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Chromatography of nucleoside mono- and polyphosphates on hydroxyapatite

Chromatography on hydroxyapatite has been established as a useful tool in protein chemistry by TISELIUS, LEVIN AND HJERTÉN[1]. Many applications to protein purification problems have been reported so far; this technique appears to be particularly interesting in the fractionation of phosphoproteins[2]. Work carried out in this laboratory over the past four years has shown the usefulness of this method in the field of polynucleotides[3–6]. During an investigation aiming at elucidating the nature of the process of polynucleotide adsorption on hydroxyapatite the chromatographic behaviour of nucleoside mono- and polyphosphates was studied in detail. The results obtained will be briefly reported here because of their potential use-

fulness in several fractionation problems, particularly those involving both nucleoside phosphates and proteins or polynucleotides.

Hydroxyapatite was prepared according to TISELIUS *et al.*[1]. Chromatographic experiments were performed at room temperature, generally on 1×7-cm columns, which were loaded with 1–5 mg of the substances under investigation (these were commercial products purchased from Calbiochem, Los Angeles, Calif., or Pabst, Milwaukee, Wisc.). Elution was carried out, at a flow rate of about 35 ml/h, with a linear molarity gradient of potassium phosphate buffer (pH 6.8) (0.001 to 0.5 M); the total eluent volume was 200 ml. The ultraviolet absorption of the fractions was used to follow the elution curve, except in the case of phosphoribose pyrophosphate where ribose assay was used[7]. In the above experimental conditions, purine and pyrimidine bases (adenine, thymine, cytosine, methylcytosine and uracil), ribo- and deoxyribonucleosides (adenosine, cytidine, uridine), and coenzyme derivatives, like thiamine and riboflavin, were not retained on the columns equilibrated with 0.001 M phosphate.

Nucleoside monophosphates (2',3' mixed isomers of AMP, CMP, UMP, GMP, 5' isomer of AMP), thiamine monophosphate and riboflavin 5'-phosphate were also eluted by 0.001 M buffer, but they were slightly retarded.

On the other hand, nucleoside polyphosphates were strongly adsorbed and needed high molarities of phosphate buffer to be eluted. As an example, Fig. 1 shows a chromatogram obtained with a mixture of AMP, ADP, ATP and adenosine tetraphosphate. Rechromatography experiments of the peaks obtained indicated that separation was complete. The peaks were identified, on the basis of their typical elution molarity, by running the single compounds, separately.

Similar experiments showed that other nucleoside 5'-diphosphates (CDP, UDP) and thiamine pyrophosphate were eluted at the same phosphate molarity as ADP; whereas nucleoside 5'-triphosphates (deATP, CTP, UTP) were eluted like ATP. An interesting exception is that GDP and GTP, and, at a smaller extent, IDP and ITP, are eluted at higher phosphate molarities than the other di- and triphosphates, respectively. This finding is probably related to phenomena of intermolecular association, of the type described by GELLERT, LIPSETT AND DAVIES[8] for GMP.

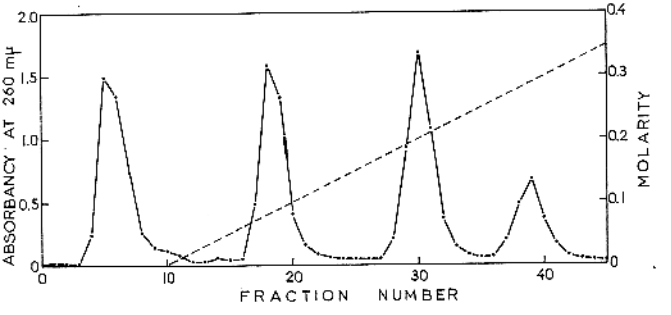

Fig. 1. Chromatography of a mixture of AMP, ADP, ATP, and adenosine tetraphosphate on a 1×10-cm hydroxyapatite column. The yields were 133 %, 113 %, 113 % and 50 %, respectively, owing to the fact that ADP, ATP, and, at a much larger extent, adenosine tetraphosphate were contaminated by the lower phosphate, as indicated by separate experiments. The overall recovery was 101 %. Fractions of 3 ml were collected. Elution was carried out with a molarity gradient of potassium phosphate buffer (pH 6.8) (0.001 to 0.5 M; 100+100 ml); this was followed by refractometry and is indicated by the broken line (right-hand ordinate).

Compounds in which the pyrophosphate has no free secondary acid group, like ADPribose, NAD, FAD, UDPglucose, were not retained on the column at 0.001 M phosphate buffer. NADP and coenzyme A were retarded only slightly more than nucleoside monophosphates; phosphoribose pyrophosphate was eluted at a higher molarity than triphosphates.

While the significance of these findings in relationship with the adsorption process will be discussed elsewhere in a detailed presentation of this work, one basic conclusion appears to be immediately evident, namely that adsorption on hydroxyapatite of the compounds investigated here is only related to the ionization of phosphate groups and that the organic moieties of the molecules do not interfere with the process.

Several fractionations may be performed on the basis of the above observations. Although other available methods (particularly ion-exchange chromatography and zone electrophoresis) also will be useful, hydroxyapatite may prove more convenient in several instances because of its ease and rapidity. In one particular case this method has unique advantages, namely when nucleoside mono- or polyphosphates, or coenzymes, have to be separated from high-molecular-weight substances like polynucleotides and proteins.

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