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High-performance capillary zone electrophoresis of carbohydrates in the presence of alkaline earth metal ions

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ABSTRACT

1-Phenyl-3-methyl-5-pyrazolone (PMP) derivatives of reducing carbohyrates were analyzed by capillary zone electrophoresis with on-tube ultraviolet detection, by using a capillary tube of fused silica and carriers containing alkaline earth metal salts. The direction (from cathode to anode) of electro-osmotic flow was the reverse of that observed for ordinary carriers not containing such metal salts, and the PMP derivatives of isomeric aldopentoses were completely separated from each other by the interaction with these metal ions. The order of mobility for the derivatives of aldopentose isomers was different from that observed in borate buffer, suggesting formation of different types of complexes. Examples of the application of this method to other monosaccharides and several oligosaccharides are also presented. It was demonstrated that this method allows quantification of reducing carbohydrates with high accuracy and high reproducibility.

INTRODUCTION

High-performance capillary electrophoresis (HPCE) is one of the epoch-making methodologies in separation science, and has been gaining in popularity for the analysis of biological substances. Application to carbohydrates, however, has the following two problems. First, carbohydrates lack functional groups which give signals in the detection system. Secondly, they have no electric charge, and hence cannot be separated by plain zone electrophoreis mode in the intact state. Micellar electrokinetic chromatography, another separation mode of HPCE, also does not allow separation of carbohydrates, since they are too hydrophilic to be solubilized in ionic surfactants as additives.

One of the solutions to the first problem is the introduction of chromophores or fluorophores by pre-column derivatization, such as condensation with 1-phenyl-3-methyl-5-pyrazolone [1], reductive pyridylamination [2] or reductive amination followed by coupling to 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde [3].

The second problem was partly solved by *in situ* conversion to ionic derivatives such as borate complexes [4,5]. In this case carbohydrate derivatives introduced into a capillary tube filled with borate buffer were almost instantaneously converted to anionic borate complexes, which were separated by means of the differences in their electric charge and molecular size, as well as by the differences in the ease of complexation of the complexes formed. This principle of *in situ* conversion to ionic species permits analysis of neutral molecules by zone electrophoresis. In this paper we describe another attempt to analyze carbohydrates by utilizing interaction with metal ions.

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EXPERIMENTAL

Chemicals

1-Phenyl-3-methyl-5-pyrazolone (PMP) was purchased from Kishida (Osaka, Japan) and used without further purification. This reagent is also available from Aldrich (Milwaukee, WI, USA) under the name 3-methyl-1-phenyl-2-pyrazolin-5-one. Other reagents and carbohydrates samples were of the highest grade commercially available.

Pre-column derivatization of reducing carbohydrates with PMP

This was done according to our published procedure [1]. In short a 0.5 *M* methanolic solution (50 μl) of PMP and 0.3 *M* sodium hydroxide (50 μl) were added to a reducing carbohydrate (100 nmol) or a mixture of reducing carbohydrates (100 nmol each), and the mixture was maintained at 70°C for 30 min with occasional swirling. The reaction mixture was cooled to room temperature, 0.3 *M* hydrochloric acid (50 μl) was added for neutralization, and the whole was evaporated to dryness. The residue was dissolved in water (200 μl) and the solution was extracted with chloroform (200 μl). The aqueous layer was analyzed by HPCE.

Apparatus for HPCE

A laboratory-made apparatus was used, which was constructed from a high-voltage power supply from Yamabishi Electric (Tokyo, Japan), a Σ 873 UV spectrometer from Irika Instruments (Kyoto, Japan), initially designed for high-performance liquid chromatography but slightly modified for HPCE, and a Chromatopak RC-6A data processor from Shimadzu (Kyoto, Japan). The greater part of the UV beam of the spectrometer was cut off by setting a narrow slit (50 μm × 500 μm) in the center of the beam. A capillary tube of fused silica (50 μm I.D.) was obtained from Polymicro Technologies (Phoenix, AZ, USA). A small portion of the polyimide coating was removed by burning at 15 cm from one end of the tube, and the transparent portion was glued to the slit. Sample solutions were introduced into the tube by gravity flow by raising one end of the tube 10 cm higher than the level of the opposite electrode solution for 10 s. The PMP derivatives of reducing carbohydrates were monitored by measuring UV absorption at 245 nm.

RESULTS AND DISCUSSION

Reducing carbohydrates react with excess PMP to give bis-PMP derivatives, which strongly absorb UV light at 245 nm [1]. When they are introduced into the anodic end of a capillary tube of fused silica filled with 100 m*M* sodium acetate and a high voltage is applied, they migrate to the cathodic end by the combined effects of electrophoresis and electro-osmosis. Electro-osmotic flow drives them to the cathode at a uniform velocity regardless of carbohydrate species, because the inner wall of the capillary tube is negatively charged under these conditions. Electrophoresis pulls them back to the anode, because they are negatively charged, presumably as a result of dissociation of the enolic hydroxyl group (s). Under these conditions the PMP derivatives of isomeric aldopentoses have the same electric charge and molecular size. Therefore, they were held back at the same velocity without separation, as shown in Fig. 1.

The PMP derivatives of aldopentoses (pentose-PMPs) were migrated at the same velocity, giving a single peak at 9.3 min. The peak at 10.0 min is due to the remaining reagent which was not extracted with chloroform. An aqueous 100 m*M* solution of potassium acetate as well as a 100 m*M* solution of ammonium acetate, used as carrier, gave similar electropherograms.

The carrier was changed to an aqueous 20 m*M* solution of calcium acetate and the tube was allowed to stand for 2 h. Introduction of a mixture of PMP derivatives of some monosaccharides from the anodic end, followed by application of a high voltage, gave an electropherogram as shown in Fig. 2a, in which the PMP derivatives of these monosaccharides were well separated from each other.

The separation was presumably due to interaction, possibly complexation, of these derivatives with the calcium ion in the carrier. However, there was a tendency for migration time to become longer as time elapsed after changing carrier. The tube was rinsed with 20 m*M* calcium acetate for 0.3 h and allowed to stand for a further 6.7 h. Analysis of the same sample gave only the peak of mesityl oxide (an internal neutral marker) in 60 min (Fig. 2b). Repeated analysis after a further 0.3-h rinse and 4.7 h standing finally gave no peaks in 60 min (Fig. 2c). Nevertheless, introduction of this sample from the

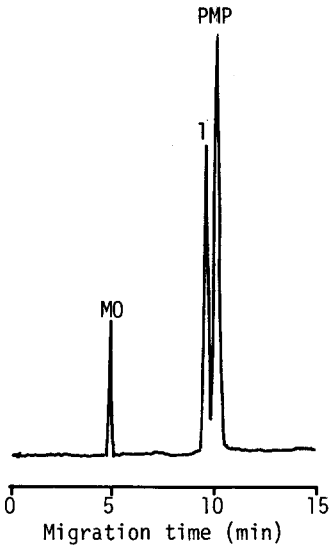

Fig. 1. Analysis of a mixture of pentose-PMPs in 100 m*M* sodium acetate. Capillary, fused silica (49 cm × 50 μm I.D.); applied voltage, 10 kV; detection, UV absorption at 245 nm. An aqueous solution was introduced from the anodic end of the tube. Peaks: MO = mesityl oxide (internal neutral marker); PMP = 1-phenyl-3-methyl-5-pyrazolone (reagent); 1 = pentose-PMPs ($1 \cdot 10^{-3}$ *M* each).

cathodic end gave rise to peaks of the monosaccharide derivatives in *ca.* 10 min, fairly well separated from each other (Fig. 2d). The remaining reagent and mesityl oxide gave peaks before and after, respectively, this group of peaks.

This series of experiments suggests that the velocity of electro-osmotic flow gradually reduced to zero, then the direction was reversed and the velocity continued to increase to a maximal value. Use of higher concentrations of calcium acetate facilitated such a change of electro-osmotic flow; continuous rinsing with 100 m*M* calcium acetate allowed rapid reversal of direction to give a steady-state flow in the opposite direction in only 2 h. Incidentally the original state was restored by continuous rinsing of the tube with the original carrier. The reversal of the direction of electro-osmotic flow is phenomenon similar to that observed when quaternary ammonium salts are added to carrier [6], and is considered to be the result of localization of the divalent metal ions on the surface of capillary inner wall by binding to the silanol group.

Fig. 3a shows complete separation of pentose-PMPs, achieved by introducing the sample solution from the cathodic end after equilibration of the inner wall of the tube with 100 m*M* calcium acetate. The reproducibility of migration time was high (*e.g.*

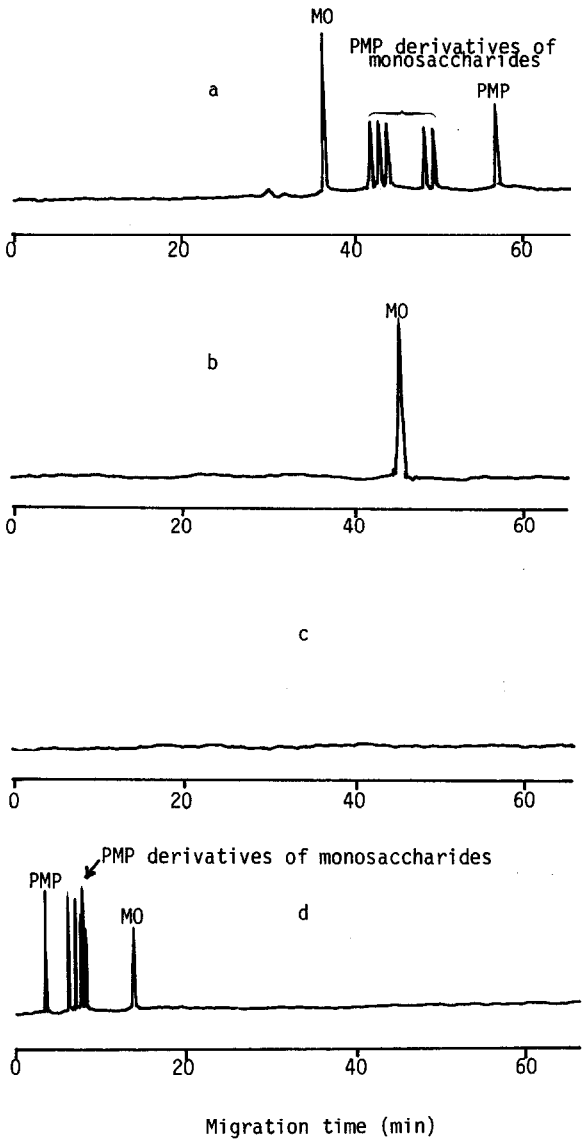

Fig. 2. Change in the electropherogram of the analysis of PMP derivatives of several monosaccharides with time after changing the carrier from 100 m*M* sodium acetate to 20 m*M* calcium acetate. (a) 2 h, (b) 7 h, (c) 12 h and (d) 13 h after the change of carrier. For details see the text. Sample, a mixture of PMP derivatives of arabinose, ribose, galactose, glucose and mannose ($1 \cdot 10^{-3}$ *M* each). An aqueous sample solution was introduced from the anodic (a–c) or cathodic (d) end of the tube. Other analytical conditions and abbreviations as in Fig. 1.

the relative standard deviation of the xylose-PMP peak was 1.5% for $n = 8$) under such equilibrated conditions.

The good separation of the peaks of pentose-PMPs is presumably because of complexation with the calcium ion. Since complexation naturally gives rise to positive charge around the calcium nucleus of the complexes, it causes a reduction in total negativity. As a result the migration velocities of the complexes (from cathode to anode) are lower than that of the reagent. The difference in the ease of complexation is at least a cause of difference in apparent electrophoretic mobility, which leads to separation of peaks.

The reversal of the direction of electro-osmotic flow and good separation of the peaks of pentose-PMPs were also observed with carriers containing other kinds of alkaline earth metal salts. Fig. 3b and c shows the electropherograms of pentose-PMPs in aqueous 100 m*M* solutions of barium acetate and strontium acetate. The barium salt-containing carrier gave sharper peaks and better separation of pentose-PMPs than the calcium salt-containing carrier. The strontium salt-containing carrier also gave good separation, but migration times were rather longer. A magnesium salt-containing carrier showed a tendency to tailing, and separation was rather worse, possibly because of weaker binding (Fig. 4).

The variation in the migration times of these metal salts is the result of variations in the velocities of electro-osmotic flow caused by the difference in the positivity of the capillary inner wall. The variation in peak resolution is mainly attributable to the ease of complexation based on the difference in electronegativity and valence angle among metal nuclei.

Table I lists the difference between apparent mobility of each pentose-PMP and that of PMP ($\Delta\mu_{ep}$), since it compares the effects of metal salts on sugar moieties.

The order of $\Delta\mu_{ep}$ values in divalent metal salt-containing carriers (ribose-PMP, lyxose-PMP, arabinose-PMP, xylose-PMP) is the same for all these metal salts, but is different from that in borate buffer (ref. 5, xylose-PMP, arabinose-PMP, ribose-PMP, lyxose-PMP). This discordance is obviously due to the difference in the mode of interaction. Since the present mode of separation is comparable to ligand exchange in liquid chromatography (*e.g* ref. 7), a mixture of pentose-PMPs was applied to a column of Shodex Sugar SP-0810, a sulfonated styrene–divinylbenzene copolymer, in the calcium form. However, elution with 100 m*M* calcium acetate gave no peaks because of strong retention on the column. Therefore, direct comparison of the present mode with ligand exchange was unsuccessful. Further discussion of this problem will be published elsewhere based on additional data.

The foregoing results indicate that aqueous solutions of the acetates of some divalent metals, especially barium, give excellent separation of pentose-PMPs. Therefore, other combinations of monosaccharides were analyzed as PMP derivatives in barium salt-containing carrier. Fig. 5 shows an example obtained for a mixture of five reducing monosaccharides (galactose, mannose, fucose, N-acetylgalactosamine and N-acetylglucosamine), ubiquitously found in animal glycoproteins.

Mannose gave a single peak with the shortest migration time (5.51 min), but the N-acetylgalactosamine–N-acetylglucosamine and gallactose–fucose couples were not separated from each other, giving

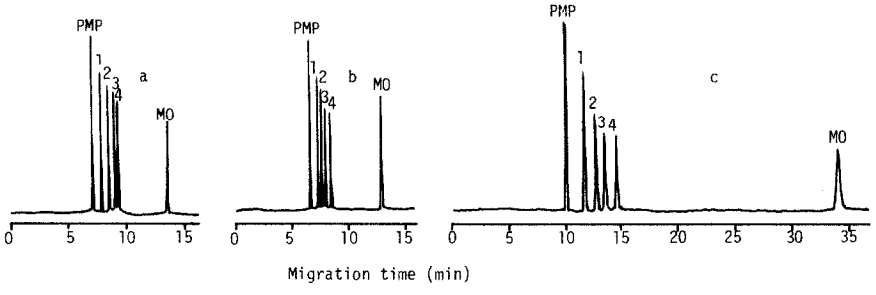

Fig. 3. Separation of pentose-PMPs in aqueous 100 m*M* solutions of calcium acetate (a), barium acetate (b) and strontium acetate (c). Capillary, fused silica (53 cm × 50 μm I.D.). An aqueous sample solution was introduced from the cathodic end of the tube. Other analytical conditions and abbreviations as in Fig. 1. Peaks: 1 = ribose-PMP; 2 = lyxose-PMP; 3 = arabinose-PMP; 4 = xylose-PMP.

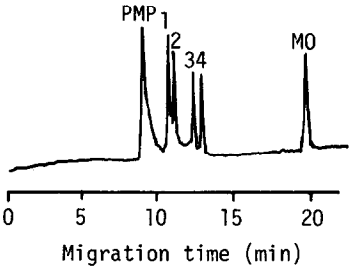

Fig. 4. Separation of pentose-PMPs in 100 m*M* magnesium acetate. Other analytical conditions, abbreviations and peak assignment as in Fig. 3.

superimposed peaks at 5.75 and 5.84 min, respectively.

Fig. 6 shows an electropherogram of partially hydrolyzed dextran containing isomaltooligosaccharides having various values of degree of polymerization (d.p.), analyzed under the same conditions as those used for the component monosaccharides of glycoproteins.

It is observed that these homologous series of oligosaccharides were separated from each other up to d.p. 9, but the separation was not so good as that in borate buffer (ref. 5, separable up to d.p. 13).

Fig. 7 shows an example of disaccharide analysis obtained under the same conditions. Maltose was separated from other disaccharides, but the cellobiose–melibiose and gentiobiose–lactose couples failed to be separated. Thus, the barium salt-containing carrier was unfavorable for differentiation of the 1,4-β-glucose–glucose and 1,6-β-galactose–glucose pair as well as the 1,6-β-glucose–glucose and 1,4-β-galactose–glucose pair.

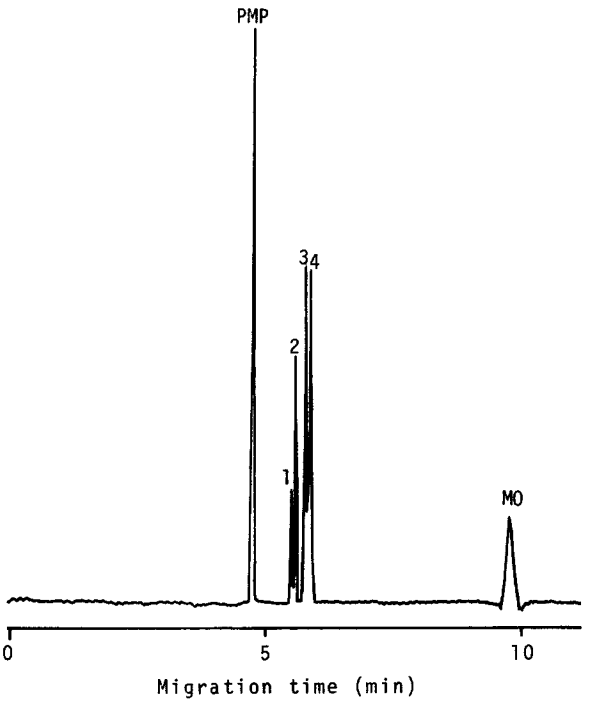

Fig. 5. Separation of the PMP derivatives of the component monosaccharides commonly found in glycoproteins. Capillary, fused silica (49 cm × 50 μm I.D.). Other analytical conditions as in Fig. 3d. Peaks: 1 = rhamnose (internal standard); 2 = mannose; 3 = N-acetylgalactosamine + N-acetylglucosamine; 4 = galactose + fucose.

Determination of reducing carbohydrates by this method was promising. In this case a reducing car-

TABLE I

μ_{ep} VALUES OF PENTOSE-PMPs

Ion in carrier[a]	$\Delta\mu_{ep}$[b] (× 10^{-2}, cm^2 min^{-1} V^{-1})			
	Ribose-PMP	Lyxose-PMP	Arabinose-PMP	Xylose-PMP
Ca^{2+}	3.0	4.9	6.2	6.7
Ba^{2+}	2.6	3.4	4.3	5.5
Sr^{2+}	3.3	4.8	5.5	6.9
Mg^{2+}	3.4	3.9	5.9	6.5
BO_2^-	2.9	3.5	2.2	1.7

[a] The concentration of ions were commonly 100 m*F*.

[b] $\Delta\mu_{ep}$ is the difference between the apparent mobility of pentose-PMP and that of PMP.

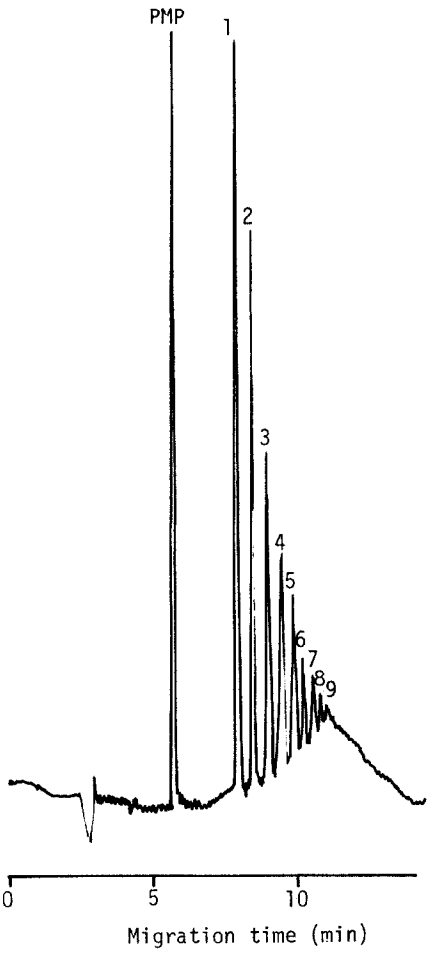

Fig. 6. Separation of the PMP derivatives of isomaltooligosaccharides. Analytical conditions and abbreviations as in Fig. 5. The peak numbers correspond d.p.s.

bohydrate or a mixture of reducing carbohydrates was derivatized with PMP in a similar manner as that described in the Experimental section, and derivatives were analyzed in divalent metal salt-containing carriers. Calibration curves showed excellent linearity over wide ranges of sample amount and they passed through the origin. For example, linearity was observed for galactose and glucose at least in the range 10–200 nmol (injected amount, 50–1000 fmol) under the conditions in Fig. 5. The respective coefficients of correlation were 0.997 and 0.999. The relative standard deviations (R.S.D., n = 8) of galactose and glucose at the 50-nmol level were 2.36 and 2.11%, respectively. The R.S.D. values of galactose and glucose, relative to rhamnose (internal standard) were much smaller: 0.69 and 0.85%, respectively. Based on these results, commercial samples of lactose and melibiose were hydrolyzed in 2 *M* trifluoroacetic acid at 100°C for 6 h under a nitrogen atmosphere, and the monosaccharides in the hydrolysates were determined. The galactose-to-glucose molar ratios obtained under the conditions in Fig. 5 were 1.05 and 1.08, respectively, both in fairly good agreement with the theoretical values. An example of the electropherograms is shown in Fig. 8.

The present method employing solutions of divalent metal salts as carrier offers a new mode of separation for carbohydrate derivatives, based on the interaction of solutes with the metal ions and the reversal effect of electro-osmotic flow. Some ex-

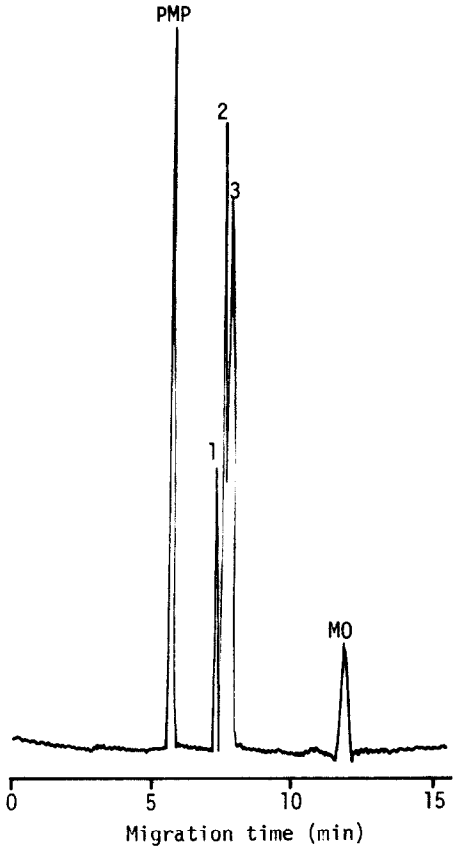

Fig. 7. Separation of the PMP derivatives of a few disaccharides. Analytical conditions and abbreviations as in Fig. 5. Peaks: 1 = maltose; 2 = cellobiose + melibiose; 3 = gentiobiose + lactose.

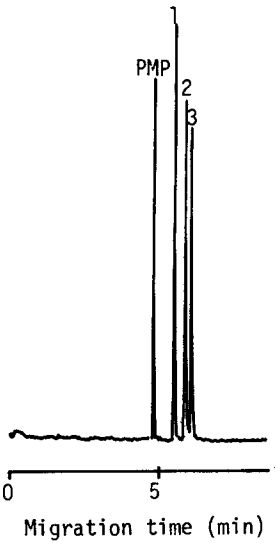

Fig. 8. Analysis of the component monosaccharides in melibiose. Capillary, fused silica (49 cm × 50 μm I.D.); carrier, 100 m*M* calcium acetate; applied voltage, 10 kV; detection, UV absorption at 245 nm.

amples presented above did not give complete separation of PMP derivatives of carbohydrates, but separation will be improved by changing the analytical conditions by using buffer solutions or additives.

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