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Determination of Mn(II) and Co(II) with Arsenazo III

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Abstract—Complex formation between Arsenazo III and Mn^{2+} and Co^{2+} at equilibrium has been investigated at pH 7.2, and the stoichiometry and stability of the complexes have been determined. The data indicate that Arsenazo III is suitable for determination of Mn^{2+} and Co^{2+} on the micromolar scale. The dissociation constants of the phosphate complexes of Mn^{2+} and Co^{2+} at pH 7.2 were estimated with Arsenazo III as 3.6 and 10 mM, respectively.

Key words: Arsenazo III, manganese (II), cobalt (II), phosphate, complex formation, spectrophotometry

Determination of free metal ion concentrations is an important task in biochemistry because about one third of known enzymes and a great many other proteins somehow depend on metals [1]. Use of metal indicators, employing spectral differences between free indicators and their metal complexes, is one of the most popular approaches [2]. Arsenazo III (2,2′-[1,8-dihydroxy-3,6-disulfo-2,7-naphthalene-bis(azo)]dibenzenearsonic acid) is one of the most widely used metal indicators. The symmetrical molecule of Arsenazo III contains two groups capable of metal binding (Fig. 1). The structure of metal complexes of Arsenazo III is still a matter of debate [3], yet it is known that the complexes can have stoichiometries of 1 : 1, 2 : 1, and 1 : 2 [4]. The merits of Arsenazo III include its high stability, high extinction coefficients, and stability of its metal complexes [4], allowing determination of metals at low concentrations. Arsenazo III is most often used to assay Ca^{2+} in biological systems, and its complex formation with this cation has been extensively characterized [2]. Arsenazo III can also bind other metal ions [4-7] but the stoichiometry and stability of the resulting complexes have been poorly characterized. In particular, this is true with respect to biochemically important metals such as manganese, zinc, and cobalt.

The present work fills in this gap with respect to Mn^{2+} and Co^{2+}. To illustrate the proposed procedure for Mn^{2+} and Co^{2+} determination, we have measured the dissociation constants for their phosphate complexes at pH 7.2. Phosphate is an obligate component of living organisms and a substrate for a number of enzymes using Mn^{2+} and Co^{2+} as cofactors. Knowledge of the dissociation constants for the phosphate complexes formed of Mn^{2+} and Co^{2+} would allow a quantitative analysis of the interactions between such enzymes and phosphate. Such an analysis is currently underway for the enzyme pyrophosphatase.

MATERIALS AND METHODS

Reagents and solutions. Tes, Mops, $N(CH_3)_4OH$, and Dowex chelating anion exchange resin were from Sigma (USA), $MnCl_2{\cdot}4H_2O$ was from Fisher Scientific (USA), and $CoCl_2{\cdot}6H_2O$ was from Baker Chemical Co. (USA). Arsenazo III (Voikov Plant, Moscow) of analytical grade was further purified by passing through a column (2 × 10 cm) of the anion exchange resin in Na^+ form [8]. Arsenazo III concentration was determined from the absorbance of its solution in concentrated sulfuric acid at 675 nm, using an extinction coefficient of 52,800 $M^{-1}{\cdot}cm^{-1}$ [9]. Other reagents were obtained from local sources and were at least of "chemically pure" grade.

The buffers used to maintain pH 7.2 and ionic strength of 0.1 M were 83 mM Tes-KOH containing 17 mM KCl in experiments with Mn^{2+} and 100 mM Mops-KOH in experiments with Co^{2+} (Tes binds Co^{2+} [10]). K^+ concentration was 50 mM in both buffer systems. H_3PO_4 solution (0.3 M) was incubated for 6 h in a

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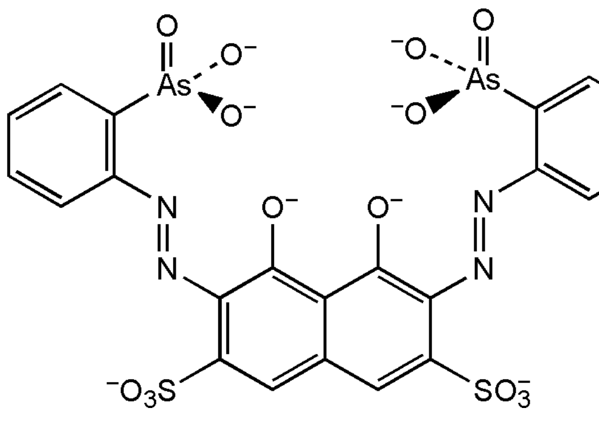

Fig. 1. Structure of Arsenazo III.

boiling water bath to hydrolyze contaminating pyrophosphate, diluted to 50 mM with water, supplemented with 17 mM KCl and 33 mM KOH and titrated to pH 7.2 with a solution of tetramethylammonium hydroxide. KCl was omitted from the H_3PO_4 solution used in experiments with Co^{2+}. The concentration of P_i solutions was measured spectrophotometrically at 650 nm, using a color solution containing ammonium molybdate, H_2SO_4, and the Malachite Green dye [11]. Ca^{2+} was removed from the buffer and P_i solutions by storing them on chelating anion exchanger (0.5 ml of the resin per 200 ml solution). The resin had been converted into H^+ form by treatment with 1 M hydrochloric acid, washed with water, and incubated with three portions of 20 mM buffer solution, whose pH was adjusted to 7.2 after resin addition, 1 h each incubation. All solutions were made on deionized water with a resistance of greater than 15 MΩ·cm.

Light absorbance measurements. Light absorbance was measured in a Pharmacia-LKB Ultraspec Plus spectrophotometer (Sweden), using cuvettes of 0.5 or 1 cm pathlength. Reagent concentrations were chosen such that light absorbance did not exceed one unit and no precipitation of metal phosphates was observed. As Arsenazo III was unstable in the presence of Co^{2+}, measurements were carried out as fast as possible with this cation—each measurement was done with a fresh sample and Co^{2+} was added last. With the Arsenazo III–Mn^{2+} system, Mn^{2+} and P_i concentrations were varied in the same sample. All measurements were conducted at 25°C unless noted otherwise.

Data treatment. Calculations were done with the program SCIENTIST (MicroMath, USA), allowing nonlinear regression analysis of sets of equations to determine the best-fit values for parameters. The Gauss elimination algorithm [12] was used to determine absorbance matrix rank; the corresponding program was written in Qbasic (Microsoft, USA).

RESULTS AND DISCUSSION

Complexing of Arsenazo III with Mn^{2+} and Co^{2+}. Addition of Mn^{2+} or Co^{2+} to Arsenazo III solution changed its spectrum due to complex formation (Fig. 2). The spectral changes were maximal at 650 nm with Mn^{2+} and at 615 nm with Co^{2+}. The Mn^{2+}-induced changes were very fast (<3 sec), and the spectrum was stable afterwards for at least 30 min. The Mn^{2+} concentration dependence curve of this effect exhibited a maximum (Fig. 3). The stoichiometry of the complexes formed at low Mn^{2+} concentrations was determined by an isomolar series method in which light absorbance is measured as a function of the concentration of one of the two interacting components at a constant sum of their concentrations [13]. A symmetrical curve with a maximum at [Arsenazo III]/[Mn^{2+}] = 1 : 1 obtained at the sum of their concentrations of 40 µM (Fig. 4) indicated formation of an MD complex in these conditions. The decrease in absorbance at greater than 1 mM metal concentration in Fig. 4 points therefore to M_2D complex formation. Thus, MD and M_2D complexes are stoichiometrically significant in the Arsenazo III–Mn^{2+} system (Scheme 1), the extinction coefficient being lower for the M_2D complex by comparison with the MD complex.

Changes in Arsenazo III spectrum in the presence of Co^{2+} exhibited several peculiarities. First, the time-course of absorption change upon addition of Co^{2+} to Arsenazo III solution exhibited two phases: fast (<3 sec) and slow (>30 min). The slow phase appeared to refer to Co^{2+} oxidation and/or Co^{2+}-induced Arsenazo III oxidation by air oxygen. This conclusion was confirmed by an increase in the rate of the slow phase from 0.003 to 0.008

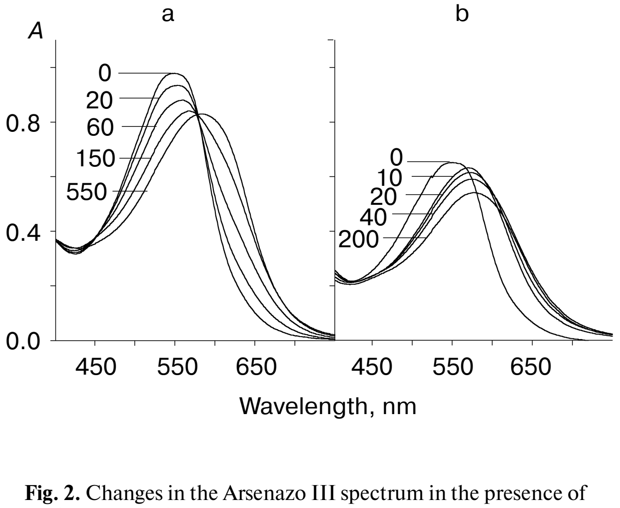

Fig. 2. Changes in the Arsenazo III spectrum in the presence of Mn^{2+} (a) or Co^{2+} (b). Metal ion concentration in micromoles per liter is indicated on the curves. Arsenazo III concentration was 60 µM (a) or 20 µM (b); 0.5 cm (a) or 1 cm (b) cuvette path length; 30°C.

absorbance unit/min in the presence of 0.3 mM ammonium persulfate (40 µM Arsenazo III, 100 µM Co^{2+}; 680 nm). For this reason, a fresh assay mixture was prepared for each measurement, $CoCl_2$ solution was added last, and absorbance measurements were done as rapidly as possible. Second, the results obtained by the isomolar series method indicated a 2 : 1 binding stoichiometry in the Arsenazo III–Co^{2+} system (40 µM total Arsenazo III and Co^{2+} concentration) (Fig. 4), i.e., MD_2 formation. Like in the Arsenazo III–Mn^{2+} system, the Co^{2+} concentration dependence of the absorbance was bell-shaped (Fig. 3), suggesting formation of at least two complexes between Arsenazo III and Co^{2+}: MD_2 and MD or MD_2 and M_2D. Computer analysis of this dependence (see below) has provided evidence for all three complexes: MD_2, MD, and M_2D.

Thus, Scheme 1 is a general scheme describing complex formation, with MD_2 being stoichiometrically insignificant in the case of Mn^{2+}.

$$\begin{tikzcd}
D \arrow[r, shift left, "K_{MD}"] & MD \arrow[l, shift left] \arrow[r, shift left, "K_{M_2D}"] \arrow[d, shift left] & M_2D \arrow[l, shift left] \\
& MD_2 \arrow[u, shift left, "K_{MD_2}"] &
\end{tikzcd}$$

Scheme 1. Complex formation between Arsenazo III (D) and metal ions (M). K_{MD}, K_{M_2D}, and K_{MD_2} are dissociation constants for the respective complexes

Dissociation constants for the Arsenazo III complexes of Co^{2+} and Mn^{2+}. The dependence of solution absorbance (A) on solution composition for Scheme 1 is described by Eq. (1), where ε_D, ε_{MD}, ε_{M_2D}, and ε_{MD_2} are the molar extinction coefficients for the respective Arsenazo III species. The concentrations of these species are obtained as solutions of the set of equations (Eqs. (2)-(6)), in which $[M]_0$ and $[D]_0$ are total concentrations of the metal ion and Arsenazo III. Equations (2) and (3) are mass balance equations. With Mn^{2+}, K_{MD_2} is vanishingly low (MD_2 is absent) and the equations are simplified accordingly. The complexity of the mathematical treatment of this system results from the necessity to take into account the decrease in free Arsenazo III and metal ion concentrations caused by complex formation.

$$A = \varepsilon_D[D] + \varepsilon_{MD}[MD] + \varepsilon_{M_2D}[M_2D] + \varepsilon_{MD_2}[MD_2]; \quad (1)$$

$$[M]_0 = [M] + \frac{[M][D]}{K_{MD}} + \frac{[M][D]^2}{K_{MD}K_{MD_2}} + 2\,\frac{[M]^2[D]}{K_{MD}K_{M_2D}}; \quad (2)$$

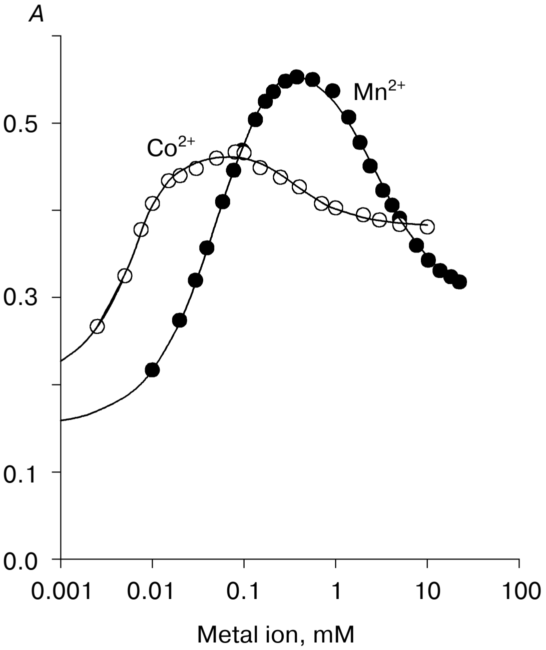

Fig. 3. Mn^{2+} and Co^{2+} concentration dependence of Arsenazo III absorbance. Arsenazo III concentration was 40 µM (Mn^{2+}) or 20 µM (Co^{2+}); 650 nm (Mn^{2+}) or 615 nm (Co^{2+}) wavelength; 1 cm cuvette pathlength.

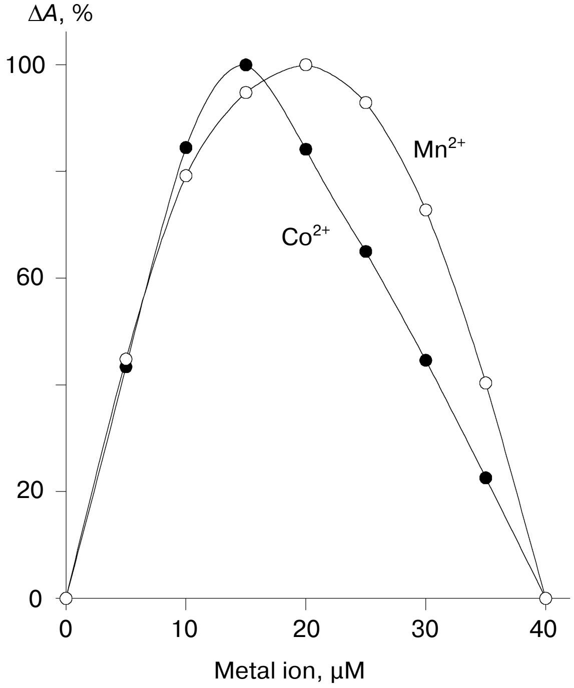

Fig. 4. Use of isomolar series method for the Arsenazo III–Mn^{2+} and Arsenazo III–Co^{2+} systems. The ordinate shows absorbance change caused by metal ions in percent of the maximal value (0.078 for Mn^{2+} and 0.28 for Co^{2+}). Total Arsenazo III and metal ion concentration was 40 µM; 650 nm (Mn^{2+}) or 615 nm (Co^{2+}) wavelength; 1 cm cuvette pathlength.

$$[D]_0 = [D] + \frac{[M][D]}{K_{MD}} + 2\frac{[M][D]^2}{K_{MD}K_{MD_2}} + \frac{[M]^2[D]}{K_{MD}K_{M_2D}}; \quad (3)$$

$$[MD] = \frac{[M][D]}{K_{MD}}; \quad (4)$$

$$[M_2D] = \frac{[M]^2[D]}{K_{MD}K_{M_2D}}; \quad (5)$$

$$[MD_2] = \frac{[M][D]^2}{K_{MD}K_{MD_2}}. \quad (6)$$

Equations (1)-(6) contain six unknown parameters (dissociation constants and molar extinction coefficients for the complexes). Their best-fit values were calculated from the dependencies shown in Fig. 3 by nonlinear regression analysis with the program SCIENTIST, which allows simultaneous use of several equations, some of which may be implicit. The obtained values are summarized in Tables 1 and 2. One can see that the second molecule of Arsenazo III binds to Co^{2+} more tightly than the first one ($K_{MD_2} < K_{MD}$). This explains why the isomolar series method showed the [Arsenazo III]/[Co^{2+}] stoichiometry of 2 : 1 (Fig. 4)—MD_2 is the dominating complex form in the conditions used. The theoretical curves simulated for complex formation models involving only two complexes between Arsenazo III and Co^{2+} poorly described the dependence presented in Fig. 3.

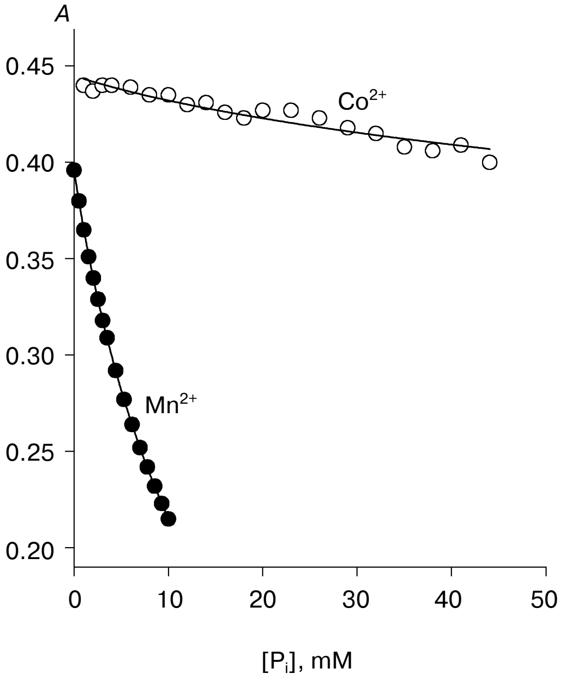

Fig. 5. P_i concentration dependence of Arsenazo III absorbance in the presence of Mn^{2+} or Co^{2+}. Arsenazo III concentration was 40 µM (Mn^{2+}) or 20 µM (Co^{2+}); metal ion concentration was 50 µM (Mn^{2+}) or 20 µM (Co^{2+}); 650 nm (Mn^{2+}) or 615 nm (Co^{2+}) wavelength; 1 cm cuvette pathlength.

Table 1. Dissociation constants for the Mn(II) and Co(II) complexes of Arsenazo III and P_i

Parameter	Mn(II)	Co(II)
K_{MD}, µM	26.5 ± 0.8	10 ± 4
K_{M_2D}, mM	2.3 ± 0.1	0.25 ± 0.10
K_{MD_2}, µM		1.0 ± 0.5
K_{MP}, mM	3.6 ± 0.1	10 ± 2

Table 2. Molar extinction coefficients ($mM^{-1}·cm^{-1}$) for the metal ion complexes of Arsenazo III. Wavelength was 650 nm for Mn(II) and 615 nm for Co(II)

Parameter	Mn(II)	Co(II)
ε_D	3.63 ± 0.03	10.01 ± 0.02
ε_{MD}	16.0 ± 0.1	23.9 ± 1.4
ε_{M_2D}	8.45 ± 0.05	19.0 ± 0.1
ε_{MD_2}		48.4 ± 1.4

Dissociation constants for MnP_i and CoP_i complexes. Phosphate addition to the Arsenazo III–metal system resulted in a decrease in absorbance, indicating a decrease in free metal ion concentration due to metal–P_i complex formation (Fig. 5). This effect allowed estimation of the dissociation constants for these complexes. The dependencies shown in Fig. 5 were also described by Eqs. (1)-(6) with the term $[M][P_i]/K_{MP}$, corresponding to the contribution of the MP_i complex into total metal ion concentration, added to right side of Eq. (2) ($K_{MP} = [M][P_i]/[MP_i]$ is the dissociation constant for this complex). The resulting K_{MP} values are summarized in Table 1 and refer to metal interaction with the HPO_4^{2-} species, which predominates in phosphate solution at pH 7.2 (pK_a value for $H_2PO_4^-$ is equal to 6.96 [14]). The published values of the dissociation constants for the $MnHPO_4$ complex are 1.3 mM (polarography) [15] and 2.5 mM (potentiometry) [14], and for the $CoHPO_4$ complex is 6.6 mM (potentiometry) [16], which are in a good agreement with the values obtained in this work.

A potential source of error in complex stability measurements employing metal indicators is a ternary a

0,977	0,863	0,883	0,892	0,899	0,902	0,905	0,906	0,900
1,022	0,925	0,941	0,947	0,953	0,955	0,957	0,957	0,950
0,970	0,914	0,921	0,923	0,925	0,926	0,927	0,925	0,917
0,683	0,749	0,734	0,724	0,720	0,716	0,713	0,709	0,698
0,370	0,549	0,511	0,489	0,476	0,466	0,459	0,453	0,438
0,231	0,433	0,389	0,362	0,347	0,335	0,327	0,319	0,303
0,201	0,401	0,358	0,331	0,314	0,303	0,294	0,287	0,271
0,106	0,270	0,236	0,215	0,201	0,192	0,185	0,178	0,164
0,051	0,164	0,141	0,127	0,118	0,112	0,108	0,103	0,092

b

1,022	0,925	0,950	0,957	0,947	0,953	0,957	0,941	0,955
0,000	0,224	0,088	0,111	0,148	0,132	0,103	0,176	0,119
0,000	0,000	0,012	0,009	0,005	0,006	0,009	0,002	0,008
0,000	0,000	0,000	0,003	0,003	0,002	0,003	0,002	0,003
0,000	0,000	0,000	0,000	0,002	0,000	0,001	0,001	0,001
0,000	0,000	0,000	0,000	0,000	0,001	–0,001	–0,001	0,000
0,000	0,000	0,000	0,000	0,000	0,000	–0,001	–0,001	0,000
0,000	0,000	0,000	0,000	0,000	0,000	0,000	–0,001	0,001
0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000

c

0,005	0,005	0,005	0,005	0,005	0,005	0,005	0,005	0,005
0,000	0,003	0,003	0,003	0,003	0,003	0,003	0,003	0,003
0,000	0,000	0,007	0,008	0,008	0,008	0,007	0,009	0,008
0,000	0,000	0,000	0,003	0,003	0,003	0,003	0,003	0,003
0,000	0,000	0,000	0,000	0,006	0,006	0,007	0,006	0,007
0,000	0,000	0,000	0,000	0,000	0,015	0,018	0,018	0,019
0,000	0,000	0,000	0,000	0,000	0,000	0,014	0,014	0,014
0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,021	0,020
0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,028

Fig. 6. Determination of the number of light absorbing species in the Arsenazo III–Mn^{2+}–P_i system. a) The initial absorbance matrix; b) the rearranged absorbance matrix; c) the rearranged matrix of the standard errors. The lines correspond to 530, 550, 570, 590, 610, 625, 630, 650, and 670 nm wavelengths (from top to bottom). The rows correspond to P_i concentrations of 0, 0, 3.4, 6.3, 9.0, 11.4, 13.6, 15.6, and 19 mM (from left to right). Mn^{2+} concentration was zero for the upper line and 50 μM for the other lines. Arsenazo III concentration was 60 μM; 0.5 cm cuvette pathlength; 30°C.

M–D–P_i complex formation. This possibility was checked by determining the number of light absorbing species in the Mn^{2+}–Arsenazo III–P_i system. The approach used [12, 17] is based on the determination of the rank of the absorbance matrix A, whose elements are absorbances of nine solutions of different composition at nine wavelengths (Fig. 6a). Matrix rank is equal to the number of non-zero lines of the rearranged matrix A′ (Fig. 6b). An element of matrix A′ was assumed to be non-zero if its absolute value exceeded more than threefold the corresponding element of the matrix of the standard errors, rearranged according to the error addition rule [17] (Fig. 6c). The absorbance values were assumed to be precise within 0.5% in this analysis. That the matrix rank was two indicated the occurrence of two light absorbing species [12]. As the contribution of the M_2D complex is negligible in the conditions used (60 μM Arsenazo III, 50 μM $MnCl_2$, 0-19 mM P_i), the light absorbing species are clearly free Arsenazo III and the MD complex. Hence, no ternary Mn^{2+}–Arsenazo III–P_i complex is formed.

Thus, Arsenazo III is suitable for quantitative determination of Mn^{2+} and Co^{2+}. The method is simpler (and more precise) with Mn^{2+} because at low concentrations this cation forms only a 1 : 1 complex with Arsenazo III. Both cations form rather stable complexes with Arsenazo III (Table 1), resulting in a significant contribution of their complex into indicator and metal ion mass balance even at their micromolar concentrations. This inevitably makes calibration curves nonlinear even in their initial parts (the nonlinearity of the initial part seen in Fig. 3 mainly results from using logarithmic concentration scale), but this problem is easily solved by applying computer data treatment. It should be also noted that Arsenazo III spectrum is sensitive to various polyvalent cations [5], which narrows the field of its application to systems containing only one cation to be determined.

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