

## Alginate Synthesis in *Pseudomonas aeruginosa* Mutants Defective in Carbohydrate Metabolism

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Mutant cells of mucoid *Pseudomonas aeruginosa* isolated from cystic fibrosis patients were examined for their ability to synthesize alginate in resting cell suspensions. Unlike the wild-type strain which synthesizes alginate from glycerol, fructose, mannitol, glucose, gluconate, glutamate, or succinate, mutants lacking specific enzymes of carbohydrate metabolism are uniquely impaired. A phosphoglucose isomerase mutant did not synthesize the polysaccharide from mannitol, nor did a glucose 6-phosphate dehydrogenase mutant synthesize the polysaccharide from mannitol or glucose. Mutants lacking the Entner-Doudoroff pathway dehydratase or aldolase failed to produce alginate from mannitol, glucose, or gluconate, as a 3-phosphoglycerate kinase or glyceraldehyde 3-phosphate dehydrogenase mutant failed to produce from glutamate or succinate. These results demonstrate the primary role of the Entner-Doudoroff pathway enzymes in the synthesis of alginate from glucose, mannitol, or gluconate and the role of glyceraldehyde 3-phosphate dehydrogenase reaction for the synthesis from gluconeogenic precursors such as glutamate. The virtual absence of any activity of phosphomannose isomerase in cell extracts of several independent mucoid bacteria and the impairment of alginate synthesis from mannitol in mutants lacking phosphoglucose isomerase or glucose 6-phosphate dehydrogenase rule out free mannose 6-phosphate as an intermediate in alginate biosynthesis.

Alginate is a polymer of selectively acetylated D-mannuronic and L-gulonic acids secreted by strains of *Azotobacter vinelandii* (5) and *Pseudomonas aeruginosa* (4). Some strains of the latter, particularly those isolated from cystic fibrosis patients, synthesize the exopolysaccharide in copious amounts (16) during exponential growth on various carbon sources. During growth on glucose, at least half of the dissimilated sugar appears as alginate. It is curious that the biosynthetic pathway of this major metabolite has not been worked out.

Based on a proposal by Lin and Hassid (10), a pathway of alginate biosynthesis has been suggested to operate in *P. aeruginosa* (20,22) as it operates in *A. vinelandii* (21). The basic feature of the pathway is the conversion of the glycolytic intermediate fructose 6-phosphate to mannose 1-phosphate via mannose 6-phosphate through the enzymes phosphomannose isomerase and phosphomannose mutase, followed by the formation of GDP-mannose which is subsequently oxidized, polymerized, epimerized, and acetylated to alginate.

We describe here results of some of our experiments with resting-cell suspensions of mucoid *P. aeruginosa* that permit synthesis of alginate acid from several precursor compounds such as sugars, dicarboxylic acids, etc. We have examined in this system the synthesis of the polysaccharide in mutants affected in various enzymes of carbohydrate metabolism. Our results indicated that triose phosphates or their condensation product, such as fructose 1,6-diphosphate rather than mannose 6-phosphate, serve as the precursor of alginate in a mucoid cystic fibrosis isolate of *P. aeruginosa*.

### MATERIALS AND METHODS

**Stock cultures.** Mucoid strain FRD1 of *P. aeruginosa* was isolated from the sputum of a cystic fibrosis patient. A histidine auxotroph isolated by mutagenesis with ethyl methane sulfonate (17) and designated FRD7 was used as the strain in which further mutations were derived. Cultures were maintained on LB agar plates (12), and stocks were stored in sterile skim milk (10 g/100 ml) at -35°C. Mucoid bacteria require frequent transfers to prevent outgrowth by nonmucoid derivatives; cultures were transferred every 4 days. Incubation was at 37°C unless mentioned otherwise.

**Isolation of mutants.** For the isolation of sugar-negative mutants, a single mucoid colony of FRD7 was grown overnight in the synthetic medium described by

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Ohman and Chakrabarty (17) with 50 mM sodium succinate as the carbon source and 0.2 mM histidine. It was mutagenized with ethyl methane sulfonate, and the washed culture was subjected to an alternate cycle of growth in the permissive succinate medium followed by counterselection of mannitol-positive clones in a medium containing mannitol and carbenicillin (as in reference 17). A reasonable enrichment of carbohydrate-negative mutants was achieved after four cycles. The culture was plated on the same minimal medium with 50 mM mannitol and 0.5 mM sodium succinate as the sources of carbon (19). Colonies that remained small after 3 days at 37°C were picked; those which grew on succinate but not on gluconate were tested for defects in specific enzymes of carbohydrate metabolism. Mutants were maintained on LB agar plates lacking glucose.

To isolate mutants blocked in the enzymes of the lower trunk of the Embden-Meyerhof pathway, gluconate was chosen as the permissive substrate and succinate was chosen as the impermissible substrate (1); counterselection with carbenicillin was done in succinate medium. Gluconate-positive colonies failing to grow on succinate or on glutamate were examined for enzyme lesions. These mutants were maintained on plates of gluconate minimal medium (17).

The mucoid parent strain FRD7 was prone to become nonmucoid during mutagenesis and antibiotic selection particularly when the alginate-promoting growth medium (17) was used with a supplementation of 30 mM  $(\text{NH}_4)_2\text{SO}_4$ , called modified Mian medium. Mutagenized cultures were streaked daily on LB agar plates to monitor the appearance of nonmucoid revertants. Runs were abandoned whenever a particular culture contained more than 5% of nonmucoid colonies.

For the isolation of revertants, the mutants were grown overnight in permissive medium, and an appropriate sample of the washed bacterial suspension was plated on the medium in which the mutants were unable to grow. The mannitol-negative succinate-positive mutants were reverted on media containing glucose, mannitol, or gluconate, whereas the glutamate-negative gluconate-positive mutants were reverted on plates containing either glutamate or succinate as the sole source of carbon. Infrequent colonies appearing on these plates were purified and kept for further studies.

**Alginate synthesis in resting cells.** Cells grown overnight in liquid media were washed twice with saline and suspended in 10 mM phosphate buffer in 150 mM NaCl (pH 7.4) containing 0.5 mM  $\text{MgCl}_2$  at a concentration of 1 to 2 mg of wet cells per ml. Gluconate, glutamate, glycerol, etc., used as precursors of alginate, were present at a concentration of 10 to 20 mM. Incubation was done in 50-ml sterile Erlenmeyer flasks containing 5 ml of the bacterial suspension in a gyratory shaker at 37°C. Samples of 0.5 ml were taken at intervals and centrifuged, and the alginate acid was estimated in the supernatants after dialysis for 18 h at room temperature with several changes of distilled water. The modified procedure of Knutson and Jeanes (7) was used to estimate alginate.

**Enzyme assays.** The activities of 6-phosphogluconate dehydrase and 2-keto 3-deoxy 6-phosphogluconate aldolase were estimated by the procedure of Kovachovich and Wood (8, 9). Phosphomannose isomerase was

assayed by the method of Kang and Markovitz (6). All other enzyme assays have been described previously (11). Reactions were conducted in 1-ml cuvettes in a Gilford 2600 spectrophotometer system at 340 nm. Cell extracts were prepared by sonication in 50 mM potassium phosphate buffer (pH 7.4) containing 0.5 mM EDTA and 0.2 mM dithiothreitol, followed by centrifugation at  $15,000 \times g$  for 10 min. For the assay of 6-phosphogluconate dehydrase and phosphomannose isomerase activities, EDTA was omitted. Results for enzyme assays have been expressed as milliunits of enzyme activity, i.e., nanomoles of substrate consumed per minute per milligram of protein.

**Chemicals.** 2-Keto 3-deoxy 6-phosphogluconate aldolase was prepared from *Pseudomonas putida* PRS2015 by the method of Meloche et al. (15). The first  $(\text{NH}_4)_2\text{SO}_4$  precipitate was used as the source of the enzyme; it was free of dehydrase activity. 2-Keto 3-deoxy 6-phosphogluconate was prepared from glyceraldehyde 3-phosphate and pyruvate by using this aldolase preparation (14). All other enzymes and substrates were from Sigma Chemical Co.

## RESULTS

**Synthesis of alginate by resting cells of wild-type strain.** Results in Fig. 1 show a typical experiment which describes the synthesis of alginate by a resting-cell suspension of wild-type strain FRD1 from the following six precursors: glycerol, fructose, mannitol, glucose, gluconate,

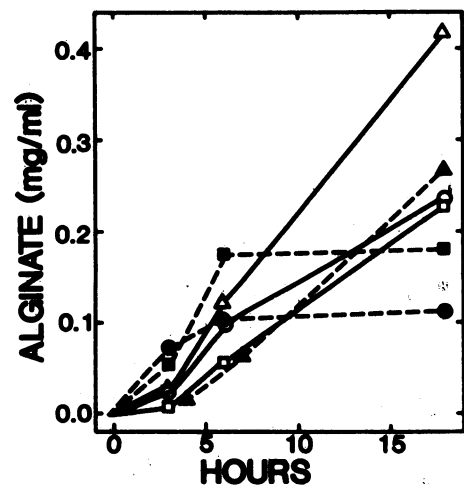


FIG. 1. Time course of alginic acid synthesis in a wild-type strain of *P. aeruginosa*. Mucoid strain FRD1 was grown overnight in modified Mian medium with 100 mM glutamate and 50 mM gluconate. The cells were washed and suspended in phosphate saline, as mentioned in the text, at a concentration giving a Klett reading of 145 with the red filter. Experimental points refer to the amount of alginic acid synthesized by the resting-cell suspensions during the incubation periods indicated on the abscissae. Symbols: ○, glucose; △, fructose; ●, gluconate; □, mannitol; ■, glutamate; ▲, glycerol. Concentrations were 10 mM in every case.

and glutamate. The synthesis from gluconate took place without any delay but was not sustained very long. Although the synthesis from fructose was delayed, the amount of alginate exceeded that made from the other precursors. The plateauing of the synthesis from gluconate or glutamate perhaps reflected the exhaustion of the precursor pool, since higher initial concentrations elicited more extensive synthesis.

**Characterization of carbohydrate dissimilation-defective mutants.** *P. aeruginosa* utilizes the Entner-Doudoroff pathway for the breakdown of hexose sugars. A sugar such as mannitol is converted to 6-phosphogluconate through the enzymes phosphoglucose isomerase and glucose 6-phosphate dehydrogenase (19). Mutants unable to grow on mannitol are therefore expected to carry lesions affecting either the Entner-Doudoroff pathway or these two enzymes. We have obtained mutants lacking the following enzymes in a collection of succinate-positive mannitol-negative clones: phosphoglucose isomerase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrase, and 2-keto 3-deoxy 6-phosphogluconate aldolase. The mutants were grown overnight in LB broth lacking glucose, followed by dilution in fresh medium containing 50 mM gluconate and a further incubation for a 6-h period to induce the enzymes of carbohydrate metabolism (19). The enzyme deficiencies were associated with defective growth on specific substrates. Although all the four classes of mutants were unable to grow on mannitol (Table 1), the one lacking phosphoglucose isomerase grew on all the other substrates tested. Its growth on fructose was, however, slower than that of the wild-type strain.

The mutant lacking glucose 6-phosphate dehydrogenase was characterized additionally by a very slow rate of growth on fructose; it failed to make visible colonies in 2 days. Longer incubation led to a faint growth on fructose plates. Mutants lacking the dehydrase and the aldolase of the Entner-Doudoroff pathway were impaired in growth on most substrates, the dehydrase mutant being glycerol positive (2).

The growth of mutants in a permissive medium was inhibited by the addition of an impermissible carbon source such as gluconate. This was found to be associated with the accumulation inside cells of deleterious levels of substrates of the blocked reaction. This is illustrated for the mutants lacking the dehydrase and the aldolase (Fig. 2). The wild-type strain in contrast had much reduced amounts of these metabolites. The results showed that the enzyme activity measured in cell extracts reflected the situation in vivo; the dehydrase mutant accumulated 6-phosphogluconate, whereas 2-keto 3-deoxy 6-phosphogluconate accumulation was observed in the aldolase mutant. In contrast, mutants lacking glucose 6-phosphate dehydrogenase were not found to accumulate glucose 6-phosphate when incubated with glucose.

The mutants presumably harbored defects in a single gene determining the activities of the respective enzymes. A thick suspension of each of the mutants was plated on minimal media containing the impermissible substrates, such as mannitol (mutants 60 and 24), glucose or gluconate (mutants 13 and 1), or a mixture of succinate and glutamate (mutants 1518 and 1504). Spontaneous revertant colonies appeared at frequencies of the order of  $10^{-6}$  to  $10^{-7}$ . These

TABLE 1. Growth phenotype of sugar-negative mutants of mucoid *P. aeruginosa*

Mutant no.	Enzyme deficiency <sup>a</sup>	Growth on: <sup>b</sup>						
		Mtl	Fru	Gly	Glu	Gcn	Suc	Gtm
60	PGI (6)	-	+	+	+	+	+	+
24	ZWF (1)	-	- <sup>c</sup>	+	+	+	+	+
13	EDD (3)	-	- <sup>c</sup>	+	-	-	+	+
1	EDA (0.4)	-	- <sup>c</sup>	-	-	-	+	+
1518	GAP (0.6)	+	+	+	+	+	-	-
1504	PGK (0.3)	+	+	+	+	+	-	-
FRD7 (wild type)	None	+	+	+	+	+	+	+

<sup>a</sup> PGI, Phosphoglucose isomerase; ZWF, glucose 6-phosphate dehydrogenase; EDD, 6-phosphogluconate dehydrase; EDA, 2-keto 3-deoxy 6-phosphogluconate aldolase; GAP, NADP-linked glyceraldehyde 3-phosphate dehydrogenase; PGK, 3-phosphoglycerate kinase. The numbers within parentheses record the respective mutated enzyme activity as a percentage of that of the wild-type strain grown under identical conditions. The enzyme activities in milliunits per milligram of protein in the wild-type strain were: PGI, 52; ZWF, 208; EDD, 150; EDA, 250; GAP, 254; and PGK, 344.

<sup>b</sup> Growth was scored on day 2 in minimal plates containing 50 mM carbon sources: Mtl, mannitol; Fru, fructose; Gly, glycerol; Glu, glucose; Gcn, gluconate; Suc, succinate; Gtm, glutamate. +, Growth; -, no growth.

<sup>c</sup> Very little growth in 2 days; longer incubation allowed slow growth on fructose.

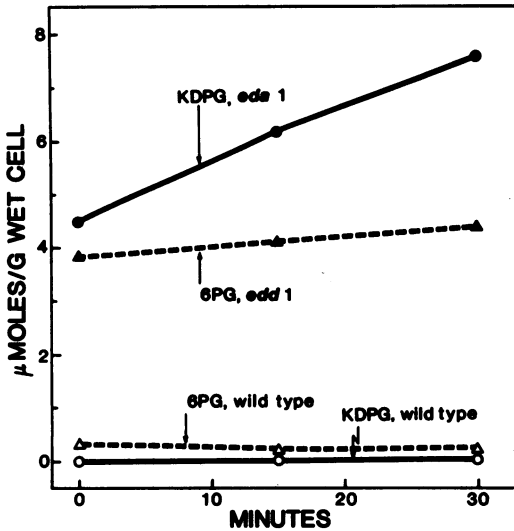


FIG. 2. Kinetics of intracellular accumulation of metabolites in *P. aeruginosa* mutants lacking 6-phosphogluconate dehydrase (*edd-1*) and 2-keto 3-deoxy 6-phosphogluconate aldolase (*eda-1*). The mutants and the wild-type strain were grown overnight in modified Mian medium with 100 mM glutamate and induced with 50 mM gluconate for 4 h in fresh medium before harvesting. The washed cells were suspended in 50 mM potassium phosphate buffer (pH 7.6) and bubbled with a stream of air over a magnetic stirrer. At time zero, 20 mM gluconate was added. Samples were withdrawn into a final concentration of 0.5 N HClO<sub>4</sub> and left for 15 min for extraction, and metabolites in the neutralized supernatants were measured (11) spectrophotometrically with 6-phosphogluconate dehydrogenase for the assay of 6-phosphogluconate (6PG) and 2-keto 3-deoxy 6-phosphogluconate aldolase and lactic dehydrogenase for the estimation of the 2-keto 3-deoxy 6-phosphogluconate (KDPG). Cell concentrations in milligrams (wet weight) per milliliter of suspensions were: wild-type strain FRD7, 32; *edd-1*, 40; *eda-1*, 36.

were purified and tested for growth on media containing the other carbon sources. The spontaneous revertants regained the ability to grow on all the compounds for which the mutants were negative. Separate experiments indicated that the revertants of mutants lacking phosphoglycerate kinase and 2-keto 3-deoxy 6-phosphogluconate aldolase had regained the corresponding enzyme activities. We concluded that the mutants had possibly no other enzyme defects besides the one in question. The reversion events also conferred the ability to synthesize alginic acid from the carbon sources, which were impermissible for the mutants.

**Alginate synthesis by resting cells of carbohydrate dissimilation-defective mutants.** Results in Fig. 3 depict the kinetics of alginic acid synthesis by buffered suspension of the mutant bacteria.

Synthesis of alginate from glutamate continued in all the mutants at rates comparable to that seen in the wild-type strain. Rates from the glucose 6-phosphate dehydrogenase mutant (Fig. 3) were the most rapid of all, namely, 40 mg of alginate per h per g of wet cells, corresponding to ca. 50 nmol of guluronic or mannuronic acid equivalent per mg of protein per min. The synthesis from the other carbohydrate precursors depended on the metabolism of the sugar. Thus, the phosphogluconate isomerase mutant synthesized alginic acid from all the sugar precursors except mannitol. The glucose 6-phosphate dehydrogenase mutant synthesized the polysaccharide from all but mannitol and glucose. In the mutants affected in the Entner-Doudoroff dehydrase and aldolase (Fig. 3C and D), the synthesis from gluconate was arrested as well. Glycerol permitted synthesis in all the mutants, and so did fructose; however, in the dehydrase mutant, the synthesis from fructose was found to plateau off after 4 h. This was seen in another independent isolate of the dehydrase mutant. Since the dehydrase mutant accumulated 6-phosphogluconate (Fig. 2), it is possible that the cessation of alginate synthesis from fructose in the dehydrase mutant reflected the inhibition of some step in alginate biosynthesis by a metabolite that accumulates in the dehydrase mutant rather than a metabolic block that prevents the flux of the sugar along the pathway of alginate biosynthesis. The extensive synthesis permitted in the aldolase mutant from fructose justifies this assumption. These results provided definitive evidence that the Entner-Doudoroff pathway enzymes play a major role for alginic acid biosynthesis from mannitol, glucose, or gluconate in mucoid *P. aeruginosa*.

**Alginate synthesis in mutants of *P. aeruginosa* affected in 3-phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase.** By looking for mutants that could grow on gluconate but were unable to grow on gluconeogenic precursors, we isolated several mutants that grew on the following compounds as the single source of carbon: mannitol, fructose, glycerol, glucose, and gluconate. However, they failed to grow on all gluconeogenic compounds such as lactate, glutamate, succinate, or their mixtures.

Assay of enzymes in crude cell extracts indicated that most of these mutants were deficient in 3-phosphoglycerate kinase, whereas one mutant was deficient in the activity of glyceraldehyde 3-phosphate dehydrogenase. Isolate 1504, lacking the kinase, was taken as a prototype of the former class, whereas isolate 1518 represented the mutant with the dehydrogenase deficiency. Their growth behavior is recorded in Table 1. The mutated enzyme activities are also indicated. Both the mutants were defective in glu-

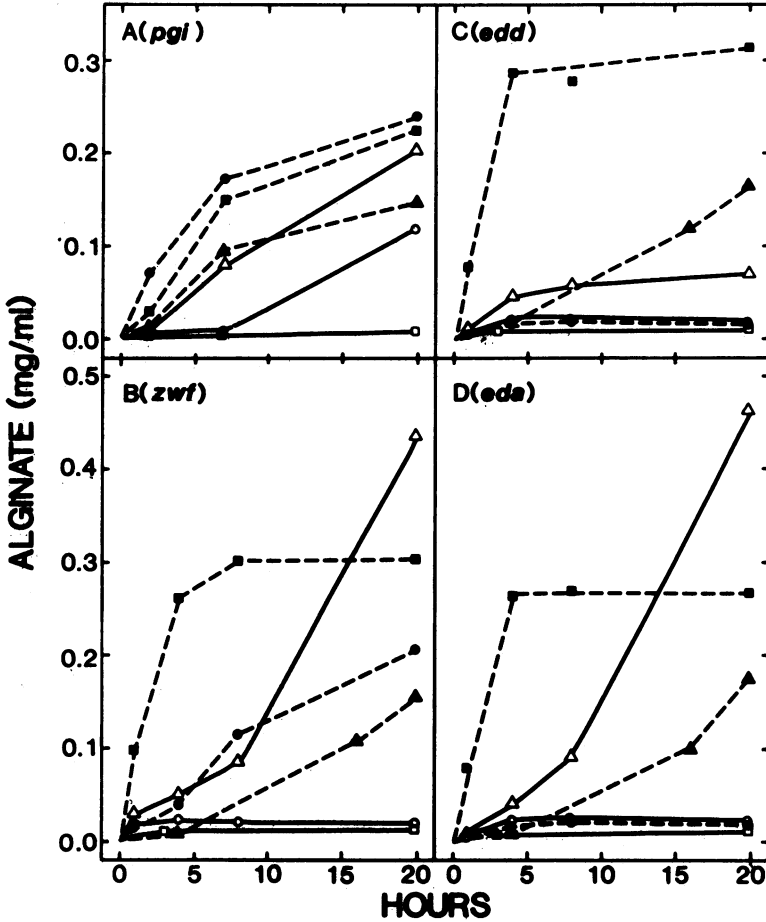


FIG. 3. Kinetics of alginate synthesis by a resting-cell suspension of *P. aeruginosa* mutants. (A), (B), (C), and (D) refer, respectively, to mutants lacking phosphoglucose isomerase (*pgi*), glucose 6-phosphate dehydrogenase (*zwf*), 6-phosphogluconate dehydrase (*edd*), and 2-keto 3-deoxy 6-phosphogluconate aldolase (*eda*). The strains were grown on modified Mian medium with 100 mM gluconate and 100 mM glutamate for 14 h; the *pgi* mutant was grown for 60 h. The final cell density was adjusted for all suspensions to  $148 \pm 2$  Klett units. Other details are as described in the legend to Fig. 1. Symbols: ○, glucose; ●, gluconate; ■, glutamate; □, mannitol; Δ, fructose; ▲, glycerol. Substrate concentrations were 20 mM each.

coneogenic growth but were otherwise normal. Spontaneous reversion of these mutants yielded colonies that were able to grow on lactate, succinate, and glutamate, leading to the restoration of the 3-phosphoglycerate kinase activity. None of the revertants from the dehydrogenase mutant, however, showed any activity of the glyceraldehyde 3-phosphate dehydrogenase with NADP as the electron acceptor in crude cell extracts. Whether this was due to the restitution of a particularly labile enzyme activity as a result of mutations in the structural gene has not been determined. The revertant extracts, however, continued to have an NAD-linked glyceraldehyde 3-phosphate dehydrogenase activity like wild-type strains FRD1 or FRD7. The NAD-linked enzyme constituted only about 5%

of the activity of the NADP enzyme in the wild-type strain grown on glutamate.

Figure 4 depicts the kinetics of alginate synthesis in resting-cell suspensions of these two mutants. Although alginate synthesis continued as in the wild-type strains from sugars, glycerol, or gluconate, no synthesis was observed from either glutamate or succinate in these mutants. This result indicated that glyceraldehyde 3-phosphate must be generated by way of the glyceraldehyde 3-phosphate dehydrogenase reaction so that gluconeogenic precursors may synthesize alginic acid in mucoid *P. aeruginosa*. Together with the results in Fig. 3, these experiments demonstrate the indispensability of glyceraldehyde 3-phosphate or some metabolite derived from it or both in alginic acid biosynthesis.

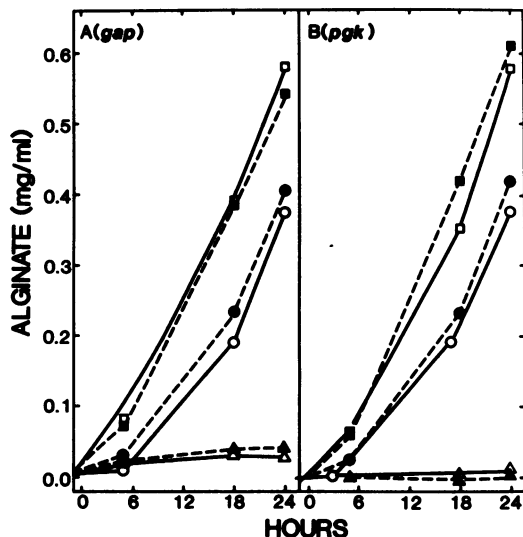


FIG. 4. Kinetics of alginate synthesis by resting-cell suspension of *P. aeruginosa* mutants lacking glyceraldehyde 3-phosphate dehydrogenase (*gap*) or phosphoglycerate kinase (*pgk*). The mutants were grown overnight in modified Mian medium containing 50 mM gluconate and 50 mM glutamate. The density of cell suspensions was  $150 \pm 2$  Klett units with filter 66. Symbols: ●, fructose; ■, glycerol; □, gluconate; ▲, glutamate; △, succinate; ○, mannitol. Other details are as described in the legend to Fig. 1.

**Role of phosphomannose isomerase in alginate biosynthesis.** Earlier work on bacteria (20, 21) had implicated mannose 6-phosphate as an intermediate in alginic acid synthesis. Since this compound could be produced from glyceraldehyde 3-phosphate through the successive intervention of triose phosphate isomerase, fructose 1,6-diphosphate aldolase, fructose diphosphatase, and phosphomannose isomerase, we examined the activity of the enzyme catalyzing the isomerization of fructose 6-phosphate to mannose 6-phosphate. Cell extracts of several independent isolates of mucoid bacteria from cystic fibrosis patients were examined for the activity of phosphomannose isomerase. To our surprise, this activity was absent in all mucoid *P. aeruginosa* strains including FRD1, FRD7, PCF1 (17), and V209 (13) as well as their spontaneous nonmucoid derivatives either in cell extracts or in a  $10,000 \times g$  pellet. A strain of *P. aeruginosa* PAO also behaved likewise. In contrast, the isomerase activity was present in *Escherichia coli* strains under these conditions. Although the *E. coli* strain had 41 MU of phosphomannose isomerase per mg of protein, the *Pseudomonas* strains had less than 1 MU of this activity per mg of protein in cell extracts. The fact that the

absence of activity was not due to the presence of an inhibitor was confirmed by mixing the extracts of mucoid or nonmucoid *Pseudomonas* strains with that of *E. coli*. The *Pseudomonas* extracts also lacked the activity of phosphomannose mutase that has been suggested to convert mannose 6-phosphate to mannose 1-phosphate (20, 21).

## DISCUSSION

The experiments with sugar-negative mutants of *P. aeruginosa* reported here revealed an unexpected feature of alginic acid biosynthesis (Table 2). The primary role of the Entner-Doudoroff pathway enzymes in the synthesis from glucose, gluconate, and mannitol is evident. The role of these enzymes in the metabolism of carbohydrates in *P. aeruginosa* is also well documented (19). This shows that the alginate biosynthetic pathway deviates from the route of carbohydrate breakdown only after the hexose skeleton is cleaved into three-carbon intermediates. Is metabolic conversion of glyceraldehyde 3-phosphate along the Embden-Meyerhof pathway or that of pyruvate along the tricarboxylic acid cycle required for alginate biosynthesis? Work with mutants lacking the enzymes 3-phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase provides important clues to this question. Results in Table 2 show clearly that glyceraldehyde 3-phosphate is an intermediate in the biosynthesis of alginate from gluconeogenic precursors. The observation that both the 2-keto 3-deoxy 6-phosphogluconate aldolase and the glyceraldehyde 3-phosphate dehydrogenase reactions need to be executed from the direction of either carbohydrate catabolism or anabolism points to glyceraldehyde 3-phosphate or a compound metabolically derived from it, such as fructose 1,6-diphosphate, as the precursor of alginic acid in *P. aeruginosa*.

TABLE 2. Alginate synthesis by resting-cell suspension of mutants defective in carbohydrate metabolism<sup>a</sup>

Enzymatic defect	Alginate made in carbon source						
	Mtl	Fru	Gly	Glu	Gcn	Suc	Gtm
PGI	-	+	+	+	+	+	+
ZWF	-	+	+	-	+	+	+
EDD	-	±	±	-	-	+	+
EDA	-	+	+	-	-	+	+
GAP	+	+	+	+	+	-	-
PGK	+	+	+	+	+	-	-
None	+	+	+	+	+	+	+

<sup>a</sup> Data on alginate synthesis are compiled from Fig. 1, 3, and 4. Abbreviations are as in Table 1. +, Alginate made; -, alginate not made; ±, limited synthesis.

The earlier studies by Carlson and Matthews (3) on the incorporation in alginate of carbon from specifically labeled glucose are in accord with our results. When C-6 of guluronic and mannuronic acid residues was decarboxylated by heating the labeled alginate with HCl, a very strong asymmetry in the pattern of labeling in alginate from [1-<sup>14</sup>C]glucose versus [6-<sup>14</sup>C]glucose was observed; the <sup>14</sup>CO<sub>2</sub> obtained from [6-<sup>14</sup>C]glucose exceeded that from [1-<sup>14</sup>C]glucose by greater than two orders of magnitude. This suggested that C-1 of glucose hardly gets incorporated in C-6 of guluronic and mannuronic acids. This would be expected if carbon atoms 1, 2, and 3 of glucose are lost as pyruvate via the 2-keto 3-deoxy 6-phosphogluconate aldolase reactions, and carbon atoms 4, 5, and 6 are retained for alginate synthesis.

Although our results throw no light on the actual route of alginic acid synthesis from glyceraldehyde 3-phosphate, the earlier suggestion (20) that mannose 6-phosphate is an intermediate is rendered untenable. This is borne out not only by the absence of phosphomannose isomerase activity in several *Pseudomonas* strains, including a mucoid variant of *P. aeruginosa* PAO (kindly provided by A. Darzins, of this laboratory), but also by the failure of mannitol to support alginate synthesis in both the mutants lacking phosphoglycerate kinase and glucose 6-phosphate dehydrogenase, respectively. We are unable to explain the discrepancy between the results of Piggott et al. (20) and ours. The metabolism of mannitol and fructose has been suggested by the work of Phibbs et al. (19) to proceed by the pathway shown in Fig. 5. The growth properties of mutants described in this work are consistent with the proposed pathway. If mannose 6-phosphate produced from fructose 6-phosphate by phosphomannose isomerase were the precursor of alginate, its synthesis from mannitol would have been unimpaired in phosphoglycerate kinase mutant; so would have been the synthesis from glucose in the glucose 6-phosphate dehydrogenase mutant. The fact that glucose fails to elicit synthesis in this mutant shows that glucose 6-phosphate is channeled not via phosphoglycerate kinase but rather through the Entner-Doudoroff pathway (Fig. 5).

That none of the six enzyme deficiencies (Table 2) affects the synthesis from glycerol and fructose suggests strongly that these two precursors follow a metabolic pathway to alginate that is not used during the metabolism of glucose or gluconate. The loss of 2-keto 3-deoxy 6-phosphogluconate aldolase had practically no effect on alginate synthesis from either glycerol or fructose, whereas that from glucose or gluconate came to a halt. Together with the pattern of fructose metabolism suggested by Phibbs et al.

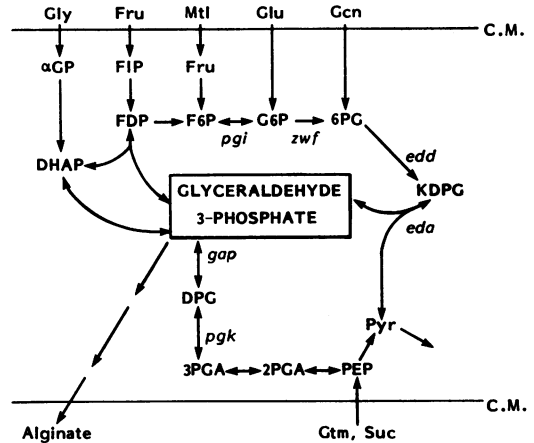


FIG. 5. Suggested metabolic route of sugars and carboxylic acids in *P. aeruginosa*.  $\alpha$ GP,  $\alpha$ -Glycerophosphate; DHAP, dihydroxyacetone phosphate; F1P, fructose 1-phosphate; FDP, fructose 1,6-diphosphate; F6P, fructose 6-phosphate; G6P, glucose 6-phosphate; 6PG, 6-phosphogluconate; KDPG, 2-keto 3-deoxy 6-phosphogluconate; Pyr, pyruvate; PEP, phosphoenol pyruvate; 2PGA, 2-phosphoglycerate; 3PGA, 3-phosphoglycerate; DPG, 1,3-diphosphoglycerate; C.M., cytoplasmic membrane. Other abbreviations are as indicated in Table 1. Mutants are italicized.

(19), this result may mean that fructose is also metabolized via fructose diphosphate aldolase to produce the precursor of alginate biosynthesis. Glycerol metabolism also culminates in the production of glyceraldehyde 3-phosphate as depicted in Fig. 5 through glyceral kinase,  $\alpha$ -glycerophosphate dehydrogenase, and triose phosphate isomerase (2). It would be interesting to examine the behavior of mutants lacking fructose diphosphatase and fructose diphosphate aldolase. Loss of the former enzyme should not affect alginate synthesis from fructose, since triose phosphates can be produced directly (Fig. 5). Loss of fructose diphosphate aldolase, on the other hand, should serve to define the alginate precursor as being a triose or hexose; if a triose, then glycerol but not fructose would permit synthesis in a double mutant lacking both the fructose diphosphate aldolase and any of the enzymes of the Entner-Doudoroff pathway. If the precursor is a hexose, such a mutant would make alginate from fructose but not from glycerol. The critical role of fructose 1,6-diphosphate during alginate synthesis from mannitol, glucose, or gluconate will be the subject of a future communication.

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