Regulation of Fructose Metabolism and Polymer Synthesis by *Fusobacterium nucleatum* ATCC 10953

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Energy for the anaerobic growth of *Fusobacterium nucleatum* ATCC 10953 can be derived from the fermentation of sugar (fructose) or amino acid (glutamate). During growth on fructose, the cells formed large intracellular granules which after extraction yielded glucose by either acid or enzymatic hydrolysis. The endogenous polymer was subsequently metabolized, and after overnight incubation of the cells in buffer, the glucan granules were no longer detectable by electron microscopy. Anaerobically, washed cells grown previously on fructose fermented this sugar to a mixture of lactic, acetic, and butyric acids, and little intracellular glucan was formed. Aerobically, the cells slowly metabolized fructose to acetate. Provision of glutamic acid as an additional energy (ATP) source elicited rapid synthesis of polymer by glycozyling cells. Intracellular granules were not present in glutamate-grown cells, and under anaerobic conditions, the resting cells failed to metabolize \(^{14}C\) fructose. However, the addition of glutamic acid to the suspension resulted in the rapid accumulation of sugar by the cells. Approximately 15% of the \(^{14}C\)-labeled material was extractable with boiling water, and by \(^{31}P\) nuclear magnetic resonance spectroscopy, this phosphorylated derivative was identified as \(^{14}C\)fructose-1-phosphate. The nonextractable material represented \(^{14}C\)glucan polymer. Fructose-1-phosphate kinase activity in fructose-grown cells was fivefold greater than that in glutamate-grown cells. We suggest that the activity of fructose-1-phosphate kinase and the availability of ATP regulate the flow of fructose into either the glycolytic or polymer-synthesizing pathway in *F. nucleatum*.

**MATERIALS AND METHODS**

**Organism and culture maintenance.** *F. nucleatum* ATCC 10953 was obtained from the American Type Culture Collection (Rockville, Md.). The organism was maintained by semimonthly transfer in modified thiglycolate medium (Fisher Scientific Co., Springfield, N.J.) supplemented with 20% (wt/vol) horse meat infusion and solid CaCO\(_3\). Cultures were grown at 37°C under anaerobic conditions (GasPak; BBL Microbiology Systems, Cockeysville, Md.) and were later stored at 4°C.

**Growth of cells.** *F. nucleatum* was grown in a medium of the following composition (grams per liter): Myosate (BBL), 6; Neopeptone (Difco Laboratories, Detroit, Mich.), 20; NaCl, 2; Na\(_2\)HPO\(_4\), 0.4; and Na\(_2\)CO\(_3\), 0.8. A salts-vitamin mixture was added separately from a concentrated stock solution (stored at 4°C) to give the following final concentrations (milligrams per liter): FeSO\(_4\) · 7H\(_2\)O, 4; MnCl\(_2\)·4H\(_2\)O, 1.9; MgSO\(_4\) · 7H\(_2\)O, 490; dithiothreitol, 100; biotin, 0.015; thiamine, 2.5; riboflavin, 2.5; pyridoxine, 5; pantothenic acid, 2.5; nicotinic acid, 5; folic acid, 0.025; and \(\alpha\)-aminobenzoic acid, 0.5. Fructose or ammonium glutamate was provided as an energy source at a final concentration of 5 g/liter.

**Preparation of cells.** A 10-ml culture of *F. nucleatum* grown previously overnight at 37°C in an anaerobic jar was transferred to 200 ml of medium supplemented with salts-vitamin mixture and an appropriate energy source (glutamate or fructose). The culture was grown under anaerobic conditions until the late log or early stationary phase (ap-
proximately 22 h) and then was quickly transferred to a capped 250-ml polycarbonate bottle. The headspace was flushed with an anaerobic gas mixture (5% CO₂, 5% H₂, 90% N₂), and the cells were collected by centrifugation at 5,000 × g for 20 min at 4°C. The supernatant fluid was discarded, and the cell pellet was suspended as quickly as possible in 35 ml of anaerobically prepared buffer (referred to as Coles buffer [7]) containing 50 mM potassium phosphate (pH 7), 0.7 mM MgCl₂, 0.1 mM AMP, and 0.1 mM NAD⁺. After being flushed with anaerobic gas mixture, the tube was capped and the cells were collected by centrifugation. The cell pellet was suspended in 5 ml of Coles buffer to yield a homogeneous thick cell suspension, which was then maintained under anaerobic conditions at 0°C until required.

**Preparation of cell extracts.** Cells were harvested from 1 liter of stationary-phase culture, washed, and suspended to 5 ml with 50 mM potassium phosphate (pH 7) buffer containing 0.7 mM MgCl₂. The cells were disrupted by sonic oscillation treatment (at 0°C, under anaerobic gas) with the microtip of a Branson sonifier operating at 70% of maximum power. After a total of 3 min of sonication (with intermittent cooling), the suspension was centrifuged at 27,000 × g for 30 min at 4°C. The supernatant fluid was removed and used for enzymatic analyses.

Assays for sugar phosphorylation (kinase activities) were performed at room temperature and contained the following in a final volume of 200 μl: 100 mM potassium phosphate buffer (pH 7); 5 mM ATP, phosphoenolpyruvate (PEP), or acetylphosphate; 5 mM MgCl₂; 2.5 mM [¹⁴C]sugar (specific activity, 0.2 μCi/μmol); and cell extract (1 to 2 mg of protein). At 0-, 5-, 10-, 20-, and 30-min intervals, 25-μl samples were transferred to Whatman DE-81 (2.5-cm-diameter) filter disks. The disks were placed first in water to remove free sugar, then in acetone and air dried. Radiolabeled sugar phosphate retained on the filters was determined by liquid scintillation spectrometry.

**Sugar transport studies.** The procedure used to monitor [¹⁴C]sugar uptake by resting (washed) cells of *F. nucleatum* has been described previously (31). In brief, 4.8-ml volumes of Coles buffer containing appropriate additions (e.g., sodium glutamate, 8 mM; [¹⁴C]fructose, 0.5 mM; specific activity, 0.2 μCi/μmol) were transferred to screw-cap tubes (16 by 125 mm) filled with anaerobic gas and capped with butyl rubber septa (Bellco Glass, Inc., Vineland, N.J.). Transport experiments (at 37°C) were initiated by the addition of 0.2 ml of anaerobic cell suspension (equivalent to 1.5 to 2.5 mg [dry weight] of cells) to the transport assay. At intervals, 0.5 ml of suspension was withdrawn with a tuberculin syringe (previously flushed with anaerobic gas), and cells were collected by vacuum filtration through membrane filters (diameter, 25 mm; pore size, 0.45 μm; type HA; Millipore Corp., Bedford, Mass.). Cells retained on the filter were rinsed with 4 ml of Coles buffer and dried, and cell-associated radioactivity was determined by liquid scintillation spectrometry (31).

**Fructose utilization by resting cells.** Resting (washed) cells were suspended (to the equivalent of 12 to 15 mg of total protein) in 5 ml of Coles buffer containing 2 mM fructose and, when required, 20 mM sodium glutamate. At intervals, 0.5-ml samples were removed from each system with an anaerobic syringe and cells were removed by filtration through Millex-GS (0.22-μm pore size; Millipore Corp.) filter units. Filtrates were collected, and fructose was determined by the Nelson-Somogyi Kit 115 (Sigma Chemical Co., St. Louis, Mo.) to which was added phosphoglucoisomerase (EC 5.3.1.9). In some experiments, detection or confirmation of the presence of fructose was provided by the resorcinol procedure of Roe (34).

**Extraction and identification of glycolytic intermediates.** Washed cells of *F. nucleatum* grown previously on either fructose or glutamate were suspended (to the equivalent of 20 mg of total protein) in 5 ml of Coles buffer containing 20 mM sodium glutamate. After 10 min of incubation at 37°C, 0.5 mM [¹⁴C]fructose (specific activity, 2 μCi/μmol) was added to the suspensions and incubation was continued for a further 10 min. Cells were collected by membrane filtration (47-mm-diameter membranes, 0.45-μm pore size) and rinsed briefly with distilled water at 0°C, and filters plus adhering cells were transferred to 8 ml of boiling water for 6 min. The suspensions were clarified by centrifugation, and the supernatant fluids were removed and lyophilized. The residues were reconstituted with 0.5 ml of water, and the solutions were passed through PD-10 gel filtration columns. Fractions containing low-molecular-weight compounds were pooled, frozen, and lyophilized. Each residue was reconstituted with 50 μl of water, and 10 μl of solution (containing approximately 60,000 cpm) was applied to thin-layer sheets of polyethyleneimine (PEI)-cellulose. Radiolabeled glycolytic intermediates were then separated by chromatography as described previously (41, 42).

**Extraction and composition of intracellular granules.** Fructose-grown cells (37 mg [dry weight]) were suspended in 5 ml of Coles buffer containing 25 mM sodium glutamate and 1 mM [¹⁴C]fructose (specific activity, 0.5 μCi/μmol). After 20 min of incubation at 37°C (to allow for polymer synthesis, the cells were collected by membrane filtration and then extracted in 8 ml of boiling water for 10 min. The suspension was centrifuged (10,000 × g for 15 min), supernatant fluid was removed, and the cell pellet was taken up in 2 ml of water. Radiolabeled cell-associated polymer was extracted by a modification of the Somogyi procedure (39), in which the 2-ml cell suspension was mixed with 4 ml of 30% (w/v) KOH and heated in a sealed tube for 3 h at 100°C. After cooling, the solution was clarified by centrifugation and supernatant fluid was removed and mixed with 2 volumes of 95% (vol/vol) ethanol and left to stand overnight at 4°C. The white precipitate collected by centrifugation was washed twice by resuspension and centrifugation from 5 ml of 95% (vol/vol) ethanol. The residue of [¹⁴C]-labeled polymer was dried in vacuo and subsequently dissolved in 500 μl of water (total of ~7 × 10⁵ cpm). In the acid hydrolysis procedure, 200 μl of polymer solution was diluted with 200 μl of 2 N HCl and the mixture was heated in a sealed tube for 5 h at 100°C. The hydrolysate was lyophilized. In the enzymatic hydrolysis procedure, 200 μl of polymer solution was mixed with 500 μl of 50 mM acetic buffer (pH 4.6) containing 20 mg (approximately 48 U) of amylase and 1 U of sodium dodecyl sulfate and the solution was left at room temperature for 4 h before lyophilization. The two residues obtained by acid and enzymatic hydrolysis were each reconstituted in 1 ml of water. The samples were applied to and eluted from PD-10 columns with distilled water, and fractions containing low-molecular-weight compounds were collected, pooled, and lyophilized. Each residue was dissolved in 300 μl of water, and the two samples were desalted by passage through small, mixed-bed ion-exchange columns (0.5 ml each of Dowex-1-formate and Dowex 50 H⁻ form resins). The water eluents (total, 5 ml) were lyophilized, and each residue (approximately 3 × 10⁶ cpm) was redissolved in 100 μl of water. Each chromatographic analysis, 3 ml of the specific glycolytic (10,000 cpm) was applied to 3MM chromatography paper (Whatman Inc., Clifton, N.J.) together with 7 μl (approximately 28,000 cpm)
of a mixture of $^{14}$Cglucose and $^{14}$Cfructose (5 mM each sugar; specific activity, 0.2 μCi/μmol) as standards. The solvent used for descending chromatography (40 h) contained butyl acetate-ethanol-pyridine-H$_2$O (8:2:2:1; vol/vol) and sugars were visualized by autoradiography.

**Extraction of fructose derivative.** Cells of *F. nucleatum* 10953 were grown to the stationary phase in 2.4 liters of medium supplemented with 0.5% (wt/vol) ammonium glutamate as the energy source. The culture was divided, and the glutamate-grown cells were harvested and washed (anaerobically) with 50 mM potassium phosphate (pH 7) buffer containing 1 mM MgCl$_2$. Each pellet was suspended in 20 ml of buffer, and sodium glutamate was added to each anaerobic suspension to a final concentration of 200 mM. The suspensions were incubated for 10 min at 37°C, and then 1 ml of anaerobic buffer was added to one suspension (control) and 1 ml of 100 mM fructose solution was added to the other. Twenty minutes later, the suspensions were chilled rapidly in ice-water and the cells were collected by centrifugation. The pellets were suspended with 20 ml of water (0°C), and then the cells were extracted with 10 ml of boiling water for 10 min. After clarification by centrifugation (10,000 × g for 30 min), the two supernatant solutions were removed, frozen, and lyophilized. The residues were dissolved in 1 ml of 1 N acetic acid, and precipitated (proteinaic) material was removed by centrifugation. The supernatant solutions were passed through PD-10 columns, and fractions containing low-molecular-weight compounds were pooled, frozen, and lyophilized.

**$^{31}$P-NMR spectroscopy.** Prior to $^{31}$P-nuclear magnetic resonance (NMR) analysis, residues were reconstituted with 0.4 ml of D$_2$O (>99.8 atom %). Merck Sharpe & Dohme, Montreal, Quebec, Canada) and 0.1 ml of 1 M HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (pH 8) containing 25 mM EDTA was added to each solution. $^{31}$P-NMR spectra were recorded on a JEOL GSX-500 spectrometer by using a 90° pulse-free induction decay acquisition-pulse delay), sequence, with continuous broadband proton decoupling. Spectral acquisition parameters included 11-μs 90° pulse, 15-KHz sweep width, 32,768 data points, 1.1-s data acquisition, 6.0-s pulse delay. Seventy-two free induction decay signals were averaged for each spectrum. Before Fourier transformation, the free induction decay signal was zero filled and then exponentially broadened to result in an additional 1.5-Hz line broadening in the frequency domain spectrum.

**Electron microscopy.** Cells of *F. nucleatum* were collected by centrifugation from 200 ml of medium containing either fructose or glutamate as the energy source. The cells were washed twice by suspension and centrifugation from phosphate-buffered saline and then were fixed for 4 h in 0.1 M cacodylate buffer (pH 7.4) containing 2% (vol/vol) each of formaldehyde and glutaraldehyde. Fixed cells were rinsed in cacodylate buffer and then were postfixed in cacodylate buffer containing 2% osmium tetroxide and 1.5% (wt/vol) potassium ferrocyanide (20). The samples were dehydrated through a graded series of increasing concentrations of ethanol. The preparations were embedded in Spurr resin (40), and thin sections were cut with a diamond knife. After being mounted on copper grids, the sections were stained lightly with Reynolds lead citrate (30) and examined under an electron microscope (110-CX; JEOL U.S.A. Inc., Peabody, Mass.).

**Analytical procedures.** Cell proteins were determined by a combination of the NaOH solubilization and Lowry procedures described by Herbert et al. (14). L-Glutamic acid was assayed with a glutamate dehydrogenase kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Fructose-1-phosphate (F1P) kinase activity was determined by assay II described by Gottschalk and von Hugo (13):

\[
\text{F1P + ATP} \rightleftharpoons \text{F1P kinase (in extract)} + \text{ADP + ADP} \\
1 \rightleftharpoons \text{F1P + PEP pyruvate kinase ATP + pyruvate} \\
2 \rightleftharpoons \text{Pyruvate + NADH + H}^+ \text{ lactate dehydrogenase lactate + NAD}^+
\]

where FDP is fructose-1,6-diphosphate. The spectrophotometric determination of F1P kinase activity by these sequential reactions relies on the formation of ADP in reaction 1. Cell extracts of *F. nucleatum* exhibit ATPase activity, and ADP is also formed via hydrolysis of ATP. The contribution of and correction for ATPase activity were determined by using a complete assay lacking F1P.

**Analysis of fermentation products.** Fermentation products were quantitatively identified on an Aminex HPX-87H (300 by 7.8 mm) organic acid separation column (Bio-Rad Laboratories, Richmond, Calif.). For each analysis, 5 μl of a standard solution (containing 20 μg each of unlabeled glucose, fructose, and lactic, formic, acetic, and butyric acids) was mixed with 100 μl of appropriate filtrate. Then 20 μl of the mixture was injected onto the column, and 0.01 N H$_2$SO$_4$ was used as the carrier solvent. Standard compounds were detected and elution profiles were recorded with an LKB Uvicord S detector (LKB, Bromma, Sweden) fitted with a 206-nm interference filter. Column effluents were collected (200-μl fractions), and radioactivity present in 100 μl of each fraction was determined by liquid scintillation spectrometry. The $^{3}P$-labeled fermentation products were identified by comparison (and coelution) with absorbance peaks of the unlabeled internal standards.

**Reagents.** Radiolabeled sugars were purchased from Dupont, NEN Research Products (Boston, Mass.). α-Amylase-glucosidase [1,4-α-d-glucan glycolhydrolyase, EC 3.2.1.3] and alkaline phosphatase (calf intestinal, EC 3.1.3.1) were obtained from Boehringer Mannheim Biochemicals. Precoated plastic-backed layers of polyethyleneimine cellulose (Bakerflex) were purchased from J. T. Baker Chemical Co. (Phillipsburg, N.J.). The PD-10 gel filtration columns were obtained from LKB-Pharmacia Inc. (Piscataway, N.J.).

**RESULTS**

**Growth studies.** Experiments presented in Fig. 1 demonstrate the capacity of *F. nucleatum* to utilize either glutamate (Fig. 1A) or fructose (Fig. 1B) as the energy source for growth. In each case, the increase in cell mass was concomitant with the utilization of the appropriate substrate. The background growth found in the absence of either fructose or glutamic acid (Fig. 1B) can be attributed to low concentrations of fermentable amino acids in the complex growth medium (e.g., glutamate, lysine, serine, and threonine [2, 3, 10, 23]).

**Polymer formation.** Electron microscopic examination of fructose-grown cells revealed large, densely-packed intracellular granules (Fig. 2A). These inclusions were not formed by the organism during growth on glutamic acid (Fig. 2C). The latter finding, and the fact that the granules became radiolabeled when the cells were grown on $^{14}$Cfructose, suggested that they were formed from either fructose or a fructose derivative. Radiolabeled granules were extracted by NaOH treatment and ethanol precipitation, and the purified material was acid hydrolyzed. Paper chromatography revealed $^{14}$Cglucose as the sole radiolabeled sugar in...
the hydrolysate (Fig. 3, lane 2). The same result was obtained after incubation of the granules with amylglucosidase (Fig. 3, lane 3). The intracellular granules (which apparently consist of a glucan [glycogen?]) polymer were subsequently metabolized, and after overnight incubation of the cells in buffer, the granules were no longer detectable (Fig. 2B). Enzymatic and gas chromatographic analyses showed that acetate, butyrate, and D-lactic acids are the products of polymer fermentation (unpublished data).

Fructose uptake by resting cells. Fructose-grown organisms, when suspended in Coles buffer containing [\(^{14}\)C]-fructose, failed to accumulate significant levels of the radiolabeled sugar under aerobic or anaerobic conditions (Fig. 4A). However, the addition of Na\(^+\)-glutamate to the anaerobic suspension elicited rapid uptake of sugar by the cells. Of the total radioactivity accumulated by the organisms, approximately 10 to 15% was extractable with boiling water and was found to consist primarily of radiolabeled glycolytic intermediates, free sugar, and fermentation products (Fig. 5A). The remainder (~85 to 90%) was recovered as [\(^{14}\)C]-labeled polymer. Previously, we have shown that the fermentation of glutamate by \textit{F. nucleatum} is inhibited by exposure of the cells to air (31). The presence of the amino acid caused little stimulation of [\(^{14}\)C]-fructose uptake by cells maintained under aerobic conditions (Fig. 4A). The consti-

tutivity of the fructose transport system was demonstrated by the finding of glutamate-stimulated transport of [\(^{14}\)C]-fructose by glutamate-grown cells (Fig. 4B). As with fructose-grown cells, >85% of the accumulated radioactivity was recoverable as [\(^{14}\)C]-labeled polymer. However, the glutamate-grown cells were found to contain unusually high levels of a derivative which, by its migration position on PEI-thin-layer chromatography (TLC), was tentatively identified as a hexose monophosphate (Fig. 5B, arrow).

**Product of fructose transport by \textit{F. nucleatum}**. The putative sugar phosphate (Fig. 5B, arrow) was eluted from the PEI-TLC and purified by ion-exchange chromatography. After incubation of the sample with alkaline phosphatase, all the radioactivity was recovered and identified by paper chromatography as [\(^{14}\)C]-fructose (Fig. 5C). The identity of the fructose phosphate(s) (fructose-6-phosphate [F6P], F1P, or a mixture of the two) was established by comparative \(^{31}\)P-NMR spectroscopy. Extracts were prepared from cells metabolizing (i) glutamate alone (Fig. 6A) or (ii) glutamate plus fructose (Fig. 6B). In the presence of fructose, two additional phosphorylated compounds were formed (peaks 1 and 2) with resonances centered at 1.85 and 1.90 ppm, respectively. Addition of authentic F6P to this extract generated a new peak in the spectrum at 1.39 ppm (Fig. 6C). However, the addition of F1P resulted in the enhancement of the signal at 1.90 ppm (i.e., peak 2), and this spectral co-incidence established that the [\(^{14}\)C]-fructose derivative formed by \textit{F. nucleatum} (Fig. 5B, arrow) was exclusively the 1-phosphate isomer. The phosphorylated compound responsible for the resonance at 1.85 ppm (peak 1) has not yet been identified, but our TLC results suggest that it is not radiolabeled and probably is not directly derived from [\(^{14}\)C]-fructose.

**Fructose metabolism by resting cells**. Previous studies (Fig. 4A) showed that in the absence of glutamic acid, little [\(^{14}\)C]-fructose was accumulated by fructose-grown cells under either aerobic or anaerobic conditions. Since the cells appeared unable to transport the sugar, it was thus perplexing to find that under the anaerobic conditions used for the transport experiments, the cells (i) depleted fructose from the medium (10 nmol of sugar consumed per mg of protein per min [Fig. 7A]), and (ii) metabolized [\(^{14}\)C]-fructose (Fig. 8) to a mixture of labeled fermentation products comprising D-lactate, butyrate, and acetate. The paradox was resolved by experiments presented in Fig. 9, in which resting cells were incubated with [\(^{14}\)C]-fructose and rapidly extracted with boiling water. Although the cell extract contained relatively low levels of radioactivity, several glycolytic intermediates (including FDP, PEP, and 2- and 3-phosphoglyceric acids), hexose monophosphate(s), and lactic acid were detected by PEI-TLC (Fig. 9A). The low level of cell-associated radioactivity was consistent with both the earlier transport data (Fig. 4A) and the fermentation of intracellular fructose (Fig. 7A and 8). The metabolism of [\(^{14}\)C]-fructose was confirmed by the dramatic (and immediate) increase in the intracellular concentrations of FDP and hexose monophosphate upon the addition of iodoacetate (a glycolytic inhibitor [41]) to the cell suspension (Fig. 9B). The inclusion of glutamate in the medium (Fig. 4A) stimulated the synthesis of intracellular polymer, and the overall rate of fructose utilization by the cells increased by almost 100% (~20 nmol of fructose consumed per mg of protein per min [Fig. 7A]). In sharp contrast to fructose-grown organisms, the rate of fructose utilization by glutamate-grown organisms was minimal (Fig. 7B). Although the presence of glutamate stimulated the rate of fructose disappearance from the medium (Fig. 7B), this
effect was largely due to the deposition of intracellular polymer rather than to an increase in the rate of sugar fermentation.

Mechanism(s) of fructose phosphorylation. The formation of F1P by cells of F. nucleatum (Fig. 5B) suggests the operation of a PEP-dependent fructose:phosphotransferase system (PTS) (28) in this organism. However, all attempts to demonstrate fructose-PTS activity in permeabilized cells or via the in vitro reconstitution of membrane and cytoplasmic components have been unsuccessful (data not shown). The possibility of kinase-mediated phosphorylation of fructose involving ATP, acetyl phosphate, or PEP as the phosphoryl donor has also been examined, again without success. Thus, under conditions in which the ATP-dependent phosphorylation of glucose and galactose was readily demonstrable (Table 1), phosphorylation of fructose was not detected. That F1P is an intermediate in the metabolism of fructose is indicated by the presence of ATP-dependent F1P kinase activity in cell extracts of F. nucleatum. Significantly, the activity of ATP-dependent F1P kinase in fructose-grown

FIG. 2. Thin-section electron micrographs of fructose-grown cells of F. nucleatum (A) and the same cells after overnight incubation in buffer (B). Cells grown previously on glutamate are shown in panel C. Note the absence of the granular inclusions in panels B and C. Magnification, ×32,500. Bar, 0.5 μm.
cells (~79 nmol of F1P converted to FDP per mg of protein per min) was approximately fivefold greater than that determined in glutamate-grown cells (~17 nmol of F1P converted to FDP per mg of protein per min).

**DISCUSSION**

The probable pathways for metabolism of fructose and glutamic acid by *F. nucleatum* ATCC 10953 are presented in

![Image of paper chromatography](attachment:image1.png)

**FIG. 3.** Identification by paper chromatography of the radiolabeled sugar produced by acid hydrolysis (lane 2) or by α-amylglucosidase treatment (lane 3) of the [*14C*]fructose-derived polymer in *F. nucleatum*. Lanes 1 and 4 contain radiolabeled fructose (Fru.) and glucose (Glu.) standards.

![Image of TLC chromatograms](attachment:image2.png)

**FIG. 5.** Identification by PEI-TLC of intracellular metabolites formed from [*14C*]fructose by cells of *F. nucleatum* grown previously on fructose (A) or glutamate (B). Washed cells were incubated with [*14C*]fructose, and intracellular metabolites were obtained by boiling water extraction (see Materials and Methods for details). SF.1 and SF.2 refer to solvent fronts 1 (H₂O) and 2 (0.5 M LiCl-2 N formic acid, 1:1), respectively. 3-PGA, 3-Phosphoglyceric acid. Arrow in panel B indicates position of [*14C*]-labeled sugar phosphate formed by glutamate-grown cells. This derivative when treated with alkaline phosphatase yielded [*14C*]fructose as shown in the paper chromatogram (C, lane 1). The migration positions of the standards [*14C*]glucose (Glu.) and [*14C*]fructose (Fru.) are shown in lanes 2 and 3, respectively, of panel C.

![Image of accumulation graph](attachment:image3.png)

**FIG. 4.** Accumulation of [*14C*]fructose (and a fructose-derived product[s]) by washed cells of *F. nucleatum* grown previously on fructose (A) or glutamate (B) as the energy source. Experimental conditions are indicated by the following symbols: □, aerobic; ■, aerobic plus glutamate; ○, anaerobic; ●, anaerobic plus glutamate. Sampling and assay procedures are described in Materials and Methods.

![Image of experimental results](attachment:image4.png)

**Fig. 10.** This illustration provides the basis for discussion of our results and depicts the interrelationships between sugar and amino acid fermentations in the oral anaerobe. The salient results from our investigation are the findings (i) that *F. nucleatum* has the capacity to utilize fructose as an
energy source for growth, (ii) that the transport and metabolism of fructose by resting (i.e., nongrowing) cells is not dependent on provision of a fermentable amino acid as energy source, and (iii) that excess ATP (generated via amino acid fermentation) promotes the diversion of fructose from the glycolytic pathway toward the polymer-synthesizing pathway of \textit{F. nucleatum} (Fig. 10). Studies with resting cells, grown previously on either fructose or glutamic acid, were instrumental to elucidation of the mechanism(s) of fructose dissimilation by \textit{F. nucleatum}. Under anaerobic conditions, washed (i.e., resting) cells grown previously on fructose rapidly fermented the sugar to a mixture of d-lactate, butyrate, and acetate. The capacity of the cells to transport fructose is in marked contrast to results from our earlier studies (31) which revealed an obligate requirement for a fermentable amino acid (e.g., glutamate, lysine, or histidine) for the transport of glucose and galactose by \textit{F. nucleatum}. Since the fermentation of fructose occurs via the Embden-Meyerhof (glycolytic) pathway, it is not clear why glucose and galactose are not also rapidly metabolized by \textit{F. nucleatum}. The presence of intracellular FIP in the cells suggests that this is the first derivative of fructose metabolism and that after phosphorylation via an ATP-dependent FIP kinase, fructose enters the glycolytic pathway at the level of FDP (Fig. 10). Glucose and galactose are phosphorylated by ATP-dependent kinases to yield glucose-6-phosphate and galactose-1-phosphate, respectively, and these compounds would be expected to enter the glycolytic pathway at the level of glucose-6-phosphate. The capacity of \textit{F. nucleatum} to rapidly ferment a particular sugar may depend on the point at which the various sugar phosphates enter the glycolytic pathway.

Although glutamate-grown cells failed to ferment fructose, the resting cells accumulated high levels of FIP (when glutamate was supplied as an energy source [Fig. 4B]), and the fructose monophosphate was rapidly transformed into polymer. Surprisingly, the polymer granules formed by growing and resting cells were composed of glucose rather than fructose. \textit{\alpha}-Amyloglucosidase cleaves \textit{\alpha}(1\rightarrow4)- and \textit{\alpha}(1\rightarrow6)-glucosidic linkages, and the polymer is evidently a glucan and most probably glycogen. Experiments conducted with glutamate-grown cells revealed the constitutivity of the
FIG. 8. Products of the anaerobic fermentation of fructose by fructose-grown cells of *F. nucleatum*. Washed cells were suspended in Coles buffer (1.6 mg of protein per ml) containing 2 mM [U-14C]fructose (specific activity, 2 µCi/µmol). Filtrates were collected immediately after addition of the cells (zero time, Control) and after 90 min of incubation. High-performance liquid chromatographic analyses revealed only [14C]fructose (peak 1) in the control filtrate (●). After 90 min of incubation (shaded areas, ●), labeled sugar was barely detectable and radioactivity was recovered as the following fermentation products: peak 2, d-lactate (~60%); peak 3, acetate (~7%); and peak 4, butyrate (~20%). Approximately 15% of the initial radioactivity was not recovered in our analyses, and this deficiency is attributed to the release of [14C]CO₂, which would accompany the formation of acetic and butyric acids (12). In this experiment, the rate of fructose utilization by cells under anaerobic conditions was 12.7 nmol of sugar per mg of protein per min. Under aerobic conditions, the rate of sugar fermentation decreased by 60% [5 nmol of fructose used per mg of protein per min], and only [14C]acetate was detected in the filtrates.

FIG. 9. Qualitative analysis and identification (by PEI-TLC) of intracellular metabolites of cells of *F. nucleatum*. Cell extracts were prepared from organisms during metabolism of [14C]fructose (A) and after addition of 5 mM iodoacetic acid (a glycolytic inhibitor) to the glycolyzing cell suspension (B). 3-PGA, 3-Phosphoglycerate.

enzymes involved in polymer synthesis and of those systems required for the transport and phosphorylation of fructose. However, the nature of the latter system(s), and the identity of the phosphoryl donor, have thus far proved elusive. Many bacteria translocate and simultaneously phosphorylate fructose via the fructose:PEP-PTS (28), and the product of this group translocation reaction is invariably FIP. Formation of this derivative by *F. nucleatum* suggests operation of the fructose-PTS, but our attempts to detect such activity in permeabilized cells or via subcellular complementation have failed. Fox et al. (8) suggest that acetylphosphate serves in lieu of PEP as the phosphoryl donor for PTS-mediated sugar phosphorylation in *Escherichia coli*. Since acetylphosphate is generated during glutamate fermentation by *F. nucleatum*, we tried this high-energy compound as a potential donor for the fructose-PTS, but again without success (data not reported).

**TABLE 1. In vitro phosphorylation of sugars** by a cell extract of *F. nucleatum* ATCC 10953**

<table>
<thead>
<tr>
<th>Sugar substrate</th>
<th>Result* with the following phosphoryl donor:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.76</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.33</td>
</tr>
<tr>
<td>Fructose</td>
<td>ND*</td>
</tr>
</tbody>
</table>

* Assay conditions were as described in Materials and Methods.

† The cell extract was prepared from glutamate-grown cells.

‡ Results are expressed as nanomoles of [14C]sugar phosphorylated per milligram of protein per minute.

§ ND, Not detectable.
Although it is generally regarded as an anaerobe, studies by Loesche (22) showed growth of \textit{F. nucleatum} in oxygen tensions approaching 6%; however, the cells died quickly when exposed to air. Our attempts to detect fructose phosphorylation were not performed under strict anaerobic conditions, and oxygen sensitivity of the putative fructose-PTS must be considered. In this context, we note that the rate of fructose metabolism by aerobic cells is only 40% of the rate determined under anaerobic conditions (Fig. 8, legend). It is conceivable that fructose accumulation occurs via active transport and that the intracellular (free) sugar is phosphorylated by an ATP-dependent kinase to yield F1P, as described recently in \textit{Halobacterium vallismortis} (1). However, we were unable to detect F1P (or F6P) synthesis in cell-free preparations using ATP, PEP, and acetylphosphate as potential phosphoryl donors (Table 1).

Our important contribution from our studies is the finding that the rate of intracellular F1P is determined by the availability and cofermentation of glutamic acid. Whereas in the absence of glutamate, fructose was metabolized almost exclusively via the glycolytic pathway, in the presence of the amino acid, intracellular F1P was also channeled into polymer synthesis (Fig. 10). The biochemical basis for this diversion has yet to be established, but it is significant that the rate of glutamate fermentation (and ATP generation) by \textit{F. nucleatum} is very high (~0.12 \(\mu\)mol/mg [dry weight] of cells per min [31]). If ATP production exceeds cellular demand, then the surplus may be consumed in the reaction: ATP + \(\alpha\)-glucose-1-phosphate \(\rightarrow\) ADP-glucose + PP. An increase in the activity of ADP-glucose pyrophosphorylase (ATP:\(\alpha\)-glucose-1-phosphate adenyl transferase, [29]) would thus pull the branchpoint metabolite (FDP) away from the glycolytic pathway and into the polymer-synthesizing pathway.

Fructose-grown cells rapidly ferment fructose (Fig. 7A), whereas fermentation of the sugar by glutamate-grown organisms is barely detectable (Fig. 7B). The inability of the latter cells to ferment fructose may, in part, be attributable to the low level of F1P kinase activity in these cells (approximately 20% of that determined in fructose-grown cells). If, because of the reduced activity of F1P kinase, the rate of FDP formation becomes comparable to the rate at which FDP is dephosphorylated to F6P (Fig. 10, step 3), then continued transport and metabolism of fructose will not be possible. In summary, we believe that F1P kinase levels and ATP availability are the two factors which regulate the entry of fructose into either the catabolic (glycolytic) or anabolic (polymer-synthesizing) pathway in \textit{F. nucleatum}.

Previously (32), we showed that concomitant fermentation of glutamate by \textit{F. nucleatum} prevented the degradation of intracellular polymer derived from glucose or galactose. In the present study, by comparative electron microscopy (Fig. 2A and B), we provided evidence for the converse, i.e., that fructose-derived granules are rapidly metabolized by the cells in the absence of glutamate (starvation overnight). The results of this investigation and of previous studies (31–33) demonstrate the capacity of \textit{F. nucleatum} (i) to form the same intracellular polymer from glucose, galactose, and fructose under conditions of amino acid excess and (ii) to ferment this sugar reserve under conditions of amino acid deprivation. The biochemical synergism between sugar and amino acid fermentations may contribute to the survival of \textit{F. nucleatum} in the feast-or-famine environment of the oral cavity and to the persistence of this organism in periodontal disease.

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**LITERATURE CITED**


