SOME ASPECTS OF INORGANIC SULPHUR METABOLISM

Part I. Production and Degradation of Sulphur Nucleotides in Microorganisms and Plants

Part II. Uptake and Utilization of Inorganic Sulphur Compounds in *Thiobacillus ferrooxidans*

by

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A thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

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PREFACE

Part of the work described in this thesis has been presented to the Australian Biochemical Society Annual Conferences (La Trobe, 1975; Armidale, 1976). Some of the results have been published or submitted for publication in the journals listed:

1. "A bioluminescence method for determining adenosine 3'-phosphate 5'-phosphate (PAP) and adenosine 3'-phosphate 5'-sulfatophosphate (PAPS) in biological materials"
   P.E. Stanley, B.C. Kelley, O.H. Tuovinen and D.J.D. Nicholas
   Analytical Biochemistry 67, 540-551 (1975)

2. "The use of a bioluminescence method to study the metabolism of adenosine 3'-phosphate 5'-sulphatophosphate (PAPS) in microorganisms and plants"
   B.C. Kelley, P.E. Stanley, O.H. Tuovinen and D.J.D. Nicholas
   Proceedings of the Australian Biochemical Society 8, 39 (1975)

3. "Fate of adenosine 5'-sulphatophate (APS) and adenosine 3'-phosphate 5'-sulphatophosphate (PAPS) in various biological materials"
   B.C. Kelley, O.H. Tuovinen, S.K. Sawhney and D.J.D. Nicholas
   Proceedings of the Australian Biochemical Society 8, 34 (1975)

4. "The uptake and assimilation of sulphate by *Thiobacillus ferrooxidans*"
   O.H. Tuovinen, B.C. Kelley and D.J.D. Nicholas
   Archives of Microbiology 105, 123-127 (1975)

5. "Enzymic comparisons of the inorganic sulphur metabolism in autotrophic and heterotrophic *Thiobacillus ferrooxidans*"
   O.H. Tuovinen, B.C. Kelley and D.J.D. Nicholas
   Canadian Journal of Microbiology 22, 109-113 (1976)

6. "The degradation of sulphur nucleotides in cell-free extracts of *Thiobacillus ferrooxidans*"
   O.H. Tuovinen, B.C. Kelley and D.J.D. Nicholas
   Zeitschrift für Allgemeine Mikrobiologie (in press)

7. "Utilization of ^{35}S-thiosulphate and an appraisal of the role of ATP-sulphurylase in chemolithotrophic *Thiobacillus ferrooxidans*"
   B.C. Kelley, O.H. Tuovinen and D.J.D. Nicholas
   Archives of Microbiology (in press)

8. "Utilization of thiosulphate by *Thiobacillus ferrooxidans*"
   B.C. Kelley, O.H. Tuovinen and D.J.D. Nicholas
   Proceedings of the Australian Biochemical Society 9, 30 (1976)

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Finally, I wish to sincerely thank my parents, Mr. and Mrs. R.S. Kelley.

DECLARATION

I hereby declare that the work presented in this thesis has been performed by myself between March 1973 and February 1976. To the best of my knowledge, no material described herein has been submitted in any previous application for a degree in any university, or reported by any other person, except where due reference is made in the text.

BRUCE KELLEY

NOMENCLATURE AND ABBREVIATIONS

The major enzymes mentioned in this thesis are listed below with their numbers and systematic names as recommended by the Enzyme Commission (Florkin & Stortz, 1973).

Trivial Name	*E.C. Name and Number*
Adenosine triphosphatase	ATP phosphohydrolase E.C. 3.6.1.3
Adenylate kinase	ATP : AMP phosphotransferase E.C. 2.7.4.3
ADP-sulphurylase	ADP : sulphate adenylyltransferase E.C. 2.7.7.5
AMP-independent sulphite oxidase	sulphite : oxygen oxidoreductase E.C. 1.8.3.1
APS-kinase	ATP : adenylylsulphate-3'-phosphotransferase E.C. 2.7.1.25
APS-reductase	Adenylylsulphate reductase E.C. 1.8.99.2
ATP-sulphurylase	ATP : sulphate adenylyltransferase E.C. 2.7.7.4
Inorganic pyrophosphatase	Pyrophosphate phosphohydrolase E.C. 3.6.1.1
NADPH-sulphite reductase	Hydrogen-sulphide : $NADP^+$ oxido-reductase E.C. 1.8.1.2
Rhodanese	Thiosulphate : cyanide sulphur-transferase E.C. 2.8.1.1

The standard abbreviations for chemicals and symbols in general follow either the tentative rules of the IUPAC-IUB Commission on Biochemical Nomenclature [Biochem. J. (1966) 101, 1-7] or the Instructions to Authors for The Biochemical Journal [Biochem. J. (1973) 131, 1-20].

Chemicals

ADP	adenosine 5'-diphosphate
5'-AMP	adenosine 5'-monophosphate
3'-AMP	adenosine 3'-monophosphate
APS	adenosine 5'-phosphosulphate
ATP	adenosine 5'-triphosphate
CCl_4	carbon tetrachloride
C_6H_6	hexane
CTP	cytidine 5'-triphosphate
CO_2	carbon dioxide
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
DNP	2,4-dinitrophenol
DD-H_2O	double glass-distilled water
EDTA	ethylenediamine tetracetic acid (Na salt)
ETSH	β-mercaptoethanol
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
GSH, GSSG	glutathione and its oxidized form
GTP	guanosine 5'-triphosphate
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine phosphodinucleotide

NEM	N-ethylmaleimide
PAP	adenosine 3'-phosphate 5'-phosphate
PAPS	adenosine 3'-phosphate 5'-phosphosulphate
PCA	perchloroacetic acid
Pi	inorganic phosphate
PPi	inorganic pyrophosphate
POPOP	1,4-bis-[2-(4-methyl-5'-phenyloxazolyl)]-benzene
PPO	2,5-diphenyloxazole
S_8	elemental sulphur
-SH	sulphydryl
TCA	trichloroacetic acid
Tris	Tris(hydroxymethyl)aminomethane
TTP	thymidine 5'-triphosphate

Symbols and Units

A	absorbance
c.p.m.	counts per minute
d.p.m.	disintegrations per minute
E	extinction
E'_o	standard electrode potential at a given pH
g	gram
g	unit of gravitation
h	hour(s)
K_m	Michaelis constant
ℓ	litre
µg	microgram
µmole	micromole
µl	microlitre

mg	milligram
mmole	millimole
ml	millilitre
µM	micromolar
mM	millimolar
M	molarity
N	normality
nmole	nanomole
pmole	picomole
min	minute
M.W.	molecular weight
%	per cent
lb	pound
in.	inch
p.s.i.	pounds per square inch
r.p.m.	revolutions per minute
sec	second
u.v.	ultra-violet
λ	wavelength
Ci	curie
mCi	millicurie
µCi	microcurie
S_{10}	supernatant fraction obtained after centrifuging homogenate at 10,000 x *g* for 30 min
P_{10}	pellet fraction obtained after centrifuging homogenate at 10,000 x *g* for 30 min
S_{105}	supernatant fraction obtained after centrifuging S_{10} at 105,000 x *g* for 90 min
P_{105}	pellet fraction obtained after centrifuging S_{10} at 105,000 x *g* for 90 min

Temperatures are expressed as degrees Centigrade (°C).

TABLE OF CONTENTS

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

SUMMARY

SUMMARY

PART I. Production and Degradation of Sulphur Nucleotides in Microorganisms and Plants

1. A rapid, sensitive, bioluminescence technique has been developed for detecting PAPS (adenosine 3'-phosphate 5'-phosphosulphate) in biological materials. PAPS is first hydrolysed in 0.2 N HCl to PAP (adenosine 3'-phosphate 5'-phosphate) and is then assayed by the luciferin-luciferase system of the sea pansy, *Renilla reniformis*, which is specific for PAP. This bioluminescence system produces light at a rate that is proportional to the amount of PAP present. Light emission is measured in a liquid scintillation spectrometer with the two photomultipliers out of coincidence. Amounts as low as 10 pmoles PAP may be determined by this procedure.

2. The method has been used to follow PAPS formation from sulphate and ATP in a variety of biological materials. Thus, PAPS produced was detected in very small amounts (50-180 pmoles/min/mg protein) in extracts of green plants. On adding 5'-AMP to the reaction mixture, the production of PAPS was enhanced about ten-fold.

PAPS produced in extracts of baker's yeast from (a) sulphate and ATP and (b) APS and ATP was readily monitored by the bioluminescence method.

3. PAPS was rapidly degraded in extracts of young wheat leaves and baker's yeast via APS to sulphate whereas, in cell-free extracts of *Thiobacillus ferrooxidans*, sulphate was cleaved first from PAPS. The

fate of APS was also investigated in extracts of this bacterium. The stabilizing effects of nucleotides, phosphate and pyrophosphate on the degradation of these sulphur nucleotides were examined.

PART 2. Uptake and Utilization of Inorganic Sulphur Compounds in *Thiobacillus ferrooxidans*

1. Differentially labelled ^{35}S-thiosulphate [$^{35}S.SO_3$, $S.^{35}SO_3$] was taken up by washed cells of *Thiobacillus ferrooxidans* grown on thiosulphate. The uptake, which was proportional to the biomass over the range 0.5-4.0 mg dry weight, showed typical saturation kinetics, with an estimated K_m value of 0.5 mM for thiosulphate. Dithionate and Group VI anions inhibited the uptake, which was under pH control and had a temperature optimum of 50°.

In the absence of thiosulphate, the cells rapidly bound ^{35}S-sulphate (within 15 seconds) but this effect did not increase on incubating further. Moreover, the label was removed completely by washing with dilute sulphuric acid, indicating that sulphate was not assimilated.

Over a 60 minute period, increasing amounts of the label were incorporated into cellular materials from the [outer-^{35}S]thiosulphate. There was little or no incorporation of the label from the [inner-^{35}S]thiosulphate.

2. The following enzymes which mediate the oxidation of thiosulphate to sulphate and the assimilation of sulphate to sulphide were assayed in various cell-free fractions of *Thiobacillus ferrooxidans* grown autotrophically with either ferrous-iron or thiosulphate, or

heterotrophically with glucose: Thiosulphate-oxidizing enzyme, rhodanese, AMP-independent sulphite oxidase, APS-reductase, ATP-sulphurylase, ADP-sulphurylase, adenylate kinase, and NADPH-linked sulphite reductase. Thiosulphate-oxidizing enzyme was not detected in extracts of bacteria grown with ferrous-iron. Comparable activities for ATP-sulphurylase, ADP-sulphurylase and adenylate kinase were found in bacteria grown autotrophically with either ferrous-iron or thiosulphate and heterotrophically with glucose.

3. The kinetic properties of partially purified ATP-sulphurylase from bacteria grown with thiosulphate and ferrous-iron were similar. This enzyme has an assimilatory function only when the bacteria are grown with ferrous-iron. The possible function of the enzyme in thiosulphate-grown cells is discussed.

1. GENERAL INTRODUCTION

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The chemical changes which sulphur compounds undergo in living organisms are numerous and varied in character. The electronic structure of sulphur is such that the element exhibits a range of valency states [sulphate (+6) to sulphide (-2)] in various compounds, as shown in Table 1. Many of these compounds which occur in nature undergo oxidation-reduction reactions, often mediated by enzymes.

Sulphur, in its most oxidized form (sulphate), is found in living organisms as mucopolysaccharides (e.g. keratosulphates, chrondroitin sulphates and heparin). Sulphur is an important constituent of amino acids such as cysteine and methionine, of vitamins such as biotin and thiamin, and of cofactors such as coenzyme A and lipoic acid. Microorganisms and plants derive inorganic compounds of sulphur directly from the environment and incorporate them into organic compounds which are then utilized by animal systems. The biological changes mediated by the sulphur cycle (Figure 1) can be considered as follows:

1. Reduction of sulphate to sulphide, which is either released into the atmosphere as H_2S (dissimilatory sulphate reduction, e.g. *Desulphovibrio* and *Desulphomaculum*), or is further converted to thiols, and then incorporated into cellular material (assimilatory sulphate reduction, e.g. yeast and green plants).
2. Biochemical decomposition and conversion of the organic sulphur compounds into inorganic forms such as sulphides, thiosulphates, polythionates and elemental sulphur.
3. Oxidation of reduced inorganic sulphur compounds to sulphates, mainly by chemolithotrophic (e.g. *Thiobacillus*) and

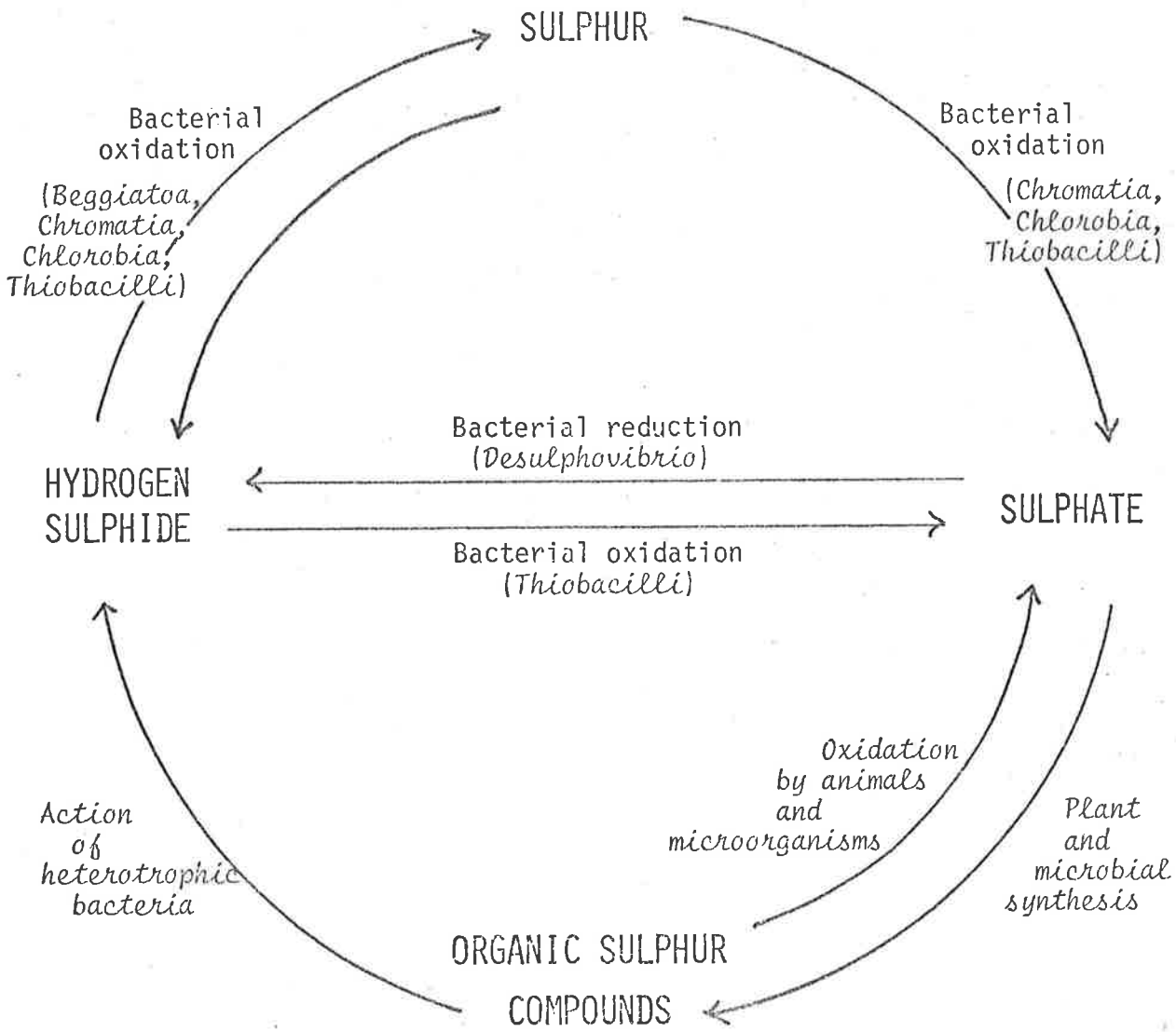
TABLE 1

Oxidation-reduction states and potentials of some sulphur compounds

[Data taken from Latimer (1952), Long (1961), Nicholas (1967) and Loach (1968)]

Compound	Formula	Oxidation-reduction state of sulphur atom	Oxidation-reduction potential	
			Couple	E_0' volts (pH 7.0)
Sulphate	SO_4^{2-}	+6	SO_4^{2-}/SO_3^{2-}	-0.454
Dithionate	$S_2O_6^{2-}$	+5		
Sulphite	SO_3^{2-}	+4	$SO_3^{2-}/S_2O_4^{2-}$	-0.527
Tetrathionate	$S_4O_6^{2-}$	+4		
Dithionite	$S_2O_4^{2-}$	+3	$S_2O_4^{2-}/S_2O_3^{2-}$	+0.484
Sulphur dioxide	SO_2	+3	$S_4O_6^{2-}/S_2O_3^{2-}$	-0.240
Sulphur monoxide	SO	+2	S^0/S^{2-}	-0.280
Thiosulphate	$S_2O_3^{2-}$	+2	S^0/H_2S	-0.243
Elemental sulphur	S^0	0	S^0/SH^-	-0.272
Sulphide	S^{2-}	-2	GSSG/GSH	-0.340
			Cystine/Cysteine	-0.340
			Lipoic acid	-0.289

FIGURE 1

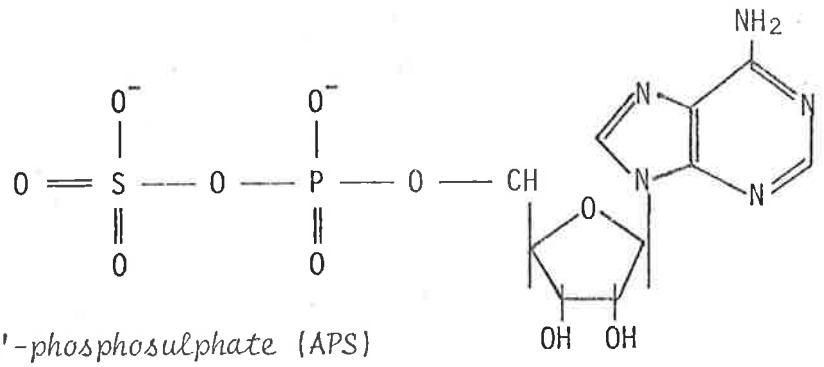


The Sulphur Cycle

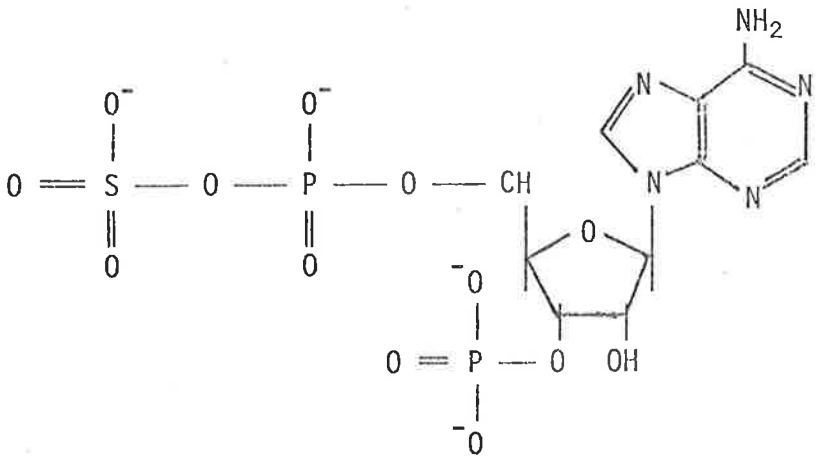
[Adapted from Young & Maw (1958)]

photolithotrophic bacteria (e.g. *Chromatium*). Such oxidations produce the energy required for synthetic processes.

The synthesis of sulphate esters involves the formation of an "active sulphate", a process which requires the utilization of two molecules of ATP per molecular of sulphate. The key intermediates in the activation of sulphate are the two sulphate-containing nucleotides, adenosine 5'-phosphosulphate (APS) and adenosine 3'-phosphate 5'-phosphosulphate (PAPS). The structures of these two compounds are as follows:-



Adenosine 5'-phosphosulphate (APS)



Adenosine 3'-phosphate 5'-phosphosulphate (PAPS)

These activated forms of sulphate (APS and PAPS) participate not only in the formation of sulphate esters but also in the activation and reduction of sulphate via sulphite to sulphide (dissimilatory and assimilatory sulphate reduction).

In animal systems, PAPS functions as a donor for sulphate transfer reactions, resulting in sulphate ester formation. The esters include phenol, steroid, polysaccharide, choline, cerebroside and flavonoid sulphates. These reactions are catalysed by sulphotransferase enzymes. Aspects of sulphate transfer have been reviewed by Roy & Trudinger (1970), Schiff & Hodson (1973) and De Meio (1975).

In yeast, PAPS is reduced to sulphite by a complex reducing system, "PAPS-reductase" (Wilson *et al.*, 1961; Asahi *et al.*, 1961; Torii & Bandurski, 1964,1967). Recent studies of assimilatory reduction in *Chlorella* suggest that APS, rather than PAPS, is the main substrate for reduction (Schmidt, 1972; Schiff & Hodson, 1973). It has been suggested that a combination of APS : thiol sulphotransferase and thiosulphonate reductase is involved (Schmidt, 1972; Schiff & Hodson, 1973). APS-reductase is present in all the dissimilatory sulphate-reducing bacteria which have so far been studied and it appears to be absent from assimilatory sulphate-reducing organisms (Peck, 1961*a*,1962). The roles of APS and PAPS during assimilatory sulphate reduction, especially in plant systems, have not been fully elucidated. Studies on the metabolism of these sulphur nucleotides are further complicated by their instability in crude extracts of biological materials.

Important advances have been made with the metabolism of inorganic sulphur compounds in microorganisms, which utilize a wide range of these substances at diverse points in the sulphur cycle, e.g. the assimilation and dissimilation of sulphate and the oxidation of reduced sulphur compounds to sulphate by chemolithotrophic bacteria. Even though most of the thiobacilli are chemolithotrophic bacteria, some can also grow heterotrophically. *Thiobacillus ferrooxidans*, for example, is a versatile bacterium since it can grow chemolithotrophically

on ferrous sulphate or thiosulphate, and also heterotrophically on glucose. Studies with this bacterium cover a wide range of metabolic activity.

The present thesis is sub-divided into two parts, namely

PART I : Production and Degradation of Sulphur Nucleotides in Microorganisms and Plants (page 30)

and PART II : Uptake and Utilization of Inorganic Sulphur Compounds in *Thiobacillus ferrooxidans* (page 57).

The Materials and Methods section (page 5) covers both parts. The aims of the work are given in the introductions to Parts I and II.

2. MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 Preparation of solutions and buffers

All aqueous solutions and buffers were dispensed in double glass-distilled water at room temperature. The stock solutions of 0.25 M phosphate, Tris-HCl and citrate buffers were prepared by the method of Gomori (1955) and were stored at 2°. The pH of the buffers was determined at room temperature (20-25°) except for Tris-HCl buffers, where temperature adjustments were necessary (Sigma Technical Bulletin, 106 B, 1967). The pH was determined with a Beckman H5 pH meter, and the electrode was standardized routinely before use with buffer (pH 7.0) provided by Beckman Instruments Inc. (Fullerton, California, U.S.A.).

2.2 Biological materials

2.2.1 Bacteria

A strain of *Thiobacillus ferrooxidans* (Tuovinen & Kelly, 1973) was grown in 50 ℓ of a medium supplemented with ferrous sulphate (Tuovinen & Kelly, 1973). The thiosulphate-oxidizing culture of *T. ferrooxidans* was originally derived from this strain (Tuovinen & Kelly, 1974b) and maintained in the liquid thiosulphate-containing medium (Bounds and Colmer, 1972) for about three years before the experiments described in the present study were commenced. A heterotrophic culture of *T. ferrooxidans* (Shafia & Wilkinson, 1969; Shafia *et al.*, 1972) was kindly provided by Dr. F. Shafia (Department of Biological Sciences, California State Polytechnic College, Pomona, California, U.S.A.).

The culture of *Desulfotomaculum nigrificans* (N.T.C.C. 8351) was

a generous gift from Dr. P.A. Trudinger (Bureau of Mineral Resources and Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization, Canberra, A.C.T., Australia).

2.2.2 Yeast

Baker's yeast (*Saccharomyces cerevisiae*), obtained as frozen blocks (Pinnacle) from Mauri Bros. and Thompson (Adelaide, South Australia), was stored at -15° before use.

2.2.3 Higher plants

The plant materials used in this study were as follows:- (i) shoots of wheat (*Triticum vulgare* cv Insignia), (ii) shoots of sorghum (*Sorghum bicolor* x Sudan grass hybrid), (iii) young green leaves of spinach (*Spinacea oleracea*), and (iv) explants from artichoke tuber (*Helianthus tuberosus*), kindly supplied by Dr. J.F. Jackson of this Department.

2.2.4 *Renilla reniformis*

The acetone powder of *Renilla reniformis* and the luciferyl sulphate (Hori *et al.*, 1972) were kindly donated by Professor Milton Cormier (Department of Chemistry, University of Georgia, Athens, Georgia, U.S.A.).

2.2.5 Enzymes

Alcohol dehydrogenase, glucose-6-phosphate dehydrogenase and desiccated firefly lanterns (*Photinus pyralis*) were purchased from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.).

2.3 Radioisotopes

Carrier-free ^{35}S-sulphate, ^{35}S-sulphite, differentially labelled ^{35}S-thiosulphate and U-^{14}C-5'-AMP were purchased from The Radiochemical Centre (Amersham, Bucks., England); ^{35}S-PAPS was from New England Nuclear (Boston, Massachusetts, U.S.A.). U-^{14}C-APS and ^{35}S-APS were prepared from either sulphite and U-^{14}C-5'-AMP or ^{35}S-sulphite and 5'-AMP using a partially purified APS-reductase enzyme from *T. denitrificans* (Adams *et al.*, 1971). The radiochemical purity and the specific activity of all radioisotopes were checked routinely by separating them on 3MM paper by high-voltage electrophoresis (Section 2.9.1) followed by radioassay (Section 2.9.3). Radioactive sulphite was kept sealed under O_2-free N_2 at -15° to prevent oxidation.

2.4 Chromatographic materials

Whatman DEAE-cellulose (types 11 and 32) and Whatman 3MM chromatographic paper were supplied by H. Reeve Angel and Co. Ltd. (London, England), and Sephadex G-25, G-100 and G-200, by Pharmacia (Uppsala, Sweden).

2.5 Chemicals

3'-AMP, 5'-AMP, ADP, ATP, NADH, NADPH, APS, FMN, FAD, adenosine and D-luciferin were obtained from the Sigma Chemical Co. (St. Louis, Missouri, U.S.A.). Adenine was purchased from Koch-Light Laboratories Ltd. (Colnbrook, Bucks., England) and PAP was from Boehringer Mannheim GmbH (Tutzing, West Germany). Na_2SO_3 (anhydrous) was from By-Products and Chemicals Pty. Ltd. (Alexandria, New South Wales, Australia). Anhydrous $Na_2H_2P_2O_7$ and $NH_4H_2PO_4$ were purchased from British Drug Houses Ltd. (Poole, Dorset, England).

2,5-Diphenyloxazole (PPO) and 1,4-*bis*[2-(4-methyl-5-phenyloxazol-2-yl)]-benzene (POPOP) were obtained from Packard Instrument Co. (Chicago, Illinois, U.S.A.).

All other chemicals were of the best quality and purity available, obtained from one or more of the following sources: Ajax Chemical Co. (Auburn, New South Wales, Australia), May and Baker (Dagenham, England) and BDH Chemicals Ltd. (Poole, Dorset, England).

2.6 Culture of bacteria

A strain of *T. ferrooxidans* was grown at 28° in 55 ℓ carboys containing 50 ℓ of a medium supplemented with ferrous sulphate (Tuovinen & Kelly, 1973). The cultures were aerated with 4 ℓ of sterilized air per min. The thiosulphate- and glucose-oxidizing strains were grown at 28° in defined culture solutions (Shafia & Wilkinson, 1969; Bounds & Colmer, 1972) in 8 ℓ batches sparged with 0.5 ℓ of sterilized air per min per ℓ medium. The compositions of the three culture solutions are presented in Table 2. The bacteria, maintained in liquid culture, were sub-cultured once a week. Microscopic examinations and plating experiments were conducted periodically by Dr. O.H. Tuovinen of this Department to check the purity of the cultures.

Desulfotomaculum nigrificans was sub-cultured in a culture solution containing (g/ℓ distilled water):- "Bacto-Peptone" or "Bacto-Tryptone" (Difco), 5; yeast extract (Difco), 4; Na_2SO_4, 1.5; $MgSO_4 \cdot 7H_2O$, 1.5, and glucose, 10, at 28° for 48 h in 5 ℓ batches (Postgate, 1951).

The bacteria, at the late exponential phase of growth, were harvested in a Sorvall Superspeed RC-2 refrigerated centrifuge fitted with a continuous-flow rotor (SS-34). *T. ferrooxidans*, harvested from the ferrous-iron and glucose cultures respectively, was washed once with cold 0.01 N H_2SO_4 and then with 50 mM Tris-HCl buffer (pH 7.5)

TABLE 2

Compositions of culture solutions for T. ferrooxidans

The glucose and thiosulphate media were dispensed, autoclaved (Smith Industries, South Australia), cooled and then inoculated. The ferrous-iron medium, prepared in 55 ℓ carboys, was not autoclaved. The conditions for growing the bacteria are given in Section 2.6.

NUTRIENT		
a)	Glucose-grown culture	Addition/8 ℓ
	Glucose	80 g
	KH_2PO_4	8 g
	$(NH_4)_2SO_4$	16 g
	$MgSO_4 \cdot 7H_2O$	1.6 g
	Trace metal solution	80 ml
	N H_2SO_4	80 ml
b)	Thiosulphate-grown culture	Addition/8 ℓ
	KH_2PO_4	24 g
	$(NH_4)_2SO_4$	24 g
	$MgSO_4 \cdot 7H_2O$	4 g
	$CaCl_2 \cdot 2H_2O$	2 g
	$Na_2S_2O_3 \cdot 5H_2O$	40 g
c)	Ferrous-iron-grown culture	Addition/8 ℓ
	36 N H_2SO_4	154 ml
	K_2HPO_4	20 g
	$(NH_4)_2SO_4$	20 g
	$MgSO_4 \cdot 7H_2O$	20 g
	$FeSO_4 \cdot 7H_2O$	1700 g

containing 1 mM Na-EDTA. The bacteria grown on thiosulphate were washed twice in the Tris-EDTA buffer described above. All operations were carried out at 2°. *D. nigrificans* was washed twice in 100 mM potassium phosphate buffer (pH 6.8). If the cells were not used immediately, they were stored as a paste at -15°.

2.7 Growth of plant material

Seeds of wheat and sorghum were surface sterilized by soaking in a 0.02% (w/v) solution of mercuric chloride. Excess mercuric chloride was removed by throughly washing the seeds with double-distilled water. The seeds, transferred to moist vermiculite in trays, were then grown in the dark for 4 days at 28° following germination. After 4 days, young shoots were harvested in the dark and washed twice with cold double-distilled water. Plants were, in the first instance, grown under dark conditions to minimize any colour quenching (from chlorophyll) in the bioluminescence assay. However, this was subsequently found to have no effect because the substantial dilutions employed eliminated any interference by colour quenching.

Chloroplast preparations were made from plants grown under identical conditions, except that they were exposed to light for 10 days following germination at 28°.

Young green leaves of spinach harvested from a local garden were washed thoroughly with cold double-distilled water.

Explants from artichoke tuber were grown for 48 h at about 20° according to the procedure of Yeoman & Evans (1967). The explants were washed with cold double-distilled water after harvest to remove any residual traces of the mineral salts from the growth medium.

2.8 Enzyme techniques

2.8.1 Preparation of homogenates and cell-free extracts

2.8.1.1 Bacteria

All operations were carried out at 2°. After the cells had been thoroughly washed with the appropriate solutions (Section 2.6), they were suspended in four volumes (w/v) of 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM Na-EDTA, and the homogenates were prepared by passing the cell suspension three times through a chilled Aminco French Pressure Cell (American Instrument Co., Maryland, U.S.A.) at 20,000 p.s.i. (Hughes *et al.*, 1971). The pH of the homogenate was adjusted to pH 7.5 with M NaOH and the homogenate was centrifuged at 10,000 x *g* for 30 min in a Sorvall-SS3 automatic superspeed centrifuge (SS-34 rotor) to sediment unbroken cells and cell debris. The resultant supernatant fraction (S_{10}) was used and is referred to as the crude extract.

The crude extract (S_{10}) was centrifuged at 105,000 x *g* for 90 min (Spinco, Model L, rotor type 30), resulting in a supernatant fraction (S_{105}) and a pellet or membrane fraction (P_{105}). The pellet fraction (P_{105}) was washed by resuspending it in 50 mM Tris-HCl buffer (pH 7.5) and recentrifuging it at the same speed for 60 min. A homogeneous suspension of the washed pellet was then prepared in a glass-glass homogenizer.

2.8.1.2 Yeast

A cell-free extract (S_{60}) from baker's yeast was prepared by the method of Hawes & Nicholas (1973). All procedures were carried out at 2° in 50 mM Tris-HCl buffer (pH 7.5) containing

1 mM Na-EDTA. Dried yeast (50 g) was suspended in 100 ml of the cold buffer. The cell suspension was then passed three times through a chilled Aminco French Pressure Cell at 20,000 p.s.i. as described previously (Section 2.8.1.1). The pH of the homogenate was adjusted to pH 7.5 with M NaOH, and the extract was centrifuged at 27,000 x *g* for 1 h in a Sorvall SS-3 refrigerated automatic superspeed centrifuge (SS-34 rotor). The supernatant fraction (S_{27}) was then centrifuged at 60,000 x *g* for 30 min (Spinco model L, rotor type 50 Ti or type 30). The bulk of the enzyme activity was located in the supernatant fraction (S_{60}). The pH of this fraction was adjusted to pH 7.5 with M NaOH.

2.8.1.3 Higher plants

Cell-free extracts were prepared by freezing 10 g of the fresh plant material in liquid nitrogen, pulverizing in a top-drive homogenizer (Sorvall Omnimixer, Ivan Sorvall Inc., Newton, Connecticutt, U.S.A.) and extracting in 40 ml of 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM Na-EDTA and 1 mM β-ETSH. The supernatant fraction, prepared by centrifuging the extract at 20,000 x *g* for 40 min, was used for enzyme assays.

Intact chloroplasts were prepared by an aqueous technique as described by Hadziyev *et al.* (1968). Freshly harvested wheat leaves were cooled at 2°, cut and mixed with Honda medium [Ficoll, 2.5% (w/v); dextran, 5% (w/v); sucrose, 0.25 M; Tris-HCl buffer (pH 7.8), 25 mM; $MgCl_2$, 1 mM; β-ETSH, 4 mM] in a ratio 1:2 (w/v), and then homogenized in a mortar and pestle at 2°. The resultant slurry was pressed through eight layers of cheese cloth to yield a debris-free homogenate. The latter was centrifuged at 2,300 x *g* for 10 min. The chloroplast pellet was suspended in Honda medium and again centrifuged for 10 min. The washed chloroplast pellet was resuspended in Honda

medium and was layered onto a discontinuous gradient containing 7 ml each of 2.5, 2.0, 1.5 and 1.0 M sucrose (Sager & Ishida, 1963) in Honda medium. The gradient was centrifuged at 20,000 x *g* for 40 min. The various bands obtained were examined under a light microscope and by phase contrast microscopy. The main band, consisting of more than 90% of the applied solution, was positioned as the third band from the top of the gradient and contained the intact chloroplasts. This third band was not present when a broken chloroplast preparation was used with the continuous gradient technique. The intact chloroplasts from the third layer were collected and diluted with Honda medium lacking sucrose, and again centrifuged at 2,300 x *g* for 10 min. This washing procedure was repeated three times at 2°, resulting in a clean preparation of intact chloroplasts.

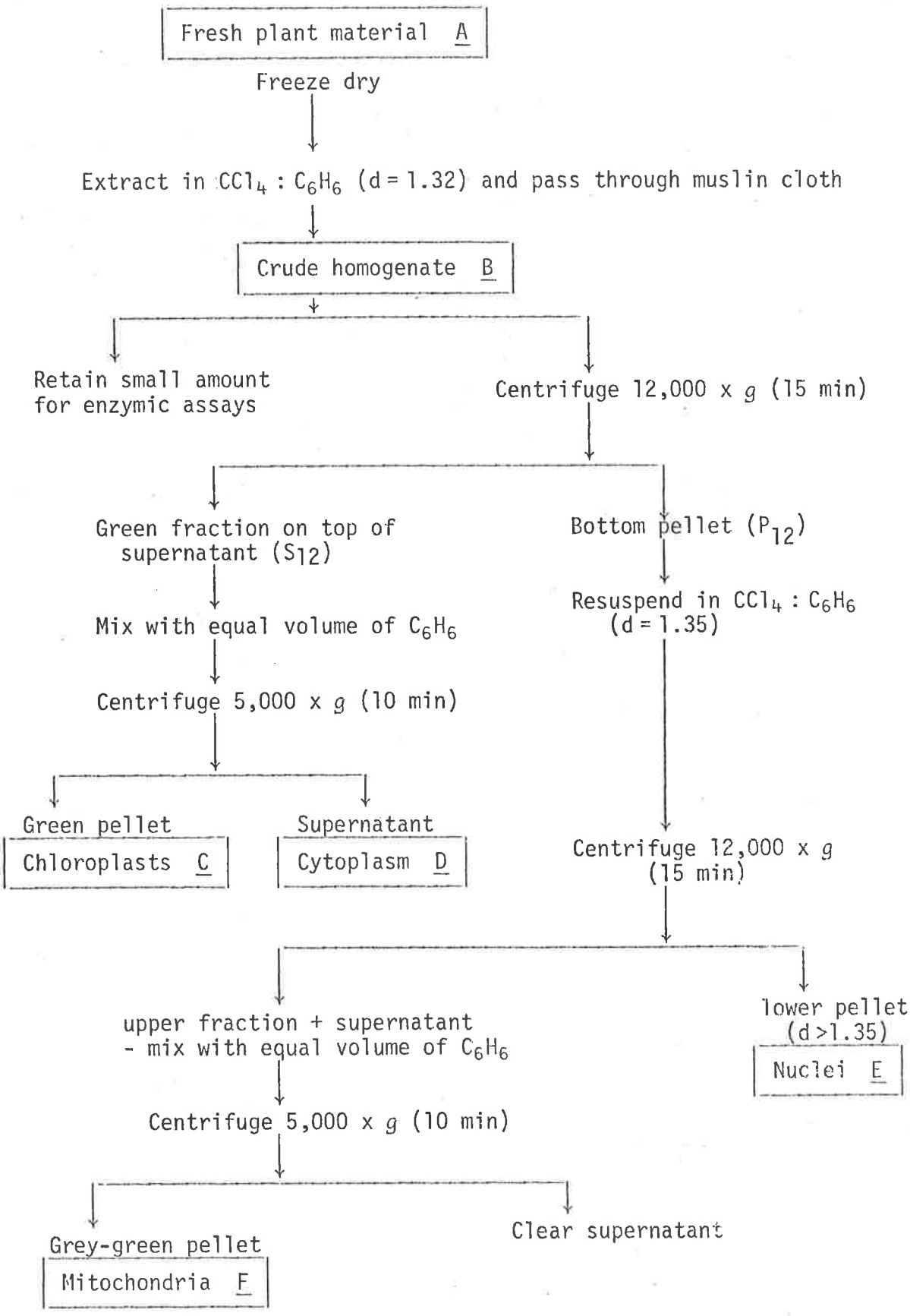
Various fractions of wheat leaves were also prepared by a non-aqueous technique as described by Stocking (1959), except that the densities of $CCl_4 : C_6H_6$ used were different. The method has been summarized as a flow chart in Figure 2. The freeze-dried wheat leaves (A) were extracted in $CCl_4 : C_6H_6$ mixture (d = 1.32) at -15° by gentle maceration in a chilled mortar and pestle. A small amount of the homogenate (B) was retained for enzyme assay and the remainder was centrifuged at 12,000 x *g* for 15 min. Layered on top of the supernatant fraction was a green band of chloroplasts. This fraction, together with the supernatant, was further resolved into chloroplast and cytosol fractions by mixing with an equal volume of C_6H_6 and recentrifuging at 5,000 x *g* for 10 min. The pellet fraction (C) predominantly contained intact chloroplasts and the supernatant fraction (D) was primarily cytosol. Other cellular components were prepared from the bottom pellet fraction obtained by centrifuging the crude homogenate (B) ($CCl_4 : C_6H_6$, d = 1.32) at 12,000 x *g* for 15 min as described previously.

FIGURE 2

Non-aqueous preparation of various cell fractions of wheat leaves

Various fractions of wheat leaves were prepared by a non-aqueous technique as described by Stocking (1959), except that the densities of $CCl_4 : C_6H_6$ used were different. The technique is described in Section 2.8.1.3.

FIGURE 2


Fresh plant material A
Freeze dry
Extract in CCl_4 : C_6H_6 (d = 1.32) and pass through muslin cloth
Crude homogenate B
Retain small amount for enzymic assays
Centrifuge 12,000 x g (15 min)
Green fraction on top of supernatant (S_{12})
Bottom pellet (P_{12})
Mix with equal volume of C_6H_6
Resuspend in CCl_4 : C_6H_6 (d = 1.35)
Centrifuge 5,000 x g (10 min)
Green pellet
Chloroplasts C
Supernatant
Cytoplasm D
Centrifuge 12,000 x g (15 min)
upper fraction + supernatant - mix with equal volume of C_6H_6
lower pellet (d>1.35)
Nuclei E
Centrifuge 5,000 x g (10 min)
Grey-green pellet
Mitochondria F
Clear supernatant

This pellet was resuspended in $CCl_4 : C_6H_6$ mixture of density 1.35 and centrifuged at 12,000 x *g* for 15 min. The resultant pellet (E) contained sub-cellular components having densities greater than 1.35, while the upper pellet fraction and supernatant contained components with densities between 1.32 and 1.35. The supernatant and upper pellet were recovered as a pellet by mixing with an equal volume of hexane and were centrifuged at 5,000 x *g* for 10 min. The pellets from the fractions with densities between 1.32 and 1.35, and greater than 1.35, were combined and designated as the sub-cellular components. All the fractions were evaporated under vacuum on ice and resuspended in 6 ml of 25 mM Tris-HCl buffer (pH 7.6), containing 0.3 M sucrose, and used for studying the degradation of sulphur nucleotides.

The chloroplasts prepared by this procedure were intact, as determined by phase contrast microscopy and a discontinuous sucrose gradient (Hadziyev *et al.*, 1968). For some experiments, intact chloroplasts were disrupted by ultrasonication for 5 min at 2° (MSE Ultrasonic Titanium Probe, 20 kilocycles/sec). In addition, the disrupted chloroplasts were centrifuged at 16,000 x *g* for 30 min and the supernatant fraction examined for PAP and PAPS degradation (Section 2.8.7).

2.8.2 Preparation of the firefly luciferin-luciferase system

The luciferin-luciferase extract was prepared by the method of Stanley & Williams (1969) with some modifications as follows. Six firefly lanterns were homogenized in a glass homogenizer (Kontas Glass Co., New Jersey, U.S.A.) with 3 ml cold 50 mM arsenate buffer pH 7.5). The homogenate was allowed to stand at 20° for 15 h and then placed on ice for 1 h before centrifuging at 12,000 x *g* for 30 min at

2° in a Sorvall-SS3 automatic superspeed centrifuge. A few crystals of D-luciferin were added to the supernatant fraction and the extract stored on ice until use.

2.8.3 Preparation of the *Renilla reniformis* luciferin-luciferase system

Ten g of the acetone powder of *Renilla reniformis* were added to 40 ml of cold 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM Na-EDTA and 1 mM β-ETSH, and the mixture was stirred continuously for 2 h at 2°. The resulting slurry was centrifuged at 35,000 x *g* for 20 min and the precipitate discarded. The supernatant fraction (S_{35}) was dialysed at 2° against four 1 ℓ changes of 10 mM potassium phosphate buffer (pH 7.5) containing 1 mM Na-EDTA and 1 mM β-ETSH, allowing at least 3 h between buffer changes. After dialysis, the extract was again centrifuged at 35,000 x *g* for 20 min and the pellet discarded. The luciferin sulphokinase and luciferase activities were retained in the supernatant fraction, which can be used at this stage for assay. The PAP content of this preparation was reduced by passing it through a Sephadex G-25 column equilibrated with the same buffer used for dialysis. The enzyme system was stable over several months when stored at -15°. When stored at 0-4°, the luciferase activity was quite stable but the sulphokinase activity decreased with a half-life of about 3 days.

2.8.4 Preparation of luciferyl sulphate

The luciferyl sulphate was supplied by Professor Milton Cormier (Section 2.2.4) as a stock solution dissolved in 10 mM potassium phosphate buffer (pH 7.5) containing 50% (v/v) ethanol to avoid bacterial

contamination. Luciferyl sulphate is acid-labile and must always be kept at pH 7.0 or above. It was maintained in potassium phosphate buffer (pH 7.5) at a concentration which delivered 150 pmoles/0.5 µl for each assay. The stock solutions and dilutions were stable when kept frozen. Any precipitate (phosphate) which formed during storage of the luciferyl sulphate was removed by centrifuging, although this is not critical.

An attempt was also made to extract luciferyl sulphate from luminescent alcyonarians (*Cavernularia obesa*), which were gathered from Sydney Harbour by Dr. J.R. Paxton, Department of Ichthyology, The Australian Museum, Sydney, Australia. The extractions were carried out using the methods of Hori & Cormier (1965) and Cormier *et al.* (1970). Unfortunately, the luciferyl sulphate product did not substitute in the *Renilla reniformis* bioluminescence reaction.

2.8.5 Preparation of PAP

At the beginning of this study, PAP was not commercially available and it was generously supplied by Professor Milton Cormier. PAP was dissolved in 10 mM potassium phosphate buffer (pH 7.0). E^{260}_{mM} for PAP is 14.6. This solution was stable indefinitely when kept frozen. During the assay, PAP (120 pmoles) was added in a volume of 2 µl as an internal standard.

PAP was also isolated and purified from aged commercial preparations of ATP. Amounts of PAP of up to 3% (w/w) were found as an impurity in these ATP samples, using the *Renilla* bioluminescence assay. A solution of the ATP sample (Na-salt) was adjusted to pH 7.5 with 2 N KOH. The sample was then loaded onto a DEAE-11 formate column (30 cm x 4 cm) and eluted with a linear gradient of 5 ℓ of

0.1 M NH_4HCO_3 and 5 ℓ of 0.6 M NH_4HCO_3, and 15 ml fractions were collected using an L.K.B. Ultrarac fraction collector. The position of the elution peak ($E_{254\ nm}$) corresponding to PAP was determined by the *Renilla* bioluminescence assay. Refractometer readings indicated that PAP was eluted at a concentration of approximately 0.2 M NH_4HCO_3. Samples containing PAP were pooled, evaporated to dryness in a rotary evaporator at 60°, thoroughly washed with double-distilled water and stored at 2°. The purity of the PAP preparation was checked at each stage of the purification by high-voltage electrophoresis (Tate, 1968) (Section 2.9.1). A pure preparation of PAP, suitable for use as an internal standard, was obtained after preparative paper chromatographic separation from contaminating nucleotides, using the method of Cornier (1962).

Commercial preparations of PAP are now available from Boehringer Mannheim GmbH (Tutzing, West Germany).

2.8.6 Synthesis of PAPS and PAP

The reaction mixture for the production of PAPS contained (μmoles/ml):- ATP, 25; Tris-HCl buffer (pH 8.5), 100; $MgCl_2$, 20; Na_2SO_4, 40, and 0.5 ml of the extract. After incubating in a reciprocating water bath for various periods up to 1 h at 30°, 0.1 ml aliquots were removed from the incubation mixtures and hydrolysed in two volumes (0.2 ml) of 0.2 N HCl at 37° for 30 min. Samples were then stored on ice prior to PAP determination by the *Renilla* bioluminescence assay (Section 3.2.1.1).

2.8.7 Incubation of cell-free extracts with ^{35}S-PAPS and ^{35}S-APS

The enzyme assays were carried out at 30° in a reciprocating water bath. Details of the incubation mixtures are given in the legends

to the tables in the Results section 3.2.2. The reactions were started by adding the cell-free extract. Each series of experiments had appropriate controls, including boiled extracts. At intervals during the incubation of wheat extracts, 50 µl samples were mixed with an equal volume of 99% (v/v) ethanol and centrifuged at 2,000 x *g* for 5 min, then the supernatant fraction applied onto Whatman 3MM paper. Samples (20 µl) from the incubation mixtures of yeast and *T. ferrooxidans* were applied directly onto the paper. The appropriate standards for the various nucleotides and labelled sulphur compounds were applied to separate lanes of the paper. The degradation of sulphur nucleotides in the controls containing boiled extract was found to be negligible in all experiments.

2.8.8 Binding and uptake of ^{35}S-thiosulphate and ^{35}S-sulphate by thiosulphate-grown *Thiobacillus ferrooxidans*

Cells grown with thiosulphate were washed twice with and resuspended in the following mineral salts solution (g/ℓ):- KH_2PO_4, 3; $(NH_4)_2SO_4$, 3; $MgSO_4 \cdot 7H_2O$, 0.5, and $CaCl_2$, 0.25 (pH 4). The washed cell suspensions were starved for 16 h at 30° in a gyratory shaker at 200 r.p.m. and standardized by dry-weight determinations (105°). Cell homogenates, in 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM Na-EDTA, were prepared on ice with an MSE ultrasonic probe at 20 kilocycles/sec for 15 min. The homogenate standardization was based on protein determination (Lowry *et al.*, 1951).

The uptake experiments were carried out in a reciprocating water bath at 30°. The properties of the uptake system were studied by pre-incubating the cell suspensions for 2 min (or for 5 min with inhibitors) before adding a mixture of cold thiosulphate and ^{35}S-

thiosulphate. After a further incubation of 5 min, 75-100 µl samples were filtered through Millipore discs (GSWP 02500) and washed with 10 ml of cold mineral salts solution. In experiments with ^{35}S-sulphate, cells were incubated with carrier-free ^{35}S-sulphate (0.64 µCi/ml culture/20 mg dry wt.) with or without 20 mM thiosulphate. Cultures grown with 20 mM thiosulphate received carrier-free ^{35}S-sulphate (0.028 µCi/ml culture) at inoculation, and the sulphur compounds ($S_2O_3^{2-}$, $S_4O_6^{2-}$) were determined at intervals during the growth cycle by cyanolysis (Kelly *et al.*, 1969) as described in Section 2.9.6.

For the washing experiments, ^{35}S-labelled cells were collected by centrifuging at 10,000 x *g* for 10 min at 4° and the radioactivity in the supernatant fraction after successive washings was determined.

2.8.9 Fractionation of the ^{35}S-labelled cells and homogenates

Cells labelled with ^{35}S from [outer-^{35}S]thiosulphate and [inner-^{35}S]thiosulphate respectively were washed once with cold 200 mM potassium phosphate buffer (pH 8.0), followed by 0.01 N sulphuric acid, and fractionated (1 ml fractions) as described previously (Tuovinen & Kelly, 1974*a*; Tuovinen *et al.*, 1975). The sequential fractionation was as follows:- (a) 2 ml of 10% (w/v) TCA, 15 h at 4°; (b) 70% (v/v) ethanol, 45°, 30 min; (c) ethanol : diethyl ether (1:1), 30°, 30 min; (d) 5% (w/v) TCA, 90°, 30 min; (e) 2 N HCl, 90°, 45 min; (f) 1 N NaOH, cell residue. Aliquots (0.1 ml) from each fraction were applied onto glass-fibre filter discs (Whatman GF/A), dried and radioassayed as described in Section 2.9.3.

Samples (0.5 ml) of cell homogenates labelled with ^{35}S-thiosulphate or ^{35}S-sulphate were mixed with 1.25 ml of 99% (v/v) ethanol and 10% (w/v) TCA respectively and kept on ice for 45 min. The radioactivity

of these solutions was determined before and after centrifuging at 10,000 x *g* for 15 min.

The radioactive samples on filters and glass-fibre filter discs were placed in 20 ml scintillation vials and dried at 105° for 16 h. After cooling, they were immersed in scintillation fluor and the radioactivity determined as described in Section 2.9.3.

2.8.10 Determination of enzyme activities in *T. ferrooxidans*

2.8.10.1 Thiosulphate-oxidizing enzyme

Homogenates and the various fractions were prepared from cells grown autotrophically and heterotrophically respectively (Section 2.8.1.1). The thiosulphate-oxidizing enzyme was assayed spectrophotometrically by following the reduction of ferricyanide at 420 nm. Reaction mixture (20°) contained in µmoles:- Sodium acetate buffer (pH 5), 250; $K_3(FeCN)_6$, 2.5; $Na_2S_2O_3$, 10, in a final volume of 3 ml. After a 1 min preincubation, the assays were started by adding the homogenate or cell extract (approximately 1-5 mg protein), and the change in optical density at 420 nm was monitored for 5 min against appropriate controls without thiosulphate.

2.8.10.2 Rhodanese

Homogenates and the various fractions were prepared from cells grown both autotrophically and heterotrophically (Section 2.8.1.1). Rhodanese activity was assayed by the method of Bowen *et al.* (1965). The reaction mixture contained in µmoles:- potassium phosphate buffer (pH 7.5), 40; $Na_2S_2O_3$, 50; and enzyme (approximately 1-7 mg protein), in a final volume of 2.25 ml. After preincubating in a reciprocating water bath at 30° for 10 min, 0.5 ml

0.1 M KCN (50 µmoles) was added. The mixture was incubated for a further 10 min and the reaction stopped by adding 0.25 ml of 40% (v/v) sodium formaldehyde; 2 ml of this mixture were then added to 2.75 ml 10% (w/v) $Fe(NO_3)_3$ in 65% (v/v) HNO_3. After centrifuging at room temperature at 2,000 x *g* for 10 min, the optical density of the supernatant fraction was determined at 470 nm against a control sample without enzyme.

2.8.10.3 AMP-independent sulphite oxidase

Homogenates and the various fractions were prepared from cells grown both autotrophically and heterotrophically (Section 2.8.1.1). A modification of the assay described by Aminuddin & Nicholas (1974) was employed. The reaction mixture contained in µmoles:- Tris-HCl buffer (pH 8), 100; Na_2SO_3, 5; $K_3(FeCN)_6$, 2.5, and enzyme (approximately 1-5 mg protein), in a final volume of 3 ml. The reaction (30°) was started by adding sulphite to the sample cuvette (quartz, 1 cm). The reference cuvette contained all components except sulphite. Changes in optical density were measured at 420 nm.

2.8.10.4 APS-reductase

a) Spectrophotometric method

Homogenates and the various fractions were prepared from cells grown both autotrophically and heterotrophically (Section 2.8.1.1). A modification of the method of Peck (1961b) was employed. The reaction mixture contained in µmoles:- Tris-HCl buffer (pH 8), 100; Na_2SO_3, 5; $K_3(FeCN)_6$, 2.5; 5'-AMP, 5, and enzyme (approximately 1-5 mg protein), in a final volume of 3 ml. The reaction (30°) was started by adding the enzyme to the sample cuvette (quartz, 1 cm).

The reference cuvette contained all components except the enzyme, which was replaced by buffer. Changes in optical density were measured at 420 nm.

b) Radioisotope method

The incubation mixtures, as described in Section 2.8.10.4(a) [except that ^{35}S-Na_2SO_3 (2 µCi) was also included], were placed in a reciprocating water bath at 30°. The reaction was started by adding the enzyme. After a 30 min incubation, aliquots (50 µl) were applied to Whatman 3 MM paper. The labelled sulphur compounds were separated by high-voltage electrophoresis (Section 2.9.1), scanned (Section 2.9.2) and radioassayed (Section 2.9.3).

2.8.10.5 ATP-sulphurylase

Homogenates and the various fractions were prepared from cells grown both autotrophically and heterotrophically (Section 2.8.1.1). ATP-sulphurylase activity was determined by the following static assay. Reaction mixtures contained in µmoles:- Tris-HCl buffer (pH 7.5), 40; $Na_2H_2P_2O_7$, 0.2; $MgCl_2$, 0.5; APS, 0.25, and enzyme (0.1 ml, 0.6-3.0 mg protein), in a total volume of 1 ml. The reaction (30°) was started by adding the enzyme and terminated after 1 min with 1 ml of 5% (v/v) perchloric acid. After centrifuging at 3,000 x *g* for 10 min, an 0.1 ml aliquot of the supernatant fraction was mixed with 1.9 ml ice-cold distilled water. ATP was determined in a suitable aliquot (0.1 ml) of the diluted sample as described in Section 2.9.4.

ATP-sulphurylase activity was also determined by the continuous method of Balharry & Nicholas (1971). The reaction vial contained in µmoles:- potassium phosphate buffer (pH 7.4), 10; sodium arsenate

buffer (pH 7.5) neutralized with HCl, 50; $MgCl_2$, 0.5; $Na_2H_2P_2O_7$, 5 nmoles, and APS, 10 nmoles, in a final volume of 3 ml made up with double-distilled water. The reaction mixture was then incubated at 20° in a Packard Tri-Carb liquid scintillation spectrometer (Model 3375), which was set up as follows. The circuit was switched out-of-coincidence so that the photomultiplier detected individual light flashes (photons). A single channel was used and was set with a gain of 100%; the discriminator settings were between 70 and 300. Counts were recorded for 0.1 min at 20°.

The enzyme reaction was initiated by adding 0.1 ml of firefly extract, prepared as described in Section 2.8.2; the vial was swirled and placed in the well of the spectrometer. Six counts, each of 0.1 min, were recorded at 0.3 min intervals. The homogenate or cell fraction (20 µl) was next added to the vial 2 min after adding the firefly extract and another six counts recorded. After a further 2 min, an internal standard of ATP (10 pmoles in 0.1 ml) was added to the vial and the counting sequence repeated.

A computer programme developed by Balharry & Nicholas (1971) was used to analyse the results of the assay. Specific activity of the enzyme is expressed in nmoles ATP produced/min/mg protein.

2.8.10.6 ADP-sulphurylase

Homogenates and the various fractions were prepared from cells grown both autotrophically and heterotrophically (Section 2.8.1.1). ADP-sulphurylase was assayed using a method developed by Dr. R.G. Nicholls in this Department which is essentially an adaptation of a polynucleotide phosphorylase assay described by Kimhi & Littauer (1968). The reaction mixture contained in µmoles:-

Tris-HCl buffer (pH 7.5), 50; double-distilled water, 0.1 ml; APS, 0.5; ^{32}Pi, 2 (0.23 μCi), and enzyme (0.3 ml, 2-10 mg protein), in a final volume of 0.7 ml. After incubating at 30° in a reciprocating water bath for 10 min, the reaction was stopped by adding 0.1 ml of 20% (w/v) TCA, and denatured protein removed by centrifuging at 3,000 x g for 10 min. Next, 0.3 ml of 10 N H_2SO_4 was added to 0.2 ml of the supernatant fraction and mixed, followed immediately by 1.5 ml of 5% ammonium molybdate and the components further mixed. After 2 min, double-distilled water (3 ml) was added to give a 5 ml total volume, followed by the addition of 5 ml iso-butanol; the contents of the tube were then mixed vigorously in a Vortex mixer and the top layer which formed was removed by suction. Then 5 ml of 0.2 M potassium phosphate buffer (pH 7.5) and another 5 ml of iso-butanol were added, the tube shaken and allowed to stand for 2 min. The top layer was again removed. The aqueous layer (3 ml) was added to 7 ml of double-distilled water in a scintillation vial and the amount of radioactivity determined (Packard liquid scintillation spectrometer, ^{3}H push button). The control included a reaction mixture in which the 20% (w/v) TCA (0.1 ml) was added before the enzyme.

2.8.10.7 Adenylate kinase

Homogenates and the various fractions were prepared from cells grown both autotrophically and heterotrophically (Section 2.8.1.1). The reaction mixture contained in μmoles:- Tris-HCl buffer (pH 7.5), 40; $MgCl_2$, 0.5; ADP, 0.4, and extract (0.1 ml, approximately 0.6-3.0 mg protein), in a total volume of 1 ml. The reaction (30°) was started by adding ADP. After incubating the mixture in a reciprocating water bath for 1 min, the reaction was terminated by adding 1 ml 5% (v/v) PCA. The mixture was then centrifuged for

5 min at 3,000 x *g* and a 0.1 ml sample of the supernatant fraction combined with 1.9 ml of ice-cold double-distilled water. ATP was then determined in a suitable aliquot (0.1 ml) of the diluted sample as described in Section 2.9.4.

2.8.10.8 Sulphite reductase

Homogenates and the various fractions were prepared from cells grown both autotrophically and heterotrophically (Section 2.8.1.1). Sulphite reductase activity was monitored by the method of Ellis (1964). The reaction mixtures contained in μmoles:- Tris-HCl buffer (pH 8), 10; $MgCl_2$, 1; Na_2SO_3, 10; FAD, 0.1; an NADPH-generating system consisting of glucose-6-phosphate, 5; $NADP^+$, 0.5; glucose-6-phosphate dehydrogenase, 0.06 international units; and enzyme (0.2 ml, 2-8 mg protein), in a total volume of 1 ml. Incubations were carried out anaerobically in test-tubes fitted with rubber septa (Suba-seal; Wm. Freeman, Barnsley, Yorks., England) at 37° in a reciprocating water bath. The tubes were evacuated for 2 min. The reaction was started by adding the Na_2SO_3; after 50 min, 0.5 ml of 0.2 M NEM was added to the tubes. The mixtures were thoroughly mixed and further incubated at 37° for 10 min. Finally, 1.5 ml of 2 M Na_2CO_3 was added, the contents mixed, and the tubes allowed to stand at 37° for 10 min. After centrifuging at 3,000 x *g* for 10 min, the optical density at 520 nm was read against boiled enzyme blanks.

2.9 General techniques

2.9.1 High-voltage paper electrophoresis

The apparatus of Tate (1968) was used. Standard solutions or aliquots of reaction mixtures were spotted 2.5 cm apart at the

origin on Whatman 3MM chromatographic paper (15 cm x 55 cm) near the cathode. The paper was moistened with 100 mM sodium citrate buffer (pH 5) and then lightly blotted to remove surface moisture. It was then laid out on a polythene frame and placed into a ceramic tank filled with CCl_4 in such a way that the ends of the paper were connected by wicks to buffer chambers containing 100 mM sodium citrate buffer (pH 5). The CCl_4 was cooled by passing cold tap water through a copper coil placed in the centre of the tank. The current from a stabilized power pack (Paton Industries Ltd., South Australia) was applied to the buffer chambers, maintaining a voltage gradient of 30 volts/cm along the paper. The electrophoresis was usually carried out for 1 h with appropriate marker compounds.

After electrophoresis, the paper was dried by hot air and the lanes (30-35 cm x 2.5 cm) were separated by cutting with a razor blade. Nucleotides were detected on the dried paper by U.V. absorption using a hand monitor (Ultra-Violet Products Inc., South Pasadena, California, U.S.A.). Radioactive nucleotides and compounds were detected as described in Section 2.9.2 and radioassayed as described in Section 2.9.3.

2.9.2 Radiochromatogram scanner

Electrophoretograms containing radioisotopes were thoroughly dried and separated into lanes, which were then joined end to end. This strip was run through a Packard 7201 radiochromatogram scanner. Small aliquots of radioactive material were spotted as markers behind the origin to align the chromatogram with the recorded chart data.

2.9.3 Liquid scintillation spectrometry

For quantitative determinations, the radioactive areas on the electrophoretograms were cut into small portions (1.5 x 2 cm) so that the pieces were horizontal at the base of the glass scintillation vials (Packard Instrument Co., Chicago, Illinois, U.S.A.). Then, 3 ml of scintillation fluor, consisting of PPO (0.5%, w/v) and POPOP (0.3%, w/v) in toluene, was pipetted into the vials, and these were assayed in a Packard Tri-Carb liquid scintillation spectrometer (Model 3375).

Radioactivity in standard solutions or assay mixtures (^{14}C or ^{35}S) was measured either by spotting aliquots onto squares (1.5 cm x 1.5 cm) of Whatman 3MM chromatography paper and drying and counting as above, or by dispensing aliquots into a scintillation mixture consisting of 2 ml of 95% (v/v) ethanol and 5 ml of toluene fluor.

Channels ratios were used to determine the counting efficiency (usually about 80%), by comparison with ^{35}S-sulphate. This standard had previously been calibrated against a standard ^{14}C-toluene sample (5.73 x 10 d.p.m./g), assuming equal efficiency of ^{14}C and ^{35}S.

2.9.4 ATP

The amount of ATP present in reaction mixtures or solutions was determined using the luciferin-luciferase system of the firefly, *Photinus pyralis*. The light emitted, measured in a Packard Tri-Carb liquid scintillation spectrometer, is directly proportional to the amounts of ATP present. The settings for the spectrometer were as described for the ATP-sulphurylase continuous assay (Section 2.8.10.5). The reaction vial contained 1 ml 10 mM potassium phosphate buffer (pH 7.5), 0.1 ml 5 mM $MgCl_2$ and 0.9 ml double-distilled water. The following additions were made at 2 min intervals:-

A : 0.1 ml firefly extract;

B : 0.1 ml aliquot of sample containing ATP;

C : 0.1 ml internal standard of ATP (10 pmoles).

The counts per min were determined after each addition and ATP was calculated from the expression

$$\text{ATP(pmoles)} = \frac{C_{\text{c.p.m.}} - B_{\text{c.p.m.}}}{B_{\text{c.p.m.}} - A_{\text{c.p.m.}}} \times 10 \ .$$

2.9.5 Inorganic phosphate

Inorganic phosphate was determined by the method of Chen *et al.* (1956). An aliquot of the solution under investigation was placed in a test-tube and made up to 0.9 ml with double-distilled water. Then, 2.1 ml of a solution containing 1 part 10% (w/v) ascorbic acid to 6 parts 0.42% (w/v) ammonium molybdate in N H_2SO_4 were added to the tube. The contents were mixed and incubated at 45° for 20 min. The optical density was determined in a 1 cm cell at 820 nm in a QR-50 Shimadzu spectrometer against a water blank. A standard phosphate stock solution of 1 mg/ml was prepared by dissolving 1.0867 g of analytical reagent KH_2PO_4 (dried in an air oven) in distilled water and diluting to 250 ml.

2.9.6 Thiosulphate and tetrathionate

Thiosulphate and tetrathionate were determined by cyanolysis using the methods of Kelly *et al.* (1969). The first method described determines tetrathionate alone. To a 25 ml volumetric flask, 0-8 µmoles of $Na_2S_4O_6$ (1-5 ml) were added. Next, 4 ml of 0.2 M NaH_2PO_4-NaOH buffer (pH 7.4) were added, followed by enough double-distilled water to give a total volume of about 10 ml. Then, 5 ml of

100 mM KCN were dispensed from a burette into the flask; the contents were mixed rapidly and allowed to stand for 20 min at room temperature. After adding 3 ml of 1.5 M $Fe(NO_3)_3$ in 4 N $HClO_4$, the contents were again mixed and then made to 25 ml with double-distilled water. The optical density at 460 nm was read against a reagent blank using water as a base.

The second method can be used to determine both tetrathionate and thiosulphate. $Na_2S_2O_3$ (0-8 µmoles) was placed in a 25 ml volumetric flask, together with 4 ml 0.2 M NaH_2PO_4-NaOH buffer (pH 7.4). Double-distilled water was added to give an approximate volume of 10 ml. Then, 5 ml of 100 mM KCN were dispensed from a burette into the flask; the contents were mixed rapidly and allowed to stand at room temperature for 10 min. Next, 1.5 ml of 100 mM $CuSO_4$ were added and, after mixing, the flasks were left at room temperature for 10 min. Finally, after adding 3 ml of 1.5 M $Fe(NO_3)_3$ in 4 N $HClO_4$, the contents were mixed and made up to 25 ml with double-distilled water. The optical density at 460 nm was read against a reagent blank using water as a base.

2.9.7 Protein

Proteins were determined by the Folin-Ciocalteau method as modified by Lowry *et al.* (1951), using bovine serum albumin as a standard. The optical density was measured in 1 cm cuvettes at 750 nm in the Shimadzu spectrophotometer (Section 2.9.9). When determining the protein content of plant materials, samples were first treated with cold 10% TCA to separate polyphenols which would interfere with the colour reaction. The presence of dilute TCA does not affect the final colour or the protein colour development (Lowry *et al.*, 1951).

2.9.8 Chlorophyll

Chlorophyll was determined by the method of Arnon (1949).

2.9.9 Absorption spectra

Absorption measurements were made using a Shimadzu MPS-50L recording spectrophotometer, at room temperature (20-25°), using 1 cm glass microcuvettes (0.4 ml or 3.5 ml volumes). Absorption maxima were corrected by referring to the spectrum of a Holmium filter (Unicam) with maxima at 418.4, 453.2, 536.2 and 637.5 nm.

Fixed wavelength measurements (O.D.) were made in 1 cm glass or quartz cuvettes (4 ml) in a Shimadzu QR-50/1 spectrophotometer.

3. PART I. PRODUCTION AND DEGRADATION OF SULPHUR NUCLEOTIDES IN MICROORGANISMS AND PLANTS

3. PRODUCTION AND DEGRADATION OF SULPHUR NUCLEOTIDES IN MICROORGANISMS AND PLANTS

3.1 Introduction

The activation of sulphate to PAPS was first characterized in yeast (Wilson & Bandurski, 1956; Segal, 1956) and is mediated by the following enzymes (Bandurski *et al.*, 1956; Robbins & Lipmann, 1956; Wilson & Bandurski, 1958; Robbins & Lipmann, 1958*a*,*b*):-

$$ATP + SO_4^{2-} \xrightleftharpoons{\text{ATP-sulphurylase}} APS + PPi$$

[*ATP : sulphate adenylyltransferase*]

$$APS + ATP \xrightleftharpoons{\text{APS-kinase}} PAPS + ADP$$

[*ATP : adenylylsulphate 3'-phosphotransferase*]

Studies leading to the elucidation of the metabolic sequence for the biosynthesis of PAPS have been recently reviewed by De Meio (1975).

Roy & Trudinger (1970) and De Meio (1975) consider that the ATP-sulphurylase and APS-kinase are present in all tissues which form PAPS, and similarly in those tissues containing sulphate esters. It is suggested that the sulphate-activating system appears to be present in most mammalian tissues. However, only in a few cases have the enzymes been isolated or even identified. There is a deal of evidence that this pathway operates in a variety of other organisms. Sulphate activation and PAPS reduction have been demonstrated in *Escherichia coli* (Mager, 1960); Fujimoto & Ishimoto, 1961; Pasternak, 1962; Pasternak *et al.*, 1965), in *Salmonella* (Dreyfuss & Monty, 1963*a*,*b*), in *Bacillus subtilis* (Pasternak, 1962; Pasternak *et al.*, 1965), in

Nitrosomonas europaea (Varma & Nicholas, 1970), and in *Chlorella* (Levinthal & Schiff, 1965), while evidence has been found for the formation of PAPS during sulphate reduction by *Penicillium chrysogenum* (Segel & Johnson, 1963), by *Rhodospirillum rubrum* chromaphores (Ibanez & Lindstrom, 1962), by *Euglena gracilis* (Abraham & Bachhawat, 1963; Davies *et al.*, 1966) and in marine algae (Goldberg & Selbruck, 1959).

The way in which sulphate is activated in plants however is not so clear. Nissen & Benson (1961) used ^{35}S-labelled sulphate to demonstrate choline sulphate production in root tissue from corn (*Zea mays*), barley (*Hordeum vulgare*), and sunflower (*Helianthus annus*). However, the presence of ^{35}S-labelled APS or PAPS was not reported in their study. Neither Asahi (1964) nor Balharry & Nicholas (1970) were able to detect PAPS during the reduction of ^{35}S-labelled sulphate by spinach chloroplasts; the formation of ^{35}S-labelled APS was readily demonstrated. Similarly, Ellis (1969) and Onajobi *et al.* (1973) have isolated ATP-sulphurylase from a variety of higher plant tissues but failed to detect either PAPS formation or APS-kinase activity.

Mercer & Thomas (1969) detected PAPS in chloroplast preparations from French bean (*Phaseolus vulgaris*) and maize (*Zea mays*). In a labelled experiment (100 µCi ^{35}S-labelled sulphate), they found a relatively small area on their chromatogram where ^{35}S-labelled PAPS was reputed to run. However, the product was not unequivocally identified as PAPS. This sulphur nucleotide has been recorded in cell-free preparations of *Chlorella* (Hodson *et al.*, 1968; Tsang *et al.*, 1971; Schmidt, 1972). Similarly, PAPS production has been shown to occur in spinach chloroplasts (Schmidt & Trebst, 1969; Burnell & Anderson, 1973). The properties and distribution of the sulphate-

activating enzymes from some plants and microorganisms have been reviewed by Schiff & Hodson (1973). In mammals and plants, little is known of the quantitative distribution of the sulphate-activation system or its activity under physiological and pathological conditions. Such plant and mammalian systems have been briefly reviewed by Roy & Trudinger (1970) and De Meio (1975).

In several studies, PAPS production has been determined by the combined activities of ATP-sulphurylase and APS-kinase. Indeed, over the past few years, much of the work on the sulphate-activation pathway has been primarily aimed at the isolation and purification of ATP-sulphurylase. This may relate to the importance of this enzyme as the first step in the activation of sulphate, and also to the relationship between ATP-sulphurylase and sulphate uptake studies. The properties of ATP-sulphurylases from various sources have been extensively reviewed (Roy & Trudinger, 1970; De Meio, 1975).

APS-kinase appears to possess some unusual properties. Robbins & Lipmann (1958*a*) partially purified the enzyme from yeast. The enzyme, which required Mg^{2+} for optimum activity, was completely saturated with APS at 5 μM. Higher concentrations resulted in a substrate inhibition. Little is known about the distribution and properties of APS-kinase [see reviews by Roy & Trudinger (1970) and De Meio (1975)].

Studies on the control of sulphate activation in microorganisms have been discussed by De Meio (1975). In *Escherichia coli*, the formation of PAPS was repressed by cysteine, while in *Bacillus subtilis*, both cysteine and glutathione had this effect (Pasternak, 1962). Further studies (Wheldrake & Pasternak, 1965) showed that both ATP-sulphurylase and APS-kinase were repressed simultaneously

by cysteine in *E. coli* and probably also in *B. subtilis*. Wheldrake (1967) has suggested that the intracellular cysteine concentration in *E. coli* controls the rate of synthesis of these enzymes. A similar control system was not observed in *Desulfovibrio desulfuricans*. where APS-kinase is absent (Wheldrake & Pasternak, 1965) and where ATP-sulphurylase was not repressed by either cysteine or sulphite. Studies on the metabolic regulation of ATP-sulphurylase of yeast (de Vito & Dreyfuss, 1964) indicate that the enzyme was strongly inhibited by low concentrations of APS, PAPS and sulphide. Sulphide was a competitive inhibitor of ATP-sulphurylase. Cysteine, methionine, sulphite and thiosulphate did not inhibit the enzyme. ATP-sulphurylase was repressed when yeast was grown with methionine, and derepressed when yeast was grown with cysteine. In contrast, the enzyme sulphite reductase was repressed in cells grown with cysteine. Thus, the sulphate-reducing pathway in yeast appears to be regulated at its first step by feedback inhibition (sulphide) and a repression (methionine).

The reductive pathways of APS in algae and PAPS in some bacteria and fungi have been reviewed in detail by Schiff & Hodson (1973) and Siegel (1975). Extracts of *Chlorella* contain two enzymes, namely APS : thiol sulphotransferase (Abrams & Schiff, 1973) and thiosulphonate reductase (Schmidt, 1973), which catalyse the reduction of the sulphonate moiety of APS to the level of thiol, with reduced ferredoxin as the electron donor in the presence of a thiol carrier. The pathway of sulphate reduction in spinach chloroplasts may be similar to that in *Chlorella* (Schmidt & Schwenn, 1972). However, both contain APS-kinase and form PAPS from sulphate and ATP. Schmidt (1972) suggests that PAPS is first converted to APS in these organisms by a Mg^{2+}-dependent 3'-nucleotidase (Schmidt, 1972; Hodson & Schiff, 1971;

Burnell & Anderson, 1973). Similarly, *Salmonella pullorum* contains 3'-nucleotidase which degrades PAPS to APS, and during sulphate reduction in cell-free extracts, the formation of PAPS occurs only on adding a 3'-nucleotidase (Kline & Schoenhard, 1970). The presence of this enzyme may explain the difficulty in observing PAPS formation from sulphate and ATP in many higher plants under conditions in which the formation of APS is easily detected. Abrams & Schiff (1973) suggest that PAPS is used for sulphate ester formation in algae and higher plants, while APS is the "active" substrate for sulphate reduction, thus indicating a regulatory role for the 3'-nucleotidase.

In dissimilatory sulphate-reducing bacteria, APS reduction to the level of sulphite is mediated by APS-reductase, as reviewed by Roy & Trudinger (1970).

In enterobacteria and fungi, PAPS serves as the substrate for sulphate reduction to hydrogen sulphide, with NADPH as the electron donor. This pathway requires the combined activities of PAPS-reductase and sulphite reductase (Roy & Trudinger, 1970; Siegel, 1975).

Problems arise when metabolic studies with APS and PAPS are conducted in crude extracts of biological materials because of their instability. Robbins & Lipmann (1957) found that PAPS was hydrolysed to APS by purified 3'-nucleotidase of rye grass. They also reported that, in bull semen, 5'-nucleotidase hydrolysed APS to adenosine, phosphate and sulphate. PAPS is also degraded by a sulphatase enzyme, which catalyses the hydrolysis of PAPS to PAP and sulphate (Roy & Trudinger, 1970). In addition, another type of hydrolysis of APS to AMP and sulphate by a sulphohydrolase enzyme has been described (Armstrong *et al.*, 1970).

Some of the techniques used to assay PAPS production listed in

Table 3 are primarily based on the use of radioisotopes and coupled enzyme assays. Unlike ATP-sulphurylase, the properties of APS-kinase and its distribution in biological materials are not well known. In recent years, the bioluminescence system of the firefly, which is specific for ATP, has been used to assay ATP-sulphurylase in plant tissues (Balharry & Nicholas, 1971). In the field of analytical bioluminescence, two basic reactions have been developed: (i) ATP, using the firefly (*Photinus pyralis*) luciferin-luciferase system (Stanley & Williams, 1969; Schram *et al.*, 1971) and (ii) FMN and NADH, using the dehydrogenase-luciferase complex of the marine bacterium *Photobacterium fischeri* (Stanley, 1971*a*,*b*; Schram *et al.*, 1971). These bioluminescence systems have been used mainly for measuring static levels of these compounds, but more recently the use of enzyme-coupled reactions in which ATP or NADH is either produced or utilized has enabled dynamic measurements to be made, e.g. ammonia (Nicholas & Clarke, 1971) and APS (Balharry & Nicholas, 1971). The bioluminescence mechanisms have been considered in detail in a number of reviews (Cormier & Totter, 1964; Hastings, 1968; Schram *et al*, 1971; Stanley, 1974; Cormier *et al.*, 1974). Cormier & Totter (1964) have classified bioluminescence systems into four general classes on the basis of type of reactions (Table 4).

The present study is primarily concerned with the development of a bioluminescence assay for PAP based on the luciferin-luciferase system of the sea pansy, *Renilla reniformis*. This organism is a colonial soft coral (Anthozoan) normally found resting on the ocean floor. Cormier (1959,1960,1961) has described methods for preparing luminescent extracts from *Renilla*. The overall reaction leading to light emission by this organism has been shown to proceed via a two-step reaction (Cormier *et al.*, 1970) as follows:-

TABLE 3

Enzymological methods for determining PAP and PAPS in biological materials

Nucleotide assayed	Method	Assay system	Reference
1. PAPS	Radiotracer	Labelled substrates, e.g. ^{35}S-sulphate, ^{35}S-APS, ^{14}C-ATP, ^{32}P-ATP Separation by high-voltage electrophoresis and subsequent radioassay	Asahi (1964) Mercer & Thomas (1969) Ellis (1969) Balharry & Nicholas (1970) Onajobi *et al.* (1973)
2. PAP, PAPS	Coupled enzymes	p-NPS + phenol $\xrightarrow[\text{PAP}]{\text{sulphokinase}}$ p-NP + phenyl sulphate The transfer of sulphate from p-nitrophenol sulphate to phenol is dependent upon PAP.	Gregory & Lipmann (1957) Robbins & Lipmann (1958*a*)
3. PAPS	Coupled enzymes	PAPS $\xrightarrow[\text{+ phenol sulphotransferase}]{\text{2-naphthol}}$ PAP + Naphthol 2-sulphate APS-kinase activity is measured as synthesis of naphthol 2-sulphate from PAPS in presence of purified phenol sulphotransferase from rabbit liver.	Burnell & Anderson (1973)
4. APS-kinase	Coupled enzymes	APS + ATP $\xrightarrow{\text{APS-kinase}}$ PAPS + ADP ADP produced is linked to oxidation of NADH + H^+ via pyruvate kinase and lactate dehydrogenase measured by NADH oxidation at 340 nm.	Burnell & Whatley (1975)

TABLE 4

A classification of known bioluminescence reactions
(adapted from Cormier & Totter, 1964)

Type reactions	Examples	References
A : Pyridine-nucleotide linked:		
(1) $NADH + H^+ + FMN \xrightleftharpoons{\text{NADH-oxidase}} FMNH_2 + NAD$	*Photobacterium fischeri*	Stanley (1971*a*,*b*) Schram *et al.* (1971)
$FMNH_2 + O_2 \xrightarrow[\text{long-chain aldehyde}]{\text{luciferase}} \text{light}$		
Also adapted to measure ammonia: $\alpha KG + NH_2 + NADH \rightleftharpoons L(+)\text{-glutamic acid} + NAD$		Nicholas & Clarke (1971)
(2) $NADH + H^+ + L \xrightleftharpoons{\text{NADH-oxidase}} LH_2 + NAD$	Fungi	Stanley (1971*a*,*b*) Schram *et al.* (1971)
$LH_2 + O_2 \xrightarrow{\text{luciferase}} \text{light}$		
B : Adenine-nucleotide linked:		
(1) $LH_2 + ATP + O_2 \xrightarrow[Mg^{2+}]{\text{luciferase}} \text{light}$	Firefly	Stanley & Williams (1969)
(2) $L.SO_4 + PAP \xrightleftharpoons{\text{luciferin sulphokinase}} L + PAPS$	Sea Pansy	Cormier *et al.* (1974)
$L + O_2 \xrightarrow[Ca^{2+}]{\text{luciferase}} \text{light}$		
C : Simple enzyme-substrate systems $LH_2 + O_2 \xrightarrow{\text{luciferase}} \text{light}$	Cypridina (crustacean), Apogon (fish), Odontosyllis (annelid), Pholas (clam), Gonyaulax (protozoan)	Cormier & Totter (1964)
D : Peroxidation systems $2LH_2 + H_2O_2 \xrightarrow{\text{luciferase}} \text{light}$	Balanoglossus (acorn worm)	Cormier & Totter (1964)

$$\text{Luciferyl sulphate} + \text{PAP} \xrightarrow[\text{sulphokinase}]{\text{luciferin}} \text{luciferin} + \text{PAPS} \quad (1)$$

$$\text{Luciferin} + O_2 \xrightarrow{\text{luciferase}} \text{oxyluciferin} + CO_2 + \text{light} \quad (2)$$

Renilla stores luciferin, an easily auto-oxidizable compound, as a stable sulphonated derivative termed luciferyl sulphate (Cormier *et al.*, 1970). Luciferyl sulphate is not a substrate for luciferase and must therefore be converted to luciferin. In *Renilla*, this process is mediated by the enzyme luciferin sulphokinase in the presence of PAP (Cormier *et al.*, 1970; Hori *et al.*, 1972), as shown in equation 1. Luciferase has been purified to homogeneity and has a molecular weight of approximately 24,000 (Hori *et al.*, 1973; Cormier *et al.*, 1974). In the presence of oxygen, luciferase catalyses the oxidation of luciferin, resulting in the production of blue light with an emission maximum of 490 nm (equation 2). The mechanism of the light reaction has recently been reviewed by Cormier *et al.* (1974).

The bioluminescence reaction is sensitive and quite specific for PAP, since both 2',5'-diphosphoadenosine and 3',5'-diphosphoinosine are inactive. Similarly, no activity was found with DNP, 3',5'-cyclic-AMP, or deoxy-AMP (Cormier, 1962).

The specificity of these reactions leading to the production of light by *Renilla* allows for the development of very sensitive assays for PAP and thus PAPS, after an acid hydrolysis to PAP. Such assay systems have proved useful in studies on the utilization, synthesis and role of both PAP and PAPS in biological systems.

Aim of the study. The present study is concerned with

1. The development of a sensitive and specific assay system for determining picomole amounts of PAP and thus PAPS (which can be

hydrolysed to PAP) in biological materials.

2. The stability of sulphur nucleotides in tissue extracts and the use of various compounds to offset the effects of enzymes which degrade PAP and PAPS.

3.2 Results

3.2.1 Development of a bioluminescence assay for PAP and PAPS

3.2.1.1 Quantitative measurement of PAP

The technique for determining PAP is shown in Figure 3 and is essentially similar to that employed for estimating ATP by the luciferin-luciferase system of the firefly, *Photinus pyralis*, using a liquid scintillation spectrometer (Balharry & Nicholas, 1971).

Two ml of 10 mM potassium phosphate buffer (pH 7.5) containing 1 mM Na-EDTA and 1 mM β-ETSH were added to a standard scintillation vial and equilibrated at 20° for 15 min in the liquid scintillation spectrometer. After equilibrating, 150 pmoles of luciferyl sulphate (0.5 µl) in 10 mM potassium phosphate buffer (pH 7.5) containing 50% ethanol, and 50 µl of the *Renilla reniformis* enzyme preparation (see Section 2.8.3) were added. The vial was shaken and quickly replaced in the well of the spectrometer. Five counts, each of 0.1 min duration, were recorded at intervals of 0.3 min, as shown in Figure 3 (Section A). The vial was then unloaded and an aliquot (5-20 µl) of the hydrolysate (see Section 2.8.6) containing PAP was added to the vial, which was shaken and lowered into the spectrometer. A further five counts of 0.1 min duration were recorded at intervals of 0.3 min

FIGURE 3

Reaction sequence for the determination of PAP by the Renilla luciferin-luciferase system

The experimental details of the bioluminescence assay are given in Section 3.2.1.1. "In" and "out" refer to the loading and unloading of the scintillation vial in the Model 3375 Packard Tri-Carb liquid scintillation spectrometer, as described in Section 3.2.1.1.

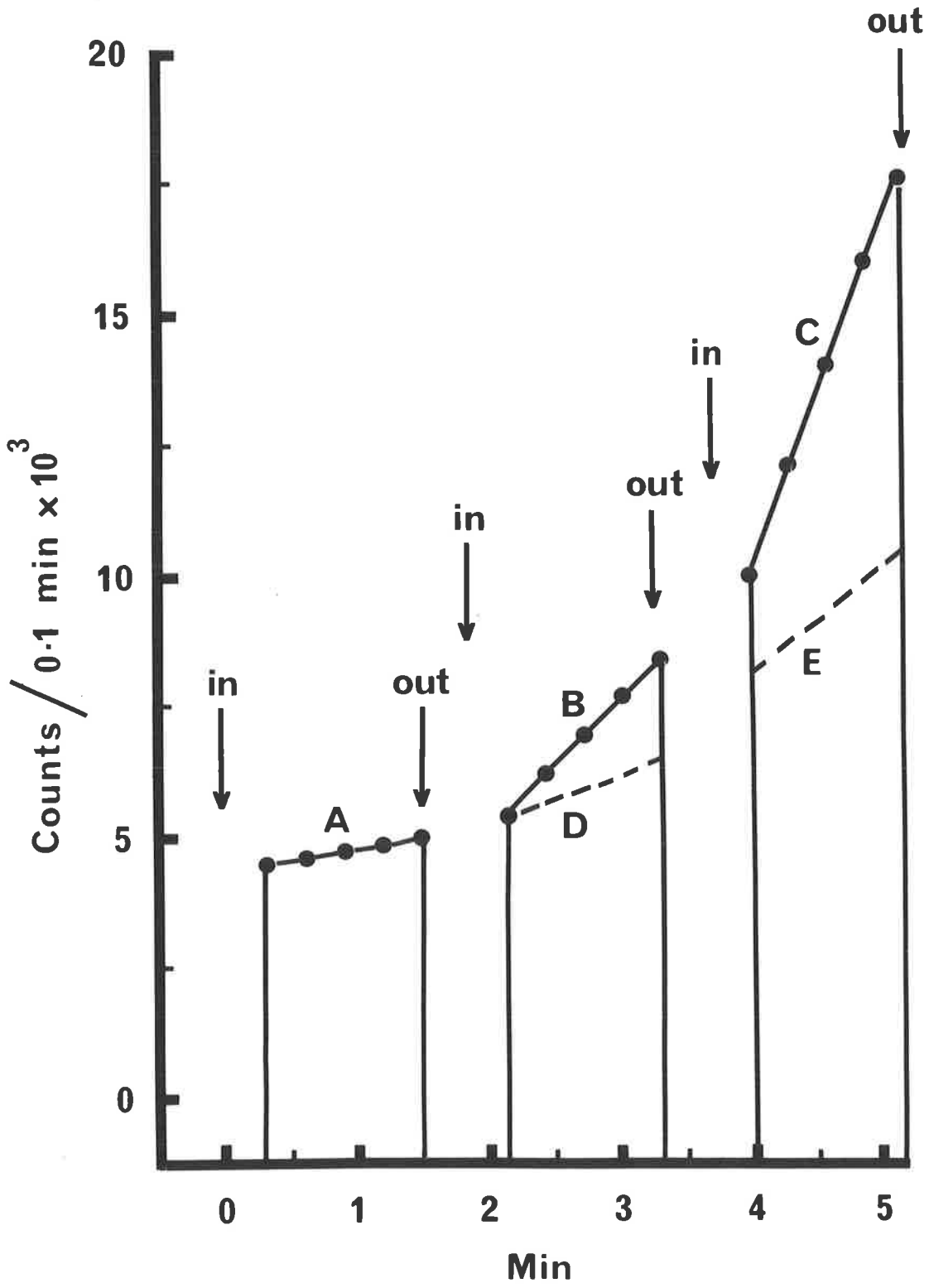

20
15
10
5
0
Counts / 0·1 min × 10³
0
1
2
3
4
5
Min
in
out
in
out
in
out
A
B
C
D
E

Figure 3

(Figure 3 - Section B). The vial was again unloaded and an internal standard of 120 pmoles of purified PAP was added in a volume of 2 µl (Figure 3 - Section C). The counting sequence was repeated.

All procedures were carried out in a room at 25° illuminated only with tungsten light units to avoid phosphorescence in glass scintillation vials from other types of light source. In addition, vials were equilibrated in the same room for at least 2 h under tungsten light prior to use.

The assay was standardized by adding graded amounts of PAP (1-150 pmoles) to vials containing luciferyl sulphate and the *Renilla* enzyme extract.

All measurements of light output were made in a Model 3375 Packard Tri-Carb liquid scintillation spectrometer with the two photomultipliers switched out-of-coincidence (Stanley & Williams, 1969; Stanley, 1971*a*,*b*). One channel was used at 100% gain, with the two discriminators set at 75 and 300.

A computer program, RENILLA, has been developed to analyse the data output of the bioluminescence assays. The program prints out quantitative results for PAP, as well as specific activities for the enzymic production of PAPS in extracts of biological materials and presents these in a graphical form. The FORTRAN listing of the program is shown in Appendix I.

The small initial light output in the blank (Figure 3 - Section A) was due to endogenous PAP present in the *Renilla* enzyme preparation (Section 2.8.3). PAP was removed from all such enzyme fractions by passing the samples through a Sephadex G-25 column either prior to assay or before storage at -15°. Thus, the luciferin sulphokinase and luciferase activities were essentially free of PAP so that the

light emission by the blank (Figure 3 - Section A) during the assay was reduced.

An internal standard of PAP (120 pmoles) was always included in the assays to check for any variations which might affect light output. The addition of large volumes of sample (> 50 µl) decreased the light output both by the sample (Figure 3 - Section D) and the internal standard (Figure 3 - Section E). This effect was probably associated with temperature changes, since the test samples were stored on ice, or with slight alterations in pH within the assay vial due to the acidic nature of the added hydrolysate. Thus the test aliquots used were always maintained at 20 µl or less, which did not affect the light output of the internal standard. The internal standard also provided a convenient way of checking that the light output increased linearly with time and that the luciferin sulphokinase was not saturated with PAP from the test sample. High levels of PAP were assayed by either decreasing the amount of PAP in the internal standard (Figure 3 - Section C) or by reducing the sample size (Figure 3 - Section B).

A calibration curve for PAP against light output is given in Figure 4. As little as 10 pmoles PAP can be accurately measured by this procedure. The extreme sensitivity of the assay, involving the use of small volumes, necessitates a precision in all operations of the assay, especially in the preparation and dispensing of all components. Careful experimentation resulted in a reproducibility of replicates within 1%.

3.2.1.2 Stability of PAP under the assay conditions

There was no degradation of PAP when samples containing graded amounts of this nucleotide in Tris-HCl buffer (pH

FIGURE 4

A calibration curve for PAP

The assay was standardized by adding graded amounts of PAP (0-150 pmoles), instead of the hydrolysate, to a scintillation vial containing the luciferyl sulphate and the *Renilla* enzyme extract (Section 3.2.1.1). Five counts, each of 0.1 min at 0.3 min intervals, were taken for each level of PAP used.

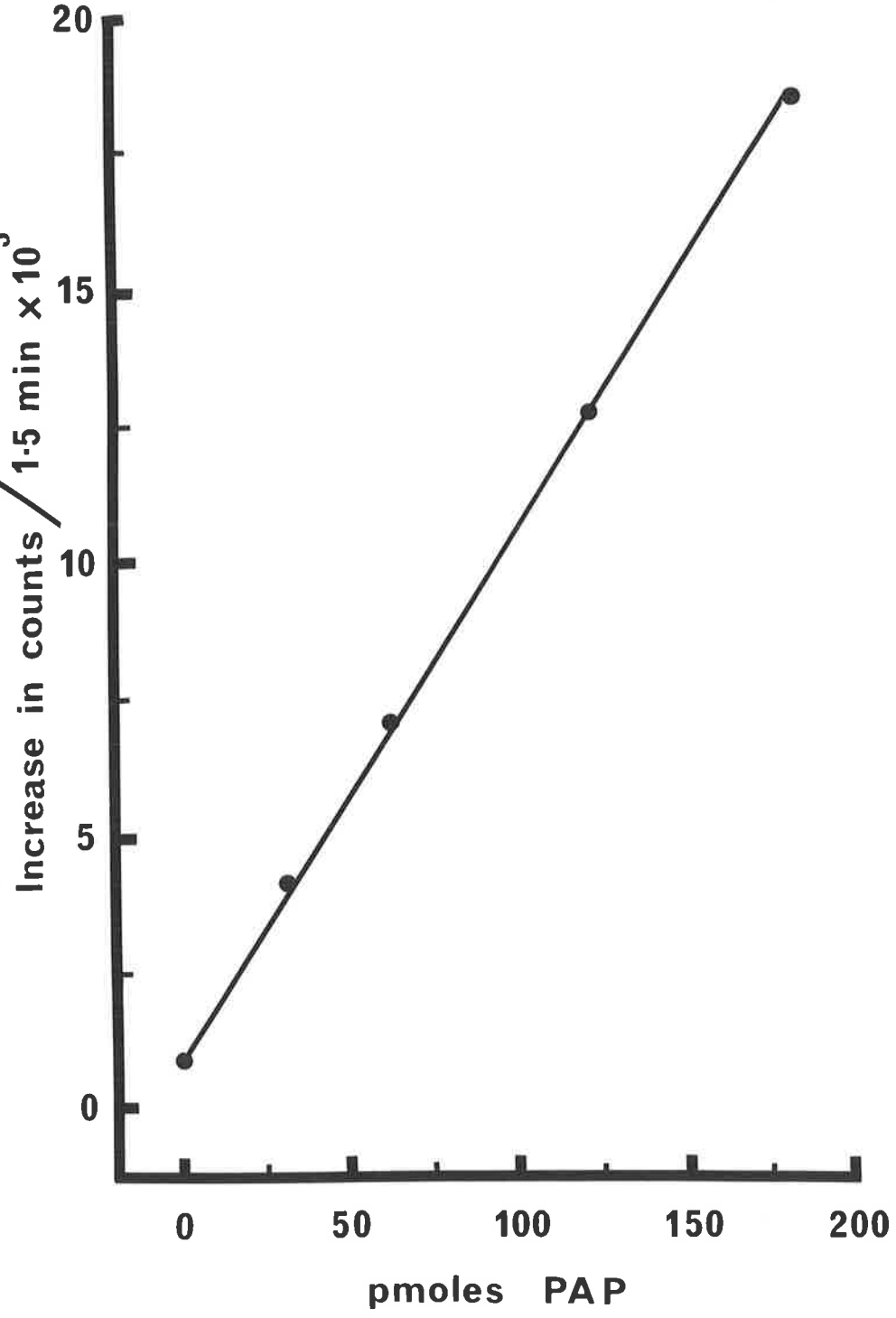

20
15
10
5
0
Increase in counts / 1·5 min x 10^3
0
50
100
150
200
pmoles PAP

Figure 4

8.0) were hydrolysed in two volumes (0.2 ml) of 0.2 N HCl at 37° for 1 h. Under these conditions, the conversion of PAPS to PAP was complete after 30 min. For all studies reported herein, the hydrolysis of PAPS to PAP was carried out for 30 min.

Similarly, PAP was stable when incubated with yeast extract under the conditions used for acid hydrolysis (Table 5). The presence of phosphate, pyrophosphate and a range of nucleotide phosphates did not affect the stability of PAP over the standard 30 min hydrolysis period. However, when PAP was incubated with extracts of yeast prior to acid hydrolysis, there was a rapid degradation of this compound (as presented later in Table 14). The degradation was significantly reduced in the presence of 32 mM 5'-AMP, ADP or ATP. These nucleotides appeared to protect the PAP, presumably from cleavage by a 5'-nucleotidase (to be considered later) which does not appear to operate under the conditions used for acid hydrolysis. Boiled extracts did not degrade PAP.

3.2.1.3 Purity of nucleotides and other compounds

PAP was found as a trace impurity in many of the biochemical compounds used in this study. Less than 0.003% of PAP was detected in commercial preparations of 3'-AMP, 5'-AMP and ATP. The highest level of PAP (0.01%) was detected in ADP; the amounts of PAP in ADP and ATP increased with storage at -15°. Aged commercial preparations of ATP were found to be a good source of PAP, as described in Section 2.8.5. In addition, the amounts of PAP occurring as an impurity in a variety of biochemical reagents were determined by the *Renilla* bioluminescence system both before and after acid hydrolysis. No change was detected in the amount of PAP over the 30 min incubation

TABLE 5

Stability of PAP in extracts of yeast during acid hydrolysis

Details of the preparation of the yeast extract (S_{60}) are given in Section 2.8.1.2. The incubations were carried out at 37°. Reaction mixtures contained in μmoles: Tris-HCl buffer (pH 7.5), 5; Na_2EDTA, 1; $MgCl_2$, 4; nucleotides, 4; NaH_2PO_4, 4; $Na_2H_2P_2O_7$, 4; 0.2 ml of 0.2 N HCl and yeast extract (S_{60}, 4.4 mg protein) in a total volume of 0.32 ml. After preincubating at 37° for 1 min, the reaction was started by adding PAP (600 pmoles). At 0 and 30 min, 0.02 ml samples were withdrawn and the amount of PAP in these samples determined immediately by the *Renilla* bioluminescence procedure as described in Section 3.2.1.1. Boiled yeast extracts were used as controls for each treatment.

Treatment	nmoles of PAP remaining/ml of incubation mixture	
	Time of acid hydrolysis (min)	
	0	30
Extract	1.7	2.0
Extract + 3'-AMP	1.9	1.8
Extract + 5'-AMP	2.8	2.7
Extract + ADP	5.0	5.1
Extract + ATP	1.7	2.1
Extract + NaH_2PO_4	1.8	2.0
Extract + $Na_2H_2P_2O_7$	2.0	2.2

period. The contamination of nucleotides with PAP was taken into account in all assays by including appropriate boiled extracts and other controls. In addition, controls were included to check for possible phosphorylation of the adenosine mononucleotides to PAP and for contributions to the endogenous PAP levels from compounds such as coenzyme A, which is easily degraded by acid and enzymic hydrolysis to PAP.

3.2.2 Production and degradation of PAPS and PAP in biological materials, determined by bioluminescence and radiotracer methods

3.2.2.1 Higher plants

The synthesis of PAPS from ATP and sulphate was studied in extracts of green plants using the bioluminescence procedure. The production of PAPS in the absence of 5'-AMP was very low in all plant extracts tested (Table 6), reaching a maximum after a 10 min incubation period followed by a rapid decline (Figure 5). The addition of 5'-AMP (25 mM final concentration) to the reaction mixture prior to adding the plant extract greatly enhanced the production of PAPS (Table 6, Figure 5). In most plant extracts tested, this stimulation of PAPS production by 5'-AMP was about ten-fold. When 5'-AMP was added to a leaf extract, the initial production of PAPS was curvilinear, reaching a maximum after a 30 min incubation period (Figure 5). There was no further increase in the level of PAPS over a longer incubation time. An enhanced production of PAPS was also recorded when 3'-AMP (25 mM final concentration) was added to reaction mixtures prior to adding the wheat extract (Table 7), although the effect of 3'-AMP was not as marked as that of 5'-AMP. The addition of sulphite, which is an

TABLE 6

PAPS production by various plant species

Artichoke (A) represents cells at the beginning of the cell cycle. The extract for artichoke (B) was prepared from tuber explants that had been grown in culture medium for 48 h using the method of Yeoman & Evans (1967). The plant materials and preparation of the plant extracts used in this experiment are given in Sections 2.2.3 and 2.8.1.3. The extracts were prepared immediately before assay. The reaction mixtures contained in μmoles/ml of incubation mixture: Tris-HCl buffer (pH 8.5), 100; $MgCl_2$, 20; Na_2SO_4, 40; ATP, 25; and 5'-AMP, 25, where shown. The reaction (30°) was started by adding the plant extract (approximately 12 mg protein). Samples (0.1 ml) were taken over a 60 min incubation period and were immediately hydrolysed in 0.2 ml of 0.2 N HCl at 37° for 30 min. After hydrolysis, samples were stored on ice and the amount of PAP determined with the *Renilla* bioluminescence procedure (Section 3.2.1.1).

Plant type	Specific activity (nmoles of PAPS produced/min/mg of protein)	
	Without 5'-AMP	With 5'-AMP
Artichoke (A)	0.18	0.23
Artichoke (B)	0.09	1.05
Wheat	0.14	1.52
Sorghum	0.05	0.78
Spinach	0.06	0.62

FIGURE 5

The effect of 5'-AMP on the production of PAPS from sulphate and ATP by an extract of young wheat leaves

The preparation of the plant extract used in this experiment is described in Section 2.8.1.3. The reaction mixtures contained in μmoles/ml incubation mixture: Tris-HCl buffer (pH 8.5), 100; $MgCl_2$, 20; Na_2SO_4, 40; ATP, 25; and 5'-AMP, 25. The reaction (30°) was started by adding an extract of the wheat leaves (0.5 ml, 1.1 mg of protein). Samples (0.1 ml) were withdrawn at the time intervals shown and immediately hydrolysed in two volumes (0.2 ml) of 0.2 N HCl at 37° for 30 min. After hydrolysis, samples were stored on ice and the amount of PAP determined using the *Renilla* bioluminescence procedure (Section 3.2.1.1). Extract alone (o——o); extract with 5'-AMP (●——●).

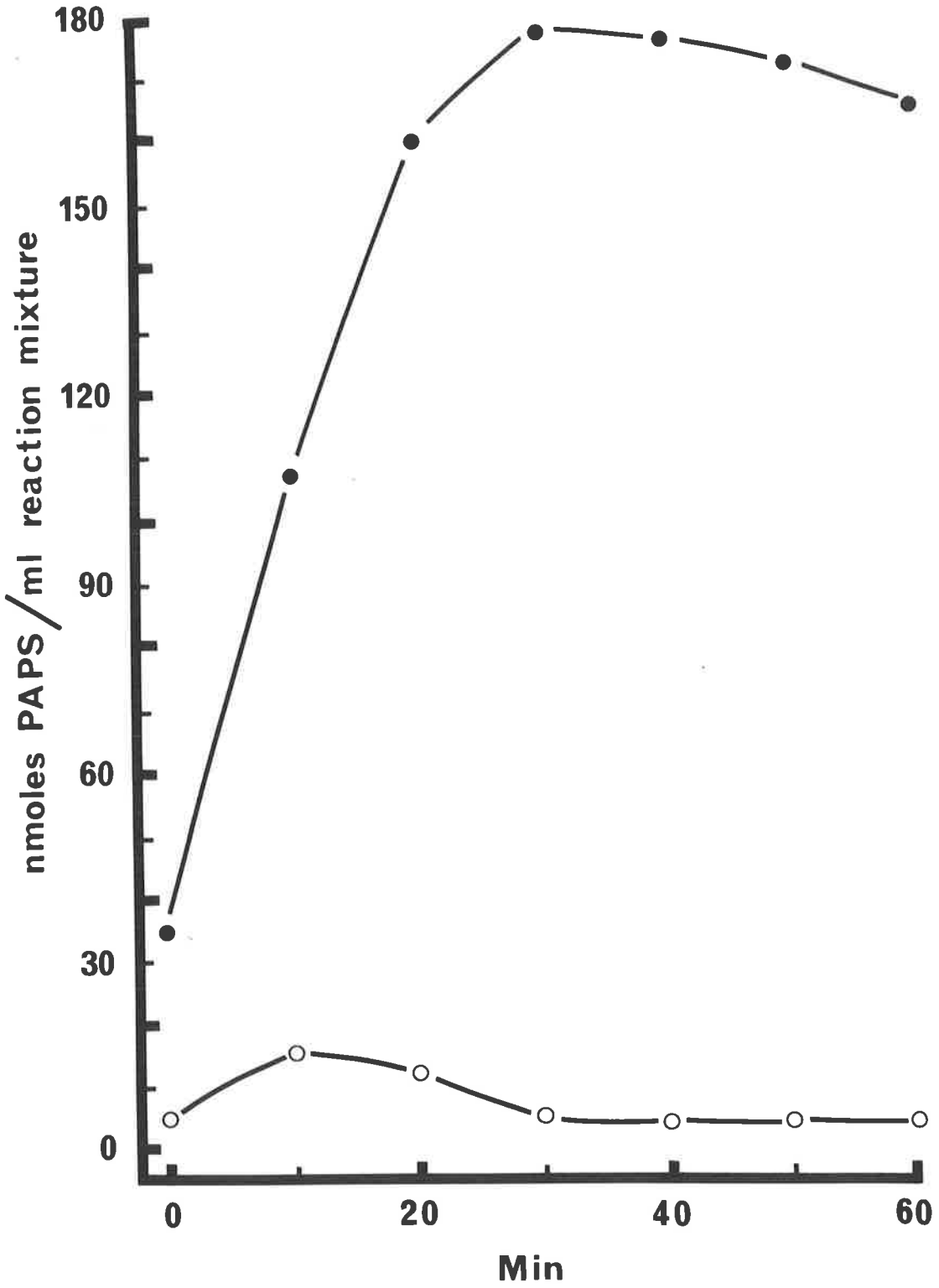

180
150
120
90
60
30
0
nmoles PAPS/ml reaction mixture
0
20
40
60
Min

Figure 5

TABLE 7

The effect of 3'-AMP, 5'-AMP and Na_2SO_3 on the production of PAPS in extracts of young wheat leaves

The preparation of the plant extract used in this experiment is described in Section 2.8.1.3. The reaction mixtures contained in μmoles: Tris-HCl buffer (pH 8.5), 225; $MgCl_2$, 50; Na_2SO_4, 50; ATP, 50; and 3'-AMP, 25; 5'-AMP, 25; or Na_2SO_3, 100; in a total volume of 5 ml. The reaction (30°) was started by adding an extract of the wheat leaves (10 mg protein). Samples (0.1 ml) withdrawn from the reaction mixtures at 10 min intervals over a period of 1 h were hydrolysed in two volumes (0.2 ml) of 0.2 N HCl at 37° for 30 min. After hydrolysis, samples were stored on ice and the amount of PAP determined using the *Renilla* bioluminescence procedure (Section 3.2.1.1).

Treatment	Specific activity (nmoles PAPS produced/min/mg of protein)
Extract	0.03
Extract + 5'-AMP	1.75
Extract + 3'-AMP	0.79
Extract + Na_2SO_3	0.06

inhibitor of sulphatase activity, had virtually no effect on PAPS production (Table 7). Attempts to demonstrate PAPS production by plant extracts using various radioactive substrates (^{35}S-sulphate, ^{14}C-ATP, ^{35}S-APS), as an alternative method to the *Renilla* assay, were unsuccessful, even in the presence of 5'-AMP. The use of ^{14}C-5'-AMP also showed that this compound was not phosphorylated to PAP, which could then contribute to the amounts of PAP determined by the *Renilla* bioluminescence procedure.

The apparent instability of PAPS in plant extracts led to a study of its protection from degradation using ^{35}S-PAPS, as described in Section 2.8.7. Figure 6 shows the time course and the degradation products of ^{35}S-PAPS in crude extracts of wheat leaves. Thus, ^{35}S-APS was produced very rapidly from ^{35}S-PAPS and it was further degraded to ^{35}S-sulphate. The data in Table 8 show a similar fate for ^{35}S-PAPS in various fractions of the leaves, except for the cytosol, where labelled APS persisted even after a 30 min incubation. There was no difference in the pattern of degradation of either PAPS or APS in intact and broken chloroplasts (Table 8). These results were similar even after the removal of membrane material from the broken chloroplasts by centrifuging at 16,000 x *g* for 30 min (Table 8).

The conversion of ^{35}S-PAPS via ^{35}S-APS to ^{35}S-sulphate in homogenates of young wheat leaves was not affected by including 3'-AMP (20 mM) in the incubation mixture (Table 9). Although there was some stabilizing effect by 3'-AMP on ^{35}S-PAPS after 0.3 min, this was not sustained over a 30 min incubation period. In contrast, when 5'-AMP (20 mM) was incubated with leaf homogenates in the presence of ^{35}S-PAPS over a 30 min incubation period, ^{35}S-APS was the major labelled compound detected (93%). This was also the case when increased amounts

FIGURE 6

Time course for the degradation of ^{35}S-PAPS in extracts of young wheat leaves

The preparation of the leaf homogenate used in this experiment is described in Section 2.8.1.3. The reaction mixtures contained in μmoles: Tris-HCl buffer (pH 7.5), 22.5; Na_2-EDTA, 0.9; ^{35}S-PAPS, 360 pmoles (0.58 μCi); and the leaf extract (0.1 ml, 2.2 mg protein), in a total volume of 1 ml. The incubations, carried out at 30° in a reciprocating water bath, were started by adding the leaf extract. Samples (0.05 ml) were taken at the time intervals shown and mixed with an equal volume of 99% (v/v) ethanol, centrifuged at 3,000 x *g* for 5 min and then aliquots (0.05 ml) of the supernatant fraction were applied to 3MM Whatmann paper. Details of the electrophoretic separation and radioassay of labelled compounds are presented in Section 2.9.1 and Section 2.9.3 respectively. ●——●, ^{35}S-PAPS; o——o, ^{35}S-APS; ■——■, $^{35}S\text{-}SO_4^{2-}$.

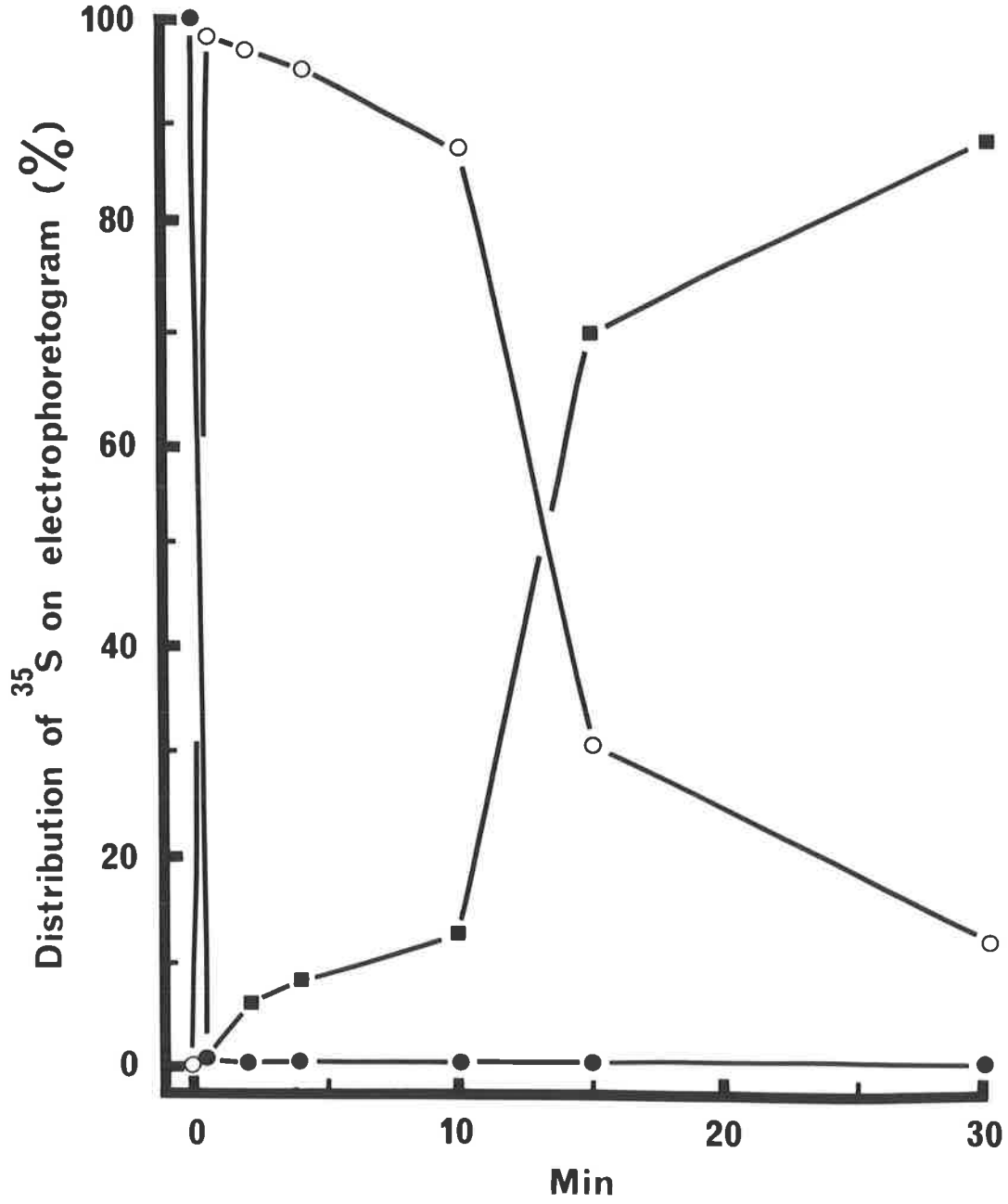

100
80
60
40
20
0
Distribution of ^{35}S on electrophoretogram (%)
0
10
20
30
Min

Figure 6

TABLE 8

Fate of ^{35}S-PAPS in various fractions of young wheat leaves

The preparation of the various leaf fractions used in this experiment are described in Section 2.8.1.3. Intact chloroplasts were disrupted by ultrasonication for 5 min at 2° (MSE Ultrasonic Probe, 20 kilocycles/sec) and the extract assayed before (A) and after (B) centrifugation at 16,000 x *g* for 30 min. The reaction mixtures contained in μmoles: Tris-HCl buffer (pH 7.5), 12.5; Na_2-EDTA, 0.5; ^{35}S-PAPS, 360 pmoles (0.58 μCi); and the leaf fractions (0.5 ml, approximately 1 mg of protein) in a total volume of 1 ml. The incubations, carried at 30° in a reciprocating water bath, were started by adding the leaf extract. Samples (0.05 ml) were taken at the three time intervals shown and mixed with an equal volume of 99% (v/v) ethanol, centrifuged at 3,000 x *g* for 5 min and then aliquots (0.05 ml) of the supernatant fraction were applied to 3MM Whatmann paper. Details of the electrophoretic separation and radioassay of labelled compounds are presented in Section 2.9.1 and Section 2.9.3 respectively.

Leaf preparation	mg chlorophyll per ml leaf extract	Incubation time (min)	Distribution of ^{35}S on electrophoretogram (%)		
			^{35}S-PAPS	^{35}S-APS	^{35}S-SO_4^{2-}
Homogenates of leaves	0.103	0	100	-	-
		0.3	1.7	87.5	10.8
		30	0.6	0.4	99.0
Cytosol	0.010	0	100	-	-
		0.3	71.4	27.2	1.4
		30	5.8	89.9	4.3
Intact chloroplasts	0.104	0	100	-	-
		0.3	2.1	90.6	7.3
		30	1.3	6.0	92.7
Broken chloroplasts (A)	0.113	0	100	-	-
		0.3	5.6	90.1	4.3
		30	0.8	23.4	75.8
Broken chloroplasts (B)	-	0	100	-	-
		0.3	0.9	92.3	6.8
		15	3.5	17.2	79.3
		30	1.5	3.2	95.3
Sub-cellular components	0.132	0	100	-	-
		0.3	0.7	24.9	74.4
		30	0	0.2	99.8

TABLE 9

Effect of 3'-AMP and 5'-AMP on the fate of ^{35}S-PAPS in homogenates of young wheat leaves

The preparation of the leaf homogenate used in this experiment is described in Section 2.8.1.3. The reaction mixtures contained in μmoles: (A) and (B), Tris-HCl buffer (pH 7.5), 22.5; Na_2-EDTA, 0.9; 3'-AMP, 20; ^{35}S-PAPS, 360 pmoles (0.58 μCi); and the leaf homogenate (0.1 ml, 0.22 mg protein) in a total volume of 1 ml; (C) Tris-HCl buffer (pH 7.5), 12.5; Na_2-EDTA, 0.5; 5'-AMP, 20; ^{35}S-PAPS, 360 pmoles (0.58 μCi) and the leaf homogenate (0.5 ml, 1.1 mg of protein) in a total volume of 1 ml. The incubations, carried out at 30° in a reciprocating water bath, were started by adding the leaf homogenate. Samples (0.05 ml) were taken at the three time intervals shown and mixed with an equal volume of 99% (v/v) ethanol, centrifuged at 3,000 x g for 5 min and then aliquots (0.05 ml) of the supernatant fraction were applied to 3MM Whatmann paper. Details of the electrophoretic separation and radioassay of labelled compounds are presented in Section 2.9.1 and Section 2.9.3 respectively.

	Addition	Protein (mg/ml of incubation mixture)	Incubation time (min)	Distribution of ^{35}S on electrophoretogram (%)		
				^{35}S-PAPS	^{35}S-APS	^{35}S-SO_4^{2-}
A.	Extract	0.22	0	100	-	-
			0.3	4.6	73.3	22.1
			15	1.0	19.6	79.4
			30	0.6	4.6	94.8
B.	Extract + 3'-AMP	0.22	0	100	-	-
			0.3	32.5	22.2	45.3
			15	3.0	20.8	76.2
			30	1.4	12.9	85.7
C.	Extract + 5'-AMP	1.10	0	100	-	-
			0.3	12.6	81.4	6.0
			15	2.9	93.0	4.1
			30	2.0	93.0	5.0

of leaf extract were used as shown in Table 9.

^{35}S-PAPS was stabilized when phosphate, pyrophosphate or nucleotide triphosphates were included in the reaction mixtures (Table 10). In the absence of protective compounds, labelled PAPS was degraded only as far as APS (Table 10). Thus, storage of the plant extract on ice for 24 h altered the pattern of degradation (see Table 9), suggesting that the enzyme(s) responsible for the cleavage of these sulphur nucleotides may be labile. Since these studies were only concerned with a qualitative comparison between the effects of various compounds on the stability of ^{35}S-PAPS, extracts aged up to 24 h were still used in the experiments. After a 30 min incubation period with 90 mM of either phosphate or pyrophosphate, 64% and 95% respectively of the radioactivity was retained in PAPS (Table 10). Under similar conditions using 20 mM nucleotide triphosphates, the residue levels of ^{35}S-PAPS were (%) 70, 51, 51, 37 and 34, in the presence of ATP, GTP, TTP, CTP and UTP respectively (Table 10).

Labelled PAPS was not degraded when incubated with either leaf extracts or chloroplast fractions prepared in an aqueous medium which contained dextran sulphate (Type 500-S), as used by Hadziyev *et al.* (1968). Thus, the effect of dextran sulphate in stabilizing ^{35}S-PAPS was investigated further using extracts of wheat leaves prepared by the non-aqueous technique of Stocking (1959) (see Section 2.8.1.3). Increased protection for ^{35}S-PAPS was well correlated with the amount of dextran sulphate in the incubation mixtures, as shown in Table 11 and Figure 7. The stabilization of ^{35}S-PAPS was probably associated with the phosphate content of dextran sulphate. Thus, 1 µmole of the dextran sulphate contained approximately 20 µmoles of phosphate (see Section 2.9.5). The stabilizing effect of phosphate on ^{35}S-PAPS in

TABLE 10

Effect of nucleotide triphosphates, phosphate and pyrophosphate on the stability of ^{35}S-PAPS in homogenates of young wheat leaves

The preparation of the leaf homogenate used in this experiment is described in Section 2.8.1.3. The reaction mixtures contained in µmoles: Tris-HCl buffer (pH 7.5), 20; Na_2-EDTA, 0.8; nucleotides, 20; ^{35}S-PAPS, 360 pmoles (0.58 µCi); and the leaf homogenate (0.1 ml, 0.2 mg of protein) in a total volume of 1 ml. For the NaH_2PO_4 and $Na_2H_2P_2O_7$ treated extracts, the reaction mixtures contained in µmoles: phosphate buffer (pH 7.6), 90; or pyrophosphate buffer (pH 7.6), 90; ^{35}S-PAPS, 360 pmoles (0.58 µCi) and the leaf homogenate (0.1 ml, 0.2 mg protein). The incubations, carried out at 30° in a reciprocating water bath, were started by adding the leaf homogenate. Samples (0.05 ml) were taken at the time intervals shown and prepared for radioassay as described in Table 9.

Addition	Incubation time (min)	Distribution of ^{35}S on electrophoretogram (%)		
		^{35}S-PAPS	^{35}S-APS	^{35}S-SO_4^{2-}
Extract	0	100	-	-
	0.3	68.6	30.3	1.1
	30	2.8	82.7	14.5
Extract + NaH_2PO_4	0	100	-	-
	0.3	96.1	1.8	2.1
	30	64.0	33.6	2.4
Extract + $Na_2H_2P_2O_7$	0	100	-	-
	0.3	94.5	1.9	3.6
	30	95.0	1.7	3.3
Extract + ATP	0	100	-	-
	0.3	71.3	27.1	1.6
	30	69.9	27.1	2.4
Extract + UTP	0	100	-	-
	0.3	48.8	35.3	15.9
	30	35.2	56.7	8.1
Extract + GTP	0	100	-	-
	0.3	56.3	40.0	3.7
	30	51.4	37.7	10.9
Extract + CTP	0	100	-	-
	0.3	52.0	42.6	5.4
	30	37.3	59.6	3.1
Extract + TTP	0	100	-	-
	0.3	65.5	32.9	1.6
	30	51.3	45.9	2.8

TABLE 11

Effect of dextran sulphate on the stability of ^{35}S-PAPS in homogenates of wheat leaves

The preparation of the leaf homogenate used in this experiment is described in Section 2.8.1.3. The reaction mixtures contained in μmoles: Tris-HCl buffer (pH 7.5), 22.5; Na_2-EDTA, 0.9; dextran sulphate, 0.05, 0.1 and 0.2 respectively; ^{35}S-PAPS, 360 pmoles (0.58 μCi) and the leaf homogenate (0.1 ml, 0.2 mg of protein) in a total volume of 1 ml. The incubations, carried out at 30° in a reciprocating water bath, were started by adding the leaf homogenate. Samples (0.05 ml) were taken at the time intervals shown and prepared for radioassay as described in Table 9.

Addition	Incubation time (min)	Distribution of ^{35}S on electrophoretogram (%)		
		^{35}S-PAPS	^{35}S-APS	^{35}S-SO_4^{2-}
Extract	0	100	-	-
	0.3	4.6	73.3	22.1
	15	1.0	19.6	79.4
	30	0.6	4.6	94.8
Extract + 0.05 mM dextran sulphate	0	100	-	-
	0.3	88.8	10.6	0.6
	15	77.2	20.0	2.8
	30	25.3	6.4	68.3
Extract + 0.1 mM dextran sulphate	0	100	-	-
	0.3	85.1	4.4	10.5
	15	80.1	16.3	3.6
	30	48.0	12.2	39.8
Extract + 0.2 mM dextran sulphate	0	100	-	-
	0.3	86.6	5.4	8.0
	15	78.2	13.3	8.5
	30	66.6	14.7	18.7

FIGURE 7

Effect of dextran sulphate on the stability of ^{35}S-PAPS *in homogenates of wheat leaves*

The experimental details are described in Table 11. The amount of residual ^{35}S-PAPS, determined after a 30 min incubation, has been plotted as a function of the increasing dextran sulphate concentration.

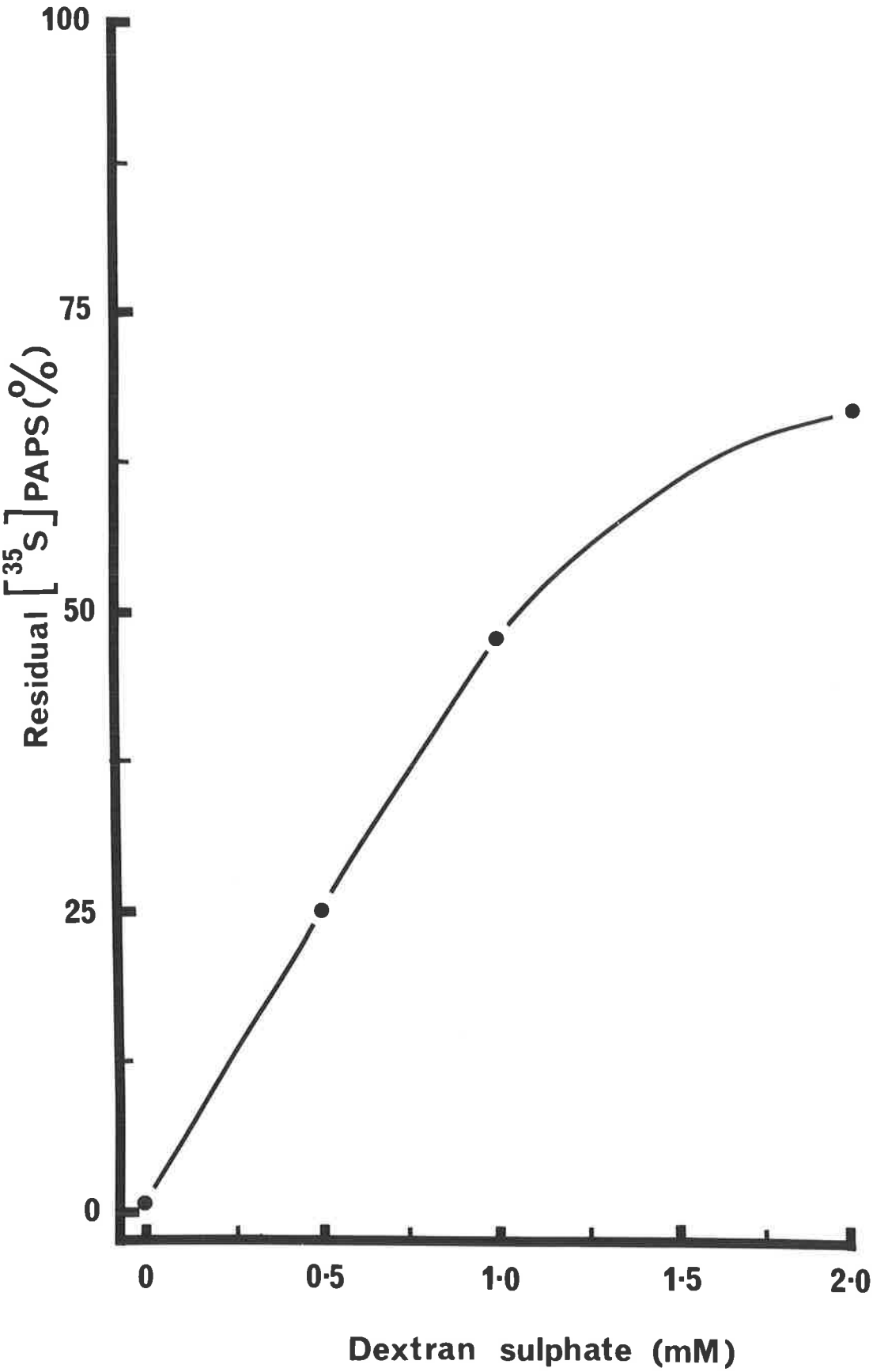

100
75
50
25
0
Residual [35S]PAPS(%)
0
0·5
1·0
1·5
2·0
Dextran sulphate (mM)

Figure 7

the presence of leaf homogenates has already been presented (Table 10).

The stability of PAP in leaf homogenates and chloroplast fractions of wheat was investigated using the *Renilla* bioluminescence assay procedure. The products of PAP degradation were not identified. PAP was unstable in leaf homogenates as shown in Table 12. The addition of 5'-AMP (25 mM) afforded complete protection for PAP at the lowest level of homogenate tested (0.01 mg protein), but increasing amounts of the homogenate offset the effect of 5'-AMP after a 20 min incubation period (Table 12). In contrast, PAP was found to be virtually stable over a 20 min period when incubated with intact chloroplasts in the absence of 5'-AMP. The chloroplast preparation was prepared in the absence of any stabilizing compound (Section 2.8.1.3).

3.2.2.2 Yeast

The production of PAPS from sulphate and ATP in a yeast extract (S_{60}), determined by the *Renilla* bioluminescence procedure, is shown in Figure 8. The synthesis of PAPS was curvilinear over a 60 min period. Similarly, PAPS was produced by incubating APS and ATP with the yeast extract (Figure 9). The product, PAPS, was confirmed by following the incorporation of ^{35}S-sulphate and ^{35}S-APS into ^{35}S-PAPS in the presence of ATP. The labelled compounds from an aliquot of the reaction mixture were separated by high-voltage electrophoresis in citrate buffer (pH 5) (Section 2.9.1) and compared with a standard of ^{35}S-PAPS run under identical conditions. The specific activity for PAPS produced from (a) ATP and sulphate and (b) APS and ATP was 0.24 and 0.32 nmoles/min/mg protein respectively, as determined by the bioluminescence method.

The bioluminescence technique was further tested as an assay

TABLE 12

Stability of PAP in leaf homogenates and in chloroplast fractions of wheat

The preparations of the leaf homogenate and intact chloroplasts are described in Section 2.8.1.3. The reaction mixtures contained in μmoles/ml of incubation mixture: Tris-HCl buffer (pH 8.0), 100; $MgCl_2$, 1; 5'-AMP, 25; and either the homogenate of wheat leaves (0.01-0.20 mg of protein) or intact chloroplast preparation (0.06 mg of protein). After preincubating at 30° for 1 min, the reaction (30°) was started by adding PAP (5 nmoles). Samples (0.05 ml) were taken at 0, 10 and 20 min and the amount of PAP in these samples determined immediately by the *Renilla* bioluminescence procedure as described in Section 3.2.1.1. Boiled preparations were used as controls for each treatment.

Treatment	Protein (mg/ml)	nmoles of PAP remaining/ml of incubation mixture		
		Sampling time (min)		
		0	10	20
Homogenate	0.01	11	0	0
	0.04	4	0	0
	0.20	0	0	0
Boiled homogenate	0.20	19	13	11
Homogenate + 5'-AMP (25 mM)	0.01	33	31	35
	0.04	32	13	1
	0.20	34	2	0
Boiled homogenate + 5'-AMP (25 mM)	0.20	30	27	30
Intact chloroplasts	0.06	36	36	29

FIGURE 8

Production PAPS from sulphate and ATP in a yeast extract (S_{60})

The preparation of the yeast extract (S_{60}) is described in Section 2.8.1.2. The reaction mixture contained in µmoles: Tris-HCl buffer (pH 8.5), 500; $MgCl_2$, 50; ATP, 50; and Na_2SO_4, 50, in a total volume of 5 ml. The reaction, started by adding the yeast extract (12.2 mg of protein), was carried out at 30° in a reciprocating water bath. Samples (0.1 ml) withdrawn from the incubation mixture at the time intervals shown were immediately hydrolysed in two volumes (0.2 ml) of 0.2 N HCl at 37° for 30 min. The hydrolysates were stored on ice and aliquots (0.02 ml) taken and PAP determined by the *Renilla* bioluminescence procedure as described in Section 3.2.1.1.

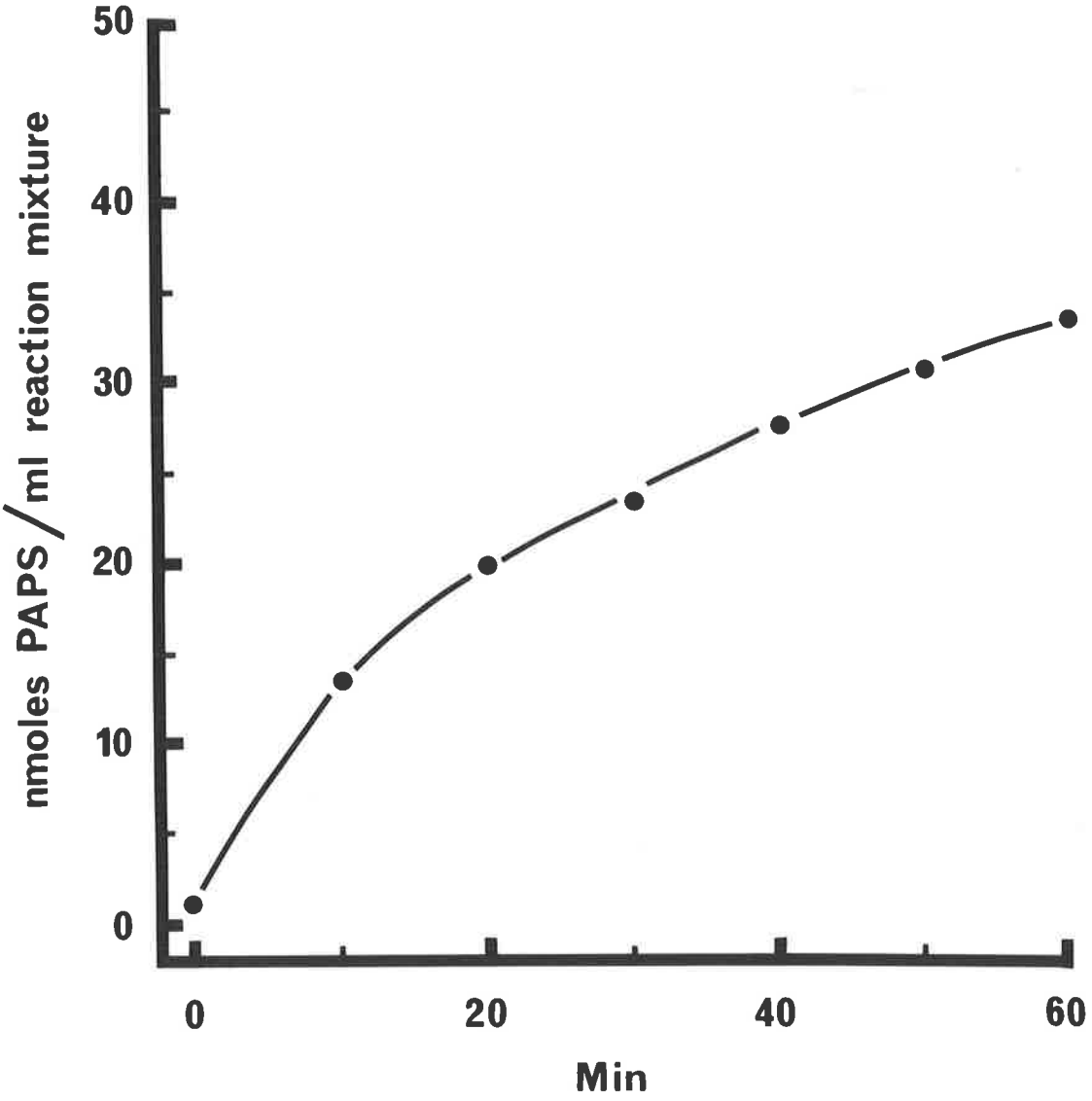


Figure 8

FIGURE 9

Production of PAPS from APS and ATP in a yeast extract (S_{60})

The preparation of the yeast extract (S_{60}) is given in Section 2.8.1.2. The reaction mixture contained in µmoles: Tris-HCl buffer (pH 8.0), 120; $MgCl_2$, 50; ATP, 60; and APS, 18 nmoles, in a total volume of 1.2 ml. The reaction, started by adding the yeast extract (5 mg of protein), was carried out at 30° in a reciprocating water bath. Samples (0.1 ml), withdrawn from the incubation mixture at the time intervals shown, were immediately hydrolysed in two volumes (0.2 ml) of 0.2 N HCl at 37° for 30 min. The hydrolysates were stored on ice and aliquots (0.02 ml) taken and PAP determined by the *Renilla* bioluminescence procedure as described in Section 3.2.1.1.

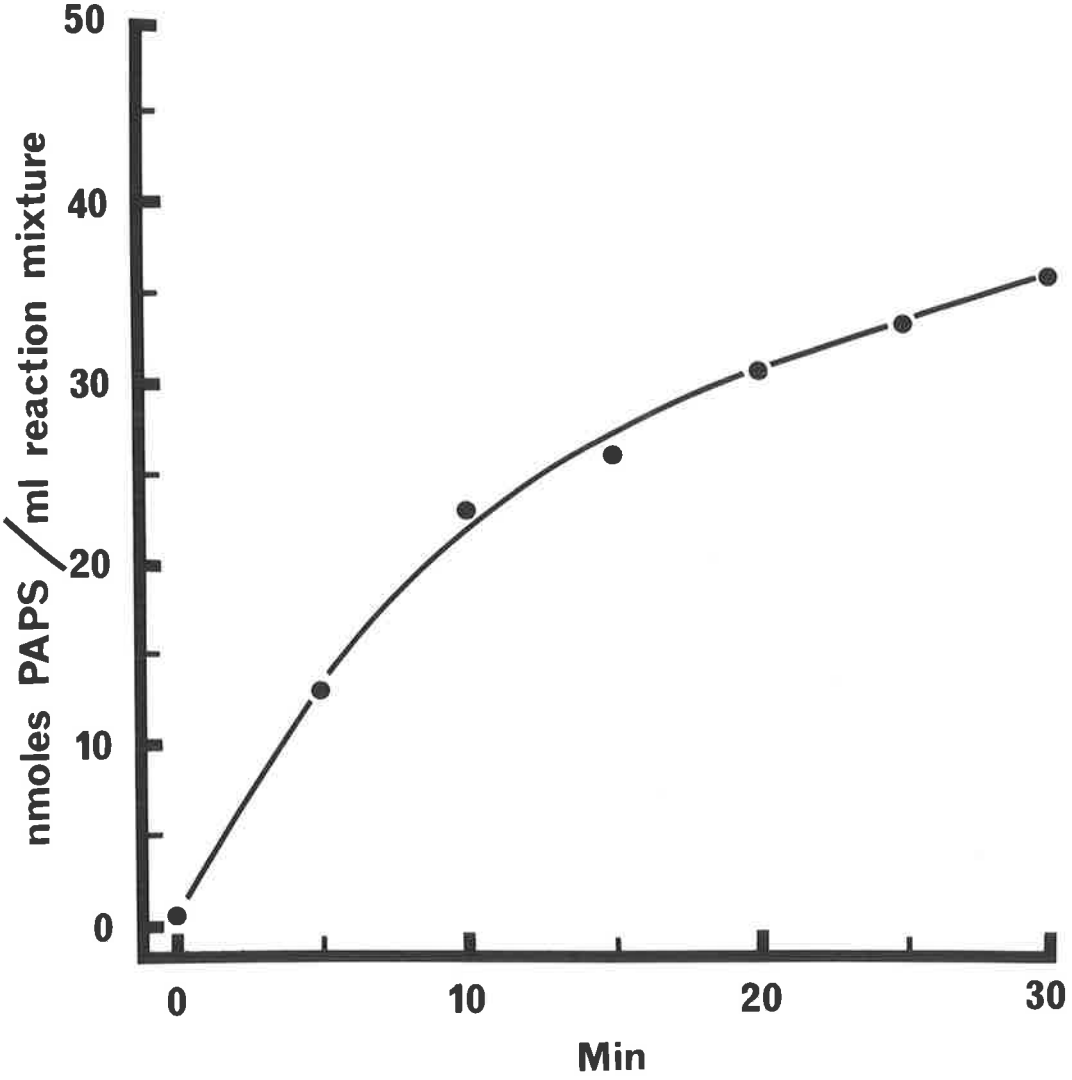


Figure 9

system for APS-kinase activity in yeast extracts (S_{60}). Figure 10 shows the effect of increasing APS concentration on APS-kinase activity. A marked inhibition of enzymic activity was observed in extracts of yeast (S_{60}) at APS concentrations greater than 75 µM. The *Renilla* assay was also used to follow the partial purification of APS-kinase in yeast. A purification of approximately ten-fold was obtained by treating the high-speed supernatant fraction (S_{60}) with 2% (w/v) protamine sulphate.

The production of PAPS from sulphate and ATP in a yeast extract (Figure 8) differs markedly from that in extracts of young wheat leaves (Figure 5). Thus, the effect of mixing the two extracts on PAPS production was investigated. The synthesis of PAPS by the yeast extract was curvilinear over a period of 60 min. When an extract of wheat leaves was added to that of yeast at zero time prior to incubation, the production of PAPS was completely inhibited (Figure 11). The addition of the same amount of wheat extract to the yeast preparation which had been previously incubated with sulphate and ATP for 20 min produced a rapid decline in PAPS synthesis, as shown in Figure 11. Control tubes without wheat extract showed no PAPS disappearance (Figure 11).

The time course and the products of the degradation of ^{35}S-PAPS in yeast extracts (S_{60}) were similar to those already presented for leaf homogenates (Figure 6). Thus, ^{35}S-PAPS was almost quantitatively converted via ^{35}S-APS to ^{35}S-sulphate. A marked protection of ^{35}S-PAPS was again observed by including either ATP or ADP (20 mM) in the incubation mixtures (Table 13). In the presence of UTP and TTP (20 mM), 73% and 80% respectively of the ^{35}S-PAPS remained after 30 min incubation. In contrast to the leaf homogenates (Table 9), 5'-AMP did not protect either ^{35}S-PAPS or ^{35}S-APS (Table 13). Similarly, the labelled sulphur nucleotides were not protected by including 3'-AMP, phosphate

FIGURE 10

Effect of graded amounts of APS on the production of PAPS from APS and ATP in a yeast extract (S_{60})

The preparation of the yeast extract (S_{60}) is given in Section 2.8.1.2. The reaction mixtures contained in μmoles: Tris-HCl buffer (pH 8.0), 6-30; $MgCl_2$, 12; ATP, 15; APS, 0-45 nmoles; and yeast extract (1.3 mg protein), in a final volume of 0.3 ml. The reaction, started by adding the yeast extract, was carried out in a reciprocating water bath at 30°. Samples (0.1 ml), withdrawn from the incubation mixtures at 0, 5 and 10 min time periods, were hydrolysed in two volumes (0.2 ml) of 0.2 N HCl at 37° for 30 min. The hydrolysates were stored on ice and aliquots (0.02 ml) taken and PAP determined by the *Renilla* bioluminescence procedure as described in Section 3.2.1.1.

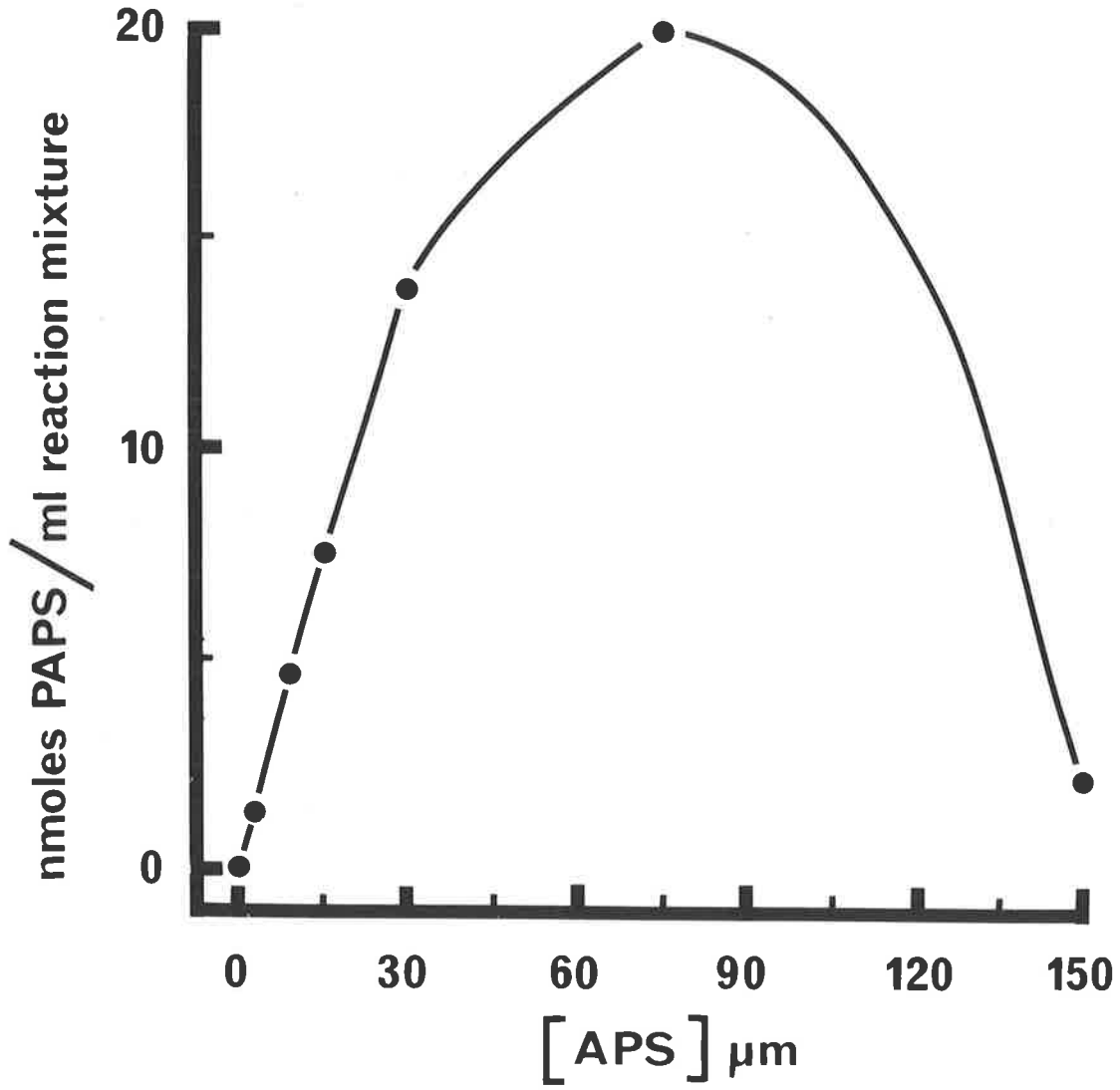

20
10
0
nmoles PAPS / ml reaction mixture
0
30
60
90
120
150
[APS] µm

Figure 10

FIGURE 11

PAPS production by combined extracts of yeast and wheat leaves

The preparations of the yeast extract and the leaf homogenate are described in Section 2.8.1.2 and Section 2.8.1.3 respectively. The reaction mixtures contained in μmoles: Tris-HCl buffer (pH 8.5), 500; $MgCl_2$, 50; Na_2SO_4, 50; and ATP, 50, in a total volume of 5 ml. The reaction, started by adding the yeast (12 mg of protein) was carried out at 30° in a reciprocating water bath. An aliquot (1 ml containing 2.2 mg protein) of the leaf extract was then added to the separate yeast-containing incubation mixtures at 0 min (■——■) and at 20 min (●——●) respectively. Control tubes were started by adding the yeast extract as above, but the wheat extract was replaced by an equal volume of 0.2 M Tris-HCl buffer at 0 min (□——□) and at 20 min (o——o). Samples (0.7 ml) were withdrawn at the time intervals shown and immediately hydrolysed in two volumes (0.2 ml) of 0.2 N HCl at 37° for 30 min. After storing the hydrolysates briefly on ice, the amounts of PAP in aliquots of these samples (0.02 ml) were determined by the *Renilla* bioluminescence procedure (Section 3.2.1.1).

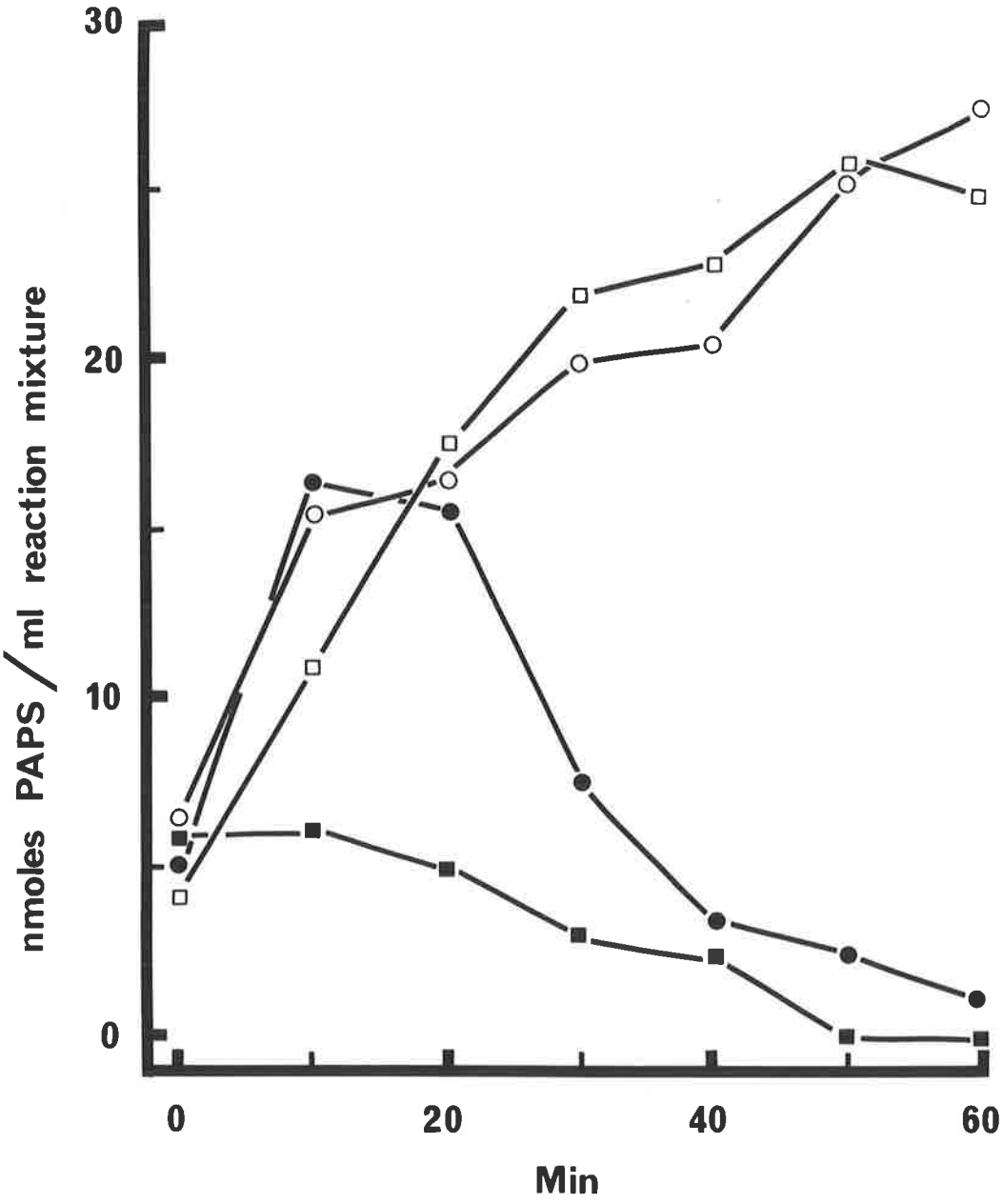


Figure 11

TABLE 13

Effect of various compounds on the degradation of ^{35}S-PAPS in extracts of baker's yeast

The preparation of the yeast extract (S_{60}) is described in Section 2.8.1.2. The reaction mixtures contained in µmoles: Tris-HCl buffer (pH 7.5), 25; Na_2-EDTA, 1.0; nucleotides, NaH_2PO_4, $Na_2H_2P_2O_7$, Na_2SO_3, Na_2SO_4, 20; ^{35}S-PAPS, 52 pmoles (0.09 µCi) and the yeast extract (0.4 ml, 15.2 mg of protein). The NADH-generating system contained in µmoles: NAD, 0.4; ethanol, 20; alcohol dehydrogenase, 444 units of activity, in a total volume of 0.15 ml. The NADPH-generating system contained in µmoles: NADP, 0.5; glucose-6-phosphate, 5; glucose-6-phosphate dehydrogenase, 0.06 units of activity, in a total volume of 0.15 ml. The incubations, carried out at 30° in a reciprocating water bath, were started by adding the cell-free extract. Samples (0.02 ml), taken from the incubation mixtures, were applied directly to 3MM Whatmann paper. Details of the electrophoretic separation and radioassay of labelled compounds are presented in Section 2.9.1 and Section 2.9.3 respectively.

Addition	Incubation time (min)	Distribution of ^{35}S on electrophoretogram (%)				
		^{35}S-PAPS	^{35}S-APS	^{35}S-SO_3^{2-}	^{35}S-SO_4^{2-}	Origin
Extract alone	0	100	-	-	-	-
	0.3	7.4	90.7	-	1.9	-
	15	3.3	11.0	10.1	71.4	4.2
	30	11.0	3.8	7.3	71.6	6.3
Extract + NaH_2PO_4	0	100	-	-	-	-
	0.3	23.7	72.5	-	2.3	-
	15	3.1	6.4	16.6	67.4	6.5
	30	5.9	4.6	12.2	67.6	9.7
Extract + $Na_2H_2P_2O_7$	0	100	-	-	-	-
	0.3	30.5	49.5	-	20.0	-
	15	8.2	2.3	7.0	76.1	6.4
	30	1.9	5.2	8.6	77.2	7.1
Extract + 3'-AMP	0	100	-	-	-	-
	0.3	40.4	36.8	-	22.8	-
	15	26.8	22.5	0.1	50.5	0.1
	30	15.5	26.7	0	57.7	0.1
Extract + 5'-AMP	0	100	-	-	-	-
	0.3	47.9	27.6	-	24.5	-
	15	5.3	3.4	6.5	79.2	5.6
	30	1.7	5.2	7.1	79.7	6.3

TABLE 13 (*cont'd*)

Addition	Incubation time (min)	Distribution of ^{35}S on electrophoretogram (%)				
		^{35}S-PAPS	^{35}S-APS	^{35}S-SO_3^{2-}	^{35}S-SO_4^{2-}	Origin
Extract + ADP	0	100	-	-	-	-
	0.3	62.4	21.8	-	15.8	-
	15	62.0	12.8	0.2	24.8	0.2
	30	48.5	17.2	0.1	34.0	0.2
Extract + ATP	0	100	-	-	-	-
	0.3	82.1	9.2	-	8.7	-
	15	83.2	5.7	0	11.1	0
	30	78.1	7.2	0.1	14.5	0.1
Extract + NADH	0	100	-	-	-	-
	0.3	34.5	16.2	4.0	41.3	4.0
	15	12.3	5.3	8.8	64.0	9.6
	30	20.3	11.9	12.6	41.4	13.8
Extract + NADPH	0	100	-	-	-	-
	0.3	23.7	7.3	19.7	40.2	9.1
	15	22.2	7.1	15.2	38.9	16.6
	30	24.1	13.6	16.7	28.1	17.5
Extract + adenine	0	100	-	-	-	-
	0.3	25.8	27.3	3.2	40.3	3.4
	15	14.9	4.6	10.9	64.8	4.8
	30	17.9	6.7	14.2	51.1	10.1
Extract + adenosine	0	100	-	-	-	-
	0.3	37.9	16.8	3.3	39.7	2.3
	15	11.2	5.0	11.4	67.2	5.2
	30	16.8	5.4	15.1	51.5	11.2
Extract + SO_3^{2-}	0	100	-	-	-	-
	0.3	62.9	11.1	-	26.0	-
	15	14.5	3.3	50.7	26.8	4.7
	30	4.6	5.0	12.8	75.1	2.5
Extract + SO_4^{2-}	0	100	-	-	-	-
	0.3	36.7	20.6	-	42.7	-
	15	8.5	2.6	8.7	74.4	5.8
	30	3.2	11.9	9.2	70.0	5.7

or pyrophosphate in the incubation mixtures (Table 13). After 30 min incubation with sodium fluoride (10 mM), 27% of the ^{35}S-PAPS remained.

Small amounts of sulphite were detected in the incubation mixtures (Table 13). Labelled ^{35}S, probably as membrane-bound ^{35}S-sulphate (Moriarty & Nicholas, 1969), was found in small amounts at the origin of the electrophoretograms of the yeast samples (Table 13) but not in those of wheat leaves.

There was a rapid degradation of PAP when it was incubated with extracts of yeast, as determined by the bioluminescence procedure (Table 14). The degradation was significantly reduced in the presence of 5'-AMP, ADP or ATP. Boiled extracts did not degrade PAP. The higher estimates of PAP in the ADP-treated sample resulted from the large amounts of PAP present as an impurity in commercial preparations of ADP (Section 3.2.1.3).

3.2.2.3 Bacteria

In cell-free extracts of either *Desulfotomaculum nigrificans* or *Thiobacillus ferrooxidans*, grown autotrophically or heterotrophically, PAPS production from ATP and either sulphate or APS was not detected by the bioluminescence method, even in the presence of 3'-AMP or 5'-AMP.

Cell-free extracts (S_{10}) of ferrous-iron-grown *T. ferrooxidans* produced very small amounts of ^{35}S-APS from ^{35}S-sulphate. Less than 0.6% of the tracer was found in ^{35}S-APS after a 30 min incubation in the presence of inorganic pyrophosphatase and an ATP-generating system. Similarly, ATP-sulphurylase activity, obtained by assaying ATP production from APS and PPi with the firefly luciferin-luciferase system, was very low (230 pmoles ATP produced/min/mg protein). Thus, APS appears to be the activated form of sulphate in *T. ferrooxidans*.

TABLE 14

Stability of PAP in extracts of yeast (S_{60})

The preparation of the yeast extract (S_{60}) is described in Section 2.8.1.2. The reaction mixtures contained in μmoles: Tris-HCl buffer (pH 7.5), 25; Na_2-EDTA, 2.5; $MgCl_2$, 20; nucleotides, 20; NaH_2PO_4, 20; $Na_2H_2P_2O_7$, 20; and yeast extract (21.7 mg of protein) in a total volume of 0.62 ml. After preincubating at 30° for 1 min, the reactions were started by adding PAP (900 pmoles). The reactions were carried out at 30° in a reciprocating water bath. Samples (0.1 ml), taken from the incubation mixtures at 0, 15, 30 and 45 min, were added to two volumes (0.2 ml) of 0.2 N HCl and hydrolysed at 37° for 30 min. After hydrolysis, samples were stored on ice and aliquots (0.02 ml) taken for determining the amount of PAP using the *Renilla* bioluminescence procedure, as described in Section 3.2.1.1. Boiled extracts were included as controls for each treatment.

Treatment	nmoles of PAP remaining/ml of incubation mixture			
	Sampling time (min)			
	0	15	30	45
Extract	1.6	0	0	0
Extract + 3'-AMP	0.2	0	0	0
Extract + 5'-AMP	2.6	0.9	0.5	0.4
Extract + ADP	6.8	1.2	0.5	0.7
Extract + ATP	1.9	0.6	0.6	0.6
Extract + NaH_2PO_4	1.7	0	0	0
Extract + $Na_2H_2P_2O_7$	1.4	0	0	0

The fate of ^{35}S-APS was followed in cell-free extracts (S_{10}) of *T. ferrooxidans* grown with ferrous-iron. After a 20 sec incubation of ^{35}S-APS with the extract, about 80% of the label appeared in free sulphate (Table 15). ^{35}S-APS could be effectively stabilized in extracts by including PPi, 5'-AMP, ATP or ADP, or to a lesser extent, Pi, in the incubation mixtures (Table 15). The presence of an NADPH- or NADH-generating system was without effect. No ^{35}S-PAPS was produced from ^{35}S-APS by the various treatments described in Table 15.

The only labelled degradation product of U-^{14}C-APS in cell-free extracts incubated for 30 min appeared to be ^{14}C-AMP; neither ^{14}C-adenosine nor ^{14}C-adenine was detected. Protection was again afforded by the compounds listed in Table 15 where ^{35}S-APS was used as substrate.

In cell-free extracts of *T. ferrooxidans*, ^{35}S-PAPS was degraded at a much slower rate than was ^{35}S-APS (Table 16). ^{35}S-PAPS was well protected by ATP and Pi, whereas PPi and 5'-AMP, which offset the hydrolysis of ^{35}S-APS, were now less effective (Table 16). The increase in radioactivity at the origin in the electrophoretograms in some treatments was probably associated with the formation of membrane-bound sulphate (Moriarty & Nicholas, 1969), since the amount of free ^{35}S-sulphate present after 30 min incubation was markedly decreased. It is also of interest that in the presence of ATP, ADP or phosphates, ^{35}S-PAPS was resynthesized after an initial cleavage of the ^{35}S-sulphate moiety. Only trace amounts of ^{35}S-APS were detected during the incubations, while the major labelled products were free and membrane-associated ^{35}S-sulphate.

The stability of PAP was studied in the cell-free extracts. The initial amounts of PAP were higher in the presence of ATP and PPi, suggesting an immediate breakdown of PAP in the absence of these

TABLE 15

Effect of various compounds on the degradation of ^{35}S-APS in extracts (S_{10}) of T. ferrooxidans

The preparation of the cell-free extracts (S_{10}) of iron-grown *T. ferrooxidans* is described in Section 2.8.1.1. The reaction mixtures contained in µmoles: Tris-HCl buffer (pH 7.5), 25; Na_2-EDTA, 1; $MgCl_2$, 20; ^{35}S-APS, 370 nmoles (0.19 µCi) and the extract (S_{10}) (10.7 mg of protein) in a total volume of 0.64 ml. The NADH- and NADPH-generating systems are described in Table 13. The incubations, carried out at 30° in a reciprocating water bath, were started by adding the cell-free extract. Samples (0.02 ml) were taken at the time intervals shown and applied directly to 3MM Whatmann paper. The labelled compounds were separated by high-voltage electrophoresis followed by radioassay as described in Section 2.9.1 and Section 2.9.3 respectively.

Addition	Incubation time (min)	Distribution of ^{35}S on electrophoretogram (%)		
		^{35}S-PAPS	^{35}S-APS	^{35}S-SO_4^{2-}
Extract alone	0	100	-	-
	0.3	18.2	0	81.8
	30	5.2	0	94.8
Extract + NaH_2PO_7	0	100	-	-
	0.3	38.4	0	61.6
	30	24.1	0	75.9
Extract + $Na_2H_2P_2O_7$	0	100	-	-
	0.3	94.3	0	5.7
	30	86.9	0	13.1
Extract + 5'-AMP	0	100	-	-
	0.3	87.7	0.4	11.9
	30	98.0	0	2.0
Extract + ADP	0	100	-	-
	0.3	71.5	0.1	28.4
	30	97.8	0	2.2
Extract + ATP	0	100	-	-
	0.3	74.5	0.1	25.4
	30	98.5	0.1	1.4
Extract + NADH	0	100	-	-
	0.3	67.5	0.2	32.3
	30	10.7	0	89.3
Extract + NADPH	0	100	-	-
	0.3	75.4	2.6	22.0
	30	8.8	0.4	90.8

TABLE 16

Effect of various compounds on the degradation of ^{35}S-PAPS in extracts (S_{10}) of T. ferrooxidans

The preparation of the cell-free extracts (S_{10}) of iron-grown *T. ferrooxidans* is described in Section 2.8.1.1. The reaction mixtures contained in µmoles: Tris-HCl buffer (pH 7.5), 25; Na_2-EDTA, 1; $MgCl_2$, 20; nucleotides, 20; NaH_2PO_4, 20; $Na_2H_2P_2O_7$, 20; ^{35}S-PAPS, 360 pmoles (0.58 µCi) and the extract (10.7 mg of protein) in a total volume of 0.6 ml. The NADH- and NADPH-generating systems are described in Table 13. The incubations, carried out at 30° in a reciprocating water bath, were started by adding the cell-free extract. Samples (0.02 ml) were taken at the time intervals shown and applied directly to 3MM Whatmann paper. The labelled compounds were separated by high-voltage electrophoresis followed by radioassay, as described in Section 2.9.1 and Section 2.9.3 respectively.

Addition	Incubation time (min)	Distribution of ^{35}S on electrophoretogram (%)			
		^{35}S-PAPS	^{35}S-APS	^{35}S-SO_4^{2-}	Origin
Extract alone	0	100	-	-	-
	0.3	55.1	0.8	41.9	2.2
	30	40.1	0.5	12.8	46.6
Extract + NaH_2PO_4	0	100	-	-	-
	0.3	70.1	0.8	70.1	5.2
	30	88.1	2.0	88.1	5.6
Extract + $Na_2H_2P_2O_7$	0	100	-	-	-
	0.3	62.1	0.8	36.2	0.9
	30	65.2	0.9	3.5	30.4
Extract + 5'-AMP	0	100	-	-	-
	0.3	35.3	0.4	62.4	1.9
	30	40.6	0.6	37.6	21.2
Extract + ADP	0	100	-	-	-
	0.3	59.0	0.9	38.6	1.5
	30	69.4	2.5	26.4	1.7
Extract + ATP	0	100	-	-	-
	0.3	79.7	0.7	17.4	1.9
	30	93.0	2.0	2.8	2.2
Extract + NADH	0	100	-	-	-
	0.3	53.4	1.0	44.3	1.3
	30	41.6	0.9	3.1	54.4
Extract + NADPH	0	100	-	-	-
	0.3	62.3	0.5	36.0	1.2
	30	42.1	2.3	3.0	52.6

compounds (Figure 12). Over a 45 min incubation period, however, a further breakdown of PAP did not occur in any of the treatments (Figure 12), suggesting that PAP was stable in cell-free extracts of ferrous-iron-grown *T. ferrooxidans*.

3.3 Discussion

3.3.1 The bioluminescence method for PAP and PAPS

Previous techniques (Mercer & Thomas, 1969; Roy & Trudinger, 1970; Burnell & Anderson, 1973) used to detect PAP in extracts of microorganisms and plants have been based on the use of radioisotopes and coupled enzyme assays. The use of radioactive techniques offers some flexibility since various labelled substrates can be used, e.g. ^{14}C-ATP, ^{14}C-APS, ^{35}S-sulphate, ^{35}S-APS and ^{32}P-ATP. However, adequate controls are essential to take into account any non-specific isotopic exchange reactions that might occur. The synthesis of PAPS by radiotracer methods has been demonstrated in cell-free preparations of *Chlorella* (Hodson *et al.*, 1968; Tsang *et al.*, 1971; Schmidt, 1972). In the present study, the production of PAPS in extracts of higher plants could not be demonstrated by employing radioactive substrates, e.g. ^{35}S-sulphate and ^{14}C-ATP. Similarly, many workers have found radioisotope techniques insufficiently sensitive to detect the picomole amounts of PAPS produced in extracts of higher plants (Asahi, 1964; Ellis, 1969; Balharry & Nicholas, 1970; Onajobi *et al.*, 1973). This may be associated with such factors as the insufficiently high specific activity of the labelled substrate and the presence in crude extracts of enzymes which readily degrade PAPS.

FIGURE 12

The stability of PAP in extracts of T. ferrooxidans

The preparation of the cell-free extracts (S_{10}) of iron-grown *T. ferrooxidans* is described in Section 2.8.1.1. The reaction mixtures contained in μmoles: Tris-HCl buffer (pH 7.5), 25; Na_2-EDTA, 1; $MgCl_2$, 20; ATP, 20; NaH_2PO_4, 20; $Na_2H_2P_2O_7$, 20; PAP, 5 nmoles; and extract (10 mg of protein), in a total volume of 0.7 ml. The incubations, carried out at 30° in a reciprocating water bath, were started by adding the cell-free extract. Samples (0.1 ml) were withdrawn at the time intervals shown and cooled on ice. PAP was immediately determined in 0.02 ml aliquots of these samples using the *Renilla* bioluminescence procedure, as described in Section 3.2.1.1. Extract only, (o——o); extract + NaH_2PO_4, (□——□); extract + $Na_2H_2P_2O_7$, (●——●); extract + ATP, (■——■).

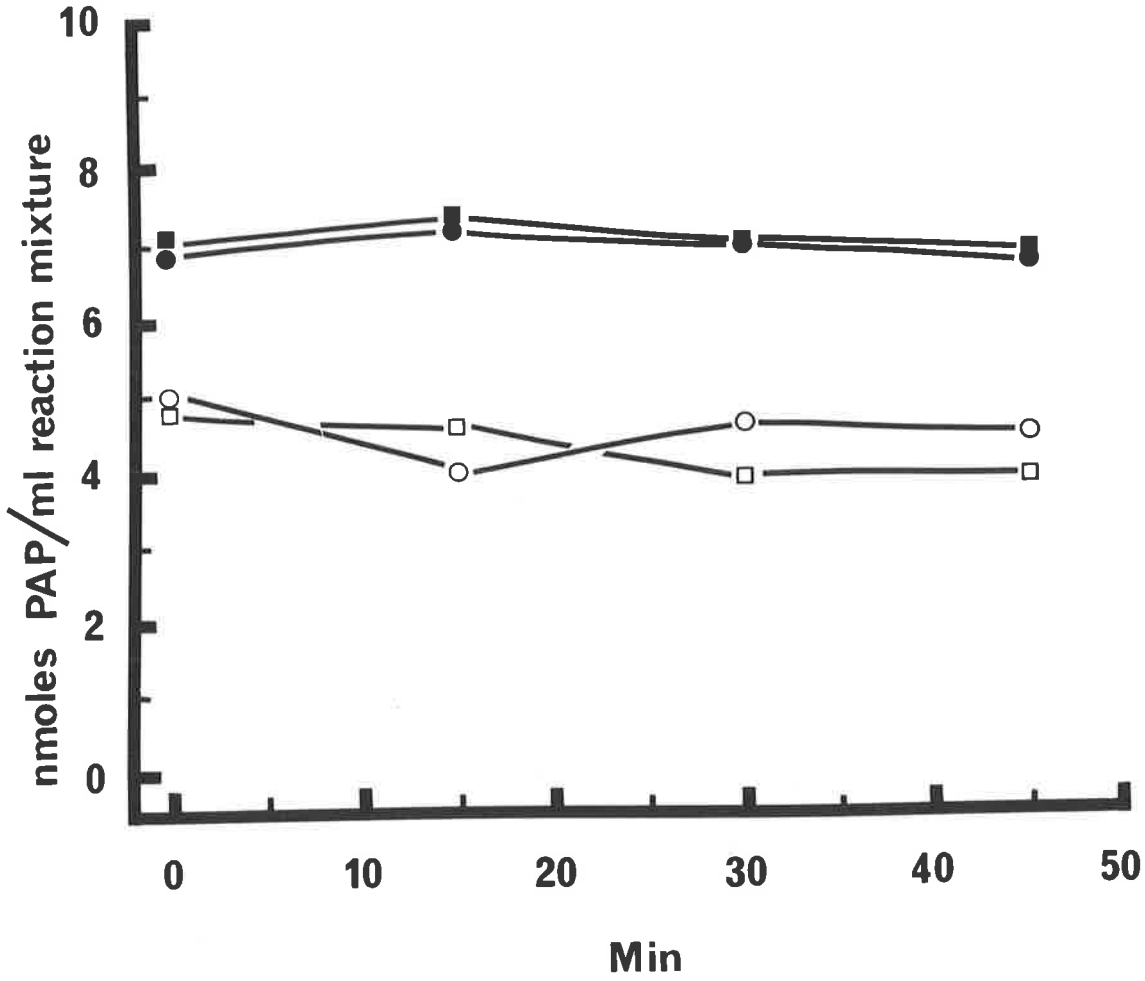


Figure 12

Small amounts of PAPS can be detected by coupled assay systems. A sensitive coupled enzyme system for determining PAP has been described for sulphate transfer among phenolic compounds (Gregory & Lipmann, 1957). However, the specificity of this technique may be questioned since coenzyme A was found to have more than 10% of PAP activity when tested as a substrate. It was not established whether PAP was an original contaminant or derived from a cleavage of the pyrophosphate linkage of coenzyme A, which contains the PAP configuration (Wang *et al.*, 1954).

Recently, a coupled enzyme system was used to study the properties of APS-kinase in spinach chloroplasts (Burnell & Anderson, 1973). The specificity of this technique for PAPS necessitates the preparation of purified phenolsulphotransferase from animal sources, since PAPS production is coupled to this enzyme. Burnell & Whatley (1975) have employed coupled enzyme systems which rely on the sulphate- or APS-dependent production of ADP via APS-kinase, in the presence of pyruvate kinase and lactate dehydrogenase. Thus, ADP production was followed spectrophotometrically by measuring the rate of NADH oxidation at 340 nm. This coupled system can be carried out in a single reaction mixture and so represents a rapid and sensitive method for continuously monitoring APS-kinase activity. The assay, however, is subject to interference from contaminating enzymes and compounds (e.g. NADH oxidase, ATP-ase, 5'-AMP, sulphate); this can be overcome by using a variety of controls. Coupled enzyme assays for PAPS have also been successfully employed in various studies with animal tissues (Robbins & Lipmann, 1957; Roy & Trudinger, 1970).

The bioluminescence technique described herein provides a simple, sensitive and reproducible coupled enzyme assay for determining PAP

and PAPS in biological materials. When PAPS is to be determined, it is first hydrolysed to PAP, which is then assayed by the bioluminescence technique. The method detects picomole amounts of PAP and PAPS, provided that appropriate controls are included to assay the endogenous PAP prior to acid hydrolysis.

The bioluminescence technique is extremely sensitive, since about 10 pmoles of PAP and PAPS can be determined quantitatively (± 1%). The sensitivity of the assay can probably be increased by using saturating levels of luciferyl sulphate. Care must be taken to ensure that the luciferin-luciferase system is not saturated by excessive amounts of PAP. The K_m for PAP in the reaction mediated by luciferin sulphokinase in the *Renilla* bioluminescence system is 7.3×10^{-8} M (Cormier, 1962). The PAP internal standard, which was routinely included in the liquid scintillation spectrometer assay vial, provided a convenient way of checking that the light output increased linearly with time and that the luciferin sulphokinase was not saturated with PAP from the test sample. Thus, high levels of PAP were assayed by either decreasing the amount of PAP in the internal standard or by reducing the sample size. In addition, the internal standard was used as a check for any variations which might affect light output, such as sample hydrolysate size, pH, temperature or quenching.

Another major advantage of the bioluminescence system is its specificity for PAP (Cormier, 1962; Cormier *et al.*, 1970), as discussed in Section 3.1. Many of the adenosine nucleotides tested appeared to be slightly active in the *Renilla* system at high concentrations. However, this activity was shown to be due to the presence of PAP as a trace impurity in many of these compounds. Indeed, PAP was isolated from aged commercial preparations of ATP and purified by column and

paper chromatography prior to the availability of commercially prepared PAP. Gregory & Lipmann (1957) also found that PAP is present in a wide range of commonly used biochemicals, especially in a preparation of ADP containing up to 2% PAP. High levels of PAP (50%) have been reported as an impurity in crude preparations of ATP (Gregory, 1962). The contamination of nucleotides with PAP was taken into account in all assays by including boiled extracts and other appropriate controls.

The extreme sensitivity of the technique necessitates a precision in all operations of the assay, especially in the preparation and dispensing of all components. Under these conditions, replicate samples were within 1%.

The bioluminescence system also offers flexibility since PAP and PAPS can be assayed as either substrates or products of a reaction. Other coupled enzyme assays only measure PAPS production since it must be coupled to a phenol acceptor using a purified phenol sulphotransferase (Roy & Trudinger, 1970). Thus, the *Renilla* assay provides a new and sensitive method to study the production of both PAPS and PAP and their role in intermediary sulphur metabolism. Very small amounts of PAPS were indeed produced from sulphate and ATP in leaf extracts of various plants. The low levels of PAPS produced in the present study may also explain why previous workers (Asahi, 1964; Ellis, 1969; Balharry & Nicholas, 1970; Onajobi *et al.*, 1973), using radioisotope techniques, were unable to demonstrate synthesis of this sulphur nucleotide. In addition, the small amounts of PAPS produced by crude extracts disappeared after very short incubation periods. Thus, the use of prolonged incubations up to 1 h (Asahi, 1964) may be unreliable for measuring PAPS production, especially in crude extracts (De Meio, 1975). The rapid disappearance of PAPS in plant extracts

recorded in the present study suggests an instability of this compound because of degradative enzymes. This was further supported by the observation that the curvilinear production of PAPS by a yeast extract was completely inhibited in the presence of plant extracts. This inhibition results from the enzymic degradation of PAPS (Roy & Trudinger, 1970; De Meio, 1975). This contrasts with the findings of Ellis (1969), who suggests that the failure to detect PAPS formation in several species of higher plants was not due to the presence of degradative enzymes, but rather to the lability of APS-kinase activity. In the present study, a similar lability of this enzyme was observed in a yeast extract preparation, stored at 2°.

The role of PAPS in the assimilation of sulphate is still uncertain. Thompson (1967) has concluded from data for microorganisms that PAPS is the substrate for reduction by assimilatory sulphate reducers, whereas APS is the substrate used by dissimilatory sulphate reducers. Schiff & Hodson (1973), however, suggest that both types of sulphur reducers appear to use APS as the activated intermediate for sulphate reduction. This is based on the observations of Schmidt (1972), who has demonstrated sulphate reduction in *Chlorella pyrenoidosa* from the level of APS only. Although PAPS is produced in this organism, in order for this compound to be reduced it must first be converted to APS (Schmidt, 1972). This conversion is catalysed by a Mg^{2+}-dependent 3'-nucleotidase activity found in extracts of both *Chlorella* and spinach chloroplasts (Hodson & Schiff, 1971; Schmidt, 1972; Burnell & Anderson, 1973). The presence of this enzyme may also explain the relative difficulty in observing PAPS formation from sulphate in many higher plants under conditions in which formation of APS is readily detected (Schmidt & Trebst, 1969; Burnell & Anderson,

1973). Similarly, Goldschmidt *et al.* (1975) have demonstrated that APS is an intermediate in the conversion of PAPS to acid-volatile radioactivity. Sulphate reduction can also occur from the level of PAPS in many bacteria and fungi (De Meio, 1975). In addition, PAPS may function as a donor for sulphate transfer, as discussed by Schiff & Hodson (1973). The present study has not attempted to establish an absolute function for PAPS in biological systems. However, it has introduced a new specific bioluminescence technique with the sensitivity and flexibility required for further investigations of the role of PAPS and PAP in sulphate activation, reduction and transfer.

The stimulation of PAPS production in plant extracts by 3'-AMP, and particularly by 5'-AMP, is of interest. There is no evidence for a direct phosphorylation of 3'-AMP or 5'-AMP to PAP, suggesting that these nucleotides may either function to protect PAPS from enzymic degradation by acting as alternative substrates for degradative enzymes or they may act in a regulatory role. Burnell & Anderson (1973) demonstrated PAPS production in crude extracts and isolated chloroplasts of spinach only when 3'-AMP was added. However, they have discounted the possibility that 3'-AMP might act as an alternative substrate for an enzyme(s) that would hydrolyse PAPS, since 3'-nucleotidase activity was virtually absent from isolated chloroplasts, where PAPS is synthesized. In the absence of 3'-AMP, APS is still formed, indicating a possible regulatory function of 3'-AMP on APS-kinase activity. Indeed, the production of PAPS may be under strict regulation by more than one nucleotide. Hodson *et al.* (1968) have found an inhibition of PAPS utilization in *Chlorella* by high amounts of ATP; this was subsequently shown to be due to contamination of ATP with traces of PAP. PAP would be a likely product of the reduction of

PAPS, and its role as a product inhibitor, and thus as a regulator of PAPS levels, may be important in the metabolism and availability of PAPS. In addition, Hodson & Schiff (1969) have demonstrated higher levels of PAPS production in *Chlorella* in the presence of 5'-AMP, which is in accord with the findings of the present study. It is not known whether 5'-AMP might regulate the amount of PAPS produced at the level of APS-kinase or at some step involved in PAPS reduction.

Recent observations have indicated that phosphonucleotides may inhibit or regulate other enzymes involved in either the activation of sulphate or the reductive pathway of APS and PAPS. Increased ATP-sulphurylase activities, assayed by the production of ^{35}S-APS from ^{35}S-sulphate and ATP, have been detected in the presence of 5'-AMP in extracts of rice roots (Onajobi, 1975). Regulation of ATP-sulphurylase activity by phosphonucleotides may be important in conserving cellular ATP, since this enzyme represents the initial energy-requiring step for sulphate activation and reduction. Schmidt (1975) has also postulated a regulatory role for 5'-AMP. The APS-sulphotransferase activity from spinach leaves was unaffected by the addition of increasing amounts of 3'-AMP. However, this enzyme was drastically inhibited in the presence of 5'-AMP. The inhibition of PAPS utilization by 5'-AMP in *Chlorella* extracts (Hodson & Schiff, 1969) may thus be due to the 5'-AMP inhibition of APS-sulphotransferase (Schmidt, 1975), since PAPS is converted to APS prior to sulphate reduction (Schmidt, 1972). The various interactions and effects of 5'-AMP, coupled with the use of crude plant and bacterial extracts, make it difficult to establish a unifying role for 5'-AMP, and clearly, further work is required. In addition, the role of PAP in sulphate activation and reduction warrants investigation and the *Renilla* bioluminescence method should prove invaluable in these studies.

3.3.2 Stability of PAP and PAPS in biological materials

An alternative role for phosphonucleotides would be in protecting PAPS and APS from degrading enzymes present in crude extracts. In the present study, experimental conditions are described for stabilizing PAPS and APS against hydrolytic enzyme activities. The results indicate that extracts of wheat, yeast and *T. ferrooxidans* contain enzymes which hydrolyse PAPS and APS quite rapidly. In extracts of leaves, ^{35}S-PAPS was hydrolysed via ^{35}S-APS to ^{35}S-sulphate. 3'-AMP did not protect ^{35}S-PAPS from the enzymic degradation, whereas in the presence of 5'-AMP, high levels of ^{35}S-APS persisted. In the present work, no attempt has been made to characterize the enzymes which degrade these sulphur nucleotides. However, the production of ^{35}S-APS from ^{35}S-PAPS in leaf extracts may suggest a 3'-nucleotidase which could not utilize 3'-AMP as a substrate, whereas nucleotide triphosphates, phosphate and pyrophosphate afforded protection for ^{35}S-PAPS. The occurrence of such hydrolytic enzymes has been reported in extracts of various organisms. The degradation of PAPS has been attributed to 3'-nucleotidase in rabbit liver (Brunngraber, 1958), rye grass (Robbins & Lipmann, 1957) and *Chlorella* (Goldschmidt *et al.*, 1975). Similarly, in other biological materials, both 3'-nucleotidase and 5'-nucleotidase activities have been found to hydrolyse PAPS (Lewis & Spencer, 1962; Koizumi *et al.*, 1969; Fry & Koritz, 1972). PAPS hydrolysis, attributable to PAPS-sulphatase, has been reported in hen oviduct (Suzuki & Storminger, 1960) and sheep brain (Balasubramanian & Bachawat, 1962). The recent report of Sawhney & Nicholas (1976) has extended PAPS degradation studies to include extracts of *Anabaena cylindrica*. Their results suggest that PAPS may be subjected to both sulphatase and 3'-nucleotidase activity. The possible involvement of the various nucleotidase activities in intermediate sulphur nucleotide

metabolism and sulphate reduction has been discussed at length (Burnell & Anderson, 1973; Schiff & Hodson, 1973; Anderson, 1975).

In yeast, the pattern of degradation of ^{35}S-PAPS was similar to that observed in extracts of wheat leaves. The effects of 3'-AMP and 5'-AMP, however, were now different to those found in wheat extracts but again suggest the action of nucleotidases. The relatively slow rate of ^{35}S-PAPS degradation in extracts of *T. ferrooxidans* may relate to previous observations in this study that PAPS is not involved as an active intermediate during sulphate assimilation in this bacterium. Only trace amounts of ^{35}S-APS were detected during PAPS hydrolysis while the major labelled products were free and membrane-associated ^{35}S-sulphate. These observations suggest that PAP was the other immediate product of the hydrolysis of PAPS, probably mediated by a sulphatase-type activity. It is of interest that the extracts degraded ^{35}S-APS, the sulphur nucleotide involved in sulphate reduction in this bacterium, more readily than ^{35}S-PAPS. Pyrophosphate and 5'-AMP, which offset the hydrolysis of ^{35}S-APS, were less effective in stabilizing ^{35}S-PAPS.

The present study indicates that hydrolytic enzymes play an important role in regulating the amounts of sulphur nucleotides in crude extracts of biological materials. Although no attempt has been made to identify the types of hydrolytic activities operating, the results reported herein serve to emphasize the differences in such activities between various biological materials and the effect that these enzymes have on studies on intermediary sulphur metabolism. In addition, this study considers the types of compounds which stabilize sulphur nucleotides. Whether compounds such as 5'-AMP are acting in a protective or regulatory role, or both, is not established, and merits further consideration.

4. PART II. UPTAKE AND UTILIZATION OF INORGANIC SULPHUR COMPOUNDS IN *THIOBACILLUS FERROOXIDANS*

4. UPTAKE AND UTILIZATION OF INORGANIC SULPHUR COMPOUNDS IN *THIOBACILLUS FERROOXIDANS*

4.1 Introduction

The genus *Thiobacillus* comprises a small number of closely related species of gram-negative bacteria which are found in a wide variety of marine, freshwater and terrestrial environments. The thiobacilli are considered to be chemolithotrophic bacteria (Kelly, 1971) since their energy requirement is met by the oxidation of inorganic sulphur compounds and they can thus grow in simple salt media containing an oxidizable sulphur compound and dissolved carbon dioxide. The bacteria closely resemble photosynthetic organisms since they utilize carbon dioxide as a sole source of carbon for the synthesis of cellular material (Santer & Vishniac, 1955; Trudinger, 1955,1956; Peck, 1962, 1968; Roy & Trudinger, 1970; Lundgren *et al.*, 1974; Suzuki, 1974). Some thiobacilli, previously regarded as obligate chemolithotrophs, are capable of growth on organic compounds and assimilate a range of organic substances. Such thiobacilli include *T. novellus* (Vishniac & Santer, 1957); *T. perometabolis*, which is not a strict autotroph yet derives energy from the oxidation of inorganic sulphur compounds (London & Rittenberg, 1967); *T. neopolitanus* strain C (Kelly, 1965, 1966,1967*a*,*b*,*c*); *T. denitrificans* (Taylor *et al.*, 1971; Taylor & Hoare, 1971) and *T. ferrooxidans* (Tabita & Lundgren, 1971*a*,*b*; Shafia *et al.*, 1972). Indeed, the whole concept of obligate chemoautotrophy has been questioned by Rittenberg (1972) since facultative autotrophs would have a greater metabolic adaptability. The concept of autotrophy in relation to the lithotrophic bacteria has been reviewed by Kelly (1971).

The classification of the thiobacilli has not been satisfactorily resolved. The multivariate analysis by Hutchinson *et al.* (1965,1966, 1967,1969) suggests the thiobacilli may be subdivided into six groups without any overlapping intermediates. However, because of the wide variety of growth conditions employed and the ability of these microorganisms to adapt to these different growth conditions, the method does not effectively analyse the relationships between all species of thiobacilli. An alternative approach (Jackson *et al.*, 1968) to the classification of the thiobacilli has been the DNA-base composition analyses, which divide the nine species of thiobacilli into three groups, regardless of culture conditions. The recent characterization of a *Thiobacillus* species by gas-liquid chromatography of its cellular fatty acids provides yet another approach to this problem (Agate & Vishniac, 1972).

The thiobacilli oxidize sulphide, elemental sulphur, thiosulphate, tetrathionate and sulphite to sulphate (Roy & Trudinger, 1970). In addition, *T. ferrooxidans* derives energy from the oxidation of ferrous-iron (Colmer *et al.*, 1950; Temple & Colmer, 1951; Razzell & Trussell, 1963). Aspects of the general physiology and biochemistry of the thiobacilli have been reviewed by Vishniac & Santer (1957), Lees (1960,1962), Peck (1962) and Kelly (1968,1971). The oxidative pathways of inorganic sulphur compounds have been discussed by Peck (1968), Trudinger (1967,1969), Roy & Trudinger (1970), Lundgren *et al.* (1974), Suzuki (1974) and Aleem (1975).

The acidophilic iron-oxidizing bacteria include those organisms previously referred to as *Ferrobacillus ferrooxidans* and *F. sulpho-oxidans*. These species were distinguished from *T. ferrooxidans* because the former did not grow on sulphur or thiosulphate (Leathen

et al., 1956) whereas the second did not utilize thiosulphate (Kinsel, 1960). However, Kelly & Tuovinen (1972) have demonstrated that these differences between the three species are invalid, since all of them grow on either sulphur or thiosulphate. Thus, the names *F. ferrooxidans* and *F. sulphooxidans* are now regarded as being synonymous with *T. ferrooxidans*.

The general reaction for biological iron oxidation is:-

$$4Fe^{2+} + O_2 + 4H^+ \longrightarrow 4Fe^{3+} + 2H_2O \ .$$

Even though the production of energy from inorganic compound oxidation is a separate function from that of the fixation of carbon dioxide, the two processes are closely linked and regulated in these chemolithotrophic bacteria. During iron oxidation, the electron released is transported via the electron transport system of the cell. *T. ferrooxidans* contains cytochromes $c + c_1$, cytochrome *a* and cytochrome *b* (Vernon *et al.*, 1960; Blaylock & Nason, 1963). The energy-producing steps of this system occur between cytochromes $c + c_1$ and cytochrome *a* (Vernon *et al.*, 1960; Blaylock & Nason, 1963; Yates & Nason, 1966; Din *et al.*, 1967*a*). A similar finding has been reported for *T, novellus* (Aleem, 1965). Coenzyme Q_6 and other quinoid compounds have been detected in *T. ferrooxidans* (Dugan & Lundgren, 1964) and these probably function as electron carriers in the electron transport chain (Dugan & Lundgren, 1965). The mechanism of the iron oxidase system has been reviewed by Lundgren *et al.* (1974). The ATP produced by oxidative phosphorylation provides energy for the synthesis of cellular materials and also reduces NAD and NADP. An ATP-dependent reduction of pyridine nucleotides was first demonstrated in chemolithotrophic organisms (Aleem *et al.*, 1963), and these results were later extended by Aleem (1966). Thus, the ATP-dependent reduction

of pyridine nucleotides provides the source of cellular reducing power, which is used in the reduction of carbon dioxide for cell growth in the Calvin cycle. This process, studied extensively in other chemolithotrophic bacteria (Aleem, 1970), has been reviewed by Peck (1968) and Kelly (1971).

T. ferrooxidans also grows on inorganic sulphur compounds as an energy source. The generation of cellular energy, via oxidative phosphorylation, is similar to that described for iron-grown cells, except that two electrons are transported via the electron transport chain. Energy is also conserved by means of substrate-level phosphorylation during the oxidation of inorganic substrates, as will be discussed later in this section.

T. ferrooxidans has been grown on either elemental sulphur or ferrous-iron (Margalith *et al.*, 1966). These workers suggest that the iron-oxidizing enzyme is constitutive, since iron is oxidized as rapidly by sulphur-grown cells as it is by iron-grown cells. However, sulphur-grown cells have a better capacity for oxidizing sulphur than those grown with iron, suggesting that the sulphur-oxidizing system is not inducible. Silver (1970) has reported that all components oxidized by *T. ferrooxidans* — e.g. elemental sulphur, sulphite, dithionite, thiosulphate, tetrathionate and sulphide — produce energy and thus support carbon dioxide fixation in the bacterium.

The similarity of carbon dioxide fixation by chemolithotrophic and photosynthetic bacteria to the Calvin cycle of green plants has been suggested in a review by Kelly (1971). Evidence for the existence of the Calvin cycle as a route for carbon dioxide fixation in *T. ferrooxidans* has been presented by Maciag & Lundgren (1964), Din *et al.* (1967b), and Gale & Beck (1967). Lundgren *et al.* (1974) have

recently reviewed the current knowledge on the assimilation of carbon dioxide via the Calvin cycle in *T. ferrooxidans*. Heterotrophic growth results in a varied repression of ribulose-1,5-diphosphate carboxylase, depending on the organic substrate metabolized. Indeed, Tabita & Lundgren (1971*a*) reported that, in *T. ferrooxidans*, cell-free extracts from iron-fructose- and iron-glutamate-grown cells had about 70% of the carboxylase level of autotrophically grown cells, whereas extracts from iron-glucose- and iron-sucrose-grown cells showed a 95% repression of carboxylase activity. Lundgren *et al.* (1974) suggest that the controlling points for the assimilation of carbon dioxide via the Calvin cycle are at the levels of the synthesis of the carboxylase enzyme and the inhibition and activation of preformed Calvin cycle enzymes.

T. ferrooxidans also grows heterotrophically on glucose as a source of energy, as first reported by Lundgren *et al.* (1964). Shafia & Wilkinson (1969) found that the bacterium grew on glucose, mannitol, and several other sugars, as well as on a few amino acids in the absence of an oxidizable iron source and after a brief adaptation to glucose. Glucose has been shown to inhibit the oxidation of iron and elemental sulphur in manometric experiments with resting cells prepared from cultures grown on either inorganic substrate (Silver *et al.*, 1967). Growth in a glucose medium was only achieved after the cells had been grown in a ferrous sulphate medium containing 0.5% glucose (Shafia & Wilkinson, 1969). Once adapted to glucose, *T. ferrooxidans* grew on glucose as its sole carbon and energy source. Shafia & Wilkinson (1969) reported that adaptation to glucose did not initially interfere with autotrophic mechanisms, since glucose-adapted cells continued to oxidize iron and grew autotrophically. In contrast to these findings, Tabita & Lundgren (1971*a*) found that the adaptation

to glucose had a pronounced effect on the ability of the bacterium to derive energy from the oxidation of ferrous sulphate. The extent of the inhibition of iron oxidation is dependent on the particular organic energy source (Tabita & Lundgren, 1971*a*). In addition to the inhibition of substrate oxidation, the fixation of carbon dioxide is inhibited by high concentrations of glucose (Silver *et al.*, 1967). Similarly, Silver (1970) demonstrated that glucose inhibited carbon dioxide fixation during the oxidation of inorganic sulphur compounds. The relationship between the oxidation of inorganic iron and ribulose-1,5-diphosphate carboxylase (Tabita & Lundgren, 1971*a*) has already been discussed. London & Rittenberg (1966) have also reported a repression of thiosulphate oxidation in *T. intermedius*, coupled with a decreased level of the carboxylase enzyme when cells were grown in a medium containing thiosulphate and an organic supplement. Similarly, when *T. novellus* was grown under heterotrophic conditions, reduced activities of the carboxylase enzyme were recorded (Aleem & Huang, 1965). Lundgren *et al.* (1974) have suggested that some common product(s) of heterotrophic catabolism is responsible for the repression of iron oxidation. The only by-products common to both lithotrophic and organotrophic metabolism are ATP and NADH.

The transition from autotrophic to heterotrophic growth results in drastic changes in the metabolism of *T. ferrooxidans*. Thus, glucose-6-phosphate dehydrogenase and the enzymes of the Entner-Doudoroff pathway are induced during heterotrophic growth of the bacteria (Tabita & Lundgren, 1971*b*). These workers suggest that glucose is dissimilated through this pathway, with the Embden-Meyerhof pathway probably playing a minor role in providing carbon skeletons for biosynthesis. Thus, the products of the Entner-

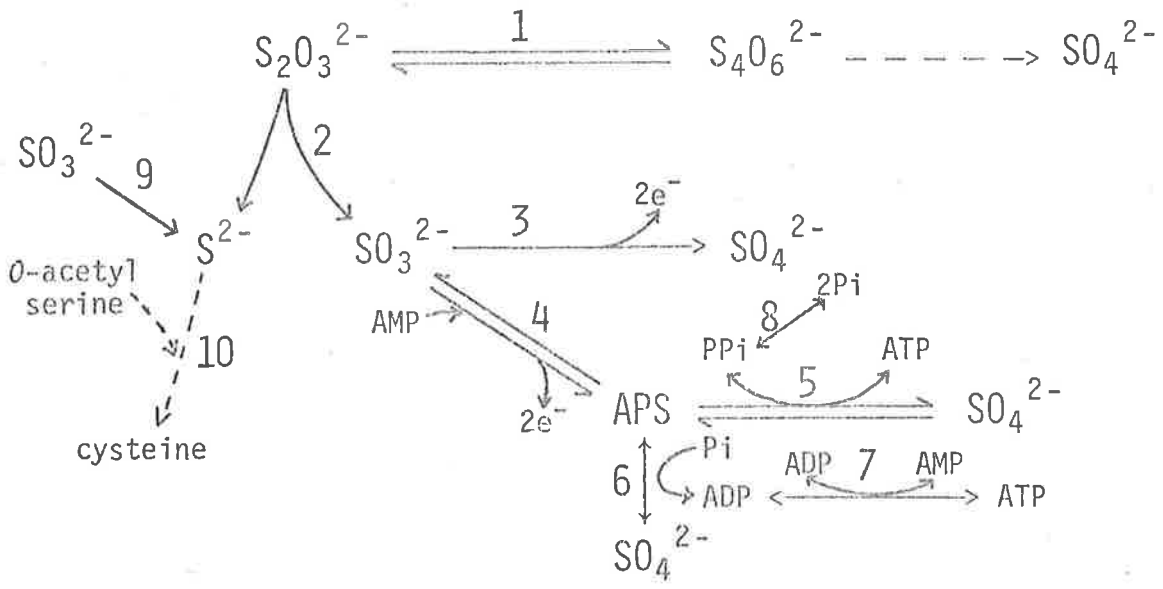
Doudoroff pathway, pyruvate and 3-phosphoglyceraldehyde, must be utilized via the tricarboxylic acid (TCA) cycle for energy production. This is in accordance with the findings of Lundgren *et al.* (1974), who found increased levels of the TCA cycle enzymes when the organisms metabolized glucose. Smith *et al.* (1967) have suggested that the elimination of $NADH_2$ oxidase, which is linked to the TCA cycle, in chemoautotrophs would have a selective advantage, since this enzyme would dissipate reducing equivalents. The TCA cycle in autotrophically grown *T. ferrooxidans* is incomplete (Tabita & Lundgren, 1971b) because both α-ketoglutarate dehydrogenase and reduced NAD oxidase are not present.

Lundgren *et al.* (1974) suggest that the most important enzymic step in the conversion from autotrophy to heterotrophy is that catalysed by glucose-6-phosphate dehydrogenase. The purified enzyme from *T. ferrooxidans* has a much greater affinity for $NADP^+$ than NAD^+. ATP selectively inhibits the NAD-linked reaction and Tabita & Lundgren (1971c) propose that this may be a means of controlling the flow of carbon through the Entner-Doudoroff pathway. In addition, during autotrophic growth, relatively high levels of ATP and NADH are required for carbon dioxide fixation, and both of these compounds inhibit the glucose-6-phosphate dehydrogenase from *T. ferrooxidans*. The regulation of this enzyme may be an important factor during changes from an autotrophic to a heterotrophic mode of life. However, Guay & Silver (1975) have recently isolated a new species called *Thiobacillus acidophilus* from a culture of iron-grown *T. ferrooxidans* which was briefly exposed to glucose. Unlike the glucose-6-phosphate dehydrogenase from *T. ferrooxidans*, the enzyme from *T. acidophilus* was specific for NAD. Thus, changes in enzyme composition accompanying the transition from autotrophy to heterotrophy are complex and need more investigation.

The current status of the pathways of the oxidation of reduced inorganic compounds of sulphur by the thiobacilli has been reviewed in detail (Peck, 1968; Kelly, 1968,1971; Roy & Trudinger, 1970; Suzuki, 1974; Aleem, 1975) and is summarized in part in Scheme I (page 65). It is likely that different species of thiobacilli have different oxidative pathways. In *T. ferrooxidans*, knowledge of the pathways for the oxidation of inorganic sulphur compounds remains incomplete. Elemental sulphur is oxidized to sulphite by the sulphur-oxidizing enzyme, as described by Silver & Lundgren (1968*a*). The enzyme has also been found in *T. thioparus* (Suzuki & Silver, 1966) and *T. novellus* (Charles & Suzuki, 1966), and closely resembles the enzyme from *T. thiooxidans* (Suzuki, 1965). Thiosulphate can be produced chemically at physiological pH values from sulphur and sulphite during sulphur oxidation (Lundgren *et al.*, 1974). In some organisms, thiosulphate undergoes a reductive cleavage in the presence of glutathione to form sulphide and sulphite (Peck & Fisher, 1962). This reaction is catalysed by the enzyme thiosulphate reductase, and the end product, sulphite, is then oxidized to sulphate (Peck, 1960; Charles & Suzuki, 1966). No thiosulphate reductase activity has been detected in iron-oxidizing bacteria. The enzyme rhodanese is also responsible for the enzymic cleavage of the S–S bond of thiosulphate. Rhodanese activity has been found in sulphur-grown *T. ferrooxidans* (Tabita *et al.*, 1969). It is not established whether thiosulphate reductase and rhodanese are separate enzymes.

Tetrathionate is produced from thiosulphate by the thiosulphate-oxidizing enzyme (Silver & Lundgren, 1968*b*). This reaction is reversed under anaerobic conditions (Kelly & Tuovinen, 1975). Studies with other thiobacilli suggest that polythionates are formed during thiosulphate oxidation. Trudinger (1959) has demonstrated the

SCHEME I



REACTION	ENZYME
1	Thiosulphate-oxidizing enzyme
2	Rhodanese
3	AMP-independent sulphite oxidase
4	APS-reductase
5	ATP-sulphurylase
6	ADP-sulphurylase
7	Adenylate kinase
8	Pyrophosphatase
9	Sulphite reductase
10	O-acetylserine sulphhydrase

formation and utilization of polythionates during thiosulphate oxidation by cells of *T. thioparus*. Similar findings have been reported for cell-free extracts of *T. thiooxidans* and *T. thioparus* when oxidizing sulphide and thiosulphate (London & Rittenberg, 1964). In addition, Trudinger (1964*a*,*b*) found that variations in experimental conditions, e.g. oxygen tension and cell or protein concentration, affected tetrathionate oxidation. Thiosulphate and tetrathionate oxidation have recently been reviewed by Aleem (1975).

Vestal & Lundgren (1971) isolated, partially purified and characterized the AMP-independent sulphite oxidase (sulphite : cytochrome c oxidoreductase) from sulphur-grown *T. ferrooxidans*. Since the enzyme activity was not stimulated by adding 5'-AMP, they postulated that the enzyme is principally associated with ATP production in sulphur-grown cells. Since the enzyme catalysed the reduction of cytochrome c, the electrons released during the oxidation of sulphite would pass through the electron transport chain to oxygen. Thus, ATP would be produced by oxidative phosphorylation as in the ferrous-iron-grown *T. ferrooxidans*.

The contribution of substrate-level phosphorylation to the energy balance in *T. ferrooxidans* was not fully explored by Vestal & Lundgren (1971). These workers did not unequivocally establish that APS-reductase was absent from this bacterium. Indeed, their results suggest that, if APS-reductase were present, it would be a separate protein independent of the sulphite reductase activity. Energy production during sulphite oxidation may involve the APS-reductase pathway as originally proposed by Peck (1962,1968). APS-reductase activity has been found in extracts of *T. thioparus* (Peck *et al.*, 1965) and it has been purified from *T. denitrificans* (Bowen *et al.*, 1966). Thus, sulphite in the presence of 5'-AMP is oxidized to APS by APS-reductase. The electron acceptor for the two electrons released during

this oxidation is not known. The two electrons may be transferred to oxygen via the cytochrome system or may be used to provide reducing conditions for intermediate sulphur reactions such as the reduction of elemental sulphur to sulphide. The substrate-level phosphorylation pathway, involving APS-reductase, ADP-sulphurylase and adenylate kinase (Peck, 1968), also presents a way of conserving energy in chemolithotrophic bacteria. The link between ATP-sulphurylase and ADP-sulphurylase during the substrate-level phosphorylation is likely to be influenced by inorganic pyrophosphatase activity, as found in *T. ferrooxidans* (Howard & Lundgren, 1970). The contribution of substrate-level phosphorylation to the overall energy balance of *T. ferrooxidans* remains to be determined.

It is likely that some enzymes involved in the oxidation of inorganic sulphur compounds would also function in the incorporation of sulphur into cellular materials. During the oxidation of inorganic sulphur compounds by chemolithotrophic thiobacilli, intermediate APS may be formed by APS-reductase (Peck, 1968). Sulphate is then produced from APS by ADP-sulphurylase or ATP-sulphurylase (Peck, 1968; Suzuki, 1974). Tuovinen *et al.* (1975) found that sulphate is actively taken up and assimilated via its initial activation to APS in *T. ferrooxidans* grown on ferrous-iron.

In the sulphur-oxidizing thiobacilli, reduced sulphur for cellular synthesis is probably derived from sulphide, which is an intermediate in the oxidation of inorganic sulphur compounds. It has been found that the adaptation of *T. intermedius* to heterotrophy can only be accomplished when reduced organic sulphur compounds are included in the culture medium (Smith & Rittenberg, 1974). This suggests that the bacterium is lacking one or more of the enzymes which mediate the

activation and reduction of sulphate. In contrast, *T. ferrooxidans* has no requirement for reduced sulphur compounds during its grown on ferrous-iron or glucose. Little is known about the assimilation of sulphur compounds in *T. ferrooxidans* and its relation to the oxidative sulphur pathways.

Aim of the study. The aims of the present study are as follows:-

1. To investigate the uptake of sulphate and thiosulphate by thiosulphate-grown *T. ferrooxidans*.

2. To compare the enzymes involved in the intermediary metabolism of inorganic sulphur compounds in *T. ferrooxidans* grown autotrophically on both thiosulphate and ferrous-iron and heterotrophically on glucose.

3. To compare the properties of ATP-sulphurylase from bacteria grown with both ferrous-iron and thiosulphate.

4.2 Results

4.2.1 Binding, uptake and assimilation of ^{35}S-sulphate and ^{35}S-thiosulphate by *T. ferrooxidans* grown on thiosulphate

4.2.1.1 Uptake of ^{35}S-sulphate by washed cells

During growth on thiosulphate, cells bound increasingly more ^{35}S-sulphate after thiosulphate and tetrathionate were completely oxidized (Figure 13). Up to 1.7% of the total ^{35}S-sulphate added was bound by the cells. The sulphate concentration for calculating the specific activity was derived from the original values for thiosulphate and tetrathionate, assuming that no other intermediates were formed.

FIGURE 13

Uptake of ^{35}S-sulphate by cultures of T. ferrooxidans growing with thiosulphate

The composition of the mineral salts solution (containing 20 mM thiosulphate) is given in Section 2.8.8. Carrier-free ^{35}S-sulphate (0.028 µCi/ml culture) was added to 50 ml of the mineral salts solution in a 250 ml conical flask. At the same time, the flask was inoculated with 0.5 ml of a suspension of freshly harvested cells (approximately 12 mg dry weight). The growth experiments were carried out by placing the flask in a gyratory shaker at 200 r.p.m. at 30°. At the specified time intervals, the following samples were taken from the culture: (a) 0.1 ml was applied to glass-fibre discs; (b) 0.2 ml was centrifuged at 10,000 x *g* for 10 min and 0.1 ml of the resulting supernatant was applied to glass-fibre filter discs; (c) a 1 ml sample was filtered (Millipore GSWP 02500) and then washed with 10 ml of cold mineral salts solution (Section 2.8.8); (d) 0.5 ml samples were taken and the residual sulphur compounds ($S_2O_3^{2-}$ and $S_4O_6^{2-}$) were determined by cyanolysis (Kelly *et al.*, 1969) as described in Section 2.9.6. The radioassay of labelled compounds (e.g. ^{35}S-sulphate) on filters and glass-fibre discs has been described in Section 2.9.3. The figure shows the variations in the amounts of ^{35}S-sulphate (■----■), $S_2O_3^{2-}$ (●——●), and $S_4O_6^{2-}$ (o——o) over the growth period.

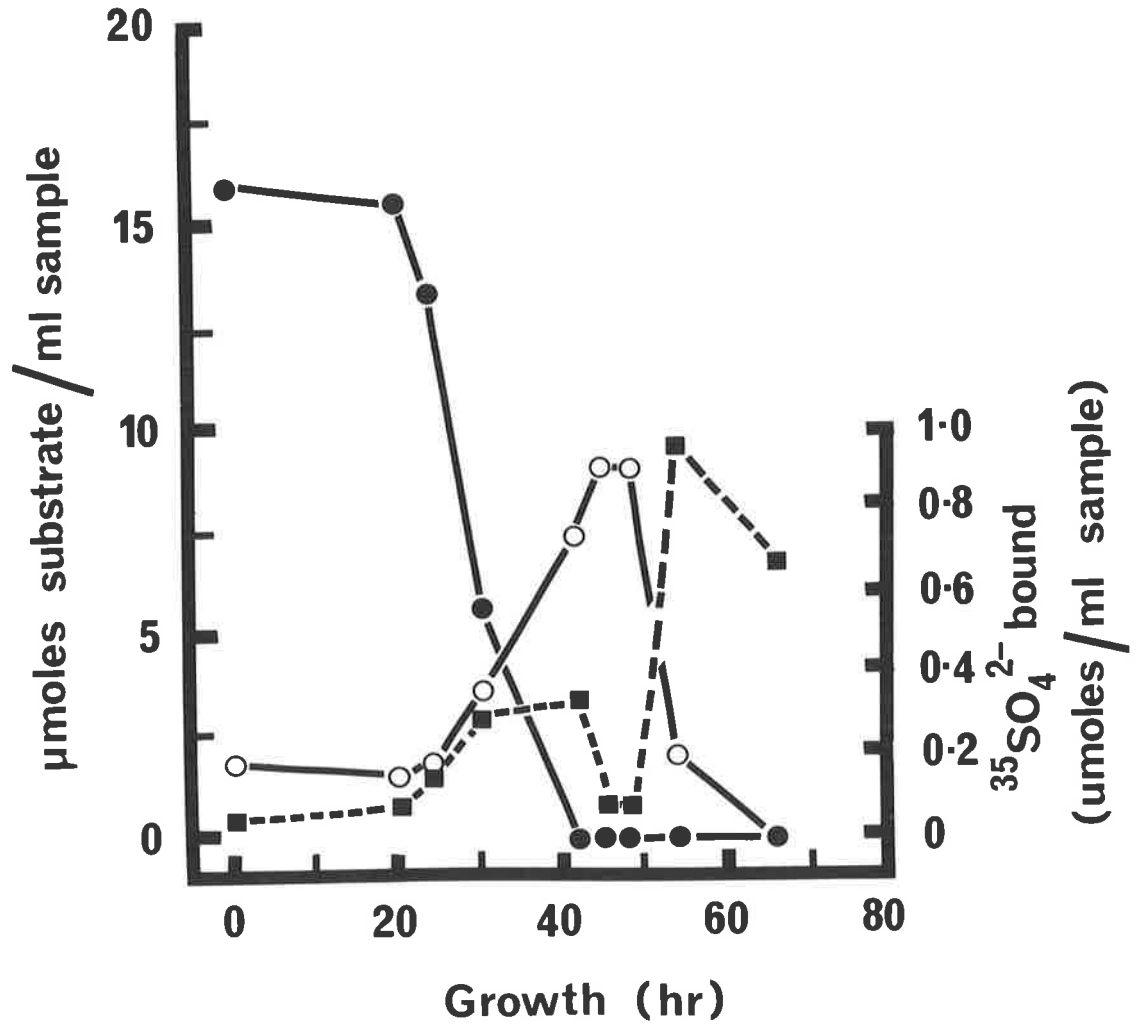

20
15
10
5
0
µmoles substrate / ml sample
1·0
0·8
0·6
0·4
0·2
0
$^{35}SO_4^{2-}$ bound
(umoles / ml sample)
0
20
40
60
80
Growth (hr)

Figure 13

Only small amounts of ^{35}S-sulphate were bound by cell suspensions (0.7% of the total ^{35}S-sulphate added) (Figure 14). There was no evidence that ^{35}S-sulphate was incorporated into the cells, since the amount of tracer bound within 15 sec did not increase further over a 10 min period (Figure 14). Indeed, increasing the incubation period to 60 min did not enhance the binding of ^{35}S-sulphate by intact cells. Moreover, the tracer was completely removed from the cells by three successive washings with either 0.01 N sulphuric acid or 10% (w/v) trichloroacetic acid at 25°. In boiled cell preparations, a relatively high level of passive binding of ^{35}S-sulphate was observed (Figure 14). The addition of 20 mM thiosulphate repressed the binding of ^{35}S-sulphate by both washed cells and boiled preparations (Figure 14).

4.2.1.2 Uptake of ^{35}S-thiosulphate by washed cells

The data in Figure 15 demonstrate that both [inner-^{35}S]thiosulphate and [outer-^{35}S]thiosulphate were taken up by washed cells, with a maximum uptake after a 15 min incubation. Only 4% of the label was removed from the cells by a single wash with mineral salts solution at 4° (see Section 2.8.8), and there was no further increase in the amounts of the tracer removed on washing with 0.01 N sulphuric acid.

Since the pattern of ^{35}S uptake was similar for thiosulphate labelled in either S atom, subsequent experiments were performed with the outer-labelled thiosulphate preparation only, unless otherwise stated. The different amounts of ^{35}S-thiosulphate bound (see Figure 15) were due to different radiochemical specific activities of the stock [inner-^{35}S]thiosulphate and [outer-^{35}S]thiosulphate.

The uptake of thiosulphate by washed cells was proportional to

FIGURE 14

Effect of thiosulphate on the binding of ^{35}S-sulphate by washed cells of T. ferrooxidans grown with thiosulphate

Details of the preparation of the cell suspension are given in Section 2.8.8. Incubations were carried out in 10 ml conical flasks in a reciprocating water bath at 30°. Incubation mixtures contained per ml: washed cells, 20 mg dry wt.; mineral solution (Section 2.8.8), 0.89 ml; carrier-free ^{35}S-sulphate (0.64 µCi/ml culture/20 mg dry wt.) and either 0.1 ml of cold thiosulphate (20 µmoles) or 0.1 ml of the mineral salts solution. Boiled cell samples were included as controls. At the specified time intervals, duplicate samples (0.5 ml), withdrawn from the incubation mixtures, were mixed with 0.2 ml of cold 0.2 N HCl. The resulting solutions were centrifuged at 10,000 x *g* for 10 min at 2°. The pellets remaining after centrifuging were resuspended in 1 ml of cold mineral salts solution, allowed to stand for 40 min and then centrifuged again as above. The washing process was repeated once more before finally resuspending the pellets in 0.5 ml of mineral salts solution and applying 0.1 ml aliquots from these suspensions directly onto glass-fibre filter discs. The radioassay of the filter discs has been described in Section 2.9.3. Cells + ^{35}S-sulphate (●——●); boiled cells + ^{35}S-sulphate (o——o); cells + $S_2O_3^{2-}$ (20 mM) + ^{35}S-sulphate (■——■); boiled cells + $S_2O_3^{2-}$ (20 mM) + ^{35}S-sulphate (□——□).

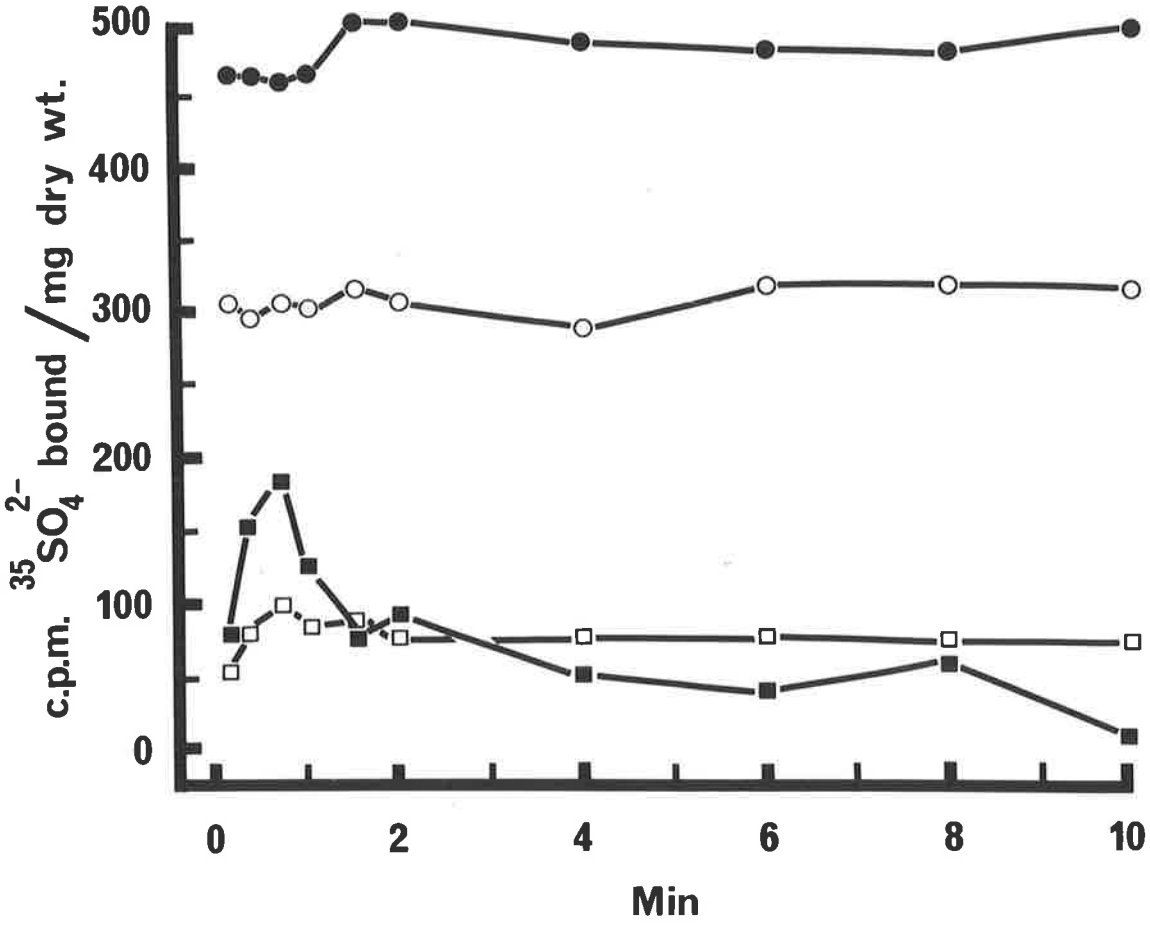


Figure 14

FIGURE 15

Uptake of differentially labelled ^{35}S-thiosulphate by whole cells

The incubation mixtures contained per ml: washed cells, 5.4 mg dry wt.; mineral salts solution (Section 2.8.8), 0.97 ml; [outer-^{35}S]thiosulphate, 0.11 µmoles (3.3 µCi) (o——o) or [inner-^{35}S]thiosulphate, 0.72 µmoles (3.3 µCi) (●——●). Samples (0.1 ml) were taken at time intervals over a 30 min period. Samples were filtered, washed, dried and the radioactivity determined as described in Section 2.9.3. The results have been corrected for controls, which included boiled cells incubated under identical conditions.

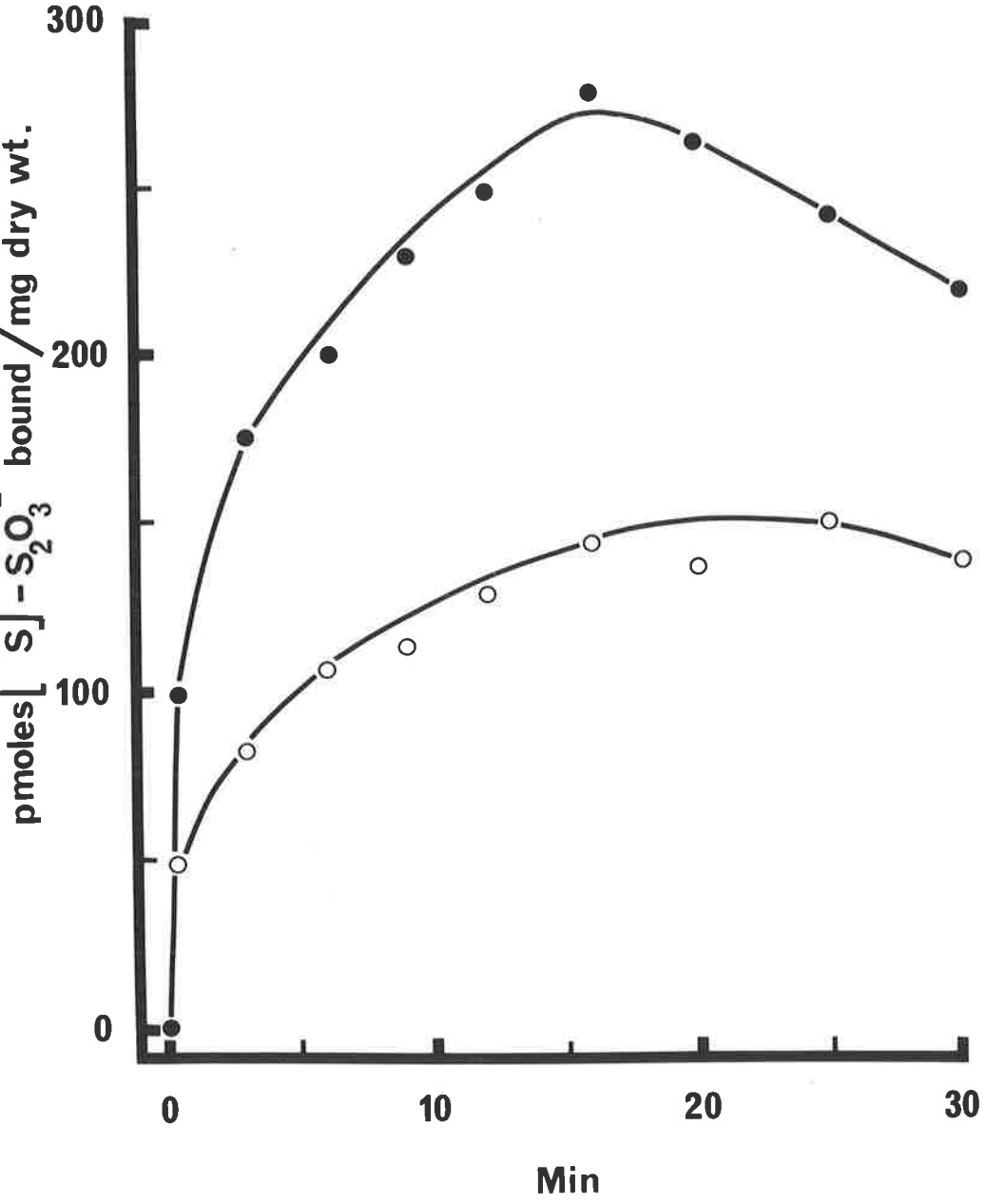


Figure 15

the cell density over the range 0.5-4.0 mg dry wt. bacteria (approximately 500 pmoles ^{35}S were bound per 5 min per mg dry wt.) as shown in Figure 16, and the optimum temperature for uptake was 50° (Figure 17). Boiled cell controls showed no uptake of [outer-^{35}S]thiosulphate, even under the optimum conditions discribed in the present study. The uptake system was relatively sensitive to changes at low pH. When 11 mM citrate buffer was used, maximum uptake was observed at pH 3.6 (Figure 18). In acetate buffer of identical molarity, the uptake of [outer-^{35}S]thiosulphate by washed cells increased at acid pH values down to pH 3.5 (Figure 18). Further decreases in pH using citrate and acetate buffers to study the optimum pH for the uptake system were not possible due to the limitations of the pH range available when using these buffers.

The uptake showed typical saturation kinetics, with a K_m value of approximately 0.5 mM for thiosulphate estimated from the velocity substrate curve (Figure 19). The shape of the Lineweaver-Burk plot (Figure 20) indicated an inhibition of the uptake system at high thiosulphate concentrations. A number of Group VI anions, e.g. molybdate, selenate and tungstate, inhibited the uptake of thiosulphate (Table 17), while dithionate and 2,4-dinitrophenol showed less inhibition. Cysteine and glucose enhanced uptake (Table 17).

4.2.1.3 Incorporation of ^{35}S from differentially labelled thiosulphate by washed cells and cell homogenates

Increasing amounts of ^{35}S were incorporated from [outer-^{35}S]thiosulphate into washed cells over a 60 min period. The fractionation of the cells showed that the lipid fraction contained about ten times greater amounts of the tracer than did the protein,

FIGURE 16

Effect of increasing amounts of cells on the uptake of [outer-^{35}S]thiosulphate by whole cells

The incubation mixture contained per ml: washed cells, 1.1-5.3 mg dry wt.; mineral salts solution (Section 2.8.8), 0.90 ml; [outer-^{35}S]thiosulphate, 0.11 µmoles (3.3 µCi) and cold thiosulphate, 0.39 µmoles. The incubations, at 30° in a reciprocating water bath, were started by adding the labelled thiosulphate and the amounts of the tracer taken up by washed cells were determined after a 5 min incubation period as described in Section 2.8.8. The results have been corrected for controls, which included boiled cells incubated under identical conditions.

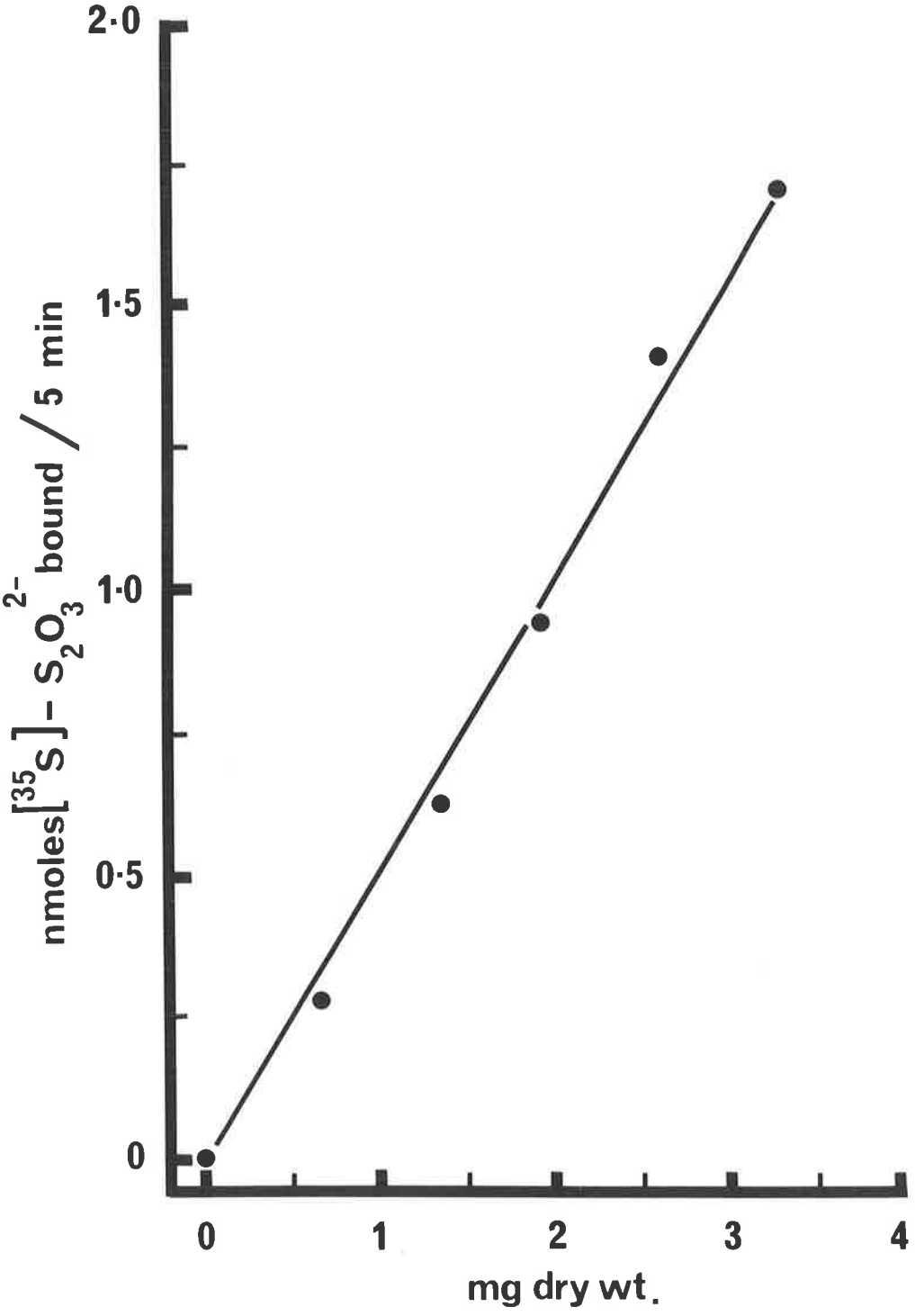


Figure 16

FIGURE 17

Effect of temperature on the uptake of [outer-^{35}S]thiosulphate by whole cells

The incubation mixture contained per ml: washed cells, 6.5 mg dry wt.; mineral salts solution (Section 2.8.8), 0.93 ml; [outer-^{35}S]thiosulphate, 0.11 µmoles (3.3 µCi) and cold thiosulphate, 0.39 µmoles. The incubations, at 30° in a reciprocating water bath, were started by adding the mixture of cold and labelled thiosulphate, and the amount of tracer taken up by washed cells (●——●) was determined after a 5 min incubation period as described in Section 2.8.8. (Specific activity : pmoles [outer-^{35}S]thiosulphate bound/ 5 min/mg dry wt.)

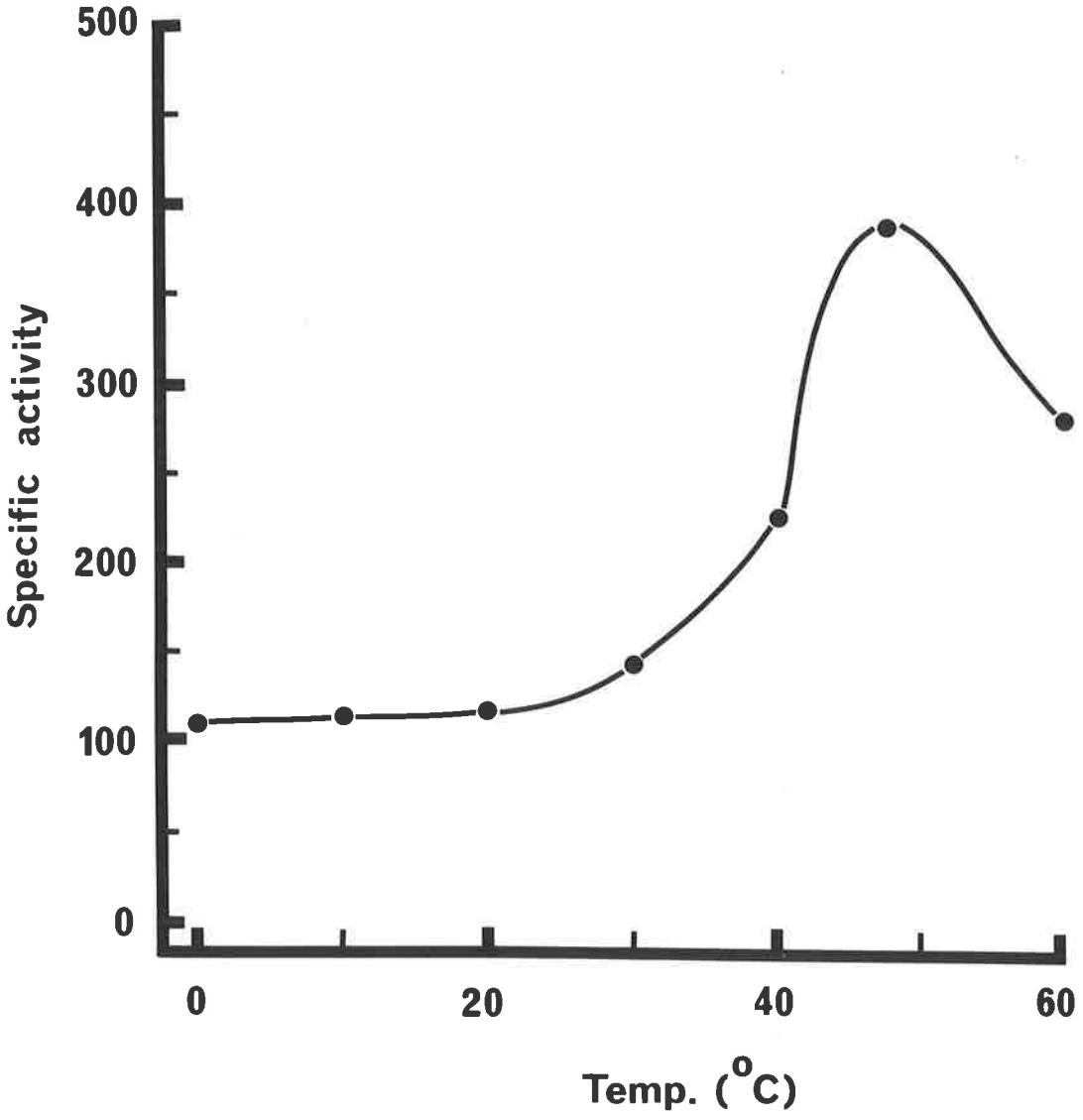


Figure 17

FIGURE 18

Effect of pH on the uptake of [outer-^{35}S]thiosulphate by whole cells

The incubation mixtures contained per ml: washed cells, 6.5 mg dry wt.; acetate or citrate buffer at various pH values, 11 µmoles; [outer-^{35}S]thiosulphate, 0.11 µmoles (3.3 µCi) and cold thiosulphate, 0.39 µmoles. The incubations, at 30° in a reciprocating water bath, were started by adding the labelled and cold thiosulphate, and the amounts of the tracer taken up by washed cells in acetate (o——o) and citrate (●——●) buffers were determined after a 5 min incubation, as described in Section 2.8.8. (Specific activity : nmoles [outer-^{35}S]-thiosulphate bound/5 min/mg dry wt.)

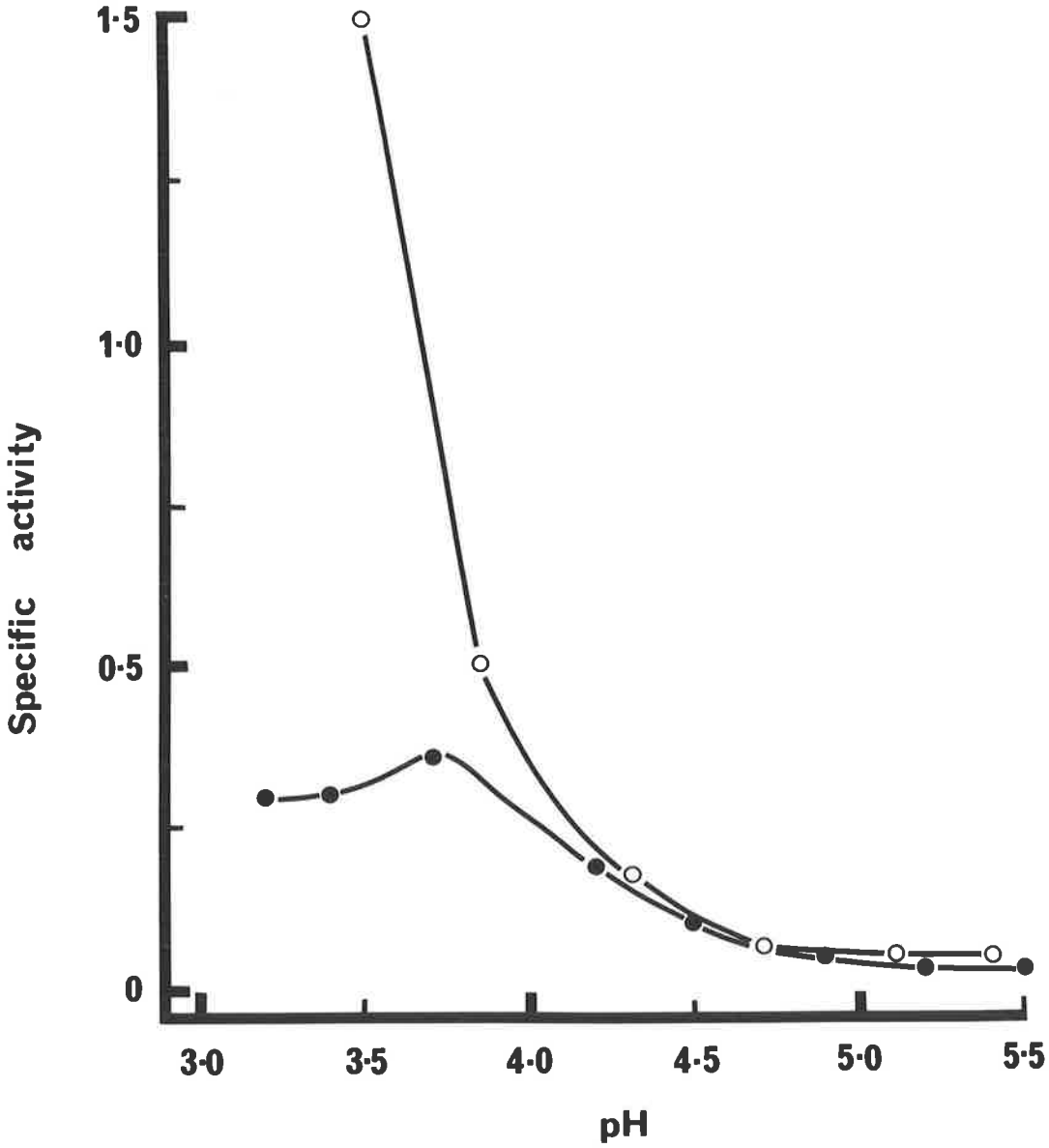


Figure 18

FIGURE 19

Effect of thiosulphate concentration on the uptake of [outer-^{35}S]thiosulphate by whole cells

The incubation mixtures contained per ml: washed cells, 3.1 mg dry wt.; mineral salts solution (Section 2.8.8), 0.90-0.98 ml; [outer-^{35}S]thiosulphate, 0.06-0.11 µmoles (1.65-3.3 µCi) and increasing amounts of cold thiosulphate, to give final concentrations between 0.06 mM and 2.6 mM. The incubations, at 30° in a reciprocating water bath, were started by adding the mixtures of cold and labelled thiosulphate. The amounts of the tracer taken up by the cells were determined after a 5 min incubation period, as described in Section 2.8.8. The estimated K_m value for thiosulphate is 0.5 mM. (Specific activity : nmoles [outer-^{35}S]thiosulphate bound/5 min/mg dry wt.)

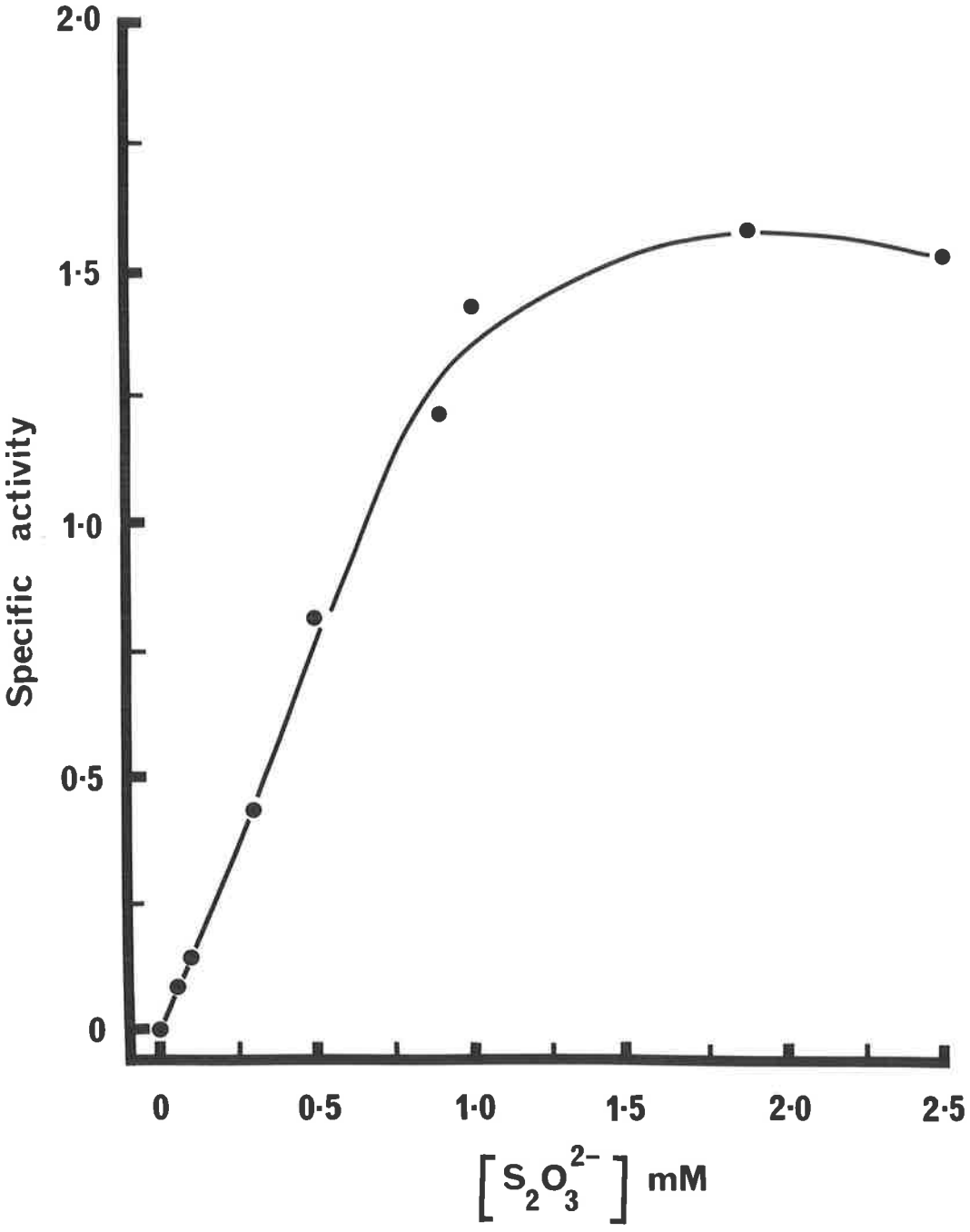


Figure 19

FIGURE 20

Effect of thiosulphate concentration on the uptake of [outer-^{35}S]thiosulphate by whole cells

The experimental conditions have been described in the legend for Figure 19. The results are presented as a Lineweaver-Burk plot and are a replot of the data presented in Figure 19.

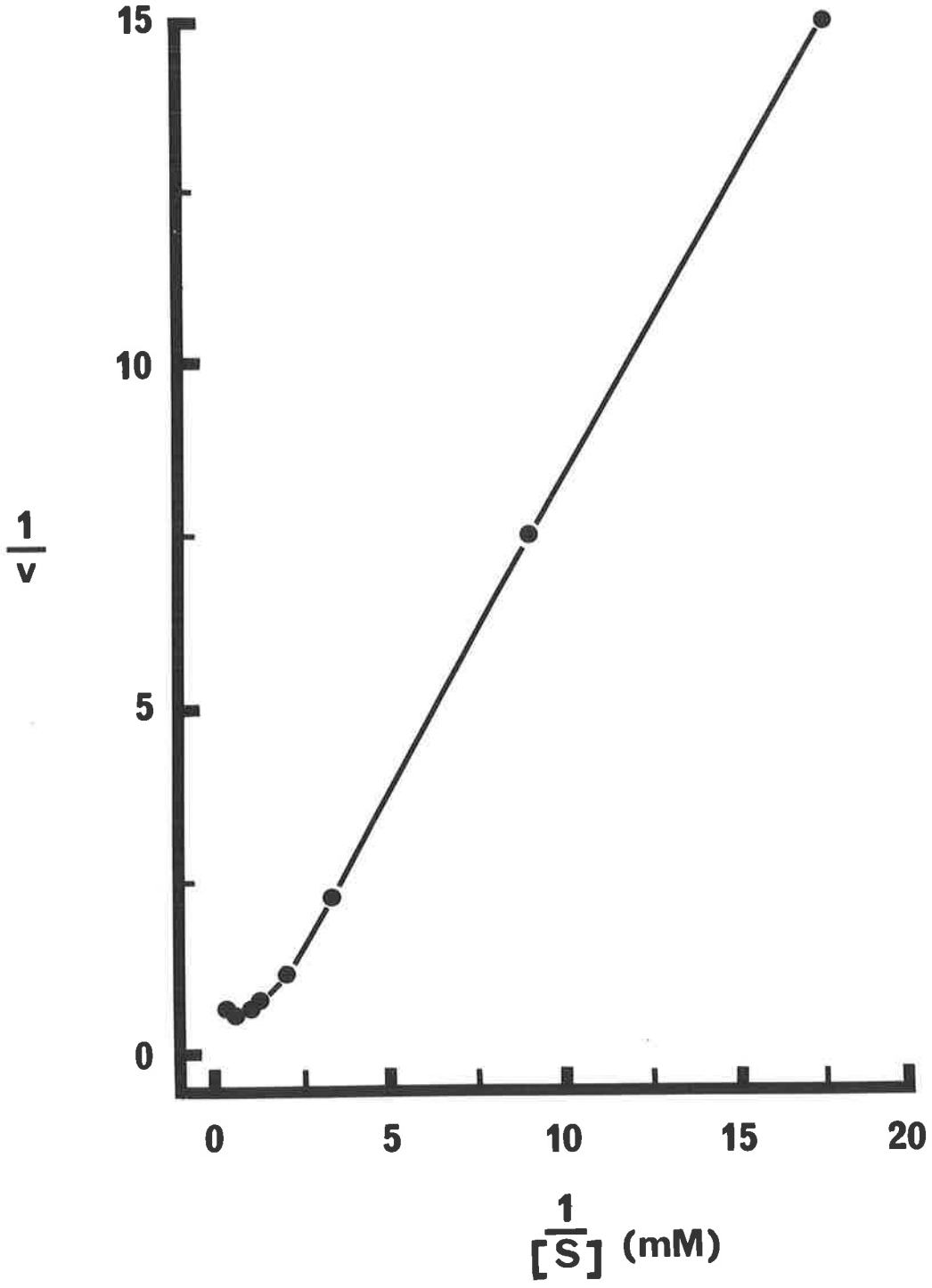


Figure 20

TABLE 17

Effect of various compounds on uptake of [outer-^{35}S]thiosulphate by whole cells

Experimental details are given in the legend for Fig. 16, except that acetate buffer (pH 4.0) (11 µmoles/ml incubation mixture) was used instead of the mineral salts solution. The various compounds were added to the cell suspensions and preincubated at 30° for 5 min before the reaction was started by adding thiosulphate. Washed cells and boiled controls bound 1.2 and 0.1 nmoles of ^{35}S-thiosulphate/5 min/mg dry weight respectively.

Treatment of washed cells	Final concentration (mM)	Percentage of control (%)
Washed cells		100
Molybdate	0.1	76
	0.5	31
	1.0	31
Dithionate	0.5	69
	1.0	32
	5.0	16
Tungstate	1.0	94
	5.0	6
	10.0	4
Selenate	1.0	98
	5.0	76
	10.0	67
2,4-Dinitrophenol	1.0	91
	5.0	79
Cysteine	0.5	130
	1.0	139
Glucose	0.5	113
	1.0	109
	5.0	104

polysaccharide or cell-residue fractions (Figure 21). In parallel experiments, much smaller amounts of ^{35}S were incorporated into the various cell fractions from [inner-^{35}S]thiosulphate (Figure 21).

In cell homogenates, larger amounts of ^{35}S were incorporated from the [outer-^{35}S]thiosulphate, over a 60 min period, into ethanol- or TCA-precipitated protein, than from the [inner-^{35}S]thiosulphate (Table 18). The incorporation of the label into the TCA fraction was enhanced in the presence of *O*-acetylserine (Table 18). There was no evidence for the incorporation of ^{35}S from ^{35}S-sulphate into the protein fractions in cell homogenates, even in the presence of *O*-acetylserine, as shown in Table 18.

4.2.2 Enzymic comparisons of the inorganic sulphur metabolism in autotrophically and heterotrophically grown cells

4.2.2.1 Thiosulphate-oxidizing enzyme and rhodanese

Thiosulphate-oxidizing enzyme was present in extracts of bacteria grown on thiosulphate and glucose (Table 19). In particular, relatively high activities of this enzyme were recorded in the supernatant fractions (S_{10}, S_{105}) of the glucose-grown cells. Thiosulphate-oxidizing enzyme was not detected in any of the cellular fractions from bacteria grown on ferrous-iron (Table 19).

Rhodanese activity was found in extracts of the bacteria grown both autotrophically and heterotrophically (Table 20). The highest activity of this enzyme was recorded in the low-speed pellet fraction P_{10}) of thiosulphate-grown cells. Similarly, higher activities of rhodanese were clearly associated with the pellet fractions (P_{10}) of bacteria grown on ferrous-iron or glucose (Table 20), although enzymic activities were still found in the supernatant fractions.

FIGURE 21

Incorporation of ^{35}S from differentially labelled thiosulphate into cellular fractions of T. ferrooxidans

The incubation mixtures contained per ml: washed cells, 6.7 mg dry wt.; mineral salts solution (Section 2.8.8), 0.68-0.79 ml; [outer-^{35}S]thiosulphate, 4.5 µmoles (8.5 µCi), or [inner-^{35}S]thiosulphate, 4.5 µmoles (10.5 µCi). The incubations, at 30° in a reciprocating water bath, were started by adding the labelled thiosulphate. Samples (0.5 ml) were withdrawn after 1, 10, 30 and 60 min incubation periods and the cells were fractionated as described in Section 2.8.9. The lipid layer represents the combined label extracted with the ethanol and ethanol : diethyl ether (1:1) treatments, as described in Section 2.8.9. □, [outer-^{35}S]thiosulphate; ▒, [inner-^{35}S]-thiosulphate.

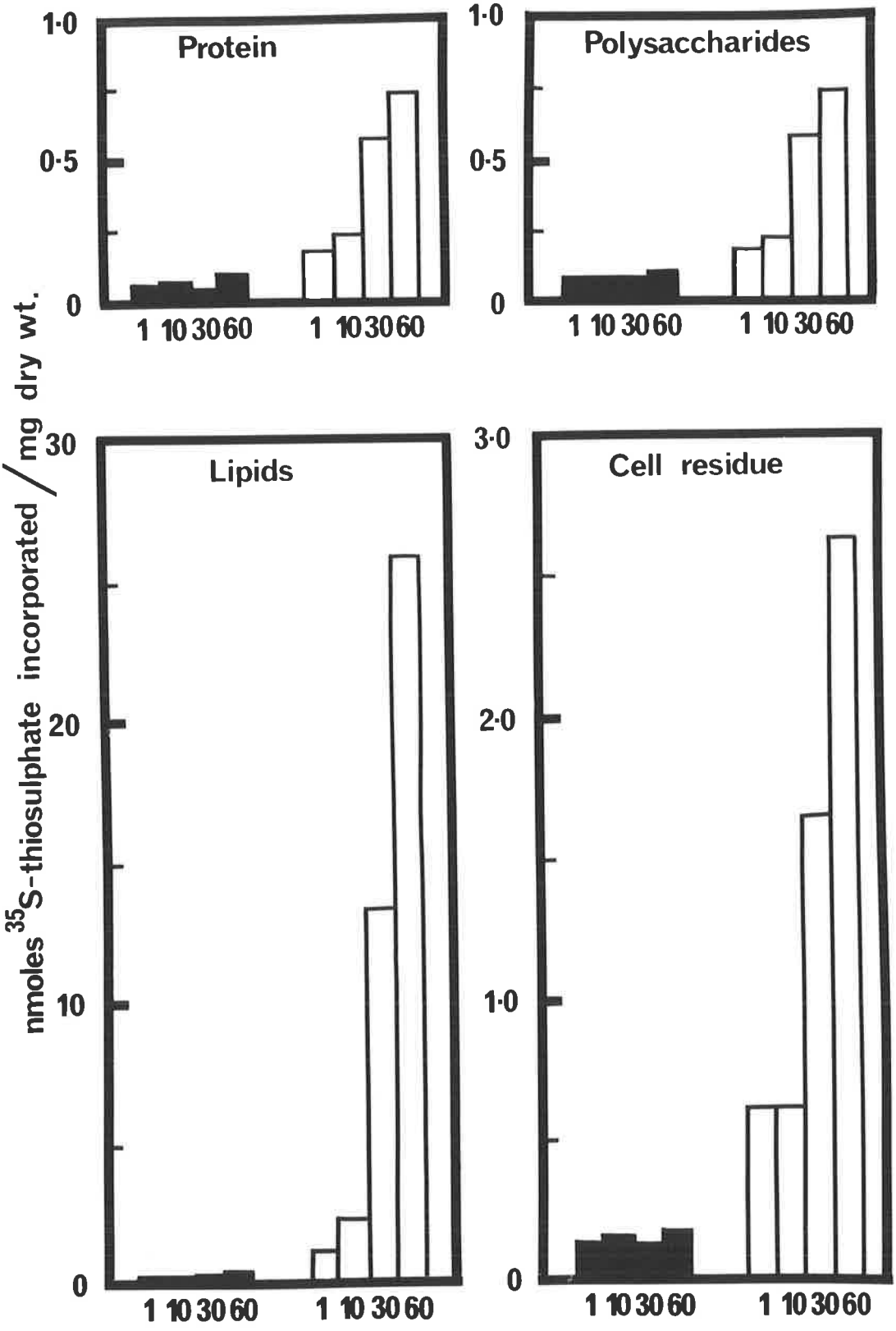

Protein
Polysaccharides
Lipids
Cell residue
1·0
0·5
0
30
20
10
3·0
2·0
1·0
1 10 30 60
nmoles 35S-thiosulphate incorporated / mg dry wt.
Min

Figure 21

TABLE 18

Incorporation of ^{35}S into cellular protein in cell homogenates of T. ferrooxidans

The incubation mixtures contained per ml: Tris-HCl buffer (pH 8.0), 44 µmoles; Na-EDTA, 0.8 µmoles; *O*-acetylserine, 45.5 µmoles; cell homogenate, 6.2 mg protein; thiosulphate, 4.5 µmoles, mixed with either [inner-^{35}S]thiosulphate, 0.13 µmoles (0.6 µCi), [outer-^{35}S]thiosulphate, 0.02 µmoles (0.6 µCi), or ^{35}S-sulphate (4.5 µmoles, 2.4 µCi). The incubations, carried out at 30° in a reciprocating water bath, were started by adding the tracer. After incubating for 30 and 60 min, samples (0.5 ml) were taken and mixed with A, 1.25 ml cold 99% (v/v) ethanol, or B, 1.25 ml cold 10% (v/v) TCA, and kept on ice for 45 min before centrifuging at 10,000 x *g* for 10 min at 4°. Aliquots (0.1 ml) were withdrawn from the samples before and after centrifugation and radioassayed as described in Section 2.9.3. The results have been corrected for the incorporation of ^{35}S by boiled cell homogenates under identical conditions.

Addition	Time (min)	^{35}S incorporated (nmoles/mg protein)					
		[inner-^{35}S]thiosulphate		[outer-^{35}S]thiosulphate		^{35}S-sulphate	
		A	B	A	B	A	B
Homogenate	30	0	0	0	0	0	0
	60	0	32	128	43	0	0
Homogenate and *O*-acetylserine	30	0	0	112	112	0	0
	60	0	37	53	251	0	0

TABLE 19

Distribution of thiosulphate-oxidizing enzyme in various cell fractions of T. ferrooxidans grown autotrophically and heterotrophically

The preparation of the homogenates and the various cell fractions (S_{10}, P_{10}, S_{105}, P_{105}) from cells grown autotrophically and heterotrophically has been described in Section 2.8.1.1. Thiosulphate-oxidizing enzyme was assayed spectrophotometrically by following the reduction of ferricyanide at 420 nm, as described in Section 2.8.10.1.

Cell fraction	Specific activity [nmoles $Fe(CN_6)^{3-}$ reduced/min/mg protein]		
	Autotrophic		Heterotrophic
	$S_2O_3^{2-}$	Fe^{2+}	Glucose
Homogenate	8.2	0	42.9
S_{10}	3.1	0	24.6
P_{10}	11.0	0	9.4
S_{105}	12.0	0	33.8
P_{105}	12.7	0	2.3

TABLE 20

Distribution of rhodanese activity in various cell fractions of bacteria grown autotrophically and heterotrophically

The preparation of the homogenates and the various cell fractions (S_{10}, P_{10}, S_{105}, P_{105}) from cells grown autotrophically and heterotrophically has been described in Section 2.8.1.1. Rhodanese activity was assayed by a slightly modified method of Bowen *et al.* (1965), which has been described in Section 2.8.10.2.

Cell fraction	Specific activity (nmoles CNS^- produced/min/mg protein)		
	Autotrophic		Heterotrophic
	$S_2O_3^{2-}$	Fe^{2+}	Glucose
Homogenate	14.8	7.1	6.6
S_{10}	7.1	6.0	5.6
P_{10}	110.0	30.4	28.4
S_{105}	17.8	6.5	13.7
P_{105}	12.9	4.7	4.4

4.2.2.2 AMP-independent sulphite oxidase and APS-reductase

The activity of AMP-independent sulphite oxidase was highest in the thiosulphate-grown bacteria and was clearly associated with the low-speed pellet fraction (P_{10}) (Table 21). A similar trend was observed in the glucose-grown bacteria, although the enzymic activities were lower. Very low activities were found in various fractions of iron-grown bacteria (Table 21). The addition of 5'-AMP did not enhance the activity of the AMP-independent sulphite oxidase, as shown in Table 22. It appears that it was largely the AMP-independent sulphite oxidase activity that was measured in preparations to which 5'-AMP had been added for the APS-reductase assay. Experiments with ^{35}S-sulphite and 5'-AMP indicated that ^{35}S-APS was produced at similar rates in comparable cell fractions of the bacteria grown autotrophically and heterotrophically. Between 0.5 and 1.8% of ^{35}S-sulphite was incorporated into ^{35}S-APS (0.4-1.44 nmoles) during a 5 min incubation.

4.2.2.3 ATP-sulphurylase, ADP-sulphurylase and adenylate kinase

The distribution and activity of ATP-sulphurylase were similar in cells grown autotrophically with both ferrous-iron and thiosulphate and heterotrophically with glucose (Table 23). Higher activities were recorded in the supernatant fractions (S_{10}, S_{105}) than in the pellet fractions (P_{10}, P_{105}). ADP-sulphurylase was also widely distributed in the cell fractions of autotrophic and heterotrophic *T. ferrooxidans*, with higher activities recorded in the low-speed pellet fraction (P_{10}) of bacteria grown on thiosulphate and glucose (Table 24). There was also little difference in the distribution of

TABLE 21

Distribution of AMP-independent sulphite oxidase activity in various cell fractions of bacteria grown autotrophically and heterotrophically

The preparation of the homogenates and the various cell fractions (S_{10}, P_{10}, S_{105}, P_{105}) from cells grown autotrophically and heterotrophically has been described in Section 2.8.1.1. AMP-independent sulphite oxidase activity was assayed by a slightly modified method of Aminuddin & Nicholas (1974), which has been described in Section 2.8.10.3.

Cell fraction	Specific activity [nmoles $Fe(CN_6)^{3-}$ reduced/min/mg protein]		
	Autotrophic		Heterotrophic
	$S_2O_3^{2-}$	Fe^{2+}	Glucose
Homogenate	14.9	1.3	1.1
S_{10}	2.9	0.7	2.1
P_{10}	219	0	26.9
S_{105}	5.6	1.8	5.7
P_{105}	2.7	0	1.6

TABLE 22

Distribution of APS-reductase activity in various cell fractions of bacteria grown autotrophically and heterotrophically

The preparation of the homogenates and the various cell fractions (S_{10}, P_{10}, S_{105}, P_{105}) from cells grown autotrophically and heterotrophically has been described in Section 2.8.1.1. APS-reductase was assayed by a modification of the method of Peck (1961), which has been described in Section 2.8.10.4.

Cell fraction	Specific activity [nmoles $Fe(CN_6)^{3-}$ reduced/min/mg protein]		
	Autotrophic		Heterotrophic
	$S_2O_3^{2-}$	Fe^{2+}	Glucose
Homogenate	12.6	1.8	0.9
S_{10}	2.3	0.3	2.7
P_{10}	210	0	18.7
S_{105}	5.4	1.8	3.6
P_{105}	2.2	0	1.3

TABLE 23

Distribution of ATP-sulphurylase activity in various cell fractions of bacteria grown autotrophically and heterotrophically

The preparation of the homogenates and the various cell fractions (S_{10}, P_{10}, S_{105}, P_{105}) from cells grown autotrophically and heterotrophically has been described in Section 2.8.1.1. ATP-sulphurylase activity was determined by the static firefly bioluminescence assay outlined in Section 2.8.10.5

Cell fraction	Specific activity (nmoles ATP produced/min/mg protein)		
	Autotrophic		Heterotrophic
	$S_2O_3^{2-}$	Fe^{2+}	Glucose
Homogenate	0.35	0.11	0.19
S_{10}	0.19	0.59	0.27
P_{10}	0.11	0.06	0.04
S_{105}	0.34	0.28	0.33
P_{105}	0.07	0.02	0.06

TABLE 24

Distribution of ADP-sulphurylase activity in various cell fractions of bacteria grown autotrophically and heterotrophically

The preparation of the homogenates and the various cell fractions (S_{10}, P_{10}, S_{105}, P_{105}) from cells grown autotrophically and heterotrophically has been described in Section 2.8.1.1. ADP-sulphurylase was assayed using a method developed by Dr. R.G. Nicholls in this Department, which is essentially an adaptation from a polynucleotide phosphorylase assay described by Kimhi & Littauer (1968) and which is outlined in Section 2.8.10.6.

Cell fraction	Specific activity (nmoles [^{32}P]ADP produced/min/mg protein)		
	Autotrophic		Heterotrophic
	$S_2O_3^{2-}$	Fe^{2+}	Glucose
Homogenate	0.32	0.06	0.34
S_{10}	0.28	0.24	0.25
P_{10}	4.70	0.66	3.20
S_{105}	0.73	0.23	1.10
P_{105}	0.05	0.18	0

activity of adenylate kinase between autotrophically and heterotrophically grown bacteria; higher activities of this enzyme were found in the supernatant fractions (S_{10}, S_{105}) (Table 25). In general, the specific activity of adenylate kinase (Table 25) was higher than either ATP-sulphurylase (Table 23) or ADP-sulphurylase (Table 24).

4.2.2.4 NADPH-linked sulphite reductase

Under the conditions used for assaying NADPH-linked sulphite reductase (see Section 2.8.10.8), sulphide production was observed in the high-speed pellet fraction (P_{105}) of bacteria grown with thiosulphate (0.02 nmoles sulphide produced/min/mg protein) and in the high-speed supernatant fractions (S_{105}) of bacteria grown with ferrous-iron and with glucose (0.03-0.05 nmoles sulphide produced/min/mg protein) (Table 26). Under similar conditions, including appropriate controls, a 250-fold increase in the rate of sulphide production (5 nmoles sulphide produced/min/mg protein) was obtained when the low-speed pellet fraction (P_{10}) of thiosulphate-grown bacteria was assayed (Table 26). This high rate of sulphide production, however, is unlikely to result from an active sulphite-reducing system but rather from the precipitated sulphur in the P_{10} fraction carried over from the growth medium; this sulphur may react with sulphite added in the assay mixture producing thiosulphate. A high rhodanese activity, capable of enzymically cleaving thiosulphate to yield sulphide and sulphite, has already been recorded (Table 20).

TABLE 25

Distribution of adenylate kinase activity in various cell fractions of bacteria grown autotrophically and heterotrophically

The preparation of the homogenates and the various cell fractions (S_{10}, P_{10}, S_{105}, P_{105}) from cells grown autotrophically and heterotrophically has been described in Section 2.8.1.1. Adenylate kinase activity was assayed using the static firefly bioluminescence method as outlined in Section 2.8.10.7.

Cell fraction	Specific activity (nmoles ATP produced/min/mg protein)		
	Autotrophic		Heterotrophic
	$S_2O_3^{2-}$	Fe^{2+}	Glucose
Homogenate	5.73	5.44	4.01
S_{10}	5.75	8.04	5.53
P_{10}	0.27	2.10	0.27
S_{105}	12.71	12.50	9.03
P_{105}	0.29	0.42	1.11

TABLE 26

Distribution of NADPH-linked sulphite reductase activity in various cell fractions of bacteria grown autotrophically and heterotrophically

The preparation of the various cell fractions (S_{10}, P_{10}, S_{105}, P_{105}) from cells grown autotrophically and heterotrophically has been described in Section 2.8.1.1. Sulphite reductase activity was determined using the method of Ellis (1964) which has been outlined in Section 2.8.10.8.

Cell fraction	Specific activity (nmoles S^{2-} produced/min/mg protein)		
	Autotrophic		Heterotrophic
	$S_2O_3^{2-}$	Fe^{2+}	Glucose
S_{10}	0	0	0
P_{10}	5.0	0	0
S_{105}	0	0.03	0.05
P_{105}	0.02	0	0

4.2.3 A comparison of the properties of partially purified ATP-sulphurylase from cells grown with ferrous-iron and with thiosulphate

4.2.3.1 Purification

A similar procedure was used for the purification of ATP-sulphurylase from cells grown with either ferrous-iron (Table 27) or thiosulphate (Table 28). All operations were carried out at 2° unless otherwise stated.

Washed cells, suspended (25%, w/v) in 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM Na-EDTA, were disrupted in a French pressure cell as described in Section 2.8.1.1. The resultant homogenate (Fraction I, Table 27; Fraction I, Table 28) was used as the starting material for the fractionation of the enzyme. The homogenate was then centrifuged at 10,000 x *g* for 30 min and the supernatant fraction (S_{10}) retained (Fraction II, Table 27; Fraction II, Table 28). The supernatant fraction (S_{105}, Fraction III, Table 27) left after centrifuging the S_{10} extract at 105,000 x *g* for 90 min was immersed in a water bath at 58° for 5 min. The precipitated protein was removed by centrifuging at 12,000 x *g* for 20 min. The supernatant fraction (Fraction IV, Table 27; Fraction III, Table 28) was then treated with solid ammonium sulphate to give 30-40% saturation. The precipitate recovered by centrifuging at 12,000 x *g* for 20 min was dissolved in a minimal volume of the cold Tris-HCl buffer and dialysed for 16 h at 2° in two changes of the buffer (1 *ℓ*). This fraction (Fraction V, Table 27; Fraction IV, Table 28) was used as the source of ATP-sulphurylase. Details of the purification procedure for ATP-sulphurylase from ferrous-iron- (6.5-fold purification) and thiosulphate- (8-fold purification) grown cells are given in Tables 27 and 28 respectively.

TABLE 27

Partial purification of ATP-sulphurylase from T. ferrooxidans grown on ferrous-iron

Washed cells, suspended in 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM Na-EDTA, were disrupted by three passages through the French Pressure Cell at 4° (Section 2.8.1.1). The resulting cell homogenate was used as the starting material (Fraction I). ATP-sulphurylase activity was determined by the static bioluminescence method, as described in Section 2.8.10.5. (Specific activity : pmoles ATP produced/min/mg protein).

Fraction	Procedure	Protein (mg/ml)	Specific activity (pmoles/ min/mg protein)	Fold purification
I	Cells disrupted after three passages through French Pressure Cell, 20,000 p.s.i.	59.7	122.8	1
II	Crude extract (S_{10}) left after centrifuging I at 10,000 x *g* for 30 min	29.3	222.0	1.8
III	Supernatant fraction (S_{105}) left after centrifuging II at 105,000 x *g* for 90 min	19.5	200.6	1.6
IV	Fraction III heated at 58° for 5 min, then centrifuged at 12,000 x *g* for 20 min	18.2	283.1	2.3
V	Fraction IV precipitated with ammonium sulphate (30-40% saturation) and dialysed against 2 *ℓ* of 50 mM Tris-HCl buffer (pH 7.5) + 1 mM Na-EDTA	8.4	792.8	6.5

TABLE 28

Partial purification of ATP-sulphurylase from T. ferrooxidans grown on thiosulphate

Washed cells, suspended in 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM Na-EDTA, were disrupted by three passages through the French Pressure Cell (Section 2.8.1.1). The resulting cell homogenate was used as the starting material (Fraction I). ATP-sulphurylase activity was determined by the static bioluminescence method, as described in Section 2.8.10.5. (Specific activity : pmoles ATP produced/min/mg protein).

Fraction	Procedure	Protein (mg/ml)	Specific activity (pmoles/ min/mg protein)	Fold purification
I	Cells broken after three passages through French Pressure Cell, 20,000 p.s.i.	19	110.5	1
II	Crude extract (S_{10}) left after centrifuging I at 10,000 x *g* for 30 min	25	74.0	-
III	S_{105} fraction heated at 58° for 5 min, then centrifuged at 12,000 x *g* for 20 min	8.0	380.7	3.5
IV	Fraction III precipitated with ammonium sulphate (30-40% saturation) and dialysed against 2 *ℓ* of 50 mM Tris-HCl buffer (pH 7.5) + 1 mM Na-EDTA	6.0	881.4	8.0

4.2.3.2 Distribution

Most of the enzymic activity was found in the supernatant fractions produced by centrifuging the cell homogenate at 10,000 x *g* for 30 min (S_{10}) and then centrifuging the S_{10} fraction at 105,000 x *g* for 90 min (S_{105}) (see Table 23). Only very low ATP-sulphurylase activity was detected in the high-speed pellet fraction (P_{105}) which had been washed once with the 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM Na-EDTA (Table 23).

4.2.3.3 Purity of the enzyme

At each stage of the purification of ATP-sulphurylase from ferrous-iron-grown cells (Table 27), samples of the enzyme preparation were taken and the stability of ^{35}S-APS, the substrate of the ATP-sulphurylase assay, was examined. Maximum stability was observed with Fraction V (Table 27), even over a 30 min incubation period (Table 29). Since ATP-sulphurylase assays were conducted over a 2 min incubation period, there was no degradation of APS. Similarly, the product of the assay, ATP, was found to be stable on adding it to this same fraction (Fraction V, Table 27), as determined by the continuous bioluminescence assay of the firefly (see Section 2.8.10.5). The partially purified enzyme (Fraction V, Table 27) was also devoid of inorganic pyrophosphatase and ATP-ase enzymes.

Similarly, ATP-sulphurylase from thiosulphate-grown cells (Fraction IV, Table 28) did not degrade either APS or ATP, and was devoid of the other interfering enzymes described above.

TABLE 29

Stability of ^{35}S-APS in the various cell fractions containing partially purified ATP-sulphurylase from T. ferrooxidans grown on ferrous-iron

The reaction mixtures contained: Tris-HCl buffer (pH 7.5), 17 µmoles; ^{35}S-APS, 0.74 µmoles (0.38 µCi) and extracts as shown in Table 27 (Fraction III, 2.0 mg; Fraction IV, 1.8 mg; Fraction V, 0.84 mg protein), in a total volume of 0.5 ml. Incubations were carried out at 30°. Samples (20 µl), taken at 10 min and 30 min intervals, were applied straight to 3MM Whatmann paper. Electrophoresis was in citrate buffer (pH 5.0) at 1500 V for 60 min (Section 2.9.1). Radioactive areas were determined as described in Sections 2.9.2 and 2.9.3.

Fraction	Time (min)	Distribution of ^{35}S (%)	
		APS	SO_4^{2-}
S_{105}, Fraction III from Table 27	0	100	0
	10	78.5	21.5
	30	76.5	23.5
Heated, Fraction IV from Table 27	0	100	0
	10	74.5	25.5
	30	78.8	21.2
Ammonium sulphate Fraction V from Table 27)	0	100	0
	10	88.9	10.1
	30	93.0	7.0

4.2.3.4 Effect of incubation time, composition of reaction mixture and enzyme concentration

The effect of incubation time on ATP-sulphurylase from ferrous-iron- and thiosulphate-grown cells is shown in Figure 22a and Figure 22b respectively. For both enzyme preparations, the reaction was linear for 5 min. Thus, a 2 min incubation was used routinely to assay the enzyme.

Little ATP was produced in the absence of magnesium chloride, pyrophosphate, APS or when phosphate was substituted for pyrophosphate (Table 30). Thus, there was no difference in assay requirements between the two partially purified enzyme preparations, as shown in Table 30.

The effect of enzyme concentration on the rate of ATP formation measured by the static bioluminescence technique is shown in Figures 23a and 23b. Both enzyme preparations displayed linearity with respect to enzymic activity as the protein concentration increased to 1 mg protein per ml incubation mixture. The enzyme from cells grown with thiosulphate exhibited higher specific activity than that isolated from ferrous-iron-grown cells, as shown in Figure 23a and Figure 23b.

4.2.3.5 Effect of divalent cations, pH and temperature

The metal cofactor requirements for maximum enzymic activity were similar for both ATP-sulphurylase preparations. Magnesium was essential for activity (Table 31) and indeed all kinetic studies were carried out in the presence of this cation (0.5 mM). Cobalt (0.5 mM) was as effective as magnesium (Table 31) in both enzyme preparations (Fraction V, Table 27; Fraction IV, Table 28). ATP-sulphurylase was inactive in the presence of copper, calcium, manganese,

FIGURE 22

Effect of incubation time on ATP-sulphurylase activity from bacteria grown on (a) ferrous-iron and (b) thiosulphate

ATP-sulphurylase was assayed by the static bioluminescence procedure as described in Section 2.8.10.5. The ATP-sulphurylase preparation was incubated with the substrates at 30° for time periods from 0 to 5 min. a): Fraction V (Table 27) from ferrous-iron-grown bacteria (0.35 mg protein per incubation mixture. b): Fraction IV (Table 28) from thiosulphate-grown bacteria (0.33 mg protein per incubation mixture).

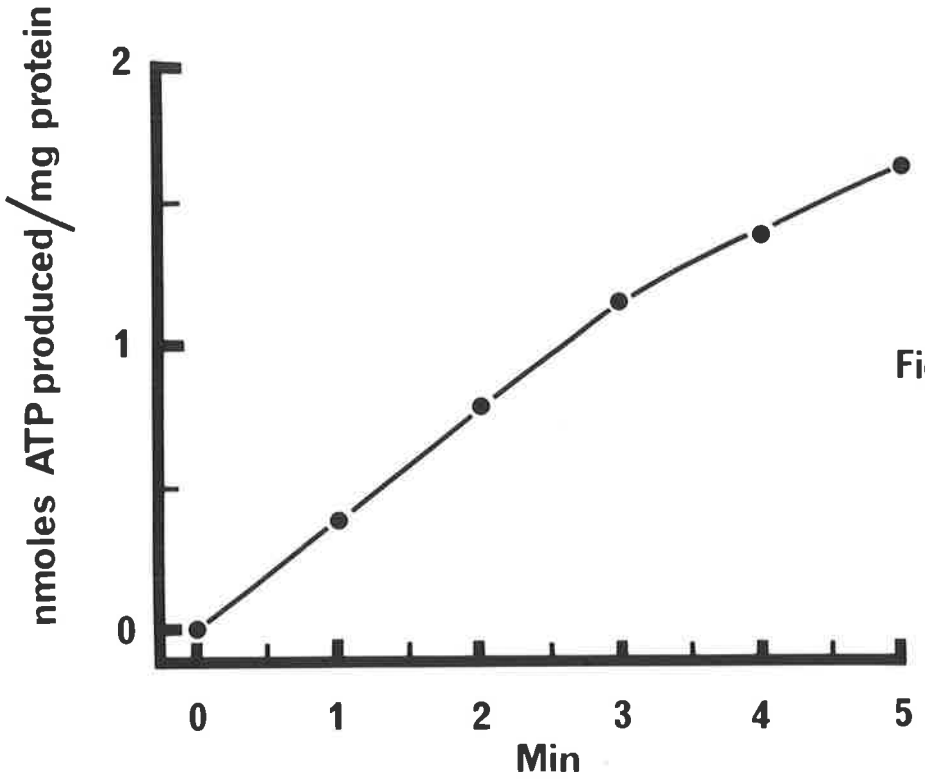


Figure 22a

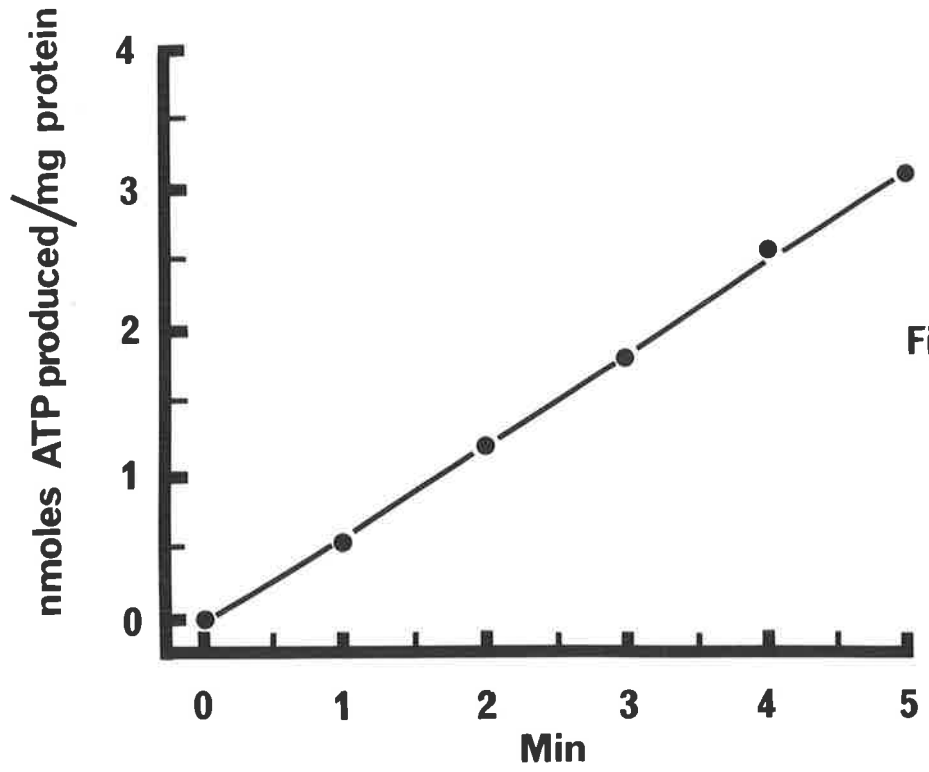


Figure 22b

TABLE 30

Effect of composition of the reaction mixture on ATP-sulphurylase from bacteria grown on ferrous-iron and thiosulphate

The complete reaction mixture contained in μmoles: Tris-HCl buffer (pH 7.5), 37; $MgCl_2$, 0.5; APS, 0.25; PPi, 0.5; and the partially purified ATP-sulphurylase preparation, either A — Fraction V (Table 27), 0.84 mg protein, or B — Fraction IV (Table 28), 0.6 mg protein, in a final volume of 1 ml. The enzyme was assayed as described in Section 2.8.10.5. Substrates omitted were replaced with suitable aliquots of the buffer. Boiled enzyme preparations were prepared by immersing them in a water bath at 100° for 5 min. After cooling, the boiled samples were homogenized and then assayed as above.

Assay conditions	Specific activity (nmoles ATP produced/min/mg protein)	
	A (Ferrous-iron)	B (Thiosulphate)
Complete	0.35	0.80
Boiled enzyme	0.10	0.18
Omit enzyme	0.08	0.03
Omit $MgCl_2$	0.08	0.19
Omit PPi	0.10	0.15
Omit APS	0.08	0.07
Pi substituted for PPi	0.09	0.18

FIGURE 23

Effect of enzyme concentration on ATP-sulphurylase activity from bacteria grown on (a) ferrous-iron and (b) thiosulphate

ATP-sulphurylase was assayed by the static bioluminescence procedure as described in Section 2.8.10.5. Increasing amounts of the enzyme over the range 0-1 mg protein were used in the assay mixtures. a): Fraction V (Table 27) from ferrous-iron-grown bacteria (0-1 mg protein). b): Fraction IV (Table 28) from thiosulphate-grown bacteria (0-0.7 mg protein).

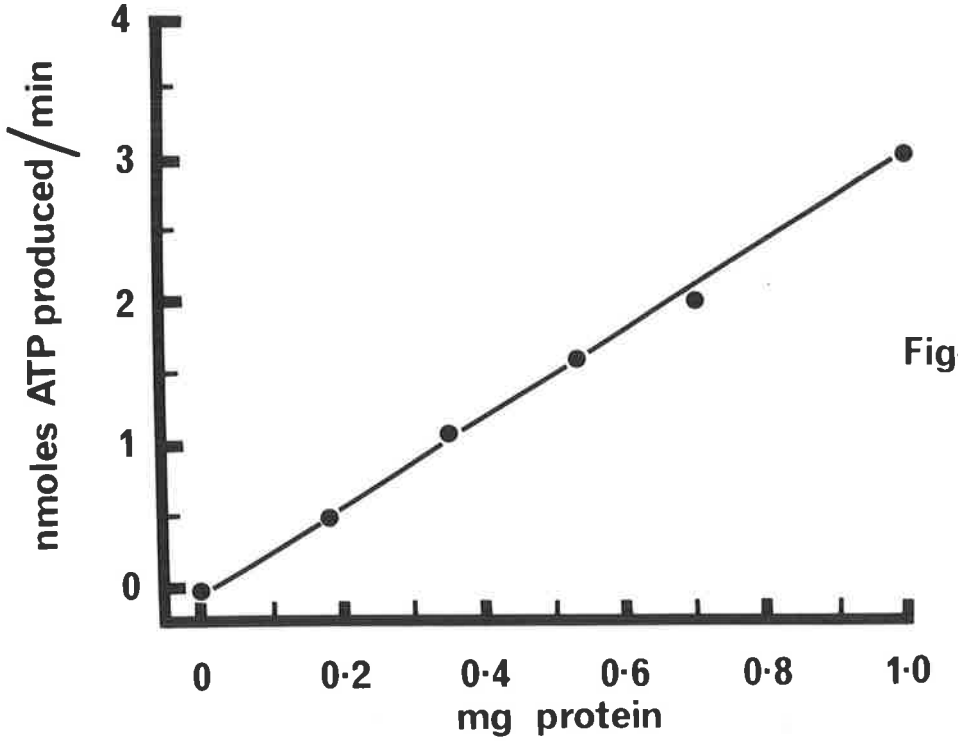


Figure 23a

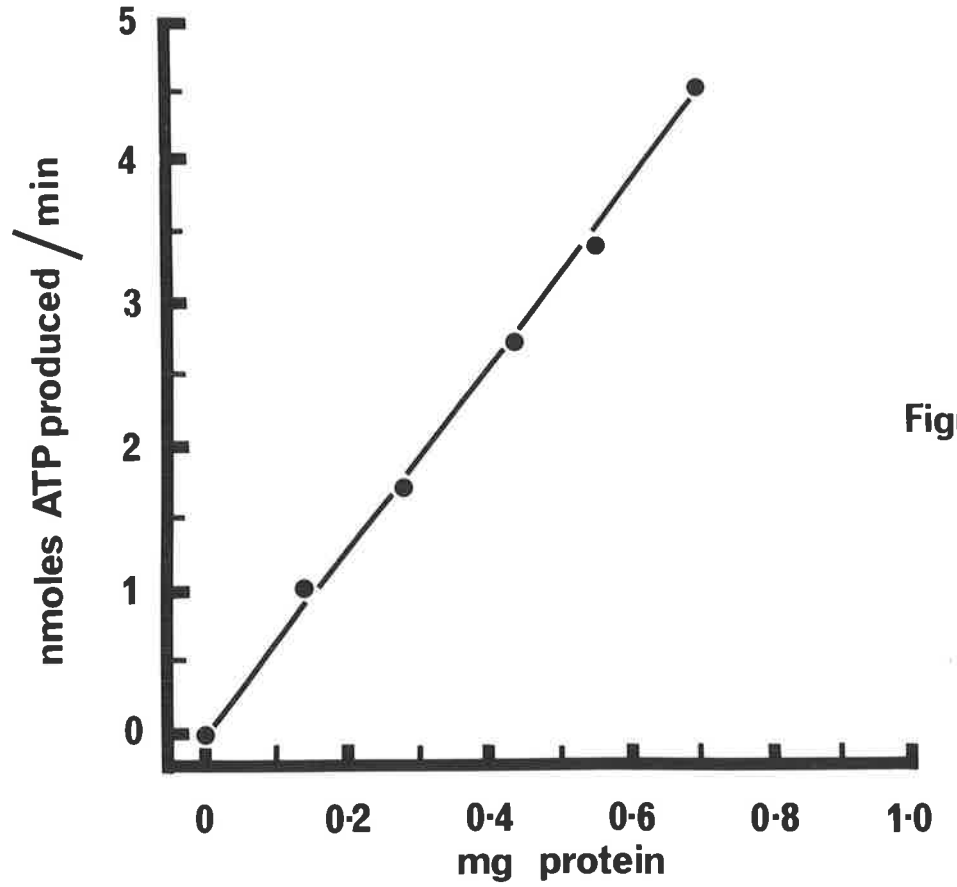


Figure 23b

TABLE 31

Effect of divalent cations on ATP-sulphurylase activity from bacteria grown on ferrous-iron and thiosulphate

The complete reaction mixture contained in µmoles: Tris-HCl buffer (pH 7.5), 37; $MgCl_2$, 0.5; APS, 0.25; PPi, 0.5; and the partially purified ATP-sulphurylase preparation, either A – Fraction V (Table 27), 0.84 mg protein, or B – Fraction IV (Table 28), 0.6 mg protein, in a final volume of 1 ml. The enzyme was assayed by the static bioluminescence technique as described in Section 2.8.10.5. The various cations listed (0.5 µmoles/ml incubation mixture) were substituted for $MgCl_2$ in the assay mixtures.

Cation added	Specific activity (nmoles ATP produced/min/mg protein)	
	A (Ferrous-iron)	B (Thiosulphate)
Enzyme + Mg^{2+}	0.18	0.72
Boiled enzyme + Mg^{2+}	0.03	0.13
CO^{2+}	0.21	0.89
Cu^{2+}	0.07	0.13
Ca^{2+}	0.07	0.06
Mn^{2+}	0.09	0.19
Ba^{2+}	0.07	0.14
Zn^{2+}	0.07	0.14
Hg^{2+}	0.06	0.11

barium, zinc and mercury cations (0.5 mM) (Table 31).

Both ATP-sulphurylase preparations showed similar responses to pH changes (Figure 24a,b). The buffer for routine assay was 50 mM Tris-HCl, and optimum activity was obtained between pH 7.6 and pH 8.2 (Figure 24a,b).

The effect of temperature on enzymic activity is shown in Figures 25a and 25b. The optimum temperature for enzyme activity from ferrous-iron-grown cells was sharply defined between 55° and 60° (Figure 25a). However, the enzyme from cells grown with thiosulphate had a much broader temperature optimum over the range 40° to 55° (Figure 25b).

The temperature stability of the two enzymes at 30° and 60° is presented in Figures 26a and 26b. Exposure of the enzyme from ferrous-iron-grown cells to 60° for 30 min resulted in 55% loss of activity (Figure 26a). Under identical conditions, a 57% loss of activity was recorded for ATP-sulphurylase from cells grown with thiosulphate (Figure 26b).

4.2.3.6 Effect of pyrophosphate

The rate of production of ATP was measured at various concentrations of pyrophosphate using partially purified ATP-sulphurylase preparations (Fraction V, Table 27; Fraction IV, Table 28). The results show normal Michaelis-Menten kinetics and are presented for the ferrous-iron-grown bacteria (Figure 27a,b) and the thiosulphate-grown bacteria (Figure 28a,b). At an APS concentration fixed at 0.25 mM, the K_m for the pyrophosphate from ATP-sulphurylase in cells grown with ferrous-iron is 0.77 mM and for those grown with thiosulphate, 0.53 mM.

FIGURE 24

Effect of pH on ATP-sulphurylase activity from bacteria grown on (a) ferrous-iron and (b) thiosulphate

ATP-sulphurylase was assayed by the static bioluminescence procedure as described in Section 2.8.10.5. Tris-malate buffer (50 mM) (o——o) and Tris-HCl buffer (50 mM) (●——●) at pH values ranging from 5.8 to 7.0 and 7.4 to 8.8 respectively were used for the assay of the enzyme by the above method. a): Fraction V (Table 27) from ferrous-iron-grown bacteria (0.93 mg protein/ml incubation mixture). b): Fraction IV (Table 28) from thiosulphate-grown bacteria (0.33 mg protein/ml incubation mixture). (Specific activity : nmoles ATP produced/min/mg protein)

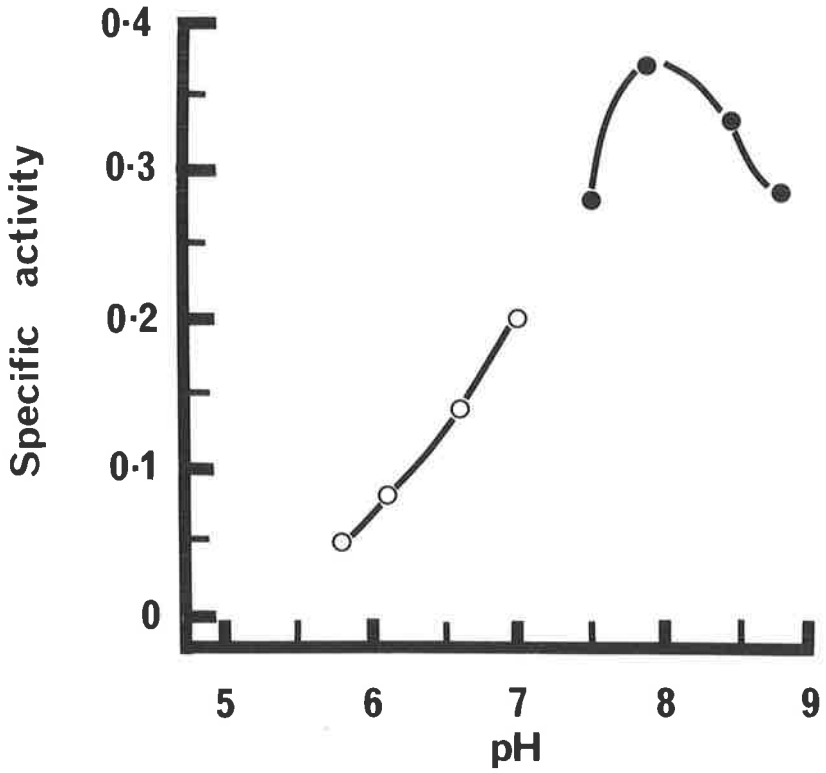


Figure 24a

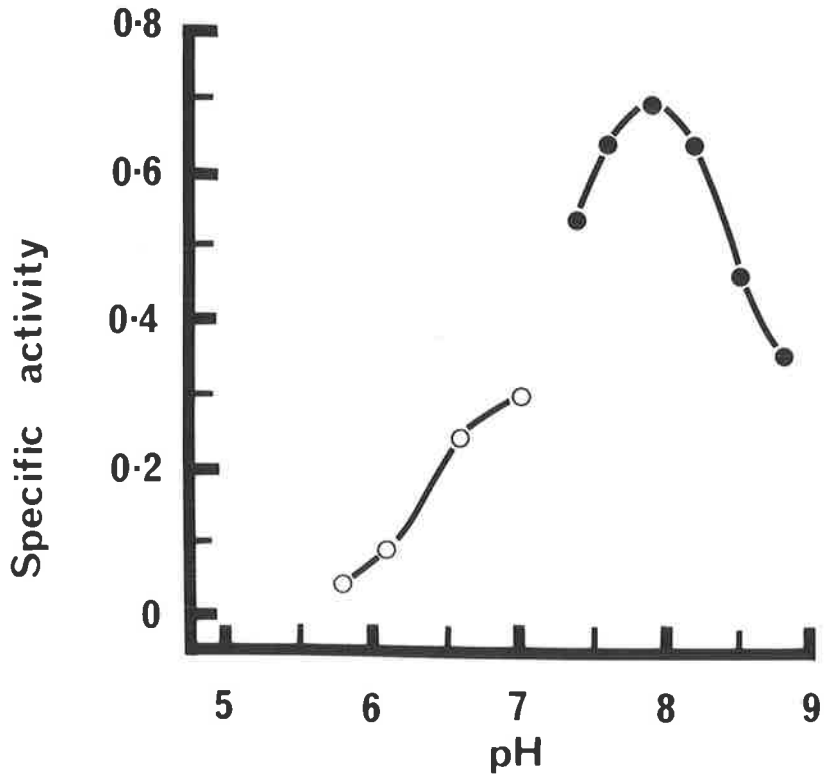


Figure 24b

FIGURE 25

Effect of temperature on ATP-sulphurylase activity from bacteria grown on (a) ferrous-iron and (b) thiosulphate

ATP-sulphurylase was assayed by the static bioluminescence procedure as described in Section 2.8.10.5. The enzyme was pre-incubated for 3 min at the temperature specified prior to starting the assay. a): Fraction V (Table 27) from ferrous-iron-grown bacteria (0.8 mg protein/ml incubation mixture). b): Fraction IV (Table 28) from thiosulphate-grown bacteria (0.5 mg protein/ml incubation mixture). (Specific activity : nmoles ATP produced/min/mg protein)

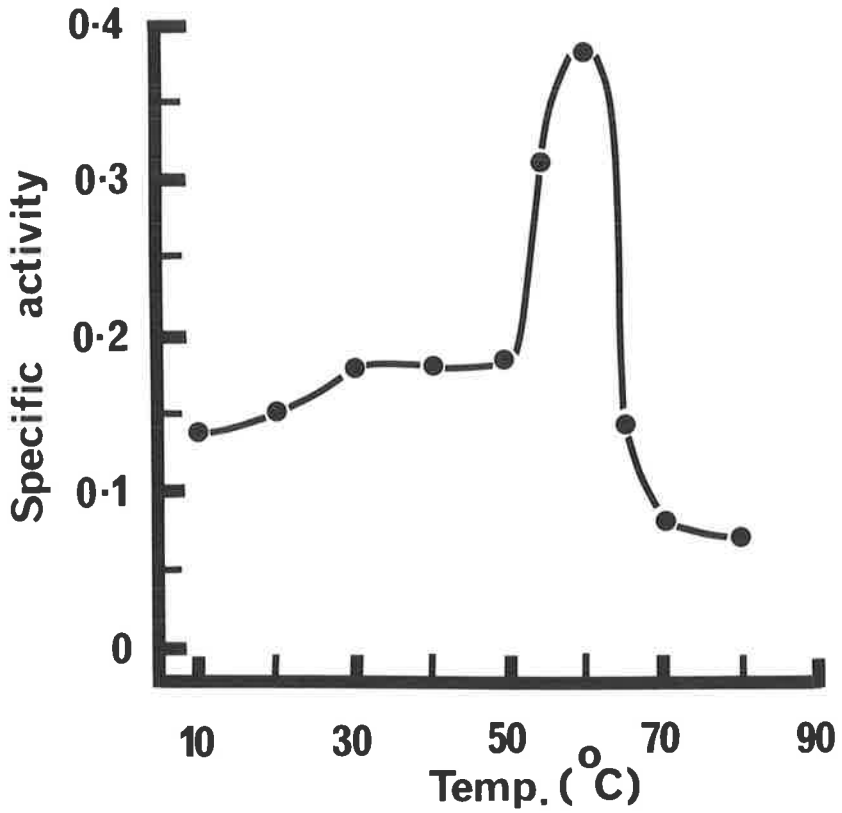


Figure 25a

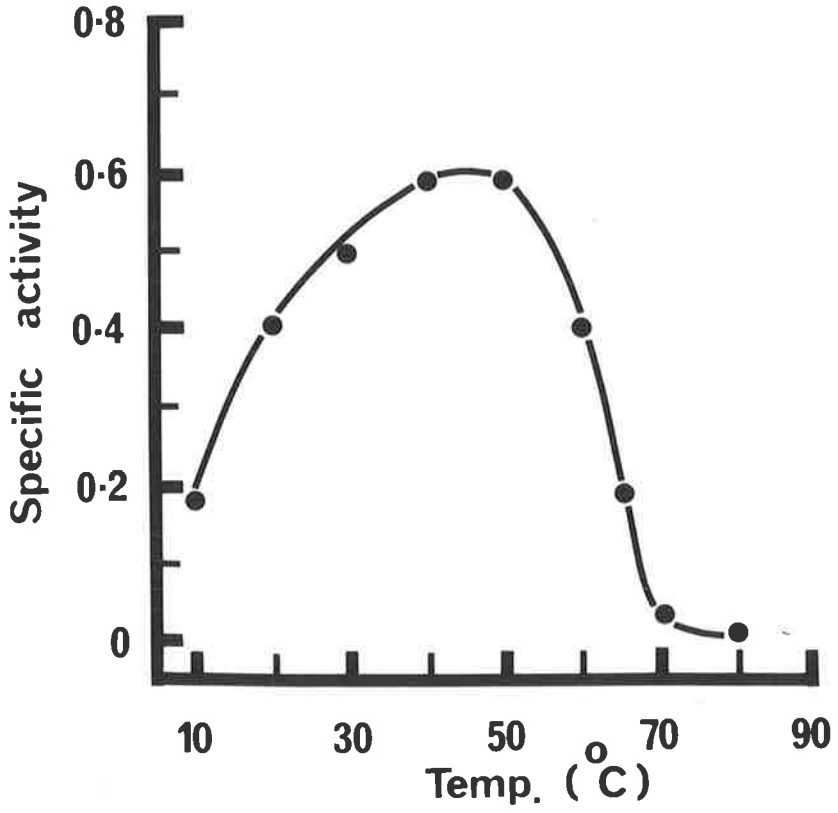


Figure 25b

FIGURE 26

Temperature stability of ATP-sulphurylase from bacteria grown on (a) ferrous-iron and (b) thiosulphate

ATP-sulphurylase was assayed by the static bioluminescence procedure as described in Section 2.8.10.5. The enzyme was incubated for up to 30 min at 30° (o——o) and 60° (●——●) in reciprocating water baths. Aliquots of the enzyme from the incubated samples at the two temperatures were taken at the specified time intervals and assayed under normal conditions at 30° (Section 2.8.10.5). a): Fraction V (Table 27) from ferrous-iron-grown bacteria (0.8 mg protein/ml incubation mixture). b): Fraction IV (Table 28) from thiosulphate-grown bacteria (0.5 mg protein/ml incubation mixture). (Specific activity : nmoles ATP produced/min/mg protein)

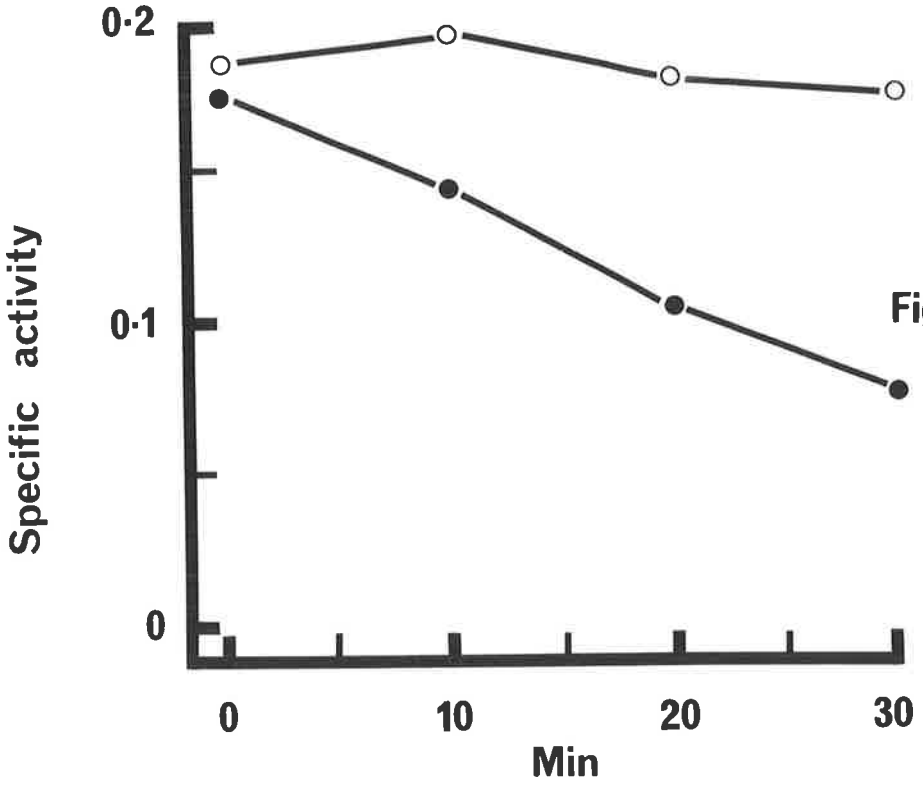


Figure 26a

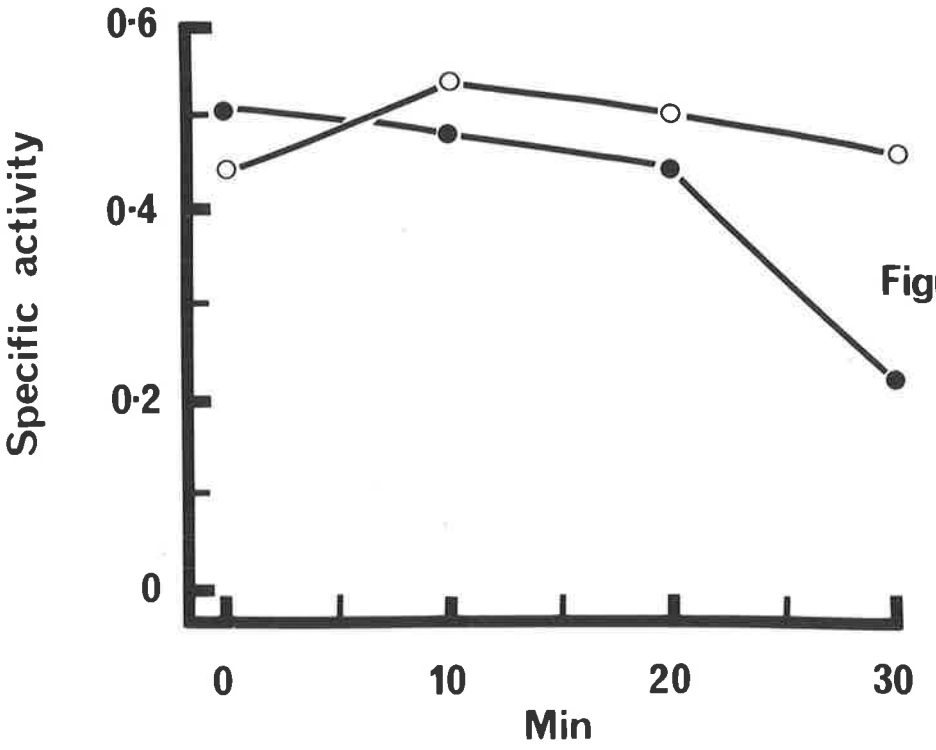


Figure 26b

FIGURE 27

Effect of pyrophosphate (PPi) concentration on ATP-sulphurylase from bacteria grown on ferrous-iron

The activity of ATP-sulphurylase (Fraction V, Table 27) was determined by the static bioluminescence procedure, outlined in Section 2.8.10.5, in the presence of varying amounts of pyrophosphate (0-1.25 µmoles/ml incubation mixture). The amount of APS was fixed at 0.25 mM. The results are presented as a) a velocity substrate curve and b) a Lineweaver-Burk plot. The K_m for PPi is 0.77 mM. (Specific activity : nmoles ATP produced/min/mg protein)

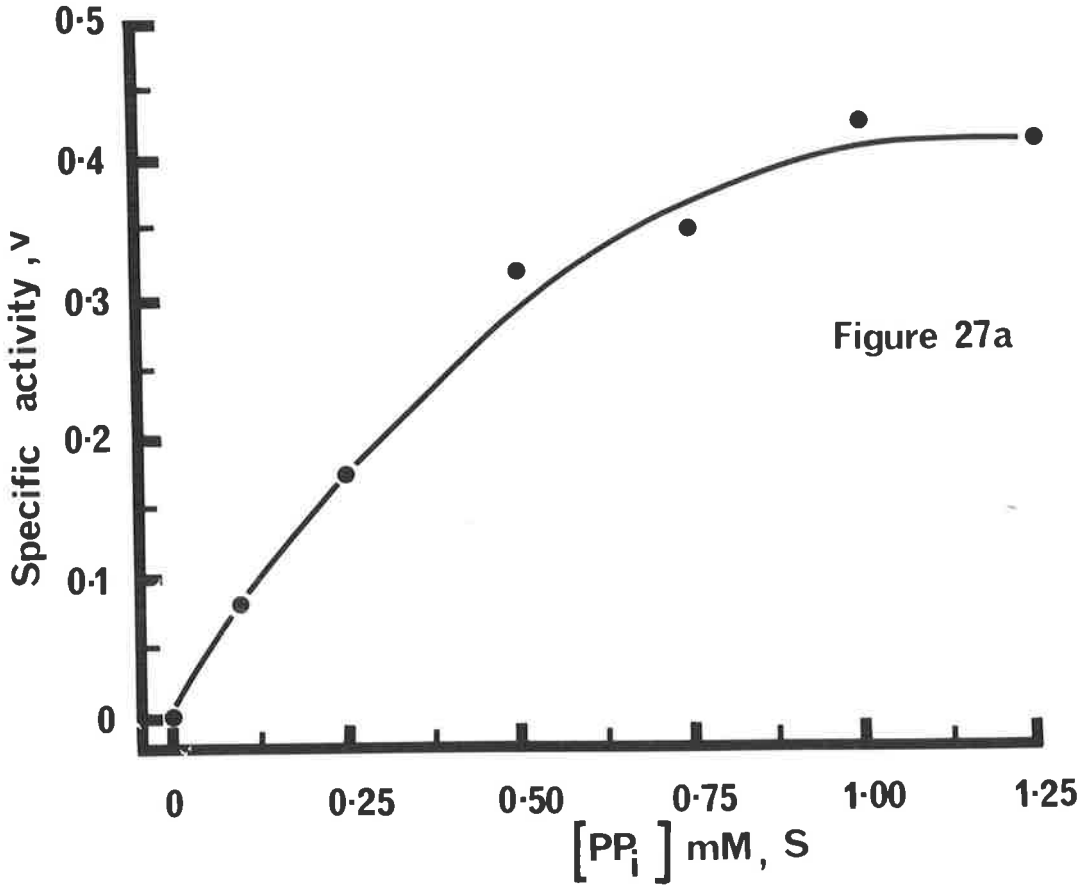


Figure 27a

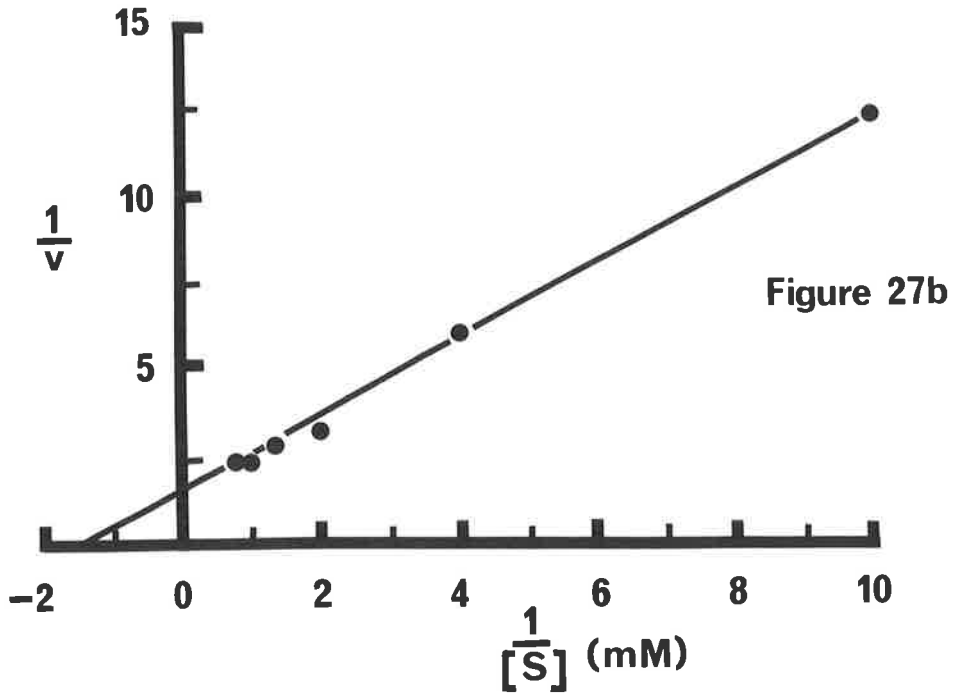


Figure 27b

FIGURE 28

Effect of pyrophosphate (PPi) concentration on ATP-sulphurylase from bacteria grown on thiosulphate

The activity of ATP-sulphurylase (Fraction IV, Table 28) was determined by the static bioluminescence procedure, outlined in Section 2.8.10.5, in the presence of varying amounts of pyrophosphate (0-1.25 μmoles/ml incubation mixture). The amount of APS was fixed at 0.25 mM. The results are presented as a) a velocity substrate curve and b) a Lineweaver-Burk plot. The K_m for PPi is 0.53 mM. (Specific activity : nmoles ATP produced/min/mg protein)

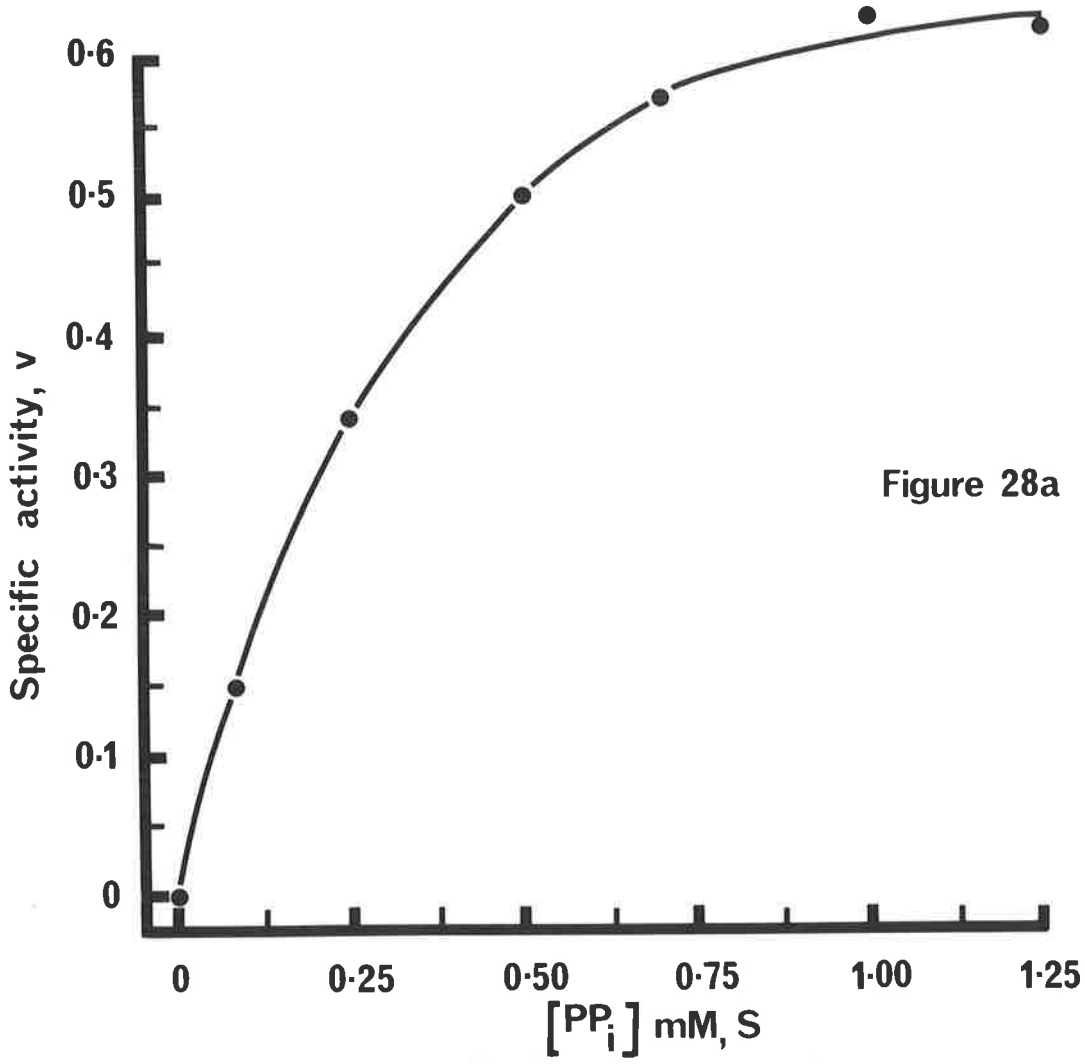


Figure 28a

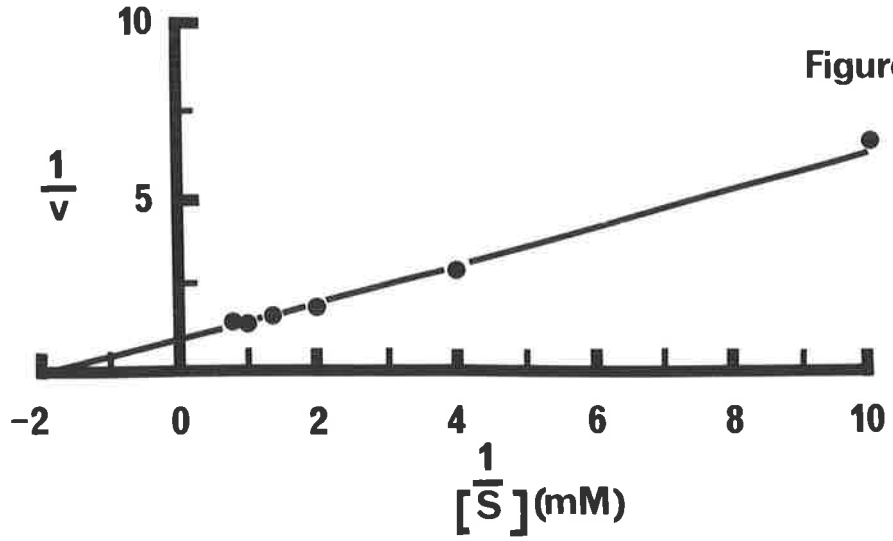


Figure 28b

4.2.3.7 Effect of APS

The rate of production of ATP was measured at various concentrations of APS using the partially purified ATP-sulphurylase preparations (Fraction V, Table 27; Fraction IV, Table 28). The enzyme from cells grown with ferrous-iron exhibited negative cooperativity as the APS concentration increased (pyrophosphate concentration fixed at 0.5 mM), as shown in Figures 29a and 29b. For this reason, estimation of the K_m for APS was difficult and a K_m value has not been presented. For the enzyme isolated from thiosulphate-grown cells, normal Michaelis-Menten kinetics were established (Figure 30a,b) and, at a fixed pyrophosphate concentration of 0.5 mM, the K_m for APS was estimated at 0.14 mM.

FIGURE 29

Effect of APS concentration on ATP-sulphurylase from bacteria grown on ferrous-iron

The activity of ATP-sulphurylase (Fraction V, Table 27) was determined by the static bioluminescence procedure, outlined in Section 2.8.10.5, in the presence of varying amounts of APS (0-1.25 µmoles/ml incubation mixture). Pyrophosphate was fixed at 0.5 mM. The results are presented as a) a velocity substrate curve and b) a Lineweaver-Burk plot. (Specific activity : nmoles ATP produced/min/mg protein)

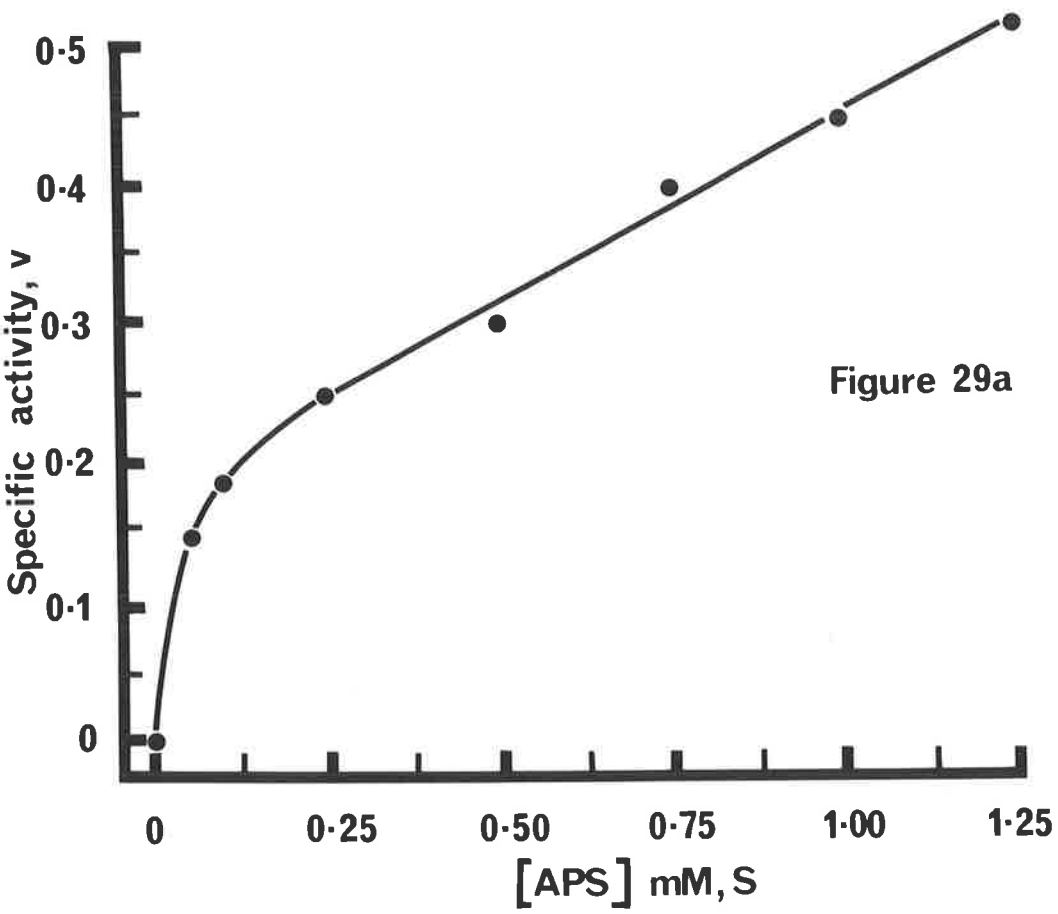


Figure 29a

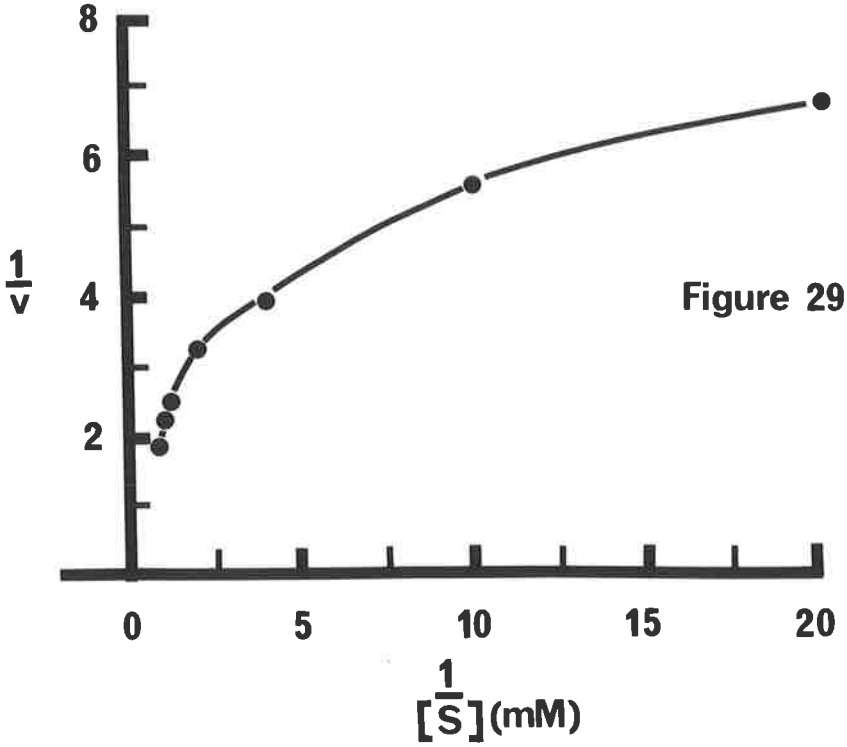


Figure 29b

FIGURE 30

Effect of APS concentration on ATP-sulphurylase from bacteria grown on thiosulphate

The activity of ATP-sulphurylase (Fraction IV, Table 28) was determined by the static bioluminescence procedure, outlined in Section 2.8.10.5, in the presence of varying amounts of APS (0-1.25 µmoles/ml incubation mixture). Pyrophosphate was fixed at 0.5 mM. The results are presented as a) a velocity substrate curve and b) a Lineweaver-Burk plot. (Specific activity : nmoles ATP produced/min/mg protein)

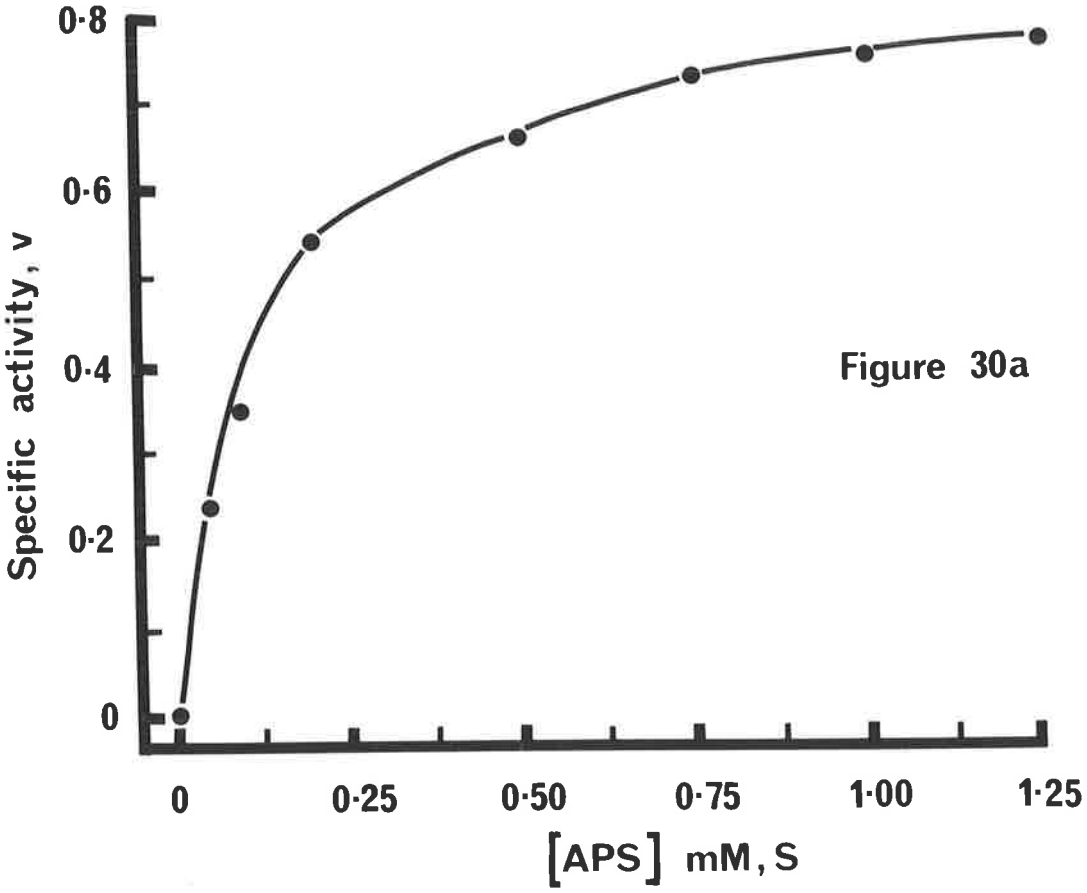


Figure 30a

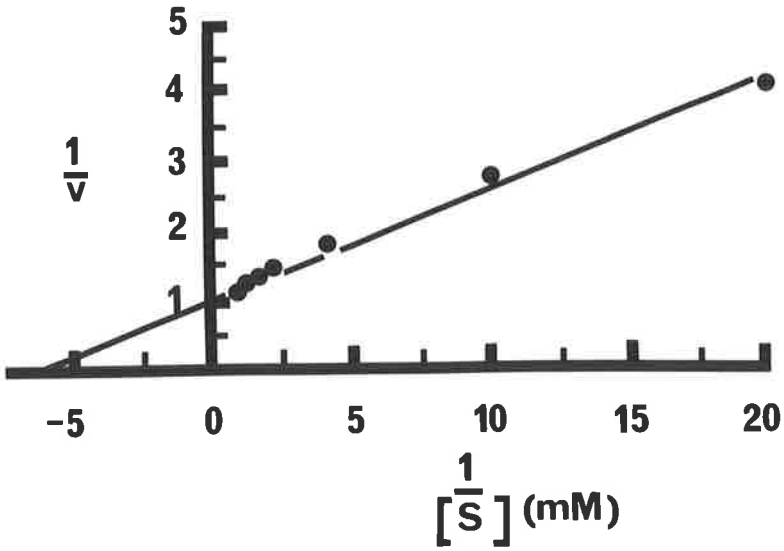


Figure 30b

4.3 Discussion

4.3.1 Utilization of ^{35}S-thiosulphate and ^{35}S-sulphate by *T. ferrooxidans* grown with thiosulphate

Although a great deal of work has centred around the two proposed pathways for the oxidation of thiosulphate to sulphate by thiobacilli, little is known about the thiosulphate uptake system or the subsequent fate of the two sulphur atoms of thiosulphate after its reductive cleavage, with particular reference to its assimilation into cellular materials.

Thiosulphate was readily taken up and incorporated into washed cells of *T. ferrooxidans* grown with thiosulphate. This uptake was independent of and preceded thiosulphate oxidation, since there was no difference in the pattern of uptake using thiosulphate labelled in either the outer or inner sulphur atom. The production of sulphate has been shown to be more rapid from the inner-labelled sulphur atom of thiosulphate than from the outer one during thiosulphate oxidation by *Thiobacillus* X (Trudinger, 1964c), *Chromatium* strain D (Smith, 1965) and *Thiobacillus* strain c (Kelly & Syrett, 1966). Thiosulphate was firmly bound by cells of *T. ferrooxidans* grown on thiosulphate, and only small amounts of label (4%) were removed by successive washings with a cold mineral salts solution and 0.01 N sulphuric acid. The uptake of thiosulphate was under pH control with a maximum at around pH 3.6, which is also the optimum pH for growth (Tuovinen & Kelly, 1974b). Thiosulphate spontaneously decomposes in acid solution (Moeller, 1952) but is stable above pH 3.5 (Tuovinen & Kelly, 1974b).

Thiosulphate uptake was optimal at 50°, which is higher than the growth temperature (30°). Above 50°, the growth of the bacterium was reduced.

The K_m for thiosulphate in the uptake system was 0.5 mM. Bounds & Colmer (1972) found that the K_m for thiosulphate during thiosulphate oxidation by *T. ferrooxidans* was 50 mM. This suggests a greater affinity of the uptake system for thiosulphate than for its subsequent oxidation. However, it is difficult to compare these interrelated studies because different strains of the bacteria may show quite distinct kinetic responses, as observed by Bounds & Colmer (1972). These workers have also reported variations in K_m values between different batches of cells of the same strain. Trudinger (1964*d*) has shown that during thiosulphate oxidation by *Thiobacillus X* the fate of the individual sulphur groups is influenced by changes in the oxygen tension during the growth of the organism as well as by the concentrations of substrate, cells and oxygen used in the assays. Thus, these factors, in addition to the previous growth history of the bacteria, must be carefully evaluated when comparing data for different organisms.

Dreyfuss (1964) has described a sulphate transport system in *Salmonella typhimurium* which functions in transporting thiosulphate. He postulates, however, that even though thiosulphate is a structural analogue of sulphate and a single system transports both ions, sulphate is the most likely "*in vivo*" substrate for the transport system in view of its dominant role in cysteine biosynthesis. Aspects of sulphate uptake and its control have been reviewed by Schiff & Hodson (1973).

In the present study, ^{35}S-sulphate was rapidly bound by thiosulphate-grown bacteria, as reported for those cells grown with ferrous-iron (Tuovinen *et al.*, 1975). Unlike labelled thiosulphate, however, ^{35}S-sulphate was completely removed from the cells grown with

thiosulphate, by washing with either 0.01 M sulphuric acid or 10% (w/v) trichloroacetic acid. In addition, sulphate was not taken up and incorporated by cells growing with thiosulphate.

In the dual sulphate-thiosulphate transport system described by Dreyfuss (1964), thiosulphate is a competitive inhibitor of sulphate uptake. Segel & Johnson (1961) have also shown a thiosulphate inhibition of sulphate accumulation in *Penicillium chrysogenum*. Sulphate may also affect thiosulphate utilization as described by Ragland & Liverman (1958), using the thiosulphate uptake system present in certain mutants of *Neurospora crassa*. In the present study, sulphate binding by thiosulphate-grown cells was almost completely inhibited by thiosulphate (20 mM). The inhibition of uptake systems may be more complex in the thiobacilli, where sulphate is the product of thiosulphate oxidation. The sulphate produced via this oxidation may be actively extruded from the cell in order to preserve the cellular pH balance and thus may affect the uptake of thiosulphate. It is likely that the transport system accumulating thiosulphate into the cell might also function to extrude sulphate from it.

The absence of a sulphate uptake system in *T. ferrooxidans* growing with thiosulphate is of interest in relation to the previous growth history of the bacterium. The thiosulphate-oxidizing culture was originally adapted from the ferrous-iron-grown culture (Tuovinen & Kelly, 1974b), which was subsequently shown to possess an active sulphate uptake system (Tuovinen *et al.*, 1975). The inability to demonstrate the latter uptake system in thiosulphate-grown cells may not be unequivocal evidence that it is absent. The adaptation of the ferrous-iron-grown bacteria to growth on thiosulphate may have suppressed or inhibited the sulphate uptake system, which is then not

apparent during thiosulphate oxidation. Indeed, only one transport site for sulphate and thiosulphate may be involved and its function may well be determined by the immediate growth conditions and the previous cultural history. This can be further investigated by transferring the thiosulphate-grown cells to ferrous-iron cultures, to see whether sulphate and thiosulphate uptake systems operate.

The pattern of incorporation of ^{35}S from differentially labelled thiosulphate into cellular material of cells grown on thiosulphate is consistent with the idea that the first step in thiosulphate oxidation is the cleavage of the S–S bond in the molecule, as proposed by Peck (1960,1962) and Peck & Fisher (1962). The present study demonstrates a discriminative incorporation of ^{35}S, depending on the position of the label within the thiosulphate molecule. The oxidative pathways of thiosulphate were not investigated. ^{35}S-Thiosulphate is probably cleaved to sulphite and sulphide, since the label was recovered in cellular materials only when thiosulphate was labelled in the outer position. The enzymic cleavage of thiosulphate has been widely investigated. Rhodanese activity has been found in *T. ferrooxidans* (Tabita *et al.*, 1969) and will be discussed in more detail later (see Section 4.3.2). Thiosulphate reductase activity has been reviewed by Roy & Trudinger (1970). Whether or not thiosulphate reductase and rhodanese are separate enzymes is not known. However, both enzyme activities function by cleaving thiosulphate.

There was virtually no incorporation of ^{35}S when the [inner-^{35}S]-thiosulphate was used as a substrate, suggesting that the source of cellular sulphur for the bacteria is from the sulphide moiety resulting from the enzymic cleavage of thiosulphate. The fate of sulphite produced from this cleavage has not been in doubt, since many workers

have noted a more rapid formation of sulphate from [inner-^{35}S]thiosulphate than from [outer-^{35}S]thiosulphate (Peck & Fisher, 1962; Trudinger, 1964*c*,*d*; Kelly & Syrett, 1966). Although most of the sulphide produced by the cleavage of thiosulphate would also be eventually oxidized to the level of sulphate (Peck & Fisher, 1962), the slower rate of oxidation of sulphide would probably result in its accumulation within the cell. Thus, Smith (1965) has reported that, during thiosulphate oxidation by *Chromatium* strain D, the radioactivity from [outer-^{35}S]thiosulphate accumulates intracellularly at a rate similar to that of thiosulphate cleavage. In the present study, since the incorporation of ^{35}S from [outer-^{35}S]thiosulphate into cellular materials was enhanced by *O*-acetylserine, the product of sulphur incorporation was probably cysteine. There was no incorporation of ^{35}S when cell extracts were provided with ^{35}S-sulphate, even in the presence of *O*-acetylserine. The results suggest that sulphate may not be assimilated in bacteria oxidizing thiosulphate, but rather that sulphur for cellular synthesis is derived from the outer sulphur moiety of thiosulphate.

4.3.2 Enzymic comparisons of the inorganic sulphur metabolism in autotrophically and heterotrophically grown cells

In this work, activities of a number of enzymes which mediate the oxidation of thiosulphate to sulphate and the assimilatory reduction of sulphate to sulphide were assayed with a view to establishing similarities and differences relating to the growth history of *T. ferrooxidans*.

Relatively high activities of rhodanese and 5'-AMP-independent sulphite oxidase were found in extracts of bacteria grown

with thiosulphate. This is further evidence that the first step in thiosulphate oxidation involves a cleavage of the S–S bond of the thiosulphate molecule, as discussed in Section 4.3.1. The high activity for the 5'-AMP-independent sulphite oxidase would be one route for the oxidation of sulphite to sulphate. Sulphide would be oxidized to sulphate, possibly via various intermediates, e.g. polysulphides as in *T. concretivorus* (Moriarty & Nicholas, 1969) and in *T. neapolitanus* (Saxena & Aleem, 1973). In the latter organism, the second stage of oxygen uptake, associated with the oxidation of polysulphide, is catalysed by the supernatant (S_{144}) fraction.

The thiosulphate-oxidizing enzyme may have been repressed by ferrous-iron in iron-grown *T. ferrooxidans*. This lack of activity correlates with previous observations (Tuovinen & Kelly, 1974b) that ferrous-iron-grown bacteria do not readily grow on thiosulphate, whereas the transition from growth on ferrous-iron to that on tetrathionate takes place without difficulty. In manometric and growth experiments, however, it has been shown (Tuovinen & Kelly, 1974b; Kelly & Tuovinen, 1975) that tetrathionate is formed during thiosulphate oxidation. Thus, it is reasonable to speculate that the initial product of thiosulphate oxidation is tetrathionate, but its further metabolism has yet to be determined. Other factors, such as the greater stability of tetrathionate at acid pH values and the lability of the thiosulphate-metabolizing system when bacteria are removed from thiosulphate cultures, should also be considered (Tuovinen & Kelly, 1974b).

The relatively high activity for the thiosulphate-oxidizing enzyme in heterotrophic *T. ferrooxidans* suggests that the enzyme is not induced by thiosulphate. It is possible that it is not essential

for thiosulphate oxidation since there is another route for its utilization via a cleavage to sulphide and sulphite. Indeed, rhodanese was detected in the glucose-grown bacteria. Similarly, in ferrous-iron-grown cells, even though the thiosulphate-oxidizing enzyme was not detected, the relatively high rhodanese activity suggests an alternative route for thiosulphate utilization. The subsequent fate of the products (sulphide and sulphite) of this cleavage of thiosulphate in ferrous-iron-grown cells warrants further investigation, since only low activities for 5'-AMP-independent sulphite oxidase and APS-reductase were recorded.

Preliminary scanning of the cytochromes showed that components *c* and *a* were present in cell extracts of both autotrophic and heterotrophic cells of *T. ferrooxidans*. Cytochromes of the c type act as electron acceptors for partially purified thiosulphate-oxidizing enzyme systems from *T. neapolitanus* (Trudinger, 1961) and from *T. novellus* (Aleem, 1965). Aleem (1965) has shown that thiosulphate oxidation is linked to molecular oxygen through cytochromes of the c and *a* types, with no evidence for the involvement of flavins or cytochrome *b*. Similarly, Blaylock & Nason (1963) consider that the electron transport system in *T. ferrooxidans* involves cytochromes of the c and *a* types only, in agreement with the present work.

The spectrophotometric method for determining APS-reductase activity proved unsuitable for use in the present study. It appears that it was largely the 5'-AMP-independent sulphite oxidase activity that was measured in preparations to which 5'-AMP had been added for the APS-reductase assay. Thus, APS-reductase was assayed using ^{35}S-sulphite and 5'-AMP, and results indicate that ^{35}S-labelled APS was produced at similar rates in comparable cell fractions of the bacteria

grown autotrophically or heterotrophically. High 5'-AMP-independent sulphite oxidase activity in thiosulphate- and glucose-grown cells was associated with the particulate fraction (P_{10}), as was found in *T. denitrificans* (Aminuddin & Nicholas, 1974). These high activities suggest that this enzyme has an important function in the oxidative metabolism of *T. ferrooxidans* and may be closely associated with rhodanese function. Only a very low activity of 5'-AMP-independent sulphite oxidase was recorded in ferrous-iron-grown bacteria, where an active sulphate uptake and activation system has been demonstrated (Tuovinen *et al.*, 1975).

The production of ATP from APS via ATP-sulphurylase or by a combination of ADP-sulphurylase and adenylate kinase was similar in cells grown autotrophically or heterotrophically. In general, the enzymes mediating the latter pathway had higher specific activities. This observation suggests that ATP-sulphurylase contributes little to the substrate-level phosphorylation, whereas its reverse reaction to activate sulphate to APS at the expense of ATP is essential for bacteria grown on either ferrous-iron or glucose. The link between ATP-sulphurylase and ADP-sulphurylase during the substrate-level phosphorylation is likely to be influenced by inorganic pyrophosphatase activity, previously described in *T. ferrooxidans* (Howard & Lundgren, 1970).

A relatively high sulphite reductase activity has recently been reported in extracts from *T. denitrificans* (Schedel *et al.*, 1975), although its physiological role is uncertain. In the present work, only very small activities of NADPH-linked sulphite reductase were recorded in cell extracts of heterotrophic and autotrophic *T. ferrooxidans*. The higher activity of this enzyme recorded in the low-speed pellet fraction of thiosulphate-grown cells has already been discussed

(see Section 4.2.2.4). Schedel *et al.* (1975) suggest that the sulphite reductase from *T. denitrificans* may take part in the overall oxidation of reduced sulphur compounds. A similar role may be postulated for sulphite reductase from thiosulphate-grown *T. ferrooxidans*, where sulphate assimilation may not occur and would be energetically expensive if it did. The reductive pathway of sulphur compounds leading to sulphur incorporation into cysteine remains to be elucidated in bacteria which oxidize reduced sulphur compounds, e.g. thiobacilli.

The present results confirm and extend the recent observations of Guay & Silver (1975) on the comparative metabolism of inorganic sulphur compounds in autotrophic and heterotrophic *T. ferrooxidans*. These workers propose that the heterotrophic bacteria derived from *T. ferrooxidans* be assigned to a new species, *Thiobacillus acidophilus*, which also grows on sulphur compounds but not on ferrous-iron. They also suggest that this new species was isolated by means of a selection rather than an adaptation. This conclusion is based on DNA analysis, enzyme studies and cultural requirements of the new species.

The results of the present study suggest that the enzymes involved in intermediary sulphur metabolism play no regulatory role in the transition between growth from one substrate to another. Rather, the transition from autotrophy to heterotrophy is likely to be dependent on the regulation of the carbon metabolism, in particular the Calvin cycle, which has been shown to be repressed during heterotrophic growth (Tabita & Lundgren, 1971*a*,*b*,*c*).

4.3.3 A comparison of the properties of partially purified ATP-sulphurylase from cells grown with ferrous-iron and with thiosulphate

The properties of ATP-sulphurylase in bacteria grown with either thiosulphate or ferrous-iron are very similar. When grown with ferrous-iron, ^{35}S-sulphate was actively taken up by the cells, and the presence of labelled APS within the cells (Tuovinen *et al.*, 1975) demonstrates that the function of ATP-sulphurylase is primarily to activate sulphate. Indeed, this is the only source of sulphur available for synthesis of cellular meterials. However, when the bacteria were grown with thiosulphate, externally supplied sulphate was not taken up and thus an alternative source of sulphur is required. This is provided by thiosulphate, which cleaved to produce sulphide, which in turn is incorporated into cellular materials. Since sulphate was not assimilated in bacteria oxidizing thiosulphate, the ATP-sulphurylase from these bacteria, which has similar properties to that from ferrous-iron grown cells, may not be required to activate sulphate to APS. Indeed, the process of sulphate activation and assimilation would be unnecessary in thiosulphate-grown cells because of the readily available source of sulphur for cellular synthesis from the cleavage of thiosulphate. Such an assimilatory process would require ATP for both sulphate activation and sulphite reduction, in the latter case to produce NADH by reverse electron flow (Aleem *et al.*, 1963).

The ATP-sulphurylase of *T. ferrooxidans* may be a constitutive enzyme; this aspect would be advantageous to the bacteria when growing in an environment in which thiosulphate was depleted but ferrous-iron was available. Under these conditions, sulphate would be readily

assimilated via the constitutive ATP-sulphurylase already present in the cells, without resorting to the longer process of enzyme induction required of adaptive enzymes. Other enzymes described previously (Section 4.3.2), e.g. APS-reductase, have comparable activities in cells grown with ferrous-iron, thiosulphate or glucose. The presence of these enzymes in bacteria with different growth histories may suggest that they are, like ATP-sulphurylase, constitutive.

The contribution of ATP-sulphurylase to the overall energy balance of the cell is not clear. The equilibrium for the reaction catalysed by this enzyme is strongly in favour of ATP production. The apparent equilibrium constant was found to be of the order of 10^{-8} (Robbins & Lipmann, 1958b; Akagi & Campbell, 1962). Thus, in thiosulphate-grown cells, a possible function of the enzyme would be to produce ATP, although this would require an adequate supply of pyrophosphate within the cell. Such a supply would be unlikely to be derived via a pyrophosphatase, since the reaction would be energetically unfavourable. Indeed, *in vitro* studies have shown that ATP production by ATP-sulphurylase requires the addition of pyrophosphate. The operation of this enzyme would also be dependent on a supply of 5'-AMP, which is required with sulphite for the production of APS, mediated by the APS-reductase enzyme.

A fully functional APS-reductase/ADP-sulphurylase/adenylate kinase pathway would require an adequate supply of ADP within the cell. The utilization of ADP by the adenylate kinase reaction would provide 5'-AMP, a substrate required for APS production by APS-reductase. Apart from supplying ATP for processes such as carbon dioxide fixation and the generation of reduced pyridine nucleotides, the substrate-level phosphorylation pathway as described by Peck (1968) would prevent a

build-up of 5'-AMP, thus alleviating the potential inhibition of carbon dioxide fixation by 5'-AMP, which may compete with the normal substrate ATP for the phosphoribulokinase enzyme (Gale & Beck, 1966). In addition, the inhibition of carbon dioxide fixation by 5'-AMP may represent an important control mechanism for temporarily maintaining a level of ATP within the cell in the absence of an oxidizable substrate (Johnson & Peck, 1965; Peck, 1968). Alternatively, if 5'-AMP and ATP accumulate, the adenylate kinase enzyme may function to produce ADP. The adenylate energy charge may provide useful information in this context.

The contributions to the energy balance of the cell by oxidative phosphorylation and substrate-level phosphorylation remain to be investigated. The two processes may act independently to produce ATP during inorganic substrate oxidation or may be coordinated to operate either simultaneously or under some form of regulation. Energy production may be controlled at the level of sulphite oxidation. In the absence of 5'-AMP, the energy requirements for growth and carbon dioxide fixation would be met by the oxidative phosphorylation pathway. If 5'-AMP is available, substrate-level phosphorylation would provide a way of conserving energy during the oxidation of inorganic substrates, probably in association with oxidative phosphorylation.

5. BIBLIOGRAPHY

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6. APPENDICES

APPENDIX I

Computer program RENILLA (FORTRAN listing)

[developed by Dr. P.E. Stanley, Mr. P.J. Malcolm and Mr. B.C. Kelley]

APPENDIX II

Reprints of published papers

```
ATTACH(FTNLIB,MR=1)
FTN(L=0)
LIBRARY(FTNLIB)
LGO.
      PROGRAM RENILL(INPUT,OUTPUT)
      DIMENSION XTITLE(7), XX(25)
      DIMENSION SUM(612),VOLS(20)
      DIMENSION BTITLE(7), BX(25) ,STITLE(2),ACTNL(20)
      COMMON TIMES(20), SEB
5     FORMAT(1H0)
100   CONTINUE
      READ 33, NBLANK,BTITLE, NB
33    FORMAT(I2,7A10,I2)
      IF(BTITLE(1) EQ 10H          ) STOP " RENILLR COMPUTATION "
      IF(NB.LE.0) NB=5
      IF(NBLANK.LE.0) NBLANK=1
      READ 3, (BX(I),I=1,NB)
      CALL REG(NB,BX,BLANK,1)
      DO 34 KK=1,NBLANK

      PRINT 30
      PRINT 30
      PRINT 11, BTITLE,NB
11    FORMAT(1H0,5X,*BLANK DATA*,/,5X,7A10,/,5X,I2,* DATA POINTS*)
      PRINT 12, (BX(I),I=1,NB)
12    FORMAT(5X,12F8.0)
      IF(NB.NE.2) GO TO 202
      PRINT 203, BLANK
203   FORMAT(5X,*SLOPE = *,F9.2)
      GO TO 204
202   PRINT 13, BLANK,STD
13    FORMAT(5X,*SLOPE = *,F9.2,* (PER CENT STD. ERROR = *,F5.2,*)*)
204   PRINT 30


      READ 20, NSET, PROT, STD, NGRAPH, M,N
      TEST=50.0
      IF(NSET.LE.0) NSET=1
      IF (PROT.LE.0.0) PROT=TEST=1.0
      IF (STD.LE.0.0) STD=120.0
      READ 40, STITLE
40    FORMAT(2A10)
20    FORMAT(I2,2F8.0,3I2)
      IF(M.EQ.0) M=5
      IF(N.EQ.0) N=5
      IF(NSET.LE.1) GO TO 42
      READ 21,(TIMES(I),I=1,NSET)
42    READ 21,(VOLS(I),I=1,NSET)
      PRINT 22, (VOLS(I),I=1,NSET)
22    FORMAT (* *,20F5.0)
21    FORMAT(20F3.0)
      NCOUNT=1
      DO 29 JJ=1,NSET
      ACTNL(JJ)=0.0
      READ 1, XTITLE
1     FORMAT(7A10,I2)
      READ 3, (XX(I),I=1,N)
3     FORMAT(16F5.0)
      CALL REG(N,XX,SLOPE1,1)
      NCOUNT=NCOUNT+1
      IF(NCOUNT.NE.5) GO TO 17
      PRINT 5
```

```
      NCOUNT=1
   10 PRINT 4, XTITLE, N
    4 FORMAT(2X,7A10,//,2X,'NUMBER OF DATA POINTS = ',I2)
      PRINT 12, (X(I),I=1,N)
      IF(N.NE.2) GO TO 206
      PRINT 207, SLOPE1
      GO TO 207
  206 PRINT 13, SLOPE1,SE2
  207 CONTINUE
      READ(3,*)(X(I),I=1,N)
      CALL REG(N,X,SLOPE2,I)
      PRINT 6,N
    6 FORMAT(2X,'THE INTEGRAL STD  ORDER OF ',I2,' POINTS WITH VALUES
      PRINT 12, (X(I),I=1,N)
      IF(N.NE.2) GO TO 209
      READ (99),SLOPE2
      GO TO 210
  209 PRINT 13, SLOPE2,SE2
  210 CONTINUE
      SLOPE1=SLOPE1-SLOPE
      SLOPE2=SLOPE2-SLOPE
      IF(SLOPE1.LE.0.02) GO TO 14
      RATIO=SLOPE1/(SLOPE2-SLOPE1)
      PRINT 7, SLOPE1,SLOPE2,RATIO
    7 FORMAT  (/2X,'INITIAL SLOPE = ',F14.2,/,2X,'INT. STD. SLOPE = ',
     1F 12.2,/,2X,'RATIO OF SLOPES = ',F12.5)
      GO TO 9
   14 PRINT 17, SLOPE1
   17 FORMAT(2X,'INITIAL SLOPE NOT SIG. DIFF  FROM BLANK',/,2X,
     1'INTERNAL STANDARD SLOPE = ',F12.6)
      ACTUAL(J0)=0.0
      PRINT 30
      GO TO 34

    9 CONTINUE
      ACT=RATIO*STD
      PRINT 24, PCI
   24 FORMAT(2X,'THIS REPRESENTS ',G7.1,' PICOMOLES PER ML IN THIS SAMPLE
      ACTUAL(J0)=ACT*1.0E03/VOLS(J0)
      PRINT 26,ACTUAL(J0)
   26 FORMAT (1H ,25X, ' OR ',F10.1,' PICOMOLES PER ML',///)
      IF(UNIT.NE.1) GO TO 27
      PRINT 30,
   30 FORMAT(/,//)
      GO TO 34
   22 CONTINUE
      IF (TEST.NE.1.0) GO TO 47
      PRINT 48, QTITLE
      GO TO 34
   47 CONTINUE
      IF(CHANNEL.LE.0) GO TO 44
      PRINT 11, QTITLE
   43 FORMAT(1H1,20X,6A10)
      BUF(1)=BUF(2)=0.0
      BUF(3)=BUF(4)=BUF(5)=-1000.
      CALL LPBRIP(BUF,1,1)
      CALL LPLINE(BUF,TIMES,ACTUAL,NSET,IR,)
      CALL LPPLOT(BUF,TIMES,,CTRL,NSET,1,ENN)
      CALL LPOUT (BUF,'A=TIME',10X,'ACTIVITY',1,55)
      GO TO 16
   44 PRINT 45, QTITLE
   45 FORMAT(2X,6A10)
   46 IF(NSET.LE.0) GO TO 215
      CALL SPS(NSET,ACTUAL,SLOPE3,2)
      SLOPE3=SLOPE3/FOOT
      PRINT 214, SLOPE3
```

```
214   FORMAT(1X,*SPECIFIC ACTIVITY = *,F7.2,* PICOMOLES PER MINUTE PE
     1R MG. PROTEIN*)
      GO TO 102
215   CONTINUE
      CALL REG(NACT,ACTBL,SLOPE3,2)
      SLOPE3=SLOPE3/PROT
      SED=SED/PROT
      PRINT 27,SLOPE3,SED
27    FORMAT(1X,*SPECIFIC ACTIVITY = *,F7.2,* (S.E. = *,F7.2,
     1*) PICOMOLES PER MINUTE PER MG. PROTEIN*)
      PRINT 28
28    FORMAT(1H+,87X,*(ALL POINTS USED)*)
      CALL REG(3,ACTBL,SLOPE4,2)
      SLOPE4=SLOPE4/PROT
      SED=SED/PROT
      PRINT 27,SLOPE4,SED
      PRINT 29
29    FORMAT(1H+,87X,*(USING FIRST 3 POINTS)*)
102    CONTINUE
54    CONTINUE
      GO TO 100
      END
      SUBROUTINE REG(N,X,SLOPE,L)
      DIMENSION X(25), Y(25)
      COMMON TIMES(20), SED
      SED=50
      YMEAN=0.0
      SUMX=0.0
      IF(L.GT.1) GO TO 3
      DO 1 I=1,N
      Y(I)=FLOAT(I)
      YMEAN=YMEAN+Y(I)
      SUMX=SUMX+X(I)
1     CONTINUE
      GO TO 5
3     CONTINUE
      DO 4 I=1,N
      Y(I)=TIMES(I)
      YMEAN=YMEAN+Y(I)
      SUMX=SUMX+X(I)
4     CONTINUE
5     CONTINUE
      XMEAN=SUMX/FLOAT(N)
      YMEAN=YMEAN/FLOAT(N)
      SUM=0.0
      YSQ=0.0
      XSQ=0.0
      DO 2 I=1,N
      XX=X(I)-XMEAN
      YY=Y(I)-YMEAN
      SUM=SUM+(XX*YY)
      YSQ=YSQ+(YY*YY)
      XSQ=XSQ+(XX*XX)
2     CONTINUE
      SLOPE=SUM/YSQ
      IF(L.EQ.2) RETURN
      VAR=(XSQ-SUM*SUM/YSQ)/(FLOAT(N)-2.0)
      SED=SQRT(VAR/YSQ)
      IF(L.EQ.1) SED=SED*100./SLOPE
      RETURN
      END
```

ANALYTICAL BIOCHEMISTRY 67, 540–551 (1975)

A Bioluminescence Method for Determining Adenosine 3′-Phosphate 5′-Phosphate (PAP) and Adenosine 3′-Phosphate 5′-Sulfatophosphate (PAPS) in Biological Materials

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A rapid, sensitive, bioluminescence technique for detecting PAPS (adenosine 3′-phosphate 5′-sulfatophosphate) in biological materials is described. PAPS is first hydrolysed in 0.2 N HCl to PAP (adenosine 3′-phosphate 5′-phosphate) and is then assayed by the luciferin–luciferase system of the sea pansy, *Renilla reniformis*, which is specific for PAP. This bioluminescence system produces light at a rate that is proportional to the amount of PAP present. Light emission is measured in a liquid scintillation spectrometer with the two photomultipliers out of coincidence.

Very low amounts of PAPS (10–100 pmoles) have been determined in extracts of yeast and various plant tissues by this method. The production of PAPS in extracts of young wheat leaves is enhanced by including either 5′-AMP or 3′-AMP in the reaction mixture. It is possible that these nucleotides protect PAPS from enzymes that degrade this compound, e.g., a nucleotidase.

The activation of sulfate to PAPS (adenosine 3′-phosphate 5′-sulfatophosphate) was first characterized in yeast and is mediated by the following enzymes (1–6):

$$ATP + SO_4 \xrightarrow{Mg^{2+}} APS + PP_i$$

ATP-sulfurylase: ATP:sulfate adenylyltransferase (EC 2.7.7.4);

$$APS + ATP \xrightarrow{Mg^{2+}} PAPS + ADP$$

APS-kinase: ATP:adenylylsulfate 3′-phosphotransferase (EC 2.7.1.25).

It has recently been demonstrated that small amounts of PAPS are produced from sulfate and ATP in isolated spinach chloroplasts (7). APS kinase activity was measured by linking PAPS production to the

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synthesis of naphthol 2-sulfate in the presence of purified phenol sulfotransferase. A sensitive coupled enzyme system for the determination of PAP (adenosine 3′-phosphate 5′-phosphate) has been described for sulfate transfer amongst phenolic compounds (8).

The production of PAPS can also be demonstrated by the use of radioactive labeling techniques. When extracts of chloroplasts from *Zea mays* and *Phaseolus vulgaris* were incubated with ATP and $^{35}SO_4^{2-}$ or [^{35}S]APS of high specific activity, a 2% conversion to PAPS was obtained (9). The product [^{5}S]PAPS was identified by standard electrophoretic and radiotracer methods. Similarly, PAPS synthesis has been demonstrated in cell-free preparations of *Chlorella* (10–12). However, many workers have found radioisotope techniques insufficiently sensitive to detect picomole amounts of PAPS produced in extracts of higher plants (13–16).

The luciferin–luciferase bioluminescence system of the sea pansy, *Renilla reniformis*, is sensitive and specific to PAP (17,18). It also offers a convenient and specific method for assaying PAPS. In the assay, PAPS is hydrolysed to PAP and assayed in that form. This luciferin–luciferase system produces light at a rate that is proportional to the amount of PAP present (19) and is mediated by the following enzymes:

$$\text{PAP} + \text{luciferyl sulfate} \xrightarrow{\text{luciferin sulfokinase}} \text{luciferin} + \text{sulfate};$$

$$\text{luciferin} + O_2 \xrightarrow{\text{luciferase}} \text{products} + h\nu\ (\lambda_{max},\ 485\ \text{nm}).$$

We now report on a suitable method employing the luciferin–luciferase bioluminescence system of *Renilla reniformis* for measuring PAPS in extracts of yeast and higher plants.

MATERIALS AND METHODS

Chemicals

3′-AMP, 5′-AMP, ADP, ATP, adenosine and adenine (grade A) were obtained from Sigma Chemical Co. (St. Louis, MO); PAP from Boehringer Mannheim GmbH (Tutzing, West Germany). Sodium sulfite (anhydrous) was from By-Products and Chemicals Pty. Ltd. (Alexandria, N.S.W., Australia); anhydrous $Na_2H_2P_2O_7$ and $NH_4H_2PO_4$ were purchased from British Drug Houses Ltd. (Poole, England). All other reagents used were of Analar grade.

Baker's yeast (*Saccharomyces cerevisiae*) was obtained as frozen blocks (Pinnacle) from Mauri Bros. and Thompson (Adelaide, S.A., Australia) and was stored at −15°C before use.

[^{35}S]PAPS (155 μCi/100 μmoles) was purchased from New England

Nuclear (Boston, MA). Carrier-free $^{35}SO_4^{2-}$ was obtained from The Radiochemical Centre (Amersham, U.K.). [^{35}S]APS was prepared as described previously (20).

The acetone powder of *Renilla reniformis* and luciferyl sulfate (21) were generously donated by Professor Milton Cormier, Athens, GA.

Preparation of Enzymes

Luciferin–luciferase system. Ten grams of the acetone powder of *Renilla reniformis* was added to 40 ml of cold 0.1 M potassium phosphate buffer (pH 7.5) containing 1 mM Na-EDTA and 1 mM β-mercaptoethanol and stirred continuously for 2 hr at 2°C. The resulting slurry was centrifuged at 35,000g for 20 min and the precipitate discarded. The supernatant fraction, dialysed at 2°C against four 1-liter changes of 0.01 M potassium phosphate buffer (pH 7.5) containing 1 mM Na-EDTA and 1 mM β-mercaptoethanol, was then centrifuged at 35,000g for 20 min. The luciferin sulfokinase and luciferase activities were retained in the supernatant fraction. The PAP content of this preparation was reduced by passing it through a Sephadex G-25 column equilibrated with the same buffer used for dialysis. The enzyme system was stable over several months when stored at −15°C.

Cell-free extracts of plant materials. The plant materials used in this study were as follows: (i) Shoots of wheat (*Triticum vulgare* cv. Insignia) and of sorghum (*Sorghum bicolor* × *Sudan grass* hybrid) grown in the dark for 4 days at 28°C following germination; (ii) green leaves of spinach (*Spinacea oleracea*) obtained from a local garden; (iii) explants from artichoke tuber (*Helianthus tuberosus*) grown for 48 hr at about 20°C according to the procedure of Yeoman and Evans (22) and kindly supplied by Dr. J. F. Jackson of this department.

Cell-free extracts were prepared by freezing 10 g of the fresh plant material in liquid nitrogen, pulverising in a top-drive homogeniser (Sorvall Omni-mixer, Ivan Sorvall Inc., Newton, CT) and extracting in 40 ml of 0.1 M potassium phosphate buffer (pH 7.5) containing 1 mM Na-EDTA and 1 mM β-mercaptoethanol. The supernatant fraction, prepared by centrifuging the extract at 20,000g for 40 min, was used for enzyme assays.

A cell-free extract (S_{60}) from baker's yeast (*S. cerevisiae*) was prepared by a method described previously (23).

Synthesis of PAPS and PAP

The reaction mixture for the production of PAPS contained per milliliter: 25 μmoles of ATP, 100 μmoles of Tris–HCl buffer (pH 8.5), 20 μmoles of $MgCl_2$, 40 μmoles of Na_2SO_4 and 0.5 ml of the extract. After

incubating in a reciprocating water bath for various periods up to 1 hr at 30°C, 0.1-ml aliquots were removed and hydrolysed in two volumes (0.2 ml) of 0.2 N HCl at 37°C for 30 min and then stored on ice.

Determination of PAP

A standard scintillation vial containing 2 ml of 0.01 M potassium phosphate buffer (pH 7.5), 1 mM Na-EDTA and 1 mM β-mercaptoethanol was equilibrated at 20°C for 15 min in the liquid scintillation spectrometer. After equilibrating, 150 pmoles of luciferyl sulfate (0.5 μl) in 0.01 M potassium phosphate buffer (pH 7.5) containing 50% ethanol and 50 μl of the *Renilla* enzyme preparation was added. The vial was shaken and quickly placed in the well of the spectrometer. Five counts, each of 0.1 min, were recorded at intervals of 0.3 min. The vial was unloaded and an aliquot (5–20 μl) of the hydrolysate containing PAP was added to the vial which was shaken and lowered into the spectrometer. A further five counts, each of 0.1 min, were recorded at intervals of 0.3 min. The vial was again unloaded and an internal standard of 120 pmoles of purified PAP was added in a volume of 2 μl. The same counting sequence was repeated.

All procedures were carried out in a room lit only with tungsten lights so as to avoid problems of phosphorescence in glass scintillation vials.

The assay was standardised by adding graded amounts of PAP (1–150 pmoles), instead of the hydrolysate, to vials containing luciferyl sulfate and the *Renilla* enzyme extract.

All measurements were made in the Model 3375 Packard Tri-Carb liquid scintillation spectrometer with the two photomultipliers switched out of coincidence (24,25). One channel was used at 100% gain with the two discriminators set at 75 and 300, respectively.

A computer program, RENILLA, has been developed to analyse the data output from this bioluminescence assay. The program prints out quantitative results for PAP, as well as the specific activities for the enzymic production of PAPS in extracts of biological materials and presents these in a graphical form. The FORTRAN listing of the program is available on request.

High-Voltage Paper Electrophoresis

Labeled sulfur nucleotides were separated on 3 MM Whatman paper by high-voltage electrophoresis at room temperature (26). The electrophoresis was maintained at 1500 V for 1 hr in 0.1 M citrate buffer (pH 5.0).

Nucleotides were detected on the dried paper by UV absorption. Labeled compounds were detected by scanning the paper in a Packard 7201 radiochromatogram unit.

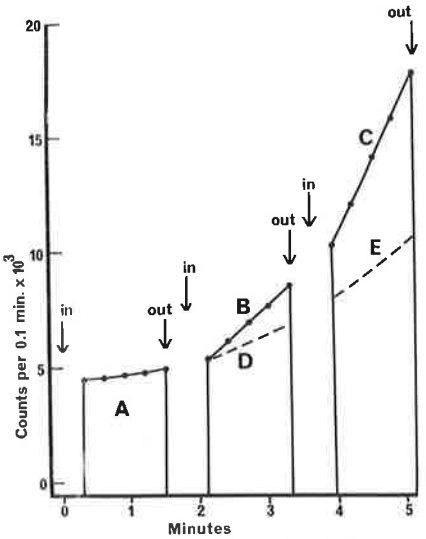

FIG. 1. Reaction sequence for the determination of PAP by *Renilla* luciferin–luciferase system. The details of the bioluminescence assay have been outlined in Materials and Methods.

RESULTS

Quantitative Measurement of PAP

The reaction sequence for determining PAP is shown in Fig. 1. The small initial light output in the blank (Section A, Fig. 1) was due to endogenous PAP present in the *Renilla* enzyme preparation. PAP was removed from this sample by passing it through a Sephadex G-25 column, so that the light emission in Section A was reduced.

An internal PAP standard (120 pmoles) was always included in the assays to check for any variations which might affect light output. The addition of large volumes of sample (>50 μl) decreased the light output by the internal standard (Fig. 1, Sections D,E), presumably because of temperature changes since the test samples were stored on ice. Thus, the test aliquots used were always 20 μl or less.

A calibration curve for PAP against light output is given in Fig. 2. As few as 10 pmoles of PAP can be accurately measured by this procedure, as reported in a preliminary communication (27) and a review (28).

Stability of PAP

There was no degradation of PAP when samples containing graded amounts of this compound were hydrolysed in two volumes of 0.2 N HCl at 37°C for up to 1 hr. Conversion of PAPS to PAP was complete

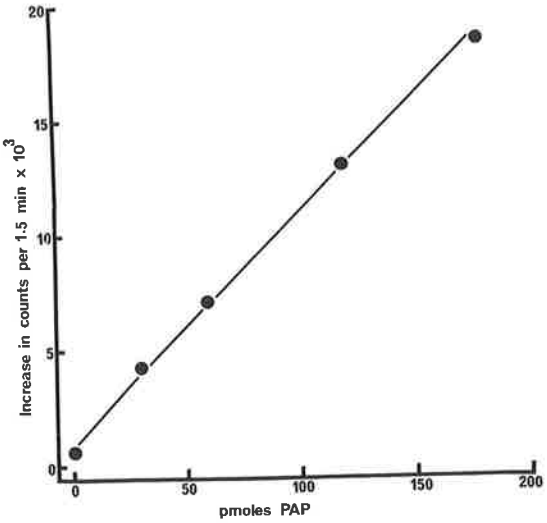

FIG. 2. A calibration curve for PAP. The assay was standardised by adding graded amounts of PAP (0–150 pmoles), instead of the hydrolysate, to a scintillation vial containing the luciferyl sulfate and the *Renilla* enzyme extract. Five counts, each of 0.1 min at 0.3-min intervals, were taken for each level of PAP used.

TABLE 1
STABILITY OF PAP IN EXTRACTS OF YEAST[a]

Treatment	Sampling time (min)			
	0	15	30	45
	(nmoles of PAP/ml of incubation mixture)			
Extract	1.57	0	0	0
Extract + 3′-AMP	0.15	0	0	0
Extract + 5′-AMP	2.59	0.86	0.51	0.38
Extract + ADP	6.77	1.15	0.48	0.69
Extract + ATP	1.90	0.55	0.60	0.64
Extract + P_i	1.66	0	0	0
Extract + PP_i	1.37	0	0	0

[a] The reaction mixtures contained (in μmoles): $MgCl_2$ (20); nucleotides (20); P_i (20); PP_i (20) and yeast extract (21.7 mg of protein) in a total volume of 0.62 ml. After preincubating at 30°C for 1 min, the reaction was initiated by adding PAP (900 pmoles). Samples (0.1 ml) were taken at 0, 15, 30 and 45 min and were added to 0.2 ml of 0.2 N HCl and hydrolysed for 30 min. After hydrolysis, samples were stored on ice and suitable aliquots not exceeding 20 μl were used for PAP determination using the *Renilla* bioluminescence system. Boiled extracts were used as controls for each treatment.

TABLE 2
STABILITY OF PAP IN EXTRACTS OF YEAST DURING ACID HYDROLYSIS[a]

Treatment	Time of hydrolysis (min)	
	0	30
	(nmoles of PAP remaining/ml of incubation mixture)	
Extract	1.66	1.97
Extract + 3′-AMP	1.87	1.79
Extract + 5′-AMP	2.83	2.69
Extract + ADP	4.95	5.07
Extract + ATP	1.73	2.14
Extract + P_i	1.81	2.01
Extract + PP_i	1.97	2.22

[a] Incubations were carried out at 37°C. Reaction mixtures contained (in μmoles): $MgCl_2$ (4.0); nucleotides (4.0); P_i (4.0); PP_i (4.0); 0.2 ml of 0.2 N HCl and yeast extract (4.4 mg) in a total volume of 0.32 ml. After preincubating at 37°C for 1 min, the reaction was initiated by adding PAP (600 pmoles). At 0 and 30 min, 20-μl samples were withdrawn and PAP levels determined immediately by the bioluminescence procedure, as outlined in the text. Boiled extracts were used as controls for each treatment.

after 30 min. For all studies reported herein, the hydrolysis was routinely carried out for 30 min.

When PAP was incubated with extracts of yeast prior to acid hydrolysis, there was a rapid degradation of this compound (Table 1). The degradation was significantly reduced in the presence of 32 mM 5′-AMP, ADP or ATP. These nucleotides appeared to protect the PAP from cleavage by a 5′-nucleotidase. Boiled extracts did not degrade PAP. Similarly, PAP was stable when incubated with yeast extract under the conditions used for acid hydrolysis (Table 2).

Purity of Nucleotides

The possibility that PAP was an impurity in the reagents was checked by the *Renilla* system both before and after acid hydrolysis. Less than 0.003% of PAP was found in the commercial preparations of 3′-AMP, 5′-AMP and ATP. The highest level of PAP (0.01%) was detected in ADP (Table 2); the amounts of PAP in ADP and ATP increased with storage at −15°C. The contamination of nucleotides with PAP is accounted for in the assay by including appropriate controls.

Synthesis of PAPS

Yeast. The production of PAPS from SO_4^{2-} and ATP in a yeast extract (S_{60}) was determined by the bioluminescence assay, as shown in Fig. 3. The synthesis of PAPS was curvilinear over a 1-hr period. Similarly, PAPS was produced by incubating APS and ATP with the

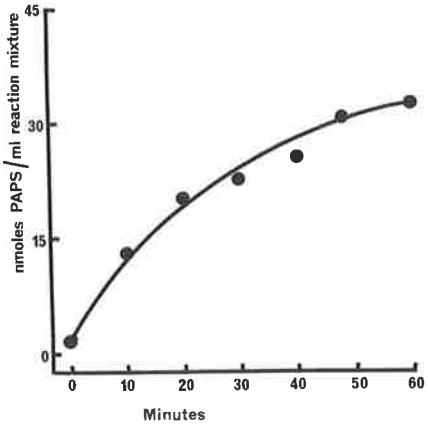

FIG. 3. Production of PAPS from SO_4^{2-} and ATP in a yeast extract (S_{60}). The reaction mixture (30°C) contained (in μmoles): Tris–HCl, pH 8.5, (500); $MgCl_2$ (50); ATP (50); and Na_2SO_4 (50) in a total volume of 5.0 ml. The reaction was initiated by adding the yeast extract (12.2 mg of protein). Sample aliquots (0.1 ml) were withdrawn from the incubation mixture at the times shown and immediately hydrolysed in two volumes of 0.2 N HCl at 37°C for 30 min. The samples were stored on ice and PAP was determined by the *Renilla* bioluminescence procedure.

TABLE 3
PAPS PRODUCTION BY VARIOUS PLANT SPECIES[a]

Plant type	Specific activity (nmoles of PAPS produced/min/mg of protein)	
	Without 5′-AMP	With 5′-AMP
Artichoke (A)	0.18	0.23
Artichoke (B)	0.09	1.05
Wheat	0.14	1.52
Sorghum	0.05	0.78
Spinach	0.06	0.62

[a] Artichoke (A) represents cells at the beginning of the cell cycle. The extract for artichoke (B) was prepared from tuber explants that had been grown in culture medium for 48 hr (22). Plant extracts were prepared immediately before assay as described in the text. The reaction mixtures (30°C) contained (in μmoles/ml): Tris–HCl, pH 8.5, (100); ATP (25); $MgCl_2$ (20); Na_2SO_4 (40); and 5′-AMP (25) where indicated. The reaction was initiated by adding the plant extract (approximately 12 mg of protein). Samples (0.1 ml) were taken at ten intervals over a 60-min period and were immediately hydrolysed in 0.2 ml of 0.2 N HCl at 37°C for 30 min. After hydrolysis, samples were stored on ice, and PAP levels were determined using the *Renilla* bioluminescence procedure.

yeast extract. The product PAPS was independently confirmed by following the incorporation of $^{35}SO_4^{2-}$ and [^{35}S]APS into [^{35}S]PAPS in the presence of ATP. The labeled compounds from an aliquot of the reaction mixture were separated by high-voltage paper electrophoresis in citrate buffer (pH 5.0) (26) and compared with a standard of [^{35}S]PAPS run under identical conditions. The specific activity for PAPS produced from ATP and SO_4^{2-} or APS was 0.24 and 0.32 nmoles/min/mg of protein, respectively, as determined by the bioluminescence method.

Green plants. The synthesis of PAPS was studied in extracts of green plants using the bioluminescence procedure. The production of PAPS was very low in all plant extracts (Table 3), reaching a maximum after a 10-min incubation period followed by a decline (Fig. 4). The addition of 5′-AMP (25 μmoles) to the reaction mixture prior to adding the plant extract greatly enhanced the production of PAPS (Table 3, Fig. 4) but 3′-AMP (25 μmoles) was less effective (Table 4). Previous workers (7) found that PAPS could be detected in isolated spinach chloroplasts only

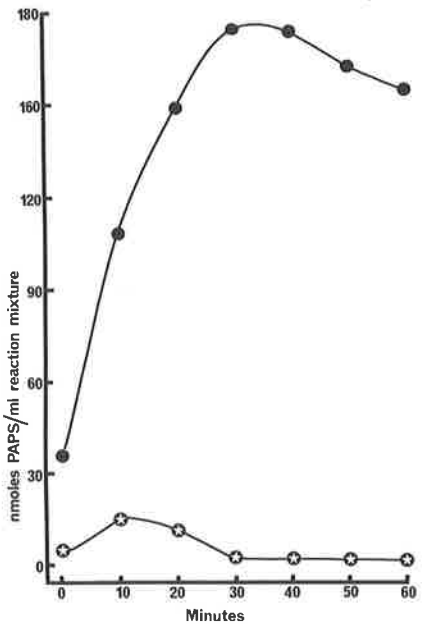

FIG. 4. The effect of 5′-AMP on the synthesis of PAPS from SO_4^{2-} and ATP by an extract of young wheat leaves. The reaction mixture (30°C) contained (in μmoles/ml): Tris–HCl, pH 8.5, (100); ATP (25); $MgCl_2$ (20); Na_2SO_4 (40); and 5′-AMP (25). The reaction was initiated by adding 0.5 ml of an extract of young wheat leaves. Sample aliquots (0.1 ml) were withdrawn at the times shown and immediately hydrolysed in two volumes of 0.2 N HCl at 37°C for 30 min. The samples were stored on ice and PAP was determined by the *Renilla* bioluminescence procedure. (✪—✪), Extract alone; (●—●), extract with 5′-AMP.

TABLE 4
THE EFFECT OF 3′-AMP, 5′-AMP AND SO_3^{2-} ON THE PRODUCTION OF PAPS IN EXTRACTS OF YOUNG WHEAT LEAVES[a]

Treatment	Specific activity (nmoles of PAPS produced/min/mg of protein)
Extract	0.03
Extract + 5′-AMP	1.75
Extract + 3′-AMP	0.79
Extract + SO_3^{2-}	0.06

[a] Reaction mixtures (30°C) contained (in μmoles): ATP (50), Na_2SO_4 (50), $MgCl_2$ (50) and 3′-AMP (25), 5′-AMP (25) or Na_2SO_3 (100) in a volume of 5.0 ml. The reaction was initiated by the addition of the young wheat leaf extract (10 mg of protein). Sample aliquots (0.1 ml) were withdrawn from the reaction mixtures at 10-min intervals for 1 hr and were hydrolysed in two volumes of 0.2 N HCl at 37°C for 30 min. The level of PAPS was determined by the *Renilla* bioluminescence procedure as described in the text.

in the presence of 3′-AMP but they did not test 5′-AMP. The addition of sulfite, which is a potent nonspecific inhibitor of sulfatase activity, had virtually no effect on PAPS production (Table 4).

The production of PAPS by combined extracts of yeast and young wheat leaves. The production of PAPS from SO_4^{2-} and ATP in yeast extract is curvilinear over a period of 1 hr. When an extract of young wheat leaves was added to the yeast extract, the production of PAPS was inhibited (Fig. 5). When a wheat extract was added to yeast extract

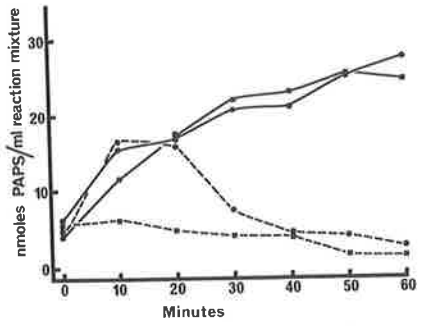

FIG. 5. PAPS production by combined extracts of yeast and young wheat leaves. The reaction mixtures (30°C) contained (in μmoles): Tris-HCl, pH 8.5, (500); $MgCl_2$ (50); Na_2SO_4 (50); and ATP (50) in a total volume of 5.0 ml. The reaction was initiated by adding the yeast extract (12 mg of protein). A portion (1.0 ml, 2.2 mg) of an extract of young wheat leaves was added to separate reaction mixtures at 0 (■---■) and at 20 min (●---●), respectively. Control tubes received an equal volume of 0.2 M Tris–HCl, pH 8.5, at 0 (■—■) and at 20 min (●—●). Sample aliquots (0.1 ml) were withdrawn at various time intervals and immediately hydrolysed in two volumes of 0.2 N HCl at 37°C for 30 min. The samples were stored on ice, and PAP was determined by the *Renilla* bioluminescence procedure.

which had been incubated with SO_4^{2-} and ATP for 20 min, PAPS rapidly declined (Fig. 5). The wheat extract contained enzymes that degraded PAPS. This is consistent with the data presented in Tables 3 and 4, which suggest a protection of PAPS by adenosine mononucleotides.

DISCUSSION

The previous techniques (7,9,29) used to detect PAPS in extracts of microorganisms and different plant tissues are laborious and time consuming for routine work. Furthermore, the methods based on radioisotopes are not sensitive enough to detect picomole amounts of PAPS. Recently a coupled enzyme system was used to study the properties of APS-kinase in spinach chloroplasts (7). The specificity of this technique for PAPS necessitates purified enzyme preparations since it relies on the coupling of PAPS to a phenol sulfotransferase.

A bioluminescence assay system described herein provides a simple and reproducible technique for determining PAPS in biological materials by hydrolysing it first to PAP, which produces light proportional to its concentration in the bioluminescence system of *Renilla reniformis*. The assay is sensitive and specific for PAPS provided that appropriate controls are included to assay the endogenous PAP prior to acid hydrolysis.

The sensitivity of the assay can probably be increased by using saturating levels of luciferyl sulfate. Care must be taken to ensure that the luciferin–luciferase system is not saturated by excessive levels of PAP. The K_m for PAP in the reaction mediated by luciferin sulfokinase in the *Renilla* bioluminescence system is 7.3×10^{-8} M (17). High levels of PAP can be assayed by decreasing the amount of PAP in the internal standard. The internal standard provided a convenient method for checking that light output increased linearly and that the luciferin sulfokinase enzyme was not saturated with PAP from the test sample.

The *Renilla* luciferin–luciferase system is extremely sensitive since about 10 pmoles of PAP and PAPS can be accurately determined. We have been able to demonstrate that very small amounts of PAPS are indeed produced from SO_4^{2-} and ATP in leaf extracts of various plants in the absence of any added nucleotides. The presence of enzymes in plant tissues that actively degrade PAPS may explain why previous workers (13–16), using less sensitive techniques, were unable to demonstrate its synthesis. Although the production of small amounts of PAPS in leaf extracts has now been unequivocally established, its role in the assimilation of sulfate is still uncertain.

3′-AMP and 5′-AMP may protect PAPS from enzymic degradation by acting as alternative substrates for a general nucleotidase. Recent experiments (unpublished data) support this idea since 5′-[U-^{14}C]AMP and [U-^{14}C]ATP, when incubated with extracts of young wheat leaves,

are rapidly degraded to [^{14}C]adenosine. There is no evidence for a direct phosphorylation of 3′-AMP or 5′-AMP to PAP. The bioluminescence assay was not affected by any component in plant extracts since the light production by internal standards of PAP was unchanged.

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