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A Sensitive Chromatographic Method for the Detection of Pyruvyl Groups in Microbial Polymers from Sediments

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Abstract. A method was developed for the quantitation of pyruvyl groups in microbial polymers using mild acid hydrolysis, o-phenylenediamine labeling, reversed-phase high-performance liquid chromatography (RP-HPLC), and fluorescence detection. The method was used to determine the pyruvate content of various microbial exopolysaccharides and to estimate the abundance of polymeric pyruvate in freshwater sediments. The results of this method were compared with those of several other pyruvate assays. The detection limit of the method was 1.6 nmol pyruvate. As little as 3.7 μg of the bacterial polysaccharide xanthan gum, or from 5 to 22 mg of sediment (depending on polymeric pyruvate content), were needed for detection and quantitation of polymeric pyruvate. The results should be useful in determining the contribution of polymeric pyruvate to total metal-binding ligands in sediments.

Introduction

Many microbial strains, particularly those found in aqueous environments, elaborate extracellular polymers. These polymers are most common composed of polysaccharide and protein and seem to exist as either firmly bound capsular polymers or loosely associated slime. The majority of research on these polymeric substances has focused on the polysaccharide fraction, its sugar composition, and structure [1, 2, 7, 11, 25, 31]. Some polysaccharides contain uronic acids and sugars substituted with ester-linked succinate and ketal-linked pyruvate, each of which contains an ionizable carboxyl group [17, 18, 23, 27–29, 32]. It is likely that these groups contribute to or are responsible for the polyanionic character of many of the microbial polymers described to date.

Stone [30] has found that a large fraction of the total reductant capacity of sediments is contributed by organic compounds with carboxyl, carbonyl, alcoholic, and phenolic groups. Metallic cations tend to interact with the electron-donating carbonyl oxygen of carboxyl groups [12]. Microbial polymers containing carboxyl groups have been shown to interact with and promote reductive dissolution of metallic cations in aqueous environments [8]. Such reactions may be particularly important in geochemical transformations of metals in sediments [3]. To better understand the extent to which microbial

exopolysaccharides interact with metals in sediments, it would be useful to be able to determine the abundance of carboxyl groups contributed by polymeric pyruvate, succinate, and uronic acids in sediment samples.

Various approaches have been used to quantitate pyruvate. The enzymatic assay for pyruvate using lactic dehydrogenase described by Duckworth and Yaphe [9] has been used to determine pyruvate content of polysaccharide preparations that have relatively high levels of pyruvate. Chromatographic methods for the identification and quantitation of α-keto acids have been developed using underivatized and derivatized samples [5, 14, 15, 21, 24, 33, 34]. Derivatized α-keto acids can be detected with increased selectivity and sensitivity compared to underivatized material. Of the commonly used derivatives, 3,4-dinitrophenylhydrazine (DNPH) derivatives can exist as syn- and anti-isomers, yielding multiple peaks for each α-keto acid [16, 23], whereas, o-phenylenediamine (OPD) derivatives yield a single peak for each acid [13]. OPD derivatives also yield greater specificity and sensitivity with fluorescence detection than that obtained with DNPH derivatives [19, 20]. We present a method for polymeric pyruvate detection and quantitation in freshwater sediments using OPD derivatives of pyruvate, high-performance liquid chromatographic (HPLC) separation from other α-keto acids, and fluorescence detection. The method is compared with other commonly used methods for pyruvate analysis.

Methods

Reagents and Materials

Chromatography grade methanol, reagent grade ethyl acetate, β-mercaptoethanol, and $NaHCO_3$ were obtained from Baxter Health Care Corp. (Los Angeles, California). Sodium salts of α-keto acids, anhydrous sodium sulfate, $Na_3PO_4 \cdot 12H_2O$, $CaCO_3$, oxalic acid (free acid) reagent grade triethanolamine, β-nicotinamide adenine dinucleotide (Grade II, disodium salt, reduced form), L-lactic dehydrogenase (Type XI, 970 U/mg, salt free), and o-phenylenediamine (OPD, free base) were purchased from Sigma Chemical Co. (St. Louis, Missouri). Technical grade humic acid (sodium salt) was obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin). All water used was double-distilled (dd-H_2O). Spectra/Por wet cellulose dialysis membranes (2,000 dalton molecular weight cutoff) were obtained from VWR Scientific Products (Cerritos, California).

Samples

Commercial grade xanthan gum (Keltrol) prepared from the bacterium *Xanthomonas campestris* was obtained from Kelco Co. (San Diego, California). Exopolymer from a bacterial isolate (FRI) recovered from the Fraser River at New Westminster, British Columbia, was prepared according to the procedure of Platt et al. [25]. Nearshore surface samples collected in October 1989 from El Dorado Park Lake, Long Beach, California and bottom deposits from Still Creek in Burnaby, British Columbia and from the Fraser River at New Westminster, British Columbia were collected in December 1986 using a plastic coring tube. The cores were sealed with neoprene stoppers, transported to the laboratory on dry ice, and stored at $-80°C$ prior to analysis. Characteristics of the Fraser River and Still Creek sediments have been reported previously [10]. The sediments of El Dorado Park Lake have not yet been characterized.

Standards

α-Keto acids (20 nmol/ml) were prepared fresh on the day they were derivatized.

Hydrolysis of Pyruvyl Residues in Bacterial Exopolymers

Hydrolysis of pyruvyl groups from polymer preparations was performed according to the procedure of Duckworth and Yaphe [9]. Oxalic acid (0.04 N) was added to achieve a final volume of 3 ml for every 3 to 5 mg of polymer in the sample. Hydrolysis was carried out at 100°C for 4 hours in teflon-lined screw-capped tubes. After cooling to room temperature, the hydrolysate was neutralized and oxalate precipitated by addition of 0.23 g $CaCO_3$. The sample was vortexed for 2 min, and then centrifuged at 1,000 × *g* for 10 min to pellet the precipitated calcium oxalate. A 1-ml portion of the supernatant was subsequently derivatized with o-phenylenediamine as described below.

Sediments

Sediment cores were thawed and the top 1 cm was recovered, refrozen, and lyophilized to dryness. Three grams of the lyophilized sediment were added to 10 ml of 0.01 M sodium phosphate (extraction solution) in plastic 100-ml centrifuge tubes. The solution was sonicated (Tekmar Sonic Disruptor with microprobe) during addition of 3 g of lyophilized sediment over a 2-min period to release adsorbed organic matter from sediment particles. Replicate sediment slurries prepared as above were spiked with 1.0, 2.5, or 5.0 mg xanthan gum. The final pH of the slurries was 7.5 to 8.0. The slurries were then dialyzed for 24 hours against approximately 100 volumes of extraction solution using dialysis tubing with a molecular weight cutoff of 2,000 daltons. The contents of the dialysis sacks were mixed frequently to facilitate diffusion of free pyruvate and other low molecular weight material out of the tubing. Polymeric pyruvate is defined as that pyruvate retained in the dialysis sacks following dialysis. The contents of the dialysis sacks were then centrifuged at 12,000 × *g* for 20 min, the supernatant and pellet recovered, and each dialyzed separately for 24 hours against 3 liters dd-H_2O to remove the sodium phosphate. Supernatant and pellet were then frozen at −80°C and lyophilized. Dried pellet and supernatant fractions were weighed and treated with 6 ml of 0.04 N oxalic acid per 3 g pellet, or 9 ml of 0.04 N oxalic acid per 15 mg lyophilized supernatant. After hydrolysis both fractions were pooled in screw-capped glass tubes and centrifuged at 2,000 × *g* for 20 min. The supernatant was transferred to a clean tube, while the pellet was washed successively two times with 0.5 ml of 0.04 N oxalic acid. The wash fractions were then pooled with the respective supernatant fraction, and the combined hydrolysate was neutralized with $CaCO_3$ as described above for bacterial exopolymer hydrolysates, and 1 ml of the resulting supernatant subjected to derivatization with o-phenylenediamine.

In order to determine the efficiency of dialysis of free pyruvate from sediment samples, sediments were spiked with 65.3, 130.6, and 261.2 μg pyruvic acid instead of xanthan. One sample was left unspiked as a control.

Derivatization of α-Keto Acids

A volume (50 μl) of α-ketoisocaproic acid (2.2 mg/ml) was added as an internal standard to 1 ml of each of the following: pyruvic acid standard solutions, solutions containing pyruvate hydrolyzed from xanthan, and solutions containing pyruvate hydrolyzed from xanthan-spiked sediments. Initially, no internal standard was added to a sediment subsample in order to ensure that there were no detectable levels of naturally occuring α-ketoisocaproic acid present. Samples were subsequently derivatized with o-phenylenediamine using the method of Koike and Koike [20]. This derivatization procedure was maximized for pyruvate by increasing the incubation time to 40 min.

If neutralized hydrolysates were not to be immediately labeled with OPD, they were frozen at −40°C to prevent possible degradation or polymerization during storage.

Chromatography

HPLC was performed using a Hewlett-Packard 1090 liquid chromatograph fitted with a 25-μl automatic sample injector. A Whatman 0.46 × 25-cm Partisil ODS-3 (C_{18}, 5 μm particle size) analytical reversed-phase column, and ODS CSK guard column (Baxter Health Care Corp., Los Angeles, California) were used to separate the organic acids. Chromatographic parameters were those of Koike and Koike [20]. The eluant was a 6:4 mixture of methanol : double-distilled water, the flow rate was 1 ml/min, and the column temperature was 35°C. Absorbance of effluent of underivatized sample was measured at 210 ± 10 nm as performed by Wormersley et al. [34] and Cheetham and Punruckvong [5], while that of the derivatized sample was measured at 335 ± 20 nm as performed by Hayashi et al. [15] using a Hewlett-Packard 1040 diode array detector with a 5-μl flow cell. Fluorescence measurements were made using a Hewlett-Packard 1046-A programmable fluorescence detector with a 5-μl flow cell. Detection of the fluorescent 2-quinoxalinol derivative was maximized by using excitation and emission wavelengths of 329 nm and 403 nm, respectively. This was determined by "peak trapping" (stopping eluant flow when the pyruvate peak entered the detector and then adjusting excitation and emission wavelengths for maximum signal). Samples were chromatographically analyzed in triplicate. The sample injection volume was 10 μl. The pyruvate in all samples, except for underivatized samples, was compared to a computerized internal standard calibration program. Since only 1 ml of the original x ml of acid hydrolysate was derivatized, the values were multiplied by x to provide the amount of polymeric pyruvate in the original sample. Water blanks were prepared and analyzed along with each set of samples. HPLC parameters, data collection, and analysis were controlled using a Hewlett-Packard 79994A Analytical Workstation with a CSA 760 interface.

Lactic Dehydrogenase Assay

A 2-ml volume of neutralized sediment hydrolysate or pyruvate standard was analyzed for pyruvate by the enzymatic assay described by Duckworth and Yaphe [9], except that samples were not diluted to 10 ml prior to analysis. Absorbance was measured with a Shimadzu UV-160 recording spectrophotometer. All samples were analyzed in triplicate.

Results

When OPD derivatives of pyruvic acid and five other α-keto acids (glyoxylic, glutaric, butyric, isovaleric, and isocaproic acid) were applied as a mixture to a reversed-phase column, all were resolved as distinct, symmetrical peaks with a smooth, flat baseline when a fluorescence detector was used (Fig. 1). Controls composed of water blanks produced chromatograms with no peaks and a baseline with little or no fluctuation. Freshly prepared pyruvic acid standards produced a linear fluorescence response over the range 1.6 nmol (178 ng) to 2 μmol (225 μg) (Fig. 2). Recovery of pyruvate from xanthan was linear over the range of 2 to 15 mg dry weight polymer (Fig. 3). The oxalate concentration in the sample was significantly reduced after $CaCO_3$ precipitation. Precipitation of oxalate had no detectable effect on pyruvate recovery. On the basis of peak areas obtained from pyruvate standards, the xanthan preparation contained

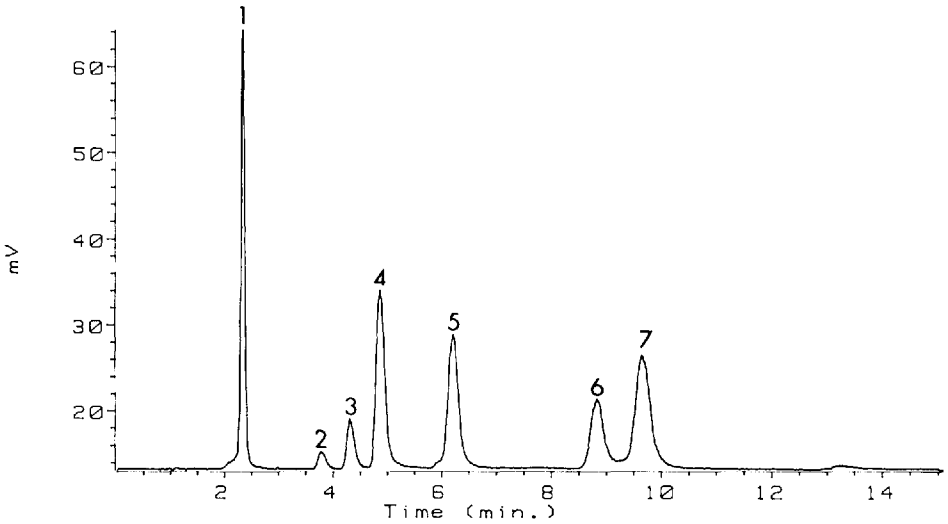

Fig. 1. Chromatogram of the quinoxalinol derivatives of a standard mixture of six α-keto acids (220 nmol each). Peaks: 1, α-ketoglutarate; 2, α-ketoisovalerate breakdown product/oxalate; 3, glyoxalate; 4, pyruvate; 5, α-ketobutyrate; 6, α-ketoisovalerate; 7, α-ketoisocaproate.

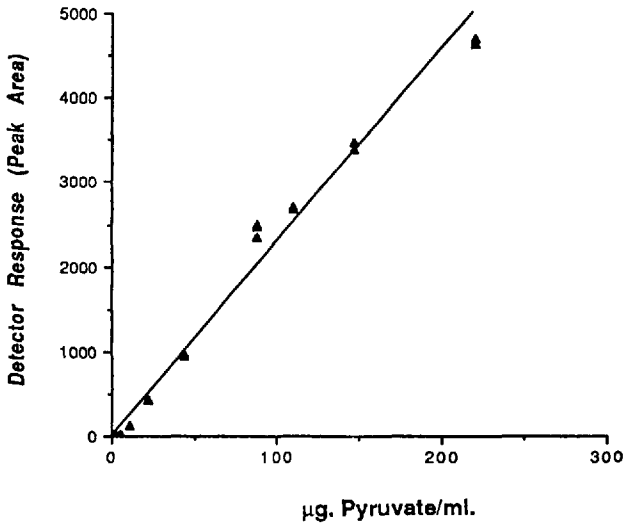

Fig. 2. Detector response (peak area) versus amount of pyruvate subjected to OPD-labeling, and subsequent chromatography.

48.3 ± 0.58 µg pyruvate/mg dry weight of polymer which corresponds closely with the 4 to 5% (wt/wt) pyruvate content reported by the commercial vendors.

Exopolymer isolated from a bacterium (FRI) recovered from sediments of the Fraser River was found to contain 49 µg pryuvate/mg polysaccharide, when subjected to this method of polymeric pyruvate analysis. The method was also successful in detecting pyruvate in exopolymer isolated from the marine bacteria *Alteromonas atlantica* and *Alteromonas colwelliana,* LST-D (data not shown).

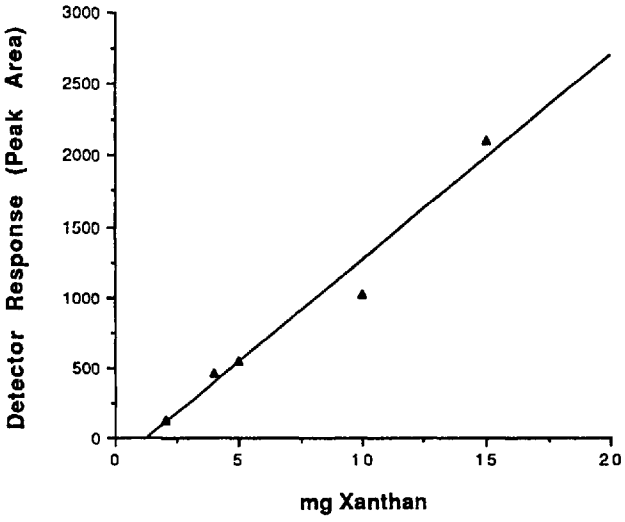

Fig. 3. Detector response (peak area of pyruvate) versus amount of xanthan treated. Linear regression showed that the recovery of polymeric pyruvate from xanthan was linear in the range tested.

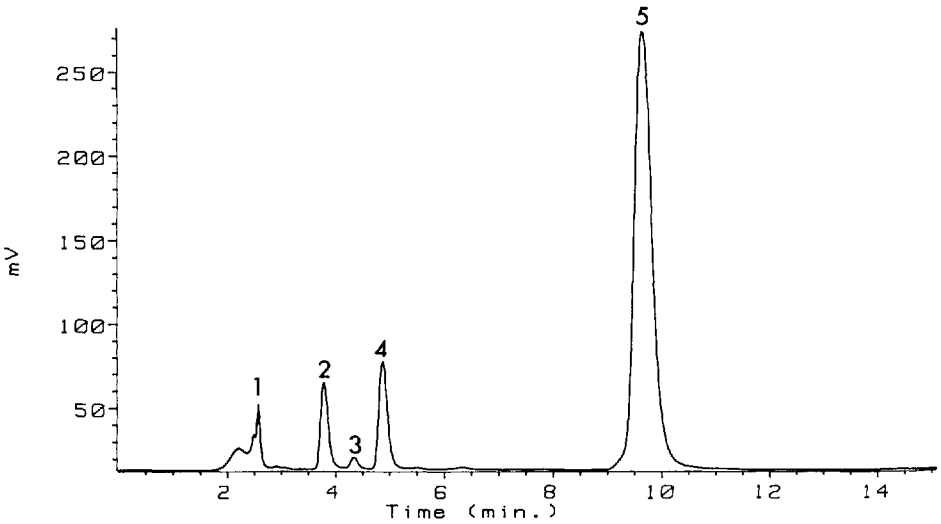

Fig. 4. Chromatogram of polymeric pyruvate derived from Fraser River sediment. Peaks: 1, unresolved α-keto acids/α-ketoglutarate; 2, oxalate (reagent); 3, glyoxalate; 4, polymeric pyruvate; 5, α-ketoisocaproate (internal standard).

Sediment samples from the Fraser River yielded chromatograms with a small group of unresolved peaks, which coeluted with α-ketoglutarate at 2 to 2.5 min, and peaks corresponding to oxalate (3.8 min), glyoxalate (4.4 min), and pyruvate (5.0 min) (Fig. 4). Since the sediment samples with no added internal standard yielded no peak with a retention time corresponding to α-ketoisocaproic acid, this acid was selected as an internal standard.

Chromatograms of sediment samples from the Fraser River spiked with xanthan provided an estimate of the amount of polymeric pyruvate that was present in unspiked sediment (Fig. 5). Extrapolation of the linear regression

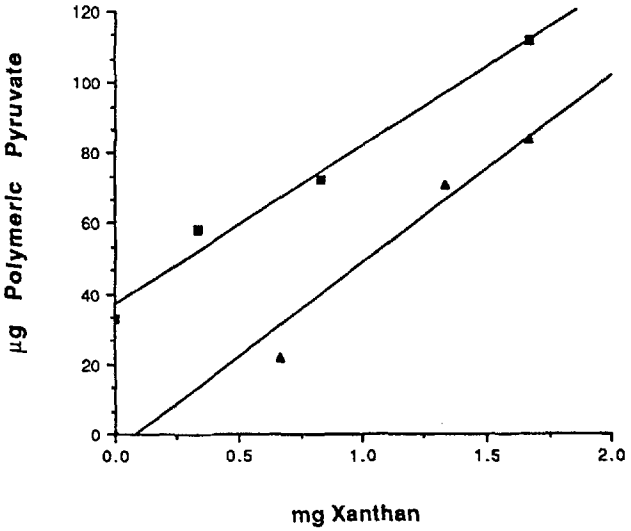

Fig. 5. Recovery of polymeric pyruvate from xanthan alone (triangles) and from Fraser River sediment spiked with xanthan (squares).

obtained from sediment spiked with different amounts of xanthan to the y-axis indicated the presence of 36.9 µg polymeric pyruvate/g dry weight sediment. The linear regression of the measured values was compared with predicted values based on the summation of pyruvate recovery from xanthan alone. This, and the amount of polymeric pyruvate determined in sediments by standard additions, indicated that recovery of polymeric pyruvate from sediment samples, to which 16 to 81 µg xanthan-derived polymeric pyruvate/g dry weight sediment were added, varied from 104 to 93%, respectively.

When sediments were spiked with free pyruvate instead of xanthan, no free pyruvate was recovered from the polymeric fraction after dialysis, when added at concentrations corresponding to 2×, 4×, and 8× the amount of polymeric pyruvate present (Fig. 6).

To determine whether humic acids contributed significantly to the polymeric pyruvate recovered from sediment, a commercial preparation was analyzed in the same manner as xanthan. It was found that the preparation contained 0.23 µg pyruvate/mg humic acid.

Fluorescence detection of OPD-labeled, HPLC-separated derivatives was also successful in quantitating polymeric pyruvate in sediments from other aquatic environments. Surface sediment collected from Still Creek, an urban tributary of the Fraser River, was found to contain 23.3 µg polymeric pyruvate/g dry weight sediment. Surface sediments from El Dorado Park Lake in Long Beach, California contained 8.1 µg polymeric pyruvate/g dry weight sediment.

Detection and quantitation of OPD-labeled pyruvate with fluorescence detection was found to be more sensitive and selective than other chromatographic detection methods. Oxalate-hydrolyzed sediments from El Dorado Park Lake were prepared as both underivatized and OPD-derivatized samples, and the column effluents were evaluated by absorbance at 210 and 335 nm, respectively. The peak areas of both xanthan-spiked and unspiked portions were significantly less than that obtained with OPD-labeled derivatives detected by fluorescence

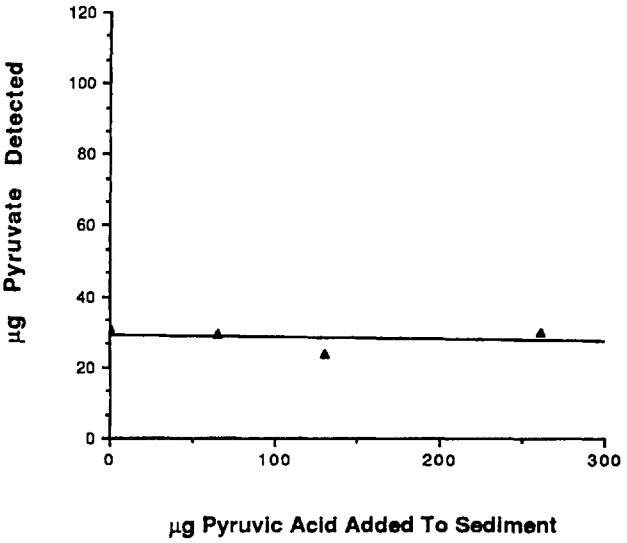

Fig. 6. Recovery of polymeric pyruvate from sediment samples spiked with different amounts of free pyruvate.

Table 1. Comparison of methods for detecting polymeric pyruvate in sediments from El Dorado Park Lake

Method[a]	Sample	Peak area (relative units)	Polymeric pyruvate (µg/g dry sediment)	Detection limit (nmol)
1	Spiked sediment[b]	611		
	Unspiked sediment	*[c]		
2	Spiked sediment	1,694	77.9 ± 2.4	
	Unspiked sediment	207	6.7 ± 0.2	
3	Spiked sediment	7,657	91.5 ± 1.0	
	Unspiked sediment	801	8.1 ± 0.3	1.6
4	Spiked sediment		121.4 ± 20.0	32
	Unspiked sediment		9.2 ± 3.9	

[a] Method 1, HPLC separation of underivatized sample, absorbance detection at 210 ± 10 nm (see [34]); method 2, HPLC separation of OPD-derivatized sample, absorbance detection at 335 ± 20 nm (see [5]); method 3, same as method 2 except that fluorescence detection was used; and method 4, lactic dehydrogenase enzyme assay (see [9])

[b] All sediment samples were spiked with 1.67 mg xanthan gum/g dry sediment

[c] Pyruvate peak was unable to be resolved from background

(Table 1). With underivatized samples, it was not possible to resolve the pyruvate peak in the spiked subsample from that contributed by other sediment components or from the internal standard α-keto isocaproic acid. There was no detectable absorbance of pyruvate in the unspiked, underivatized subsample.

Enzymatic analysis of polymeric pyruvate from hydrolyzed subsamples of sediment from El Dorado Park Lake was found to be less sensitive, and the values deviated more among replicates than occurred with the method of de-

tection and quantitation of OPD-labeled pyruvate with fluorescence detection (Table 1). The amount of pyruvate detected in the unspiked subsample by the lactic dehydrogenase assay described by Duckworth and Yaphe [9] was less than two times that of the detection limit of the method.

Discussion

Previous determinations of pyruvate in sediments have been restricted to that which exist as free pyruvate [30]. Another potentially important form of pyruvate in sediments is that which is covalently linked to polysaccharides produced by sessile algae and bacteria. The procedure described in this study provides a means of determining the amount of polymeric pyruvate that exists in freshwater sediments. Extraction of sediments with sodium phosphate and subsequent dialysis against distilled water separated all of the free pyruvate from the sediment particles and polymeric pyruvate. A similar extraction procedure has been shown to be effective in releasing ATP from sediments [4]. Subsequent treatment of the sediments with oxalic acid proved to be effective in quantitatively hydrolyzing the ketal-linkage in xanthan and several other pyruvate-containing bacterial exopolymers, including one from a bacterium (FRI) isolated from sediments of the Fraser River. Nevertheless, it is possible that the hydrolysis conditions found to be effective in liberating pyruvate from the polymers tested in this study are not equally effective in releasing pyruvate from all naturally occuring polymers. Whether the hydrolysis conditions described here achieve the same degree of efficiency when applied to other polymeric forms of pyruvate can only be evaluated after determining total release of pyruvate from samples subjected to several different hydrolysis conditions.

Neutralization and removal of excess oxalate from sediment extracts was carried out in order to minimize pyruvate degradation reported to occur under acidic conditions [6, 22] and to maximize pyruvate labeling with OPD. Since oxalate competes with pyruvate for OPD, reducing the residual oxalate concentrations after hydrolysis was found to increase the range of pyruvate concentrations over which OPD labeling was linear.

Since the data indicate that the levels of naturally occurring polymeric pyruvate in the spiked sediment subsamples are similar, the recovery of polymeric pyruvate from river sediment appears to be quantitative (93 to 104% efficiency) using the procedures described above. A maximum of approximately 2.25 μmol of α-keto acids could be effectively OPD-labeled before the response became nonlinear.

It has previously been determined that the sediments used in this study contained approximately 12.6 mg organic carbon/g sediment [10], and that humic substances generally contribute up to 71% of sediment organic carbon [26]. On the basis of these data, it was calculated that the Fraser River sediment contained approximately 2.0 μg humic acid-associated pyruvate per gram sediment. Since it was determined in this study that the Fraser River sediment contained 36.9 μg polymeric pyruvate per gram sediment, as much as 5% of that which was detected as polymeric pyruvate may be contributed by humic substances. We are presently in the process of isolating the humic fraction from

these sediments to obtain a more precise value for the humic acid-associated polymeric pyruvate.

The detection limit of pyruvate using the chromatographic method which employed fluorescence detection of OPD derivatives was 1.6 nmol. This corresponds to 32 nmol pyruvate in the sample at the derivatization step. Based on this detection limit of pyruvate, a minimum of 3.7 μg xanthan is needed for analysis. From the amount of polymeric pyruvate present in the sediment sample collected from the Fraser River, a minimum of 5 mg of sediment is required for detection of polymeric pyruvate by this technique. Pyruvyl groups in polymeric material derived from algal and bacterial cultures were detected at levels as low as 0.41% (wt/wt).

This chromatographic approach should lead to a better understanding of the amount of polymeric pyruvate associated with microbial exopolymers in sediments.

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