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RESERVED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF ADENINE NUCLEOTIDES: APPLICATION TO THE KINETICS OF AN ADENOSINE 3'-PHOSPHATE 5'-SULPHATOPHOSPHATE SULPHOTRANSFERASE FROM PLANTS

J. D. SCHWENN and H. G. JENDER

Lehrstuhl für Biochemie der Pflanzen, Abteilung Biologie der Ruhr-Universität Bochum, P.O. Box 102148, 4630 Bochum 1 (G.F.R.)

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SUMMARY

The common adenine nucleotides (adenosine mono-, di-, triphosphate) have been separated by paired-ion chromatography from their sulphated derivatives (adenosine 5'-sulphatophosphate, adenosine 3'-phosphate 5'-sulphatophosphate). With tetrabutylammonium hydroxide as the ion-pair forming reagent, nicotinamide–adenine dinucleotide phosphate, flavin–adenine dinucleotide and adenosine 3',5'-bisphosphate were also separated from these nucleotides by rapid isocratic elution. The method is highly reliable as shown by the capacity factors (k'), and its compatibility with the requirements of a continuous-flow radio detection for ^{35}S-labelled nucleotides is demonstrated. It has been applied to an investigation of the kinetics of a PAPS sulphotransferase reaction involved in higher plant assimilative sulphate reduction.

INTRODUCTION*

Ion-exchange chromatography has widely been used for the analysis of nucleosides and nucleotides. The application of ion exchange in high-performance liquid chromatography (HPLC) has thoroughly been investigated by Brown and coworkers[1]. In the last three years the introduction of quaternary ammonium alkylates as ion-pair forming counter-ions has enabled the rapid separation of polar water-soluble compounds on reversed phases[2,3]. Compared to ion exchange, paired-ion chromatography on reversed phases needs less sophisticated chromatographic equipment. Moreover, regeneration of the column is no longer required and the time of analysis is thereby considerably reduced.

The study of biological sulphate activation or sulphate transfer in higher plants is hampered by the extremely low concentrations of the reaction products

* Abbreviations: AMP, ADP, ATP = adenosine 5'-mono-, di-, tri-phosphate; APS = adenosine 5'-sulphatophosphate; FAD = flavin–adenine dinucleotide; NADP = nicotinamide–adenine dinucleotide phosphate; PAPS = adenosine 3'-phosphate 5'-sulphatophosphate; 2,5'- or 3',5'-PAP = adenosine 2',5'- or 3',5'-bisphosphate; TBAH = tetrabutylammonium hydroxide.

such as APS, PAPS or PAP in the presence of ATP, ADP and AMP[4–7]. Previous methods for the determination of these adenine compounds are cumbersome and often too slow for routine analysis. Therefore a rapid separation of the nucleotides on the basis of reversed-phase paired-ion chromatography has been developed, with the emphasis on the separation of sulphated adenine nucleotides from their phosphorylated precursors. The method has been applied to a study of the reaction kinetics of a PAPS sulphotransferase from higher plant tissue.

MATERIALS AND METHODS

Chromatographic equipment

An HPLC pump (Waters Assoc., Model 6000A) in combination with a pressureless injection system (Waters Assoc., Model U6K) was used for solvent delivery and sample application. The analytical column (250 × 4.6 mm) mounted with a precolumn (40 × 4.6 mm) (Knauer) was packed with LiChrosorb RP-18 (10 μm; E. Merck, Darmstadt, G.F.R.). The absorbance of the nucleotides was monitored with a dual wavelength photometer (Waters Assoc., Model 440), and peak areas and retention times were calculated with a laboratory integrator (Spectra-Physics, Model Minigrator). For continuous flow measurements of ^{35}S-labelled compounds a modified flow analyser was used (Packard, Model Tri Carb Flow Analyzer).

Solvent for paired-ion chromatography

A concentration of 9.4% of 2-propanol was found to be suitable for the ion-pairs investigated. The solution was made 3×10^{-3} with respect to TBAH and the pH was adjusted as indicated with 1 *N* orthophosphoric acid. The solution was filtered through a membrane filter (pore size 0.45 μm; SM Göttingen, Type 162 60) and degassed under reduced pressure. The column required 60 min for conditioning at a flow-rate of 1.5 cm^3/min when stored under 30% methanol. Due to the low concentration of organic solvent, the system built up a back pressure of 76 bar which increased to 80 bar at a flow-rate of 2.0 cm^3/min. The separation of compounds was usually completed after 15 min (at 1.5 cm^3/min) and no recycling or flushing was required.

Sample clean up and preparation

Nucleotides were extracted from the assay mixtures either by 1,1,2-trichlorotrifluoroethane/trioctylamine according to Khym[8] or by rapid filtration through micro collodion bags (exclusion limit 12,000 daltons; SM Göttingen, Type 165 37) employing NADP as internal standard. Pure biochemicals were purchased from Boehringer (Mannheim, G.F.R.) (AMP, ADP, ATP) or Sigma (St. Louis, MO, U.S.A.) (2′,5′- and 3′,5′-PAP). Sulphated nucleotides (APS or PAPS) were prepared enzymatically[9]. Further purification of ^{35}S-labelled nucleotides was achieved by chromatography on DEAE-cellulose[10] and desalting by passage through Bio-Gel P-2 (Bio-Rad Labs, Richmond, CA, U.S.A.). Tetrabutylammonium hydroxide was purchased from Fluka (Buchs, Switzerland).

Crude samples of the enzyme PAPS sulphotransferase were extracted from plant cell cultures (*Nicotiana tabacum* var. Samsun) as described earlier (*cf.*, ref. 11). The plant extract was liberated from low-molecular-weight UV-absorbing material by

filtration through a column packed with Bio-Gel P-6 (exclusion limit 6000 daltons). The enzymatic reaction was terminated by rapidly forcing an aliquot of the assay through a SM micro filter at 6 bar under nitrogen. The bag retained the enzyme protein, but almost complete recovery of the nucleotides was observed as compared to NADP as internal standard.

RESULTS AND DISCUSSION

Adenine nucleotides have been well separated by isocratic elution with a single eluent (Table I). Under the chromatographic conditions selected (reversed-phase RP-18, 10 μm) the nucleotides were eluted by 9.4% 2-propanol, 3.0 m*M* TBAH at pH 9.4 in the order of their negative charge: AMP^{2-}, APS^{2-}, ADP^{3-}, ATP^{4-}, PAP^{4-}, $PAPS^{4-}$ (Table I and Fig. 1A). The corresponding capacity factors k' (Table I) showed that the introduction of a sulphate group into the molecule led to a higher retardation compared to the equivalent phosphate compound. APS is retained more efficiently than AMP, which was also found for PAPS *versus* PAP. This may reflect either that the sulphate group is fully dissociated (pK_{a2} 1.92) at pH 9.4 whereas the phosphate group has a much higher pK_{a3} (10.3 as found in 3',5'-PAP, details not given) for complete dissociation, or that for sterical reasons two alkylates cannot bind properly to a single fully dissociated phosphate group.

The formation of ion-pairs was strongly affected by the pH. At pH 10.0 the efficiency of the column was better than at pH 9.4 considering the number of theoretical plates, but at more alkaline pH values the silica was found to deteriorate after a few weeks of continuous operation of the column. A pH of 8.0, which would improve the lifetime, resulted in a loss of resolution for ATP and band broadening,

TABLE I

PROPERTIES OF THE INVESTIGATED NUCLEOTIDES RELATED TO THEIR CHROMATOGRAPHIC BEHAVIOUR

The nucleotide standards (each $5 \cdot 10^{-10}$ mol) were dissolved in the eluent 9.4% 2-propanol, 3 m*M* TBAH, pH as indicated; total volume injected 50 mm^3. Flow-rate, 1.5 cm^3/min; temperature, ambient; pressure, 76 bar (*ca.* 1100 p.s.i.); column packing, LiChrosorb RP-18/10, column, 250 × 4.6 mm, precolumn, 40 × 4.6 mm. For other conditions see Materials and methods.

Nucleotide	pK_{a2}*	*Charge at pH ≈ 9.4*	k'	
			pH 9.4	*pH 10.0*
5'-AMP	6.2–6.4	−2	0.29 ± 0.0029**	0.345 ± 0.0022
3'-AMP	5.92	−2	—	0.431
ADP	6.1–6.7	−3	1.217 ± 0.0042	1.015 ± 0.0056
3',5'-PAP	6.5 (pK_{a3} 10.3)	−4	2.895 ± 0.0079	2.556
2',5'-PAP	6.7 (pK_{a3} 10.7)	−4	—	2.670
ATP	6.0–6.95 (pK_{a3} 11.3)	−4	—	2.697 ± 0.0105
APS	not known	−2	0.536 ± 0.0031	0.479 ± 0.0021
PAPS	not known	−4	3.914 ± 0.0138	3.914

* From ref. 12.
** $n = 10$.

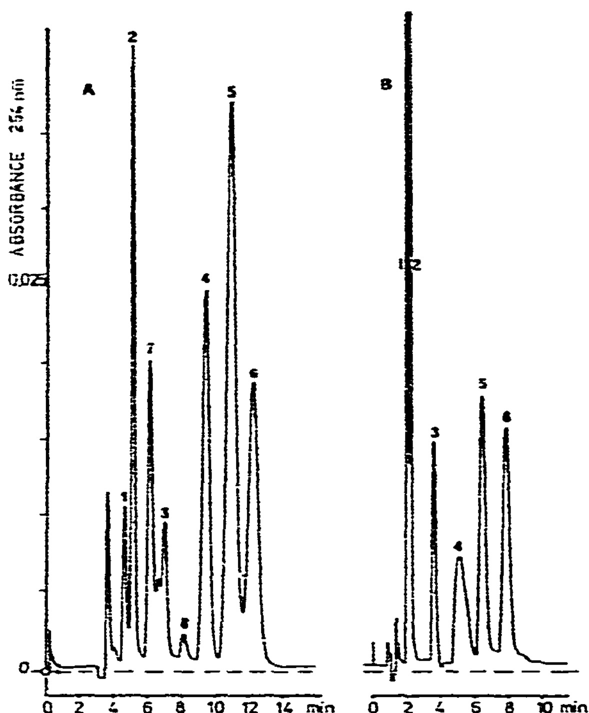

Fig. 1. Separation of adenine nucleotides (mixture of authentic compounds) at pH 9.4 (A) and pH 8.0 (B). Column: Knauer RP-18/10, operated with a precolumn. Eluent: 9.4% 2-propanol, 3 m*M* TBAH, pH adjusted with 1 *N* phosphoric acid. Other conditions as in Materials and methods. Peaks: 1 = 5'-AMP; 2 = APS; 3 = ADP; 4 = ATP; 5 = 3',5'-PAP; 6 = PAPS; 7 = NADP; 8 = FAD.

with decreases in k' [from 2.697 to 2.139 (Fig. 1B)] and in the number of theoretical plates (from 1248 to 493).

The solvent system used for paired-ion chromatography was found compatible with the continuous measurement of radioactivity by an anthracene flow cell. The solid anthracene particles, which serve to convert β-radiation into photons, were not dissolved by 2-propanol at low concentrations. Moreover, the eluent did not interfere with the measurement of the radioactivity in the effluent as compared to water. There were no increased memory or background effects. The simultaneous recording of the UV absorbance and the radioactivity of a labelled nucleotide (Fig. 2) was required for the investigation of an unidentified PAPS sulphotransferase in plants. The enzyme is believed to transfer the activated sulpho group from PAPS onto an unknown acceptor thiol, according to:

$$[^{35}S]PAPS + RSH \rightarrow RS\text{–}^{35}SO_3H + PAP$$

The amounts of reaction products of this enzyme are in the sub-nanomolar range, so that ^{35}S-labelled PAPS has to be used to detect its activity in crude extracts from plants. Previous investigations only considered the labelled reaction product $RS\text{–}^{35}SO_3H$, because it was difficult to determine traces of PAP, breakdown products

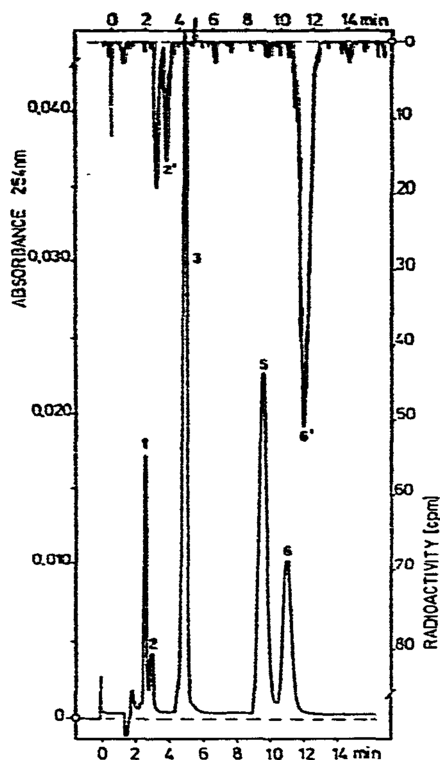

Fig. 2. ^{35}S-labelled compounds formed from [^{35}S]PAPS during the assay of a PAPS sulphotransferase in crude extracts from plants. Simultaneous recording of UV absorbance and radioactivity (upper curve) in the same sample. Column and other operating conditions as in Fig. 1A. Enzymatic assay as in Fig. 3. Peaks as in Fig. 1, except for 2' = [^{35}S]APS and 6' = [^{35}S]PAPS; first peak in the upper trace identified as [^{35}S]sulphate.

thereof (*e.g.*, 3'-AMP and 5'-AMP) or APS formed during the assay. In order to obtain a complete pattern of the reaction products and products of degradation the UV-photometer was connected with the continuous flow analyser and both absorbance and radioactivity of the sample were monitored. The areas of the UV-peaks were calculated by the internal standard method with NADP as standard, radioactive peaks were sampled and measured directly in a liquid scintillation counter and the bound sulphite was determined after isotope exchange as described earlier (*cf.* ref. 13).

The distribution pattern of compounds formed from [^{35}S]PAPS either by the transferase reaction or by accompanying breakdown reactions* is given in Fig. 3. The kinetics of the metabolic fate of PAPS as estimated by radioactivity resembled those obtained by measurement of the UV-absorption. Both measurements agreed in that PAPS was rapidly degraded into [^{35}S]sulphate and 5'-AMP, Moreover, the kinetics of the UV-absorbing compounds showed that the resynthesis of PAPS (presumably from APS) was paralleled by the formation of ADP. This rapid re-

* A more detailed study of the enzymatic reactions involved will be published separately.

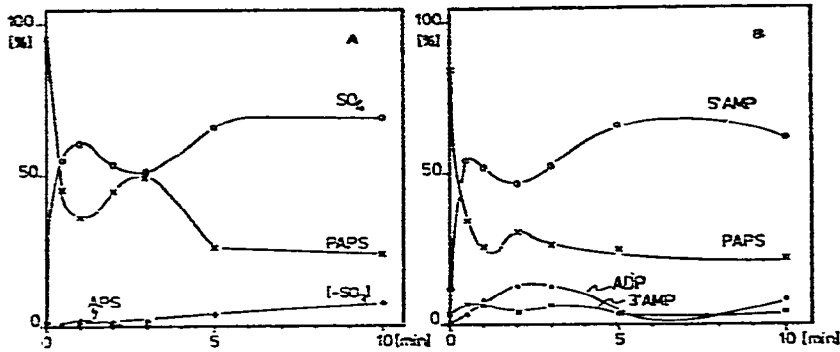

Fig. 3. Kinetics of a PAPS sulphotransferase from plants with [^{35}S]PAPS as substrate. A, Distribution of radioactivity in the samples; B, distribution of adenine compounds in the samples withdrawn at intervals as indicated. Chromatographic conditions as in Fig. 1A. The enzymatic assay contained 50 m*M* Tris-Cl (pH 8.0), 10 m*M* $MgCl_2$, 0.5 m*M* glutathione, 0.1 m*M* [^{35}S]PAPS (specific activity 42,617 sec^{-1} $nmol^{-1}$) and 1.22 mg/cm^3 partially purified protein. Incubation was carried out at 30 °C and samples of 100 mm^3 were withdrawn for HPLC analysis.

synthesis may explain why APS did not accumulate. 3',5'-PAP only occurred in the presence of NADP during the enzymatic assay, but in its absence the breakdown products 3'- and 5'-AMP were found. The formation of bound sulphite as the reaction product of the transferase appeared to be unaffected by 3'- or 5'-AMP.

ACKNOWLEDGEMENT

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