CHARGE-TRANSFER CHROMATOGRAPHY

An efficient method for separation of nucleosides, nucleotides and cyclic nucleotides on acriflavin–Sephadex

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1. Introduction

For investigations on the degradation or synthesis of nucleotides a rapid qualitative and quantitative method for the separation of these substances from each other is needed. In the past, a great number of methods have been developed for the extraction and purification of these nucleotides by ion exchange resins (e.g., Dowex 50, QAE–Sephadex, polyethylene imine cellulose, alumina) or by co-precipitation procedures using inorganic salts (reviewed [1]).

We have devised here a new procedure based on charge-transfer chromatography for the detection and estimation of small quantities of nucleosides or nucleotides. Purines and pyrimidines are able to form complexes with different aromatic derivatives by intervention of charge-transfer phenomena [2] which consist in an electron displacement or transfer from one component the donor, to the other component, the acceptor.

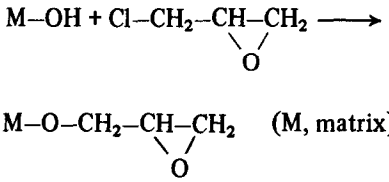
Donor and acceptor properties of each component are functions of their conjugated system and of electron-attracting or electron-releasing substituents on the aromatic ring. If one complex-forming component is anchored to an insoluble matrix, such as a polysaccharide, the latter will be converted to an adsorbent for the other component of the charge-transfer complex. The interaction may be more or less strong [3,4], and the strength of the complex could be determined by the association constant [5].

In a precedent work, different aromatic derivatives coupled to Sephadex, were studied for their abilities to interact with nucleotides. Since acriflavin–Sephadex yielded the largest interaction for the tested substances [4] and as acriflavin has good electron-acceptor properties toward nucleotide derivatives [6], it was selected for this present study.

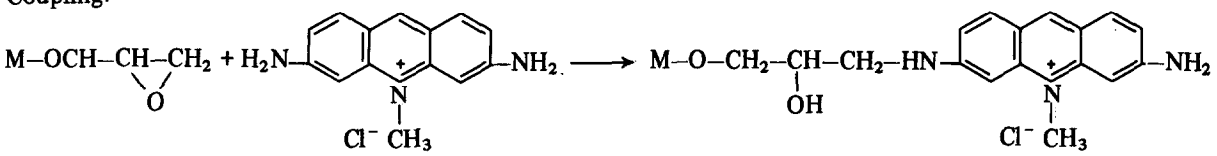
2. Materials and methods

The preparation of acriflavin–Sephadex G-25 is effected according to the following reaction:

Activation:



Coupling:



Sephadex G-25 (100 ml; Pharmacia Fine Chemicals) was swollen, washed on a glass filter funnel with distilled water and introduced, along with 50 ml NaOH 2 M, 5 ml epichlorydrin (Merck, Darmstadt) and 0.2 g sodium borohydride (Merck, Darmstadt), into a 3-necked 2 liter flask. An additional 50 ml NaOH (2 M) and 25 ml epichlorydrin were slowly and continuously introduced simultaneously over a period of 4–5 h with moderate stirring [7]. Stirring was continued overnight at room temperature. The gel was then collected on a glass filter funnel and washed with distilled water and ethanol–KOH (0.02 M, v/v) by stirring at 50°C overnight. The gel was then extensively washed with ethanol (50%), acetic acid (0.05 M) and water on the glass filter funnel until a colourless effluent was obtained. The gel used in the chromatographic experiments contained 125 μmol acriflavin/dry wt.

3. Results and discussion

Considering for example one single series of nucleotides, like adenosine-derivatives, each of these can serve as the product or the substrate of different types of enzymes (see below).

ATP + Adenosine $\xrightarrow[\text{adenosine kinase}]{\text{EC 2.7.1.20}}$ AMP + ADP

AMP $\xrightarrow[\text{5′ nucleotidase}]{\text{EC 3.1.3.5}}$ Adenosine + P_i

Cyclic AMP $\xrightarrow[\text{3′, 5′ cyclic AMP phosphodiesterase}]{\text{EC 3.1.4.17}}$ AMP

ADP $\xrightarrow[\text{nucleoside diphosphate kinase}]{\text{EC 2.7.4.6}}$ ATP

ATP $\xrightarrow[\text{adenylate cyclase}]{\text{EC 4.6.1.1}}$ Cyclic AMP + PP_i

The chromatographic behaviour of adenosine and related compounds was surprisingly complex, and dependent on the composition of the eluate buffer. A previous study [4] has revealed that increasing ionic strength or salt concentration of the elution buffer abolishes the electrostatic interaction between solute and ligand. At low ionic strength (fig.1), the different solutes were eluted or retarded as functions of their charge transfer and electrostatic properties toward the acriflavin ligand. The electrostatic interaction occurs between the positive charge on acriflavin (on N-position 9) and the negative charge due to the phospho-group. Increases in both size and polarity of the N_9 substituent of the base, especially the anionic phosphate group of AMP, ADP or ATP, increased the interaction with acriflavin. With 0.1 M ethyl-morpholine buffer pH 7, a good separation of the adenosine nucleotide series was obtained (fig.1), and it was possible to purify some AMP preparations (NEN Chemicals, Boston) which still contained about 6% adenosine as impurity.

At high ionic strength, the AMP–acriflavin interaction will be weaker when expressed as a V_e/V_t value, reduced elution volume (V_e, elution volume of the sample; V_t, total volume of the column). This phe-

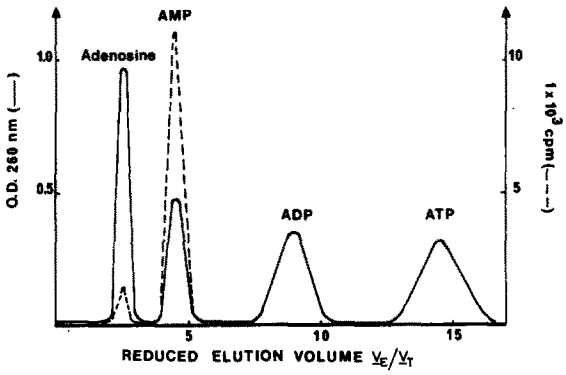


Fig.1. Chromatography on acriflavin–Sephadex. 50 μl adenosine and adenosine nucleotides (Sigma, St Louis) at 3 mg/ml were run separately and simultaneously on acriflavin–Sephadex. Column dimensions 10 × 1 cm. Flow rate 10 ml/h; temp. 24°C. V_e/V_t reduced elution volume of sample chromatographed. Eluate buffer 0.1 M ethyl morpholine, pH 7. Chromatography was followed spectrophotometrically. 2 μl AMP (NEN Chemicals, Boston, spec. act. 13.5 μCi/μmol) were run on the same column and fractions of 1.5 ml were collected which were mixed with 1 ml water and 5 ml Instagel (Packard) with vigorous shaking and the radioactivity was measured by liquid scintillation spectrometry.

nomenon occurs when a 0.1 M ammonium acetate buffer, pH 6, containing 0.25 M ethanolamine, or 0.1 M ethyl-morpholine buffer, pH 7, containing 0.1 M NaCl is used for elution (fig.2). This technique has been used to determine the 3′, 5′-cyclic AMP phosphodiesterase esterase activity of a whole homogenate of rat brain. Usually, this enzymatic activity was determined following the 2-step radiometric method [8]: (1) The enzyme transforms tritiated cyclic AMP into 5′ tritiated AMP; (2) An excess of snake venom 5′-nucleotidase cleaves AMP to form adenosine and inorganic phosphate. The substrate and product of the enzymatic reaction are then separated by one of the techniques above [1] using ion exchange chromatography. Chromatography on acriflavin–Sephadex gel allows one to omit the second step of the described procedure and to obtain a good separation between AMP and cyclic AMP, with low blanks (see table 1).

This method offers a great number of advantages: e.g., it minimizes time and manipulation, and renders unnecessary some other experimental steps which involve other enzymatic actions and other products, thereby decreasing the % error. The eluate buffer is relatively simple, and the chromatographic procedure can provide good separations from pH 5–8.5 [4,5]. Since this buffer is volatile, the use of salt is not necessary in many cases; separated nucleotides can be recuperated after lyophilisation. Concerning cyclic AMP–acriflavin interaction, the formation of a complex is possible, and the phospho-group bound at the 3′- and 5′-position of the sugar does not obstruct the so-called charge-transfer overlap recognized between flavin and indol rings [2]. Under these experimental conditions, the V_e/V_t value of cyclic AMP remains high and permits a good separation between AMP and cyclic AMP (fig.2). It seems that in the formation of the charge-transfer complex, the phospho-group of AMP does not play a direct role but that it is related to a configuration of the nucleotide molecule. At high ionic strength, an intramolecular arrangement could be favored in which the phospho-group in monoester form can partially shield the aromatic ring of adenosine, thus decreasing the acriflavin–nucleotide interaction. With this chromatographic method, low molecular weight compounds are linearly retained and desorption by variation of pH, salt or temperature is not necessary. Column regeneration after use is not absolutely needed. Some of the protein extracts applied with the chromatographed sample can be either excluded with the void volume of the column or completely retained; in the latter case, the column can be regenerated by washing with 1 M NaSCN or 0.4% SDS (unpublished results). This gel can work as charge-transfer gel, but as mentioned above and in

Table 1

Expts[a]	cAMP	AMP	% cAMP hydrolysed
Blank	43 100	200	0.5
Control	42 010	300	0.7
Expt. 1	1750	42 480	96
Expt. 2	31 500	11 220	26

[a] Average of 3 expts

The incubation mixture contained 80 mM Tris–HCl buffer with $MgSO_4$ 2 mM, pH 8, 100 μl tritiated 3′, 5′-cyclic AMP (NEN, Boston) diluted with non-labelled cyclic AMP to final conc. 10 μM and 60 μl cell homogenate of rat brain. In controls, 20 μl cell homogenate containing Tris/HCl buffer were boiled for 3 min to denature the enzyme, and 100 μl tritiated AMP was added. Then, each tube was incubated for 10 min and kept in ice before being applied to acriflavin–Sephadex. Column dimensions 10 × 1 cm. Flow rate 10 ml/h; room temp.; eluate buffer 0.1 M ammonium acetate, pH 6, containing 0.15 M ethanolamine. For blanks 100 μl aliquots of cyclic [^{3}H]AMP were pre-run.

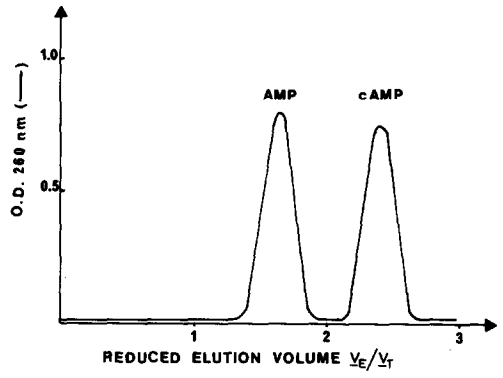


Fig.2. Chromatography on acriflavin–Sephadex: 50 μl AMP and 3′, 5′-cyclic AMP at 3 mg/ml were run on acriflavin–Sephadex, equilibrated with 0.1 M ammonium acetate buffer, pH 6, containing 0.25 M ethanolamine and on the same type of column as in fig.1.

a precedent study [4], some ionic interaction due to the positive charge on acriflavin can be favoured by using buffers at low ionic strength. This versatile method can serve many purposes using appropriate buffer composition (e.g., separation, detection and purification of nucleosides or nucleotides).

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