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Review

Isotachophoresis of nucleic acid constituents

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ABSTRACT

Determination methods for purine and pyrimidine bases, nucleosides, nucleotides and related compounds using analytical capillary isotachophoresis are reviewed. First, the isotachophoretic characterization of these compounds, as well as methods for sample preparation prior to analysis, and the different ways of detecting unknown substances in complex biological systems are described. Then applications of isotachophoretic analysis in medical diagnosis and biomedical research are reviewed. In particular, the analysis of purines, pyrimidines, nucleosides and related compounds in blood and serum for the diagnosis and treatment of inherited diseases and cancer is described. Selected applications of nucleotide analysis in biomedical research using different tissue extracts are also reviewed, and some examples of nucleotide-dependent enzymic reactions, which were performed by means of analytical isotachophoresis, are presented.

CONTENTS

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LIST OF ABBREVIATIONS

2′,5′A	2′,5′-Oligoadenylates
ADA	Adenosine deaminase
Ap4A	Diadenosine 5′,5″,p^1,p^4-tetraphosphate
2′,5′A-PDE	2′,5′-Oligoadenylate phosphodiesterase
A-PRT	Adenine phosphoribosyltransferase
2′,5′A-synthetase	2′,5′-Oligoadenylate synthetase
BAP	Bacterial alkaline phosphatase
BES	N,N-Bis-(2-hydroxylethyl)-2-aminoethanesulphonic acid
C	Conductivity
DMD	Duchenne muscle disease
5-dFUR	5′-Deoxy-5-fluorouridine
EPPS	N-Hydroxyethylpiperazinepropanesulphonic acid
5-FdUMP	5-Fluorodeoxyuridylate
5-FU	5-Fluorouracil
GlcUA	Glucuronic acid
HEPES	N-2-Hydroxyethylpiperazine-N′-2-ethanesulphonic acid
HG-PRT	Hypoxanthine-guanine phosphoribosyltransferase
HPLC	High-performance liquid chromatography
HPMC	Hydroxypropylmethyl cellulose
IMP	Inosine monophosphate
ITP	Isotachophoresis
LE	Leading electrolyte
NAC	Nucleic acid constituent
PAP	Adenosine 3′,5′-diphosphate
PAPS	Adenosine 3′-phosphate 5′-phosphosulphonate
PCA	Perchloric acid
PNP	Purine nucleoside phosphorylase
pNP	4-Nitrophenol
pNPS	4-Nitrophenyl sulphate
RNase	Ribonuclease
SCID	Severe combined immunodeficiency disease
SV-PDE	Snake venom phosphodiesterase
TE	Terminating electrolyte
UDPGlcUA	UDP–glucuronic acid
UDP-HexNAc	UDP–N-acetylhexosamine
UV	Ultraviolet
XO	Xanthine oxidase

1. INTRODUCTION

The determination of nucleic acid constituents (NACs) by capillary analytical isotachophoresis (ITP) is one of the most successful areas of application of this powerful analytical method. Many reports appeared between 1970 and 1980, and these have been reviewed [1,2]. Virtually all of the main nucleotides, nucleosides and purine/pyrimidine bases have been characterized by analytical capillary ITP. Unfortunately, although accepted as a very precise analytical method, ITP has never reached the popularity and wide distribution of high-performance liquid chromatography (HPLC) or, more recently, capillary zone electrophoresis [3].

In this review some of the principal early investigations and some new applications of ITP for the analysis of NACs will be discussed. NACs are defined here as purine and pyrimidine nucleotides, nucleosides and bases that are compounds of RNA and DNA. Furthermore, NAC-related compounds, such as their precursors or degradation products, NAC-related therapeutics, and substances that are structurally related to them without being NACs in the strict sense of the word will also be considered.

1.1. Basic principles of isotachophoresis

The theory of ITP and the different types of equipment for ITP analysis have been reviewed in several articles and books [4–11]. Therefore, only the most basic features of this method will be briefly summarized. Separations are carried out in small capillaries using a discontinuous electrolyte system consisting of a leading electrolyte (LE) and a terminating electrolyte (TE). The LE has the highest mobility of all ions, and the TE the lowest. Usually, 1–10 μl of the sample is injected between the LE and the TE zones and allowed to be separated. In order to help prevent diffusion of the separated sample zones, substances such as methylcellulose are included to enhance the viscosity. ITP analysis is performed at constant current. Since the separated zones will experience different electric field strengths, there is an efficient counteraction of diffusion, leading to sharp zone boundaries (zone-sharpening effect). According to Kohlrausch's law [12], the lengths of the separated homogeneous sample zones are a function of the LE concentration. Depending on the concentration of the LE electrolyte, the compounds to be separated will be either concentrated or diluted. Consequently, compounds present in only small amounts can be detected more easily in complex mixtures than in most other separation procedures (concentrating effect).

NAC show a strong absorption at 254 (and 280) nm, therefore the homogeneous zone lengths can be measured easily by column-integrated ultraviolet (UV) detectors. The limit for quantification of samples is *ca.* 50 pmol using UV zone-length measurements [14]. Under certain conditions, it may also be possible to quantitate very small amounts of NACs, which do not fill the UV slit completely, by estimating the peak height. Arlinger [13] reported that the detection limit for ATP by measuring UV absorption at 254 nm (slit width: 0.2 mm) was *ca.* 25 pmol at an LE concentration of 0.01 *M*, and a few picomoles at 0.0005 *M*. Wielders and Everaerts [14] estimated very small amounts of ADP by the addition of acetate, which formed a mixed zone with ADP under distinct LE/TE conditions; the fine UV peak within the acetate zone, representing ADP, could be estimated by integration.

1.2. Biosynthesis and degradation of nucleic acid constituents

Fig. 1a and b give a simplified overview of the main steps of biosynthesis and degradation of NACs (according to ref. 15). All the purine and pyrimidine bases, nucleosides and nucleotides of these metabolic pathways can be determined by analytical capillary ITP. Some of the NACs that have often been analysed by ITP (see below) are specially mentioned in order to facilitate their localization within the metabolic pathways.

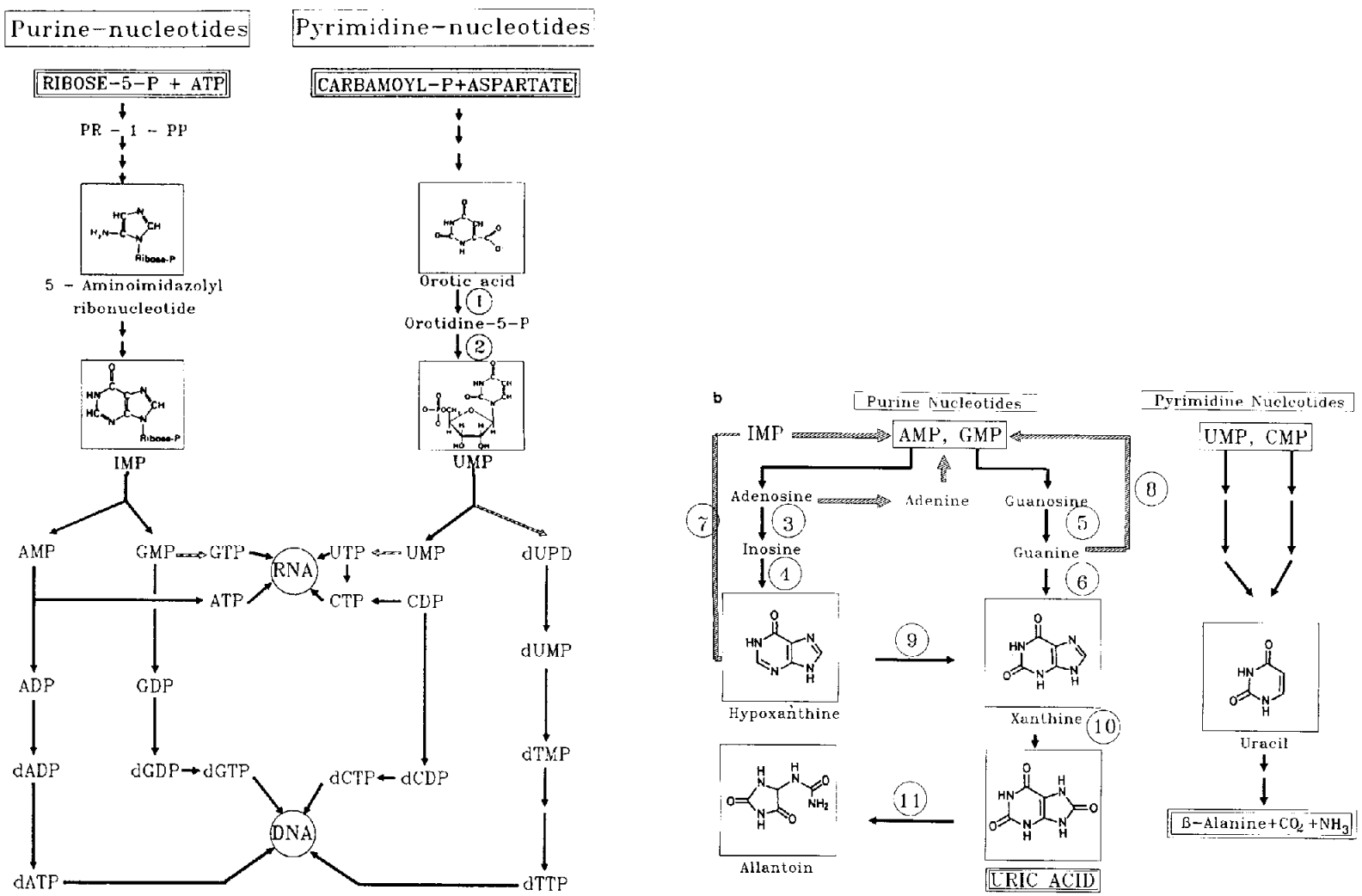

Fig. 1. (a) Simplified scheme of purine and pyrimidine nucleotide biosynthesis. Some central steps are emphasized with respect to their role in the diagnosis of inborn errors or other diseases. Steps: 1 = orotate phosphoribosyltransferase; 2 = orotidylate decarboxylase; PR-1-PP = 5-phosphoribosyl-1-pyrophosphate. (b) Simplified scheme of the degradation (3,4,5,6,9,10) and salvage (7,8) pathways of purine and pyrimidine nucleotides. Steps: 3 = adenosine deaminase (ADA); 4 and 5 = purine nucleoside phosphorylase (PNP); 6 = guanine deaminase; 7 and 8 = hypoxanthine-guanine phosphoribosyltransferase (HGPRT); 9 and 10 = xanthine oxidase (XO); 11 = uricase. Unnumbered steps, adenine phosphoribosyltransferase (APRT) (adenine → AMP).

2. ISOTACHOPHORETIC CHARACTERIZATION OF NUCLEIC ACID CONSTITUENTS

In this section, some of the conditions commonly used for the separation of nucleotides, nucleosides and purine and pyrimidine bases by ITP in synthetic mixtures are presented. In many cases, these LE/TE systems can also be used directly for analysis in biological systems. However, it may be advantageous to optimize the separation conditions for distinct biological samples, depending on the substances of interest. Some recommendations for the choice of a suitable operating system for the separation of NACs are given in refs. 1 and 2.

2.1. Nucleotides

Separation of 5′-substituted mono-, di- and triphosphates of adenosine, guanosine, uridine and cytidine (and of cAMP and 2′,3′-cyclic CMP), using 5 m*M* HCl (adjusted to pH 3.89 with β-alanine) as the LE and 5 m*M* caproic acid as the TE, has been described [2]. The compounds could be characterized by their UV absorption at 254 nm (Fig. 2).

The separability of these substances with regard to optimal pH conditions was determined by Beckers and Everaerts [16] by measuring the thermal step heights in different electrolyte systems. Fig. 3 shows that separation improved when the pH of the chosen system was lower. However, at low pH the analysis time will be prolonged. The original measurements shown in Fig. 3 were carried out using 0.01 *M* HCl as the LE with different counter-ions in order to get the desired pH values (pH 3.4, adenosine; pH 3.7, α-naphthylamine; pH 4.2, aniline; pH 4.6, aniline; pH 5.0, pyridine; pH 6.0, histidine; pH 7.0, imidazole). Because adenosine, α-naphthylamine and aniline show a strong UV absorption at 254 nm, Kopwillem [17] suggested the use of other, non-UV-absorbing counter-ions (pH 3.4–4.2, β-alanine; pH 4.6–5.0, creatinine). Under these conditions analyses can be carried out using UV detection at 254 nm, the preferential detection method for NAC analysis in ITP. For reasons of clarity, the step-heights of a given compound measured at different pH values are connected by lines in Fig. 3. However, this interpolation is not absolutely correct because the different counter-ions used for adjusting the pH also influence net mobility [17].

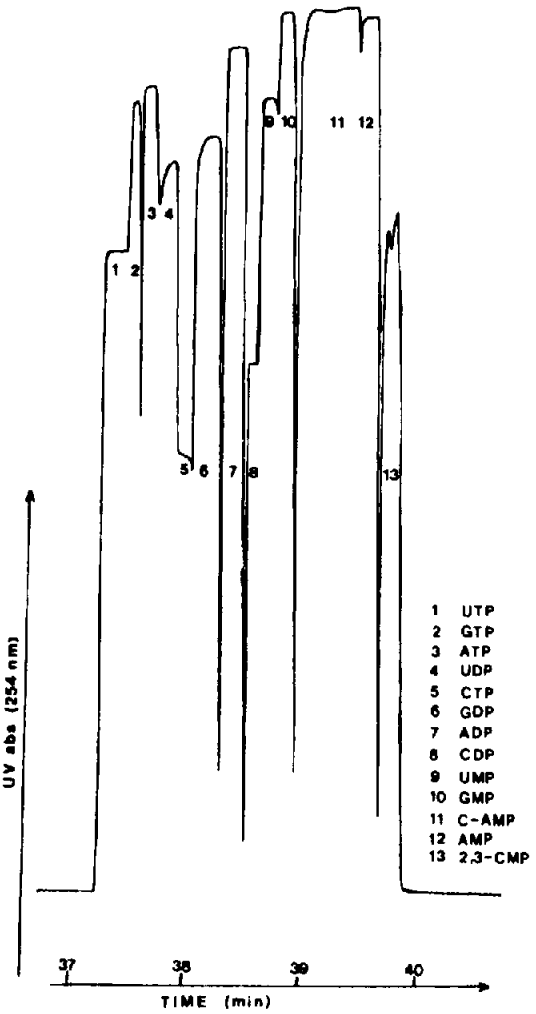

Fig. 2. Separation of thirteen nucleotides in a synthetic mixture (cAMP *ca.* 4.5 nmol, all others *ca.* 1.5 nmol) on a 63-cm capillary. For the LE/TE system see the text. (From *LKB Isotachophoresis News* No. 1, 1977, cited in ref. 2.)

Bours *et al.* [18] later extended these analyses of synthetic nucleotide mixtures. They analysed twenty-two 5′-nucleotides and four nicotinamide-adenine dinucleotides using different spacers by recording the UV absorption at 254

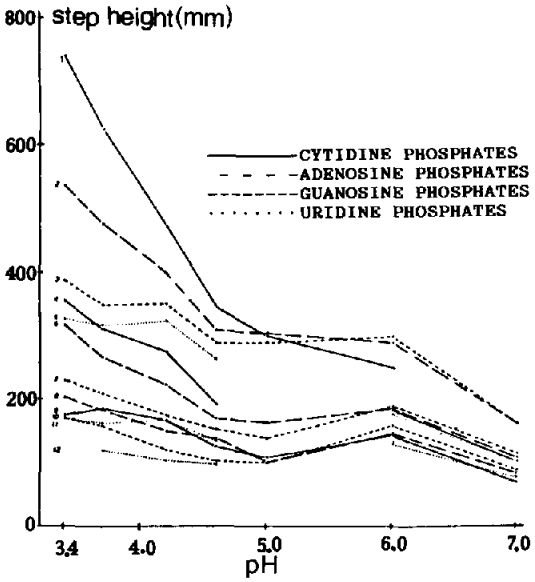

Fig. 3. Influence of pH on the separability of cytidine phosphates (CTP, CDP, CMP), adenosine phosphates (ATP, ADP, AMP), guanosine phosphates (GTP, GDP, GMP) and uridine phosphates (UTP, UDP, UMP), measured by registration of step heights. Sequence of step heights: nucleoside triphosphates < nucleoside diphosphates < nucleoside monophosphates. (From ref. 16.)

nm (LE, 0.005 *M* HCl–aminobutyric acid (GABA), pH between 4 and 5; TE, 0.005 *M* caproic acid, pH 5). The sequence of mobility and the extinction coefficients of these nucleotides at 254 nm were determined and are presented in Table 1.

2.2. Nucleosides and purine and pyrimidine bases

Oerlemans *et al.* [19] separated a mixture of seventeen purine and pyrimidine derivatives with the help of non-UV-absorbing spacers (Fig. 4). Because nucleosides and bases lack the highly charged phosphate groups, electrolyte systems with higher pH values are necessary for ITP of these compounds. The authors used 0.005 *M* HCl–ammediol (pH 8.55) as the LE, 0.02 *M* β-alanine, adjusted to pH 10.4–10.5 with Ba $(OH)_2$, as the TE. UV absorption at 254 nm was used as the detection system. The mixture to be separated was composed of the following substances (where the numbers in parentheses indicate the positions in the tachogram in Fig. 4): the

TABLE 1

ELECTROPHORETIC ORDER AND EXTINCTION COEFFICIENTS OF 5'-NUCLEOTIDES AND NICOTINAMIDE ADENINE DINUCLEOTIDES

Order	Nucleotide	Amount[a] (nmol)	$E^{254\ nm}_{0.1\%}$	Order	Nucleotide	Amount[a] (nmol)	$E^{254\ nm}_{0.1\%}$
1	UTP	1.82	13.04	13a	FAD	1.21	32.23
2	TTP	1.82	11.05	14	CDP	2.18	11.96
3	ITP	1.81	16.29	15	NADP	1.27	18.68
4	GTP	1.70	21.18	16	NADH	1.41	16.47
5	ATP	1.81	21.38	17	cGMP	2.57	30.78
6	UDP	2.13	14.96	18	UMP	2.72	19.85
7	TDP	2.14	12.96	19	TMP	2.73	16.45
8	IDP	2.02	17.33	20	IMP	2.00	19.78
9	CTP	1.82	7.34	21	GMP	2.46	28.34
10	GDP	2.05	25.60	22	cAMP	2.68	30.75
11	NADPH	1.20	14.19	23	AMP	2.63	30.03
12	UDPG	1.64	11.21	24	NAD	1.51	22.93
13	ADP	2.17	25.58	25	CMP	2.72	13.27

[a] Amount injected, 2.01 ± 0.48 nmol (mean ± S.D.).

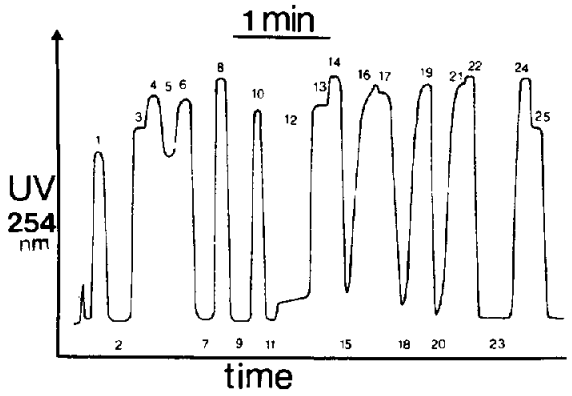

Fig. 4. Analysis of a mixture of seventeen nucleosides, purine and pyrimidine bases and related compounds absorbing at 254 nm and eight non-UV-absorbing spacers (2,7,9,11,15,18,20,23); *ca.* 1.5 nmol of each compound were used. Substances and conditions for ITP are indicated in the text. (From ref. 19.)

purine nucleosides guanosine (22), inosine (16) and xanthosine (8); the purines guanine (21), adenine (24), xanthine (4), hypoxanthine (14), 3-methylxanthine (10), 1,3-dimethylxanthine (theophylline) (13), 3,7-dimethylxanthine (theobromine) (25), uric acid (the final product of purine metabolism in humans) (3) and its degradation product allantoin (12), which is formed in the uricase reaction and is not a purine. Furthermore, allopurinol (19), an analogue of hypoxanthine, and its oxidation product oxopurinol (6), were included. Allopurinol, a substrate and inhibitor of xanthine oxidase, is used in the therapy of gout because it inhibits formation of uric acid (see Fig. 1). The pyrimidines orotic acid (1) and uracil (17) were also present in this mixture. Additionally, substances that were non-absorbing at 254 nm were included in order to better separate the pyrimidines and purines (spacers: aspartic acid (2), N,N-bis-(2-hydroxyethyl)-2-aminoethanesulphonic acid (BES) (7), N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES) (9), N-hydroxyethylpiperazinepropanesulphonic acid (EPPS) (11), 1-methylhistidine (15), histidine (18), 3-methylhystidine (20) and α-alanine (23).

The ribonucleosides guanosine, uridine and thymidine were separated using 0.005 *M* HCl–ammediol (pH 8.6) as the LE and ε-aminocaproic acid as the TE. Cytidine and adenosine were separated using 0.005 *M* potassium acetate–acetic acid (pH 5.2) as the LE and β-alanine as the TE. The purine bases xanthine and guanine and the pyrimidine bases uracil and thymine were separated using 0.005 *M* HCl–Tris (pH 7.8) as the LE and β-alanine as the TE. Cytosine and adenine were separated using 0.005 *M* potassium acetate–acetic acid (pH 5.2) as the LE and 0.01 *M* α-alanine as the TE. Thermal step-heights and UV detection at 280 nm were used in all these investigations as detection systems (data from LKB-Application Laboratory, Bromma, Sweden, presented in ref. 2, pp. 302–303).

2.3. Nucleic acid constituent-related substances

The ITP characterization of some nucleotide-, nucleotide-, purine- and pyrimidine-related substances has already been described in Sections 2.1 and 2.2, although they are not NACs in a strict sense of the word. Among them were the nucleotide analogous compounds $NAD(P)^+$, NAD(P)H, cAMP, cGMP and UDP-glucuronic acid (Table 1) and the purine analogous compounds allopurinol, oxopurinol, theophylline and theobromine (Fig. 4). Furthermore, ITP analyses of some other derivatives have been reported, such as the cytostatic drugs 5-fluorouracil [20,21], 5′-deoxy-5-fluorouridine [22] and arabinofuranosylcytosine triphosphate [23] (see Section 3.2.2).

Analysis of some oligonucleotides (di-, tri- and tetrameric forms of 2′,5′-oligoadenylates) by capillary analytical ITP has been described [24,25]. These oligoadenylates (pppA2′p5′A, pppA2′p5′A2′p5′A, pppA2′p5′A2′p5′A2′p5′A), which are generated by the action of interferon in some virus-infected celles [26], are characterized by connection of their adenosine residues via phosphodiester bridges in the unusual 2′,5′ position (Fig. 5). They contain either triphosphate groups on their 5′-end (2′,5′-oligoadenylates) or not (2′,5′-oligoadenylate cores). Using Ampholines as spacers, the separation of the di-, tri and tetrameric forms of 2′,5′-oligoadenylate cores from each other was possible, but not of the tri- and tetrameric forms of the 5′-triphosphates (Fig. 6).

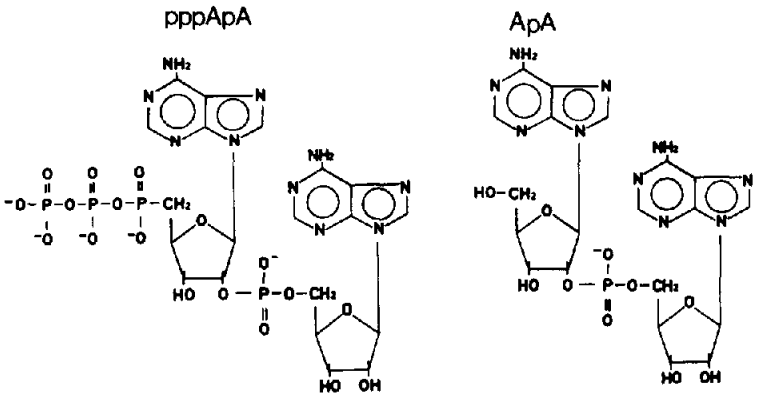

Fig. 5. Structures of 2',5'-oligoadenylates (dimeric form, pppA2'p5'A, or pppApA) and 2',5'-oligoadenylate cores (dimeric form, A2'p5'A, or ApA).

Summarizing the results obtained by analysis of complex synthetic mixtures, it can be stated that ITP proved to be a very powerful separation technique for nucleotides as well as for nucleosides and purine and pyrimidine bases. The separation systems of these synthetic mixtures can also be used for the investigation of biological samples. However, depending on the substances of interest, the separation conditions, especially the choice of the most suitable LE/TE system, have to be adapted to individual problems. In contrast to the analysis of nucleotides, analytical capillary ITP is less suitable for the separation of oligonucleotides. For analysis of these NACs, the related technique of capillary zone electrophoresis is much more successful [27].

The following sections deal with the application of analytical capillary ITP to the determination of nucleotides, nucleosides and pyrimidine/purine bases in different fields of medicine and biology.

3. DETERMINATION OF NUCLEIC ACID CONSTITUENTS IN BIOLOGICAL SAMPLES BY ANALYTICAL CAPILLARY ISOTACHOPHORESIS

3.1. Identification of nucleic acid constituents in complex systems

A wide array of methods can be used for isotachophoretic detection and quantification of NACs in complex synthetic mixtures and in biological material. In many cases the column-integrated detection systems allow direct identification and quantification of the substances of interest. They can be identified by comparing the thermal step-heights or the conductivity signals

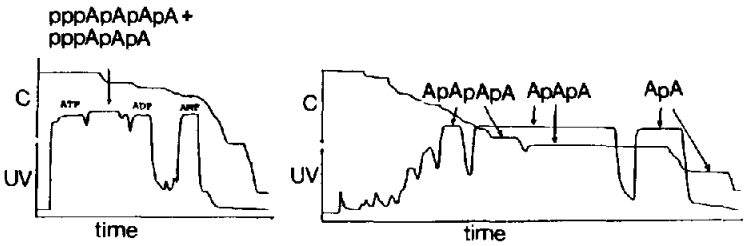

Fig. 6. Tachograms of a mixture of ATP, ADP, AMP and the trimeric and tetrameric forms of 2',5'-oligoadenylates (pppApApA, ppppApApA), and of the dimeric, trimeric and tetrameric 2',5'-oligoadenylate cores (ApA, ApApA, ApApApA) in the presence of spacers (polyamino-polycarboxylic acids, ampholines). The LE was 0.01 *M* HCl–β-alanine (pH 3.89); the TE was 0.01 *M* caproic acid. A UV detector set at 254 nm and a conductivity detector were used. Only the oligoadenylate cores could be separated.

(a more sensitive method) with those of pure substrates. The strong UV absorption of NACs at 254/280 nm was most suitable for characterization, especially when the analyses were carried out in the presence of non-UV-absorbing spacers (see Fig. 4). In some cases imporant information may be obtained by performing the analyses using UV detection at 254 nm *and* at 280 nm. This procedure was successfully applied to the identification of nucleoside mono-, di- and triphosphates and related compounds in synthetic mixtures and mollusc extracts [28], in liver extracts [29] and to the identification of purines and pyrimidines in urine samples [30].

Radiolabelled NACs were identified after ITP separation by collecting the separated substances on a cellulose acetate strip by means of the Tachofrac system [31]. After passing the detection units, the separated ions are pushed out of the capillary via a T-tube using a counter-flow of LE. The substances are transferred onto the moving cellulose acetate strip, allowing an exact correlation between the peak positions registered at the column-integrated detectors and at the strip. Using this system, studies concerning the metabolism of [^{3}H]orotic acid in liver cells [32] or the metabolism of [^{35}S]sulphate and [^{3}H]glucosamine in human fibroblasts were performed [33]. Recently, an on-column radiometric detector based on solid scintillation counting was constructed allowing the registration of ^{14}C-labelled NACs with a detection limit of *ca.* 16 Bq [34]. In combination with a conductivity detector, studies on ^{14}C-labelled CTP have been carried out. This detection system may allow some biotransformation studies of radiolabelled NAC precursors and metabolites in a more convenient way than with the Tachofrac system.

Another procedure commonly used for identification of unknown NACs consists of repeating the analysis using the same sample, but spiked with the substance of interest. Only the zone length of the respective substance will be enlarged. In contrast, the suspected substance can often be identified in a complex mixture by repeating the analysis after treatment with an enzyme that metabolizes this compound. The signal of the substance in question disappears and, under appropiate conditions, even the product signal may become visible in the tachogram. This method has been widely used for the identification of different purine metabolites in urine samples [19]. Addition of xanthine oxidase (XO) led to a decrease of hypoxanthine and xanthine, but to an increase of uric acid (see Figs. 1 and 4); addition of purine nucleoside phosphorylase (PNP), which converts xanthine into xanthosine and hypoxanthine into inosine, led to a decrease of xanthine and hypoxanthine, but to an increase of the zone lengths of xanthosine and inosine; adenine was localized after treatment with adenine phosphoribosyltransferase (A-PRT), which converted it into AMP. Following addition of uricase, uric acid disappeared and its non-UV-absorbing (at 254 nm) reaction product allantoin appeared.

Although recording of the UV signal at 254 or 280 nm is the preferred method for the detection of NACs in ITP, it is sometimes difficult to get separate signals of two compounds because of their very similar extinction coefficients. The use of spacers that do not absorb at 254 or 280 nm may then allow separation of the individual compounds, provided that their net mobilities are sufficiently different. In the case of very similar mobilities it may be advantageous to change the LE/TE system in order to get a clear separation (see Fig. 2). However, especially in complex mixtures where a high number of compounds are to be separated, it may be better not to change an optimized LE/TE system. Then, identification and quantification of two compounds in a mixed zone can be assessed by enzymic conversion of one of these compounds. Anhalt and Holloway [35] reported that adenosine-2′-monophosphate and adenosine-3′-monophosphate, which form mixed zones, could be analysed quantitatively after treatment with 3′-ribonucleotide phosphohydrolase. Another example is shown in Fig. 7: Ap4A (diadenosine 5′,5″,p^1,p^4-tetraphosphate) and ATP have almost identical extinction coefficients at 254 nm, and form mixed zones [0.01 *M* HCl–β-alanine (pH 3.89) as the LE; 0.01 *M* ε-caproic acid as the TE and, therefore, cannot be

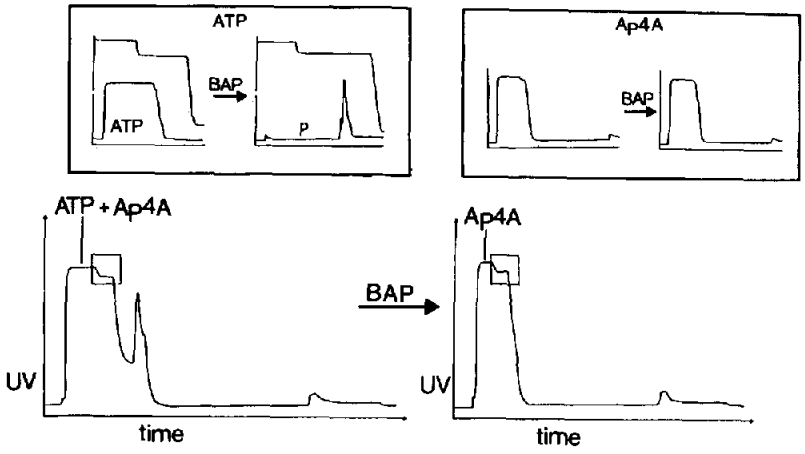

Fig. 7. Identification of ATP and diadenosine 5′,5″-p^1–p^4 tetraphosphate (Ap4A) in a mixed zone by treatment with 1 I.U. of bacterial alkaline phosphatase (BAP) for 1 h at 37°C. As shown in the insets, ATP [ATP → adenosine + 3 P (inorganic phosphate)] is a substrate of BAP, but Ap4A is not. The ATP zone represents the mixed ATP–Ap4A zone minus the remaining Ap4A zone after treatment with BAP. The square area highlights contamination (by Ap3A?) of the commercial ATP–Ap4A mixture. The LE/TE system was as described in Fig. 6.

analysed separately. However, bacterial alkaline phosphatase (BAP, EC 3.1.3.1) specifically converts ATP into phosphate and adenosine (which cannot be detected in this LE/TE system), whereas Ap4A is not used as a substrate. Therefore, both compounds can be estimated qualitatively and quantitatively after treatment with BAP.

Sometimes, it is not possible to obtain separate zones of two compounds, *e.g.* 1- and 2-naphthyl glucuronides formed in the UDP-glucuronyltransferase reaction

UDP-glucuronic acid + naphthol → naphthyl glucuronide + UDP

are not separable. However, the compounds have different UV extinctions and a quantification of both compounds was possible at pH 3.5 because the naphthyl glucuronides formed a uniformly mixed zone. A prerequisite for their quantification was the fact that both substances showed identical zone lengths per unit of sample [36].

3.2. Sample preparation for isotachophoresis

Sample preparation prior to ITP analysis is often much less laborious than for other analytical procedures. Many purines and pyrimidines can be estimated directly in diluted urine and, in some cases, also in serum. For the determination of nucleotides in biological material, proper tissue extraction is a crucial step because of the high turnover rate of many nucleotides. Among the extraction media reported are methanol, methanol–EDTA, methanol–perchloric acid and perchloric acid (PCA). Reijenga *et al.* [37] compared a methanol extraction procedure in the presence and absence of EDTA with PCA extraction. The nucleotides ATP/ADP, GTP/GDP/GMP, UTP/UDP and IMP were analysed. The total recovery of nucleoside tri- plus diphosphates was almost equal for methanol with (14.8 nmol/μl) and without (14.8 nmol/μl) EDTA but significantly lower than for PCA (33.2 nmol/μl). Furthermore, the total triphosphate/diphosphate ratios were lower for methanol alone (1.04) than for the methanol–EDTA group (1.43), indicating that enzymic decomposition occurs in the absence of EDTA. In these experiments (using a preparation of *Nassarius reticulatus* as the biological source) the PCA procedure gave the best recovery of nucleotides. The experiments described were carried out using the column-coupling method [38,39], which allows analysis in the presence of the high ionic strength associated with the PCA extraction procedure. This method is based on the use of two

capillaries, a wider one (0.8 mm I.D.), in which the (fast) pre-separation step is carried out, and a narrower one (0.2 mm I.D.), in which the final separation and analytical detection occur. At the connection of both capillaries a bifurcation block – – equipped with a side channel with an auxiliary electrode — is integrated, allowing the elimination of the zones of ballast. With this equipment, a high sample load is possible, and high ratios of sample constituents are tolerated. The column-coupling method offers great advantages in ITP analysis. It enables the determination of samples with high ionic strength, *e.g.* PCA extracts. It may be that in other cases the methanol–EDTA method is more suitable than PCA extraction, especially if no column-coupling system is available. Furthermore, PCA may lead to some hydrolysis of nucleoside triphosphates [40]. Woledge and Reilly [41] used the methanol–EDTA procedure for determination of nucleotides and other metabolites in muscle extracts. For unknown biological samples the best way is obviously to test different extraction procedures prior to analysis. The most important methods for preparation of biological samples were recently reviewed [42].

3.3. Determination of nucleic acid constituents in urine and blood samples

3.3.1. Analysis of inborn errors of purine and pyrimidine metabolism

Several inborn errors of the purine metabolism are known. Patients suffering from these diseases have abnormal levels of different purine metabolites in their urine and serum: Lesch–Nyhan syndrome patients show elevated uric acid levels due to decreased activity of hypoxanthine-guanine phosphoribosyltransferase (HG-PRT, EC 2.4.2.8) [43]. This leads to a diminished resynthesis of GMP and IMP from hypoxanthine and guanine in the salvage pathway (see Fig. 1). Other inborn errors of the purine metabolism are due to decreased activities of adenine phosphoribosyltransferase (A-PRT, EC 2.4.2.7), leading to abnormal levels of 2,8-dihydroxyadenine [44], of xanthine oxidase (EC 12.32) (abnormal levels of xanthine and hypoxanthine) [45], and of purine nucleoside phosphorylase (PNP, EC 2.4.2.1) (abnormal levels of deoxyguanosine and deoxyinosine) [45]. Defective adenosine deaminase (ADA, EC 3.5.4.4) (abnormal levels of deoxyadenosine, detectable as adenine after acid hydrolysis) [46] leads to a selective killing of lymphocytes, resulting in severe combined immunodeficiency disease (SCID). Inborn errors are also known in pyrimidine metabolism, *e.g.* indicated by elevated levels of orotic acid due to decreased activities in orotidine-5′-phosphate pyrophosphorylase and of orotidine-5′-phosphate decarboxylase [47] (see Fig. 1).

Simmonds *et al.* [48] and Sabota *et al.* [30] have successfully investigated these metabolites in diluted urine samples of patients suffering from the respective diseases by analytical capillary ITP: the method was considered to be superior for these investigations to HPLC analysis.

Oerlemans *et al.* [19] carried out analyses in patients with Lesch–Nyhan syndrome. The elevated levels of uric acid were characterized in diluted urine samples. Addition of spacers that do not absorb at 254 nm were included in order to improve the separation of the different compounds (see Fig. 4). Uric acid, which is present in high concentration even in normal persons, tends to precipitate in Lesch–Nyhan patients, leading to gout and uric acid stone formation. (Uric acid as well as xanthine, oxalate, phosphate and cystine in urinary stones were determined by analytical capillary ITP using two different LE/TE systems [49].) Therefore, patients are treated with allopurinol, an inhibitor of xanthine oxidase. Consequently, uric acid levels drop and xanthine and hypoxanthine increase. Fig. 8 shows a urine sample from a Lesch–Nyhan patient before and after treatment with allopurinol (separation in the absence of spacers). The different compounds were identified according to the methods described in Section 3.1. Since allopurinol is oxidized by xanthine oxidase, oxipurinol can also be detected in the tachogram.

The inborn errors described above are characterized by the fact that the respective enzyme defect is expressed in all tissues. Urine and serum

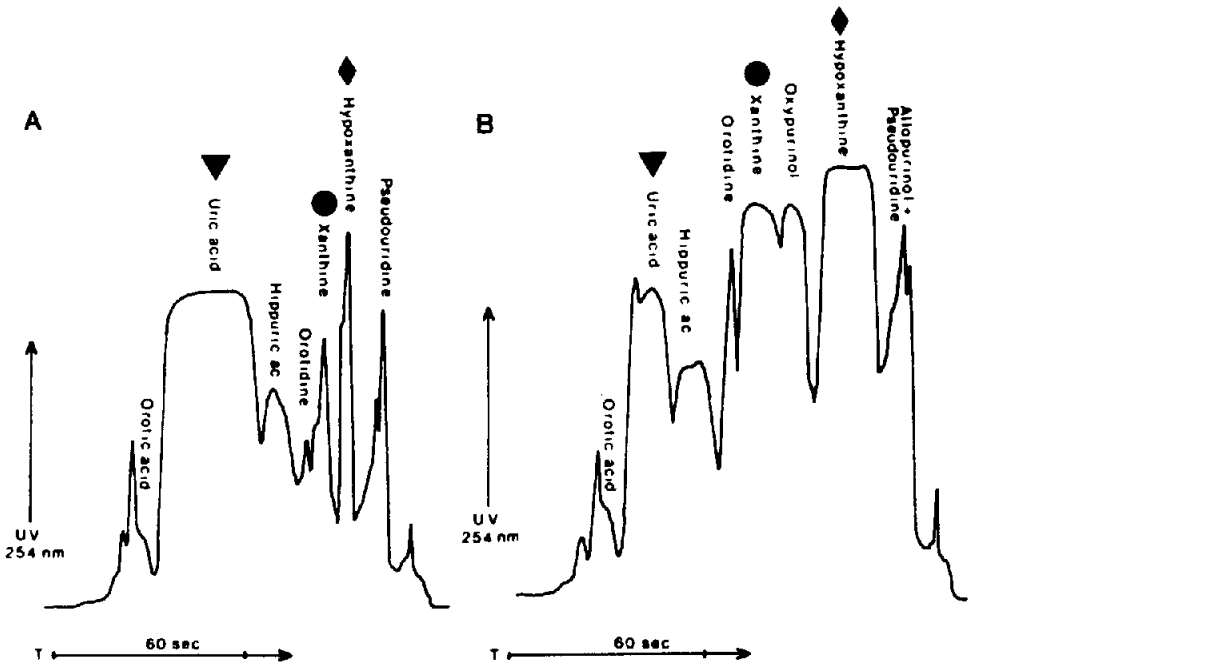

Fig. 8. UV signals of an 1:5 diluted urine samples obtained from a patient suffering from Lesch Nyhan disease (A) before and (B) after five days treatment with allopurinol (10 mg/kg body weight per 24 h). Allopurinol, as an inhibitor of xanthine oxidase, decreases the uric acid signal and increases the levels of xanthine and hypoxanthine. The LE was 0.0005 *M* HCl–ammediol (pH 8.55); the TE was 0.002 *M* β-alanine–$Ba(OH)_2$ (pH 10.4 10.5). (From ref. 106.)

samples can be used for detection. In some other diseases, however, the enzyme defect may be restricted to certain cell types: therefore, the respective tissue material has to be investigated for its characterization. An example of this is the Duchenne muscle disease (DMD) [50,51], an X-chromosome-linked degenerative disease that is invariably fatal by around age 25. In muscle extracts from patients suffering from this disease almost no adenine nucleotides are present compared with normal persons, as shown in Fig. 9.

3.3.2. Determination of nucleic acid constituents in cancer diagnosis and therapy

Buhl *et al.* [52] measured the urine excretion of hypoxanthine, xanthine, pseudouridine, methylguanidine, 1-methylhypoxanthine and orotic acid in nude mice heterotransplanted with a human mesothelioma. The modified purines 7-methylguanidine and 1-methylhypoxanthine as well as pseudouridine occur in tRNA. Owing to an increased tRNA turnover [53] and an increased degree of tRNA methylation [54] in many types of cancer, the urinary excretion of these modifed purine and pyrimidine compounds is considerably increased, because they are not subject to further catabolism or to metabolic reincorporation. Especially elevated levels were observed in patients with various forms of leukemia as well as with Hodgkin's disease [55,56], and in solid tumours [57]. They have been found to be a sensitive parameter to tumour proliferation *in vivo* and as a convenient follow-up control to judge the success of chemo/radiotherapy and surgery.

ITP analysis showed that the urine levels of pseudouridine and hypoxanthine, xanthine, 7-methylguanine and 1-methylhypoxanthine were increased in mesothelioma-bearing nude mice. The determination of hypoxanthine and pseudouridine proved to be a useful marker of tumour expansion. The levels of both substances normalized during chemotherapy. It was con-

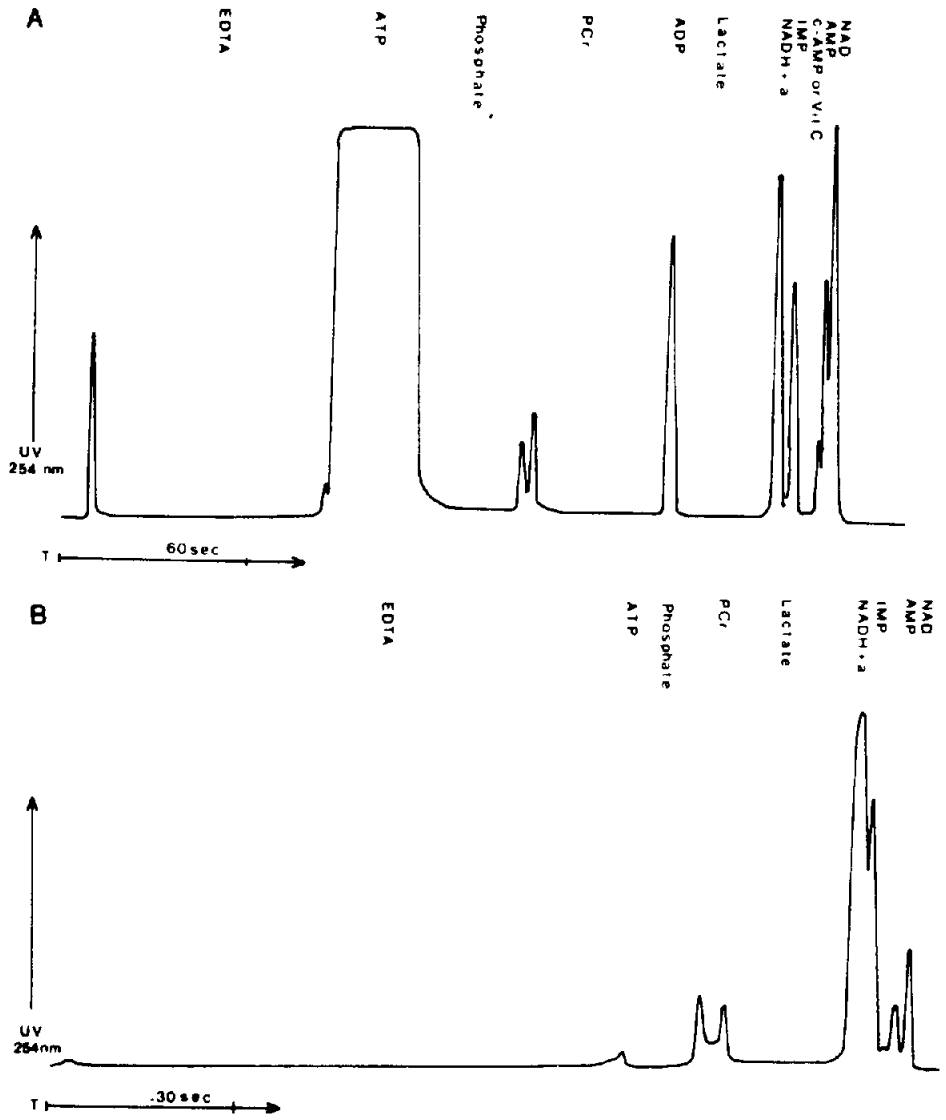

Fig. 9. Tachogram of muscle extracts from (A) a normal person and (B) a patient with Duchenne disease. The LE was 0.005 *M* HCl–β-alanine (pH 3.89); the TE was 0.005 *M* caproate (pH 6.0). (From ref. 106.)

cluded that the determination of pseudouridine may be suitable as a follow-up parameter during cancer therapy. Using 0.005 *M* HCl–2-amino-2-methyl-1,3-propanediol (pH 9.8) as the LE and 20 m*M* β-alanine–$Ba(OH)_2$ (pH 10.5) as the TE, analysis was carried out in urine samples without any pretreatment.

Another example of the analysis of NAC-related substances in cancer therapy by ITP was reported by Gustavsson and co-workers [20,21]. They determined 5-fluorouracil (5-FU) in serum. 5-FU is a widely distributed chemotherapeutic drug [58], which is operative after metabolic conversion into 5-fluorodeoxyuridylate (5-FdUMP) by inhibition of thymidylate synthase. 5-FdUMP irreversibly inhibits thymidylate synthase, thereby preventing formation of dTMP from dUMP. The aim of the ITP study was to find a method that allows the determination of serum levels of 5-FU in order to adapt an optimal therapy to

individual persons. Serum was pretreated by anion exchange and 5-FU was eluted with formic acid. After evaporation the dry sample was dissolved in water [LE, 0.005 *M* HCl–Tris (pH 7.45); TE, 0.04 *M* glycine–$Ba(OH)_2$ (pH 9.4). Samples from patients were measured before and after therapy, with a recovery rate of 82%. A simultaneous determination of xanthine was possible using the same method. Because in tumour-bearing individuals purine turnover is elevated, additional information may be drawn from these data.

These investigations were later extended by the determination of 5′-deoxy-5-fluorouridine (5-dFUR) [22]. This substance showed antineoplastic activity against several tumours in rodents [59,60]. Compared with 5-FU, a dramatic reduction in host toxicity was observed. Using 0.005 *M* HCl–hydroxypropylmethyl cellulose (HPMC) (pH 8.4) as the LE and 0.04 *M* glycine-$Ba(OH)_2$ (pH 9.4) as the TE, simultaneous determination of 5-FU and 5-dFUR in pretreated plasma samples was possible using non-UV-absorbing spacers.

3.3.3. Other determinations of nucleic acid constituents in blood samples

Theophylline (1,3-dimethylxanthine) is a bronchodilator and, therefore, widely used in the treatment of asthma. Its therapeutical effect depends on its serum concentration. For the detection of the small therapeutical theophylline levels, the peak-height method [13] was used. Prior to analysis, serum was deproteinized by polyethylene glycol, and ITP was carried out using glycylglycine and serine as spacers [61]. Alternatively, ITP analysis was performed in ultrafiltered serum using serine and bicine as non-absorbing spacers [62]. Theophylline was also determined using conductometric detection [63].

Hypoxanthine, a marker of tissue hypoxia, was measured in the serum of normal persons and in umbilical blood, after deproteinization and ion-exchange chromatography [64].

Uric acid was determined in serum samples without any sample pretreatment using the column-coupling technique [65]. The amount of uric acid bound to serum proteins was estimated by comparing ultrafiltered and non-ultrafiltered samples: *ca.* 15% of uric acid bound to pooled serum proteins with a molecular mass exceeding 25 000.

Nucleotides have been determined in the cellular compounds of blood by analytical capillary ITP. Different nucleotides at concentrations down to 50 pmol have been determined in lyophilized acetonitrile–water extracts of human red blood cells, mononuclear phagocytes and platelets [66].

Talbot [67] used the ATP (as well as lactate, phosphate and diphosphoglycerate) concentration of erythrocytes as a blood storage parameter. A decrease of ATP levels was observed in patients with anemia and in aged erythrocytes [68].

Another study was carried out to determine nucleotides in lymphocytes [69]. The particular aim of this investigation was to study the influence of γ-radiation (30 Gy) and hyperthermia on the NAD^+ pool: γ-radiation causes DNA-strand breaks, a process associated with the activation of poly(ADP-ribose) polymerase. This enzyme transfers poly(ADP-ribose) residues to histones and other proteins, thereby consuming NAD^+ as a substrate [70]. Consequently, the NAD^+ pool dropped significantly after γ-radiation, but recovered to the original level within 5 h. In contrast, recovery was only small under hyperthermic conditions.

3.4. Determination of nucleic acid constituents in different biological materials

In contrast to nucleosides and their respective bases, nucleotides are found almost exclusively inside the cells. They have been determined in different tissue extracts. ITP investigations on nucleotides carried out before 1980 were reviewed by Holloway and Lüstorff [1].

Many experiments have been performed in order to characterize the nucleotide content of muscle tissues of humans [40,71,72], frogs [73,74], pigs [75], rats [76] and even in the body wall musculature of the lugworm *Arenicola marina* [77]. Using muscle biopsies from pigs, Van Bennekom *et al.* [75] found that the amount of IMP (as well

as creatine phosphate, creatine and lactate) can be used as a diagnostic parameter for susceptibility to malignant hyperthermia, a complication in anaesthesia with a high mortality rate [78]. In another investigation, ^{31}P NMR spectra of rabbit portal vein, urinary bladder, and taenia coli smooth muscles were investigated for ATP, phosphocreatine and phospho monoester. These substances were analysed in parallel in tissue extracts by ITP. Phosphocreatine/ATP ratios were found to be similar in both systems, whereas ADP and inorganic phosphate levels were found to be much lower in tissue extracts [79].

Nucleotides have been investigated not only in muscles, but also in many other tissues. Perez *et al.* [76] measured ATP and ADP levels in muscles, heart, liver, kidney and lung of rats. The highest levels were in skeletal and smooth muscles, and the lowest in the kidney and lung.

A number of interesting experiments have been performed by Eriksson *et al.* [29]. In rat liver, nucleotides were determined by recording the UV absorption at 254 and 280 nm. Twenty-four different nucleotides (and ascorbic acid) could be clearly detected. Quantitative determinations were carried out of ATP (3000 nmol/g wet liver tissue), ADP (715), AMP (160) and UTP (360). Eriksson and Strath [80] established optimal methods for liver sampling in order to prevent nucleotide decomposition during tissue preparation, and observed that UDP-GlcUA levels decreased after diethyl ether and divinyl ether narcosis [80].

Eriksson [81] also measured the base composition of rat liver RNA. Extracted liver was hydrolysed with 0.3 *M* KOH, and the 2′(3′)-monophosphates formed were determined by capillary ITP using the column coupling method. In agreement with other assays, a G + C/U + A ratio of 0.75 was found in DNA-like RNA and of 1.64 in ribosomal-type RNA.

By using the Tachofrac technology, different investigations were carried out with radiolabelled nucleotide precursors as well as with substrates that interact with uridine nucleotides. The effect of 5-fluoroorotic acid on early labelling of nucleotides in white rats was investigated in the presence of [^{3}H]orotic acid [32]. Radiolabelled UTP, UDP-GlcUA, UDP, UMP, UDP-Hex and UDP-HexNAc were identified on the cellulose acetate strip.

In cultured human fibroblasts, the incorporation of [^{3}H]glucosamine into UDP-N-acetylhexosamine, as well as of [^{35}S]sulphate, leading to the formation of 3′-phosphoadenosine-5′-phosphosulphate (PAPS) was determined [32]. Treatment with the glucocorticoid budesonide led to an increase of the UDP-N-acetylhexosamine pool. The rate of equilibration of [^{3}H]glucosamine with the UDP-N-acetylhexosamine pool was decreased by glucocorticoid treatment [82].

Nucleotide ITP analysis was also used for many other applications, for example in studies concerning age-related changes in different parts of the lenses of the eye [83,84], in a development study using mollusc *Nassarius reticulatus* [23], in food technology to evaluate the best storage conditions for frozen carp muscles [85], and to follow the nucleotide release of microorganisms after treatment with voltage pulse and ultrasonic treatment [86]. The results of these investigations usually correspondend well with those of other established methods. In many cases ITP analysis allowed the simultaneous estimation of many different nucleotides and metabolically related compounds, such as inorganic phosphate, lactate or creatine phosphate, in a single run.

4. NUCLEIC ACID CONSTITUENTS AND RELATED SUBSTANCES AS SUBSTRATES FOR ENZYMIC REACTIONS

First experiments concerning the use of analytical capillary ITP for enzymic determinations using NAC as substrates have been carried out by Kopwillem [87] and Everaerts and Verheggen [88]. They investigated the two-step enzymic conversion of glucose into 6-phosphogluconate as model system to demonstrate the separability of the involved ions ATP, ADP, $NADP^{+}$, NADPH, glucose-6-phosphate and 6-phosphogluconate.

Many well established photometric assays exist for the determination of enzymes that use NAD

(P) (H) or ATP as substrates. However, in some cases, the use of ITP for the determination of NAC-dependent enzymic reactions may be superior to photometric methods, because several substrates and products of an enzymic reaction can be monitored at the same time. This is important in complex biological systems, for example, if one substrate is simultaneously metabolized by different enzymes. The simultaneous estimation of the enzymically generated products may offer additional information concerning the relative activities of the enzymes in the reaction mixture. The group of Holloway performed several investigations concerning the use of NAC and related compounds as substrates for enzymic determination using capillary ITP. They investigated in detail the enzymic reaction of UDP–glucuronate with different substrates using microsomes [89,90]. UDP–glucuronate is both the substrate of UDP glucuronyltransferase (EC 2.4.1.17), which transfers glucuronate to many xenobiotics and endogeneous toxins:

$$\text{UDPGlcUA} + \text{A-OH} \rightarrow \text{A-GlcUA} + \text{UDP}$$

and of a non-specific nucleotide pyrophosphatase (EC 3.6.1.9), which splits UDP–glucuronate into UMP and glucuronate-1-phosphate:

$$\text{UDPGlcUA} \rightarrow \text{UMP} + \text{GlcUA-1-P}$$

Most of the standard assays for UDP glucuronyltransferase activity take no account of the loss of UDP–glucuronate through phosphatase activity. By using paracetamol as glucuronide acceptor, it could be shown that this substrate was metabolized slowly by UDP glucuronyltransferase of rat liver microsomes, therefore, the pyrophosphatase activity became more important. With the help of ITP both activities could be determined simultaneously. Based on the substances identified by ITP it was further concluded that an additional phosphatase activity was present in the reaction mixture, which finally led to the disappearance of UDP, UMP and GlcUA-1-P (Fig. 10). Because in conventional assays the hydrolytic pathways were often not considered to be part of the glucuronidation reaction, false results occurred. ITP proved to be an excellent method for

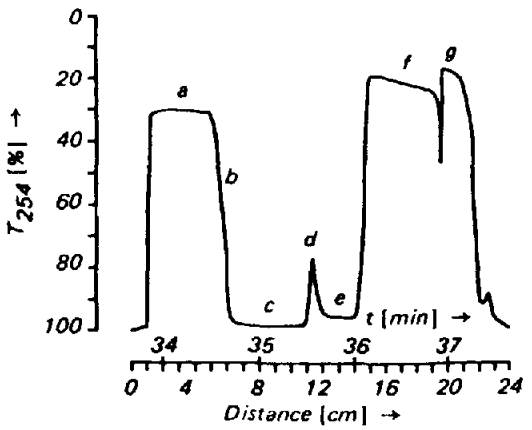

Fig. 10. Tachogram from a mixture of (a) UDP–glucuronate, (b) UDP and glucuronic acid-1-phosphate, (c) inorganic phosphate, (e) glucuronate, (f) UMP, (g) 4-acetamidophenyl glucuronoside, (d) impurity of the electrolyte system. The LE was 0.005 *M* HCl–*β*-alanine (pH 3.89); the TE was 0.005 *M* caproic acid. (From ref. 89.)

the complete analysis of this system. Furthermore, the turnover rate of different substrates (*e.g.* *p*-nitrophenol, 4-methylphenol, 1-naphthol and 2-naphthol) in the glucuronidation reaction could be estimated simultaneously [91,92]. As already mentioned (see Section 3.1), a special technique has been used for the determination of the glucuronidation products of 1- and 2-naphthol, since they form inseparable mixed zones in the tachogram.

The determination of phenolsulphotransferase (EC 2.8.2.1), which catalyses the transfer of sulphate to phenolic hydroxy groups, is another example of the use of ITP in the analysis of complex enzymic reactions [93]. The usual photometric assay, which is normally carried out in the "reverse reaction", was compared with the ITP procedure. In this assay, adenosine 3′,5′-diphosphate (PAP) and 4-nitrophenyl sulphate (pNPS) are converted into adenosine 3′-phosphate 5′-phosphosulphonate (PAPS) and 4-nitrophenol (pNP). Subsequently, transfer of sulphate groups from PAPS to different phenolic acceptor molecules was followed by ITP, leading to the regeneration of PAP. ITP analysis proved to be superior to the

photometric assay, which detects only the formation of pNP. A recently introduced ITP method allowed the simultaneous determination of glucuronidation and sulphation (as well as glutathione conjugation). In contrast to other techniques, the ITP method could be carried out directly without any additional analytical steps [94].

ITP was also used to investigate the activity of adenylate kinase (EC 2.7.4.3), which converts two molecules of ADP into AMP and ATP. Using both physiological substrates and substrate-analogous compounds, these investigations showed that the enzyme required ADP as phosphate donor, whereas the specifity of the phosphate acceptor was found to be less strict [95].

In another study, the specificity and kinetic characteristics of different enzymes (3'-nucleotidase, EC 3.1.3.6; 5'-nucleotidase, EC 3.1.3.5; nucleotide pyrophosphatase, EC 3.6.1.9) for different nucleotides (ATP, ADP, AMP, adenosine 2',5'-diphosphate, adenosine 3',5'-diphosphate, adenosine 2'-monophosphate, adenosine 3'-monophosphate, adenosine 5'-phosphosulphate) have been investigated [96].

Analytical capillary ITP was used for the determination of 2',5'-oligoadenylate synthetase (2',5'A-synthetase) [25], and 2',5'-oligoadenylate phosphodiesterase (2',5'A-PDE) [24]. Both enzymes are part of the so-called 2-5A system, which can be induced in virus-infected cells by interferon [26]. Interferon stimulates the synthesis of 2',5'A-synthetase which generates 2',5'-oligoadenylates (2',5'A) from ATP after binding to partially double-stranded virus RNA. Trimeric and tetrameric forms (see Fig. 6) are the most important 2',5'-oligoadenylates: they activate a latent RNase, which splits preferentially the single-stranded regions of the partially double-stranded RNA [97]. Consequently, synthesis of the respective proteins is inhibited. This reversible reaction is stopped by a 2',5'A-PDE, which splits 2',5'-oligoadenylates into AMP and ATP [98], thereby leading the RNase back to its inactive form. The formation and degradation of 2',5'-oligoadenylates (trimeric form as an example) are shown below:

$$\underset{(3\ \mathrm{ATP})}{3\ \mathrm{ppp5'A}} \xrightarrow{\text{2',5'A-synthetase}} \underset{(\text{2',5'A-trimer} + 2\ \text{pyrophosphate})}{\mathrm{ppp5'A2'p5'A2'p5'A} + 2\ \mathrm{pp}}$$

$$\underset{(\text{2',5'A-trimer})}{\mathrm{ppp5'A2'p5'A2'p5'A}} \xrightarrow{\text{2',5'A-PDE}} \underset{(\mathrm{ATP} + 2\ \mathrm{AMP})}{\mathrm{ppp5'A} + \mathrm{p'A} + \mathrm{p'A}}$$

2',5'A-synthetase acitivity in lysates of interferon-treated mononuclear blood cells was determined by monitoring the consumption of ATP [25]. 2',5'A-PDE activity was followed by the degradation of A2'p5'A2'p5'A (or ApApA). Enzyme assays were carried out using snake venom phosphodiesterase (SV-PDE, EC 3.1.4.1), which is known to have 2',5'A-PDE activity, and in lysates of mononuclear blood cells.

The determination of 2',5'A-PDE activity by analytical capillary ITP proved to be superior to many other reported methods [99]. Fig. 11 shows as an example the time course of the degradation of ApApA after 5 and 60 min incubation time with 0.04 I.U./ml SV-PDE. Beside unmetabolized ApApA, ApA and small amounts of AMP are detectable after 5 min. After 60 min, ApApA is completely degraded to inorganic phosphate and adenosine (not visible under the test conditions) because of the additional 5'-exonuclease activity of SV-PDE. The metabolites in these tachograms were identified by comparison with pure substrates.

In summary, ITP proved to be an excellent method for the determination of special enzymes that use nucleotides and their analogues as substrates. The method often allows the simultaneous determination of different reactions that frequently occur in parallel in complex biological systems.

5. MISCELLANEOUS APPLICATIONS

Analytical capillary ITP has often been used for purity control of commercially available NACs. Frequently, significant levels of impuri-

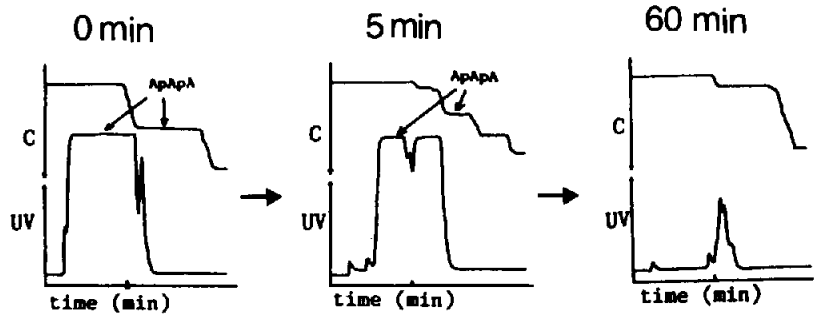

Fig. 11. Time course of the degradation of ApApA (3 m*M*) by incubation with 0.04 I.U./ml snake venom phosphodiesterase at 37°C, leading finally at 60 min to inorganic phosphate and adenosine (not visible in the LE/TE system). The LE was 0.01 *M* HCl *β*-alanine (pH 3.89); the TE was 0.01 *M* caproic acid. After 5-min incubation, as well as unmetabolized ApApA, it was possible to detect inorganic phosphate, AMP and ApA by comparison with the respective pure substrates. (From ref. 24.)

ties could be detected, often in contrast to the manufacturer's data (*e.g.*, ADP contaminated with AMP and ATP) [100], UDP (contaminated with UMP [101], 2′,5′-oligoadenylate cores (A2′p5′A2′, A2′p5′A2′p5′A, A2′p5′A2′p5′A2′p5′A) [24,25] and Ap4A, Fig. 7). Capillary analytical ITP, equipped with an integrated radio detector, has been used for quality control of ^{14}C-labelled nucleotides: contaminations with CDP, CMP and unknown compounds could be detected [34].

Another application for analytical capillary ITP was the control of the synthesis of nucleotide analogous compounds, among them N^6-(6-aminohexyl)carbamoylmethyl-ATP and N^6-(6-aminohexyl)carbamoylmethyl-NAD [102]. The purity of other synthesized NAD^- derivatives (8-bromo-NAD^-, N^1-(2-aminoethyl)-NAD^+ and N^1-(3-chloro-2 hydroxy-propyl)NAD^+ was investigated by Buret and Cox [103].

Single nucleotides are well suited to analysis by analytical ITP, but oligonucleotides are not. The differences in net mobilities of oligonucleotides with similar numbers of bases are often too small for them to be separated. For example, as shown in Fig. 6, separation of the dimeric, trimeric and tetrameric forms of 2′,5′-oligoadenylate cores was possible, but not of the corresponding 5′-triphosphates. Oligoadenylates with even small differences in their number of bases can be successfully analysed by a related method, capillary zone electrophoresis [27]. However, preparative carrier ITP has been used for the complete recovery of DNA fragments (ranging from oligonucleotides up to 20 000 base pair fragments) from agarose and polyacrylamide gels [104].

6. CONCLUSION

Nucleotides, nucleosides and purine and pyrimidine bases and their analogues are among the most suitable substrates for analysis by capillary ITP. The strong UV absorption of purines and pyrimidines at 254 nm allows convenient, sensitive and reproducible analyses using only small amounts of samples. Comparisons between analytical capillary ITP and many other established methods (HPLC, fluorimetric and photometric assays, microbial analysis or ^{31}P NMR spectroscopy) consistently confirmed the high quality of NAC determinations by ITP. In many cases, ITP analysis proved to be superior, because the substance of interest and a number of related metabolites could be estimated simultaneously. The method is flexible, because LE/TE systems can easily be adapted to a special separation problem. Furthermore, the preparation of biological samples prior to analysis is often more convenient in ITP than in comparable methods. By using ITP systems with column-coupling, the analysis of sample constituents even in unfavourable extraction systems such as PCA is further facilitated.

Despite these advantageous features, analytical capillary ITP is not a widely used analytical technique. For routine analysis in medical laboratories, many other methods are available which are similarily sensitive and accurate, but easier and faster to perform. However, for some special problems in medical diagnosis, the high specificity and sentivity of ITP may be of benefit. In particular, the analysis of purine and pyrimidine metabolites in patients with inborn errors, in diseases in which cellular energy supply cannot be maintained [105], or in the determination of NAC-related antimetabolites in cancer diagnosis and therapy, may well be susceptible to this technique.

For research problems that deal with nucleotide metabolism, ITP is a powerful method. Furthermore, for some problems in the expanding field of gene technology (*e.g.* purity-control studies), capillary analytical ITP may also be helpful. However, in oligonucleotide research and in many other related areas, capillary zone electrophoresis is increasingly the method of choice.

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