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preservative.

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# Measurement of adenosine triphosphate and some other metabolites in blood cells by isotachopheresis. II. Application to monitoring changes during storage in acid-citrate-dextrose preservative.\*

Alan Talbot

## Abstract

The applicability and accuracy of isotachopheresis in measuring the concentrations of some of the intermediates of red cell metabolism during blood bank storage in acid-citrate-dextrose preservative was assessed. Preparative treatment consisted of washing with isotonic sucrose solution and centrifugation to remove serum, but white cells and platelets were not separated out from red blood cells. The results for 2,3-diphosphoglycerate, ATP, inorganic phosphate and lactate were also confirmed enzymatically. The technique had good reproducibility (variation less than 3%) and was extremely convenient compared to traditional enzyme assays of the same components. It enabled simultaneous measurement of components with concentrations greater than 1  $\mu\text{mole/red}$  blood cell.

**KEYWORDS:** ATP, organic acids, red blood cells, isotachopheresis, ACD storage

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**MEASUREMENT OF ADENOSINE TRIPHOSPHATE AND SOME OTHER METABOLITES IN BLOOD CELLS BY ISOTACHOPHORESIS. II. APPLICATION TO MONITORING CHANGES DURING STORAGE IN ACID-CITRATE-DEXTROSE PRESERVATIVE.**

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*Abstract.* The applicability and accuracy of isotachophoresis in measuring the concentrations of some of the intermediates of red cell metabolism during blood bank storage in acid-citrate-dextrose preservative was assessed. Preparative treatment consisted of washing with isotonic sucrose solution and centrifugation to remove serum, but white cells and platelets were not separated out from red blood cells. The results for 2,3-diphosphoglycerate, ATP, inorganic phosphate and lactate were also confirmed enzymatically. The technique had good reproducibility (variation less than 3%) and was extremely convenient compared to traditional enzyme assays of the same components. It enabled simultaneous measurement of components with concentrations greater than 1  $\mu$ mole / red blood cell.

*Key words :* ATP, organic acids, red blood cells, isotachophoresis, ACD storage.

Erythrocytes contain a number of organic phosphate compounds, most of which are metabolic intermediates of the glucose metabolism of the cell. The red cell glycolytic rate has been shown to be interdependent on the concentrations of 2,3-diphosphoglycerate (2,3-DPG) and adenosine triphosphate (ATP), red cell survival (1), and the delivery of oxygen (2,3). The organic phosphate compounds are usually maintained at constant levels (4), but gradually disappear to be replaced by inorganic phosphate (Pi) when blood is stored in acid citrate dextrose (ACD) preservative (5).

The concentrations of individual phosphate compounds have been studied during the storage of human (6, 7) and rabbit (8, 9) blood in ACD. The methods have been enzymatic (4), a combination of a recording spectrophotometer and enzymatic (10), or chromatographic on ion-exchange resins (4, 11). In the present study, the applicability and accuracy of isotachophoresis in measuring the concentrations of some of the intermediates of red cell metabolism during blood bank storage in ACD preservative are assessed.

MATERIALS AND METHODS

Blood was drawn by venipuncture from 15 healthy male volunteer donors (age range :

25-37). Approximately 100 ml was collected into each plastic bag containing 15 ml of ACD anticoagulant. After thorough mixing, samples were obtained, using an aseptic technique, immediately following collection (Day 0) and on days 1, 3, 5, 7, 9, 12, 21, and 30.

Blood collected into heparin (1 mg heparin/100 ml blood) from patients for other clinical tests was also analyzed as for the Day 0 ACD anticoagulated blood.

Each blood sample was prepared for isotachopheresis as described previously (12). The technique is summarized in Fig. 1. The conditions of isotachopheresis are shown in

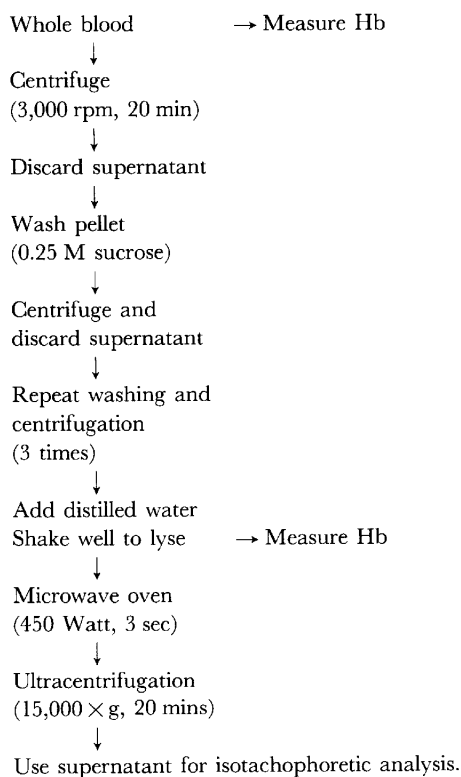


Fig. 1. Preparation of blood specimens for isotachopheresis.

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

Leading electrolyte	10 mM HCl- $\beta$ -alanine (pH 4.2) containing 0.5% Triton X-100.
Terminating electrolyte	10 mM n-caproic acid solution
Potential gradient attenuation	256 mV
Migration current	100 $\mu$ A
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 $^{\circ}$ C

Table 1. The sample volume used for isotachopheresis was usually  $10\ \mu\text{l}$  injected by microsyringe, but this was increased to  $20\ \mu\text{l}$  at times to give better definition of the zones of components present at only low concentrations. The four zones previously identified (12) as 2,3-DPG, ATP, Pi, and lactate were measured for each sample.

The blood sample after preparation for isotachopheresis was also measured enzymatically for 2,3-DPG, ATP, Pi, and lactate on days 0, 1, 5, 9, 12, 21, and 30. Standard enzyme kits (Boehringer-Mannheim Co., Tokyo, Japan) were used to measure 2,3-DPG, ATP, and lactate. Inorganic phosphate was measured colorimetrically with a P-test kit (Wakō Pure Chemical Industries Co., Osaka, Japan).

The hemoglobin concentration of each sample was measured colorimetrically using a Hemoglobin-Test kit (Wakō Pure Chemical Industries Co., Osaka, Japan).

### RESULTS

Fig. 2 shows the characteristic isotachopheretic patterns for blood on Days 0 (left) and 15 (right). The accuracy and reproducibility of the IP-1B Isotachopheretic Apparatus have already been verified (12, 13); but, in the present ex-

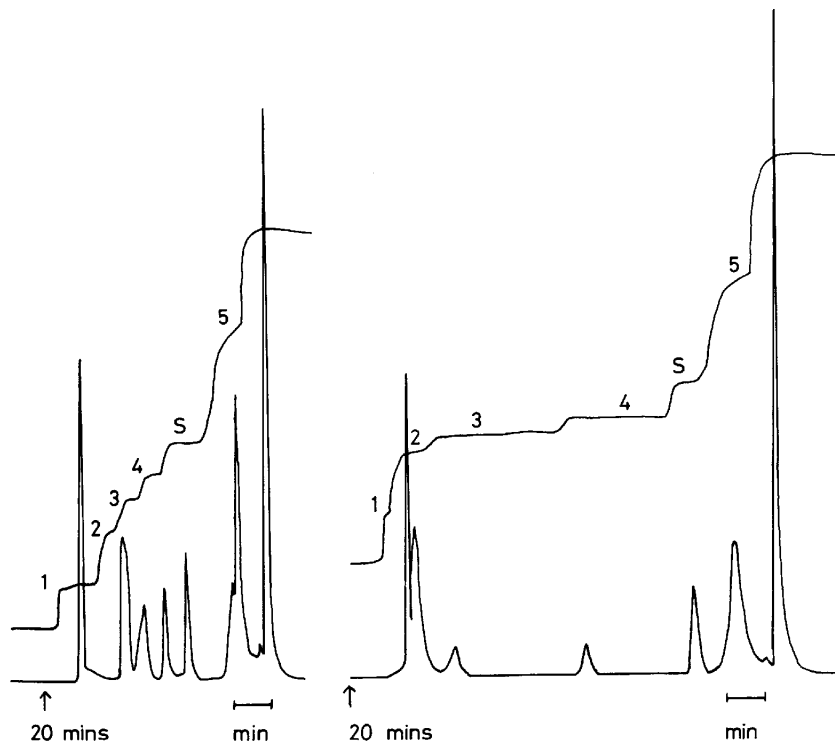


Fig. 2. Isotachopheretic patterns for normal blood prepared as in Fig. 1 and analyzed under the conditions in Table 1. Left: Day 0, Hb 9.7. Injection volume  $20\ \mu\text{l}$ . Right: Day 15, Hb 13.9, injection volume  $20\ \mu\text{l}$ . Both specimens stored in ACD preservative. A marker of  $2\ \mu\text{l}$  of succinate (zone S) has been added to both specimens.

periment, a known marker (2  $\mu$ l of 0.01 M succinate) was added to each sample (the zone "S" in Fig. 2, left and right) to monitor the reproducibility. The results for this zone under the conditions in Table 1 were a PU value (14) of 0.47 and a zone distance of 11 mm at a chart speed of 10 mm/min. The variations in this reading were very small ( $< 3\%$ ) and were the result of reading variations less than 1 mm with a scale rather than actual variations in the zone distance.

Zones 1, 2, 3 and 4 were identified and confirmed enzymatically in a previous report (12) and correspond to 2,3-DPG, ATP, Pi and lactate, respectively. Their PU values were, in order: 0.11, 0.24, 0.36, and 0.38. Zone 5 had a PU value of 0.77 and is, as yet, unidentified.

For each test, a mixed standard sample of 0.01 M solutions of 2,3-DPG, ATP, Pi, lactate and succinate was run (results not shown) to allow calculation of the concentration of each component in  $\mu$ mol of component/g hemoglobin as described previously (12). Fig. 2 (left) shows that, on Day 0, the main component was 2,3-DPG ( $5.5 \pm 0.8$ ). ATP was also always clearly present but at much lower concentrations ( $2.5 \pm 0.7$ ). Pi ( $5.9 \pm 1.4$ ) showed a wide variation from person to person, as did lactate ( $3.8 \pm 2.6$ ). As Fig. 1 (right) shows, zones 3 (Pi) and 4 (lactate) become the dominant feature with increase in the duration of storage. At Day 15, zone 1 (2,3-DPG) has almost disappeared, and zone 2 (ATP) has not shown much change. Zone 5 remained constant throughout the period of storage.

The actual values obtained on Days 0, 1, 3, 5, 7, 9, 12, 15, 21 and 30 are plotted in Fig. 3. The study group consisted of 15 apparently healthy males, 9 of whom were smokers and 6 who were not. The results for each person, however, showed more variation from person to person than from day to day for any particular person, but the differences were not related to smoking; therefore, the results for all 15 were pooled (Fig. 3).

The concentration of 2,3-DPG, the main component on Day 0, fell sharply over the first 24 h to a concentration of  $3.6 \pm 0.7$  on Day 1, that is, a 40% loss. This fall continued, although at a more gradual rate, over the first 15 days. 2,3-DPG was detected at low concentrations in only some of the samples on Day 15 and in none of the samples on Day 21.

ATP remained fairly constant (around 2.5) until Day 12, then fell gradually over the latter half of the storage period. The fall was more marked after 2,3-DPG had reached low levels, but ATP was still present to a certain extent ( $0.2 \pm 0.2$ ) on Day 30.

Lactate was present in most specimens on Day 0 although at only just detectable concentrations in some, giving a wide range of values ( $3.7 \pm 2.6$ ). It rose sharply over the first 24 h ( $11.4 \pm 2.2$ ) and proceeded to increase gradually throughout the rest of the storage period. Apart from Day 0, there was very little variation in values for all 15 giving a standard deviation in most cases

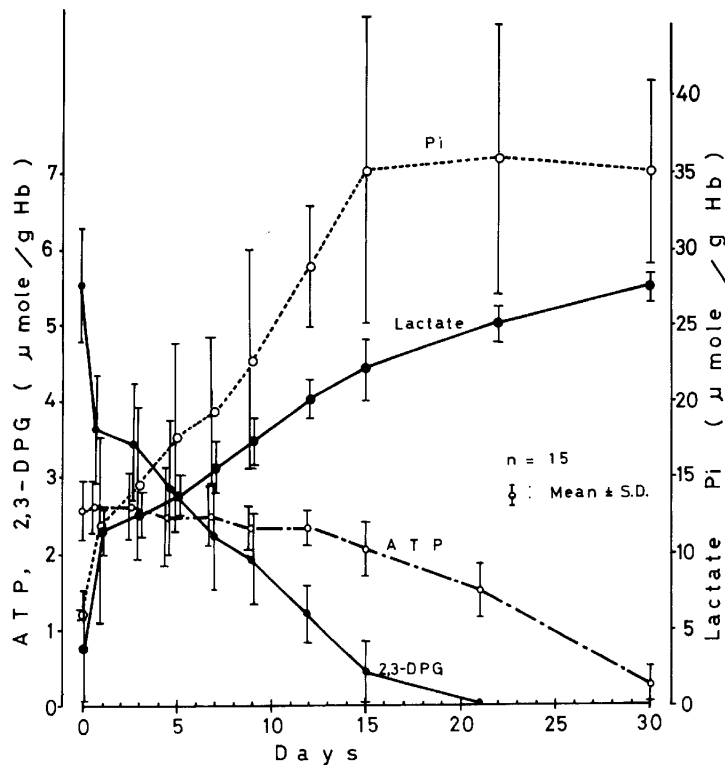


Fig. 3. Changes in red blood cell components during storage in ACD preservative. Conditions for isotachophoretic analysis as in Table 1. Specimens prepared for analysis as in Fig. 1.

of less than  $2.0 \mu\text{mol/g Hb}$ .

Pi showed great variation between different samples on the same day; hence the standard deviation values are high, but the general trend can be seen in Fig. 3: a sharp rise from Day 0 ( $5.9 \pm 1.4$ ) to Day 1 ( $11.5 \pm 3.5$ ), which continued to Day 15 ( $35 \pm 10.2$ ) and then levelled off over the later half of the storage period ( $35 \pm 6.3$ ) at Day 30.

Table 2 shows a comparison of results for enzymatic confirmation of the isotachophoretic results. The marked individual variation meant that the results could not be pooled for this phase. One of the 15 samples was studied in this way. The four components, 2,3-DPG, ATP, Pi and lactate were measured enzymatically on Days 0, 3, 7 and 15. The results coincide well with the isotachophoretic results for 2,3-DPG, ATP and lactate. The results for Pi, however, were significantly different at Days 7 and 15, with the isotachophoretic result being much higher than the enzymatic value. The previous report (12) had already obtained good correlation between the two methods. This discrepancy was investigated and it was found that, although experimental factors such

TABLE 2. ENZYMATIC CONFIRMATION OF THE RESULTS FOR ISOTACHOPHORETIC ANALYSIS OF CHANGES IN RED BLOOD CELL COMPONENTS DURING STORAGE IN ACD PRESERVATIVE

Component	Method	Amounts ( $\mu$ mole of component/g Hb)			
		Days of Storage			
		0	3	7	15
2,3-DPG	E	6.12	3.94	1.76	0.02
	I	6.24	4.06	1.81	
ATP	E	3.17	2.97	2.51	1.30
	I	3.27	3.06	2.56	1.32
Pi	E	6.88	9.42	19.35	23.2
	I	7.24	9.87	23.19*	37.5*
Lactate	E	6.74	11.43	16.8	23.5
	I	6.81	11.66	17.3	23.2

E: enzymatic, I: isotachophoresis. \*: result significantly different ( $p < 0.01$ ) for the two methods.

TABLE 3. COMPARISON OF RED BLOOD CELL COMPONENTS USING HEPARIN AND ACD AS ANTICOAGULANTS

Component	Amounts ( $\mu$ mole of component/g Hb, mean $\pm$ S.D.)	
	Heparin	ACD
2,3-DPG	10.19 $\pm$ 1.2	5.49 $\pm$ 0.76
ATP	4.66 $\pm$ 0.9	2.53 $\pm$ 0.66
Pi	4.8 $\pm$ 1.08	5.99 $\pm$ 1.39
Lactate	8.24 $\pm$ 2.7	3.76 $\pm$ 2.64

All samples taken and prepared for isotachophoretic analysis within two hours of being collected into the anticoagulant. For heparin, blood was taken from 30 male volunteers presenting to the dental outpatient clinic. For ACD, blood was taken from 15 male volunteers (age range: 25-35). Conditions of isotachophoresis as in Table 1.

as inadequate warming up of the apparatus or freshly made leading and terminal solutions were not related, sometimes a poorly separated zone appeared to be present with zone 4. In such cases, the result obtained by including the poorly separated zone in the calculation was the cause of the erroneously high value for Pi. In the preliminary experiment (13) to decide the conditions for isotachophoresis, various phosphate containing compounds and organic acids had been studied. At pH 4.2, it was possible that this zone close to Pi might be citrate (PU value of 0.33 at pH 4.2). A repeat experiment with particular care on the washing stage to remove the ACD anticoagulant resulted in the loss of this irregularity and an even plateau for the Pi zone. This then gave good correlation with the enzymatic results for Pi.

Table 3 shows a comparison of the results for blood taken into different anticoagulants. There is a marked fall in 2,3-DPG and ATP under the acidic conditions of the ACD preservative ( $p < 0.01$ ). The difference in phosphate



levels is significant at the  $p < 0.05$  level, with the ACD level being higher. Lactate is significantly elevated in the heparinized red blood cells ( $p < 0.01$ ).

#### DISCUSSION

Isotachopheresis has already been shown to be accurate and reliable for the *in vitro* situation, but has yet to find its place in the study of clinical specimens. The present study presents a method for its application to the measurement of the organic acids and phosphate-containing compounds that occur in red blood cells. Until the present report, the convenience of not having to pretreat specimens of blood before injection into the isotachophoretic apparatus had been praised (15), but it was found that the use of hemolyzed specimens had poor reproducibility. The values for each zone varied in consecutive analyses, hence the schedule shown in Fig. 1 was developed.

The blood sample is centrifuged and the supernatant (serum) set aside for other purposes. The pellet of blood cells is then thoroughly washed, not with saline but with isotonic sucrose solution because saline has been shown to affect the nucleotide content of the red cells (16). White blood cells and platelets are not removed since their presence does not affect the results for nucleotides (10, 16). Hemolysis is known to cause a decrease in ATP with an increase in ADP (10). Although ADP was not measured in this experiment, ATP expressed as  $\mu\text{mol}$  of ATP/g hemoglobin gave comparable values for lysed blood and whole blood (12). To avoid the hemolytic step would involve using whole blood. This poses the problem of interference with the conditions for isotachopheresis from chloride ions in the serum (with the leading electrolyte) and citrate ions in the ACD preservative (with the inorganic phosphate as in Fig. 3). Studies of blood not taken into ACD preservative would solve the problem of citrate interference, but a new system with a different leading electrolyte would be needed to cope with the chloride ions in serum.

The many glycolytic intermediates and nucleotides in human blood listed by Minakami *et al.* (10) are better measured enzymatically because of their low concentrations. The concentrations measured with the present isotachophoretic method were limited by the manual measurement of the isotachophoretic pattern. This limited its accuracy to  $\pm 0.5$  mm at a chart speed of 10 mm/min. Electronic measurement at this step would improve the results, but the lowest concentrations detected were of the order of 1000  $\text{m}\mu\text{mole/ml}$  red cells in the table given by Minakami *et al.* (10). The concentrations of ADP and AMP are much lower; therefore, when present, the peaks thought to represent them could not be sampled by the withdrawal cell, or be identified by their PU values because of the absence of a definite plateau. Identification of these components by UV absorption (15) has only been deduced from the results of standard solutions and needs more conclusive confirmation. Advantages of this isotachophoretic

phoretic method over conventional enzymatic techniques are the small volume of sample needed (with practice, less than 2 ml of blood for one analysis) and the fact that all of the identified components can be read from this one analysis. Conclusive identification of other peaks representing components present in lower concentrations will further increase the usefulness. One run took 25-30 mins. The preparative stages can be performed for a number of specimens at the same time, and automation of the final readout would remove the only tedious step. This was very evident when making the comparison with conventional enzyme techniques (Table 2).

The changes in the components of red blood cells during storage in ACD anticoagulant have been studied by column chromatography on ion-exchange resins (17) and enzymatically (6, 7, 10). The rapid fall for 2,3-DPG during the first 15 days was confirmed in the present experiment. ATP is reported to decrease slowly during the same period, then fall more rapidly after the 2,3-DPG has reached a very low level (17), or to decrease at a constant rate from Day 0 (18, 19). The present study showed an almost constant level during the first two weeks followed by a steady decrease thereafter.

The concentrations of both 2,3-DPG and ATP were lower on Day 0 for ACD preservative than for heparin. These differences have been documented before (18, 19) and the rapid initial breakdown is thought to account for the higher levels of inorganic phosphate in stored blood. Actually, Day 0 refers to two or three hours of *in vitro* life for the red cell, including the period for the washing and centrifugation of cells. The time the first sample was taken after blood was collected into the ACD packs also varied, and allowed metabolic changes that would account for the spread in values for lactate and inorganic phosphate.

The results for the present study are consistent with those of other methods (6, 10) and were verified enzymatically (Table 2). For the major components studied, isotachopheresis proved to be a very convenient, rapid and accurate method for measuring them simultaneously, only needing additional estimation of hemoglobin to convert the results to conventional units. The results for isotachopheresis tended to be higher than the enzymatic ones, but the difference was not significant. Care was needed in reading the zones (especially for inorganic phosphate) to ensure that only a pure zone was present, the citrate preservative being one source of likely error. This method is now available for other investigations such as improving blood storage (20, 21) and oxygen delivery where organic phosphates are being studied in relation to red cell glycolysis and hemoglobin-oxygen affinity (3, 22).

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