Journal of Chromatography, 391 (1987) 67–77
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 309

ALLOWANCE FOR "ANALYTE VALENCE" IN THE RETENTION MODEL OF ION-EXCHANGE CHROMATOGRAPHY

STUDIES OF ADENOSINE 5'-PHOSPHATES ON DEAE CELLULOSE

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(First received June 27th 1986; revised manuscript received November 27th, 1986)

SUMMARY

Quantitative expressions are derived for various retention models of the ion-exchange behaviour of multivalent analytes. Their effectiveness as descriptions of experimental results is then tested by application to partition equilibrium studies of the effect of phosphate concentration on the interaction between the three adenosine 5'-phosphates and diethylaminoethylcellulose at pH 4.4. A completely general multi-state model has pointed to quantitative deficiencies in the currently accepted co-operative two-state model, which is, however, superior to a multi-state model devoid of cooperative effects. A similar situation pertains to the ion-exchange behaviour of cytochrome *c* on carboxymethylcellulose at pH 7.0.

INTRODUCTION

Retention models of ion-exchange chromatography[1–5] have in common a parameter for the number of sites (f) on the analyte (A) interacting with stationary phase sites (X). Although described as the analyte valence[1–4], this parameter does not necessarily refer to the overall net charge on the molecule. In one sense coordination number might be regarded as a more appropriate term for the parameter in question; but it is felt that more confusion would result from describing an analyte as being tetradentate rather than tetravalent, since adoption of the former adjective could mistakenly be taken to connote covalent bond formation. We shall therefore continue the practice[1–5], albeit open to criticism, of referring to f as the analyte valence, a term which at least conveys the freely reversible and electrostatic nature of the interactions between sites on analyte and ion-exchange resin.

Since the valence f, so defined, is seldom known in advance, there has been little attention paid to the validity of the assumption[1–5] that description of the analyte distribution requires consideration of only a single immobilized analyte species, AX_f. Anderson and Walters[6] recently drew attention to this potential deficiency of the retention models of chromatography by rediscovering the fact that in affinity chromatography it is necessary to consider the formation of AX_f as a stepwise process

0021-9673/87/$03.50

involving complexes with intermediate stoichiometries AX_i ($1 \leqq i < f$). From the outset[7–9] the need to consider all such complexes in quantitative treatments of affinity chromatography of multivalent solutes was recognized; and although the retention model was at one stage proposed to account for the NADH-dependent desorption of lactate dehydrogenases from 10-carboxydecylamino-Sepharose[10,11], the results were subsequently reappraised in terms of the more general model[12].

On the only occasion that the retention model of ion-exchange chromatography was subjected to any stringent test[3], results for the behaviour of guanosine 5′-phosphates (GMP, GDP, GTP) on an anion exchanger, Perisorb AN, were described reasonably well by this model with extreme cooperativity of analyte–matrix interactions. However, such a finding would provide much more compelling evidence if this extent of cooperativity were shown to be mandatory for description of the anion-exchange chromatographic behaviour of the nucleoside phosphates. The aims of the present investigation are (i) to develop more general theoretical expressions for ion-exchange chromatography of an analyte with f equivalent sites for interaction with matrix sites; (ii) to examine the interactions of the three adenosine 5′-phosphates (AMP, ADP, ATP) with diethylaminoethyl (DEAE)-cellulose by a recycling partition equilibrium technique[13], chosen because it allows the partition behaviour to be studied over a much wider range of eluting conditions than is practicable with conventional column chromatography; and (iii) to compare the relative merits of describing the results in terms of the newly developed and existing retention models of ion-exchange chromatography.

THEORY

Non-cooperative multi-state retention model

Consider the interaction of f-valent analyte, A, with univalent matrix sites, X, that occurs via ion-exchange in the presence of a uni-univalent electrolyte comprising analyte counterion C and non-counterion N, the successive equilibria being represented as

$$\begin{array}{rcrcl} AC_f & + & XN \rightleftarrows XAC_{f-1} & + & NC \\ XAC_{f-1} & + & XN \rightleftarrows X_2AC_{f-2} & + & NC \\ \cdot & & \cdot \quad \cdot & & \cdot \\ \cdot & & \cdot \quad \cdot & & \cdot \\ X_{f-1}AC & + & XN \rightleftarrows X_fA & + & NC \end{array} \qquad (1)$$

In addition, the solute–matrix interactions are considered to be governed by a single intrinsic association constant[14], k_{AX}, irrespective of whether the remaining A sites are occupied by X or C. By introducing a parameter P_{AX} to denote the probability that an A site has reacted with a matrix site, this intrinsic association constant may be written in terms of total solute concentration [$\bar{A}$] as

$$k_{AX} = \frac{P_{AX} f [\bar{A}][NC]}{(1 - P_{AX}) f [\bar{A}][XN]} \cong \frac{P_{AX}[NC]}{(1 - P_{AX})[\bar{X}]} \qquad (2)$$

the simplification that [XN] $\cong$ [$\bar{X}$] being made[15] on the grounds that the analyte

concentration is much smaller than the effective total concentration of ion-exchange sites, $[\bar{X}]$. From reacted-site probability theory[16,17], the liquid-phase concentration of solute ($[AC_f]$) is related to its total concentration ($[\bar{A}]$) by the expression

$$[\bar{A}] = [AC_f]/(1 - P_{AX})^f \tag{3}$$

Eqns. 2 and 3 may be combined and rearranged[15] to give

$$\{1 - ([AC_f]/[\bar{A}])^{1/f}\}/([AC_f]/[\bar{A}])^{1/f} = k_{AX}[\bar{X}]/[NC] \tag{4a}$$

or, in logarithmic and simplified form,

$$\log \{([\bar{A}]/[AC_f])^{1/f} - 1\} = \log k_{AX}[\bar{X}] - \log [NC] \tag{4b}$$

Conformity of experimental data with this situation is thus recognized by a slope of -1 for a plot of the left-hand side of eqn. 4b *versus* the logarithm of the uniunivalent electrolyte concentration.

Adaptation of eqn. 4b for analysis of column chromatographic data is effected by noting[18] that $V^*[\bar{A}] = \bar{V}[AC_f]$, where V^* is the elution volume of A in the absence of any interaction with matrix, and $\bar{V}$ is the elution volume in the presence of a concentration [NC] of uniunivalent electrolyte. Thus

$$[\bar{A}]/[AC_f] = \bar{V}/V^* = t_r/t_0 \tag{5}$$

where t_r and t_0 are the respective retention times[3–6] corresponding to $\bar{V}$ and V^*. In acquisition of results by the recycling partition equilibrium procedure[8,13,18], V^* refers to the volume of liquid phase, which changes, however, due to successive additions of concentrated NC solution. The consequent change in effective matrix-site concentration is countered by expressing $[\bar{X}]$ in terms of its initial value. On making the substitution $[\bar{X}] = (V_0^*/V^*)[\bar{X}]_0$, where zero subscripts now refer to intitial values, eqn. 4b becomes

$$\log [V^*\{([\bar{A}]/[AC_f])^{1/f} - 1\}] = \log (V_0^* k_{AX}[\bar{X}]_0) - \log [NC] \tag{6}$$

which again predicts a linear plot of the left-hand side *versus* log [NC].

Two-state retention model

The previous approach to the retention model of chromatography has been to consider that analyte is distributed between two states, the soluble form (AC_f) and a single immobilized species (AX_f). In these terms the analogue of eqn. 1 is

$$AC_f + fXN \rightleftarrows AX_f + fNC \tag{7a}$$

for which an equilibrium constant may be written as

$$K = [AX_f][NC]^f/[AC_f][XN]^f \tag{7b}$$

As before, we consider that the total concentration of matrix sites to approximate the corresponding free concentration, whereupon it follows that

$$[\bar{A}] = [AC_f] + [AX_f] = [AC_f]\{1 + (K[\bar{X}]^f/[NC]^f)\} \tag{8a}$$

of which a convenient logarithmic transform is

$$\log \{([\bar{A}]/[AC_f]) - 1\} = \log (K[\bar{X}]^f) - f \log [NC] \tag{8b}$$

Thus a plot of $\log \{([\bar{A}]/[AC_f]) - 1\}$ *versus* log [NC] has a slope of $-f$ in the event that this model pertains to ion-exchange chromatography of the analyte. Inasmuch as $K[\bar{X}]^f$ is a constant, we note that eqn. 8b is formally identical with the expression used in earlier studies[3,6] for the determination of valence from a plot of $\log \{(t_r/t_0) - 1\}$ *versus* $\log (1/[NC])$; but that others[4,5] have considered the slope to define $-2f$.

Adaptation of eqn. 8b to the situation pertaining in recycling partition equilibrium studies again requires expression of $[\bar{X}]$ in terms of its initial value, the expression analogous to eqn. 8b being

$$\log [(V^*)^f\{([\bar{A}]/[AC_f]) - 1\}] = \log \{K(V_0^*[\bar{X}]_0)^f\} - f \log [NC] \tag{9}$$

Eqn. 9 describes an inverse linear relationship between $\log [(V^*)^f\{([\bar{A}]/[AC_f]) - 1\}]$ and log [NC] in the event that the ion-exchange chromatographic behaviour of the analyte conforms with the two-state model.

Multistate retention model with cooperativity

Finally we return to the possibility that ion-exchange chromatography of a multivalent analyte requires description in terms of the successive equilibria presented in eqn. 1; but now make allowance for cooperativity of binding by removing the previous restraint that all steps be governed by the same intrinsic binding constant. The approach is illustrated by consideration of divalent and trivalent analytes.

For a divalent analyte the respective concentrations of singly-linked (ACX) and doubly-linked (AX_2) analyte–matrix complexes are considered to be given by the relationships

$$[ACX] = 2k_{AX}[AC_2][XN]/[NC] \tag{10a}$$

$$[AX_2] = Qk_{AX}^2[AC_2][XN]^2/[NC]^2 \tag{10b}$$

The total ($[\bar{A}]$) and free ($[AC_2]$) concentrations of analyte are then related by the expression

$$[\bar{A}] = [AC_2]\{1 + (2k_{AX}[XN]/[NC]) + (Qk_{AX}^2[XN]^2/[NC]^2)\} \tag{10c}$$

in which the parameter Q (a constant) has been introduced to allow the intrinsic binding constant for the second interaction of an analyte molecule with matrix (Qk_{AX}) to differ from that for the first (k_{AX}). After substituting $[\bar{X}]$ for [XN], as before, eqn. 10c is readily transformed to

$$[NC]\{([\bar{A}]/[AC_2]) - 1\} = 2k_{AX}[\bar{X}] + Q(k_{AX}[\bar{X}])^2/[NC] \quad (11a)$$

or, in the case of recycling partition equilibrium (where $[\bar{X}] = V_0^*[\bar{X}]_0/V^*$, to

$$V^*[NC]\{([\bar{A}]/[AC_2]) - 1\} = 2k_{AX}V_0^*[\bar{X}]_0 + \{Q(k_{AX}V_0^*[\bar{X}]_0)^2/V^*[NC])\} \quad (11b)$$

In the latter type of experiment a plot of $V^*[NC]\{([\bar{A}]/[AC_2]) - 1\}$ *versus* $1/(V^*[NC])$ should thus be linear with an ordinate intercept of $2k_{AX}V_0^*[\bar{X}]_0$ and a slope of $Q(k_{AX}V_0^*[\bar{X}]_0)^2$; and hence allow evaluation of Q, the ratio of intrinsic binding constants.

For a trivalent analyte the concentrations of the three analyte–matrix complexes are considered to be given by the expressions

$$[AC_2X] = 3k_{AX}[AC_3][XN]/[NC] \quad (12a)$$

$$[ACX_2] = 3Qk_{AX}^2[AC_3][XN]^2/[NC]^2 \quad (12b)$$

$$[AX_3] = Rk_{AX}^3[AC_3][XN]^3/[NC]^3 \quad (12c)$$

The relationship between total ($[\bar{A}]$) and free ($[AC_3]$) concentrations of analyte then becomes

$$[\bar{A}] = [AC_3]\{1 + (3k_{AX}[XN]/[NC]) + (3Qk_{AX}^2[XN]^2/[NC]^2) + \\ + (Rk_{AX}^3[XN]^3/[NC]^3)\} \quad (12d)$$

in which the additional constant, R, is introduced to allow all three successive interactions of analyte with matrix to be governed by different intrinsic association constants. The expressions analogous to eqns. 11a and 11b respectively then become

$$[NC]\{([\bar{A}]/[AC_3]) - 1\} = 3k_{AX}[\bar{X}] + \{3Q(k_{AX}[\bar{X}])^2/[NC])\} + \\ + \{R(k_{AX}[\bar{X}])^3/[NC]^2\} \quad (13a)$$

$$V^*[NC]\{([\bar{A}]/[AC_3]) - 1\} = 3(k_{AX}V_0^*[\bar{X}]_0) + \\ + \{3Q(k_{AX}V_0^*[\bar{X}]_0)^2/(V^*[NC])\} + \{R(k_{AX}V_0^*[\bar{X}]_0)^3/(V^*[NC])^2\} \quad (13b)$$

Provided that a value of $3k_{AX}V_0^*[\bar{X}]_0$ may be obtained in a recycling partition equilibrium experiment from the ordinate intercept of the curvilinear plot of $V^*[NC]\{([\bar{A}]/[AC_3]) - 1\}$ *versus* $1/(V^*[NC])$, eqn. 13b may be linearized by the following transformation.

$$V^*[NC](V^*[NC]\{([\bar{A}]/[AC_3]) - 1\} - 3k_{AX}V_0^*[\bar{X}]_0) \\ = 3Q(k_{AX}V_0^*[\bar{X}]_0)^2 + \{R(k_{AX}V_0^*[\bar{X}]_0)^3/(V^*[NC])\} \quad (14)$$

Thus a plot of the left-hand side of eqn. 14 *versus* $1/(V^*[NC])$ is linear with an ordinate intercept of $3Q(k_{AX}V_0^*[\bar{X}]_0)^2$ and a slope of $R(k_{AX}V_0^*[\bar{X}]_0)^3$: the cooperativity constants, Q and R, may therefore be determined by combining these two values with the estimate of $3k_{AX}V_0^*[\bar{X}]_0$ used for their delineation.

EXPERIMENTAL

Materials

Diethylaminoethylcellulose (DEAE-cellulose) and carboxymethylcellulose (CM-cellulose) were obtained in microgranular form (DE52 and CM52 respectively) from Whatman Biochemicals (Maidstone, U.K.). Horse-heart cytochrome *c* (Type III), adenosine 5'-monophosphate (AMP; Type II), adenosine 5'-diphosphate (ADP; Grade I) and adenosine 5'-triphosphate (ATP; Grade I) were all products of Sigma (St. Louis, MO, U.S.A.). All other chemicals were of reagent grade.

Prior to use, both cellulosic ion-exchangers were pretreated with 0.5 *M* hydrochloric acid and 0.5 *M* sodium hydroxide in accordance with the manufacturer's recommendation; and then washed with 4 *M* sodium chloride. The DEAE-cellulose was then equilibrated with 10 m*M* sodium dihydrogen phosphate (pH 4.4), and the CM-cellulose with 10 m*M* phosphate buffer (6.1 m*M* Na_2HPO_4–3.9 m*M* NaH_2PO_4), pH 7.0. Between experiments the ion exchangers were washed with 4 *M* sodium chloride to ensure complete removal of all analyte, and then re-equilibrated with the appropriate phosphate medium.

Cytochrome *c* was dissolved in the 10 m*M* phosphate buffer (pH 7.0) and then dialyzed for 24 h at 4°C against more of the same buffer, the final diffusate being used to equilibrate the CM-cellulose for the partition equilibrium study. Solutions of the three adenosine phosphates were prepared by direct dissolution of the nucleotides in 10 m*M* sodium dihydrogen phosphate (pH 4.4) in readiness for partition equilibrium studies using DEAE-cellulose.

Partition equilibrium studies

The effect of univalent phosphate (NaH_2PO_4) on the affinity of DEAE-cellulose for the three nucleoside phosphates (AMP, ADP, ATP) was studied at 20°C by a recycling partition equilibrium procedure[8,13,18] in which the liquid phase of a stirred slurry of ion-exchanger and analyte was analyzed spectrophotometrically at 260 nm by means of a flow cell placed in the line returning the liquid phase to the slurry. A peristaltic pump was used to maintain a flow-rate of 3 ml/min, and an overhead stirrer used to ensure adequate mixing of the slurry. To a stirred mixture containing 5 g DEAE-cellulose in 20–30 ml of 10 m*M* sodium dihydrogen phosphate (pH 4.4) was added nucleoside phosphate (500 μl, 4–5 m*M*) followed by aliquots (100–500 μl) of concentrated (2 *M*) sodium dihydrogen phosphate, sufficient time (approximately 25 min) being allowed between successive additions for attainment of a constant absorbance reading. At the stage when the absorbance began to decrease upon addition of further phosphate solution, a large volume (20 ml) of the concentrated sodium dihydrogen phosphate solution was added to displace all remaining adsorbed analyte. The resulting absorbance reading was then combined with the amount of nucleotide added to determine the appropriate value of V^* (the volume of liquid phase) corresponding to each partition equilibrium experiment.

A second partition equilibrium study at 20°C employed CM-cellulose (5 g in 20 ml of 10 m*M* phosphate buffer, pH 7.0) as the ion-exchanger and cytochrome *c* (700 μl, 1.0 m*M*) as analyte, displacement being effected by addition of aliquots (100–500 μl) of 2 *M* phosphate buffer (1.22 *M* Na_2HPO_4–0.78 *M* NaH_2PO_4), pH 7.0. In these experiments the flow-rate was maintained at 5 ml/min, and the liquid phase monitored at 280 nm. As above, a large volume (20 ml) of the concentrated phosphate buffer was added to achieve total displacement of cytochrome *c* and hence allow determination of the liquid phase volume (V^*) appropriate to each partition measurement.

RESULTS AND DISCUSSION

Interactions of nucleoside phosphates with DEAE-cellulose

To test the relative merits of the various retention models of ion-exchange chromatography a recycling partition procedure[8,13] was used to determine the effect of sodium dihydrogen phosphate concentration on the interactions between DEAE-cellulose and the three adenosine 5′-phosphates at pH 4.4, conditions under which pH-titration and electrophoretic studies[19,20] have shown that these analytes should act in "univalent" (AMP), "divalent" (ADP) and "trivalent" (ATP) manner. Fig. 1 summarizes analyses of the results in accordance with the two-state retention model (eqn. 9), which was considered[3] to describe adequately the column chromatographic behaviour of the corresponding guanosine 5′-phosphates on the anion-exchanger Perisorb AN. In agreement with those earlier findings (Fig. 3 of ref. 3), these plots are essentially linear; but only in the case of AMP is the slope in reasonable agreement with the predicted value (Table I). Although Jandera *et al.*[3] considered their results for GDP and GTP to also be characterized by the required slope, those conclusions

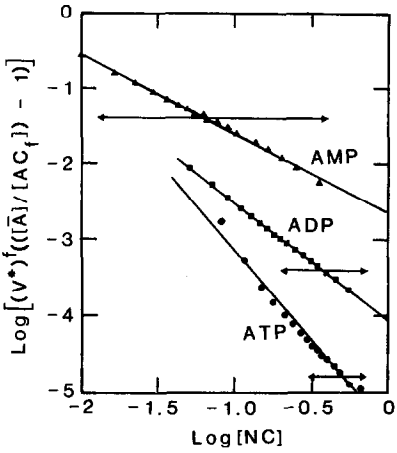

Fig. 1. Effect of NaH_2PO_4 concentration on the interaction of adenosine 5′-phosphates with DEAE-cellulose (pH 4.4). Results of partition equilibrium measurements are plotted in accordance with eqn. 9, the quantitative expression for the two-state retention model of ion-exchange chromatography: analyte valence (*f*) is taken as unity for AMP (▲), two for ADP (■) and three for ATP (●). Horizontal arrows denote the range of phosphate concentration used by Jandera *et al.*[3] in corresponding column chromatographic studies with guanosine 5′-phosphates on Perisorb AN.

TABLE I

COMPARISON OF EXPERIMENTAL AND PREDICTED EFFECTS OF A UNIUNIVALENT ELECTROLYTE (NaH_2PO_4) ON THE ELUTION OF ADENOSINE 5'-PHOSPHATES FROM DEAE-CELLULOSE AT pH 4.4

Results of recycling partition equilibrium experiments on the three nucleotides were tested for conformity with the two-state and non-cooperative multi-state models of ion-exchange chromatography by plotting results in accordance with eqns. 6 and 9 respectively. Reported slopes and uncertainties (±2 S.E.M.) inherent therein were obtained by least-squares calculations. Numbers in parentheses denote predicted values.

Analyte	*Valence (f)*	*Slope of relevant plot*	
		Two-state model	*Multi-state model*
AMP	1	−1.06 ± 0.03 (−1.0)*	−1.06 ± 0.03 (−1.0)*
ADP	2	−1.54 ± 0.01 (−2.0)	−1.30 ± 0.06 (−1.0)
ATP	3	−2.36 ± 0.15 (−3.0)	−1.76 ± 0.08 (−1.0)

* Models are identical for a univalent analyte.

do not withstand the more stringent test afforded by the present partition results over a much wider range of sodium dihydrogen phosphate concentration (Fig. 1).

Table I also summarizes tests of the results for conformity with the non-cooperative multi-state retention model (eqn. 6); and again only the results for AMP are characterized by the required slope. In that regard the adequacy of description by both the two-state and the multi-state models is mandatory, since both describe the same situation for a univalent analyte. However, the non-cooperative multi-state model yields experimental slopes for ADP and ATP that are also in poor agreement with the values predicted by eqn. 6. It is therefore necessary to consider the results for these two multivalent analytes in terms of the completely general multi-state model in which no restrictions are placed on the magnitudes of binding constants for successive interactions of an analyte molecule with ion-exchange sites.

Analysis of the partition results for ADP in accordance with eqn. 11b is shown in Fig. 2a. Least-squares calculations yield an ordinate intercept of 3.6 ± 0.3 mmol and a slope of (2.17 ± 0.12) · 10^{-5}: a value of 6.7 ± 1.5 for Q is thereby indicated. The concentration of doubly-bound ADP (AX_2) is thus some seven-fold greater than that predicted by the simple multi-state model. From the thermodynamic viewpoint it is immaterial whether this finding is regarded as signifying the existence of co-operative binding (intrinsic constant a function of site-occupancy) or a requirement for description of the second interaction between analyte and matrix in terms of localized surface concentrations instead of their bulk solution counterparts. The important point to emerge from this thermodynamic analysis of the ion-exchange behaviour of ADP on DEAE-cellulose is the existence of a finite proportion of the divalent analyte as singly-linked complex AXC, a species neglected in the two-state model but overemphasized in the simple multi-state model involving only a single intrinsic binding constant.

Fig. 2b presents a plot of results for ATP in the format suggested by eqn. 14, for which a value of $3k_{AX}V_0^*[\bar{X}]_0$ was first required to determine the ordinate parameter appropriate to each $1/(V^*[NC])$ value. Since the curvilinear plot of

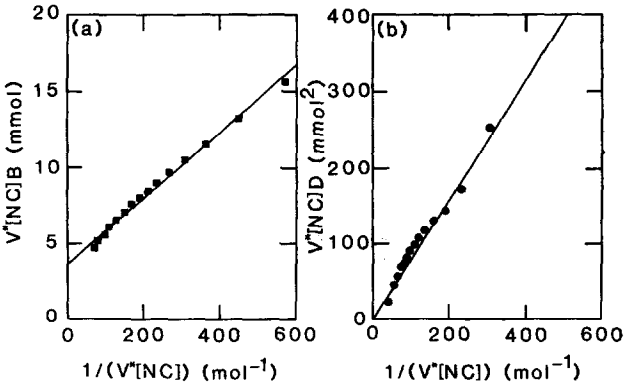

Fig. 2. Analysis of the ion-exchange behaviour of ADP and ATP on DEAE-cellulose (pH 4.4) by means of the general retention model. (a) Partition equilibrium results for ADP (a divalent analyte) plotted in accordance with eqn. 11b ($B = ([\bar{A}]/[AC_2]) - 1$). (b) Plot of corresponding results for ATP (a trivalent analyte) according to eqn. 14 with $D = V^*[NC]\{([\bar{A}]/[AC_3]) - 1\} - 3k_{AX}V_0^*[\bar{X}]_0$ and $k_{AX}V_0^*[\bar{X}]_0$ taken as 1.8 mmol (see text).

$V^*[NC]\{([\bar{A}]/[AC_3]) - 1\}$ *versus* $1/(V^*[NC])$ (eqn. 13b) extrapolated to an ordinate intercept ($3k_{AX}V_0^*[\bar{X}]_0$) in the vicinity of 5 mmol, $k_{AX}V_0^*[\bar{X}]_0$ was taken as 1.8 mmol, the corresponding value for ADP with the same batch of DEAE-cellulose (Fig. 2a). Least-squares calculations on the results shown in Fig. 2b yield an ordinateintercept, $3Q(k_{AX}V_0^*[\bar{X}]_0)^2$, of $(0.007 \pm 0.011) \cdot 10^{-3}$ mol², and a slope, $R(k_{AX}V_0^*[\bar{X}]_0)^3$, of $(7.8 \pm 0.8) \cdot 10^{-7}$ mol³. From eqn. 14 $Q = 0.7 \pm 1.1$ and $R = 133 \pm 14$, the value of Q being indistinguishable experimentally from zero. This completely general analysis thus leads to the conclusion that the ion-exchange behaviour of ATP on DEAE-cellulose at pH 4.4 requires description in terms of a three-state (AC_3, AXC_2 and AX_3) retention model, the subsequent interactions after initial binding (to form AXC_2) being sufficiently cooperative for the concentration of doubly-linked analyte–matrix species (AX_2C) to be effectively zero. This observation coupled with the large value of R signifies preponderance of AX_3 species, which accounts for the observation (Table I) that the system is described better (though not well) by the two-state model than by the simple multi-state model with a statistical distribution of all four analyte states.

Interaction of cytochrome c with CM-cellulose

Although results obtained with ADP and ATP indicate the likelihood of a relatively large extent of cooperativity in interactions between multivalent analytes and ion-exchangers, the potential for greater spacing of the charged groups on a protein molecule could well modify the situation in ion-exchange chromatography of protein analytes. This aspect has been explored by examining the interaction of cytochrome *c* with CM-cellulose at pH 7.0, a system similar to that which led Boardman and Partridge[1] to postulate the two-state model of ion-exchange chromatography. Fig. 3 summarizes the effect of sodium ion concentration on the distribution of cytochrome *c*, the results being plotted in accordance with the two-state model: although eqn. 9 refers specifically to effects of a uniunivalent electrolyte NC, the "valence" of the non-counterion does not appear to influence unduly the chromato-

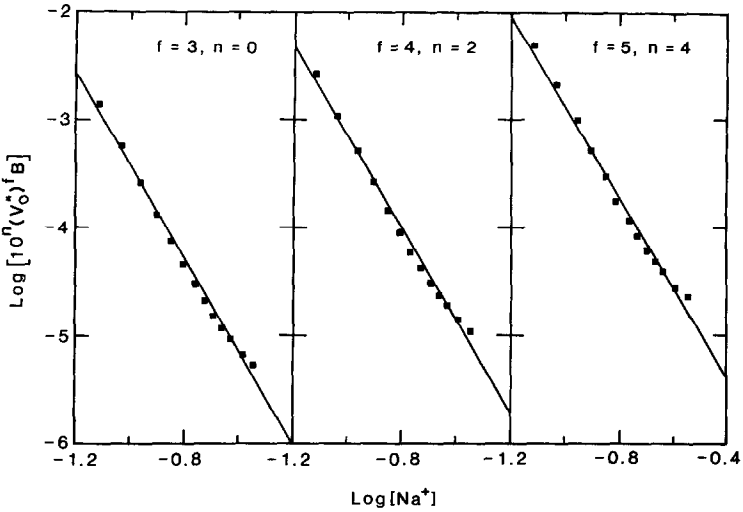

Fig. 3. Test of partition equilibrium results for the CM-cellulose–cytochrome *c* system (pH 7.0) for conformity with the two-state retention model of ion-exchange chromatography. Results are plotted according to eqn. 9 with the indicated values of analyte valence (*f*) and *n*, a parameter introduced to effect vertical displacement of plots for use of a common ordinate scale.

graphic behaviour of cytochrome *c* on cation-exchangers at this pH[1]. For cytochrome *c*, the "valence", *i.e.*, the effective number of cationic protein sites involved, is not known; and accordingly plots for a range of *f* values (3–5) are presented. Whereas the results could not be reconciled with the non-cooperative multi-state model for any value of *f*, Fig. 3 indicates reasonable conformity of the results to a system with $f = 4$ inasmuch as the experimental slope (-4.2 ± 0.3) matches fairly closely the value of *f* used for its evaluation. From that viewpoint trivalency of cytochrome *c* is certainly precluded (slope = -4.3 ± 0.3), whereas pentavalency would imply a lesser degree of cooperativity (slope = -4.2 ± 0.3). Since resort to the general multi-state retention model becomes impracticable for analytes with $f > 3$, it is concluded that the ion-exchange behaviour of cytochrome *c* on CM-cellulose at pH 7.0 is reasonably rationalized on the basis of largely cooperative interactions between matrix-bound carboxymethyl groups and a small cluster of four or five basic groups on the protein. Inspection of the three-dimensional structure of horse-heart cytochrome *c*[21] indicates the sequence of residues 86–91 (lys-lys-lys-thr-glu-arg) as a likely cluster of cationic sites responsible for the ion-exchange behaviour of this protein on CM-cellulose (Fig. 3) and, presumably, on IRC-50 (Fig. 3 of ref. 1) at neutral pH.

CONCLUSIONS

This examination of the effectiveness of various retention models to describe the ion-exchange behaviour of adenosine 5′-phosphates on DEAE-cellulose at pH 4.4 has pointed to quantitative deficiencies in the currently accepted two-state model for multivalent analytes, based on extreme cooperativity of binding such that only a single immobilized species, AX_f, exists. However, from the viewpoint of providing an approximate estimate of effective analyte valence, the two-state model is clearly

superior to the non-cooperative multi-state model for the adenosine phosphates (Table I) and also cytochrome *c* (Fig. 3). In this regard we note that the present theoretical and experimental findings confirm that the slope of the two-state plot should be identified with the valence, *f*, a conclusion reached by Jandera *et al.*[3]; but disputed by others[4,5], who have evaluated effective protein valences on the basis that the slope defines 2*f*. Undoubtedly the major contributions of this investigation have been the development of the general retention model of ion-exchange chromatography for divalent and trivalent analytes; and the demonstration that its application to the ion-exchange behaviour of nucleoside phosphates on DEAE-cellulose rationalizes use of the two-state model for obtaining approximate (minimal) estimates of analyte valence.

ACKNOWLEDGEMENTS

The partial support of this investigation by the Australian Research Grants Scheme is gratefully acknowledged, as is the receipt (by D.A.B.) of a Commonwealth Postgraduate Research Award.

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