Journal of Chromatography, 186 (1979) 371–403
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CHROM. 11,984

MEASUREMENT OF ASSOCIATION CONSTANTS FOR COMPLEXES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

CSABA HORVÁTH, WAYNE MELANDER and AVI NAHUM

Chemical Engineering Group, Department of Engineering and Applied Science, Yale University, New Haven, Conn. 06520 (U.S.A.)

SUMMARY

Association constants for metal-binding by various nucleotides, crown ethers and nitroso-naphtholsulfonic acids in solution were measured by high-performance liquid chromatography employing appropriate eluents and non-polar bonded stationary phases. The results are in excellent agreement with pertinent literature data. The procedure is applicable to the measurement of stability constants of complexes which contain one solute molecule and are formed in the solution used as the eluent, provided species bound to the stationary phase are not involved in the complex formation and the chromatographic system is linear. The concentration of the metal ion in the appropriate mobile phase was varied and small quantities of the above substances were injected into the column. The hyperbolic dependence of the retention factor, k, on the concentration of Na^+, K^+, Mg^{2+} or Zn^{2+} in the eluent allowed the use of various linear plots for the evaluation of the stability constants for complexes formed by such ions with substances mentioned above. The effect of complexation on retention is conveniently measured by the retention modulus, η, which is given for the complex, η_c, by the ratio of the retention factors of the complex and the uncomplexed solute, both measured under otherwise identical conditions. With nucleotides chromatographic retention increases upon metal binding due to reduction of electronic charge on the molecule and $\eta > 1$. On the other hand nitroso-naphtholsulfonates and crown ethers eluted faster in the form of a complex with metal ions so that $\eta < 1$. Theoretical and practical limitations of the chromatographic approach to evaluation of stability constants are extensively discussed and tests are proposed to assure the applicability of the method to a given system.

INTRODUCTION

Recently, significant advances have been made toward understanding the physico-chemical basis of solute retention in reversed-phase high-performance liquid chromatography (HPLC)[1]. It has been shown that the effect of solvent is predominant in determining solute interactions with the stationary phase, and can be solely responsible for the differences in the retention of closely related substances[2,3]. Thus, measurement of retention values in reversed-phase HPLC under appropriate con-

ditions should give quantitative information on certain physico-chemical phenomena which take place in solution[4,5]. The usefulness of this approach has already been demonstrated by chromatographic evaluation of protonic dissociation constants[4]. The present work is an attempt to augment this concept and to broaden the scope of physico-chemical measurements by using HPLC for the quantitative evaluation of equilibrium constants for metal binding by various substances.

A wide variety of techniques has been described for the determination of stability constants of such complexes[6,7]. Spectroscopic methods and titration are by far the most popular whereas electrochemical techniques and calorimetry find less extensive use. Sometimes distribution methods based on extraction or ion-exchange are also employed. These traditional methods all share the requirements that complex-forming substances be available in highly pure form and have sufficiently high solubility in the liquid medium used in the investigation. Such constraints play a much lesser role in the measurement of equilibrium constants by gas chromatography and electrophoresis, which has been reviewed recently[8]. On the other hand with these methods the substances investigated have to be volatile and special experimental skills of the researcher are needed, respectively. Whereas exclusion chromatography has been employed in an ingenious way to obtain association constant of complexes involving relatively large and polar molecules[9,10] our literature search has yielded only one report that employed retention data obtained in liquid chromatography for the measurement of stability constants of complexes by ion-exchange paper chromatography[11].

Precision instruments with highly sensitive detectors, which are presently available in HPLC, facilitate rapid gathering of highly accurate retention data. Reversed-phase HPLC employing columns packed with "inert", non-polar stationary phase of relatively uniform surface appears to be particularly suitable to study equilibrium phenomena which take place in solution, *i.e.*, in the mobile phase. In many respects the physico-chemical basis of reversed-phase HPLC is similar to that of extraction that has also been employed for the measurement of stability constants. In fact both processes feature solute distribution between a polar and a non-polar phase. Reversed-phase HPLC with bonded hydrocarbonaceous stationary phases, however, has the advantage over liquid–liquid partition systems used in extraction that the phases can be considered to be completely immiscible. Whereas the chromatographic technique offers convenience and accuracy, it has the disadvantage that the properties of bonded stationary phases are still ill-defined and the presently available column materials do not always behave as completely inert non-polar phases. Therefore, as discussed later, certain tests are mandatory to assure that the chromatographic system under consideration is suitable for the measurement of certain type of stability constants.

Naturally, solute complexation in the eluent is frequently used in analytical work to manipulate the selectivity of liquid chromatographic systems[12] and thereby improve resolution. The employment of such secondary equilibria is a particularly powerful tool in reversed-phase HPLC where retention is governed by solute–solvent interactions. Correspondingly, protonic equilibria and "ion-pair" formation between solutes carrying electronic charge and oppositely charged "hydrophobic" ions[4,13] are widely used to adjust the retention of ionizable eluites. We have the term "hetaeron", the Greek word for companion (*ἑταῖρον*), for the complexing agent added to the

eluent in order to denote an intentional use of secondary equilibria for the modulation of selectivity in reversed-phase chromatography[5].

The direct use of metal ions to form complexes in the eluent and thereby modulate selectivity was first explored in ion-exchange chromatography[14,15]. At present the method is rapidly gaining importance in reversed-phase HPLC. In certain cases, such as when charge-transfer complexes are formed[16], the metal complex has a greater polarity and elutes faster than the non-complexed solute[17]. Thus, Ag^+ complexes of various unsaturated and heterocyclic solutes have been successfully used to enhance the selectivity of the separation[18]. In other cases, however, metal complexation may result in a reduction of the electronic charge on the eluite molecule with a concommitant increase in retention on non-polar stationary phases. It should be pointed out that this approach is different from ligand-exchange chromatography[19], where the metal is anchored to, and therefore is a part of, the stationary phase and its variants[20].

The aim of the present investigation is to demonstrate that complexation of the solute with a hetaeron in the mobile phase is not only useful to change selectivity and thereby facilitate separation, but also allows chromatographic measurement of the pertinent stability constant, provided stationary phase properties are independent of the hetaeron concentration in the eluent. As with most other methods, when different complexes are present and they are rapidly interconverted on the time scale of the chromatographic separation, an average formation constant is obtained.

THEORY

Phenomenological model: effect of hetaeron concentration

In the simplest case *one* solute molecule, S, forms a complex with *one* hetaeron molecule, H, and the equilibria involved in the chromatographic process can be represented by the following scheme

$$\begin{array}{ccccc} S + H & \overset{K}{\rightleftharpoons} & SH & \overset{K}{\rightleftharpoons} & H + S \\ \Big\downarrow\uparrow k_0 & & \Big\downarrow\uparrow k_c & & \Big\downarrow\uparrow k_h \\ S^* + H & \overset{K_1^*}{\rightleftharpoons} & (SH)^* & \overset{K_2^*}{\rightleftharpoons} & H^* + S \end{array}$$

where the asterisk denotes that the species are bound to the stationary phase. The respective stability constants of the complex in the mobile phase and on the stationary phase are K, K_1^* and K_2^*. The retention (capacity) factors for the free solute, the hetaeron and the complex, are denoted by k_0, k_h and k_c, respectively. The equilibrium constant for binding the hetaeron to the stationary phase, K_h, is related to k_h by

$$k_h = \varphi K_h \tag{1}$$

where φ is the phase ratio of the column. In reversed-phase chromatography with bonded phases φ can be related to the "concentration" of the hydrocarbonaceous ligates, such as the stearyl functions in octadecyl-silica, which are accessible to the solute molecules, in the column.

According to a previous more detailed treatment of a similar set of chromatographic equilibria[5] the dependence of the retention factor for the eluite, k, on the hetaeron concentration, $[H]$ can be expressed by

$$k = \frac{k_0 + k_c K[H]}{1 + K[H]} \tag{2}$$

provided that (i) the concentration of the free solute is much smaller than $[H]$, (ii) only a small fraction of the stationary phase surface, *i.e.*, the accessible ligates on the surface of a bonded phase, is occupied by the bound eluite, and (iii) that solute interaction with the bound hetaeron is relatively small, that is, $K_2^*[H] \ll 1$. It is seen from eqn. 2 that by measuring the retention factor as a function of the hetaeron concentration, the stability constant, k, can be evaluated provided the retention factors of the free solute and complex are known.

Alternatively, when $K[H] \ll 1$, retention of the eluite can be expressed by the following equation

$$k = \frac{k_0 + k_h K_2^*[H]}{1 + K_2^*[H]} \tag{3}$$

where K_2^* is the stability constant of the complex formed by the solute with the hetaeron bound to the stationary phase and k_h is the retention factor of the hetaeron.

Both eqns. 2 and 3 represent limiting cases as far as the mechanism of the retention process is concerned and reflect a hyperbolic dependence of the retardation factor for the eluite on the hetaeron concentration in the eluent. When such a limiting behavior is observed, then either eqn. 2 or eqn. 3 can describe the system depending on the mechanism of the retention process. Therefore in any attempt to evaluate the stability constant, K, by using eqn. 2 we must make sure that not only the dependence of k on $[H]$ is hyperbolic but also that the mechanism of the chromatographic process is represented by eqn. 2 and not by eqn. 3.

Eqn. 1 can be generalized for the case, when the solute molecule has n binding sites and the complex contains j monovalent hetaeron species, as follows

$$k = \frac{k_0 + \sum_{i=1}^{n} k_i [H]^i \prod_{j=1}^{i} K_j}{1 + \sum_{i=1}^{n} [H]^i \prod_{j=1}^{i} K_j} \tag{4}$$

where k_0 is the retention factor of the uncomplexed solute and k_i is the retention factor of a complex containing i hetaeron. The stability constant of the complex containing j hetaeron is K_j and the total number of hetaerons that can bind is n.

If the differences between the k_i and k_j values for a given multi-complex are sufficiently large, eqn. 4 can be used to evaluate the parameters. More than often, however, the conditions do not allow the extraction of accurate stability constants

when $n > j > 3$. Nevertheless a recent study on the retention behavior of pteroyl-oligo-γ-L-glutamates in reversed-phase chromatography[21] showed that the appropriate form of eqn. 4 could be used to estimate the protonation constants of the α-carboxylic groups in molecules containing up to seven glutamyl moieties.

Plots of retention factor against the dimensionless hetaeron concentration according to eqn. 2 yield rectangular hyperbolas such as that depicted in Fig. 1A for the case of $k_c > k_0$. When $K[H] = 0$ there is no complex formation and the retention factor is that of the free solute, k_0. For $K = 1/[H]$ the hetaeron concentration equals the dissociation constant of the complex so that $K[H] = 1$ and the retardation ratio of the eluite is given by $(k_c + k_0)/2$. Thus, the hetaeron concentration that yields the mean value of the retardation factor is numerically equal to the stability constant. The upper limiting value of the retardation factor, k_c, is obtained at sufficiently high hetaeron concentrations, $K[H] \gg 1$, when practically all eluite is present in the form of the complex.

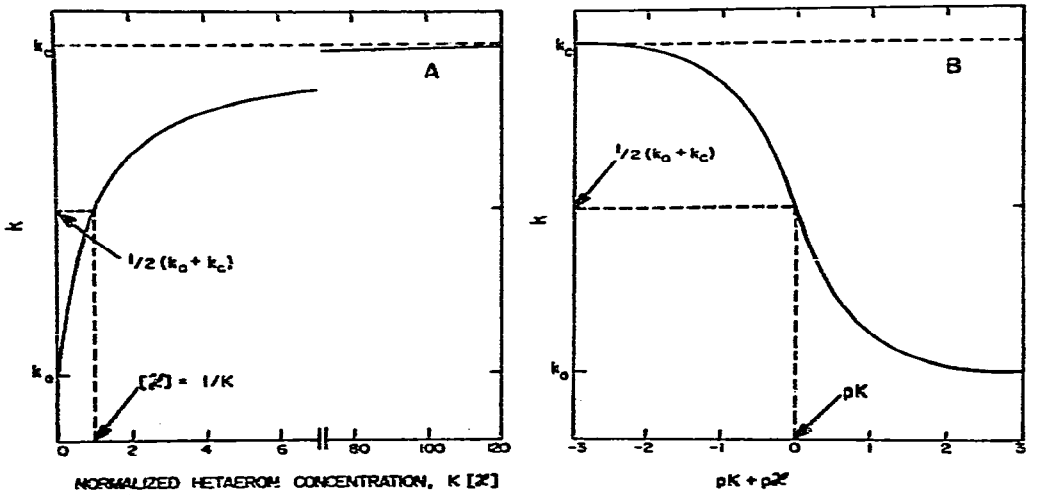


Fig. 1. A, Schematic illustration of the hyperbolic dependence of the retention factor, k, on the concentration of complexing agent (hetaeron) in the mobile phase, $[H]$, according to eqn. 2. The retention factor obtained at the hetaeron concentration that is numerically equal to the reciprocal of the stability constant of the complex, $1/K$, is indicated. The limiting retention factors k_0 and k_c are those of the free solute and the complex, respectively, under otherwise identical chromatographic conditions and are assumed to be $k_0 = 1$ and $k_c = 6$, so that the retention modulus for the complex, η_c, is 6. B, Schematic illustration of the sigmoidal dependence of the retention factor on the logarithm of the hetaeron concentration under the above conditions.

Upon plotting the retention factor against log $[H]$ according to eqn. 2 sigmoidal curves are obtained as illustrated in Fig. 1B for the case of $k_c < k_0$. The retardation factor shown on the ordinate is normalized to the retention factor of the free solute whereas the abscissa is given by the negative logarithm of the normalized stability constant $K[H]$. The hetaeron concentration at which the inflection occurs in the sigmoidal curve corresponds to the dissociation constant of the complex. Plateau regions of the sigmoidal graphs are usually reached at hetaeron con-

centrations two orders of magnitude around the stability constant. The enhancement or attenuation of retention upon complex formation is conveniently expressed by the modulus, η, that is defined by

$$\eta = k/k_0 \tag{5}$$

where k_0 and k are the retention factors of the free solute and the eluite complex, respectively. The limiting value of the modulus, η_c, is obtained when complex formation is complete at sufficiently high hetaeron concentrations, that is,

$$\eta_c = k_c/k_0 \tag{6}$$

Obviously when complexation reduces retention η_c is smaller than unity, whereas when the complex is longer retarded η_c is greater than unity.

The modulus can be considered as a reduced retention factor and substituted in eqn. 2. Thus by using the dimensionless hetaeron concentration, β, defined by $\beta = K[H]$, it can be shown that

$$\eta = \frac{\beta\eta_c + 1}{\beta + 1} \tag{7}$$

It is seen that if complexation has no effect on the retention, that is, $\eta_c = 1$, the value of k cannot be measured chromatographically by using the present methods. The magnitude of η_c can shed light on the physico-chemical basis of the complexation process[5] and change in selectivity upon complexation can be measured by comparing the appropriate moduli.

Linearized graphs for parameter estimation

Although parameter estimation has been greatly facilitated by high speed computers, linearized forms of eqn. 2 allow the use of graphical methods for the evaluation of stability constants from chromatographic data. Some of such equations are listed in Table I and designated by capital letters for convenience. If the dependence of k on $[H]$ is hyperbolic according to eqns. 2 or 3, plots of the experimental variables or groups of variables according to eqns. A to E generate straight lines. When the retention mechanism corresponds to the model underlying eqn. 2, the slopes and intercepts yield the stability constant of the complex, K, as shown in Table II. The value of k_c can be obtained graphically whereas the magnitude of k_0 is readily evaluated from chromatographic measurements in the absence of complexing agent.

Even if in chromatographic experiments the hetaeron concentration, pH and ionic strength can accurately be controlled, the measured retention factors therefore, the calculated equilibrium constants are subject to experimental error. The accuracy of parameter estimation, however, depends not only on the magnitude and type of experimental error but also on the type of linearized equation used. Therefore, eqns. A–E are not expected to yield the same estimate for the stability constant.

In order to establish which linear transformation of eqn. 2 yields the most accurate estimates of chromatographic parameters, the linear transformation, eqns. A–E, were analyzed in a fashion similar to that done by Dowd and Riggs[22] in a study

TABLE I

LINEARIZED FORMS OF EQN. 2 FOR GRAPHICAL EVALUATION OF FORMATION CONSTANTS FOR COMPLEXATION IN THE MOBILE PHASE

$$\frac{[H]}{k-k_0} = \frac{1}{k_c}\frac{k[H]}{k-k_0} + \frac{1}{k_cK} \quad \text{(A)}$$

$$\frac{k-k_0}{[H]} = -Kk + k_cK \quad \text{(B)}$$

$$\frac{1}{k-k_0} = \frac{1}{(k_c-k_0)K}\frac{1}{[H]} + \frac{1}{k_c-k_0} \quad \text{(C)}$$

$$\frac{[H]}{k-k_0} = \frac{1}{k_c-k_0}[H] + \frac{1}{(k_c-k_0)K} \quad \text{(D)}$$

$$k-k_0 = -\frac{1}{K}\frac{k-k_0}{[H]} + (k_c-k_0) \quad \text{(E)}$$

of the linearized forms of the Michaelis–Menten equation which is formally similar to eqn. 2. In that study three kinds of experimental uncertainties in the observed dependent variable were considered: (i) small uncertainties of constant magnitude, (ii) large uncertainties of constant magnitude, and (iii) large uncertainty proportional in magnitude to the dependent variable. We examined the same type of uncertainties in the retention factor, the observed dependent variable in a chromatographic measurement, assuming the hetaeron concentration, pH and ionic strength in the mobile phase are controlled without any error.

TABLE II

USE OF EQNS. A–E FOR LINEARIZED PLOTS ACCORDING TO THE FORMULA $y = mx + b$ IN ORDER TO EVALUATE THE STABILITY CONSTANTS, K, OF COMPLEXES FORMED IN THE MOBILE PHASE FROM RETENTION DATA OBTAINED IN REVERSED-PHASE CHROMATOGRAPHY

Equation	y	x	K
A	$\frac{[H]}{k-k_0}$	$\frac{[H]k}{k-k_0}$	$\frac{m}{b}$
B	$\frac{k-k_0}{[H]}$	k	$-m$
C	$\frac{1}{k-k_0}$	$\frac{1}{[H]}$	$\frac{b}{m}$
D	$\frac{[H]}{k-k_0}$	$[H]$	$\frac{m}{b}$
E	$k-k_0$	$\frac{k-k_0}{[H]}$	$-\frac{1}{m}$

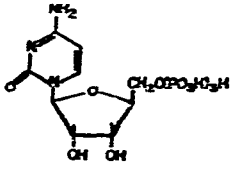
For a small uncertainty of constant magnitude in the retention factor a standard deviation $\sigma = 0.002$ was assumed so that the coefficient of variation, σ/k, ranged from 0.004 to 0.006. For a large uncertainty of constant magnitude in the retention factor a standard deviation $\sigma = 0.01$ was assumed so that the coefficient of variation ranged from 0.022 to 0.029. For small and large uncertainties of constant magnitude at 95% probability level the assumed uncertainties correspond to 0.8–1.2% and 4.4–5.8% of the retention factor, respectively. The coefficient of variation was chosen to be 0.03 for the uncertainty proportional to the retention factor corresponding to 6% of the retention factor at 95% probability level.

For small uncertainty of constant magnitude all linear transformations, with the exception of eqn. C, gave equally accurate estimates of the parameters. Since the error in measurement of retention factors is usually greater than 3% in practical applications eqn. C is inferior to the other linear transformations. The inferiority of eqn. C was further corroborated by results obtained upon examining the other two cases, *i.e.* that with large uncertainty of constant magnitude and with error proportional to the retention factor. In fact for all types of errors investigated, eqn. C was clearly the poorest of all linear transformations. Among the rest of the linear transformations it is not easy to choose the best one for the type of uncertainties considered. Nevertheless, for errors having a relatively large constant value or being proportional to the retention factor, eqns. A and B yield the most close estimates for the true values of the parameters.

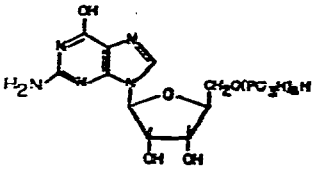
Error analysis also showed plots made according to eqns. B or E give the biggest scatter of experimental points. On the other hand eqn. C not only yields the least accurate estimates of the parameters but it is also gives a misleadingly good fit of the data points to a straight line.

Equilibria under investigation

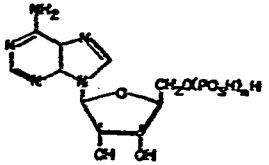
Metal binding by nucleotides. The properties of nucleotides in general have been the object of extensive study ever since the central role of their bases in the storage and transmittal of genetic information via DNA and RNA was recognized. Investigation of the binding of metals by nucleotides has constituted an important part of this research. These were prompted by the recognition of the fact that Ca^{2+} and Mg^{2+} as well as alkali metals ions play important roles in controlling the rates of various *in vivo* phosphoryl-transfer reactions which determine cellular energy flow. Adenosine-5′-triphosphate (ATP) has a special significance due to its central role in energy metabolism. In the present study metal binding by adenosine and guanosine nucleotides having two or three phosphate groups and by cytidinetriphosphate (CTP), was investigated. The chemical structure of these compounds is illustrated. The acronyms ADP, GDP and GTP stand for adenosine-5′-diphosphate, guanosine-5′-diphosphate and guanosine-5′-triphosphate, respectively.



CTP



n=2, GDP
n=3, GTP



n=2, ADP
n=3, ATP

The nucleotides can exist in a variety of ionic forms as the protons on the phosphate moiety can dissociate and the amino groups and nitrogens of the rings can be protonated. For our purpose the negatively charged species are of interest and by using ATP as an example the possible ionization steps leading to such species are shown as follows

$$ATP^{4-} + H^+ \overset{K_4}{\rightleftharpoons} ATP^{3-} \tag{8a}$$

$$ATP^{3-} + H^+ \overset{K_3}{\rightleftharpoons} ATP^{2-} \tag{8b}$$

$$ATP^{2-} + H^+ \overset{K_2}{\rightleftharpoons} ATP^{1-} \tag{8c}$$

$$ATP^{1-} + H^+ \overset{K_1}{\rightleftharpoons} ATP^{0} \tag{8d}$$

The corresponding acid ionization constants, $K_{a,t}$, are usually given as the appropriate $pK_{a,t}$ values and the two highest, $pK_{a,4}$ and $pK_{a,3}$ have the approximate values of 6.5 and 4.1, respectively at 25° and 0.1 ionic strength[23].

The several ionic forms can combine with cations to form complexes[23–25]. If ATP^{4-} and ATP^{3-} complex with a divalent metal, M^{2+}, the corresponding equilibria can be written as follows

$$ATP^{4-} + M^{2+} \overset{K_{M,1}}{\rightleftharpoons} M\cdot ATP^{2-} \tag{9a}$$

$$ATP^{3-} + M^{2+} \overset{K_{M,2}}{\rightleftharpoons} M\cdot ATP^{1-} \tag{9b}$$

At a particular pH value, one can define an apparent equilibrium constant for the formation of cation–ATP complex as the ratio of the sum of the concentrations of all cation–ATP complexes to the sum of the concentrations of all free ATP complexes and free cation. Thus, $K_{M,app}$, the apparent stability constant for formation of complex between ATP and metal, M^{2+}, is given as

$$K_{M,app} = \frac{([M\cdot ATP^{2-}] + [M\cdot ATP^{1-}])}{[M^{2+}]([ATP^{4-}] + [ATP^{3-}] + [ATP^{2-}] + [ATP^{1-}] + [ATP^{0}])} \tag{10a}$$

On the other hand with the equilibrium constants defined in eqns. 8a–9b, the apparent equilibrium constant for ATP is given by

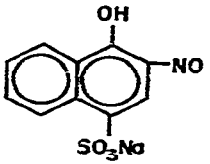
$$K_{M,app} = \frac{(K_{M,1} + K_{M,2}K_4[H^+])}{1 + K_4[H^+] + K_3K_4[H^+]^2 + K_2K_3K_4[H^+]^3 + K_1K_2K_3K_4[H^+]^4} \tag{10b}$$

Eqn. 10b demonstrates that the apparent binding constant is an average constant which is weighted by the fraction of each free species and the relative amount of each cation-binding form present. In this fashion the equilibrium constant appropriate to experimental conditions can be calculated if the stability constant for each reaction is known. All methods used to measure metal binding by ATP, including the chro-

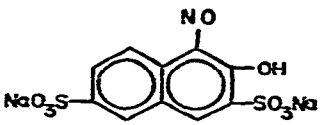
matographic approach described here yield $K_{M,app}$ whose value, of course, depends on the experimental conditions. Therefore in its calculation the individual equilibrium constants appropriate to the ionic strength and temperature of the experimental conditions are used. The theoretically expected values of the association constant presented in Table IV were calculated by use of eqn. 10b and the critical compilation of stability constants of Smith and Martell[26].

The above treatment can be extended to the interaction of other nucleotides with metals since all nucleotides investigated in this study have several ionizable groups. However, due to their different chemical structure for ADP, GTP, GDP and CTP the formula of the apparent stability constant will have a different form than that given in eqn. 10b for ATP.

Metal binding by nitrosonaphthol sulfonates. Nitroso-*R*-salt (NRS) and 2-nitroso-1-naphthol-4-sulfonic acid (NNSA) are well known analytical reagents for trace analysis of several metal ions and their chemical structure is shown below.



2-Nitroso-1-Naphthol-4-Sulfonic acid



Nitroso-R-Salt

The detection of metal ions is facilitated by complex formation between the particular nitrosonaphthol and the given metal ion. Consequently, the metal chelating properties of nitrosonaphthols have been examined by various investigators under different experimental conditions such as ionic strength, temperature and mixed aqueous and organic solvents[27,28].

The molecular structures of NNSA and NRS contain two and three ionizable groups, respectively. Both the sulfonyl and hydroxyl groups attached to the naphthalene ring can be ionized. The pK_a values of the hydroxyl groups for NNSA and NRS are 6.20 and 6.88, respectively[29]. The pK_a value of the sulfonic group for NNSA is 2.63. All pK_a values have been measured at an ionic strength of 0.1 and a temperature of 25°. With the symbol H_2A standing for NNSA its ionization equilibria can be expressed

$$H_2A \overset{K_1'}{\rightleftharpoons} HA^- + H^+ \tag{11a}$$

$$HA^- \overset{K_2'}{\rightleftharpoons} A^{2-} + H^+ \tag{11b}$$

where the equilibrium constants K_1' and K_2' correspond to the protonation of the sulfonyl and the hydroxyl groups, respectively. In the case of NRS one more equilibrium for the ionization of the second sulfonic acid group must be included.

The number of ligand molecules which will complex with a given metal ion will depend both on the ligand and the particular metal ion. For NNSA and NRS with different metal ions 1:1 and 2:1 complexes have been reported[28,30]. Denoting

NNSA or NRS by A^{n-} and assuming that both types of complexes are formed, the complex formation with a divalent metal can be represented by the following equilibria

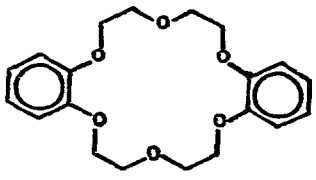
$$A^{n-} + M^{2+} \overset{K'_{M11}}{\rightleftharpoons} MA^{(n-2)-} \tag{12a}$$

$$A^{n-} + MA^{(n-2)-} \overset{K'_{M12}}{\rightleftharpoons} MA_2^{2(n-1)-} \tag{12b}$$

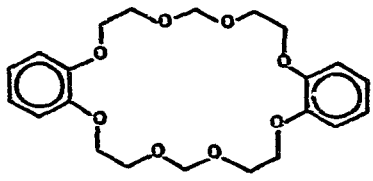
where $1 < n < 3$ for NRS and $1 < n < 2$ for NNSA.

When only 1:1 complex is formed, as in the case of NNSA–Zn complexation[29], the stability constant, K_{M_11}, is given by the ratio of the concentration of the complex to the total free nitrosonaphthol and can be evaluated using the chromatographic approach outlined in this work. When 2:1 complex is present the stability constant is dependent on the solute concentration and our linear model does not apply to the system.

Crown ether–alkali ion adducts. Crown ethers are cyclic polyethers in which oxygen atoms are separated by two aromatic or aliphatic carbons. A review of their properties and applications in analytical chemistry has recently appeared[31]. The term "crown" arises from the general structure of these compounds that resembles a crown in which the carbons form the circlet and the oxygens the points[31,32]. A large number of crowns have been prepared which vary in number of atoms in the circlet and the number of heteroatoms in the ring. In fact, some crowns use heteroatoms other than oxygen[33]. The nomenclature generally describes the number of atoms and heteroatoms in the cyclic ether and the groups substituted for ethylene groups. Thus, dibenzo-18-crown-6 (DBC), the structure of which is illustrated, consists of an 18-member ring with six oxygens with two phenyl groups. The molecular formula of the other compound, dibenzo-24-crown-8 (DB24C8), examined here, is also shown.



Dibenzo-18-crown-6



Dibenzo-24-crown-8

Crown ethers form very specific and stable complexes with alkali metal ions[32]. Since most crown ethers are soluble in organic solvents, they can be used to increase the concentration of salts in organic solvents. This has been exploited in various syntheses which require a nucleophile such as a halide ion or thiocyanate inorganic solvents since their salts can be "solubilized" by using an appropriate crown ether.

Although some crown ethers bind a few large monovalent ions such as Ag^+ and Hg^+, their most interesting feature is a strong complexation with alkali metal but not with most alkaline earth ions. The specificity is due to geometric factors; the

ring is large enough to enclose only relatively small cations[34]. In the simplest case, a 1:1 complex is formed between the crown and cation. In the particular case where KNO_3 combines with a crown ether, $\mathscr{C}$, the reaction can be written as

$$KNO_3 + \mathscr{C} \underset{}{\overset{K}{\rightleftharpoons}} K\mathscr{C}^+NO_3^- \qquad (13)$$

where the species on the right-hand side of eqn. 13 form either a "contact" or "solvent-separated" ion pair. If the species indicated by $\mathscr{C}$ and $K\mathscr{C}^+$ are distinguishable chromatographically, the association constant for the "ion pair" formed in the above reaction can be determined by using the approach described here.

EXPERIMENTAL

A Model 601 liquid chromatograph (Perkin-Elmer, Norwalk, Conn., U.S.A.) with a Model 7010 sampling valve with a 20-ml injection loop (Rheodyne, Berkeley, Calif., U.S.A.) was used. The effluent was monitored by either a Perkin-Elmer Model LC-55 variable wavelength photometric detector or a Model FS-970 fluorometric detector (Kratos-Schoeffel, Westwood, N.J., U.S.A.) with excitation wavelength and emission filter cut-offs set at 218 and 300 nm, respectively. Chromatograms were obtained with a Perkin-Elmer Model 123 strip-chart recorder. In all cases the column temperature was kept at 25° by water circulation through an insulated stainless-steel jacket from Model K-2/R thermostated water bath (Messgeraetewerk, Lauda, G.F.R.). In all experiments the flow-rate of the eluent was maintained at 2 ml/min.

Metal binding of nucleotides was investigated with a 250 × 4.6 mm LiChrosorb RP-18 column (Knauer, Berlin, G.F.R.) packed with 10-μm octadecyl-silica. The eluent was 0.04 *M* acetate buffer, pH 4.75, containing the corresponding metal sulfate and Na_2SO_4 so that the ionic strength was maintained constant at 0.1 upon changing the concentration of the complexing metal. In this part of the study the UV detector was set at 254 nm.

The stability constants of the nitroso-naphthol-sulfonic acids were determined by using a 250 × 4.6 mm 5-μm Partisil ODS column. (Whatman, Clifton, N.J., U.S.A.). The eluent was $4 \cdot 10^{-2}$ *M* piperazine-N,N'-bis (2-ethane sulfonic acid) (PIPES) buffer, pH 6.80, containing Na_2SO_4 as the background electrolyte and $ZnSO_4$ so that the ionic strength was maintained constant at 0.1 when the concentration of Zn^{2+} was varied. The UV detector was set at 220 nm to monitor the column effluent in these experiments. The stability constants of DBC complexes with potassium and sodium ions, as well as that of DB24C8 complex with cesium ion were determined from experiments in which the fluorescence detector was used. The complexation of DBC with K^+ and Na^+ was investigated by using a 5-μm perfluoroheptyl-silica column and a 10-μm LiChrospher RP-8 column, respectively. Both columns were 250 × 4.6 mm I.D. For the study of complex formation between DB24C8 and cesium ion a 10-μm Spherisorb ODS 250 × 4.6 mm column was used. The three columns and the column materials with the exception of LiChrospher RP-8 (E. Merck, Darmstadt, G.F.R.) were prepared in our laboratory (to be published). Samples of DBC and DB24C8 were prepared in the eluent and had concentrations of 10^{-5} and 10^{-4} *M*, respectively.

ATP was obtained from Worthington (Freehold, N.J., U.S.A.), ADP, CDP and GTP from Sigma (St. Louis, Mo., U.S.A.), CTP from Calbiochem (La Jolla, Calif., U.S.A.), DBC from Eastman (Rochester, N.Y., U.S.A.), DB24C8 from Strem Chemicals (Newburyport, Mass., U.S.A.) and "distilled-in-glass" methanol from Burdick & Jackson (Muskegon, Mich., U.S.A.). 2-Nitroso-1-naphthol-4-sulfonic acid and nitroso-*R*-salt (1-nitroso-2-naphthol-3,6-disulfonic acid disodium salt) were purchased from Tridom/Fluka (Happauge, N.Y., U.S.A.). All other chemicals were reagent grade.

Retention times were measured from the distance between the injection point and the peak maxima on the chromatogram. The mobile phase hold-up times were measured at 230 nm by injecting $NaNO_2$ into the eluent and the retention time of the $NaNO_2$ peak was taken as t_0 and the retention factors, k, have been calculated in the usual way[35]. The analysis of the chromatographic data was performed with a HP-25 programmable calculator, and the equilibrium constants were evaluated by using the specified equations and linear regression on the data.

RESULTS

We have found that with the reversed-phase systems at our disposal stability constants for complexes described above could be evaluated by using the present approach when certain precautionary measures, which are discussed later, were taken. In each case linearized forms of eqn. 2 have yielded association constants that are in good agreement with literature data.

Figs. 2 and 3 show plots of typical experimental data according to the scheme depicted in Fig. 1B. In both cases the sigmoidal behavior is expected and the graphs

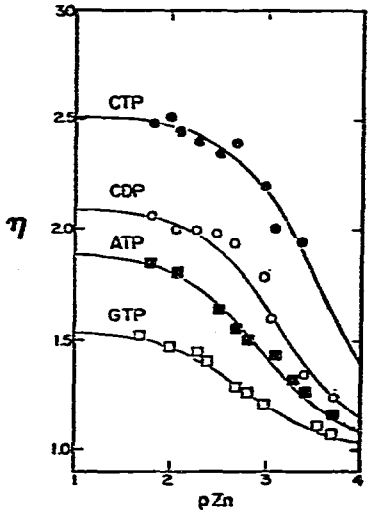


Fig. 2. Graph illustrating plots of the retention moduli of nucleotides against the negative logarithm of molar Zn^{2+} concentration, pZn, in reversed-phase HPLC on octadecyl-silica with 0.04 *M* acetate buffer, pH 4.75, the ionic strength of which was maintained at 0.1 with Na_2SO_4 and $ZnSO_4$. The solid lines were calculated from eqn. 2 by using appropriate parameter values given in Table IV.

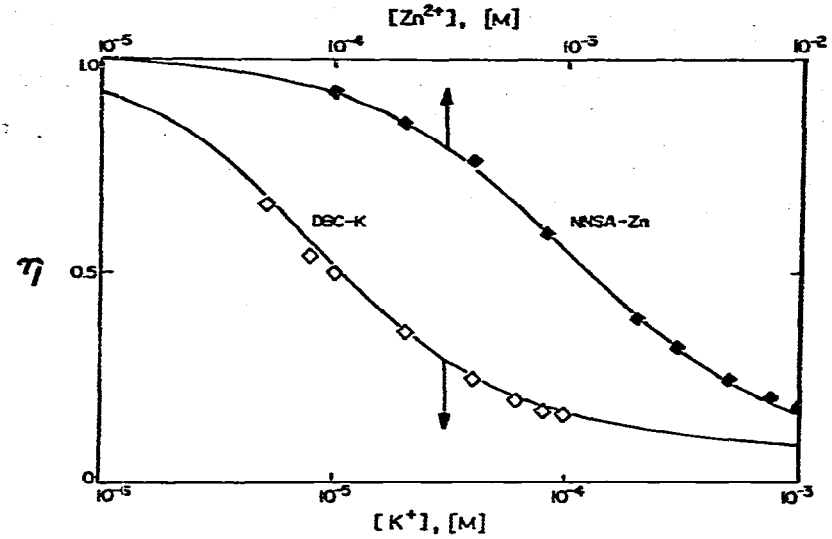


Fig. 3. Graph illustrating plots of the retention moduli of 2-nitroso-1-naphthol-4-sulfonic acid (NNSA) and dibenzo-18-crown-6 (DBC) as functions of the molar concentration of Zn^{2+} and K^+ on logarithmic scales. The stationary and mobile phases were: NNSA, octadecyl-silica, and 0.04 *M* PIPES buffer, pH 6.8; DBC, perfluoroheptyl-silica and methanol. The solid lines were calculated from eqn. 2 by using appropriate parameter values given in Table IV.

permit a direct estimation of the magnitude of the formation constants from the inflection points. The use of eqns. A–E given in Table I for plotting the experimental data according to the forms shown in Table II is illustrated in Figs. 4–9 for the various complexation reactions. The chromatographic conditions employed are summarized in Table III.

By using these graphical methods we have evaluated the stability constants of various complexes from the slopes and intercepts of the linear plots according to the formulae given in Table II. In general the least squares fit of the experimental points to theoretical lines is very good; in each case the confidence limit is given at the 95% level. As expected from the results of the statistical analysis discussed previously, the scatter of the experimental points is the greatest in Figs. 4 and 9, which show plots according to eqns. A and E.

Stability constants of the various systems have been evaluated from the experimental data by using eqns. A and B. They are listed in Table IV together with the retention parameters as well as with the literature value of the equilibrium constants evaluated under different experimental conditions. The literature values of the stability constants in Table IV were recalculated to experimental conditions of ionic strength and pH whenever appropriate as the chromatographically determined overall stability constants are pH dependent and the literature values were usually reported in terms of pH independent absolute values for the individual species.

The good fit of experimental data to yield linear plots in Figs. 4–9 indicate that the chromatographic retention of the eluite is modified by the secondary equilibria in agreement with the theoretical model developed here. As the stability constants estimated chromatographically are very similar to those measured under

TABLE III

EXPERIMENTAL CONDITIONS USED IN THE DETERMINATION OF METAL ION-ELUITE COMPLEX FORMATION CONSTANTS

The stability constants are presented in Table IV. All columns were 25 cm long. Sample volume. 20 μl; temperature, 25°; flow-rate. 2. 0 ml/min.

Exp. No.	*Solute*	*Metal ion*	*Mobile phase*	*Column*	*Detection*	*Sample concentration (M)*
1	ATP	Mg^{2+}	0.04 *M* Sodium acetate, pH 4.75	LiChrosorb RP-8, 10 μm	Absorbance at 254 nm	$1.0 \cdot 10^{-3}$
2	GTP	Mg^{2+}	0.04 *M* Sodium acetate, pH 4.75	Knauer, LiChrosorb RP-18, 10 μm	Absorbance at 254 nm	$1.1 \cdot 10^{-3}$
3	ATP	Zn^{2+}	0.04 *M* Sodium acetate, pH 4.75	Knauer, LiChrosorb RP-8, 10 μm	Absorbance at 254 nm	$1.0 \cdot 10^{-3}$
4	ADP	Zn^{2+}	0.04 *M* Sodium acetate, pH 4.75	Knauer, LiChrosorb RP-8, 10 μm	Absorbance at 254 nm	$0.92 \cdot 10^{-3}$
5	GTP	Zn^{2+}	0.04 *M* Sodium acetate, pH 4.75	Knauer, LiChrosorb RP-8, 10 μm	Absorbance at 254 nm	$0.98 \cdot 10^{-3}$
6	CTP	Zn^{2+}	0.04 *M* Sodium acetate, pH 4.75	Knauer, LiChrosorb RP-8, 10 μm	Absorbance at 254 nm	$1.1 \cdot 10^{-3}$
7	CDP	Zn^{2+}	0.04 *M* Sodium acetate, pH 4.75	Knauer, LiChrosorb RP-8, 10 μm	Absorbance at 254 nm	$2.2 \cdot 10^{-3}$
8	NNSA	Zn^{2+}	0.04 *M* PIPES, pH 6.80	Knauer, LiChrosorb RP-8, 10 μm	Absorbance at 220 nm	$1.1 \cdot 10^{-3}$
9	NNSA	Zn^{2+}	0.04 *M* PIPES, pH 6.80	Knauer, LiChrosorb RP-8, 7 μm	Absorbance at 220 nm	$1.1 \cdot 10^{-3}$
10	NNSA	Zn^{2+}	0.04 *M* PIPES, pH 6.80	Partisil ODS-2, 5 μm	Absorbance at 220 nm	$1.1 \cdot 10^{-3}$
11	NRS	Zn^{2+}	0.04 *M* PIPES, pH 6.80	Partisil ODS-2, 5 μm	Absorbance at 220 nm	$1.0 \cdot 10^{-3}$
12	DBC	Na^{+}	Methanol	LiChrospher RP-8, 10 μm	Fluorescence above 300 nm with excitation at 218 nm	$1.0 \cdot 10^{-5}$
13	DBC	K^{+}	Methanol	Perfluoroheptyl-silica, 5 μm	Fluorescence above 300 nm with excitation at 218 nm	$1.0 \cdot 10^{-5}$
14	DB24C	Cs^{+}	Methanol	Spherisorb ODS, 10 μm	Fluorescence above 300 nm with excitation at 218 nm	$1.0 \cdot 10^{-4}$

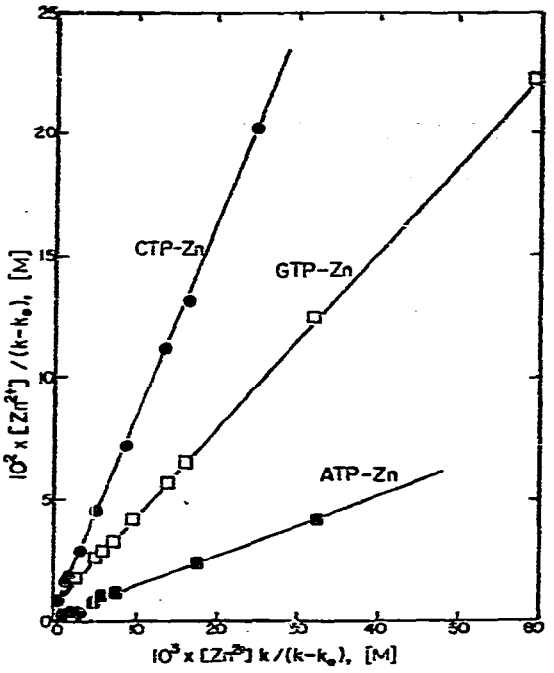


Fig. 4. Linear plots according to eqn. A for ATP, CTP, and GTP complexation with Zn^{2+}. The solid lines were calculated by least squares analysis. Chromatographic conditions are given in Table III.

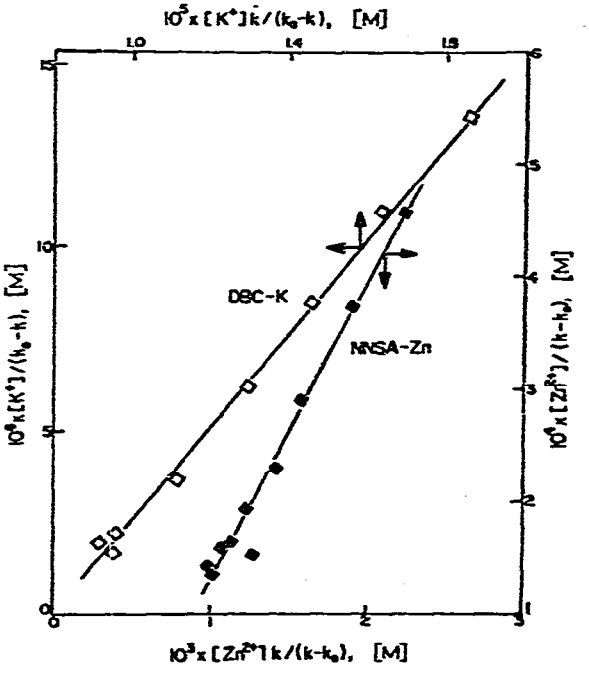


Fig. 5. Linear plots according to eqn. A for NNSA–Zn complexes in aqueous PIPES buffer, pH 6.8, and DBC–K complexes in methanol. The solid lines were calculated by least squares analysis. Chromatographic conditions are given in Table III.

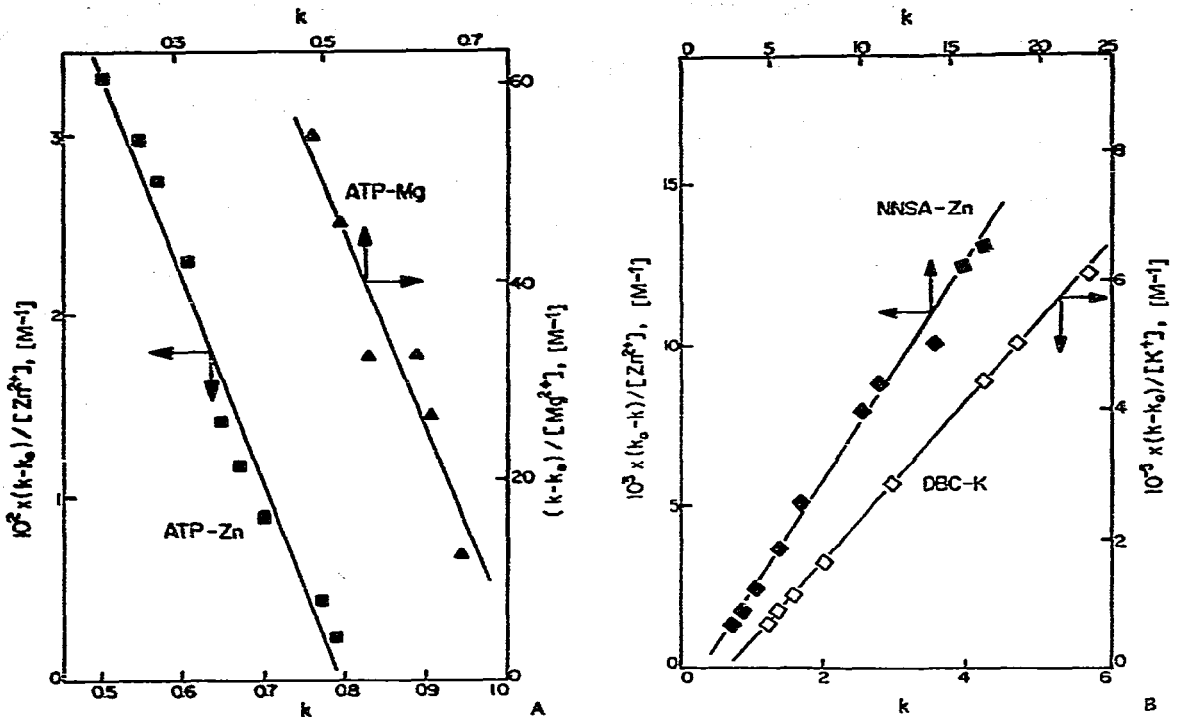


Fig. 6. A, Linear plots according to eqn. B for the binding of Mg and Zn by ATP. The solid lines were calculated by least squares analysis. Chromatographic conditions are given in Table III. B, Linear plots according to eqn. B for NNSA–Zn and DBC–K complexes in aqueous and methanolic solutions, respectively. Chromatographic conditions are given in Table III.

different experimental conditions and reported in the literature, both the usefulness of HPLC as a tool for physico-chemical measurement and the broad applicability of the phenomenological model, *cf.* eqn. 2, are demonstrated. Nevertheless, certain precautionary measures, which will be discussed later, are necessary to obtain satisfactory results.

DISCUSSION

Physico-chemical basis of changes in retention upon complexation

Solvophobic theory of retention in reversed-phase chromatography. The chromatographic method described here for the measurement of stability constants requires a marked augmentation or attenuation of the retention due to complex formation. Some insight into the physico-chemical basis of this phenomenon can be obtained by recourse to the solvophobic theory[36,37] that has been successfully adapted to account for retention behavior in reversed-phase chromatography[2,3].

The changes occurring in the molecular properties of the eluite upon com-

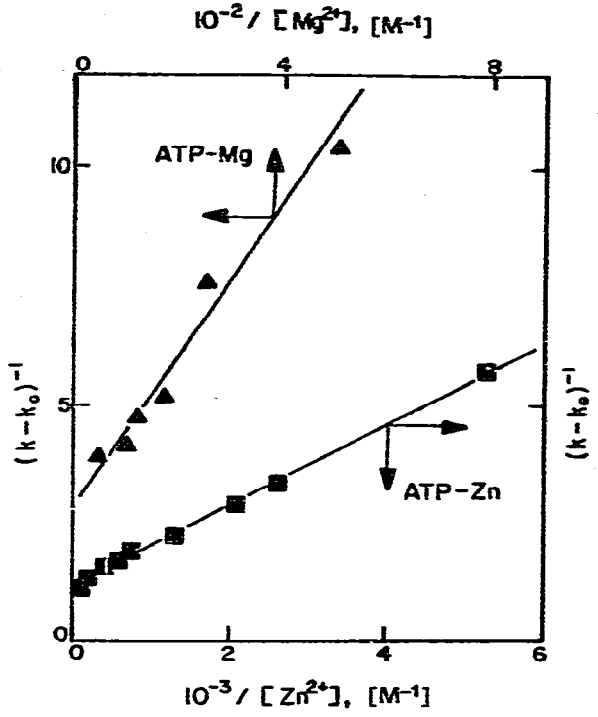


Fig. 7. Linear plots according to eqn. C for ATP–Mg and ATP–Zn complexes. The solid lines were calculated by least squares analysis. Chromatographic conditions are given in Table III.

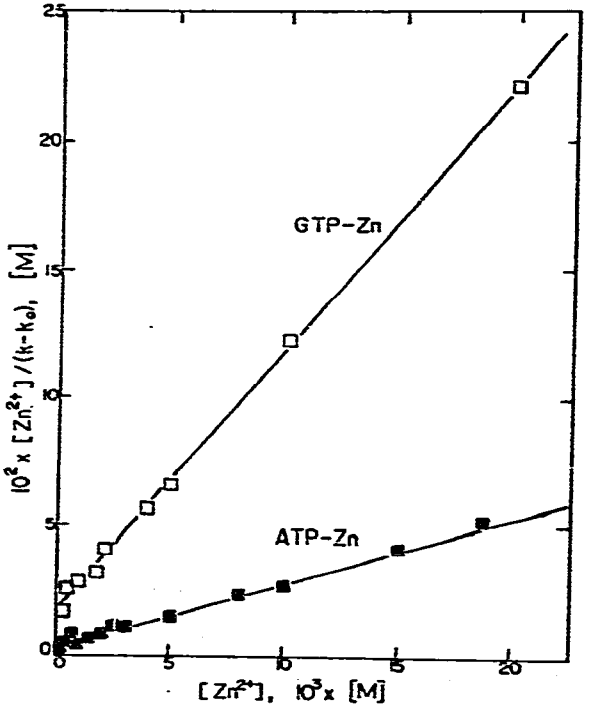


Fig. 8. Linear plots according to eqn. D for the binding of Zn by ATP and GTP. The solid lines were calculated by least squares analysis. Chromatographic conditions are given in Table III.

TABLE IV

COMPARISON OF STABILITY CONSTANTS OBTAINED FROM CHROMATOGRAPHIC MEASUREMENTS WITH LITERATURE VALUES

The table shows the retention factor of the eluite obtained in the absence of metal ion, k_0, the retention modulus for the complex, η_c, and the logarithm of the stability constant of eluite-metal complex measured in units of M^{-1}. The best values of the equilibrium constant available from the literature are also shown together with the means by which they were determined.

Exp. No.	*Eluite*	*Metal ion*	k_0	*Modulus* (η_c)	*Logarithm of the stability constant*					
					This work			*Other studies*		
					Eqn. A	*Eqn. B*	*Mean value*	*log K*†	*Method*††	*Ref.*
1	ATP	Mg^{2+}	0.432	1.81	2.342 ± 0.109	2.254 ± 0.100	2.298	2.213 ± 0.2	a	26
2	GTP	Mg^{2+}	0.196	1.53	2.445 ± 0.212	2.442 ± 0.205	2.444	2.269	a	26
3	ATP	Zn^{2+}	0.429	1.91	3.028 ± 0.063	2.984 ± 0.065	3.006	3.003 ± 0.4	a	26
4	ADP	Zn^{2+}	0.682	1.54	2.640 ± 0.215	2.481 ± 0.136	2.561	2.517 ± 0.1	a	26
5	GTP	Zn^{2+}	0.178	1.55	2.766 ± 0.087	2.779 ± 0.040	2.773	—	—	—
6	CTP	Zn^{2+}	0.050	2.52	3.522 ± 0.107	3.515 ± 0.126	3.519	—	—	—
7	CDP	Zn^{2+}	0.067	2.10	3.025 ± 0.061	3.208 ± 0.078	3.117	—	—	—
8	NNSA	Zn^{2+}	4.74	0.220	2.97 ± 0.05	3.07 ± 0.12	3.02	2.98	a	28
9	NNSA	Zn^{2+}	3.25	0.161	2.99 ± 0.04	3.04 ± 0.10	3.02	2.98	a	28
10	NNSA	Zn^{2+}	17.25	0.068	2.96 ± 0.03	3.00 ± 0.19	2.98	2.98	a	28
11	DBC	Na^+	6.30	0.182	4.35 ± 0.09	4.45 ± 0.30	4.40	4.367 ± 0.04	b	34
12	DBC	K^+	8.667	0.085	5.094 ± 0.016	5.092 ± 0.016	5.093	5.00 ± 0.04	b	34

† The measured constant for nucleotides is equivalent to $K_{M,app}$, defined by eqn. 10b for ATP, for nitrosonaphthol sulfonates to $K_{M,1}$ in eqn. 12a, and for crown ethers K in eqn. 13.

†† a = Determined from pH titration data; b = determined by ion-selective electrodes.

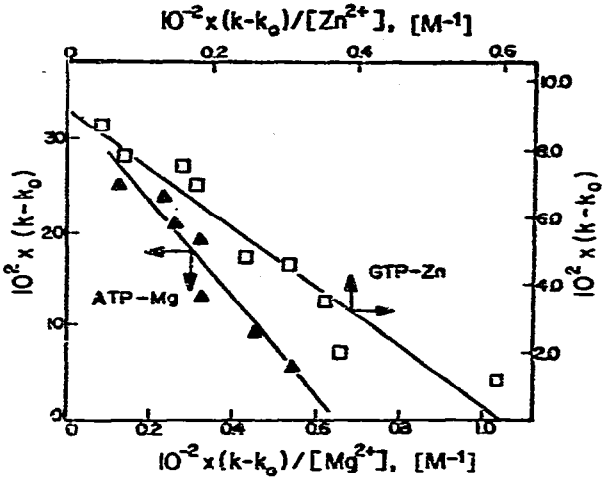


Fig. 9. Linearized plots according to eqn. E for ATP–Mg and GTP–Zn complexes. The solid lines were calculated by least squares analysis. Chromatographic conditions are given in Table III.

plexation can be manifold. In most cases, however, it suffices to consider one or more of the following alterations in eluite properties. (i) As a result of complex formation the molecular surface area of the eluite changes and thereby the contact area of the complex upon binding to the non-polar surface of the stationary phase will be different from the corresponding contact area of the uncomplexed eluite. (ii) Upon complexation the dipole moment of the eluite becomes greater or smaller than that of the free solute. (iii) The process produces an alteration in the net electronic charge of the eluite molecule.

The consequences of changes in the molecular surface area of eluites on the retention have been discussed earlier in a treatment of "ion-pair" reversed-phase chro matography[5]. It was shown that the retention modulus for the complex, η_c, is linearly dependent on the molecular surface area of "hydrophobic" hetaerons such as long-chain alkyl sulfonates or quaternary alkyl amines when the ionized solute forms a complex with the hydrophobic ion in the mobile phase and the enhancement of retention arises from a stronger binding of the complex to the non-polar stationary phase with respect to the binding of the "free" solute. The results of experiments with biogenic amines and alkyl sulfate as the hetaerons having different chain-lengths ndeed showed the maximum value of the modulus to be linearly dependent on the carbon number of the alkyl chain, *i.e.*, on the increase in the surface area of the eluite complex.

In many other practical situations, however, the change in the size of the eluite is small upon complex formation. The complexing of proton or metal ions by relatively bulky organic molecules, for instance, is accompanied by negligible changes in the molecular volume and surface area. Under such conditions variations occurring in the dipole moment or electronic charge of the eluite molecules upon complexation have to be considered.

As shown previously[2] the effect of dipole moment of the eluite on the reten-

tion factor at temperature T can be approximated for a given chromatographic system by

$$\ln k = \text{const.} + B\Delta A - C\mu_e^2 \tag{14}$$

where the constant and B are defined by eqns. 48 and 50 in ref. 2, μ_e is the dipole moment of the eluite and C is calculated by

$$C = \frac{1 - \lambda}{2k_B T\lambda v_e} \frac{\mathscr{D}}{1 - \mathscr{D}(\bar{\alpha}_e/v_e)} \tag{15}$$

The reduction in the molecular surface area upon binding the eluite to the stationary phase is given by ΔA and k_B is the Boltzmann constant. The symbol λ represents the volume ratio of the eluite–stationary phase conjugate and the eluite, v_e and $\bar{\alpha}_e$ are the molecular volume and average molecular polarizability of the eluite, respectively. The value of λ can be taken as 2 in reversed-phase chromatography with octadecyl-silica. The expression for $\mathscr{D}$ is given by

$$\mathscr{D} = \frac{2(\varepsilon - 1)}{2\varepsilon + 1} \tag{16}$$

where ε is the dielectric constant of the medium.

Assuming that the change in v_e upon complex formation is negligible and $\bar{\alpha}_e$ is a small fraction of v_e, we can express the retention modulus for the complex as

$$\ln \eta_c \approx \text{const.} + B'\Delta\Delta A - C'(\mu_c^2 - \mu_0^2) \tag{17}$$

where $\Delta\Delta A$ is the increase in ΔA upon complex formation, μ_c and μ_0 are the dipole moments of the complex and the free solute. The constant C' can be evaluated from eqn. 15.

For cases when $\Delta\Delta A$ is sufficiently small eqn. 17 shows that if the complex has a smaller dipole moment than the free solute the modulus is greater than unity and the complex is retarded longer. In contradistinction, if complexation is accompanied by an increase in the dipole moment the modulus is smaller than unity so that it results in decreasing retention.

In most instances, changes in the net electronic charge upon complex formation have the greatest effect on altering chromatographic retention. In an earlier report[4] the modulus for eluite retention arising from the conversion of a monopole to a dipole, when no changes occur in the molecular surface area, was given as

$$\ln \eta_c = C\mu_e^2 - \frac{Z^2e^2}{k_B T\varepsilon}\left[\frac{\varepsilon - \varepsilon^*\lambda}{\varepsilon^*\lambda^{1/3}b_e} - \frac{\varepsilon - \varepsilon^*}{\varepsilon^*}(BI^{1/3} + CI)\right] \tag{18}$$

where e is the electronic charge of the eluite, Z is the charge of the eluite, b_e is its

radius and ε^* is the dielectric constant at the stationary phase surface. B and C are constants for a given eluent and their values are tabulated for a variety of aqueous salt solutions[38]. Eqn. 18 is valid at relatively high ionic strengths, I, usually employed in chromatographic experiments. The estimation of changing retention upon protonic association or dissociation according to eqn. 18 showed good agreement with the experimentally observed values for aromatic acids and bases[4].

Estimation of the retention moduli for the complexes under study. In the present work the stability constant of the complexes formed by DBC with Na^+ and K^+ were measured. When the counter ions of Na^+ and K^+ are only loosely associated with the complex cation[33], the process of complex formation from the neutral crown ether by alkali ions could be considered as the formation of a monopole from a dipole. The phenomenon is similar to the protonic dissociation of neutral ionogenic substances where complexation also results in decreasing retention and the use of eqn. 18 would allow us to make a crude estimate of the retention modulus for complexation.

However, it is more likely that the counter ions of the Na^+ or K^+ will remain strongly associated with the crown ether–alkali metal ion complex in a medium of relatively low dielectric constant such as methanol. If the distance between the ions is assumed to be the same as that observed in the salt, the energy required for dissociation of NaCl–crown ether to Na^+–crown ether and chloride ion is 7.4 k_BT, using 2.3 Å as the interionic distance[39]. The barrier is rather large compared to the value of k_BT calculated for water at 25°. As a consequence, complex formation can be viewed as the creation of a large dipole moment in the crown ether molecule. When NaCl is the salt, the crown ether complex could have a moment greater than 11 Debye ($2.3 \cdot 10^{-3}$ cm $\times$ $4.8 \cdot 10^{-10}$ e.s.u.).

In the first approximation we can assume that the dipole moment of the crown ether is zero because of its centrosymmetric structure. Then the approximate expression for the modulus of the complex given by eqn. 17 can be further simplified to

$$\ln \eta_c = - C' \mu_c^2 \tag{19}$$

provided the first two terms on the right-hand side of eqn. 17 are the same for both the uncomplexed crown ether and the complex. The term C' can be evaluated from the molecular properties so that we obtain

$$\ln \eta_c = - 2.81 \cdot 10^{-2} \mu_c^2 \tag{20}$$

In order to arrive at eqn. 20 the following values were employed: molecular weight, 328; density, 0.8; refractive index, 1.46. The value of $\mathscr{D}$ was calculated[2] as 0.92. This crude approach yields an estimated value of 1/14 for the modulus of the complex that compares to the observed value of 1/6. Thus we obtain the right order of magnitude which is quite acceptable considering the simplifying assumptions employed here.

When the number of charges change in oligo-electrolyte molecules upon complexation the situation is more complicated. In the present work the binding of divalent metal ions by nucleotide triphosphates is investigated. Similar problems, however, are frequently encountered in reversed-phase chromatography as demon-

strated recently by the effect of pH on the retardation of pteroyl-oligo-γ-L-glutamates[21]. In the case of molecules, which carry more than one charge, the mathematical expression for the retention factor depends on the way the charges are treated. If the charges are in close proximity they may be added and treated as one charge located in the center of the molecule. The last term of eqn. 18 can then be used for calculation of the modulus for the retention of differently charged species.

If charge separation in the eluite molecule is substantial, individual charges should be considered independently. In such cases the total electronic charge is thought to be the sum of unit charges acting independently and Z^2 in eqn. 18 is replaced by Z, the number of charges in the molecule. This accounts for the effect of Z unit charges on the corresponding free energy change and as a result, the logarithm of the retention factor is a linear function of Z, a behavior that was recently observed with dissociated pteroyl-oligo-γ-L-glutamates in reversed-phase chromatography[21].

As the charge density of nucleotides is localized on the phosphate oxygens, the model based on independent unit charges in the molecule appears to be more reasonable than the assumption of additive charges. Therefore we shall use it to estimate the retention modulus for metal binding to nucleotides. In the case investigated both the free solute and the complex are charged. The difference in net charge, ΔZ, is given by

$$\Delta Z = Z_c - Z_0 \tag{21}$$

where Z_c and Z_0 are the net charges on the eluite in the complex and in the free form, respectively. In our case the retention modulus is calculated for the formation of a species comprised of a dipole (the complex between Mg^{2+} and two basic oxygens) and n monopoles from the reaction of a species composed of $(n+2)$ monopoles with Mg^{2+} so that $\Delta Z = 2$. An extension of eqn. 18 appropriate to this situation yields

$$\ln \eta_c = C\mu_c^2 - \frac{\Delta Z e^2}{k_B T \varepsilon} \frac{\varepsilon - \varepsilon^*}{\varepsilon^* \lambda^{1/3} b_c} \tag{22}$$

With values $\lambda = 2$ and $\varepsilon^* = 35$, which are appropriate in RPC with octadecyl-silica or octyl-silica as the stationary phase, by using a molecular weight of 550 for ATP and assuming that the dipole moment is about 12 Debye as in the crown ether calculation by using eqns. 15 and 22 we find that $\ln \eta_c$ has the value of 0.79, *i.e.*, the modulus of the complex, η_c, is 2.2. This value shows a much better agreement with the observed value, $\eta_c = 1.6$, than that one would expect considering the crudity of the various assumptions used in the calculation.

The above examples demonstrate that the solvophobic theory can indeed be used to predict the effect of complex formation on eluite retention in reversed-phase HPLC. Whereas the estimates are at present not accurate enough to predict retention data in practical work, they are remarkably good considering the approach taken to their calculation.

Selection of proper experimental conditions

Deviations from linearity

Sample overloading. The dependence of the retention factor on the hetaeron concentration can be described by eqn. 2 only if the behavior of the chromatographic system is close to the ideal behavior represented by the underlying model. It implies that the sorption isotherm of the eluite is linear in the concentration range investigated and the properties of both the stationary and mobile phases remain invariant upon introducing the sample into the system. Sample concentrations well within the purview of HPLC usually suffice to avoid column overloading or depletion of the hetaeron in the mobile phase, thus, maintaining a linear system represented by eqn. 2. Under circumstances, however, when the value of K is relatively high, the latter restriction may require the use of very small samples and the utmost detector sensitivity available in HPLC.

Testing for the appropriate sample size is necessary by varying in a wide range the amount of solute injected also at the highest detector sensitivity possible. The sample size used in measurements of equilibrium constants should be so small that the retention values are truly independent of the sample amount even at the lowest concentration of hetaeron in the eluent, *i.e.*, working with practically zero sample size is required. In fact, the great advantage of HPLC is that highly sensitive detectors allow the use of very low sample concentrations, thus, the chromatographic process with or without secondary equilibria can be maintained linear. Consequently, easily measurable retention values can be directly used to extract thermodynamic information from the data.

In such physico-chemical measurements by HPLC deviation from linearity may occur due to the employment of too concentrated sample solution. In this case a significant fraction of the hetaeron in the eluent volume containing the eluite is bound to the solute and the concentration of the free hetaeron is larger than the assumed value in and in the vicinity of the eluite band. As the measured retention ratios do not correspond to the hetaeron concentration in the eluent as prepared, the relationship in eqn. 2 does not hold.

This effect can be eliminated if an upper bound is set for the eluite concentration which is equal to the concentration of the hetaeron in the mobile phase. The permissible concentration of the solute in the sample, [S], can be estimated by

$$[S] \approx \frac{1 + K[H]}{K} \tag{23}$$

where both [S] and $[H]$ are molar concentrations. It is seen that the permissible solute concentration in the sample increases with the concentration of the hetaeron and decreases with increasing values of K. Hetaeron depletion can be eliminated over the whole length of the column and for any hetaeron concentration in the mobile phase if the concentration of the injected sample is adjusted via eqn. 23 with $[H] = 0$, *i.e.* $[S] = 1/K$. Of course, the sample is usually dissolved in the eluent having the appropriate hetaeron concentration. It may be desirable to use the largest possible sample volume that still affords acceptable band spreading and the smallest amount of solute that affords a well detectable peak.

The employment of higher than the permissible sample concentration can

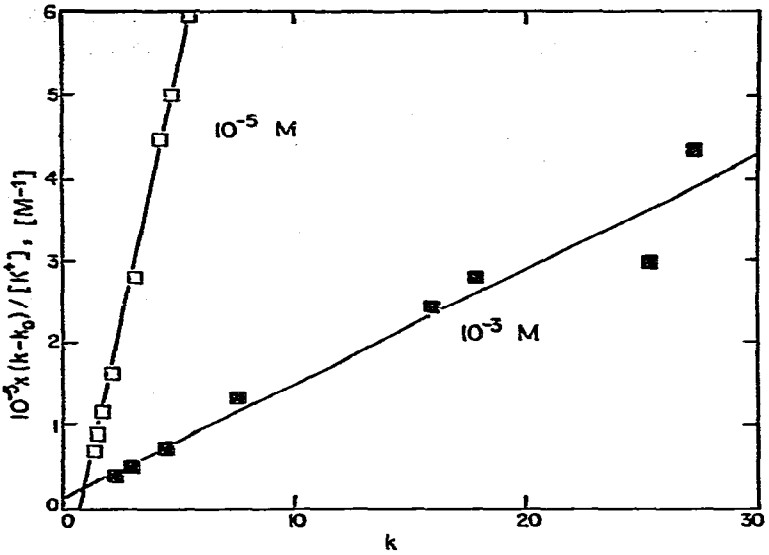


Fig. 10. Plots of retention data obtained at different sample loadings for the formation of the K^+ complex of dibenzo-18-crown-6 (DBC) in methanol according to eqn. B. Injected amount of DBC: ■, 0.072 μg, □, 72 μg. At high DBC load the ordinate intercept is positive indicating that the data do not follow the phenomenological model described by eqn. 2. For chromatographic conditions, see Table III.

readily lead to false results because the data can yield misleading quasi-linear plots such as shown in Fig. 10. The data measured for the complexation of DBC with K^+ are plotted according to eqn. B. Under otherwise identical conditions in one case the concentration of DBC in the sample was 10^{-3} *M*, and in the other 10^{-5} *M*. In both cases straight lines could be drawn through the points although the data obtained with samples having lower DBC concentrations show superior fit. Considering that the stability constant for the complex is 10^{-5} l/mole, *cf.* Table IV, the permissible sample concentration is about 10^{-5} *M* according to eqn. 23. Thus it is expected that data measured with samples having such a solute concentration yield a straight line with the appropriate slope and intercept. Yet, the data obtained with hundred times more concentrated samples give also a quasi-straight line on a plot according to eqn. B. The fallacy, however, is easy to recognize as the intercept of this line obtained with 10^{-3} *M* DBC samples in Fig. 10 has the wrong sign (see eqn. B in Table I).

Multiple equilibria. Eqn. 2 does not reflect the dependence of the retention factor on the hetaeron concentration when in the concentration range investigated complex formation cannot be described by a single, concentration independent equilibrium constant because the complex contains more than one solute molecule. The deviation, however, is not easy to recognize in many cases so that great care has to be employed not to calculate some "equilibrium constant" that falls short of the physico-chemical meaning of an association constant for the complex in solution and would not represent an apparent constant such as given in eqn. 10a.

For instance, NRS forms not only 1:1 but also 2:1 complexes with zinc[28,30] and crown ethers are known to form "sandwich" complexes when the radius of the alkali metal ion such as that of Cs^+ is relatively large[33]. In these cases the system is

not linear because the association constant and consequently the retention factor depend on the analytical concentration of the eluite. Moreover this concentration is not constant but changes along the column. A rigorous treatment of such problems leads to an equation with at least seven parameters.

Plots of data according to the form shown in Fig. 1B, for instance, do not necessarily reveal that the dependence of the retention factor on the logarithm of hetaeron concentration does not satisfy eqn. 2. This is illustrated for the case of NRS–Zn^{2+} complexes in Fig. 11 and a curvilinear regression analysis of the data yields a value of $10^{3.85}$ $[M^{-1}]$ for the equilibrium constant that is to be compared with the literature value of $10^{4.04}$ $[M^{-1}]$ corrected for total eluite concentration and the interaction between Zn^{2+} and SO_4^{2-}. Fortunately some of the linearized forms of eqn. 2 can be used for the diagnosis of such deviations from the model. Fig. 12 shows linearized plots according to eqn. B for the retention data obtained in the course of investigating the formation of NRS–Zn^{2+} and DB24C8–Cs^{+} complexes. Four and two data points obtained with NRS and DB24C8, respectively, at low hetaeron concentrations are far off from the apparently straight lines obtained with data measured at higher metal ion concentrations. In the case of NRS if only those data points are considered which fall on the apparent straight line the calculated stability constant would be 1.75 times higher than the literature value thus the estimate would be in serious error. For DB24C8 the linear part of the plot is not as deceptive because the nonconformance of the data with eqn. 2 is obvious from the positive ordinate intercept. The above example clearly demonstrates the necessity of taking into account retention data obtained at low hetaeron concentrations even if the temptation to discard the data points as the result of experimental error would be great.

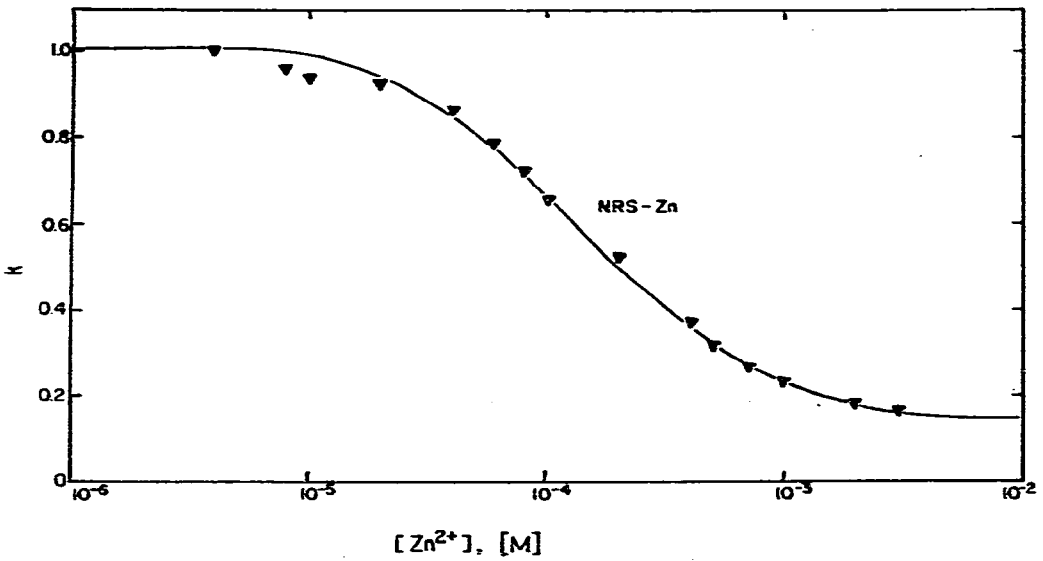


Fig. 11. Plot of retention data obtained for the formation of Zn-complex of nitroso-R-salt at a sample load of 6 μg. The solid line obtained by non-linear regression analysis of eqn. 2 corresponds to a stability constant of $10^{3.85}$ l/mole, which is 1.5 times smaller than the corrected literature value of $10^{4.04}$ l/mole.

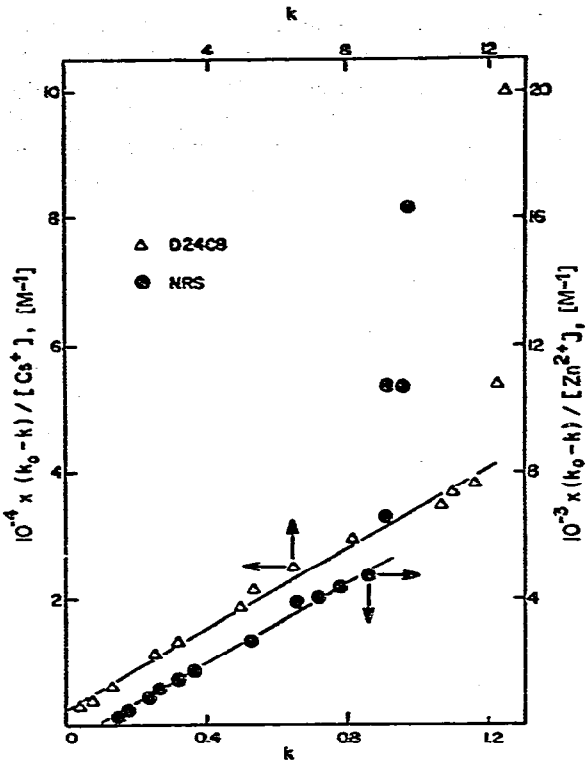


Fig. 12. Plots illustrating the effect of a second complexation equilibrium of the eluite with the hetaeron according to eqn. B for NRS–Zn and DB24C8–Cs in aqueous and methanolic solutions, respectively. The solid lines were drawn by a least squares analysis of eqn. B ignoring the last four and two data points with the highest retention factors for NRS and DB24C8, respectively. For NRS this procedure gives a stability constant that is 1.75 times higher than the literature value. The negative intercept of the line for DB24C8 clearly shows that the behavior of the chromatographic system is not described by eqn. 2. For chromatographic conditions, see Table III.

Observation of the peak shape as a function of the sample concentration may shed light on the behavior of the system because departure from linearity often manifests itself in strongly asymmetric peaks or even in peak splitting.

Effect of strong hetaeron binding to the stationary phase

In practice the inhomogeneity of the stationary phase surface, and with reversed-phase chromatography the presence of accessible silanol groups at the surface in particular, is blamed most commonly for peak asymmetry at low sample concentrations.

The selection of the stationary phase could be an important factor when specific interactions between the hetaeron and the stationary phase surface, *e.g.* free silanol groups, can cause deviations from our equilibrium model so that eqn. 2 does not describe the retention behavior of the eluite as a function of the hetaeron concentration. In our study of complexation with metal ions, bonded phases of high surface coverage have given satisfactory results as experiments performed under appropriate conditions with three different commercially available columns, RP-8,

RP-18 and Partisil ODS, yielded for NNSA–Zn complexes the same stability constant within experimental error. Thus, different type of reversed-phase columns can be used in such studies. Indeed, an experimental perfluoroheptyl-silica stationary phase has also been found suitable for such measurements. Although for metal binding by organic substances such chromatographic systems appear to behave in a regular manner, in other cases the selection of the stationary phase might have an effect on the accuracy of the measurements.

Our simple model, *cf.*, eqn. 2, is based on a mechanism that entails complex formation in the mobile phase, and only in the mobile phase, with reversible binding of the complex by the stationary phase. However, in many cases the chromatographic process may be different. Under many practical conditions the hetaeron, *e.g.* a "hydrophobic" ion, may be bound to the stationary phase to such an extent that complex formation with the bound hetaeron is appreciable and, as a result, eqn. 2 is not applicable to the dependence of the retention factor on the hetaeron concentration. For instance hydrophobic hetaerons such as hexadecyltrimethyl ammonium ions used in ion-pair reversed-phase HPLC for the enhancement of the retention of acidic components can be strongly adsorbed on the stationary phase so that $K[H] \ll 1$ and the mechanisms of the chromatographic process lead to eqn. 3. Since formally both eqns. 2 and 3 are the same, the linear plots described above would yield straight lines also. However, if complexation with the hetaeron bound to the stationary phase is predominant, the parameter estimation would not yield the stability constant for the complex in solution. Consequently it is necessary to assure, often by using extra-chromatographic means, that the system parameters are represented by the constants of eqn. 2 and not by those of eqn. 3.

Certain hetaerons such as divalent metal ions, can irreversibly change the surface of some bonded siliceous stationary phases over a prolonged time interval. Then retention might occur due to the metal ions at the surface and the retention behavior does not obey the simple model described by eqn. 2. In order to recognize alterations of the column surface during a given series of experiments, the hetaeron concentration in the mobile phase should be changed in a random fashion. Care should be taken to equilibrate the respective mobile phase with the column surface every time the concentration of hetaeron in the mobile phase is changed.

In our study modification of the column surface was observed with Zn^{2+} as the hetaeron when a home-made octadecyl-silica column was used in preliminary experiments. As a result ATP retention factors were not reproducible at low concentrations of Zn^{2+} after the column was exposed to high ($2 \cdot 10^{-2}$ *M*) concentrations of Zn^{2+}. The retention factor of ATP at low (10^{-4} *M*) concentrations of Zn^{2+} increased by almost a factor of ten after the column was exposed to high concentrations of Zn^{2+} relative to the value obtained before exposure of the column to high concentrations of Zn^{2+}. On the basis of this experience with a column packed with a home-made octadecyl-silica having substantial silanol concentration at the surface other types of columns have been used. The above effect was not observed with Partisil ODS 2 and other commercially available columns.

With increasing hetaeron concentrations both the formation constant of the complex in the mobile phase, K, and the equilibrium constant for hetaeron binding by the stationary phase, K_h, may affect the dependence of the retention factor on

the hetaeron concentration[4] that follows a parabolic relationship in such a case and is given by

$$k = \frac{k_0 + k_c K[H]}{(1 + K[H])(1 + K_h[H])} \tag{24}$$

Eqn. 24 can also be used for the evaluation of the stability constant in solution, K, when the equilibrium constant for the binding of the hetaeron to the stationary phase, K_h, is known.

Linear plots and departures from the simple model

In order to illustrate the effect of departures from the simple model such as those manifested in eqn. 24 on linearized plots over a wide range of conditions we have performed computer simulation of the expected behavior. Eqn. 24 can be written in dimensionless form as

$$\eta = \left(\frac{\eta_c \beta + 1}{\beta + 1}\right)\left(\frac{1}{1 + \alpha\beta}\right) \tag{25}$$

where the parameter α expresses the magnitude of hetaeron binding to the stationary phase relative to complex formation and is given by

$$\alpha = K_h/K \tag{26}$$

Comparison of eqn. 25 to eqn. 7 shows that the factor $(1+\alpha\beta)^{-1}$ is the measure of the deviation from hyperbolic behavior.

When $\alpha = 0$, there is no deviation and the forms of eqn. 25 linearized according to the schemes represented by eqns. A–E yield straight lines. On the other hand the effect of non-zero α values is illustrated in Fig. 13 in which $(\eta-1)/\beta$ is plotted against η since this type of plot is equivalent to that obtained by using eqn. B that has frequently been employed in the course of the present study. Simulation was carried out by assuming that the retention modulus of the complex is either 4.0 (Fig. 13A) or 0.25 (Fig. 13B). Comparison of the two plots shows that the magnitude of η_c has a significant effect on system behavior. The plots depicted in Fig. 13A reveal why apparently straight lines could be obtained by plotting data measured in a narrow range of conditions. It is seen that the intercept and slope, even the sign of the slope, is different for such "straight lines" from the ideal line at $\alpha = 0$. When complex formation reduces retention (see Fig. 13B), straight lines, which are almost paralliel to the ideal line, are obtained for $\alpha < 0.5$ at sufficiently high values of the retention modulus.

The effect of deviations from the simple mechanism, *cf.* eqn. 2, was also examined by using all linear transformations of eqn. 25, that is, those equivalent to eqns. A–E, in a fashion similar to that shown in Fig. 13. As expected, not all equations are equally sensitive to deviations from the "ideal" behavior, therefore, their utility as diagnostic tools for discerning changes in the physico-chemical mechanism of the chromatographic process are also different. When complex formation enhances retention, *i.e.*, $\eta_c > 1$, plots according to eqns. A and B are the most sensitive to

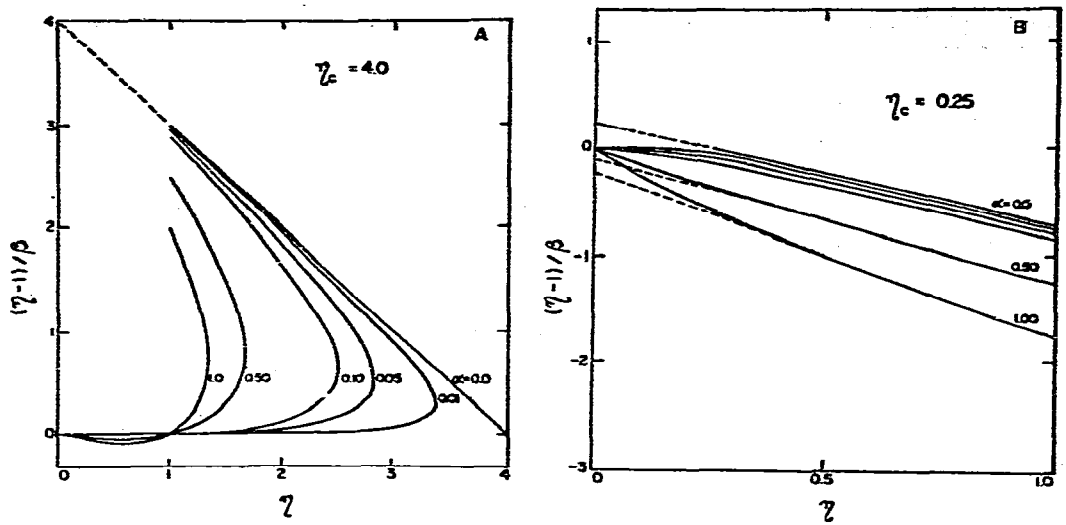


Fig. 13. A, Effect of deviations from the complexation model described by eqn. 2 due to appreciable hetaeron binding by the stationary phase as illustrated by departure from linearity in plots according to eqn. B. In the case shown, $\eta_c = 4$, that is, complex formation increases the retardation of the eluite. The parameter $\alpha = K_h/K$ measures the extent of hetaeron binding to the column surface relative to the eluite. B, Illustration of the same effect when $\eta_c = 0.25$, that is, complexation decreases the retardation factor. Deviation from ideal behavior is measured by the parameter α. Note that for $\alpha > 0.5$, *i.e.*, when both mechanisms become equally important, extrapolation of the linear portion of the curves give negative intercepts indicating that eqn. 2 does not hold despite the apparent linearity of the plot under certain conditions.

deviations from linearity as measured by the value of α and are strongly curved if a wide enough concentration range is examined. In the range of $0.1 \leqslant \beta \leqslant 10$, *i.e.*, when the range of hetaeron concentration encompasses two orders of magnitude around the equilibrium constant, plots according to eqns. A and B show a reversal of slopes even at $\alpha = 0.01$, whereas the other plots exhibit very small deviations from linear behavior. As expected, however, all plots strongly deviate from linearity in certain domain of η when $\alpha > 0.5$, *i.e.*, the two equilibrium constants, K and K_h are about the same and both mechanisms are equally important.

For illustrating the result obtained by using eqn. A, plots of the dimensionless form of eqn. 24 linearized according to eqn. A are depicted in Fig. 14. It is seen that for sufficiently high α values negative intercepts of the quasi-straight lines are obtained and thus the departure from the model can be diagnosed.

The diagnostic value of all plots is very low when complex formation decreases retardation, *i.e.*, $\eta_c < 1$. In such cases, the departure from linearity is relatively small so that plots of experimental data may be erroneously taken as straight lines yielding incorrect "apparent" values of the parameters. Only when both mechanisms are equally important, *i.e.* $\alpha \geqslant 0.5$, can deviations from the simple model be recognized because the linear portions of the plots give negative intercepts when extrapolated to $\eta = 0$.

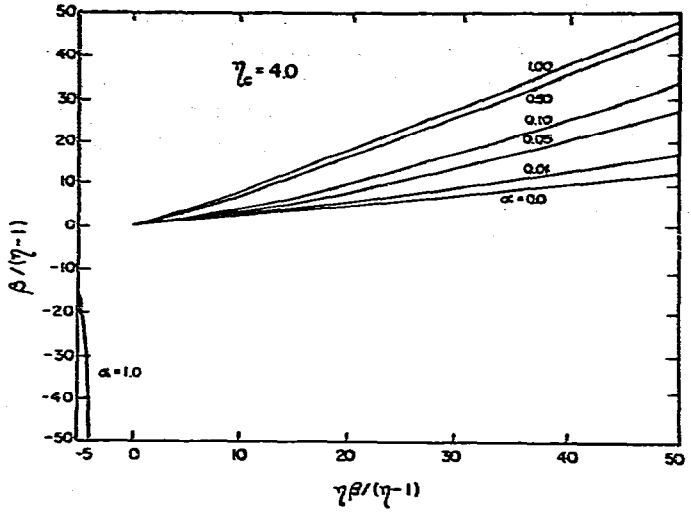


Fig. 14. Deviations from linearity in plots according to eqn. A due to the effect of appreciable hetaeron binding by the stationary phase. In the case illustrated $\eta_c = 4$, that is, complex formation increases the retardation factor. The relative magnitude of hetaeron binding is expressed by the parameter α.

Secondary equilibria in analytical work

A distinguishing feature of liquid chromatography is the wide range of mobile phase properties which can be exploited to obtain desirable retention factors with a given column. Secondary equilibria such as those investigated here may be used for "fine tuning" of the selectivity of a chromatographic system.

The effect of suitable secondary equilibria may be used to advantage in two ways. It may be possible to adjust the mobile phase composition by varying hetaeron composition in such a way that certain sample components form complexes and thereby retention or detectability can be manipulated in order to attain higher resolution or more convenient retention values. As shown in an earlier study the protonation of simple conjugate bases to form neutral acids results in a significant enhancement of the capacity factor. Thus, the retention of a charged eluite can be increased by converting it into a neutral species, and *vice versa*, by simply adjusting the pH of the eluent. The effect of Mg^{2+} in the eluent on the retention of ATP is not as dramatic, yet, it results in an increase of the retention by a factor of 1.8 at 0.02 *M* $MgCl_2$ (see Table III). On the other hand the presence of Mg^{2+} has virtually no effect on the retardation of AMP. Hence, ATP, which is otherwise only slightly retained on non-polar stationary phases could be separated from AMP with divalent metal ions in the eluent.

On the other hand addition of 10^{-4} and 10^{-2} *M* of K^+ and Zn^{2+} ions, respectively, to the eluent results in up to a tenfold decrease of the retention factor of DBC and NNSA. This effect can be used to bring about faster elution of these substances, *e.g.*, in order to reduce separation time or detect relatively low concentrations of these solutes. Alternatively the manipulation of retention may facilitate separations which otherwise would be impossible.

As shown in a recent review[12] the scope of the analytical applications of secondary equilibria in reversed-phase HPLC is rapidly expanding. Undoubtedly various novel hetaerons will be introduced in order to bring about specific changes in the retention behavior of certain sample components. Of course, secondary equilibria also may adversely affect analytical results due to poor peak shape. An understanding of the physico-chemical basis of such phenomena, however, can be of great help in adjusting conditions to get symmetrical peaks and high column efficiency.

CONCLUSIONS

HPLC with non-polar stationary phases offers a precision method for the evaluation of stability constant of complexes formed in solution as demonstrated by various examples. The advantages of the chromatographic method stem from the high precision and detector sensitivity of present day instruments and relative simplicity of reversed-phase chromatography both operationaliy and theoretically.

Modern HPLC apparatus, which enables the investigator to control accurately and quickly experimental conditions, offers means for convenient and rapid measurements. The use of precision eluent mixing devices can be particularly advantageous in measuring retention factors at various eluent compositions in order to evaluate the moduli.

Unlike all other methods, the use of HPLC affords measurements with impure samples without further purification because with properly chosen efficient chromatographic systems impurities are separated from the eluite and hetaeron–eluite complex. In our experiments commercially available substances were employed without further purification and in all cases peaks representing impurities were present on the chromatogram.

Because of the low solute concentrations in the eluent the solubility does not impose constraints on measurements by HPLC, whereas poor sample solubility often limits the employment of conventional techniques. Submicrogram quantities of a substance may be adequate to measure equilibrium constants by HPLC, in contradistinction to conventional methods.

The advantages of HPLC can be exploited for such physico-chemical measurements when a chromatographic system with an eluent, which is a proper medium for the equilibrium constant to be evaluated and yields conveniently measureable retention ratios, as well as a stationary phase, which is inert for the hetaeron, are available and very low eluite concentrations in the effluent can be monitored by a sensitive detector.

Nevertheless the method is applicable for the measurement of association constants only in those cases when the rate of equilibration in the mobile phase is rapid on the time scale of the chromatographic run. Otherwise poor peak shape and peak splitting render the evaluation of retention factors difficult without the computer. This may be one of the rare instances where the high speed of HPLC is an apparent disadvantage.

ACKNOWLEDGEMENTS

One of us (Cs.H.) wishes to thank M. Lederer for stimulating discussions and hospitality during sabbatical leave at the Laboratorio di Cromatografia, C.N.R., Rome, Italy. This work was supported by the respective Grants No. CA21948 and No. GM20993 from the National Cancer Institute and the National Institute for General Medical Sciences, U.S. Public Health Service, Department of Health, Education and Welfare.

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