# SOME STUDIES ON THE REDUCTION OF SULFATE TO SULFIDE IN *DESULFOVIBRIO DESULFURICANS*

by

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# ABSTRACT

The sulfate-activating ATP-sulfurylase of Desulfovibrio desulfuricans was purified 26-fold by a combination of $(NH_4)_2SO_4$ precipitation, TEAE column chromatography and alumina $C_\gamma$ adsorption. Disc electrophoresis of the final enzyme preparation showed one major and two minor bands. The pH optimum of the enzyme was 8.7. When $MoO_4^=$ was used as the variable substrate, kinetic analysis gave a Km value of $1.2 \times 10^{-3}$ M for the same. When ATP or $SO_4^=$ was used as a variable substrate at low concentrations the enzyme exhibited non linear kinetics yielding Km values of $3.5 \times 10^{-4}$ M and $1.43 \times 10^{-3}$ M for ATP and $2.5 \times 10^{-3}$ M and $10^{-2}$ M for $SO_4^=$. However, at higher substrate concentrations the lines became linear and described the classical Michaelis-Menten kinetics. The possible mechanisms applicable to the system are discussed.

## ABBREVIATIONS

| | |
|---|---|
| APS | - Adenosine-5'-phosphosulfate |
| ATP | - Adenosine-5'-triphosphate |
| Bi Bi | - Binary Binary |
| Cyt $\underline{c}_3$ | - Cytochrome $\underline{c}_3$ |
| EDTA | - Ethylene diamine tetraacetic acid |
| NADP | - Nicotinamide-adenine dinucleotide phosphate |
| PAPS | - 3'-phosphoadenosine-5'-phosphosulfate |
| Pi | - Phosphate |
| PPi | - Pyrophosphate |
| TCA | - Trichloroacetic acid |

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# HISTORICAL INTRODUCTION

All organisms contain sulfur and are involved in diverse transformations of sulfur to some degree. With some microorganisms these transformations are primarily reactions of assimilation of sulfur; with others, sulfur compounds act as sources of energy for growth, or function as specific hydrogen donors or acceptors.

The reduction of sulfur compounds such as sulfate, tetrathionate, thiosulfate, and sulfite (and also elemental sulfur) is much more common and many bacteria and fungi are concerned in such transformations. Of particular importance among the reductions of inorganic sulfur compounds is the reduction of $SO_4^=$ and accumulation of $H_2S$ that is brought about principally by specific groups of bacteria, primarily the *Desulfovibrio* species.

Clark and Tanner (1938) obtained results which suggested the reduction of sulfate by thermophilic bacteria. Prevot (1948) reported that many anaerobic bacteria produce sulfide from sulfite, and that two even produce sulfide from sulfate. Challenger *et al*. (1933, 1934, 1937) reported the methylation of organically linked sulfur compounds in the fungus *Penicillium brevicaule* but the first instance of the direct methylation of inorganic sulfur by microorganisms was

observed (Birkinshaw, Finlay, and Webb, 1942) in the fungus Schizophyllum commune which produces hydrogen sulfide and methyl mercaptan from sulfate during growth in a glucose medium.

The most important and specialized microorganisms that use sulfate as a terminal electron acceptor for their respiration are divided into two groups, Desulfovibrio spp. and Clostridia. The first species of one group was described as Spirillum desulfuricans (Biejerinck, 1895), now known as Desulfovibrio desulfuricans. A second type of sulfate reducing bacterium is Clostridium nigrificans. The latter was originally described as a thermophilic strain of D. desulfuricans (Starkey, 1938) and was later identified as C. nigrificans (Campbell, Frank and Hall, 1957). The name Desulfomaculum was proposed (Campbell and Postgate, 1965) as a genus for the classification of three sporogenous types of sulfate reducing bacteria: Desulfomaculum nigrificans, earlier Clostridium nigrificans; Desulfomaculum orientis, earlier Desulfovibrio orientis; Desulfomaculum ruminis, earlier "Coleman's organism" (Coleman, 1959, 1960). Generally, the name Desulfovibrio is retained for the non sporulating type of sulfate reducer while sporulating types are to be found among the Clostridia.

It appears that a rational classification of sulfate reducing organisms may now be realized (Postgate, 1965).

Studies using various strains of D. desulfuricans showed that the buoyant density of the DNA, growth in saline, and resistance to certain inhibitors, could be used as bases to divide the organisms into three groups (Saunders, Campbell and Postgate, 1964; Postalek and Kvet, 1964; Saleh, 1964; Saleh, Macpherson and Miller, 1964):

Group 1 organisms, with a guanine and cytosine content of 60-62% of the total DNA base, is composed mainly of fresh water organisms unable to dismute pyruvate or to metabolize choline; they are inhibited by hibitane, and CTAB (cetyltrimethylammonium bromide).

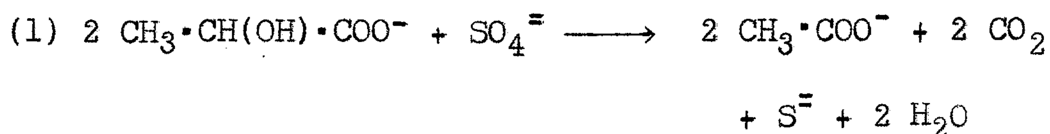
Group 2 organisms with a guanine and cytosine content of 54-56% of the total DNA base are mostly salt-water forms, though adaptable in all cases but one to an NaCl-free medium, and are able to dismute pyruvate and metabolize choline; they are somewhat more resistant to hibitane and CTAB than are Group 1 organisms (within a narrow concentration range).

Group 3 organisms with a guanine and cytosine content of 45.6-46.6% of the total DNA base are strictly salt water strains, and appear to be most resistant of the three groups to hibitane and CTAB. It was also observed by Saleh (1964) that the spore-forming sulfate reducers in general show greater sensitivity to these inhibitors

compared to the non-sporulating organisms.

D. desulfuricans is a mesophile that can grow at temperatures as high as 50° C under certain conditions. It is a non-sporulating curved rod that develops best at nearly neutral reactions (pH 6.5 - 8.0) and has a growth range from pH 5.5 to 9.0 (Starkey and Wight, 1945; Postgate, 1951a, 1952).

Various organic substances are used as hydrogen donors for sulfate reducing bacteria. Baars (1930) observed that sulfate reducing bacteria could oxidize many organic compounds including simple carbohydrates, organic acids, alcohols and amino acids; he noted that there were differences in the kinds of compound oxidized by different cultures. The bacteria can be cultured in media containing only a simple organic compound and minerals, but growth may be enhanced by the addition of supplements such as yeast extract, peptone, liver extract, protein hydrolysate, and other complex mixtures (Starkey, 1938; Miller, 1949; Butlin, Adams and Thomas, 1949; Postgate, 1951a,b). A typical overall reaction of D. desulfuricans is the following:



$$(1)\quad 2\ CH_3{\cdot}CH(OH){\cdot}COO^- + SO_4^{=} \longrightarrow 2\ CH_3{\cdot}COO^- + 2\ CO_2 + S^{=} + 2\ H_2O$$

It was long believed that *Desulfovibrio* could grow autotrophically, using molecular hydrogen as the hydrogen donor. In 1931, Stephenson and Stickland reported that washed cells reduce sulfate in the presence of hydrogen. Starkey and Wight (1943, 1945) reported growth of cultures on molecular cathodic hydrogen alone as a source of energy and reducing power and observed that the cultures grew in an inorganic medium containing metallic iron and mineral salts where bicarbonate was the only carbon source. Hydrogen was produced through electro-chemical reaction between the water and iron, and the hydrogen thus formed supported the development of the bacteria according to the equation:-

(2) $4\ H_2 + SO_4^{=} \longrightarrow 4\ H_2O + S^{=}$

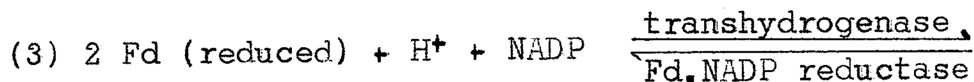
($H_2/S$ ratio = 4.0)

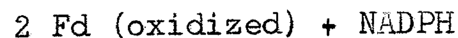
Postgate (1951b) reported that whole cells of *D. desulfuricans* rapidly reduce sulfate, sulfite and thiosulfate to sulfide in the presence of molecular hydrogen.

Ferredoxin (Mortenson *et al.*, 1962) is present in *D. desulfuricans* (Tagawa and Arnon, 1962; Akagi, 1967). Ferredoxins are relatively small, iron containing proteins. For instance, bacterial ferredoxin is a protein with a M.W. of approximately $5.5 \times 10^3$, a very low redox potential,

($E_o$ ca - 0.425 volt) and a characteristic wide absorption maximum in the region of 390 mμ. It contains 4-7 iron atoms/molecule (depending on species e.g., 7 in Clostridia), probably linked to an equal number of cysteine residues (Mahler and Cordes, 1966). Ferredoxins have shown to be present in the chloroplasts of spinach, in blue-green (Nostoc) and green algae, photosynthetic bacteria (Chromatium, Rhodospirillum rubrum) and many anaerobic bacteria. The word "ferredoxin" was first coined by D. C. Wharton of the DuPont Co., and applied to an "iron protein" obtained from Clostridium pasteurianum, a nitrogen fixing organism (Mortenson, 1962). The requirement of ferredoxin for nitrogen fixation was demonstrated in cell free extracts (freed from ferredoxin by DEAE-cellulose) of C. pasteurianum by Mortenson and co-workers (Mortenson et al., 1962; Mortenson, 1964) who showed that the pyruvate dehydrogenase of this organism generates reduced ferredoxin which serves as a source of electrons for the conversion of molecular nitrogen to ammonia. A survey of pyruvate clastic reactions in several anaerobic bacteria indicated that a ferredoxin-linked pyruvate cleavage may occur generally (Valentine, 1964). Addition of ferredoxin to extracts of C. pasteurianum, Micrococcus lactilyticus, Peptostreptococcus elsdenii, C. lactoacetrophilum, C. acidiurici, C. thermosaccarolyticum,

*Butyribacterium rettgeri*, *Diplococcus glycinophilus* and *D. desulfuricans* gave marked stimulation of pyruvate oxidation. Tagawa and Arnon (1962) showed the similarity of bacterial and spinach ferredoxin by demonstrating that *C. pasteurianum* ferredoxin could replace the "photosynthetic pyridine nucleotide reductase" (the name first given to ferredoxin previously isolated from spinach by San Pietro and Lang, 1958) in the chloroplast reactions resulting in the reduction of NADP (Fd indicates ferredoxin):-



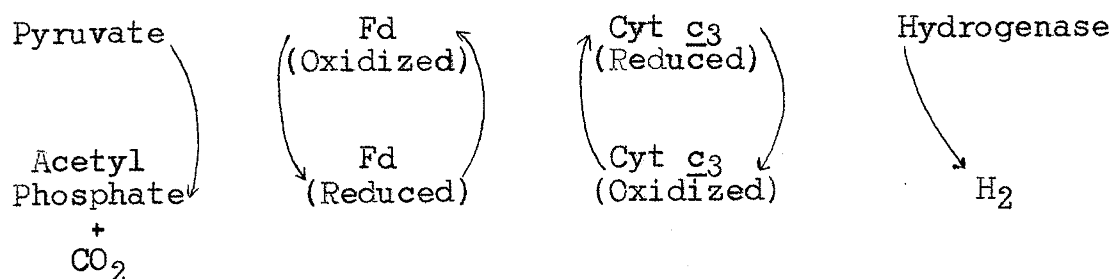


$$(3)\ 2\ \text{Fd (reduced)} + H^{+} + \text{NADP} \xrightarrow[\text{Fd.NADP reductase}]{\text{transhydrogenase}} 2\ \text{Fd (oxidized)} + \text{NADPH}$$

Another electron carrier produced by *D. desulfuricans* is cytochrome (cyt $c_3$; Postgate, 1956) which has been reported to function as an essential component in the reduction of sulfate to sulfide (Postgate, 1956; Postgate, 1961; Ishimoto, Koyama and Yagi and Shiraki, 1957; Ishimoto, Kondo, Kameyama, Yagi and Shiraki, 1958). It can donate electrons to the hydrogenase (studied and purified by Sadana and Jogannathan, 1956; and by Krasna, Riklis and Rittenberg, 1960) of *D. desulfuricans* in a hydrogen evolution assay of the enzyme pyruvic dehydrogenase (Peck and Gest, 1956) and participate as an electron carrier in the formic hydrogenlyase system of the organism (Ishimoto

et al., 1957; Williams and Peck, 1962).

A proposed sequence of electron transfer between pyruvic acid dehydrogenase and hydrogenase is shown below (Akagi, 1967):-




The two electron carriers then participating in this process are ferredoxin and cyt $c_3$. The ferredoxin accepts the electrons from the pyruvic dehydrogenase complex whereas cytochrome $c_3$ does not. Although both the electron carriers are capable of donating electrons to the hydrogenase of *D. desulfuricans*, it is believed that the sequential transfer of electrons is as shown above (Akagi, 1967).

Desulfoviridin (a soluble porphyroprotein) and cytochrome $c_3$ have been considered (Postgate, 1956, 1957) to be characteristic pigments of all strains of the sulfate-reducing bacterium *D. desulfuricans*, with the exception of the strain Norway 4, which has cyt $c_3$ but no desulfoviridin (Miller and Saleh, 1964), and which is regarded as a mutant of *D. desulfuricans* that has lost this pigment. No

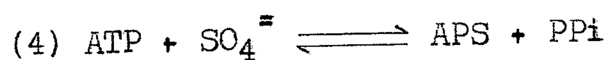
metabolic abnormality has yet been discovered in this strain that might reveal the function of desulfoviridin, which has a strong absorption band at about 630 mμ. It undergoes no oxido-reduction reactions, and decomposes readily with acids and alkalis or heat to yield, not a haem pigment, but a free porphyrin (Postgate, 1961).

Even though sulfate reducing bacteria were widely believed to be facultative autotrophs, the work of Mechalas and Rittenberg (1960) and Postgate (1960) throws some doubt on their truly autotrophic status. The incorporation of labelled $CO_2$ during autotrophic growth never exceeded 25% of the cell carbon, indicating that organic carbon compounds were available to the cell probably from organic detritus not excluded by normal bacteriological sterile techniques.

It was originally demonstrated by Bernstein and McGilvery (1952) and DeMeio et al. (1952, 1953), working with a system from liver, that sulfate must be "activated" by ATP before further participating in enzymic reactions. The actual mechanism of this activation was finally clarified by Robbins and Lipmann (1956a,b, 1957) and Bandurski et al. (1956) who showed that a two step reaction is involved. Two sulfur containing nucleotides, adenosine-5'-phosphosulfate (APS) and 3'-phosphoadenosine-5'-phosphosulfate (PAPS) have been established as the activated

forms of sulfate in biological systems (Robbins and Lipmann, 1956a,b, 1957; Wilson and Bandurski, 1958).

The initial reaction requires ATP for the primary activation of the sulfate by the enzyme ATP-sulfurylase according to the following:-

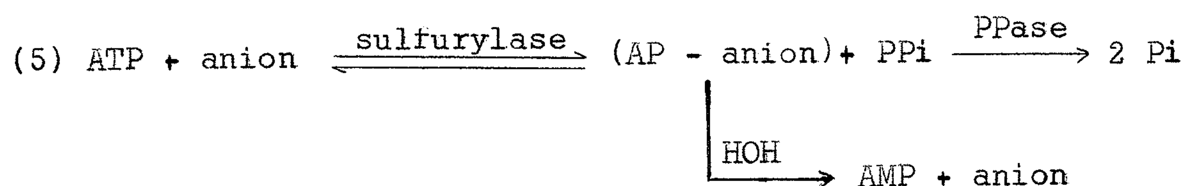


(4) $ATP + SO_4^{=} \rightleftharpoons APS + PPi$

The enzyme requires $Mg^{++}$ for activity and has been characterized and purified from Bakers yeast (Robbins and Lipmann, 1958; Wilson and Bandurski, 1958), from _D. desulfuricans_ (Baliga, Vartak and Jagannathan, 1961; Akagi and Campbell, 1962) and from _Clostridium nigrificans_ (Akagi and Campbell, 1962). The equilibrium of this reaction lies far to the left, i.e., towards the formation of ATP and sulfate ($K = 10^{-8}$, pH 8.0, 37° C), therefore to observe a net formation of APS pyrophosphatase is required to remove the PPi and thus to drive the reaction to the right.

Postgate (1949) demonstrated that the reduction of sulfate by whole cells of _Desulfovibrio_ spp. was competitively inhibited by selenate while the reduction of sulfite or thiosulfate was unaffected. Ishimoto, Koyama and Omura (1954) found that sulfate reduction in whole cells was also inhibited by molybdate while sulfite reduction was again unaffected. This inhibition of sulfate-, but not

sulfite-, reduction by $SeO_4^=$ and $MoO_4^=$ suggested that the enzyme ATP-sulfurylase was involved in the reduction of sulfate by D. desulfuricans, especially when Wilson and Bandurski (1958) reported that the same enzyme isolated from yeast also catalyzed a rapid release of phosphate from ATP in the presence of group VI anions and pyrophosphatase (PPase), according to the following reactions:-




When selenate was used as the anion, the formation of phosphoselenate was demonstrated by the incorporation of $P^{32}$ into ATP from labelled PPi; however when chromate, molybdate or tungstate was used no "AP - anion" could be shown, indicating that these anions form very unstable intermediate complexes. Peck (1959) showed the stimulatory effect of molybdate, tungstate and chromate on the release of inorganic phosphate from ATP in cell-free extracts of D. desulfuricans. Further experiments showed that if ATP was omitted from the reaction mixture there was no liberation of phosphate. When extracts from this organism were supplemented with ATP and sulfate, hydrogen was utilized; but if either ATP or sulfate was omitted there

was no measurable hydrogen uptake. Ishimoto (1959) showed that extracts of Desulfovibrio incubated in the presence of ATP and $S^{35}O_4^=$ formed radioactive acid-volatile sulfur (sulfite or sulfide).

In yeast, and in certain mammalian cells (Wilson and Bandurski, 1956, 1958; Lipmann, 1958; Robbins and Lipmann, 1958a,b), APS is further phosphorylated in the 3'-position of the ribose moiety by ATP to form adenosine-3'-phosphate-5'phosphosulfate (PAPS) and ADP (Robbins and Lipmann, 1958a,b):

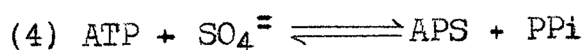
(6) APS + ATP $\rightleftharpoons$ PAPS + ADP

The enzyme responsible for this phosphorylation has been termed APS kinase. The product of the reaction, PAPS is the form of "active sulfate" in mammalian tissue and yeast, and in the presence of the enzymes termed "sulfokinases" can transfer sulfate to phenols, lipids and carbohydrates (Lipmann, 1958).

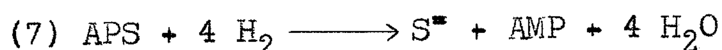
The formation of PAPS has been observed in extracts of Escherichia coli and other bacteria (Hilz, Kittler and Knape, 1959; Peck, 1962) and many fungi (DeMeio and Wizerkaniuk, 1956; Hilz and Lipmann, 1955; Spencer and Harada, 1960). The presence of the choline ester of sulfuric acid in fungi has been known for a number of years

(Woolley and Peterson, 1937), and Kaji and Gregory (1959) have shown that the sulfate donor for the synthesis of sulfacoline is PAPS, probably due to the action of a specific sulfokinase (Spencer and Harada, 1960); evidence for the occurrence of sulfated compounds in bacteria seems however to be scanty, although a sulfate-containing polysaccharide has been isolated and characterized (Taylor and Novelli, 1961) from a soil diphtheroid.

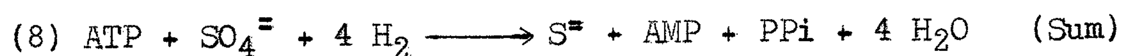
The reduction of sulfate to sulfide in the presence of hydrogen by D. desulfuricans proceeds through APS as the intermediate (Peck, 1959):



(4) $ATP + SO_4^{=} \rightleftharpoons APS + PPi$



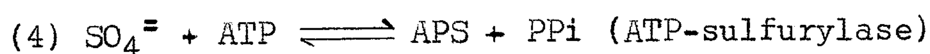
(7) $APS + 4\ H_2 \longrightarrow S^{=} + AMP + 4\ H_2O$



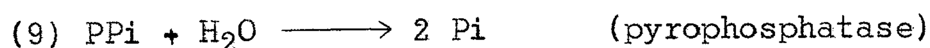
(8) $ATP + SO_4^{=} + 4\ H_2 \longrightarrow S^{=} + AMP + PPi + 4\ H_2O$ (Sum)

APS is the form of sulfate that is reduced by D. desulfuricans (Peck, 1959) rather than PAPS (the form that is reduced in assimilatory sulfate reducers). The formation of one single labelled nucleotide when cell free extracts were incubated in the presence of ATP and $S^{35}O_4^{=}$ has been reported (Peck, 1959, 1962). Peck (1961) also showed that an APS-reductase was present in a Thiobacillus species and

that it is characteristic of organisms whose metabolism is based on a gross turnover of sulfur. The reduction of APS is "pulled" in sulfate-reducing bacteria by a pyrophosphatase (Peck, 1959) which has also been extracted and purified (Ishimoto and Fujmoto, 1961; Akagi and Campbell, 1963). Thus, the initial steps of sulfate reduction in D. desulfuricans can be expressed by the following equations:



(4) $SO_4^{=} + ATP \rightleftharpoons APS + PPi$ (ATP-sulfurylase)



(9) $PPi + H_2O \longrightarrow 2\ Pi$ (pyrophosphatase)

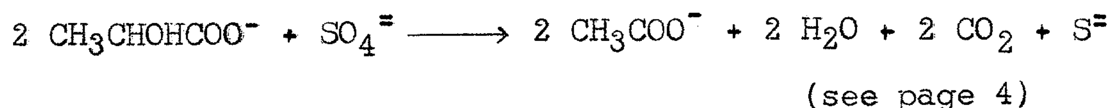
(10) $APS + 2\ e^- \rightleftharpoons AMP + SO_3^{=}$ (APS reductase)

The sulfate-reducing bacterium D. desulfuricans is commonly found in the rumen of sheep, in soils, waters, industrial effluents and sewage. In fact, it can be detected (Postgate, 1961) in almost any aqueous environment, although it multiplies most rapidly when the oxygen tension is very low and the activities of other microorganisms have reduced the Eh to about -0.1 V. It is of considerable importance in the formation of sulfur and certain mineral deposits, and causes much concern in pollution (poisonous odour), oil technology (mercaptan formation during oil cracking) and in corrosion (Postgate, 1959).

Von Wolzogen Kuhr in Holland was the first to report on anaerobic bacterial activity as a factor in underground corrosion (1923, 1934, 1937). Romanoff (1945) found that the pH of a poorly aerated sulfide-containing soil could be considerably decreased by oxidation of sulfur compounds while Beckwith (1941) reported that some aerobic bacteria may cause or accelerate corrosion.

The problems of corrosion arising from the activities of D. desulfuricans result primarily from its ability to reduce sulfate to sulfide. There are many reactions by which $SO_4^{=}$ can be reduced to sulfide. Three such examples are:

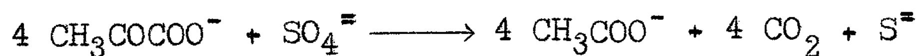
(1) Lactate as electron donor,



$$2\ CH_3CHOHCOO^- + SO_4^{=} \longrightarrow 2\ CH_3COO^- + 2\ H_2O + 2\ CO_2 + S^{=}$$

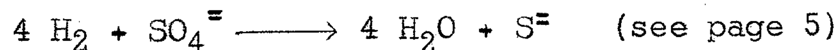
(see page 4)

(11) Pyruvate as electron donor,



$$4\ CH_3COCOO^- + SO_4^{=} \longrightarrow 4\ CH_3COO^- + 4\ CO_2 + S^{=}$$

(2) Hydrogen as electron donor,

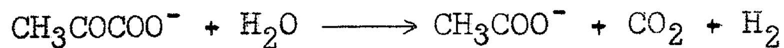


$$4\ H_2 + SO_4^{=} \longrightarrow 4\ H_2O + S^{=}$$

(see page 5)

Both reactions (1 and 11) are analogous. If sulfate is absent from the system pyruvate dismutation can take place

to yield gaseous hydrogen:-



$$CH_3COCOO^- + H_2O \longrightarrow CH_3COO^- + CO_2 + H_2$$

For sulfate reduction to take place, certain conditions must be fulfilled:

1. Organic matter must be present to provide electrons for this reduction.
2. Oxygen or nitrates must be absent because these would be used preferentially to $SO_4^=$ as electron acceptors.
3. Sulfates must be present as a source of sulfur to accept electrons from the electron donors.
4. Temperature must be favorable because all bacteria grow only within an optimal temperature range.

The first step in sulfate reduction is the activation of sulfate by the enzyme ATP-sulfurylase (p. 9) which is inhibited by group VI anions (p. 10). Zeal (1966) isolated and partially purified D. desulfuricans, and showed that it was inhibited by sodium molybdate. An extension of this work by the present author resulted in experiments on the addition of molybdate to septic tanks in various sites in the Province of Manitoba. It was found (unpublished results) that the levels of sulfide production in molybdate treated tanks did decrease somewhat, but only for a period of three to four weeks. These results indicate

that the molybdate in the tank either was washed away in the effluent or the process of inhibition was only transitory. It was then decided to work at the molecular level on the enzymes concerned with sulfate reduction in Desulfovibrio spp; this decision led to the purification of the enzyme ATP-sulfurylase in an attempt to find the affinity of the enzyme for $MoO_4^=$, ATP and $SO_4^=$ (an affinity expressed, in terms of kinetic parameters, as a Km value), and perhaps to elucidate the enzyme mechanism, or possible mechanisms, applicable to the systems studied. The enzyme ATP-sulfurylase is the first enzyme (and probably the key enzyme) involved in the reduction of sulfate to sulfide. Although a great deal of work has been carried out on this enzyme, with $MoO_4^=$ as a substrate, there appears to be no sound quantitative data available on the actual reactions of the enzyme with $SO_4^=$. If these data were available, they might well shed light on the mechanism whereby $MoO_4^=$ "stimulates" ATP-sulfurylase activity yet at the same time inhibits $H_2S$ production in natural habitats, e.g., in septic tanks. The work reported in this thesis is an attempt to provide such data.

# METHODS AND MATERIALS

## Culture

The organism D. desulfuricans obtained from American Type Culture Collection (ATCC 7757) was used in this investigation; it displays a characteristic ability to produce hydrogen sulfide from sulfate as reported in Bergey's Manual of Determinative Bacteriology (7th Edition).

## Liquid Medium

A modification of the medium recommended by Postgate (1963 - medium #3) for the isolation and enumeration of sulfate-reducing bacteria was used to grow these organisms and had the following compositions:-

| | Postgate's medium | Modified medium |
|---|---|---|
| $KH_2PO_4$ | 0.5 g | 0.5 g |
| $NH_4Cl$ | 1.0 g | 1.0 g |
| $Na_2SO_4$ | 1.0 g | 2.5 g |
| $CaCl_2 \cdot 2H_2O$ | - | 0.1 g |
| $CaCl_2 \cdot 6H_2O$ | 1.0 g | - |
| $MgSO_4 \cdot 7H_2O$ | 2.0 g | 0.5 g |

| | Postgate's medium | Modified medium |
|---|---|---|
| Na lactate | 3.5 g | 6.0 g |
| $FeSO_4 \cdot 7H_2O$ | 0.5 g | 0.02 g |
| Yeast extract | 1.0 g | 1.0 g |
| Peptone | - | 1.0 g |
| Ascorbic acid | 0.1 g | 0.2 g |
| Thioglycollic acid | 0.1 g | 0.2 g |
| Agar | 15.0 g | - |

Distilled water to a total of 1000 ml

(pH adjusted to 7.6)

Maintenance, Inoculation, and Bulk Cultivation of Sulfate-Reducing Organisms

*D. desulfuricans* stock cultures were maintained in the modified medium (plus trace amounts of agar to make the medium slightly viscous thus promoting anaerobiosis) contained in screw-capped tubes. The tubes were autoclaved for twenty minutes at 15 $lb/in^2$ and the caps immediately tightened after removal from the autoclave. The tubes were inoculated upon cooling and incubated at 30° C for 72 hours before being used as inocula for the 1 liter cultures described below.

Two 1 liter Erlenmeyer flasks each containing 1000

ml of the liquid medium and fitted with glass and rubber tubing inserted in rubber stoppers to facilitate flushing with hydrogen after autoclaving were used to serve as inocula for two 20 liter carboys. The flasks were autoclaved at 15 lb/in$^2$ for a full 20 minutes after peak pressure had been attained, and upon removal, sealed with the rubber stoppers, cooled, aseptically flushed with hydrogen gas, inoculated with 5.0 ml of the semi-solid medium, again flushed with hydrogen gas, resealed and incubated at 30$^{\circ}$ C for 72 hours.

The treatment of the carboys was the same as above, except that the autoclaving time was extended to 1 hour at 15 lb/in$^2$.

Cell Preparation

Cells cultured in the final medium were harvested on a Sharples centrifuge and washed three times with 0.1 M Tris-HCl buffer (pH 8.0).

Extract Preparation

Cell free extracts were obtained by sonication of a 30% wet weight by volume cell suspension for 45 minutes in a Raytheon Sonic oscillator in 0.1 M Tris-HCl buffer (pH 8.0) under a nitrogen atmosphere. The cell debris was

removed by centrifugation at 27,000 x g for 30 minutes.

Special Reagents

The following chemicals were obtained from commercial sources:

Adenosine-5'-triphosphate, disodium - Sigma Chemical Company, St. Louis, Mo.

The purified frozen aqueous inorganic pyrophosphatase (Sigma Chemical Company, St. Louis, Mo) contained 600 units of enzyme/vial (Ca 0.5 cc), a unit defined as that amount which will liberate one µmole of orthophosphate from pyrophosphate per min at pH 7.2, 25$^{\circ}$ C.

The EDTA was obtained from Sigma Chemical Company.

Triethylaminoethyl cellulose (TEAE) with an exchange capacity of 0.89 meq/g was obtained from Sigma Chemical Company. It was suspended in 100 g quantities in 4 liters of 0.1 NaOH and stirred for 1 hour. After a settling time of 1 hour, the alkali was decanted and the procedure repeated once. Distilled water (4 liters) was added and the suspension stirred for 20 minutes and allowed to settle for 30 minutes. This procedure was repeated twice. The pH was then adjusted to 8.0 by the addition of 6 N HCl and the suspension washed twice with distilled water with 30 minute settling periods. Two liters of 0.5 M KCl - 0.05 M Tris

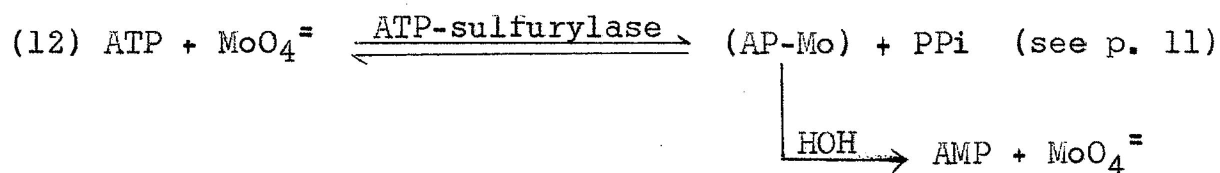
buffer (pH 8.0) were added and the slurry was stirred for 4 hours. The suspension was then washed three times with 4 liters of distilled water (with 30 minute settling periods) and stored in water at $4^{\circ}$ C until used.

Sulfuric acid ($H_2S^{35}O_4$) was obtained from the Radiochemical Center, Amersham, Bucks., England and neutralized by the addition of NaOH before used.

APS (used as a standard control nucleotide for Fig. 10) was kindly supplied as a gift from I. Suzuki, Dept. of Microbiology, University of Manitoba, Canada.

Enzyme Assay

Routine assays were carried out by the molybdate method described by Bandurski, Wilson and Squires (1956), which depends on the following reactions:



The reaction mixture contained ATP, 5 µmoles; $MgCl_2$, 5 µmoles; $Na_2MoO_4$, 10 µmoles; Tris buffer (pH 8.0), 50 µmoles; purified yeast inorganic pyrophosphatase and enzyme, in a total volume of 1.0 ml. The incubation time was 10 minutes at

$30^{o}$ C. The reaction was stopped by the addition of 8.0 ml of 10% TCA and the necessary reagents were added for the determination of inorganic phosphate according to the method of Fiske and SubbaRow (1925). Control experiments were carried out in the absence of $NaMoO_4$ or enzyme (enzyme replaced by boiled enzyme).

## Definition of Unit and Specific Activity

One unit of enzyme is defined as that amount which produces 1 µmole of inorganic phosphate per hour under the conditions specified (pH 8.0, $30^{o}$ C). Specific activity is expressed as the number of units per mg of protein. Protein was measured by the method of Lowry *et al.* (1951).

## APS Formation

The $APS^{35}$ formed from $S^{35}O_4^{=}$ ($5.0 \times 10^6$ cpm/µmole $SO_4^{=}$) was isolated by paper electrophoresis (Fig. 10) on Whatman 1 MM paper in 0.03 M citrate buffer (pH 5.5), with a Beckman model R paper electrophoresis system operating at 15 V/cm for 4 hours at $4^{o}$ C. The nucleotide $APS^{35}$ was detected with ultraviolet light and eluted from the paper with water and counted for radioactivity in a Packard Tri-Carb liquid scintillation spectrometer.

Enzyme Purification Procedure

This was a modification of that used by Akagi and Campbell (1962). After the cell debris was removed by centrifuging at 27,000 x g for 30 minutes the crude extract was centrifuged at 105,000 x g for 2 hours and the supernatant fluid decanted from the black pellet. Solid $(NH_4)_2SO_4$ was slowly added with gentle stirring to the supernatant liquid to a saturation of 45%. After standing for one hour at 0° C, the mixture was centrifuged at 27,000 x g for 30 minutes. The precipitate was discarded, and additional $(NH_4)_2SO_4$ was added (with stirring) until a 65% saturation level was obtained. After a settling period of 30 minutes at 0° C, the mixture was centrifuged as above. The precipitate was dissolved in a minimal amount of 0.1 M Tris buffer (pH 8.0) and dialyzed for 3 hours against 200 volumes of 0.025 M tris-0.001 M EDTA buffer (pH 8.0). The dialyzed enzyme was applied to a TEAE column (1.8 x 20 cm) and eluted with 0.1 M KCl-0.05 M Tris buffer (pH 8.0). The tubes (25-35) containing sulfurylase (Fig. 1) were pooled and 1 mg of $C_\gamma$ alumina gel was added per mg of protein. The alumina-enzyme mixture was dialyzed for 3 hours at 0° C against 50 volumes of 0.025 M tris-0.001 M EDTA buffer (pH 7.0). The sulfurylase

was eluted from the gel by successive treatment with small portions of 0.01 M ATP in 0.1 M Tris buffer (pH 7.0).

Disc Electrophoresis

Acrylamide gel electrophoresis of the alumina C$\gamma$ gel eluate was performed as described by Davis (1964) using the Canalco-Disc Electrophoresis Trial Kit supplied by Canal Industrial Corporation, Bethesda, Maryland. Samples (150 μg protein) mixed with equal volumes of upper gel in a total volume of 0.3 ml, were layered on the spacer gel. Electrophoresis was carried out for 60 minutes at 25 V/cm. After the run, the gel columns (0.5 x 6 cm) were removed, stained in acidified aniline black, electrophoretically destained with acetic acid, and then photographed.

RESULTS

## Purification of Enzyme

A 26-fold purification of ATP-sulfurylase was obtained with a combination of $(NH_4)_2SO_4$ precipitation, TEAE chromatography and alumina $C_\gamma$ adsorption. The eluates from the alumina gel were stable for several months when stored at $-20^{\circ}$ C under a hydrogen atmosphere. The purity of the enzyme was tested by disc electrophoresis on polyacrylamide gel; one major and two minor bands were observed at distances 2.5, 1.5 and 3.4 cms respectively behind the tracking band (an albumen dye complex; Fig. 2). Table 1 presents the purification steps of ATP-sulfurylase from *D. desulfuricans*.

## Activity as a Function of Time

In a study of the relation between enzyme activity and incubation time, a straight line plot was obtained (Fig. 3).

## Activity as a Function of Enzyme Concentration

A straight line relation was observed (Fig. 4).

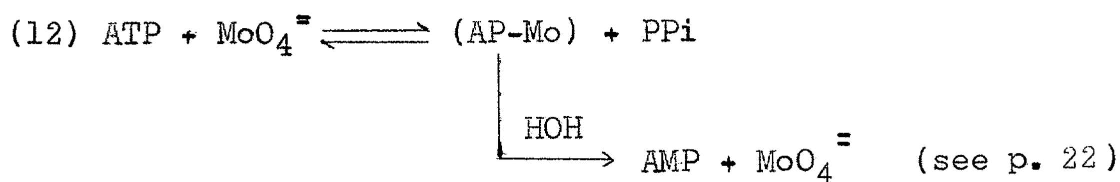
Effect of pH on ATP-Sulfurylase Activity

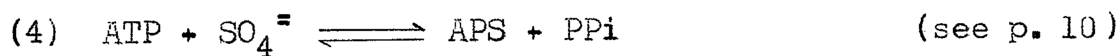
The pH optimum of the enzyme was determined in a series of maleate (pH 5-6.5), Tris-HCl (pH 7-9) and glycine (pH 9.5-10.5) buffers. The optimum pH for the enzyme as shown in Fig. 5 was approximately 8.7.

Kinetics of ATP-Sulfurylase

The initial velocity data were plotted in the double reciprocal form (1/v versus 1/s) according to Lineweaver and Burk (1934) where v is the initial velocity and s is the variable substrate concentration. The nomenclature of reaction mechanisms and definitions of kinetic constants were the same as those proposed by Cleland (1963a,b,c).

The enzyme ATP-sulfurylase apparently catalyses two reactions:

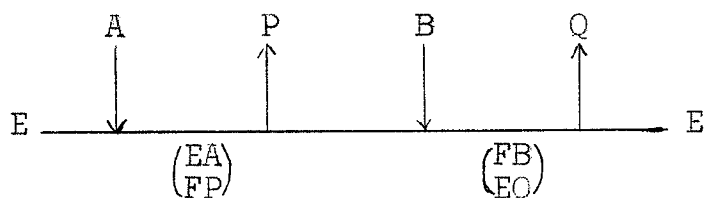


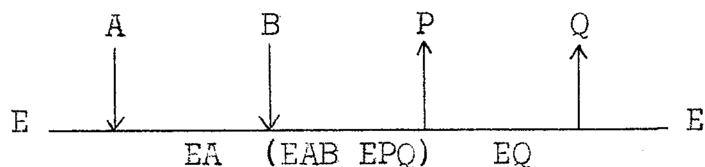

If we adopt the nomenclature of Cleland (1963a) the above equations can be written as:- $A + B \rightleftharpoons P + Q$

where A and B are the two substrates and P and Q are the two products. The second reaction (4) is inhibited by molybdate which is used as the substrate in the first reaction (used in all routine assays).

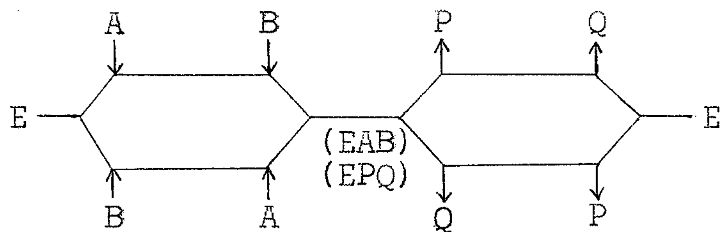
The above reactions are of Binary-Binary type of Bi Bi type (2 substrates, 2 products) and can, at first glance, be explained either by a ping pong Bi Bi mechanism,




or, by a sequential ordered,




or a rapid equilibrium random Bi Bi mechanism,



Cleland (1963a) derived the following steady state rate equation for a Ping Pong Bi Bi reaction mechanism when P and Q are zero:-

$$v = \frac{V_1 AB}{K_b A + K_a B + AB}$$

which in the double reciprocal form becomes

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1}\left(\frac{1}{A}\right) + \frac{K_b}{V_1}\left(\frac{1}{B}\right)$$

where A and B are the concentrations of substrates, $K_a$ and $K_b$ the Michaelis constants for A and B respectively, v = the initial velocity of reaction and $V_1$ = the maximum velocity of reaction.

For a sequential ordered, or rapid equilibrium random Bi Bi reaction mechanism (when P and Q are again zero) the steady state rate equation is:

$$v = \frac{V_1 AB}{K_{ia}K_b + K_b A + K_a B + AB}$$

where the additional parameter, $K_{ia}$ is the dissociation constant for the enzyme -A complex. The reciprocal of the last equation given is transformed to:

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1}\left(\frac{1}{A}\right) + \frac{K_b}{V_1}\left(\frac{1}{B}\right) \frac{K_{ia}K_b}{V_1}\left(\frac{1}{AB}\right)$$

The $K_m$ values were determined by measuring the initial velocities of reactions when one substrate was varied and the second substrate held at several fixed concentrations. Fig. 6 (where $MoO_4^=$ was the varying substrate and ATP the fixed variable) shows an intersecting pattern of lines where the point of intersection at the x-axis (apparent $K_m$) is unaffected by the ATP concentrations used. The results from this study then give a $K_m$ value = $1.2 \times 10^{-3}$ M for $MoO_4^=$. A replot of the intercepts versus the reciprocal of ATP concentrations of the lines from Fig. 6 yields two straight lines (Fig. 7a) that intersect at the x-axis at two points yielding two $K_m$ values for ATP = $3.5 \times 10^{-4}$ M and $1.43 \times 10^{-3}$ M (a replot of slopes versus the reciprocal of ATP concentrations from Fig. 6 yields a line curved downward towards the origin shown in Fig. 7b). The same two $K_m$ values for ATP can also be obtained from Fig. 8 (where ATP is the varying substrate and $MoO_4^=$, the fixed variable) which substantiates those taken from Fig. 7a. The replot of the intercepts from Fig. 8 versus $MoO_4^=$ concentrations is shown in Fig. 9 and two straight lines intersecting at the x-axis are observed, the point

of intersection being equal to the $K_m$ value previously assigned to $MoO_4^=$ ($1.2 \times 10^{-3}$ M) from Fig. 6.

The formation of a single labelled nucleotide when cell-free extracts of D. desulfuricans are incubated with ATP and $S^{35}O_4^=$ has been reported (Peck, 1959, 1962). A nucleotide was isolated (p. 44) from the present culture that had the same electrophoretic mobility as APS, moved faster than ADP but more slowly than ATP. Fig. 10 shows the effects of various treatments on the formation of this labelled nucleotide. Heating the extract for 90 minutes in a boiling water bath completely destroyed the ability of the enzyme to form the nucleotide. ATP and $SO_4^=$ both were required for its formation, and the presence of $MoO_4^=$ inhibited its formation.

The formation of $APS^{35}$ in the absence and presence of $MoO_4^=$ when $S^{35}O_4$ was the variable substrate and ATP the fixed substrate was followed and used as a criterion for the measure of the activity of the enzyme ATP-sulfurylase. Non-linear plots were obtained in the absence, and in the presence of lower concentrations ($5.0 \times 10^{-5}$ M) of $MoO_4^=$ but higher concentrations of $MoO_4^=$ ($1.0 \times 10^{-4}$) completely eliminated this non-linear effect and the plot becomes linear. If one assumes that the line obtained, when $MoO_4^=$

was absent from the system is straight in the region of low $S^{35}O_4^{=}$ concentration, an extrapolation of this line toward the x-axis (dotted line) yields a $K_m$ value for $SO_4^{=}$ = $2.5 \times 10^{-3}$ M. If linearity is also assumed when the $SO_4^{=}$ concentration is raised towards infinity (near the x-axis) a second $K_m$ value for $SO_4^{=}$ would be obtained (intersection of this segment at the x-axis) and would be in the region of $10^{-2}$ M. However, if non-linearity is assumed, the latter $K_m$ assigned for $SO_4^{=}$ ($10^{-2}$ M) would not be valid. Nevertheless, the results appear to indicate the existence of more than one binding site for $SO_4^{=}$ on the enzyme surface. The intersection of the three lines on the y-axis suggests that the inhibition observed is competitive.

Fig. 12 shows the replot of slope (at high $SO_4^{=}$ concentrations) versus $MoO_4^{=}$ concentrations from Fig. 11 in which $SO_4^{=}$ was the varying substrate and $MoO_4^{=}$ the inhibitor. The line obtained is curved concave upward which indicates that there is also more than one binding site for $MoO_4^{=}$ as well as for $SO_4^{=}$ on the enzyme surface.

Fig. 13 shows the plots of velocity (in cpm) versus varying $MoO_4^{=}$ concentrations in the presence of a fixed concentration of ATP, and $S^{35}O_4$ ($4.0 \times 10^{-4}$ M). Low concentrations of $MoO_4^{=}$ appear to activate the enzyme slightly, but as the inhibitor concentrations of $MoO_4^{=}$ are

raised, the inhibition of $APS^{35}$ formation is apparent. The curve seen in Fig. 13 indicates also that Fig. 12 should probably be drawn toward the origin as shown by the dotted line.

Table I.

Purification of ATP-sulfurylase from *Desulfovibrio desulfuricans*

| Fraction | Volume (ml) | Total Protein (mg) | Total Units | Specific Activity (units/mg protein) | Yield % | Purity |
|---|---|---|---|---|---|---|
| 1. 105,000 x g supernatant | 37.5 | 1200 | 82,500 | 68.5 | 100 | 1.00 |
| 2. 45-65% $(NH_4)_2SO_4$ | 7.0 | 151.2 | 108,000 | 716.0 | 103.1 | 10.40 |
| 3. Dialysis | 10.0 | 272.0 | 79,200 | 230 | 96 | 3.36 |
| 4. TEAE eluate | 54 | 42.3 | 36,072 | 850 | 43.8 | 12.4 |
| 5. Alumina eluate | 13.9 | 10.92 | 19,682 | 1800 | 23.8 | 26.2 |

Fig. 1. Chromatographic purification of ATP-sulfurylase of _D. desulfuricans_ on TEAE.

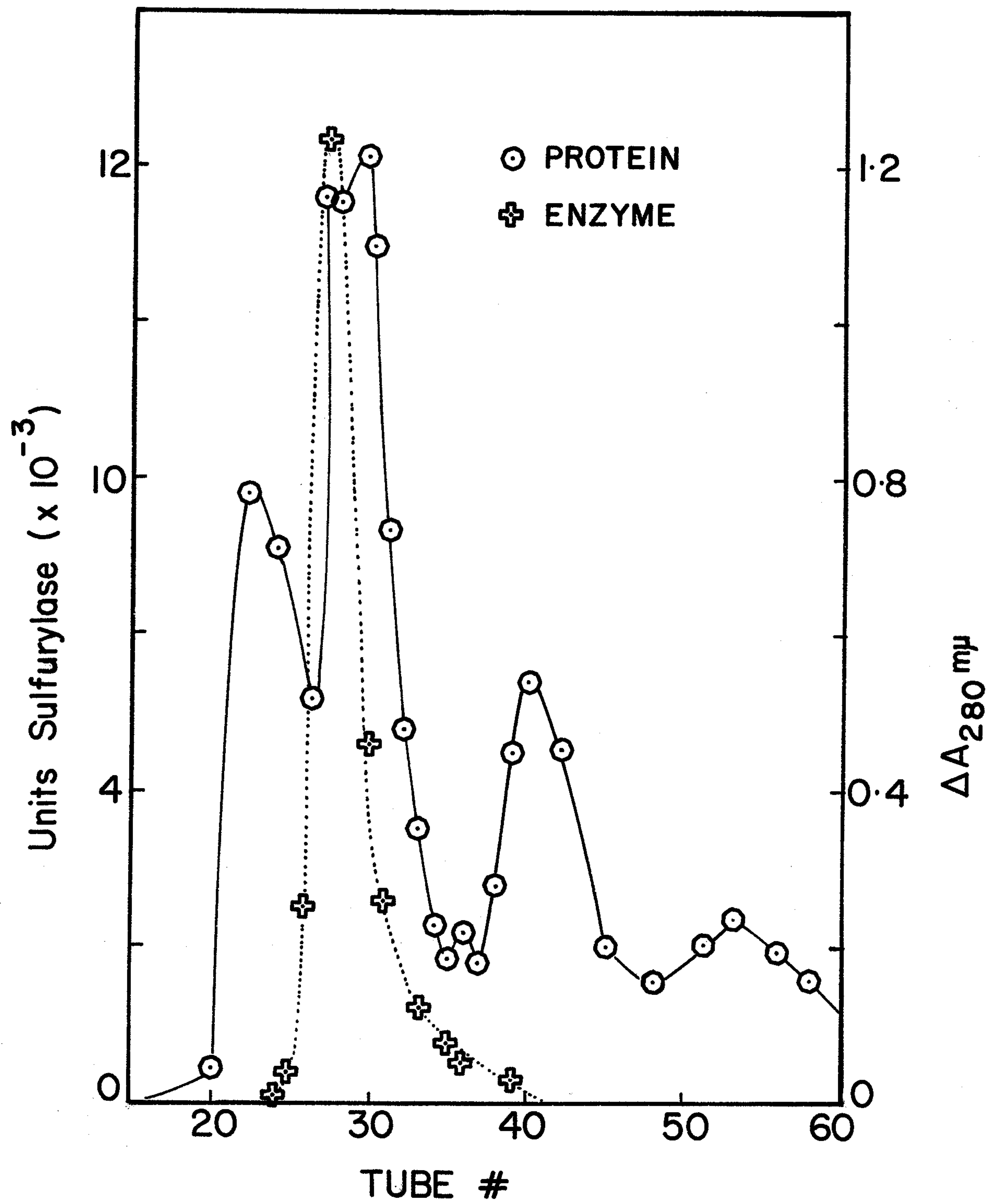

PROTEIN
ENZYME
Units Sulfurylase ( x 10$^{-3}$)
$\Delta A_{280}$ mμ
12
10
4
0
1·2
0·8
0·4
0
20
30
40
50
60
TUBE #

Fig. 2. Disc electrophoresis of ATP-sulfurylase. Disc electrophoresis was carried out as described in Methods and Materials. The dark band at the bottom of the tube is the tracking dye.

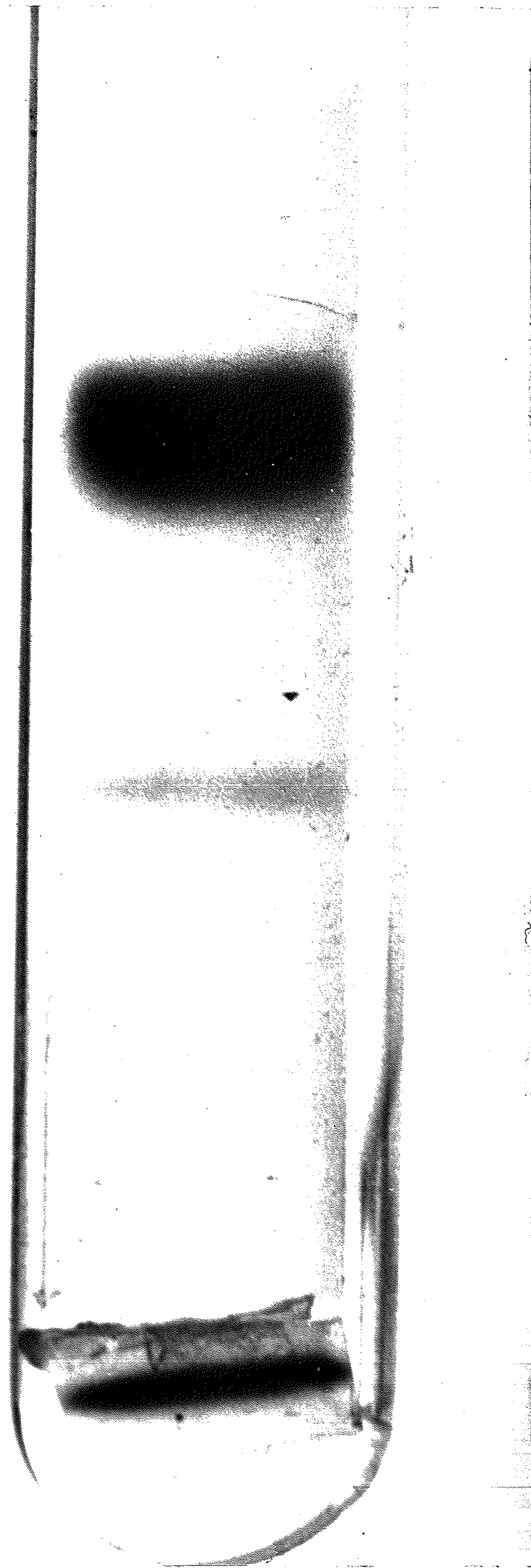


Fig. 3. Time course of ATP-sulfurylase reaction.

Each tube contained:

ATP - $5.0 \times 10^{-3}$ M

$Na_2MoO_4$ - $1.0 \times 10^{-2}$ M

$MgCl_2$ - $5.0 \times 10^{-3}$ M

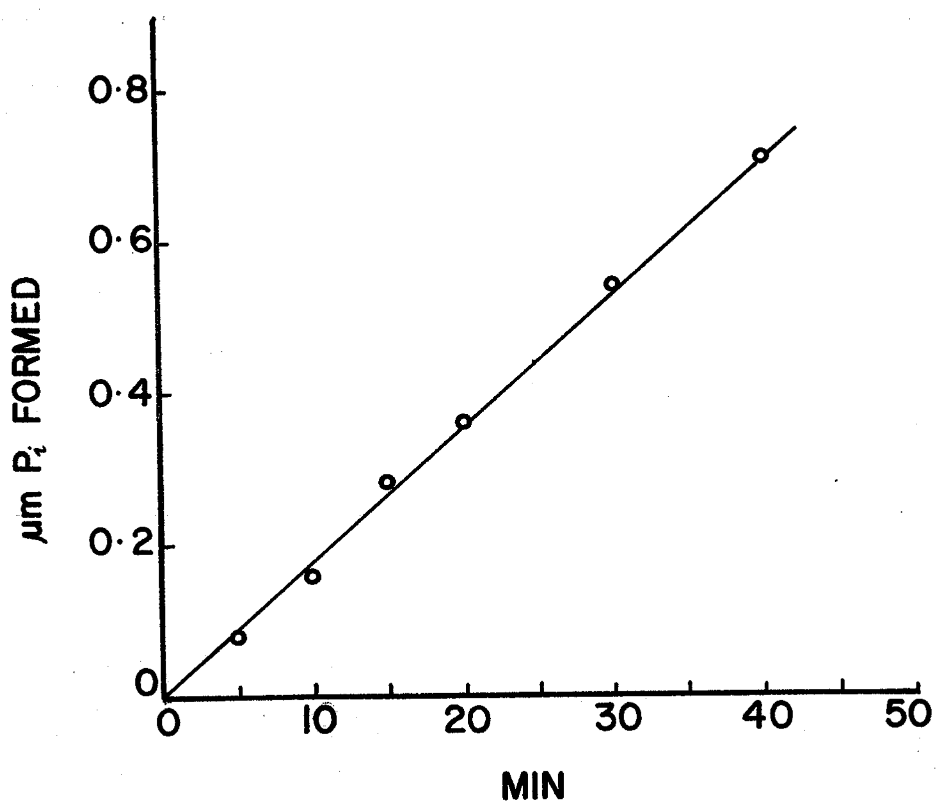
Tris-HCl (pH 8.0) - $5.0 \times 10^{-2}$ M

Pyrophosphatase - 4 mg

Enzyme - 2 mg

$H_2O$ - (to a total volume of 1.0 ml)

The tubes were incubated at 30° C for the times shown, the reactions stopped by the addition of 8.0 ml of 10% TCA, and the amount of inorganic phosphate formed determined by the method indicated in Methods and Materials by observing the color formation in a Klett-Summerson Photoelectric colorimeter with a 66 KS filter.



µm Pi FORMED
0·8
0·6
0·4
0·2
0
0
10
20
30
40
50
MIN

Fig. 4. Activity as a function of ATP-sulfurylase concentration (for experimental conditions, see Fig. 3).

The incubation time was 15 min at 30° C.

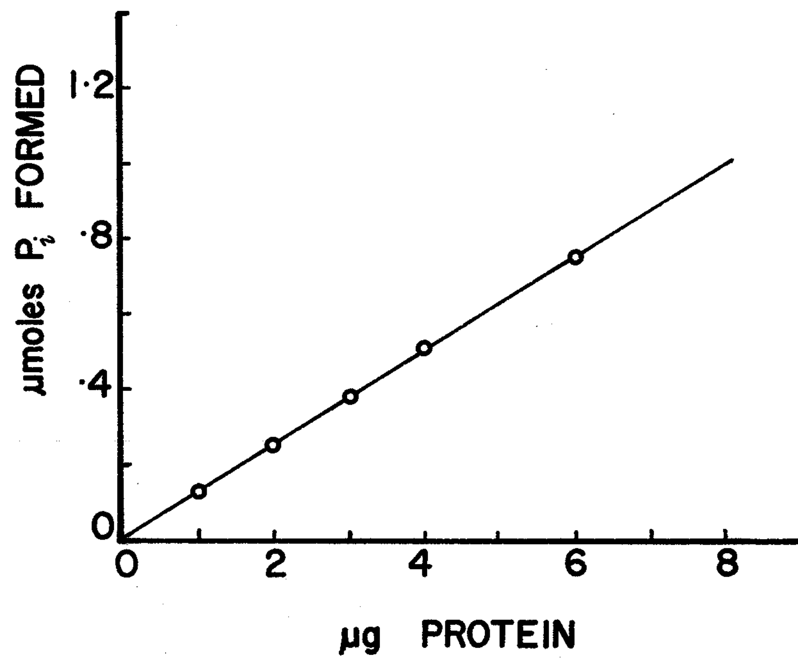
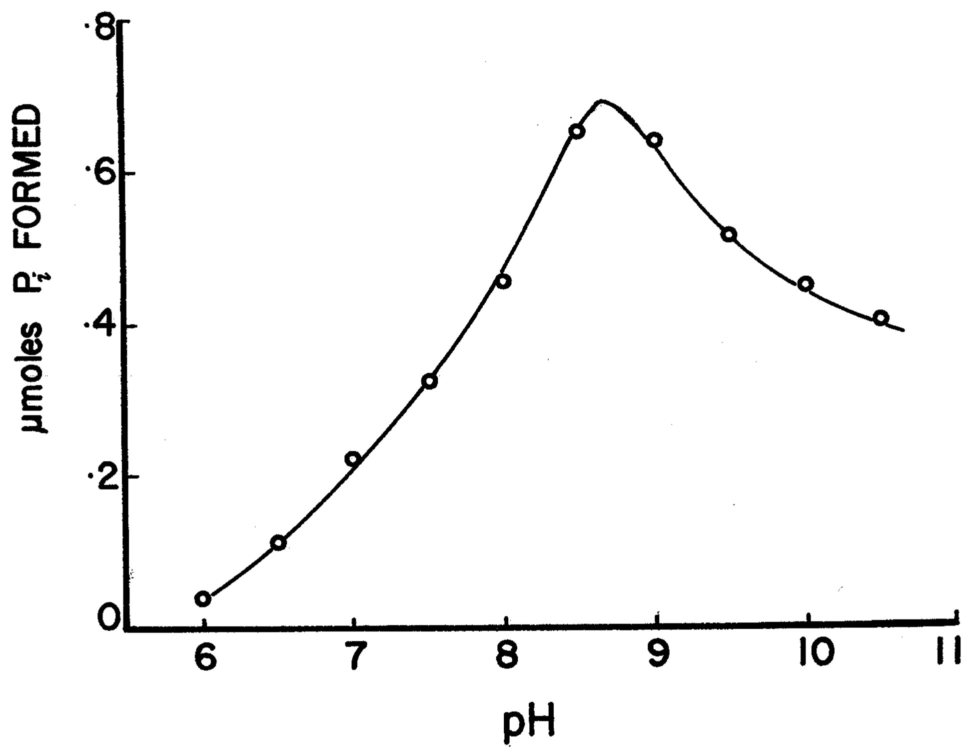

µmoles $P_i$ FORMED
1·2
·8
·4
0
0
2
4
6
8
µg PROTEIN

Fig. 5. Effect of pH on ATP-sulfurylase activity. The experimental conditions were the same as those outlined in Fig. 3 except that pyrophosphatase was not initially present in the reaction mixture and the buffer concentration was increased to $10^{-1}$ M.

The incubation time was 15 min at $30^{\circ}$ C. At the end of the reaction time 1.0 ml of 2 M Tris-HCl buffer (pH 7.2) was added to each tube, and the tubes immediately immersed in a boiling water bath for 90 sec. The tubes were then cooled in ice, pyrophosphatase (4.0 µg) added, and incubated again for 30 min at $30^{\circ}$ C and subsequently analyzed for inorganic phosphate as described earlier, except that the color formation was observed in a Unicam SP 700 Recording Spectrophotometer at 740 mµ in a 4 cm light-path cuvette.



·8
·6
·4
·2
0
6
7
8
9
10
11
pH
µmoles Pi FORMED

Fig. 6. Double reciprocal plots of velocity versus varying $Na_2MoO_4$ concentrations with ATP as the fixed variable.

The reaction mixture contained:

ATP (1) 0.25 mM (2) 0.5 mM (3) 0.75 mM (4) 1.0 mM (5) 1.25 mM (6) 1.5 mM

$Na_2MoO_4$ varied as indicated.

$MgCl_2$ - $5.0 \times 10^{-3}$ M

Tris-HCl (pH 8.0) - $5.0 \times 10^{-2}$ M

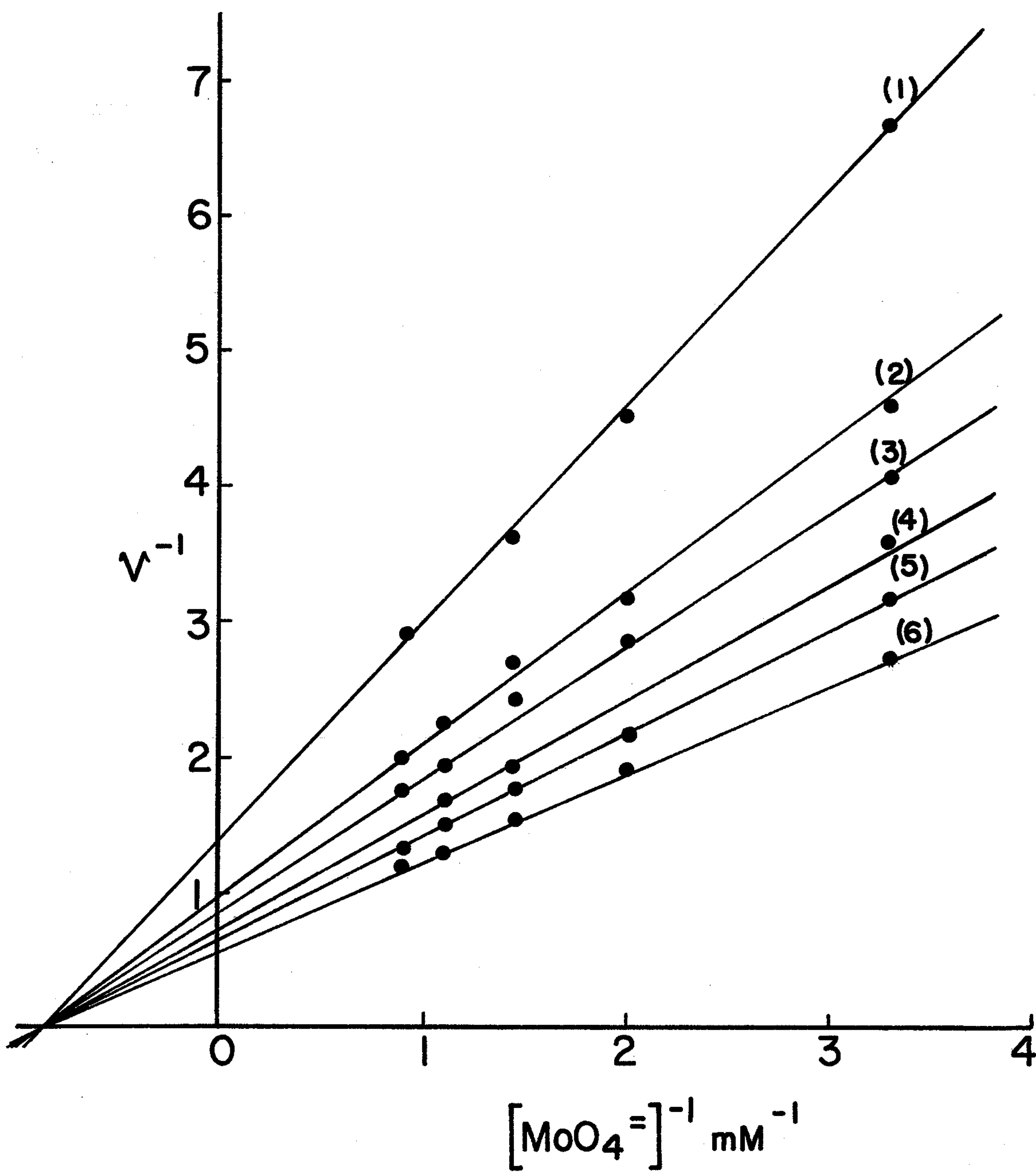
Pyrophosphatase - 4.0 µg

Enzyme - 4.0 µg

$H_2O$ to a total volume of 1.0 ml

The incubation time was 15 min at 30° C.

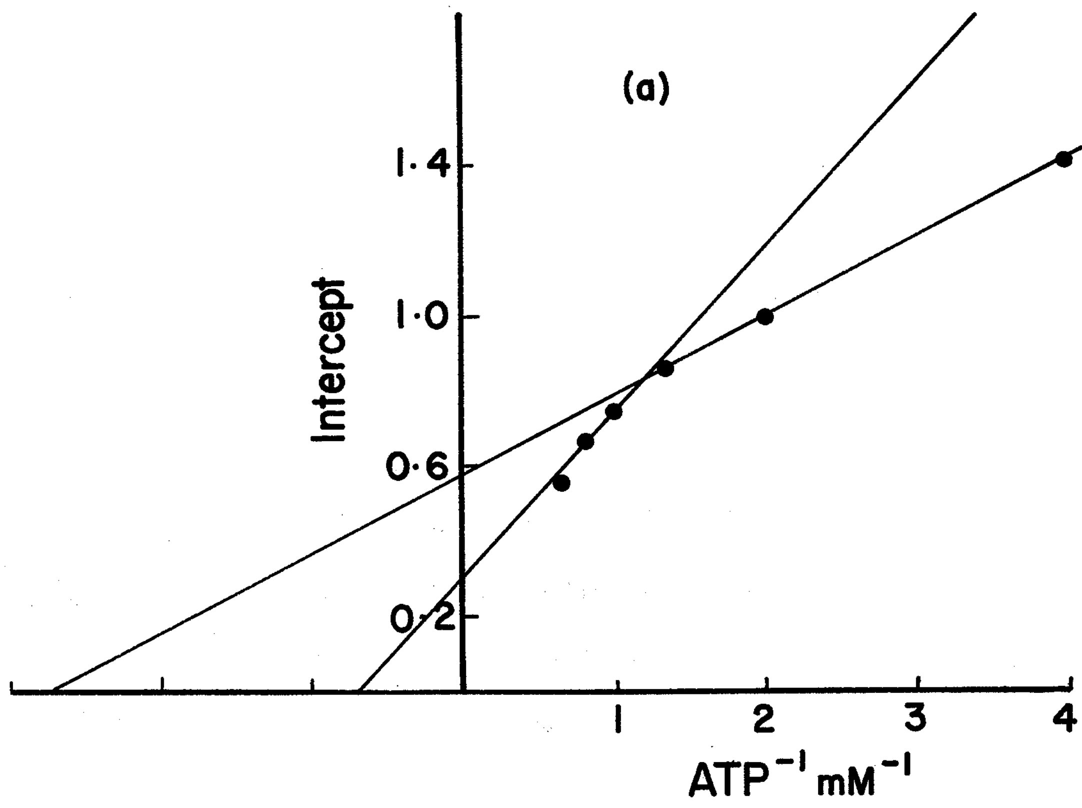
At the end of this reaction time, the reactions were stopped by the addition of 8.0 ml of TCA and the tubes analyzed for inorganic phosphate as described in Methods and Materials. The amount of color formation was detected in a Unicam SP 700 Recording Spectrophotometer at 740 mµ in 4 cm light-path cuvetts. The velocity of reaction is expressed as the number of µmoles Pi formed in the 15 min incubation period.



7
6
5
4
3
2
1
0
1
2
3
4
$V^{-1}$
(1)
(2)
(3)
(4)
(5)
(6)
$[MoO_4^{=}]^{-1}$ $mM^{-1}$

Fig. 7a. Replot of intercepts versus reciprocal of ATP concentrations from Fig. 6 in which $MoO_4^{=}$ was varying substrate, and ATP the fixed variable.

Fig. 7b. Replot of slope versus reciprocal of ATP concentrations from Fig. 6 in which $MoO_4^{=}$ was varying substrate and ATP the fixed substrate.


(a)
Intercept
1·4
1·0
0·6
0·2
1
2
3
4
ATP$^{-1}$ mM$^{-1}$

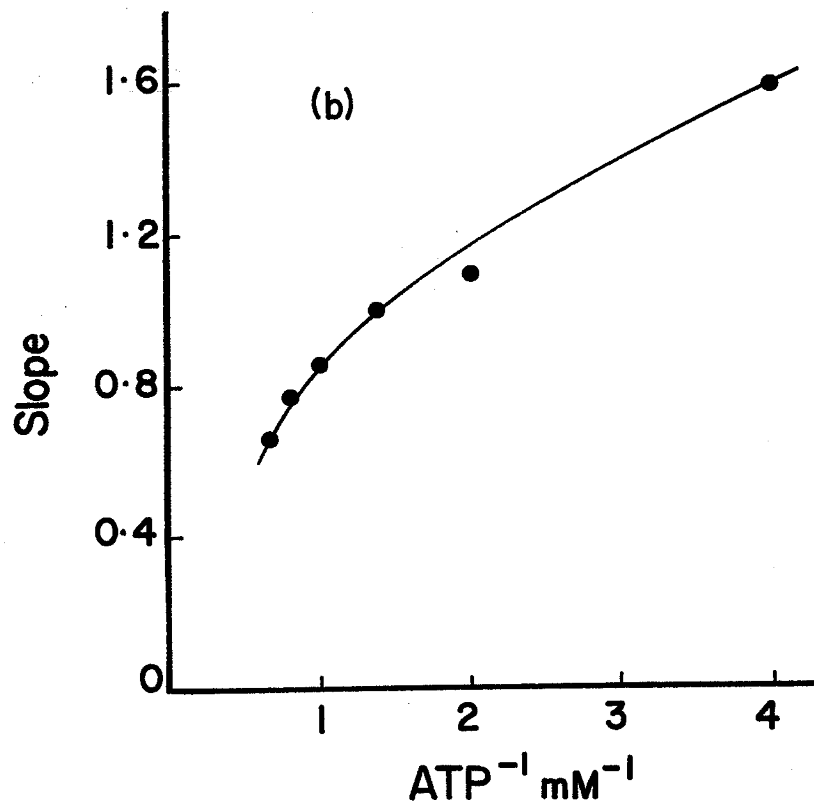
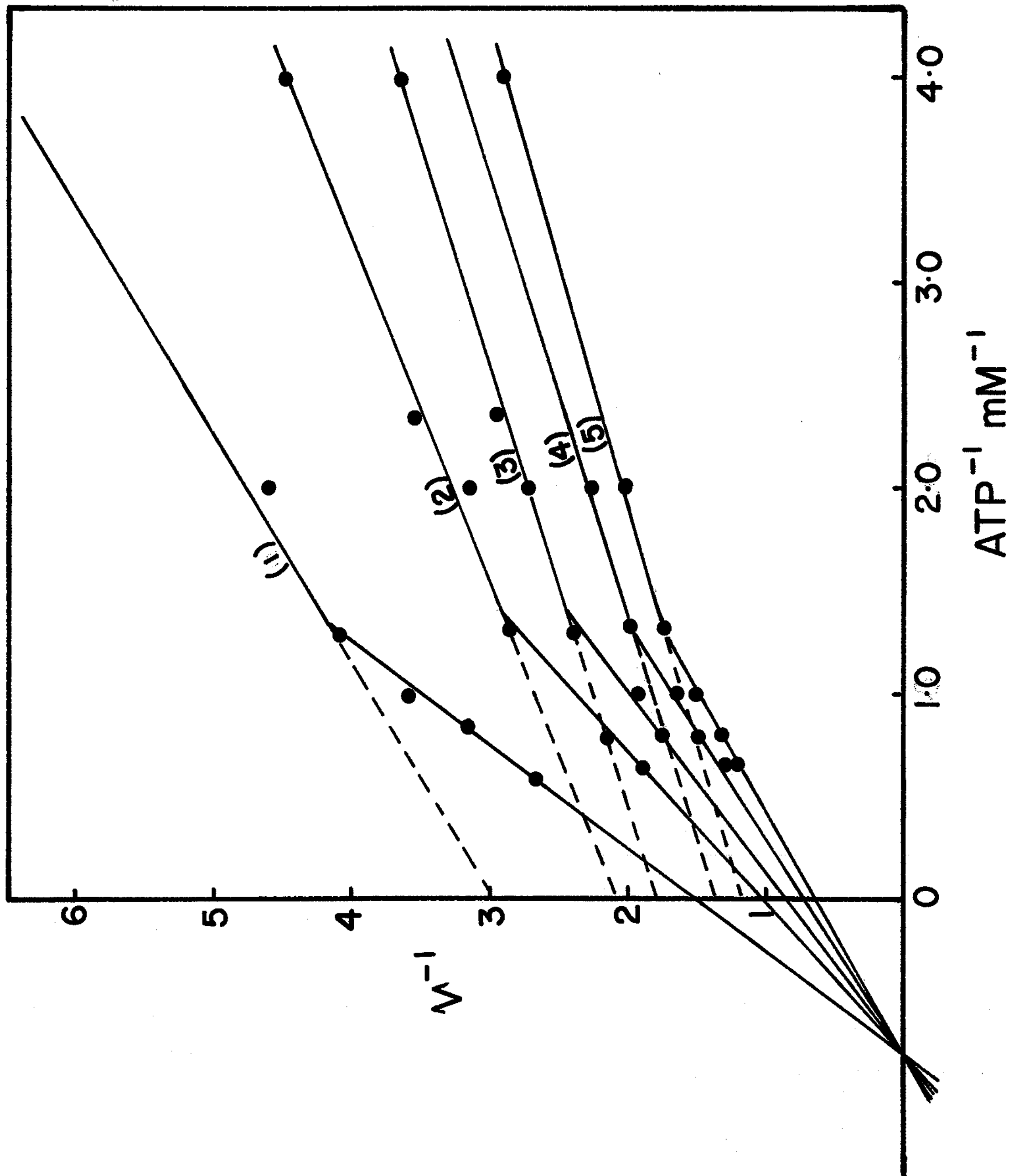

(b)
Slope
1·6
1·2
0·8
0·4
0
1
2
3
4
ATP$^{-1}$ mM$^{-1}$

Fig. 8. Double reciprocal plots of velocity versus varying ATP concentrations with $MoO_4^{=}$ as the fixed variable.

The experimental conditions were the same as those described in Fig. 6. The $MoO_4^{=}$ concentrations used were (1) 0.3 mM (2) 0.5 mM (3) 0.7 mM (4) 0.9 mM (5) 1.1 mM.



V⁻¹
ATP⁻¹ mM⁻¹
(1)
(2)
(3)
(4)
(5)
0
1
2
3
4
5
6
1·0
2·0
3·0
4·0

Fig. 9. Replot of intercepts versus reciprocal of $MoO_4^{=}$ concentrations from Fig. 8 in which ATP was the varying substrate and $MoO_4^{=}$ the fixed variable.

The solid line represents the extrapolated intercepts obtained at high ATP concentrations, while the dotted line represents those at low ATP concentrations.

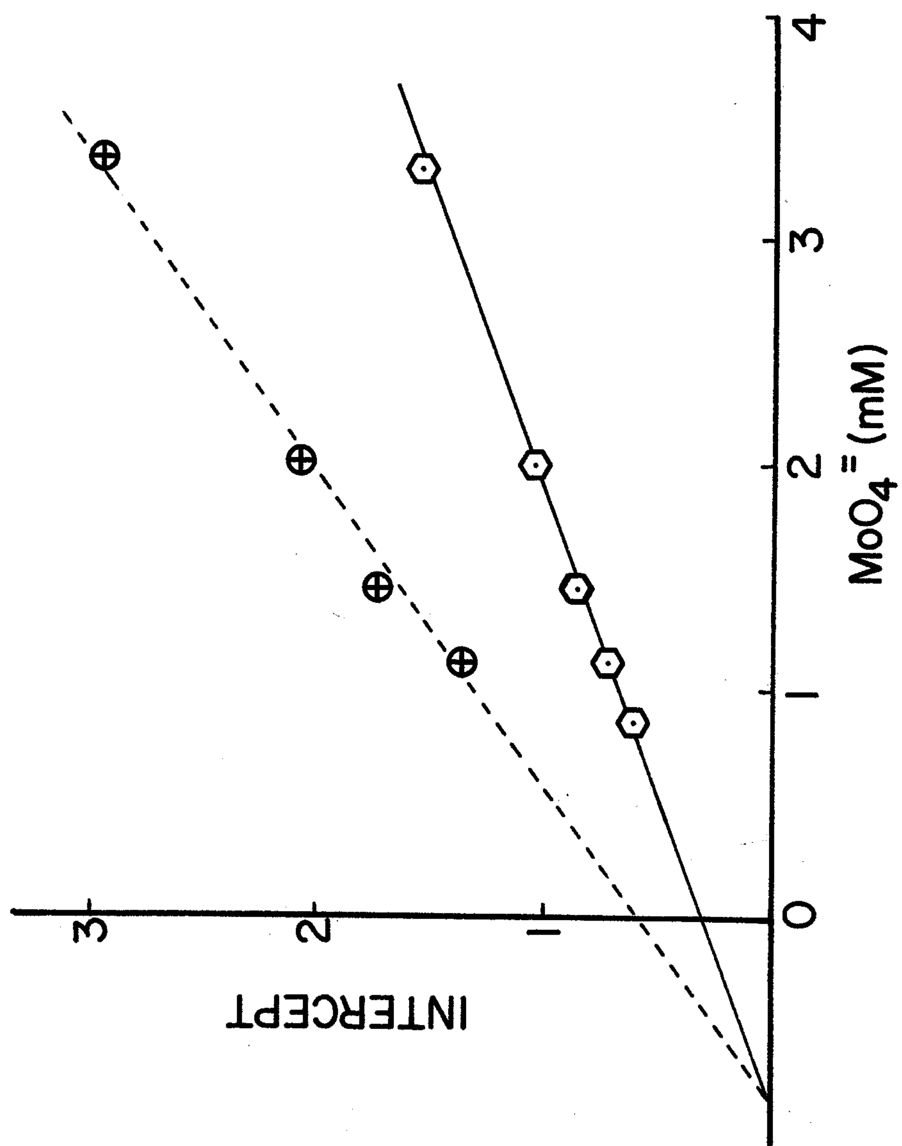

INTERCEPT
3
2
1
0
1
2
3
4
MoO$_4^=$ (mM)

Fig. 10. APS formation.

The complete reaction mixture contained:

$MgCl_2$ - $5.0 \times 10^{-3}$ M

Tris-HCl (pH 8.0) - $5.0 \times 10^{-2}$ M

ATP - $5.0 \times 10^{-3}$ M

$Na_2S^{35}O_4$ - $2.0 \times 10^{-4}$ M ($5.0 \times 10^6$ cpm per µmole)

Pyrophosphatase - 4 µg

Enzyme - 16 µg

$H_2O$ to a total volume of 1.0 ml

and when indicated:

$Na_2MoO_4$ - $2.5 \times 10^{-3}$ M

After 20 minutes, the reactions were stopped by boiling for 90 secs. The nucleotides were then separated by electrophoresis (4 hr) on a Beckman model R apparatus in 0.03 M citrate (pH 5.5). The outlined areas indicate ultraviolet quenching, the shaded areas, radioactivity.

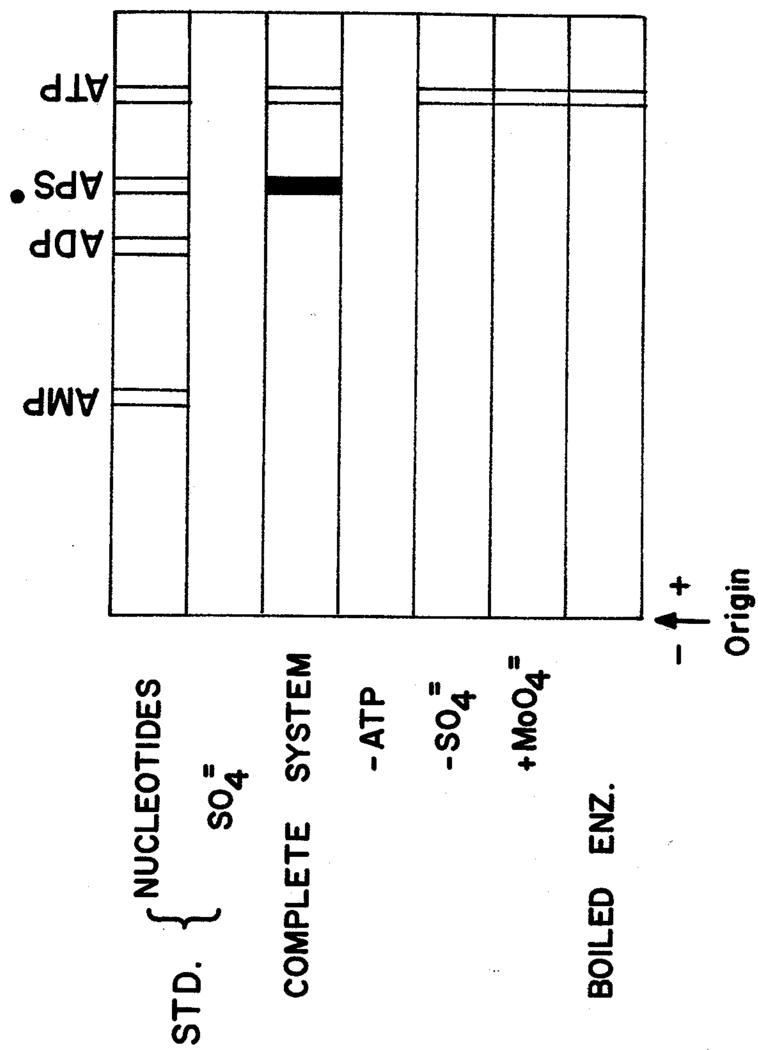

ATP
APS
ADP
AMP
STD.
NUCLEOTIDES
$SO_4^=$
COMPLETE SYSTEM
-ATP
$-SO_4^=$
$+MoO_4^=$
BOILED ENZ.
− +
Origin

Fig. 11. Double reciprocal plots (m = slope) of cpm versus varying $SO_4^=$ concentrations (with ATP as the fixed substrate) in the absence and in the presence of $MoO_4^=$, the inhibitor.

The reaction mixture contained:

ATP - $5.0 \times 10^{-3}$ M

$Na_2MoO_4$ - (1) 0.1 mM (2) 0.05 mM (3) nil

$S^{35}O_4^=$ - $(0.1 - 4.0) \times 10^{-3}$ M

$MgCl_2$ - $5.0 \times 10^{-3}$ M

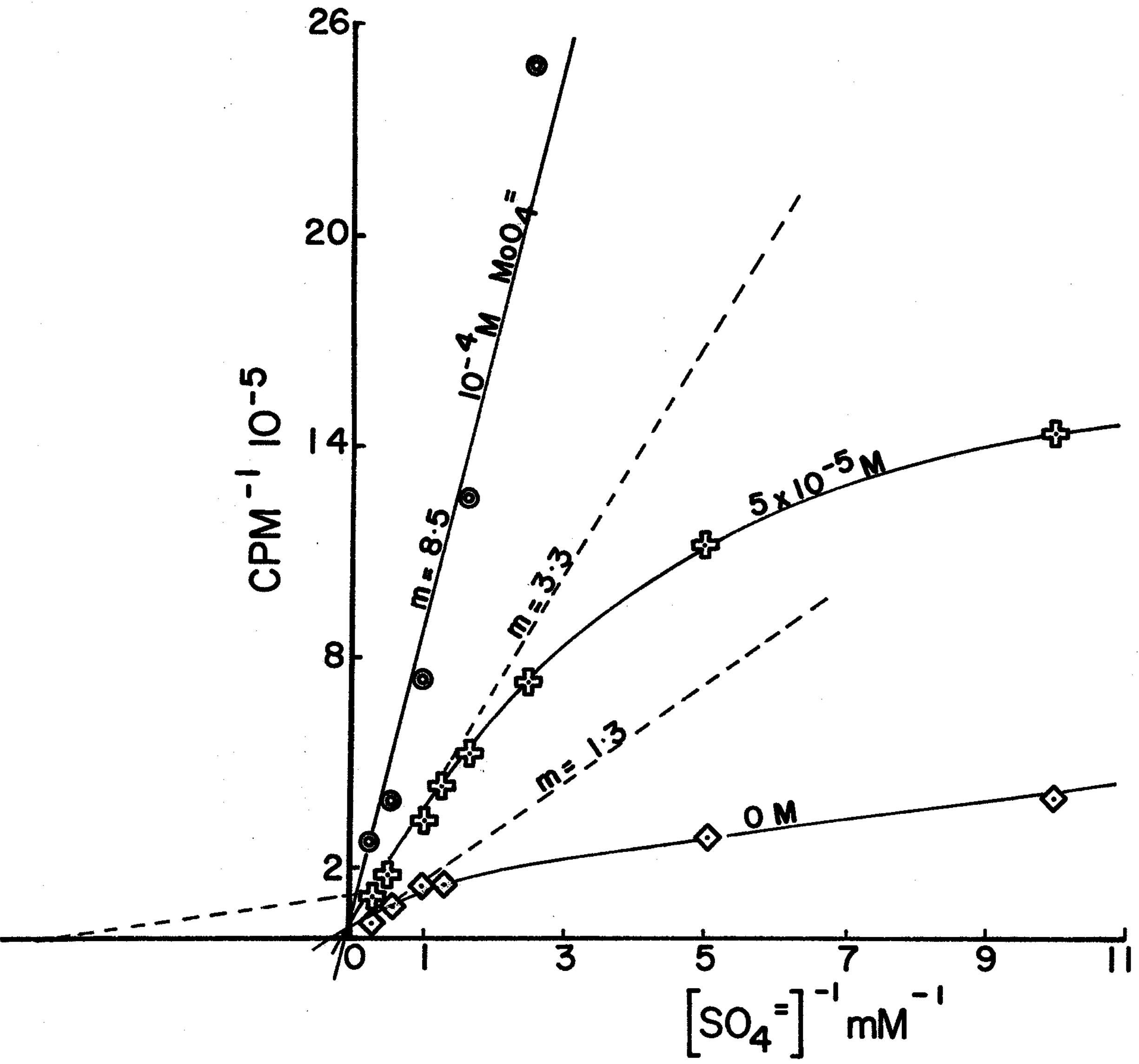
Tris-HCl (pH 8.0) - $5.0 \times 10^{-2}$ M

Pyrophosphatase - 4.0 µg

Enzyme - 16 µg

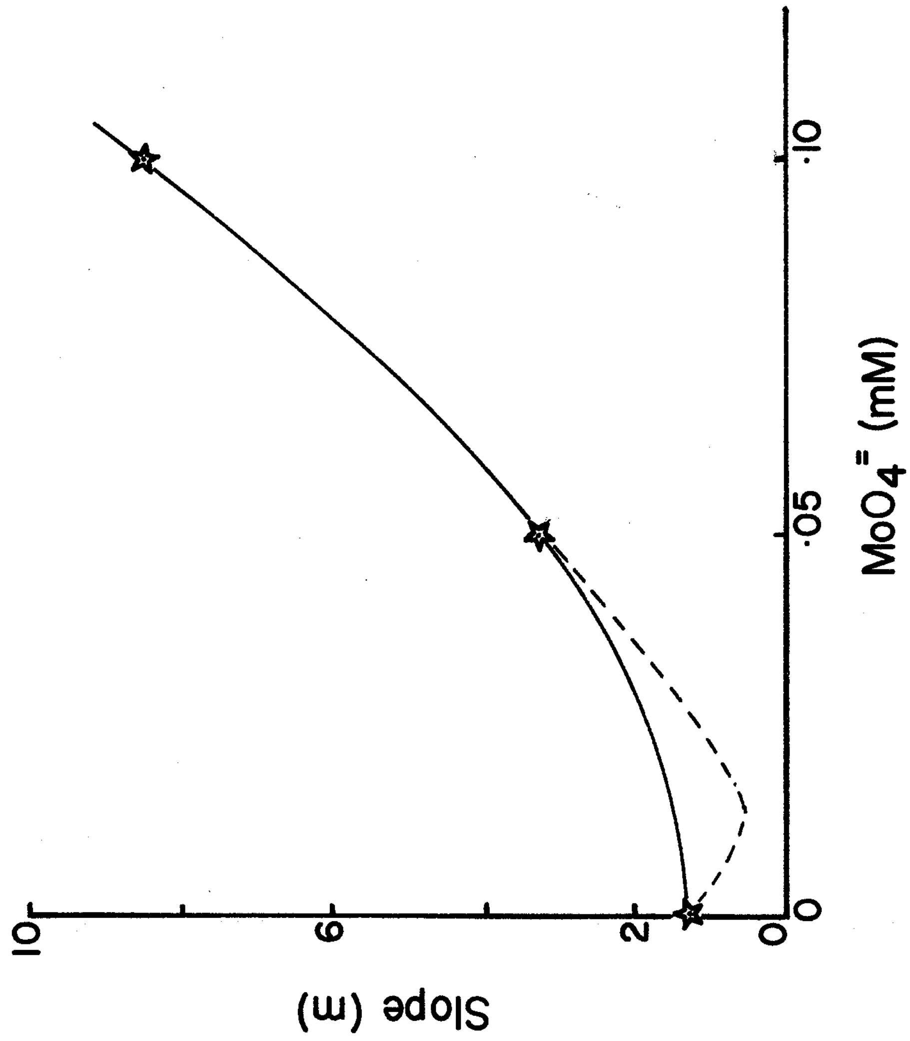
$H_2O$ to a total volume of 1.0 ml

The incubation time was extended to 20 min at 30° C. At the end of the reaction time the reactions were stopped by placing tubes in a boiling water bath for 90 secs., and immediately cooled upon removal. Then 50 µl of the reaction mixture from each tube was applied at one end of a paper electrophoresis strip which was placed on a Beckman model R electrophoresis system and run for 4 hr at 4° C. The strips were air-dried, cut into a V-shape at one end and eluted overnight with water (in a trough supported above the strips) in a decending manner. The next day an aliquot was taken and counted in a Packard tri-carb scintillation spectrometer.



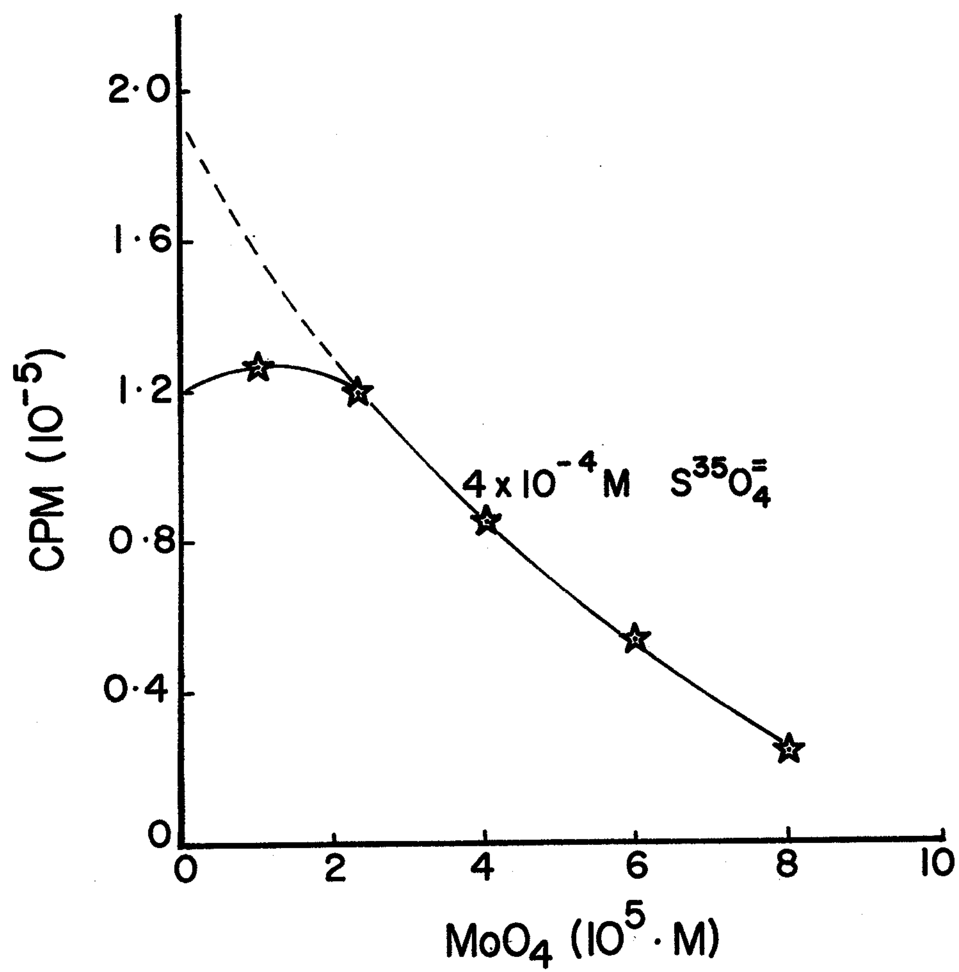
CPM $^{-1}$ $10^{-5}$
26
20
14
8
2
0
1
3
5
7
9
11
$[SO_4^=]^{-1}$ mM $^{-1}$
$10^{-4}$M $MoO_4^=$
m = 8.5
m = 3.3
m = 1.3
$5 \times 10^{-5}$ M
0 M

Fig. 12. Replot of slope (m) versus $MoO_4^=$ concentrations from Fig. 11 in which $SO_4^=$ was the varying substrate, ATP the fixed substrate, and $MoO_4^=$ the inhibitor. For the significance of the dotted line see Fig. 13, p. 47.



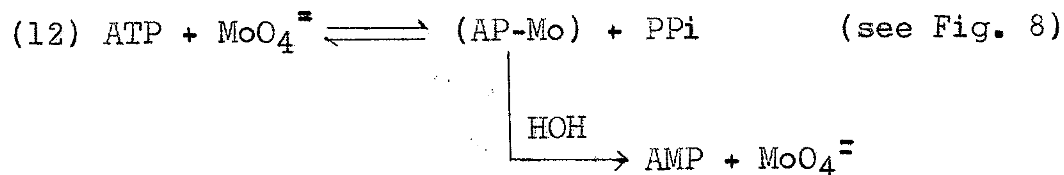
Slope (m)
10
6
2
0
0
·05
·10
MoO4= (mM)

Fig. 13. Plots of cpm versus inhibitor ($MoO_4^=$) concentrations in the presence of a fixed $SO_4^=$ concentration ($4.0 \times 10^{-4}$ M). The reaction mixture and procedure is the same as that described for Fig. 11.


2·0
1·6
1·2
0·8
0·4
0
CPM (10^-5)
4 x 10^-4 M S^35 O_4^=
0
2
4
6
8
10
MoO_4 (10^5 · M)

## DISCUSSION

The kinetic data in this work were a direct measure of the rates of product formation when one substrate was varied and the other held at a fixed concentration. This was accomplished by following, in the presence of pyrophosphatase, either one of the two subsequent reactions catalyzed by ATP-sulfurylase:



The results shown in Figs. 1-9 inclusive were obtained by determining the amount of phosphate formed (as a result of the action of pyrophosphatase on PPi) according to equation (12) and to the experimental procedure outlined in Methods and Materials. An attempt to follow the phosphate formed through equation (4) was also made, but was unsuccessful. The results disclosed in Figs. 10-13 were acquired then by measuring the amount of $APS^{35}$ formation with the use of electrophoretic and radioactive techniques, thus obtaining direct quantitative data for

APS formation that was lacking, until now.

The intersecting pattern of the lines plotted in Fig. 6 indicates that the enzyme mechanism is not of the ping pong type and the non-linearity of the lines observed in Figs. 8 and 11 cannot be explained by a simple ordered or rapid equilibrium random Bi Bi mechanism. There thus appear to be two possible mechanisms applicable to the experimental data obtained:

<u>Mechanism I: Two Enzyme Hypothesis</u>

The non-linear plots observed in Figs. 8 and 11 can be explained by two enzymes catalyzing the same reaction. If the two following rate equations $v_1 = \frac{V_1 S}{K_1 + S}$ and $v_2 = \frac{V_2 S}{K_2 + S}$ are considered where $v_1$ and $v_2$ are the initial velocities, $V_1$ and $V_2$ the maximum velocities, and $K_1$ and $K_2$ the Michaelis constants for the two enzymes $E_1$ and $E_2$ respectively and S is the common substrate (for either enzyme), a resulting total velocity v can be expressed as the sum of the two initial velocities:

$$v = v_1 + v_2 = \frac{V_1 S\ (K_2 + S) + V_2 S\ (K_1 + S)}{(K_1 + S)\ (K_2 + S)}$$

$$= \frac{(V_1 + V_2)\ S_2 + (V_1 K_2 + V_2 K_1)\ S}{S_2 + (K_1 + K_2)\ S + K_1 K_2}$$

$$= \frac{(V_1K_2 + V_2K_1)(\frac{1}{S}) + (V_1 + V_2)}{K_1K_2(\frac{1}{S})^2 + (K_1 + K_2)(\frac{1}{S}) + 1}$$

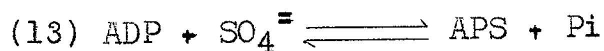
The reciprocal form of the equation then becomes:

$$\frac{1}{v} = \frac{K_1K_2(\frac{1}{S})^2 + (K_1 + K_2)(\frac{1}{S}) + 1}{(V_1K_2 + V_2K_1)(\frac{1}{S}) + (V_1 + V_2)}$$

which is of the form

$$y = \frac{ax^2 + bx + c}{dx + 1}$$

where 1/v is a 2/1 function of 1/S. When these two variables are plotted the plots will be a 2/1 function with two asymptotes, which agrees with the experimental data obtained. This, at first seemed possible because it is known that D. desulfuricans has also an enzyme ADP-sulfurylase the role of which seems unknown in whole cells but in cell-free extracts catalyzes the reaction (Peck, 1962):



(13) $ADP + SO_4^{=} \rightleftharpoons APS + Pi$

(the substrate ADP required in this reaction would be present in small amounts as an impurity in ATP solutions). In yeast, this reaction (13) was catalyzed by a protein

fraction that was free of ATP-sulfurylase activity (Robbins and Lipmann, 1958). If two such enzymes were active in the formation of APMo and PPi, APS and PPi, and APS and Pi (reactions 12, 4, and 13 respectively) a 2/1 function would result from the kinetic data.

There is, however, a certain amount of evidence which discounts the two enzyme hypothesis. First, the equilibrium of reaction (13) is unfavorable like that of ATP-sulfurylase for the formation of detectable amounts of APS but even more so than the latter enzyme (Peck, 1962). Secondly, it seems that the enzyme ADP-sulfurylase does not react with $MoO_4^=$ (the anion used as substrate in equation (12) for Fig. 8) and the other group VI anions as does the ATP-sulfurylase enzyme because heated extracts of D. desulfuricans that possess no ATP-sulfurylase enzyme are unable to catalyze the liberation of Pi from ATP (or ADP) in the presence of $MoO_4^=$ but still show pyrophosphatase and ADP-sulfurylase activities (Peck, 1962). This fact discredits the possible activity of the enzyme ADP-sulfurylase in the final purified enzyme preparation (with ADP and $MoO_4^=$ as substrates; Fig. 8, equation 13) but does appear to explain the transitory (3-4 weeks) inhibition of sulfate reduction observed in septic tanks, and a mechanism may be postulated whereby the reduction of sulfate (in the

presence of $MoO_4^{=}$) in 'whole cells' is eventually channeled through the ADP-sulfurylase enzyme which is unaffected by $MoO_4^{=}$. This would be possible if phosphate is rapidly used by 'whole cells' shifting the equilibrium of equation 13 toward APS formation; however Peck (1962) claims that the complete inhibition of sulfate reduction in 'whole cells' of D. desulfuricans by $MoO_4^{=}$ and other VI anions indicates that ADP-sulfurylase does not participate in the sulfate-reducing system. Finally, the lack of ADP-sulfurylase activity and the detection of only one major protein band in gel electrophoresis excludes the possibility of the presence of ADP-sulfurylase in the purified ATP-sulfurylase preparation. This latter finding also discredits the possibility that the non-linear plots obtained were due to the presence of two ATP-sulfurylase enzymes in the preparation.

## Mechanism II: Partial Allosterism

This mechanism (ordered) postulates that the enzyme "E" (unmodified) has two affinity sites for ATP (Fig. 8) and assumes that both the E•ATP and E•ATP•ATP complexes possess activity. The following rate equation is the sum of the activities of these two forms (Sanwal, unpublished results):

$$( \text{ i}) \quad v = V_1\left(\frac{ATP}{K_{ATP_1} + ATP}\right)\left(\frac{K_1}{K_1 + ATP}\right) + \frac{V_2 ATP^2}{(K_{ATP_2} + ATP)(K_1 + ATP)}$$

where $v$ = initial velocity of reaction, $K_1$ = the dissociation constant of the enzyme substrate (ATP) complex, $V_1$ and $K_{ATP_1}$, and $V_2$ and $K_{ATP_2}$, are the maximum velocities and the kinetic affinity constants (for the substrate ATP) for the E•ATP and E•ATP•ATP forms respectively. This equation then reduces to:

$$( \text{ ii}) \quad v = \frac{V_1K_1K_{ATP_2}ATP + (V_1K_1 + V_2K_{ATP_1})(ATP)^2 + V_2(ATP)^3}{K_1K_{ATP_1}K_{ATP_2} + (K_1K_{ATP_1} + K_1K_{ATP_2} + K_{ATP_1}K_{ATP_2})ATP + (K_1 + K_{ATP_1} + K_{ATP_2})(ATP)^2 + (ATP)^3}$$

which can be transformed into the reciprocal form:

$$(\text{iii}) \quad \frac{1}{v} = \frac{1 + (K_1 + K_{ATP_1} + K_{ATP_2})\left(\frac{1}{ATP}\right) + K_1K_{ATP_1} + K_1K_{ATP_2} + K_{ATP_1}K_{ATP_2})\left(\frac{1}{ATP}\right)^2 + K_1K_{ATP_1}K_{ATP_2}\left(\frac{1}{ATP}\right)^3}{V_2 + (V_1K_1 + V_2K_{ATP_1})\left(\frac{1}{ATP}\right) + V_1K_1K_{ATP_2}\left(\frac{1}{ATP}\right)^2}$$

which is of the form:

$$y = \frac{ax^3 + bx^2 + cx + d}{ex^2 + fx + g}$$

which is a 3/2 function. The plot of slope or/and intercepts versus the reciprocal of ATP concentrations can give a curve which curves either upward or downward depending on whether $V_2$ is greater or less than $V_1$ and $K_{ATP_1}$ greater or less than $K_{ATP}$. When $V_1$ and $V_2$ are the same for the E•ATP and E•ATP•ATP complexes (when the substrate ATP concentration is very high so $\left(\frac{1}{ATP}\right)^3$ is approximately = 0) the equation then becomes:-

$$\frac{1}{v} = \frac{a\left(\frac{1}{ATP}\right)^2 + b\left(\frac{1}{ATP}\right) + c}{d\left(\frac{1}{ATP}\right) + 1}$$

which is a 2/1 function where a, b, c, and d are combinations of the rate constants shown in equation three. The results shown in Fig. 11 suggest that there are also two or more $SO_4^=$ sites which would result in a more complex rate equation for sulfate. Also if the enzyme has two more sites for sulfate addition it would be expected that the same sites would exist for molybdate which cannot only be used as substrate, but also as an inhibitor, for the enzyme ATP-sulfurylase. However, Fig. 6 does not show this - possibly because of the higher concentrations of molybdate used as substrate (due to limiting sensitivity of the phosphate determination) to obtain the straight line plot.

The intersecting patterns observed then indicate

that the substrates ATP and $MoO_4^{=}$ (or $SO_4^{=}$) react at two points in the sequence connected by reversible reactions. This pattern, however, does not give us any information about the order of addition of the substrates (ATP and $MoO_4^{=}$ or $SO_4^{=}$) because both random and sequential mechanisms would show the same effect, and to distinguish between the two, product inhibition and equilibrium binding studies are necessary (not attempted in this thesis) which would be technically difficult to carry out with this system.

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