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Separation of Nucleotides on Ecteola Cellulose

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A chromatographic method is described for separating nucleotides. The method is based on ion-exchange on ecteola cellulose. The nucleotides are separated by elution with a linear gradient of triethylammonium acetate at pH 6.

The method is suitable for the analysis of solutions or extracts containing labile compounds, since the separation is performed at a pH not far from neutrality and since the eluent is easily removed by freeze-drying.

Determination of the nucleotide content of different organs and organisms is chiefly based upon separation of the nucleotides by ion-exchange chromatography on different anion-exchange resins.

In 1950 Cohn [1] developed a method in which the nucleotide components of nucleic acid were separated by elution from Dowex-2 columns with hydrochloric acid. In 1953 Cohn and Volkin [2] extended the versatility of the method by introducing formic acid — formate mixtures as eluents. Several authors have since modified this method in various respects. For example Bergkvist and Deutsch [3] and Hurlbert *et al.*[4] developed different formic acid — formate elution systems and Pontis and Blumson [5] a system with a gradient of calcium chloride and dilute hydrochloric acid.

Very complex nucleotide mixtures can be resolved with these methods. However, there remain two major problems; the quantitative recovery of the components and the recovery of labile compounds in intact form.

The compounds can be recovered in two different ways from the dilute solution in which they are obtained from the column; the eluent can be removed or the eluted nucleotides can be removed selectively. In some cases the eluent is easily removed, *e.g.* formic acid by freeze-drying and ammonium formate by sublimation, but in other cases, *e.g.* sodium formate, it is difficult to remove the eluent.

The best method for removing the nucleotides from the eluates is the procedure developed by Crane and Lipmann [6]. The nucleotides are adsorbed on charcoal from acid solution and can then be eluted with ammoniacal ethanol. However, even if the removal of the nucleotides is quantitative the method has a drawback in that the elution is not quantitative and in some cases only little of the compounds is recovered.

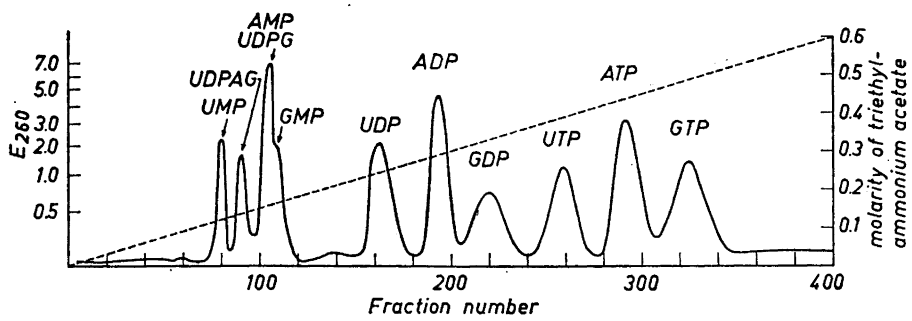

Fig. 1. Ion-exchange chromatography of nucleoside polyphosphates. Column: ecteola cellulose, 1 cm × 25 cm. Eluent: a linear gradient of triethylammonium acetate, pH 6. The adsorbed material had a total optical density $E_{260} = 825$. Recovery 98 %.

Furthermore, in most of the methods used, the nucleotides are exposed to rather extreme acid and alkaline conditions which may destroy labile compounds. The advantages gained by using a mild extracting agent such as phenol or ethanol are therefore more or less lost on exposing of the extract to drastic changes in pH either during the ion-exchange chromatography or in the concentration step by the charcoal method.

To avoid the difficulties mentioned, the ion-exchange chromatography of extracts containing acid or alkali labile compounds should be performed at a pH around pH 7 and one should use an eluent that can be quantitatively removed at low temperatures. The method described below appears largely to satisfy these requirements.

The ion-exchange material which promised to give the best results was the weakly basic ecteola cellulose, which has been used by several authors [7–11] for fractionation of oligonucleotides and nucleic acids. Michelson [11] also used ecteola columns for separation of different adenosine monophosphates.

In previous investigations, sodium chloride or lithium chloride were used as eluents. In preliminary experiments we have also found a good separation of AMP *, ADP and ATP by using a gradient of lithium chloride at pH 6. But since it was difficult to remove the lithium chloride — even if it can be done by extraction with absolute ethanol after freeze-drying — we sought an eluent more suitable for our purposes.

We found triethylammonium acetate to serve the purpose well. It gives good separations, it is easily removed by freeze-drying, and since it has a very low light absorption at wavelengths over 250 mμ it does not interfere with the measurements of the fractions.

Fig. 1 shows the separation of 11 nucleotides on an ecteola column using a linear gradient of triethylammonium acetate at pH 6 as eluent. The gradient

* A = adenosine
G = guanosine
C = cytidine
U = uridine
UDPG = UDP-glucose
UDPAG = UDP-acetylglucosamine
MP = 5′-monophosphate
DP = 5′-diphosphate
TP = 5′-triphosphate
DPN = diphosphopyridine nucleotide

was applied in two steps. The first step running from 0 to 0.3 M triethylammonium acetate eluted the nucleoside mono- and diphosphates with the exception of GDP; in the next step a gradient from 0.3 to 0.6 M eluted the remaining compounds.

In each group of nucleotides, *viz.* the different mono-, di- and triphosphates, the substances were eluted in the order uridine, adenosine and guanosine compounds. As judged from the elution position for CTP between ADP and UTP, the cytidine derivatives are eluted before the corresponding uridine derivatives. DPN is eluted before UMP. Bases and nucleosides are only weakly retained on the column; part of them runs straight through the column and the rest is eluted in the first fractions.

Very complex mixtures of nucleotides are not completely resolved by this method. The overlapping of UDPG, AMP and GMP is seen in Fig. 1, and with an increased number of components in the solution to be chromatographed there should be still more chances for overlapping. However, heterogeneous fractions can be resolved into individual components by rechromatography of the freeze-dried fraction, using a less steep gradient.

The method is very useful for the separation of extracts containing only a few components. The nucleotides in extracts from the rabbit's vagina [12] were thus well and rapidly separated. The method has also been used for resolving complex fractions obtained by chromatography on Dowex-1. The uric acid peak from human milk [13] was thus resolved into three components when rechromatographed on ecteola [14].

The recovery of each of the compounds is quantitative. However, the ecteola cellulose seemed rapidly to alter its exchange properties in that respect. The first four or five runs gave a recovery of 95—105 %, but in the following runs it decreased and after eight runs only 65 % of the adsorbed optical density was eluted. The irreversible adsorption might be due to the accumulation of metal ions on the column, as was reported by Lipkin *et al.*[15] to occur on Dowex-1. However, treatment of the ecteola columns with ethylenediaminetetraacetic acid did not restore the original ion-exchange properties as it did with Dowex-1.

Triethylammonium acetate is easily removed by freeze-drying, but the formate is still better in that respect and should therefore presumably be more suitable as eluent. However, substituting formate for acetate impaired the separation; therefore the acetate was preferred.

The advantages of the present method can be summarized as follows: (1) the separation is performed under mild conditions, at almost neutral pH, (2) the eluent is easily removed also under mild conditions, as *e.g.* by freeze-drying, (3) the separation is very rapid, a complete run not taking more than 24—36 h and (4) the individual compounds appear as sharp and high peaks, which make it possible to use rather small samples.

EXPERIMENTAL

Nucleotides were obtained from Sigma Chemical Co. Some samples of UMP, UDPG, UDPAG and UDP were, however, substances isolated from cow's colostrum [16].

Triethylamine was obtained from Eastman Kodak Co. It contained a component with high absorption in the ultraviolet range but could be purified by distillation through a 35 cm Vigreux column.

Acetic acid and *formic acid* were of Merck's analytical grade.

Chromatography. Ecteola cellulose (Whatman) in powder form was suspended in water and freed from fines by decantation. A dilute suspension of the ecteola powder was poured into a column and allowed to settle under gravity. The column (1 cm × 25 cm) was washed with 200 ml of M triethylammonium acetate pH 6 and then with water to remove the excess triethylammonium acetate.

The substances to be separated were adsorbed from a dilute water solution at a pH between 6 and 7. After adsorption the column was washed with three bed volumes of water; the optical density of the effluent at 260 mμ was then usually below 0.05.

The elution was performed with a linear gradient of triethylammonium acetate with the following composition: 500 ml of distilled water (in a flask, No. 1, fitted with a magnetic stirrer and connected to the column) and 500 ml of 0.3 M triethylammonium acetate, pH 6 (in another flask, No. 2, connected with flask No. 1 by a siphon). The two flasks were placed at the same level. If the separation was not finished by this solution it was continued with 500 ml of 0.3 M triethylammonium acetate in flask No. 1 and 500 ml of 0.6 M solution in flask No. 2.

The flow rate was 1—2 ml per min.

The optical density of the eluate was continuously read at 254 mμ in a LKB Uvicord Ultraviolet Absorptiometer. The eluate was then collected in 5 ml fractions in a fraction collector.

The identification of the components was based on ultraviolet spectra and R_F-values in several paper chromatographic solvent systems. Appropriate fractions from the ion-exchange were pooled and freeze-dried; the residue was taken up in a small volume of water and used for paper chromatography before and after hydrolysis.

The purine or pyrimidine part of the nucleotides was identified after hydrolysis for 1 h in N hydrochloric acid at 100°C by paper chromatography in *iso*butyric acid — ammonia (*d* 0.880) — water (66:1:33).

The sugar moiety was identified after hydrolysis in N hydrochloric acid for 10 min at 100°C, by chromatography in ethylacetate — pyridine — water (20:7:5) and butanol — pyridine — water (6:4:3). The carbohydrate spots were detected by spraying with aniline hydrogen phthalate and with the Elson-Morgan reagent.

The identification was finally checked by chromatography in *iso*butyric acid — ammonia (*d* 0.880) — water (66:1:33) and ethanol — M ammonium acetate, pH 7.5 (70:30).

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