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Adenosine 5'-Sulphatophosphate Kinase Activity in Spinach Leaf Tissue

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1. An F^--insensitive 3'-nucleotidase was purified from spinach leaf tissue; the enzyme hydrolysed 3'-AMP, 3'-CMP and adenosine 3'-phosphate 5'-sulphatophosphate but not adenosine 5'-nucleotides nor PP_i. The pH optimum of the enzyme was 7.5; K_m (3'-AMP) was approx. 0.8mM and K_m (3'-CMP) was approx. 3.3mM. 3'-Nucleotidase activity was not associated with chloroplasts. Purified Mg^{2+}-dependent pyrophosphatase, free from F^--insensitive 3'-nucleotidase, catalysed some hydrolysis of 3'-AMP; this activity was F^--sensitive. 2. Adenosine 5'-sulphatophosphate kinase activity was demonstrated in crude spinach extracts supplied with 3'-AMP by the synthesis of the sulphate ester of 2-naphthol in the presence of purified phenol sulphotransferase; purified ATP sulphurylase and pyrophosphatase were also added to synthesize adenosine 5'-sulphatophosphate. Adenosine 5'-sulphatophosphate kinase activity was associated with chloroplasts and was released by sonication. 3. Isolated chloroplasts synthesized adenosine 3'-phosphate 5'-sulphatophosphate from sulphate and ATP in the presence of a 3'-nucleotide; the formation of adenosine 5'-sulphatophosphate was negligible. In the absence of a 3'-nucleotide the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate was negligible, but the formation of adenosine 5'-sulphatophosphate was readily detected. Some properties of the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate by isolated chloroplasts are described. 4. Adenosine 3'-phosphate 5'-sulphatophosphate, synthesized by isolated chloroplasts, was characterized by specific enzyme methods, electrophoresis and i.r. spectrophotometry. 5. Isolated chloroplasts catalysed the incorporation of sulphur from sulphate into cystine/cysteine; the incorporation was enhanced by 3'-AMP and L-serine. It was concluded that adenosine 3'-phosphate 5'-sulphatophosphate is an intermediate in the incorporation of sulphur from sulphate into cystine/cysteine.

Yeast incorporates the sulphur of sulphate into cysteine. In this organism, sulphate is activated by two molecules of ATP to form adenosine 3'-phosphate 5'-sulphatophosphate, which is then reduced to sulphide by the sulphate-reduction complex (Roy & Trudinger, 1970). The formation of adenosine 3'-phosphate 5'-sulphatophosphate is catalysed by the enzymes ATP sulphurylase (ATP–sulphate adenylyltransferase, EC 2.7.7.4) and adenosine 5'-sulphatophosphate kinase (ATP–adenylyl sulphate 3'-phosphotransferase, EC 2.7.1.25) respectively:

$$ATP + SO_4^{2-} \xrightleftharpoons{Mg^{2+}} \text{adenosine 5'-sulphatophosphate} + PP_i$$

$$ATP + \text{adenosine 5'-sulphatophosphate} \xrightleftharpoons{Mg^{2+}} \text{adenosine 3'-phosphate 5'-sulphatophosphate} + ADP$$

Higher plants also incorporate sulphur from sulphate into cysteine. The synthesis of adenosine 5'-sulphatophosphate by crude extracts and isolated chloroplasts from higher plants has been described (Asahi, 1964; Ellis, 1969; Balharry & Nicholas, 1970) and ATP sulphurylase has been purified from leaf tissue (Shaw & Anderson, 1972). The presence of adenosine 5'-sulphatophosphate kinase in the tissues of higher plants, however, is uncertain. Mercer & Thomas (1969) reported that fragmented chloroplasts from *Phaseolus vulgaris* and *Zea mays* synthesized adenosine 3'-phosphate 5'-sulphatophosphate when incubated with ATP, sulphate and pyrophosphatase; the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate from adenosine 5'-sulphatophosphate was also described, but in neither case was the product identified. Asahi (1964) and Balharry & Nicholas (1970), however, were unable to demonstrate the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate either in intact or fragmented chloroplasts. Ellis (1969) reported that crude extracts of plants synthesized adenosine 5'-sulphatophosphate but not adenosine 3'-phosphate 5'-sulphatophosphate;

the latter compound, however, was synthesized when an extract of *Escherichia coli* mutant 4-2, which lacks ATP sulphurylase, was added. Ellis (1969) concluded that the lack of synthesis of adenosine 3'-phosphate 5'-sulphatophosphate by plant extracts in the absence of the extract of *E. coli* mutant was not due to metabolism of adenosine 3'-phosphate 5'-sulphatophosphate by other enzymes present in the plant extracts. From their studies, Ellis (1969) and Balharry & Nicholas (1970) raised the question whether adenosine 5'-sulphatophosphate might be the activated form of sulphate for reduction in higher plants, as has been established for the dissimilatory sulphate-reducing bacteria (Roy & Trudinger, 1970).

Adenosine 3'-phosphate 5'-sulphatophosphate is synthesized by *Chlorella* (Hodson & Schiff, 1969), but Tsang *et al.* (1971) and Schmidt (1972) postulated that adenosine 5'-sulphatophosphate is the activated form of sulphate that is reduced in this organism. Schmidt (1972) reported that extracts of *Chlorella* were unable to reduce adenosine 3'-phosphate 5'-sulphatophosphate unless a 3'-nucleotidase was present. Schmidt (1972) also described an adenosine 5'-sulphatophosphate-specific sulphotransferase in *Chlorella* which catalysed the transfer of the sulphate moiety of adenosine 5'-sulphatophosphate to glutathione or dithiothreitol; he suggested that an unidentified endogenous thiol acted as the acceptor *in vivo* and that the bound form was the substrate for reduction.

In studies with extracts of *Salmonella pullorum*, Kline & Schoenhard (1968) were unable to demonstrate the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate. The extracts, however, contained very high 3'-nucleotidase activity, the 3'-phosphate of adenosine 3'-phosphate 5'-sulphatophosphate being hydrolysed by 3'-nucleotidase (Robbins & Lipmann, 1958). Kline & Schoenhard (1968) reported that adenosine 3'-phosphate 5'-sulphatophosphate was synthesized by *S. pullorum* extracts when 3'-AMP was included in the incubation mixture as an alternative substrate for 3'-nucleotidase activity.

Plants are rich sources of 3'-nucleotidase activity (Sung & Laskowski, 1962; Loring *et al.*, 1966; Hanson & Fairley, 1969) and the activity of this enzyme might account for the conflicting reports on the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate in higher plants. The present paper describes a reinvestigation of the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate in plant tissue with special reference to the characterization of adenosine 3'-phosphate 5'-sulphatophosphate and to 3'-nucleotidase activity. We report that isolated chloroplasts prepared essentially by the method of Spencer & Wildman (1964) catalyse the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate; the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate was greatly enhanced by 3'-AMP, but the effect of 3'-AMP is probably not due to its activity as an alternative substrate of 3'-nucleotidase, since the 3'-nucleotidase of isolated chloroplasts was negligible.

Experimental

Chemicals

Adenosine 3'-phosphate 5'-sulphatophosphate was synthesized biologically from sulphate and ATP by using an extract of rat liver as a source of sulphate-activating enzymes as described by Roy (1960) except that the concentration of ATP was increased to 15mM. Adenosine 3'-phosphate 5'-sulphatophosphate was isolated and purified essentially by the method of Hodson & Schiff (1969). The reaction was terminated with ethanol (75%, v/v) and the supernatant solution was concentrated to approx. 20ml. The concentrated solution was adjusted to pH7.2 and then filtered. The filtered solution was applied to an anion-exchange resin [15cm×1.5cm column of Dowex AG 1 (X2, 200–400 mesh, Cl^- form)]. The column was washed with 0.3M-NaCl until the extinction at 260nm was less than 0.05; adenosine 3'-phosphate 5'-sulphatophosphate was eluted with a linear gradient of NaCl (0.3–0.8M). Adenosine 3'-phosphate 5'-sulphatophosphate was adsorbed on charcoal as described by Roy (1960) and the charcoal was washed with water to remove Cl^-. Adenosine 3'-phosphate 5'-sulphatophosphate was eluted from the charcoal with approx. 1 litre of aq. 50% (v/v) ethanol containing 10ml of aq. 1M-NH_3. The eluted material was filtered through a layer of Kieselgel to remove particulate material (mostly charcoal) and the solution was evaporated to dryness. The residue was dissolved in 20ml of water and the pH of the solution was adjusted to 7.2. The concentration of adenosine 3'-phosphate 5'-sulphatophosphate was determined from the extinction at 259nm by using the molar extinction coefficient cited by Baddiley *et al.* (1957); the concentration was confirmed by the formation of naphthol 2-sulphate in the presence of purified phenol sulphotransferase.

[β,γ-^{32}P]ATP was synthesized from [^{32}P]PP_i by sulphate-dependent PP_i–ATP exchange catalysed by purified ATP sulphurylase (Shaw & Anderson, 1972). Reaction mixtures contained 2μmol of [^{32}P]PP_i (approx. 200μCi), 2μmol of Na_2K_2ATP, 40μmol of K_2SO_4, 20μmol of $MgCl_2$, 200μmol of Tris–HCl buffer, pH8.0, and 120 units of purified ATP sulphurylase. Reactions were conducted at 35°C for 2h and terminated by the addition of 2.0ml of 7.5% (w/v) trichloroacetic acid. [^{32}P]ATP was extracted from the incubation mixtures and identified as [^{32}P]-ATP by chromatography in solvent II as described by Shaw & Anderson (1971).

Adenosine 5'-[^{35}S]sulphatophosphate was synthesized enzymically as described by Shaw & Anderson (1972).

All other chemicals were obtained from the sources described by Shaw & Anderson (1972).

Methods

Preparation of crude spinach extracts and of chloroplast suspensions. Spinach leaves were washed with water and the midribs removed. The leaf tissue was extracted with 100mM-Tris–HCl buffer, pH8.0, containing 20mM-$MgCl_2$ (2ml/g fresh wt.) in a Waring Blendor. The extract was squeezed through muslin and the cell debris removed by centrifugation at 500*g* for 10min. The supernatant solution was recentrifuged at 30000*g* for 30min and the high-speed supernatant solution (referred to as the spinach crude extract) was recovered. The crude extract was dialysed against extracting medium for 12h and used as the source of 3'-nucleotidase activity. All operations were conducted at 2°C.

Chloroplasts were isolated, washed and disrupted as described by Burnell & Anderson (1973). Washed chloroplasts were suspended in the medium described by Spencer & Wildman (1964) except that Ficoll and dextran were omitted and $MgCl_2$ was added instead of magnesium acetate. Washed chloroplast suspensions contained approx. 0.8–1.3mg of chlorophyll/ml.

Preparation of purified enzymes. ATP sulphurylase was purified from spinach leaf tissue as described by Shaw & Anderson (1972); the specific activity of the purified enzyme was 102 units/mg of protein by using the definition of an enzyme unit described by Shaw & Anderson (1972). ADP sulphurylase, pyrophosphatase, 3'-nucleotidase and adenosine 5'-sulphatophosphate kinase activities were absent from purified ATP sulphurylase.

F^--insensitive 3'-nucleotidase was purified from spinach. All purification procedures were conducted at 2°C. Leaf tissue was extracted and fractionated with $(NH_4)_2SO_4$ and acetone as described by Shaw & Anderson (1972) for the purification of pyrophosphatase; the 36–55%-acetone fraction contained both pyrophosphatase and 3'-nucleotidase activities. The 36–55%-acetone fraction was passed through a column of Sephadex G-200 (60cm×3.5cm) equilibrated with 20mM-Tris–HCl buffer, pH8, and 3'-nucleotidase and pyrophosphatase activities were monitored. F^--insensitive 3'-nucleotidase was eluted from the column just before pyrophosphatase, and some 3'-nucleotidase fractions were contaminated with pyrophosphatase (Fig. 1); only those fractions containing negligible pyrophosphatase activity were used as a source of 3'-nucleotidase. Residual pyrophosphatase was removed by passage through a DEAE-cellulose column (25cm×2.0cm) equilibrated with 20mM-Tris–HCl buffer, pH8.0; the column was developed with a linear gradient of KCl (0–0.35M) in 20mM-Tris–HCl buffer, pH8.0; 3'-nucleotidase was eluted at a lower concentration of KCl than was pyrophosphatase. Fractions containing 3'-nucleotidase and free of pyrophosphatase activity were pooled and dialysed to remove KCl and then applied to another DEAE-cellulose column (3.0cm×1.5cm) equilibrated with 20mM-Tris–HCl buffer, pH8.0. The enzyme was eluted with 10ml of 20mM-Tris–HCl buffer, pH8.0, containing 1M-KCl; the second DEAE-cellulose treatment effected a 4–6-fold concentration of the enzyme. The concentrated enzyme was dialysed against 20mM-Tris–HCl buffer, pH8.0, and used as the source of purified 3'-nucleotidase. The enzyme was purified approx. 37-fold and the specific activity of the purified enzyme was 3.4 units/mg of protein.

Mg^{2+}-dependent alkaline pyrophosphatase was purified from spinach leaf as described for 3'-nucleotidase except that only those fractions eluted from the Sephadex G-200 column and which contained negligible F^--insensitive 3'-nucleotidase activity (Fig. 1) were subjected to further purification on DEAE-cellulose as described by Shaw & Anderson (1972). ATP sulphurylase, ADP sulphurylase, adenosine 5'-sulphatophosphate kinase and F^--insensitive 3'-nucleotidase activities were absent from purified pyrophosphatase, but the purified enzyme catalysed a weak F^--sensitive hydrolysis of 3'-AMP (Table 1). The specific activity of purified pyrophosphatase was 9 units/mg of protein by using the definition of an enzyme unit described by Shaw & Anderson (1972).

Phenol sulphotransferase was extracted and partially purified from rabbit liver as described by Gregory (1962); this procedure, however, did not remove 3'-nucleotidase activity. Accordingly the enzyme was further purified by gel filtration on a Sephadex G-200 column (30cm×2.5cm) equilibrated with 20mM-Tris–HCl buffer, pH7.5. The fractions containing phenol sulphotransferase were pooled and dialysed against 20mM-Tris–HCl buffer, pH7.5, for 5h. The specific activity of purified phenol sulphotransferase was 0.7 unit/mg of protein; purified enzyme was uncontaminated with 3'-nucleotidase, ATP sulphurylase and adenosine 5'-sulphatophosphate kinase activities. The enzyme could be stored at −15°C for many weeks without loss of activity.

Adenosine 5'-sulphatophosphate kinase was purified from baker's yeast as described by Robbins & Lipmann (1958).

Assay of enzyme activities. ATP sulphurylase and pyrophosphatase activities were measured as described by Shaw & Anderson (1972) and ADP sulphurylase was assayed by the method of Burnell & Anderson (1973). 3'-Nucleotidase activity was measured as described for pyrophosphatase except that incubation mixtures contained 50μmol of 3'-AMP instead of PP_i and the pH was altered to 8.0. 3'-Nucleotidase activity (corrected for zero-time control) is expressed as μmol of P_i produced/min (3'-nucleotidase units).

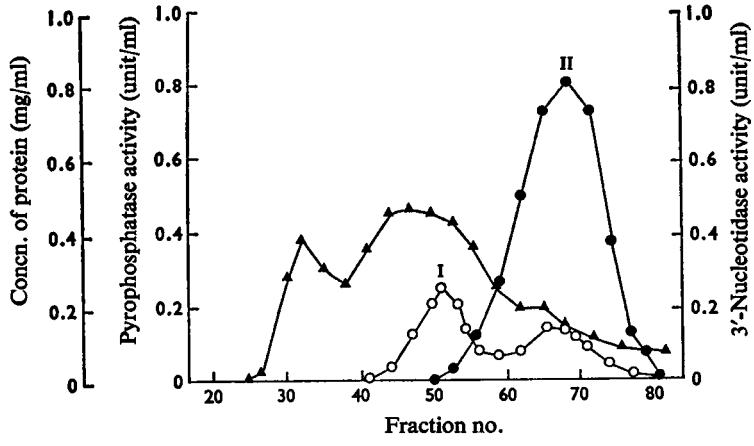


Fig. 1. *Separation of 3'-nucleotidase and Mg^{2+}-dependent pyrophosphatase activities*

The activities were separated by gel filtration on Sephadex G-200 after $(NH_4)_2SO_4$ and acetone fractionation as described for the purification of pyrophosphatase by Shaw & Anderson (1972). Fractions (41–54 and 59–78) were collected and designated peak I and peak II respectively. Enzyme activities were measured as described in the text. Peak I was a specific 3'-nucleotidase and peak II was a pyrophosphatase that catalysed weak unspecific hydrolysis of 3'-AMP. ○, 3'-Nucleotidase activity; ●, pyrophosphatase activity; ▲, protein.

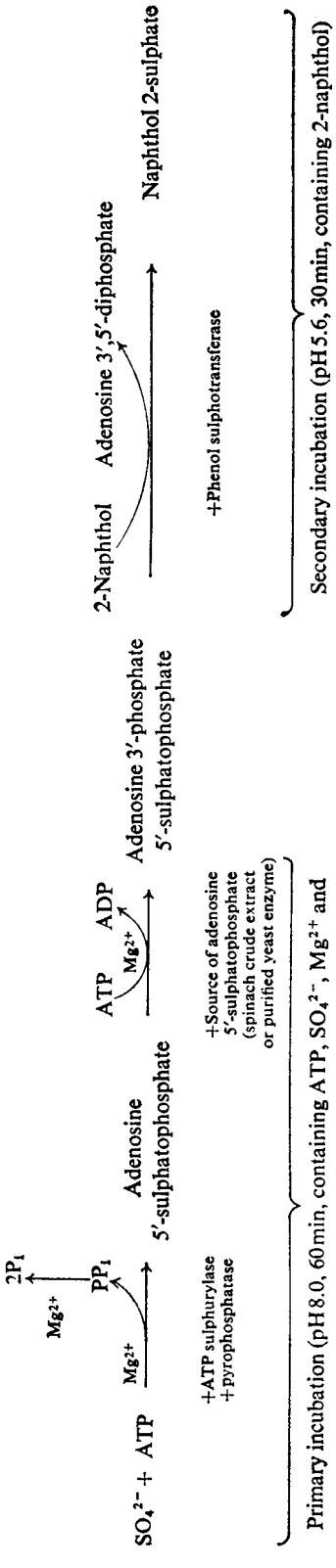
Table 1. *Substrate specificity and the effect of F^- on the phosphatase activity of peaks I and II after gel filtration on Sephadex G-200*

Phosphatase peaks I and II were separated by gel filtration as described in Fig. 1. Phosphatase activities were measured in the presence of 20mM-$MgCl_2$ as described in the text for the assay of 3'-nucleotidase except that the standard amount of 3'-AMP was replaced with the substrates specified.

Substrate	Activity (μmol of P_i/min per mg of protein)	
	Peak I	Peak II
PP_i (20μmol)	0.01	3.11
3'-AMP (20μmol)	1.90	0.25
3'-AMP (20μmol)+NaF (10μmol)	1.86	0.02
PP_i (20μmol)+NaF (10μmol)	0	0.05

Phenol sulphotransferase activity was determined essentially as described by Roy (1956) except that adenosine 3'-phosphate 5'-sulphatophosphate was used as the sulphate donor. Standard incubation mixtures contained 500μmol of sodium acetate buffer, pH5.6, 0.5μmol of 2-naphthol, 0.3μmol of adenosine 3'-phosphate 5'-sulphatophosphate and 0.5ml of enzyme in a total volume of 2.5ml. Reactions were conducted for 60min at 35°C and samples (1ml) were removed in duplicate immediately after the addition of enzyme and deproteinized with 5ml of ethanol; further samples were removed after 60min. The supernatant solutions were recovered by centrifugation and evaporated to dryness. The residue was redissolved in water (1ml) and sulphate ester was measured by the method of Roy (1956). Phenol sulphotransferase activity is expressed as the synthesis of sulphate ester (corrected for zero time) in pmol/min (phenol sulphotransferase units).

The principle underlying the assay of adenosine 5'-sulphatophosphate kinase activity is summarized in Scheme 1. A primary incubation was conducted at pH8.0 to synthesize adenosine 5'-sulphatophosphate by the coupled enzyme method of Shaw & Anderson (1972). Spinach crude extract or purified yeast was added as a source of adenosine 5'-sulphatophosphate kinase. Since spinach crude extracts contain high 3'-nucleotidase activity, 3'-AMP was included in primary incubation mixtures to inhibit hydrolysis of adenosine 3'-phosphate 5'-sulphatophosphate by 3'-nucleotidase. The primary incubation was followed by a secondary incubation at pH5.6 in the presence of phenol sulphotransferase and 2-naphthol to synthe-



Scheme 1. *Summary of the theory of the assay for adenosine 5'-sulphatophosphate kinase activity*

Adenosine 5'-sulphatophosphate kinase activity was measured as the synthesis of naphthol 2-sulphate from adenosine 3'-phosphate 5'-sulphatophosphate in the presence of purified phenol sulphotransferase from rabbit liver; adenosine 5'-sulphatophosphate was synthesized from ATP and sulphate by purified ATP sulphurylase and pyrophosphatase from spinach leaf tissue.

size naphthol 2-sulphate from any adenosine 3'-phosphate 5'-sulphatophosphate formed in the primary assay. Specifically, the primary incubation was conducted at 35°C for 60min and contained 20.4 units of purified spinach ATP sulphurylase, 12 units of purified spinach pyrophosphatase, 20μmol of Na_2K_2ATP, 30μmol of $MgCl_2$, 40μmol of K_2SO_4, 100μmol of Tris–HCl buffer, pH8.0, and a source of adenosine 5'-sulphatophosphate kinase in a final volume of 1.5ml. The secondary incubation (final volume 2.5ml) was initiated after 60min by the addition of 0.15ml of purified phenol sulphotransferase, 0.5μmol of 2-naphthol and 500μmol of sodium acetate buffer, pH5.6, in that order. Immediately after the addition of acetate buffer, a 1ml sample was removed and deproteinized and its sulphate ester content determined, as described for the assay of phenol sulphotransferase. The secondary incubation was terminated after 30min when a further 1ml sample was withdrawn and deproteinized and its sulphate ester content measured. Adenosine 5'-sulphatophosphate kinase activity of spinach crude extracts is expressed as the time-dependent synthesis of sulphate ester during the secondary incubation in nmol/min (adenosine 5'-sulphatophosphate kinase units). In some experiments, 10μmol of $[^{35}S]SO_4^{2-}$ (40μCi) was used; the procedures for the formation of $[^{35}S]$sulphate ester were the same as those described for unlabelled experiments except that the ^{35}S-labelled Methylene Blue salt was evaporated on to stainless-steel planchets and its radioactivity counted with a gas-flow detector.

Synthesis of adenosine 3'-phosphate 5'-sulphatophosphate by isolated chloroplasts. Assays were conducted at 35°C for 2h and contained 20μmol of Na_2K_2ATP, 20μmol of $MgCl_2$, 20μmol of 3'-AMP, 10μmol of $K_2{}^{35}SO_4$ (50μCi), 200μmol of Tris–HCl buffer, pH8.5, and 1ml of a suspension of washed chloroplasts in a final volume of 2ml. Reactions were terminated and deproteinized with 5ml of ethanol. The supernatant solutions were evaporated to dryness and the residues dissolved in water; nucleotides were adsorbed on charcoal and recovered as described by Shaw & Anderson (1972). The nucleotides were subjected to paper chromatography in solvent I and the dried chromatograms were scanned for radioactivity in a gas-flow scanner. The synthesis of adenosine 3'-phosphate 5'-sulphatophosphate was assessed from the radioactivity of the ^{35}S-labelled nucleotide associated with the R_F of adenosine 3'-phosphate 5'-sulphatophosphate (Fig. 2).

Chromatography and electrophoresis. Paper chromatograms developed in solvents I, II and III were run on acid-washed Whatman 3MM paper; chromatograms developed in solvent IV, V, VI and VII were run on unwashed paper. 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

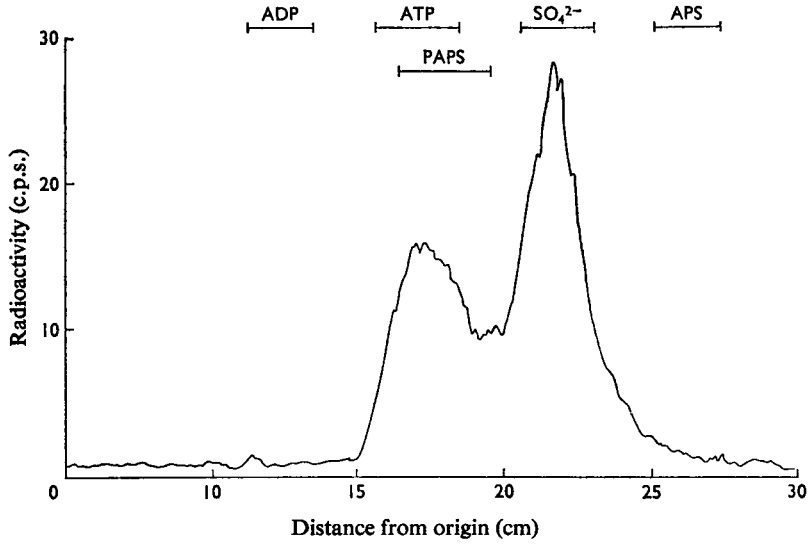


Fig. 2. *Radiochromatogram trace of the ^{35}S-labelled nucleotides synthesized from [^{35}S]sulphate by isolated chloroplasts*

The nucleotides were isolated from reaction mixtures containing [^{35}S]sulphate, ATP, 3′-AMP and chloroplasts as described for the synthesis of adenosine 3′-phosphate 5′-sulphatophosphate by isolated chloroplasts, and subjected to chromatography in solvent I. Marker spots of ATP, ADP, SO_4^{2-}, adenosine 5′-sulphatophosphate (APS) and adenosine 3′-phosphate 5′-sulphatophosphate (PAPS) are shown. The radioactivity associated with the R_F of adenosine 3′-phosphate 5′-sulphatophosphate was eluted and used to measure the incorporation of sulphate into adenosine 3′-phosphate 5′-sulphatophosphate.

(sp.gr. 0.88)–water (66:33:1, by vol.) (Shaw & Anderson, 1971): III, satd. $(NH_4)_2SO_4$–0.1M-ammonium acetate–propan-2-ol (79:19:2, by vol.) (Robbins, 1963); IV, butan-1-ol–acetic acid–water (4:1:5, by vol., top phase) (Ellis, 1963); V, water-saturated phenol; VI, ethanol–aq. NH_3 (sp.gr. 0.88)–water (18:1:1, by vol.) (Block *et al.*, 1958).

Paper electrophoresis was conducted on acid-washed paper (Whatman 3MM) at 2°C at 5.5V/cm. The following systems were used: I, 0.1M-sodium citrate buffer, pH5, for 3.5h (Balharry & Nicholas, 1970); II, 0.05M-ammonium acetate buffer, pH8.5, for 3h (Baddiley *et al.*, 1957).

Incorporation of sulphate into cystine by isolated chloroplasts. Standard assays were conducted at 35°C for 2h and contained 20μmol of Na_2K_2ATP, 20μmol of $MgCl_2$, 20μmol of 3′-AMP, 2.5μmol of $K_2{}^{35}SO_4$ (50μCi), 20μmol of L-serine, 100μmol of Tris–HCl buffer, pH8, and 1ml of chloroplast suspension in a final volume of 2ml. Reaction mixtures were illuminated at 5.8 klx supplied by two tungsten-filament lamps. Reactions were terminated and the amino acids extracted as described by Ellis (1963) except that the pH of the extraction medium was lowered to 6.5 to minimize hydrolysis of *N*-ethylmaleimide (Gregory, 1955).

Terminated reaction mixtures were evaporated to dryness and dissolved in 2ml of water; samples were subjected to descending paper chromatography in solvent IV for 24h and the dried chromatograms monitored for radioactivity in a gas-flow strip scanner. The synthesis of the *N*-ethylmaleimide adduct of [^{35}S]cysteine by isolated chloroplasts was not detected; all the radioactivity was associated with cystine and sulphate, which were not resolved in solvent IV. The radioactivity was eluted and subjected to rechromatography in solvent I, which resolved [^{35}S]cystine and [^{35}S]sulphate; the radioactivity associated with the R_F of cystine was eluted, evaporated on to stainless-steel planchets and counted with a gas-flow detector. The ^{35}S-labelled compound with the R_F of cystine in solvent I was confirmed as [^{35}S]cystine by rechromatography in solvents V and VI.

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).

Results

Purification and properties of spinach leaf 3'-nucleotidase

Spinach leaf tissue is a rich source of 3'-nucleotidase activity; it contains approx. 1.5 units/g fresh wt. Purified pyrophosphatase, prepared by the method of Shaw & Anderson (1972), was heavily contaminated with 3'-nucleotidase, but the two activities could be separated by careful monitoring and selection during gel filtration on a Sephadex G-200 column (Fig. 1). Two peaks of activity catalysing the hydrolysis of 3'-AMP were resolved by gel filtration. Peak II material catalysed the hydrolysis of both PP_i and 3'-AMP (Table 1). The relative rate of hydrolysis of PP_i was approximately 12 times the rate of hydrolysis of 3'-AMP; neither PP_i nor 3'-AMP were hydrolysed in the presence of F^-. The hydrolysis of 3'-AMP catalysed by peak II material was not resolved from pyrophosphatase activity by ion-exchange chromatography on DEAE-cellulose. Weak F^--sensitive 3'-nucleotidase activity was always associated with pyrophosphatase activity, suggesting that the hydrolysis of 3'-AMP catalysed by peak II material was due to unspecific activity of the pyrophosphatase; we cannot, however, rule out the possibility that peak II contained two independent phosphatase activities of different substrate specificity.

The rate of hydrolysis of 3'-AMP catalysed by peak I material was constant for at least 15 min and activity was proportional to the concentration of peak I protein up to 9 mg of protein/ml. Peak I material did not catalyse the hydrolysis of PP_i, ATP, ADP or AMP, but 3'-CMP and adenosine 3'-phosphate 5'-sulphatophosphate were hydrolysed; the K_m of peak I material for 3'-AMP was approximately one-quarter of that for 3'-CMP (Fig. 3). The pH optimum of peak I material for the hydrolysis of 3'-AMP was 7.5 (Fig. 4); addition of ATP inhibited the hydrolysis of 3'-AMP, but hydrolysis of 3'-AMP was not inhibited by F^- (Table 1). We concluded that peak I contained a specific 3'-nucleotidase similar to the enzyme described by Shuster & Kaplan (1953) in rye-grass leaf tissue.

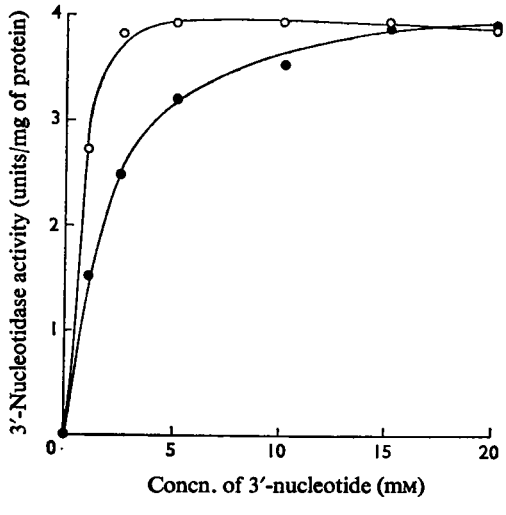


Fig. 3. *Effect of concentration of 3'-AMP and 3'-CMP on the activity of purified 3'-nucleotidase activity*

3'-Nucleotidase activity was measured as described in the text except that the standard amount of 3'-AMP was replaced with the amounts of 3'-AMP and 3'-CMP specified. ○, 3'-AMP; ●, 3'-CMP. K_m values for 3'-AMP and 3'-CMP were computed from measurements at eight concentrations of substrate (0–3 mM-3'-AMP and 0–10 mM-3'-CMP) in separate experiments. K_m (3'-AMP) was 0.8 mM and K_m (3'-CMP) was 3.3 mM.

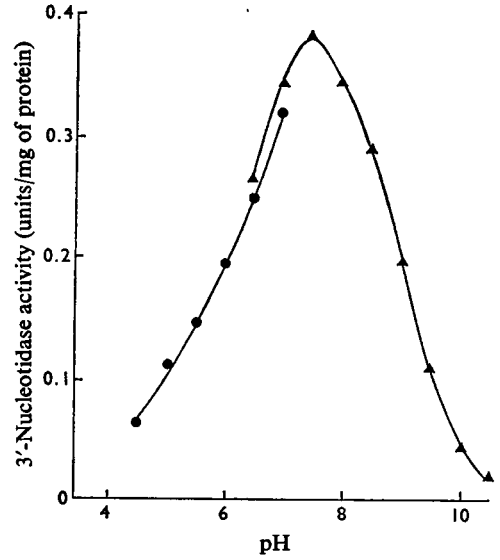


Fig. 4. *Effect of pH on the activity of purified spinach leaf 3'-nucleotidase*

3'-Nucleotidase activity was measured as described in the text except that Tris–HCl buffer, pH 8.0, was replaced with 0.5 ml of Tris–HCl buffer or Tris–maleic acid–KOH buffer as described by Colowick & Kaplan (1955). ▲, Tris–HCl buffer; ●, Tris–maleic acid–KOH buffer.

Crude extracts of spinach catalysed the metabolism of adenosine 3'-phosphate 5'-sulphatophosphate (Table 2). The metabolism of adenosine 3'-phosphate 5'-sulphatophosphate was inhibited by 50mM-3'-AMP and 50mM-3'-CMP; the inhibition by 3'-AMP was twice that by 3'-CMP. Although the K_m of 3'-nucleotidase for 3'-AMP is lower than that with 3'-CMP, the enzyme is saturated by 50mM-3'-AMP and 50mM-3'-CMP (Fig. 3). The inhibition of adenosine 3'-phosphate 5'-sulphatophosphate metabolism by 3'-AMP and 3'-CMP is consistent with the hydrolysis of adenosine 3'-phosphate 5'-sulphatophosphate by the 3'-nucleotidase in crude spinach extracts, but the different degrees of inhibition with 50mM-3'-AMP and 50mM-3'-CMP suggest that 3'-nucleotidase is not the only enzyme involved in the metabolism of adenosine 3'-phosphate 5'-sulphatophosphate by crude extracts.

When chloroplasts and the crude supernatant solution were examined for 3'-nucleotidase, activity was found in the supernatant solution (Table 3); high recovery of ATP sulphurylase from chloroplasts demonstrated that isolated chloroplasts were intact. 3'-Nucleotidase, unlike pyrophosphatase, was not released from chloroplasts by sonication (Burnell & Anderson, 1973; Table 3).

Table 2. *Effect of 3'-AMP and 3'-CMP on the metabolism of adenosine 3'-phosphate 5'-sulphatophosphate by crude spinach extracts*

Crude spinach extract (0.5ml) was incubated for 60min at 35°C with 9nmol of adenosine 3'-phosphate 5'-sulphatophosphate and 50μmol of Tris–HCl buffer, pH7.5, in a volume of 1.0ml; the values include additions introduced with the crude extract. Residual adenosine 3'-phosphate 5'-sulphatophosphate was determined by the formation of naphthol 2-sulphate at pH5.6 in a secondary assay (30min) containing 0.5μmol of 2-naphthol, 5.4 units of phenol sulphotransferase and 200μmol of acetate buffer, pH5.6, in a final volume of 2.5ml. Sulphate ester was measured as described in the text for the measurement of phenol sulphotransferase activity. Treatments refer to additions to or deletions from the primary assay mixture.

Treatment	Adenosine 3'-phosphate 5'-sulphatophosphate metabolized (%)
Complete mixture minus crude extract	0
Complete mixture	72
Complete mixture plus 50μmol of 3'-AMP	20
Complete mixture plus 50μmol of 3'-CMP	59

Table 3. *Subcellular distribution of adenosine 5'-sulphatophosphate kinase, ATP sulphurylase, 3'-nucleotidase and Mg^{2+}-dependent alkaline pyrophosphatase in spinach leaf tissue*

The fractionation procedure was as described in the text; subcellular fractions were prepared from 60g fresh wt. of leaf tissue. The coarse particulate material (cell walls, nuclei etc.) which was removed by centrifugation before isolation of the unwashed chloroplasts was not analysed for enzyme activities. Enzyme activities were measured as described in the text except that assays were conducted in the medium used to extract the chloroplasts and ATP sulphurylase and 3'-nucleotidase were measured in the presence of 10mM-NaF. F^- was added to ATP sulphurylase assays to prevent interference by pyrophosphatase (Shaw & Anderson, 1971), and F^- to 3'-nucleotidase assays to prevent unspecific hydrolysis of 3'-AMP by pyrophosphatase. All enzyme activities are expressed with respect to the weight of tissue extracted.

Treatment	Fraction	ATP sulphurylase (units)	3'-Nucleotidase (units)	Pyrophosphatase (units)	Adenosine 5'-sulphatophosphate kinase (units)
Extraction	P1 (Unwashed chloroplasts)	2.37	0.41	0.13	0.146
	S1 (Crude supernatant solution)	0.19	1.52	1.11	0.043
Wash P1	P2 (Washed chloroplasts)	4.97	0.0	0.18	0.251
	S2 (Chloroplast washings)	0.31	0.01	0.04	0.053
Sonicate P2	P3 (Chloroplast fragments)	0.03	0	0.07	0.035
	S3 (Chloroplast supernatant solution)	4.63	0	1.94	0.191

Adenosine 5'-sulphatophosphate kinase activity in crude extracts of spinach

The standard method described for the assay of adenosine 5'-sulphatophosphate kinase activity was examined by using purified yeast adenosine 5'-sulphatophosphate kinase. Sulphate ester was only formed in the presence of phenol sulphotransferase, the adenosine 5'-sulphatophosphate-synthesizing system and adenosine 5'-sulphatophosphate kinase (Table 4); sulphate ester was not synthesized in the absence of adenosine 5'-sulphatophosphate kinase, thereby showing that adenosine 5'-sulphatophosphate kinase activity was absent from purified ATP sulphurylase, pyrophosphatase and phenol sulphotransferase.

Crude extracts catalysed the synthesis of the sulphate ester of 2-naphthol from ATP and sulphate in the presence of 3'-AMP, phenol sulphotransferase, ATP sulphurylase and pyrophosphatase (Table 5). Sulphate ester synthesis in the absence of 2-naphthol, ATP, sulphate, phenol sulphotransferase or crude extract was negligible, showing that spinach leaf tissue contains adenosine 5'-sulphatophosphate kinase activity. Some ester was synthesized in the absence of purified pyrophosphatase and ATP sulphurylase; this effect was attributed to the presence of ATP sulphurylase and pyrophosphatase activity in the crude spinach extract (Shaw & Anderson, 1971). The rate of synthesis of sulphate ester in standard incubation mixtures was proportional to the amount of crude extract added to the primary assay.

One problem associated with the standard assay was that the amount of sulphate ester synthesized was small in relation to the amount of endogenous sulphate ester. Addition of [^{35}S]sulphate instead of unlabelled sulphate minimized this problem and enhanced the sensitivity of the assay; studies with [^{35}S]sulphate confirmed the results with unlabelled sulphate.

Adenosine 5'-sulphatophosphate kinase activity of

Table 4. *Demonstration of adenosine 5'-sulphatophosphate kinase activity by the standard assay with purified yeast enzyme*

Purified yeast enzyme was used as the source of adenosine 5'-sulphatophosphate kinase. Assay conditions and the measurement of adenosine 5'-sulphatophosphate kinase activity were as described in the text except that some components of the standard incubation mixture were omitted in some treatments as described in the table.

Treatment		Adenosine 5'-sulphatophosphate kinase activity
Primary incubation	Secondary incubation	(pmol/min per mg of protein)
Complete mixture minus adenosine 5'-sulphatophosphate kinase	Complete mixture minus phenol sulphotransferase	0.1
Complete mixture minus adenosine 5'-sulphatophosphate kinase	Complete mixture	0.2
Complete mixture	Complete mixture	321

Table 5. *Adenosine 5'-sulphatophosphate kinase activity of crude extracts of spinach with and without substrates and coupling enzymes*

Crude extracts of spinach were used as the source of adenosine 5'-sulphatophosphate kinase. Assay conditions and the measurement of adenosine 5'-sulphatophosphate kinase activity by the formation of sulphate ester were as described in the text except that some components of the standard incubation were omitted in some treatments as described in the table.

Treatment		Adenosine 5'-sulphatophosphate kinase activity
Primary incubation	Secondary incubation	(pmol/min per mg of protein)
Complete mixture	Complete mixture	506
Complete mixture minus ATP	Complete mixture	7
Complete mixture minus sulphate	Complete mixture	38
Complete mixture minus spinach extract	Complete mixture	2
Complete mixture minus ATP sulphurylase	Complete mixture	190
Complete mixture minus pyrophosphatase	Complete mixture	213
Complete mixture	Complete mixture minus phenol sulphotransferase	1

spinach leaf tissue was associated with chloroplasts. Activity was recovered in the chloroplast supernatant solution when isolated chloroplasts were sonicated; only a small proportion of the activity was associated with chloroplast fragments (Table 3). Accordingly, isolated chloroplasts were used for studies on the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate.

Synthesis of adenosine 3'-phosphate 5'-sulphatophosphate by isolated chloroplasts

The synthesis of adenosine 3'-phosphate 5'-sulphatophosphate was studied by incubating chloroplasts in iso-osmotic medium with [^{35}S]sulphate and ATP (or [^{32}P]ATP and sulphate) in the presence of 3'-AMP and examining the labelled nucleotides by chromatography. [^{35}S]Sulphate was incorporated into adenosine 3'-phosphate 5'-sulphatophosphate, but the accumulation of adenosine 5'-[^{35}S]sulphatophosphate was negligible (Fig. 2). In the absence of 3'-AMP, however, only a small amount of adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate was detected and a large amount of adenosine 5'-[^{35}S]sulphatophosphate was found. The distribution of the ^{35}S label between the two nucleotides in the absence of 3'-AMP was similar to that described by Mercer & Thomas (1969). The adenosine 5'-sulphatophosphate-synthesizing system was not included in the reaction mixture, since this did not increase the yield of adenosine 3'-phosphate 5'-sulphatophosphate.

The optimum pH for the accumulation of adenosine 3'-phosphate 5'-sulphatophosphate was 8.5 (Fig. 5); the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate was studied at this pH value in all subsequent experiments. The rate of accumulation of adenosine 3'-phosphate 5'-sulphatophosphate by isolated chloroplasts was approximately constant for 2h; longer incubation times caused a net loss of the adenosine 3'-phosphate 5'-sulphatophosphate (Fig. 6). One explanation for the net loss of adenosine 3'-phosphate 5'-sulphatophosphate accumulated could be that chloroplasts contain enzymes which both synthesize and degrade adenosine 3'-phosphate 5'-sulphatophosphate and the synthetic enzymes are more labile. Purified spinach pyrophosphatase and ATP sulphurylase were stable at 35°C for 4h suggesting that adenosine 5'-sulphatophosphate kinase might be a labile enzyme.

The rate of accumulation of adenosine 3'-phosphate 5'-sulphatophosphate was very low in the absence of 3'-AMP; addition of 3'-CMP instead of 3'-AMP enhanced the accumulation of adenosine 3'-phosphate 5'-sulphatophosphate but was less effec-

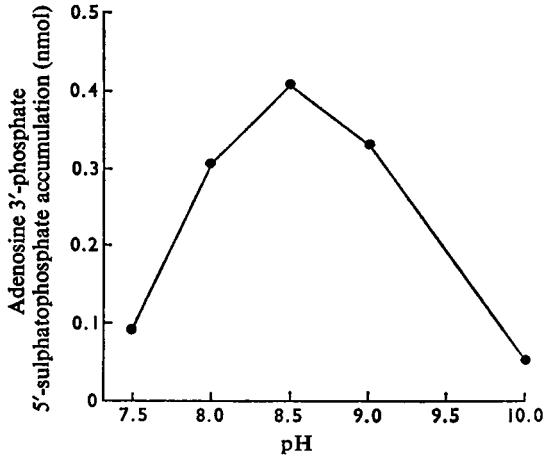


Fig. 5. *Effect of pH on the accumulation of adenosine 3'-phosphate 5'-sulphatophosphate by isolated chloroplasts*

The accumulation of adenosine 3'-phosphate 5'-sulphatophosphate was measured as described in the text except that Tris–HCl buffer, pH8.5, was replaced with the Tris–HCl buffers specified.

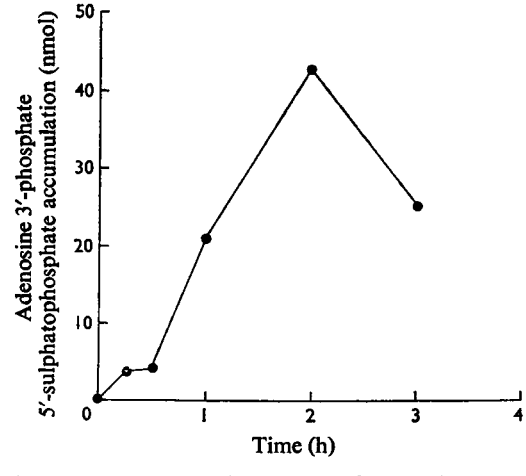


Fig. 6. *Time-course of the accumulation of adenosine 3'-phosphate 5'-sulphatophosphate by isolated chloroplasts*

The accumulation of adenosine 3'-phosphate 5'-sulphatophosphate was measured as described in the text except that the amount of adenosine 3'-phosphate 5'-sulphatophosphate was measured at the times specified.

tive than 3'-AMP (Table 6). The increased accumulation of adenosine 3'-phosphate 5'-sulphatophosphate in the presence of 3'-AMP or 3'-CMP cannot be attributed to the action of 3'-AMP or 3'-CMP, as alternative substrates, to inhibit the hydrolysis of adenosine 3'-phosphate 5'-sulphatophosphate by 3'-nucleotidase, since chloroplasts contain negligible 3'-nucleotidase activity (Table 3). Further, the apparent affinity of 3'-nucleotidase for 3'-AMP is low; increasing the concentration of 3'-AMP from 0.5mM to 5mM did not increase the accumulation of adenosine 3'-phosphate 5'-sulphatophosphate by an amount predicted from the substrate-concentration curve for 3'-nucleotidase (Fig. 3 and Table 6). Since neither pyrophosphatase nor ATP sulphurylase activities are affected by either 3'-AMP or 3'-CMP, this suggests either that adenosine 5'-sulphatophosphate kinase activity of spinach chloroplasts is regulated by 3'-AMP and 3'-CMP and possibly other 3'-nucleotides or that the metabolism of adenosine 3'-phosphate 5'-sulphatophosphate by chloroplasts is inhibited by 3'-AMP.

The incorporation of $[^{35}S]SO_4^{2-}$ into adenosine 3'-phosphate 5'-sulphatophosphate by isolated chloroplasts was increased by 50% when the chloroplasts were illuminated as described under 'Methods' for cystine biosynthesis.

Table 6. *Effect of 3'-AMP and 3'-CMP on the accumulation of adenosine 3'-phosphate 5'-sulphatophosphate by isolated spinach chloroplasts*

Incubation mixtures and the determination of adenosine 3'-phosphate 5'-sulphatophosphate were as described in the text except that the standard amount of 3'-AMP was omitted.

Additions to incubation mixtures	Adenosine 3'-phosphate 5'-sulphatophosphate synthesized (nmol/min per mg of chlorophyll)
No additions	0.01
3'-AMP (1.0μmol)	0.58
3'-AMP (10μmol)	0.63
3'-CMP (10μmol)	0.36

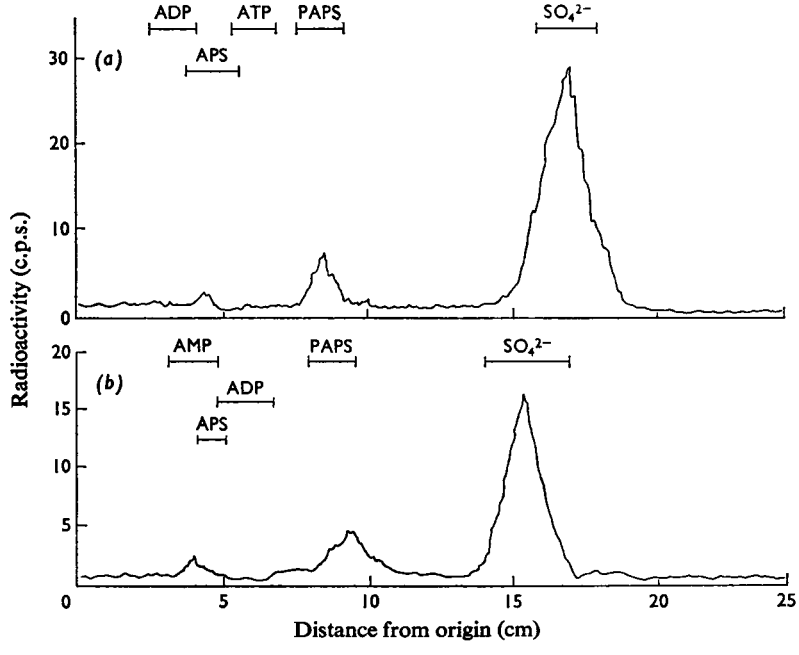


Fig. 7. *Electrophoresis of the ^{35}S-labelled nucleotide synthesized from $[^{35}S]$sulphate by isolated chloroplasts*

Chloroplasts were incubated with $[^{35}S]$sulphate and the ^{35}S-labelled nucleotides were isolated by chromatography in solvent I as described for the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate by isolated chloroplasts; the ^{35}S-labelled nucleotide with the R_F of adenosine 3'-phosphate 5'-sulphatophosphate in solvent I was eluted and subjected to electrophoresis. (*a*), Radioelectrophoretogram trace after electrophoresis in system I; (*b*) radioelectrophoretogram trace after electrophoresis in system II. Marker spots of ATP, ADP, AMP, adenosine 5'-sulphatophosphate (APS), sulphate and adenosine 3'-phosphate 5'-sulphatophosphate (PAPS) are shown.

Characterization of adenosine 3'-phosphate 5'-sulphatophosphate synthesized by isolated spinach chloroplasts

The ^{35}S-labelled nucleotide formed in reaction mixtures containing chloroplasts, ATP, [^{35}S]sulphate and 3'-AMP and which ran with an R_F similar to that of adenosine 3'-phosphate 5'-sulphatophosphate during paper chromatography in solvent I (Fig. 2) was eluted and subjected to electrophoresis. The mobility of the ^{35}S-labelled nucleotide was similar to that of adenosine 3'-phosphate 5'-sulphatophosphate in systems I and II (Fig. 7). The ^{35}S-labelled nucleotide with an R_F similar to that of adenosine 3'-phosphate 5'-sulphatophosphate in solvent I was tested as a substrate of purified phenol sulphotransferase with 2-naphthol as sulphate acceptor. [^{35}S]Sulphate ester was synthesized only in the presence of phenol sulphotransferase and 2-naphthol; [^{35}S]sulphate ester was not synthesized when adenosine 5'-[^{35}S]-sulphatophosphate or [^{35}S]sulphate was added to incubation mixtures instead of the ^{35}S-labelled product from chromatograms. Since rabbit liver phenol sulphotransferase is specific for adenosine 3'-phosphate 5'-sulphatophosphate (Roy & Trudinger, 1970)

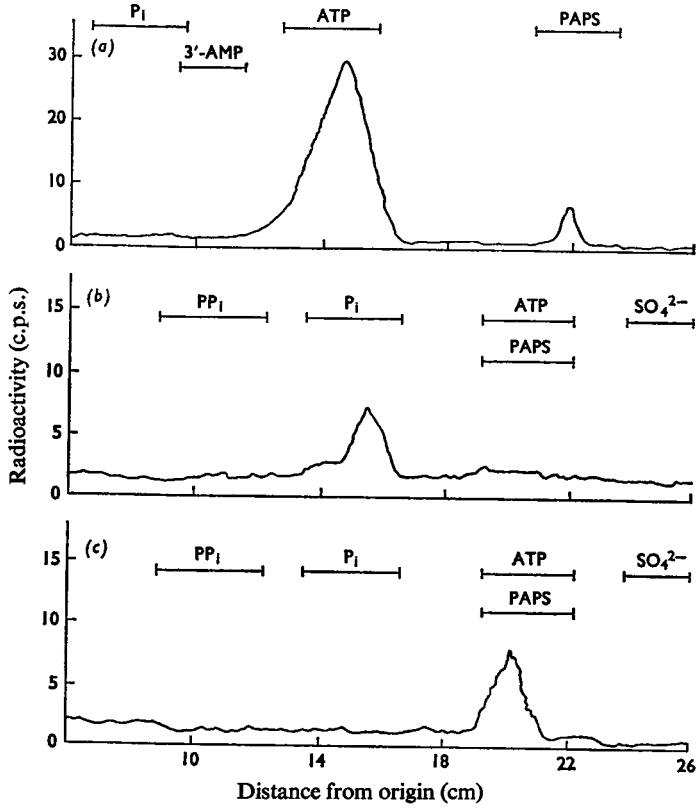


Fig. 8. *Radiochromatography of the ^{32}P-labelled nucleotide synthesized from [β,γ-^{32}P]ATP by isolated chloroplasts and the effect of purified 3'-nucleotidase on the isolated product*

Chloroplasts were incubated as described for the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate by isolated chloroplasts except that [β,γ-^{32}P]ATP and sulphate were used instead of [^{35}S]sulphate and ATP. The ^{32}P-labelled nucleotides were extracted by adsorption on charcoal, eluted and subjected to paper chromatography in solvent III (*a*). The ^{32}P-labelled nucleotide with an R_F similar to adenosine 3'-phosphate 5'-sulphatophosphate in solvent I was eluted and incubated with purified 3'-nucleotidase as described in the text; the products were subjected to rechromatography in solvent I (*b*), and the untreated ^{32}P-labelled product was also subjected to rechromatography in solvent I (*c*). Marker spots of ATP, ADP, 3'-AMP, P_i, PP_i, sulphate and adenosine 3'-phosphate 5'-sulphatophosphate (PAPS) are shown. In solvent I, adenosine 5'-sulphatophosphate ran with an R_F greater than that of sulphate.

Table 7. *Effect of* L-*serine and* 3'-*AMP on the incorporation of* [^{35}S]*sulphate into cystine by illuminated spinach chloroplasts*

Reaction mixtures for the study of sulphate incorporation were as described in the text except that the standard amounts of L-serine and 3'-AMP were omitted.

	Sulphate incorporated (pmol/min per mg of chlorophyll)
No additions	11
L-Serine (20 μmol)	36
3'-AMP (10 μmol)	147
L-Serine (20 μmol)+3'-AMP (10 μmol)	223
L-Serine (20 μmol)+3'-AMP (20 μmol)	391

it was concluded that the ^{35}S-labelled nucleotide synthesized by chloroplasts was adenosine 3'-phosphate 5'-sulphatophosphate.

The ^{35}S-labelled nucleotide with an R_F similar to that of adenosine 3'-phosphate 5'-sulphatophosphate in solvent I was subjected to ion-exchange chromatography. A ^{35}S-labelled compound was co-eluted with a compound with a u.v. spectrum characteristic of adenosine when the ion-exchange column was developed with 1 M-KCl; the elution pattern of the ^{35}S-labelled adenosine nucleotide was similar to the elution pattern for adenosine 3'-phosphate 5'-sulphatophosphate as described by Hodson & Schiff (1971). Since the ratio of sulphur to adenosine was 0.94 as determined from the specific radioactivity of the [^{35}S]sulphate and the extinction coefficient of adenosine, it was concluded that the ^{35}S-labelled nucleotide was adenosine 3'-phosphate 5'-sulphatophosphate. The purified adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate synthesized by isolated chloroplasts and the adenosine 3'-phosphate 5'-sulphatophosphate synthesized by the rat liver extract were examined in a Perkin–Elmer 257 i.r. spectrophotometer by using KCl discs; the absorption bands of the two samples were essentially the same.

When chloroplasts were incubated with [^{32}P]ATP and sulphate in the presence of 3'-AMP, a ^{32}P-labelled nucleotide was synthesized which ran with a similar R_F to that of adenosine 3'-phosphate 5'-sulphatophosphate in solvent III; the ^{32}P-labelled nucleotide was resolved from [^{32}P]ATP and unlabelled 3'-AMP (Fig. 8). The ^{32}P-labelled nucleotide was eluted and incubated with purified 3'-nucleotidase and the reaction products were examined by chromatography in solvent I. The ^{32}P label of the labelled nucleotide was quantitatively recovered as P_i after treatment with 3'-nucleotidase; adenosine 5'-sulpha[^{32}P]phosphate was not detected. Untreated ^{32}P-labelled nucleotide ran with an R_F similar to that of adenosine 3'-phosphate 5'-sulphatophosphate in solvent I (Fig. 8). The results show that adenosine 3'-[^{32}P]phosphate 5'-sulphatophosphate was synthesized.

Incorporation of sulphur from [^{35}S]*sulphate into cystine by isolated illuminated chloroplasts*

Cysteine was quantitatively oxidized to cystine under the experimental conditions (pH 8) used to study sulphate incorporation; cysteine was not recovered from reaction mixtures as the *N*-ethylmaleimide adduct. We are therefore unable to distinguish between the biosynthesis of cysteine and cystine.

The synthesis of cystine was enhanced by addition of L-serine (Table 7), suggesting that the enzyme serine sulphhydrase (L-serine hydro-lyase, EC 4.2.1.22), previously detected in spinach chloroplasts (Brüggemann *et al.*, 1962) and in beetroot (Ellis, 1963), is operative in cystine biosynthesis in spinach chloroplasts. The enhanced synthesis of cystine in the presence of L-serine also suggests that chloroplasts reduce sulphate to sulphide. The synthesis of cystine by spinach chloroplasts was enhanced by 3'-AMP, in both the presence and the absence of serine (Table 7).

Discussion

The demonstration of adenosine 5'-sulphatophosphate kinase activity and the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate by isolated spinach chloroplasts confirms and extends the results of Mercer & Thomas (1969). The adenosine 3'-phosphate 5'-sulphatophosphate synthesized was characterized enzymically by the formation of naphthol 2-sulphate in the presence of purified phenol sulphotransferase and by the liberation of P_i in the presence of purified 3'-nucleotidase (Fig. 8). The formation of adenosine 3'-phosphate 5'-sulphatophosphate was confirmed by electrophoresis (Fig. 7), ion-exchange chromatography and i.r. spectrophotometry.

The synthesis of adenosine 3'-phosphate 5'-sulphatophosphate and the activity of adenosine 5'-sulphatophosphate kinase in both crude extracts and isolated chloroplasts was negligible in the absence of 3'-AMP (Table 6), though adenosine 5'-sulphatophosphate was synthesized; this might explain why many previous authors have been unable to demonstrate the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate in crude extracts and chloroplasts from higher plants. Crude extracts of spinach contain a powerful F^--insensitive 3'-nucleotidase (Table 1 and Fig. 3) that hydrolyses adenosine 3'-phosphate 5'-sulphatophosphate, but 3'-nucleotidase is virtually absent from isolated chloroplasts (Table 3). The

increased synthesis of adenosine 3'-phosphate 5'-sulphatophosphate by isolated chloroplasts in the presence of 3'-AMP cannot therefore be attributed to 3'-AMP acting as an alternative substrate to inhibit the hydrolysis of adenosine 3'-phosphate 5'-sulphatophosphate by 3'-nucleotidase. Even the inhibition of the metabolism of adenosine 3'-phosphate 5'-sulphatophosphate by crude spinach extracts (Table 2) could not be fully accounted for by 3'-AMP acting as an alternative substrate of 3'-nucleotidase. We conclude that the effect of 3'-AMP on the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate by spinach chloroplasts differs from the explanation for a similar effect in extracts of *S. pullorum* (Kline & Schoenhard, 1968). Since neither Mg^{2+}-dependent pyrophosphatase nor ATP sulphurylase from spinach are activated by 3'-AMP, this suggests that the most likely explanation for the effect of 3'-AMP on the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate is that 3'-AMP regulates spinach leaf adenosine 5'-sulphatophosphate kinase activity. We cannot, however, discount the possibility that 3'-AMP might inhibit other enzymes such as ADP sulphurylase (Burnell & Anderson, 1973) that metabolize intermediates in the biosynthesis of adenosine 3'-phosphate 5'-sulphatophosphate, or other enzymes such as sulphatases and non-specific phosphatases that hydrolyse adenosine 3'-phosphate 5'-sulphatophosphate (Roy & Trudinger, 1970). The hydrolysis of adenosine 3'-phosphate 5'-sulphatophosphate by the various plant phosphatases, including chloroplast pyrophosphatase, has not been studied.

The methods used to demonstrate the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate and to demonstrate adenosine 5'-sulphatophosphate kinase activity are not suitable for a direct study of the properties of adenosine 5'-sulphatophosphate kinase, since the methods involve coupled enzyme systems involving at least two other enzymes in addition to adenosine 5'-sulphatophosphate kinase. Some information on the properties of adenosine 5'-sulphatophosphate kinase, however, can be inferred from studies on the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate by isolated chloroplasts, since certain features of the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate cannot be attributed to pyrophosphatase and ATP sulphurylase activities. This approach suggests that adenosine 5'-sulphatophosphate kinase is labile (Fig. 6) and is regulated by 3'-AMP. The pH optimum for the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate by isolated chloroplasts (Fig. 5) presumably represents the optimum for the coupled enzyme system consisting of ATP sulphurylase, pyrophosphatase and adenosine 5'-sulphatophosphate kinase. The pH optimum is similar to that for spinach leaf ATP sulphurylase (Shaw & Anderson, 1972) and spinach leaf pyrophosphatase (El-Badry & Bassham, 1970). The pH optimum for the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate by isolated chloroplasts is also similar to that reported for adenosine 5'-sulphatophosphate kinase of yeast (Robbins & Lippmann, 1958) suggesting that the pH optimum for the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate by isolated chloroplasts reflects the pH optimum of spinach chloroplast adenosine 5'-sulphatophosphate kinase.

Incorporation of sulphate into cystine/cysteine in isolated chloroplasts was enhanced by 3'-AMP (Table 6). The possibility that 3'-AMP might act as an alternative substrate for an enzyme(s) that hydrolyses adenosine 3'-phosphate 5'-sulphatophosphate during the incorporation of sulphur from sulphate into cystine/cysteine has already been discounted. The increased incorporation of sulphur from sulphate into cystine/cysteine in the presence of 3'-AMP is consistent with the hypothesis that adenosine 5'-sulphatophosphate kinase is regulated by 3'-AMP. Although the presence of adenosine 5'-sulphatophosphate kinase activity in chloroplasts does not in itself imply that adenosine 3'-phosphate 5'-sulphatophosphate is an intermediate in sulphate reduction, the enhanced incorporation of sulphur from sulphate into cystine/cysteine by spinach chloroplasts in the presence of 3'-AMP suggests that it is unlikely that adenosine 5'-sulphatophosphate is the activated form of sulphate, as has been suggested for *Chlorella* (Schmidt, 1972). If adenosine 5'-sulphatophosphate was the activated form, addition of 3'-AMP should inhibit the formation of adenosine 5'-sulphatophosphate from adenosine 3'-phosphate 5'-sulphatophosphate by 3'-nucleotidase activity and stimulate the formation of adenosine 3'-phosphate 5'-sulphatophosphate from adenosine 5'-sulphatophosphate by adenosine 5'-sulphatophosphate kinase; both effects would cause decreased cysteine.

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