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STUDY OF THE COLOURED SUBSTANCES IN MOLYBDENUM BLUE USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Methods for the separation of reduction products of phosphomolybdate and silicomolybdate were established by high-performance liquid chromatography using Styragel 60 Å (Waters Assoc.) as a packed material and acetonitrile solution containing sulphuric acid as an eluent. Reduction products of phosphomolybdate were separated into two species, Product I (P) and Product II (P). Reduction products of silicomolybdate were also separated into two component species, Product I (Si) and Product II (Si). From the investigation of phosphomolybdenum blue complexes produced via various different colour reactions, it was observed that the molybdenum blue reaction for phosphate proceeded in two stages as follows: $H_3PO_4 \cdot 12MoO_3 \rightarrow H_3PO_4 \cdot 10MoO_3 \cdot Mo_2O_5 \rightarrow H_3PO_4 \cdot 8MoO_3 \cdot 2Mo_2O_5$. The apparent molar absorptivity of Product I (P) was 7.4 times larger than that of Product II (P). Similarly, the molybdenum blue reaction for silicate was studied and discussed.

INTRODUCTION

Molybdenum blue methods, which are used to determine orthophosphate and silicate in biological materials such as plasma and urine, have been studied and modified by many workers[1–6]; but each of them has some disadvantages. One of the most serious disadvantages is that the absorbance of the reaction mixture increases with the lapse of time after the addition of reagents, and the analytical results are therefore not reliable. As a cause of the disadvantage it has been considered that there are some reduction products of molybdocomplexes which differ from one another in their absorption spectra in the visible region and their ratios vary with a slight change of reaction conditions. Bamann *et al.*[7] pointed out that, in the reduction of phosphomolybdate with 1-amino-2-naphthol-4-sulphonic acid–sodium bisulphite (ANS–$NaHSO_3$) in acidic solution, even the reduced molybdenum blue complexes contain two species, $H_3PO_4 \cdot 10MoO_3 \cdot Mo_2O_5$ and $H_3PO_4 \cdot 8MoO_3 \cdot 2Mo_2O_5$. The former was prepared by reducing phosphomolybdate for 30 min; the latter was prepared by reducing it for 3 days. Elucidation of the two coloured complexes was based on the average valence, potassium permanganate consumption and stoichiometric structure.

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For the evaluation of several molybdenum blue methods, it is necessary to separate and determine the reduced blue complexes. Previously, we established a method for the simultaneous determination of phosphomolybdate and silicomolybdate by high-performance liquid chromatography (HPLC)[8]. In this paper, we describe the separation of reduction products of phosphomolybdate and silicomolybdate by the use of HPLC. Further, we discuss the reaction mechanism of molybdenum blue methods.

EXPERIMENTAL

Reagents

Acetonitrile for HPLC was purchased from Wako Pure Chemicals. Tetrabutylammonium hydrogen sulphate (TBA) was purchased from Tokyo Kasei Kogyo. All other chemicals were of reagent grade and reagent solutions were prepared by dissolving in deionized and distilled water. The standard solution of phosphate (1 mg P/ml) was prepared by dissolving potassium phosphate monobasic in distilled water. The standard solution of silica (1 mg Si/ml) for atomic absorption spectrophotometry (Wako Pure Chemicals) was diluted with distilled water before use.

Preparation of reductant solution. 1-Amino-2-naphthol-4-sulphonic acid–sodium bisulphite (ANS–$NaHSO_3$) was prepared as follows: 15 g of sodium bisulphite, 0.5 g of sodium sulphite and 0.3 g of ANS were dissolved in 100 ml of distilled water and the solution was filtrated with filter paper. The filtrate was stored in a plastic bottle at room temperature in the dark. Stannous chloride solution was prepared by dissolving 1.0 g of stannous chloride in 10 ml of 6.0 *M* hydrochloric acid and diluting with distilled water before use.

Apparatus

The high-performance liquid chromatographic system consisted of a reciprocating pump (PSU-2.5, Seishin Seiyaku, Tokyo), a loop injector (VMU-5, Seishin Seiyaku), UV–VIS detector (UVILOG-7, Oyo-Bunko Kiki, Tokyo) and a recorder (SS-250F, Sekonic, Tokyo). A pyrex-glass tube (200 × 5 mm I.D.) was packed with Styragel 60 Å (Waters Assoc.).

Procedure

A 1-ml aliquot of the solution containing orthophosphate or silicate was placed in a plastic tube and 1 ml of 2% ammonium molybdate and 1 ml of reductant solution were added. After shaking vigorously, the solution was left to stand for more than 1 min and submitted to HPLC. Reduction products of molybdocomplexes were detected at 650 and 340 nm; the reducing reagents have no absorption at these wavelengths.

RESULTS AND DISCUSSION

Separation of molybdenum blue complexes

In the previous paper[8], the separation of phosphomolybdate and silicomolybdate was performed on a column packed with Styragel 60 Å using an aqueous acetonitrile solution containing sulphuric acid and TBA as an eluent. In the solution

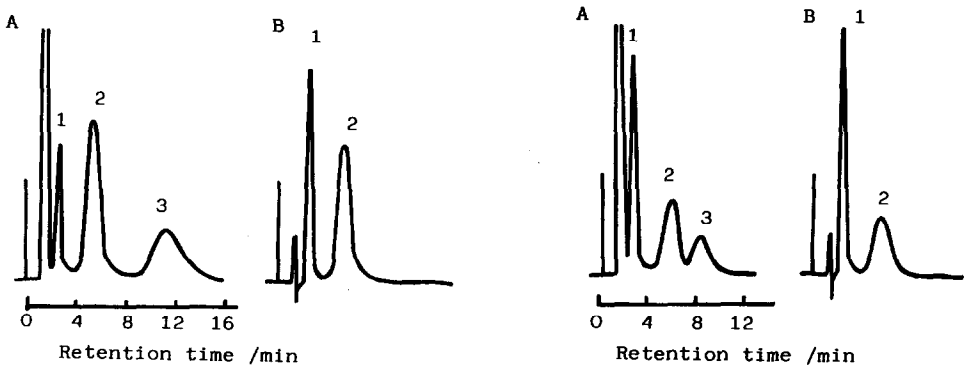


Fig. 1. Chromatograms of products obtained on reduction of phosphomolybdate: Peaks: 1 = Product I (P); 2 = Product II (P); 3 = phosphomolybdate. Column, Styragel 60 Å (200 × 5 mm I.D.); eluent, 55% (v/v) acetonitrile containing 0.75 *M* sulphuric acid and 20 m*M* TBA; flow-rate, 1.0 ml/min; detection, 340 nm (A) and 650 nm (B); sample size, 50 μl; sample, phosphomolybdate (5 μg/ml as P) reduced by 0.02% hydroquinone.

Fig. 2. Chromatograms of products obtained on reduction of silicomolybdate. Peaks: 1 = Product I (Si); 2 = Product II (Si); 3 = silicomolybdate. Column, Styragel 60 Å (200 × 5 mm I.D.); eluent, 50% (v/v) acetonitrile containing 0.75 *M* sulphuric acid and 20 m*M* TBA; flow-rate, 1.0 ml/min; detection, 340 nm (A) and 650 nm (B); sample size, 50 μl; sample, silicomolybdate (5 μg/ml as Si) reduced by 0.1% ascorbic acid.

containing organic solvents which are miscible with water, dissociation of molybdocomplexes did not occur. During the investigation of molybdenum blue complexes by the use of HPLC, reduction products of phosphomolybdate were separated into two species and those of silicomolybdate were also separated into two species. Chromatograms and chromatographic conditions are shown in Figs. 1 and 2.

Table I shows that molybdenum blue complexes, which are prepared by reducing phosphomolybdate and silicomolybdate with other reductants, are also separated into two species and their retention times are the same as those prepared by

TABLE I

RETENTION TIMES OF PRODUCTS OBTAINED FROM PHOSPHOMOLYBDATE AND SILICOMOLYBDATE BY REDUCTION WITH SEVERAL REDUCTANTS

P–Mo (2 μg/ml as P) and Si–Mo (2 μg/ml as Si) were reduced by each reductant in 0.2 *N* sulphuric acid.

Reductant		*Retention time (min)*					
		Reduced P–Mo			*Reduced Si–Mo*		
ANS	(0.02%)	2.5	5	11.5	2.5	5.9	8.1
Hydroquinone	(0.02%)	2.5	5	11.5	2.5	5.9	8.1
Hydrazine	(0.02%)	2.5	5	11.5	2.5	5.9	8.1
Ascorbic acid	(0.02%)	2.5	5	11.5	2.5	5.9	8.1
Uric acid	(0.02%)	2.5	5	11.5	2.5	5.9	8.1
$FeSO_4$	(1.0%)	2.5	5	11.5	2.5	5.9	8.1
Na_2SO_3	(1.0%)	2.5	5		2.5	5.9	8.1
KI	(1.0%)	2.5	5		2.5	5.9	8.1
$SnCl_2$	(0.01%)	2.5	5		2.5	5.9	8.1

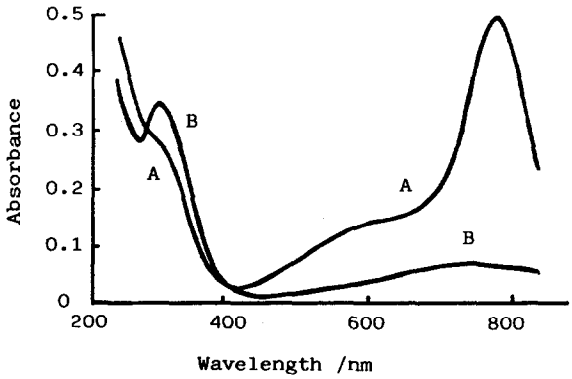


Fig. 3. Absorption spectra of products obtained on reduction of phosphomolybdate. (A) Product I (P), 0.90 μg/ml as P; (B) Product II (P), 0.94 μg/ml as P.

reduction with ANS–$NaHSO_3$. The reduction proeduct of phosphomolybdate eluted at 2.5 min is termed Product I (P) and that eluted at 5.0 min is termed Product II (P). The reduction products of silicomolybdate eluted at 2.5 and 5.9 min are correspondingly termed Product I (Si) and Product II (Si), respectively.

Bamann *et al.*[7] have shown that the phosphomolybdenum blue complexes used in the colorimetric determination of orthophosphate consists of two compounds, $H_3PO_4 \cdot 10MoO_3 \cdot Mo_2O_5$ and $H_3PO_4 \cdot 8MoO_3 \cdot 2Mo_2O_5$. When two molybdenum blue complexes obtained by the method of Bamann *et al.* were submitted to HPLC, $H_3PO_4 \cdot 10MoO_3 \cdot Mo_2O_5$ was eluted at the same position as Product II (P) and $H_3PO_4 \cdot 8MoO_3 \cdot 2Mo_2O_5$ at the same position as Product I (P).

Absorption spectra of molybdenum blue complexes

The absorption spectra of the reduction products separated by HPLC are shown in Figs. 3 and 4. The spectra of Product I (P) and Product II (P) were identical with those of $H_3PO_4 \cdot 8MoO_3 \cdot 2Mo_2O_5$ and $H_3PO_4 \cdot 10MoO_3 \cdot Mo_2O_5$ as shown

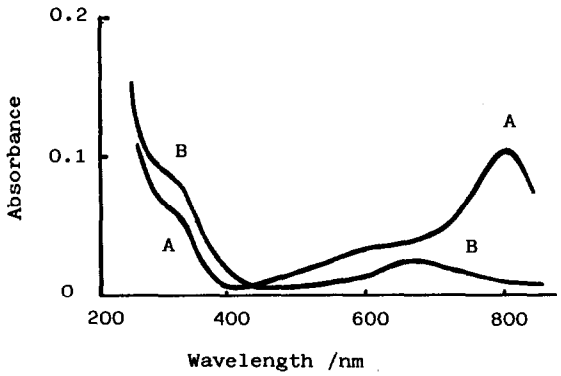


Fig. 4. Absorption spectra of products obtained on reduction of silicomolybdate. (A) Product I (Si), 0.14 μg/ml as Si; (B) Product II (Si), 0.19 μg/ml as Si.

TABLE II

APPARENT MOLAR ABSORPTIVITIES OF PRODUCT I (P), PRODUCT II (P), PRODUCT I (Si) AND PRODUCT II (Si) AT EACH ABSORPTION MAXIMUM

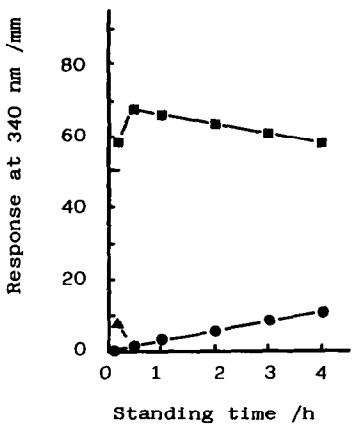
Product	*Apparent molar absorptivity at absorption maximum*	
Product I (P)	$1.7 \cdot 10^4$	λ_{max} 790 nm
Product II (P)	$2.3 \cdot 10^3$	λ_{max} 730 nm
Product I (Si)	$2.1 \cdot 10^4$	λ_{max} 800 nm
Product II (Si)	$3.5 \cdot 10^3$	λ_{max} 660 nm

by Bamann *et al.*[7], although their absorption maxima slightly shift to the long-wavelength region because of measurement in a different solvent. Silicomolybdenum blue complexes obtained by the molybdenum blue method were separated by HPLC as Product I (Si) and Product II (Si), the absorption spectra of which were very similar to Product I (P) and Product II (P).

Phosphorus and silicon in molybdenum blue complexes were determined as follows: to 5.0 ml of the fractions containing each molybdenum blue complex, 50 µl of 10% (m/v) bromine in ethanol were added. After shaking vigorously, phosphomolybdate or silicomolybdate produced from the corresponding molybdenum blue complex was determined by the HPLC method[8]. Table II shows the apparent molar absorptivity of molybdenum blue complexes in the eluent. Apparent molar absorptivity of Product I is 6–7 times larger than that of Product II. When Product II is converted to Product I after a lapse of time, the absorbance of the reaction mixture in the visible region increases.

Reaction mechanism of molybdenum blue method

Figs. 5 and 6 show the formation rate of molybdenum blue complexes. Within 30 min, phosphomolybdate is perfectly reduced to Product II (P) and is then gradu-



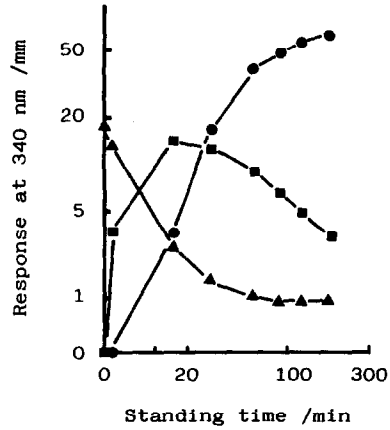


Fig. 5. Effect of standing time on levels of products obtained from phosphomolybdate by reduction in the reaction mixture. (■) Product II (P), (●) Product I (P), (▲) phosphomolybdate. Phosphomolybdate (2 µg/ml as P) was reduced by ANS–$NaHSO_3$. The procedure is described in the Experimental section.

Fig. 6. Effect of standing time on levels of products obtained from silicomolybdate by reduction in the reaction mixture. (■) Product II (Si), (●) Product I (Si), (▲) Silicomolybdate. Silicomolybdate (2 µg/ml as Si) was reduced by ANS–$NaHSO_3$. The procedure was described in the experimental section.

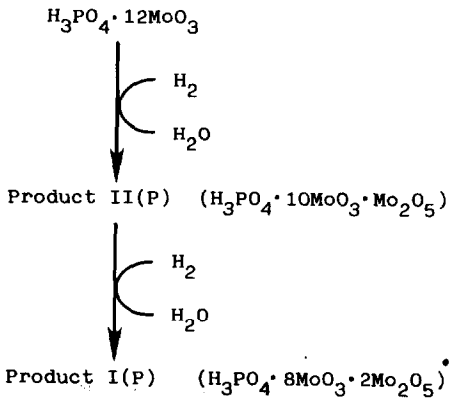


Fig. 7. Reduction mechanism of phosphomolybdate.

ally converted to Product I (P). Silicomolybdate decreases after the addition of reductant, while Product II (Si) increases. After 15 min, Product II (Si) decreases and Product I (Si) increases with lapse of time. These results suggest that the molybdenum blue reaction occurs in two stages and Product II is an intermediate.

Unoura *et al.*[9] reported that in the study of phosphomolybdate reduction by cyclic voltammetry, phosphomolybdate was reduced to the two-electron and the four-electron reduction products but not to the six-electron reduction product. This information supports the view that Product II (P) is the two-electron reduction product of phosphomolybdocomplex and Product I (P) is the four-electron reduction product.

Fig. 7 shows the mechanism of the molybdenum blue reaction inferred from the results described above.

The structure of silicomolybdenum blue complexes has not been made clear, but it may be concluded that Product II (Si) is the two-electron reduction product ($H_4SiO_4 \cdot 10MoO_3 \cdot Mo_2O_5$) and Product I (Si) is the four-electron reduction product ($H_4SiO_4 \cdot 8MoO_3 \cdot 2Mo_2O_5$).

Molybdenum blue complexes consist of two compounds whose apparent molar absorptivities differ from one another. Their ratio varies with a slight change of reaction conditions. This is the reason why some of the molybdenum blue methods are not reliable. To establish the reliable molybdenum blue method, it is necessary to choose a condition under which a single molybdenum blue is produced and the absorbance of the reaction mixture does not increase with a lapse of time. The present method for the separation of coloured substances is available for investigations into improving the molybdenum blue methods.

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