

Improved Synthesis and Rapid Isolation of Millimole Quantities of Adenylylsulfate

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An improved enzymatic method for the synthesis of adenylylsulfate (APS) from adenosine 5'-phosphate using APS-reductase from *Thiobacillus denitrificans* is described. Isolation of millimole quantities of this sulfur nucleotide is achieved rapidly by means of ion exchange chromatography on a strongly basic ion exchange resin. A facile and reproducible desalting procedure is described.

Numerous procedures have been described for both the chemical and enzymatic synthesis and the isolation of APS*. De Meio [1] offers a review of these methods. The chromatographic procedures described thus far have proved to be unsatisfactory for routine preparative work in our laboratory. Ion exchange chromatography on Dowex 1 resin, the method originally used by Baddiley *et al.* [2], involved large quantities of salt for elution. Desalting using activated charcoal and silica gel has proved, however, to be a tedious and unreliable procedure. Ion exchange chromatography using DEAE-cellulose and volatile buffers is now widely practiced and has proved to be an excellent method for the isolation of micromole amounts of this nucleotide. Preparation of millimole amounts of the nucleotide was, however, time-consuming, unwieldy and expensive due to the large columns and volumes of buffer needed for elution [3].

It was therefore our goal to develop a rapid and inexpensive method for the isolation of millimole quantities of APS for research work in our laboratory. We sought to improve an existing enzymatic synthesis procedure and investigated the possibility of retaining Dowex 1 resin for ion exchange chromatography.

Materials and Methods

Thiobacillus denitrificans strain RT was obtained from M. Schedel (this institute) and grown according to Baldensperger and Garcia [4]. The concentra-

tions of $\text{Na}_2\text{S}_2\text{O}_3$ and KNO_3 were raised to 50 mM and 60 mM, respectively, for large scale cultures. Cells were harvested after having reached an optical density of 1.3 (450 nm) by centrifugation in a Ceba continuous flow centrifuge at 40,000 rpm, washed twice with 50 mM Tris-HCl buffer, pH 7.5, and finally suspended in the same buffer (20% w/v). If not used immediately the cell suspension was stored at -18°C . Cell-free extracts were obtained by passage of the cell suspension through an Aminco French pressure cell at 80 bars pressure and centrifugation. The cell suspension could be stored frozen for several months with no loss of activity.

APS was synthesized in preliminary work according to Adams *et al.* [5]. The APS synthesis was finally modified as follows to obtain a higher yield. For each 100 ml final volume 0.5 mmol Na_2AMP , 0.05 mmol EDTA and then 1.0 mmol Na_2SO_3 were dissolved in 80 ml of 80 mM Tris-HCl, pH 7.5, and then tempered in a water bath at 30°C . Ten ml of the phosphate-free enzyme extract containing 100 units APS-reductase were added followed by ten successive additions of 1 ml each of 150 mM $\text{K}_3\text{Fe}(\text{CN})_6$ at intervals of 2 min. After 30 min the reaction was terminated by boiling for 90 sec. The reaction mixture was cooled in an ice bath and precipitated protein removed by centrifugation.

Protein was determined according to Lowry *et al.* [6] using bovine serum albumin as standard. APS-reductase activity was determined according to Peck [7]. High performance thin layer chromatography (HPTLC) was carried out on HPTLC plates (Merck, art.-no. 5628) using a mixture of iso-propanol, ammonia (33%) and water (6 : 3 : 1). High voltage electrophoresis was carried out in 25 mM sodium citrate buffer, pH 5.8, on Schleicher and Schüll paper

* Abbreviation: Adenylylsulfate, APS; adenosine 5'-monophosphate, AMP; adenosine 5'-diphosphate, ADP; adenosine 5'-triphosphate, ATP; DEAE-cellulose, diethylaminoethyl-cellulose and EDTA, ethylenediaminetetraacetate.

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no. 2043 B in a Camag electrophoretic unit. 5000 volts were applied for 10 min. Nucleotides were detected on silica plates and on paper by UV quenching under a UV lamp and identified by comparison with known nucleotides used as references. NaCl in column eluates containing bicarbonate was determined qualitatively with acid silver nitrate solution.

APS in column eluates was initially determined enzymatically. The test solution contained in a volume of 1 ml 100 mM Tris-HCl buffer, pH 8.0, 5 mM MgCl_2 , 20 mM glucose, 0.25 mM NADP (disodium salt), 1 mM $\text{Na}_4\text{P}_2\text{O}_7$, 10 units each of hexokinase and glucose-6-phosphate dehydrogenase, an APS sample containing up to 100 nmol and one unit of ATP-sulfurylase. The reaction was followed spectrophotometrically at 340 nm and 25 °C. Comparison of the absorbance of the APS solution and the APS concentration determined enzymatically yielded an extinction coefficient of 15.4 $\text{cm}^2/\text{micromol}$ which was used for all further measurements. The concentration of AMP in solution was determined spectrophotometrically. Ferrocyanid was first oxidized to Ferricyanid with H_2O_2 and then determined colorometrically. Inorganic sulfate was determined according to Dodgson [8]. Urea was detected by measuring the absorbance at 230 nm.

Preparative column chromatography was carried out on Dowex 1 X 8 resin, chloride form, 200–400 mesh. 160 g of resin were washed with 500 ml of 2 M NaCl solution four times and then with two liters of distilled water. The resin was then packed into a sintered glass column (45 cm \times 2.6 cm) and washed with water until the washings were free of chloride ions. Up to 1000 ml of APS synthesis mixture containing up to five millimoles APS were passed through the column at a flow rate of 0.6 cm/min. The column was then washed with 200 ml of water and developed with a linear salt gradient composed of 2.4 l each of 7 M urea + 50 mM Tris-HCl, pH 8.0, and 1.2 M NaCl + 7 M urea + 50 mM Tris-HCl buffer, pH 8.0. Fractions of 26.2 ml were collected and analysed.

Desalting was carried out basically according to Cohn and Bollum [9] by diluting the pooled APS fractions from the Dowex 1 column with four volumes of water and passing them through a column (30 cm \times 2.2 cm) of Bio-Rex 5, chloride form, 200–400 mesh. Urea and Tris are not bound and thus removed by a simple passage through the

column. APS was bound quite strongly. The column was then converted to the bicarbonate form by passing 0.1 M NH_4HCO_3 through the column until no more chloride could be detected (about 2 l). The column was then eluted with 2 l of a linear NH_4HCO_3 gradient from 0 to 1.5 M. Fractions of 26.2 ml were collected and analysed. Those fractions containing APS were pooled and lyophilized. The freeze dryer was equipped with a heatable plexiglass hood. After attaining a vacuum of 0.05 mbar the hood temperature was raised from room temperature to 40 °C to accelerate the decomposition of the remaining NH_4HCO_3 . The oil pump was run with full gas ballast during the entire procedure. The dry salt of APS was stored dry at –18 °C. Alternatively the APS fractions were pooled and diluted with water to a concentration of 10 mM. This solution was stored frozen in small portions until needed and was suitable for most purposes.

Hexokinase, glucose-6-phosphate dehydrogenase and the disodium salts of AMP, ADP, ATP and APS as well as adenosine were obtained from Boehringer & Söhne, Mannheim. Dowex ion exchange resin was from Serva, Heidelberg. Bio-Rex 5 resin was purchased from Bio-Rad Laboratories, Richmond, California. Yeast ATP-sulfurylase was obtained from Sigma, München. HPTLC plates and all other chemicals used were from Merck, Darmstadt. Spectral measurements were carried out in a Zeiss PM 6 spectrophotometer at 25 °C. The freeze dryer used was a model LO 5-60 from WKF, Brandau/Darmstadt. All operations were carried out at room temperature unless stated otherwise.

Results and Discussion

Adams *et al.* [5] reported that too high sulfate concentrations made chromatography of their APS synthesis mixture on DEAE-cellulose difficult and that too high a concentration of ferricyanide inhibited the APS-reductase used significantly. They therefore used a slight excess of sulfite and ferricyanide. We found that increasing the sulfite concentration by 100% and the ferricyanide concentration by 50% led consistently to the conversion of over 98% of the AMP used to APS. This is in comparison to a variable yield of 60–90% reported by these authors. Ferricyanide was added in small portions and did not impair the reaction rate. The high sulfate and ferrocyanide concentrations did not impair the ion exchange chromatography on Dowex 1 resin. Fig. 1

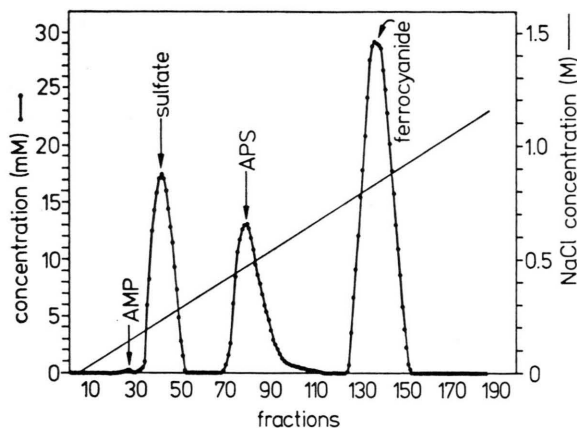


Fig. 1. Preparative column chromatography of 1000 ml APS synthesis mixture (5 mmol APS) on Dowex 1 resin. For experimental details see Materials and Methods.

shows the elution profile of a typical run. Sulfate (5 mmol), APS (4.95 mmol), ferrocyanide (12 mmol) and a trace of AMP (0.05 mmol) were eluted as distinct and well-separated peaks. The use of 7 M urea was necessary due to the fact that APS and ferrocyanide were eluted simultaneously in its absence. Some of the ferrocyanide is oxidized on the column and remains bound tightly.

The desalting procedure described above is based on the fact that APS is bound more tightly to anion exchangers than are chloride ions. It is thus possible to dilute the APS/chloride solution and rebind the APS to a second smaller column. The chloride ions on the column are displaced by bicarbonate ions in dilute solution while APS remains tightly bound to

the column. After removal of all chloride ions the APS is then eluted with a more concentrated volatile NH_4HCO_3 solution. Initial attempts to employ the desalting procedure of Cohn and Bollum [9] failed due to the fact that APS could not be eluted from Dowex with concentrated carbonate solutions. Fig. 2 shows the course of a typical desalting procedure.

The APS fraction obtained by chromatography on Bio-Rex 5 resin contained no other UV-absorbing material and no inorganic sulfate. It was sufficiently concentrated to be used for most purposes. The purity of the APS fraction after lyophilization was estimated by high performance thin layer chromatography, high-voltage electrophoresis and enzymatic determinations. One major UV-absorbing component was obtained which was identified enzymatically as APS (90–93% w/w). Two minor UV-absorbing impurities were detected consistently. One was identified as AMP; the other was not any of the known nucleotides used as references in high performance thin layer chromatography and high voltage electrophoresis. Research on its structure and mode of formation is being carried out presently. The chromatographic data for all substances involved are listed in Table I.

Table I. Chromatographic data for the lyophilized APS preparation isolated as described in text, the two minor impurities and the reference substances used.

Substance	R_f -value (high performance thin layer chromatography)	Distance of migration (high voltage electrophoresis) [cm]
adenosine	0.80	0.0
AMP	0.42	4.8
ADP	0.23	8.5
ATP	0.15	10.3
commercial APS	0.68	8.0
APS (as synthesized above)	0.68	8.0
first impurity	0.72	2.4
second impurity	0.42	4.8

Although the procedure described above is suitable for the preparation of up to 5 mmol APS, it should be feasible to scale it up to accommodate even greater quantities if desired.

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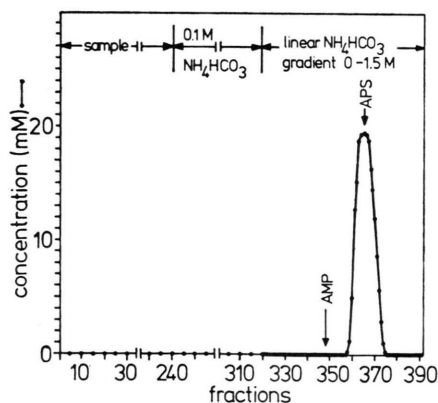


Fig. 2. Desalting of the Dowex 1 APS fraction on Bio-Rex 5 resin. 6.3 liters of APS/chloride/urea solution were applied and desalted as described in Materials and Methods. The APS fraction was lyophilized to obtain the diammonium salt of APS.

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