STUDIES ON ADENOSINE 5'-PHOSPHOSULFATE REDUCTASE AND ENZYMES INVOLVED IN GLUTAMATE SYNTHESIS IN *THIOBACILLUS THIOPARUS*

BY

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"STUDIES ON ADENOSINE 5'-PHOSPHOSULFATE REDUCTASE AND ENZYMES INVOLVED IN GLUTAMATE SYNTHESIS IN THIOBACILLUS THIOPARUS"

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A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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TO MY PARENTS

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ABSTRACT

(Adenosine 5'-phosphosulfate Reductase)

ABSTRACT

The reaction mechanism of adenosine 5'-phosphosulfate reductase (APS reductase) from Thiobacillus thioparus was studied using difference spectrum and stopped-flow techniques.

The results of these studies suggested that two components of the enzyme, FAD and nonheme iron, were involved in this oxido-reduction reaction. The formation of a red (anionic) semiquinone was postulated as an intermediate from the stopped-flow spectrophotometric studies.

A mechanism has been proposed for the reaction catalyzed by APS reductase based on these studies. The important steps of the mechanism are the reduction of FAD to $FADH_2$ by sulfite and the partial reoxidation of $FADH_2$ to semiquinone ($FADH\cdot$) with concomitant reduction of nonheme iron by the addition of AMP. The electron flow in the presence of cytochrome c was assumed to be as follows: sulfite $\longrightarrow$ FAD $\longrightarrow$ nonheme iron $\longrightarrow$ cytochrome c.

The formation of superoxide radicals (O_2^-) was also detected during the enzyme reaction. It was concluded, however, that there definitely was a direct

binding or reduction of cytochrome c in this system because under the anaerobic conditions the enzyme retained 65% of its rate of cytochrome c reduction obtained in the presence of air.

ABSTRACT

(Enzymes involved in Glutamate Synthesis in *Thiobacillus thioparus*)

ABSTRACT

Studies were made of enzymes involved in glutamate synthesis in T. thioparus. The enzymes of the glutamine pathway, glutamine synthetase and glutamate synthase, as well as two distinct glutamate dehydrogenases (NAD- and NADP-dependent) were detected in this organism. The NADP-dependent glutamate dehydrogenase was partially purified and kinetic parameters were determined. The K_m values of glutamate dehydrogenases for ammonia were very high (23 mM for the NADP-dependent enzyme). The levels of glutamate synthase were similar in T. thioparus grown on 0.7 mM or 7.0 mM $(NH_4)_2SO_4$. Since the sum of the activities of both glutamate dehydrogenases was only 1/25 of that of glutamate synthase, it was concluded that glutamate was synthesized mainly through the glutamine pathway in T. thioparus.

Glutamate synthase was purified more than 500-fold from the T. thioparus sonicate and was characterized. The molecular weight was estimated by a gel filtration method as 280,000 g/mole. It was established by the SDS-polyacrylamide gel electrophoresis that T. thioparus glutamate synthase possessed two dissimilar subunits and the molecular weight of the smaller subunit was 72,000 g/mole. The enzyme was specific for NADPH and

α-ketoglutarate, but L-glutamine was partially replaced by ammonia as amino donor. The ammonia activity obtained with 100 mM NH_4Cl, however, was only 6% of the glutamine activity with 5 mM L-glutamine. The Km values of T. thioparus glutamate synthase for NADPH, α-ketoglutarate and glutamine were determined as 3.0 μM, 50 μM and 1.1 mM, respectively. The enzyme had a pH optimum between 7.3 and 7.8.

Glutamate synthase from T. thioparus was relatively insensitive to feedback inhibition by amino acids. Only four amino acids, L-serine, L-methionine, L-histidine and L-glutamate, produced more than 50% inhibition at 50 mM.

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ABBREVIATIONS

ADP ..... adenosine 5'-diphosphate

AMP ..... adenosine 5'-monophosphate

APS ..... adenosine 5'-phosphosulfate

ATP ..... adenosine 5'-triphosphate

BIS ..... N,N'-methylenebisacrylamide

c-AMP ..... adenosine 3',5'-cyclic monophosphate

Cyt.c ..... cytochrome c

DEAE ..... diethylaminoethyl

EDTA ..... ethylenediaminetetraacetic acid

FAD ..... flavin adenine dinucleotide

FADH· ..... flavin adenine dinucleotide semiquinone (half-reduced radical)

$FADH_2$ ..... dihydroflavin adenine dinucleotide

FMN ..... flavin mononucleotide

HEPES ..... N-2-hydroxyethyl-piperazine-N'-2-ethane

α-KG ..... α-ketoglutarate

ms ..... millisecond

NAD^+ ..... oxidized nicotinamide adenine dinucleotide

NADH ..... reduced nicotinamide adenine dinucleotide

$NADP^+$ ..... oxidized nicotinamide adenine dinucleotide phosphate

NADPH ..... reduced nicotinamide adenine dinucleotide phosphate

PEP ..... phosphoenolpyruvate

SDS ..... sodium dodecyl sulfate

TEMED ..... N,N,N',N'-tetramethylethylenediamine

Tris ..... tris(hydroxymethyl)aminomethane

PART I

ADENOSINE 5'-PHOSPHOSULFATE REDUCTASE

INTRODUCTION

INTRODUCTION

APS reductase was first detected in a sulfate-reducing bacterium, Desulfovibrio (Peck, 1959; Ishimoto and Fujimoto, 1959), where it was involved in the reduction of sulfate to sulfite. Peck (1960) proposed a role for APS reductase in the oxidation of sulfite by T. thioparus.

D. vulgaris APS reductase was purified and identified as a flavoprotein (Peck et al., 1965). In 1970, Michaelis et al. studied the spectral change of the D. vulgaris enzyme caused by the addition of its substrates and proposed a three step mechanism in the absence of an added electron acceptor. The postulated mechanism included the formation of a flavin-sulfite adduct at the N(5) position of the isoalloxazine ring as an intermediate. The D. vulgaris enzyme like the enzyme from T. thioparus (Lyric and Suzuki, 1970b) could use cytochrome c as electron acceptor (Michaelis et al., 1971), but superoxide radicals were responsible for the reduction of cytochrome c (Bramlett and Peck, 1975).

T. thioparus APS reductase involved in the oxidation of sulfite to sulfate was purified and characterized by Lyric and Suzuki (1970b). The T. thioparus enzyme was also a flavoprotein and it was demonstrated that the enzyme-bound FAD was reduced by its substrate, sulfite. Lyric and Suzuki (1970d) proposed an Ordered Quad Ter mechanism from the initial velocity and product inhibition studies.

However, these studies on the mechanism of APS reductase were carried out under steady state conditions. The present investigation using stopped-flow equipment was undertaken with the hope of studying short-life intermediates formed during the enzyme reaction which could not be detected under the steady state kinetic conditions. It was of particular interest to find out whether or not T. thioparus APS reductase possessed the same mechanism as the D. vulgaris enzyme in spite of its different biological role. Attempts were also made to compare the properties of APS reductases from the two organisms in relation to their respective physiological function.

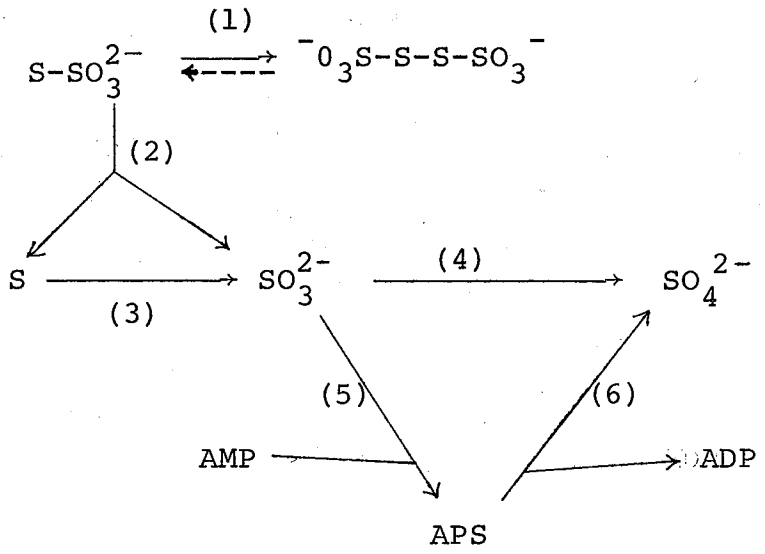
HISTORICAL

HISTORICAL

1. General

An obligate autotroph Thiobacillus thioparus oxidizes thiosulfate to sulfate through the pathway shown in Scheme I (Lyric and Suzuki, 1970 a, b, and c; Suzuki, 1974).

Scheme 1 Inorganic Sulfur Oxidation by T. thioparus.



(1) Thiosulfate-oxidizing enzyme
(2) Thiosulfate-cleaving enzyme (Rhodanese)
(3) Sulfur-oxidizing enzyme
(4) Sulfite oxidase
(5) APS reductase
(6) ADP sulfurylase

For the oxidation of sulfite two enzymes were found in T. thioparus, namely APS reductase (Adenylyl sulfate reductase) and sulfite oxidase (sulfite dehydrogenase).

Sulfite oxidase (E.C.1.8.2.1, sulfite: ferricytochrome c oxidoreductase) catalyzes the oxidation of sulfite to sulfate: $SO_3^{2-} + H_2O \longrightarrow SO_4^{2-} + 2H^+ + 2e^-$. Sulfite oxidase purified from T. thioparus (Lyric and Suzuki, 1970 a) resembles the T. novellus enzyme (Charles and Suzuki, 1965) and liver sulfite oxidase (Howell and Fridovich, 1968).

APS reductase (E.C.1.8.99.2, AMP, sulfite: (acceptor) oxidoreductase) was first described in a sulfate-reducing bacterium, Desulfovibrio desulfuricans (Peck, 1959; Ishimoto and Fujimoto, 1959). The enzyme catalyzes the reduction of APS to sulfite and AMP in the presence of reduced methyl viologen (MVH); APS + 2MVH $\longrightarrow$ SO_3^{2-} + AMP + 2MV, and the oxidation of sulfite to APS in the presence of AMP and an electron acceptor: SO_3^{2-} + AMP $\longrightarrow$ APS + $2e^-$.

Peck (1960) reported finding APS reductase, ADP sulfurylase and adenylate kinase in T. thioparus and suggested that the oxidation of sulfite involves substrate level phosphorylation. The reaction sequence suggested by Peck (1960) is shown in equations below.

$$SO_3^{2-} + AMP \longrightarrow APS + 2e^- \quad \text{(APS reductase)}$$

$$APS + Pi \longrightarrow ADP + SO_4^{2-} \quad \text{(ADP sulfurylase)}$$

$$2ADP \longrightarrow AMP + ATP \quad \text{(Adenylate kinase)}$$

APS reductase was so far purified and characterized only from four sources: D. vulgaris (Peck et al., 1965; Michaelis et al., 1971; Bramlett and Peck, 1975). T. denitrificans (Bowen et al., 1966), T. thioparus (Lyric and Suzuki, 1970 b) and Thiocapsa roseopersicina (Trüper and Rogers, 1971). An abbreviated comparison of the properties of APS reductase from these four sources is presented in Table 1.

APS reductases from all four sources were iron-flavoprotein and had similar molecular size. It was also found that the D. vulgaris enzyme exists in either monomer (mol. wt. 220,000) or dimer and both forms are active (Michaelis et al., 1971). A major structural difference was noted when the T. roseopersicina enzyme was found to be membrane-bound (Trüper and Peck, 1970) and contained c-type cytochrome as an additional coenzyme (Trüper and Rogers, 1971). When assayed with ferricyanide, these four APS reductases had similar specific activities and substrate Km's. The optimum pH for the

Table 1. Comparison of the properties of purified APS reductases.

Source (Reference)	*D. vulgaris* (1) (2) (3)	*T. denitrificans* (4)	*T. thioparus* (5)	*T. roseopersicina* (6)
Structure				
mol. wt.	218,500	N	170,000	180,000
$S_{20,w}$	9.8×10^{-13}	9.5×10^{-13}	N	9.7×10^{-13}
FAD/mole	1	(0.69 0.77/10^5g)	1	1
NHI[a]/FAD	12.7	6-11[c]	8-10	4
Labile Sulfide/mole	12.3	N	4-5	6
Heme/mole	0	0	0	2
Kinetic Parameter				
a) $Fe(CN)_6^{3-}$ assay				
K_m for SO_3^{2-}	2×10^{-3}M	1.5×10^{-3}	2.5×10^{-3}	1.5×10^{-3}
AMP	2.4×10^{-4}	4.1×10^{-5}	10^{-4}	7.3×10^{-5}
$Fe(CN)_6^{3-}$	N[b]	N	N	1.3×10^{-4}
pH Optimum	7.4	7.2	7.4	8.0
Specific Activity	7.3	8.5	6.4	8.7
b) Cyt.*c* assay				
K_m for SO_3^{2-}	N	N	1.7×10^{-5}	9.3×10^{-5}
AMP	1.8×10^{-4}	N	2.5×10^{-6}	5.9×10^{-5}
Cyt.*c*	N	N	1.4×10^{-5}	3.3×10^{-5}
pH Optimum	9.5	N	9.5	9.5
Activity Ratio to $Fe(CN)_6^{3-}$ assay	1/1070	N	1/80	N

Table 1. Continued

Source (Reference)	D. vulgaris (1) (2) (3)	T. denitrificans (4)	T. thioparus (5)	T. roseopersicina (6)
Inhibition by Anaerobiosis	100%	N	N	N

(a) NHI = Nonheme iron, (b) N= Not known, (c) Assuming one FAD per mole.

(1) Peck et al., 1965 (2) Michaelis et al., 1971.

(3) Bramlett and Peck, 1975 (4) Bowen et al., 1966.

(5) Lyric and Suzuki, 1970 b (6) Trüper and Rogers, 1971.

enzyme from T. roseopersicina was somewhat higher (8.0) than those for the other enzymes (7.2-7.4). Like APS reductase from T. thioparus, the T. roseopersicina enzyme was found to be capable of using the Candida krusei cytochrome c as the electron acceptor as well as ferricyanide. The horse heart cytochrome c was only 30% as active as the yeast cytochrome c in the T. thioparus system while it was not at all active in T. roseopersicina system. Peck et al. (1965) and Bowen et al. (1966) were not able to show any reactivity of the enzymes from D. vulgaris and T. denitrificans with cytochrome c. Recently, however, Michaelis et al. (1971) succeeded in demonstrating the reduction of cytochrome c using the D. vulgaris enzyme. The turnover rates for APS reductase from D. vulgaris using cytochrome c and oxygen as electron acceptors were very similar and were approximately $^{1}/1000$ of that assayed with ferricyanide (Michaelis et al., 1971) as compared to $^{1}/80$, the value for the T. thioparus enzyme (Lyric and Suzuki, 1970 b). Bramlett and Peck (1975) demonstrated the activity of the D. vulgaris enzyme assayed with cytochrome c was inhibited 100% by anaerobiosis and 84% with 10^{-6}M of superoxide dismutase. From these observations, these authors suggested that superoxide radicals were responsible for the reduction of cytochrome c in this system as in xanthine oxidase

(McCord and Fridovich, 1969). Bramlett and Peck (1975) noted that the K_m values for AMP and sulfite of the D. vulgaris enzyme did not vary appreciably with ferricyanide and cytochrome c as electron acceptors while the K_m values for sulfite were over 10-fold lower with cytochrome c than those with ferricyanide in microorganisms where the physiological function of the reductase was the oxidation of sulfite. These facts may indicate that with APS reductases from sulfur-oxidizing bacteria there is direct reduction or binding of added cytochrome c to the enzymes.

APS reductase has been found only in bacteria living on the basis of a dissimilatory metabolism of sulfur compounds (Peck, 1961; Trüper and Peck, 1970). The presence of the enzyme has been proven for the sulfate-reducing bacteria D. desulfuricans (Peck, 1961), D. vulgaris (Peck et al., 1965), D. africanus (Campbell et al., 1966), Desulfotomaculum nigrificans (Peck, 1961) and D. orientis (Lanigan and Peck, 1963).

Although APS reductase was first detected in the sulfate-reducing bacterium, Peck (1960) has proposed a role for APS reductase in the oxidation of sulfite by Thiobacilli. In the thiobacilli, definite evidence has been obtained only for T. denitrificans (Bowen et al, 1966) and T. thioparus (Lyric and Suzuki, 1970 b).

It has become clear that APS is also an intermediate of photosynthetic sulfite oxidation in Thiorhodaceae and Chlorobiaceae and is formed in the reaction catalyzed by APS reductase (Peck, 1961; Thiele, 1968a and b; Trüper and Peck, 1970; Trüper and Rogers, 1971).

It was also found that a very high content of protein in the cell was in a form of APS reductase in D. vulgaris (1-2%), in T. denitrificans (4-5%) and in T. thioparus (3%). Since APS reductase from T. roseopersicina was reported to be almost pure after 60-80 fold purification (Trüper and Rogers, 1971), this phototrophic sulfur bacterium also seemed to contain a high concentration of the enzyme.

It appears then that APS reductase must play an important role in energy metabolism of these bacteria. In thiobacilli it was suggested to conserve energy both through reduction of cytochrome c (Lyric and Suzuki, 1970b) followed by its oxidation with coupled oxidative phosphorylation and substrate level phosphorylation (Peck, 1961).

It might be generalized that the sulfate-reducing bacteria, which utilize sulfate as a terminal electron acceptor in an anaerobic respiration, contain APS reductase. The phototrophic sulfur bacteria which also

contain APS reductase are strict anaerobes and do not possess sulfite oxidase (Thiele, 1968b). It seems odd then that APS reductase was also found in two species of thiobacilli which possess another sulfite-oxidizing enzyme, sulfite oxidase.

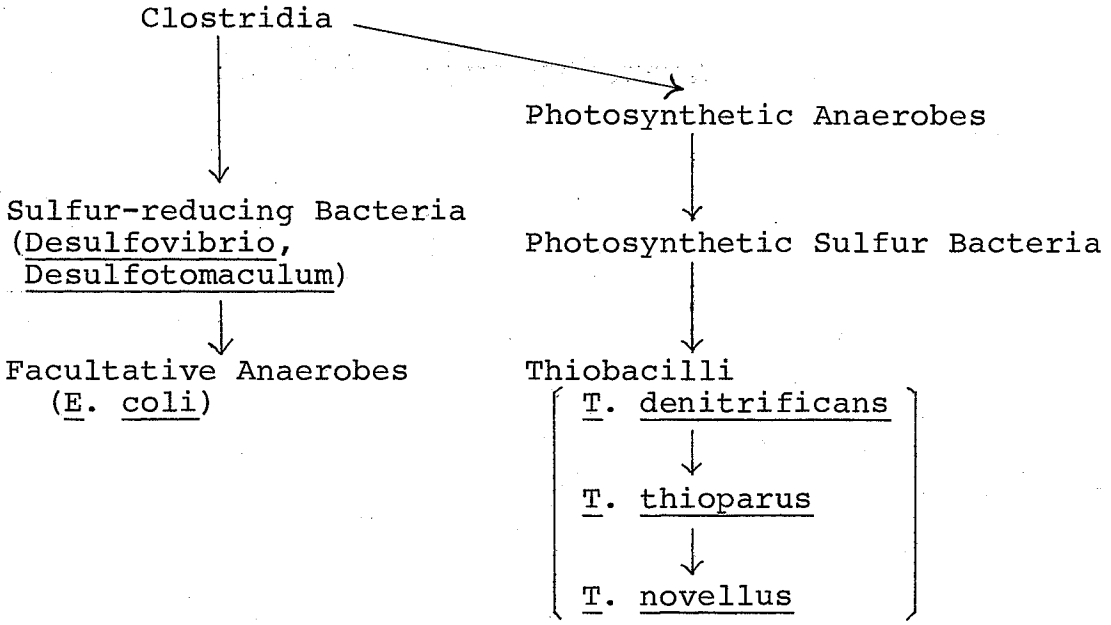
Broda (1970) has suggested in a review on the evolution of microorganisms from a bioenergetic point of view that the sulfate-reducing bacteria and photosynthetic sulfur bacteria developed independently from an assumed photosynthetic anaerobe which was developed from clostridia, and the thiobacilli then developed from the phototrophic sulfur bacteria. It seems likely then that a facultative anaerobe (with nitrate as electron acceptor), T. denitrificans links an aerobe T. thioparus to the phototrophic sulfur bacteria which are strict anaerobes.

Trüper and Rogers (1971) suggested that the common ancestor of these three groups already possessed APS reductase and that the enzyme lost its binding to the particle fraction together with its heme portion during the change from the phototrophic to the chemoorganotrophic (Desulfovibrio, Desulfotomaculum) or chemolithotrophic (thiobacilli) way of life. Within the group of the thiobacilli, the enzyme was lost completely in sev-

eral species, for example in T. novellus, and functionally replaced by sulfite oxidase during the change to aerobiosis.

However, Desulfovibrio and Desulfotomaculum are very similar to clostridia and it is generally believed that the sulfate-reducing bacteria were developed directly from the strict anaerobic bacteria. According to Egami (1974), the photosynthetic anaerobes were probably developed independently from the anaerobic respirators (Scheme II).

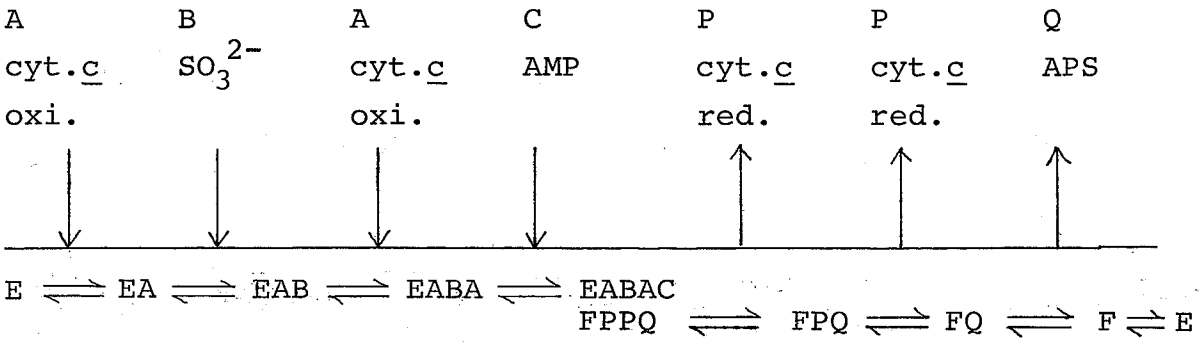
Scheme II. Evolution of thiobacilli and sulfate-reducing bacteria.



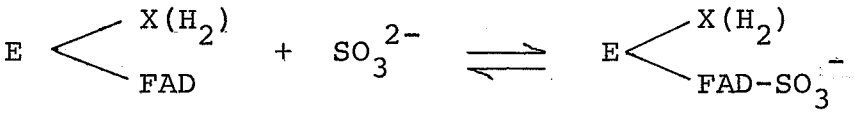
2. Enzyme Mechanism

The enzyme mechanism of APS reductase has been of particular interest because the oxidation of sulfite in the presence of AMP results in the formation of a phosphosulfate bond of adenosine 5'-phosphosulfate (APS) which was shown to have a high ΔF^{o} of 18-19 kcal per mole at pH 8.0 (Robbins and Lipmann, 1958).

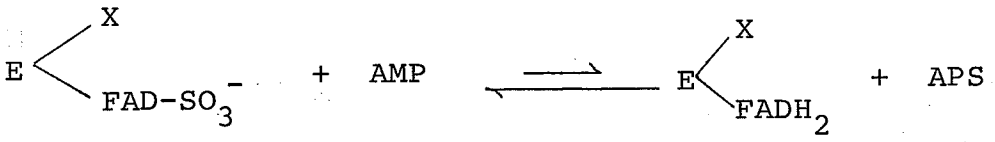
Peck et al (1965) first purified APS reductase from D. vulgaris and showed that the enzyme was an iron-flavoprotein but could not confirm the involvement of these components in the enzyme reaction. Recently Lyric and Suzuki (1970 b) reported that the enzyme-bound FAD was reduced by the addition of sulfite using the T. thioparus enzyme. These authors proposed an Ordered Quad Ter reaction for the kinetic mechanism of APS reductase from the initial velocity and product inhibition studies using cytochrome c as electron acceptor as below (Lyric and Suzuki, 1970 d).



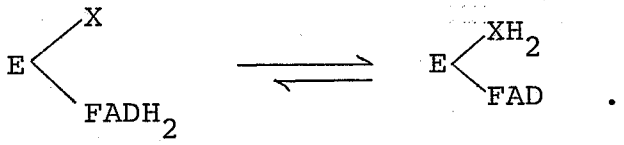
Michaelis et al (1970, 1971) studied the absorption spectrum change of the enzyme from D. vulgaris by the addition of its substrates without an added electron acceptor. These authors also observed the reduction of the enzyme-bound FAD and an increase of absorbance around 320 nm by the addition of sulfite. It has been shown by Massey's group that the flavin-sulfite complex at the N(5) position of isoalloxazine ring has a typical absorbance at 320 nm (Swoboda and Massey, 1966; Massey et al., 1969b; Müller and Massey, 1969). It was also reported by Michaelis et al. (1970, 1971) that there was a reduction of another chromophore, probably a nonheme iron, by the addition of AMP to the sulfite-reduced enzyme. From these observations, Michaelis et al. (1970, 1971) proposed a three step mechanism for APS reductase as follows: The first step involves the reversible association of sulfite with the enzyme-bound FAD to form an adduct at the N(5) position:



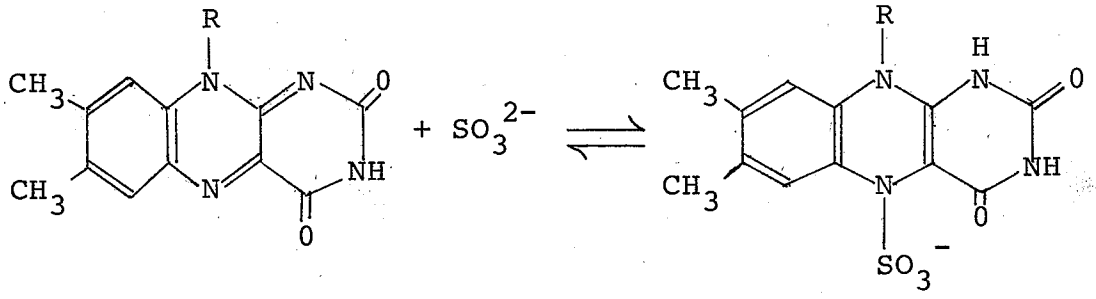
X represents the unknown chromophore, probably nonheme iron, either in the oxidized or reduced (H_2) state. The second step is the transfer of the sulfur moiety from FAD to AMP:



and the reduction of the chromophore was assumed as the last step:



The formation of the flavin-sulfite adduct at the N(5) position of the isoalloxazine ring was first reported for glucose oxidase (Swoboda and Massey, 1966) and was subsequently studied on several flavoproteins (Massey et al., 1969b) and on model compounds (Müller and Massey, 1969; Hevesi and Bruice, 1973).



The absorption spectra of the N(5) sulfite adducts of various flavin compounds appeared to have a common peak at 320 nm which is also common for the flavin-acetate complex at the N(5) position (Müller and Massey, 1969).

In all these cases the formation of the adduct took rather long periods of time, some cases as long as 30 min (Müller and Massey, 1969). Hevesi and Bruice (1973) noted that among the flavoenzymes investigated (oxidases, reductases, dehydrogenases, and hydroxylases) only the oxidases were found to yield flavin-sulfite adducts and the complex was enzymatically inactive. Thus a correlation exists between the ability of fully reduced flavoproteins to react with oxygen and the ability of their oxidized forms to react with sulfite.

Since APS reductase is not an oxidase, it seems strange that the enzyme from D. vulgaris formed the N(5) sulfite adduct which was catalytically active as an intermediate (Michaelis et al, 1970; 1971).

METHODS AND MATERIALS

(Adenosine 5'-phosphosulfate Reductase and Enzymes involved in Glutamate Synthesis in *Thiobacillus thioparus*)

MATERIALS AND METHODS

Materials

All chemicals and reagents used in this investigation were of analytical grade and obtained commercially. Bovine serum albumin (Fraction V), horse heart cytochrome c (Type III), all amino acids used, bovine hemoglobin, α-ketoglutarate (monopotassium salt), pyruvate, phosphoenolpyruvate, cis-aconitate, 2-mercaptoethanol, AMP, ADP, ATP, c-AMP, Tris (Trizma Base^{R}), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, γ-glutamylhydroxamate, rabbit muscle phosphorylase a, yeast alcohol dehydrogenase, bovine liver catalase, and milk xanthine oxidase were obtained from Sigma Chemical Co., St. Louis, Missouri. Superoxide dismutase was purchased from Miles Laboratories, Inc., Kankakee, Illinois. Dithiothreitol, NAD^{+}, NADH, $NADP^{+}$ and NADPH were products of P-L Biochemicals, Inc., Milwaukee, Wisconsin. Ammonium sulfate (special enzyme grade) used for ammonium sulfate fractionation was supplied by Schwarz/Mann, Orangeburg, New York. HEPES and cytochrome c from Candida krusei (prepared by Sankyo Co., Ltd., Tokyo, Japan) were purchased from Calbiochem., San Diego, California. Acrylamide, BIS, TEMED, ammonium

persulfate, and fumarate were obtained from J. T. Baker, Phillipsburg, New Jersey, L-(—)-malic acid from Eastman Kodak Co., Rochester, New York, and succinate (disodium salt) from Coleman and Bell, Norwood, Ohio. Sodium lauryl (dodecyl) sulfate, citrate (trisodium salt), NH_4Cl, $(NH_4)_2SO_4$ used for the medium, Na_2SO_3, and $K_3Fe(CN)_6$ were supplied by Fisher Scientific Co., Fair Lawn, New Jersey. Sephadex G-200, Blue Dextran 2000, and AH-Agarose 4B were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

All the reagents and the media were prepared in distilled water.

Organism and Medium

Thiobacillus thioparus was obtained from American Type Culture Collection (A.T.C.C. 8158). Crude extracts of Thiobacillus novellus and Nitrosomonas europaea were generously donated by Mr. J. K. Oh and Mr. S. C. Kwok in this laboratory, respectively.

T. thioparus was grown at 26°C on Starkey's No. 2 medium (Starkey, 1934) which contained 4 g KH_2PO_4, 4 g K_2HPO_4, 0.1 g $CaCl_2$, 0.1 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4.7H_20$, 0.02 g $FeCl_3.6H_20$, 0.02 g $MnCl_2$, and 10 g $Na_2S_2O_3.5H_20$ per liter. Phenol red was added as pH indicator to the

medium except for the pH-stat culture. Sodium thiosulfate was autoclaved separately and mixed together before inoculation. The solid medium contained 2% agar.

Stock cultures were maintained in agar slants made in 25 ml screw cap test tubes. The liquid culture was regularly streaked on agar plates and a sulfur-forming colony after 14 days of incubation was transferred to the slant as stock culture. In order to start a liquid culture, the cells grown on a slant were suspended in 2 ml of the medium and transferred to a 50 ml culture in 125 ml flask. The culture was successively inoculated into 150 ml (250 ml flask) and 1.5 l (2 l flask) cultures. These flask cultures were grown on a rotary shaker (150 r.p.m.) until pH of the cultures became lower than 5.5 (orange-yellow in color). The incubation time was usually 2 days for 50 ml cultures, 3 days for 150 ml cultures and 6 days for 1.5 l cultures.

The cells used in the investigation of adenosine 5'-phosphosulfate (APS or adenylyl sulfate) reductase and glutamate dehydrogenase were grown in a batch culture of 18 l Pyrex^{R} glass carboy which contained 15 liters of the medium with forced aeration. The pH of the culture was maintained between 6 and 7 by the addition of a 15% K_2CO_3 solution as required. The cells were harvested

after 4 days of growth. The cells used in the investigation of glutamate synthase and the levels of enzymes involved in glutamate synthesis were grown on 20 liters of the medium in 22 ℓ Quickfit^{R} (England) glass carboys with forced aeration. The carboy was fitted with a Radiometer (Denmark) pH-stat unit (Model 28 pH meter, Model GK 2303 C combination electrode, Model TTT 11 titrator, and Model MNV 1 magnetic valve). The pH of the culture was maintained above 6.5 by the addition of a 15% K_2CO_3 solution by the pH-stat unit. After 84 hours (3½ days) of growth, the cells were harvested. A Sharples centrifuge (England) or a Cepa centrifuge (West Germany) was used to harvest the cells with the flow-rate of 250 ml or 150 ml per minute, respectively. About 8 g of cells were obtained per 20 liters.

The purity of the culture was routinely checked by streaking it on agar plates of Starkey's No. 2 medium and Difco Nutrient Agar. A contamination of heterotrophic bacteria was detected on the Nutrient Agar plates after 48 hours of incubation at 28^{o}. Contaminating autotrophic bacteria were detected by their formation of non-sulfur-forming colonies on the autotrophic medium plates after 7 to 10 days of growth at 26^{o}C.

The harvested cells were suspended in 0.1 M potassium phosphate buffer (pH 7.5) and stirred for 15 min at 4^{o}C. The cell suspension was then centrifuged at

27,000 x g for 20 min and the packed cells were separated from the sulfur layer and stored at -20°C until needed.

Preparation of Crude Extract

1. T. thioparus - The methods used to prepare the T. thioparus crude extract are specified in each experiment ("Results").

2. T. novellus - The cells were suspended in 0.05 M potassium phosphate buffer (pH 7.5) at the ratio of 200 mg cells (wet weight) per ml. The cell suspension was sonicated for 15 min under nitrogen atmosphere in a Raytheon sonic oscillator at 7°C. The sonicate was centrifuged at 2,000 x g for 20 min and the supernatant was taken as crude extract.

3. N. europaea - The suspension of cells (30 mg wet weight per ml) in 0.1 M potassium phosphate buffer (pH 7.5) containing 20 mg bovine serum albumin per ml was passed through a French Pressure Cell (Aminco) at 18,000 p.s.i.. The homogenate was centrifuged at 2,000 x g for 20 min and the supernatant was carefully collected as crude extract. The crude extract usually contained 3.8 mg of cell protein per ml.

Determination of Protein

The protein content of the enzyme solution was determined by the colorimetric method of Lowry et al. (1951) at room temperature using bovine serum albumin as the reference protein. The extent of color formation was read in a Klett-Summerson photoelectric colorimeter using a red (#66) filter.

Enzyme Assay Procedures

1. APS reductase - APS reductase was assayed with ferricyanide or cytochrome c as electron acceptor by the method of Lyric and Suzuki (1970 b) with a little modification.

In the ferricyanide assay the reaction mixture contained 1 mM $K_3Fe(CN)_6$, 1 mM Na_2SO_3, 50 mM potassium phosphate buffer (pH 7.5), 0.5 mM AMP and the enzyme. The reduction of ferricyanide was followed at 420 nm.

Using the cytochrome c coupled assay, the standard reaction mixture contained 0.1 mM yeast cytochrome c, 0.1 mM Na_2SO_3, 0.1 mM AMP, 10 mM Tris-Cl (pH 8.7) and the enzyme. The reduction of cytochrome c was followed at 550 nm.

AMP was added last to initiate the reaction. The activity obtained without AMP was subtracted from that with AMP to calculate the actual activity.

2. NADP-dependent glutamate dehydrogenase - This enzyme was assayed in both directions by following either the oxidation of NADPH or the reduction of $NADP^+$ at 340 nm. The standard reaction mixture for the reductive amination procedure contained 20 mM α-ketoglutarate, 160 mM NH_4Cl, 0.15 mM NADPH, 100 mM Tris-Cl (pH 8.5) and the enzyme.

For the oxidative deamination assay the reaction mixture contained 33 mM L-glutamate, 0.33 mM $NADP^+$ and 100 mM glycine-NaOH (pH 9.5).

Glutamate was added last to initiate the reaction. One unit of enzyme was defined as that amount causing the oxidation of 1 μmole NADPH or the reduction of 1 μmole $NADP^+$ per minute using the proper assay mixture.

3. NAD-dependent glutamate dehydrogenase - For the reductive amination assay the standard reaction mixture contained 20 mM α-ketoglutarate, 200 mM NH_4Cl, 0.15 mM NADH, 100 mM Tris-Cl (pH 8.0) and the enzyme.

The same reaction mixture as the NADP-dependent enzyme was used for the oxidative amination assay except using NAD^+ instead of $NADP^+$.

One unit of enzyme was defined as for the NADP-dependent enzyme.

4. Glutamate synthase - The rate of glutamate synthase activity was determined by following the oxidation of NADPH at 340 nm. The reaction mixture contained 0.5 mM α-ketoglutarate, 5 mM L-glutamine, 0.075 mM NADPH, 50 mM Tris-Cl (pH 7.8) and the enzyme. The reaction was initiated by the addition of either α-ketoglutarate or glutamine.

A unit of the glutamate synthase activity was defined as the amount of enzyme that oxidized 1 μmole of NADPH per minute.

5. Glutamine synthetase - The activity of glutamine synthetase was determined by γ-glutamyltransferase assay described in Shapiro and Stadtman (1970). A standard curve was prepared with commercially obtained γ-glutamyl-hydroxamate. Both ADP and potassium arsenate were omitted for controls. The samples were read in a Klett-Summerson photoelectric colorimeter using a green (#54) filter after centrifugation to remove the precipitate.

One unit of enzyme was defined as that amount required to catalyze the synthesis of 1 μmole of γ-glutamylhydroxamate per minute.

6. Xanthine oxidase - Xanthine oxidase was obtained commercially. The reaction mixture contained 0.05 mM xanthine, 0.01 mM horse heart cytochrome c, 0.1 mM

NaEDTA, 50 mM potassium phosphate buffer (pH 7.8) and the enzyme sufficient to produce a rate of reduction of the cytochrome c at 550 nm of 0.025 absorbance unit per min.

7. Superoxide dismutase - This enzyme was also obtained commercially. The assay conditions were specified in each experiment ("Results"). One unit of enzyme was defined as described by McCord and Fridovich (1969) except the reaction was performed at room temperature (approximately 23^{o}C). That is, under the defined assay conditions for xanthine oxidase, the amount of superoxide dismutase required to inhibit the rate of reduction of cytochrome c by 50% was one unit.

8. Anaerobic enzyme assay - Anaerobic assays of APS reductase and xanthine oxidase were performed in an anaerobic cuvette. The system was evacuated with a water pump and filled up with argon gas. In order to remove the air from the system thoroughly, this procedure was repeated 10 times.

Spectrophotometry

Routine spectrophotometric determinations were performed at room temperature using Unicam SP500 or SP700 spectrophotometer, Gilford Model 2000 or Model 2400

spectrophotometer, Beckman Acta III spectrophotometer, or a Shimadzu MPS-50 spectrophotometer as indicated. One centimeter lightpath silica cuvettes were used in all experiments.

Stopped-flow Spectrophotometry

For the determination of enzymic spectrum change of APS reductase, a Durrum stopped-flow spectrophotometer was used. This stopped-flow spectrophotometer was equipped with a Durrum photometric log amplifier Model D-133 and a Tektronix storage oscilloscope. The lightpath length of the stopped-flow cuvette was 20 mm and it held 0.3 ml of the reaction mixture. This instrument was built by Durrum Instrument Co., California, on the basis of the work of Gibson and Milnes (1964) and was described by Gibson (1969) and Schechter (1970).

Preparation of Affinity Gel

Two types of affinity gels were prepared from AH-Sepharose 4B (Pharmacia) with either α-ketoglutarate or L-glutamine as ligand. In routine carbodiimide coupling procedure, 2.5 g of freeze-dried AH-Sepharose 4B were placed in a 125 ml beaker and washed with 80 ml of 0.5 M NaCl five times and then with 80 ml of distilled water

five times. The coupling mixture contained washed AH-Sepharose 4B (2.5 g dry weight), 3.7 g α-ketoglutarate (monopotassium salt) or 1.7 g L-glutamine, 0.77 g 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and distilled water to the volume of 40 ml. The mixture was incubated at room temperature for 36 hours with gentle propeller stirring. The pH of the solution was kept between 4.5 and 6.0. After the incubation, the gel was washed thoroughly with 0.1 M Tris-Cl buffer (pH 9.0) containing 1.0 M NaCl and 0.1 M acetate buffer (pH 4.0) containing 1.0 M NaCl alternately to remove non-covalantly bound ligands. After a further washing with water the gel was equilibrated with a buffer of choice and was packed into a column (1 x 10 cm). When the gel was not in use, it was stored in 0.1 M acetate buffer (pH 4.0) with 1.0 M NaCl at 4°C.

Sucrose Density Gradient Centrifugation

Sucrose density gradient centrifugation was performed as described by Martin and Ames (1961). Sucrose gradients (5 to 20%) were prepared in 0.1 M potassium phosphate buffer (pH 7.5). Samples (0.1 ml in the same buffer containing 5% sucrose) were layered on 5 ml gradients in Beckman cellulose nitrate tubes (½ x 2 inch).

The tubes were centrifuged in a Beckman ultracentrifuge Model L2-65B at 50,000 r.p.m. for 4 hours at 4°C using a SW 50 rotor. At the end of the run the tubes were ruptured at the bottom and the gradient was passed through a flow-through cuvette (Hellma) by means of a Buchler Polystaltic^{R} pump. The absorbance at 280 nm or at 450 nm was recorded on a Gilford Model 2000 spectrophotometer. The top of the gradient was conveniently recorded as a sharp jump of the pen on the chart paper caused by air bubbles as the sample run out.

Molecular Weight Determination

The molecular weight of glutamate synthase was determined by means of gel filtration as described by Andrews (1964). A Sephadex G-200 column (2.5 x 92 cm) was equilibrated with 0.05 M potassium phosphate buffer containing 5 mM NaEDTA and 1 mM dithiothreitol. The void volume was measured with Blue dextran 2000 (Pharmacia) dissolved in the same buffer. Reference proteins used were bovine serum albumin (Molecular weight; 68,000), yeast alcohol dehydrogenase (151,000), bovine liver catalase (250,000) and milk xanthine oxidase (290,000) (Avis et al.,1956). The proteins were dissolved in the same buffer at the ratio of 25 mg per 10 ml.

Disc Polyacrylamide Gel Electrophoresis

The electrophoresis was performed at pH 8.5 according to the method of Baker et al. (1972). Upper gel was omitted. The gels were polymerized from a mixture containing 5% acrylamide, 0.13% BIS, 25 mM DL-asparagine, 19 mM Tris, 0.07% ammonium persulfate, 0.05% ($^{v}/_{v}$) TEMED and 25% glycerol. The upper and lower electrode buffers were identical and contained 25 mM DL-asparagine,19 mM Tris, and pH was 8.5. Gels were stained with 0.025% Coomassie brilliant blue in 10% acetic acid and 50% methanol. Destaining solution contained 7.5% acetic acid and 5% methanol.

For the routine preparation of gels the following stock solutions were used:

a) Gel buffer (pH 8.5)
   1.32 g DL-asparagine
   0.92 g Tris
   0.2 ml TEMED
   Water to 100 ml.

b) 20 g Acrylamide
   0.52 g BIS
   Water to 100 ml.
   (Stored in a dark and cool place)

c) 0.14 g Ammonium persulfate

50 ml Glycerol

Water to 100 ml

The solutions were mixed at the ratio of a: b: c = 1:1:2 and poured into glass tubes. The gel size of 6 x 70 mm was usually used. Protein solution was prepared in 25% glycerol and bromophenol blue (BPB) was used as the tracking dye. Electrophoresis was performed with constant current of 2 mA per tube until the dye reached the bottom of the tubes.

Sodium Dodecyl Sulfate (SDS) Gel Electrophoresis

Polyacrylamide gel electrophoresis in the presence of 0.1% SDS at pH 7.5 was performed according to the procedure of Weber and Osborn (1969). The protein solution was boiled for 5 min with 1% SDS and 1% ($^{v}/_{v}$) 2-mercaptoethanol before application.

For the routine preparation of gels the following stock solutions were prepared:

a) Gel Buffer (pH 7.5)

7.8 g of $NaH_2PO_4.H_20$

38.6 g of $Na_2HPO_4.7H_20$

2.0 g of SDS

Water to 1 liter

b) 22.2 g of acrylamide

0.6 g of BIS

Water to 100 ml

(Stored in a dark and cool place)

c) 0.15 g ammonium persulfate

Water to 10 ml

The solutions were mixed at the ratio of a : b : c = 15 : 13.5 : 1.5 and poured into glass tubes. The gel size was usually 6 x 70 mm.

Samples were prepared for each gel as follows: 50 µl of boiled protein solution (prepared as mentioned above containing about 50 to 100 µg protein), 50 µl of the gel buffer, 5 µl of 2-mercaptoethanol, 1 drop of tracking dye (0.05% BPB), and a drop of glycerol.

The following proteins were used as the standard: Hemoglobin (15,500), yeast alcohol dehydrogenase (37,000), serum albumin (68,000) and rabbit muscle phosphorylase a(94,000).

RESULTS

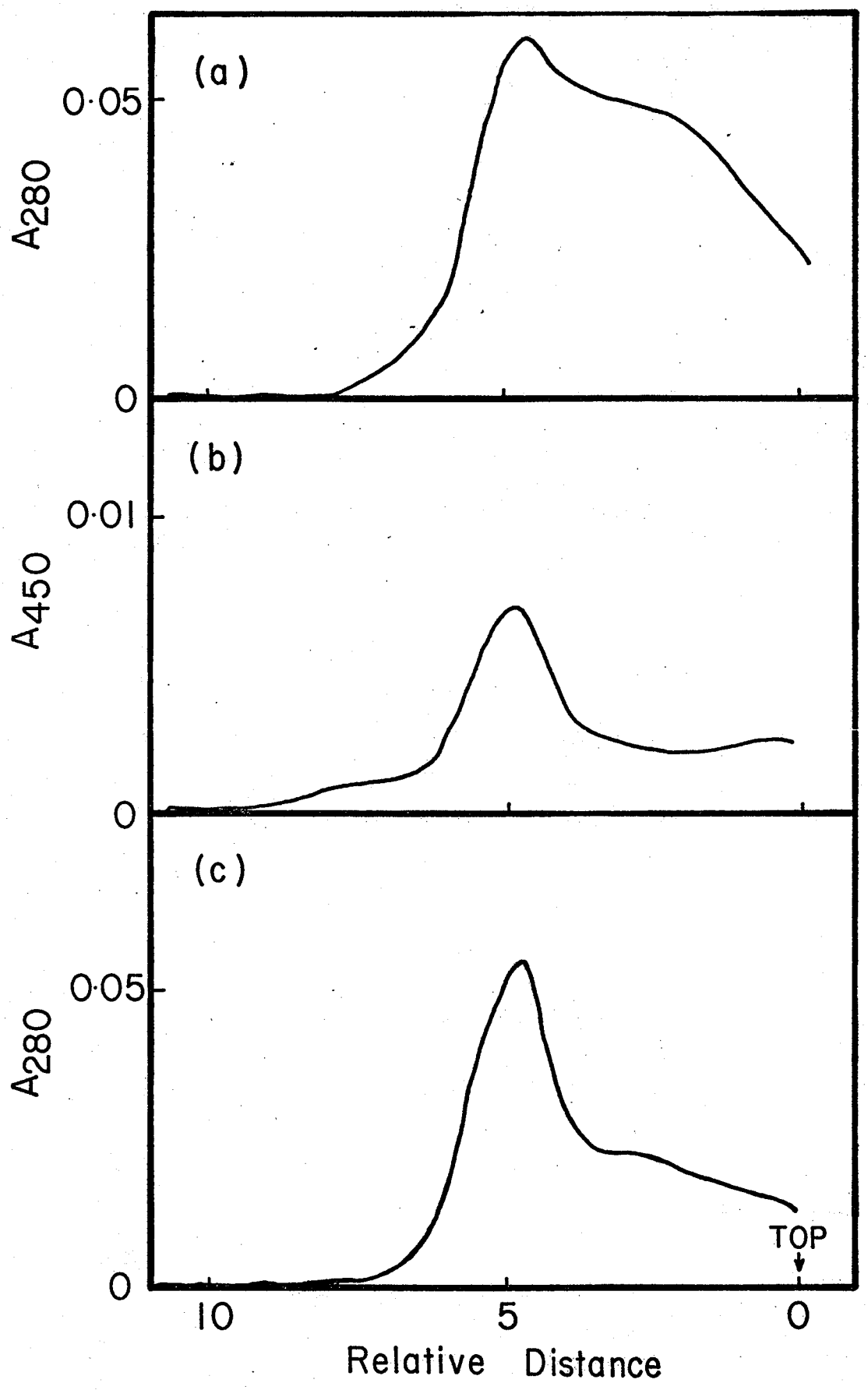
Figure 1. Sedimentation profile of APS reductase in sucrose density gradients.

a) A_{280} profile of APS reductase before the heat treatment.

b) A_{450} profile of APS reductase before the heat treatment.

c) A_{280} profile of APS reductase after the heat treatment.

Details are described in "Methods".


(a)
0·05
A280
0
(b)
0·01
A450
0
(c)
0·05
A280
0
TOP
10
5
0
Relative Distance

the relative distance scale and a broad shoulder around 2.5. It has been shown that APS reductase is an iron-sulfur flavoprotein and is yellow in color (Lyric and Suzuki, 1970 b). Fig. 1b shows the position of a yellow band in the gradient after the original enzyme was centrifuged. The protein sedimentation pattern of the heat-treated enzymes, shown in Fig. 1c, indicates that a large portion of the contaminating proteins was removed by this treatment.

II. Enzyme Mechanism

The mechanism of APS reductase has been of particular interest because the oxidation of sulfite in the presence of AMP results in the formation of a phosphosulfate bond of adenosine 5'-phosphosulfate (APS) which was shown to have a high ΔF^{o} of formation of 18-19 kcal per mole at pH 8.0 (Robbins and Lipmann, 1958).

An Ordered Quad Ter reaction has been proposed as a kinetic mechanism of APS reductase from T. thioparus based on the initial velocity and product inhibition studies with cytochrome c as an electron acceptor (Lyric and Suzuki, 1970 d). These authors observed the reduction of the enzyme-bound FAD by sulfite and also further decrease of absorbance between 400 and 500 nm, by the addition of dithionite to the enzyme (Lyric and Suzuki, 1970 b).

Michaelis et al. (1970, 1971) have carried out the spectral studies on the enzyme from D. vulgaris without an added electron acceptor and proposed a three step mechanism which involved a FAD-sulfite adduct formation at the N(5) position of the isoalloxazine ring as an intermediate.

1. Cytochrome c Reduction

The formation of superoxide radicals - Superoxide dismutase which catalyzes the dismutation of superoxide radicals ($O_2^- + O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2$; McCord and Fridovich, 1969) would inhibit the reduction of cytochrome c by superoxide radicals which are generated, for example, during the reaction of xanthine oxidase (Fridovich and Handler, 1962). The formation of superoxide radicals by nonheme irons and flavins has been discussed by various workers (Knowles et al., 1969; Komai et al., 1969; Massey et al., 1969a; Ballou et al., 1969; Nakamura, 1970; Fridovich, 1975).

Bramlett and Peck (1975) have reported that APS reductase from D. vulgaris was inhibited 100% by anaerobiosis and 85% by 10^{-6}M superoxide dismutase in the presence of air. These authors suggested that superoxide radicals are responsible for the reduction of cytochrome c in this enzyme system.

In order to find out whether superoxide radicals are involved in the reaction catalyzed by APS reductase from T. thioparus, a study of the effects of anaerobiosis and superoxide dismutase on the reduction of cytochrome c was performed. The results are shown in Fig. 2.

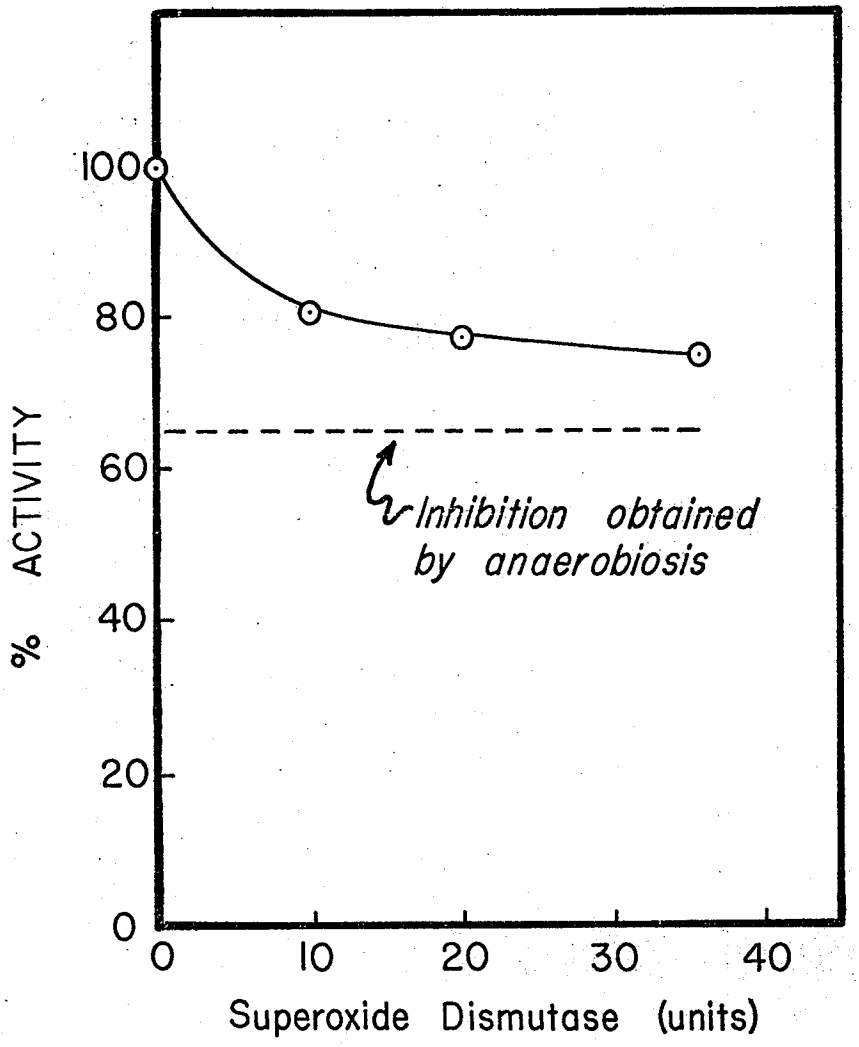
Anaerobic enzyme assays were performed in an anaerobic cuvette. The air was removed from the system by repeating the cycle of evacuating the cuvette with a water pump and filling it up with argon gas 10 times.

Anaerobiosis resulted in only 35% inhibition of the reductase activity with cytochrome c as electron acceptor unlike the enzyme from D. vulgaris which was 100% inhibited. Under the same anaerobic conditions xanthine oxidase activity was totally inhibited indicating that the air removal was complete.

In the presence of air, the addition of increasing amounts of superoxide dismutase displayed increasing degree of inhibition, but only up to 26% with 36 units of the dismutase (Fig. 2). No inhibition of the ferricyanide assay was observed at the superoxide dismutase concentration of 10 units.

The unit for the dismutase activity was defined as that of McCord and Fridovich (1969) except room temperature was used for the assays. One unit of superoxide dismutase activity was usually obtained at

Figure 2. Effect of superoxide dismutase and anaerobiosis on the APS reductase activity. The cytochrome c coupling assay was performed using 0.22 mg enzyme per 3 ml under the standard conditions except either superoxide dismutase was added at the concentration indicated or it was assayed under the anaerobic conditions. The conditions of anaerobiosis and the determination of the superoxide dismutase unit were as described in "Methods". The results are presented as a percentage of the activity under the standard conditions.


100
80
60
40
20
0
% ACTIVITY
Inhibition obtained
by anaerobiosis
0
10
20
30
40
Superoxide Dismutase (units)

the enzyme concentration of 1.6×10^{-8}M or 0.5 µg/ml.

These results show that there definitely is a direct reduction or binding of cytochrome c by the T. thioparus enzyme although superoxide radicals are formed during the aerobic reaction.

Bramlett and Peck (1975) noted that the K_m values for AMP and sulfite of the D. vulgaris enzyme did not vary appreciably with ferricyanide and cytochrome c as electron acceptors while the K_m values for sulfite were over 10-fold lower with cytochrome c than those with ferricyanide in microorganisms where the physiological function of the reductase was the oxidation of sulfite.

It was also reported that the ratio of the enzyme activities measured using ferricyanide and cytochrome c was 80:1 in T. thioparus system (Lyric and Suzuki, 1970 b) while it was 1000:1 in D. vulgaris system (Michaelis et al., 1971).

From these observations it was concluded that the mechanism of electron transfer to cytochrome c by T. thioparus enzyme differs from that by D. vulgaris enzyme.

The effect of the phosphate buffer concentration - During the course of experiments performed to find out whether cytochrome c could be used as electron acceptor in potassium phosphate buffer (pH 7.5), it was found that the concentration of the buffer had a significant effect on the enzyme activities measured with cytochrome c from both Candida krusei and horse heart (Fig. 3).

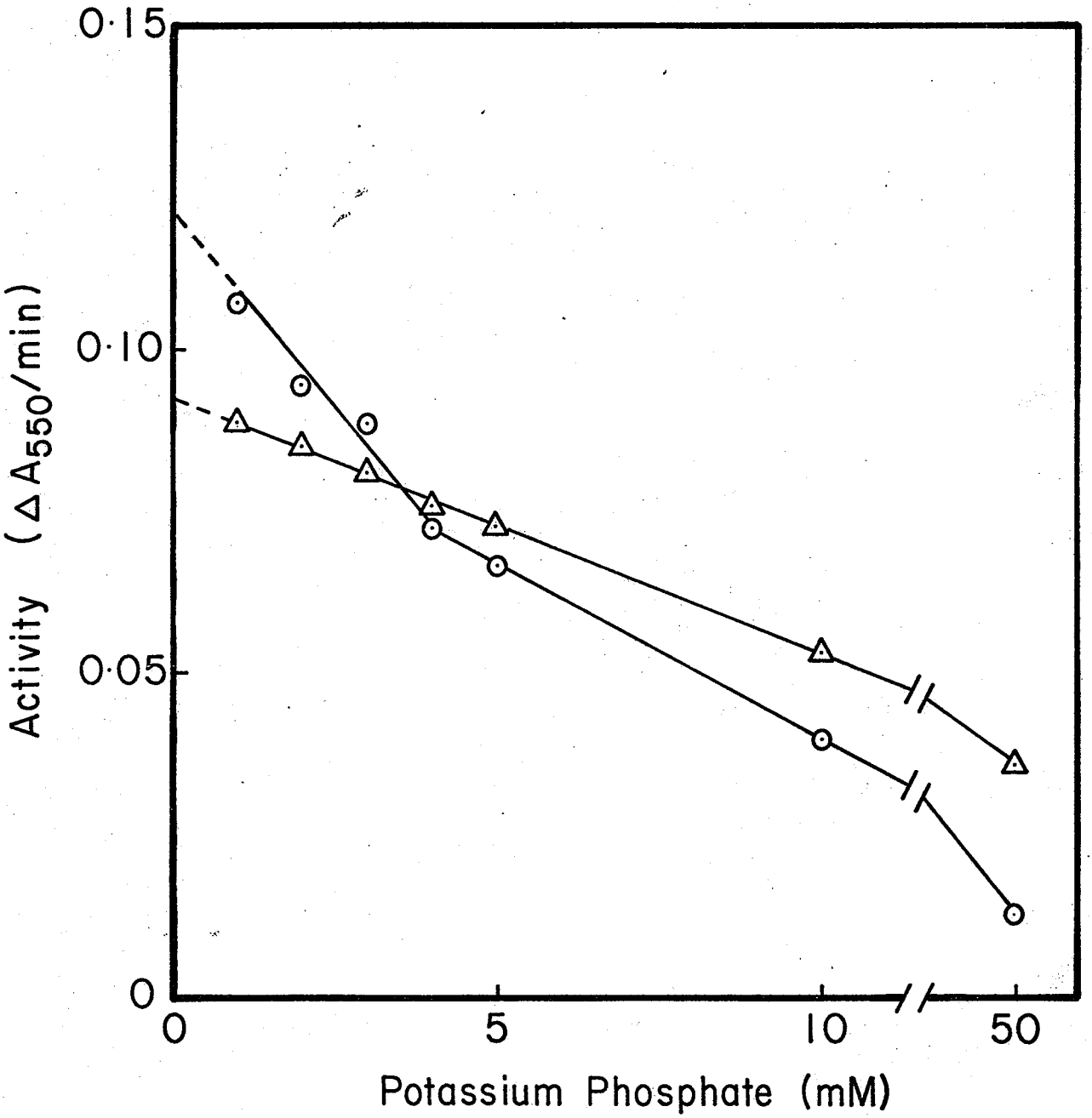
In the horse heart cytochrome c assay the activity increased 8 times when the buffer concentration was reduced from 50 mM to 1 mM while it increased only 2.5 times in the yeast cytochrome c assay.

In 50 mM potassium phosphate, the horse heart cytochrome c was only 30% as active as the yeast cytochrome c as the electron acceptor, but when the buffer concentration was lower than 3 mM the horse heart cytochrome c became a slightly better electron acceptor than the yeast cytochrome c (Fig. 3).

It is possible that the differences in reactivity of two cytochromes reported on APS reductase from T. thioparus (Lyric and Suzuki, 1970 b) and that from D. vulgaris (Bramlett and Peck, 1975) were due to the different responses by these cytochromes towards the salt concentration.

The effect of different conditions on cytochrome c assay was not investigated any further.

Figure 3. Effect of potassium phosphate concentration on the cytochrome c reduction by APS reductase. The assay was performed using either yeast (-△-△-) or horse heart (-⊙-⊙-) cytochrome c as electron acceptor. Phosphate buffer (pH 7.5) was used at the concentration as indicated in the figure. The concentrations of substrates were the same as described in "Methods".


0·15
0·10
0·05
0
Activity (Δ A550/min)
0
5
10
50
Potassium Phosphate (mM)

2. Difference Spectra

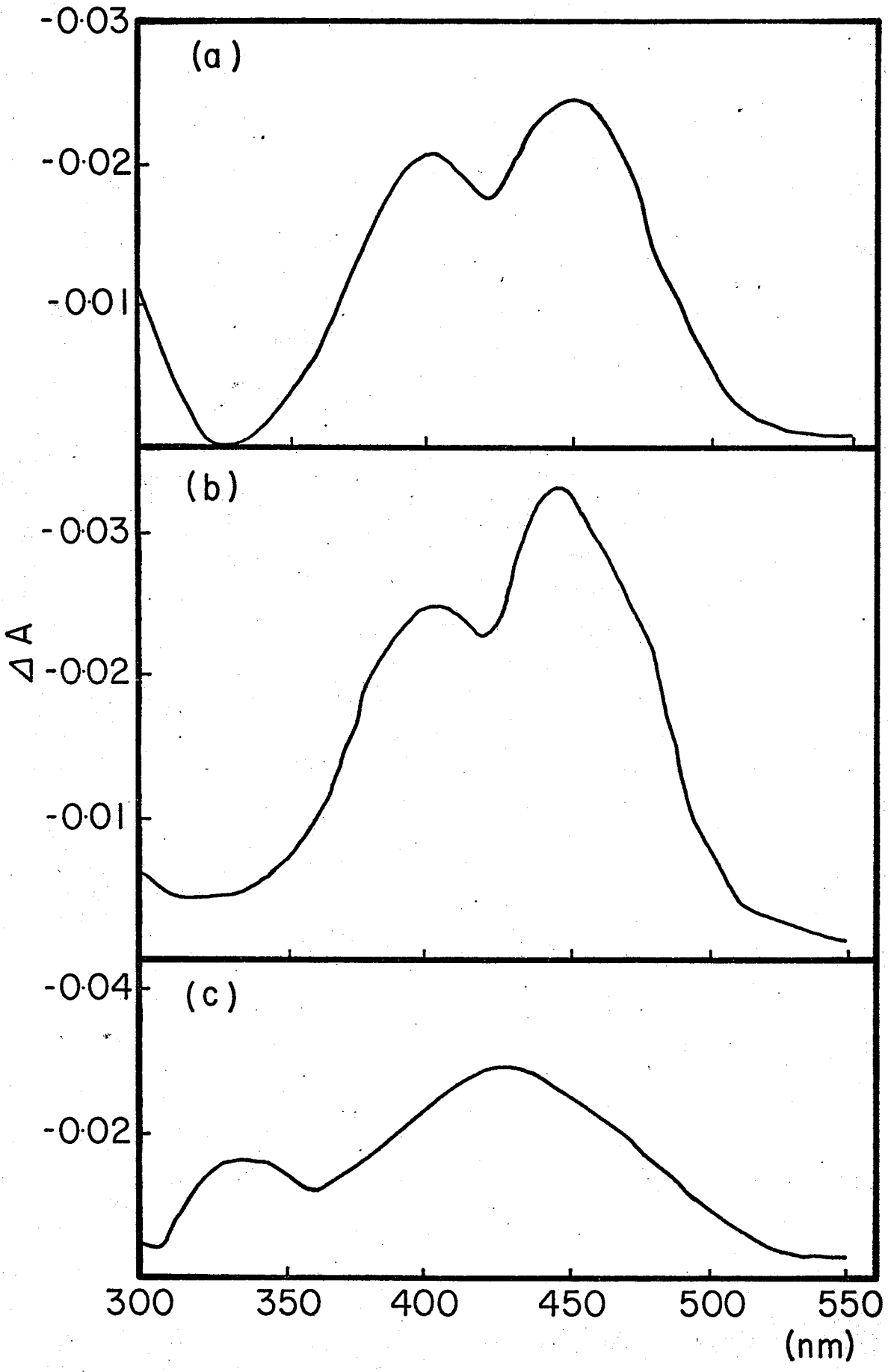
APS reductase has been shown to be an iron-sulfur flavoprotein. The enzyme from T. thioparus possessed one mole of FAD per mole of protein and the ratio of iron to sulfide to FAD was 8-10: 4-5 : 1 (Lyric and Suzuki, 1970 b).

Spectral changes of the enzyme caused by the addition of substrate(s) in the absence of an added electron acceptor were studied in a Shimadzu MPS spectrophotometer. The results are shown in Fig. 4.

The enzyme was partially bleached by the addition of sulfite alone. The difference spectrum is shown in Fig. 4 a which exhibits maximum at 395 and 450 nm. This result indicates that the enzyme bound FAD was reduced by sulfite. In the absence of sulfite, the addition of AMP caused no significant change to the enzyme spectrum. These results were consistent with those obtained by Lyric and Suzuki (1970 b) with the same enzyme and Michaelis et al. (1970, 1971) with the D. vulgaris enzyme.

When AMP was added to the enzyme which had been previously mixed with sodium sulfite a further decrease in absorbance was observed. Fig. 4 b shows the difference spectrum caused by the addition of both sulfite

Figure 4. Difference spectra resulting from the sequential addition of sulfite and AMP to APS reductase. Enzyme concentrations were 3.6 mg per ml in (a) and (b), and 7.2 mg per ml in (c). Curve a, ($NaSO_3$, $3x10^{-3}M$) minus (oxidized); Curve b, ($NaSO_3$, $3x10^{-3}M$ plus AMP, $3x10^{-4}M$) minus (oxidized); Curve c, ($NaSO_3$, $3x10^{-3}M$ plus AMP, $3x10^{-4}M$) minus ($NaSO_3$, $3x10^{-3}M$).


-0·03
-0·02
-0·01
(a)
(b)
-0·03
-0·02
-0·01
ΔA
-0·04
(c)
-0·02
300
350
400
450
500
550
(nm)

and AMP. In order to record accurately the spectral change induced by AMP to the sulfite-reduced enzyme, the difference spectrum (Fig. 4 c) was taken with a higher enzyme concentration (7.2 mg per ml). The spectrum exhibits maxima at 330-340 nm and 420-440 nm and is quite different from that in Fig. 4 a obtained with sulfite alone. It was concluded that one component, at least, of the enzyme other than FAD was reduced by this process. The difference spectrum obtained by Michaelis et al. (1970, 1971) in a comparable experiment had similar two peaks, but at slightly shorter wavelengths (320 nm and 400-430 nm). This spectral change (Fig. 4 c) may be caused by the reduction of nonheme iron and the overall features of the difference spectrum resemble those obtained upon the reduction of ferredoxin (Tagawa and Arnon, 1962, peaks at 315 nm and 420 nm; Mayhew et al., 1968, 315 nm and 415 nm) and high potential iron proteins from Rhodopseudomonas and Chromatium (Dus et al., 1967, 340 nm and 475 nm).

Since these different spectra were measured rather long periods of time (several minutes) after the addition of substrates compared to the rapid rate of enzymic reactions, they would not reveal the spectral features of the initial intermediates, if any, generated in the course of reactions before reaching a new

equilibrium. In order to study each step of reactions initiated by the addition of substrates in a short period of time, a fast rate measurement of the spectral change was necessary. A series of experiments were carried out in a stopped-flow spectrophotometer for this purpose.

3. Stopped-Flow Experiments

Fast rate spectral changes of APS reductase induced by its substrates were measured by a stopped-flow spectrophotometer. The enzyme concentration after mixing in the apparatus with the substrate(s) was 1.7 mg per ml, or 10^{-5}M assuming the enzyme was pure, throughout the experiments.

Instrumental time constants were set at 1/10 of oscilloscope sweep rates per division or the closest settings available on the equipment. All the stopped-flow experiments were carried out at the room temperature. The solutions were well equilibrated with the room temperature before mixing to minimize absorbance changes caused by temperature equilibration process.

A. Control Experiments

Determination of the mixing time - A model reaction was performed in order to determine the mixing time of the stopped-flow equipment. The following solutions were prepared and set into the reservoir syringes separately.

Solution A: 0.01 M $Fe(NO_3)_3$ in 0.1 N H_2SO_4

B: 0.01 M KCNS

The production of a deep red color was followed by the stopped-flow spectrophotometer at 650 nm and recorded on the oscilloscope. The results are shown in Fig. 5. The total mixing volume was adjusted as 0.5 ml in a and 0.3 ml in b.

The lower straight lines in both pictures were produced after the mixing of the reactants and they were set as 100% transmission. A line in the upper part of Fig. 3 b was produced with the distilled water.

From the figures, the mixing time was determined as 15 ms with 0.5 ml sample and 12 ms for 0.3 ml. The total mixing volume of 0.3 ml was used in all stopped-flow experiments hereafter.

Detection of the absorbance self-zero adjustment pulse from the log amplifier - In order to find out the effect of the absorbance self-zero pulse from the log amplifier

on the results, the oscilloscope was activated manually by pushing the trigger button on the log amplifier. Fig. 6 shows the results obtained with the time setting of 0.5 ms per division (a) and with 5 ms per division (b). The width of the pulse was measured as 2 ms and it was concluded that it would not have any effect on the results of experiments performed in this stopped-flow system with 12 ms mixing time.

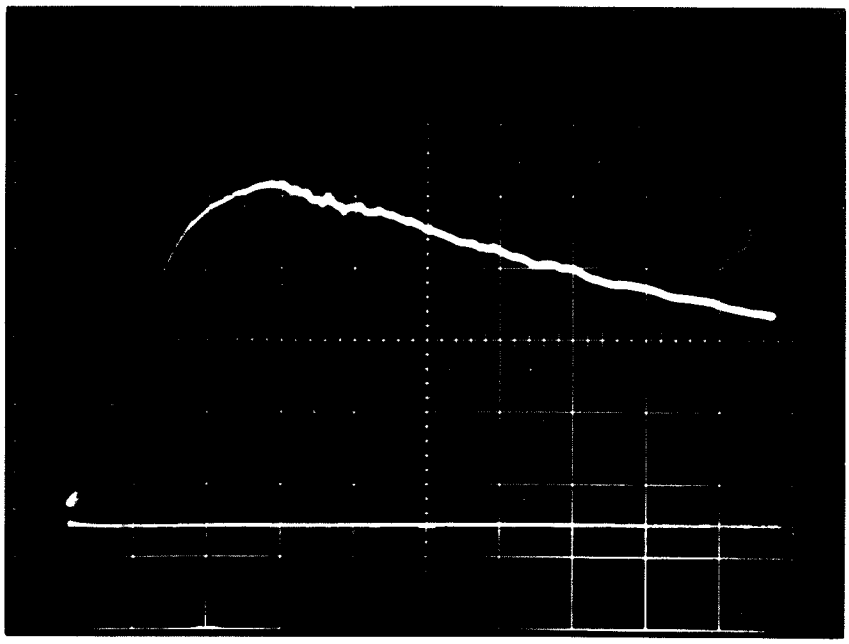
Quick dilution of the enzyme - APS reductase (3.4 mg per ml) was mixed with the same amount of potassium phosphate buffer (0.1 M, pH 7.5) and the absorbance at 450 nm (a) and 550 nm (b) were recorded. The results are shown in Fig. 7. In both cases a sharp increase in absorbance was detected between 80 ms and 100 ms after the dilution (0.003 at 450 nm and 0.0015 at 550 nm).

Quick dilution of the cytochrome c - Fig. 8 shows the result of cyt. c dilution on the absorbance at 550 nm. Cyt. c concentration was 10^{-4}M before the mixing with phosphate buffer. The signal obtained also exhibits a sharp absorbance jump between 80 ms and 100 ms.

A signal obtained with water - The absorbance change at 450 nm was followed with distilled water in both syringes. The result is shown in Fig. 9 and

Figure 5. Oscilloscope trace of transmission change process at 650 nm occurring for the reaction: $Fe^{3+} + SCN^{-} \longrightarrow FeSCN^{2+}$. The concentration of each reactant was 10 mM. Oscilloscope sweep rate was set at 5 milliseconds per division. The sweep was made from left to right. The vertical scale was set at 0.5 V per division which was identical to 5 % transmission change per division. T% increased towards the top of oscilloscope. The total volume of the sample per experiment was 0.5 ml in (a) and 0.3 ml in (b).

a



b

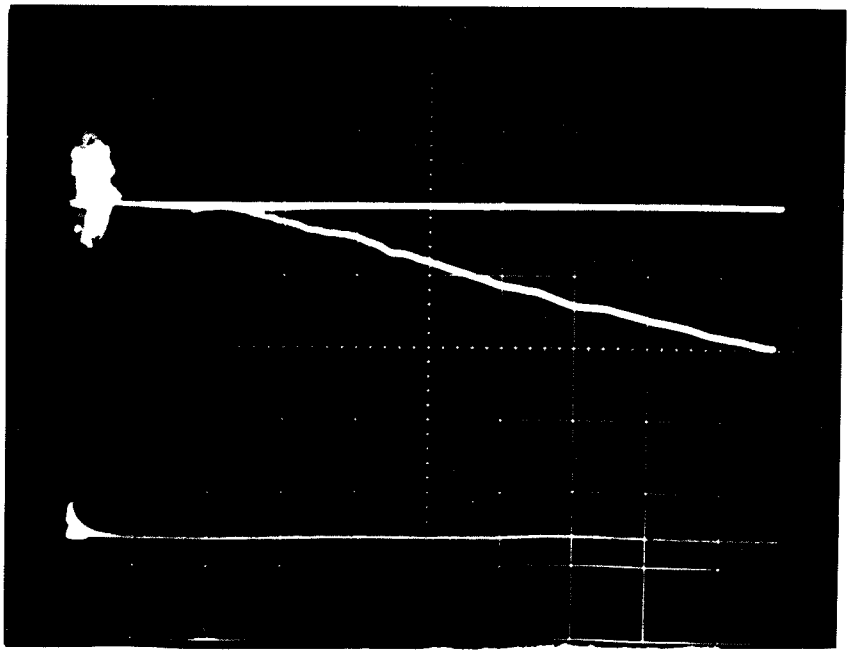
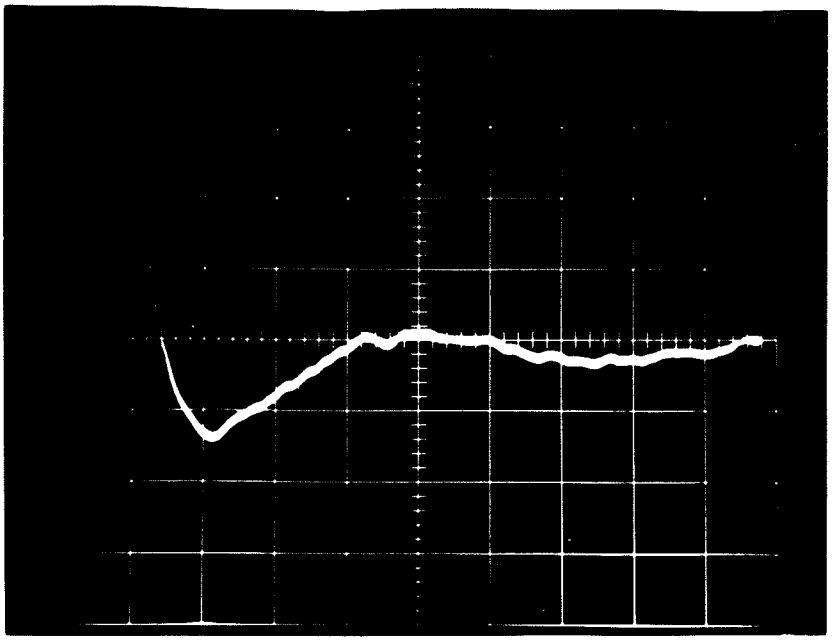


Figure 6. Oscilloscope trace of the absorbance self-zero adjustment pulse from the photometric log amplifier. The oscilloscope was activated by a trigger button on the log amplifier. The sweep rate was set at : (a) 0.5 ms/div.; (b) 5 ms/div.. The vertical scale was set at 50 mV/div. for both which was identical to the absorbance change of 0.005 per division. The absorbance increased towards the top.

a



b

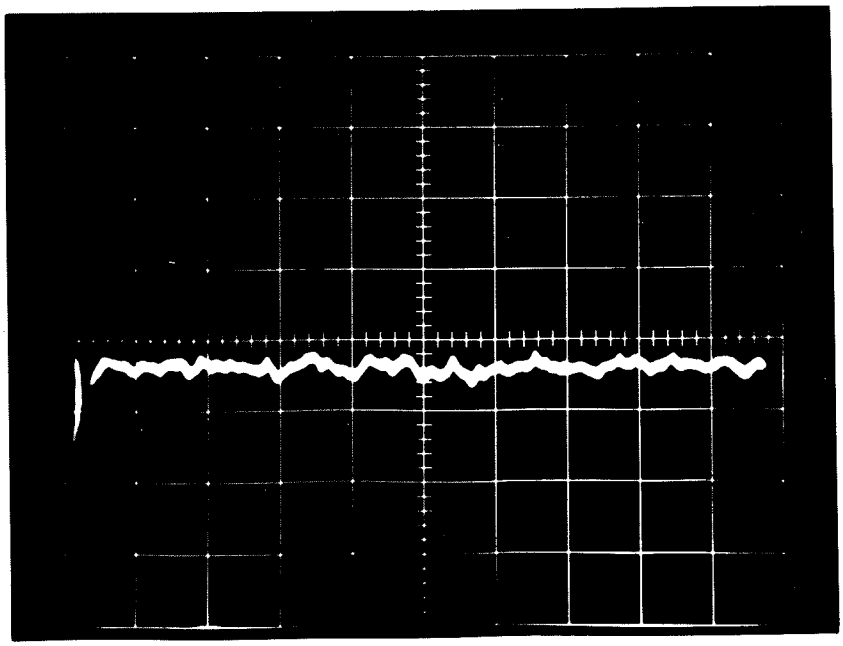
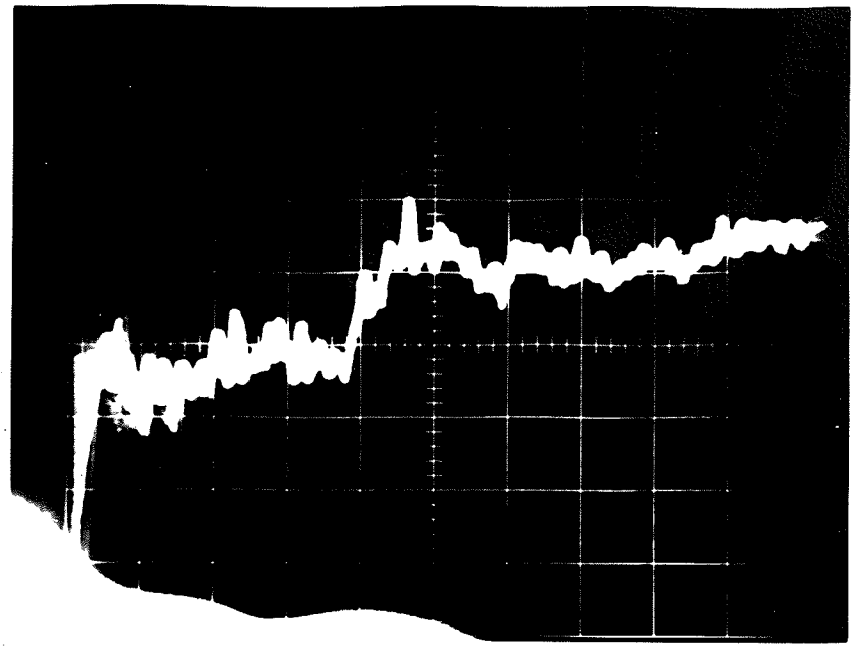


Figure 7. Effect of quick dilution on the absorbance of APS reductase. APS reductase (3.4 mg per ml) was mixed with the same volume of phosphate buffer (0.1 M, pH 7.5). The processes of absorbance changes at 450 nm (a) and 550 nm (b) are shown. The scales used were: (a) 20 ms/div., and 0.002A/div.; (b) 20 ms/div., and 0.001A/div..

a



b

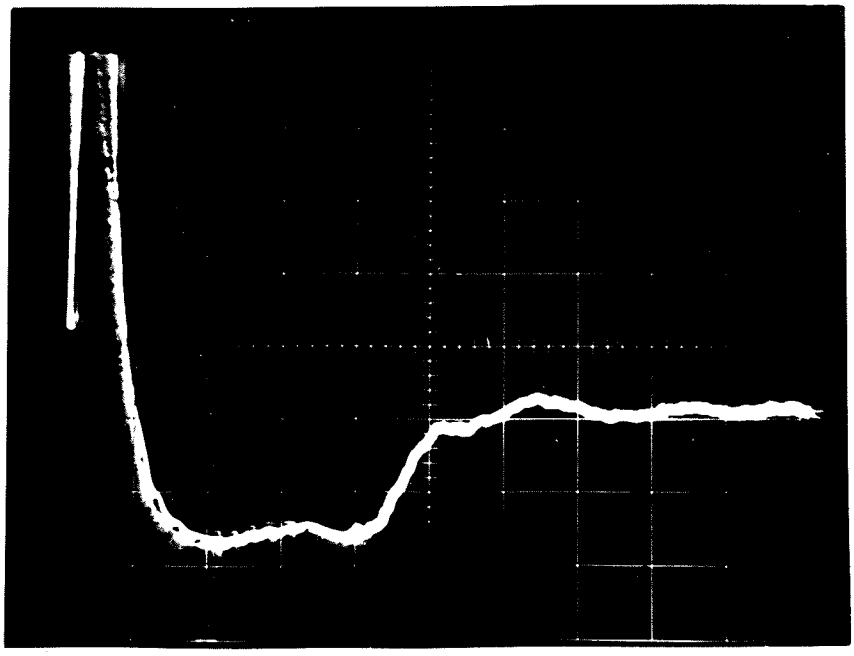


Figure 8. Effect of quick dilution on the absorbance of cytochrome c. Yeast cytochrome c (0.1 mM) was mixed with the same volume of phosphate buffer. The measurement was done at 550 nm with the scales set at 20 ms/div., and 0.002A/div..

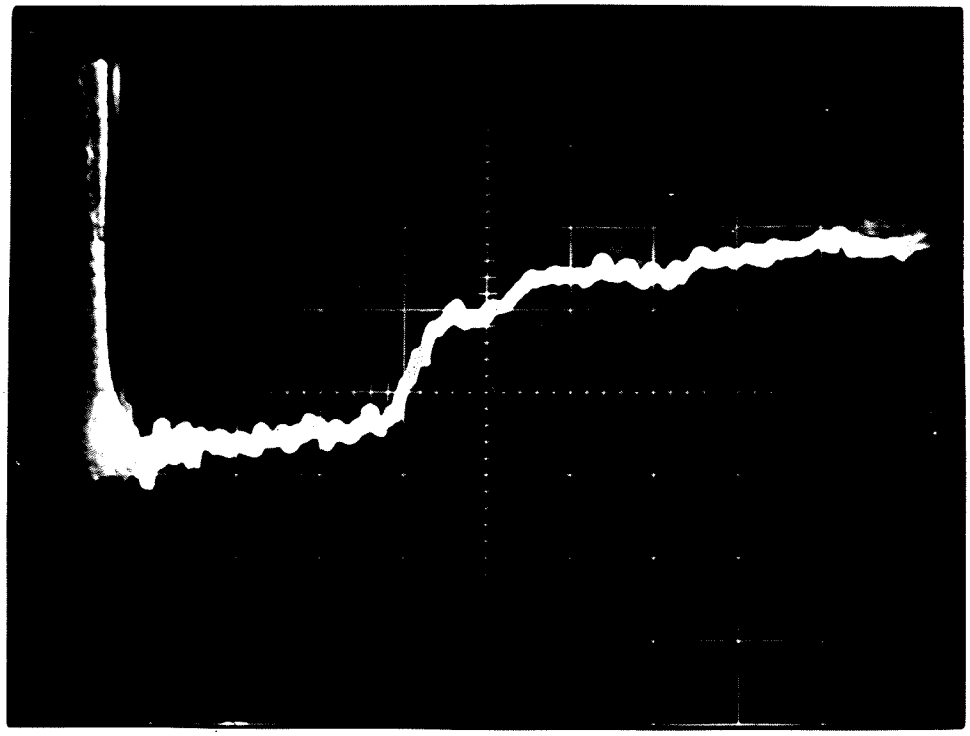
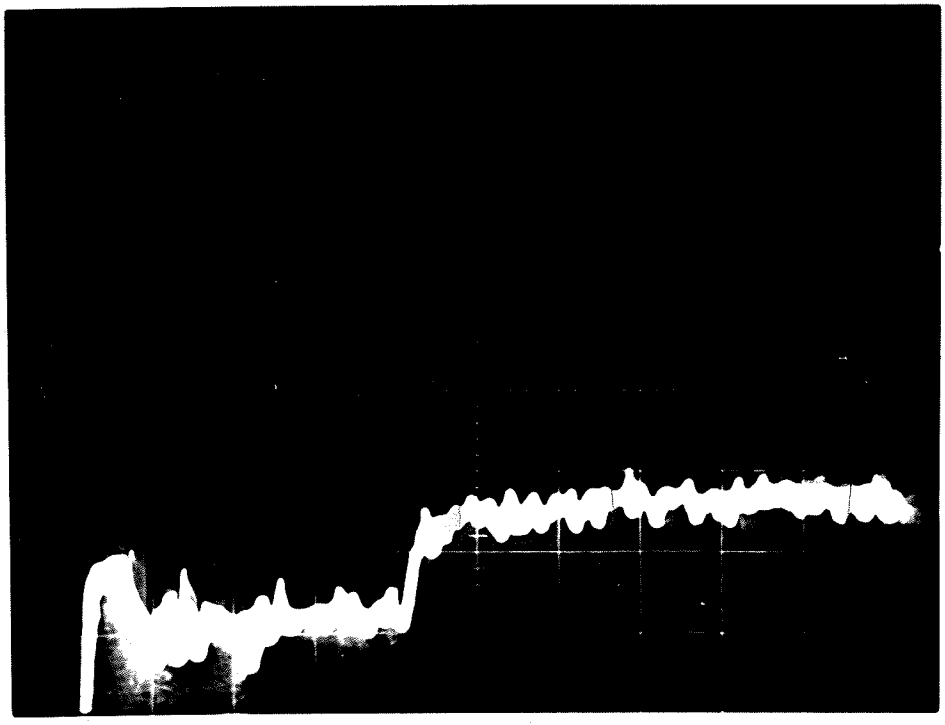


Figure 9. Oscilloscope trace of the signal obtained for the mixing of water. The absorbance change at 450 nm was recorded using distilled water in two syringes for mixing. The scales were set at 20 ms/div., and 0.001A/div..



it is surprisingly similar to those of Fig. 7 and Fig. 8. ΔA_{450} between 80 ms and 100 ms was determined as 0.0014.

Since this sudden absorbance increase between 80 and 100 ms after mixing was observed even when the two syringes contained distilled water, it was concluded that the increase was due to either mechanical or electronic artifact of the instrument and not due to the actual absorbance change of the mixed sample.

B. Effect of Substrates on the Enzyme Spectra (General)

The absorbance changes at 320 nm, 450 nm and 600 nm were measured as a function of time with the different combination of substrates.

The wavelength of 320 nm was used to detect the formation of the flavin-sulfite complex at the N(5) position of the isoalloxazine ring which was proposed as an intermediate of APS reductase reaction by Michaelis et al. (1970, 1971).

The reduction of FAD and nonheme iron was followed at 450 nm.

The absorbance change at 600 nm was used for the purpose of detecting the formation of the flavin semiquinone which was reported in many cases to have a broad absorption peak between 550 nm and 650 nm.

Sulfite - The effect of sulfite on the enzyme spectrum was measured at 320 nm, 450 nm and 600 nm with a time scale of 20 ms per division. The enzyme (3.4 mg per ml) was mixed with 1.5 x 10^{-4}M sodium sulfite, and the results are shown in Fig. 10. The absorbance after 1 min was recorded and shown as a line in each experiment. It was found in Fig. 10 a that there was a decrease in the absorbance at 320 nm with the addition of sulfite instead of an increase expected by the N(5) FAD-sulfite complex formation. This result suggests that either the complex was not formed in this experiment or an absorbance decrease caused by other reactions has overcome the small increase caused by the complex formation.

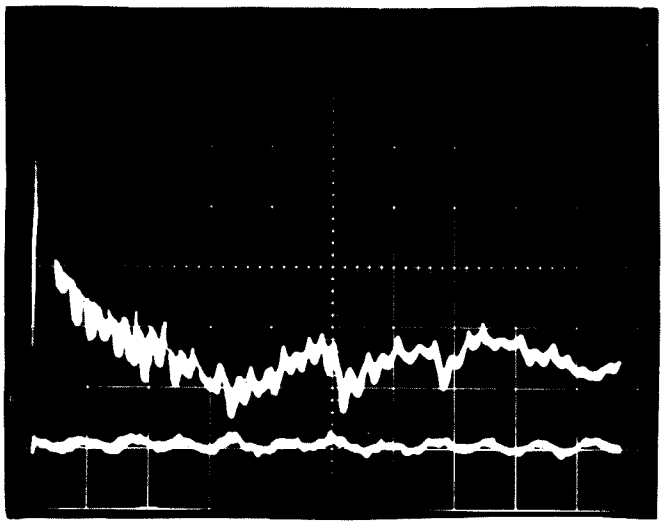
Fig. 10 b (450 nm) shows that the FAD reduction by sulfite was a fast one-step reaction. No further decrease in absorbance was detected after 60 ms.

From Fig. 10 c, it was concluded that there was no significant absorbance change at 600 nm (also see Fig. 9).

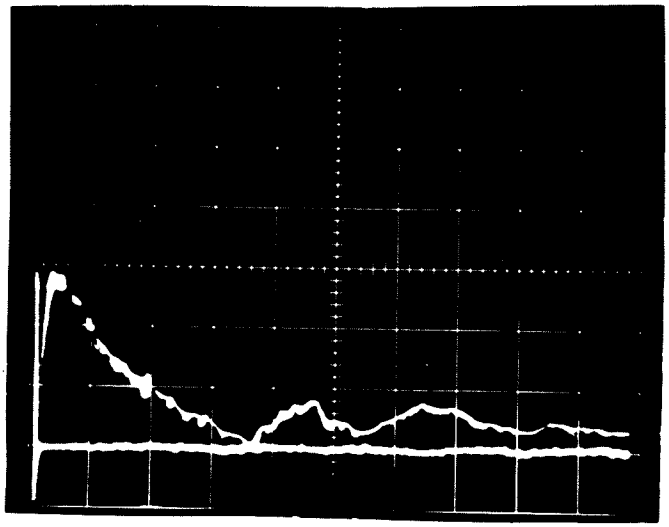
AMP - In the absence of sulfite no significant change in absorbance at 450 nm was detected with the addition of AMP to the enzyme (not shown).

Figure 10. Spectral change of APS reductase by the addition of sulfite. APS reductase was mixed with sodium sulfite at the final concentrations of 10^{-5}M and 7.5 x 10^{-5}M, respectively. The absorbance changes were followed at 320 nm (a), 450 nm (b), and 600 nm (c). The scales used were 20 ms/div., and 0.005A/div. for all experiments.

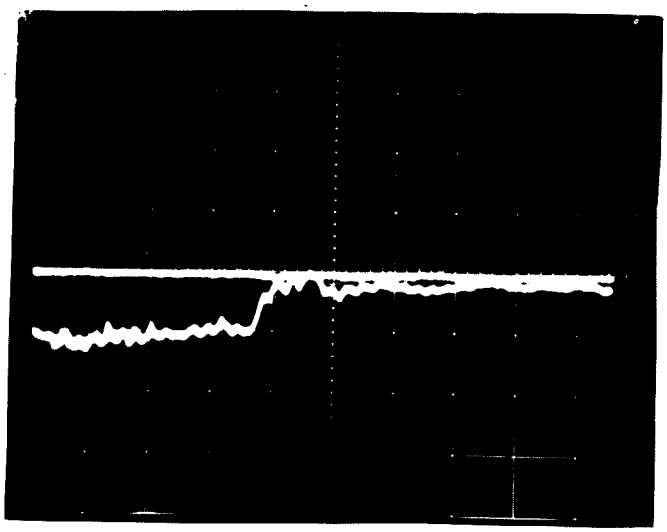
a



b



c



Sulfite and AMP - APS reductase was mixed with the mixture of 1.5 x 10^{-4}M sodium sulfite and 1.5 x 10^{-4}M AMP and the absorbance changes at 320 nm, 450 nm and 600 nm were recorded as shown in Fig. 11.

Fig. 12 shows the results obtained from a similar experiment except sulfite was added to the enzyme which had been pre-incubated with AMP. The results in Fig. 12 are similar to those in Fig. 11.

The results obtained either at 320 nm or 600 nm were similar in Figs. 10-12. At 450 nm, however, the results in Fig. 11 b and Fig. 12 b which were almost identical, were different from that in Fig. 10 b. There was no absorbance change recorded after 60 ms in Fig. 10 b with sulfite alone while there was a further decrease in absorbance in Fig. 11 b and Fig. 12 b after the initial reaction.

These results strongly suggest that sulfite has reacted with the enzyme before AMP which could then react with the sulfite-reduced enzyme.

Figure 11. Spectral change of APS reductase by the addition of sulfite and AMP. APS reductase was mixed with the mixture of sodium sulfite and AMP at the final concentration of 10^{-5}M, 7.5 x 10^{-5}M and 7.5 x 10^{-5}M, respectively. The scale settings were the same as those in Fig. 10.

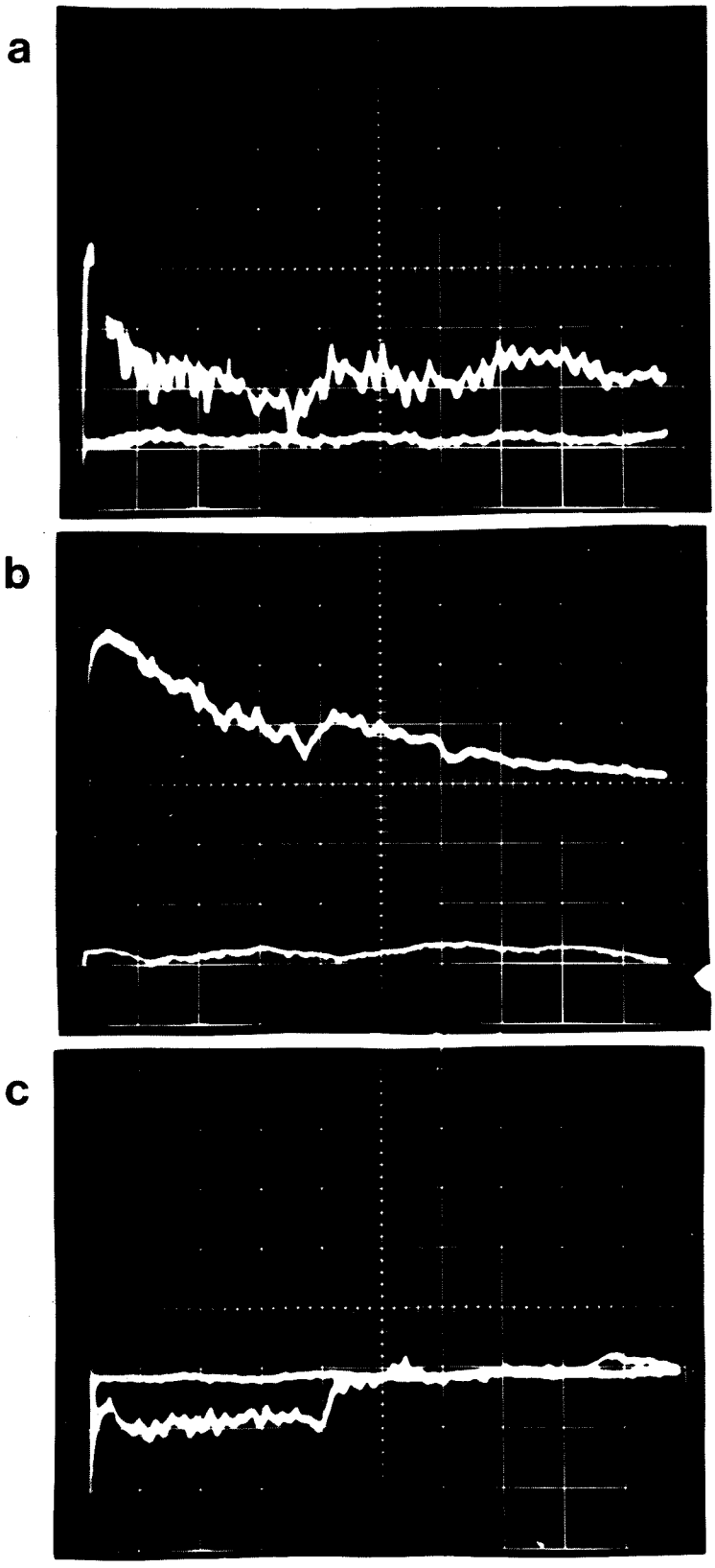
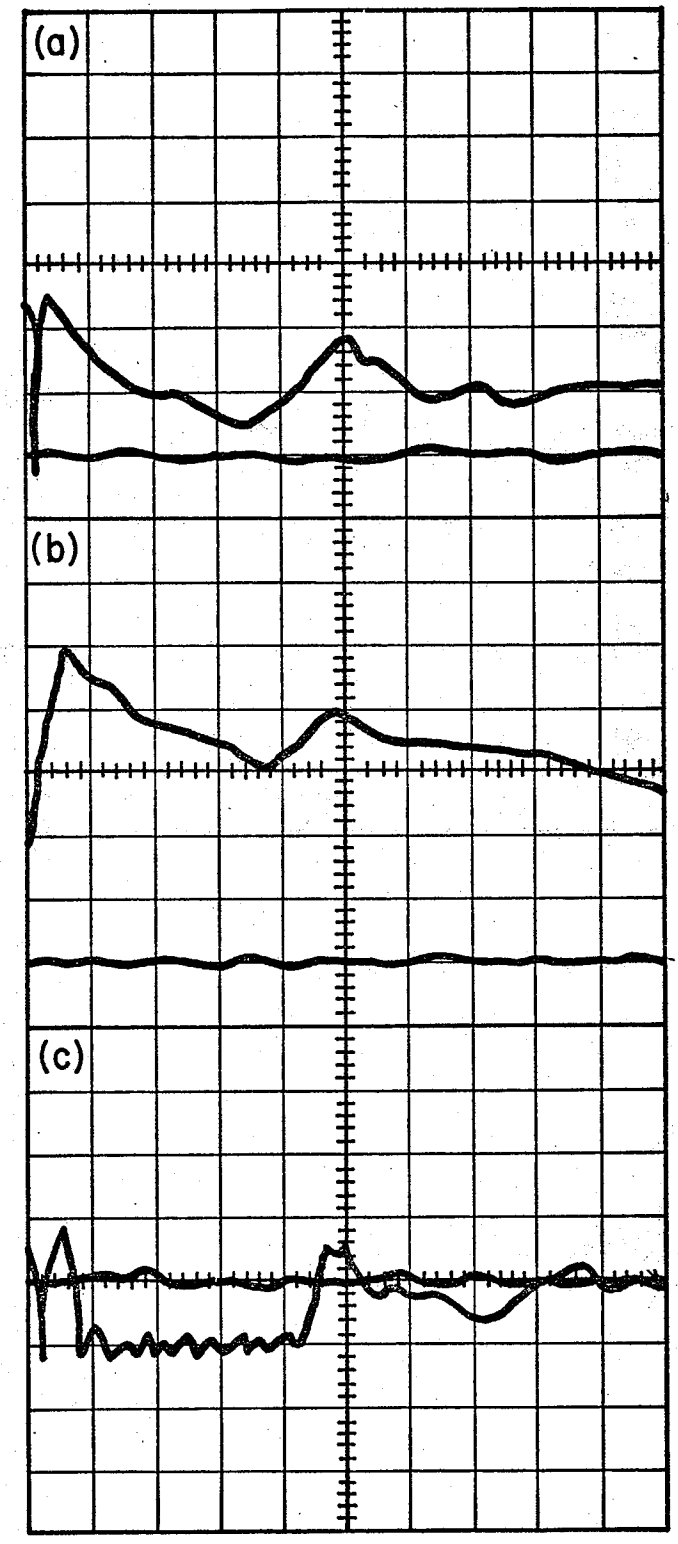

a
b
c

Figure 12. Spectral change of APS reductase by the addition of AMP and sulfite. The mixture of APS reductase (2×10^{-5}M) and AMP (1.5×10^{-4}M) was mixed with sodium sulfite (1.5×10^{-4}M). The wavelength, the scale settings and the final concentration of substrate and enzyme were the same as those in Fig. 11.


(a)
(b)
(c)

C. Partial Reaction with Sulfite

Enzyme spectrum change - APS reductase was mixed with sodium sulfite at the final concentration of 10^{-2}M and the absorbance changes were measured between 360 nm and 470 nm. The difference spectra shown in Fig. 13 were reconstructed from the data obtained at 10, 20, 30, 40, and 50 ms after the mixing and also between 10 ms and 50 ms. All the spectra indicate that the enzyme-bound FAD was converted to the fully reduced 1, 5-dihydro level and FAD was the only chromophore contributing to this spectral change. Concomitant with the FAD reduction sulfite must have been oxidized. Since free sulfate or APS is not formed by this enzyme without AMP, the oxidation product could be an enzyme-bound active sulfate. This experiment did not supply any information with regard to the nature of the enzyme-sulfite complex. The reaction observed could be written as the equation (1).

$$\text{Enz-FAD}+SO_3^{2-} + H_2O \rightleftharpoons \text{Enz-FADH}_2(SO_4^{2-}) \qquad (1)$$

The effect of sulfite concentration on the FAD reduction - Eq. (1) indicates that there are only two enzyme species observed. If it is assumed that the binding of sulfite to Enz-FAD is much faster than the conversion of Enz-FAD (SO_3^{2-}) to Enz-FADH$_2$$(SO_4^{2-})$, then it should be possible to calculate the dissociation constant for the enzyme-

sulfite complex by studying the rate of flavin reduction at various sulfite concentrations. The absorbance changes at 450 nm were recorded with varied concentrations of sodium sulfite. Fig. 14a shows the result obtained with 1 mM Na_2SO_3. The semilog plot of this result shown in Fig. 14b gives a slope equal to -k'/2.3 (k'= first order rate constant = 51.6 sec^{-1}). The reciprocal first order rate constants were plotted against the reciprocal of sodium sulfite concentration to obtain the rate constant at extrapolated infinite sulfite concentration and the dissociation constant ($K_D=\frac{(E)(S)}{(ES)}$) of sulfite from the enzyme-sulfite complex. Fig. 15 shows this graph. The value of the extrapolated rate constant is 97.1 sec^{-1} and K_D is 0.92 mM. In the calculation of K_D the total concentration of sulfite plus bisulfite has been used instead of the sulfite content (approx. 80% at pH 7.5).

The half reaction time ($t_{\frac{1}{2}}$) was calculated using the equation:

$\log(S) = -\frac{k}{2.3}t + \log(S)_o$, or $t_{\frac{1}{2}} = \frac{0.693}{k}$.

$t_{\frac{1}{2}}$ is 13.4 ms at the sulfite concentration of 1 mM (k'=51.6 sec^{-1}). At this sulfite concentration 30.6% of the enzyme-bound FAD were reduced.

Effect of dilution on the enzyme-sulfite complex - The enzyme (3.4 mg per ml) mixed with 3 x 10^{-3}M sodium sulfite was subjected to a quick dilution with the buffer

Figure 13. Difference spectra occurring during the reduction of APS reductase by sulfite. Enzyme and sulfite were rapidly mixed at the final concentration of 10^{-5}M and 10^{-2}M, respectively. The spectral change was recorded at the wavelength indicated. Difference spectra were reconstructed at 10 ms, 20 ms, 30 ms, 40 ms, and 50 ms after the mixing and also between 10 ms and 50 ms as indicated in the figure.

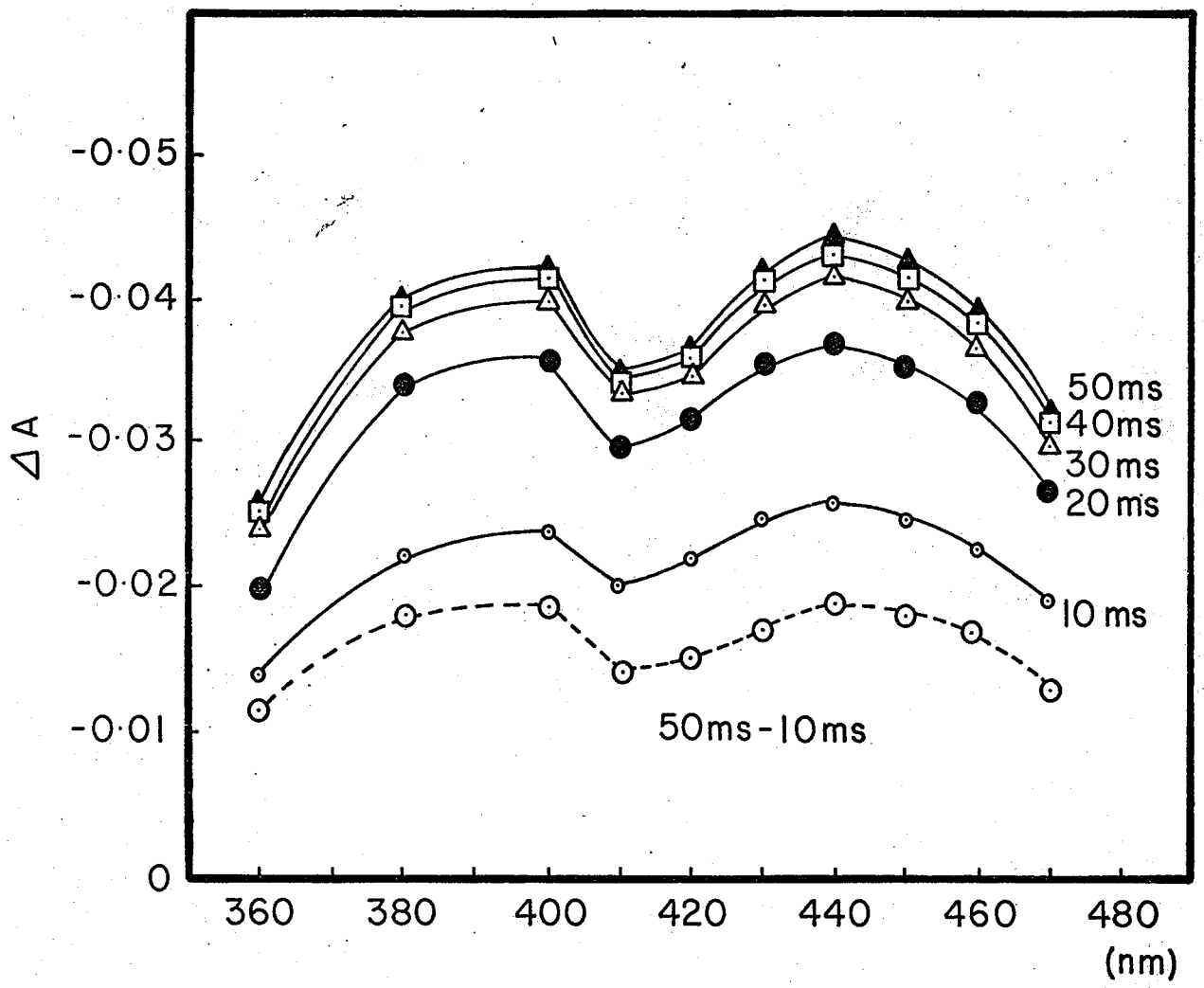

-0·05
-0·04
-0·03
-0·02
-0·01
0
ΔA
360
380
400
420
440
460
480
(nm)
50ms
40ms
30ms
20ms
10 ms
50ms-10ms

Figure 14. Reduction process of the enzyme-bound FAD by the addition of sulfite. The result obtained with 1 mM sodium sulfite is shown in (a). Semilogarithmic plot of the data is shown in (b) which gives a slope of $-k'/2.3$.

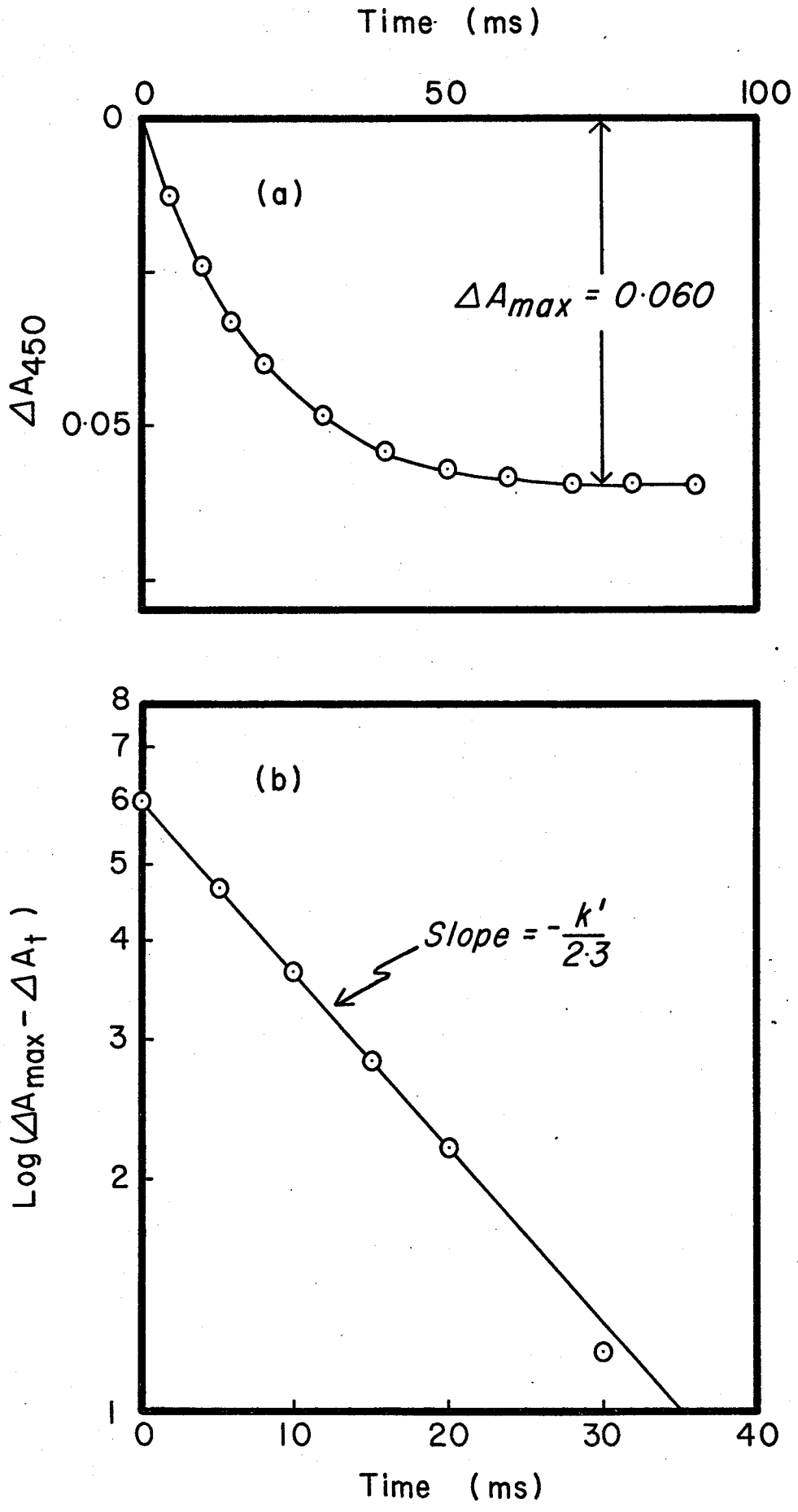

Time (ms)
0
50
100
0
(a)
ΔA_{max} = 0·060
ΔA_{450}
0·05
8
7
6
5
4
3
2
1
(b)
Slope = $-\frac{k'}{2.3}$
Log ($\Delta A_{max} - \Delta A_t$)
0
10
20
30
40
Time (ms)

Figure 15. Double reciprocal plots of first order rate constants and sulfite concentrations. The rates are expressed as first order rate constants (sec^{-1}) of the ΔA_{450} as measured in a stopped-flow spectrophotometer. The extrapolated first order rate constant was 97.1 sec^{-1}, and the K_D value for sulfite was 0.92 mM.

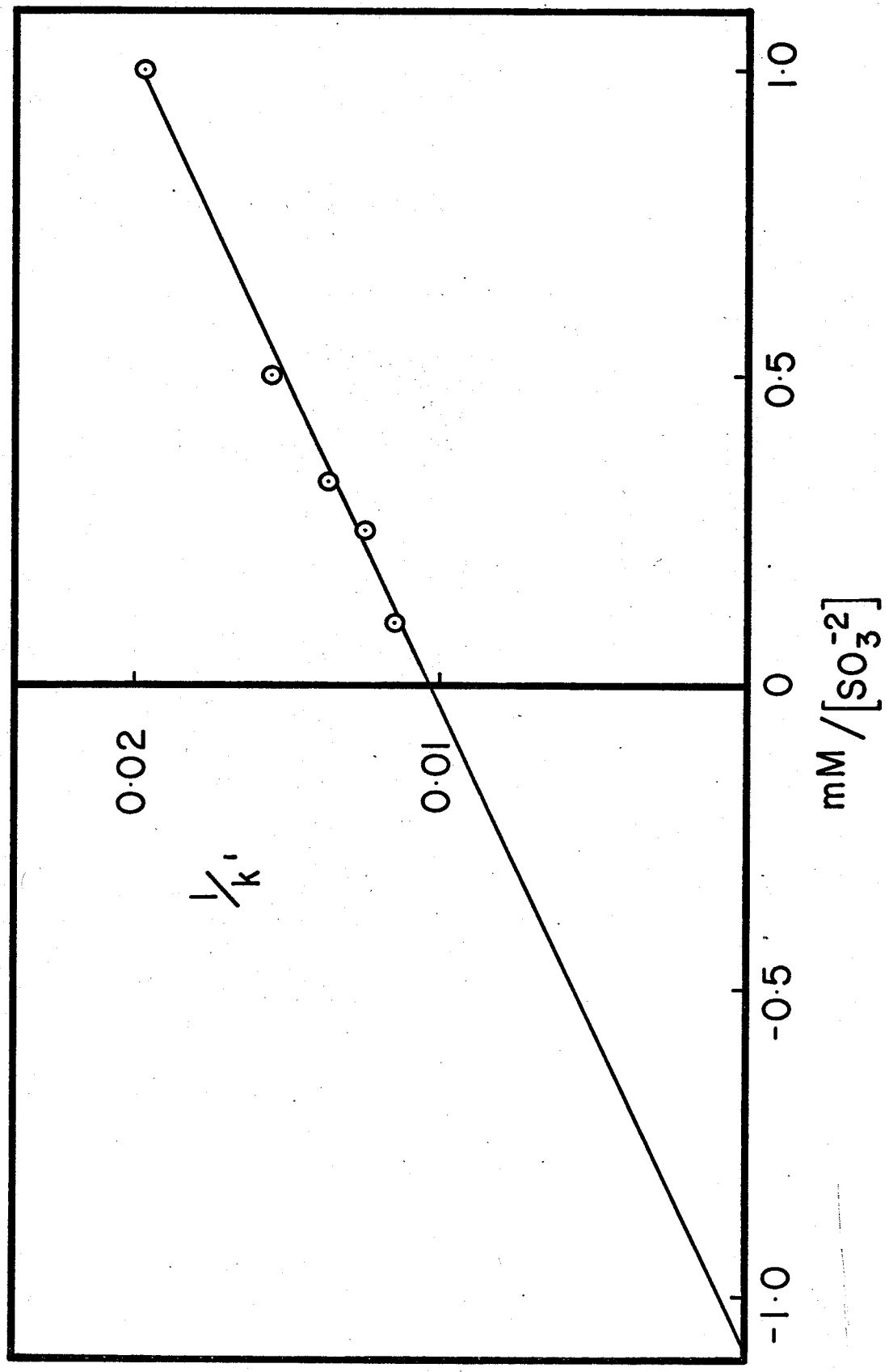
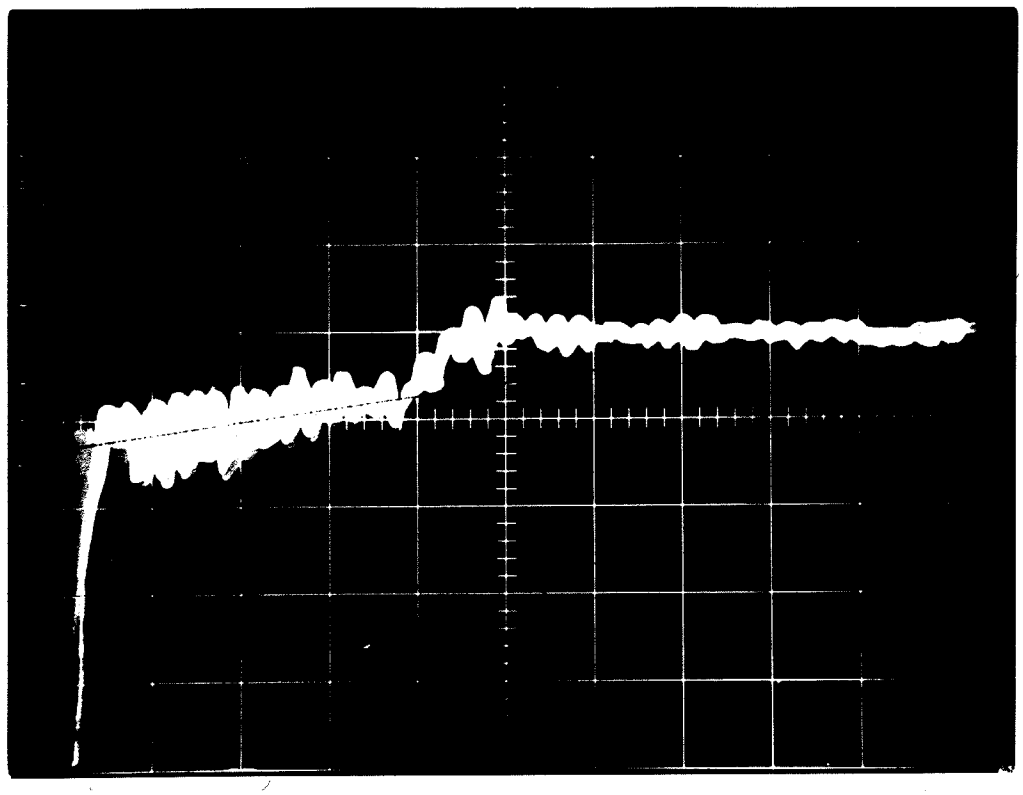

0·02
0·01
1/k'
-1·0
-0·5
0
0·5
1·0
mM /[SO3-2]

Figure 16. Oscilloscope trace of the absorbance change at 450 nm caused by the twofold dilution of the sulfite-reduced enzyme. The mixture of APS reductase (2×10^{-5}M) and sodium sulfite (3×10^{-3}M) was mixed with the same volume of phosphate buffer and the absorbance change was followed at 450 nm. The scale settings were at 20 ms/div. and 0.002A/div..



and the absorbance change at 450 nm was recorded (Fig.16).

A slight increase in absorbance was observed during the first 80 ms at each wavelength used. A typical signal jump was also observed between 80 ms and 100 ms as in previous mixing experiment.

The gradual increase in absorbance observed during the first 60 ms was possibly caused by the 2-fold dilution of enzyme and substrate concentrations which slightly shifted the equilibrium away from the enzyme - sulfite complex. It seems that the new equilibrium was gradually established during the first 60 ms after the dilution.

D. Partial Reaction with AMP

Since AMP alone caused no significant change in the APS reductase spectrum as mentioned previously, the sulfite-reduced enzyme (3.4 mg enzyme reduced with 3×10^{-3}M sodium sulfite) was used to study the role of AMP in the enzyme mechanism.

Enzyme spectrum change - The sulfite-reduced enzyme was mixed with the same volume of AMP solution (2×10^{-5}M) and the spectral changes between 350 nm and 480 nm were recorded. Fig. 17 shows the absorbance changes for two critical wavelengths (370 nm and 440 nm) as a function of time.

Figure 17. Oscilloscope traces of the absorbance changes caused by the addition of AMP to sulfite-reduced APS reductase. AMP was added to the sulfite-reduced enzyme and the absorbance change was recorded. The wavelengths and the scales used were as follows:

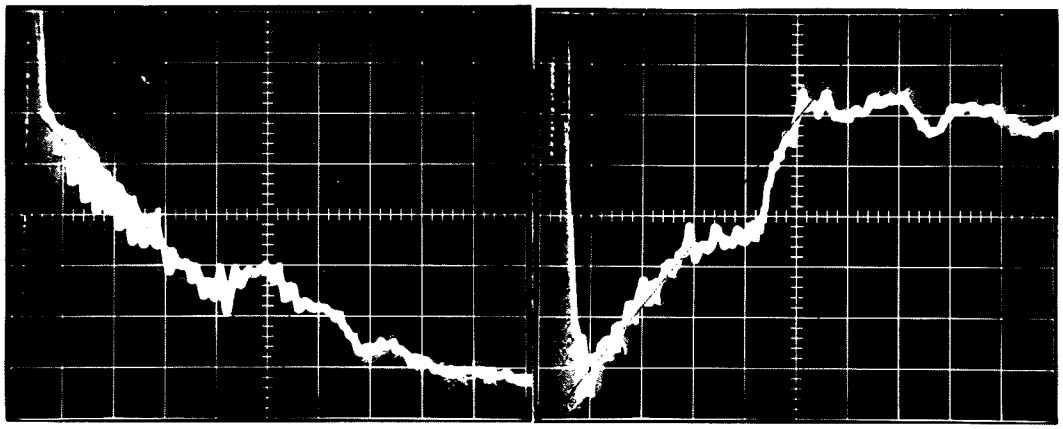
(a) 440 nm, 20 ms/div. and 0.002A/div.;

(b) 370 nm, 20 ms/div. and 0.002A/div.;

(c) 440 nm, 100 ms/div. and 0.002A/div.;

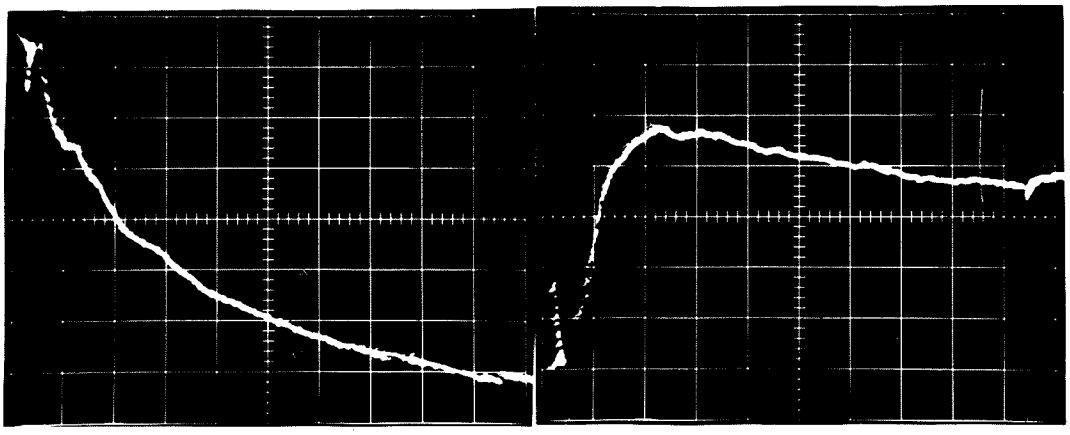
(d) 370 nm, 100 ms/div. and 0.002A/div..

a



b

c



d

The results obtained at 440 nm (Figs. 17a and c) were similar to those obtained at 450 nm in Fig. 11b and Fig. 12b. The decrease in absorbance was observed for 20 sec at 450 nm under the same conditions (not shown).

The absorbance changes at 370 nm (Figs. 17b and d) were, however, completely different from those obtained at 440 nm. The absorbance increased quickly for the first 180 ms and then decreased slowly after that period.

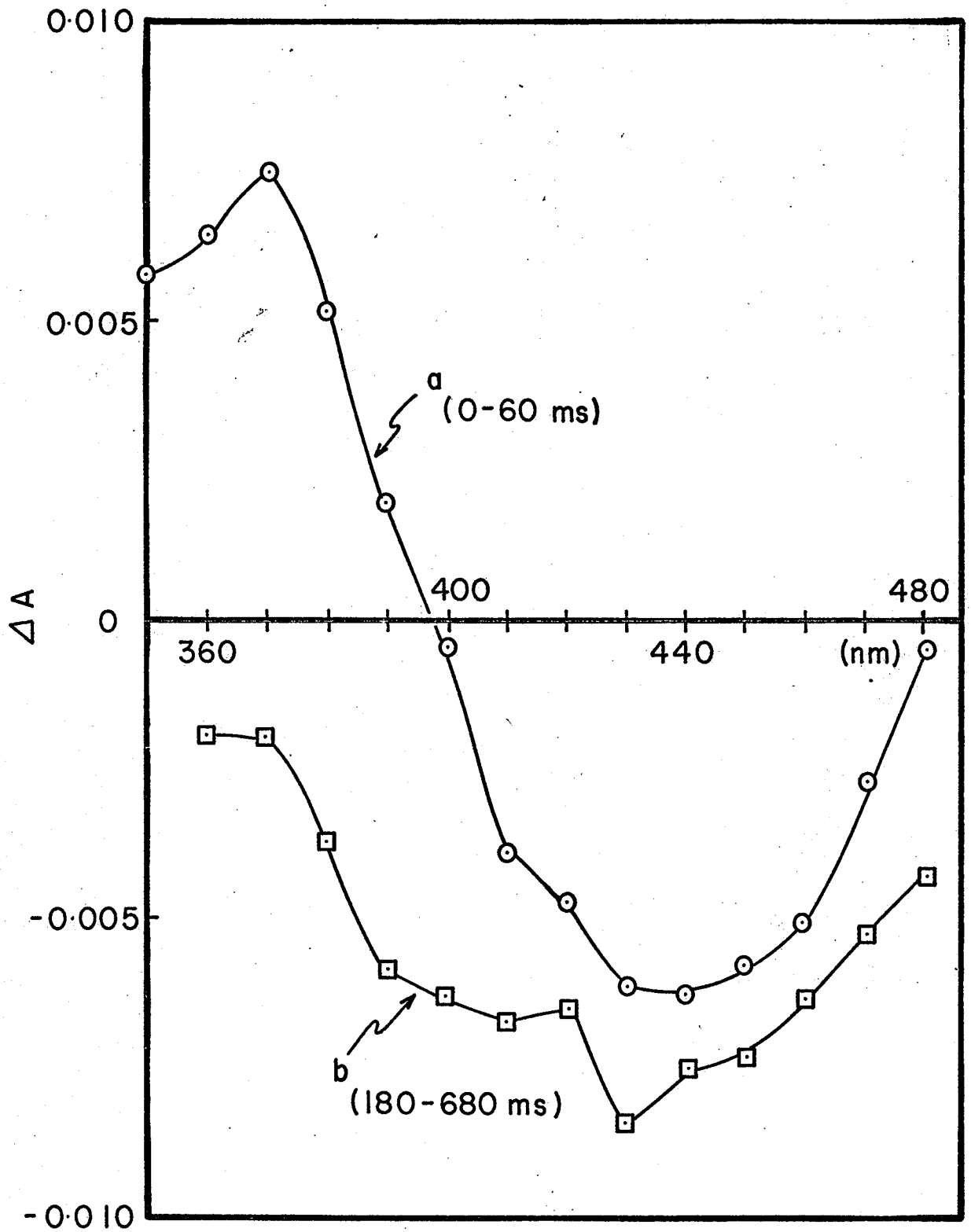
The difference spectra between time 0 and 60 ms (a) and between 180 ms and 680 ms (b) were plotted in Fig. 18. These two spectra are quite different and suggest that two reactions were initiated by the addition of AMP to the sulfite-reduced enzyme without an added electron acceptor.

Since the enzyme-bound FAD was reduced to the dihydro level ($FADH_2$) with sulfite prior to the addition of AMP (Eq. 1) and only nonheme iron was considered to be involved in these redox reactions other than FAD, the following two possible reactions (Eq. 2 and Eq. 3) were postulated for the first step reaction after the addition of AMP to the sulfite-reduced enzyme.

$$FADH_2 + 2Fe^{3+} \longrightarrow FAD + 2Fe^{2+} + 2H^{+} \qquad (2)$$

$$FADH_2 + Fe^{3+} \longrightarrow FADH\cdot + Fe^{2+} + H^{+} \qquad (3)$$

Figure 18. Difference spectra induced by the addition of AMP to sulfite-reduced APS reductase. Difference spectra (a) (between 0 and 60 ms) and (b) (between 180 ms and 680 ms) were reconstructed from oscilloscope traces recorded at various wavelengths as indicated in the figure.


0·010
0·005
0
-0·005
-0·010
ΔA
360
400
440
480
(nm)
a
(0-60 ms)
b
(180-680 ms)

FADH· in Eq. 3 designates the flavin semiquinone.

The oxidized FAD is electrophilic in nature and would react quickly with sulfite which is a strong nucleophile (Page 71, the half reaction time = 13.4 ms). However, the rate of the second step reaction was found to be slow (Figs. 17 a and c) and the difference spectrum obtained for the second reaction (Fig. 18 b) does not indicate the reduction of FAD to $FADH_2$. It seems then that the equation (2), where FAD is one of the products, is unlikely.

In order to find out whether the difference spectrum shown in Fig. 18 a could be caused by the reaction proposed in Eq. 3, a possible spectral change was simulated with the spectra of nonheme iron and flavin semiquinone appeared in some reports. Eq. 3 was divided into two equations to simplify the attempt.

$$Fe^{3+} \longrightarrow Fe^{2+} \qquad (3a)$$

$$FADH_2 \longrightarrow FADH\cdot \qquad (3b)$$

Reaction (3a), the reduction of nonheme iron, could cause a decrease in absorbance similar to that in Fig. 4 c with maxima at 330-340 nm and at 420-440 nm (Tagawa and Arnon, 1962; Dus et al., 1967; Mayhew et al., 1969). The molar extinction coefficient for nonheme iron at 440 nm ($\varepsilon 440$) is approximately 10,000.

Reaction 3b, the oxidation of dihydroflavin to the half-oxidized semiquinone, may result in an increase of absorbance with maxima around 380 nm and 480 nm. In most cases, ΔA380 is much larger than ΔA480 in the formation of the semiquinone from the fully reduced flavin. For example, the molar extinction coefficients at 385 nm and at 485 nm of L-amino acid oxidase were calculated from the data given by DeKok et al. (1971) for the formation of the semiquinone from the dihydroflavin as 12,500 and 2,400, respectively.

The absorbance increase obtained by the semiquinone formation at 450 nm region (ε = 2,400) could be easily overcome by the decrease caused by the reduction of nonheme iron (ε = 10,000) while the increase around 370 nm (ε = 12,500) should remain largely unaffected.

From these observations, it was concluded that Eq. 3 could account for the spectral change in Fig. 18 a, where the absorbance increased around 370 nm and decreased around 440 nm.

Since the flavin semiquinone is still slightly electrophilic, it is expected to react with sulfite slowly. Nonheme iron could be the acceptor of another electron. The second step reaction is then written as Eq. 4.

$$FADH^{\cdot} + Fe^{3+} + SO_3^{2-} + H_2O \longrightarrow FADH_2(SO_4^{2-}) + Fe^{2+} + H^{+} \quad (4)$$

These reactions written in Eqs. (3) and (4) could occur alternatively to form a cycle (Scheme III). The overall reaction (Eq. 3 followed by Eq. 4) becomes quite simple as shown in Eq. 5.

$$2Fe^{3+} + SO_3^{2-} + H_2O \longrightarrow 2Fe^{2+} + SO_4^{2-} + 2H^+ \qquad (5)$$

The difference spectrum shown in Fig. 18 b, which indicates the reduction of nonheme iron, should be the result of the reaction in Eq. 5.

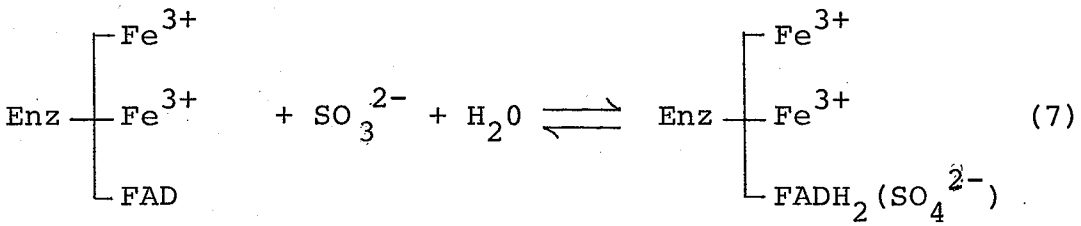
The overall reaction, which was initiated by the addition of AMP to the sulfite-reduced enzyme, could be written as Eq. 6.

$$nFe^{3+} + {}^{n}/_{2}\,SO_3^{2-} + {}^{n}/_{2}\,H_2O + {}^{n}/_{2}\,AMP \longrightarrow nFe^{2+} + {}^{n}/_{2}\,APS + nH^+ \qquad (6)$$

The number of nonheme iron reduced is not known but qualitatively the spectral change shown in Fig. 4 c should be the result of this reaction (Eq. 6) and it is in good agreement with other reports on the reduction of nonheme iron (Tagawa and Arnon, 1962; Dus et al., 1967; Mayhew et al., 1969).

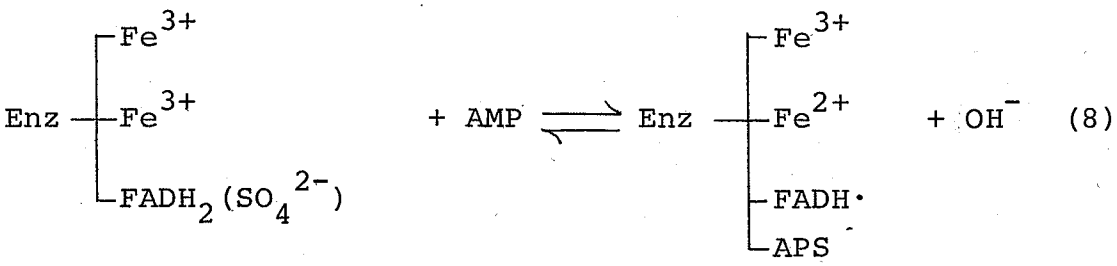
The mechanism of APS reductase is now postulated to include at least three steps in the absence of an added electron acceptor. The first step involves the reversible association of sulfite with the enzyme (Eq. 7).

Although the results shown in Fig. 4 and Figs. 10-12 are not sufficient to establish the exact form and nature of the enzyme-sulfite complex, sulfite is shown as FAD bound. In this reaction FAD is reduced fully to the dihydro level and sulfite is oxidized to sulfate level.



(7)

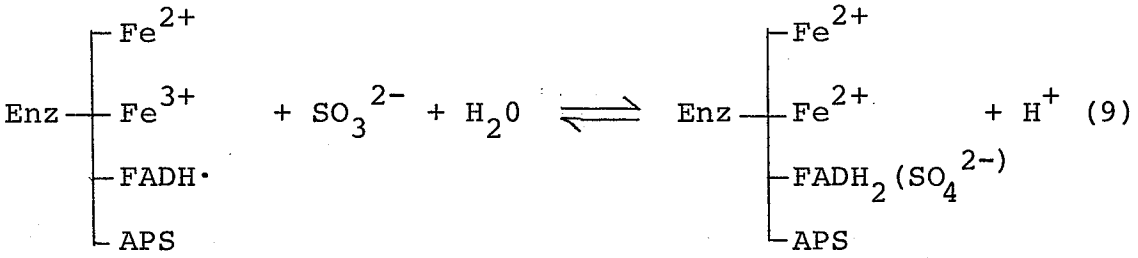
The second reaction is initiated by the addition of AMP and transfers an electron from the reduced FAD to nonheme iron to form flavin semiquinone (Eq. 8).



(8)

The typical semiquinone signal in long wavelength could not be detected in Fig. 11 c and Fig. 12 c, but it has been reported that some flavoprotein semiquinones have little absorbance in this region. For example, Old Yellow Enzyme exhibits very little absorption in this region when it is reduced by dithionite to semiquinone form (Matthews and Massey, 1971).

The last step (Eq. 9) is a slow reduction of FADH· to $FADH_2$ and a transfer of an electron to nonheme iron.



(9)

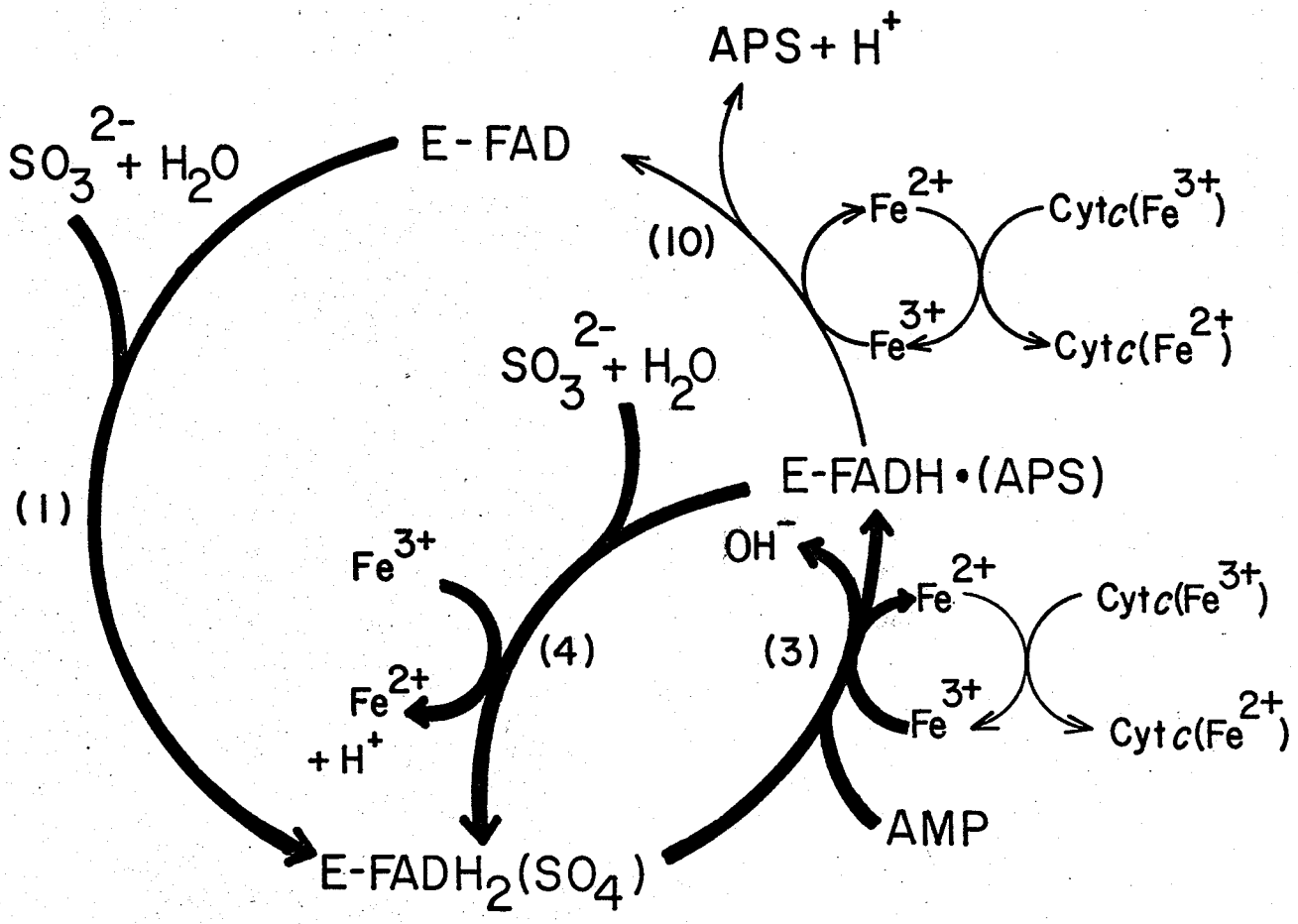
Lyric and Suzuki (1970 d) have proposed a mechanism of APS reductase from the initial velocity and product inhibition studies where APS was released from the enzyme only after it was completely oxidized by cytochrome c. Since in this stopped-flow experiment there was no added electron acceptor, APS is shown as enzyme-bound and not to be released at the end of the reaction.

Scheme III shows a postulated reaction mechanism of APS reductase. In order to complete the reaction cycle, the reaction shown in Eq. 10 was assumed.

$$E\text{-}FADH\cdot(APS) + Fe^{3+} \longrightarrow E\text{-}FAD + APS + Fe^{2+} + H^{+} \quad (10)$$

Thick arrows in Scheme III indicate the reactions observed in the stopped-flow experiment reported here.

Scheme III. Proposed Reaction Cycle of APS Reductase



The reactions observed in the stopped-flow spectrophotometer are shown as thick arrows. Slim arrows indicate the reactions assumed. The numbers in the scheme correspond to the equations shown in text.

DISCUSSION

DISCUSSION

APS reductase was originally found in Desulfovibrio where the physiological role of the enzyme is the reduction of APS to form sulfite and AMP. However, the T. thioparus enzyme catalyzes the reaction in favour of the formation of APS (oxidation of sulfite). APS reductases from D. vulgaris and T. thioparus were extensively studied and it subsequently became clear that the properties of the enzymes from these two sources are different in many aspects as well as their physiological functions.

Peck et al. (1965) purified and studied APS reductase from D. vulgaris but could not obtain any activity of the enzyme using cytochrome c as the electron acceptor. In 1970, Lyric and Suzuki (1970 b) purified the reductase from T. thioparus and reported that it could use cytochrome c as the electron acceptor as well as ferricyanide. Peck's group reinvestigated the D. vulgaris enzyme and succeeded in demonstrating the reduction of cytochrome c by the enzyme (Michaelis et al., 1971). However, the activity obtained with cytochrome c was only $^{1}/_{1000}$ of that using ferricyanide as compared to $^{1}/_{80}$, the value for the T. thioparus enzyme (Lyric

and Suzuki, 1970 b).

Bramlett and Peck (1975) reported that the cytochrome c assay of the D. vulgaris enzyme was inhibited 100% by anaerobiosis and up to 84% with 10^{-6}M superoxide dismutase and suggested that superoxide radicals are responsible for the reduction of cytochrome c in this system.

The reduction of cytochrome c by APS reductase from T. thioparus was inhibited only 35% by anaerobiosis and up to 26% with 36 units of superoxide dismutase. Since under these anaerobic conditions the reduction of cytochrome c by xanthine oxidase was completely inhibited, it was concluded that there is a direct reduction or binding of cytochrome c by the T. thioparus enzyme although superoxide radicals are found during the aerobic reactions.

The concentration of superoxide dismutase used by Bramlett and Peck (1975) to confirm the involvement of superoxide anions was questionably high (1 μM). However the complete inhibition of the cytochrome c reduction by anaerobiosis is reminiscent of the xanthine oxidase system and superoxide radicals were probably responsible for the reduction of cytochrome c in the D. vulgaris system.

These differences in the properties and the physiological function of the enzyme suggest that there might be a difference in the mechanisms of APS reductases from these two sources.

A difference was also noticed in the specificity of the enzymes to different types of cytochrome c. With the T. thioparus enzyme, the horse heart cytochrome c was only 30% as active as that from Candida krusei (Lyric and Suzuki, 1970 b) while the yeast cytochrome c was 70% as active as the other in the D. vulgaris system (Bramlett and Peck, 1975). However, when the T. thioparus enzyme was assayed in the potassium phosphate buffer (pH 7.5), the activity of the enzyme with cytochrome c was dependent on the concentration of the buffer (Fig. 3).

Labeyrie (1971) suggested that the difference in reactivity between two cytochromes would disappear at zero salt concentration in the APS reductase system.

Therefore it is possible that the apparent difference in specificities of APS reductases from D. vulgaris and T. thioparus to the cytochrome c was derived from the different responses of the enzymes to the salt concentration.

Michaelis et al. (1970, 1971) proposed the reaction mechanism for APS reductase from D. vulgaris

involving the N(5) flavin-sulfite adduct as an intermediate. They observed an increase at 320 nm region of the enzyme spectrum by the addition of sulfite and also demonstrated the existence of this adduct in the supernatant of the sulfite-reduced enzyme treated with 0.244 N trichloroacetic acid.

In the T. thioparus system, however, the absorbance at 320 nm did not increase by the addition of sulfite in either the difference spectrum or the stopped-flow experiments. Although these experiments did not supply enough information to establish the form of the intermediate, it is doubtful that the N(5) flavin-sulfite adduct was formed by the addition of sulfite to the T. thioparus enzyme.

The formation of the N(5) flavin-sulfite adduct was extensively studied by Massey's group (Swoboda and Massey, 1966; Massey et al.,1969b; Müller and Massey, 1969). Hevesi and Bruice (1973) noted that among the flavoenzymes investigated (oxidases, reductases, dehydrogenases, and hydroxylases) only the oxidases were found to yield flavin-sulfite adducts. Thus a correlation exists between the ability of fully reduced flavoenzymes to react with oxygen and the ability of their oxidized forms to react with sulfite.

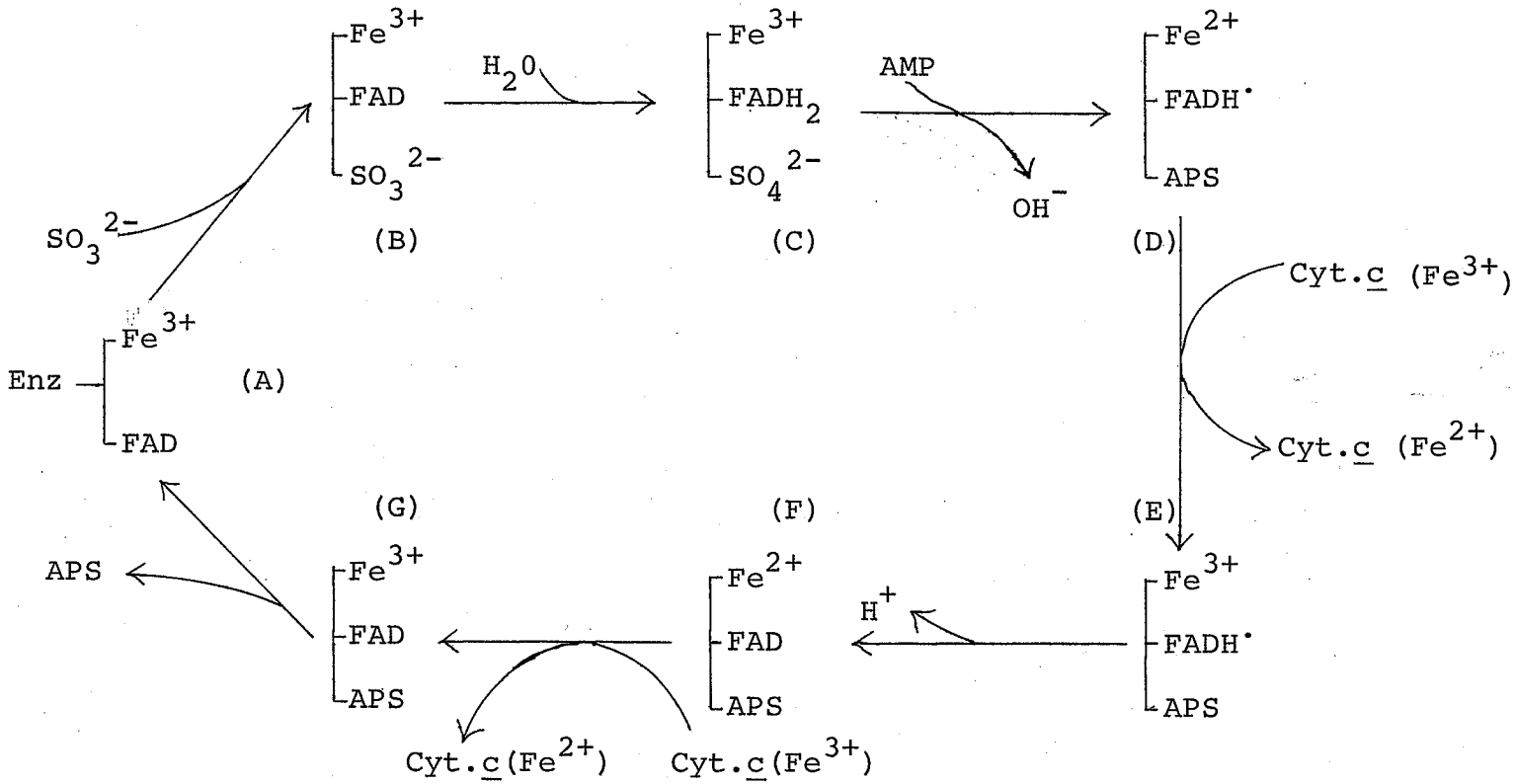
All the sulfite adducts of flavoenzymes tested by Massey's group were so far found enzymatically inactive. Swoboda and Massey (1966) stated that a major difference between the reduced flavoenzyme and the enzyme-sulfite adduct is the failure of the latter compound to react with electron acceptors such as oxygen, ferricyanide, or sulfate.

Since APS reductase is not an oxidase, it seems strange that the enzyme from D. vulgaris formed the N(5) adduct and it was active as an intermediate of the enzyme reaction. It might be related to the fact that the D. vulgaris enzyme could indeed use oxygen as the electron acceptor and its activity using cytochrome c was totally depending on the presence of oxygen as in the xanthine oxidase system (Bramlett and Peck, 1975).

A reaction mechanism is now proposed for APS reductase from T. thioparus and shown in Scheme IV.

The reaction shown in Eq. 1 (in "Results") involves two steps, the binding of sulfite to the enzyme followed by the reduction of FAD with concomitant oxidation of sulfite to sulfate. The addition of AMP to the sulfite-reduced enzyme induces a transfer of an electron from $FADH_2$ to nonheme iron (Fe^{3+}).

Scheme IV Proposed Reaction Mechanism of APS Reductase



Since the addition of sulfite caused only the reduction of FAD and in the absence of the electron acceptor nonheme iron could be reduced by the substrates, the electron flow in the APS reductase system was assumed as follows:

SO_3^{2-} ⟶ FAD ⟶ Nonheme iron ⟶ Cytochrome c .

The electron transfer from FAD to cytochrome c via nonheme iron is shown twice in Scheme IV to oxidize the two components of the enzyme. The last step is the release of APS from the fully oxidized enzyme, which completes the reaction cycle.

According to the kinetic mechanism of APS reductase proposed by Lyric and Suzuki (1970 d), the binding of cytochrome c occurs directly before and after the binding of sulfite. Although only the cytochrome c reduction site is shown in Scheme V, the binding of cytochrome c could occur at these steps proposed by these authors without contradicting the mechanism proposed here.

The generation of superoxide was found in APS reductase from T. thioparus. The site of electron transfer, however, was not identified and the mechanism of the superoxide production is not clear in this system. For these reasons the formation of superoxide is not shown in Scheme IV.

The mechanism of superoxide generation in metalloflavoproteins is not yet clearly understood but both the flavin moiety and the iron-sulfur moiety have been reported to be responsible in separate cases.

In the xanthine oxidase system the reports on the site of superoxide generation are contradictory. According to Fridovich and Handler (1962) and Knowles et al. (1969) the iron-sulfur moiety of the enzyme was responsible for the superoxide formation. On the other hand, Komai et al. (1969) prepared "deflavo" xanthine oxidase and proposed that the FAD moiety is the site for superoxide production.

The following three possibilities exist for the superoxide generation in metalloflavoproteins (Nakamura, 1970).

(a) $FADH_2 + O_2 \longrightarrow FADH\cdot + O_2^- + H^+$

(b) $FADH\cdot + O_2 \longrightarrow FAD + O_2^- + H^+$

(c) $Fe^{2+} + O_2 \longrightarrow Fe^{3+} + O_2^-$

Since the reaction (a) would result in the AMP-independent cytochrome c reduction in the APS reductase system it should be eliminated from the possibilities for the superoxide formation by T. thioparus APS reductase.

There are two possible sites for the reaction (b) in Scheme IV as follows:

$$(D) + O_2 \longrightarrow (F) + O_2^- + H^+ \qquad \text{and}$$

$$(E) + O_2 \longrightarrow (G) + O_2^- + H^+ \ .$$

The reaction (c), nonheme iron as the electron donor, has also two possible sites:

$$(D) + O_2 \longrightarrow (E) + O_2^- \text{ and } (F) + O_2 \longrightarrow (G) + O_2^- \ .$$

A further investigation is required to identify the exact site for superoxide formation in APS reductase.

The formation of flavin semiquinone was assumed as an intermediate from the enzyme spectrum change caused by the addition of AMP to the sulfite-reduced enzyme (Eq. 3 in "Results"). Under these conditions the absorbance at 600 nm did not change significantly (Figs. 11 c and 12 c).

Two different types of semiquinone exist depending on its ionic state (Müller et al., 1970). One type is a blue (neutral) semiquinone which has considerable extinction in the region of 600 nm and is consistent with $F\ell H_2^{\cdot}$, the two labile protons occupying positions N(3) and N(5). The other type is a red (anionic) semiquinone which has an absorption peak at 480-490 nm

and always has very pronounced extinction in the region of 370 nm. The red type of flavin semiquinone is due to the corresponding anion formation by one acidic proton remaining at N(3) which is designated as $F\ell H^{\bullet}$ or to the tautomeric neutral species.

If the semiquinone was formed as an intermediate of the APS reductase reaction, it should be in the form of a red semiquinone. In order to confirm the existence of the semiquinone, the EPR measurement should be carried out with a rapid freezing technique after the addition of AMP to the sulfite-reduced enzyme. It is known that aromatic radicals, e.g. flavin semiquinone, display a typical EPR signal at g = 2.00.

PART II

ENZYMES INVOLVED IN GLUTAMATE SYNTHESIS IN *THIOBACILLUS THIOPARUS*

INTRODUCTION

INTRODUCTION

Microorganisms in a medium with ammonia as the sole source of nitrogen must synthesize amino acids from ammonia to grow. For many years it was assumed that the primary metabolic pathway for the synthesis of glutamate involved the reductive amination of α-ketoglutarate catalyzed by glutamate dehydrogenase. Recently, however, an alternate pathway for glutamate synthesis was discovered in K. aerogenes (Tempest et al., 1970). This pathway involved two reactions. The first reaction was the formation of glutamine from ammonia and endogenous glutamate catalyzed by glutamine synthetase. The second reaction was the reductive transfer of the glutamine amido-nitrogen to α-keto-glutarate to form glutamate. This reaction was catalyzed by a previously unknown enzyme, glutamate synthase.

In K. aerogenes, it was found that glutamate dehydrogenase operated only under the ammonia excess conditions. The ammonia-limited cultures of K. aerogenes repressed glutamate dehydrogenase and produced a high level of glutamate synthase (Meers et al., 1970; Senior, 1975). It was suggested that due to the high

affinity of glutamine synthetase for ammonia (K_m < 0.2 mM) this pathway had the capacity to function at levels of free ammonia far below those necessary for the production of glutamate by glutamate dehydrogenase (Tempest et al., 1970; Miller and Stadtman, 1972).

In E. coli, however, Senior (1975) demonstrated that glutamate dehydrogenase was progressively induced under the ammonia-limited conditions instead of glutamate synthase which remained much the same level during the ammonia starvation. Senior concluded from these observations that E. coli unlike K. aerogenes used glutamate dehydrogenase to incorporate ammonia into glutamate during the ammonia-limited growth.

In the present investigation an attempt was made to survey the enzymes involved in glutamate synthesis in T. thioparus in order to study the mechanism of ammonia assimilation in this autotrophic bacterium.

Although glutamate sythase has been detected in a large number of prokaryotic microorganisms and some eukaryotes, it was purified from only two sources, E. coli (Miller and Stadtman, 1972) and K. aerogenes (Trotta et al., 1974). In the present study an attempt

was also made to purify glutamate synthase from *T. thioparus* and to characterize it with regard to its kinetic properties and structure.

HISTORICAL

HISTORICAL

1. Glutamate Dehydrogenase

Glutamate dehydrogenase (L-glutamate:NAD(P) oxidoreductase(deaminating)) catalyzes the reversible reductive amination of α-ketoglutarate to form L-glutamate (Eq.11).

$$\alpha\text{-ketoglutarate} + NH_3 + NAD(P)H + H^+ \rightleftharpoons \text{L-glutamate} + NAD(P)^+ + H_2O \quad (11)$$

The reaction is of importance in ammonia incorporation and linking the nitrogen metabolism to the carbon metabolism.

The enzyme has been widely studied in animals, higher plants, fungi and bacteria. The properties of the enzyme from animals, especially from bovine liver mitochondria, have been studied extensively with regard to kinetic property, control mechanism and physical structure. In order to limit the subject in the area of microorganisms, especially bacteria, the historical aspects of the enzymes from animals and plants are not discussed here. The reader is referred to excellent reviews on glutamate dehydrogenases from animals by Frieden (kinetic properties; 1970), Goldin and Frieden (control mechanisms; 1971) and Fisher (physical structure; 1973) and also fungi by

LéJohn (kinetic control and phylogeny; 1974, 1975).

Frieden (1965) found that the enzymes from plants and microorganisms, unlike the animal enzymes, were not affected by low concentration of purine nucleotides, nor were they inhibited by zinc, apparently due to the lack of these specific binding sites. Of particular note in Frieden's studies is the discovery that all the non-animal enzymes are specific for one of the two pyridine nucleotide coenzymes which are used non-specifically by the animal enzymes.

It has been shown that some microorganisms possess two distinct glutamate dehydrogenases, one specific for NAD^+(NADH) and the other specific for $NADP^+$(NADPH). This situation has been demonstrated in a number of fungi (Holzer and Schneider, 1957; Sanwal and Lata, 1961; Dennen and Neiderpruem, 1967; Casselton, 1969; LéJohn and Stevenson, 1970; also see LéJohn, 1971) and in facultative autotrophic bacteria, T. novellus (LéJohn and McCrea, 1968) and Hydrogenomonas H16 (Krämer, 1970). In 1971, LéJohn reported that only NAD-dependent glutamate dehydrogenase was found in the lower fungi, while the higher fungi possessed both the NAD- and NADP-dependent forms (also see Casselton, 1969). It was suggested that in the lower fungi, Phycomycetes, there existed three types of NAD-dependent glutamate dehydrogenases with respect to control

mechanism which were closely related to the order of evolution (LéJohn, 1971).

Sanwal and Lata (1961) suggested that the occurrence of two enzyme forms in Neurospora crassa, each specific for a different coenzyme, reflected their separate role in catabolism and biosynthesis. Studies of the regulation of the relative activities of the two enzymes indicated that the NADP-dependent form of the enzyme operated in the direction of glutamate synthesis, while the NAD-dependent form functioned in the catabolism of glutamate to form α-ketoglutarate and ammonia (Sanwal and Lata, 1962; Hollenberg et al., 1970). This concept seems to be supported by the studies on bacteria which possess only one form of the enzyme. The NAD-dependent enzyme from clostridia functions to supply α-ketoglutarate for fermentation and transamination (see Goldin and Frieden, 1971). On the other hand, the function of NADP-dependent glutamate dehydrogenases from E. coli (Halpern and Umbarger, 1960) and from an obligate autotroph, N. europaea (Hooper et al., 1967) appears to be the ammonia assimilation in the synthesis of glutamate.

Allosteric regulation has been found to affect NAD-dependent glutamate dehydrogenase from T. novellus which possessed both types of the enzyme (LéJohn, 1967).

The regulation was unidirectional and was specifically controlled by AMP (LéJohn et al., 1968). In the presence of AMP, the NAD-linked enzyme was modified so as to favour glutamate breakdown and showed linear kinetics. NADP-dependent glutamate dehydrogenase from T. novellus was not affected by AMP and showed normal Michaelis-Menten kinetics. LéJohn et al. (1968) suggested that AMP could be acting as a signal for a requirement of NADH for the generation of ATP.

2. Glutamate Synthase

Until recently it was assumed that in bacteria the glutamate dehydrogenase-catalyzed synthesis of glutamate from ammonia and α-ketoglutarate provided the major pathway for ammonia assimilation. In bacteria lacking this enzyme, e.g. Bacillus megaterium, alternative routes for ammonia assimilation were postulated (Shen et al., 1959). These included reactions catalyzed by alanine dehydrogenase, leucine dehydrogenase, valine dehydrogenase and aspartate ammonia-lyase.

In 1970, Tempest and co-workers studied on the enzyme system of ammonia assimilation in Klebsiella aerogenes using chemostat cultures. Substantial levels of glutamate dehydrogenase were detected in extracts of

organisms which had been limited in growth by the availability of either glucose, phosphate or magnesium. However, when growth was limited by the availability of ammonia the level of glutamate dehydrogenase in K. aerogenes extract was markedly reduced indicating that glutamate synthesis in ammonia-limited cells proceeded by a route other than the reaction catalyzed by glutamate dehydrogenase. Other known potential enzymes for ammonia assimilation in bacteria were not detected in extracts of the ammonia-limited organisms. Tempest and co-workers, therefore, concluded that ammonia was incorporated into amino acids by an unknown route in the ammonia-limited culture of K. aerogenes.

It was found that when a small amount of ammonia was added to the ammonia-limited culture of K. aerogenes there was a 25-fold increase in free cellular glutamine within 2 minutes of the pulse and the glutamine level returned towards the original level after 10 minutes. In contrast, no other amino acid increased as much as twofold. A comparison of glutamine synthetase level in ammonia-limited K. aerogenes with that in glucose-limited cells demonstrated marked derepression of the enzyme under the former growth conditions (Wu and Yuan, 1968; Rebello and Straus, 1969; Tempest et al., 1970). This

suggested to Tempest and co-workers that ammonia assimilation in ammonia-limited K. aerogenes proceeded via the glutamine synthetase-catalyzed synthesis of glutamine from glutamate, NH_3 and ATP (Eq. 12), and that a novel enzyme existed for the conversion of glutamine and an α-ketoacid to glutamate and the corresponding amino acid. This enzyme was shown to catalyze the transfer of the α-amino group of glutamine to α-ketoglutarate to form 2 molecules of glutamate (Glutamate synthase, Eq. 13) (Tempest et al., 1970).

$$\text{L-Glutamate} + NH_3 + \text{ATP} \longrightarrow \text{L-glutamine} + \text{ADP} + P_i \quad (12)$$

$$\text{L-Glutamine} + \alpha\text{-ketoglutarate} + \text{NAD(P)H} + H^+ \longrightarrow 2\ \text{L-glutamate} + \text{NAD(P)}^+ \quad (13)$$

$$\text{L-Glutamate} + \alpha\text{-ketoacid} \longleftrightarrow \alpha\text{-ketoglutarate} + \alpha\text{-amino acid} \quad (14)$$

Coupled with the reaction catalyzed by glutamine synthetase (Eq. 12), the glutamate synthase-catalyzed reaction (Eq. 13) provides a previously unknown pathway of glutamate synthesis similar to the glutamate dehydrogenase reaction (Eq. 11) except ATP is involved (Eq. 12 + Eq. 13): NH_3 + α-ketoglutarate + NAD(P)H + H^+ + ATP ⟶ L-glutamate + NAD(P)^+ + ADP + P_i. Furthermore, it provides an ATP-dependent pathway for the assimilation of

ammonia into all amino acids by coupling with α-ketoacid-glutamate transaminase (Eq. 14). The overall reaction (Eq. 12 + Eq. 13 + Eq. 14) is : NH_3 + α-ketoacid + NAD(P)H + H^+ + ATP ⟶ α-amino acid + $NAD(P)^+$ + ADP + P_i.

Since the discovery of glutamate synthase in K. aerogenes (Tempest et al., 1970), its existence in a large number of microorganisms has been established. Meers et al. (1970) measured the enzyme in K. aerogenes, Erwinia carotovara, Pseudomonas fluorescens, B. subtilis, and B. megaterium. Nagatani et al. (1971) in a study of the mechanism of ammonia assimilation in nitrogen-fixing bacteria detected glutamate synthase in K. pneumonia, Azotobacter vinelandii, Clostridium pasteurianum, Chromatium D., Chlorobium thiosulfatophilum, Rhodospirillum rubrum, Rhizobium japonicum (bacteroids), and in the non-nitrogen fixing bacterium Escherichia coli K12. Savageau et al. (1972) found the enzyme activity in Salmonella typhimurium and Acinetobacter calco-aceticus. Glutamate synthase activity has also been detected in twelve strains of pseudomonas-like, nitrate grown, marine bacteria (Brown et al., 1972), in several species of blue-green algae and thermophilic bacteria (Tempest et al., 1973), and in free living cultures of Rhizobium spp. (Brown and Dilworth, 1975). This pathway catalyzed by glutamine synthetase and

glutamate synthase also provided the major, if not the only, route for ammonia assimilation in B. megaterium (Elmerich and Aubert, 1971) which lacks glutamate dehydrogenase and mutants of B. subtilis lacking both glutamate dehydrogenase and alanine dehydrogenase (Tempest et al., 1973).

Although the occurrence of glutamate synthase in bacteria has been well documented and the enzyme appears to be widely distributed, there is presently little information concerning the distribution of this enzyme in eukaryotic organisms. Tempest et al. (1973) have reported their inability to detect the enzyme in a variety of eukaryotes including yeasts (Candida utilis, Saccharomyces cerevisiae) as well as mammalian liver and brain from rat, mouse, and guinea pig. Neilson and Doudoroff (1973) also reported that the enzyme activity could not be detected in blue-green algae. Although glutamate synthase seems not to occur either in fungi, N. crassa and Aspergillus nidulans or in green alga Chlorella vulgaris, Brown et al. (1973) succeeded in detecting the activity in fission yeast, Schizosaccharomyces pombe, for the first time in a eukaryote. Glutamate synthase was also detected in yeast, S. cerevisiae (Roon et al., 1974). The level of glutamate synthase in yeast was approximately 10 fold lower than the maximum

level of activity for either glutamate dehydrogenase. This low activity and the presence of NAD(P)H oxidase in crude extracts could be the reasons for a previous report (Tempest et al., 1973) indicating that yeast does not contain glutamate synthase. Dougall (1974) reported of finding this enzyme in carrot cell culture, but the properties of the carrot glutamate synthase, in particular the substrate specificity, differed markedly from those of the other enzymes. The carrot enzyme could use either NADH or NADPH as coenzyme and L-asparagine could substitute for L-glutamine as amino doner, while the enzymes from all other sources were specific to one of the two pyridine nucleotide coenzymes and could not use L-asparagine as amino donor. It is not clear at this stage whether this enzyme from carrot possesses low specificity to its substrates or the activity detected was a mixture of several enzymes.

Glutamate syntase has been purified, so far, from only two sources, E. coli (Miller and Stadtman, 1972) and K. aerogenes (Trotta et al., 1974). The purified E. coli enzyme was an iron-sulfide flavoprotein and had a molecular weight of 800,000. Sodium dodecyl sulfate gel electrophoresis revealed the existence of two types of subunits with molecular weights of 135,000 and 53,000.

Since the enzyme in an ammonium sulfate fraction of crude extract sedimented much more slowly in the sucrose density gradient centrifugation than the purified enzyme, it was suggested that the purified enzyme might be composed of four catalytically active dimers of non-identical subunits (Miller and Stadtman, 1972). Miller and Stadtman (1972) demonstrated that the enzyme-bound flavin could be reduced with NADPH and the addition of both α-ketoglutarate and L-glutamine partially reoxidized the dithionite-reduced enzyme, and concluded that flavin was actively involved in the enzyme reaction.

The K. aerogenes enzyme was purified unexpectedly in the course of studies on glutamine-dependent carbamyl phosphate synthetase by Trotta et al. (1974). Glutamate synthase from K. aerogenes had a molecular weight of 227,000 and was also an iron-sulfide flavoprotein composed of dissimilar subunits exhibiting molecular weights of 175,000 and 51,500. It was found by the use of a radioactive chloroketone analog of glutamine that only the heavy subunit possessed a binding site for glutamine. The sites for flavin and iron-sulfide were also located on the heavy subunit (Trotta et al., 1974). The function of the light subunit has not been determined.

3. Enzyme Levels

Glutamate occupies a central position in bacterial amino acid metabolism, acting as amino donor of transamination in the synthesis of practically all other amino acids. Therefore, control of glutamate synthesis is fundamental to the control of amino acid synthesis and growth. The effects of availability of nitrogen or energy sources on the enzymes of glutamate synthesis were studied in K. aerogenes by Tempest and co-workers (Tempest et al., 1970; Meers et al., 1970). In extracts of K. aerogenes that had been grown in chemostat culture with growth limited by the availability of either glucose or phosphate, substantial amounts of glutamate dehydrogenase could be detected, while almost no activity of glutamate synthase was found. But in the ammonia-limited culture of K. aerogenes, the level of glutamate dehydrogenase was greatly decreased, while a considerable amount of glutamate synthase was found (Meers et al., 1970). There seems to be a clear reciprocal relationship between the levels of glutamate dehydrogenase and glutamate synthase depending on the availability of ammonia in K. aerogenes.

However, it has gradually become evident that this type of control on glutamate synthesis in response to the concentration of ammonia is not ubiquitous among organ-

isms which possess glutamate synthase as well as glutamate dehydrogenase. The level of glutamate synthase in B. megaterium decreased, instead of increasing, when the growth was limited by the availability of ammonia, while the glutamate dehydrogenase level was constant (Meers et al., 1970; Tempest et al., 1973). In E. coli grown in batch culture, levels of these two enzymes were relatively unaffected by ammonia concentration (Miller and Stadtman, 1972). However, Senior (1975) demonstrated that growth of E. coli in the ammonia-limited chemostat induced glutamate dehydrogenase progressively during the nitrogen starvation.

Although there appears to be no common enzyme level control in response to the availability of ammonia, it was generally suggested on the basis of kinetic data that during ammonia-limited growth glutamate dehydrogenase would not operate because of its low affinity for ammonia (K_m for $NH_4^+ \geq 5$ mM, Sanwal and Zink, 1961; 1.5 to 3.0 mM, Miller and Stadtman, 1972). Under the conditions of ammonia starvation, therefore, it was suggested that glutamate was synthesized via reactions catalyzed by glutamine synthetase and glutamate synthase (glutamine pathway) because of its overall high affinity for ammonia (K_m of glutamine synthetase for $NH_4^+ \leq 0.2$ mM, Tempest

et al., 1970; Miller and Stadtman, 1972). On the other hand, it was suggested by Prusiner and Stadtman (1973) that when there was an ample supply of ammonia in the growth medium the glutamine pathway would not operate because glutamine synthetase would be repressed (Woolfolk et al., 1966; Wu and Yuan, 1968; Pateman, 1969). Under these conditions glutamate dehydrogenase acting biosynthetically is probably the key enzyme in ammonia assimilation.

Senior (1975) studied on the levels of enzymes involved in glutamate biosynthesis with chemostat cultures of K. aerogenes and E. coli grown under the various conditions of nitrogen supply. This author concluded that this proposal for the enzyme level control by the availability of ammonia is valid in K. aerogenes. It was found, however, that the levels of glutamate dehydrogenase, glutamine synthetase and glutamate synthase in E. coli changed differently from the K. aerogenes system. Under the ammonia-limited growth conditions, glutamate dehydrogenase was progressively induced in E. coli and its level became much higher than that of glutamate synthase which remained at the same level during the ammonia starvation. It was, therefore, concluded that E. coli uses glutamate dehydrogenase to incorporate ammonia during the ammonia-limited conditions unlike K. aerogenes (Senior, 1975).

The control mechanism of the glutamate dehydrogenase level has been studied extensively in fungi

(Sanwal and Lata, 1961, 1962; Holzer, 1966; Pateman, 1969; Strickland 1971; Fawole and Casselton, 1972) which seem not to possess strong glutamate synthase, if any (Tempest et al., 1973; Brown et al., 1973; Roon et al., 1974). It appears that regulation of the two enzyme forms is affected both by nitrogen metabolism and by catabolite effects of carbon sources (Hynes, 1974). As the glutamate dehydrogenase reaction is the main link between the tricarboxylic acid cycle and amino acid metabolism in fungi, such a regulation pattern might be expected.

The effect of glutamate on the enzymes of nitrogen assimilation in bacteria was studied in K. aerogenes and E. carotovora (Meers et al., 1970). When glutamate was provided as a sole source of nitrogen either growth limiting or in excess quantity, the synthesis of both glutamate dehydrogenase and glutamate synthase was suppressed in K. aerogenes. E. carotovora totally lacked glutamate dehydrogenase and the level of glutamate synthase decreased under these conditions. However, glutamate is apparently not the corepressor of these enzymes. When glutamate was provided as the sole nitrogen source to a nitrogen-limited culture of K. aerogenes instead of ammonia the intracellular glutamate level was only slightly changed yet the synthesis of glutamate synthase was still totally repressed. The pool glutamate level was greatly

increased by the addition of NaCl to the ammonia-limited K. aerogenes culture, but the levels of glutamate synthase and glutamate dehydrogenase were only partially affected (Meers et al., 1970). Roon et al. (1974) reported that in yeast, S. cerevisiae, NAD-dependent glutamate dehydrogenase was repressed by ammonia, while the activities of NADP-dependent glutamate dehydrogenase and glutamate synthase were increased. Glutamate induced only NAD-dependent glutamate dehydrogenase without affecting the levels of NADP-dependent glutamate dehydrogenase and glutamate synthase significantly.

In K. aerogenes there appears to be a clear reciprocal relationship between the activities of glutamine synthetase and glutamate dehydrogenase. Magasanik et al. (1973) have proposed that the regulation of glutamate dehydrogenase synthesis is controlled by the glutamine synthetase molecule itself in a fashion similar to a recently proposed regulation of histidase activity in a mutant of K. aerogenes (Prival et al., 1973) and in Salmonella typhimurium (Tyler et al., 1974). It was noted by Senior (1975) that the absence of close linkage in repression and induction between glutamate dehydrogenase, glutamine synthetase and glutamate synthase in E. coli may be because there is no close genetic

linkage between the structural genes of E. coli K12 coding for these enzymes (Berberich, 1972).

RESULTS

RESULTS

A. GLUTAMATE DEHYDROGENASE

1. Evidence for Existence of Two Distinct Glutamate Dehydrogenases

It was found that both NADH and NADPH were active as coenzyme when the spinco supernatant ("Purification of NADP-dependent GDH") was assayed for glutamate dehydrogenase. In order to separate the NADH activity from the NADPH activity, if possible, DEAE cellulose column chromatography and ammonium sulfate fractionation were performed.

The spinco supernatant (40 ml) was applied to a DEAE cellulose column (2.5 x 40 cm) previously equilibrated with 0.02 M phosphate buffer (pH 7.5) and the proteins were eluted with a linear gradient of phosphate buffer (0.05 M to 0.20 M). It was found, however, that the fractions which contained the NADH activity also had most of the NADPH activity as well as a large amount of flavoprotein known as APS reductase (Lyric and Suzuki, 1970 b).

The pooled DEAE eluate was made 40% saturated in ammonium sulfate. After 20 min of stirring, the solution

was centrifuged at 27,000 x g for 20 min. The pellet was discarded and the supernatant was further fractionated with ammonium sulfate stepwise. The precipitates which formed at 50%, 60% and 70% saturation in $(NH_4)_2SO_4$ were collected separately and dissolved in 0.1 M phosphate buffer (pH 7.5). These fractions were dialyzed against the same buffer and assayed for glutamate dehydrogenase. The results are shown in Table 2.

The activity of glutamate dehydrogenase depending on NADH was found mainly in the fraction 1, while most of the NADPH-dependent activity was found in the fraction 2. From these results it was concluded that an obligate autotroph, T. thioparus, possesses two distinct species of glutamate dehydrogenases. A similar situation has been reported for a facultative autotroph, T. novellus (LéJohn and McCrea, 1968) and also for yeast (Holzer, 1957), Neurospora (Sanwal and Lata, 1961), and Schizophyllum (Dennen and Neiderpruem, 1967).

Although two glutamate dehydrogenases were assayed in both directions, the oxidative deamination reaction catalyzed by the NAD-dependent enzyme could not be detected. It was speculated that there might be a control mechanism on this activity, but no effector was found. AMP had no effect. The NADP-dependent

Table 2. Separation of two species of glutamate dehydrogenases.

Fraction (% saturation $(NH_4)_2SO_4$)	Glutamate Dehydrogenase Activity* (Total Units)	
	NADPH activity	NADH activity
1. 40 ~ 50	0.052	0.152
2. ~ 60	0.222	0.072
3. ~ 70	0.005	0.000

* Standard assay system was used ("Methods")

glutamate dehydrogenase was chosen for further characterization studies.

2. Purification of NADP-Dependent Glutamate Dehydrogenase

NADP-dependent glutamate dehydrogenase was partially purified (38-fold) with the recovery of 47% from the crude extract. The pertinent data of the purification obtained by the standard procedure are presented in Table 3. Potassium phosphate buffer (0.1 M, pH 7.5) was used throughout the purification which was performed at 4°C.

Frozen T. thioparus cells were suspended in the buffer in a ratio of 1 g of the cells (wet weight) for each 9 ml of buffer. The cells were disrupted by sonication in a Raytheon sonic oscillator for 30 min. The sonicate was centrifuged at 27,000 x g for 20 min to remove residual debris. The supernatant (crude extract) was further centrifuged at 160,000 x g for 90 min in a Beckman ultracentrifuge Model L or L2-65B. The supernatant (spinco supernatant) was stored at -20°C until needed.

The spinco supernatant (approximately 40 ml) was applied to a DEAE cellulose column (2.5 x 40 cm) which had previously been equilibrated in 0.02 M phosphate buffer. The column was washed with 0.05 M phosphate buffer to remove sulfite oxidase fraction (Lyric and

Table 3. Purification of NADP-dependent glutamate dehydrogenase from *T. thioparus*

Fraction	Total[1] protein (mg)	Total[2] activity (units)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
Crude extract	2427	(2.65)[3]	(0.0011)[3]	1.0	(100)[3]
Spinco supernatant	1120	2.65	0.0023	2.2	100
DEAE eluate	209.0	1.85	0.0089	8.3	70.5
1st $(NH_4)_2SO_4$ 40 - 60%	78.0	1.68	0.0233	20.3	64.5
2nd $(NH_4)_2SO_4$ 50 - 58%	30.6	1.25	0.0409	38.3	47.2

(1) Seventeen grams of cells were used.
(2) The oxidative deamination assay was performed under the standard conditions.
(3) Activity in the spinco supernatant was taken as 100%.

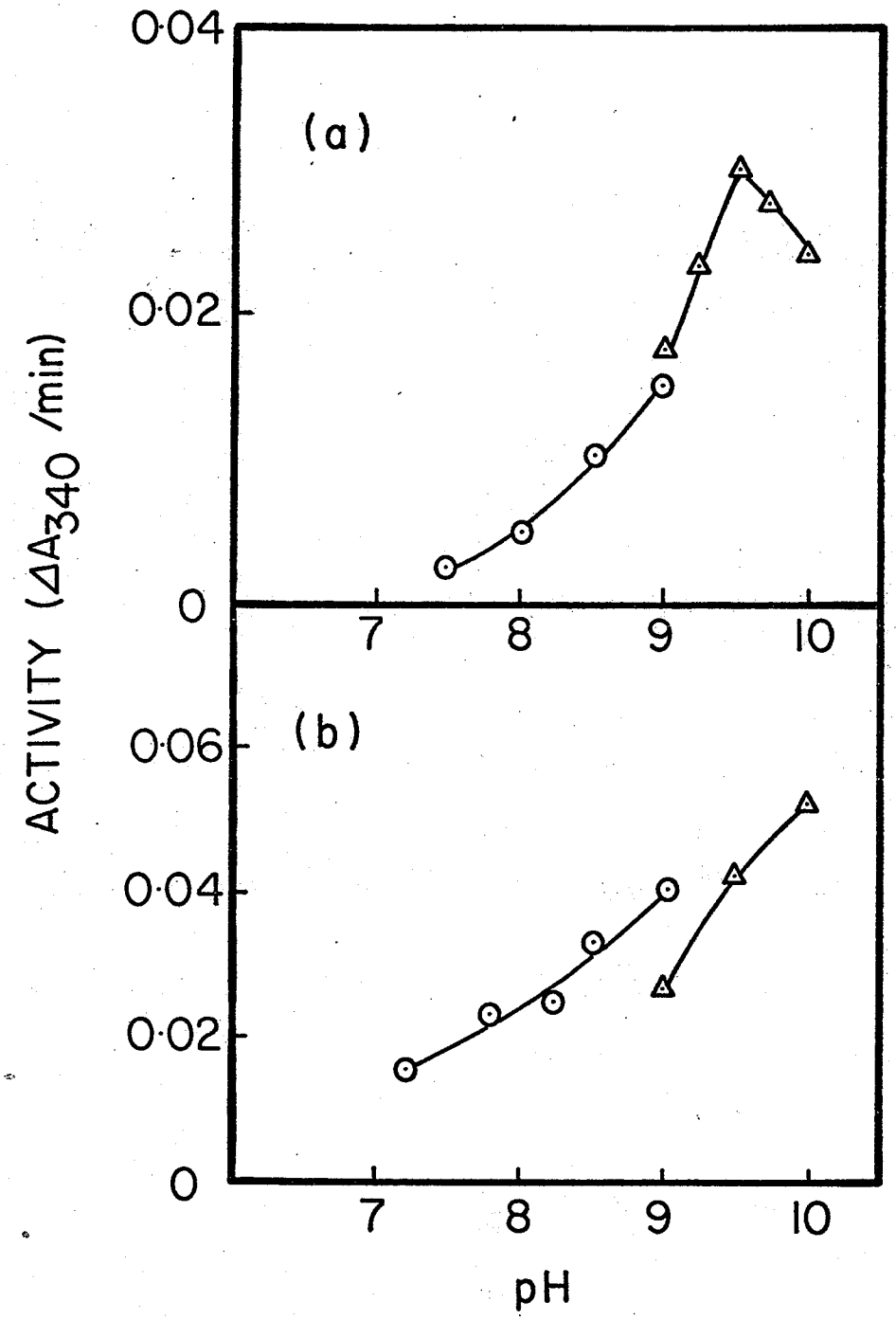
Suzuki, 1970 a) and then the glutamate dehydrogenase was eluted with 0.15 M phosphate buffer.

The pooled eluate was fractionated with ammonium sulfate and the precipitation formed between 40% and 60% saturation was collected by centrifugation and re-suspended in 0.1 M phosphate buffer. The solution was dialyzed against 50% saturated $(NH_4)_2SO_4$ in the buffer and the precipitate was pooled as NAD-dependent glutamate dehydrogenase. The supernatant was dialyzed against 58% saturated $(NH_4)_2SO_4$ and the precipitate was collected and dissolved in the buffer. The solution was dialyzed against the same buffer and pooled as NADP-dependent glutamate dehydrogenase.

3. Effect of pH

The variation in NADP-dependent glutamate dehydrogenase activity in response to changing pH was studied and is presented in Fig. 19. The optimum pH for $NADP^+$ assay (oxidative deamination) was determined to be 9.5. The enzyme was assayed for the reductive amination reaction with NADPH between pH 7 and 10. Although the highest activity was obtained at pH 10, it was found that the enzyme activity was not linear with time above pH 9. It might be caused by a poor stability of the

Figure 19. Effect of varying pH on NADP-dependent glutamate dehydrogenase. The assay conditions were as described in "Methods" for both oxidative deamination (a) and reductive amination (b) reactions except various pH was used as indicated. The assays were carried out in Tris-Cl (—⊙—⊙—), and Glycine-NaOH (—△—△—).


0·04
0·02
0
(a)
7
8
9
10
0·06
0·04
0·02
0
(b)
7
8
9
10
ACTIVITY (ΔA340 /min)
pH

enzyme above this pH. Since glutamate dehydrogenase from other organisms usually had the optimal pH between 7.5 and 8.5 for the reductive amination reaction, pH 8.5 was used for the NADPH assay throughout this report.

4. Effect of Substrate Concentration

The effect of varying substrate concentration on the reaction velocity was studied and presented as the velocity (v) versus the substrate concentration (S) plots. The apparent Michaelis constant was calculated from double reciprocal plots.

Effect of ammonia concentration

The effect of varying NH_4Cl concentration on the activity is presented in Fig. 20. The K_m value for ammonia was determined as 23 mM from Fig. 2 b. This high value is similar to 16 mM, the value obtained for NADP-dependent glutamate dehydrogenase from an obligate autotroph, Nitrosomonas europaea (Hooper et al., 1967), but somewhat higher than that for the T. novellus enzyme (7.5 mM; LéJohn et al., 1968).

Effect of α-ketoglutarate concentration

The velocity concentration plot obtained with varying α-ketoglutarate concentration was a standard rectangular hyperbola (Fig. 21 a). The apparent

Figure 20. Effect of ammonium chloride concentration on NADP-dependent glutamate dehydrogenase activity. Reductive amination assays were performed under the standard conditions ("Methods") with the exception of that the concentrations of NH_4Cl were varied as indicated. (a) The activity (ΔA_{340} per min) was plotted against the concentration of NH_4Cl. (b) Double reciprocal plots of the results obtained in (a).

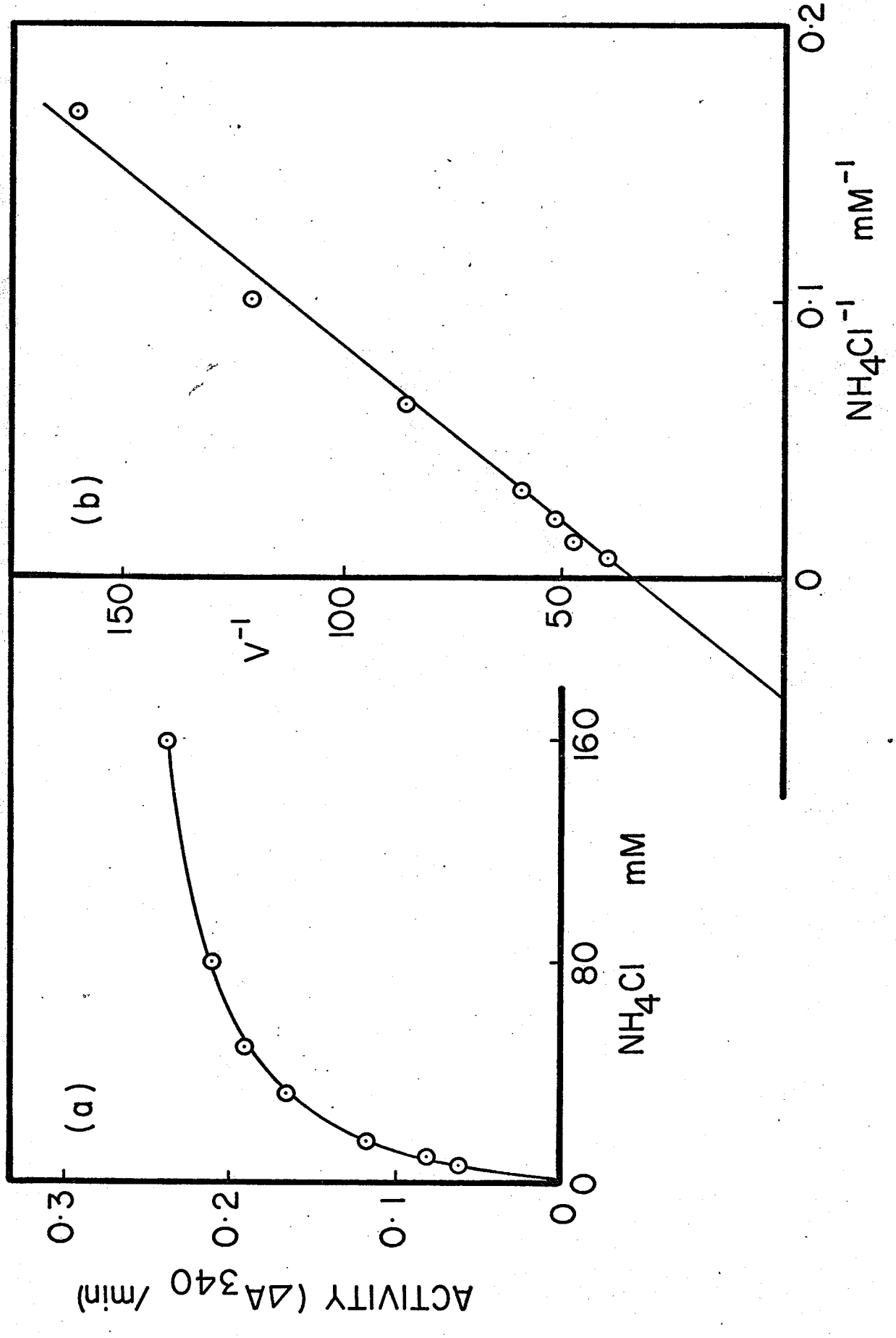
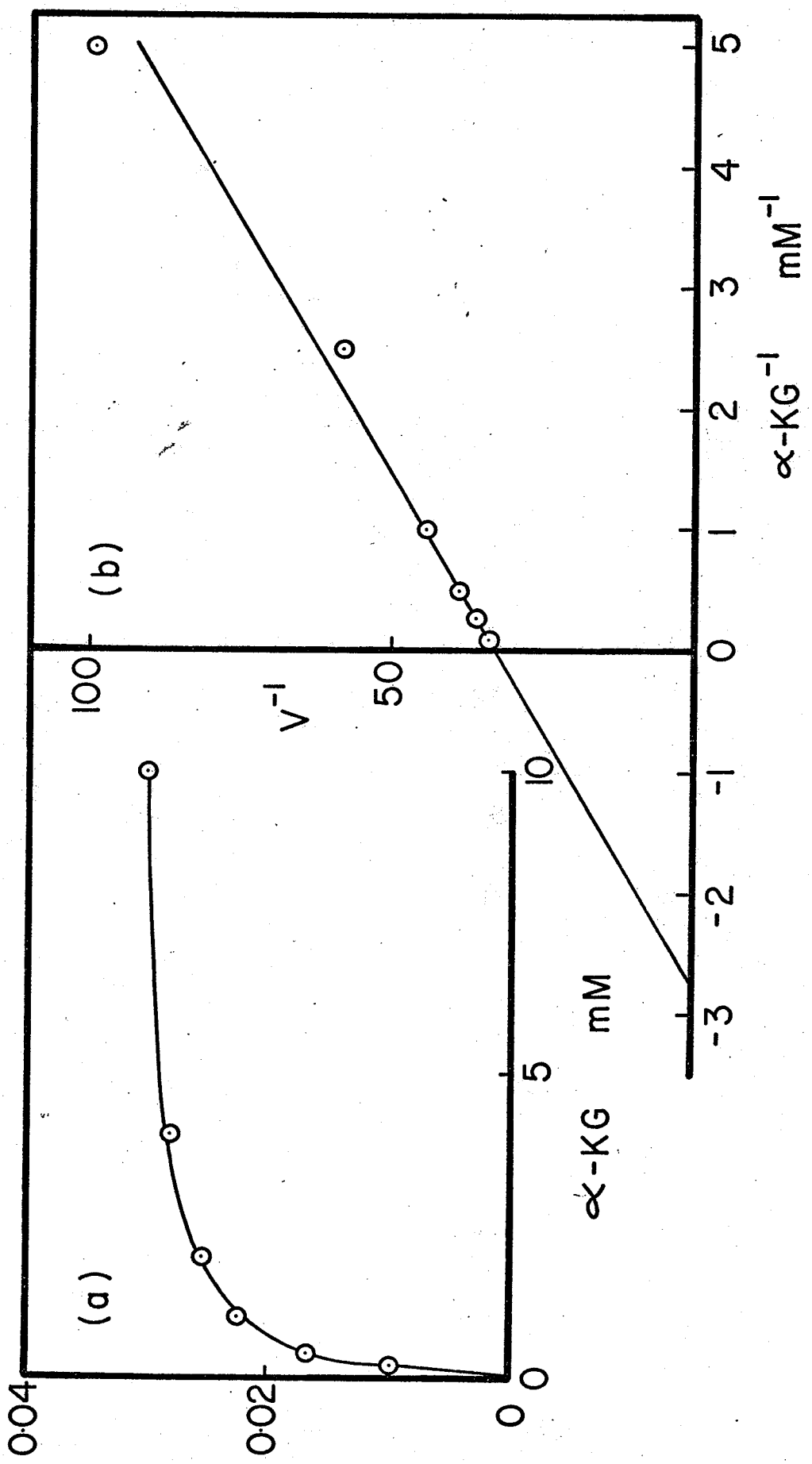

(a)
ACTIVITY (ΔA_{340} /min)
0·3
0·2
0·1
0
0
80
160
NH_4Cl mM
(b)
V^{-1}
150
100
50
0
0·1
0·2
NH_4Cl^{-1} mM^{-1}

Figure 21. Effect of α-ketoglutarate concentration on NADP-dependent glutamate dehydrogenase activity. Reductive amination assays were performed under the standard conditions as described in "Methods" except varying concentration of α-ketoglutarate was used as indicated.


ACTIVITY (ΔA_{340} /min)
0.04
0.02
0
(a)
0
5
10
α-KG mM
(b)
100
50
V^{-1}
-3
-2
-1
0
1
2
3
4
5
α-KG^{-1} mM^{-1}

Michaelis constant was calculated from the double reciprocal plot as 370 μM (Fig. 21 b). This figure is much lower than the values of 4.3 mM and 7.4 mM reported for NADP-dependent glutamate dehydrogenases from N. europaea (Hooper et al., 1967) and T. novellus (LéJohn et al., 1968), respectively.

Effect of NADPH concentration

The effect of varying NADPH concentration on the velocity is shown in Fig. 22. The apparent K_m value was calculated as 80 μM which is similar to those for the enzymes from N. europaea and T. novellus (49 μM and and 77 μM, respectively).

Effect of glutamate concentration

Fig. 23 a shows the velocity concentration plot with varying glutamate concentration. The values were replotted in Fig. 23 b double reciprocally yielding K_m value for glutamate as 870 μM. It was noted that this value is much lower than those for the N. europaea (6.7 mM; Hooper et al., 1967) and the T. novellus enzyme (36 mM; LéJohn et al., 1968).

Effect of $NADP^+$ concentration

The effect of varying $NADP^+$ concentration on the activity is presented in Fig. 24. The apparent K_m for $NADP^+$ was calculated as 50 μM which is similar to 61 μM,

Figure 22. Effect of NADPH concentration on NADP-dependent glutamate dehydrogenase activity. Reductive amination assays were performed under the standard conditions ("Methods") except varying concentration of NADPH was used as indicated.

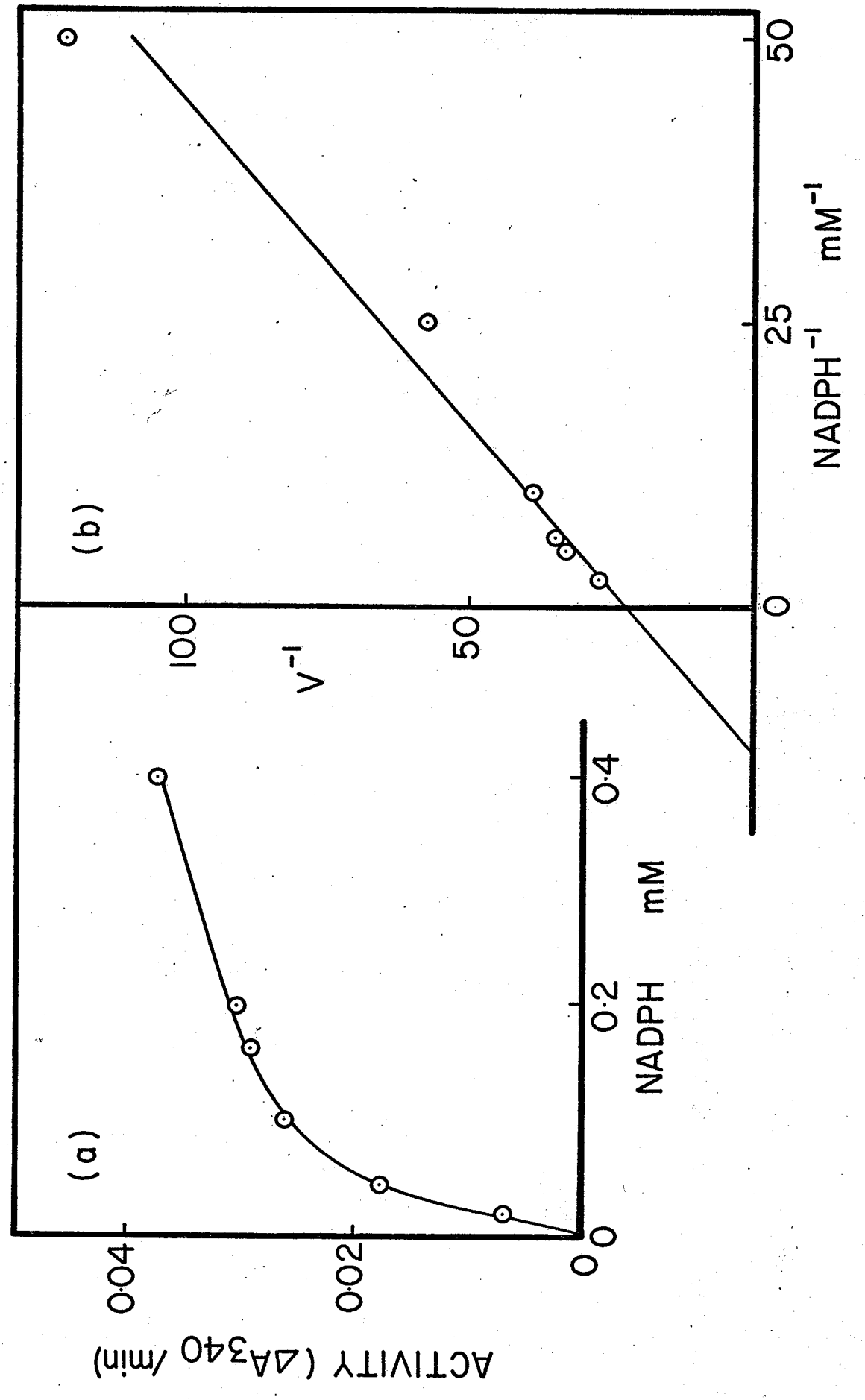

ACTIVITY (ΔA_{340} /min)
0·04
0·02
0·0
0·2
0·4
NADPH mM
(a)
V^{-1}
100
50
0
25
50
$NADPH^{-1}$ mM^{-1}
(b)

Figure 23. Effect of L-glutamate concentration on NADP-dependent glutamate dehydrogenase activity. Oxidative deamination assays were performed under the standard conditions ("Methods") except varying concentration of L-glutamate was used as indicated.

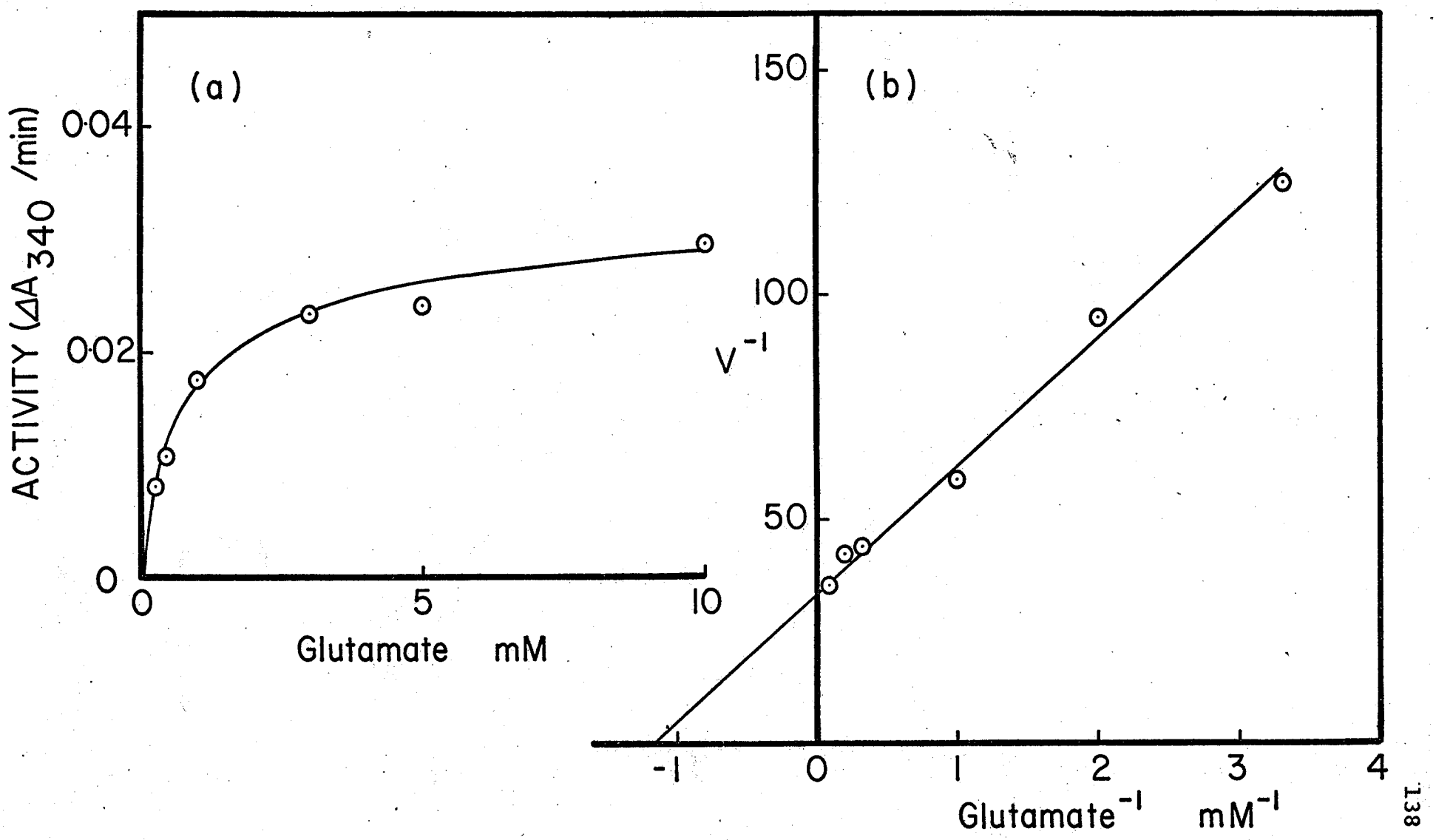
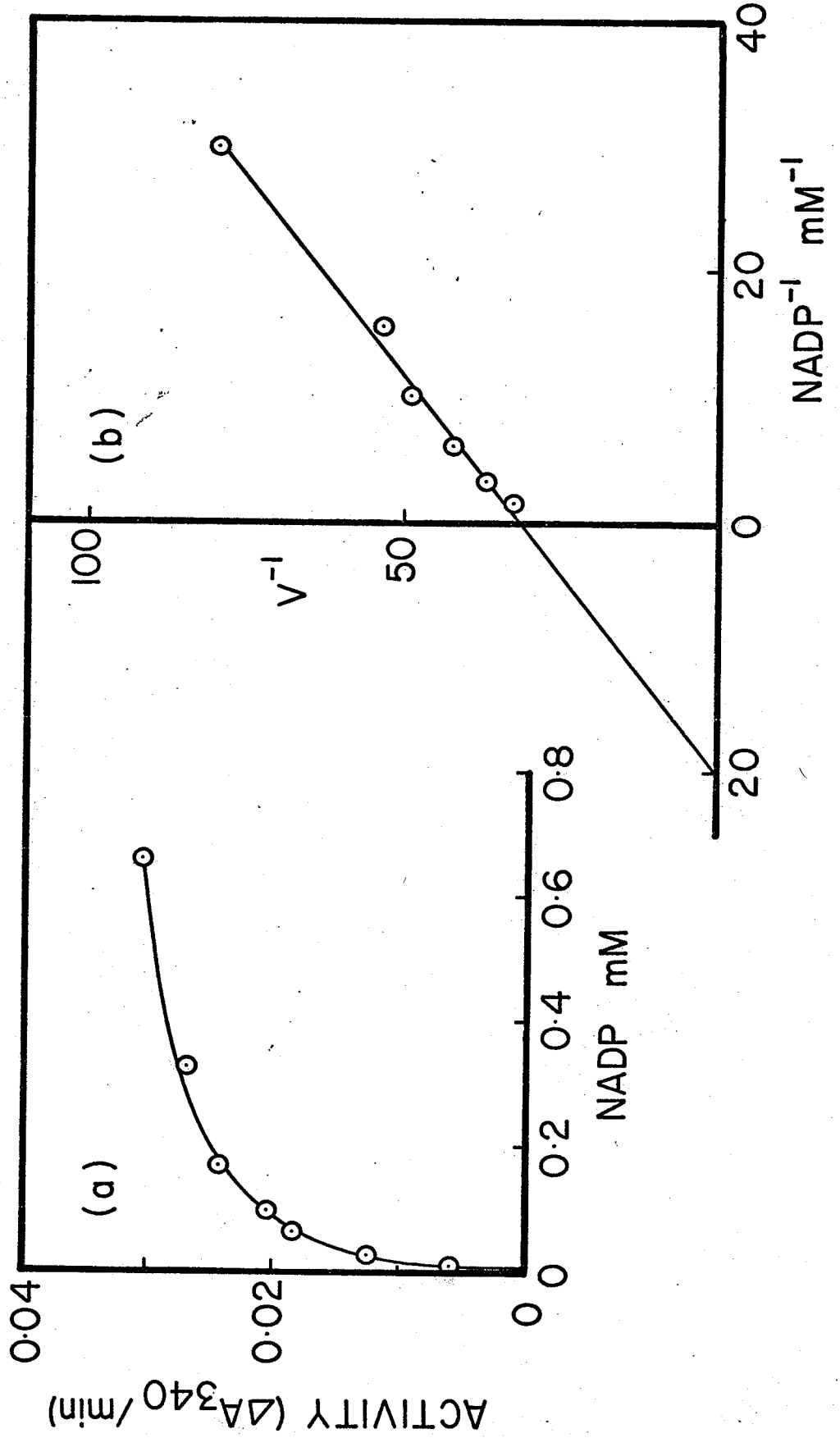

(a)
ACTIVITY (ΔA_{340} /min)
0·04
0·02
0
0
5
10
Glutamate mM
(b)
150
100
50
V^{-1}
-1
0
1
2
3
4
Glutamate^{-1} mM^{-1}

Figure 24. Effect of $NADP^+$ concentration on NADP-dependent glutamate dehydrogenase activity. Oxidative deamination assays were performed under the standard conditions ("Methods") except varying concentration of $NADP^+$ was used as indicated.


(a)
(b)
ACTIVITY (ΔA_{340}/min)
0·04
0·02
0
0
0·2
0·4
0·6
0·8
NADP mM
100
50
0
20
40
V^{-1}
$NADP^{-1}$ mM^{-1}

the value reported for the T. novellus enzyme by LéJohn et al. (1968), but is much higher than that for the N. europaea enzyme (7.9 μM; Hooper et al., 1967).

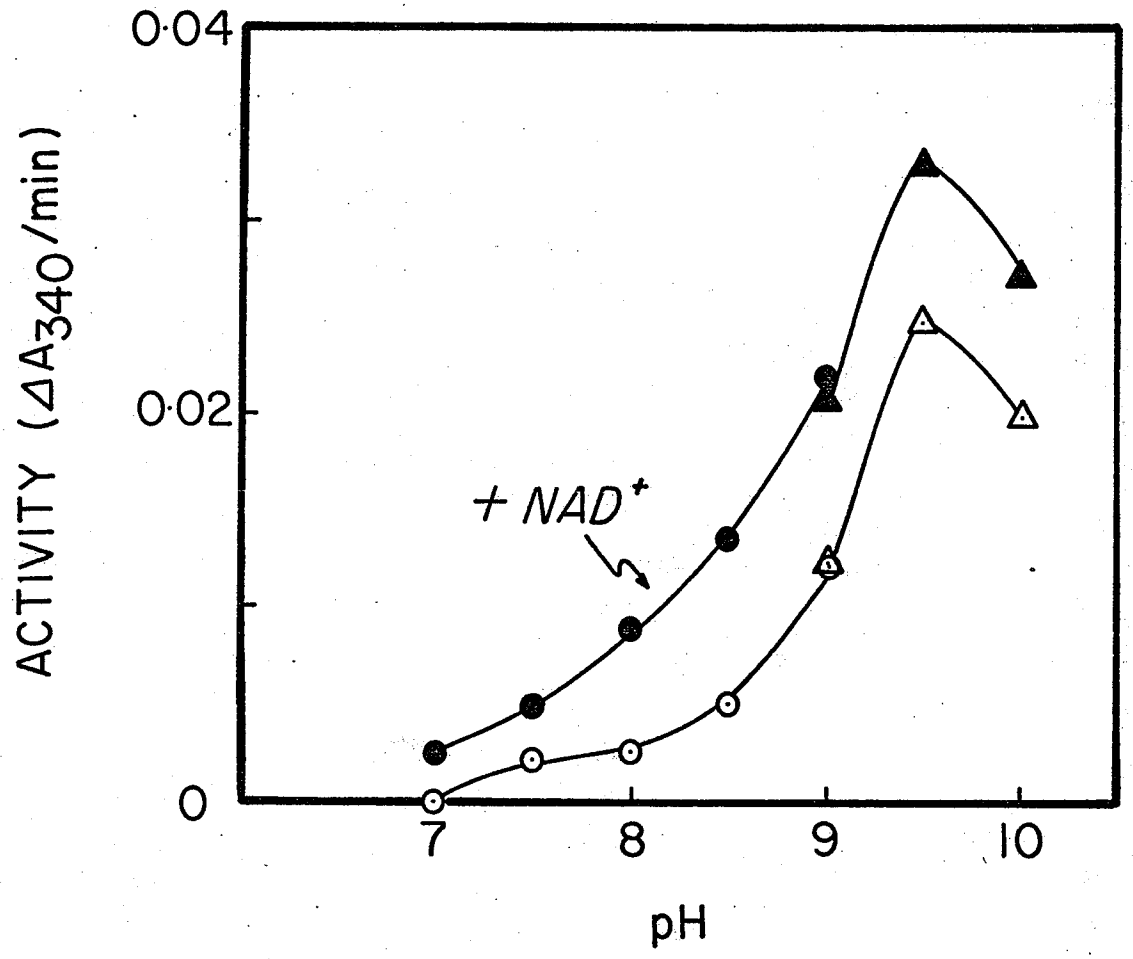
5. Activation of NADP-Dependent Glutamate Dehydrogenase by NAD^+.

Electron transfer between pyridine nucleotides could occur through the catalytic action of transhydrogenase (Kaplan et al., 1956). The existence of NAD-and NADP-dependent isocitrate and glutamate dehydrogenases in many organisms has been considered as a possible route through which such electron transfer may occur (Kaplan, 1963; Greville, 1969).

Attempts were made with no success to detect transhydrogenase activity in the first ammonium sulfate fraction which contained both glutamate dehydrogenases. It was found, however, that NAD^+ stimulated the oxidative deamination activity of NADP-dependent glutamate dehydrogenase (Fig. 25). The partially purified enzyme was activated with 0.4 mM NAD^+ by 32% at pH 9.5 and by 180% at pH 8.5. Since the enzyme preparation was not completely free from NAD-dependent glutamate dehydrogenase, the possibility of NADH formation through the oxidative deamination could not be eliminated entirely.

However, the NAD-dependent enzyme showed no activity when it was assayed for the oxidative deamination with NAD^+ alone and therefore this possibility was considered less likely.

Figure 25. Effect of NAD^+ on the oxidative deamination activity of NADP-dependent glutamate dehydrogenase at various pH. Oxidative deamination assays were performed at various pH indicated. The following buffers were used: Tris-Cl (—⊙—⊙—), Tris-Cl in the presence of 0.4 mM NAD^+ (—●—●—), Glycine-NaOH (—△—△—), Glycine-NaOH in the presence of 0.4 mM NAD^+ (—▲—▲—).


0·04
0·02
0
ACTIVITY (ΔA340/min)
+ NAD+
7
8
9
10
pH

B. GLUTAMATE SYNTHASE

1. Purification

Glutamate synthase was purified over 500-fold from the T. thioparus sonicate. Unless otherwise stated, the buffer contained 50 mM potassium phosphate (pH 7.5), 5 mM EDTA and 1 mM dithiothreitol and all steps in the purification were performed at 4°C. The purification obtained by following the standard procedure, is presented in Table 4.

Step 1 : Preparation of sonicate

Frozen T. thioparus cells were suspended in 0.1 M potassium phosphate buffer (pH 7.5) in a ratio of 1 g of cells for each 4 ml of buffer. Cells were disrupted by sonic treatment for 30 min in a Raytheon sonic oscillator. Routinely, four batches of frozen cells, about 32 g in total wet weight, were used for the purification.

Step 2 : Preparation of crude extract

The sonicate was centrifuged at 48,200 x g for 20 min to remove cellular cebris. The supernatant was pooled as crude extract.

Step 3 : Ultracentrifugation

The crude extract was further clarified by centrifugation at 113,000 x g for 60 min in a Beckman ultracentrifuge (Model L2-65B or L3-50). The supernatant

was kept frozen at -20°C until needed.

Step 4 : First ammonium sulfate fractionation

The spinco supernatant from Step 3 was made 35% saturated in $(NH_4)_2SO_4$ with the solid salt. The solution was stirred for 20 min and then centrifuged at 48,200 x g for 15 min. The pellet was discarded and the supernatant was adjusted to 50% saturated ammonium sulfate. After stirring for 20 min the suspension was centrifuged and the supernatant was discarded. The pellet was dissolved in a minimum amount of the buffer. The solution was dialyzed against about 100 volumes of the buffer for at least 8 hours. Insoluble material was removed by centrifugation.

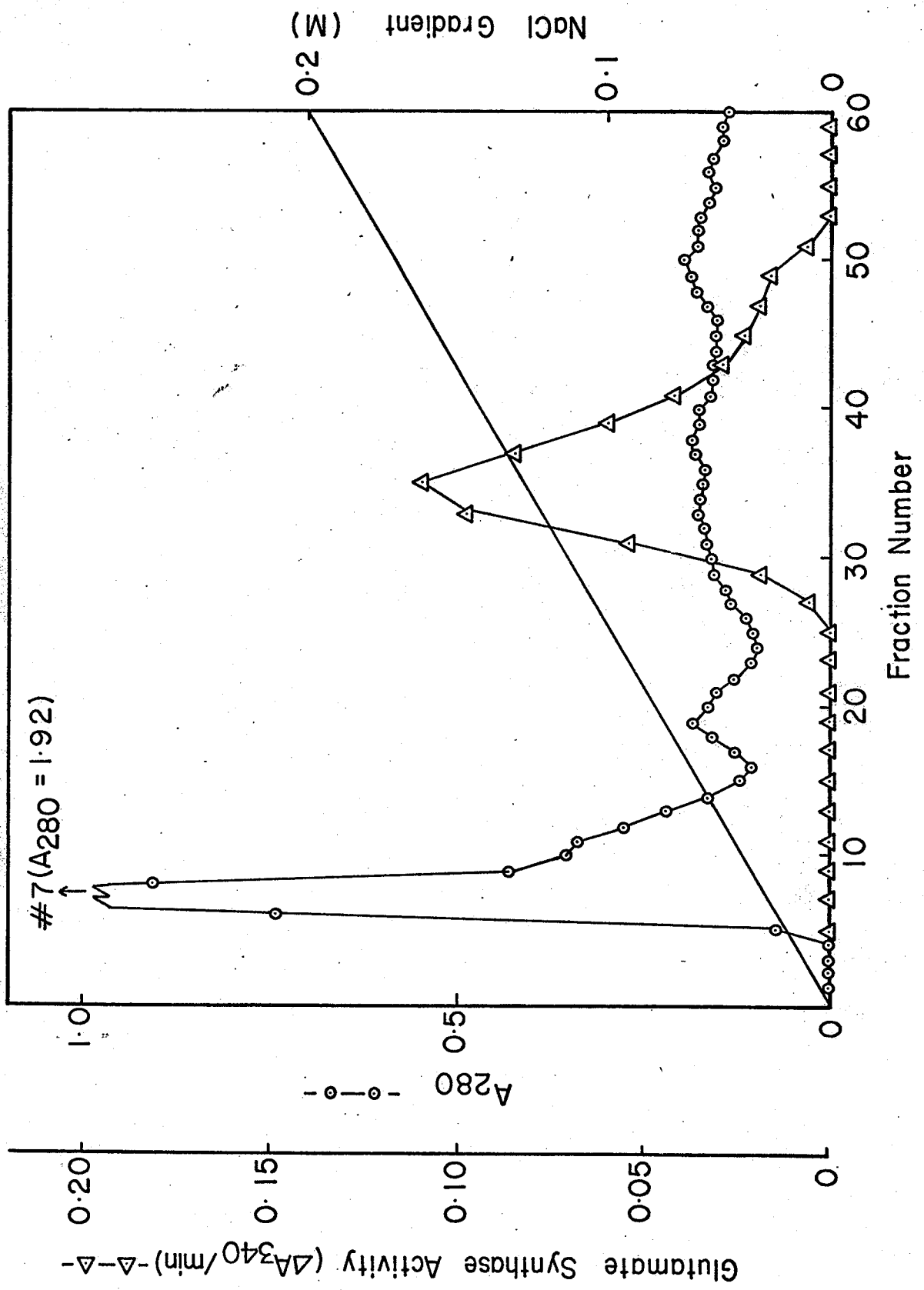
Step 5 : First DEAE-cellulose column chromatography

The solution from Step 4 was applied to a DEAE cellulose column (2.5 x 40 cm) previously equilibrated with the buffer. The enzyme was eluted with a linear sodium chloride gradient (0 to 0.2 M NaCl made in the buffer). Fractions which contained most of the enzyme activity were pooled (fractions 29 to 45 in Fig. 26).

Step 6 : Second ammonium sulfate fractionation

The pooled enzyme in Step 5 was made 70% saturated in $(NH_4)_2SO_4$ and stirred for 20 min. The precipitate was collected by centrifugation and the pellet was suspended

Figure 26. The elution profile of glutamate synthase during the first DEAE-cellulose column chromatography. A sample containing about 700 mg of protein in 40 ml of the first ammonium sulfate fraction was subjected to chromatography as described in text. Glutamate synthase activity was assayed under the standard conditions ("Methods") using 20 µl of each fraction.


Glutamate Synthase Activity (ΔA_{340}/min) -Δ-Δ-
A280 -o-o-
NaCl Gradient (M)
#7 (A_{280} = 1·92)
Fraction Number
0·20
0·15
0·10
0·05
0
1·0
0·5
0
0·2
0·1
0
10
20
30
40
50
60

in 10 ml of 50% saturated ammonium sulfate prepared in the buffer and stirred for 30 min. The turbid solution was centrifuged and the supernatant was discarded. The pellet was suspended in 5 ml of 35% saturated $(NH_4)_2SO_4$ and stirred for 30 min. The solution was centrifuged and the supernatant was dialyzed against 1 ℓ of the buffer. The buffer was changed once after 3-4 hours and the dialysis was continued overnight.

Step 7 : Sephadex G-200 column chromatography

The enzyme solution from Step 6, usually about 10 ml in volume, was applied to a column of Sephadex G-200 (2.5 x 92 cm) previously equilibrated with the buffer. The protein was eluted with the same buffer and the highest activity was found in the fraction which eluted when the ratio of elution volume (Ve) to void volume (Vo) was 1.46 (Fig. 27).

Step 8 : Second DEAE-cellulose column chromatography

The pooled fractions from Step 7 (fractions 71 to 85 in Fig. 27) were applied to a DEAE cellulose column (1.5 x 27 cm). The column was eluted in the same manner as that for the first DEAE cellulose column in Step 5. The fractions with the enzyme activity (fractions 23 to 33 in Fig. 28) were pooled and dialyzed overnight against 2 ℓ of the buffer.

Figure 27. The elution profile of glutamate synthase during Sephadex G-200 column chromatography. A sample containing about 60 mg protein in 12 ml of the second ammonium sulfate fraction was subjected to chromatography as described in text. Glutamate synthase activity was measured as described in "Methods" using 20 µl of each fraction.

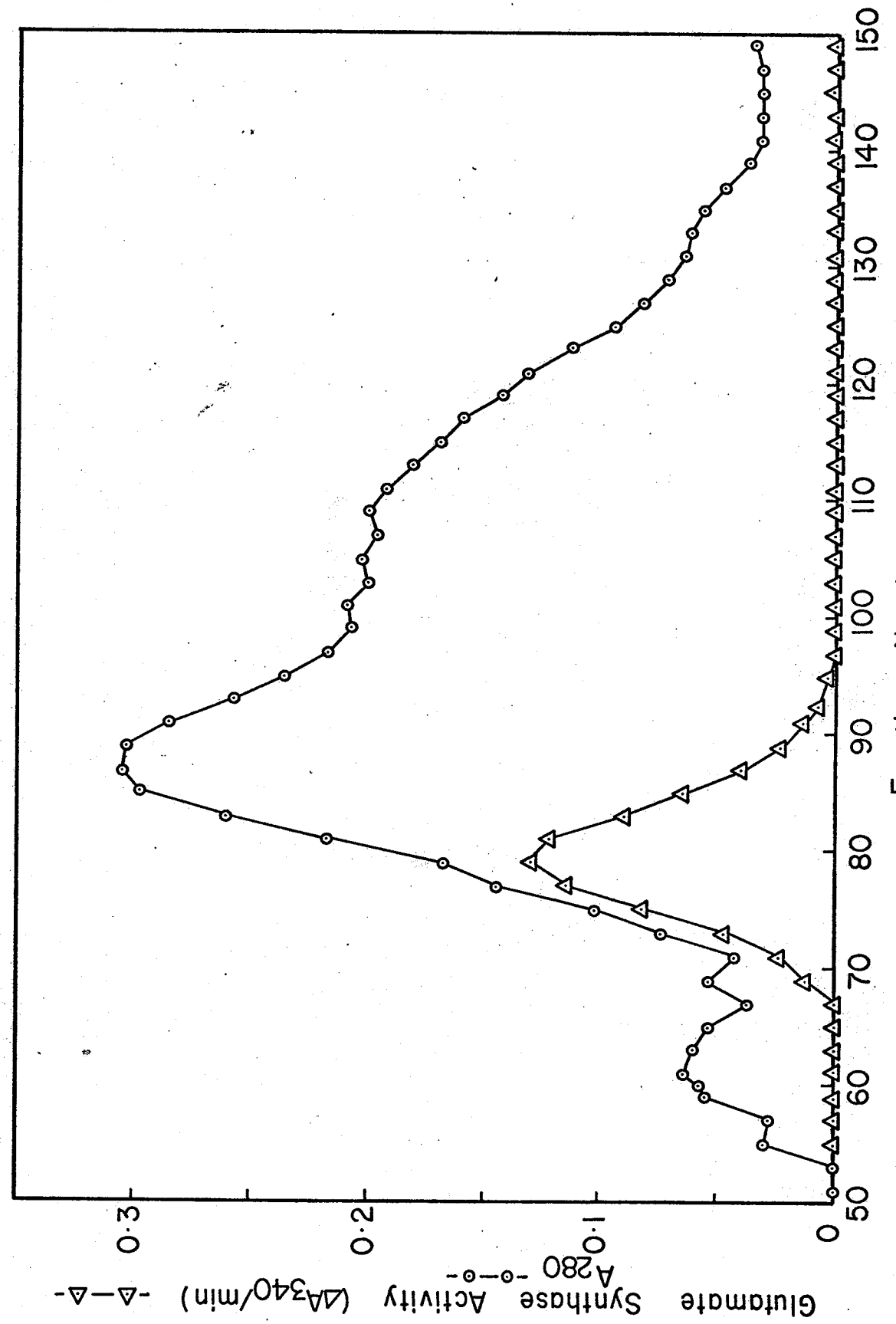

Glutamate Synthase Activity (ΔA_{340}/min) -Δ—Δ-
A_{280} -o—o-
0·3
0·2
0·1
0
50
60
70
80
90
100
110
120
130
140
150
Fraction Number

Figure 28. The elution profile of glutamate synthase during the second DEAE-cellulose column chromatography. A sample containing 13.5 mg protein in 47 ml was subjected to chromatography under the conditions described in text. Glutamate synthase activity was measured as described in "Methods" using 20 µl of each fraction.

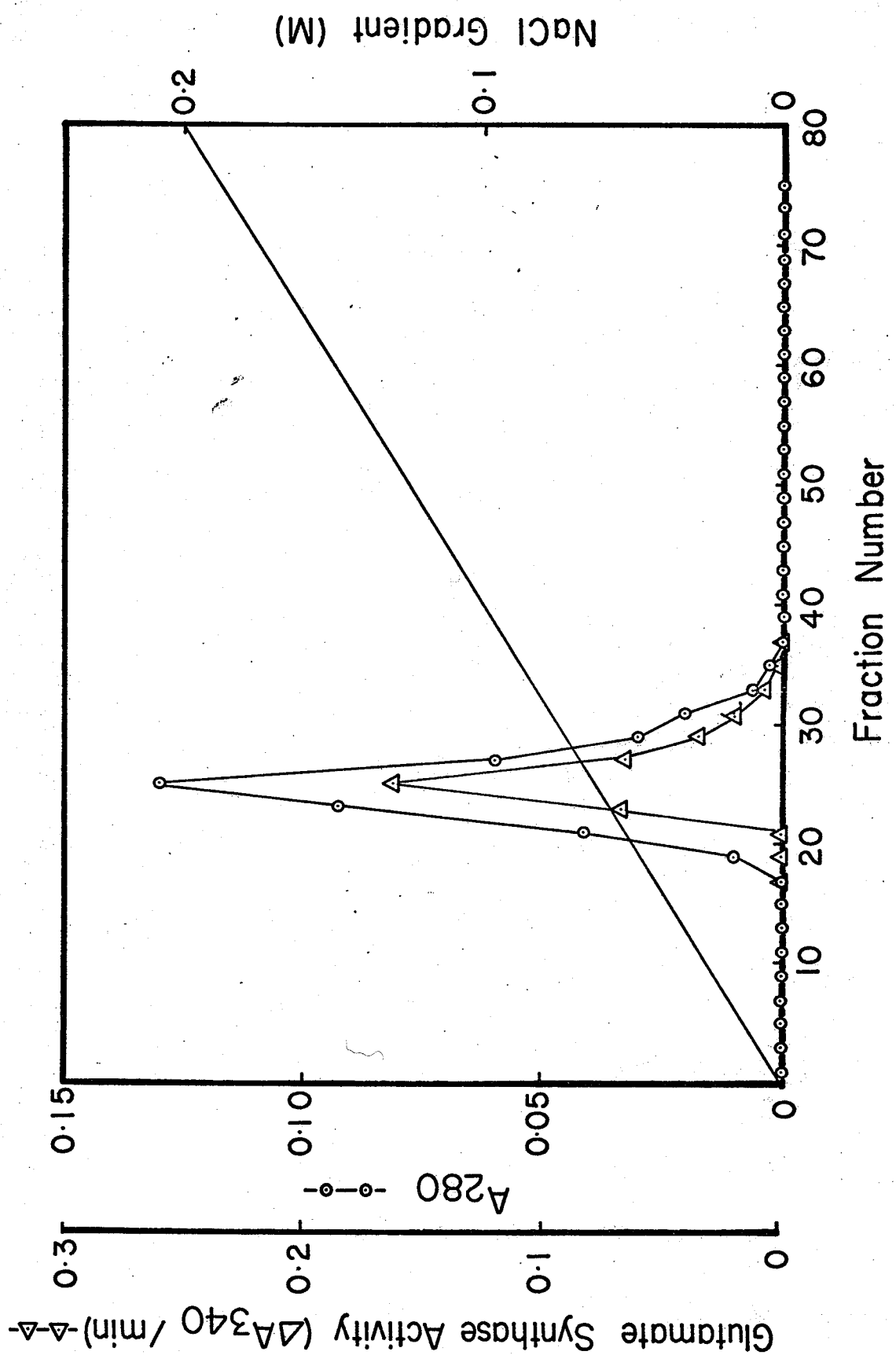

Glutamate Synthase Activity (ΔA340 /min)-▵-▵-
A280 -⊙—⊙-
NaCl Gradient (M)
Fraction Number
0
0·1
0·2
0·3
0·05
0·10
0·15
10
20
30
40
50
60
70
80

Table 4. Purification of glutamate synthase from *T. thioparus*

Fractionation step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
Sonicate	6790	(117)*	(0.0173)*	(1.00)*	(100)*
Crude extract	3120	117	0.0376	2.17	100
Spinco supernatant	2360	100	0.0424	2.45	85.5
1st $AmSO_4$ 35 - 50%	723	77.3	0.114	6.60	66.2
1st DEAE eluate	110	61.0	0.670	38.8	52.2
2nd $AmSO_4$ fractionation	59.2	58.7	0.990	57.3	50.2
Sephadex G200 eluate	13.5	39.2	2.90	168	33.5
2nd DEAE eluate	3.60	33.6	9.30	538	28.7

*Calculated from total activity in the crude extract.

Table 4 summarizes the results of a typical enzyme purification by the above procedure. A 538-fold purification of the enzyme was achieved with an overall yield of 29%.

2. Enzyme Purity

The enzyme prepared by the standard method displayed several bands in polyacrylamide gel electrophoresis indicating an incomplete purification. Attempts were made to purify the enzyme further using affinity gel chromatography. L-Glutamine and α-ketoglutarate were used to prepare two types of affinity gels ("Methods"). The protein was eluted by several methods including linear gradients of NaCl, KCl and NH_4Cl and also 10 mM NADPH solution. The best result was obtained when α-ketoglutarate-hexahydrocarbon-agarose gel was used and the protein was eluted with a linear gradient of NaCl (0 to 0.3 M prepared in the buffer) (Fig. 29). The enzyme purified by this method (fractions 15 to 22 in Fig. 29) displayed one major band and a minor band in the polyacrylamide gel electrophoresis (Fig. 30). Unfortunately, the enzyme activity decreased considerably during this step and it was not included in the standard purification procedure.

Figure 29. Affinity gel chromatography of glutamate synthase. A sample containing 3.6 mg protein in 60 ml was subjected to chromatography under the conditions as described in text. Glutamate synthase was assayed as described in "Methods" using 20 µl of each fraction.

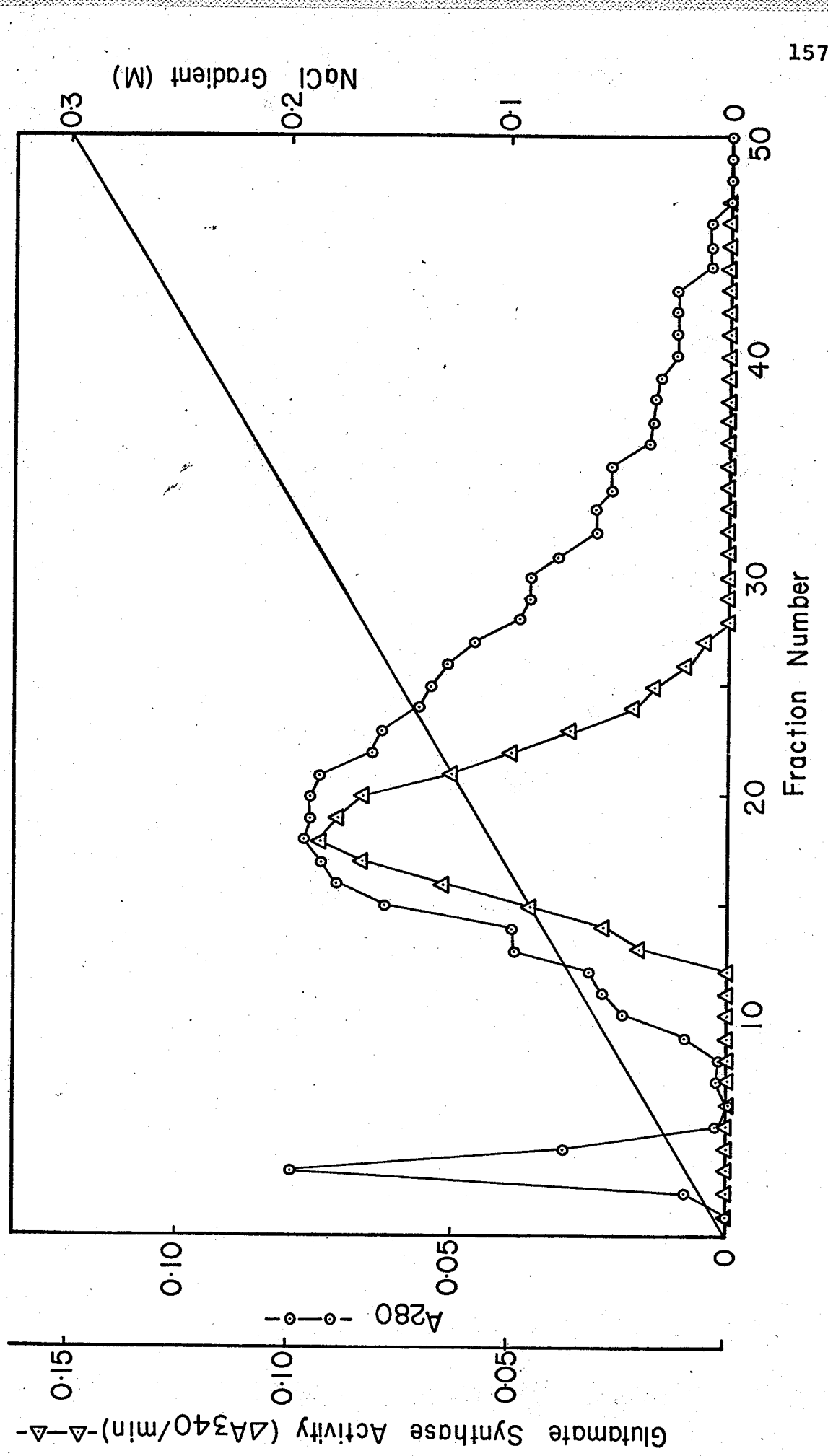
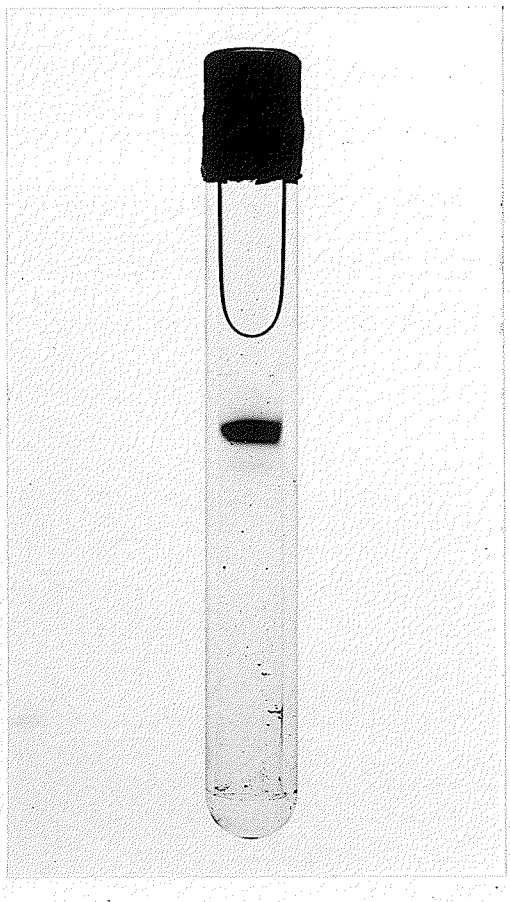

Glutamate Synthase Activity (ΔA340/min)-Δ—Δ-
A280 -o—o-
NaCl Gradient (M)
Fraction Number
0·15
0·10
0·05
0·10
0·05
0
0·3
0·2
0·1
0
10
20
30
40
50

Figure 30. Electrophoresis in polyacrylamide gel of glutamate synthase purified by affinity gel chromatography. Electrophoresis was performed as described in "Methods". Approximately 60 μg of protein was applied to the gel. The direction of migration was from top to bottom.



The yellow colored component in the enzyme preparation moved as a single band through the gel and after staining and destaining it was revealed that the position of the yellow band corresponded to that of the major protein band in Fig. 30. The yellow portion of the polyacrylamide gel was cut into small pieces and the protein was eluted with the buffer. This yellow protein preparation was assayed for glutamate synthase and was also subjected to sodium dodecyl sulfate (SDS) gel electrophoresis to determine the subunit molecular weight. It was found that the activity of glutamate synthase was also associated with this yellow protein fraction. Since glutamate synthases from E. coli (Miller and Stadtman, 1972) and K. aerogenes (Trotta et al., 1974) were identified as flavoprotein containing both FMN and FAD, it is likely that the yellow color of T. thioparus glutamate synthase is also derived from the enzyme-bound flavin.

3. Determination of Molecular Weight of Glutamate Synthase and its Subunit

Molecular weight determination

The molecular weight of glutamate synthase was estimated by a gel filtration method (Andrews, 1964) using a Sephadex G-200 column (2.5 x 92 cm). The following proteins were used as standard : milk xanthine oxidase (molecular weight 290,000; Ve/Vo = 1.45), bovine liver

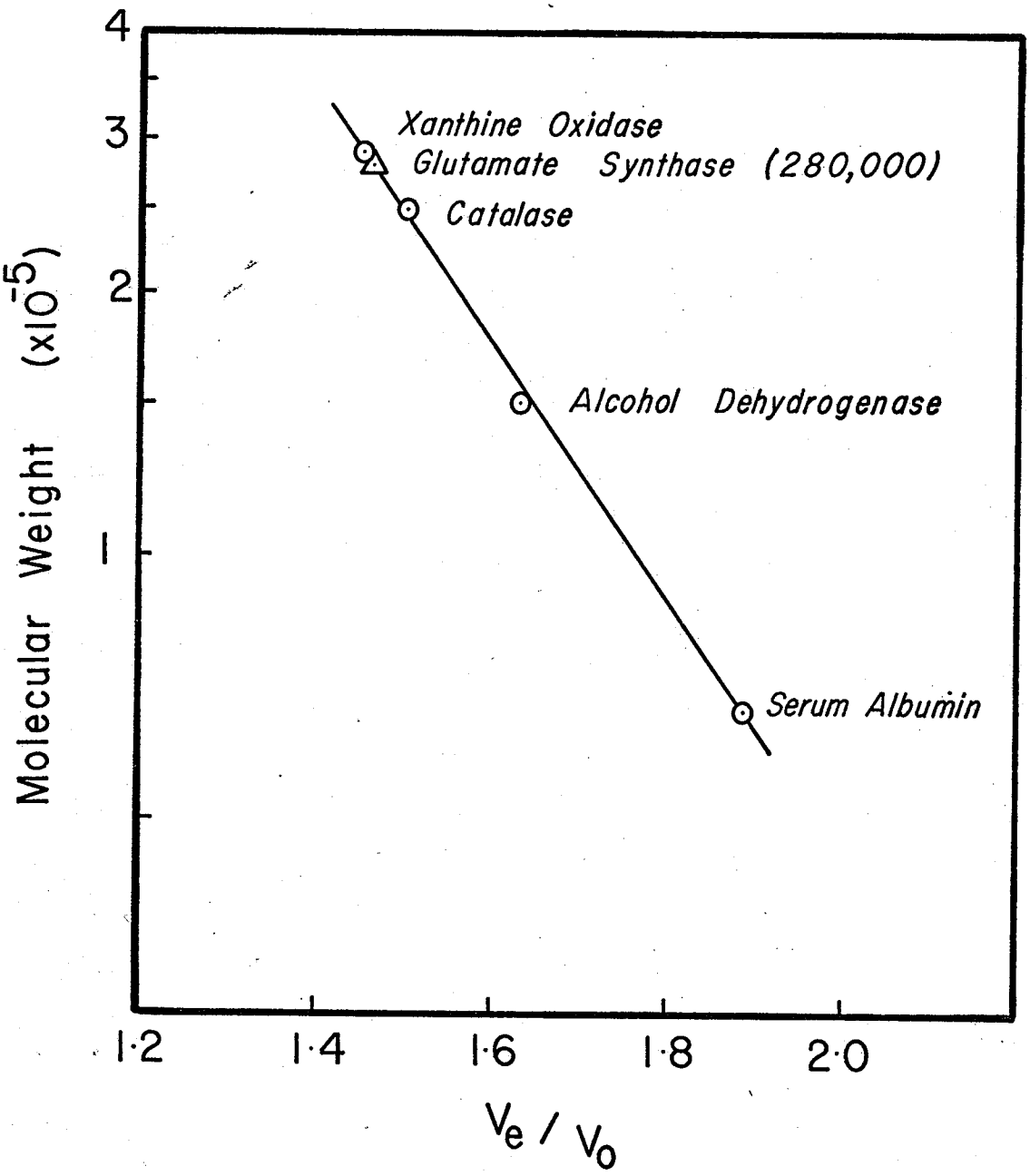
catalase (250,000 ; 1.50), yeast alcohol dehydrogenase (151,000 ; 1.63), bovine serum albumin (68,000 ; 1.89). The results of the determination are shown in Fig. 31 and the molecular weight of glutamate synthase was estimated to be 280,000 g/mole.

Glutamate synthase from T. thioparus is larger than the K. aerogenes enzyme (molecular weight 227,000 ; Trotta et al., 1974), but is significantly smaller than the purified enzyme from E. coli (800,000 ; Miller and Stadtman, 1972). However, a possibility that the purified glutamate synthase from E. coli might be composed of four catalytically active subunits was suggested by the fact that enzyme activity in a cruder preparation sedimented much more slowly in sucrose density gradient than the purified enzyme (Miller and Stadtman, 1972).

Subunit composition

In order to determine the molecular weight of subunit, SDS gel electrophoresis was performed as described by Weber and Osborn (1969). The sample protein was boiled for 5 min with 1% SDS and 1% 2-mercaptoethanol. As marker proteins, phosphorylase a (94,000), serum albumin (68,000), alcohol dehydrogenase (37,000) and hemoglobin (15,500) were used. Glutamate synthase, eluted from the

Figure 31. Molecular weight determination of glutamate synthase by gel filtration on a Sephadex G-200 column. Details were described in "Methods".


Molecular Weight (x10^-5)
4
3
2
1
Xanthine Oxidase
Glutamate Synthase (280,000)
Catalase
Alcohol Dehydrogenase
Serum Albumin
1·2
1·4
1·6
1·8
2·0
Ve / Vo

polyacrylamide gels (see "2. Enzyme purity"), displayed one major and one minor bands after staining as shown in Fig. 32. The approximate molecular weight of the faster moving minor band was 72,000 as judged by its mobility relative to the standard protein markers (Fig. 33). The molecular weight of the major (slower moving, mobility 0.033) band could not be calculated from the standards used because of its high molecular weight. Since glutamate synthases from two other sources, E. coli (Miller and Stadtman, 1972) and K. aerogenes (Trotta et al., 1974) had two dissimilar subunits, it was assumed that the slower moving major band represented the heavy subunit of the T. thioparus enzyme. The approximate molecular weight of the heavy subunit was calculated as 208,000 from the glutamate synthase molecular weight of 280,000 obtained by the gel filtration and the light subunit molecular weight of 72,000, assuming the presence of one of each subunit in the enzyme molecule. The subunits of the T. thioparus enzyme were larger than those of the E. coli enzyme, 53,000 and 135,000 (Miller and Stadtman, 1972) or the K. aerogenes enzyme, 51,500 and 175,000 (Trotta et al., 1974).

4. Enzyme Stability

The stability of glutamate synthase in the absence and in the presence of substrates, products and other com-

Figure 32. Electrophoresis of glutamate synthase in 0.1% SDS-polyacrylamide gel. The direction of migration was from top to bottom. Details were described in text. The molecular weight of the faster-moving species in the gel was 72,000 g/mole (Fig. 33). The molecular weight of slower-moving species could not be determined (text).

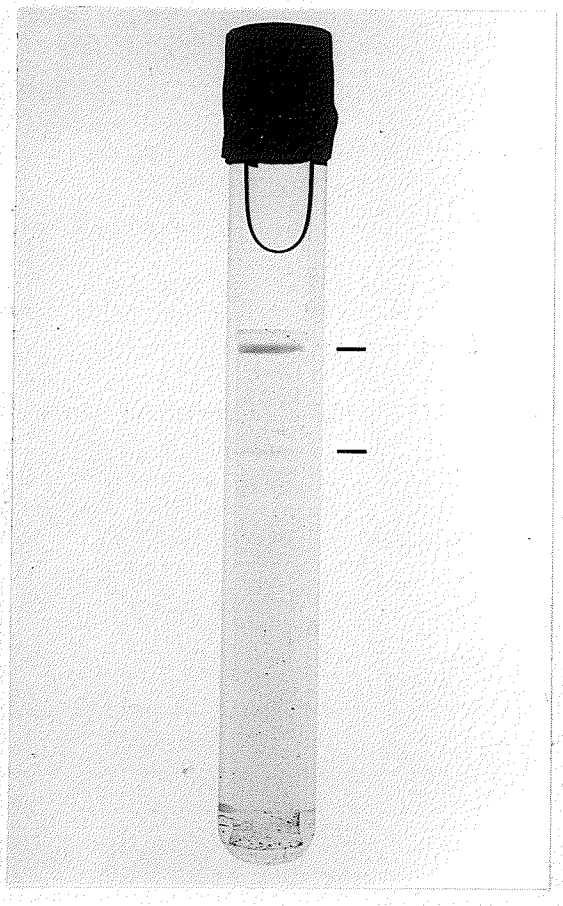
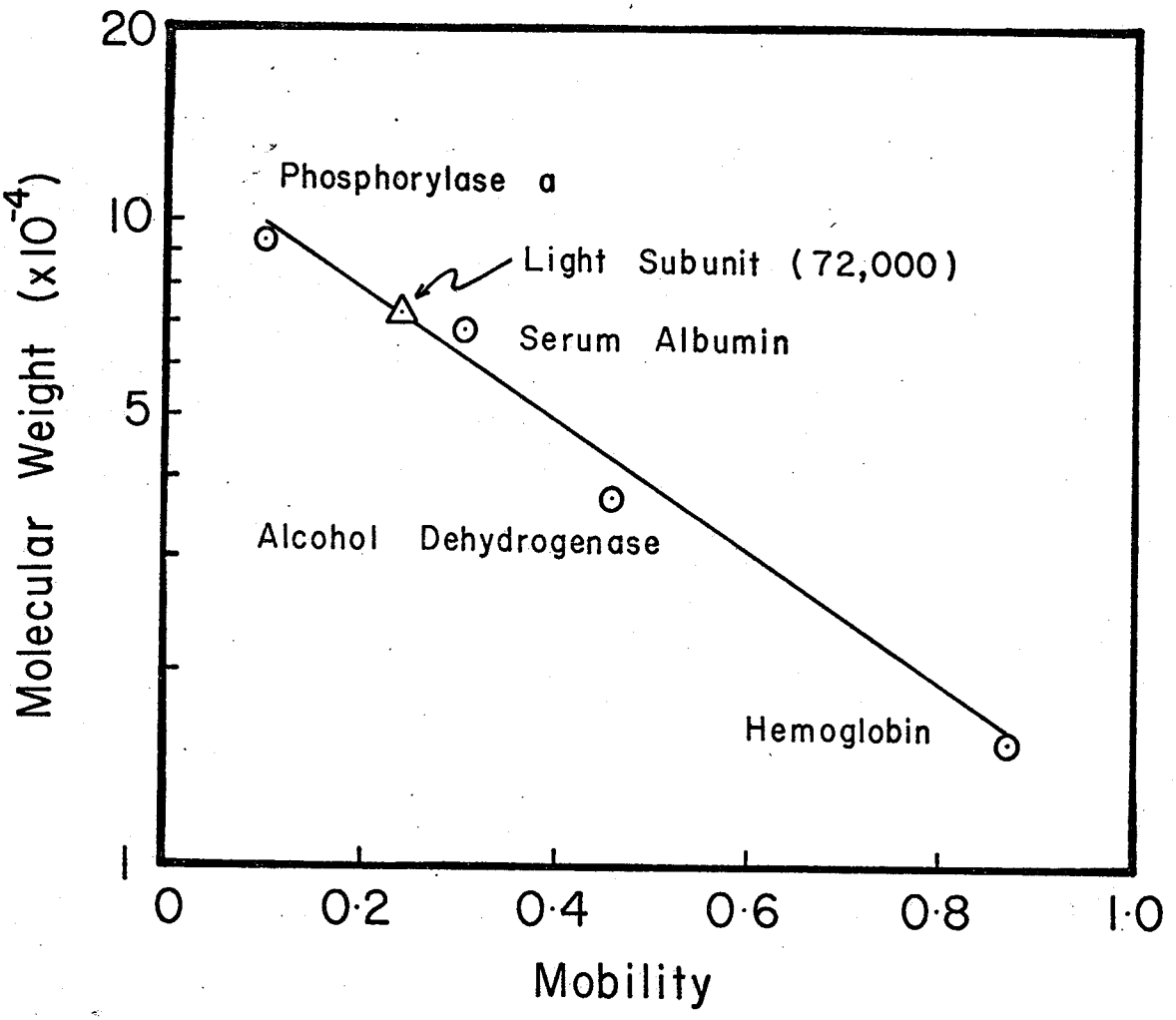


Figure 33. Semilog plot of the monomer molecular weights against mobility in SDS gel electrophoresis. Details were described in "Methods".


20
10
5
1
Molecular Weight (x10⁻⁴)
Phosphorylase a
Light Subunit (72,000)
Serum Albumin
Alcohol Dehydrogenase
Hemoglobin
0
0·2
0·4
0·6
0·8
1·0
Mobility

pounds was studied before the purification procedures were established. The enzyme obtained from the first ammonium sulfate fractionation ("Purification") prepared without EDTA and dithiothreitol was used. The data in Table 5 show that the unprotected enzyme was unstable and the addition of EDTA, 2-mercaptoethanol and dithiothreitol resulted in enhancement of the enzyme activity and its protection during storage at $4^{o}C$. NADPH or α-KG afforded a relatively slight degree of protection and L-glutamine, L-glutamate or $NADP^{+}$ did not protect the enzyme at all from inactivation. The enzyme was therefore purified in the presence of 5 mM EDTA and 1 mM dithiothreitol ("Purification").

Storage of purified glutamate synthase at $4^{o}C$ in the presence of 5 mM EDTA, 1 mM dithiothreitol and 50 mM potassium phosphate (pH 7.5) resulted in about a 40% loss of activity in 10 days. Dialysis against the freshly prepared buffer partially restored the lost activity and occasional addition of dithiothreitol during storage protected the enzyme to some extend from inactivation. Freezing and storing the purified enzyme at $-20^{o}C$ resulted in a total loss of activity when examined after 10 days. Boiling the enzyme solution for 30 sec destroyed the enzyme activity completely.

Table 5. Stability of glutamate synthase

Additions	Relative Activity (%)*					
	1 hr	3 hrs	6 hrs	12 hrs	24 hrs	48 hrs
None**	90	57	42	30	19	9
NADPH (2 mM)	90	68	56	48	49	36
α-KG (2 mM)	95	68	55	43	30	14
L-Glutamine (2 mM)	85	61	38	22	15	6
L-Glutamate (2 mM)	72	38	27	19	13	8
$NADP^+$ (2 mM)	65	47	34	27	25	14
EDTA (5 mM)	114	105	95	96	94	91
2-Mercapto-ethanol (5 mM)	129	133	119	115	120	90
Dithiothreitol (5 mM)	143	147	135	125	124	115
Dithiothreitol (2 mM)	128	145	123	127	124	109
Dithiothreitol (1 mM)	133	132	129	128	128	120

*The enzyme activity at zero time before additions was taken as 100% and the relative activity was determined after incubation at 4°C for various lengths of time. The protein concentration was adjusted at 6.2 mg per ml, and 25 µl of preliminary incubation mixture were used for each assay.

**The enzyme was incubated in 0.1 M potassium phosphate buffer (pH 7.5) without additions.

5. Substrate Requirement

The substrate requirements for glutamate synthase of T. thioparus are shown in Table 6. It was noted that the enzyme had a strict requirement for NADPH as pyridine nucleotide coenzyme and α-ketoglutarate as amino acceptor. A slow oxidation of NADPH observed with NH_4Cl as amino donor instead of glutamine could be explained either by the presence of a small amount of contaminating NADP-dependent glutamate dehydrogenase or by the lack of complete specificity for glutamine by glutamate synthase. A similar degree of the ammonia activity (about 6% of the glutamine activity with 100 mM NH_4Cl) was also found in the apparently pure enzyme from K. aerogenes (Trotta et al., 1974). The NADP-dependent glutamate dehydrogenase of T. thioparus precipitated mainly between 50% and 60% saturation in $(NH_4)_2SO_4$ ("Results of GDH") while glutamate synthase precipitated between 35% and 50% saturation. Since the specific activity of the glutamate dehydrogenase was only 3-4% of that of glutamate synthase in the spinco supernatant to begin with (Table 6, "Results of Glutamate synthase") and the ammonium sulfate fractionation was performed twice to purify glutamate synthase, the latter explanation was considered more likely for this ammonia activity.

Table 6. Substrate requirements of glutamate synthase

Assay conditions*	Specific activity (units/mg protein)
Complete	2.7
- NADPH, + NADH (75 μM)	0
- Glutamine	0
+ NH_4Cl (5 mM)	0.05
+ NH_4Cl (100 mM)	0.16
+ Asparagine (5 mM)	0
- α-Ketoglutarate	0
+ Pyruvate (5 mM)	0
+ Oxalacetate (5 mM)	0
- Glutamine, - α-Ketoglutarate	0
+ Asparagine (5 mM), + Oxalacetate (5 mM)	0

*Standard assay conditions (75 μM NADPH, 5 mM glutamine, 0.5 mM α-ketoglutarate) were used with 0.45 μg of purified enzyme per assay.

6. Effect of pH

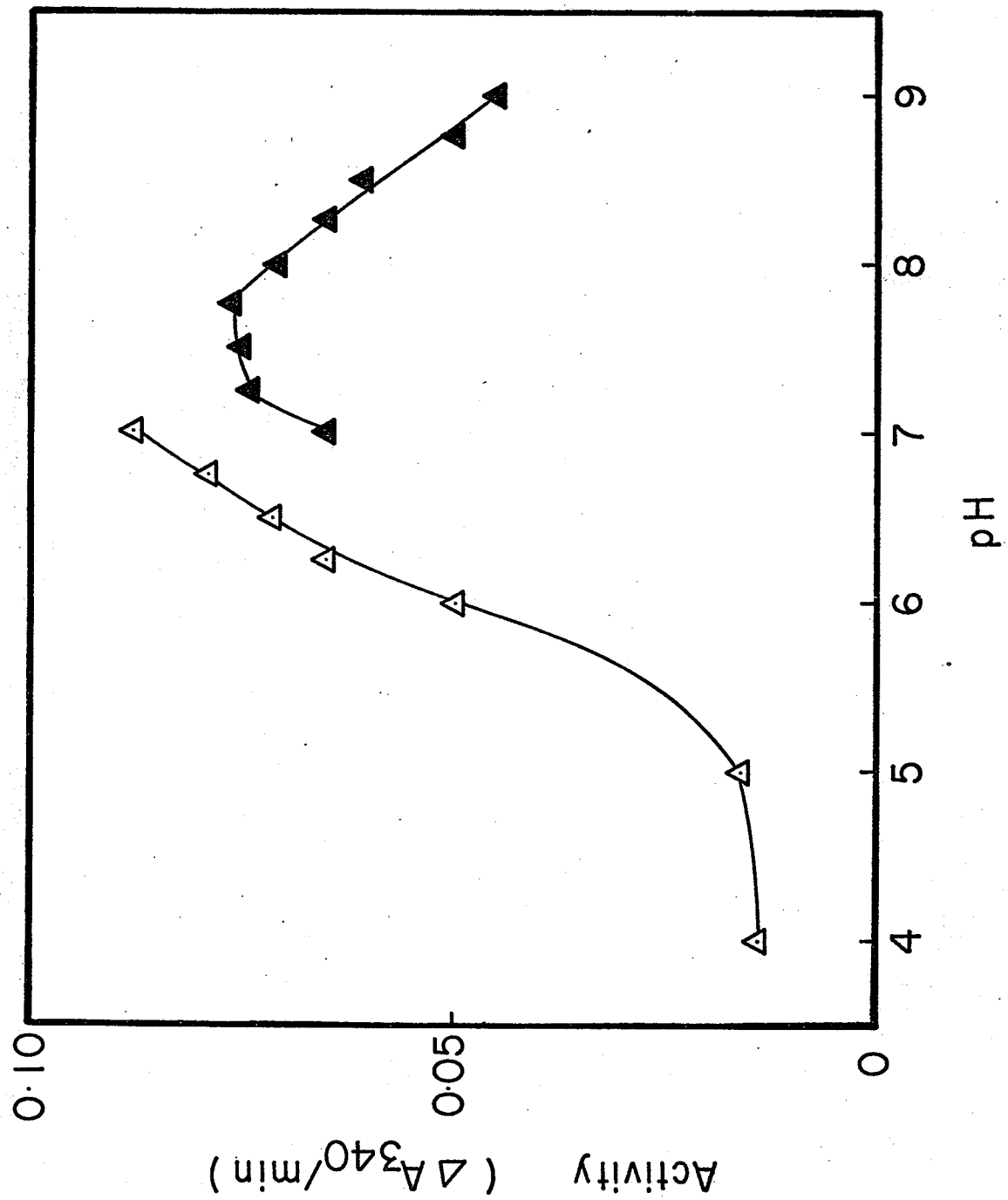
The effect of pH on the activity of glutamate synthase was determined and is shown in Fig. 34. The pH activity profile of the enzyme was relatively broad with the optimum around 7.5. This figure is similar to those obtained with the enzymes from K. aerogenes (7.6; Meers et al., 1970), E. coli (7.6; Miller and Stadtman, 1972) and S. cerevisiae (7.1-7.7; Roon et al., 1974). Since the maximum activity in the crude extract was obtained at pH 7.8 (not shown), this pH was used for the assay throughout this course of investigation.

It was noted that the enzyme had a higher activity in Na_2HPO_4 - Citric acid buffer than in Tris-Cl at pH 7.0. It was also found that the enzyme was 10% less active in HEPES buffer (Good et al., 1966) than Tris-Cl or potassium phosphate at pH 7.8. Tris-Cl buffer (pH 7.8) was used for the standard assay of glutamate synthase.

7. Effect of Substrate Concentration

Studies on the effect of varying concentration of substrate on velocity were performed and presented as standard velocity (v) versus substrate concentration (s)

Figure 34. Effect of varying pH on glutamate synthase activity. Buffers used were Na_2HPO_4-citric acid (—△—△—) between pH 4 and pH 7, and Tris-Cl (—▲—▲—) between pH 7 and pH 9. The assay conditions were as described in "Methods" with the exception of buffers.


Activity (ΔA_{340}/min)
0·10
0·05
0
4
5
6
7
8
9
pH

plots. The apparent Michaelis constant (K_m) for the varying substrate concentration was calculated from double reciprocal plots.

Effect of glutamine concentration

A plot of velocity versus L-glutamine concentration resulted in the normal rectangular hyperbola (Fig. 35 a) with the double reciprocal plot yielding a straight line (Fig. 35 b). The apparent K_m for L-glutamine was calculated to be 1.1 mM. This figure is somewhat higher than the value of 0.25 mM for the E. coli enzyme (Miller and Stadtman, 1972) but is very close to 1.0 mM, the value obtained for the S. cerevisiae (Roon et al., 1974).

Effect of α-ketoglutarate concentration

The effect of varying α-ketoglutarate concentration on the activity of glutamate synthase is presented in Fig. 36. The apparent K_m was calculated as 50 μM. This value is again higher than that for the E. coli enzyme (7.3 μM), but is lower than the value obtained for the S. cerevisiae enzyme (140 μM).

Effect of NADPH concentration

The effect of varying NADPH concentration on activity is shown in Fig. 37. The K_m value for NADPH was determined to be 3.0 μM. It was difficult to obtain accurate velocity data at low concentrations of NADPH

Figure 35. Effect of varying L-glutamine concentration on glutamate synthase activity.

(a) Rate-concentration plots

(b) Double reciprocal plots

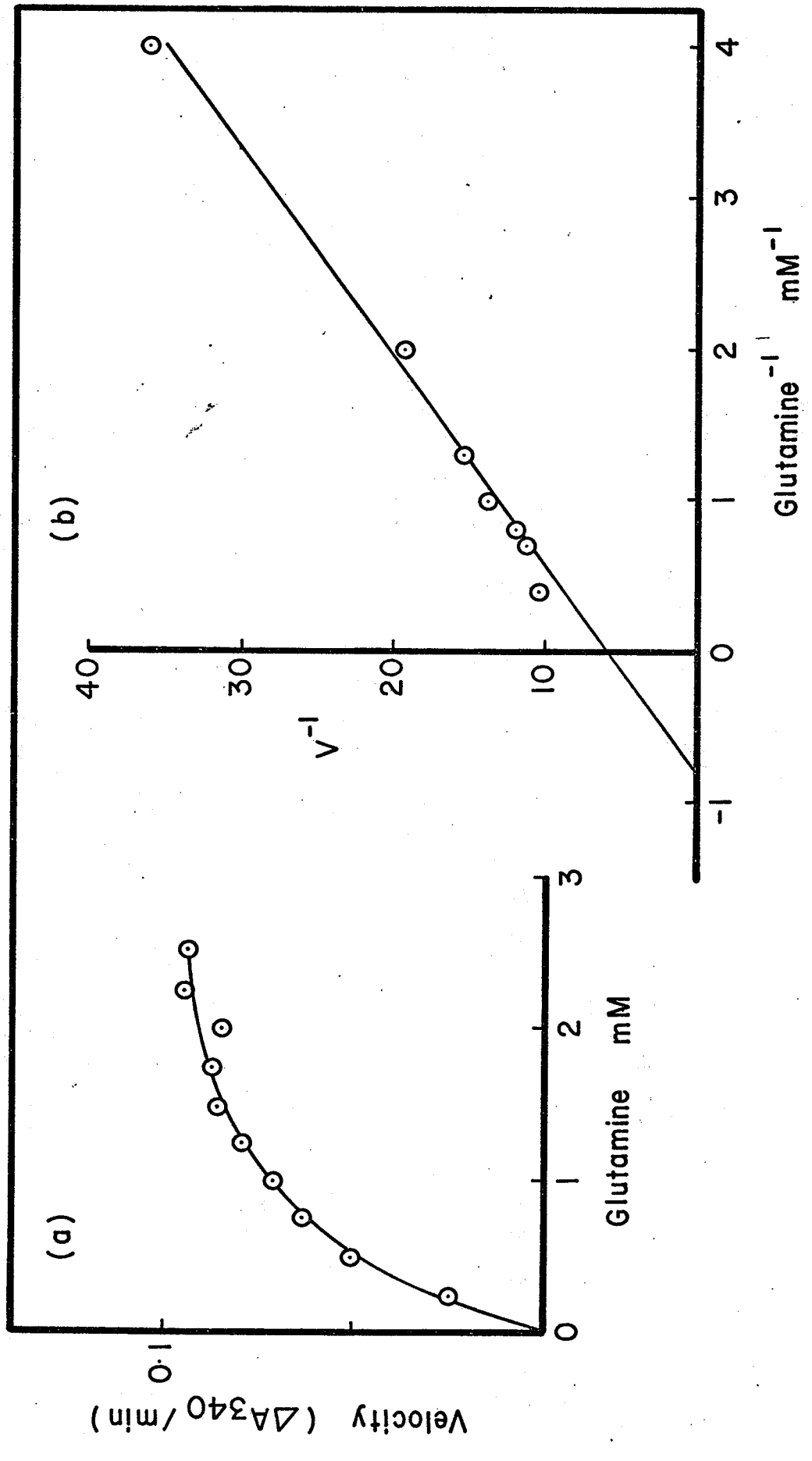

(a)
Velocity (ΔA340/min)
1.0
0
1
2
3
Glutamine mM
(b)
40
30
20
10
V-1
0
1
2
3
4
Glutamine-1 mM-1

Figure 36. Effect of varying α-ketoglutarate concentration on glutamate synthase activity.

(a) Rate-concentration plots

(b) Double reciprocal plots

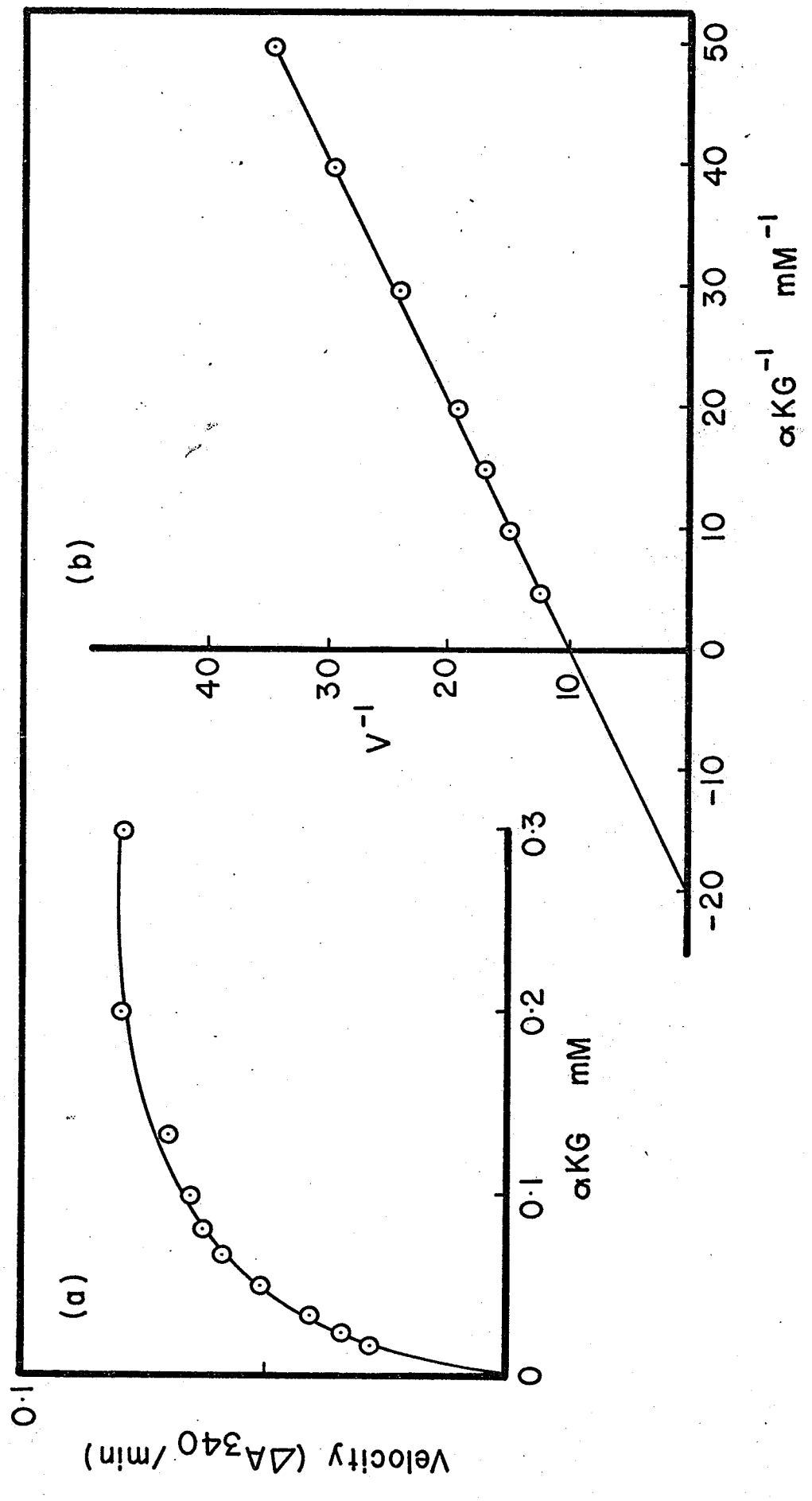
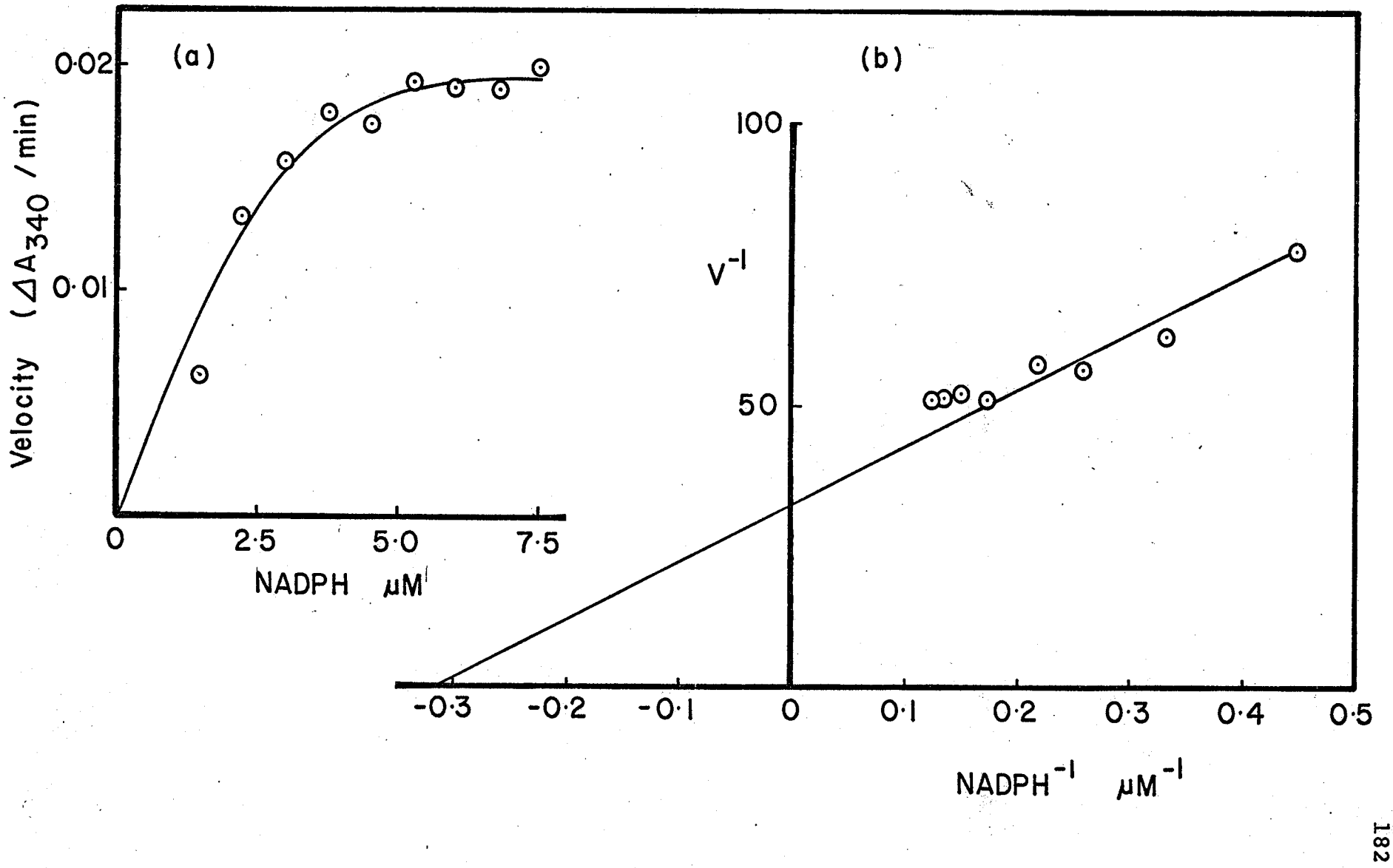

(a)
Velocity (ΔA_{340}/min)
0·1
0
0·1
0·2
0·3
αKG mM
(b)
V^{-1}
40
30
20
10
0
-10
-20
10
20
30
40
50
αKG^{-1} mM^{-1}

Figure 37. Effect of varying NADPH concentration on glutamate synthase activity.

(a) Rate-concentration plots

(b) Double reciprocal plots


(a)
0·02
0·01
Velocity (ΔA340 / min)
0
2·5
5·0
7·5
NADPH μM
(b)
100
V-1
50
-0·3
-0·2
-0·1
0
0·1
0·2
0·3
0·4
0·5
NADPH-1 μM-1

due to the optical limitations inherent in the assay system. For this reason it should be expected that the data obtained in this experiment bear a certain degree of error. This figure, however, is similar to the values of 7.7 μM and 2.6 μM for the enzymes from E. coli and S. cerevisiae, respectively.

8. Effect of Metabolites

The effect of various amino acids on the activity of glutamate synthase is presented in Table 7.

Glutamate synthase, the key enzyme in amino acids synthesis, was expected to be subject to feedback inhibition by various amino acids. It was, however, found that only L-serine inhibited the enzyme activity more than 40% at 10 mM. Only four amino acids L-serine, L-methionine, L-histidine and L-glutamate, produced more than 50% inhibition at 50 mM.

According to Miller and Stadtman (1972), amino acids which produced more than 50% inhibition at 50 mM in the E. coli system included ten L-form amino acids, L-aspartate, L-methionine, glycine, L-cysteine, L-glutamate, L-serine, L-homoserine, L-asparagine, L-alanine and L-histidine. The first two amino acids inhibited the enzyme from E. coli more than 90% while only L-serine inhibited

Table 7. Effect of various amino acids on the glutamate synthase activity

Amino Acid	% Activity	
	10 mM	50 mM
L-Aspartate	89	62
L-Glutamate	81	44
L-Methionine	64	31
L-Serine	56	24
L-Alanine	79	54
Glycine	83	50
L-Histidine	93	43
L-Cysteine	86	63
L-Asparagine	95	72
DL-Homoserine	92	52
L-Tryptophan	91	81
L-Phenylalanine	95	80
L-Valine	92	96
L-Leucine	100	82
L-Isoleucine	97	93
L-Lysine	88	61
DL-Threonine	102	93
L-Proline	91	76
L-Arginine	93	67

*Assay mixtures (1 ml) contained 75 μM NADPH, 0.1 mM α-ketoglutarate, 2 mM L-glutamine, 50 mM Tris (pH 7.8), the indicated compound, and 0.45 μg of purified enzyme. The activity obtained without addition was taken as 100%.

more than 70% in the T. thioparus system. These results indicate that the E. coli enzyme is more susceptible to feedback inhibition than the T. thioparus enzyme.

The effect of other metabolites on glutamate synthase activity was studied and is presented in Table 8.

Of the tricarboxylic acid cycle intermediates tested only oxalacetate produced more than 50% inhibition at 20 mM. Pyruvate did not produce significant inhibition at the same concentration, but isocitrate inhibited the enzyme by 32%. Among adenine nucleotides tested the inhibition by ATP (33% at 20 mM) was the highest. $NADP^+$ at 1 mM produced 59% inhibition while NAD^+ had no effect. The inhibition pattern of these metabolites was similar to that of the E. coli enzyme (Miller and Stadtman, 1972), although a higher concentration of the metabolites was used in the E. coli system.

Table 8. Effect of various metabolites on the glutamate synthase activity

	* % Activity	
	5 mM	20 mM
Cyclic AMP	102	-
AMP	102	99
ADP	105	85
ATP	92	67
PEP	93	92
Pyruvate	99	88
Citrate	94	89
cis-Aconitate	95	77
DL-Isocitrate	91	68
Succinate	93	77
Fumarate	88	80
Malate	87	81
Oxalacetate	82	41
$NADP^+$ (1 mM)	41**	-
NAD^+ (1 mM)	103**	-

*Assay conditions are identical with those given in the legend to Table 7.
**At the concentration of 1 mM.

C. LEVELS OF GLUTAMATE DEHYDROGENASE, GLUTAMATE SYNTHASE AND GLUTAMINE SYNTHETASE IN T. THIOPARUS

Recently, Tempest and co-workers (1970) identified a previously unknown pathway, catalyzed by glutamine synthetase and glutamate synthase, for glutamate biosynthesis in K. aerogenes. Since then, existence of the glutamine pathway (NH_4^+ ⟶ glutamine ⟶ glutamate) for glutamate synthesis in a large number of microorganisms has been established (Meers et al., 1970; Nagatani, 1971; Miller and Stadtman, 1972; Brown et al., 1973; Roon et al., 1974). The significance of the glutamine pathway in ammonia assimilation was demonstrated by the changing levels of enzyme under different conditions. Under the conditions of nitrogen excess K. aerogenes contained almost no glutamate synthase activity but had a high glutamate dehydrogenase level. However, when growth was limited by the availability of ammonia the glutamate synthase activity was relatively high whereas glutamate dehydrogenase activity became low (Meers et al., 1970).

It has also been suggested on the basis of kinetic data that glutamate dehydrogenase would not operate during nitrogen-limited growth of either E. coli (Miller and Stadtman, 1972) or K. aerogenes (Tempest et al.,

1970) because of high K_m of glutamate dehydrogenase for ammonia. It was therefore proposed that the glutamine pathway catalyzed by glutamine synthetase, which characteristically has a low K_m for ammonia, and glutamate synthase would be the major route of nitrogen assimilation (Tempest et al., 1970; Miller and Stadtman, 1972).

In 1975, Senior studied with continuous cultures of K. aerogenes and E. coli and confirmed that this is indeed the case in K. aerogenes. However, the enzyme composition of E. coli during nitrogen limitation differed markedly from that of K. aerogenes. In E. coli, glutamate dehydrogenase was progressively induced during nitrogen limitation while glutamate synthase, which had almost identical specific activity at zero time, remained at the same level during the nitrogen starvation. It was therefore concluded by this author that E. coli, unlike K. aerogenes, uses glutamate dehydrogenase to incorporate ammonia during the nitrogen-limited growth.

In order to find out whether or not the glutamine pathway exists in T. thioparus, levels of glutamate dehydrogenases, glutamate synthase and glutamine synthetase were measured. The levels of these enzymes were also tested in T. novellus (autotrophically grown) and N. europaea for comparison. The results are shown in Table 9.

Table 9. Levels of glutamate dehydrogenase, glutamate synthase and glutamine synthetase in various sources.

Enzyme	Coenzyme	Specific Activity[1] (unit/mg)			
		T. thioparus[2]		*T. novellus*[3]	*N. europaea*[3]
		Standard conditions	High[4] ammonia		
Glutamate Dehydrogenase	NADPH	0.0018	0.0016	0.0083	0.613
	NADH	0.0009	0.0008	[5]	-
Glutamate Synthase	NADPH	0.0626	0.0593	0.0152	-
	NADH	-[6]	NT[7]	[5]	-
Glutamine Synthetase		0.115	NT	NT	+[8]

(1) Assay conditions were those described in "Methods".
(2) The spinco supernatant was prepared as described in "Purification of Glutamate Synthase".
(3) The crude extract was prepared as described in "Methods".
(4) Ten times higher $(NH_4)_2SO_4$ (7.6 mM) was added to the medium.
(5) Could not be detected because of the presence of strong NADH oxidase (see LéJohn amd McCrea, 1968).
(6) - = Not detected.
(7) NT= Not tested.
(8) Some activity was detected in the presence of 3 mM hydrazine sulfate (inhibitor of hydroxylamine-oxidizing system).

The activity of glutamine synthetase was determined only by the γ-glutamyl transfer assay ("Methods") because of its relative lack of interference by contaminating enzymes compared to other methods (Shapiro and Stadtman, 1970). The specific activity found in the spinco supernatant of T. thioparus (0.115 units per mg protein) was somewhat lower than those of Rhodospirillum rubrum (grown with 3.8 mM $(NH_4)_2HPO_4$) (0.29; Nagatani et al., 1971) and E. coli (grown with 20 mM NH_4Cl) (0.188; Miller and Stadtman, 1972). However, since glutamine synthetase activity is controlled by a complicated manner (Stadtman et al., 1972), the result should be considered tentative until the enzyme from T. thioparus is well studied.

In T. thioparus, it was also found that the sum of the levels of both glutamate dehydrogenases was only 4% of that of glutamate synthase. This value did not change when T. thioparus was grown in "high NH_4^+" medium (10 times higher than the standard medium (Table 9). This apparent lack of response to "high NH_4^+" could have been due to the presence of high enough NH_4^+ in the standard medium (0.1 g $(NH_4)_2SO_4$ per liter) for the growth. Since the content of glutamate dehydrogenases was very low under these conditions, it appears that glutamate is synthesized mainly by the catalytic action of glutamate synthase through the glutamine pathway in T. thioparus grown under the standard or "high NH_4^+" conditions. The effect of ammonia starva-

tion must be studied in a chemostat continuous culture before any firm conclusion is made on the control of the glutamine pathway by the availability of ammonia in T. thioparus.

A preliminary experiment was performed to determine the effect of glutamate on the glutamine pathway of T. thioparus. The cells were grown in a medium which contained glutamate (2 mM, equivalent to 0.142 g $(NH_4)_2SO_4$ per liter as nitrogen source) as a sole nitrogen source. The growth rate was slow under these conditions and the cells were harvested after 10 days of incubation. The activities of glutamate synthase, glutamine synthetase and APS reductase were measured and compared to those in the normally grown cells. The level of APS reductase was 62% of that in the normal cells. The levels of the glutamine pathway enzymes were changed significantly. The glutamate synthase activity decreased to 23% of the normal level, while the glutamine synthetase activity increased to 286% of that in the normal cells. A similar case was reported for the culture of E. coli grown with glutamate as a sole source of nitrogen (Miller and Stadtman, 1972). A huge amount of glutamine synthetase was synthesized in the glutamate-nitrogen-limited culture of E. coli while almost no glutamate synthase was detected (Senior, 1975).

Senior (1975) proposed a two-step pathway of nitrogen assimilation in the glutamate-nitrogen-limited culture of E. coli. The first step is the decomposition of glutamate catalyzed by glutamate dehydrogenase to ammonia and α-ketoglutarate followed by the formation of glutamine catalyzed by glutamine synthetase (glutamate ⟶ NH_4^+ ⟶ glutamine).

More sophisticated experiments are required to determine the control mechanism of nitrogen assimilation by various factors in an obligate autotroph, T. thioparus.

The enzyme composition in T. novellus differed from that of T. thioparus. There were a lower level of glutamate synthase and a higher level of glutamate dehydrogenases. Although, in the T. novellus crude extract, NAD-dependent glutamate dehydrogenase could not be detected because of the presence of strong NADH oxidase, two types of glutamate dehydrogenase were purified (LéJohn and McCrea, 1968) and characterized (LéJohn, 1967; LéJohn et al, 1968). The level of NAD-dependent glutamate dehydrogenase was approximately a half of that of the NADP-dependent enzyme (LéJohn and McCrea, 1968). Therefore the sum of the levels of two glutamate dehydrogenases, assuming a specific activity of 0.0042 for the NAD-dependent enzyme, was calculated as 80% of the glutamate synthase activity. This figure is much higher than the value, 4%, for

the T. thioparus system. It seems that T. novellus is more dependent on the glutamate dehydrogenase system for ammonia assimilation than T. thioparus under these conditions.

In N. europaea, no activity was found when the crude extract was assayed for glutamate synthase using either NADPH or NADH as coenzyme. However, a strong activity of NADP-dependent glutamate dehydrogenase was detected (220 times as strong as the combined activities of both glutamate dehydrogenases in T. thioparus). This enzyme was first studied and reported by Hooper and co-workers (1967) in N. europaea. NAD-dependent glutamate dehydrogenase was not detected in this organism. Since N. europaea inhabits an ammonia-rich environment, using ammonia as the sole source of both energy and nitrogen, the absence of glutamate synthase and the existence of a high level of glutamate dehydrogenase seem to be reasonable. It is possible, however, that the synthesis of glutamate synthase is only repressed by the high level of ammonia in the medium (20 mM $(NH_4)_2SO_4$) and it could be induced together with glutamine synthetase during ammonia starvation as is the case in K. aerogenes. It was impossible to detect glutamine synthetase activity by the standard γ-glutamyl transfer assay system because of the

existence of hydroxylamine-oxidizing activity in the N. europaea extract. However, some activity was detected when hydrazine sulfate, an inhibitor of the hydroxylamine-oxidizing system, was added to the assay mixture (3 mM). Since the effect of this compound on glutamine synthetase is not known, the result should be regarded as tentative.

DISCUSSION

DISCUSSION

An obligate autotroph, T. thioparus, has been shown by this work to have two distinct glutamate dehydrogenases. A similar case has been reported in facultative autotrophs, T. novellus (LéJohn and McCrea, 1968) and Hydrogenomonas H 16 (Krämer, 1970) and in a number of fungi (Holzer and Schneider, 1957; Sanwal and Lata, 1961; Dennen and Neiderpruem, 1967; Casselton, 1969; LéJohn and Stevenson, 1970; LéJohn, 1971). It has been suggested that NADP-dependent glutamate dehydrogenase operates biosynthetically, while the function of NAD-dependent glutamate dehydrogenase is the catabolism of glutamate (Sanwal and Lata, 1962; Hollenberg et al., 1970; also see "Historical").

Partially purified T. thioparus NADP-dependent glutamate dehydrogenase showed normal and linear kinetics. Under the experimental conditions employed at pH 8.0, the maximum rate of glutamate formation per mole of the NADP-dependent enzyme was 8 times the corresponding rate of glutamate oxidation suggesting its possible biosynthetic function. However, the K_m of the enzyme for ammonia is very high (23 mM) and it is not expected to operate biosynthetically unless there is an ample supply of ammonia.

On the other hand, the K_m values of the enzyme for L-glutamate and $NADP^+$ (870 μM and 50 μM, respectively) are much lower and it could supply ammonia from glutamate, if needed, under the conditions of ammonia starvation or growing on glutamate as a sole nitrogen source. A preliminary experiment indicated that T. thioparus could grow on glutamate as nitrogen source instead of $(NH_4)_2SO_4$. Although the levels of glutamate dehydrogenases were not determined, a high level of glutamine synthetase and a low level of glutamate synthase were found in the crude extract of glutamate-grown T. thioparus. These preliminary results indicate that the pathway of glutamate incorporation could be the same as that proposed for glutamate-grown E. coli by Senior (1975) : glutamate $\longrightarrow$ NH_4^+ $\longrightarrow$ glutamine. This pathway of nitrogen assimilation was catalyzed by glutamate dehydrogenase and glutamine synthetase.

T. thioparus NAD-dependent glutamate dehydrogenase was assayed for both directions, but the activity of the enzyme could be detected only in the direction of glutamate formation. It was demonstrated that NAD-dependent glutamate dehydrogenase from T. novellus was an allosteric enzyme and specifically controlled by AMP (LéJohn et al., 1968). In the presence of AMP the affinity of the

T. novellus enzyme to substrates was modified so as to favour glutamate breakdown. Although AMP had no effect on the T. thioparus enzyme, it was speculated that there could be an unknown control mechanism on this activity.

The level of glutamate synthase detected in T. thioparus was approximately 25-fold higher than the sum of both activities of two glutamate dehydrogenases measured in the direction of glutamate synthesis. Since the activity of glutamine synthetase was also detected in the crude extract (results not shown) and the spinco supernatant of T. thioparus (Table 9, "Results"), it was concluded that T. thioparus possessed the glutamine pathway to synthesize glutamate. The activity of glutamate synthase was not detected in the cells of K. aerogenes grown under the ammonia excess conditions (Meers et al., 1970). However, T. thioparus grown in the high ammonia medium produced approximately the same levels of glutamate synthase and glutamate dehydrogenases as in the standard medium (Table 9, "Results"). It appears then that T. thioparus unlike K. aerogenes incorporates ammonia through the glutamine pathway under the ammonia excess conditions. The K_m of NADP-dependent glutamate dehydrogenase for ammonia was very high (23 mM) and it is not expected to operate under the conditions of ammonia starvation. Although the K_m of NAD-dependent glutamate dehydrogenase for ammonia was not determined, it is unlikely to be much lower than 23 mM

because its maximum velocity was obtained with 200 mM or higher NH_4Cl concentration. The available information seems to indicate that T. thioparus assimilates ammonia generally through the glutamine pathway catalyzed by glutamine synthetase and glutamate synthase. However, the effect of the ammonia-limited conditions on the enzyme composition should be studied in the chemostat continuous culture of T. thioparus before any conclusion is made. The E. coli levels of glutamate synthase and glutamate dehydrogenase were relatively unaffected by the concentration of ammonia in batch cultures (Miller and Stadtman, 1972). Nevertheless, it was demonstrated that the ammonia-limited chemostat culture of E. coli progressively produced increasing levels of glutamate dehydrogenase to incorporate ammonia (Senior, 1975).

In spite of the existence of two distinct glutamate dehydrogenases, the function of the T. thioparus glutamate dehydrogenase system is obscure. One possibility is the activity of transhydrogenase to produce NADPH at the expense of NADH. The low K_m's of the NADP-dependent enzyme for glutamate and $NADP^+$ and the NAD^+ activation of $NADP^+$ reduction seem to fit to this idea. However, the originally proposed function of pyridine nucleotide transhydrogenase was the oxidation of NADPH by NAD^+ to form

NADH (Kaplan et al., 1952) which, in turn, was oxidized in oxidative phosphorylation to generate ATP (Kaplan et al., 1956). It has been suggested that NADPH was used primarily as the reducing power in synthetic reactions (Kaplan, 1963). The possibility of $NADP^+$ activating NAD-dependent glutamate dehydrogenase in the direction of glutamate breakdown could not be excluded because the partially purified NADP-dependent glutamate dehydrogenase preparation used in this NAD^+ activation experiment was not completely free from the NAD-dependent enzyme. The latter possibility, the formation of NADH, should be tested by following the decrease in absorbance at 340 nm after the addition of a NADH specific dehydrogenase, e. g. alcohol dehydrogenase, and its substrate to the reaction mixture.

Levels of glutamate synthase and glutamate dehydrogenase were also measured in T. novellus and N. europaea (Table 9, "Results"). The specific activity of glutamate synthase in the crude extract of T. novellus was about ¼ of that in the T. thioparus spinco supernatant. Calculations indicate that the sum of the reductive amination activities of both glutamate dehydrogenases is as high as 80% of the glutamate synthase activity in T. novellus. This figure is much higher than 4%, the value obtained for the T. thioparus system. It was concluded that the synthesis of glutamate in T. thioparus was more depen-

dent on glutamate synthase than T. novellus.

A strong activity of NADP-dependent glutamate dehydrogenase was detected in the N. europaea crude extract, while no activity of glutamate synthase was found (Table 9, "Results"). According to Hooper et al. (1967), the maximum rate of reductive amination of α-ketoglutarate by Nitrosomonas glutamate dehydrogenase may be as much as 80-fold greater than the rate needed to account for all the organic nitrogen of the organism. Although N. europaea might totally lack glutamate synthase, the possibility that the enzyme was only repressed by the high concentration of ammonia in the medium (23 mM $(NH_4)_2SO_4$, 30 times higher than the standard medium for T. thioparus) could not be eliminated. However, it might be difficult to study with the ammonia-limited culture of N. europaea because it would create both nitrogen and energy-limited conditions to this organism.

Since the physiological significance of glutamate synthase is well documented, its properties are of particular interest. Table 10 shows an abbreviated comparison of the properties of the enzyme from T. thioparus and three other sources, E. coli (Miller and Stadtman, 1972), K. aerogenes (Meers et al., 1970; Trotta et al., 1974) and S. cerevisiae (Roon et al., 1974). The pH

Table 10. Comparison of the properties of glutamate synthase.

Source (Reference)	E. coli (1)	K. aerogenes (2) (3)	S. cerevisiae (4)	T. thioparus
Structure		(2)		
Mol. wt.	800,000	227,000	N	280,000
Heavy subunit	135,000	175,000	N	208,000*
Light subunit	53,000	51,500	N	72,000
Flavin/mole	7.8	1.7	N	N
Kinetic Parameter		(3)		
pH Optimum	7.6	7.6	7.1 - 7.7	7.2 - 7.8
K_m for NAD(P)H	7.7×10^{-6} M	N	2.6×10^{-6}	3.0×10^{-6}
α-Ketoglutarate	7.3×10^{-6}	2.0×10^{-3}	1.4×10^{-4}	5.0×10^{-5}
L-Glutamine	2.5×10^{-4}	1.8×10^{-3}	1.0×10^{-3}	1.1×10^{-3}
Coenzyme	NADPH	NADPH	NADH	NADPH
Specific activity	26.2	18.3 (2)	N	9.3

N = not known, * Tentative value, see "Results".
(1) Miller and Stadtman, 1972; (2) Trotta et al., 1974
(3) Meers et al., 1970; (4) Roon et al., 1974

optimum of T. thioparus glutamate synthase (a plateau from pH 7.2 to 7.8) was in the same range as that reported for the partially purified enzyme from S. cerevisiae (7.1 - 7.7; Roon et al., 1974). These values were also similar to the pH optimum of the E. coli enzyme (pH 7.6; Miller and Stadtman, 1972) which was identical with that for the enzyme in crude extract of K. aerogenes (Meers et al., 1970).

The K_m of T. thioparus glutamate synthase for NADPH was very low (3.0 μM) and was comparable to those reported for the enzyme from E. coli (7.6 μM; Miller and Stadtman, 1972) and S. cerevisiae (2.6 μM for NADH; Roon et al., 1974). The K_m's of the T. thioparus enzyme for α-ketoglutarate (50 μM) and L-glutamine (1.1 mM) were much higher than those for the E. coli enzyme (7.3 μM and 250 μM, respectively) (Miller and Stadtman, 1972), but were similar to those reported for the yeast enzyme (140 μM and 1.0 mM, respectively) (Roon et al., 1974). The K_m's of K. aerogenes glutamate synthase for α-keto-glutarate (2.0 mM) and L-glutamine (1.8 mM) (Meers et al., 1970) were the highest among the enzymes compared, while those of the E. coli enzyme were the lowest. Since K. aerogenes appears to use glutamate synthase with the high K_m's under the ammonia starvation conditions and E. coli seems to use glutamate dehydrogenase instead of glutamate synthase (Senior, 1975) which has the low K_m's,

the physiological significance of these K_m values are not clear.

T. thioparus glutamate synthase was purified more than 500-fold by the standard procedure (Table 4, "Results"). However, this enzyme preparation displayed one major and several minor bands in the polyacrylamide gel electrophoresis. Since no significant competing reaction was found in this preparation (Table 6, "Results"), it was used for the kinetic studies. However, attempts were made to purify the enzyme further by means of affinity chromatography. The enzyme eluted from an affinity gel column with the linear NaCl gradient displayed one major and one minor bands in the electrophoresis (Fig. 30, "Results"). It was observed that the yellow color component and the activity of glutamate synthase corresponded to the position of the major protein band. It has been demonstrated that the enzymes purified from E. coli (Miller and Stadtman, 1972) and K. aerogenes (Trotta et al., 1974) are flavoproteins containing both FMN and FAD. It seems likely that the yellow T. thioparus enzyme is also a flavoprotein. The amount of cells (about 30 g in wet weight) used for the routine purification could never supply a sufficient quantity of enzyme to study the nature of the yellow component spectrophotometrically. A larger scale of purification is required to confirm the existence of the

enzyme-bound flavin in T. thioparus glutamate synthase.

Sodium dodecyl sulfate (SPS) gel electrophoresis of E. coli glutamate synthase revealed the existence of two dissimilar subunits with molecular weights of 135,000 and 53,000 (Miller and Stadtman, 1972). It was reported that the K. aerogenes enzyme was also composed of non-identical subunits with molecular weights of 175,000 and 51,500 (Trotta et al., 1974). The yellow T. thioparus enzyme was eluted from the polyacrylamide gel after electrophoresis and was subjected to SDS gel electrophoresis to determine the size of subunit (Fig. 32, "Results"). This revealed the existence of two distinct subunits in T. thioparus glutamate synthase. The molecular weight of the light subunit was 72,000 (Fig. 33, "Results"). Although the molecular weight of the heavy subunit could not be determined directly because of its large molecular weight, it was calculated as 208,000 assuming that two dissimilar subunits occur in the native protein in equimolar amounts. No further attempt was made to determine the molecular weight of the heavy subunit of T. thioparus glutamate synthase by SDS gel electrophoresis. The use of 5% gel, instead of regular 10% gel, was recommended by Weber and Osborn (1969) with myosin (200,000), β-galactosidase (130,000) and paramyosin (100,000) as the standards for

the determination of molecular weight of large proteins.

It was found that T. thioparus glutamate synthase was highly specific for NADPH as coenzyme and α-keto-glutarate as amino acceptor. However, it was found that NH_4Cl could substitute for L-glutamine to a small degree. The ammonia activity (100 mM NH_4Cl) was about 6% of the glutamine activity (5 mM L-glutamine). This ratio of the activities is similar to that reported for the purified K. aerogenes enzyme which was apparently free from glutamate dehydrogenase (Trotta et al., 1974). The ammonia activity could be considered as a partial reaction of overall reaction catalyzed by glutamate synthase.

Among 19 amino acids tested none produced more than 50% inhibition at 10 mM. Only L-serine (44%) and L-methionine (36%) inhibited the enzyme more than 30% at this concentration (10 mM). Four amino acids, L-serine (76%), L-methionine (69%), L-histidine (57%), L-glutamate (56%), produced more than 50% inhibition at 50 mM (Table 7, "Results"). This relative lack of susceptibility of glutamate synthase to inhibition by amino acids, the presumed end products of the pathway, is consistent with its physiological significance. The glutamine pathway is the major assimilation route of ammonia which is the sole source of nitrogen in T. thioparus.

Since glutamate synthase is essential for the growth of T. thioparus, the organism possibly can not "afford" to inhibit this enzyme.

The effect of other metabolites on glutamate synthase was also tested (Table 8, "Results"). $NADP^+$ was the strongest inhibitor and produced 59% inhibition at 1 mM, while NAD^+ had no effect. Inhibition of T. thioparus glutamate synthase by oxalacetate (59%) and ATP (33%) at 20 mM was noted, while pyruvate and AMP did not produce significant inhibition.

However, there is a possibility that the inhibition of glutamate synthase is cumulative as described by Woolfolk and Stadtman (1967) for glutamine synthetase. That is, each inhibitor is able to cause only partial inhibition, but collectively they can cause almost complete inhibition of the enzyme. This possibility has not been tested in the T. thioparus system and a further investigation is required to clarify the control mechanism of glutamate synthase.

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