Determination of Total Phosphorus in Biological Samples by Flow Injection Analysis with Spectrophotometric Detection

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Synopsis. Flow injection analysis with spectrophotometric detection has been developed for the determination of phosphorus in biological samples. Potassium peroxodisulfate was used as an oxidizing reagent with aid of a platinum wire as the catalyst, and organophosphorous compounds were converted to orthophosphate which reacted with ammonium molybdate in the presence of ascorbic acid to form the molybdenum blue complex. The absorbance of the complex was detected at 880 nm.

Analysis and monitoring of pollutants in environmental and biological samples are important from the viewpoint of environmental protection. For such a purpose, flow injection analysis (FIA) is one of the promising analytical methods because FIA is relatively a convenient technique.[1] The determination of phosphorus by FIA with spectrophotometric detection has been reported in terms of plant materials,[2,3] biological samples,[4,5] waste waters,[6,7] sea water,[8] and river waters.[9,10] In these studies, the spectrophotometric methods using blue colored phosphomolybdate[11] and yellow colored complex produced in a vanadomolybdate solution[12] have been employed for detection. More recently the yellow complex formed with malachite green was described by Motomizu et al.[13] Furthermore, Toei et al.[14] reported more sensitive method using Guinea Green B.

As mentioned above, many workers have reported phosphorus determination, but only a few authors have described the determination of organic or biological phophorous compounds. Hence, in the present paper the applicability of the flow injection system to the analysis of total phosphorous compounds, i. e., inorganic and organic phosphorus present in biological samples, have been investigated by using a microflow technique with on-line sample decomposition and molybdenum blue complex formation.

Experimental

Chemicals. All reagents used in the present work were of analytical reagent grade or better, which were obtained from Wako Pure Chemicals Co. (Osaka), Sigma (St. Louis, USA) and E. Merck (Darmstad, Germany). AMP, CMP, ITP, GMP, TMP, UMP, UTP, ammonium molybdate, ascorbic acid, sulfuric acid, perchloric acid, and potassium hydrogencarbonate were obtained from Wako Pure Chemical Co; ADP, ATP, CDP, CTP, IMP, IDP, GDP, GTP, TDP, TTP, and UDP from Sigma; and potassium dihydrogenphosphate and potassium peroxodisulfate from E. Merck. The abbreviation of nucleotides are given in Table 2. Purified water was prepared with a Millipore Milli-Q water purification system (Nihon Millipore Co., Tokyo).

Apparatus. A schematic diagram of the FIA-spectrophotometry system used is shown in Fig. 1. The instrumental components and operating conditions are given in Table 1.

Recommended Procedures. For the determination of total phosphorus concentration, the sample was first mixed with the oxidizing reagent and passed through the 8 m×0.5 mm i.d. Teflon reaction coil heated in an aluminum block bath for the decomposition of phosphorous compounds, where all phosphorous compounds were converted to orthophosphate. A platinum wire with a 0.2 mm diameter was inserted into the oxidation reaction tube to serve as a catalyst. The orthophosphate formed was then mixed with a color-forming reagent in the 5 m×0.5 mm i.d. Teflon reaction coil at room temperature. The molybdenum blue complex formed was introduced into a flow cell for the spectrophotometric detection at 880 nm.

Pretreatment of Biological Samples. The biological samples were prepared as follows: The samples (8—40 g) were digested by using 40 ml of 1 M (M=mol dm^{-3}) perchloric acid, followed by shaking vigorously before centrifugation at 3,000 rpm (1600 G, 1 G=6.6726×10^{-11}m^{3}kg^{-1}s^{-2}) for 5 min. A 20 ml volume of the supernatant was taken and its pH was adjusted to 6.5 with 1.5 M potassium hydrogencarbonate. A portion of the supernatant was finally injected into the FIA system.

Results and Discussion

Optimization of Flow Rate of Reagents. In flow injection analysis, generally, the flow rate of reagent solution plays an important role in achieving better sensitivity. The flow rates of 4% potassium per-

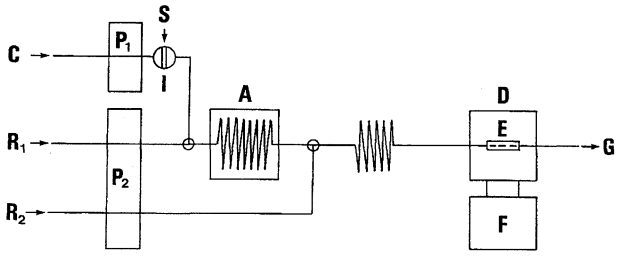

Fig. 1. Schematic diagram of the flow injection spectrophotometric apparatus. C, carrier; R_1, oxidizing reagent; R_2, color-forming reagent; P_1, HPLC pump; P_2, peristaltic pump; S, sample; I, injector; A, aluminum bath; D, detector; E, flow cell; F, recorder; G, waste.

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Table 1. Instrumental Components and Experimental Conditions

Component	Specification and experimental conditions
Pump 1	Shimadzu LC-6AD HPLC pump
Pump 2	Gilson Miniplus 2 peristaltic pump
Injector	Rheodyne model 7000 switching valve
Bath	Eyela EMG-1 aluminum block bath
Detector	Jasco model UVIDEC-100IVUV spectrophotometer
Flow cell	10 mm light path length, 8 μl volume
Wavelength	880 nm
Recorder	Yokogawa Electronic Work Ltd, YEW type 3056 pen recorder

Table 2. Effect of Decomposition Temperature on Signal Intensity

Compounds	Relative signal intensity%[a)]		
	100 °C	120 °C	140 °C
AMP (adenosine-5′-monophosphate)	60.3	85.1	99.6
ADP (adenosine-5′-diphosphate)	43.6	65.2	99.3
ATP (adenosine-5′-triphosphate)	26.7	53.4	99.1
CMP (cytidine-5′-monophosphate)	55.3	78.3	99.7
CDP (cytidine-5′-diphosphate)	50.0	79.0	99.2
CTP (cytidine-5′-triphosphate)	24.0	45.0	99.5
IMP (inosine-5′-monophosphate)	71.0	85.5	99.8
IDP (inosine-5′-diphosphate)	59.6	79.4	99.4
ITP (inosine-5′-triphosphate)	35.5	51.5	99.3
GMP (guanosine-5′-monophosphate)	65.4	87.5	100.1
GDP (guanosine-5′-diphosphate)	50.0	70.3	99.6
GTP (guanosine-5′-triphosphate)	30.5	54.0	99.5
TMP (thymidine-5′-monophosphate)	61.0	78.0	99.4
TDP (thymidine-5′-diphosphate)	41.0	71.5	99.7
TTP (thymidine-5′-triphosphate)	19.0	39.5	97.0
UMP (uridine-5′-monophosphate)	71.0	81.5	99.8
UDP (uridine-5′-diphosphate)	58.5	79.2	99.5
UTP (uridine-5′-triphosphate)	31.0	43.4	99.3

a) Calculated values based on the signal response of phosphorus in comparison with that of potassium dihydrogenphosphate.

oxodisufate solution as the oxidizing reagent and 2% ammonium molybdate solution containing 0.36% ascorbic acid and 1.5 M sulfuric acid as the color-forming reagent were optimized for the phosphorus determination. The oxidizing and color-forming reagent solutions were supplied into the flow of sample solution with a peristaltic pump through the manifolds R_1 and R_2 in Fig. 1, respectively. When the total flow rate was changed from 50 to 300 μl min^{-1}, the response increased with increasing the flow rate up to 150 μl min^{-1}, and reached almost a constant value in the range from 150 to 200 μl min^{-1}, while it decreased at the higher flow rate because of the short reaction time for production of the molybdenum blue complex. Consequently, 200 μl min^{-1} was selected as the optimum flow rate of the reagent solutions, that is, each flow rate of the oxidizing and color-forming reagent solutions was 100 μl min^{-1}.

Effect of Potassium Peroxodisulfate on Decomposition of Organophosphorous Compounds. The previous papers[15,16)] showed that platinum was an effective catalyst for converting phosphorous compounds to orthophosphate. Hence, a platinum wire was utilized in the oxidation reaction tube shown in Fig. 1.

In order to convert organophosphorous compounds to the phosphate ion, use of peroxodisulfate is usually recommended, and thus the effect of potassium peroxodisulfate in decomposition process was examined, where AMP, ADP, and ATP were chosen as the analytes. In the presence of 4% potassium peroxodisulfate the signal was enhanced by the factors of 3, 2.5, and 2 for AMP, ADP, and ATP, respectively. The difference in the enhancement factor may be ascribed to the structures of the nucleotides examined. It is clear from these results that AMP is more easily decomposed than ADP and ATP.

Effect of Decomposition Temperature. In order to obtain the uniform response signals for inorganic and organic phosphorous compounds, the oxidation reaction temperature was examined because the decomposition efficiency depends on the reaction temperature. Decomposition temperature was varied to achieve better signal sensitivity. The effect of decomposition temperature was examined for 18 kinds of nucleotides. The results are summarized in Table 2. It is noted here that

the signal intensities of all analytes examined increased at the higher decomposition temperature and that the responses observed at 140 °C were almost similar to that of potassium dihydrogenphosphate. At the higher temperature, the air bubbles were generated in the reaction tubing and interfered with the signal detection.

Analytical Figures of Merit. The analytical figures of merit obtained by the present flow injection analysis system were evaluated under the optimized conditions, where ATP was chosen as a model compound. Figure 2 shows typical signal responses obtained by injection of 5 μl of the sample solution in the concentration range from 0.5 to 20 μg ml^{-1} as phosphorus (P). The calibration graph was linear with an equation of A (absorbance) $=0.0001+0.0244$ P [μg ml^{-1}], and its correlation coefficient was 0.99. The detection limit of phosphorus estimated from the peak height measurement was about 50 ng ml^{-1}. By increasing the sample volume up to 20 μl, the detection limit was improved down to about 16 ng ml^{-1}.

Application to Determination of Phosphorus in Biological Samples. The present method was applied to the determination of total phosphorus concentration contained in biological samples such as livers of cow, pork, chicken and porgy fish, heart of chicken, eggs of yellowtail fish and salmon. In sample pretreatment by acid digestion with perchloric acid, as described in the experimental section, phosphorus still existed as the nucleotide compounds such as mono-, di-, and triphosphates.[17] Thus potassium peroxodisulfate for sample decomposition in an oxidation reaction tube was required to produce orthophosphate ion. The analytical results of total phosphorus contents in the biological samples investigated are summarized in Table 3, where

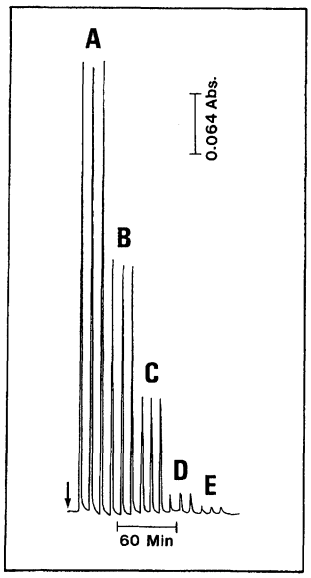

Fig. 2. FIA signals obtained for ATP. Sample volume, 5 μl; decomposition temperature, 140 °C; concentration (μg ml^{-1} as P), A: 20, B: 10, C: 5, D: 1, E: 0.5.

Table 3. Analytical Results of Phosphorus Contents in Biological Samples

Samples	Total phoshorus (mg g^{-1})[a]
Liver-Cow	0.10
-Pork	0.12
-Chicken	0.16
-Porgy fish	0.17
Heart-Chicken	0.13
Egg-Yellowtail fish	0.014
-Salmon	0.017

a) Calculated based on fresh weight.

the analytical values were consistent within 5% in the measurement of 3 duplicate analysis for each sample and the reproducibility of instrumental analysis was ca. 3%. These results were also examined by inductively coupled plasma atomic emission spectrometry, and the data obtained by both methods were in good agreement with each other within 3%.

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