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Purification and properties of adenylyl sulphate : ammonia adenylyltransferase from *Chlorella* catalysing the formation of adenosine 5′-phosphoramidate from adenosine 5′-phosphosulphate and ammonia

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Extracts of *Chlorella pyrenoidosa*, *Euglena gracilis* var. *bacillaris*, spinach, barley, *Dictyostelium discoideum* and *Escherichia coli* form an unknown compound enzymically from adenosine 5′-phosphosulphate in the presence of ammonia. This unknown compound shares the following properties with adenosine 5′-phosphoramidate: molar proportions of constituent parts (1 adenine : 1 ribose : 1 phosphate : 1 ammonia released at low pH), co-electrophoresis in all buffers tested including borate, formation of AMP at low pH through release of ammonia, mass and i.r. spectra and conversion into 5′-AMP by phosphodiesterase. This unknown compound therefore appears to be identical with adenosine 5′-phosphoramidate. The enzyme that catalyses the formation of adenosine 5′-phosphoramidate from ammonia and adenosine 5′-phosphosulphate was purified 1800-fold (to homogeneity) from *Chlorella* by using $(NH_4)_2SO_4$ precipitation and DEAE-cellulose, Sephadex and Reactive Blue 2–agarose chromatography. The purified enzyme shows one band of protein, coincident with activity, at a position corresponding to 60000–65000 molecular weight, on polyacrylamide-gel electrophoresis, and yields three subunits on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of 26000, 21000 and 17000 molecular weight, consistent with a molecular weight of 64000 for the native enzyme. Isoelectrofocusing yields one band of pI 4.2. The pH optimum of the enzyme-catalysed reaction is 8.8. ATP, ADP or adenosine 3′-phosphate 5′-phosphosulphate will not replace adenosine 5′-phosphosulphate, and the apparent K_m for the last-mentioned compound is 0.82mM. The apparent K_m for ammonia (assuming NH_3 to be the active species) is about 10mM. A large variety of primary, secondary and tertiary amines or amides will not replace ammonia. One mol.prop. of adenosine 5′-phosphosulphate reacts with 1 mol.prop. of ammonia to yield 1 mol.prop. each of adenosine 5′-phosphoramidate and sulphate; no AMP is found. The highly purified enzyme does not catalyse any of the known reactions of adenosine 5′-phosphosulphate, including those catalysed by ATP sulphurylase, adenosine 5′-phosphosulphate kinase, adenosine 5′-phosphosulphate sulphotransferase or ADP sulphurylase. Adenosine 5′-phosphoramidate is found in old samples of the ammonium salt of adenosine 5′-phosphosulphate and can be formed non-enzymically if adenosine 5′-phosphosulphate and ammonia are boiled. In the non-enzymic reaction both adenosine 5′-phosphoramidate and AMP are formed. Thus the enzyme forms adenosine 5′-phosphoramidate by selectively speeding up an already favoured reaction.

During investigations of sulphate reduction with adenosine 5′-phosphosulphate as a substrate, a new compound was found among the products when a partially purified enzyme preparation from *Chlorella pyrenoidosa* containing $(NH_4)_2SO_4$ was employed (Tsang & Schiff, 1976*a*). Although many properties of this compound resembled those of cyclic AMP, separation of a new entity from cyclic AMP was eventually achieved.

In the present paper we show that the new compound is adenosine 5′-phosphoramidate and describe the purification and properties of an enzyme,

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adenylyl sulphate:ammonia adenylyltransferase, that catalyses its formation. Brief reports in abstract of the work have appeared previously (Fankhauser *et al.*, 1979; Fankhauser & Schiff, 1980).

Experimental

Organisms and enzyme preparations

Chlorella pyrenoidosa Chick Emerson strain 3 from slants was grown phototrophically on the medium described previously (Schiff & Levinthal, 1968) in 15-litre cultures in carboys or in 150-litre lots in barrels (Lyman & Siegelman, 1967) for enzyme purification. Growth took place at 24°C. For carboys light from a mixture of red and daylight fluorescent lamps was employed at an intensity of 3.8 klx (350 ft.-candles); barrels were placed in growth chambers at an intensity of 11 klx (1000 ft.-candles) from overhead mixed white fluorescent and incandescent lamps, and additional white fluorescent lamps contained in large test tubes were inserted into the barrels to provide supplementary light as previously described (Lyman & Siegelman, 1967). From this point on, everything was done at 4°C. Cells were harvested in the late exponential phase of growth by centrifugation either in the Szent-Györgyi–Blum continuous-flow attachment in a Sorvall model RC2-B centrifuge or in a CEPA Schnell continuous centrifuge. The yield from the barrels was about 2 g (fresh wt.)/litre and from the carboys was about 3 g/litre.

The *Chlorella* cells were then washed with 0.1 M-Tris/HCl buffer, pH 7.0, containing 50 mM-2-mercaptoethanol (buffer I) by centrifugation at 10000 *g* for 10 min and were resuspended in a minimum amount of the same buffer. The cells were usually frozen and stored at −18°C at this point. After being thawed, the cells were diluted to a concentration of about 1 g/10 ml in the same buffer and were passed through a chilled French pressure cell at 41–48 MPa (6000–7000 lbf/in^2). The suspension was then centrifuged at 10000 *g* for 30 min and the supernatant fluid was used as the crude enzyme preparation.

The crude *Chlorella* extract was fractionated further to purify the adenylyl sulphate:ammonia adenylyltransferase activity by using procedures based on previous methods (Tsang & Schiff, 1976*a*). $(NH_4)_2SO_4$ was added with stirring over the course of 1 h to the crude extract to a concentration of 40% saturation determined for 25°C but performed at 4°C. After being stirred for an additional 1 h the suspension was centrifuged at 16000 *g* for 15 min and the precipitate was discarded. More $(NH_4)_2SO_4$ was added to the supernatant fluid in the same manner to yield a final concentration of 60% saturation. After centrifugation and isolation of the pellet, $(NH_4)_2SO_4$ was added to the supernatant fluid to a concentration of 80% saturation, and the precipitate was isolated by centrifugation; the supernatant fluid contained negligible activity and was discarded. The pellets obtained from 40–60%-saturation and 60–80%-saturation $(NH_4)_2SO_4$ precipitations were each resuspended in the minimum amount of 20 mM-Tris/HCl buffer, pH 8.0, containing 50 mM-2-mercaptoethanol (buffer II) and were dialysed with stirring against 2 litres of the same buffer for 4 h and against another 2 litres of the same buffer overnight. The dialysis tubing was washed before use by boiling for 1 h in 5% (w/v) $NaHCO_3$ containing 0.1 mM-EDTA, rinsing with distilled water for 2 h, boiling for 1 h in distilled water, rinsing in distilled water for 2 h and storage at 4°C in distilled water. The contents of each dialysis sac were centrifuged at 16000 *g* for 15 min, the pellets were discarded and the supernatant fluids were subjected, separately, to column chromatography on DEAE-cellulose (Whatman DE-52).

Chromatography of the 40–60%-satn.-$(NH_4)_2SO_4$ fraction was on a 24 cm × 2.2 cm column (bed volume 88 ml); a 10 cm × 1.0 cm column (bed volume 9.0 ml) was used for the 60–80%-satn.-$(NH_4)_2SO_4$ fraction. DE-52 DEAE-cellulose, suspended in buffer II, was poured into the columns and washed with additional buffer II until the effluent reached a pH of 8.0. The supernatant fluids from $(NH_4)_2SO_4$ fractionation were then passed through their respective columns followed by 3 vol. of buffer II containing 50 mM-NaCl, and the proteins were eluted with a linear gradient of 50 mM-0.27 M-NaCl in buffer II. After determination of enzyme activity directly on each fraction, the active fractions were pooled and dialysed against buffer II in the same manner as before. The active fractions from the two columns were then combined and subjected to rechromatography on DE-52 DEAE-cellulose. Rechromatography on DE-52 DEAE-cellulose was performed in the same manner as before on a 10 cm × 1.0 cm column. The active fractions (Fig. 1*a*) were again pooled and dialysed as before against buffer II, centrifuged at 16000 *g* for 15 min and the supernatant fluid was used for further purification.

The supernatant fluid from the previous step was subjected to chromatography on Sephadex G-200. Sephadex G-200 was swelled in 0.1 M-Tris/HCl buffer, pH 9.0, containing 50 mM-2-mercaptoethanol (buffer III) overnight at room temperature. The swelled gel was then poured as a 78 cm × 2.2 cm column. The enzyme supernatant was added to the column in a total volume of 7.2 ml, and the column was developed with buffer III. The most active fractions (Fig. 1*b*) were isolated individually and further purified by Reactive Blue 2–agarose chromatography.

Reactive Blue 2–agarose from Sigma Chemical

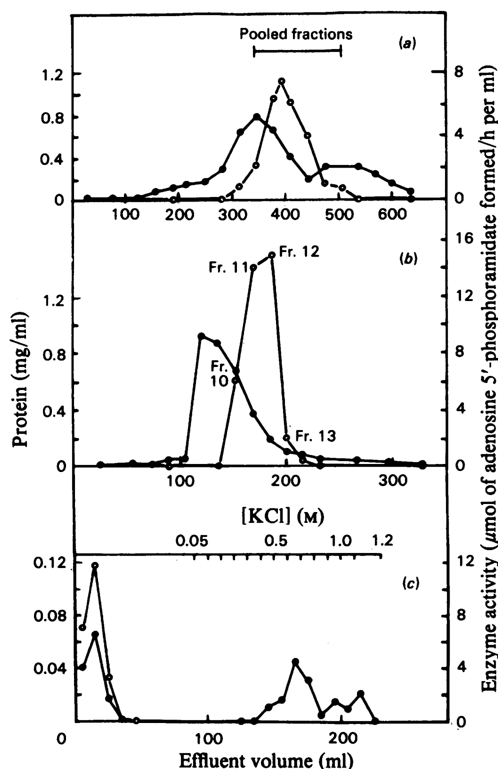


Fig. 1. *Chromatography of adenylyl sulphate: ammonia adenylyltransferase during various steps of purification*
For experimental details see the text. (*a*) Step IV: DEAE-cellulose chromatography of the 40–60%-satn.-$(NH_4)_2SO_4$ fraction from step III. (*b*) Step V: Sephadex G-200 chromatography of pooled fractions from step IV. (*c*) Step VI: Reactive Blue 2–agarose chromatography of fraction V12. O, Adenylyl sulphate: ammonia adenylyltransferase activity; ●, protein.

Co. (St. Louis, MO, U.S.A.) was poured as a 5.5 cm × 1.5 cm column and was then equilibrated by passage of a large volume of buffer II. Each active fraction was processed separately. The fraction (16 ml) was applied to the column, followed by 74 ml of buffer II, which removes all of the adenylyl sulphate: ammonia adenylyltransferase activity (Fig. 1). This was followed by 10 ml of 50 mM-KCl in buffer II, 10 ml of 0.1 M-KCl in buffer II and 10 ml portions of buffer II successively increasing in 0.1 M increments of KCl to 1.0 M-KCl, and finally with two successive 10 ml volumes of 1.2 M-KCl in buffer II. The adenosine 5′-phosphosulphate sulphohydrolase activity appears in the first 1.2 M-KCl eluate (Fig. 1*c*). Selection of the appropriate fractions for Reactive Blue 2–agarose chromatography yields a maximum purification of adenylyl sulphate: ammonia adenylyltransferase of 1791-fold with a recovery of 14% based on the activity in the crude extracts, when all fractions from the Reactive Blue 2–agarose column containing the activity are pooled. These pooled fractions from Reactive Blue 2–agarose chromatography of Sephadex fractions 11, 12 and 10 + 13 were used in the experiments reported in the present paper.

Euglena gracilis Klebs var. *bacillaris* Cori was grown in light and darkness as previously described (Greenblatt & Schiff, 1959; Stern *et al.*, 1964) on Hutner's pH 3.5 medium (Greenblatt & Schiff, 1959). Cultures containing 10^6 cells/ml were harvested by centrifugation at 16000 g for 15 min at 4°C. The cells were resuspended in a minimum volume of 0.1 M-Tris/HCl buffer, pH 7.5, and were broken by pulse sonication in ice in a Branson model 200 Sonifier at 50 W for 15 s.

$(NH_4)_2SO_4$ fractionation was performed on the supernatant fluid followed by dialysis as described above, and the various fractions were assayed for adenylyl sulphate: ammonia adenylyltransferase activity.

Barley (*Hordeum vulgare* L.) and spinach (*Spinacia oleracea* L.) were grown from seed for 30 days in vermiculite/potting soil (1:1) in a growth chamber on a 10 h day (22°C)–14 h night (18°C) cycle at 20 klx (1850 ft.-candles) provided by white fluorescent and incandescent lamps. The plants received distilled water as needed. A 500 g batch of washed leaves was cut into small pieces and ground in a chilled mortar with washed and ignited sand and 0.1 M-Tris/HCl buffer, pH 7.5. The homogenate was then centrifuged at 12000 g for 15 min at 4°C, the supernatant was subjected to $(NH_4)_2SO_4$ fractionation and dialysis as before, and the fractions were then assayed for adenylyl sulphate: ammonia adenylyltransferase activity.

Dictyostelium discoideum was cultured as described by Pederson (1977), and was harvested when the cell concentration was 10^7 cells/ml. The cells were isolated and broken by using the same methods as for *Euglena*. $(NH_4)_2SO_4$ fractionation followed by dialysis was as before, followed by assay of the fractions for adenylyl sulphate: ammonia adenylyltransferase activity.

Escherichia coli B/r was grown as described previously (Tsang & Schiff, 1976*b*). The cells were harvested by centrifugation at 10000 g for 10 min at 4°C and were washed with 0.1 M-Tris/HCl buffer pH 7.0, containing 50 mM-2-mercaptoethanol. After resuspension in this buffer the mixture was passed twice through a chilled French pressure cell at 41–48 MPa (6000–7000 lbf/in^2). The suspension was centrifuged at 16000 g for 30 min at 4°C, and $(NH_4)_2SO_4$ fractionation was performed on the supernatant in the usual way. The dialysed fractions

were then assayed for adenylyl sulphate:ammonia adenylyltransferase activity.

Enzyme assays

Assay of adenylyl sulphate:ammonia adenylyltransferase and adenylyl sulphate sulphohydrolase activities. Two assay methods were used to measure adenylyl sulphate:ammonia adenylyltransferase activity. When both adenylyl sulphate:ammonia adenylyltransferase and adenylyl sulphate sulphohydrolase activities were present in the same enzyme preparation, an assay based on the conversion of [U-^{14}C]adenosine 5′-phosphosulphate into [^{14}C]-adenosine 5′-phosphoramidate or [^{14}C]adenosine 5′-phosphate was used. When the highly purified enzymes were assayed sulphate release from adenosine 5′-phospho[^{35}S]sulphate was measured. Ammonia was added when the transferase or transferase plus sulphohydrolase activity was to be measured; ammonia was omitted when only the sulphohydrolase activity was assayed.

For the ^{14}C-based assay [^{14}C]adenosine 5′-phosphosulphate was used and the protocols are shown in the individual Figures and Tables. The incubations took place in tubes covered with Parafilm and placed at 30°C for 1h. The reaction was started by adding the enzyme. At the end of the incubation period, the tubes were chilled to 0°C and acid-washed charcoal (15mg/ml) was added. After a thorough mixing by vortex, the mixture was centrifuged at 5000**g** for 10min at 4°C. The charcoal was resuspended in the supernatant fluid, which was them re-centrifuged. The supernatant fluid was discarded and the charcoal was extracted three times with 2ml of 1% (w/v) aq. NH_3 in 50% (w/v) ethanol by centrifugation at 5000**g** for 10min at 4°C. The eluates were combined and were concentrated *in vacuo* at 30°C to a volume of about 0.5ml. A sample was applied to Whatman 3MM filter paper (57cm × 20cm) and was subjected to electrophoresis (EC apparatus) in 0.1M-sodium borate buffer, pH8.0, at 1800V for 1.5h at 0°C. After the paper had been dried, the metabolites were located under 253.7nm u.v. light, and the spots were excised from the paper, cut into small pieces, placed in scintillation vials with a mixture containing 0.4% 2,5-diphenyloxazole and 0.012% 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene/Triton X-100 (2:1, v/v) and their radioactivities were measured in a Beckman model LS-150 scintillation counter.

For the ^{35}S-based assay adenosine 5′-phospho-[^{35}S]sulphate was used and the procedure was the same up to the adsorption on charcoal. After adsorption of the nucleotides on the charcoal, a sample of the supernatant fluid was added to the same scintillation mixture and its radioactivity was counted to determine the amount of $^{35}SO_4^{2-}$ released.

Without added enzyme no adenosine 5′-phosphoramidate or AMP was formed in either assay procedure.

Assay of other enzyme activities. Adenylyl sulphate sulphotransferase activity was assayed as described previously (Fankhauser & Brunold, 1978). Conditions for assay of adenylyl sulphate kinase (EC 2.7.1.25) were as previously described (Burnell & Anderson, 1973*b*), but adenosine 5′-phospho[^{35}S]sulphate (195000c.p.m./μmol) was used as substrate and the formation of adenosine 3′-phosphate 5′-phospho[^{35}S]sulphate was assayed after purification by electrophoresis as described above for the adenylyl sulphate:ammonia adenylyltransferase assay. ADP sulphurylase (EC 2.7.7.4) activity was measured as phosphate uptake during the formation of ADP as previously described (Burnell & Anderson, 1973*a*), but, instead of the generation of adenosine 5′-phosphosulphate *in situ* with ATP, sulphate and ATP sulphurylase, synthetic adenosine 5′-phosphosulphate was used as the substrate. The formation of ADP was confirmed by paper electrophoresis in 25mM-sodium citrate buffer, pH5.8, on Whatman 3MM filter paper (57cm × 20cm) at 1800V for 1h. ATP sulphurylase (EC 2.7.7.4) was measured in the forward direction as described previously (Wilson & Bandurski, 1958). Assay of alkaline phosphatase activity with *o*-nitrophenyl phosphate as substrate followed established methods (Bessey *et al.*, 1946; Sigma Technical Bulletin 104). The phosphodiesterase treatment of adenosine 5′-phosphoramidate was as previously described (Tsang & Schiff, 1976*a*). Protein was measured turbidimetrically by the method of Schmidt (1975), but for chlorophyll-containing samples a wavelength of 750nm was used rather than 436nm.

Identification of adenosine 5′-phosphoramidate as a product of the adenylyl sulphate:ammonia adenylyltransferase

A partially purified enzyme (pooled fractions from step V; Table 1) was used in a prolonged incubation to obtain enough adenosine 5′-phosphoramidate for identification. A reaction mixture containing adenosine 5′-phosphosulphate (10μmol), NH_4Cl (500μmol), Tris/HCl buffer, pH9.0 (100μmol), and enzyme (30μg of protein) in a total volume of 1.0ml was incubated at 30°C for 5h. Adenosine 5′-phosphosulphate (10μmol) was then added and the incubation was continued for a further 12h, at which time another 10μmol of adenosine 5′-phosphosulphate was added. After incubation for another 4h the mixture was chilled to 0°C and acid-washed charcoal (20mg/ml) was added. After a vigorous mixing, the suspension was centrifuged at 5000**g** for 10min at 4°C and the charcoal was resuspended in the same supernatant fluid by

vigorous mixing. After re-centrifugation, the supernatant fluid was discarded and the charcoal pellet was eluted four times with 4 ml portions of 1% (w/v) NH_3 in 50% (v/v) ethanol by centrifugation as before. The combined eluates were then concentrated *in vacuo* at 30°C to 0.5 ml. This concentrate was streaked across sheets of Whatman 3MM filter paper (57 cm × 20 cm) and electrophoresis was performed in 0.1 M-sodium borate buffer, pH 8.0, at 1800 V for 1.5 h at 4°C. The papers were dried at room temperature and the adenosine 5′-phosphoramidate region was located by its quenching properties under 253.7 nm illumination and comparison with an authentic sample. These regions were cut from the papers and cut into small pieces, which were suspended in glass-distilled water and stirred for 30 min at 4°C. After centrifugation at 10000 *g* for 10 min at 4°C the paper was re-extracted two more times and the extracts were combined. Charcoal adsorption from the combined extracts was done as before. The charcoal pellet was resuspended in 10 ml of water adjusted to pH 7.0 with LiOH, and the suspension was then centrifuged at 5000 *g* for 10 min at 4°C. This was repeated twice [at which point the washings were free of borate as judged by the colorimetric reaction with Carmine (Taras *et al.*, 1971)] and the charcoal was resuspended in 10 ml of 50% (v/v) ethanol adjusted to pH 10.3 with LiOH. After centrifugation as before, the extraction was repeated five times. The supernatant fluids were combined and concentrated to about 1 ml *in vacuo* at 30°C. After filtration through a Millipore type HA filter (pore size 0.45 μm) to remove fine residual charcoal, the preparation was freeze-dried and the powder was washed twice with ethanol by centrifugation at 10000 *g* for 10 min at 4°C. The pellet was then dried *in vacuo*.

This pellet was used for all analyses except mass spectrophotometry. For mass spectrophotometry, excess salts were removed as follows. Bio-Gel P-2 (100–200 mesh) was poured as a 2.6 cm × 110 cm column as a slurry in water adjusted to pH 7.5 with LiOH. The samples in 0.5 ml of the same liquid was loaded on to the column, which was then developed with the same solution. The fractions emerging between 205 and 220 ml of effluent were collected and were freeze-dried.

Analysis for adenine was done by measuring the absorption of the sample at 259 nm in water adjusted to pH 7.0 with LiOH and using a molar absorption coefficient of $15.4 \times 10^3\,M^{-1}\cdot cm^{-1}$ (Bock *et al.*, 1956). Ribose was determined by using the orcinol reaction (Ashwell, 1957). Analysis for total phosphate employed a previously published method (Chen *et al.*, 1956).

Analysis for acid-releasable ammonia (Moffatt & Khorana, 1961) was done with the Nessler reagent. A sample (0.92 ml) was mixed with 0.08 ml of conc. (10 M) HCl and was incubated for 2 h at 30°C. Water (3.5 ml) was then added, followed by 0.5 ml of Nessler reagent (Sigma Ammonia Color Reagent). After 10 min at 30°C the absorption of this solution was measured at 420 nm, with a blank containing water rather than sample. The ammonia content was determined from a standard curve prepared under identical conditions.

The radioimmunoassay for cyclic AMP was done as previously described (Yalow & Berson, 1971) with a kit from New England Nuclear Corp. (Boston, MA, U.S.A.).

For i.r. spectra the dry sample (0.5–1.0 mg) was ground in an agate mortar with 0.8–1.0 g of i.r.-grade KBr and was then compressed into a pellet, which was scanned in a Perkin–Elmer model 567 grating infrared spectrophotometer.

Field desorption mass spectrometry, performed by Dr. C. E. Costello in the laboratory of Dr. K. Biemann, Massachusetts Institute of Technology, was employed in the present work to establish an identity between authentic lithium adenosine phosphoramidate and the lithium salt of the unknown.

Paper electrophoresis in sodium borate buffer (as above) was used to compare the enzyme product with authentic adenosine 5′-phosphoramidate. Some samples of this compound and of the enzyme product were treated with acid before electrophoresis, by adjusting a sample to pH 1.0 with HCl and incubating it for 2 h at 30°C. The pH was then adjusted to 7.0 with NaOH, and the compound was adsorbed on charcoal and eluted with ammonia in ethanol as described above. The combined eluates were concentrated *in vacuo* at 30°C and were then subjected to electrophoresis.

Characterization of the adenylyl sulphate : ammonia adenylyltransferase protein

Polyacrylamide-disc-gel electrophoresis was performed as described previously (Davis, 1964) with the following modifications. A 10% gel was used and the buffer was Tris/EDTA/borate described in a previous publication (Tsang & Schiff, 1976*a*). Electrophoresis was performed at 2.5–5 mA for about 4 h at 4°C. The gels were stained overnight with Coomassie Brilliant Blue R (from Sigma Chemical Co.) and were then destained at room temperature (Vaisberg *et al.*, 1976). For enzyme determinations, the unstained gel was sliced into 0.5 cm segments. Each segment was ground with 0.5 ml of 0.3 M-Tris/HCl buffer, pH 9.0, at 4°C and was then kept overnight at 4°C. The suspension was then centrifuged at 10000 *g* for 10 min at 4°C. The pellet was washed with the same buffer by centrifugation, and the extracts were combined and assayed with adenosine 5′-phospho[^{35}S]sulphate.

Sodium dodecyl sulphate/polyacrylamide-disc-gel electrophoresis was performed by standard methods

as described previously (Weber & Osborn, 1969). The sample was incubated at 37°C in a solution containing sodium dodecyl sulphate (1%, w/v), Bromophenol Blue (0.015%) and urea (36%, w/v) in 10mM-sodium phosphate buffer, pH 7.2. Before application of the sample, the gel was subjected to 2mA per tube for 0.5h; after application of the sample electrophoresis took place at 5mA for 3h at room temperature. Standards obtained from Sigma received the same treatment. Staining and destaining employed the same procedures as previously given for electrophoresis in the absence of sodium dodecyl sulphate.

Isoelectrofocusing was accomplished with polyacrylamide gel (7.5% acrylamide) with Biolyte 3-10 and Biolyte 3-5 (from Bio-Rad Laboratories, Richmond, CA, U.S.A.) in equal amounts at a final total concentration of 2% (v/v). The protein was loaded in 200μl of 25% (w/v) sucrose and was overlaid with 200μl of 20% (w/v) sucrose and 100μl of 10% (w/v) sucrose. Isoelectrofocusing was performed at 200V for 17h at 4°C, with 60mM-NaOH and 30mM-H_2SO_4 as the electrolytes. The gels were stained and destained as described above, for polyacrylamide-gel electrophoresis. For measurement of pH the gel was sliced into 0.5cm pieces, each slice was left to stand with 2ml of water and the liquid was measured in an Orion model 701A pH-meter.

Non-enzymic reaction of adenosine 5′-phosphosulphate and ammonia

The complete reaction mixture contained in a total volume of 1.0ml: Tris/HCl buffer, pH 7-9 (depending on the experiment) (100μmol), [^{14}C]-adenosine 5′-phosphosulphate (221320c.p.m./μmol; 2.0μmol) and NH_4Cl (500μmol). The reaction took place in a small test tube (capped with a rubber stopper) placed at 95°C for various times. In some experiments the reaction mixture was sealed into ampoules that were then autoclaved for 0.5h at 14kPa (15lbf/in^2 above atmospheric pressure) followed by an exhaust time of 0.5h. The reaction mixtures were then chilled in an ice bath, and from this point the measurement of adenosine 5′-phosphoramidate formation followed the same methods used for the enzymic assay, including adsorption on charcoal, elution with ethanolic ammonia and electrophoresis in borate.

Properties of adenylyl sulphate: ammonia adenylyltransferase

All assays were done under conditions where activity was proportional to enzyme concentration and time of incubation. The measurement of the stoicheiometry of the adenylyl sulphate:ammonia adenylyltransferase reaction was done by using the standard ^{14}C-based and ^{35}S-based assays described above. After paper electrophoresis, the radioactive spots were excised from the paper, cut into small pieces and the radioactivity was measured by scintillation counting as described above. Non-radioactive spots were eluted in a similar manner and the absorption at 259nm was measured against the eluate of a similar area of paper lacking the compounds as a blank. For determination of ammonia consumed and sulphate formed, an incubation mixture was adsorbed on charcoal and the charcoal was washed and eluted in the usual way. The radioactivity of the supernatant fluid plus the washes from the charcoal adsorption was counted to indicate the amount of sulphate and the Nessler reaction provided a measure of the ammonia needed. Unincubated blanks were measured in all cases to provide the values at zero time of incubation. The eluates from the charcoal were subjected to paper electrophoresis in borate to confirm that adenosine 5′-phosphoramidate was the only u.v.-absorbing product and to be sure that no AMP was formed from its breakdown.

Nucleotide specificity of the adenylyl sulphate: ammonia adenylyltransferase reaction was measured by using the standard assay conditions with non-radioactive substrates. Incubation of adenosine 3′-phosphate 5′-phosphosulphate, ATP, ADP or AMP at the same concentrations normally used for adenosine 5′-phosphosulphate yielded no detectable products on borate electrophoresis.

Specificity for ammonia was demonstrated by using the standard ^{35}S-based assay conditions; each nitrogen compound was added at either 10 or 100μmol/ml of reaction mixture. Sulphate released was measured as radioactivity in the supernatant fluid from the charcoal adsorption, and borate electrophoresis of the charcoal eluates was used to confirm the presence or absence of other products. The following compounds were tested.

Primary amines included: amino acid mixture (alanine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophan, glycine, serine, threonine, cysteine, tyrosine, aspartic acid, asparagine, glutamic acid, glutamine, lysine, histidine, arginine and proline); aniline; arginine; creatine; cyclohexylamine; *NN*-dimethyl-*p*-phenylenediamine; ethanolamine; ethylamine; ethylenediamine; hexamethyleneamine; histidine; hydroxylamine; lysine, methylamine hydrochloride; *N*-(napth-1-yl)ethylenediamine dihydrochloride; ornithine; *o*-phenylenediamine; *p*-phenylenediamine; protamine sulphate; putrescine dihydrochloride; pyridoxamine dihydrochloride; spermidine; spermine.

Among the secondary amines tested were: phosphocreatine; diethylamine hydrochloride; dimethylamine hydrochloride; diphenylamine; histidine; *N*-(naphth-1-yl)ethylenediamine dihydrochloride; spermidine; spermine.

Tertiary amines included: choline chloride; creatine; phosphocreatine; *p*-dimethylaminobenzaldehyde; *NN*-dimethylaniline; *NN*-dimethyl-*p*-phenylenediamine; histidine; pyridine; pyridoxal 5′-phosphate; pyridoxamine dihydrochloride; triethanolamine; triethylamine.

Amides tested were: acetamide; asparagine; formamide; glutamine; iodoacetamide; urea.

The possibility that a nitrogen function on a high-molecular-weight compound could serve as an acceptor for the adenylyl group of adenosine 5′-phosphosulphate under catalysis by adenylyl sulphate:ammonia adenylyltransferase in the absence of ammonia was tested as follows. The 1 ml reaction mixture contained [^{14}C]adenosine 5′-phosphosulphate (444000 c.p.m./μmol; 4μmol), Tris/HCl buffer, pH 9.0 (100μmol), dialysed crude extract [before $(NH_4)_2SO_4$ fractionation] (300μl; 2.96 mg of protein) and highly purified adenylyl sulphate:ammonia adenylyltransferase (fraction V 11 from step VI; 20μg). The incubation took place at 30°C for 3h. After being chilled to 0°C in an ice bath, the suspension was centrifuged at 10000g for 10min. The supernatant fluid was divided into two equal portions. One portion was subjected to charcoal treatment and borate electrophoresis in the usual manner, and it showed no radioactive materials other than adenosine 5′-phosphosulphate and AMP; about 80% of the adenosine 5′-phosphosulphate had been converted into AMP. The other portion was subjected to separation on Sephadex G-25 that had been swollen in 0.1M-Tris/HCl buffer, pH 8, overnight at room temperature. The column was washed with the same buffer until the effluent reached a pH of 8.0. The sample (0.4 ml) was applied to the column, followed by the same buffer. The excluded volume was collected in 6 ml fractions; no radioactivity was found in any of them.

Materials

All water used was glass-distilled, and all chemicals were reagent grade. Acid-washed charcoal was prepared as described previously (Kornberg & Stadtman, 1957).

Adenosine 5′-phospho[^{35}S]sulphate and adenosine 3′-phosphate 5′-phospho[^{35}S]sulphate were prepared as described previously (Hodson & Schiff, 1969; Tsang *et al.*, 1976), except that the final product was passed through 2.0ml of Dowex 50W-X8 (100–200 mesh) cation-exchange resin in the Na^+ form in a Pasteur pipette to convert the products into the sodium salts. The resin was received from Bio-Rad Laboratories in the H^+ form and was converted into the Na^+ form by washing with 0.1M-NaOH and then with water until neutral. The sample was applied as 5.0ml of solution, and the column was washed with water until no more of the desired product appeared in the effluent. The effluent was then adjusted to pH 7.5 with Tris base. Occasionally, non-radioactive adenosine 5′-phosphosulphate was purchased from Sigma Chemical Co.

[^{14}C]Adenosine 5′-phosphosulphate and [^{14}C]-adenosine 3′-phosphate 5′-phosphosulphate were prepared by the same methods previously used to obtain the ^{35}S-labelled compounds with the following modifications. The incubation mixture for adenosine 3′-phosphate 5′-phosphosulphate formation contained in a total volume of 1.3 ml: magnesium acetate (30μmol), Tris/HCl buffer, pH 9.0 (70μmol), 2-mercaptoethanol (20μmol), Na_2SO_4 (34μmol), levamisole (20μmol), disodium ATP (29.5μmol), disodium [^{14}C]ATP (569 Ci/mol; 250 mCi in 200μl of 0.1M-$KHCO_3$) and enzyme extract (1.0ml). A smaller DEAE-cellulose column (1.0cm × 35cm) was used for separation of the products. The ATP was re-isolated from the column effluents and was used for another incubation. This was repeated two more times, the four preparations combined yielding about 40% conversion of labelled ATP into labelled adenosine 3′-phosphate 5′-phosphosulphate. For conversion of this product into [^{14}C]adenosine 5′-phosphosulphate everything was scaled down proportionately by 10–15-fold. After isolation of the [^{14}C]adenosine 5′-phosphosulphate, it was converted into the sodium salt as described above for the ^{35}S-labelled material.

Results

We have been able to purify an enzyme (adenylyl sulphate:ammonia adenylyltransferase) from *Chlorella* that catalyses the reaction of adenosine 5′-phosphosulphate and ammonia to form adenosine 5′-phosphoramidate. The purification scheme and the results achieved are shown in Table 1. By using a combination of $(NH_4)_2SO_4$ fractionation and column chromatography on DEAE-cellulose, Sephadex G-200 and Reactive Blue 2–agarose (see Fig. 1), a purification of more than a 1000-fold over the crude extract was achieved, with a yield of about 33%. Unless stated otherwise, the highly purified enzyme from Reactive Blue 2–agarose chromatography (Table 1, step VI) was used in all experiments.

The requirements for the reaction catalysed by adenosine 5′-phosphosulphate:ammonia adenylyltransferase are shown in Table 2. Adenosine 5′-phosphosulphate, ammonia and enzyme are required; heating destroys the enzyme activity. On incubation of the enzyme with adenosine 5′-phosphoramidate and highly radioactive sulphate, no radioactive adenosine 5′-phosphosulphate was detectable after purification by charcoal adsorption and borate paper electrophoresis. Thus the back reaction is undetectable, as might be expected from

Table 1. *Purification of adenylyl sulphate: ammonia adenylyltransferase*

For enzyme assays the reaction mixture contained, in a total volume of 1.0ml, [^{14}C]adenosine 5′-phosphosulphate (193500c.p.m./μmol, 2.35μmol), NH_4Cl (500μmol), Tris/HCl buffer, pH 9.0 (100μmol), and an appropriate amount of enzyme. Incubation was for 1.0h at 30°C. The formation of [^{14}C]adenosine 5′-phosphoramidate was measured after paper electrophoresis.

	Step	Enzyme activity (μmol of [^{14}C]adenosine 5′-phosphoramidate formed/h)	Protein (mg)	Enzyme specific activity (μmol of [^{14}C]adenosine 5′-phosphoramidate formed/h per mg of protein)	Purification (fold)	Recovery (%)	
I	Crude extract	1575	15750	0.100		100	
II	$(NH_4)_2SO_4$ fractionation						
	40–60% saturation	562	2079	0.27	3	36	61
	60–80% saturation	400	197	2.03	20	25	
III	DEAE-cellulose column chromatography of $(NH_4)_2SO_4$ fractions						
	40–60% saturation	421	171	2.5	25	27	44
	60–80% saturation	260	22	11.8	118	17	
IV	Rechromatography on DEAE-cellulose of combined active fractions from III	618	69	9.0	90	39	
V	Sephadex G-200 chromatography of combined active fractions from IV						
	Fraction 10	98	11	8.9	89	6	37
	Fraction 11	224	5.7	39.3	393	14	
	Fraction 12	239	3.0	80.0	806	15	
	Fraction 13	31	1.5	20.7	207	2	
VI	Reactive Blue 2–agarose chromatography of fractions from V						
	Fraction V 11	215	1.7	126.4	1264	14	33
	Fraction V 12	215	1.2	179.1	1791	14	
	Fraction V 10+13	86	1.9	45.2	452	5	

Table 2. *Minimal requirements for the adenosine 5′-phosphosulphate: ammonia adenylyltransferase reaction*

The complete assay mixture contained, in a total volume of 0.5ml, adenosine 5′-phospho[^{35}S]-sulphate (195000c.p.m./μmol; 1.2μmol), NH_4Cl (250μmol), Tris/HCl buffer, pH 9.0 (50μmol), and enzyme (fraction V 11 from step VI; see Table 1) (4μg of protein). The incubation was for 1.0h at 30°C. The release of [^{35}S]sulphate was measured as described in the Experimental section.

	Enzyme activity (μmol of $^{35}SO_4^{2-}$ released/h per mg of protein)
Complete assay mixture	105.6
NH_4Cl omitted	0.1
Enzyme heated (95°C, 3min)	0

the observation that the forward reaction goes essentially to completion. Separation of the products of the forward reaction by borate electrophoresis (Fig. 2) shows that almost all of the adenosine 5′-phosphosulphate has disappeared and is replaced by a compound that co-migrates with adenosine 5′-phosphoramidate. Small amounts of 5′-AMP are present, but this is attributable to contaminating AMP in the adenosine 5′-phosphosulphate preparation, as may be seen from the incubation with heated enzyme. The purified enzyme product also co-migrates with authentic adenosine 5′-phosphoramidate, and like this compound is converted into 5′-AMP by incubation at low pH or by treatment with a phosphodiesterase that also hydrolyses cyclic AMP. It was difficult to separate adenosine 5′-phosphoramidate from 3′:5′-cyclic AMP in previous experiments (Tsang & Schiff, 1976*a*), but borate electrophoresis provides an excellent separation because adenosine 5′-phosphoramidate, with its vicinal hydroxy groups, can form a negatively charged borate complex whereas cyclic AMP cannot. Adenosine 5′-phosphoramidate cross-reacts with anti-(cyclic AMP) antibody, but 10^4 as much of the

compound is required, relative to cyclic AMP, to give the same amount of reaction.

The results of analysis of the enzyme product for adenine, ribose and acid-hydrolysable phosphate and for release of ammonia at low pH are shown in Table 3. The molar proportions found are in excellent agreement with those expected (1.0 adenine : 1.0 ribose : 1.0 phosphate : 1.0 ammonia) and found

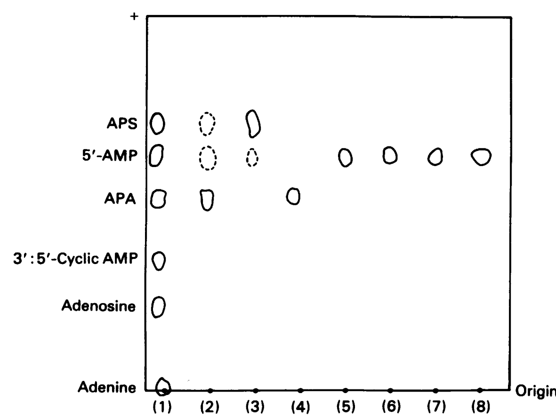


Fig. 2. *Paper electrophoresis in borate buffer, pH 8.0, of adenosine 5′-phosphoramidate and the enzyme product of the adenylyl sulphate : ammonia adenylyltransferase reaction compared with the same compounds subjected to various treatments*

For experimental details see the text. (1) Standards [adenine, adenosine, 3′:5′-cyclic AMP, adenosine 5′-phosphoramidate (APA), 5′-AMP and adenosine 5′-phosphosulphate (APS)]; (2) incubation mixture containing enzyme + adenosine 5′-phosphosulphate + ammonia; (3) incubation mixture containing boiled enzyme + adenosine 5′-phosphosulphate + ammonia; (4) purified product (of the enzyme reaction) + adenosine 5′-phosphoramidate; (5) purified product after acidification; (6) adenosine 5′-phosphoramidate after acidification; (7) purified product + 3′:5′-cyclic AMP phosphodiesterase; (8) adenosine 5′-phosphoramidate + 3′:5′-cyclic AMP phosphodiesterase.

for authentic adenosine 5′-phosphoramidate. I.r.-absorption spectra of the lithium salts of the enzyme product and authentic adenosine 5′-phosphoramidate in KBr pellets also agree quite well (Fig. 3); the u.v.-absorption spectra are identical (results not shown).

Mass spectra of authentic lithium adenosine phosphoramidate and the lithium salt of the unknown also established their identity (results not shown). Although the behaviour of the compounds was somewhat anomalous (not infrequent with nucleotide salts of this nature), several peaks corresponding to adenine and adenosine derivatives were detected. The major peak at 358 mass units could be ascribed to a dilithium salt of the molecular ion, but a somewhat smaller peak was found at 352 mass units corresponding to the expected molecular ion for the monolithium salt of adenosine phosphoramidate. Since both samples behaved similarly, this evidence, taken together with the analysis for adenosine, ribose, phosphate and acid-releasable ammonia, the identity of the i.r. and u.v. spectra and the co-electrophoresis data, leaves little doubt that the unknown material is adenosine 5′-phosphoramidate.

Polyacrylamide-gel electrophoresis of the highly purified adenylyl sulphate : ammonia adenylyltransferase yields one band that stains for protein and contains all of the enzyme activity (Fig. 4). The movement of this band relative to markers of known molecular weight indicates a molecular weight of about 60000–65000 for the enzyme protein. The same material subjected to denaturation with sodium dodecyl sulphate and electrophoresis on sodium dodecyl sulphate/polyacrylamide gel (Fig. 5) indicates the presence of three subunits of mol.wts. 26000, 21000 and 17000, consistent with a molecular weight of 64000 for the native enzyme protein. Isoelectrofocusing also yields one band that stains for protein and contains the activity; this band occupies a position at pI 4.2.

The activity of the highly purified enzyme at various pH values is shown in Fig. 6. The determinations were made in two different ways. In one, the total ammonia added ($NH_4^+ + NH_3$) was kept constant at 0.5 M; in the other the total ammonia

Table 3. *Analysis of the product of the adenylyl sulphate : ammonia adenylyltransferase-catalysed reaction*
Values for adenine are mol/mol of compound; other values in each column are mol/mol of adenine in that column.

	Adenosine 5′-phosphoramidate (from Sigma)		Enzyme product
	Expected	Found	Found
Adenine	1.00	0.98	0.97
Ribose	1.00	1.01	0.95
Total P	1.00	1.00	0.98
NH_3 released at low pH	1.00	0.95	0.98

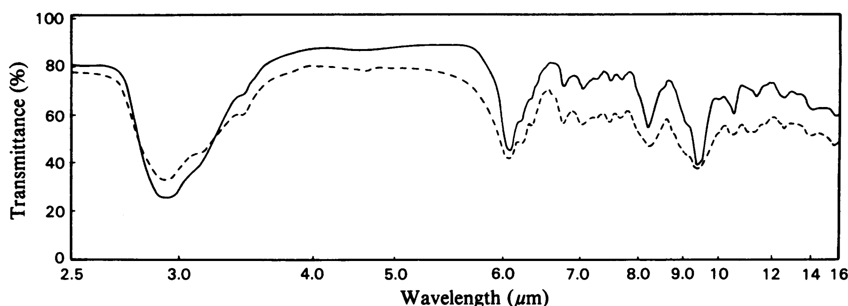


Fig. 3. *I.r. spectra of adenosine 5'-phosphoramidate (----) and the product of the adenylyl sulphate:ammonia adenylyltransferase reaction (——), in KBr pellets*
For experimental details see the text.

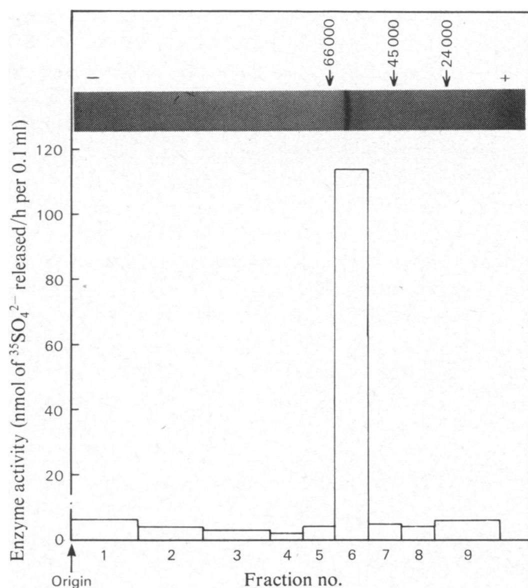


Fig. 4. ***Polyacrylamide-gel electrophoresis of the native highly purified adenylyl sulphate:ammonia adenylyltransferase in Tris/EDTA/borate buffer, pH 9.2***
For experimental details see the text. The photograph shows the single protein band stained by Coomassie Blue. The molecular weights of standards are indicated: bovine serum albumin (66000), egg albumin (45000) and trypsinogen (24000). The histogram shows the enzyme activity determined in successive slices cut from the gel.

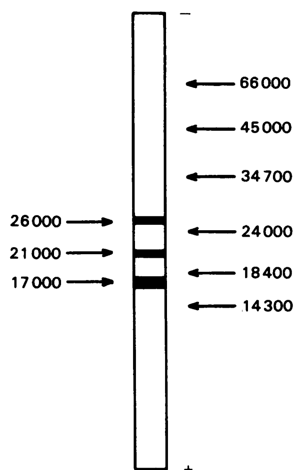


Fig. 5. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the highly purified adenylyl sulphate: ammonia adenylyltransferase protein subjected to treatment with sodium dodecyl sulphate and urea to denature and release the subunits*
For experimental details see the text. The molecular weights to the left of the diagram of the gel represent the values for the enzyme subunits, which are present in equal amounts. The molecular weights of the standards are indicated to the right: bovine serum albumin (66000), egg albumin (45000), pepsin (34700), trypsinogen (24000), subunit of β-lactoglobulin (18400) and lysozyme (14300).

added ($NH_4^+ + NH_3$) was varied to provide a constant amount of NH_3 (89mM) at each pH as calculated from the Henderson–Hasselbach equation. The two curves are quite similar, since NH_3 is saturating at all pH values under both sets of conditions. The curve obtained with a constant concentration of total ammonia ($NH_4^+ + NH_3$) has an optimum at pH 8.8 with a shoulder at about pH 8.0. With a constant concentration of NH_3 there is a broad maximum at pH 8–9.

The apparent K_m of the enzyme for adenosine 5'-phosphosulphate is 0.82mM (Fig. 7). The apparent K_m for NH_3 at two different pH values

(7.9 and 8.5), assuming that the active species is NH_3, is 10–11 mM (Fig. 7), consistent with the assumption that NH_3 is the reactive species. The $V_{max.}$ is also the same at the two pH values, in accord with the assumption that NH_3 is the reactive form. The stoicheiometry of the reaction indicates that 1 mol of adenosine 5′-phosphosulphate reacts

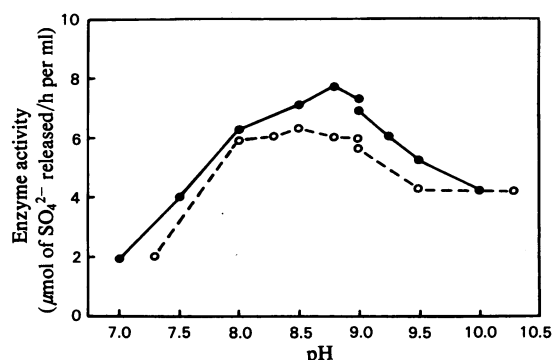


Fig. 6. *Plots of activity of highly purified adenylyl sulphate : ammonia adenylyltransferase against pH*

For the upper curve (●) the concentration of total ammonia ($NH_3 + NH_4^+$) was held constant at 0.5 M for all pH values. For the lower curve (O) the concentration of total ammonia was varied to provide a constant concentration of NH_3 (0.089 M) at each pH value. The reaction mixtures contained, in a total volume of 0.75 ml, adenosine 5′-phospho[^{35}S]sulphate (235000 c.p.m./μmol; 1.8 μmol), Tris/HCl buffer (pH 7.0–9.0) or $NaHCO_3/Na_2CO_3$ buffer (pH 9.0–10.3) of the indicated pH value (75 μmoles), enzyme (fraction V12 from step VI; Table 1) (0.006 mg of protein) and NH_4Cl at concentrations already indicated. Incubation was at 30°C for 30 min.

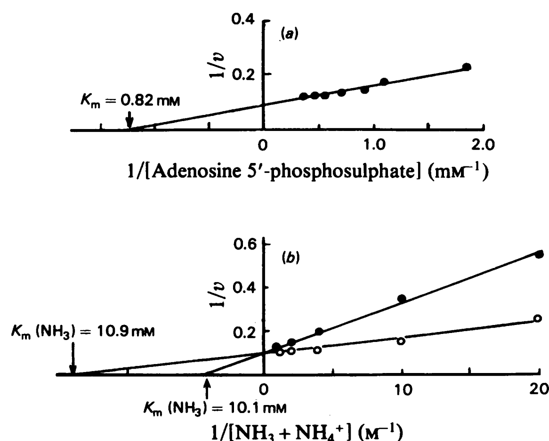


Fig. 7. *Apparent K_m values of highly purified adenylyl sulphate : ammonia adenylyltransferase (a) for adenosine 5′-phosphosulphate and (b) for NH_3 at two different pH values*

The incubation mixtures contained, in a total volume of 0.75 ml, Tris/HCl buffer, pH 9.0 for (*a*) or pH 7.9 (●) or 8.5 (O) for (*b*) (75 μmol), enzyme (fraction V12 from step VI; Table 1) (0.006 mg of protein), adenosine 5′-phospho[^{35}S]sulphate [235000 c.p.m./μmol; (*a*) as indicated; (*b*) 2 μmol] and NH_4Cl [(*a*) 375 μmol; (*b*) as indicated]. Incubation was at 30°C for 30 min to obtain the initial rates of reaction, *v*, expressed as μmol of SO_4^{2-} released/h per ml.

Table 4. *Stoicheiometry of the reaction catalysed by adenylyl sulphate : ammonia adenylyltransferase*

Reaction mixture (1) contained in a total volume of 1.0 ml, [^{14}C]adenosine 5′-phosphosulphate (110650 c.p.m./μmol; 5.56 μmol), NH_4Cl (500 μmol), Tris/HCl buffer, pH 8.5 (100 μmol), and enzyme (fraction V 12 from step VI; Table 1) (0.007 mg of protein). Incubation was at 30°C for 1.0 h. Reaction mixture (2) was the same as (1) except that [^{14}C]adenosine 5′-phosphosulphate was replaced by adenosine 5′-phospho[^{35}S]sulphate (195000 c.p.m./μmol; 4.85 μmol). Reaction mixture (3) contained, in a total volume of 0.5 ml, adenosine 5′-phospho[^{35}S]sulphate (195000 c.p.m./μmol; 4.0 μmol), NH_4Cl (0.42 μmol), Tris/HCl buffer, pH 8.0 (50 μmol), and enzyme (fraction V 12 from step VI; Table 1) (0.0035 mg of protein). Incubation was at 30°C for 56 h. Reaction mixture (4) was the same as (3) except that Tris/HCl buffer, pH 8.5 (50 μmol), replaced Tris/HCl buffer, pH 8.0. Incubation was at 30°C for 24 h.

Labelled substrate and pH of incubation	Adenosine 5′-phosphosulphate utilized (μmol)	NH_3 utilized (μmol)	Adenosine 5′-phosphoramidate formed (μmol)	SO_4^{2-} formed (μmol)
(1) [^{14}C]Adenosine 5′-phosphosulphate pH 8.5	1.24		1.18	
(2) Adenosine 5′-phospho[^{35}S]sulphate, pH 8.5	1.01		0.96	0.97
(3) Adenosine 5′-phospho[^{35}S]sulphate, pH 8.0		0.36		0.32
(4) Adenosine 5′-phospho[^{35}S]sulphate, pH 8.5		0.37		0.38

with 1 mol of ammonia (assumed to be NH_3) to yield 1 mol of adenosine 5′-phosphoramidate plus 1 mol of sulphate (Table 4). The formation of 1 mol of H^+ ion must be assumed in order to balance the equation.

The enzyme will not utilize adenosine 3′-phosphate 5′-phosphosulphate, ATP, ADP or 5′-AMP as substrates in place of adenosine 5′-phosphosulphate. It also rejects a wide variety of organic analogues of ammonia (see the Experimental section), including primary, secondary and tertiary amines, amino acids and amides. Thus the enzyme appears to be highly specific for ammonia and adenosine 5′-phosphosulphate as substrates.

To test whether the enzyme might transfer the adenylyl moiety of adenosine 5′-phosphosulphate to an amino function on a macromolecule (either the enzyme itself, or some other large molecule in the crude enzyme extracts), purified adenylyl sulphate: ammonia adenylyltransferase and [^{14}C]adenosine 5′-phosphosulphate were incubated with the crude extract as the acceptor in place of ammonia. After incubation and separation of macromolecules from small molecules by Sephadex G-25 chromatography, no radioactivity was detected in the excluded fraction. This indicates that, under these conditions, no transfer of the adenylyl group from adenosine 5′-phosphosulphate to macromolecular amino groups takes place to yield a stable product.

The highly purified enzyme does not catalyse any of the known biochemical reactions of adenosine 5′-phosphosulphate (Table 5), including the ATP sulphurylase (Wilson & Bandurski, 1958), adenylyl sulphate kinase (Burnell & Anderson, 1973*b*), ADP

Table 5. *Assay of crude extracts of Chlorella and highly purified adenylyl sulphate:ammonia adenylyltranferase for various enzymic activities utilizing adenosine 5′-phosphosulphate*

For assay of ATP sulphurylase activity the reaction mixture contained, in a total volume of 0.5 ml, ATP (5 μmol), sodium molybdate (5 μmol), $MgCl_2$ (1.0 μmol), Tris/HCl buffer, pH 7.5 (50 μmol), EDTA (3 μmol), inorganic pyrophosphatase (1.8 μg of protein) and crude enzyme extract (316 μg of protein) and/or purified adenylyl sulphate:ammonia adenylyltransferase (fraction V 11 from step VI; Table 1) (5 μg of protein). For assay of adenylyl sulphate kinase activity the reaction mixture contained, in total volume of 1.0 ml, adenosine 5′-phospho[^{35}S]-sulphate (235000 c.p.m./μmol; 4.5 μmol), $MgCl_2$ (20 μmol), ATP (10 μmol), Tris/HCl buffer, pH 8.0 (100 μmol), and crude enzyme extract (316 μg of protein) and/or purified adenylyl sulphate:ammonia adenylyltransferase (fraction V 11 from step VI; Table 1) (5 μg of protein). For assay of ADP sulphurylase activity the reaction mixture contained, in a total volume of 1.0 ml, adenosine 5′-phosphosulphate (2 μmol), Na_2HPO_4 (10 μmol), Tris/HCl buffer, pH 8.0 (100 μmol), and crude enzyme extract (190 μg of protein) and/or purified adenylyl sulphate:ammonia adenylyltransferase (fraction V 11 from step VI; Table 1) (5 μg of protein). For assay of adenylyl sulphate sulphotransferase activity the reaction mixture contained, in a total volume of 0.4 ml, adenosine 5′-phospho[^{35}S]sulphate (234000 c.p.m./μmol; 0.5 μmol), Tris/HCl buffer, pH 9.0 (50 μmol), dithiothreitol (4 μmol), Na_2SO_4 (400 μmol) and crude enzyme extract (190 μg of protein) and/or purified adenylyl sulphate:ammonia adenylyltransferase (fraction V 11 from step VI; Table 1) (5 μg of protein). For assay of adenylyl sulphate sulphohydrolase activity the reaction mixture contained, in a total volume of 0.5 ml, adenosine 5′-phospho[^{35}S]sulphate (235000 c.p.m./μmol; 1.2 μmol), Tris/HCl buffer, pH 9.0 (50 μmol), and crude enzyme fraction (158 μg of protein) and/or purified adenylyl sulphate:ammonia adenylyltransferase (fraction V 11 from step VI; Table 1) (5 μg of protein). For assay of adenylyl sulphate:ammonia adenylyltransferase activity the conditions were the same as for assay of adenylyl sulphate sulphohydrolase except that NH_4Cl (250 μmol) was added. For assay of alkaline phosphatase activity the reaction mixture contained, in a total volume of 1.1 ml, glycine (50 μmol), $MgCl_2$ (0.5 μmol), disodium *p*-nitrophenyl phosphate (8 μmol) and crude enzyme fraction (316 μg of protein) and/or purified adenylyl sulphate:ammonia adenylyltransferase (fraction V 11 from step VI; Table 1) (5 μg of protein). Before the addition of the enzymes the pH was adjusted to pH 10.5 by addition of NaOH.

	Enzyme activity		
	Crude extract	Crude extract + purified enzyme	Purified enzyme
ATP sulphurylase (nmol of P_i released/h per mg of protein)	2362	2236	Not detected
Adenylyl sulphate kinase (nmol of adenosine 3′-phosphate 5′-phosphosulphate/h per mg of protein)	171	227	Not detected
ADP sulphurylase (nmol of P_i consumed/h per mg of protein)	186	163	Not detected
Adenylyl sulphate sulphotransferase (nmol of SO_3^{2-} formed/h per mg of protein)	221	179	Not detected
Adenylyl sulphate sulphohydrolase (nmol of SO_4^{2-} released/h per mg of protein)	164	219	Not detected
Adenylyl sulphate:ammonia adenylyltransferase (nmol of adenosine 5′-phosphoramidate formed/h per mg of protein)	174	1887	171000
Alkaline phosphatase (nmol of P_i released/h per mg of protein)	228	254	Not detected

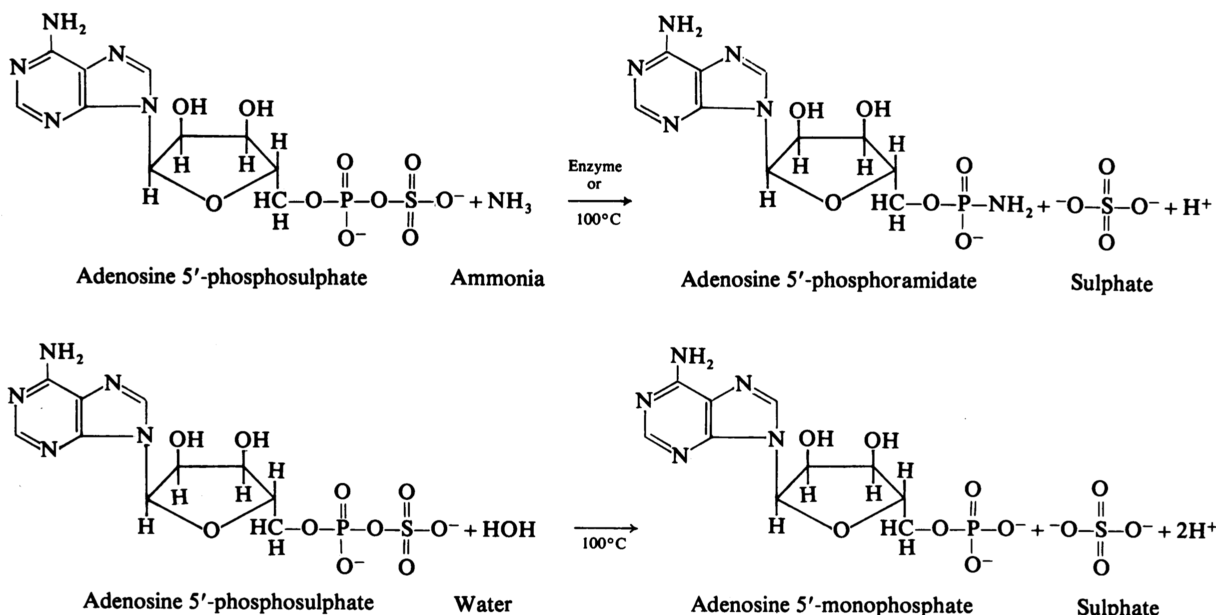
sulphurylase (Burnell & Anderson, 1973*a*), adenylyl sulphate sulphotransferase (Brunold & Schmidt, 1976; Schmidt *et al.*, 1974), and adenylyl sulphate sulphohydrolase reactions (Tsang & Schiff, 1976*a*). It also lacks alkaline phosphatase activity (Bessey *et al.*, 1946). The last step in purification separates adenylyl sulphate : ammonia adenylyltransferase from an adenylyl sulphate sulphohydrolase activity that catalyses the formation of sulphate and 5′-AMP from adenosine 5′-phosphosulphate (Tsang & Schiff, 1976*a*). This sulphohydrolase is not specific for adenosine 5′-phosphosulphate since it also catalyses the hydrolysis of adenosine 3′-phosphate 5′-phosphosulphate and ATP.

Adenylyl sulphate : ammonia adenylyltransferase activity has been found in the indicated $(NH_4)_2SO_4$ fractions from the following organisms: barley and spinach (90–100% saturation), *Chlorella pyrenoidosa* (40–80% saturation), light-grown or dark-grown *Euglena gracilis* var. *bacillaris* (30–50% saturation), *Dictyostellium discoideum* (50–60% saturation) and *Escherichia coli* B/r (60–80% saturation).

Although there is no detectable non-enzymic reaction of adenosine 5′-phosphosulphate and ammonia at 30°C (Table 2), these compounds will react non-enzymically at elevated temperatures (results not shown). At pH values between 7 and 9 almost all of the adenosine 5′-phosphosulphate is converted within 1 h at 95°C. Unlike the enzymic reaction, which forms only adenosine 5′-phosphoramidate, the non-enzymic reaction forms both this compound and AMP. With 89 μmol of NH_3 and 1.5 μmol of adenosine 5′-phosphosulphate per ml in the reaction mixture the ratio of adenosine 5′-phosphoramidate to AMP formed is about 2.5 : 1. Prolonged storage (2 years at −18°C) of the ammonium salt of adenosine 5′-phosphosulphate also results in the formation of detectable amounts of adenosine 5′-phosphoramidate. Other workers have found that storage of adenosine 5′-phosphosulphate or adenosine 3′-phosphate 5′-phosphosulphate in the presence of NH_4HCO_3 yields the corresponding phosphoramidates (Cooper & Trüper, 1979; Cooper *et al.*, 1980).

Discussion and conclusions

In the present paper we have described a new enzyme activity, adenylyl sulphate : ammonia adenylyltransferase, widely distributed in living systems, that catalyses the reaction of adenosine 5′-phosphosulphate with ammonia to form adenosine 5′-phosphoramidate (Scheme 1). The enzyme protein was purified to homogeneity and its properties were determined. We have written the



Scheme 1. *Enzymic and non-enzymic reactions concerned with formation of adenosine 5′-phosphoramidate*
The transfer of the adenylyl group of adenosine 5′-phosphosulphate to ammonia to form adenosine 5′-phosphoramidate is shown. This reaction, when catalysed by adenylyl sulphate : ammonia adenylyltransferase, forms only adenosine 5′-phosphoramidate as the nucleotide product; the non-enzymic reaction forms both adenosine 5′-phosphoramidate and 5′-AMP.

reaction with NH_3 rather than NH_4^+ as the reacting species because increasing pH, which would increase the NH_3/NH_4^+ ratio, favours the reaction. By analogy to other reactions that involve ammonia, and for mechanistic reasons, NH_3 appears to be the more likely substrate leading to the formation of an H^+ ion during the reaction.

Since the forward reaction goes essentially to completion, and since the back reaction is undetectable, the reaction must involve a substantial loss of free energy. The free energy of hydrolysis of the phosphosulphate bond has been estimated to be about −80kJ/mol (−19kcal/mol) (pH 7.5–9) (Roy & Trudinger, 1970). The free energy of hydrolysis of the phosphoramide bond in phosphocreatine is −43.1kJ/mol (−10.3kcal/mol) (pH 7.3 and 7.5), and that of phosphoarginine is −32.2kJ/mol (−7.7kcal/mol) (pH 8.0) (Kuby & Noltmann, 1962; Uhr *et al.*, 1966; Jencks, 1968). Thus one would estimate that the energy liberated in the reaction of adenosine 5′-phosphosulphate and ammonia to yield adenosine 5′-phosphoramidate should be of the order of, or perhaps somewhat less than, 42kJ/mol (10kcal/mol). This would account for the observation that the forward reaction is far to the right. A free energy of hydrolysis of about −38kJ/mol (−9kcal/mol) for the phosphoramide bond of adenosine 5′-phosphoramidate would also be in agreement with the known usefulness of this compound in the synthesis of phosphate anhydrides of various nucleotides, since the free energy of hydrolysis of the phosphoramide bond would have to be higher than that of the phosphate anhydride bond, which is estimated to be about −34kJ/mol (−7.5kcal/mol). A study of the mechanism of hydrolysis of phosphoramidic acid has been published (Jencks & Gilchrist, 1964, 1965). The many properties shared by adenosine 5′-phosphoramidate and cyclic AMP made it difficult to separate these two compounds in earlier work (Tsang & Schiff, 1976*a*). Both compounds comigrate in the usual electrophoresis buffers, have the same molar proportions of adenine to ribose to phosphate, show some reaction with anti-(cyclic AMP) antibody and react with a cyclic AMP phosphodiesterase. As we show in the present paper, however, they are distinguishable by different migration rates on borate electrophoresis and by the ready release of ammonia from adenosine 5′-phosphoramidate at low pH. The i.r. spectra and mass spectra show that the product of the adenylyl sulphate:ammonia adenylyltransferase reaction is adenosine 5′-phosphoramidate.

The enzyme shows a very high specificity for its substrates adenosine 5′-phosphosulphate and ammonia. Adenosine 3′-phosphate 5′-phosphosulphate or other nucleotides such as ATP or ADP will not replace adenosine 5′-phosphosulphate; amines, amino acids or amides will not replace ammonia. Since the enzymic reaction forms only adenosine 5′-phosphoramidate whereas the non-enzymic reaction of adenosine 5′-phosphosulphate and ammonia yields both this compound and 5′-AMP (Scheme 1), it is tempting to speculate that the enzyme excludes water from the active site but allows ammonia to enter. Since the reaction of adenosine 5′-phosphosulphate with ammonia can be conducted either enzymically or non-enzymically, further studies of the mechanism of this reaction may yield interesting results.

Since adenylyl sulphate:ammonia adenylyltransferase does not catalyse any of the other known enzymic reactions of adenosine 5′-phosphosulphate, it must be regarded as a new enzyme activity. Its wide distribution among living organisms indicates that the enzyme and its product, adenosine 5′-phosphoramidate, may perform some important cellular functions.

One problem in finding a role for this enzyme is the relatively high apparent K_m for ammonia (10–11mM). If we have isolated the native enzyme molecule and if the reaction were to proceed *in vivo* as it does *in vitro*, relatively high ammonia concentrations would be required to achieve significant rates of reaction in intact cells. If adenosine 5′-phosphoramidate were to serve as a regulatory molecule, however, perhaps lower rates of formation would be appropriate. Adenosine 5′-phosphoramidate is known to replace 5′-AMP as a regulator of certain enzyme reactions (see below). Other explanations of the relatively high apparent K_m for ammonia can be suggested. Perhaps the form of the enzyme that we have isolated is not the native form in size or subunit composition, or perhaps an allosteric activator or coenzyme present *in vivo* is missing. There is no loss of enzyme activity during extensive purification, however, which would argue against the loss of an activator molecule unless this were lost in the preparation of the crude extracts. Another possibility is that ammonia is not the normal substrate. Although no other compound containing an amino group among the many we have tested will replace ammonia, it is not excluded that the enzyme might use some other, unrelated, nucleophile, normally present in the cells, more efficiently. Further studies of the enzyme *in vivo* and *in vitro* are necessary to explore these questions.

Various enzymic reactions related to the adenylyl sulphate:ammonia adenylyltransferase reaction are known. Phosphate transfer from ATP (catalysed by phosphokinases) to form phosphoramides of amino acids are well known, since the phosphoramides are energy-rich compounds that serve as phosphagens in various systems (Baldwin, 1967). Adenylyl sulphate:ammonia adenylyltransferase

does not seem to belong to this group, since it will not catalyse the reaction of ATP with ammonia. Another type of reaction is adenylate transfer, as in the DNA ligase reaction, where ATP or NAD serves as a donor of an adenylate group that is transferred to the ε-amino group of lysine on the enzyme (Gumport & Lehmann, 1971). The evidence given in the present paper shows that adenylyl sulphate : ammonia adenylyltransferase will not transfer the adenylate group of adenosine 5′-phosphosulphate to macromolecules under the conditions used and argues against this type of activity for the enzyme. It is not ruled out, of course, that a transient adenylate–enzyme intermediate may exist in the adenylyl sulphate : ammonia adenylyltransferase reaction itself that subsequently reacts with ammonia.

Since none of the known reactions seem to apply, we must regard the enzyme reaction described in the present paper as being a new type of adenylyl transfer from a nucleotide phosphosulphate to ammonia. To our knowledge, no other direct activation reactions of ammonia or amino groups of this type are known. This may be because such reactions require a higher free-energy investment than is available from the phosphate anhydride bond of ATP and related molecules; perhaps there are other biochemical reactions requiring the higher energy provided by phosphosulphate anhydrides waiting to be discovered.

We have already noted that adenosine 5′-phosphoramidate is used as an intermediate in the organic synthesis of various phosphate anhydride compounds (Moffatt & Khorana, 1961). The only enzymic reactions, to our knowledge, that are regulated by adenosine 5′-phosphoramidate are phosphorylase *b* (Okazaki *et al.*, 1968; Black & Wang, 1970), threonine deaminase from *Escherichia coli* (Nakazawa *et al.*, 1967) and adenylate cyclase from mammalian skin (Hzuka *et al.*, 1976), where adenosine 5′-phosphoramidate replaces AMP as an enzyme activator. The search for other biochemical roles for adenosine 5′-phosphoramidate is an important next step in determining the function of adenylyl sulphate : ammonia adenylyltransferase in living systems. That this compound and the enzyme that forms it are of physiological significance is strengthened by our finding that adenosine 5′-phosphoramidate can be isolated from the soluble fraction of intact *Chlorella* cells (Berkowitz *et al.*, 1981).

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