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KEYWORDS: isotachophoresis, 2, 3-diphosphoglycerate, nucleotides, preparation for isotachophoresis, ion mobility

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MEASUREMENT OF ADENOSINE TRIPHOSPHATE AND SOME OTHER METABOLITES IN BLOOD CELLS BY ISOTACHOPHORESIS. I. PREPARATIVE TECHNIQUE AND ENZYMATIC CONFIRMATION

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Abstract. This study investigated the optimal conditions for detection of nucleotides in blood using an IP-1B capillary isotachophoretic apparatus. The system used 10 mM HCl-β-alanine (pH 4.2) as the leading electrolyte and *n*-caproic acid as the terminal electrolyte. Direct application of lysed red blood cells was shown to be inaccurate, and a method of deproteinization based on heat in a microwave oven was developed. The zones for 2,3-diphosphoglycerate, ATP, inorganic phosphate, and lactate were identified enzymatically by withdrawal of pure samples of each zone via a special withdrawal cell. The quantitative values obtained by isotachophoresis were also confirmed enzymatically. The technique is now available for convenient and accurate identification of these metabolites simultaneously.

Key words : isotachophoresis, 2,3-diphosphoglycerate, nucleotides, preparation for isotachophoresis, ion mobility.

Analytical isotachophoresis in capillary tubes is capable of identifying and quantitating a number of ions at the same time, and has already been used for clinical specimens such as urine (1, 2, 3), plasma (4, 5), cerebro-spinal fluid (6, 7), and to monitor serum drug concentrations (8, 9). The convenience of the technique, its accuracy, and the fact that little or no pre-treatment of the specimen is needed have all been commended (10, 11).

Nucleotides have been studied at some length, but the samples studied have usually been made from pure reagent solutions (12-16), or, clinically, from such tissue as muscle (17, 18). Apart from one forensic study of bloodstains (19), blood samples involving red blood cells have been little investigated by isotachophoresis, and such study of the nucleotides in red blood cells has only been reported by one group (20, 21). Identification of the nucleotides involved has so far rested on the potential gradient values of individual components in a sample as compared with known standards. Although this is how the technique would be used if it comes into clinical use, the results for capillary isotachophoresis must first be confirmed by other traditional techniques such as enzyme analysis.

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The present study investigates the optimal conditions for detection of nucleotides in blood using an IP-1B capillary isotachophoresis apparatus and uses the special withdrawal described previously (22) to confirm the results enzymatically.

MATERIALS AND METHODS

Instruments. Capillary isotachophoresis was performed with a Shimadzu Isotachophoretic Analyzer Model IP-1B. The migration capillary tube was a Teflon tube 30 cm long with an internal diameter (I. D.) of 0.5 mm. The special cell used for withdrawal of individual components during isotachophoresis was a Shimadzu ipp-1 model withdrawal cell which includes a potential gradient detector (PGD). This cell and the technique of withdrawal have been described previously (22).

Isotachophoretic conditions for separation of nucleotides and organic acids. The composition of the electrolyte solutions used for isotachophoresis is shown in Table 1. The standards used in this preliminary experiment were lithium lactate, sodium succinate, adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), sodium citrate, 2,3-diphosphoglycerate (2,3-DPG), adenosine 5'-monophosphate (AMP) and inorganic phosphate (Pi). These chemicals were odtained from the Wakō Pure Chemical Industries Co., Osaka, Japan. Each chemical was made up as a 0.01 M solution, then 1 μl of each solution was used as the volume injected into the isotachophoresis apparatus by microsyringe. The net mobilities were plotted against the pH of the leading electrolyte over a pH range of 2.7 to 4.2. The pH of the leading electrolyte was adjusted by the addition of β-alanine, the final pH being monitored with a pH meter.

Characteristics of the IP-1B isotachophoresis apparatus. The characteristics of the IP-1B isotachophoresis apparatus were studied using various standard solutions. The standards studied were 2,3-DPG, ATP, lactate, and inorganic phosphate using a pH of 4.2 for the leading solution and the conditions listed in Table 1. Firstly, standard curves were constructed by using each of the reagent solutions separately. Each reagent was made up as a 0.01 M solution. Varying volumes (from 1 μl to 20 μl) of each were then injected individually and the distance between the peaks of the record was measured. Next, the process was repeated using a fixed injection volume (10 μl) and graded dilutions of each of the standard solutions. Finally, equal volumes of the 0.01 M standard solutions were mixed. This mixture of standards was used as the test sample in order to investigate the possibility of interaction between the various substances during isotachophoresis.

Pre-treatment of blood specimens. Blood was withdrawn into standard acid-citrate-dextrose (ACD) transfusion bags (30 ml ACD anticoagulant/200 ml blood) and stored at 4℃ until used. The treatment thereafter was identical for all tests: blood was withdrawn aseptically from the transfusion pack, placed into a centrifuge tube, centrifuged once at 3,000 rpm for 30 min and the supernatant (serum) discarded. The pellet of blood cells was resuspended in 0.25 M sucrose, mixed well, and the suspension recentrifuged at 3,000 rpm for 20 min. This washing procedure was repeated three times. The pellet of blood cells thus obtained was lysed by adding approximately two times its volume of distilled water and shaking constantly.

The hemoglobin of the original whole blood specimen and of this hemolysed stage was measured colorimetrically using a Hemoglobin-test (Wakō Pure Chemical Industries Co., Osaka, Japan). The hemolysate was then placed in a microwave oven to remove protein

by heating. The procedure was terminated when colour change and clumping occurred (3-7 seconds). The sample was then centrifuged at $15,000 \times g$ for 20 min, and the clear straw-coloured supernatant thus obtained used as the sample for analysis by isotachophoresis.

Isotachophoresis of the blood sample. Firstly, under the conditions shown in Table 1, the hemolysate and the straw-coloured deproteinized specimen were subjected to isotachophoretic analysis. The volume injected in each case was 10 μl. All later experiments used the deproteinized sample. A mixed standard was always analyzed first to tentatively identify the components. The mixed standard was made up of equal volumes of each standard (0.01 M) solution. This was then taken in volumes that resulted in 2 μl of each standard being present in the sample injected for isotachophoresis; that is, if four standards had been mixed, 8 μl was taken as the injection volume. The deproteinized blood sample (20 μl) was then injected and the result recorded.

Identification and quantitation of the isotachophoretic pattern. The potential gradient (PG) of each component (for example, in Fig. 1 on the right, the plateaus 2,3,4,5,6,7,8) and of the leading (plateau 1) and terminal (plateau 9) electrolytes was measured. The ratio of the PG of a component to the PG of the terminal electrolyte was calculated, taking the PG of the leading electrolyte as zero (for plateau 2, this would be: $(PG_2 - PG_1)/(PG_9 - PG_1)$. This ratio is called the potential unit (PU) value (23) and is used to identify the component.

The distance between the peaks for each zone (for example, in Fig. 1 on the right, the

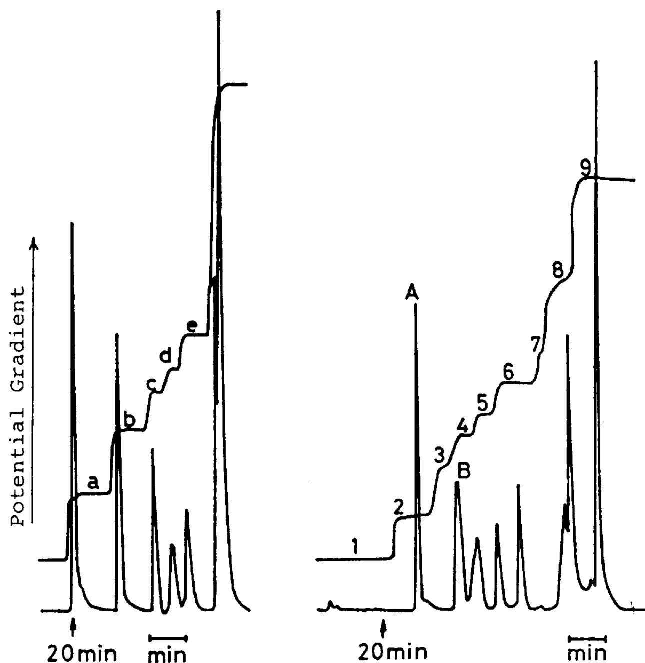

Fig. 1. Typical pattern for isotachophoretic analysis of nucleotides in red blood cells (right). Blood taken into ACD anticoagulant and measured on the same day. Hemoglobin of lysed red blood cells: 12.8 g/dl, Injection volume: 20 μl. Plateau 1: leading electrolyte, Plateau 9: terminal electrolyte. Left: Mixed standards (0.02 μmole each). a: 2,3-DPG, b: ATP, c: inorganic phosphate, d: lactate, e: succinate.

distance between A and B for zone 2, D_2) was measured. The distance for the standard (Fig. 1, left) that had the same PU value was measured similarly (D_S). Each standard contained 0.02 μmole. When the volume of test sample injected was 20 μl, the concentration was calculated as: $(D_2/D_S) \times (0.02/20)$ μmole of component / μl blood. This was then expressed as concentration of component per g hemoglobin.

Confirmation of the identity of isotachophoretic peaks. The zones in the analyzed blood sample that had the same PU values as the four standards were selected for further study using the special Shimadzu ipp-1 withdrawal cell. Each zone was sampled via this withdrawal cell using the technique described previously (22). The specimen withdrawn was resubmitted to isotachophoretic analysis to ensure that it had the same PU value as the zone under investigation and to confirm that it did not contain adjoining zones. After the timing of withdrawal was determined in this manner ensuring that a single zone was sampled, the withdrawal specimen was then analyzed by routine enzyme test kits to test for the presence of 2,3-DPG, ATP, lactate (Boehringer-Mannheim Co., Tokyo, Japan) and inorganic phosphate (P-test, Wakō Pure Chemical Industries Co., Osaka, Japan).

For quantitation, withdrawal fractions from each zone were first reanalysed by isotachophoresis to (a) ensure that they were pure, and to (b) calculate the fraction (F %) of the parent zone (P) that each represented. Isotachophoresis of the original blood specimen was then repeated and a second withdrawal made using the same technique. This was then measured enzymatically (E). The two withdrawal specimens were considered equivalent because of the high degree of reproducibility of the results for isotachophoresis, so the enzymatic result was then recalculated to 100 % (I.e. 100E/F) for comparison with the isotachophoretic result.

RESULTS

Isotachophoretic conditions for separation of nucleotides and organic acids. Fig. 2 shows the results for the mobilities of various ions analyzed as pure solutions of each ion and plotted as the PU value against variations in pH of the leading electrolyte. There is a straight line relationship between the value and the inverse of the mobility (23). 2,3-DPG was the first component to appear and was well separated from the other components. ATP and Pi had parallel slopes that were very close in the pH range 2.7 - 3.6. From pH 3.6 to 4.2, good separation was obtained. Citrate was a straight line curve in this pH range, but crossed with ADP at pH 3.0 and with Pi at pH 4.2. The slope for ADP crossed the slopes for citrate and lactate at pH 3.0 and 4.2 respectively.

The pH that appeared to give the best resolution for all of the components in Fig. 2 was 3.75. This was confirmed by analyzing a mixed sample at pH 3.75 which gave the predicted result. With the objective of examining nucleotides from red blood cells, the best separation of phosphate compounds was desired so a pH of 4.2 was chosen. A mixed solution of nucleotides and organic acids confirmed this pH as giving good separation. The PU values for each component at pH 4.2 were: 2,3-DPG 0.11, ATP 0.22, citrate 0.28, Pi 0.29, lactate 0.37, ADP 0.39, succinate 0.46, and AMP 0.78.

Characteristics of the IP-1B isotachophoresis apparatus. Fig. 3 shows the results

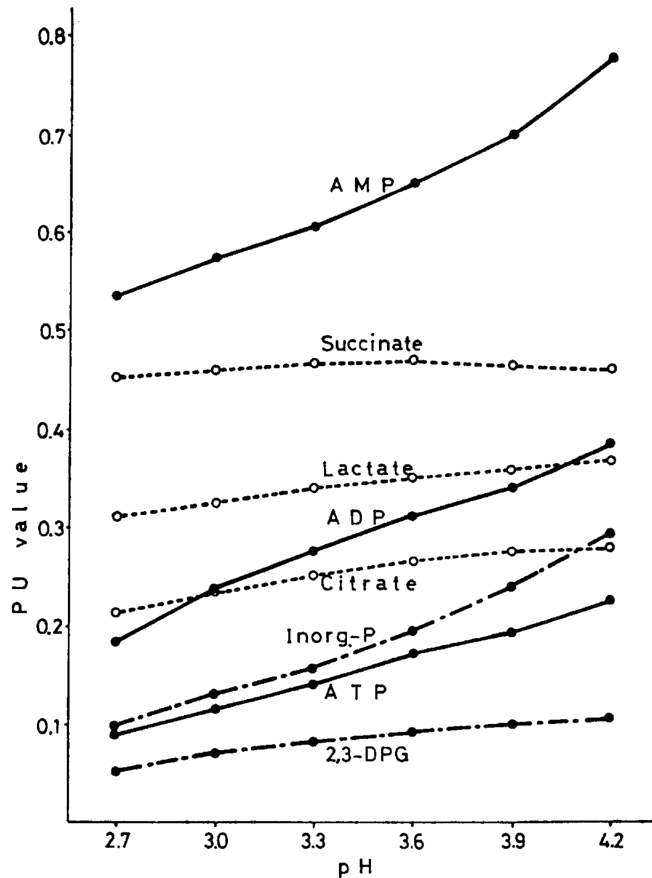

Fig. 2. Mobilities of nucleotides and organic acids over the pH range 2.7-4.2. Conditions of isotachophoresis as in Table 1. PU values calculated as explained in Materials and Methods. Each injection volume: 1 μl of 0.01 M solution.

TABLE 1. MIGRATION CONDITIONS FOR CAPILLARY ISOTACHOPHORESIS (IP-1B MODEL).

Leading electrolyte	10 mM HCl-β-alanine (pH 4.2) containing 0.5% Triton X-100.
Terminating electrolyte	10 mM *n*-caproic acid solution
Potential gradient attentuation	256 mV
Migration current	100 μA
Migration tube	30 cm long Teflon tube of 0.5 mm internal diameter
Chart speed	10 mm/min
Temperature of the potential gradient detector	20℃

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for standard curves of Pi, ATP, 2,3-DPG, and lactate under the conditions shown in Table 1. The width of the isotachophoresis plateau of each component increased in linear proportion to the nanomole quantity injected. This was true both when the reagent was injected as a pure solution and when it was injected as a mixture of standard solutions. The plateau width varied only with the quantity of component (nmole) injected, and not with the volume; that is, a 2 μl sample of 0.01 M standard gave the same plateau width as a 20 μl sample of 0.001 M standard. Injection of samples in divided doses also proved accurate; for example, injection of 10 μl + 5 μl of solution gave the same plateau width as one injection of 15 μl of the same solution. This means that components present in only low concentrations (for example, lactate in blood during the first few hours of storage) can be measured by injecting larger volumes of sample than can be delivered by the microsyringe as a divided injection sample.

The upper limit of this system for nucleotides was 3 μmole, that is a width of approximately 20 mm on the chart paper at a speed of 10 mm/min. At higher concentrations, mixed zones appeared and the accuracy of the plateau width was lost. The smallest concentrations that could be measured were of the order of 2 nmole. The slopes for each component (Fig. 3) pass through the origin if extrapolated; however, concentrations giving a plateau width of less than 1 mm (at 10 mm/min) were difficult to measure accurately. For known components, the distance between peaks could be measured to a certain degree, but the lack of a plateau rendered identification of unknown components inaccurate.

Pre-treatment of blood samples. Standard solutions tested with enzyme kits before and after heat treatment in a microwave oven showed no change in concentration, and no new peaks on isotachophoresis, indicating that the heat treatment did not cause any degradation of the substances involved. Blood samples were also measured by enzyme kits before and after heat treatment (Table 2). For the four substances studied, this was a valid technique for removing protein from the sample.

The sample after heat treatment gave clear peaks that were easy to measure on isotachophoresis (Fig. 1 right). The sample in this state could be stored at 4 ℃ for at least 3 days without significant change in the values obtained.

Isotachophoresis of the blood sample. Fig. 1 shows the isotachophoretic pattern for a blood sample of nucleotides and organic acids (right) with the pattern for a mixed standard sample (left). From the standard PU values, zones represented by plateaus 2, 3, 4 and 5 were tentatively identified as 2,3-DPG, ATP, Pi, and lactate respectively. This was further supported by the fact that addition of each standard separately caused lengthening of the relevant zone; for example, with the addition of 1 μl of 2,3-DPG, to the blood sample, the distance between the peaks (AB) for zone 2 lengthened correspondingly.

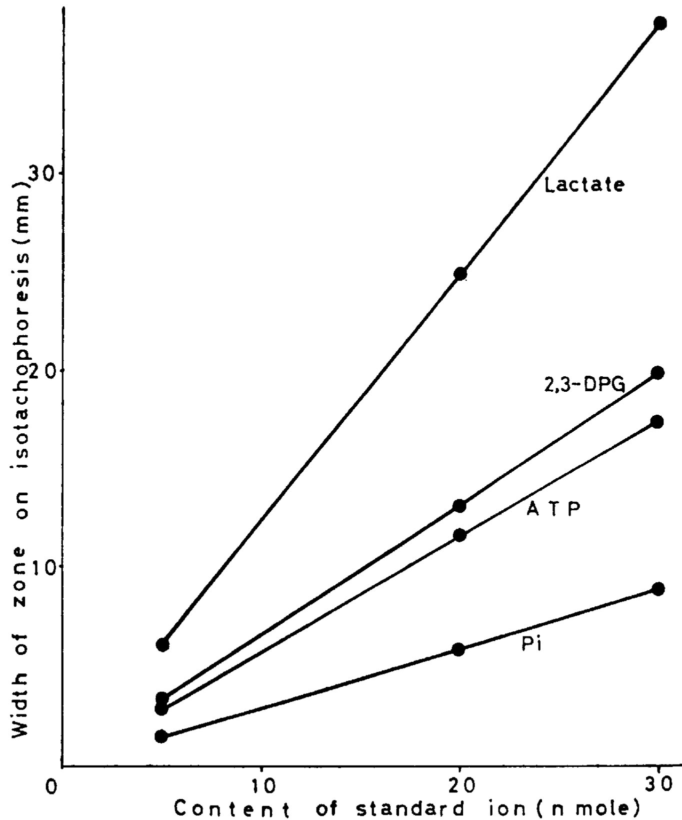

Fig. 3. Standard curves on IP-1B isotachophoretic analyzer. Equal volumes of a 0.01 M solution of each standard were mixed, and 8 μl, that is 2 μl of each standard, of this mixture was injected as the sample. Conditions of analysis as in Table 1. Pi: inorganic phosphate.

TABLE 2. EFFECT OF PRE-TREATMENT (WASHING, LYSIS, HEAT DEPROTEINIZATION) ON SAMPLE COMPONENTS.

Treatment stage	2,3-DPG	ATP	Pi	Lactate
Whole blood	14.19	2.424	*	*
After lysis	14.31	2.417	0.230	0.430
After deproteinization with perchloric acid	14.57	2.425	0.233	0.438
After deproteinization in microwave oven	14.55	2.420	0.229	0.441

Pi: inorganic phosphate. Concentrations expressed in mg of component/g hemolobin. * : not measured because component not confined to blood cells.

Each zone was then sampled via the withdrawal cell. The sample thus obtained was first reanalyzed by isotachophoresis to ensure that it was a single

zone and did not contain adjoining zones (Fig. 4), then identical samples were tested by enzyme test kits. In each case, the zone thus tentatively identified was confirmed enzymatically.

Quantitation. The results for isotachophoresis were compared with the results for the same sample as analyzed enzymatically (Table 3). Pi and lactate, both of which are present in serum as well, were measured as the intracellular content. The results showed good correlation between the two methods. The

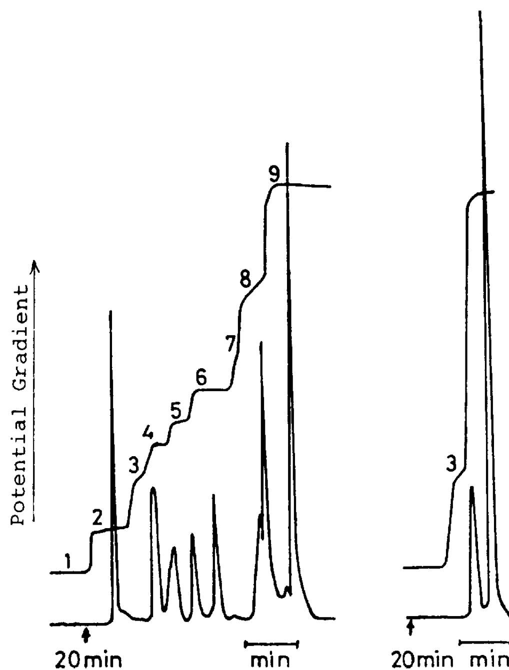

Fig. 4. Withdrawal of pure sample from zones. Left: pattern of blood taken under conditions as for Fig. 1. Right: pattern for fraction withdrawn from zone 3 (left) and re-injected for isotachophoretic analysis as explained in Materials and Methods.

TABLE 3. COMPARISON OF RESULTS OF ISOTACHOPHORESIS WITH ENZYME TEST KITS.

Component	Sample	Enzymatic	Isotach.
2,3-DPG	Whole blood	14.56	14.89
ATP	Whole blood	2.42	2.57
Pi	Hemolysate	0.27	0.28
Lactate	Hemolysate	0.43	0.44

Blood taken into ACD anticoagulant and analyzed on the same day. Units : mg of component/g hemoglobin. Pi : inorganic phosphate. Isotach. : isotachophoresis. Enzyme results are recalculated to represent the parent zone (100%) as explained in Materials and Methods.

results for isotachophoresis tended to be higher but the difference was not statistically significant.

DISCUSSION

The technique of capillary type isotachophoresis has been recommended for the qualitative and quantitative analysis of a wide range of ions. Essentially, it consists of introducing a sample into the boundary between the leading electrolyte (which has a larger mobility than the sample) and the terminal electrolyte (which has a smaller mobility), then electrophoresis is performed by applying a constant current. The sample ions are separated into zones of pure ions in the order of mobilities.

For any particular sample, however, it is necessary to investigate the conditions that give optimal results, that is, the choice of leading and terminal electrolytes, the pH at which analysis is performed, and the sample preparation needed (if any). These have already been determined for clinical specimens of urine (1-3), plasma (4, 5), and cerebro-spinal fluid (6, 7), as well as to monitor drug concentrations (8, 9). The present study investigated the conditions for analysis of the nucleotides within red blood cells and supplements the work of Sakagishi *et al.* (20, 21).

The composition of the electrolyte solutions used for this analysis of nucleotides (Table 1) was chosen by consideration of the practical results reported in the literature (12-18). Computor plots for theoretical values suggested various possibilities, but also supported this selection (24). The use of *n*-caproic acid with the addition of Triton X-100 minimized the diffusion between adjacent zones, helping to give clear-cut boundaries (14).

The pH of the leading solution was selected by studying the mobilities of pure solutions of relevant ions over a pH range of 2.7 to 4.2 (Fig. 2). In this range, compounds containing phosphate (2,3-DPG, ATP, ADP, AMP, Pi) ran approximately parallel to each other, but at a sharper gradient than the family of parallel lines for citrate, lactate, and succinate. A pH of 4.2 was chosen from these results to give the best resolution of nucleotides, and was confirmed by analysis of a mixture of the ions at pH 4.2.

Red blood cells were originally given no pre-treatment other than centrifugation to remove the serum, then lysis with distilled water following the report of Sakagishi *et al.* (21). However, the pattern of numerous peaks was difficult to identify and measure; moreover, repeated measurements gave conflicting results for the same sample indicating a lack of reproducibility even over the time taken to collect and prepare the sample. Removal of the protein was indicated but the use of perchlorate was not possible because the high chloride content interfered with the isotachophoresis. Rapid denaturation of the protein by heat (in a microwave oven) proved satisfactory. Chloride in the serum also interfered with the results. This was removed by careful repeated (three times) wash-

ing of the red blood cells.

The technique of isotachophoresis has been investigated theoretically (23) and experimentally using pure reagent solutions (12-16), both of which show that the zones have widths proportional to the quantities of the ions, and that identification can be achieved by measuring the porential gradient. However, in mixed samples (especially clinical specimens) the results have not been confirmed by other traditional methods such as enzyme analysis. The present report succeeded in sampling pure zones via the special withdrawal cell described previously (22, 25). This gave positive identification of the four substances studied (2, 3-DPG, ATP, inorganic phosphate, lactate) and confirmed the accuracy of identifying individual components by their PU values. This technique proved much simpler than the collection of zones on a moving cellulose acetate strip (10, 11).

Quantitatively, the samples taken via the withdrawal cell and measured enzymatically correlated well with calculations based on the distance between peaks (Table 3). The withdrawal samples were shown to be pure zones on reanalysis by isotachophoresis (Fig. 4); moreover, addition of known amounts of a given reagent gave proportional increases in the distance between peaks that, by extrapolation, suggested strongly that the zone was comprised entirely of that particular substance. Changing the pH of the leading solution would help separate minor amounts of any other substance that may be present at a given potential gradient (for example, inorganic phosphate and ADP may be separated at a pH of 3.2). Under the present conditions, however, calculations based on the distance between peaks (that is, the assumption that the zones contained pure components) gave satisfactory quantitative results, although electrical attachments to measure the zone distance more accurately would improve the results for low concentrations (distance between peaks less than 1 mm). The results showed excellent reporducibility (variation less than 3%) and now make it possible to measure accurately and conveniently the identified substances simultaneously.

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