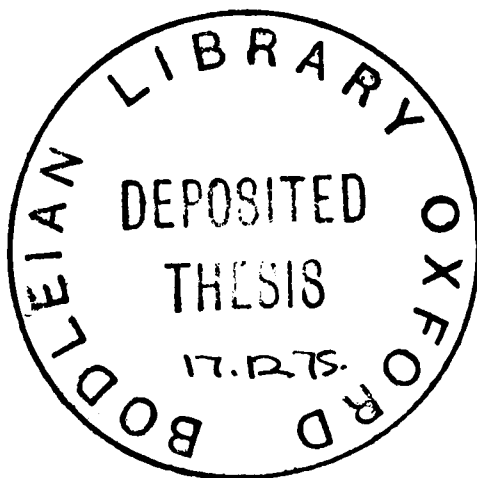


SULPHUR METABOLISM
IN PARACOCCLUS DENITRIFICANS

A thesis presented for the degree of
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by
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ABSTRACT

This thesis describes the pathway of sulphur metabolism in Paracoccus denitrificans (NCIB 8944). The compounds involved in the sulphur metabolic pathway were determined in growth experiments (Chapter 2) and time-course and pulse-chase experiments, using radioactively labelled sulphate (Chapter 3).

P. denitrificans is an assimilatory sulphate-reducing organism with a pathway of sulphate reduction involving inorganic intermediates. P. denitrificans can not utilise cysteine as a sole sulphur; cysteine inhibits amino acid metabolism at low concentrations and respiration at higher concentrations.

Sulphate is taken up by P. denitrificans against a concentration gradient (Chapter 4). The mechanism of sulphate uptake was investigated using right side out and inside out membrane vesicles prepared from P. denitrificans. The uptake mechanism involves an uncoupler-sensitive transport mechanism driven either by respiration, or by a transmembrane pH gradient (alkaline inside). The active transport of sulphate was shown to be carrier-mediated, by its sensitivity to sulphhydryl-group reagents. It is proposed that the sulphate carrier operates by a mechanism of electroneutral proton symport, and is capable of transporting sulphate in either direction across the plasma membrane (Chapter 5).

ATP Sulphurylase, the initial enzyme involved in the activation of sulphate, was purified, and its kinetic and regulatory properties investigated (Chapter 6). ATP Sulphurylase activity was repressed by sulphite, sulphide and cysteine, and inhibited by sulphide. Accumulation of APS, the end-

product of the ATP sulphurylase-catalysed reaction, could only be detected in the presence of inorganic pyrophosphatase, an enzyme which removed pyrophosphate, another end-product of sulphate activation.

Inorganic pyrophosphatase was purified and its substrate specificity, kinetics and regulatory properties examined, in relation to its part in sulphate metabolism (Chapter 7). Inorganic pyrophosphatase is a constitutive enzyme which functions equally well with either Mg^{2+} or Co^{2+} as the cofactor.

APS Kinase activity was detected in crude extracts of P. denitrificans. A new assay is described for measuring APS kinase activity (Chapter 8). APS Kinase was purified. Coupled enzyme assays, with purified ATP sulphurylase, inorganic pyrophosphatase and APS kinase, indicated that all three enzymes were necessary for the synthesis, and accumulation of PAPS (Chapter 8). No 3'-nucleotidase or enzyme "A" activity was detected.

Serine transacetylase and O-acetyl serine sulphydrylase were purified and the kinetics and regulation of these two enzymes, investigated (Chapter 9).

O-Acetyl serine lyase activity was detected in crude extracts of P. denitrificans, representing the first report of this enzyme in bacteria (Chapter 9).

β -Cystathionase was purified and its kinetic and regulatory properties investigated; the unidirectionality of the cysteine to methionine pathway was confirmed (Chapter 10).

Cysteinyl- and methionyl-tRNA synthetases were purified and the kinetics and regulation of these enzymes studied (Chapters 11 and 12 respectively). Both these enzymes possess

different substrate specificities to the aminoacyl-tRNA synthetases from other organisms. Both enzymes appear to be constitutive.

During this investigation of the sulphur metabolism of P. denitrificans, the substrate specificity of the different enzymes, to the selenium analogues of the respective sulphur-containing substrates, were investigated. Selenate competitively inhibits sulphate uptake and ATP sulphurylase, with respect to sulphate, but no APSe or PAPSe synthesis could be detected in the coupled enzyme assays (Chapters 7 and 8).

Purified O-acetyl serine sulphydrylase catalysed the synthesis of selenocysteine from selenide and O-acetyl serine (Chapter 9).

Both selenocysteine and selenomethionine are activated by the respective aminoacyl-tRNA synthetase (Chapters 11 and 12, respectively).

ACKNOWLEDGEMENTS

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I am also indebted to the Royal Commissioners for the Exhibition of 1851, for a scholarship.

NOMENCLATURE AND ABBREVIATIONS

This thesis is written in accordance with the conventions of the Biochemical Journal (1975) (Biochem. J. 145, 1-20). The journals cited in the References are abbreviated in accordance with the system used in the Chemical Abstracts Service Index (1969). In addition to the abbreviations listed in the Biochemical Journal (1975) the following abbreviations are also used:-

APS	Adenosine 5'-phosph ^u sulphate
PAPS	3'-phosphate adenosine 5'-phosphosulphate
FCCP	Carbonyl cyanide <u>p</u> -trifluoromethoxyphenylhydrazine
EDTA	Ethylene diamine tetracetic acid
P _i	Orthophosphate
PP _i	Pyrophosphate
TMPD	Tetramethyl- <u>p</u> -phenylenediamine
Tris	Tris-(hydroxymethyl)-methylglycine
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
BSTFA	Bis(trimethylsilyl) trifluoroacetamide

ENZYMES

<u>Trivial Name</u>	<u>Systematic Name</u>	<u>Enzyme Commission No.</u>
Alcohol dehydrogenase	Alcohol:NAD ⁺ oxidoreductase	1.1.1.1
Lactate dehydrogenase	L-lactate:ferricytochrome c oxidoreductase	1.1.2.3
NADH	NADH:(acceptor)oxidoreductase	1.6.99.3
Serine transacetylase	Acetyl-CoA:L-serine O-acetyl transferase	2.3.1.30
Myokinase	ATP:AMP phosphotransferase	2.7.4.3
APS kinase	ATP:adenylylsulphate 3'-phosphotransferase	2.7.1.25
ATP sulphurylase	ATP-sulphate adenylyltransferase	2.7.7.4
ADP sulphurylase	ADP:sulphate adenylyltransferase	2.7.7.5
3'-Nucleotidase	3'-Ribonucleotide phosphohydrolase	3.1.3.6
Deoxyribonuclease	Deoxyribonucleate 5'-oligonucleotidohydrolase	3.1.4.5
Lysozyme	Mucopeptide N-acetylmuramoyl-hydrolase	3.2.1.17
Inorganic pyrophosphatase	Pyrophosphate phosphohydrolase	3.6.1.1
ATPase	ATP phosphohydrolase	3.6.1.3
L-Serine dehydratase)	L-serine hydro-lyase	4.2.1.13
L-Serine deaminase)	(deaminating)	4.2.1.13
Cystathionine -syn-)	L-serine hydro-lyase	
thase)	(adding homocysteine)	
Serine sulphydrase)		4.2.1.22
O-Acetyl serine sulphydrylase	O-Acetyl L-serine-lyase (adding hydrogen sulphide)	4.2.99.8

Cystathionine γ - synthase	O-Succinyl-L-homoserine succinate lyase (adding cysteine)	4.2.99.9
δ -Cystathionase	L-Cystathionine cyst- eine-lyase (deaminating)	4.4.1.1
β -Cystathionase	L-Cystathionase L-homo- cysteine-lyase (deaminating)	4.4.1.8
Methionyl-tRNA syn- thetase	L-Methionine:tRNA ^{met} ligase (AMP-forming)	6.1.1.10
Cysteiny-tRNA syn- thetase	L-Cysteine:tRNA ^{cys} ligase (AMP-forming)	6.1.1.16

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CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION

Paracoccus denitrificans Davis, previously Micrococcus denitrificans Beijerinck (Davis et al., 1969) is a gram-negative, non-sporulating immotile coccus (Verhoeven, 1957; Kocus et al., 1968; Vogt, 1965). It is a facultative autotroph and is capable of growing on a wide variety of organic compounds (Davis et al., 1969).

In addition to being able to grow aerobically when supplied with reduced carbon compounds such as succinate, glucose and ethanol, P. denitrificans can readily adapt to anaerobic conditions in the presence of nitrate, nitrite or nitrous oxide (Kluyver, 1956; John, 1969).

Whilst P. denitrificans is capable of growing under anaerobic conditions it is unable to ferment (Davis et al., 1969) i.e. intermediates of its carbon metabolism do not act as terminal electron acceptors and growth is dependent upon the addition of a suitable electron acceptor.

According to Verhoeven (1957) thiosulphate can be used as the oxidizable substrate.

The experiments described in this thesis were carried out to determine the pathway, and investigate the control, of sulphur metabolism in P. denitrificans.

This chapter serves as a general introduction to the known pathways of sulphur metabolism in both microorganisms, plants and animals.

SULPHUR METABOLISM

"Sulphur-containing compounds are essential for the structure and function of all living cells" (Roy & Trudinger, 1970). Important sulphur-containing compounds which are found in cells include the amino acids cysteine and methionine, the cofactors lipoic acid, thiamine pyrophosphate and Coenzyme A, and the methyl group donor S-adenosyl-methionine. Sulphur-containing lipids are essential for the structure of many organelles, including chloroplasts. In addition, essential vitamins such as biotin, also contain sulphur.

The most important sulphur-containing compounds are the two amino acids cysteine and methionine. The occurrence of cysteine in proteins has special importance in determining the shape of proteins since three-dimensionally adjacent cysteine residues form -S-S- linkages via their -SH moieties. Furthermore, a substantial percentage of enzymes are inhibited by sulphhydryl-group reagents such as N-ethyl maleimide and p-chloromercuribenzoate suggesting that the point of attachment of the substrate to such enzymes involves a sulphhydryl moiety associated with a cysteinyl residue.

The subject of sulphur metabolism has been periodically reviewed since 1959; Challenger; 1959, Gregory & Robbins, 1960; Wilson, 1962; Nicholas, 1967; Thompson, 1967; Dziewiatkowski, 1970; Roy & Trudinger, 1970; Smith, 1971, Schiff & Hodson, 1973; Peck, 1974*.

*

The survey of the literature pertaining to this thesis was concluded on June 1st, 1975.

Sulphur is in the same group of the periodic table as oxygen; its chemical behaviour is, however, quite different. Sulphur resembles nitrogen in its ability to exist in a variety of valency states from +6 to -2. Furthermore, sulphur occurs in widely different types of compounds, polysulphides, sulphates, thiosulphates, sulfoxamates etc. The diverse valency states of sulphur, coupled with the facile interconversions and the multiplicity of compounds, have rendered the investigation of sulphur compounds difficult.

Sulphur metabolism involves both reductive and oxidative processes and the co-operative action of these processes in nature gives rise to the so-called "sulphur cycle" (Fig. 1.1) whereby sulphur is continually recycled between sulphate and reduced forms such as sulphide and sulphur-containing amino acids (Bunker, 1936; Young & Maw, 1958; Gibbs & Schiff, 1960). The sulphur cycle embraces an eight-electron change between sulphate and sulphide and may well involve the formation of intermediates which have no stable counterparts in chemistry (Trudinger, 1969).

The work described in this thesis is restricted to the assimilation of sulphur; oxidative processes involving sulphur compounds will not be discussed in any detail. Readers are referred to Trudinger (1969) and Roy & Trudinger (1970) for reviews on sulphur oxidation.

By analogy with Kluver's (1953) classification of bacterial nitrate reductions, Postgate (1959b) distinguished the following two classes of biological sulphate reduction:-

Fig.1.1

The sulphur cycle in nature showing the oxidation - reduction reactions that sulphur undergoes in various organisms.

REDUCTION (+ΔG).

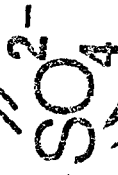
Assimilatory reduction (Plants, microorganisms)

Dissimilatory reducing bacteria

Polysaccharide

suphate

Steroidol sulphate



Chemosynthetic sulphur bacteria

PROTEIN



Animals, plants (?), microorganisms.

OXIDATION (-ΔG).

- 1) assimilatory, small-scale reductions of sulphate to sulphur-containing amino acids, and
- 2) dissimilatory, large-scale transformations of sulphate to sulphide which are linked to energy-yielding reactions in the organism.

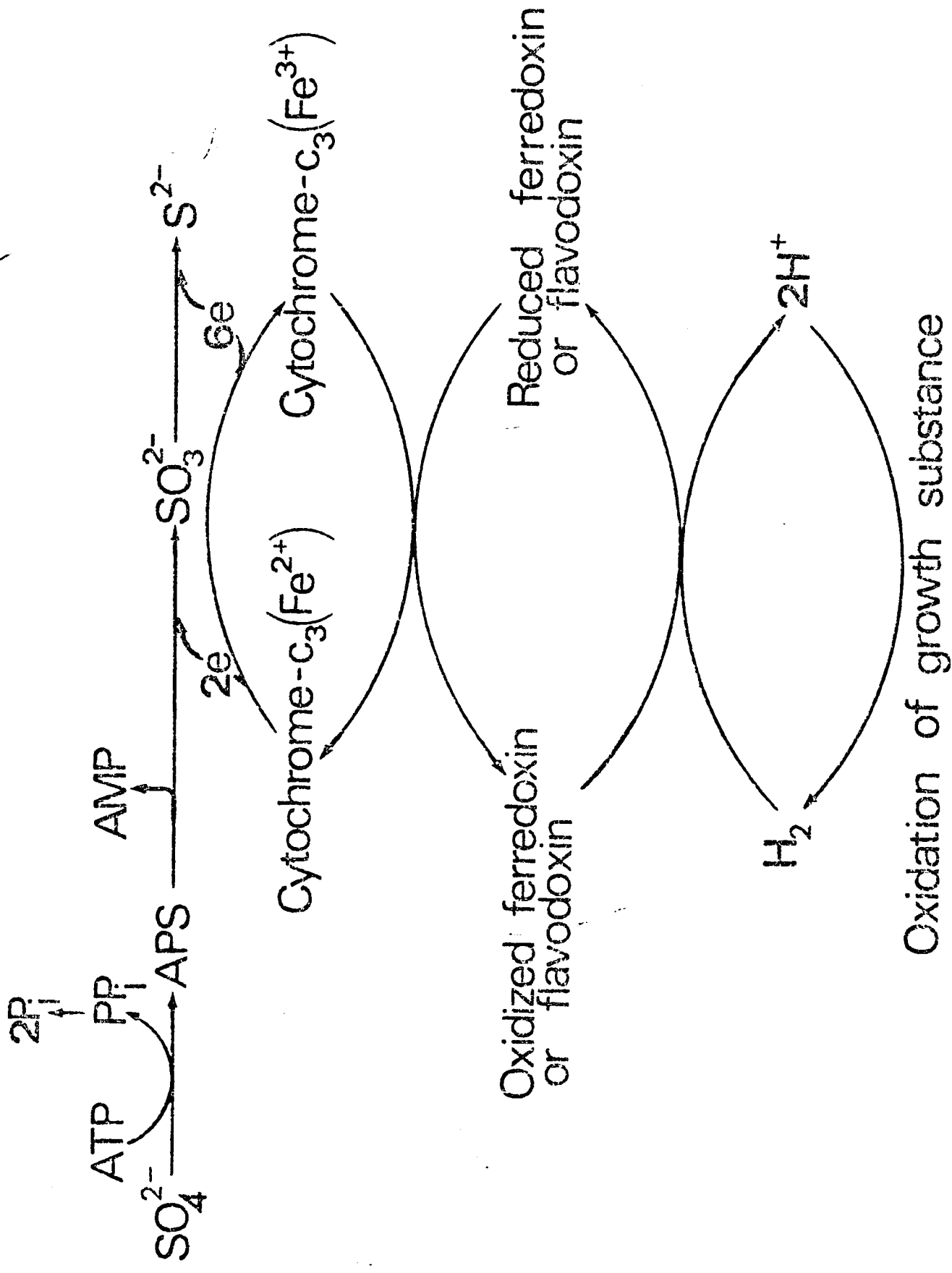
Dissimilatory sulphate reduction

Dissimilatory sulphate reduction is found in only two genera of bacteria as far as is known; Desulfovibrio and Desulfotomaculum (Ishimoto & Fujimoto, 1959; Peck, 1959, 1962a, 1962b, 1970). These bacteria are strict anaerobes which utilise sulphate as an electron acceptor in place of oxygen, thereby reducing sulphate to sulphide during oxidation of respiratory substrate. Oxidative phosphorylation of ADP to ATP is associated with these reactions. The dissimilatory sulphate reducing pathway is summarized in Fig. 1.2.

During dissimilatory sulphate reduction, sulphate is initially activated by ATP, a reaction catalyzed by ATP sulphurylase, to form adenosyl 5'-phosphosulphate (APS); this reaction, being thermodynamically unfavourable for the production of APS is "pulled" towards the production of APS by hydrolysis of pyrophosphate (PP_i) by inorganic pyrophosphatase. APS is the substrate for reduction to sulphite, the sulphite ultimately being reduced to sulphide. The immediate electron donor in these reactions is the very low potential cytochrome c_3 ($E'_0 = 205 \text{ mV}$) which ultimately receives electrons from molecular hydrogen via ferredoxin as a carrier. ATP formation from ADP is coupled to the reduction process with P_i/H_2 ratios of 0.18:1. Although the sulphur intermed-

Fig. 1.2.

Pathway of dissimilatory sulphate reduction in Desulfovibrio
(Roy & Trudinger, 1970)



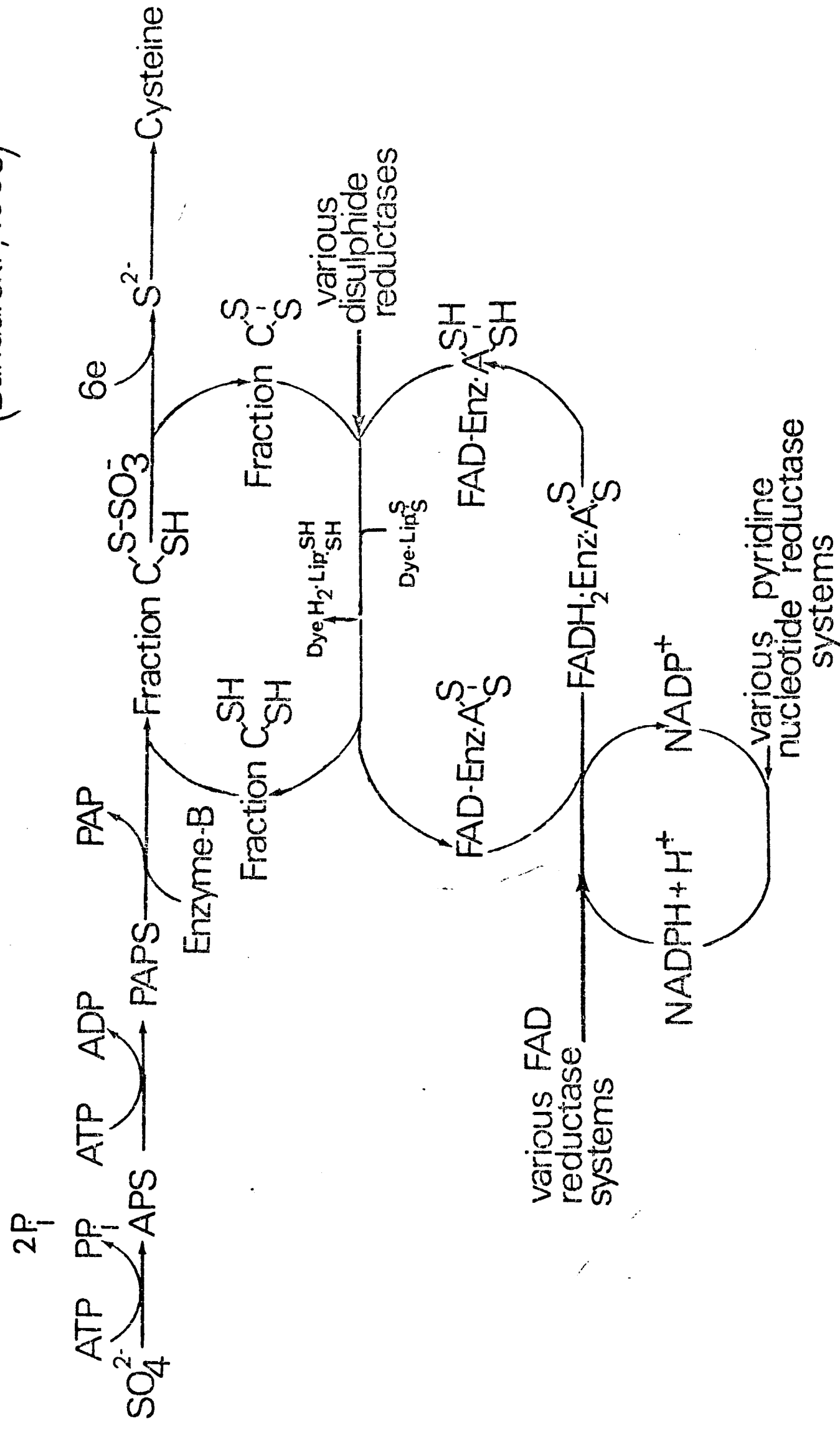
iates in this pathway seem to accumulate as the free, unbound compounds, there is evidence for the formation of a flavin-sulphite adduct in the APS-reductase reaction of Desulfovibrio (Michaels et al., 1970). Since ATP formation is the goal of dissimilatory sulphate reduction, the reduction of APS rather than PAPS (as in some assimilatory sulphate-reducing organisms - see below) conserves available energy even though APS formation is not favoured (Gregory & Robbins, 1960; Lipmann, 1958). In addition to utilizing sulphate as a terminal electron acceptor during respiration, some of the sulphide produced during respiration is utilized in the synthesis of essential compounds.

Assimilatory sulphate reduction

Dissimilatory sulphate reduction is restricted to a small group of anaerobic bacteria whereas assimilatory sulphate reduction is widespread throughout procaryotes and plants (Roy & Trudinger, 1970; Schiff & Hodson, 1973).

Sulphate reduction in animals. Some animals have been shown to reduce small amounts of sulphate to sulphide; Dohlman, (1957) demonstrated the incorporation of the sulphur of sulphate into cystine and methionine by mammalian corneal tissue. Wortman (1963) and Robinson (1965) have demonstrated the reduction of the sulphate of PAPS to sulphite using extracts from bovine and rat tissues, respectively. Although animals are capable of reducing small amounts of sulphate they are, in the absence of any other form of sulphur, incapable of producing sufficient amounts of reduced sulphur essential for survival. Therefore animals must obtain their

Pathway of assimilatory sulphate reduction in yeast
(Bandurski, 1965)



reduced sulphur from plants or microorganisms which are capable of reducing sulphate in large amounts. The physiological significance of sulphate reduction by animals is unknown. Animals are, however, capable of incorporating large amounts of sulphate into compounds such as sulphate esters. They are also capable of oxidizing reduced sulphur (e.g. cysteine and methionine) to sulphate, which is then incorporated into sulphate esters (Roy & Trudinger, 1970).

Sulphate reduction by microorganisms and plants

Assimilatory sulphate reduction is a property of the majority of bacteria, fungi, algae and higher plants since they utilize sulphate as the sole sulphur source.

Pathway of cysteine biosynthesis in *Escherichia coli*

The pathway of cysteine biosynthesis has been extensively studied in *E. coli* and *Salmonella typhimurium* and the steps, at present believed to be involved in the biosynthesis of cysteine are shown in Fig. 1.3. A great deal of nutritional, genetic, isotope competition and enzymic evidence, much of which has been previously reviewed in detail (Wilson, 1962; Thompson, 1967; Nicholas, 1967) leaves little doubt that the scheme is basically correct and that similar pathways operate in a number of bacteria and fungi. Perhaps the most convincing evidence in support of the pathway has been the isolation of cysteine-requiring mutants of *E. coli*, *S. typhimurium*, *Aspergillus nidulans* and *Neurospora crassa* which lack specifically either ATP sulphurylase, APS kinase, PAPS reductase, sulphite reductase, or O-acetyl serine sulphhydrylase (Ragland, 1959; Naiki, 1964; Dreyfuss & Monty, 1963a; Leinweber & Monty,

1965; Wheldrake & Pasternak, 1965; Hussey et al., 1965; Metzenberg & Parsons, 1966; Kredich & Tomkins, 1966; Yoshimoto et al., 1967; Spencer et al., 1968).

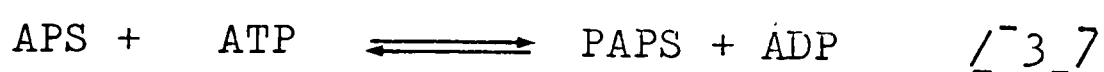
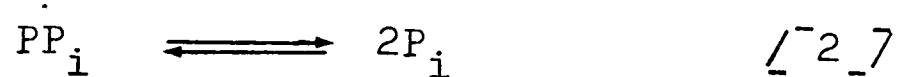
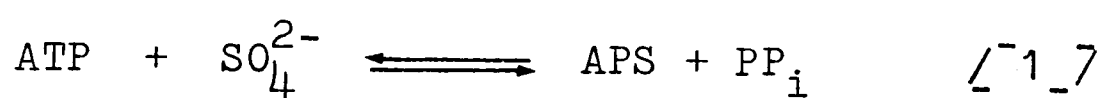
The enzymes of cysteine biosynthesis

(i) Sulphate uptake. Sulphate is transported into a number of microorganisms by an active process rather than a passive one (Roy & Trudinger, 1970). Sulphate uptake and its control has been studied in bacteria (Dreyfuss & Pardee, 1966; Jones-Mortimer, 1967; Kredich, 1971; Springer & Huber, 1973), fungi (Bradfield et al., 1970; Metzenberg & Parsons, 1966; McCready & Din, 1974), Chlorella (Wedding & Black, 1960), higher plants (Vange et al., 1974a, 1974b; Holmern et al., 1974; Leggett & Epstein, 1956; Nissen, 1974) and in plant cell suspension cultures (Hart & Filner, 1969). Sulphate uptake, in general, is accomplished through active transport mediated by a carrier with the properties of an enzyme (Schiff & Hodson, 1973). The transport of sulphate seems to be unidirectional, from the outside of the cell to the inside, with negligible exchange of internal sulphate with external sulphate or other anions. The sulphate carrier will mediate the transport of other group VI anions. The rate of transport is governed by pH, temperature, ionic strength, sulphate concentration, and the availability of metabolic energy. The K_m for sulphate is in the range of 10^{-5} to 10^{-4} M; free intracellular sulphate depresses the rate of sulphate uptake and endogenous cysteine and methionine have an inhibitory effect upon the rate of sulphate uptake. Pardee and co-workers (Langridge et al., 1970; Ohta et al., 1971; Pardee, 1966, 1967;

Pardee & Watanabe, 1968) have extensively studied the control of sulphate uptake in S. typhimurium. They have also purified, crystallized and characterized a sulphite-binding protein from a membrane fraction, but the absence of the binding protein does not necessarily correlate with the absence of transport activity.

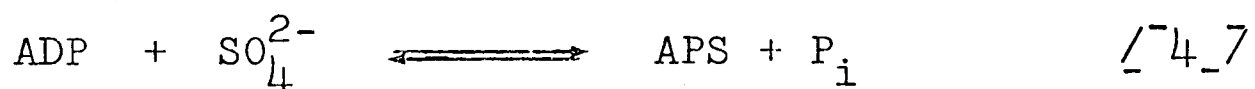
Sulphate activation. Thermodynamically, the reduction of sulphate to sulphide is an extremely unfavourable process. The reduction of sulphate to sulphide has a standard free energy change of +59 kJ/mole (Postgate, 1956) and the reduction of sulphate to sulphide, +218 kJ/mole. Three enzymes are required for the activation of sulphate in assimilatory reducing organisms; ATP sulphurylase, inorganic pyrophosphatase and APS kinase.

Sulphate first reacts with ATP to form APS (reaction [1]); the reaction is catalysed by ATP sulphurylase. This reaction proceeds with an unfavourable equilibrium for APS formation. The hydrolysis of pyrophosphatase partly overcomes this unfavourable equilibrium (reaction [2]); a further activation of sulphate by another ATP, a reaction which is thermodynamically favourable, to form PAPS, catalysed by APS kinase, helps to "pull" the overall reaction of sulphate activation in the direction of PAPS synthesis (reaction [3]);

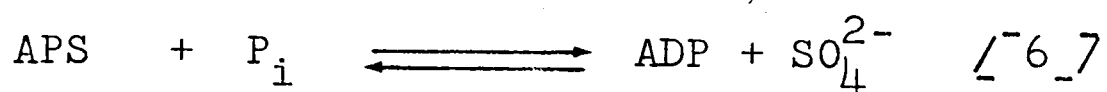
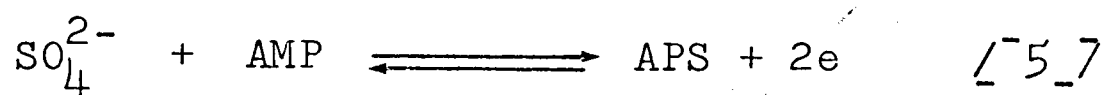


In many systems, PAPS is the sulphate donor for sulphate ester formation (Peck, 1970; Roy & Trudinger, 1970; Suzuki & Strominger, 1960).

Robbins & Lipmann (1958) first demonstrated the presence of another sulphate-activating enzyme, ADP sulphurylase, which catalyses the following reaction (reaction /⁻4₇);



Since the group potential of the sulphate is approximately twice that of analogous phosphate in ATP, the reaction can be considered to be essentially irreversible i.e. functioning only in the direction of ADP formation. ADP sulphurylase activity has been demonstrated in the sulphate-reducing bacteria (Peck, 1962b), Thiobacilli (Peck, 1960), Thiorhodaceae (Theile, 1968; Truper & Rogers, 1971), Saccharomyces cerevisiae (Robbins & Lipmann, 1958; Hawes & Nicholas, 1973) and higher plants (Burnell & Anderson, 1973a). Its function in yeast, higher plants and the sulphate-reducing bacteria is completely unknown, but it is conceivable that it regulates the size of the APS pool. The function of ADP sulphurylase in the Thiorhodaceae (Truper & Rogers, 1971) and certain of the Thiobacilli (Peck, 1962b) has been postulated to be the generation of ADP from APS during the oxidation of sulphide by APS-reductase according to reactions /⁻5₇ & /⁻6₇;



The reduction of sulphate. One of the most important steps in the utilization of sulphate in plant and bacterial systems is the reduction of sulphate to sulphite. Hilz et al., (1959) demonstrated that cell-free extracts of yeast suitably fortified with ATP, Mg^{2+} and NADPH formed labelled sulphite from labelled sulphate; PAPS was implicated as the immediate precursor of sulphite. Subsequently Wilson, Bandurski and co-workers fractionated this system (Asahi, Bandurski & Wilson, 1961; Torii & Bandurski, 1967; Wilson, Asahi & Bandurski, 1961) into three fractions which they called "A", "B", and "C". They proposed that fraction A was reduced by NADPH and then fraction A reduced fraction C (a heat-stable protein disulphide). Fraction B, an enzyme, acted upon reduced fraction C and PAPS to yield sulphite, PAP and oxidized fraction C. Until recently it was thought that all dissimilatory sulphate reducing organisms utilized APS, and that all assimilatory sulphate reducing organisms used PAPS, as the immediate sulphate donor for reduction (Peck, 1961). Dissimilatory sulphate reducers have been shown to utilize APS via APS reductase and it was suggested that all assimilatory sulphate reducers use PAPS via the enzyme PAPS-reductase (Peck, 1961).

Schiff and his co-workers found, however, that among assimilatory sulphate reducing organisms, Chlorella pyrenoidosa uses APS as the substrate for reduction, while E. coli uses PAPS (Tsang et al., 1971; Tsang & Schiff, 1974; Goldschmidt et al., 1975). Thus the correlation between the use of PAPS as a substrate for reduction and assimilatory sulphate reduction does not appear to hold. Furthermore, Schiff and his co-workers have shown that the reactions catalysed by the enzymes previously known as "APS-(or PAPS-) reductase" was the

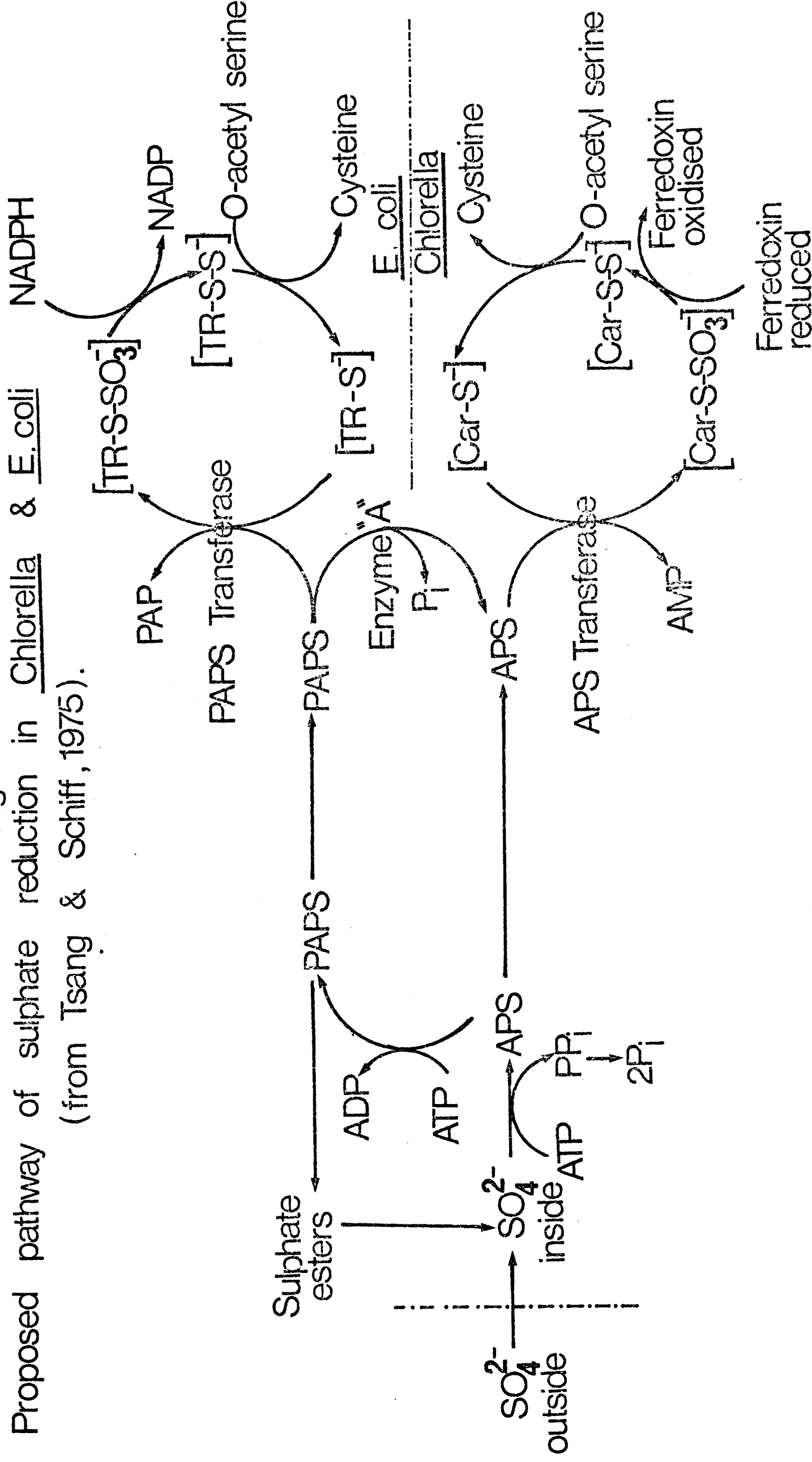
transfer of the sulpho-group from APS (or PAPS) to a protein carrier to form carrier bound sulphite.

In a survey conducted to investigate the substrate for sulphate reduction, Schiff and his co-workers found that APS appears to be the preferred substrate for the reduction of sulphate in all forms of photosynthetic organisms studied while non-photosynthetic organisms use PAPS.

They found an enzyme which converts PAPS to APS. This enzyme they termed fraction 'A', because it could be adsorbed to alumina C_γ, (and not be confused with the enzyme fraction "A" from yeast) is different from the widely distributed enzyme described by Shuster & Kaplan (1953). The rye grass 3'-nucleotidase will cleave 3'-nucleotides to yield inorganic phosphate without regard to substituents in the 5'-position. Fraction 'A' will cleave the 3'phosphate efficiently from nucleotides containing a phosphate as the 5'-position. As a source of APS, the reaction catalysed by fraction 'A' might provide several advantages over the ATP sulphurylase-pyrophosphatase activation step alone. As mentioned above, the ATP sulphurylase forms APS against an unfavourable equilibrium. It may be that it is more convenient for the cell to accumulate PAPS and form APS as needed by the nucleotidase reaction. A proposed pathway for the assimilatory reduction of sulphate in Chlorella is shown in Fig. 1.4.

Sulphite reduction. The reduction of sulphite to sulphide is a complex six-electron reaction; the E. coli sulphite reductase is one of the most complex soluble enzymes known (Siegel, Davis & Kamin, 1974). The reaction has been shown to be catalysed by extracts of E. coli (Mager, 1960; Leinweber &

Fig. 14

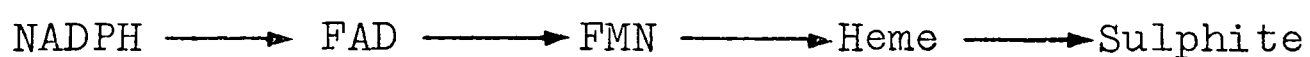


Monty, 1961; 1962; Ellis, 1964; Murphy et al., 1973, 1974; Siegel & Davis, 1974; Faeder et al., 1974; Davis & Siegel, 1974), S. typhimurium (Dreyfuss & Monty, 1963b), Aspergillus nidulans (Yoshimoto et al., 1961, 1967), Neurospora crassa (Leinweber et al., 1965; Leinweber & Monty, 1965), yeast (Wainwright, 1961; Okuda & Uemura, 1965; Naiki, 1965; Yoshimoto & Sato, 1965, 1968a, 1968b) and of a number of higher plants (Tamura, 1964, 1965; Asada, 1967).

The possibility that an intermediate electron carrier is required to link sulphite reduction with NADPH oxidation was first suggested by Siegel & Monty (1964) and Siegel, Click & Monty (1964).

The yeast sulphite reductase has been studied in detail by Yoshimoto & Sato (1968a, 1968b). They purified a protein to homogeneity which catalysed the complete reduction of sulphite to sulphide. Their results indicated that the yeast sulphite reductase is a multicomponent flavoprotein, the flavin being responsible for transferring electrons from NADPH to sulphite.

Kamin et al., (1968) first purified NADPH-linked sulphite reductase from E. coli to a homogeneous state. Studies with inhibitors (Siegel et al., 1974) have indicated that electrons are transferred from NADPH to sulphite via the minimum sequence;



From this sequence then, much of the complexity of NADPH-sulphite reductase from E. coli is associated with the transfer of electrons from NADPH to the site of sulphite reduction, itself.

The enzyme can catalyse a variety of reactions other than NADPH-dependent sulphite reduction (Siegel & Kamin, 1968; Siegel et al., 1974; Siegel & Kamin, 1971); among these are an NADPH-cytochrome c reductase activity (one of a group of NADPH-"diaphorase"-type reactions) which involves electron flow through the FAD and FMN groups, but not the heme, and a reduced methyl viologen-sulphite reductase activity, which requires integrity of the heme, but not the flavin prosthetic groups. This enzyme resembles the sulphite reductase from yeast in containing flavins, non-labile sulphide and a chromophore(s) absorbing at 385 and 587 nm. Flavins (FAD and FMN) have also been reported to be present in purified sulphite reductase from A. nidulans (Yoshimoto et al., 1967) and have been shown to stimulate NADPH-linked sulphite reduction by yeast (Prabhakararoa & Nicholas, 1968), E. coli (Mager, 1960) and N. crassa (Leinweber & Monty, 1965; Siegel et al., 1965).

The inability of purified reduced methyl viologen (MVH)-linked sulphite reductase from spinach (Asada et al., 1968), and possibly that from Allium odorum (Tamura, 1965) to catalyse NADPH-linked sulphite reduction may be attributed to the absence of flavins in these enzymes.

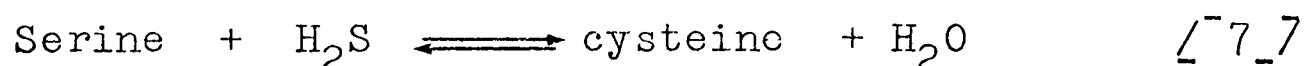
A chromophore absorbing in the region of 587-589 nm appears to be a general property of sulphite reductases. In addition to being present in the enzymes from yeast and E. coli it is present in purified sulphite reductases from spinach. (Asada et al., 1968), Allium odorum (Tamura, 1965) and Aspergillus nidulans (Yoshimoto et al., 1967).

There is some evidence that free sulphite may not be a normal intermediate in sulphate reduction. Torii & Bandurski (1967), for example, showed that "bound sulphite" formed from

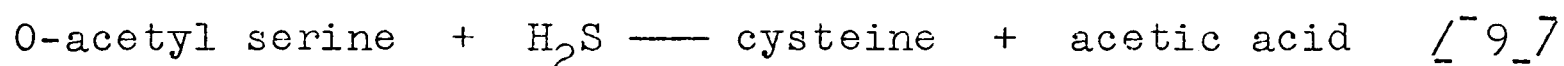
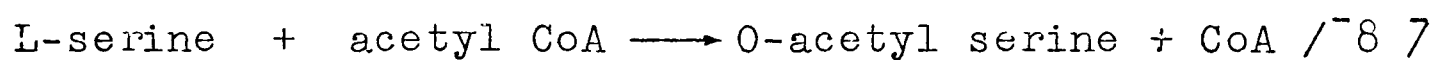
PAPS reductase is reduced to sulphide by yeast extracts in the presence of NADPH and Bandurski, Asada & Tamura (quoted by Thompson, 1967) obtained evidence that sulphite must be bound to a thiol group before being reduced. Results with certain mutants of Salmonella have been interpreted as indicating that enzymes A and B and fraction C of the PAPS-reductase system are required also for sulphite reduction by the intact cell, and that they may be concerned with the formation of "bound sulphite" (Thompson, 1967). In this connection it is interesting that of the six "resolved" sulphite reductase proteins purified by Wainwright (1962) from yeast, two proteins had properties similar to the A and C proteins required for PAPS reduction. It is also of interest that sulphite strongly represses synthesis of the sulphate-activating enzymes of E. coli but has a relatively slight effect on the synthesis of PAPS reductase (Pasternak et al., 1965). Thus it is possible that no free intermediates exist between PAPS and sulphide.

Cysteine synthesis. It is believed that the incorporation of sulphide into cysteine was a simple condensation reaction involving serine and sulphide; the reaction being catalysed by serine sulphydrase. The assumption that sulphide is the immediate precursor of cysteine-sulphur in a number of organisms is supported, not only by the results on sulphite reduction discussed above, but also by the existence of mutants of E. coli and S. typhimurium which are unable to grow on sulphide and which are simultaneously unable to utilise more oxidised forms of sulphur (Lampen et al., 1947; Clowes, 1958; Mizobuchi et al., 1962; Dreyfuss & Monty, 1963a).

A mechanism for cysteine biosynthesis from sulphide was demonstrated by Schlossman & Lynen (1957), who isolated from S. cerevisiae the enzyme, serine sulphydrase which catalyses the condensation of serine and sulphide to form cysteine (reaction /⁻7_7)



Serine sulphydrase is fairly widely distributed amongst bacteria, fungi and plants (Bruggeman et al., 1962). In S. typhimurium however, cysteine synthesis has been reported to involve the elimination of acetate from O-acetyl serine by sulphide; O-acetyl serine is formed from acetyl CoA and serine in a reaction catalysed by serine transacetylase (Kredich & Tomkins, 1966) (reactions /⁻8_7 & /⁻9_7).

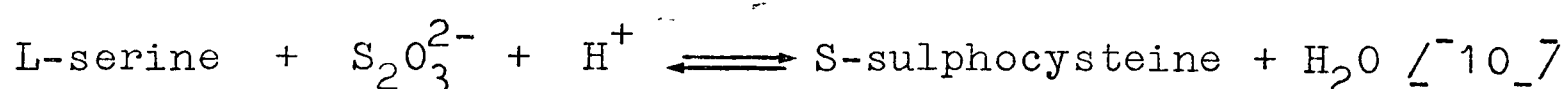


Kredich & Tomkins (1966) isolated several mutants of S. typhimurium which were unable to use sulphide and which contained negligible amounts of serine transacetylase and low O-acetyl serine sulphydrylase activity, indicating an essential role for these enzymes in cysteine biosynthesis from sulphide.

Both serine sulphydrase (Bruggeman et al., 1962; Pasternak et al., 1965) and O-acetyl serine sulphydrylase (Kredich & Tomkins, 1967; Jones-Mortimer et al., 1968) are present in E. coli together with serine transacetylase. Pasternak et al., (1965) reported specific activities (nmoles of cysteine synthesised per mg of protein per min at 37°C) of about 0.8 for serine sulphydrase of E. coli, compared with specific activities

of the order of 18,000 for O-acetyl serine sulphydrylase in the same organism (Jones-Mortimer et al., 1968). This indicates that the latter enzyme is the main catalyst of the terminal stage of cysteine biosynthesis in E. coli. Furthermore, O-acetyl serine sulphydrylase (Jones-Mortimer et al., 1968), but not serine sulphydrase (Pasternak et al., 1965), is repressed by the end-product, cysteine. Similar high activities of O-acetyl serine sulphydrylase and repression by cysteine occur in S. typhimurium (Kredich & Tomkins, 1966, 1967). The specific activities of serine sulphydrase in a number of other bacteria, yeasts, fungi and higher plants (see Schlossmann & Lynen, 1957; Bruggemann et al., 1962) are of a low order, comparable with that of the enzyme in E. coli.

Studies with certain mutants of Aspergillus nidulans suggested that, in this organism, S-sulphocysteine might be an intermediate in cysteine synthesis (Nakamura & Sato, 1962). Subsequently the enzymic condensation of serine and thiosulphate (reaction $\angle^{-10}_7$) in A. nidulans was described (Nakamura & Sato, 1963a, 1963b).



An NADPH-linked reduction of S-sulphocysteine to cysteine and sulphite in A. nidulans was briefly reported by Nakamura & Sato (1965). A second mechanism for the formation of cysteine from S-sulphocysteine involving a chemical reaction of the latter with reduced glutathione, was demonstrated by Woodin & Segel (1968).

A survey of the O-acetyl serine sulphydrylase and serine transacetylase activities in bacteria was conducted by Chambers

& Trudinger (1971), who concluded that the reaction between O-acetyl serine and sulphide, originally demonstrated in E. coli and S. typhimurium, may be a general mechanism for cysteine biosynthesis in bacteria. Furthermore, the low or undetectable serine sulphydrase activities accord with those previously reported in bacteria and other organisms (Bruggermann et al., 1962; Pasternak et al., 1965) and they indicate that this enzyme may have only a minor role in the formation of cysteine.

The physiological role of S-sulphocysteine is uncertain but the possibility that it may be involved in an alternate pathway of cysteine biosynthesis must be considered, particularly in the case of pseudomonads and thiobacilli where its activity is in excess of 10% that of O-acetyl serine sulphydrylase (Chambers & Trudinger, 1971).

Involvement of thiosulphate in assimilatory sulphate reduction. Early nutritional evidence from mutants of E. coli, S. typhimurium and A. nidulans, was interpreted as indicating an intermediate role for thiosulphate in sulphite reduction. Although the presence in S. typhimurium and other organisms of enzymes catalysing the direct reduction of thiosulphate to sulphite and sulphide (Artman, 1956; Kawakami et al., 1957; Kaji & McElroy, 1959; Woolfolk, 1962; Leinweber & Monty, 1963) lends weight to the contention of Leinweber & Monty (1963) - that thiosulphate is reduced to sulphide and sulphite prior to incorporation - Hilz et al., (1959) obtained evidence that thiosulphate is not an intermediate in sulphate reduction. They showed that, although thiosulphate inhibits the activation of sulphate by yeast extracts, it does not compete with ^{35}S -

labelled "active sulphate" in the formation of sulphide. Furthermore, thiosulphate is apparently not formed by purified sulphite reductase preparations from yeast.

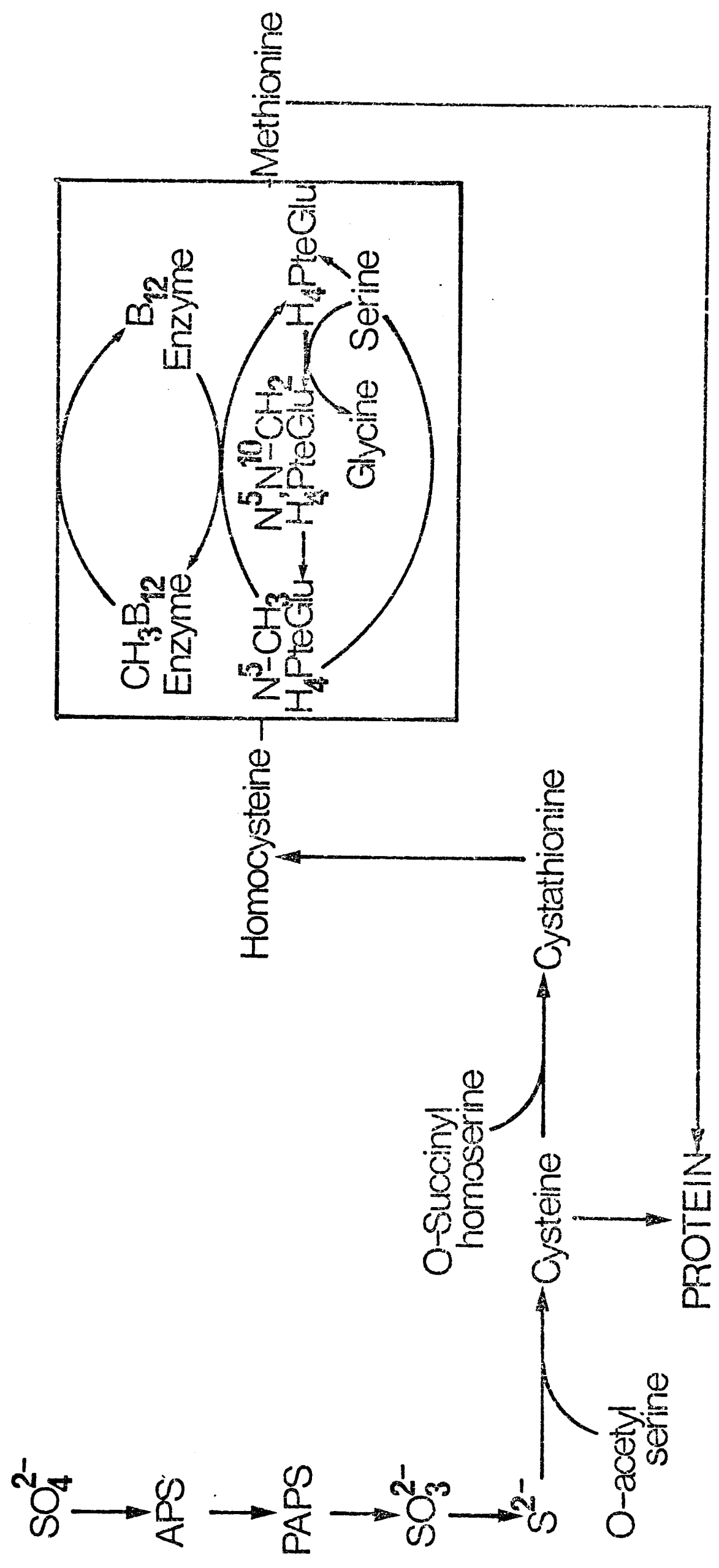
There is strong evidence, however, for an intermediate role for thiosulphate in sulphate reduction by A. nidulans (Nakamura & Sato, 1962; 1963a, 1963b).

The biosynthesis of methionine. The synthesis of methionine by microorganisms and plants assumes important biological significance since animals are unable to synthesise this essential amino acid. A number of different pathways have been described for the synthesis of methionine in bacteria, fungi and higher plants (Smith, 1970; Thompson, 1967; Guggenheim, 1971; Paszewski & Grabski, 1974); the relative contributions of the different pathways to the gross synthesis of methionine is not known (Giovanelli et al., 1973). The pathways differ in the reactions leading to the synthesis of homocysteine, a key intermediate in the biosynthesis of methionine. A description of the known pathways of methionine synthesis in S. typhimurium and E. coli and a description of deviations from this pathway follows.

The pathway of methionine synthesis, as it is known in E. coli and S. typhimurium, is illustrated in Fig. 1.5.

Homocysteine, the immediate precursor of methionine, is synthesised in two stages. O-Succinyl homoserine reacts with cysteine to give the thioether, cystathionine, and succinic acid; the reaction is catalysed by cystathionine γ -synthase (Kaplan & Flavin, 1966a; Rowbury & Woods, 1964b; Smith & Childs, 1966). Cystathionine is then hydrolysed to homocysteine, pyruvic acid and ammonia; the reaction is catalysed by β -cystathionase (Delavier-Klutchko & Flavin, 1965a, 1965b;

Fig.1.5 Methionine synthesis in E. coli & S. typhimurium (from Smith, 1970)



Rowbury & Woods, 1964b; Smith, 1961; Wijesundera & Woods, 1962).

The methyl group required for the synthesis of methionine from homocysteine may originate from either of two sources; from methylated derivatives of the folic acid cofactors tetrahydropteroyl monoglutamate ($H_4PteGlu_1$) or tetrahydropteroyl triglutamate ($H_4PteGlu_3$). These compounds are in turn synthesised in two steps. First N^5, N^{10} -methylene $H_4PteGlu_1$ (or Glu_3) is formed by the transfer of the hydroxymethyl group from serine by the action of serine hydroxymethyl transferase, glycine being formed as a by-product. And second, the methylene derivatives are reduced by N^5, N^{10} -methyl tetrahydrofolate reductase to give N^5 -methyl $H_4PteGlu_1$ (or Glu_3) (Cauthen et al., 1966; Smith, 1971). There are alternative pathways for the actual methylation of homocysteine (Woods et al., 1964).

1) A cobalamin-dependent methylation of homocysteine in which either N^5 -methyl $H_4PteGlu_1$ (or Glu_3) can serve as the methyl donor (Childs & Smith, 1969; Guest et al., 1964; Katzen & Buchanan, 1965; Taylor & Weissbach, 1967a, 1967b).

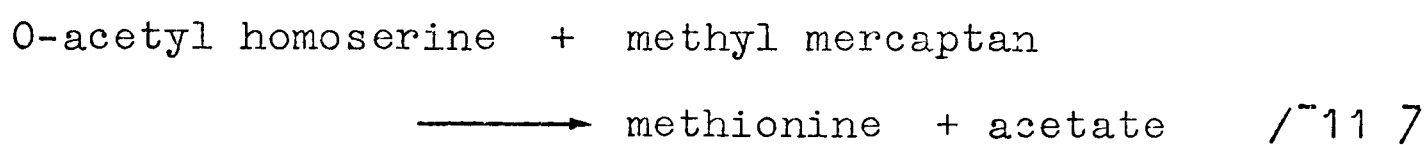
2) A cobalamin-independent methylation in which only N^5 -methyl $H_4PteGlu_3$ is the methyl donor (Cauthen et al., 1966; Whitfield & Weissbach, 1968).

In addition it has been shown that, in E. coli B, S-adenosyl methionine and methyl cobalamin can also act as methyl donors for methionine synthesis from homocysteine (Taylor & Weissbach, 1967a, 1967b).

Deviations from this pathway. In plants, it appears that the activated homoserine derivative is the O-acetyl- rather than the O-succinyl- form as found in E. coli and S. typhimurium.

An alternative pathway, in fungi and higher plants, for the synthesis of homocysteine, includes a direct sulphurylation of the activated form of homoserine (probably O-acetyl homoserine) using sulphide, produced by the sulphate reducing pathway, as the sulphurylating agent (Giovannelli & Mudd 1967, 1968; Paszewski & Grabski, 1974); the pathway involving the intermediate formation of cystathionine is, however, the major physiological pathway for methionine biosynthesis.

A mechanism for the synthesis of methionine has been described in plants which does not involve transmethylation of homocysteine (Giovannelli & Mudd, 1968). Again, an activated form of homoserine is the substrate and the sulphurylating agent is methyl mercaptan, reaction [11].



The importance of this reaction in methionine biosynthesis cannot be established until the biosynthetic origin of methyl mercaptan (a common metabolite in at least some plants) is established; the reaction has no significance in the biosynthesis of methionine unless methyl mercaptan is derived from sources other than methionine.

Control of the enzymes of the sulphate assimilation pathway differs in different organisms; in E. coli and S. typhimurium there is no evidence that cysteine affects the synthesis of methionine enzymes and the effect of methionine on cysteine enzymes is probably an indirect one (Smith, 1971).

Transulphurylation

Transulphurylation, the name originally given to describe the transfer of sulphur from homocysteine to cysteine via

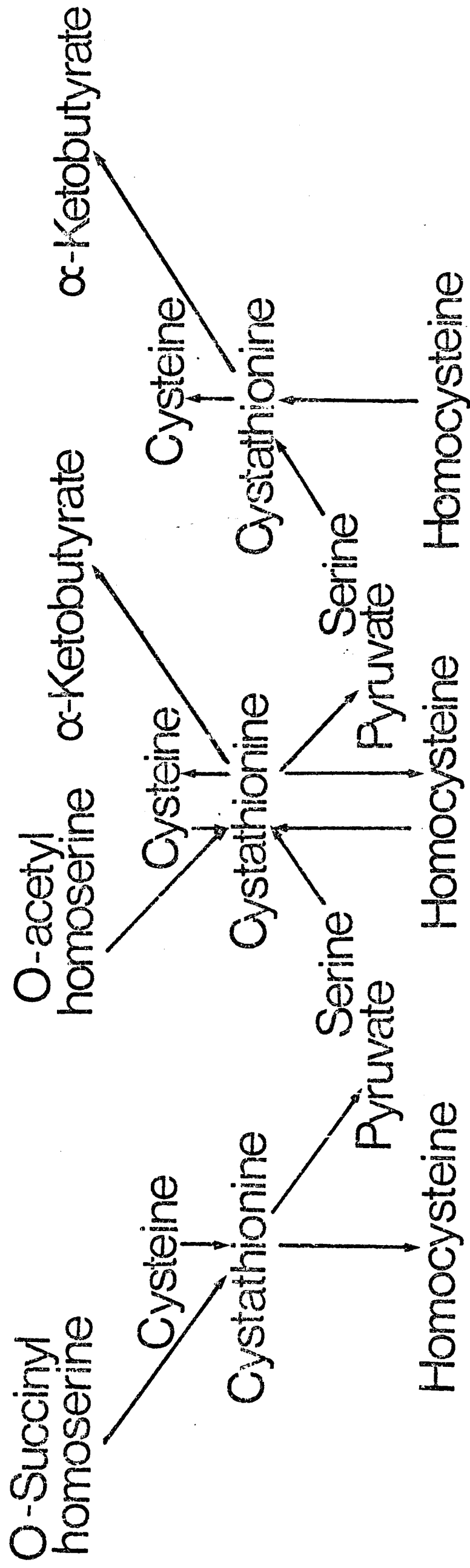
cystathionine in mammalian liver (Du Vigneaud, 1952) has recently been extended to include the reactions mediating the comparable transfer from cysteine to homocysteine in bacteria (Kaplan & Flavin, 1966a) and to the reactions in fungi (Nagai & Flavin, 1967) by which sulphur is transferred in both directions (Fig. 1.6).

The conversion of homoserine to homocysteine via cystathionine is believed to be the main pathway of methionine biosynthesis in Salmonella (Flavin & Slaughter, 1967) and Neurospora (Kerr & Flavin, 1968). However, synthesis of homocysteine (or methionine) also occurs from the appropriate homoserine derivative and H_2S (or CH_3SH) with extracts from both bacteria (Flavin & Slaughter, 1967b) and fungi (Nagai & Flavin, 1967; Kerr & Flavin, 1969). Genetic evidence has shown that these reactions do not function as a major role in methionine biosynthesis in bacteria (Flavin & Slaughter, 1967).

Metabolism of cysteine and methionine. Cysteine and methionine are incorporated into proteins; they are activated to form cysteinyl- and methionyl-tRNA; the reactions being catalysed by their respective aminoacyl-tRNA synthetases (Novelli, 1967). Methionine has an important role in the initiation of protein biosynthesis (Clarke & Marcker, 1965; Adams & Capecchi, 1966; Webster et al., 1966) in the form of N-formyl methionine tRNA.

Schematic representation of transsulphurylation mechanisms

Fig. 1.6



E. coli

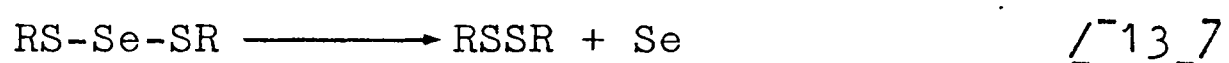
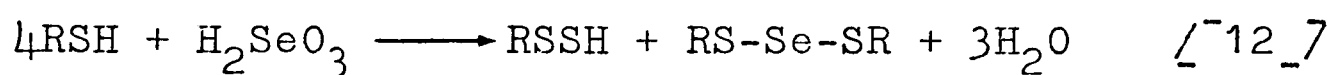
Neurospora

Mammals

SELENIUM METABOLISM

Chemical properties. Selenium and sulphur are both group VI elements; sulphur and selenium can exist in a relatively large number of valency states. The outer electron shells of selenium is $3d^{10}4s^24p^6$ as compared with $3s^23p^6$ for sulphur. Selenium is therefore larger and less electronegative than sulphur and therefore H_2Se is less stable than H_2S . All selenium oxides are less stable than their sulphur analogues; selenium differs from sulphur in showing tendency to become oxidised to the +6 valency state.

Selenite readily reacts with cysteine and glutathione according to reactions [12] and [13] (Painter, 1941).



Selenium as an essential trace element. The importance of selenium, as a trace element to ruminants, is widely recognised and is reviewed by Underwood (1971). Much of the work supporting its recognition has come from the study of white muscle disease in lambs (Muth et al., 1958; Godwin et al., 1974; Schwartz, 1961). The epidemiology of this malady, which affects mainly lambs and calves, is now understood (Hartley & Grant, 1961) and the pathology of white muscle disease is also well documented (Muth, 1955); biochemical changes of selenium are not understood.

Rotruck et al., (1973) reported that selenium is an essential constituent of glutathione peroxidase. Similarly, the normal development of formic dehydrogenase in E. coli requires

the presence of selenite in culture media (Pinsent, 1954). The biochemical implications of these selenium requirements are not fully understood.

Selenium toxicity (a) As a sulphur analogue. It is well established that in several systems selenium compounds compete with the corresponding sulphur compound. The intracellular concentration of selenium in Chlorella vulgaris remains constant if the sulphate:selenate ratio is kept constant even when the concentration of each is increased 20-fold (Shrift, 1954a). A similar result is obtained for methionine and selenomethionine (Shrift, 1954b). Similarly Leggett & Epstein (1956) obtained competitive kinetics for sulphate and selenate uptake by barley roots. Shaw & Anderson (1972) showed that selenate competed for the same enzyme site as sulphate on ATP sulphurylase; they were, however, unable to demonstrate the synthesis of APSe (APSe appears to be much more labile than APS).

Nissen & Benson (1964) reported that they were unable to find any selenate esters of lipids in several plants grown in the presence of selenate, and they attributed this to the inability of plants to make PAPSe (because of the lability of APSe). Transport of methionine is competitively inhibited by selenomethionine (Spencer & Blau, 1962) and competitive inhibition of methionyl-tRNA synthetase, by selenomethionine, has been reported (Hoffman et al., 1970).

Microorganisms have been shown to react to selenium compounds in a number of ways. Inhibition of growth by selenium compounds is well documented and has been reviewed by Rosenfeld & Beath (1964) and Shrift (1969). Complete replacement of some sulphur nutrients with their selenium analogues has also been claimed (Mautner & Gunther, 1959; Cowie & Cohen, 1957).

(b) Other sites of action. Selenate and selenite have been shown to inhibit respiration in yeast (Moxon & Franke, 1935; Potter & Elvehjem, 1936) and oxygen uptake (Wright, 1939). Selenite, but not selenate, uncouples oxidative phosphorylation in rat liver mitochondria (Whitchouse, 1964).

Selenium-containing compounds inhibit succinic dehydrogenase (Collett, 1924), urease (Wright, 1940), cell division (Scott, 1964; Shrift, 1961) and cell growth (Holland & Humphrey, 1953; Pengra & Berry, 1953; Fels & Cheldelin, 1955).

Selenium tolerance. Some plant species (some species of the genus Astragalus and Neptunia), accumulate and tolerate high concentrations of selenium; indeed some plant species are restricted to growth on selenium-rich soils. In contrast, other plants are extremely sensitive to high concentrations of selenium in the soil. It has been suggested that plants which grow on, and accumulate selenium, are capable of tolerating high selenium concentrations because of the presence of either (1) enzyme systems which can distinguish selenium from sulphur and therefore incorporate sulphur-containing compounds into biologically important cell constituents (Fowden et al., 1967), (2) a detoxification mechanism (i.e. a mechanism which renders selenium-containing compounds inert as regards further metabolism) or (3) the presence of both types of mechanisms.

Objects of this investigation. The experiments described in this thesis were conducted to determine the mode and pathway of sulphur metabolism in Paracoccus denitrificans.

Growth experiments (Chapter 2) were conducted to determine the possible modes of sulphur metabolism. After determining

a possible pathway of sulphur metabolism in in vivo experiments (Chapter 3) individual enzymes, implicated in the sulphur metabolic pathway, were investigated. These included sulphate uptake, and the mechanism thereof (Chapters 4 & 5), ATP sulphurylase (Chapter 6), inorganic pyrophosphatase (Chapter 7), APS kinase (Chapter 8), serine transacetylase and O-acetyl serine sulphydrylase (Chapter 9), β -cystathionase (Chapter 10), and the cysteine- and methionine- activating enzymes (Chapters 11 & 12). Possible control mechanisms of sulphur metabolism were investigated.

Since it has been suggested that selenate is metabolised by the same metabolic pathway as sulphate, the activity of selenium analogues, of the respective sulphur-containing compounds were investigated.

CHAPTER 2

GROWTH EXPERIMENTS WITH PARACOCCLUS DENITRIFICANS

INTRODUCTION

The pathway of cysteine biosynthesis from inorganic sulphate was initially proposed for a variety of micro-organisms including Escherichia coli (Lampen et al., 1947; Roberts et al., 1955; Simmonds, 1948), Neurospora crassa (Horowitz, 1950, 1955; Phinney, 1948), Aspergillus nidulans (Hockenhull, 1949; Shepherd, 1956), and Aspergillus niger (Steinberg, 1941). In most of the studies the data are consistent with an inorganic pathway of sulphate reduction through sulphite and sulphide and only after passing through these inorganic intermediates is the reduced sulphur atom combined with a carbon skeleton (serine) to form cysteine. The irreversibility of the cysteine-methionine pathway in E. coli was apparent from the results of both Simmonds (1948) and Roberts et al., (1955).

Detailed information on sulphur metabolism in micro-organisms was initiated by Steinberg's (1941) investigation into the utilization of various sulphur-containing compounds by Aspergillus niger.

This chapter reports results of experiments conducted to determine which sulphur-containing compounds can be utilized by Paracoccus denitrificans and thus gain an overall view of the pathway of sulphur metabolism in this organism. The effect of selenate, an analogue of sulphate, on growth of P. denitrificans, is also reported. It has been reported that P. denitrificans can utilize thiosulphate as a terminal electron acceptor for respiration (Verhoeven, 1957). Results of experiments to determine if P. denitrificans can reduce sulphate via the dissimilatory sulphate-reducing pathway are reported. Finally experiments designed to determine the mech-

anism of inhibition by cysteine are reported.

MATERIALS AND METHODS

Chemicals. Oxoid Agar No. 3 was obtained from Oxoid Ltd., London, U.K. and yeast extract was obtained from Difco Laboratories, Detroit, Mich., U.S.A. L- $^{-1}$ - ^{14} C-7 Methionine was obtained from the Radiochemical Centre, Amersham, Bucks., U.K. DL-Homocysteine thiolactone-HCl was obtained from Koch-Light Laboratories Ltd., Bucks., U.K. and was converted to DL-homocysteine by heating at 100°C for 3 min in the presence of two equivalents of NaOH (Flavin, 1962). Alcohol dehydrogenase (salt free), gramicidin D and all amino acids were obtained from the Sigma Chemical Co., London, U.K. Napthalene (scintillation grade) was obtained from Koch-Light Laboratories, U.K. Butyl-PBD was obtained from Ciba-Geigy Ltd., Cambridge, U.K. N,N'-Dimethyl-p-phenylene diamine sulphate was obtained from Eastman Organic Chemicals, N.Y., U.S.A. NNN'N'-tetramethyl-p-phenylene diamine dihydrochloride (TMPD) was obtained from British Drug Houses, Dorset, U.K. All other chemicals were of analytical reagent grade and were obtained from either the Sigma Chemical Co., London, U.K. or from British Drug Houses, Dorset, U.K.

Culture conditions. Cells of Paracoccus denitrificans (NCIB 8944) were grown in liquid medium at 30°C . The basic medium was a modified version of that used by John & Whatley (1970), and contained per litre; 0.68g KH_2PO_4 , 0.60g $(\text{NH}_4)_2\text{HPO}_4$, 0.1 ml modified Hoagland's trace element solution (Collins, 1969), 2mg FeEDTA, 54mg Na_2SO_4 , 25mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 25^{mg} $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, and 13.5g sodium succinate hexahydrate. The Na_2SO_4 and CaCl_2 solutions were autoclaved separately and added after cooling.

Aerobically grown cells were grown in 500 ml of culture medium in 2 litre flasks and incubated in an orbital incubator at 30°C at 185 rpm describing a 40 cm diameter circle. Nitrate medium, in which nitrate was added as the terminal electron acceptor for respiration, was the same as the aerobic medium with the addition of 10.1 g of potassium nitrate per litre. Cells were grown in two litre flat-bottomed flasks almost filled with medium.

Cultures were maintained on the nitrate medium with daily subculturing. All media were inoculated with an 0.1 (v/v) inoculum of the previous day's nitrate grown cells.

Sulphur sources, tested as substrates for growth, were prepared fresh each day and sterilised by filtration through a Millipore membrane filter (pore size 0.22 μ). L-Cysteine was prepared as the hydrochloride and neutralized with KOH immediately prior to use.

Growth of bacterial cultures was followed by measuring the optical density at 550 nm in a Beckman DB spectrophotometer.

In experiments where the pH and the $A_{550\text{ nm}}$ of the culture solution were measured, an apparatus was designed to allow the two variables to be continuously monitored. The pH was measured using a Pye Unicam Model 292 pH meter. The optical density of the culture solution was measured using a Pye Unicam SP600 spectrophotometer fitted with a continuous flow cuvette. A peristaltic pump maintained a constant flow (6ml/min) of culture solution through the cuvette. The pH meter and the spectrophotometer were linked to a Servoscribe double pen recorder.

In experiments conducted to determine whether P. denitrificans can utilize sulphate, in lieu of nitrate, as a terminal electron acceptor, and thus reduce sulphate in a way similar

to dissimilatory sulphate-reducing organisms, nitrate was replaced by sulphate, sulphite or thiosulphate (0.1M). Argon (10ml/min) or CO_2/N_2 (5% CO_2 in 95% N_2 , v/v) was bubbled through spargers into the culture media to maintain an oxygen-free environment. L-ascorbic acid (5mM) and/or H_2S (5mM) were added to the medium to adjust the redox potential of the medium (see discussion).

Preparation of cell-free extracts. Unless otherwise stated cell-free extracts were prepared from cells grown on nitrate media. Cells were harvested during the exponential growth phase ($A_{550}=2.9$) by centrifugation, washed with medium containing 20mM -Tris-HCl, pH 8.0, resuspended with the washing medium in 0.01 of the original volume and then frozen. After thawing and degassing for 10 min, cells were disrupted by sonication using a Dawe Soniprobe Type 7530A at full output. Cells were sonicated for 10 min in 30 sec bursts and the temperature of the cell suspension was maintained between $0-4^\circ\text{C}$ with the use of an acetone-ice bath. Cell debris was removed by centrifugation at 30,000 g for 60 min. The resultant clear, brown supernatant was decanted, dialysed against 20mM -Tris-HCl buffer, pH 8.0, at 4°C and refrozen at -15°C .

Measurement of methionine uptake. Whole cells of P. denitrificans, grown on succinate-nitrate media plus yeast extract (0.5g/litre), were used to study methionine uptake. Cells were harvested by centrifugation, washed twice with growth media minus sulphate and yeast extract and resuspended in 50% vol. of the culture solution minus sulphate and yeast extract. Incubation mixtures contained 3.0 ml of washed cell suspension, 1.5 ml of washing solution, 0.1 ml L- ^{14}C -7 methionine (10mM and $1\text{ }\mu\text{Ci}/\mu\text{mol}$) in a total volume of 5.0 ml.

The γ - ^{14}C -methionine was added at zero time. Experiments were conducted at 30°C . Samples (0.1 ml) were withdrawn at intervals and placed in 2 ml of 10mM-L-methionine at 2°C . The diluted samples were allowed to stand for 5 min and then the cells were collected by filtration using an Oxoid membrane filter (0.45 μ pore size, 4.7 cm diam.). The filters were first washed with 10 ml of 10 mM-L-methionine (at 2°C) and the 2.1 ml samples then filtered. The filters were quickly washed with five 10 ml washes of 10 mM-L-methionine at 2°C and were placed in scintillation vials, dried (to reduce quenching by water) and the radioactivity counted following the addition of scintillation fluid (7 g butyl-PBD, 80 g naphthalene, made up to one litre with a 6:4 (v/v) mixture of toluene and 2-methoxyethanol, respectively). Vials were allowed to stand for 12 h prior to counting in a scintillation counter.

Assay for cysteine desulphydrase. This enzyme may be assayed by measuring the rate of production of any of its products; sulphide, pyruvate or ammonium ions. Two methods were used to detect cysteine desulphydrase activity in extracts of P. denitrificans.

The first assay method involved following the rate of production of sulphide. Reactions were conducted at 30°C in plastic Eppendorf mini-centrifuge tubes containing 100 μ moles of Tris-HCl, pH 8.5, and 0.1 ml of crude enzyme (cell-free extract) (1 to 10 mg of protein per ml) in a total volume of 0.9 ml. The reaction was initiated by the addition of 0.1 ml of L-cysteine (varying concentrations were used) and the reaction was terminated after 10 min by the addition of 0.2 ml of 0.02 M-N, N'-dimethyl-p-phenylenediamine sulphate in 7.2 M-HCl followed immediately by 0.2 ml of 0.03 M- FeCl_3 in HCl (Siegel, 1965).

The tubes were capped, vigourously shaken, and after storage in the dark for 20 min, were centrifuged to remove any precipitated protein. The $A_{650 \text{ nm}}$ of the clear supernatant was determined in a Pye Unicam SP 8000 spectrophotometer. The measured E_{650} for sulphide is $1.56 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

The second assay method involved measuring the rate of production of pyruvate. Pyruvate was measured by a modification of the procedure of Friedeman (1957). Into an uncapped Eppendorf mini-centrifuge tube containing 100 μmoles Tris-HCl pH 8.5, water, and crude enzyme extract (0.5 mg of protein) was added 2.0 μmoles of L-cysteine in a final volume of 1 ml. After incubation at 30°C for 10 min, the reaction was terminated by the addition of 0.2 ml of 15% (w/v) trichloroacetic acid. The terminated reaction mixture was centrifuged to remove precipitated protein and 1.0 ml of the supernatant added to 0.5 ml of an 0.1% (w/v) solution of 2,4-dinitrophenylhydrazine in 2M -HCl. This was allowed to react for 10 min after which 2.0 ml of 2M -NaOH was added. The brownish-orange colour was allowed to develop for 20 min, after which the absorbance at 520 nm was measured. The measured E_{520} for pyruvate 2,4-dinitrophenylhydrazone is $3.74 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

Preparation of membrane vesicles. The procedure for the preparation of membrane vesicles from cells grown using succinate as substrate and nitrate as the terminal electron acceptor is described in Chapter 5.

Assay of electron transport in membrane vesicles. The reaction was carried out at 30°C in a Rank Type oxygen electrode of 3 ml capacity. Reaction mixtures contained, in a total volume of 3 ml, 20mM -Tris- PO_4 buffer, (pH 7.3), 5mM -magnesium acetate, 3 μg of gramicidin D, 30mM -ammonium acetate,

a respiratory substrate, and membrane vesicles (0.1 mg of protein per ml). When NADH was the substrate, 0.1 mg of alcohol dehydrogenase (EC 1.1.1.1) and 30 μ l of absolute ethanol were added, and the reaction was initiated by the addition of 0.6 mM -NAD⁺. When succinate was the substrate, sodium succinate, pH 7.3, to a final concentration of 10 mM , was added to initiate the reaction. When ascorbate-TMPD was the substrate, sodium ascorbate (20 mM) and TMPD (0.1 mM) were added prior to the addition of membrane vesicles and the reaction initiated by the addition of membrane vesicles. There was no significant non-enzymic oxidation of ascorbate-TMPD in the reaction mixture.

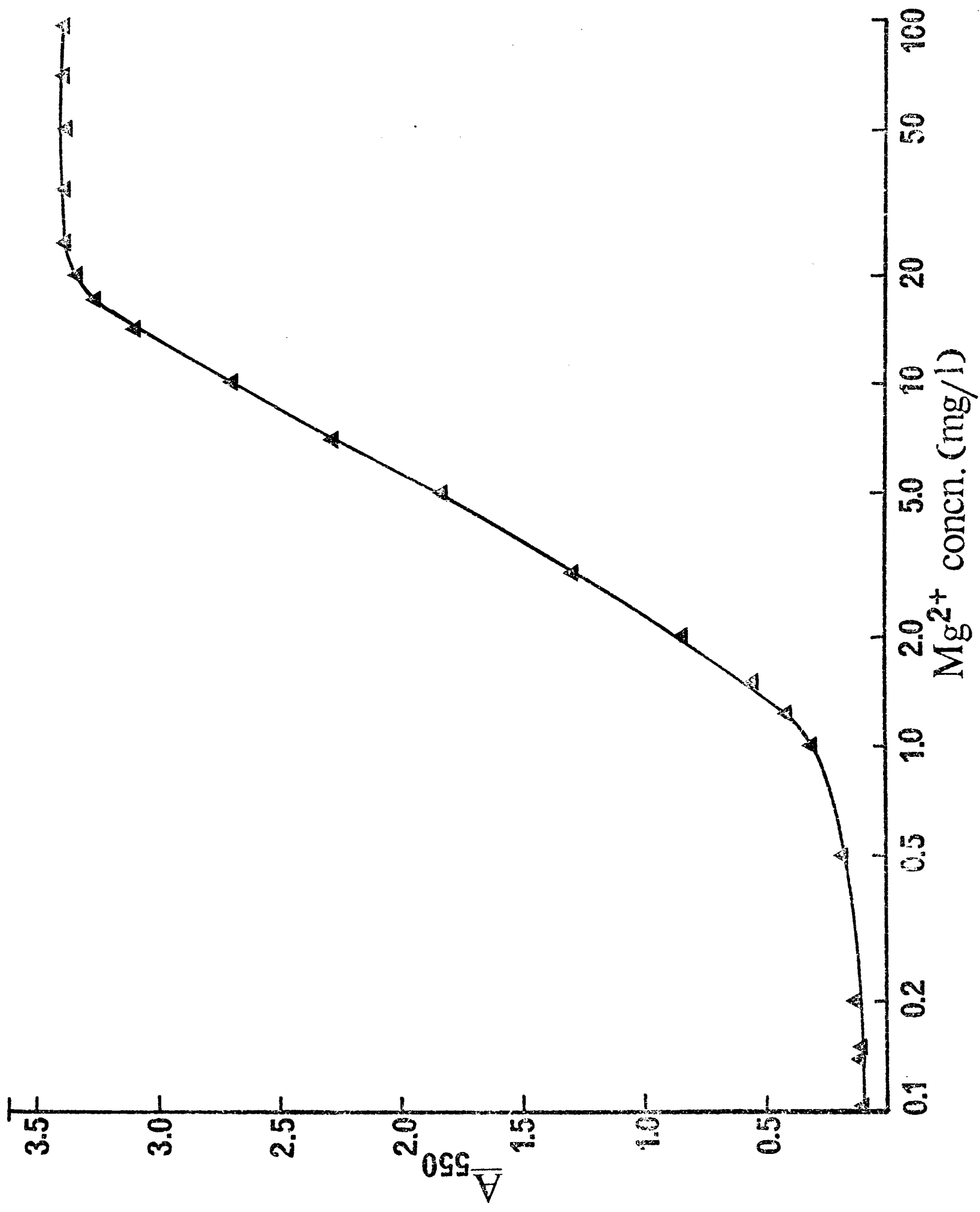
RESULTS

The effect of Mg^{2+} concentration on the growth of *Paracoccus denitrificans*. The basic medium used in the culture solutions was a modification of the minimal medium used by John & Whatley (1970). The modifications included titrating the $(\text{NH}_4)_2\text{HPO}_4$ with HCl in lieu of H_2SO_4 , and adding magnesium as the chloride salt instead of as the sulphate salt; these changes minimized the concentration of endogenous sulphate and enabled a greater control of the sulphur concentration in the culture solution. The effect of Mg^{2+} concentration upon the growth of *P. denitrificans* was studied to find the appropriate level of Mg^{2+} to add to the culture solution. Fig. 2.1 illustrates the effect of Mg^{2+} upon the growth of *P. denitrificans*. Growth is limited at low Mg^{2+} concentrations but is not inhibited by high concentrations of Mg^{2+} . The concentration of Mg^{2+} added to all subsequent culture solutions was therefore maintained at 25 mg/litre.

Fig. 2.1

The effect of Mg^{2+} concentration on the growth of Paracoccus denitrificans.

Cells were grown as described in Materials and Methods with succinate as substrate and nitrate as the terminal electron acceptor. The sulphate concentration was maintained at 54 mg S/litre.



Typical growth pattern of *P. denitrificans*. A typical growth curve for cells grown on succinate as substrate, and oxygen as the terminal electron acceptor, is shown in Fig. 2.2. A lag phase of between 6 and 8 hours and an optimal A_{550} of between 3.8 and 4.2 was obtained for these cells. Cells grown on succinate, with nitrate as the terminal electron acceptor, exhibited a lag phase of 8 to 10 hours and reached a maximum A_{550} of 3.2 to 3.7.

Relationship between pH and cell density. The pH of the culture medium roughly follows the curve for the A_{550} indicating that as the cell density increases, the medium becomes increasingly alkaline (Fig. 2.3). The increase in the alkalinity is due to the removal of the anion, succinate, from the medium, and, in cultures containing nitrate as the terminal electron acceptor, anaerobic growth also removes the anion, nitrate, from the medium replacing it with hydroxyl ions. A pH increase during denitrification has been previously noted by Allen & Van Neil (1955), Bovell (1967), and John (1969).

The effect of sulphur compounds on growth. The rate of growth increases as the sulphate concentration increases up to 10 mg of S/litre (0.21 mM). Increasing the sulphate concentration above this level has no effect upon growth (Fig. 2.4). At lower concentrations of sulphate, the cells deplete the medium of sulphate and the final cell density (A_{550}) is dependent on the total amount of sulphate available. These data show that the sulphur concentration in the basic medium is both non-limiting and non-inhibitory.

The effect of sulphite and sulphide concentration upon the growth of *P. denitrificans* is also illustrated in Fig. 2.4. The effect of these two inorganic sulphur compounds is very

Fig. 2.2

Growth curve for P. denitrificans grown on succinate as substrate and oxygen as the terminal electron acceptor.

The culture was grown as described in Materials and Methods.

Samples were withdrawn every hour, diluted, and the A₅₅₀ determined. The culture was inoculated with 0.01 of the culture volume of succinate-nitrate grown cells in mid log phase.

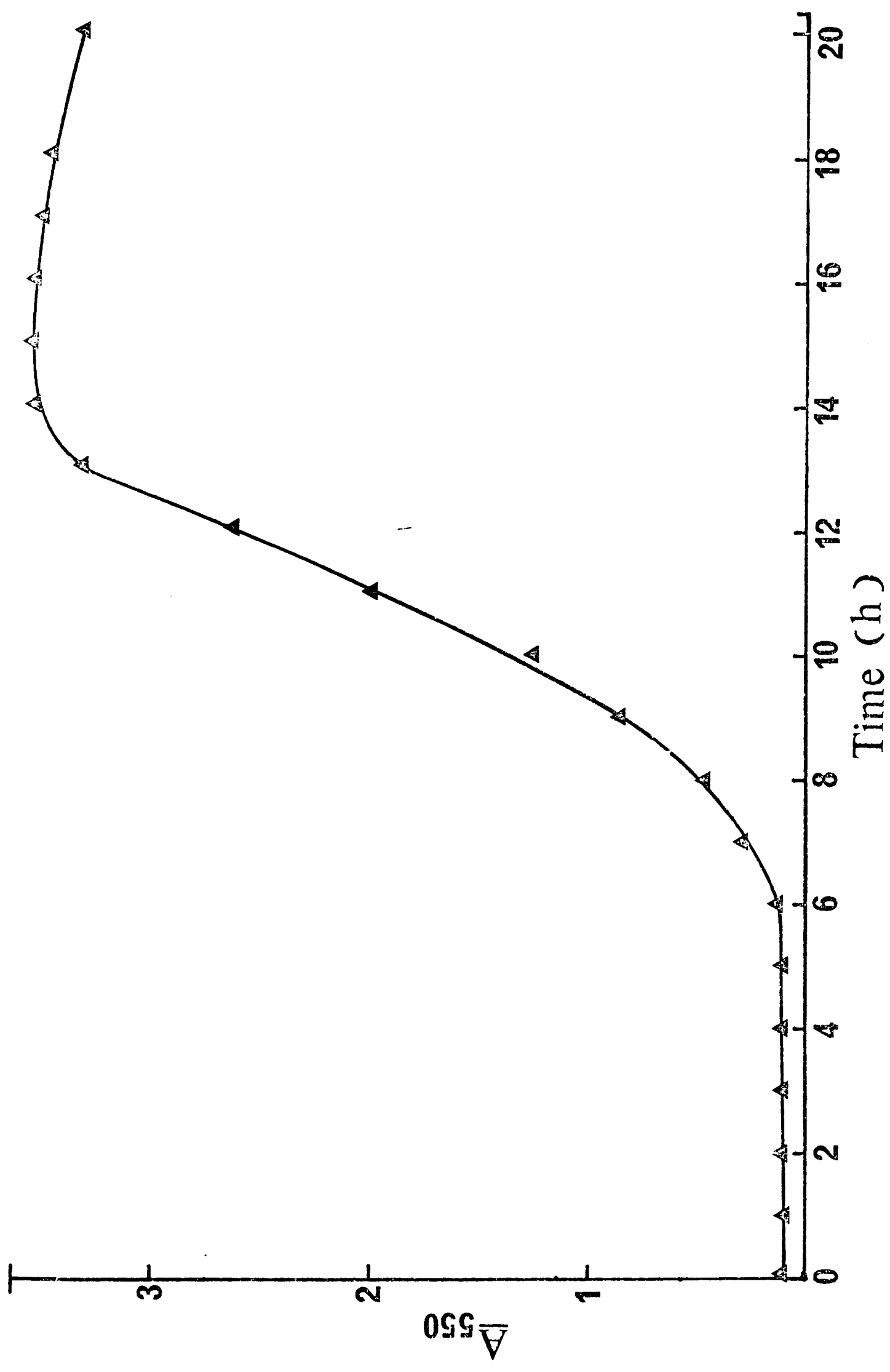


Fig. 2.3

The relationship between pH and the growth of P. denitrificans.

Cells were grown on succinate as substrate and nitrate as the terminal electron acceptor. The pH and the A_{550} of the culture medium were continuously monitored as described in Materials and

Methods.

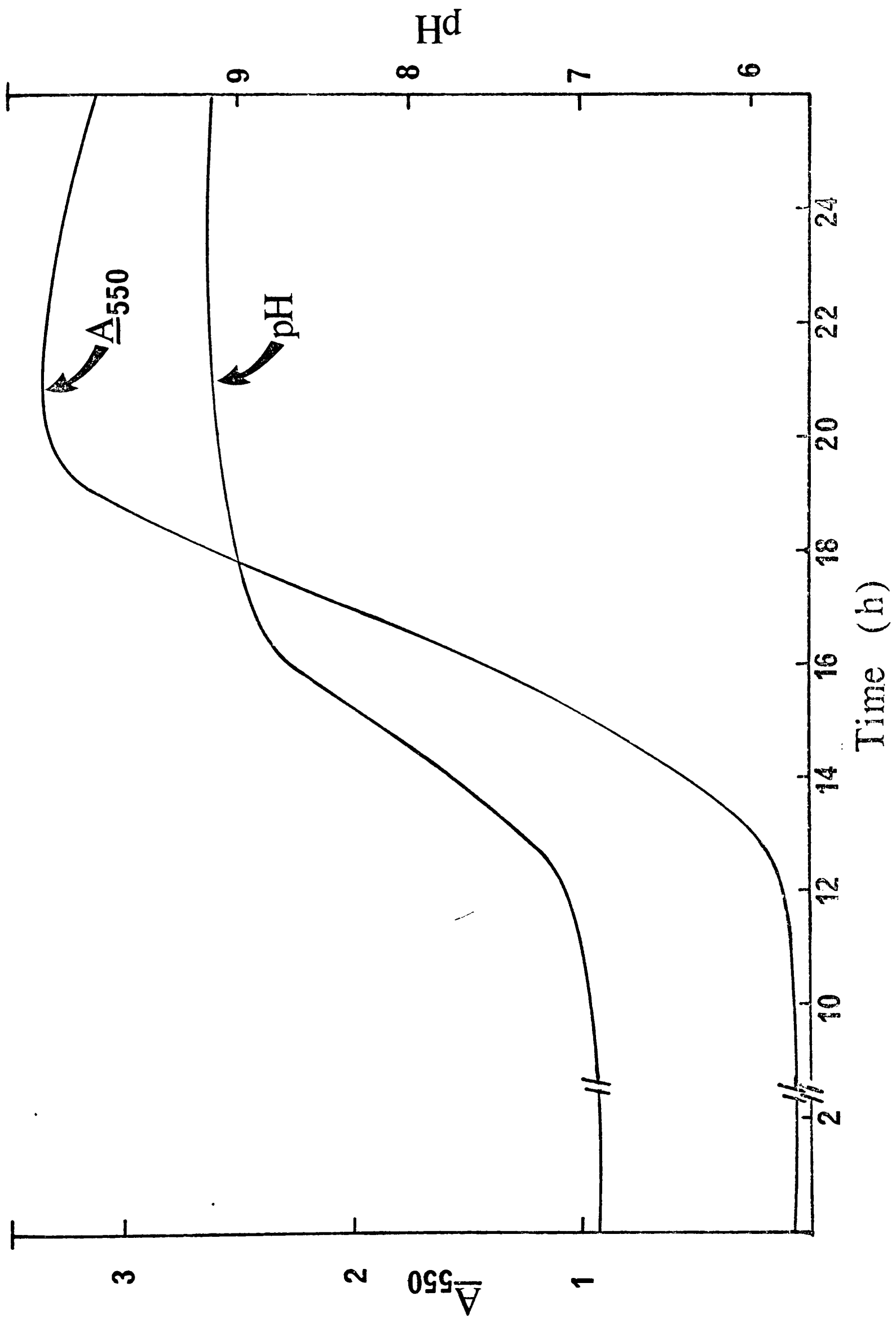


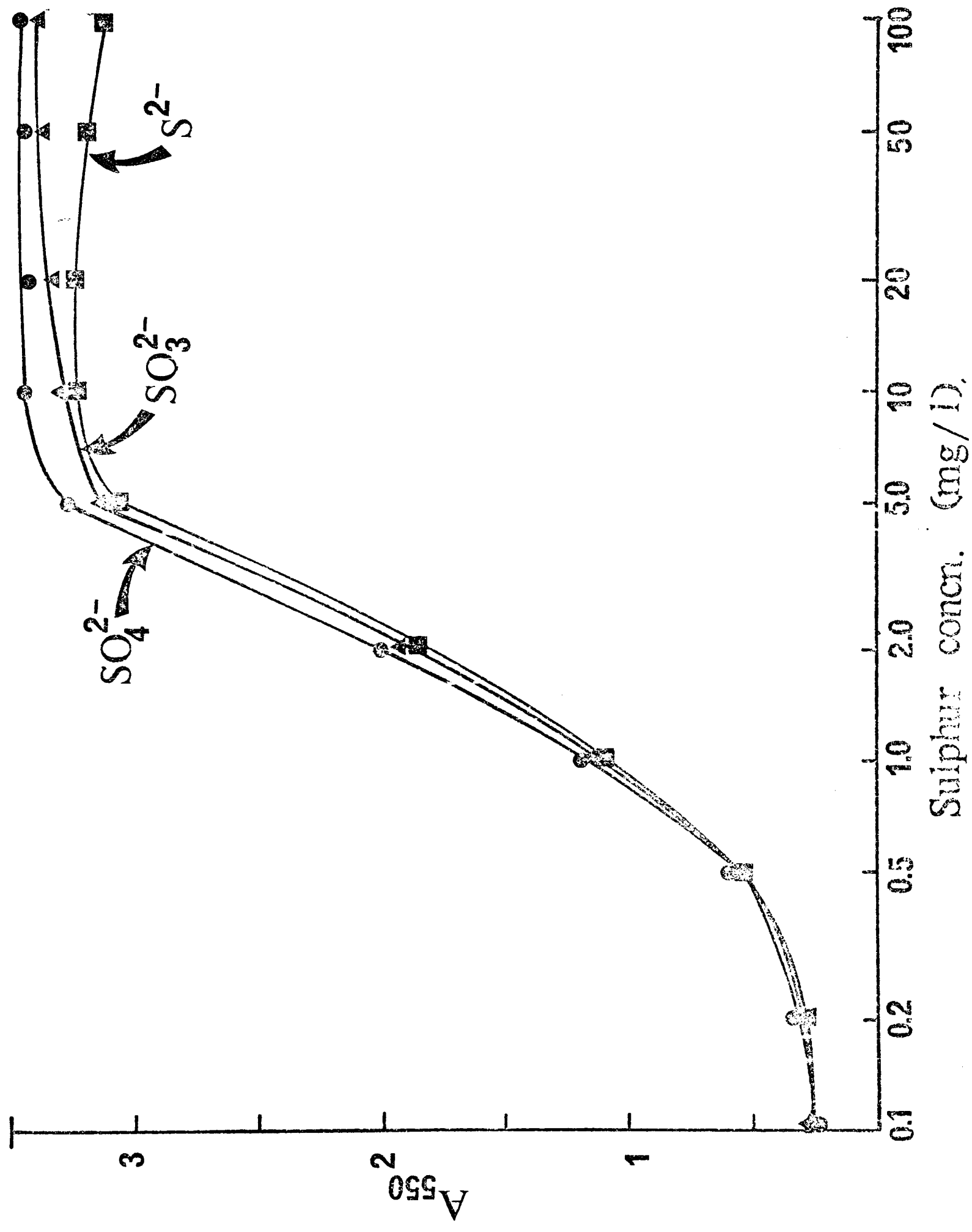
Fig. 2.4

Growth of P. denitrificans as a function of sulphur concentration.

Cells were grown as described in Materials and Methods using nitrate

as the terminal electron acceptor, except that the sulphur source was

as indicated; ●, Na_2SO_4 ; ▲, Na_2SO_3 ; ■, Na_2S .



similar to that of sulphate, except that, increasing the sulphide concentration above 50 mg of S/litre inhibits growth. This is not surprising since sulphide inhibits many enzymes (Dixon & Webb, 1965). The concentrations of sulphide shown in Fig. 2.4 are only approximate since some sulphide will be lost from the growth medium together with the N₂ and NO released by P. denitrificans growing on succinate and using nitrate as the terminal electron acceptor.

The effect of methionine and homocysteine upon the growth of Paracoccus denitrificans. P. denitrificans is unable to utilise either homocysteine or methionine as a sole source of sulphur for growth. However, the addition of methionine to growth media containing limiting concentrations of sulphate, caused an increased rate of growth (Table 2.1). This sparing effect

TABLE 2.1

Effect of methionine concentration upon the growth of P. denitrificans in the presence and absence of sulphate.

Cells were grown and the growth measured as described in the text except that the concentrations of sulphate and methionine were as specified in the table. Cells were grown and the A₅₅₀ measured 20 h after inoculation. Values are expressed as the A₅₅₀ of the culture.

Sulphate concentration mg of S/1	Methionine concentration mg of S/1		
	0	5	25
0	0	0	0
2	1.8	2.3	2.4
20	3.5	3.6	3.6

was also observed in culture solutions to which homocysteine was added. Growth of P. denitrificans was not inhibited by higher concentrations (50 mg of S/litre) of either methionine or homocysteine. The failure of P. denitrificans to utilise methionine and homocysteine as sole sulphur sources for growth might be due to its inability either, (a) to take up methionine or homocysteine from the culture solution, or, (b) to convert these amino acids, after absorption, into utilizable forms of sulphur for growth.

The possibility that P. denitrificans was incapable of transporting methionine was investigated further. A brief study of the uptake of ^{14}C -labelled methionine was conducted. Whole cells of P. denitrificans accumulated [^{14}C] methionine (Fig. 2.5). Furthermore the rate of methionine uptake was not affected by the addition of sulphate, sulphite, thiosulphate or sulphide but was decreased by cysteine (Table 2.2).

TABLE 2.2

The effect of sulphur-containing compounds upon the uptake of methionine by whole cells of P. denitrificans

Additions	Methionine uptake (% of control)
Methionine (10mM)	100
Methionine (10mM) plus sulphate (10mM)	98
Methionine (10mM) plus sulphite (10mM)	103
Methionine (10mM) plus thiosulphate (10mM)	101
Methionine (10mM) plus sulphide (10mM)	95
Methionine (10mM) plus cysteine (10mM)	33
Methionine (10mM) plus homocysteine (10mM)	96

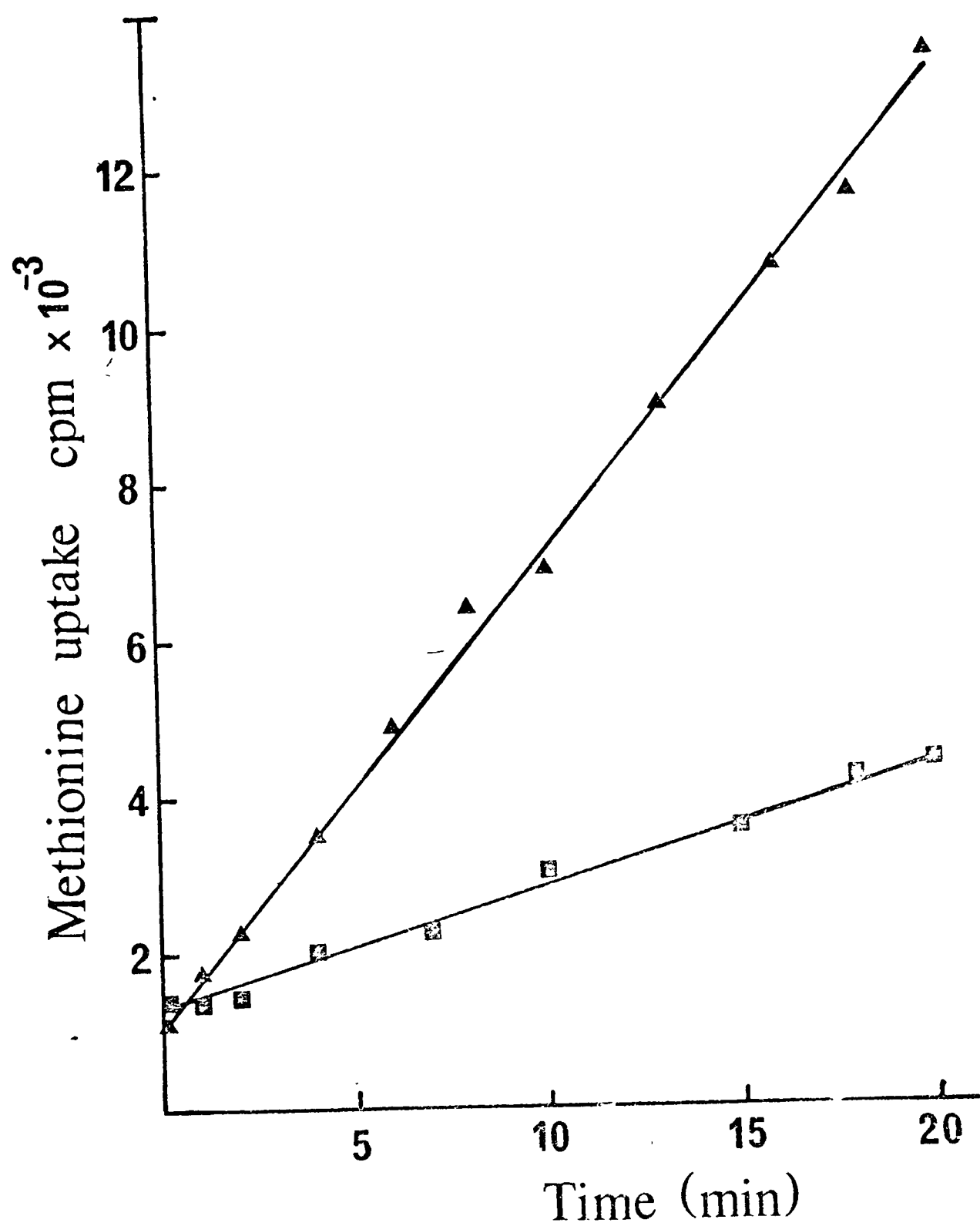


Fig. 2.5

Methionine uptake by whole cells of P. denitrificans. Methionine uptake was measured as described in Materials and Methods in the presence (■) and absence (▲) of 10 mM-L-cysteine.

Although P. denitrificans can accumulate methionine, it is incapable of converting methionine to utilisable forms of sulphur and, therefore, is unable to utilise it as a sole source of sulphur. These results, together with those reported in the literature, indicate a difference between strains of P. denitrificans, for in growth experiments with P. denitrificans strain 11, Banerjee (1966) detected growth of both mutant and wild type cells on methionine. He concluded from his results that cysteine could be made from methionine but not via the reversal of the methionine biosynthetic route. Animals and fungi are capable of converting methionine to homocysteine, and via cystathionine, to cysteine (Guggenheim, 1971). P. denitrificans (NCIB 8944) appears similar to both E. coli and S. typhimurium in possessing a metabolic pathway involving a unidirectional flow of sulphur from cysteine to methionine and being unable to utilise methionine as a sole sulphur source for growth. This unidirectional flow of sulphur will be discussed in more detail in Chapter 10.

Growth of P. denitrificans on other sulphur-containing compounds.

The inorganic (dithionate or bisulphite) and organic (L-cysteamine, L-cysteic acid, reduced glutathione, L-cystathionine, L-allo cystathionine and taurine) sulphur-containing compounds could neither support nor, had they any effect upon, the growth when added to culture solutions containing a utilisable sulphur source. L-Djenkolic acid could act as a sole sulphur source for growth, but the rate of growth was very slow and the final cell yields were approximately 35% of those grown with sulphate.

Effect of selenate upon the growth of *P. denitrificans*.

In the presence of low concentrations of sulphate, the growth of *P. denitrificans* is stimulated by low concentrations and inhibited by high concentrations of selenate (Fig. 2.6). The inhibition of growth by selenate could be completely removed by increasing the concentration of sulphate in the medium thus indicating that the inhibition of growth by selenate was competitive with respect to sulphate. In the absence of added sulphate no growth was observed with increasing selenate concentrations. Growth in the presence of selenate was characterised by a longer lag phase, a linear, rather than an exponential, growth phase and a lower final cell yield (Fig. 2.7). The stimulation of growth by selenite was approximately 30% as great as with equivalent concentrations of selenate and, further increasing the selenite concentration inhibited growth more efficiently than equivalent concentrations of selenate. Growth was estimated by light absorbance rather than by a direct viable cell count. Consequently, a long lag phase would also be observed if only a small portion of the cells in the inoculum were viable.

The long lag phase may reflect a requirement for some essential sulphur compound before growth can begin. In the presence of selenate, an analogue of sulphate, the synthesis of any critical sulphur compound can only occur after sulphate has been taken up by the cell and metabolised; selenate is a competitive inhibitor of both sulphate uptake (Chapters 4 & 5) and ATP sulphurylase (Chapter 6). Furthermore, the rate of synthesis of sulphur metabolites in the presence of high intracellular levels of selenium analogues may be extremely slow.

Fig. 2.6

The effect of selenate concentration on the growth of P. denitrificans.

Anaerobic culture solutions (succinate as substrate and nitrate as the terminal electron acceptor) were grown as described in Materials and

Methods. Culture solutions contained either no sulphate (Φ), or 5 mg S/litre (Φ). The concentration of selenate added to the culture solutions was as indicated in the figure. The increase in cell number was monitored as the A_{550} after 20 h after inoculation.

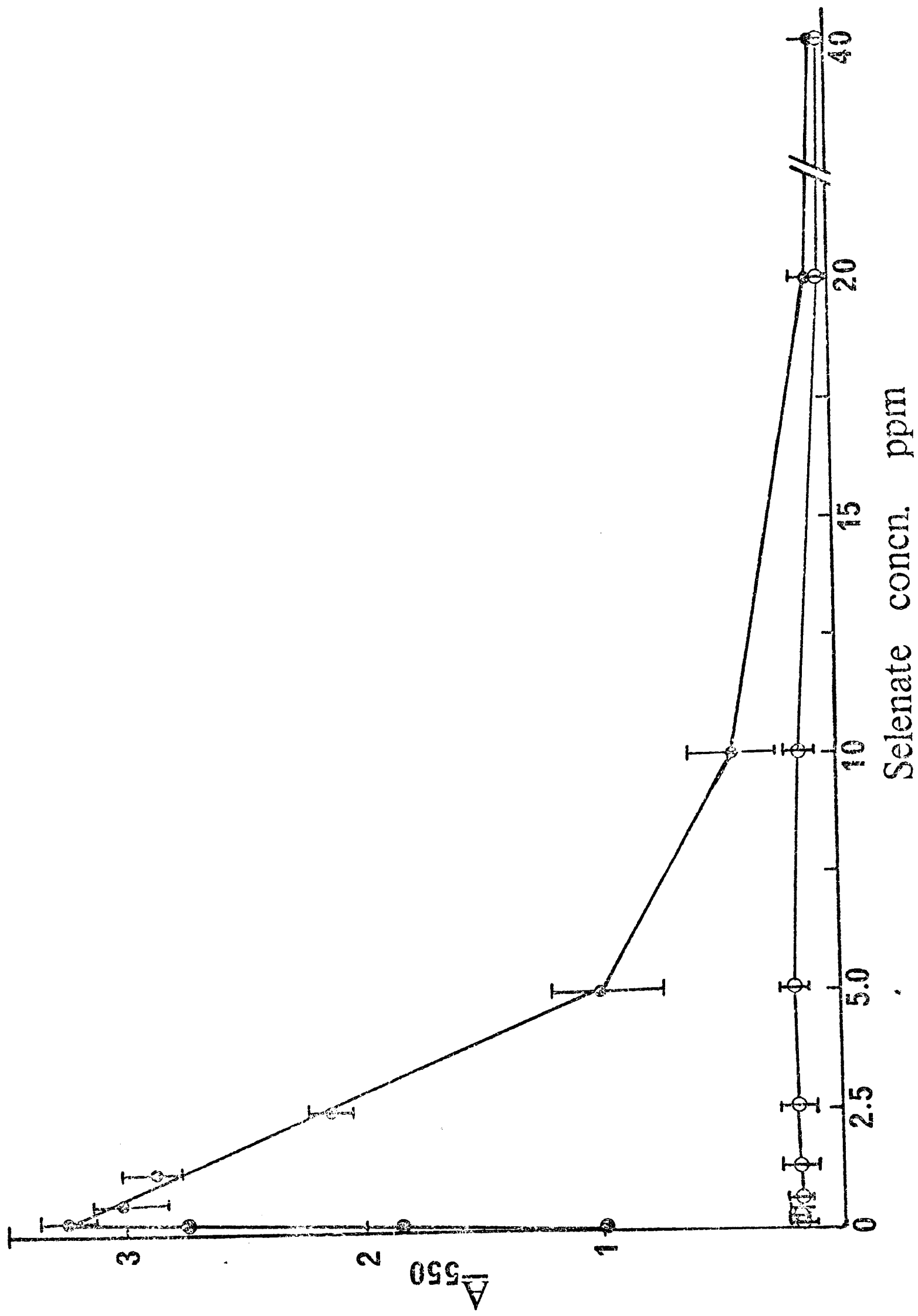
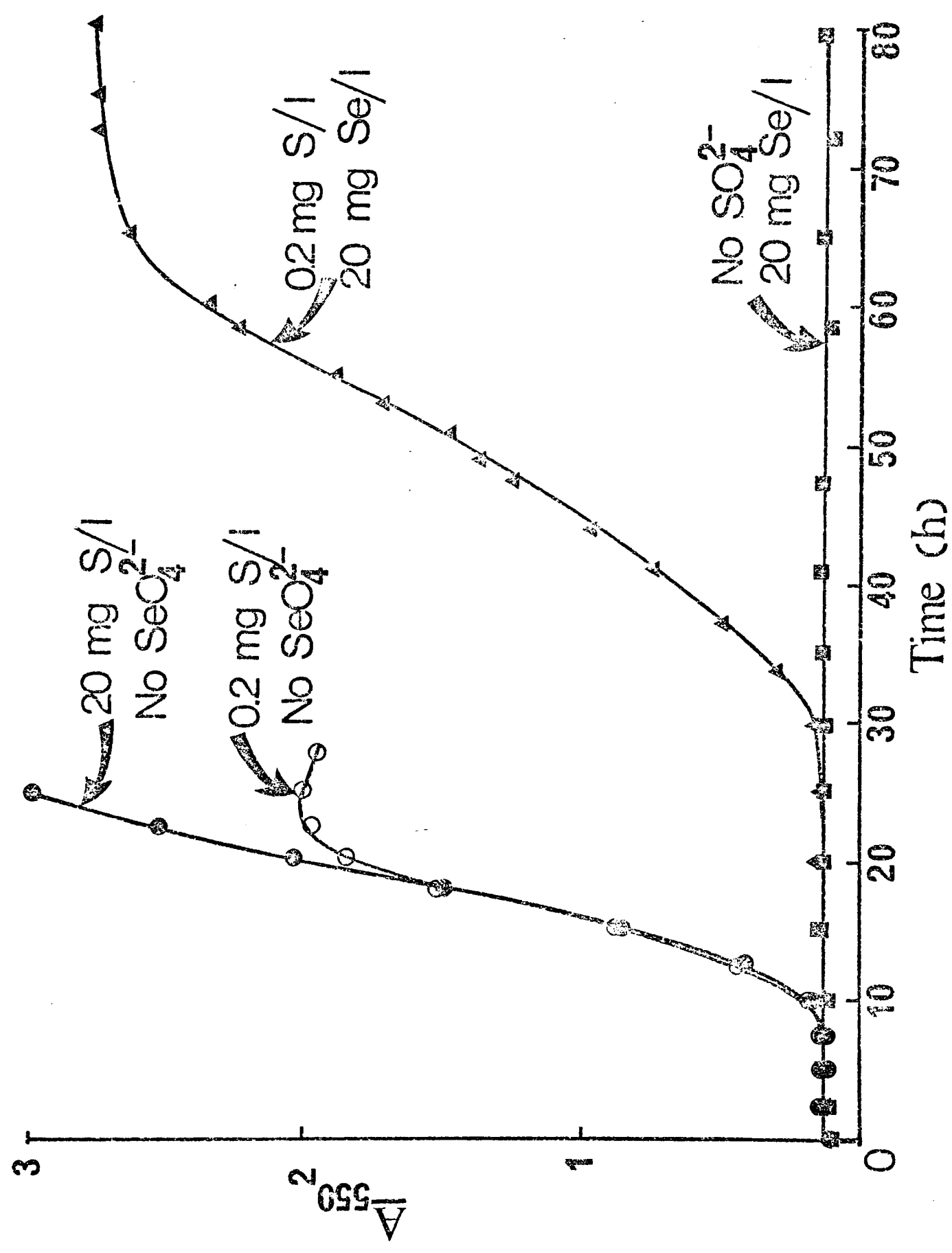


Fig. 2.7

Growth curves of P. denitrificans grown on sulphate and selenate. Cells were grown as described in Materials and Methods with succinate as substrate and nitrate as the terminal electron acceptor with; 20 mg of S/litre supplied as sulphate, (●); 0.2 mg of S/litre supplied as sulphate, (○); 20 mg of Se/litre supplied as selenate, (■); or, 20 mg of Se/litre supplied as selenate plus 0.2 mg of S/litre supplied as sulphate, (▲).



The linear growth observed in media containing inhibitory levels of selenate is reminiscent of the type of bacterial growth observed in the presence of amino acid analogues (Richmond, 1962; Cohen & Munier, 1959; Munier & Cohen, 1959; Cowie et al., 1959; Neale & Tristram, 1963). The explanation for the linear growth curve is unknown.

Factors required in trace amounts for growth are generally considered to play a catalytic role in the metabolism of the organism. Pinsent (1954) found that selenite and molybdate are specifically required by members of the coli-aerogenes group of bacteria in the formation of formic dehydrogenase. More recently, Rotruck et al., (1973) demonstrated the biochemical role of selenium as a component of glutathione peroxidase. The evidence presented in Fig. 2.6 suggests that the stimulatory effect of growth of low selenium concentrations may be supplying an essential trace element, and only at higher concentrations does it interfere with sulphur metabolism and (or) other metabolic routes.

Shrift & Kelly (1962) described experimental conditions under which E. coli K12 was adapted to grow in the presence of otherwise toxic levels of K_2SeO_4 (0.01M) and in the absence of sulphate. These cells were found to grow exponentially after a short lag period. Subsequent data of Huber et al., (1967) suggests that, in experiments conducted by Shrift & Kelly, the extent of contamination of the chemicals by sulphur was high enough to allow E. coli to grow in the presence of selenate. Furthermore, Huber et al., (1967) found that growth, in the presence of selenate, is essentially linear rather than exponential, a characteristic also of the growth of P. denitrificans when grown on selenate (Fig. 2.7).

Shrift & Kelly (1962) reported that E. coli K12 adapted to growth on selenate. However, P. denitrificans was unable to adapt to growth on either selenate or selenite in the absence of sulphate. In several microorganisms including, Saccharomyces cerevisiae (Fels & Cheldelin, 1949; Pengra & Berry, 1953), Aspergillus nidulans (Weissman & Trelease, 1955), Chlorella vulgaris (Shrift, 1954) and Desulfovibrio desulfuricans (Postgate, 1952) growth inhibition by selenate is a function of selenate concentration and cells do not adapt to the levels of selenium added to the culture solutions.

Selenium, in the form of selenite, has been included in enrichment media for the isolation of Salmonellae (Leifson, 1936; Shrift & Boullette, 1974). Salmonellae are one of a few groups of organisms (a restricted number of Astragalus spp. form another such group), in which so many species show a natural tolerance to selenium, usually a very toxic element (see reviews by Shrift, 1967, 1969). The biochemical basis for this tolerance, though investigated to some extent (McCready et al., 1966), is incompletely understood.

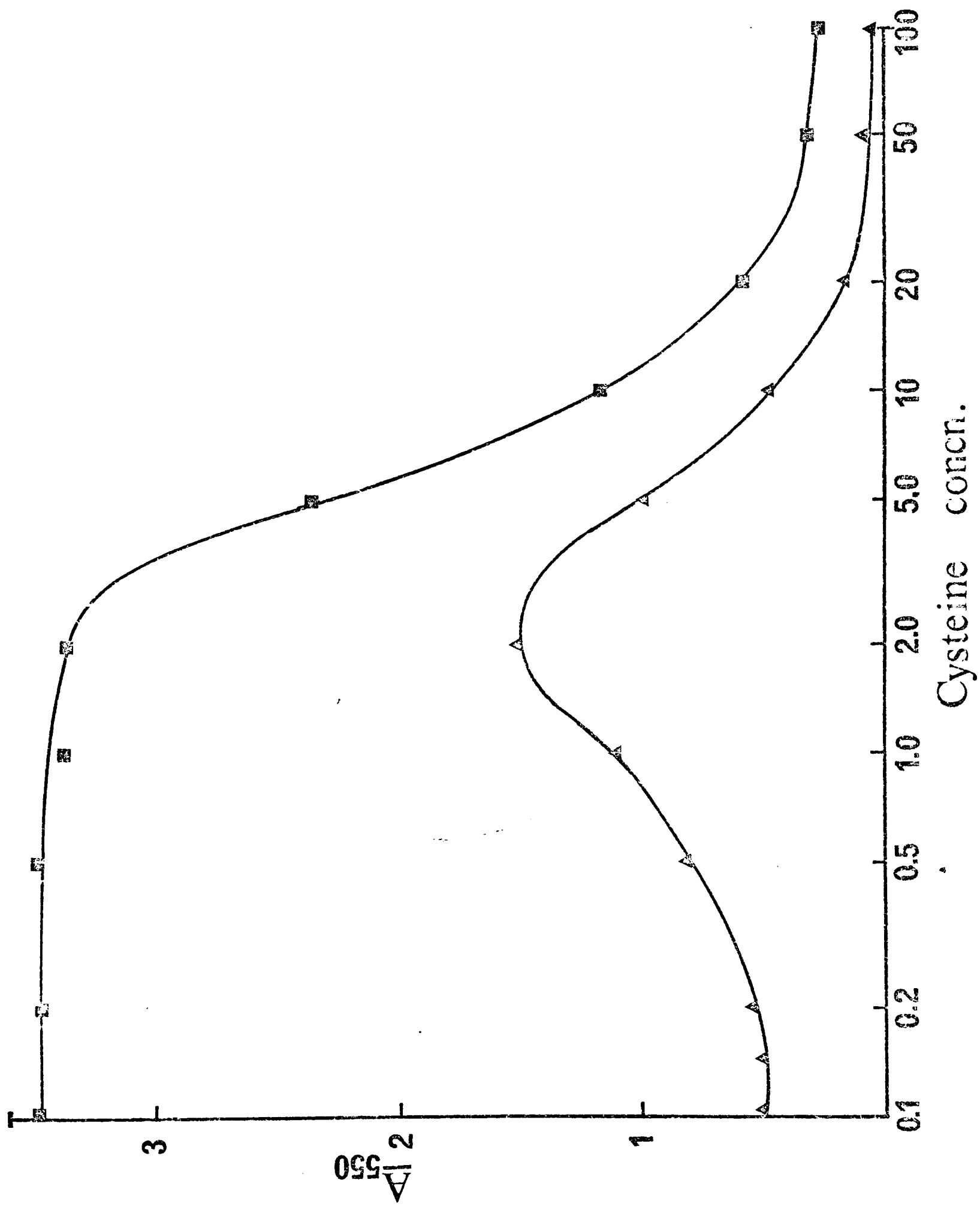
Effect of L-cysteine upon the growth of P. denitrificans.

L-Cysteine, added to a culture medium, inhibits cell growth. Fig. 2.8 shows the growth of P. denitrificans as a function of cysteine concentration added to a sulphur-free culture medium. A small amount of growth is obtained at low cysteine concentrations; this may be due to the slow oxidation of cysteine to cystine, a compound which P. denitrificans can readily utilise as a sole sulphur source for growth. At concentrations in excess of 3.0 mg of S/litre (supplied as cysteine) growth of P. denitrificans is depressed.

Fig. 2.8

Effect of L-cysteine on the growth of P. denitrificans.

Cell cultures were inoculated and grown as described in Materials and Methods except that sulphate was replaced with increasing concentrations of L-cysteine(▲), or increasing concentrations of cysteine were added to culture solutions containing sulphate (20 mg/1)(■). Nitrate was included in the culture solution as a terminal electron acceptor.



Growth inhibition involves more than the inability of the bacterium to use cysteine as a sole sulphur source; inhibition occurs when adequate sulphur (added as sulphate) is also present. Thus in Fig. 2.8 the sulphate concentration was maintained at 20 mg of S/litre while the concentration of cysteine was increased. The lack of inhibition at cysteine concentrations below 2 mg of S/litre may be due to the oxidation of cysteine to cystine (which it is able to utilise as a sole sulphur source), it may be due to cysteine concentrations being below the critical level for it to exhibit a depressing effect upon cellular metabolism, or it may also be due to a combination of these factors.

Inhibition of growth by cysteine has been reported for E. coli (Roberts et al., 1955; Nagy et al., 1968, 1969, 1970; Kari et al., 1971), Salmonella typhimurium (Collins et al., 1973) and yeast (Bhuvaneswaran et al., 1964). Collins and co-workers (1973) reported that cysteine, added to logarithmically growing cultures of S. typhimurium, caused an immediate cessation of growth, followed by a long lag phase before growth again resumed. They reported an enzyme, cysteine desulphydrase (L-cysteine hydrogen sulphide-lyase, deaminating EC 4.4.1.1) which was induced by high exogenous cysteine concentrations and degraded cysteine to H_2S , pyruvate and ammonia; S. typhimurium can then utilise H_2S as a sulphur source, so growth continued after induction of the enzyme, cysteine desulphydrase.

The inhibition of growth of E. coli by cysteine was found to be due to two separate effects (Kari et al., 1971). At low cysteine concentrations an antagonism between cysteine and leucine, isoleucine, threonine and valine could be detected and it was suggested that the biosynthesis of these amino acids

was inhibited by cysteine. At higher cysteine concentrations, a second effect was observed which was not relieved by the addition of leucine, isoleucine, threonine and valine. Kari et al., (1971) suggested that this effect was due to the inhibition of membrane-bound respiratory enzymes and, as a consequence of this action, caused an energy depletion of the cells of E. coli.

Both the presence of cysteine desulphydrase in P. denitrificans and the growth inhibitory effect of cysteine were investigated further.

Cysteine desulphydrase. No cysteine desulphydrase activity was detected in assays containing dialysed or undialysed cell-free extracts of P. denitrificans where enzyme activity was monitored by measuring both sulphide and pyruvate production. No cysteine-dependent pyruvate or sulphide production was detected in assays where the crude extract concentration (0.1 to 10 mg of protein/ml), the cysteine concentration (0.1 to 10.0 mM) and the pH (4.0 to 12.0) were all varied in turn. Cell-free extracts prepared from cells grown in the presence of cysteine did not possess cysteine desulphydrase activity. These cells were grown on sulphate (0.2 mM) and varying concentrations of L-cysteine (0, 1, 2, 5, and 10 mM) were added to the culture media during the log phase of growth. Cells were harvested after 8 h and cell-free extracts, in addition to toluene-treated (0.02%, v/v) cells, examined for cysteine desulphydrase activity. No cysteine desulphydrase activity was found in assays containing dithiothreitol (1 mM), MgCl_2 (10 mM), or pyridoxal phosphate (0.1 mM) added separately, or in combination.

Cysteine-dependent pyruvate and sulphide production were detected in assays containing cysteine-grown, toluene-treated cells of S. typhimurium, thus confirming the validity of the assay.

Banerjee (1966) has reported the presence of cysteine desulphydrase activity in crude extracts of P. denitrificans strain 11 grown in the presence of yeast extract. (Yeast extract contains cysteine). Examination of P. denitrificans NCIB 8944 crude extracts, prepared from cells grown as described by Banerjee (1966), also proved negative. Therefore, cysteine dissimilation in the two strains of P. denitrificans is not the same.

The existence of a specific, inducible cysteine desulphydrase in S. typhimurium is firmly established (Guarneros & Ortega, 1970; Kredich et al., 1973). However, the desulphurylation of cysteine to hydrogen sulphide, by extracts of bacteria and animal tissues, has often been attributed to cysteine desulphydrase although there is strong evidence to indicate that cystathionase (Rowbury & Woods, 1964a), tryptophanase (Crawford and Ito, 1964), tryptophan synthetase B (Newton et al., 1965) and more complicated pathways (Fanshier & Kun, 1962) may also contribute to the pathway of cysteine dissimilation. Therefore, the absence of cysteine desulphydrase activity in P. denitrificans NCIB 8944 does not only reflect the absence of a specific desulphurylating enzyme, but it also reflects the inability of other enzymes to dissimilate cysteine as an alternative substrate.

Relief of the inhibitory effect of cysteine. The growth inhibitory effect of 2 mM-cysteine was partially relieved by

the addition of leucine, isoleucine, threonine and valine; leucine and isoleucine were more effective than threonine or valine. The most significant reversal of cysteine inhibition was obtained with a mixture of all four amino acids (Table 2.3). Only the L-isomers of these amino acids were effective. The growth inhibitory effect was not relieved by either norleucine or norvaline.

TABLE 2.3

Effect of leucine, threonine, isoleucine and valine on inhibition of growth of *Paracoccus denitrificans* by cysteine.

P. denitrificans was grown with succinate as substrate and nitrate as the terminal electron acceptor at 30°C. Logarithmically growing cultures were treated with compounds indicated. Leucine, isoleucine, threonine and valine were each added to a concentration of 50µg/ml. The degree of growth inhibition was calculated as described by Nagy et al., (1968).

Treatments	Growth inhibition (%)	
	with 2mM cysteine	with 10mM cysteine
Cysteine	82	9
Cysteine plus L-leucine	56	11
Cysteine plus L-isoleucine	58	12
Cysteine plus L-threonine	64	8
Cysteine plus L-valine	63	10
Cysteine plus L-leucine, L-isoleucine, L-threonine and L-valine	15	14

Growth inhibition (%)= 100 -

growth rate of bacteria in the presence of cysteine

growth rate of bacteria without cysteine

x100

In the presence of a mixture of the four amino acids growth of P. denitrificans was inhibited by 10 mM-cysteine (Table 2.3).

The growth inhibitory effect of cysteamine, the decarboxylated product of L-cysteine was also examined. Cysteamine at a concentration of 0.1 to 10 mM, can neither act as a sole sulphur source for growth, nor does it affect the growth of cells in the presence of added sulphate. Furthermore, the addition of any, singly, or all, of the amino acids antagonistic to cysteine inhibition did not promote growth.

Similar results for the inhibition of cell growth by cysteine have been reported for E. coli (Nagi et al., 1968, 1969, 1970; Kari et al., 1971). These authors have suggested that the inhibition due to cysteine can be due to two effects.

- 1) At low concentrations (2 mM), two enzymes concerned with the biosynthesis of leucine, isoleucine, threonine and valine are inhibited in vitro by cysteine; homoserine dehydrogenase (Datta, 1967, 1969) and acetohydroxy acid synthetase (Leavitt & Umbarger, 1961). This inhibition is overcome by the addition of the four amino acids due to the fact that if both enzyme activities are simultaneously inhibited, the bacteria will be starved for the four amino acids (Umbarger & Davis, 1962).
- 2) At higher concentrations, inhibition by cysteine is due to inhibition of the membrane-bound respiratory enzymes and, via this action, causes energy depletion of the cells (Kari et al., 1971). Since little is known about the effect of cysteine on the respiratory pathway, the effects of cysteine and cysteamine upon respiration in membrane vesicles of P. denitrificans, isolated from cells grown with succinate as substrate and nitrate as the terminal electron acceptor, were examined.

Cysteine inhibition of respiration in membrane vesicles.

Increasing concentrations of cysteine inhibit respiration in membrane vesicles of P. denitrificans Table 2.4 and Fig. 2.9).

TABLE 2.4

Inhibition of respiration in membrane vesicles of P. denitrificans by cysteine.

Respiratory substrate	Respiratory rates (% inhibition)							
	Cysteine concn. (mM).				Cysteamine concn. (mM).			
	0	5	10	20	0	5	10	20
Succinate	0	60	90	100	0	0	16	30
NADH	0	19	65	90	0	0	0	0
Ascorbate-TMPD	0	0	17	93	0	0	0	0

The inhibition of respiration by cysteine varies with the substrate used; the inhibition of respiration is greatest when succinate is the respiratory substrate, less with NADH and least when ascorbate-TMPD is used as the respiratory substrate (Fig. 2.9). Cysteamine only inhibits respiration when succinate is the respiratory substrate and this inhibition is much less than the inhibition observed with cysteine. Bhuvaneshwaran et al., (1964) reported that cysteine inhibited the uptake of oxygen in yeast; this was relieved by the addition of metal ions; Fe^{3+} being the most effective. Bhuvaneshwaran et al., (1964) also reported that inhibition of respiration by cysteine is due to cysteine forming complexes with metal ions. This view is supported by Albert (1952) who found that cysteine has a high affinity for metal ions.

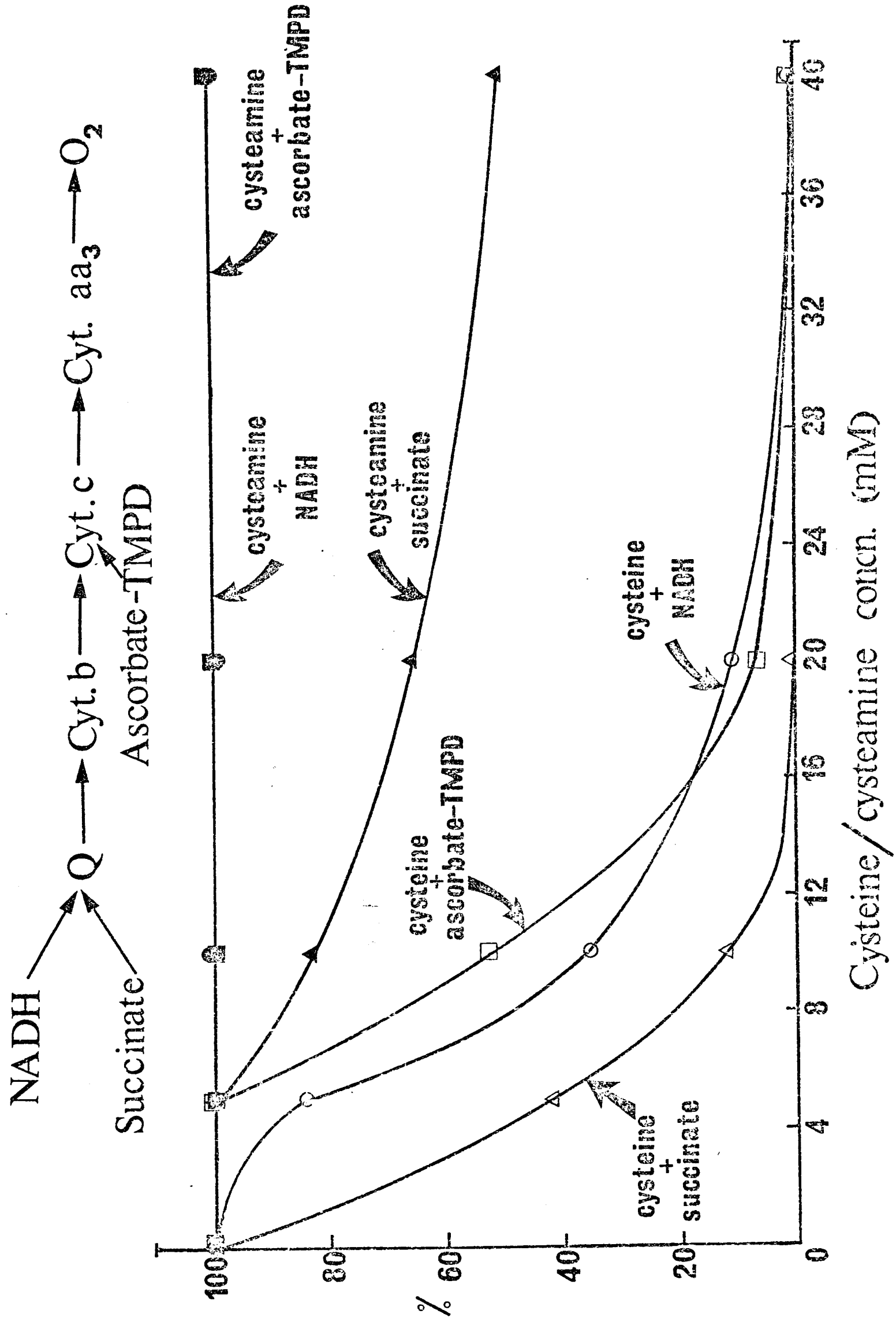
From the results in Table 2.4 and Fig. 2.9, it can be

Fig. 2.9

Effect of cysteine and cysteamine on the respiration in membrane vesicles of P. denitrificans.

The effect of cysteine and cysteamine upon the respiratory rates of membrane vesicles were studied as described in the Materials and Methods using the respiratory substrates indicated. Open symbols, cysteine; closed symbols, cysteamine; Δ , succinate; $\circ$, $\bullet$, NADH; $\square$, $\blacksquare$, ascorbate-TMPD.

The respiratory chain of P. denitrificans (John & Whatley, 1975) is also illustrated.



suggested that the inhibition by cysteine of respiration, using succinate as substrate, may be due to the complexing of a non-haem iron associated with succinate dehydrogenase and essential for its activity (Scholes & Smith, 1968b). A similar explanation can be advanced to explain the inhibition by cysteine of NADH-dependent respiration; NADH dehydrogenase also requiring a non-haem iron for its activity (Imai et al., 1968), but being less sensitive to inhibition by cysteine. Inhibition of ascorbate-TMPD - dependent respiration may be due to cysteine complexing with the Cu^{2+} associated with cytochrome $a + a_3$ oxidase (Lemberg, 1969). The ascorbate-TMPD - dependent respiration is the least sensitive of the three respiratory enzymes, implicated in this study of inhibition due to cysteine, suggesting that cysteine has a higher affinity for the non-haem iron associated with succinate- and NADH-oxidation than for the Cu^{2+} associated with the cytochrome oxidase. Since leucine, isoleucine, threonine and valine do not affect cysteine-metal ion complexing, they cannot remove the inhibition of cell growth at high concentrations of cysteine (Table 2.3).

Assimilatory/dissimilatory growth of *Paracoccus denitrificans*.

In the presence of the carbon sources formate, succinate, glucose, or $\text{H}_2\text{-CO}_2$, *P. denitrificans* is able to grow when nitrate or oxygen are supplied as the terminal electron acceptors but is unable to grow when sulphate, sulphite or thiosulphate are supplied as the terminal electron acceptors. Addition of yeast extract, ascorbic acid or H_2S had no effect upon cell cultures containing inorganic sulphur compounds as terminal electron acceptors (Table 2.5).

TABLE 2.5

The effect of growth conditions upon the assimilatory/
dissimilatory growth of *P. denitrificans*.

SUBSTRATE	TERMINAL ELECTRON ACCEPTOR				
	NO_3^-	SO_4^{2-}	SO_3^{2-}	$\text{S}_2\text{O}_3^{2-}$	O_2
Formate or succinate	✓	x	x	x	✓
Formate or succinate plus yeast	✓	x	x	x	✓
Formate or succinate plus yeast and a.a.	✓	x	x	x	✓
Formate or succinate plus yeast, a.a., H_2S	✓	x	x	x	✓
Glucose	✓	x	x	x	✓
Glucose plus yeast	✓	x	x	x	✓
Glucose plus yeast and a.a.	✓	x	x	x	✓
Glucose plus yeast, a.a. and H_2S	✓	x	x	x	✓
$\text{H}_2\text{-CO}_2$	n.d.	x	n.d.	n.d.	✓
$\text{H}_2\text{-CO}_2$ plus yeast	n.d.	x	n.d.	n.d.	✓
$\text{H}_2\text{-CO}_2$ plus yeast and a.a.	✓	x	n.d.	x	✓
$\text{H}_2\text{-CO}_2$ plus yeast, a.a. and H_2S	✓	x	n.d.	x	✓

n.d. indicates not determined

✓ indicates growth

x indicates no growth

Growth conditions were as described in the Methods and Materials.

Ascorbic acid was added to a final concentration of 5 mM and
yeast extract 0.5 g/l.

Dissimilatory growth on sulphate by Desulfovibrio desulphuricans, Desulfotomaculum orietis and Dm. nigrificans is increased by the addition of yeast extract to culture medium (Adams & Postgate, 1959; Butlin et al., 1949). Furthermore ascorbic acid, and H_2S were added to lower the redox potential of the culture solution since all dissimilatory sulphate-reducing bacteria require the redox potential of the culture media to be poised between 0 to -200 mV, (Grossman & Postgate, 1953; Abd-el-Malek & Rizk, 1959, 1960, Postgate, 1959a; Alico & Liegey, 1966). Grossman & Postgate (1953) have used cysteine to lower the redox potential of culture solutions for growing dissimilatory sulphate-reducing bacterium. However, cysteine could not be used to lower the E_h of the cultures because earlier experiments demonstrated that cysteine inhibits the growth of P. denitrificans. Therefore only ascorbic acid and H_2S were used to lower the E_h of the culture solutions.

Anaerobic conditions were maintained in cultures of P. denitrificans by sparging the culture solutions with a gas mixture of CO_2/N_2 (5%, v/v).

From this study, it may be concluded that P. denitrificans is an assimilatory sulphate reducing organism according to Postgate's (1958) classification. (Sulphate-reducing bacteria cannot reduce nitrate; in fact nitrate inhibits the growth of some of the sulphate-reducing bacteria (Allen, 1949; Senez et al., 1956).

SUMMARY

The growth experiments reported in this chapter are consistent with an inorganic pathway of sulphate reduction through sulphite and sulphide. The inability of P. denitrificans to

utilise methionine, homocysteine or cystathionine as sole sources for growth is consistent with the proposal that the cysteine-methionine pathway is irreversible.

The growth of P. denitrificans is inhibited by high concentrations of selenate; this inhibition is competitive with respect to sulphate. Cysteine depresses the growth of P. denitrificans: at low concentrations cysteine inhibits amino acid metabolism, and at high concentrations cysteine inhibition is due to the inhibition of respiration by cysteine.

Finally this chapter reports experiments which demonstrate that P. denitrificans is strictly an assimilatory sulphate-reducing organism.

CHAPTER 3

STUDIES OF THE INCORPORATION OF $^{35}\text{SO}_4^{2-}$ INTO CELLS

INTRODUCTION

The metabolism of sulphur-containing compounds has been studied in many microorganisms but relatively little is known about sulphur metabolism in Paracoccus denitrificans. Evidence advanced from growth experiments described in Chapter 2 indicates that P. denitrificans is an assimilatory sulphate-reducing organism according to the classification of Postgate (1959).

Since this thesis is primarily concerned with the control and regulation of sulphur metabolism, insight into the overall pathway of sulphur assimilation in P. denitrificans is desirable for a study of the effects of sulphur-containing compounds, known to be involved in sulphur metabolism in P. denitrificans, upon various systems within the pathway.

Many microorganisms can use inorganic sulphate and other inorganic sulphur compounds as their sole sulphur source for growth. Several pathways have been proposed for the conversion of sulphate to sulphide. These proposals have been based on evidence obtained by indirect experimental techniques which include:-

- (1) growth studies: including growth studies with a series of related biochemical mutants, each one supposedly blocked at a single reaction, and cross-feeding experiments using these mutants,
- (2) the isolation of a proposed intermediate from cells,
- (3) isotopic or substrate competition experiments with growing cells, and
- (4) in vitro reactions carried out with cell-free extracts and partially purified enzymes.

While the last method (4) provides the most convincing evidence for a proposed pathway, the presence of specific enzymes in

cells does not per se establish the existence of a given reaction in vivo. Ideally, the metabolic pathway in the whole, living organism should be investigated under physiological conditions.

This chapter reports experiments in which the utilisation of inorganic sulphate was studied in whole, living cells of P. denitrificans. Time-course and pulse-chase experiments were used in this study.

MATERIALS AND METHODS

Chemicals APS and all L-amino acids were obtained from the Sigma Chemical Co., London, U.K. Na γ - ^{35}S - SO_4 (carrier free) was obtained from the Radiochemical Centre, Amersham, Bucks., U.K. N-Ethyl maleimide was obtained from British Drug Houses, Dorset, U.K. PAPS was synthesised as described below. All other chemicals were obtained from the sources described in Chapter 2.

Preparation of PAPS γ - ^{35}S PAPS was prepared from ATP and carrier free γ - ^{35}S - SO_4^{2-} by the method described by Roy (1960) except that the concentration of ATP was increased to 20 mM and a cell-free extract from Escherichia coli, strain 9723, grown on L-djenkolic acid (Jones-Mortimer, 1965) was used as the source of sulphate-activating enzymes. PAPS formed after 12 h at 30° was isolated and purified by the method of Burnell & Anderson (1973).

Preparation of cells Cells of P. denitrificans were grown on medium containing succinate as substrate and nitrate as the terminal electron acceptor, as described in Chapter 2. Cells were harvested during the exponential phase of growth by centrifugation in an M.S.E. 18 centrifuge at 9 000g.

Bacteria were washed in succinate-nitrate medium minus sulphate (washing medium), resuspended in 5% vol. of washing medium, and reincubated at 30°C for 30 min to deplete the extracellular culture solution of sulphate and to reduce the amount of intracellular sulphate present.

Incorporation of γ - ^{35}S - SO_4^{2-} into whole cells. The pathway of sulphate metabolism was examined by following the incorporation of γ - ^{35}S - SO_4^{2-} into whole cells in time-course and pulse-chase experiments.

Time-course experiments. Incubation mixtures containing 9.9 ml of washed cell suspension were incubated at 30°C for 30 min to remove endogenous sulphate. At zero time 0.1 ml of $\text{Na}_2\gamma$ - ^{35}S - SO_4 containing 30 $\mu\text{Ci}/\mu\text{mole}$ was added. At various time intervals 1 ml aliquots were removed and added to 4 ml of washing medium at 2°C. The cell suspension was thoroughly mixed and the cells collected by centrifugation. The supernatant was discarded. The cells were resuspended in 1.0 ml of 95% (v/v) ethanol containing 25 μmoles of N-ethyl maleimide (Ellis, 1966). After 30 min incubation at 40°C 1.0 ml of water was added and the cells were sonically disrupted, and centrifuged at 20 000g for 20 min. The supernatant fraction was evaporated to dryness in a rotary evaporator at 30°C. The residue was dissolved in 0.5 ml of 50% (v/v) ethanol, and streaked onto acid-washed Whatman 3MM paper and subjected either to high voltage electrophoresis in Solvent I and solvent II, as described by Burnell & Anderson (1973), or to two-dimensional chromatography in solvents III and IV as described by Margolis & Mandl (1958). Following development, the electrophoretograms strips were cut into sections (1 cm) and the radioactivity of the strips counted by scintillation count-

ing as described in Chapter 2.

Pulse-chase experiments. Incubation mixtures were the same as those used for the time-course experiments. The ^{35}S -labelled sulphate was added at time zero. After 5 min 1.0 ml of $3\text{M}-\text{Na}_2\text{SO}_4$ was added, the incubation mixture thoroughly mixed, and samples (1 ml) were withdrawn at intervals, extracted and analysed by high voltage paper electrophoresis as described above.

Chromatography and electrophoresis. High voltage paper electrophoresis was conducted on acid-washed Whatman 3MM paper. Separation was carried out at 50 v/cm for 1 to 3 h using a Shandon Southern Model 3200 high voltage electrophoresis apparatus. Two-dimensional paper chromatography was conducted as described by Margolis & Mandl (1958). Chromatograms were run in solvent III, dried, and after rotation through 90° run in solvent IV. Amino acids were visualised by spraying chromatograms with an ethanolic solution of ninhydrin (0.25% w/v) and heating for 10 min at 110°C . Nucleotides were visualised by u.v. fluorescence quenching and sulphite and sulphide, by spraying with a solution of HgNO_3 (5% w/v) and heating. The N-ethyl maleimide derivatives were identified by co-chromatography with authentic compounds.

Solvent systems

- I. 0.1M-sodium citrate buffer, pH 5.0 (Balharry & Nicholas, 1970)
- II. formic acid (90% v/v)—glacial acetic acid (1:2, v/v), pH 2.0 (Smith, 1961)
- III. ethanol—t-butanol (58% v/v)—ammonia—water (120:40:10:30, by vol) (Varma & Nicholas, 1971).
- IV. t-butanol (88% v/v)—formic acid—water (140:30:30, by

vol) (Varma & Nicholas, 1971).

RESULTS

Separation and identification of ^{35}S -labelled compounds

Amino acid are readily separated from nucleotides and the N-ethyl maleimide derivatives of sulphite and sulphide by high voltage paper electrophoresis in both solvents I and II. The nucleotides and inorganic sulphur compounds migrated towards the anode whilst all the ^{35}S -labelled amino acids, except cysteic acid, migrated towards the cathode. Greatest resolution of nucleotides, NEM-SO_3^{2-} and NEM-S^{2-} was achieved in solvent I (see Table 3.1 for R_f values). Greatest resolution

TABLE 3.1

High voltage electrophoresis.

R_f values of ^{35}S -labelled cell metabolites which migrated towards the anode in solvent I. R_f values are expressed as the distance of migration relative to cysteic acid.

Compound	R_f value
Cysteic acid	1.00
Sulphate	0.93
Sulphite-NEM	0.77
Sulphide-NEM	0.66
PAPS	0.49
APS	0.32

of amino acids was achieved by using solvent II (see Table 3.2 for R_f values). Therefore, high voltage electrophoresis using solvents I and II was used as the routine method for resolving ^{35}S -labelled, ethanol-soluble metabolites in both

TABLE 3.2

High voltage electrophoresis.

R_f values of ^{35}S -labelled cell metabolites which migrate towards the cathode in solvent II. R_f values are expressed as the distance of migration relative to methionine.

Compound	R_f values
Cystathionine	1.12
Methionine	1.00
Methionine sulphone	0.93
Methionine sulfoxide	0.87
Cysteine-NEM	0.74
Homocysteine-NEM	0.68
Glutathione-NEM	0.50
Cystine	0.13

the time-course and pulse-chase experiments. Such compounds such as methionine sulfoxide, methionine sulphone and cystathionine were eluted from electrophoretograms and their identity confirmed by "finger-printing" with the authentic compounds using two-dimensional descending paper chromatography.

Incorporation of γ - ^{35}S sulphate by cell suspensions.

The uptake of γ - ^{35}S sulphate cells resuspended in the washing medium was linear for 2 h showing that the concentration of sulphate in the medium was not limiting. The total incorporation of γ - ^{35}S sulphate by cells is shown in Fig. 3.1.

The distribution of ^{35}S in various sulphur-containing compounds as a function of time.

(1) Time-course experiments. The quantitative distribution

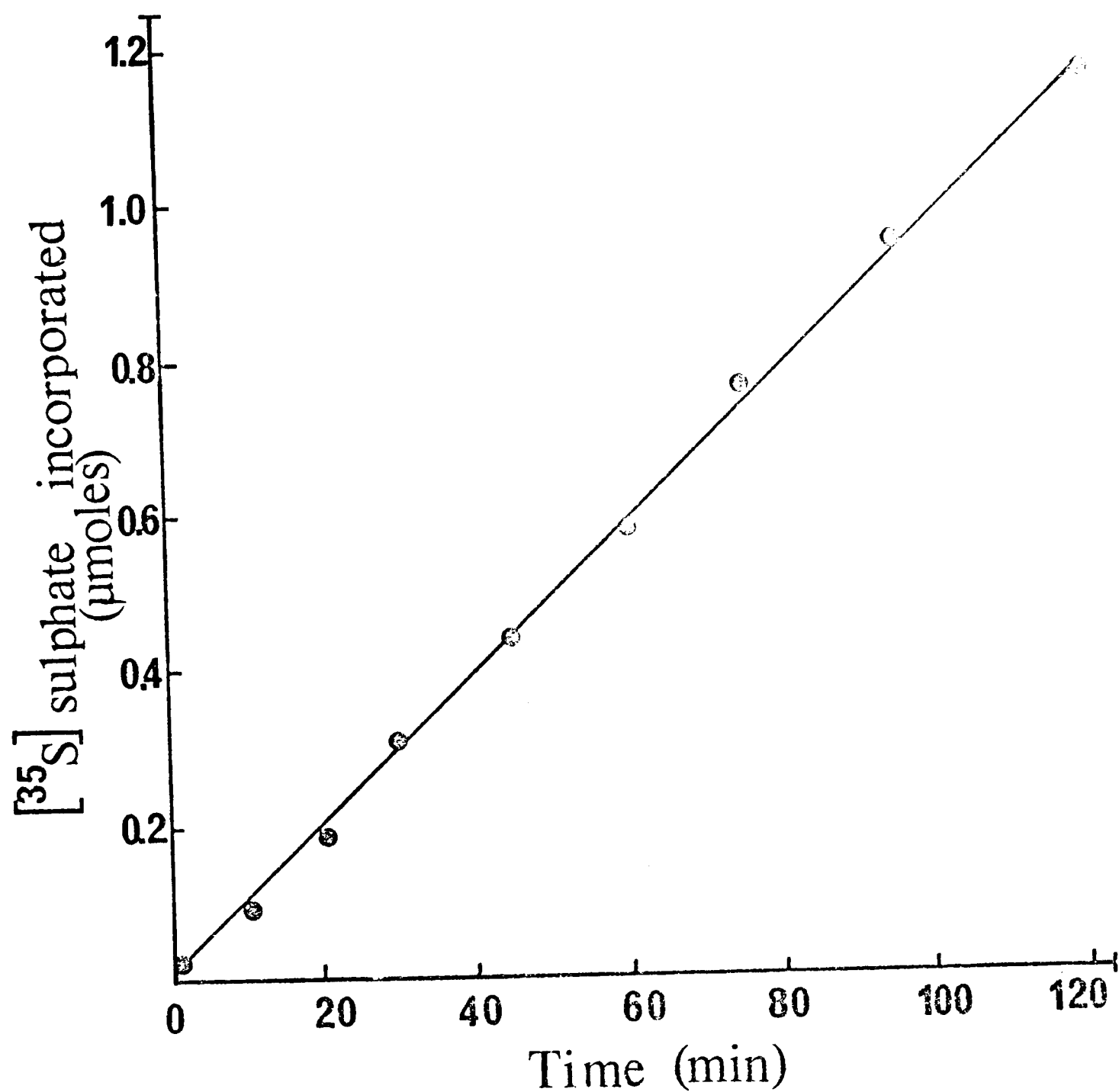


Fig. 3.1

Uptake and incorporation of $[^{35}\text{S}]\text{SO}_4^{2-}$ by whole cells of *P. denitrificans*.

Experimental conditions were as described in the text.

of ^{35}S in various compounds separated by high voltage electrophoresis after exposing the cells to $[-^{35}\text{S}]$ sulphate for different lengths of time is shown in Table 3.3. This

TABLE 3.3

Time-course for experiment.

Percentage distribution of ^{35}S in various ethanol-soluble compounds.

^{35}S -labelled compound	Incubation period (min)						
	2	5	10	20	40	60	120
Bound SO_4^{2-}	100	72	66	55	41	21	13
APS		4	3	5	5	2	3
PAPS		13	14	11	7	6	3
NEM- SO_3^{2-}		8	5	7	7	4	5
NEM- S^{2-}		3	3	2	2	3	2
NEM-Cysteine			9	12	22	29	31
Cystathionine				3	3	5	2
NEM-Homocysteine				5	4	9	7
Methionine					6	11	16
NEM-Glutathionine					3	5	7
Methionine sulfoxide						3	3
Methionine sulphone						2	3
Cysteic acid							3
Cystine							2

experiment was conducted four times and the figures expressed in Table 3.3 are results of a typical experiment. After 2 min, all the ^{35}S -label was recovered from the cells in the form of

sulphate. After a 5 min incubation, ^{35}S -label was detected in APS, PAPS and sulphite. The first ^{35}S -labelled amino acid to be detected was cysteine, after 10 min. ^{35}S -Labelled cystathionine and homocysteine were detected after 20 min incubation, methionine and glutathione after 40 min, and methione sulfoxide and methionine sulphone after 60 min. ^{35}S -Labelled cysteic acid and cystine were detected after 2 h.

(2) Pulse-chase experiments. As found for the time-course experiments, all the ^{35}S -label was extracted after 2 min in the form of sulphate. Excess, unlabelled sulphate was added to the incubation mixtures after 5 min, at which time ^{35}S -labelled APS, PAPS and sulphite were detected. After 10 min, ^{35}S -labelled sulphide and cysteine were detected. After 20 min no ^{35}S -labelled APS, PAPS, sulphite or sulphide was detected, the amount of ^{35}S -labelled cysteine had increased, and $\gamma\text{-}^{35}\text{S}$ cystathionine, homocysteine and methionine were detected for the first time. ^{35}S -Labelled glutathione, methionine sulfoxide and methionine sulphone were detected after 40 min. After 60 min incubation, no ^{35}S -cysteine or cystathionine was detected and the amount of ^{35}S -label in homocysteine had decreased. $\gamma\text{-}^{35}\text{S}$ Cysteic acid was detected after 60 min and $\gamma\text{-}^{35}\text{S}$ cystine after 2 h. (Table 3.4). The amount of ^{35}S -label in the ethanol-soluble fraction decreased with time, probably due to the incorporation of soluble ^{35}S -labelled compounds into insoluble compounds such as proteins, polysaccharides and esters.

DISCUSSION

Time-course and pulse-chase experiments with whole cells of P. denitrificans have shown that APS, PAPS and sulphite were the first ^{35}S -labelled intermediates synthesised from inorganic

TABLE 3.4

Incorporation of γ - ^{35}S sulphate by cell suspensions

Pulse-chase experiment

Results are expressed as counts per sec.
(Sections of chromatogram strips were counted for 5 min)

^{35}S -labelled compound	Incubation time (min)						
	2	5	10	20	40	60	120
SO_4^{2-}	178	305	164	72	69	58	49
APS		15.8	2.6				
PAPS		18.9	11.3				
NEM-SO_3^{2-}		3.2	6.2				
NEM-S^{2-}			3.3				
NEM-Cysteine			4.0	14.1	0.3		
Cystathionine				4.8	2.9		
NEM-Homocysteine				5.3	7.2	0.6	
Methionine				2.2	9.4	4.5	2.4
NEM-Glutathionine					4.1	3.2	1.6
$\text{Methionine sulphoxide}$					0.8	1.2	0.4
$\text{Methionine sulphone}$					0.2	0.3	1.7
Cysteic acid						0.9	0.6
Cystine							1.2

The pulse-chase experiment was conducted six times. The results in this table represent typical results obtained in one experiment.

sulphate. The data indicate that inorganic sulphite is an intermediate in sulphate reduction but give no information on the reduction pathway. Sulphite may be formed by a pathway not involving APS or PAPS; it may be formed by the action of an APS reductase, as in dissimilatory reducing bacteria (Peck, 1962a), or it may be formed from PAPS by an enzyme similar to that found in yeast (Hilz et al., 1959; Wilson et al., 1961; Asahi et al., 1961).

After sulphate has been reduced to sulphide, through the intermediate formation of sulphite, sulphide is incorporated into cysteine. Cysteine is then converted to methionine through the successive formation of cystathionine and homocysteine. Homocysteine is converted to methionine, which in turn, is metabolised to methionine sulfoxide and methionine sulphone.

The major ethanol-soluble ^{35}S -labelled compounds detected in the time-course study were cysteine and methionine; similar results have been found for yeast (Roberts et al., 1955) and Neurospora (Horowitz, 1950, 1955).

Taurine was not found in P. denitrificans although it has been detected in other bacteria (Varma & Nicholas, 1970).

Sulphide has been implicated as the immediate precursor of cysteine in a number of microorganisms (Schlossmann & Lynen, 1957; Varma & Nicholas, 1970a, 1970b). ^{35}S -Labelled thiosulphate was not detected in the present study and is not implicated as an intermediate in sulphate reduction in P. denitrificans. Thiosulphate is thought to be an intermediate in the assimilation of sulphate in a variety of organisms (Hockenhull, 1949; Nakamura, 1962; Nakamura & Sato, 1960, 1962; Shepherd, 1956). In Chapter 2 it was reported that P. denitrificans

can utilise thiosulphate as a sole sulphur source for growth. However, the ability of an organism to utilise a particular compound does not mean that the compound is a normal intermediate in a metabolic pathway. Many bacteria are capable of utilising thiosulphate (Ishimoto et al., 1955; Kawakami et al., 1957; Artman, 1956; Woolfolk, 1962; Peck, 1960) by splitting thiosulphate into the constituents, sulphite and sulphide, which enter the sulphate reducing pathway.

Cystathionine and homocysteine are known to be precursors of methionine in E. coli (Lampen et al., 1947; Guest et al., 1962; Foster et al., 1961), S. typhimurium (Kaplan & Flavin, 1966; Flavin & Slaughter, 1966) and Nitrosomonas europaea (Varma & Nicholas, 1970a), and the results of the time-course and pulse-chase experiments indicate that cysteine is converted to methionine through the successive formation of cystathionine and homocysteine. The sequence of synthesis of ^{35}S -labelled compounds formed by whole cells of P. denitrificans incubated with $\gamma\text{-}^{35}\text{S}$ sulphate is summarised in Fig. 3.2.

The results reported in this chapter suggest that the pathway for the incorporation of $\gamma\text{-}^{35}\text{S}$ sulphate into P. denitrificans are similar to those suggested by Wilson (1962), Thompson (1967) and Varma & Nicholas (1970a) for other assimilatory sulphate reducing organisms. By comparing the results presented in this chapter with the postulated pathways for sulphur metabolism in other bacteria, a possible scheme for the pathway of sulphur metabolism in P. denitrificans was constructed (Fig. 3.3).

Fig. 3.2

A time-sequence pathway for the incorporation of labelled sulphate into Paracoccus denitrificans.

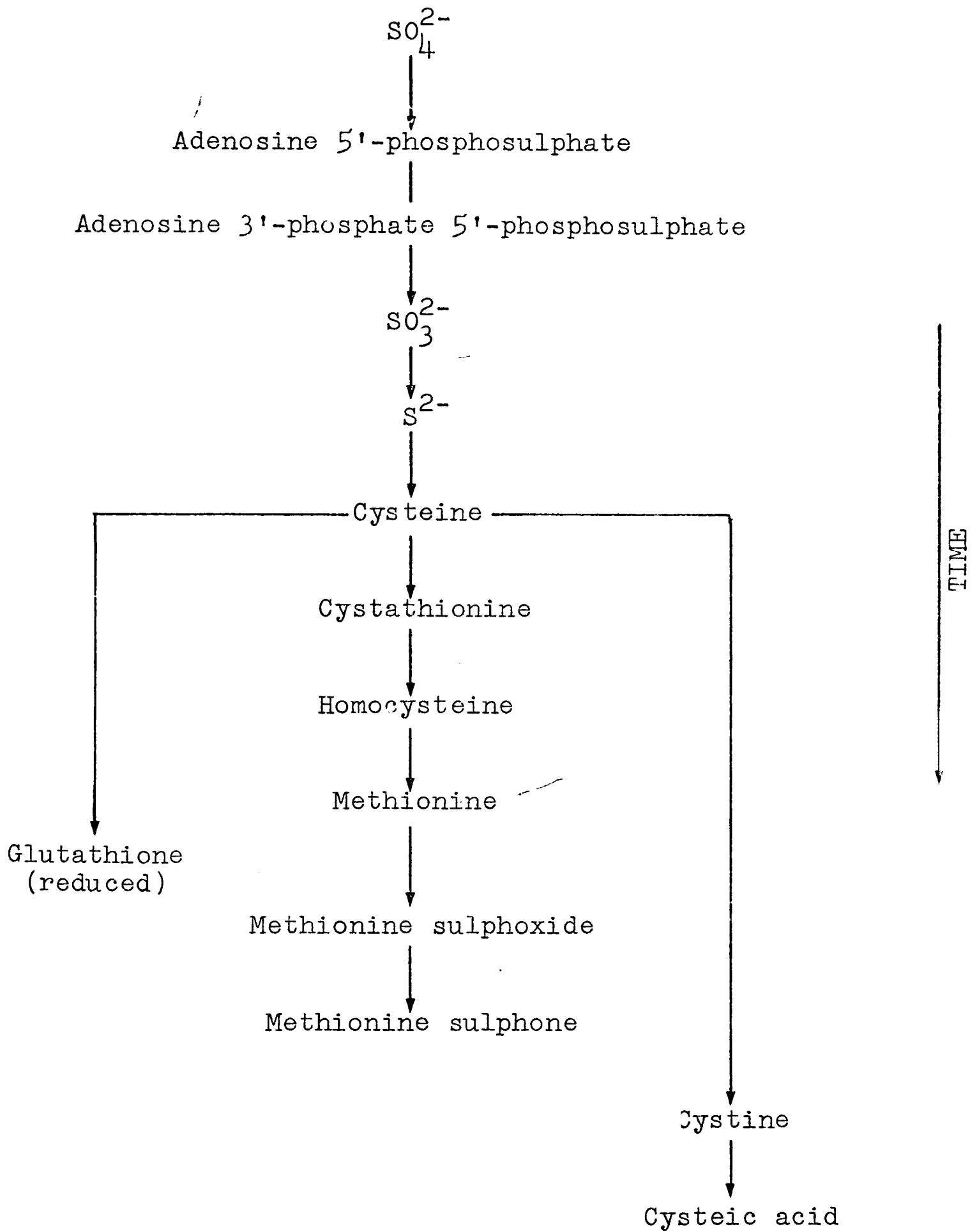
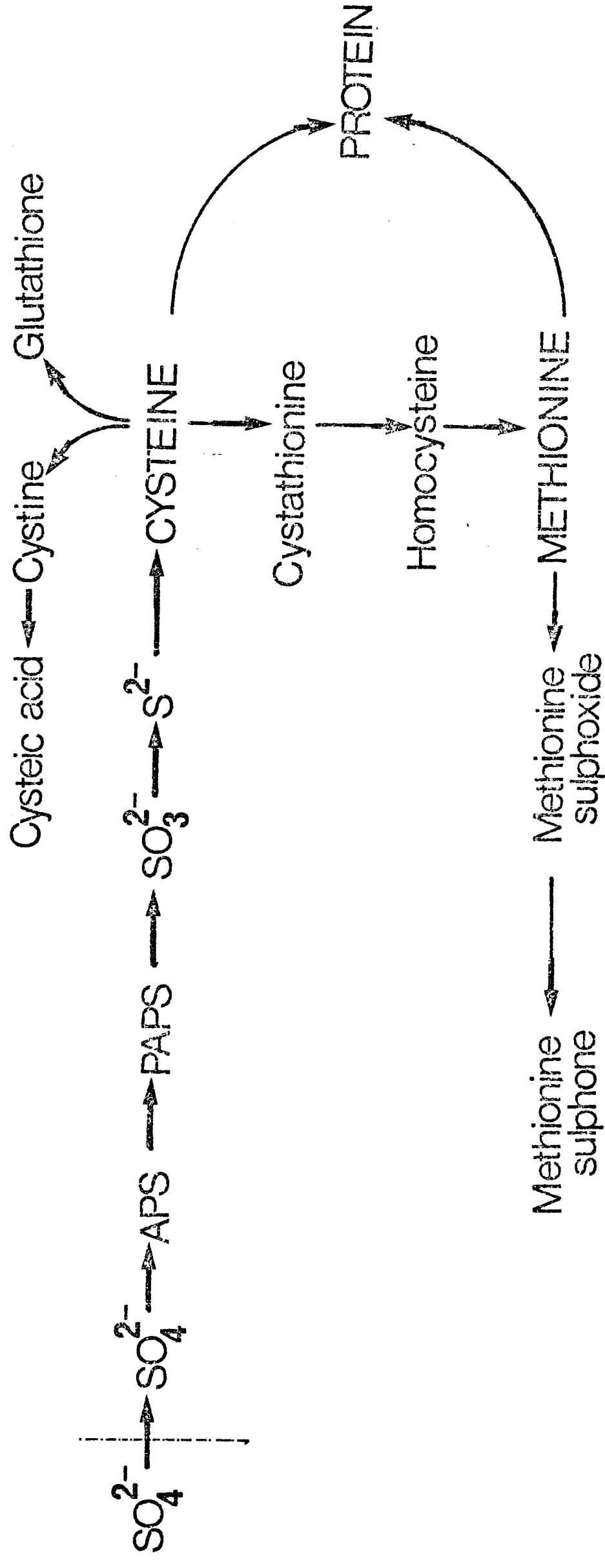


Fig. 3.3

A possible pathway of sulphur metabolism in Paracoccus denitrificans.



CHAPTER 4

SULPHATE UPTAKE INTO WHOLE CELLS

INTRODUCTION

In Chapter 2, Paracoccus denitrificans was shown to be able to utilise inorganic sulphate as a sole sulphur source. The uptake of sulphate from the external environment therefore represents the first process in the assimilation of sulphate. It also represents a possible control site for sulphate metabolism.

Mechanisms for the active uptake of sulphate have been suggested for Escherichia coli and Salmonella typhimurium (Dreyfuss, 1964; Ellis, 1964; Dreyfuss & Pardee, 1965; Pardee, 1967; Springer & Huber, 1972).

In this chapter evidence is presented for the existence of an active uptake mechanism for the uptake of sulphate in P. denitrificans.

MATERIALS AND METHODS

Chemicals. L-Amino acids and Triton X-100 were obtained from the Sigma Chemical Co., London, U.K., carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) was obtained from Boehringer und Soehne, G.m.b.H., Mannheim, Germany, and blue dextran 2000 from Pharmacia Fine Chemicals, U.K. All other chemicals were of Analytical Reagent Grade and were obtained from the sources as stated in the previous chapters.

Growth of cells. Cells of P. denitrificans were maintained and grown as described in Chapter 2. Aerobic cultures, (succinate as substrate, oxygen as the terminal electron acceptor and sulphate as the sole sulphur source) were grown in 500 ml of culture solution in 2 litre flasks (0.5 ml inoculum) and incubated in an orbital incubator at 30°C at 185 rpm describing a 40 cm diameter circle. Anaerobic cultures (succinate as substrate, nitrate as the terminal electron acceptor and sulphate as the sole sulphur source) were grown

in 2 litre flasks, filled with culture medium.

Cells were harvested during the exponential phase (16 h after inoculation) by centrifugation in an M.S.E. 18 centrifuge at 9 000g. Bacteria were washed twice in a washing medium (culture medium minus sulphate), resuspended in 50% volume of washing medium and reincubated for 30 min at 30°C to deplete the cells of intracellular sulphate.

Measurement of sulphate uptake. Anaerobic incubation mixtures (total volume 5 ml) contained 3 ml of washed cell suspension, 1.5 ml of washing solution, 0.1 ml $\text{Na}_2\text{S}_2\text{O}_4$ - ^{35}S SO_4 (10mM; 1 $\mu\text{Ci}/\mu\text{mole}$), and appropriate additions. The radioactive sulphate was added at zero time. Experiments were conducted at 30°C. Samples (0.1 ml) were withdrawn at 15 sec intervals for 2 min and added to 2 ml of 10mM- Na_2SO_4 at 2°C. The diluted samples were allowed to stand at 2°C for 5 min and the cells were then collected by filtration, onto an Oxoid Membrane Filter (0.45 micron pore size- 4.7 cm diam.) as follows; the filters were first washed with 10 ml of 10mM- Na_2SO_4 (at 2°C) and the diluted samples then applied. Following drainage of the sample onto the filter, the filter was quickly washed with five 10 ml washes of 10mM- Na_2SO_4 (at 2°C). Filters with washed cells were individually placed in scintillation vials, dried (to reduce quenching by water) and counted, following the addition of 5 ml of scintillation fluid (see Chapter 2). The vials were allowed to stand for 12 h prior to counting the radioactivity in a scintillation counter.

Aerobic incubation mixtures were similar to those described for anaerobic conditions except that nitrate was omitted from the washing solution. Uptake of ^{35}S sulphate was studied in open 25 ml flasks in a Warburg apparatus at 30°C.

Sulphate uptake was assayed as described for the anaerobic experiments.

Chromatography and identification of $\text{[}^{-35}\text{S}_7\text{SO}_4^{2-}\text{]}$ sulphate.

Hot water washings of cells (see Table 4.1) were pooled,

TABLE 4.1

Removal of intracellular sulphate by washing		
Procedure	Percentage of $\text{[}^{-35}\text{S}_7\text{SO}_4^{2-}\text{]}$	
Total $\text{[}^{-35}\text{S}_7\text{SO}_4^{2-}\text{]}$ taken up	100	
Activity removed by first washing	97.8	
second "	0.8	
third "	0.5	
fourth "	0.2	
fifth "	0.1	
Total $\text{[}^{-35}\text{S}_7\text{SO}_4^{2-}\text{]}$ removed by washing	99.4	
Total $\text{[}^{-35}\text{S}_7\text{SO}_4^{2-}\text{]}$ remaining after 5 washes	0.3	

Cells were incubated for 2 min in a standard incubation mixture containing $\text{[}^{35}\text{S}_7\text{SO}_4^{2-}\text{]}$. After washing the cells with $10\text{mM-Na}_2\text{SO}_4$ to remove extracellular $\text{[}^{35}\text{S}_7\text{SO}_4^{2-}\text{]}$ the cells were washed with boiling water and the radioactivity of each of the washings counted.

The washings were then pooled, concentrated and chromatographed. All the ^{35}S recovered by the five washes was in the form of sulphate.

evaporated to dryness in a rotary evaporator, and dissolved in 0.5 ml of water. Fractions (0.05 ml) were applied to Whatman 3MM paper and developed for 16 h by descending chroma-

tography in propan-1-ol— ammonia (sp. gr. 0.88)— water; 6 : 3 : 1, by vol. Dried chromatogram strips were cut into 1 cm sections and their radioactivity determined by scintillation counting. γ - ^{35}S Sulphate was identified by comparing the R_f value of the radioactivity of the hot water washings with the R_f value of authentic γ - ^{35}S sulphate.

Calculation of cell volume and intracellular sulphate concentration. The intracellular concentration of sulphate was calculated from the values obtained from the volume of a cell, and from the total amount of sulphate accumulated by the cell after 2 min. The volume of a P. denitrificans cell was determined using blue dextran 2000, a compound, which because of its large size (mol. wt. = 2×10^6), is presumably excluded by the cell wall (Gerhardt & Judge, 1964). When a cell suspension was added to a solution of blue dextran, less dilution of the blue dextran was observed than when the same volume of cell-free suspending medium was added. From the difference, the dextran-impermeable volume of the cell suspension was calculated and this volume was taken as the volume occupied by the cells themselves.

Cells were grown anaerobically, harvested and washed as described above. A range of volumes of cell suspension (0.5 - 4.0 ml) was added to a standard solution (0.1%, w/v) of blue dextran, mixed thoroughly and centrifuged, after which the extinction of the supernatant was measured at 630 nm. Samples (0.1 ml) of the stock cell suspension were diluted and inoculated on 4 duplicate sets of agar plates to allow an estimation of the cell number by counting the viable colonies.

RESULTS

Sulphate uptake. In studying a transport of uptake system

in intact cells it is important to determine sulphate uptake separately from sulphate incorporation by ascertaining the extent to which sulphate is free to leave the cell after it has been taken up. In experiments with Salmonella typhimurium, Dreyfuss (1964) used mutants which lacked the sulphate-activating enzymes, thereby avoiding confusing sulphate uptake and sulphate incorporation. The results in Table 4.1 indicate that all the γ - ^{35}S sulphate taken up by P. denitrificans during a two minute incubation can be quantitatively removed by washing the cells with water at 100°C.

The method of measuring the rate of sulphate uptake is essentially the same as that of Yamamoto & Segel (1966) in their studies with Penicillium, being a determination of the amount of free γ - ^{35}S sulphate within the cells following a brief incubation period. Unlike Yamamoto & Segel (1966) however, the sulphate extracted by boiling water was not measured, but the time of the assay was restricted to a period over which all the sulphate taken up by the cells could have been quantitatively extracted as free inorganic sulphate and the radioactivity of the cells was counted. The validity of this method therefore rests on the demonstration that practically all the sulphate taken up in 2 min can be extracted as sulphate by boiling water (Table 4.1).

Experiments in Chapter 2 indicated that P. denitrificans is unable to utilise any organic sulphur source as a source of sulphur for growth, except cystine. Since cystine/cysteine has been found to inhibit (Ellis, 1964) or repress (Dreyfuss, 1964) sulphate uptake in E. coli and S. typhimurium respectively, and may therefore play some part in the regulation and/or inhibition of the uptake of sulphate in P. denitrificans,

growth on cystine would be unsuitable. It was for this reason that P. denitrificans was grown on sulphate, harvested, washed and then resuspended in a sulphate-free medium. The cells were then incubated for 30 min prior to their use in experiments; a procedure that ensured that the concentration of sulphate was low.

Linearity of sulphate uptake. Fig. 4.1 shows that sulphate uptake is linear with respect to time up to 5 min. Sulphate uptake was also observed into cells under anaerobic conditions.

The linearity of sulphate uptake by P. denitrificans contrasts with the three phase uptake system exhibited by S. typhimurium, which consists of an immediate rapid uptake, followed by an equally rapid excretion of sulphate, and then by a much more gradual, but sustained, uptake (Dreyfuss, 1964).

The fact that the curve for sulphate uptake by P. denitrificans does not pass through the origin (Fig. 4.1) is probably due to the adsorption of a small amount of $\gamma\text{-}^{35}\text{S}_7$ sulphate onto the cell surface. P. denitrificans cells, which do not take up sulphate at 2°C show the adsorption of $\gamma\text{-}^{35}\text{S}_7$ sulphate onto the cell surface and have the same intercept.

Sulphate uptake is also directly proportional to the cell density (Fig. 4.2).

Effect of pH. The effect of pH on sulphate uptake is shown in Fig. 4.3. Sulphate remains as the divalent ion (SO_4^{2-}) throughout the pH range examined. Consequently any effect on external pH must be either on the cell surface, on the uptake system itself, or on both. As shown in Fig. 4.3, sulphate uptake is pH-dependent and exhibits a sharp pH optimum at pH 8.0. The rate of sulphate uptake was relatively low between pH 2.5 and 5.5 but showed a sharp increase between

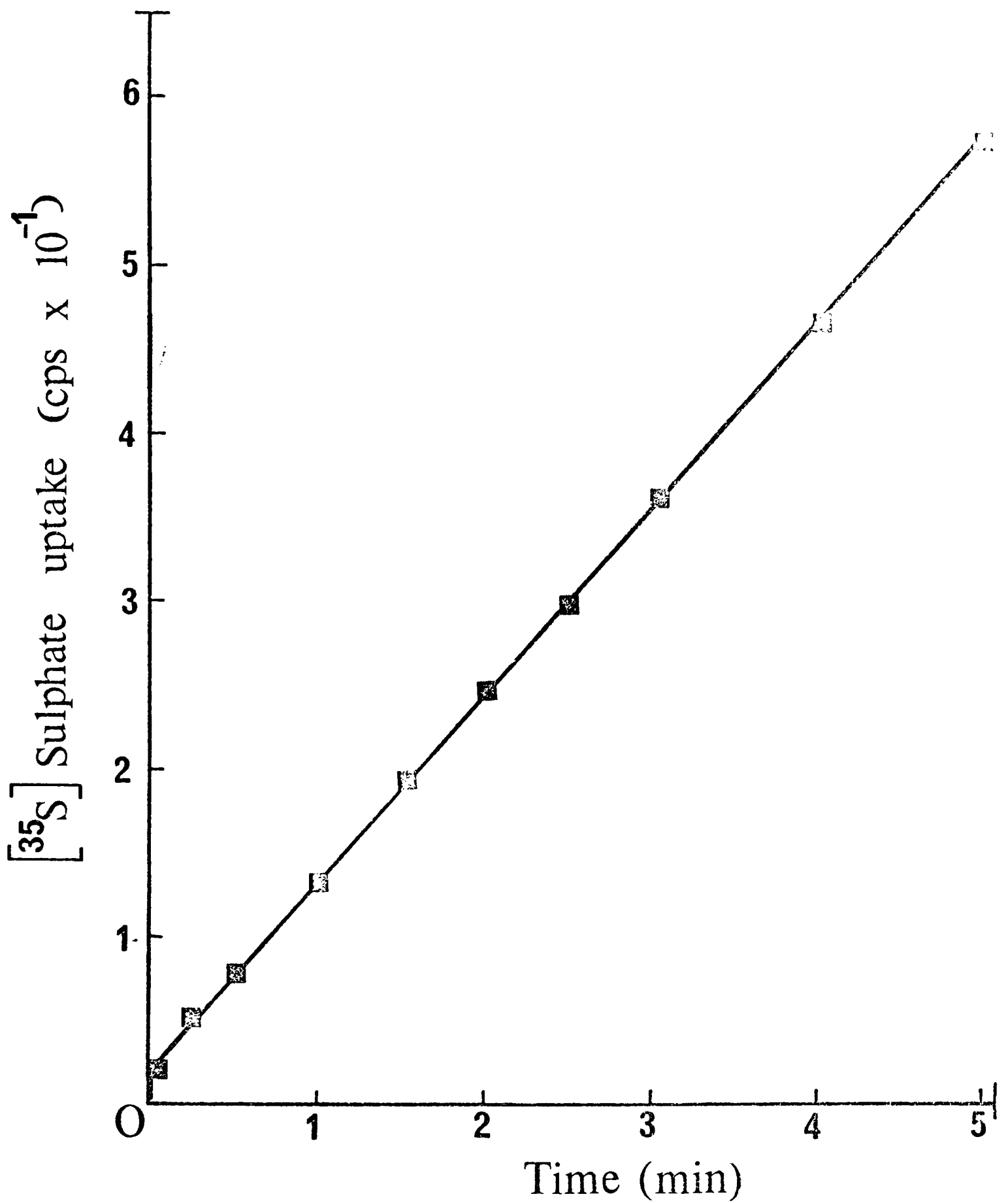


Fig. 4.1

Time-course of sulphate uptake by whole cells of P. denitrificans. Incubation mixtures contained 3 ml of washed cell suspension, 1.5 ml of washing medium and 1 μ mole $[-^{35}\text{S}]$ sulphate (1 $\mu\text{Ci}/\mu\text{mole}$). Sulphate uptake was conducted under aerobic conditions.

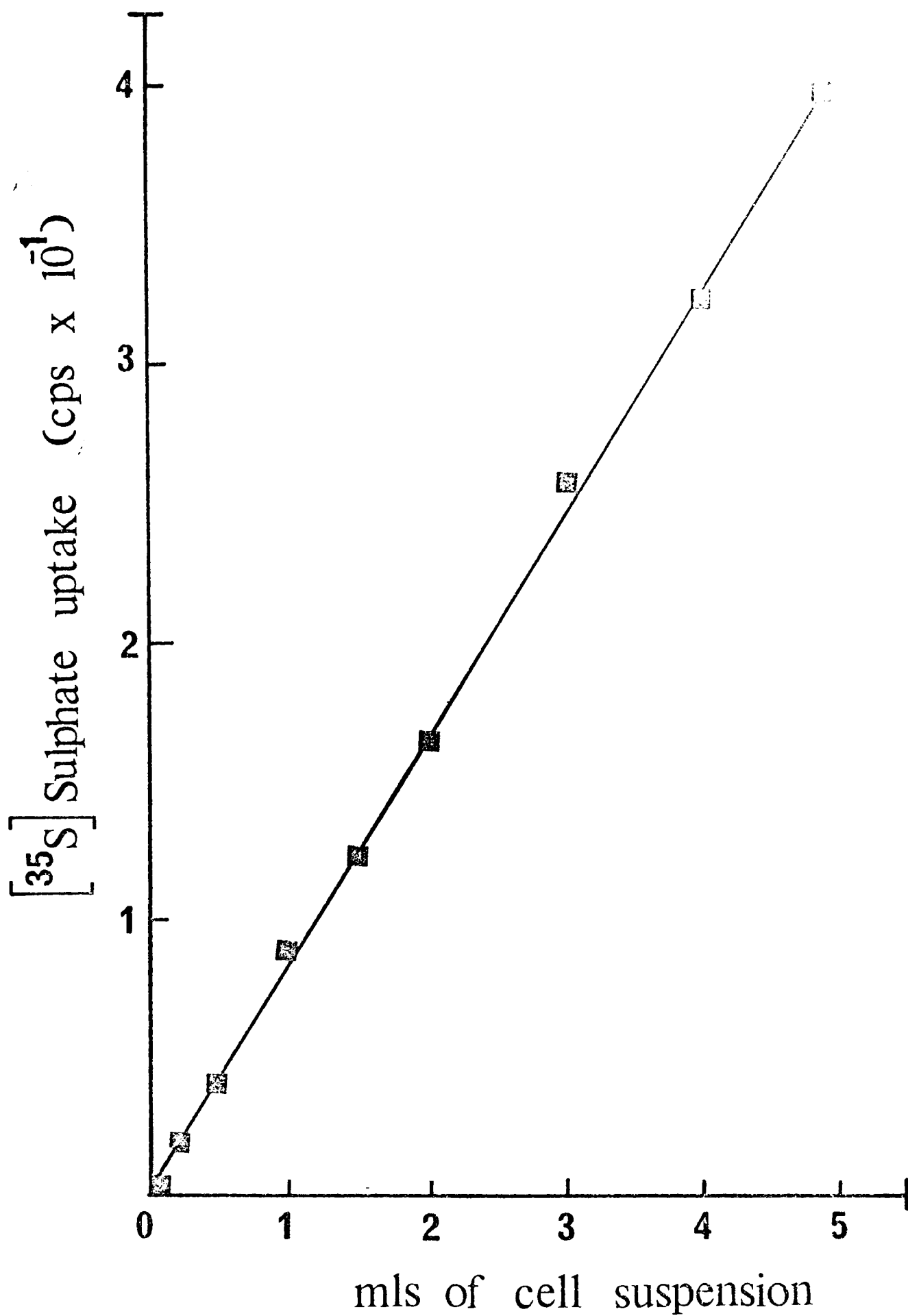


Fig. 4.2

Linearity of sulphate uptake as a function of cell density. Reaction mixtures were as described in the Fig. 4.1 except that the amount of cell suspension, and the aerobic washing medium added to the incubation mixtures were varied. Sulphate uptake was studied under aerobic conditions.

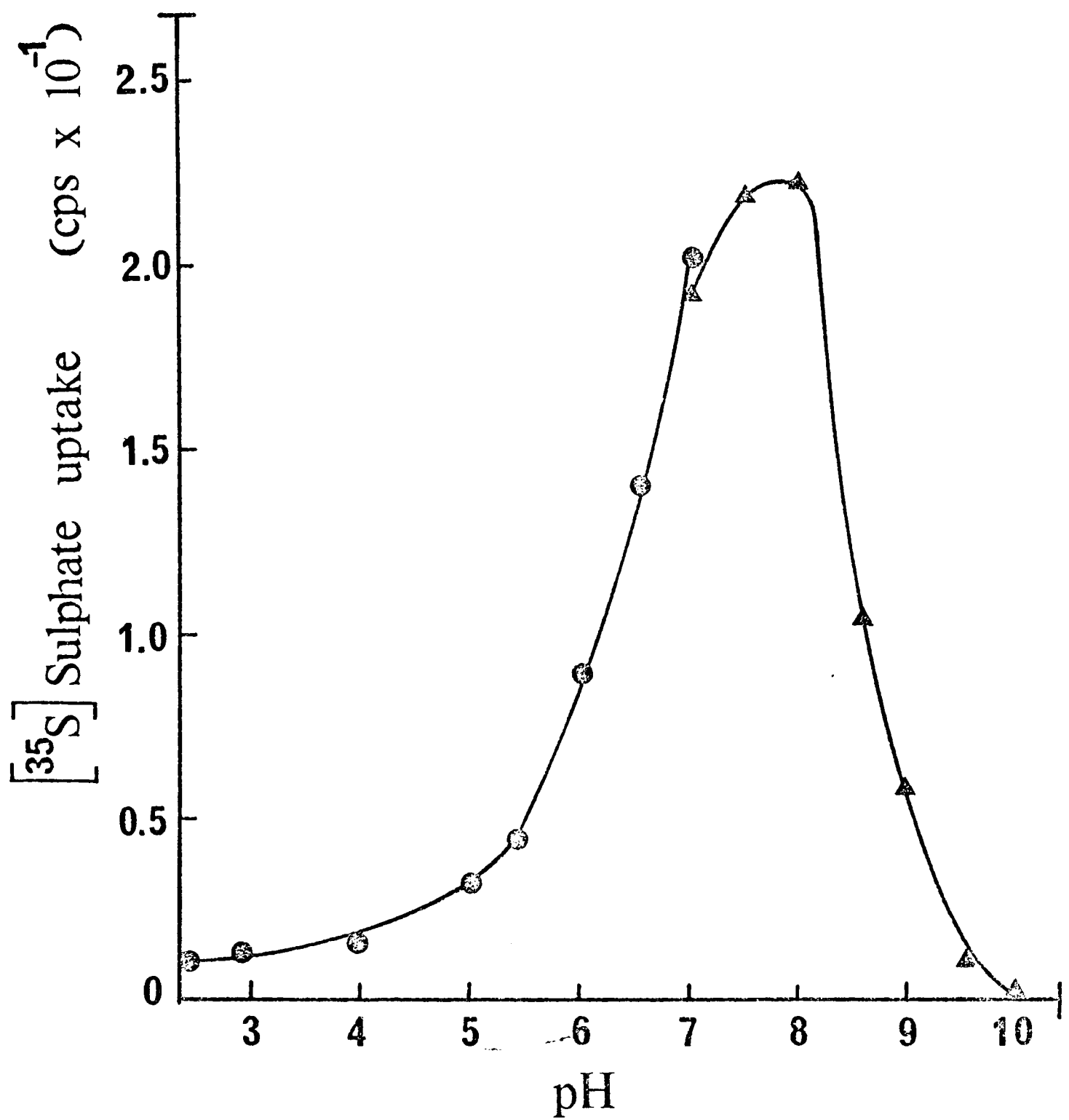


Fig. 4.3

Effect of pH on sulphate uptake by whole cells.

Sulphate uptake into whole cells was measured as described in Fig. 4.1 (aerobic conditions) except that the pH of the incubation mixtures was altered with either HCl or NaOH. The pH of each incubation mixture was constant for the duration of the assay (2 min).

pH 5.5 and 7.5. There was a sharp decrease in the rate of sulphate uptake above pH 8.0, with no uptake at pH 10.0. All subsequent assays of the sulphate uptake system in whole cells were conducted at pH 8.0.

Effect of temperature. As shown in Fig. 4.4 the sulphate uptake system shows a marked temperature dependence with a maximum at 40°C. The decrease in the uptake rate at low temperatures was reversible. However if the cells were preincubated for 2 min at 50°C, and then assayed at 30°C, no uptake was observed.

Effect of sulphate concentration. The effect of sulphate concentration on sulphate uptake was determined over a 10,000-fold range of extracellular concentration (1 μM - 10 mM). The rate of sulphate uptake was found to be dependent on the concentration of sulphate and exhibited Michaelis-Menten kinetics (Fig. 4.5). The "transport system K_m values" were found to be the same for different bacterial cultures, provided they were washed and reincubated in a sulphate-free medium for a constant time; this ensured that the concentration of free sulphate in the medium and the intracellular and adsorbed sulphate was low and, therefore, did not interfere with the exogenous sulphate added to study the effect of sulphate concentration.

The K_m for sulphate in the sulphate uptake system was found, graphically, to be $5 \times 10^{-5} \text{M}$ sulphate.

Effect of analogues. The uptake of sulphate by P. denitrificans is inhibited by selenate and molybdate but not by tellurate (Table 4.2). The inhibition of sulphate uptake by selenate can be overcome by increasing the concentration of sulphate, indicating that selenate is a competitive inhibitor

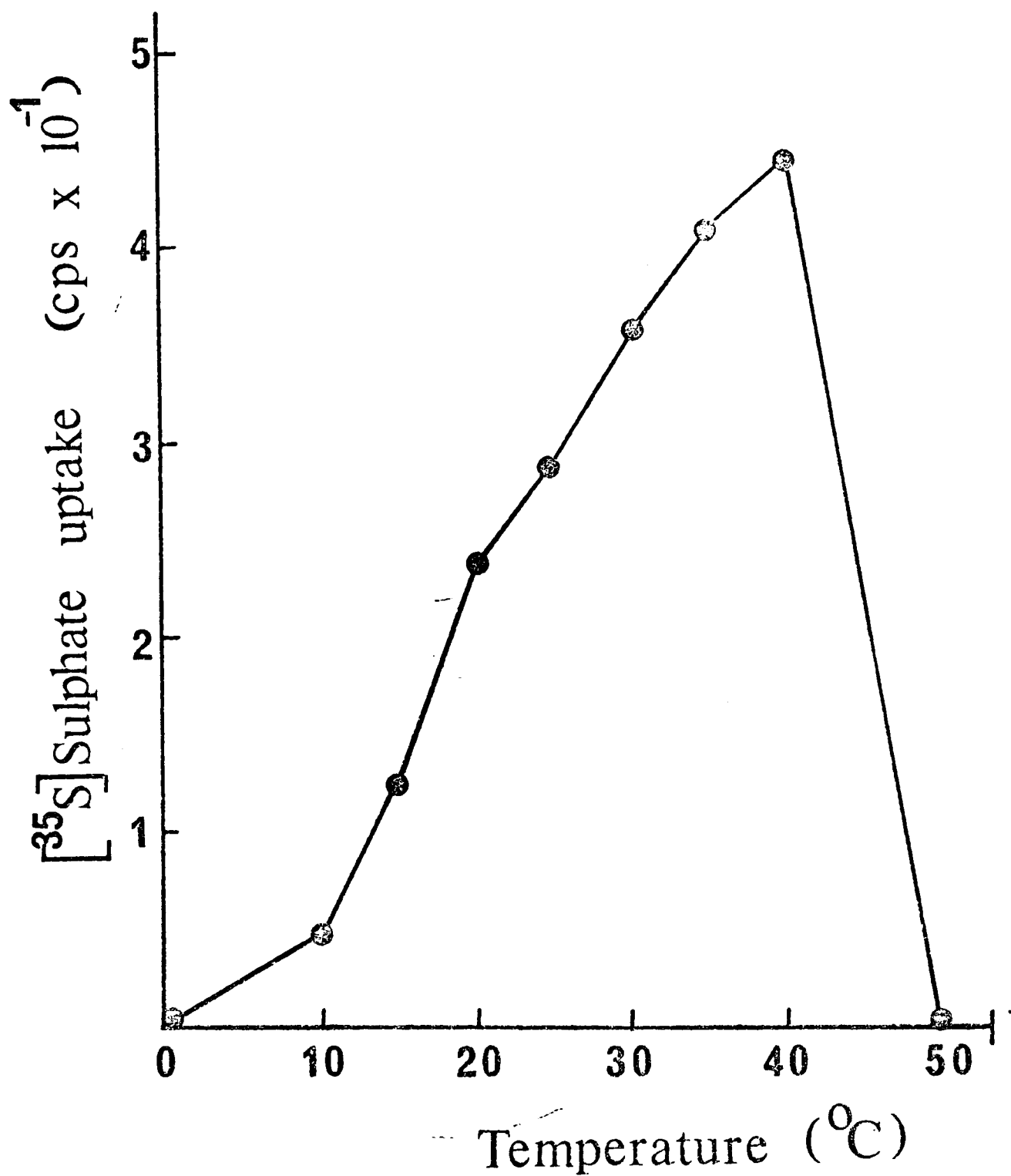


Fig. 4.4

Effect of temperature upon sulphate uptake into whole cells. Cell suspensions were added to pre-warmed or pre-cooled washing medium and immediately assayed for sulphate uptake under aerobic conditions as described for Fig. 4.1.

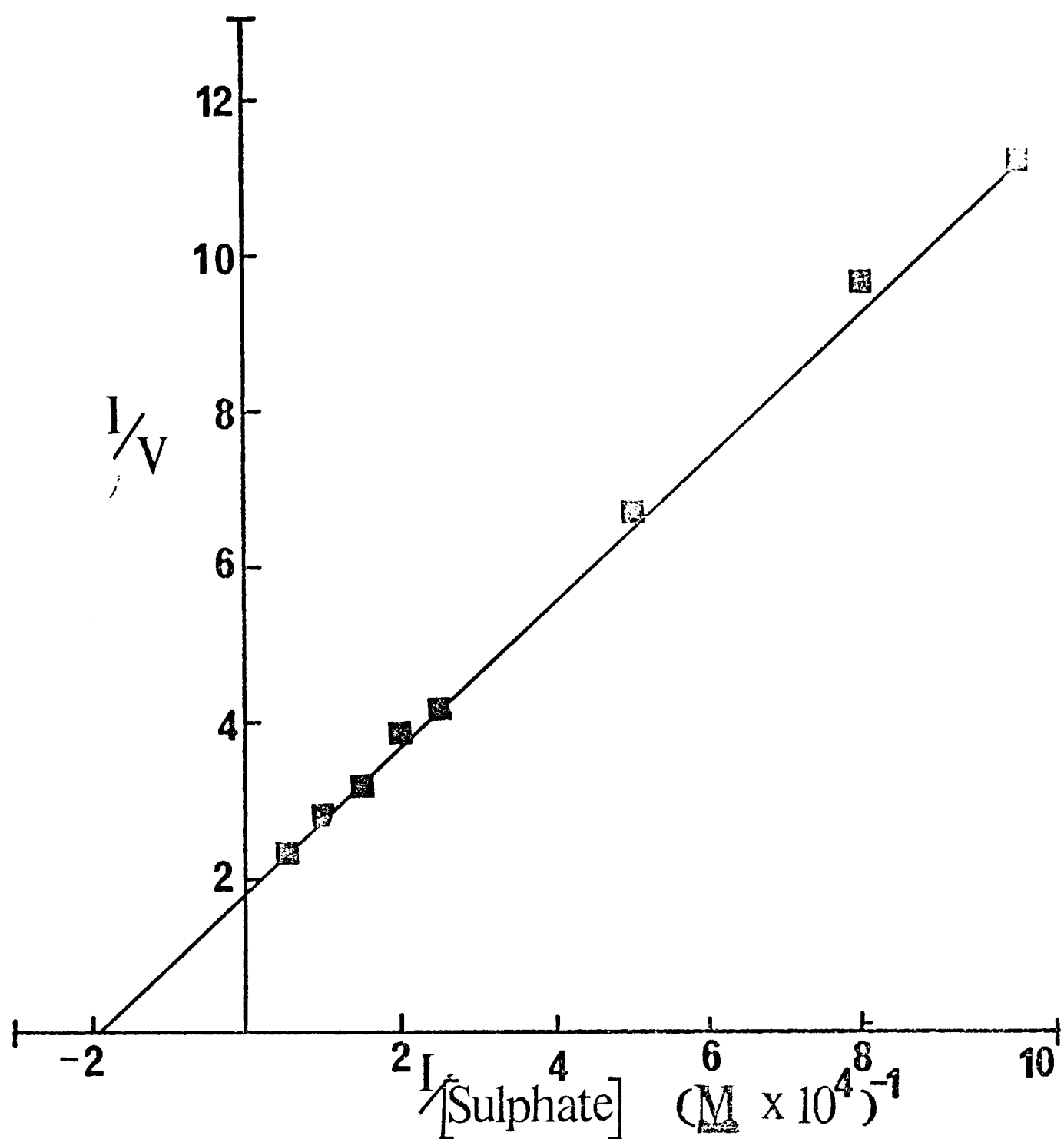


Fig. 4.5

Double reciprocal plot of the sulphate concentration versus the rate of sulphate uptake by whole cells.

Sulphate uptake was measured under aerobic conditions as described in Fig. 4.1 except that the sulphate concentration was varied. The specific activity of the ^{35}S -labelled sulphate was constant throughout.

TABLE 4.2

Effect of analogues on sulphate uptake	
Additions	Sulphur uptake (% of control)
None (control)	100%
MoO ₄ ²⁻	48
SeO ₄ ²⁻	47
TeO ₄ ²⁻	97

Reaction mixtures contained, in a total volume of 5 ml, 3 ml of washed cell suspension, 1.5 ml of succinate-nitrate washing medium, 1.0 umoles γ -³⁵S- γ -SO₄²⁻ (1 μ Ci/ μ mole) and 0.4 ml of additions. Sulphate uptake was measured as described in Materials and Methods, under aerobic conditions.

of sulphate uptake in P. denitrificans, as has been found in a number of microorganisms including E. coli (Huber et al., 1967 ; Springer & Huber, 1967), S. typhimurium (Pardee et al., 1966) and fungi (Yamamoto & Segel, 1966; Bradfield et al., 1973; Tweedie & Segel, 1970).

Several inorganic and organic sulphur-containing compounds were examined as potential inhibitors of sulphate uptake. None of the common sulphur-containing amino acids, including cysteine, cystine, methionine, cysteic acid or taurine, had any effect on sulphate uptake. A similar situation has been found in S. typhimurium. This contrasts with the rapid inhibition of sulphate uptake in E. coli by cysteine observed by Ellis (1964), who postulated that L-cysteine may be a specific allosteric inhibitor of the sulphate uptake system in E. coli.

Reduced glutathione was consistently found to stimulate the rate of sulphate uptake in P. denitrificans slightly (Table

4.3). In contrast to the sulphur-containing amino acids,

TABLE 4.3

Effect of sulphur compounds on sulphate uptake

Reaction mixtures were as described in Table 4.2, except for the addition of sulphur-containing compounds as indicated. All sulphur-containing compounds were added to give a final concentration of 0.05 mM in the incubation mixture. The concentration of $\text{[}^{35}\text{S]}$ sulphate was maintained at 0.2 mM throughout.

<u>Additions (0.05 mM)</u>	<u>Rate of sulphate uptake (% of control)</u>
Na_2SO_4 (control)	100
Na_2SO_3	11
Na_2S	63
$\text{Na}_2\text{S}_2\text{O}_3$	54
Cysteine	87
Cystine	90
Methionine	91
Taurine	100
Djenkolic acid	89
Glutathione (red.)	117
Cysteamine	107

the inorganic sulphur compounds, sulphate, thiosulphate and sulphide are potent inhibitors of sulphate uptake. Kinetic studies involving the inhibition by sulphite, thiosulphate and sulphide suggest that both sulphite and sulphide may be acting as feedback inhibitors ($1/K_m$ remains constant with varying sulphite and sulphide concentration), while thiosulphate is acting as a competitive inhibitor ($1/V$ remains constant with varying thiosulphate concentration) of sulphate up-

take. The inhibition of sulphate uptake by thiosulphate and sulphite has also been described in S. typhimurium (Dreyfuss, 1964) although sulphide actually exhibits a slight stimulatory effect upon sulphate uptake in S. typhimurium (Dreyfuss, 1964). It was confirmed that the sulphate uptake rates, observed in the presence of sulphite, sulphide and thiosulphate, were linear during the 2 min assay.

Effect of inhibitors. The effect of various inhibitors on the uptake of sulphate by aerobic cells is shown in Table 4.4.

TABLE 4.4

Effect of inhibitors on the rate of sulphate uptake by aerobic cells of <u>P. denitrificans</u>		
Reaction mixtures were as described in Table 4.2, except that the compounds were added as indicated in the Table.		
<u>Inhibitor</u>	<u>Final concn.</u>	<u>Rate of sulphate uptake (% of control)</u>
None (control)		0%
FCCP	10 μ <u>M</u>	96
KCN	1 m <u>M</u>	98
<u>p</u> -CMB	10 μ <u>M</u>	57
<u>p</u> -CMB + glutathione	10 μ <u>M</u>	0
EDTA	1 m <u>M</u>	48

All inhibitors were incubated with the cells for 30 min at 30°C prior to assaying for the rate of sulphate uptake. Both FCCP and KCN inhibited the uptake of sulphate. The inhibition of sulphate uptake by p-chloromercuribenzoate (p-CMB) was

reversed by the addition of reduced glutathione. It must be remembered, however, that glutathione has a slight stimulatory effect upon sulphate uptake in the absence of inhibitors (Table 4.3).

Effect of extracellular ionic concentration. The rate of uptake was significantly decreased by lowering the total ionic strength of the incubation mixture while maintaining a constant sulphate concentration. Sulphate uptake could be restored to its maximal rate by the addition of KCl, NaCl, MgCl_2 , or CaCl_2 to give final concentrations of 10 mM.

Effect of sulphur source during growth. Cells of P. denitrificans were grown on a variety of sulphur compounds, added as the sole sulphur source, and the rate of sulphate uptake by cells grown on the different sulphur-containing substrates was examined. The same amount of sulphur was added to each growth medium. The results are shown in Table 4.5.

TABLE 4.5

Effect of sulphur source during growth on the rate of sulphur uptake

The sulphate of the basic growth medium was replaced by a variety of sulphur-containing compounds and the effect of these compounds, on the rate of sulphate uptake, was examined. All the sulphur compounds were at a final concentration of 31.2 mM in the growth medium.

<u>Sulphur source during growth</u>	<u>Rate of sulphate rate (% of control)</u>
SO_4^{2-}	100%
SO_3^{2-}	38
S^{2-}	25
Cystine	7

Sulphate uptake appears to be repressed during growth on the four sulphur-containing compounds examined. Cystine significantly represses the sulphate uptake mechanism, while sulphite and sulphide have a less significant effect upon the rate of sulphate uptake. These results suggest that two possible mechanisms may be operating. Firstly a mechanism of "differential repression" may be in operation i.e., possible intermediates involved in the pathway of sulphate assimilation, reduction and incorporation into amino acids, repress the uptake mechanism more severely the closer the compound is to the end of the metabolic pathway. A second possible mechanism may involve repression by intracellular cystine alone and the different levels of repression observed with the addition of sulphite and sulphide may only reflect the relative rates of incorporation of sulphite and sulphide to form cysteine, in which form it may repress the sulphate uptake system.

Effect of sulphur starvation. The rate of sulphate uptake increased only slightly if cells were starved of extracellular sulphate for periods of time exceeding 30 min. If, however, cells were grown on cystine (and their sulphate uptake mechanism repressed) the sulphate uptake system developed if cells were harvested, washed and resuspended in a sulphur-free medium and reincubated. Maximal rates of sulphate uptake were obtained following two hours of sulphur starvation. This suggests that the sulphate uptake mechanism is not a sulphate-inducible system. This would confer an obvious advantage upon P. denitrificans if it is capable of developing a sulphate uptake system in the absence of sulphate, since it would be able to utilise exogenous sulphate when it became available to the sulphur-starving cells.

Effect of cell wall and membrane integrity. The effect of FCCP and Triton X-100 upon the uptake of sulphate by whole cells of P. denitrificans was investigated. Individual control incubations confirmed that sulphate uptake remained linear for the duration of the assay (2 min) in the absence of FCCP or Triton X-100 (Fig. 4.6). Addition of Triton X-100 (0.1%, v/v in the assay) allowed the sulphate, already accumulated, to be leached out during washing. FCCP ($5 \mu\text{M}$ in the assay) inhibited the uptake of sulphate, but on washing, the cells retained the accumulated sulphate taken up prior to the addition of FCCP. These results suggest that Triton X-100 is causing physical damage to the cells which allows sulphate to be washed out of the cell. FCCP, however, appears to act only by inhibiting the uptake mechanism and does not cause physical damage to the cell. The effect of FCCP on sulphate uptake by P. denitrificans is discussed more fully in the following chapter.

Cell volume and intracellular sulphate concentration. The volume of a cell of P. denitrificans was determined in three separate experiments and found to be $2 \times 10^{-8} \mu\text{l}$ ($\pm 7\%$). Knowing the specific radioactivity of the added sulphate and the radioactivity of the cells, the internal sulphate concentration can now be calculated. Experiments using four separate preparations of cell suspensions indicated that sulphate-starved cells accumulated sulphate to an internal concentration of $8 (\pm 2) \text{ mM}$ in two minutes. This represents a 40-fold increase in the intracellular sulphate concentration over the concentration in the medium (0.2 mM). Since blue dextran cannot penetrate the cell wall of P. denitrificans, this value for the estimated internal concentration will be, at worst, an underestimate. It is clear that the cells

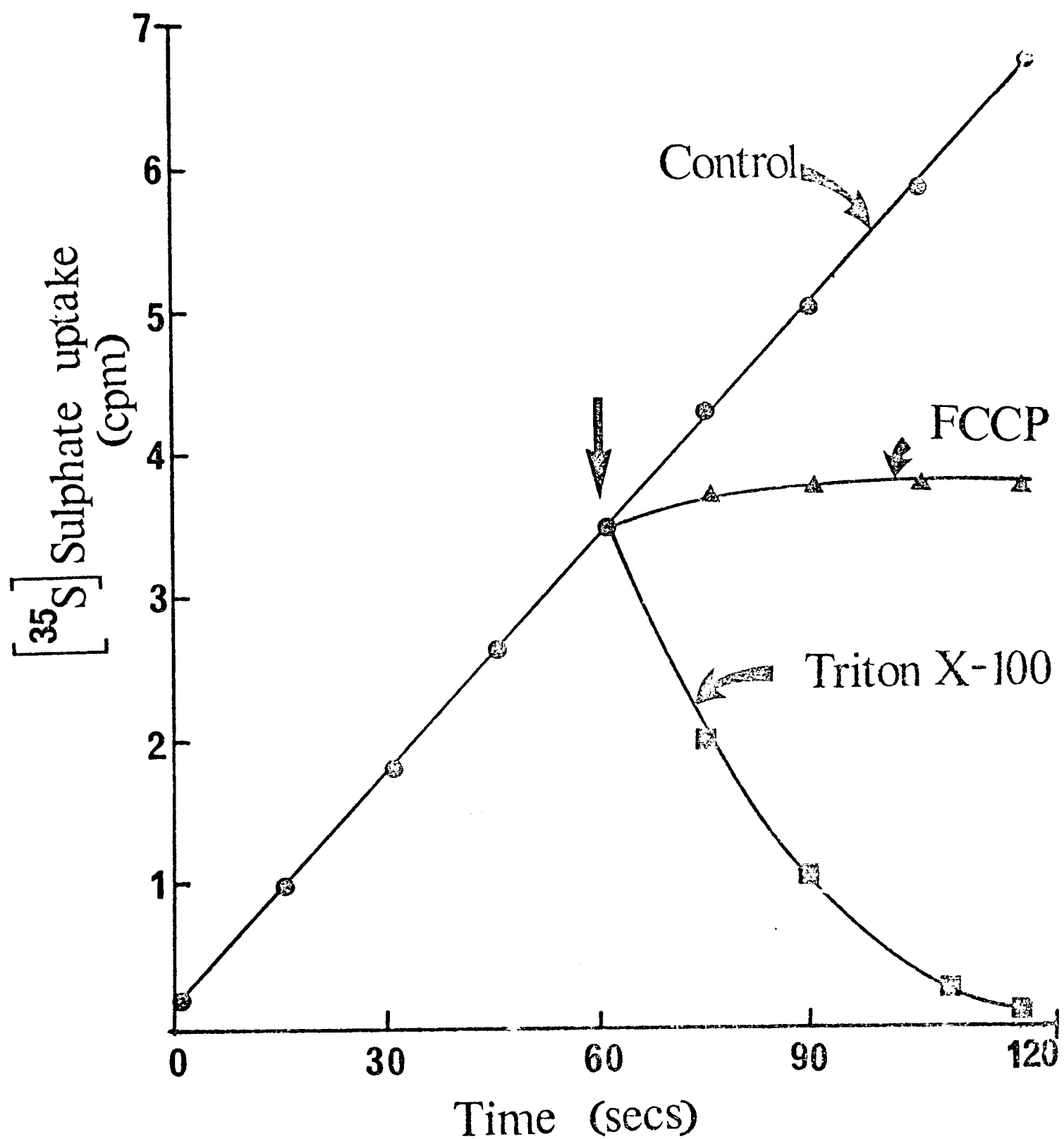


Fig. 4.6

Effect of FCCCP and Triton X-100 on sulphate uptake by whole cells of P. denitrificans.

Sulphate uptake was measured under aerobic conditions as described in Fig. 4.1, except that FCCCP (5 μ M in the assay) and Triton X-100 (0.1%, v/v in the assay) were added to the incubation mixtures as indicated by the arrow.

accumulate sulphate against a concentration gradient and show that activated or passive diffusion cannot account for the uptake of sulphate into whole cells of P. denitrificans.

DISCUSSION

The results presented in this chapter show that the uptake system for sulphate is an active one. Evidence in support of this assertion may be summarised as follows:-

- (1) sulphate is accumulated against a concentration gradient
- (2) sulphate uptake is dependent upon an energy source
- (3) sulphate uptake is both pH- and temperature-dependent
- (4) the uptake system has a high affinity for its substrate (sulphate).

An active uptake system for sulphate would confer a distinct physiological advantage upon the cell; such a system provides a means by which the sulphur required for esters, amino acids, coenzymes, vitamins and lipids can be concentrated from a relatively dilute external sulphate pool. Since the first reaction involved in the assimilation of inorganic sulphate into organic sulphur compounds in many organisms involves an activation reaction possessing a highly unfavourable equilibrium constant / free energy change (ΔG^0) = +11 kcal/mole; Robbins & Lipmann, 1958_7, the intracellular accumulation of sulphate would tend to favour the forward direction of the reaction.

Sulphite and sulphide both exhibit non-competitive inhibition suggesting that they may actually function as physiological feedback inhibitors of sulphate uptake. In light of experiments conducted with membrane vesicles prepared from P. denitrificans (see Chapter 5) which demonstrates the presence of a bi-directional sulphate carrier located in the

membrane, the effect of extracellular sulphite and sulphide appears to reflect the effect of intracellular compounds upon the sulphate uptake mechanism. Similarly the fact that extracellular sulphur-containing amino acids have no immediate effect upon sulphate uptake does not exclude them as intracellular effectors.

The sulphate uptake system appears capable of transporting thiosulphate, a conclusion supported by the observation that:-

- (1) cells are capable of growth on sulphate or thiosulphate as the sole source
- (2) thiosulphate competitively inhibits sulphate uptake
- (3) the percentage of inhibition of sulphate uptake by thiosulphate remains constant during the derepression of the uptake system, in the course of sulphur starvation following growth on cystine.

Since thiosulphate is a structural analogue of sulphate it is possible that a single uptake system could transport both ions. Despite the dual function of the transport system however, sulphate is most likely the physiological substrate for the uptake system considering its primary role in cysteine biosynthesis and its role in sulphate ester formation. Furthermore, the time-course and pulse-chase experiments described in the previous chapter (Chapter 3) indicate that, although thiosulphate may be utilised as a sole sulphur source by P. denitrificans, it is not an intermediate in the sulphate metabolic pathway.

Although the sulphate uptake system in S. typhimurium initially appeared to be controlled by sulphite and thiosulphate (Dreyfuss, 1964), Dreyfuss & Pardee (1966) have subsequently suggested that the true inhibitor is PAPS, the activated

form of sulphate, which is the substrate for sulphate reduction. Ellis (1964, 1966) suggested that cysteine controlled sulphate uptake in E. coli by feedback inhibition, and Jones-Mortimer (1968) has presented a scheme for the control of sulphate metabolism with cysteine acting as both a feedback inhibitor and a repressor of sulphate uptake. Evidence is accumulating to suggest that there are active mechanisms for the uptake of sulphate by a variety of microorganisms. Segel & Johnson (1961) showed that Penicillium chrysogenum is able to concentrate sulphate to levels considerably above those of the external medium, and later Yamamoto & Segel (1966) reported that sulphate uptake in P. chrysogenum is temperature-, pH- and concentration dependent, is suppressed by inhibitors of energy metabolism such as dinitrophenol or azide, and is independent of sulphate reduction.

Recent evidence indicates that there are many differences between the properties of uptake systems encountered in the study of microorganisms. Differences between bacteria and fungi have been described earlier (Yamamoto & Segel, 1966; Bradfield et al., 1970).

Of most importance in relation to this chapter are the similarities and differences which have been encountered during studies on the sulphate uptake systems of S. typhimurium, E. coli and P. denitrificans. These differences may reflect differences in the overall intracellular control of sulphate reduction by assimilatory sulphate-reducing organisms.

CHAPTER 5

SULPHATE UPTAKE INTO MEMBRANE VESICLES

INTRODUCTION

The uptake of sulphate by whole cells of P. denitrificans has been described in Chapter 4. This chapter describes the mode of operation of the sulphate carrier present in membrane vesicles prepared from the plasma membrane of P. denitrificans.

Previously, sulphate uptake has been studied with whole cells of Salmonella typhimurium and Escherichia coli (Pardee et al., 1966; Springer & Huber, 1973). Sulphate uptake into bacterial membrane vesicles has not been previously reported (see Kaback, 1974).

However, membrane vesicles prepared from a variety of bacteria (Kaback, 1974) including P. denitrificans (White et al., 1974) have proved to be useful systems for analysing the mechanism of bacterial active transport (Harold, 1974). Such vesicles lack the cytoplasmic contents of intact cells (Kaback, 1974) and thus the transport of a particular solute can be studied in the absence of cell metabolism, and without the complications arising from the possible movement of other solutes normally present in the intact bacterial cell. The accumulative uptake of amino acids and β -galactosides into membrane vesicles of Escherichia coli has been shown to be driven by the energy released by respiration (see Kaback, 1974). Evidence that this energy is in the form of a pH gradient and membrane potential across the membrane, as postulated by the chemiosmotic theory (Mitchell, 1970), has come from the observation that an accumulative uptake of amino acids and β -galactosides can be driven in the absence of respiration by an electrogenic efflux of K^+ from the vesicles (Hirata et al., 1973; Altendorf et al., 1974).

In the present work two types of vesicle are used: right side out vesicles, in which the vesicle membrane has the same orientation as the plasma membrane of the intact cell; and inside out vesicles in which inversion has occurred during their preparation so that the orientation of the vesicle membrane is the reverse of that of the plasma membrane of the intact cell. In previous studies of active transport in bacterial vesicles, respiration-driven uptake has been observed with right side out vesicles but not with inside out vesicles (Harold, 1972; Kaback, 1974; Hare et al., 1974; Mevel-Ninio & Yamamoto, 1974). However, Ca^{2+} appears to be an exception, since respiration drives the uptake of Ca^{2+} into inside out vesicles but not into right side out vesicles (Rosen & McClees, 1974). Active transport driven by artificially induced ion gradients has been observed previously with preparations consisting predominantly of right side out membrane vesicles (Hirata et al., 1973; Altendorf et al., 1974) and with intact cells (Kashket & Wilson, 1973; Asgar et al., 1973; Niven & Hamilton, 1974) but not with inside out vesicles.

In the present work it is reported that when a pH gradient (alkaline inside) is applied across the vesicle membrane both right side out and inside out membrane vesicles are equally capable of transporting sulphate, but that respiration is effective in driving uptake of sulphate only with the right side out membrane vesicles.

The specificity of respiration-driven sulphate transport for right side out membrane vesicles of P. denitrificans extends the findings of other authors using different bacteria and different solutes; the demonstration of sulphate uptake driven by an artificially induced transmembrane pH gradient in

both right side out and inside out membrane vesicles provides the first indication of the reversibility of active transport across a bacterial membrane. From a comparison of the relative effectiveness of electrogenic and electroneutral ionophores (see Harold 1972; Hamilton, 1975) required in the induction of sulphate transport, it can be inferred that the sulphate carrier in the plasma membrane of P. denitrificans operates by a process of electroneutral proton symport (see Mitchell, 1970).

Reagents

$\text{Na}_2 \text{ } \gamma\text{-}^{35}\text{S}\text{-SO}_4$ and $\gamma\text{-}^{32}\text{P}\text{-P}_i$ were obtained from the Radiochemical Centre, Amersham, Bucks., U.K. $\gamma\text{-}^{32}\text{P}\text{-P}_i$ was converted into $\gamma\text{-}^{32}\text{P}\text{-PP}_i$ by pyrolysis (Lee Peng, 1956) and adjusted to 0.25 Ci/mol. Bee venom (grade I), NADH (grade III) deoxyribonuclease (EC 3.1.4.5), lysozyme (EC 3.2.1.17) and Triton X-100 were obtained from Sigma (London) Chemical Co Ltd., Kingston-upon-Thames, Surrey, U.K.; valinomycin from Calbiochem Ltd., London, W.I., U.K. Nigericin was a gift from Professor Lester Packer. All other reagents were of analytical reagent grade and were obtained either from Sigma (London) Chemical Co. Ltd., or from British Drug Houses Ltd., Poole, Dorset, U.K.

Culture of Bacteria

Paracoccus denitrificans (NCIB 8944) was maintained and grown with succinate as the substrate and nitrate as the added terminal electron acceptor under the growth conditions described in Chapter 2. Cells were also grown with H_2 as the reductant, CO_2 as the carbon source and O_2 as the terminal electron acceptor in a medium of similar composition to that containing succinate (see Chapter 2) except that succinate and nitrate

were omitted and 0.5% (w/v) NaHCO_3 was added. The 1 litre culture solutions, contained in 1.5 litre cylindrical flasks (7.5 cm diam.), were flushed with a gas mixture composed of 60% H_2 , 30% O_2 and 10% CO_2 . The gas mixture was supplied at a rate of 660 ml. min^{-1} through a sintered glass "sparger" which ensured a thorough distribution of gas through the medium. The cultures were maintained at 30°C.

Cells grown with H_2 as the reductant were obtained from cultures inoculated with a 1% inoculum of cells previously adapted to growth with H_2 . The mean generation time of P. denitrificans grown with succinate and nitrate was 2h, and that with H_2 and O_2 was about 8h.

Preparation of Membrane Vesicles

The procedure employed in the preparation of the membrane vesicles was based on a method described by John and Whatley (1970). All the solutions and apparatus used were maintained at 1-4°C and all operations, except the lysozyme treatment, were carried out at these temperatures. Stock buffer solutions at a concentration of 0.4M were adjusted to pH 7.3 at room temperature, and then diluted to 10 or 100mM as required.

Cells from 21. of mid log phase culture were sedimented by centrifugation at 5 000g for 30 min and were washed in 800 ml of 50mM-NaCl containing 10mM-Tris-HCl buffer. The cells were then suspended in 400 ml of 0.5M-sucrose containing 10mM-Tris-HCl buffer so that a 0.1 ml aliquot of the suspension diluted to 2.5 ml with water had an absorbance at 550 nm of 0.3 when read in a Beckman DB Spectrophotometer. This was equivalent to about 25 mg of wet weight of cells per ml of suspension in the 0.5M sucrose. Lysozyme (E.C. 3.2.2.17) was added at a concentration of 250µg/ml to this suspension which

was then allowed to stand in a water bath at 30°C for 20-30 min until the absorbance of an aliquot diluted 1/25th with water decreased from 0.3 to 0.05.

After treatment with lysozyme the cells were sedimented by centrifugation at 40 000g for 10 min and resuspended in 40 ml of 100mM-Tris-acetate buffer using a Potter-Elvehjem homogeniser. The suspension was then diluted with 360 ml of water to disrupt the cells. After allowing to stand for 20 min a trace of deoxyribonuclease (E.C. 3.1.4.5) and 3mM-magnesium acetate were added. The suspension was shaken gently to distribute the deoxyribonuclease and magnesium acetate. When the viscosity of the suspension was sufficiently lowered, the suspension was centrifuged at 40 000g for 40 min to yield a double-layered pellet and a clear supernatant, which was discarded. The upper, red layer of the pellet was resuspended in 400 ml of 1mM-magnesium acetate containing 10mM-Tris-acetate buffer. The lower, white layer of the pellet which probably consists of poly- β -hydroxybutyrate (Schöles and Smith, 1968a) was discarded. The suspension was centrifuged at 40 000g for 40 min and the resulting pellet was resuspended in 1mM-magnesium acetate containing 10mM-Tris acetate buffer to a concentration of about 5 mg membrane protein/ml.

The suspension of membrane vesicles was stored at 1-4°C and could be used for experiments over a period of 3 days.

Measurement of sulphate uptake by whole cells

Cells were grown aerobically, harvested, washed, resuspended and reincubated as described in Chapter 4. Sulphate uptake was measured under aerobic conditions as described in Chapter 4.

Measurement of sulphate uptake by membrane vesicles

Assays were conducted at 30°C in a small flat-bottomed,

cylindrical flask (1.5 x 3.5 cm) which was stirred magnetically. Standard incubation mixtures contained, in a total volume of 2 ml, 1 μ mole γ - ^{35}S - Na_2SO_4 (1 μ Ci/ μ mol) 0.2 ml. of membrane vesicles (1.0-1.5 mg protein) and 100 μ moles Tris-HCl (pH 7.3). Membrane vesicles were added to start the experiment. Samples (0.1 ml) were withdrawn at intervals and membrane vesicles collected by filtration on a Millipore filter (0.22 μ diam. pores). Membrane vesicles collected on the filters were washed twice with 5 ml portions of 50mM-Tris-HCl (pH 7.3) at 30°C. Where uptake was driven by respiration the appropriate respiratory substrate was included in the wash solution at the same concentration as employed in the reaction medium, and a water-saturated atmosphere of O_2 was maintained in the reaction flask.

Filters with washed vesicles were individually placed in scintillation vials, dried (to reduce quenching by water), and 5 ml of scintillation fluid was added. The scintillation fluid contained 7g Butyl-PBD and 80g naphthalene made up to 1 litre with a 6:4 (v/v) mixture of toluene and 2-methoxyethanol, respectively. The vials were allowed to stand for 12 h prior to counting in a scintillation counter. The complete composition of both the reaction mixtures and wash solutions are given in the legends of the appropriate figures.

Identification of sulphate by electrophoresis

Vesicles were incubated with 0.1mM-phenazine methosulphate and 20mM-sodium ascorbate for 20 min in the presence of $\text{Na}_2\gamma$ - ^{35}S - SO_4 in a standard incubation mixture for measuring sulphate uptake with right side out membrane vesicles. An aliquot (0.5 ml) was filtered and the vesicles washed with 20 ml of hot water. The washings were evaporated to dryness

by rotary evaporation and the residue dissolved in 0.5 ml of water. 50 μ l samples were subjected to paper electrophoresis on Whatman 3MM chromatography paper in 0.1M-sodium citrate buffer (pH 5.0) at 5.5 V per cm for 3 h. Paper electrophoresis strips were cut into sections (1.0 cm) and the radioactivity counted in a scintillation counter as described above following the addition of 1 ml of scintillation fluid.

Determination of NADH dehydrogenase activity

The oxidation of NADH was linked to the reduction of 2,6-dichlorophenol-indophenol and the initial rate of decrease in absorption at 600nm monitored spectrophotometrically at 30°C in a Pye Unicam SP 8000. The reaction mixture contained in 3 ml: 50mM-Tris HCl (pH 8.0), 33 μ M-2,6-dichlorophenol-indophenol, 2mM-KCN (pH 7.5) and membrane vesicles (0.1 mg protein). The reaction was started by the addition of 1mM-NADH. In the absence of membrane vesicles a slow rate of NADH-dependent reduction of 2-6-dichlorophenol-indophenol was observed. The nonenzymic rate (12% of the maximum rate observed in the presence of membrane vesicles) was subtracted from the rates observed in the presence of membrane vesicles to give a measure of the NADH dehydrogenase activity of the membrane vesicles. The extinction coefficient for 2,6-dichlorophenol-indophenol was taken to be 21000 litre.mol⁻¹.cm⁻¹ (King, 1963).

Protein

Protein was determined by the Folin method of Lowry et al., (1951) with bovine serum albumin as a standard.

Results

Orientation of the Membrane Vesicles

As in other bacteria (Hare et al., 1974; Hampton & Freese,

1974) the plasma membrane of P. denitrificans (Scholes & Smith, 1968b) is effectively impermeable to NADH, and the NADH dehydrogenase of the respiratory chain is located on the inside of the plasma membrane so that it is accessible to NADH only from the inside of the intact cell. Thus those membrane vesicles which have retained the orientation of the plasma membrane of the intact cell (right side out membrane vesicles) would be expected to oxidise NADH added to the reaction mixture only slowly unless the permeability of the plasma membrane to NADH was increased. On the other hand membrane vesicles which have the reverse orientation of the plasma membrane to that of the intact cell (inside out membrane vesicles) would be expected to oxidise NADH added to the reaction mixture rapidly and this rate of oxidation would not be much affected by treatments which increase the permeability of the plasma membrane to NADH.

In the present study, bee venom (Habermann, 1972; Verma et al., 1974) was used to increase the permeability of the plasma membrane to NADH. Fig. 5.1 shows that in the presence of increasing concentrations of bee venom there is an increase in the observed NADH dehydrogenase activity of membrane vesicles prepared from cells grown with H_2 as the reductant. On the other hand similar concentrations of bee venom have relatively little effect upon the observed NADH dehydrogenase activity of the membrane vesicles prepared from cells grown with succinate as the substrate. In the presence of saturating levels of bee venom the observed NADH dehydrogenase activities of the two types of membrane vesicles are essentially the same.

Thus the membrane vesicles isolated from cells grown with succinate are mainly inside out vesicles, and those

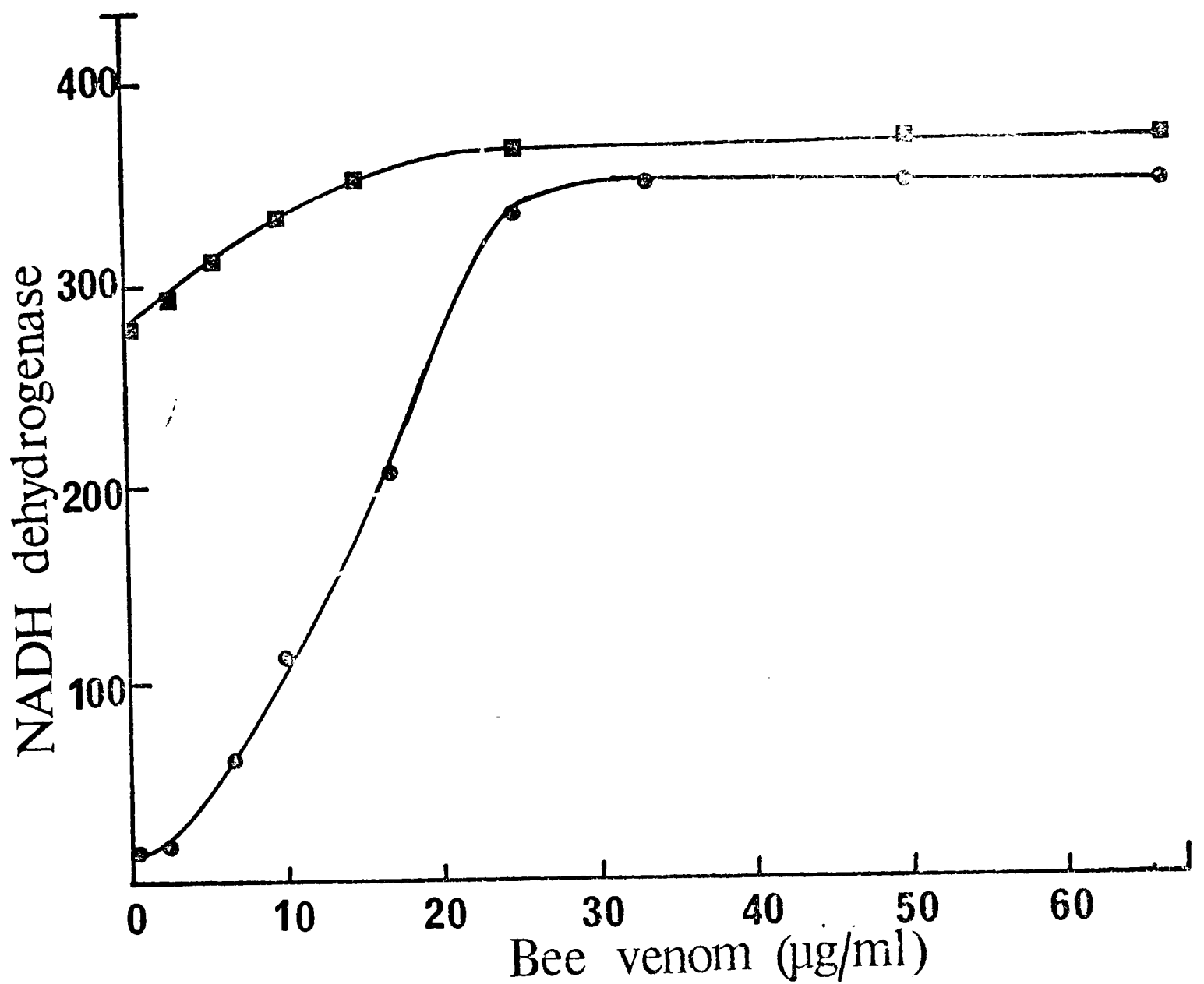


Fig. 5.1

Effect of bee venom on the NADH dehydrogenase activity of membrane vesicles prepared from the plasma membrane of P. denitrificans.

Membrane vesicles were prepared from cells grown with either hydrogen as the reductant (●), or with succinate as the substrate (■). NADH dehydrogenase activities were measured as described in Materials and Methods and is expressed umoles of NADH oxidised per min per mg of protein.

isolated from cells grown with H_2 are essentially all right side out vesicles.

The orientation of the vesicle membrane is discussed at length below, but for convenience in the remainder of the Results section of this chapter the two types of membrane vesicle will be referred to by the inferred orientation of the membrane rather than by reference to the growth substrates of the cells from which they were prepared.

Sulphate uptake driven by respiration

Sulphate uptake into right side out vesicles was observed in the presence of a variety of respiratory substrates (Fig. 5.2). However inside out vesicles did not accumulate sulphate under these conditions (Fig. 5.2). The uptake of sulphate into the right side out vesicles was dependent upon the presence of a respiratory substrate (Fig. 5.3), and was abolished by the inclusion of the uncoupling agent FCCP (Fig. 5.2). When this uncoupler was added to right side out vesicles which had already accumulated sulphate there was a rapid efflux of the accumulated sulphate from the vesicles (Fig. 5.3). Sulphate efflux was also observed upon depletion of the respiratory substrate.

Sulphate uptake driven by additions of salts.

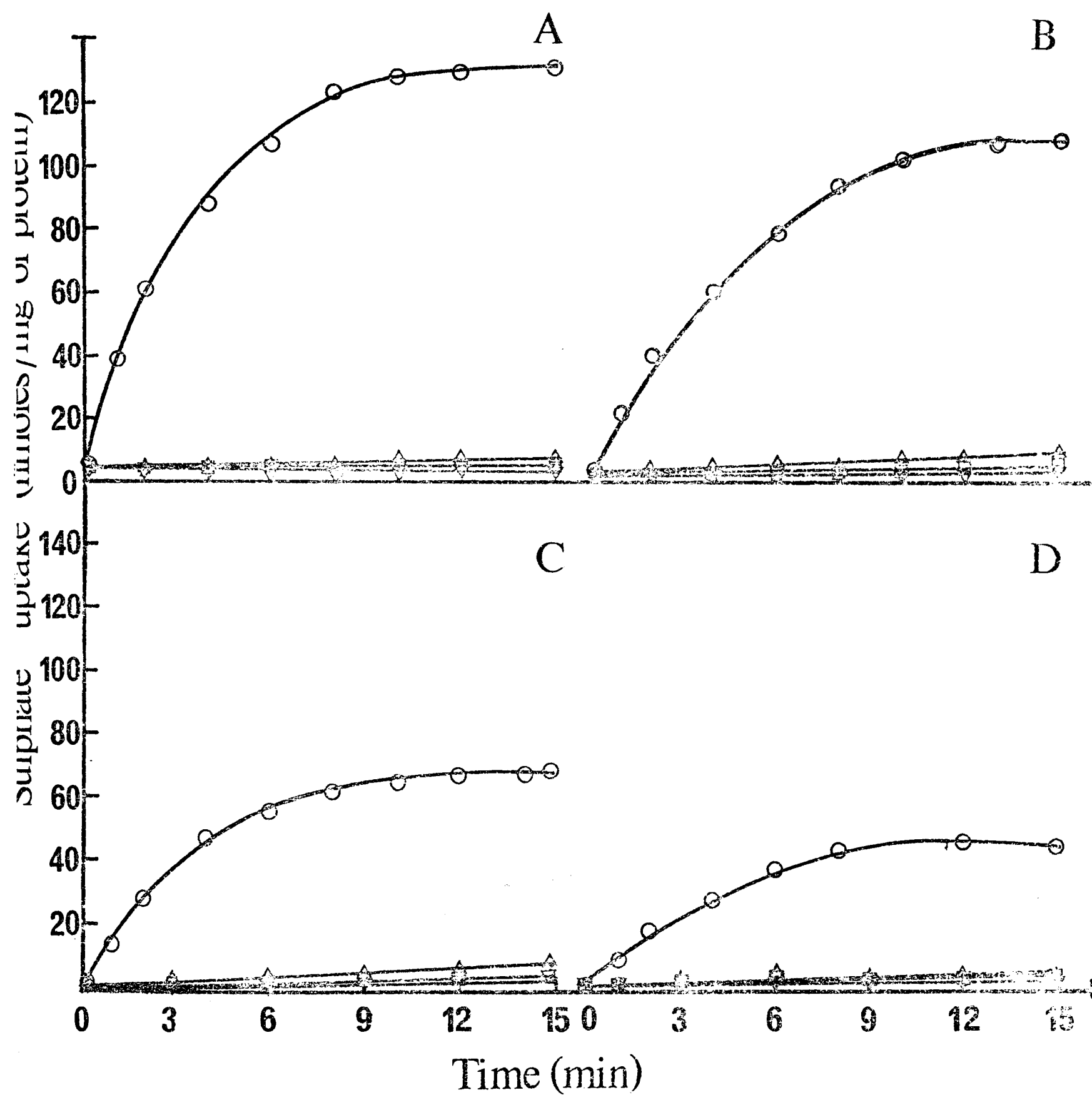
In the absence of respiratory substrates, the addition of a pulse of $150mM$ -KCl to a suspension of membrane vesicles containing nigericin resulted in transient accumulation of sulphate. This accumulation occurred to a similar extent when inside out or right side out membrane vesicles were used (Fig. 5.4A, B). No uptake was observed when nigericin was omitted and valinomycin could not substitute for nigericin (Fig. 5.4C, D). Furthermore, in the presence of nigericin the inclusion of valinomycin resulted in no uptake (Fig. 5.4C, D).

Fig. 5.2

Effect of membrane orientation in respiration-driven uptake of sulphate into membrane vesicles of P. denitrificans. Sulphate uptake into right side out ($\square$) and inside out (Δ, ∇) membrane vesicles was measured as described in Materials and Methods in a medium which contained in 2 ml: 1 μ mole $\text{Na}_2\text{S}_2\text{O}_4$ ($1 \mu\text{Ci}/\mu\text{mole}$), 0.2 ml of the membrane vesicle suspension (1.5 mg protein) and 100 μ moles of Tris-HCl buffer (pH 7.3). Vesicles collected on the filter were washed twice with 5 ml portions of a solution which contained 50 mM-Tris-HCl and the respective respiratory substrate at the concentration used in the reaction mixture. The respiratory substrates were:-

- (A) 20 mM-sodium ascorbate plus 0.1 mM-phenazine methosulphate,
- (B) 20 mM-sodium ascorbate plus 0.1 mM- TMPD,
- (C) 10 mM-sodium D-lactate, (D) 10 mM-sodium succinate.

5 μM -FCCP was added ($\square, \nabla$), or omitted ($\circ, \Delta$) as indicated.



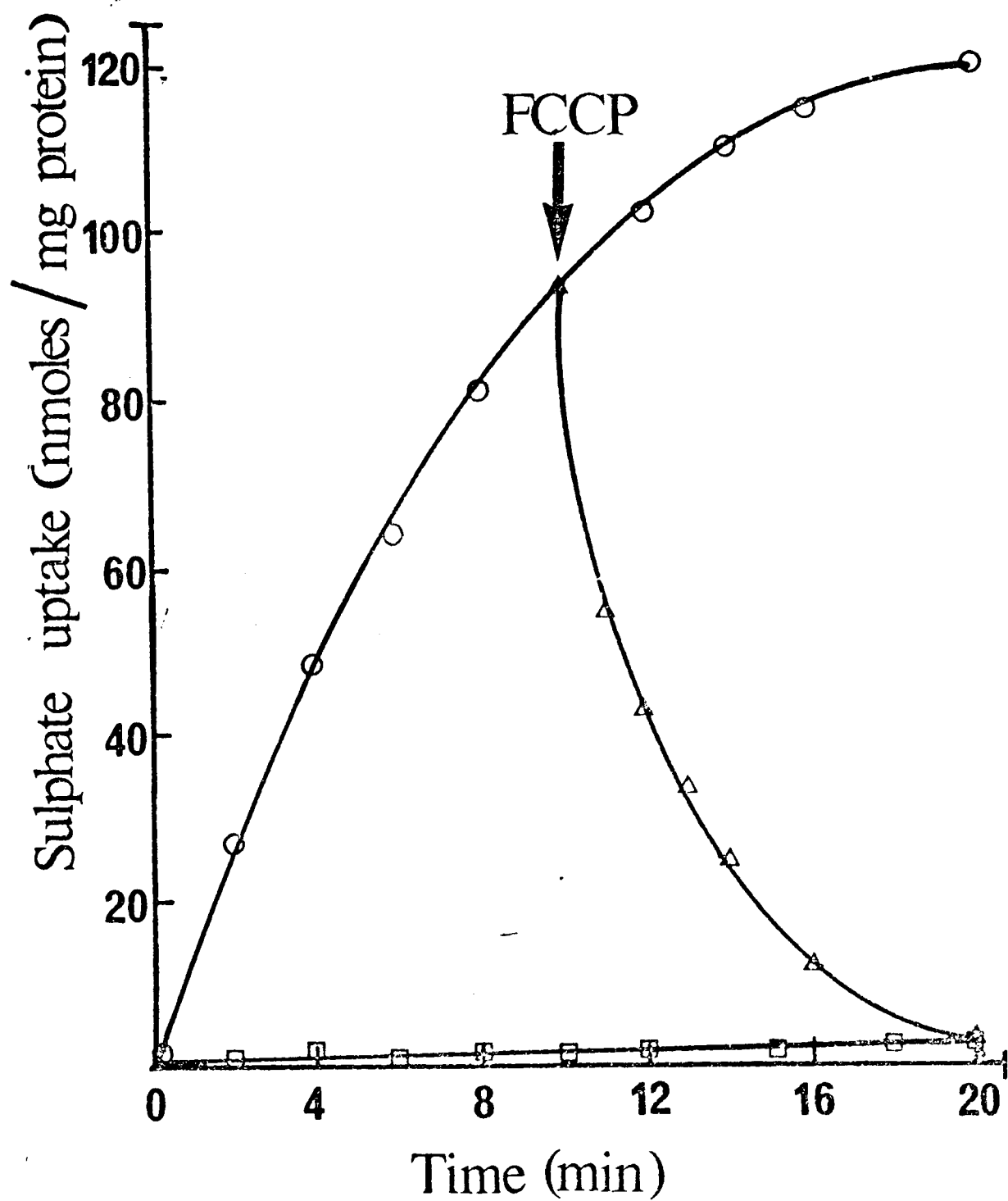


Fig. 5.3

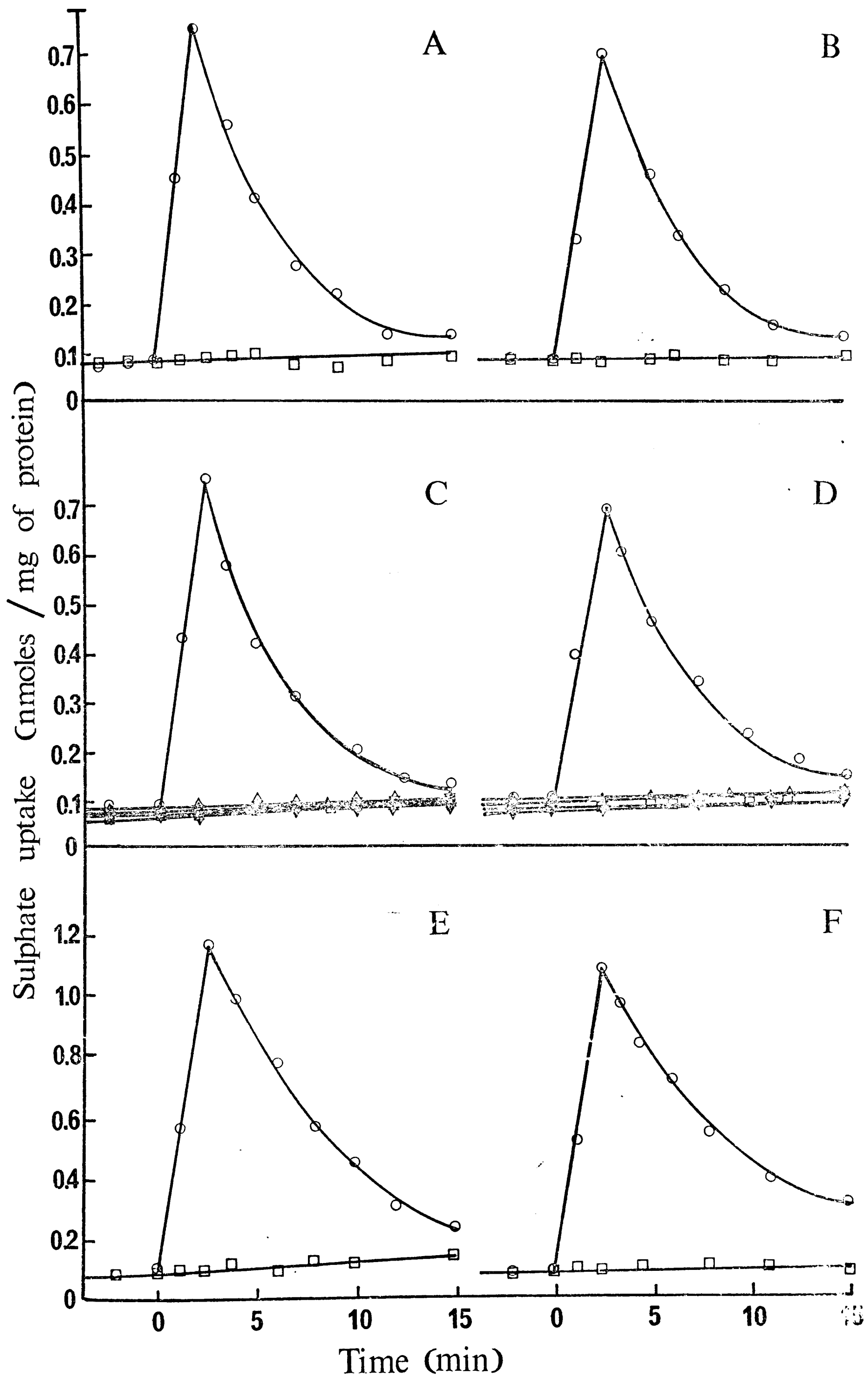
Sulphate uptake by right side out membrane vesicles of P. denitrificans.

Sulphate uptake was measured as described in Fig. 5.2A in the presence ($\Delta, \square$) and absence (o) of 20 mM-sodium ascorbate plus 0.1 mM-phenazine methosulphate. 5 μ M-FCCP was added at the time indicated by the arrow to one of the reaction mixtures which contained the respiratory substrates (o).

Fig. 5.4

Transient uptake of sulphate into membrane vesicles of P. denitrificans driven by addition of salts.

Sulphate uptake into right side out (A, C, E) and inside out (B, D, F) membrane vesicles was measured as described in Materials and Methods in a reaction mixture (2 ml) which contained 1 μ mole $\text{Na}_2 \text{ } ^{35}\text{S}_7\text{SO}_4$ (1 $\mu\text{Ci}/\mu\text{mole}$), 0.2 ml of membrane vesicle suspension (1.5 mg of protein) and 100 μ moles of Tris-HCl buffer (pH 7.3) (0). At time 0, either 0.15 M-HCl (A,B,C,D,) or 0.15 M- NH_4Cl (E, F) were added. In A, B, C, D, 1 μg -nigericin was included. Additions and subtractions to the reaction mixtures were as follows: $\square$, plus 5 μg -FCCP; Δ , plus 1 μg -valinomycin (added in the presence of 1 μg -nigericin) ∇ , plus 1 μg -valinomycin (minus 1 μg -nigericin). $\blacktriangle$, minus 1 μg -nigericin.



Similarly, addition of a pulse of $150\text{mM-NH}_4\text{Cl}$ caused uptake to a similar extent in both right side out and inside out membrane vesicles even in the absence of any ionophore (Fig. 5.4E, F). The extent of NH_4Cl -induced sulphate uptake was similar to that observed on addition of KCl to a suspension of membrane vesicles containing nigericin (Fig. 5.4).

Inclusion of the sulphhydryl-group reagents, N-ethyl-maleimide, p-chloromercuribenzoate (pCmb) and methyl mercuric chloride prevented the KCl-induced sulphate uptake. When these compounds were added to a suspension of membrane vesicles which had accumulated sulphate, the rate of efflux of the accumulated sulphate was decreased (Fig. 5.5).

The inclusion of selenate in the reaction mixture decreased the rate and extent of sulphate uptake and also decreased the rate of efflux of sulphate (Fig. 5.6).

Validity of the assay of sulphate uptake

The sulphate accumulated by right side out membrane vesicles during a 20 min incubation period (as described for Fig. 5.2) could be quantitatively recovered (98%) by washing the cells in hot water, and identified as sulphate by electrophoretic examination. Similarly the sulphate accumulated by both inside out and right side out membrane vesicles in response to a pulse of KCl (in the presence of nigericin) was quantitatively extracted (96%) and electrophoretically identified as sulphate. Furthermore, the initial enzyme involved in the assimilation of sulphate, ATP sulphurylase (ATP-sulphate adenylyl transferase EC 2.7.7.4) was not detected whether in inside out or in right side out vesicles (Table 5.1). These observations show that the sulphate accumulated by membrane vesicles could not be further metabolized but remained as sulphate.

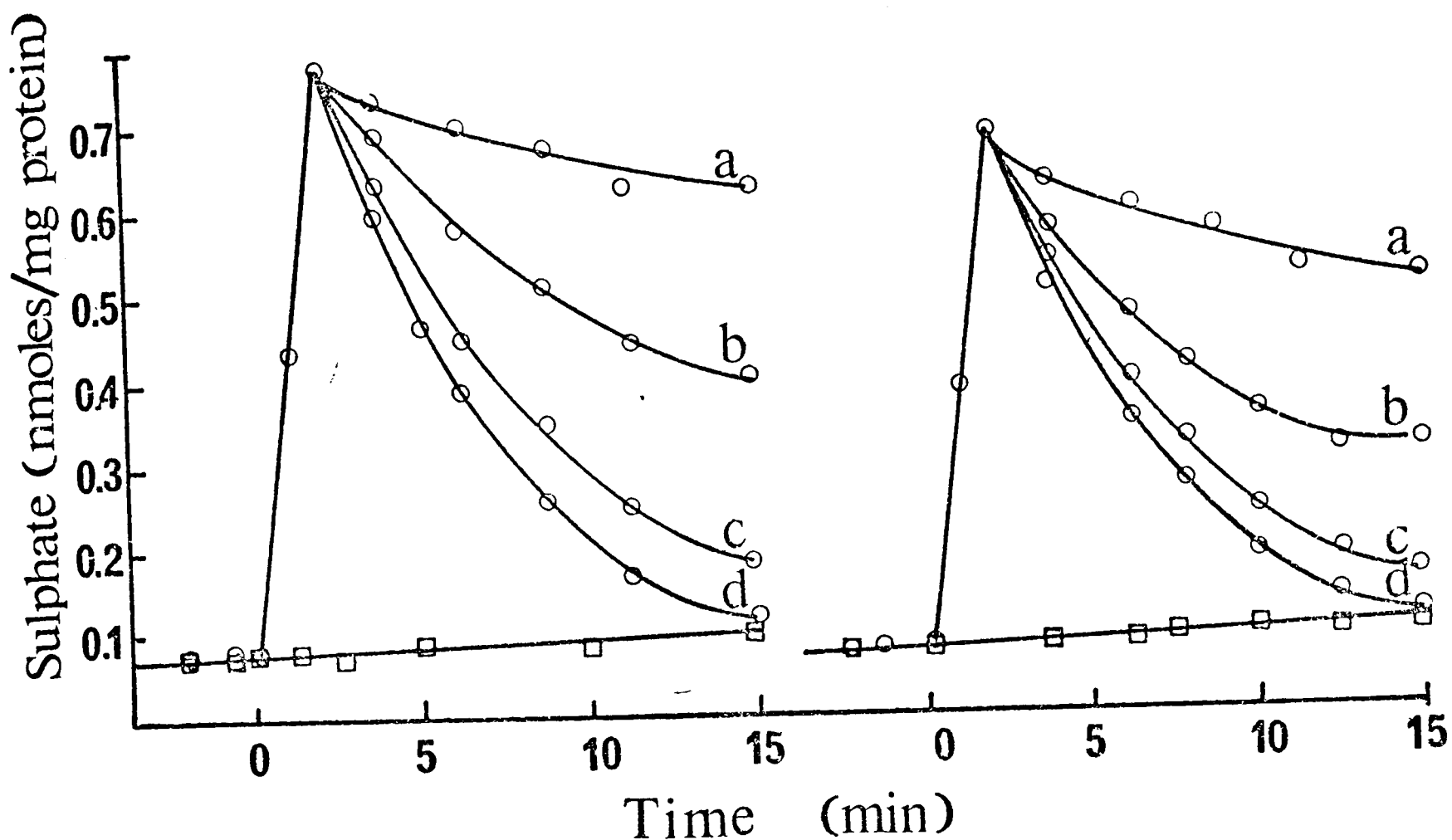


Fig. 5.5

Effect of sulphhydryl-group reagents on the efflux of sulphate accumulated by membrane vesicles.

Transient uptake into right side out (A) and inside out (B) membrane vesicles was driven by the addition of 0.15 M-KCl in the presence of 1 μ g-nigericin (curved) as in Fig. 5.4A and Fig. 5.4B in the presence ($\square$) or absence ($\circ$) of 5 μ M-FCCP. Further additions were made at time 2 min as indicated: (a) none, (b) 8 μ M-p-chloromercuribenzoate; (c) 10 mM-N-ethyl maleimide; (d) 10 mM-methyl mercuric chloride.

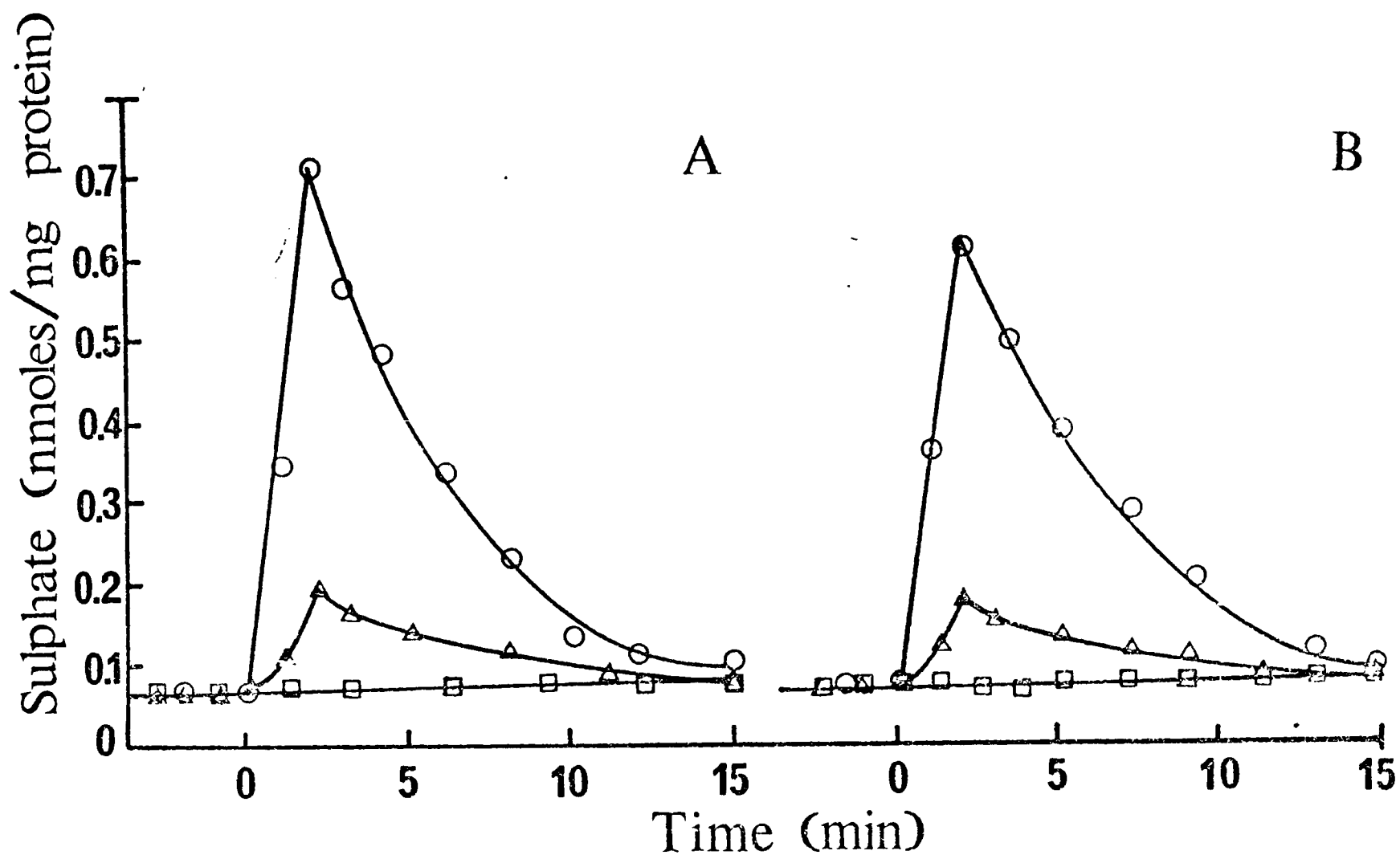


Fig. 5.6

Effect of selenate on KCl-induced transient sulphate uptake into membrane vesicles.

Uptake was driven by the addition of 0.15 M-KCl in the presence of 1 μg-nigericin as in Fig. 5.4A and Fig. 5.4B (0) except that 1 mM-Na₂SeO₄ (Δ) or 5 μM-FCCP (□) were added where indicated to right side out (A) or inside out (B) membrane vesicles.

TABLE 5.1

The levels of ATP sulphurylase in whole cells and membrane vesicles of P. denitrificans

ATP Sulphurylase activity.
(nmoles ATP-PP_i exchange/min per ng protein)

	<u>Substrate for cell growth</u>	
	H ₂	Succinate
Whole cells	4.23	4.47
Membrane vesicles	0.01	0.01

Cells were grown with either hydrogen as the reductant or with succinate as substrate, and membrane vesicles prepared from these cells as described in "Materials and Methods". The ATP sulphurylase of cells (22mg protein/ml) and membrane vesicles (7mg/ml) was monitored by sulphate-dependent ATP-PP_i exchange as described by Shaw & Anderson (1971) in the presence of Triton X-100 (0.3% v/v).

Sulphate efflux from whole cells

The accumulation of sulphate by whole cells was linear for 2 min. Addition of FCCP or KCN or the addition of FCCP followed by KCN stopped sulphate uptake but did not result in the loss of the accumulated sulphate (Fig. 5.7). However, the addition of sodium acetate following the addition of FCCP resulted in a rapid efflux of sulphate from the cells. A similar efflux of sulphate was observed on the addition of sodium formate, but no efflux was observed on the addition of the same concentration of NaCl (Figure 5.8).

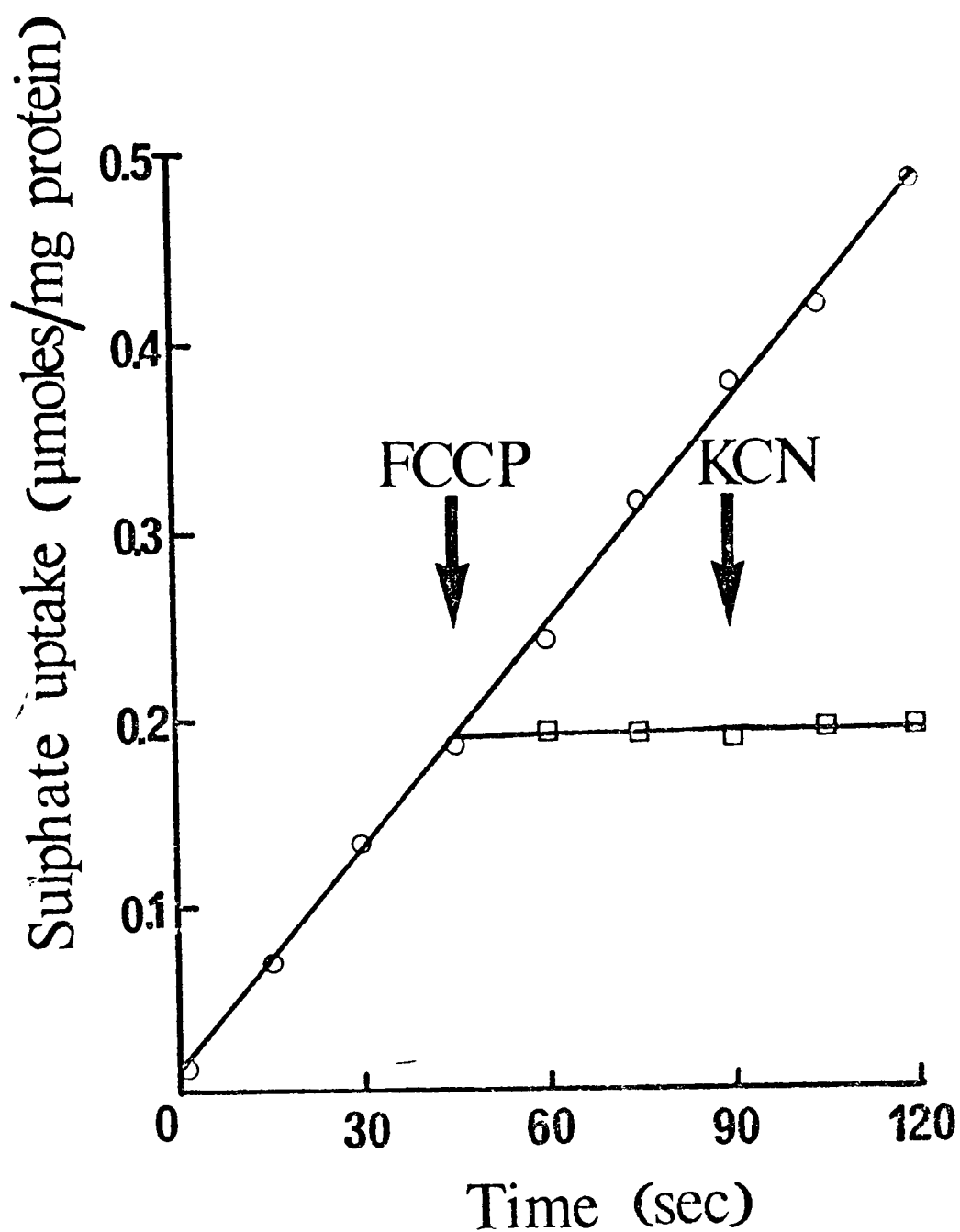


Fig. 5.7

Effect of FCCP and KCN upon sulphate accumulation by intact cells of P. denitrificans.

Sulphate uptake was measured as described in Chapter 4 (0). To one reaction flask no inhibitor was added (0). To the other reaction flask (□), FCCP and KCN were added as indicated. Reaction mixtures contained in 5 ml; 3 ml of washed cell suspension, 1.5 ml of succinate-oxygen growth medium (minus sulphate) and 1 μmole γ - ^{35}S - Na_2SO_4 (1 μCi/μmole). Sulphate uptake was measured under aerobic conditions.

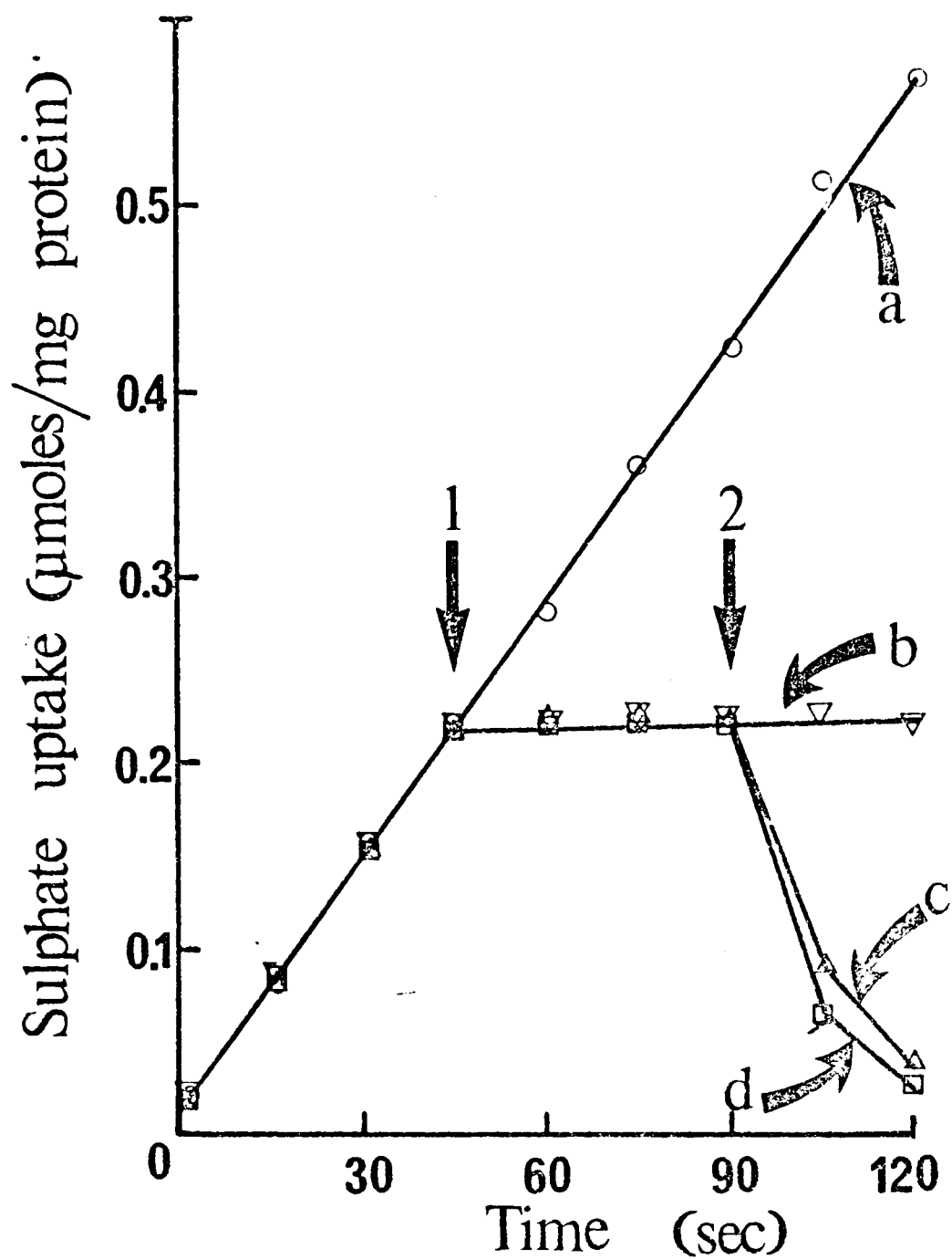


Fig. 5.8

The effect of salts upon the efflux of sulphate accumulated by whole cells of P. denitrificans. All reaction flasks were initially identical. At Arrow 1, 5 μ M-FCCP was added to flasks b, c, and d, and at Arrow 2, a further addition of salt was made as follows; (b) 0.15M-NaCl, (c) 0.15M-sodium formate and (d) 0.15M-sodium acetate. Reaction mixtures were initially as described in Fig. 5.7 and sulphate uptake and efflux was measured under aerobic conditions.

Discussion

In the Results Section of the present chapter it has been stated that membrane vesicles obtained from cells grown with succinate consist predominantly of inside out vesicles and that membrane vesicles obtained from cells grown with H_2 consist predominantly of right side out vesicles. The importance of this conclusion to the interpretation of the rest of the data presented in this chapter justifies further comment.

There is sufficient evidence from previous work with a variety of bacteria for the following two generalisations to be made: (1) NADH, supplied at a concentration of approximately 1mM in the suspending medium, is oxidised at an appreciable rate by inside out membrane vesicles (or by non-vesicular membranes), but not by right side out membrane vesicles (Eisenberg et al., 1970; Harold, 1972; Hare et al., 1974; Hampton & Freese, 1974; Futai, 1974a, b), and (2) respiration drives the active uptake of substances normally accumulated within the bacterial cell into right side out vesicles, but not into inside out vesicles (Hirata & Brodies, 1972; Harold, 1972; Hampton & Freese, 1974; Hare et al., 1974; Mevel-Ninio & Yamamoto, 1974). With the membrane vesicles isolated from P. denitrificans described in the present chapter the following observations were made. Firstly, the rate of NADH oxidation observed in the absence of bee venom with the vesicles isolated from cells grown with H_2 is only 5% of the maximum rate observed in the presence of bee venom; whereas the vesicles isolated from cells grown with succinate give 80% of the maximum rate without treatment with bee venom. Secondly, the rate and extent of respiration-driven sulphate uptake by the vesicles isolated from cells grown with succinate are only 10%

of the rate of the respiration-driven sulphate uptake by the vesicles isolated from cells grown with H_2 . Thus it is concluded that the vesicles isolated from cells grown with succinate are predominantly inside out, while the vesicles isolated from cells grown with H_2 are predominantly right side out. From the rates and extent of sulphate uptake, and from the degree to which bee venom stimulates NADH oxidation it can be inferred that right side out vesicles contribute no more than 10-20% of the total vesicle population in the vesicles isolated from cells grown with succinate. It is unlikely that non-vesicular membranes constitute a significant proportion of the membrane material of the preparation obtained from cells grown with succinate since these preparations show a high degree of respiratory control (John & Whatley, 1975). Bee venom contains mellitin, a haemolytic agent with mild detergent properties (Haberman, 1972; Verma et al., 1974; Mollay & Kreil, 1974), and presumably this component is responsible for destroying the integrity of the plasma membrane of P. denitrificans thus enabling NADH to gain access to its dehydrogenase, even when the NADH is orientated towards the inner face of right side out vesicles. Other authors using a variety of techniques have detected the presence of both inside out and right side out vesicles in membrane preparations obtained by osmotic lysis of Escherichia coli (Hare et al., 1974), Bacillus subtilis (Hampton and Freese, 1974) and Micrococcus lysodeikticus (Gorneva and Ryabova, 1974).

The reason for the different orientation of the vesicles in the preparations obtained from cells grown under the two conditions is unknown. An autotrophic mode of growth or a

slower growth rate do not appear to be directly related to the production of right side out vesicles, since other authors (using different conditions of lysozyme treatment and of osmotic lysis) have isolated right side out vesicles from cells of P. denitrificans grown heterotrophically with high growth rates in rich media (Scholes & Smith, 1968a, b; White et al., 1974). Membrane vesicles isolated by the procedure described here from cells grown in rich media are predominantly of the right side out variety (P. John, unpublished work), and resemble the membrane vesicles isolated from cells grown with H_2 in the latency of their NADH dehydrogenase, but such vesicles could not be used in the present study because of the absence of a sulphate carrier in cells grown in the presence of S-containing amino acids (chapter 4).

It is also important for the further interpretation of the present data to emphasise that while the two types of membrane preparation used in the present study differ in the orientation of the membrane-vesicles, other differences between the two types of preparation are not likely to be of significance for the active transport of sulphate. Thus both types of preparation contain a fully functional constitutive respiratory chain (John & Whatley, 1970; Knobloch et al., 1971), which closely resembles the mitochondrial respiratory chain. (John & Whatley, 1975).

The transient accumulation of sulphate driven by the addition of KCl (in the presence of nigericin) or of NH_4Cl can be explained most readily by assuming that additions of the appropriate salts generate an electroneutral pH gradient (alkaline inside) across the vesicle membrane, and that this gradient drives sulphate uptake as implied by the chemiosmotic

theory (Mitchell, 1970), discussed in detail for the uptake of other substrates by Harold (1974) and Hamilton (1975), and represented diagrammatically in Fig. 5.9. Since valinomycin did not substitute for nigericin in these experiments it may be concluded that sulphate is not taken up electrophoretically. The effectiveness of valinomycin as a conductor of K^+ across the vesicle membrane under the conditions of these experiments is demonstrated by its ability to prevent the sulphate uptake driven by addition of KCl (in the presence of nigericin).

The extent of sulphate uptake observed in response to the addition of KCl (in the presence of nigericin) or of NH_4Cl is only 1% of that observed with respiration as the driving force. The sensitivity of the sulphate uptake driven by KCl (in the presence of nigericin) to FCCP, to sulphydryl-group reagents and selenate can be readily explained only if this sulphate uptake involves a carrier.

It is possible, however, that the relatively limited, transient uptake of sulphate, observed upon the addition of salts, is due solely to an energy-dependent binding of sulphate to the sulphate carrier, without the sulphate entering the internal aqueous phase of the membrane vesicle. An analogous energy-dependent, uncoupler-sensitive binding of non-transportable dansyl- β -galactosides to the β -galactoside carrier of membrane vesicles of E. coli has already been described (Reeves et al., 1973; Schuldiner et al., 1975). However, by comparing the results using the sulphate carrier of P. denitrificans with those obtained with the β -galactoside carrier of E. coli we may identify the sulphate uptake, driven by addition of salts, as sulphate transport rather than sul-

phate binding as follows. Addition of sulphydryl-group reagents causes a retention of transported lactose, (Kaback & Barnes, 1971); but causes an apparent release of bound dansyl- β -galactoside (Reeves et al., 1973). The sulphate accumulated in response to an addition of KCl (in the presence of nigericin) is normally rapidly lost from the vesicles, but an appropriately timed addition of a sulphydryl-group reagent results in the accumulated sulphate being retained (Fig. 5.5). The results may be interpreted as showing sulphate transport rather than sulphate binding, in response to an imposed pH gradient.

The observation of an energised uptake of sulphate into both inside out and right side out membrane vesicles demonstrates the reversibility of the sulphate carrier present in the plasma membrane of P. denitrificans. Presumably the direction in which this carrier operates in whole cells is determined by the polarity of the pH gradient imposed across the plasma membrane. In whole cells of P. denitrificans respiration is associated with an outward movement of protons (Scholes & Mitchell, 1970). The return of these protons to the cell interior via the sulphate carrier would drive sulphate accumulation by the whole cells.

Having established that the sulphate carrier present in membrane vesicles of P. denitrificans is reversible, indications were sought of its reversibility in the plasma membrane of intact cells. However, intact cells (unlike membrane vesicles) retain the accumulated sulphate when the driving force, respiration, is abolished by the addition of KCN. In the presence of FCCP the accumulated sulphate is rapidly lost from vesicles (Fig. 5.3) but retained by whole cells (Fig. 5.7). But it was

found that an addition of sodium acetate to cells which had already accumulated sulphate and had been subsequently treated with FCCP and KCN caused a rapid efflux of sulphate (Fig. 5.8). An interpretation of this phenomenon is that undissociated acetic acid enters the cell where it dissociates generating an electroneutral pH gradient across the bacterial plasma membrane. This gradient, which reverses the polarity of the pH gradient maintained by respiration (Scholes & Mitchell, 1970) drives sulphate efflux (Fig. 5.9).

Fig. 5.9

Sulphate uptake into inside out and right side out membrane vesicles of P. denitrificans.

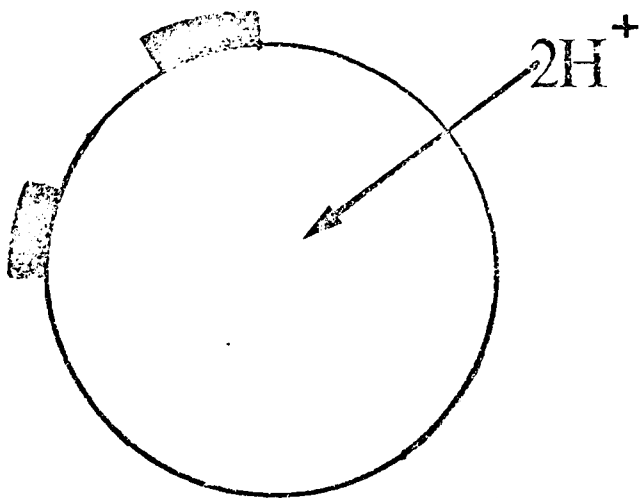
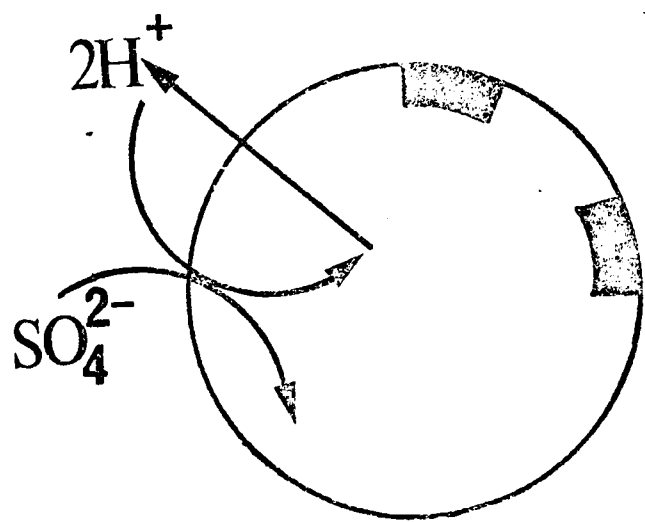
The orientation of the NADH dehydrogenase on the inner face, on the right side out membrane vesicles and on the outer face, of the inside out membrane vesicles, is indicated. During respiration (upper row), in right side out membrane vesicles, protons are pumped outwards and their return is coupled to sulphate uptake. In inside out membrane vesicles protons are pumped inwards and sulphate accumulation cannot occur, but a coupled-sulphate expulsion would be possible. Influx of K^+ , via nigericin, (middle row) results in an efflux of an equivalent number of protons and their return is coupled to sulphate uptake. This is true for both inside out and right side out membrane vesicles. Influx of ammonia (bottom row) causes a proton deficit inside the membrane vesicles and the consequential influx of protons is coupled to sulphate uptake. This is true for both inside out and right side out membrane vesicles.



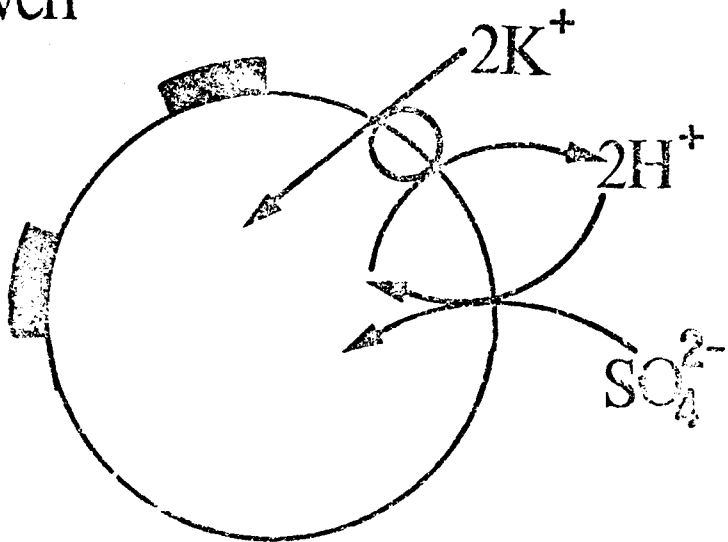
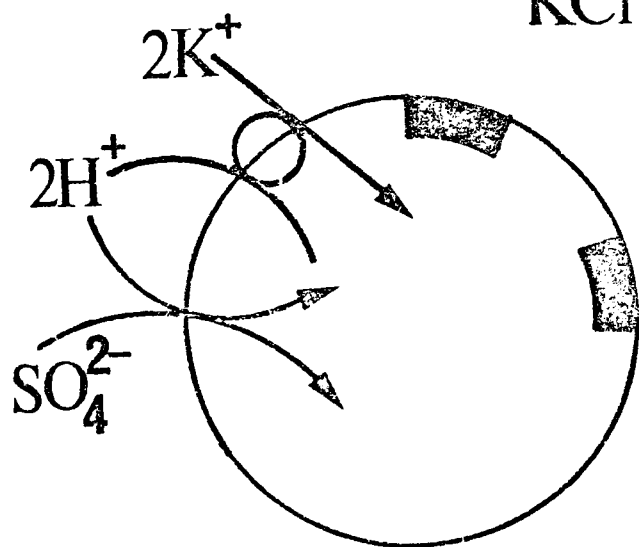
RIGHT SIDE OUT

INSIDE OUT

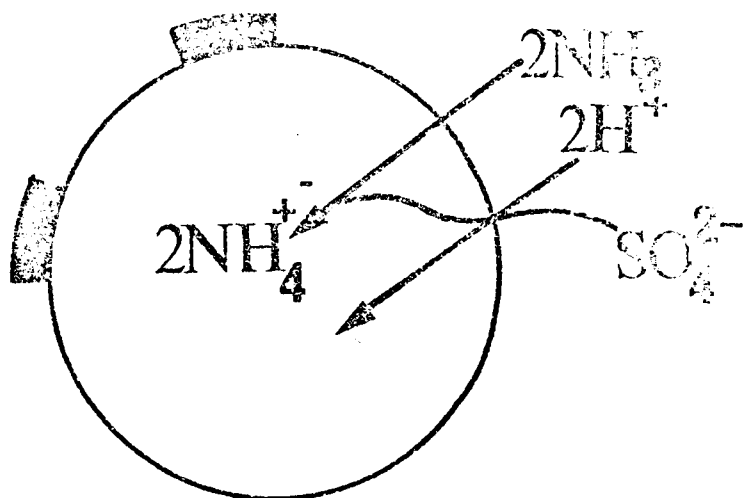
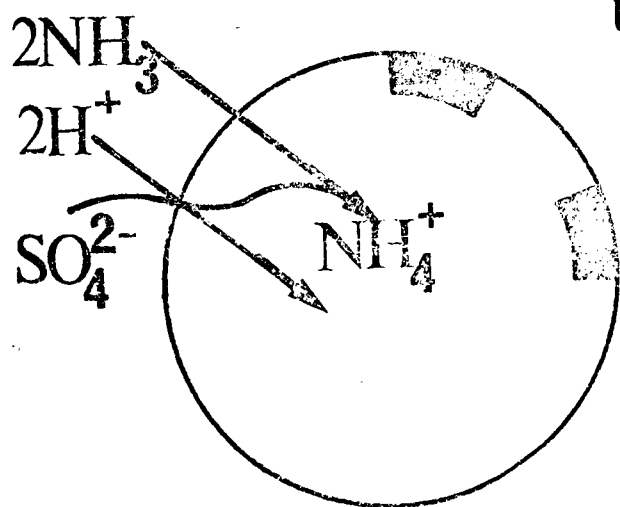
Respiration - driven uptake



KCl pulse - driven uptake



NH_4Cl pulse-driven uptake



CHAPTER 6

ATP SULPHURYLASE

INTRODUCTION

Paracoccus denitrificans can utilise sulphate as a sole source of sulphur (Chapter 2). It is capable of reducing sulphate and incorporating reduced sulphur into amino acids (Chapter 3). The regulation and control of the sulphate uptake mechanism has been described in Chapters 4 and 5.

Sulphate is a relatively unreactive compound and must be activated before it can be metabolised (De Meio et al., 1955; Hilz & Lipmann, 1955; Peck, 1970; Robbins & Lipmann, 1957, 1958). In yeast, sulphate is activated by two molecules of ATP in two separate reactions catalysed by the enzymes ATP sulphurylase (ATP-sulphate adenylyl transferase, EC 2.7.1.25), respectively. Another enzyme, ADP sulphurylase, has also been described in yeast (Robbins & Lipmann, 1958; Burnell & Anderson, 1973a, Hawes & Nicholas, 1973).

ATP Sulphurylase catalyses the nucleophilic displacement of the pyrophosphate moiety in ATP by the sulphate anion resulting in the formation of APS (Akagi & Campbell, 1962). This reaction proceeds with an unfavourable equilibrium for APS formation, undoubtedly because sulphuric is a stronger acid than phosphoric, and the free energy of hydrolysis of the phosphate-sulphate bond is higher than the phosphate-phosphate bond (Hodson & Schiff, 1973). Roy & Trudinger (1970) estimate the group potential of the phosphate-phosphate bond in APS to be approximately 19 kcal/mole, compared with 8 kcal/mole for the phosphate-phosphate bond of ATP. Consequently, the unfavourable equilibrium must be offset if APS is to be produced. This offset may occur in several ways:-

- (1) the second activating step, catalysed by APS kinase, removes synthesised APS, and

(2) inorganic pyrophosphatase (EC 3.6.1.1) cleaves the pyrophosphate released in the ATP sulphurylase reaction (Hodson & Schiff, 1973; Roy & Trudinger, 1970) thus removing one of the reaction products.

The level of ATP sulphurylase has been shown to be under metabolic control in fungi and bacteria (Tweedie & Segel, 1971a; Wheldrake, 1969; Jones-Mortimer, 1968; Jones-Mortimer et al., 1968; Dreyfuss & Pardee, 1966; Wheldrake & Pasternak, 1965).

This chapter reports the purification, some properties and substrate specificity of the enzyme, in addition to its control and regulation. It also reports the properties of the coupled enzyme system, ATP sulphurylase and inorganic pyrophosphatase (the latter also purified from P. denitrificans as described in Chapter 7), that was used to measure the synthesis of APS with γ - ^{35}S -sulphate and ATP as substrates.

MATERIALS AND METHODS

Chemicals. γ - ^{32}P - P_i (in dilute HCl, carrier free), γ - ^{35}S - Na_2SO_4 (carrier free) and γ - ^{75}Se - Na_2SeO_4 (carrier free) were obtained from the Radiochemical Centre, Amersham, Bucks., U.K. γ - ^{32}P - P_i was converted to γ - ^{32}P - PP_i by pyrolysis as described by Lee Peng (1956). ATP and ADP were obtained from Boehringer und Soehne, Mannheim, Germany; APS and activated Norit A charcoal from the Sigma Chemical Co., London, U.K. Na_2MoO_4 , K_2CrO_4 , K_2SeO_4 and sodium propionate from British Drug Houses, Dorset, U.K. Dowex AG 11A8, 50-100 mesh, ion retardation resin from Bio Rad Laboratories, Richmond, California, U.S.A. All other chemicals were of Analytical Reagent Grade and were obtained from the sources stated in previous chapters.

Pyrolysis of γ - ^{32}P - γP_i to γ - ^{32}P - γPP_i . γ - ^{32}P - γP_i was converted to γ - ^{32}P - γPP_i by the method described by Lee Peng (1956). γ - ^{32}P - γP_i was obtained from commercial sources in dilute HCl solution. 1 mCi γ - ^{32}P - γP_i was added to 2 ml of water in a 10 ml pyrex test tube. The solution was evaporated to dryness under a tungsten filament lamp in a fume cupboard. Two ml of 100 mM- Na_2HPO_4 was added and the solution again evaporated to dryness. After cooling the test tube, the test tube was stoppered (ground glass stopper), wrapped in aluminium foil, and heated at 400°C for 2 h. After cooling, the white precipitate was dissolved in 5 ml of water to give a solution of approximately 20 mM- $\text{Na}_2^{32}\text{P}_2\text{O}_7$. This stock solution was diluted with 20 mM- $\text{Na}_4\text{P}_2\text{O}_7$ to a specific activity of 0.25 $\mu\text{Ci/ml}$.

Enzyme assays. Crude extracts of P. denitrificans contained a powerful, fluoride-insensitive inorganic pyrophosphatase which interfered with measuring ATP sulphurylase activity of the ATP- PP_i exchange method of Shaw & Anderson (1971, 1972) since it removed pyrophosphate, one of the substrates, from the reaction mixtures. Although this method is more sensitive and easier to use for routine measurements of ATP sulphurylase activity than the less sensitive molybdate method of Bandurski et al., (1956) ATP sulphurylase activity in crude extracts of P. denitrificans had to be measured by the molybdate method. Only after separation of the ATP sulphurylase and inorganic pyrophosphatase activities could the ATP- PP_i exchange method be used to assay ATP sulphurylase activity.

Method (1) Molybdolysis assay. A modification of the method of Bandurski et al., (1956) was used. Reaction mixtures

contained 100 μ moles Tris-HCl buffer, pH 8.0, 10 μ moles MgCl_2 , 2 μ moles ATP, 2 μ moles Na_2MoO_4 , 5 units purified inorganic pyrophosphatase, and enzyme extract in a total volume of 1.0 ml. Controls without molybdate, and with boiled enzyme, were performed. The reaction mixtures were equilibrated at 30°C for 1 min before the reaction was initiated by the addition of enzyme. After 10 min, reactions were terminated by the addition of 1.0 ml of ice cold trichloroacetic acid (7.5%, w/v) and placed in ice to minimise the acid-catalysed hydrolysis of ATP. Phosphate was determined in the reaction mixtures by the method of Allen (1940). When crude extracts were used, the acid-precipitated protein was removed by centrifugation and a sample (1 ml) of the supernatant assayed for phosphate by the method of Allen, (1940).

Method (2) Sulphate-dependent ATP-PP_i exchange. ATP sulphurylase was assayed by the method of Shaw & Anderson (1971, 1972). Incubations were conducted at 30°C for 10 min and the reaction mixtures contained 2 μ moles ATP, 40 μ moles K_2SO_4 , 2 μ moles $\gamma\text{-}^{32}\text{P}\text{-}7\text{PP}_i$, 10 μ moles MgCl_2 , 100 μ moles Tris-HCl buffer, pH 8.0 and enzyme in a final volume of 1 ml. In control assays K_2SO_4 was replaced by KCl. Reactions were terminated by the addition of 2 ml of 7.5% (w/v) trichloroacetic acid. The procedures for the separation of $\gamma\text{-}^{32}\text{P}\text{-}7\text{ATP}$ and $\gamma\text{-}^{32}\text{P}\text{-}7\text{PP}_i$ were as described by Anderson (1968). ATP Sulphurylase activities were calculated by the method of Davie et al., (1956) and are expressed as sulphate-dependent ATP-PP_i exchange units in nmoles/min. Specific activities are expressed as units/mg of protein. The adsorption of nucleotides by charcoal was 97 - 100% efficient; no correc-

tion was therefore applied for incomplete adsorption.

Method (3). ATP sulphurylase activity was also measured by the incorporation of $\gamma\text{-}^{32}\text{P}\text{-PP}_i$ into ATP in the presence of APS. This is essentially the same as the method described by Shaw & Anderson (1974a). Reaction mixtures contained 1 mM-MgCl₂, 0.4 mM- $\gamma\text{-}^{32}\text{P}\text{-PP}_i$ (0.5 μCi), 0.2 mM-APS and enzyme, in a final volume of 1.0 ml; APS was omitted from control assays. Reactions were run for 10 min at 30°C and terminated by the addition of 2 ml of 7.5% (w/v) trichloroacetic acid. $\gamma\text{-}^{32}\text{P}\text{-ATP}$ was separated from $\gamma\text{-}^{32}\text{P}\text{-PP}_i$ as described for method (2) and counted for radioactivity with a Geiger-Muller end-window counter. ATP sulphurylase activity, measured by method (3), is expressed as the APS-dependent synthesis of ATP in nmoles/min. The ^{32}P -labelled compound synthesised in method (3) was confirmed as $\gamma\text{-}^{32}\text{P}\text{-ATP}$ by electrophoresis as described below.

Assay of ATP-ase and inorganic pyrophosphatase activities.

ATP-ase and inorganic pyrophosphatase activities were measured in reaction mixtures containing 100 μmoles Tris-HCl buffer, pH 8.0, 20 μmoles MgCl, 5 μmoles ATP or Na₄P₂O₇ and enzyme extract in a total volume of 1 ml. Assays were conducted at 30°C and terminated after 10 min by the addition of 2 ml of 12% (v/v) perchloric acid. The terminated reaction mixtures were centrifuged to remove precipitated protein and the supernatant analysed for phosphate by the method of Allen (1940). All values were corrected for phosphate present in reaction mixtures terminated at zero time and all results are means of duplicate determinations.

Assay of the ATP sulphurylase-inorganic pyrophosphatase coupled enzyme system. Incubations were conducted at 30°C for 90 min in the presence of 30 units of purified ATP sulphurylase and 5 units of purified inorganic pyrophosphatase. In addition, reaction mixtures contained 10 μ moles $\text{Na}_2\text{K}_2\text{ATP}$, 40 μ moles $\gamma\text{-}^{35}\text{S}\text{-Na}_2\text{SO}_4$ (100 μCi), 20 μ moles MgCl_2 , and 100 μ moles Tris-HCl buffer, pH 8.0 in a total volume of 1 ml. The reactions were terminated by heating the reaction mixtures at 100°C for 1 min in an oil bath. $\gamma\text{-}^{35}\text{S}\text{-APS}$ was separated from $\gamma\text{-}^{35}\text{S}\text{-sulphate}$ by adsorption of the APS to charcoal and counting the radioactivity; the method was the same as that used for the separation of $\gamma\text{-}^{32}\text{P}\text{-ATP}$ from $\gamma\text{-}^{32}\text{P}\text{-PP}_i$ in the assay of ATP sulphurylase, except that the charcoal, collected by filtration, was dried prior to counting the radioactivity of the adsorbed ^{35}S -labelled nucleotide with a thin end-window counter. Activity of the coupled enzyme system is expressed as nmoles of APS synthesised per hour.

High voltage paper electrophoresis. High voltage paper electrophoresis was conducted on acid-washed Whatman 3MM paper. Separation was done at 50 v/cm for 2 h at 15°C in 0.1 M-sodium citrate buffer, pH 5.0. After drying at room temperature, electrophoretograms were scanned by u.v. light to locate the position of nucleotides. The electrophoretograms were then cut into 1 cm sections, placed in scintillation vials with 1.0 ml of Ciba 4 scintillation fluid, and the radioactivity counted.

Purification of ATP sulphurylase. All operations were conducted at 0 to 4°C. The buffer used throughout the purifi-

cation procedure was composed of 20 mM-Tris-HCl, pH 8.0, containing 5 mM-MgCl₂ unless specified otherwise.

Crude extract. Crude extract was prepared from cells grown with succinate as substrate and nitrate as the terminal electron acceptor as described in Chapter 2, except that cells were resuspended in buffer prior to freezing (Fraction I).

Ammonium sulphate fractionation. Fraction I was brought to 30% (w/v) saturation with solid ammonium sulphate at 4°C. The mixture was stirred at 2°C for 20 min and the precipitate collected by centrifugation at 30 000g for 30 min at 2°C. The precipitate was discarded, the supernatant brought to 80% (w/v) saturation with solid ammonium sulphate and stirred for 20 min at 2°C. The precipitate was collected by centrifuging at 30 000g for 30 min at 2°C and then dissolved in a minimal volume of buffer (Fraction II).

Dowex ion retardation resin treatment. Fraction II was passed through a Dowex AG 11A8, 50 - 100 mesh, ion retardation resin column (30 x 1.5 cm) at 1.0 ml per min. Fractions (5 ml) were collected in tubes, in each of which had been placed 0.1 ml of 1 M-Tris-HCl buffer, pH 8.0, containing 5 mM-MgCl₂. The fractions containing the bulk of the protein were pooled to give fraction III.

Sephadex G-200 gel filtration. Fraction III was applied to a Sephadex G-200 column (75 x 3.5 cm) equilibrated with buffer. Five ml fractions were collected at a rate of 0.4 ml per min. Fractions were assayed for ATP sulphurylase and inorganic pyrophosphatase activities and only those fractions containing ATP sulphurylase activity and no inorganic pyrophosphatase activity were pooled to give Fraction IV.

DEAE-52 column chromatography. Fraction IV was applied to a DEAE-52 cellulose column (7.5 x 3.5 cm) equilibrated with buffer and washed with 50 ml of 0.2 M-KCl dissolved in buffer. A 500 ml KCl gradient from 0.2 M to 0.5 M-KCl was developed and 5 ml fractions were collected at a rate of 0.55 ml per min. Fractions (45 - 55) containing sulphurylase activity, were pooled to give Fraction V.

DEAE-52 concentration step. Fraction V was dialysed against buffer, applied to a DEAE-52 column (5.0 x 1.0 cm) and washed with 10 ml of buffer. ATP sulphurylase was then eluted with 5 ml of 0.5 M-KCl dissolved in buffer. The concentrated enzyme solution was dialysed against 20 mM-Tris-HCl buffer, pH 8.0 for 6 h and then stored frozen at -15°C - Fraction VI.

Purification of inorganic pyrophosphatase. Inorganic pyrophosphatase was purified as described in Chapter 7.

RESULTS

Purification of ATP sulphurylase. The crude enzyme extract contained a powerful inorganic pyrophosphatase activity which interfered with ATP sulphurylase estimations measured by the ATP-PP_i exchange method (Method (2)). Fluoride is a strong inhibitor of plant inorganic pyrophosphatase (El-Badry & Bassham, 1970; Shaw & Anderson, 1971) but the inorganic pyrophosphatase present in crude extracts of P. denitrificans was insensitive to fluoride. Therefore, to allow the more sensitive exchange assay (Method 2) to be used, ATP sulphurylase had to be separated from inorganic pyrophosphatase activity.

ATP sulphurylase and inorganic pyrophosphatase activities could not be separated by ammonium sulphate, pH (acid), NaCl, or acetone fractionation. However, ATP sulphurylase and

inorganic pyrophosphatase activities were separated by gel filtration on Sephadex G-200 (Fig. 6.1). ATP sulphurylase was purified further and concentrated by DEAE-cellulose chromatography. ATP sulphurylase was purified approximately 150-fold and was free of all inorganic pyrophosphatase and ATP-ase activities.

Validity of the ATP-PP_i exchange assay. The radioactivity adsorbed by charcoal in standard reaction mixtures, with and without added sulphate, was eluted with 0.1 M-NH₃ in 50% (v/v) ethanol. High voltage paper electrophoresis of the eluates demonstrated that the sulphate-dependent radioactivity adsorbed to charcoal was not pyrophosphate, but ran with an R_f similar to ATP (Fig. 6.2). When the radioactive compound was eluted from the electrophoretogram with water and its u.v. absorption examined, a u.v. absorption spectrum identical with ATP was observed. No γ -³²P-ADP or γ -³²P-AMP was detected.

Linearity of the assay. The rate of sulphate-dependent ATP-PP_i exchange was constant up to 20 min (Fig. 6.3). The rate of sulphate-dependent ATP-PP_i exchange increased linearly with increase in protein concentration (Fig. 6.4). Preincubation of the purified ATP sulphurylase at 80°C for 2 min totally destroyed both endogenous and sulphate-dependent ATP-PP_i exchange. The enzyme retained 93% of its activity when stored at -15°C for 8 months.

Effect of pH. No single buffer system was satisfactory for examining the pH optimum of purified ATP sulphurylase. However, maximum activity was found over the range of pH 7.0 to pH 9.0 (Fig. 6.5) when a series of buffers was used. All subsequent assays were conducted at pH 8.0 in Tris-HCl buffer.

Fig. 6.1

Separation of ATP sulphurylase and inorganic pyrophosphatase activities.

The activities were separated by Sephadex G-200 gel filtration after a 0 - 80% $(\text{NH}_4)_2\text{SO}_4$ fractionation was done as described in the text. ATP Sulphurylase activity was measured by Method 1.

▲, protein; ●, ATP sulphurylase; ■, inorganic pyrophosphatase

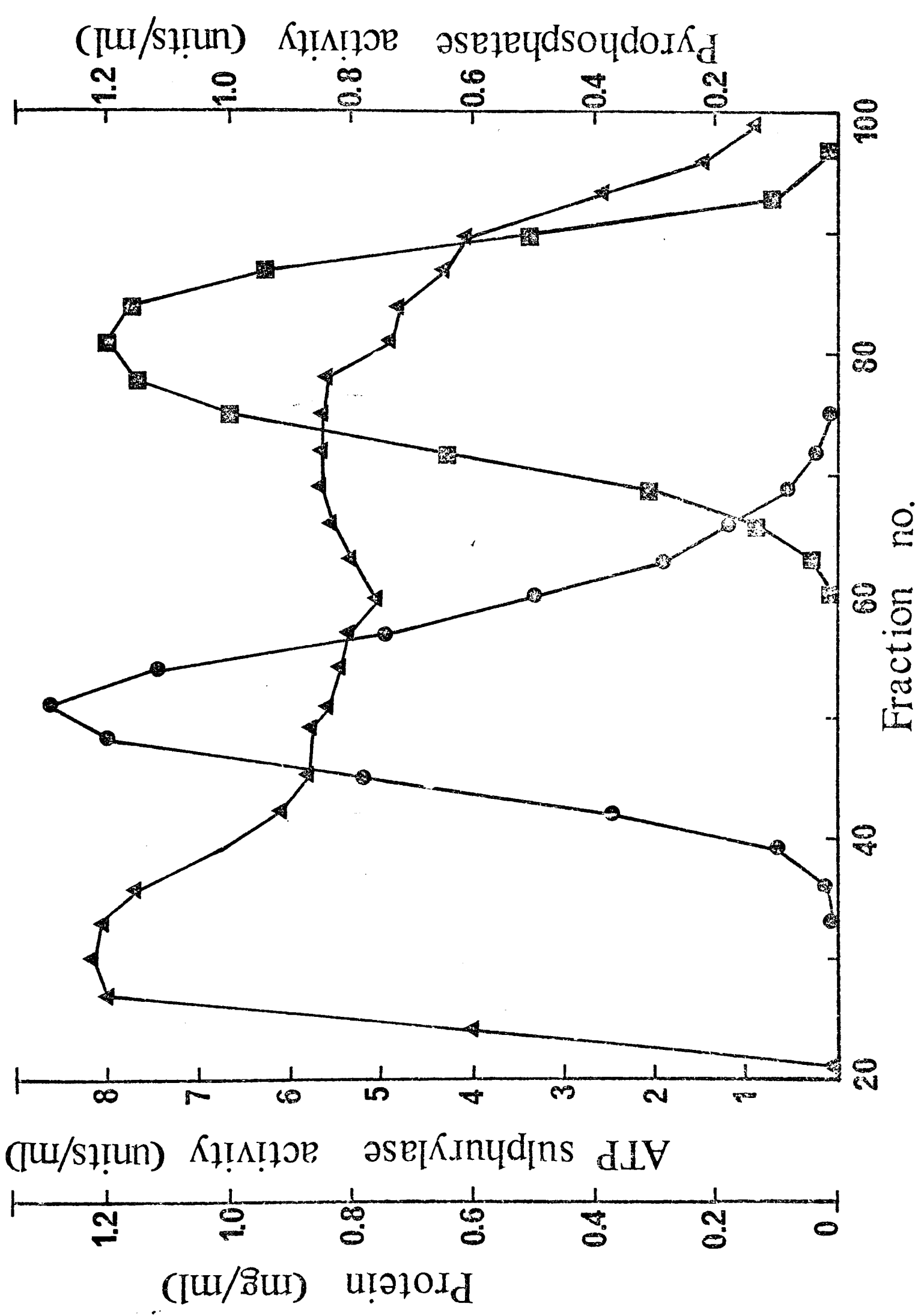


Fig. 6.2

Verification of the validity of the PP_i - ATP exchange assay.
Radiochromatogram trace of the material eluted from charcoal with
 0.1M-NH_3 in 50% (v/v) ethanol following a standard assay containing
sulphate.

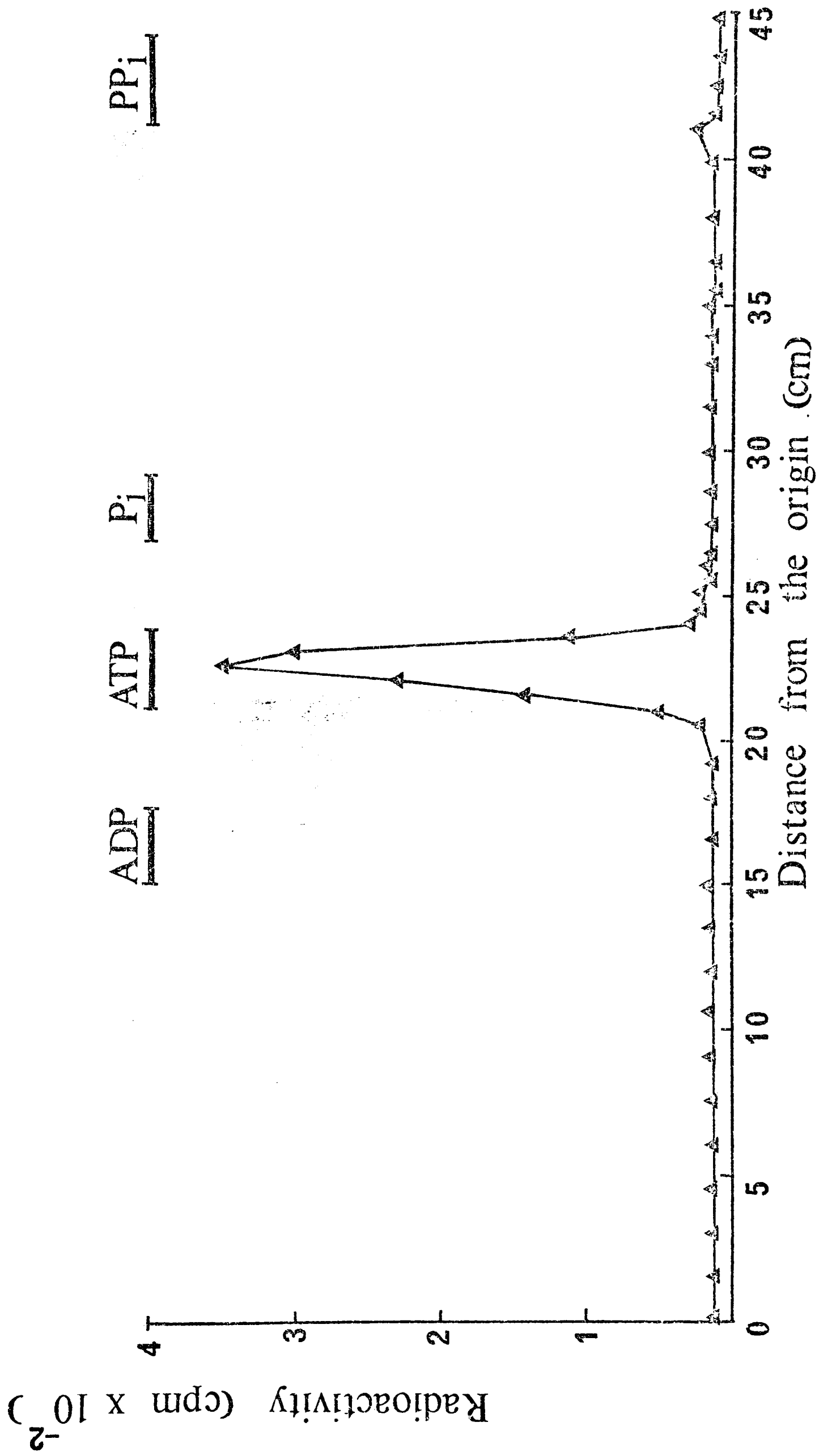


Fig. 6.3

Time-course of sulphate-dependent exchange

Purified ATP sulphurylase was used and the enzyme was assayed by the ATP-PP_i exchange method.

ATP Sulphurylase activity is expressed in units per assay.

Fig. 6.4

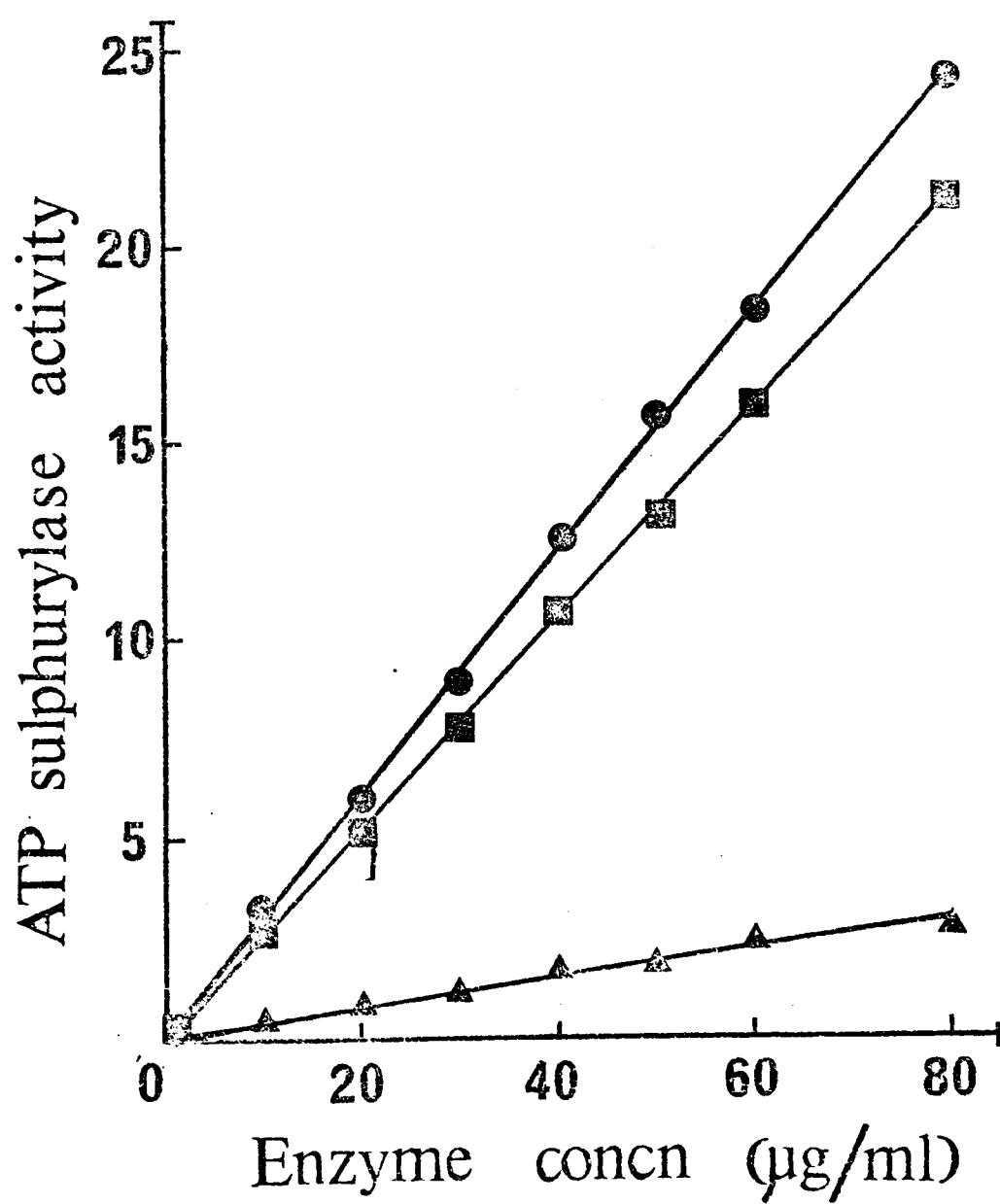
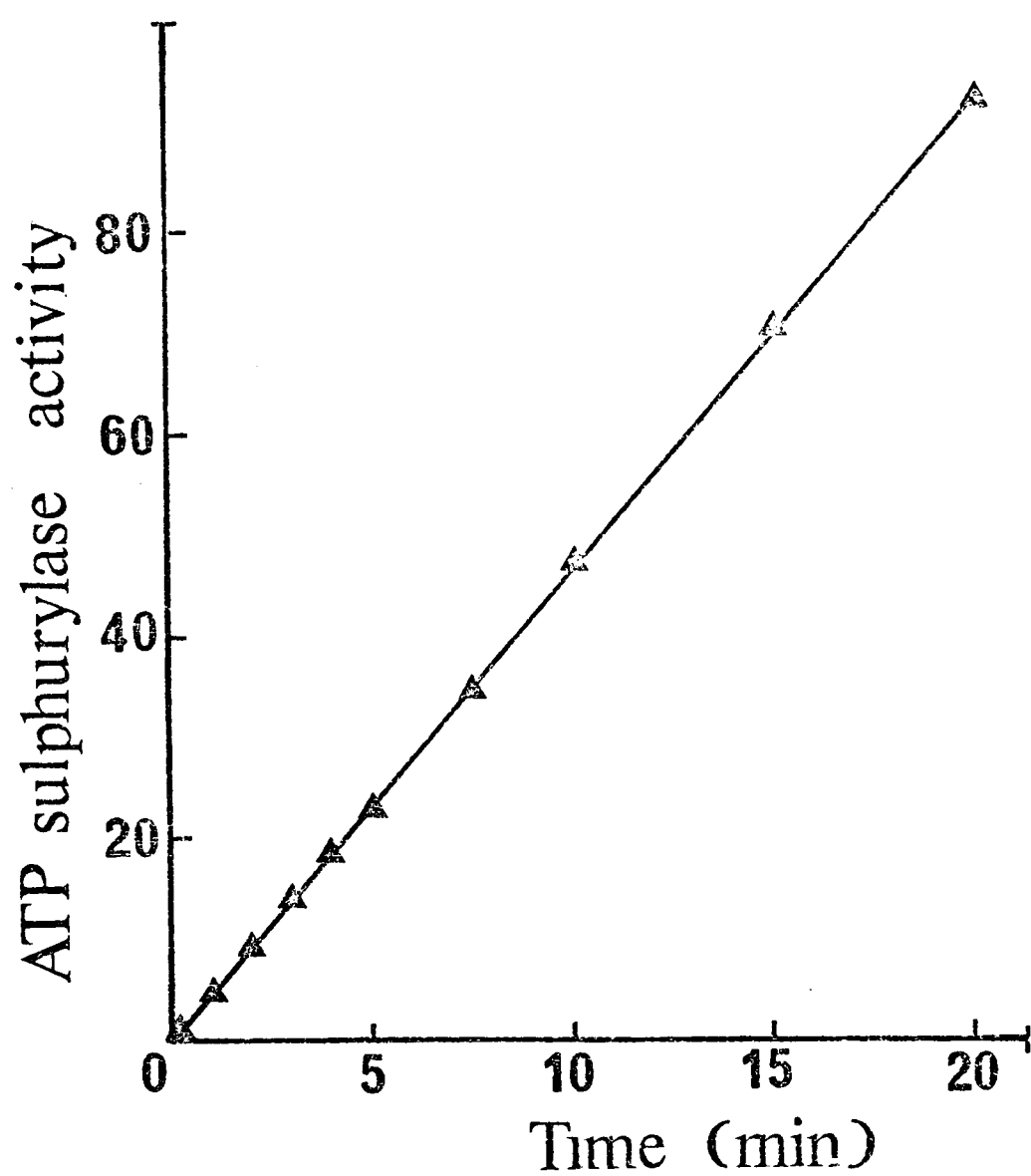
Effect of enzyme concentration upon sulphate-dependent ATP-PP_i exchange activity

Purified ATP sulphurylase was used;

⊙; ATP-PP_i exchange with K₂SO₄

▲; ATP-PP_i exchange with KCl in lieu of K₂SO₄

■; Sulphate-dependent ATP-PP_i exchange



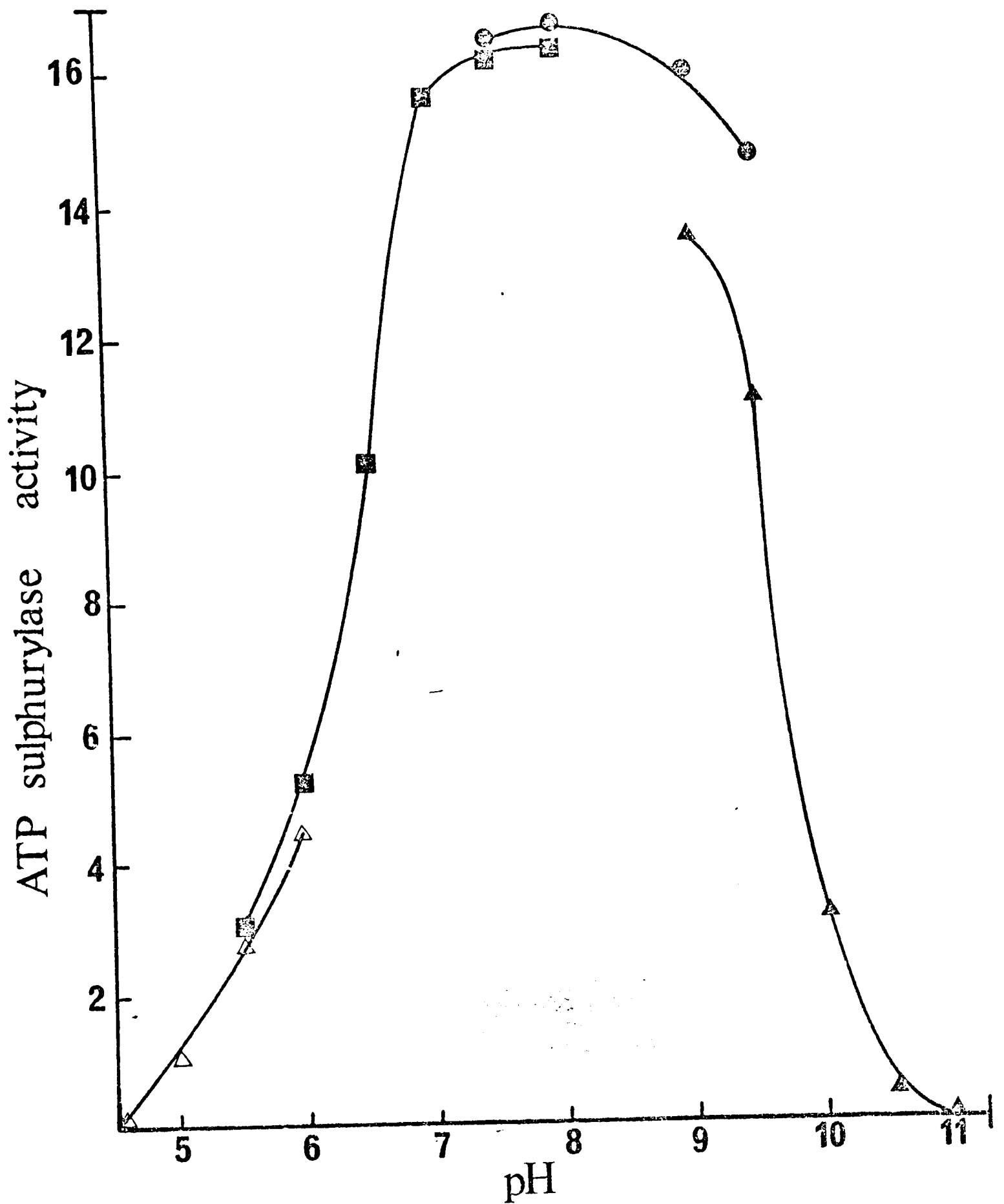


Fig. 6.5

Effect of pH upon ATP sulphurylase

The enzyme was assayed by Method 2 and all conditions were as described in Materials and Methods except that the buffer used was as specified;

- △, citrate
- , Tris-maleate
- , Tris-HCl
- ▲, Glycine-NaOH

Effect of γ - ^{32}P - PP_i concentration. The sulphate-dependent ATP- PP_i exchange reaction catalysed by purified ATP sulphurylase is essentially independent of the γ - ^{32}P - PP_i concentration above 0.2mM as shown in Fig. 6.6. The concentration of γ - ^{32}P - PP_i was, therefore, routinely maintained at 2.0 mM in all assays in which ATP sulphurylase activity was monitored by Method (2).

Effect of sulphate concentration. A Lineweaver-Burk plot of the effect of sulphate concentration upon sulphate-dependent ATP- PP_i exchange is shown in Fig. 6.7. High concentrations of sulphate were not inhibitory. The apparent K_m for sulphate obtained from this plot is 1.2 mM.

Effect of ATP concentration. A Lineweaver-Burk plot of the effect of ATP concentration on sulphate-dependent ATP- PP_i exchange is shown in Fig. 6.8. At the higher concentrations of ATP examined, ATP sulphurylase activity was inhibited. The apparent K_m for ATP obtained from this graph is 0.4 mM.

Interaction of ATP and magnesium. It has previously been observed that a divalent cation (Mg^{2+} , Mn^{2+} , Co^{2+} or Zn^{2+}) is necessary for the activity of ATP sulphurylase in spinach leaves (Shaw & Anderson, 1972) and *P. chrysogenum* (Tweedie & Segel, 1971a; Hawes & Nicholas, 1973). Fig. 6.9 shows that the rate of sulphate-dependent ATP- PP_i exchange at low Mg^{2+} concentrations increases as the ATP concentration is increased up to a maximum. Increasing the ATP concentration above this level causes inhibition. At a higher concentration of Mg^{2+} the concentration of ATP required to inhibit sulphate-dependent ATP- PP_i exchange is increased. Examination of the Mg^{2+} - ATP ratio which gives optimal rates of sulphate-dependent ATP- PP_i exchange gives a constant ratio of 1:1. Therefore,

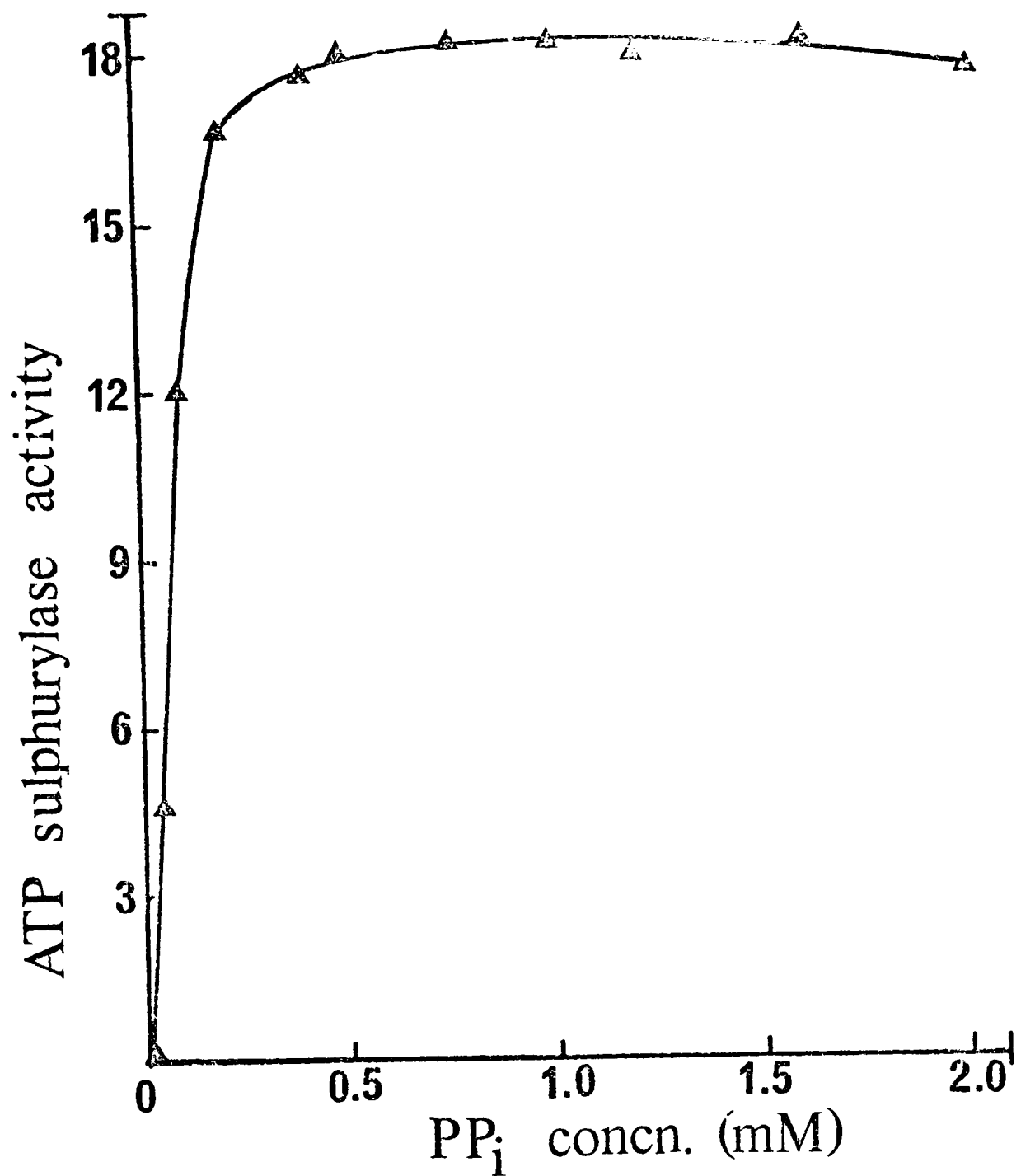


Fig. 6.6

The effect of $[^{32}\text{P}] \text{PP}_i$ concentration on sulphate-dependent ATP- PP_i exchange.

Reaction mixtures were as described in Materials and Methods except that the concentration of $[^{32}\text{P}] \text{PP}_i$ was varied as specified. ATP Sulphurylase activity is expressed in nmoles of sulphate-dependent ATP- PP_i exchange.

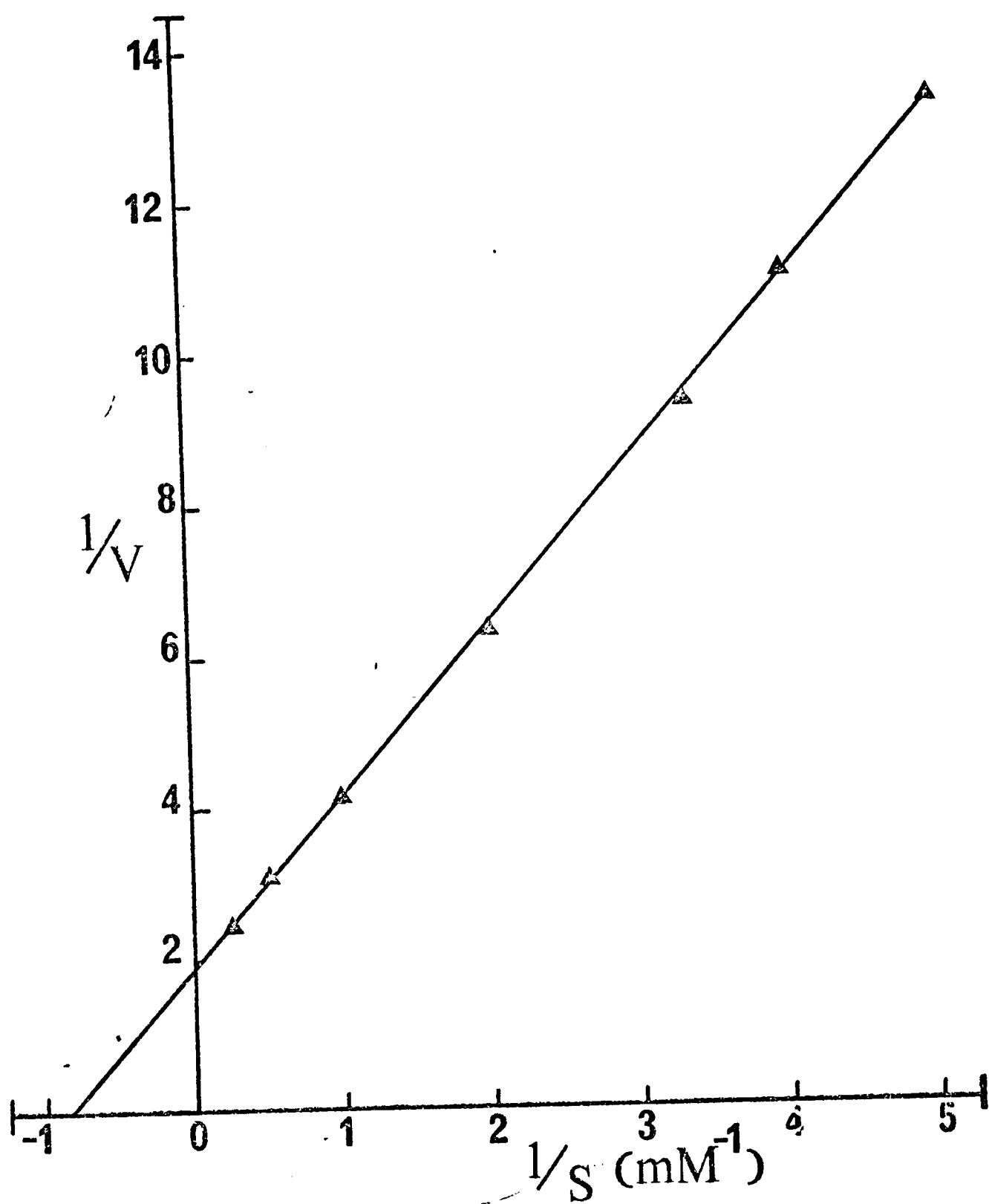


Fig. 6.7

Double reciprocal plot of the dependence of the rate of reaction of ATP sulphurylase on sulphate concentration.

Enzyme activity was determined by Method 2.

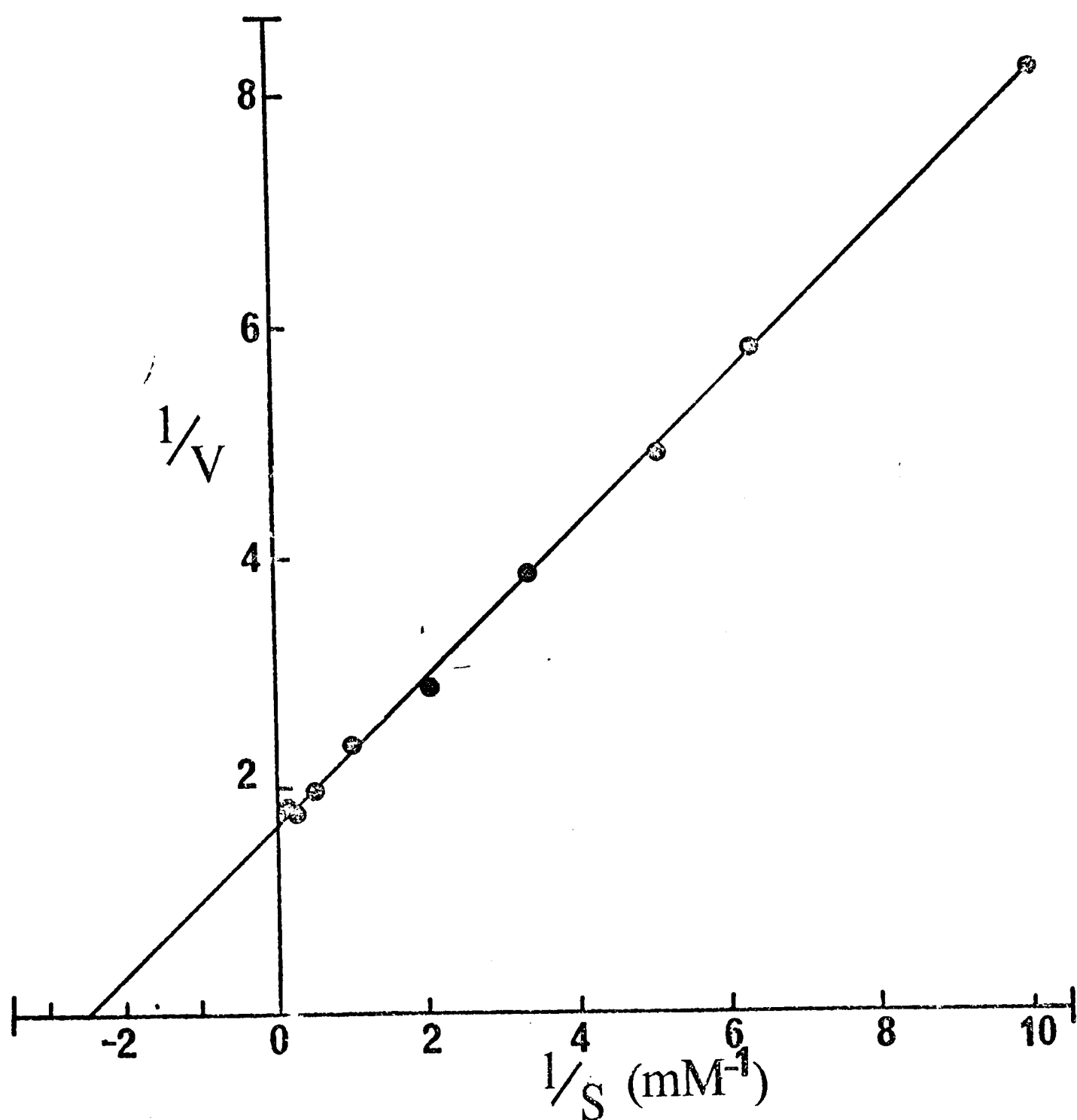


Fig. 6.8

Double reciprocal plot of the effect of ATP concentration on the rate of sulphate-dependent ATP-PP_i exchange catalysed by ATP sulphurylase.

ATP sulphurylase activity was measured by Method 2. Reaction mixtures were as described in Materials and Methods except that the ATP concentration was varied as indicated.

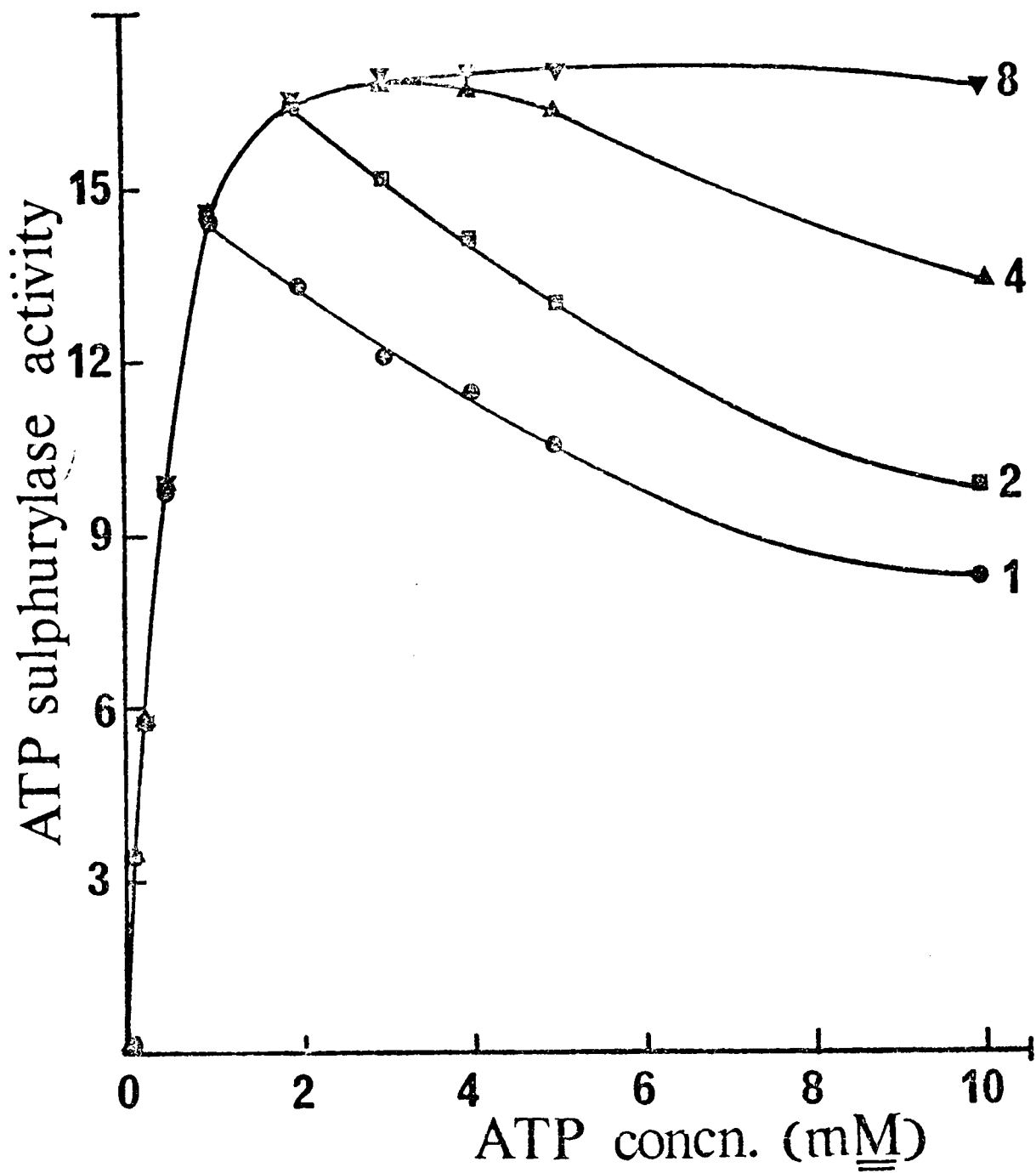


Fig. 6.9

The effect of ATP concentration on the rate of sulphate-dependent ATP-PP_i exchange catalysed ATP sulphurylase, at several $MgCl_2$ concentrations.

The $MgCl_2$ concentrations were ; ; ●, 1mM;
 ■, 2mM; ▲, 4mM; ▼, 8mM.

the probable substrate for the reaction, catalysed by ATP sulphurylase, is a 1 : 1 complex of Mg^{2+} and ATP. Fig. 6.9 also shows that free ATP is an inhibitor of the enzyme. A double reciprocal plot of the free ATP concentration, calculated by the difference between the total ATP and the MgATP^{2-} concentration, extrapolated to the same V_{max} for all free ATP concentrations, as expected for a competitive inhibitor.

Effect of inhibitors. The effect of some common inhibitors on the activity of purified ATP sulphurylase, as measured by method (2), is shown in Table 6.1. Inhibition by

TABLE 6.1

Effect of some common inhibitors on the
activity of purified ATP sulphurylase

ATP sulphurylase activity was measured by method (2). The results are expressed as a percentage of a control assay in which no inhibitor was added.

<u>Addition</u>	<u>Activity (%)</u>
Iodoacetamide (10 <u>mM</u>)	94%
N-Ethyl maleimide (10 <u>mM</u>)	87
p-chloromercuribenzoate (8 <u>μM</u>)	83
Potassium fluoride (10 <u>mM</u>)	89
Phenylhydrazine (10 <u>mM</u>)	6
EDTA (10 <u>mM</u>)	2

the sulphydryl-group reagents was less than 20%. The carbonyl-group inhibitor, phenylhydrazine, almost completely inhibited ATP sulphurylase activity. EDTA also inhibited ATP-PP_i exchange; this would be expected since EDTA would chelate out Mg^{2+} and

thus prevent the formation of the true substrate (MgATP^{2-}).

A number of sulphur-containing compounds which might be inhibitors of ATP sulphurylase activity were examined. Only sulphide and APS inhibited the enzyme significantly. Sulphide is the end-product of the pathway of sulphate activation and reduction and, as such, would be a likely feedback inhibitor controlling the first step in the sequence. Inhibition by APS is also expected, since it is a product of the reaction. None of the sulphur compounds tested (Na_2SO_3 , $\text{Na}_2\text{S}_2\text{O}_3$, L-cysteine, L-cystine, L-methionine, reduced glutathione, cystathionine, and homocysteine) had any effect upon the enzyme. Similarly, neither L-homoserine, L-serine or O-acetyl L-serine had any effect upon ATP sulphurylase activity. Fig. 6.10 shows a Lineweaver-Burk plot of the effect of sulphide on the rate of sulphate-dependent ATP- PP_i exchange. Sulphide is a competitive inhibitor of ATP sulphurylase activity with respect to sulphate and is probably functioning as a feedback inhibitor: At pH 8.0 sulphide exists mainly as SH^- ($\text{pK}_1 = 7.04$, $\text{pK}_2 = 11.96$; Handbook of Chemistry and Physics, 1969 - 1970) thus it is probably this entity that is inhibiting the enzyme.

Effect of nucleotides. Purified ATP sulphurylase did not catalyse the PP_i exchange reaction when ATP was replaced by either ADP, 5'-AMP, 3'-AMP, or 3'-CMP. Similarly, the enzyme did not catalyse exchange of P_i with ATP, ADP, 5'-AMP, 3'-AMP, or 3'-CMP when the $\gamma\text{-}^{32}\text{P}\text{-PP}_i$ was replaced with $\gamma\text{-}^{32}\text{P}\text{-P}_i$ showing that ADP sulphurylase was absent. ADP competitively inhibits ATP sulphurylase activity with respect to ATP (Fig. 6.11) and non-competitively with respect to sulphate (Fig. 6.12). ATP, ADP, and PP_i , in the presence of 10 mM-MgCl_2

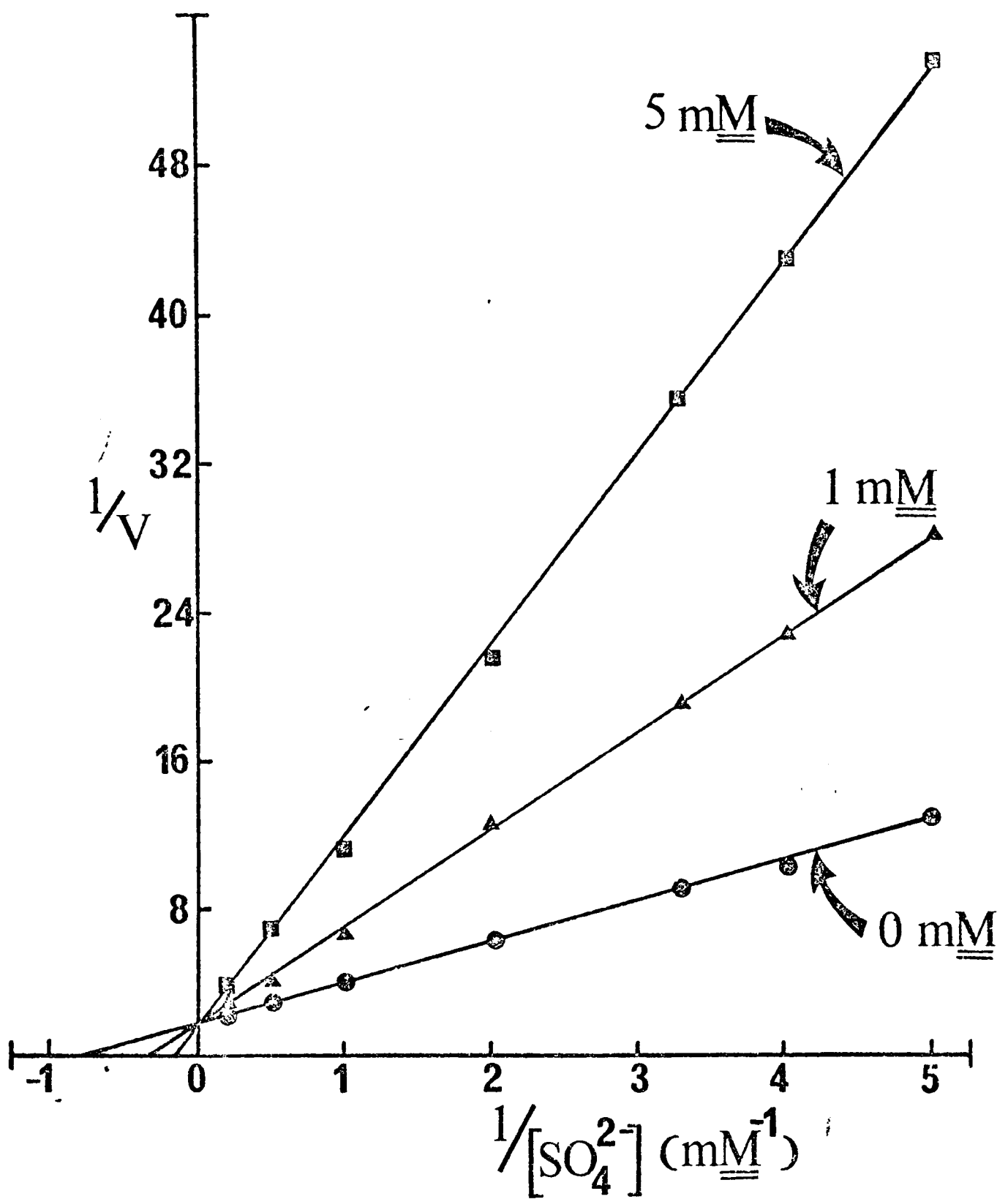


Fig. 6.10

Double reciprocal plots of the inhibition of ATP sulphurylase.

Enzyme activity was measured in the presence of saturating amounts of ATP and at various concentrations of sulphate; ●, no sulphide; ▲, with 1mM-sulphide; ■, with 5 mM-sulphide..

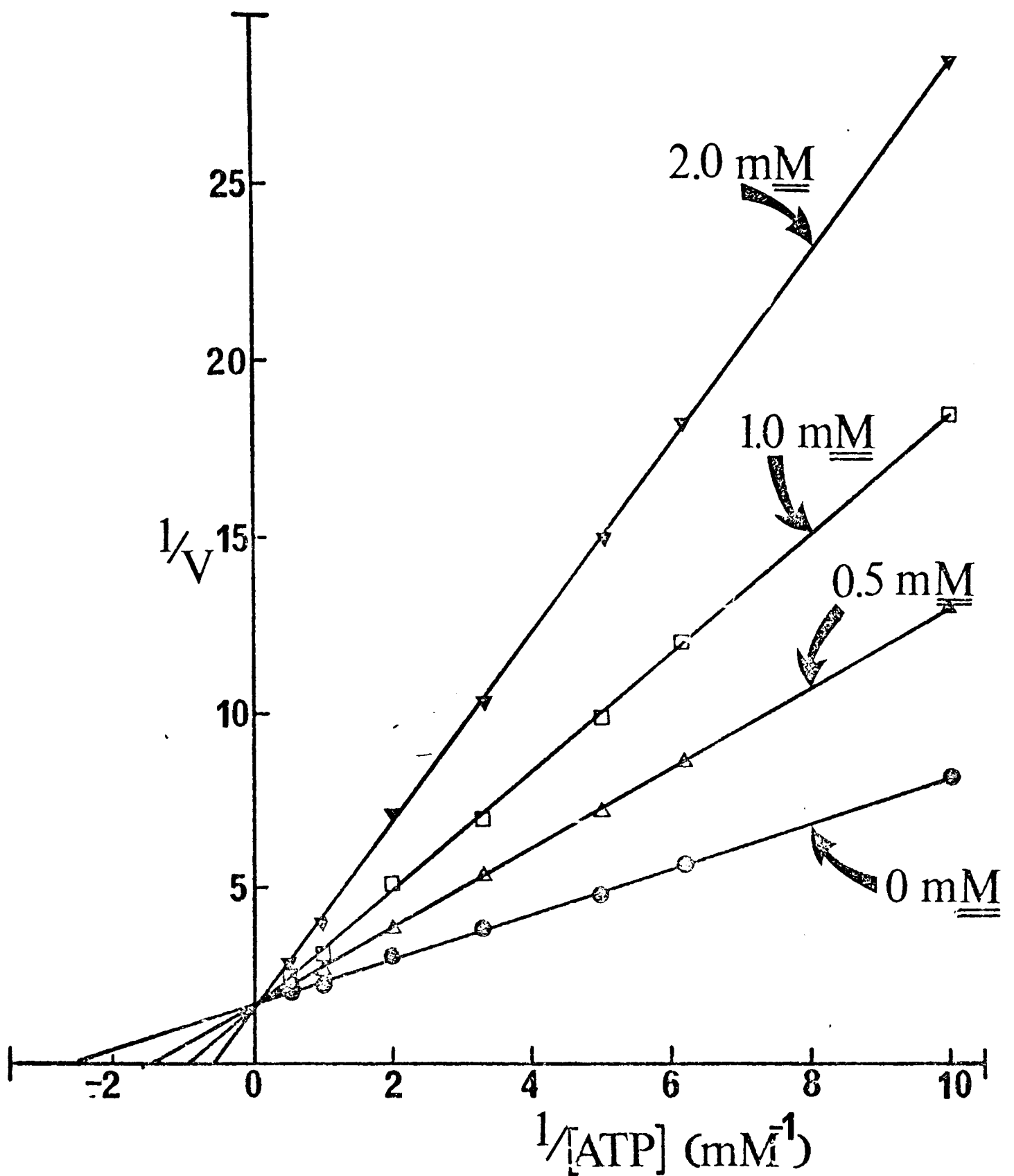


Fig. 6.11

Double reciprocal plot of the effect of ATP concentration upon ATP sulphurylase activity in the presence and absence of ADP.

ATP sulphurylase activity was measured by Method 2 as described in Materials and Methods except that the standard amount of ATP was replaced by the amounts specified and ADP was added in the following concentrations:

●, no ADP; Δ , 0.5mM-ADP; $\square$, 1.0mM-ADP; $\blacktriangledown$, 2mM-ADP.

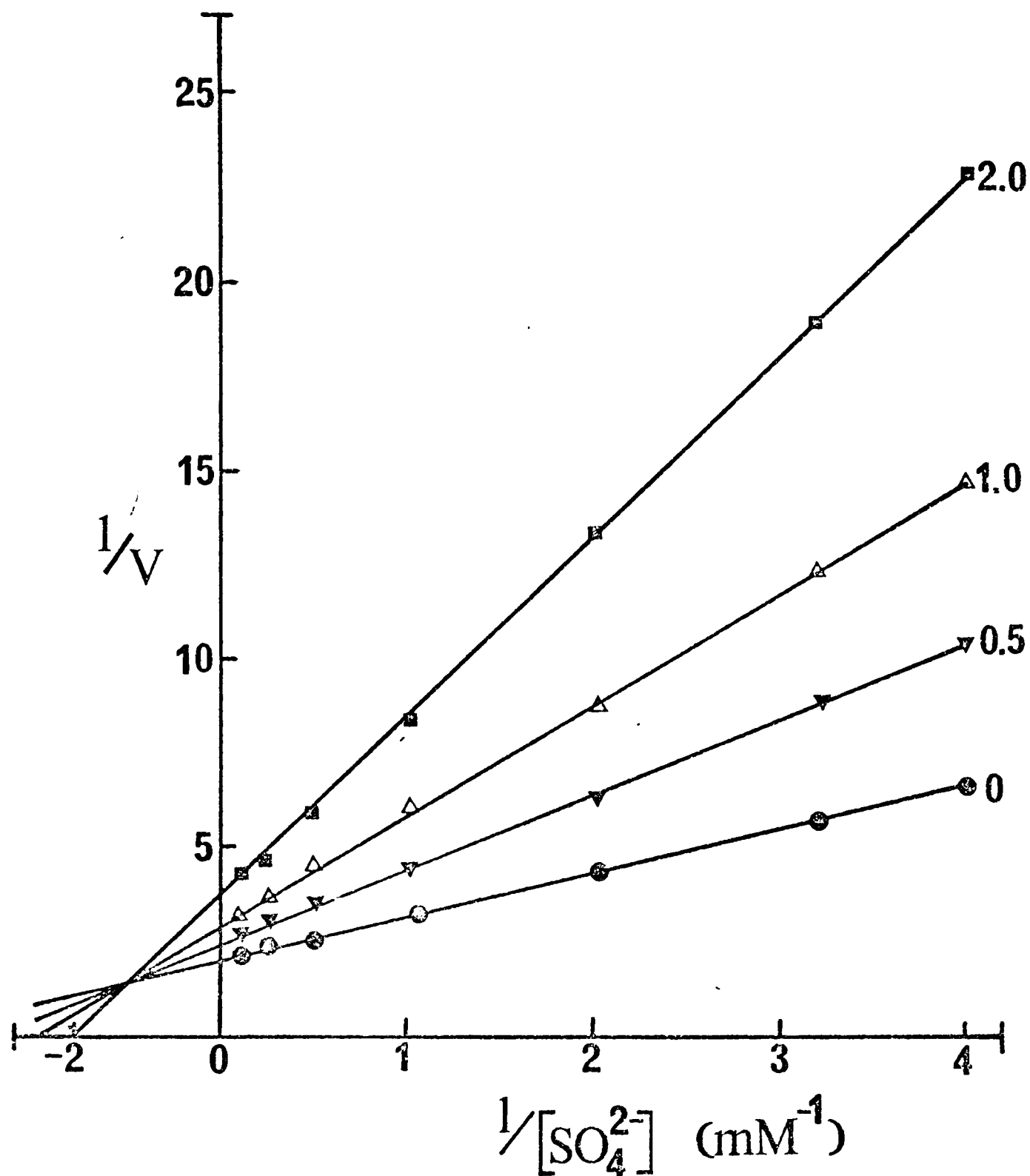


Fig. 6.12

Double reciprocal plot of the effect of concentration of sulphate on ATP sulphurylase in the presence and absence of ADP.

ATP sulphurylase activity was measured by Method 2 as described in Materials and Methods except that the standard amount of sulphate was replaced by the amounts specified and ADP was added as indicated:

●, no ADP, ▼; 0.5 mM-ADP; △, 1.0mM-ADP; ■, 2.0mM-ADP.

were present at MgATP^{2-} , MgADP^{2-} , and $\text{MgP}_2\text{O}_7^{2-}$ respectively, so that the inhibition of exchange by ADP could not be accounted for by a decrease in the concentration of MgATP^{2-} and $\text{MgP}_2\text{O}_7^{2-}$.

Substrate specificity. Purified ATP sulphurylase did not catalyse ATP-PP_i exchange when sulphate was replaced with 10 mM-L-cysteine, L-methionine or a mixture of the L-amino acids (2 mM each), indicating that the purified enzyme was not contaminated with amino-acyl tRNA synthetases. γ -Amino-acyl tRNA synthetases catalyse amino acid-dependent ATP-PP_i exchange (Marcus, 1959)7.

The enzyme was also free of short-chain fatty acid thio-kinases (Millerd & Bonner, 1953; Young & Anderson, 1974) since no ATP-PP_i exchange occurred when sulphate was replaced by 40 mM-sodium acetate or 40 mM-sodium propionate. No ATP-PP_i exchange was observed in the presence of sulphite, thiosulphate, dithionate, metabisulphite or selenite (each at 40 mM). However, ATP-PP_i exchange was observed in the reaction mixtures containing selenate. A Lineweaver-Burk plot of the effect of selenate concentration on the rate of ATP-PP_i exchange is shown in Fig. 6.13. The apparent K_m for selenate, obtained from the graph, is 0.6 mM. The affinity of ATP sulphurylase for selenate ($K_m = 0.6$ mM) is greater than that for sulphate ($K_m = 1.2$ mM) but the V (selenate) is only 30% of the V (sulphate). The ^{32}P -labelled product, adsorbed to charcoal in reaction mixtures containing selenate instead of sulphate, was identified as γ - ^{32}P -ATP by high voltage paper electrophoresis. No ^{32}P -labelled ADP or AMP was detected in reaction mixtures containing selenate and no unlabelled ADP or AMP was detected by u.v. fluorescence quenching when electrophoretogram strips

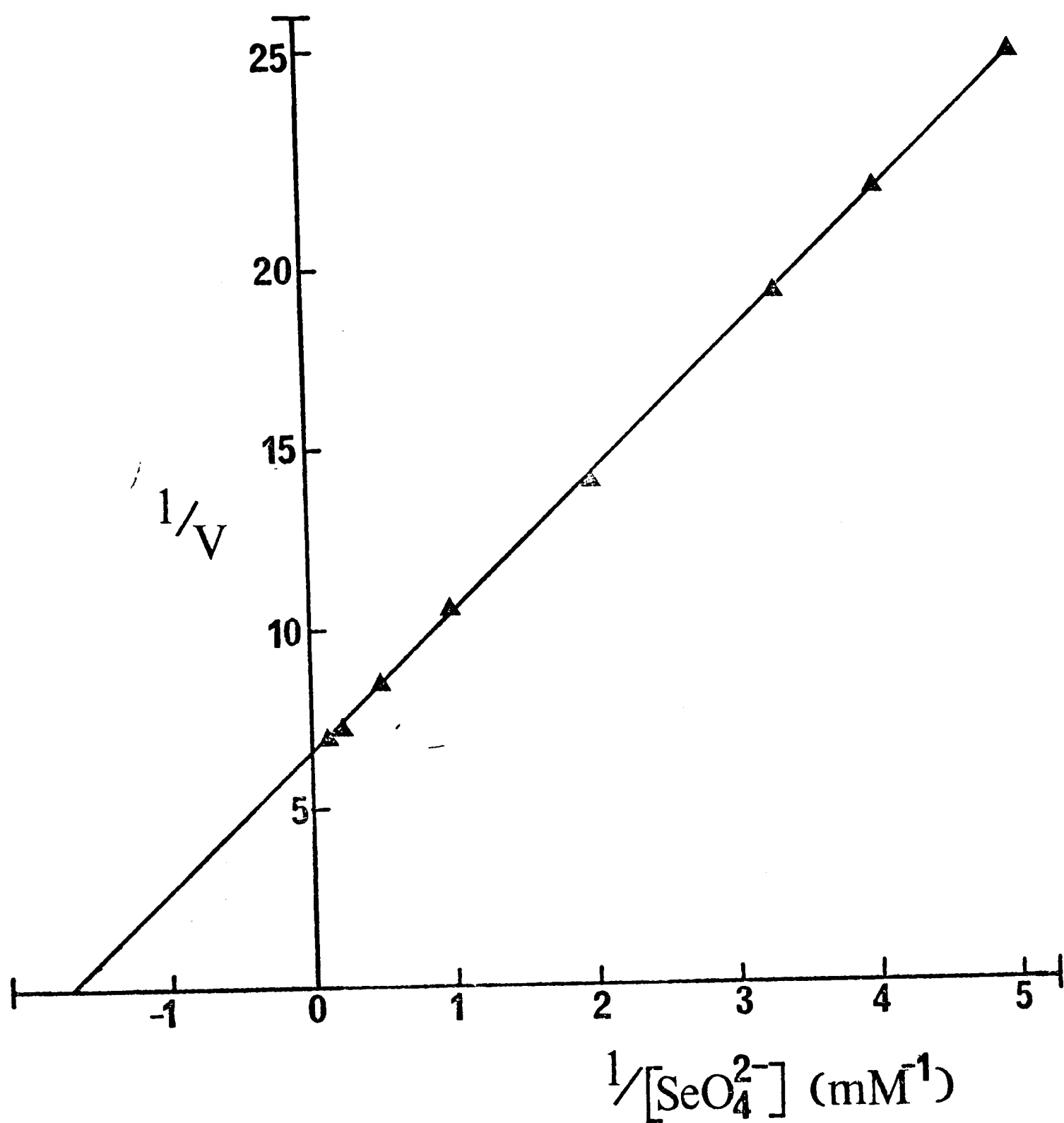


Fig. 6.13

Double reciprocal plot of the effect of selenate concentration upon ATP sulphurylase activity.

Reaction mixtures were as described in Materials and Methods except that sulphate was replaced by selenate at various concentrations. ATP sulphurylase activity was measured by Method 2.

were scanned with u.v. light.

Addition of selenate to reaction mixtures containing sulphate and purified ATP sulphurylase decreased the rate of ATP-PP_i exchange (Fig. 6.14). The kinetics of sulphate/selenate competition experiments are consistent with the kinetics of two substrates competing for one enzyme (Pocklington & Jeffrey, 1969).

Molybdate is an analogue of sulphate for ATP sulphurylase but, unlike sulphate, it does not form a stable adenylate compound. ATP sulphurylase catalyses the release of AMP from ATP in the presence of molybdate (Wilson & Bandurski, 1958; Shaw & Anderson, 1972). ATP sulphurylase did not catalyse ATP-PP_i exchange in the presence of molybdate.

Chromate is also an analogue of sulphate and has been found to inhibit spinach leaf ATP sulphurylase (Shaw & Anderson, 1974). Purified ATP sulphurylase from P. denitrificans was inhibited (84% inhibition) by 1 mM-chlorate.

Effect of sulphur source during growth on ATP sulphurylase activity. P. denitrificans was grown with several different sulphur compounds as the sole sulphur source, and the ATP sulphurylase activity of the cells was determined by the molybdolysis assay. The initial concentration of the different sulphur sources added to the growth medium was 30 mg of sulphur per ml. Sulphur sources at this concentration are not limiting over a 24 hour period (see Chapter 2). The results of this experiment are shown in Table 6.2. The most dramatic difference in the level of enzyme was shown by cells grown on sulphide or cysteine. The level of ATP sulphurylase activity was unaffected by the addition of L-methionine or L-homocysteine to the growth medium. The ATP sulphurylase activity per cell is

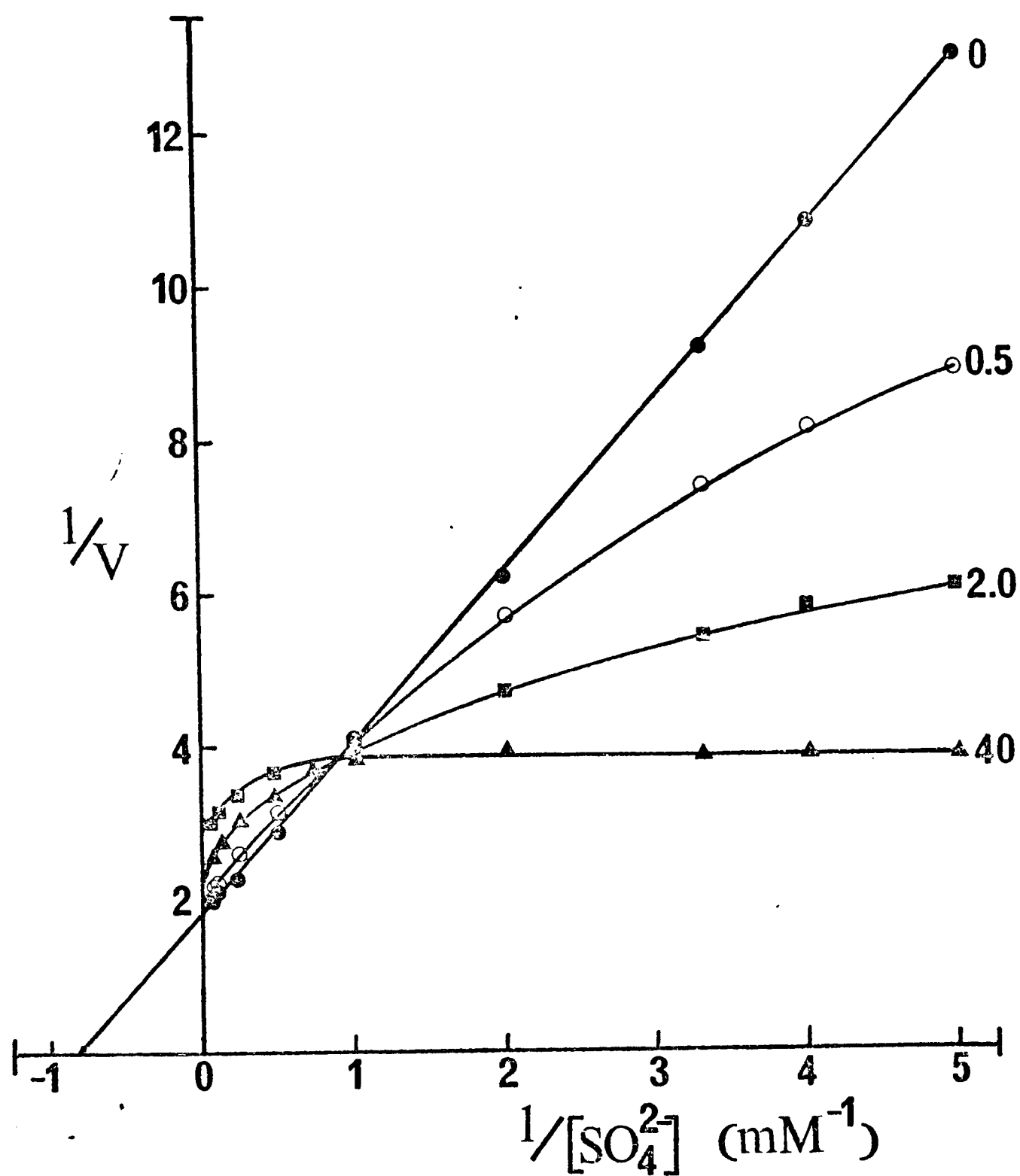


Fig. 6.14

Double reciprocal plot of the effect of sulphate concentration on ATP sulphurylase activity in the presence and absence of selenate.

ATP sulphurylase activity was measured by Method 2 as described in Materials and Methods except that the amount of sulphate added to reaction mixtures was as indicated and selenate was added as specified.

●, no selenate; ○, 0.5mM-selenate; ■, 2.0mM-selenate
▲, 40 mM-selenate.

TABLE 6.2

Activity of ATP sulphurylase in P. denitrificans
grown in various sulphur sources

Cells were grown (2 x 2 litres) and cell-free extracts prepared as described in Materials and Methods, except that the growth media contained the sulphur source(s) as specified. Cell-free extracts were dialysed against 20 mM-Tris-HCl buffer, pH 8.0, containing 5 mM-MgCl₂ for 15 h. ATP sulphurylase activity was determined using Method (1).

<u>Sulphur source during growth</u>	<u>ATP sulphurylase activity (units/mg)</u>
Na ₂ SO ₄	0.27
Na ₂ SO ₃	0.13
Na ₂ S	0.06
L-Cystine	0.02
Na ₂ SO ₄ + L-Methionine	0.23
Na ₂ SO ₄ + L-Homocysteine	0.25
Na ₂ SO ₄ + L-Cystine	0.04

Enzyme activity is expressed in μ moles of P_i released per min per mg of protein x 0.5; i.e., μ moles of PP_i released from ATP per min per mg of protein.

lower in cultures with sulphide. This is probably due to cysteine, which is known to repress the ATP sulphurylase in other organisms (Pasternak, 1962; Kredich, 1971).

It may be significant that ATP sulphurylase activity is never completely repressed, even in the presence of cysteine. Therefore, the organism is always assured of a supply of APS for the synthesis of PAPS; the sulphate donor for the produc-

tion of sulphate esters (Roy & Trudinger, 1970).

Formation of ATP using PP_i and APS as substrates.

Balharri & Nicholas (1970) described the synthesis of ATP from APS and PP_i ; they used the luciferin-luciferase assay to measure the amount of ATP synthesised. Shaw & Anderson (1974a) measured the synthesis of ATP from APS and PP_i by counting the radioactivity of $\gamma\text{-}^{32}\text{P}\text{-ATP}$ formed from $\gamma\text{-}^{32}\text{P}\text{-}PP_i$ and APS in the presence of magnesium ions. Purified ATP sulphurylase from *P. denitrificans* catalysed the synthesis of $\gamma\text{-}^{32}\text{P}\text{-ATP}$ from $\gamma\text{-}^{32}\text{P}\text{-}PP_i$ and APS confirming the findings of Shaw & Anderson (1974a); the synthesis of $\gamma\text{-}^{32}\text{P}\text{-ATP}$ was confirmed by high voltage paper electrophoresis. The synthesis of ATP was dependent on the presence of magnesium ions since addition of excess EDTA inhibited the synthesis of ATP; EDTA, itself, is not inhibitory. These properties are in general agreement with the properties described by Balharri & Nicholas (1970) and Shaw & Anderson (1974a) for ATP sulphurylase from spinach leaf tissue and yeast, respectively.

The effect of concentration of APS and PP_i on the activity of the reverse reaction was studied in the presence of 1 mM-MgCl₂. The effect of PP_i concentration upon the activity of the reverse reaction is shown in Fig. 6.15 and the apparent K_m value for PP_i obtained from this plot is $1.0 \times 10^{-4}\text{M}$. The effect of APS concentration on the reverse reaction is shown in Fig. 6.16 and the apparent K_m value obtained from this graph is $4.9 \times 10^{-5}\text{M}$. Both PP_i and APS are inhibitory at higher concentrations.

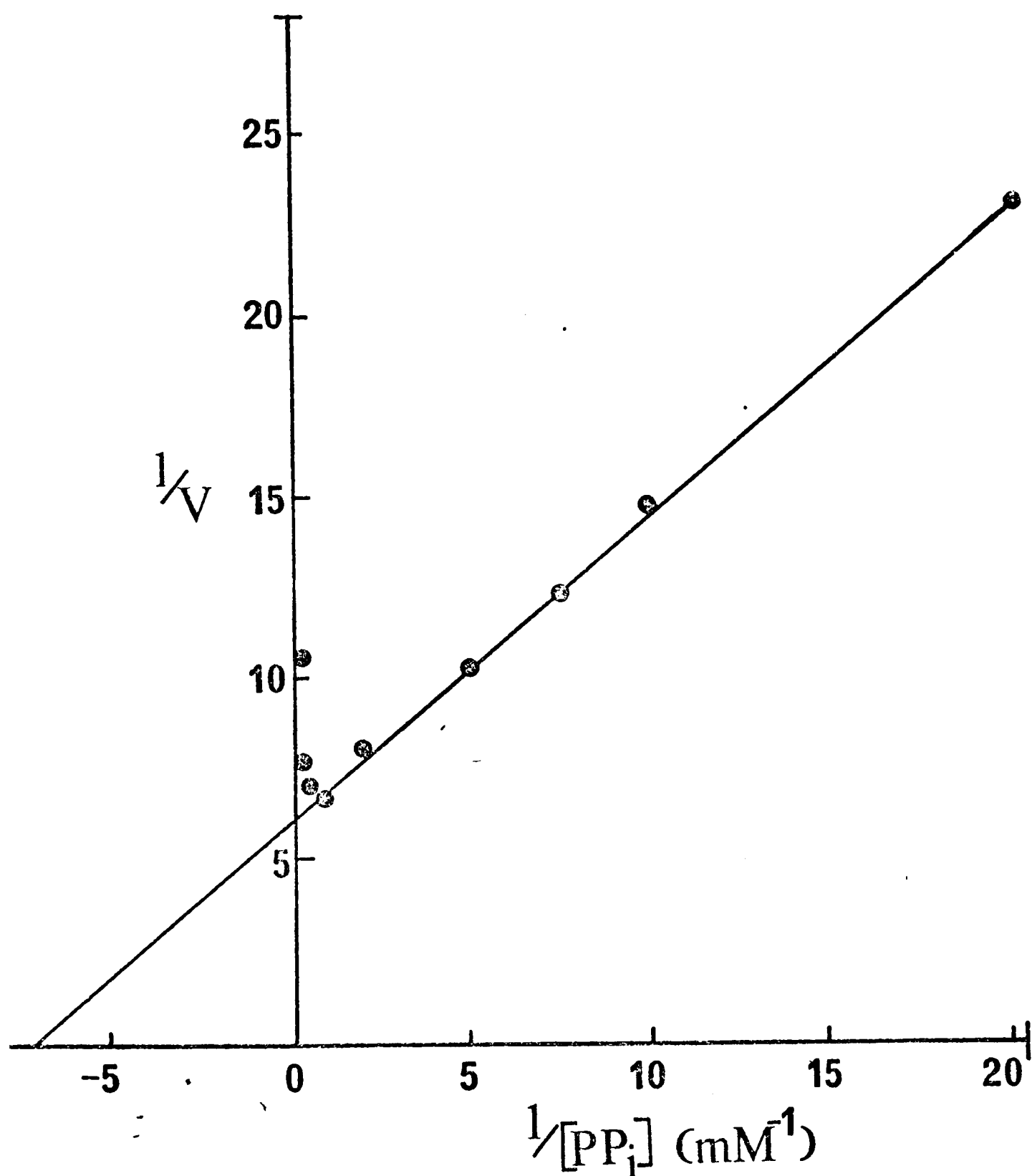


Fig. 6.15

Double reciprocal plot of the rate of reaction catalysed by ATP sulphurylase (in the direction of ATP synthesis) as a function of PP_i concentration. Reaction mixtures were as described in Materials and Methods except that the amount of $\gamma\text{-}^{32}\text{P}\text{-}PP_i$ was varied as specified.

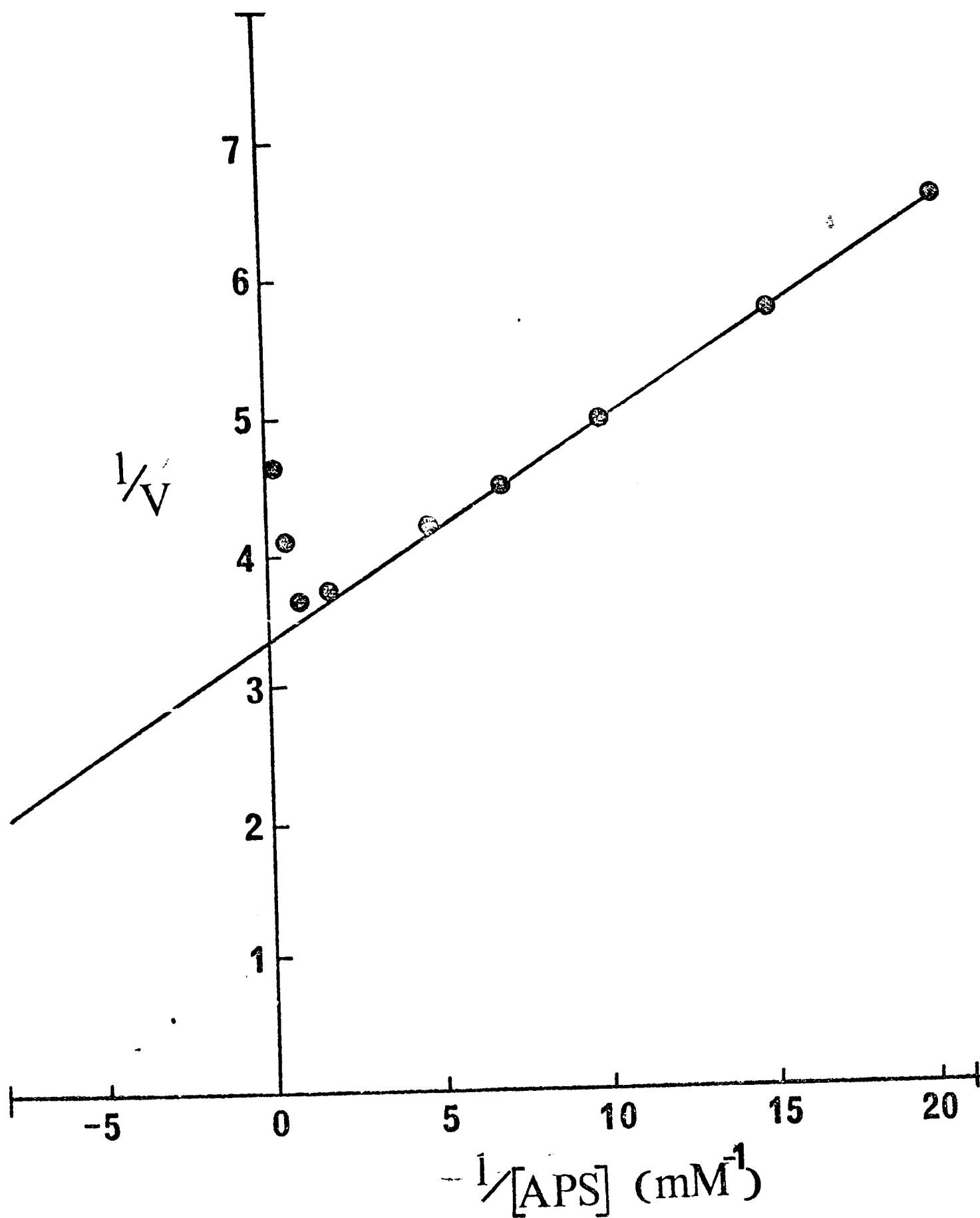


Fig. 6.16

Double reciprocal plot of the rate of incorporation of $\gamma\text{-}^{32}\text{P}\text{-PP}_i$ into ATP as a function of APS concentration. Reaction mixtures were as described in Materials and Methods except that the concentration of APS was as specified.

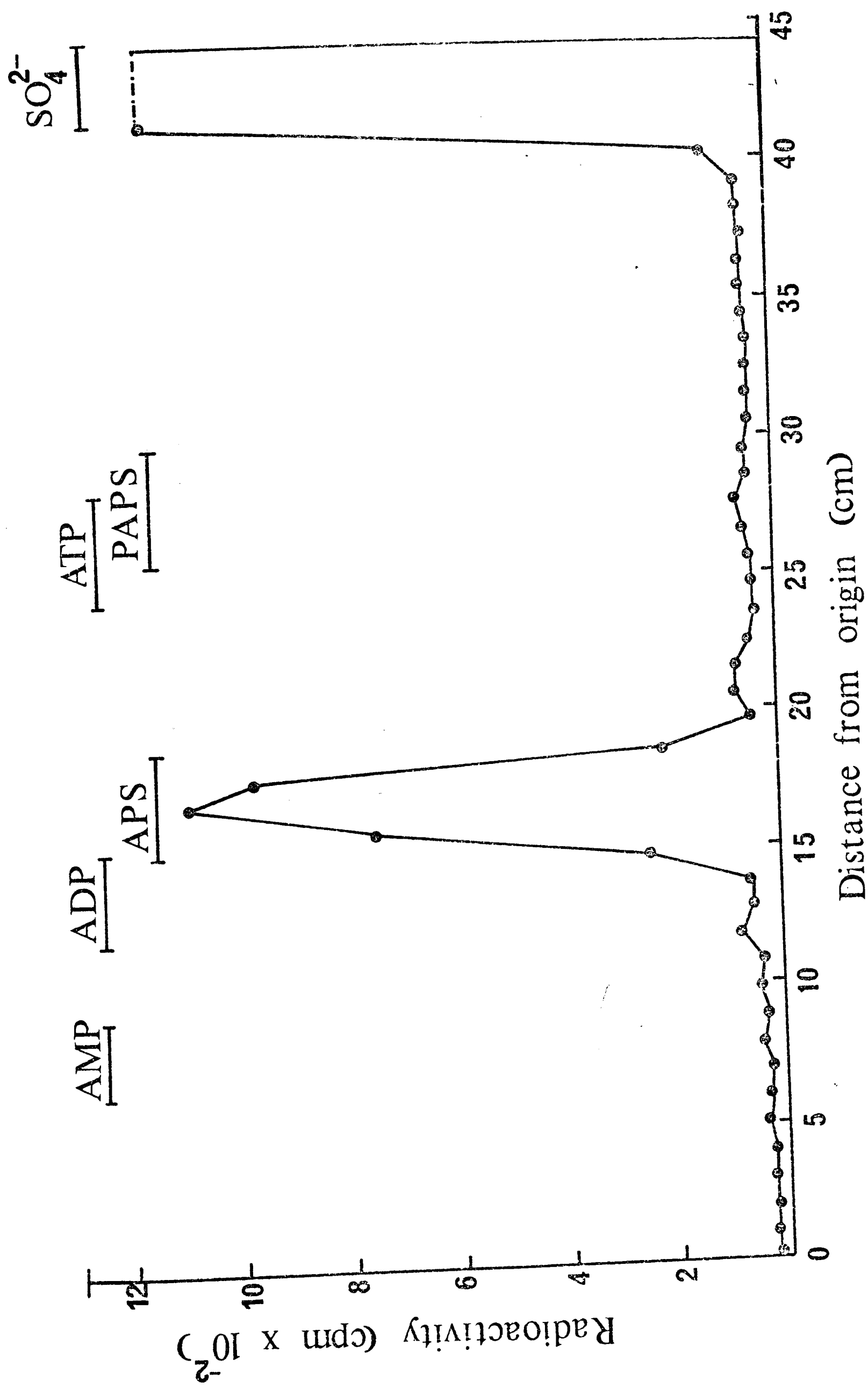
Properties of ATP sulphurylase-inorganic pyrophosphatase coupled enzyme system. The synthesis of $\gamma\text{-}^{35}\text{S}_7\text{APS}$ could not be demonstrated in reaction mixtures containing purified ATP sulphurylase with $\gamma\text{-}^{35}\text{S}_7\text{sulphate}$ and ATP as substrates, in the presence of Mg^{2+} . However, by including purified inorganic pyrophosphatase in the reaction mixtures, $\gamma\text{-}^{35}\text{S}_7\text{APS}$ synthesis was detected. The $\gamma\text{-}^{35}\text{S}_7\text{APS}$ was adsorbed to charcoal, eluted with 0.1 M NH_3 in 50% (v/v) ethanol and subjected to high voltage paper electrophoresis; the $\gamma\text{-}^{35}\text{S}_7\text{APS}$ was separated from ATP and $\gamma\text{-}^{35}\text{S}_7\text{sulphate}$ and ran with an R_f value identical to that of synthetic APS (Fig. 6.17). No $\gamma\text{-}^{35}\text{S}_7\text{APS}$ was detected in reaction mixtures if any of the components of the "complete" system were omitted. No ^{75}Se -labelled nucleotide was synthesised in reaction mixtures in which $\gamma\text{-}^{75}\text{Se}_7\text{selenate}$ was included in place of $\gamma\text{-}^{35}\text{S}_7\text{sulphate}$.

DISCUSSION

The molybdolysis assay is based on the finding of Bandurski et al., (1956) that ATP sulphurylase produces AMP and PP_i from ATP in the presence of molybdate. In the presence of inorganic pyrophosphatase, the PP_i released is hydrolysed to, and is measurable as, P_i . By analogy with the physiological activity of the enzyme, it is assumed that an extremely unstable anhydride, AMP-MoO_4 , is produced. The disadvantages of using the molybdolysis assay have been discussed by Shaw & Anderson (1971) and Hawes & Nicholas (1973); the major disadvantages being (1) that the assay is conducted with the wrong substrate, and (2) the need to ensure that there is sufficient inorganic pyrophosphatase activity present to remove pyrophosphate as soon as it is formed. In the present study, the molybdolysis assay was only used to assay

Fig. 6.17

Identification of the ^{35}S -labelled nucleotide adsorbed to charcoal, synthesised in reaction mixtures containing purified ATP sulphurylase and purified inorganic pyrophosphatase (i.e. the coupled enzyme assay). Reaction mixtures were conducted as described in Materials and Methods and the ^{35}S -labelled nucleotide adsorbed to charcoal was eluted from the charcoal with 0.1M-NH_3 in 50% (v/v) ethanol. The eluate was concentrated by rotary evaporation and then subjected to high voltage paper electrophoresis in 0.1M -sodium citrate buffer, pH 5.0, for 2 h at 50 v/cm.



crude extracts of P. denitrificans. The more sensitive assay, in which sulphate, the true substrate for the enzyme, is used, could not be used to assay ATP sulphurylase activity in crude extracts due to high endogenous inorganic pyrophosphatase activities which removed PP_i , one of the substrates, from the reaction mixture.

ATP sulphurylase activity was demonstrated in extracts of P. denitrificans using both assay methods.

ATP sulphurylase was purified free from inorganic pyrophosphatase and ATP-ase activities. The most important result of the purification was the separation of ATP sulphurylase from inorganic pyrophosphatase, since inorganic pyrophosphatase, with a similar pH optimum and requirement for magnesium and pyrophosphate as ATP sulphurylase, interferes with the measurement of ATP sulphurylase activity by the ATP- PP_i exchange method of Shaw & Anderson (1971). Neither ADP sulphurylase (Robbins & Lipmann, 1958; Burnell & Anderson, 1973a) nor an enzyme catalysing ADP- P_i exchange (Grunberg-Manago et al., 1966) was detected in the purified ATP sulphurylase.

The ATP sulphurylase of P. denitrificans is similar to the yeast enzyme (Robbins & Lipmann, 1958; Wilson & Bandurski, 1958; Hawes & Nicholas, 1973), the spinach leaf enzyme (Shaw & Anderson, 1971, 1972, 1974a), the P. chrysogenum enzyme (Tweedie & Segel, 1971a, 1971b), the Desulfovibrio desulfuricans enzyme (Akagi & Campbell, 1962; Baliga et al., 1961) and the enzyme from Desulfotomaculum nigrificans (formerly Clostridium nigrificans (Roy & Trudinger, 1970; Akagi & Campbell, 1962)). The enzymes from all the above sources are stable when frozen, require magnesium ions as a cofactor, are inhibited by free ATP, have a broad pH optimum, and are insensitive to sulphydryl-group reagents. The properties of purified

Nitrobacter agilis ATP sulphurylase (Varma & Nicholas, 1971) differ in a number of properties from the ATP sulphurylase studied in other organisms. The N. agilis enzyme has a less broad and lower pH optimum (pH optimum pH 7.3 - 7.5), is sensitive to sulphydryl-group reagents and loses its activity when stored at -15°C .

The ATP sulphurylase from sheep liver (Panikkar & Baccawat, 1968) and rat liver (Levi & Wolf, 1969) also differ in that they are unstable enzymes, sensitive to sulphydryl-group reagents. The mouse mastocytoma enzyme (Shoyab et al., 1972) however, was not affected by sulphydryl-group reagents.

ATP sulphurylase shows an absolute requirement for, or activation by, Mg^{2+} . That the actual substrate is the MgATP^{2-} complex has also been suggested by Tweedie & Segel (1971a) for the P. chrysogenum enzyme.

Inhibition by APS has also been observed for the yeast enzyme (De Vito & Dreyfuss, 1964; Hawes & Nicholas, 1973). Hawes & Nicholas (1973) examined the inhibition by APS more closely and suggested that the APS is an allosteric inhibitor (Monod et al., 1965) of ATP sulphurylase. This contrasts with the findings of Tweedie & Segel (1971a), for the P. chrysogenum enzyme, who found that APS was a non-competitive inhibitor with respect to molybdate. Both groups of workers assayed the enzyme activity using the molybdolysis assay method.

Inhibition by high PP_i concentration has been previously observed (Levi & Wolf, 1969; Segel, 1956) and shown to be non-competitive with respect to sulphate using the spinach enzyme (Shaw & Anderson, 1974).

L-Cysteine and L-methionine, end-products of sulphate activation, reduction and incorporation into amino acids, in

which ATP sulphurylase is the first enzyme of the reaction sequence, did not inhibit the enzyme from P. denitrificans. Sulphide, however, is an inhibitor (Fig. 6.10). The P. denitrificans enzyme is competitively inhibited by sulphide with respect to sulphate (Fig. 6.10).

Sulphate is the only form of inorganic sulphur activated by purified ATP sulphurylase from P. denitrificans. No ATP-PP_i exchange was detected when molybdate was added to reaction mixtures in place of sulphate. The addition of molybdate to assays containing sulphate caused a decrease in the sulphate-dependent ATP-PP_i exchange; this decrease can only be partially explained by the decrease in the substrate concentration of ATP. This suggests that molybdate competes for the sulphate-binding site of ATP sulphurylase and the results are consistent with the model proposed by Wilson & Bandurski (1958).

Chromate inhibits purified ATP sulphurylase from P. denitrificans. Chromate is an analogue of sulphate and forms an unstable adenylate intermediate which causes the release of PP_i from ATP in a similar manner to molybdate. The inhibition by chromate of spinach leaf ATP sulphurylase, as monitored by the ATP-PP_i exchange method, was reported to be uncompetitive with respect to ATP, and competitive with respect to sulphate (Shaw & Anderson 1974a).

Wilson & Bandurski (1958) initially reported that selenate was an analogue of sulphate for ATP sulphurylase, but that it differed from molybdate in that a more stable adenylate was formed. Shaw & Anderson (1972, 1974a, 1974b) reported that spinach ATP sulphurylase catalysed selenate-dependent ATP-PP_i exchange and demonstrated that selenate and sulphate competed for the same active site on the enzyme. A similar

situation has been found with the ATP sulphurylase from P. denitrificans.

Selenate has been reported to act as an analogue of sulphate in various animal and microbial systems (Rosenfeld & Beath, 1964). The uptake of sulphate by both whole cells and membrane vesicles of P. denitrificans is inhibited by selenate (see Chapters 4 and 5).

Synthesis of APS in a coupled enzyme system was dependent on the presence of inorganic pyrophosphatase. Since the equilibrium constant for the reaction catalysed by ATP sulphurylase is extremely unfavourable for the synthesis of APS (Roy & Trudinger, 1970), one of the functions of inorganic pyrophosphatase might be to augment the yield of APS by hydrolysing pyrophosphate as it is released from ATP, by ATP sulphurylase, in the presence of sulphate. Although synthesis of APS by the coupled enzyme system was detected, no APSe synthesis was detected when γ - ^{35}S sulphate was replaced with γ - ^{75}Se selenate. The inclusion of selenate in reaction mixtures containing sulphate decreased the amount of APS synthesised by the coupled enzyme system. This agrees with the competitive antagonism of sulphate and selenate and may explain the toxic effect of selenate to whole cells of P. denitrificans (see Chapter 2, Fig. 2.6), because a stable selenate-adenylate complex does not form and addition of selenate thus causes a decrease in the synthesis of APS.

The affinity of the enzyme for sulphate, molybdate, chromate and selenate must be very specific because thiosulphate, a structural analogue of sulphate, selenate, molybdate and chromate, and which competitively inhibits the uptake of sulphate by whole cells of P. denitrificans (see Chapter 4, Table 4.3), does not inhibit ATP sulphurylase, and neither does sul-

phite.

In addition to being regulated by feedback inhibition by APS, PP_i and sulphide, ATP sulphurylase is regulated by enzyme repression. The level of enzyme is repressed by both sulphide and cysteine (Table 6.2). Repression of sulphate activation by cysteine has been observed for E. coli, B. subtilis (Pasternak, 1962) and S. typhimurium (Kline & Schoenhard, 1970; Kredich, 1971). The ATP sulphurylase of Desulfovibrio desulfuricans, in which sulphate reduction is linked to the production of energy, is not repressed by growth in the presence of L-cyst(e)ine (Nakamura, 1962; Ohta et al., 1971; Wheldrake & Pasternak, 1965). The repression observed in the presence of sulphide is probably due to the formation of cysteine from sulphide and the enzyme is actually repressed by cysteine. Repression of ATP sulphurylase in cells grown in the presence of cystine and sulphate indicates that cysteine is used in preference to sulphate as a sulphur source.

CHAPTER 7

INORGANIC PYROPHOSPHATASE

INTRODUCTION

Activities which hydrolyse inorganic pyrophosphate have been demonstrated in a wide variety of natural sources, and the enzyme from yeast has been extensively investigated. Although the role of these activities in overall cell metabolism is not certain, inorganic pyrophosphatases (pyrophosphate phosphohydrolase EC 3.6.1.1.) may be of considerable importance as they influence the equilibria of several vital synthetic reaction sequences.

As reviewed by Kornberg (1962) pyrophosphate is a by-product of numerous important enzymic syntheses including the reactions of deoxyribo- and ribo-nucleic acid polymerization, coenzyme synthesis and amino acid-, fatty acid- and sulphate-activation. Although some of these reactions are themselves exergonic, the free energy change is often not great, as, for example, in amino acid activation. However, by coupling these reactions with the hydrolysis of concurrently produced PP_i (ΔG / "standard free energy change" = -5 kcal/mole), the overall equilibrium can be markedly shifted in favour of synthesis.

During an investigation of the properties and regulation of ATP sulphurylase from Paracoccus denitrificans in which the ATP- PP_i exchange method of Shaw & Anderson (1971) was used to monitor the enzyme, it was found that cell-free extracts of P. denitrificans contained a high level of inorganic pyrophosphatase activity; this activity interfered with the ATP sulphurylase assay by rapidly lowering the PP_i concentration in the reaction mixture. This interference of the ATP sulphurylase assay could not be inhibited by the method of Shaw

& Anderson (1971) (addition of fluoride to the reaction mixtures) since the inorganic pyrophosphatase was only partially inhibited by fluoride.

Shoyab & Marx (1971), whilst investigating the properties of ATP sulphurylase and APS synthesis, noticed that the inorganic pyrophosphatase of mouse mastocytoma was inhibited by APS. The inorganic pyrophosphatase from P. denitrificans was therefore investigated, in particular, with respect to the effect of inhibitors and compounds involved in the sulphate reduction pathway.

MATERIALS AND METHODS

Chemicals. Analar $\text{Na}_4\text{P}_2\text{O}_7$ was obtained from British Drug Houses, Dorset, U.K. and was used without further purification; 2,4-diaminophenol hydrochloride (amidol) was also obtained from British Drug Houses. O-Acetyl L-serine was obtained from Calbiochem, York, U.K., 3'-AMP, 5'-AMP, and APS from the Sigma Chemical Co., Ltd., London, U.K., ATP, ADP, NAD, NADH, NADP, NADPH, PAP and Coenzyme A from Boehringer und Soehne, G.m.b.H., Mannheim, Germany. Amino acids were obtained from the Sigma Chemical Co Ltd., London, U.K. $\gamma\text{-}^{32}\text{P}_7\text{P}_i$ (in dilute HCl solution, pH 2 - 3) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were reagent grade and were obtained from the sources stated in the previous chapters.

Assay of inorganic pyrophosphatase. Catalytic hydrolysis of pyrophosphate was monitored by the appearance of phosphate as detected by the method of Allen (1940). The reaction mixtures contained, in a total volume of 1 ml, 5 μmoles $\text{Na}_4\text{P}_2\text{O}_7$ (PP_i), 10 μmoles MgCl_2 , 200 μmoles Tris-HCl buffer, pH 8.0, and

sufficient enzyme, diluted in 20 mM-Tris-HCl buffer, pH 8.0, to hydrolyse 0.1 to 1.0 μ mole of substrate after 10 min at 30°C. The reaction was terminated by plunging the test tube into an ice bath and adding 2 ml of ice cold trichloroacetic acid (7.5%, w/v) followed by the addition of 1.0 ml of amidol reagent (1.28 g of amidol and 25.6 g of $\text{Na}_2\text{S}_2\text{O}_5$ made up to 200 ml with distilled water) and then 0.5 ml of sodium molybdate (6.6%, w/v). After 10 min at room temperature the A_{750} was determined.

These slight modifications of the procedure of Allen (1940) allowed full colour development due to P_i , with negligible acid hydrolysis (non-enzymic hydrolysis) of PP_i , and negligible breakdown of more labile substrates such as nucleotides. The rate of liberation of P_i in the assay was proportional to the amount of enzyme up to 2 μ moles of PP_i hydrolysed per 10 min.

All results presented in this chapter are means of duplicate determinations and variability between duplicate assays was consistently less than $\pm 4\%$.

A unit of enzyme activity is defined as that amount of enzyme which will hydrolyse 1 μ mole of PP_i per min in the above assay. (Amidol reagent was prepared fresh daily).

Stoichiometry of the reaction. The stoichiometry of the enzymic hydrolysis of pyrophosphate to orthophosphate was followed by monitoring the concentrations of $\text{[}^{-32}\text{P]PP}_i$ and $\text{[}^{-32}\text{P]P}_i$ during a time-course experiment.

$\text{[}^{-32}\text{P]P}_i$ was pyrolysed to $\text{[}^{-32}\text{P]PP}_i$ as described in Chapter 6. The pyrolysed phosphate was examined by high voltage paper electrophoresis in 0.1 M-sodium citrate buffer, pH 5.0, and the pyrolysis of P_i to PP_i determined to be 99.4%

efficient. Incubation mixtures contained, in a total of 1 ml, 1 μ mole γ - ^{32} P-PP_i (0.25 Ci/mole), 2 μ moles MgCl₂, 100 μ moles Tris-HCl buffer, pH 8.0, and purified enzyme. Reactions were run at 30°C. At 0, 10, 20, and 30 min, 0.01 ml samples were removed, deproteinised with ethanol, and streaked onto Whatman 3MM paper. After 30 min at 50 v/cm, electrophoretograms were dried and the electrophoretogram strips cut into 1.0 cm sections, placed in scintillation vials containing 1.0 ml of scintillation fluid and the radioactivity counted in a scintillation counter.

Assay of ATP sulphurylase. ATP sulphurylase activity was measured by the sulphate-dependent ATP-PP_i exchange method of Shaw & Anderson (1971).

Purification of inorganic pyrophosphatase. All operations were conducted at 0 - 4°C and the buffer used throughout the purification procedure was composed of 20 mM-Tris-HCl, pH 8.0, containing 5 mM-MgCl₂.

Crude extract. Crude extract was prepared from cells of P. denitrificans (NCIB 8944) grown in liquid culture with succinate as substrate, nitrate as the terminal electron acceptor and sulphate as the sole sulphur source as described in Chapter 2.

Ammonium sulphate fractionation. The crude extract was brought to 80% saturation with the slow addition of solid ammonium sulphate at 4°C. The mixture was stirred for 20 min and the precipitate collected by centrifugation at 30 000g for 30 min. The supernatant was discarded, the precipitate dissolved in a minimal volume of buffer and the solution extensively dialysed against buffer.

Sephadex G-200 gel filtration. The dialysed, concentrated extract was applied to a column (75 x 3.5 cm) of Sephadex G-200 equilibrated with buffer, and then eluted with buffer at a rate of 0.45 ml/min. Fractions (5 ml) were assayed for ATP sulphurylase and inorganic pyrophosphatase activities and only those fractions containing inorganic pyrophosphatase activity and devoid of ATP sulphurylase activity were pooled.

DEAE-cellulose column chromatography. The pooled Sephadex G-200 fractions were applied to a DEAE-cellulose column (8.0 x 3.5 cm) equilibrated with buffer and washed with 50 ml of 0.15 M-KCl dissolved in buffer. A 250 ml KCl-gradient from 0.15 to 0.40 M-KCl was developed and 5 ml fractions collected at a rate of 0.60 ml/min. Fractions containing the highest specific activity of inorganic pyrophosphatase were pooled and dialysed against buffer.

DEAE-cellulose concentration step. The dialysed inorganic pyrophosphatase was applied to a small column (5.0 x 1.0 cm) of DEAE-cellulose equilibrated with 0.15 M-KCl in buffer. The active fraction (5 ml) was dialysed against 20 mM-Tris-HCl buffer, pH 8.0, and stored at -15°C.

RESULTS

Linearity of the assay. The standard curve for P_i concentration versus A_{750} was linear up to at least 1 mM- P_i .

The inorganic pyrophosphatase reaction was linear with time up to at least 60 min, and linear with protein concentration up to at least 0.75 mg of protein/ml.

Purification of inorganic pyrophosphatase. Inorganic pyrophosphatase was purified approximately 160-fold by Sephadex G-200 gel filtration and DEAE-cellulose ion exchange chromatography. The purified enzyme preparation was free of ATP

sulphurylase activity; the separation of ATP sulphurylase and inorganic pyrophosphatase activities was accomplished by gel filtration as illustrated in Fig. 6.1.

The inorganic pyrophosphatase from P. denitrificans was stable during dialysis; in addition it was neither activated by cysteine or glutathione, nor inhibited by sulphhydryl-group reagents. Furthermore, the enzyme was stable at room temperature for at least a week or at 60°C for 10 min.

Metal requirement for inorganic pyrophosphatase activity.

No activity was detected in the absence of a divalent cation. Of the salts tested as possibly active divalent cations, only those with Mg^{2+} , Mn^{2+} , Zn^{2+} or Co^{2+} permitted enzymic activity (Table 7.1). The effect of the above mentioned cations was

TABLE 7.1

The effect of cations upon inorganic pyrophosphatase activity

Reaction mixtures were as described in Materials and Methods except that MgCl_2 was replaced by the cations specified in the table.

<u>Cation</u>	<u>Activity</u> (% of control)
Mg^{2+}	100
Mn^{2+}	6.3
Co^{2+}	98
Zn^{2+}	13.5
None	0

All cations were added as the chloride salts at a concentration of 10 mM.

identical no matter what salts were used. No activity was observed with Na^+ , K^+ , Ca^{2+} , Ni^{2+} , Fe^{2+} or Ba^{2+} .

Purified inorganic pyrophosphatase from P. denitrificans functions at maximum activity with either Mg^{2+} or Co^{2+} ; the amount of inorganic pyrophosphatase decreases in the order

$$\text{Mg}^{2+} > \text{Co}^{2+} > \text{Zn}^{2+} > \text{Mn}^{2+} > \text{Ca}^{2+}.$$

The Mg^{2+} - and the Co^{2+} - dependent activities were catalysed by one enzyme; the two activities were not separated by Sephadex G-200 gel filtration, DEAE-cellulose ion exchange chromatography or heat denaturation.

The addition of metal ions, except Co^{2+} , to reaction mixtures containing Mg^{2+} , inhibited the release of P_i from PP_i . Addition of Co^{2+} to reaction mixtures containing Mg^{2+} consistently increased the rate of PP_i hydrolysis by almost 20%. Again, the anion of the added salt was of no consequence as long as the salt remained soluble.

Effect of pH. The effect of pH upon both Mg^{2+} - and Co^{2+} -dependent inorganic pyrophosphatase activity was examined. No single buffer system was suitable for studying the pH optimum of the enzyme. However, maximum activity was found over the range pH 7 - 9 (Fig. 7.1) when a series of buffers was used. Although the ionisation state of PP_i substrate is important, this does not explain the pH optimum since the pK of a solution containing 5mM- Na_4PP_i and 10 mM- MgCl_2 (identical with the reaction mixture except that the buffer was omitted) indicated that ionisation to PP_i^{4-} was more than 95% complete at pH 8.5. The pH optimum in reaction mixtures containing Co^{2+} instead of Mg^{2+} was identical with that observed in the presence of MgCl_2 (pH 7.8).

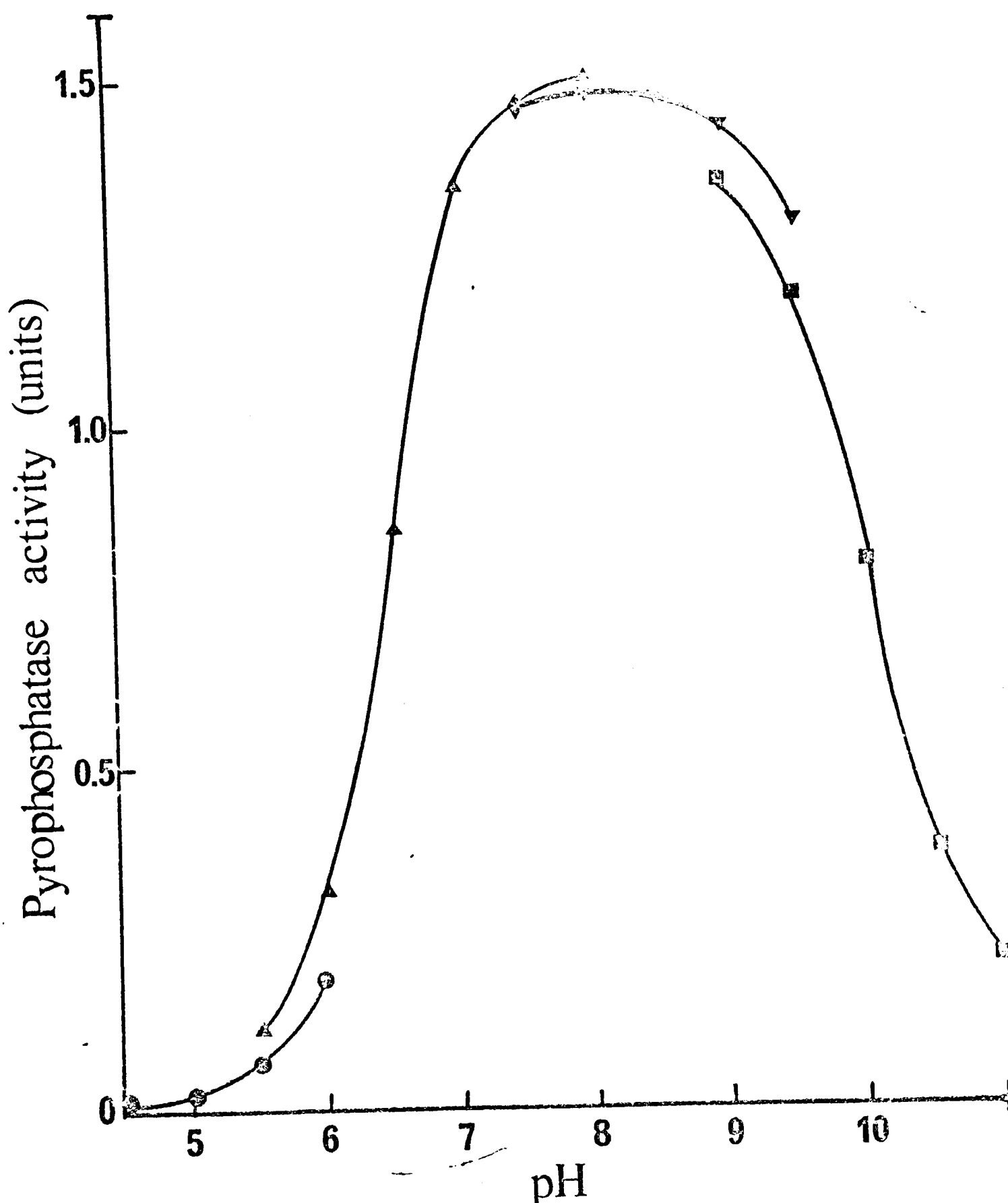


Fig. 7.1

Effect of pH on purified inorganic pyrophosphatase activity. Inorganic pyrophosphatase activity was measured as described in Materials and Methods except where specified. Tris-HCl buffer was replaced with the buffers specified. Buffers were prepared as described by Gomori (1955) and 0.5 ml of the buffer was used in each assay.

●, citrate; ▲, Tris-maleate; ▼, Tris-HCl; ■, Glycine.

Substrate specificity. A wide variety of phosphate esters were examined for activity with the enzyme as measured by the release of P_i . Of the compounds tested, only PP_i operated as a substrate. The following phosphate-containing compounds were not affected by large amounts of enzyme in the presence of Mg^{2+} , Zn^{2+} , Mn^{2+} or Co^{2+} at pH 8.0:-

- (a) nucleotide coenzymes (NAD, NADH, NADP, NADPH)
- (b) the phosphomonoester (glucose-6-phosphate)
- (c) nucleotides (ATP, ADP, 3'-AMP, 5'-AMP, APS, PAPS and PAP.

Effect of Mg^{2+} , PP_i and $MgPP_i^{2-}$ concentrations

(i) Effect of ion concentration. Prior to examining the effect of Mg^{2+} , PP_i and $MgPP_i^{2-}$ concentrations on inorganic pyrophosphatase activity, experiments were conducted with increasing concentrations of KCl to determine the effect of ionic concentration. Enzyme activity was independent of ionic concentration up to 1.5 M-KCl.

(ii) Effect of Mg^{2+} concentration. The effect of Mg^{2+} concentration upon inorganic pyrophosphatase activity is illustrated in Fig. 7.2. This graph shows that there is an absolute requirement for Mg^{2+} for activity. Furthermore, it indicates a decrease in activity at low Mg^{2+} concentrations; this effect has been observed for a number of inorganic pyrophosphatases (Block-Frankentahl, 1954; Botts, 1958; El Badry & Bassham, 1970) and it has been suggested that excess PP_i binds Mg^{2+} and so lowers the effective concentration of the enzyme activator so that the velocity falls, and when the concentration of free Mg^{2+} is reduced to zero, the enzyme becomes inactive. Heppel & Hilmoie (1951) and Block-Frankenthal (1954) initially suggested that the true substrate of inorganic pyrophosphatase is the complex, $MgPP_i^{2-}$ and not free PP_i . It has subsequently been

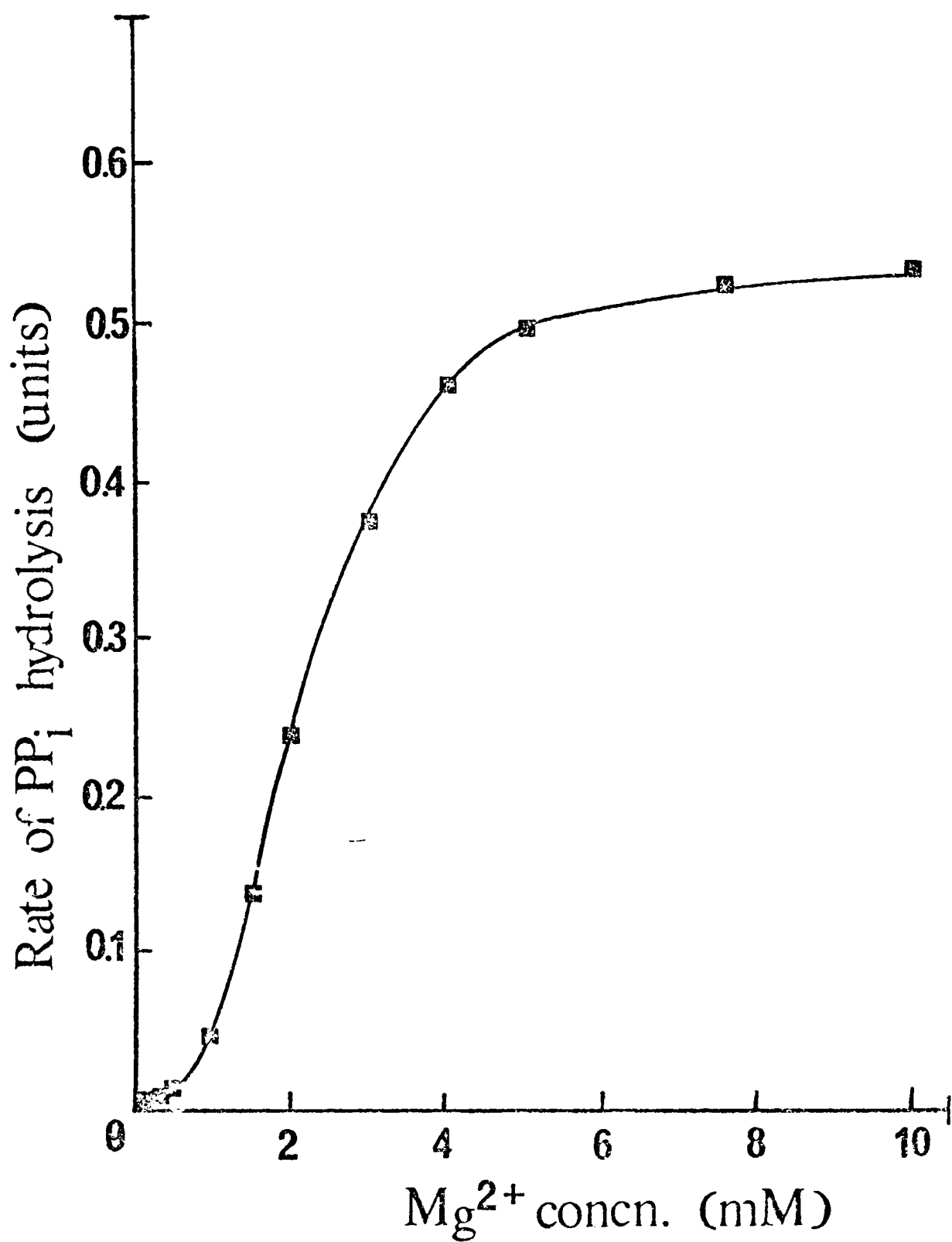


Fig. 7.2

The effect of Mg²⁺ concentration upon inorganic pyrophosphatase activity. Reaction mixtures were as described in Materials and Methods except that the concentration of Mg²⁺ was varied as shown on the graph.

shown that the combination of preformed enzyme-metal and substrate-metal complexes can lead to the reactive species (Baykov & Avaeva, 1974; Moe & Butler, 1972; Rapoport et al., 1972; Braga & Avaeva, 1972).

(iii) Effect of PP_i concentration. The effect of PP_i concentration on the activity of inorganic pyrophosphatase was examined at four concentrations of Mg^{2+} ; enzymic hydrolysis of PP_i was inhibited by high concentrations of PP_i (Fig. 7.3). Continuing the above argument as regards the true substrate of the enzyme, the inhibition of PP_i hydrolysis by high concentrations of PP_i could be due to competitive inhibition by PP_i with the true substrate, $MgPP_i^{2-}$.

(iv) Effect of $MgPP_i^{2-}$ concentration. The effect of $MgPP_i^{2-}$ concentration on the enzymic hydrolysis of PP_i was investigated while the $Mg^{2+} : PP_i$ ratio was maintained at a constant value of 3 : 1. A double-reciprocal plot (Fig. 7.4) produced a straight line; this supported the latter argument presented above for the true substrate of the enzyme. An apparent K_m for PP_i in the presence of non-limiting concentrations of Mg^{2+} was 2.0mM; this K_m value was similar to the values obtained for other inorganic pyrophosphatases isolated from different sources (Bailey & Webb, 1944; Marsh & Militzer, 1956).

Effect of Co^{2+} and $CoPP_i^{2-}$ concentration. Since equivalent rates of PP_i hydrolysis were observed in the presence of Co^{2+} , added in place of Mg^{2+} , the effect of Co^{2+} concentration upon the enzymic hydrolysis of PP_i was examined. When the PP_i concentration was maintained at a constant level, the effect of Co^{2+} upon the rate of P_i release from PP_i was almost identical to the effect observed when the effect of Mg^{2+} was examined (Fig. 7.5). A double-reciprocal plot of the rate of

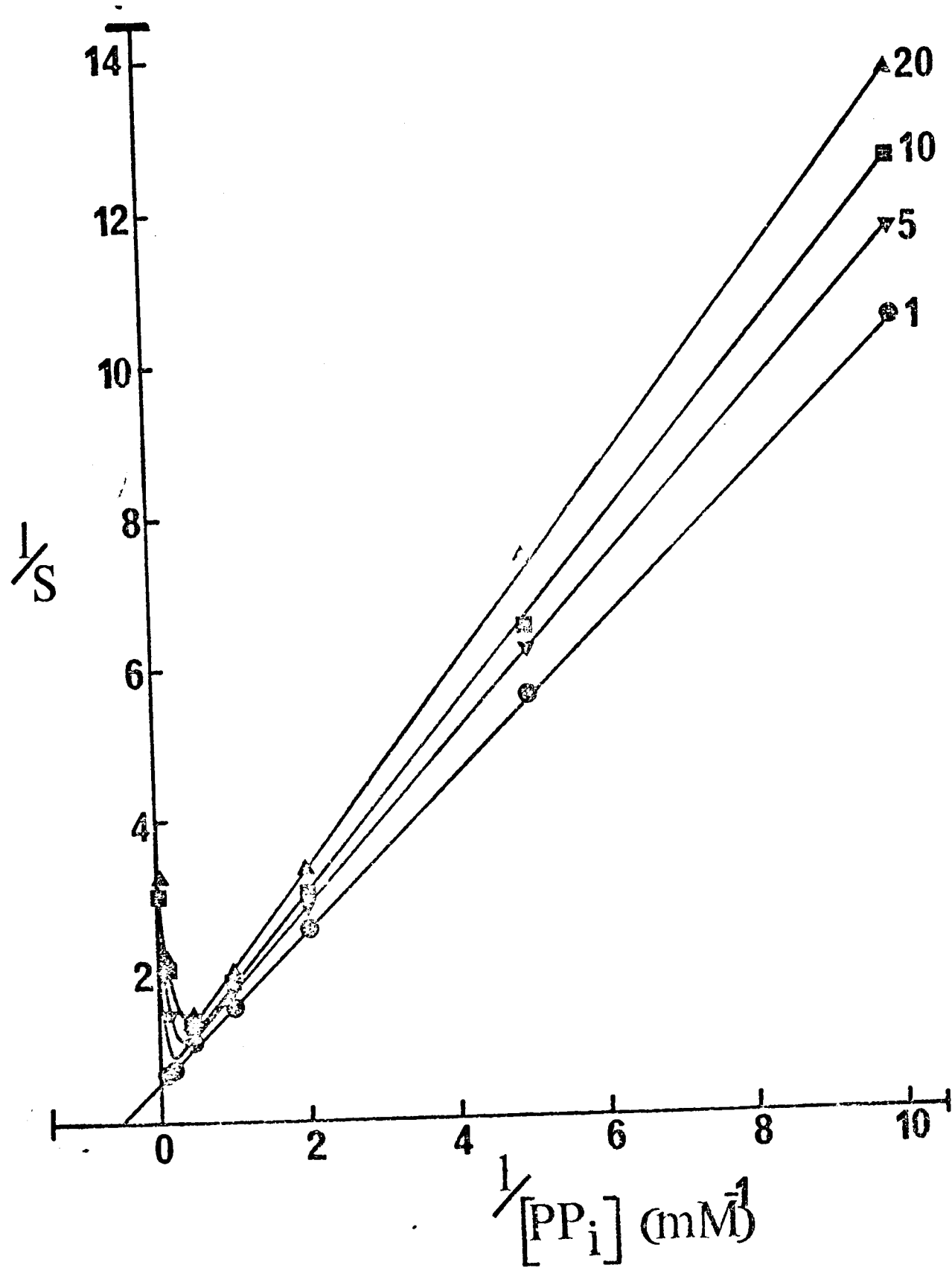


Fig. 7.3

The effect of PP_i concentration upon inorganic pyrophosphatase activity. The $MgCl_2$ and PP_i concentrations were added to reaction mixtures as specified.

●, 1mM- $MgCl_2$; ▼, 5mM- $MgCl_2$; ■, 10mM- $MgCl_2$; ▲, 20mM- $MgCl_2$.

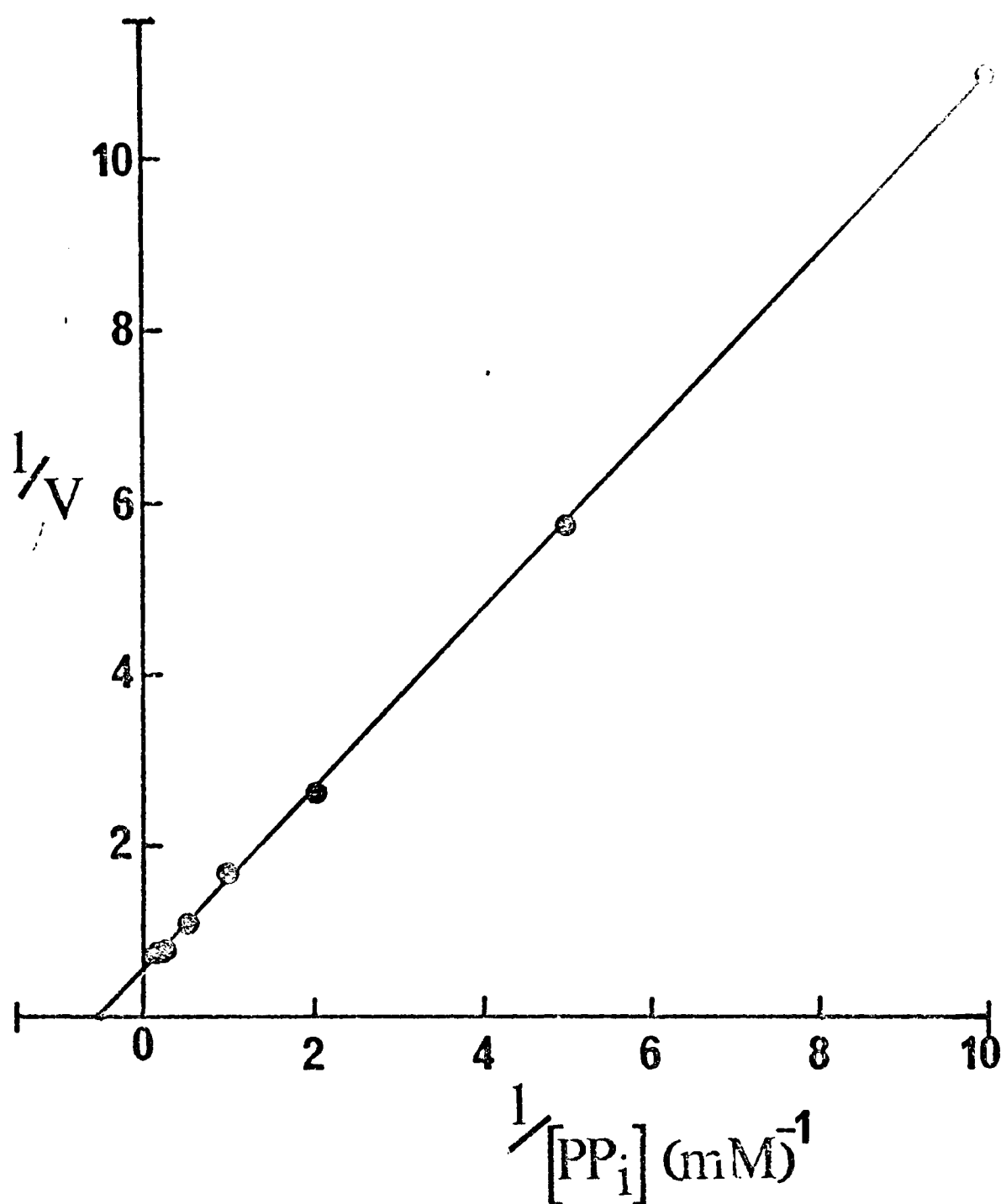


Fig. 7.4

Effect of PP_i concentration upon the activity of purified inorganic pyrophosphatase at a constant Mg^{2+} : PP_i ratio. Reaction mixtures were as described in Materials and Methods except that the Mg^{2+} and the PP_i concentrations were varied as indicated. A constant ratio of 3 : 1 of Mg^{2+} to PP_i , respectively was maintained throughout.

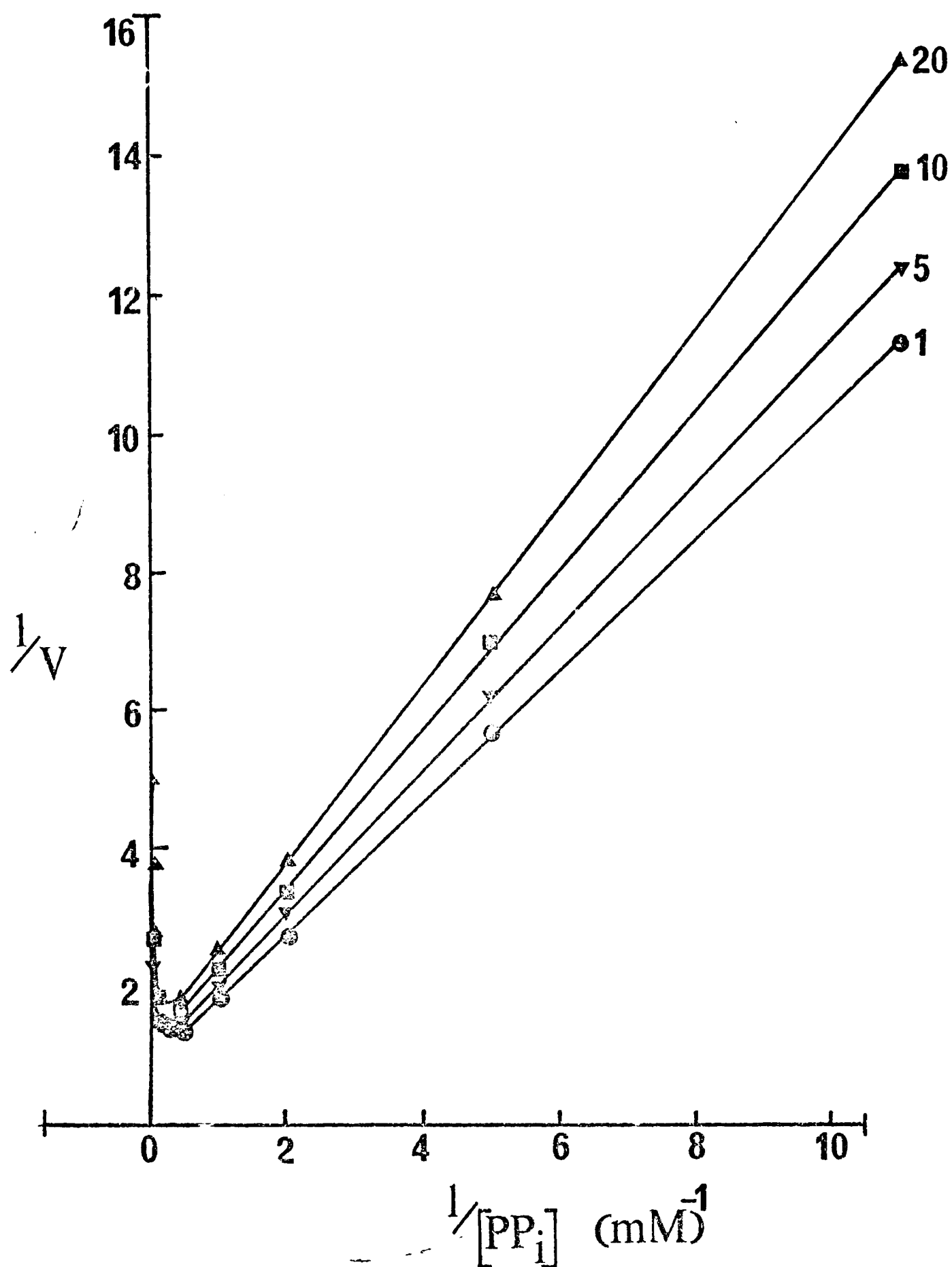


Fig. 7.5

Effect of PP_i concentration upon inorganic pyrophosphatase activity in the presence of Co^{2+} .

Reaction mixtures were as described in Materials and Methods except that Mg^{2+} was replaced by Co^{2+} at the specified concentrations.

PP_i hydrolysis versus $CoPP_i^{2-}$ is shown in Fig. 7.6; the Co^{2+} : PP_i ratio was maintained at 3 : 1. The apparent K_m for PP_i , in the presence of non-limiting concentrations of Co^{2+} , was 0.15 mM. Purified inorganic pyrophosphatase thus has a higher affinity for the $CoPP_i^{2-}$ complex than for the $MgPP_i^{2+}$ complex.

Effect of inhibitors. Unlike the inorganic pyrophosphatase from many other sources, the P. denitrificans enzyme was only partially inhibited by fluoride. Preincubation of the enzyme with 0.1 M-NaF caused no further inhibition. Purified inorganic pyrophosphatase activity was essentially unaffected by the sulphhydryl-group reagents-N-ethylmaleimide and p-chloromercuribenzoate.

Purified inorganic pyrophosphatase from E. coli is insensitive to sulphhydryl-group reagents (Josse, 1966a) but it is almost completely inhibited by 10 mM-fluoride. Inorganic pyrophosphatase activity from Desulfovibrio desulfuricans (Akagi & Campbell, 1963) was similar to the inorganic pyrophosphatase from P. denitrificans in that the activity of the enzyme is unaffected by both sulphhydryl-group reagents and fluoride.

Stoichiometry of the reaction. Two peaks of radioactivity only were detected following electrophoretic separation of the substrates and product of the reaction catalysed by purified inorganic pyrophosphatases; these corresponded to P_i and PP_i possessing relative electrophoretic mobilities of 1.0 and 1.54, respectively. The data in Table 7.2 shows that the disappearance of PP_i could be entirely accounted for by the appearance of P_i . There was no detectable condensation of the reactants or product to form P_{3i} or P_{4i} .

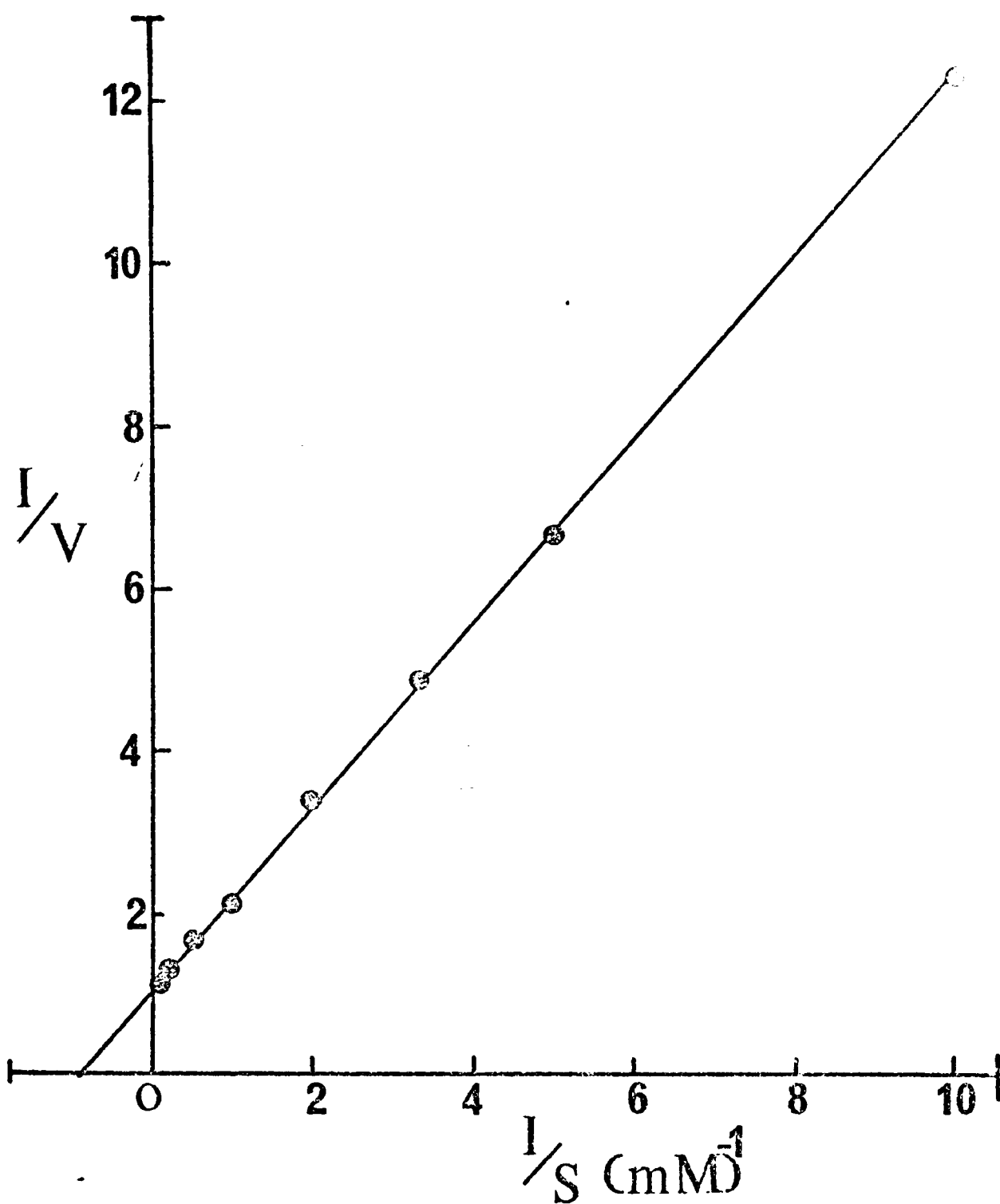


Fig. 7.6

Effect of PP_i concentration upon the activity of purified inorganic pyrophosphatase in the presence of Co^{2+} . The reaction mixtures were as described in Materials and Methods except that the concentration of PP_i and Co^{2+} were varied as specified; a 3 : 1 ratio of Co^{2+} to PP_i was maintained throughout.

TABLE 7.2

Stoichiometry of the reaction catalysed by
purified inorganic pyrophosphatase

Reaction mixtures contained, in a total volume of 1 ml, 1 $\mu\text{mole } \gamma\text{-}^{32}\text{P}\text{-PP}_i$ (0.25 $\mu\text{Ci}/\mu\text{mole}$), 2 $\mu\text{moles MgCl}_2$, 100 $\mu\text{moles Tris-HCl}$ buffer, pH 8.0, and purified inorganic pyrophosphatase. Reactions were run at 30°C and samples (0.01 ml) removed and examined by electrophoresis. Radioactivity was measured after electrophoretograms were cut into sections (1 cm) and placed in scintillation vials containing 1 ml of scintillation fluid.

Time (min)	PP _i (μmoles)	P _i (μmoles)
0	1.0	0
10	0.872	0.229
20	0.782	0.436
30	0.698	0.612

Effect of nucleotides. The effect of ATP, ADP, 3'-AMP and 5'-AMP upon inorganic pyrophosphatase activity was examined in reaction mixtures which contained either a fixed, or an increasing concentration of Mg^{2+} . When a constant Mg^{2+} concentration was maintained, all four nucleotides inhibited inorganic pyrophosphatase activity; this was presumably due to the removal of Mg^{2+} by the nucleotides. This argument is supported by the observation that when the Mg^{2+} concentration was increased as the nucleotide concentration was increased, no inhibition was detected.

Effect of sulphur-containing compounds upon the activity of inorganic pyrophosphatase. A variety of sulphur-containing compounds were surveyed as possible regulators of inorganic pyrophosphatase activity. Sulphate, thiosulphate, methionine, homocysteine, cystathionine, homocystine, glutathione, and cysteic acid had no effect upon inorganic pyrophosphatase activity, whilst sulphite, sulphide and cysteine inhibited inorganic pyrophosphatase activity (Fig. 7.7). Neither APS nor PAPS had any effect. Shoyab & Marx (1971) reported that nucleotidyl-sulphates, which included guanylylsulphate and adenylyl-sulphate, inhibited inorganic pyrophosphatase activity from yeast. The levels of APS used by Shoyab & Marx (1971) and found to be inhibitory, would be unlikely to occur in the cell as APS is rapidly converted to PAPS via APS kinase. Neither O-acetyl serine nor serine had any effect upon the activity of inorganic pyrophosphatase of P. denitrificans.

Thermal stability studies. In the absence of cations, exposure of purified inorganic pyrophosphatase to a temperature of 60°C for 60 min resulted in complete loss of enzyme activity. In the presence of either Mg^{2+} or Co^{2+} , exposure of purified inorganic pyrophosphatase to a temperature of 60°C for 10 min resulted in 8% loss of activity in the presence of 10 mM- Co^{2+} and 13% loss of activity in the presence of 10 mM- Mg^{2+} .

Effect of sulphur source during growth upon inorganic pyrophosphatase activities in P. denitrificans. P. denitrificans was grown in liquid culture containing succinate as substrate, nitrate as the terminal electron acceptor and a variety of sulphur-containing compounds as the sulphur source. Crude extracts, prepared from cells grown on a variety of

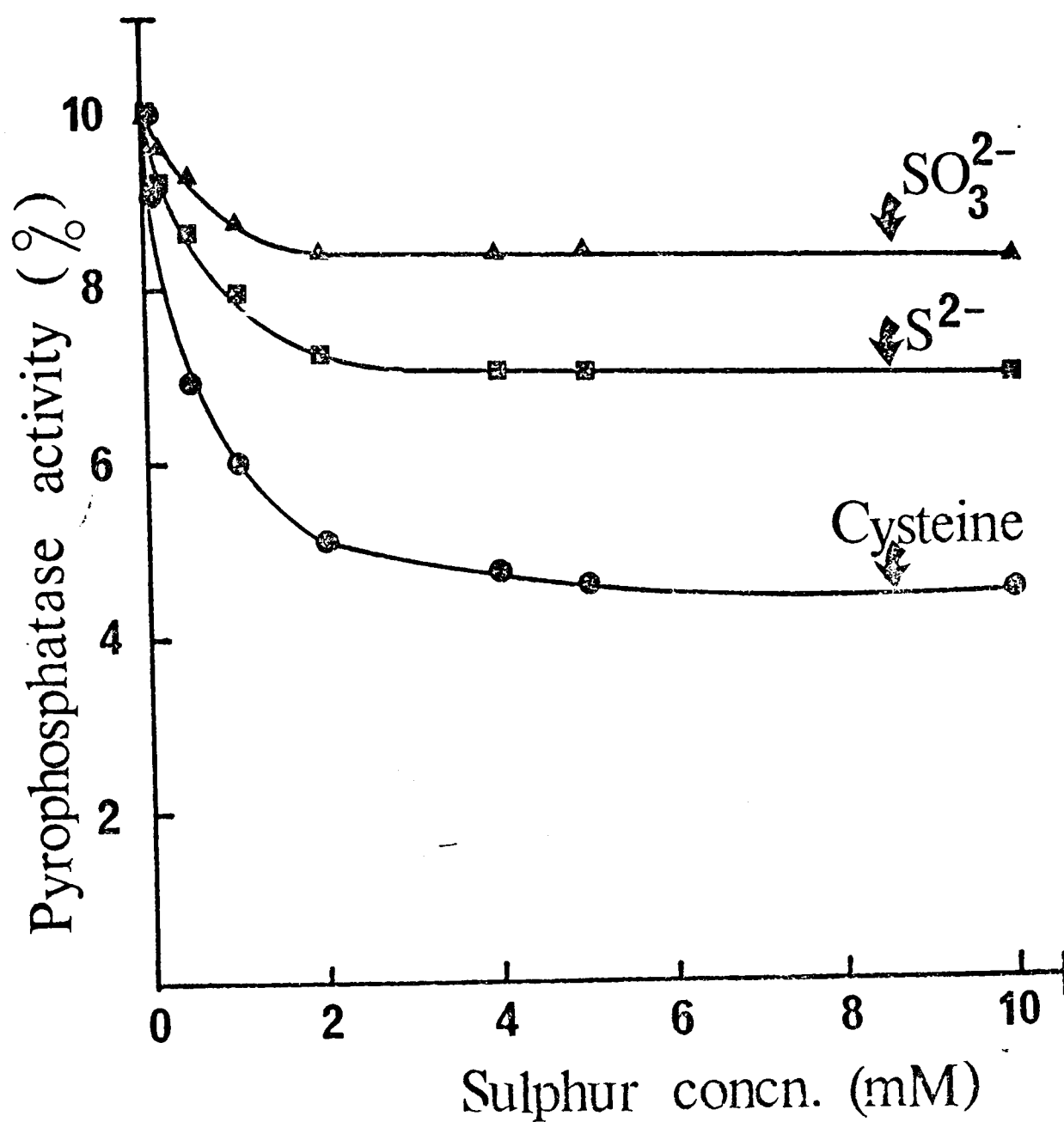


Fig. 7.7

Effect of sulphite, sulphide and cysteine upon the activity of purified inorganic pyrophosphatase.

Reaction mixtures were as described in Materials and Methods except that the sulphite, sulphide and cysteine were added at the concentrations specified.

sulphur-containing compounds, contained almost identical levels of inorganic pyrophosphatase activities.

DISCUSSION

Inorganic pyrophosphatases are widely distributed in nature, having been found in a variety of yeasts, fungi, bacteria, higher plants and animal tissues (Schmidt, 1951; Kunitz & Robbins, 1961). With several microbial inorganic pyrophosphatases, Mg^{2+} is required for maximal enzyme activity whereas Mn^{2+} , Co^{2+} , and Ca^{2+} are either ineffective, or have only slight activity (Kunitz, 1952; Murrell, 1952; Marsh & Militzer, 1956; Johnson & Johnson, 1959). Oginsky and Rumbaugh (1955) reported that Streptococcus faecalis contains two inorganic pyrophosphatases; one with an alkaline pH optimum, activated by Mg^{2+} , and the other with an acid pH optimum, activated primarily by Co^{2+} . Baliga et al., (1961) described two inorganic pyrophosphatase enzymes in Desulfovibrio vulgaris; one was a soluble enzyme which showed maximum activity at pH 8.5 and required Mg^{2+} , and the other one was an insoluble enzyme which showed maximum activity at pH 7.0 and required Co^{2+} , Mn^{2+} , or Zn^{2+} , although the last two cations gave less activity.

Inorganic pyrophosphatases are often unstable and sometimes show some degree of reactivation by reducing agents (Ware & Postgate, 1971). Gordon (1957) showed that the inorganic pyrophosphatase from rat brain, whose activity was increased 18% by cysteine, was 90% inactivated by dialysis at pH 7.4 and that 42% of this activity was restored by cysteine.

Josse (1966a, 1966b) described a constitutive inorganic pyrophosphatase in E. coli but reported no instability; indeed this enzyme was stable at 80°C for 10 min, and at room temper-

ature for a year in neutral buffer. Similarly, Kunitz (1952) reported no instability in the inorganic pyrophosphatase from yeast at 5°C, although the enzyme denatured above 40°C.

Akagi and Campbell (1963) reported only a single inorganic pyrophosphatase in a substrain of D. vulgaris which required Mg^{2+} or Mn^{2+} for optimum activity at pH 8.0; Co^{2+} gave 65% of the activity obtained with Mg^{2+} . The enzyme from E. coli requires Co^{2+} , Mg^{2+} or Mn^{2+} but the activity with each metal ion is influenced by pH, molar ratio of metal ion to pyrophosphate, and solubility of the metal ion·pyrophosphate complex (Josse, 1966a, 1966b). Josse's work shows that a knowledge of the saturation solubilities of the relevant metal ion·pyrophosphates is essential for useful studies of metal ion cofactor activity.

Inorganic pyrophosphatase is a constitutive enzyme in P. denitrificans. Inorganic pyrophosphate is a reaction product of many reactions which are thermodynamically unfavourable and, by hydrolysing PP_i , inorganic pyrophosphatase serves to increase the yield of products in these reactions. It is not unexpected, therefore, that, since inorganic pyrophosphatase plays an indirect role in so many biosynthetic reactions, it is a constitutive enzyme.

In P. denitrificans there appears to be only one inorganic pyrophosphatase which is activated equally by both Mg^{2+} and Co^{2+} and which has almost identical pH profiles for activity in the presence of Mg^{2+} and Co^{2+} . The alkaline pyrophosphatase of D. desulfuricans is activated equally well by Mg^{2+} or Mn^{2+} whilst Co^{2+} is about 65% as effective.

The optimal ratio of Mg^{2+} to PP_i for enzyme activity has been found to vary from 0.5 to 5.0, depending on the source of the enzyme and the pH (Bailey & Webb, 1944; Kunitz, 1952; Bloch-Frankentahl, 1954). A ratio of Mg^{2+} (or Co^{2+}) to PP_i of 1.0 was most effective for the inorganic pyrophosphatase from P. denitrificans at pH 8.0.

The purified inorganic pyrophosphatase from P. denitrificans exhibited stricter substrate specificity than other pyrophosphatases. For example, there was no cleavage of nucleotide di- or triphosphates in any of the pH or ionic environments tested, whereas yeast inorganic pyrophosphatase readily hydrolyses these substrates in the presence of Zn^{2+} or Co^{2+} (Schlesinger & Coon, 1960). A more extreme contrast is apparent on comparison with the inorganic pyrophosphatase of rat liver microsomes which has several different activities (Nordlie & Arion, 1964; Stetton & Taft, 1964).

E. coli possesses two distinct pyrophosphatase activities operative at neutral and alkaline pH values (Josse, 1966a). One is present only in phosphate-deprived cells (or in certain mutants) and is identical with the inducible, non-specific alkaline phosphatase (orthophosphoric monoester phosphohydrolase EC 3.1.1.1.), whilst the other is the constitutive enzyme of highly restricted specificity. The inorganic pyrophosphatase isolated from P. denitrificans also appears to be a constitutive enzyme.

It appears, from a number of experiments, that Mg^{2+} serves two functions with respect to inorganic pyrophosphatase activity (Rapoport et al., 1973; Braga & Avaeva, 1972; Moe & Butler, 1972). Firstly, Mg^{2+} forms one or more metal-enzyme complexes (Baykov & Avaeva, 1974) which are required for activity. Secondly, Mg^{2+} forms a complex with inorganic

pyrophosphate ($\text{MgP}_2\text{O}_7^{2-}$) which is the true substrate for inorganic pyrophosphatase (Moe & Butler, 1972; Baykov & Avaeva, 1974).

The results presented in this chapter are consistent with a model where MgPP_i^{2-} is the true substrate and Mg^{2+} is an essential cofactor. It is apparent that Mg^{2+} plays two roles in the activity of inorganic pyrophosphatase. The role of free Mg^{2+} in the activity of an inorganic pyrophosphatase has been reported for the pyrophosphatase isolated from yeast (Moe & Butler, 1972; Rapoport *et al.*, 1972; Braga & Avaeva, 1972) and spinach chloroplasts (El Badry & Bassham, 1970).

The initial enzymic reaction involved in the metabolism of sulphate by P. denitrificans is the ATP-dependent activation of sulphate. The products of this reaction are APS and PP_i . Since the equilibrium of this reaction lies far towards the direction of ATP and sulphate (Peck, 1962; Akagi & Campbell, 1962) removal of the PP_i , by the action of inorganic pyrophosphatase, serves to pull the reaction, catalysed by ATP sulphurylase, in the direction of APS formation. Similarly, the initial steps in protein synthesis catalysed by the aminoacyl-tRNA synthetases, which activate amino acids prior to the synthesis of a peptide bond according to the reaction:

Amino acid + ATP $\xrightleftharpoons{\hspace{1.5cm}}$ Aminoacyl adenylate + PP_i

has a $\Delta G = 0$ kJ/mole. Hydrolysis of the PP_i formed during this reaction serves to pull the reaction in the direction of aminoacyl adenylate formation.

CHAPTER 8

APS KINASE

INTRODUCTION

The substrate for sulphate reduction in most organisms and all sulphotransferases is PAPS. PAPS is formed by the transfer of phosphate from ATP to the 3'-position of APS. The reaction,



is catalysed by APS kinase (ATP:adenylylsulphate 3'-phosphotransferase, EC 2.7.1.25).

APS kinase has been rarely studied and the work of Robbins & Lipmann (1958) still remains the most complete description of its activity. There have been few determinations of levels of APS kinase activity, but APS kinase activity is assumed to be present in all tissues having ATP sulphurylase and capable of forming PAPS from ATP and sulphate. These include most bacteria (assimilatory sulphate-reducing bacteria), plants and animals. APS kinase is not present in the dissimilatory sulphate-reducing bacteria which reduce sulphate in the form of APS, rather than PAPS (Peck, 1962).

The occurrence of APS kinase in higher plants has been questioned (Asahi, 1964; Ellis, 1969) but it has clearly been shown to be present in leaf tissue (Burnell & Anderson, 1973b).

Research of APS kinase activity has been restricted by the unavailability of a simple, sensitive and rapid assay. The radioactive assay, in which $\gamma\text{-}^{35}\text{S}\text{-PAPS}$ is synthesised from $\gamma\text{-}^{35}\text{S}\text{-SO}_4^{2-}$ in the presence of ATP, Mg^{2+} and the enzymes ATP sulphurylase, pyrophosphatase and APS kinase, has been utilised to determine APS kinase activity in a limited number of organisms. This assay has often proved both inaccurate and misleading for both plant and bacterial extracts due to the interference by a powerful 3'-nucleotidase activity present in

crude extracts (Kline & Schoenhard, 1968; Burnell & Anderson, 1973b). This effect has been partially overcome by including 3'-nucleotides in the reaction mixtures (Burnell & Anderson, 1973b).

An alternative assay system involves the transfer of sulphate from PAPS to a suitable acceptor, usually a phenol derivative, in the presence of a suitable sulphotransferase (from animal tissue) to form a sulphate ester which can be colorimetrically determined (Banerjee & Roy, 1966). This assay was also found to be both unreliable and too time-consuming for routine work (Burnell & Anderson, 1973b; Jansen & Van Kempen, 1973).

Robbins & Lipmann (1958) determined the stoichiometry of the APS kinase-catalysed reaction by measuring the amount of ADP that had accumulated in a reaction mixture after a given time and comparing the amount of PAPS accumulated and the ATP used. They added pyruvate kinase and lactate dehydrogenase and measured the amount of ADP accumulated by following the amount of NADH oxidised according to the method of Bucher & Pfleider (1955).

This chapter reports the identification of APS kinase activity in cell free extracts of P. denitrificans. It also describes a new, rapid and sensitive method for continuously monitoring APS kinase activity by linking the APS-dependent production of ADP to the oxidation of NADH via pyruvate kinase and lactate dehydrogenase, and monitoring the reaction spectrophotometrically. In the method described, the formation of ADP by APS kinase is linked to the oxidation of NADH in a single reaction mixture. Furthermore, 3'-nucleotidase, an interfering enzyme in experimental methods previously used

(Kline & Schoenhard, 1968; Burnell & Anderson, 1973b) is useful in this assay since it regenerates APS, thus maintaining a low, non-inhibitory level of the sulphate-nucleotide. Indeed, when extracts to be assayed do not contain 3'-nucleotidase, it is advisable to add it to produce a linear rate of NADH oxidation.

MATERIALS AND METHODS

Chemicals. ATP (disodium salt), PAP, and coenzyme A were supplied by C.F. Boehringer und Soehne, G.m.b.H., Mannheim, Germany; solutions of ATP were adjusted to pH 8.0 with KOH prior to use. APS, 3'-AMP, 5'-AMP, phosphoenolpyruvate (PEP), pyruvate, NADH, and Tris buffer were obtained from the Sigma Chemical Co., London, U.K. APS was also synthesised as described by Baddiley et al., (1957) from sulphur trioxide and AMP in pyridine. The APS was separated either by high voltage paper electrophoresis on Whatman 3MM paper in 0.1 M-sodium phosphate buffer, pH 7.0, at 3 kV for 45 min, or by DEAE-cellulose chromatography, following the procedure of Adams et al., (1971). Sephadex G-200 was supplied by Pharmacia, Uppsala, Sweden, and DEAE-cellulose was obtained from Whatman Biochemicals Ltd., Kent, U.K. γ - ^{35}S -PAPS was prepared as described in Chapter 3. α - β , γ - ^{32}P -ATP was prepared as described by Burnell & Anderson (1973b).

Enzymes. Pyruvate kinase, lactate dehydrogenase and myokinase were supplied as solutions in 50% glycerol by C.F. Boehringer und Soehne, G.m.b.H., Mannheim, Germany. ATP sulphurylase and inorganic pyrophosphatase were purified from P. denitrificans as described in Chapters 6 and 7, respectively. Fluoride-insensitive 3'-nucleotidase was purified from spinach

leaf tissue as described by Burnell & Anderson, (1973b).

The purified enzymes were free of APS kinase and ADP sulphurylase activities.

Cells of P. denitrificans were grown in liquid culture medium with succinate as substrate, nitrate as the terminal electron acceptor and sulphate as the sole sulphur source, as described in Chapter 2. Cells were harvested during the exponential growth phase ($A_{550} = 2.9$) by centrifugation, washed with medium containing 20 mM-Tris-HCl buffer, pH 8.0, and 1mM-dithiothreitol, resuspended with washing medium in 0.01 of the original culture volume and then frozen. After thawing and degassing, cells were disrupted by sonication and cell debris removed by centrifugation. The resultant clear, brown supernatant was dialysed against 20 mM-Tris-HCl buffer, pH 8.0, containing 5 mM-MgCl₂ and 1 mM-dithiothreitol to remove endogenous sulphate. The sulphate-activating enzymes of P. denitrificans were separated from myokinase by adsorption on alumina C γ as described by Gregory (1962). The sulphate-activating enzymes were eluted from the alumina C γ with 0.5 M-(NH₄)₂SO₄ and the alumina C γ removed by centrifugation. The supernatant was extensively dialysed against 20 mM-Tris-HCl buffer, pH 8.0, containing 5mM-MgCl₂ and 1mM-dithiothreitol to remove the (NH₄)₂SO₄.

Purification of APS kinase. All procedures were conducted at temperatures between 0 and 4°C. The buffer used throughout the purification procedure contained 20 mM-Tris-HCl, pH 8.0, 5 mM-MgCl₂ and 1 mM-dithiothreitol.

The fraction containing the sulphate-activating enzymes (after adsorption and elution from alumina C γ), was brought to 80% saturation with solid ammonium sulphate. The mixture

was stirred for 20 min and then centrifuged at 30 000g for 20 min; the supernatant was discarded. The precipitate was dissolved in a minimal volume of buffer and dialysed against buffer to remove the endogenous sulphate.

Sephadex G-200 filtration. The dialysed enzyme preparation was applied to a column (75 x 3.5 cm) of Sephadex G-200, equilibrated with buffer and the enzyme was eluted at a rate of 0.40 ml/min with the same buffer. Fractions of 5 ml were collected and assayed for APS kinase activity and those fractions (55 - 68) which contained the highest specific activity of APS kinase were pooled.

DEAE-cellulose ion exchange chromatography. The pooled Sephadex G-200 fractions were applied to a column of DEAE-52 cellulose (7.5 x 3.5 cm) equilibrated with buffer and the column was washed with 50 ml of buffer. The APS kinase was then eluted with 400 ml of a linear KCl gradient from 0 to 0.4 M-KCl (dissolved in buffer) at a flow rate of 0.55 ml/min. Fractions of 5 ml were collected and assayed for both ATP sulphurylase and APS kinase activity and those fractions containing APS kinase activity, but no ATP sulphurylase activity, were pooled and dialysed against buffer (to remove the KCl).

DEAE-cellulose concentration step. The dialysed DEAE-cellulose fractions were applied to a column of DEAE-cellulose (5.0 x 1.0 cm) equilibrated with buffer. The APS kinase was eluted with 5 ml of 0.5 M-KCl in buffer. Following dialysis against buffer (to remove the KCl) the enzyme was stored at -15°C.

Enzyme assays. ATP sulphurylase was measured by the sulphate-dependent ATP-PP_i exchange method of Shaw & Anderson (1971) as described in Chapter 6. ADP sulphurylase activity

was measured by the sulphate-dependent ADP- P_i exchange method of Burnell & Anderson (1973a). Inorganic pyrophosphatase and 3'-nucleotidase activities were measured as described by Burnell & Anderson (1973b) (see also Chapter 7). Myokinase, lactate dehydrogenase and pyruvate kinase were measured as described in the Boehringer Catalogue (1970). NADH dehydrogenase activity was measured as described in Chapter 5.

Instrumentation. Assays were conducted at 30°C in cuvettes (1 cm light path) placed in a Pye Unicam SP 8000 spectrophotometer linked to an AR25 linear recorder.

Assay of APS kinase. The reaction mixture contained 10 μ moles $MgCl_2$, 0.4 μ moles PEP, 2.5 μ moles ATP, 200 μ moles Tris-HCl buffer, pH 8.0, 10 μ moles NaF, 5 μ moles KCN, 10 units lactate dehydrogenase, 10 units pyruvate kinase and 0.1 ml of crude extract in a total volume of 0.95 ml. The reaction mixture was preincubated at 30°C in a water bath for 2 min to equilibrate the temperature. then placed in the thermostatted cell holder of the spectrophotometer at 30°C and the A_{340} examined for 1 min to ensure that a steady baseline was obtained. Following this, 0.3 μ moles of reduced NADH (at 30°C) in a volume of 48 μ l, was added and the endogenous rate of NADH oxidation monitored for 1 min. This was followed by the addition of 20 μ l of a 1mM solution of APS. The rate of NADH oxidation, in the presence of APS, was recorded for 2 min. The rate of endogenous NADH oxidation was subtracted from the rate observed in the presence of APS to give the APS-dependent rate of NADH oxidation. This is a direct measure of the APS-dependent rate of ADP production.

Identification and purification of γ - ^{35}S -7 PAPS by Dowex ion exchange chromatography. γ - ^{35}S -7PAPS was synthesised in reaction mixtures containing 10 μmoles ATP, 20 μmoles MgCl_2 , 200 μmoles Tris-HCl buffer, pH 8.5, 40 μmoles $\text{Na}_2^{35}\text{SO}_4$ (50 $\mu\text{Ci}/\mu\text{mole}$), 55 units purified ATP sulphurylase, 10 units purified inorganic pyrophosphatase and 20 units purified APS kinase, in a total volume of 2.5 ml. Reactions were run for 2 h at 30°C . Reactions were terminated and mixtures deproteinised by the addition of 5 ml of ethanol. The supernatant solutions were recovered by centrifugation (5 min at 5 000g) and evaporated to dryness. The residue was dissolved in 2.0 ml of water and applied to a Dowex 1-X2 column (Cl^- form) (20 x 1.5 cm). (A 15 x 2.5 cm column of Dowex 1-X2 will completely adsorb 1 mmole of adenine nucleotide -Brunngraber 1958). The elution followed the method described by Hodson & Schiff (1971). The column was washed with water until the extinction at 259 nm fell below 0.05 and then washed with 0.5 M-KCl until the extinction at 259 nm again fell below 0.05. γ - ^{35}S -7PAPS was subsequently eluted with 1.0 M-KCl. Radioactivity was monitored by placing 0.1 ml of the eluate into 5.0 ml of scintillation fluid (see Chapter 2) and counted in a scintillation counter. The eluted material was continually monitored at 259 nm, for nucleotides.

Identification of PAPS by hydrolysis using 3'-nucleotidase

α - β γ - ^{32}P -7PAPS was synthesised in reaction mixtures containing 20 μmoles MgCl_2 , 40 μmoles K_2SO_4 , 200 μmoles Tris-HCl buffer, pH 8.0, 10 μmoles γ - ^{32}P -7ATP, 50 units purified ATP sulphurylase, 20 units purified inorganic pyrophosphatase and 40 units purified APS kinase in a final volume of 2 ml. The reaction was run at 30°C for 2 h and terminated, and the mix-

ture deproteinised, by the addition of ethanol. The deproteinised supernatant was evaporated to dryness, the residue dissolved in 2.0 ml of water and the γ - ^{32}P -PAPS purified by ion exchange chromatography on Dowex 1-X2 (Cl^- form) 200-400 mesh. The fractions containing γ - ^{32}P -PAPS were combined and stirred for 15 min with acid-washed charcoal (1 g/3 μmoles of PAPS based on the extinction at 259 nm, assuming $\epsilon_{259} = 15.4 \text{ mM}^{-1}$ (Baddiley et al., 1957) to absorb the γ - ^{32}P -PAPS. The charcoal was filtered and washed with water to remove Cl^- , and PAPS was then eluted from the charcoal with 500 ml of ethanol (50%, v/v), containing 7.5 ml of 1 M - NH_4Cl . The eluted material was filtered through a Millipore filter (pore size, 0.22 μ) to remove particulate material (mostly charcoal), and evaporated to dryness in a rotary evaporator. The residue was dissolved in 2.0 ml of water and the pH of the solution adjusted to 8.0 with 1 M -HCl.

One ml of the solution was incubated with 10 units of purified spinach leaf 3'-nucleotidase in an assay mixture containing 100 μmoles of Tris-HCl, pH 8.0, and 20 μmoles of MgCl_2 ; another 1 ml aliquot of γ - ^{32}P -PAPS was incubated similarly except that the 3'-nucleotidase was replaced with an equal volume of 20 mM -buffer. Reactions were conducted at 30°C and terminated, and the mixtures deproteinised, by the addition of ethanol. The supernatant solutions were recovered by centrifugation, evaporated to dryness, dissolved in 0.25 ml of water and applied to Whatman 3MM paper. Electrophoretograms were run at 50 v/cm for 1 hr in 0.1 M -sodium citrate buffer, pH 5.0, dried and scanned for radioactivity as described above.

Electrophoretic identification of PAPS. Electrophoresis

in Chapter 3. Two solvent systems were used:-

- I. 0.1 M-sodium citrate, pH 5.0 (Balharry & Nicholas, 1970)
- II. 0.5 M-ammonium acetate, pH 8.5.

Radioactivity was located by cutting the electrophoretogram strips into 1 cm sections, and counting the radioactivity in a scintillation counter. Nucleotides were located by u.v. fluorescence quenching.

RESULTS AND DISCUSSION

Development of the new assay for measuring APS kinase activity in crude extracts. During initial experiments interference due to a number of contaminating enzymes and substrates was observed. (I) High endogenous rates of NADH oxidation were obtained when crude extracts were used as a source of APS kinase due to the presence of endogenous NADH dehydrogenase activity. Since NADH dehydrogenase is a membrane-bound enzyme (Scholes & Smith, 1968), centrifugation at 30 000g for 1 h at 2°C, followed by careful decantation of the supernatant removed most of the interfering activity. The addition of 5 μ moles of KCN to the reaction mixture almost completely inhibited the remaining NADH dehydrogenase activity without affecting the APS kinase assay system. (II) Crude extracts contained a small amount of ATP-ase activity which was inhibited by the addition of 10 μ moles of NaF without detrimental effects to the assay. (III) 5'-AMP, a contaminant in some preparations of APS, reacted with added ATP when endogenous myokinase activity was present in crude extracts and this caused rapid ADP synthesis and, consequently, high rates of NADH oxidation. This interference could be overcome without the need to remove myokinase, by purification of APS either, (IIIa), by high voltage electrophoresis (Burnell &

Anderson, 1973b) or, (IIIb), by DEAE-cellulose chromatography, prior to use. It was confirmed that the purified APS was free from AMP by mixing a sample with pure ATP and purified myokinase, and testing for the production of ADP by the coupled pyruvate kinase/lactate dehydrogenase assay. Alternatively, (IIIc) the APS could be continuously generated in the reaction vessel by the addition of purified ATP sulphurylase, purified inorganic pyrophosphatase, ATP and sulphate. (IV) Crude enzyme preparations were dialysed to remove the endogenous sulphate which supported endogenous APS synthesis. Thus, in the presence of cyanide and fluoride, and after taking precautions to remove effects of possible interfering enzymes, the APS-dependent production of ADP, measured as NADH oxidation, proved to be a rapid and sensitive technique for assaying APS kinase activity in both crude and purified extracts. Furthermore, the assay was found to be reproducible and the variation between replicate experiments was consistently less than $\pm 3.5\%$. The lower limit of APS-dependent NADH oxidation that can be accurately measured with this method is 0.5 nmoles/min.

Properties of APS kinase. The rate of APS-dependent NADH oxidation was linear for 2 min under the standard assay conditions, provided 3'-nucleotidase activity was present in the reaction mixture (Fig. 8.1). Crude extracts of P. denitrificans, prepared as described in Materials and Methods, do not contain 3'-nucleotidase activity and it was therefore necessary to add purified spinach 3'-nucleotidase to the reaction cuvettes to obtain a linear rate of APS-dependent NADH oxidation. The rate of NADH oxidation was directly proportional to the amount of APS kinase added up to at least 4 μ moles NADH

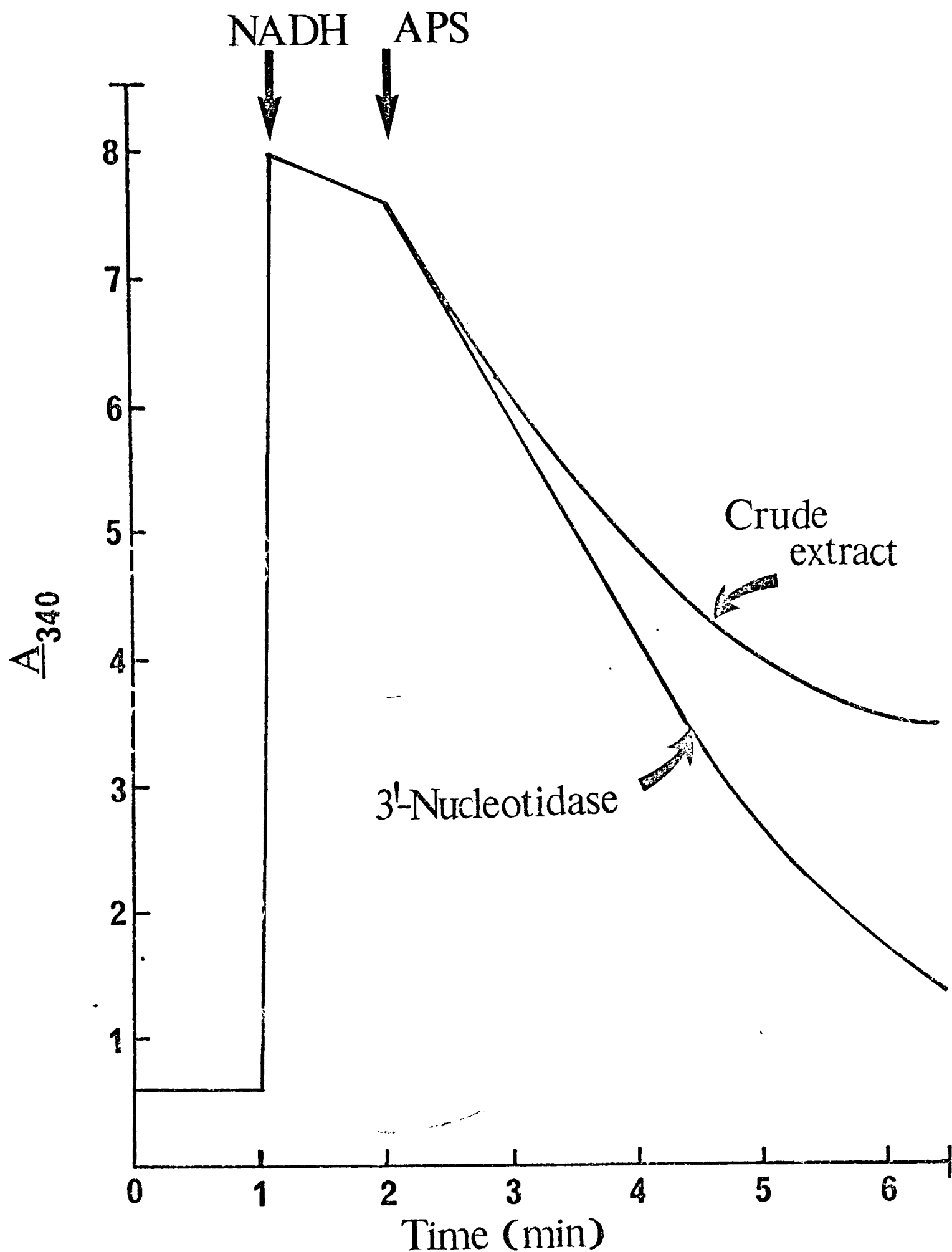


Fig. 8.1

Progress curves of APS-dependent NADH-oxidation by crude extracts of Paracoccus denitrificans.

The reaction mixture in A was as described in Materials and Methods. In B reaction mixtures also included 5 units of purified spinach leaf 3'-nucleotidase.

oxidised/min. The non-linearity of the reaction in the absence of 3'-nucleotidase is due to the decrease in the concentration of APS. The non-linearity of the reaction in the presence of 3'-nucleotidase after 4 min is due to the decrease in NADH concentration, and not the instability of the 3'-nucleotidase; doubling the concentration of 3'-nucleotidase in the assay does not affect the progress of the reaction.

The rate of APS-dependent NADH oxidation is directly proportional to the amount of enzyme protein added. The dependence of the initial rate of ADP synthesis on ATP concentration is shown in Fig. 8.2; during these experiments the APS concentration was maintained at $20 \mu\text{M}$. The apparent K_m for ATP read from the Lineweaver-Burk plot in this graph is $1.3 \times 10^{-3} \text{M}$. The effect of APS concentration upon NADH oxidation is illustrated in Fig. 8.3. The initial reaction rate increases with increasing APS concentration up to $20 \mu\text{M}$, and higher concentrations are inhibitory. The apparent K_m value for APS obtained from the Lineweaver-Burk plot is $5 \times 10^{-6} \text{M}$ (Fig. 8.3) when the ATP concentration was maintained at 2.5mM .

The substrate specificity of ATP sulphurylase and APS kinase. The extent to which the pathway of selenate follows that of sulphate is open to question. The formation of APSe can be detected by the ATP-PP_i exchange method (Shaw & Anderson, 1972; Wilson & Bandurski, 1958) but it is difficult to show its accumulation (Roy & Trudinger, 1970).

In animals (Roy & Trudinger, 1970) and, perhaps, also in plants, the sulphate of sulpholipids and sulphate esters is derived from PAPS. However, Nissen & Benson (1964) reported that selenate is not incorporated into the sulpholipid or sulphate ester fraction of plants and Burnell *et al.*, (1974)

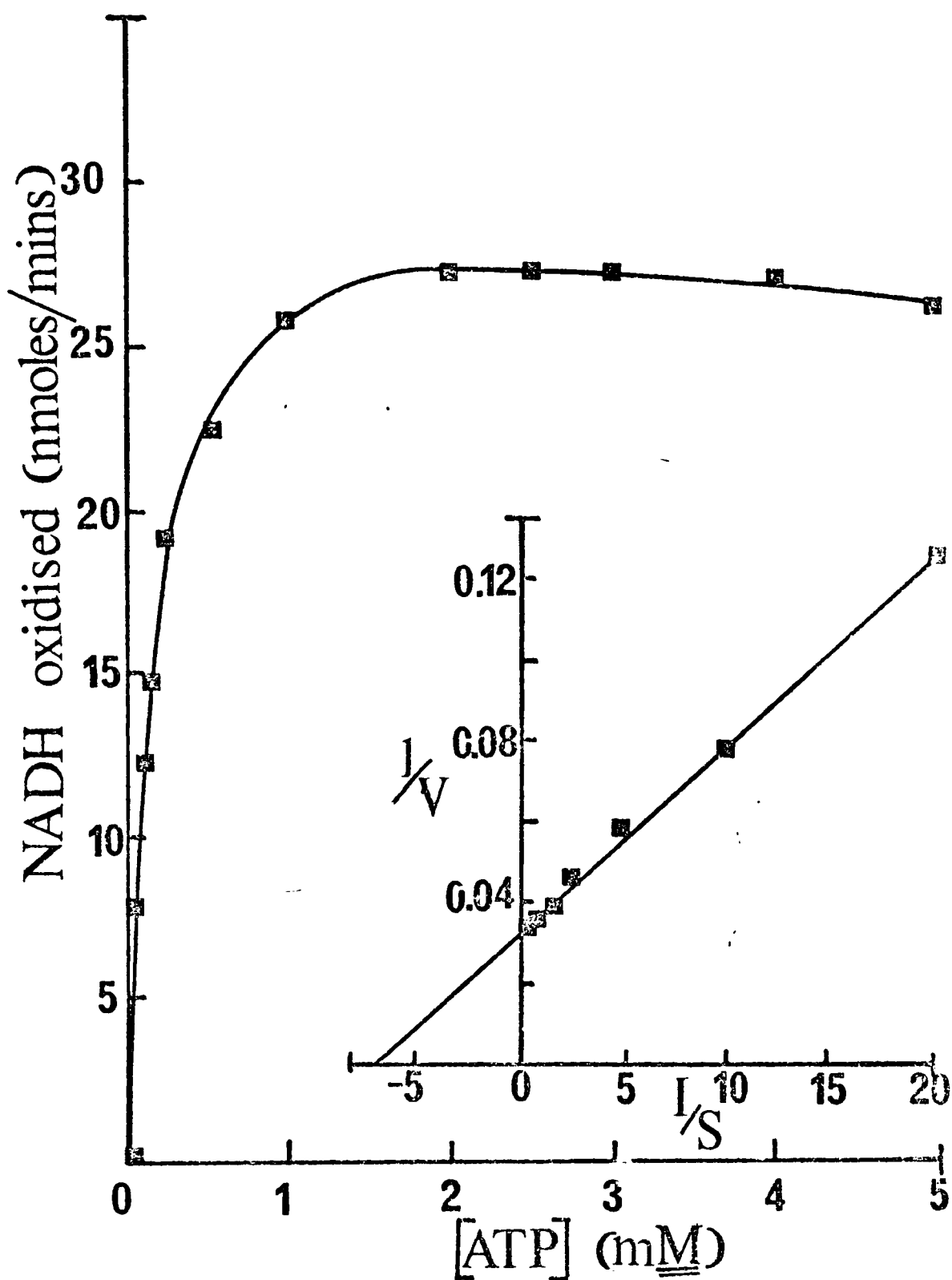


Fig. 8.2

Effect on ATP concentration on APS-dependent NADH oxidation (ADP production) of crude extracts of P. denitrificans.

The reaction mixtures were as described in the Materials and Methods except that the ATP concentration was varied and 3'-nucleotidase was added to ensure linearity.

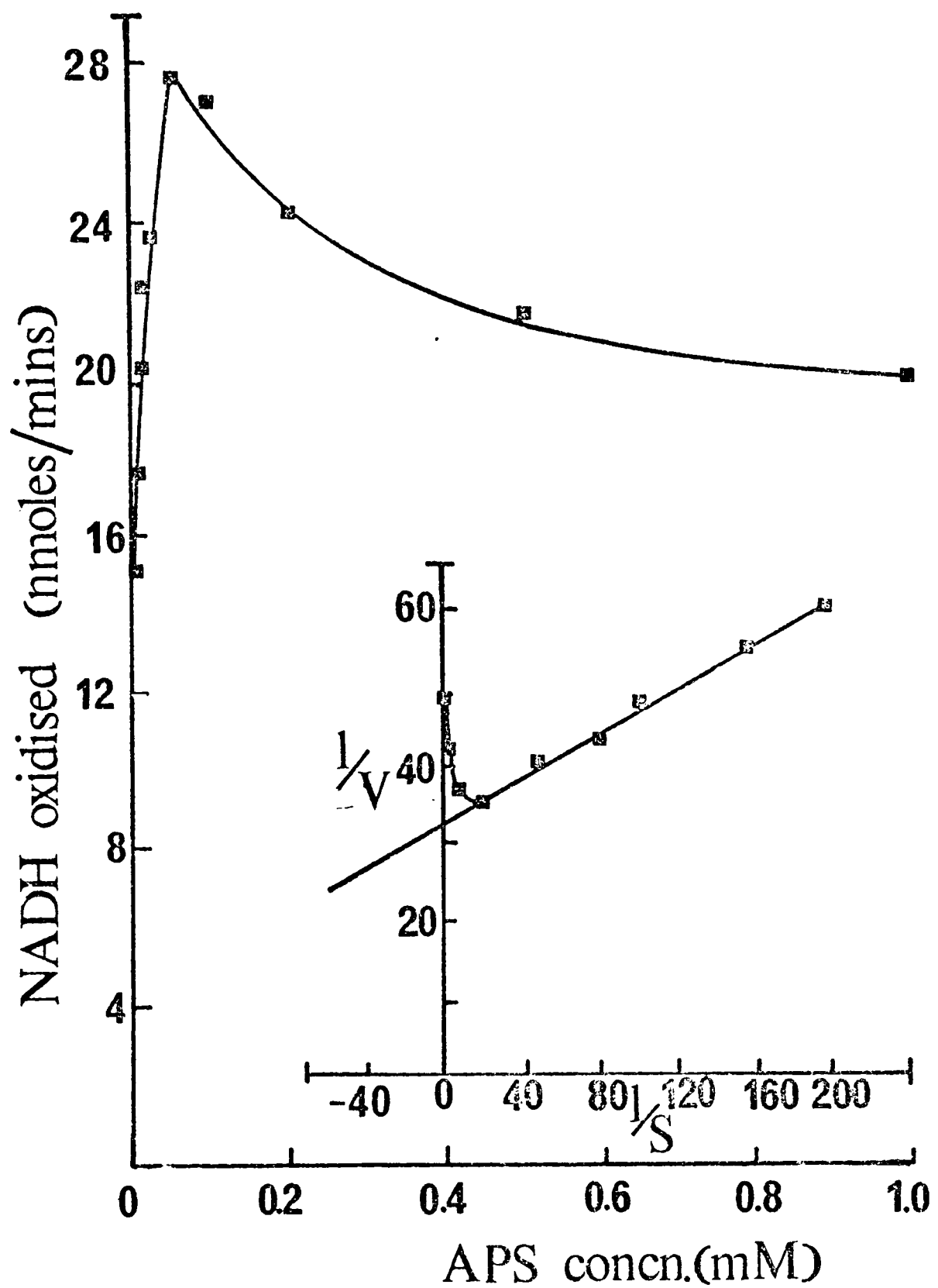


Fig. 8.3

Effect of concentration of APS on APS-dependent NADH oxidation (ADP production) of crude extracts of P. denitrificans.

The reaction mixtures were as described in Materials and Methods except that the concentration of APS was varied and 3'-nucleotidase was added to maintain the APS concentration.

have shown that selenate is not incorporated in the selenolipids of PAPSe either in selenium accumulator or in selenium non-accumulator plants.

The results may have been due to the instability of APSe or PAPSe, so that no accumulation of the final products (APSe, PAPSe or selenolipids) occurred. However, APSe could be continuously supplied by synthesis in situ, using the alternative system, (IIIc), above, by replacing sulphate by selenate, thus ensuring that some APSe will always be available for the synthesis of PAPSe via APS kinase. Any formation of PAPSe in this system would necessarily be accompanied by ADP production (a stable compound) which would be detected as NADH oxidation.

Sulphate-dependent NADH oxidation was observed in crude extracts of P. denitrificans. When sulphate was replaced by selenate, selenate-dependent NADH oxidation was observed but this was found to be due to the selenate-dependent release of AMP from ATP which, in the presence of myokinase, reacts with ATP to produce ADP. The sulphate-activating enzymes were separated from myokinase activity as described in Materials and Methods. Following this separation sulphate-dependent, but not selenate-dependent, NADH oxidation could be detected when ATP concentrations were varied between 0.1 and 5mM and selenate concentrations varied from 1 to 40 mM. Since selenate-dependent NADH oxidation could not be detected it must be concluded either that APSe is not being synthesised, or that it has an exceedingly short half-life, or that it does not function as a substrate for APS kinase. The latter explanation seems to be the most probable.

Purification of APS kinase. APS kinase was purified to

remove ATP sulphurylase activity; this was accomplished by ion exchange chromatography on DEAE-cellulose (Fig. 8.4). During the purification procedure, APS kinase was purified approximately 370-fold with a recovery of 33%. APS kinase activity was lost during the purification procedure if either MgCl_2 or dithiothreitol was omitted from the buffer solution. Purified APS kinase lost 21% of its activity during storage at -15°C for 6 weeks, and 73% of its activity during storage at 4°C for 1 week. The enzyme was completely inactivated by heat treatment at 80°C for 2 min.

Synthesis of $\gamma\text{-}^{35}\text{S}_7\text{PAPS}$ using purified ATP sulphurylase, inorganic pyrophosphatase and APS kinase. Synthesis of $\gamma\text{-}^{35}\text{S}_7\text{PAPS}$ from ATP and $\gamma\text{-}^{35}\text{S}_7\text{SO}_4^{2-}$ was detected in reaction mixtures which contained purified ATP sulphurylase, inorganic pyrophosphatase and APS kinase. Omission from the reaction mixture of any of the purified enzymes, singly or in combination, prevented synthesis of detectable amounts of $\gamma\text{-}^{35}\text{S}_7\text{PAPS}$; this indicated that the purified enzyme preparations were not cross-contaminated. No $\gamma\text{-}^{35}\text{S}_7\text{PAPS}$ was synthesised in reaction mixtures which did not contain inorganic pyrophosphatase activity; this confirmed that inorganic pyrophosphatase is a necessary component of the sulphate activation pathway.

Identification of PAPS by electrophoresis. Two electrophoretic analyses of the ^{35}S -labelled nucleotide isolated from the coupled enzyme assay indicated that the compound possessed an electrophoretic mobility identical to PAPS (Fig. 8.5, Fig. 8.6).

Identification of $\gamma\text{-}^{35}\text{S}_7\text{PAPS}$ by Dowex ion exchange chromatography. The ^{35}S -labelled nucleotide synthesised in the coupled assay system described above, was subjected to ion

Fig. 8.4

Separation of APS kinase and ATP sulphurylase activities

The activities were separated by ion exchange chromatography on DEAE-cellulose after adsorption and elution from alumina C_γ and gel filtration on Sephadex G200. Enzyme activities were measured as described in the text. ■, protein; ●, ATP sulphurylase; ▲, APS kinase.

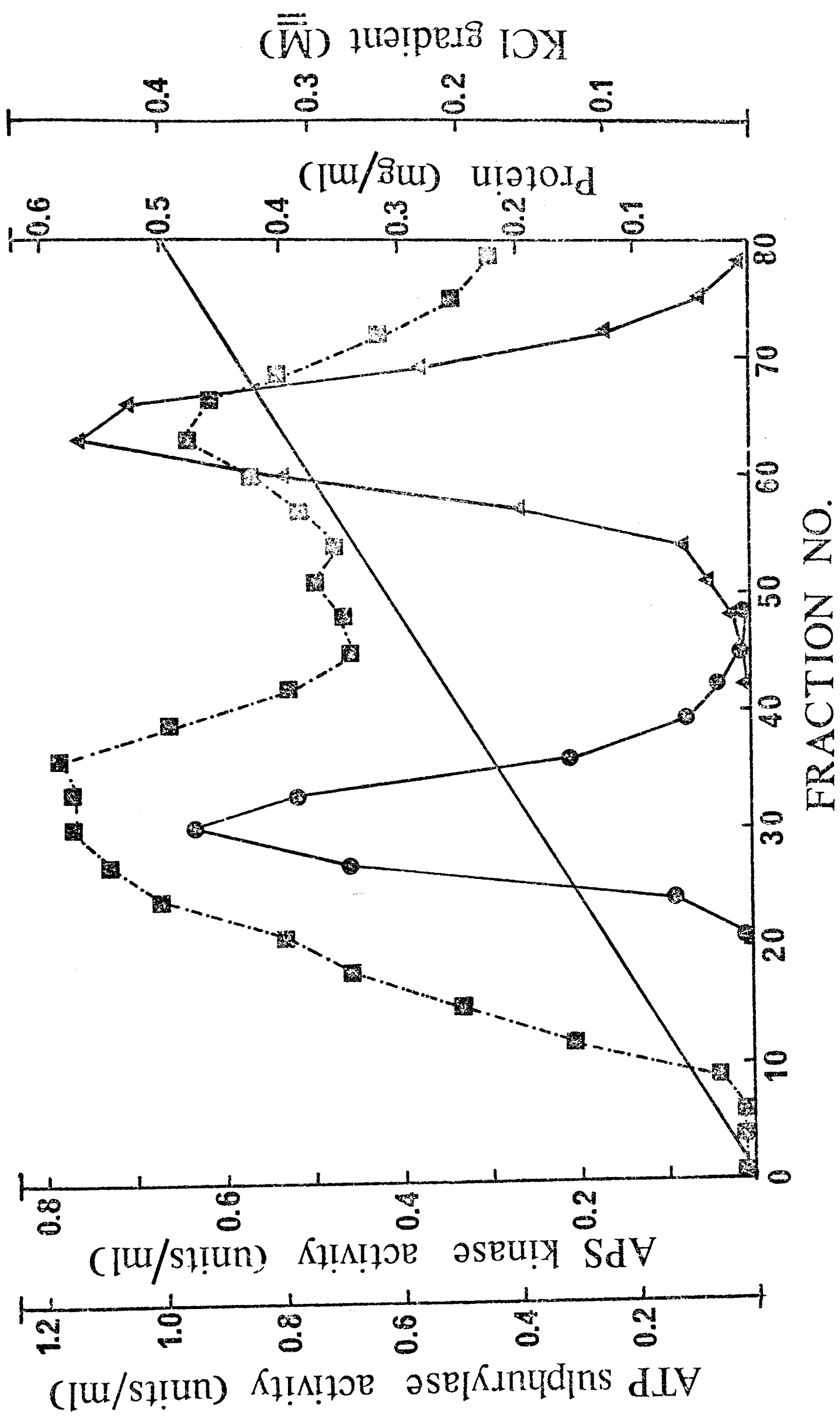


Fig. 8.5

Synthesis of γ - ^{35}S -PAPS from γ - ^{35}S - SO_4^{2-} and ATP.

The reaction mixtures contained, in a total volume of 1 ml, 200 μmoles of Tris-HCl buffer, pH 8.0, 10 μmoles of ATP, 20 μmoles of MgCl_2 , 40 μmoles of γ - ^{35}S - Na_2SO_4 (50 $\mu\text{Ci}/\mu\text{mole}$), 55 units of purified ATP sulphurylase, 10 units of purified inorganic pyrophosphatase and 20 units of purified APS kinase. Reactions were run for 2 h at 30°C , and terminated by the addition of 5 ml of ethanol. The deproteinised fractions were subjected to high voltage paper electrophoresis in 0.1M-sodium citrate buffer, pH 5.0, for 2 h at 60 v/cm. Nucleotide spots were visualised by u.v. fluorescence quenching. Radioactivity was measured in a scintillation counter after the electrophoretograms were cut into 1 cm sections and placed in scintillation vials containing 1 ml of scintillation fluid.

No significant radioactivity above background was detected in minus enzyme or boiled enzyme controls.

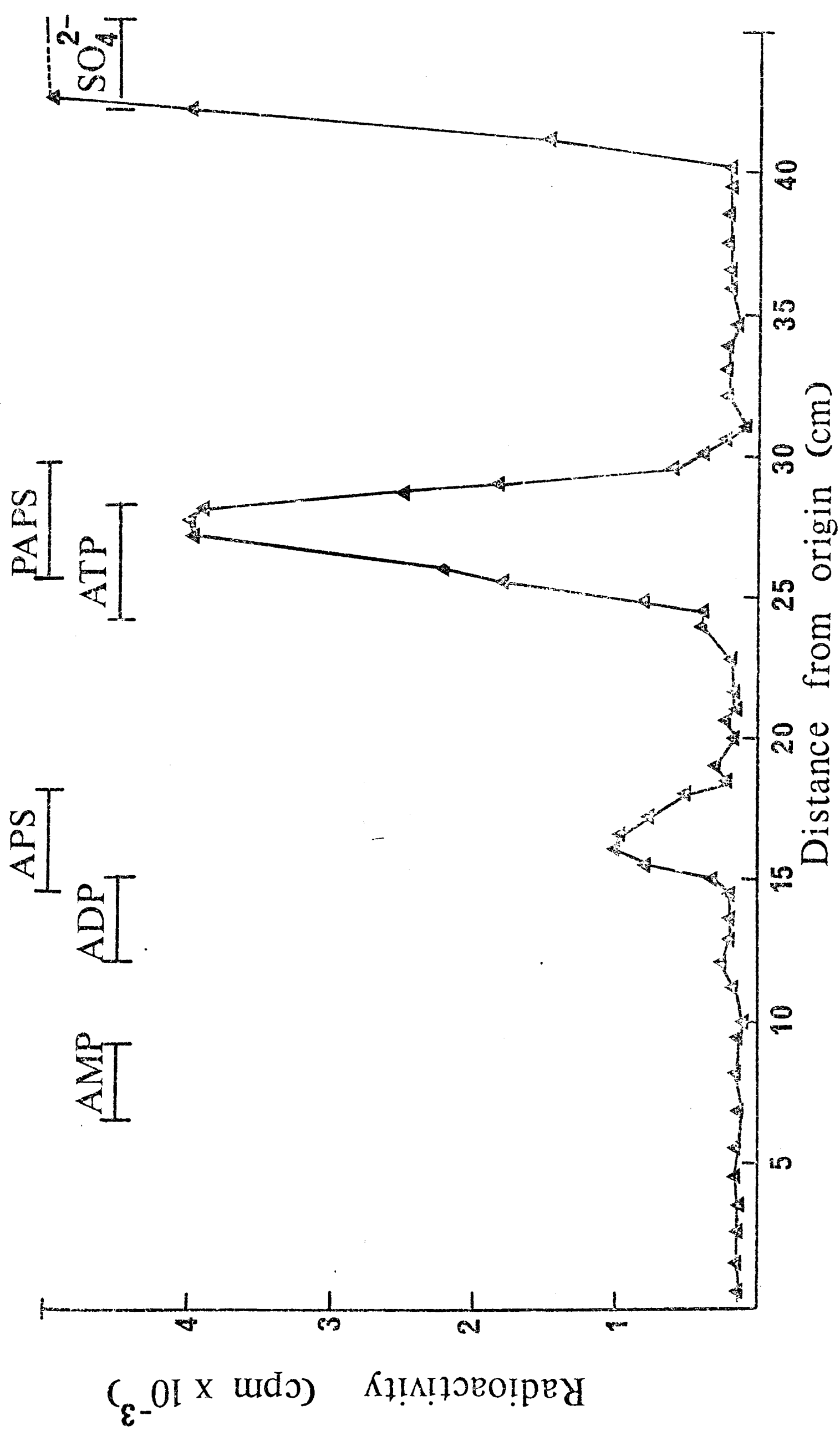


Fig. 8.6

Electrophoretic identification of the products of the coupled enzyme assay.

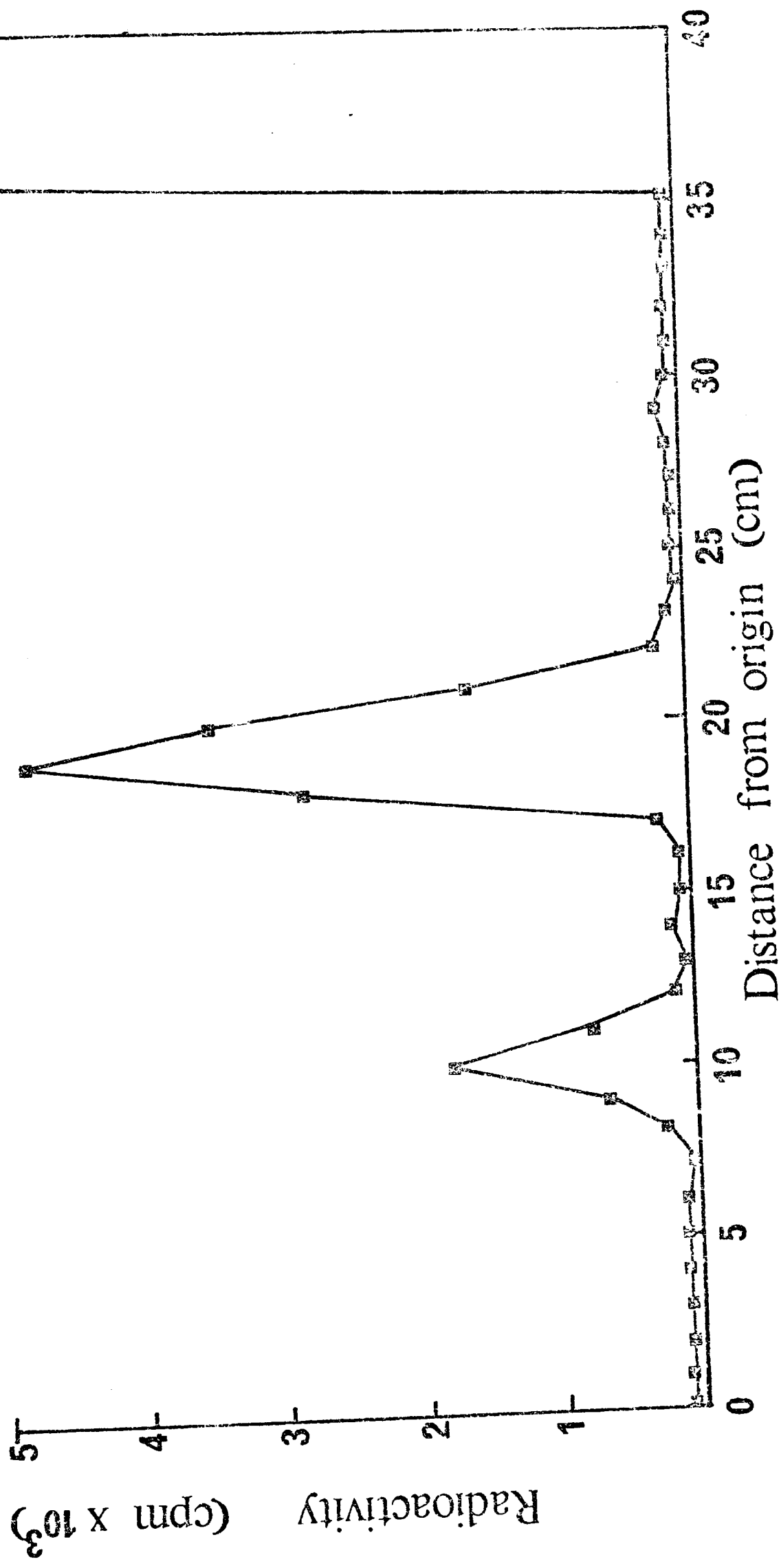
Reaction mixtures were as described in the legend to Fig. 8.5.

Electrophoresis was conducted on acid-washed Whatman 3MM paper, in 0.5M-ammonium acetate buffer, pH 8.5 at 50v/cm for 45 min. Nucleotides and radioactivity were located as described in the legend to

Fig. 8.5.

[³⁵S] sulphate

ADP APS ATP PAPS



exchange chromatography as described in Materials and Methods; contaminating $\gamma\text{-}^{35}\text{S}_7\text{SO}_4^{2-}$ and nucleotides (ATP and ADP) were eluted with 0.5 M-KCl (Fig. 8.7). A ^{35}S -labelled compound coeluted with a compound possessing a u.v. spectrum characteristic of adenosine, when the column was developed with 1M-KCl; the elution pattern was similar to the elution pattern described for PAPS by Hodson & Schiff (1971). Since the ratio of sulphate to adenosine was 1 : 1, as determined from the specific radioactivity $\gamma\text{-}^{35}\text{S}_7\text{SO}_4^{2-}$ and the u.v. absorption (assuming that $\epsilon_{259} = 15.4 \text{ mM}^{-1}$ for PAPS; Roy & Trudinger, 1970) it was concluded that the ^{35}S -labelled compound was PAPS.

(When the ^{35}S -labelled nucleotide eluted from the Dowex ion exchange column was examined by electrophoresis, it ran with an electrophoretic mobility identical to that of PAPS).

Identification of PAPS by hydrolysis using 3'-nucleotidase

Robbins & Lipmann (1957) identified the 3'-phosphate of PAPS with purified rye grass 3'-nucleotidase; 3'-nucleotidase has also been used to determine the position of the 3'-phosphate in Coenzyme A (Wang et al., 1957). (3'-Nucleotidase was purified from spinach leaf tissue as described by Burnell & Anderson, 1973b).

$\gamma\text{-}^{32}\text{P}_7\text{PAPS}$ was synthesised as described in Materials and Methods, purified by Dowex ion exchange chromatography, and incubated with purified 3'-nucleotidase. Synthesis of $\gamma\text{-}^{32}\text{P}_7\text{PAPS}$ from $\alpha\text{-}\beta\text{-}\gamma\text{-}^{32}\text{P}_7\text{ATP}$ and sulphate, theoretically yields $\gamma\text{-}^{32}\text{P}_7\text{PAPS}$. Incubation of $\gamma\text{-}^{32}\text{P}_7\text{PAPS}$ with purified 3'-nucleotidase quantitatively released $\gamma\text{-}^{32}\text{P}_7\text{P}_i$ (Fig. 8.8) thus demonstrating that the $\gamma\text{-}^{32}\text{P}_7\text{P}_i$ was attached to the 3'-position of PAPS.

Fig. 8.7

Elution profile from a Dowex ion exchange column showing the u.v. extinction and the radioactivity of the eluate.

The reaction mixture was the same as described in the legend to Fig. 8.5. The reaction was run for 2 h at 30°C and terminated by the addition of 5 ml of ethanol. The terminated reaction mixture was filtered, evaporated to dryness, the residue dissolved in water, and applied to a Dowex 1-X2 column, 200 - 400 mesh. The column was developed with 0.5M-KCl as indicated by the arrows. The curve shows the A_{259} of the eluate; the radioactivity eluted from the column is indicated (~~graph~~).

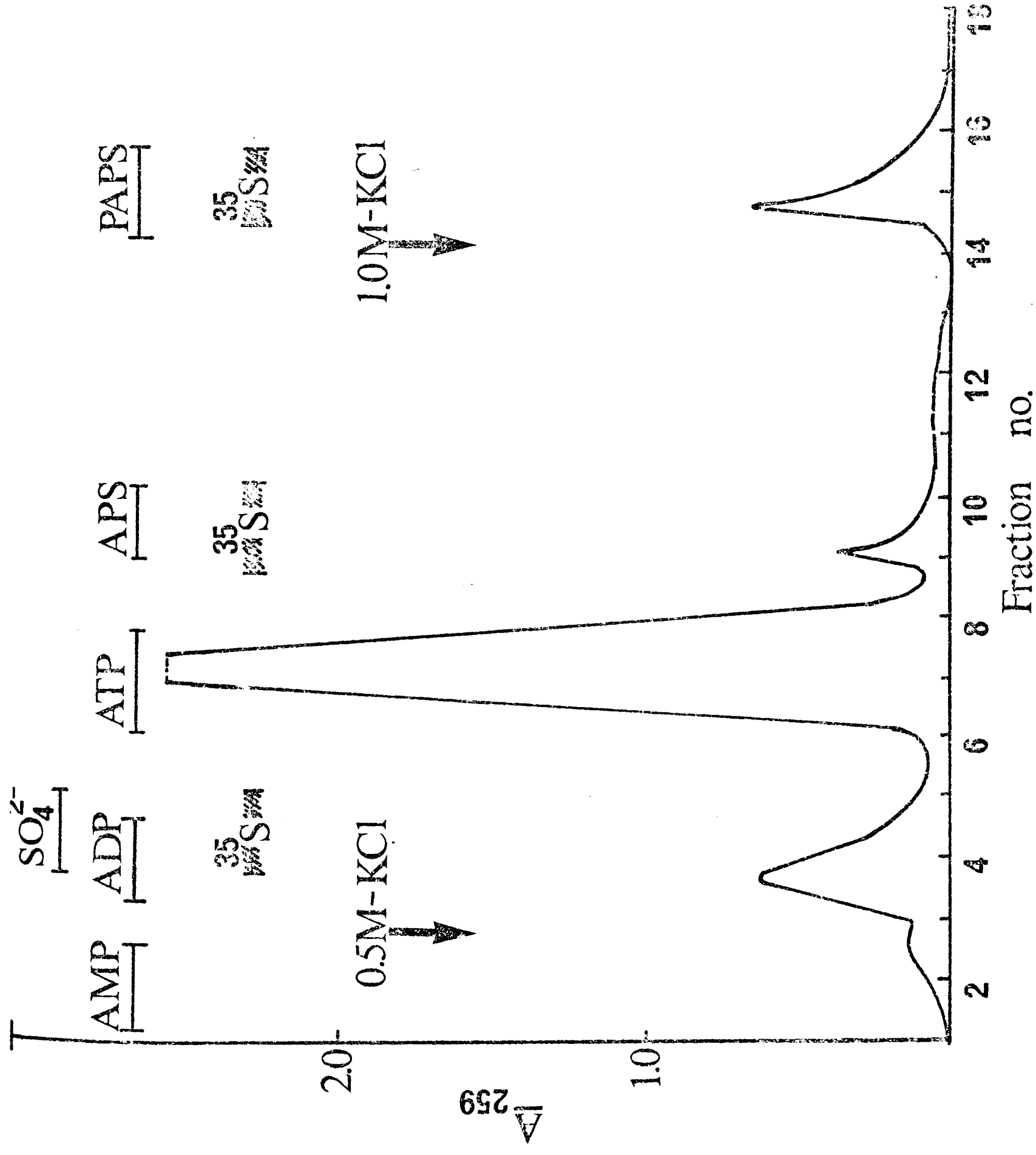
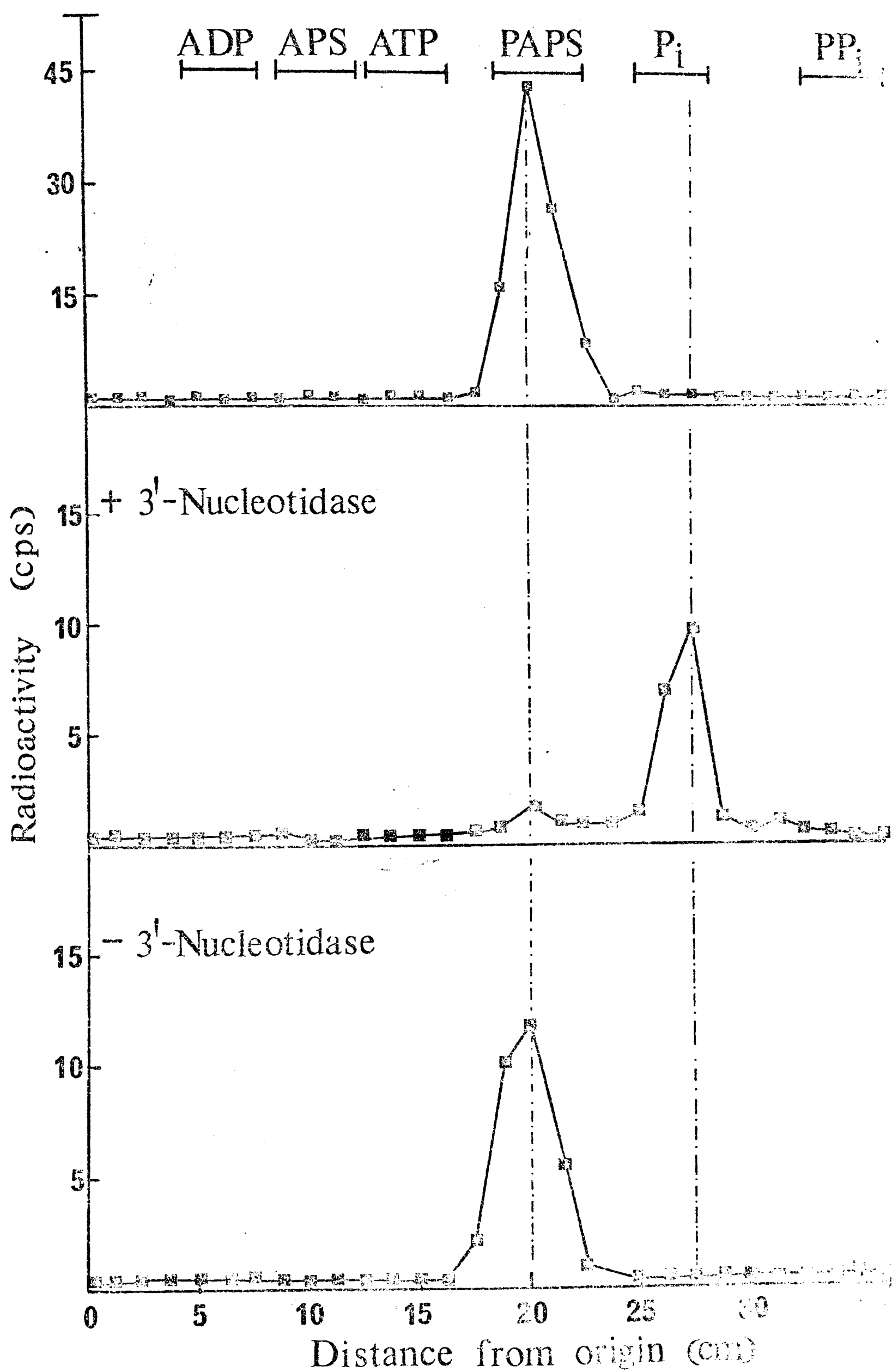


Fig. 8.8

Hydrolysis of γ - ^{32}P -7PAPS by purified 3'-nucleotidase

γ - ^{32}P -7PAPS was synthesised in reaction mixtures containing 20 μmoles MgCl_2 , 40 μmoles Na_2SO_4 , 200 μmoles Tris-HCl buffer, pH 8.0, 10 μmoles γ - ^{32}P -7 ATP, 50 units of purified ATP sulphurylase, 20 units of purified inorganic pyrophosphatase and 40 units of purified APS kinase in a final volume of 1 ml. Reactions were run for 2 h and the reaction terminated and the mixture deproteinised by the addition of 5 ml of ethanol. The supernatant was filtered, evaporated to dryness, the residue dissolved in water, and applied to a column of Dowex 1-2X (Cl^- form), 200-400 mesh. The γ - ^{32}P -7PAPS was eluted free of γ - ^{32}P -7PP_i, γ - ^{32}P -7P_i and γ - ^{32}P -7ATP. The γ - ^{32}P -7PAPS was concentrated by rotary evaporation and divided into three aliquots. One aliquot was treated with 3'-nucleotidase, one was incubated in an identical reaction mixture without 3'-nucleotidase, the third aliquot was used as a control and marker for electrophoresis.



Effect of sulphur source during growth upon the specific activity of APS kinase. Crude extracts were prepared from cells of P. denitrificans grown in the presence of a variety of sulphur sources and the APS kinase activity determined. The specific activity of APS kinase varied with the sulphur source included in the culture media (Table 8.1); cells grown

TABLE 8.1

Effect of sulphur source during growth upon the specific activity of APS kinase in cells of P. denitrificans.

Cells were grown on a variety of sulphur sources and crude extracts prepared and treated as described in Materials and Methods. APS kinase activity was measured by the spectrophotometric assay method.

<u>Sulphur source</u> <u>during growth</u>	<u>APS kinase activity</u> <u>(nmoles/mg of protein/min)</u>
Sulphate	7.62
Sulphite	3.83
Sulphide	0.49
L-Cystine	0.15
Sulphate plus L-homocysteine	7.34
Sulphate plus L-methionine	7.68
Sulphate plus L-cystine	0.13

in the presence of cystine had a very low level of APS kinase activity whilst APS kinase activity in cells grown in culture medium containing either homocysteine or methionine (in the presence of sulphate), was approximately the same as for cells grown on sulphate alone. APS kinase activity was significantly

less in cells grown in the presence of sulphite or sulphide as compared with cells grown in the presence of sulphate; this may have been due to repression of APS kinase activity by either sulphite or sulphide directly, or by cysteine, after conversion of sulphite and sulphide to cysteine.

The repression of APS kinase activity by growth on a number of different sulphur sources corresponds closely with the repression of ATP sulphurylase activity (c.f. Table 6.2).

* A note regarding APS kinase activity, PAPS synthesis and sulphate reduction. Schiff and his co-workers (Tsang et al., 1971; Hodson & Schiff, 1971) found an enzyme in Chlorella which converts PAPS to APS. APS rather than PAPS, appears to be the substrate for reduction in Chlorella (Schmidt, 1972; Tsang et al., 1971; Schmidt et al., 1974). This enzyme, called fraction "A" because it could be adsorbed to alumina C_g gel (and not to be confused with enzyme fraction A from yeast), is a 3'-nucleotidase which is different from the widely distributed enzyme described by Shuster & Kaplan (3'-nucleotide phosphohydrolase: EC 3.1.3.6.) (Shuster & Kaplan, 1955), from a variety of tissues and purified by them from rye grass. The rye grass enzyme will cleave 3'-nucleotides to yield inorganic phosphate without regard to substituents in the 5'-position. Fraction "A" will only cleave the 3'-phosphate efficiently from nucleotides containing, as far as is known, a phosphate in the 5'-position (Tsang et al., 1971). Unlike the Shuster & Kaplan enzyme, fraction "A" will not act on 3'-AMP very effectively, but like this fraction "A" will cleave the 3'-phosphate from PAPS, coenzyme A, and PAP. Its K_m for PAPS is in the vicinity of 10^{-4}M (Schiff & Hodson, 1973).

Although E. coli possesses a 3', 5'-diphosphonucleotide 3'-phosphohydrolase which converts PAPS to APS; PAPS is the substrate for the sulphate reducing pathway.

Crude extracts of P. denitrificans were examined for both 3'-nucleotidase and fraction "A" activities in reaction mixtures containing: 100 μ moles of Tris-HCl buffer, pH 8.0, 20 μ moles $MgCl_2$, crude enzyme extract (0.1 to 10 mg of protein/ml) and substrate (either 3'-AMP - 1 to 50 μ moles; Coenzyme A - 1 to 20 μ moles or PAPS - 1 to 5 μ moles) in a total volume of 1 ml. Neither 3'-nucleotidase nor fraction "A" activity was detected.

Since P. denitrificans does not contain an active 3'-nucleotidase or an active fraction "A" enzyme, the substrate for reduction is more likely to be PAPS, rather than APS.

SUMMARY

A new method for the determination of APS kinase activity using a spectrophotometric assay, is described. This procedure involves the spectrophotometric determination of sulphate- or APS- dependent production of ADP in the presence of pyruvate kinase and lactate dehydrogenase. Methods are described that overcome interference from contaminating enzymes and compounds. This method also provides a means for a critical examination of the substrate specificity of the sulphate-activating enzymes.

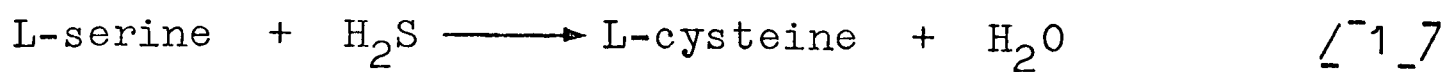
APS kinase activity was demonstrated in both crude and purified enzyme extracts. PAPS, the end-product of the reaction catalysed by APS kinase, was synthesised from sulphate and ATP in the presence of purified ATP sulphurylase, inorganic pyrophosphatase and APS kinase, and was identified as PAPS by a variety of methods. PAPS appears to be the substrate for sulphate reduction in P. denitrificans.

CHAPTER 9

CYSTEINE SYNTHESIS

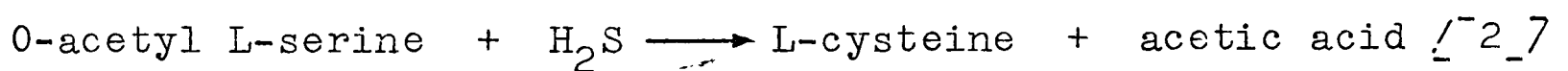
INTRODUCTION

For many years it was thought that cysteine synthesis in microorganisms involved the pyridoxal phosphate - dependent enzyme, serine sulphydrase, which catalyses the sulphydrylation of serine according to reaction [1];



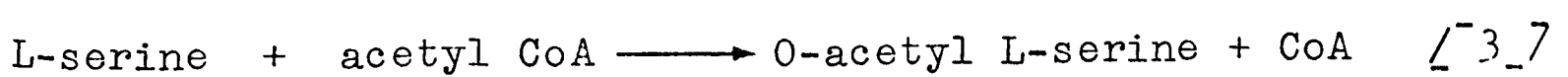
Although serine sulphydrase activity has been demonstrated in a number of organisms (Dreyfuss & Monty, 1963; Bruggemann *et al.*, 1962; Schlossman & Lynen, 1957; Schlossmann *et al.*, 1962; Thompson & Moore, 1968; Wiebers & Garner, 1963, 1967; Leinweber & Monty, 1965; Pasternak *et al.*, 1965; Pieniazek *et al.*, 1973) it now seems that serine sulphydrase does not play a major role in the total synthesis of cysteine.

An alternative mechanism for the synthesis of cysteine involving the sulphydrylation of O-acetyl serine was first reported by Kredich & Tomkins (1966). This reaction is catalysed by the enzyme O-acetyl serine sulphydrylase according to reaction [2];



Subsequently, O-acetyl serine sulphydrylase has been reported in a variety of organisms (Kredich & Tomkins, 1966; Jones-Mortimer *et al.*, 1968; Chambers & Trudinger, 1971; Wiebers & Garner, 1967; Thompson & Moore, 1968; Giovanelli & Mudd, 1968).

The synthesis of O-acetyl serine from serine and acetyl CoA is catalysed by serine transacetylase according to reaction [3];



Serine transacetylase activity has been demonstrated in a variety of organisms (Kredich & Tomkins, 1966; Becker et al., 1969; Kredich et al., 1969).

This chapter describes the presence and purification of serine transacetylase and O-acetyl serine sulphydrylase activities in P. denitrificans. The enzymes were purified free from each other and their kinetics, control and regulation is reported. In addition, the synthesis of selenocysteine from O-acetyl serine and selenide, catalysed by purified O-acetyl serine sulphydrylase, is reported.

Finally, this chapter describes the presence of O-acetyl serine lyase activity in crude extracts of P. denitrificans; this is the first report of this enzyme in bacteria.

MATERIALS AND METHODS

Chemicals. O-Acetyl L-serine was obtained from Calbiochem. Ltd., London, U.K; acetyl CoA was a gift from C.F. Boehringer und Soehne, G.m.b.H., Mannheim, Germany; DL-selenocysteine, 5,5'-dithiobis (2-nitrobenzoic acid), Coenzyme A and 1,2,4-triazole were obtained from the Sigma Chemical Co., London, U.K.; triethylamine and aluminium selenide from British Drug Houses, Dorset, U.K.; L-[3-¹⁴C] serine from the Radiochemical Centre, Amersham, Bucks., U.K., and silica gel TLC plates (20 x 20 cm, type 6060 with fluorescent indicator) from Eastman Kodak Co., Rochester, N.Y.

O-Acetyl homoserine and O-acetyl L-threonine were chemically synthesised by the method of Nagai & Flavin (1967). O-Acetyl L-serine and O-acetyl D-serine were synthesised by the method of Sheehan et al., (1965) and their purity examined by gas-liquid chromatography. N-Acetyl L-serine was prepared by incubation of O-acetyl L-serine at pH 8.0 for 1 h. Under this

treatment the L-configuration of serine is conserved (Kredich & Tomkins, 1966). Other chemicals were obtained from the sources stated in previous chapters.

Assay for serine transacetylase. Two assays were used to determine serine transacetylase activity.

Method /⁻1_7. The first method was adapted from the procedure of Alpers et al., (1965) for the assay of thiogalactoside transacetylase. It is based on a disulphide exchange between the Coenzyme A, liberated from O-acetyl Coenzyme A during the reaction, and dithiobis (2-nitrobenzoic acid) (DTNB). The production of thionitrobenzoic acid is followed spectrophotometrically at 412 nm. Reactions were conducted in cuvettes (1 cm light path) at 30°C in a final volume of 1.0 ml which contained 100 µmoles Tris-HCl buffer, pH 7.6, 1.0 µmoles DTNB, 1 µmole EDTA, 2 µmole acetyl Coenzyme A, 2.5 µmoles L-serine and an appropriate amount of enzyme. The reaction was initiated by the addition of L-serine and the initial rate of reaction was followed at 412 nm in a Pye Unicam SP 8000 spectrophotometer linked to an AR 25 linear recorder.

Method /⁻2_7. When serine transacetylase activity was studied in the presence of compounds containing free sulphydryl groups, another assay was required because of the reactivity of DTNB with many thiols. This second assay method is based on the rate of loss of absorbance at 232 nm, followed spectrophotometrically, which occurs upon cleavage of the thioester bond of acetyl Coenzyme A (Stadtman, 1953). The reaction mixtures were identical with those in Method /⁻1_7 except for the omission of DTNB. Both assays gave identical rates indicating that DTNB had no effect on the reaction.

All determinations of serine transacetylase activity were done by Method [1] unless thiols were added to the reaction mixtures, in which case Method [2] was used.

Assay of O-acetyl serine sulphydrylase activity. O-Acetyl serine sulphydrylase activity was determined by a modification of the procedure of Kredich & Tomkins (1966). Assays were conducted in capped, plastic minicentrifuge tubes. Reaction mixtures at pH 7.5, contained 10 μ moles O-acetyl L-serine, 10 μ moles Na_2S , 100 μ moles Tris, HCl, 10 μ moles pyridoxal phosphate, 5 μ moles dithiothreitol in a final volume of 0.9 ml. (Dithiothreitol was added to prevent oxidation of cysteine). Reaction mixtures were incubated at 30°C for 2 min to allow temperature equilibration. Reactions were initiated by the addition of 0.1 ml of enzyme and terminated, after 15 min, by the addition of 0.2 ml of 7.5% (w/v) trichloroacetic acid. When crude extracts were used as a source of enzyme, the protein precipitated by the addition of trichloroacetic acid was removed by centrifugation. Cysteine was estimated by a modification of the method of Gaitonde (1967). A suitable sample (0.5 ml) of the supernatant was added to 0.5 ml glacial acetic acid and 0.5 ml acid ninhydrin (Gaitonde's reagent 2). The solution was thoroughly mixed, heated in an oil bath at 100°C for 10 min and cooled in ice. Solutions were made up to 5 ml with 95% (v/v) ethanol and the absorbance at 560 nm read against a reagent blank. The standard curve for the estimation of cysteine (Fig. 9.1) was not based on weighed portions of cysteine, since commercial preparations of cysteine are never 100% free cysteine because of its oxidation to cystine. Instead, determinations of free cysteine concentrations in standard mixtures were performed using Ellman's

method (1958). Samples (0.2 ml), were mixed with 2.8 ml of 0.1 mM-DTNB solution in 0.1 M-Tris-HCl buffer, pH 7.6. The concentration of free cysteine was calculated from the molar extinction coefficient of Ellman's reagent (ϵ_m at 412 nm = 13,600).

Assay of selenocysteine. Selenocysteine was measured by the method described for the determination of cysteine. DL-Selenocystine, obtained from commercial sources, was reduced to selenocysteine by incubation with excess dithiothreitol at pH 8.0 in Tris-HCl buffer. The sensitivity of the method for determining selenocysteine was approximately 15% that for cysteine, but the A_{560} was directly proportional to selenocysteine concentration (Fig. 9.1) and could therefore be readily used provided no cysteine was present.

Preparation of Gaitonde's acid ninhydrin reagent 2. 500 mg of ninhydrin was shaken in a mixture of 12 ml glacial acetic acid and 8 ml conc. HCl, at room temperature until it dissolved (in 20 to 30 min). This reagent was prepared fresh daily.

Assay of O-acetyl serine lyase. O-Acetyl serine lyase activity was followed by measuring the release of pyruvate from O-acetyl serine as described by Mazelis & Fowden (1972). Reactions were conducted at 30°C in Eppendorf minicentrifuge tubes. Reaction mixtures contained 200 μ moles O-acetyl serine, 100 μ moles Tris-HCl buffer, pH 7.5, 10 nmoles pyridoxal phosphate, and enzyme in a total volume of 1 ml. Reactions were started by the addition of enzyme and stopped, after 30 min, by adding 0.2 ml 7.5% (w/v) trichloroacetic acid. Precipitated protein was removed by centrifugation and 0.5 ml of the supernatant was assayed for pyruvate using 2,4-dinitroph-

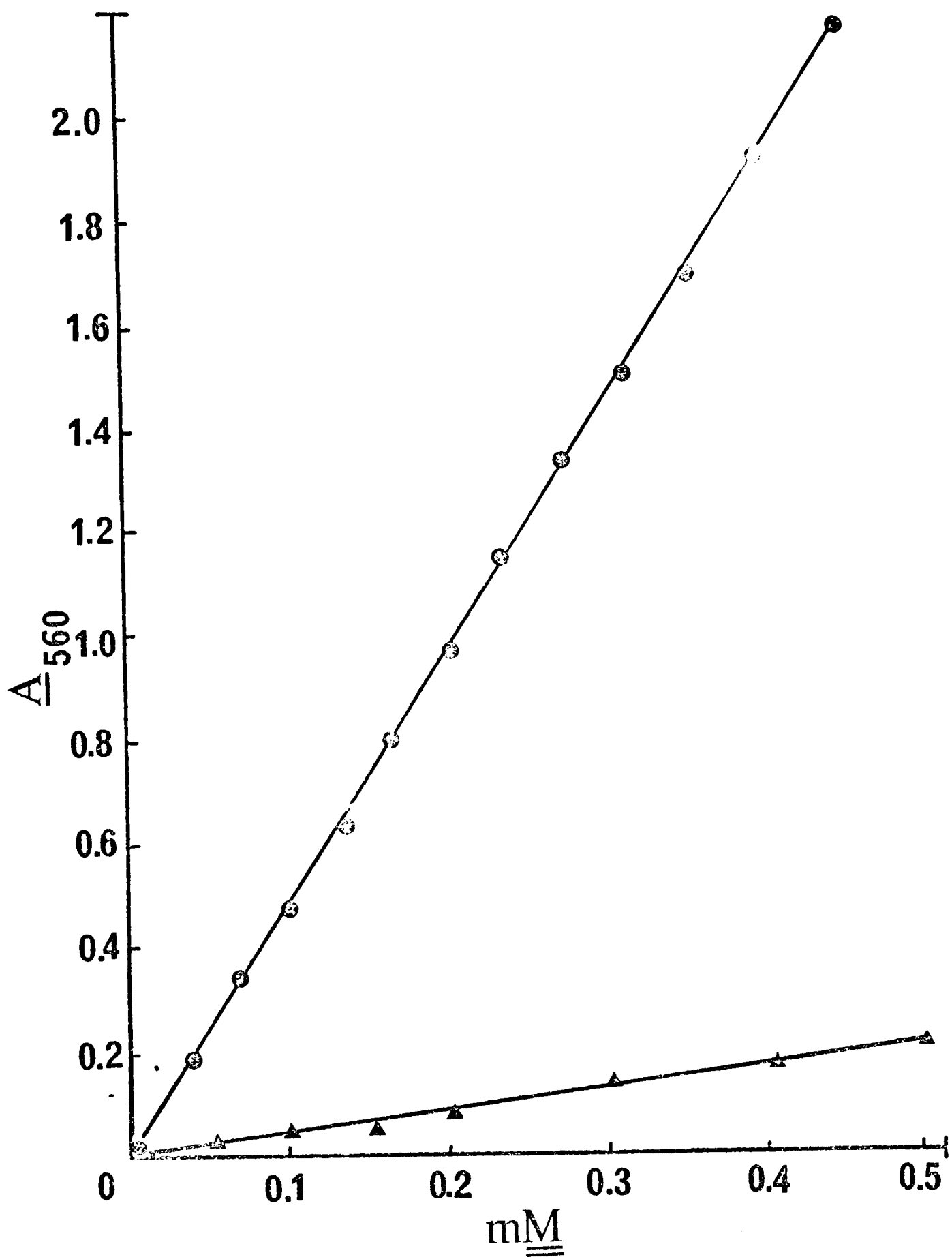


Fig. 9.1

Standard curve for L-cysteine and L-selenocysteine..
 L-Cysteine and L-selenocysteine were assayed, in the presence of 5 mM-dithiothreitol, by the ninhydrin method of Gaitonde (1967) as described in Materials and Methods;
 ●, L-cysteine; ▲, L-selenocysteine.

enylhydrazine as described in Chapter 10.

Gas-liquid chromatographic determination of the purity of chemically synthesised O-acetyl serine. GLC determinations of the purity of O-acetyl serine were conducted in a Pye Series 104 Gas Chromatogram linked to a Servoscribe type RE 541.20 linear recorder. A glass column (1.5 m x 4 mm I.D.) packed with OV 17 (5%, w/w) on Gas Chrom Q, 100 - 120 mesh, was used. Oxygen free nitrogen, at a flow rate of 40 ml/min, was used as the carrier gas. The detector oven was set at 250°C and the injector port setting at 4. A temperature program of 5 min hold at 110°C with a temperature gradient of 3°C/min to 150°C was used. O-Acetyl serine (1 mg) was derivatised in 0.2 ml of a 50/50 (v/v) mixture of acetonitrile and bis (trimethyl silyl) trifluoroacetamide (BSTFA) at 100°C for 10 min. Internal standards (0.1 µl of a 50/50, v/v, mixture of both dodecane and hexadecane) were injected together with 0.3 µl of the derivatised sample and 0.1 µl of the ethyl acetate (Fig. 9.2). The relative response of the flame ionisation detector to O-acetyl serine and serine was 1 and 2.17, respectively.

Thin layer chromatography. Thin layer chromatography was conducted in plates (20 x 10 cm) of silica gel (with fluorescent indicator) and developed with chloroform-ethanol-glacial acetic acid-water; 50 : 32 : 10 : 8, by vol. When cysteine was analysed by thin layer chromatography, the solution containing cysteine was treated with an excess of N-ethyl maleimide for 10 min at pH 6.8 prior to its application to the chromatogram. The cysteine-NEM derivative was thus protected from thiol oxidation and ran as a single spot separate from the remaining compounds examined in this study (Table 9.1).

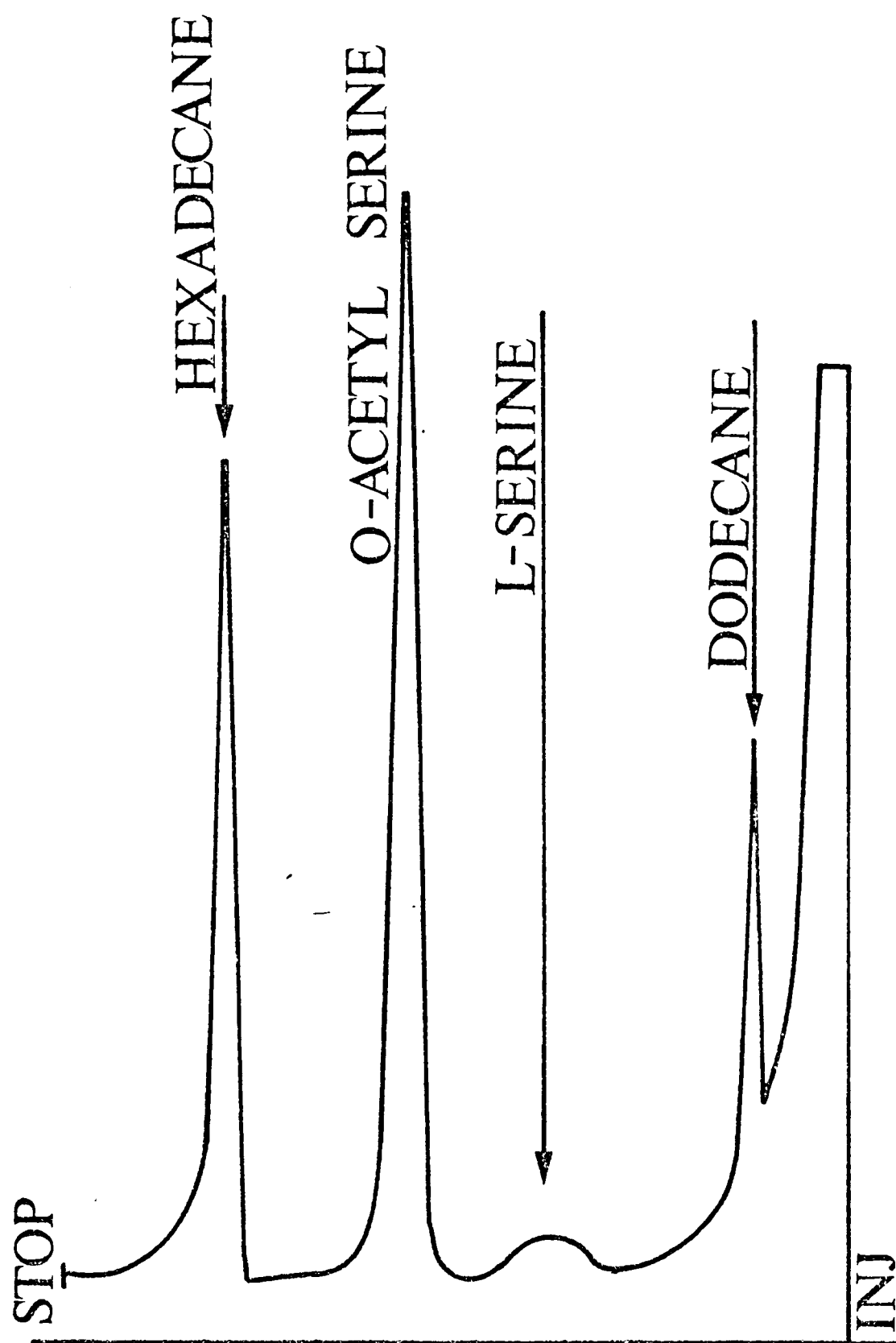


Fig. 9.2

Examination of the purity of the chemically synthesised O-acetyl L-serine.

O-Acetyl L-serine was synthesised as described in Materials and Methods. O-Acetyl L-serine (1 mg) was derivatised in a mixture 50/50 (v/v) of BSTFA/acetonitrile and examined by gas-liquid chromatography as described in Materials and Methods.

Compounds were located with ninhydrin spraying or by u.v. fluorescence quenching.

TABLE 9.1

Thin layer chromatography

Thin layer chromatograms of silica gel were spotted with 0.1 - 1.0 μ moles of compound and developed in chloroform-ethanol-glacial acetic acid-water; 50:32:10:8, by vol. After drying compounds were detected with ninhydrin and u.v. fluorescence quenching.

<u>Compound</u>	<u>R_f value</u>
L-Serine	0.21
O-Acetyl L-serine	0.38
N-Acetyl L-serine	0.69
L-Cysteine	0.41
L-Cysteine-NEM	0.64

Purification of serine transacetylase and O-acetyl serine sulphydrylase. Both serine transacetylase and O-acetyl serine sulphydrylase were purified from P. denitrificans cells grown on succinate as substrate, nitrate as the terminal electron acceptor and sulphate as the sole sulphur source. All steps of the purification were conducted at 0 - 4°C and the standard buffer used throughout, unless stated otherwise, contained 20 mM-Tris HCl, pH 7.5 and 1mM-dithiothreitol.

Purification of serine transacetylase.

Step 1 Crude extract. Sixteen litres of culture medium containing log phase cells were harvested, the cells were washed twice with buffer, resuspended in 160 ml of buffer and frozen. The frozen cell suspension was thawed and degassed for 10 min

in vacuo. Cells were sonically disrupted for 10 min (30 sec burst at 1 min intervals) at maximum output, and the resultant suspension centrifuged at 9 000g to remove whole cells. The supernatant was then centrifuged for 60 min at 30 000g and the supernatant decanted (Fraction I).

Step 2 Ammonium sulphate fractionation. Ammonium sulphate was added slowly to Fraction I to 50% saturation with stirring, and stirring was continued for 20 min after all the ammonium sulphate was added. The suspension was centrifuged at 30 000g for 20 min. The precipitate was dissolved in a minimal volume of buffer and dialysed against 5 litres of buffer for 12 h (Fraction II).

Step 3 Sephadex G-200 gel filtration. Fraction II was applied to a Sephadex G-200 column (3.5 x 75 cm) equilibrated with buffer and the enzyme was eluted at a flow rate of 0.45 ml/min. Fractions (5 ml) were collected, assayed for O-acetyl serine sulphydrylase, serine deaminase, and serine transacetylase activities, and those fractions containing serine transacetylase and no O-acetyl serine sulphydrylase activity were pooled (Fraction III) (see Fig. 9.3).

Step 4 Hydroxylapatite column chromatography. Fraction III was applied to a column of hydroxylapatite (15 x 1.5 cm) equilibrated with buffer. The column was washed with 20 ml of buffer and the enzyme eluted with 500 ml of a linear KCl gradient dissolved in buffer (0 to 0.45 M-KCl) at a flow rate of 0.5 ml/min. Fractions (5 ml) were collected and assayed. Those fractions containing serine transacetylase activity were pooled and dialysed against 10 litres of buffer for 12 h (Fraction IV).

Step 5 Concentration step. Fraction IV was concentrated by

dialysing against a saturated solution of polyethylene glycol, Fraction IV was thereby reduced in volume from 55 ml to 6 ml.

Purification of O-acetyl serine sulphydrylase.

Step 1 Crude extract. Crude extract (Fraction I) was prepared as described for the purification of serine transacetylase.

Step 2 Ammonium sulphate fractionation. Solid ammonium sulphate was added to Fraction I to 35% saturation with stirring. The suspension was stirred for 20 min. After centrifuging the suspension at 30 000g for 20 min the supernatant was brought to 70% saturation with ammonium sulphate. The suspension was stirred for 20 min and then centrifuged for 20 min at 30 000g. The supernatant was discarded and the precipitate was dissolved in a minimal volume of buffer. This solution was dialysed against 8 l of buffer for 10 h (Fraction II).

Step 3 Sephadex G-200 Gel filtration. Fraction II was applied to a column of Sephadex G-200 (75 x 3.5 cm) equilibrated with buffer; the enzyme was eluted at a flow rate of 0.45 ml/min with buffer. Fractions of 5 ml were collected and assayed for O-acetyl serine sulphydrylase activity, and those fractions containing the highest specific activity of O-acetyl serine sulphydrylase were pooled (Fraction III).

Step 4 DEAE-cellulose column chromatography. Fraction III was applied to a DEAE-52 cellulose column (8.5 x 3.0 cm) equilibrated with buffer. The column was washed with 20 ml of buffer and then with 0.2 M-KCl dissolved in buffer. The enzyme was eluted with 500 ml of a linear gradient of 0.2 M- to 0.45 M-KCl dissolved in buffer, at a flow rate of 0.55 ml/min.

Fractions of 5 ml were collected and assayed and those fractions with the highest specific activity of O-acetyl serine sulphydrylase were pooled (Fraction IV) and dialysed against 8 l of buffer for 12 h.

Step 5 DEAE-cellulose concentration step. Fraction IV was applied to a second DEAE-cellulose column (5 x 1 cm) and the column washed with 10 ml of 0.2 M-KCl (dissolved in buffer). After dialysing the concentrated enzyme against 4 l of buffer for 12 h, this preparation was stored at -15°C.

RESULTS

In initial experiments conducted to determine the parameters of an assay system for O-acetyl serine sulphydrylase using crude extract as a source of enzyme, O-acetyl serine sulphydrylase activity was found not to be linear with time or protein concentration. Upon investigation, it was found that O-acetyl serine was being removed from reaction mixtures because of the activity of O-acetyl serine lyase, an enzyme found previously only in plants. Mazelis & Fowden (1972) described the occurrence of O-acetyl serine lyase in crude extracts of higher plants; the enzyme cleaves O-acetyl serine by β -elimination into pyruvic acid, ammonia and acetate.

A brief study of O-acetyl serine lyase, present in crude extracts of P. denitrificans, demonstrated that the bacterial enzyme resembled the plant enzyme in many of its properties. The enzyme was heat sensitive; pretreatment at 80°C for 3 min totally destroyed activity. The enzyme was quite specific for O-acetyl serine; L-serine, N-acetyl L-serine, O-acetyl homoserine, and O-acetyl threonine could not act as substrates for the enzyme. The enzyme had a very high K_m (O-acetyl serine); a number of experiments gave a K_m of 50 - 100 mM.

The enzyme was probably a pyridoxal-phosphate-requiring enzyme being slightly stimulated by the addition of pyridoxal phosphate and strongly inhibited by the addition of hydroxylamine and KCN to reaction mixtures. Since this enzyme removed O-acetyl serine from reaction mixtures used to measure O-acetyl serine sulphydrylase activity, it was necessary to remove O-acetyl serine lyase from enzyme preparations prior to investigating the kinetic and regulatory properties of O-acetyl serine sulphydrylase.

Similarly, serine deaminase activity, present in crude extracts of P. denitrificans, had to be removed from enzyme preparations used to investigate the properties of serine transacetylase activity, since serine deaminase rapidly removed serine, one of the substrates of the enzyme, from the reaction mixtures.

So, O-acetyl serine sulphydrylase and serine transacetylase were purified, not only to separate the two activities, but also to remove O-acetyl serine lyase and serine deaminase activities, enzymes which catalysed the removal of substrates of O-acetyl serine sulphydrylase and serine transacetylase - catalysed reactions.

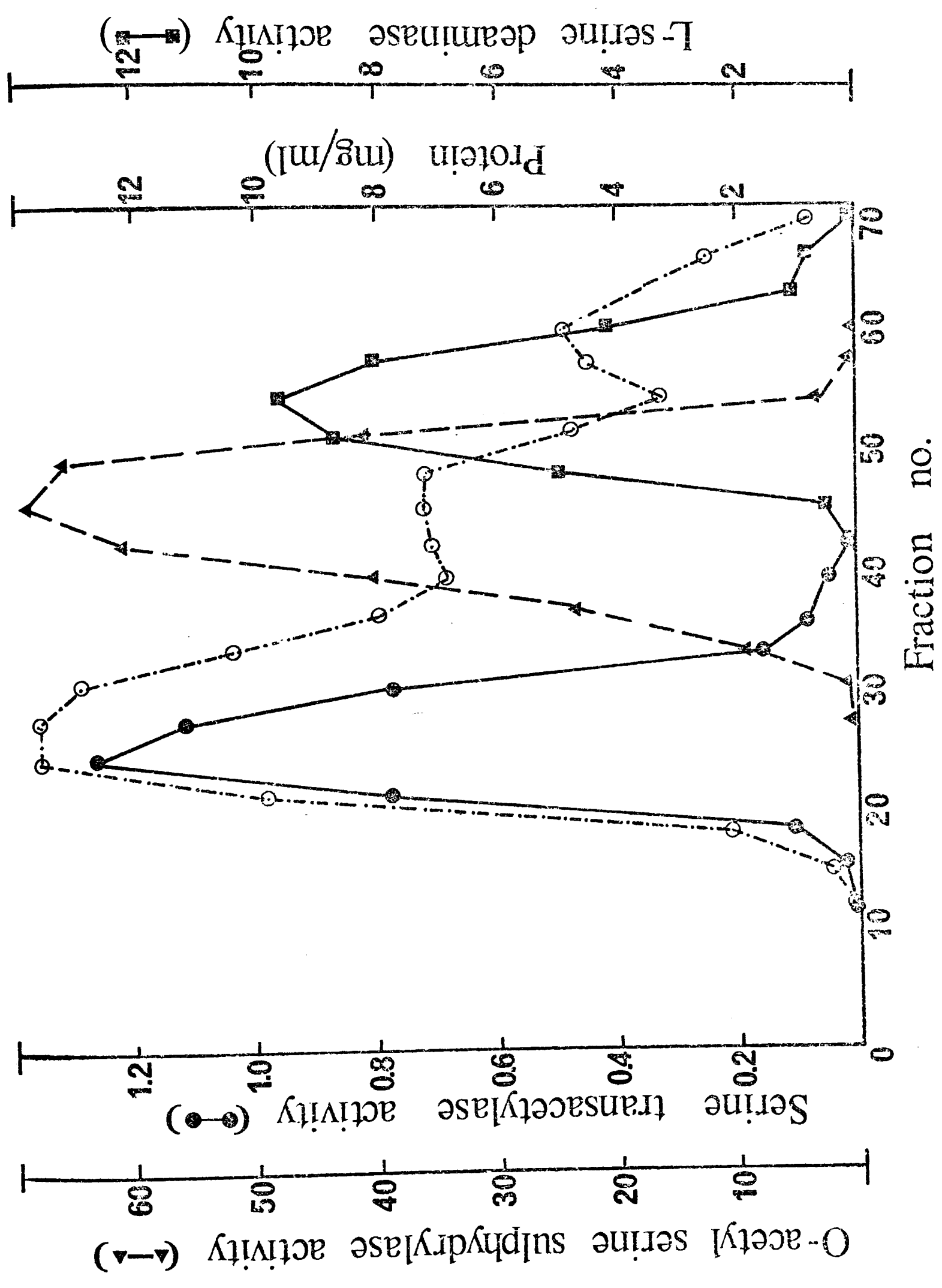
Purification of serine transacetylase. Serine transacetylase activity was separated from O-acetyl serine lyase, O-acetyl serine sulphydrylase and serine deaminase activities during the four step purification procedure. Separation of the enzyme activities on Sephadex G-200 is shown in Fig. 9.3. During the purification procedure serine transacetylase was purified approximately 150-fold.

Identification of the product of serine transacetylase activity. To determine the product of the reaction catalysed

Fig. 9.3

Separation of serine transacetylase, O-acetyl serine sulphydrylase and serine deaminase activities.

The activities were separated by gel filtration on Sephadex G-200 after concentration of crude enzyme by 0 to 80% ammonium sulphate fractionation. Serine transacetylase activity was assayed by Method 2. All other conditions of the assays of the three enzymes were as described in Materials and Methods. (0), protein; ● serine transacetylase; ▲, O-acetyl serine sulphydrylase, ■, L-serine deaminase.



by purified serine transacetylase, serine was replaced by ^{14}C -labelled serine, and the end products of the reaction examined by thin layer chromatography. The results are shown in Fig. 9.4. Both O-acetyl serine and N-acetyl serine were detected. It was found, however, that O-acetyl serine is chemically converted to N-acetyl serine at pH values above 7.6, whereas N-acetyl serine is quite stable under the same conditions (Flavin & Slaughter, 1965). Since there is much more O-acetyl serine than N-acetyl serine (Fig. 9.4), and since O-acetyl serine can only be converted to the N-acetyl form and not vice versa, it was concluded that the physiological product of the reaction was O-acetyl serine. The end-product was further identified by its ability to function as substrate for purified O-acetyl serine sulphydrylase, whereas N-acetyl serine can not.

Linearity of the assay. Serine transacetylase activity, as measured by both Method 1 and 2, was found to be linear with time (0 to 10 min) and protein concentration (0.01 to 0.68 mg of protein). The linearity of the time course experiments indicate that the enzyme is not inhibited by the end-product, O-acetyl serine.

Effect of L-serine concentration. The rate of Coenzyme A release from acetyl CoA, as measured by Method 1, increased with concentration up to 2mM . Increasing the concentration of serine above 2.5mM caused inhibition. Michealis-Menten kinetics were observed up to a concentration of 2.0mM -serine. The apparent K_m for L-serine is $4.0 \times 10^{-4}\text{M}$ (Fig. 9.5)

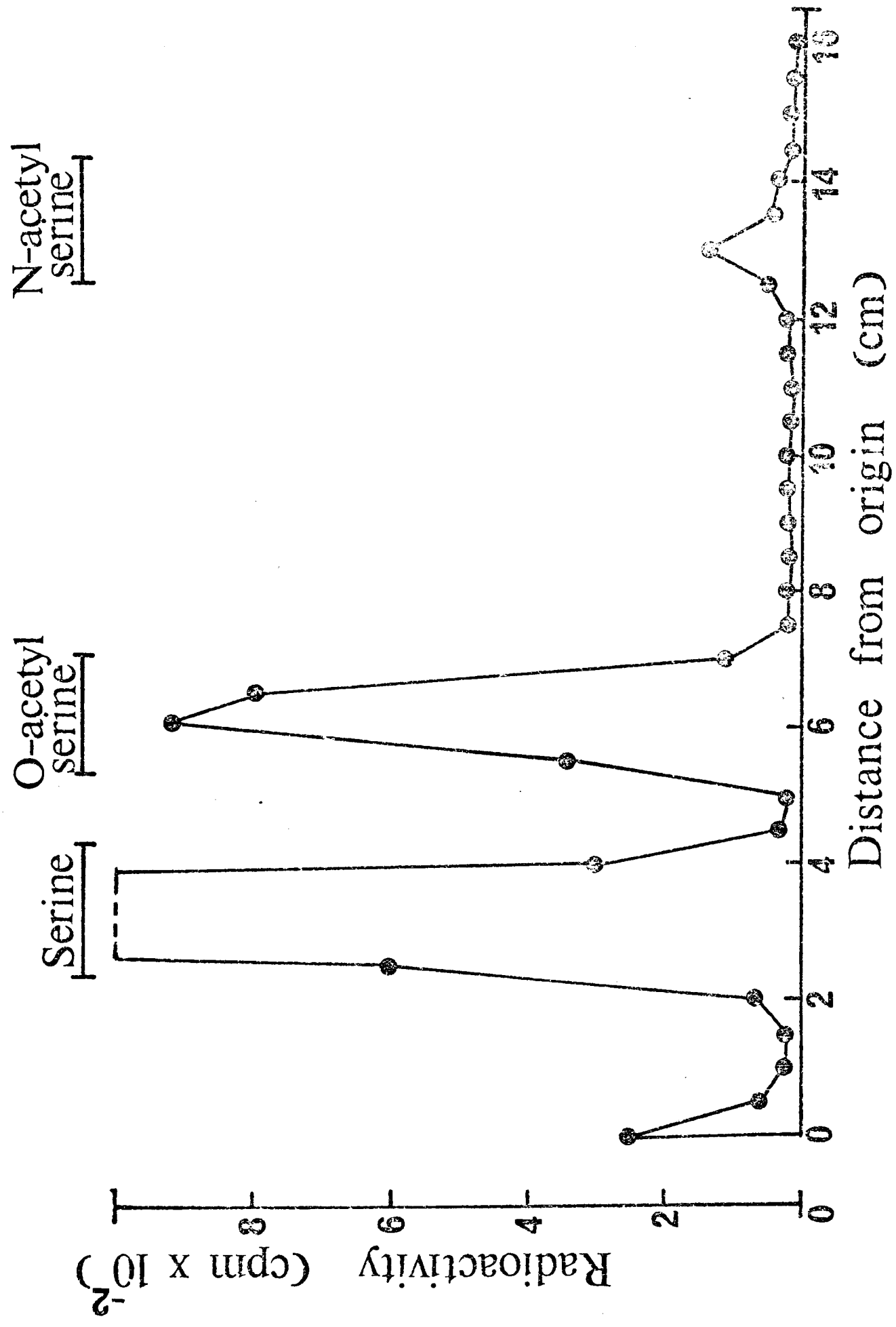
Effect of acetyl CoA concentration. A double reciprocal plot of the effect of increasing acetyl CoA concentration upon the rate of Coenzyme A release showed Michealis-Menten

Fig. 9.4

Identification of the product of the serine transacetylase -catalysed reaction.

Reaction mixtures were as described in Materials and Methods except that ^{14}C -labelled L-serine was included, and DTNB was omitted.

Aliquots of the reaction mixture were spotted onto thin layer plates of silica gel, developed, dried, and the radioactivity of the plates counted.



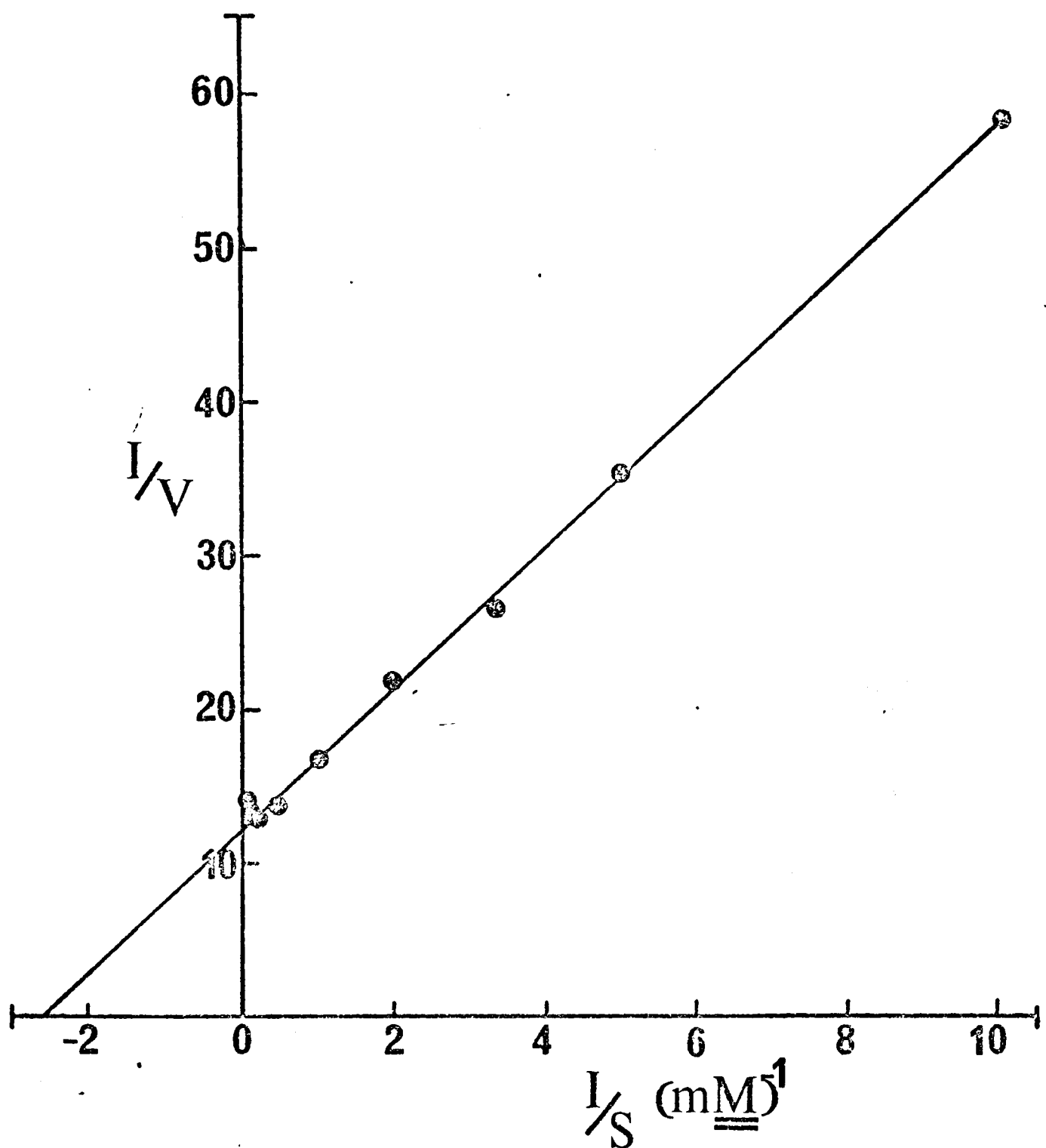


Fig. 9.5

Double reciprocal plot of the effect of concentration of L-serine on serine transacetylase activity.

The reaction mixtures contained 2.0mM-acetyl CoA and the reaction was monitored by Method 1.

The apparent K_m for L-serine is 4×10^{-4} .

kinetics with an apparent K_m of $1 \times 10^{-4} M$ (Fig. 9.6).

Effect of pH. Serine transacetylase has a pH optimum between 7.5 and 8.0. (The effect of pH upon cysteine synthesis is discussed later in the chapter). However, due to the chemical properties of one of the products, O-acetylserine, the pH of the reaction mixtures was maintained at pH 7.5.

Whilst studying the effect of pH upon serine transacetylase activity it was observed that the enzyme was inhibited by salt concentrations above $0.25 M$.

Substrate specificity. Serine transacetylase was apparently specific for L-serine and did not catalyse the acetylation of homoserine, threonine, ethanolamine, taurine, or D-serine. The enzyme did not catalyse the exchange of the acetyl group from O-acetylserine or O-acetylhomoserine to serine or homoserine, respectively. By contrast, N. crassa homoserine transacetylase catalysed an exchange between homoserine and O-acetylhomoserine (Nagai & Flavin, 1967).

Effect of inhibitors. The effect of various compounds on serine transacetylase activity is shown in Tables 9.2 and 9.3. The enzyme was inhibited by p-chloromercuri-benzoate, N-ethylmaleimide and iodacetamide, components known to react with sulphhydryl groups. The enzyme was inhibited by hydroxylamine; this may be due to a lowering of the acetyl CoA concentration by the formation of the hydroxamate.

Since serine transacetylase appears to be involved in the biosynthetic pathway for cysteine, and therefore for methionine, the effect of various sulphur-containing amino acids on serine transacetylase activity was investigated.

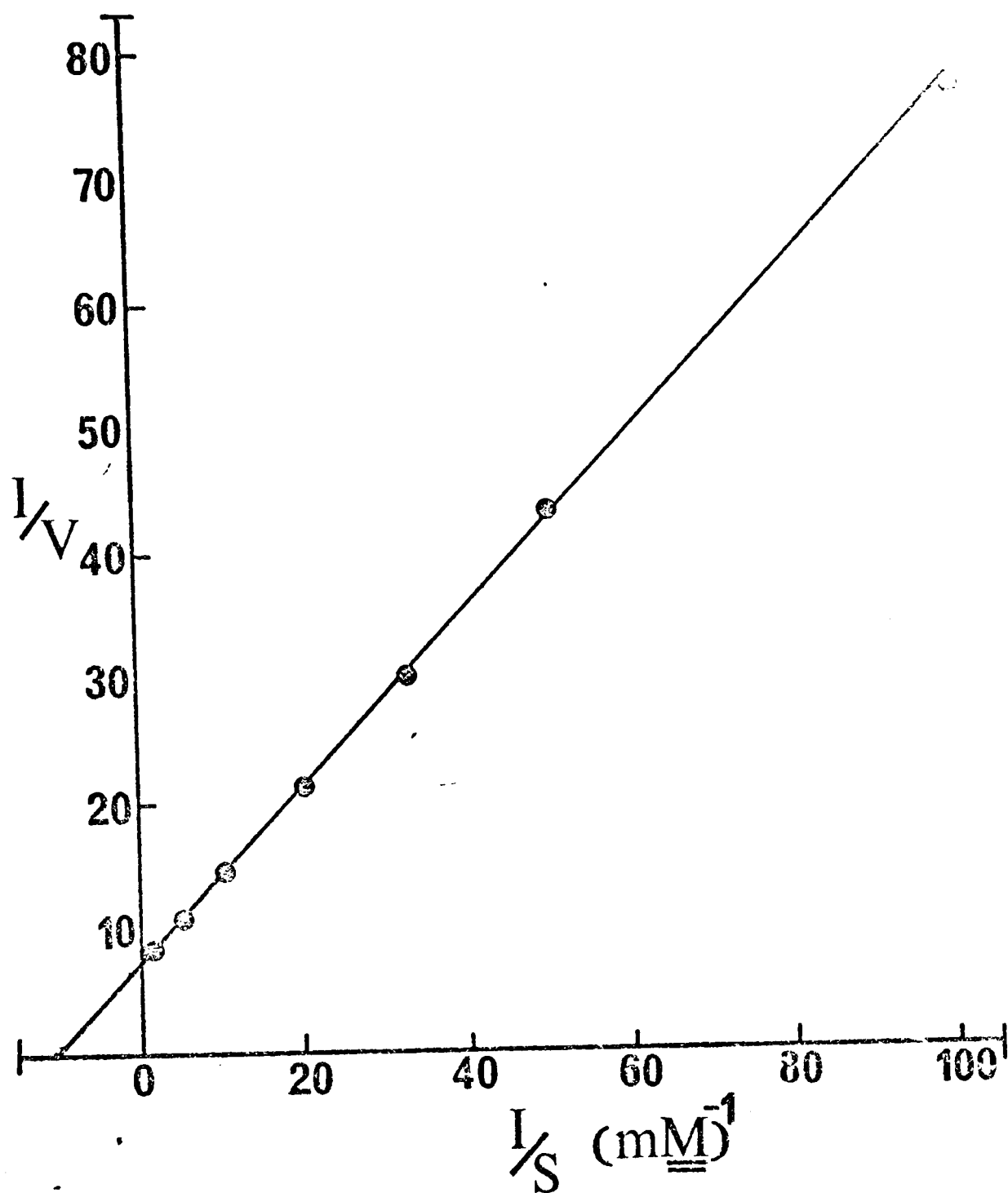


Fig. 9.6

Lineweaver-Burk plot of the serine transacetylase - catalysed reaction with acetyl CoA as the variable substrate.

The reaction was followed by Method 1 and the L-serine concentration was maintained at 2.0mM .

The apparent K_m for acetyl CoA is 1×10^{-4} .

TABLE 9.2

Effect of inhibitors on serine transacetylase activity.
Serine transacetylase activity was measured by Method 2.

<u>Compound</u>	<u>Inhibition</u> <u>(%)</u>
N-ethylmaleimide	88
p-chloromercuribenzoate	95
Iodoacetamide	76
Hydroxylamine	64
Potassium cyanide	85
Phenylhydrazine	6

L-Methionine and L-homocysteine inhibited serine transacetylase activity at relatively high concentrations only. L-Cysteine was an effective inhibitor of serine transacetylase activity, inhibiting 78% of the activity at a concentration of $1 \times 10^{-5} \underline{\underline{M}}$. In E. coli and S. typhimurium, Kredich & Tomkins (1966) demonstrated a 50% inhibition of serine transacetylase activity at a concentration of cysteine of $1.1 \times 10^{-6} \underline{\underline{M}}$. Serine transacetylase activity was also inhibited by Coenzyme A, an end-product of the reaction.

Purification of O-acetyl serine sulphydrylase. O-Acetyl serine sulphydrylase was purified to separate it from serine transacetylase, serine deaminase and O-acetyl serine lyase activities. The separation of O-acetyl serine sulphydrylase and serine transacetylase activities was achieved by Sephadex G-200 gel filtration (Fig. 9.3). Although serine deaminase, O-acetyl serine sulphydrylase and O-acetyl serine lyase activities could not be completely separated by Sephadex G-200 gel filtration, they were readily separated by DEAE-cellulose

ion exchange chromatography. O-Acetyl serine sulphydrylase was purified approximately 500-fold (Table 9.4) and was free of detectable serine deaminase, O-acetyl serine lyase and serine transacetylase activities. The enzyme was stable at room temperature for several days and retained 93% of its activity at -15°C after 10 months.

Identification of the product of the O-acetyl serine sulphydrylase-catalysed reaction. To 50 ml of 0.20 M-Tris HCl buffer, pH 7.5, containing 0.5 μmoles pyridoxal phosphate, 5 mmoles of O-acetyl serine and 20 mmoles of Na_2S were added 150 units of purified O-acetyl serine sulphydrylase. The reaction mixture was placed in a stoppered volumetric flask. The reaction was run for 3 h at 30°C and was terminated by the addition of 5.0 ml of 15% (v/v) trichloroacetic acid. The remaining sulphide was removed with a stream of nitrogen. The volume of the solution was reduced by rotary evaporation at 30°C . The solution was filtered to remove precipitated protein. The pH of the solution was then adjusted to pH 10 by the addition of NaOH, and the solution quickly filtered. The solution was then bubbled with oxygen for 12 h.; a fine, white precipitate formed during bubbling. The precipitate was collected by filtration and was dissolved in warm 1 M-HCl, filtered and recrystallized by the addition of ammoniacal ethanol (5%, v/v) to pH 3.5. After standing at 4°C overnight, the precipitate was collected by filtration. The final product was washed with ether and dried in vacuo. The purified product was identified as L-cystine by infra red spectrophotometry (see Appendix). Following treatment with excess (0.25M) dithiothreitol at pH 8.0, the compound gave a positive test with Gaitonde's reagent and also functioned as a substrate for purified cysteinyl-tRNA synthetase (purified from P. denitrificans - see Chapter 11). Since the enzymic

TABLE 9.4
Purification profile of O-acetyl L-serine sulphydrylase
Units are expressed as the amount of cysteine synthesized
per min. (μmole/min)

Fraction No.	Purification step	Total Protein conc. (mg)	Specific activity (units/ml)	Total units	Recovery (%)
I	Crude extract	6590	3.31	21812	100
II	(NH ₄) ₂ SO ₄ fractionation	1573	12.51	19831	91
III	Sephadex G-200 gel filtration	108.4	150.4	16300	75
IV	DEAE-52 cellulose chromatography I	8.3	1496	12429	57
V	DEAE-52 cellulose chromatography II	8.1	1498	12133	56

TABLE 9.3

Effect of inhibitors upon serine transacetylase activity

Serine transacetylase activity was measured by Method (2)

<u>Compound</u>	<u>Concn.</u> <u>(mM)</u>	<u>Inhibition</u> <u>(%)</u>
L-Methionine	10.0	45
	1.0	11
L-Homocysteine	10.0	34
	1.0	0
L-Cysteine	10.0	100
	1.0	100
	0.1	93
	0.01	78
D-Cysteine	10.0	0
	1.0	0
L-Threonine	10.0	0
	1.0	0
L-Homoserine	10.0	12
	1.0	9
L-Cystine	2.0	0
	0.2	0
Coenzyme A	5.0	12
	0.5	5
L-Selenocysteine	1.0	100
	0.1	96
L-Selenocystine	2.0	0

product of the O-acetyl serine sulphydrylase-catalysed reaction is a thiol, the product is confirmed as L-cysteine.

Properties of O-acetyl serine sulphydrylase. The time course of the synthesis of cysteine from sulphide and O-acetyl serine is shown in Fig. 9.7. The rate of cysteine synthesis is linear for at least 1 h indicating that the product, L-cysteine, is not inhibitory during this period. The rate of cysteine synthesis is directly proportional to the enzyme concentration (Fig. 9.8).

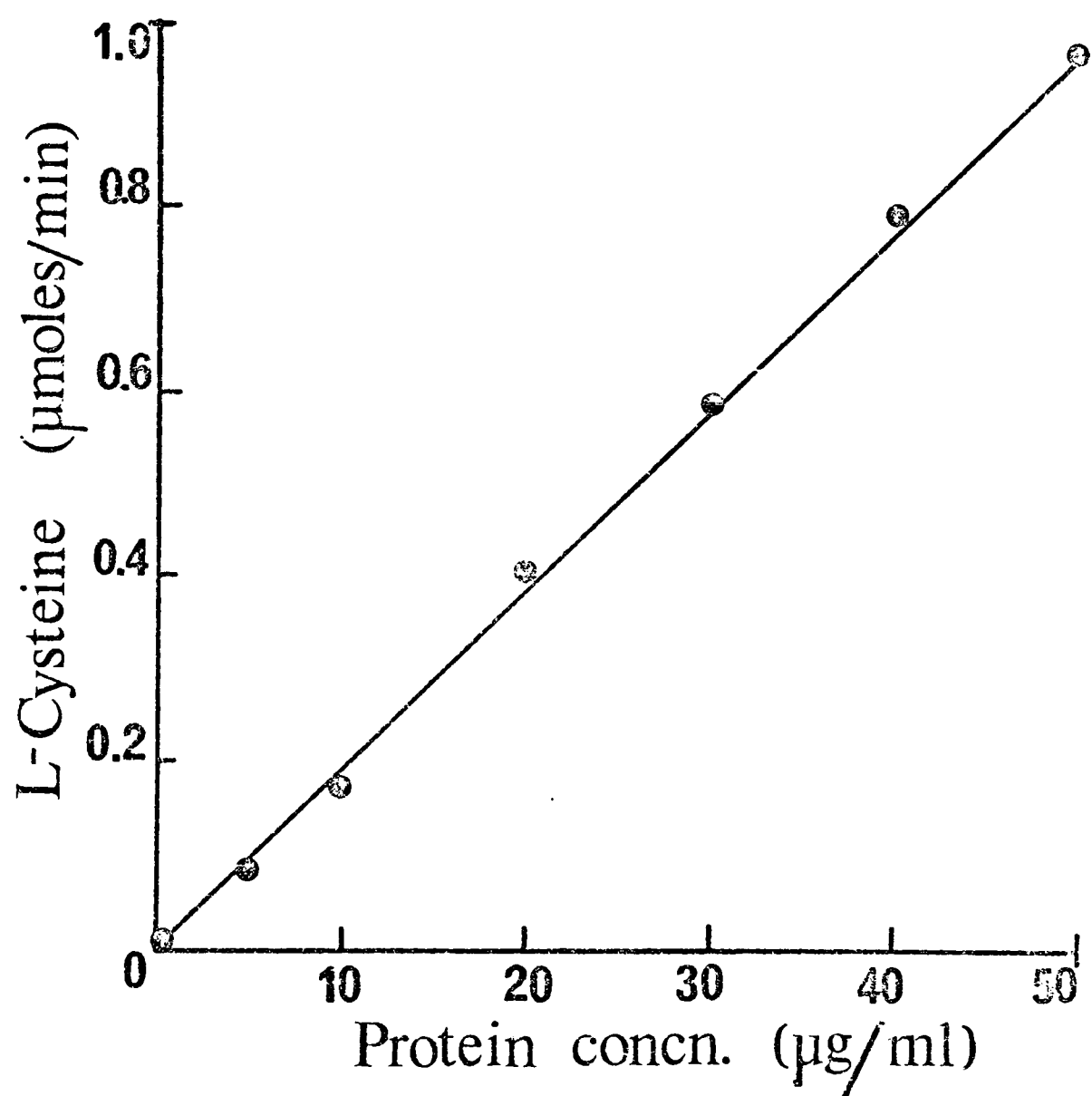
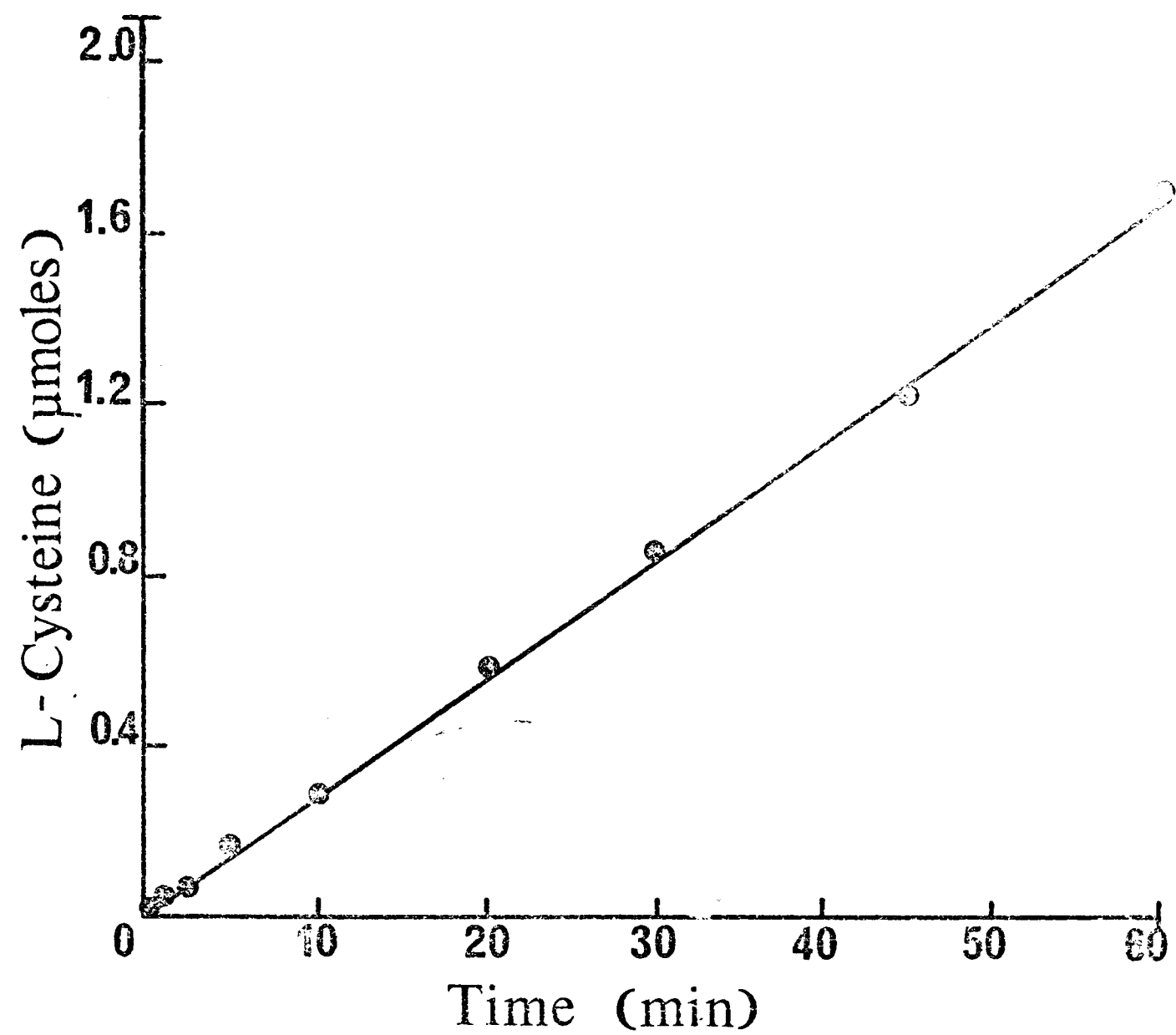
Substrate specificity. L-Serine, N-acetyl serine, O-acetyl threonine, O-acetyl DL-serine and O-acetyl D-serine gave no activity when substituted for O-acetyl serine in the standard reaction mixture. β -Mercaptoethanol could not replace sulphide in the reaction. Selenide did, however, replace sulphide in the reaction and this is discussed below.

Effect of pH. The effect of pH is shown in Fig. 9.9. The chemical properties of the substrates involved in the reaction make it difficult to obtain meaningful data for the effect of pH on O-acetyl serine sulphydrylase activity. Maximum rates of cysteine synthesis occurred at pH 7.6 with Tris-HCl as the buffer. Enzyme activity is restricted, however, to a limited range of pH, for at acid pH sulphide volatilizes and is rapidly lost from incubation mixtures, and at alkaline pH, O-acetyl serine is converted to N-acetyl serine, which is not a substrate for this enzyme (Flavin & Slaughter, 1965; Kredich & Tomkins, 1966). All subsequent enzyme assays were conducted at pH 7.5 in Tris-HCl buffer.

Effect of cofactors upon O-acetyl serine sulphydrylase activity. O-Acetyl serine sulphydrylase activity was not affected by the addition of Mg^{2+} or by EDTA (Table 9.5). A

Fig. 9.7 Time-course of cysteine synthesis by O-acetyl serine sulphydrylase

Fig. 9.8 The effect of enzyme protein concentration upon the rate of cysteine synthesis by O-acetyl serine sulphydrylase. The reaction mixtures were as described in Materials and Methods and the assays were run for 10 min.



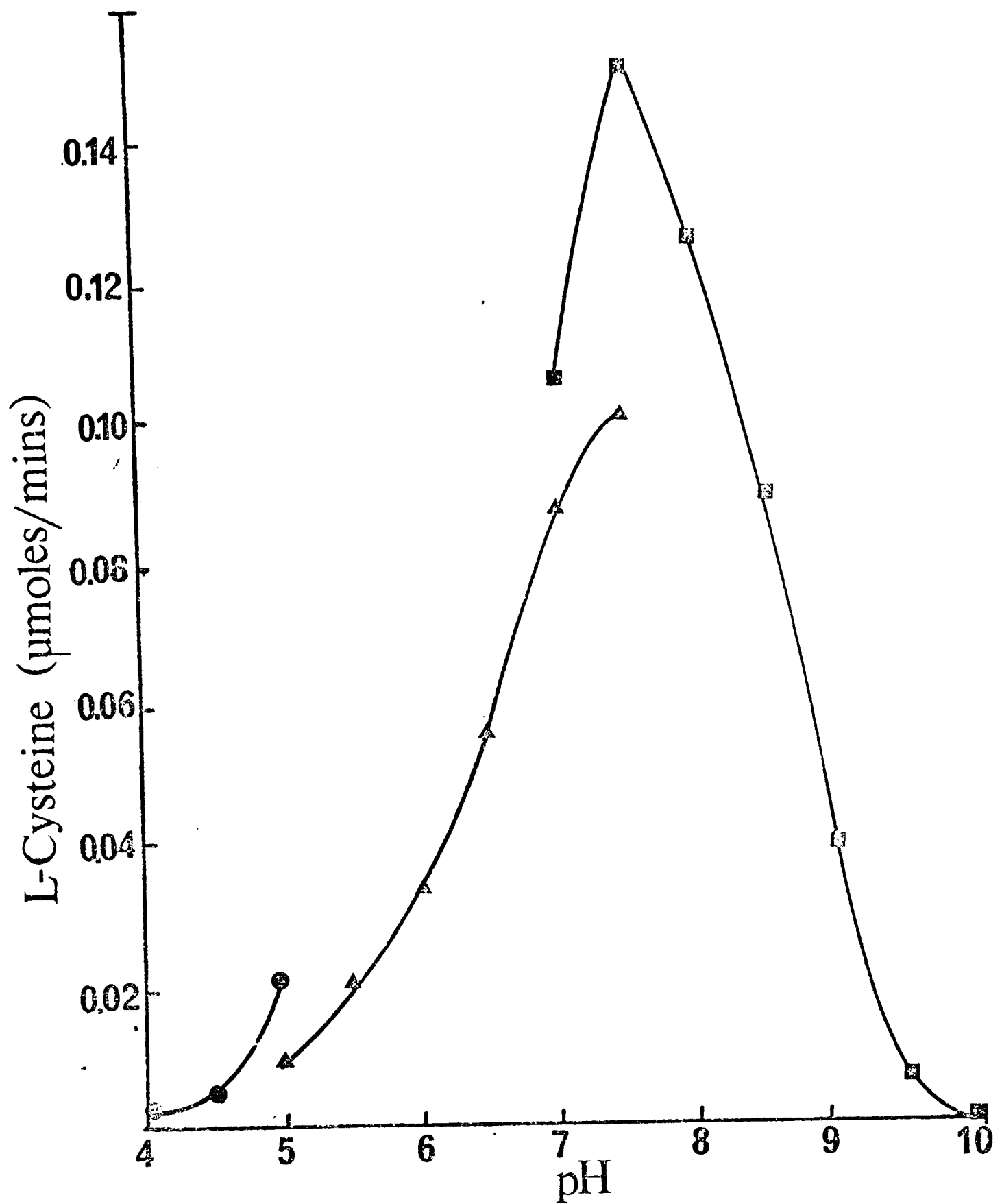


Fig. 9.9

Effect of pH upon O-acetyl serine sulphydrylase activity. O-Acetyl serine sulphydrylase activity was measured as described in the text except that Tris-HCl buffer was replaced with the buffers specified. The buffers were prepared as described by Gomori (1955).

●, Citrate; ▲, Tris-maleate-KOH; ■, Tris-HCl.

TABLE 9.5

Effect of cofactors upon purified O-acetyl serine
sulphydrylase activity

O-Acetyl serine sulphydrylase activity was assayed as described in the text. All the additions to the reaction mixtures were preincubated for 2 min prior to initiating the reaction by the addition of O-acetyl L-serine.

<u>Additions</u>	<u>Activity</u> (%)
None	100
MgCl ₂ (10mM)	102
EDTA (10mM)	97
Pyridoxal 5'-phosphate (0.1mM)	179
Hydroxylamine (5mM)	62
Dithiothreitol (5mM)	175
ATP (5mM)	100
ADP (5mM)	102

stimulation of enzyme activity by pyridoxal phosphate, and an inhibition by hydroxylamine, which was relieved by the addition of pyridoxal phosphate, indicated that O-acetyl serine sulphydrylase has a pyridoxal phosphate requirement. The stimulatory effect of dithiothreitol may be due to either (1) the reduction, by dithiothreitol, of thiol groups important to enzymic function, or (2) the protection by dithiothreitol of the enzymically produced cysteine from oxidation, thus allowing a greater percentage of the cysteine synthesised to be measured by the method of Gaitonde (1965), (Gaitonde's method does not detect cystine), or (3) to a combination

of these factors.

Pyridoxal phosphate (10 nmoles) and dithiothreitol (5mM) were therefore routinely included in all subsequent reaction mixtures.

Effect of sulphide concentration. A double reciprocal plot of the rate of cysteine synthesis versus sulphide concentration is shown in Fig. 9.10; the apparent K_m value obtained from this plot is 2.7×10^{-3} M.

Effect of O-acetyl serine concentration. The apparent K_m for O-acetyl serine was obtained from a double reciprocal plot and was found to be 1.25×10^{-3} M (Fig. 9.11).

Effect of sulphur-containing compounds. The effect of a number of sulphur-containing compounds upon O-acetyl serine sulphydrylase activity were investigated to determine some of the regulatory properties of the enzyme.

O-Acetyl serine sulphydrylase activity was not affected by sulphate (0.1 to 10 mM) when the concentration of either O-acetyl serine (0.1 to 10mM) or sulphide (0.1 to 10 mM) was varied. The effects of sulphite upon O-acetyl serine sulphydrylase activity are shown in Figs. 9.12 and 9.13. Sulphite non-competitively inhibits enzyme activity with respect to O-acetyl serine and un-competitively with respect to sulphide. L-Homocysteine competitively inhibited O-acetyl serine sulphydrylase activity with respect to sulphide (Fig. 9.14) and non-competitively with respect to O-acetyl serine (Fig. 9.14). The inhibition of O-acetyl serine sulphydrylase activity by methionine was non-competitive with respect to O-acetyl serine (Fig. 9.16) and competitive with respect to sulphide (Fig. 9.17.) Cysteic acid was found to inhibit O-acetyl serine sulphydrylase activity; this inhibition was non-

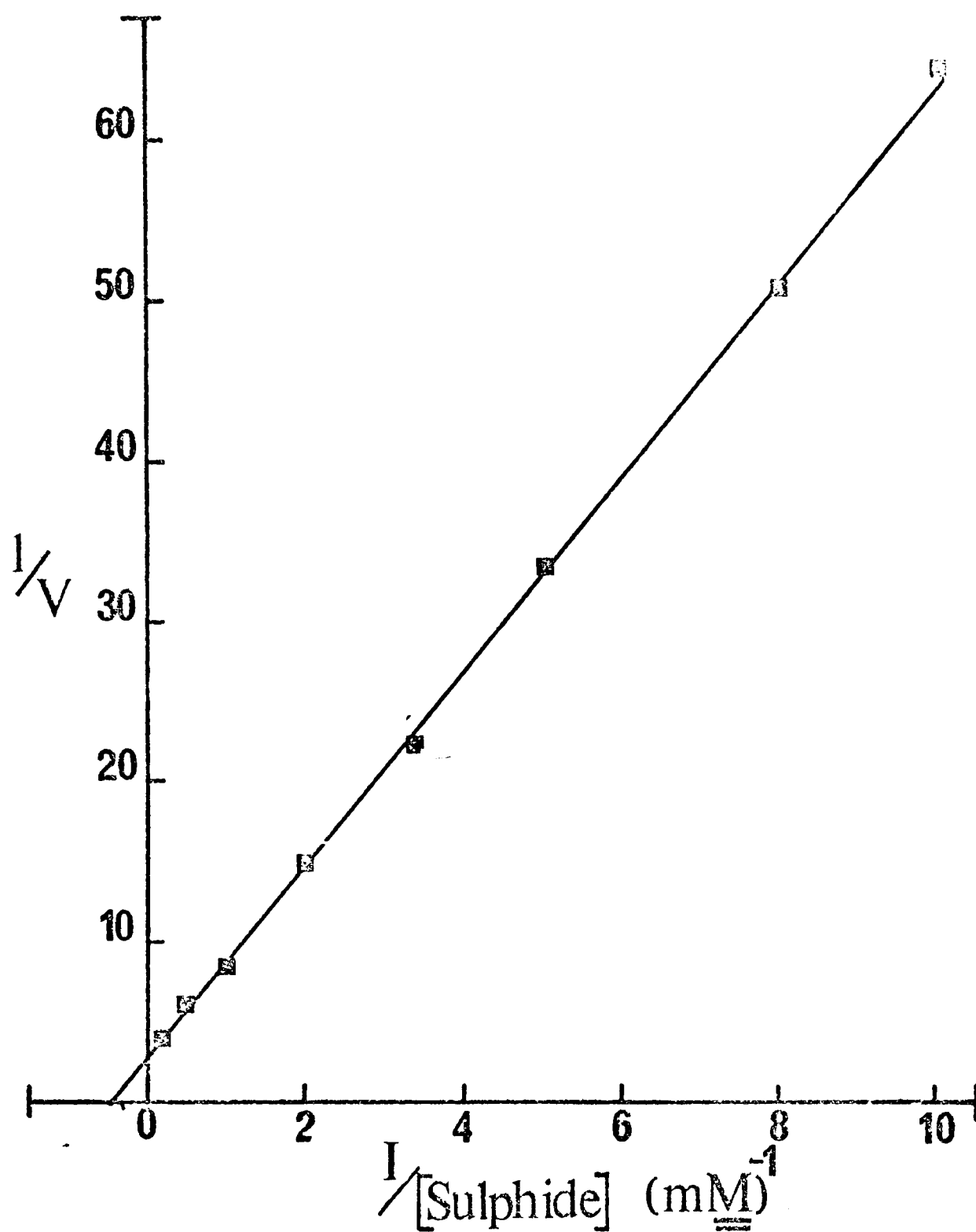


Fig. 9.10

Lineweaver-Burk plot of the rate of cysteine synthesis versus sulphide concentration.

The apparent K_m , obtained from this plot, is 2.7mM.

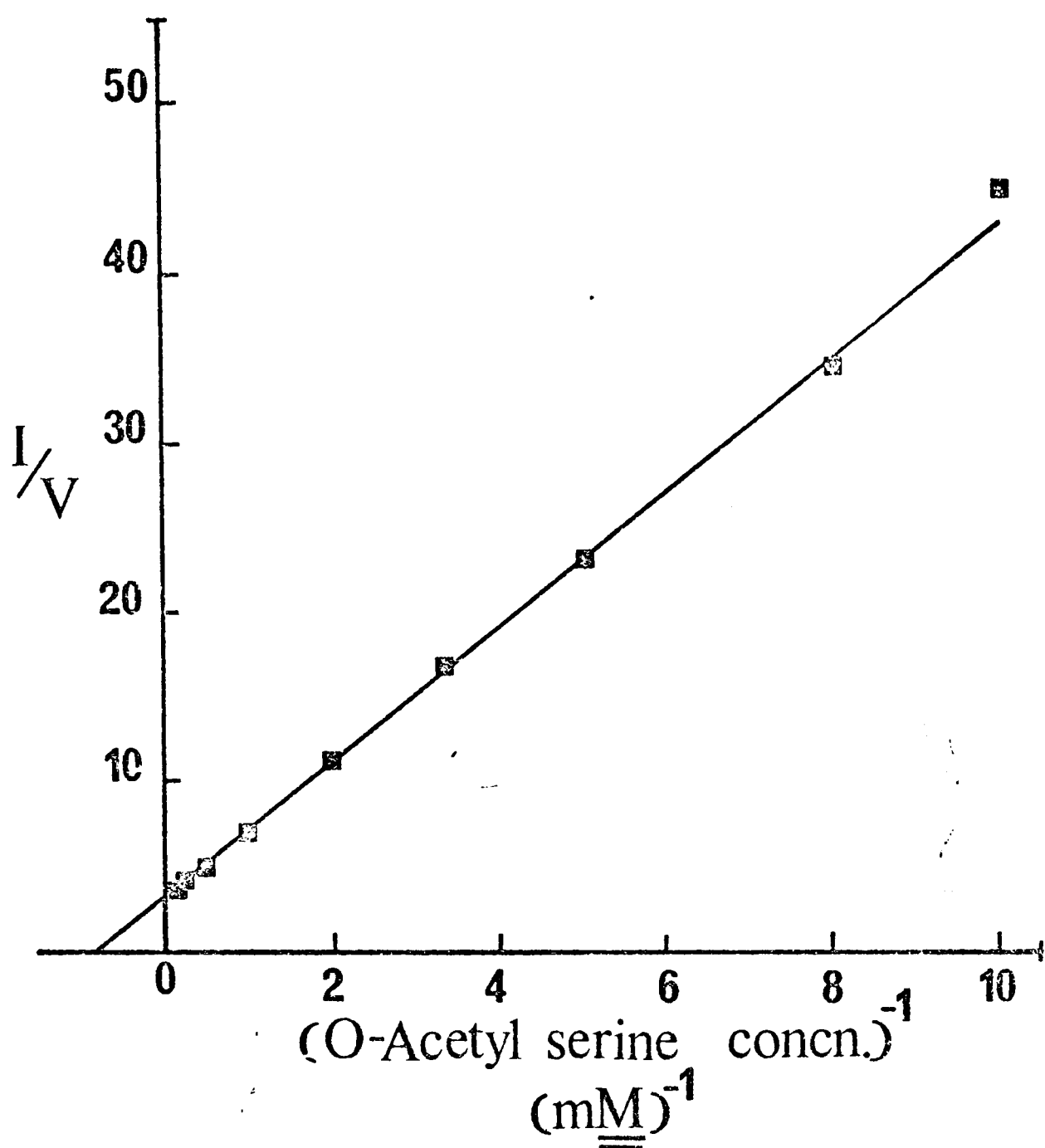


Fig. 9.11

Lineweaver-Burk plot of the effect of O-acetyl concentration upon O-acetyl serine sulphydrylase activity. The apparent K_m , obtained from this graph, is 1.25 mM.

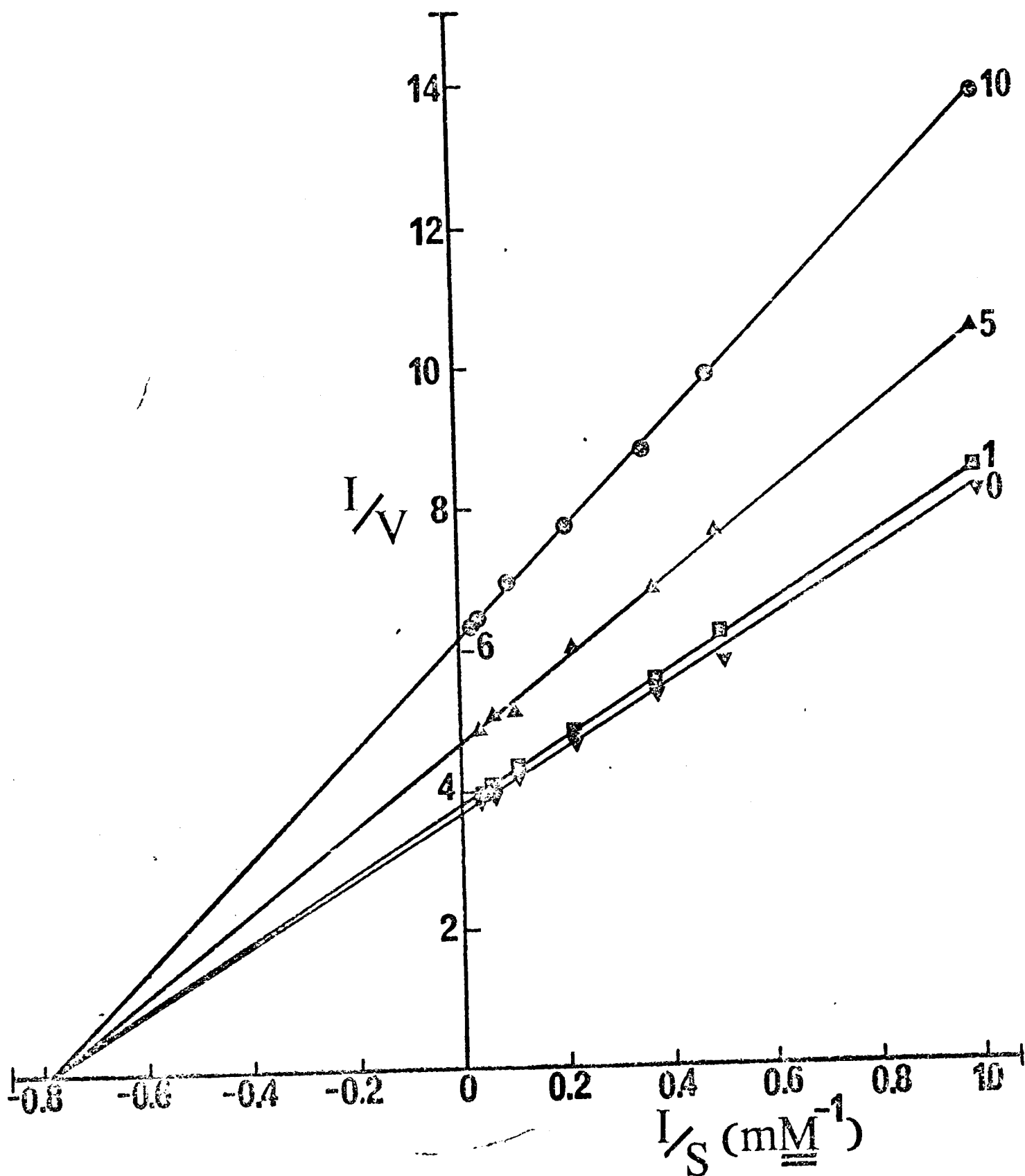


Fig. 9.12

Lineweaver-Burk plot of the inhibition of O-acetyl serine sulphydrylase activity by sulphite.

O-Acetyl serine sulphydrylase activity was measured in the presence of saturating amounts of sulphide at various concentrations of O-acetyl serine.

▼, without sulphite; ■, 1mM-sulphite; ▲, 5mM-sulphite; ●, 10mM-sulphite.

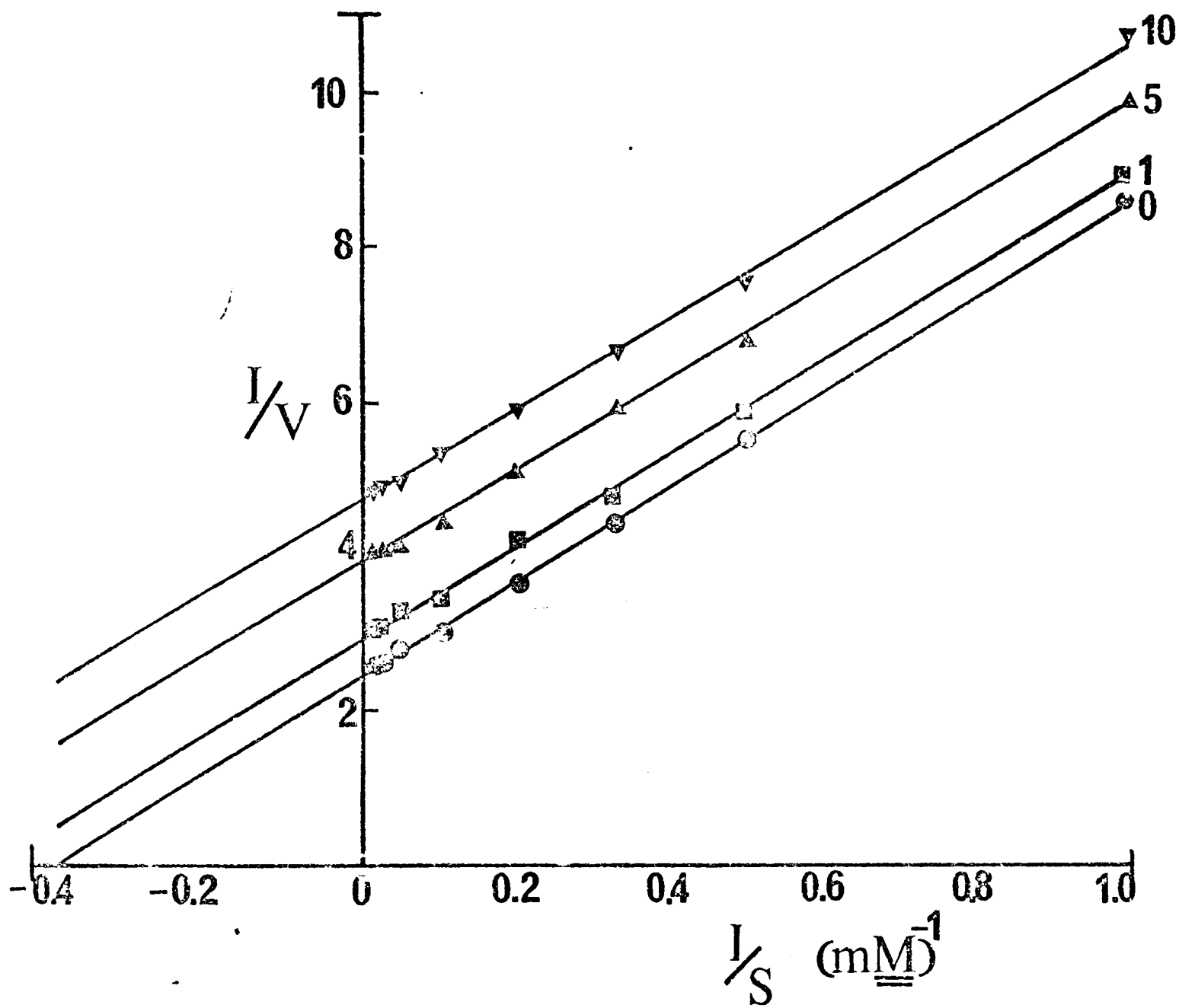


Fig. 9.13

Lineweaver-Burk plots of the inhibition of 0-acetyl serine sulphydrylase activity by sulphite.

0-Acetyl serine sulphydrylase activity was measured in the presence of saturating amounts of 0-acetyl serine and various concentrations of sulphide.

●, without sulphite; ■, 1mM-sulphite; ▲, 5mM-sulphite; ▼, 10mM-sulphite.

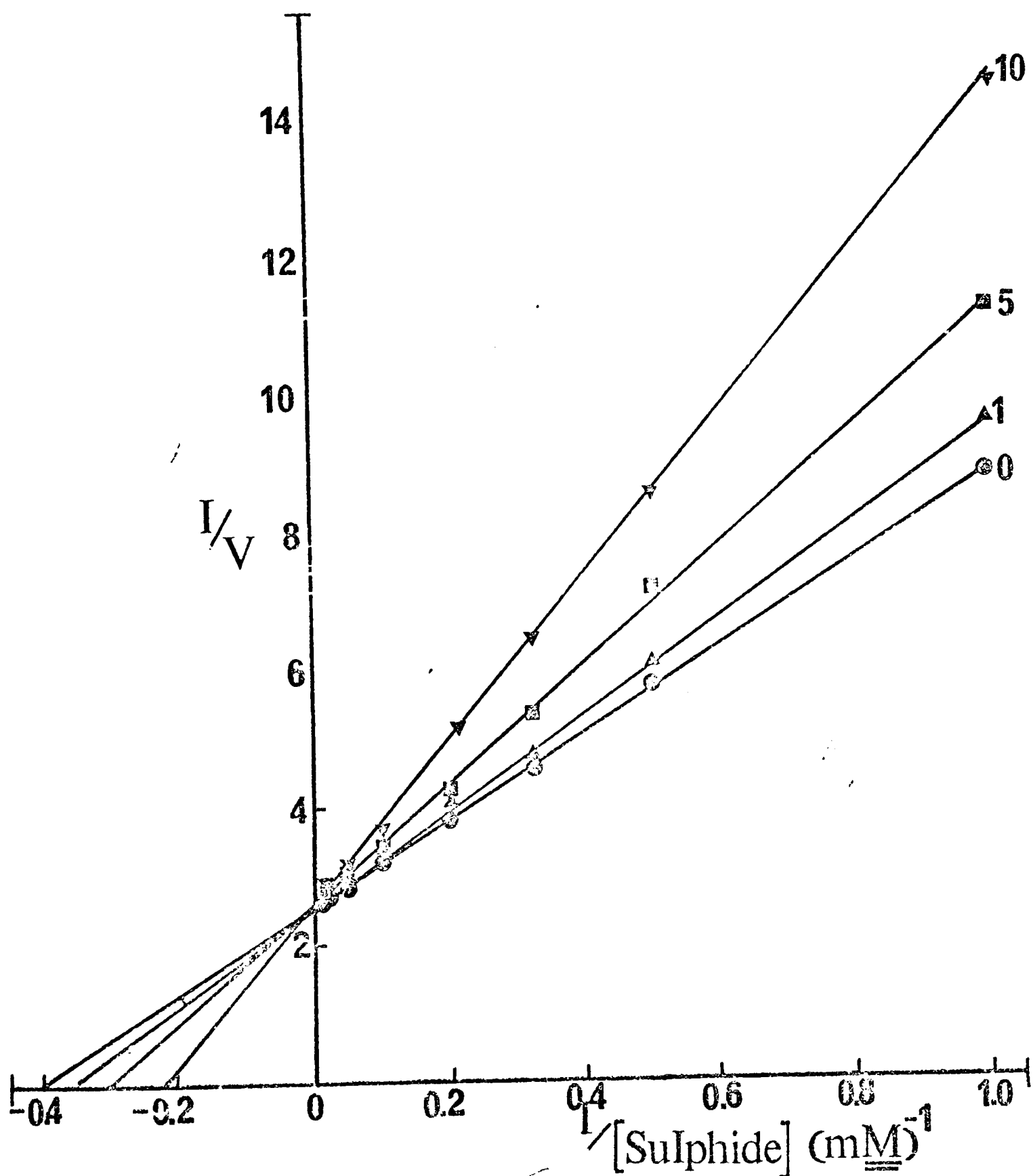


Fig. 9.14

Lineweaver-Burk plots of the inhibition of O-acetyl serine sulphydrylase activity by L-homocysteine.

O-Acetyl serine sulphydrylase activity was measured in the presence of saturating amounts of O-acetyl serine and various concentrations of sulphide.

●, without L-homocysteine; ▲, 1mM-L-homocysteine; ■, 5mM-L-homocysteine; ▼, 10mM-L-homocysteine.

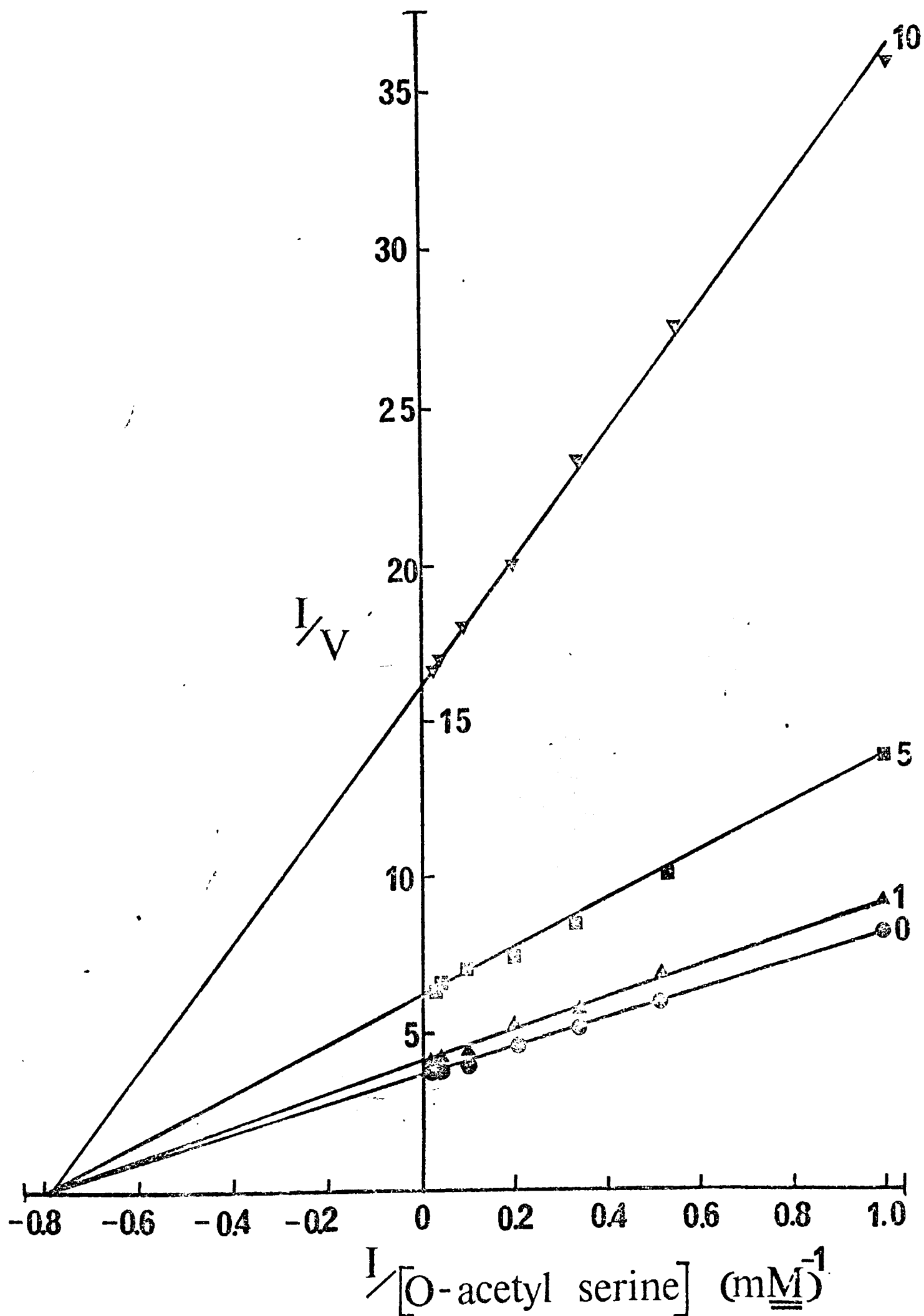


Fig. 9.15

Lineweaver-Burk plots of the inhibition of O-acetyl serine sulphydrylase activity by homocysteine.

O-Acetyl serine sulphydrylase activity was measured in the presence of saturating amounts of sulphide and various concentrations of O-acetyl serine.

●, without homocysteine; ▲, 1mM-homocysteine; ■, 5mM-homocysteine; ▼, 10mM-homocysteine.

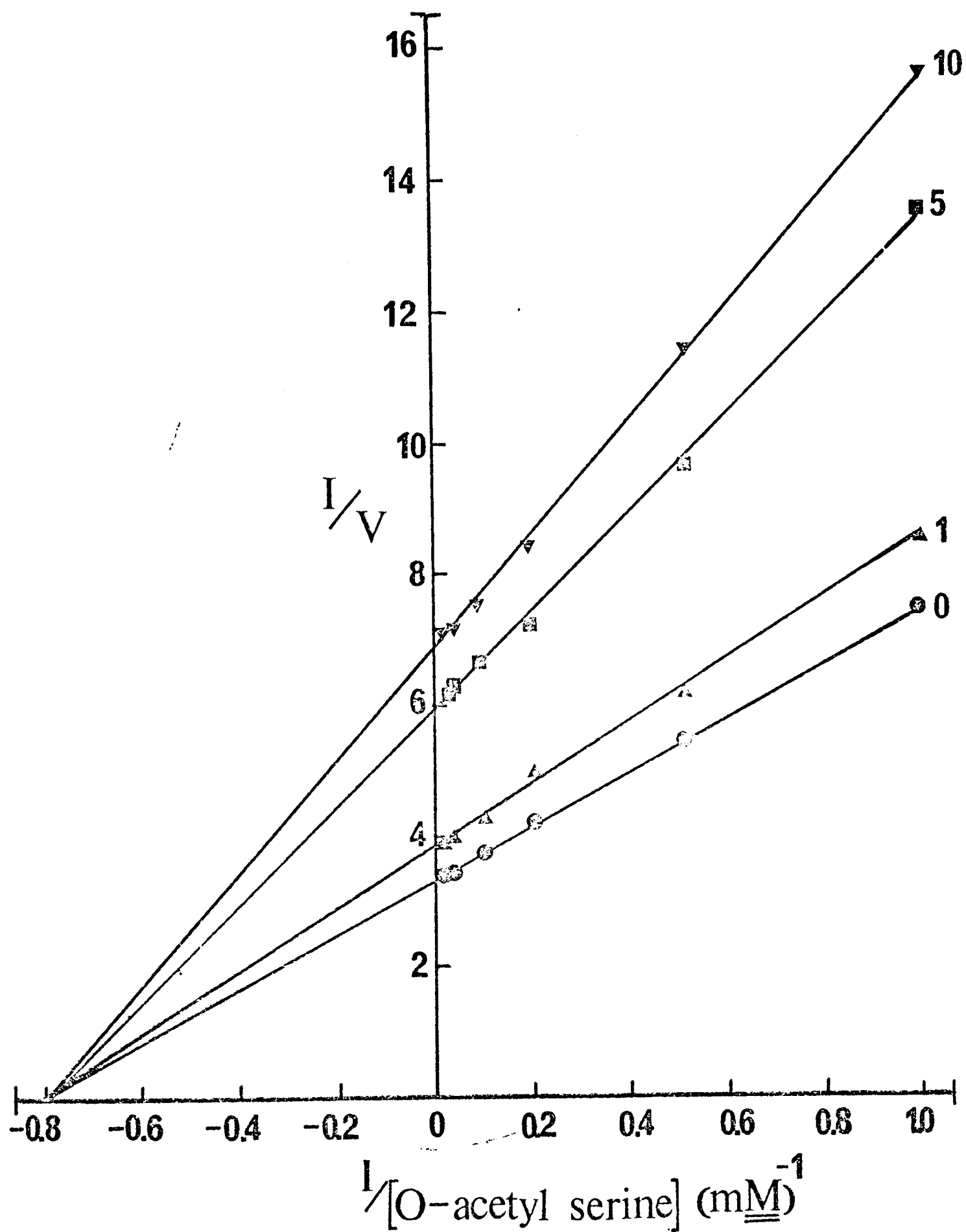


Fig. 9.16

Lineweaver-Burk plots of the effect of methionine upon O-acetyl serine sulphydrylase activity.

Enzyme activity was measured in the presence of saturating amounts of sulphide and various concentrations of O-acetyl serine.

●, without methionine; ▲, 1mM-methionine; ■, 5mM-methionine;

▼, 10mM-methionine.

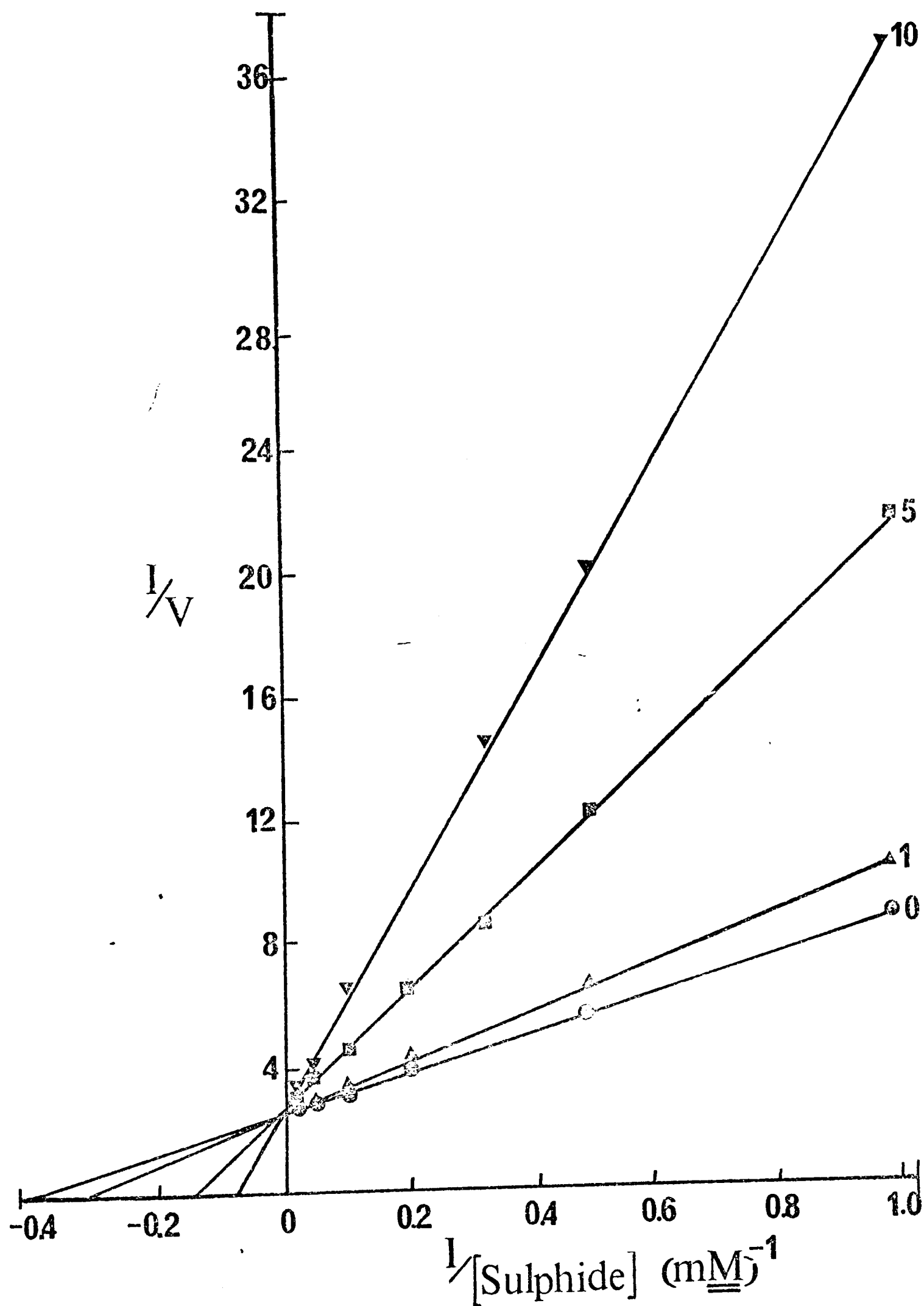


Fig. 9.17

Lineweaver-Burk plots of the inhibition of O-acetyl serine sulphydrylase activity by methionine.

Enzyme activity was measured in the presence of saturating amounts of O-acetyl serine and various concentrations of sulphide. ●, without methionine; ▲, 1mM-methionine; ■, 5mM-methionine; ▼, 10mM-methionine.

competitive with respect to O-acetyl serine (Fig. 9.18) and competitive with respect to sulphide. Cystathionine inhibits O-acetyl serine sulphydrylase activity un-competitively (Fig. 9.19).

Miscellaneous observations. L-Serine is not a substrate for O-acetyl serine sulphydrylase but competitively inhibits cysteine synthesis with respect to O-acetyl serine (Fig. 9.20), and non-competitively with respect to sulphide.

O-Acetyl serine sulphydrylase activity was not affected by acetate, an end-product of the reaction. None of the nucleotides examined had any effect on O-acetyl serine sulphydrylase activity; those tested included ATP, ADP, 5'-AMP, 3'-AMP and adenosine.

1,2,4-Triazole (0.1 to 10 mM) did not affect the activity of the purified enzyme.

A study of the effect of sulphydryl-group inhibitors upon O-acetyl serine sulphydrylase is not likely to be particularly meaningful since experiments with such compounds as p-chloro-mercuribenzoate and N-ethylmaleimide are complicated by the reaction of the compounds with cysteine.

Properties of the serine transacetylase and O-acetyl serine sulphydrylase coupled enzyme system. Cysteine synthesis was not detected in reaction mixtures containing [^{14}C]serine, acetyl CoA, sulphide, pyridoxal phosphate and either purified serine transacetylase or purified O-acetyl serine sulphydrylase (Table 9.6); this demonstrated that both enzymes were free from cross contamination. When both enzymes were included in the reaction mixture, cysteine synthesis was detected, although the amount of cysteine formed was small. However, by conducting a two stage incubation, the amount of

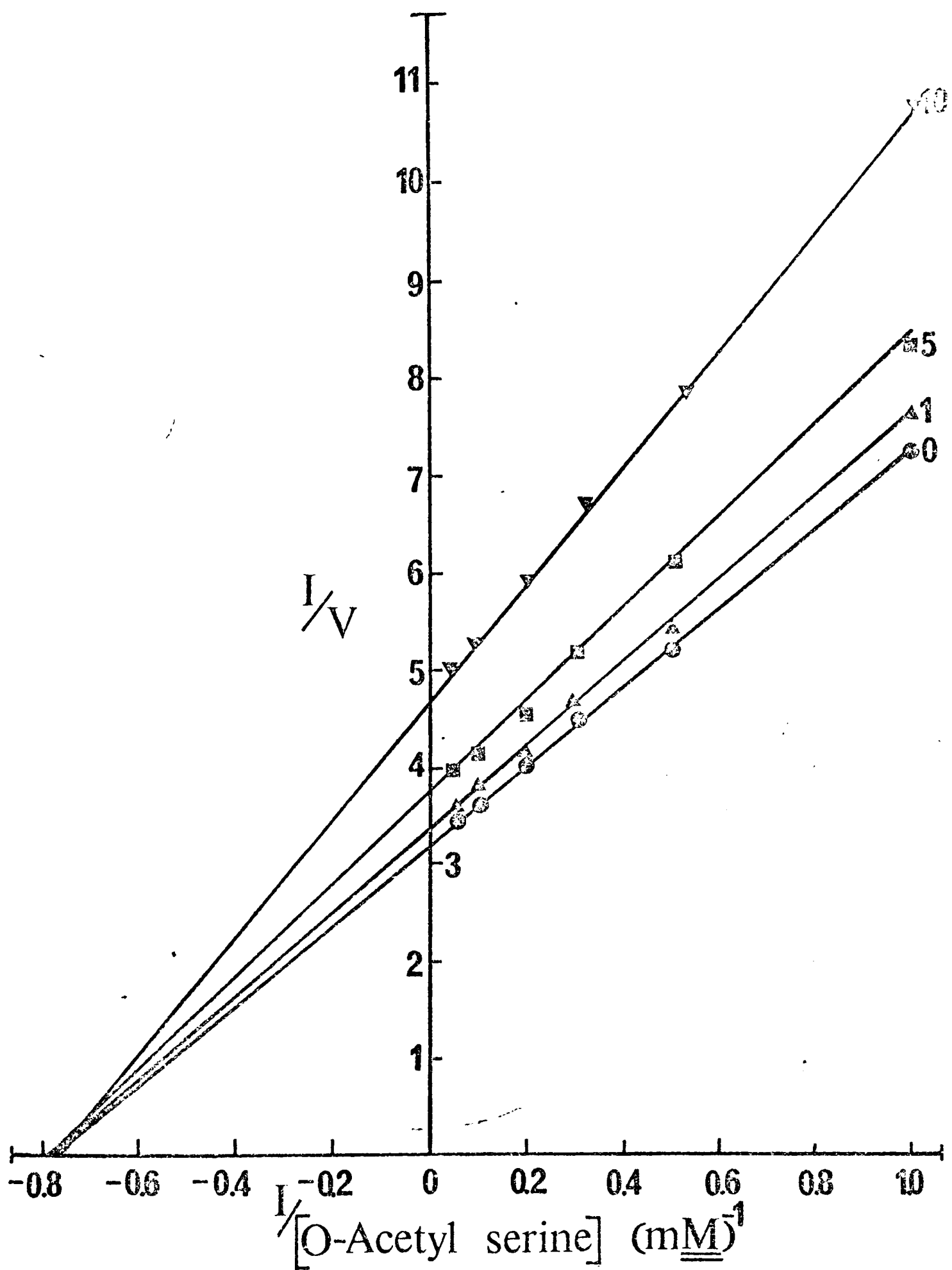


Fig. 9.18

Lineweaver-Burk plots of the inhibition of O-acetyl serine sulphydrylase activity by cysteic acid. Enzyme activity was measured in the presence of saturating amounts of sulphide and various concentrations of O-acetyl serine. ●, Without cysteic acid; ▲, 1mM-cysteic acid; ■, 5mM-cysteic acid; ▼, 10mM-cysteic acid.

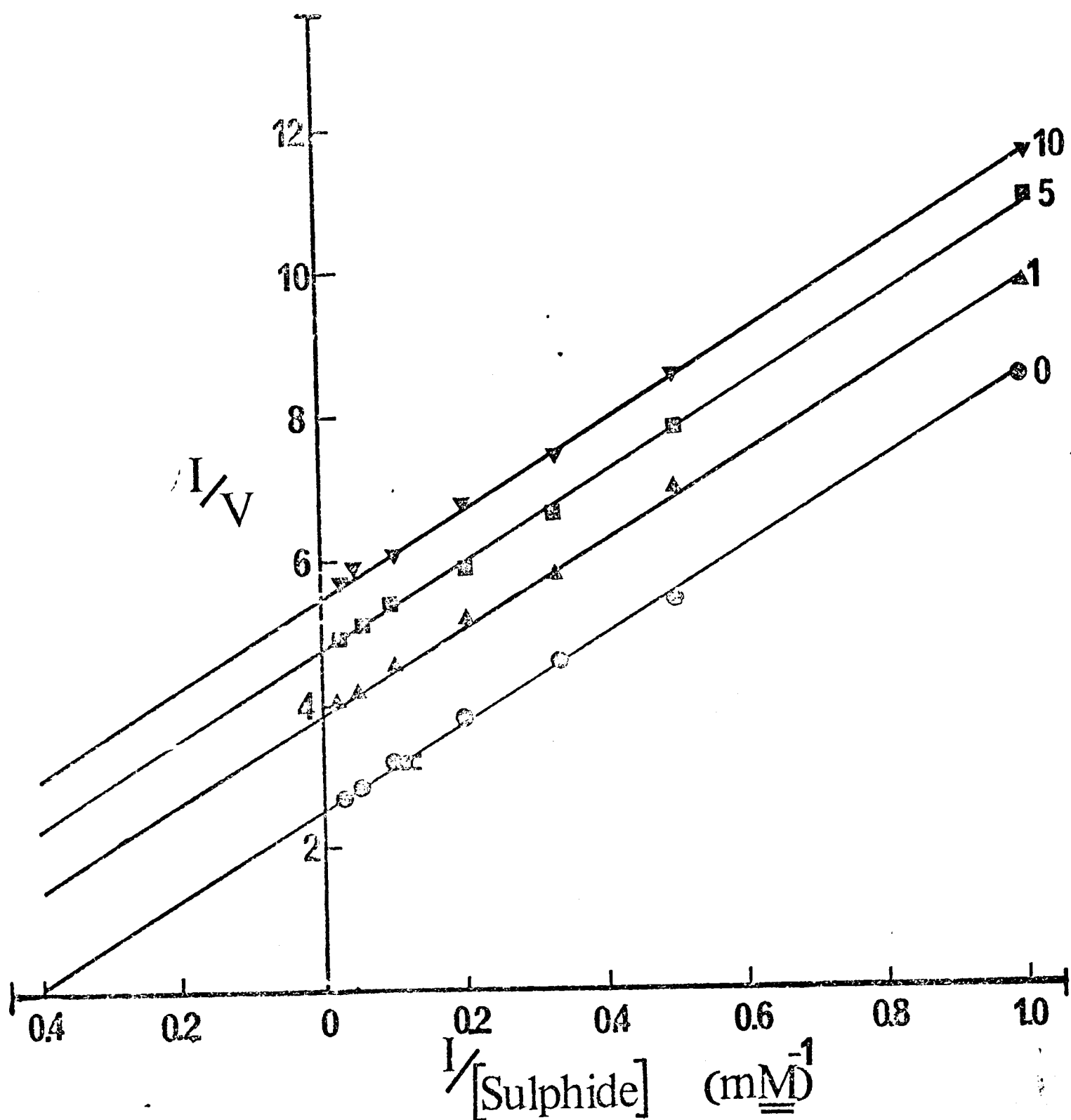


Fig. 9.19

Lineweaver-Burk plots of the inhibition of O-acetyl serine sulphydrylase activity by L-cystathionine. Enzyme activity was measured in the presence of saturating amounts of O-acetyl serine and various concentrations of sulphide. ●, without cystathionine; ▲, 1mM-cystathionine; ■, 5mM-cystathionine; ▼, 10mM-cystathionine.

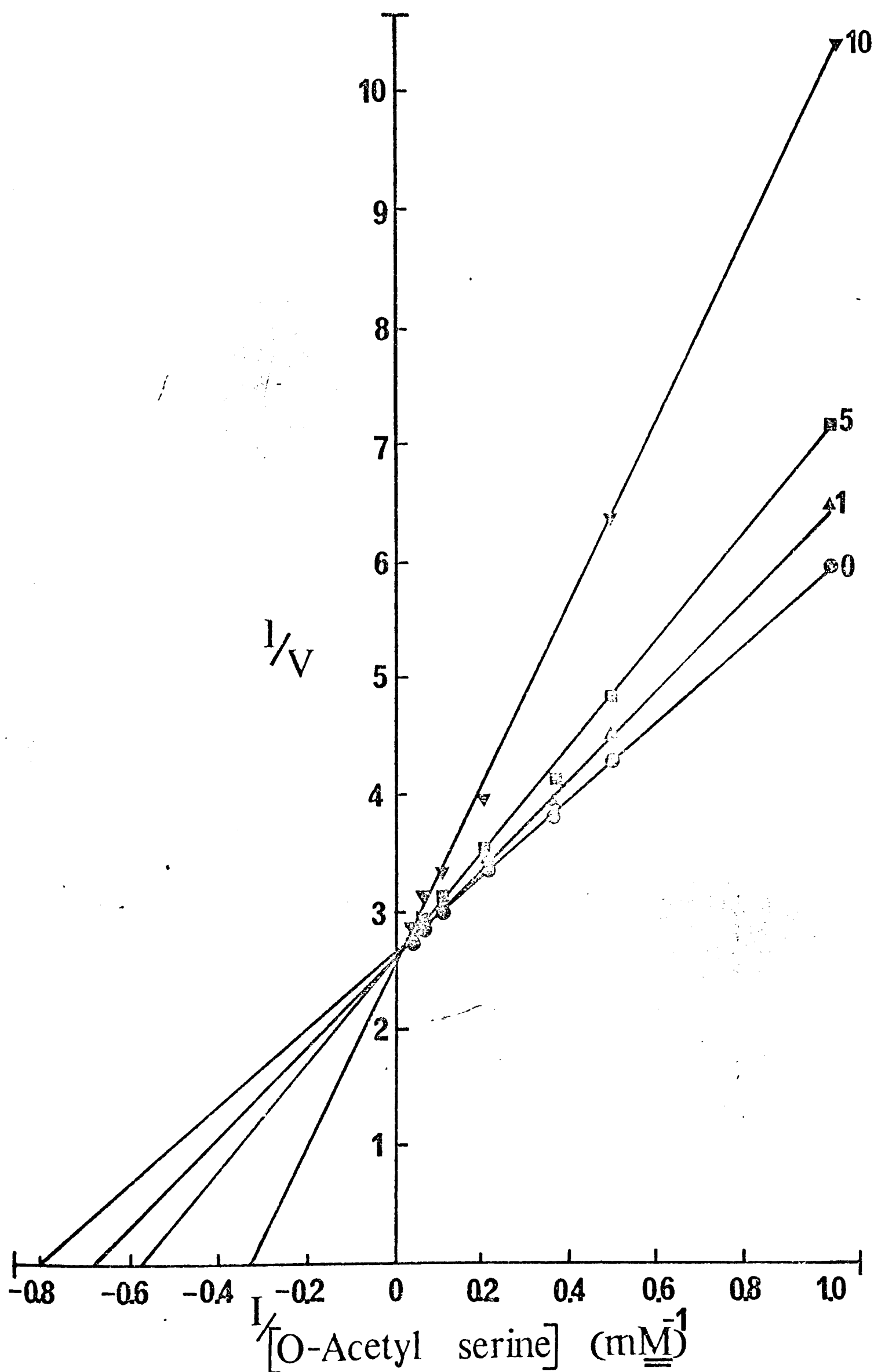


Fig. 9.20

Lineweaver-Burk plots of the inhibition of O-acetyl serine sulphydrylase activity by serine. O-Acetyl serine sulphydrylase activity was measured in the presence of saturating amounts of sulphide and various concentrations of O-acetyl serine; $\circ$, without serine; $\blacktriangle$, 1mM-serine; $\blacksquare$, 5mM-serine; $\blacktriangledown$, 10mM-serine.

TABLE 9.6

Requirements of the serine transacetylase and O-acetyl L-serine sulphydrylase coupled enzyme system for the synthesis of cysteine.

Reaction mixtures contained, in a total of 1.0 ml. 2.5 μ moles acetyl CoA, 10 μ moles sulphide, 2.5 μ moles L-serine, 100 μ moles sulphide, 2.5 μ moles L-serine, 100 μ moles Tris-HCl buffer, pH 7.5, 10nmoles pyridoxal phosphate, 5 units purified serine transacetylase and 5 units purified O-acetyl L-serine sulphydrylase. Reactions, in the absence of sulphide, were run for 15 min, at 30° and then the sulphide added and the reaction continued for a further 15 min.

<u>System</u>	<u>μmoles cysteine synthesized</u>
Complete	0.269
Complete minus acetyl CoA	0.018
Complete minus sulphide	0.017
Complete minus serine	0.027
Complete minus pyridoxal phosphate	0.214
Complete minus serine transacetylase	0.015
Complete minus O-acetyl L-serine sulphydrylase	0.017

cysteine synthesis could be greatly increased. This reconfirmed an earlier result of the inhibition of serine transacetylase by cysteine (see Table 9.3). The terminated complete reaction mixture was evaporated to dryness in a rotary evaporator and the residue dissolved in 0.5 ml of H₂O. A sample of the residue (25 μ l) was subjected to thin layer chromatography (Fig. 9.21) as described in Materials and Methods; the

Fig. 9.21

Identification of the product of the serine transacetylase and O-acetyl serine sulphydrylase coupled enzyme system by thin layer chromatography. Reaction mixtures were as described in Materials and Methods except that serine was replaced by $[^{-14}\text{C}]$ serine. The products were separated on TLC plates as described in Materials and Methods.

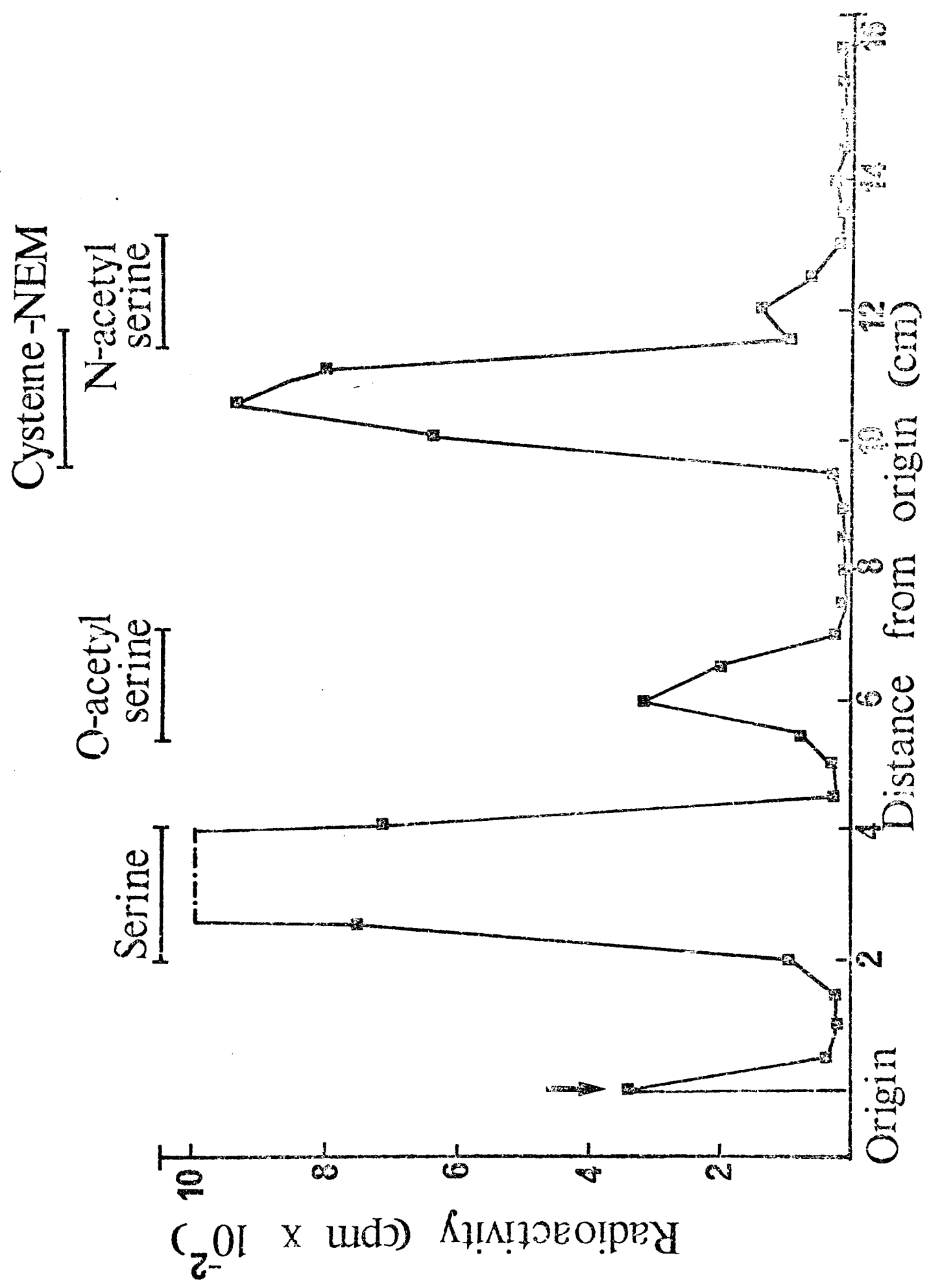


TABLE 9.7

Effect of sulphur source during growth upon serine transacetylase and O-acetyl L-serine sulphydrylase activity.

Crude extracts were prepared from 4 litres of culture medium containing log phase cells, as described in the text. Crude extracts were dialysed for 12 h against 20mM-Tris-HCl buffer, pH 7.5. Serine transacetylase activity was assayed by Method (2).

Sulphur source during growth	Specific activity (units/mg of Protein)	
	Serine transacetylase	O-Acetyl L-serine sulphydrylase
SO_4^{2-}	0.14	3.31
SO_3^{2-}	0.16	3.76
S^{2-}	0.13	3.64
SO_4^{2-} plus L-homocysteine	0.11	3.38
SO_4^{2-} plus L-methionine	0.10	3.17
SO_4^{2-} plus, 1,2,4-triazole	0.15	3.43
L-Cystine	0.36	6.68
L-Cystine plus 1,2,4-triazole	0.41	6.49

^{14}C -labelled product was separated from serine and O-acetyl serine and ran with an R_f value identical with that of cysteine-NEM (Table 9.1).

Synthesis of selenocysteine. Selenide was found to be an alternate substrate for O-acetyl serine sulphydrylase; selenide replaces sulphide. This section reports results of a brief investigation of the synthesis of selenocysteine from O-acetyl serine and selenide, catalysed by purified O-acetyl serine sulphydrylase.

Identification of selenocysteine. To 40 ml of 0.2 M-Tris-HCl buffer, pH 7.5 containing 0.5 μ moles of pyridoxal phosphate and 5 mmoles of O-acetyl serine were added 10 ml of a saturated solution of selenide in 0.2 M-Tris-HCl buffer, pH 7.5, and 200 units of purified O-acetyl serine sulphydrylase. The reaction was allowed to proceed for 5 h at 30°C and terminated by the addition of 5 ml of 15% (w/v) trichloroacetic acid. The remaining selenide was removed by bubbling with nitrogen. Selenocystine was precipitated and recrystallised from HCl by the method described for the isolation of cystine. The purified product was identified as L-selenocystine by (1) comparison of its infra red spectrum with the infra red spectrum of authentic selenocystine (see Appendix) and (2) by allowing it to react, after treatment with excess dithiothreitol at pH 8.0, confirming it as a substrate for purified cysteinyl-tRNA synthetase (see Chapter 11).

Kinetics of selenocysteine synthesis. Selenide, but not selenite or selenate, could replace sulphide as a substrate for purified O-acetyl serine sulphydrylase. Since selenide and selenocysteine are toxic and extremely unpleasant compounds to work with, only a limited number of experiments involving selenide were conducted. The following experiments

described were conducted in duplicate and the results presented are means of the duplicates. Variation between duplicates was not greater than $\pm 7\%$.

The rate of selenocysteine synthesis was linear as a function of both time and protein concentration (Figs. 9.22 and 9.23 respectively). Linearity of the reaction with respect to time was only achieved when dithiothreitol was included in reaction mixtures, presumably because it prevented oxidation of selenocysteine to selenocystine - selenocystine does not react with the acid ninhydrin reagent. Nevertheless, linearity of the selenocysteine synthesis with respect to time indicated that O-acetyl serine sulphydrylase activity was not inhibited by selenocysteine.

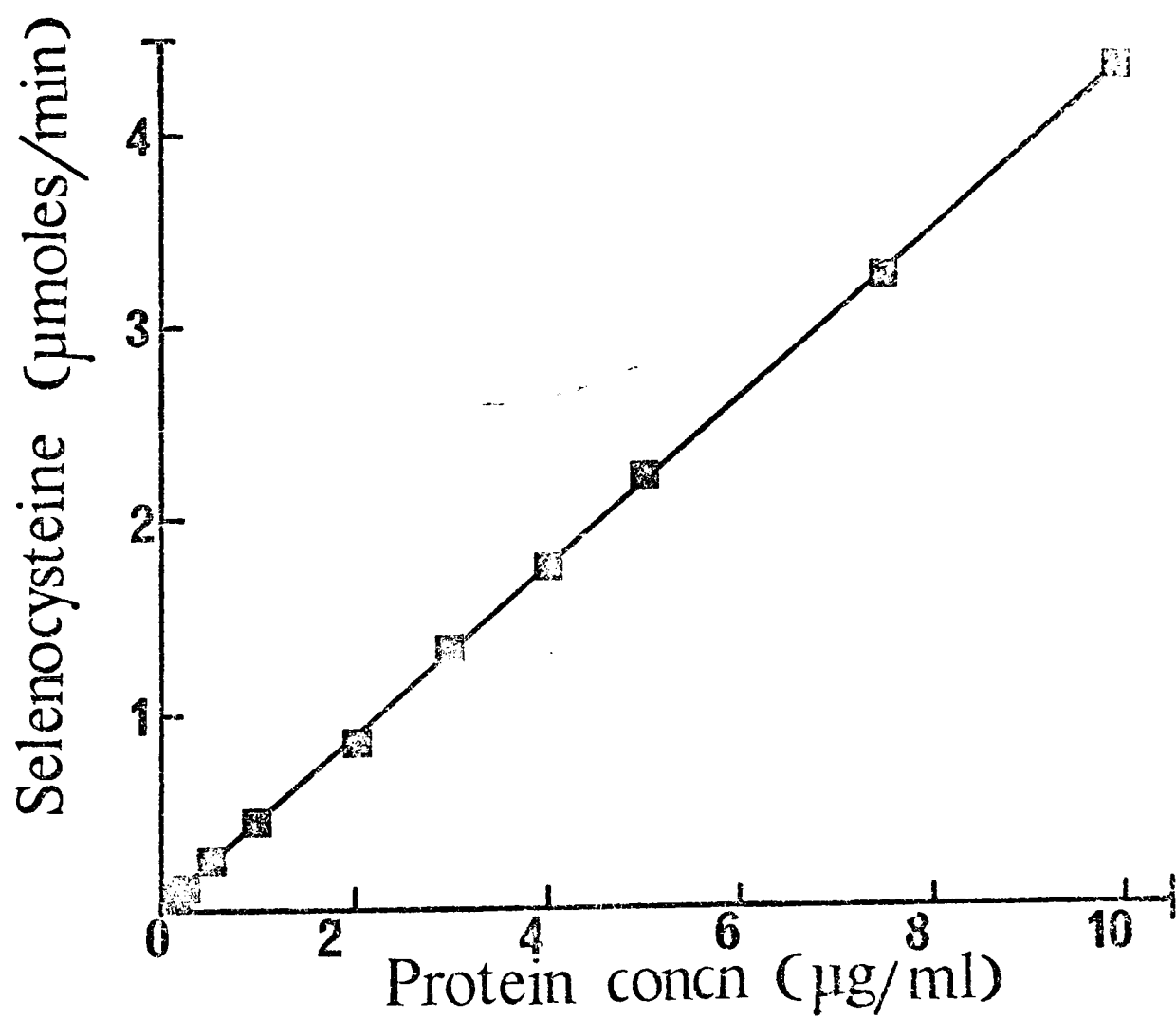
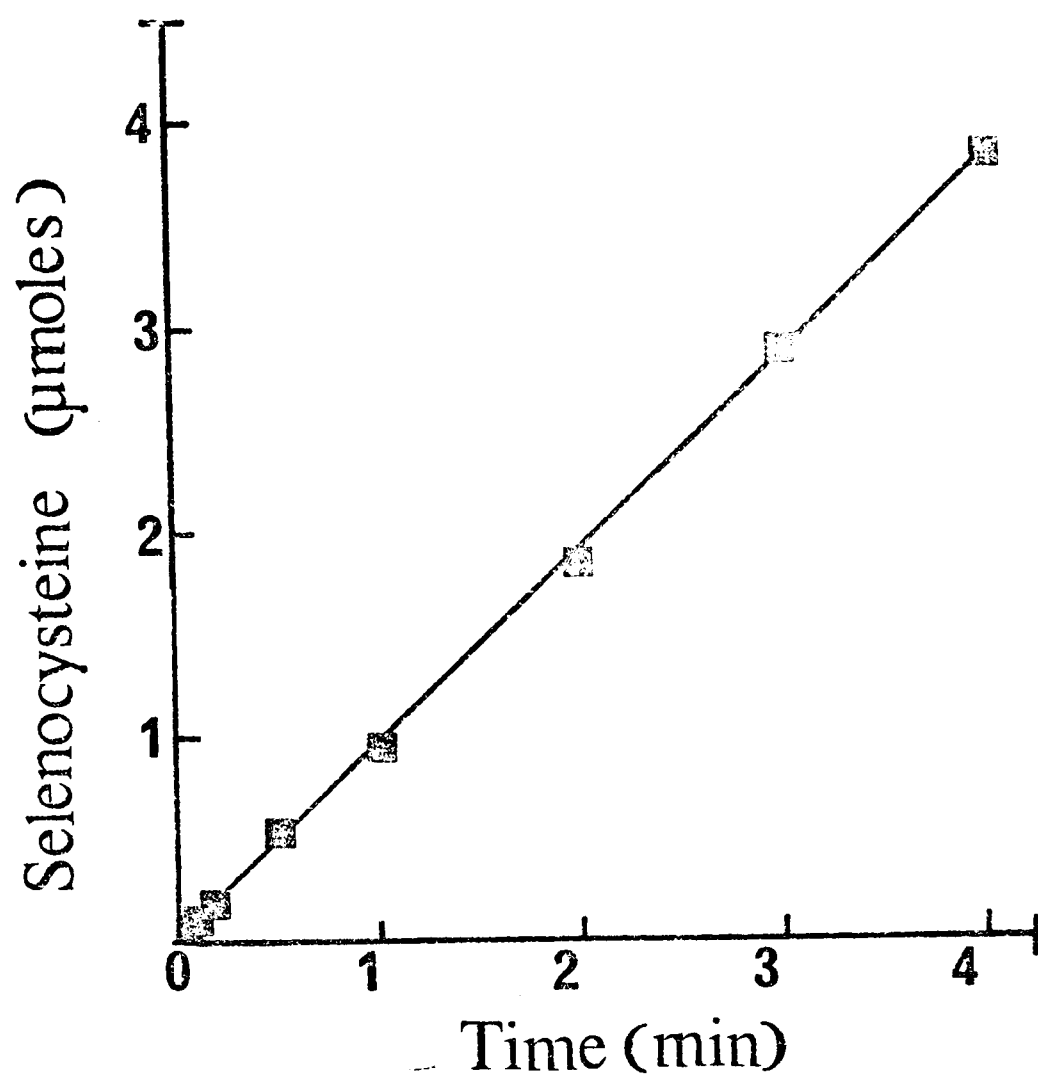
The addition to selenide, to reaction mixtures containing sulphide, inhibited cysteine synthesis, but the kinetics of this inhibition were not investigated.

In an experiment to determine the relative rate of selenocysteine synthesis with respect to the rate of cysteine synthesis, increasing amounts of a saturated solution of selenide were added to reaction mixtures until no further increase in the rate of selenocysteine synthesis was detected; this ensured that the selenide concentration was not limiting. Under these conditions, the rate of selenocysteine synthesis, catalysed by purified O-acetyl serine sulphydrylase was approximately 40% of the rate of cysteine synthesis from sulphide under the same conditions.

Effect of sulphur source during growth on the specific activity of serine transacetylase and O-acetyl serine sulphydrylase. Except when L-cystine was the sole sulphur source for growth, the specific activity of both serine transacety-

Fig. 9.22 Time-course of the synthesis of selenocysteine by purified O-acetyl serine sulphhydrylase.

Fig. 9.23 Effect of protein concentration upon the rate of selenocysteine synthesis by purified O-acetyl serine sulphhydrylase.



lase and O-acetyl serine sulphydrylase remained approximately the same, no matter what the sulphur source included in the growth medium (Table 9.7). Cells grown on sulphate plus homocysteine, or sulphate plus methionine, possessed levels of serine transacetylase and O-acetyl serine sulphydrylase activity similar to cells grown on sulphate alone. When P. denitrificans was grown on L-cystine as the sole sulphur source, the specific activity of both enzymes was double that of cells grown on sulphate as the sole sulphur source.

Growth of P. denitrificans on sulphate, as the sole sulphur source, and in the presence of 1,2,4-triazole, did not affect the specific activity of either serine transacetylase or O-acetyl serine sulphydrylase.

All of the O-acetyl serine sulphydrylase activity was present in the soluble fraction. During the above study, the specific activity of serine transacetylase, from culture grown on the same sulphur source, was found to vary. This appeared to be due to the variable release of serine transacetylase from the plasma membrane during sonication, a conclusion varified by examining the serine transacetylase activity associated with membrane vesicles prepared from cells of P. denitrificans grown in culture medium containing succinate as substrate, nitrate as the terminal electron acceptor, and sulphate as the sole sulphur source, as described in Chapter 2. From these studies it was found that serine transacetylase was bound to the plasma membrane and, since sonication released serine transacetylase from the plasma membrane, it appeared to be loosely bound.

Therefore, in order to obtain accurate estimations of the specific activity of serine transacetylase in crude extracts

prepared from cells grown in the presence of a variety of sulphur sources, cells were treated with 0.1% (v/v) Triton X-100 immediately prior to sonication to ensure a quantitative (96%) release of serine transacetylase activity into the soluble crude extract fraction.

DISCUSSION

Cysteine synthesis. Although O-acetyl serine has not been isolated from a biological source, it has been widely used as a substrate for the enzymic synthesis of cysteine. Enzymes isolated from a variety of bacteria (Chambers & Trudinger, 1971; Kredich & Tomkins, 1966; Kredich et al., 1969; Kredich, 1971) and plants (Giovanelli & Mudd, 1968; Thompson & Moore, 1968; Smith & Thompson, 1971; Smith, 1972) all utilise O-acetyl serine and sulphide for in vitro synthesis of cysteine. The occurrence of serine transacetylase in bacteria and plants, and the fact that O-acetyl serine is the most active substrate for the enzymic synthesis of cysteine, strongly suggests that O-acetyl serine is the most likely substrate for the synthesis of cysteine. Cell-free extracts of P. denitrificans synthesised cysteine from O-acetyl serine and sulphide at a much faster rate than from serine and sulphide.

The occurrence of both serine transacetylase and O-acetyl serine sulphydrylase has been demonstrated in a wide variety of assimilatory and dissimilatory sulphate-reducing bacteria. The enzymes isolated from P. denitrificans exhibited many similar properties to the enzymes isolated from a number of different sources.

Substrate specificity. The reaction catalysed by O-acetyl serine sulphydrylase is a fairly specific one. Only when O-acetyl serine was used as the substrate, providing the

carbon skeleton, was there significant synthesis of cysteine. Since serine is a poor substrate for this reaction, the role of the O-acetyl moiety of O-acetyl serine is, perhaps, to serve as a better leaving group than the hydroxyl group of serine. The acetyl group may also be involved in the binding of substrate to the enzyme by creating a more polar environment. Steric hindrance may also be a factor in the poor utilisation of other than O-acetyl derivatives of serine and homoserine.

Pyridoxal phosphate stimulation. The O-acetyl serine sulphydrylase activity from P. denitrificans is stimulated by pyridoxal phosphate. Although this stimulation has not been demonstrated in many organisms, O-acetyl serine sulphydrylase is generally believed to be a pyridoxal phosphate - requiring enzyme.

Enzyme kinetics. O-Acetyl serine sulphydrylase, purified from P. denitrificans, has K_m values for O-acetyl serine and sulphide similar to those found in other organisms. Becker et al., (1969) obtained two different K_m values for the binding of O-acetyl serine to the enzyme isolated from S. typhimurium; a value of $5 \times 10^{-3} M$ was obtained using a colorimetric assay method, while using a spectrometric method, a value of $6 \times 10^{-7} M$, was obtained. Becker et al., (1969) were unable to explain these two vastly different K_m values. Ngo and Shargool (1973) obtained a K_m value for O-acetyl serine of $1.7 \times 10^{-6} M$ with O-acetyl serine sulphydrylase isolated from rape seed. The two lower K_m values (of Becker et al., 1969 and Ngo & Shargool, 1973) may be more realistic in the living organism. Support for this idea comes from the fact that, in spite of O-acetyl serine being the most likely sub-

strate in vivo for the biosynthesis of cysteine (Giovanelli & Mudd, 1967; Smith & Thompson, 1971; Thompson & Moore, 1968; Thompson et al., 1969) it has not, as yet, been isolated. It is acknowledged that the K_m value of an enzyme for a substrate is an indication not only of the affinity of the enzyme for the substrate, but also for the availability of the substrate in vivo (Cleland, 1970). Thus the low K_m value obtained for O-acetyl serine may be indicative of a very low overall, but perhaps localised concentration of that compound in plant tissues, perhaps explaining the previous lack of success on the part of those who have attempted to isolate it.

Methionine synthesis and enzyme specificity. Two alternative mechanisms have been proposed for the incorporation of sulphur into methionine in microorganisms and plants. One is that sulphur is initially incorporated into cysteine and then transferred to homocysteine via cystathionine (by trans-sulphurylation - see Chapter 10). The other is that sulphide (or methylmercaptan) attaches directly onto a 4-carbon acceptor to form homocysteine (or methionine) (by direct sulphydrylation). The enzymes that operate both these pathways have been identified and found to have a high specificity for a particular hydroxyl amino acid, but a low specificity for sulphide, methylmercaptan or ethylmercaptan (Giovanelli & Mudd, 1968). However, in vivo function and in vitro experiments with enzymes purified from different organisms, do not always correlate.

A discrepancy between the function in vivo and in vitro experiments with this group of reactions was noted in the sulphydrylation of succinyl-homoserine in Salmonella (Flavin

& Slaughter, 1967a). Cystathionine- γ -synthase, previously purified to homogeneity (Kaplan & Flavin, 1966) was also found to catalyse direct formation of homocysteine (or methionine) from O-succinyl homoserine and sulphide (or methylmercaptan). However, evidence that these reactions do not account for homocysteine or methionine synthesis in vivo, was provided by a mutant blocked specifically in the cleavage of cystathionine to homocysteine which was unable to form homocysteine. Therefore homocysteine and methionine synthesis by cystathionine- γ -synthase appears to be primarily an in vitro phenomenon, reflecting the lack of specificity for cysteine as the replacing agent. Biosynthesis of homocysteine by direct sulphydrylation does, however, appear to be consistent with data obtained from yeast (Cherest et al., 1970).

The literature on the pathway of homocysteine synthesis in fungi and plants is confused and requires some clarification. The use of biochemical studies, in conjunction with experiments involving specific bacterial mutants, has helped in the clarification of the understanding of the biosynthesis in bacteria (i.e., sulphide is initially incorporated into cysteine, and is then transferred to homocysteine via cystathionine. Pulse-chase and time-course experiments (Chapter 3) indicated that cysteine was synthesised prior to cystathionine which was, in turn, synthesised prior to homocysteine thus showing that cysteine is synthesised by direct sulphydrylation of O-acetyl serine in P. denitrificans.

At present, the pathway of methionine synthesis is not clearly understood. All the enzymes that might be required to synthesise methionine from serine, acetyl CoA and sulphide are present in plants (Giovanelli & Mudd, 1966, 1967, 1968; Granroth & Sarnesto, 1974; Smith, 1972). Giovanelli & Mudd

(1966, 1967, 1968) have, however, reported the presence of an enzyme which catalyses the synthesis of homocysteine from O-acetyl homoserine and sulphide without the intermediate formation of cysteine or cystathionine. There is, at present, some argument as to the major pathway of homocysteine biosynthesis in plants; synthesis of homocysteine via the intermediate formation of cysteine and cystathionine, or synthesis of homocysteine directly from sulphide and O-acetyl homoserine.

Results presently available suggest, that, in plants, homocysteine is synthesised directly from O-acetyl homoserine and sulphide. Supporting evidence for this argument is provided by (1) the presence of both homoserine transacetylase (Acetyl CoA:L-homoserine O-acetyl transferase EC 2.3.1.31) and O-acetyl homoserine sulphydrylase (O-Acetyl L-homoserine acetate lyase EC 4.2.99.10) (Giovanelli & Mudd, 1967) activities, (2) the very low specific activities of enzymes involved in homocysteine synthesis via cystathionine (especially cystathionine- γ -synthase and β -cystathionase, and (3) the observation by Giovanelli & Mudd (1966) that crude extracts of spinach converted O-acetyl homoserine to S-adenosyl-homocysteine at a rate at least ten times greater than they converted it to cystathionine.

The results of pulse-chase and time-course experiments (see Chapter 3), the high specific activities of both serine transacetylase and O-acetyl sulphydrylase (this Chapter), the high specific activity of β -cystathionase (see Chapter 10), and the absence of detectable homoserine transacetylase or O-acetyl homoserine sulphydrylase activities in crude extracts of P. denitrificans (see this Chapter) indicates

that the synthesis of homocysteine from sulphide in P. denitrificans involves the successive formation of cysteine and cystathionine both acting as intermediates.

Effect of 1,2,4-triazole. It has been reported that 1,2,4-triazole inhibited the growth of S. typhimurium and that this inhibition was overcome by the addition of serine, cysteine or methionine (Bugusloewski et al., 1967; Hulanika et al., 1972). Subsequently, this growth inhibition effect has been found to be due to the lowering of serine transacetylase activity by 1,2,4-triazole, and this causes a lowering of the intracellular concentration of O-acetyl serine. Jones-Mortimer et al., (1968a) have reported that O-acetyl serine is required in E. coli to induce the enzymes of sulphate assimilation; sulphate permease, ATP sulphurylase, APS kinase and sulphite reductase. The inhibition of bacterial growth by 1,2,4-triazole was, therefore, believed to be due to the lowering of serine transacetylase activity, which lowered the intracellular concentration of O-acetyl serine, and thus removed the inducer of enzymes involved in the initial steps of sulphate assimilation (ATP sulphurylase, APS kinase and sulphite reductase). In P. denitrificans, neither the growth, nor the specific activity, of serine transacetylase was affected when cells were grown in the presence of 1,2,4-triazole. In addition, examination of the specific activity of ATP sulphurylase and APS kinase in crude extracts prepared from cells grown in the presence of 1,2,4-triazole and with sulphate as the sole sulphur source, showed that the specific activity of neither enzyme was altered.

Control and regulation of cysteine biosynthesis.

Kredich (1971) reported that serine transacetylase activity

of S. typhimurium was not repressed or derepressed by either cysteine or sulphur starvation; it was, however, inhibited by cysteine. In E. coli (Jones-Mortimer et al., 1968a, Chambers & Trudinger, 1971), Rhodopseudomonas spheroides (Chambers & Trudinger, 1971) and S. typhimurium (Kredich & Tomkins, 1966) the formation of O-acetyl serine sulphydrylase is strongly repressed by the presence of cysteine in growth medium. This is not, however, a general phenomenon, since the levels of O-acetyl serine sulphydrylase in Pseudomonas aeruginosa and Bacillus megaterium were apparently not correlated with the nature of the sulphur source (Chambers & Trudinger, 1971). The specific activity of both serine transacetylase and O-acetyl serine sulphydrylase were almost doubled in cells grown in the presence of cystine. Increase in the specific activity of O-acetyl serine sulphydrylase, when grown with cysteine as the sole sulphur source, has been observed for P. aeruginosa (Chambers & Trudinger, 1971).

Both serine transacetylase and O-acetyl serine sulphydrylase appear to be constitutive enzymes in P. denitrificans but are both controlled by feedback inhibition. Serine transacetylase activity is strongly inhibited by cysteine; this appears to be a property of serine transacetylase in bacteria (Kredich et al., 1969) and plants (Smith & Thompson, 1971). Feedback inhibition of serine transacetylase regulates the amount of O-acetyl serine available for the synthesis of cysteine and therefore plays an important role in regulating the intracellular concentration of cysteine; regulation of the intracellular concentration of cysteine is important since quite low concentrations of cysteine inhibit respiration (see Chapter 2). If serine transacetylase is inhibited by cysteine,

the accumulation of another toxic compound, sulphide, will be prevented by the feedback inhibition by sulphide upon ATP sulphurylase, the initial enzyme in sulphate metabolism (see Chapter 6).

O-Acetyl serine sulphydrylase is also under strict control by feedback inhibition. Accumulation of cystathionine, homocysteine, methionine and cysteic acid - all metabolites of cysteine (see Chapter 3) inhibit O-acetyl serine sulphydrylase activity.

O-Acetyl serine sulphydrylase activity is also inhibited by serine; this could be important since intracellular accumulation of serine would occur if there was a deficiency of acetyl CoA, required to acetylate serine prior to its condensation with sulphide.

The fact that both serine transacetylase and O-acetyl serine sulphydrylase are constitutive enzymes in P. denitrificans provides a means of removing sulphide, a compound extremely toxic to respiration, should it be produced intra- or extra-cellularly.

In S. typhimurium serine transacetylase and O-acetyl serine sulphydrylase are, under certain conditions, associated into a multifunctional complex, cysteine synthetase (Kredich et al., 1969). Smith (Smith, 1972; Smith & Thompson, 1971) showed that serine transacetylase and O-acetyl serine sulphydrylase do not associate, by the observation that a fraction of serine transacetylase activity was firmly bound to mitochondria, whereas all the O-acetyl serine sulphydrylase activity was present in the soluble fraction. In P. denitrificans, no evidence was found to indicate that serine transacetylase and O-acetyl serine sulphydrylase activities were

associated in a protein complex. On the contrary, a situation similar to that in plants was found; serine transacetylase activity was associated with the plasma membrane and the O-acetyl serine sulphydrylase activity was found entirely in the soluble fraction of the cell. In a survey conducted by Chambers & Trudinger (1971) it was found that P. aeruginosa also possessed a membrane bound serine transacetylase.

Synthesis of selenocysteine. This chapter includes the first report of the synthesis of selenocysteine by a purified enzyme preparation, although its synthesis by whole cells of yeast has previously been reported (Blau, 1961). The rate of selenocysteine synthesis in the presence of purified O-acetyl serine sulphydrylase was approximately 40% of the rate of cysteine synthesis under the same conditions. Therefore selenide can substitute for sulphide in the cysteine-synthesising system. This result should be treated with caution since, as mentioned above, O-acetyl serine sulphydrylase has a loose specificity for the sulphide donor in vitro, and this does not reflect the function of the enzyme in vivo.

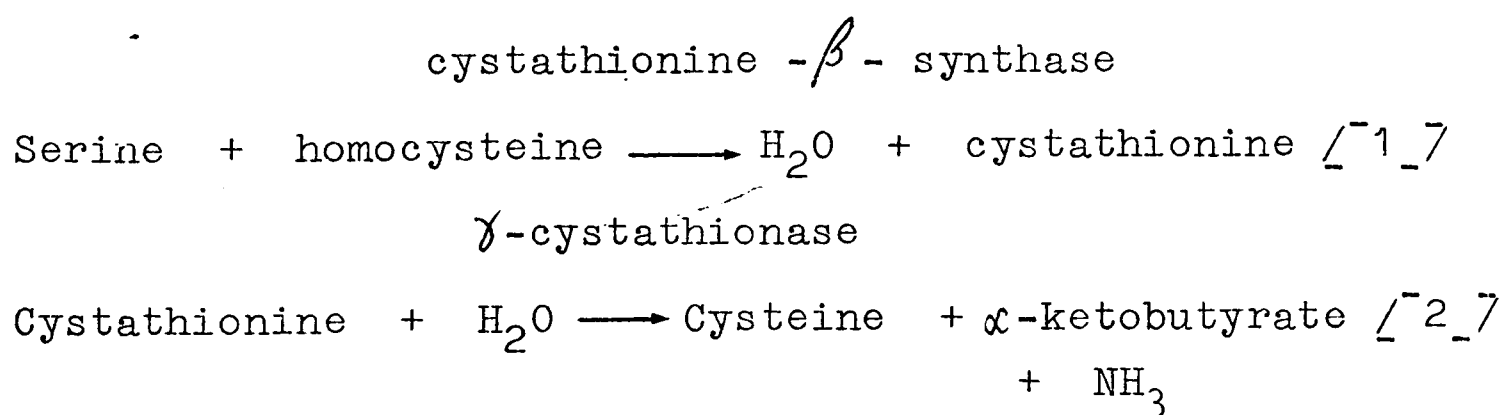
O-Acetyl serine lyase. O-Acetyl serine lyase activity has been previously reported only in plant extracts (Mazelis & Fowden, 1972) but is now reported for the first time in bacterial extracts. Since β -cystathionase, cysteinyl- and methionyl-tRNA synthetases are inhibited by O-acetyl serine (see Chapters 10, 11 and 12, respectively) the function of O-acetyl serine may be to regulate the intracellular concentration of O-acetylserine, and therefore, play a role in the overall regulation of sulphur metabolism.

CHAPTER 10
 β -CYSTATHIONASE

INTRODUCTION

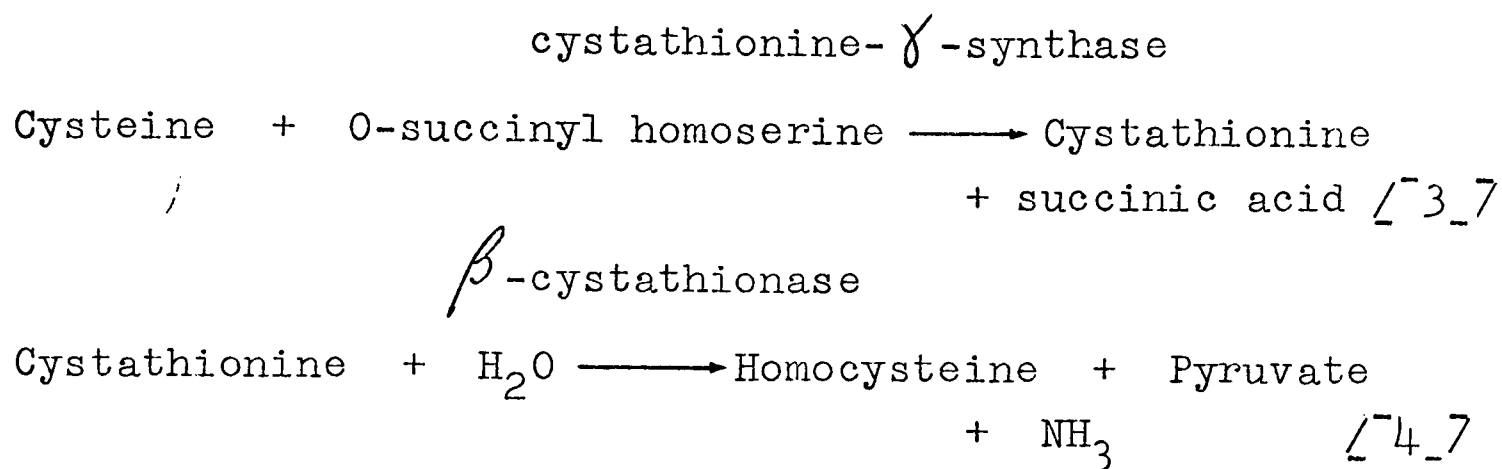
In Chapter 2, results of growth experiments showed that P. denitrificans (NCIB 8944) is unable to utilise cystathionine, homocysteine or methionine as sole sulphur sources for growth and this indicates that P. denitrificans is unable to convert these three sulphur-containing amino acids to forms in which the sulphur can be utilised for growth. In Chapter 3, results from time-course and pulse-chase experiments indicated that cysteine was converted to methionine through homocysteine and cystathionine.

In animals the transfer of sulphur between cysteine and homocysteine (known as transsulphurylation) has been found to occur only in the direction homocysteine to cysteine, through the mediation of two pyridoxal phosphate requiring enzymes (Reactions γ -1 and γ -2) (Binkley et al., 1942; Carroll et al., 1949; Du Vigneaud, 1952; Matsuo & Greenberg, 1958a, 1958b; Selim & Greenberg, 1959);



In a number of fungi, the transfer of sulphur between cysteine and homocysteine is reversible (Horowitz, 1947; Fischer, 1957; Flavin, 1952; Murray, 1960; Flavin & Slaughter, 1964; Delavier-Klutchko & Flavin, 1965). In the bacteria studied to date, which includes E. coli (Rowbury & Woods, 1964; Wijesundera & Woods, 1953, 1962; Delavier-Klutchko & Flavin, 1965), S. typhimurium (Guggenheim, 1971), Proteus morganii (Binkley & Hudgins, 1953; Binkley, 1955) and P. denitrificans strain 11

(Banerjee, 1966), the transfer of sulphur is unidirectional in the direction of cysteine to homocysteine (Reactions Γ_3 and Γ_4), and also occurs by a different mechanism to that in animals;



Banerjee (1966) demonstrated the presence of β -cystathionase activity in cell free extracts of P. denitrificans strain 11, but he did not examine the regulatory properties of the enzyme.

This chapter reports the presence of β -cystathionase activity in cell free extracts and partially purified extracts of P. denitrificans strain NCIB 8944, and reports on some of the regulatory properties of the enzyme.

MATERIALS AND METHODS

Chemicals. Pyruvate (sodium salt), α -ketobutyrate (sodium salt), pyridoxal 5'-phosphate, L-cystathionine, hydroxylamine, homoserine, and NADH were all obtained from the Sigma Chemical Co., London, U.K. DL-Lanthionine, 2,4-dinitrophenyl-hydrazine and $(\text{NH}_4)_2\text{SO}_4$ (specially low in heavy metal ions) were obtained from British Drug Houses, Dorset, U.K. Thin layer chromatography plates (20 x 20 cm; 6060 silica gel with fluorescent indicator) were obtained from Eastman, Kodak Ltd., Liverpool, U.K. β -Cyanoalanine was obtained from Calbiochem Ltd., London, U.K. Dowex AG1-X2 (200 - 400 mesh) chloride form, was obtained from Biorad Labs.,

Richmond, California, U.S.A. Nitrogen (oxygen free) gas was obtained from Air Products Ltd., U.K. Bis(trimethylsilyl) trifluoroacetamide (BSTFA) was obtained from the Sigma Chemical Co., London, U.K, and acetonitrile was obtained from British Drug Houses, Dorset, U.K. OV 1, OV 17 and Gas Chrom Q, 100- 200 mesh were obtained from Applied Science Laboratories, U.S.A.

Instruments. Infra red spectral studies were done with a Hilger-Watts Infracgraph Mk 2 Model H 1200. KCl discs (13 mm diam) of the compounds to be examined were pressed using a minidie (obtained from Spectroscopic Accessories Company, Kent, U.K.).

Assay of β -cystathionase activity. β -Cystathionase activity was measured by two methods.

Method 1_7. Pyruvate was measured by a modification of the procedure of Friedemann (1957). Reaction mixtures, in a total volume of 1 ml, were conducted in Eppendorf mini-centrifuge tubes and contained; 200 μ moles of Tris-phosphate buffer, pH 9.0, 10 nmoles of pyridoxal phosphate, 10 μ moles of L-cystathionine and enzyme extract. After incubation at 30°C for 15 min, reactions were terminated by the addition of 0.2 ml of 15% (w/v) trichloroacetic acid. Terminated reaction mixtures were centrifuged to remove the protein and 1.0 ml of the supernatant was added to 0.5 ml of a 0.1% (w/v) solution of 2,4-dinitrophenylhydrazine in 2M-HCl. Following thorough mixing, solutions were allowed to stand for 10 min at 30°C after which 2.0 ml of 1.5M-NaOH was added. After shaking, the brownish-orange colour was allowed to develop for 20 min at 30°C, and then the absorbance at 520 nm was read. Control reaction mixtures, stopped at time zero, were conducted for

each experiment to allow for any non-enzymic production of pyruvate. A sample containing 288 nmoles/ml of the 2,4-dinitrophenylhydrazone of pyruvate gives an absorbance of 1.0 at 520 nm, while a sample containing 408 nmoles/ml of the 2,4-dinitrophenylhydrazone of α -ketobutyrate gives an absorbance of 1.0 at 520 nm.

This assay method can be used with crude extracts but has the disadvantage of being a slow assay technique. A second method, which allows β -cystathionase activity to be continuously monitored, spectrophotometrically, has been previously described (Guggenheim, 1971). Due to interference by high endogenous levels of NADH dehydrogenase activity in crude extracts, Method 2_7 can only be used to assay β -cystathionase activity in partially purified preparations.

Method 2_7. The enzyme activity was determined by the spectrophotometric method of Guggenheim (1971) in which pyruvate formation was coupled to the oxidation of NADH in the presence of excess lactate dehydrogenase. Reactions were conducted in 1 ml cuvettes of 1 cm light path at 30°C in a thermoregulated Pye Unicam SP 8000 spectrophotometer. Reaction mixtures contained, in a final volume of 1 ml; 200 μ moles of Tris-phosphate buffer, pH 9.0, 10 nmoles of pyridoxal phosphate, 0.3 μ moles of NADH, 10 μ moles of L-cystathionine, 10 units of lactate dehydrogenase and enzyme extract. The reaction was initiated by the addition of L-cystathionine and the optical density at 340 nm, versus a water blank, was recorded continuously on a Pye Unicam AR 25 linear recorder linked to the spectrophotometer.

Preparation and identification of 2,4-dinitrophenylhydrazone derivatives of pyruvate and α -ketobutyrate. 2,4-Dini-

trophenyldiazine derivatives of pyruvate were prepared and separated as described by Smith (1961). An excess of 2,4-dinitrophenyldiazine in 2M-HCl (0.1%, w/v solution) was added to 0.5 g of the keto acids in 10 ml of water. Yellow crystals of the hydrazones formed immediately. After standing at 30°C for 2 h, and at 4°C overnight, the crystals were filtered off and recrystallised from ethanol three times. The infra red and u.v. spectra, and the extinction coefficients, were consistent with the reported data for both the pyruvate and the α -ketobutyrate 2,4-dinitrophenyldiazine derivatives.

A similar method was used for isolating the keto acid 2,4-dinitrophenyldiazine derivatives from the reaction mixtures. Five ml of a 0.1% (w/v) solution of 2,4-dinitrophenyldiazine in 2M-HCl was added to a 5 ml reaction mixture and the solution left at 30°C for 1 h. 10 ml of ethyl acetate was added to the solution and mixed thoroughly by vigorous bubbling with nitrogen. The two layers were allowed to settle and were further separated by centrifugation. The aqueous layer was removed and reextracted with 2 ml of ethyl acetate. The ethyl acetate fractions were pooled and this solution extracted with three successive 2 ml volumes of Na_2CO_3 (10%, w/v, in water). The Na_2CO_3 layers were combined and extracted once with 1 ml of ethyl acetate to remove traces of 2,4-dinitrophenyldiazine. The pH of the solution was adjusted to pH 2.0 with HCl and the solution extracted three times with 2 ml of ethyl acetate. The ethyl acetate fractions were pooled and evaporated to dryness, and dissolved in 0.5 ml of solvent 1. The keto acid dinitrophenyldiazine derivatives were separated and identified by thin layer chromatography in solvent I. Chromatograms were developed by

ascending chromatography in solvent I for 8 h. Plates were then dried at room temperature, dipped in a 5% (w/v) solution of KOH in ethanol, and dried again. Keto acid dinitrophenolhydrazone derivatives appear as brown spots on a white background. Alternatively, derivatives can be detected under a u.v. lamp at 366 nm, either before or after treatment with KOH.

Separation or reaction products by high voltage paper electrophoresis. Electrophoretic separation of reaction products was conducted on acid-washed Whatman 3 MM paper soaked in solvent II. Electrophoresis was conducted for 2 h with a voltage gradient of 42 v/cm.

Amino acids were visualised as described in Chapter 3.

Solvents

I, n-butanol — ethanol — water (70 : 20 : 10, by vol),

II, 78 ml formic acid (90%, v/v) and 148 ml glacial acetic acid, titrated to pH 1.8 with HCl and made up to 2.5 l.

Identification of reaction products by gas-liquid chromatography. A 50 ml reaction mixture was run for 16 h at 30°C and the reaction was terminated, and the protein precipitated, by boiling for 3 min. The precipitated protein was removed by filtration and the filtrate applied to a Dowex 1-X2 (200 - 400 mesh) column in the OH⁻ form. The column (15 x 1.5 cm) was washed with distilled water until the pH returned to neutral. The amino and keto acids were then eluted with 0.2 M-HCl. Fractions were collected and monitored for pH, amino and keto acids. Both amino and keto acids were eluted in a small volume (approx. 7 ml) when the pH of the column eluent dropped sharply from neutrality to 1. The fraction containing the amino and keto acids was evaporated to dryness.

Approximately 1 mg of the resultant powder was derivatised in 0.1 ml of a 50/50 (v/v) mixture of BSTFA and acetonitrile in a sealed derivatisation tube, in an oil bath, at 150°C for 1 h. 0.5 μ l of the resultant solution was examined by gas-liquid chromatography.

Gas chromatographic identifications were carried out with a Pye Series 104 Gas Chromatogram with a flame ionisation detector. Nitrogen (oxygen free) was used as the carrier gas. Glass columns 1.5 m long and with 5mm internal diameter, were used.

Two different column packings were used; Column 1 was packed with 5% (w/w) OV 17 on Gas Chrom Q, 100 - 120 mesh, and Column 2 was packed with 5% (w/w) OV 1 on Gas Chrom Q, 100 - 120 mesh. The carrier gas flow rate was maintained at 40 ml/min for both columns.

When Column 1 was used for separation of the reaction products a temperature program starting at 100°C and finishing at 250°C, with a temperature gradient of 5°C/min was employed. When Column 2 was used, a temperature program of an initial 5 min hold at 80°C, a temperature gradient of 5°C/min, and a final hold at 250°C for 5 min was employed. The injector heater was set at 5, and the detector oven set at 250°C. The Pye Gas Chromatogram was linked to a Hewlett-Packard Integrator.

Partial purification of β -cystathionase. Cells of P. denitrificans were grown, harvested and a cell-free extract prepared from them as described in Chapter 5. All subsequent procedures were conducted at 4°C. The cell-free extract was brought to 80% saturation by the slow addition of solid ammonium sulphate whilst the extract was rapidly stirred. After all the ammonium sulphate was added, the solution was

stirred for a further 30 min. Precipitated protein was recovered by centrifugation, dissolved in 15 ml of 20 mM-Tris-phosphate buffer, pH 9.0, applied to a Sephadex G-200 column (90 x 3.5 cm), previously equilibrated with buffer, and eluted with 20 mM-Tris-phosphate buffer, pH 9.0. Fractions were assayed and the active fractions were pooled and loaded onto a DEAE-cellulose column (10 x 2.5 cm) and the column washed with 50 ml of Tris-phosphate buffer. A KCl gradient of 0 to 0.5M was developed and the enzyme eluted at a flow rate of 0.55 ml/min. The fractions were assayed and the active fractions were pooled, dialysed against 20 mM-Tris-phosphate buffer, pH 9.0 for 12 h, and then applied to another DEAE-cellulose column (3 x 1 cm) equilibrated with Tris-phosphate buffer, pH 9.0. The enzyme was eluted with 0.5M-KCl (in buffer) in a volume of 8 ml. The enzyme fraction was dialysed against 20 mM-Tris-phosphate buffer, pH 9.0 for 8 h (to remove the KCl) and then stored at -15°C.

RESULTS

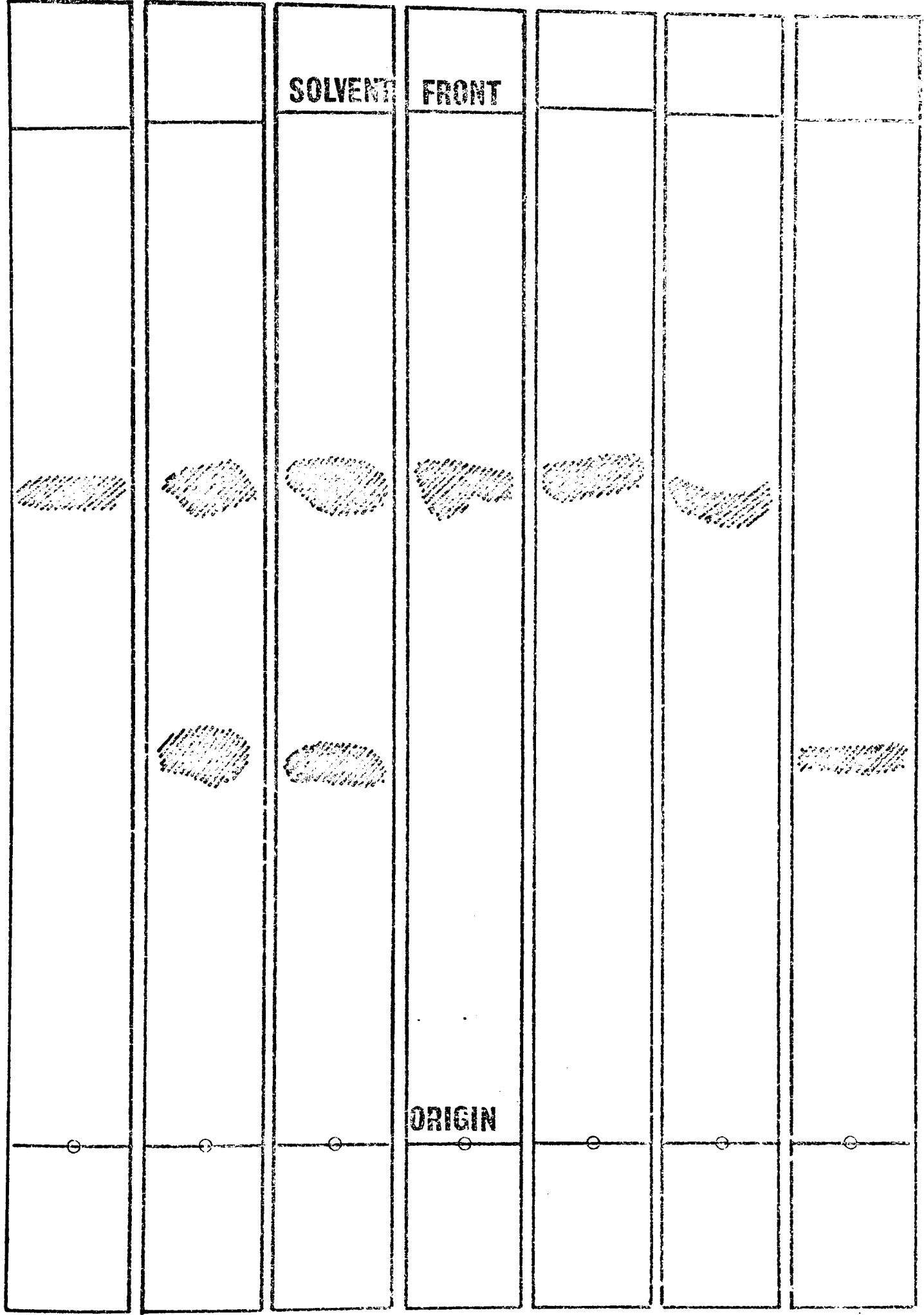
Identification of reaction products. The enzymic release of pyruvate and homocysteine was confirmed by a variety of methods.

Cysteine and α -ketobutyrate, which are also possible products of cystathionine metabolism, were not detected. Thin layer chromatography of the keto acid 2,4-dinitrophenylhydrazones extracted from reaction mixtures ran with the same R_f value as pyruvate-2,4-dinitrophenylhydrazone and ran well separated from the α -ketobutyrate-2,4-dinitrophenylhydrazone (Fig. 10.1). The keto acid derivative extracted from the reaction mixtures had a light absorption spectrum identical to the pyruvate derivative in both the visible and

Fig. 10.1

Identification of pyruvate 2,4-dinitrophenylhydrazone
by thin layer chromatography

The keto-acid formed during incubation mixtures containing β -cystathionase was isolated, purified and concentrated as described in the text. 2,4-Dinitrophenylhydrazone derivatives of pyruvate and α -keto butyrate were prepared as described in the text. Samples (10 μ l) were streaked onto the chromatogram using a glass micropipette. The applied samples were dried and the chromatogram developed by ascending chromatography in solvent 1 for 8 h. Following development the plate was dried at room temperature and dipped in an ethanolic solution of KOH and again dried. The 2,4-dinitrophenylhydrazone derivatives were visualised by UV absorption at 366nm.



PYRUVATE
+
SAMPLE

α-KETO BUTYRATE
+
SAMPLE

α-KETO BUTYRATE
+ PYRUVATE
+ SAMPLE

SAMPLE

PYRUVATE

SAMPLE

α-KETO BUTYRATE

the u.v. regions. In the presence of lactate dehydrogenase, pyruvate is rapidly oxidised whilst α -ketobutyrate is oxidised at a much slower rate. The rate of pyruvate release from L-cystathionine, catalysed by purified β -cystathionase, as measured by the direct method (Method [17]) and the indirect method (Method [27]), was in close agreement and therefore showed that the keto acid released from cystathionine was pyruvate.

L-Homocysteine was identified as a reaction product by high voltage electrophoresis; the absence of cysteine was also confirmed.

The presence of both pyruvate and homocysteine and the absence of α -ketobutyrate and cysteine in terminated reaction mixtures, was also demonstrated by gas-liquid chromatography (Fig. 10.2 and 10.3).

Enzyme kinetics Effect of time and protein concentration

Unpurified cell-free extracts of P. denitrificans catalysed the cleavage of L-cystathionine to pyruvate, as measured by method 1. In early experiments, the release of pyruvate from L-cystathionine was not linear with time or protein concentration, and it was found that pyruvate was being removed from reaction mixtures at a significant rate. In reaction mixtures in which L-cystathionine was replaced by pyruvate, removal of pyruvate from reaction mixtures was observed (Table 10.1). The cell-free extract was therefore partially purified to remove the contaminating enzyme(s) responsible for the removal of pyruvate. Following partial purification of the cell-free extract, the release of pyruvate from L-cystathionine was linear as a function of time (Fig. 10.4), and protein concentration (Fig. 10.5). The enzyme was sensitive to heat;

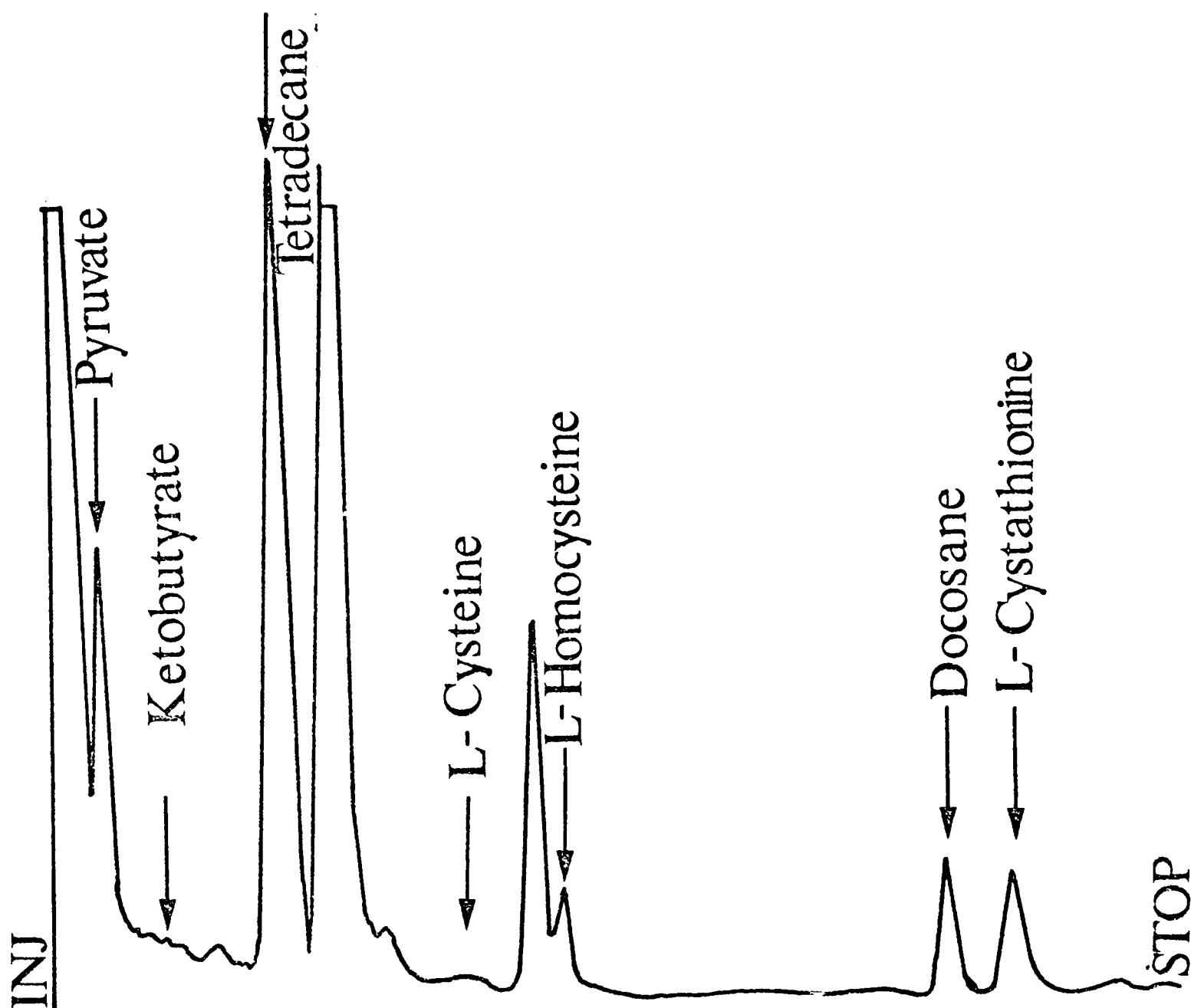


Fig. 10.2

Identification of the reaction products by gas-liquid chromatography

A 50 ml reaction mixture, containing cell-free extract as the source of β -cystathionase activity, was run for 16 h, deproteinated and the amino- and keto-acids purified, concentrated and derivatized as described in Materials and Methods. 0.5 μ l Of the derivatized solution was subjected to gas liquid chromatography. The 1.5m x 4mm glass column was packed with 5% (w/w) OV17 on Gas Chrom Q 100-120 mesh. The temperature program was begun at 100 $^{\circ}$ and increased at a gradient of 5 $^{\circ}$ /min up to 250 $^{\circ}$.

Fig. 10.3

Identification of the reaction products by gas-liquid chromatography

The isolation and purification of the keto- and the amino- acids was the same as described in Figure 11.6. 0.5 μ l of the derivatized solution was subjected to gas-liquid chromatography. The 1.5m x 4mm I.D. glass column was packed with 5% (w/w) OV1 on Gas Chrom Q 100-120 mesh. The temperature program consisted of a 5 min hold at 80°, a temperature gradient of 5°/min and a final hold of 5 min at 250°.

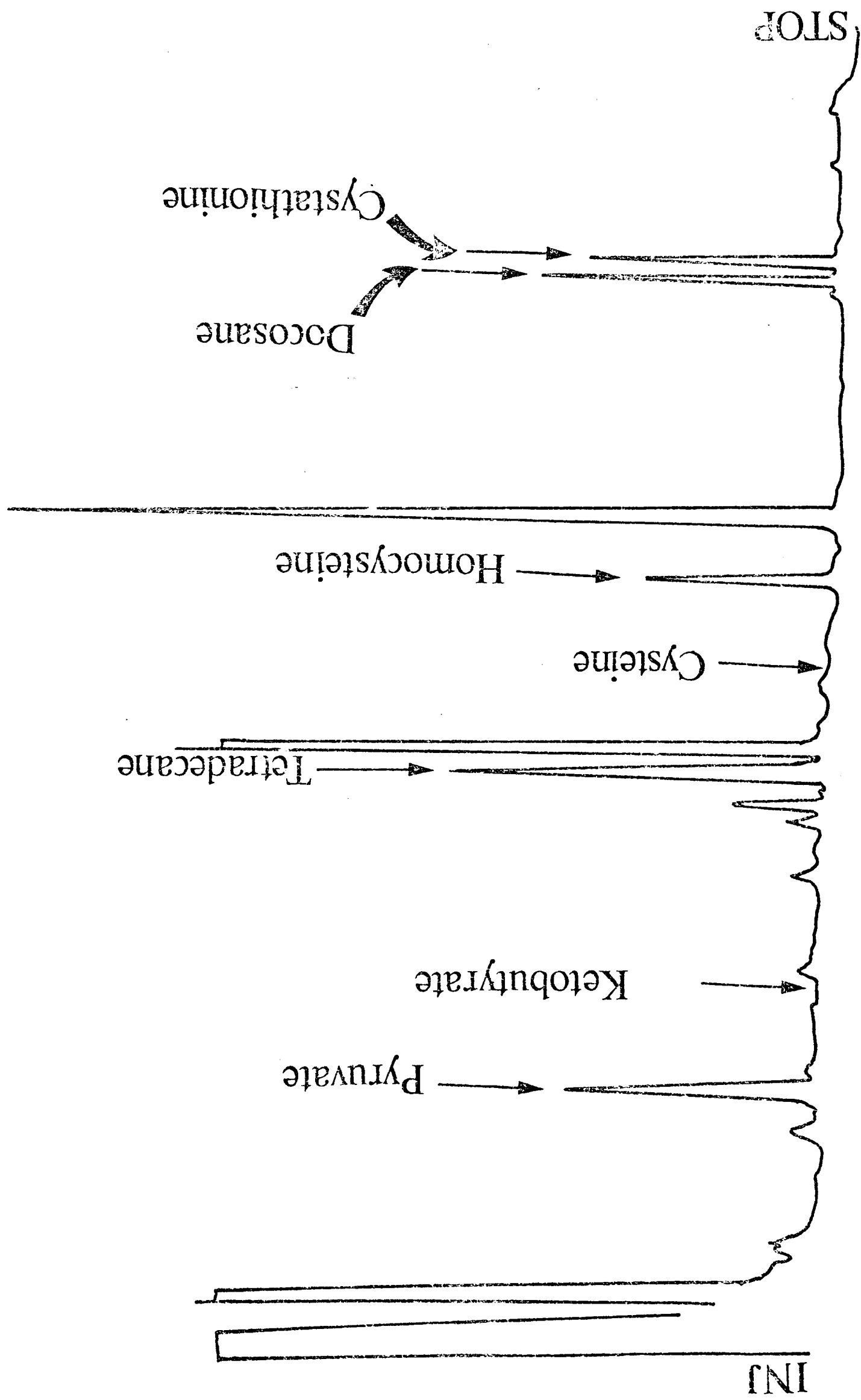


Fig. 10.4 The effect of time on β -cystathionase activity.

Reaction mixtures were as described in Materials and Methods. Partially purified β -cystathionase was used as the source of enzyme and the protein concentration in the reaction mixtures was maintained at 0.063 mg per ml. β -Cystathionase activity was measured by Method 1.

Figure 10.5 The effect of protein concentration on the rate of release of pyruvate from L-cystathionine.

Reaction mixtures were as described in Materials and Methods except that the amount of enzyme added was as stated. The partially purified β -cystathionase was used as the source of enzyme and the reactions were run for 15 min. β -Cystathionase activity was measured by Method 1.

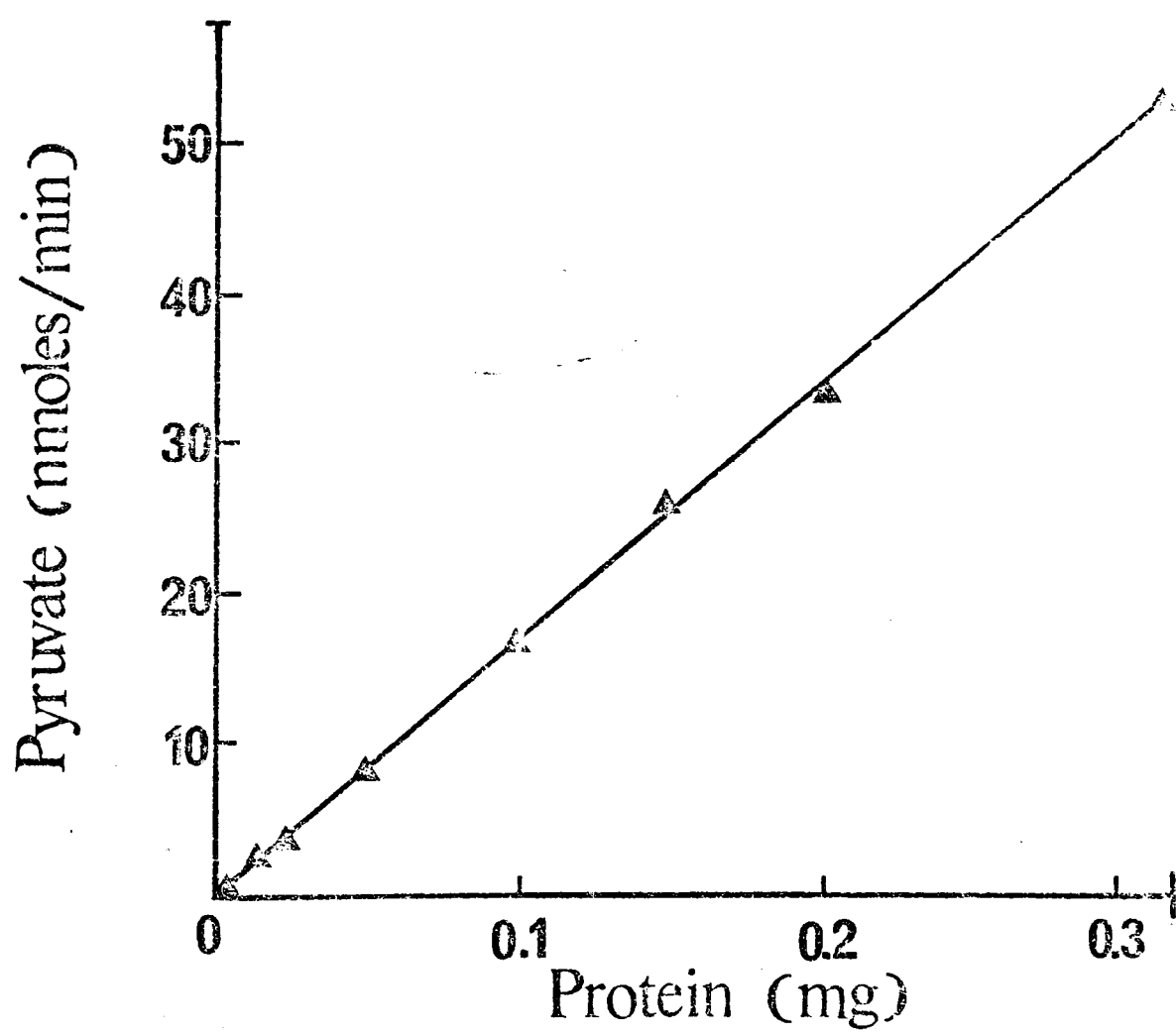
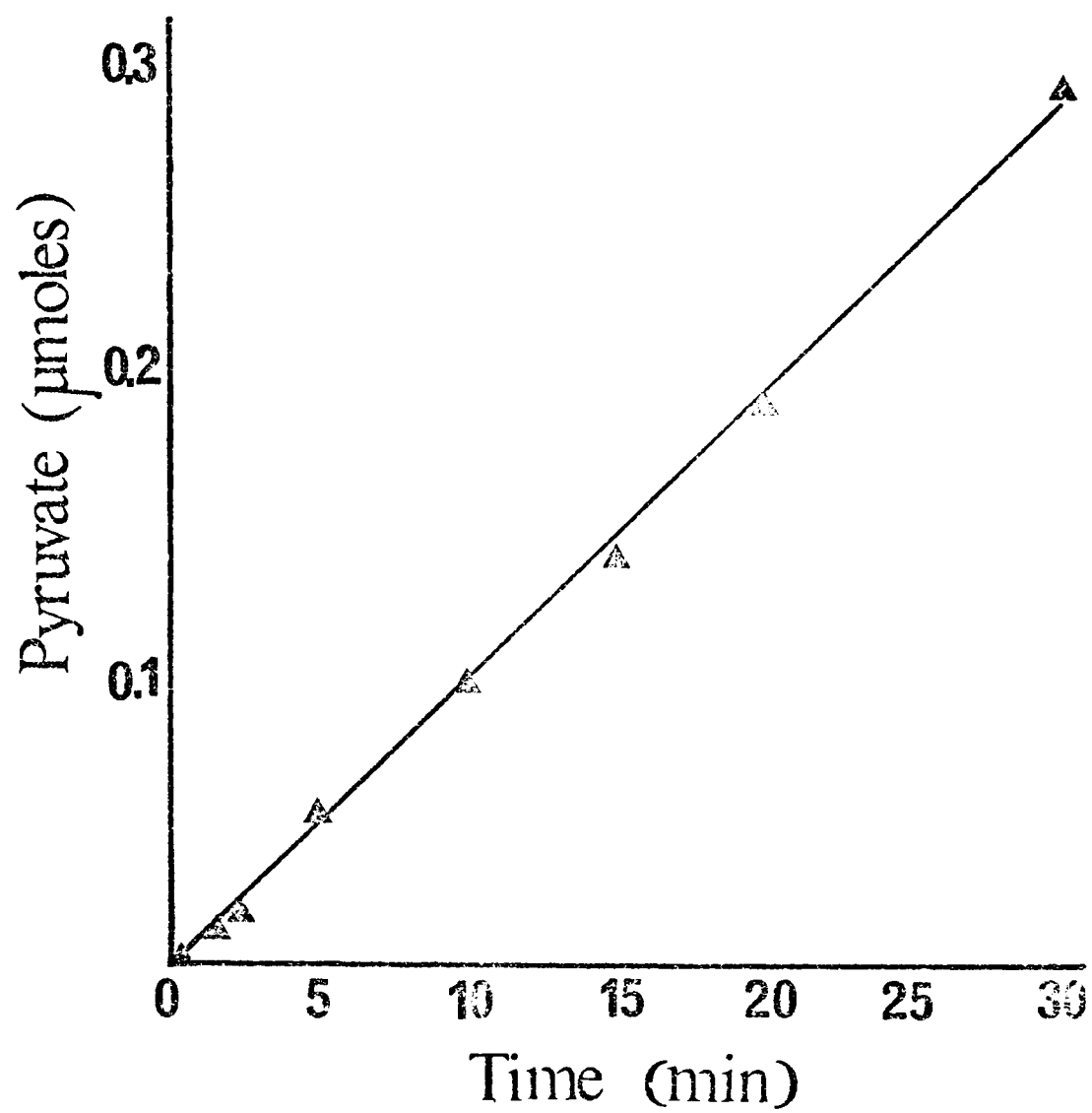


TABLE 10.1

Enzymic removal of pyruvate from incubation
mixtures containing cell-free extracts and partially-
purified enzyme preparations

Reaction mixtures were as described in Materials and Methods except that L-cystathionine was replaced by pyruvate as specified in the Table. The cell-free extract and the partially-purified enzyme preparation were used as the source of enzyme. Reactions were conducted at 30° and the amount of pyruvate remaining in the incubation mixtures was determined using the 2,4-dinitrophenylhydrazine method.

Addition to assay	Enzyme source	nmoles of pyruvate remaining in reaction mixtures after		
		0 min	15 min	30 min
Nil	Cell-free extract	0	3	7
100 nmoles of pyruvate	" " "	98	67	52
200 nmoles of pyruvate	" " "	207	157	123
Nil	Partially-purified enzyme	0	0	2
100 nmoles of pyruvate	"	103	100	101
200 nmoles of pyruvate	"	209	206	204

pretreatment at 100°C for 3 min abolished the release of pyruvate from L-cystathionine.

Effect of L-cystathionine concentration. The rate of pyruvate release from L-cystathionine as measured both by methods Γ^{-1}_7 and Γ^{-2}_7 , increased with concentration up to 9 mM. A double reciprocal plot of the effect of varying L-cystathionine concentration, upon the rate of pyruvate production, gave an apparent K_m of 4.2 mM (Fig. 10.6). 10 mM-L-Cystathionine was included in all subsequent reaction mixtures.

Effect of pH. No single buffer system was satisfactory for studying the pH optimum of β -cystathionase. However, maximum activity was found to lie between pH 9.0 and 9.5 when a number of buffer systems were used. β -Cystathionase activity was measured by method Γ^{-1}_7 . In the presence of 0.2M-glycine-NaOH buffer, β -cystathionase activity was only 20% of that found in both the Tris-phosphate and the Tris-pyrophosphate buffers (Fig. 10.7) at the same molarity and pH. This inhibitory effect was found to be due to glycine, which strongly inhibits β -cystathionase activity. Notwithstanding this effect, β -cystathionase activity decreased sharply at pH values above pH 9.5. In all subsequent reaction mixtures, the pH was maintained at pH 9.0 using 0.2M-Tris-phosphate buffer.

Effect of inhibitors. β -Cystathionase activity was inhibited by the sulphydryl-group reagents, iodoacetamide, p-chloromercuribenzoate and N-ethylmaleimide and by the carbonyl-group reagent, phenylhydrazine (Smythe, 1963). Inhibition of pyruvate release from L-cystathionine, by phenylhydrazine, may have been caused by the reaction between phenylhydrazine and

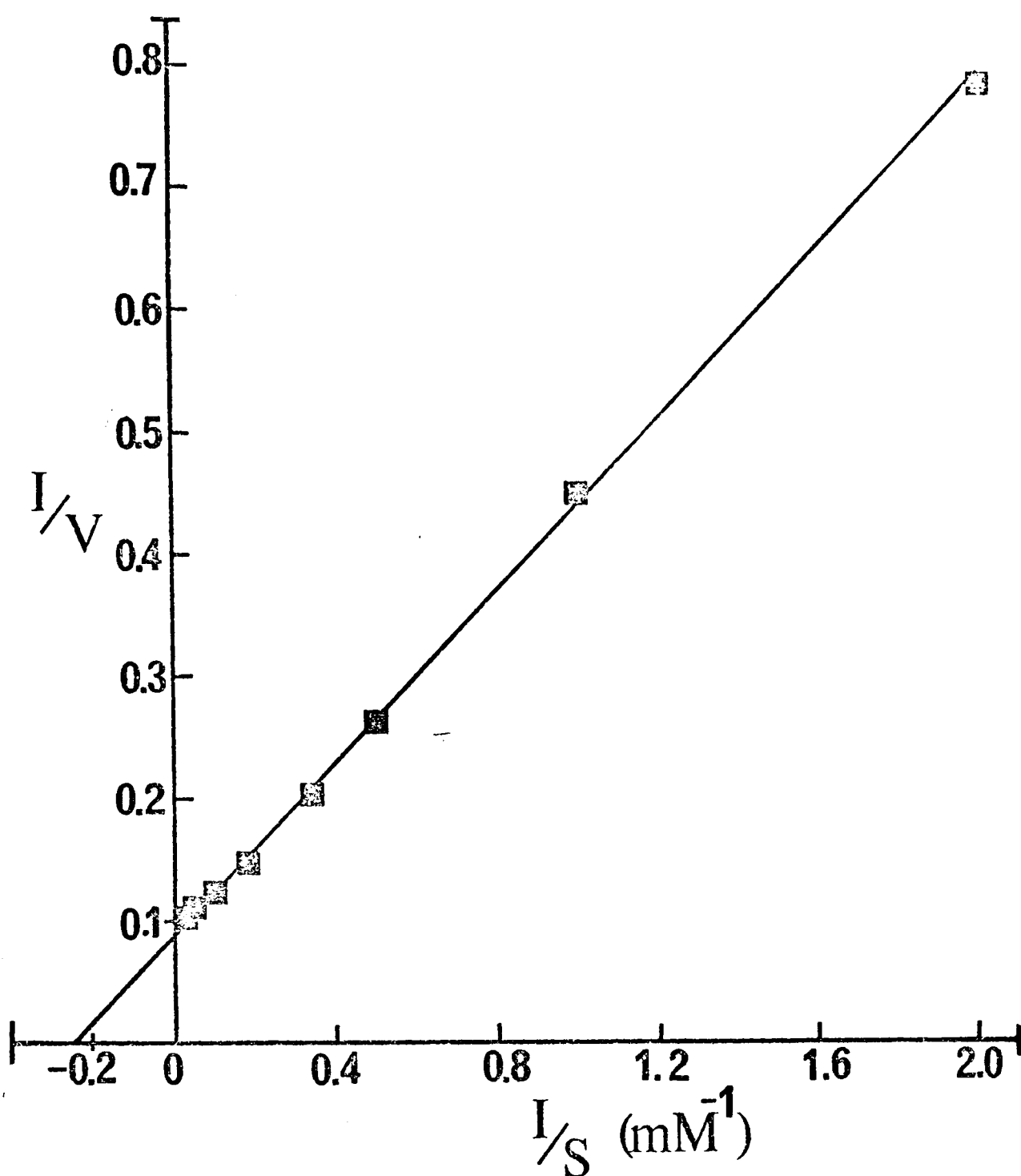


Fig. 10.6

Double reciprocal plot of the effect of concentration of L-cystathionine on β -cystathionase activity.

Incubation mixtures contained 0.063 mg of protein and assays were conducted in the presence of 5mM dithiothreitol; all other conditions of the assay were as described in the text except that the standard amount of L-cystathionine was replaced with the amounts specified.

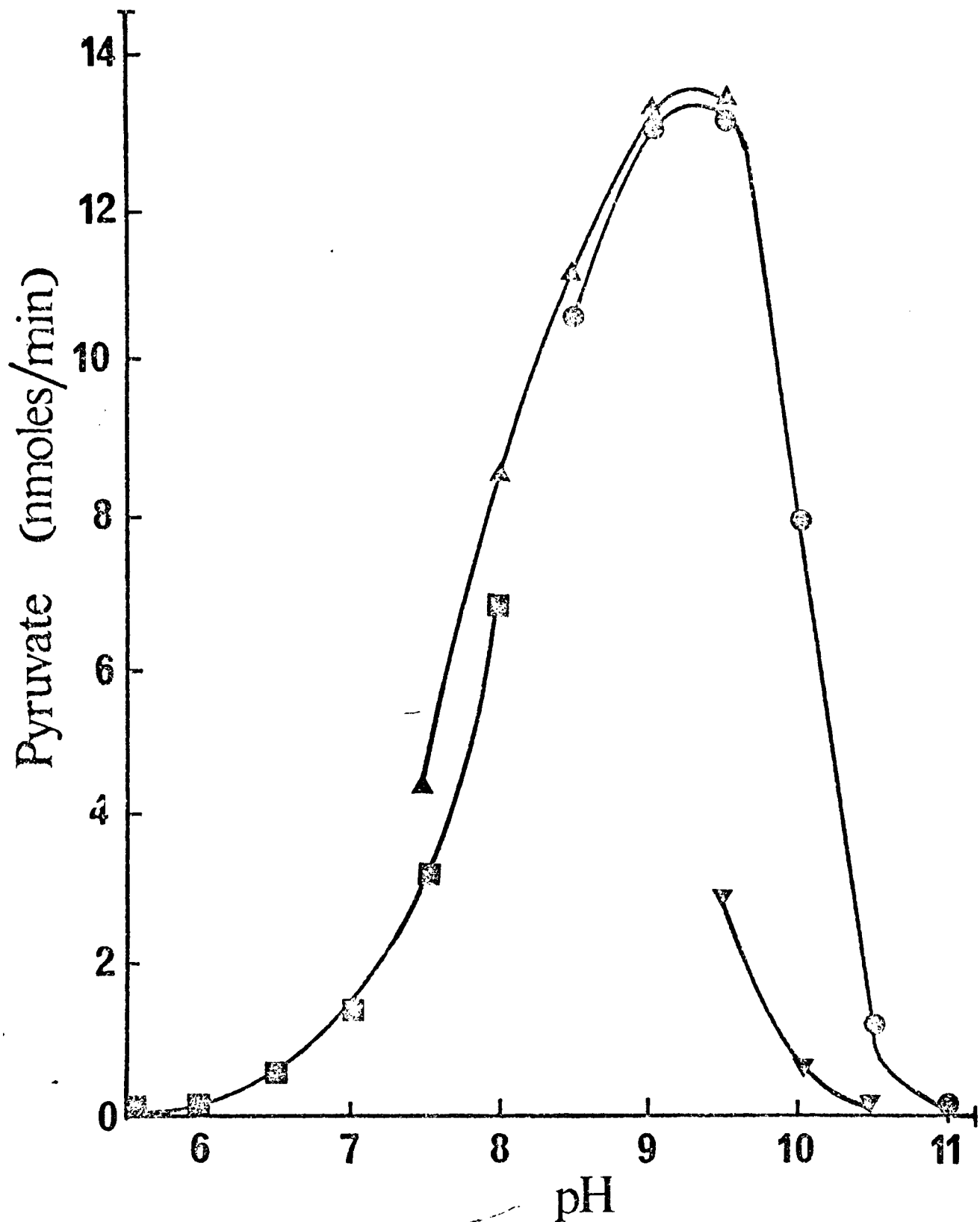


Fig. 10.7

The effect of pH on β -cystathionase activity

β -Cystathionase activity was measured by Method 1; the incubation mixtures were as described in the text except that Tris-phosphate buffer, pH 9.0 was replaced by the buffers specified. The buffers were prepared as described by Gomori (1955) and 0.5ml of the buffer was used in each assay.

■, Tris-maleate - KOH buffer; ▲, Tris-phosphate buffer;

●, Tris-pyrophosphate buffer; ▼, Glycine-NaOH buffer. The partially purified β -cystathionase was used as the source of enzyme.

the carbonyl group of L-cystathionine, thus preventing cystathionine from acting as a substrate. β -Cystathionase activity was completely inhibited by KCN (Table 10.2).

TABLE 10.2

Effect of some common inhibitors on the activity of partially-purified β -cystathionase

β -Cystathionase activity was measured by Method 1₇. The absolute activity of the partially purified β -cystathionase preparation in standard assays, as measured by Method 2₇, was 5.03 units/mg of protein. Results are expressed as a percentage of the activity measured by Method 2₇.

<u>Addition</u>	<u>Activity</u> (%)
Iodoacetamide (10m <u>M</u>)	45
p-Chloromercuribenzoate (5 <u>μM</u>)	5
N-Ethylmaleimide (10m <u>M</u>)	6
Potassium cyanide (10m <u>M</u>)	0
Potassium fluoride (10m <u>M</u>)	84
Phenylhydrazine (10m <u>M</u>)	0

Effect of cofactors. In the absence of pyridoxal phosphate, the rate of enzymic release of pyruvate from L-cystathionine as measured by both methods 1₇ and 2₇, was approximately 6% of the control (Table 10.3). Addition of 10 nmoles of pyridoxal phosphate allowed maximum β -cystathionase activity. The addition of hydroxylamine almost completely inhibited β -cystathionase activity but Mg^{2+} , EDTA,

ATP, ADP or dithiothreitol had no effect.

TABLE 10.3

Effect of cofactors upon partially-purified
 β -cystathionase activity

β -Cystathionase activity was measured by Method 1_7.

All additions to the reaction mixtures were preincubated for 5 min prior to initiating the reaction by the addition of L-cystathionine. The enzyme activity was the same as that described in Table 10.2

<u>Additions</u>	<u>Activity</u> (%)
Hydroxylamine (<u>10mM</u>)	0
EDTA (<u>10mM</u>)	102
MgCl ₂ (<u>10mM</u>)	97
Pyridoxal phosphate (<u>0.1mM</u>)	100
Dithiothreitol (<u>5mM</u>)	106
ATP (<u>5mM</u>)	102
ADP (<u>5mM</u>)	100

Substrate specificity. The partially purified β -cystathionase preparation catalysed the cleavage and release of pyruvate from a number of substrates other than cystathionine. DL-Lanthionine was the most active substrate; β -cystathionase also catalysed the hydrolytic cleavage of L-djenkolic acid and L-cystine. L-Cysteine and L-homocysteine, in the presence of dithiothreitol, L-homoserine and β -cyano alanine could not be shown to be substrates for the partially purified β -cystathionase when measured by both methods 1_7 and 2_7

(Table 10.4).

TABLE 10.4

Substrate specificity of the partially purified
 β -cystathionase.

Reaction mixtures were as described in the text except that L-cystathionase was replaced by the compounds specified.

<u>Substrate</u>	<u>Activity</u> (%)
L-Cystathionase (10mM) *	100
L-Cysteine (10mM)	0
L-Cystine (5mM)	28
DL-Lanthionine (20mM)	136
L-Djenkolic acid (10mM)	79
L-Homoserine (10mM)	0
L-Homocysteine (5mM)*	5
L-Glutathionine (10mM)	0
L-Methionine (10mM)	0
β -Cyano L-alanine (10mM)	0

* denotes assays containing 5mM-dithiothreitol.

Effect of sulphur-containing compounds, homoserine and
O-acetyl serine. A number of compounds were tested for the
 effect on β -cystathionase activity. Sulphate, sulphite,
 thiosulphate and sulphide had no effect upon β -cystathionase
 activity. Cysteine, O-acetyl serine, homocysteine and reduced
 glutathione were strong inhibitors of β -cystathionase activity.
 Methionine and homoserine were weaker inhibitors (Table 10.5).

TABLE 10.5

Effect of sulphur-containing compounds and homoserine on partially purified β -cystathionine activity

Reaction mixtures were as described in the text except that the additions were made as specified. The L-cystathionine concentration was maintained at 10mM.

<u>Addition</u>	<u>Activity</u> (%)
L-Methionine (<u>10mM</u>)	95
L-Methionine (<u>20mM</u>)	62
L-Homocysteine (<u>10mM</u>)*	5
L-Homocysteine (<u>20mM</u>)*	0
L-Homoserine (<u>10mM</u>)	100
O-Acetylserine (<u>10mM</u>)	12
L-Homoserine (<u>20mM</u>)	86
L-Cysteine (<u>10mM</u>)*	6
L-Cysteine (<u>20mM</u>)*	0
L-Cystine (<u>5mM</u>)	93
L-Glutathione (<u>10mM</u>)	27
L-Glutathione (<u>20mM</u>)	6

* 5mM Dithiothreitol was included in the assay mixtures.

L-Cystine slightly inhibited β -cystathionase activity, probably due to competition with the substrate (L-cystathionine). The release of pyruvate from cystathionine was measured by method /27.

Effect of sulphur source in growth media upon the specific activity of β -cystathionase. β -Cystathionase activity of dialysed, cell-free extracts of cells grown in the presence

of sulphate, sulphite, sulphide, L-cystine or sulphate and L-cystine together showed little variation. However, in extracts prepared from cells grown with sulphate, in the presence of L-homocysteine or L-methionine, the specific activity of β -cystathionase was significantly lower (Table 10.6). The repression of β -cystathionase was significantly

TABLE 10.6

Effect of sulphur source in the growth medium upon the specific activity of β -cystathionase.

Cells were grown (2 x 21 litres) and cell-free extracts prepared as described in Materials and Methods, except that the growth media contained the sulphur source(s) as specified. Cell-free extracts were dialysed against 20mM-Tris-phosphate buffer, pH 9.0 for 12 h. β -Cystathionase activity was determined using Method 17.

<u>Sulphur source for growth</u>	<u>β-Cystathionase activity (nmoles of puruvate produced per min per mg of protein)</u>
Na ₂ SO ₄	5.23
Na ₂ SO ₃	5.61
Na ₂ S	4.92
L-Cystine	5.41
Na ₂ SO ₄ + L-Cystine	5.07
Na ₂ SO ₄ + L-Homocysteine (10mM)	3.87
Na ₂ SO ₄ + L-Methionine (10mM)	0.93

greater in those cells grown with methionine than in those grown with homocysteine. Since methionine and homocysteine are known to inhibit β -cystathionase activity directly (Table 10.5) the cell-free extracts were dialysed for 12 h to ensure removal of these potential inhibitors.

A non-enzymic cleavage of L-cystathionine to homocysteine, pyruvate and ammonia, at 37°C, catalysed by pyridoxal phosphate, was observed by Binkley (1955). Similarly, De Marco and coworkers (De Marco et al., 1963; Cavallini et al., 1963; De Marco & Renaldi, 1970) have reported the pyridoxal phosphate-catalysed, non-enzymic production of pyruvate from a number of thioethers including L-cystine, DL-allocystathionine and DL-lanthionine, in slightly alkaline media in the absence of added metal ions. This non-enzymic production of pyruvate from thioethers, in the assay conditions described in this chapter, were significant (less than 3 nmoles of pyruvate per 30 min incubation) since reactions were conducted at a lower temperature and the reactions were run for 15 min only. Notwithstanding these steps, control reaction mixtures, in which reactions were killed at time zero, were conducted for each experiment.

DISCUSSION

The results of time-course and pulse-chase experiments indicated that cystathionine is an intermediate in the biosynthesis of methionine (see Chapter 3). The growth experiments in Chapter 2 did not, however further support this argument. The results presented in this chapter indicate that P. denitrificans actively converts cystathionine to homocysteine. The enzymic mechanism is repressed in cells grown in the presence of homocysteine and methionine. Homocysteine can control β -cystathionase activity by direct feedback inhibition. It may also control β -cystathionase activity by direct repression, or it may be repressed by methionine (after conversion of homocysteine to methionine).

The results reported in Chapter 2 indicated that P. denitrificans cannot utilise either methionine or homocysteine as sole sulphur sources for growth. This could be due to the inability of P. denitrificans to take up homocysteine and/or methionine, or the inability to convert homocysteine and methionine to forms of sulphur which can be utilised. In Chapter 2 methionine uptake by P. denitrificans was demonstrated. In this chapter it was found that the addition of homocysteine or methionine to culture solutions containing sulphate repressed β -cystathionase activity indicating that both methionine and homocysteine can be taken up and metabolised by P. denitrificans.

The repression, caused by homocysteine, may be due either to methionine formed from homocysteine by the growing organism, or to homocysteine itself.

Cystathionine could be degraded in either of two ways; (1) cleavage between the sulphur atom and the three carbon chain (β -elimination) resulting in the formation of homocysteine, pyruvate and ammonia (Reaction [4]), or (2) cleavage between the sulphur atom and the four carbon chain (γ -elimination) resulting in the formation of cysteine, α -ketobutyrate and ammonia (Reaction [2]). There is a stable and highly active β -cystathionase present in extracts of P. denitrificans. With cell-free extracts or partially purified preparations, no evidence for mechanism (2), the mechanism which operates in animals and fungi, was found. The possibility remains that an enzyme catalysing the γ -elimination is destroyed during preparation of enzyme extracts.

The relatively high pH optimum of β -cystathionase activity has been previously reported for E. coli (Wijesudera &

Woods, 1962), S. typhimurium (Guggenheim, 1971) and P. denitrificans (strain 11) (Banerjee, 1966).

Similar effects of inhibitors upon β -cystathionase activity were reported for β -cystathionase isolated from E. coli (Wijesundera & Woods, 1962). β -Cystathionase activity, from both P. denitrificans and E. coli were inhibited by reagents which react with sulphydryl-groups and by cyanide.

The β -cystathionase of P. denitrificans, like that of E. coli (Wijesundera & Woods, 1962) and Proteus morganii (Binkley & Hudgins, 1953) and β -cystathionase from fungi (Delavier-Klutchko & Flavin, 1965) and mammalian liver (Matsuo & Greenberg, 1959a) require pyridoxal phosphate as coenzyme.

The occurrence of enzyme repression provides further evidence bearing on the status of a possible intermediate in a biosynthetic pathway. The present results show that the formation of β -cystathionase is repressed by methionine. It seems reasonable to take this as a priori evidence for the involvement of cystathionine as an intermediate in the biosynthesis of methionine.

So, the results presented in this chapter indicate that homocysteine and methionine can be utilised, but cannot be metabolised, to forms of sulphur which are essential for growth (i.e., neither homocysteine nor methionine can be converted to cysteine, which is the sulphydryl-group donor for such compounds as t-RNA and biotin (Smith, 1970). Therefore cells are capable of utilising exogenous homocysteine and methionine only in the presence of a sulphur source which can be converted to cysteine.

CHAPTER 11

CYSTEINYL-tRNA SYNTHETASE

INTRODUCTION

Cysteine is amongst the twenty amino acids commonly found in proteins and is of special importance because of its particular chemical properties. The sulphhydryl groups involved in the active sites of many enzymes is supplied by cysteine. Furthermore, cysteine is an important protein amino acid since it often plays a major role in controlling the three-dimensional configuration of proteins.

The incorporation of amino acids into proteins marks the end of the pathway of sulphur metabolism and a study of the incorporation of cysteine and methionine into proteins may give some idea of the overall involvement of these compounds in the regulation of sulphur metabolism. Furthermore, a study of the substrate specificity of these two aminoacyl-tRNA synthetases, with respect to selenocysteine and selenomethionine, may give some idea of the mechanism of the inhibition of growth caused by selenium and also the toxicity of selenium-containing compounds.

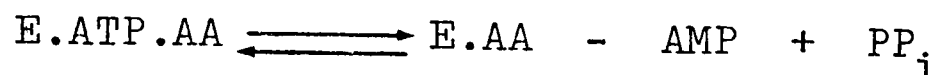
During the last two years, the study of the enzymic mechanisms involved in protein synthesis has received considerable attention (for reviews see; Novelli, 1967; Soffer, 1974; Moldave, 1965; Lea & Norris, 1972; Kisselev & Favarova, 1974; Soll & Schimmel, 1974).

Aminoacyl-tRNA synthetases (amino acid:t-RNA ligase (AMP), EC 6.1.1.-) catalyse the initial step in protein synthesis by attaching specific amino acids to their cognate tRNA molecules. The activity of an aminoacyl-tRNA synthetase, as now visualised, takes place in several reversible steps (Novelli, 1967; Owens & Bell, 1970).

(1) An ordered binding reaction



(2) A carbonyl activation reaction



(3) a tRNA aminocylation reaction



During amino acid activation, an activating enzyme (an aminoacyl-tRNA synthetase), specific for one of the 20 amino acids commonly found in proteins, catalyses a reaction between the carboxyl group of the amino acid and the phosphoryl group of ATP. With the elimination of inorganic pyrophosphate, a mixed anhydride is formed between the carboxyl group of the amino acid and the 5'-phosphate of AMP. This aminoacyl adenylate remains firmly attached to the enzyme surface and does not dissociate, or accumulate, as a free intermediate (Novelli, 1967).

Recently, an increasingly large number of different aminoacyl-tRNA synthetases, from a variety of sources, have been obtained in highly purified form. While studies using these preparations have greatly expanded our knowledge relating to the catalytic functions and other general properties of aminoacyl-tRNA synthetases, very little progress has been achieved towards a knowledge, or an understanding, of their control and regulation. Only in the last few years has the importance of the aminoacyl-tRNA synthetases in the regulation of biosynthetic pathways been realised.

This investigation was undertaken with particular emphasis on the problem of regulation and control of cysteinyl-tRNA synthetase activity. This chapter describes the purification of cysteinyl-tRNA synthetase from P. denitrificans, the regu-

lation and control of the enzyme, and a study of the substrate specificity of the enzyme, with particular reference to selenocysteine.

MATERIALS AND METHODS

Chemicals. All amino acids (D-, L- and DL- isomers), streptomycin and cysteine hydroxamic acid were obtained from the Sigma Chemical Co., London, U.K. Hydroxylapatite was obtained from BioRad Labs., Richmond, California, U.S.A. All other chemicals were obtained from the sources stated in previous chapters.

Enzymes. D-Amino acid oxidase (EC 1.4.4.3) and catalase (EC 1.11.1.6) were obtained from the Sigma Chemical Co., London, U.K.

Preparation of L-selenocysteine. L-Selenocysteine was prepared from commercially available DL-selenocystine. DL-Selenocystine was reduced by incubation with excess dithiothreitol in 100 mM-Tris-HCl buffer, pH 8.0 at 30°C for 30 min. The DL-selenocysteine was treated with D-amino acid oxidase, in the presence of catalase, and the L-selenocysteine isolated and purified by ion-exchange chromatography on Dowex 1-X2 (Cl⁻ form) 200 - 400 mesh. The column eluate was concentrated by rotary evaporation. A standard solution of 10 mM-L-selenocysteine, in 10 mM-Tris-HCl buffer, pH 8.0, and 5mM-dithiothreitol, was prepared fresh and used immediately.

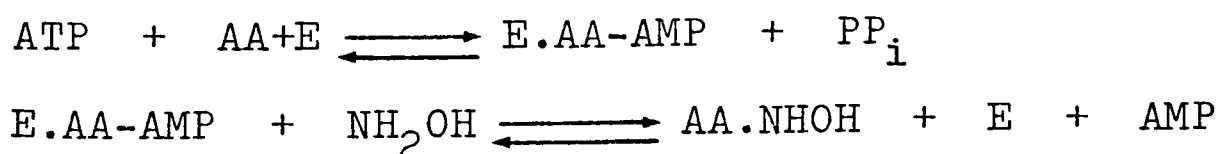
Assay of cysteinyl-tRNA synthetase. Two assay methods were used.

Method 1 Cysteine-dependent ATP-PP_i exchange. The capacity of cysteinyl-tRNA to form a cysteinyl-adenylate was measured by the cysteine-dependent exchange of ATP and γ -³²P-PP_i. In as much as the rate of cysteinyl-adenylate formation

is the rate determining step in the overall exchange reaction (Berg, 1956, 1958; Berg et al., 1961) the cysteine-dependent incorporation of $\gamma\text{-}^{32}\text{P}\text{-PP}_i$ into ATP actually measures the rate of cysteinyl-adenylate formation (Berg et al., 1961).

The reaction mixtures contained 100 μmoles of Tris-HCl buffer, pH 8.0, 10 μmoles of MgCl_2 , 5 μmoles of dithiothreitol, 4 μmoles of $\text{Na}_2\text{K}_2\text{ATP}$, 100 nmoles of L-cysteine, 2 μmoles of $\gamma\text{-}^{32}\text{P}\text{-PP}_i$ (0.25 $\mu\text{Ci}/\mu\text{mole}$) and a limiting amount of enzyme in a total volume of 1 ml. Reactions were run for 10 min at 30°C and were terminated by the addition of 2 ml 7.5% (w/v)-trichloroacetic acid. The $\gamma\text{-}^{32}\text{P}\text{-ATP}$ was separated from the $\gamma\text{-}^{32}\text{P}\text{-PP}_i$, and the radioactivity of the $\gamma\text{-}^{32}\text{P}\text{-ATP}$ counted, as described in Chapter 6. Cysteinyl-tRNA synthetase activities are expressed as cysteine-dependent ATP- PP_i exchange in nmoles/min (cysteinyl-tRNA synthetase units).

Method 2 Formation of cysteine hydroxamic acid. Aminoacyl-AMP anhydrides can transfer the amino acid residue non-enzymically to a variety of acceptors including ammonia, hydroxylamine, amino acids, proteins, and RNA (Dixon & Webb, 1964). The general reaction between amino acid (AA) and enzyme (E) in the presence of hydroxylamine (NH_2OH) may be formulated as:



In method 2, hydroxylamine was used as the acceptor, since the formation of amino acid hydroxamic acid can be easily followed. Reaction mixtures contained 200 μmoles of Tris-HCl buffer, pH 8.0, 10 μmoles of MgCl_2 , 4 μmoles of $\text{Na}_2\text{K}_2\text{ATP}$, 10 μmoles of L-cysteine, 5 μmoles of dithiothreitol, 2.5 nmoles

of hydroxylamine and purified enzyme in a total volume of 1 ml. After incubation at 30°C for 30 min, reactions were terminated by the addition of 1 ml of trichloroacetic acid (7.5%, w/v). Cysteine hydroxamic acid was determined colorimetrically as the ferric complex. To the terminated reaction, 1.0 ml of a 10% (w/v) solution of FeCl_3 , in 0.2M-trichloroacetic acid and 6.6M-HCl, was added. The mixture was shaken and, after 5 min, the A_{540} was determined using a Pye Unicam SP 8000 spectrophotometer. In experiments in which the enzymic formation of cysteine hydroxamic acid was measured, synthetic cysteine hydroxamic acid was added to control tubes (minus ATP and cysteine) as an internal standard.

Purification of cysteinyl-tRNA synthetase. All operations were conducted at 0 to 4°C. The buffer used throughout the purification procedure contained 20 mM-Tris-HCl buffer, pH 8.0, 5.0 mM-MgCl₂ and 1 mM-dithiothreitol; the dithiothreitol was added to the buffer solution immediately prior to use.

Step I Crude extract. Cells of *P. denitrificans* were grown and harvested as described in the earlier chapters, except that sulphate was the sole sulphur source present in the culture medium, and 48 litres of cell culture were used. Crude extracts (Fraction 1) were prepared as described previously (see Chapter 6).

Step II Alumina C_γ treatment. Alumina C_γ was added, with constant stirring, to Fraction I to a final concentration of 10 mg/ml. The solution was stirred for 15 min and then centrifuged at 30 000g for 40 min. The supernatant (Fraction II) was decanted; the precipitate was discarded.

Step III Streptomycin treatment. Streptomycin (5 g) was slowly added to Fraction II, with constant stirring. The

suspension was stirred for 6 h and then centrifuged at 30 000 g for 30 min. The supernatant was decanted (Fraction III) and the precipitate was discarded.

Step IV Ammonium sulphate fractionation. Solid ammonium sulphate was added to Fraction III, with stirring, to give 30% saturation. The mixture was stirred for 30 min and then centrifuged at 30 000g for 30 min. The precipitate was discarded. The supernatant was brought to 60% saturation by the addition of ammonium sulphate, and the suspension was again stirred for 30 min and then centrifuged. The precipitate was dissolved in a minimal volume of buffer and the solution (Fraction IV) dialysed against 12 litres of buffer for 12 h.

Step V Sephadex G-200 gel filtration. Fraction IV was loaded onto a Sephadex G-200 column (85 x 3.5 cm) equilibrated with buffer, and eluted with buffer at a flow rate of 0.45 ml/min. Fractions of 5 ml were collected and assayed for enzyme activity and fractions containing cysteinyl-tRNA synthetase activity were pooled (Fraction V).

Step VI DEAE-cellulose chromatography. Fraction V was applied to a DEAE-52 cellulose column (10.0 x 3.5 cm) equilibrated with buffer. The column was washed with 60 ml of buffer and then with 50 ml of 0.2 M-KCl in buffer. The enzyme was then eluted with 500 ml of a linear gradient of KCl, in buffer, from 0.2 to 0.5 M-KCl at a flow rate of 0.65 ml/min. Fractions (5 ml) were collected and assayed for cysteine-dependent and methionine-dependent ATP-PP_i exchange. Those fractions catalysing cysteine-dependent ATP-PP_i exchange were pooled (Fraction VI) and dialysed against 10 litres of buffer for 6 h.

Step VII Hydroxylapatite column chromatography.

Fraction VI was applied to a column of hydroxylapatite (10.0 x 1.5 cm) equilibrated with buffer. The enzyme was eluted with a 500 ml linear gradient from 0 to 0.3 M-KCl, in buffer, at a flow rate of 0.4 ml/min. Those fractions containing the highest specific activity were pooled (Fraction VII) and dialysed against 10 litres of buffer for 6 h.

Step VIII Concentration on DEAE-cellulose.

Fraction VII was concentrated using a small DEAE-cellulose column (5 x 1 cm) equilibrated with buffer. The enzyme was adsorbed to the column and then eluted with 10 ml of buffer containing 0.4 M-KCl (Fraction VIII). Fraction VIII was dialysed against 5 litres of buffer to remove the KCl, and was then stored at -15°C.

Chromatography of the ^{32}P -labelled product adsorbed to charcoal in exchange assays. The ^{32}P -labelled compound formed in reaction mixtures containing $\gamma\text{-}^{32}\text{P}\text{-PP}_i$ and ATP, with either, cysteine, selenocysteine or α -aminobutyrate as the other substrate, and which adsorbed to charcoal, was eluted from the charcoal with 0.1 M- NH_3 in 50% (v/v) ethanol. The eluted material was evaporated to dryness, by rotary evaporation at 30°C, and the residue dissolved in water. Samples were streaked onto acid-washed Whatman 3MM paper and subjected to high voltage paper electrophoresis in 0.1 M-sodium citrate, pH 5.0, for 2 h, as described in Chapter 3.

Determination of protein. Protein in crude extracts, alumina C γ , streptomycin, ammonium sulphate, and Sephadex G-200 fractions was determined by the Folin method of Lowry et al., (1951). Purer protein, from DEAE-cellulose and hydroxylapatite fractions, was measured by the method of Warburg &

Christian (1941).

RESULTS

The location of aminoacyl-tRNA synthetases in micro-organisms is not well defined. Activation of 20 commonly occurring amino acids has been reported using both soluble and particulate fractions prepared from bacteria (McCorguodale & Zellig, 1959; Nisman & Hirsch, 1958). Extracts prepared by sonication of cells of P. denitrificans in 0.1 M-Tris-HCl buffer had significant synthetase activity associated with both the membrane and the soluble fraction. However, activity was completely removed from the membrane fraction by sonication of cells in 20 mM-Tris-HCl buffer.

Purification of cysteinyl-tRNA synthetase. The six-step purification procedure yielded an enzyme preparation that had been purified between 1200- and 1300-fold (Table 11.1). The overall yield of enzyme activity recovered was close to 60%. The crude extract contained very high inorganic pyrophosphatase activity which interfered with the estimation of cysteinyl-tRNA synthetase activity measured by the ATP-PP_i exchange method (Method 1). The values shown in Table 11.1, for both the specific and total activities for the fractions I to IV, may be an underestimate. A large proportion of the inorganic pyrophosphatase activity was removed by streptomycin and ammonium sulphate fractionation, and it was completely removed from both cysteinyl- and methionyl-tRNA synthetases by Sephadex G-200 gel filtration. Fractionation of the active ammonium sulphate fraction with acetone or acid (pH) was unsatisfactory because of high losses of cysteinyl-tRNA synthetase activity, and also because the specific activity was not increased, and no separation of cysteinyl-tRNA synthetase

TABLE 11.1

Purification of cysteinyl-tRNA synthetase

Fraction No.	Purification step	Protein (mg)	Total activity (units)	Specific Activity (units/mg)	Recovery (%)
I	Crude extract	13259	30496	2.3	100
II	Alumina C _γ fractionation	8295	29865	3.6	97.3
III	Streptomycin fractionation	2293	28438	12.4	93
IV	(NH ₄) ₂ SO ₄ fractionation	430	27362	63.6	90
V	Sephadex G-200 gel filtration	105	25945	247.5	85
VI	DEAE-52 Cellulose column chromatography	11.5	22780	1976	75
VII	Hydroxylapatite column chromatography	6.5	18430	2849	60

from inorganic pyrophosphatase was affected. Cysteinyl- and methionyl-tRNA synthetases were separated by Sephadex G-200 gel filtration (Fig. 11.1) and contamination completely removed by ion exchange chromatography on DEAE-52 cellulose or hydroxyl-apatite.

Criteria of Purity. Rechromatography of the purified enzyme on Sephadex G-200, gave rise to a single, sharp and symmetrical peak of enzyme activity with constant specific activity across the peak.

Linearity and validity of the cysteine-dependent ATP-PP_i exchange assay. In the presence of purified cysteinyl-tRNA synthetase and 5mM-dithiothreitol, cysteine-dependent ATP-PP_i exchange was linear for at least 20 min (Fig. 11.2). In the absence of dithiothreitol, the rate of ATP-PP_i exchange decreased with time and this was presumably due to the oxidation of the substrate, cysteine. Cysteine-dependent ATP-PP_i exchange was stimulated by dithiothreitol indicating that the cysteinyl-tRNA synthetase is probably a sulphhydryl enzyme, a property common to almost all aminoacyl-tRNA synthetases (DeLuca & McElroy, 1965; Novelli, 1967). Exceptions to this generalisation include the lysyl-tRNA synthetase isolated from some organisms (Stern & Mehler, 1965; Stern et al., 1966; Mustafa, 1964; Hele, 1961), cysteinyl-tRNA synthetase from yeast (James & Bucavaz, 1969), and the methionyl-tRNA synthetase from Sarcina lutea (Hahn & Brown, 1967).

The rate of cysteine-dependent ATP-PP_i exchange increased linearly with increase in protein concentration (Fig. 11.3).

The radioactivity, adsorbed by charcoal in incubations containing cysteine, was eluted and subjected to high voltage electrophoresis. All the radioactivity in the charcoal eluate ran with the same R_f value as ATP. No γ -³²P-PP_i, γ -³²P-P_i, γ -³²P-ADP, or γ -³²P-AMP was detected; no un-labelled AMP was detected.

Purified cysteinyl-tRNA synthetase was heat-sensitive (pretreatment at 80°C for 2 min totally destroyed activity) but it could be stored at -15°C for at least 10 months without

Fig. 11.1

Separation of cysteinyl- and methionyl-tRNA synthetase activities.

The activities were separated by ion exchange chromatography on DEAE-cellulose after Sephadex G-200 gel filtration as described in the text for the purification of cysteinyl-tRNA synthetase. Cysteinyl-tRNA synthetase and methionyl-tRNA synthetase activities were measured by cysteine- and methionine-dependent ATP-PP_i exchange, respectively. ■ , protein; ● , methionyl-tRNA synthetase activity; ▲ , cysteinyl-tRNA synthetase activity.

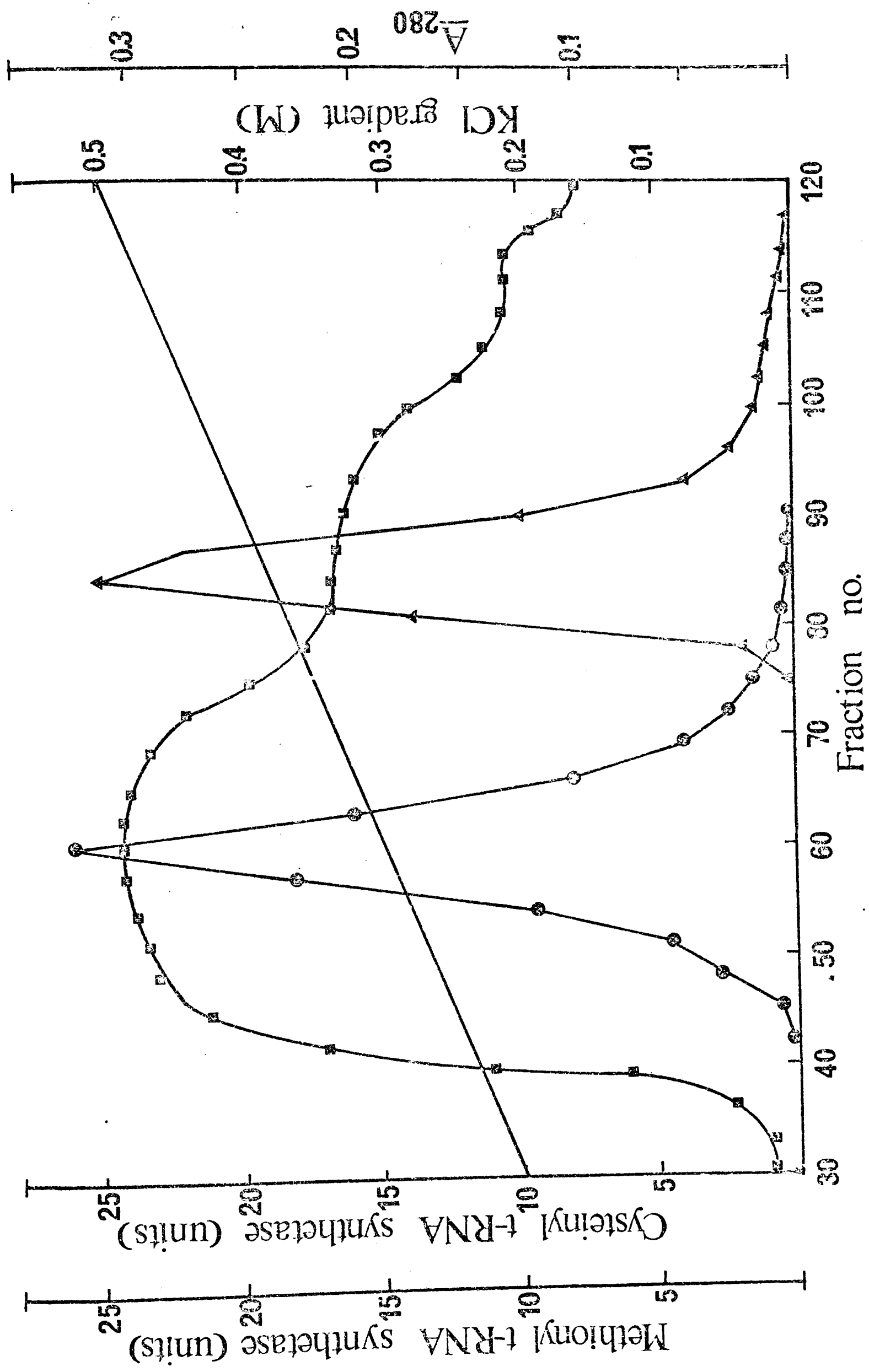
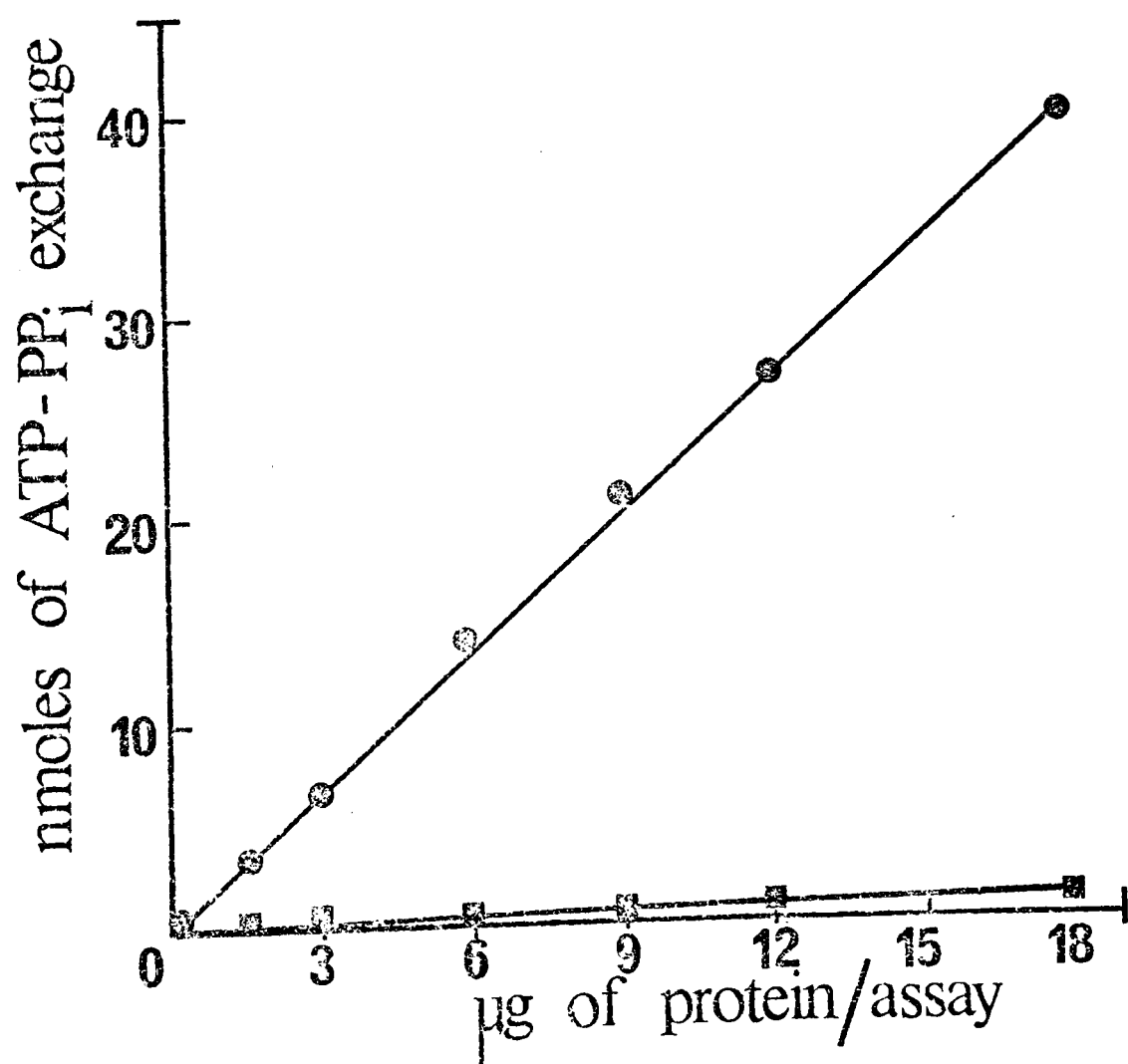
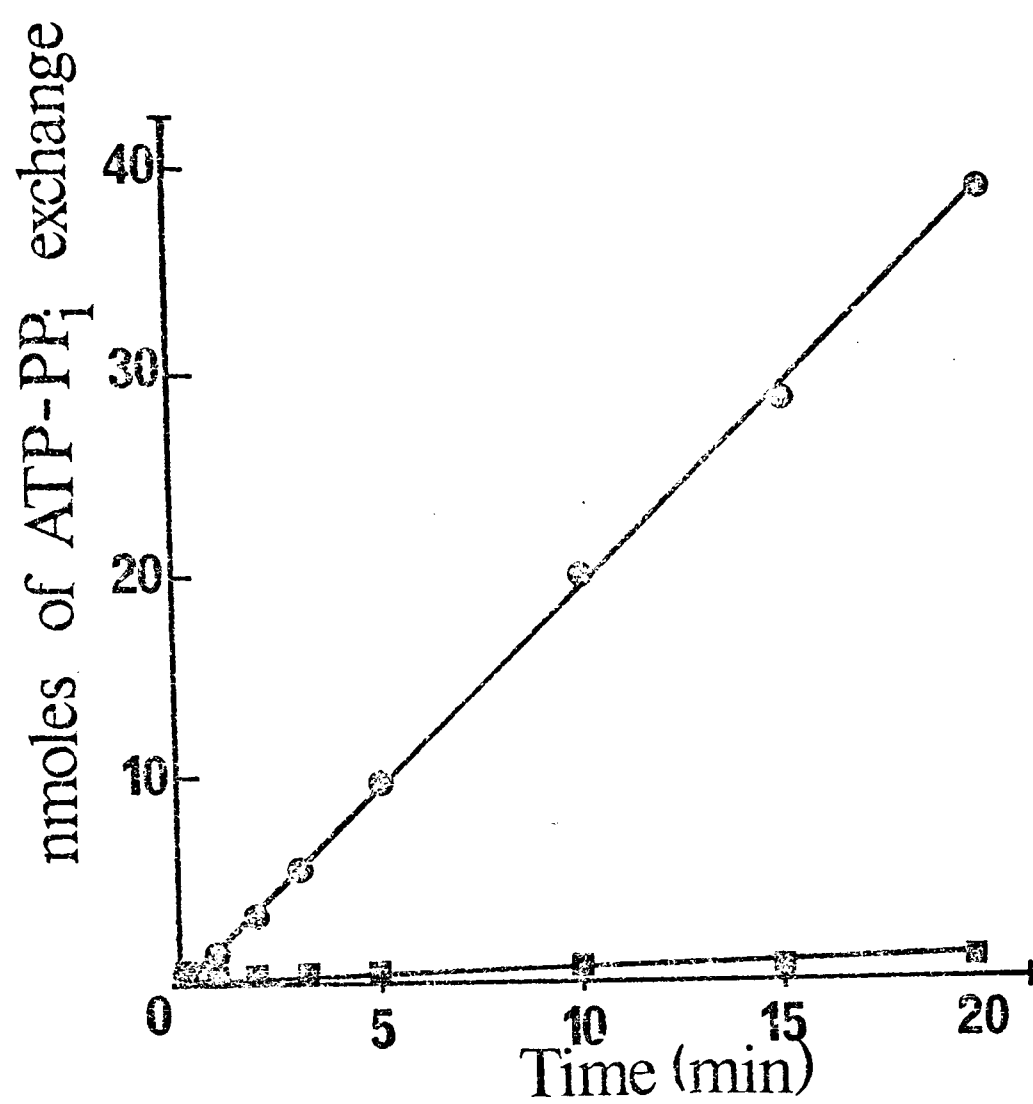


Fig. 11.2 Time-course of endogenous (■) and cysteine dependent (●) ATP-PP_i exchange. Assays were described in Materials and Methods and contained 9.0μg of enzyme protein.

Fig. 11.3 Effect of enzyme concentration upon endogenous (■) and cysteine-dependent (●) ATP-PP_i exchange. Assays were conducted for 10 min.



loss of activity.

Effect of pH. When a series of buffers were used to study the pH optimum of cysteinyl-tRNA synthetase, the pH optimum was found to lie between pH 7.5 and 8.5 (Fig. 11.4). No activity was detected below pH 4.0 or above pH 11.0. The fact that endogenous ATP-PP_i exchange remained low in assays conducted in glycine-NaOH buffer indicated that no glycyl-tRNA synthetase activity was present in the purified cysteinyl-tRNA synthetase preparation.

Effect of Mg²⁺ concentration. The cysteine-dependent ATP-PP_i exchange reaction, catalysed by purified cysteinyl-tRNA synthetase, is magnesium ion-dependent. The effect of Mg²⁺ concentration upon the rate of cysteine-dependent ATP-PP_i exchange is shown in Fig. 11.5. At a fixed concentration of ATP the rate of cysteine-dependent ATP-PP_i exchange increased with increase in the Mg²⁺ concentration up to a maximum; further increase in the Mg²⁺ concentration resulted in the inhibition of enzyme activity. Examination of the effect of Mg²⁺ concentration upon cysteinyl-tRNA synthetase activity, at different concentrations of ATP, indicates that maximum enzyme activity occurs when the Mg²⁺: ATP ratio is 2.5 : 1. As stated in Chapter 6, in an assay containing 10 mM-MgCl₂, 2 mM-ATP, and 2mM-PP_i, the ATP and PP_i will be in the form of MgATP²⁻ and MgPP_i²⁻ respectively. Therefore the substrate for cysteinyl-tRNA synthetase is probably MgATP²⁻. The method of Perrin & Sayce (1967) was used to calculate the forms of PP_i, ATP and Mg²⁺ at various concentrations of MgCl₂ in the incubation mixtures, and the concentration of free Mg²⁺ did not correlate with the rate of cysteinyl-tRNA synthetase activity; the concentration of the

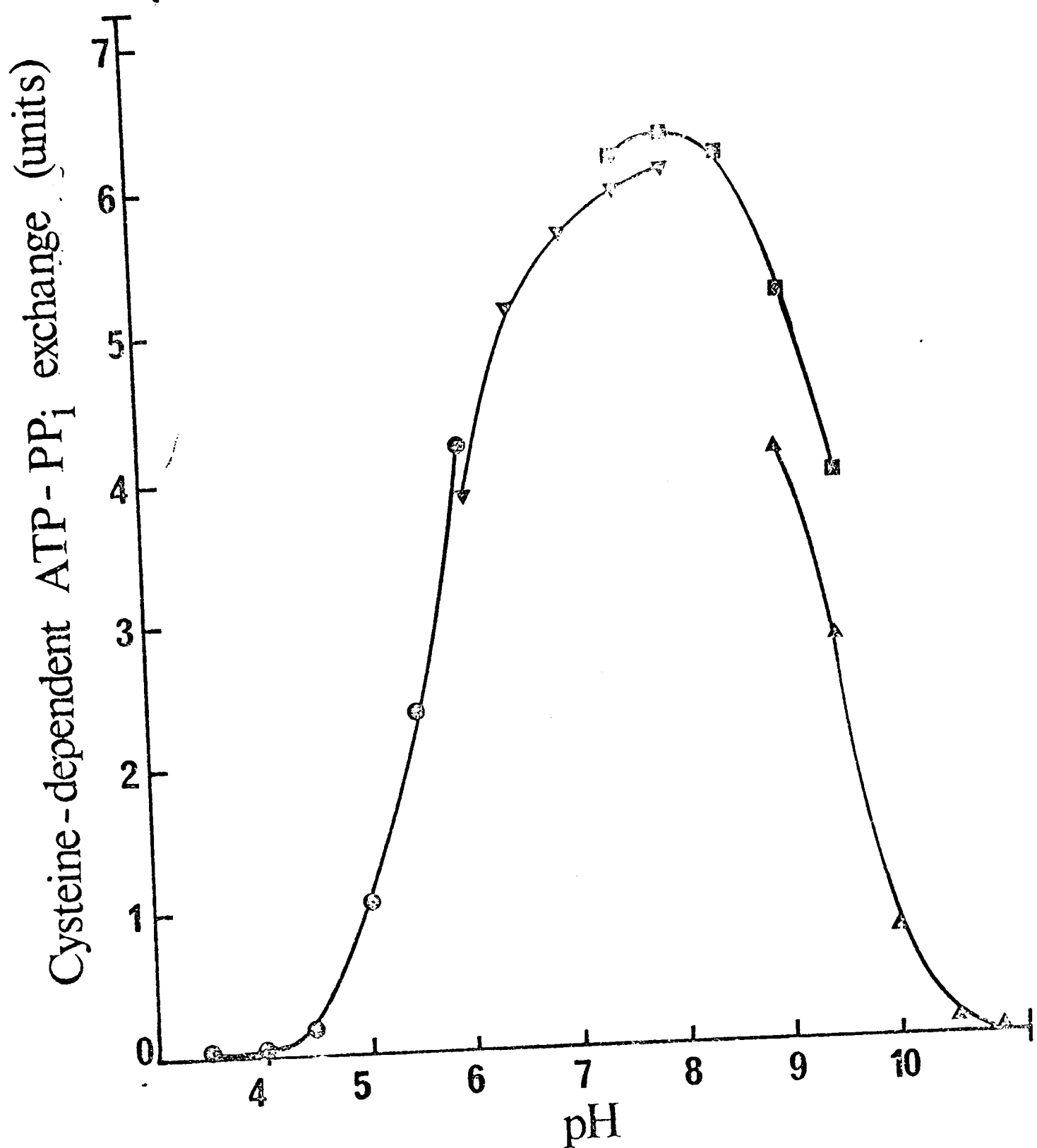


Fig. 11.4

Effect of pH upon cysteine-dependent ATP-PP_i exchange catalysed by purified cysteinyl-tRNA synthetase.

Reaction mixtures were as described in Materials and Methods except that Tris-HCl buffer was replaced by the buffers as specified; ●, citrate buffer; ▼, Tris-maleate buffer; ■, Tris-HCl buffer; ▲, glycine buffer.

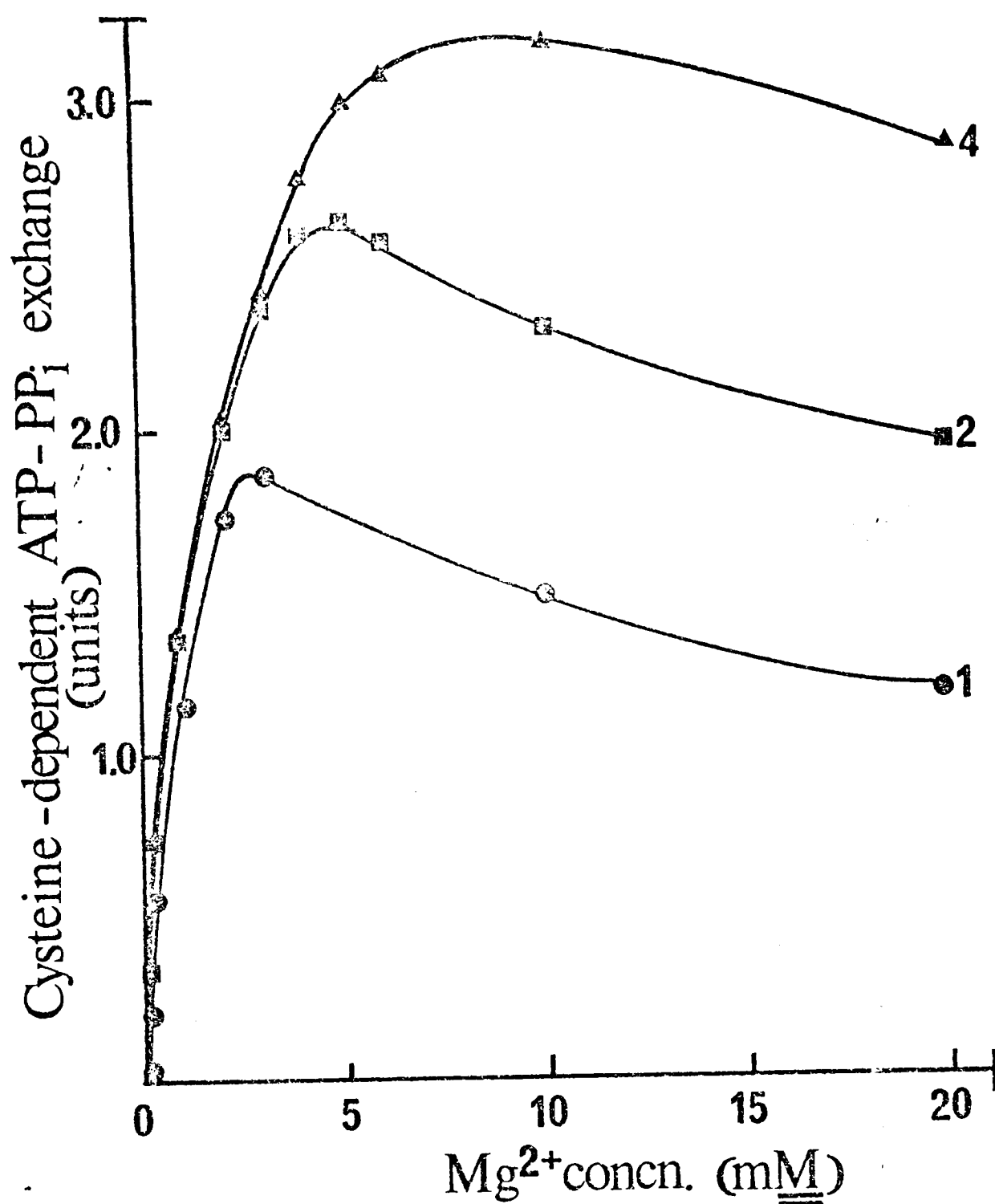


Fig. 11.5

Effect of magnesium ion concentration upon cysteine-dependent ATP-PP_i exchange at different concentrations of ATP. Reaction mixtures were as described in Materials and Methods except that the Mg²⁺ concentrations were as specified; ●, 1mM-ATP; ■, 2mM-ATP; ▲, 4mM-ATP.

MgATP²⁻ complex did, however, correlate with the rate of cysteine-dependent ATP-PP_i exchange. Mg²⁺ is therefore required to form MgATP²⁻ complex and probably MgPP_i²⁻, which are the active substrates for cysteinyl-tRNA synthetase. Cole & Schimmel (1970) reached a similar conclusion with respect to the isoleucyl-tRNA synthetase of E. coli, though Santi et al., (1971) concluded that Mg²⁺ itself was the active form of magnesium for the phenylalanyl-tRNA synthetase of E. coli. Novelli (1967) emphasised that the optimal Mg²⁺/ATP ratio for different aminoacyl-tRNA synthetases varies both within and between species.

Effect of ATP concentration. In the presence of 5 mM-MgCl₂, the rate of cysteine-dependent ATP-PP_i exchange increased with increase in ATP concentration up to 2mM; increasing the ATP concentration above 3 mM caused inhibition of enzyme activity. Therefore, free ATP, as well as free Mg²⁺, inhibits cysteinyl-tRNA synthetase activity. A linear Lineweaver-Burk plot of ATP concentration versus cysteine-dependent ATP-PP_i exchange, was obtained when the Mg²⁺/ATP ratio was maintained at 2.5 (Fig. 11.6); the K_m (ATP) obtained by extrapolation of the graph is 1.33 mM. This K_m value for ATP is the same as the K_m (ATP) obtained for the cysteinyl-tRNA synthetase isolated from yeast (James & Bucovaz, 1969). The concentrations of Mg²⁺ and ATP in reaction mixtures were routinely maintained at 10 mM and 4mM, respectively, in all subsequent assays.

Effect of L-cysteine concentration. Due to the rapid oxidation of cysteine to cystine in reaction mixtures containing no other reducing agent, cysteine was maintained in the reduced form by the addition of excess dithiothreitol. Puri-

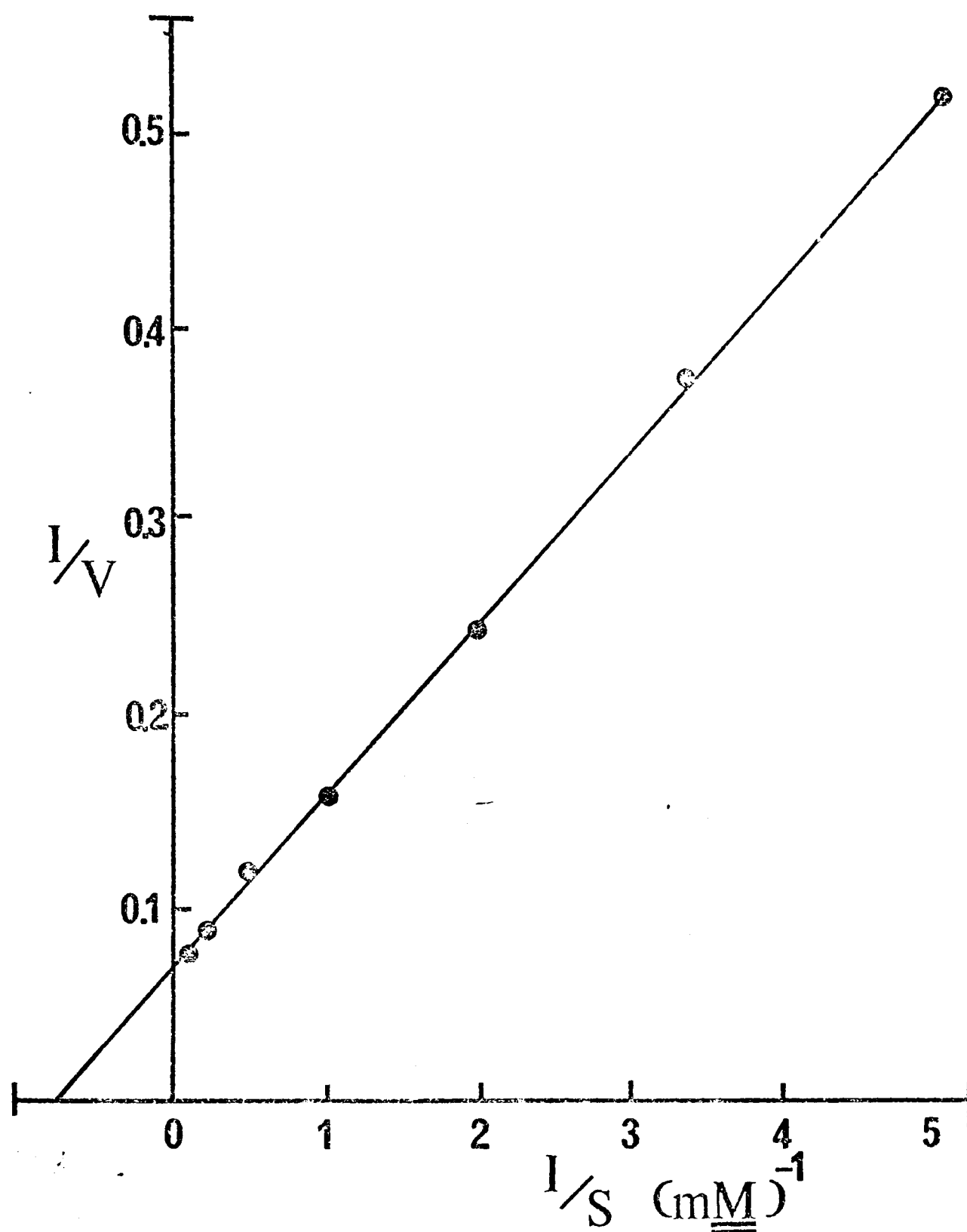


Fig. 11.6

Double reciprocal plot of the effect of ATP concentration on cysteine-dependent ATP-PP_i exchange. Reaction mixtures were as described in Materials and Methods except that the ATP concentration was as described. The Mg²⁺ : ATP ratio was maintained at 2.5 : 1, throughout.

purified cysteinyl-tRNA synthetase had a very high affinity for L-cysteine. A Lineweaver-Burk plot of L-cysteine concentration versus the rate of cysteine-dependent ATP-PP_i exchange, is shown in Fig. 11.7. The K_m (cysteine) obtained from this graph was $12.5 \mu M$. The concentration of L-cysteine subsequently added to reaction mixtures was routinely maintained at $0.10 mM$.

Substrate specificity. Purified cysteinyl-tRNA synthetase did not catalyse γ -³²P-PP_i exchange when ATP was replaced with either ADP or AMP. Similarly, the enzyme did not catalyse the incorporation of P_i into ATP, ADP, or AMP, when γ -³²P-PP_i was replaced with γ -³²P-P_i. Purified cysteinyl-tRNA synthetase did not catalyse ATP-PP_i exchange when cysteine was replaced with sulphate or selenate, indicating that the enzyme was not contaminated with ATP sulphurylase. The enzyme did not catalyse propionate- or acetate-dependent ATP-PP_i exchange, indicating that the enzyme was free from short-chain fatty acid thiokinases present in crude extracts. Only L-cysteine, of the 20 amino acids commonly found in proteins, could act as substrate for ATP-PP_i exchange, indicating that the purified cysteinyl-tRNA synthetase was not contaminated with other aminoacyl-tRNA synthetases.

The only true alternative substrates of L-cysteine in the ATP-PP_i exchange assay of cysteinyl-tRNA synthetase were L-selenocysteine and α -L-aminobutyric acid. Examination of the structure of L-cysteine, L-selenocysteine and α -L-aminobutyric acid shows that the only difference between the two alternative substrates and L-cysteine, the true substrate, is an alteration to the terminal -SH group; in L-selenocysteine, this is replaced by an -SeH group, and in α -aminobutyric acid, it is replaced by a -CH₃ group. Alteration to any other part

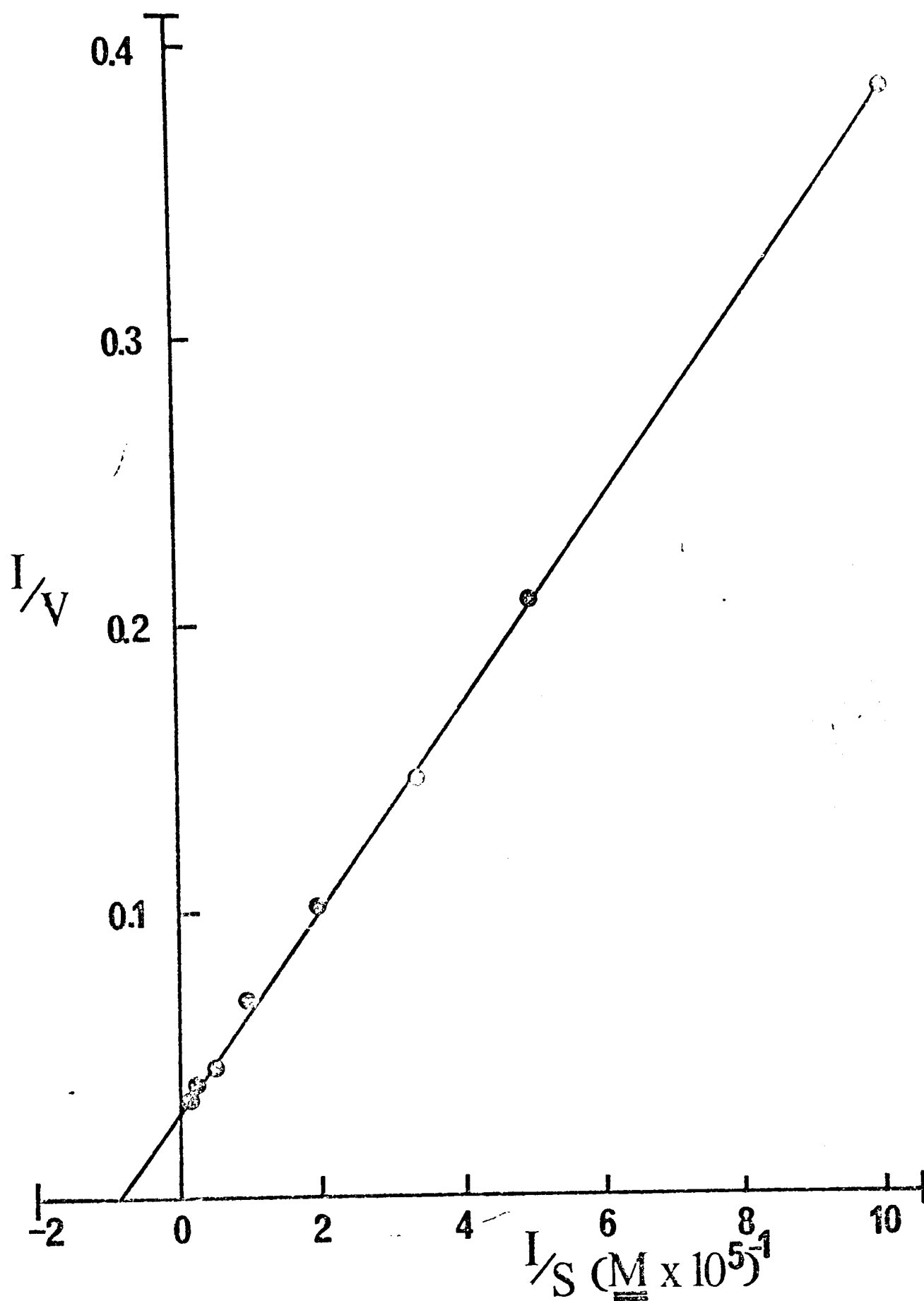


Fig. 11.7

Double reciprocal plot of the effect of cysteine concentration upon cysteine-dependent ATP-PP_i exchange.

Reaction mixtures were as described in Materials and Methods except that the concentration of cysteine was as specified.

of the substrate resulted in the failure of that compound to act as a substrate. Other amino acids examined as potential substrates included; L-aspartic acid, L-cysteic acid, D-cysteine, cysteamine, L-serine, taurine, cysteine sulphinic acid, α -L-aminobutyric acid, L-homoserine, and L-homocysteine.

Double reciprocal plots of L-selenocysteine and α -L-aminobutyric acid concentration versus the rate of L-selenocysteine- and α -L-aminobutyric acid-dependent ATP-PP_i exchange are shown in Figs. 11.8 and 11.9, respectively; the K_m values for these substrates, obtained from the graphs, were $1.59 \mu M$ and $10 mM$, respectively. While the affinity of cysteinyl-tRNA synthetase for selenocysteine ($K_m = 1.59 \mu M$) was greater than that for L-cysteine ($K_m = 12.9 \mu M$) the V (selenocysteine) was approximately the same as the V (cysteine). The affinity of cysteinyl-tRNA synthetase for α -L-aminobutyric acid ($K_m = 10.0 mM$) was much less than that for cysteine, but the V (α -aminobutyric acid) was approximately three times the V (cysteine). Evidence that L-selenocysteine and α -L-aminobutyric acid-dependent ATP-PP_i exchange, catalysed by the purified cysteinyl-tRNA synthetase preparation, was not due to contaminating enzymes, is provided by a number of observations; (1) the similarity in the structure of all three compounds, (2) the ratio of L-selenocysteine-, α -aminobutyric acid- and cysteine -dependent ATP-PP_i exchange activities was approximately constant during purification, and the three activities were not separated during gel filtration on Sephadex G-200 or ion exchange chromatography on either DEAE-52 cellulose or hydroxylapatite, (3) double reciprocal plots obtained from experiments with various concentrations of the substrates.

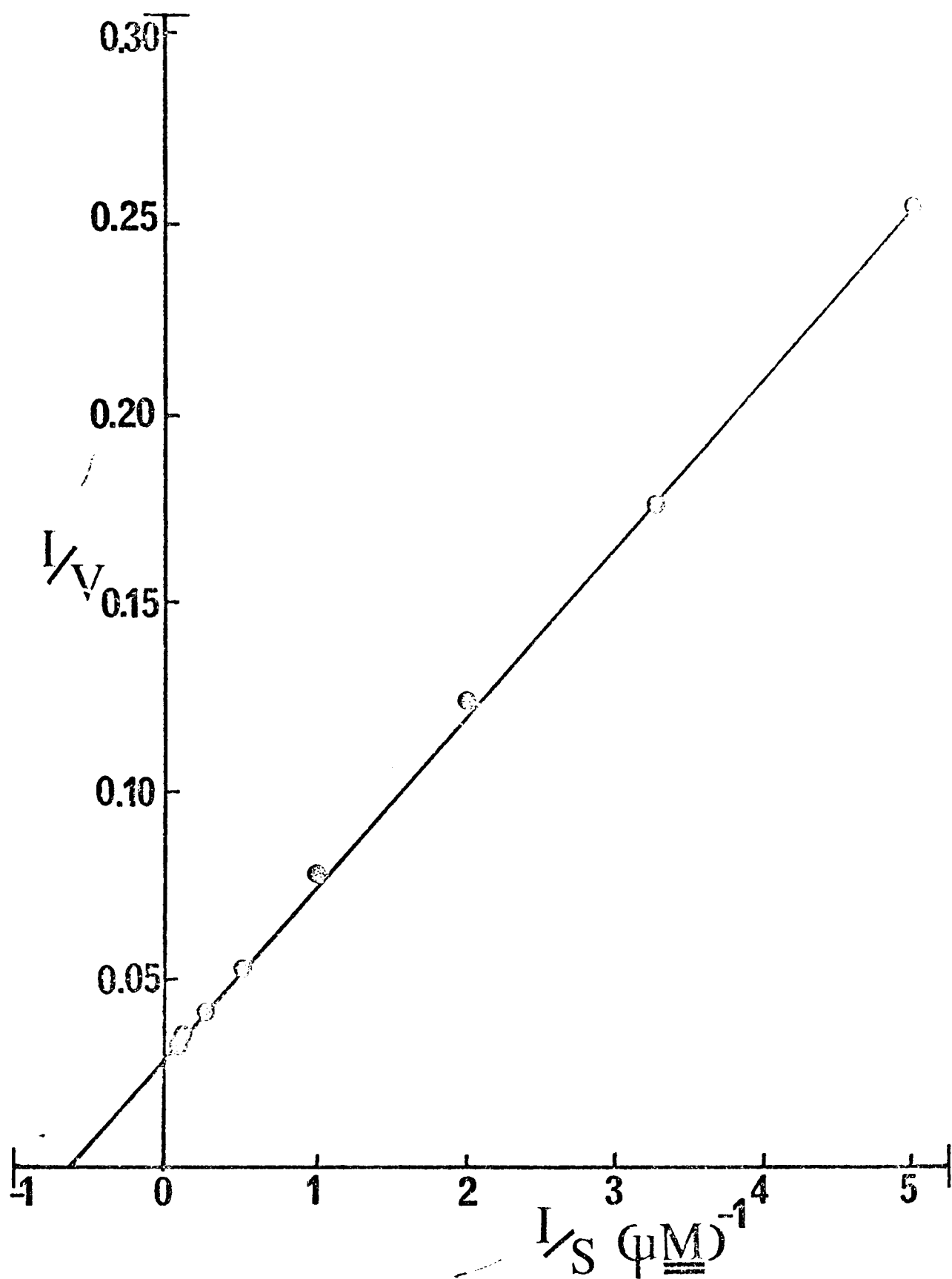


Fig. 11.8

Double reciprocal plot for the rate of selenocysteine-dependent ATP-PP_i exchange versus the concentration of L-selenocysteine. Reaction mixtures were as described in Materials and Methods except that cysteine was replaced by selenocysteine at the concentration specified.

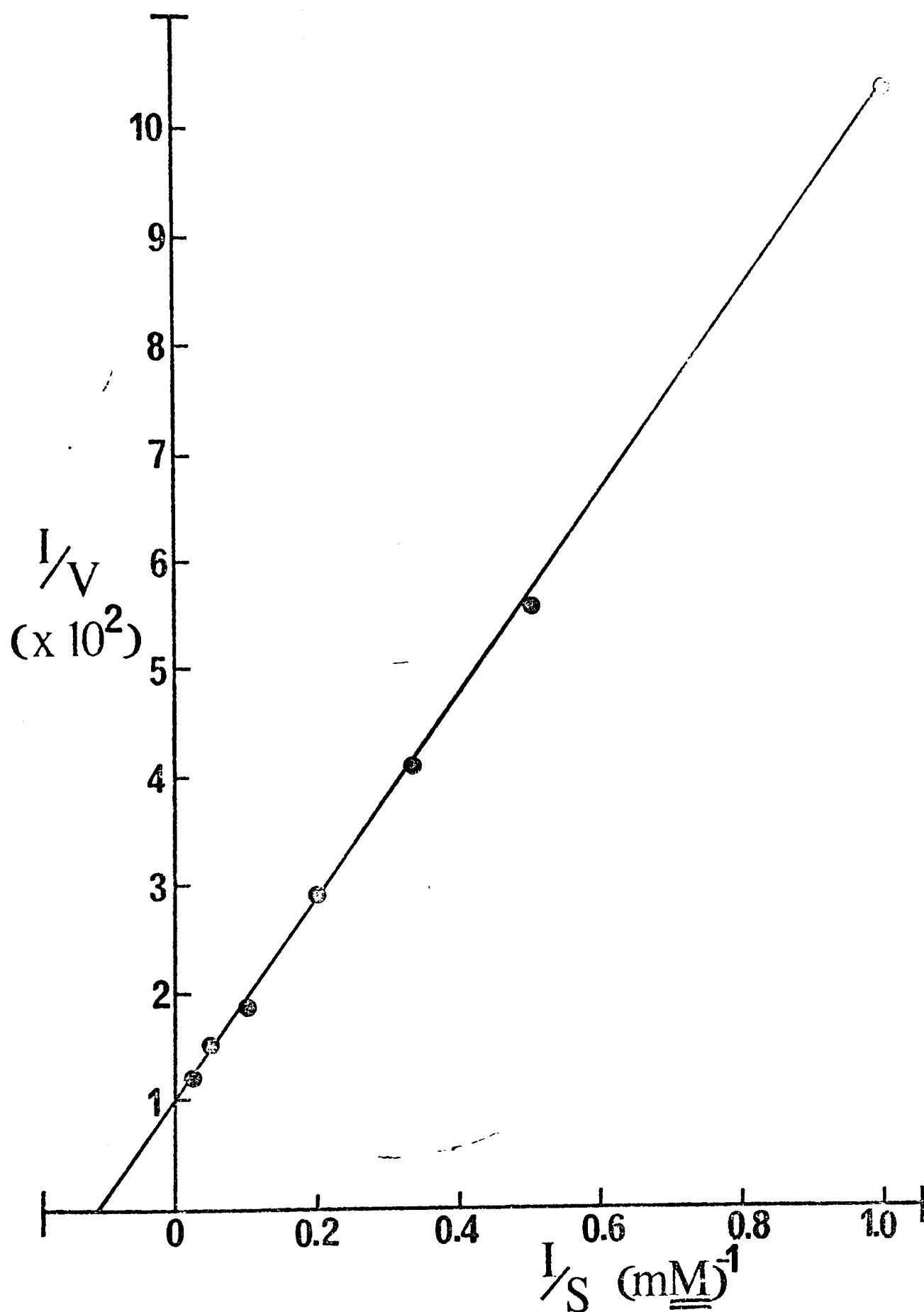


Fig. 11.9

Double reciprocal plot of the effect of L- α - amino- butyric acid concentration upon L- α -aminobutyric acid- dependent ATP-PP_i exchange.

Assays were conducted as described in the Materials and Methods except that L-cysteine was replaced by L- α -aminobutyric acid at the concentrations specified.

Double reciprocal plots of L-cysteine and L-selenocysteine concentration versus the rate of ATP-PP_i exchange, are shown in Fig. 11.10; the kinetics of this experiment are similar to those obtained for the competition between sulphate and selenate for ATP sulphurylase (see Chapter 6) indicating competition between two substrates for one enzyme (Pocklington & Jeffery, 1968). The ³²P-labelled product formed in the presence of purified enzyme and L-selenocysteine or α-L-aminobutyric acid, ATP and γ -³²P-PP_i as substrates and which adsorbed to charcoal, was eluted and identified, using high voltage paper electrophoresis, as γ -³²P-ATP; γ -³²P-ADP and unlabelled AMP were not detected.

Effect of cations. Cysteine-dependent ATP-PP_i exchange was negligible in the absence of Mg²⁺. Only Co²⁺ (13%) and Zn²⁺ (6%) supported cysteine-dependent ATP-PP_i exchange. Ni²⁺, Ba²⁺, Fe²⁺, Ca²⁺, Cu²⁺ and Mn²⁺ were examined but did not substitute for Mg²⁺.

Effect of inhibitors. The study of the effect of inhibitors upon cysteine-dependent ATP-PP_i exchange was confused by the reaction of cysteine with a number of the commonly used sulphhydryl-group reagents. The sensitivity of purified cysteinyl-tRNA synthetase to some common inhibitors was studied by using the alternative substrate, α-aminobutyric acid, which did not react directly with the inhibitors. The effect of some common inhibitors upon cysteinyl-tRNA synthetase activity are summarised in Table 11.2. The enzyme is sensitive to sulphhydryl-group reagents; almost all aminoacyl-tRNA synthetases have been found to be sulphhydryl-group reagent sensitive (Novelli, 1967). The enzyme was inhibited by EDTA; this was presumably due to the chelation of Mg²⁺, since EDTA was, itself, not inhibitory.

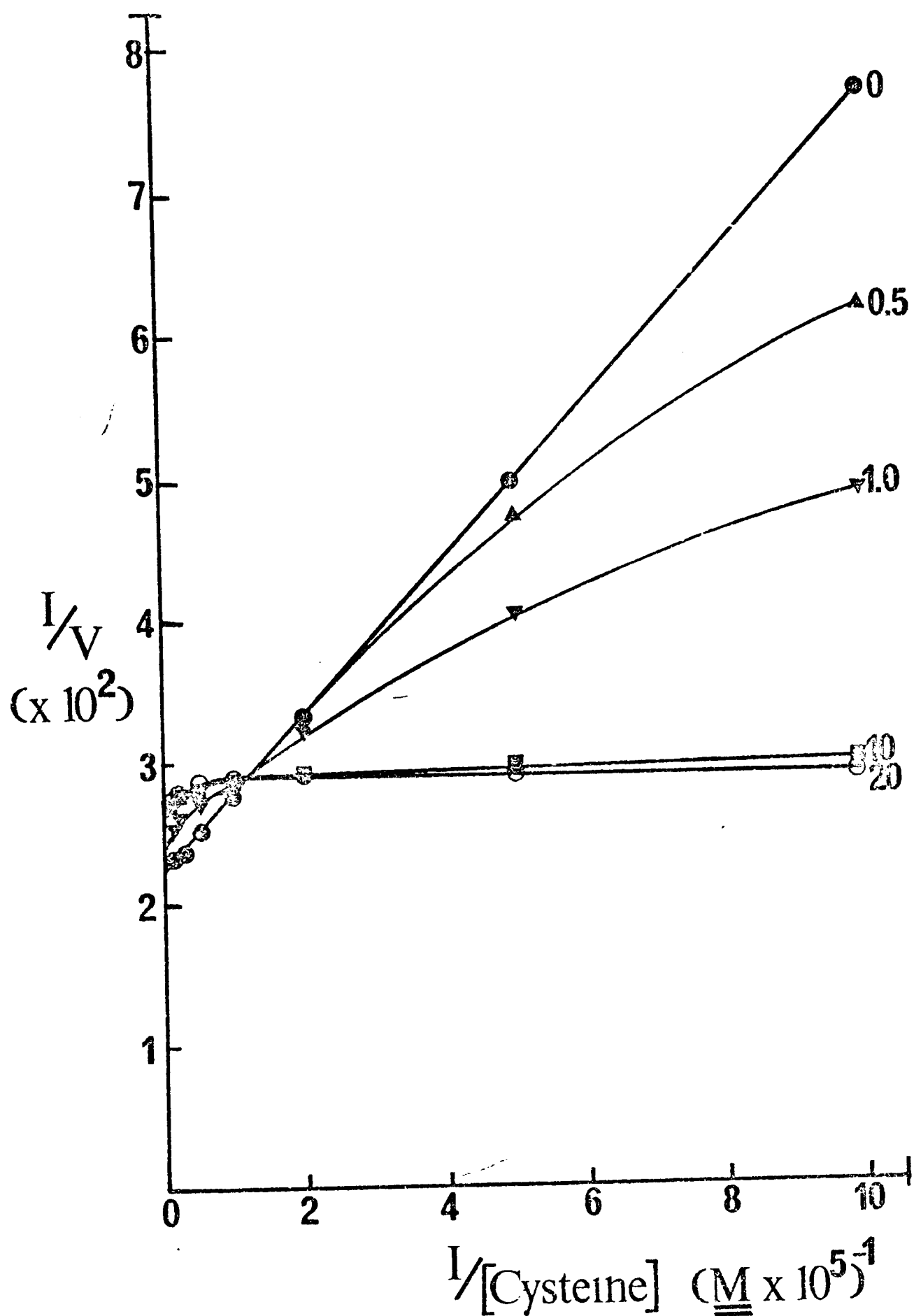


Fig. 11.10

Double reciprocal plot of the effect of cysteine concentration upon cysteine-dependent ATP-PP_i exchange in the presence or absence of selenocysteine.¹ Incubation mixtures contained 21 units of purified cysteinyl-tRNA synthetase and assays were conducted in the presence of 5mM-dithiothreitol; all other conditions of the assays were as described in Materials and Methods except that the amount of cysteine was replaced with the amounts specified and the concentrations of selenocysteine were as specified. ●, without selenocysteine; ▲, 0.5mM-selenocysteine; ▼, 1.0mM-selenocysteine; ■, 10mM-selenocysteine; O, 20mM-selenocysteine.

TABLE 11.2

Effect of some common inhibitors upon cysteine- and
 α -aminobutyric acid-dependent ATP-PP_i exchange

The compounds were incubated with the enzyme for 1 min before the assay was initiated by the addition of cysteine or α -aminobutyric acid.

Inhibitor	% inhibition	
	Cysteine	Substrate α -aminobutyric acid
Nil (control)	0	0
Phenylhydrazine (10mM)	8	5
N-Ethyl maleimide (10mM)	100	18
p-chloromercuribenzoate (8 μ M)	100	100
Iodoacetamide (10mM)	100	87
EDTA (10mM)	97	95
NaF (10mM)	11	7

Inhibition studies of cysteine-dependent ATP-PP_i exchange.

A variety of nucleotides, sulphur-containing anions, amino acids and peptides, were surveyed as possible regulators of cysteinyl-tRNA synthetase activity. Both sulphite and sulphide inhibited cysteine-dependent ATP-PP_i exchange. The kinetics of the inhibition, by sulphite and sulphide, were studied in factorial experiments containing cysteine as the substrate. The inhibition of cysteine-dependent ATP-PP_i exchange was competitive with respect to cysteine (Figs. 11.11 and 11.12, respectively).

D-Cysteine competitively inhibited L-cysteine-dependent exchange (Fig. 11.13) with respect to L-cysteine concentration. The inhibition of cysteine-dependent exchange by L-

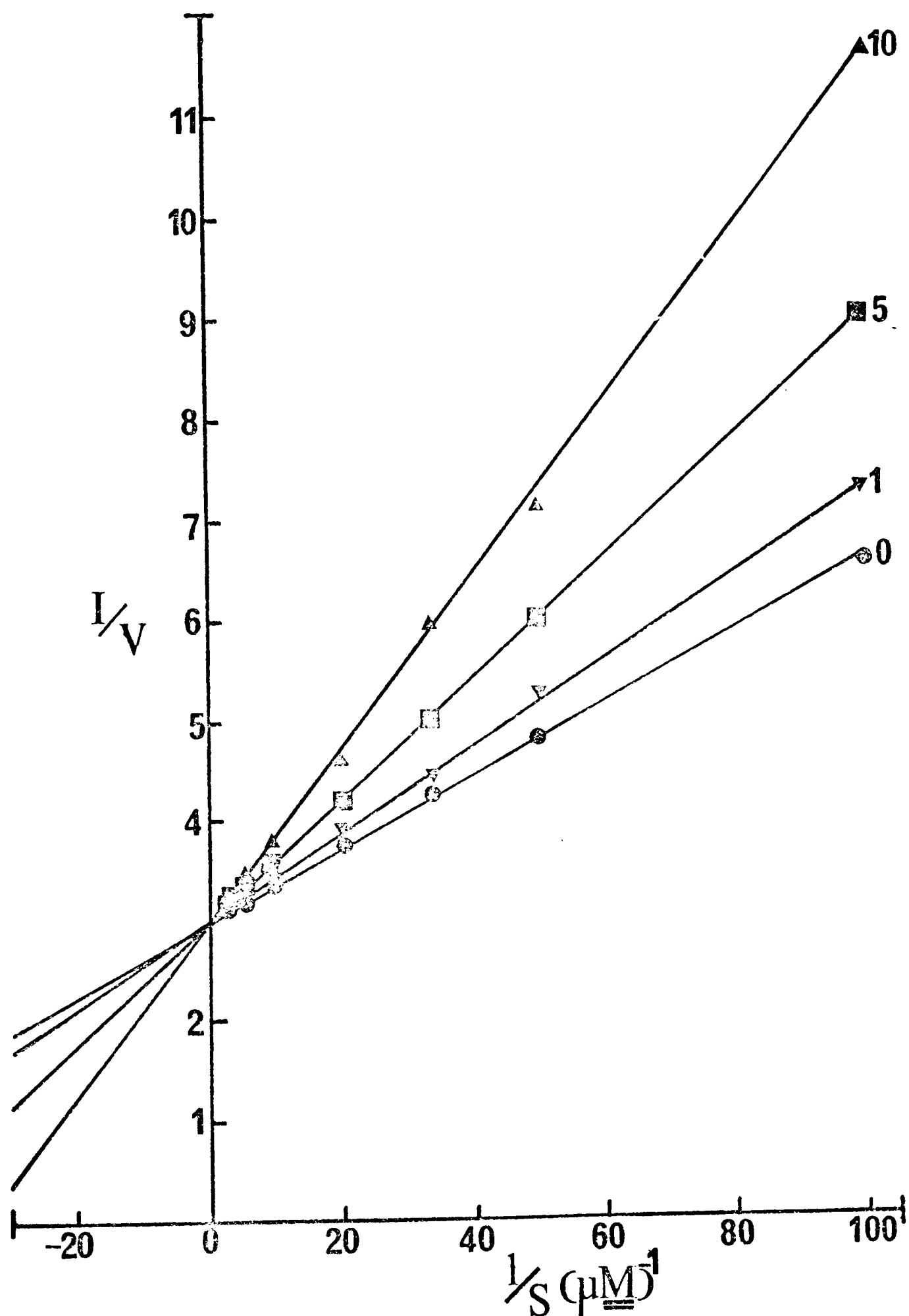


Fig. 11.11

Double reciprocal plots of the effect of cysteine concentration upon cysteine-dependent ATP-PP_i exchange in the presence or absence of sulphite. Reaction mixtures were as described in Materials and Methods except that the concentrations of sulphite and cysteine were as specified.

●, without sulphite; ▼, 1mM-sulphite; ■, 5mM-sulphite; ▲, 10mM-sulphite.

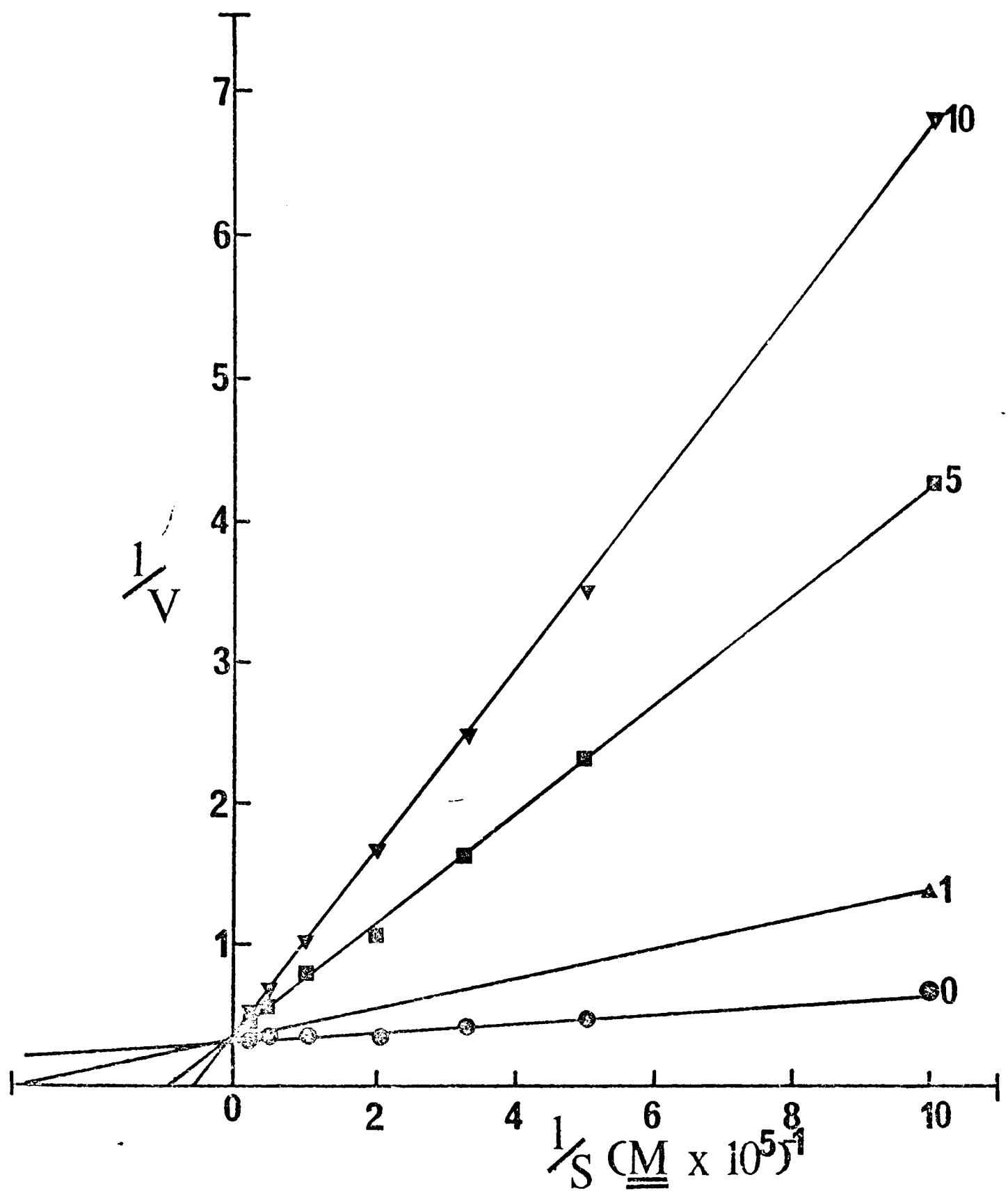


Fig. 11.12

Double reciprocal plots of the effect of sulphide concentration upon cysteine-dependent ATP-PP_i exchange.

Assays were conducted as described in Materials and Methods except that the concentration of sulphide and cysteine in the reaction mixtures were as specified.

●, without sulphide; ▲, 1mM-sulphide; ■, 5mM-sulphide; ▼, 10mM-sulphide.

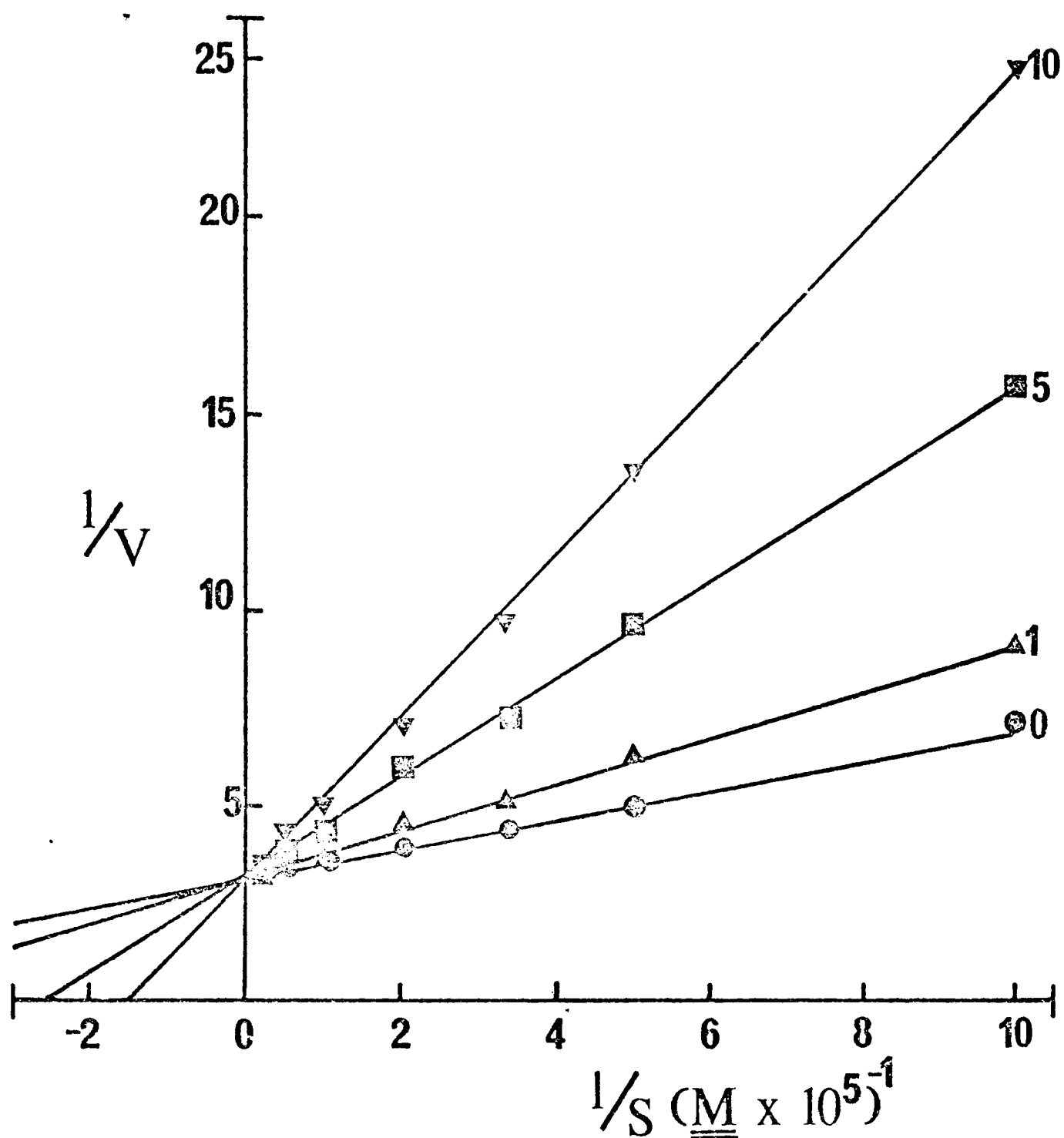


Fig. 11.13

Double reciprocal plots of the effect of L-cysteine concentration upon cysteine-dependent ATP-PP_i exchange in the presence or absence of D-cysteine.

Reaction mixtures were as described in Materials and Methods except that the concentrations of L-cysteine and D-cysteine were as specified.

●, without D-cysteine; ▲, 1mM-D-cysteine; ■, 5mM-D-cysteine; ▼, 10mM-D-cysteine.

homocysteine was non-competitive with respect to cysteine (Fig. 11.14).

Methionine stimulated cysteinyl-tRNA synthetase activity (Fig. 11.15); methionine lowered the apparent K_m of the enzyme for cysteine, but did not alter the V_{max} .

Reduced glutathione competitively inhibited cysteinyl-tRNA synthetase activity with respect to cysteine (Fig. 11.16).

The inhibition of cysteinyl-tRNA synthetase by O-acetylserine was non-competitive with respect to cysteine (Fig. 11.17). Cysteamine, the decarboxylated form of cysteine, strongly inhibited cysteinyl-tRNA synthetase activity (Fig. 11.18); inhibition was non-competitive with respect to cysteine. Cysteic acid competitively inhibited cysteinyl-tRNA synthetase activity with respect to cysteine (Fig. 11.19).

Cysteine-dependent ATP-PP_i exchange was inhibited by ADP, 5'-AMP and 3'-AMP; with respect to ATP, inhibition by ADP was non-competitive (Fig. 11.20), inhibition by 5'-AMP was un-competitive (Fig. 11.21) and inhibition by 3'-AMP was competitive (Fig. 11.22).

Phosphate ions, considered an end-product of amino acid activation, non-competitively inhibited cysteinyl-tRNA synthetase activity with respect to cysteine (Fig. 11.23). Strong inhibition of purified E. coli phenylalanyl-tRNA synthetase by phosphate ions has been observed by Stulberg (1967). Makman & Cantoni (1966) similarly reported phosphate inhibition of seryl-tRNA synthetase from yeast.

L-Alanine, the amino acid which is one methyl group shorter than α -aminobutyric acid, competitively inhibited cysteine-dependent ATP-PP_i exchange (Fig. 11.24) with respect to cysteine.

Factorial experiments were conducted with several concen-

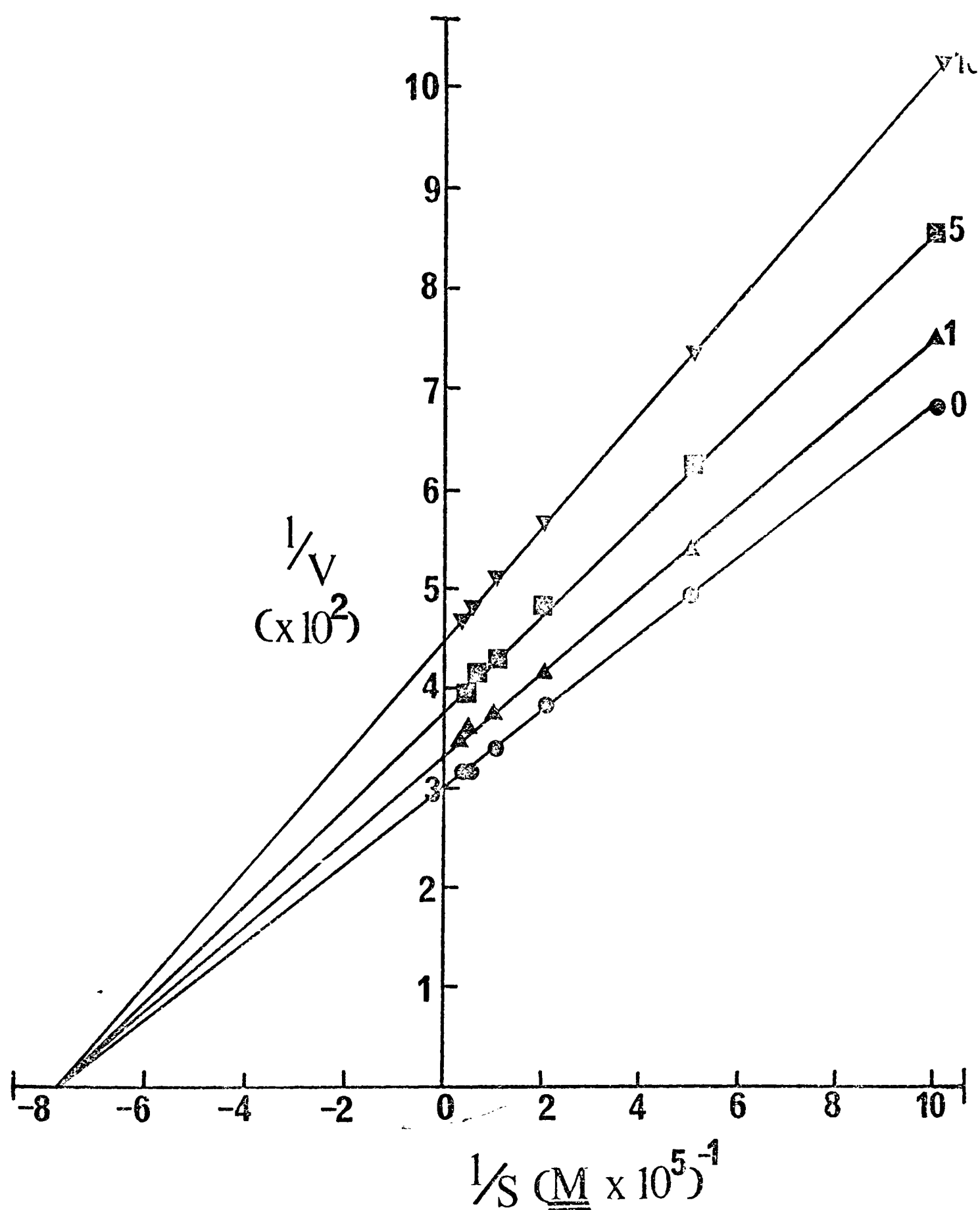


Fig. 11.14

Double reciprocal plots of the effect of cysteine concentration on cysteine-dependent ATP-PP_i exchange in the presence or absence of homocysteine. Reaction mixtures were as described in Materials and Methods except that cysteine and homocysteine were added to the reaction mixtures in the concentrations specified. ●, without homocysteine; ▲, 1mM-homocysteine; ■, 5mM-homocysteine; ▼, 10mM-homocysteine.

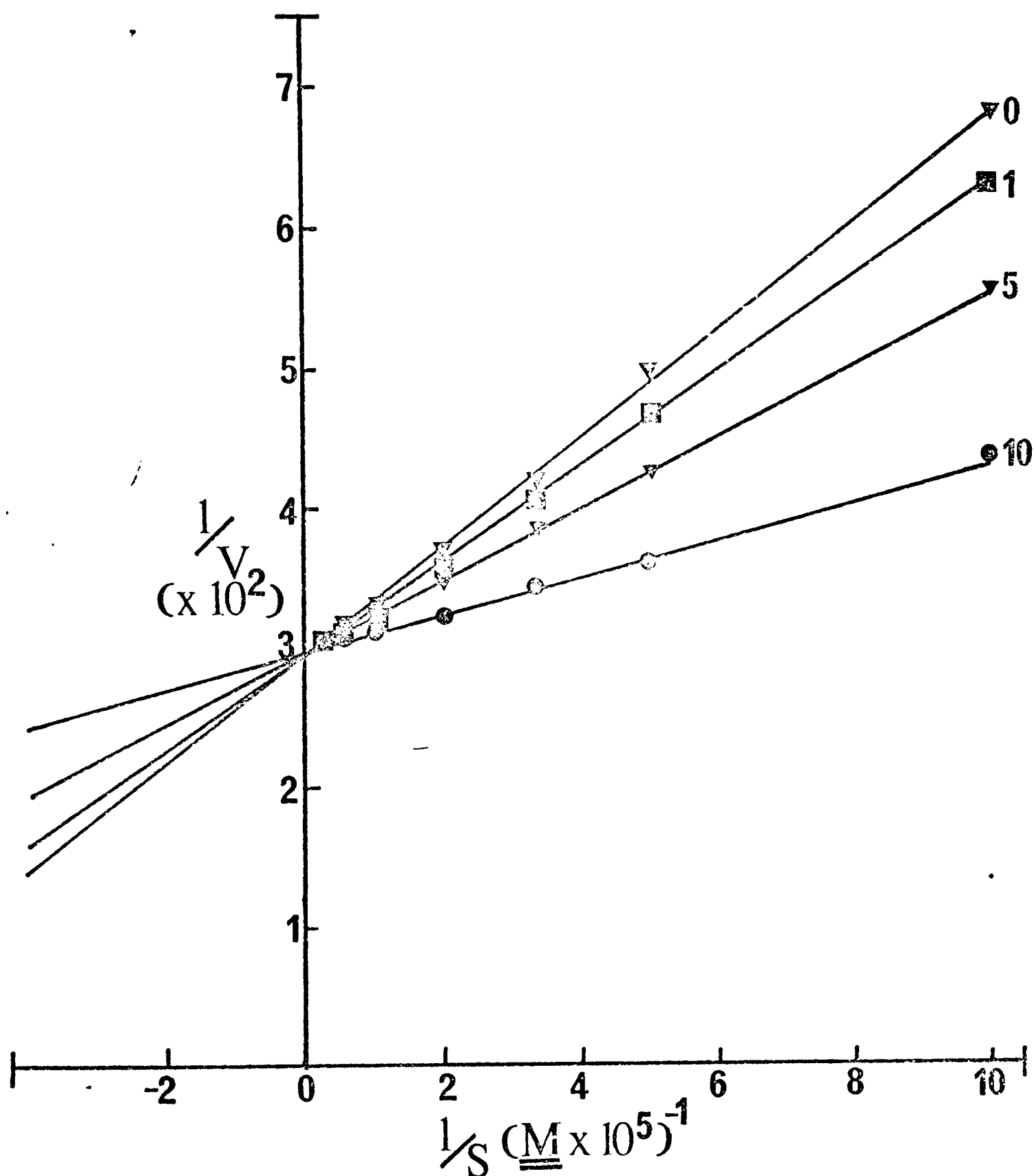


Fig. 11.15

Double reciprocal plots of the effect of cysteine concentration upon cysteine-dependent ATP-PP_i exchange in the presence or absence of methionine.

Reaction mixtures were as described in Materials and Methods except that cysteine and methionine were added to the reaction mixtures at the concentrations specified.

▼, without methionine; ■, 1mM-methionine; ▼, 5mM-methionine; ●, 10mM-methionine.

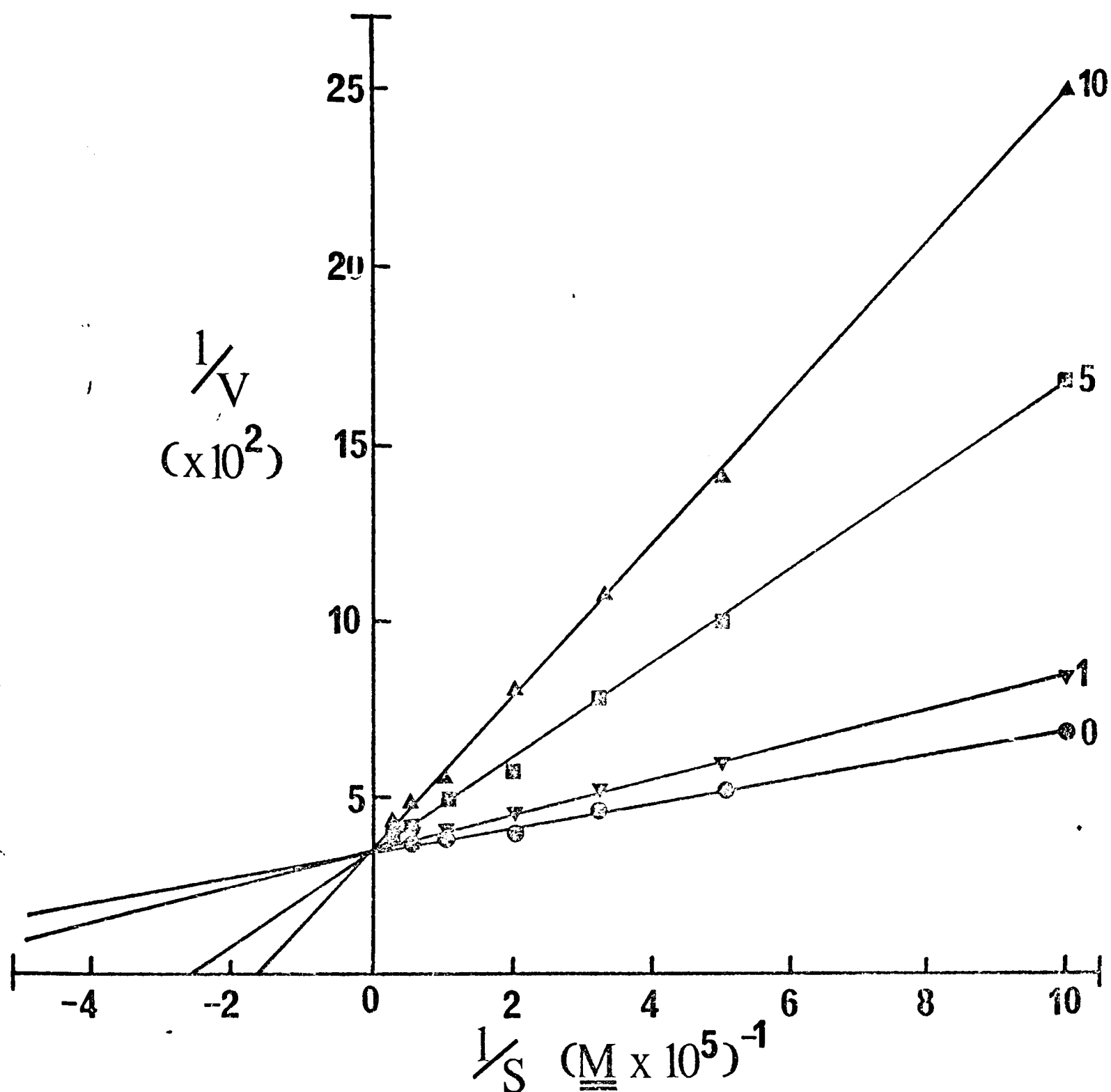


Fig. 11.16

Double reciprocal plots of the effect of cysteine concentration upon cysteine-dependent ATP-PP_i exchange in the presence or absence of glutathione.

Reaction mixtures were as described in Materials and Methods except that cysteine and glutathione were added to the reaction mixtures in the concentrations specified.

●, without glutathione; ▼, 1mM-glutathione; ■, 5mM-glutathione; ▲, 10mM-glutathione.

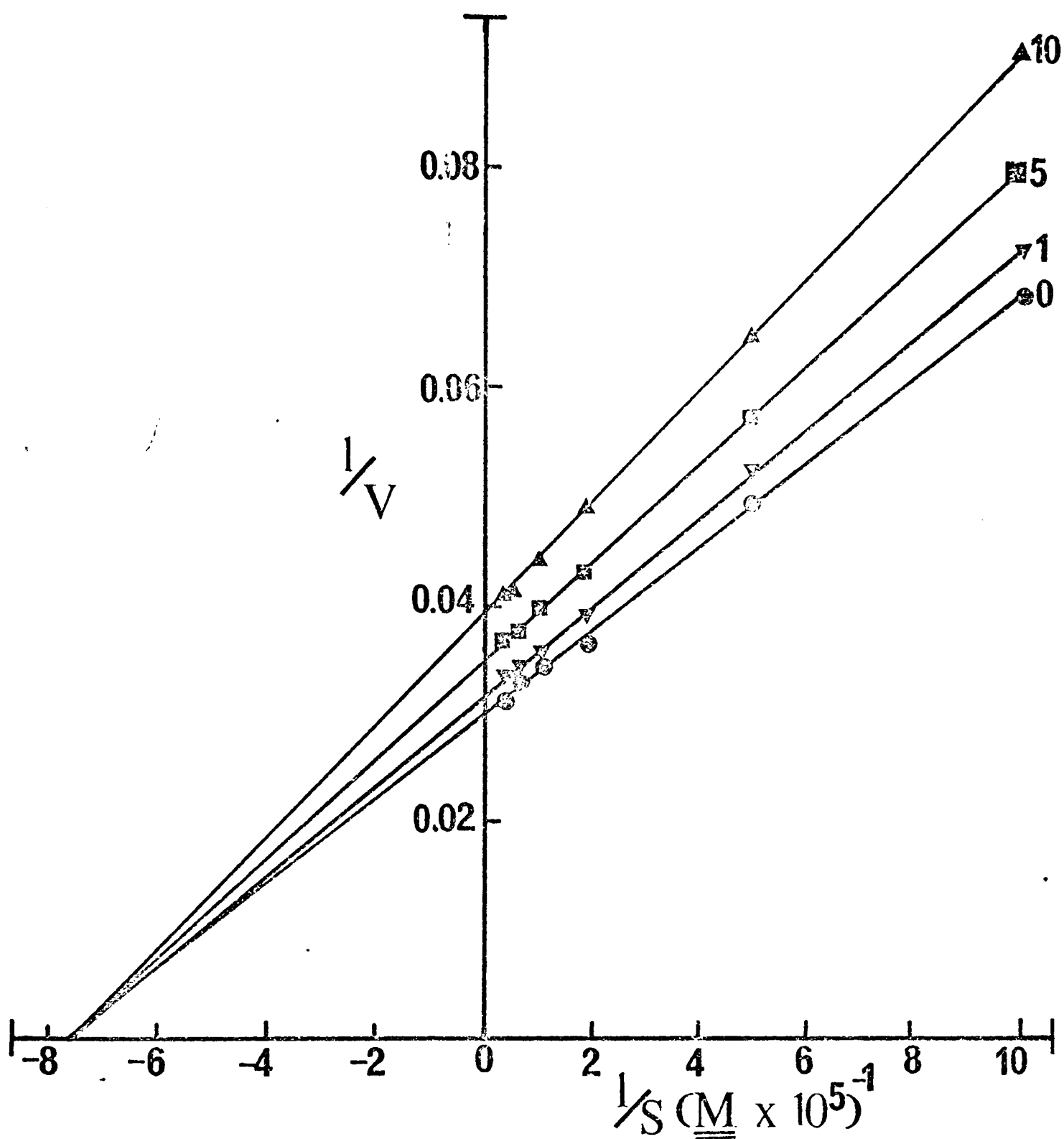


Fig. 11.17

Double reciprocal plots of the effect of cysteine concentration upon cysteine-dependent ATP-PP_i exchange in the presence or absence of O-acetyl serine.

Reaction mixtures were as described in Materials and Methods except that cysteine and O-acetyl serine were added to the reaction mixtures in the concentrations specified.

●, without O-acetyl serine; ▼, 1mM-O-acetyl serine; ■, 5mM-O-acetyl serine; ▲, 10mM-O-acetyl serine.

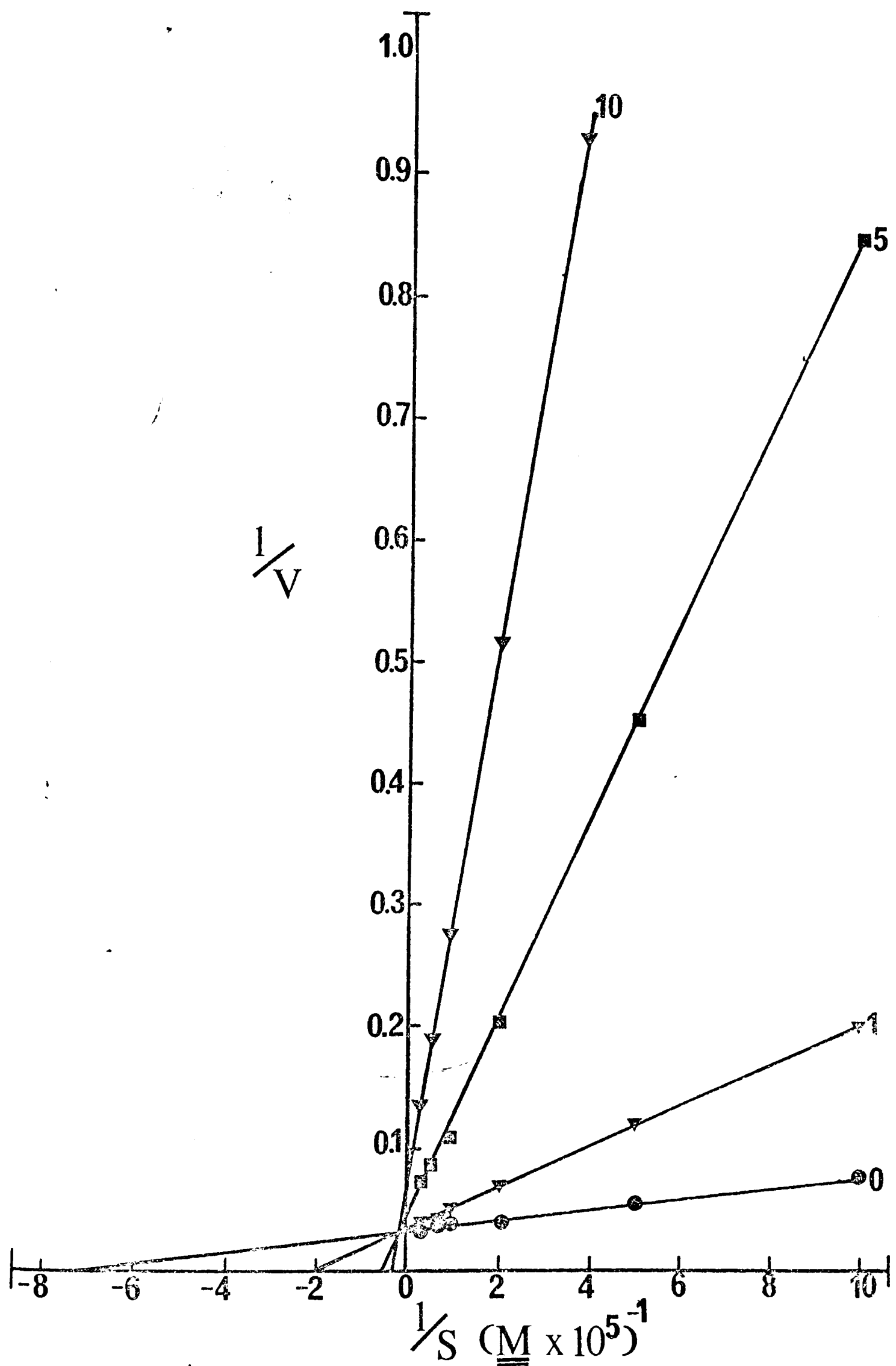


Fig. 11.18

Double reciprocal plots of the effect of cysteine concentration upon cysteine-dependent ATP-PP_i exchange in the presence or absence of cysteamine. Reaction mixtures were as described in Materials and Methods except that cysteine and cysteamine were added to the reaction mixtures at the concentrations specified. ●, without cysteamine; ▼, 1mM-cysteamine; ■, 5mM-cysteamine; ▽, 10mM-cysteamine.

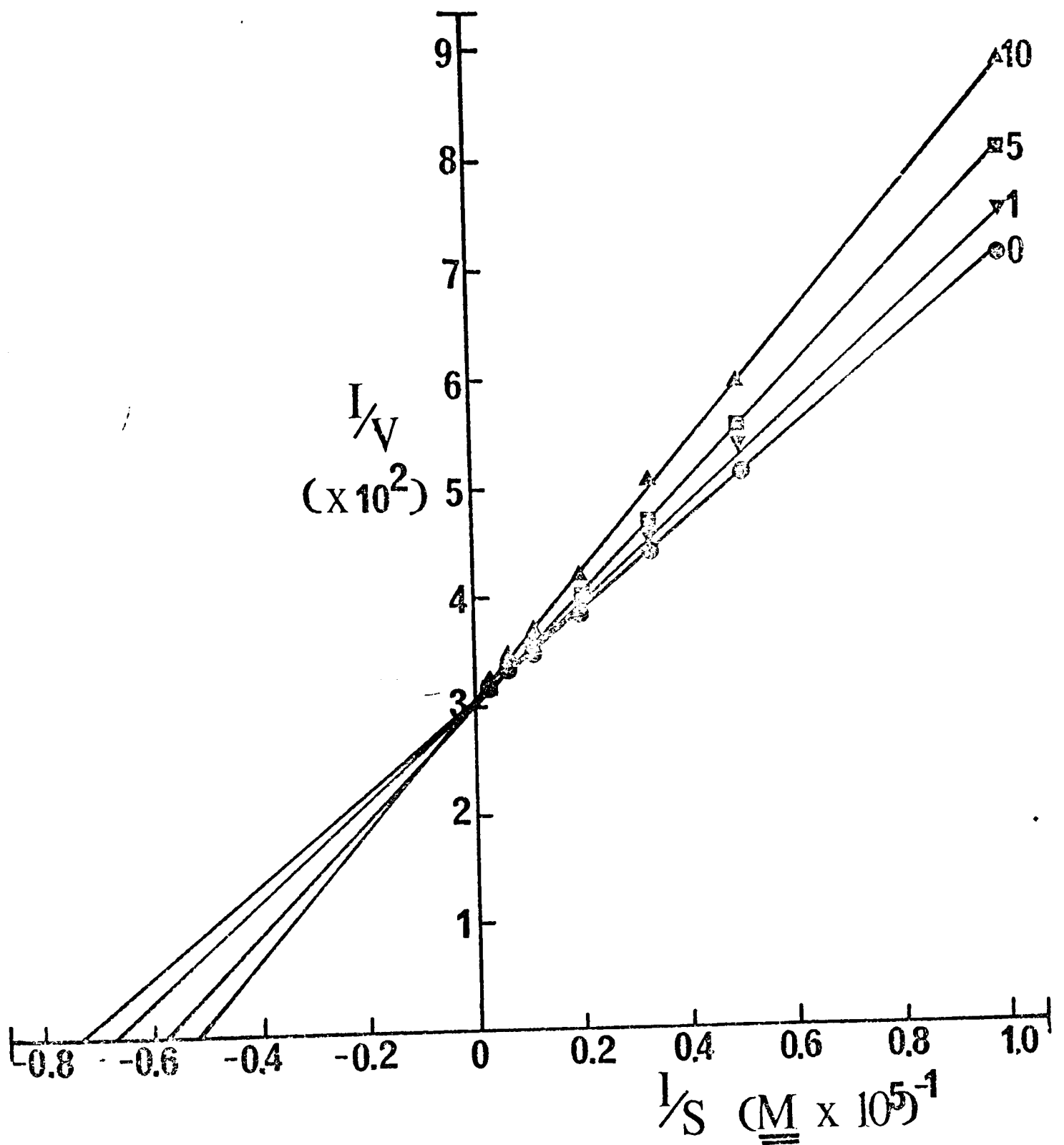


Fig. 11.19

Double reciprocal plots of the effect of cysteine upon cysteine-dependent ATP-PP_i exchange in the presence or absence of cysteic acid. Reaction mixtures were as described in Materials and Methods except that cysteine and cysteic acid were added at the concentrations specified. ●, without cysteic acid; ▼, 1mM-cysteic acid; ■, 5mM-cysteic acid; ▲, 10mM-cysteic acid.

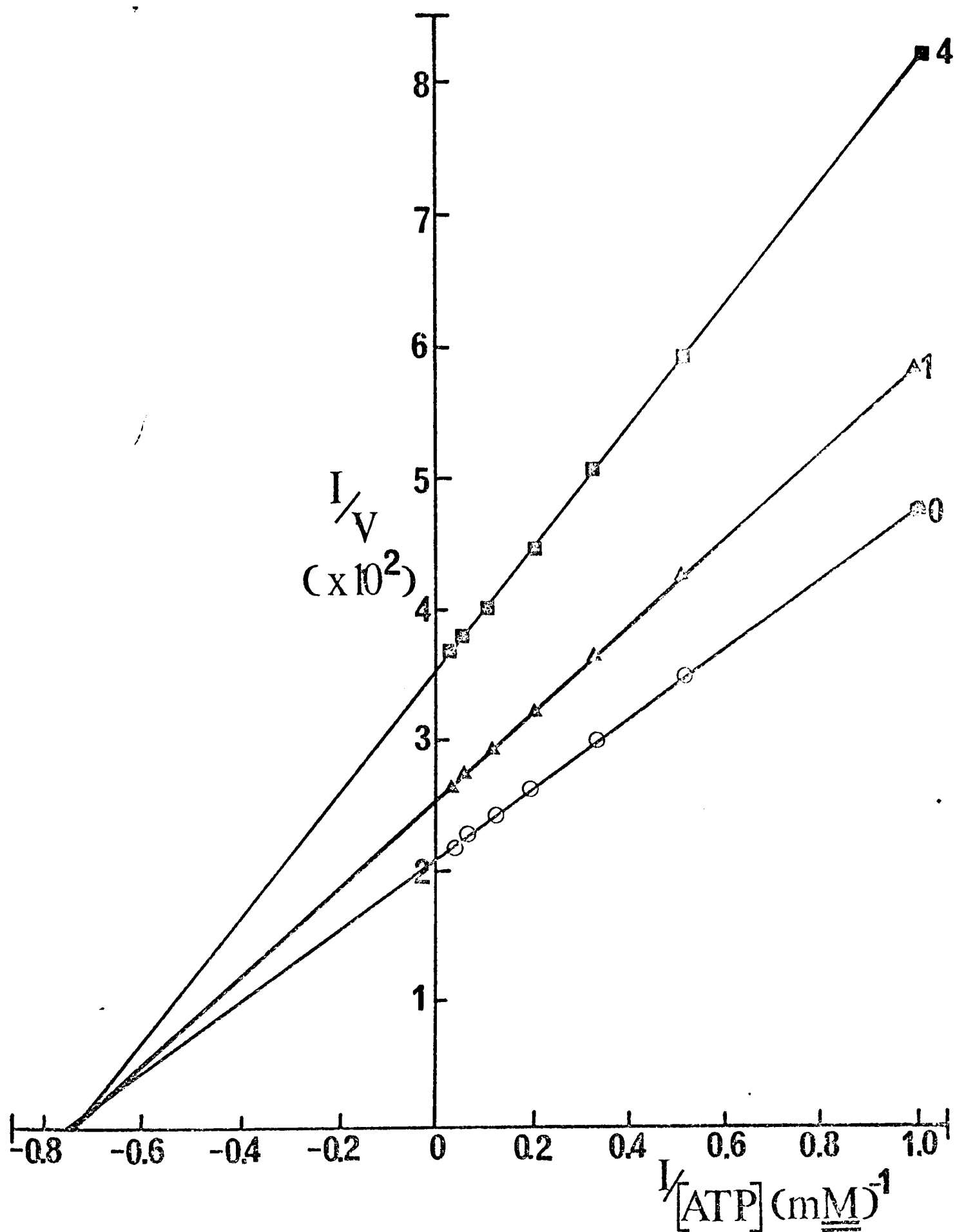


Fig. 11.20

Double reciprocal plots of the effect of ATP concentration upon cysteine-dependent ATP-PP_i exchange in the presence or absence of ADP. . Reaction mixtures were as described in Materials and Methods except that ADP and ATP were added to the reaction mixtures at the concentrations specified.

●, without ADP; ▲, 1mM-ADP; ■, 4mM-ADP.

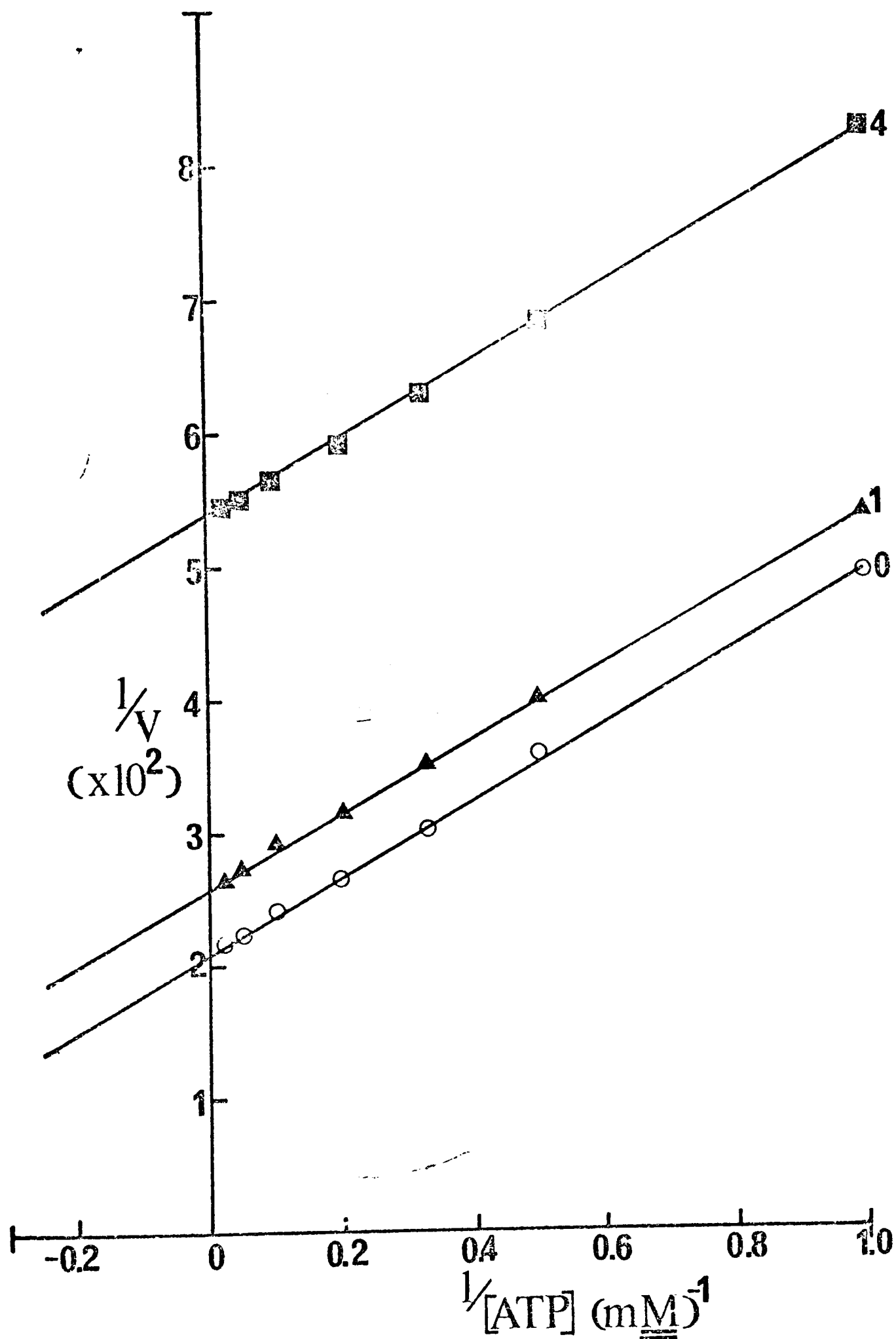


Fig. 11.21

Double reciprocal plots of the effect of ATP concentration upon cysteine-dependent ATP-PP_i exchange in the presence or absence of 5'-AMP. Reaction mixtures were as described in Materials and Methods except that ATP and 5'-AMP were added to reaction mixtures at the concentrations specified. $\circ$, without 5'-AMP; $\blacktriangle$, 1mM-5'-AMP; $\blacksquare$, 4mM-5'-AMP.

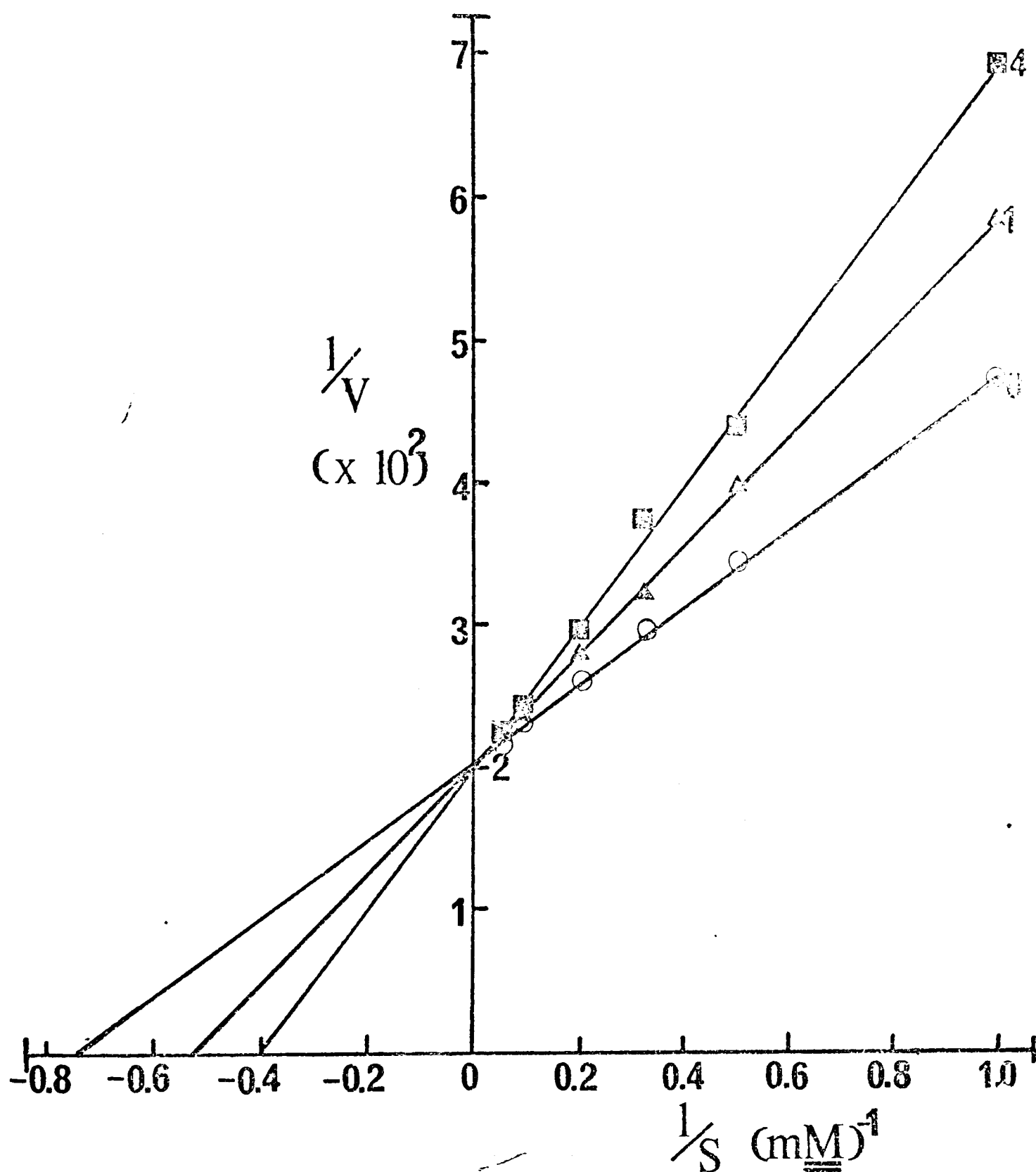


Fig. 11.22

Double reciprocal plots of the effect of ATP concentration upon cysteine-dependent ATP-PP_i exchange in the presence or absence of 3'-AMP. Reaction mixtures were as described in Materials and Methods except that ATP and 3'-AMP were added to reaction mixtures at the concentrations specified.

O, without 3'-AMP; Δ , 1mM-3'-AMP; $\square$, 4mM-3'-AMP.

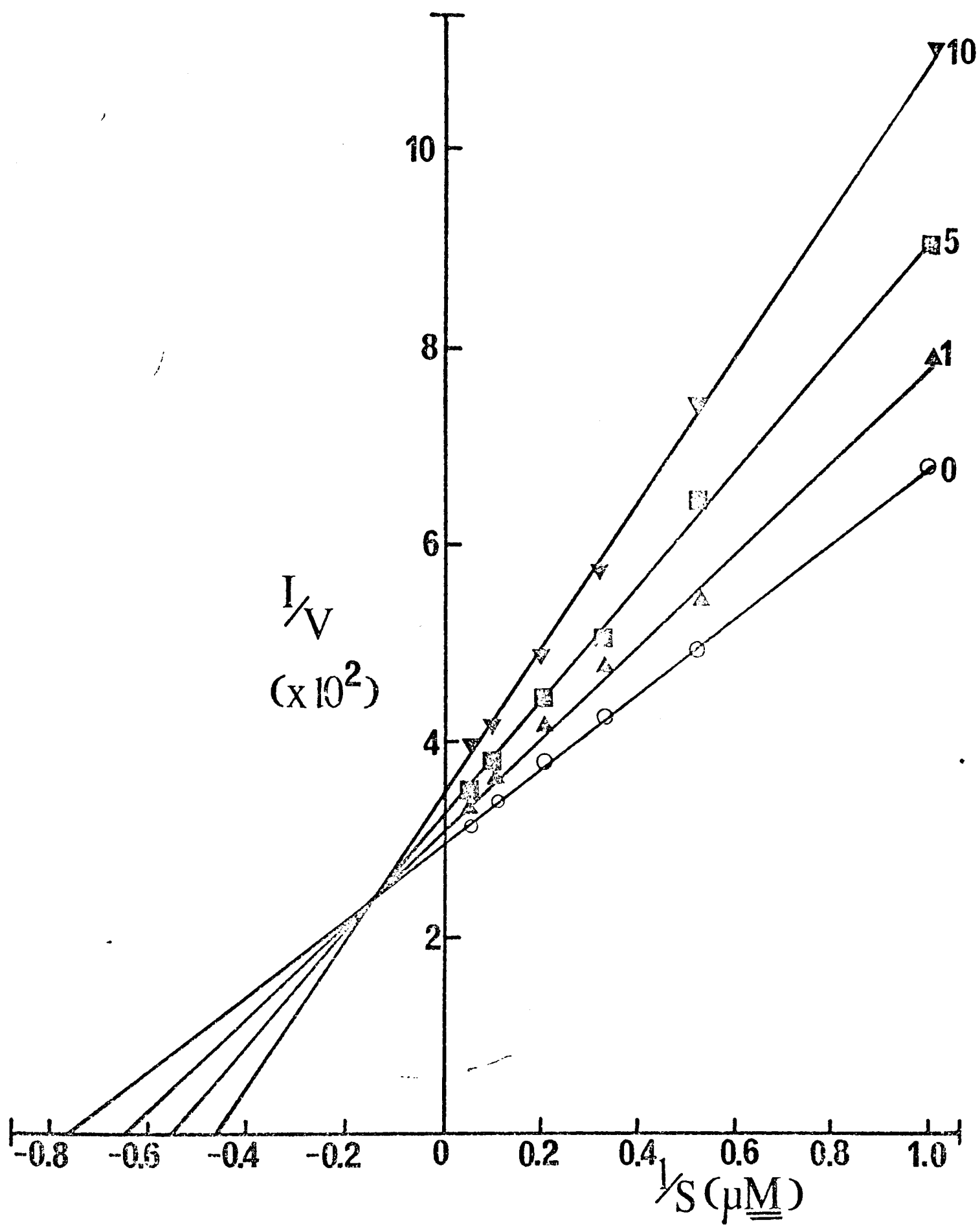


Fig. 11.23

Double reciprocal plots of the effect of cysteine concentration upon cysteine-dependent ATP-PP_i exchange in the presence or absence of phosphate ions. Reaction mixtures were as described in Materials and Methods except that cysteine and phosphate were added to reaction mixtures at the concentrations specified. O, without phosphate; $\blacktriangle$, 1mM-phosphate; $\blacksquare$, 5mM-phosphate; $\blacktriangledown$, 10mM-phosphate.

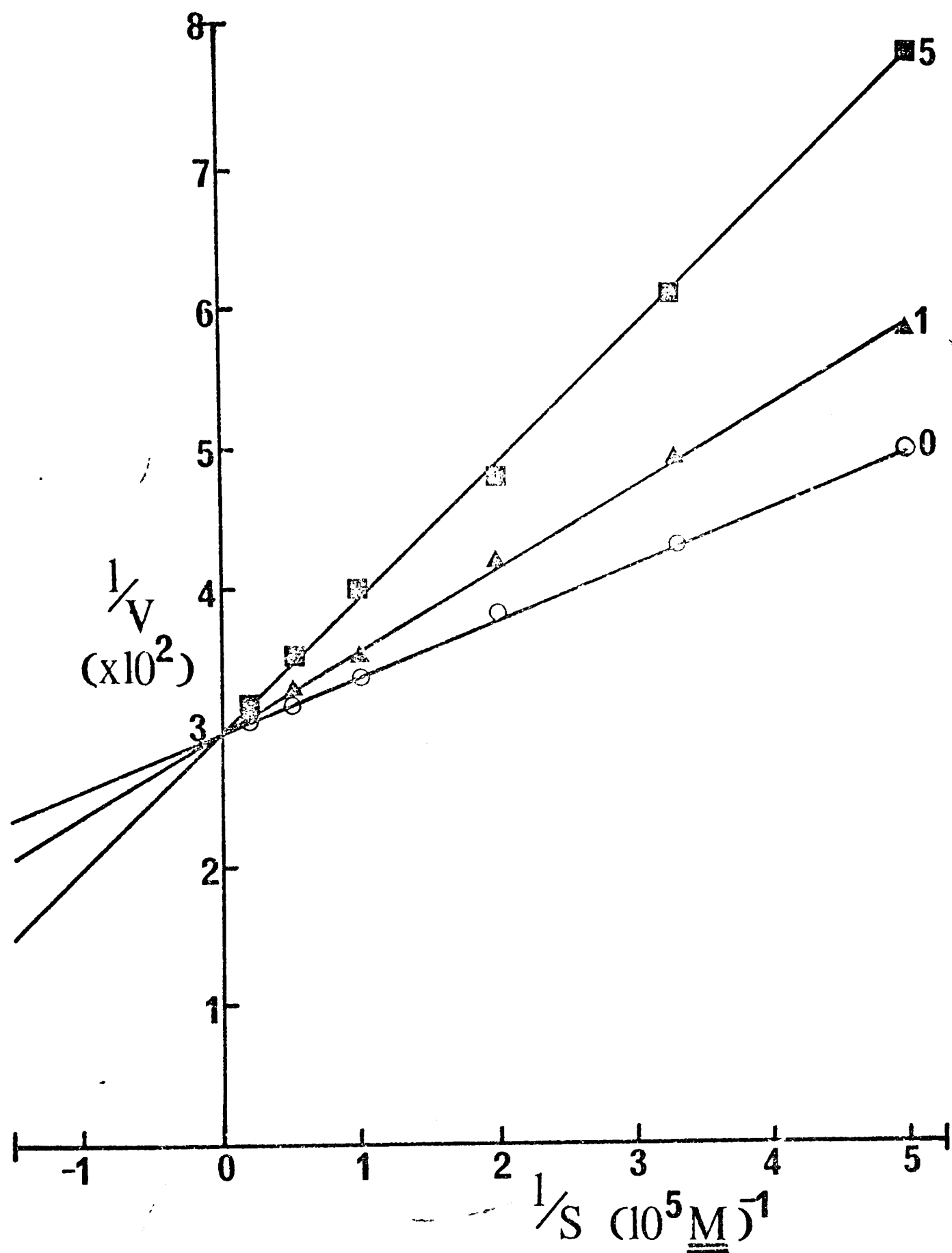


Fig. 11.24

Double reciprocal plots of the effect of cysteine concentration upon cysteine-dependent ATP-PP_i exchange in the presence or absence of L-alanine. Reaction mixtures were as described in Materials and Methods except that cysteine and alanine were added to reaction mixtures at the concentrations specified. O, without alanine; ▲, 1mM-alanine; ■, 5mM-alanine.

trations of sulphate, NAD, NADH, NADP, NADPH, L-serine, taurine, β -alanine or aspartic acid, against several concentrations of ATP or cysteine. None of the former group of compounds had any effect upon cysteinyl-tRNA synthetase activity.

Synthesis of cysteine hydroxamic acid. The synthesis of cysteine hydroxamic acid is analagous to the system described by Berg (1956); he used hydroxylamine to trap the acetyl group of adenyl acetate as acetylhydroxamic acid.

With ATP, cysteine, enzyme, and a high concentration of hydroxylamine, synthesis of cysteine hydroxamic acid was detected. Cystine hydroxamic acid synthesis was linear with time and was dependent on the presence of ATP, Mg^{2+} , enzyme and cysteine (Fig. 11.25). The rate of cysteine hydroxamic acid synthesis was dependent on enzyme concentration. Under these assay conditions, the formation of cysteine hydroxamic acid was accompanied by the liberation of an equivalent amount of AMP and PP_i (Table 11.3). Cysteinyl-tRNA synthetase also catalysed the synthesis of selenocysteine hydroxamic acid; the rate of selenocysteine hydroxamic acid formation was slightly less than that for cysteine (Table 11.3).

Effect of sulphur source during growth upon the specific activity of cysteinyl-tRNA synthetase. Cells were grown in the presence of sulphur sources including sulphate, sulphite, sulphide, cystine, sulphate plus homocysteine and sulphate plus methionine. Dialysed cell-free extracts prepared from cells harvested during mid log phase showed little variation of the specific activity of the cysteinyl-tRNA synthetase.

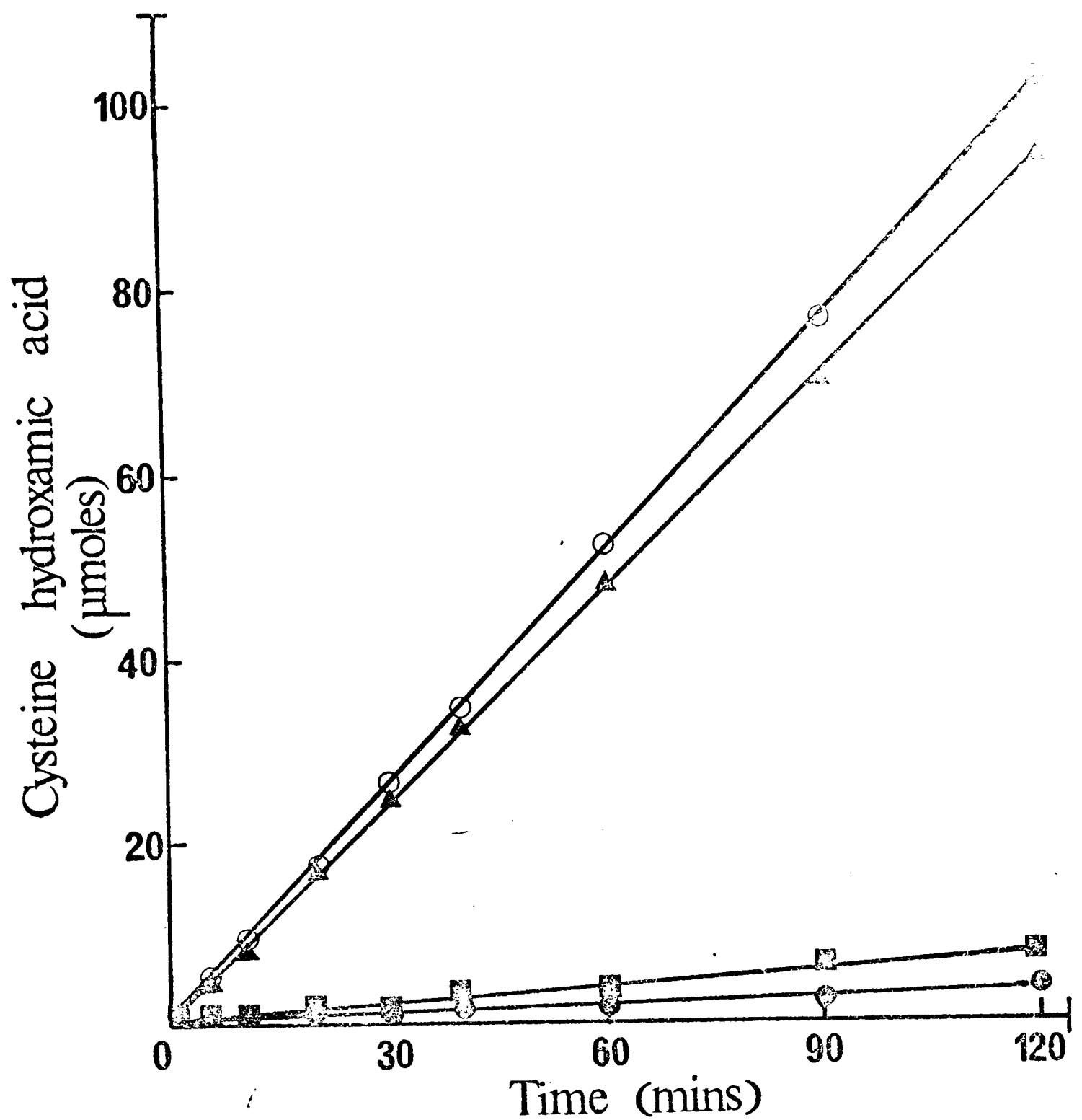


Fig. 11.25

The effect of time upon the formation of cysteine and selenocysteine hydroxamic acid.

The control reaction mixture was as described in Materials and Methods (O). Alterations to the reaction mixture were as described below;

- $\blacktriangle$, selenocysteine as substrate; $\bullet$, plus cysteine minus ATP;
- $\blacksquare$, plus cysteine minus Mg^{2+} .

TABLE 11.3

<u>Stoichiometry of products formed in ATP-cysteine reactions in the presence of hydroxylamine</u>			
<u>Components</u>	<u>μmoles per h</u>		
	<u>Amino acid hydroxamate</u>	<u>PP_i</u>	<u>AMP</u>
Complete (with cysteine)	0.47	0.45	0.45
Complete minus ATP	0.01	0.01	0.01
Complete minus Mg ²⁺	0.01	0.01	0.01
Complete minus cysteine	0.03	0.02	0.04
Complete (with seleno-cysteine)	0.38	0.37	0.39
Complete minus ATP	0.01	0.01	0.01
Complete minus Mg ²⁺	0.01	0.01	0.01
Complete minus seleno-cysteine	0.03	0.03	0.04

Six duplicates reaction mixtures were conducted for each of which two were assayed for each compound. Cysteine hydroxamic acid was assayed as the ferric complex as described in the text; PP_i was measured as P_i after treatment with purified inorganic pyrophosphatase (see Chapter 7); AMP was measured using an ADP/AMP Test Kit obtained from Boehringer und Soehne G.m.b.H., Mannheim, Germany.

DISCUSSION

Considering the importance of cysteine in proteins;

(a) of providing the sulphhydryl-groups necessary for the function of a large proportion of enzymes, and

(b) of providing the sulphhydryl-groups which form disulphide bridges in many proteins and, therefore, play an important role in determining the shape of proteins, remarkably little work has been conducted on the study of cysteinyl-tRNA synthetase. (The only reported study to date, of a highly purified preparation of cysteinyl-tRNA synthetase has been of the enzyme from yeast - James & Bucovaz, 1969).

Cysteinyl-tRNA synthetase has a high affinity for its true substrate, cysteine. It also exhibits a high affinity for selenocysteine. This is the first report of a purified cysteinyl-tRNA synthetase catalysing the activation of selenocysteine. Purified cysteinyl-tRNA synthetase from P. denitrificans not only catalyses selenocysteine-dependent ATP-PP_i exchange, but in the presence of hydroxylamine, also catalyses the synthesis of selenocysteine hydroxamic acid. The ratios of the Vmax of cysteine - and selenocysteine-dependent ATP-PP_i differs from the ratio of the rate of formation of the corresponding hydroxamic acid derivatives. It is, however, recognised that when amino acid activation is measured both by hydroxamic acid formation and ATP-PP_i exchange, the ratio of activities, determined by these two methods, does not remain constant, but varies with each amino acid (Novelli, 1958; Schweet et al., 1957; Hoagland et al., 1956; Hahn & Brown, 1967a).

The activation of selenocysteine, catalysed by cysteinyl-tRNA synthetase, may possibly explain the toxicity of selenate

to P. denitrificans at higher selenate concentrations (see Chapter 2), since selenate is taken up by bacteria (Chapters 4 and 5), reduced to selenide (by an unknown pathway), and condensed with O-acetyl serine to form selenocysteine (Chapter 9), which is then capable of replacing cysteine in proteins (via the cysteinyl-tRNA synthetase). A substitution of the sulphur atom, by a selenium atom in the sulphhydryl group of an enzyme site, would be expected to drastically alter the properties of the enzyme, and this may, in turn, lead to inactivation of a number of enzymes vital for survival.

Purified cysteinyl-tRNA synthetase also catalyses α -amino butyric acid-dependent ATP-PP_i exchange; the affinity of the enzyme for α -amino butyric acid was much lower than for either cysteine or selenocysteine.

Considering the structures of the alternative substrates of cysteinyl-tRNA synthetase, the requirements of a compound, capable of acting as an alternative substrate were:

- (i) an α -amino acid group
- (ii) the amino acid must be the L-isomer
- (iii) a terminal sulphhydryl- or selenohydryl-group is required for maximum activity.

The only substitution of the sulphhydryl- or selenohydryl-group which was almost as active was a replacement by a -CH₃ group (α -aminobutyric acid). Compounds in which the sulphhydryl group was replaced by -COOH (aspartic acid), an -OH (serine), an -HSO₂ (cysteine sulphinic acid) or an HSO₃ (cysteic acid) could not act as substrates, even at high concentrations.

The relatively low affinity of the enzyme for α -aminobutyric acid, as compared with the high affinity of the enzyme for its true substrate, cysteine, are in agreement with

Novelli's (1967) proposal that the K_m of an aminoacyl-tRNA synthetase for the "wrong" amino acid, or the analogue, is several orders of magnitude larger than the K_m for the natural substrate (c.f. K_m (cysteine) = $12.5 \mu M$ and K_m (α -aminobutyric acid) = $10.0 mM$).

Selenocysteine is an exception to the proposed rule of Novelli (1967). This exception is, however, in agreement with the proposal of Fowden et al., (1967), who have suggested that the high levels of selenium by certain groups of selenium-accumulating plants is due to their possession of enzymes which are able to discriminate between the sulphur- and the selenium-containing amino acids. Since P. denitrificans is unable to grow on culture medium containing high concentrations of selenate, but is able to incorporate selenide into selenocysteine (see Chapter 9) and is able to catalyse the activation of selenocysteine in the initial steps of its incorporation into proteins, it is reasonable to suppose that the toxicity of selenate to P. denitrificans may be due to its ultimate incorporation into proteins.

Unlike the purified cysteinyl-tRNA synthetase from yeast, the enzyme from P. denitrificans is sulphhydryl-group reagent sensitive (like almost all other aminoacyl-tRNA synthetases; Novelli, 1967).

Sulphite, sulphide and O-acetyl serine all inhibited cysteinyl-tRNA synthetase activity; intracellular increases in the concentration of any of these compounds would occur if there was a lack of O-acetyl serine (when sulphide was in excess) or a lack of sulphide (if O-acetyl serine was in excess).

Methionine stimulated cysteinyl-tRNA synthetase activity, indicating that an intracellular accumulation of methionine

stimulates the incorporation of cysteine into proteins.

Since cysteine not only inhibits a number of reactions, including respiration (see Chapter 2) but also is a substrate for a number of different reactions (see Fig. 3.3), there must be a strict control mechanism regulating both, the intracellular concentration of cysteine, and the synthesis of compounds from cysteine. Furthermore, since cysteine plays an important part in controlling both the structure and function of a large number of proteins, the affinity of the initial enzyme, involved in the incorporation of cysteine into proteins, should be high, and the control of this enzyme activity must be strict.

Results of experiments to determine the effect of different sulphur sources during growth, upon the specific activity of cysteinyl-tRNA synthetase, suggested that the enzyme may be constitutive.

The effect of tRNA upon the activity of the aminoacyl-tRNA synthetases has been found to be important in the overall control and regulation of the activity of aminoacyl-tRNA synthetases (Williams & Neidhardt, 1969) but no attempt was made in the study to investigate this phenomenon.

CHAPTER 12

METHIONYL-tRNA SYNTHETASE

INTRODUCTION

Methionine is utilised as a constituent in proteins, as a source of methyl groups (Shapiro & Schlenk, 1965; Greenberg, 1963; Taylor, 1970), and is important in the initiation events of protein synthesis (Waller, 1963; Marcker & Sanger, 1964; Webster et al., 1966; Nathans, 1964; Zamir et al., 1966; Bachmeyer & Kreil, 1969; Nomura & Lowry, 1967). It has also been tentatively implicated in the catalytic mechanism of several enzymes which appear to lose activity after modification of a limited number of their methionine residues (Ray & Koshland, 1962; Margoliash et al., 1966; Colman, 1967, 1968; Rose, 1966).

Administration of organic and inorganic selenium compounds to a variety of organisms often results in the appearance of selenium in various proteins of these organisms (Jauregui-Adell, 1966). Cowie and Cohen (1957) have shown that the methionine requirement of a mutant strain of Escherichia coli could be satisfied by selenomethionine. These, and other studies, have led to the assumption that, in protein, as in other biochemical pathways, selenium metabolism occurs via seleno-analogues of the relevant sulphur-containing metabolites; methionine and cysteine in the case of protein synthesis.

Nisman and Hirsch (1958) have shown that selenomethionine can stimulate ATP-PP_i exchange in E. coli extracts. Hoffman et al., (1970) have shown that selenomethionine is incorporated into the first step of protein aminoacylation of tRNA at much the same rate as the normal substrate, methionine.

The purpose of this investigation was to study the regulation and control of methionine activation by methionyl-tRNA

synthetase. The purification of methionyl-tRNA synthetase from P. denitrificans is reported. The ability of the amino acid analogues to serve as substrates, or to act as inhibitors of aminoacyl adenylate formation was studied to establish which groups of the amino acid were necessary for binding to the enzyme. To provide further insight into the mechanism and control of methionine activation, the effects of added sulphhydryl-group reagents, nucleotides, various metal cations, and a number of intermediates of sulphur metabolism, on aminoacyl adenylate formation, were investigated.

MATERIALS AND METHODS

Chemicals. Methionine hydroxamic acid, and DL-selenomethionine were obtained from the Sigma Chemical Co., London, U.K. DL-Homocysteine thiolactone-HCl was obtained from Koch-Light Labs., Bucks., U.K. All other chemicals were obtained from the sources stated in previous chapters.

Preparation of L-selenomethionine. DL-Selenomethionine was treated with D-amino acid oxidase, in the presence of catalase, and the L-selenomethionine was isolated and purified by ion exchange chromatography on Dowex 1-2X (Cl⁻ form), 200 - 400 mesh. The concentration of L-selenomethionine was adjusted to 0.1 M; the concentration of L-selenomethionine was measured, after dilution, by the ninhydrin method of Yemm & Cocking (1965).

Assay of methionyl-tRNA synthetase. Methionyl-tRNA synthetase activity was measured by two assay methods similar to those used for the measurement of cysteinyl-tRNA synthetase activity (see Chapter 11).

Method / 1 7 Methionine-dependent ATP-PP_i exchange. The reaction mixture contained, in a volume of 1 ml, 100 μ moles

of Tris-HCl buffer, pH 8.0, 8 μ moles of $MgCl_2$, 4 μ moles of Na_2K_2ATP , 5 μ moles of dithiothreitol, 2 μ moles γ - ^{32}P - PP_i (0.25 μ Ci/ μ mole), 10 μ moles of L-methionine and a limiting amount of enzyme. Reactions were started by the addition of enzyme. After incubation at 30°C for 10 min the reaction was stopped by the addition of 2 ml of trichloroacetic acid (7.5%, w/v). The γ - ^{32}P -ATP was separated from γ - ^{32}P - PP_i and counted as described in Chapter 6. Methionyl-tRNA synthetase activities are expressed as methionine-dependent exchange in nmoles/min (methionyl-tRNA synthetase units).

Method γ -2-7 Formation of methionine hydroxamic acid.

Reaction mixtures contained 200 μ moles of Tris-HCl buffer, pH 8.0, 8 μ moles of $MgCl_2$, 4 μ moles of Na_2K_2ATP , 10 μ moles of L-methionine, 5 μ moles of dithiothreitol, 2.5 mmoles of hydroxylamine and purified enzyme in a total volume of 1 ml. After incubation for 60 min at 30°C, reactions were terminated by the addition of 1 ml of trichloroacetic acid (7.5%, w/v). Methionine hydroxamic acid was determined colorimetrically as the ferric complex, as described in Chapter 11.

Purification of methionyl-tRNA synthetase. All operations were conducted at 0 to 4°C. The buffer used throughout the purification procedure was composed of 20 mM-Tris-HCl, pH 8.0, 1 mM-dithiothreitol and 5 mM- $MgCl_2$.

The first five steps in the purification procedure (Steps I to V) which included preparation of crude extract, alumina C_γ , streptomycin and ammonium sulphate fractionation and Sephadex G-200 gel filtration, were the same as those described for the purification of cysteinyl-tRNA synthetase (see Chapter 11).

Step VI DEAE-cellulose chromatography. Fraction V was applied to a DEAE-cellulose column (3.5 x 10.0 cm), equilibrated

with buffer. The column was washed with 60 ml of buffer, followed by 50 ml of 0.2 M-KCl, in buffer. The enzyme was then eluted with a 500 ml gradient of KCl in buffer, from 0.2 to 0.5M, at a flow rate of 0.65 ml/min. Five ml fractions were collected and assayed for methionine-dependent and cysteine-dependent ATP-PP_i exchange. Only those fractions which catalysed methionine-dependent and not cysteine-dependent ATP-PP_i exchange, were pooled and dialysed (Fraction VI).

Step VII Hydroxylapatite column chromatography.

Fraction VI was applied to a column of hydroxylapatite (1.5 x 10 cm), equilibrated with buffer. The enzyme was eluted with a 500 ml linear gradient of 0 to 0.3 M-KCl in buffer, at a flow rate of 0.4 ml/min. Those fractions containing the highest specific activity of methionyl-tRNA synthetase were pooled (Fraction VII) and dialysed.

Step VIII Concentration on DEAE-cellulose. Fraction VII was concentrated by the same procedure as described for the concentration of cysteinyl-tRNA synthetase (see Chapter 11).

RESULTS

Remarks on enzyme stability and purification. Methionyl-tRNA synthetase was purified to remove contaminating enzymes which catalysed ATP-PP_i exchange in the presence of their respective substrates. No evidence for the existence of more than one type of methionyl-tRNA synthetase was obtained during the purification procedure (c.f. Allende et al., 1966). The enzyme in the crude extract, stored at -15°C during the purification procedure, exhibited identical elution properties compared with the purified enzyme when examined by gel filtration on Sephadex G-200. The enzyme was purified approximately 1000-fold with a yield of 59% (Table 12.1). Methionyl-tRNA

TABLE 12.1

Purification of methionyl-tRNA synthetase

Fraction No.	Purification Step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)
I	Crude extract	13259	26380	1.98	100
II	Alumina C _γ fractionation	8295	25845	3.1	98
III	Streptomycin fractionation	2293	24764	10.8	94
IV	(NH ₄) ₂ SO ₄ fractionation	430	23521	54.7	89
V	Sephadex G-200 gel filtration	104	22259	212.4	84
VI	DEAE-52 cellulose column chromatography	14.7	17080	1162.1	65
VII	Hydroxylapatite column chromatography	8.04	15675	1950.5	59

synthetase was purified free of inorganic pyrophosphatase. It was also purified free of cysteinyl-tRNA synthetase; separation of methionyl- and cysteinyl-tRNA synthetases by DEAE-cellulose ion exchange chromatography is shown in Fig. 11.1.

The stability of methionyl-tRNA synthetase was increased by the presence of a reducing agent (dithiothreitol). A similar observation has been reported for the methionyl-tRNA synthetase from wheat germ (Mustafa, 1964) and *E. coli* (Heinrickson & Hartley, 1967) and it was suggested that a reduced thiol might be involved in the catalytic function of the enzyme. Although the stability dependence on dithiothreitol displayed by many of the aminoacyl-tRNA synthetases suggests that an active-site cysteine residue might be a common feature of these enzymes, at least two of the synthetases, namely lysyl- (Stern & Mehler, 1965) and alanyl-tRNA synthetase (Webster, 1961), do not require a reducing medium for stability and, indeed, may be inactivated by mercaptoethanol.

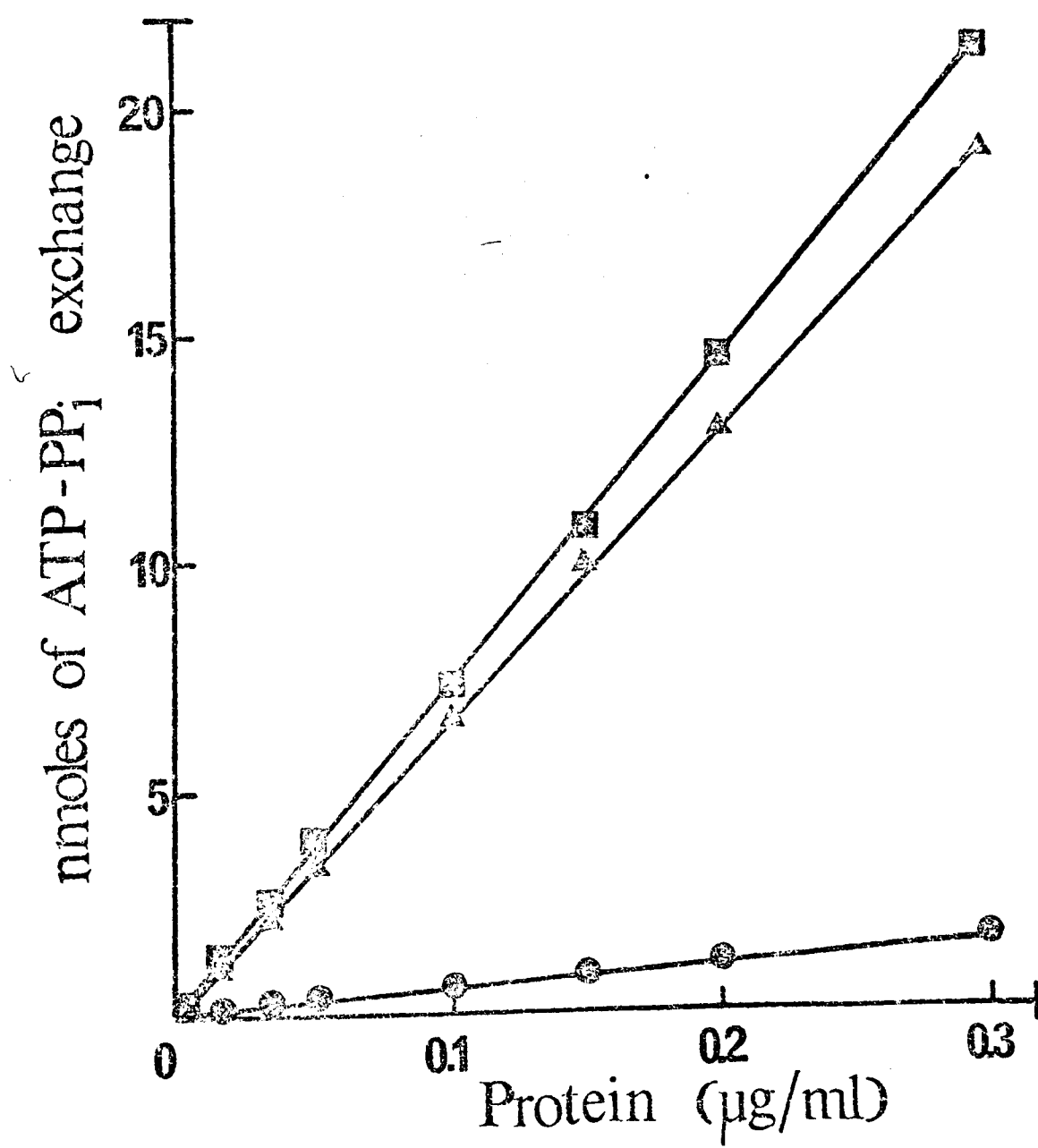
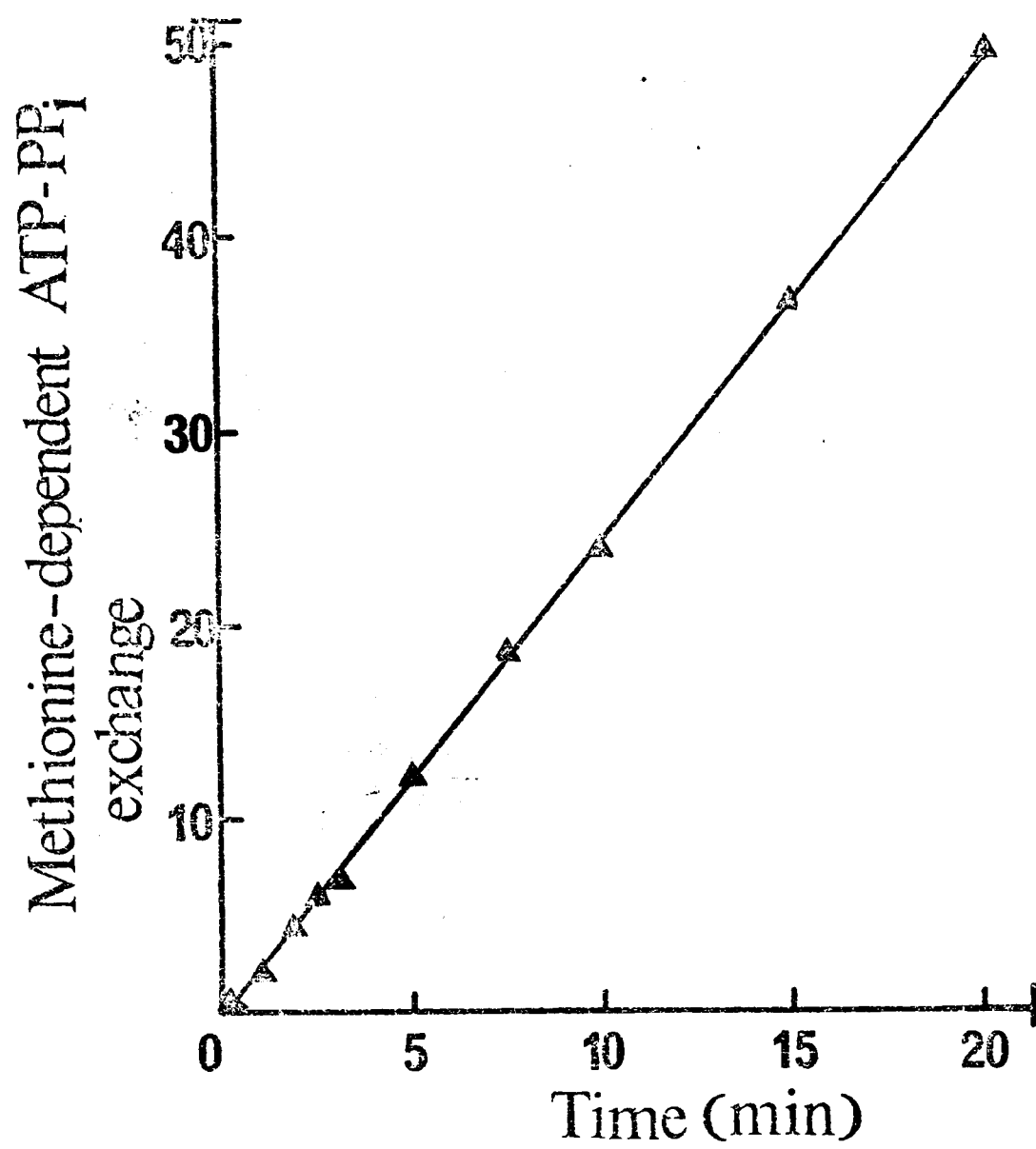
Linearity of the assay. In the presence of 5-mM-dithiothreitol, the rates of methionine-dependent ATP-PP_i exchange were linear with both time and protein concentration (Fig. 12.1 and Fig. 12.2 respectively). All subsequent assays were conducted for 10 min in the presence of 5 mM-dithiothreitol.

Validity of the assay. The radioactivity adsorbed by charcoal was eluted and examined by high voltage paper electrophoresis. The methionine-dependent radioactivity adsorbed to, and eluted from the charcoal was identified as γ -³²P-ATP. No γ -³²P-ADP or AMP was detected.

The enzyme was heat sensitive; pretreatment at 80°C for 1 min totally destroyed methionyl-tRNA synthetase activity as measured by both Methods 1 and 2.

Fig. 12.1 Time-course of methionyl-tRNA synthetase activity. Assays were conducted as described in Materials and Methods.

Fig. 12.2 Effect of enzyme concentration upon endogenous (●) methionine-dependent (▲) and total (■) ATP-PP_i exchange. Assays were conducted for 10 min.



The enzyme was stored, in the presence of 5 mM-dithiothreitol at -15°C for 8 months without loss of activity.

Effect of pH. No single buffer system was satisfactory for studying the effect of pH on methionyl-tRNA synthetase activity. However, maximum activity was found to lie between pH 8.0 and 8.5 (Fig. 12.3) when a series of buffers were used. The Tris-maleate buffer did not inhibit methionyl-tRNA synthetase from P. denitrificans : Tris-maleate buffer has been found to inhibit yeast methionyl-tRNA synthetase (Boman & Svensson, 1961); the inhibition was due to maleic acid. This effect appears to be specific for yeast methionyl-tRNA synthetase, since other synthetases appear to function properly in the presence of maleate buffer (Arca et al., 1964; Cherayil & Block, 1965). In the present study, all assays were conducted at pH 8.0 in Tris-HCl buffer.

Effect of $\gamma\text{-}^{32}\text{P}\text{-PP}_i$ concentration. The methionine-dependent ATP- PP_i exchange reaction, catalysed by purified methionyl-tRNA synthetase was essentially independent of $\gamma\text{-}^{32}\text{P}\text{-PP}_i$ concentration above 0.15 mM. The concentration of $\gamma\text{-}^{32}\text{P}\text{-PP}_i$ was routinely maintained at 2.0 mM in all assays where Method 1 was used to measure enzyme activity. (Crude extracts of P. denitrificans contain a powerful inorganic pyrophosphatase activity. This was removed during the purification procedure).

Effect of Mg^{2+} and ATP concentration. The methionine-dependent ATP- PP_i exchange reaction, catalysed by purified methionyl-tRNA synthetase is both Mg^{2+} - and ATP-dependent. The effect of ATP concentration, at various Mg^{2+} concentrations, upon the rate of methionine-dependent ATP- PP_i exchange is shown in Fig. 12.4. As found with cysteinyl-tRNA synthe-

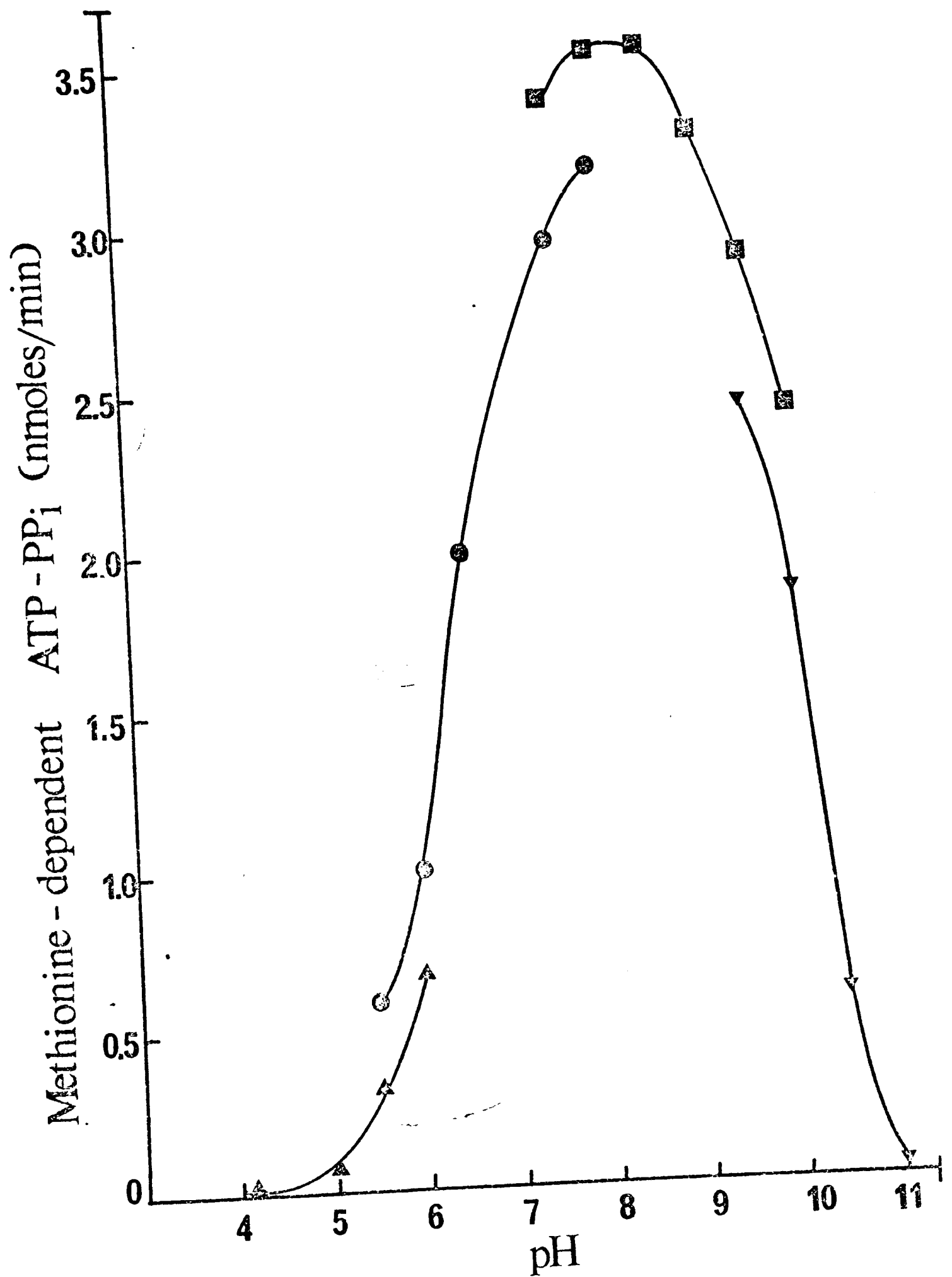


Fig. 12.3

Effect of pH upon the activity of purified methionyl-tRNA synthetase. Reaction mixtures were as described in Materials and Methods except that Tris-HCl buffer was replaced by the buffers as specified; ▲, citrate buffer; ⊗, Tris-maleate buffer; ■, Tris-HCl buffer; ▼, Glycine-NaOH buffer.

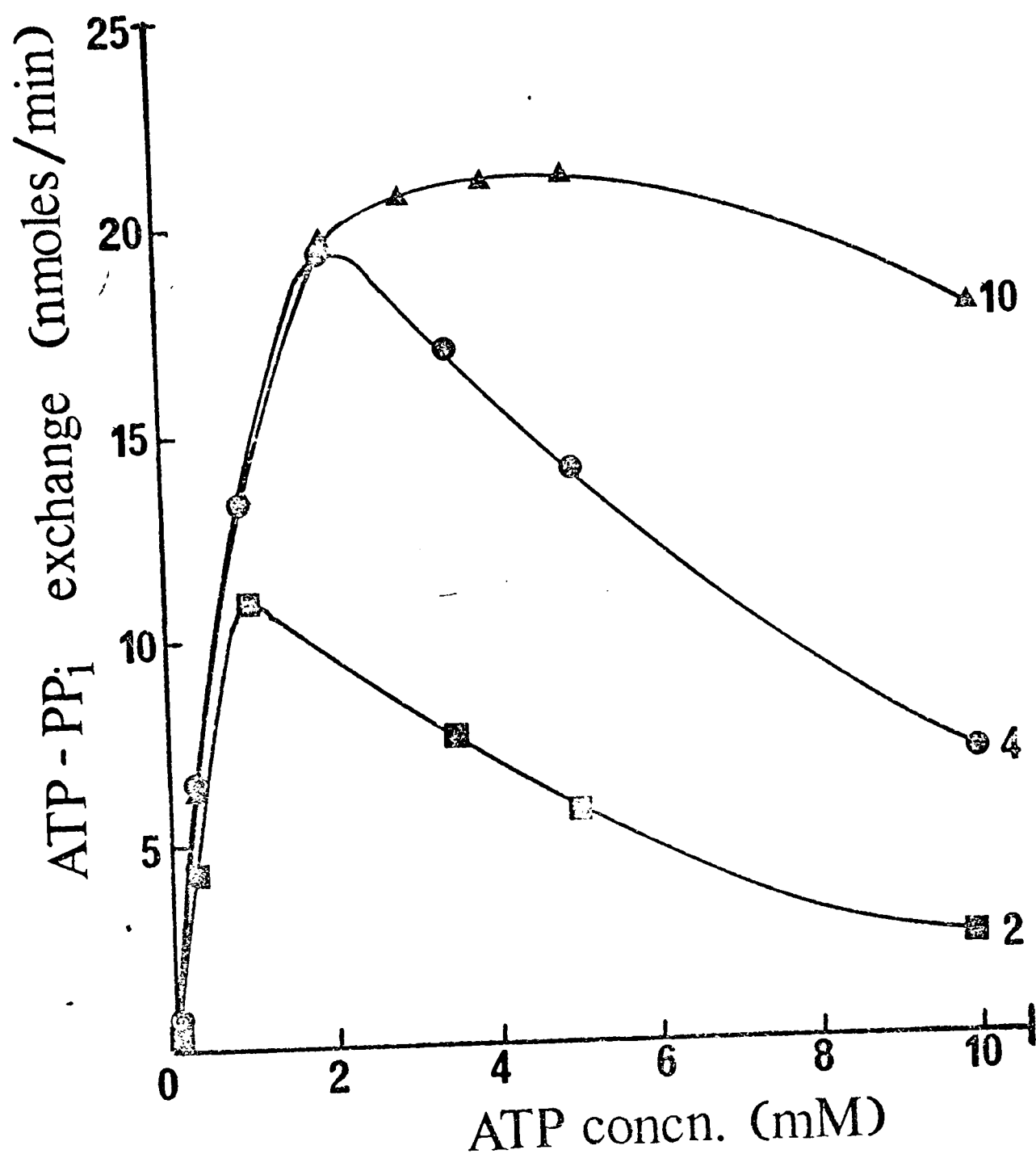


Fig. 12.4

Effect of ATP concentration upon methionine-dependent ATP-PP_i exchange in the presence or absence of Mg²⁺.

Reaction mixtures were as described in Materials and Methods except that ATP and Mg²⁺ were added at the concentrations specified. ■, 2mM-Mg²⁺; ●, 4mM-Mg²⁺; ▲, 10mM-Mg²⁺.

tase from P. denitrificans (Chapter 11), methionine-dependent ATP-PP_i exchange is both inhibited by excess ATP and maximal at a certain Mg²⁺/ATP ratio; for methionyl-tRNA synthetase, the optimal Mg²⁺/ATP ratio is 2 : 1. Calculation of the concentration of PP_i, ATP and Mg²⁺ in the reaction mixture, by the method of Perrin & Sayce (1967), shows that the concentration of free Mg²⁺ does not correlate with the rate of methionine-dependent ATP-PP_i exchange. Fig. 12.5 illustrates the effect of ATP concentration upon methionyl-tRNA synthetase; the Mg²⁺/ATP ratio was maintained at 2 : 1. The K_m for ATP, as calculated from three separate experiments, was $0.87 \text{ mM} \pm 0.02 \text{ mM}$. The ATP concentration in reaction mixtures was subsequently maintained at 4 mM and the Mg²⁺ concentration at 8 mM .

Effect of L-methionine concentration. Unlike the tyrosyl-tRNA synthetase from E. coli (Calender & Berg, 1966), which catalyses the activation and subsequent transfer of both D- and L-tyrosine to tRNA, purified methionyl-tRNA from P. denitrificans is specific for the L-isomer of methionine. Purified methionyl-tRNA synthetase exhibited Michaelis-Menten kinetics (Fig. 12.6) with a K_m for methionine of $0.26 \text{ mM} \pm 0.02 \text{ mM}$. The K_m value of 0.26 mM for methionine, as measured by the ATP-PP_i exchange method, is approximately the same as that obtained by Heinrikson & Hartley (1967) for the E. coli enzyme, and approximately half that obtained by Hahn & Brown (1967) for the Sarcina lutea enzyme under comparable conditions.

Substrate specificity. Purified methionyl-tRNA synthetase did not catalyse $\gamma\text{-}^{32}\text{P}\text{-}7\text{PP}_i$ exchange when ATP was replaced with ADP, 5'-AMP or 3'-AMP. Similarly, the enzyme did

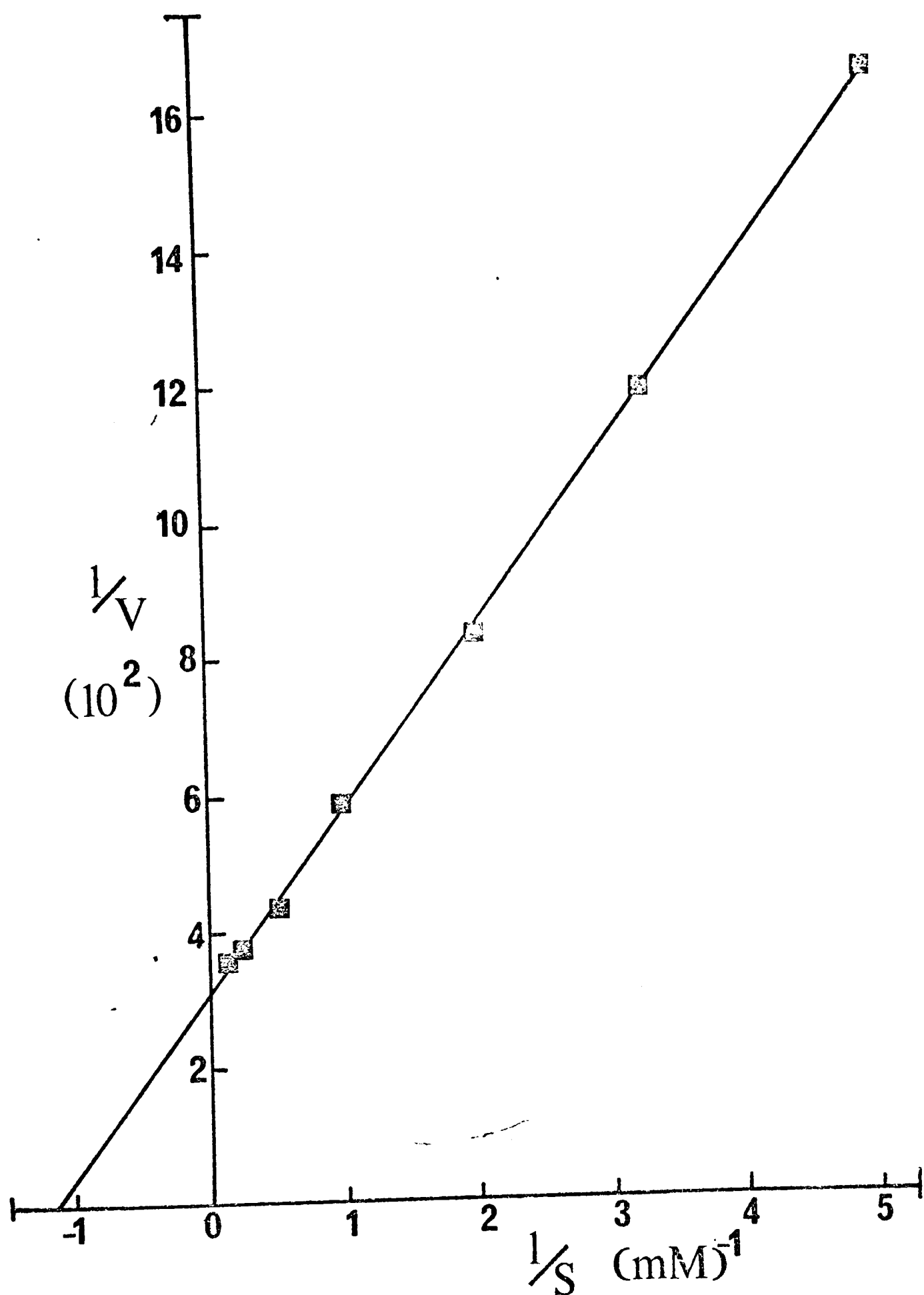


Fig. 12.5

Double reciprocal plots of the effect of ATP concentration on Methionine-dependent ATP-PP_i exchange catalysed by purified methionyl-tRNA synthetase. Reaction mixtures were as described in Materials and Methods except that the ATP concentration was varied and the Mg²⁺: ATP ratio was maintained at 2 : 1.

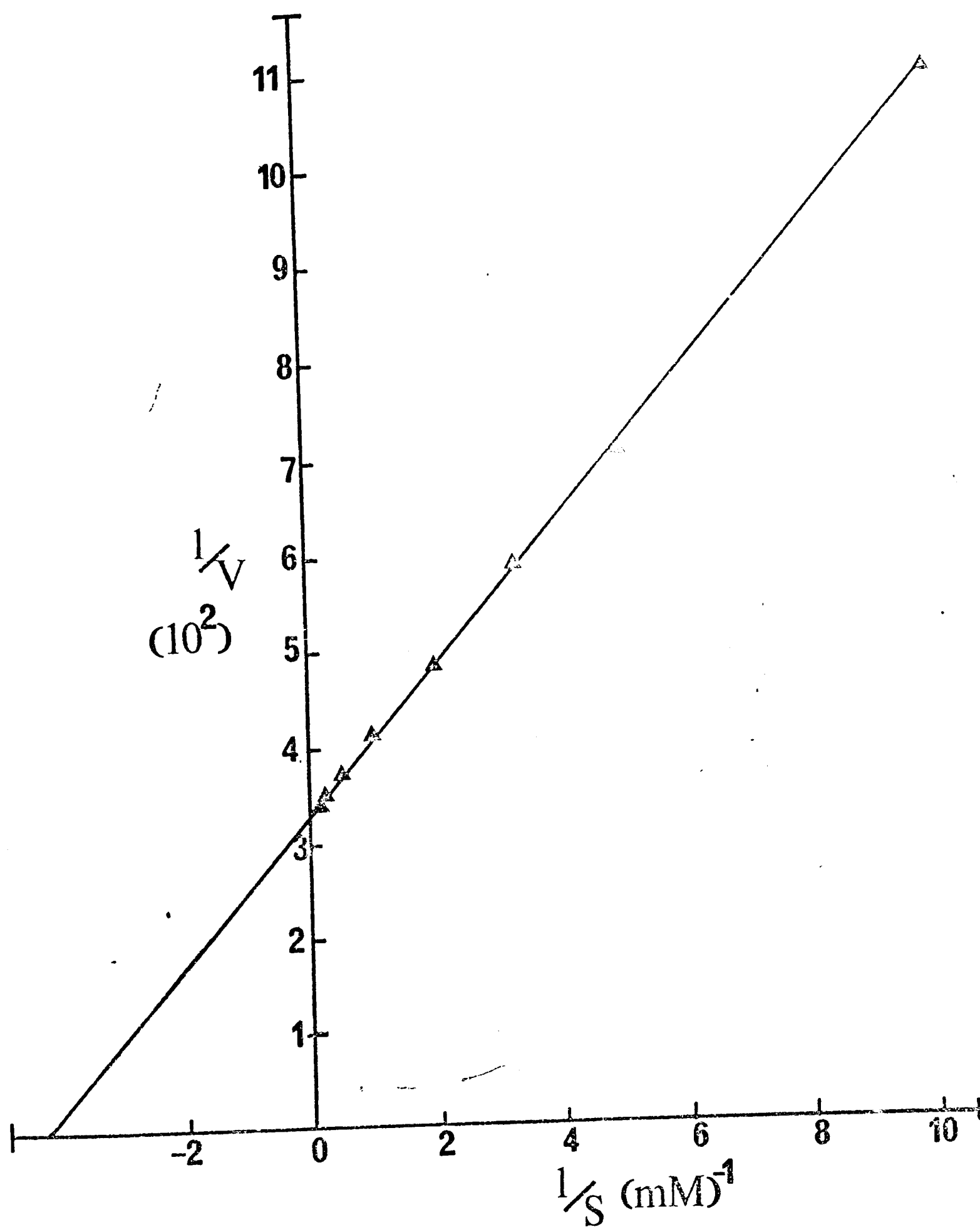


Fig. 12.6

Double reciprocal plot of the effect of L-methionine concentration upon methionine-dependent ATP-PP_i exchange.

Reaction mixtures were as described in Materials and

Methods except that the methionine concentration was varied.

not catalyse the incorporation of P_i into ATP, ADP 5'-AMP or 3'-AMP when $\gamma\text{-}^{32}\text{P}\text{-PP}_i$ was replaced with $\gamma\text{-}^{32}\text{P}\text{-P}_i$.

The enzyme did not catalyse sulphate- or selenate-, or acetate- or propionate- dependent ATP- PP_i exchange indicating that the purified methionyl-tRNA synthetase was not contaminated with ATP sulphurylase or short chain fatty acyl thiokinase activities, respectively. Only L-methionine of the 20 amino acids, commonly found in proteins, could act as substrate for ATP- PP_i exchange indicating that purified methionyl-tRNA synthetase was not contaminated with other aminoacyl-tRNA synthetases and that none of the other 19 amino acids could act as substrate for the methionine-activating enzyme.

Purified methionyl-tRNA synthetase did, however, catalyse homocysteine-dependent ATP- PP_i exchange. A Lineweaver-Burk plot of L-homocysteine concentration versus reaction velocity (Fig. 12.7) exhibits Michaelis-Menten kinetics with an apparent K_m of 3.83 mM. D-Homocysteine did not stimulate ATP- PP_i exchange but inhibited both L-homocysteine- and L-methionine-dependent ATP- PP_i exchange. DL-Homocysteine was therefore treated with D-amino acid oxidase to remove the D-isomer. Although the affinity of methionyl-tRNA synthetase for L-methionine is almost 15 times greater than the affinity for L-homocysteine, the V (homocysteine) is twice the V (methionine).

Purified methionyl-tRNA synthetase also catalysed norleucine-dependent ATP- PP_i exchange. A Lineweaver-Burk plot of norleucine concentration versus reaction rate, exhibited Michaelis-Menten kinetics and gave an apparent K_m for norleucine of 28.6 mM (Fig. 12.8).

Purified methionyl-tRNA synthetase also catalysed L-selenomethionine- dependent ATP- PP_i exchange. Methionyl-tRNA

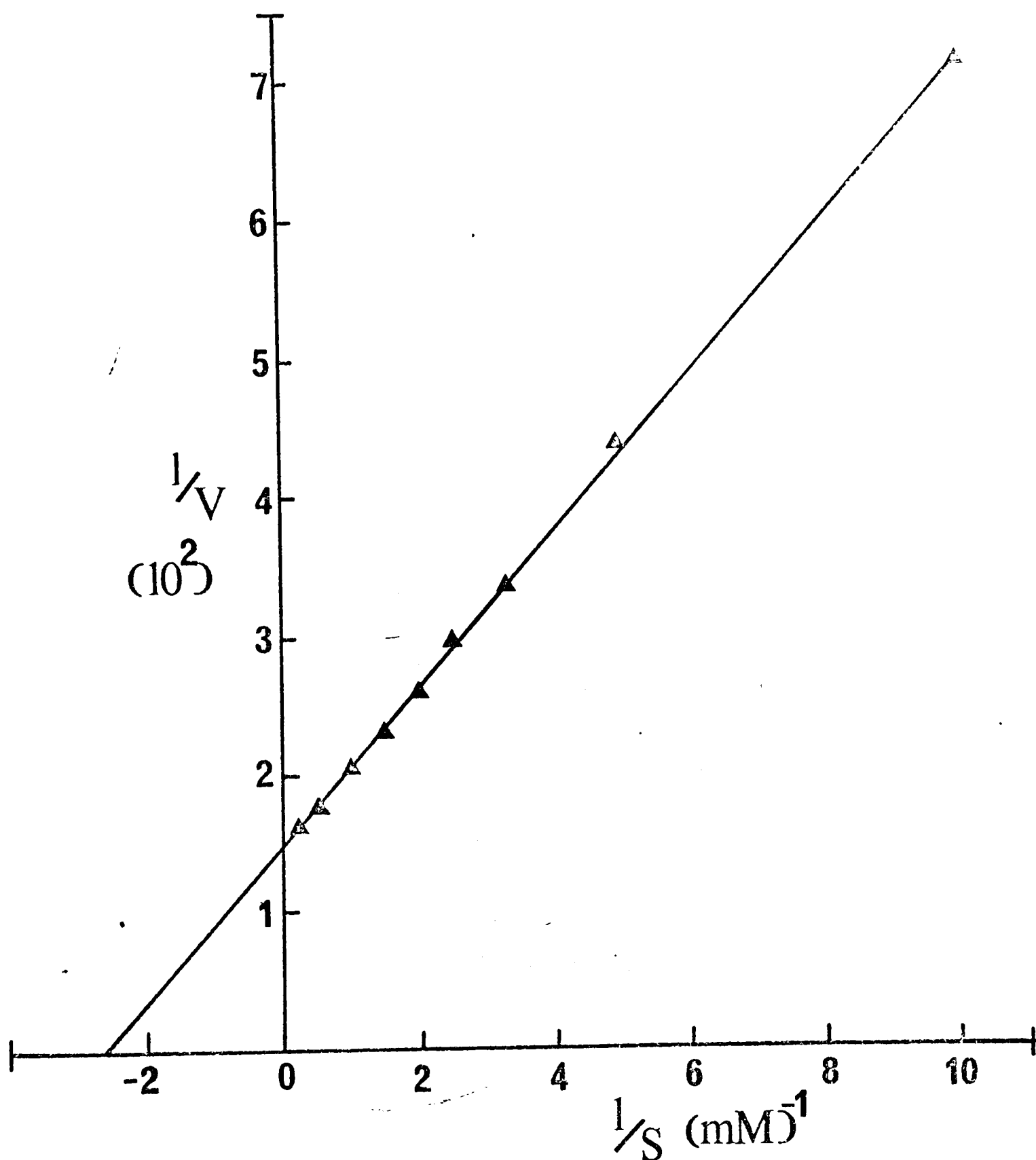


Fig. 12.7

The effect of L-homocysteine concentration upon the rate of homocysteine-dependent ATP-PP_i exchange as catalysed by purified methionyl-tRNA synthetase. Reaction mixtures were as described in Materials and Methods except that methionine was replaced with homocysteine.

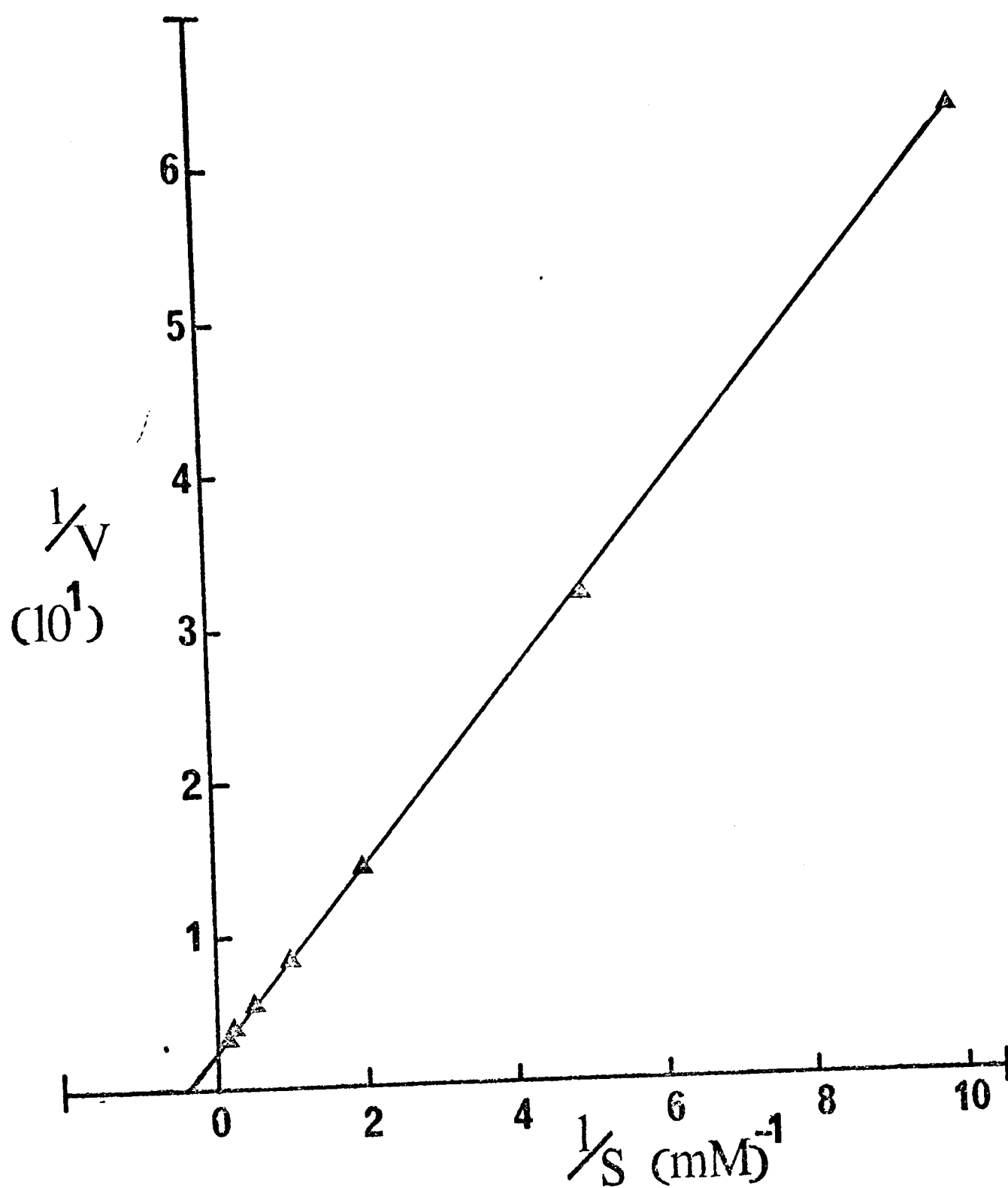


Fig. 12.8

Double reciprocal plot of the effect of norleucine concentration upon norleucine-dependent ATP-PP_i exchange catalysed by purified methionyl-tRNA synthetase. Reaction mixtures were as described in Materials and Methods except that methionine was replaced with norleucine.

synthetase exhibited Michaelis-Menten kinetics with an apparent K_m for L-selenomethionine of 0.31 mM (Fig. 12.9); this is a slightly higher K_m value than for methionine ($K_m = 0.26 \text{ mM}$). A double reciprocal plot of the effect of methionine concentration upon methionyl-tRNA synthetase activity, in the presence of increasing concentrations of L-selenomethionine, is shown in Fig. 12.10. Addition of L-selenomethionine, to incubation mixtures containing L-methionine, decreased the ATP-PP_i exchange, and the kinetics of methionine/selenomethionine competition experiments (Fig. 12.10) are consistent with the kinetics of two substrates competing for one enzyme (Pocklington & Jeffery, 1969). The ratio of methionine-dependent to selenomethionine-dependent ATP-PP_i exchange rates was approx. constant during purification, and the methionine- and selenomethionine-dependent activities were not separated during gel filtration on Sephadex G-200, or ion exchange chromatography on DEAE-cellulose (Fig. 12.11A and 12.11B).

D-Methionine was not a substrate for purified methionyl-tRNA synthetase. D-Methionine did, in fact, un-competitively inhibit L-methionine-dependent ATP-PP_i exchange with respect to L-methionine concentration (Fig. 12.12). Consequently, DL-selenomethionine, the commercially available form of selenomethionine was treated with D-amino acid oxidase to remove the D-isomer, and therefore allow a more critical study of the effect of L-methionine and its selenium-analogue upon methionyl-tRNA synthetase activity.

Other compounds examined as possible substrates of purified methionyl-tRNA synthetase included, L-methionine sulphone, L-methionine sulphoxide, L-ethionine, α -methyl methionine, γ -L-aminobutyric acid, L-homoserine, L-cysteine

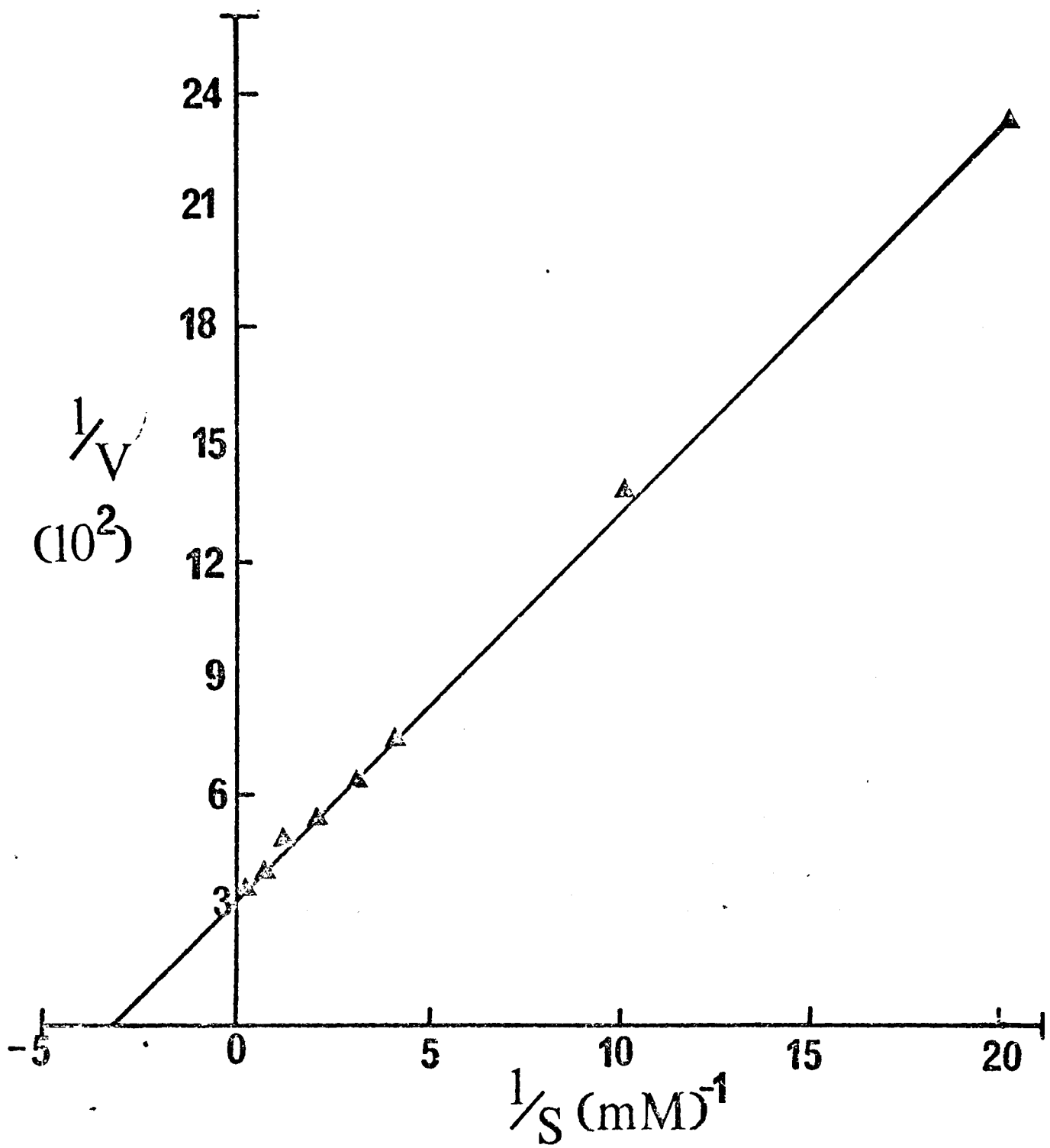


Fig. 12.9

Double reciprocal plot of the effect of selenomethionine concentration upon selenomethionine-dependent ATP-PP_i exchange catalysed by purified methionyl-tRNA synthetase. Reaction mixtures were as described in Materials and Methods except that methionine was replaced by selenomethionine.

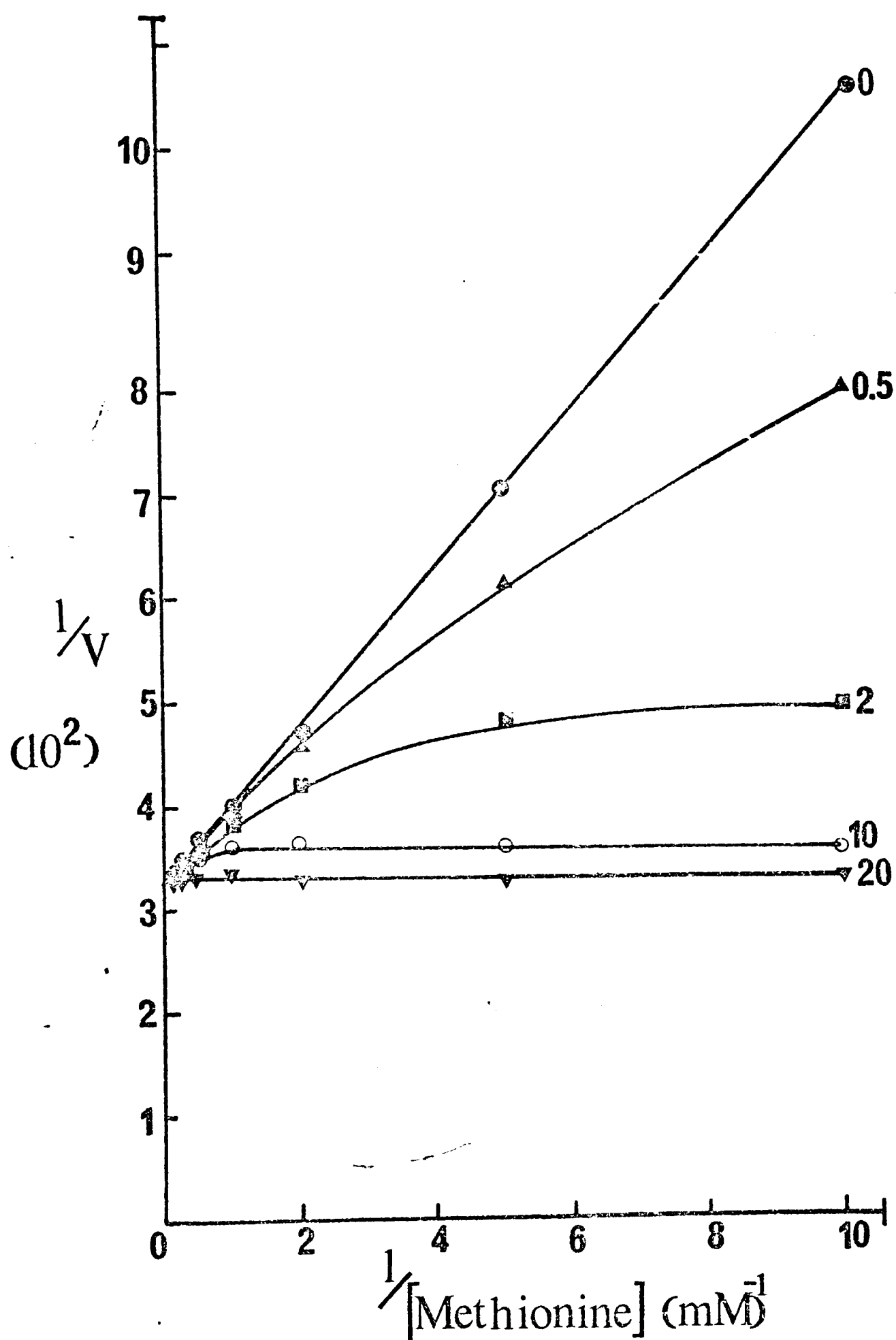


Fig. 12.10

Double reciprocal plots of the effect of methionine concentration on methionine-dependent ATP-PP_i exchange in the presence or absence of selenomethionine.

Reaction mixtures were as described in Materials and Methods except that methionine and selenomethionine were added as specified. ●, without selenomethionine; ▲, 0.5mM-selenomethionine; ■, 2mM-selenomethionine; ○, 10mM-selenomethionine; ▼, 20mM-selenomethionine.

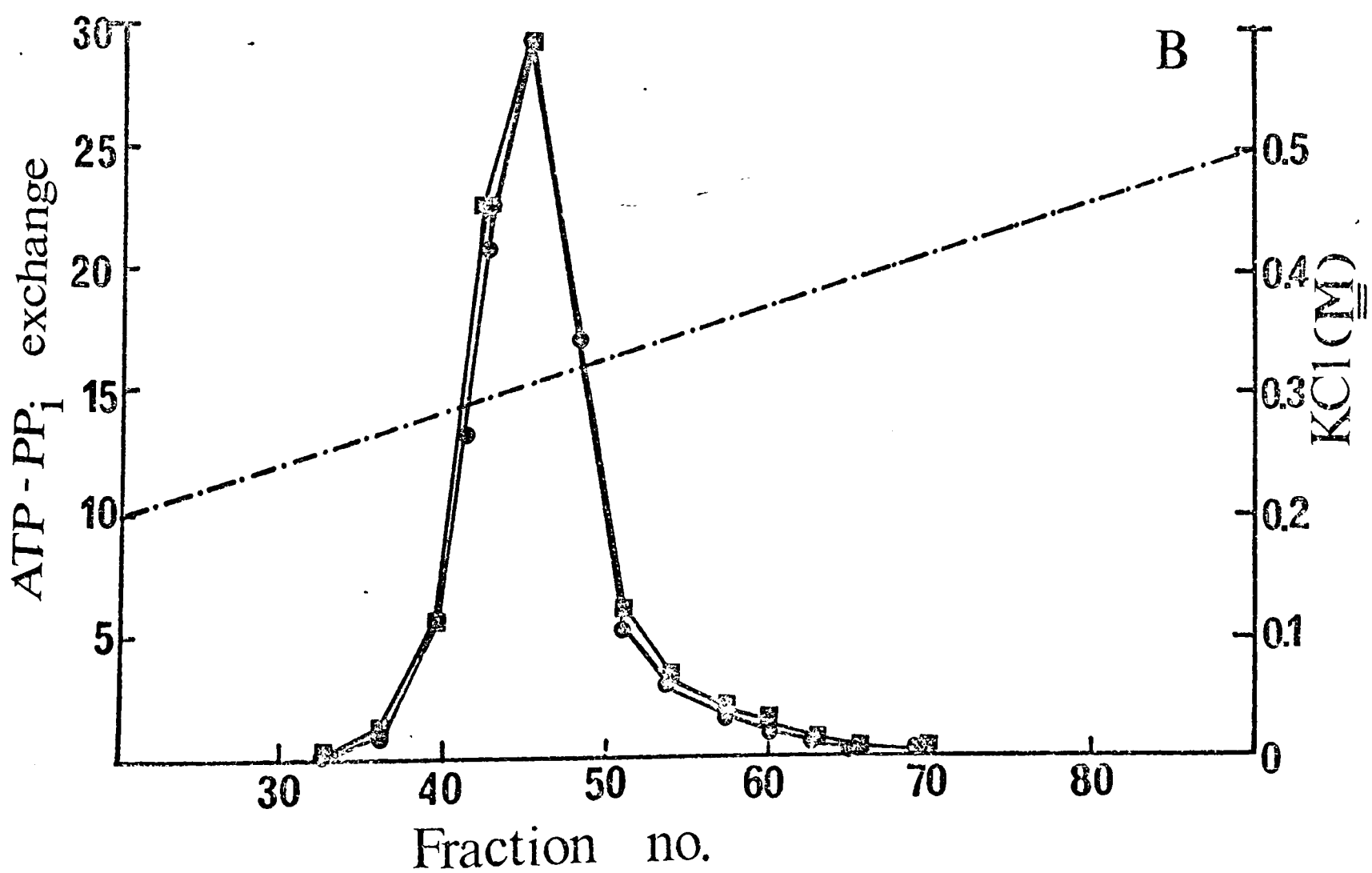
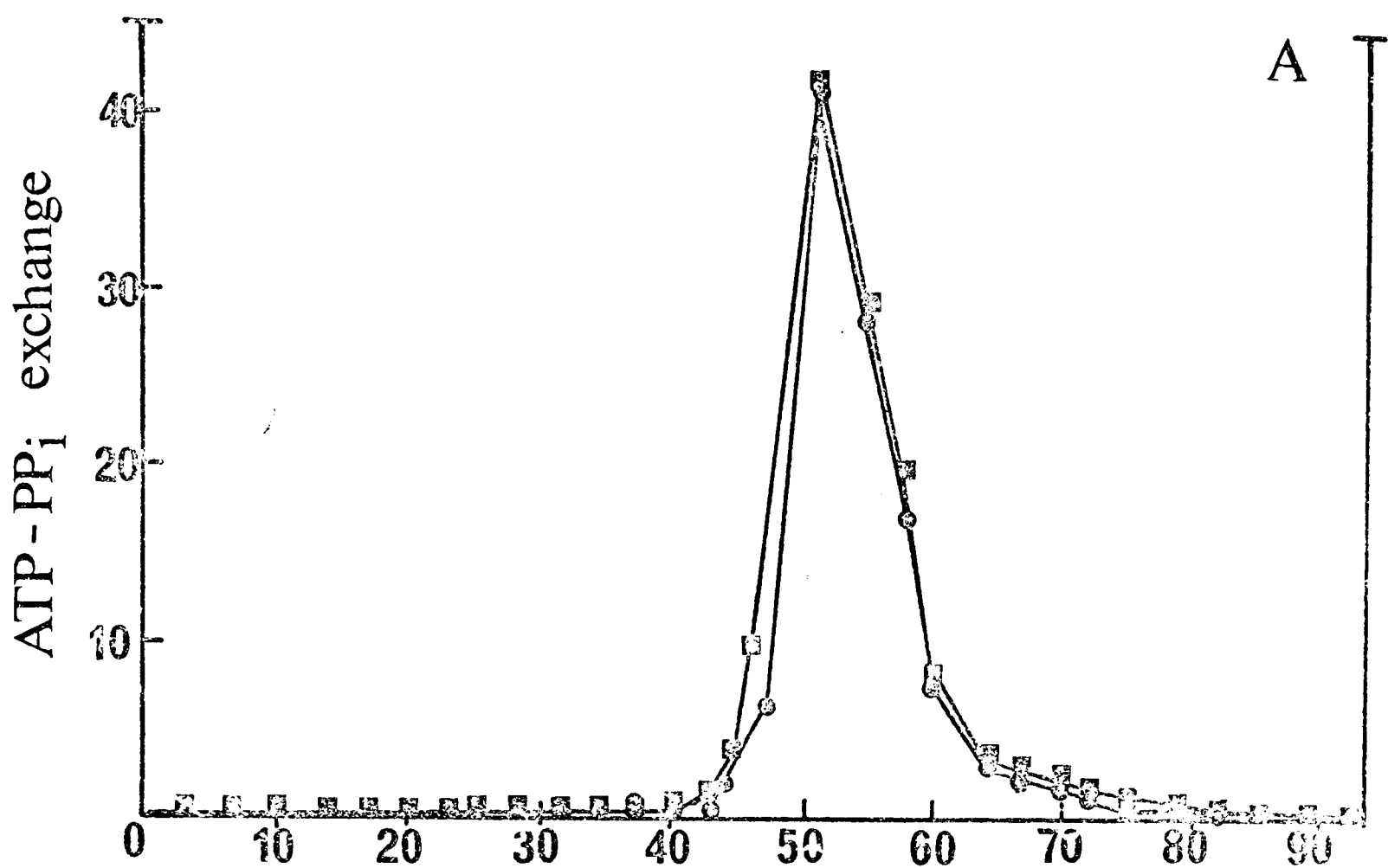
Fig. 12.11

Sephadex G-200 gel filtration and DEAE-cellulose ion exchange chromatography of purified methionyl-tRNA synthetase.

Fig. 12.11A. Sephadex G-200 gel filtration. The 30 to 60% ammonium sulphate fraction (Fraction IV) was applied to a Sephadex G-200 column (3.5 x 85 cm) equilibrated with 20mM-Tris-HCl buffer, pH 8.0, containing 5mM-MgCl₂, and eluted with the same buffer at a flow rate of 4.5 ml/min. Fractions of 5 ml were collected and assayed for both methionine- and selenomethionine-dependent ATP-PP_i exchange.

Methionine-dependent ATP-PP_i exchange (■)
Selenomethionine-dependent ATP-PP_i exchange (●)

Fig. 12.11B. DEAE-cellulose ion exchange chromatography. Fractions 42 to 60 from (A) were pooled (Fraction V) and applied to a 3.5 x 10 cm column of DEAE-cellulose. The column was washed with 60 ml of buffer and then with 50 ml of 0.2M-KCl in buffer. The enzyme was eluted with a 500 ml linear gradient of KCl from 0.2 to 0.5M, and the fractions were collected and assayed as in (A) except that the flow rate of the column was 0.65 ml/min.



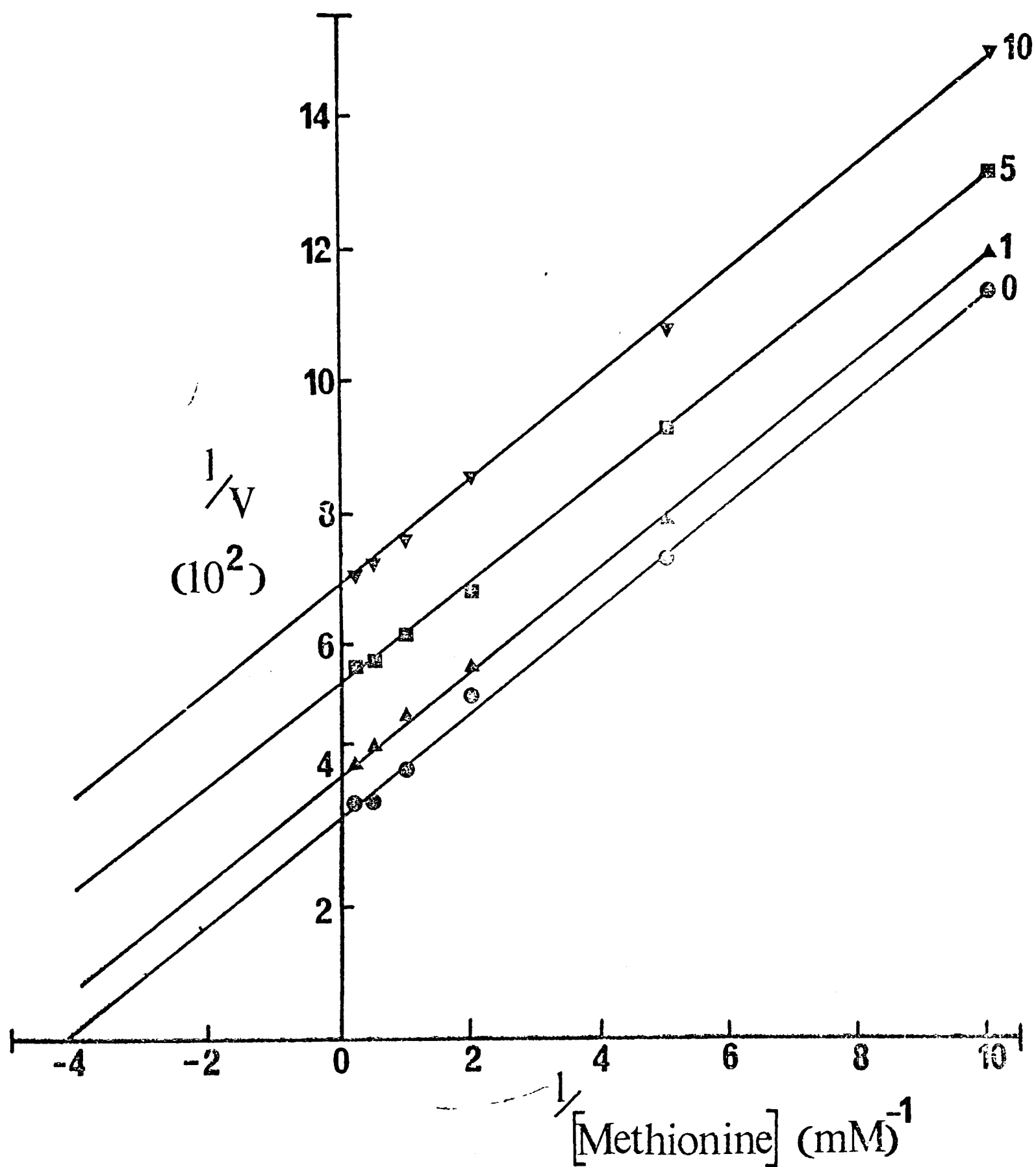


Fig. 12.12

Double reciprocal plots of the effect of L-methionine concentration upon methionine-dependent ATP-PP_i exchange in the presence or absence of D-methionine.

Reaction mixtures were as described in Materials and Methods except that D- and L-methionine were added to the reaction mixtures as specified. ●, without D-methionine; ▲, 1mM-D-methionine; ■, 5mM-D-methionine; ▼, 10mM-D-methionine.

and L-cystathionine; none of these compounds acted as alternative substrates for methionyl-tRNA synthetase.

The ^{32}P -labelled product formed in the presence of purified methionyl-tRNA synthetase and L-senomethionine, L-norleucine or L-homocysteine, ATP and $\gamma\text{-}^{32}\text{P}\text{-PP}_i$ as substrates and which adsorbed to charcoal was eluted and identified as $\gamma\text{-}^{32}\text{P}\text{-ATP}$; no $\gamma\text{-}^{32}\text{P}\text{-ADP}$ or AMP was detected.

Effect of cations. Methionine-dependent ATP-PP_i exchange was negligible in the absence of Mg^{2+} . In a study of the effect of other cations on methionine-dependent ATP-PP_i exchange only Mn^{2+} (15%), Co^{2+} (20%) and Ca^{2+} (14%) supported ATP-PP_i exchange. Ni^{2+} , Ba^{2+} , Fe^{2+} , Zn^{2+} , Hg^{2+} , and Cu^{2+} also were tested, but none of these ions stimulated activity of the enzyme. Additions of cations, other than Mg^{2+} , to incubation mixtures containing Mg^{2+} , decreased methionine-dependent ATP-PP_i exchange.

Effect of inhibitors. Purified methionyl-tRNA synthetase was inhibited by a number of sulphydryl-group reagents (Table 12.2). These data are consistent with the generalisation, proposed by Novelli (1967) that almost all aminoacyl-tRNA synthetases are sulphydryl reagent sensitive. Methionine-dependent ATP-PP_i exchange was decreased by the addition of EDTA to reaction mixtures, probably due to the chelation of Mg^{2+} by EDTA; EDTA did not, itself, inhibit methionyl-tRNA synthetase activity.

Inhibition studies of methionine-dependent ATP-PP_i exchange. A variety of nucleotides, sulphur-containing anions and amino acids were surveyed as possible regulators of methionyl-tRNA synthetase activity.

TABLE 12.2

Effect of some common inhibitors upon methionine-dependent ATP-PP_i exchange catalysed by purified methionyl-tRNA synthetase.

Inhibitors were pre-incubated in reaction mixtures for 1 min prior to initiating the reaction by the addition of ATP.

<u>Compound</u>	<u>Inhibition (%)</u>
Phenylhydrazine (10 <u>mM</u>)	2
N-Ethylmaleimide (10 <u>mM</u>)	0
Iodacetamide (10 <u>mM</u>)	0
p-Chloromercuribenzoate (8 <u>μM</u>)	98
Potassium fluoride (10 <u>mM</u>)	6
EDTA (10 <u>mM</u>)	97

3'-AMP, 5'-AMP and ADP all inhibited methionine-dependent ATP-PP_i exchange. Inhibition by 3'-AMP, 5'-AMP and ADP was non-competitive (Fig. 12.13), competitive (Fig. 12.14) and uncompetitive (Fig. 12.15), respectively, with respect to ATP. 5'-AMP competitively inhibited methionyl-tRNA synthetase from S. lutea (Hahn & Brown, 1967); the mechanism of this inhibition presumably resides in the ability of 5'-AMP to compete with γ -³²P-PP_i in the nucleophilic displacement reaction with enzyme bound aminoacyl adenylate, the reverse of the aminoacyl activation reaction.

Sulphate (0.1 to 10.0 mM) and sulphite (0.1 to 10.0 mM) had no effect upon the rate of methionine-dependent ATP-PP_i exchange at high (20 mM) or low (0.1 mM) concentrations of L-methionine. Addition of sulphide to reaction mixtures increased methionine-dependent ATP-PP_i exchange (Fig. 12.16);

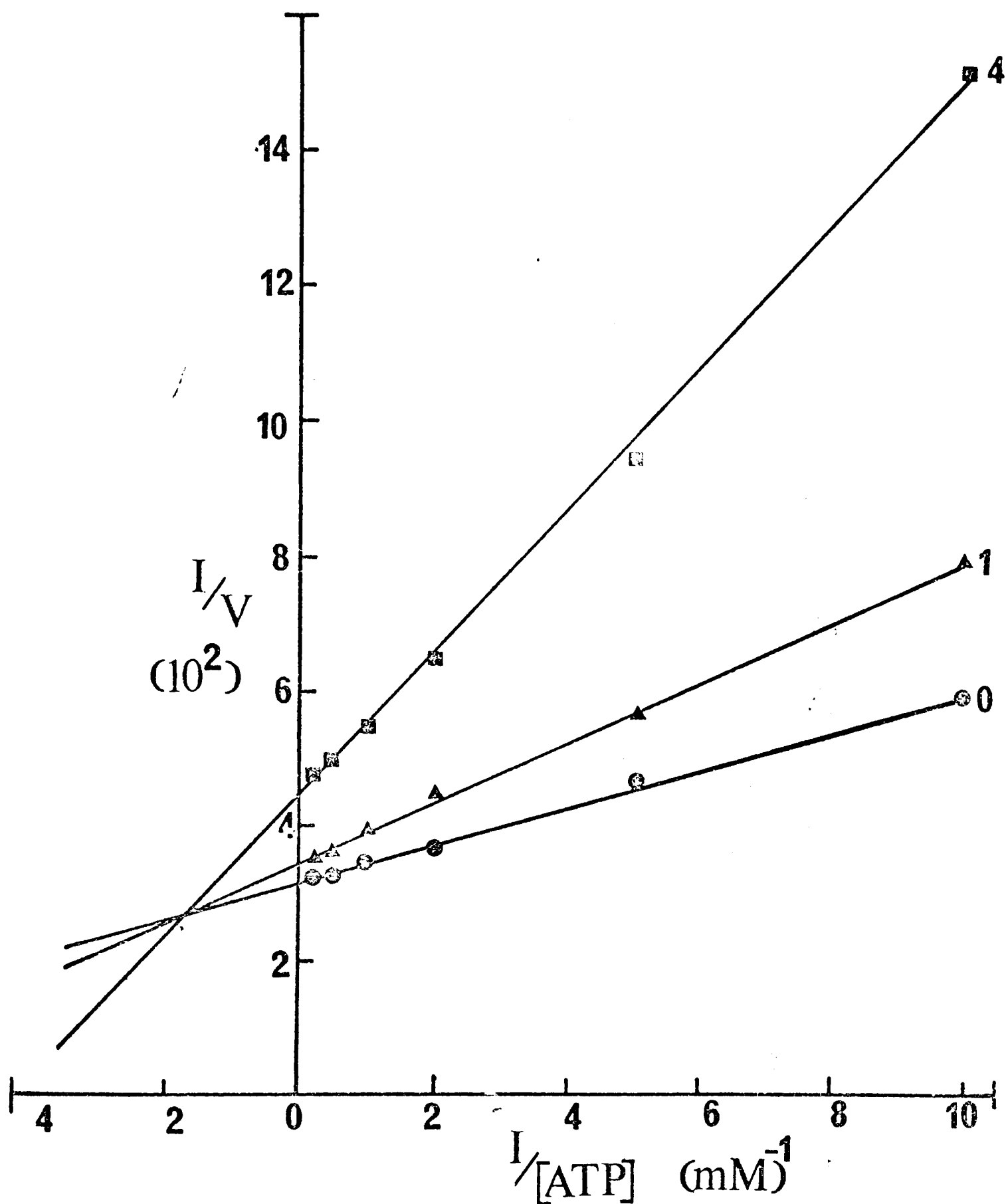


Fig. 12.13

Double reciprocal plots of the effect of ATP concentration upon methionine-dependent ATP-PP_i exchange in the presence and absence of 3'-AMP.

Reaction mixtures were as described in Materials and Methods except that ATP and 3'-AMP were added as specified.

●, without 3'-AMP; ▲, 1mM-3'-AMP; ■, 4mM-3'-AMP.

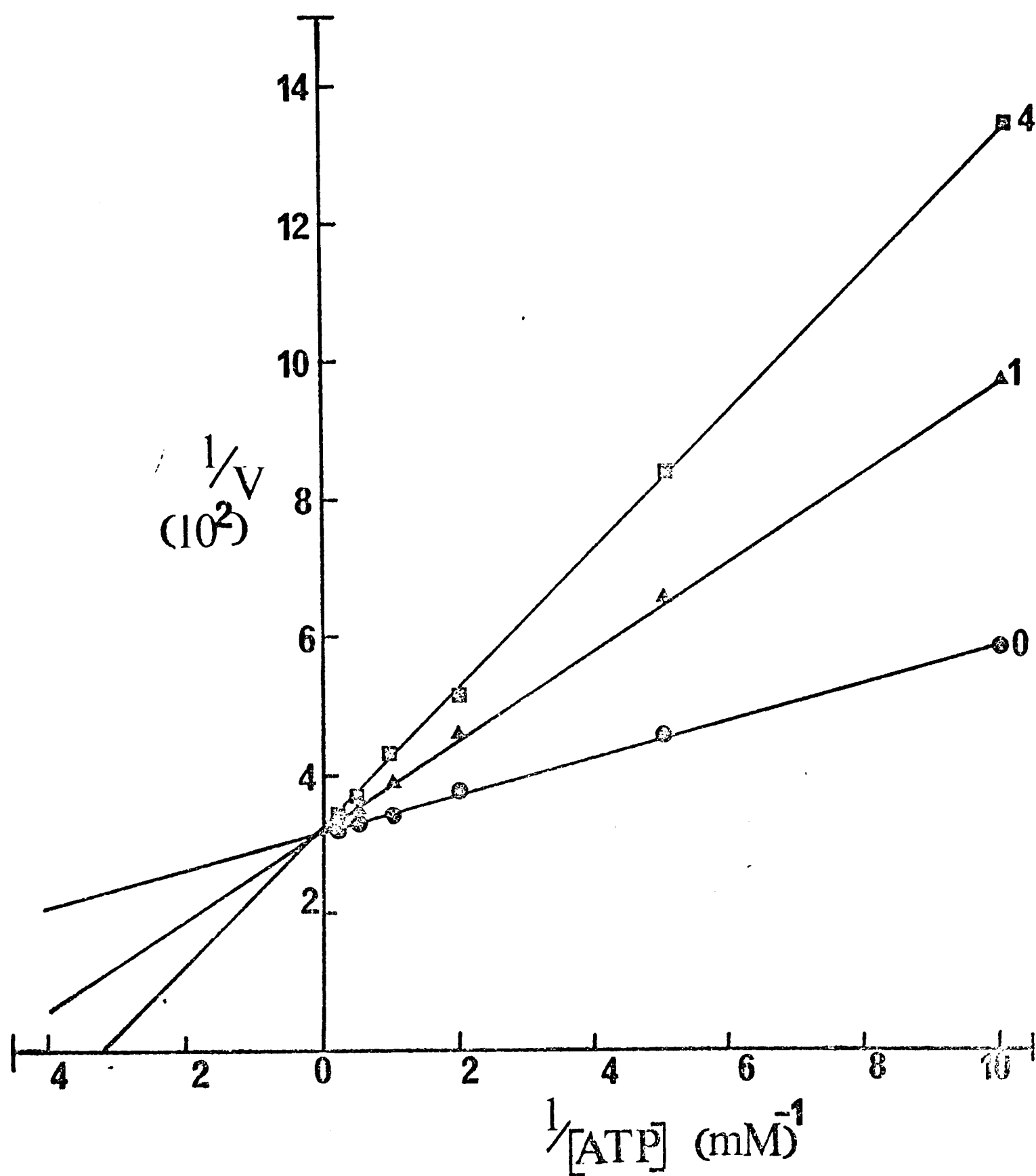


Fig. 12.14

Double reciprocal plot of the effect of ATP concentration upon methionine-dependent ATP-PP_i exchange in the presence or absence of 5'-AMP. Reaction mixtures were as described in Materials and Methods except that ATP and 5'-AMP were added as specified. ●, without 5'-AMP; ▲, 1mM-5'-AMP; ■, 4mM-5'-AMP.

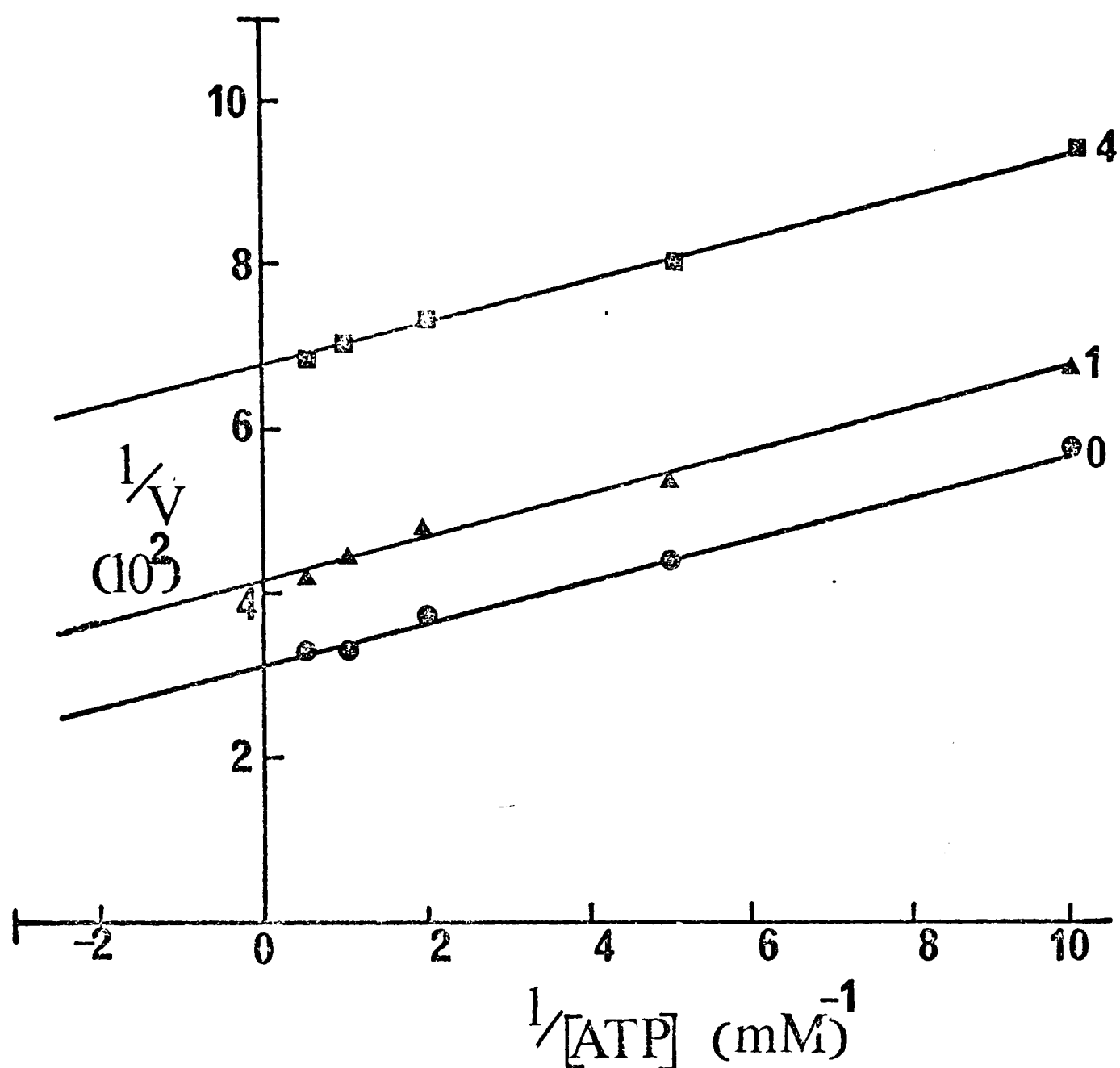


Fig. 12.15

Double reciprocal plot of the effect of ATP concentration upon methionine-dependent ATP-PP_i exchange in the presence or absence of ADP. Reaction mixtures were as described in Materials and Methods except that ATP and ADP were added as specified. ●, without ADP; ▲, 1mM-ADP; ■, 4mM-ADP.

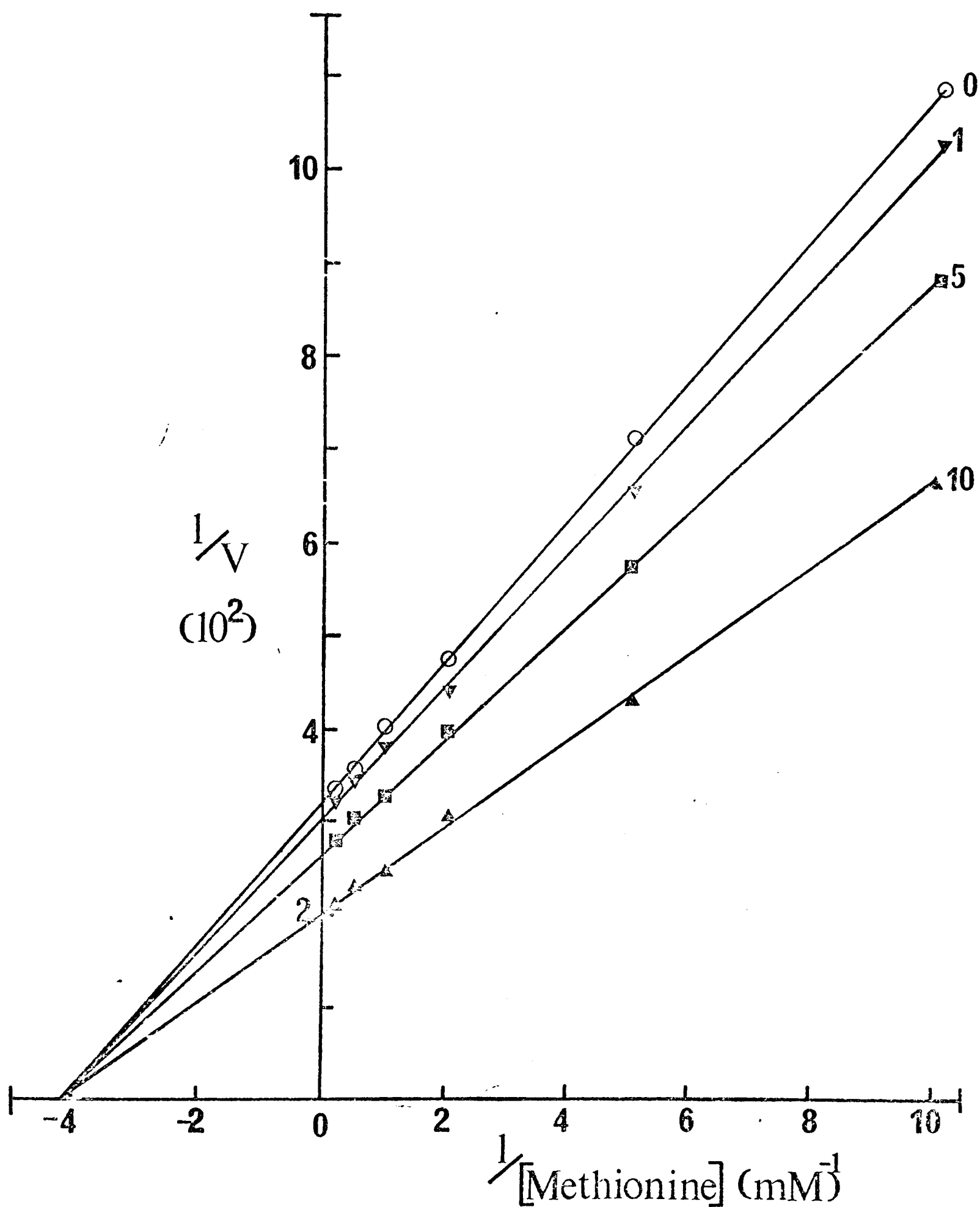


Fig. 12.16

Double reciprocal plots of the effect of methionine concentration upon methionine-dependent ATP-PP_i exchange in the presence or absence of sulphide. Reaction mixtures were as specified in Materials and Methods except that methionine and sulphide were added as specified. ○, without sulphide; ▼, 1mM-sulphide; ■, 5mM-sulphide; ▲, 10mM-sulphide.

addition to reaction mixtures caused an increase in the V_{max} without altering the K_m of the enzyme for methionine. This contrasted to the stimulation of enzyme activity caused by cysteine (Fig. 12.17); L-cysteine had no effect upon the V_{max} but lowered the K_m of the enzyme for methionine. Cysteic acid stimulated methionyl-tRNA synthetase in a way similar to cysteine (Fig. 12.18).

O-Acetyl serine (Fig. 12.19) and reduced glutathione (Fig. 12.20) non-competitively inhibited methionine-dependent ATP-PP_i exchange with respect to methionine. Other compounds investigated as possible regulators, but which had no effect upon methionyl-tRNA synthetase activity, included phosphate ions, L-methionine sulfoxide, L-methionine sulphone, L-cystine, L-serine, NAD, NADH, NADP and NADPH.

Effect of sulphur source for growth upon the specific activity of methionyl-tRNA synthetase. A variety of inorganic sulphur-containing compounds were added to culture media as sole sulphur sources for growth. These included; sulphate, sulphite, sulphide, L-cystine, sulphate plus L-homocysteine and sulphate plus L-methionine. Dialysed cell-free extracts prepared from cells harvested during mid log phase showed little variation in the specific activity of the methionyl-tRNA synthetase.

Formation of methionine and selenomethionine hydroxamic acid. In a reaction mixture containing a high concentration of hydroxylamine, ATP, L-methionine and enzyme, the formation of L-methionine hydroxamic acid was detected. The synthesis of L-methionine hydroxamic acid was linear with time and was dependent upon the presence of ATP, Mg²⁺, enzyme and L-methionine (Fig. 12.21). The formation of L-methionine hydroxamic acid was accompanied by the liberation of an equivalent

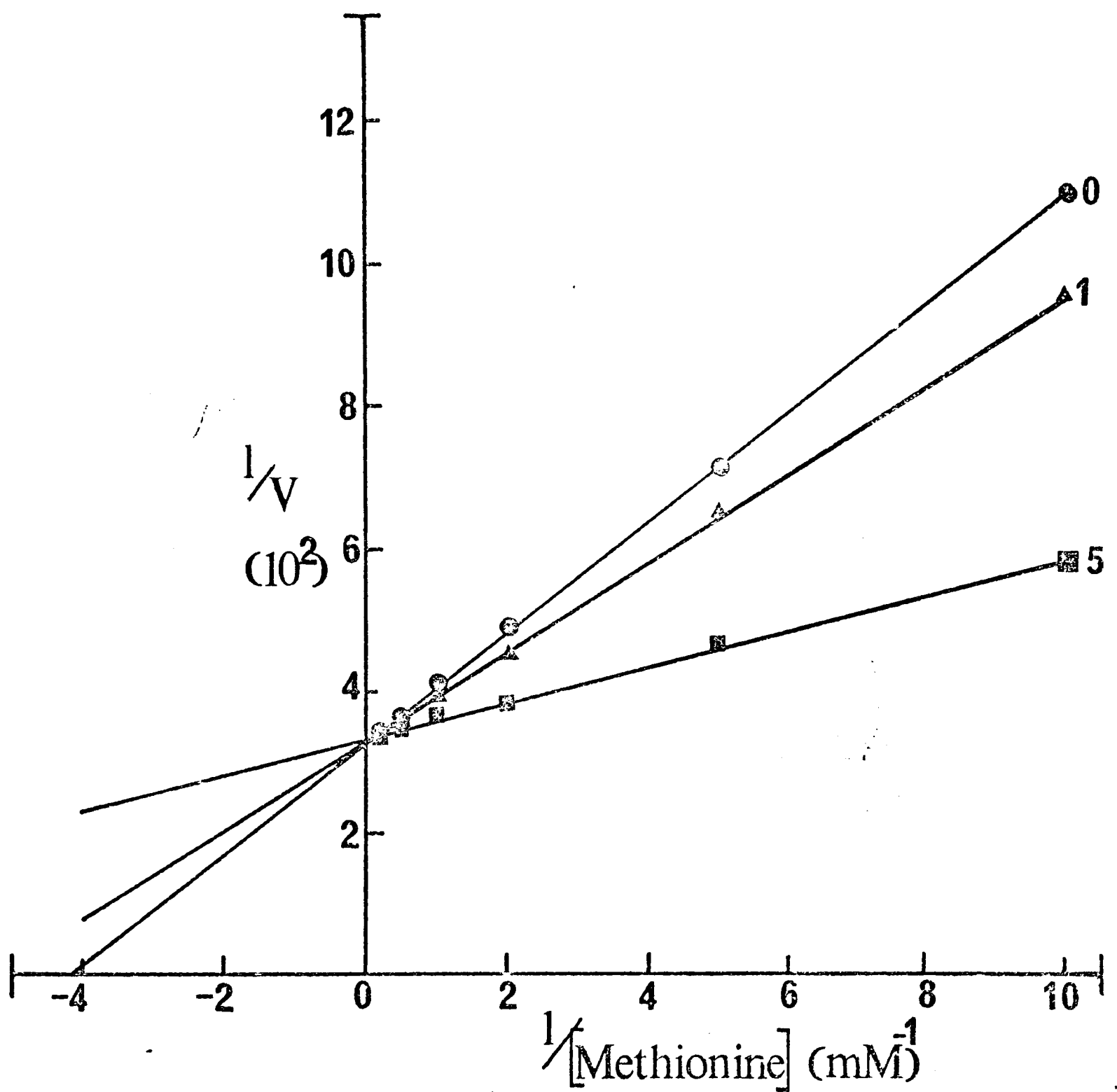


Fig. 12.17

Effect of methionine upon the methionine-dependent ATP- PP_i exchange in the presence or absence of cysteine. Reaction mixtures were as described in Materials and Methods except that methionine and cysteine were added as specified. ●, without cysteine; ▲, 1mM-cysteine; ■, 5mM-cysteine;

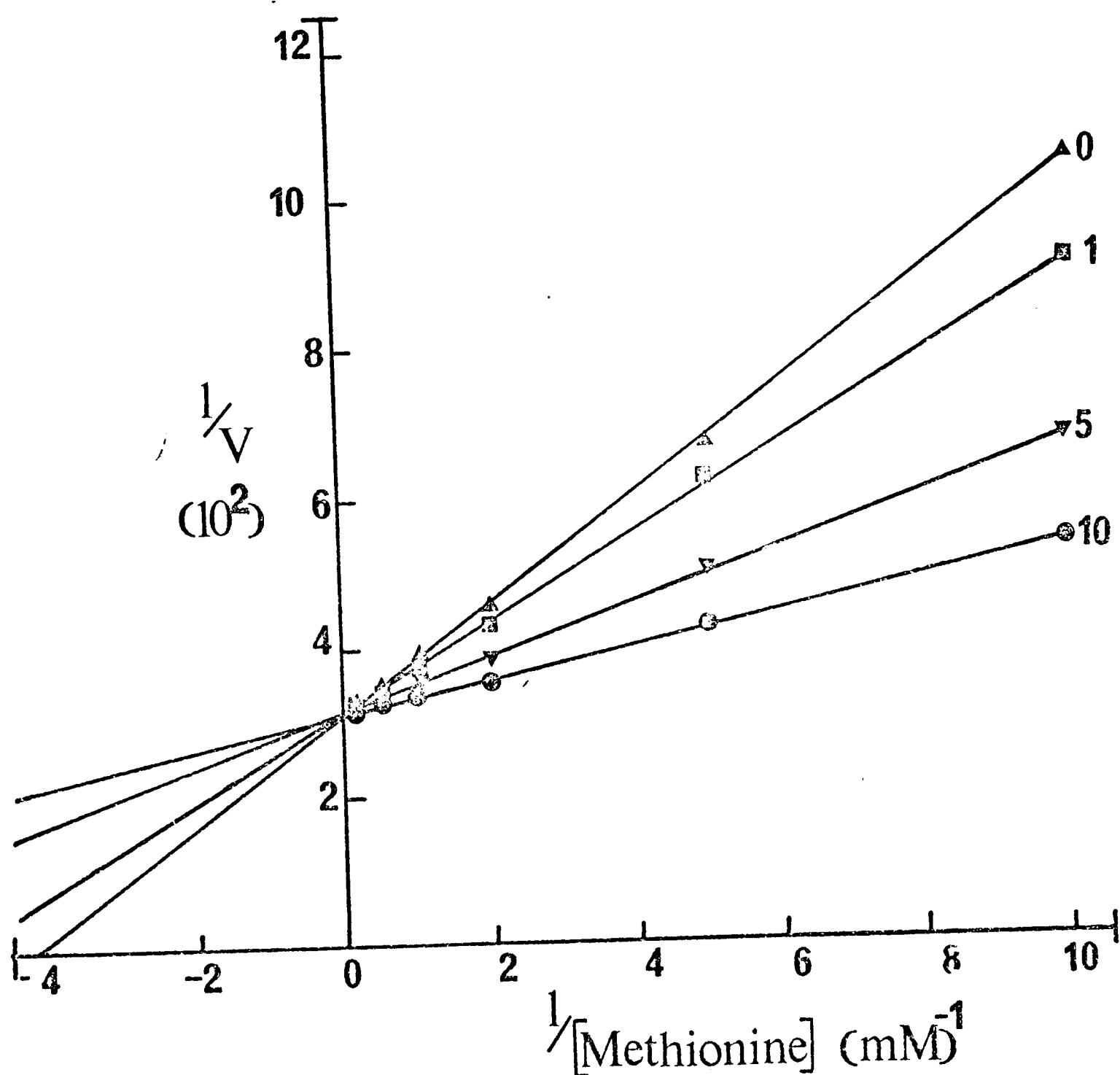


Fig. 12.18

Double reciprocal plots of the effect of methionine concentration upon methionine-dependent ATP-PP_i exchange in the presence or absence of cysteine acid. Reaction mixtures were as described in Materials and Methods except that methionine and cysteine acid were added as specified.

▲, without cysteine acid; ■, 1mM-cysteine acid; ▼, 5mM-cysteine acid; ●, 10mM-cysteine acid.

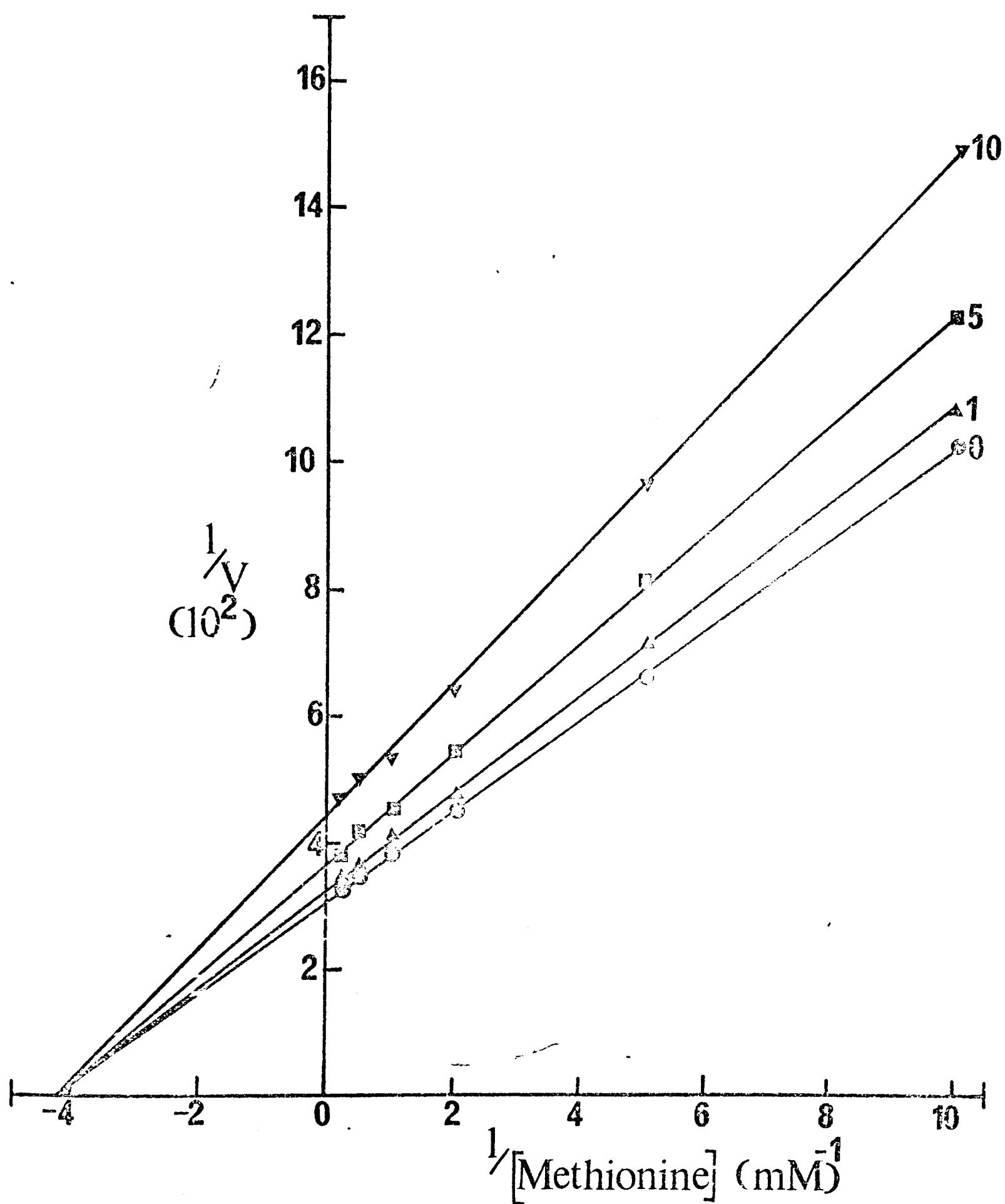


Fig. 12.19

Double reciprocal plots of the effect of methionine concentration upon methionine-dependent ATP-PP_i exchange in the presence or absence of O-acetyl serine. Reaction mixtures were as described in Materials and Methods except that methionine and O-acetyl serine were added as specified.

●, without O-acetyl serine; ▲, 1mM-O-acetyl serine; ■, 5mM-O-acetyl serine; ▼, 10mM-O-acetyl serine.

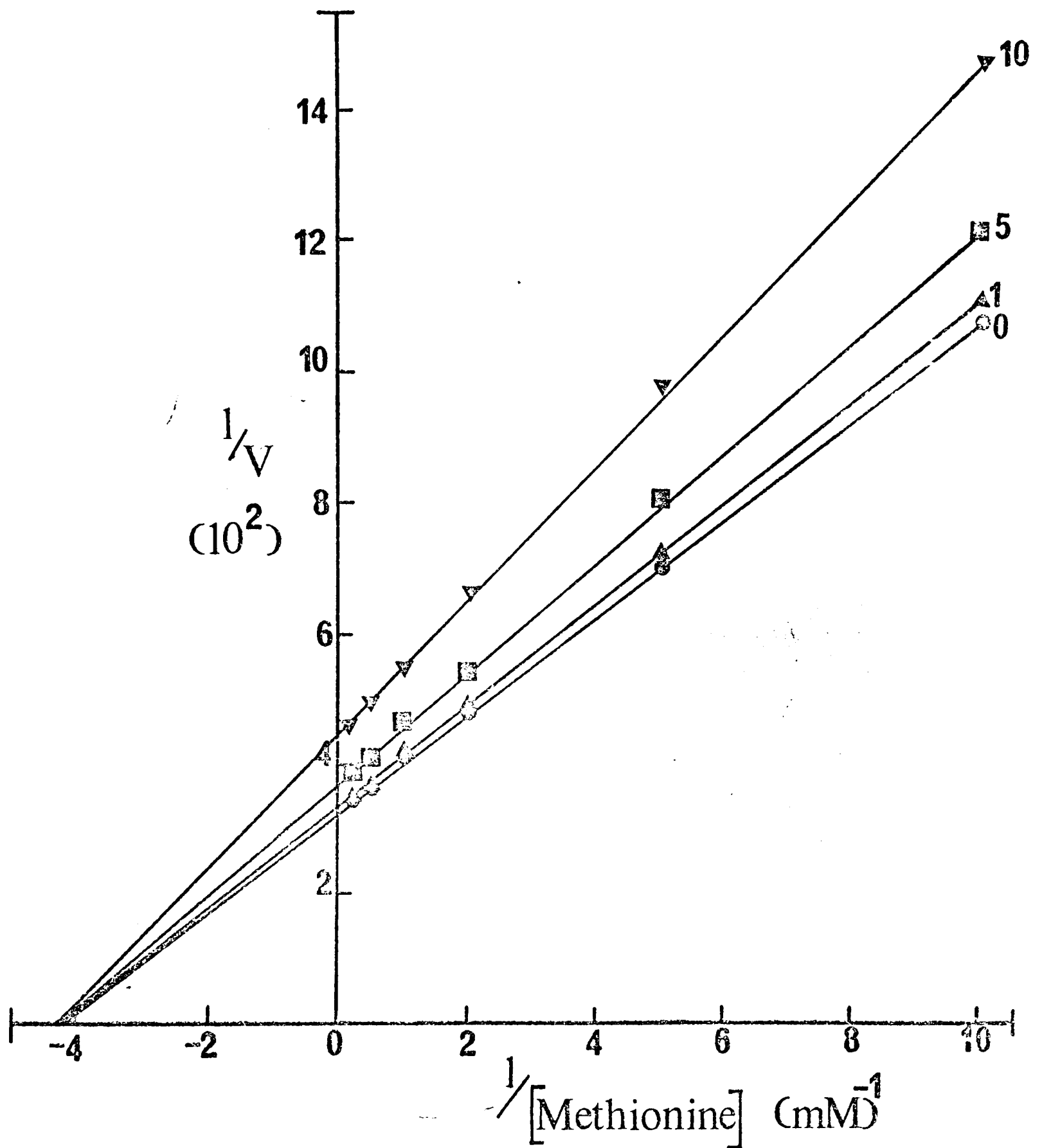


Fig. 12.20

Double reciprocal plot of the effect of methionine concentration upon methionine-dependent ATP-PP_i exchange in the presence or absence of glutathione. Reaction mixtures were as described in Materials and Methods except that methionine and glutathione were added as specified. ●, without glutathione; ▲, 1mM-glutathione; ■, 5mM-glutathione; ▼, 10mM-glutathione.

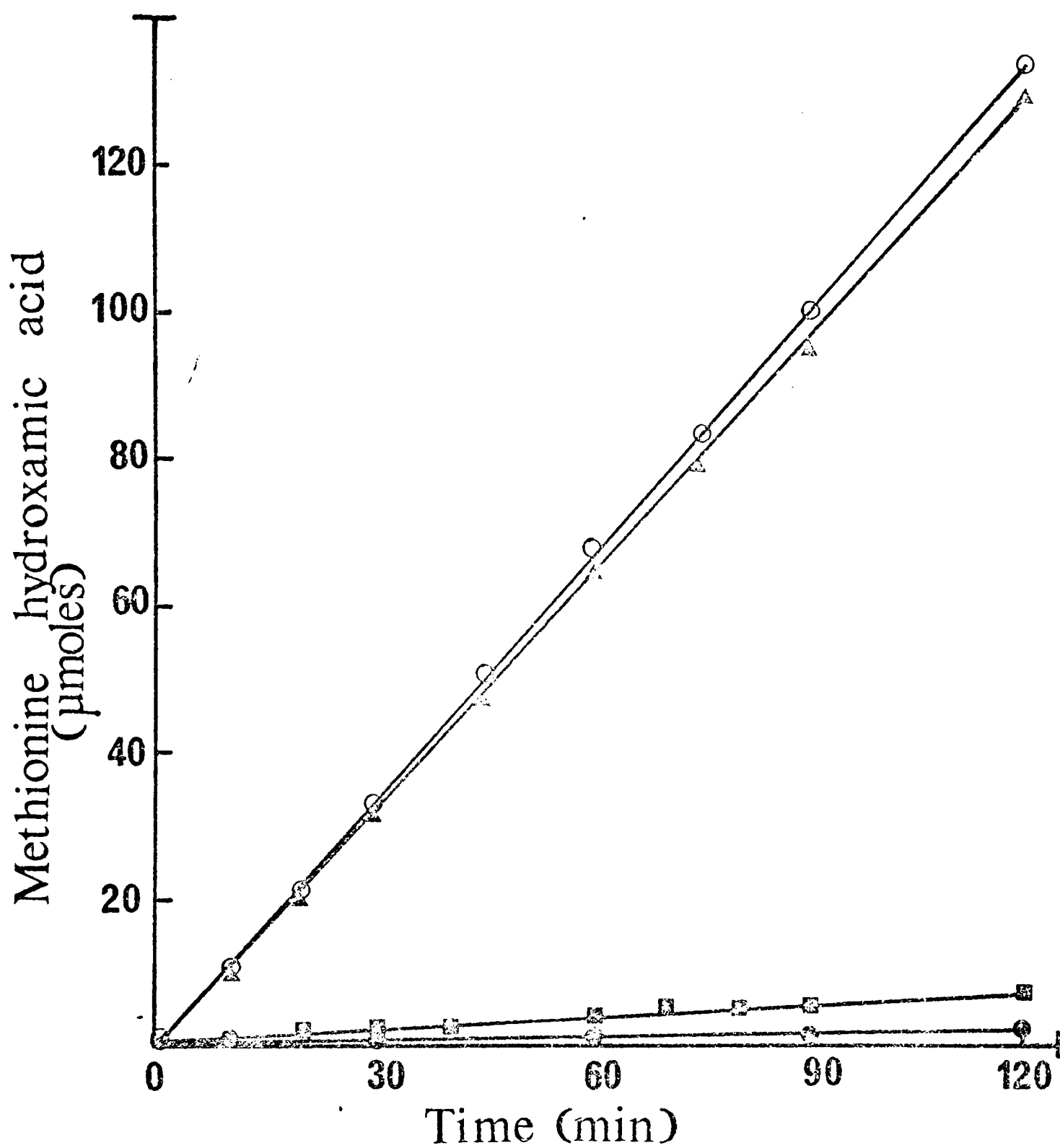


Fig. 12.21

The effect of time upon the formation of methionine and selenomethionine hydroxamic acid. The control reaction mixture was as described in Materials and Methods (O). Alterations to the reaction mixture were as described below; ▲, selenomethionine as substrate; ●, plus methionine minus ATP; ■, plus methionine minus Mg^{2+} .

amount of AMP and PP_i (Table 12.3). When L-methionine was replaced by L-selenomethionine in the reaction mixtures, L-selenomethionine hydroxamic acid formation was detected. L-Selenomethionine hydroxamic acid and L-methionine hydroxamic acid were formed at almost equivalent rates.

No ethionine or α -methyl methionine hydroxamic acid was formed when methionine was replaced with ethionine or α -methyl methionine. The rates of norleucine and homocysteine hydroxamic acid formation were very low as compared with the rate of methionine or selenomethionine hydroxamic acid formation.

When L-methionine was replaced with D-methionine at concentrations between 0.1 and 50 mM, no D-methionine hydroxamic acid was detected.

DISCUSSION

A comparison of the aminoacyl-tRNA synthetases from P. denitrificans with the synthetases from other bacteria, and a comparison of the cysteinyl- and methionyl-tRNA synthetases of P. denitrificans, shows that the synthetases differ widely in their K_m values for amino acids, ATP and Mg^{2+} , and are especially sensitive to Mg^{2+} /ATP ratio. Although the optimal Mg^{2+} /ATP ratio for methionyl-tRNA synthetase, as measured by the ATP- PP_i exchange method is 2 for both P. denitrificans and E. coli (Svensson, 1967), other aminoacyl-tRNA synthetases differ drastically with respect to their Mg^{2+} /ATP ratios; the leucyl-tRNA synthetase from E. coli has an optimal Mg^{2+} /ATP of 10, while the prolyl-tRNA synthetase is 30 (Novelli, 1967).

Examination of the structure of the alternative substrates of purified methionyl-tRNA synthetase from P. denitrificans indicated that the requirements for substrate activity were;

(i) the presence of an α -amino acid group,

TABLE 12.3

Stoichiometry of products formed in ATP-methionine
(selenomethionine) reactions in the presence of
hydroxylamine.

Components of reaction mixture	Amino acid hydroxamate	$\mu\text{mols/hr}$	
		PP_i	AMP
Complete (with methionine)	0.68	0.64	0.67
Complete minus ATP	0.01	0.01	0.01
Complete minus Mg^{2+}	0.01	0.01	0.01
Complete minus methionine	0.05	0.04	0.05
Complete (with selenometh- ione)	0.62	0.64	0.64
Complete minus ATP	0.01	0.01	0.01
Complete minus Mg^{2+}	0.01	0.01	0.01
Complete minus selenometh- ione)	0.06	0.04	0.04

Six duplicate reaction mixtures were run for each treatment of which two were assayed for either amino acid hydroxamate, PP_i or AMP. Methionine hydroxamate was assayed as the ferric complex as described in the text. PP_i was measured as 2P_i after treatment with purified inorganic pyrophosphatase (see Chapter 8). AMP was measured using an ADP/AMP Test Kit from Boehringer und Soehne, G.m.b.H. Mannheim, Germany.

- (ii) the α -amino acid must be the L-isomer,
- (iii) the amino acid must not exceed the chain length of methionine, and
- (iv) the requirement of a -S- or -Se- substituent on the α -position for maximum enzyme activity.

The substrate specificity of the methionyl-tRNA synthetase from P. denitrificans differs from that of E. coli (Lemoine et al., 1968; Nisman & Hirsch, 1958), S. lutea (Hahn & Brown, 1967) and yeast (Berg, 1956). The P. denitrificans methionyl-tRNA synthetase catalyses homocysteine-dependent ATP-PP_i exchange, but not ethionine- or cystathionine-dependent exchange, whereas the S. lutea enzyme does not catalyse homocysteine-dependent ATP-PP_i exchange but does catalyse both ethionine- and cystathionine-dependent exchange. Methionyl-tRNA synthetase from P. denitrificans, E. coli and S. lutea catalyse selenomethionine- and norleucine-dependent ATP-PP_i exchange. The K_m values for methionine and selenomethionine are essentially the same in P. denitrificans, whereas the K_m value for norleucine is considerably higher than that for methionine; a similar situation is found in E. coli (Lemoine et al., 1968; Cassio et al., 1967, S. lutea (Hahn & Brown, 1967) and yeast (Berg, 1956).

The effects of ethionine in biological systems have been extensively studied (Levine & Tarver, 1951) and ethionine has been found to inhibit protein synthesis in a variety of studies (Halvorsen & Spiegelman 1952; Simpson et al., 1950; Younathan et al., 1956). Yoshida & Yamasaki (1959) found that ethionine can be incorporated into the normal peptide bond sequence of amylase in place of methionine, and that α -amylase containing ethionine, has the same physicochemical properties, and enzyme activity, as those of the normal protein. On the contrary, incorporation of norleucine into

proteins by E. coli, apparently in place of methionine (Cohen & Munier, 1959; Munier & Cohen, 1959), results in loss of enzyme activity of that protein.

It appears that for both E. coli and P. denitrificans, selenomethionine is a more satisfactory substitute for methionine than is norleucine. The substrate specificity of methionine adenylyltransferase (ATP:L-methionine S-adenosyltransferase EC 2.5.1.6), another methionine-activating enzyme, also activates selenomethionine (Mudd & Cantoni, 1957). The similarity of the K_m values for methionine and selenomethionine in the amino acid activation reaction presents a major exception to the safeguard against incorporating an amino acid analogue into protein, previously noted by Novelli (1967), whereby the K_m for the "wrong" amino acid or the analogue is several orders of magnitude larger than the K_m for the natural substrate. So it appears that the amount of selenomethionine in proteins in P. denitrificans would be governed by the ratio of selenomethionine to methionine in the cell.

Huber & Criddle (1967) isolated β -galactosidase from E. coli grown on selenate; approx, 50% of the methionine residues of the enzyme were replaced by selenomethionine. Although the stability of the enzyme was less for the selenium-substituted enzyme, the catalytic parameters (the K_m and V_{max}) were not changed.

β -Cystathionase, cysteinyl-tRNA synthetase and methionyl-tRNA synthetase are all inhibited by O-acetyl serine. This may represent an important mechanism in the control of the metabolic pathway of sulphur metabolism in P. denitrificans. An increase in the intracellular concentration of O-acetyl serine might be expected if there were a sulphur deficiency, especially a deficiency in sulphide. This would, in turn,

lead to a decrease in the rate of cysteine synthesis. Therefore, when there is an O-acetyl serine surplus, and a sulphide deficiency, incorporation of cysteine into protein, lysis of cystathionine (by β -cystathionase) and incorporation of methionine into proteins would be inhibited. Conversely, when the intracellular levels of sulphide and cysteine are increased, methionyl-tRNA synthetase activity is stimulated.

How the formation of aminoacyl-tRNA synthetases is regulated is an intriguing question because of their critical role in the growth of the cell, and because they constitute a significant fraction of the cell's protein. All together, the 20 aminoacyl-tRNA synthetase in E. coli constitute about 10% of the cell's enzymically active protein under all growth conditions. Nevertheless, the mechanism by which the cellular level of these important molecules is controlled is not well understood. Early reports in the literature point out the most easily observed feature of synthetase regulation - during growth in a great variety of media, E. coli cells maintain a nearly constant level of the individual amino acid-activating enzymes (Boman et al., 1961; Coles & Rogers, 1964; Ames & Hartman, 1962). This has been found to be the case with cysteinyl- and methionyl-tRNA synthetases in P. denitrificans.

CHAPTER 13

CONCLUSIONS

CONCLUSIONS

This thesis reports on experiments conducted to determine the pathway, and the control and regulation, of sulphur metabolism in Paracoccus denitrificans. Like many other bacteria, P. denitrificans is an assimilatory sulphate reducing organism and, except for a number of regulatory properties, has a similar metabolic pathway to a number of other bacteria (e.g. E. coli, S. typhimurium).

P. denitrificans possesses all the enzymes required to activate, reduce and incorporate inorganic sulphate, into cysteine and methionine.

The reduction of sulphate by P. denitrificans is similar to that in E. coli in that the inferred substrate for sulphate reduction is PAPS; P. denitrificans possesses APS kinase activity but neither 3'-nucleotidase nor enzyme "A" activities. It further resembles E. coli in that the conversion of cysteine to methionine (transsulphurylation) is irreversible.

From experiments reported in this thesis, it appears that cysteine plays a central role in the control and regulation of sulphur metabolism in P. denitrificans. Cysteine is involved in, or has an effect upon, almost every enzyme system studied in the present investigation.

O-Acetyl serine and methionine also play important roles in the sulphur metabolic pathway and its regulation.

No conclusions can be drawn on the role of sulphite and sulphide, separate from cysteine, in the repression of enzymes of sulphur metabolism in P. denitrificans, for both sulphite and sulphide will, ultimately, be incorporated into cysteine, and the extent of enzyme repression by sulphite and sulphide may only reflect the relative rates of incorporation of sul-

phite and sulphide into cysteine. (Experiments with mutants lacking either sulphite reductase or O-acetyl serine sulphydrylase would aid in the clarification of this point).

Although cysteine, methionine, and O-acetyl serine are not the only compounds involved in the regulation of sulphur metabolism in P. denitrificans, they do appear to be the most important. A scheme summarising the role of cysteine, methionine and O-acetyl serine in sulphur metabolism in P. denitrificans is presented in Fig. 13.1.

Selenium metabolism. It has been suggested that selenate is metabolised via the enzymes of the sulphate reduction pathway. Selenium compounds competitively inhibit sulphate uptake, ATP sulphurylase, O-acetyl serine sulphydrylase, cysteinyl- and methionyl-tRNA synthetase, with respect to the respective sulphur analogue. However, since the activation of selenate to PAPSe (the selenium analogue of PAPS) could not be detected, it is suggested that selenate is reduced via a separate, as yet unknown pathway, and only in the latter stages of selenium metabolism does it follow the same metabolic pathway as sulphur (i.e. after reduction of selenate to selenide, selenide is incorporated into selenocysteine and then selenium follows the same metabolic pathway as the sulphur analogues).

Fig. 13.1

A schematic representation showing the role of cysteine, methionine and O-acetylserine in the regulation of sulphur metabolism in Paracoccus denitrificans.

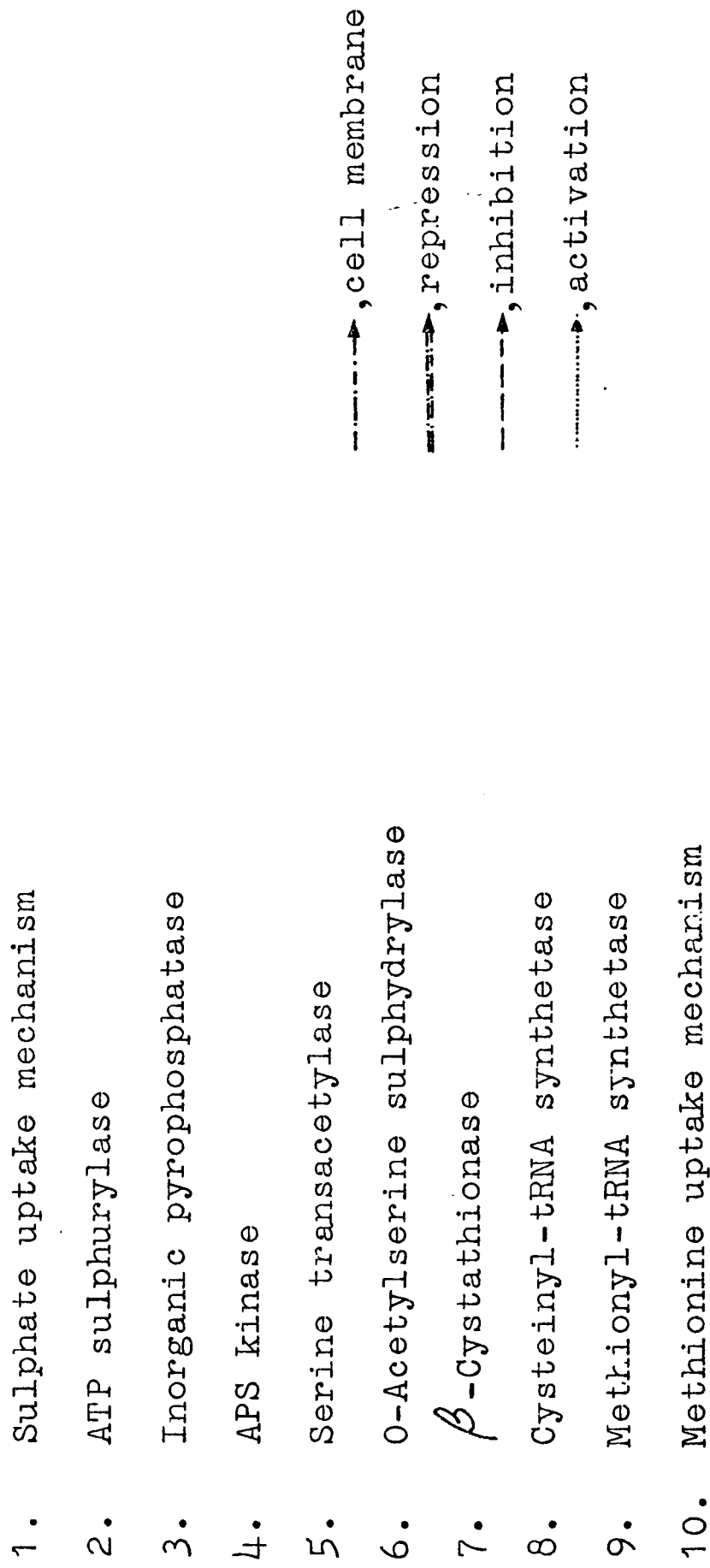
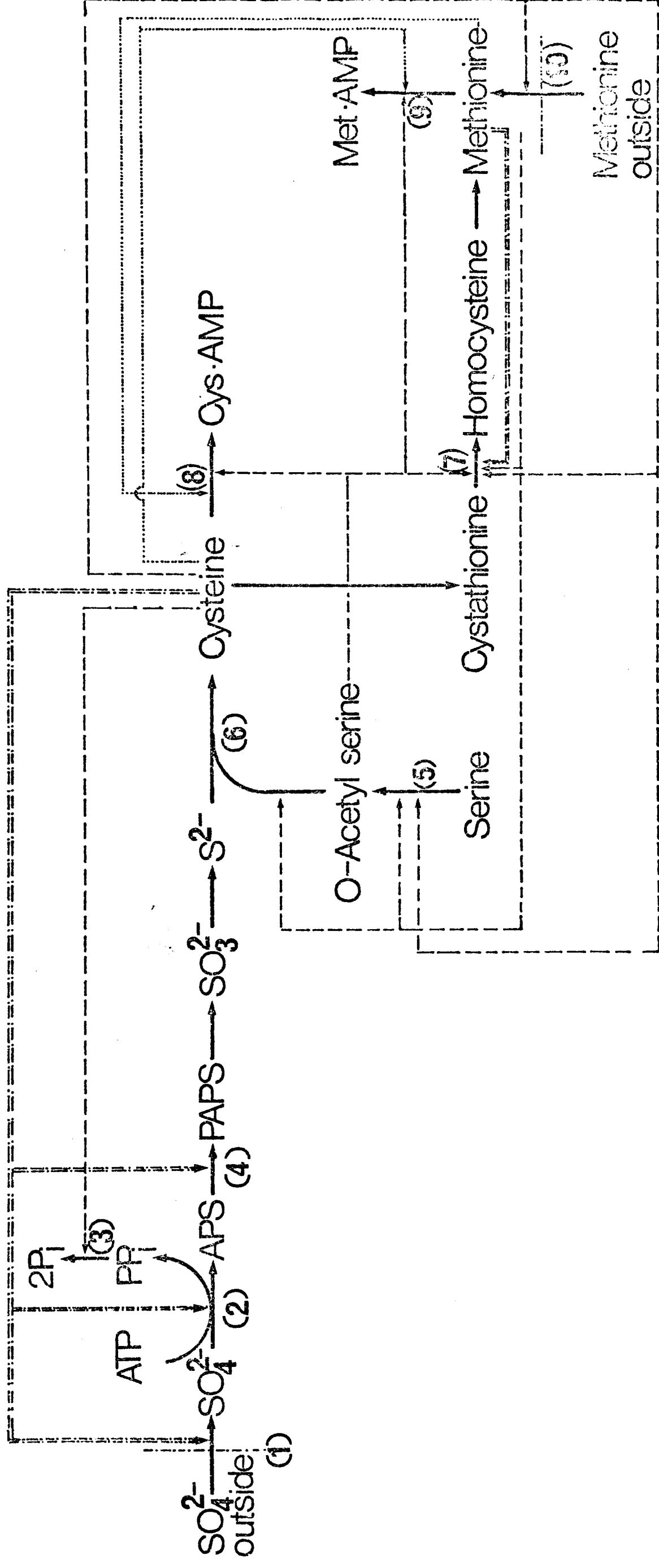


Fig.13.1

The role of cysteine, methionine, and O-acetyl serine in the regulation of sulphur metabolism in Paracoccus denitrificans.



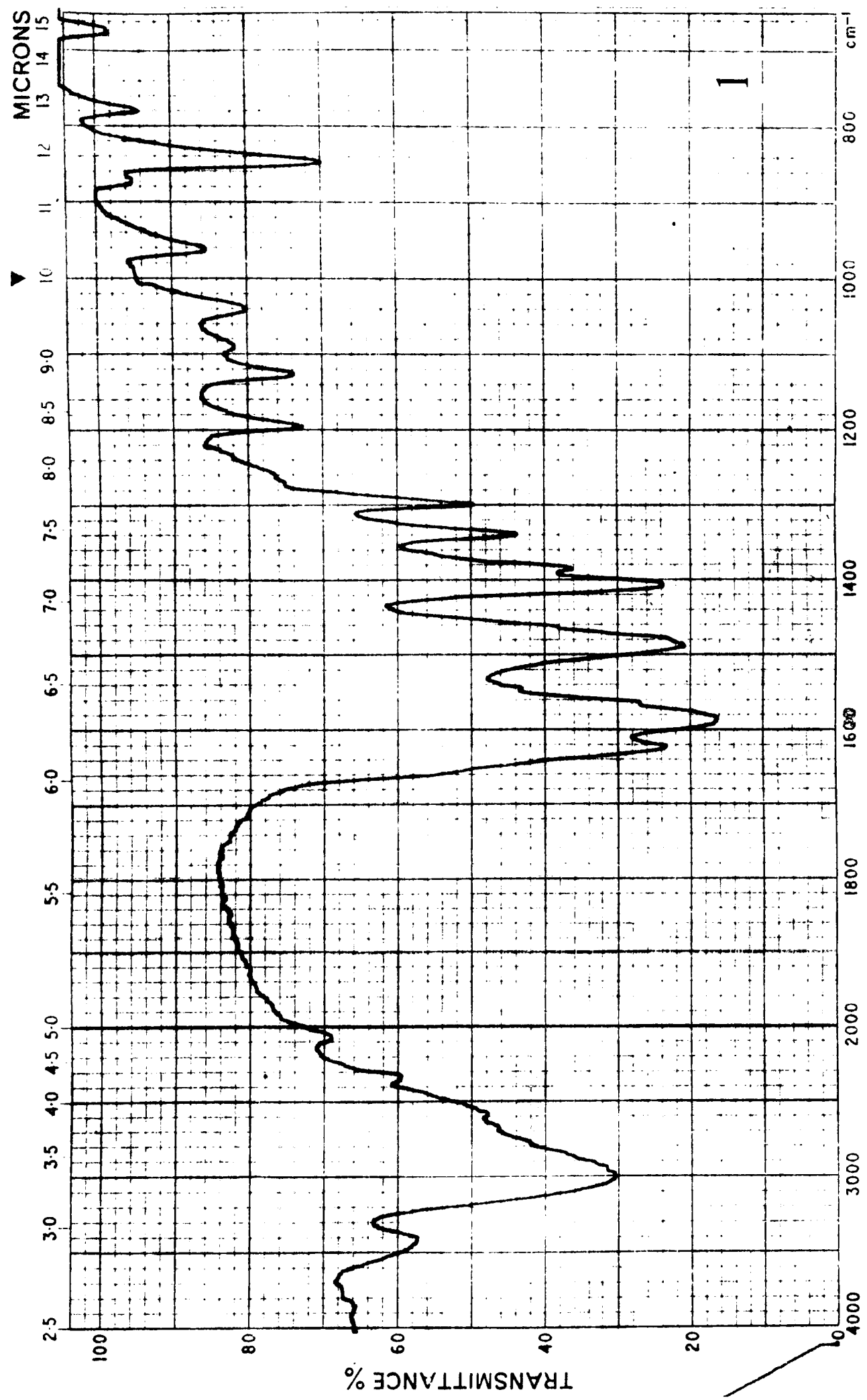
APPENDIX

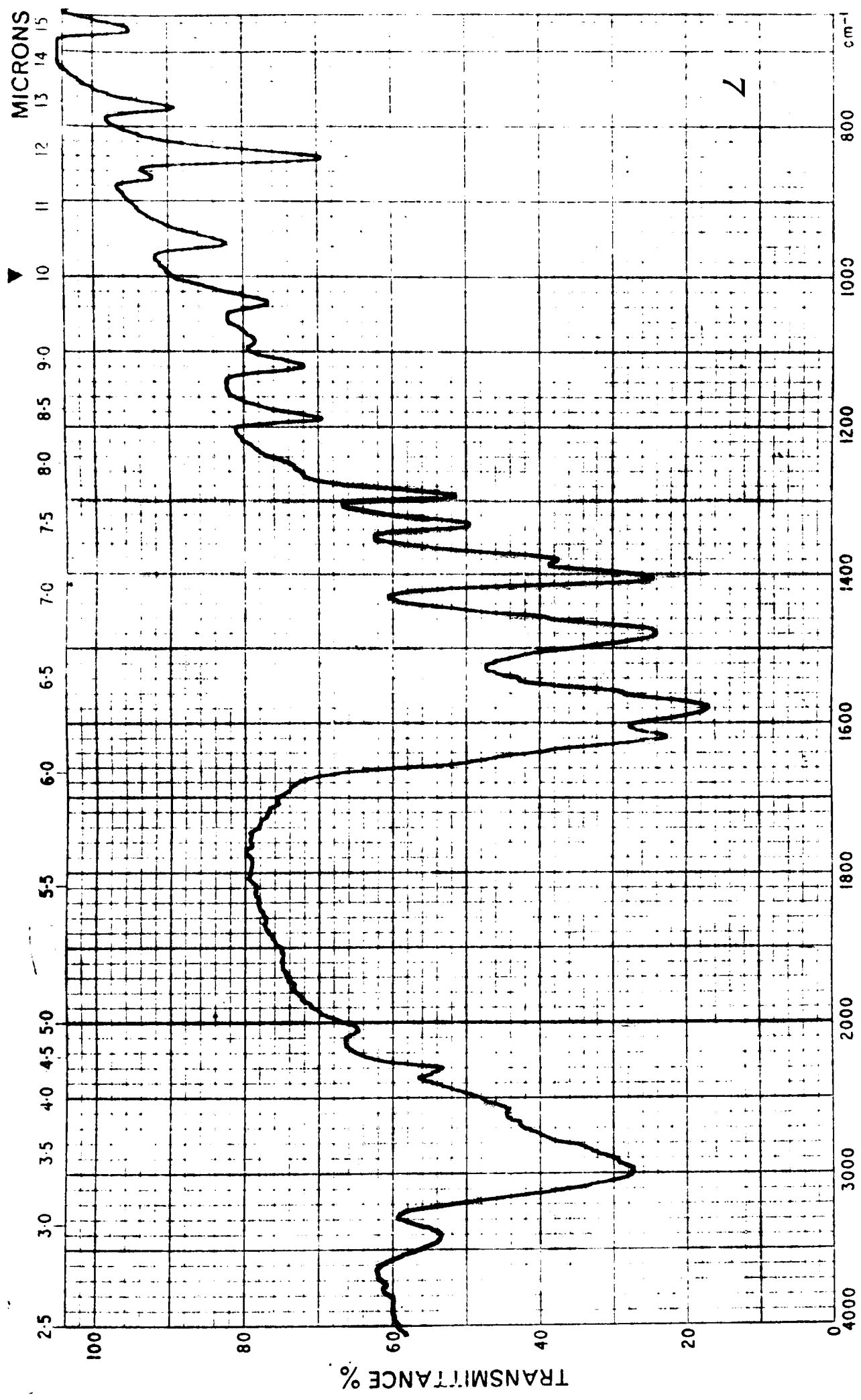
All infra red examinations were conducted using a Hilga-Watts Infragraph Model H1200. Hydrochloride compounds were pressed into 13 mm discs containing KCl (80 - 100 mg); other discs contained KBr (80 - 100 mg). Discs contained between 0.5 - 1.0 mg of the compound under examination.

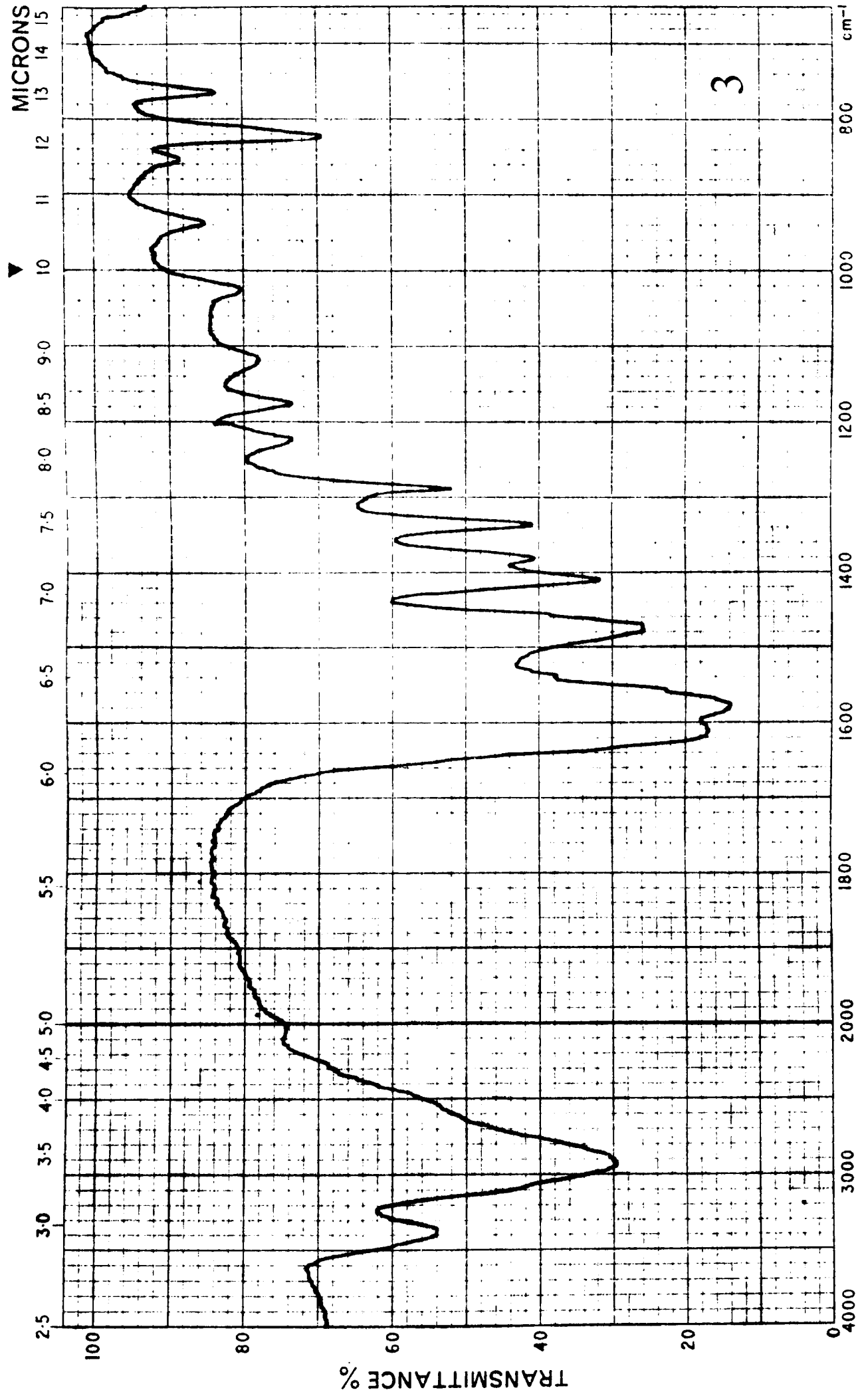
All infra red spectra were run at scan speed 1, with an attenuated reference.

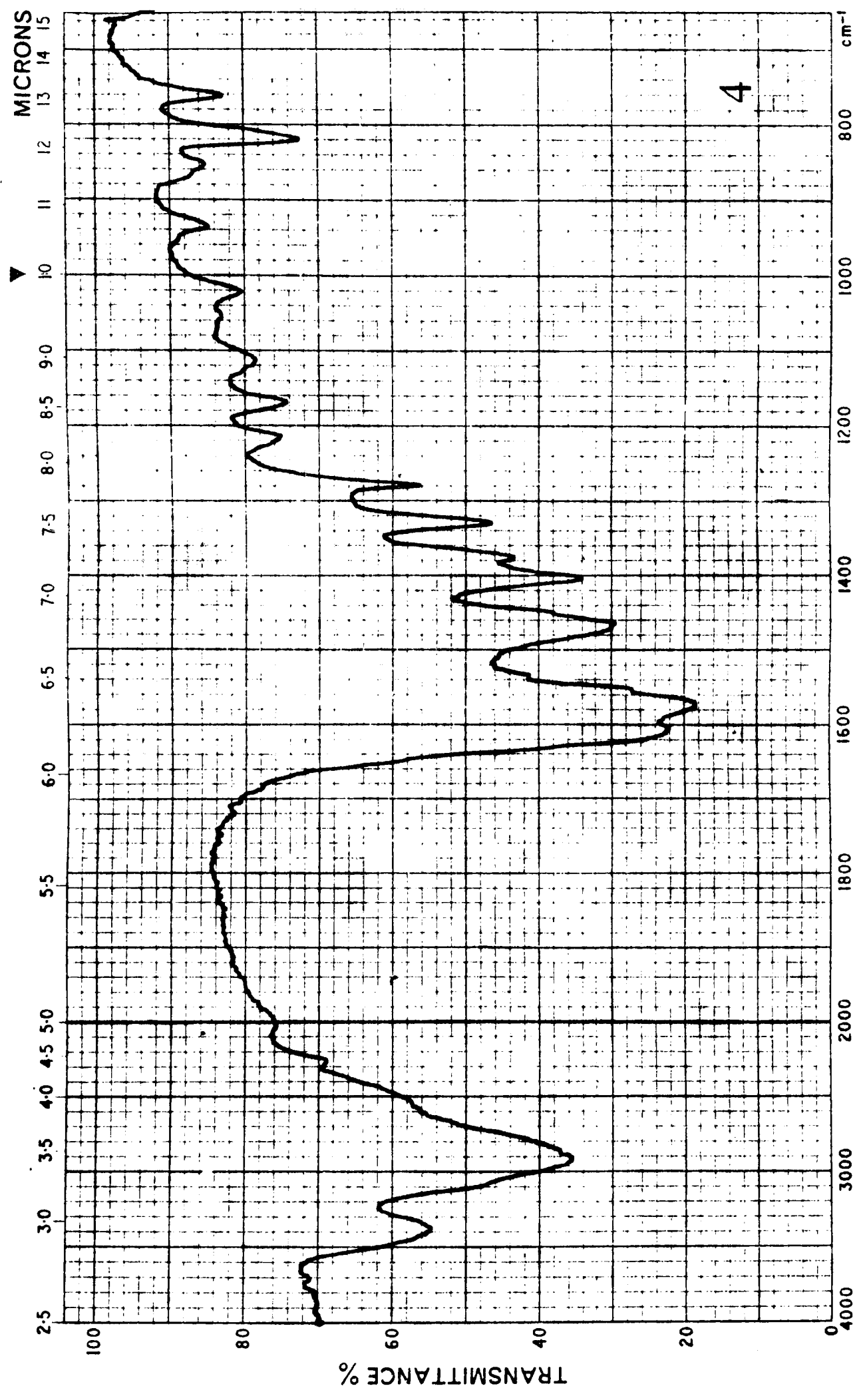
Spectra

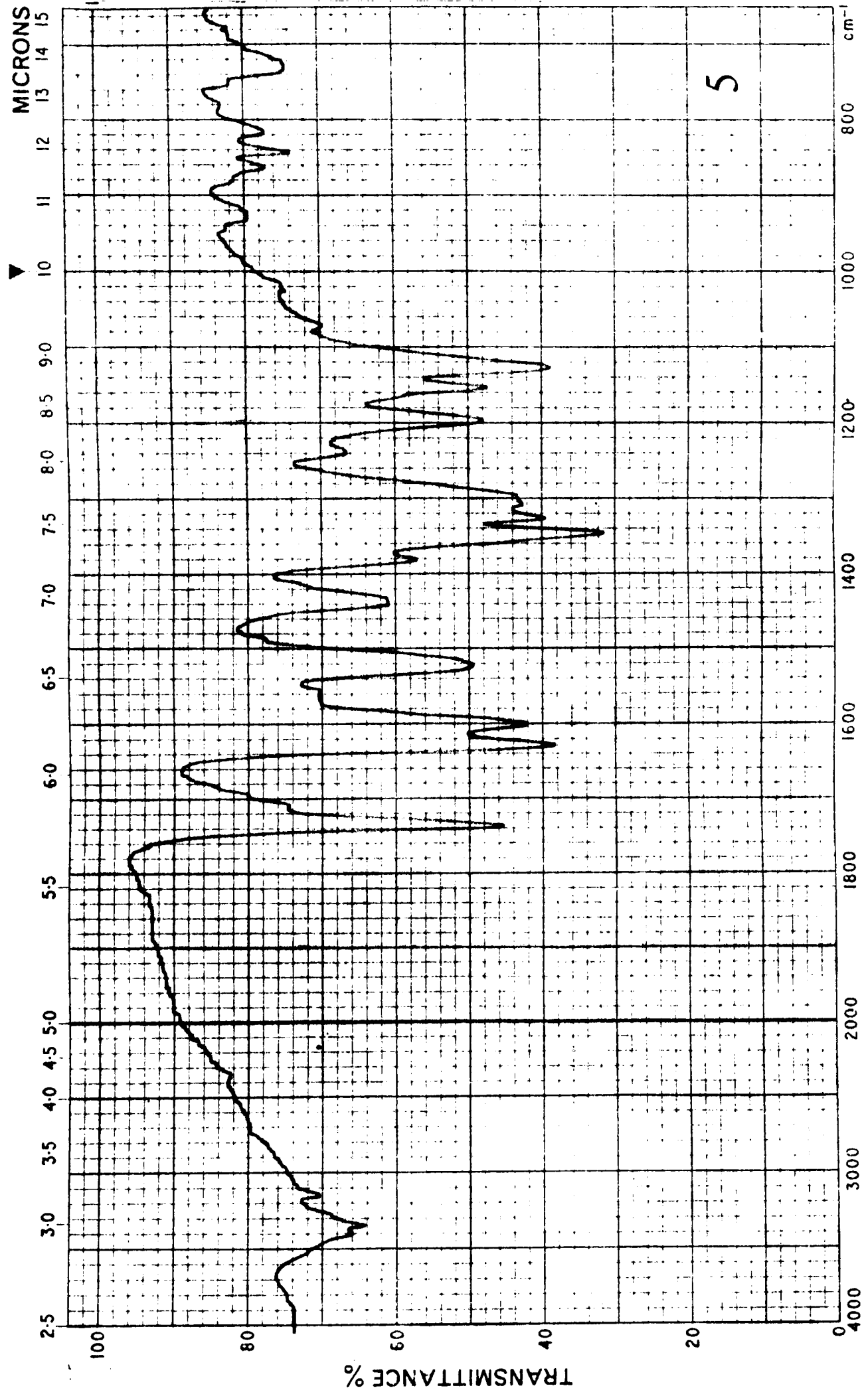
1. L-Cystine reference
2. Enzymically synthesised L-cystine (see Chapter 9)
3. L-Selenocystine reference
4. Enzymically synthesised L-selenocystine (see Chapter 9)
5. Pyruvate 2,4-dinitrophenylhydrazone reference
6. 2,4-Dinitrophenylhydrazone derivative of enzymically synthesised pyruvate (see Chapter 10)
7. L-Homocysteine thiolactone-HCl reference
8. L-Homocysteine reference
9. L-Homocysteine; chemically synthesised from L-Homocysteine thiolactone-HCl (see Chapter 10)
10. L-Homocysteine thiolactone-HCl; chemically synthesised from L-Homocysteine (see Chapter 10).

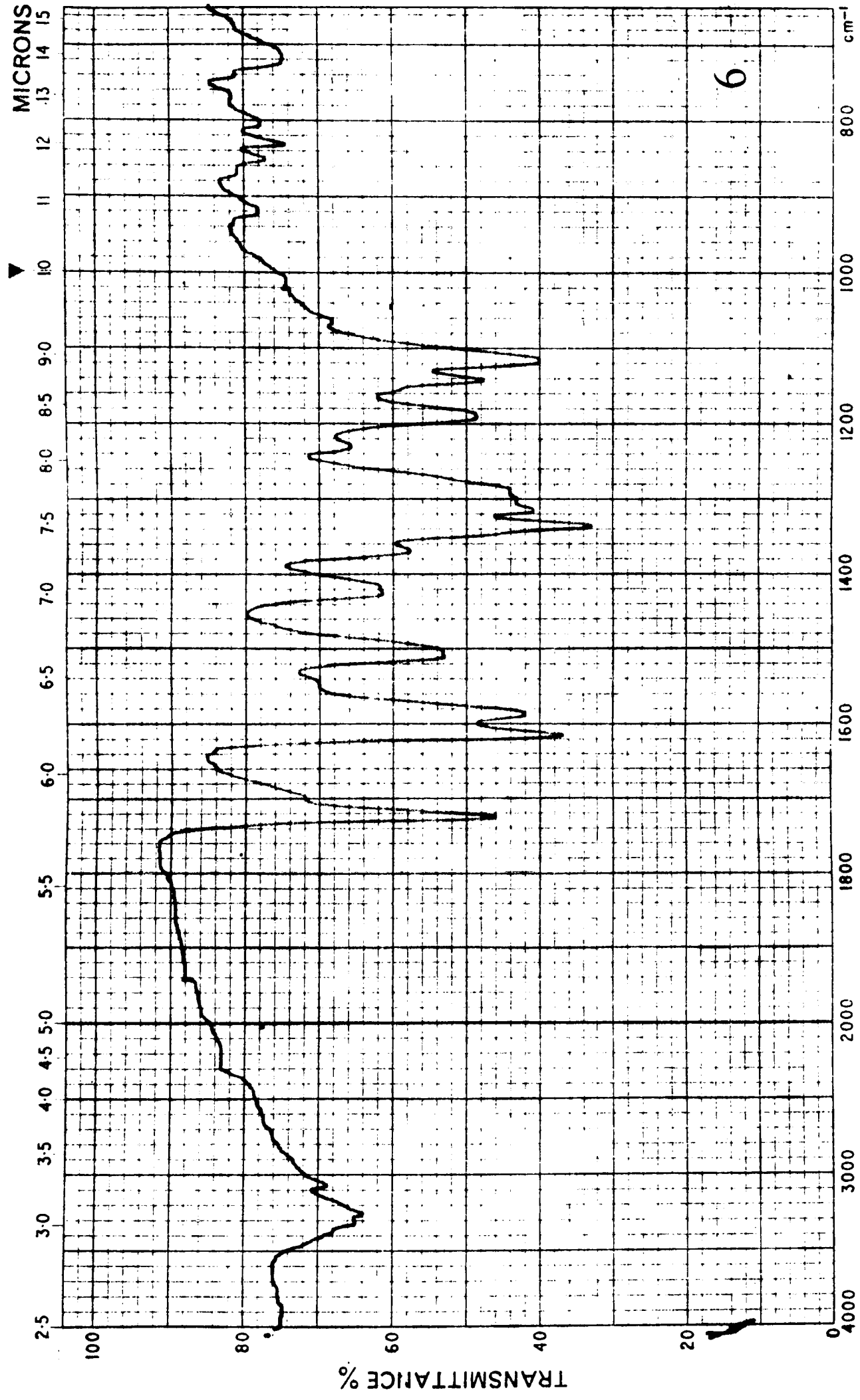


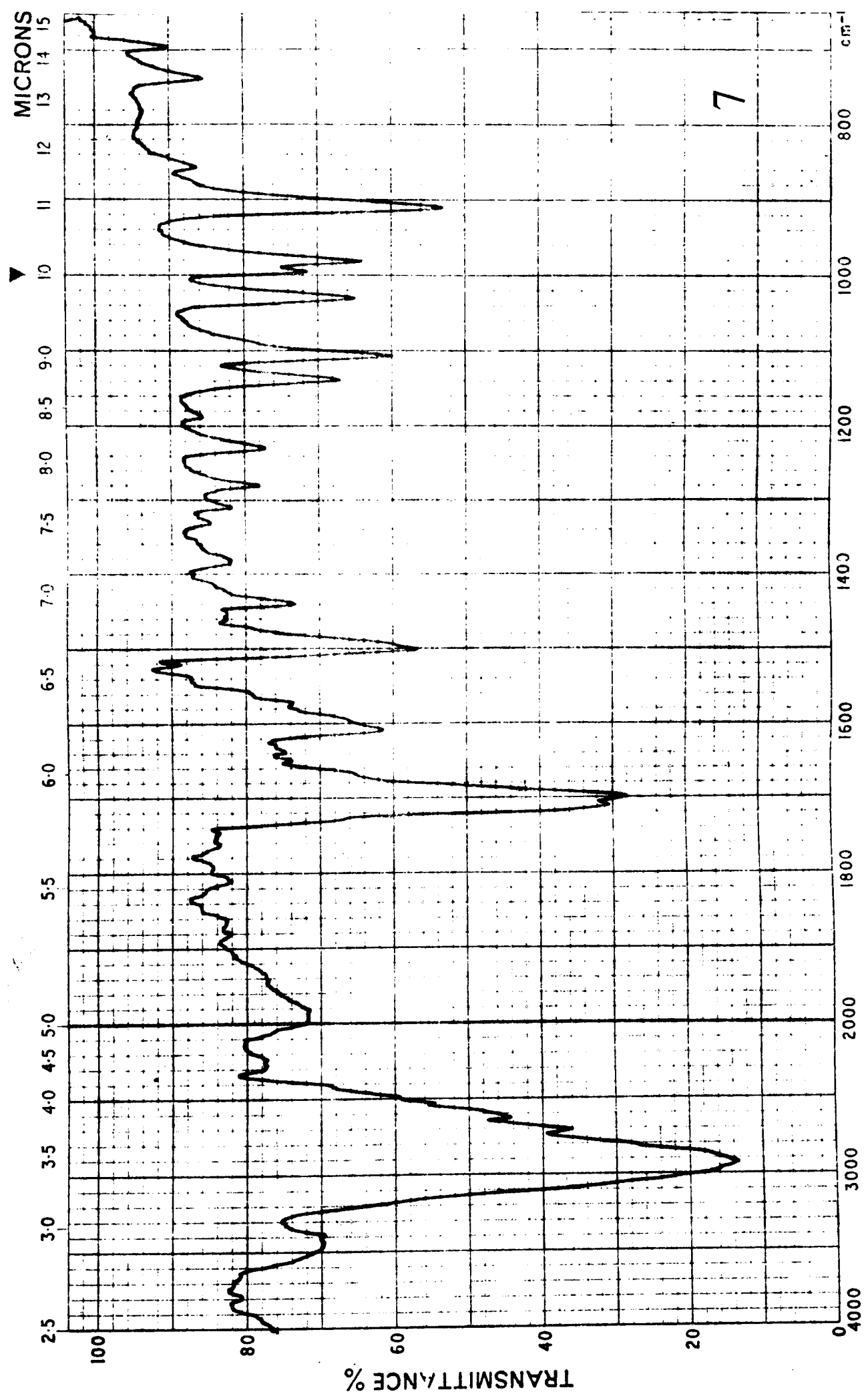


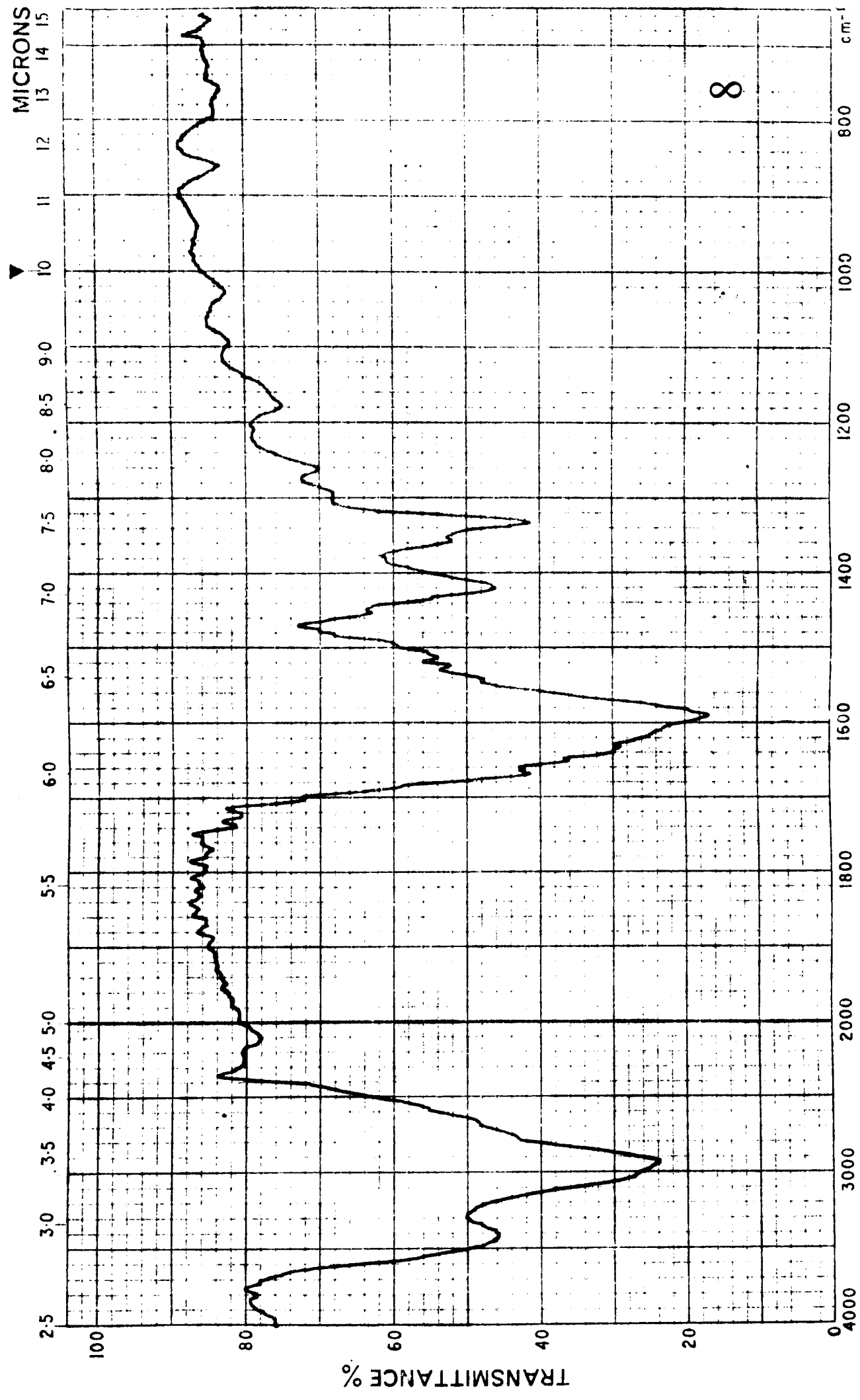


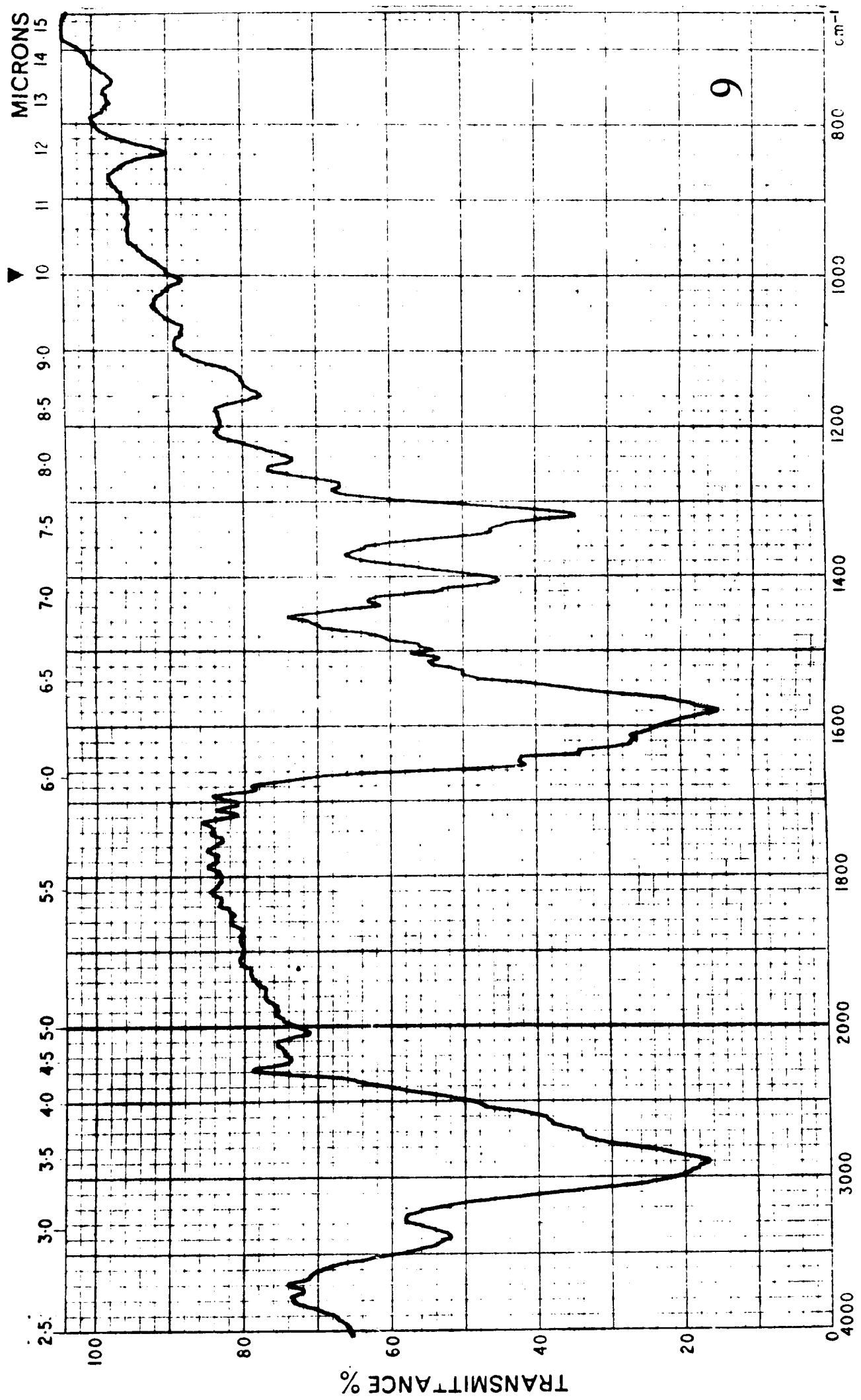


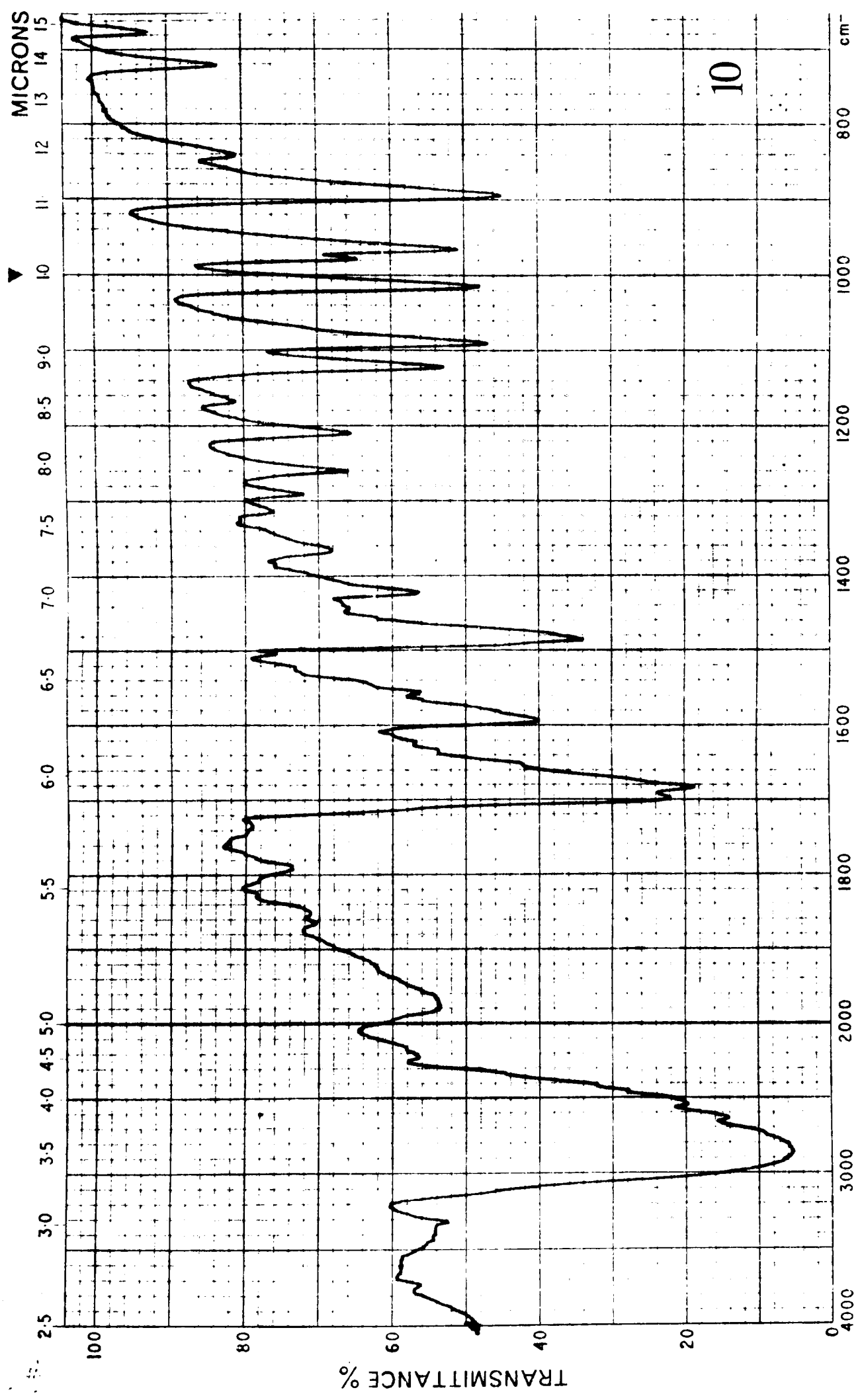












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