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NEW METHOD FOR THE QUANTITATIVE ASSAY OF THE ENZYMATIC HYDROLYSIS OF UDPGLUCURONIC ACID USING ANALYTICAL CAPILLARY ISOTACHOPHORESIS

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SUMMARY

The microsomal fraction of mammalian liver homogenate contains a pyrophosphatase activity which catalyses the hydrolysis of UDPglucuronic acid to UMP and glucuronic acid 1-phosphate. A further phosphatase activity cleaves these products to uridine and phosphate, and glucuronic acid and phosphate, respectively. A new method for the assay of these reactions is described using analytical capillary isotachophoresis. This technique permits the simultaneous quantitative analysis of mixtures containing UDPglucuronic acid, the primary products of hydrolysis, UMP and glucuronic acid 1-phosphate, and the secondary products, phosphate and glucuronic acid. The sensitivity of the method is sufficient for the accurate determination of 0.1 nmole of these substances applied to the isotachophoresis instrument in a volume of 1–10 μl, *i.e.*, for solutions of concentration as low as 10 μmole/l. The sample-to-sample analysis time, including washing of the capillary and resetting of the instrument is of the order of 40 min. The major advantages of this method are that a complete analysis is possible without the need for radioactively labelled compounds, and the analysis time is considerably less than for a conventional chromatographic separation.

INTRODUCTION**

UDPglucuronic acid is the key substance in the so-called D-glucuronic acid pathway[1]. There are two enzyme systems of mammalian liver endoplasmic reticulum which employ UDPglucuronic acid as substrate. First, the biosynthesis of glucuronides catalysed by UDPglucuronyltransferase (E.C. 2.4.1.17) uses this nucleotide as the source of glucuronic acid for conjugation to a wide range of acceptors[2]. This process

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** Abbreviations: UDPGA = uridine 5′-diphosphate glucuronic acid; UMP = uridine 5′-monophosphate; GA-1-P = α-D-glucuronic acid 1-phosphate; GA = D-glucuronic acid; P_i = inorganic phosphate. Enzymes: nucleotide pyrophosphatase (dinucleotide nucleotidohydrolase, E.C.3.6.1.9), alkaline phosphatase (nucleotide nucleotidohydrolase, E.C.3.1.3.1.)

is vital in hepatic detoxification mechanisms. Second, UDPglucuronic acid can be hydrolysed to UMP and glucuronic acid 1-phosphate by a pyrophosphatase activity, believed to be the unspecific nucleotide pyrophosphatase (E.C. 3.6.1.9), which also cleaves UDPglucose, NAD^+, NADH, $NADP^+$, and $NADPH$[3]. Free D-glucuronic acid is important in the biosynthesis of D-glucaric acid, L-xylulose and other pentoses, and can also be converted into L-ascorbic acid in species which synthesize this vitamin. D-Glucuronic acid can be produced through either of the two above-mentioned pathways, by hydrolysis of glucuronides (catalysed by β-glucuronidase, E.C. 3.2.1.31) or by hydrolysis of glucuronic acid 1-phosphate (probably catalysed by an unspecific alkaline phosphatase, E.C. 3.1.3.1). These pathways are summarized in Fig. 1.

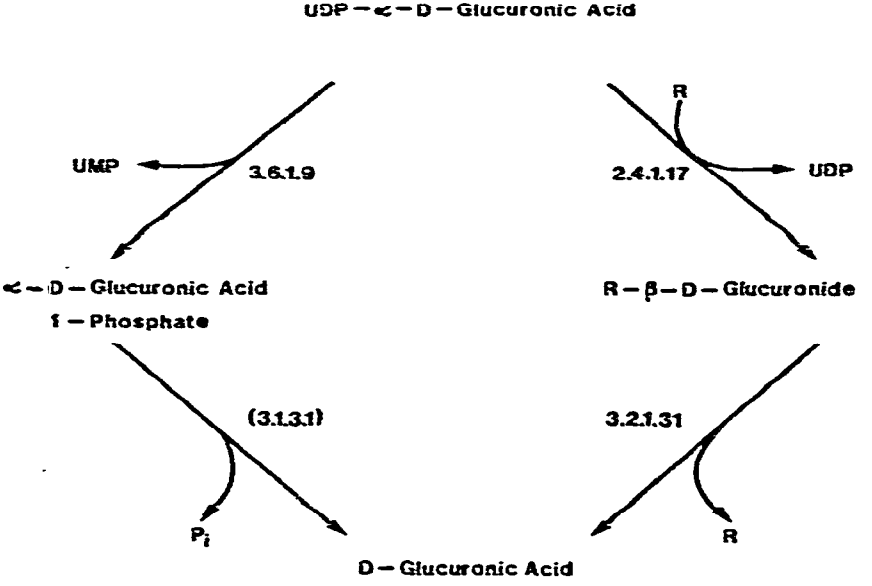

Fig. 1. Scheme showing the formation of D-glucuronic acid from UDPglucuronic acid. The first pathway is via dinucleotide nucleotidohydrolase (E.C.3.6.1.9) and phosphatase (probably E.C.3.1.3.1. activities with intermediate product D-glucuronic acid 1-phosphate. The alternative pathway is via UDPglucuronyltransferase (E.C.2.4.1.17) and β-glucuronidase (E.C.3.2.1.31) activities with a β-D-glucuronide as intermediate metabolite.

To date, two basic methods for the assay of UDPglucuronic acid hydrolysis have been reported. A complete analysis has only been possible using labelled UDP-glucuronic acid and a paper-chromatographic separation[4]. The major advantage of this method is that the metabolism of UDPglucuronic acid through both routes can be followed simultaneously. However, the analysis is somewhat laborious and time-consuming. A more rapid assay of the pyrophosphatase activity is possible by the further hydrolysis of the products, UMP and glucuronic acid 1-phosphate, with the aid of added pyrophosphatase-free alkaline phosphatase (E.C. 3.1.3.1). The total inorganic phosphate liberated is estimated by the method of Fiske and SubbaRow, and the amount of UDPglucuronic acid hydrolysed at the pyrophosphate linkage is calculated directly as half the total phosphate formed[5]. Although this assay is accurate

and easy to perform, the method is only applicable to the pyrophosphatase reaction; the rate-limiting process of glucuronic acid 1-phosphate hydrolysis in microsomal preparations cannot be followed.

The aim of the present work was to develop a method for the isotachophoretic separation of UDPglucuronic acid from the products of hydrolysis to provide a rapid and accurate technique for the analysis of the pyrophosphatase/phosphatase route to glucuronic acid. Analytical capillary isotachophoresis has already been employed for the qualitative and quantitative analysis of other nucleotides and organic anions[6,7], and for the study of some enzymatic processes[8,9]. The major advantages which could be offered by this technique are: (a) a rapid analysis, based on the separation times for the other nucleotides, (as the separation is carried out in carrier-free buffer media, very little time is wasted in cleaning and resetting the instrument for the next analysis), and (b) detection of the samples is carried out photometrically so that radioactively labelled compounds are not required. For a more detailed description of the background to isotachophoresis see refs. 10 and 11.

MATERIALS AND METHODS

The isotachophoretic analyses were carried out on the LKB 2127 Tachophor (LKB-Produkter AB, Bromma 1, Sweden), fitted with a PTFE capillary (61 cm × 0.5 mm I.D.). The capillary was maintained at a temperature of 10° during the measurements. The separated zones were detected photometrically at 254 nm.

The reference samples for the experiments, UDPGA, UMP, GA-1-P and GA were purchased from Boehringer (Mannheim, G.F.R.), and potassium dihydrogen phosphate from Merck (Darmstadt, G.F.R.), and were of the highest available commercial purity. Solutions of these substances were prepared to a concentration of 1 mmole/l in 100 mmole/l Tris–HCl buffer at pH 8 as standards for calibration of zone lengths. The leading electrolyte system was 5 mmole/l HCl containing 0.25% (w/v) HPMC (hydroxypropylmethylcellulose; Dow Chemical, Midland, Mich., U.S.A.), and corrected to pH 3.89 by the addition of β-alanine. The terminating electrolyte was 5 mmole/l caproic acid.

The separation was performed under conditions of constant current, at 100 μA for the first 20 min, and then at 50 μA. The total separation time was *ca.* 30 min, and the samples passed the UV detector at a potential of 17–20 kV. Calibration curves were derived from the zone lengths of various amounts of the standard solutions defined above. The range employed was 1–10 nmole of substance injected into the instrument in a volume no greater than 10 μl. A number of small absorbing and non-absorbing zones due to impurities in the electrolyte systems and in the samples were detected in the isotachopherograms. These were differentiated by running a separation in the absence of added sample.

Incubations of UDPGA with microsomal enzyme preparations were carried out in 100 mmole/l Tris–HCl buffer at pH 8, and a temperature of 25°. The UDPGA concentration employed was 0.5 mmole/l, and incubations were carried out in the presence and absence of $MgCl_2$ at a concentration of 1 mmole/l. The microsomal fraction of homogenates of rabbit, rat, and pig liver were prepared by differential centrifugation according to standard procedures[12]. The protein concentration in the incubations was adjusted to about 1 mg/ml, determined by the method of Lowry

et al.[15] with bovine serum albumin as reference. Immediately after initiation of the incubation, 10 μl of the mixture were removed in a microsyringe and injected into the LKB-Tachophor. The separation was carried out according to the conditions described for the calibration experiments. Further aliquots of 10 μl were removed from the incubation mixture at intervals of 30–40 min (*i.e.*, after completion of the preceding analysis) until no more UDPGA was detectable in the isotachopherogram. Control experiments were carried out to ensure that no further enzymatic hydrolysis occurred after injection of the sample into the instrument. In addition to the aliquot removed for direct application to the apparatus, a further sample was taken at the same intervals of time, and the protein was precipitated by the addition of one tenth of the sample volume of 70% perchloric acid. On completion of the direct kinetic experiment, 10-μl portions of the supernatant of the perchloric acid-treated samples were analysed. The results of the two methods were similar to within the normal experimental accuracy, demonstrating that injection of a sample into the electrolyte system in the instrument leads to effective quenching of the enzymatic reactions.

Fig. 2 depicts the principles of analysis of a UV-absorbing and non-absorbing zone. A prerequisite for the accurate quantitative determination of an absorbing zone is symmetry of the peak. The width of the zone (D_x) was measured at half its height (H_x), and was converted into units of time according to the set chart speed. The width of a non-absorbing zone (D_y) was estimated at half the height (H_y) of the lower absorbing neighbouring peaks, which can be either sample zones, or a peak due to

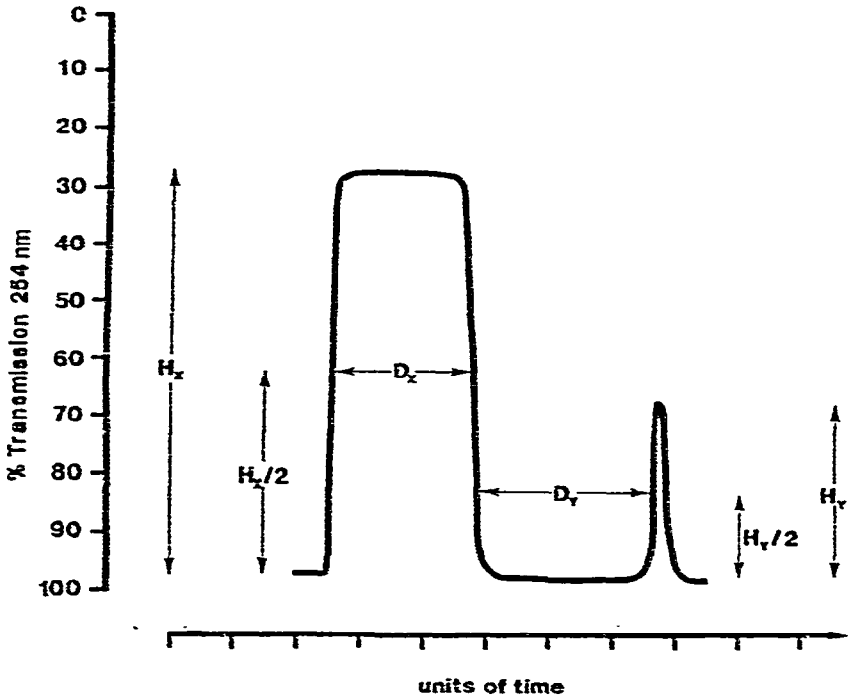

Fig. 2. The principles of quantification of isotachophoretic zones. A UV-absorbing peak is assessed by the width of the zone, D_x, at half the peak height, H_x, and a non-absorbing zone by the width, D_y, at half the height of the lower absorbing neighbouring zone, H_y.

TABLE I

RESULTS OF CALIBRATION PLOTS FOR THE ANIONIC SPECIES AT pH 3.89

The zone widths were measured in centimetres of chart paper, and converted into units of time from the chart speed.

Substance injected	*Slope of plot (sec/nmole)*
Uridine 5′-diphosphate glucuronic acid	1.12
Glucuronic acid 1-phosphate	0.44
Inorganic phosphate	0.58
Glucuronic acid	0.60
Uridine 5′-monophosphate	0.65

an impurity. Calibration curves for the UV-absorbing species UDPGA and UMP, and the non-absorbing species, GA-1-P, GA, and P_i, were constructed as zone widths in seconds with respect to the amount of injected sample in nanomoles. These plots were all linear within the range studied, and passed through the origin of the axes.

RESULTS

The order of migration of the anions involved in this study from high to low mobility was UDPGA, GA-1-P, P_i, GA, and UMP, as derived from isotachophero-

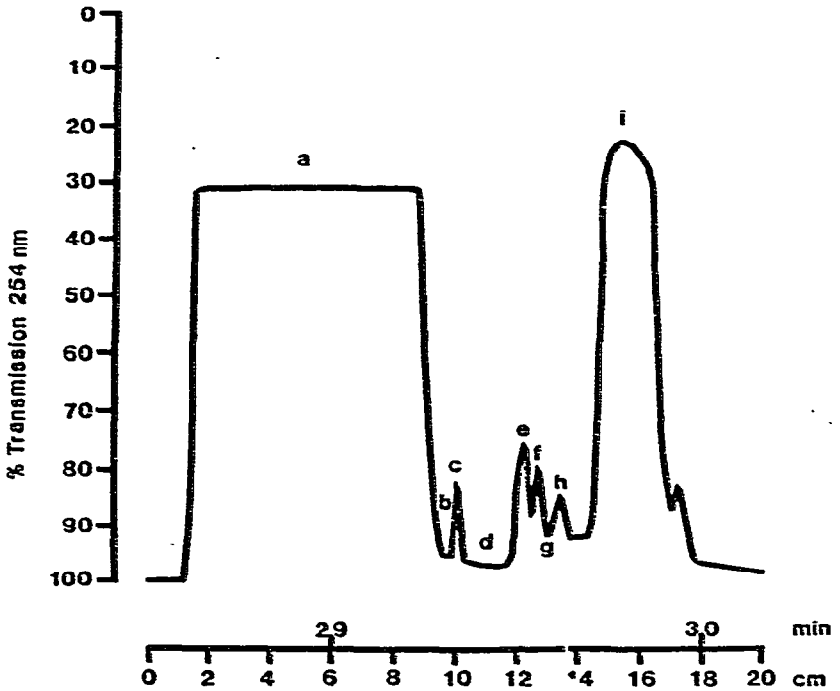

Fig. 3. Isotachopherogram demonstrating the early stage of hydrolysis of UDPGA. Peaks a and i are due to UDPGA and UMP, respectively. The non-absorbing zones, b, d, and g due to GA-1-P, P_i, and GA are minimal at this stage. The remaining zones are due to impurities in the buffer systems. The chart speed employed was 6 cm/min, and the scale is shown both in time and paper-length units.

grams of the reference substances. The calibration curves were linear for the range 1–10 nmole of substance injected, and the slopes of the calibration plots expressed in seconds per nmole resulting from these plots are given in Table I.

Figs. 3 and 4 are examples of isotachopherograms at an early and later stage of hydrolysis, respectively. The source of microsomes in this case was rabbit liver, and the incubation was carried out in the absence of magnesium ions, which gave slower kinetics than in the presence of magnesium. The small peaks denoted by c, e, f, and h were due to impurities, and were also present in a blank run. These impurities were thus present in the electrolyte systems. UDPGA is the UV-absorbing peak, a, with the highest mobility of the anions present. The non-absorbing zones, b, d, and g, are due to GA-1-P, P_i, and GA, respectively. These zones are definable through the small absorbing impurity peaks, which are often used as markers in isotachophoretic experiments. UMP is the UV-absorbing zone, i, with the lowest mobility of the anions present. The two isotachopherograms demonstrate that although the width of the UDPGA zone is reduced by more than half in the period between Fig. 3 and Fig. 4, the UMP zone is only slightly wider. The more dramatic increases are observed in the non-absorbing zones, particularly P_i, which is explained by the further hydrolysis of UMP. The final product of phosphatase cleavage, uridine, is not detectable under these conditions. The width of the UDPGA, GA-1-P, P_i, GA, and UMP zones were

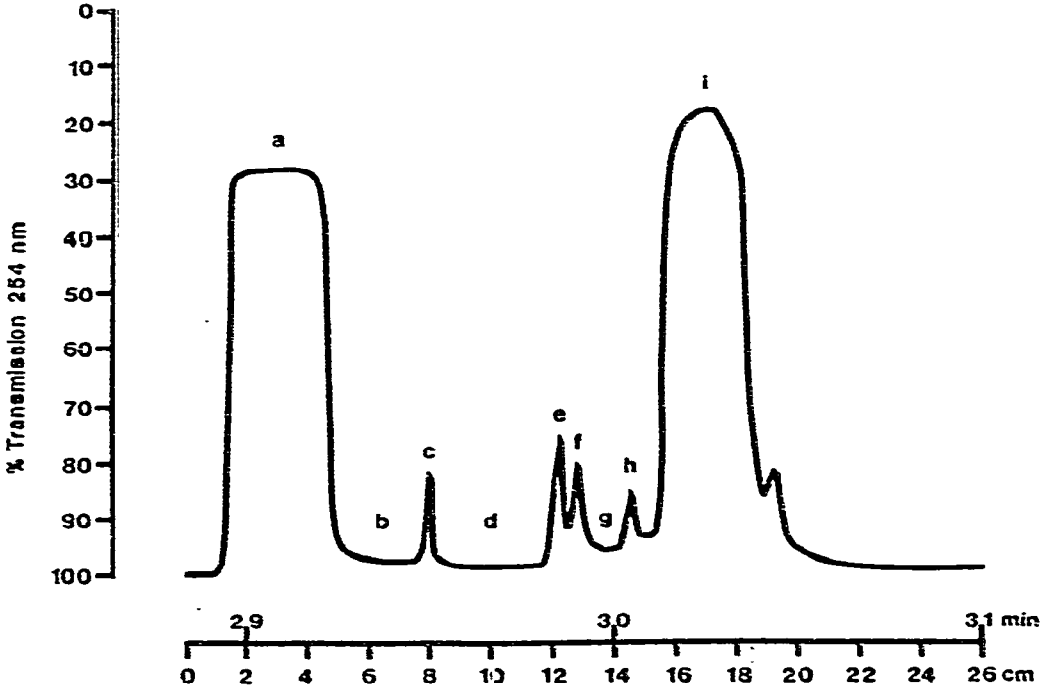

Fig. 4. Isotachopherogram of the same incubation mixtures as in Fig. 3, but at a later stage of the reaction. The UDPGA peak is reduced in width from the hydrolysis. The UMP zone, however, is only minimally wider than at the early stage of the reaction, demonstrating that this product of UDPGA hydrolysis is itself further subject to hydrolysis. The three non-absorbing zones, and particularly P_i (zone d) are seen to have increased considerably.

determined according to the aforementioned method, and were converted into concentrations from the calibration curves. A kinetic profile of the concentration changes is shown in Fig. 5. The conditions were the same as those for the isotachopherograms in Figs. 3 and 4. UDPGA was completely hydrolysed within *ca.* 200 min.

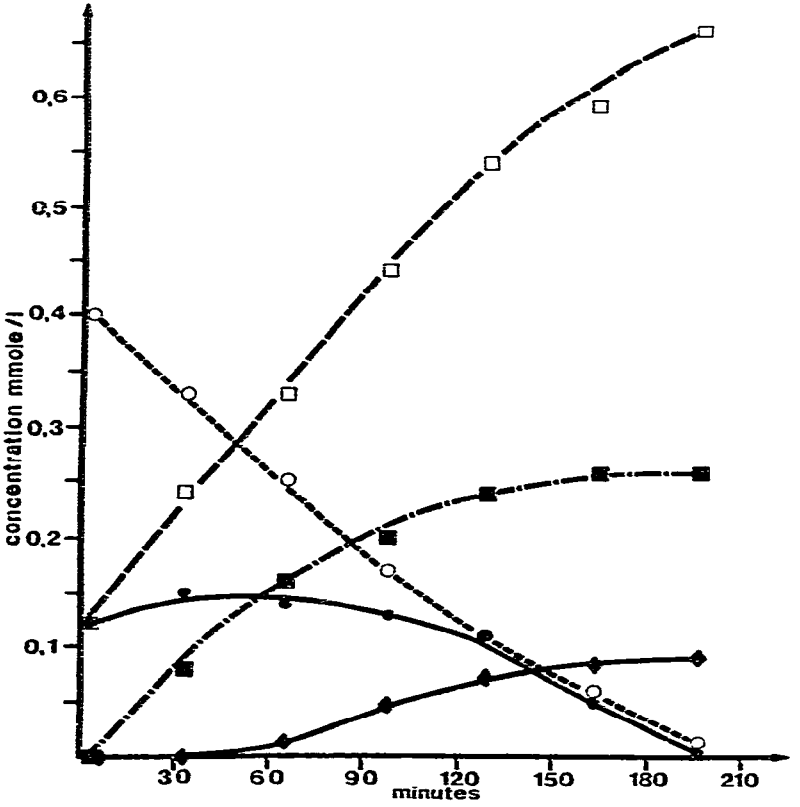

Fig. 5. Kinetic profile derived from the isotachophoretic analysis of UDPGA hydrolysis. The substrate UDPGA (○) is hydrolysed within *ca.* 200 min under our experimental conditions. UMP concentrations (●) are more or less constant during the first 40% of the reaction time, demonstrating similar rates of formation from UDPGA and further hydrolysis. The other primary product of UDPGA hydrolysis, GA-1-P (■), displays increasing concentration for the first 2 h, and levels off when 80% of the UDPGA has been hydrolysed. The final hydrolysis product, P_i (□) increases rapidly in concentration, linearly for the first 2 h of reaction. Glucuronic acid (◆) is formed only when a considerable amount of GA-1-P has accumulated in the reaction mixture.

Inorganic phosphate is produced through hydrolysis both of GA-1-P and of UMP. It is interesting to resolve the phosphate components P_i^g and P_i^u (derived from the cleavage of GA-1-P and UMP, respectively) from the total phosphate $\overline{P}_i$ observed in the isotachopherogram. A further component, P_i^o must be defined for the small amount of inorganic phosphate present as impurity in the system. This amount was estimated from the measurement at initiation of the incubation, which in the case of the kinetic profile shown in Fig. 5 was 0.12 mmole/l.

Thus,

$$\bar{P}_i = P_i^g + P_i^u + P_i^0$$

The inorganic phosphate component of GA-1-P hydrolysis, P_i^g, is identical with the concentration of GA, the other product of hydrolysis of GA-1-P.
Thus,

$$P_i^u = \bar{P}_i - P_i^0 - GA$$

By the same argument, P_i^u is equal to the uridine concentration. The UDPGA concentration at a particular stage of the hydrolysis, $UDPGA_t$ can be estimated in three ways:

(1) Directly from the width of the UDPGA peak.

(2) From the sum of the GA-1-P and GA concentrations deducted from the initial UDPGA concentration, $UDPGA_0$.

(3) From the sum of the UMP and P concentrations deducted from $UDPGA_0$.

Table II summarizes the values of $UDPGA_t$, GA-1-P, GA, $\bar{P}_i$, and UMP concentrations measured directly from the zone widths, and the concentrations P_i^g and P_i^u calculated indirectly as described above. The three calculations of $UDPGA_t$ are listed in Table III. The values derived from the GA-1-P and GA concentrations are in good agreement with the direct measurements of $UDPGA_t$ at the later stages of hydrolysis. At the early stages of the reaction, the amounts of GA-1-P and GA generated are too small to be estimated accurately from the zones. Thus, the amount deducted from $UDPGA_0$ is underestimated, and the resulting value of $UDPGA_t$ is too high. As the inorganic phosphate component of UMP hydrolysis, P_i^u, is derived from a deduction of the GA concentration from total phosphate, one would thus expect the values of P to be overestimated during the early stage of hydrolysis. Thus, the values of $UDPGA_t$ derived from UMP and P_i^u should be lower than the direct values. This is indeed found to be the case. The errors can be compensated by calculating a mean value for the $UDPGA_t$ values derived by the two indirect methods. These results are found to be in good agreement with the direct determination of $UDPGA_t$, as shown in Table III.

TABLE III

THE CONCENTRATIONS OF UDPGA, GA-1-P, GA, UMP, AND TOTAL PHOSPHATE P_i, DETERMINED DIRECTLY FROM THE ZONE WIDTHS AT VARIOUS TIME INTERVALS

The phosphate component from GA-1-P hydrolysis, P_i^g, was taken to be equal to the GA concentration, and the component from UMP hydrolysis, P_i^u, was calculated from $\bar{P}_i - P^0 - GA$ as explained in the text. For this experiment, $UDPGA_0$ was 0.5 mmole/l and the initial phosphate concentration, present as impurity, P^0, was 0.12 mmole/l. The conditions of this experiment are defined in the text (see also Fig. 5).

Time (min)	*$UDPGA_t$ (mmole/l)*	*GA-1-P (mmole/l)*	*$GA = P_i^g$ (mmole/l)*	*UMP (mmole/l)*	*$\bar{P}_i$ (mmole/l)*	*P_i^u (mmole/l)*
33	0.33	0.08	0.00	0.15	0.24	0.12
65	0.25	0.16	0.03	0.14	0.33	0.18
97	0.17	0.20	0.10	0.13	0.44	0.22
129	0.11	0.24	0.15	0.12	0.54	0.27
163	0.06	0.26	0.17	0.05	0.59	0.30
196	0.03	0.26	0.18	0.00	0.66	0.36

TABLE III

THE UDPGA CONCENTRATIONS DURING THE COURSE OF HYDROLYSIS, ESTIMATED ACCORDING TO THREE METHODS FROM THE DATA OF TABLE II

Method A is from the direct analysis of the UDPGA zone. Method B is derived from the sum of the GA-1-P and GA concentrations deducted from the initial UDPGA concentration, $UDPGA_0$, and method C from the sum of the UMP and P_i^a concentrations deducted from $UDPGA_0$, as explained in the text.

Time (min)	*$UDPGA_t$, method A (mmole/l)*	*$UDPGA_t$, method B (mmole/l)*	*$UDPGA_t$, method C (mmole/l)*	*$UDPGA_t$, D (mean of B and C) (mmole/l)*
33	0.33	0.42	0.23	0.33
65	0.25	0.31	0.18	0.25
97	0.19	0.20	0.15	0.17
129	0.11	0.11	0.11	0.11
163	0.06	0.07	0.15	0.11
196	0.03	0.06	0.14	0.10

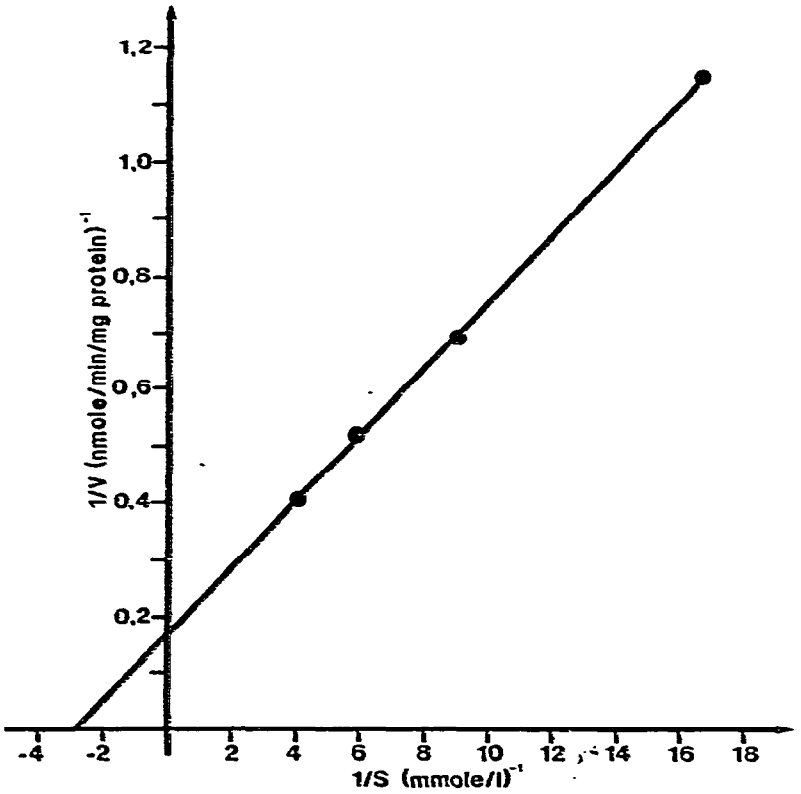

Fig. 6. Lineweaver–Burk plot showing the dependence of rate of hydrolysis of the pyrophosphate bond as a function of the initial UDPGA concentration. The incubations were carried out at 25° and in the absence of magnesium ions. The K_m from the plot is 0.36 mmole/l.

Fig. 6 is a Lineweaver–Burk plot for the rabbit liver microsomal enzyme with varying initial concentrations of UDPGA. The incubations were carried out at 25°, and in the absence of added magnesium. The K_m from the plot was 0.36 mmole/l, which is of the same order as that found by Puhakainen *et al.*[5], (0.21 mmole/l), albeit with rat liver microsomes, and in the presence of magnesium ions. The initial rates of UDPGA hydrolysis were derived from the direct calculation of UDPGA concentrations from the UV-absorbing zone, and the method was thus shown to be accurate enough to permit the use of substrates in a concentration range which is of biochemical relevance. Liver microsomes from all three mammalian sources were found to have both pyrophosphatase activity towards UDPGA, and phosphatase activity towards UMP and GA-1-P. The hydrolysis, particularly of the primary products UMP and GA-1-P was found to be accelerated in the presence of magnesium ions. These results are in agreement with previous reports in the literature[3–5].

DISCUSSION

These results demonstrate that analytical capillary isotachophoresis is a very sensitive method for the separation and quantitative determination of mixtures of nucleotides, nucleotide analogues, and anionic derivatives, and can be employed for the study of enzymatic reactions involving such substances. The hydrolysis of UDPGA catalysed by a liver microsomal pyrophosphatase activity can be monitored by this technique, and the fate of the primary products UMP and GA-1-P which are further cleaved by a phosphatase activity can be followed simultaneously. The basic principle of the isotachophoretic separation of nucleotides with high resolution has already been reported in the literature[6,7], but very little work has previously been invested in the use of this method for the assay of enzymatic processes involving nucleotides.

As far as the hydrolysis of UDPGA is concerned, analytical capillary isotachophoresis offers considerable advantages over more conventional methods. Thin-layer or paper-chromatographic separation techniques are useful for the complete analysis of reaction substrates and products, but are generally sensitive enough for an accurate quantitative assay only if radioactively labelled substances are employed. Moreover, such methods can be tedious and time-consuming. Simpler methods are available for the analysis of one of the reactants or products, but such measurements can be subject to considerable errors due to side reactions which are not detected by the direct analytical technique. An example of this problem is the competition between microsomal nucleotide pyrophosphatase and hydroxylation or conjugation processes[14], all of which act on nucleotides with pyrophosphate linkages. The pyrophosphatase hydrolyses not only UDPGA as described in this work, but also apparently dinucleotides such as NADP, NADPH, NAD, and NADH, which are also involved in glucuronidation and hydroxylation reactions, respectively. Thus, it has been suggested that hydrolysis of the nucleotides should always be investigated parallel to studies of drug metabolism reactions[14], in order to ensure correct saturating conditions with respect to substrate. The correction would be of even more importance in end-point methods for the analysis of the equilibrium state. The method described here would be applicable to such studies.

Under the conditions described for the isotachophoretic separations, most of the more common nucleotides, and many of the products of metabolism can be

analysed simultaneously. Thus, all but one of the products in the complete phosphatase hydrolysis of UDPGA, namely uridine, can be assayed. The pH employed, 3.89, was found to be most suitable in this case. As the net mobility of an ionic species is defined as the product of the ionic mobility (a constant), and the degree of dissociation of the species, it is clear that the net mobility will be pH-dependent. In the application of this technique to the study of other nucleotide mixture, therefore, it would be necessary to investigate the pH at which the net mobilities of the species in a given mixture differ as widely as possible. The highest isotachophoretic resolution is obtained under such conditions.

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