A METHOD FOR THE SEPARATION OF NUCLEOTIDES BY CONCAVE GRADIENT ELUTION

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Since the introduction and development of ion-exchange chromatography in the nucleic acid field by COHN[1], improved methods of preparation and analysis of nucleotides, nucleosides, and their bases have led to the discovery of many new substances. In most of these separations there have been two main problems, the resolution of the mixtures and the recovery of the nucleotides separated by the chromatographyl.

This paper describes a method which allows separation of most of the mono-, di-, and triphosphates of adenosine, guanosine, cytidine and uridine as well as the sugar-containing nucleotides in a single chromatographic run on an anion-exchange resin column, and at the same time permits an almost quantitative recovery of the nucleotides separated in the column. It is based on the use of a pH-decreasing concave gradient of calcium chloride and on the solubility of calcium chloride in a mixture of absolute alcohol and ether.

EXPERIMENTAL

Materials

DPN** and the adenosine, guanosine, cytidine and uridine nucleotides were purchased from Pabst Laboratories, Wisconsin, U.S.A. The sugar-containing nucleotides were a generous gift from Dr. E. CABIB. The latter were as barium salts and, prior to chromatography, the barium was removed by double decomposition with sodium sulphate.

Preparation of the columns

Dowex-2 X10 resin was used in the preliminary experiments. Better results were obtained by using Dowex-1, 2% (X2) or 4% (X4) cross-linked resin, and this type of resin has been used in all subsequent experiments.

The resin in the chloride form was freed from fines by repeated decantation from water. It was classified according to the sedimentation time in a 2-l beaker, into "5, 10, 15 and 20 minutes" resin, then washed twice, alternatively with 1 *N* sodium hydroxide, and 1 *N* hydrochloric acid. Then the resin was washed with water until the pH was above 5. The columns used were 120 cm long, 1 cm in diameter and were prepared by allowing a suspension of "5 minutes resin" to settle in the bottom followed by portions of the "10, 15 and 20 minutes resin" until a bed 100 cm high was obtained.

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** The following abbreviations are used: AMP, ADP and ATP for the mono-, di-, and triphosphates of adenosine. Similar symbols are used in guanosine (G), uridine (U) and cytidine (C). DPN is used for diphosphopyridine nucleotide; UDPG for uridine diphosphate glucose; UDPAG for uridine diphosphate acetylglucosamine; GDPM for guanosine diphosphate mannose.

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Elution

The nucleotide solution, adjusted to pH 7.5, was passed through the column which was washed with water and the elution started.

The nucleotides were eluted with a concave gradient of calcium chloride and dilute hydrochloric acid. A concave gradient can be obtained by different experimental arrangements[2-4]. The system[2] used to produce it consists of two parallel-sided containers of cross-sectional areas A_1 and A_2. The larger bottle (A_1) is the mixing chamber and contains a magnetic stirrer, while the smaller (A_2) is the reservoir. They are joined, through their lower outlets by glass-polythene tubing, which allows the passage of the concentrated solution, from the reservoir to the mixing chamber. Simultaneously, the eluent flows from the mixing chamber through a glass outlet into the column.

This system, according to BOCK AND NAN-SING LING[2] delivers a solution of concentration:

$$C = C_2 - (C_2 - C_1)\ (1 - a)^{A_2/A_1}$$

where C_1 and C_2 are the initial concentrations in the vessels of cross-sectional area A_1 and A_2.

$a = v/V$ where v is the volume of eluent which has passed through the system, and V is total initial volume present in the system.

A concave gradient is obtained when the ratio A_2/A_1 is less than unity. The degree of concavity of the curve will depend on the value of this ratio. We have found that with a ratio of 0.6 a suitable concavity is obtained.

In the separation shown in Fig. 1 the reservoir (aspirator bottle of 5 l) was filled with 3 l of 0.15N calcium chloride in 0.01N hydrochloric acid, and the mixing chamber (aspirator bottle of 10 l) with 5 l of 0.0001N hydrochloric acid. The flow rate was regulated by air pressure, controlled by a mercury monostat. A rate of 0.5 ml/min was used in these experiments and 10 ml fractions were collected by a Tower's Model A Automatic Fraction Collector.

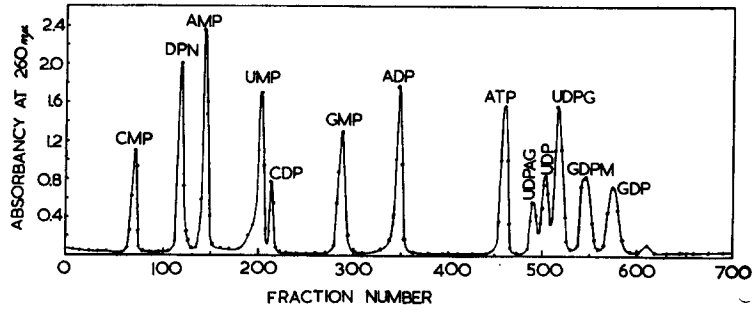

Fig. 1. Separation of nucleotides on Dowex-1 X2 in the chloride form. For details see EXPERIMENTAL.

Concentration

The fractions under a peak were pooled and the pH adjusted to 7 by addition of a suspension of calcium hydroxide. The neutral solution was concentrated *in vacuo* by means of a rotary evaporator[5] to a small volume. Concentration to dryness was achieved by freeze drying in an Edwards Freeze-Dryer Model 10P. The calcium

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chloride was dissolved by adding a mixture of absolute alcohol–ether (1:1 v/v) leaving in suspension the calcium salt of the nucleotide. The suspension was kept in the deep-freeze overnight, the precipitate centrifuged, washed twice with the alcohol–ether mixture and twice with the ether. Then the tube was placed in a desiccator and the remaining ether pumped out. After several hours, the desiccator was opened, the substance weighed, suspended in water and the calcium removed by double decomposition with ammonium oxalate. These operations can be carried out conveniently if the freeze drying is done in a centrifuge tube. However, care must be taken to avoid material passing into the pump trap. The solutions, free of calcium, were used for analyses.

Note

We have found that the percentage recovery of the nucleotide, as the calcium salt, is influenced by the presence of traces of water in the freeze-dried powder. We attribute the low recovery values of AMP and GDP (Table I) to the above cause since in other runs higher recoveries were obtained.

Analytical procedures

Optical densities were measured in a Unicam SP, 500 Spectrophotometer. The following analytical methods were used: FISKE AND SUBBAROW[6] for phosphate, PARK AND JOHNSON[7] for glucose and mannose liberated from UDPG and GDPM after hydrolysis, REISIG *et al.*[8] for acetylglucosamine liberated from UDPAG, and CLARK *et al.*[9] for chloride.

Paper chromatography was carried out according to PALADINI AND LELOIR[10], together with the above methods, to ascertain the identity and purity of the isolated nucleotides.

RESULTS AND DISCUSSION

The principle of continuously increasing the concentration of the eluent has been referred to as gradient elution by TISELIUS[11], who has pointed out its advantages over the stepwise elution method. The former technique was used by ALM, WILLIAMS AND TISELIUS[12] for the resolution of carbohydrate mixtures. Following this work, gradient elution has been applied to the separation of mixtures of organic acids[13,14] oligosaccharides[15], amino acids[16], peptides[17] and proteins[4]. More recently this method has been used for some separations of nucleotides[18,19] and, in this field, a slightly different procedure has been developed by HURLBERT *et al.*[20], which has been referred to as extended gradient chromatography. A common feature of all these procedures is that the plot of eluent concentration against effluent volume describes an exponential curve, which is characterized by a steep ascent at first, which subsequently flattens and approaches asymptotically the value of the concentrated solution. This type of elution tends to crowd together or cause mixing of the more easily eluted substances, while as the rate of increase of eluent concentration decreases with time, the elution of the later substances is unduly delayed. These disadvantages of the convex exponential gradient were apparent when it was applied to the separation of complex mixtures of nucleotides. In order to obtain resolution of the mixtures which appeared as one peak from a column, it was necessary to rechromatogram them[20,21]. Alternatively, use has been made of only the flat part of the gradient[19]. LAKSHMANAN AND LIEBERMAN[3] have emphasised the advantage of a concave gradient where there is a gradual increase in eluent concentration, when the concentration itself is small, the rate of change increasing with the concentration. In this way the more easily eluted substances are spread apart in the chromatogram and as the concentration in the last part of the curve increases rapidly, the later substances are

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eluted without undue delay. The gradient effect is kept roughly constant throughout the chromatogram, which minimises tailing and keeps the peaks narrow.

In the separation of a mixture of nucleoside mono-, di- and triphosphates, the monophosphates are separated in the region of pH 3 by a low salt concentration, whereas a lower pH is needed to elute di- and triphosphates if very high salt concentrations are to be avoided which could be troublesome in the recovery procedures. These requirements are fulfilled by a concave gradient of calcium chloride in hydrochloric acid, which produces the desired pH values in both extremes of the chromatogram (Fig. 2).

The result obtained by the application of this procedure to a complex mixture of nucleotides is shown in Fig. 1. More effective resolution of UDP and the UDP-sugar compounds, similar to that achieved by PONTIS *et al.*[19], was obtained by means of a slightly flatter concave gradient which maintained the separation of the earlier compounds. Being more time-consuming, because of the larger volume of eluent required, it is used less frequently.

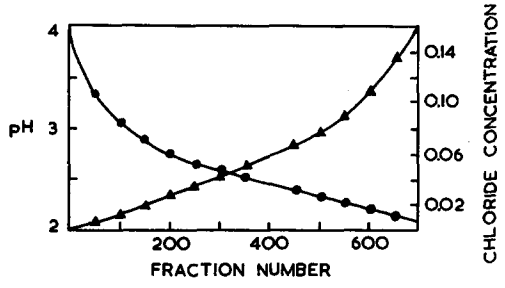

Fig. 2. Variation of pH (●), and total chloride concentration (▲), expressed as normality, in the effluent, from the chromatography shown in Fig. 1.

The use of a long column permits the separation of the nucleotides in a single run, allowing at the same time easy correlation between a chromatogram of known and unknown compounds. Concave gradient elution has been used successfully in this laboratory with shorter columns, together with smaller eluent volumes, and obviously it can be applied to a particular separation in the same way as the convex exponential gradient. Particularly, the application in this laboratory of concave gradient elution has permitted the resolution of the 2′,5′- and 3′,5′-diphosphates of adenosine[22], separation which was not achieved by other methods.

It is interesting to compare the elution order of nucleoside di- and triphosphates obtained by this method (Fig. 1) with that achieved when sodium chloride is used as eluting agent, that is GDP, UDP, ATP, GTP, UTP. We suggest that this can be ascribed to the different degree of dissociation and solubility of the calcium and sodium salts of the nucleotides. A similar inversion is seen in the case of the uridine diphosphate-sugar compounds and GDPM.

Concave gradient elution was applied initially to the separation of nucleotides on formate resin columns, using ammonium formate as eluting agent. Promising results (Fig. 3) were obtained even with resin of very high cross linking. The disadvantages in the recovery procedure led to the calcium chloride method to be developed.

The recovery of nucleotides from eluates is not easy, many different procedures

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having been used. The method of COHN[1] which makes use of small resin columns, is not applicable to all nucleotides because of the high acidity to which the substances are exposed during the process of concentration. On the other hand, formate or acetate columns have been preferred to chloride ones, because of the relative ease with which these acids and their ammonium salts can be volatilized. However, during this removal some hydrolysis of labile compounds can occur[20,21].

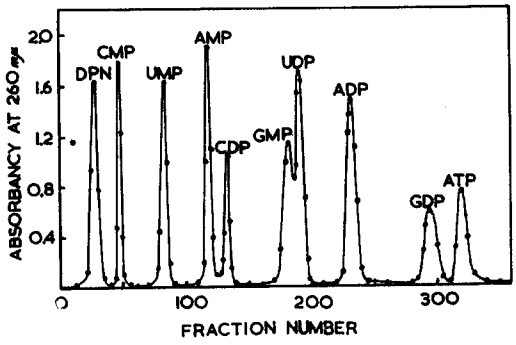

Fig. 3. Separation of nucleotides on Dowex-2 X10 in the formate form. Mixing chamber 3750 ml of water. Reservoir 1500 ml of 3 M ammonium formate pH 4.6, $A_2/A_1 = 0.5$.

Adsorption on activated charcoal has been used for the concentration of nucleotides from effluents either from chloride or formate columns[20,23-28]. Although this is a very convenient technique for the removal of salts, its use could lead, depending on the charcoal batch, to irregular results, poor recovery, and hydrolysis of labile compounds even with eluting agents as mild as neutral dilute alcohol[29]. However, improved nucleotide elution is obtained when the charcoal is washed with a solution of ethylenediaminetetraacetic acid after adsorption of the nucleotides, and prior to the elution[19].

Attempts to precipitate nucleotides as mercury salts from the effluent fractions[30], following the procedure of CAPUTTO *et al.*[31] for the isolation of yeast nucleotides, has not been very successful. This is probably because of the low concentration of nucleotides, together with the high salt concentration, which is attained in the last steps of ion-exchange chromatography. Moreover, recently it has been found that mercury precipitation leads to high losses[19].

In some cases the nucleotides have been precipitated as the barium salts, by addition of barium acetate or barium chloride[32-34] in the presence of alcohol, to the concentrated effluent fractions. In others, lithium hydroxide has been used to neutralize acid effluents from columns, followed by concentration and precipitation of the nucleotide lithium salt on addition of acetone and alcohol[35]. No attempt has been made to use these procedures as general methods of recovery.

LELOIR AND CARDINI[36] have eluted hexosamine phosphates from resin columns with calcium chloride and they have shown that the sugar phosphates can be freed from the inorganic salt, after concentration, by dissolving the latter in alcohol. This recovery procedure can be improved and applied to nucleotides, since all deliquescent calcium salts[37] are soluble not only in alcohol but also in an absolute alcohol–ether mixture. Consequently it is possible to separate a solid mixture of calcium chloride

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and calcium salt of the nucleotides by solution of the former in a mixture of equal volumes of absolute alcohol and ether, owing to the insolubility of the latter in these solvents. The application of this technique following the procedure described under EXPERIMENTAL permits an almost quantitative recovery of the nucleotides (Table I), with the further advantage that this isolation has been achieved at pH 7, thus avoiding hydrolysis of labile compounds. In particular UDPG recovered by this procedure is free of UMP which usually arises from hydrolysis when other methods have been used[19,31].

TABLE I

AMOUNT OF NUCLEOTIDES RECOVERED

The figures represent the total μmoles of nucleotide in the effluent, under a peak, and the total μmoles of nucleotide after recovery, as the ammonium salt, calculated from the absorbancy at their respective maxima.

Nucleotide	*μmoles of nucleotide in the effluent*	*μmoles of nucleotide recovered*	*Per cent recovery*
CMP	11.5	11.0	96
DPN	6.9	6.8	99
AMP	9.7	8.4	87*
UMP	12.7	11.4	90
CDP	5.7	5.4	95
GMP	10.8	10.0	93
ADP	7.7	7.2	94
ATP	7.8	7.5	96
UDPAG	4.4	4.0	92**
UDP	6.0	5.7	95**
UDPG	12.1	11.1	93**
GDPM	4.3	4.3	100
GDP	6.7	5.7	85*

* See Note in EXPERIMENTAL section.
** Only the main part of the peak was recovered.

The method described in this paper has been primarily concerned with the separation of known nucleotides and their recovery from the effluent fractions by a very gentle procedure with the aim of applying it to the study of nucleotides present in biological materials.

We should like to point out that this chromatographic method is applicable to organic acids, phosphoric esters, particularly sugar phosphates and in general to all anionic substances which can form calcium salts insoluble in an alcohol–ether mixture.

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SUMMARY

A method is presented for the separation of nucleotides on ion-exchange resin, by concave gradient elution. An almost quantitative procedure for the recovery of the nucleotides in the effluents is described.

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THE DIFFUSION OF VARIOUS SUBSTANCES THROUGH RAT DIAPHRAGM

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Earlier studies have reported that the diffusion constant holding for the movement of potassium in the extracellular space of rat muscle and brain appears reduced below the free solution value[1,2]. By contrast, the diffusion of other substances, both ions and neutral molecules, is unimpeded in this way. In the present series of experiments a

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