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ISOTACHOPHORESIS

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INTRODUCTION

Electrophoretic separation techniques nowadays seem to have an inevitable liaison with protein chemistry and chromatography. The early developments on electrophoresis however were in the field of low molecular weight substances and colloids. Moreover chromatography at the turn of the century was still in its infancy.

A very important discovery was made by Hardy[1] in 1899. He noticed that proteins may be either negatively or positively charged, depending on the acidity of the solution. It was in fact Michaelis[2] who revealed the potential strength of electrophoresis on the separation and characterisation of proteins. Substantial experimental improvements in electrophoretic techniques were introduced by Svedberg and Tiselius in 1926[3]. The real importance of electrophoresis for protein chemistry was stipulated by the wonderful work of Tiselius[4], who in 1937 described in detail his moving boundary equipment, which won him the NOBEL PRIZE. Although Tiselius was convinced of the general analytical applicability of

electrophoresis, from that time a particularly close link between electrophoresis and proteins was established. In the early sixties this association was reinforced by the ingenious work of Ornstein[5] and Davis[6], with their development of discontinuous electrophoresis. Isoelectric focusing, developed about the same time by Svensson[7], in addition, suggests a strong relation.

From the start however there was a problem which always worried research workers: stabilisation in electrophoresis. In fact the liaison electrophoresis-chromatography was born out of this. It must be emphasised that that liaison in many respects was quite beneficial for electrophoresis.

After World War II chromatography developed in a rather spectacular way. Although Tswett[8] was first to introduce liquid chromatography, it was Martin and Synge[9] who perceived the requirements for a new analytical method: gas chromatography. Since then the gas chromatography has progressed through a phenomenal development. The potential of liquid chromatography nevertheless was appreciated, hence the reason for this technique to develop so extensively.

Electrophoresis however stopped about at the same level of utility as that of paper chromatography. The development of detection systems with high response and sensitivity however can be the turning point to the development of electrophoresis. As a result Isotachophoresis has reached a point where it competes with chromatographic techniques. Isotachophoresis has applications in many fields, especially with low molecular weight ionic substances, and has advantages due to its high resolution capabilities, short time of analysis, high accuracy and tremendous flexibility. It now seems that the general applicability of electrophoretic separation techniques may become evident and be given the attention it deserves.

. THE CONCEPT OF MOBILITY.

.0. Introduction.

The concept of mobility plays an important role in lectrophoresis, since the effective mobility determines n many cases (with the exception of isoelectric focuing), whether electric charged species can be separated. he effective mobility, i.e. the actual mobility, μ, nder operational conditions, is given by the ratio of lectrophoretic velocity, v, and electric field strength, .

$$v_{electrophoresis} = \mu_{effective} E \qquad (1)$$

onsisting of two well defined experimental parameters, he effective mobility, in practice, is a rather easily easurable quantity.

There have been several theoretical approaches for he calculation of electrophoretic mobilities. From the hysical point of view, they must be defined in terms f electric attraction forces, Stokes friction, relaxaion effects, etc[10,11]. Following the lines of fluid ynamics the theoretical models, for the moment, still how a lack of practical value. Considering only ionic lectrolyte solutions, correlation of mobility to paraeters, such as charge, shape, and size, gives a somehat more useful theory of ionic mobility[12].

From the analytical point of view however, it is ather obvious that electrophoretic mobility is strongly elated to physico-chemical parameters, such as dissoiation, solvation and complex formation. These are arameters that are, in common practice, easily measurble and that are, as will be shown, of decisive and nvaluable importance.

1.1. Charge transport and conductance.

In electrophoresis we are dealing with the migration, i.e. directed movement, of electrically charged species in a solvent. This means that it is essentially a charge transport process and that Ohm's law is valid:

$$V = I\ R \tag{2}$$

In this form however the relation might appear to have little connection with the chemical nature of electrolyte solutions. The relevancy of Ohm's law can be regained using the specific conductance of a solution.

$$\kappa = \frac{1}{R}\frac{\ell}{O} \tag{3}$$

Now Equation (2) may be rearranged to its "chemical equivalent", that is called a modified Ohm's law.

$$j = \kappa\ E \tag{4}$$

The advantage of the modified Ohm's law is that the electric field strength, an electrophoretic parameter, can easily be converted into a chemical one, i.e. the specific conductance.

In general, the electrical conductance of a solution is the summation of contributions from all charged species present. In spite of the fact that it is a non-specific property, conductance gives useful information of the charged species present in a solution and the interactions with the solvent. Obviously electrical conductance is strongly influenced by the number of charged particles. Moreover conductance is a function of temperature. Contrary to electronic (metallic) conductance, which most generally decreases with increasing temperature, the opposite is true for most electrolytic, or ionic, conductors.

From Table I we can see that the specific conductance is almost doubled by a temperature increase of 25°C.

1.2. Conductance and mobility.

As can be seen from Equation (3), the specific conductance has some geometric dimensions. In addition however the specific conductance is strongly influenced by the number of electrically charged particles per unit of volume, i.e. by concentration. Since this is a chemical important parameter, it seems beneficial to use the specific conductance per unit of concentration, which is known as the equivalent conductance Λ_c,

$$\Lambda_c = \frac{1000\ \kappa}{c} \qquad (5)$$

At the end of the last century Kohlrausch established two important features concerning electrolytic solutions.
i. There exists a limiting value for the equivalent conductance. This he expressed in a semi-empirical relation that is valid for strong electrolytes up to 10 meq/l.

$$\Lambda_c = \Lambda_\infty - k\sqrt{c} \qquad (6)$$

TABLE I

The temperature dependence of the specific conductance of a 0.01 M. KCl solution[13]

T(°C)	0	15	25	35	45	50	75
$\kappa * 10^5$ ($\Omega^{-1}cm^{-1}$)	77.4	114.3	141.3	169.8	199.6	215.2	295.2

This relation has been extended by various other scientists, e.g. Debye, Hückel, Falkenhagen, Fuoss[14]. Weak electrolytes do not show this behaviour and should be treated according to the theory of Arrhenius.

ii. At infinite dilution the equivalent conductance contains two additive components:

$$\Lambda_\infty = \lambda_\infty^+ + \lambda_\infty^- \qquad (7)$$

This law of Kohlrausch states that at infinite dilution the conductance of an electrolyte, NaCl for example, depends on the independent contributions from Na^+ and Cl^-. This principle can be successfully applied for the determination of the equivalent conductance at infinite dilution of weak electrolytes, for which Equation (6) is not valid.

In comparing the dimensions of both electrophoretic mobility and equivalent conductance, we must conclude that they differ only in the Faraday constant:

$$\frac{cm^2}{\Omega eq} = \frac{coul}{eq} * \frac{cm^2}{Vsec} \qquad (8)$$

$$\lambda_\infty = F\,|\mu_\infty|$$

TABLE II

The additivity principle

Species	$(cm^2\Omega^{-1}eq^{-1})$	Species	$(cm^2\Omega^{-1}eq^{-1})$	Δ
HCl	377.0	NaCl	108.1	268.9
HIO_3	345.1	$NaIO_3$	76.7	268.4
Δ	31.9		31.4	

TABLE III

The determination of the equivalent conductance of acetic acid.

$\Lambda_{\infty,CH_3COOH}$	=	$\Lambda_{\infty,CH_3COONa}$	+ $\Lambda_{\infty,HCl}$	- $\Lambda_{\infty,NaCl}$	
$\Lambda_{\infty,CH_3COOH}$	=	91.0	+ 426.0	- 126.5	= 390.5

The mobilities at infinite dilution are called ionic mobilities and can be obtained, accordingly to Equation (8) from conductance measurements. In Table IV are given some ionic mobilities derived in this way. The temperature coefficient of ionic mobilities again is positive and lies between +1 and +2 % per ^{o}C of temperature increase. Furthermore ionic mobilities are dependent on the nature of the solvent. In fact the ionic mobility

TABLE IV

Ionic mobilities at 25°C[13].

Cationic species	$\mu_\infty * 10^5$	Anionic species	$\mu_\infty * 10^5$
H^+	362.5	OH^-	-204.1
Li^+	40.1	F^-	- 56.4
Na^+	52.4	Cl^-	- 79.2
K^+	76.2	Br^-	- 81.0
Rb^+	79.2	I^-	- 79.6
Cs^+	82.8	ClO_3^-	- 67.0
NH_4^+	77.0	ClO_4^-	- 70.4
Mg^{2+}	55.0	SO_4^{2-}	- 83.7
Ca^{2+}	61.7		

is a rather complicated function of viscosity, dielectric constant and structure of the solvent, as well as the charge, shape and size of the ionic species.
Now ions are not the only particles that migrate in an electric field. It should however be obvious that in the case of colloids, cells etc., the mobility at infinite dilution has no real significance.

1.3. The effective mobility.

In electrophoresis we are never working under the ideal conditions of infinite dilution. This of course means that we are dealing with "effective" mobilities, that may substantially differ from ionic mobilities. Accordingly to Equation (1) the effective mobility in the absence of hydrodynamic flow of solvent is given by:

$$\mu_{eff} = \frac{v}{E} \tag{9}$$

The causes of the deviations can be classified according to their origin.

i. Deviations are caused by the electrolyte system. This means that corrections for dissociation, complex formation and concentration must be taken into account.

ii. Deviations are caused by the supporting medium. Corrections for adsorption, permeability, tortuosity and viscosity must be taken into account.

In practice all causes interact in a rather complicated way. Nevertheless an approximation, which has a practical value, can be given, Equation (10):

$$\mu_{eff} = \sum_i (\alpha_i \gamma_i f_i \mu_{i,\infty})_T \tag{10}$$

μ_{eff} is the effective mobility at temperature T.
α_i is the degree dissociation for the species i.
γ_i is the activity coefficient for the species i.
f_i is the retardation coefficient for the species i.
$\mu_{i,\infty}$ is the ionic mobility for the species i.

2. ELECTROPHORETIC PRINCIPLES.

2.0. Introduction.

In all electrophoretic processes there will be a transport of electrically charged species. The theoretical aspects of boundary formation and concentration distribution were first studied by Kohlrausch[15], as early as 1897. He showed that the change of concentrations in electrophoresis was not an arbitrary process, but a strongly regulated one.
Electrophoresis can be considered as a transport-process of charge carrying species in a conductor, that consists of a solution containing different electrolytes with an arbitrary local concentration[16,17,18]. It can be shown that for this electrically conducting system a function must hold, regardless of the intensity of the electric driving current and the geometric dimensions of the conductor. This function he called "die beharrliche Funktion" (i.e. "the regulating function").

$$\frac{\delta}{\delta t}(\omega) = \frac{\delta}{\delta t}\left(\sum_i \frac{c_i}{\mu_i}\right) = 0 \qquad (11)$$

or

$$\sum_i \frac{c_i}{\mu_i} = \text{constant} \qquad (12)$$

2.1. Parameters.

In electrophoresis there are three important parameters:

i. The electric field.
ii. The supporting medium.
iii. The electrolyte system.

Before we discuss these various principles of electrophoresis, some attention will be given to the parameters and their importance.

2.1.1. The electric field.

Electrophoresis always implies the use of an electric field in order to perform a separation. Because in electrophoretic separations we try to obtain a well defined movement of the charged particles, there are three different ways open to generate the electric field:

i. Using a current stabilised power supply. During the electrophoretic experiment the electric current will be constant, which means that the voltage will vary as a function of time.
ii. Using a voltage stabilised power supply. Consequently this implies that the electric current will vary as a function of time, while the voltage remains constant.
iii. Using a wattage stabilised power supply. During the experiment the output wattage of the power supply is constant, both the current and the voltage output may vary as a function of time.

The electric current, together with the resistance of the electrolyte system, induces a temperature effect. According to Joule's law the amount of heat produced is:

$$w = j\ E \qquad (13)$$

nce, temperature differences can exist in an elec-
ophoretic system, depending on the local electrical
eld strength and the current density. These tempe-
ture differences influence the electrophoretic
ocess: effective mobilities will change, solvent
y evaporate, denaturation and/or decomposition of
nstituents may occur. In some cases temperature
fects can be levelled by effective thermostating.
nce power dissipation is a local phenomenon, neither
the three modes for generating the electrical
eld guarantees a uniform temperature. It seems that
e constant current mode has some advantages in
lytical electrophoresis, while the constant power
le is more applicable in preparative methods.

1.2. Supporting media in electrophoresis.

In electrophoresis a supporting medium is always
ded. In addition stabilisation against convective
turbances is often necessary. Convection in electro-
resis is mainly caused by density and temperature
ferences, which are generated by the electrical field
by other operational conditions such as gravity,
fficient thermostating etc. Nevertheless in most
es convection can be limited using appropriate
porting media and stabilising agents. Both however,
selectively influence the mobility of the electro-
ic constituents. Stabilisation may be achieved in
ious ways. It can be obtained by increasing the
cosity of the solution by the use of soluble polymers.
monly used linear polymers are: agarose, hydroxy-
yl cellulose, polyacrylic amide, etc. More stabili-
ion is obtained by using crosslinked gels such as:
yacrylic amide and Sephadex^{R}.

In addition such gels can increase the selectivity according to the mole-sieve principle. Another possibility is the use of density gradients, for which components without electrical charge must be chosen as for example sucrose, glycerol, ethylene-glycol etc. Stabilisation can be obtained by the use of capillary systems like paper or cellulose-acetate. A comparable type of stabilisation is obtained when packed columns are employed. Rotation of the separation chamber also gives stabilisation against convective disturbances due to gravity. We, in our laboratory working on the analytical scale, use a large inner surface-area to volume ratio of narrow bore tubes as an anticonvective "carrier". In addition the use of solvent soluble polymers increases the performance and the selectivity.

2.1.3. The electrolyte system.

The composition of the electrolyte system plays a dominant role in all electrophoretic separation principles. One of the most important demands is an appropriate electrical conductance, because there is always a transport of electrical current. Required of the solvent is generally a high ionizing power with a low inherent conductance. Water seems to be the most promising compromise between these two seemingly contradictory requirements. The electrolytes used, must have two important parameters; the specific conductance (Equation 14) and the ionic strength (Equation 15).

$$\kappa = F \sum_i c_i |\mu_i| \qquad (14)$$

$$J = \tfrac{1}{2} \sum_i c_i z_i^2 \qquad (15)$$

n most experiments the specific conductance (κ) will ie between 10^{-2} and $10^{-5}\Omega^{-1}cm^{-1}$. The ionic strength J) will vary mostly between 10^{-1} and 10^{-3}. Within hese limits the choice is problem-dependent. Further- ore it is important that the electrophoretic process s not influenced by electrochemical effects, caused by lectrodereactions: hence buffered systems are generally referred. Stability, purity and solubility of all com- onents involved are obvious requirements.

s has been shown in Section 1.3, the separation of two omponents is strongly dependent on the difference in heir effective mobilities. The effective mobility can e regulated by parameters such as dissociation, com- lexformation and solvatation. In Figure 1 is shown the nfluence of these parameters on the effective mobility f some components.

e can see, for example, that citric acid, and α-keto- lutaric acid have equal effective mobilities in the ystem A and therefore can not be separated in this ystem. Using a complexing agent as in system B, these ids now show a large difference in their effective obilities. Changing the solvent as in system C induces ly a slight difference. The effect of dissociation n be seen from the systems C and D. It is again ob- ous that the choice of an appropriate electrolyte ystem is determined by the analytical problem that has be solved. It is therefore important to note that by ese physico-chemical parameters selectivity in electro- oresis can be varied. In practice this means that ffective mobilities deliberately may be varied in the nge of $+100 * 10^{-5}$ to $-100 * 10^{-5}$ $cm^2V^{-1}sec^{-1}$.

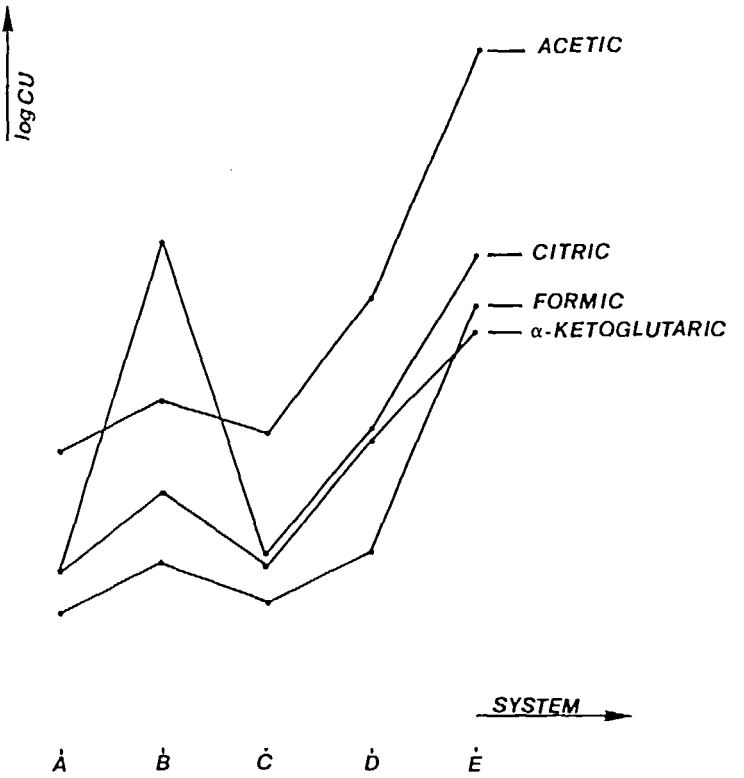

FIGURE 1
The influence of an electrolyte system on the effective mobility. A; pH = 6, solvent water. B; pH = 6, complexing agent Ca^{2+}. C; pH = 6, solvent heavy water. D; pH = 4.5, solvent water. E; pH = 3, solvent water.

2.2. Basic principles.

There are several criteria that can be chosen to classify the various electrophoretic separation techniques[19]:

i. The level at which the separation will be performed: analytical or preparative, high voltage or low voltage, etc.

ii. The method of stabilisation: paper, polyacrylic-amide gel, density gradient, etc.

iii. The choice of the electrolyte system and its arrangement with respect to the electrical field.

Only the last criterion allows for a simple and systematic classification. According to this criterion the basic principle never can be determined by the available equipment. The choice of the electrolytes, their composition and their position in the available equipment determines which basic principle of electrophoresis will be applied. In this way all electrophoretic techniques can be classified into essentially four principles.

ZONE ELECTROPHORESIS
MOVING BOUNDARY ELECTROPHORESIS
ISOTACHOPHORESIS
ISOELECTRIC FOCUSING

Combinations of these principles with one another can be made: e.g. "disc" electrophoresis is a combination of isotachophoresis and zone electrophoresis. Moreover combinations with other separation techniques and methods for detection can be made: with chromatography, immunochemistry, etc.

2.2.1. Zone electrophoresis.

In zone electrophoresis the entire system, i.e. anode, cathode and separation compartment, is filled with the same electrolyte mixture. The composition of this so-called "background" electrolyte is such that the introduction of a sample hardly changes the local composition of the background electrolyte.
At place S, Figure 2a., a sample consisting of cations A and B and the anionic species C, has been introduced. Applying an electric field, (Fig. 2b.) the various ionic

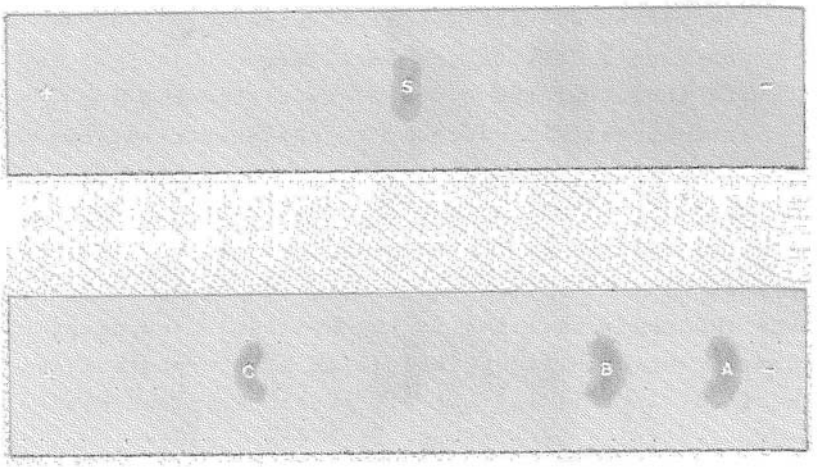

FIGURE 2
Zone electrophoresis.

species will start to move with a velocity that is determined by their respective effective mobilities and the electric field strength. Obviously both the ions of the background electrolyte and of the sample will move simultaneously. During the electrophoretic process ions of the background electrolyte will move continuously through the zone(s) formed by the sample ions. This is one of the most important characteristics of zone electrophoresis; zone electrophoresis is comparable with the chromatographic elution principle.

It is rather obvious that the anionic and the cationic species of the sample will separate without many problems. Whether the cationic constituents of the sample, A and B will separate, depends on the difference in their effective mobilities. As has been discussed in Section 1.3, this is determined by the ionic mobility and various operational conditions. In zone electrophoresis dispersion plays an important role, peak-broadening and

diffusivity of the zone-boundaries increases with time. Adsorption to the supporting medium may cause tailing. Because the ratio of the concentration of sample ions to carrier ions is small, in general only specific methods of detection can be applied. This is almost always done after the experiment has been carried out, using a sample specific chemical reaction.

If the buffering capacity and the concentration of the background electrolyte is high enough, the conductance, the electric field strength, the pH and the temperature over the entire system will be constant in time and place. Some of these electrophoretical important parameters are schematically shown in Figure 3, valid only under specific assumptions.

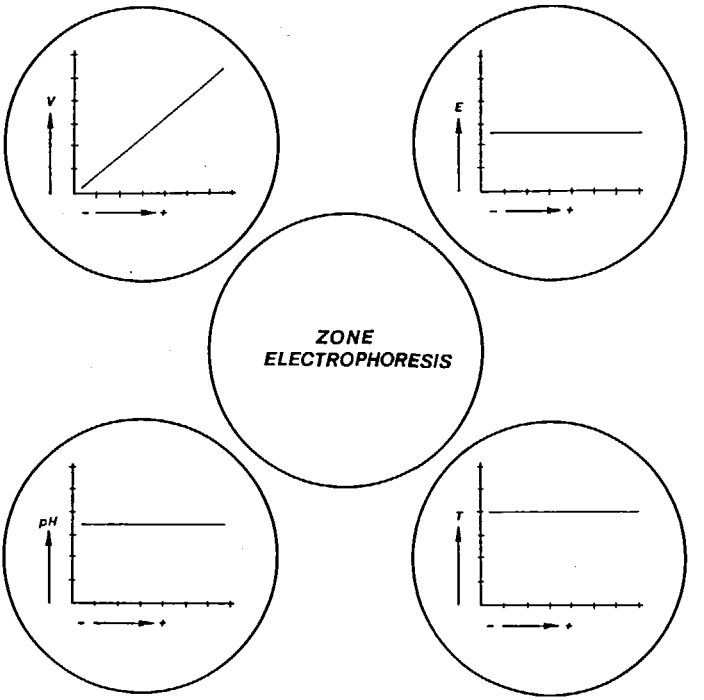

FIGURE 3
Parameters in zone electrophoresis. V: potential, E:

2.2.2. Moving boundary electrophoresis.

In moving boundary electrophoresis one has to decide whether anionic or cationic species are going to be separated. We will discuss the separation of two cations. The anode compartment, Figure 4, is initially filled, with a sample containing the cationic species A and B and an appropriate counterionic species. The separation and cathode compartment are filled with a so-called "leading electrolyte". The effective mobility of the "leading ion" L has to be higher than the effective mobilities of the cationic sample constituents. So, for the example shown in Figure 4, we assume:

$$\mu_{eff,L} > \mu_{eff,A} > \mu_{eff,B}$$

Both the concentration of the leading ion and its anionic counter-ion are chosen for an optimal buffering capacity. Due to the fact that the leading ion L has the

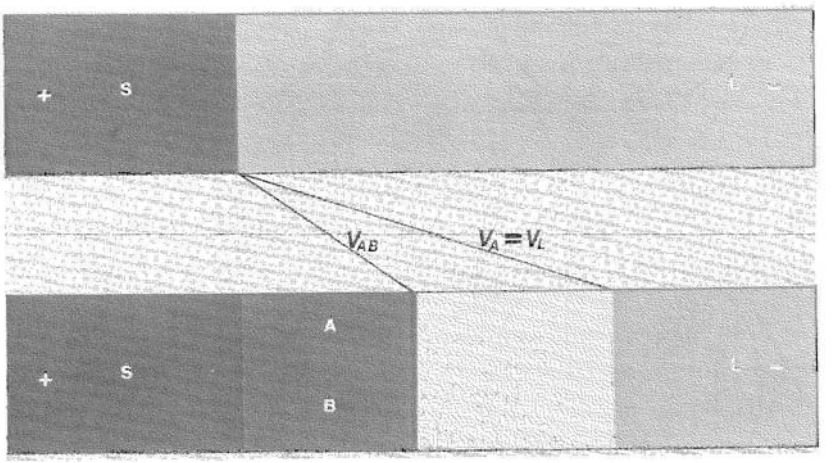

FIGURE 4
Moving boundary electrophoresis.

highest effective mobility, this ionic species will migrate in front of all other cationic species, when an electric gradient is applied. Behind this "leading zone" two other zones will be formed, within a frame of two migrating zone boundaries. The first of these two zones contains only the cationic species A and the counter-ionic species from the leading electrolyte. The second zone will contain both the cations A and B and again the counterion from the leading electrolyte. Since the anions all migrate in anodic direction the counterionic species of the sample can not enter the separation compartment. Since the supply of sample-constituents is not limited there will never be a complete separation.
This principle of separation can be compared with frontal analysis in chromatography.
We now will focus our attention on the development of a moving boundary process with respect to time. Figure 5a shows the situation in which the leading electrolyte fills the separation compartment. We will consider a volume-element, indicated by α.
Due to the action of an external electric field this volume has been refilled, after some time, with another electrolyte, Figure 5b. The question now is, whether the composition of this "new" electrolyte can be arbitrary or not. Kohlrausch[15] concluded that the changing of electrolyte concentrations by means of an external electrical field is a strictly regulated process.

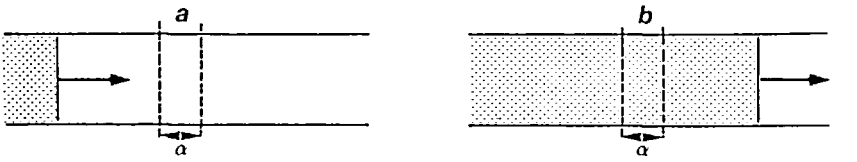

FIGURE 5
Electrolyte changes due to an electric field.

For simplicity we will make some assumptions, that are however immaterial for the regulating function concept. We assume a constant current density, a uniform cross section, no temperature effects and no hydrodynamic flow of solvent. Because the volume α has to conduct a well defined current, there will always be present, in this volume, a certain amount of charge carriers, for example ions, with their own characteristic velocity. Now the concentration of charge carriers in the volume α is a problem of in- and outflow of charge carriers. Velocity in electrophoresis is the product of effective mobility and electric field strength. The last quantity is related to the specific conductance by the modified Ohm's law, whereas the effective mobility is determined by chemical equilibria and ionic mobility. Hence, during the electrophoretic process four fundamental laws have to be obeyed: conservation of mass, the principle of electroneutrality, the chemical equilibria and the Ohm's law. All this, results in a regulating function with a very discrete output in terms of chemical and physical variables such as concentration, pH, conductance and boundary velocity. For the example of Figure 5 this means that the numerical value of the regulating function, ω_α, for the given volume α can not be changed by the electrophoretic process. The original electrolyte, which determines, ω_α has been replaced by a new electrolyte in such a way that the numerical value for both situations is the same.

The velocity with which this process proceeds is mainly determined by the current-density.

For the moving boundary process, as shown in Figure 4, this implies that all electrophoretic variables in the separation compartment are matched with those of the leading electrolyte. Of course the regulating function

concept is also applicable for the other compartments, though the numerical values may differ.
Since the composition of the leading electrolyte does not change, due to the presence of an electric field, the adapted zones will not do this either. In Figure 6, are shown some important characteristics in moving boundary electrophoresis. The separation procedure as a function of time for moving boundary electrophoresis

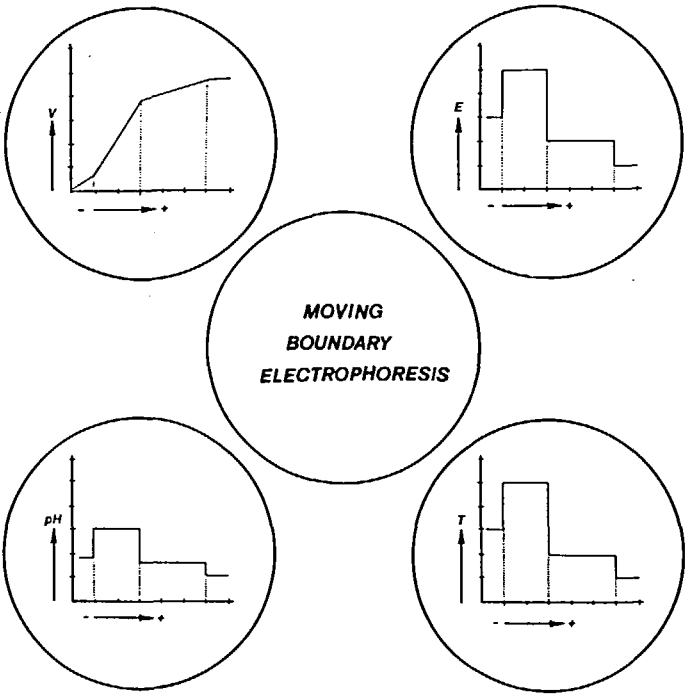

FIGURE 6
Characteristics of moving boundary electrophoresis.
V: potential, E: electric field strength, T: temperature.
Special attention is called to the constancy per zone boundary of the electric field strength, the temperature and pH. This not only holds for the first sample zone migrating behind the leading zone, but also for the zones where more sample constituents are present.
In isotachophoresis these zones are called "mixed-zones".

is given in Figure 7. Again, assuming a constant current density, it is easily shown that, according to the modified Ohm's law and the regulating principle, the electric field strength can be used as a characteristic property.

Starting from the original situation, Figure 7.1, two boundaries are formed. The cationic species L in the leading zone will migrate with a velocity given by:

$$v_L = \mu_{eff,L} E_L \qquad (16)$$

This of course means that the first boundary will migrate at a constant velocity. The first sample zone consists

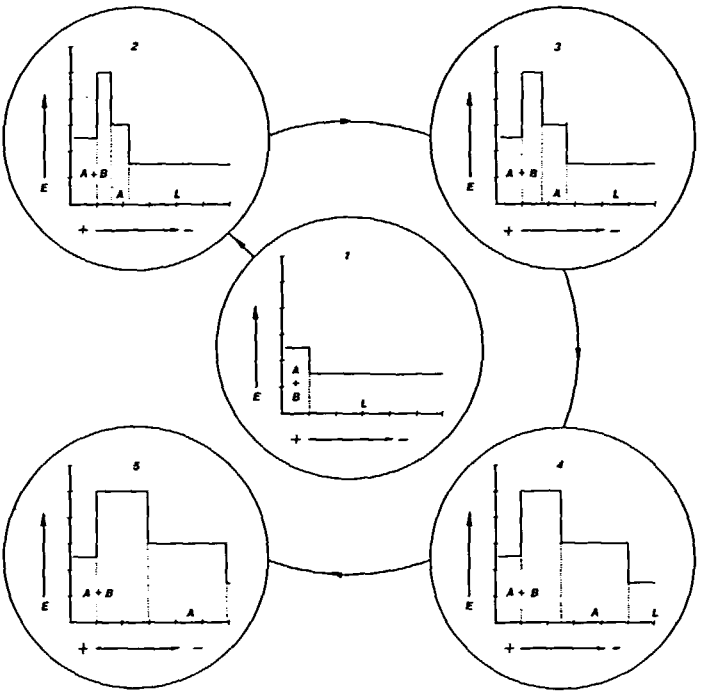

FIGURE 7
Moving boundary electrophoresis as a function of time.

of the cationic species A and the counterions from the leading electrolyte. Due to the electroneutrality principle, the ionic constituent A will have to migrate directly behind the leading zone, and therefore has to migrate with the same velocity (see Figure 4: $v_A = v_L$), as shown by Eq. (17):

$$v_A = \mu_{eff,A} E_A = v_L \quad (17)$$

Since the effective mobility of the leading constituent is higher, we must conclude that:

$$E_A > E_L \quad (18)$$

By applying the modified Ohm's law it follows that the electrical conductance of zone A must be lower than the electric conductance of the leading electrolyte. According to Joules law the heat production in zone A will be higher, resulting in a higher temperature. Considering the transition boundary of L and A, it can be shown that this boundary must be very sharp and that any disturbance of it will be counteracted by the electric field. This effect of "self correction" will be discussed later on in Section 2.2.3. Since the supply of both sample cations is unlimited, a mixed zone, containing the cationic species A and B and the counterions from the leading electrolyte, will follow the zone of "pure" A. Due to the fact that the ionic species B has the lowest effective mobility, the zone boundary A/A+B will move with a lower velocity than the leading boundary. As a result the zone-length of "pure" A will increase with time, a complete separation however never will be achieved (see Figure 4: $v_{AB} \neq v_A$). According to Equation (16) the velocity of the leading boundary L/A is constant and independent of the sample.

The velocity of the second boundary again is constant but is determined by both the leading electrolyte and the sample. As can be seen from Figure 6 all electrophoretic parameters in moving boundary electrophoresis are constant in time, and therefore may be used for specific of universal detection.

2.2.3. Isotachophoresis.

In isotachophoresis we must decide whether a separation of anionic or of cationic species will be performed. Again we will restrict ourselves to a separation of the cationic species A and B.
In Section 2.2.2. we have seen that in moving boundary electrophoresis a complete separation of the sample could not be achieved. Obviously this was due to the fact that we did not limit the amount of sample.
Following the lines of isotachophoresis however a complete separation can be achieved.
In isotachophoresis clearly three different electrolytes can be recognised at three different places, Figure 8a:

i. The leading electrolyte, which is situated in the case of a cationic separation, in the separation compartment and the cathode compartment.
ii. The terminating electrolyte, which is situated in the anode compartment.
iii. The sample, that has been introduced between the leading and the terminating electrolyte.

The cationic species of these electrolytes have to match an important requirement. The leading ionic species L must have the highest effective mobility, whereas the terminating ionic species must have the lowest effective mobility. The sample contains constituents with intermediate effective mobilities:

$$\mu_{eff,T} < \mu_{eff,B} < \mu_{eff,A} < \mu_{eff,L}$$

The first stage of the electrophoretic process will proceed along the lines of the moving boundary principle, Figure 8b. Since the amount of sample is limited, there will be a moment after which the sample is separated. In this so-called steady state, Figure 8c, there will be two zones, each of them containing one sample species sandwiched between the leading- and the terminating electrolyte.

Hence isotachophoresis can be compared with chromatographic displacement. Once the steady state has been

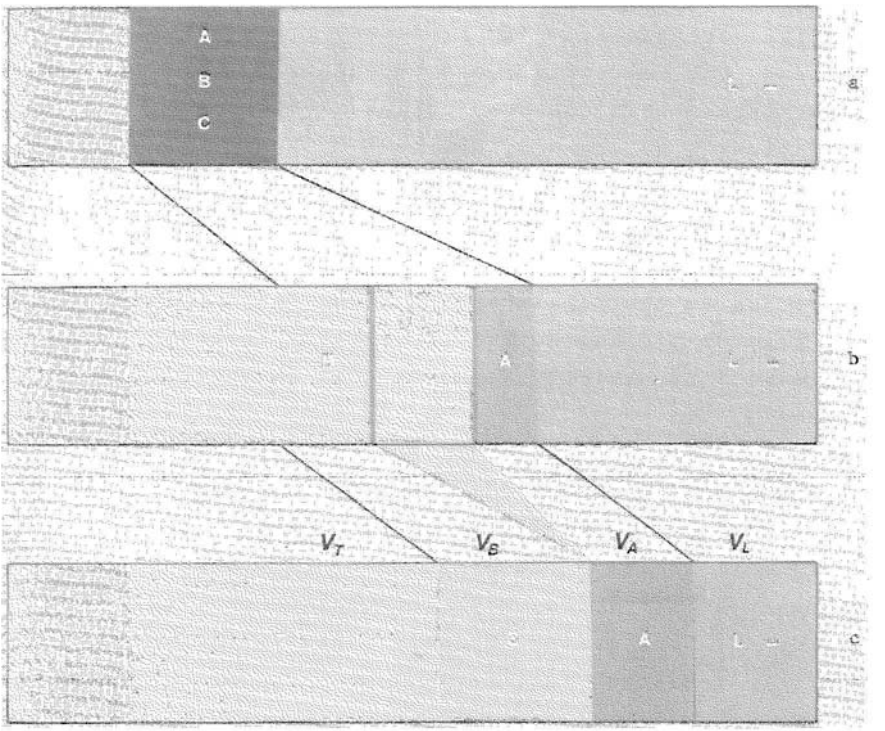

FIGURE 8
Isotachophoresis.

reached there will be present, in the separation compartment, four different zones, within a frame of three zone boundaries, that move with equal velocity. In fact it was this phenomenon that was the basis for naming this electrophoretic principle isotachophoresis as follows:

ισος = equal, ταχος = velocity, φορεεσται = to be moved

That the velocities must be equal is easily understood. When, for example, the leading zone moves faster then the following zone, there will be a cationic "vacuum" between the two zones. This "vacuum" will contain only anions, which is against the principle of electroneutrality. Therefore, once the steady state has been reached, the velocities must be equal. In electrophoretic terms, this has been called the isotachophoretic condition[19], as shown by Eq. (19):

$$v_L = v_A = v_B = v_T \qquad (19)$$

According to the regulating function concept the electrophoretic characteristics of the steady state again will be strictly regulated, as is shown in Figure 9.

These characteristics of the steady state can be calculated. For convenience we make the same assumptions as in the case of moving boundary electrophoresis and suppose in addition the presence of only strong single charged ionic species, with constant properties, i.e. $\mu_{eff} = \mu_\infty$. Considering the steady state, the isotachophoretic condition requires:

$$\mu_{eff,L}\, E_L = \mu_{eff,A}\, E_A = \mu_{eff,B}\, E_B = \mu_{eff,T}\, E_T \qquad (20)$$

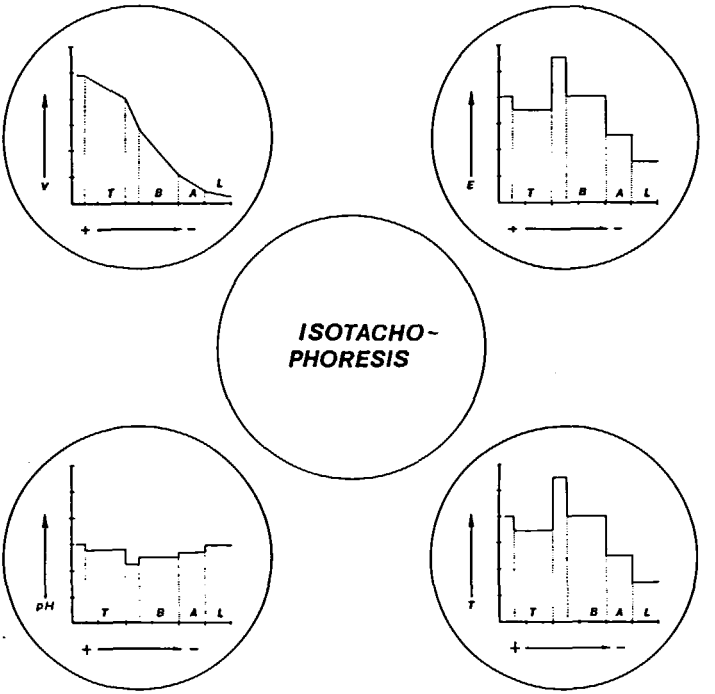

FIGURE 9
Characteristics of Isotachophoresis. V: potential, E: electric field strength, T: temperature.

Since the current density is assumed to be constant, the product of electric field strength and specific conductance will be constant too:

$$j = E \kappa = \text{constant} \tag{21}$$

The specific conductance is a summation of all ionic contributions, so according to Equation (14) we may write:

$$\kappa_L = F (c_L \mu_L - c_C \mu_C) \tag{22}$$

Using the electroneutrality principle:

$$c_L = c_C \tag{23}$$

Equation (22) becomes:

$$\kappa_L = F\, c_L(\mu_L - \mu_C) \tag{24}$$

In a similar way we find for the other zones:

$$\kappa_A = F\, c_A(\mu_A - \mu_C) \tag{25}$$

or:

$$\kappa_i = F\, c_i(\mu_i - \mu_C) \tag{26}$$

The isotachophoretic condition now may be combined with Equation (24), and (26) to give:

$$\frac{c_i}{c_L} = \frac{\mu_i}{\mu_i - \mu_C} * \frac{\mu_L - \mu_C}{\mu_L} \tag{27}$$

This last equation is equivalent to the "Kohlrausch regulation function" for the case of isotachophoresis. From this relation several important conclusions can be drawn. Firstly we see that this equation contains for a fixed leading electrolyte two quantities that may vary, though not independently. Since for a given ionic species the ionic mobility is a characteristic property, Eq. (27) states that the concentration for that ionic species, once the steady state has been reached, is invariable related to the leading electrolyte and therefore will be constant for a given leading electrolyte. Hence the concentration can be calculated

if all ionic mobilities involved, and the concentration of the leading ionic species are known.
Furthermore the Equation (27) means that the length of a zone is a measure of the absolute amount of ionic species. If, for example, the absolute amount of the constituent A in the sample is doubled, the zone length, occupied by the constituent A in the steady state, will similarely be doubled, Figure 10. So we must conclude:

The length of a zone in isotachophoresis contains information concerning quantity.

From the isotachophoretic condition, Equation (20), we can derive another important feature of isotachophoresis. Because of the fact that in the steady state all velocities are equal and because of the discrete order of mobilities, the electric field strength increases stepwise from zone to zone, going from the leading zone to the terminating zone, Eq. (29,30).

$$E_L < E_A < E_B < E_T \tag{29}$$

and

$$E_i = \frac{\mu_L}{\mu_i} E_L \tag{30}$$

The right hand side of Equation (30) contains a species specific property and the relation therefore can be used for identification purposes. So we must conclude:

Qualitative information in isotachophoresis is obtained from the electric field strength.

Since the heat produced in a zone and hence the temperature of a zone is directly related to the electrical field strength, measurement of the zone temperature

also may be used for qualification[20]. The use of the electrical conductance with respect to identification is obvious. Calculating the different electrophoretic characteristics, we did make some simplifications. The concept of course, can be extended to more complex situations, in which allowance can be made for multiple charge, dissociation, temperature effects etc.[21]. In fact there are several computerprograms available[22,23,24,25], that can be used for the calculation of isotachophoretic steady state configurations. Moreover it should be emphasized that the constant current density mode is not a necessity for isotachophoresis, though in practice it is the most convenient one. In Figure 10

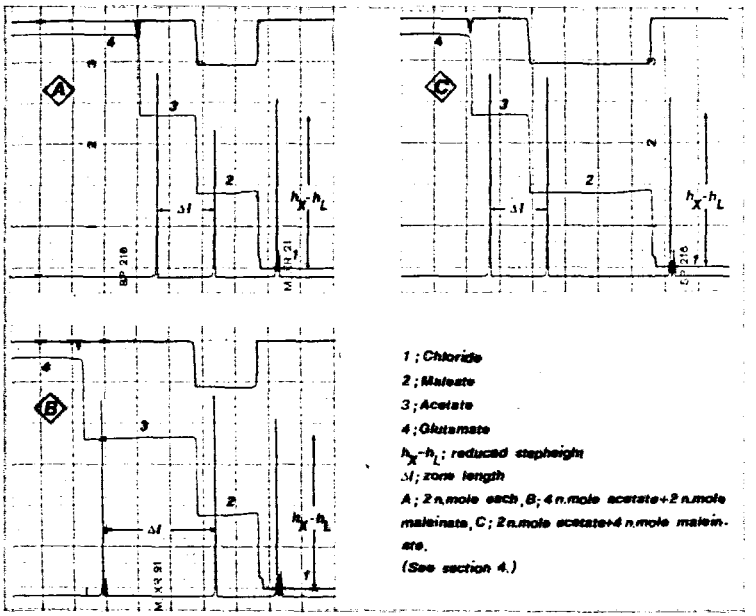

FIGURE 10
Quantity and quality.

the aspects of quantification and identification are shown in a practical example.

From the Figure 10 we can see that the step height for a particular species, e.g. acetate, is constant and hence characteristic. Furthermore the figure shows the quantitative information of zone-length.

Obviously the transition-boundary between two consecutive zones must be very small. This is caused by the so-called "self correcting" effect of the zone boundaries. Suppose the species A, Figure 11, has migrated, for some reason, in the zone of species B. There it will be confronted by an electrical gradient that is relatively high compared with the gradient of its own zone. Due to the higher gradient the lost species will move out of the B-zone back into the zone where it belongs. This of course means that dispersion, for example by diffusion, in isotachophoresis is optimally minimized and that the zone-boundaries are extremely sharp and remain in this manner irrespective of the time of analysis.

This self correcting phenomenon is of course closely related to the process of separation. As has been already mentioned, the separation process in isotacho-

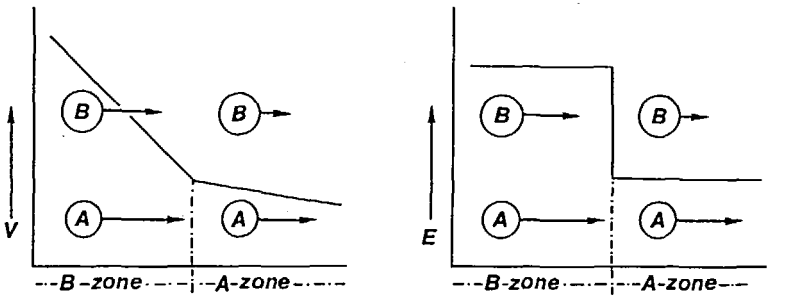

FIGURE 11
Self-correction.

phoresis proceeds according to the moving boundary principle. The process as a function of time is illustrated in Figure 12. The steady state in isotachophoresis is a strictly regulated state, the same of course holds for the separation process. In practice this means that all electrophoretic characteristics, as long as they exist, are constant in time. The time needed for a full separation, steady state, is determined by the current density, the leading electrolyte *and* the sample. It must be emphasized that within the total system, Figure 12.1, there are three regions, that may have their own regulating function Eq. (12),

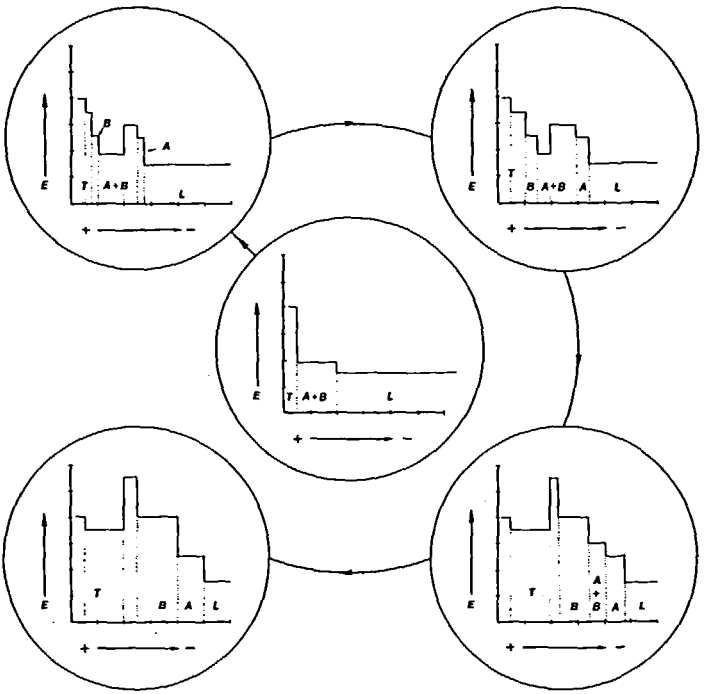

FIGURE 12
Isotachophoresis as a function of time.

anode compartment (T), sampling compartment (A + B), separation compartment (L). These regulating functions are locally invariable and need not necessarily be matched to one another.

This of course may give rise to discontinuities, that are generally not migrating. The various parameters again can be calculated, though the mathematical intricacy increases exponentially with the number of components involved.

2.2.4. Isoelectric focusing.

According to this principle only amphoteric substances can be separated. Being amphoteric means for an electrolyte, that this substance will have a net possitive, zero or negative charge, depending on the pH of the solution. Moreover it means that for such a substance there will be a pH at which the effective mobility is zero: this pH is called the isoelectric point.

$$\mu_{eff} = \sum_i \alpha_i \mu_i = 0 \text{ at } pH = pI \qquad (31)$$

At a pH above the isoelectric point the substance will migrate in anodic direction; below it, in cathodic direction.

Due to the existence of the pH gradient an amphoteric substance will focus at its isoelectric point, as is shown in Figure 13. Moreover from the figure can be seen that the electrophoretical velocity, with which the substance moves towards its isoelectric point, continuously decreases. Hence the time needed for complete focusing, will be rather long.

Of course there are numerous substances, that show amphoteric behaviour. The most pronounced group is formed by the amino acids, peptides and proteins.

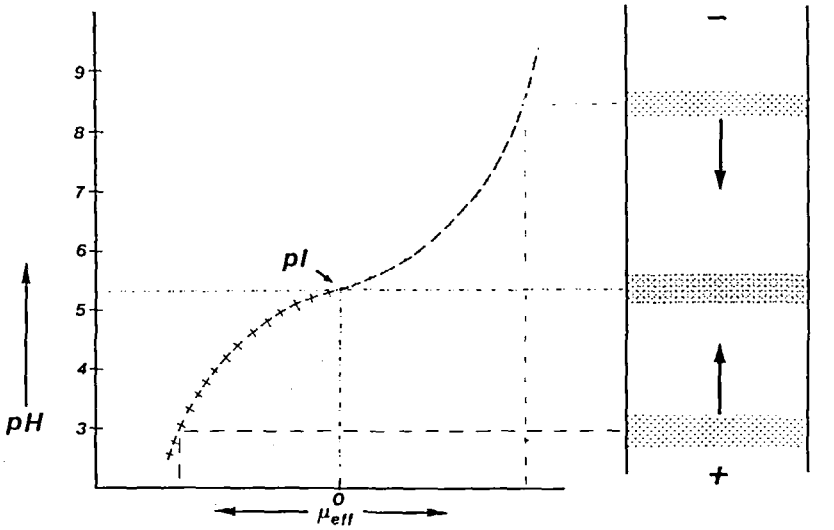

FIGURE 13
An amphoteric substance in a pH gradient.

In isoelectic focusing (Figure 14) we, again, can recognise three different electrolytes at three different places.

i. The separation compartment is filled with a homogeneous mixture of amphoteric substances: the carrier ampholytes.

ii. The anode compartment contains a protogenic electrolyte, The pH of this electrolyte solution should be lower than the pI of the most acidic ampholyte.

iii. The cathode compartment is filled with a protophylic electrolyte, of which the pH has to be higher than the pI-value of the most basic ampholyte.

Important in isoelectric focusing is the formation of a stable pH-gradient with uniform conductance. In

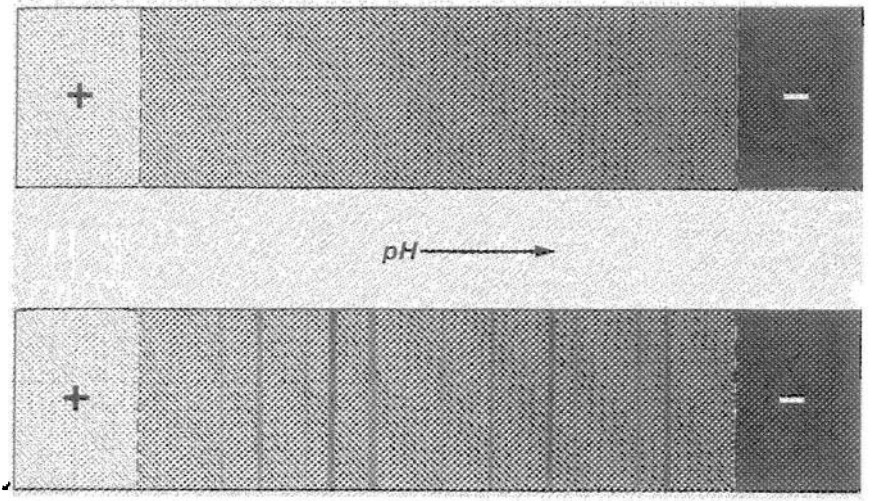

FIGURE 14
Isoelectric focusing.

principle any homogeneous mixtures of amphiprotic substances could be used. In practice however synthetic polymeric substances are used. The structure of such a polymer of low molecular weight, a polyaminopolycarboxylic acid, is shown below:

$$-CH_2-N-(CH_2)_x-N-CH_2-$$
$$\qquad\quad|\qquad\qquad\quad|$$
$$\qquad(CH_2)_x\qquad\quad R$$

$x = 2,3 \quad R = H, (CH_2)_x COOH$

Other synthetic carrier ampholytes are based upon the presence of amino- and sulphonic- and/or phosphonic and/or carboxylic groups. These ampholytes are commercially available in several ranges of isoelectric points:

Ampholine^R, Biolyte^R, Servolyte^R.

The formation of the pH-gradient is based upon the differential charge of the ampholytes.
Suppose we have a homogeneous ampholyte mixture at pH = 7, that contains a great number of constituents that have their isoelectric point within the range of pH 4 to pH 9. Applying an external electric field, components with low pI values will migrate in anodic direction, whereas components with high pI values migrate as cations. The component with the lowest pI value will migrate until it is immobilised by the protons, that migrate out of the anode compartment in the separation compartment. In fact it will focus in its isoelectric state at some distance from the anode. The opposite of course will happen with the most basic carrier ampholyte. For the complete mixture the process results in a separation, in which the carrier ampholytes are focused in order of their isoelectric points. As a consequence of this a pH gradient has been created. The nature of the gradient will depend on the range of isoelectric points, the number of ampholyte species, their concentration and their buffer capacity.
Amphoteric sample species, introduced in such a gradient, of course will focus at their isoelectric point, according to the same principle, as is shown in Figure 15.
The principal application of isoelectric focusing is the resolution of high molecular weight proteins. The resolved proteins can be identified through their pI-value. Detection by means of a staining procedure is the most commonly used method. UV-absorption, autoradiography and zymogram methods are other possibilities.
In practice the formation of a stable pH-gradient and the separation procedure may give rise to some problems. Since the ampholytes are moving towards their isoelectric state, their electrophoretic velocity gradually decreases. As a result the time for reaching the

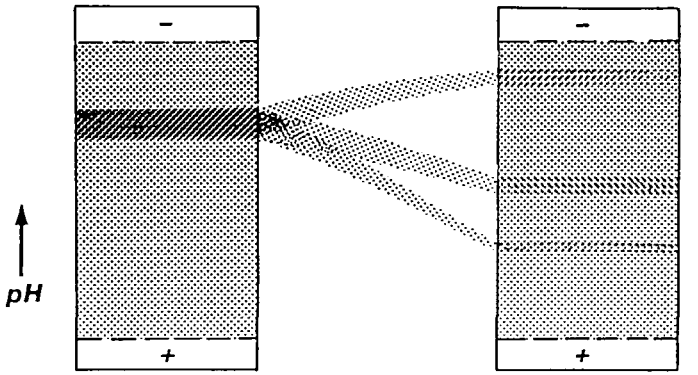

FIGURE 15
Separation of a sample via isoelectric focusing.

focused state will be rather long. Moreover, this decrease in mobility implies also a decrease in electrical conductance. Moreover since focusing times for different ampholytes may substantially differ, this decrease in conductivity can result in local overheating. Therefore the carrier ampholytes should have a good electrical conductance at their isoelectric points. For the carrier ampholytes this demand may be well satisfied for components to be separated however this can be a problem. In the case of proteins precipitation and/or denaturation may occur. Since local temperature effects may occur and density differences arise, stabilisation against thermal and gravitational convection must always be made.

A more fundamental problem in isoelectric focusing is the instability of the pH-gradient. In spite of the fact that the "cathodic drift"[26,27] seems incompatable with the concept of isoelectric focusing, it should be emphasised that this phenomenon is inherent in the principle. It can be shown that the pH-gradient according to the regulating-function concept, can not be considered

as a fixed gradient, but rather should be considered as a steady state configuration, with a very low cathodic drift velocity.

3. INSTRUMENTATION

3.0. Introduction.

In this section attention will be paid only to instruments for analytical purposes, as developed at the Eindhoven University of Technology.
In principle Isotachophoresis can be carried out on strips of cellulose acetate or paper, in gels and other stabilizing media. We will focus our attention to separations carried out in narrow bore tubes of Teflon ^{R} .

3.1. Instrumentation.

The equipment as constructed at the department of Instrumental Analysis of the Eindhoven University of Technology, is schematically given in the Figure 16. An extensive description of the equipment, suitable for isotachophoretic analyses, is given in the reference 25. It will suffice therefore to give only a brief description here.
The equipment consists of two electrode compartments (2,16), which are directly connected with the current stabilizing power supply (1), an injection block (4) and/or an injection valve (7) and a narrow bore tube (8). To prevent a hydrodynamic flow between the two electrode compartments, a semipermeable membrane (17) is mounted between the electrode compartment (16) and the central block (18), in which various connections are drilled. One of these connections is joined with the separation compartment (8) and the reservoir of the leading electrolyte (24). The separation compartment

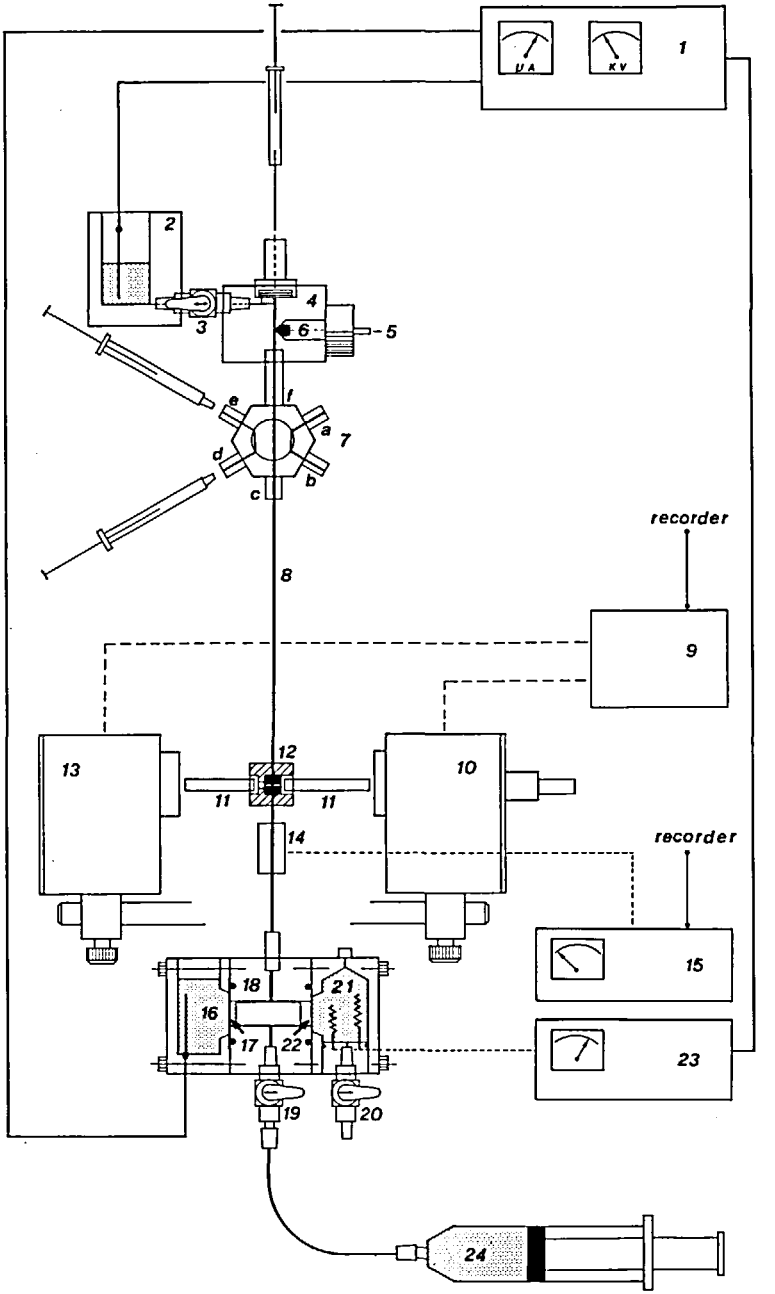

FIGURE 16
Isotachophoretic equipment.

is a narrow bore tube of Teflon^{R} (PTFE) with an inside diameter of 0.2 mm and an outside diameter of 0.4 mm. This diameter was found to be optimal, because the temperature difference between the various zones is small. Moreover the convective disturbances are small and the curvature of the zone profile is suppressed[25]. The sample can be introduced via a microlitre syringe in the injection block (4) or via a six way valve (7). In the position such that 7d is connected with 7a (7e - 7f and 7c - 7b) a sample can be introduced; the narrow bore tube can be rinsed and refilled; the terminating reservoir can be rinsed and refilled. In the position drawn in Figure 16 the valve is in its operational mode. Because in isotachophoretic analyses the sample zones are separated in consecutive zones according to their effective mobilities, all zones have their characteristic features: temperature, conductance, pH and potential gradient. Moreover a zone may have UV-absorption, optical rotation, fluorescence or radioactive compounds may be present. A thermometric detector, a thermocouple made of Cu-Constantan wires with a diameter of approximately 25 µm and a micro beat thermistor, was first developed as a detection system. This detector is mounted at the outside of the narrow bore tube. The response of the thermometric detector is rather low, its sensitivity however is still comparable with the high resolution detectors such as conductimetric, potential gradient and UV-absorption detector. From thermometric detection universal information can be derived. More recently a potential gradient detector has been developed as well as a conductivity detector (14) with micro sensing electrodes (10 µm Pt-Ir 10%) in direct contact with the electrolytes.

In most cases the conductivity probe is made of acrylic polymer. The electrodes are mounted axially, such that the electrolyte remains surrounded by an uninterrupted cylindrical wall. A contact cement has been used for the construction of the probe. The cell volume is a few nanolitres. In our equipment a UV absorption detector is also mounted. The UV source is an microwave mercury electrodeless lamp. The UV light is guided by a quartz rod of optical quality (11) into a slit of 0.1 mm diameter. The UV light passes the narrow bore tube and is guided again by another quartz rod towards a UV sensitive photodiode. More information can be found in the reference 25.

Teflon lined valves are applied at various places in the equipment, for the connection with the electrolyte reservoirs and the drain. For experiments with counterflow of electrolyte a special regulated pump has been constructed[25]. The reservoir (21) can be filled with an electrolyte such as KCl (0.1 N). Between this reservoir and the central chamber of the electrophoretic equipment a thin membrane (22) (e.g., a rubber contraceptive) is mounted with pre-stressing. If gas is now produced in the electrolysis cell (21), the volume increase will be accommodated by the expansion of the thin membrane. This results in a counterflow of electrolyte inside the narrow bore tube. The amount of gas produced is regulated by electronics (23) and the isotachophoretic moving zones can be stopped somewhere in the narrow bore tube.

The important parts of the equipment are identified in Figure 16 and 17 as follows:

- The reservoir for the terminating electrolyte (2);
- Sample introduction part (4, 7);
- The separation compartment (8);
- The detectors (12, 14).

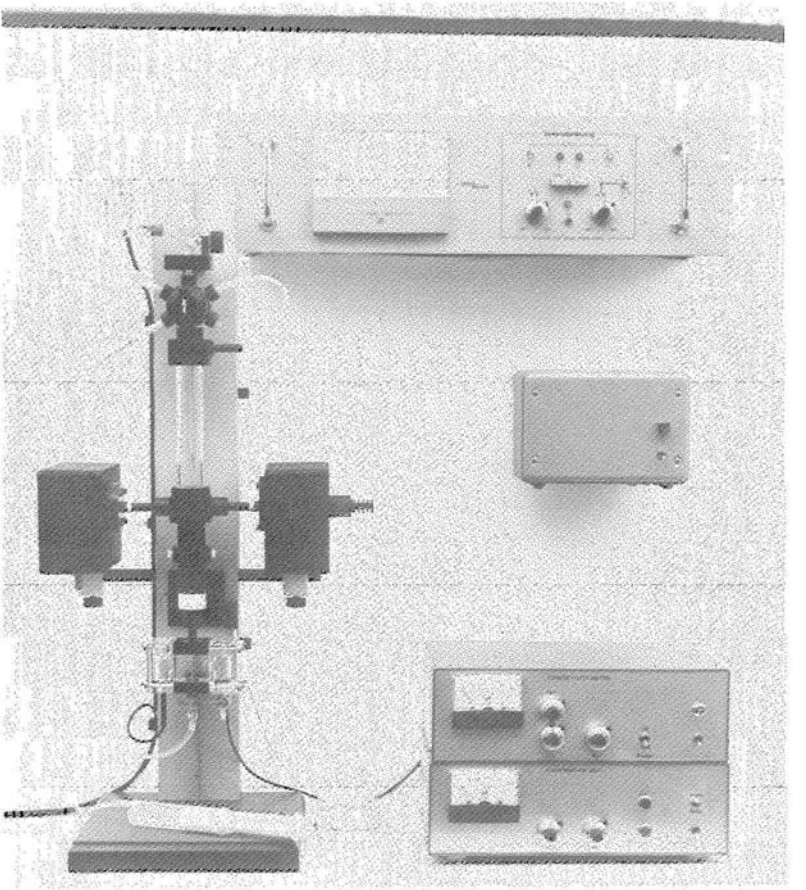

FIGURE 17
Isotachophoretic equipment.

Furthermore in Figure 16 (17) we have: a Teflon-lined two way valve (3), the drain (5), a tap with a silicone tip (6), the power supply for the UV lamp and the UV detector (9), the UV source (10), the UV slit (12), the UV detector with the set of filters (13), the electronics for the conductimeter (15), and two Teflon-lined two-way valves (19, 20).

4. APPLICATIONS.

4.0. Introduction.

It is difficult to describe all possible fields of application of analytical Isotachophoresis. Only a few arbitrarily chosen examples will be given in the following pages. Special attention will be paid to the choice of the electrolyte system and the qualitative and quantitative aspects of this separation technique. To predict feasibility for isotachophoresis as an analytical method, the ratio of molecular weight to effective charge can be used. Generally this ratio should not exceed 300, although examples can be given in which it can exceed 3000. Needless to say the compound must have a sufficient solubility in the solvent chosen. A brief survey of possible fields in which analytical Isotachophoresis can be applied is given in the Figure 18. This figure summarizes the various applications published in the scientific literature up to 1976.

Isotachophoresis, as it is applied presently (Figure 18), can be compared with liquid chromatography. Shortage of commercial equipment makes Isotachophoresis rather unknown among biochemists and analytical chemists.

4.1. Qualitative and quantitative aspects.

4.1.0. Introduction.

As is shown in the Figures 10 and 19 the signals derived form a conductivity detector (potential gradient, thermometric) gives general characteristics. From these signals qualitative and quantitative information can be deduced. Various ways can be given to list the qualitative information, such that various laboratories working with other equipment can make use of it.

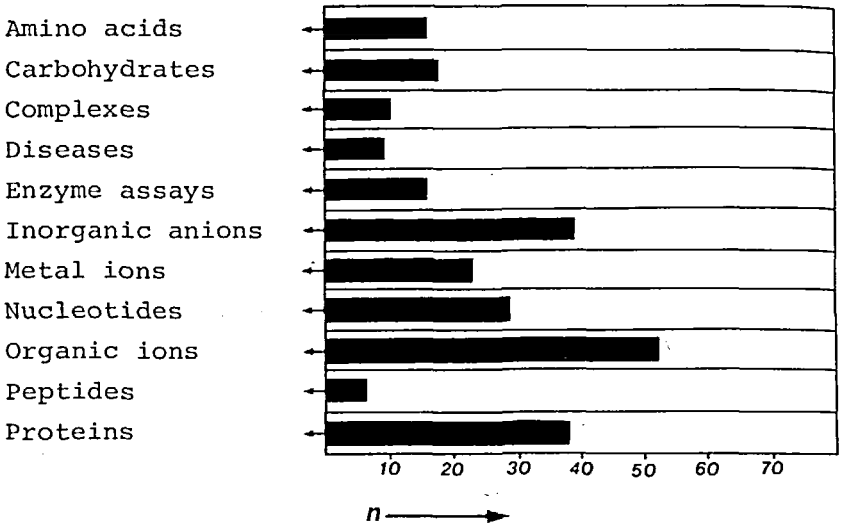

FIGURE 18
Publications about Isotachophoresis up to 1976.
n = amount of papers on the subjects given.

For a correct qualitative evaluation it is always important to know whether the temperature difference between leading electrolyte and terminating electrolyte is large or small: e.g. the temperature difference between the chloride zone and the morpholino-ethane sulphonate zone of Table VIII. The final temperature not only influences the effective mobility (2% per ^{o}C), but also the pK_a values, especially of cationic species. For quantitative evaluation the temperature is of less influence, as long as calibration curves are used, (section 4.1.2.). If ionic species are present that are migrating faster than the leading ion this can be seen in the linear signal of the conductivity detector, which indicates a conductivity lower than that of the leading ion. The qualitative and quantitative informa-

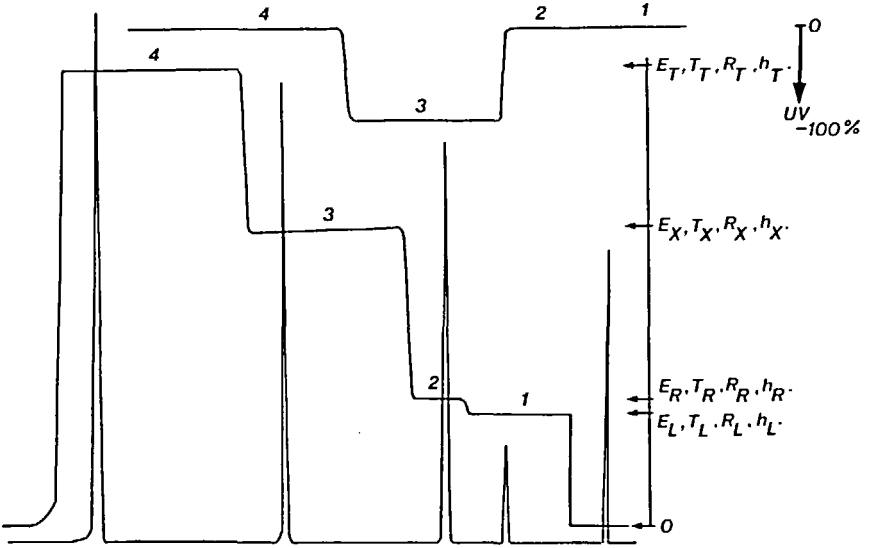

FIGURE 19
Isotachophoretic separation of perchlorate and p-amino-benzoate, carried out in the system listed in Table VIII. For explanation see text. 1 = chloride; 2 = perchlorate; 3 = p-amino-benzoate; 4 = MES.

tion of all other ions, migrating in between the leading zone and the terminating zone is not lost. If desired a leading ion can be chosen with a higher effective mobility, in order to have all sample constituents between the leading and terminating zone. If an ion is present in the sample with an effective mobility equal to that of the leading ion the conductivity of the leading electrolyte is not changed. Quantitative information still can be obtained by measuring the retardation of the appearance of the first separation boundary. Of course in this case the conductivity and zone lengths of all other zones are not influenced by this ionic species.

4.1.1. Qualitative aspects.

In the Figure 19 an isotachopherogram is given as obtained with a potentiometric detector. A comparable result can be obtained from a thermometric detector, or a conductimetric detector. For that reason not only the potential gradient (E) is indicated, but also the values for the conductimetric detector (R) and the thermometric detector (T). In this figure is shown the linear trace of the potential gradient detector[25], the differential of this and the linear trace of the UV absorption detector. Qualitative information is derived from the linear trace of the potential gradient detector. The quantitative information is obtained by measuring the steplengths (distances between peaks of the differential trace). The UV absorption detector gives additional qualitative (specific) information.

For listing the qualitative data in operational systems, four different methods are given:

i The stepheight h_X;

ii The reduced stepheight $h_X - h_L$;

iii The stepheight-unit value or S.U.-value

$$100 \frac{h_X}{h_L} ;$$

iiii The reference-unit value or R.U.-value

$$100 \frac{h_X - h_L}{h_R - h_L}$$

From these possibilities h_X does give obscure information, especially if qualitative information, obtained from the various detectors, is compared. The conductimeter indicates R_L, which is constant at various current densities, assuming the temperature differences are

small enough. The potential gradient detector and the thermometric detector does not give any signal at I = 0 μA. Both T_L and E_L are determined by the current density applied. T_L has a square relation and E_L has a linear relation with the current density. The stepheights and the reduced stepheights vary, moreover, if the amplification is changed. The S.U.-values, the potential unit values, the temperature unit values and the conductivity unit values, are applicable, as they give a ratio in $\mu_{effective}$. Therefore we prefer these S.U.-values to work with in practice. Moreover a correction is made for the amplification of the electric circuitry applied, although this holds also for the R.U.-value. The R.U.-value is less dependent on the current density than the S.U.-value, because two reference species are considered. Therefore the accuracy of a R.U.-value can be greater than that of a S.U.-value.

For listing qualitative data it is always important that the conditions of the operational electrolyte systems applied are well defined, the current density is fixed, the outputsignals of the detectors are linearised and, for a high accuracy, the diameter of the narrow bore tube is well chosen. We found an optimum for this diameter at 0.2 mm inside diameter.

4.1.2. Quantitative aspects.

As Figure 20 shows, the quantitative information in isotachophoretic analyses can be obtained by measuring the zonelengths. There is a linear relationship between the zone length of an ionic species and the amount of that ionic species introduced as a sample, assuming the electric current is stabilised.

The calibration curves of these ionic species are given in the Figure 21.

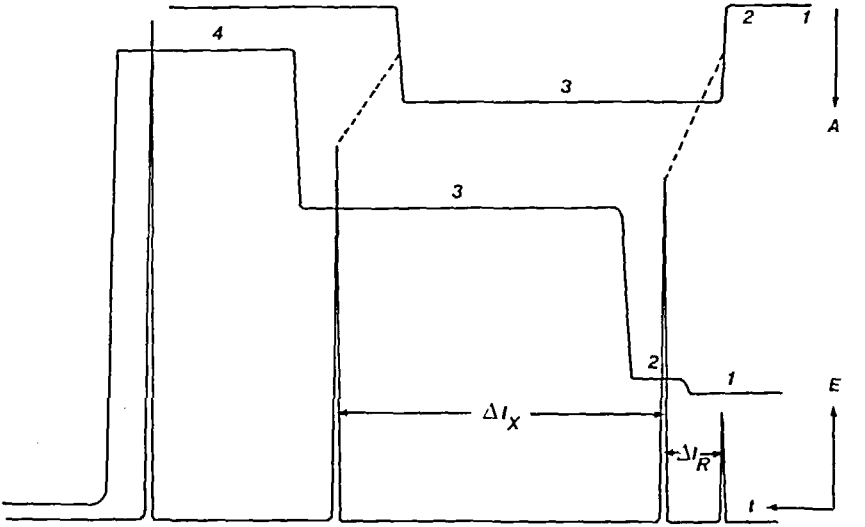

FIGURE 20
Isotachophoretic separation of perchlorate and p-amino-benzoate, carried out in the system listed in Table VIII. From this figure (and Figure 19) data are used for the calibration curve (Figure 21). Perchlorate has been used as an internal standard. 1 = chloride; 2 = perchlorate; 3 = p-amino-benzoate; 4 = MES; R = increasing resistance; A = increasing UV absorption and t = increasing time; E = electric field strength.

If mobility and pK_a values are known a calibration constant can be determined[25]. This is a constant in each operational system, if the operational conditions are fixed, for all ionic species. The use of an internal standard for quantitative evaluation is described in the reference[28].

At present we prefer to work with calibation curves. We found that this way is more accurate, because it is practically determined and corrects for dissociation, complexation, temperature, solvation, activity and other factors which may influence Equation 27 sufficiently. Table V gives some general characteristics for

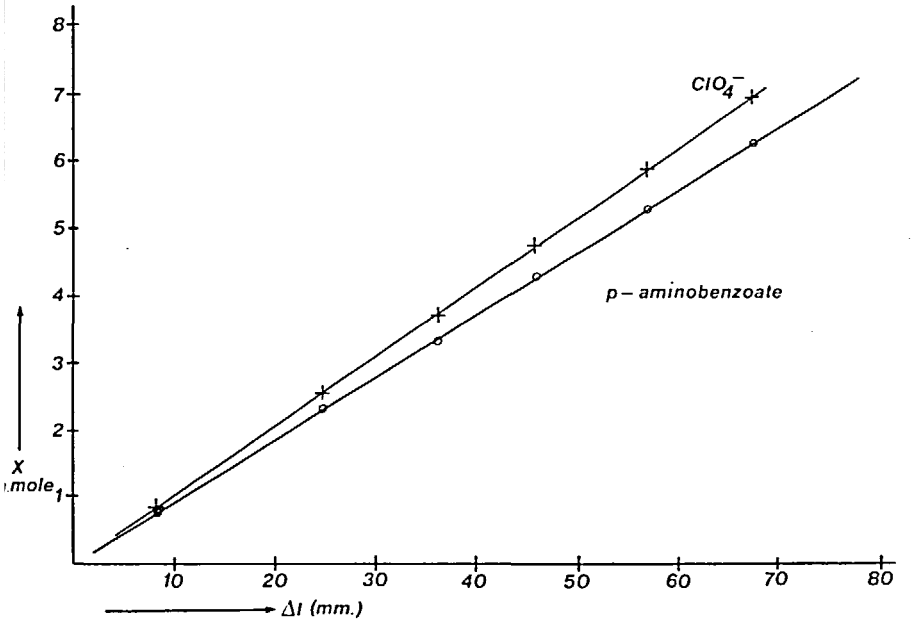

FIGURE 21
Calibration curve for quantitative evaluation.

detectors, as currently used in analytical isotachophoresis. Nearly all values, especially the minimal detectable amounts, refer to the equipment as shown in Figure 17 using a narrow bore tube of Teflon having an inside diameter of 0.2 mm and an outside diameter of 0.35 - 0.40 mm.

4.2. Separation of cationic species.

4.2.1. Electrolyte systems.

As is discussed in the section 2.2.3 the leading cation, in a separation of cationic species, must have a high effective mobility. For most experiments K^+ is

TABLE V

Survey of the detectors as currently used in analytical Isotachophoresis.

Type	Performance	L_{min} mm	Q_{min}	t_{av} min
Thermal	Thermocouple 15 μm (Cu-constantan) Thermistor (Philips micro beat)	5	$\frac{1}{2}10^{-9}$[a]	30
Conductivity	Microsensing electrodes (1-20 kHz)	0.05	10^{-12}[b]	10
Potential gradient	Microsensing electrodes in combination with the AC-DC converter (ref. 25)	0.05	10^{-12}[b]	10
UV	Microwell wavelengths: 256,280 nm	0.05	10^{-12}[b]	10

L_{min} = minimum detectable zone length; Q_{min} = minimum detectable amount of ionic component in gram equivalents; t_{av} = average time for analysis.

[a] In gram equivalents (0.4 mm I.D. narrow bore tube).

[b] In gram equivalents (0.2 mm I.D. narrow bore tube).

found to be sufficient. The counterion is chosen for optimal buffering capacity, this means that:

$$pK_C - 0.5 < pH_L < pK_C + 0.5$$

Generally the counterionic species should have low UV-absorption, low mobility and high stability. Its dissociation constant must vary as little as possible with temperature and concentration. Moreover the counterion should not form (insoluble) complexes with the sample constituents and the terminating electrolyte. In Table VI the operational conditions for Isotachophoretic analysis are given at pH_L is 5.0. For a good qualitative evaluation of the results it is important to match the concentration of the leading ion and the pH of the leading electrolyte to those values given in

TABLE VI

Operational system at pH 5.0, suitable for cationic separations.

Solvent	: H_2O	
j	: 0.08 A cm^{-2}	
Purification:	The additive is purified	
Electrolyte	Leading	Terminating
Cation	K^+	H^+
Concentration	0.01 N	
Counterion	CH_3COO^-	CH_3COO^-
pH	5.00	ca. 4.0
Additive	0.05% PVA or HEC	none

PVA: polyvinylalcohol; HEC: hydroxy-ethylcellulose

the Table VI. Although to create an optimal difference in effective mobility between a given pair of sample constituents, the pH of this system may be varied between pH_L = 4.5 and pH_L = 5.4: hence a new system is created. The concentration of the leading ion is normally kept at 0.01 N. Without problems this concentration can be varied from 1 centimolar to 1 millimolar, if the influence of activity is wanted or the concentration of (some) sample constituents is low: again a new system is created. High molecular weight poly-vinyl-alcohol or hydroxy-ethyl-cellulose is added in low concentration for optimal stabilisation and depression of the electro-endosmotic flow, if any. Sometimes chemicals, even of analytical grade, are not pure enough for Isotachophoretic experiments. Distillation or recrystallisation from water, acetone or alcohol is often necessary. Especially the constituents applied for the terminating electrolyte need to be of highest purity. The concentration and pH must be adjusted to the concentration and/or pH of the leading electrolyte. If the concentration and/or pH is too different from those of the leading electrolyte both quantitative and qualitative information from the sample can be obscured. Especially if the concentration is high, impurities form the terminating electrolyte may influence the Isotachophoretic analysis. Even the sample ions, mixed with the terminating electrolyte, may not reach their appropriate zones in time, due to a poor dissociation or the low field strength present in the highly concentrated terminating electrolyte. In these cases a part of the sample constituents may have already passed the detector(s), before the steady state has been reached. Only some examples of terminating ionic species will be discussed. In the system, listed in Table VI, ε-amino caproic acid has been applied. The effective

mobility is sufficiently low. So it can be used as terminator for the separation of atropine. Its effective mobility is, however, too high to be used for the separation of vitamin B-complex (see section 4.2.2). In this case the H^+ can be applied as terminating ion. Although the conductimeter indicates now that the resistance of the terminating zone is lower than that of the zone of nicotine-amide (Figure 24), the H^+ ion migrates behind the zone of nicotine-amide due to a pH-shift[25].

4.2.2. Some examples.

In the system listed in Table VI, many components such as metals and organic bases can be analysed. In the left hand side of Figure 22 an analysis of atropine (1.79 nanomol) is shown. The atropine-sulphate solution has been badly sterilized (30 minutes, 130°C). In this isotachopherogram the cationic degradation products tropane and methylamine are clearly visible. The right hand side of Figure 22 shows the analysis of the anionic species of the atropine solution. As expected the degradation product tropanoic acid is clearly visible. The sulphate zone is a measure for the total amount of the atropine originally present. From a calibration curve we calculated that the amount of atropine has been reduced to 60% due to the bad sterilisation.
The cationic separation was carried out in the operational system of Table VI, the anionic separation in the system of Table VIII.
In the Figure 23 the analysis of atropine is shown, sterilised at 115°C for about 15 minutes. Again the analyses were carried out in the systems listed in the Tables VI and VIII. No degradation products are present.

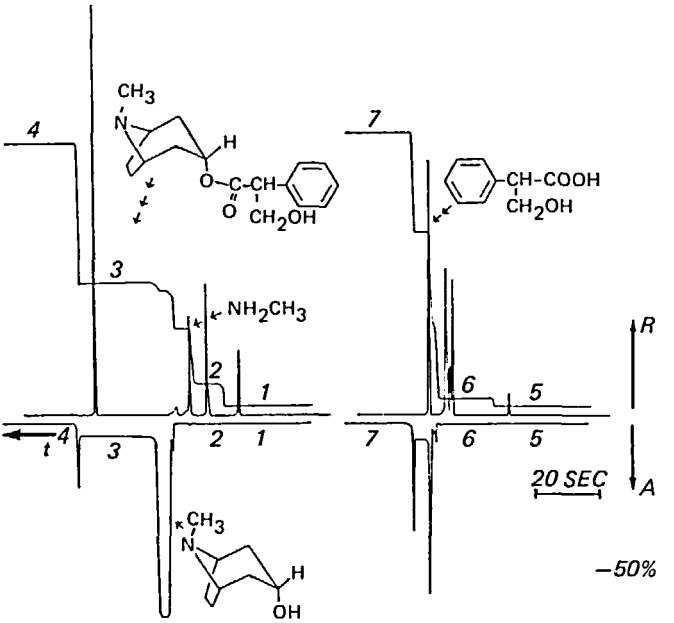

FIGURE 22
The isotachophoretic separation of atropine badly sterilised. 1 = K^+; 2 = Na^+; 3 = atropine; 4 = ε-aminocaproate; 5 = chloride; 6 = sulphate; 7 = MES; R = increasing resistance; A = increasing UV absorption and t = increasing time. Time for analysis ca. 12 minutes.

In the left hand side of Figure 23 2.98 nanomol atropine has been analysed. The right hand side shows the analysis of the anionic species (the injected amount being doubled). From such isotachopherograms (Figures 22 and 23) a mass-balance can be made to study the degradation (in other cases kinetics) as a function of time, but also to check the accuracy of an analysis. Figure 24 shows the isotachophoretic separation of vitamin B-complex. The separation has been carried out by the system listed in Table VI and the time for analysis was 15 minutes.

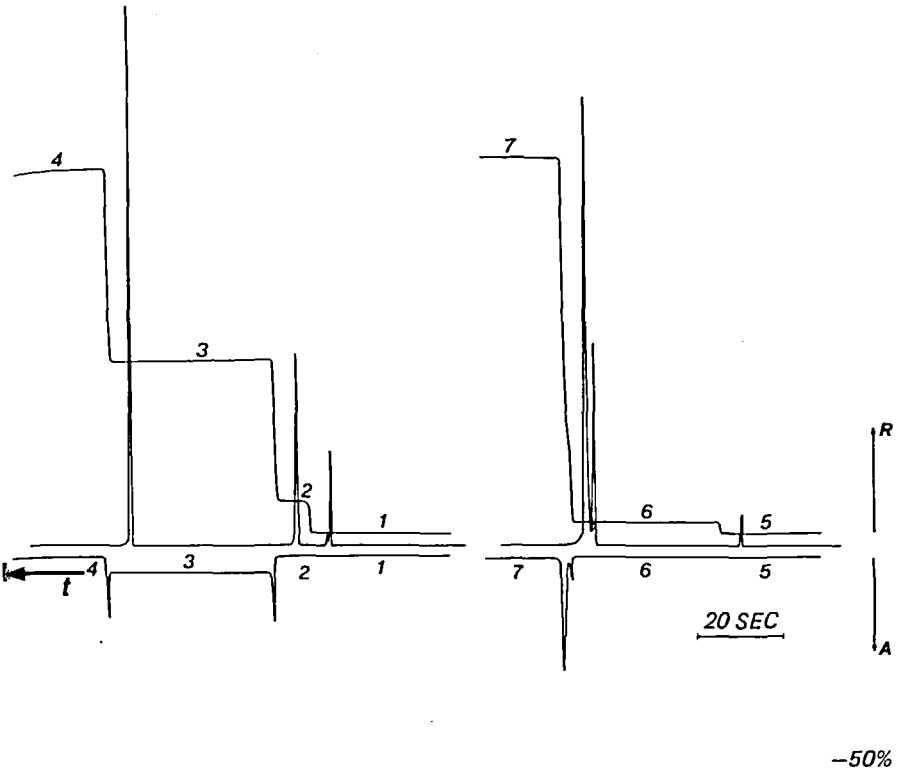

FIGURE 23
The isotachophoretic separation of atropine, properly sterilised (see Figure 22).

Besides the components mentioned in Figure 24, the amount of panthothenic acid can be determined, because it is present as a Ca-salt. The linear traces of both conductimeter and UV absorption detector were found to be reproducible for the nicotine amide zone even if products of various origins were used. It can be seen that the nicotine-amide does not form a homogeneous zone. Sometimes cations can be separated as complexes, if there are problems in using the free cations such as poor solubility, or too small a difference in effective

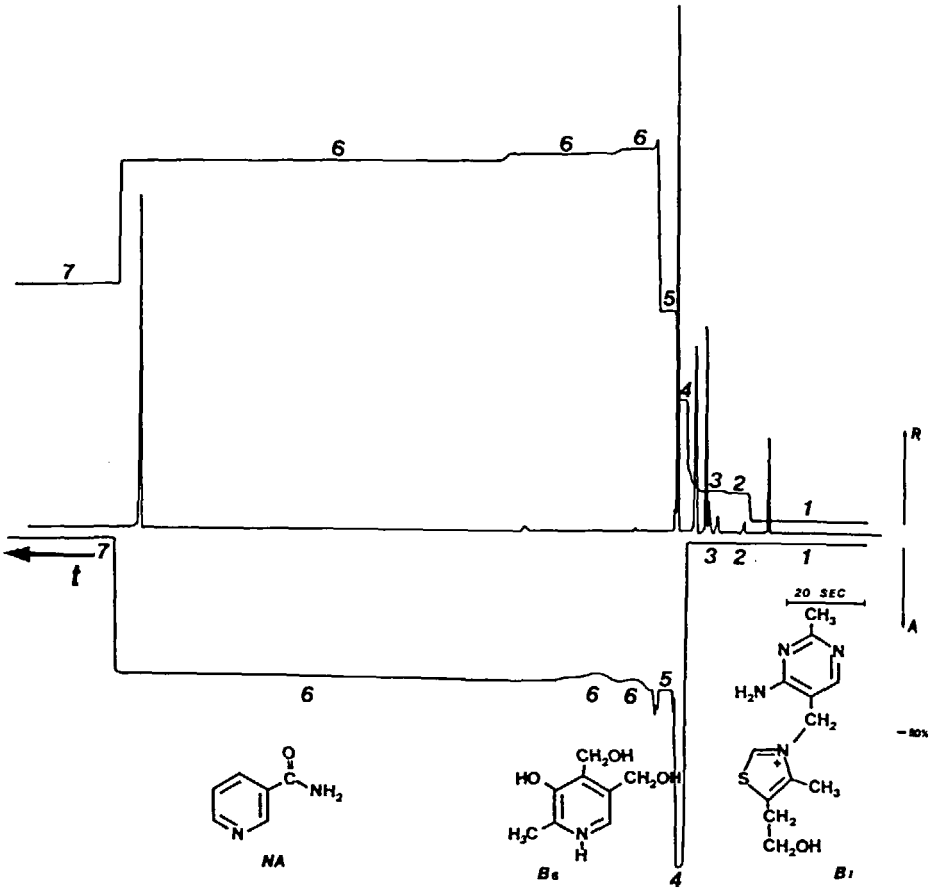

FIGURE 24
The isotachophoretic separation of vitamine B complex. 1 = K^+; 2 = Ca^{++} (originating from panthothenic acid); 3 = Na^+; 4 = vitamine B_1; 5 = vitamine B_6; 6 = nicotineamide; 7 = H^+; R = increasing resistance; A = increasing UV absorption and t = increasing time.

mobility. In section 4.3.2 a cation example is given, migrating as EDTA complexes. In addition other solvents can be chosen for the separation of cations: especially methanol is suitable. Detailed information can be found in the reference 25.

4.3. Separation of anionic species.

4.3.1. Electrolyte systems.

For most anionic separations as the leading anion chloride can be chosen. Its optimal concentration was found to lay between 1 centimolar and 1 millimolar. As for cationic species the pK_a values of the sample constituents determine at which pH and hence, which counterion with buffering capacity needs to be chosen. In the Tables VII, VIII, IX and X some examples of electrolyte systems are given. We refer to section

TABLE VII

Operational system at pH 7.5, suitable for anionic separations.

Solvent :	H_2O	
j :	0.08 A cm^{-2}	
Purification:	Morpholinoethanesulphonic acid is recrystallized three times and the crystals are washed with acetone. The additive is purified.	
Electrolyte	Leading	Terminating
Anion	Cl^-	MES^-
Concentration	0.01 N	ca. 0.01 N
Counterion	$TrisH^+$	$TrisH^+$
pH	7.50	ca. 6
Additive	0.05% PVA or HEC	none

PVA: polyvinylalcohol; HEC: hydroxy-ethylcellulose.
Tris: tris(hydroxymethyl)-aminomethane.

TABLE VIII

Operational system at pH 6, suitable for anionic separations.

Solvent	: H_2O	
j	: 0.08 A cm^{-2}	
Purification:	Morpholinoethanesulphonic acid (MES) is recrystallized three times and the crystals are washed with acetone. The additive is purified.	
Electrolyte	Leading	Terminating
Anion	Cl^-	E.g., MES^-
Concentration	0.01 N	Ca. 0,01 N
Counterion	$HistidineH^+$	$TrisH^+$
pH	6.02	Ca. 6
Additive	0.05% PVA or HEC	none

PVA: polyvinylalcohol; HEC: hydroxy-ethylcellulose.

4.2.1. for the importance of the concentration and pH of the various electrolytes. The addition of complexing counterions, such as Ca^{++}, Cd^{++}, Al^{+++}, Pb^{++}, etc. or non-ionic components with complexing capacity can give these systems total different characteristics (Figure 1). It must be emphasized that most of the systems can also be applied in other solvents or mixture of solvents.

TABLE IX

Operational system at pH 4.5, suitable for anionic separations.

Solvent :	H_2O	
j :	0.08 A cm^{-2}	
Purification:	Morpholinoethanesulphonic acid (MES) is recrystallized three times and the crystals are washed with acetone. ε-Aminocaproic acid is recrystallized. The additive is purified.	
Electrolyte	Leading	Terminating
Anion	Cl^-	MES^-
Concentration	0.01 N	Ca. 0.01 N
Counterion	$COOH-C_4H_8-CH_2N^+H$	Tris H^+
pH	4.50	Ca. 7
Additive	0.05% PVA or HEC	none

PVA: polyvinylalcohol; HEC: hydroxy-ethylcellulose.

4.3.2. Some examples.

Many tissue metabolites can be analysed in the systems VII, VIII, IX and X, even at serum level. No pretreatment is often needed.

In Figure 25 a separation of Ni and Al, present in a Raney-Nickel catalyst is given. These cations were analysed in the system, listed in Table VIII, migrating as EDTA complexes. The time for analysis is approximate-

TABLE X

Operational system at pH 3, suitable for anionic separations.

Solvent	:	H_2O
j	:	0.08 A cm^{-2}
Purification:		β-Alanine must be purified by recrystallization from a methanol-water mixture and the crystals are washed with acetone. The additive is purified.

Electrolyte	Leading	Terminating
Anion	Cl^-	E.g., CH_3COO^-
Concentration	0.01 N	Ca. 0.01 N
Counterion	$COOH-CH_2-CH_2N^+H$	Na^+
pH	3.00	Ca. 6
Additive	0.05% PVA or HEC	none

PVA: polyvinylalcohol; HEC: hydroxy-ethylcellulose.

ly 12 minutes. In Figure 24 an example is shown of the separation of some anionic species in the system listed in Table VIII. A poor separation is obtained because formic acid, α-keto-glutaric acid, tartaric acid and citric acid have nearly equal effective mobilities at pH_L = 6. By the addition of 2mM $CaCl_2$ to the leading

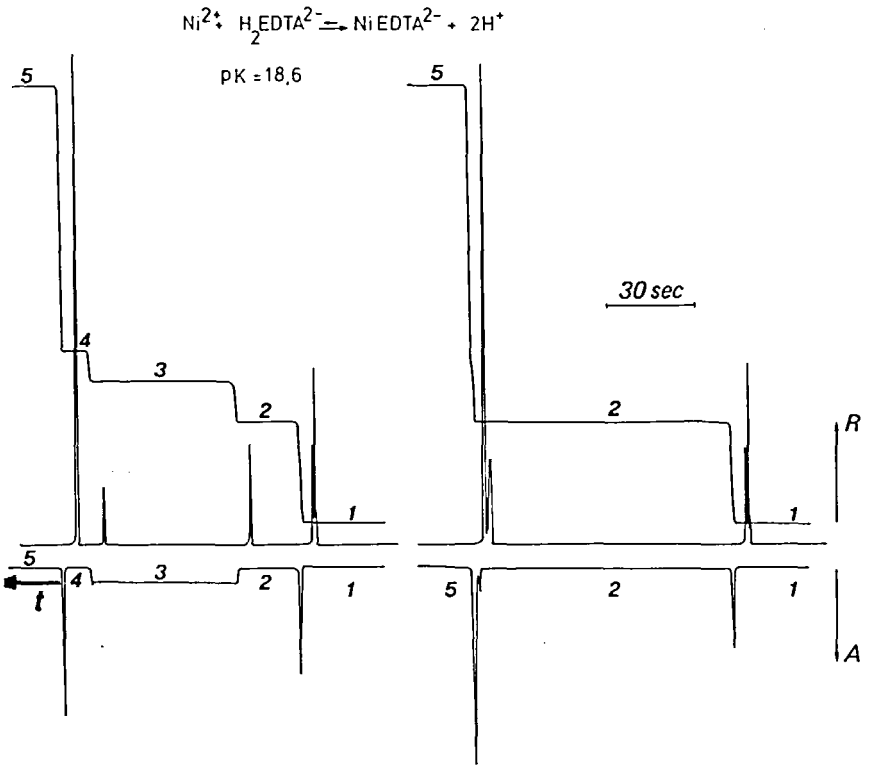

FIGURE 25
The analysis of a Raney-Nickel catalyst by complex formation. 1 = chloride; 2 = EDTA; 3 = NiEDTA; 4 = AlEDTA; 5 = MES; R = increasing resistance; A = increasing UV adsorption and t = increasing time.

electrolyte an effective difference in mobility could be established so that a complete separation could be obtained. The times for analyses were in both cases approximately 12 minutes.

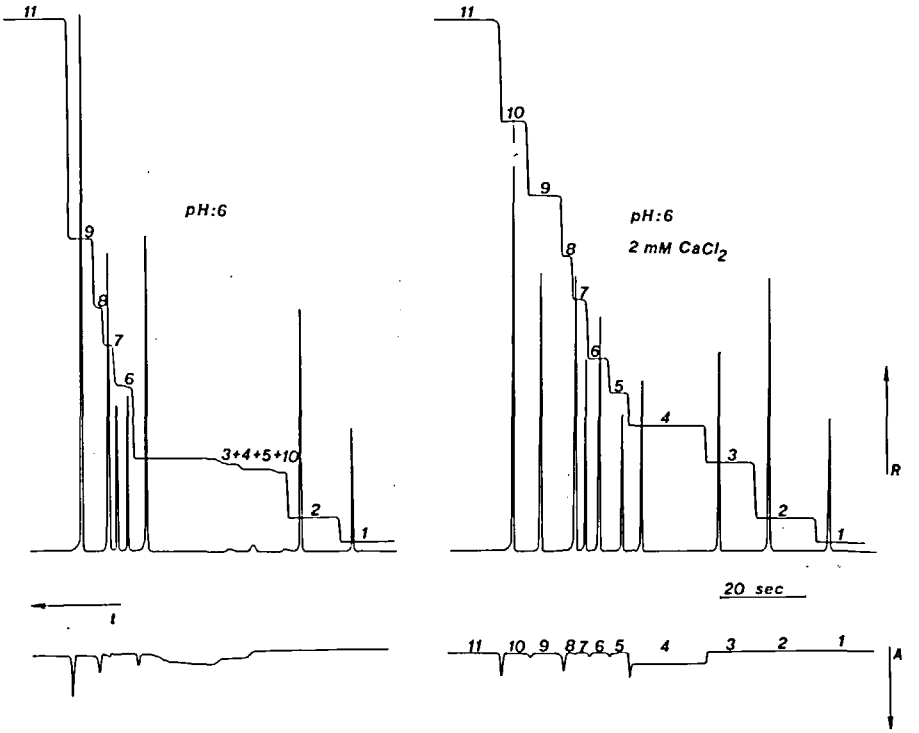

FIGURE 26
The influence of complexing counterions on the effective mobilities of some anions. 1 = chloride; 2 = perchlorate; 3 = formate; 4 = α-keto-glutarate; 5 = tartrate; 6 = acetate; 7 = lactate; 8 = 2-hydroxy-butyrate; 9 = capronate; 10 = citrate; 11 = MES. R = increasing resistance; A = increasing UV absorption and t = increasing time. The left hand side shows the separation obtained in the system, listed in Table VIII. The right hand side shows the separation after addition 2mM $CaCl_2$ to the leading electrolyte.

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LIST OF SYMBOLS

subscript
linescript
superscript

α	●	degree of dissociation	
γ	●	activity coefficient	
δ	●	derivative	
κ	●	specific electric conductance	$(\Omega^{-1}cm^{-1})$
λ	●	equivalent ionic conductance	$(cm^2\Omega^{-1}eq^{-1})$
μ	●	mobility	$(cm^2V^{-1}cm^{-1})$
ω	●	numerical	
Δ	●	difference	
Λ	●	equivalent electric conductance	$(cm^2\Omega^{-1}eq^{-1})$
Ω	●	Ohm	(Ω)
∞	●	infinite dilution	

A	●	●		ionic species	
B	●	●		ionic species	
C	●	●		ionic species	
E		●		electric field strength	(Vcm^{-1})
F		●		Faraday constant	(coul eq^{-1})
I		●		electric current	(A)
J		●		ionic strength	
L	●	●		ionic species	
O		●		cross section	(cm^2)
R		●		electric resistance	(Ω)
T		●		temperature	(^oC)
T	●			ionic species	
V		●		electric potential difference	(V)
c	●	●		concentration	(eq cm^{-3})
f		●		retardation coefficient	
i	●			species	
j		●		current density	(Acm^{-2})
k		●		numerical	
ℓ		●		length	(cm)
t		●		time	(sec)
v		●		linear velocity	(cm sec^{-1})
w		●		wattage	(watt cm^{-3})
z		●	●	number of equivalents per mole	