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Ion Exchange Chromatography of Inosine Phosphates

ADAM DEUTSCH and ROY NILSSON

Institute of Chemistry, University of Lund, Sweden

A method for the quantitative separation of the different adenosine-5'-phosphates, *viz.* adenosine monophosphate (AMP), adenosine diphosphate (ADP) and adenosine triphosphate (ATP) by ion-exchange chromatography has been devised by Cohn and Carter [1]. After adsorption on a column of the strong base anion-exchange resin Dowex-1 in the chloride form, the individual phosphates are removed as separate fractions by successive elution with 1) 0.003 *M* hydrochloric acid (AMP), 2) 0.02 *M* sodium chloride in 0.01 *M* hydrochloric acid (ADP plus any inorganic orthophosphate (P_0)) and 3) 0.2 *M* sodium chloride in 0.01 *M* hydrochloric acid (ATP).

For the last two years, extensive use was made of this method in our laboratory both for analytical and preparative purposes. Although the exchange resin Dowex-2 was employed, the eluting solvents of Cohn and Carter could be used unchanged, at variance with the findings of Leuthardt and Bruttin [2]. A drawback of the method as originally described, namely the contamination of the ADP fraction by P_0, could be eliminated by continued elution with 0.003 *M* hydrochloric acid after the removal of AMP; P_0 appears then in the effluent as a separate fraction following AMP. In several large-scale experiments with mixtures of P_0, ADP and ATP, where elution with 0.003 *M* hydrochloric acid was omitted, a sharp separation of P_0 and ADP could nevertheless be obtained. P_0 and ADP appeared either in the 0.02 *M* sodium chloride solvent as separate fractions or else — depending on the volume of the eluents applied — P_0 was obtained in the 0.02 *M* sodium chloride solvent, and ADP in the 0.2 *M* sodium chloride solvent as a separate fraction preceding ATP, without any cross-contamination of the components. Removal of P_0 can also be achieved by elution with 0.025 *M* ammonium chloride in 0.0025 *M* ammonium hydroxide solution, prior to the removal of AMP, according to the method of Khym and Cohn [3] for the separation of sugar phosphates. In some experiments with preparations of high P_0 content, a selective adsorption of the organic phosphates could be obtained by making the solution *ca.* 0.025 *M* with respect to ammonium chloride prior to adsorption.

Inorganic pyrophosphate (PP) — if present — is eluted after ADP and can be collected by elution with 0.2 *M* sodium chloride in 0.01 *M* hydrochloric acid solution as a separate fraction preceding ATP.

Application of the method to the separation of inosine-5′-phosphates, *viz.* inosine monophosphate (IMP), inosine diphosphate (IDP) and inosine triphosphate (ITP) gave — as expected — an equally clear separation of all components as in the case of the related adenosine phosphates (Fig. 1). In agreement with the observations of Cohn [4] on the relative elution positions of the isomeric inosinic and adenylic acids, each inosine phosphate is eluted after the corresponding adenosine compound and can be collected in the solvent which elutes the next higher adenosine phosphate. Thus, on chromatography of mixtures, containing both adenosine and inosine phosphates, IMP and ADP on the one hand and IDP and ATP on the other, are removed by the same solvents from the column. But whereas IMP precedes ADP as a separate fraction, no appreciable separation of IDP and ATP occurs. A separation of IDP and ATP can be achieved by a slight modification of the elution system. After removal of ADP by 0.02 *M* sodium chloride in 0.01 *M* hydrochloric acid, elution is continued with 0.05 *M* sodium chloride in 0.01 *M* hydrochloric acid, whereby IDP appears in the effluent as a separate fraction preceded by PP. ATP is eluted thereafter with 0.2 *M* sodium chloride in 0.01 *M* hydrochloric acid (Fig. 2). In this way, a mixture of AMP, ADP, ATP, IMP, IDP, ITP, P_0 and PP can be resolved into all its components. Analysis of mixtures, prepared from the chromatographically purified components and containing 1—5 mg total phosphorus, gave recoveries of at least 95 but mostly about 98 % of the individual compounds. Cross-contamination of successive fractions was under 5 %. In preparative runs, the individual compounds could generally be isolated in a yield of about 90 %, without contamination by other components.

EXPERIMENTAL

Test materials. ADP and ATP were prepared as the barium salts from rabbit muscle [5]. AMP was a commercial preparation, free from ultraviolet-absorbing and phosphorus-containing contaminants. IMP, IDP and ITP were prepared as the barium salts from AMP, ADP and ATP by application of the method of Kleinzeller, described for ITP [6]. IMP was purified by two recrystallizations of the barium salt from water and ADP, ATP, IDP and ITP by ion-exchange chromatography. For this purpose, the barium salts were first converted to the free acids. 5—20 ml Dowex-50 resin (250—500 mesh) in the hydrogen form were added to the ice-cooled solution or suspension of 0.5—2 g of the barium salt in 5—20 ml water and after vigorous stirring for 5—10 minutes at 0° C, the resin was filtered and washed with 5—20 ml water of 0° C. The solution of the free acid thus obtained was adjusted to a pH of about 8.5 with ammonia and passed through an ion-exchange column (see below). The appropriate fractions of the effluent were pooled and the barium salt precipitated by addition to the neutralized pooled fractions at 0° C of a small excess of 2 *N* barium acetate solution, followed by 1—2 volumes of ethanol in the case of ADP and IDP. The barium salts were centrifuged, washed with water/ethanol, reconverted to the free acids with Dowex-50 and after neutralization stored at —20° C. Their identity was further established by chemical analysis (purine : pentose : acid labile phosphorus : total phosphorus) and two-dimensional paper chromatography [7].

Ion-exchange chromatography. Columns of the anion-exchange resin Dowex-2 (250—500 mesh) in the chloride form were used throughout. In analytical runs, columns of bed size 0.3 cm^2 × 2.5 cm and 0.8 cm^2 × 5 cm (flow rate 0.5—1 ml/min) were used for the chromatography of material with a total phosphorus content of under 1 mg and 1—5 mg respectively. In preparative runs, as in the purification of the test materials, columns of bed size 4 cm^2 × 15 cm and 8 cm^2 × 30 cm (flow rate 3 ml/min) were used at 0° C for amounts of 50—100 mg and 200—400 mg total phosphorus respectively. The test substances were adsorbed from dilute ammoniacal solution at about pH 8.5 followed by

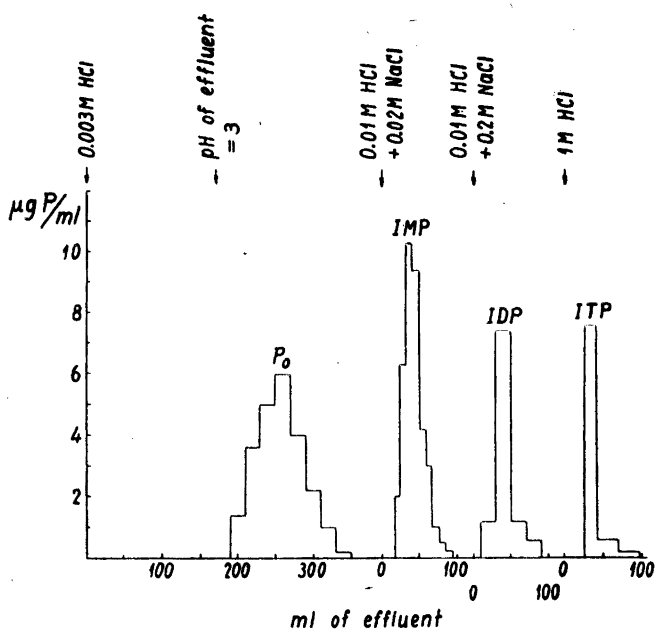

Fig. 1. Ion-exchange separation of inosine phosphates and orthophosphate. Exchanger: Dowex-2, 250—500 mesh, 0.8 cm² × 5 cm, chloride form; flow rate 0.5 ml/min. Test materials: orthophosphate (543 µg), IMP (349 µg), IDP (240 µg), ITP (248 µg) (calculated as total phosphorus present). Recoveries (based on phosphorus analysis): orthophosphate, 98 %; IMP, 98 %; IDP, 99 %; ITP, 97 %.

washing the column with water until the effluent became neutral. The eluting solvents of Cohn and Carter were used except in the separation of mixtures containing both adenosine and inosine phosphates, where elution with 0.05 *M* sodium chloride in 0.01 *M* hydrochloric acid solution was interposed between the eluting agents of next lower and next higher anion content. Fractions of approximately 10 ml in analytical runs and of 50—100 ml in large-scale experiments were taken with an automatic fraction collector adjustable to different time intervals. Each fraction was analysed for total phosphorus and the different fractions were further identified by determination of the acid labile phosphorus, measurement of ultraviolet-absorption and paper chromatography. Individual column runs were carried out with each test substance in order to ascertain the elution position and as an additional test for purity in the final preparations, prior to the analysis of mixtures. Eventual cross-contamination of two successive fractions can be detected by the ratio of total phosphorus : acid labile phosphorus, the ratio of the optical densities at 250 : 260 mµ and by one-dimensional paper chromatography in saturated ammonium sulphate solution : water : *iso*propanol (79 : 19 : 2). This solvent system gives a sharp separation of the adenosine series of phosphates from the inosine series, although no adequate resolution occurs in either series [7]. It is particularly suitable for the present purpose, as cross-contamination by only one compound from each series can be expected.

Analytical procedures. Phosphorus was determined by the method of Allen [8] using a Klett Summerson photoelectric colorimeter with a red filter and was differentiated in separate analyses into total phosphorus, inorganic phosphorus and acid labile phosphorus determined after 10 minutes hydrolysis in *N* hydrochloric acid at 100° C. Pentose was determined by the method of Mejbaum [9] as modified by Albaum and Umbreit [10]. A Klett Summerson photoelectric colorimeter was used with a red filter. Optical densities

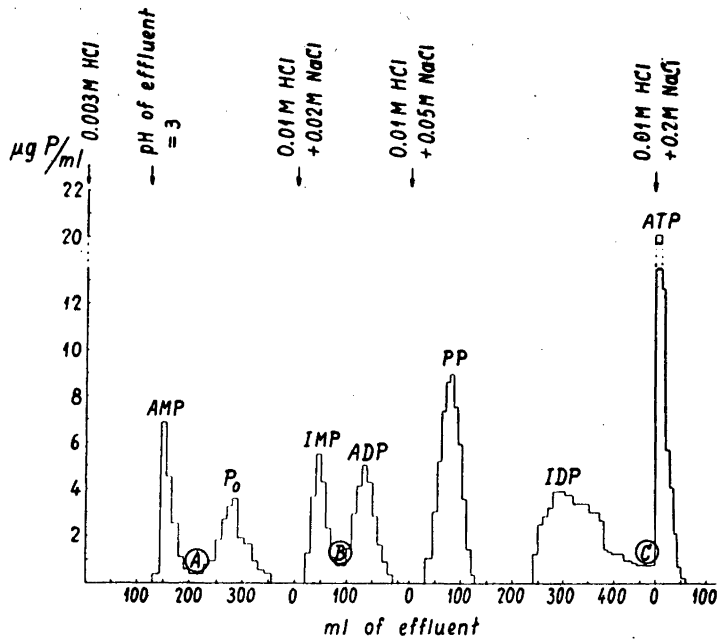

Fig. 2. Ion-exchange separation of inosine phosphates, adenosine phosphates, orthophosphate and pyrophosphate. Exchanger: Dowex-2, 250—500 mesh, 0.8 cm² × 5 cm, chloride form; flow rate 0.5 ml/min. Test materials: orthophosphate (240 µg), pyrophosphate (462 µg), AMP (200 µg), ADP (250 µg), ATP (465 µg), IMP (200 µg), IDP (578 µg) (calculated as total phosphorus present). Recoveries (based on phosphorus analysis): orthophosphate, 102 %; pyrophosphate, 95.5 %; AMP, 97.5 %; ADP, 97.5 %; ATP, 99 %; IMP, 98 %; IDP, 98 %. Cross-contaminated material: 2.3 % at (A), 4.0 % at (B), 2.9 % at (C).

were measured in 0.01 *N* hydrochloric acid solution in a Beckman Universal Spectrophotometer, Model DU, the molecular extinction coefficients of 14 200 at 260 mµ for adenosine phosphates [1] and 13 200 at 250 mµ for inosine phosphates [11] being used.

SUMMARY

By slight modifications of the method of Cohn and Carter for the resolution of adenosine phosphates, a method has been developed for the resolution of a mixture of orthophosphate, pyrophosphate, inosine phosphates and corresponding adenosine phosphates into the individual components.

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