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Review

Application of capillary isotachophoresis in peptide analysis

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ABSTRACT

This paper gives a broad and detailed review of the applications of one of the modern high-performance electromigration separation techniques — capillary isotachophoresis (ITP) — in peptide analysis. Examples are presented of the utilization of capillary ITP for peptide analysis in the fields of chemistry, general and clinical biochemistry, biology, biotechnology, pharmacy and the food industry. The complete composition of all the electrolyte systems used for peptide ITP analyses in both cationic and anionic techniques is given in tabular form. According to the purpose of analysis the applications are divided into several sections: model studies, determination of physico-chemical characteristics, purity control of both intermediate and final peptide preparations, including the determination of low-molecular-mass ionogenic admixtures, and the analysis of peptides in biological fluids and tissue extracts. In addition to the main applications the theoretical and methodological aspects of peptide ITP analysis are discussed. The basic electromigration properties of peptides (their polyampholyte character, effective and absolute mobilities, acid–base equilibria) are explained and the selection of parameters for peptide ITP analysis is described in detail. The advantages and disadvantages of ITP compared with other electrophoretic and chromatographic methods used for peptide analysis are discussed.

CONTENTS

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LIST OF ABBREVIATIONS

AABA	α-Aminobutyric acid
ACES	N-2-Acetamido-2-aminoethanesulphonic acid
ACTH	Adrenocorticotropic hormone
AMED	2-Amino-2-methyl-1,3-propanediol (ammediol)
AMPOL	2-Amino-2-methyl-1-propanol
BALA	β-Alanine
BIS-TRIS	1,3-bis[tris(hydroxymethyl)methylamino]propane
BPTI	Basic pancreatic trypsin inhibitor
BUSI	Bull seminal isoinhibitor of trypsin
CACOD	Cacodylic acid
CAPR	Caproic acid
CHAPS	3-[(Cholamidopropyl)dimethylammonio]-1-propanesulphonate
CHAPSO	3-[(Cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulphonate
CM-Cys	Carboxymethylcysteine
CTI	Cow colostrum trypsin inhibitor
CZE	Capillary zone electrophoresis
DEBA	Diethylbarbituric acid
EA	Ethanolamine
EACA	ε-Aminocaproic acid
ES	Electrolyte system
GABA	γ-Aminobutyric acid
GC	Gas chromatography
GSH	Glutathione
GSSG	Glutathione disulphide
HAc	Acetic acid
HEPES	N-2-Hydroxyethylpiperazine-N′-2-ethanesulphonic acid
HEPPS	N-2-Hydroxyethylpiperazine-N′-2-propanesulphonic acid
HPC	Hydroxypropylcellulose

HPCE	High-performance capillary electrophoresis
HPLC	High-performance liquid chromatography
HPMC	Hydroxypropylmethylcellulose
IEC	Ion-exchange chromatography
IEF	Isoelectric focusing
IMID	Imidazole
LTH	Lipotropic hormone
ITP	Isotachophoresis
MC	Methylcellulose
MEC	Methylethylcellulose
MES	2-(N-Morpholino)ethanesulphonic acid
MOPS	3-(N-Morpholino)propanesulphonic acid
Mur	Muramic acid
Mpa	Mercaptopropionic acid
PCA	Pyrrolid-2-one-5-carboxylic acid (pyroglutamic acid)
PEG	Polyethyleneglycol
PTFE	Polytetrafluorethylene
PVA	Polyvinylalcolhol (Mowiol)
RP-HPLC	Reversed-phase high-performance liquid chromatography
SB 12	N-Dodecyl-N,N-dimethyl-3-ammonium-1-propanesulphonate (sulphobetain 12)
SDS	Sodium dodecylsulphate
TART	Tartaric acid
TES	2-{[Tris-(hydroxymethyl)methyl]amino}ethanesulfonic acid
TMAPS	Trimethyl-3-ammonium-1-propanesulphonate
Tricine	N-[Tris(hydroxymethyl)methyl]glycine
TRIS	Tris-(hydroxymethyl)aminomethane
TX-100	Triton X-100
VIP	Vasoactive intestinal peptide

For three-letter amino acid abbreviations see *Biochemistry*, 14 (1975) 449.

1. INTRODUCTION

Peptides are by their chemical composition amides of amino acids linked by peptide bonds or cross-linked by disulphide bonds. The chemical nature of a peptide is determined not only by the type and number of amino acid residues present, but also by their sequence in the peptide chain.

The occurrence and function of peptides in nature are both very varied and extremely important. Peptides act as co-enzymes, inhibitors of enzymes, hormones, immunity and neuronal regulators, drugs, antibiotics and poisons.

Most peptides are soluble ionogenic substances and can therefore be studied by electromigration separation methods. An almost infinite number of variations of amino acid sequences in the polypeptide chain gives rise to an extraordinary

variety of peptides differing in size (relative molecular mass), steric arrangement, charge value in a given medium, and thus also in electrophoretic mobility, which allows the use of capillary isotachophoresis (ITP) – one of the modern electromigration separation methods – for their separation. Peptides, together with proteins, form one of the largest classes of compounds which can be determined by ITP [1]. The first part of this paper describes the methodological aspects of peptide determination by ITP, *i.e.* the electromigration properties of peptides and the selection of conditions for ITP analysis. However, the details of the principles, theory and instrumentation for ITP will not be given here and can be found elsewhere [2–6].

2. ELECTROMIGRATION PROPERTIES OF PEPTIDES

The electromigration properties of peptides are those properties which are related to their electrophoretic mobility, which is the most important parameter in electromigration separation methods [7]. As peptides are amphoteric (poly) electrolytes or (poly)ampholytes, the most useful characteristic for their rate of movement in an electrical field is the effective mobility, *i.e.* the mobility at a given pH, ionic strength, solution composition and temperature. Only mobilities defined in this way and referred to the same conditions can be compared with each other.

The relationship between the electrophoretic mobility of peptides and their relative molecular mass and charge is decribed by Offord's equation, derived empirically [8] from peptide separations by paper zone electrophoresis:

$$m = k \cdot z \cdot M_r^{-2/3} \tag{1}$$

where m is the relative electrophoretic mobility of the peptide (relative with respect to a standard), z is the peptide charge in elementary units, M_r is the relative molecular mass and k is a constant.

The effective mobility of peptides can be influenced by changing the charge to which the mobility is directly proportional (see eqn. 1). The peptide charge strongly depends on the medium in which it occurs. This is a result of the fact that peptides contain in their chains various amounts of different ionogenic groups, the degree of dissociation of which is dependent on the pH of the medium. A survey of these groups of individual amino acids and the approximate extent of their dissociation constants is given in Table 1.

As is seen in simple ampholytes, the total charge (the sum of all charges irrespective of sign) and the effective (resulting, net) charge, *i.e.* the sum of the charges including their sign, must also be differentiated in polyampholytes.

At a known amino acids composition, it is possible to approximately determine, from the dissociation constants of the individual ionogenic groups, which groups are dissociated at a given pH and how they contribute to the effective charge. Generally, a given group is dissociated to 50% in a solution with a pH

TABLE 1

APPROXIMATE RANGE OF pK VALUES OF INDIVIDUAL IONOGENIC GROUPS IN PEPTIDES [9,10]

Ionogenic group		pK value
Type	Amino acid	
$-SO_3H$	Cysteic acid	1.3
α-COOH	C-Terminus of peptide chain	2.0–3.7
β-COOH	Aspartic acid	3.5–4.5
$-S-CH_2-COOH$	S-Carboxymethylcysteine	3.4–4.0
γ-COOH	Glutamic acid	4.0–4.5
Imidazolium	Histidine	5.6–6.9
α-NH_3^+	N-Terminus of peptide chain	7.5–8.6
ε-NH_3^+	Lysine	9.0–11.0
–SH	Cysteine	9.0–10.5
Phenol	Tyrosine	9.8–11.0
Guanidinium	Arginine	10.0–12.0

equal to the pK value of that group. For pH = pK+1, the group is 90% dissociated, whereas for pH = pK−1 the group is 10% dissociated [11]. The amino acid residues of arginine and lysine are the main carriers of the positive charge, and partly also those of histidine, whereas the negative charge is contributed mainly by the residues of aspartic and glumatic acid, and, at higher pH values, cysteine.

From the effective charge z, the specific charge z_s, *i.e.* the charge referred to the unit of relative molecular mass M_r can be determined:

$$z_s = z/M_r \tag{2}$$

Specific charge can be used for an approximate estimation of the effective mobility.

An important electromigration characteristic of peptides is their isoelectric point (pI), *i.e.* the pH value of the solution in which the effective charge and the effective mobility of the peptide is zero. The isoelectric point differs from the similar characteristic, the isoionic point, in that it is referred to a given buffer composition in which the measurement of the electrophoretic mobility is carried out, and it comprises electrostatic interactions with all the ions present, whereas the isoionic point only takes into account interactions with protons. The isoelectric and isoionic points are mostly similar in value, but are not generally identical. In a solution where pH < pI, an amphoteric substance moves in a direct current electric field as a cation, whereas in a solution where the pH is higher than pI, it moves as an anion. The resulting peptide charge can thus be changed by changing the pH from a positive value to zero, then to a negative value. The

isoelectric points of numerous peptides and proteins are tabulated [9,12,13], or they can be determined experimentally by isoelectric focusing [14,15], chromatofocusing [16], or they can be calculated [17–19]. On the basis of the pI values of the given peptides, a suitable regimen may be selected (cationic or anionic) as well as the pH of their ITP separation (see Section 3.2).

For a more precise selection and optimization of the pH of the leading electrolyte, the dependence of the effective mobility or effective charge of the peptides to be determined on pH is important. This dependence can be determined experimentally by the acid–base titration curve method [20], the electrophoretic titration curve method [21,22], or it can be calculated theoretically [17–19,23].

From the pH dependence of the effective mobility, effective charge and specific charge, important conclusions can be inferred for the selection of conditions for the ITP separation of peptides and proteins. For ITP separation, the only suitable pH is that at which the effective mobilities are sufficiently high, *i.e.* approximately higher than $1–2 \cdot 10^{-9}$ m^2 V^{-1} s^{-1} [24], with an equal sign, *i.e.* the peptides to be separated move in the same direction, either cationic or anionic, and the differences Δm in their mobilities are sufficient for their separation ($\Delta m \approx 1 \cdot 10^{-9}$ m^2 V^{-1} s^{-1} [25,26].

If only the course of the dependence of specific charge on pH is known, then the pH for separation is selected so that the absolute value of the specific charge of the separated compound is higher than about $2–5 \cdot 10^{-4}$e [27].

Fig. 1 shows the calculated pH dependence of the effective charge of pig insulin [19]. For the calculation the average values from the pK ranges given in Table 1 were used as the dissociation constants of the ionogenic groups of the known amino acid sequence of insulin [28]. This leads to a certain inaccuracy because the pK values of individual ionogenic groups are different, not only in various polypeptides, but also in the molecule of the same peptide, as a result of the electro-

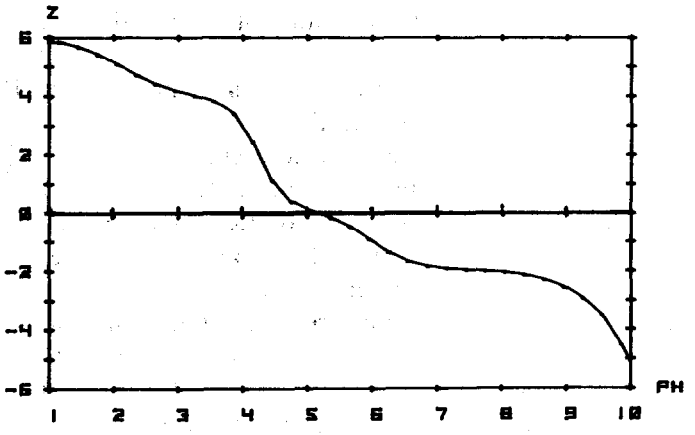


Fig. 1. Calculated dependence of effective charge z (in elementary units, e) of pig insulin on pH.

static and configurational effects of their environment. In spite of this inaccuracy the calculation gives some important information: on the basis of the calculated isoionic point and the course of the dependence of the effective charge on pH, conditions for the ITP analysis of insulin have been selected [19].

3. SELECTION OF CONDITIONS FOR ITP SEPARATION OF PEPTIDES

3.1. General

When selecting the conditions for the ITP separation of peptides the general rules for the selection of conditions for ITP separation should be followed [2,5,29]; however, the following specific properties of peptides should also be borne in mind:

1. Wide range of mobilities, including relatively low mobilities.
2. Wide range of solubilities, including relatively low solubilities.
3. High density, viscosity and sedimentation of polypeptide solutions.
4. Biological activity.
5. Chemical lability.
6. Thermal lability.
7. Complexity of the polypeptide mixtures, mutual interactions.
8. Interaction with a contact detector.

All these special properties result in some limitations for the selection of parameters for ITP analysis; this is discussed in detail in the following sections.

3.2. Regimen of separation and pH of leading electrolyte

The cationic or anionic regimen of separation and the pH of the leading electrolyte for ITP analysis are selected according to the character of the peptides to be determined, *i.e.* according to the dependence of their effective mobilities or effective and specific charges on pH. The isoelectric points, amino acid composition and approximate pK values of the ionogenic groups present (see Table I), or the data obtained during the characterization of a given sample by other methods (see Section 3.9.) are also important parameters in the selection procedure.

A cationic regimen (predominantly in the acidic pH region) is suitable for basic peptides (see Tables 5 and 6), whereas an anionic regimen (predominantly in the alkaline pH region) is used for neutral and acidic peptides (see Tables 3 and 4). The pH value of the leading electrolyte should be at least 1–2 units away from the isoelectric point, because in this region better conditions for solubility and sufficient effective mobility of polypeptides can generally be assumed than in close proximity to the isoelectric point.

The regimen of separation and the pH of the leading electrolyte should be

selected depending on the solubility, stability and biological activity of the peptides to be determined. For the correct choice of pH for the leading electrolyte, the following rules should be respected:

let pH_1 be the pH interval generally used in ITP (about 2–10);
let pH_2 be the pH interval in which the given polypeptides are soluble (at least about 1 mmol/l);
let pH_3 be the pH interval in which the given polypeptides are chemically stable;
let pH_4 be the pH interval in which the biological activity of the given peptides is preserved;
let pH_5 be the pH interval in which the given polypeptides possess a sufficient effective mobility of the same sign;
let pH_6 be the pH interval in which relative differences of effective mobilities of polypeptides (Δm_{ef}) are sufficient for their separation (about $\Delta m_{ef} > 2$–3%);

then the pH_{ITP} interval suitable for the ITP separation of the given polypeptides is given by the multiplication in the sense of algebra of classes (logical product $\cap$) of the above intervals:

$$pH_{ITP} = \bigcap_{i=1}^{6} (pH_i) \tag{3}$$

If the conservation of the biological activity of the peptides is not required (*e.g.* for amino acid sequence analysis even denatured polypeptides can be used), then the pH interval (preservation of biological activity) is not included in the logical product (eqn. 3).

3.3. Leading ion

In the anionic regimen the chloride anion is most frequently used as the leading ion, whereas in the cationic regimen potassium or sodium cations are used.

The use of a slower leading ion, *e.g.* 2-(*N*-morpholino)ethanesulphonic acid (MES) in the anionic regimen, is justified if only the sample components with a relatively low mobility are of interest. In this instance the components with a higher mobility than that of the leading ion do not move isotachophoretically, but follow the rules of zone electrophoresis in the zone of the leading electrolyte [30]. In this way extremely rapid components can be eliminated from the ITP analysis and the interpretation of the isotachopherogram is simplified.

The concentration of the leading ion, together with further parameters in Kohlrausch's regulation function [31], determines the concentration of the separated substances in the steady state and thus the concentration of the compounds can be controlled as required. For analytical purposes low concentrations

of the leading ion are suitable (0.01–0.005 mol/l), because lower concentrations of this ion in the analytical zone decrease the total amount of the sample required for the analysis, or increase the real length of the zone for an equal amount of analyte, hence increasing the sensitivity or accuracy of the analysis. It is therefore more advantageous theoretically to work at still lower concentrations of the leading ion, but in practice there are difficulties caused by the low buffering capacity of solutions diluted in this manner and by the presence of minor impurities in the electrolyte system. The concentration of the leading ion also determines the ionic strength of a given electrolyte system, which should be selected with respect to the solubility of the analysed peptides. The concentration effect causes the concentration of polypeptides in the steady state to be relatively high and their precipitation may take place in the ITP zone. For this reason it is also more suitable to select lower concentration of the leading ion.

3.4. Counter-ion

The counter-ion of the leading electrolyte should be selected so that it has sufficient buffering capacity at the given pH of the leading electrolyte, pH_L. Under the anionic (cationic) regimen this requirement is fulfilled by a weak base (acid), the pK_a of which is in the interval ($pH_L \pm 0.5$), *i.e.*:

$$pK_a \doteq pH_L \pm 0.5 \tag{4}$$

The concentration of the counter-ion c_c ranges, in agreement with the condition given by eqn. 4, in the following interval:

$$1.3 \cdot c_L \leqslant c_c \leqslant 4c_L \tag{5}$$

where c_L is the concentration of the leading ion.

In addition to the function of the buffering component the counter-ions may also fulfil further roles. The addition of counter-ions which form complexes with selected components and which influence the mobility of certain peptides and proteins selectively may increase the separation power of ITP. The use of mixtures of counter-ions in the leading electrolyte increases the buffering capacity within a broader pH interval [32].

Zwitter-ionic substances used as biological buffers may be recommended as counter-ions [33] and also as terminating ions, *e.g.* MES, ACES, MOPS, TES, TMAPS, BIS-TRIS (for chemical compositions, see list of abbreviations), which are characterized by the facts that their pK value is generally within the range 6–8, they are soluble in water, have a minimum salting-out effect, their dissociation constants are not very dependent on temperature and sample composition, they do not form complexes with inorganic cations, are chemically stable, non-toxic and they are easily available in the pure form. As a result of all these properties they are especially suitable for the separation of biologically active substances.

3.5. Terminating ion

In view of the relatively low effective mobility of a large number of peptides, extremely slow terminating ions must be selected for their ITP separation, *i.e.* ions with a very small specific charge. A low specific charge may be achieved by the selection of an ionogenic substance with a low degree of dissociation at the given pH of the ITP separation and/or with a large relative molecular mass. When a very slow terminating ion is used the range of mobilities between the leading and the terminating ion is also increased, which in turn increases the amount of information which can be obtained from the isotachopherogram [34]. The use of a more rapid terminating ion eliminates very slow sample components from the ITP separation, which travel under the regimen of zone electrophoresis in the zone of the terminating electrolyte, thus simplifying the interpretation of the isotachopherogram.

Under the anionic (cationic) regimen an anion of a weak acid (base) is suitable as the terminating ion; the pK_a value of this acid (base) should be 1–2 pH units higher (lower) than the pH of the leading electrolyte (pH_L) so that at pH_L their degrees of dissociation and effective mobilities are low.

Low effective mobilities may also be attained by using a derivatized polymer with a high mass-to-charge ratio. Thus derivatized poly(ethylene glycol) was used (M_r = 6000–7500) with two carboxyl groups with a pK_a value of approximately 3.7 [27]. The mobility of the fully ionized polymer was approximately $1.3 \cdot 10^{-9}$ $m^2\ V^{-1}\ s^{-1}$, which is the value considered to be the lower limit of mobility of substances separable by ITP [24].

The total concentration range of the terminating ion is usually 0.01–0.03 mol/l (see Tables 3–8).

3.6. Electrolyte system solvents

ITP analyses are mainly carried out in aqueous solutions of leading and terminating electrolytes. However, other solvents can also be used to influence the mobility and solubility of analysed substances, as well as their mixtures with or without water, *e.g.* methanol [36–38] and other alcohols (C_2–C_4) [19,36,39], acetone, dioxane [36], acetonitrile [40], dimethylformamide [41] and dimethyl sulphoxide [42–44]. The solvents used in ITP must fulfil the condition of low autodissociation and their own electrical conductivity must also be low. Their ionic product should be comparable to the ionic product of water (pK_{H_2O} = 14) or higher, because the concentration of ions originating from the solvent must be negligible compared to the concentration of ions in the electrolyte system.

3.7. Additives

3.7.1. Additives to the leading electrolyte

These additives fall into the classes listed below.

1. Agents decreasing electro-osmosis, *e.g.* surface active substances (polyvinyl alcohol, Triton X-100) [2,45,46] which increase the sharpness of the boundary zones.

2. Agents increasing the viscosity of the solution (derivatives of cellulose such as -methyl, -hydroxyethyl, -hydroxypropylmethyl [46] and poly(ethylene glycol) [2]). These anticonvective agents stabilize the zones of the separated peptides and proteins and thus contribute to maintaining the sharpness of zone boundaries.

3. Agents increasing the solubility of peptides: chaotropic agents (urea and its derivatives [45]), non-ionogenic detergents (Triton X-100, Nonidet P 40, Tween 20), β-octyl-D-glucopyranoside [45] and amphoteric (zwitter-ionic) detergents of the sulphobetaine type (SB-12, SB-14, TMAPS) [45,47] and amphoteric derivatives of cholic acid [CHAPS, CHAPSO] [47,48]. The last mentioned group has suppressed denaturation effects and they are especially suitable for the solubilization of membrane and hydrophobic polypeptides and proteins. A survey of detergents wich can be used and their chemical composition is given in Table 2.

4. Agents increasing the separation power and selectivity. These are non-ionogenic substances forming complexes or conjugates with selected components of the mixture and affecting their mobility (*e.g.* propanal [2], cyclodextrins and crown ethers [49–51]).

3.7.2. Additives to the sample

These additives fall into the classes listed below.

1. Agents increasing solubility: chaotropic agents, non-ionogenic and zwitter-ionic detergents (see Section 3.7.1), solvents (see Section 3.6), and spacers forming mixed zones with peptides and decreasing their concentration in the steady state.

2. Agents increasing the separation power and selectivity such as ionogenic and non-ionogenic complexing agents. These agents form complexes with selected mixture components; they influence their mobility selectively and enable their separation (see Section 3.7.1.). By forming complexes, some undesirable components may be eliminated from the ITP analysis.

3. Agents increasing the sensitivity of the detection from the point of view of the minimum detectable amount; compounds forming mixed zones with the determined components prolong their zones and thus decrease the detection limits.

4. Agents enabling identification and quantification. The addition of internal standards to the sample enables, in some instances, a more accurate qualitative and quantitative evaluation of the isotachopherogram.

5. Agents increasing the resolving power of the UV detector. Spacers which

TABLE 2

SURVEY OF DETERGENTS USED IN ITP AND THEIR CHEMICAL COMPOSITION

Non-ionogenic detergents		Amphoteric detergents	
Polyoxyethylene-			
alcohol ether	Brij 35 (monolauryl ether)	Alkyl sulphobetaine	N-Alkyl-N,N-dimethyl-3-ammonio-1-propanesulphonate
sorbitol ester	Tween 20 (monolaurate)		(alkyl = octyl–cetyl)
	Tween 80 (mono-oleate)		Zwittergent 3-08–3-16
alkylphenol ether	Triton X-100	Cholamidopropyl	3-[(3-Cholamidopropyl-dimethyl-ammonio]-2-hydroxy)*-1-
	Nonidet P-40 (*p-tert.*-octyl phenol ether)	sulphobetaine	propanesulphonate
			CHAPS, CHAPSO*
Alkyl-			
β-D-hexosopyranoside	Octyl-β-D-glycopyranoside		
N-methylglucamide	MEGA-8 (Octanoyl-N-methylglucamide)		

have a low absorption in the UV region and intermediary mobilities with respect to the separated polypeptides are inserted between their zones and allow their resolution by UV detection.

3.7.3. Additives to the terminating electrolyte

Addition of barium hydroxide or strontium hydroxide [52] decreases the concentration of carbonates in the terminating electrolyte, which limits the effect of these undesirable ions which travel out from the space of the terminating electrolyte and disturb the ITP analysis under the anionic regimen in the neutral and alkaline pH regions. The amount of hydroxide added is chosen so that the pH of the terminating electrolyte is in the range 10.0–10.8.

Examples of the additives used and their concentrations in the electrolyte systems are given in Tables 3–8.

The procedure for the choice of a suitable solvent and additives to the leading electrolyte for the ITP separation of peptides and proteins is shown in Fig. 2 in flow-chart form.

3.8. Detection

The interaction of polypeptides with the microelectrodes of the conductivity or potential gradient detector is the cause of low resolving power and poor reproducibility, so that zones with a low difference in conductivity cannot be sufficiently resolved and some components of the mixture cannot be identified reliably. For this reason contact detectors do not suffice for the determintation of polypeptides. However, they can be used for smaller peptides which do not interact with the electrodes. For these small peptides a universal detector is indispensable if the instrument is not provided with a photometric detector for the wavelength in the region of absorption of the peptide bond, *i.e.* in the short-wavelength UV region (200–220 nm).

Of the various universal detectors, the contactless high-frequency conductivity detector [53–55] seems to have prospects. Of the specific detectors, the contactless photometric detector is most frequently used. Its great advantage is that the contactless detector keeps its high resolving power and reproducibility even with complex mixtures of polypeptides.

The photometric detector is suitable for the detection of peptides absorbing light at the given wavelength of the detector. For the most frequently used wavelength of 254 nm, the absorption is due to the presence of aromatic amino acid residues (tyrosine, tryptophan, phenylalanine) in the polypeptide chain.

A specific photometric detector can be used universally under the assumption that the counter-ion of the leading electrolyte absorbs light of the detection wavelength. Owing to different counter-ion concentrations in the ITP zones, a different detector signal is obtained for each zone.

A universal detector for peptides is that for the 206-nm wavelength [56,57], *i.e.*

TABLE 3

ITP ANALYSIS OF PEPTIDES IN ANIONIC REGIMEN: AQUEOUS ELECTROLYTE SYSTEMS (ES)

See list of abbreviations for definitions. n.g. = Not given.

ES No.	Sample	Leading electrolyte[a]	Terminating electrolyte[b]	Ref.
1	Reduced and oxidized forms of GSH	L: Cl^- (0.01) C: BALA pH: 3.1	T: CAPR (0.01) pH: n.g.	121
2	Isovalerylglycin in urine	L: Cl^- (0.01) C: BALA $CuCl_2$ (0.001) pH: 3.1	T: CAPR (0.01) pH: n.g.	122
3	Middle molecule peptides in uraemic plasma and urine	L: Cl^- (0.005) C: BALA (0.02) A: HPMC (0.04) pH: 4.0	T: CAPR (0.005) pH: n.g.	123
4	Asp–Glu–Gly	L: Cl^- (0.01) C: His pH: 5.8	T: MES (0.001) P: TRIS pH: 5.8	124
5	Asp–Gln	L: Cl^- (0.01) C: BIS–TRIS A: HPMC (0.4) pH: 6.0	T: MES (0.01) P: TRIS pH: $\approx$ 6	57
6	Ala–Glu Ala–Gln	L: Cl^- (0.01) C: His A: HPMC (0.4) pH: 6.0	T: MES (0.01) P: TRIS pH: 6.0	100
7	Gly–Pro Pro–Gly	L: Cl^- (0.01) C: AMPOL A: PVA (0.05) pH: 6.0	T: GABA (0.01) P: $Ba(OH)_2$ pH: 10.9	125
8	Muramindipeptide (Mur–Ala–Gln)	L: Cl^- (0.005) C: His (0.01) A: PVA (0.2) pH: 6.1	T: HEPES P: $Ba(OH)_2$ pH: 8.1	19
9	Tryptic fragments of human haemopexin	L: Cl^- (0.01) C: His (0.02) pH: 6.1	T: HEPES P: $Ba(OH)_2$ pH: 8.1	19
10	Reduced and oxidized forms of GSH	L: Cl^- C: AMED (0.02) A: HPMC (0.5) pH: 6.52	T: Phenol (0.005) P: $Ba(OH)_2$ pH: 10.0	36, 86
11	Angiotensin I and II, reduced and oxidized forms of GSH	L: Cl^- (0.007) C: TRIS A: HPMC (0.3) pH: 7.0	T: Phenol (0.005) P: $Ba(OH)_2$ pH: 10.0	86, 89
12	Oxidized and reduced forms of GSH, GSH conjugates with electrophilic components	L: Cl^- (0.005) C: TRIS A: HPMC (0.25) pH: 7.0	T: Phenol (0.005) P: TRIS (0.01) $Ba(OH)_2$ pH: 10.0	126,127

TABLE 3 *(continued)*

ES No.	Sample	Leading electrolyte	Terminating electrolyte	Ref.
13	Angiotensin I and II	L: Cl^- C: AMED (0.02) A: MC (0.05) pH: 7.05	T: Phenol (0.005) P: $Ba(OH)_2$ pH: 10.0	86
14	Synthetic fragments of human fibrin (decapeptide and undecapeptide)	L: Cl^- (0.005) C: TRIS pH: 7.2	T: Val (0.01) P: $Ba(OH)_2$ pH: 9.0	97
15	Ala–Ala, Ala–AABA, Ala–Asn, Ala–Gly, Ala–Leu, Ala–Met, Ala–Phe, Ala–Ser, Ala–Val, Gly–Ala, Gly–AABA, Gly–Asn, Gly–GLy, Gly–Ile, Gly–Leu, Gly–Phe, Gly–Pro, Gly–Ser, Gly–Thr, Gly–Trp, Gly–Tyr, Gly–Val, Leu–Gly, Leu–Leu, Leu–Phe, Leu–Tyr, Leu–Val	L: Cl^- (0.01) C: IMID (0.02646) A: HPC (0.02) pH: 7.41	T: Tau (0.01) P: $Ba(OH)_2$ pH: 10	26
16	Gly–Pro, Gly–Hyp, Val–Pro, Leu–Pro	L: Cl^- (0.01) C: AMPOL pH: 7.5	T: GABA (0.01) P: $Ba(OH)_2$ pH: 10.9	128
17	Glu–Pro, Asp–Pro, Thr–Pro, Gly–Pro, Leu–Pro, Ile–Pro, Ala–Pro, Val–Pro, Phe–Pro, Tyr–Pro, Ser–Pro, Pro–Pro in urine	L: Cl^- (0.01) C: AMPOL pH: 7.5	T: GABA (0.01) P: $Ba(OH)_2$ pH: 10.9	129
18	Synthetic fragment of human growth hormone HGH (125–156)	L: Cl^- (0.01) C: TRIS (0.02) A: TX-100 (0.2) pH: 7.65	T: Gly (0.05) P: $Ba(OH)_2$ pH: 9.0	93, 94
19	Aspartame (Asp–Phe–methyl ester)	L: HAc (0.005) C: TRIS pH: 7.7	T: His (0.005) P: TRIS pH: 7.8	111
20	Human apo-HDL-polypeptides	L: Cl^- (0.005) C: TRIS (0.02) A: TX-100 (0.2) pH: 8.05	T: BALA (0.04) P: $Ba(OH)_2$ pH: 8.7	130
21	Bull seminal trypsin isoinhibitors (BUSI IA, IB1, BUSI IB2) Cow colostral isoinhibitors of trypsin (CTI A, B, C), cyanogen bromide fragments of human haemopexin	L: Cl^- (0.005) C: TRIS (0.01) A: PVA (0.02) pH: 8.1	T: Gly (0.01) P: $Ba(OH)_2$ pH: 10.5	19, 96
22	Cyanogen bromide fragments of human haemopexin	L: Cl^- (0.005) C: TRIS (0.01) pH: 8.1	T: Gly (0.01) P: $Ba(OH)_2$ pH: 10.5	96
23	Cyanogen bromide fragments of human haemopexin	L: Cl^- (0.005) C: TRIS (0.01) A: SB 12 (0.1–0.2) pH: 8.1	T: Gly (0.01) P: $Ba(OH)_2$ pH: 10.5	96
24	Ala–Ala, Ala–Gly, Gly–Asn, Gly–Gly, Gly–Ile, Gly–Leu, Gly–Phe, Gly–Pro, Gly–Ser, Gly–Trp, Leu–Gly	L: Cl^- (0.01) C: AMED (0.01301) A: HPC (0.02) pH: 8.30	T: BALA (0.01) P: $Ba(OH)_2$ pH: 10	26

(Continued on p. 138)

TABLE 3 *(continued)*

ES No.	Sample	Leading electrolyte[a]	Terminating electrolyte[b]	Ref.
25	Bull seminal trypsin isoinhibitors (BUSI IA, IB1, IB2) Cow colostral trypsin isoinhibitors (CTI A, B, C)	L: Cl^- (0.01) C: TRIS (0.02) A: PVA (0.02) pH: 8.3	T: Gly (0.01) P: $Ba(OH)_2$ pH: 10.5	96
26	Ala–AABA, Ala–Asn, Ala–Leu, Ala–Met, Ala–Phe, Ala–Ser, Ala–Val, BALA–His, Gly–Ala, Gly–AABA, Gly–Thr, Gly–Tyr, Gly–Val, Leu–Leu, Leu–Phe, Leu–Tyr, Leu–Val	L: Cl^- (0.01) C: AMED(0.01354) A: HPC (0.02) pH: 8.37	T: BALA (0.01) P: $Ba(OH)_2$ pH: 10	26
27	Methotrexate	L: Cl^- (0.008) C: TRIS A: PVA (0.05) pH: 8.4	T: His (0.008) P: TRIS pH: 9.5	131
28	Pig and beef insulins	L: Cl^- (0.005) C: AMED pH: 8.5	T: EACA (0.01) P: $Ba(OH)_2$ pH: 10.5	116
29	Cyanogen bromide fragments of human heamopexin	L: Cl^- (0.01) C: TRIS (0.02) A: Urea (7.2 mol/l) pH: 8.5	T: Gly (0.01) P: $Ba(OH)_2$ pH: 10.2	96
30	Ala–Ala, Ala–Gly, Gly–Ala, Gly–Asn, Gly–Gly, Gly–Ile, Gly–Leu, Gly–Phe, Gly–Pro, Gly–Ser, Gly–Trp, Gly–Val, Leu–Gly, Leu–Tyr	L: Cl^- (0.01) C: AMED (0.01586) A: HPC (0.02) pH: 8.59	T: BALA (0.01) P: $Ba(OH)_2$ pH: 10	26
31	The same as in ES 15	L: Cl^- (0.01) C: AMED (0.01886) A: HPC (0.01) pH: 8.77	T: BALA (0.01) P: $Ba(OH)_2$ pH: 10	26
32	Peptides from pregnant sow ovary tissue extracts	L: Cl^- (0.005) C: AMED A: HPMC (0.5) pH: 8.83	T: BALA (0.02) P: $Ba(OH)_2$ pH: 10	132
33	Gly–Gly, Gly–Gly–Gly	L: Cl^- C: AMED (0.05) pH: 8.97	T: BALA (0.01) P: $Ba(OH)_2$ pH: 10.9	120
34	Ala–Tyr, Ala–Val, Ala–Glu, Gly–Ala, Gly–Val, Gly–Tyr, Gly–Leu, Gly–Gly, Gly–Gly–Gly, Ala–Ala, Ala–Ala–Ala	L: Cl^- C: AMPOL (0.05) A: MC (0.5) pH: 8.97	T: BALA (0.01) P: $Ba(OH)_2$ pH: 10.9	86
35	Disarcosine	L: Cl^- (0.01) C: AMED A: PVA (0.2) pH: 8.97	T: BALA (0.01) P: $Ba(OH)_2$ pH: 10.9	36
36	GSH, Leu–Tyr, Gly–Gly, Gly–Gly–Gly–Gly	L: 5-Br-2,4-dihydroxybenzoic acid (0.04) C: Lys pH: 9.0	T: Ala P: $Ba(OH)_2$ pH: 9.8	85

TABLE 3 *(continued)*

ES No.	Sample	Leading electrolyte[a]	Terminating electrolyte[b]	Ref.
37	Aspartame (Asp–Phe–methyl ester)	L: Cl^- (0.01) C: AMED (0.02) A: HPMC (0.025) TX-100 (0.1) pH: 9.0	T: Ala (0.01) P: $Ba(OH)_2$ pH: 9.8–10.0	112
38	Drug forms of insulins	L: Cl^- (0.005) C: AMED A: HPMC (0.2) pH: 9.0	T: BALA (0.01) P: $Ba(OH)_2$ pH: 10.4	116
39	Insulin	L: Cl^- (0.01) C: TRIS A: TX-100 (0.2) pH: 9.0	T: m-Cresol (0.01) P: $Ba(OH)_2$ pH: 11.0	36
40	The same as in ES 15 and BALA–His	L: Cl^- (0.01) C: EA (0.01333) A: HPC (0.02) pH: 9.06	T: BALA (0.01) P: $Ba(OH)_2$ pH: 10	26
41	Ala–Gln, Ala–Ala	L: MES (0.005) C: AMED A: HPMC (0.4) pH: 9.1	T: BALA (0.01) P: AMED $Ba(OH)_2$ pH: ~10	57
42	N-Benzoyl–Ala–Gln N-Benzoyl–Ala	L: Cl^- (0.01) C: AMED pH: 9.1	T: Gln (0.01) P: $Ba(OH)_2$ pH: 10	101
43	N-Benzoyl–Ala–Gln N-Benzoyl–Ala	L: Cl^- (0.01) C: AMED pH: 9.1	T: BALA (0.01) P: $Ba(OH)_2$ pH: 10	101
44	Pig insulin	L: Cl^- (0.005) C: AMED A: HPMC (0.2–0.3) pH: 9.1	T: EACA (0.01) P: $Ba(OH)_2$ pH: 10.5	116
45	Ala–Gln, N-2-Tyr–N-6-Tyr–Lys	L: MES (0.0055) C: AMED A: HPMC (0.4) pH: 9.1	T: BALA (0.01) P: AMED (0.01) $Ba(OH)_2$ pH: 10.5	98, 99
46	Glu–Ala, Gly–Gly–Val, Gly–Gly, Gly–Gly–Gly	L: Cl^- (0.01) C: AMED pH: 9.15	T: EACA (0.01) P: $Ba(OH)_2$ pH: 10.5	133
47	Reduced and oxidized forms of GSH, Gly–Gly, Ala–Gly, Gly–BALA, Gly–Ala, Leu–Gly, Val–Val, Val–Leu, Gly–His, Leu–Val, Leu–Leu, Ala–Ala–Ala	L: Cl^- (0.005) C: AMED (0.01) A: HPMC (0.25) pH: 9.2	T: 8-Aminooctanoic acid (0.01) P: AMED (0.01) $Ba(OH)_2$ pH: 10.8	87, 134
48	Bacitracin	L: Cl^- (0.005) C: AMED A: HPMC (0.3) pH: 9.3	T: EACA (0.005) P: $Ba(OH)_2$ pH: 10.0	89

(Continued on p. 140)

TABLE 3 *(continued)*

ES No.	Sample	Leading electrolyte[a]	Terminating electrolyte[b]	Ref.
49	The same as in ES 15	L: Cl^- (0.01) C: EA (0.01619) A: HPC (0.02) pH: 9.33	T: BALA (0.01) P: $Ba(OH)_2$ pH: 10.0	26
50	The same as in ES 15	L: Cl^- (0.01) C: EA (0.01959) A: HPC (0.02) pH: 9.52	T: BALA (0.01) P: $Ba(OH)_2$ pH: 10.00	26
51	Ala–Ala, Ala–Ala–Ala, Gly–Gly, Gly–Gly–Gly	L: Cl^- C: AMED A: MC (0.5) pH: 9.63	T: Phenol (0.01) P: $Ba(OH)_2$ pH: 10.15	86
52	Monocomponent insulin	L: Cl^- (0.005) C: TRIS (0.0055) A: HPMC (0.3) pH: n.g.	T: Tricine (0.01) P: $Ba(OH)_2$ pH: 12.2	116
53	Monocomponent insulin	L: Cl^- (0.005) C: TRIS (0.0055) A: HPMC (0.3) Urea (6 mol/l) pH: n.g.	T: Trincine (0.01) P: $Ba(OH)_2$ pH: 12.2	116

[a] L: Leading ion (mol/l). C: counter-ion constituent (mol/l). A: Additive (%, w/v).
[b] T: Terminating ion constituent (mol/l). P: pH adjusting constituent (mol/l).

TABLE 4

ITP ANALYSIS OF PEPTIDES IN ANIONIC REGIMEN: MIXED SOLVENT ELECTROLYTE SYSTEMS

See list of abbreviations and Table 3 for definitions.

ES No.	Sample	Leading electrolyte[a]	Terminating electrolyte[a]	Ref.
54	Pig insulin, pig proinsulin	L: Cl^- (0.005) C: TRIS (0.01) S: Propane-2-ol–water (10:90, v/v) pH: 8.1	T: Gly (0.01) P: $Ba(OH)_2$ S: Water pH: 10.2	19
55	Pig insulin, pig proinsulin, Pig desoctapeptide–(B-22-31)–insulin	L: Cl^- (0.005) C: TRIS (0.01) S: propane-1-ol–water (20:80, v/v) pH: 8.1	T: Gly (0.01) P: $Ba(OH)_2$ S: Water pH: 10.2	96

[a] S: Solvent.

TABLE 5

ITP ANALYSIS OF PEPTIDES IN CATIONIC REGIMEN: AQUEOUS ELECTROLYTE SYSTEMS

See list of abbreviations and Table 3 for definitions.

ES No.	Sample	Leading electrolyte	Terminating electrolyte	Ref.
56	β-Endorphin	L: K^+ (0.01) C: HAc pH: 4.2	T: BALA (0.01) pH: n.g.	46
57	Aspartame (Asp–Phe–methyl ester)	L: NH_4^+ (0.05) C: HAc (0.10) pH: n.g.	T: HAc (0.05) pH: n.g.	109
58	Bovine basic pancreatic trypsin inhibitor, bull seminal trypsin isoinhibitors (BUSI II, IIb)	L: Na^+ (0.01) C: HAc A: PVA (0.02) pH: 4.8	T: BALA (0.01) P: HAc pH: 4.5	19
59	C-Terminal pentapeptide from bombinin (Gln–His–Phe–Ala–Asn–NH_2), Phe–Ala–Asn–NH_2	L: K^+ (0.005) C: HAc pH: 4.9	T: BALA (0.005) P: HAc pH: 5.1	105, 106
60	Adiuretin [(8-D-Arg)–deaminovasopressin]	L: Na^+ (0.01) C: HAc pH: 5.0	T: BALA (0.01) P: HAc pH: 4.7	19
61	Gly–Gly, His–His	L: K^+ (0.01) C: HAc A: PVA (0.05) or MEC 0.2 pH: 5.0	T: H^+ pH: n.g.	56
62	CM–Cys–His–Pro–NH_2 PCA–His–Pro–NH_2	L: K^+ (0.006) C: HAc A: HPMC (0.2) pH: 5.0	T: BALA (0.01) pH: n.g.	103
63	CM–Cys–His–Pro–NH_2 PCA–His–Pro–NH_2	L: K^+ (0.006) C: HAc A: HPMC (0.4) pH: 5.0	T: BALA (0.01) P: HAc pH: 5.0	103
64	Protirelin	L: K^+ (0.006) C: HAc A: HPMC (0.4) pH: 5.0	T: BALA (0.01) pH: n.g.	107
65	CM–Cys–His–Pro–NH_2 PCA–His–Pro–NH_2	L: K^+ (0.006) C: Pivalic acid A: HPMC (0.4) pH: 5.1	T: BALA (0.01) P: Pivalic acid pH: 4.9	103
66	Pig vasointestinal peptide	L: K^+ (0.01) C: HAc pH: 5.1	T: BALA (0.01) pH: n.g.	46
67	Vasointestinal peptide	L: Ba^{2+} (0.005) C: Val A: HPMC (0.25) pH: 5.1	T: Ala (0.005) pH: n.g.	89, 113

(Continued on p. 142)

TABLE 5 *(continued)*

ES No.	Sample	Leading electrolyte	Terminating electrolyte	Ref.
68	Triglycylvasopressin, Lys–vasopressin, *O*-methyloxytocin, bovine basic pancreatic trypsin inhibitor	L: K^+ (0.01) C: HAc pH: 5.1	T: BALA (0.02) pH: n.g.	95
69	Secretin, cholecystokinin	L: K^+ C: HAc A: HPMC (0.4) pH: 5.2	T: Ala (0.01) pH: n.g.	89
70	Pig intestinal peptide with C-terminal somatostatin	L: K^+ (0.01) C: HAc A: HPMC (0.2) Urea (4 mol/l) pH: 5.3	T: Ala (0.01) pH: n.g.	114
71	Saralasin, gonadorelin, (1-Asn, 5-Val)angiotensin II-diacetate, (1-Mpa-8-D-Arg) vasopressin, (2-*O*-Met-Tyr)oxytocin, protirelin	L: K^+ (0.006) C: CACOD A: HPMC (0.4) pH: 6.2	T: Creatinine (0.01) P: HCl pH: 5.9	107
72	Saralasin, gonadorelin	L: K^+ (0.006) C: CACOD A: HPMC (0.4) pH: 6.2	T: EACA (0.01) P: HCl pH: 4.8	107
73	Substance P (undecapeptide)	L: K^+ (0.006) C: MES A: HPMC (0.4) pH: 6.2	T: Creatinine (0.01) P: HCl pH: 5.8–6.0	108
74	Substance P (undecapeptide)	L: K^+ (0.005) C: MES A: HPMC (0.4) pH: 6.3	T: Creatinine (0.01) P: HCl pH: 5.8–6.0	108
75	Somatostatin (fourteen amino acid residues)	L: K^+ (0.005) C: CACOD A: HPMC (0.5) pH: 6.9	T: Creatinine (0.01) pH: n.g.	89, 94, 114
76	Oxytocin, Arg-vasopressin, bacitracin, ACTH, cholecystokinin fragment (six amino acid residues)	L: K-Acetate (0.005) C: CADOD A: HPMC (0.25) pH: 7.0	T: BALA (0.01) P: HCl pH: 3.8	89
77	Snake venom peptides from Chinese cobra and Siamese cobra	L: K^+ (0.005) C: CACOD pH: 7.0	T: Creatinine (0.01) P: HCl pH: 5	46
78	Basic polypeptides	L: K-Acetate (0.0025) pH: 8.0	T: Ala (0.005) P: HCl pH: 1.8	135
79	Met–Lys–bradykinin, kallidin	L: Ba^{2+} (0.01) C: Gln pH: 9.25	T: TRIS (0.02) P: HCl pH: 8.0	36, 86
80	Arg–vasopressin, Lys–vasopressin	L: Ba^{2+} (0.005) C: Val pH: 9.3	T: TRIS (0.02) P: HCl (0.005) pH: 8.4	89
81	Kallidin, bradykinin	L: Ba^{2+} (0.01) C: Met pH: 9.55	T: TRIS (0.02) P: HCl (0.005) pH: 8.0	36, 86

TABLE 6

ITP ANALYSIS OF PEPTIDES IN CATIONIC REGIMEN: MIXED AND NON-AQUEOUS SOLVENT ELECTROLYTE SYSTEMS

See list of abbreviations and Table 3 for definitions.

ES No.	Sample	Leading electrolyte	Terminating electrolyte	Ref.
82	Insulin	L: K-Acetate (0.004) C: HAc (0.1) S: Methanol–water (80:20, v/v) pH: n.g.	T: Gly (0.01) P: HAc (0.05) S: Water pH: n.g.	115
83	N-2-Acetyllysine, N-2-Acetyllysine methyl ester	L: K^+ (0.01) C: α-Ketoglutaric acid S: methanol pH: 5.32	T: Betaine · HCl (0.01) S: methanol pH: n.g.	136

in the region of the absorption of the peptide bond. More information is obtained by simultaneous detection at two wavelengths [58], or by spectrophotometric detection. Spectrophotometric detection was realized in ITP by using a classical spectrophotometer and by stoppig the zones with a counter flow [59], or with a diode-array detector [60].

TABLE 7

ITP ANALYSIS OF ANIONIC ADMIXTURES IN SYNTHETIC PEPTIDES: AQUEOUS ELECTROLYTE SYSTEMS

See list of abbreviations and Table 3 for definitions.

ES No.	Sample	Leading electrolyte	Terminating electrolyte	Ref.
84	Acetate in saralasin	L: Cl^- (0.005) C: EACA A: HPMC (0.3) pH: 4.4	T: CAPR (0.008) P: TRIS pH: 4.6	107
85	Cl^-, Br^-, I^- in peptides	L: NO_3^- C: $Cd(NO_3)_2$ (0.004) pH: 5.6	T: Tartaric acid (0.01) P: NaOH pH: 5.60	138
86	Formate, citrate, methanesulphonate, trifluoracetate, chloracetate, acetate, *p*-toluenesulphonate, 1-hydroxy-benzotriazole in ACTH (1–24), β-LPH (62–77), β-LPH (41-43)	L: Cl^- (0.01) C: His pH: 5.8	T: MES (0.001) P: TRIS pH: 5.8	124, 138
87	Formate, acetate, trifluoracetate in derivatives of oxytocin and vasopressin	L: Cl^- (0.005) C: His (0.01) pH: 6.1	T: Na-Glu (0.01) pH: 6.7	96

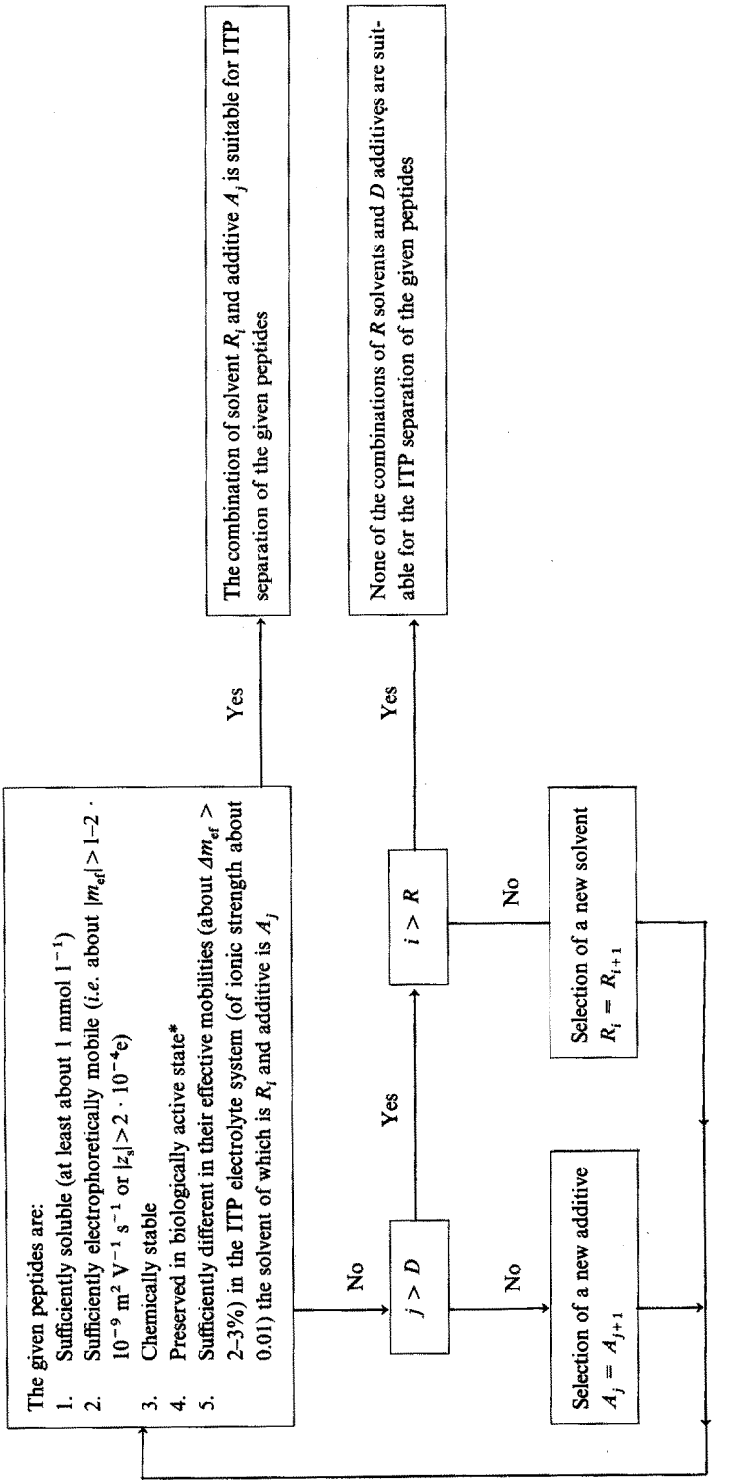


Fig. 2. Flow diagram for the choice of solvent and additives for ITP separation of peptides. The solvent R_i is selected from a set of R solvents; this set contains individual solvents (*e.g.* water, alcohols, acetone) and their mixtures (*e.g.* water–methanol, water–dimethyl sulphoxide) – see Section 3.6. The additive A_j is selected from a set of D individual additives and their mixtures (*e.g.* chaotropic agents, detergents, cellulose derivatives) – see Section 3.7. Asterisk denotes a requirement which is not always indispensable (see Section 3.2.); m_{ef} = effective mobility; z_s = specific charge; Δm_{ef} = relative difference of effective mobilities.

TABLE 8

ITP ANALYSIS OF CATIONIC ADMIXTURES IN SYNTHETIC PEPTIDES: AQUEOUS ELECTROLYTE SYSTEMS

See list of abbreviations and Table 3 for definitions.

ES No.	Sample	Leading electrolyte	Terminating electrolyte	Ref.
88	NH_4^+ in dipeptide Glu–Gly, β-endorphin (6-17), (8-L-arginine-des-9-glycinamide) vasopressin	L: H^+ C: Cl^- (0.01) pH: 2	T:Na^+ (0.01) P: Citrate pH: 8.0	138
89	Hydrazine, sodium, trimethylamine, tetramethylammonium, pyridine, piperidine, 4-dimethylaminopyridine, N-ethylmorpholine, N,N-diisopropyl-ethylamine, triethylamine, dicyclo-hexylamine in oligo- and polypeptides	L: K^+ (0.01) C: HAc pH: 4.5	T: BALA (0.01) P: HAc pH: 4.5	138
90	Benzyldimethylammonium in undecapeptide substance P	L: K^+ (0.006) C: MES A: HPMC (0.4) pH: 6.2	T: Creatinine (0.01) P: HCl pH: 5.8–6.0	108

Further types of detectors which can be used for peptides are fluorescence [61], radiometric [62] and mass spectrometric detectors [35].

3.9. Selection of conditions for ITP analysis on the basis of characteristics obtained by other methods

ITP belongs to the group of electromigration separation methods. It is therefore natural that for the choice of conditions for ITP analysis the most valuable information is obtained from these methods, which are based on a similar separation principle.

The most valuable information – the dependence of the effective mobilities of peptides and proteins on pH – is afforded by the method of electrophoretic titration curves. Valuable information on effective mobility at a given pH is afforded by capillary zone electrophoresis (CZE) in a free solution [63–65]. The data from electrophoresis on a carriers (restrictive and non-restrictive polyacrylamide gels, agarose gels, paper, cellulose acetate) must be considered critically with respect to the possible influences of the sieve effect, interaction with the carrier and electroosmotic flow on the effective mobility. The sieve (restriction) effect of the gel can be characterized by the Ferguson plot [66] – the dependence of the effective mobility on the total concentration of the gel. Extrapolation to zero concentration of the gel gives values of effective mobilities which apply during the ITP separation in free solution.

Isoelectric focusing (IEF) gives the isoelectric point, a very important charac-

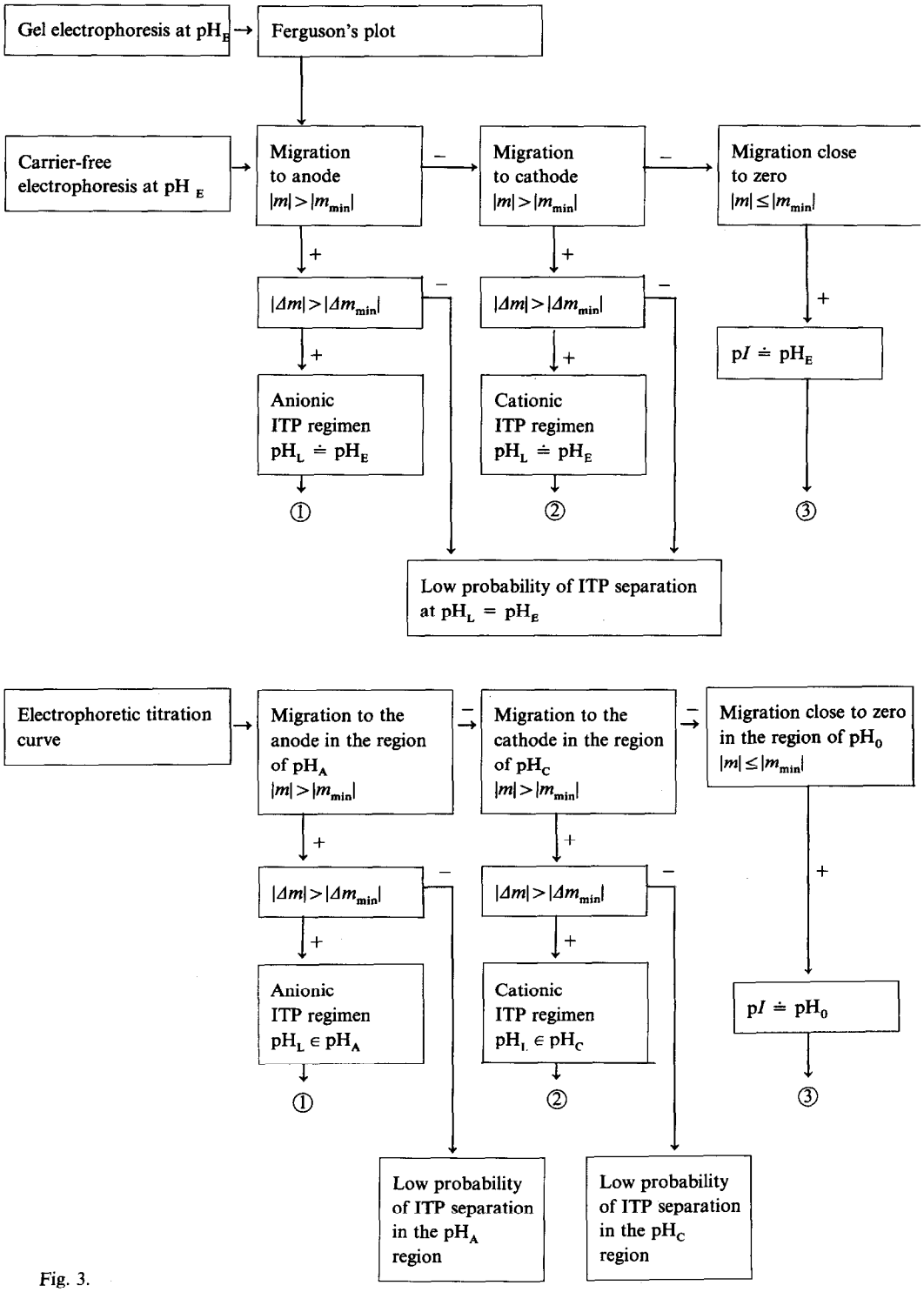

Gel electrophoresis at pH_E
Ferguson's plot
Carrier-free electrophoresis at pH_E
Migration to anode $|m| > |m_{min}|$
Migration to cathode $|m| > |m_{min}|$
Migration close to zero $|m| \leq |m_{min}|$
$|\Delta m| > |\Delta m_{min}|$
$|\Delta m| > |\Delta m_{min}|$
$pI \doteq pH_E$
Anionic ITP regimen $pH_L \doteq pH_E$
Cationic ITP regimen $pH_L \doteq pH_E$
①
②
③
Low probability of ITP separation at $pH_L = pH_E$
Electrophoretic titration curve
Migration to the anode in the region of pH_A $|m| > |m_{min}|$
Migration to the cathode in the region of pH_C $|m| > |m_{min}|$
Migration close to zero in the region of pH_0 $|m| \leq |m_{min}|$
$|\Delta m| > |\Delta m_{min}|$
$|\Delta m| > |\Delta m_{min}|$
$pI \doteq pH_0$
Anionic ITP regimen $pH_L \in pH_A$
Cationic ITP regimen $pH_L \in pH_C$
①
②
③
Low probability of ITP separation in the pH_A region
Low probability of ITP separation in the pH_C region

Fig. 3.

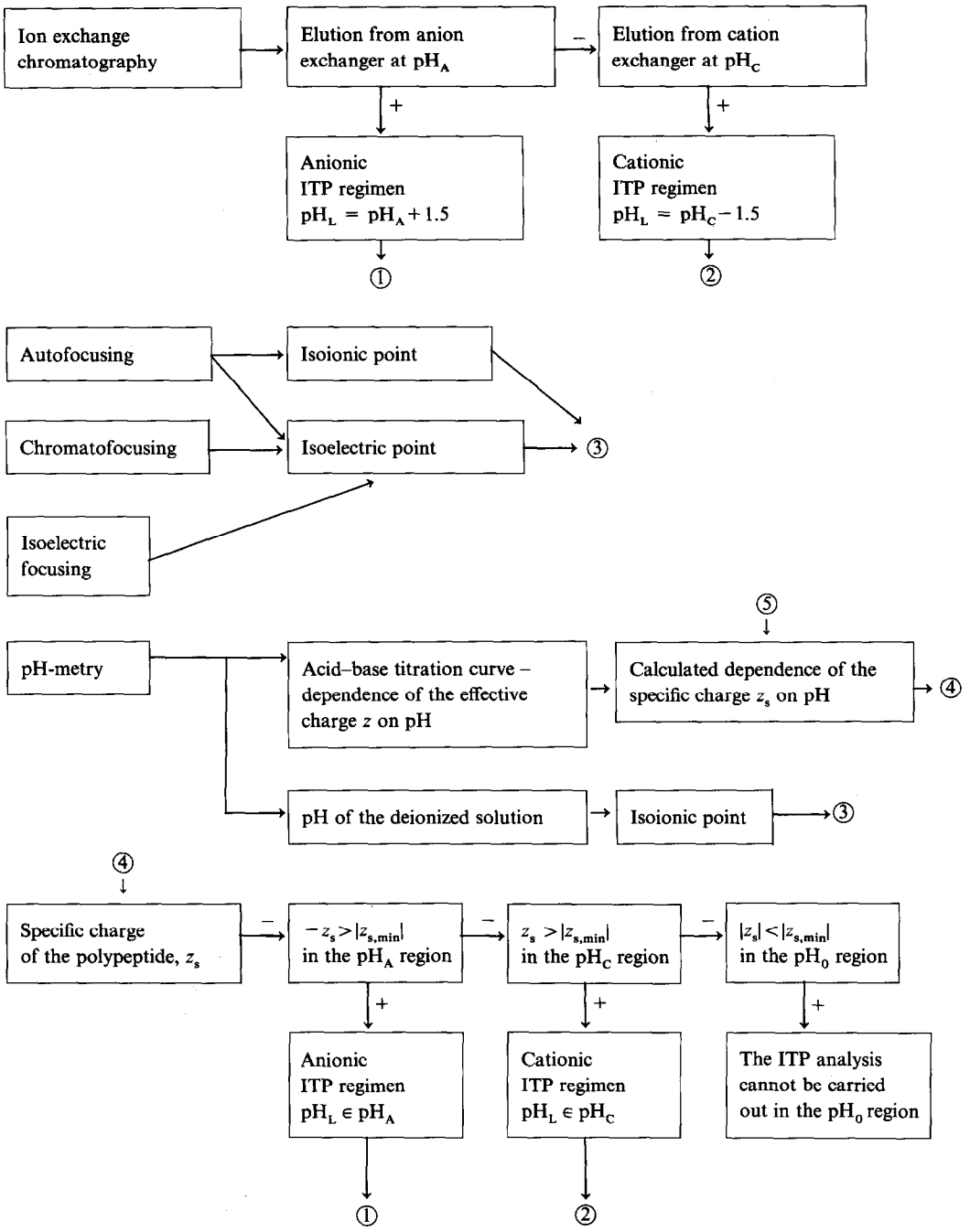
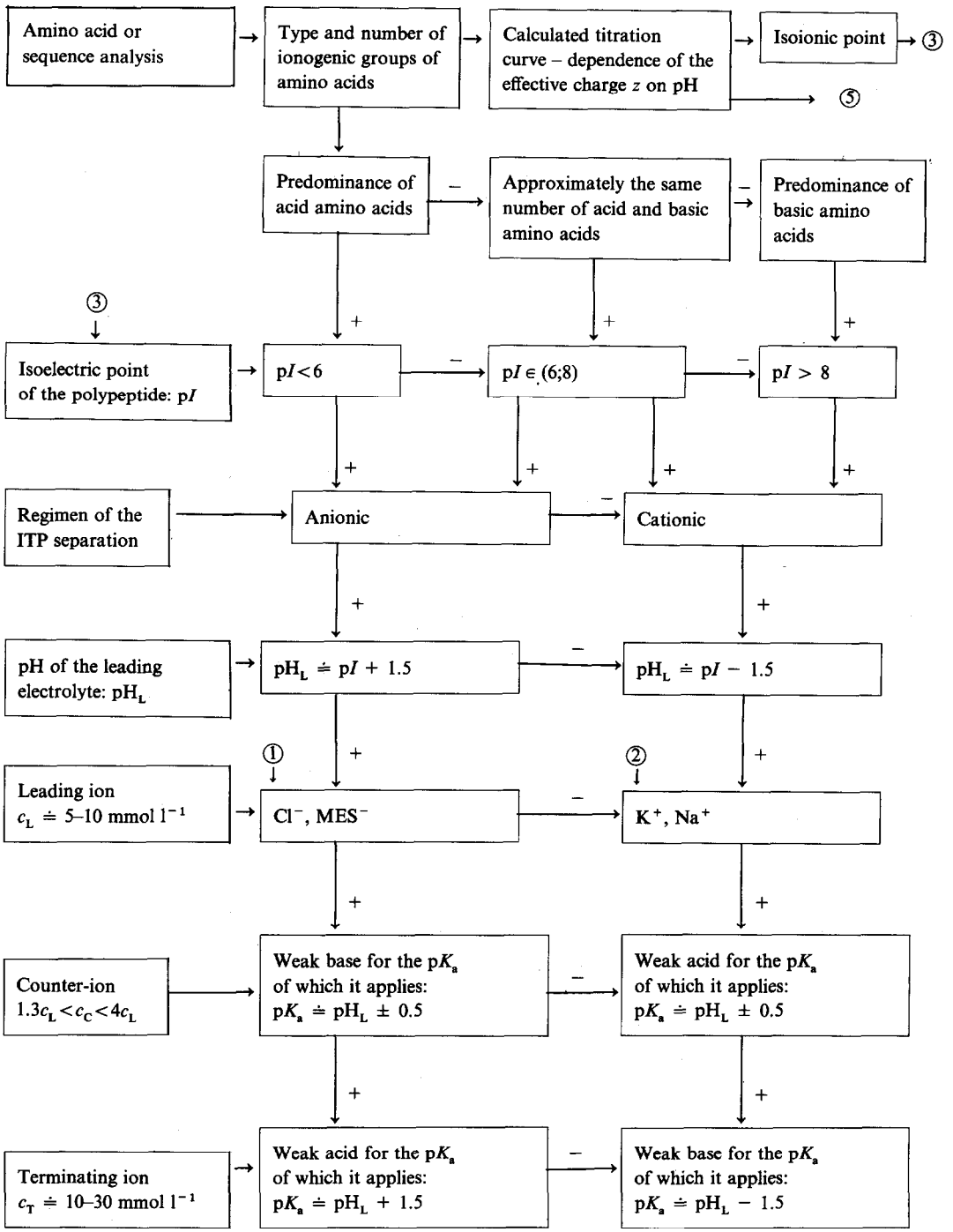


Fig. 3.

(Continued on p. 148)


Amino acid or sequence analysis
Type and number of ionogenic groups of amino acids
Calculated titration curve – dependence of the effective charge z on pH
Isoionic point
③
⑤
Predominance of acid amino acids
Approximately the same number of acid and basic amino acids
Predominance of basic amino acids
③
Isoelectric point of the polypeptide: pI
pI < 6
pI ∈ (6;8)
pI > 8
Regimen of the ITP separation
Anionic
Cationic
pH of the leading electrolyte: pH_L
pH_L ≐ pI + 1.5
pH_L ≐ pI − 1.5
①
②
Leading ion c_L ≐ 5–10 mmol l⁻¹
Cl⁻, MES⁻
K⁺, Na⁺
Counter-ion 1.3c_L < c_C < 4c_L
Weak base for the pK_a of which it applies: pK_a ≐ pH_L ± 0.5
Weak acid for the pK_a of which it applies: pK_a ≐ pH_L ± 0.5
Terminating ion c_T ≐ 10–30 mmol l⁻¹
Weak acid for the pK_a of which it applies: pK_a ≐ pH_L + 1.5
Weak base for the pK_a of which it applies: pK_a ≐ pH_L − 1.5

teristic for the choice of the regimen and the pH of the ITP separation (see Section 3.2). On the other hand, the data on mobility obtained by sodium dodecylsulphate (SDS) electrophoresis [66] have less significance for ITP, because SDS forms complexes with polypeptides, in which the original charge of the polypeptide is completely overlapped by the SDS charge and the separation does not proceed on the basis of the different mobilities of the polypeptides, but in consequence of the restriction effect of the gel on the electrophoretic mobility of the polypeptide–SDS complexes.

In addition to the related electromigration methods, further methods offer important information for the selection of conditions for ITP analysis, such as methods in which charge plays a role in the separation principle, as, for example, ion-exchange chromatography (IEC) and chromatofocusing. The elution pH of a polypeptide in chromatofocusing is equal to its isoelectric point, and the elution pH in IEC is relatively close to the isoelectric point, which can be made use of for the choice of regimen and the pH of the ITP separation. A condition of the use of elution pH in IEC is that the elution should take place on the basis of the change of charge of the protein in consequence of the pH change, and not in consequence of a change of the ionic strength.

The dependence of the effective charge on pH can be determined by studying acid–base equilibria by acid–base titration or pH metry.

A further source of information is the amino acid and sequence analysis of peptides, which give data on the quality and number of ionogenic groups present, on the basis of which it is possible to estimate or calculate the effective and specific charge of peptides.

A scheme of the procedure for the choice of ITP analysis conditions on the basis of characteristics obtained by other methods is shown in Fig. 3 in flow-chart form.

If none of the characteristics is available, on the basis of which the choice of conditions for ITP separation of peptides and proteins can be made, several

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Fig. 3. Flow diagram for the procedure for the choice of conditions of ITP analysis of peptides on the basis of data obtained during their characterization by other methods. Individual methods represent starting points from which it is possible to proceed in the direction of the arrows to the characteristics afforded by these methods. It is possible then to proceed further to individual ITP parameters according to the values of these characteristics. The arrow with the signs + (−) indicates the direction of the procedure for fulfilment (non-fulfilment) of the condition written in the frame from which the arrow leads. The numbers in circles indicate the connections by which the individual parts of the scheme are linked. pH_L = pH of the leading electrolyte; pH_E = pH of zone electrophoresis; pI = isoelectric point; m = effective mobility; m_{min} = minimal effective mobility (about $\pm 1\text{–}2 \cdot 10^{-9}$ m^2 V^{-1} s^{-1}); z_s = specific charge of the polypeptide ($z_s = z/M_r$, where z is the effective charge of the polypeptide in elementary units, including the sign, and M_r is the relative molecular mass of the given polypeptide); Δm = relative difference of mobilities; Δm_{min} = minimal relative difference of mobilities (about 2–3%); $z_{s,min}$ = minimal specific charge of polypeptide (about $\pm 2 \cdot 10^{-4}$ e); c_L = concentration of the leading ion; c_C = concentration of the counter-ion; c_T = concentration of the terminating ion. For further details, see Section 3.9.

different ITP electrolyte systems should be tried and further optimized and adjusted on the basis of the results obtained.

4. ADVANTAGES AND DISADVANTAGES OF CAPILLARY ITP IN THE ANALYSIS OF PEPTIDES

Before actual applications are discussed, the place of capillary ITP in the more general determination of peptides is considered. An analysis is presented of the advantages and disadvantages compared with related electrophoretic and chromatographic methods, and the most suitable spheres of use for ITP are discussed.

A number of advantages of capillary ITP follows from the fact that the separation takes place in a free solution, without the application of sorbents, gels or other carriers. The carrierless medium of capillary ITP allows the direct application of this technique to the analysis of even very complex mixtures of peptides and proteins, such as biological fluids and tissue extracts. Whereas in chromatography some polypeptidic and non-polypeptidic, ionogenic and non-ionogenic components, *e.g.* (lipo-, glyco-)proteins and polypeptides, lipids and polysaccharides, may be irreversibly bound to the column packing, decreasing its separation ability and reproducibility, in capillary ITP non-ionogenic compounds (and with a suitable choice of conditions even some ionogenic compounds) remain at the site of application of the sample (ionogenic components with an opposite charge move from the application site in the opposite direction from the analysed compounds), and after the termination of the analysis they are easily washed out from the separation space. A carrierless medium thus contributes to an easy restoration of the initial separation conditions and to a high reproducibility of the ITP analysis. The probability of contamination of the ITP separation space, *i.e.* the inner walls of the separation capillary, with some sample components is substantially lower than the probability of contamination of the many times larger and chemically not completely inert surface of the chromatographic column packing. Therefore ITP generally requires only a minimal preliminary treatment of the sample. In most cases dissolution, or extraction, centrifugation and filtration or ultrafiltration will suffice.

In comparison with electrophoretic methods carried out in gel, on a cellulose acetate membrane or on paper or other carriers, capillary ITP has the advantage that it is independent of the non-specific interaction of the peptides and proteins with these carriers. For example, the interactions of polypeptides with the carrier medium in paper electrophoresis greatly complicate or even prevent the separation of peptides containing more than 20–80 amino acid residues. Difficulties with the fixation and staining of peptides with a relative molecular mass lower than about 2000 make their detection more difficult when their separation by electrophoresis is performed in polyacrylamide gel. On the other hand, from this point of view capillary ITP is independent of the relative molecular mass of the analysed peptides and proteins, and therefore allows the determination of the

purity of peptides and proteins, including their low-molecular-mass ionogenic admixtures. The column-coupling ITP system [67] also allows the analysis of peptides with a high salt content, whereas in gel or paper electrophoresis the peptides must be desalted prior to analysis. In comparison with classically performed gel electrophoresis, capillary ITP in the analysis of individual samples has the advantage that, as a result of built-in on-line detectors, the tedious and elaborate operations of fixation, staining and destaining may be omitted. In advanced apparatus for gel electrophoresis in a thin layer of polyacrylamide gel at high electric field intensities, the processes connected with fixation, staining and destaining can be automated (*e.g.* Phastsystem, LKB Pharmacia) [68], the times of analyses become comparable, and the advantages of ITP remain for the areas of the detection of peptides with lower relative molecular masses.

The accuracy of quantitative ITP analysis (relative error 1–2%) considerably exceeds that of semiquantitative evaluation by gel electrophoresis, based on staining, which is dependent both on the method of staining and on the character of the analysed polypeptides. The sensitivity of the detection of polypeptides in gel by staining, especially with silver, is higher than the sensitivity of capillary ITP.

In comparison with IEF, ITP offers a comparable separation power, but a broader field of application. IEF is reserved for amphoteric compounds, the p*I* of which is in the pH range of about 2–11. Not all peptides fulfil this condition. Some peptides with a blocked nitrogen or carbon terminus completely lack an amphoteric character, and in some peptides with a prevalence of basic or acid ionogenic groups the isoelectric point reaches extreme values outside the working pH range of IEF. In ITP no such limitation exists. On the contrary, in peptides with extreme p*I* values the assumption is fulfilled that they have sufficiently high mobilities within a broad pH range, either under cationic or anionic regimen. It is true, IEF, similarly to ITP, displays a concentration and self-sharpening effect, but in the IEF steady state the concentrated peptides are at their isoelectric points where they have an increased tendency to precipitation and denaturation. The risk of the loss of biological activity is higher mainly in peptides with isoelectric points in marginal regions of the pH range of IEF.

The concentration effect of ITP permits analysis of dilute samples of peptides, and it is made use of as a concentration step even in other methods (discontinuous zone electrophoresis, ITP electrodesorption, ITP electroelution).

Owing to the concentration effect and the linear relationship between the amount of the substance and the length of its zone or the area of its peak, even complex mistures of peptides and proteins may be evaluated without their standards.

The self-sharpening effect of ITP suppresses diffusion and ensures a high sharpness of the zone boundaries. The sharpness of the separation is independent of the amount of the separated substances if the time and the separation volume suffice for the formation of the steady state. The self-sharpening effect also contributes to the high separation power of ITP. In one experiment up to 30–40

components may be resolved, while concentration ratios of these components, especially in an apparatus with column coupling [67], may be very high (1:100 up to 1:10 000).

Capillary ITP is further characterized by a relatively high sensitivity (the minimum detectable amount of peptides is in the range of tens to hundreds of picomoles), reproducibility, short time of analysis (10–30 min) and low operational costs.

The large number and changeability of the parameters of ITP analysis, *i.e.* pH, ionic strength, solvent, charge and complexation ability of the counter-ion, neutral complexation agents and additives to the leading electrolyte, spacers and additives in the sample, allow the optimization of the conditions of analysis from the point of view of separation power and the retention of the biological activity of the separated peptides.

So far the advantages of ITP have been predominantly discussed, but in the interest of an objective evaluation the disadvantages and limitations of ITP are now indicated. In comparison with high-performance liquid chromatography (HPLC), ITP has a lower separation power and lower sensitivity, which is reflected, for example, in the lower number of components which can be resolved by ITP in the separation of polypeptides formed by enzymatic hydrolysates of proteins and the inability of ITP to separate simultaneously all 20 amino acids most frequently occurring in peptides and proteins. The lower sensitivity of capillary ITP is caused by the fact that, in most cases, a quantitative evaluation is based on a linear relationship between the amount of substance and the length of its zone, and less frequently between the amount of substance and the area or height of the peak of this substance, as, for example, in chromatography or CZE.

The lower sensitivity is also partly caused by the concentration effect of ITP, in consequence of which the concentration of peptides at the ITP steady state is many times higher than the concentration which can be detected with a photometric detector. However, in a number of actual cases the separation power and the sensitivity of ITP do not represent limiting factors for the use of ITP in the analysis of peptides. A more important disadvantage is the lower solubilization ability of ITP electrolyte systems compared with the solubilization ability of the mobile phase in HPLC and the backgroud electrolyte in CZE, where even concentrated solutions of acids and bases may be used in the presence of organic solvents. In contrast to HPLC, in ITP the conversion of an analytical separation to a preparative one is instrumentally more complicated.

So far commercial ITP analysers still lack detectors with a high information content. Conductivity or photometric detectors of ITP analysers afford substantially more modest information for the identification and characterization of the separated peptides and proteins than the spectrophotometric diode-array detectors, Fourier transform infrared detectors and mass spectrometric detectors with which modern HPLC analysers are provided.

Chromatographic and electrophoretic methods in gel or other carriers afford

easier off-line identification of the separated peptides on the basis of immunochemical and enzymatic methods than capillary ITP, where these analyses must be preceded by relatively more complicated isolations of the separated peptides from the capillary. The ITP analysis of peptides is thus more suitable in cases where the quantitative relationships of the components are observed in a sample of a similar qualitative composition than in qualitative analysis of complex mixtures of peptides.

Among the disadvantages of ITP, compared with chromatography, its limitation to relatively soluble ionogenic compounds of one charge type should be mentioned. This, it is true, narrows the sphere of application of ITP even in the case of peptides. However, this disadvantage becomes an advantage in the case of the analysis of peptides in a complex matrix of non-ionogenic compounds and/or in the presence of their excess.

Other electromigration techniques for the separation of peptides, such as zone electrophoresis [63–65, 69–80], IEF with an immobilized pH gradient [81] and SDS gel electrophoresis [82,83] have recently started to be carried out in a capillary arrangement (fused-silica capillaries, I.D. 50–100 μm). These methods are now developing very rapidly and seem to be potentially advantageous because of their substantially higher sensitivities and separation efficiencies than both ITP and HPLC. Their detection limits depend on the detection method used and are in the range femtomoles to sub-attomoles; the number of theoretical plates per metre of column length is 10^5–10^7. An one-line combination of the concentration effect of ITP with subsequent separation by these methods [84] has potential, because it would further increase the separation and detection possibilities of electromigration methods.

From a comparison of capillary ITP with other methods it follows that each method has its advantages and disadvantages and the suitability of its use should be judged individually, according to the aim of the analysis and the character of the sample analysed. In most cases the individual methods should not be considered as competitive, but as mutually complementary.

5. APPLICATIONS

5.1. General

The potential of capillary ITP for the analysis of peptides is very high and can be realized in many areas such as chemistry, biochemistry, biology, biotechnology, pharmacy, human and veterinary medicine and the food and feed industry. Before actual application are considered, a short summary of the fields of application is given below.

1. Control of the purity of synthetic and natural peptides, including the determination of low-molecular-mass ionogenic admixtures.

2. Determination of the efficiency of purification procedures during the isolation of peptides.

3. Analysis of complex mixtures of peptides in biological fluids and tissue extracts.

4. Study of the interactions of peptides with low- and high-molecular-mass ligands and their use for analytical and micropreparative purposes.

5. Evaluation of enzymatic reactions (peptide cleavage and/or synthesis).

6. Determination of physico-chemical characteristics of peptides (effective mobilities, dissociation constants and relative molecular masses).

These application fields are described in more detail in the following sections. The composition of all the electrolyte systems used for peptide ITP analyses is given in Tables 3–8.

5.2. Model peptide separations

Model separations of the dipeptides Gly–Gly, Leu–Tyr, the tetrapeptide Gly–Gly–Gly–Gly and glutathione in electrolyte system (ES) 36 (see Table 3) have been demonstrated by Everaerts *et al.* [85]. More detailed studies were carried out by Miyazaki and Katoh [86], who investigated the conditions of ITP separation of the amino acids Gly and Ala and oligopeptides composed of these amino acids, *e.g.*, Gly–Gly, Gly–Gly–Gly, Ala–Ala, Ala–Ala–Ala. In electrolyte systems in acid and neutral pH regions a satisfactory separation could not be achieved. Suitable conditions for the separation were found in alkaline media under an anionic regimen at pH 8.97 or 9.63 (see ES 34 and 51 in Table 3).

Amino acids and oligopeptides appear on isotachopherograms in the following order: Gly–Gly, Gly–Gly–Gly, Gly, or Ala–Ala, Ala–Ala–Ala, Ala. This order can be explained by the fact that in consequence of the shift of the dissociation constants of oligopeptides compared with free amino acids, the specific charge (and thus the effective mobility) of oligopeptides is larger than the specific charge of free amino acids. Therefore, oligopeptides appear on the isotachopherogram before amino acids, even though their relative molecular mass is approximately double or triple that of amino acids. In the analysis of mixtures of all six components a complete separation could not be achieved because Ala–Ala and Gly–Gly–Gly formed a mixed zone. The amount of peptides in one analysis was 1–10 μg.

In the same study the relative moblities of nine oligopeptides were further determined in an anionic regimen (ES 34 in Table 3) at pH 8.97, expressed as the so-called potential unit (PU). The PU of component A, PU_A, is defined by the equation

$$PU_A = (PG_A - PG_L)/(PG_T - PG_L) \quad (6)$$

where PG_L, PG_T and PG_A are the values of the potential gradient in the zone of the leading electrolyte, terminating electrolyte and component A, respectively.

Miyazaki and Katoh [86] have also found suitable conditions for the ITP analysis of other biologically active peptides: plasmatic kinins (bradykinin, kallidin and Met–Lys–bradykinin), angiotensin I, II and the oxidized and reduced forms of glutathione (ES 10, 11 and 13 in Table 3).

Oligopeptides, which are well defined from the point of view of mobility and which are available in standard forms, were proposed [87] as discrete, non-UV-absorbing spacers for the ITP separation of complex mixtures in ES 47 (Table 3).

5.3. Determination of physico-chemical characteristics

Capillary ITP is also used in the determination of the physico-chemical characteristics of peptides, *e.g.* effective and limit mobilities, dissociation constants and relative molecular masses. In this area the advantage of the carrierless medium of capillary ITP is made use of, and this allows the description of the theoretical basis of the method fairly accurately. The mathematical models developed give more reliable characteristics of peptides than the models of the chromatographic methods, into which corrections must be included for inaccurately defined interactions with the solid phase.

Hirokawa *et al.* [26] measured the quantitative characteristics (relative heights, R_E) of 28 dipeptides [alanyl, glycyl and leucyl derivatives of amino acids and β-alanine (BALA)–His] in eight anionic ITP regimens in the pH range 7.4–9.6 (ES 15, 24, 26, 30, 31, 40, 49 and 50 in Table 3).

The relative height of component A, $(R_E)_A$, is defined by the equation:

$$(R_E)_A = E_A/E_L \tag{7}$$

where E_A and E_L are the intensities of the electrical field (potential gradients) in the zone of component A and in the zone of the leading electrolyte. From the measured data, on the basis of the mathematical model of the ITP steady state using the method of least squares, the effective mobilities m_{ef}, absolute mobilities m_0 and the dissociation constants pK_a of the given peptides were obtained. For some of the peptides the determined pK_a values were in good agreement with the tabulated data obtained by other methods, whereas for others they were determined for the first time. The values of absolute mobilities in all analysed peptides were also determined for the first time, because conductivity measurements do not allow the determination of absolute conductivities and the absolute mobilities of amphoteric substances derived from them.

The determined absolute mobilities of monovalent dipeptide ions range from $21.6 \cdot 10^{-9}$ (Leu–Leu) to $31.5 \cdot 10^{-9}$ $m^2\ V^{-1}\ s^{-1}$ (Gly–Gly), and the pK_a values from 8.269 (Leu–Gly) to 8.746 (Gly–Pro) (except for BALA–His, pK_a 9.664).

Using the determined values of m_0 and pK_a, simulations of the ITP separation of 28 dipeptides and 15 amino acids were carried out, during which their effective mobilities were calculated and compared.

On the basis of the simulated and the experimentally performed separations of

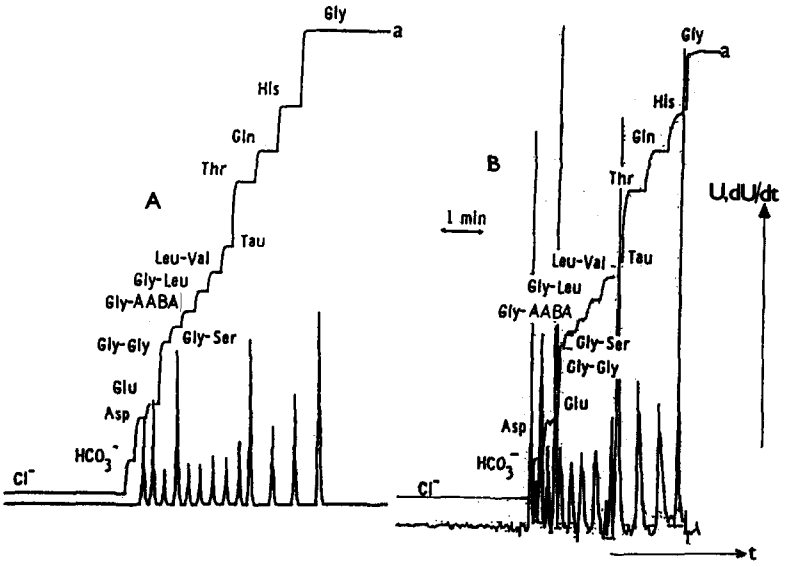

Fig. 4. Comparison of (A) calculated and (B) experimental isotachopherograms of peptides, amino acids and carbonates [26]. Anionic regimen at pH_L 8.0, leading ion Cl^- (0.01 mol/l); counter-ion, Tris; a = signal of PG detector; dU/dt = differentiated PG detector signal; U = voltage of PG detector, t = time; separation capillary length 400 mm, I.D. 0.5 mm; detection current 50 μA; cooling medium temperature 25°C.

peptides and amino acids the criterion of their separability was derived. It was shown that the differences in effective mobilities are, at least in the first approximation, a good measure of separability: two components are mutually separable if the difference between their effective mobilities is larger than about $1 \cdot 10^{-9}$ m^2 V^{-1} s^{-1}. In actual examples this value will depend, of course, on the amount of sample and the length of the separation path. A good agreement between the simulated and experimental ITP separation of a selected mixture of peptides and amino acids is demonstrated in Fig. 4.

By comparison of the ITP determined m_0 and pK_a values of peptides and the m_0 and pK_a values of constituent amino acids (also determined by ITP [25]), simple approximate relationships between these values are found. Let m_A, m_B be the mobilities of amino acids A and B; then the mobility m_{AB} of the monovalent anion of the dipeptide AB is given by the equation:

$$m_{AB} = (m_A^{-3} + m_B^{-3})^{1/3} \tag{8}$$

The pK_a value of the amino group of most monovalent anions of dipeptides is about 1.4 units lower than the pK_a value of the amino group of a free amino acid. This fact can be made use of for the approximate evaluation of the pK_a value of dipeptides.

In the series of investigated dipeptides the relationship was also derived between the absolute mobility m_0 and the relative molecular mass M_r:

$$m_0 = (306.9\, M_r^{-1/2} + 3.4) \cdot 10^{-9}\ \text{m}^2\ \text{V}^{-1}\ \text{s}^{-1} \tag{9}$$

Statistical evaluation of this relationship using a set of dipeptides gave a relatively high correlation coefficient 0.94, and the mean deviation between the calculated and observed m_0 values was 2.5%. The eqns. 8 and 9 can be used for the estimation of the absolute mobility of dipeptides on the basis of their relative molecular mass, or from the absolute mobilities of their constituent amino acids.

The ITP determination of the relative electrophoretic mobility of several tens of anions, including Gly–Gly and Gly–Gly–Gly peptides, was carried out by Carchon and Eggermont [88]. The relative mobilities were determined within a broad pH_L range of 4–10, and all the mobilities were referred to the effective mobility of acetate in the given electrolyte system.

5.4. Purity control of synthetic and natural peptides

One of the most important applications of capillary ITP in peptide chemistry is the analysis of synthetic or natural peptide preparations. The need for and util-

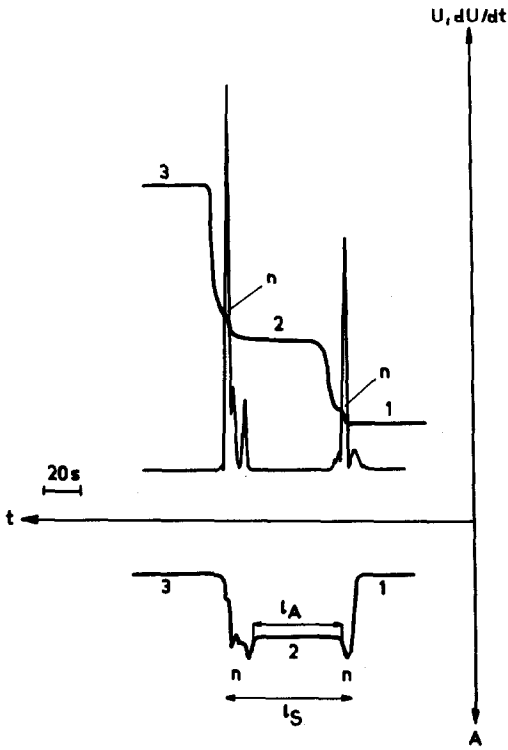

Fig. 5. Estimation of degree of purity of bull seminal trypsin isoinhibitor (BUSI II) by ITP analysis. Cationic regimen, ES 58, Table 5. 1 = Na^+; 2 = BUSI II; 3 = BALA; l_A = zone length of pure BUSI II; l_S = total length of UV-positive zones; n = unidentified sample admixture; A = absorbance at 254 nm; U = signal of PG detector; t = time; dU/dt = differentiation of PG detector signal. A 9-μg mass of BUSI II preparation in a 2-μl volume was applied. The analysis was performed in a thermostated PTFE apparatus developed in the Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague, Czechoslovakia. Separation capillary 230 mm × 0.45 mm; cooling medium temperature 20°C.

isation of synthetic peptides has risen in recent years. In biochemistry synthetic peptides are used as substrates and inhibitors of enzymes in the elucidation of the mechanism of their catalytic effect, in the modelling of the interactions of antigens with antibodies, hormones with receptors, peptides and proteins with nucleic acids, in the mapping of antigenic determinants of proteins and in the study of the dependence of the secondary and tertiary structure of a peptide chain on amino acid sequence. The utilisation of synthetic peptides in pharmaceutical research and in human and veterinary medicine is also widespread. In the food industry peptides are used as sweeteners and additives.

In most of these applications of synthetic or natural peptides capillary ITP can be used as a sensitive control method for the determination of their purity, or as a control of the efficiency of individual steps of a purification procedure, giving rapid and accurate qualitative and quantitative data on the properties of a given preparation.

As a quantitative index of electrophoretic homogeneity of peptides, the so-called ITP degree of purity has been introduced [19,89]. Let l_S represent the total length of all zones on an isotachopherogram of the analysis of sample S, and l_A the length of the zone of compound A, constituting the main component of sample S (see Fig. 5). Then the ITP degree of purity of component A, p_A, is given by the ratio of the lengths l_A and l_S:

$$p_A = l_A/l_S \tag{10}$$

However, the lengths of the zones of possible impurities from the electrolyte system must not be calculated into the total length l_S. The length of the zones of these impurities can be determined during the blank run of the ITP analysis without the application of the sample.

The degree of purity defined in this manner can be approximately identified with the molar fraction of a given compound in the analysed mixture. This approximation is more precise the closer the charges and the effective mobilities of the components present are, and the higher the proportion of the main component in the sample is. This requirement is fulfilled to a considerable extent in the case of peptide preparations purified to a relatively high degree of purity, so that the error caused by this approximation is in the percentage range and in some instances – at equal values of the charge, close effective mobilities (deviations up to several per cent) and at a relatively low mobility of the counter-ion of the leading electrolyte – it can be negligible [90,91].

The degree of purity or the ratio of concentrations of individual components may thus be approximately determined even without standards of individual compounds, merely on the basis of the zone length ratio. This is an advantage of ITP compared to HPLC, because in chromatographic analysis with photometric detection at 254 or 280 nm, the concentration ratios of individual components cannot be determined without a knowledge of their molar absorption coefficients.

The use of capillary ITP for the control of purity of synthetic peptides was

introduced by Kopwillem *et al.* [92,93] in the analysis of fragments of human growth hormone (the 32-peptide from position 125–156 of the polypeptide chain). The fragment was synthesised using Merrifield's solid-phase method. During this synthesis a complex mixture of peptides is formed. ITP analysis determined the presence of 18 components and, using the method of IEF, as many as 22 components were determined. The difference between the two methods is explained by assuming that under the conditions of the ITP analysis used (ES 18 in Table 3) some components were slower than the terminating ion, and thus they escaped the ITP analysis.

This method of evaluating peptide preparations by ITP was used by other workers in a number of analyses of synthetic or natural peptides. These are surveyed in Tables 3–6. Some of these applications will be described in more detail.

The synthetic tetradecapeptide somatostatin, which has an important physiological activity (suppressing the release of the growth hormone, insulin, glucagon and gastrin), was analysed by ITP after various purification steps [89,94]. As its character is basic due to the presence of two lysine residues in addition to other neutral amino acids in the molecule, the analysis was carried out under the cationic regimen with the leading electrolyte (ES 75 in Table 5) at pH 6.9. ITP afforded qualitative and quantitative data on the degree of purity, in contrast to the semiquantitative data obtained by thin-layer chromatography.

Among other examples let us mention the utilisation of capillary ITP in the control of purity of a number of physiologically active peptides which were mostly obtained as synthetic or natural preparations from pharmaceutical firms.

Using capillary ITP (ES 60 and 76 in Table 5) some structurally similar basic cyclic octapeptides were analysed and separated, such as the pituitary hormones oxytocin (eliciting contractions of smooth muscles), Arg–vasopressin and Lys–vasopressin (increasing blood pressure and with an antidiuretic effect) [19,89,95,96].

The ITP analyses (ES 76, Table 5) of synthetic commercial adrenocorticotropic hormone (ACTH; an 18-peptide stimulating the growth and metabolic activity of adrenal cortex) and bacitracin (a cyclic peptide with an antimicrobial effect) demonstrated a considerable heterogeneity of the samples. The degree of purity of ACTH was less than 50% [89].

ITP was used for the separation of natural cholecystokinin peptides containing 39 and 33 amino acids, the separation of peptidic fragments (di- and tetrapeptide) obtained by the enzymatic hydrolysis of a hexapeptide from cholecystokinin, separations of angiotensin I and II, of the oxidised and reduced form of glutathione [89], for the analysis of neurohormone β-endorphin and for calcitonin, a peptide affecting the metabolism of calcium [46]. In the analysis of peptide hormones ITP proved to be very suitable and helped to define new purity criteria.

In the case of synthetic fragments of human fibrin (a decapeptide and undecapeptide from the region of the "cross-linking site" of fibrin) ITP (ES 14 in

Table 3) was used not only for the control of their purity, but also for the amino acid analysis of their acid hydrolysate [97]. The agreement in the determination of the amino acid content by both ITP and amino acid analyser was very good. This application of ITP is only suitable, however, for cases when peptides contain a limited number of amino acid types, because in the electrolyte systems tested so far the 20 types of amino acids occurring most frequently in peptides and proteins could not be separated in one experiment. In the ITP determination of the amino acid composition of fibrin peptides the contents of leucine and isoleucine were obtained only as a sum, because these amino acids gave a mixed zone in the electrolyte system used.

Stehle and co-workers [98–100] used ITP in the analysis of the synthetic peptides Ala–Gly, Ala–Gln and N^2-tyrosinyl-N^6-tyrosinyllysine (ES 6 and 45 in Table 3). These peptides are used as nutritional additives in parenteral solutions of amino acids, which are administered to patients suffering from altered catabolism to achieve a more favourable utilisation of proteins. Most of the amino acids are added directly to these solutions. However, tyrosine cannot be used in this way owing to its low solubility, nor can glutamic acid owing to the formation of the toxic pyroglutamic acid during sterilization. Therefore these two amino acids are added in the form of peptides. Their degree of purity, the presence of the constituent amino acids and the monitoring of their synthesis, stability and decomposition during heat sterilization were controlled by capillary ITP.

ITP was also used to monitor the enzymatically catalysed synthesis (ES 42 and 43 in Table 3) and subsequent purification of the N-protected dipeptide N-benzoyl-Ala–Gln [101,102] and the evaluation of the amino acid composition of the acid and enzymatic hydrolysate. Compared with alternative chromatographic methods, ITP offers the advantage of a simultaneous analysis of the peptide, free amino acids, derivatives of amino acids and the organic and inorganic ions present. A sufficient amount of sample for one analysis was about 200 ng, and for quantitative evaluation on the basis of the ratio of zone lengths no standards of the determined substances were necessary, so that it was easy to follow the degree of enrichment of the main component during subsequent purification operations.

This approach, *i.e.* the ITP control (ES 62 and 65 in Table 3) of the purity of synthetic peptides after their purification by reversed-phase HPLC (RP-HPLC) was applied in the preparation of thyrotropin-releasing hormone and its analogues [103].

The usefulness of this approach, *i.e.* the combination of ITP (and recently also CZE) with RP-HPLC as a source of complementary information on peptide purity was confirmed for several other peptide preparations, *e.g.* pig insulin, ACTH fragment analogues, human β-endorphin, Arg–vasopressin, cholecystokinin [104] and the dipeptide L-His–L-Phe [74].

Friedel and Holloway [105,106] used capillary ITP to monitor the synthesis of C-terminal pentapeptide bombinin, a surface-active peptide secreted by the European toad (*Bobina variegata*). This pentapeptide, the sequence of which is Gln–

His–Phe–Ala–Asn–NH_2, is a typical example of a basic non-amphoteric peptide which cannot be analysed by isoelectric focusing, whereas ITP analysis under a cationic regimen (ES 59 in Table 5) served very well for the control of its purity. While following the course of the synthesis, it was observed that the heterogeneity of the synthesised intermediates increased distinctly after the introduction of a histidine residue into the peptide, which is in agreement with the experience obtained in the synthesis of other histidine-containing peptides.

In the analysis of peptidic drugs, such as the octapeptide saralasin (Ser–Arg–Val–Tyr–Val–His–Pro–Ala · CH_3COOH · $5H_2O$), decapeptide gonadorelin (Glu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly–NH_2 · $2CH_3COOH$ · $4H_2O$), tripeptide protirelin (Glu–His–Pro–NH_2) [107] and the so-called substance P (undecapeptide Arg–Pro–Lys–Pro–Glu–Glu–Phe–Phe–Gly–Leu–MetNH_2 · xCH_3COOH · yH_2O) [108], ITP (ES 71–74 in Table 5) served for the determination of purity of these synthetic preparations, for the determination of their concentration in injection solutions and in the control of the stability of the preparations in dependence on the method and time of storage. The content of acetates was also determined by ITP (ES 84 in Table 7).

In the food industry ITP is used in the determination of the artificial peptidic sweetener aspartame (Asp–Phe–methyl ester), both in the control of the purity of the final synthetic product and its intermediates [109,110] (Es 57 in Table 5), and in its determination in beverages and yoghurts [111,112] (ES 19 and 37 in Table 3).

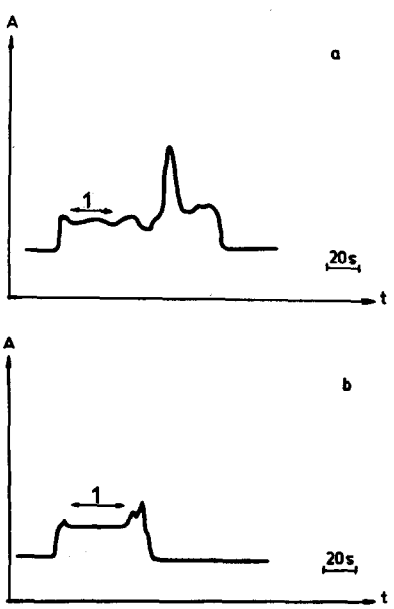

Fig. 6. ITP analysis of bovine basic pancreatic trypsin inhibitor (BPTI) in a cationic regimen, ES 58, Table 5. Samples: (a) 200-μg of crude BPTI product (precipitated by magnesium sulphate); (b) 6 μg of a purified BPTI after IEC. A = absorbance at 254 nm; t = time; 1 = BPTI zone, other conditions as in Fig. 5.

Of the naturally occurring peptides, the vasoactive intestinal peptide (VIP) [46,89,113], with a pharmacological activity related to glucagon and secretin, isolated from the upper small intestine of pigs, was determined by ITP (ES 66 and 67 in Table 5). ITP was used both for the determination of its purity after individual purification steps, and for the separation of a mixture of VIP and secretin, a hormone affecting the pancreatic free hydrogencarbonate secretion, isolated from pig intestine.

Another example, the ITP analysis of two different preparations of polypeptidic basic pancreatic trypsin inhibitor (BPTI) is shown in Fig. 6. The substantially increased purity of BPTI after IEC compared to a crude product after precipitation can be observed and quantified [19].

Pradayrol *et al* [114] used ES 70 (Table 5) for the determination of porcine intestinal peptide with C-terminal somatostatin. To increase the solubility, which appears to be a limiting factor for the use of ITP in some peptide separations, urea was added to the leading electrolyte (4 mol/l).

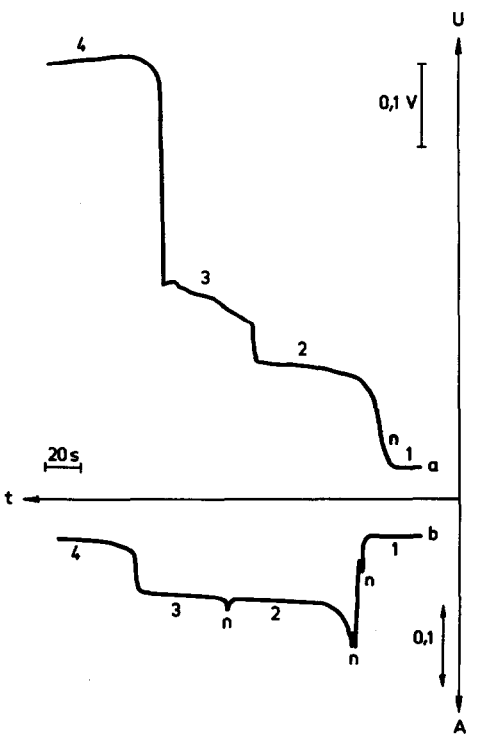

Fig. 7. ITP separation of pig insulin and proinsulin in an anionic regimen in water–propanol (ES 55, Table 4). (a) Signal of PG detector; (b) signal of photometric detector. A = absorbance at 254 nm; t = time; 1 = Cl^-; 2 = insulin; 3 = proinsulin; 4 = glycine; n = unidentified sample components. Sample: 8.8 μg of lyophilized pig insulin (Léčiva n.p., Czechoslovakia) and 8.5 μg of lyophilized pig proinsulin (Novo Industri, Denmark) in 5 μl of leading electrolyte. Separation current = 30 μA; detection current = 20 μA; analysis time = 20 min; cooling medium temperature = 20°C. Other conditions as in Fig. 5.

In the ITP determination of insulin (ES 82 in Table 6), the systems water–methanol, water–ethanol and water–ethoxyethanol were used as solvents for the leading electrolyte to increase its solubility and the separation was carried out at acid pH under the cationic regimen [115]. In spite of this, material losses occurred as a consequence of its aggregation and adsorption onto the inner walls of the capillary.

Insulin and proinsulin [19] were separated under the anionic regimen at pH 8.1 (ES 54 and 55 in Table 4). A water–propan-2-ol mixture was used as the solvent. An isotachopherogram of this separation is shown in Fig. 7.

Baldesten [116] analysed six different preparations of insulin under the anionic regimen, using systems ES 28, 38, 44, 52 and 53 (Table 3) in alkaline medium with the pH of the leading electrolyte in the range 8.5–9.1. All the samples were electrophoretically heterogeneous and on the isotachopherograms several UV-positive zones appeared at 280 nm. In addition to the longest zones of the insulin monomer, there were zones of its oligomers and derivatives (deamino, ethyl) which could be separated in the presence of mixed ampholytic spacers and 6 mol/l urea.

The construction of a UV detector for a wavelength of 206 nm [56,57,117], *i.e.* in the absorption region of a peptide bond, has led to considerable progress in the ITP analysis of peptides. A high-resolution contactless detector is now available even for peptides not containing aromatic amino acids and thus not absorbing at 254 or 280 nm, the most common wavelengths in UV detectors in commercial ITP analysers. At 206 nm the only peptide bond of the dipeptide (Gly–Gly–methyl ester) led to a 20% absorption of light (referred to ES 61 in Table 5). In

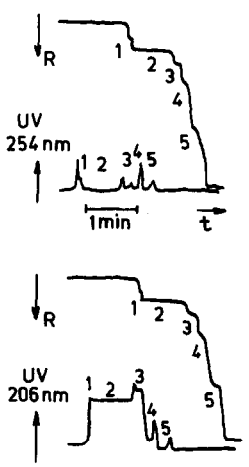

Fig. 8. ITP analysis of crude synthetic analyl–glutamin with UV photometric detection at 206 and 254 nm [57] in ES 41. (Table 3). *R* = conductivity; UV 206 nm, UV 254 nm = UV absorption at 206 or 254 nm, respectively; Sample, 20 μl of aqueous solution containing 8.68 μg of crude peptide material. 1 = Ala–Ala; 2 = Ala–Gln; 3 = unknown peptide(s); 4 = Gln; 5 = Ala.

peptides containing 10–20 amino acids the absorption increased proprotionally with the number of peptide bonds [56].

At 254 nm the use of the UV detector for peptides which do not contain aromatic amino acids is dependent on whether spacers are present in the sample which absorb at this wavelength. Such spacers will indicate the boundaries between the peptide zones; the absorption at 206 nm is universal for peptides. However, the 206 nm wavelength narrows the choice of electrolyte systems to some extent, because counter-ions with a high absorption at this wavelength (*e.g.* histidine) cannot be used.

The detection of peptides at 206 nm was used in the ITP control of the synthesis and the purification of dipeptides and tripeptides containing glutamine, tyrosine and cysteine [57,118,119]. The analysis (ES 5 and 41 in Table 3) helped to optimize the conditions of the synthesis because it allowed the simultaneous monitoring of the reactants, reaction products and contaminating organic and inorganic ions in various phases of the synthesis and the purification procedure.

A comparison of the UV detection of the dipeptide Ala–Gln at 206 and 254 nm is shown in Fig. 8. The advantage of the detection at 206 nm is that, on the basis of the absorption level, admixtures of a peptidic nature can be distinguished from free amino acids, whereas absorption at 254 nm does not allow this distinction. An increase in UV absorption by the peptide bond takes place already at 220 nm, at which wavelength the ITP separation of the peptides Gly–Gly and Gly–Gly–Gly was detected using an adapted flow-through spectrophotometric analyser [120].

5.5. Determination of low-molecular-mass ionogenic admixtures in peptide preparations

One of the special and very advantageous applications of ITP in the chemistry of peptides is the determination of low-molecular-mass ionogenic admixtures in peptide preparations [96,104,124,137,138]. For this separation the fact is made use of that ionogenic compounds can be determined simultaneously by ITP and at high concentration ratios (10^2–10^4), without regard to their relative molecular mass, *i.e.* the amount of salts present in the preparations can be determined in addition to peptides and proteins.

The quality and quantity of the low-molecular-mass ionogenic admixtures present should be known before using peptidic preparations in biological and medical tests in human and veterinary medicine. According to the results of the analysis, decisions should be made as to whether the pharmaceutical peptide preparations are suitable for use in clinical practice or whether the purification procedure should be continued.

Low-molecular-mass ionogenic admixtures occur in peptides both as electrostatically bound counter-ions (*e.g.* acids bound to basic peptides) and as non-stoichiometric admixtures of acids, bases and metal ions after individual steps of the isolation and purification procedure.

For basic peptides the analysis of the actual peptides is carried out under the cationic regimen. The determination of the admixtures of acids is carried out under the anionic regimen at which the anions of the acids travel in the opposite direction to that of the basic peptides; they can therefore be easily separated and determined. For non-basic peptides, not only the admixtures of the low-molecular-mass acids move anionically, but the peptides as well, so they can be determined simultaneously in one experiment [104,124].

The quantitative evaluation of the ITP determination of the content of acids in peptides is most commonly and accurately carried out using the calibration graph method. These graphs are obtained as plots of the zone lengths *versus* the amount of substance applied.

Let l_{A1} represent the zone length read from the calibration graph, corresponding to the unit amount of the determined component A, l_A the zone length of compound A read from the isotachopherogram of the analysis of sample S, M_A the relative molecular mass of compound A, m_S the weight of sample S, V_S the volume of the dissolved sample S and V_{ap} the applied volume of the sample in the ITP analysis. Then the absolute mass amount of compound A, m_A, in sample S is given by the equation:

$$m_A = M_A l_A V_S/(l_{A1} V_{ap}) \tag{11}$$

and the relative amount of compound A in sample S, expressed as a percentage mass fraction r_A, is given by the equation:

$$r_A = (m_A/m_S)100 \tag{12}$$

Some further examples of the use of ITP in this field are given in the following section.

In the synthesis of peptides Van Nispen *et al.* [124] used ITP for the determination of acetate in the N-terminal 24-peptide ACTH(1–24), achieving a very good agreement in the determination of the acetate by the titration method and by gas chromatography (GC). The mean mass content of acetate was 10% by titration, 9.9% by GC and 9.7% (m/m) by ITP. The reproducibility of the ITP determination depended on the absolute amount of acetate in the sample: at 2.4–17.0% acetate it was 1.4–1.5%; at 1.35–2.4% acetate it was 2.6%; at 0.21–1.35% acetate it was 4.9%. The detection limit for acetate during a routine determination was about 5 ng, *i.e.* 83 pmol.

The acetate content was also determined in the 16-peptide of human β-lipotropic hormone (β-LTH, 62–77) and the tripeptide Asp–Glu–Gly of the same hormone (position 41–43). Whereas for the basic 24-peptide ACTH and the neutral 16-peptide β-LTH only admixtures of anions were determined in the anionic ITP system, for the acid tripeptide Asp–Glu–Gly not only was the determination of acetate carried out in one experiment, but also the determination of the peptide itself.

The ITP separation of the model mixture of acids, presented in the same paper

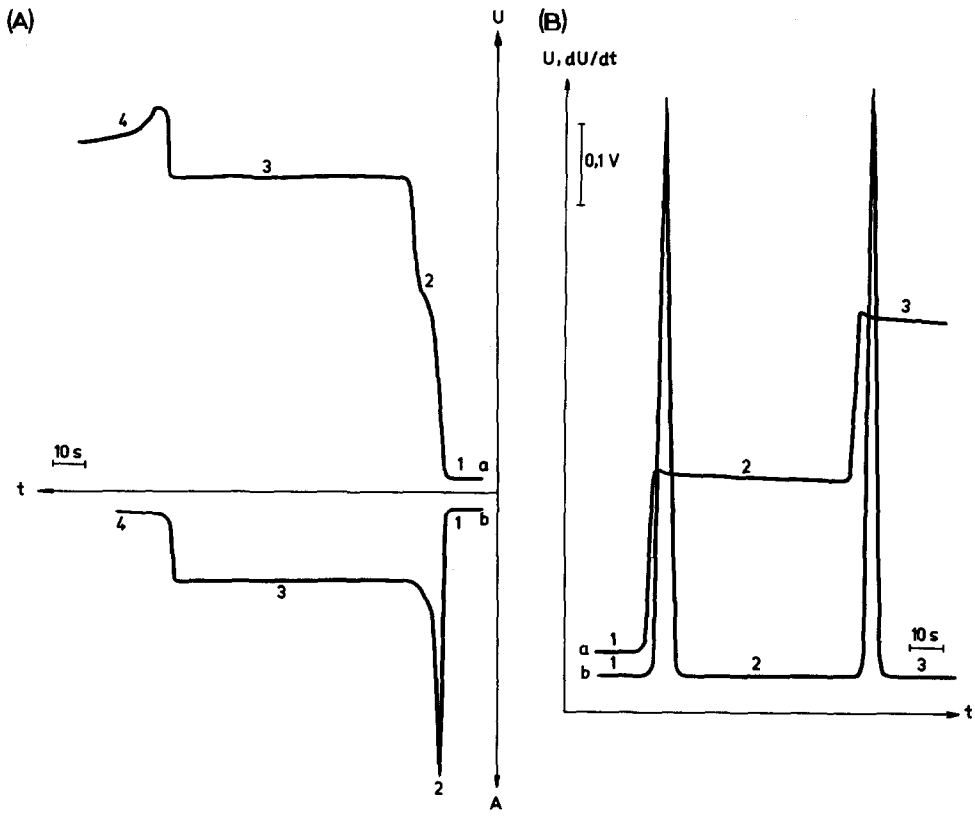

Fig. 9. (A) ITP analysis of synthetic Adiuretin (8-D-Arg)–deaminovasopressin, pharmaceutical product of n.p. Léčiva), in a cationic regimen (ES 60, Table 5). a = Signal of PG detector; b = signal of UV detector; A = absorbance at 254 nm; U = voltage of PG detector; t = time; 1 = Na^+; 2 = unidentified sample component; 3 = Adiuretin; 4 = BALA; sample, 2 μl of aqueous solution of 8 μg of dry Adiuretin product; separation current = 50 μA, detection current = 35 μA. Other conditions as in Fig. 5. (B) ITP analysis of Adiuretin in an anionic regimen (ES 87, see Table 7). a = Signal of PG detector; b = differentiation of PG detector signal; 1 = Cl^-; 2 = acetate; 3 = glutamate; detection current = 20 μA; analysis time = 15 min. All other parameters and symbols as in (A).

[124], indicated the possibility of the simultaneous determination of further anions of acids used during the synthesis and purification of the peptides (formates, citrates, methanesulphonates, trifluoroacetates, *p*-toluenesulphonates).

In a further paper [138] the ITP determination of low-molecular-mass admixtures in peptides was extended further to anions (chlorides, bromides, iodides) and cations of bases (ammonia, hydrazine, trimethylamine, tetramethylammonium, pyridine, piperidine, 4-dimethylaminopyridine, N-ethylmorpholine, N,N-diisopropylethylamine, triethylamine, dicyclohexylamine), which are also frequently used in the synthesis and purification of peptides.

The composition of electrolyte systems for the determination of anions and cations in peptides is given in Tables 7 and 8.

The determination of the anions of formic, acetic and trifluoroacetic acids in the oligopeptidic pharmaceuticals Adiuretin (Mpa–Tyr–Phe–Gln–Asn–Cys–Pro–D–Arg–Gly–NH_2), Carbetocin [$(CH_2)_3$–CONH–Tyr(Me)–Ile–Glu–Asn–Cys–Pro–Leu–Gly–NH_2], *O*-methyloxytocin (Cys–Tyr(Me)–Ile–Glu–Asn–Cys–Pro–Leu–Gly–NH_2), Remestyp (Gly–Gly–Gly–Cys–Tyr–Phe–Glu–Asn–Cys–Pro–Lys–Gly–NH_2), Gonadoliberin (γ–Glu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly–NH_2) (Léčiva Praha, Czechoslovakia) was carried out in our laboratory [96]. Fig. 9 shows the ITP determination of an Adiuretin preparation in both cationic and anionic regimens. In the cationic regimen a high degree of purity was found for Adiuretin, whereas in the anionic regimen the acetate content in the preparation was determined. The amount of sample necessary for one analysis was of the order of micrograms and the content of the determined acids ranged from tenths to units of the percentage mass fraction.

The determination of acetates or chlorides in peptide preparations has considerable practical importance as biologically active peptides are frequently converted to acetates or chlorides before use.

The advantage of ITP in these determinations consists in the simple preparation of the sample (only weighing and dissolution), the small consumption of the sample (200–300 μg), the short analysis time (10–15 min), the accuracy of the analysis (relative error 1–2%) and the possibility of the simultaneous determination of several ions of the same charge type.

5.6. Determination of peptides in biological fluids and tissue extracts

The use of ITP in the direct determination of peptides in biological fluids and tissue extracts is less frequent than for synthetic or isolated peptides as peptide hormones and other biologically active peptides are present in biological fluids and tissues in very low concentrations which do not usually suffice for a direct ITP analysis. Hence, in most cases the ITP analysis must be preceded by preseparation and preconcentration steps (precipitation, ultrafiltration, chromatographic separation and other procedures).

One of the exceptions in this context is glutathione (GSH), a tripeptide (γ–Glu–Cys–Gly in the reduced state) which is a co-enzyme of oxidoreductases. GSH is a so-called biological redox factor, which is present in cells in approximately mmol/l concentrations, *i.e.* at an optimum level for ITP. Using ITP the ratio of the reduced and oxidised forms of glutathione was determined, *i.e.* GSH and glutathione disulphide (GSSG), in various rat tissue extracts (liver, kidneys, heart, brain) and in serum [121]. Freshly obtained tissues were homogenised in four volumes of water or 1.25% sulphosalicylic acid (four volumes of sulphosalicylic acid only are added to the serum), centrifuged and 10 μl of the supernatant were directly applied to the ITP analyser. The oxidised dimeric form travelled in the electrolyte system ES 1 (Table 3) at pH 3.1 more rapidly than the reduced monomer. GSH gave a homogeneous ITP zone and its concentration was directly

determined from the length of the zone. The homogeneity of the GSH zone was confirmed by its disappearance when N-ethylmaleimide (selectively reacting with GSH) was added to the supernatant, and by the formation of a zone of a GSH–N-ethylmaleimide complex. The GSH zone also disappeared from the isotachopherogram after the addition of an extract containing γ-glutamyltranspeptidase. ITP proved to be simpler and faster than the methods currently used for the determination of GSH.

Holloway and Battersby [139] used ITP to study the spontaneous conversion of the reduced form of GSH to GSSG, the oxidised form, depending on the time and method of storage of GSH.

An increased concentration of peptides in biological fluids is present in some illnesses, for the diagnosis and monitoring of which ITP was used.

Mikasa *et al.* [129] used ITP for the determination of iminopeptides in the urine of patients with iminopeptidury. An excessive excretion of iminopeptides takes place as a result of prolidase deficiency, which is a rarely occurring defect manifested by clinical symptoms such as chronic recurrent infections, mental retardation, splenomegaly and skin lesions.

The direct analyses in ES 17 (Table 3) of normal human urine and the urine of patients with iminopeptidury gave different isotachopherograms, but for a specific determination of iminopeptides it was necessary to adjust the samples. The alkaline urine samples were separated chromatographically on Chelex 100; individual fractions were dried, redissolved in water, chromatographed on Diaion Sk-1 and then the eluates were evaporated to dryness and redissolved. Part of the samples obtained in this manner were analysed by ITP and then, after total hydrolysis, by an amino acid analyser, whereas a second part was left aside for further separation and identification.

Using this procedure almost all the peptides could be eliminated from the urine of healthy persons, whereas on the isotachopherograms of patients with iminopeptidury five zones were found, in which twelve dipeptides containing proline at the C-terminus could be identified. In addition to three homogeneous zones of dipeptides (Thr–Pro, Ser–Pro and Pro–Pro; the second, fourth and fifth zone on the isotachopherogram, respectively), two mixed zones were also detected, of which the first was a mixture of Gly–Pro and Asp–Pro and the second (the third zone on the isotachopherogram) was a mixture of Gly–Pro, Leu–Pro, Ile–Pro, Ala–Pro, Val–Pro Phe–Pro and Tyr–Pro.

The identification of the dipeptides was carried out on the basis of: (1) comparison with corresponding standards by ITP and paper chromatography; (2) amino acid analysis of the acid hydrolysate of the peptides; and (3) determination of the N-terminal amino acid by the dinitrophenylation method.

Although an accurate representation of the individual iminopeptides has not been determined, but only their total content, ITP has proved to be a rapid and simple method for the screening of patients with iminopeptidury.

The ITP analysis in ES 16 (Table 3) of iminopeptides was used both for the determination of prolidase activity itself [128] and for the simultaneous measurement of prolidase and prolinase activity in erythrocytes [125] of a patient with peptidury and in the erythrocytes of her mother. The substrates of prolidase (Gly–Pro) and prolinase (Pro–Gly) were incubated with an adjusted lysate of erythrocytes. After the end of the reaction by heating in boiling water, the reaction solution was centrifuged and the content of unreacted dipeptide substrates and one of the cleavage products (glycine) were determined in the supernatant. The amount of glycine corresponded to the sum of the decrease of the amount of both dipeptides. Whereas the prolinase activity was equal in both persons, prolidase activity was absent in the patient with iminopeptidury.

ITP was used for the study of isovaleric acidaemia, a congenital defect of leucine metabolism [122]. In the urine of patients with this disease the peptide isovalerylglycine is extracted, the amount of which was determined in ES 2 (Table 3). The advantage of ITP determination (compared with chromatographic methods) is that the urine of patients could be directly applied to the ITP analyser, without any previous treatment. This case is also interesting because for a complete separation of isovalerylglycine and aspartic acid, which is also present in the sample, the complexing effect of copper(II) ions was utilized, which were added in the form of copper(II) chloride as co-counter-ions of the leading electrolyte.

In a number of papers ITP was used in the determination of peptidic and other ionic metabolites in body fluids of uraemic patients [123,140–146]. Uraemia (derived from "urine in blood") is a consequence of a chronic failure of the kidneys. The substances which are currently eliminated by the kidneys remain in the body fluids and have a poisonous effect on the organism.

The ITP separation profiles of the sera of uraemic patients are mutually heterogeneous, but distinctly different from the profiles of normal sera. The main difference appears in the region of low- and medium-molecular-mass anionic solutes, among which oligopeptide and polypeptide components can be confirmed. These so-called "middle molecules", with a relative molecular mass of 500–5000, are considered to be the main toxic components in uraemia.

The characterization of uraemic middle molecules by ITP and other methods was investigated by Zimmerman *et al.* [123]. In the urine and plasma of uraemic patients they found characteristic components (of relative molecular mass 500–2000), which were absent in normal samples. The ITP analysis in ES 3 (Table 3) was preceded by gel chromatography on Sephadex G-25. Amino acid analysis of individual fractions before and after their acid hydrolysis and the determination of N- and C-terminal amino acids demonstrated the peptide character of the substances present. The main peptidic component was polyglycine with a blocked N-terminus.

The ITP analysis of directly applied snake venoms containing complex mixtures of peptides and proteins showed a high reproducibility of the separation profile (UV detection at 280 nm) of the venom of one species and significant

differences between snakes of different species (Siamese cobra and Chinese cobra), which can be used in their identification [46].

The ITP separation profiles also showed a species specificity in the case of the analysis of bee and wasp venoms [147,148], in which mellitin was also identified, *i.e.* the toxic (heamolytic) polypeptide containing 26 amino acid residues.

The increase in the importance of ITP in the analysis of peptides in biological fluids may only be expected after the spread of commercially accessible ITP analysers with column coupling, which will allow the application of larger volumes of samples, indispensable for the analysis of substances at very low concentrations, and also for a rapid separation of the macrocomponents from the minor components.

6. CONCLUSIONS

Capillary ITP has been demonstrated to be a fast, high-performance method of peptide microanalysis on the nanomole and sub-nanomole scale, giving both qualitative and quantitative information on peptide purity including the determination of low-molecular-mass ionogenic admixtures in peptide preparations. The technique also allows the separation of complex peptide and protein mixtures of biological origin.

In spite of this fact and in spite of the many inherent advantages of capillary ITP (such as the concentrating effect, self-sharpening effect, carrierless separation medium, built-in on-line detection, minimal and simple sample pretreatment, insensitivity to non-ionogenic admixtures, high reproducibility and relatively high sensitivity, short analysis time), the applications of capillary ITP in peptide analysis have been so far underestimated. Possible reasons for this state may be as follows:

1. A lack of knowledge of the real possibilities offered by the method.
2. More complicated and expensive instrumentation is required than for slab gel electrophoresis.
3. A shortage of commercially available apparatus with a broad choice of suitable detectors, automated computer control and data evaluation. Until now the equipment produced has fulfilled these demands, unlike HPLC equipment, only partly. This situation may be improved in the near future when the very rapidly developing instrumentation for high-performance capillary electrophoresis (HPCE) is adapted for capillary ITP.
4. Conversion of analytical separation into preparative separation is more complicated than in HPLC. This problem will be solved in a similar way as in HPCE, where micropreparative arrangements will be available.

After overcoming these "teething troubles" capillary ITP will have a chance to become an important, widely accepted counterpart or complementary method to HPLC in peptide analysis and preparation.

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