The Analysis of Adenosine Triphosphate and Adenosine Diphosphate Preparations by Paper Ionophoresis

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(Received 30 April 1953)

The importance of adenosine triphosphate (ATP) in cell metabolism and the instability of its preparations during storage have resulted in a keen interest being shown in methods for its analysis.

Until recently the most satisfactory analysis of ATP has involved the preparation and use of highly purified enzyme systems (Bailey, 1949). Other less time-consuming methods involving paper chromatography are now available. The most recent (Eggleston & Hems, 1952) involves the separation and estimation of ATP, adenosine 5-phosphate (AMP), orthophosphate and pyrophosphate + adenosine diphosphate (ADP) by 'two-solvent one-dimensional chromatography' and the individual determination of pyrophosphate by a further chromatographic separation.

During the development of a general scheme for the analysis of acid-soluble phosphates a method involving paper ionophoresis was evolved which was found to separate all the above esters in one operation and in the space of a few hours. The technique which is described below has been applied to the analysis of seven samples of ATP and one of ADP.

EXPERIMENTAL

Ionophoreses were carried out on 28 cm. widths of Whatman no. 3 paper washed three times with *n* formic acid and finally with distilled water that had been passed through Amberlite Monobed Resin MB-1 analytical grade (supplied by British Drug Houses Ltd.). Twelve sheets (28 × 45.25 cm.) were supported by a perforated sheet of Perspex built into a Perspex box so that suction could be applied from beneath the paper drawing the solvents through it. The sheets were dried in a current of warm air. This treatment reduced the phosphorus of the paper to about 0.03 μg . P/sq.cm., and removed interfering substances.

The electrodes were two lengths of 26 (s.w.g.) gauge copper wire threaded through holes pierced at 1 cm. intervals 1.3 cm. from each edge (Fig. 1). The wires extended beyond the paper and provided a means of suspending it during subsequent operations.

As the method suffers no interference from Ca^{++} or Ba^{++} the samples were applied as aqueous solutions acidified, in the instance of the Ba salts, with *n*-HCl. (If the sample is freshly precipitated as the Ba salt and in a finely divided state it can be applied as the suspension.) The solution (2.5 μl . containing about 25 μg . P) was applied to the paper 1.9 cm. from the cathode. This large quantity was necessary

in order to provide measurable amounts of the weaker components.

5% (w/v) Oxoid agar (supplied by Oxo Ltd.) and the same solution containing 5% (w/v) NaCl were delivered, in a molten state, on to the cathode and anode, respectively, to ensure intimate association of wire and paper and to provide at the anode a reservoir of Na ions.

The paper was sprayed with aqueous solution (pH 3.2) containing 4.6% (v/v) redistilled *n*-butyric acid and 0.057% (w/v) NaOH until it was just saturated with the solution. The paper was then suspended from the edge of a covered glass trough over sufficient water to maintain a saturated atmosphere.

A potential of 300–400 v (d.c.) was applied to give a current of 0.25 mA/cm. breadth. After 2 hr. the paper was withdrawn and dried in a current of warm air and the phosphates were located by spraying first with 0.1% (w/v) FeCl_3 , $6\text{H}_2\text{O}$ in 80% (v/v) aqueous ethanol and secondly, after drying, with 1% (w/v) sulphosalicylic acid in 80% (v/v) ethanol (Wade & Morgan, 1953).

The spots were cut from the paper, transferred to 4 in. × $\frac{1}{8}$ in. Pyrex test tubes and ashed on a microdigestion rack (Gallenkamp & Co. Ltd., London). After cooling, 0.5 ml. 72% (w/v) aqueous HClO_4 was added and digestion carried out using initially the minimum heat necessary from the bottom element to boil the acid and finally heat from both elements to enable the condensing acid to reach the upper parts of the tubes.

When digestion was complete the tubes were cooled. 1 ml. 5*N*-NaOH and 1 drop of 0.05% (w/v) phenolphthalein in 80% (v/v) aqueous ethanol were added to each and 20% (w/v) aqueous HClO_4 was added until the indicator was decolorized. Orthophosphate determinations were carried out by the method of King (1932), and the quantities of reagents were adjusted to give a final volume of 5 ml. The colour intensities were read on a Spekker absorptiometer (A. Hilger & Sons Ltd., London) using microcells of 2 cm. depth and 1 ml. capacity.

In the analysis of an ATP or an ADP preparation 5 mg. were treated with 0.1 ml. water and acidified where necessary with the minimum quantity of *n*-HCl. 2.5 μl . quantities were delivered alternately on to the base line (1.9 cm. from the cathode) of an ionophoresis paper (28 cm. long and 15 cm. broad) at 2.5 cm. intervals, and on to squares (0.63 cm. × 0.63 cm.) of filter paper. Three spots were applied to the ionophoresis paper and four to the paper squares. The latter were subsequently digested to determine the total P applied; blank squares similarly treated provided reagent blanks.

After ionophoresis and the detection of the phosphates, 2.5 cm. strips of the paper containing the components of each original spot were cut out and divided into rectangles

containing the individual spots. Each rectangle was digested and total P determined; from this was subtracted the quantity of P calculated from the blank to be present in the area of paper digested.

Special precautions. As other workers have stressed, it is imperative to have paper and reagents as free as possible from interfering substances. Glass-distilled water is recommended for use throughout. Unsatisfactory conditions are reflected in the nature of the ATP spot, which streaks badly.

The even spraying of the solvent is important and requires some practice. The application of more solvent than the paper can accommodate should be avoided as this results in diffused spots and a merging of the ATP spot with the orthophosphate spot. If this continues to give trouble the following alternative form of analysis can be adopted. The spot of ATP and orthophosphate are combined and extracted with 0.1N-HCl. Orthophosphate and total P in this extract are then determined. This alternative is made possible by the mild conditions employed in the location of the phosphates.

Fig. 1. Fractionation of ATP and ADP preparations by paper ionophoresis. Sample numbers correspond to those in Table 1. The total P applied to the paper was adjusted to give about 10 μ g. P in the main constituent. (1) AMP; (2) ADP, (3) ATP, (4) orthophosphate, (5) pyrophosphate. (i) Photograph of an ionophoresis paper after detection of the phosphates by FeCl₃/sulphosalicylic acid spraying. (ii) Photograph of an ultraviolet contact print of an electrophoresis paper showing the presence of two ultraviolet absorbing areas (a) and (b) in addition to those of (1), (2) and (3).

Table 1. Analyses* of preparations of adenosine triphosphate and diphosphate (ATP and ADP)

Preparation	Source	Storage temp.	Year received	Estimated as % total P					Pyro-phosphate	Total estimated
				AMP	ADP	ATP	Ortho-phosphate			
1. ATP, free acid	Light & Co. Ltd.	Room	1950	10.7 $\pm$ 0.24†	27.3 $\pm$ 0.30	28.9 $\pm$ 0.26	24.5 $\pm$ 0.12		7.8 $\pm$ 0.30	99.2 $\pm$ 0.56
2. ATP, Ca salt	Light & Co. Ltd.	Room	1950	10.9 $\pm$ 0.78	27.8 $\pm$ 1.03	31.5 $\pm$ 1.01	24.3 $\pm$ 0.90		7.9 $\pm$ 0.74	102.4 $\pm$ 2.02
3. ATP, Ba salt	Private	2°	1952	4.1 $\pm$ 0.39	18.3 $\pm$ 0.59	64.4 $\pm$ 1.45	8.8 $\pm$ 0.18		2.3 $\pm$ 0.72	97.9 $\pm$ 1.78
4. ATP, Ba salt	Light & Co. Ltd.	2°	1952	7.3 $\pm$ 1.25	26.3 $\pm$ 0.80	50.8 $\pm$ 1.14	10.6 $\pm$ 0.51		2.1 $\pm$ 0.51	97.1 $\pm$ 2.01
5. ATP, Ba salt	Schwarz Lab. Inc.	2°	1952	3.4 $\pm$ 0.94	7.5 $\pm$ 0.83	74.0 $\pm$ 2.04	4.1 $\pm$ 0.53		4.2 $\pm$ 0.57	93.2 $\pm$ 2.52
6. ATP, Na salt	Boots Pure Drug Co.	Room	1945	8.8 $\pm$ 0.33	13.7 $\pm$ 0.26	37.3 $\pm$ 1.43	15.3 $\pm$ 0.41		23.6 $\pm$ 0.37	98.7 $\pm$ 1.59
7. ATP, Ca salt	Light & Co. Ltd.	Room	1950	11.2 $\pm$ 0.23	24.9 $\pm$ 0.40	28.2 $\pm$ 0.43	23.3 $\pm$ 0.39		6.5 $\pm$ 0.24	94.1 $\pm$ 0.77
8. ADP, Ba salt	Schwarz Lab. Inc.	2°	1952	4.9 $\pm$ 0.29	76.0 $\pm$ 1.18	3.8 $\pm$ 0.14	10.8 $\pm$ 0.21		—	95.5 $\pm$ 1.24

* Analyses were carried out in March 1953.

† Standard deviation.

RESULTS

The analyses given in Table 1 represent the average of three sets of determinations carried out at the same time. Fig. 1 (i) shows the spots of AMP, ADP, ATP, orthophosphate and pyrophosphate separated by the ionophoresis of seven preparations of ATP and one of ADP.

Low concentrations of other phosphoric esters are present in most of the preparations analysed. At least two further ultraviolet-absorbing esters are present in preparations 1, 2, 4, 6 and 7 (Fig. 1 (ii)). One (a) occurs between AMP and ADP; its identity is unknown and it has not been included in this analysis. The other (b) is located just behind the ATP spot, and contaminates it.

DISCUSSION

The elementary analysis of ATP and ADP preparations, together with the estimation of acid-labile phosphate, is still used by some workers as a criterion of purity (LePage, 1945; Dounce *et al.* 1948). Other workers seeking more specific methods have used enzyme systems (Kalckar, 1943, 1944, 1947; Bailey, 1949; Rowles & Stocken, 1950); such methods, however, require the lengthy preparation of highly purified enzymes. In addition to this, discrepancies are reported to exist in the results obtained from the use of different combinations of enzymes (Rowles & Stocken, 1950) and the reactions catalysed by some of them are still questioned (Bailey, 1949; Banga, 1947). This type of analysis, in common with some others, requires the initial preparation of the free acid or the sodium salt of the ester, and if losses in ATP of up to 20% incurred by the direct precipitation of the barium as the sulphate are to be avoided, an ion-exchange or electrical transport system has to be used to bring this about (Bailey, 1949).

Ion-exchange resins have been used by Cohn & Carter (1950) for the separation and analysis of adenine, adenosine, AMP, ADP and ATP. As an analytical method this procedure suffers the disadvantage of requiring the use of large volumes of fluid. These workers also carried out this separation by paper chromatography using an *isoamyl* alcohol:disodium phosphate:water system. In both methods barium was removed as the sulphate and no account was taken of any pyrophosphate that may have been present.

The Tiselius apparatus has also been used for the ionophoretic separation of these adenine derivatives (Bock & Alberty, 1951); in this method no provision was made for the analysis of pyrophosphate or orthophosphate.

A paper-chromatographic technique, recently introduced by Eggleston & Hems (1952), involves 'two-solvent, one-dimensional chromatography' and overcomes some of the disadvantages of the above methods. The apparatus required is simple,

only small quantities of material being necessary, and the analysis is speedy. However, conversion of the preparations to the sodium salts is still necessary, and an individual determination has to be made for the pyrophosphate.

The main advantages of the method described here can be outlined as follows. The apparatus is very simple and inexpensive to construct; the analysis can be carried out completely inside a working day; no interference is caused by the presence of barium or calcium ions, thus permitting the direct analysis of any of the common forms of preparations; and the separation of mixtures into AMP, ADP, ATP, orthophosphate and pyrophosphate can be carried out in one operation.

The method, as described, cannot claim to analyse these phosphates in preparations that are heavily contaminated with other esters but it is put forward as a basic technique to which can be added more exacting steps involving the elution and subsequent analysis of the fractions separated.

SUMMARY

1. A method is described for the separation of adenosine triphosphate, adenosine diphosphate, adenosine 5-phosphate, orthophosphate and pyrophosphate by paper ionophoresis.

2. The ionophoresis technique differs from those normally used in having no reservoirs of buffer at the electrodes.

3. The method has been used in the analysis of seven samples of adenosine triphosphate and one of adenosine diphosphate. The results of this are given.

We are indebted to Dr D. Herbert for helpful criticism of this paper and to Dr K. Bailey for supplying a sample of adenosine triphosphate. The statistical treatment of results was carried out under the guidance of Mr S. Peto. Acknowledgement is made to the Ministry of Supply for permission to publish this paper.

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