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Influence of counter-ion inclusion complexation on the quality of cyclodextrin-supported separations in isotachophoresis

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ABSTRACT

The effect of counter-ion inclusion complex formation on the separation efficiency in electrolyte systems modified with β-cyclodextrin and its methyl derivatives was studied. On the basis of the analysis of complex-forming equilibria established in the sample mixed zone, it was concluded that an increasing stability of the counter-ion inclusion complex results n a decreased efficiency of the separation process. This was verified experimentally on the set of eleven aliphatic and aromatic acids used as counter-ions in slightly acidic cationic electrolyte systems.

INTRODUCTION

The ability of cyclodextrins (CDs) and their derivatives to interact with various types of compounds and form inclusion complexes, according to the sizes and shapes of the molecules, has made possible a wide range of analytical applications, especially in chromatographic and related electromigration methods [1–3]. A number of reported applications have confirmed the possibility of using CDs as structural selectors in capillary isotachophoresis (ITP) [4]. It was proved experimentally that the modification of ITP electrolyte systems by CDs and their derivatives may substantially improve the resolution of structurally related compounds [5–8] and various types of isomers, including enantiomers [7–12].

Cyclodextrins and their derivatives have proved to have many exceptional properties making them unique structural selectors in ITP. From the practical point of view it is important that CDs form most stable inclusion complexes in aqueous solutions rather than in organic solvents. In the commonly used pH range of ITP electrolyte systems the CD molecule does not carry a significant electric charge and therefore it does not migrate under the influence of an applied electric field. An average velocity of CD molecules in the separation compartment is given only by complex-

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forming equilibria with charged species from an electrolyte system. Owing to the high molecular mass, the velocity of CD molecules is estimated to be many times lower than the migration velocities of charged species. Therefore, the role of CD molecules dispersed in the separation compartment of an ITP apparatus may be compared with the role of the stationary phase in chromatography.

Although in most instances the utilization of CDs leads to a significant improvement in resolution of structurally related compounds, it may fail in some applications because of small differences in the stabilities of sample component complexes. A possible role of two limiting CD–solute interactions, introduced as effective and non-effective complex formation, on the quality of ITP resolution has been studied and widely discussed [13].

The aim of this paper is to extend the proposed model of effective and non-effective complex formation, considering the influence of competitive inclusion complexation of the components of electrolyte systems on the quality of sample resolution.

THEORETICAL

In a first approximation, let us suppose that it is possible to neglect inclusion complex formation between cyclodextrin and minor components of electrolyte systems such as anti-convective additives and possible trace impurities. Only complexation of leading ions, terminating ions and counter ions will be involved in our considerations.

The components of electrolyte systems must satisfy at least two fundamental conditions in order to alter effectively the sample–CD complex-forming equilibria. It is essential that the requirements of proper size and shape of complexed molecules or ions fit well into the cavity of the CD used and form stable inclusion complexes. Another important requirement is continuous contact with separating components of the sample in the whole volume of the sample zone. The distribution of ionic species and CD molecules in leading, sample and terminating zones during the analysis is outlined schematically in Fig. 1.

As shown in Fig. 1, leading ions are not present in the sample zone during ITP analysis. The area of coexistence of leading and sample zones where competitive complex formation may occur is restricted to the boundary B1. Chlorides, the most commonly used leading ions in anionic electrolyte systems, do not form stable

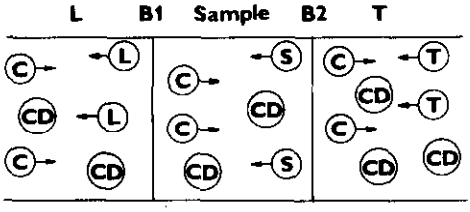

Fig. 1. Schematic distribution of ionic species and cyclodextrin molecules in leading, sample and terminating zones. C = Counter-ion; L = leading ion; T = terminating ion; CD = cyclodextrin molecule; B1 and B2 = zone boundaries.

inclusion complexes with β-CD and its methyl derivatives owing to their small ionic diameter and high charge density. Their contribution to complex-forming equilibria is considered to be negligible. For the same reasons it is not necessary to consider the complexation of NH_4^+ ions and the ions of alkali metals which are frequently used as leading ions in cationic electrolyte systems.

What is more significant is the complexation of CDs with ions of suitable size in the terminator. By using the correct injection technique ensuring minimum mixing of the sample with electrolyte system, the ions of the terminator are assumed not to be present in initial sample zone. As shown in Fig. 1, the area of competitive CD–terminator complex formation affecting the selective complexation of sample components is restricted to the moving boundary B2.

Critical for the quality of separation should be the competitive inclusion complexation of counter-ions. Many counter-ions used are able to fit well into the cavity of CDs and to form relatively strong inclusion complexes. From Fig. 1 it should be noted that the counter-ion–CD complex formation may occur in the whole volume of the sample zone during the analysis.

Simple considerations may be useful for characterizing the role of counter-ion inclusion complex formation in the ITP separation process. Let us suppose that three complex-forming equilibria with cyclodextrin are established in the mixed zone of two analyte components X and Y and the counter-ion.

$$X + CD \rightleftharpoons X\text{–}CD \quad \text{(I)}$$
$$Y + CD \rightleftharpoons Y\text{–}CD \quad \text{(II)}$$
$$C + CD \rightleftharpoons C\text{–}CD \quad \text{(III)}$$

The equilibrium reactions are characterized by conditional stability constants:

$$K'_X = \frac{[X\text{–}CD]}{[X][CD]_I} \qquad K'_Y = \frac{[Y\text{–}CD]}{[Y][CD]_{II}} \qquad K'_C = \frac{[C\text{–}CD]}{[C][CD]_{III}} \quad (1)$$

where [X–CD], [Y–CD] and [C–CD] are equilibrium molar concentrations of inclusion complexes, [X], [Y] and [C] are equilibrium molar concentrations of X, Y and C and $[CD]_I$, $[CD]_{II}$ and $[CD]_{III}$ are equilibrium molar concentrations of cyclodextrin not bound in inclusion complexes X–CD, Y–CD and C–CD.

The relationship between stability constant K and conditional stability constant K' is given by the equation

$$K'_{X(Y)} k_{X(Y)} = K_{X(Y)} \quad (2)$$

where $k_{X(Y)}$ is the coefficient of the side reaction for equilibrium I defined by

$$k_X = \frac{[CD]'}{[CD]} \quad (3)$$

The molar concentration of CD that is not bound in the X–CD complex, [CD]', may be expressed as

$$[CD]' = [CD] + [Y\text{–}CD] + [C\text{–}CD] \quad (4)$$

Analogously the coefficient of side reactions for equilibrium II is defined by

$$k_Y = \frac{[CD]''}{[CD]} \tag{5}$$

where

$$[CD]'' = [CD] + [X\text{–}CD] + [C\text{–}CD] \tag{6}$$

The degree of complexation of the component X(Y) in the mixed zone, $\alpha_{X(Y)}$, is defined by

$$\alpha_{X(Y)} = \frac{[X(Y)\text{–}CD]}{[X(Y)] + [X(Y)\text{–}CD]} \tag{7}$$

Combining the defined expression for K'_X (eqn. 1), and eqns. 2, 3 and 7, we obtain

$$\alpha_X = \frac{K_X[CD]'/k_X}{1 + K_X[CD]'/k_X} \tag{8}$$

Analogously by substituting from the defined equation for K'_Y (eqn. 1), eqns. 2 and 5 into eqn. 7, α_Y could be expressed as

$$\alpha_Y = \frac{K_Y[CD]''/k_Y}{1 + K_Y[CD]''/k_Y} \tag{9}$$

With coexistence of complex forming equilibria I–III the following condition must be satisfied:

$$[CD]'/k_X = [CD]''/k_Y = [CD]_E \tag{10}$$

where $[CD]_E$ = equilibrium molar concentration of free CD in the mixed zone.

From eqns. 8 and 9 it should be noted that the degree of complex formation, $\alpha_{X(Y)}$, is expressed as a function of the corresponding stability constant $K_{X(Y)}$ and equilibrium concentration $[CD]_E$. It can be proved that with decreasing $[CD]_E$ the degree of complex formation $\alpha_{X(Y)}$ decreases. This fact is clearly visible from computer-simulated $\alpha_{X(Y)} = f([CD]_E)$ dependence (curves 1 and 2) given in Fig. 2.

Combining eqns. 7, 8 and 11 (see ref. 13), it is theoretically possible to determine effective mobilities of complexed solutes $(U_X)_C$ and $(U_Y)_C$ for given $[CD]_E$ and known molecular masses M_X, M_Y, effective charge Z, molecular mass of used cyclodextrin M_{CD} and empirical coefficients a and b:

$$(U_X)_C = a + b \cdot \frac{|Z + \alpha_X \Delta Z|}{M_X + \alpha_X \Delta M_{CD}}$$

$$(U_Y)_C = a + b \cdot \frac{|Z + \alpha_Y \Delta Z|}{M_Y + \alpha_Y \Delta M_{CD}} \tag{11}$$

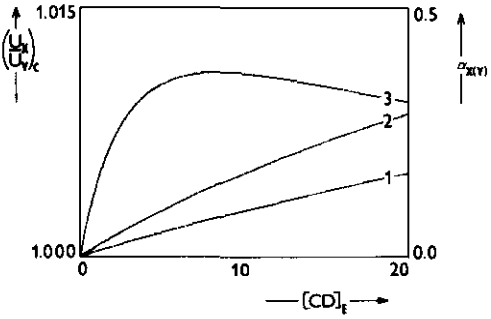

Fig. 2. Computer-simulated dependence of the degree of complexation $\alpha_{X(Y)}$ (curves 1 and 2) and of the ratio of effective mobilities $(U_X/U_Y)_C$ (curve 3) of complexed solutes X and Y on the molar concentration of free, uncomplexed cyclodextrin, $[CD]_F$ (mmol/l). Numerical values substituted in eqn. 11 (from ref. 13): $M_X = M_Y = 150$, $K_X = 10$, $K_Y = 20$, $Z = 0.5$, $M_{CD} = 1135$ (β-CD), $a = 69.85$, $b = 1558$.

The computer-simulated dependence of the ratio $(U_X/U_Y)_C$, characteristic for the separation quality, on changing the $[CD]_E$ value for the pair of enantiomers ($M_X = M_Y$) is shown in Fig. 2, curve 3. Characteristic for this dependence is the existence of a $(U_X/U_Y)_C$ maximum, a slight decrease in the mobility ratio for higher $[CD]_E$ values and a very fast decrease for low $[CD]_E$.

From eqns. 3–6 it results that $[CD]_E$ is a function of all considered stability constants and depends simultaneously on [X], [Y] and [C]. For a pair of components X and Y with given inclusion complex stability constants and concentrations in the mixed zone, the decrease in $[CD]_E$ value depends particularly on an increase in the stability of the C–CD complex caused by the choice of a counter-ion with a great affinity to the CD used. Even the simplified balance of the concentrations of components in the mixed zone during ITP separation confirms that the influence of counter-ion inclusion complex formation on the quality of CD-based separation is not marginal. In order to maintain electroneutrality, the molar concentration of counter-ions in the mixed zone must be equal to the sum of the molar concentrations of X and Y ions. Assuming similar values of the effective charges of counter-ion and sample molecules (*e.g.*, the case of enantiomers separated in a leading electrolyte buffered to a pH near to the numerical value of their dissociation constants), not only ionic but also their overall molar concentrations will be equal.

EXPERIMENTAL

Chemicals

Redistilled water was used in the preparation of the electrolyte and racemate solutions investigated. Hydroxyethylcellulose 4000 (HEC) (Serva, Heidelberg, Germany) was deionized by stirring its aqueous solution with Zerolit DM-F mixed-bed ion exchanger (BDH, Poole, UK). All other chemicals were of the highest quality commercially available and were used without any purification: hydrochloric acid (30%), acetic acid, propionic acid, butyric acid, valeric acid, caproic acid, trimethylacetic acid, 2-aminobenzoic acid, 4-aminobenzoic acid and 4-morpholinoethanesulphonic acid (MES) (Merck, Darmstadt, Germany); pyridine-2-carboxylic acid,

pyridine-3-carboxylic acid and pyridine-4-carboxylic acid (Fluka, Buchs, Switzerland); 6-aminocaproic acid (EACA) (Sigma, St. Louis, MO, USA); sodium hydrogenphosphate (Carlo Erba, Milan, Italy); and β-cyclodextrin (β-CD), heptakis(2,6-di-O-methyl)-β-cyclodextrin (diMe-β-CD) and heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin (triMe-β-CD) (Chinoin, Budapest, Hungary).

The solutes of pseudoephedrine (ΨE) and *p*-hydroxynorpseudoephedrine (NHΨE) racemates were produced by the Research Institute of Antibiotics (Roztoky, Czechoslovakia).

Methods

Isotachophoretic experiments were performed with a Tachophor 2127 system (LKB, Bromma, Sweden) equipped with a conductivity detector and poly(tetrafluoroethylene) (PTFE) capillary. Injections were made with a 10-μl Hamilton syringe.

The pH of the electrolyte solutions was measured with a Metrohm (Herisau, Switzerland) Model 605 digital pH meter using a combined glass electrode.

All computations were made on a DS-15 data station (Varian, N. Springvale, Australia) using our own software.

RESULTS AND DISCUSSION

The possible role of competitive counter-ion complex formation in ITP separation processes was experimentally verified on a set of aliphatic and aromatic acids (Table I), that could be used as counter-ions for slightly acidic cationic electrolyte systems (pH 4.0–5.5). The stabilities of inclusion complexes of the chosen acids were studied in anionic electrolyte system 1 (Table II). The leading electrolyte was consecutively modified by β-CD, diME-β-CD and triMe-β-CD in amounts corresponding to concentrations of 0.5, 1.0, 2.0, 4.0, 6.0 and 8.0 mmol/l. Details of the experimental conditions, composition and injected amounts of the sample are given in Table III.

TABLE I

ORGANIC ACIDS AND THEIR DISSOCIATION CONSTANTS, pK_A [14], CHOSEN FOR EXPERIMENTAL VERIFICATION OF THE INFLUENCE OF COUNTER-ION COMPLEX FORMATION ON THE QUALITY OF ITP SEPARATION

No.	Acid	pK_A
1	Acetic	4.756
2	Propionic	4.874
3	Butyric	4.820
4	Valeric	4.842
5	Caproic	4.857
6	Trimethylacetic	5.037
7	2-Aminobenzoic	4.939
8	4-Aminobenzoic	4.853
9	Pyridine-2-carboxylic	4.9[a]
10	Pyridine-3-carboxylic	4.819
11	Pyridine-4-carboxylic	4.8[a]

[a] Determined by titration.

TABLE II

ELECTROLYTE SYSTEMS USED[a]

No.	Leading ion	Counter-ion	pH_{LE}	Terminating ion
Anionic electrolyte system				
1	Cl^- (5 mmol/l)	EACA	4.46	MES (10 mmol/l)
Cationic electrolyte systems				
1	Na^+ (5 mmol/l)	Acetate	4.00	EACA (10 mmol/l)
–		–	4.50	
–		–	5.00	
–		–	5.50	
2		Propionate	4.49	
3		Butyrate	4.51	
4		Valerate	4.48	
5		Capronate	4.49	
6		Trimethylacetate	4.47	
7		2-Aminobenzoate	4.50	
8		4-Aminobenzoate	4.51	
9		Pyridine-2-carboxylate	4.50	
10		Pyridine-3-carboxylate	4.51	
11		Pyridine-4-carboxylate	4.49	

[a] All the leading electrolytes used contained 0.08% of HEC.

The dependence of the relative step heights, $(h_i)_{rel}$, of the zones with increasing concentration of β-CD is shown in Fig. 3A. Sodium hydrogenphosphate, added to each solute injected, was used as an internal standard for the determination of $(h_i)_{rel}$ values. The experimental points obtained were interpolated by a polynomial function of suitable degree. The quality of approximation was controlled by the sum of square deviations from the experiment, ΣS^2. The computed first root of the selected polynomial function, r_1, which represents the slope of the interpolation curve at its beginning (where the molar concentration of cyclodextrin in the leading electrolyte $c_{CD} = 0$), was utilized advantageously as a value characterizing the stability of the corresponding inclusion complex. For better orientation, the acids in Fig. 3A are ordered according to increasing stability of their β-CD inclusion complexes characterized by increasing r_1 value. In agreement with the theory of inclusion complex

TABLE III

CONDITIONS FOR ITP MEASUREMENT

Parameter	Anionic mode	Cationic mode
Capillary	250 × 0.55 mm I.D.	300 × 0.55 mm I.D.
Detection	Conductivity	Conductivity
Current	100 μA (7 min), 50 μA det.[a]	150 μA (6 min), 50 μA det.[a]
Temperature	18°C	18°C
Injected sample	1 μl of aqueous solution of acid 1–11 (20 mmol/l) + $Na_2HPO_4 \cdot 2H_2O$ (4 mmol/l)	(i) 1, 2, 3 μl of ΨE (6.34 mmol/l); (ii) 1, 2, 3 μl of HNΨE (5.43 mmol/l)

[a] det. = for detection.

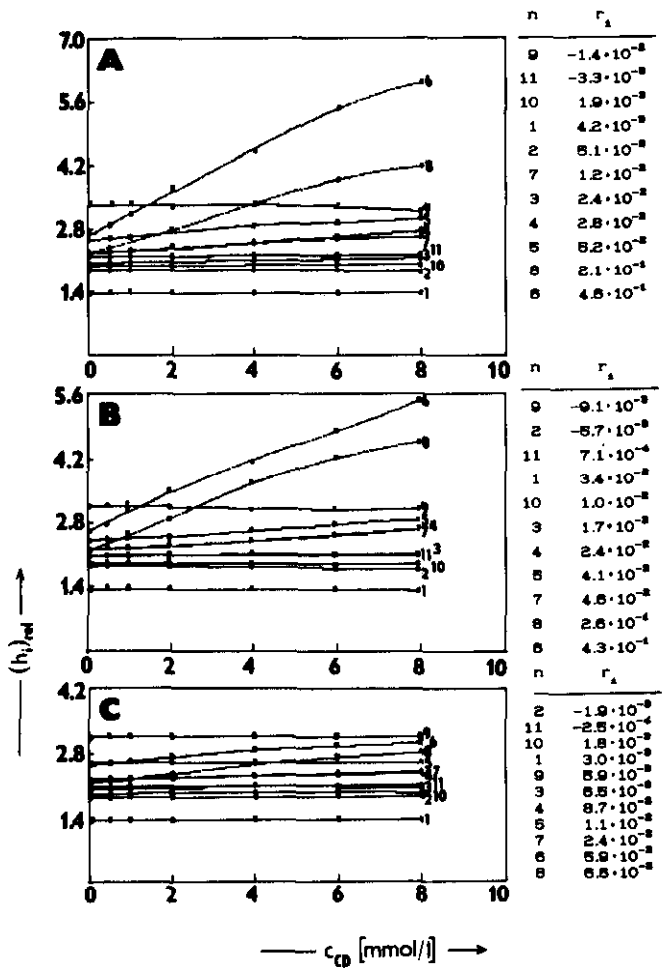

Fig. 3. Dependence of $(h_i)_{rel}$ values of acids 1–11 on increasing molar concentration, c_{CD}, of (A) β-CD, (B) diMe-β-CD and (C) triMe-β-CD in anionic electrolyte system 1. n = Index number of the acid according to Table I; r_1 = computed first root of approximating polynomial.

formation and known structural requirements on guest molecules, an increased stability of complexes may be observed for aliphatic acids with longer and especially branched chains (trimethylacetic acid). Also explained by theory is the observed difference in stability of *o*- and *p*-aminobenzoic acid complexes and the very low stability of β-CD complexes with all the studied pyridinecarboxylic acids.

The dependence of $(h_i)_{rel}$ values on increasing concentration of diMe-β-CD in the leading electrolyte, together with the list of studied acids ordered according to increasing stability of their complexes, are given in Fig. 3B. Unlike the above-mentioned complexation with β-CD, higher stabilities of *o*- and *p*-aminobenzoic acid complexes and lower stability of the trimethylacetic acid complex may be observed.

The complexation of the studied acids with triMe-β-CD is illustrated in Fig. 3C. From tabulated r_1 values it follows that only *o*-aminobenzoic, *p*-aminobenzoic, trimethylacetic and caproic acid are able to form more stable inclusion complexes.

After finishing the experiments in the anionic mode, which provided necessary data about the stabilities of inclusion complexes, the acids under examination were used as counter-ions in cationic electrolyte systems 1–11 (Table II). The racemates of ΨE and HNΨE were used as model samples for monitoring the effectiveness of the separation process with changing counter-ions. Both samples used are known to separate enantioselectively in a slightly acidic electrolyte system modified by β-CD or diMe-β-CD and, moreover, the HNΨE racemate could be resolved in the electrolyte system with triMe-β-CD [11].

In order to compare theoretical conclusions concerning the influence of counter-ion complex formation on the quality of separation with practical experiments, a suitable quantity characterizing the efficiency of the separation process must be chosen. The use of the maximum racemate load capacity, n_r [12], seems to be the most advantageous because of its availability from experiments.

The substitution of one counter-ion by another in the leading electrolyte induces changes in some experimental parameters. As follows from the theory of ITP, changes in the mobility of the counter-ion cause pH shifts in the sample mixed zone. This phenomenon may, however, alter the complex-forming equilibria with CD and change the separation capacity of the system without a contribution of competitive counter-ion complex formation. The role of changes in the pH of the leading electrolyte was verified experimentally by comparing the n_r values for ΨE obtained in β-CD-modified electrolyte system 1 and its analogues with leading electrolyte pH values ranging from 4.0 to 5.5. The slight and non-significant increase in n_r observed with increasing pH of the leading electrolyte indicates that the changes in pH in the mixed zone do not alter the quality of enantioselective resolution (Fig. 4). Therefore, all changes in n_r values in the following experiments should be assigned to the influence of competitive counter-ion complex formation.

The dependence of n_r values for ΨE and HNΨE on the increasing stability of counter-ion inclusion complexes (r_1 value) in cationic electrolyte systems 1–11 modified with β-CD (8 mmol/l) is shown in Fig. 5A. It is obvious that the best chiral resolution is achieved in electrolyte systems 1, 9, 10 and 11 containing acetate or

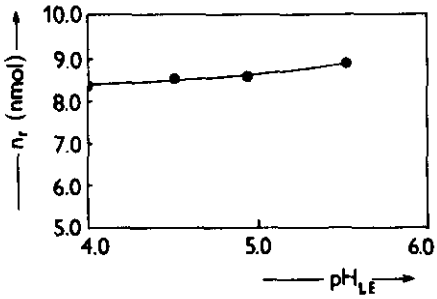

Fig. 4. Dependence of the maximum racemate load capacity n_r for ΨE on the pH of the leading electrolyte 1 modified with β-CD (8 mmol/l).

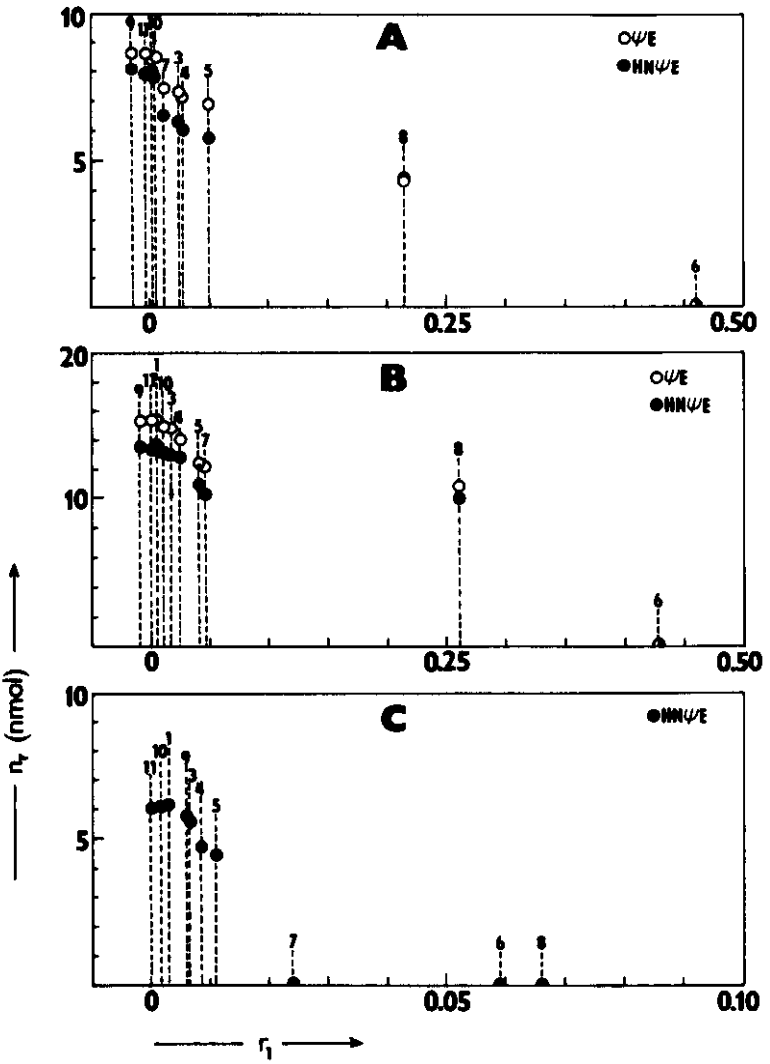

Fig. 5. Dependence of the maximum racemate load capacity n_r for ΨE and HNΨE on the stability of the counter-ion inclusion complex, characterized by the first root of the approximating polynomial function, r_1, in cationic electrolyte systems 1–11 modified with (A) β-CD, (B) diME-β-CD and (C) triMe-β-CD.

pyridinecarboxylate as the counter-ions with low affinity to β-CD. With increasing stability of the counter-ion inclusion complex, the maximum racemate load value steadily decreases. In electrolyte system 6 with the most complexed trimethylacetate, the chiral resolution of both racemates completely disappears.

The dependence of n_r for ΨE and HNΨE on the stability of counter-ion inclusion complexes, measured in electrolyte systems 1–11 modified with diMe-β-CD (8 mmol/l), is shown in Fig. 5B. As in the previously discussed example, the chiral resolution of both racemates deteriorates with increasing stability of the counter-ion

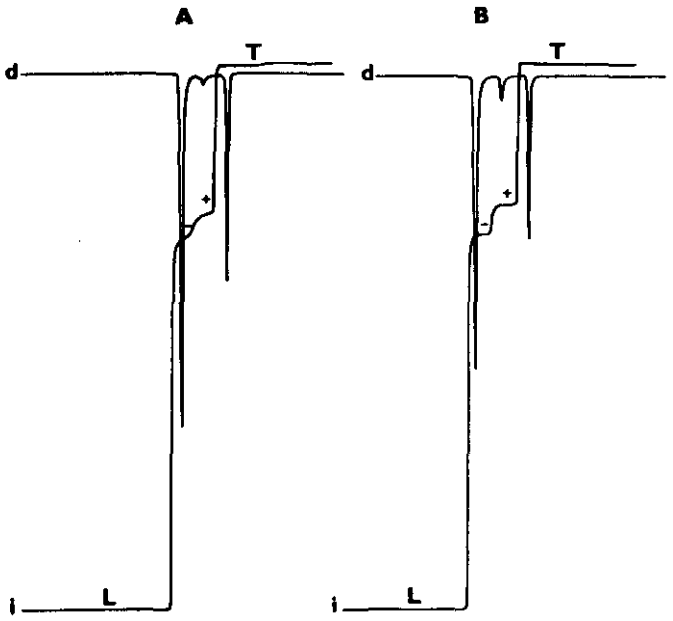

Fig. 6. Separation of ΨE enantiomers in cationic electrolyte systems (A) 9 and (B) 1 modified with β-CD (8 mmol/l). Conductivity detection.

inclusion complex. Similar conclusions may be drawn for th systems modified with triMe-β-CD (Fig. 5C). Critical is the decrease in n_r in electrolyte systems 6–8 containing the counter-ions significantly complexed by triMe-β-CD.

According to the n_r values obtained, the systems with aromatic and aliphatic uncomplexed or a few complexed counter-ions are nearly equal. By a more detailed study of the conductivity detector recordings obtained, it is possible, however, to find the differences in th boundary sharpness. As is shown in Fig. 6, the zone boundary between separated enantiomers is much sharper in an electrolyte system containing an aliphatic counter-ion than in a system with an aromatic counter-ion. The explanation of this phenomenon, which is probably connected with the decreased rate of sample inclusion complex formation in the presence of an aromatic counter-ion, must be found in the theory of kinetics of inclusion complex formation and decomposition [15].

CONCLUSIONS

On the basis of a theoretical analysis of complex-forming equilibria in the sample mixed zone and associated experiments, it was possible to demonstrate unambiguously the influence of counter-ion inclusion complex formation on the separation efficiency of the system modified with β-CD and its methyl derivatives.

The theoretically possible case when the separation in systems with more complexed counter-ions is more effective than in those with less complexed counter-ions [13], resulting from the existence of the maximum of $(U_X/U_Y)_C = f([CD]_E)$ (Fig. 2), was not confirmed experimentally. It is probable that in the range of optimized CD concentrations and possible stability constants of the sample inclusion complexes,

the $[CD]_E$ values are very low and only a significantly negative effect of counter-ion complex formation may be observed.

The measurements described made it possible to select the acids with a low affinity to β-CD and its methyl derivatives from the set examined, which could be used successfully as counter-ions in slightly acidic cationic electrolyte systems modified with β-CD and its methyl derivatives. According to the separation efficiency achieved and the sharpness of the zone boundaries, the most promising seems to be the use of electrolyte system 1 with acetate as counter-ion.

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