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Separation of Adenosine and Inosine Phosphates by Paper Chromatography

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In the course of a study on the dephosphorylation and deamination of adenosine triphosphate (ATP) by actomyosin, the need arose for the separation of the different adenosine- and inosine-5-phosphates, *viz.* ATP, adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine triphosphate (ITP), inosine diphosphate (IDP) and inosine monophosphate (IMP). Ion-exchange chromatography [1], paper chromatography [2-10] and electrophoresis on filter paper [11] or by a moving boundary method in the Tiselius apparatus [12] have been proposed for the separation of ATP, ADP and AMP but as far as we are aware, no separation of the different inosine phosphates from each other and from the corresponding adenosine phosphates has been hitherto described, except for the monophosphates [1,3,4].

In the present communication, our results on the separation of adenosine and inosine phosphates by paper chromatography are reported.

In spite of numerous trials with different solvent systems including those already reported in the literature together with a great number of other solvents, no satisfactory method has so far been found for the separation of all the adenosine and inosine phosphates by one-dimensional chromatography, although recent experiments with the *n*-propanol-1% ammonium sulphate solution-acetic acid (45 : 35 : 20) system [13] show promise in this respect. Mixtures containing only adenosine phosphates or only inosine phosphates are resolved by most of the methods recommended for the separation of adenosine phosphates. In our hands the procedure of Hanes and Isherwood [2], namely descending chromatography with *n*-propanol-ammonia (sp.gr. 0.880)-water (60 : 30 : 10) gave the best results. Mixtures, containing both adenosine phosphates and inosine phosphates are not resolved satisfactorily by this method, as ATP and IDP on the one hand and ADP and IMP on the other, give nearly identical spots (Fig. 1). It has been found, however, that a solvent

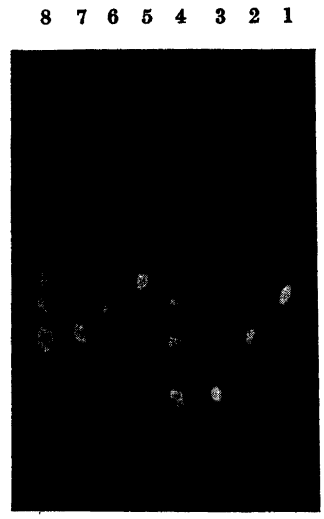

Fig. 1. One-dimensional chromatogram (Munktell No. OB paper) in n-*propanol-ammonia-water (60 : 30 : 10), 20 hours. (1) ATP; (2) ADP; (3) AMP; (4) mixture of ATP, ADP and AMP; (5) ITP; (6) IDP; (7) IMP; (8) mixture of ITP, IDP and IMP; approx. 50 μg of each compound.*

system, consisting of saturated ammonium sulphate solution-water-isopropanol (79 : 19 : 2), which had been devised [14] for the separation of nucleotides obtained by the hydrolysis of nucleic acids, separates the adenosine series of phosphates from the inosine series, although no adequate resolution occurs of the individual members in either series (Fig. 2). Combination of the two solvent systems to a two-dimensional method gives complete resolution of mixtures containing all six phosphates (Fig. 3). In the first direction, the chromatogram is developed with *n*-propanol-ammonia-water and in the second

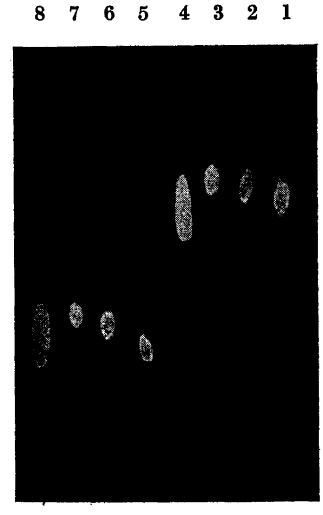

Fig. 2. One-dimensional chromatogram (Whatman No. 1 paper) in saturated ammonium sulphate solution-water-isopropanol (79 : 19 : 2), 8 hours. (1), (2), (3), (4), (5), (6), (7) and (8) same as in Fig. 1.

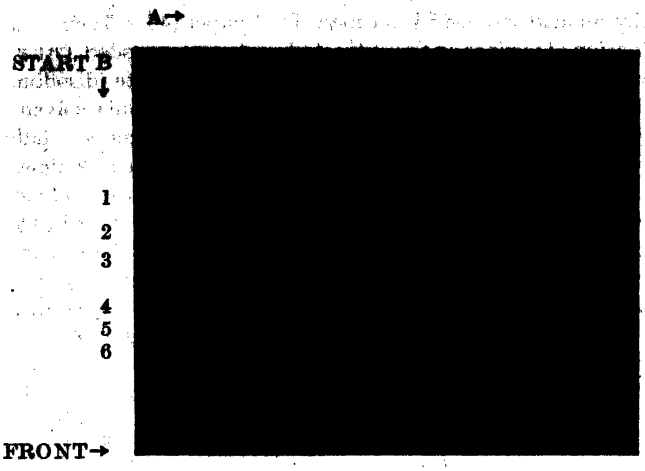

Fig. 3. Two-dimensional chromatogram (Whatman No. 1 paper) of a mixture of ATP, ADP, AMP, ITP, IDP and IMP, approx. 50 µg of each compound. (A) first solvent: n-propanol-ammonia-water (60 : 30 : 10), 40 hours; (B) second solvent: saturated ammonium sulphate solution-water-isopropanol (79 : 19 : 2), 8 hours; (1) AMP; (2) ADP; (3) ATP; (4) IMP; (5) IDP; (6) ITP.

direction, with saturated ammonium sulphate solution-water-isopropanol. This order of development gives the better results.

Application of the two-dimensional method to the separation of mixtures containing inorganic orthophosphate and pyrophosphate in addition to adenosine and inosine phosphates results in a clear separation of all components. Whereas after development in the first solvent the orthophosphate spot overlaps partly with that of ADP and IMP, and the pyrophosphate spot with that of ITP, in the second solvent both orthophosphate and pyrophosphate travel faster than any of the other components. By running the chromatogram in the ammonium sulphate system, the adenosine, inosine and inorganic phosphates appear as three sharply separated series of spots.

The results obtained by application of the method to the quantitative analysis of different adenosine and inosine phosphate preparations and the products of the reaction between ATP and actomyosin will be reported in a separate communication.

EXPERIMENTAL

ATP and ADP were prepared as the barium salts from rabbit muscle [15] and purified by ion-exchange chromatography [1]. AMP was a commercial preparation, free from ultraviolet-absorbing and phosphorus-containing contaminants. ITP, IDP and IMP were prepared from ATP, ADP and AMP by application of the method of Kleinzeller, described for ITP [16], and purified by ion-exchange chromatography [17]. From the appropriate fractions of the eluates, each compound was isolated as the barium salt and its identity confirmed by analysis (purine: acid labile P:total P). The barium salts were converted to the free acids by addition of an equivalent amount of 0.1 N sulphuric acid and the pH adjusted to 7 with ammonia. 5—10 µl of each solution, containing 10—50 µg nucleotide, were applied to the paper, the size of the starting spot not exceeding 0.5 cm in diameter. After each application, the spot was dried in a current of cold air.

Descending chromatography on acid-washed [2] Whatman No. 1 paper (46 × 53 cm) was used in all cases where determination of purines on the extracted spots was intended. Otherwise, the washing with acid was omitted and instead 0.1 % disodium versenate (disodium etylenediamine-tetraacetate dihydrate) was added [18] to the propanol-ammonia solvent. For such assays, Munktell No. OB paper has the advantage of faster running, specially in the propanol-ammonia system, whereby good resolution is obtained in shorter times. Developing times of 40 hours (20 hours with Munktell No. OB paper) in the first solvent were used without previous equilibration, by which time the solvent had overrun the paper. After previous equilibration overnight, the second solvent was run for 6—8 hours. The spots on the finished chromatogram were located by ultraviolet photography [19] or by spraying with the molybdate reagent with subsequent heating and reduction according to Hanes and Isherwood [2]. No consistent R_F-values are obtained without elaborate precautions. The relative positions of the substances, however, are reasonably constant and the spots can easily be identified by inspection. If necessary, marker substances can be run in each dimension, according to the procedure of Markham and Smith [19].

SUMMARY

A method for the separation of mixtures containing adenosine and inosine phosphates, inorganic orthophosphate and pyrophosphate by two-dimensional paper chromatography is described.

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