Journal of Chromatography, 92 (1974) 166–170

CHROM. 7305

Note

Use of bicarbonate as an easily removable eluent in preparative and analytical ion-exchange chromatography

WOLFGANG LOESCHE, RENATE BUBLITZ, ANTON HORN, WOLFRAM KOEHLER, HENNING PETERMANN and UWE TILL

Institut für Physiologische Chemie der Friedrich-Schiller-Universität, Jena (G.D.R.)

(Received December 10th, 1973)

Removing the eluent from the eluate after ion-exchange chromatography is one of the difficulties of this commonly used technique. In general, the following methods may be used. (1) Precipitation reactions of either the elution salt or the eluted substance. These have low specificity and the yield is low. (2) Lyophilization of the eluent if possible. This requires too much time and endangers labile compounds.

The present paper gives an example of the striking suitability of a third method[1]. A salt of a volatile acid is used for the elution of an anion exchanger. It is removed from the eluate by a cation exchanger in the H^+ form, which exchanges the salt cation for protons. The resulting free acid quickly evaporates. The cation-exchange resin can be filtered off. The result is the separated material in an aqueous solution which is very suitable for any further procedure, *e.g.* paper chromatography.

This principle is demonstrated by the analytical and preparative separations of nucleotides and glycolytic intermediates with DEAE-Sephadex, using $KHCO_3$ as eluent. Its removal from eluate proceeds according to the reaction:

$$KHCO_3 + \text{exchanger } (H^+) \rightarrow CO_2 + H_2O + \text{exchanger } (K^+)$$

MATERIALS AND METHODS

Nucleotides were purchased from Reanal (Budapest, Hungary), except UDP (Serva, Heidelberg, G.F.R.) and UMP (Boehringer, Mannheim, G.F.R.).

DEAE-Sephadex A-25, particle size 40–120 μm (from Pharmacia, Uppsala, Sweden) was prepared according to the producer's instructions and changed into the bicarbonate form by equilibration with 1 *M* $KHCO_3$.

Separation on DEAE-Sephadex columns

Before packing the Sephadex bed, the gel was equilibrated with 50 m*M* $KHCO_3$ solution. After samples of about 10–50 ml had been drained into the gel, it was washed with 50 m*M* $KHCO_3$ and then connected to the eluent. Flow-rates of 50 ml/h were obtained with the 30-cm columns at 30 cm water pressure, and of 15 ml/h with a pair of large columns at 200 cm water pressure (for column dimensions see legends to figures). All separations were carried out at 4°.

A check of the effective gradient shape was performed by electrolyte concentration measurements in the effluent fractions using a conductolyser (LKB).

The nucleotide peaks in the effluent were detected by measuring continuously the light transmission at 254.7 nm using a flow cell (Uvicord; LKB). The precise elution pattern was obtained by extinction measurements at 260 nm of the diluted effluent fractions. For identification of the nucleotide peaks their UV spectra recorded with a Unicam spectrophotometer were used. Additionally, ^{14}C-labelled adenine nucleotides (Radiochemical Centre, Amersham, Great Britain) were used as markers, radioactivity being measured with a Packard 2001, Tri-Carb liquid scintillation counter.

Removal of $KHCO_3$ from the eluate

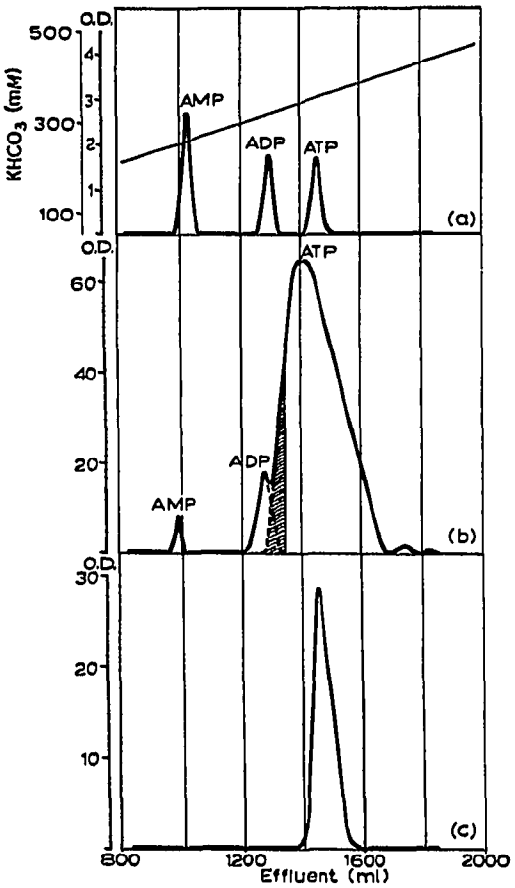
The $KHCO_3$ was removed from the effluent fractions with a cation exchanger. The procedure was performed under vacuum to facilitate the escape of CO_2 and thus prevent any significant acidification by it. Dowex 50 W-X1 (H^+), 50–100 mesh was added stepwise with stirring until bubbling ceased. The resin was then removed by filtration and the filtrate neutralized with KOH and evaporated to dryness.

Thin-layer DEAE-Sephadex chromatography

DEAE-Sephadex thin-layer plates of 1-mm thickness were prepared with dimensions of 20×80 cm. 5-μl samples were applied directly to the gel surface. A linearly increasing $KHCO_3$ concentration gradient served for development at a 15° downward inclination. The nucleotide spots were detected by means of their UV absorption at 260 nm.

RESULTS

Fig. 1a gives the elution profile of 5 mg of each of AMP, ADP and ATP separated on a 30-cm column. Fig. 1b gives a profile after preparative separation of 700 mg ATP. Contaminating AMP forms a well defined peak. The ADP peak is not clearly separated but overlaps less than 10% of the ATP peak. Re-chromatography under identical conditions of 50 mg of the remaining 90% demonstrates the purity of ATP after the first separation (Fig. 1c) and shows that Dowex 50 treatment does not cause any degradation. The recovery rate is 95%. By flame-photometric analysis 0.1–1.0 mole K^+, 1.0–3.0 mmole Ca^{2+} and 0.3–1.0 mmole Mg^{2+} per mole nucleotide can be measured after the cation exchange. In order to separate all the mono-, di- and triphosphonucleotides of adenine, guanine, cytosine, uracil and hypoxanthine an increase in the effective gel bed length is needed. This was achieved by attaching the outlet tubing of one column to the top of a second column (column pairing). The elution pattern is as shown in Fig. 2. The monophosphate of a certain nucleoside is eluted first followed by the di- and triphosphates, successively. It is noteworthy that the sequence in which the base derivatives are eluted is the same through mono-, di- and triphosphates, namely cytosine–uracil–adenine–guanine–hypoxanthine. In some cases, separated peaks were collected, freed from $KHCO_3$ by Dowex 50 and submitted to paper chromatography according to ref. 2. They formed uniform spots, indicating that the Dowex 50 treatment causes no degradation of the nucleotides.



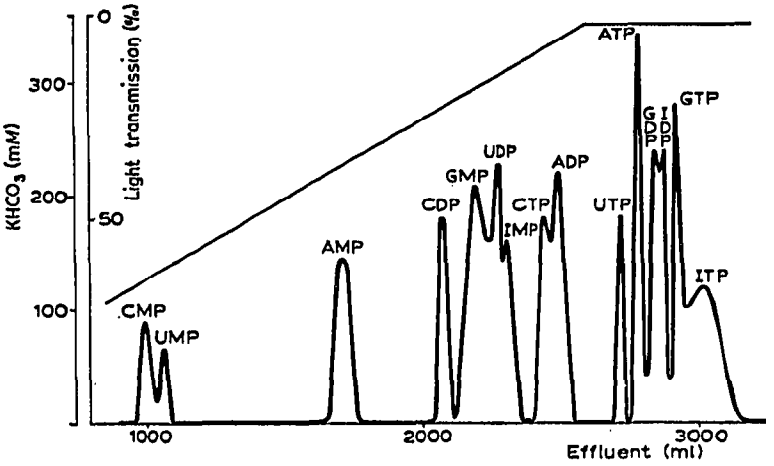


Fig. 1. Analytical and preparative separations of AMP, ADP and ATP on a DEAE-Sephadex column, 2.6 × 30 cm. The extinction at 260 nm of the effluent fractions is plotted. Elution was carried out with a linear $KHCO_3$ gradient from 50 to 500 m*M* (2000 ml), which is graphically represented only in (a), but also applies to (b) and (c). (a) Separation of 5 mg of each of AMP, ADP and ATP. (b) Preparative separation of 700 mg ATP. Contaminating ADP overlaps 10% of the ATP peak (determined by enzymatic assay of ADP concentrations in all the ATP-containing fractions). (c) Re-chromatography of 50 mg of the purified ATP of 1b.

Fig. 2. Analytical separation of mono-, di- and triphosphates of adenosine, guanosine, cytidine uridine and inosine on paired DEAE-Sephadex columns, 1 × 100 cm. The transmission at 260 nm of the effluent fractions is plotted against the elution volume. A 1-mg amount of each monophosphate, 2 mg of each diphosphate and 3 mg of each triphosphate were applied. Elution was carried out with a linear $KHCO_3$ gradient from 100 to 350 m*M*, followed by 350 m*M* $KHCO_3$ solution (thin line).

A mixture of the same nucleotides was submitted to thin-layer chromatography on DEAE-Sephadex. Fig. 3 shows the nucleotide positions after a 17-h development. As with columns, the monophosphates move quicker than the di- and triphosphates. The elution sequence of base derivatives is different from that on columns and is now cytosine–uracil–hypoxanthine–guanine–adenine, seen from the front. Only ATP breaks the rule and comes sooner than expected.

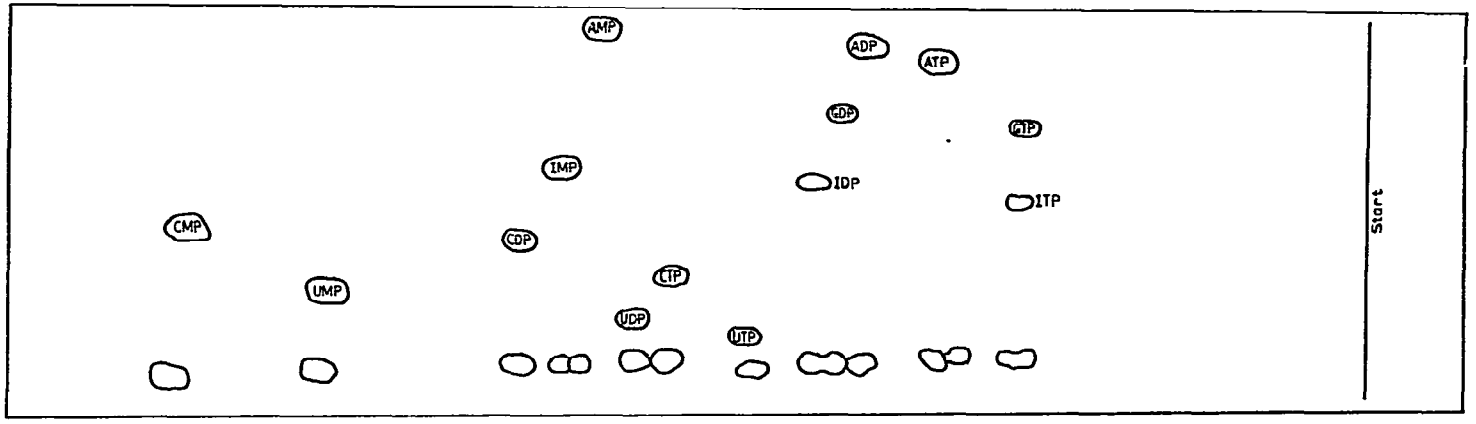


Fig. 3. Analytical separation of mono-, di- and triphosphates of adenosine, guanosine, cytidine, uridine and inosine by thin-layer DEAE-Sephadex chromatography. Nucleotide positions detected by their UV absorption after downward development for 17 h are shown. GMP is lacking. 1/3 mg of a nucleotide was applied in the case of the single reference substances, and 1/6 mg in the case of the mixed reference substances. The development was with a linear $NaHCO_3$ gradient from 50 to 330 m*M* (160 ml), followed by 330 m*M* $NaHCO_3$ solution (200 ml).

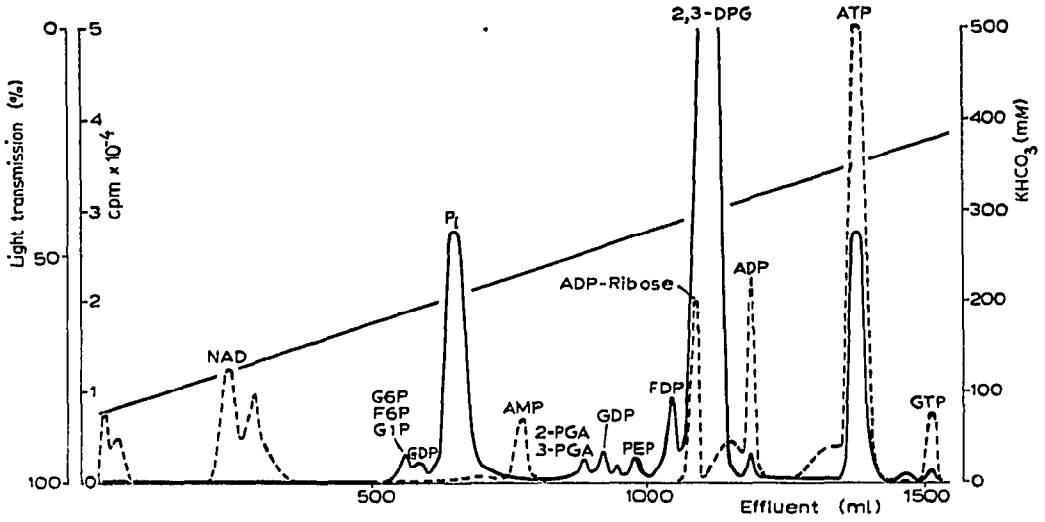


Fig. 4. Analytical separation of phosphorylated metabolites from human erythrocytes after labelling with $^{32}P_i$. After a labelling time of 4.5 h, the acid-soluble phosphorus compounds of 30 ml of red blood cells were extracted with trichloroacetic acid[3] and applied to paired DEAE-Sephadex columns, 1 × 100 cm. The elution was carried out with the plotted $KHCO_3$ gradient. Light transmission at 260 nm (– – –) and radioactivity in counts per min (———) of the eluate fractions are plotted against the eluate volume. The peaks were identified by enzymatic assays and runs of reference substances. G6P = Glucose 6-phosphate; F6P = fructose 6-phosphate; G1P = glucose 1-phosphate; P_i = inorganic phosphate; 2-PGA = 2-phosphoglyceric acid; 3-PGA = 3-phosphoglyceric acid; GDP = glucose 1,6-diphosphate; PEP = phosphoenol pyruvate; FDP = fructose 1,6-diphosphate; ADP–Ribose = splitting product of $NADH_2$.

The separation of metabolites extracted from cells is a more difficult problem, because of their often large pool concentration differences and interfering unidentified substances. Fig. 4 demonstrates the elution pattern of a trichloroacetic acid extract of $^{32}P_i$ labelled red blood cells upon separation with paired DEAE-Sephadex columns and $KHCO_3$ elution. The detected phosphorylated intermediates are eluted in the same order as with Dowex columns and formate eluent[4–7], except for the adenine nucleotides, which are retarded.

The separated peaks were collected and freed from $KHCO_3$ by Dowex 50. Measurements of the radio-counts before and after Dowex 50 treatment indicated 100% recovery of the labelled compounds.

It can be concluded from these experiments that $KHCO_3$ is a rather suitable eluting salt for analytical and preparative ion-exchange chromatography. It is very easy to remove and facilitates the subsequent purification steps.

REFERENCES

1 A. Horn, U. Till, W. Loesche and W. Achilles, *G.D.R. Pat.*, WP B 01 *d*/170 506, 1973.
2 A. M. Krebs and R. Hems, *Biochim. Biophys. Acta*, 12 (1953) 142.
3 U. Till, W. Koehler, I. Ruschke, A. Koehler and W. Loesche, *Eur. J. Biochem.*, 35 (1973) 167.
4 G. R. Bartlett, *J. Biol. Chem.*, 234 (1959) 449.
5 G. R. Bartlett, *J. Biol. Chem.*, 234 (1959) 459.
6 K. Papenberg, *Klin. Wochenschr.*, 39 (1961) 522.
7 K. Papenberg, H. E. Bock and H. Nieth, *Klin. Wochenschr.*, 40 (1962) 936.