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AN AUTOMATED SYSTEM FOR ION-EXCHANGE CHROMATOGRAPHY OF ACID-SOLUBLE NUCLEOTIDES AT THE NANOMOLE LEVEL

PERTTI VIRKOLA
Biochemistry Laboratory, State Serum Institute, Helsinki 28 (Finland)

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SUMMARY

An automated ion-exchange column chromatography system for the separation of acid-soluble nucleotides from various biological sources is presented. This system, which is a modification of earlier methods used, gave satisfactory recoveries of the nucleotides at the nanomole level.

INTRODUCTION

The study of the free nucleotides of a micro-organism is often laborious because of the complex nature of the nucleotide pool. Furthermore, the rapid growth of the organism and the technical difficulties in obtaining the extract may complicate the analysis[1-3]. In order to study the qualitative and quantitative variation of a nucleotide pool of a micro-organism under *e.g.* different growth conditions, an analytical method with good resolution and sensitivity is necessary.

Separation of nucleotides by means of ion-exchange chromatography was introduced by COHN[4,5], gradient elution was added to the procedure by HURLBERT *et al.*[6] and SCHMITZ *et al.*[7], and since then several other modifications have been published[8-13]. In addition to ion-exchange resins, cellulose based exchangers have been used[1,2,14-19]. Two-dimensional paper chromatography, paper electrophoresis combined with paper chromatography, ion-exchange paper chromatography, and recently thin-layer chromatography have also been used for nucleotide separations[1,2,20-35].

The optimum amount of nucleotides suitable for paper chromatography is 10–200 μg, for paper electrophoresis 100–500 μg, for ordinary thin-layer chromatography 0.2–30 μg per spot, and for column chromatography from 50 μg up to several hundred mg per column.

The best resolutions in the separation of complex nucleotide mixtures are obtained by thin-layer and column chromatography[34]. Column chromatography seems to be most suitable for primary separations of complex nucleotide mixtures because it can be used both as an analytical and a preparative tool simultaneously.

In our studies on the nucleotide metabolism of mycoplasma, ion-exchange column chromatography was used for the separation and partial identification of the

nucleotides. In this connection we have further modified the separation methods previously used in this laboratory[2,3]. As a result an automated, sensitive and flexible system, useful for the analysis of nucleotides of biological material, is presented.

MATERIALS AND METHODS

Chemicals

All chemicals were of analytical grade. The nucleotide standards were purchased from Calbiochem, Luzern, Switzerland, and checked for purity by means of paper chromatography.

Biological test sample

The biological test sample was a cold perchloric acid extract, 2–40 ml[2,3] of cells of *Mycoplasma laidlawii* A, grown in a liquid medium[36].

Anion-exchange columns

The resin was Dowex-2, 200–400 mesh, 8% cross linked, converted to the HCOO-form, and packed into conventional glass columns. The regeneration of the columns was performed as described previously[2,3].

All connections were made by means of small-bore teflon or acid-resistant rubber tubing (I.D. 0.045 and 0.081, "Acidflex", Technicon Corp., Tarrytown, New York, U.S.A.) and by using teflon connectors from the same manufacturer.

Components of the automatic system

A constant flow of the solutions from a gradient mixer (Varigrad, rectangular model, nine compartments, 500 ml each, Buchler Instruments Inc., Fort Lee, N.J., U.S.A.) was achieved by using a reciprocal pump (Accu-Flo, flow rate 3–700 ml/h, Beckman Instruments Inc.). The elution was followed at two wavelenghts (260 and 275 nm) with a multiple sample recorder (Gilford 2000, Gilford Instruments Laboratories Inc., Oberlin, Ohio, U.S.A.), which was used in combination with a Beckman DU Monochromator (Beckman Instruments Inc., Palo Alto, Calif., U.S.A.). The effluent was collected in 5 ml fractions (Radi Rac Universal fraction collector, with distributor and controller, LKB, Stockholm, Sweden), and these were marked on the chart paper by an event marker connected to the system.

Recovery of nucleotides

The primary identification of the components was made from the elution pattern and the ratio of E_{275} : E_{260}, which is characteristic for each individual nucleotide base. The final identification was made after the fractions were combined, lyophilized and purified as reported earlier[2,3].

EXPERIMENTAL

The improvements in the resolution and sensitivity of the nucleotide column chromatography techniques were achieved in the following manner.

Gradient

Several different formic acid–ammonium formate gradients were tested. The best resolution of the nucleotides from our biological extracts was obtained with increasing concentrations of formic acid and ammonium formate, in seven successive chambers of the gradient mixer, as follows: chamber 1: distilled water; chamber 2: 0.5 *M* formic acid; chambers 3–4: 4.0 *M* formic acid; chambers 5–7: 4.0 *M* formic acid–1.0 *M* ammonium formate. The volume of liquid in the chambers was 150–300 ml depending on the desired slope of the gradient. The best flow rate of the eluant proved to be 25–36 ml/h.

Column

Preliminary separations of large samples of biological material were performed with ordinary 1.0 × 22.0 cm glass columns. 0.8 × 12.0 cm columns proved to be practical and gave even better resolution in the separation of small amounts of nucleotides than columns with larger volumes of ion-exchange resin.

Flow cells

The detection sensitivity of the system was satisfactory with the original microcuvettes with a 10 mm light path. In practice, however, the elution of the column with a formic acid–ammonium formate gradient caused the formation of gas bubbles which were trapped in these flow cells, thus making automatic UV-monitoring impossible.

This detrimental interference was avoided by our modification of the flow cell (Fig. 1). The roof of the cell was made to slope in order to allow the bubbles to flow through the cell without entering the light path. The angle of inclination of the roof was kept as small as possible (30°, Fig. 1) in order to avoid loss of sensitivity due to any unnecessary increase of the cell volume. The final volume of the cuvette was

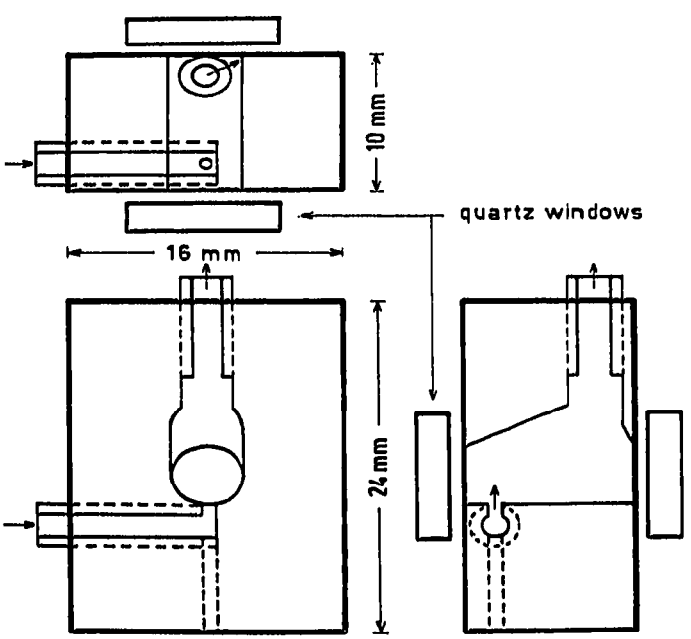

Fig. 1. A flow cell, drilled out of teflon, 0.25 ml capacity, 10 mm optical path length.

0.25 ml, the light path 10 mm, and the diameter of the cuvette window 4 mm. The flow cell was simply drilled out of a teflon block size 10 × 16 × 24 mm. Short pieces of teflon tubing were cemented to the outlet and inlet holes for attachment to the flexible tubing. The holes which were not used (dotted line in Fig. 1) were filled with cement.

UV-recording

The recorder was calibrated to the desired level of sensitivity with standard solutions of adenine of known absorbance measured with a Beckman DK-1 spectrophotometer. With maximum sensitivity a full-scale deflection on the Gilford-recorder corresponded to 0.05 absorbance units measured at 260 nm with a 10 mm light path.

The ratio between the readings at 260 and 275 nm is characteristic for each purine and pyrimidine base and therefore a valuable aid in the identification of the different peaks. For this purpose a dual-wavelength selector was connected to the UV-monitoring system, which made it possible to make recordings alternatively at 260 and 275 nm.

Background neutralization

In nucleotide separations with a formic acid–formate gradient, the increasing ammonium formate concentration of the effluent causes the base-line to drift, especially during the latter part of the run, thus disturbing the detection and quantitative evaluation of small fractions. We were able to avoid this by using two modified flow cells together with an automatic blank compensator. This assembly maintained the base-line constant when the eluant was run through the reference cell, and the effluent through the measuring cell.

Procedure in practice

The assembly of apparatus is represented schematically in Fig. 2. The recording was started and the sample was run through the column at a rate of 0.1–0.5

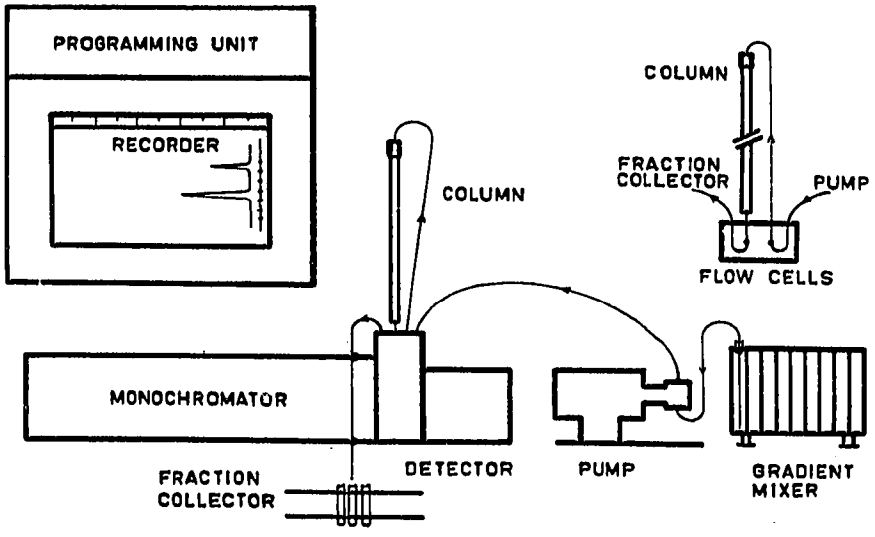

Fig. 2. Scheme for the automatic column chromatography apparatus.

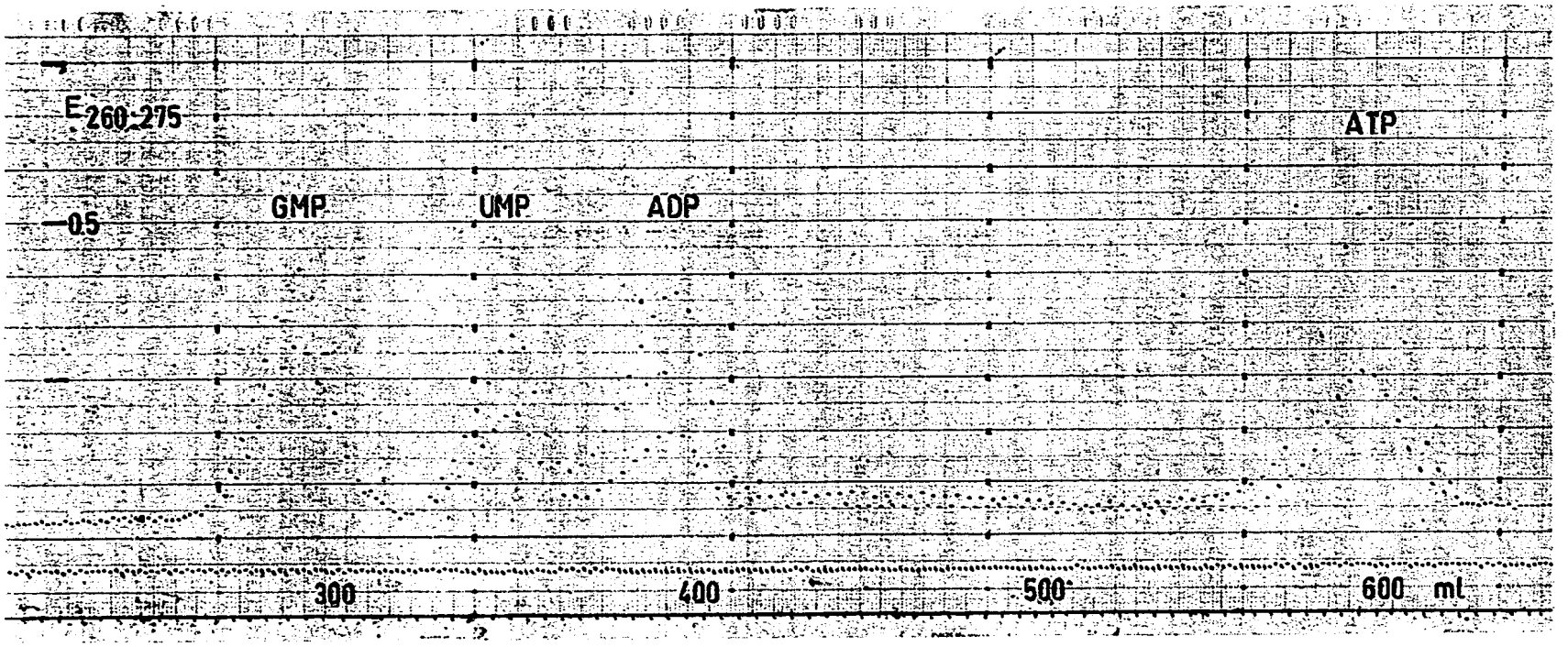

Fig. 3. Separation of 100 μg GMP, UMP, ADP and 150 μg ATP. Resin: Dowex-2, $HCOO^-$, column size: 0.8 × 12.0 cm, gradient: 7 × 150 ml (see text for further details), temperature 22°. The eluant was recorded at 2 min intervals, alternatively at 260 nm (upper curve) and at 275 nm (lower curve). The event marker line (5 ml fractions) is shown at the bottom of the chart paper.

ml/min. Occasionally, positive pressure from a nitrogen flask was used. The material which was not retained by the resin at neutral pH was washed out with distilled water. The gradient mixer was then connected to the pump. Thereafter the assembled apparatus completed the nucleotide separation unattended. The results were printed out on the chart paper, from which the relative and quantitative amounts of the nucleotides could be calculated.

Good separation and resolution of nucleotides were achieved when standard mixtures were separated with this system (Fig. 3).

Recovery of nucleotides

The recovery obtained with 100 nmoles of UMP, ADP, UDPAG and UDP added to a mycoplasma extract was satisfactory. The quantitative calculations made from the peak areas of three different separations gave the following percentages of recovery: UMP 94.0 ± 14.0%, ADP 104.0 ± 16.7%, UDPAG 104.5 ± 2.0% and

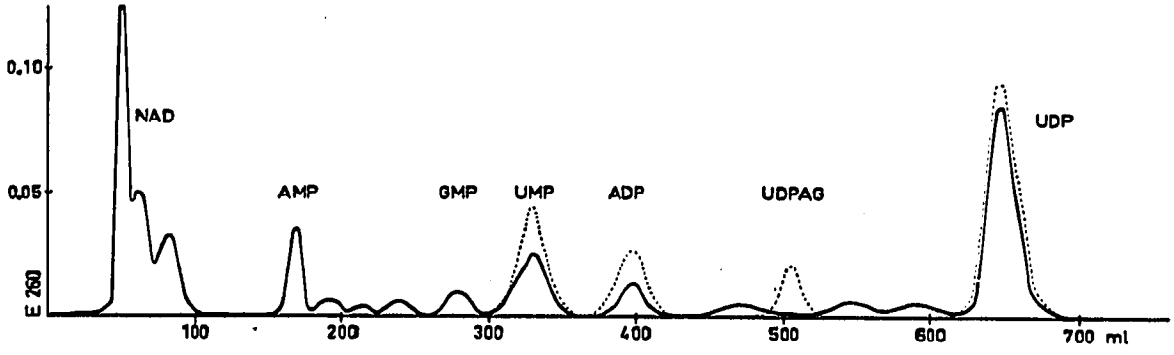

Fig. 4. Recovery of 100 nmoles UMP, ADP, UDPAG and UDP (dotted lines) added to a mycoplasma extract. Column: 0.8 × 12.0 cm, gradient: 7 × 200 ml, 22°, 24 h.

UDP 120.5 ± 8.6% (Fig. 4). The volume of the fractions was 40–70 ml. The concentration of the standard nucleotides and the mycoplasma extract could be varied between 100–1000 nmoles without significant effect on the elution pattern or the resolution of the individual nucleotides.

DISCUSSION

In several cases where good resolution has been achieved[8–10,16], long (150 cm) columns or small bore spiral plastic tubing[37] have been used. In practice, the use and servicing of these columns are laborious and difficult for multiple analysis. In our system the use of either long or short columns is possible, but because of the possible leakage of UV-absorbing material from the resin during a run[9], smaller columns are to be preferred.

A formic acid–ammonium formate gradient was chosen because of the known nucleotide elution pattern and certain other advantages[2]. The gradient mixer, baseline neutralization and modified flow cells made automation possible and eliminated the practical problems of this gradient system.

The detection sensitivity level depends on the resolution, the slope of the gradient,

the length of the light path, and on the volume of the measuring cell. With the use of capillary or cylindrical cells (Beckman), with up to a 50–100 mm light path, it would be possible to improve the sensitivity still further; practical difficulties would then, however, arise in proportion to the cell diameter and volume. The present method can be adapted for preparative work by using flat cuvettes, adjusting the recording sensitivity and splitting the effluent stream.

The application of an automatic UV-monitoring system to nucleotide column chromatography was first introduced by ÅGREN[39]. He used a monochromator connected to a photomultiplier and a recorder. ANDERSON[8] has described two useful UV-monitoring systems assembled partly on commercially available components. He used a single or double-beam system with two wavelengths and constructed a baseline compensation and flow cells. He was able to separate standard nucleotide mixtures (bases, nucleosides and nucleotides) in a single run with very good resolution[9]. The nucleotides of yeast were also separated with this nucleotide analyzer[10]. Recently, this instrumentation has been further modified and sensitized down to the 2 nmolar level in the separation of bases and nucleosides[40]. At the present time, there are also several commercial apparatus, which have been used for the partial automation of nucleotide analysis[13,41–43].

The present system was intended primarily for work with biological samples containing only small amounts of free nucleotides. The UV spectra of the nucleotides depend upon the pH, and therefore the automatic scanning cannot produce quantitative records of chromatograms with pH gradients[1]. With our modification, however, the reproducibility of chromatograms of test samples was good and the recovery of individual nucleotides satisfactory. The method has been proven useful in the quantitative determination of the changes in the concentration of different nucleotides, *e.g.* during the growth of mycoplasma[36].

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