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Adenosine 5′-Pyrophosphate Sulphurylase in Baker's Yeast

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ADP sulphurylase from baker's yeast was purified and its properties were studied. The enzyme is very heat-labile and its activity shows linear kinetics over narrow ranges of time and protein concentration. It is not activated by metals and is inhibited by thiol-reactive compounds. The enzyme, which replaces inorganic sulphate in adenosine 5′-sulphatophosphate with P_i to yield ADP, also catalyses an exchange of P_i into ADP. Kinetic studies show that the enzyme has a high affinity for adenosine 5′-sulphatophosphate, although concentrations in excess of 1.0mM are inhibitory. However, the kinetics for P_i are more complex and the enzyme is not inhibited by P_i up to 20.0mM.

ADP sulphurylase (EC 2.7.7.5, ADP–sulphate adenylyltransferase) was first assayed by Robbins & Lipmann (1956) in baker's yeast and was shown to catalyse the following reaction:

$$\text{Adenosine 5'-sulphatophosphate} + P_i \rightleftharpoons \text{ADP} + SO_4^{2-}$$

It is one of the few enzymes that utilizes adenosine 5′-sulphatophosphate as a substrate. Robbins & Lipmann (1958*b*) showed that adenosine 5′-sulphatophosphate is the immediate precursor of 3′-phosphoadenosine 5′-sulphatophosphate, which serves as a sulphate donor in many important reactions (Roy & Trudinger, 1970) and also as an intermediate during sulphate reduction (Wilson *et al.*, 1961). Consequently, enzymes regulating the concentration of adenosine 5′-sulphatophosphate are likely to be important in controlling the production of both sulphated and reduced sulphur compounds.

At least two sulphatases that convert adenosine 5′-sulphatophosphate into AMP and inorganic sulphate have been studied in animal tissues (Armstrong *et al.*, 1970; Bailey-Wood *et al.*, 1970). These may be concerned with regulating adenosine 5′-sulphatophosphate concentrations. ADP sulphurylase, however, has been detected only in micro-organisms. It has been found in *Thiobacillus thioparus* (Peck, 1960), *Desulphovibrio desulphuricans* (Peck, 1962) and the Thiorhodaceae (Thiele, 1968). An enzyme from yeast that is claimed to catalyse an ADP–$^{32}P_i$ exchange reaction has been described as ADP sulphurylase (Grunberg-Manago *et al.*, 1966), although further confirmation of this would be desirable (Roy & Trudinger, 1970).

In *T. thioparus* the enzyme appears to be involved in the oxidation of thiosulphate to sulphate with concomitant generation of ADP (Peck & Stulberg, 1962). However, in *D. desulphuricans*, yeast and in the Thiorhodaceae its function is not known, although the yeast enzyme has been compared with a nucleotide phosphorylase (Grunberg-Manago *et al.*, 1966).

Materials and Methods

Materials

Adenosine 5′-sulphatophosphate and adenosine 5′-[^{35}S]sulphatophosphate were synthesized by the method of Adams *et al.* (1971). ATP, ADP, AMP, *p*-chloromercuribenzoic acid, GSH, cysteine hydrochloride and *N*-ethylmaleimide were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). DEAE-cellulose (DE-11) and 3MM chromatography paper were purchased from N. Reeve Angel and Co. Ltd. (London, U.K.). $^{32}P_i$ in dil. HCl was supplied by the Australian Atomic Energy Commission (Lucas Heights, Sydney, N.S.W., Australia). $^{35}SO_3^{2-}$ was supplied by The Radiochemical Centre, Amersham, Bucks., U.K., as an aqueous solution (pH6–8, carrier-free). Bovine serum albumin was supplied by Calbiochem (Los Angeles, Calif., U.S.A.). All other chemicals were of analytical grade.

[^{32}P]ADP was synthesized by the ADP sulphurylase reaction. The reaction mixture contained (in a total volume of 2.5ml): tris–HCl (pH7.5), 250μmol; adenosine 5′-sulphatophosphate, 2.5μmol; P_i, 25μmol (approx. 0.1μCi/μmol); enzyme, 0.5ml (2.25mg of protein). The reaction mixture was incubated for 15min at 30°C and then heated in boiling water for 3min. After centrifuging for 10min at room temperature in a Heraeus Christ G.m.b.H. type UJ1 centrifuge at 3000rev./min, [^{32}P]ADP was isolated from the supernatant solution by column chromatography.

DEAE-cellulose (formate form) at pH6.0 was packed into a column (5cm×1.3cm) and washed

with 75ml of water. The supernatant solution was loaded on to this column, which was then eluted with 100ml of 0.05M-NH_4HCO_3 soln. This removes residual $^{32}P_i$ but not ADP. The [^{32}P]ADP was then eluted in 50ml of 0.2M-NH_4HCO_3. This eluate was then evaporated to dryness in a rotary evaporator at 50–60°C, dissolved in 20ml of water and evaporated to dryness again. This process was repeated once more to decompose the NH_4HCO_3. Finally the dry sample was dissolved in 0.5ml of water.

To check the purity of the product, 10μl of the final solution was spotted on Whatman 3MM paper. The paper was then soaked in 0.1M-sodium citrate buffer (pH5.0) and subjected to electrophoresis at 1500V (30V/cm) for 1.75h, in the apparatus described by Tate (1968), at room temperature. Radioactive areas were detected by running the dried electrophoretogram through a Packard 7201 radiochromatogram scanner. Most of the radioactivity was associated with a u.v.-quenching spot, which coincided with authentic unlabelled ADP. A second peak of radioactivity, representing about 10% of the total radioactivity, was associated with P_i. No other labelled products were apparent.

Baker's yeast (*Saccharomyces cerevisiae*) was obtained from Mauri Bros. and Thompson (Adelaide, S. Austral., Australia) and stored at −15°C.

Preparation of enzyme

Yeast cells suspended (1:2, w/v) in 0.05M-tris–HCl (pH7.5) containing 0.005M-EDTA (sodium salt) were passed twice through an Aminco French pressure cell at 718200Nm^{-2} at 4°C to give fraction I. This crude homogenate was centrifuged at 30000*g* for 30min in a Sorval SS-3 Automatic Superspeed Centrifuge at 4°C and the supernatant (fraction II) retained. Fraction II was adjusted to 60% (w/v) saturation with $(NH_4)_2SO_4$ at 2°C by slowly adding saturated $(NH_4)_2SO_4$ soln. The mixture was stirred at 2°C for 15min and the precipitate collected by centrifuging at 30000*g* for 30min at 4°C. The precipitate was dissolved in 0.05M-tris–HCl (pH7.5) containing 0.005M-EDTA (sodium salt) to give fraction III. The pH of fraction III was lowered to 5.5 with 1M-acetic acid and then solid NaCl (178.5g/l) was added slowly. The mixture was stirred for 15min and then centrifuged at 30000*g* for 30min at 4°C. The supernatant liquid was adjusted to pH7.5 with 1M-KOH to give fraction IV. This fraction was adjusted to 60% (w/v) saturation with $(NH_4)_2SO_4$ and yielded a precipitate, which was collected and dissolved in 0.05M-tris–HCl (pH7.5) containing 0.005M-EDTA (sodium salt), giving fraction V. This fraction was then used for the experiments reported below.

Enzyme assay

ADP sulphurylase was assayed by measuring the incorporation of $^{32}P_i$ into ADP with adenosine 5′-sulphatophosphate as the substrate. The assay mixture contained (in a total volume of 0.5ml): tris–HCl (pH7.5), 50μmol; adenosine 5′-sulphatophosphate, 0.5μmol; $^{32}P_i$, 2.0μmol (approx. 0.1μCi/μmol); enzyme, 0.1ml. The reaction was started by adding the enzyme, and the mixture was incubated for 10min at 30°C. The reaction was terminated by heating in boiling water for 3min, and after centrifuging for 10min in a Heraeus Christ G.m.b.H. type UJ1 centrifuge a 0.2ml sample was analysed for [^{32}P]-ADP.

DEAE-cellulose (formate form) at pH6.0 was packed into columns (2.0cm×0.8cm) and washed with 10ml of water. The 0.2ml sample of the reaction mixture was loaded on to the column, which was eluted with 15ml of 0.05M-NH_4HCO_3. This removes residual $^{32}P_i$ but not ADP. The [^{32}P]ADP was then eluted in 15ml of 0.2M-NH_4HCO_3, collected in a scintillation vial and counted by using the Čerenkov emission on a Packard 3375 Tri-Carb liquid-scintillation spectrometer. Enzyme activity was then expressed as nmol of P_i incorporated into ADP/h per mg of protein.

Identification of reaction products

Reaction mixtures were first fractionated on columns of DEAE-cellulose as described for the enzyme assay. The 0.2M-NH_4HCO_3 eluates were then evaporated to dryness in a rotary evaporator at 50–60°C, and subjected to high-voltage electrophoresis and radiochromatogram scanning as described for preparation of [^{32}P]ADP.

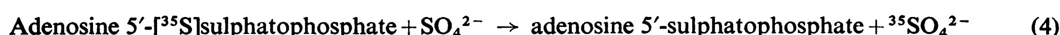
Exchange reactions

The following exchange reactions were considered:

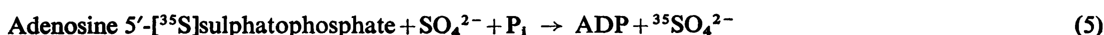
$$\text{ADP} + {}^{32}\text{P}_i \rightarrow [{}^{32}\text{P}]\text{ADP} + \text{P}_i \qquad (1)$$

$$[{}^{32}\text{P}]\text{ADP} + \text{P}_i \rightarrow \text{ADP} + {}^{32}\text{P}_i \qquad (2)$$

$$\text{Adenosine 5'-sulphatophosphate} + {}^{35}\text{SO}_4{}^{2-} \rightarrow \text{adenosine 5'-}[{}^{35}\text{S}]\text{sulphatophosphate} + \text{SO}_4{}^{2-} \qquad (3)$$



$$\text{Adenosine 5'-}[{}^{35}\text{S}]\text{sulphatophosphate} + \text{SO}_4{}^{2-} \rightarrow \text{adenosine 5'-sulphatophosphate} + {}^{35}\text{SO}_4{}^{2-} \qquad (4)$$



$$\text{Adenosine 5'-}[{}^{35}\text{S}]\text{sulphatophosphate} + \text{SO}_4{}^{2-} + \text{P}_i \rightarrow \text{ADP} + {}^{35}\text{SO}_4{}^{2-} \qquad (5)$$

The amounts of materials used in the incubation mixtures for these reactions were (in μmol): ADP, 0.5; [^{32}P]ADP, 0.5 (0.1μCi/μmol); adenosine 5′-sulphatophosphate, 0.5; adenosine 5′-[^{35}S]sulphatophosphate, 0.25 (4.5μCi/μmol); P_i, 5.0; $^{32}P_i$, 5.0 (0.1μCi/μmol); Na_2SO_4, 5.0; $Na_2{}^{35}SO_4$, 5.0 (0.2μCi/μmol). In addition all reaction mixtures contained 50μmol of tris–HCl (pH7.5) and 0.1ml of enzyme (fraction V, Table 2) in a final volume of 0.5ml.

Reaction mixtures were incubated for 15min at 30°C and then heated in boiling water for 3min. The entire reaction mixture was then loaded on to small columns of DEAE-cellulose (2.0cm×0.8cm) and eluted as described in the 'Enzyme assay' section. Thus P_i and SO_4^{2-} are eluted in 0.05M-NH_4HCO_3 but ADP and adenosine 5′-sulphatophosphate remain on the column and are eluted subsequently in 0.2M-NH_4HCO_3.

The 0.2M-NH_4HCO_3 eluates obtained from reactions (1) and (3) were concentrated and then subjected to high-voltage electrophoresis, and the electrophoretograms were passed through a radiochromatogram scanner as described under preparation of [^{32}P]ADP.

The 0.05M- and 0.2M-NH_4HCO_3 eluates obtained from reaction (2) were counted for radioactivity in a liquid-scintillation spectrometer by using their Čerenkov emissions.

Portions (0.1ml) of the 0.05M- and 0.2M-NH_4HCO_3 eluates obtained from reactions (4) and (5) were counted for radioactivity in 2.0ml of 95% (v/v) ethanol and 5.0ml of scintillation solvent. The scintillation solvent contained 8.0g of 2,5-diphenyloxazole and 0.3g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene/litre of toluene.

Determination of protein

The method of Lowry *et al.* (1951) was used with bovine serum albumin as a standard.

Results

Characterization of the assay system and enzyme purification

Enzyme activity required adenosine 5′-sulphatophosphate and enzyme. Heating the enzyme for 3min in boiling water inactivated it. The major radioactive component in the 0.2M-NH_4HCO_3 eluate of the complete reaction mixture was ADP (Fig. 1). There was also a small amount of residual $^{32}P_i$. In the absence of either adenosine 5′-sulphatophosphate or enzyme, only small peaks of $^{32}P_i$ were observed.

The enzyme was concentrated by about 300-fold. The total activity always showed an increase as purification proceeded (Table 1).

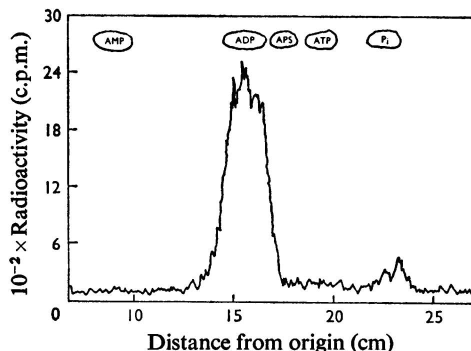


Fig. 1. *Products of ADP sulphurylase*

A reaction mixture containing 50μmol of tris–HCl (pH7.5), 0.5μmol of adenosine 5′-sulphatophosphate (APS), 2.0μmol of $^{32}P_i$ (approx. 0.1μCi/μmol) and 0.1ml of enzyme, in a final volume of 0.5ml, was incubated for 10min at 30°C. The mixture was then fractionated on a DEAE-cellulose column, subjected to high-voltage electrophoresis and scanned for radioactivity as described in the Materials and Methods section.

Influence of protein concentration, time and temperature on enzyme activity

The activity was linear over a range of protein concentration from 0.12 to 1.2mg, and with time up to 15min, when about 8% of the total $^{32}P_i$ had been incorporated. The enzyme is fairly stable at 30°C for about 20min, but it is rapidly inactivated at 40°C (Table 2). Assays were always conducted at 30°C for 10min.

Effects of substrates, pH, buffer, inhibitors and metal ions on enzyme activity

Substrates. Assays were made with adenosine 5′-sulphatophosphate replaced by equivalent amounts of AMP, ADP, ATP and PP_i. The results in Table 3 show that ADP behaved in a similar way to adenosine 5′-sulphatophosphate although with only about half the rate. With AMP, ATP and PP_i, however, there was no activity. The activity with ADP was also heat-labile, as shown in Table 2.

pH and buffer. The pH optimum is between 6.5 and 8.0, and as a routine assays were done at pH7.5. Activity declined rapidly below pH6.0 or above pH8.0. There was little difference in tris–maleate buffer compared with tris–HCl.

Inhibitors and metal cations. Arsenate and *N*-ethylmaleimide at 1mM decreased activity by 17 and 34%

Table 1. *Preparation of ADP sulphurylase*

Assays are described in the Materials and Methods section.

Fraction	10^{-3} × Total activity (nmol of P_i incorporated/h)	Total protein (mg)	Specific activity (nmol of P_i incorporated/h per mg of protein)	Relative purity
I French pressure cell extract	193	34500	6	1
II 15000*g* supernatant	1015	6500	156	20
III Precipitate from 0–60% satd. $(NH_4)_2SO_4$ fractionation	3410	4080	834	139
IV Supernatant after NaCl fractionation	2490	1365	1835	306
V Precipitate from 0–60% satd. $(NH_4)_2SO_4$ fractionation	2240	1170	1915	319

Table 2. *Influence of preincubation time and temperature on ADP sulphurylase activity*

Enzyme was preincubated at a prescribed temperature, then 0.5μmol of nucleotide and 2.0μmol of $^{32}P_i$ were added, and assays were conducted as described in the Materials and Methods section.

Preincubation temp. (°C)	Nucleotide	Specific activity (nmol of P_i incorporated/h per mg of protein) — Preincubation time (min) ... 5	10	15	20
30	Adenosine 5′-sulphatophosphate	1195	1190	1130	1100
30	ADP	581	570	658	587
40	Adenosine 5′-sulphatophosphate	896	696	670	527
40	ADP	440	320	315	201

respectively, whereas fluoride, arsenite, iodoacetamide, iodoacetic acid, sulphate, thiosulphate, sulphite, sulphide and molybdate were not inhibitory. However, *p*-chloromercuribenzoic acid was a very potent inhibitor (Table 4) and its effect could be partially reversed by various thiol-containing reagents.

Of the metal cations tested all but K^+ showed some inhibitory effect, especially Cu^{2+} and Co^{2+} (Table 5).

Effect of substrate concentration on enzyme activity

The effects of concentrations of adenosine 5′-sulphatophosphate and P_i on enzyme activity are presented as double-reciprocal plots in Figs. 2 and 3. The K_m value for adenosine 5′-sulphatophosphate varies with P_i concentration in the range 4.2×10^{-4}–6.2×10^{-5}M.

At low concentrations of adenosine 5′-sulphatophosphate (0.04mM) activity is markedly influenced by P_i concentration over the range 2–20mM. This effect diminishes with increasing concentrations of adenosine 5′-sulphatophosphate up to 0.2mM because a high concentration of P_i decreases the response to adenosine 5′-sulphatophosphate concentrations (Figs. 2 and 3). However, with adenosine 5′-sulphatophosphate concentrations in excess of 0.4mM activity is again markedly influenced by P_i concentration (Figs. 4 and 5). Conversely, at concentrations of ADP above 0.4mM, P_i concentration had little effect.

Table 3. *Comparison of ADP sulphurylase activity with adenosine 5′-sulphatophosphate and ADP as substrates*

ADP and adenosine 5′-sulphatophosphate were used at concentrations of 1.0mM and assayed as described in the Materials and Methods section.

		Specific activity (nmol of P_i incorporated/h per mg of protein)	
Reaction mixture	Substrate ...	Adenosine 5′-sulphatophosphate	ADP
Complete		688	348
Substrate omitted		72	41
Boiled enzyme		45	42
Enzyme omitted		47	47

Table 4. *Inhibition of ADP sulphurylase by p-chloromercuribenzoic acid and its reversal by thiol reagents*

Enzyme was preincubated with thiol or *p*-chloromercuribenzoic acid for 5min at 25°C; thiol reagents were then added to tubes 5, 6 and 7 and preincubated for a further 5min at 25°C. Assays were done as described in the Materials and Methods section. Activity of control was 1445nmol of P_i incorporated/h per mg of protein.

Tube no.	Compound(s) added	Final concn. (mM)	Activity (% of control)
1	GSH	1.0	93
2	Mercaptoethanol	1.0	91
3	Cysteine	1.0	93
4	*p*-Chloromercuribenzoic acid	0.1	3
5	*p*-Chloromercuribenzoic acid	0.1	
	+GSH	1.0	63
6	*p*-Chloromercuribenzoic acid	0.1	
	+mercaptoethanol	1.0	57
7	*p*-Chloromercuribenzoic acid	0.1	
	+cysteine	1.0	56

Table 5. *Effect of metal cations on ADP sulphurylase activity*

Chloride salts were used in all cases. Enzyme and cation were preincubated for 5min at 25°C and then assayed as described in the Materials and Methods section. Activity of control was 915nmol of P_i incorporated/h per mg of protein.

Metal ion	Final concn. (mM)	Activity (% of control)
Cu^{2+}	2	6
	0.2	72
K^{+}	2	99
	0.2	97
Mn^{2+}	2	75
	0.2	87
Co^{2+}	2	33
	0.2	84
Mg^{2+}	2	76
	0.2	88
Ca^{2+}	2	85
	0.2	89

Exchange reactions

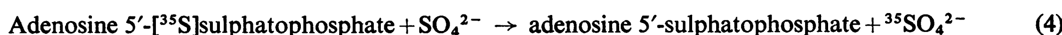
The major radioactive component obtained in the 0.2M-NH_4HCO_3 eluate from reaction (1) was [^{32}P]-ADP (Fig. 6), thus confirming reaction (1). The 0.05M-NH_4HCO_3 eluate from reaction (2) had an increased amount of radioactivity, suggesting that $^{32}P_i$ was released from [^{32}P]ADP in accordance with reaction (2) (Table 6).

There was no radioactive product on the paper electrophoretogram of the 0.2M-NH_4HCO_3 eluate from reaction (3). Under the conditions of electrophoresis used, $^{35}SO_4{}^{2-}$ would move off the paper and thus would not be detected by the scanning technique. The 0.05M-NH_4HCO_3 eluate obtained from reaction (4) did not show any significant increase in radioactivity compared with the control reaction (Table 6). These results suggest that reactions (3) and (4) do not occur. However, when unlabelled P_i was present in this reaction mixture a significant amount of $^{35}SO_4{}^{2-}$ was released from adenosine 5′-[^{35}S]sulphatophosphate, according to reaction (5) (Table 6). The radioactivity in the boiled-enzyme controls was associated

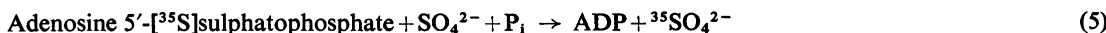
Table 6. *Exchange reactions*

Exchange reactions examined were:

$$[^{32}P]ADP + P_i \rightarrow ADP + {}^{32}P_i \quad (2)$$



$$\text{Adenosine 5'-}[^{35}S]\text{sulphatophosphate} + SO_4^{2-} \rightarrow \text{adenosine 5'-sulphatophosphate} + {}^{35}SO_4^{2-} \quad (4)$$



$$\text{Adenosine 5'-}[^{35}S]\text{sulphatophosphate} + SO_4^{2-} + P_i \rightarrow ADP + {}^{35}SO_4^{2-} \quad (5)$$

The reaction mixtures were prepared and assayed as described in the Materials and Methods section.

Reaction no.	% of total radioactivity in 0.05M-NH_4HCO_3 eluates	
	Complete mixture	Boiled-enzyme control
(2)	28	12
(4)	38	36
(5)	52	38

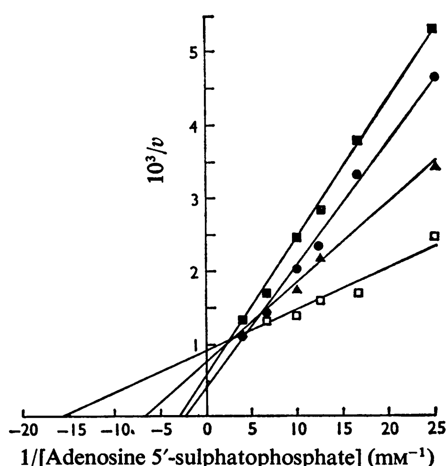


Fig. 2. *Double-reciprocal plots of ADP sulphurylase activity versus adenosine 5'-sulphatophosphate concentration*

For experimental details see the text. Velocities are nmol of P_i incorporated/h per mg of protein. ■, 2mM-P_i, $K_m = 3.57\times10^{-4}$M; ●, 6mM-P_i, $K_m = 4.17\times10^{-4}$M; ▲, 10mM-P_i, $K_m = 1.47\times10^{-4}$M; □, 20mM-P_i, $K_m = 0.62\times10^{-4}$M.

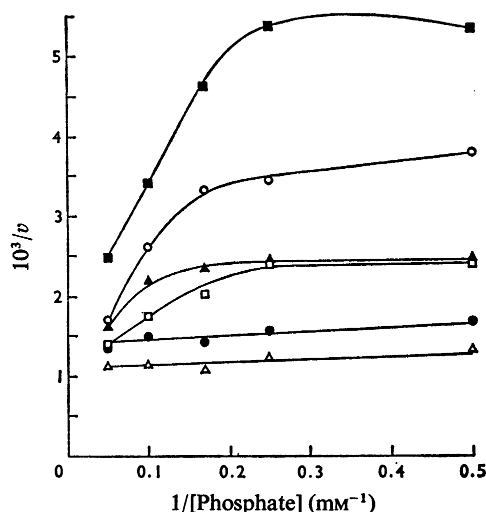


Fig. 3. *Double-reciprocal plots of ADP sulphurylase activity versus P_i concentration*

For experimental details see the text. Velocities are nmol of P_i incorporated/h per mg of protein. The concentrations of adenosine 5'-sulphatophosphate used were: ■, 0.04mM; ○, 0.06mM; ▲, 0.08mM; □, 0.10mM; ●, 0.15mM; △, 0.20mM.

with $^{32}P_i$ and $^{35}SO_4^{2-}$, which were contaminants of the [^{32}P]ADP and adenosine 5'-[^{35}S]sulphatophosphate respectively (Table 6).

Discussion

The assay system used above appears to be a valid measure of ADP sulphurylase activity based on the incorporation of $^{32}P_i$ into ADP (Fig. 1). Crude preparations also showed incorporation of $^{32}P_i$ into ATP, probably through the action of adenylate kinase.

The increase in total activity during purification (Table 1) may reflect the removal of enzymes acting on adenosine 5'-sulphatophosphate or ADP or both. The partially purified fraction V incorporated $^{32}P_i$ only into ADP when adenosine 5'-sulphatophosphate was used as substrate (Fig. 1). Attempts at further purification resulted in preparations that rapidly lost activity.

The response of enzyme activity to enzyme concentration, time and temperature is in agreement with results reported previously (Robbins & Lipmann, 1958*a*; Grunberg-Manago *et al.*, 1966). The deviation from linearity after 15 min incubation time is probably related to the extraordinary heat-lability of the enzyme. Preincubation of the enzyme at 40°C significantly decreased activity (Table 2).

The specific activity with adenosine 5′-sulphatophosphate as a substrate did not vary greatly with different batches of the enzyme, whereas that obtained with ADP as a substrate was more variable. The activity with ADP was always either equal to or more than half of the activity of the enzyme with adenosine 5′-sulphatophosphate.

The inhibition of enzyme activity by *N*-ethylmaleimide and by *p*-chloromercuribenzoic acid suggest the involvement of thiol groups (Table 4). This was further confirmed by the partial reversal of the inhibition of *p*-chloromercuribenzoic acid by thiol compounds. The potency of Cu^{2+} and Co^{2+} as inhibitors may also reflect the inactivation of thiol groups. Inhibition of thiol groups by metal ions may also explain the low activities obtained unless EDTA (sodium salt) is included in the reaction mixture. The yeast enzyme studied by Grunberg-Manago *et al.* (1966) likewise was susceptible to inhibition by heavy metals. The ineffectiveness of F^- as an inhibitor and the lack of a response to Mg^{2+} (Table 5) suggests that Mg^{2+} is not involved in the phosphate transfer catalysed by this enzyme. Robbins & Lipmann (1958*a*) were also unable to detect a stimulation of activity by Mg^{2+}.

The enzyme responds dramatically to different concentrations of adenosine 5′-sulphatophosphate when the P_i concentration is increased. The K_m for adenosine 5′-sulphatophosphate tends to decrease as P_i increases (Fig. 2) and is close to the K_m of 5.0×10^{-4} M for ADP reported by Grunberg-Manago *et al.* (1966). The double-reciprocal plots for P_i (Fig. 3) do not allow a ready evaluation of K_m.

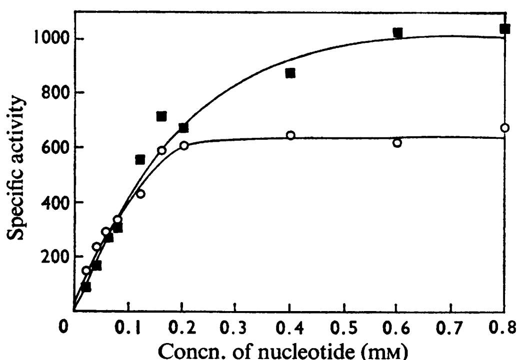


Fig. 4. *ADP sulphurylase activity with various amounts of adenosine 5′-sulphatophosphate and ADP at a constant concentration of P_i (4mM)*

For experimental details see the text. ■, Adenosine 5′-sulphatophosphate; o, ADP.

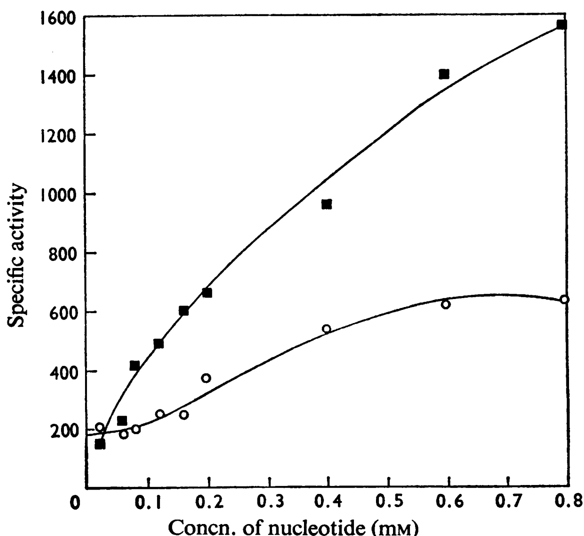


Fig. 5. *ADP sulphurylase activity with various amounts of adenosine 5′-sulphatophosphate and ADP at a constant concentration of P_i (10mM)*

For experimental details see the text. ■, Adenosine 5′-sulphatophosphate; o, ADP.

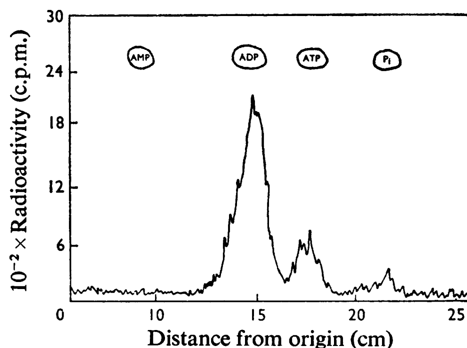


Fig. 6. *Products of ADP–$^{32}P_i$ exchange*

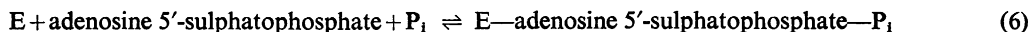
A reaction mixture was incubated and analysed as described in Fig. 1 except that adenosine 5′-sulphatophosphate was replaced by 0.5 μmol of ADP.

The unconventional double-reciprocal plots (Figs. 2 and 3) suggest a complex mechanism (Cleland, 1963) that may involve the formation of a ternary complex of enzyme, adenosine 5′-sulphatophosphate and P_i. The way in which the ternary complex may be formed, however, cannot yet be determined from the present results. This would contrast with the mechanism proposed by Grunberg-Manago *et al.* (1966), with ADP as substrate, in which an enzyme–AMP complex is proposed, involving a Ping-Pong mechanism.

Of the five exchange reactions tested only those involving exchange of P_i into ADP or adenosine 5′-sulphatophosphate were shown to proceed as indicated (reactions 1, 2 and 5). Reactions involving the exchange of SO_4^{2-} into adenosine 5′-sulphatophosphate (reactions 3 and 4) were not detectable.

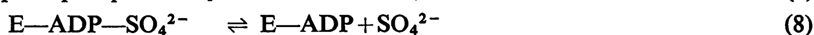
The presence of the ADP–P_i exchange reaction complicates a consideration of the adenosine 5′-sulphatophosphate-dependent activity. Unlabelled adenosine 5′-sulphatophosphate is converted into labelled ADP, which could then be transferred into unlabelled ADP through the ADP–P_i exchange reaction. Consequently the results obtained with adenosine 5′-sulphatophosphate as a substrate are a combination of the rates of conversion of adenosine 5′-sulphatophosphate into ADP and of the ADP–P_i exchange.

A possible qualitative reaction mechanism consistent with the results presented is shown below, but at this stage it is not possible to give rate equations:



$$\text{E} + \text{adenosine 5}'\text{-sulphatophosphate} + \text{P}_i \rightleftharpoons \text{E—adenosine 5}'\text{-sulphatophosphate—P}_i \quad (6)$$

$$\text{E—adenosine 5}'\text{-sulphatophosphate—P}_i \rightarrow \text{E—ADP—SO}_4^{2-} \quad (7)$$



$$\text{E—ADP—SO}_4^{2-} \rightleftharpoons \text{E—ADP} + \text{SO}_4^{2-} \quad (8)$$

$$\text{E—ADP} \rightleftharpoons \text{E} + \text{ADP} \quad (9)$$

$$\text{E—ADP} + {}^{\bullet}\text{P}_i \rightleftharpoons \text{E—AD}^{\bullet}\text{P} + \text{P}_i \quad (10)$$

$$\text{E—AD}^{\bullet}\text{P} \rightleftharpoons \text{E} + \text{AD}^{\bullet}\text{P} \quad (11)$$

In this scheme AD•P and •P_i represent substrates that undergo exchange reactions with P_i and ADP respectively. The sequence of formation of the ternary complex enzyme–adenosine 5′-sulphatophosphate–P_i is not specified and the location of the irreversible step is not known, although reaction (7) is a possibility. Reactions (9), (10) and (11) have been shown to be reversible.

Such a mechanism would allow the irreversible conversion of adenosine 5′-sulphatophosphate into ADP, releasing inorganic sulphate. This may be comparable with the adenosine 5′-sulphatophosphate sulphatases found in animal tissues (Armstrong *et al.*, 1970; Bailey-Wood *et al.*, 1970), which metabolize adenosine 5′-sulphatophosphate irreversibly into AMP and inorganic sulphate.

The significance of the ADP–P_i exchange reaction is at present obscure, but the evidence presented here and the work of Grunberg-Manago *et al.* (1966) suggest that this is mediated by ADP sulphurylase rather than by another enzyme.

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