ANALYSIS OF ADENINE POLYPHOSPHATES BY PAPER CHROMATOGRAPHY

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INTRODUCTION

The microanalytical determination of adenosine triphosphate and adenosine diphosphate is still a topic of discussion. Early methods still in use rely on the enzymic hydrolysis of phosphate groups from adenine polyphosphates[1]. Later methods have used firefly luminescence as a biological assay technique. Chemical methods are divided between those that estimate total adenine, such as that by PIRWITZ *et al.*[2], which relies on the colorimetric estimation of adenine, and the separation of adenine polyphosphates by paper chromatography and their subsequent analysis by phosphate estimation. Such a technique was first proposed by HANES AND ISHERWOOD[3].

In this paper it is proposed to discuss a technique utilizing preliminary paper chromatographic separations followed by the analysis of the adenine by absorption at λ_{max} 260. This technique has been used in this Department for the estimation of ATP* and ADP in large numbers of toad sartorii.

EXPERIMENTAL

The treatment of sartorius muscles which were taken from the toad, *Bufo marinus*, was essentially similar to that previously reported by SIMON *et al.*[4]. Special care was taken in blotting each muscle as contractions can occur. This affects the (resting) creatine phosphate level although the ATP content remains essentially unchanged[5]. At the conclusion of each experiment the muscles were frozen in liquid air. Each was then homogenized in a chilled glass homogenizer with 2 ml of 5% trichloracetic acid solution. The homogenate was centrifuged at 2° and the supernatant analysed as follows:

(a) Neat supernatant was analysed for ATP and ADP.

(b) Dilutions were estimated for creatine and creatine phosphate by a similar method to that employed by ENNOR AND ROSENBERG[6]. The cations, sodium, potassium and lithium, rubidium and caesium (if any) were measured by flame photometry[7]. 50 ml of each extract were spotted onto a Whatman No. 1 Chromatography Paper so that a pair of spots and a blank were run on the *same* muscle pair.

* Abbreviations used: ATP = adenosine triphosphate; ADP = adenosine diphosphate; AMP = adenosine monophosphate.

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Each chromatogram was run by a similar technique to that of KREBS AND HEMS[8], using isopropyl ether–90% formic acid (3:2) for 5 hours at 20° and then drying the paper in a draft cupboard at room temperature. The paper was then cut 1½ in. above the starting line and the chromatogram developed with isobutyric acid, *M* ammonia, with a trace of 0.1 *M* EDTA solution (100:60:1.6), overnight. The paper was dried for 2 hours at room temperature and photographed in the ultraviolet by a contact method similar to that suggested by MARKHAM AND SMITH[9, 10]. A B.G.E. Germicidal lamp in an industrial metal housing, fitted with a wooden front, provided the illumination and "Kodagraph" Paper (Extra Thin) was used. The photos clearly show the presence of ATP, ADP and AMP (if significant). The paper was then cut and the adenine phosphate eluted with 6 ml of water. The adenine was estimated in a Beckman Spectrophotometer at λ_{max} 260 mμ[11], using a photomultiplier attachment to increase the sensitivity of the instrument. Standard ATP samples (Sigma) of 0.020, 0.040 and 0.060 μM were also run with each determination.

RESULTS

In 64 experiments the average value obtained for muscles soaked for 4 hours in normal Ringer solution is 5.9 mmoles/kg for ATP and 2.5 mmoles/kg for ADP. This includes substantial variations in season which are correlated with creatine phosphate in the results. Normal ATP values range from about 3–8 mmoles/kg of soaked muscle. Muscles soaked in various abnormal ionic Ringer solutions and solutions of normal Ringer with added metabolic inhibitors also show good correlation. These results are given in another paper. The values correlate well with those recorded by HARRIS[13]: ATP + ADP 7.7 – 13.8 mequiv./kg.

DISCUSSION

It was necessary to design a method which could be used on a large number of muscles, which gave comparable results and where the rest of the analysis could be conducted on the same extract.

The original method tried was that of EGGLESTON AND HEMS[8], but we found that it had the following disadvantages.

(i) The separation of creatine phosphate and inorganic phosphate was not clear and besides, creatine phosphate is unstable in the acid solvent used.

(ii) The subsequent analysis was very time-consuming as the total incineration of each paper required watching and a minimum of 33 incinerations would have had to be carried out for each experiment, each of which taking 10 minutes.

(iii) The subsequent analysis of phosphate by the method of BERENBLUM AND CHAIN[14] was also laborious and the reagents used were most expensive.

(iv) It was found that most u.v. absorbing substances were removed from the paper by the first solvent and subsequent chromatography with versene forced all the remaining contaminants to the solvent front. It was therefore considered unnecessary to wash papers.

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(v) It is doubtful if the possible extra accuracy of the ATP level is warranted in the experiment, as the variation of ATP level in toad is very variable and statistical methods used in the experiment required only the variation in pairs of muscles. The photography with ultraviolet light was first suggested by MARKHAM AND SMITH[9,10]. In their method a Hanovia "Homesun" lamp was used, fitted with filters. This was not available in the laboratory and it was found that the British General Electric Germicidal lamp could be utilized for the same purpose.

The use of blue print copying paper[15] has been tested using "Ozaprint". This has the advantage of not requiring a darkroom, provided no direct sunlight falls on the paper. Against this, it requires an exposure time of 30 minutes compared with 15 seconds needed for contact paper. A paper scanner attachment for the Beckman Spectrophotometer[16] was not available so that elution methods had to be used. An

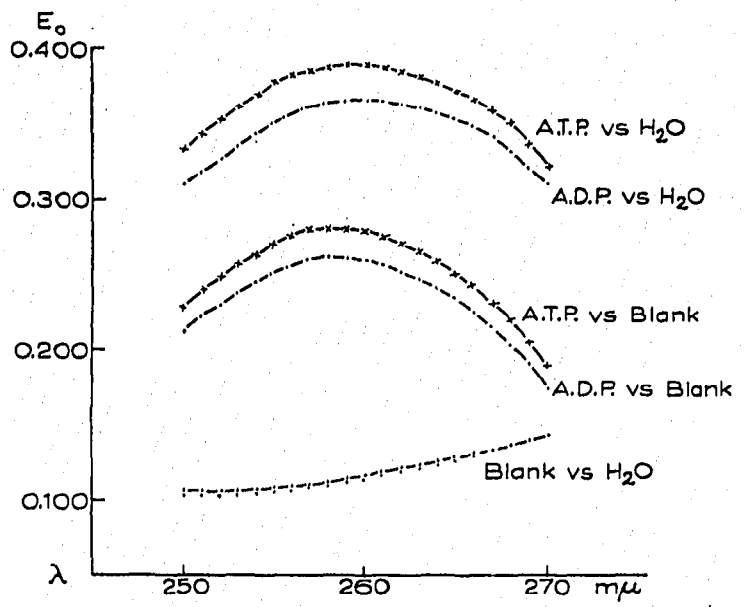

Fig. 1. Adenine spectrum.

elution with water by a capillary method[17] was found to require overnight elution. An apparatus treating up to 36 paper strips simultaneously was difficult to design. It was found that if the chromatogram was macerated in water with a plastic flanged rod in a centrifuge tube, a clear supernatant in a known volume of water was readily obtainable by centrifuging the mixture for 40 minutes at 1150 *g*. The total time for elution of 16 samples was reduced to 1 hour. Merely soaking for 1 hour[11] produced variable results. The sample was analysed at λ_{max} 260 mμ in a Beckman Spectrophotometer equipped with a photomultiplier attachment. It was found that under these circumstances, both ATP and ADP had a maximum peak of λ_{max} 258.5 mμ.

These are shown in the graph (Fig. 1). The values in both standard and muscle extract gave symmetrical curves when E_0 was plotted against the wave length. Elution with buffered solution at pH 2 (0.1 *M* HCl–KCl) gave similar E_0 readings although it was necessary to adjust the blank zero slightly.

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Choice of blank

Much of the success of the method depends on the choice of the blank. To reduce "blank" error, chromatograms 6 in. wide were divided by pencil lines into three

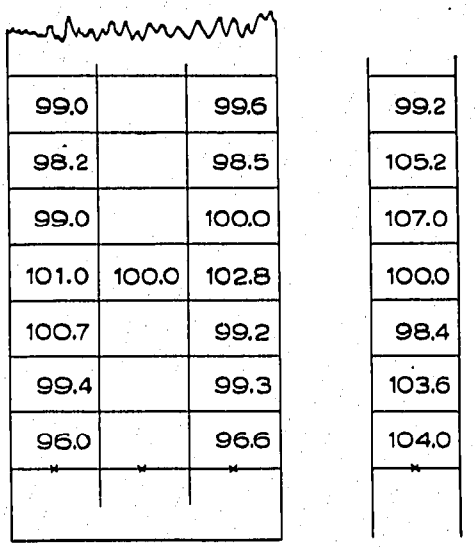

Fig. 2. Transmittance at 260 mμ. Blank: Whatman No. 1 paper.

strips. A blank was first run. The result of this can be seen in the diagram (Fig. 2). It will be seen that variation is least when horizontal blanks are used.

Advantages of the method

The greatest single advantage of the method is speed. It is possible to complete assay of 8 muscles for ATP and ADP in 24 hours, thus minimizing breakdown of ATP. The apparatus specifically designed for this method is very inexpensive. More samples can easily be analysed by the provision of more chromotanks. A similar procedure can also be used for other microanalyses. It has recently been used for determination of strychnine in *Nux vomica*[18].

Limitations and precautions

The value is relative only and depends on a number of factors.

(a) The isobutyric acid is very difficult to remove from the paper. This means that if the method is not rigidly adhered to, the absolute result may be in error, although the blank will cancel most of the effect. It may be desirable to use more easily removable solvents such as those suggested by GERLACH *et al.*[19]. These have not been tested fully in the laboratory.

(b) If the chromatograms are not placed squarely into the solvent, the spots will not run horizontally and "blank" errors are usually produced.

(c) Prior treatment of the muscle can cause a variation in the ATP content of the muscle and all the work with animal material should be done as far as possible by one analyst if comparable results are required.

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(d) The solutions do deteriorate and the chromatograms should therefore be run as soon as possible after the end of the experiment. In no case should separation be delayed for more than 24 hours, as significant breakdown occurs. Once the separation has taken place the chromatograms can be stored for a few days as the analysis is based on the adenine content.

Contamination can easily occur as many contaminants could absorb in the ultraviolet. It is necessary to observe strictest cleanliness both in the apparatus, which is specially washed with chromic acid and rinsed with distilled water, and in the laboratory generally.

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SUMMARY

A method for the microanalysis of adenine polyphosphates in toad muscles has been developed. This is based on the preliminary separation of the constituents by paper chromatography followed by the analysis of adenine by absorption at λ_{max} 260 mμ.

With this technique, it is possible to do a complete assay of eight muscles for ATP and ADP in twenty-four hours.

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