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# Adenosine-5'-Triphosphate Sulphurylase from Rice Shoots: Partial Purification and Properties

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**Key words:** **ATP-sulphurylase, purification, properties.** *Oryza sativa,* **sulphur metabolism.**

## RINGKASAN

*ATP-sulfurilase telah diperolehi dari pecahan larut sel pucuk padi. Enzim ini telah ditulinkan sebanyak 44-kali dari ekstrak kasar pucuk padi dengan menggunakan pemendakan ammonium sulfat, kromatografi DEAF-selulos dan sephadex G-200. Suhu optima enzim didapati dalam lingkungan 40°C dan pH optimanya ialah di antara 7.5–8.5. $Mg^{++}$ adalah perlu untuk aktiviti enzim sementara anion Kumpulan VI (molibdat, sulfat, selenat dan tungstat), EDTA, $Hg^{2+}$, azid, sianid, sulfid dan fluorid pula merencat ATP-sulfurilase. Nilai Km bagi APS ialah 4.5 μM dan bagi piroposfat ialah 9.0 μM.*

## SUMMARY

*ATP-sulphurplase was found in the soluble fraction of cell extracts of rice shoots. The enzyme was purified 44-fold by ammonium sulphate fractionation, DEAE-cellulose and sephadex G-200 chrmatography. The optimum temperature of the enzyme is around 40°C while its pH optimum is between 7.5–8.5. $Mg^{++}$ is required for its activity but group VI anions (molybdate, sulphate, selenate, tungstate), EDTA, $Hg^{2+}$, azide, cyanide, sulphide and fluoride are inhibitory. The Km values for APS and pyrophosphate are 4.5 μM and 9.0* μM respectively.

## INTRODUCTION

The ultimate source of sulphur-containing compounds which are essential for the growth and function of all living organisms are the inorganic compounds of sulphur. Plants and some micro-organisms have the ability to synthesize the essential sulphur-containing compounds directly from inorganic forms of sulphur but other organisms depend on plants and micro-organisms for their supply of these compounds. However, animals do have a limited capacity to utilize sulphate produced by the oxidation of compounds obtained from plants and micro-organisms (Roy and Trudinger, 1970).

The most important source of sulphur utilized by plants is sulphate (Wilson and Reuveny, 1975; Roy and Trudinger, 1970). The activation of sulphate by ATP forming adenosine-5'-phosphosulphate (APS) and liberating pyrophosphate is catalysed by ATP-sulphurylase (adenosine triphosphate – sulphate adenylyltransferase, EC 2.7.7.4.). This reaction, first reported in yeast and liver (Robbins and Lipman, 1956; Bandurski *et al,* 1956), is the first step in the metabolism of sulphate in both assimilatory and dissimilatory sulphate reducing organisms.

In spite of the biological importance of the synthesis of sulphur-containing compounds from inorganic sources of sulphur by plants, little is known about the enymology of sulphate metabolism in plants. ATP-sulphurylase has, however, been detected in crude extracts of spinach leaves (Balharry and Nicholas, 1970; Shaw and Anderson, 1971, 1972), soybean (Adams and Johnson, 1968), maize (Onajobi *et al,* 1973), beet root (Paynter and Anderson, 1974), rice (Onajobi, 1975) and in shoot and root tissues of selected plants (Ellis, 1969).

*Abbreviations:* EDTA, ethylenediamine tetracetate; DEAE, diethylamino-ethanol; APS, adenosine-5'-phosphosulphate; PPi, inorganic pyrophosphate.

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Numerous assay procedures for ATP sulphurylase have been employed by various workers. The reaction catalysed by the enzyme has a standard free-energy change of +11 kcal/mole at pH 8.0 and 37°C which corresponds to an apparent equilibrium constant of the order of $10^{-8}$ (Robbins and Lipman, 1958; Wilson and Bandurski, 1958; Akagi and Campbell, 1962). The forward reaction is thus thermodynamically unfavourable. Balharry and Nicholas, (1970, 1971) have described a direct and highly sensitive method for measuring ATP-sulphurylase by utilizing the reverse reaction. The method involves the use of APS and PPi as substrates and measurement of the ATP synthesized by the luciferin-luciferase assay in a liquid scintillation spectrometer switched out of coincidence.

In this paper we describe the partial purification and some properties of ATP-sulphurylase from extracts of rice shoots.

## MATERIALS AND METHODS

*Germination of rice seeds*

Rice seeds (*Oryza sativa,* var, MARDI Rice 7) were soaked in running water for 2 hr. It was then surface sterilised in 50% (v/v) ethanol followed by 0.05% (w/v) mercuric chloride solution and finally rinsed four times with sterile distilled water. The seeds were then transferred aseptically on to a 1 – 2 cm layer of cotton wool in aluminium trays (20 cm diameter) which had been previously sterilised by rinsing with 50% (v/v) ethanol, 0.05% (w/v) $HgCl_2$ solution and hot water. Sterilised nutrient solution was added until the seeds were submerged. The nutrient solution consisted of the following (g/l): $KNO_3$, 0.3; $(NH_4)_2SO_4$, 0.07; $Ca(NO_3)_2$, 0.6; $MgCl_2$, 0.35; $Na_2SO_4$, 0.5; $CuSO_4$, 0.02; $ZnSO_4$, 0.03 and $Fe(NO_3)_2$, 0.04. The trays were then covered with aluminium foil and incubated at 30° for a week, with periodical replenishment of the nutrient solution.

In a week-old seedling, the root and shoot measured approximately 5 cm and 6 cm respectively.

*Preparation of rice shoot extract*

Shoots, measuring approximately 5 cm were cut with scissors, rinsed with cold 50mM Tris-HCl buffer (pH 7.5) and blotted dry. They were then homogenized in 50 mM Tris-HCl buffer (pH 7.5) containing 0.2 mM EDTA (sodium salt) using a Sorvall Omni-Mixer homogenizer (4 ml of buffer were used per gram fresh weight of shoot extract). The homogenate obtained was squeezed through two layers of muslin cloth and then centrifuged at 15,000 x *g* for 30 min at 2°C in an IEC centrifuge (Model B20A). The supernatant fraction obtained ($S_{15}$), referred to as the crude extract, was used as the source of ATP-sulphurylase.

*Preparation of luciferin-luciferase enzyme*

The enzyme extract was prepared from firefly (*Photinus pyralis*) lanterns by a modification of the method of Stanley and Williams (1969) as follows: firefly lanterns were grounded with a mortar and pestel using 2 ml of cold 50 mM arsenate buffer (pH 7.5). The grounded sample was further homogenized in a glass homogenizer. The homogenate was washed into a centrifuge tube with sufficient arsenate buffer to give a final volume of 1.0 ml buffer per firefly lantern. The mixture was centrifuged at 12,000 x g for 30 min at 2°C in an IEC centrifuge. A few crystals of D-luciferin were added to the supernatant fraction. The enzyme was kept at room temperature (27°C) before use.

*Determination of ATP sulphurylase activity*

ATP sulphurylase activity was determined by the firefly bioluminescence procedure either by a continuous assay or a sampling technique. (a) *Continuous method:* The firefly bioluminescence assay of Balharry and Nicholas (1971) was employed with some modifications. The reaction vial contained Tris-HCl buffer (pH 7.5), 100 μmoles; sodium arsenate buffer, 200 μmoles; $MgCl_2$, 2.5 μmoles; $Na_4P_2O_7 . 10H_2O$, 150 nmoles and APS, 50 nmoles. The reaction mixture, nicknamed 'cocktail' in the vial was placed in the well of an ICN Caromet liquid scintillation spectrometer (model 2700) which was set up as follows: The circuit was switched out-of-coincidence. A single channel was used which was set with a gain at 32 and the two discriminators at 10 and 100 respectively. Counts were recorded at 27°C. Five counts each of 0.1 min were recorded at intervals of 0.3 min. This gave the background counts (Segment A, *Fig. 1*). The light reaction was then initiated by adding 0.1 ml of the firefly extract and a further five counts recorded (Segment B). The rate of light production in this segment is a measure of the ATP-sulphurylase activity in the firefly extract. An internal standard of ATP (50 pmoles) was added at the 4th min interval, resulting in an increase in light emission. The counting sequence was repeated (Segment C). Finally, at the 6th minute an enzyme extract was added to the vial and five counts recorded (Segment D). The activity of ATP-sulphurylase was calculated from the relationship

$$\text{ATP produced} = \frac{50\,y}{0.3x} \text{ pmoles/min}$$

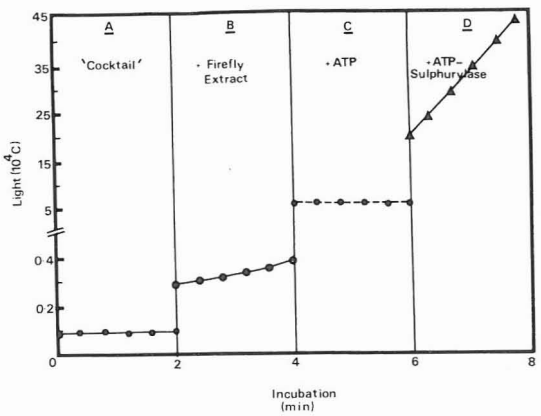


*Fig. 1. Determination of ATP-sulphurylase activity by the bioluminescence technique: a representative experiment. The procedure is described in MATERIALS AND METHODS.* A, *background counts of reaction mixture;* B, *counts on adding firefly extract;* C, *counts on adding ATP internal standard;* D, *counts due to ATP-sulphurylase activity.*

where x = increase in counts after 50 pmoles of ATP (internal standard) was added and y = counts in 0.3 min after addition of enzyme. The specific activity of the enzyme was expressed as nmoles ATP produced/min/mg protein.

(b) *Sampling method:* The reaction mixture in a test tube contained 30 μmoles Tris-HCl buffer, 5 μmoles $MgCl_2$, 0.1 μmole $Na_4P_2O_7$, 0.1 μmole APS and enzyme in a total volume of 1.0 ml. The reaction, carried out at 30°C, was initiated by adding the enzyme and incubating it for 5 min. It was terminated with 1.0 ml 10% (v/v) perchloric acid. Afer centrifuging at 3,000 x g for 5 min, 0.1 ml aliquot of the supernatant fraction was taken for the determination of ATP by the luciferin-luciferase enzyme system as follows: The reaction vial contained 1 ml of 10 mM phosphate buffer (pH 7.4), 0.1 ml of 5 mM $MgCl_2$, 1.0 ml of 50 mM arsenate buffer (pH 7.5) and 0.9 ml $H_2O$. The following additions were made at 2 min intervals: 0.1 ml firefly extract (A), 0.05 ml containing 50 pmoles ATP (B) and 0.1 ml aliquot of sample (C). The counts per min were determined after each addition and amount of ATP formed calculated from the expression

$$\frac{C_{cpm} - B_{cpm}}{B_{cpm} - A_{cpm}} \times 10$$

Enzyme activity was expressed as nmoles ATP produced/min/mg protein.

*Determination of protein*

Protein was determined with reference to a standard solution of bovine serum albumin by the Folin-Ciocalteau method as described by Lowry *et al.* (1951).

*Chemicals*

ATP, APS, bovine serum albumin, firefly lanterns, Tris-HCl, luciferin and EDTA were purchased from Sigma Chemical Co., St. Louis, Missouri, U.S.A., Whatman DEAE-cellulose (anion-exchanger type 11) was supplied by Whatman Biochemicals Ltd. (Springfield Mill, Kent, U.S.A.) and Sephadex (G200-120) was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

## RESULTS

*Purification of the enzyme*

ATP sulphurylase was purified from excised rice shoots as described below. All operations were carried out at 2°C

The shoots were treated as described in Materials and Methods to obtain the supernatant fraction ($S_{15}$) and this was used as the starting material (Fraction I, Table I). Solid ammonium sulphate was added slowly to $S_{15}$ until 40% saturation was obtained. The suspension was allowed to stand for 30 min before centrifuging at 10,000 x g for 20 min and the residue discarded. Solid ammonium sulphate was added to the supernatant fraction until 65% saturation was reached and the mixture allowed to stand for another 30 min. The precipitate, obtained after the second centrifuging, was then dissolved in a minimal volume of cold 50 mM Tris-HCl buffer (pH 7.5) and dialysed extensively against the same buffer. The dialysed fraction (Fraction II) was then loaded on to a DEAE-cellulose column (DE-11; 1.0 x 10.0 cm) which had been pre-equilibrated with 50 mM Tris-HCl buffer (pH 7.5). The column was then eluted with a linear gradient of 200 ml each of 0.05 M and 0.4 M Tris-HCl buffer (pH 7.5). The elution profile of the enzyme is shown in *Fig. 2.* Fractions (11-30, *Fig. 2*) containing ATP sulphurylase were collected in an LKB fraction collector, pooled and concentrated by ammonium sulphate precipitation (between 40% and 65% saturation) and dialysed as described above. This yielded Fraction III which was further purified by Sephadex G-200 gel chromatography. The enzyme, eluted with 50 mM Tris-HCl buffer (pH 7.5), was collected in 5 ml fractions and then bulked to give

TABLE 1
Purification of ATP-sulphurylase

| | Fraction | Total activity | Specific activity | Recovery (%) | Relative purity |
|---|---|---|---|---|---|
| I | Crude extract ($S_{15}$) left after centrifuging homogenized shoots at 15,000 x*g* for 30 min. | 1320 | 2.6 | 100 | 1 |
| II | Fraction I precipitated with amm sulphate (40 – 65%). | 616 | 9.8 | 46.7 | 3.7 |
| III | Fraction II eluted from DEAE-cellulose column. | 378 | 29.1 | 28.6 | 11 |
| IV | Fraction III eluted from Sephadex G-200 column. | 190 | 116 | 14.5 | 44 |

Total activity : nmoles ATP produced/min.
Specific activity : nmoles ATP produced/min/mg protein.

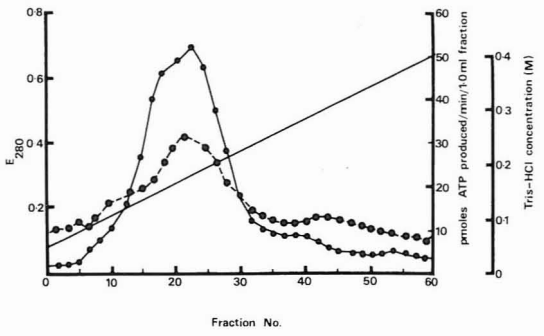


*Fig. 2. Elution of ATP-sulphurylase from DEAE-cellulose column. Fraction II (Table I) was loaded on to a DEAE cellulose column and eluted with a linear gradient 0.05M to 0.4M Tris-HCl buffer (pH 7.5). (●————●), ATP-sulphurylase activity; (●- - - - - -●), $E_{280}$; (————) concentration of Tris-HCl buffer.*

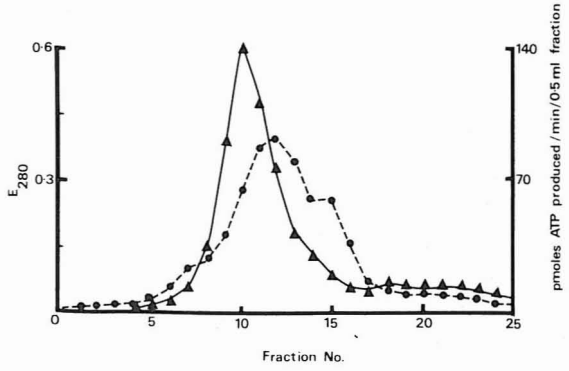


*Fig. 3. Elution of ATP-sulphurylase from Sephadex-G200 column. Fraction III (Table I) was loaded on to a Sephadex G-200 column (30 x 2.5 cm) which had been equilibrated with 50 mM Tris-HCl buffer (pH 7.5). The enzyme was eluted with the same buffer. (▲————▲), ATP-sulphurylase activity; (●- - - - - -●), $E_{280}$.*

Fraction IV (*Fig. 3*) Fraction IV was concentrated by ammonium sulphate precipitation.

The data for the entire fractionation are presented in Table I. The enzyme was purified about 44 fold over the crude ($S_{15}$) extract.

*Properties of the enzyme*

The effects of periods of incubation on the activity of ATP sulphurylase measured by the sampling technique is shown in *Fig. 4.* The reaction was linear for the initial 3 min and reached a maximum after 7 min. A five-minute incubation was used routinely for the sampling method of assay for the enzyme; this was convenient and the deviation from linearity towards the end of such an incubation was not considered serious.

Little or no ATP was produced in the absence

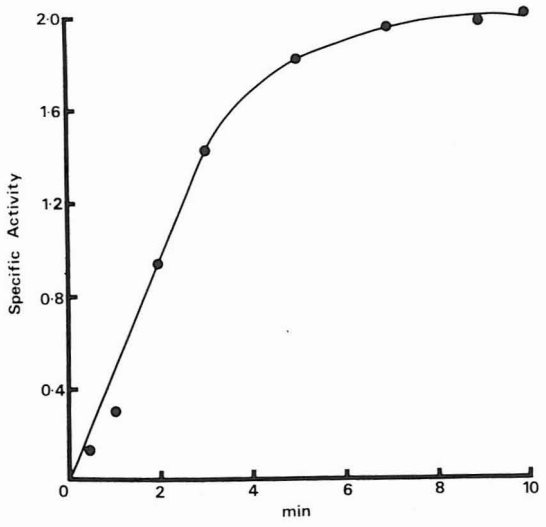


*Fig. 4. Effect of incubation time on ATP-sulphurylase activity. The enzyme was assayed by the sampling technique as described in MATERIALS AND METHOD. Fraction II (Table I) was the enzyme extract used.*
*Specific activity: nmoles ATP produced/mg protein.*

of either $Mg^{++}$, PPi or APS (Table II). The effect of temperature on enzyme activity is shown in *Fig. 5B.* The optimum temperature was around

TABLE II
Requirements for ATP-sulphurylase assay

The reaction is conducted as described in MATERIALS AND METHODS. Fraction II (Table I) was used as the enzyme source. In "boiled" preparations the enzyme was placed in a boiling water bath for 3 min and then cooled before adding it to the reaction mixture.

| Assay conditions | Specific activity |
|---|---|
| Complete | 8.6 |
| Boiled enzyme extract | 0 |
| Enzyme extract omitted | 0 |
| $MgCl_2$ omitted | 0.5 |
| PPi omitted | 0 |
| APS omitted | 0 |
| Pi substituted for PPi | 0.8 |

Specific activity: nmoles ATP produced/min/mg protein

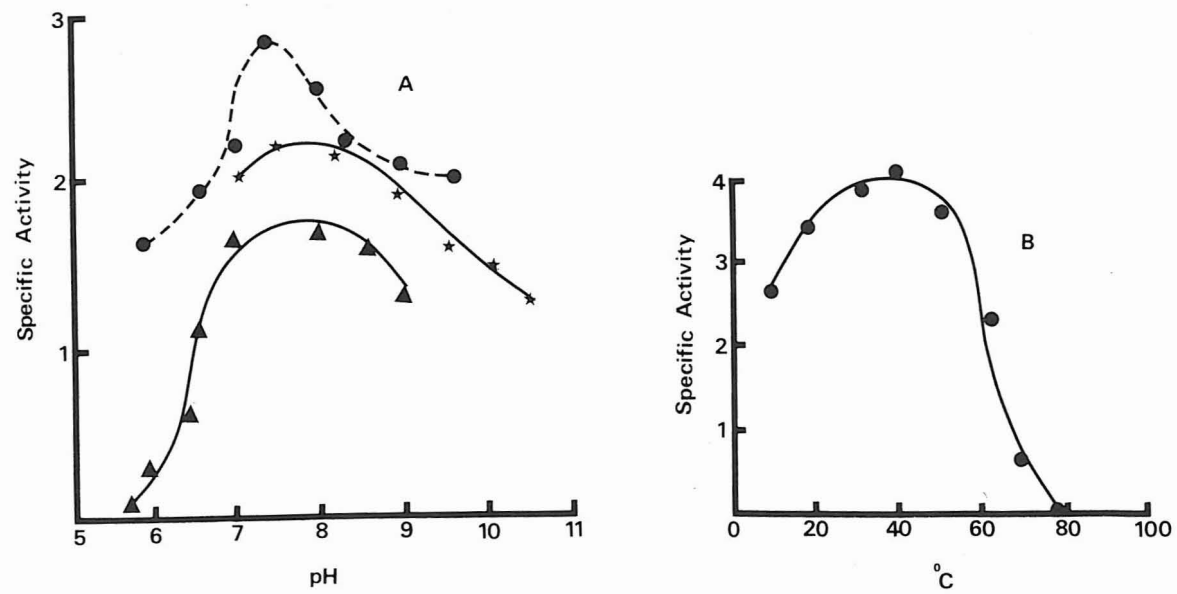


*Fig. 5. Effect of pH and temperature on ATP-sulphurylase activity.*
*A: Effect of pH. (●– – – –●), Tris-HCl buffer; (★———★), glycine-NaOH buffer; (▲———▲), citrate-phosphate buffer.*
*B. Effect of temperature.*
*Fraction II (Table I) was used as the enzyme source.*
*Specific activity; nmoles ATP produced /min/mg protein.*

30 – 40°C. Above 50°C there was a marked decline of activity and the enzyme was inactivated after 3 min incubation at 80°. ATP-sulphurylase activity varied with the buffer system used. Nevertheless, it showed maximal activity around 7.5 in all three buffer systems studied (*Fig. 5A*). The effect of inhibitors on ATP-sulphurylase is shown in Table III. Molybdate and tungstate com pletely inhibited the enzyme at 1.0 mM. Mercuric ions also inhibited the enzyme completely but at 10 mM. Sulphate and chromate are also potent inhibitors while dithionite, sulphate and thiosulphate are ineffective.

TABLE III

Effect of inhibitors on ATP-sulphurylase activity

ATP production was determined by the sampling method as described in MATERIALS AND METHODS. Fraction II (Table I) was used as the enzyme source. The inhibitor was incubated with the enzyme for 5 min at 30° before adding the other substrates. The specific activity of the control reaction (without inhibitor) was 2.4 nmoles ATP produced/min/mg protein.

| Inhibitor | Final concentration (mM) | % inhibition |
|---|---|---|
| dithionite | 10 | 0 |
| sulphite | 10 | 0 |
| sulphide | 10 | 56 |
| thiosulphate | 10 | 0 |
| sulphate | 1.3 | 60 |
| molybdate | 1.0 | 100 |
| chromate | 1.0 | 46 |
| tungstate | 1.0 | 100 |
| azide | 10 | 10 |
| cyanide | 10 | 19 |
| fluoride | 10 | 13 |
| mercuric ions | 10 | 100 |
| EDTA | 1.0 | 24 |

Crude extracts of the enzyme were also able to utilize inorganic phosphate in place of pyrophosphate (*Fig. 6*). The rate of production of ATP was measured at various concentrations of pyrophosphate and APS. The results show normal Michaelis-Menten kinetics and in each case there is a linear relation between ATP production and substrate concentration in the lower ranges (*Fig. 7A & B*). High levels of PPi inhibited the enzyme

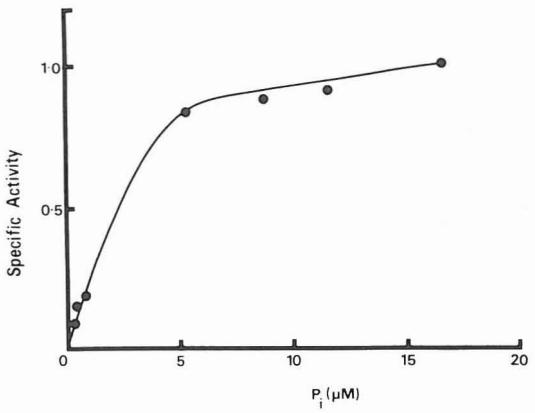


*Fig. 6. Effect of Pi on ATP-sulphurylase activity in shoot extracts. Fraction II (Table I) was used as the enzyme source. Specific activity: nmoles ATP produced/ min/mg protein.*

whereas comparative levels of APS had little effect. The calculated Km values are 4.5 μM for APS and 9.0 μM for PPi.

Partially purified enzyme (Fraction II, Table I) was comparatively stable when stored at −15°C. No loss in activity was detected after one month of storage. However, the activity of the purified enzyme (Fraction IV, Table I) decreased by 50% after two weeks of storage at the same temperature.

## DISCUSSION

ATP sulphurylase extracted from shoots of rice seedlings are found to be associated with the soluble fraction of the cell. This agrees with the findings in *Beta vulgaris* (Paynter and Anderson, 1974), yeast (Bandurski and Wilson, 1958) and spinach leaves (Shaw and Anderson, 1971, 1972). The absolute requirement of the enzyme for $Mg^{++}$ is in accord with the observations of Hawes and Nicholas (1972) and of Akagi and Campbell (1962) but contrasts with those of Robbins and Lipmann (1958).

The low Km values for APS and PPi indicate the high affinity of ATP sulphurylase for the substrates. This is in agreement with those obtained from higher plants such as spinach leaves (Balharry and Nicholas, 1970) and maize seedlings (Onajobi *et al,* 1973). In the crude extracts PPi may be substituted with Pi. The ATP generated could be due the combined action of ADP-sulphurylase and adenylate kinase as postulated by Peck (1960,

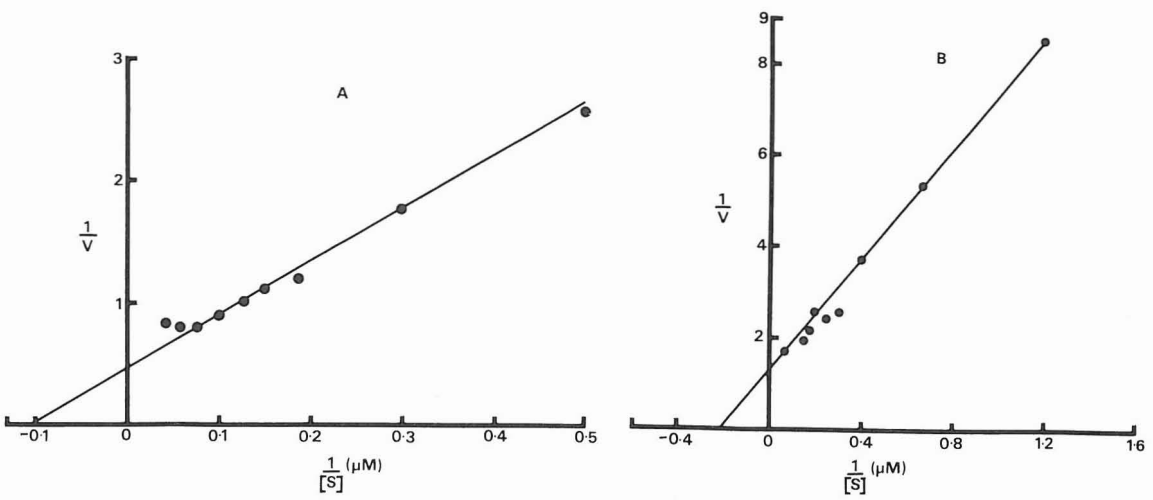


*Fig. 7. Reciprocal plots of the effect of APS and PPi concentration on ATP-sulphurylase activity. (A), Effect of PPi; (b), Effect of APS.*

1962).

The effects of pH and temperature on the enzyme are consistent with the results reported by others in other biological systems (Akagi and Campbell, 1962; Adams and Johnson, 1968; Balharry and Nicholas, 1970; Pannikar and Bacchawat, 1968; Shaw and Anderson, 1971).

Inhibitor studies on the enzyme indicate that the Group VI anions inhibit the enzyme. This is in agreement with the results reported by Varma and Nicholas (1970) and Ellis (1969). In contrast to the observations of Ellis (1969) and Shaw and Anderson (1972) the results reported herein show that EDTA and mercurial compounds are inhibitory.

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