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A Nucleotide with the Properties of Adenosine 5' Phosphoramidate from Chlorella Cells[1)]

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SUMMARY: We have extracted and purified a nucleotide from cells of Chlorella pyrenoidosa Chick which shares the following properties with adenosine 5' phosphoramidate; electrophoretic mobility in sodium bicarbonate and in sodium borate buffer (pH 8.0); retention time on high performance liquid chromatography; ultraviolet absorption spectrum at pH 1-2 and 7-9; a yield of one mole each of adenine, ribose, total phosphate and ammonia released at low pH; and formation of adenosine 5' monophosphate on acidification or treatment with 3':5'-cyclic-nucleotide phosphodiesterase (EC3.1.4.17). Although formation of APA from its precursor adenosine 5' phosphosulfate during extraction and purification is not expected this appears to be excluded by the use of low temperature throughout purification and the finding that [^{14}C] APS added before extraction does not significantly label the adenosine 5' phosphoramidate isolated. Thus adenosine 5' phosphoramidate appears to be a normal constituent of Chlorella cells like the enzyme which forms it: adenylyl sulfate: ammonia adenylyl transferase.

INTRODUCTION: We have been able to isolate an enzyme from Chlorella cells, adenylyl sulfate: ammonia adenylyl transferase which catalyzes the formation of adenosine 5' phosphoramidate (APA)[4)] from ammonia and adenosine 5' phosphosulfate (APS) (1,2,3) (Fig. 1). We have purified this enzyme to homogeneity from Chlorella and find that the activity is present in a wide variety of organisms including bacteria, algae, fungi

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4) ABBREVIATIONS: APA, adenosine 5' phosphoramidate; APS, adenosine 5' phosphosulfate; HPLC, high performance liquid chromatography.

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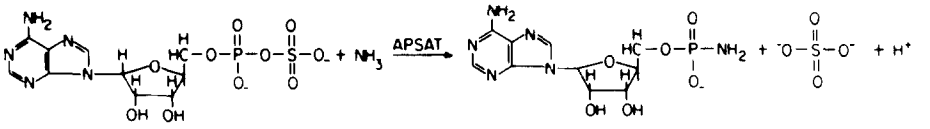

APSAT = ADENYLYL SULFATE: AMMONIA ADENYLYL TRANSFERASE

Fig. 1. Reaction catalyzed by adenylyl sulfate: ammonia adenylyl transferase (APSAT) showing structures of adenosine 5' phosphosulfate and adenosine 5' phosphoramidate.

and higher plants. Because the Km of the enzyme for ammonia is rather high (10 mM) (3) it was of interest to determine whether this enzymatic reaction ordinarily takes place in vivo. Also, nucleotide phosphoramidates such as APA have not been reported from living systems to our knowledge. The presence of APA among the soluble cellular nucleotides would lend support to the view that the enzyme forming it has a role in cellular metabolism. What follows demonstrates that a compound with the properties of adenosine 5' phosphoramidate can be isolated from the soluble fraction of Chlorella cells.

MATERIALS AND METHODS: Adenosine 5' phosphoramidate is hydrolyzed to 5' AMP and ammonia below a pH of about 6 (4,5,6); for this reason it was desirable to maintain a pH above neutrality during extraction and purification. However, various compounds catalyze the decomposition of phosphoramides or react with them even at pH values above 7 (4,5) and for this reason it was desirable to avoid high concentrations of anions, amines and other compounds that might destroy APA during extraction and purification. During this study methods were developed which were gradually improved as the work progressed. Cells of Chlorella pyrenoidosa Chick were grown phototrophically in fifteen liter carboys bubbled with 5% CO_2 in air as described previously (3).

All of the following steps were done at 4° unless otherwise noted. The cells (40-100g) were harvested in a CEPA-Schnell continuous flow centrifuge, were resuspended in 25 mM sodium phosphate buffer pH 7.4 (200-500 ml), were recentrifuged at 10,000g for 15 min. and were then resuspended in 200-500 ml of 10 mM sodium borate buffer pH 7.3. The cell suspension was allowed to freeze at -18° and was then thawed but maintained at about 4°. The pH was checked at this point and was adjusted to pH 7.3, if necessary, by addition of solid sodium tetraborate. The freezing and thawing procedure (including pH adjustment) was repeated two more times and after the final pH adjustment (if necessary) the suspension was centrifuged at 15,000g for 30 min. The supernatant fluid was concentrated about

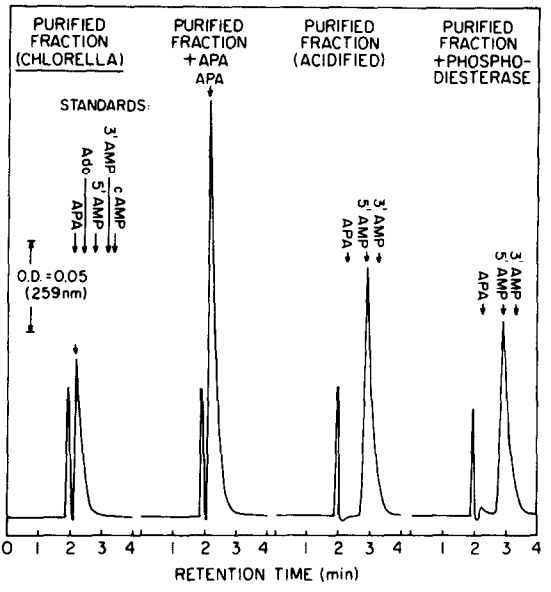

Fig. 2. Comparison of separation patterns obtained by high performance liquid chromatography (7) of: the purified fraction from Chlorella compared with standards (APA= adenosine 5' phosphoramidate, Ado=adenosine, 5' AMP= adenosine 5' monophosphate, 3' AMP=adenosine 3' monophosphate and cAMP=3'5' cyclic adenosine monophosphate); the purified fraction mixed with authentic adenosine 5' phosphoramidate; the purified fraction brought to low pH; and the purified fraction treated with phosphodiesterase. The acidified material and the phosphodiesterase-treated material cochromatographed exactly with added adenosine 5' monophosphate and showed a clear separation from added adenosine 3' monophosphate (data not shown). An analytical column of LiChrosorb RP-18 was used with 9.4% (v/v) isopropanol in water, 3 mM with respect to tetrabutylammonium hydroxide and adjusted to pH 9.4 with 1 N phosphoric acid, as solvent. The flow rate was 1.5 ml min^{-1}. The initial "peak" on the ultraviolet traces at retention time 1.9 min is found with all samples including those lacking ultraviolet absorbing compounds; thus it is not due to the presence of a nucleotide component but serves as a convenient point of rapid comparison for the nucleotides which emerge later.

forty fold in vacuo between 4° and 30° and was then frozen. This freezing step precipitates residual protein. Removal of residual protein can also be accomplished by ultra-filtration through an Amicon PM10 filter. After thawing, the concentrate was centrifuged at 15,000g for 10 min. and the supernatant fluid was used as the cell extract for subsequent steps.

Bio-Gel P-2 was equilibrated with 25 mM sodium phosphate buffer pH 8.0 and was poured as a column 2.6 x 110 cm. A five ml aliquot of the cell extract was applied to the column followed by more of the equilibrating buffer. The APA emerged from the column in the fractions between 200 and 300 ml of buffer and the precise location was determined using high performance liquid chromatography (HPLC) as previously described (7) but with an LDC constametric III pump and

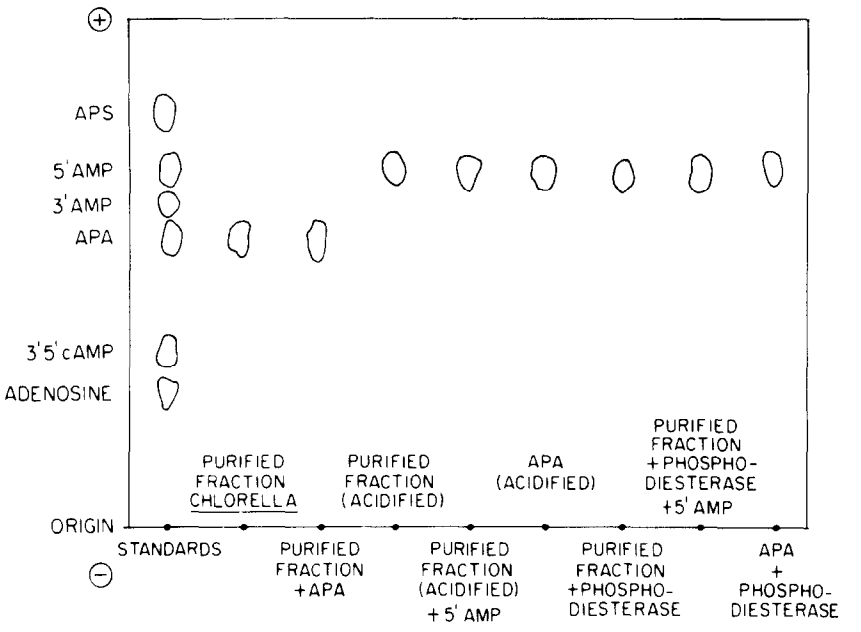

Fig. 3. Comparison of separation patterns obtained by paper electrophoresis in 0.1 M sodium borate buffer pH 8.0 of the purified fraction from Chlorella, the purified fraction after acidification and after treatment with 3'5' cyclic nucleotide phosphodiesterase, and comparisons with various standard compounds including adenosine 5' phosphosulfate (APS), 5' AMP, 3' AMP, adenosine 5' phosphoramidate (APA), 3'5' cyclic AMP (3'5' cAMP), and adenosine. Electrophoresis was performed for 1.5 hr at 1800 v at 4°.

a Gilford Model 2000 Spectrophotometer set at 259 nm equipped with a Hellma 178.320S HPLC microflowcell as detector. In this system APA emerges just ahead of AMP (see figure 2). Concomitant colorimetric tests for borate (3,8) indicated that the borate emerged from the P-2 column later than APA and did not contaminate the APA fractions with large amounts of the salt. The fractions containing APA were concentrated in vacuo between 4° and 30° to about 1 ml and the concentrate was streaked on Whatmann 3MM paper for preparative paper electrophoresis in 100 mM sodium borate buffer pH 8.0 at 1800 volts for 1.5 hrs (3). After drying at room temperature, the UV-quenching material moving to the position expected for APA (see Fig. 3) was eluted from the paper by extracting three times with glass-distilled water at 4° using a spin thimble and centrifugation to separate the liquid from the paper. The eluate was lyophyllized to about one ml. At this point HPLC showed that the APA was still contaminated with other nucleotides including small amounts of ADP and ATP, and a trace of AMP. This eluate was reapplied to the P-2 column which had been equilibrated with glass-distilled water brought to pH 8.5 with lithium hydroxide. The column was eluted with the same pH 8.5 solution; the APA emerged between 180 and 230 ml of eluant (since LiOH is not a buffer, the pH should be checked and more LiOH added if necessary). The APA-containing fractions were combined and concentrated by lyophyllization to about one ml. This concentrate was subjected to preparative paper electrophoresis as

before except that 25 mM $NaHCO_3$ pH 8.0 was substituted for the borate buffer. The pH of the $NaHCO_3$ solution used for electrophoresis should be adjusted to pH 8.0 with NaOH, if necessary, before use, since the buffer capacity at this pH is very low. For this reason the solution should ordinarily be used only once as an electrolyte; if the polarity of the apparatus is reversed on alternate electrophoreses, the solution can be used as an electrolyte up to four times. The APA region was eluted from the paper as before using glass-distilled water adjusted to pH 8 with $NaHCO_3$ solution. The combined eluates were concentrated to about one ml by lyophyllization and this preparation was used for the characterization of the APA. This method was used unless otherwise noted. In some later experiments it proved possible to shorten the procedure somewhat by omitting the preparative paper electrophoresis in borate buffer and the second P-2 column. In this shortened procedure, the concentrated eluate from the first P-2 column was used directly for preparative paper electrophoresis.

Authentic APA was obtained from Sigma.

RESULTS: Fig. 2 shows that the material purified from Chlorella moves to the same position as APA on HPLC (7) and does not separate from APA when a mixture of the isolated material and the authentic compound are subjected to HPLC. The only compound among the large number tested which also eluted near this position was adenosine but no adenosine or other nucleosides were detectable in the purified material using paper electrophoresis in either borate or bicarbonate (see above, and Fig. 3).

Another property of APA is its acid lability (1-6). The purified material was brought to pH 1.0 by addition of 10N HCl and was allowed to incubate for two hrs at 30°. The preparation was then brought to pH 7.5 by addition of NaOH; acid-washed neutral charcoal (3) (20 mg/ml) was then added. After mixing, the suspension was centrifuged at 5000g for 10 min., the charcoal resuspended again in the same supernatant fluid, and centrifuged again. The supernatant fluid was discarded and the charcoal was eluted three times with 2.0 ml of 50% (v/v) ethanol 1.0 mM with respect to tetrabutylammonium hydroxide. (Control experiments verified that this compound does not react with APS either chemically (3) or enzymatically (3) in place of ammonia.) After the last centrifugation, the eluates were combined

and concentrated to about 0.5 ml *in vacuo* at 30°. When this concentrated charcoal eluate was subjected to HPLC (Fg. 2) the formation of 5' AMP (at pH 1.0) from the APA in the purified material from *Chlorella* was verified.

Similarly, treatment with 3':5'-cyclic-nucleotide phosphodiesterase (EC 3.1.4.17) from beef heart is known to catalyze the formation of 5' AMP from APA (3,9). The reaction was carried out with the concentrated charcoal eluate as previously described (9) except that the incubation time was lengthened to one hr. Fig. 2 shows that this treatment produced 5' AMP from the APA in the purified fraction from *Chlorella.*

Further evidence for the identity of the purified material from *Chlorella* was obtained using sodium borate electrophoresis (3). This technique not only allows separation by charge differences at pH 8.0 where APA is stable, but since vicinal hydroxyl groups are necessary for the formation of negatively-charged borate complexes it distinguishes nucleotides having substituents in the 2' and 3' positions from those having only 5' substituents. The purified material shows exact coelectrophoresis with authentic APA on borate electrophoresis (Fig. 3). On treatment with acid or phosphodiesterase as for HPLC (preceding) 5' AMP is formed.

The absorption spectrum of the purified material from *Chlorella* was the same as that reported in the literature for adenine-containing nucleotides such as AMP (10) and as measured by us for authentic APA. At pH 7-9 the absorption peaks were at 259nm; at pH 1-2 the peaks shifted to 257 nm.

APA is expected to yield one mole each of ribose, total phosphate, and ammonia released at pH 1.0 for each mole of adenine determined from the absorption at 259 nm. Using established methods (orcinol reaction for ribose (11), molybdate reaction for phosphate (12) and Nessler

Tab. 1: Expected or determined molar ratios of various moieties of adenosine 5' phosphoramidate and purified material from Chlorella.

	Adenosine 5' Phosphoramidate	Isolated Compound Found*	
	Expected	Preparation 1	Preparation 2
Adenine	1.00	1.00	1.00
Ribose (Moles/Mole Adenine)	1.00	0.97	1.01
Total Phosphate (Moles/Mole Adenine)	1.00	1.04	0.92
NH_3 released at pH 1.0 (Moles/Mole Adenine)	1.00	0.94	0.94

*The values in each column were normalized to value for adenine taken as 1.00. The amount of adenine nucleotide was determined using a molar extinction coefficient of 15.4×10^3 at 259 nm (10).

reaction for ammonia (3)), the purified fraction from Chlorella yielded molar ratios in agreement with this expectation (Table 1).

Using the abbreviated procedure for isolation of the purified material from Chlorella it was possible to show that when care was taken to keep everything at 4° or less, there was no change in the preparation obtained making it very unlikely that the APA found could have been formed enzymatically or chemically during the extraction and purification procedures. One would not expect this to happen anyway, since freeze thawing releases very little protein from Chlorella (13), APS is exceedingly low or undetectable in Chlorella extracts, the Km of adenyl sulfate: ammonia adenylyl transferase for ammonia is rather high (10 mM) (3), and the chemical reaction of APS and ammonia is undetectable even at room temperature (3).

As a check on whether any APA might be formed during extraction and purification, [^{14}C-] APS (2.03×10^6 cpm; 479 cpm/nmole) (3) was added to the extraction buffer before the Chlorella cells were subjected to freezing and thawing. After purification by the abbreviated procedure the material obtained from bicarbonate or borate electro-

phoresis was found to contain negligible radioactivity indicating that essentially no APA was formed from APS during the extraction and purification procedures.

DISCUSSION AND CONCLUSIONS: Taken together, the evidence presented shows that a compound exhibiting the known properties of APA can be isolated from Chlorella cells. Since APA is found among the nucleotides of the cell it must be considered as a normally-occurring cellular constituent. The enzyme catalyzing its formation, adenylyl sulfate: ammonia adenylyl transferase has been purified to homogeneity and is a protein of 64 KD molecular weight containing three subunits of 26, 21 and 17 KD (3). The enzyme is highly specific for APS and NH_3 and is not one of the handful of enzymes known to catalyze other reactions of APS (3). Thus both the enzyme and its product are newly-discovered entities in biological systems.

The free energy of hydrolysis of APA to AMP and ammonia is substantial (3) and for this reason, among others, it has been used in the chemical synthesis of phosphate anhydrides such as ADP, ATP, and mixed dinucleotides (6). APA has also been shown to replace AMP as an activator in vitro of certain enzymes such as phosphorylase b (protein kinase, EC2.7.1.3.7), threonine deaminase (threonine dehydratase, EC4.2.1.16) and adenylate cyclase (ATP pyrophosphate-lyase, EC4.6.1.1) (14-17). Apart from its possible role as an enzyme activator in vivo, this compound might serve as a regulatory link between sulfur and nitrogen metabolism or might play a role as an intermediate or regulator in nitrogen metabolism or in nucleotide metabolism to mention only a few of the more obvious possibilities.

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