DETERMINATION OF ADENINE NUCLEOTIDES BY PAPER ELECTROPHORESIS

Z. STRÁNSKÝ

Institute of Medical Chemistry, Medical Faculty of Charles University, Hradec Krdlové (Czechoslovakia)

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INTRODUCTION

When studying the metabolism of phosphorus in red blood cells we had to choose a method which would enable us to combine high specificity with the possibility of studying the incorporation of ^{32}P into individual adenine nucleotides. Paper electrophoresis has been given preference to chromatographical methods of separation, as the latter are less rapid. Paper electrophoresis was first used for the separation of individual nucleotides by MARKHAM AND SMITH[1], who applied high voltage as did a number of subsequent authors[2–5]. This method is quick and yields good results, but because of the risk of working with high voltages and because of complications arising from the necessity of cooling the electropherograms to prevent hydrolysis, we decided in favour of low voltage electrophoresis. Several authors have applied low voltage electrophoresis for the qualitative analysis of nucleotide mixtures. Buffers ranging from pH 2.5 to 11.1 were used[6–18]. The theoretical advantages of using pH 3.5 and pH 5.0 buffers when separating various nucleotides with a different base were pointed out by SMITH[19].

We had, however, to find a most suitable electrolyte for the separation of individual adenine nucleotides differing in the content of phosphoric acid residues. The following factors had to be investigated experimentally:

1. the most suitable buffers,
2. the method of deproteinisation and the most suitable extraction agent,
3. preparation of the sample,
4. elution from the electropherogram,
5. spectrophotometric determination.

EXPERIMENTAL

Conditions of electrophoresis

We examined many buffers mentioned in the literature and the results are summed up in Fig. 1. The best separation was obtained by using citrate buffer. From Fig. 2 it can be seen that the separation of individual adenine nucleotides depends on the pH of the citrate buffer, the best results being obtained in the region of pH 4.0–5.0. Fig. 3 shows the dependence of the resolution of adenine nucleotides on the ionic strength. In the range examined separation was improved by lowering the ionic strength.

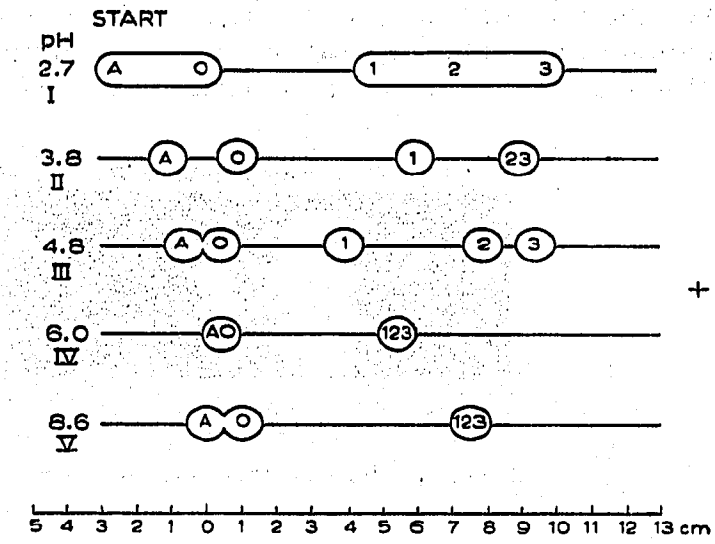

Fig. 1. Electrophoretic separation of adenine nucleotides in different buffers. I. Butyric acid–sodium hydroxide; II. acetic acid–sodium acetate; III. citric acid–sodium citrate; IV. pyridine–acetic acid; V. barbituric acid–sodium barbiturate. A = adenine; O = adenosine; 1 = AMP; 2 = ADP; 3 = ATP.

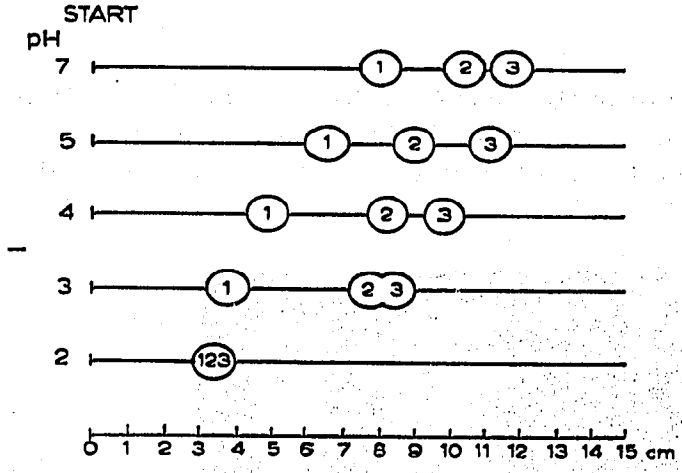

Fig. 2. Electrophoretic separation of adenine nucleotides in citrate buffers of different pH.

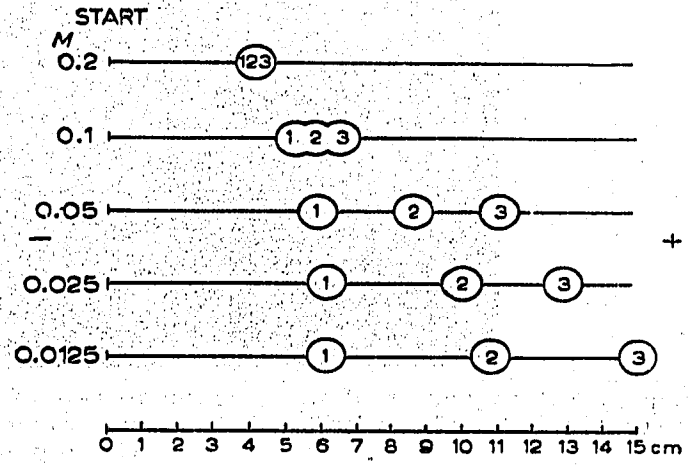

Fig. 3. Electrophoretic separation of adenine nucleotides in citrate buffers of different ionic strength (pH = 4.8).

We chose as optimum conditions: pH 4.8, 0.05 *M* citrate buffer, 3½ hours at room temperature. The separation of an adenine nucleotide mixture under these conditions is shown in Fig. 4. As citrate made chemical detection of inorganic phosphate

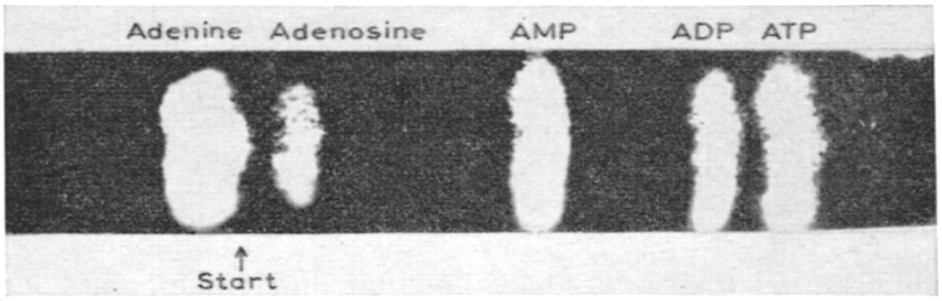

Fig. 4. Photoprint of an electropherogram of adenine nucleotides.

impossible, we used ^{32}P ortho- and pyrophosphate and examined them autoradiographically. It was found that both components migrated much faster than adenosine triphosphate (ATP) and did not interfere with the determination of the specific activity of this nucleotide (Fig. 5).

Preparation of the sample

Both ATP and inorganic phosphate are present in the acid-soluble phosphorus fraction. From the data in Table I it can be seen that extraction with perchloric acid gives a better recovery (by 50 %) than that carried out with trichloroacetic acid, which acid is still used by many authors.

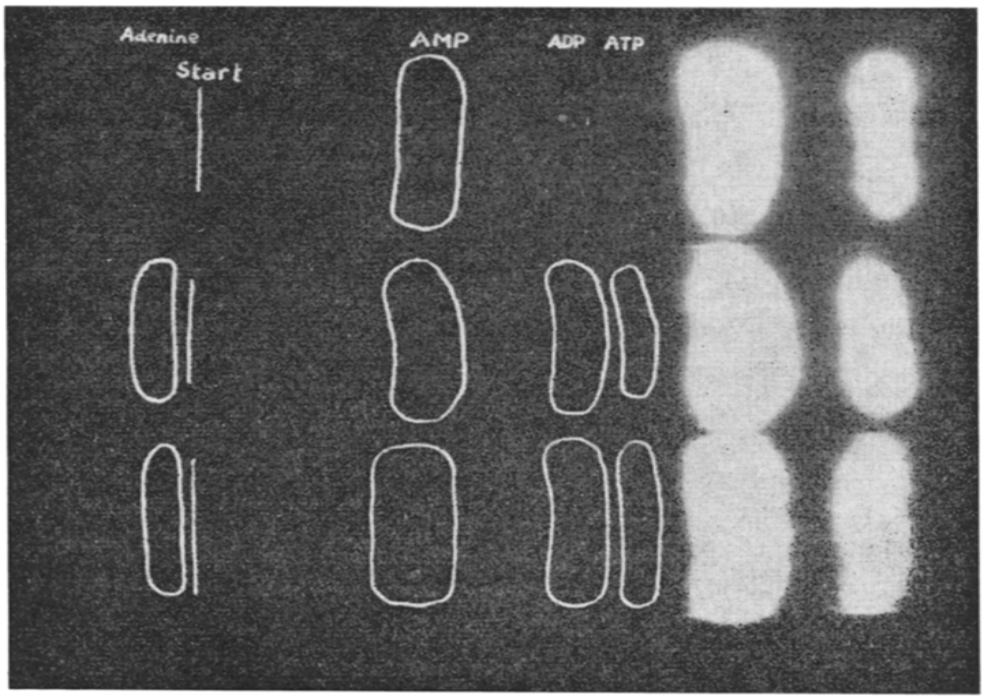

Fig. 5. Autoradiogram of an electropherogram of adenine nucleotides and radioactive ortho- and pyrophosphate. The spots of adenine nucleotides were outlined on the autoradiogram after the electropherogram.

TABLE I

DETERMINATION OF ADENINE NUCLEOTIDES IN HUMAN ERYTHROCYTES BY PAPER ELECTROPHORESIS

Extraction	*ATP (mg %)*	*ADP (mg %)*	*AMP*
Trichloroacetic acid	19.1 ± 5.5	14.7 ± 6.48	—
Perchloric acid	42.6 ± 14.5	10.7 ± 9.5	—

During the application and drying of the sample the concentration of the acid used for the extraction rises to an undesirable degree, causing hydrolysis of a part of the ATP and interfering with the results of the analysis. The excess of the acid must therefore be removed before applying the sample to the origin. Perchloric acid can be precipitated as insoluble potassium perchlorate. Fig. 6 shows that the result of the analysis is influenced by treatment of the sample before its application to the chromatogram.

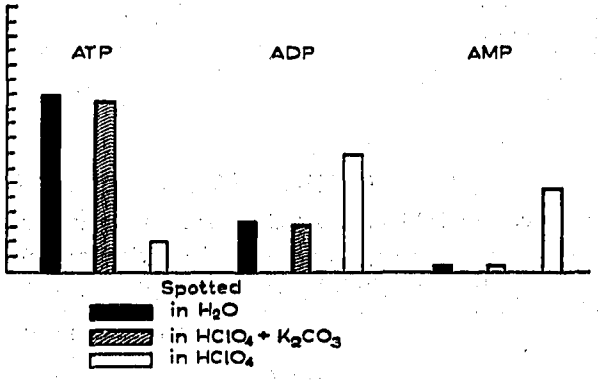

Fig. 6. Electrophoretic analysis of a sample of ATP "Fluka".

Elution

It is not necessary to carry out the elution in a special elution device. The outlined fraction may be cut out and dipped in a test-tube containing the eluent; 4 hours are sufficient for this type of elution.

Determination

The absorption maxima of the purine bases in dilute hydrochloric acid are situated around 257 mμ[20]. We measured the eluates at their maxima, *i.e.* at 257 mμ, and at 290 mμ[21]. The concentration of the nucleotides was calculated from the difference between the extinctions at these wavelengths. In this way better results could be obtained, because contamination caused by the elution of non-specific admixtures from the paper is eliminated.

The recoveries were checked with a series of samples. The results are summed up in Table II. It is obvious that in the range studied the recoveries (approximately 90 %) and the scatter of the values (approximately 3 %) remain practically unchanged. Recoveries of adenosine monophosphate (AMP) added to samples of bloodcells are given in Table III.

TABLE II

MEAN ERROR OF RECOVERY EXPERIMENTS

No.	*100 mg% solution of AMP*		*200 mg% solution of AMP*	
1	86.0		171.6	
2	88.6		171.6	
3	90.0		172.5	
4	90.0		172.8	
5	91.2		173.3	
6	91.2		173.3	
7	91.2	$\bar{x} = 91.4 \pm 2.59$ mg%	178.5	$\bar{x} = 179.7 \pm 5.9$ mg%
8	91.2		180.9	
9	91.5	*i.e.* $\sigma = 2.84$%	182.0	*i.e.* $\sigma = 3.28$%
10	92.4		183.5	
11	92.4		183.5	
12	92.4		183.5	
13	92.4		183.5	
14	92.4		183.5	
15	95.0		183.5	
16	95.0		183.5	
17			184.5	
18			189.5	

TABLE III

RECOVERY OF AMP ADDED TO SAMPLES OF BLOOD CELLS

AMP was added to water (control) and blood to give the final concentration of 150 and 300 μg in 0.1 ml.

Added AMP		*Recovered AMP*			
		Blood		*Control*	
μg	%	μg	%	μg	%
150	100	114	74	132	86
300	100	250.5	83.5	265.12	88.37

RECOMMENDED PROCEDURE

On the basis of the experiments concerning the conditions for electrophoretic determination of ATP the following method for the determination in erythrocytes was elaborated.

Preparation of haemolysate

1 ml of packed erythrocytes was added to 0.8 ml of ice-cold water with a little saponin.

Deproteinisation and extraction

To 1 ml erythrocytes haemolysed in 0.8 ml H_2O with saponin 0.2 ml of 40 % perchloric acid was added immediately after haemolysis.

After centrifugation the supernatant was transferred to a test-tube graduated to

TABLE IV

COMPARISON OF THE RESULTS OBTAINED WITH VARIOUS METHODS FOR DETERMINING ADENINE NUCLEOTIDES

Author	*ATP (µmole/ 100 ml)*	*ADP (µmole/ 100 ml)*	*AMP (µmole/ 100 ml)*	*Sample**	*Method*
PIRWITZ, *et al.*[22]	52 31–81			B	Colorimetric
OVERGAARD-HANSEN AND JØRGENSEN[23]	51–57	8.3–9.3	0	B	Enzymic
HUGHES JONES[24, 25]	63–116	12–25	3–5	RBC	Column chromatography with ion exchangers
	81	21	5	RBC	Column chromatography with ion exchangers
MILLS AND SUMMERS[26]	56.5	7.0	0.7	B	Column chromatography with ion exchangers
MANDEL AND CHAMBON[27]	82.4	14.0	2.1	RBC	Column chromatography with ion exchangers
BISHOP, *et al.*[28]	92.05	10.16	1.32	RBC	Column chromatography with ion exchangers
CARTIER, *et al.*[21]	68	31.5	10	RBC	Paper chromatography
GERLACH, *et al.*[29]	68	30	10	RBC	Paper chromatography
VOGEL[30]	45.8**	17.5**	3.7**	RBC	Paper chromatography
PRANKERD AND ALTMANN[31]	28–88	19–40		RBC	Paper chromatography
BARTLETT, *et al.*[32]	100	26	3	RBC	Paper chromatography
OVERGAARD-HANSEN, *et al.*[33]	116	24	7.5	RBC	Paper chromatography
STRÁNSKÝ, present paper	84.2	25.1	—	RBC	Paper electrophoresis
	42.6**	10.7**	—	RBC	Paper electrophoresis

* B = blood; RBC = red blood cells.
** mg%.

3 ml. The precipitate was washed twice with ice-cold 4% perchloric acid and all the supernatants were pooled in the 3 ml test-tube. A small quantity of potassium hydroxide, calculated to neutralize the perchloric acid used in the extraction and washing, was then added and subsequently the volume was made up to 3 ml.

Electrophoresis

We used a citrate buffer (pH 4.8, 0.05 *M*) and applied a voltage of 200 V for 3½ hours. On the origin 0.1 ml extract was placed. Electrophoresis was carried out in a self-constructed apparatus with horizontally placed electropherograms, which permitted simultaneous analysis of up to 18 samples.

Detection

The detection was carried out in monochromatic short wave U.V.-light, in a self-constructed detection device. The spots were outlined and cut out.

Elution

The individual fractions that had been cut out were eluted with 5 ml 0.1 *N* hydrochloric acid for at least 4 hours.

Determination

The eluates were measured in U.V.-light at wavelengths of 290 and 257 mµ. The difference in absorption at these wavelengths multiplied by a factor gave the concentration of the individual fractions. The results obtained with a series of blood samples are shown in Table I. From Table IV it can be seen that these values correspond to those reported by many authors who used other specific methods for the determination of adenine nucleotides, especially chromatography on ion exchangers.

SUMMARY

1. Low voltage paper electrophoresis in 0.05 *M* citrate buffer of pH 4.8, at a voltage of 200 V, for 3½ hours, is a suitable method for determining adenine nucleotides in erythrocytes.

2. Before applying the sample to the paper it is necessary to remove the excess of deproteinisation agent.

3. By means of the method described 42.6 ± 14.5 mg % ATP and 10.7 ± 9.5 mg % ADP were determined in the erythrocytes.

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