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ATP-SULPHURYLASE IN SPINACH LEAVES

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SUMMARY

ATP-sulphurylase (adenosine triphosphate-sulfate adenylyltransferase, EC 2.7.7.4) in spinach leaves was found to be associated with chloroplast fractions prepared in non-aqueous solvents, since the enzyme leached out of the chloroplasts during their isolation in isotonic buffer systems.

A new assay using the luciferin-luciferase enzyme system of the firefly, *Photinus pyralis*, was used to follow a 53-fold purification of the enzyme from extracts of spinach leaves. The K_m values for adenosine-5′-phosphosulfate (APS) and pyrophosphate for ATP-sulphurylase determined by the firefly assay are 0.47 μM and 3.0 μM, respectively.

^{35}S]APS was formed from $^{35}SO_4^{2-}$ in extracts of higher plants (*Spinacea oleracea*, *Lathyrus odoratus* and *Avena sativa*). ^{35}S-labeled 3′-phosphoadenosine-5′-phosphosulfate ([^{35}S]PAPS) was not detected in these plant extracts; however, both ([^{35}S]APS and ([^{35}S]PAPS) were found in extracts of *Chlorella vulgaris*. In fragmented chloroplasts from spinach leaves, about 17% of [^{35}S]APS added was converted to sulphate, but ^{35}S APS was not reduced in these preparations even in the presence of reduced glutathione.

INTRODUCTION

ATP-sulphurylase (adenosine triphosphate sulphate adenylyltransferase, EC 2.7.7.4) catalyses the activation of sulphate by ATP forming adenosine-5′-phosphosulfate (APS) and liberating pyrophosphate. This reaction, first reported in yeast and liver[1,2], is the first step in the metabolism of sulphate in both assimilatory and dissimilatory sulphate reducing organisms. A second enzyme, APS-kinase (adenosine triphosphate adenylyl sulphate 3′-phosphotransferase, EC 2.7.1.25) further activates APS to 3′-phosphoadenosine-5′-phosphosulfate (PAPS) in the assimilatory system. On the other hand, PAPS has not been detected in dissimilatory sulphate reducing bacteria[3].

Abbreviations: APS, adenosine-5′-phosphosulphate; PAPS, 3′-phosphoadenosine-5′-phosphosulphate.

The enzymes ATP-sulphurylase and APS-kinase from yeast were purified and characterized by ROBBINS AND LIPMANN[4,5] and WILSON AND BANDURSKI[6], while the ATP-sulphurylase of *Desulfovibrio desulfuricans* was studied by AKAGI AND CAMPBELL[7].

Although ASAHI[8] demonstrated that ATP-sulphurylase is present in higher plants, this activity was not accurately determined because of the high ATPase levels which interfered with the molybdolysis assay for the enzyme. ^{35}S PAPS was not detected although the thermodynamic equilibrium of the APS-kinase reaction greatly favours its formation. Recently MERCER AND THOMAS[10] claimed to have detected PAPS in chloroplast preparations from French bean (*Phascolus vulgaris*) and maize (*Zea mays*). In a heavily labelled experiment (100 μC $^{35}SO_4^{2-}$) they detected a relatively small activity in an area of their chromatogram where ^{35}S PAPS is reputed to run. Since this was not unequivocally identified as the nucleotide, it is still an open question whether PAPS is formed in these plants.

In a green alga, *Chlorella pyrenoidosa*, PAPS has been identified as an intermediate during the reduction of sulphate to sulphite[11].

This paper describes the purification of ATP-sulphurylase from spinach leaves using a new assay procedure based on the luciferin-luciferase enzyme reaction of the firefly for determining ATP formed from APS and pyrophosphate.

MATERIALS AND METHODS

Chemicals

Creatine phosphokinase (1 mg transforms 75 μmoles substrate/min at pH 7.4 and 30°), glucose-6-phosphate dehydrogenase, firefly lanterns, AMP, ADP, NADPH, bovine serum albumin and glucose 6-phosphate were purchased from Sigma Chemical Co., Mo., U.S.A.; inorganic pyrophosphatase (from *Aspergillus oryzae*, Lot 900621) and ATP were obtained from Calbiochem, Calif., U.S.A. Radiochemicals were supplied by the Radiochemical Centre, Amersham, England. Creatine phosphate was prepared from creatine (British Drug Houses, Poole, England) by the method of ENNOR[12]. APS was synthesized from pyridine-sulphur trioxide and AMP by the procedure of BADDILEY *et al.*[13]. ^{35}S PAPS was prepared by the yeast enzyme system of WILSON AND BANDURSKI[6]. All other chemicals were of analytical grade.

Protein was determined by the Folin method as modified by LOWRY *et al.*[14].

Extraction of chloroplasts from spinach leaves

Chloroplasts were prepared from spinach leaves, extracted with isotonic 0.4 M sucrose in 0.05 M Tris HCl buffer (pH 7.5) as described by JAGENDORF AND AVRON[15]. Vein-free leaves of plants (10 g), obtained fresh from the market, were ground in a mortar and pestle using 20 ml of cold buffer and about 3 g of precooled sand. The homogenate was filtered through a double layer of cheese cloth and centrifuged at 200 $\times$ *g* to remove the cell debris. Chloroplasts were then sedimented by centrifuging at 1000 $\times$ *g* for 7 min.

When leaves were extracted with buffers containing 0.33 M sorbitol, the procedure of JENSEN AND BASSHAM[16] was followed except that the buffer used was 0.05 M Tris HCl (pH 6.1). About 10 g of washed leaves were homogenized in 30 ml of the extracting buffer for 5 sec at high speed in a Sorvall Omnimixer. The extract, filtered

through six layers of cheese cloth, was centrifuged at 2000 × *g* for 50 sec to sediment the chloroplasts.

For the non-aqueous extraction of spinach leaves, the following procedure of STOCKING[17] was followed: The pale green flakes (0.2 g) obtained by freeze drying 5 g of fresh leaves were ground in 20 ml of *n*-hexane–carbon tetrachloride (69:31, by vol; s.g. 1.32). This suspension was centrifuged at 12 000 × *g* for 15 min to remove large cell debris. An equal volume of *n*-hexane was added to the supernatant fraction and the chloroplasts sedimented by centrifuging at 1000 × *g* for 5 min. All the fractions were dried under vacuum and then extracted with 1 ml of 0.05 M Tris–HCl buffer (pH 7.5). Undissolved material was removed by centrifuging at 20 000 × *g* for 15 min.

Radioassay for ATP-sulphurylase

ATP-sulphurylase was assayed by following the incorporation of $^{35}SO_4^{2-}$ into [^{35}S]APS. To counteract the effect of ATPases, an ATP-regenerating system consisting of creatine phosphate and creatine phospho-kinase was included. The reaction mixture contained (mM): Tris HCl buffer (pH 7.5), 50; $MgCl_2$, 5; disodium-EDTA, 0.3; ATP, 10; creatine phospho-kinase, 0.1 mg/ml; creatine phosphate, 15; $Na_2{}^{35}SO_4$, 10 (16 μC/ml); inorganic pyrophosphatase, 10 μg/ml, and enzyme 0.025 ml, in a final volume of 0.1 ml. The mixture was incubated at 30° for 15 min. The reaction was terminated by boiling for 90 sec, and 0.025-ml aliquots were applied to the origin of a Whatman 3 MM chromatography paper prior to electrophoresis in 0.1 M sodium citrate buffer for 1 h at 1500 V. The apparatus used was that described by TATE[18] for routine electrophoresis. [^{35}S]APS on the electrophoretograms was detected by cutting the dried paper into sections (1 cm × 2 cm) and counting in a toluene solution (0.5 ml) of 2,5-diphenyloxazole (PPO) (3.0 g/l) and 1,4-bis-(5-phenyloxazolyl-2)benzene (POPOP) (0.3 g/l) in a Packard Tri-Carb liquid scintillation spectrometer (Model 3375).

Preparation of the luciferin-luciferase enzyme system

The luciferin-luciferase extract was prepared by the method of STANLEY AND WILLIAMS[19], as modified by BALHARRY AND NICHOLAS[20]. Four dessicated firefly lanterns were homogenized in the agate mortar of a Wig-L-Bug dental homogenizer (Crescent Dental Manufacturing Co., Ill., U.S.A.) for 1 min with 0.75 ml of cold 50 mM sodium arsenate buffer (pH 7.3). This homogenate, diluted to 2 ml with the buffer, was centrifuged at 20 000 × *g* for 30 min at 2°. About 3 ml of cold distilled water were added to produce the required dilution. This extract was stable for about 48 h at 0°.

Firefly assay for ATP-sulphurylase[20]

The reaction vial contained 1 ml of 10 mM sodium phosphate buffer (pH 7.3); 50 mM $MgCl_2$; 1 ml of 50 mM sodium arsenate buffer (pH 7.3), neutralized with HCl; and 1 ml of distilled water. Sodium pyrophosphate (0.02 ml, 30 nmole) and APS (0.02 ml, 1.5 nmole) were added to the reaction mixture, which was then incubated at 20° in the Packard Tri-Carb liquid scintillation spectrometer.

The spectrometer was set up as follows: The circuit was switched out-of-coincidence so that the two photomultipliers detected individual light flashes (photons). The single channel used was set with gain at 100% and the two discriminators at 72 and 78, respectively. All counts were recorded for 0.1 min at 20°.

After initiating the enzyme reaction with 0.1 ml of firefly extract, the vial was shaken and quickly placed in the well of the spectrometer. Counting was started 15 sec later, when 6 counts each of 0.1 min were recorded at intervals of 0.3 min. Then a spinach leaf extract (0.02 ml), containing ATP-sulphurylase (producing ~ 50 pmoles ATP/min), was added to the vial 2 min after adding the firefly extract, and a further 6 counts, each of 0.1 min, were recorded at intervals of 0.3 min. After a further 2 min, an internal standard of ATP (100 pmoles in 0.1 ml) was added to the vial and the same counting sequence repeated.

In order to assay spinach extracts containing ATP-sulphurylase producing ~ 50 pmoles ATP/min, the additions of spinach extract and ATP were reversed. Thus, 100 pmoles ATP in 0.1 ml (internal standard) was added to the vial 2 min after starting the reaction with the firefly enzyme, and after a further 2 min, the leaf extract (0.02 ml) was added. Each of these additions was followed by the same counting sequence.

DEAE-cellulose and Sephadex G-100 column chromatography

A DEAE-cellulose (DE-11) column (1.6 cm × 14 cm) was equilibrated with 0.05 M Tris HCl buffer (pH 7.5). After the sample was loaded onto the column, the ATP-sulphurylase was eluted between 0.18 and 0.23 M Tris HCl (pH 7.5) using a linear gradient. The Sephadex column (1.6 cm × 44 cm) was equilibrated with 0.05 M Tris HCl buffer (pH 7.5) and the enzyme was eluted with the same buffer.

RESULTS

Formation of adenosine-5'-phosphosulphate

The products, formed on incubating a homogenate of spinach leaves in 0.05 M Tris-HCl (pH 7.5) with $^{35}SO_4^{2-}$ and ATP, were separated by electrophoresis on Whatman 3 MM paper in 0.1 M sodium citrate (pH 5.0) for 1 h at 1500 V. The distribution of labelled compounds along the electrophoretogram was detected by counting sections of the 3 MM paper in a Packard liquid scintillation spectrometer as shown in Fig. 1. The only labelled compound formed behaved as did [^{35}S]APS prepared either enzymically from yeast[2] or chemically from pyridine-sulphur trioxide and AMP[13].

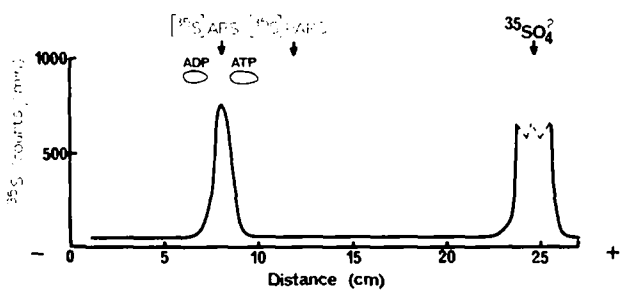


Fig. 1. Scan of an electrophoretogram of products formed on incubating $^{35}SO_4^{2-}$ and ATP with a fraction of spinach leaf extracted with 0.05 M Tris HCl (pH 7.5) containing ATP-sulphurylase (see MATERIALS AND METHODS). The reaction mixture (as for the radioassay in MATERIALS AND METHODS) was incubated at 30° for 1 h, then boiled for 90 sec. The products contained in 0.025-ml aliquots were separated by high voltage electrophoresis on Whatman 3 MM paper in 0.1 M sodium citrate buffer (pH 5.0) at 1500 V for 1 h. Segments of the electrophoretogram were analysed by scintillation counting (see MATERIALS AND METHODS).

High voltage electrophoresis in a variety of buffer systems (*e.g.* 0.1 M sodium citrate (pH 5.0), 0.1 M potassium citrate (pH 3.5), and 0.02 M sodium phosphate (pH 7.0)) confirmed that only [^{35}S]APS was present. Thus, 1600 counts/min were detected in 5 μM [^{35}S]APS formed but there was no evidence for ^{35}S PAPS formation (background 45 ± 5 counts/min).

Distribution of ATP-sulphurylase

Enzyme activity, measured by the incorporation of $^{35}SO_4^{2-}$ into ^{35}S APS, was followed during the isolation of chloroplasts from spinach leaves extracted with (a) isotonic 0.4 M sucrose in 0.05 M Tris HCl buffer (pH 7.5) and (b) *n*-hexane and carbon tetrachloride. Chloroplasts prepared in isotonic sucrose buffer contained about half the chlorophyll of the homogenate (Table I) and only 16% of the enzyme activity.

TABLE I

ATP-SULPHURYLASE IN CHLOROPLASTS ISOLATED IN ISOTONIC BUFFER

Leaves (10 g) were extracted with isotonic 0.4 M sucrose in 0.05 M Tris HCl (pH 7.5) (see MATERIALS AND METHODS). A unit of enzyme activity is the amount which produces 1 nmole of [^{35}S]APS/h in the radioassay (see MATERIALS AND METHODS). Chlorophyll was determined by the method of ARNON[21].

Fraction	*ATP-sulphurylase*		*Chlorophyll (mg) B*	*Ratio A/B*
	Total A	*Recovery (%)*		
1. Homogenate in isotonic buffer	3100	100	3.1	1000
2. Chloroplasts, sedimented by centrifuging (1) at 1000 × *g* for 7 min	560	16.2	1.5	370
3. Washed chloroplasts: (2) washed with isotonic buffer and centrifuged at 1000 × *g* for 7 min	81	1.4	1.4	58
4. Chloroplast extracts: (2) extracted with 0.05 M Tris–HCl (pH 7.5) for 45 min and centrifuged at 20 000 × *g* for 30 min to remove cell-debris	360	11.6	0	0

When these chloroplasts were washed with the isotonic sucrose buffer, only 2.4% of the enzyme activity of the homogenate was retained in these organelles. However, the specific activity of the enzyme extracted from the unwashed chloroplasts (Fraction 4, Table I) was 124 units/mg protein compared with 14.3 units/mg in the leaf homogenate.

Fractions prepared by extracting freeze dried spinach leaves with *n*-hexane and carbon tetrachloride were dried under vacuum and extracted with 0.05 M Tris HCl buffer (pH 7.5) prior to determining ATP-sulphurylase activity (Table II). The crude extract contained enzyme of lower specific activity (4.0 units/mg protein) than that of the isotonic buffer; however, under these conditions 44% of the enzyme activity and 48% of the total chlorophyll were recovered in the chloroplasts (Fraction 2, Table II). The ratio of total enzyme activity to chlorophyll in this fraction was 109, compared with 117 in the crude extract. Enzyme activity decreased after prolonged treatment with these solvents (Fraction 3).

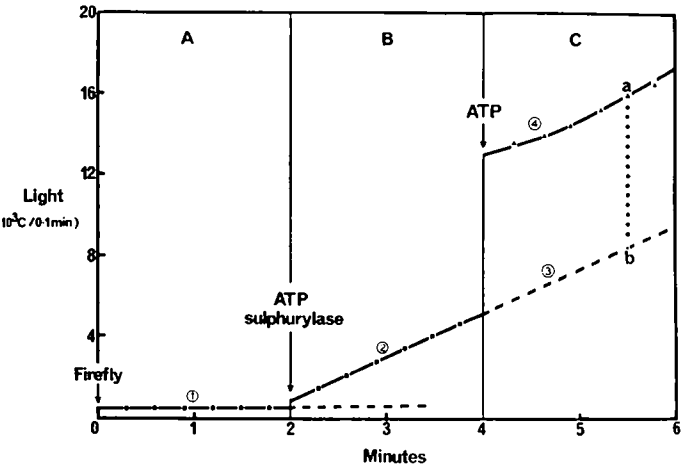


Fig. 2. Determination of ATP-sulphurylase by the firefly assay. The reaction vial contained (in μmoles): sodium phosphate buffer (pH 7.3), 10; $MgCl_2$, 50; and sodium arsenate buffer (pH 7.3), 50 in a volume of 3 ml, sodium pyrophosphate (30 nmoles), and APS (1.5 nmoles) were added and the reaction initiated by adding 0.1 ml firefly extract at 25°. Spinach ATP-sulphurylase (0.02 ml) and ATP (100 pmoles) were added as indicated. Light flashes were recorded in a Packard Tricarb liquid scintillation spectrometer (Model 3375). ATP-sulphurylase activity was calculated by a computer programme. An enzyme unit is the amount producing 1 nmole ATP/min under these conditions. Segment A. (1) ●—●, ATP-sulphurylase activity of the firefly extract (2 pmoles ATP formed/min). The line fitted to these points has been extrapolated into Segment B. Segment B. (2) ■— ■, the combined ATP-sulphurylase activities of the firefly and spinach extracts (35 pmoles ATP formed/min). The line fitted to these points has been extrapolated into Segment C. (Line 3) Segment C. (Line 4) /\ /\, a cubic function, with the inflexion at 6 min is fitted to the data so that the light produced/0.1 min by the ATP internal standard at 5.5 min can be determined 'a *minus* b'.

Firefly assay for ATP-sulphurylase

Since the activity of ATP-sulphurylase determined by the radioassay procedure is low in crude leaf extract, and the procedure is time consuming, a method has been devised to measure ATP formed in the reverse reaction of the enzyme (ATP and SO_4^{2-} formed from APS and pyrophosphate). The production of ATP was followed by linking the reaction to the luciferin-luciferase enzyme system of the firefly, *Photinus pyralis*[20]. The light emitted, measured in a Packard Tri-Carb liquid scintillation spectrometer, is related to the amounts of ATP present.

The assay of ATP formed from APS by the ATP-sulphurylase in spinach extracts by the firefly system is illustrated in Fig. 2. The enzyme reaction was initiated by adding 0.1 ml of the firefly extract to a reaction mixture containing APS and pyrophosphate (Segment A). Light produced in this segment resulted from a low ATP-sulphurylase activity in the firefly extract. A homogenate of spinach leaves (0.02 ml of Fraction I, Table I) containing ATP-sulphurylase (50 pmoles ATP formed/min) was added at 2 min (Segment B); thus, the rate of light production in this segment is a measure of the combined ATP-sulphurylases of the firefly and spinach extracts. An internal standard of ATP (100 pmoles) was added at 4 min (Segment C), resulting in an increase in light emission.

A computer programme has been developed to analyse the results of this assay. Thus, straight lines were fitted to the data of Segments A and B (Lines 1 and 2 in Fig. 2), whereas that of Segment C (Line 4) was fitted with a cubic function so that

the point of inflexion of the curve was fixed at 6 min. The line (2) in Segment B, representing the formation of ATP by the combined ATP-sulphurylases of the firefly and spinach extracts, was extrapolated into Segment C (Line 3). Then the light production by the ATP internal standard was determined at 5.5 min in Segment C – that is, 1.5 min after adding the ATP. The difference at 5.5 min in Segment C between (a) on the cubic curve, Line 4, (15 916 counts/0.1 min) and (b) on Line 3 (8277 counts/0.1 min) was the light produced by 100 pmoles of ATP (7639 counts/0.1 min). The slopes of Line 1 in Segment A (143 counts/0.1 min per min) and of Line 2 in Segment B (2125 counts/0.1 min per min) were divided by the value for the ATP internal standard. Thus, from Line 1 in Segment A, the ATP-sulphurylase activity of the firefly extract is 1.9 pmoles ATP formed/min. This value, subtracted from the activity determined from Line 2 in Segment B (27.8 pmoles ATP formed/min), yields 25.9 pmoles ATP formed/min; this is the activity of the ATP-sulphurylase in 0.02 ml of the spinach extract.

TABLE II

ATP-SULPHURYLASE IN CHLOROPLASTS ISOLATED IN NON-AQUEOUS SOLVENTS

Freeze-dried leaves (0.2 g) were extracted with *n*-hexane–carbon tetrachloride (69:31 by vol; s.g. 1.32) as described in MATERIALS AND METHODS. Fractions prepared as in the table, were dried under vacuum and extracted with 0.05 M Tris HCl (pH 7.5). The supernatant fractions obtained after centrifuging at 20 000 × *g* for 15 min were assayed for enzyme activity by following the incorporation of $^{35}SO_4^{2-}$ (see MATERIALS AND METHODS). A unit of enzyme activity is the amount which produces 1 nmole of ^{35}S-APS/h. Chlorophyll was determined by the method of ARNON[21].

Fraction	*ATP-sulphurylase*		*Chlorophyll (mg) B*	*Ratio A/B*
	Total units A	*Recovery (%)*		
1. Crude extract in *n*-hexane-carbon tetrachloride (69:31, v/v; s.g. 1.32)	520	100	4.5	117
2. Chloroplasts: (1) centrifuged at 12 000 × *g* for 15 min. An equal volume of *n*-hexane added to the supernatant; centrifuged at 1000 × *g* for 5 min	229	44	2.1	109
3. Washed chloroplasts: (2) resuspended in both solvents (s.g. 1.32) and centrifuged as in (2)	74	14	1.1	67

To measure ATP-sulphurylase activities > 50 pmoles ATP formed/min, the same procedure was followed except that the ATP internal standard was added at 2 min after initiating the reaction with the firefly extract and after a further 2 min, the enzyme from spinach was added. ATP-sulphurylase activities in the range producing 2–300 pmoles/min were readily determined by these procedures. The percentage errors were usually less than 3% (increasing to about 10% for activities < 10 pmoles ATP/min.

Purification of the enzyme

More active preparations of ATP-sulphurylase were obtained from spinach leaves extracted with hypotonic 0.05 M Tris HCl buffer (pH 7.5), which lysed the chloroplasts. Vein-free leaves (25 g) were ground in a mortar and pestle using 35 ml

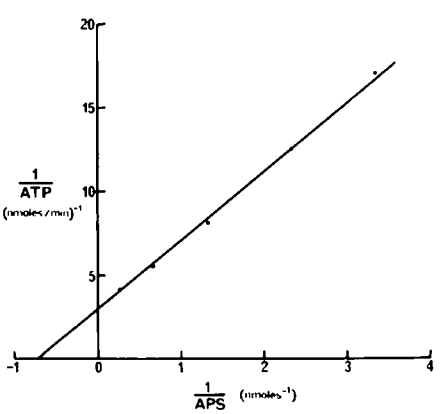
TABLE III

PURIFICATION OF ATP-SULPHURYLASE

Leaves (25 g) were extracted with 0.05 M Tris HCl (pH 7.5). Enzyme activity was determined by the firefly assay (see MATERIALS AND METHODS). One unit is the amount producing 1 nmole ATP/min. Enzyme unit: nmoles ATP/min (firefly assay). Specific activity: nmoles ATP/min per mg protein.

Fraction	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg protein)*	*Fold purification*	*Recovery (%)*
1. Homogenate in 0.05 M Tris HCl (pH 7.5)	686	760	1.11	1.0	100
2. Supernatant fraction left after centrifuging (1) at 25 000 × *g* for 30 min	309	726	2.35	2.1	96
3. Supernatant fraction left after precipitating protein from 0 to 43% $(NH_4)_2SO_4$ saturation, dialysed against 0.05 M Tris-HCl (pH 7.5) for 24 h	171	638	3.74	3.4	84
4. (3) applied to a DEAE-cellulose column, eluted by a linear gradient of Tris-HCl (pH 7.5) (0.18–0.23 M)	21	255	12.2	11.0	29.5
5. (4) concentrated by pressure dialysis, then loaded onto Sephadex G-100 column, eluted with 0.05 M Tris HCl (pH 7.5)	2.3	130	58.8	53.0	17.9

of cold 0.05 M Tris HCl buffer (pH 7.5) and about 3 g of precooled sand. The supernatant fraction, left after centrifuging the homogenate at 200 × *g* for 1 min, was used as the source of the enzyme. The firefly assay was used to determine enzyme activity during purification by ammonium sulphate fractionation and chromatography on DEAE cellulose and Sephadex G-100, as shown in Table III.



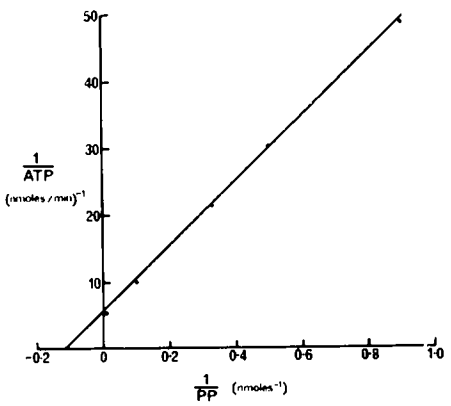


Fig. 3. Reciprocal plots of the activity of ATP-sulphurylase from spinach leaves and APS concentration (firefly assay). The reaction conditions were the same as in Fig. 2, except that the amount of APS in the reaction mixture was varied as indicated. ATP sulphurylase (Fraction 4, Table III) 0.015 mg was used.

Fig. 4. Reciprocal plots of the activity of ATP-sulphurylase from spinach leaves and pyrophosphate concentration (firefly assay). The reaction conditions were the same as in Fig. 2, except that the amount of pyrophosphate in the reaction mixture was varied as indicated. ATP-sulphurylase (Fraction 4, Table III) 0.015 mg was used.

Effect of substrate concentration in the firefly assay

The rate of production of ATP was measured at various concentrations of APS and pyrophosphate using a partially purified ATP-sulphurylase from spinach (Fraction 4, Table III, 0.015 mg protein). The results, plotted in Figs. 3 and 4 according to LINEWEAVER AND BURK[22] ($1/v$ *versus* $1/S$), show that in each case there is a linear relation between ATP formed and the substrates. The calculated K_m values are 0.47 μM for APS and 3.0 μM for pyrophosphate.

^{35}S-nucleotide formation in extracts of higher plants and Chlorella

Shoots of the sweet pea (*Lathyrus odoratus*) and root tips of seedling of oats (*Avena sativa*) were extracted in 0.05 M Tris-HCl buffer (pH 7.5) by the same method used to prepare the homogenate of spinach leaves (Fraction 1, Table III). A cell-free extract of *Chlorella vulgaris* was prepared as described in Table IV. The supernatant

TABLE IV

COMPARISON OF THE ^{35}S-NUCLEOTIDES IN *Spinaceae oleracea*, *Lathyrus odoratus*, *Avena sativa* AND *Chlorella vulgaris*

Plant tissues were extracted with 0.05 M Tris–HCl (pH 7.5). Cells of *Chlorella vulgaris* were washed 3 times in 10 vol. of 0.05 M Tris–HCl (pH 7.5), each followed by centrifugation at 20 000 × *g* for 10 min. They were then suspended in 4 vol. of buffer, disrupted in a French pressure cell (20 000 lb/inch^2) and centrifuged at 20 000 × *g* for 30 min at 2°. The supernatant fractions were incubated with $^{35}SO_4^{2-}$ (see MATERIALS AND METHODS); the products, including sulphur nucleotides were separated by high voltage electrophoresis on Whatman 3 MM paper in 0.1 M sodium citrate (pH 5.0) at 1500 V for 1 h, and then determined by liquid scintillation counting as described in MATERIALS AND METHODS. Protein was determined by the method of LOWRY *et al.*[11].

Material	*Total protein (mg)*	*[^{35}S]APS produced (nmoles/ml)*	*[^{35}S]PAPS produced (nmoles/ml)*	*Specific activity of ATP-sulphurylase (nmoles [^{35}S]APS produced/h per mg protein*
Shoots of the sweet pea (0.5 g) *(Lathyrus odoratus)*	10	10.3		1.03
Root tips of oat seedlings (0.5 g) *(Avena sativa)*	4.2	9.7		2.30
Spinach leaves (10 g) *(Spinaceae oleracea)*	14.0	10.0	-	14.3
Chlorella vulgaris cell-free extract	1.1	4.6	11.2	42.5

fractions after centrifuging the extracts at 20 000 × *g* for 30 min were incubated in reaction mixtures containing ATP, $^{35}SO_4^{2-}$ and pyrophosphatase, as in the radioassay procedure. Labelled sulphur nucleotides, separated by high voltage electrophoresis, were detected by counting sections of the paper in a liquid scintillation spectrometer. [^{35}S]APS was formed in both the pea and oat extracts (Table IV), equivalent to specific activities of 1.0 and 2.3 nmoles [^{35}S]APS/h per mg protein, respectively, compared with 14.3 nmoles [^{35}S]APS/h per mg protein in a similar fraction from spinach leaves. [^{35}S]PAPS was not detected in extracts of these plants. However, both [^{35}S]APS (4.6 nmoles/ml) and [^{35}S]PAPS (11.2 nmoles/ml) were produced in extracts of *Chlorella vulgaris* after a 15 min incubation in the reaction mixture. The

amount of ^{35}S-APS formed by ATP-sulphurylase was calculated from the sum of the amounts of ^{35}S-APS and ^{35}S-PAPS formed; thus, the specific activity was 42.5 nmoles ^{35}S-APS formed/h per mg protein.

Metabolism of ^{35}S-APS by fragmented chloroplasts

Spinach leaves were extracted with 0.33 M sorbitol in 0.05 M Tris·HCl buffer (pH 6.1) and the chloroplast fraction was isolated from the homogenate by differential centrifugation (see MATERIALS AND METHODS). Fragmented chloroplasts prepared by suspending intact organelles in 0.015 M Tris-HCl buffer (pH 7.5) were incubated with ^{35}S-APS, ATP, reduced glutathione (GSH) and NADPH together with an NADPH-glucose-6-phosphate dehydrogenase regenerating system. The results in Table V show that the decrease in radioactivity of ^{35}S-APS is accounted for in the $^{35}SO_4^{2-}$ formed. About 17% of the ^{35}S-APS was utilized either in the complete reaction mixture or in one without GSH and NADPH. When ATP was omitted from

TABLE V

FORMATION OF $^{35}SO_4^{2-}$ FROM ^{35}S-APS IN FRAGMENTED CHLOROPLASTS

The reaction mixture contained, in a final volume of 0.5 ml (μmoles): Tris·HCl (pH 7.6), 25; ^{35}S-APS, 0.25 (4500 counts/min); pyrophosphatase, 2 μg; 0.1 ml of fragmented chloroplasts containing 0.05 mg chlorophyl (prepared in 0.3 M sorbitol, see MATERIALS AND METHODS); and reduced glutathione (GSH), 1; NADPH, 0.16; glucose 6-phosphate (Glc-6-*P*), 2; and Glc-6-*P* dehydrogenase (1 unit). The reaction mixture was incubated at 30° for 30 min, and 0.05-ml aliquots were analysed by electrophoresis and scintillation counting as in the radioassay of ATP-sulphurylase (see MATERIALS AND METHODS).

Reaction mixture	$^{35}SO_4^{2-}$ *produced (nmoles)*	% ^{35}S-APS *utilized*
1. Complete	40.7	16.3
2. (1) but omit GSH, NADPH, Glc-6-*P* and Glc-6-*P* dehydrogenase	43.7	17.5
3. (1) but omit ATP	93.5	37.4

these reaction mixtures, more than twice as much ^{35}S-APS was degraded to $^{35}SO_4^{2-}$.

During similar incubations of fragmented chloroplasts with ^{35}S-PAPS (60 nmoles), about three times the amount of ^{35}S-PAPS (45 nmoles) was metabolized in reaction mixtures containing GSH and NADPH. The decrease in radioactivity in ^{35}S-PAPS was again recovered in $^{35}SO_4^{2-}$. It is likely that any $^{35}SO_3^{2-}$ formed in this reaction would be readily oxidized to $^{35}SO_4^{2-}$ under the conditions of assay.

DISCUSSION

By using the formation of ^{35}S-APS from $^{35}SO_4^{2-}$ as an assay for ATP-sulphurylase the distribution of the enzyme was followed in spinach leaves. When chloroplasts are isolated in isotonic buffer systems, it is known that soluble enzymes often leach out of these organelles[23]. In fact, only 16% of the ATP-sulphurylase in the crude homogenate was retained in isolated chloroplasts prepared in a 0.4 M sucrose solution. This loss of soluble protein may be offset by using non-aqueous media in

which this protein is insoluble[17]. Thus in the present work, by using *n*-hexane and carbon tetrachloride, as much as 44% of the ATP-sulphurylase from crude extracts was found together with 48% of the chlorophyll in the isolated chloroplasts. These results suggest that most of the enzyme is located in the chloroplasts where the specific activity is at least eight times higher than that in crude extracts.

Since the activity of ATP-sulphurylase determined by the radioassay procedure is low in crude leaf extracts (about 14 nmoles [^{35}S]APS produced/h per mg protein), a more sensitive method[20] has been devised to measure ATP formed from APS and pyrophosphate in the reverse reaction, which is thermodynamically more favourable:

$$\text{APS} + \text{pyrophosphate} \rightleftharpoons \text{ATP} + SO_4^{2-}$$

This ATP production was linked to the luciferin-luciferase enzyme reaction of the firefly (*Photinus pyralis*) resulting in a rapid and economical assay, which was geared to a computer programme developed to analyse the data. The low K_m values for APS and pyrophosphate, determined for the partially purified enzyme by the firefly assay, were 0.47 μM and 3.0 μM, respectively, indicating the very high affinity of ATP-sulphurylase for these substrates.

ASAHI[8] found a low amount of ^{35}S APS in a chloroplast preparation of spinach leaves incubated with $Na_2{}^{35}SO_4$ and ATP, but ^{35}S PAPS was not detected. In work reported herein, although the incorporation of $^{35}SO_4^{2-}$ into extracts of spinach leaves was followed by an even more sensitive electrophoresis method of resolving the products, ^{35}S PAPS was still not detected.

It has been suggested that PAPS but not APS is reduced to sulphite in chloroplasts[8]; more recently, SCHMIDT AND TREBST[24] have shown that sulphate can be reduced to sulphite by chloroplast fragments in the dark if reduced glutathione is added. In our experiments, however, ^{35}S APS was readily converted to sulphate when it was incubated with chloroplast fragments, probably *via* the reverse reaction of ATP-sulphurylase since ATP inhibited this reaction as reported by ROBBINS AND LIPMANN[5]. Since reducing agents, including glutathione, did not affect the rate of ^{35}S APS utilization, it is unlikely that the nucleotide was reduced. In contrast to this, preliminary experiments with fragmented chloroplasts indicate that ^{35}S PAPS is more readily utilized in the presence of reductants but labelled sulphate only was detected. It is likely that the labelled sulphite formed is oxidised non-enzymically to sulphate.

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