Biochem. J. (1989) 259, 295–298 (Printed in Great Britain)

The interaction of cations with the dye arsenazo III

Elizabeth ROWATT*‡ and Robert J. P. WILLIAMS†
*Department of Microbiology, School of Pathology, University College and Middlesex School of Medicine, Ridinghouse Street, London W1P 7LP, and †Inorganic Chemistry Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QR, U.K.

1. The dye arsenazo III combines with a selection of cations to give an altered absorption spectrum. 2. Large metal cations such as Ca^{2+}, La^{3+} and quadrivalent cations give a 1:1 complex with two new absorption peaks at about 610 nm and 655 nm and a K_D of about 10^{-6} M. 3. Aliphatic polyamines and complex cobalt ions give a 1:1 complex, with one absorption peak at about 610 nm and a K_D from 10^{-6} to 10^{-3}M. 4. Small metal cations finally form a 2:1 complex and also have one absorption peak at about 610 nm, but with a K_D of 10^{-5}–10^{-4} M. 5. The absorption peak at 610 nm is similar to that formed at high pH in the absence of bivalent cations and is due to ionization of phenolic groups with the dye molecule in an extended form. 6. The peak at 655 nm with 1:1 complex can be explained as a change in orientation of the diazo bonds caused by a conformational change of the molecule when it wraps around the single atom of Ca^{2+} or other large cation.

INTRODUCTION

We wish to understand the binding of different cations to a lipopolysaccharide from *Escherichia coli* in order to appreciate the combined effects of cations plus lipopolysaccharide in the control of phage reactions with *E. coli*. To this end we have been examining the binding of a wide range of cations to different organic molecules. There is already a considerable number of measurements in the literature on such complexes, to which we add here some new data concerning the binding of metal ions to arsenazo III. This ligand has been chosen since it has a specific and unusual set of charges (Fig. 1) and an absorption spectrum (Savvin, 1961) which depends on its ionization state and conformation. We can therefore inspect both binding and features of the stereochemistry of cation–ligand binding, both of which must be of importance in recognition systems such as that between a metal–lipopolysaccharide complex and a phage. The lipopolysaccharide also has a specific distribution of negative charges.

MATERIALS AND METHODS

Chemicals

Arsenazo III (98% pure) was obtained from Sigma Chemical Co., Poole, Dorset, U.K., and was used without further treatment. Other chemicals and deionized water were obtained as described by Rowatt & Williams (1985, 1987).

Arsenazo III was dissolved in water to give a 1 mM solution and kept at +4 °C in a plastic, foil-covered, container to protect it from light. It was diluted to 50 μM in 2 mM-Tris/1 mM-acetate buffer, pH 7.4, and 0.5 ml was pipetted into plastic cuvettes of 1 cm light-path, containing the desired salt concentration in 0.5 ml of water. Absorbance was measured in a Cecil 505 double-beam spectrophotometer with a CE 500 control record module. For absorption by dye at pH 1–14, spectra were measured against water. For absorption by dye + salts, spectra were measured against dye + 10 μM-EDTA in buffer at pH 7.4. The absorption by arsenazo III + buffer without EDTA against dye + buffer + EDTA was the order of 0.01, showing that the concentration of Ca^{2+} or any other contaminant and reactive cation in the reagent solutions was less than 1 μM.

Buffers used were: pH 4–10, 2 mM-Tris + varied amounts of acetic acid; pH 1, 0.1 M-HCl; pH 2–3, 0.02 M-KCl/HCl (Documenta Geigy, 1970); pH 11, 23 mM-Tris; pH 12, 13 and 14, 0.01 M, 0.1 M and 1 M-NaOH respectively.

Dissociation constants (K_D)

These were calculated by taking maximum absorbance recorded as equal to total arsenazo III added, calculating the amount of complex formed at each cation concentration, and then calculating K_D from:

$$K_D = \frac{[M^{x+}] \times [\text{arsenazo III}^{y-}]}{[M^{x+}\text{–arsenazo III}^{y-}]}$$

where M is the metal ion in question, x^+ is its charge and y^- the charge on arsenazo III.

RESULTS

Effects of pH on spectrophotometric absorption by arsenazo III

At pH 7.4, arsenazo III has an absorption peak at 540 nm with a molar absorption coefficient of $3.1 \times 10^4\ M^{-1} \cdot cm^{-1}$. The absorption is constant from pH 1 to 7.4. Above this pH the absorption moves to higher wavelengths in overlapping steps (Fig. 2). At pH 14 (1 M-NaOH), the peak has shifted to 580 nm, and in 3 M-NaOH it has moved to 600 nm. There are a series of acid dissociation constants in the pH range 0–7 (Buděšínský, 1963; see Fig. 1), but they involve the arsono groups and do not noticeably affect the spectra. The phenolate groups ionize at pH values above 8 and only they introduce observable changes in the spectrum. The pK_a values were

---

Abbreviation used: cyclo[-NH-CH_2-CH_2]$_6$, hexacyclen (1,4,7,10,13,16-hexa-azacycloactadecane).

‡ To whom correspondence and reprint requests should be sent.

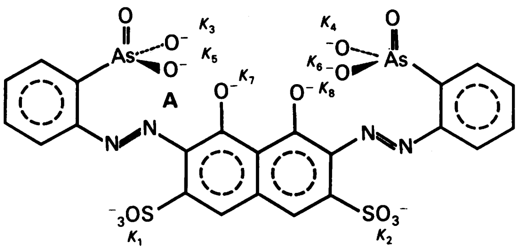

Fig. 1. Formula of arsenazo III in extended conformation

Dimensions and bond angles are taken from Phillips & Williams (1966). 'A' marks the position for binding of small cations. Ionizable groups (labelled K_1–K_8) have the following pK_a values (Buděšínský, 1963): p$K_1 = -2.5$; p$K_2 = 0$, pK_3 and p$K_4 = 2.5$; pK_5 and p$K_6 = 5.3$; p$K_7 = 7.5$; p$K_8 = 12.4$. The distance between benzene rings is 1.209 nm (12.09Å)

determined by Buděšínský (1963; and Fig. 1). These values do not concern the present work, but we notice that the values are somewhat at variance with expectation.

Reaction of arsenazo III with cations

The addition of some cations, e.g. La^{3+}, Ca^{2+} and Cd^{2+}, at pH 7.4, to arsenazo III gives a new absorption peak at about 610 nm, with a second peak at about 655 nm. Addition of other cations gives a new peak only in the region of 610 nm. The relation of absorption to cation concentration is shown in Fig. 3, and the absorption peaks of all cation complexes, including those with the polyamines spermine and hexacyclen, are given in Table 1. Absorption coefficients for the complexes are shown. With the exception of Ni^{2+}, Co^{2+} and hexacyclen (and possibly Mg^{2+}), the cations combine with arsenazo III in a 1:1 complex and clear isosbestic points are observed, showing that there is but one combined species.

Several effective K_D values, i.e. the dissociation constants at pH 7.4, were readily calculated from the 610 nm absorption for cations forming a 1:1 complex (Table 1).

The absorption with La^{3+} was also measured at pH 5.0 in 2 mM-Tris/2.4 mM-acetate buffer to avoid hydrolysis. Absorption at lower concentrations of La^{3+} was the same at pH 5 and pH 7.4. Above 15 μM-La^{3+}, the molar absorption was lower at pH 7.4, probably owing to hydrolysis. Binding of La^{3+} was so strong that the K_D could only be calculated approximately. Its association is at least 10 times as strong as that of Ca^{2+}.

Only very strong NaCl solutions (0.1 M) reduce absorption by arsenazo III in the presence of 10 μM-Ca^{2+}, showing that univalent cations bind very weakly to the reagent.

Ni^{2+} rapidly forms a blue–green colour with absorption at 640 nm, but this absorption quickly falls, to be replaced by one at 610 nm. As in the case of Co^{2+}, there is no isosbestic point, and these cations do not form a stable 1:1 complex of the expected kind, although this may be their initial form (see below). The stoicheiometry, after a long period of time, would appear to be 2 metal ions/ligand.

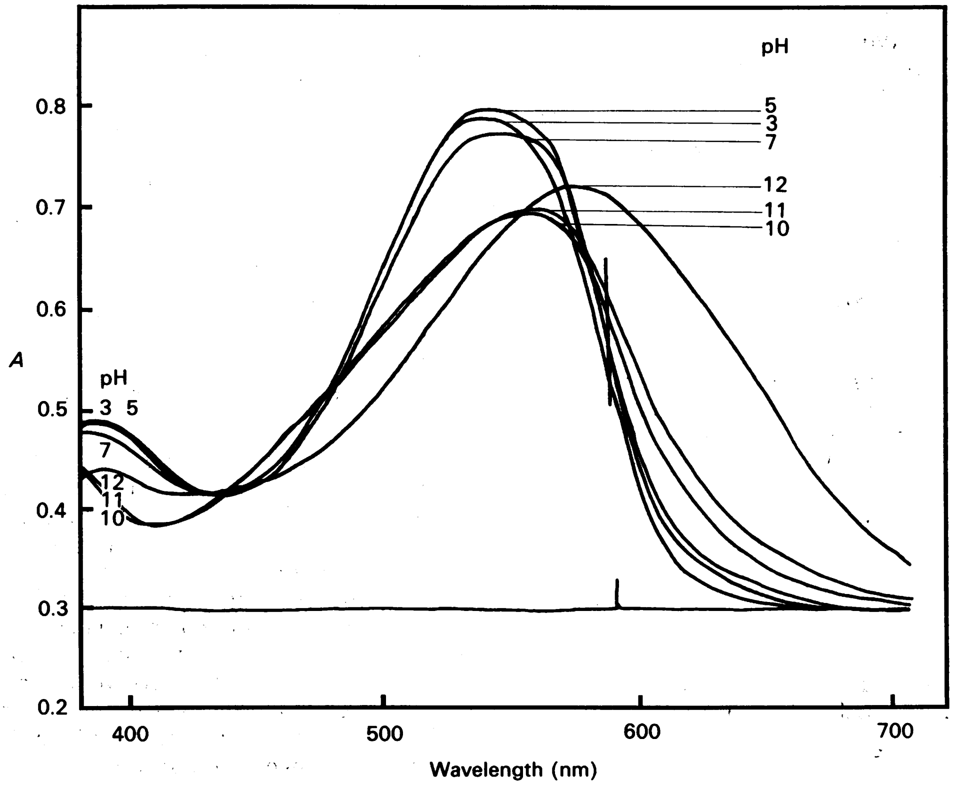

Fig. 2. Absorption spectra of arsenazo III at different pH values

Superimposed spectra from 380 to 705 nm of 25 μM-arsenazo III at pH 3, 5, 7, 10, 11 and 12 were measured against a water blank. Zero was adjusted to 0.3 at 340 nm. Buffers were as given in the Materials and methods section.

Table 1. Binding of cations by arsenazo III

(*a*) shows results for large metal cations, and (*b*) those for small metal cations, polyamines and complex cobalt ions. Some of these results are also shown in Fig. 3. Hexacyclen trisulphate was neutralized before use. The absorbance at peak maximum at about 610 nm was measured; the concentration of the cation–arsenazo III complex was calculated with reference to the absorption coefficient; K_D = ([M^{z+}] × [arsenazo III^{y-}])/[M^{z+}–arsenazo III^{y-}]. Data for Th^{4+}, Pb^{2+} and La^{3+} at the end of (*a*) are from Savvin (1961). Abbreviation: $[Co(III)]^{5+}$, $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$.

(*a*)

Cation	λ (nm)	K_D (μM)	Molar absorption coefficient ($M^{-1}\cdot cm^{-1}$)	λ (nm)	Molar absorption coefficient ($M^{-1}\cdot cm^{-1}$)
La^{3+}	610	< 0.1	$(4.33 \pm 0.07) \times 10^4$	657	$(3.53 \pm 0.06) \times 10^4$
Ca^{2+}	603	0.96 ± 0.18	$(3.47 \pm 0.009) \times 10^4$	653	$(2.64 \pm 0.40) \times 10^4$
Cd^2	606	0.97 ± 0.34	$(1.92 \pm 0.122) \times 10^4$	654	$(1.31 \pm 0.01) \times 10^4$
Th^{4+}	610	–	–	655	13×10^4
Pb^{2+}	610	–	–	655	1×10^4
La^{3+}	610	–	–	655	4×10^4

(*b*)

Cation	λ (nm)	K_D (μM)	Molar absorption coefficient ($M^{-1}\cdot cm^{-1}$)
$[Co(III)]^{5+}$	634	0.75 ± 0.28	$(2.19 \pm 0.05) \times 10^4$
$[Co(NH_3)_6]^{3+}$	618	2.79 ± 0.72	$(1.57 \pm 0.12) \times 10^4$
Mg^{2+}	616	75.9 ± 8.3	$(2.01 \pm 0.16) \times 10^4$
$[CoCl(NH_3)_5]^{2+}$	612	131 ± 3.8	$(1.24 \pm 0.58) \times 10^4$
Spermine	611	0.41 ± 0.22	$(6.92 \pm 0.04) \times 10^3$
3.3′-Imminobis-propylamine	613	7.83 ± 3.2	$(9.78 \pm 0.81) \times 10^3$
Spermidine	610	7.46 ± 2.2	$(5.70 \pm 0.19) \times 10^3$
Ni^{2+}	610	13.9 ± 6.6	$(2.20 \pm 0.17) \times 10^4$
Co^{2+}	612	10.4 ± 6.1	$(1.73 \pm 0.09) \times 10^4$
Hexacylen	610	40.4 ± 13.8	$(2.04 \pm 0.07) \times 10^4$

Those simple inorganic cations which give only a 1:1 complex have a dissociation constant of between 0.05×10^6 and 2×10^{-6} M (Table 1). Cations giving first a 1:1 and then a 2:1 complex, including Ni^{2+}, Co^{2+}, perhaps Mg^{2+} and hexacyclen, form weaker complexes.

DISCUSSION

These experiments extend the work of Tamm & Williams (1985) on metal and organic cations binding to dicarboxylic acid and phosphate anions. They investigated size-matching of different cations with these

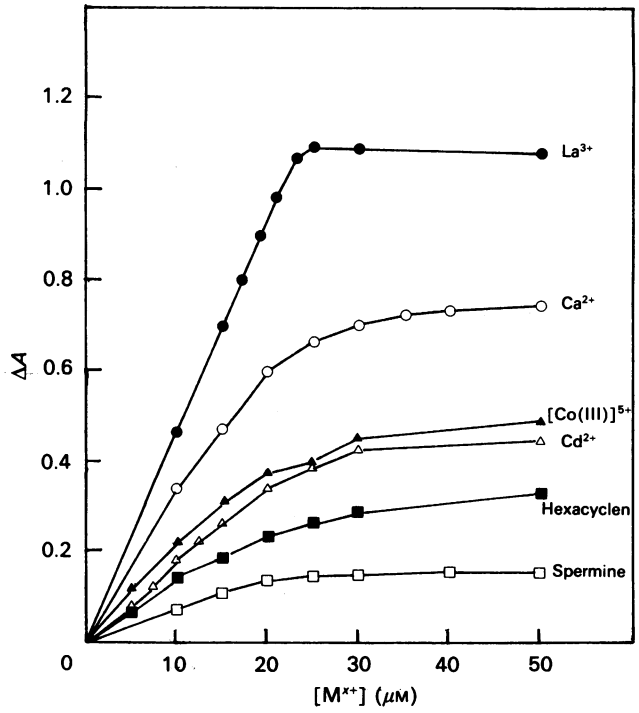

Fig. 3. Spectrophotometric absorption of arsenazo III on interaction with cations

Cations were added to 1 ml of 25 μM-arsenazo III (98% pure) in 2 mM-Tris/1 mM-acetate buffer, pH 7.4, except La^{3+}, which was in 2 mM-Tris/2.4 mM-acetate buffer, pH 5.0. All cations were in the form of chlorides, except for $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$, which was the bromide, and spermine was the free base. Absorption was scanned on a double-beam Cecil 505 spectrophotometer and values were taken for the peak at about 610 nm, measured against arsenazo III in buffer without any additions. Results are means for two or more experiments. Concentrations for Mg^{2+} and $[CoCl(NH_3)_5]^{2+}$ are too high to be included in this graph. Abbreviation: $[Co(III)]^{5+}$, $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$.

anions as a possible determinant of dissociation constants. In arsenazo III, we have a relatively rigid molecule with only two *trans* forms open to it (the *cis* form is not possible). The study of its association with cations has given values of dissociation constants and also the wavelengths of u.v./visible absorption in the complex. We shall use the latter to indicate the nature of the structure involved.

Table 1 shows that one group of large cations binds arsenazo III strongly in a 1:1 complex, and the complexes have a strong absorption at around 650 nm. Quantitatively similar observations have been made by Savvin (1961) for the complexes of Pb^{2+}, $[UO_2]^{2+}$, Th^{4+} and some lanthanides. Other cations, the small metal cations, the aliphatic and cyclic polyamines, and the complex cobalt ions do not give this absorption band, but only absorption at 610 nm, and their affinity is less. The small cations, Ni^{2+}, Co^{2+} and possibly Mg^{2+}, form a 2:1 complex. A model structure of arsenazo III is shown in Fig. 1, representing the sodium salt. This structure contains two possible types of 'socket' lined with O^- groups. The first socket (**A**, Fig. 1) can be filled by Mg^{2+}, either by bonding to the three O^- groups or, in a slightly lower position, to the three O^- groups and the nearer N atom. Ca^{2+} is too large for this socket, being of radius 0.1 nm (1.0 Å) as against the 0.06 nm (0.6 Å) of Mg^{2+}. This open conformation of the molecule is presumably similar to that found on ionization of protons, when the anionic groups of the ligand are expected to repel one another to give an extended molecule, i.e. one which absorbs at 610 nm only. The 610 nm absorption is simply due to the ionization of the phenolic groups with the arsenazo III in this conformation, whether it is free or bound to two small cations. From the absorption-spectral changes with larger cations, we know that there is another form of the molecule with an absorption maximum at 650 nm and with a 1:1 stoicheiometry. On altering the conformation of the diazo group to that shown in Fig. 4, it is seen that the large Ca^{2+} ion is just big enough to fill the cavity produced (**B**), binding to the six O^- groups. The angles and lengths of the bonds are not regular, and this also militates against small-cation binding. Small cations, such as Mg^{2+}, tend to have six evenly spaced contacts in a closely regular octahedron, and readily bond directly to nitrogen, whereas Ca^{2+} has often as many as eight contacts of variable length and bond angle in its complexes and is less likely to bond to nitrogen (Williams, 1976). The charges are more concentrated in the structure in Fig. 4 (the compact form), since in Fig. 1 they are in pairs, i.e. the arsono groups are extended relative to those in Fig. 4. The structure in Fig. 4 explains both the 1:1 complex and the high selectivity. The polyamines which give 1:1 complexes give absorption spectra like that of the Mg^{2+} complex with a 610 nm peak only. They are long enough to bind to both wings of the extended molecule, but could not pack into the hole of the compact form. Spermine has higher affinity than the other polyamines, possibly because it is quadrivalent rather than tervalent.

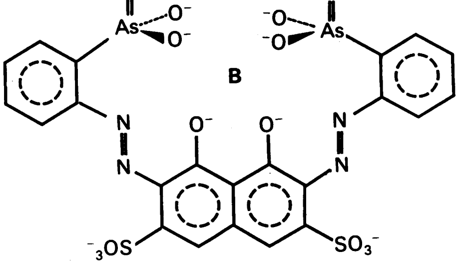

Fig. 4. Formula of arsenazo III in contracted conformation

Data are as for as Fig. 1. '**B**' marks the position that binds large cations. The distance between benzene rings is 0.989 nm (9.89 Å).

Received 1 November 1988/13 January 1989; accepted 19 January 1989

The present findings can be compared with others in the literature. The first observations concern the selectivity of Mg^{2+} and Ca^{2+} binding. Arsenazo III and the similar dye chlorophosphonazo III combine strongly with Ca^{2+}, the atom fitting between the phenol hydroxy and the arsono groups. Dyes such as Eriochrome Black T and Eriochrome Blue SE, which are selective for Mg^{2+}, have azo groups *ortho* to the phenolic groups (see Durham & Walton, 1983), in which the Mg^{2+} could bind to the nitrogen as well as to the oxygen functions. They cannot give a complex comparable with that shown above for Ca^{2+}–arsenazo III, since these reagents have no suitably placed oxyanions such as arsono groups.

Secondly, the competition between organic anions, such as spermine, and cations such as Mg^{2+} and Ca^{2+}, is of great interest. This paper shows that, while Ca^{2+} tends to concentrate charge around itself, as exemplified by arsenazo III in the compact form, the polyamines adjust themselves to fit into the extended form. It is our belief that much competition in biological systems between inorganic cations and polyamines is based on the relative stabilities of the cations with closed and extended polyanions, e.g. differently folded RNA or DNA.

E. R. thanks Professor J. R. Pattison (Department of Medical Microbiology, University College and Middlesex School of Medicine, London) for provision of facilities; Dr. E. J. Harris (Faculty of Clinical Sciences, University College, London) for suggesting the use of arsenazo III; Professor A. G. Sykes (University of Newcastle upon Tyne) for providing the complex cobalt salts; Dr. Conrad King (Department of Zoology, University College, London) for the loan of a Dore–Smith atomic-model kit; and Dr. John Stanford and members of the Department of Medical Microbiology, University College and Middlesex School of Medicine, for advice and support. E. R. is in receipt of a grant for chemicals and consumables from the Central Research Fund of the University of London.

REFERENCES

Buděšínský, B. (1963) Collect. Czech. Chem. Commun. **28**, 2902–2913

Documenta Geigy (1970) Scientific Tables (Diem, H. & Lentner, C., eds.), 7th edn., J. R. Geigy, Basle

Durham, A. C. H. & Walton, J. M. (1983) Cell Calcium **4**, 47–55

Phillips, C. S. G. & Williams, R. J. P. (1966) Inorganic Chemistry, Oxford University Press, Oxford

Rowatt, E. & Williams, R. J. P. (1985) Biochem. J. **231**, 765–768

Rowatt, E. & Williams, R. J. P. (1987) Biochem. J. **245**, 641–647

Savvin, S. B. (1961) Talanta **8**, 673–685

Tamm, S.-C. & Williams, R. J. P. (1985) Struct. Bonding (Berlin) **63**, 105–151

Williams, R. J. P. (1976) Symp. Soc. Exp. Biol. **30**, 1–17