compounds could not be positively identified but were believed to be trioses negatively charged at pH 8.6. The two compounds from the reaction mixture without TPN could have been, from their comparative mobilities, 3-phosphoglyceraldehyde and 3-phosphoglyceric acid. The intermediate from the reaction mixture containing TPN corresponded to the compound suggested as being 3-phosphoglyceric acid -- there was a considerably larger amount of this intermediate when the substrate was incubated with TPN. As some 2-ketogluconate had been converted to glucose-6-phosphate probably by way of the pentose phosphate cycle, it is reasonable that 3-phosphoglyceraldehyde would appear as an intermediate and also be oxidized to 3-phospho-glyceric acid in the presence of TPN.

GENERAL DISCUSSION

The use of cell-free extracts to demonstrate the presence of functional enzymes or metabolic pathways in an organism can offer only qualitative information and implications of enzymatic activities in the whole cell. The quantitative estimation of sequential enzymatic reactions measured in cell-free extracts, where the integrity of the whole cell has been completely disrupted, can not justifiably be applied to the situation that exists in a highly organized intact unit. Further, the amount of hexose dissimilated either by way of the pentose phosphate or Entner-Doudoroff pathways in P. aeruginosa will, unquestionably, vary according to the needs of the organism as governed by both growth conditions and stage of development.

The conversion of hexose-phosphate to pentose phosphate without CO_2 evolution has been shown to occur as a result of the relative non-specificity of the transketolase-transaldolase reactions. The pathway appears to be both freely reversible and highly economic but not necessarily of physiological importance (44), (112). Glucose dissimilation in P. aeruginosa via the Entner-Doudoroff and pentose phosphate pathways provides both the intermediates and enzymes necessary for the function of this pathway. Therefore, it is perhaps reasonable to suggest that this pathway occurs in this and many other microorganisms and tissues. The conversion of triose phosphates to hexose phosphates has been implicated in this organism. Therefore, in an organism that has two or more schemes for glucose degradation and some intermediates are common to all pathways, then certainly this must present a situation where an intermediate produced by one scheme may be further oxidized or utilized in another scheme. Glucose dissimilation can not be

a simple matter of some of the substrate being completely oxidized by one pathway and the remainder being oxidized by another without some interaction of the pathways.

The fixation of carbon dioxide by this organism was shown indirectly during attempts to measure CO_2 evolution during substrate oxidation and also as a result of substrate phosphorylation in the presence of sodium bicarbonate. Gladstone et al. (59) found that CO_2 in growth medium was a prerequisite for growth of P. aeruginosa. Using $C^{14}O_2$, Hauser and Karnovsky (68) conducted an experiment in which 17 per cent of the carbon available to P. aeruginosa represented CO_2 in the atmosphere in contact with the medium. They found atmospheric CO_2 to be incorporated well into the cell as 27 per cent of the total bacterial carbon was supplied by $C^{14}O_2$. Carbon dioxide fixation by this organism, then, is an important function and probably occurs at the triose level, maintaining an adequate supply of dicarboxyllic acids.

Ochoa (107) states that the decarboxylation of pyruvic acid to acetaldehyde is probably not reversible and, therefore, not involved in CO_2 fixation. However, the decarboxylation of malic acid is reversible and extremely effective in CO_2 fixation (108), (109). The "malic" enzyme converts pyruvic acid and CO_2 to malic acid in the presence of Mn^{++} and an adequate supply of TPNH H^+. In P. aeruginosa dehydrogenation of either glucose-6-phosphate or 6-phosphogluconate supply the cell with TPNH H^+ and the Entner-Doudoroff pathway supplies pyruvic acid, consequently, CO_2 fixation may be mediated via this scheme. The cell-free extracts of the organism also contain pyruvic kinase and this suggests phospho-enol-pyruvate as an intermediate in glucose dissimilation. This compound could also play an important role in fixing CO_2 to produce oxalacetic acid and act as a

hydrogen acceptor for reduced TPN.

The presence of the non-phosphorylated oxidative pathway of glucose degradation in P. aeruginosa has been well substantiated, however, its physiological importance relative to the phosphorylated pathways has not been ascertained. The conversion of 2-ketogluconate to glucose-6-phosphate and probably triose phosphate was demonstrated during this study but the means of conversion was not elucidated. From the previously mentioned investigations with similar organisms, one could suspect that the conversion was initiated by a specific 2-ketogluconic acid kinase followed by the conventional pentose phosphate cycle. Direct phosphorylation of the substrate, however, could not be demonstrated by cell-free extracts of glucose or 2-ketogluconate grown cells. While these results do not exclude the existence of the enzyme in whole cells, they do offer the possibility of other means of degradation of the compound.

De Ley and De Floor (36) have found that some organisms will grow in a medium containing 2-ketogluconate as the sole source of carbon and not produce a kinase for 2-ketogluconate. The possibility of 2-ketogluconate undergoing a 3:3 split to yield glyceraldehyde and β-hydroxypyruvic acid has been suggested (64), (81). Further, it has been suggested that β-hydroxypyruvate is decarboxylated and then recombines with glyceraldehyde to produce ribose (81). The suggestion of a 3:3 split offers a number of possible subsequent reactions. Firstly, the sequence involving ribose could result in phosphorylation of ribose by ribokinase followed by the activity of the pentose phosphate enzymes yielding hexose phosphate and triose phosphate. Secondly, the hydroxypyruvate could be assimilated by the cell after conversion to serine. The glyceraldehyde could also be

converted to glyceric acid and then hydroxypyruvate and be assimilated, or it could be directly phosphorylated or phosphorylated after isomerization to dihydroxyacetone and the resulting triose phosphate converted to hexose phosphate through hexose diphosphate. Thirdly, some hydroxypyruvate could possibly be reduced and phosphorylated to 3-phosphoglyceric acid. Pseudomonas aeruginosa has been reported to contain a glycolipide composed of 2 moles of rhamnose and 2 moles of β-hydroxydecanoic acid (84). The rhamnose moiety has been suggested as being derived from 2 moles of glycerol without cleavage of its carbon-carbon bonds. Conceivably, then, a portion of the glyceraldehyde formed from a 3:3 split of 2-ketogluconate could be reduced to glycerol and become a part of the glycolipide. There has been no experimental data, offered at this time, to support these postulations, however, it is felt that consideration should be given to these possibilities in subsequent investigations.

The organism under investigation has been shown to dissimilate glucose by way of the pentose phosphate, Entner-Doudoroff and non-phosphorylated oxidative pathways. The non-phosphorylated pathway can be linked to the phosphorylated pathways through phosphorylation of gluconic acid. Triose phosphate oxidation has been demonstrated and the conversion of triose phosphates to hexose phosphate has been implicated. The established pathways are shown in Figure 10.

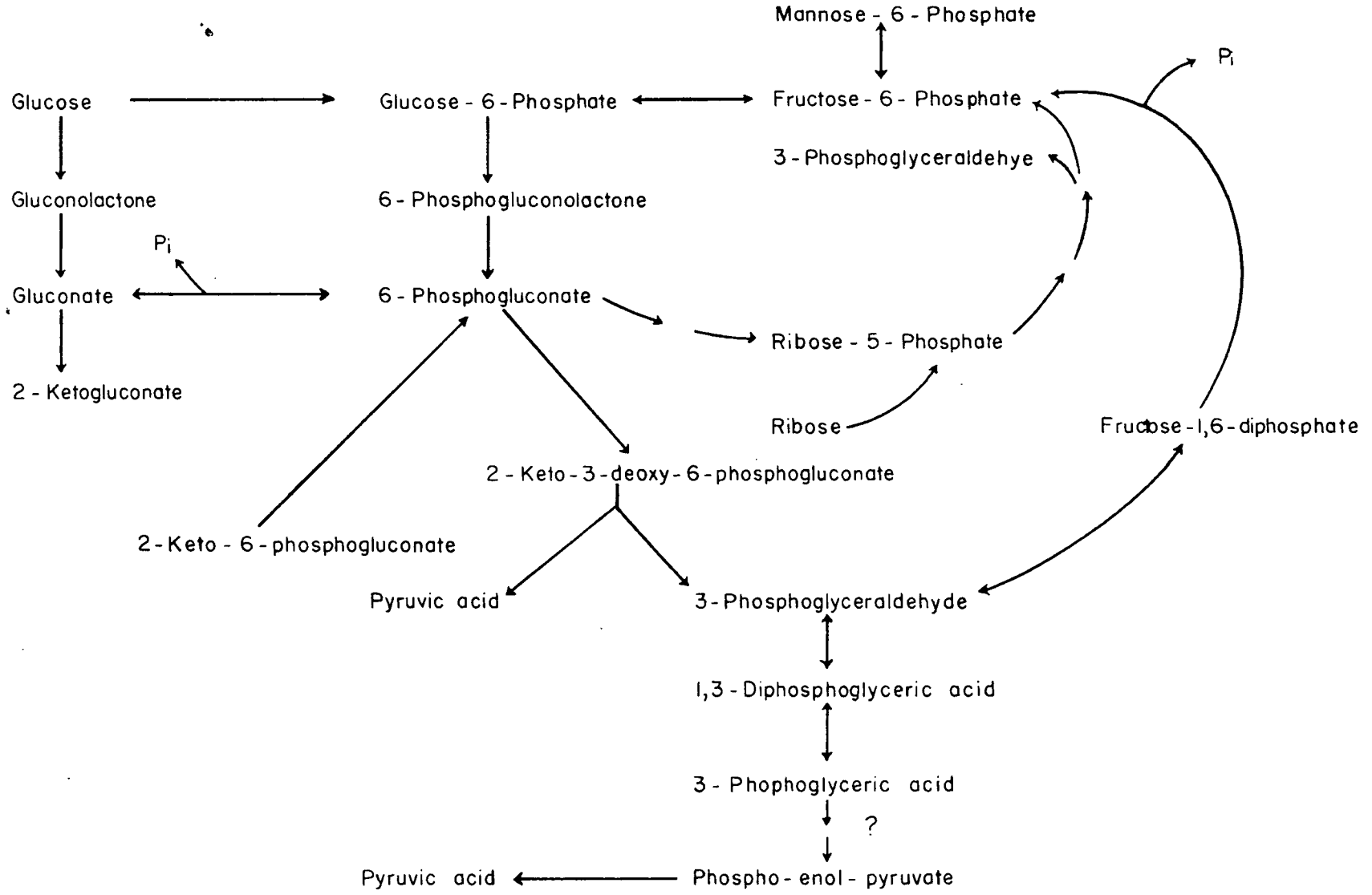

Figure 10. PATHWAYS OF GLUCOSE DISSIMILATION IN PSEUDOMONAS AERUGINOSA.

SUMMARY

The oxidation of glucose to gluconic acid in cell-free extracts of Pseudomonas aeruginosa was shown to proceed through the intermediate gluconolactone and a magnesium requirement for the enzymatic hydrolysis of the lactone was demonstrated. The product of the non-phosphorylated oxidation of glucose and gluconic acid was identified as 2-ketogluconate, as had been reported previously.

The demonstration of specific kinases for glucose and gluconic acid has provided adequate connections between the non-phosphorylated and phosphorylated oxidative pathways of glucose dissimilation.

The conversion of gluconic acid plus ATP and ribose plus ATP to glucose-6-phosphate and glyceraldehyde-3-phosphate; the presence of specific dehydrogenases for glucose-6-phosphate and 6-phosphogluconate and phosphohexoisomerase have been presented as substantial evidence for a functional pentose phosphate cycle.

The production of pyruvic acid from both 2-keto-3-deoxy-6-phosphogluconate and 6-phosphogluconate, together with glucose-6-phosphate dehydrogenase, has established the existence of a second phosphorylated oxidative system -- the Entner-Doudoroff pathway.

An aldolase for fructose-1,6-diphosphate was found and, with fructose-1,6-diphosphate phosphatase and phosphohexoisomerase, the regeneration of glucose-6-phosphate from triose phosphate has been suggested.

Evidence for the oxidation of 3-phosphoglyceraldehyde to 3-phosphoglyceric acid and conversion of phospho-enol-pyruvate to pyruvic acid was found and the possible role of trioses in CO_2 fixation in the organism has

been discussed.

A kinase for 2-ketogluconate could not be demonstrated, however, the presence of a reductase for 2-keto-6-phosphogluconate was confirmed. The dissimilation of 2-ketogluconate in cell-free extracts has been discussed.

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