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Purification, Properties and Substrate Specificity of Adenosine Triphosphate Sulphurylase from Spinach Leaf Tissue

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1. ATP sulphurylase was purified up to 1000-fold from spinach leaf tissue. Activity was measured by sulphate-dependent [^{32}P]PP_i–ATP exchange. The enzyme was separated from Mg^{2+}-requiring alkaline pyrophosphatase (which interferes with the PP_i–ATP-exchange assay) and from other PP_i–ATP-exchange activities. No ADP sulphurylase activity was detected. 2. Sulphate was the only form of inorganic sulphur that catalysed PP_i–ATP exchange; K_m (sulphate) was 3.1mM, K_m (ATP) was 0.35mM and the pH optimum was 7.5–9.0. The enzyme was insensitive to thiol-group reagents and required either Mg^{2+} or Co^{2+} for activity. 3. The enzyme catalysed [^{32}P]PP_i–dATP exchange; K_m (dATP) was 0.84mM and V (dATP) was 30% of V (ATP). Competition between ATP and dATP was demonstrated. 4. Selenate catalysed [^{32}P]PP_i–ATP exchange and competed with sulphate; K_m (selenate) was 1.0mM and V (selenate) was 30% of V (sulphate). No AMP was formed with selenate as substrate. Molybdate did not catalyse PP_i–ATP exchange, but AMP was formed. 5. Synthesis of adenosine 5′-[^{35}S]sulphatophosphate was demonstrated by coupling purified ATP sulphurylase and Mg^{2+}-dependent alkaline pyrophosphatase (also prepared from spinach) with [^{35}S]sulphate and ATP as substrates; adenosine 5′-sulphatophosphate was not synthesized in the absence of pyrophosphatase. Some parameters of the coupled system are reported.

Inorganic compounds of sulphur are the ultimate source of the sulphur-containing compounds that are essential for the growth and function of all living cells. However, only plants and some micro-organisms can synthesize the biologically essential sulphur-containing compounds directly from inorganic forms of sulphur; other organisms are dependent on plants and micro-organisms for their sources of sulphur-containing compounds, although animals have a limited capacity to utilize sulphate produced by oxidation of compounds supplied from plants and micro-organisms (Roy & Trudinger, 1970). The most important source of sulphur utilized by plants is sulphate (Wilson, 1962; Roy & Trudinger, 1970), which is obtained from soil by a specific uptake mechanism (Leggett & Epstein, 1956).

Yeast also utilizes sulphate as a sulphur source. In this organism sulphate is activated by two molecules of ATP in two separate reactions catalysed by the enzymes ATP sulphurylase (ATP–sulphate adenylyl transferase, EC 2.7.7.4) and adenosine 5′-sulphatophosphate kinase (ATP–adenylyl sulphate 3′-phosphotransferase, EC 2.7.1.25) respectively:

$$ATP + SO_4^{2-} \overset{Mg^{2+}}{\rightleftharpoons} \text{Adenosine 5′-sulphatophosphate} + PP_i$$

$$ATP + \text{adenosine 5′-sulphatophosphate} \overset{Mg^{2+}}{\rightleftharpoons} \text{Adenosine 3′-phosphate 5′-sulphatophosphate} + ADP$$

Another enzyme, ADP sulphurylase, has also been described in yeast (Robbins & Lipmann, 1958*a*).

The standard free-energy change of the reaction catalysed by ATP sulphurylase is approx. +46kJ/mol (+11kcal/mol) (Roy & Trudinger, 1970) and therefore the amount of adenosine 5′-sulphatophosphate present at equilibrium is extremely small and difficult to measure. ATP sulphurylase is most commonly assayed by coupling the enzyme with pyrophosphatase, with molybdate as substrate in lieu of sulphate and by measurement of the amount of P_i produced (Wilson & Bandurski, 1958). This method has several serious disadvantages (Roy & Trudinger, 1970; Shaw & Anderson, 1971) and is unsuitable for monitoring enzyme purification. Further, this method cannot be used to study the substrate specificity of the enzyme.

Despite the biological importance of the synthesis of sulphur-containing compounds from inorganic sources of sulphur by plants, little is known about the enzymology of sulphate metabolism in plants. Adenosine 5′-sulphatophosphate kinase has been reported in chloroplasts of *Phaseolus vulgaris* and *Zea*

mays (Mercer & Thomas, 1969), but was not detected in spinach-leaf chloroplasts (Asahi, 1964; Balharry & Nicholas, 1970). ATP sulphurylase has, however, been detected in crude extracts of plants (Adams & Johnson, 1968; Adams & Rinne, 1969) by using the molybdate-substrate method. Asahi (1964) and Ellis (1969) demonstrated the synthesis of adenosine 5′-sulphatophosphate in spinach chloroplasts and various plant extracts respectively; these authors detected the compound by chromatographic methods with [^{35}S]sulphate as substrate but only minute quantities of adenosine 5′-[^{35}S]sulphatophosphate were synthesized, presumably because of the very unfavourable free-energy change, even though these systems contained pyrophosphatase. This technique is insufficiently sensitive and too laborious for use in conjunction with purification studies of ATP sulphurylase.

Balharry & Nicholas (1970, 1971) and Shaw & Anderson (1971) have described direct and highly sensitive methods for measuring ATP sulphurylase by utilizing the back reaction to produce a favourable yield of product; neither method requires the addition of pyrophosphatase. The method of Balharry & Nicholas (1970, 1971) involves the use of adenosine 5′-sulphatophosphate and PP_i as substrates and measurement of the ATP synthesized by the luciferin–luciferase firefly assay in a scintillation spectrometer set out of coincidence. The method of Shaw & Anderson (1971) is a simple extension of the PP_i-exchange assay method used for amino acid-activation studies in which activity is measured as the sulphate-dependent incorporation of [^{32}P]PP_i into ATP; this method can be used to study substrate specificity.

In the present paper we describe the purification, some properties and substrate specificity of the ATP sulphurylase of spinach measured by using the PP_i-exchange assay method. We also describe the properties of the coupled enzyme system ATP sulphurylase plus Mg^{2+}-dependent alkaline pyrophosphatase (also prepared from spinach) that was used to measure the synthesis of adenosine 5′-sulphatophosphate with [^{35}S]sulphate and ATP as substrates.

Experimental

Chemicals

GTP and dATP (sodium salts) were obtained from the Sigma Chemical Co., St. Louis, Mo., U.S.A., ATP, ADP and AMP (sodium salts) from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, potassium selenite and potassium selenate from K & K Laboratories Inc., Plainview, N.Y., U.S.A., and L-amino acids from Mann Research Laboratories Inc., New York, N.Y., U.S.A. [^{32}P]P_i was obtained from the Australian Atomic Energy Commission, Lucas Heights, N.S.W., Australia, and converted into [^{32}P]PP_i by pyrolysis (Lee Peng, 1956) and adjusted to 0.25Ci/mol. [^{35}S]Sulphate was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., Sephadex G-200 from Pharmacia, Uppsala, Sweden, and DEAE-cellulose (DE52) from Whatman (W. and R. Balston, Maidstone, Kent, U.K.). Adenosine 5′-sulphatophosphate was a gift from Professor D. J. D. Nicholas and Dr. C. Adams.

Methods

Extraction and purification of ATP sulphurylase. Spinach (*Spinacea oleracea*) was obtained direct from a commercial market garden. The extraction procedure of Shaw & Anderson (1971) was used except that the extraction medium (medium 1) was adjusted to pH7.8 and contained potassium thioglycollate (25mM) and the extraction was performed in a Waring Blendor. Samples of the crude solution were extensively dialysed against 20mM-tris–HCl buffer, pH7.8 (medium 2), before assay; sulphate-dependent PP_i exchange was barely detectable in undialysed crude extracts (Shaw & Anderson, 1971).

The crude undialysed supernatant solution was fractionated by adding 0.277g of solid $(NH_4)_2SO_4$/ml of crude solution in two separate additions (0.196g and 0.081g/ml of crude solution), and precipitated protein was discarded after each addition of $(NH_4)_2SO_4$. Further $(NH_4)_2SO_4$ (0.032g/ml of recovered supernatant) was added and precipitated protein was again discarded. A final addition (0.033g/ml) was made, and the protein (containing ATP sulphurylase) was recovered and redissolved in medium 2 and extensively dialysed against the same buffer to remove $(NH_4)_2SO_4$ and residual thioglycollate. Addition of $(NH_4)_2SO_4$ in fewer, larger, additions caused a totally different precipitation behaviour of ATP sulphurylase, resulting in poorer purification, contamination with pyrophosphatase and phenolic compounds, low recovery and unreproducible fractionation. The dialysed $(NH_4)_2SO_4$ fraction was passed through a Sephadex G-200 column (60cm×3.5cm), equilibrated with medium 2. Fractions containing enzyme activity were pooled, solid KCl was added to adjust the concentration to 0.15M and the solution was applied to a DEAE-cellulose column (28cm×2cm) equilibrated with medium 2 containing 0.15M-KCl. After addition of the sample, the column was eluted with medium 2 containing 0.15M-KCl until the extinction of eluted material at 280nm was zero (approx. 150–250ml); this treatment removed pigmented phenolic com-

pounds in addition to inactive protein. The column was then developed with a linear gradient of KCl (0.15–0.25M) in medium 2. Fractions containing enzyme activity were pooled and dialysed to remove KCl and then applied to a DEAE-cellulose column (2cm×1.5cm) equilibrated with medium 2. The column was washed with 40ml of medium 2; the enzyme was eluted with 10ml of medium 2 containing 1M-KCl, resulting in a two- to six-fold concentration of ATP sulphurylase.

Preparation of pyrophosphatase. The procedures for the extraction and $(NH_4)_2SO_4$ fractionation of pyrophosphatase were identical with the procedures used to prepare ATP sulphurylase. Pyrophosphatase remained in solution after precipitation of the ATP sulphurylase by $(NH_4)_2SO_4$; pyrophosphatase was precipitated by adding an additional 0.067g of solid $(NH_4)_2SO_4$/ml, and the precipitate was redissolved in medium 2 (0.04ml/g original fresh weight of leaf tissue). The dissolved precipitate was extensively dialysed against medium 2 to remove $(NH_4)_2SO_4$, $MgCl_2$ and residual thioglycollate. Failure to remove these compounds and accurately control the concentration of $MgCl_2$ in the enzyme solution resulted in unreproducible and erratic acetone precipitation. $MgCl_2$ (1M; 1vol.) was added to the dialysed ammonium sulphate fraction (16vol.), and acetone was slowly added to a final concentration of 36% (v/v) while the temperature was lowered to −5°C. Precipitated material was discarded and the supernatant solution was slowly adjusted to 55% (v/v) acetone; the resulting precipitate (containing pyrophosphatase activity) was extracted with medium 2 and clarified by centrifugation. Pyrophosphatase was passed through columns of Sephadex G-200 and DEAE-cellulose as described for ATP sulphurylase, except that solid KCl was not added to the active fractions from the Sephadex G-200 column and the DEAE-cellulose column was equilibrated with medium 2 (without KCl) and developed with a linear gradient of KCl (0–0.35M) in medium 2.

Assay of ATP sulphurylase and pyrophosphatase activities. ATP sulphurylase assays were conducted at 35°C for 15min and contained 2μmol of Na_2K_2ATP, 40μmol of K_2SO_4, 2μmol of $Na_4{}^{32}P_2O_7$, 10μmol of $MgCl_2$, 100μmol of tris–HCl buffer, pH7.8, and enzyme in a final volume of 1ml. K_2SO_4 was omitted from control assays. Routine assays of dialysed crude enzyme and dialysed $(NH_4)_2SO_4$ fractions also contained 10μmol of NaF to inhibit pyrophosphatase. In some experiments tris–HCl buffer was replaced with glycine–NaOH buffer or phosphate–citrate buffer as described by Vogel (1951); 0.7ml of buffer was used in each assay. Reactions were terminated with 2ml of trichloroacetic acid (7.5%, w/v); the procedures for the separation of [^{32}P]ATP from [^{32}P]PP_i were as described by Anderson (1968). ATP sulphurylase activities were calculated by the method of Davie *et al.* (1956) on a Digital Equipment PDP 9 computer and are expressed as sulphate-dependent PP_i exchange in nmol/min (ATP sulphurylase units); specific activities are expressed as units/mg of protein. The absorption of ATP, dATP, ADP, AMP and GTP by charcoal was always 98–100% efficient and therefore no correction was applied for incomplete adsorption.

Pyrophosphatase assays were conducted at 35°C for 15min and contained 5μmol of $Na_4P_2O_7$, 20μmol of $MgCl_2$, 100μmol of tris–HCl buffer, pH7.8, and enzyme. Reactions were terminated by the addition of 2.3ml of $HClO_4$ (10%, w/v) and were analysed for P_i by the method of Allen (1940) in a final volume of 4ml. Results (corrected for zero-time control) are expressed as μmol of P_i/min (pyrophosphatase units).

Assay of coupled enzyme system. Assays were conducted at 35°C for 60min and contained approx. 50 units of concentrated purified ATP sulphurylase and approx. 1–2 units of partially purified pyrophosphatase. Standard assays also contained 10μmol of Na_2K_2ATP, 40μmol of $Na_2{}^{35}SO_4$ (approx. 50μCi), 20μmol of $MgCl_2$ and 100μmol of tris–HCl buffer, pH7.8, in a final volume of 1ml. Reactions were terminated by heating at 100°C for 1min. Adenosine 5′-[^{35}S]sulphatophosphate was separated from [^{35}S]-sulphate by adsorbing adenosine 5′-sulphatophosphate on charcoal and counting its radioactivity; the method was identical with that used for the separation of [^{32}P]ATP from [^{32}P]PP_i in the assay of ATP sulphurylase. Activity of the coupled system is expressed as nmol of adenosine 5′-sulphatophosphate synthesized/min.

Chromatography of the ^{32}P- and ^{35}S-labelled products adsorbed on charcoal in PP_i-exchange and coupled enzyme experiments. In PP_i-exchange experiments between [^{32}P]PP_i, and ATP or dATP, with either sulphate, selenate or molybdate as the other substrate, the ^{32}P-labelled product was eluted with 0.1M-NH_3 in 50% (v/v) ethanol until all the radioactivity was eluted. The eluted material was evaporated to dryness and the residue was dissolved in water; samples were subjected to descending paper chromatography on acid-washed Whatman 3MM paper. The same procedure was used for eluting the ^{35}S-labelled product synthesized by the coupled enzyme system; this procedure also eluted unlabelled ATP, which was used as substrate. The following solvents were used: I, propan-1-ol–aq. NH_3 (sp.gr. 0.88)–water (6:3:1, by vol.); II, isobutyric acid–aq. NH_3 (sp.gr. 0.88)–water (66:1:33, by vol.).

Determination of protein. Protein in crude extracts and $(NH_4)_2SO_4$ fractions was determined by the method of Ellman (1962). Purer protein from Sephadex G-200 and DEAE-cellulose columns was measured by the method of Warburg & Christian (1941).

Determination of K_m values. The procedure of Anderson & Fowden (1970) was used.

Results

Purification of ATP sulphurylase

The three-step purification procedure yielded an enzyme preparation that had been purified 700–1000-fold (Table 1). The crude enzyme preparation contained a very powerful pyrophosphatase, which interfered with ATP sulphurylase estimations measured by the PP_i-exchange technique (Shaw & Anderson, 1971). Fluoride is a powerful inhibitor of spinach leaf pyrophosphatase (El-Badry & Bassham, 1970; Shaw & Anderson, 1971), and addition of fluoride (10mM) was essential to assess ATP sulphurylase in crude extracts; it also proved necessary to include fluoride in assays of the ammonium sulphate fraction (Table 1). A large proportion of the

Table 1. *Typical purification of ATP sulphurylase from spinach and its separation from pyrophosphatase*

ATP sulphurylase activities are shown with sulphate (40mM) and selenate (40mM) as substrates with and without 10mM-NaF. All other conditions of the assays for both enzymes and details of the treatments used in the purification of ATP sulphurylase are described in the text. No pyrophosphatase activity was detected in any fraction in the presence of 10mM-fluoride.

		ATP sulphurylase (units/mg of protein)				
		With sulphate		With selenate		
Treatment	Protein (mg)	With fluoride	Without fluoride	With fluoride	Without fluoride	Pyrophosphatase (unit/mg of protein)
Dialysed crude extract	2750	4.67	3.23	1.44	0.70	0.42
$(NH_4)_2SO_4$ fraction	143	25.1	3.99	6.31	0.31	0.80
Sephadex G-200 (ATP sulphurylase peak)	26.2	105	105	29.5	29.7	0.04
DEAE-cellulose (unconcentrated)	0.75	4300	4640	1290	1300	0

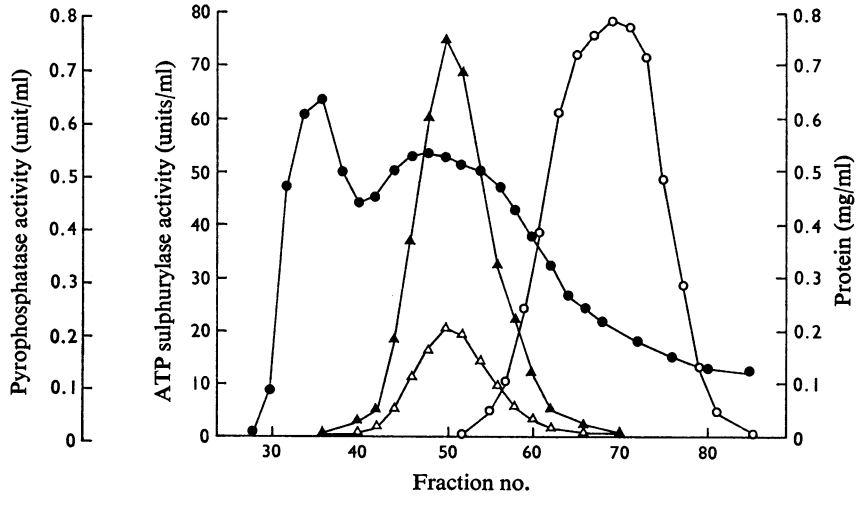

Fig. 1. *Separation of ATP sulphurylase and pyrophosphatase activities*

The activities were separated by gel filtration on Sephadex G-200 after $(NH_4)_2SO_4$ fractionation as described in the text for the purification of ATP sulphurylase. ATP sulphurylase assays were conducted in the absence of fluoride with either sulphate (40mM) or selenate (40mM) as substrates; all other conditions of the assays for both enzymes were as described in the text. ▲, Sulphate-dependent PP_i–ATP exchange; △, selenate-dependent PP_i–ATP exchange; o, pyrophosphatase activity; ●, protein.

pyrophosphatase activity (approx. 90%) was removed by fractionation with ammonium sulphate (Table 1) and the remaining activity was separated by gel filtration on Sephadex G-200 (Fig. 1). Fractionation of the active ammonium sulphate fraction with acetone was unsatisfactory, because of high losses of ATP sulphurylase, and also because the specific activity was not enhanced and no separation of ATP sulphurylase from pyrophosphatase was effected. ATP sulphurylase fractions from Sephadex G-200 and DEAE-cellulose columns were free from pyrophosphatase, and 10mM-fluoride was slightly inhibitory (7%) with these more purified fractions (Table 1); accordingly, fluoride was omitted from assays of Sephadex G-200 and DEAE-cellulose fractions.

Properties of purified ATP sulphurylase

No single buffer system was satisfactory for studying the pH optimum of the enzyme. However, maximum activity was found over the range pH 7.5–9.0 (Fig. 2) when a series of buffers was used. The enzyme was heat-sensitive (pretreatment at 75°C for 1min totally destroyed activity) but could be stored at −15°C for at least 4 months without loss of activity.

Evidence was presented (Shaw & Anderson, 1971) that the sulphate-dependent ^{32}P-labelled product synthesized by crude dialysed extracts was [^{32}P]ATP; no [^{32}P]ADP was formed. This result was confirmed with the purified enzyme; formation of unlabelled AMP was not detected. In addition to catalysing PP_i–ATP exchange, however, the enzyme also catalysed sulphate-dependent PP_i–dATP exchange. The dATP contained a negligible amount of ATP when examined by paper chromatography in solvent II; the pyrophosphate exchange with dATP could not be attributed to this low degree of ATP contamination. The ^{32}P-labelled product adsorbed on charcoal after using [^{32}P]PP_i and dATP as substrates was identified by paper chromatography in solvent II as [^{32}P]dATP (Fig. 3). The affinity of the enzyme for dATP (K_m 0.84mM) was less than that for ATP (K_m 0.35mM), and V (dATP) was 31% of V (ATP). Further, the PP_i-exchange activity with 2mM-ATP was decreased by the addition of 4mM-dATP. Accordingly, PP_i exchange was studied in a competition experiment with various concentrations of ATP and dATP (Fig. 4); the kinetics of this experiment were similar to those described by Pocklington & Jeffery (1969) for competition between two substrates competing for one enzyme. Sulphate-dependent PP_i exchange with ATP as the nucleotide was independent of PP_i concentration between 0.05 and 2.0mM. Purified ATP sulphurylase did not catalyse [^{32}P]PP_i exchange when ATP was replaced with either ADP, AMP or GTP. Similarly the

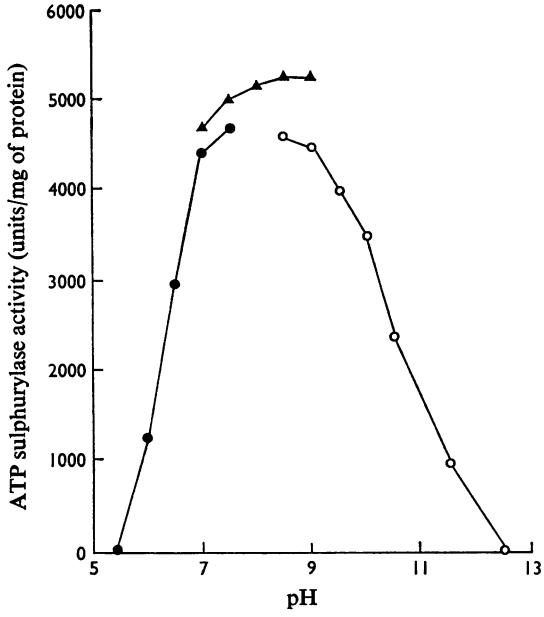

Fig. 2. *Effect of pH on the activity of purified ATP sulphurylase in the absence of fluoride*

Conditions of the assay and buffer compositions are described in the text. ▲, Tris–HCl buffer; o, glycine–NaOH buffer; ●, phosphate–citrate buffer. Glycyl-tRNA synthetase was not detected in purified ATP sulphurylase.

enzyme did not catalyse the incorporation of P_i into ATP, dATP, ADP, AMP or GTP when [^{32}P]PP_i was replaced with [^{32}P]P_i, indicating the absence of ADP sulphurylase activity. ADP sulphurylase activity was not detected in crude dialysed extracts.

Purified ATP sulphurylase did not catalyse PP_i exchange when sulphate was replaced with 1mM-L-cysteine, L-methionine, glycine or a mixture of 20 protein amino acids (L-isomers, each 1mM), indicating that the enzyme was separated from spinach-leaf aminoacyl-tRNA synthetases (Marcus, 1959). The enzyme was also separated from the short-chain fatty acid thiokinases of spinach-leaf tissue (Millerd & Bonner, 1953); no PP_i exchange occurred when sulphate was replaced with 1–40mM-acetate or propionate. The following inorganic substrates did not catalyse PP_i exchange: persulphate, thiosulphate, metabisulphite, dithionate and selenite (each 40mM). Freshly prepared sulphite (40mM) catalysed a low rate of exchange that was 5% of V (sulphate), but this activity was attributed to oxidation to sulphate (Roy & Trudinger, 1970).

The only true alternative substrate of sulphate in the PP_i-exchange assay of ATP sulphurylase was

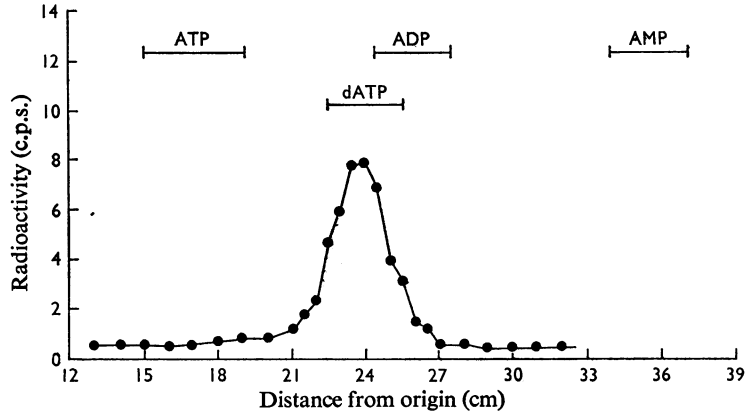

Fig. 3. *Radiochromatogram trace of the ^{32}P-labelled product*

The product was synthesized in a standard assay of ATP sulphurylase (in the absence of fluoride) containing dATP (2μmol) in lieu of ATP. The ^{32}P-labelled product was separated from [^{32}P]PP_i and applied to chromatograms as described in the text. The chromatogram was developed in solvent II for 16h; marker spots of ATP, dATP, ADP and AMP are shown.

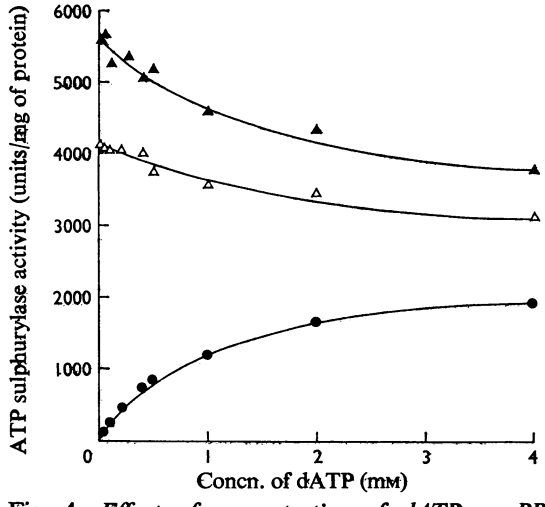

Fig. 4. *Effect of concentration of dATP on PP_i exchange in the presence and absence of ATP*

Incubation mixtures contained 9.5 units of purified ATP sulphurylase and assays were conducted in the absence of fluoride; all other conditions of the assays were as described in the text for the assay of ATP sulphurylase except that the standard amount of ATP was replaced with the amounts of dATP and ATP specified. ●, No ATP: △, 0.5mM-ATP; ▲, 1.0mM-ATP. PP_i exchange is expressed as PP_i–(ATP+dATP) exchange. K_m values for ATP and dATP were computed from measurements of v at ten concentrations of substrate in separate experiments. K_m (ATP) was 0.35±0.06mM and K_m (dATP) was 0.84±0.07mM.

selenate. The affinity of ATP sulphurylase for selenate (K_m 1.0mM) was greater than that for sulphate (K_m 3.1mM), though V (selenate) was 28% of V (sulphate). The ^{32}P-labelled product synthesized by purified enzyme with selenate, ATP and [^{32}P]PP_i as substrates was identified by chromatography in solvent II as [^{32}P]ATP; [^{32}P]ADP and unlabelled AMP were not detected. The ratio of sulphate- to selenate-dependent activities was approximately constant during purification, and the two activities were not separated during gel filtration on Sephadex G-200 or ion-exchange chromatography on DEAE-cellulose (Table 1 and Fig. 1). Addition of selenate to standard assays (containing sulphate) of purified enzyme diminished the PP_i exchange, and the kinetics of sulphate/selenate competition experiments (Fig. 5) are consistent with the kinetics of two substrates competing for one enzyme (Pocklington & Jeffery, 1969).

Wilson & Bandurski (1958) reported that molybdate was an analogue of sulphate in the ATP sulphurylase assay, though a stable adenylate of molybdate was not formed. Purified ATP sulphurylase did not catalyse molybdate-dependent PP_i exchange with ATP, but large amounts of AMP were detected when terminated standard assay mixtures containing molybdate (40μmol) in lieu of sulphate were subjected to paper chromatography in solvent II; formation of AMP was dependent on both molybdate and ATP sulphurylase. Great care was required to establish that no PP_i exchange occurs with molybdate as substrate, since a ^{32}P-labelled compound (possibly a phosphomolybdate complex) was formed non-enzymically and was weakly adsorbed

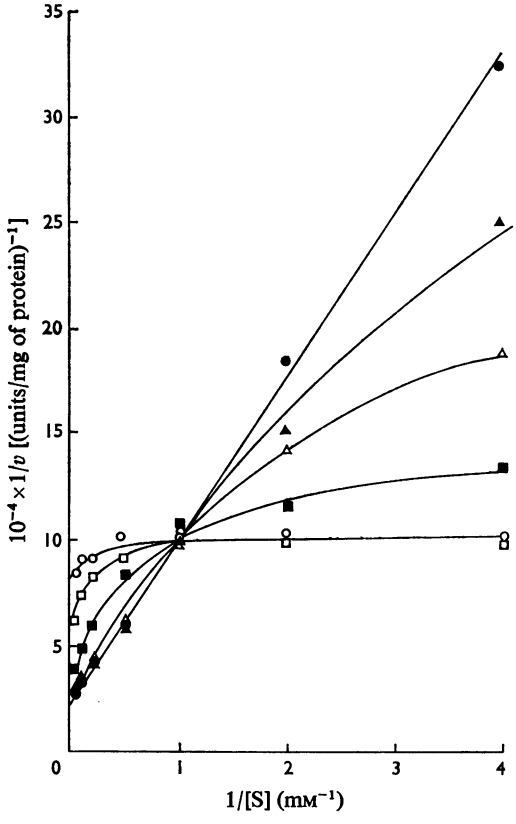

Fig. 5. *Double-reciprocal plot of the effect of concentration of K_2SO_4 substrate on ATP sulphurylase activity in the presence and absence of potassium selenate*

Incubation mixtures contained 4.4 units of purified ATP sulphurylase and assays were conducted in the absence of fluoride; all other conditions of the assays were as described in the text for the assay of ATP sulphurylase activity except that the standard amount of sulphate was replaced with the amounts of sulphate and selenate specified. ●, No selenate; ▲, 0.125mM-selenate; △, 0.5mM-selenate; ■, 2mM-selenate; □, 20mM-selenate; ○, 40mM-selenate. K_m values for sulphate and selenate were computed from measurements of v at ten concentrations of substrate in separate experiments. K_m (sulphate) was 3.1±0.3mM and K_m (selenate) was 1.0±0.3mM.

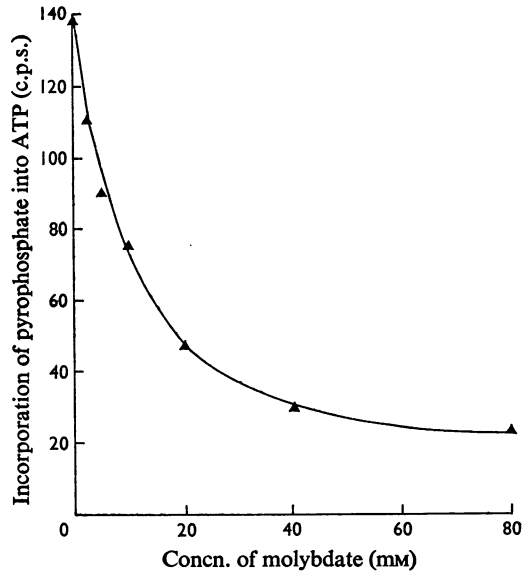

Fig. 6. *Effect of concentration of sodium molybdate on sulphate-dependent incorporation of [^{32}P]PP_i into ATP*

Incubation mixtures contained 3.6 units of purified ATP sulphurylase and assays were conducted in the absence of fluoride; all other parameters of the assays were as described in the text for the assay of ATP sulphurylase (sulphate concentration 40mM). In the absence of sulphate, ATP sulphurylase did not catalyse molybdate-dependent PP_i–ATP exchange but the enzyme catalysed molybdate-dependent hydrolysis of ATP to AMP. It is therefore impossible to calculate sulphate-dependent PP_i–ATP exchange in the presence of molybdate because of the undefined concentration of ATP produced by molybdate-dependent hydrolysis of ATP (see the text).

by charcoal; this label was removed by exhaustive washing with 50mM-acetate buffer, pH4. Addition of molybdate inhibited sulphate-dependent PP_i exchange (Fig. 6), but the exchange could not be accurately calculated by the method of Davie *et al.* (1956) because the decrease in the substrate concentration of ATP by molybdate-dependent hydrolysis of ATP to AMP was significant (up to 50% in a 15min assay containing 40mM-molybdate and 50 units of ATP sulphurylase).

Sulphate-dependent PP_i exchange was negligible in the absence of Mg^{2+}. Exchange increased with concentration of Mg^{2+} up to 5mM; higher concentrations of Mg^{2+} (up to 40mM) were not inhibitory. Co^{2+} was equally effective as Mg^{2+} at concentrations up to 10mM. Other bivalent cations also supported sulphate-dependent exchange; the activities with 10mM-Mn^{2+}, Ni^{2+} and Zn^{2+} respectively were 33%, 31% and 23% of the activity with 10mM-Mg^{2+}.

Purified ATP sulphurylase was virtually insensitive to thiol-group reagents. The following reagents

caused less than 20% inhibition: *p*-chloromercuribenzoate (8.0μM), iodoacetamide (0.1–10mM) and *N*-ethylmaleimide (0.1–10mM).

Purification and properties of pyrophosphatase

Some pyrophosphatase activity co-precipitated with ATP sulphurylase during ammonium sulphate fractionation (Table 1), but most of the activity remained in solution and was recovered by a further addition of ammonium sulphate. Only very limited purification (20–30-fold) was achieved by the acetone-precipitation and gel-filtration procedures (Table 2), though these procedures totally removed ATP sulphurylase activity. Only slight purification of pyrophosphatase was achieved by ion-exchange chromatography on DEAE-cellulose (pH7.8).

Miss G. M. Richardson of this Department has established that the enzyme is a Mg^{2+}-requiring alkaline pyrophosphatase. The pH optimum was 8.0 in tris–maleic acid–NaOH buffer (100mM) under conditions of the standard assay. No activity was detected at 0–2.0mM-$MgCl_2$, but the activity increased as the concentration of $MgCl_2$ was increased above 2mM; maximum activity was obtained at 15–25mM-$MgCl_2$. With the concentration of reagents described in the standard assay, K_m (PP_i) was approx. 0.4mM. ATP, ADP and AMP were not hydrolysed by pyrophosphatase, and 10mM-NaF generally caused 95% inhibition of activity in the standard assay.

Properties of the ATP sulphurylase–pyrophosphatase coupled enzyme system

Synthesis of adenosine 5′-sulphatophosphate could not be demonstrated with purified ATP sulphurylase with [^{35}S]sulphate and ATP as substrates. However, by including pyrophosphatase in the assay a compound labelled with ^{35}S that was adsorbed by charcoal (Table 3) was synthesized. The synthesis of the labelled product required both pyrophosphatase and ATP sulphurylase and was inhibited by 10mM-fluoride. The labelled product (as well as substrate concentrations of ATP, which were also adsorbed) was eluted from charcoal and subjected to paper

Table 2. *Typical purification of Mg^{2+}-dependent alkaline pyrophosphatase from spinach*

Details of the purification procedures and assay of pyrophosphatase are described in the text.

Treatment	Protein (mg)	Pyrophosphatase (units/mg of protein)
Dialysed crude extract	2110	2.2
$(NH_4)_2SO_4$ fraction	194	14.9
Acetone (36–58%, v/v)	49	36.0
Sephadex G-200	18	36.0
DEAE-cellulose	10.2	46.4

Table 3. *Effect of omitting components of the standard assay and the effect of including sodium fluoride* (10mM) *on the synthesis of adenosine 5′-sulphatophosphate in the coupled enzyme system*

Results are expressed as a percentage of the standard assay for each experiment; standard assay conditions of the coupled enzyme system are described in the text. The absolute activities of the standard assays for Expts. 1, 2, 3 and 4 were 23.8, 44.1, 33.2 and 33.3nmol/h respectively.

	Activity (%)			
Assay	Expt. 1	Expt. 2	Expt. 3	Expt. 4
Standard minus ATP sulphurylase	0	0	0	0
Standard minus pyrophosphatase	0	0	3	0
Standard minus ATP	—	0	0	—
Standard minus Mg^{2+}	—	—	—	3
Standard plus fluoride	15	18	50	45

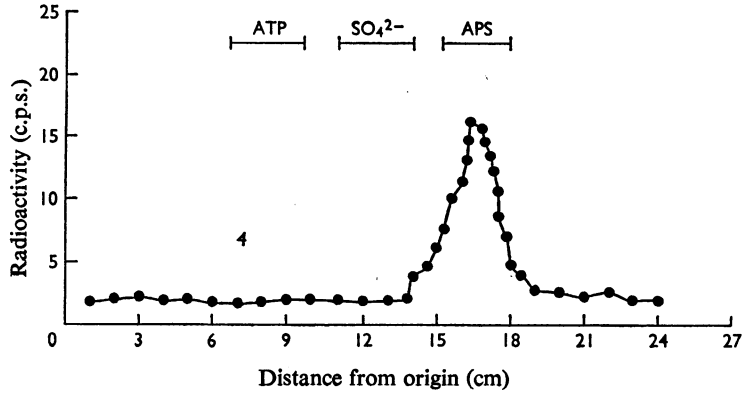

Fig. 7. *Radiochromatogram trace of the ^{35}S-labelled product synthesized in a standard coupled enzyme system*

The ^{35}S-labelled product was separated from [^{35}S]sulphate and applied to chromatograms as described in the text. The chromatogram was developed in solvent I for 24h; marker spots of ATP, adenosine 5′-sulphatophosphate (APS) and sulphate are shown.

chromatography in solvent I; the ^{35}S-labelled product was separated from ATP and sulphate (Fig. 7) and the R_F was identical with that of adenosine 5′-sulphatophosphate. The labelled product also had the same R_F as adenosine 5′-sulphatophosphate when it was eluted and re-run in solvent II.

Double-reciprocal plots of the rate of synthesis of adenosine 5′-sulphatophosphate by the coupled enzyme system versus concentration of sulphate and ATP respectively were consistent with Michaelis–Menten kinetics for a single enzyme. The affinity of the coupled enzyme system for sulphate and ATP was not high; K_m (sulphate) was approx. 8mM and K_m (ATP) was approx. 5mM.

Discussion

The most important aspect of purification was the separation of ATP sulphurylase and pyrophosphatase. Spinach leaf tissue is a rich source of Mg^{2+}-dependent alkaline pyrophosphatase (Naganna *et al.*, 1955; El-Badry & Bassham, 1970; Tables 1 and 2), and this enzyme, with its similar pH optimum and requirements for Mg^{2+} and PP_i to ATP sulphurylase, interferes with the measurement of ATP sulphurylase activity by the PP_i-exchange assay method (Shaw & Anderson, 1971; Table 1). Although we have not examined pyrophosphatase under all the permutations of Mg^{2+} and PP_i concentrations and pH, the Mg^{2+}-requiring alkaline pyrophosphatase described in the present paper is probably the same enzyme as that described by El-Badry & Bassham (1970) in spinach-leaf chloroplasts. Interference of ATP sulphurylase estimations by pyrophosphatase in fractions contaminated with pyrophosphatase was minimized by including 10mM-fluoride to inhibit pyrophosphatase, though ATP sulphurylase activity was slightly underestimated because 10mM-fluoride caused 7% inhibition of ATP sulphurylase activity in purified preparations that did not contain pyrophosphatase activity (Table 1). Neither ADP sulphurylase (Robbins & Lipmann, 1958*a*) nor an enzyme catalysing ADP–P_i/sulphate exchange (Grunberg-Manago *et al.*, 1966) was detected in crude extracts of spinach nor at any stage of purification of ATP sulphurylase.

ATP sulphurylase of spinach is similar to the yeast enzyme (Robbins & Lipmann, 1958*b*; Wilson & Bandurski, 1958). Both enzymes are stable, require Mg^{2+} as a cofactor, have similar pH optima and are insensitive to thiol-group reagents, thus differing from the ATP sulphurylases of sheep liver (Panikkar & Bachhawat, 1968) and rat liver (Levi & Wolf, 1969), which are labile and sensitive to thiol-group reagents. In addition to catalysing sulphate-dependent [^{32}P]-PP_i–ATP exchange, spinach-leaf ATP sulphurylase also catalysed sulphate-dependent [^{32}P]PP_i–dATP exchange with the formation of [^{32}P]dATP (Fig. 3). However, the nucleotide requirement was not totally unspecific, as reported for the ADP–P_i/sulphate exchange reaction in yeast (Grunberg-Manago *et al.*, 1966). The affinity of spinach leaf ATP sulphurylase for ATP was about 2.4-fold that for dATP and V(ATP) was about 3-fold that of V(dATP) (Fig. 4); since ATP sulphurylase of spinach leaf is located in chloroplasts (Asahi, 1964; Balharry & Nicholas, 1970), which are the main sites of ATP synthesis in leaf tissue, then we conclude that ATP is probably the preferred substrate *in vivo*.

The kinetic parameters of purified ATP sulphurylase measured by PP_i exchange (Figs. 4 and 5) supplement the parameters obtained for adenosine 5'-sulphatophosphate and PP_i reported by Balharry & Nicholas (1970) by using the firefly assay; neither method permits the measurement of all four parameters. The affinity of the enzyme for sulphate is low (K_m 3.1 mM), and suggests that very high concentrations of sulphate must be accumulated inside spinach-leaf chloroplasts if the synthesis of adenosine 5'-sulphatophosphate is a prerequisite for sulphate reduction *in vivo*. Ellis (1963) has reported the synthesis of [^{35}S]cysteine from [^{35}S]sulphate in beetroot slices, thereby inferring the existence of ATP sulphurylase in non-photosynthetic tissue. However, no information is available on the subcellular localization of the beetroot enzyme and its affinity for sulphate.

Sulphate was the only form of inorganic sulphur activated by spinach-leaf ATP sulphurylase. Wilson & Bandurski (1958) reported that molybdate was an analogue of sulphate with the yeast enzyme and formed an unstable adenylate that spontaneously hydrolysed to AMP and molybdate. Purified ATP sulphurylase of spinach catalysed the synthesis of AMP when molybdate was used as substrate, but did not catalyse molybdate-dependent PP_i exchange, indicating that the reaction was irreversible. Addition of molybdate to standard assay mixtures containing sulphate decreased PP_i exchange (Fig. 6); the decrease in sulphate-dependent exchange can only be partially explained by the decrease in the substrate concentration of ATP. This suggests that molybdate competes for the sulphate-binding site of ATP sulphurylase. Taken collectively, these results are consistent with the scheme proposed by Wilson & Bandurski (1958).

Wilson & Bandurski (1958) reported that selenate was also an analogue of sulphate, but that it differed from molybdate in that a more stable adenylate was formed; nevertheless ATP was still quantitatively converted into AMP. ATP sulphurylase of spinach catalysed selenate-dependent PP_i exchange. The ratio of sulphate- to selenate-dependent PP_i exchange was approximately constant during purification (Table 1 and Fig. 1) and the two activities could not be separated, suggesting that one enzyme catalysed both activities. This hypothesis was confirmed by kinetic studies with purified enzyme; selenate and sulphate competed for the same active site (Fig. 5). Formation of AMP with selenate as substrate could not be detected, suggesting that a stable adenylate of selenate was formed.

Selenate has been reported to act as an analogue of sulphate in various animal and microbial systems (Rosenfeld & Beath, 1964), but selenate does not act as a sulphate analogue in those enzyme systems from higher plants studied so far (Shrift, 1969). Some plants (e.g. *Astragalus racemosus*, *Astragalus bisulcatus* and *Neptunia amplexicaulis*) characteristically grow in selenium-rich soils. Selenium is accumulated by these species and is incorporated into non-protein amino acid derivatives of selenocysteine (Shrift, 1969). Non-accumulator species on the other hand, when supplied with selenate, typically synthesize the selenium analogues of methionine and cysteine (as well as non-protein amino acid derivatives), which are incorporated into protein in lieu of the corresponding sulphur amino acids, thereby forming inactive proteins (Peterson & Butler, 1962). ATP sulphurylase is believed to be the first enzyme in the biosynthesis of cysteine from sulphate. Since selenate is a substrate of ATP sulphurylase from spinach (a non-accumulator) and competes with sulphate for the enzyme, then one explanation for the toxicity of selenate in non-accumulator species could be that selenate enters metabolism in lieu of sulphate because the ATP sulphurylase of non-accumulator species is unable to differentiate between sulphate and selenate.

General metabolic reactions involving seleno amino acids have been described in selenium-accumulator species for which the corresponding reactions with the sulphur amino acids have not been detected, suggesting that accumulator species can differentiate between selenium and sulphur metabolites (Shrift, 1969). It is not yet known whether the ATP sulphurylases of selenium-accumulator species can differentiate between sulphate and selenate. However, several examples of substrate differentiation by enzymes from restricted members of the plant kingdom have been reported; thus the aminoacyl-tRNA synthetases of certain species differentiate between protein amino acids and amino acid analogues produced by those species (Peterson & Fowden, 1965; Anderson & Fowden, 1970).

Synthesis of adenosine 5'-sulphatophosphate in plants has been demonstrated in crude extracts and in chloroplasts with either an endogenous and/or an external source of pyrophosphatase (Asahi, 1964; Ellis, 1969; Balharry & Nicholas, 1970); synthesis of adenosine 5'-sulphatophosphate by a purified coupled enzyme system containing ATP sulphurylase and Mg^{2+}-dependent alkaline pyrophosphatase prepared from the same tissue (Table 2 and Fig. 7) has not been previously demonstrated. Synthesis of adenosine 5'-sulphatophosphate was not detected in the absence of pyrophosphatase. Fluoride inhibits pyrophosphatase (El-Badry & Bassham, 1970; Table 1), but only slightly inhibits purified ATP sulphurylase (Table 1); addition of 10 mM-fluoride to the coupled system inhibited the synthesis of adenosine 5'-sulphatophosphate, confirming the requirement for pyrophosphatase for the formation of this compound. Since the equilibrium constant of the reaction catalysed by ATP sulphurylase is extremely un-

favourable for the synthesis of adenosine 5′-sulphatophosphate (Roy & Trudinger, 1970), and since both ATP sulphurylase and Mg^{2+}-requiring alkaline pyrophosphatase are chloroplast enzymes (El-Badry & Bassham, 1970; Balharry & Nicholas, 1970), then this suggests that one of the functions of chloroplast pyrophosphatase might be to augment the yield of adenosine 5′-sulphatophosphate by removing pyrophosphate as it is formed from ATP by ATP sulphurylase.

Very high concentrations of sulphate (approx. 40mM) were required for the maximum rate of synthesis of adenosine 5′-sulphatophosphate in the coupled enzyme system; this is consistent with the low affinity of ATP sulphurylase for sulphate (Fig. 5), and again demonstrates the requirement for high concentrations of sulphate in chloroplasts before adenosine 5′-sulphatophosphate synthesis can proceed *in vivo*. We have not examined crude or fractionated extracts of spinach for adenosine 5′-sulphatophosphate kinase activity and are therefore unable to comment on whether this enzyme occurs in plants (Asahi, 1964; Mercer & Thomas, 1969; Balharry & Nicholas, 1970). Adenosine 3′-phosphate 5′-sulphatophosphate, however, was not detected as a product in the coupled enzyme system, demonstrating that adenosine 5′-sulphatophosphate kinase activity was absent from purified ATP sulphurylase and pyrophosphatase.

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