A kinetic study of hydroxylamine oxidoreductase from *Nitrosomonas europaea* using hydroxylamine, hydrazine and horse heart cytochrome *c* as substrates

by

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A KINETIC STUDY OF HYDROXYLAMINE OXIDOREDUCTASE FROM *NITROSOMONAS EUROPAEA* USING HYDROXYLAMINE, HYDRAZINE AND HORSE HEART CYTOCHROME *C* AS SUBSTRATES

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ABSTRACT

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Initial velocity kinetic studies of hydroxylamine oxidoreductase of Nitrosomonas europaea using hydroxylamine or hydrazine and horse heart cytochrome c as substrates were conducted. Oxidation of hydroxylamine seemed to have a changing stoichiometry as the hydroxylamine concentration changed. Hydrazine seemed to be oxidized with a constant four cytochrome c reduced: one hydrazine oxidized stoichiometry. The presence of two cytochrome c sites with different Km's and Vmax's was suggested by the initial velocity studies; a model for the mechanism was proposed. Some features of the model are: (a) two discrete sites of cytochrome c reduction per functional subunit of hydroxylamine oxidoreductase (b) a di-heme binding site for hydroxylamine or hydrazine (one of the hemes being P-460).

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DNase	- deoxyribonuclease I
EDTA	- ethylene diamine tetraacetic acid
tris	- tris(hydroxymethyl)aminomethane
BSA	- bovine serum albumin
DEAE	- diethylaminoethyl
HAO	- hydroxylamine oxidoreductase
HRPH	- high redox potential hemes (of HAO)
LRPH	- low redox potential hemes (of HAO)
ox. cyt.	- oxidized horse heart cytochrome c
red. cyt.	- reduced horse heart cytochrome c

INTRODUCTION

INTRODUCTION

Nitrosomonas europaea is a chemolithotroph, oxidizing ammonia:

$$NH_3 + 3/2\ O_2 \longrightarrow H^+ + NO_2^- + H_2O$$

Oxidation of ammonia proceeds by hydroxylation to hydroxylamine (Dua et al, 1979; Hollocher et al, 1981). The mechanism of hydroxylamine oxidation is not yet clear.

Whole cells are red due to large amounts of cytochromes; one of these, hydroxylamine oxidoreductase (HAO) consists of 2-3% of the total cell protein (Hooper et al, 1978). The enzyme probably consists of three 11,000 molecular weight mono-heme peptides and three 74,000 molecular weight peptides containing six c-hemes and one heme P-460 (Terry and Hooper, 1981).

HAO catalyzes the oxidation of hydroxylamine in vitro but the product of the reaction is not clear. Depending on conditions, N_2O, NO, NO_2^- and NO_3^- can be found (in vivo, NH_3 is oxidized essentially stoichiometrically to NO_2^-). Yamanaka (1980) demonstrated that hydroxylamine can be oxidized stoichiometrically to NO_2^- if an excess of electron acceptor is present; the reaction was optimal in phosphate buffer, pH 8.0. Nitrite was formed anaerobically, though transiently.

Initial velocity and inhibition studies were undertaken

in an attempt to clarify the behaviour of HAO.

HISTORICAL

HISTORICAL

Nitrification, the oxidation of ammonia to nitrate, is an integral part of the nitrogen cycle. Pasteur (1862) first suggested a biological involvement. Winogradsky (1891) established this by isolation of Nitrosomonas and Nitrobacter from soil using an inorganic medium. Nitrosomonas oxidizes ammonia to nitrite; Nitrobacter oxidizes nitrite to nitrate.

Nitrate is the preferred form of nitrogen assimilated by plants. Application of fertilizers is commonly as ammonia and compounds readily converted to ammonia. Conversion of ammonia to nitrate, though beneficial to the plant, leads to loss of nitrate by leaching and denitrification. Excess nitrate leached from agricultural soils can be a threat to water quality and even public health. Production of N_2O by denitrifiers is another source of a gas that is a potential threat to the ozone layer. (Schmidt, 1982)

Winogradsky described Nitrosomonas as a small, gram negative ovoid with a single polar flagellum. It derives all its energy from the oxidation of ammonia, and uses carbon dioxide as a carbon souce and is therefore a chemoautotroph. The organism has a generation time of around twelve hours and a growth yield of 0.1 g/L of medium. It grows in slightly alkaline medium (pH 7.8). Whole cells are red due to a high content of

cytochromes. Electron microscopy reveals extensive internal membranes, analogous to methanotrophs.

Biochemical studies on Nitrosomonas europaea have been directed at elucidating the mechanism of ammonia oxidation. The overall equation is:

$$NH_3 + 3/2O_2 \longrightarrow HNO_2 + H_2O$$

Work on N. europaea has been hampered by the difficulty of obtaining active, purified ammonia oxidizing system. Much of the work in the past has therefore been on whole cells, which is not altogether satisfactory, or on isolation and characterization of various probable components of the ammonia oxidizing system. In vitro behavior of some of the components is substantially different from in vivo behaviour. The successful preparation of active membrane fractions by Suzuki and Kwok (1981a) holds promise for the future.

Hofman and Lees (1953) demonstrated the accumulation of hydroxylamine when whole cells oxidized ammonia in the presence of hydrazine. Hydroxylamine was oxidized stoichiometrically to nitrite by washed cell suspensions (Lees, 1952). Oxidation of ammonia to hydroxylamine was inhibited by allylthiourea and thiourea, suggesting the involvement of copper (Hofman and Lees, 1953; Anderson, 1964).

Based on the free energy changes ($\Delta G'$) of:

$$NH_4^+ + 1/2O_2 \longrightarrow NH_2OH$$

$$\Delta G' = 3.7 \text{ kcal/mole}$$

$$NH_2OH + O_2 \longrightarrow NO_2^- + H^+ + H_2O$$

$$\Delta G' = -69.1 \text{ kcal/mole}$$

and on involvement of oxygen and (probably) copper, Lees (1954) suggested that oxidation of ammonia to hydroxylamine might be mediated by a type of oxidase. Since hyponitrite ($H_2N_2O_2$) was not metabolized to nitrite, he suggested that the intermediate of NH_2OH oxidation was HNO (nitroxyl).

Suzuki (1974) suggested the following scheme for ammonia oxidation:

$$NH_3 + O_2 + AH_2 \longrightarrow NH_2OH + A + H_2O$$

$$NH_2OH + 2 \text{ cyt. } \underline{c} \ (Fe^{3+}) \longrightarrow (HNO) + 2H^+ + 2 \text{ cyt. } \underline{c} \ (Fe^{2+})$$

$$HNO + A + H_2O \longrightarrow NO_2^- + AH_2 + H^+$$

$$1/2 \ O_2 + 2H^+ + 2 \text{ cyt. } \underline{c} \ (Fe^{2+}) \longrightarrow 2 \text{ cyt. } \underline{c} \ (Fe^{3+}) + H_2O$$

Oxidation of ammonia is obligatorily coupled to hydroxylamine oxidation. Prompted by the <u>in vitro</u> behavior of hydroxylamine oxidoreducatase (see below) other schemes for ammonia oxidation have been proposed, involving peroxonitrite (Suzuki <u>et al</u>, 1981c), nitrohydroxylamine (Aleem and Lees, 1964), and

dioxygenase type reactions (Hooper, 1978) (involving incorporation of both atoms of O_2 into nitrite). Current evidence suggests that the above scheme is essentially correct.

Hydroxylation of ammonia to hydroxylamine was conclusively demonstrated by Dua et al (1979) and Hollocher et al (1981) by analysis of the oxime formed from hydroxylamine using $^{15}NH_4Cl$ and $^{18}O_2$ or $H_2{}^{18}O$.

Methane, methanol, CO, ethylene, bromoethane and benzene, have been demonstrated to be substrates for ammonia monooxygenase (Drozd, 1980; Tsang and Suzuki, 1982; Hyman and Wood, 1983a and 1983b; Hyman and Wood, 1984; Jones and Morita, 1983). Tsang and Suzuki (1982) have demonstrated that the c-type cytochrome 554 is probably the electron donor for the monooxygenase (see below).

Ammonia oxidation is sensitive to near UV light (Schon and Engel, 1962) (eg. an absence of nitrification occurs in the photic zone of sea water). Hooper and Terry (1974) studied UV inactivation of cells; the hydroxylamine oxidizing apparatus was not drastically affected, and recovery required synthesis of fresh protein. Shears and Wood (1985) re-examined this effect; based on the difference spectrum of cells before and after irradiation they suggested that the monooxygenase

is a copper protein analogous to tyrosinase. Hyman and Wood (1985), using $^{14}C_2H_2$ as a suicide substrate for the monooxygenase, have demonstrated the labelling of a single membrane polypeptide of molecular weight 28,000.

Rees and Nason (1968) demonstrated the incorporation of a small amount of $^{18}O_2$ into nitrite by whole cells. Andersson et al (1982) reinvestigated this phenomenon during ammonia oxidation. Cells were found to catalyze the rapid exchange of both oxygen atoms of nitrite.

$$2H_2{}^{18}O + H^{15}N^{16}O_2 \longrightarrow H^{15}N^{18}O_2 + 2H_2{}^{16}O$$

Consequently, the use of a great excess of nitrite was necessitated in order to demonstrate the incorporation of 20% of one of the atoms of $^{18}O_2$ into nitrite.

The mechanism of hydroxylamine oxidation has not been conclusively proven. Cultures of N. europaea have been shown to produce N_2O (Ritchie and Nicholas, 1972). A kinetic analysis using ^{15}N (Poth and Focht 1985) ruled out the possibility that N_2O is a nitrification intermediate. It was concluded that N. europaea is a denitrifier, using nitrite (but not nitrate) as a terminal electron acceptor under conditions of oxygen stress.

Proton translocation studies of N_2H_4 and NH_2OH

oxidation by cells gave H^+/O ratios of 3.9 (using a suitable permeant anion and allowing for stoichiometric protons). When allowance was made for the oxygen atom incorporated by the monooxygenase, ammonia had an H^+/O ratio of 4.1 (Hollocher et al, 1982). At normal pH's of growth, it was concluded that the total proton motive force was composed of $\Delta\psi$ (Kumar and Nicholas, 1983).

Based on the rates of bromoethane oxidation in cells and the probable redox potential for NO, it was suggested that a maximum of three of the four electrons from NH_2OH oxidation are available for the monooxygenase (Hyman and Wood, 1983).

Studies on the oxidation of hydroxylamine to nitrite by more purified systems result in significant deviations from in vivo behaviour. The oxidation of hydroxylamine to nitrite by cell free extracts in the presence of electron acceptors and air was observed. Molecular oxygen was necessary for nitrite formation. Anaerobically, hydroxylamine disappeared and negligible amounts of nitrite were formed (Falcone et al, 1963; Anderson, 1964; Hooper and Nason, 1965; Aleem and Lees, 1963).

Nitric oxide and nitrous oxide were produced in amounts equivalent to hydroxylamine added. Hydroxylamine seemed to be first oxidized to HNO, then HNO was oxidized to NO_2^-, oxygen being required for the second process

(Anderson 1964; Hooper and Terry, 1979) (in vivo, NH_2OH is oxidized essentially stoichiometrically to nitrite).

Hydroxylamine oxidoreductase (HAO) catalyzes the oxidation of NH_2OH and N_2H_4 in the presence of electron acceptors (eg. phenazine methosulfate, eukaryotic cytochrome c, the native cytochrome c-554) (Hooper and Nason, 1965; Yamanaka and Shinra, 1974). It has been extensively purified and studied.

Isoelectric focusing gives a pI of 5.3 (Hooper et al, 1978a). The molecular weight has been stated to be 175,000 or 200,000 (Yamanaka et al, 1979; Rees, 1968). The reduced enzyme has an absorption spectrum typical of c-type cytochromes with peaks at 418, 524, and 553 nm, with a small absorption shoulder at 559, and also, a small peak at 463 nm (Hooper et al, 1978a; Ritchie and Nicholas, 1974; Yamanaka et al, 1979). The enzyme probably consists of three 11,000 molecular weight mono-heme peptides and three 75,000 molecular weight peptides containing six c hemes and one heme P-460. HAO comprises 2-3% of the total cell protein, 40% of the total c-type absorbance and essentially 100% of the P-460 absorbancy of the cell (Terry and Hooper, 1981).

NH_2OH, N_2H_4, and CH_3NHOH (but not NH_2OCH_3) reduce HAO from 35-50% (Ritchie and Nicholas, 1974; Hooper et al, 1978a). With a limited amount of NH_2OH, the peak at 553 nm is first reduced;

the shoulder at 559 nm appears with increased amounts of NH_2OH. After 10 minutes incubation, KCN causes an increase of the 559 nm peak relative to the 553 nm peak. KCN did not affect the oxidized or dithionite reduced spectrum of the enzyme (Yamanaka et al, 1979).

The reduced enzyme is rapidly oxidized in air; a rapid kinetic study showed a differential response of the c-type absorbances. Some of the 552 nm absorption disappeared almost as quickly as the 463 nm absorbance (P-460). The 552 and 558 nm absorbances then both disappear, followed by a 552 nm absorbance. It was concluded that electrons pass from c hemes to P-460 and then O_2; the product of O_2 reduction was H_2O_2 (Hooper and Balny, 1982). (The relevancy of H_2O_2 production to the overall mechanism of ammonia oxidation has not been conclusively shown.)

P-460 in HAO is an unusual heme. It forms a ferrous CO complex; addition of NH_2OH or NO_2^- to the ferrous enyzme results in the loss of absorbancy at 463 nm (Ritchie and Nicholas, 1974; Hooper et al, 1978a; Yamanaka et al, 1979). No natural reductant has been found for it. Micromolar concentrations of H_2O_2 destroyed P-460 when the enzyme was in the ferric state, resulting in loss of activity and substrate reducibility of the enzyme (Hooper and Terry, 1977b). Protection from H_2O_2 was given by substrates (NH_2OH, N_2H_4),

metal binding agents (eg. KCN, hydroxyurea), reductants (eg. dithiothreitol), electron acceptors (phenazine methosulfate, 2,6 dichlorophenolindophenol) and the singlet oxygen trapping agent, 1,3-diphenylfuran.

Mössbauer and electron paramagnetic resonance spectroscopy showed that the c-type hemes can be placed into at least four groups with different oxidation-reduction potentials and protein environments. Novel EPR species were noted; a tentative assignment of P-460 as a high spin heme with g-values at 6.45 and 5.6 was made using a reductive titration of the enzyme. No high spin heme was observed in the resting enyzme; it was suggested that P-460 undergoes a spin conversion as the other hemes are reduced or is EPR silent due to spin coupling or fast electronic relaxation. NH_2OH reduces approximately 45% of the hemes when complexed anaerobically with HAO (Lipscomb and Hooper, 1982a; Lipscomb et al, 1982b).

Further study indicates that one c-552 heme has a midpoint potential greater than +100 mV, one c-552 and one c-559 heme have potentials of approximately zero. One c-552 and c-559 have potentials in the range -100 to -250 mV; a change in redox potential as the state of the enzyme changes was suggested. Two heme c-552 moieties and heme P-460 have redox potentials of -320 mV, suggesting a possible involvement in reduction of

pyridine dinucleotide, activation of dioxygen for substrate oxygenation, or initiation of energy coupled electron transport. In the steady state of oxidation of hydroxylamine, roughly one third of the heme is reduced (Hooper, 1984).

The product of the oxidation of NH_2OH is not certain. Hooper et al (1977a) observed the production of nitrite and nitrate in approximately equimolar quantities accounting for 60-80% of the hydroxylamine utilized. Oxidation of diethyldithiocarbamate in equimolar quantities to nitrite was found; nitrate was not formed under these conditions.

Hooper and Terry (1979) showed the production of N_2O and NO during the oxidation of 100 uM NH_2OH using phenazine methosulfate as an electron acceptor. The presence of Mn(II) in micromolar quantities caused an increased amount of N_2O formation over NO, and an increase in the rate of oxidation of both NH_2OH and N_2H_4.

Yamanaka and Sakano (1980) demonstrated essentially stoichiometric production of NO_2^- from NH_2OH by HAO in a phosphate buffer when at least four times as much electron acceptor was present. Nitrite was formed even anaerobically, though transiently.

A partially purified complex of nitrite reductase reduced NO_2^- to NO and N_2O using NH_2OH as

electron donor aerobically or chemically reduced pyocyanine anaerobically (Hooper, 1968). Miller and Wood (1983a) purified a soluble copper oxidase (molecular weight 120,000; subunit molecular weight 35,000) which was also a nitrite reductase, utilizing ascorbate, dichlorophenolindophenol, and the native cytochrome c-552 as electron donors. DiSpirito et al (1985b) purified a "blue" copper oxidase (molecular weight 127,500; pI 4.3; subunit molecular weight 40,000) with an absorption maximum at 607 nm in the oxidized form. The enzyme catalyzed the aerobic oxidation of p-phenylenediamine, cytochrome c-554, and HAO; reduction of nitrite was with cytochrome c-552 only.

Cytochrome a_1 was purified from the organism (Erickson et al, 1972); it was shown to consist of one subunit (molecular weight 44,000) and contain two atoms of copper and two atoms of iron (DiSpirito and Hooper, 1985).

Numerous cytochromes have been identified and purified from the organism (DiSpirito et al, 1985a; Erickson et al, 1972a; Miller and Wood, 1982; Miller and Wood, 1983b; Miller et al, 1984; Rees and Nason, 1965; Tronson et al, 1973; Yamanaka and Shinra, 1974; Yamanaka et al, 1979); the uniqueness and function of many of them has not been demonstrated.

Yamanaka and Shinra (1974) have demonstrated that

electrons can flow from hydroxylamine oxidoreductase (HAO) to oxygen as follows:

$$NH_2OH \longrightarrow HAO \longrightarrow \underline{c}\text{-}554 \longrightarrow \underline{c}\text{-}552 \longrightarrow \text{cyt } \underline{a}_1 \longrightarrow O_2$$

Ninety-five percent of the soluble cytochromes (HAO, $\underline{c}$-554, $\underline{c}$-552) have been shown to be periplasmic, while cytochromes $\underline{c}_m$-553, $\underline{c}_m$-552 and cytochrome $\underline{a}$ were associated with the cell membrane (DiSpirito *et al*, 1985a). Based on heme concentration, the ratio of the components were:

HAO	$\underline{c}$-552	$\underline{c}$-554	$\underline{c}_m$-553	$\underline{c}_m$-552	cytochrome $\underline{a}$
22	11	2.9	12	1.0	10

Molar ratios of the components were:

HAO	$\underline{c}$-552	$\underline{c}$-554	$\underline{c}_m$-553	$\underline{c}_m$-552	cytochrome $\underline{a}$
0.24	2.2	0.16	1.2	0.1	1.0

Olson and Hooper (1983) proposed a mechanism for generation of a respiration dependent proton gradient which involves:

(a) periplasmic oxidation of NH_2OH with release of protons from substrate and water

(b) transmembrane transport of electrons through metal centres

(c) cytoplasmic coupling of oxygen, substrate electrons and cytoplasmic protons to form water.

Preparation of active extracts of *N. europaea* has proven to be difficult. As many as five components are necessary: ammonia oxidase protein(s), HAO, $\underline{c}$-554 and $\underline{c}$-552 and probably cytochrome $\underline{a}$.

Spheroplasts, vesicles, cell free extracts and membrane fractions plus c-554 oxidize ammonia to nitrite (Bhandari and Nicholas, 1979, 1980; Suzuki and Kwok, 1969, 1970, 1981a; Suzuki et al, 1981b). The cell free system requires activation by BSA, spermine or Mg^{+2}; BSA is most effective in 0.1 M phosphate buffer while spermine and Mg^{+2} are most effective at lower phosphate concentrations (Suzuki and Kwok, 1970; Suzuki et al, 1981b).

Initiation of ammonia oxidation seemed to require partial reduction of c-type cytochromes in the cell free systems activated with BSA, Mg^{+2}, or spermine (Suzuki et al, 1981a, 1981b). Addition of NH_2OH, N_2H_4 or NADH achieves partial reduction, suggesting a reductant is necessary for ammonia hydroxylation. NADH is not likely to act as an electron donor once ammonia oxidation is initiated.

N. europaea membrane (prepared by chromatography of cell free extracts on Sepharose 6B) plus cytochrome c-554 reconstituted ammonia oxidizing activity (Suzuki and Kwok, 1981). Hydroxylamine oxidation required both c-554 and c-552 for maximal rates and nearly stoichiometric yields of nitrite.

Cytochrome c-554 was partially reduced by NH_2OH or N_2H_4 (quickly) or by ammonia (slower) in the presence of the membrane fraction (Tsang and Suzuki, 1982).

Partially reduced c-554 is instantly partially oxidized by the addition of ammonia or CO, the extent of oxidation being related to the concentration of NH_3 or CO. Methane and methanol also oxidize the partially reduced c-554. The oxidation of c-554 is accompanied by O_2 consumption. The results were consistent with cytochrome c-554 as the donor of electrons for the monooxygenase. The stoichiometries appeared to be:

$$NH_2OH + CO + 3/2O_2 \longrightarrow HNO_2 + CO_2 + H_2O$$

$$N_2H_4 + 2CO + 2O_2 \longrightarrow N_2 + 2CO_2 + 2H_2O$$

MATERIALS AND METHODS

MATERIALS AND METHODS

MATERIALS

All chemicals were of analytical grades. Millipore "Super-Q" water was used for protein purification and kinetic experiments. Deoxyribonuclease I was obtained from Calbiochem, La Jolla, California. Trizma base and horse heart cytochrome c (Type III) were obtained from Sigma, St. Louis, Mo., U.S.A. Magnesium sulfate, ammonium sulfate, monobasic potassium phosphate, dibasic potassium phosphate and potassium cyanide were obtained from Fisher Scientific Company, Fairlawn, N.J., U.S.A.. Hydroxylamine hydrochloride, hydrazine sulfate, calcium chloride, ferric chloride, N-1-napthyl ethylene dihydrochloride and sulfanilic acid were obtained from Matheson, Coleman and Bell, Norwood, Ohio, U.S.A. Diethylaminoethyl cellulose (DEAE-cellulose) was obtained from Schleicher and Schuell Inc., Keene, New Hampshire.

Growth of Organism

A culture of Nitrosomonas europaea (Schmidt strain) was a gift of Dr. A.B. Hooper, University of Minnesota. The organism was cultivated at 28°C in a modified ATCC medium Number 221: 3.0 g $(NH_4)_2SO_4$, 0.5 g K_2HPO_4, 150 mg $MgSO_4 \cdot 7H_2O$, 12 mg $CaCl_2 \cdot 2H_2O$, 0.4 mg

chelated iron in a total of one litre of distilled water. The $MgSO_4$, $CaCl_2$, and equimolar mixture of $FeCl_3$ and EDTA were autoclaved separately.

Batch cultures of 25 L were grown in 30 L glass carboys, aerated through spargers. The pH was maintained at 7.6 with the addition of 50% K_2CO_3 (w/v) by means of a Radiometer pH stat unit.

Cells were harvested by Sharples centrifugation at 50,000 r.p.m., typically, after four days of growth (75% of the ammonia consumed). Cells were washed three times in 0.1 M potassium phosphate buffer, pH 7.5 and resuspended in same at 20 mg/mL (wet weight). Yields were typically 0.1 g/L. Cells were stored at 4 C and used within three days, or frozen and subsequently used for protein purification.

METHODS

Preparation of hydroxylamine oxidoreductase (HAO)

Two different purification schemes were used: A, which was a modification of the procedure of Yamanaka (1974), and B, which was a modification of the procedure used by Hooper (1978a).

A. A suspension of 30 mg/mL cells (wet weight), in the presence of 20 mg/mL BSA and DNase I in 0.1 M potassium phosphate buffer, pH 7.5, was passed through a French pressure cell at 18,000 p.s.i. Intact cells were removed by centrifugation at 2,000 x g for twenty min.. The

resulting cell free extract (active in ammonia oxidation) was passed through a Sepharose 6B-CL column (2 x 30 cm, equilibrated with 0.1 M potassium phosphate buffer, pH 7.5). Six milliliter fractions were collected; the bright red fraction five (HAO fraction) was collected, diluted five fold with 10 mM Tris-HCl buffer, pH 8.5 and applied to a DEAE-cellulose column (1 x 3 cm, equilibrated with the same Tris buffer). HAO bound to the column as a bright red band. The column was washed with 15 mL of the Tris buffer. Cytochrome c-552 was eluted with 10 mL of 50 mM NaCl in the Tris buffer. HAO was eluted with 150 mM NaCl in the Tris buffer, dialyzed against 50 mM pH 7.5 potassium phosphate buffer. Aliquots were frozen and withdrawn as needed.

B. Two grams of cells were suspended in 0.1 M potassium phosphate buffer, pH 7.5 (20 mg/mL) with a small amount of DNase I and were freeze-thawed three times, then spun at 2,000 x g to remove whole cells and debris. The supernatant was brought to 20% saturation with ammonium sulfate and centrifuged at 20,000 x g for 20 min. The supernatant was brought to 60% saturation with ammonium sulfate and centrifuged. The supernatant was finally brought to 75% saturation with ammonium sulfate and centrifuged. The pellet was resuspended in a small amount of 50 mM potassium phosphate buffer, pH 7.5, and applied to a Sephacryl S-200 column (1.5 x 25 cm) equilibrated with 50

mM potassium phosphate buffer, pH 7.5. HAO moved as a bright red band; appropriate fractions were collected, pooled, then dialyzed against 10 mM Tris-HCl, pH 8.5. This was then applied to a DEAE-cellulose column (1 x 4 cm) as in scheme A and a 0.0-0.5 M NaCl gradient was run. The HAO fraction collected was then diluted five fold and the DEAE step was repeated. The HAO fraction was eluted at ~0.15 M NaCl. The HAO was then precipitated with ammonium sulfate (75% saturation), dialyzed against 50 mM potassium phosphate buffer, pH 8.0, and frozen in small aliquots.

HAO activity was seemingly stable over at least several months at -20 C. At the concentration of enzyme used in kinetic experiments, there was no detectable cytochrome oxidase (nitrite reductase) activity (as assayed with reduced horse heart cytochrome c), although preparation A did show a weak oxidase activity at ten times the amount used in kinetic experiments. Preparation B showed no detectable oxidase activity; native gel electrophoresis with a 10% polyacrylamide gel (Ornstein, 1964; Davis, 1964) revealed three faint contaminating bands when HAO was overloaded.

The concentration of HAO was determined by the dithionite-reduced optical spectrum and/or by standard assay conditions (below).

Preparation of crude extracts and membrane fraction

Crude extracts and membrane fraction were prepared by the method of Tsang and Suzuki (1982). (See procedure for A, above; the membrane fraction eluted from the Sepharose 6B-CL column with the void volume).

Kinetic experiments

Standard assay conditions:

The rate of reduction of horse heart cytochrome c (50μM) by 50 μM NH_2OH in 50 mM potassium phosphate buffer, pH 7.5, was followed on a Beckman Acta III or Du-8 Spectrophotometer at 550 nm. Total volume of the reaction mixture was 1 mL (1 cm light path). Activity of the enzyme was expressed as change in absorbance in one minute.

Kinetic experiments were performed as above, using 50 mM potassium phosphate buffer, pH 7.5 or pH 8.0. Reactions were initiated by addition of enyzme. Substrates and HAO were added in microliter quantities by Hamilton micro-syringes. The rate of chemical reaction of NH_2OH or N_2H_4 with horse heart cytochrome c was essentially nil under these conditions, except at higher NH_2OH or N_2H_4 concentrations (>25 μM). Even at higher concentrations the rate of chemical reduction was ≤2% of the enzymatic rate, and was neglected. The trace of the change in absorbance at 550 nm was nearly always curved; initial velocities were calculated from the tangent drawn

to the curve. Experimental error at higher concentrations was estimated as <5%; at lower concentrations of reactants (particularly horse heart cytochrome c) error was around 15%. A set of experiments were completed within several hours as fluctuations in activity were observed over an extended period of time (probably due to the instability of reactants and the spectrophotometer). Kinetic patterns observed were confirmed by repeating experiments with a fresh set of reagents.

Typically, a 25 mM stock solution of hydroxylamine hydrochloride or hydrazine sulfate (prepared fresh daily) was used to prepare the appropriate solutions for the days' experiments. The NH_2OH or N_2H_4 solutions were the only reagents used which were not prepared in the reaction buffer; 10 μL of the appropriate dilution was added (variability was encountered when varying amounts of NH_2OH or N_2H_4 solution were added).

Horse heart cytochrome c (type III) was prepared as millimolar solutions, oxidized by a small amount of ferricyanide and dialyzed against buffer. The concentration of cytochrome c was determined by absorbance at 550 nm after reduction by dithionite, using the millimolar extinction coefficient of 29.5.

Reduced cytochrome c was prepared by ascorbate reduction, followed by dialysis in a stoppered flask which had been flushed with N_2. Typically, about 90% reduction

after dialysis was observed; allowance was made for the amount of oxidized form present.

Cytochrome solutions were used within two days after preparation.

Nitrite determination

Nitrite was determined by a modification to the method of Bratton and Marshall (1939). The reagents were:

A. 1% sulfanilic acid in 20% HCl

B. 0.12% N(1 napthyl) ethylene diamine dihydrochloride in distilled water.

For a determination of small amounts of NO_2^- in kinetic experiments, the reaction was followed on the spectrophotometer, at the appropriate time the contents of the cuvette were removed (1 mL) and placed in 1 mL of reagent A. Reagent B (1 mL) was then added, and the colour was allowed to develop for 30 min. Appropriate standards and blanks using known quantities of NO_2^- were used, and the absorbance at 530 nm was determined.

RESULTS

RESULTS

Initial velocity studies of hydroxylamine oxidation

Effects of the concentrations of substrates, horse heart cytochrome c and hydroxylamine, on the initial velocity of the reaction are presented in figures 1 and 2 as the double reciprocal plots and the intercepts and slopes replots. Both double reciprocal plots of velocity versus cytochrome c concentration (figure 1a) and hydroxylamine concentration (figure 2a) were essentially linear. The lines intersected but not to a common point. The replots were essentially linear except figure 2b. The Km for hydroxylamine was determined as 1.1 μM from figure 1b. The intercepts replot of figure 2a yielded an apparently biphasic curve (figure 2b); presuming this, the successive approximation method of Spears, Sneyd and Loten (1971) yielded:

$Vmax_1 = 0.024$ abs/min $\quad Vmax_2 = 0.333$ abs/min

$K_{m_1}^{cyt} = 18$ μM $\quad K_{m_2}^{cyt} = 670$ μM

(abs denotes the change in absorbance at 550 nm.) (This presumption will be considered further in the discussion.)

Table 1 represents an investigation into the effects of changing hydroxylamine concentration (with and without KCN) on the NO_2^- yield (at high cytochrome c concentration, 60 μM). A number of observations can be made:

Fig. 1a. Double reciprocal plots of initial velocity versus varying horse heart cytochrome c concentration at various fixed concentrations of hydroxylamine in 50 mM potassium phosphate buffer, pH 7.5, with 2.8 nM hydroxylamine oxidoreductase (Preparation A). Reaction was initiated by addition of enzyme.

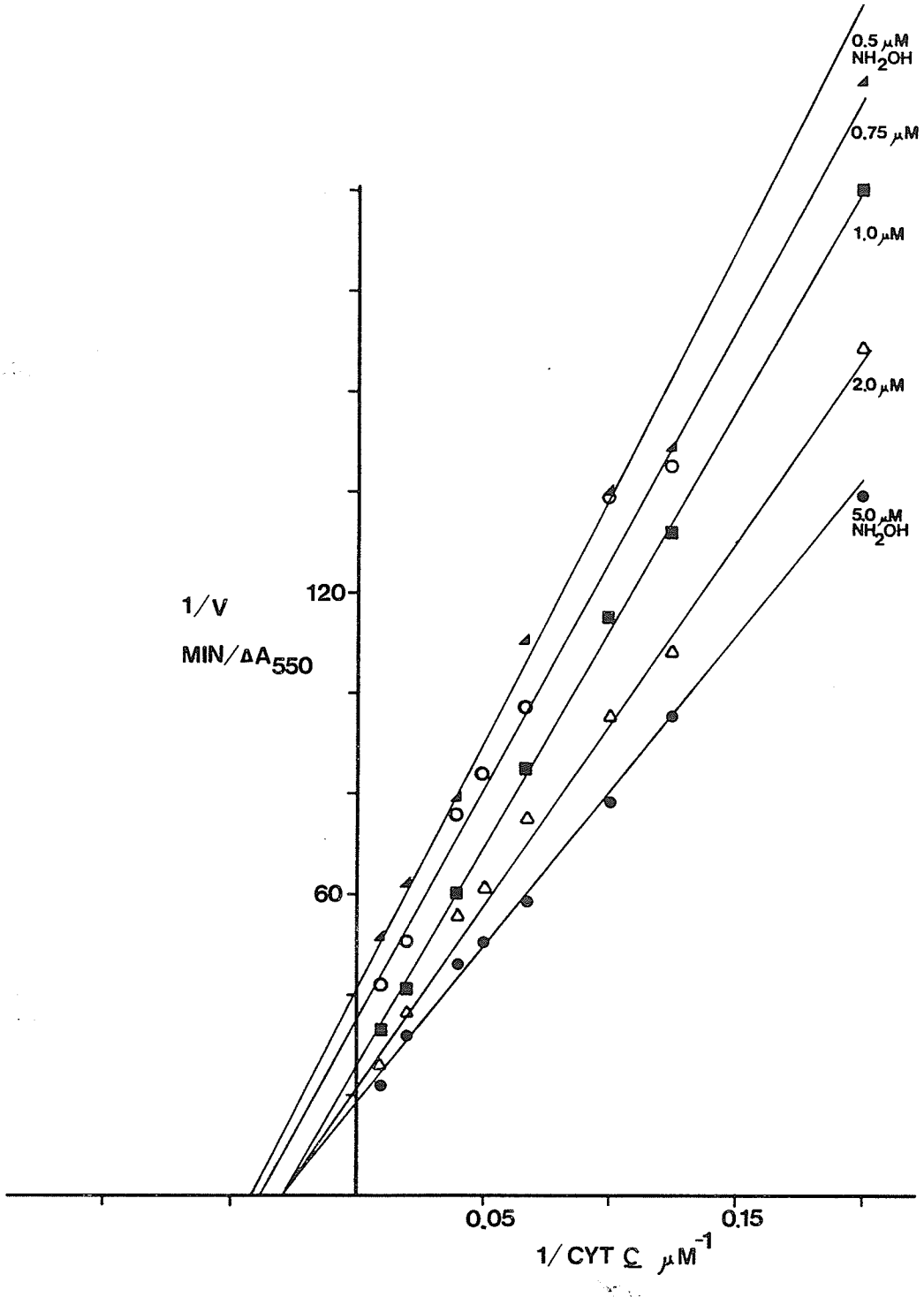

0.5 μM NH2OH
0.75 μM
1.0 μM
2.0 μM
5.0 μM NH2OH
1/V
MIN/ΔA550
120
60
0.05
0.15
1/ CYT C μM-1

Fig. 1b. Intercepts replot of Figure 1a.

Fig. 1c. Slopes replot of Figure 1a.

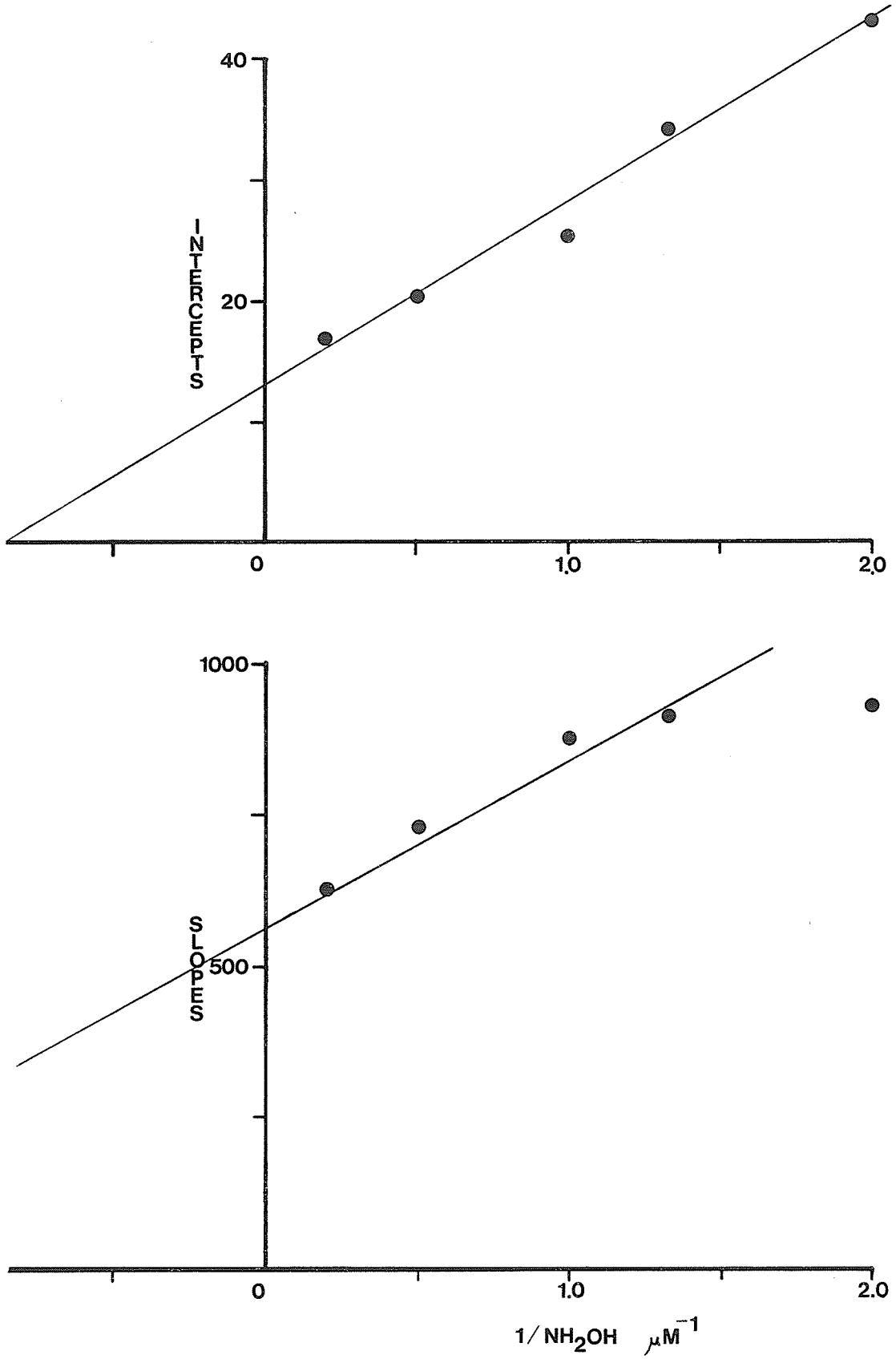

40
20
INTERCEPTS
0
1.0
2.0
1000
500
SLOPES
0
1.0
2.0
1/ NH2OH μM-1

Fig. 2a. Double-reciprocal plot of initial velocity versus varying hydroxylamine concentration at various fixed concentrations of horse heart cytochrome c. (Same data as figure 1a)

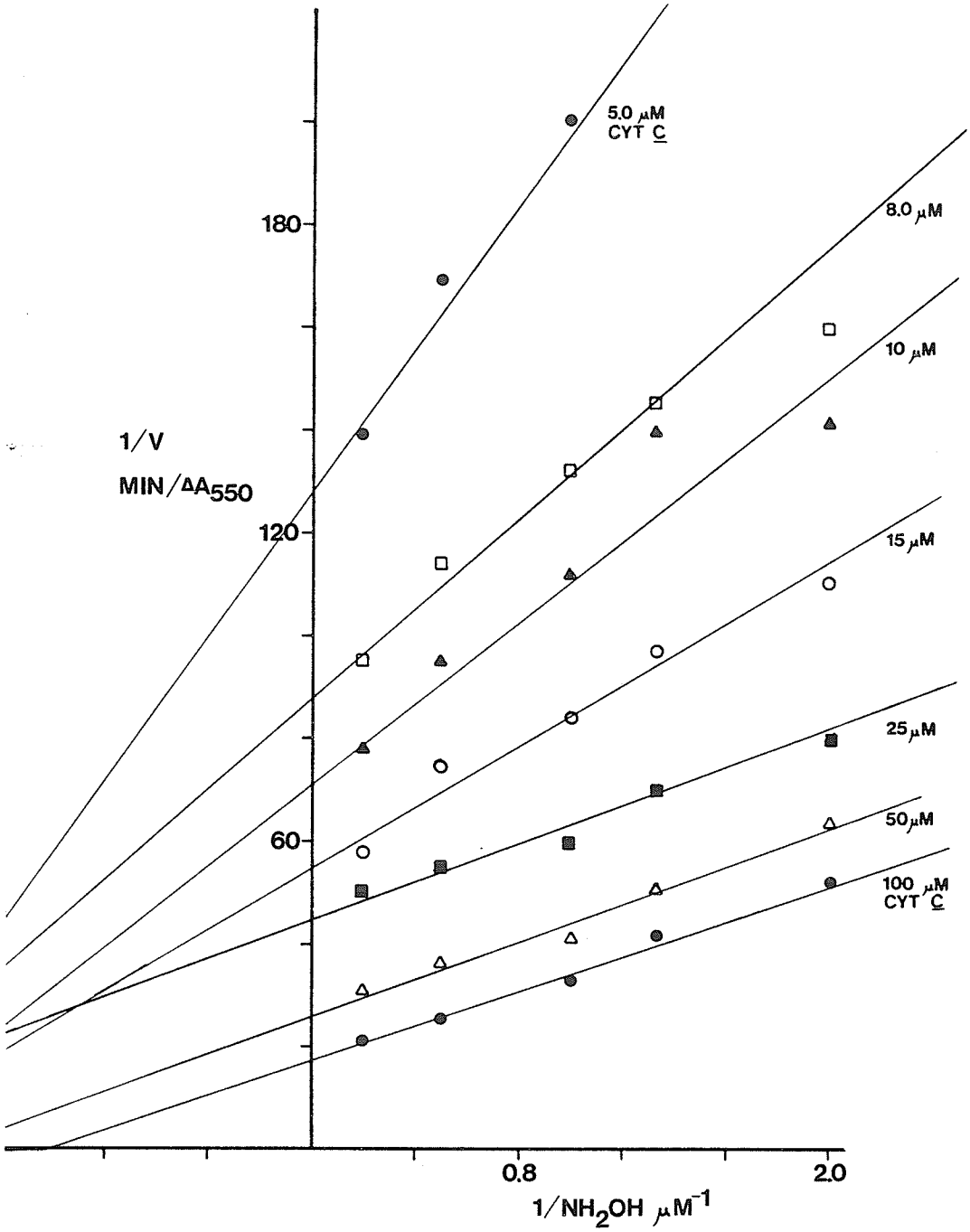

5.0 μM
CYT C
8.0 μM
10 μM
15 μM
25 μM
50 μM
100 μM
CYT C
180
120
60
1/V
MIN/ΔA550
0.8
2.0
1/NH2OH μM-1

Fig. 2b. Intercepts replot of figure 2a.

Fig. 2c. Slopes replot of figure 2a.

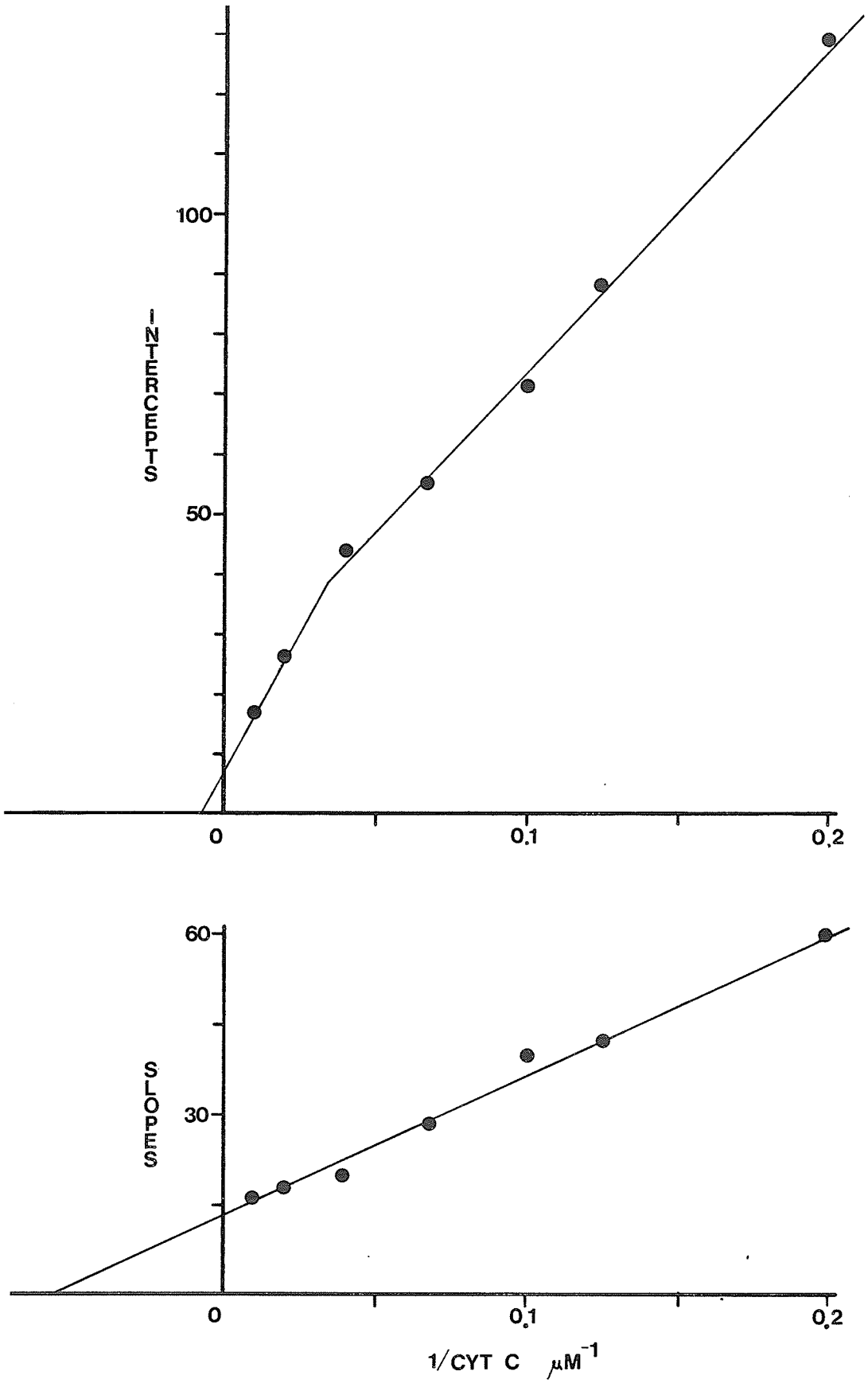

INTERCEPTS
100
50
0
0.1
0.2
SLOPES
60
30
0
0.1
0.2
1/CYT C μM⁻¹

Table 1. Effect of hydroxylamine and cyanide on nitrite formation by hydroxylamine oxidoreductase.

NH_2OH (μM)	KCN (μM)	Initial velocity (nmoles cyt. c reduced/ min.	Cytochrome c reduced (nmoles)	Nitrite formed (nmoles)	Cytochrome c reduced/ nitrite formed
50	--	3.5	30.0	3.5	8.6
50	50	3.6	28.0	2.4	12.0
10	--	3.3	22.0	3.1	7.1
10	50	2.8	17.0	1.3	13.0
5	--	2.3	4.5	0.8	5.4
3	--	1.8	2.3	0.8	2.9
3	50	1.3	1.7	0.5	3.4

The reaction mixture contained 50 mM potassium phosphate buffer pH 8.0, hydroxylamine and potassium cyanide at the concentrations indicated, 60 μM horse heart cytochrome c and 5 nM hydroxylamine oxidoreductase (preparation B) in a total volume of one ml. Reaction was initiated by addition of enzyme; nitrite was determined when the reduction of cytochrome c ceased.

1. As the hydroxylamine concentration was increased the "efficiency" of NO_2^- formation (as measured by the cytochrome c reduced:NO_2^- formed ratio) decreased. The interpretation made was that the stoichiometry of nitrite formation was changing as the hydroxylamine concentration changed.

2. KCN seemed to have an effect similar to higher hydroxylamine concentrations. It increased the cytochrome c reduced:NO_2^- formed ratio; KCN also decreased the amount of NO_2^- formed at a given hydroxylamine concentration.

3. KCN inhibited the reaction at lower hydroxylamine concentrations, but at higher hydroxylamine concentrations ($\geq$50 μM) a slight activation was seen. Although not shown in the table, activation of 15% relative to a control could be observed at 100 μM hydroxylamine with 50 μM KCN.

Initial velocity studies of hydrazine oxidation

Since the changing stoichiometry of hydroxylamine oxidation at different concentrations of hydroxylamine indicated a possible complexity, the oxidation of hydrazine, an alternate substrate for hydroxylamine oxidoreductase (HAO), was studied.

In all trials where substrate concentration permitted, 3.5-3.8 nmoles of cytochrome c were reduced for every nmole

of hydrazine initially present. This was interpreted as indicating that a 4:1 stoichiometry was followed.

The double reciprocal plots of velocity versus hydrazine concentration (figure 3a) were essentially linear and intersecting, but there was no common intersection point. The double reciprocal plots of velocity versus cytochrome c concentration (figures 4a and 5a) could be interpreted as linear or biphasic. If interpreted as linear, (figure 4a) there was no common intersection point. If interpreted as biphasic (figure 5a), the lines drawn at high cytochrome c concentrations did intersect. These results suggested an ordered addition of hydrazine and cytochrome c.

The oxidation of hydrazine to N_2 involves four electrons; the reaction is not a simple Bi-type. Hydrazine and hydroxylamine both reduce HAO; Hooper (1979) has shown that the enzyme can catalyze the oxidation of hydroxylamine albeit slowly in the absence of added electron acceptor presumably using O_2 as electron acceptor. Cytochrome cannot be binding as ordered pairs - this would yield concave up parabolic plots when cytochrome is the variable substrate. Therefore, cytochrome is reacting in a Ping Pong or Theorell-Chance sequence.

A sequence which is consistent would be a Bi Hexa Uni Bi reaction (scheme II). The results were interpreted within the framework of schemes I and II.

Fig. 3a. Double reciprocal plots of initial velocity versus varying hydrazine concentrations at various fixed concentrations of horse heart cytochrome c in 50 mM potassium phosphate, pH 8.0, with 4 nM hydroxylamine oxidoreductase (Preparation B). Reaction was initiated by addition of enzyme.

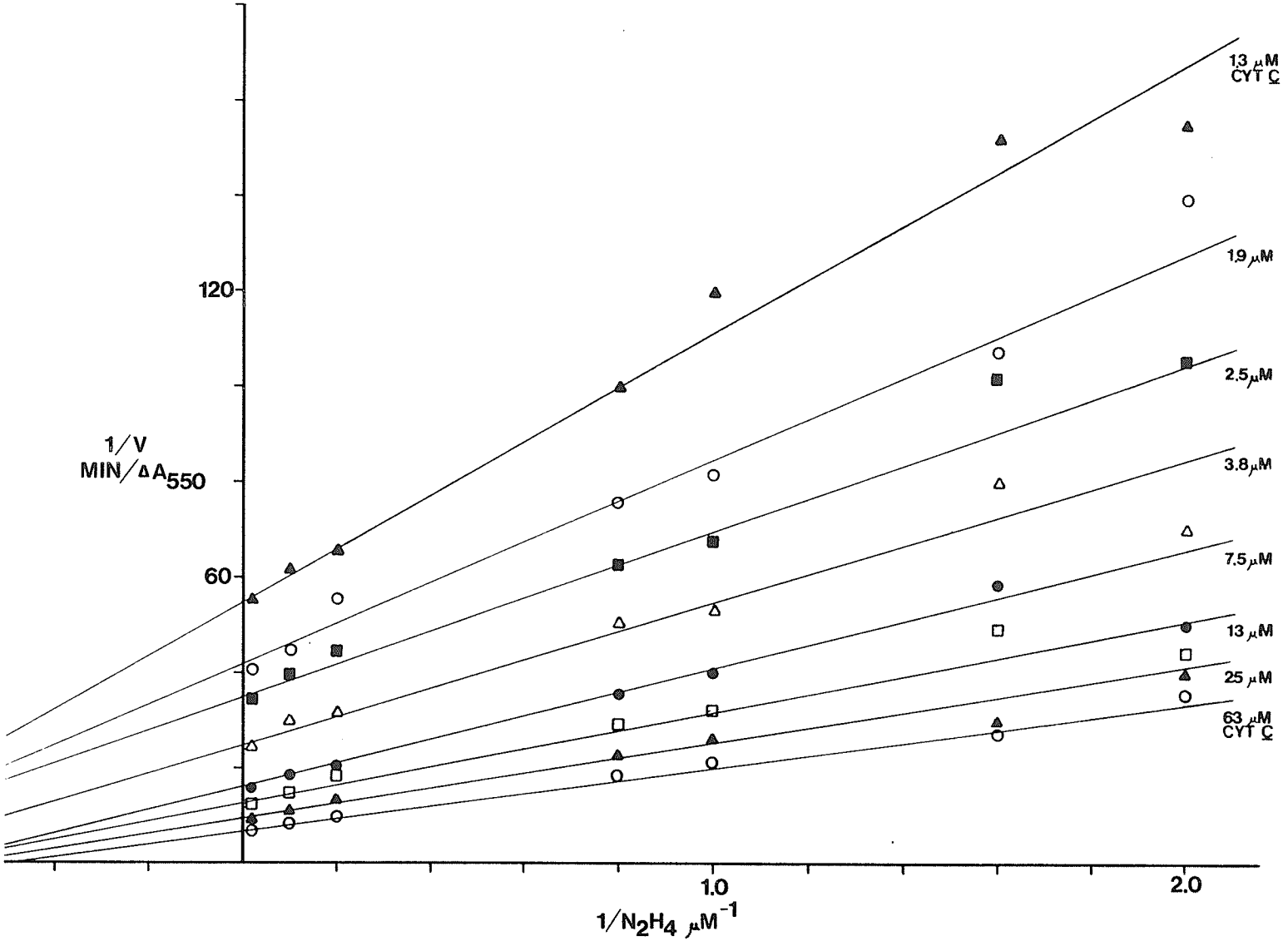
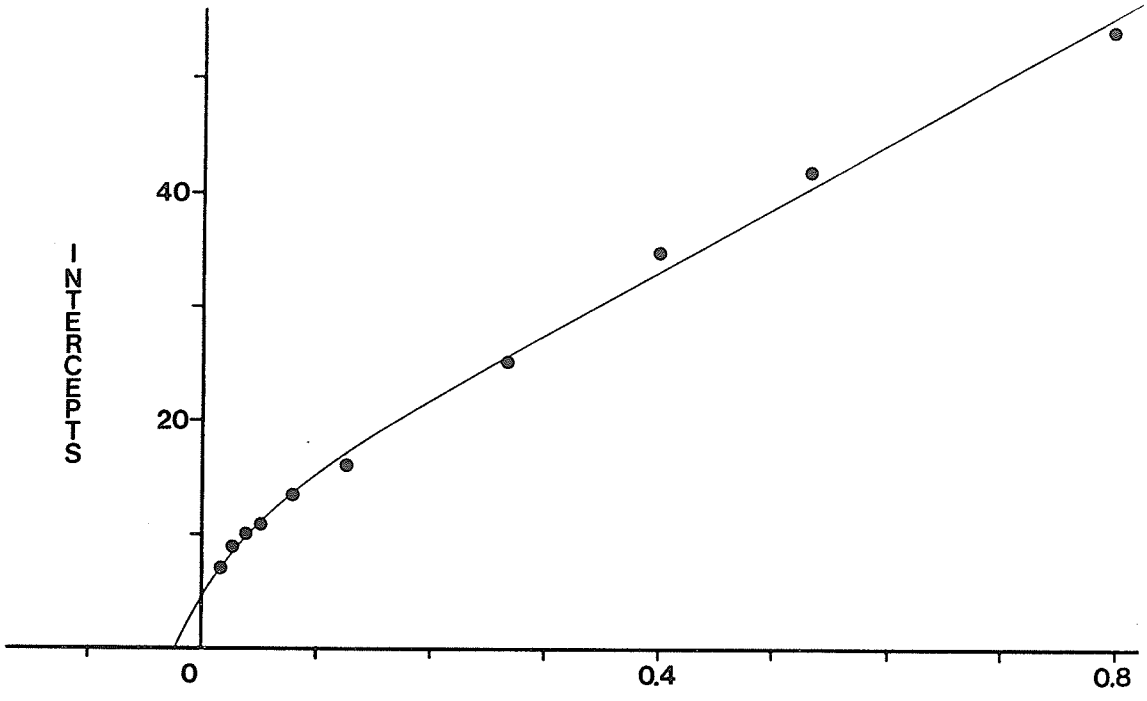

1.3 μM CYT C
1.9 μM
2.5 μM
3.8 μM
7.5 μM
13 μM
25 μM
63 μM CYT C
120
60
1/V MIN/ΔA550
1.0
2.0
1/N2H4 μM-1

Fig. 3b. Intercepts replot of figure 3a. The curve was according to equation [3].

Fig. 3c. Slopes replot of figure 3a.


INTERCEPTS
40
20
0
0.4
0.8

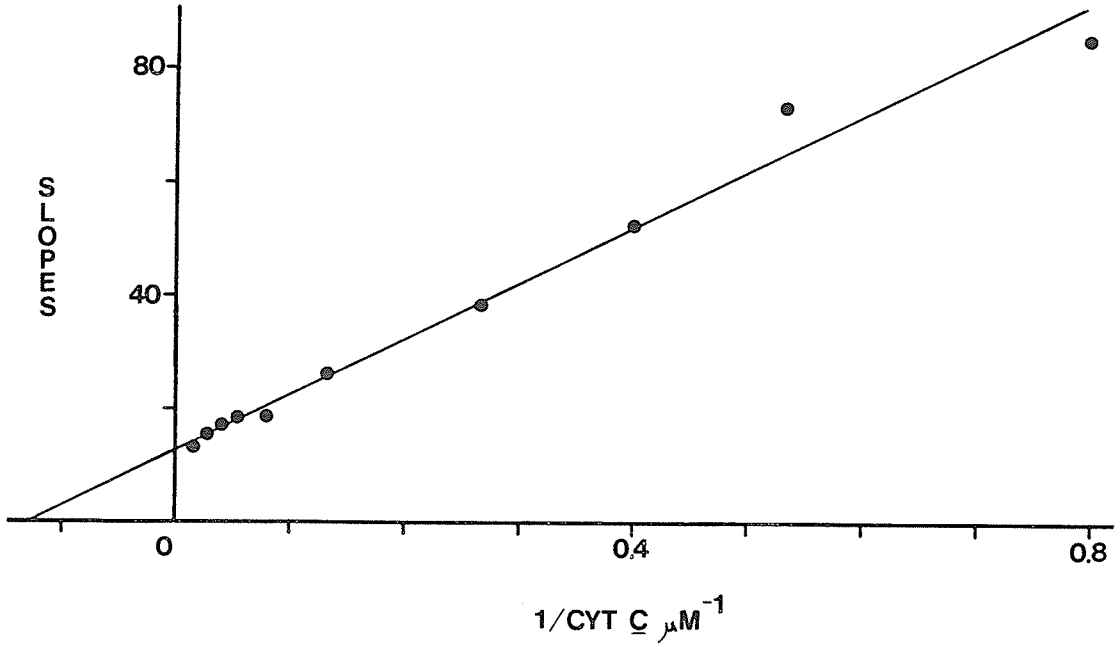

SLOPES
80
40
0
0.4
0.8
1/CYT C μM⁻¹

Fig. 4a. Double reciprocal plots of initial velocity versus varying horse heart cytochrome c concentration at various fixed concentrations of hydrazine. (Same data as figure 3a.) The best fitting line to all the points on the curve was drawn.

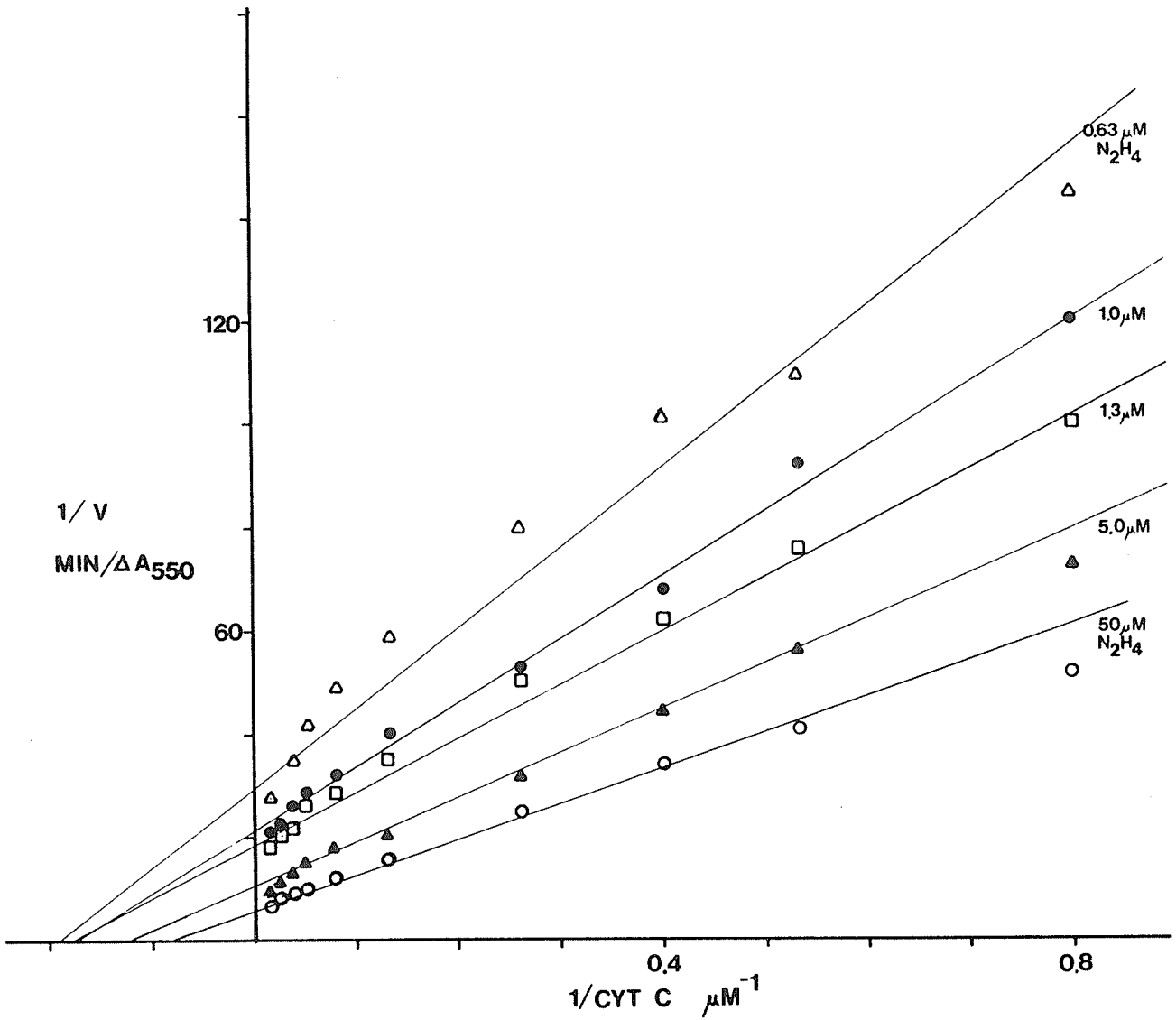

0.63 μM
N_2H_4
1.0μM
1.3μM
5.0μM
50μM
N_2H_4
120
60
1/ V
MIN/ΔA550
0.4
0.8
1/CYT C μM^{-1}

Fig. 4b. Intercepts replot of figure 4a.

Fig. 4c. Slopes replot of figure 4a.

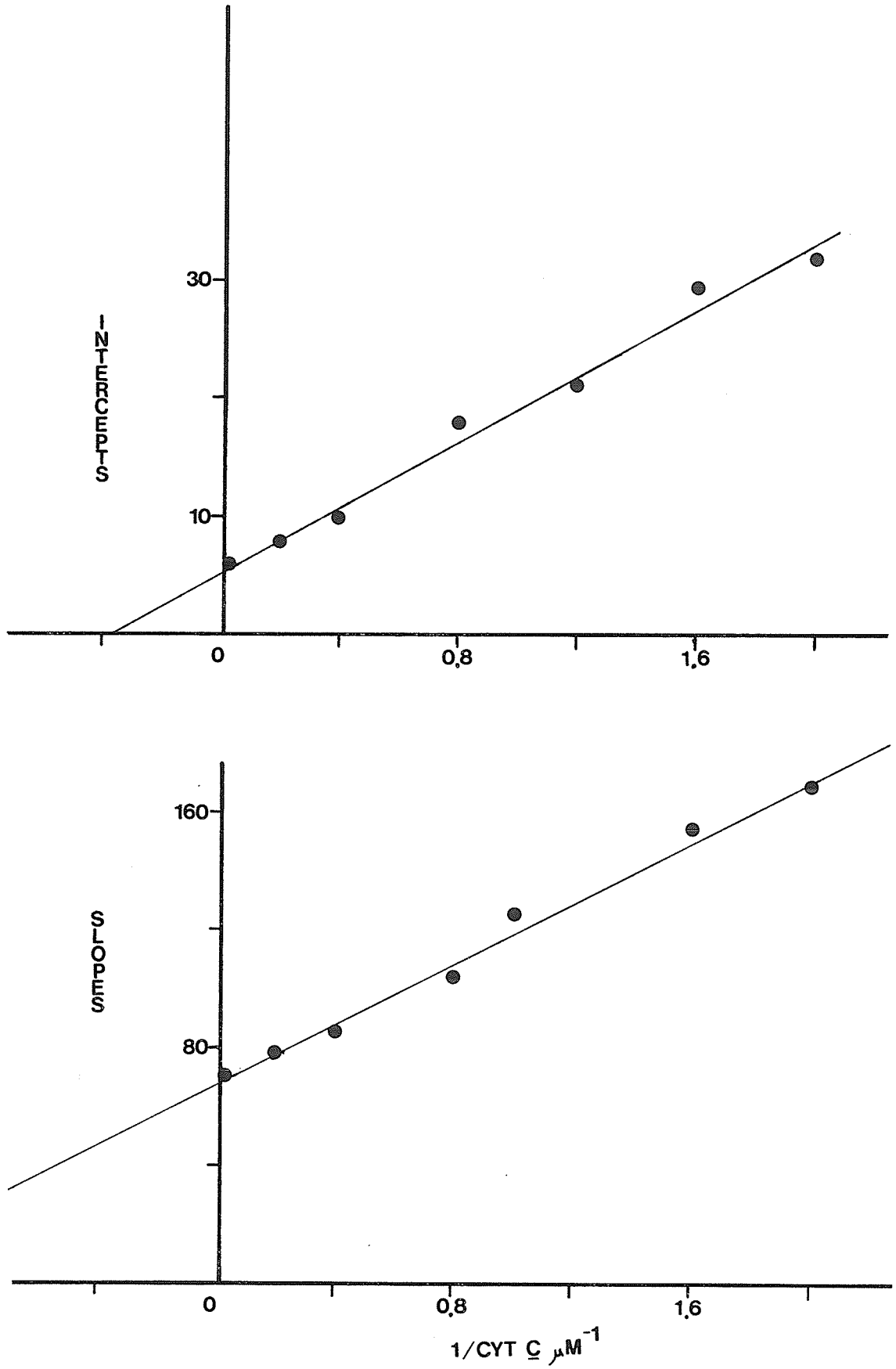

INTERCEPTS
30
10
0
0.8
1.6
SLOPES
160
80
0
0.8
1.6
1/CYT C μM⁻¹

Fig. 5a. Same graph as figure 4a, but the best fitting lines to the highest horse heart cytochrome c concentrations were drawn to emphasize the apparent biphasic nature of the lines.

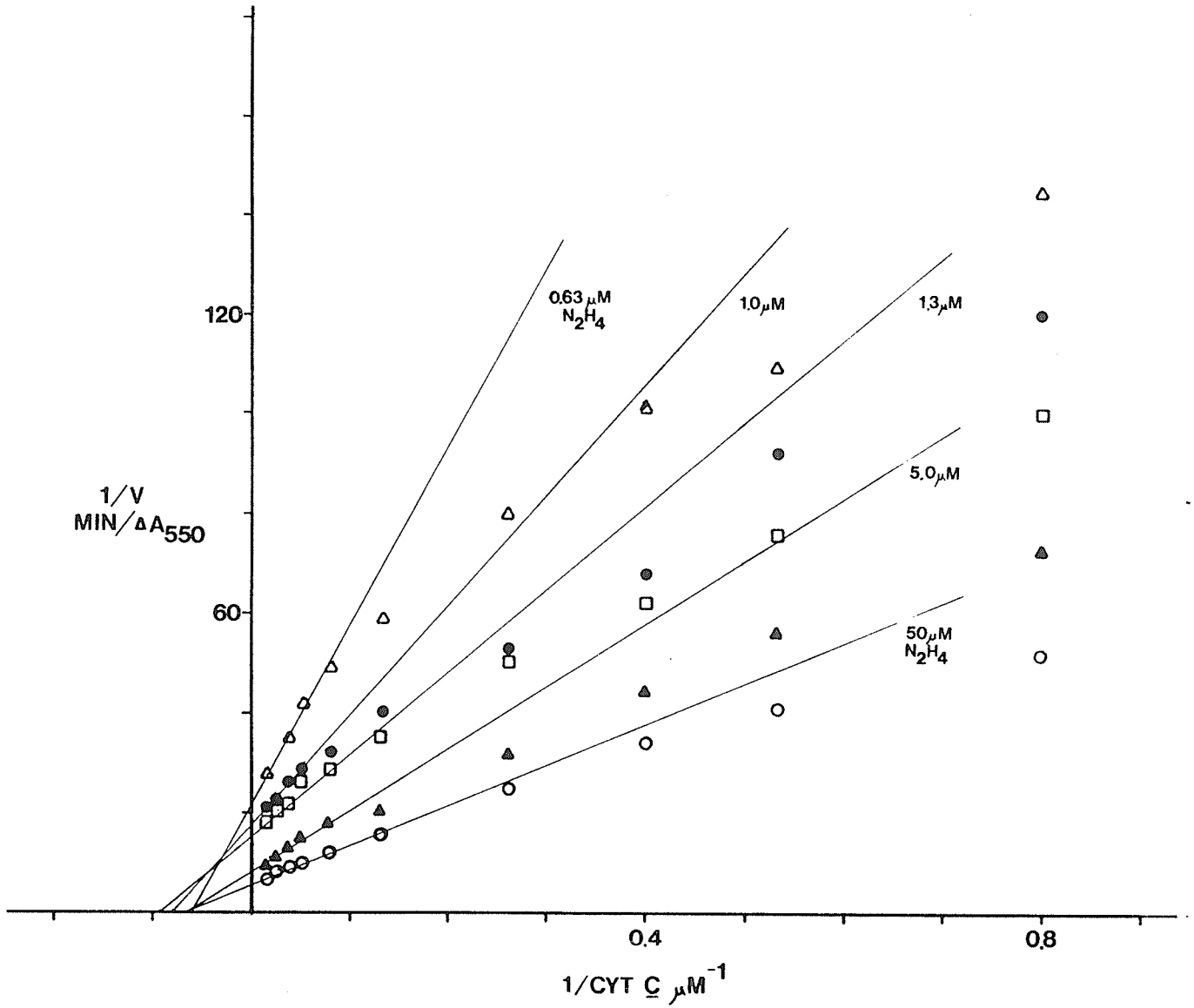
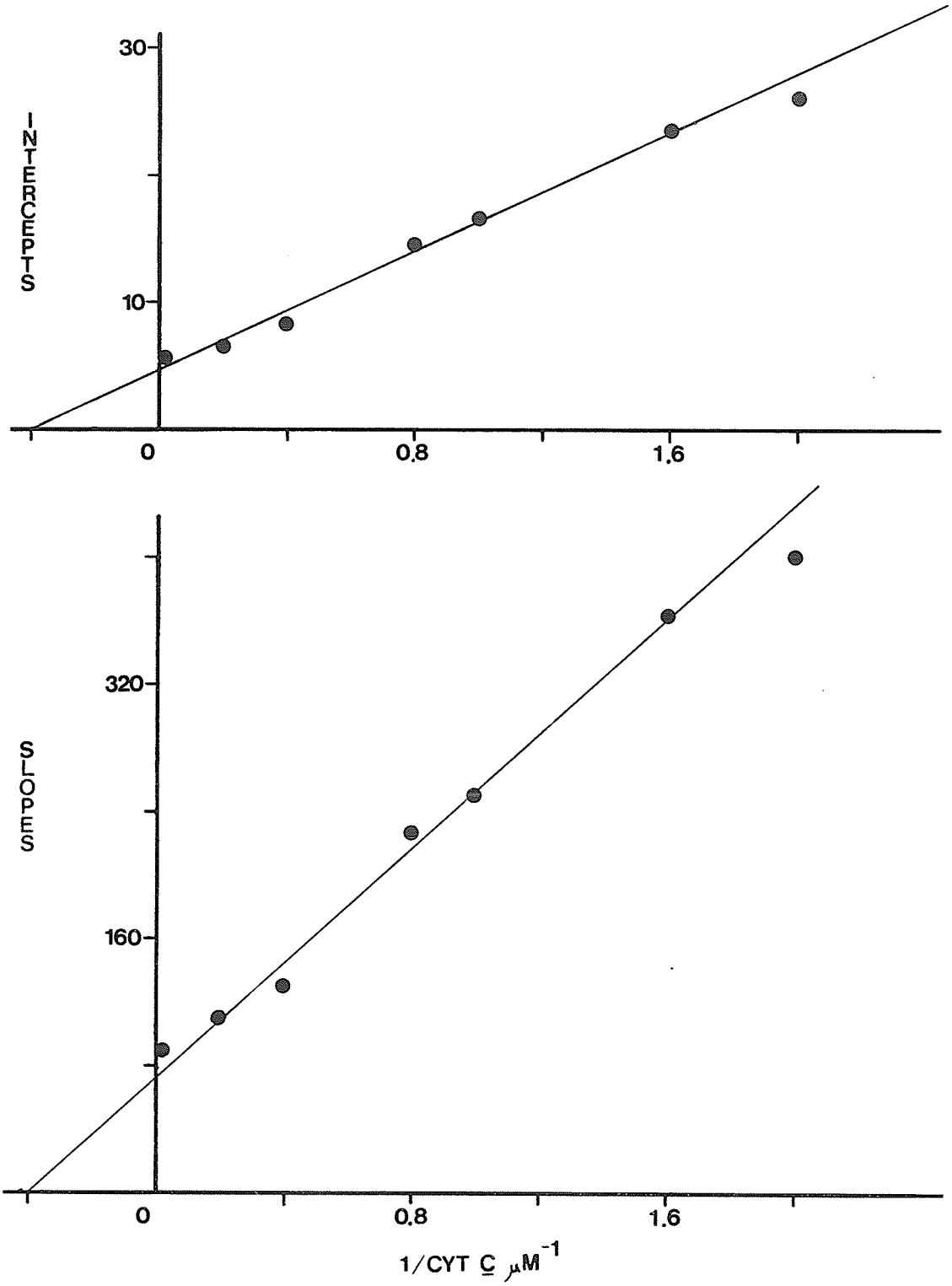

120
60
1/V
MIN/ΔA550
0.63 μM
N2H4
1.0μM
1.3μM
5.0μM
50μM
N2H4
0.4
0.8
1/CYT C μM-1

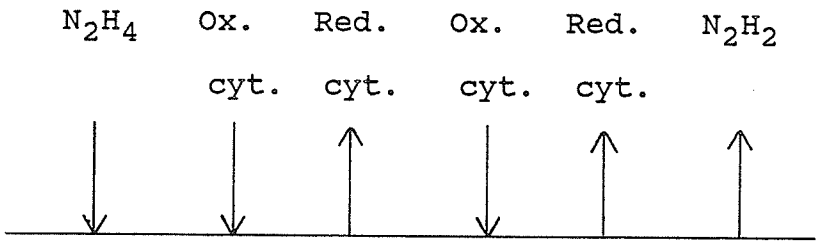
Fig. 5b. Intercepts replot of figure 5a.

Fig. 5c. Slopes replot of figure 5a.


30
10
INTERCEPTS
0
0.8
1.6
320
160
SLOPES
0
0.8
1.6
1/CYT C μM^{-1}

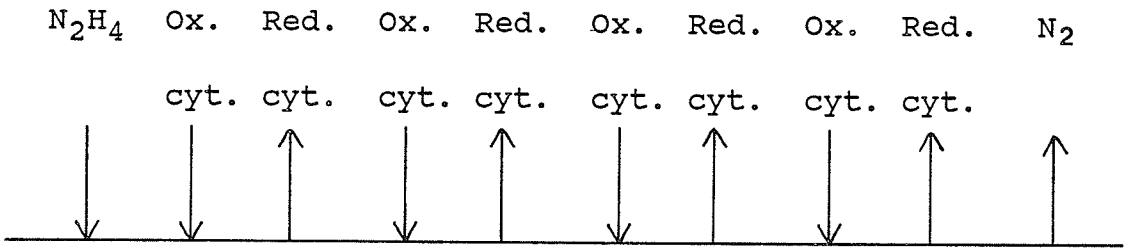
Scheme I

Bi Uni Uni Bi Ping Pong



Scheme II

Bi Hexa Uni Bi Ping Pong



where ox. cyt. or red. cyt. represents oxidized cytochrome c or reduced cytochrome c.

The equation for a Bi Uni Uni Bi reaction in the forward direction is (Segal 1975):

$$v = \frac{Vmax\ [N_2H_4][cyt]^2}{K_i^{N_2H_4}\ K_m^{cyt}\ [cyt] + 2\ K_m^{cyt}\ [N_2H_4][cyt] + K_m^{N_2H_4}\ [cyt]^2 + [N_2H_4][cyt]^2} \qquad [1a]$$

which simplifies to:

$$v = \frac{Vmax\ [N_2H_4][cyt]}{K_i^{N_2H_4}\ K_m^{cyt} + 2\ K_m^{cyt}\ [N_2H_4] + K_m^{N_2H_4}\ [cyt] + [N_2H_4][cyt]} \quad [1b]$$

The equation for a Bi Hexa Uni Bi reaction in the forward direction is:

$$v = \frac{Vmax\ [N_2H_4][cyt]^4}{3K_i^{N_2H_4}\ K_m^{cyt}\ [cyt]^3 + 4\ K_m^{cyt}\ [N_2H_4][cyt]^3 + K_m^{N_2H_4}\ [cyt]^4 + [N_2H_4][cyt]^4} \quad [2a]$$

which simplifies to:

$$v = \frac{Vmax\ [N_2H_4][cyt]}{3\ K_i^{N_2H_4}\ K_m^{cyt} + 4\ K_m^{cyt}\ [N_2H_4] + K_m^{N_2H_4}\ [cyt] + [N_2H_4][cyt]} \quad [2b]$$

The equation was derived by analogy with the Bi Uni Uni Bi system. A partial derivation of the equation in the forward direction by the method of King-Altman confirmed that there were: 3 $[cyt]^3$, 4 $[N_2H_4][cyt]^3$, 1 $[cyt]^4$ and one $[N_2H_4][cyt]^4$ terms. Complete derivation of the rate equation would have involved manipulation of some one hundred terms, and was deemed impracticable.

Both equations [1b] and [2b] are essentially identical

to an Ordered Bi Bi reaction. The only minor difference is that the intercepts replot of variable hydrazine concentrations against the reciprocal of cytochrome c concentrations will intercept at $-1/2\ K_m^{cyt}$, not $-1/K_m^{cyt}$; equation [2b] will yield $-1/4\ K_m^{cyt}$ not $-1/K_m^{cyt}$. The slopes replot of variable cytochrome c concentrations against the reciprocal of hydrazine concentrations will intercept on the x-axis at $-1/3\ K_i^{N_2H_4}$, not $-1/K_i^{N_2H_4}$ (for equation [2b]).

As with hydroxylamine, the intercepts replot of hydrazine experiments appears biphasic (figure 3b). Again assuming this reflects the presence of two sites with two Vmax's, the approximation technique of Spears et al (1971) gave:

$Vmax_1 = 0.075$ abs/min $\quad Vmax_2 = 0.16$ abs/min

$K_{m_1}^{cyt} = 4.9\ \mu M \quad K_{m_2}^{cyt} = 79\ \mu M$

The true Km's for cytochrome c will be 1/2 of these if equation [1b] is followed, or 1/4 of these if equation [2b] is followed.

Thus figure 3b can be described by the reciprocal of:

$$v = \frac{Vmax_1\ [cyt]}{4\ K_{m_1}^{cyt} + [cyt]} + \frac{Vmax_2\ [cyt]}{4\ K_{m_2}^{cyt} + [cyt]}$$

$$= \frac{0.075\ [cyt]}{4.9 + [cyt]} + \frac{0.16\ [cyt]}{79 + [cyt]} \qquad [3]$$

Equation [3] was used to draw the curve in figure 3b;

the fit is good so the assumption is consistent within the context of this hypothesis.

The Km for hydrazine from figure 5b (intercepts replot of variable cytochrome c concentrations) was 1.8 μM.

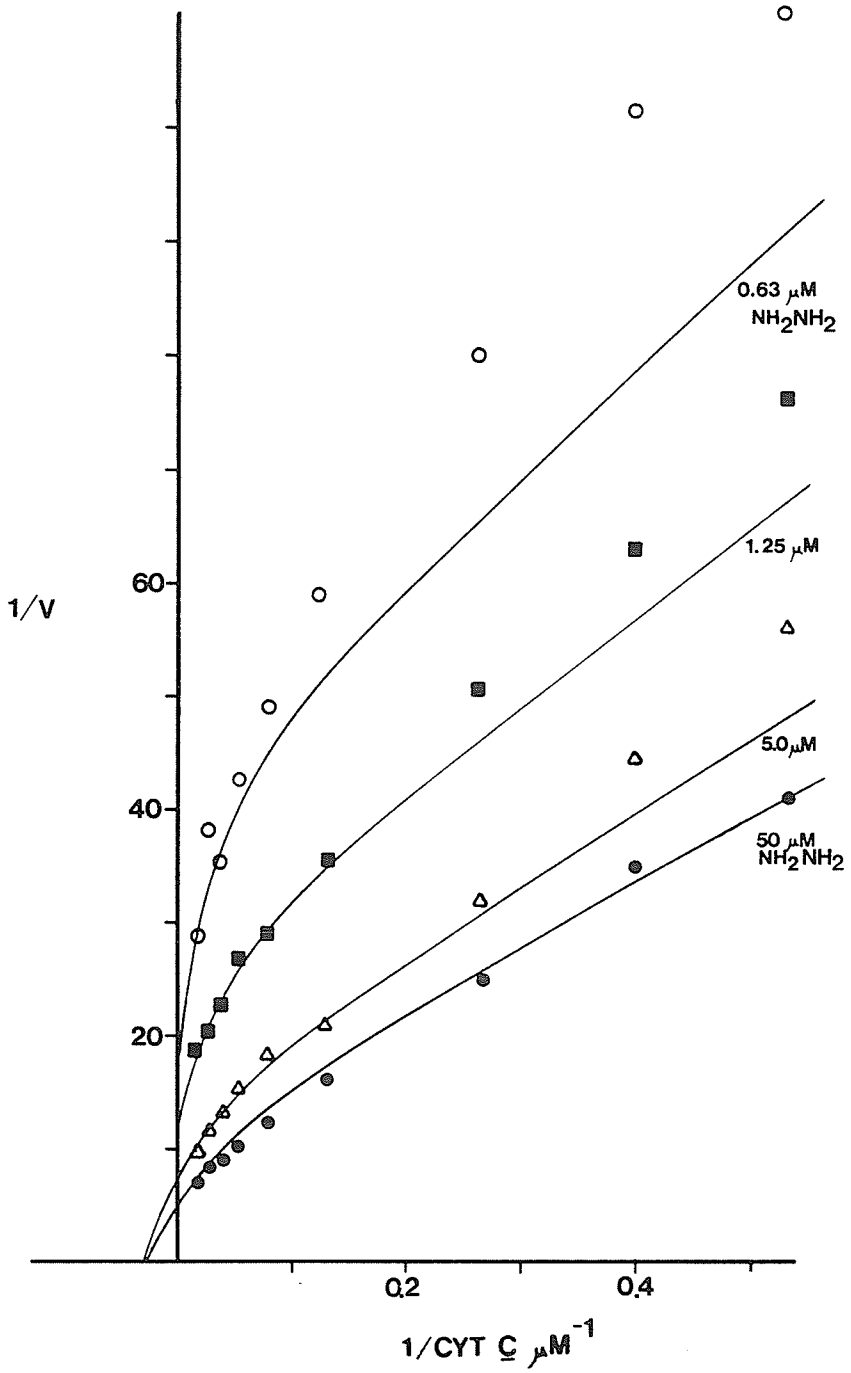
A preliminary description of the initial velocity patterns was made using:

$$v = \frac{Vmax_1[N_2H_4][cyt]}{K_{i_1}^{N_2H_4} K_{m_1}^{cyt}[cyt] + 4 K_{m_1}^{cyt}[N_2H_4] + K_{m_1}^{N_2H_4}[cyt] + [N_2H_4][cyt]} + \frac{Vmax_2[N_2H_4][cyt]}{K_{i_2}^{N_2H_4} K_{m_2}^{cyt}[cyt] + 4 K_{m_2}^{cyt}[N_2H_4] + K_{m_2}^{N_2H_4}[cyt] + [N_2H_4][cyt]}$$

$$= \frac{0.075[N_2H_4][cyt]}{1\ (4.9/4)[cyt] + 4\ (4.9/4)[N_2H_4] + 1.6[cyt] + [N_2H_4][cyt]} + \frac{0.16[N_2H_4][cyt]}{13\ (79/4)[cyt] + 4\ (79/4)[N_2H_4] + 1.6[cyt] + [N_2H_4][cyt]} \quad [4]$$

This equation describes the activity of two independent enzymes (the sum of two equations of the form of equation 2b). The values for the Vmax's and K_m^{cyt}'s were from figure 3b; $K_m^{N_2H_4}$ (1.6), $K_{i_1}^{N_2H_4}$ (1.0), $K_{i_2}^{N_2H_4}$ (13) were assumed values. Figure 6 represents a comparison of the theoretical curves from equation [4] with the experimental data. A reasonable fit resulted at higher substrate concentrations, but the model consistently predicted faster

Fig. 6. Comparison of the experimental results of figure 4 with the theoretical curves as predicted by equation [4]. (For visual clarity only some of the experimental points are included, but the overall pattern is the same for all the data.)


0.63 μM
NH2NH2
1.25 μM
5.0 μM
50 μM
NH2NH2
1/V
60
40
20
0.2
0.4
1/CYT C μM-1

initial velocities than those experimentally observed at lower substrate concentrations.

Inhibition by reduced cytochrome c

Reduced cytochrome c was used as an inhibitor of both hydroxylamine and hydrazine oxidation. Higher concentrations (>30 μM) of reduced cytochrome c inhibited in a seemingly competitive fashion with respect to oxidized cytochrome c (figure 7a) and an essentially uncompetitive fashion with respect to hydroxylamine or hydrazine (figure 7c). However, at lower (≤20 μM) reduced cytochrome c concentrations, both activation and inhibition could be observed. Figure 7a is representative of the results obtained.

At 19 μM reduced cytochrome c and high (>50 μM) concentrations of oxidized cytochrome c, activation can be observed. The general pattern of the lines is consistent with two site inhibition:

(a) an intersection point to the right of the 1/v axis,

(b) a concave up slopes replot (figure 7b)

During these experiments it was noticed that at higher reduced cytochrome c concentrations, hydroxylamine (but not hydrazine) would reoxidize the reduced cytochrome c after completion of the initial reduction phase. (The reoxidation occurred at a much faster rate than the

Fig. 7a. Double reciprocal plots of initial velocity versus varying horse heart cytochrome c concentrations using reduced horse heart cytochrome c as product inhibitor. (2 μM hydroxylamine, pH 7.5, 50 mM potassium phosphate buffer; reaction initiated by addition of enzyme (Preparation A).)

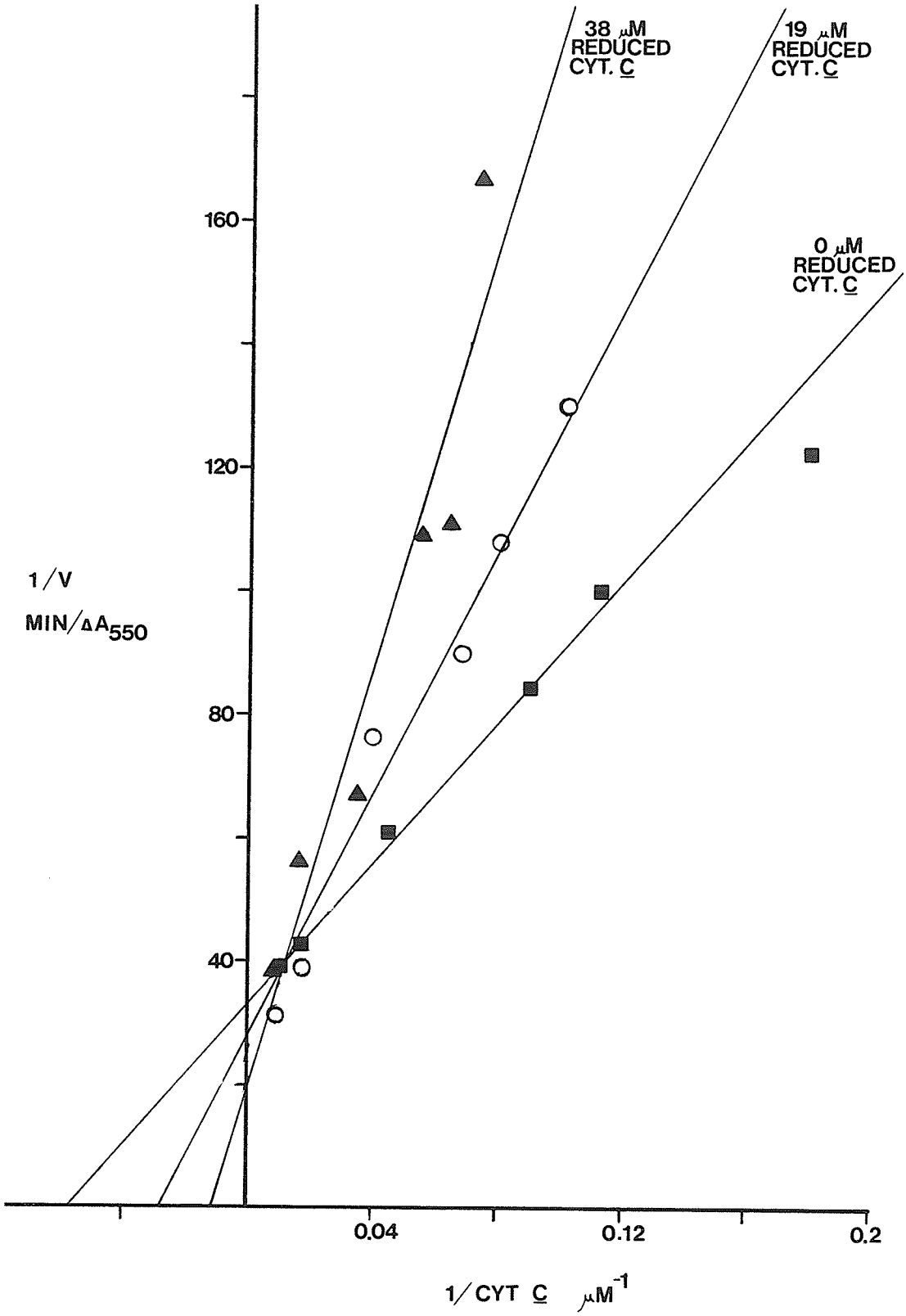

38 μM
REDUCED
CYT. C
19 μM
REDUCED
CYT. C
0 μM
REDUCED
CYT. C
160
120
80
40
1/V
MIN/ΔA550
0.04
0.12
0.2
1/CYT C μM-1

Fig. 7b. Slopes replot of figure 7a.

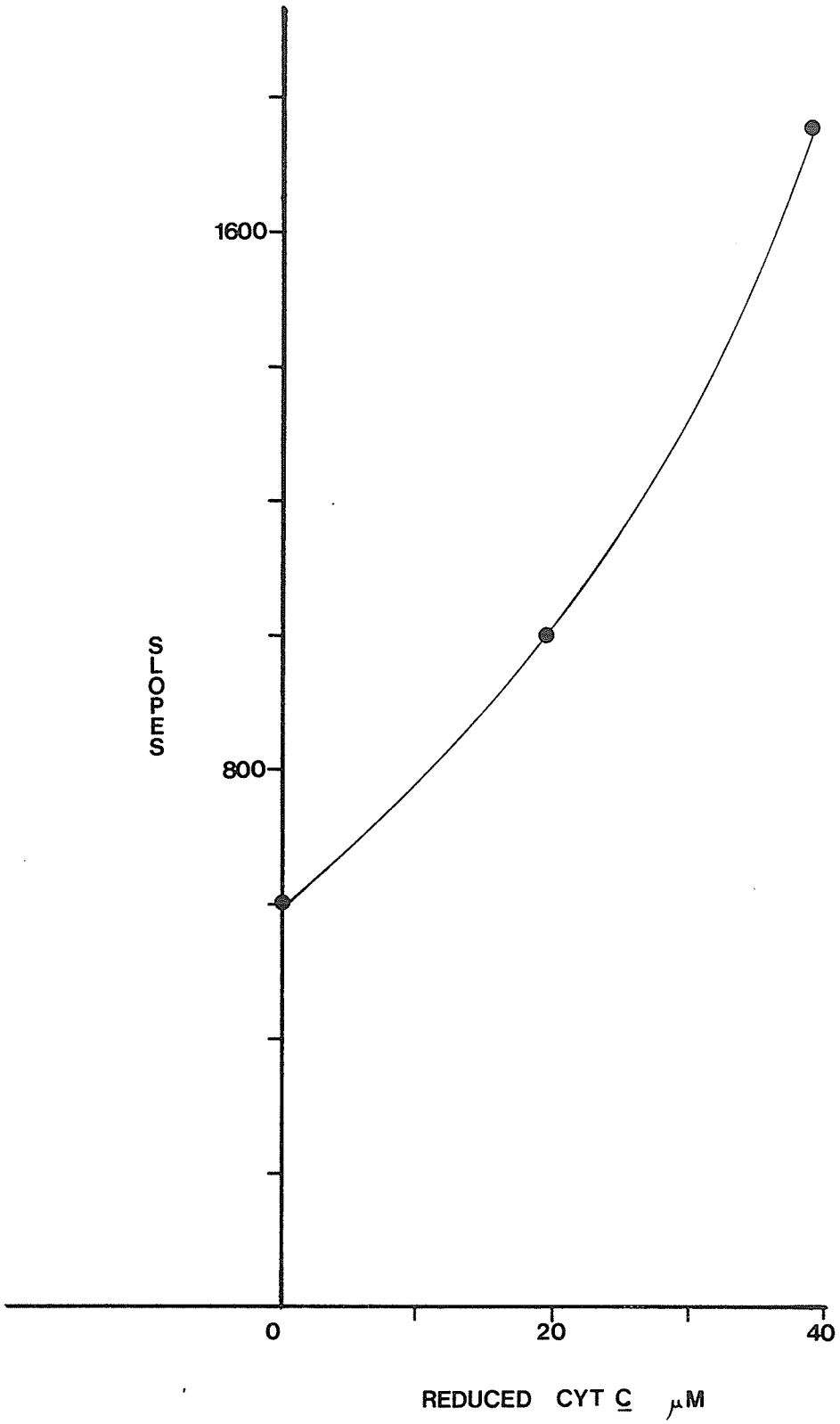
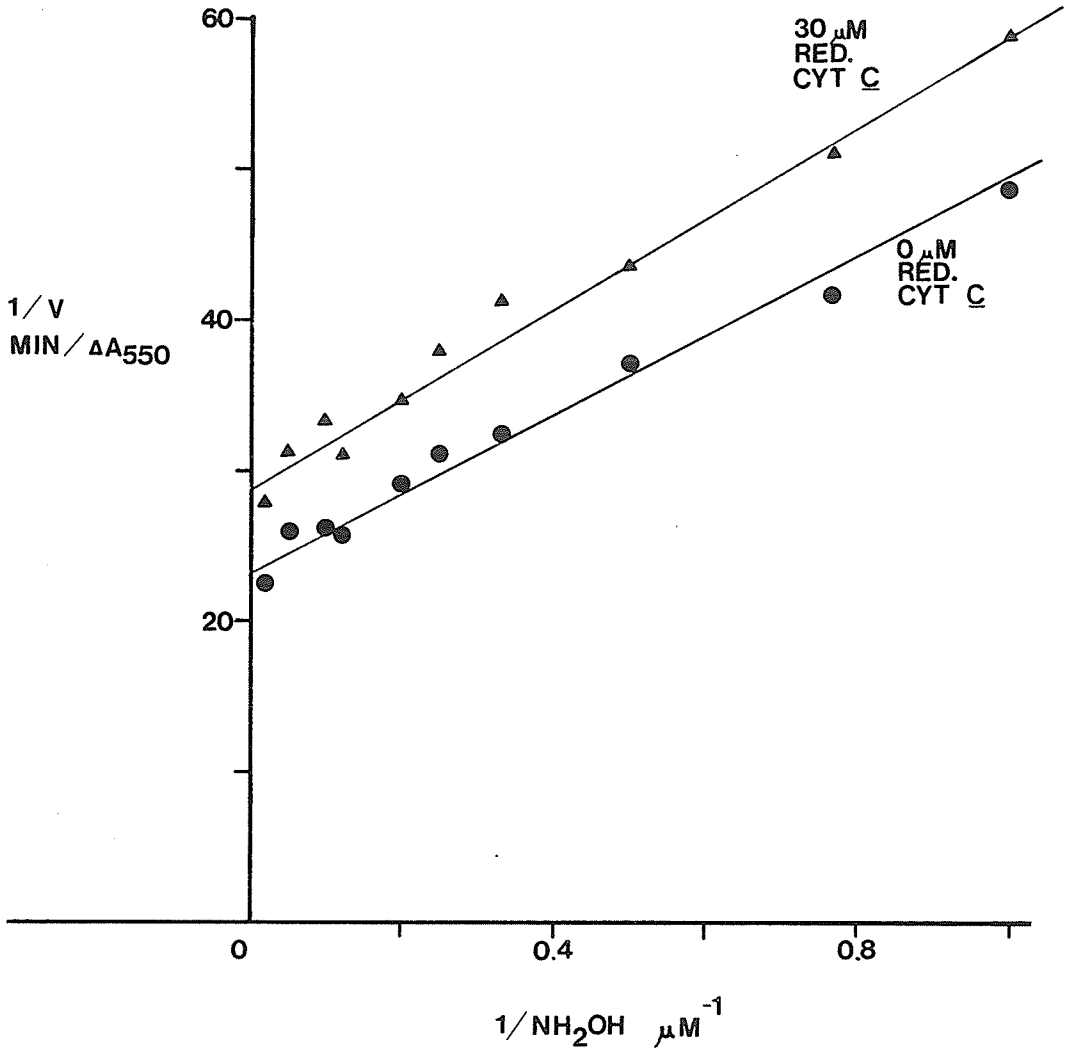

1600
800
SLOPES
0
20
40
REDUCED CYT C μM

Fig. 7c. Double reciprocal plots of initial velocity verus varying hydroxylamine concentrations using reduced horse heart cytochrome c as product inhibitor (30 μM oxidized cytochrome c, pH 7.5, 50 mM potassium phosphate buffer; reaction initiated by addition of enzyme (Preparation A)).


60
40
20
0
0.4
0.8
30 μM
RED.
CYT C
0 μM
RED.
CYT C
1/V
MIN/ΔA550
1/NH2OH μM-1

chemical reoxidation.)

Inhibition by potassium nitrite

Potassium nitrite inhibited in a mixed fashion at the relatively high concentrations of 50 and 100 mM (see figure 8). It should be noted, however, that KCl and KBr at the same concentrations inhibited in a similar fashion (not shown). Much, if not all of the inhibitory effect of KNO_2 may therefore be due to a salt effect.

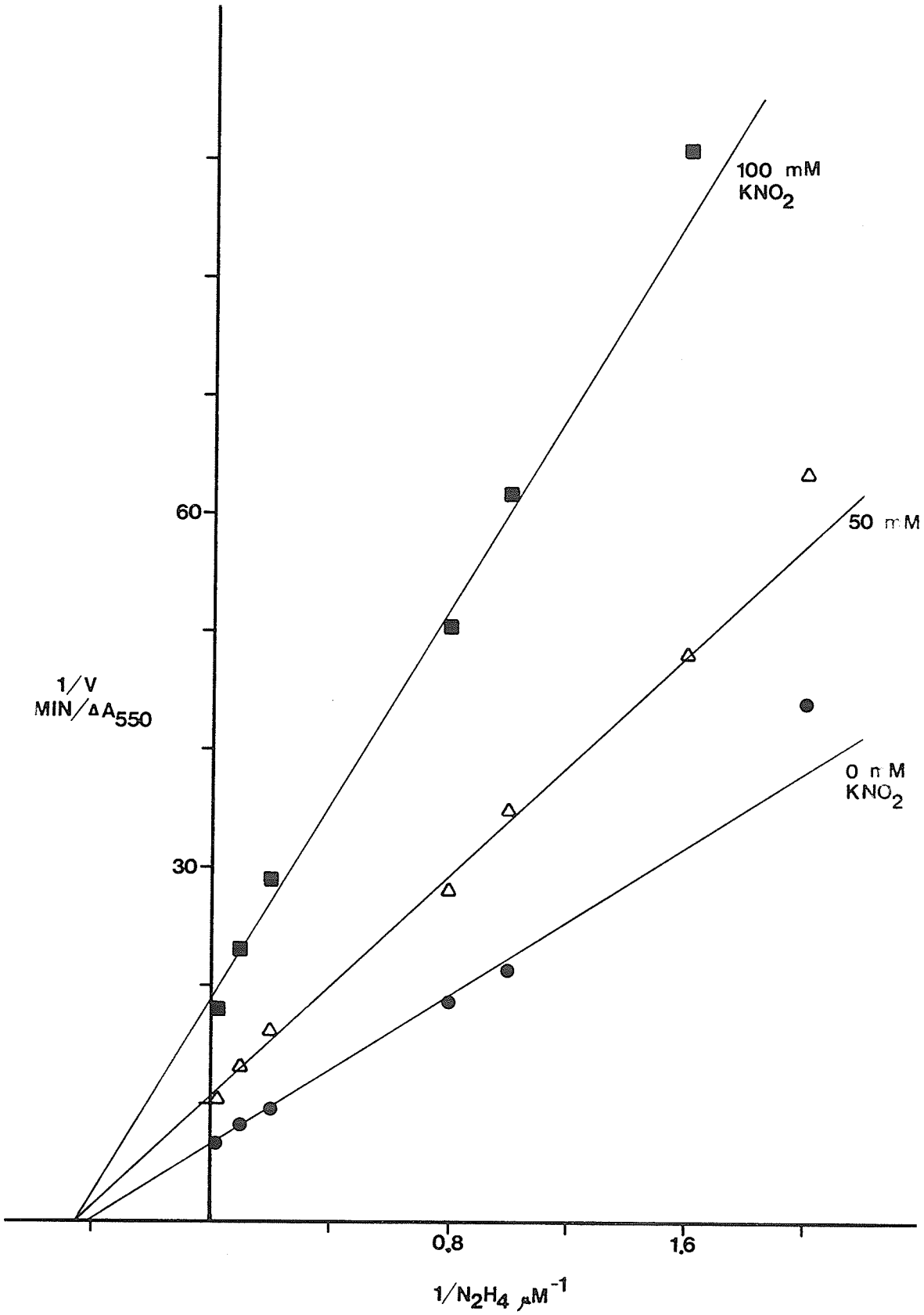
Other inhibitors

The rates of hydroxylamine oxidation at 50 and 2 μM in the presence of 100 μM of various test compounds were studied in order to find competitive inhibitors. Hydroxyurea, imidazole, methanol, sodium thiocyanate, ammonium chloride, potassium cyanate and potassium cyanide were tested. Of these, only potassium cyanide was found to inhibit in a competitive fashion (the others had little, if any, effect on hydroxylamine oxidation at the concentration used).

Inhibition by potassium cyanide

Figure 9a shows the double reciprocal plots of velocity versus hydrazine concentration at various KCN concentrations. KCN inhibited in a mixed fashion; as can be seen from the slopes replot (figure 9c) the degree of

Fig. 8. Double reciprocal plots of initial velocity versus varying hydrazine concentration at 30 μM oxidized cytochrome c and various potassium nitrite concentrations (pH 8.0, 50 μM potassium phosphate, reaction initiated by addition of enzyme (Preparation B).)


100 mM
KNO2
50 mM
0 mM
KNO2
60
30
1/V
MIN/ΔA550
0.8
1.6
1/N2H4 μM-1

inhibition approached a maximal value; this is indicative of partial inhibition.

Partial competitive inhibition occurs when inhibitor (I) and substrate (S) bind at different sites of the enzyme (E), forming ES, EI, and ESI complexes. Both ES and EI yield products, though the latter at a slower rate. As the I concentration approaches infinity, all the enzyme is in the EI form, but velocity, v, can never be driven to zero, since S can combine with EI to form ESI, which gives product (Segal 1975).

Figure 9d can be used to confirm partial competitive inhibition (Segal 1975). One anomaly was noted; at 0.625 µM hydrazine (figure 9d) almost complete inhibition at 100 µM KCN was observed.

The KCN inhibition plots with hydroxylamine as substrate exhibited the same pattern (not shown), except for the previously noted activation at high hydroxylamine concentrations.

Fig. 9a. Double reciprocal plots of initial velocity versus hydrazine concentration at varying potassium cyanide concentration in pH 8.0, 50 mM potassium phosphate buffer. Reaction initiated by addition of enzyme (Preparation B).

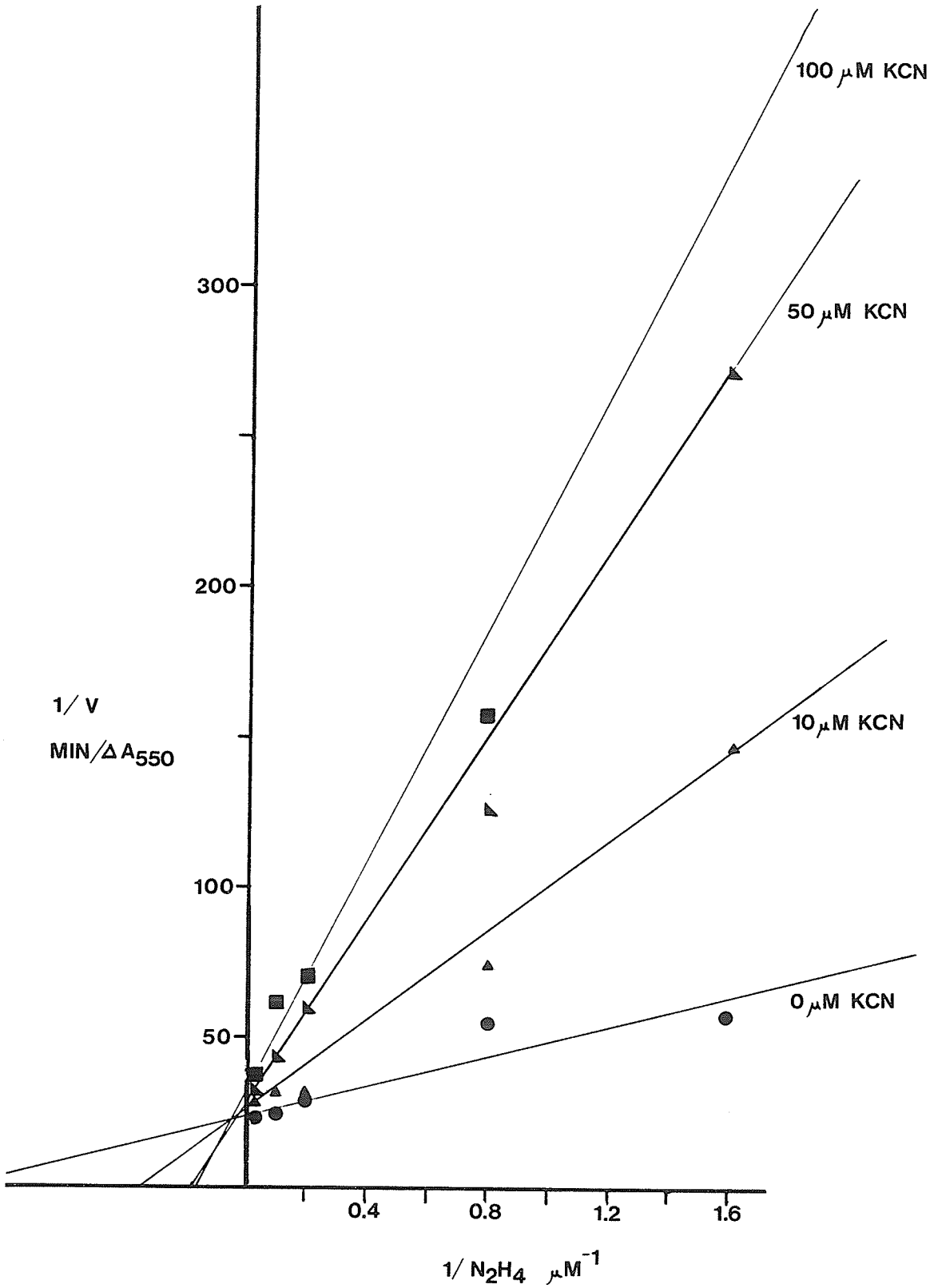

100 μM KCN
50 μM KCN
10 μM KCN
0 μM KCN
300
200
100
50
1/ V
MIN/Δ A550
0.4
0.8
1.2
1.6
1/ N2H4 μM-1

Fig. 9b. Intercepts replot of figure 8a.

Fig. 9c. Slopes replot of figure 8a.

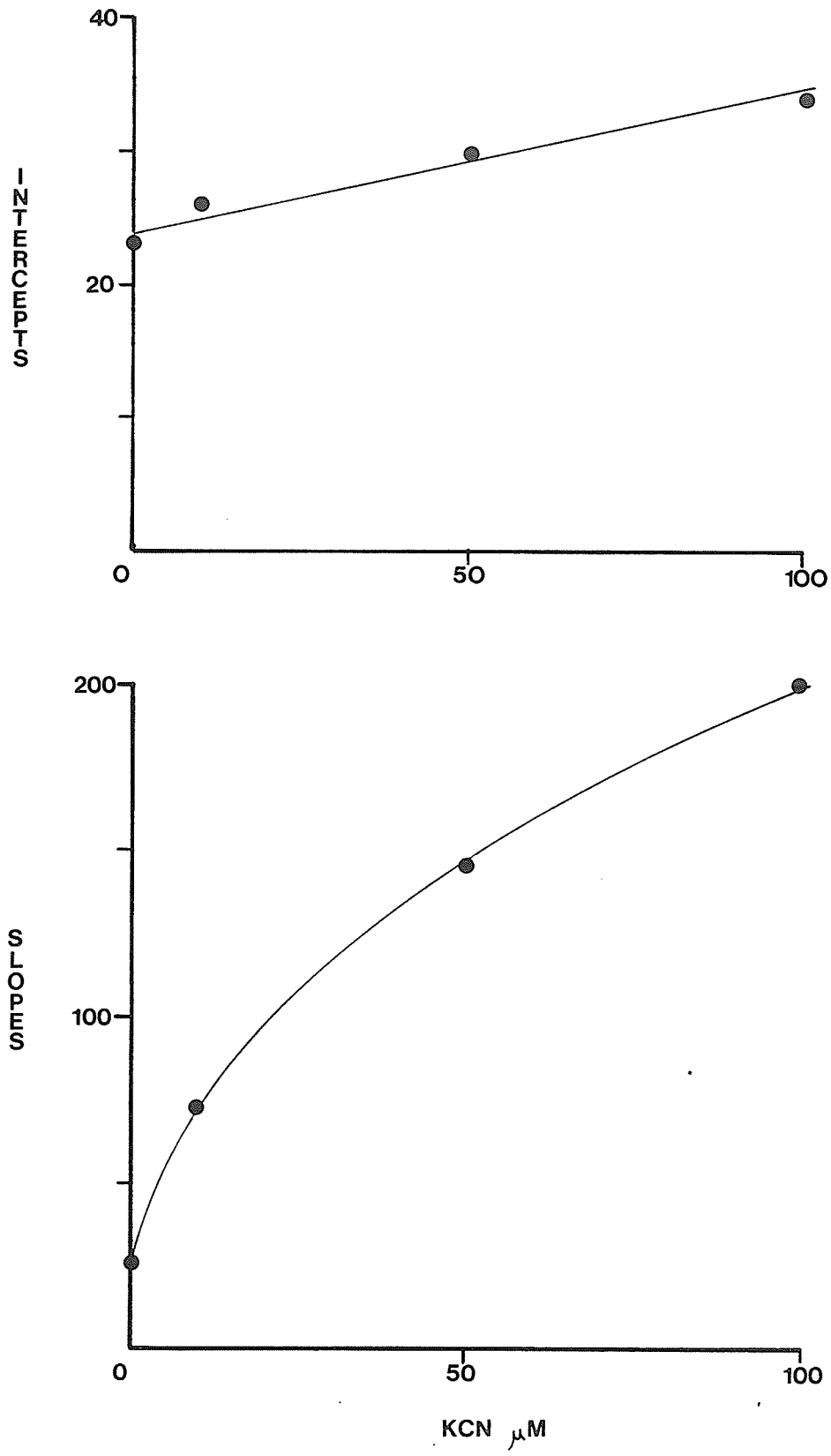
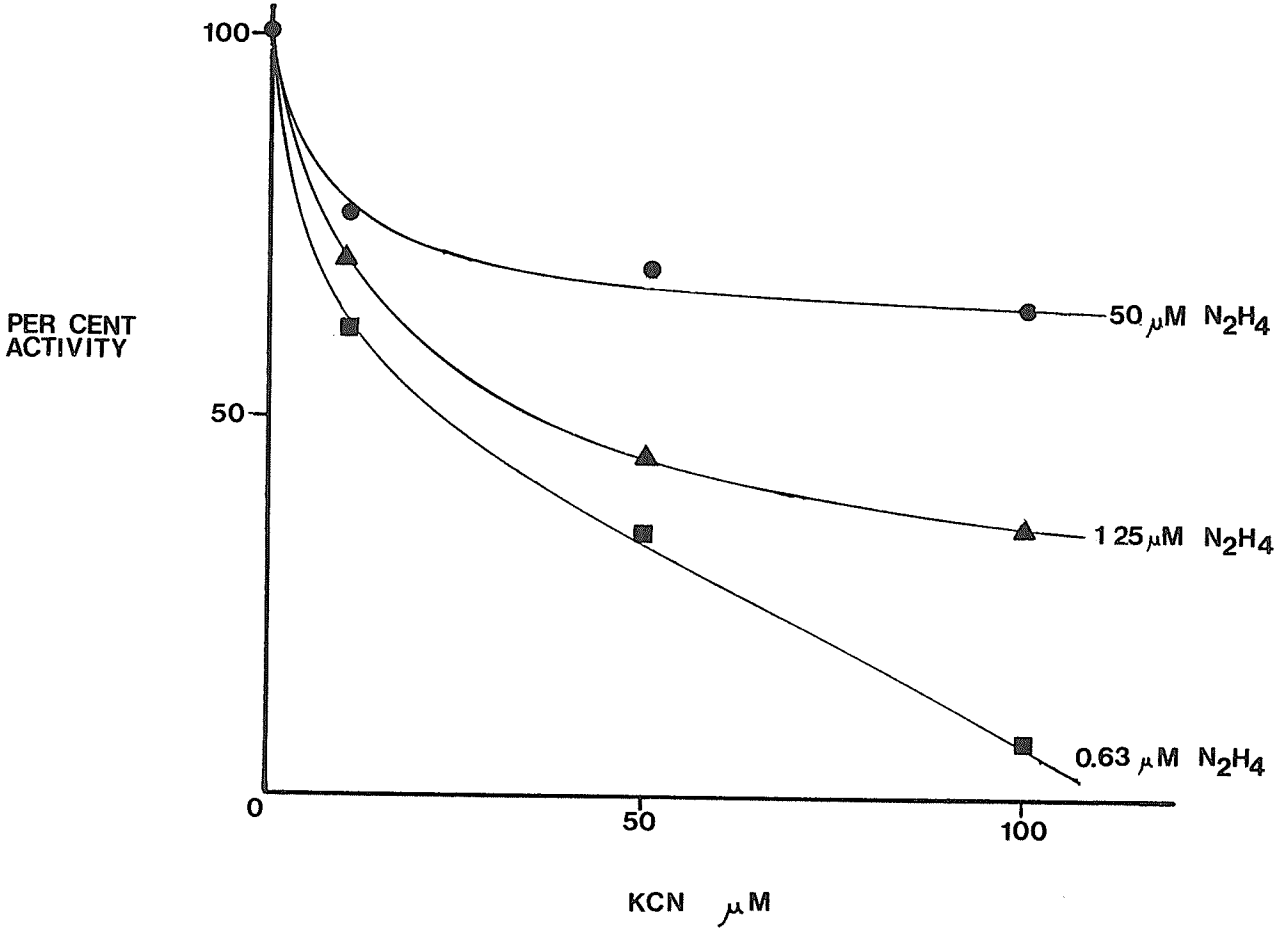

INTERCEPTS
40
20
0
50
100
SLOPES
200
100
0
50
100
KCN μM

Fig. 9d. Plot of percent inhibition against potassium cyanide concentrations at various hydrazine concentrations to confirm partial inhibition (Segal, 1975). (Same data as figure 8a.)


PER CENT
ACTIVITY
100
50
0
50
100
KCN μM
50 μM N2H4
1 25 μM N2H4
0.63 μM N2H4

DISCUSSION

DISCUSSION

A model for the oxidation of hydrazine and hydroxylamine by hydroxylamine oxidoreductase (HAO) will be presented to explain the results obtained here and the work of others. A consistent explanation is possible; it is the nature of this study that conclusive evidence is not possible. In the interests of clarity, the model for the oxidation of hydrazine will be presented first, and the results explained within the context of the model.

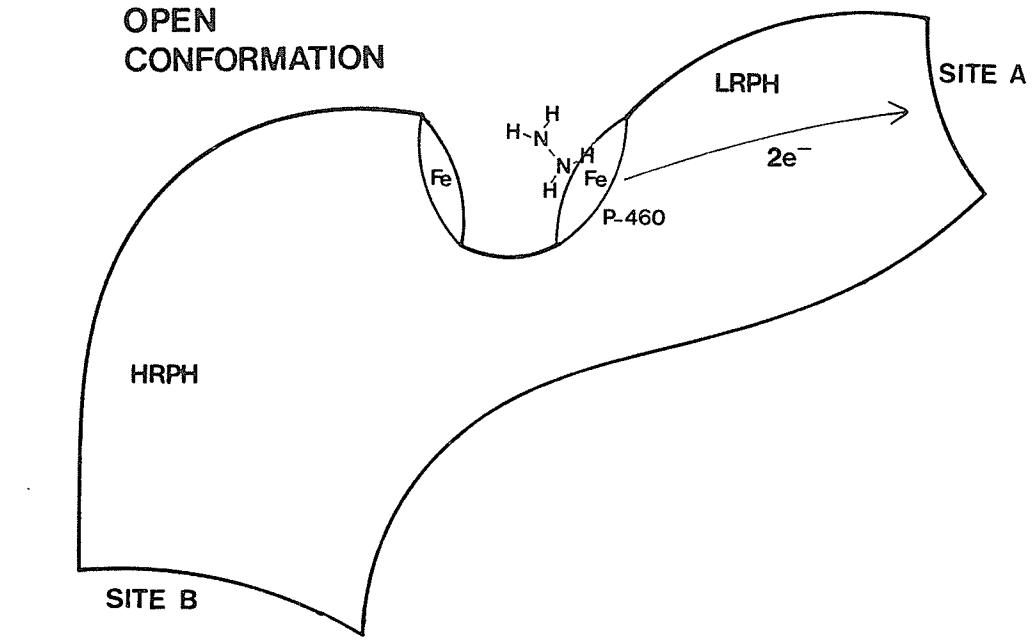
A model for hydrazine oxidation by HAO

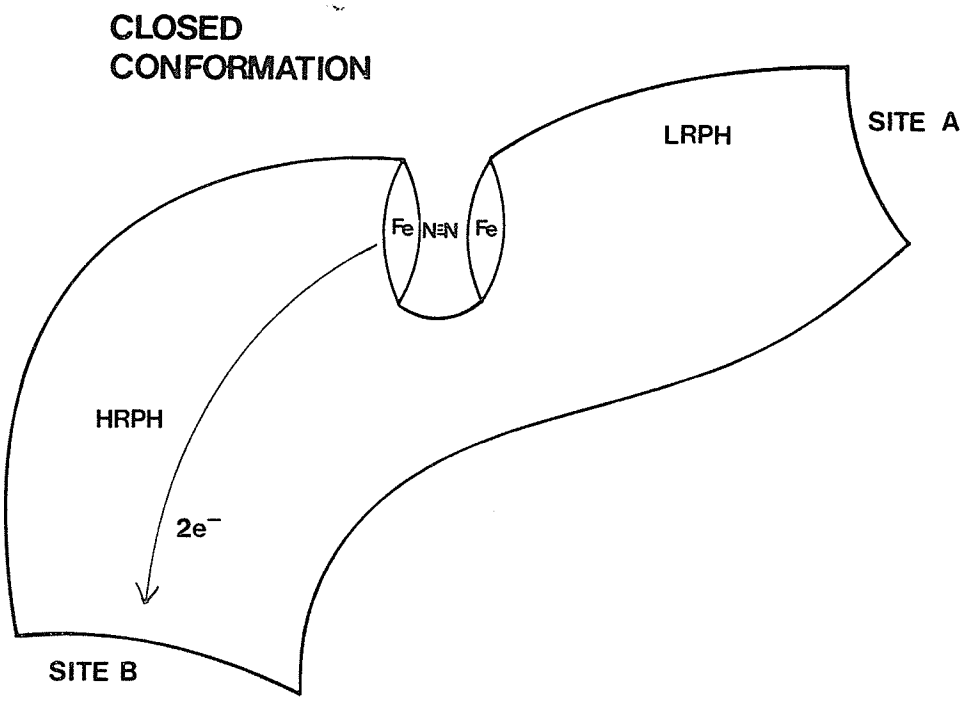
Terry and Hooper (1979) have shown that HAO probably consists of three mono-heme peptides (molecular weight 11,000) and three peptides (molecular weight 74,000) containing six c hemes and one heme P-460 in an $\alpha_3\beta_3$ arrangement. The hemes of HAO have been shown to have redox potentials ranging from +100 mV to -320 mV (Hooper, 1984).

The model is primarily concerned with one functional subunit of HAO; it is assumed there is little, if any interaction between functional subunits. The essential features are (figure 10):

(a) There are two sites of cytochrome c reduction per functional subunit of HAO. Site A (the $K_{m_2}^{cyt}$ and $Vmax_2$ of the Results) is associated with the low redox potential

Fig. 10. A highly schematic diagram depicting one functional subunit of HAO. It is proposed that the initial two electron oxidation of hydrazine occurs via P-460 and the low redox potential hemes (LRPH). These electrons are removed by cytochrome c at Site A ($Vmax_2$ = 0.16 Δ absorbance/min., $K_{m_2}^{cyt}$ = 79/2 μM). The second pair of electrons is removed via the other heme, the high redox potential hemes (HRPH) and Site B for cytochrome c ($Vmax_1$ = 0.075 Δ absorbance/min., $K_{m_1}^{cyt}$ = 4.9/2 μM).


OPEN
CONFORMATION
LRPH
SITE A
H
H
H
N
N
H
H
Fe
Fe
2e⁻
P-460
HRPH
SITE B


CLOSED
CONFORMATION
LRPH
SITE A
Fe
N≡N
Fe
HRPH
2e⁻
SITE B

hemes (LRPH) and P-460. Site B ($K_{m_1}^{cyt}$ and $Vmax_1$) is associated with the high redox potential hemes (HRPH).

(b) The active site for hydrazine (and hydroxylamine) contains two hemes: the P-460 moiety and another heme (denoted the "other heme").

(c) A normal catalytic cycle is proposed to be:

(i) binding of hydrazine to P-460

(ii) two electron oxidation of hydrazine via the LRPH, followed by reduction of two cytochrome c by a Ping Pong sequence at Site A

(iii) removal of two more electrons via the other heme and the HRPH (this may involve a conformational "closure" of the enzyme), followed by the reduction of two cytochrome c by a Ping Pong sequence at Site B.

The results of the initial velocity studies suggest that at least one and possibly four electrons are removed by cytochrome c before the release of N_2. The Bi Hexa Uni Bi sequence is therefore possible. It is proposed that the reaction sequence involves an ordered operation of Sites A and B. (Although the reduced cytochrome c studies may suggest a further complication, below.)

The simple preliminary model of the initial velocity results (equation [4]) gave a reasonable fit at high substrate concentrations, demonstrating the feasibility of a two site model in explaining the results. However, equation [4] consistently predicted faster velocities than

the experimental results, and required a relatively high $K_i^{N_2H_4}$ (12.6 μM) to obtain a fit. Both of these can be attributed to the simple nature of the model.

The system described by equation [4] is the sum of two Bi Hexa Uni Bi (or Bi Uni Uni Bi; compare equations [1b], [2b] and [4]) sequences, independent reactions catalyzed by Sites A and B. However, the real reaction sequence, based on the model in the discussion involves an ordered operation of Sites A and B (see figure 11).

The activities of Sites A and B are obligatorily coupled. Site B cannot function before Site A; Site A does not begin a new sequence until Site B has finished the cycle.

It should be intuitively apparent (and is readily demonstratable) that two individual enzymes will give a faster initial velocity than two sites on one enzyme, operating in an ordered fashion. Qualitatively, Site A of equation [4] needs a high value of $K_i^{N_2H_4}$ to slow the rate down to approximate the activity of the Site A-Site B ordered system. The activity of Site B is also too high, giving rise to faster predicted velocities than those observed. A more accurate representation of the reaction would require the derivation of an equation or the development of an algorithm which would account for the ordered operation of Sites A and B.

The results of the reduced cytochrome c inhibition

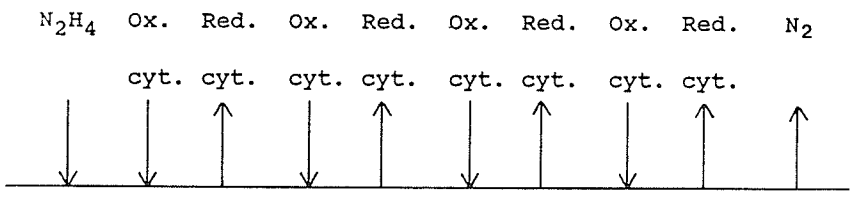
Fig. 11a. The system described by equation [4] is the sum of two independent Bi Hexa Uni Bi activities ("enzyme A" and "enzyme B").

Fig. 11b. The true reaction sequence is suggested to involve an ordered operation of Sites A and B.

ENZYME A

$Vmax_2$ 0.16 ΔA/min

$K_{m_2}^{cyt}$ 79/4 μM

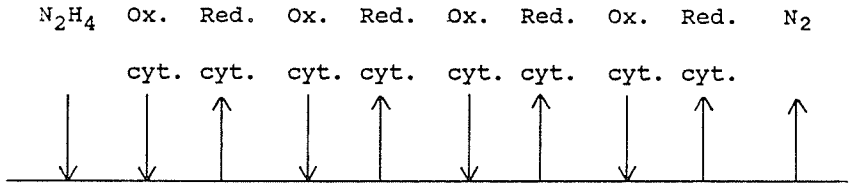


PLUS

ENZYME B

$Vmax_1$ 0.075 ΔA/min

$K_{m_1}^{cyt}$ 4.9/4 μM



SITE A

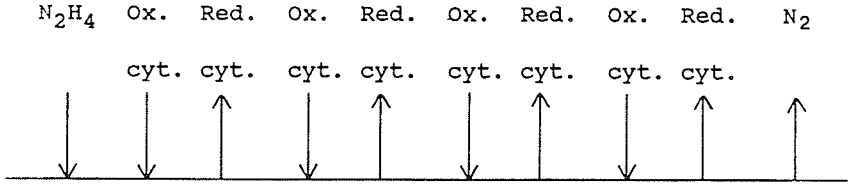
$Vmax_2$ 0.16 ΔA/min

$K_{m_2}^{cyt}$ 79/2 μM

SITE B

$Vmax_1$ 0.075 ΔA/min

$K_{m_1}^{cyt}$ 4.9/2 μM



studies are readily rationalized. The overall inhibition patterns were consistent with two site inhibition. Activation, however, occurred at moderate (~20 μM) reduced cytochrome c concentrations and higher oxidized cytochrome c concentrations. If it is assumed that K_m^{cyt} is essentially the same as K_i^{cyt} as a competitive inhibitor for both sites, and the ratio of reduced cytochrome c/oxidized cytochrome c is constant, then, according to Segal (1975) the highest degree of inhibition is expected at cytochrome c concentrations much higher than K_m^{cyt}. Thus, Site B is inhibited to a greater degree than Site A. (An alternate explanation is that the terminal hemes of the HRPH are partly reduced by cytochrome c, causing blockage of Site B.) If electrons in the HRPH can "short-circuit" to the LRPH when Site B is blocked, removal of electrons via the inherently faster Site A will result in activation at appropriate oxidized cytochrome c concentrations.

Mn (II) (Hooper and Terry, 1979) was found to activate the rate of hydroxylamine and hydrazine oxidation; this may also be explained as a selective inhibition of Site B, allowing short-circuiting to the inherently faster Site A.

If this explanation is correct, it may have significance to the initial velocity studies. Higher concentrations of substrates, such that Site B is operating at its maximal rate, may also allow short-circuiting to Site A, giving an activation.

The mixed, partial inhibition by KCN is consistent with CN^- binding to the other heme. Binding of hydrazine to P-460 is still possible (albeit with a reduced affinity), allowing two electron reduction of cytochrome c (via Site A). Presumably CN^- must dissociate from the other heme before the second pair of electrons can be removed. (If the other heme were not essential for catalysis, one would expect an activation effect similar to that observed for reduced cytochrome c (and presumably Mn (II)).)

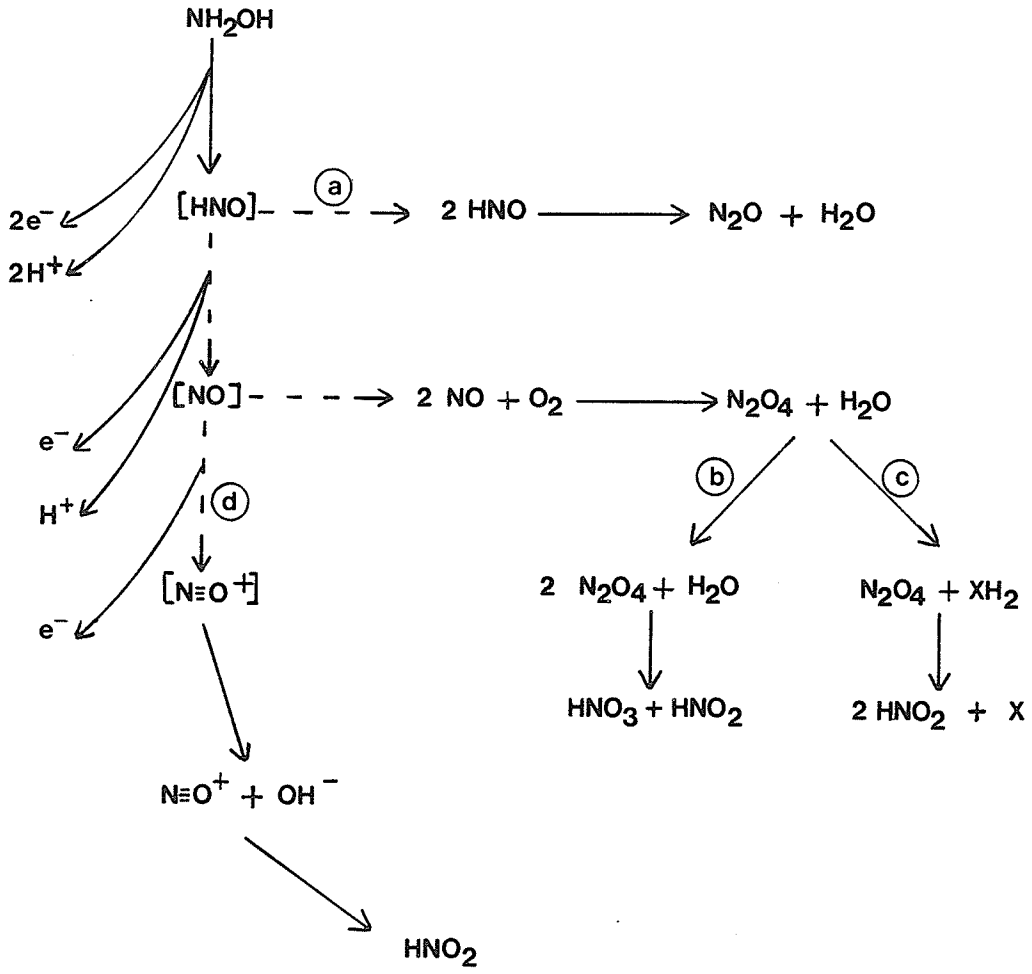
The lack of inhibition by methanol, which is essentially the same size and shape as hydroxylamine, is consistent with transient co-ordination bonds being an essential part of catalysis. The lack of inhibition by OCN^- and SCN^- may be interpreted as being due to steric reasons (ie. size).

A model for hydroxylamine oxidation by HAO

Hydroxylamine oxidation may also be understood in terms of this model.

Hydroxylamine oxidation to nitrite is proposed to be by path d (figure 12) (Hooper, 1984) by essentially the same mechanism proposed for hydrazine oxidation (above). The initial co-ordination to P-460 is probably via the oxygen (Ritchie and Nicholas, 1974, observed partial reduction of HAO by CH_3NHOH, but not by NH_2OCH_3); two electrons are removed via P-460 and Site A, followed by removal of

Fig. 12. Reactions suggested to be involved in the *in vitro* oxidation of hydroxylamine by HAO (these reactions have been previously suggested by Hooper, 1984).


NH2OH
2e−
2H+
[HNO]
a
2 HNO
N2O + H2O
e−
H+
[NO]
2 NO + O2
N2O4 + H2O
b
c
d
[N≡O+]
2 N2O4 + H2O
N2O4 + XH2
e−
HNO3 + HNO2
2 HNO2 + X
N≡O+ + OH−
HNO2

two more electrons via the other heme and Site B. It should be noted that in vivo oxidation of hydroxylamine to NO_2^- is essentially stoichiometric. (Poth and Focht, 1985, demonstrated that the N_2O production associated with cultures of Nitrosomonas is due to denitrification of NO_2^-, not decomposition of a labile intermediate of ammonia oxidation.)

Depending on the conditions, in vitro oxidation of hydroxylamine by HAO can yield N_2O, NO, NO_2^- and NO_3^-. Current evidence suggests that the scheme in figure 12 is correct (this scheme has been presented in part or whole previously by Hooper, 1984; Suzuki, 1974; and Lees, 1953). The following discussion will be conducted within the framework of figure 12.

In vitro, it is suggested that:

1. Oxidation of hydroxylamine starts with the removal of two electrons to form [HNO] and does not normally proceed past the NO level, due to the facile NO-O_2 reaction (Cotton and Wilkinson, 1981) and the possible difficulty of NO oxidation (Hyman and Wood, 1984b).

In the model, two electrons are removed via P-460 and Site A, one more electron is removed via the other heme followed by various reactions of NO or derivatives (paths b or c in figure 12).

2. The chief parameter determining the products of hydroxylamine oxidation is the hydroxylamine

concentration. Although the oxidant is also significant (Yamanaka and Sakano, 1980) it is suggested to be of secondary importance, probably only in determining whether path b or c is favored.

3. Conditions which block Site B or the other heme promote path a (figure 12) by hindering further oxidation of [HNO]. The major reaction catalyzed under these circumstances is the oxidation of hydroxylamine to [HNO] using P-460 and Site A. Because of the inherently higher Vmax of Site A, the often observed activation is understandable.

4. At hydroxylamine concentrations of 10 μM or more, dimerization reactions are promoted; depending on the conditions path a or b may be favored. Intermediates of hydroxylamine oxidation (NO or HNO) act as partial competitive inhibitors with respect to hydroxylamine oxidation, similar to the mechanism proposed for CN^- inhibition (above). That is, after the first two electrons are removed from a hydroxylamine molecule, and HNO (NO^-) has co-ordinated to the other heme, a second hydroxylamine molecule may bind to P-460 and be partially oxidized. Depending on the conditions (eg. whether or not Site B is blocked or is kinetically incompetent to keep up with the rate of the P-460-Site A reaction) either path a or b (figure 12) may be favored.

The overall reaction sequence is of a Bi $(Uni)_n$ Bi

type as suggested for hydrazine; however, the number of electrons (and hence the number of cytochrome molecules involved) can vary.

A rationale for the results of Table 1 is therefore possible. At low hydroxylamine concentrations (3 μM) oxidation to the NO (and possibly to the $N{\equiv}O^+$) level occurs (paths c and d). As the hydroxylamine concentration increases, (~10 uM) Site B is still capable of removing electrons fast enough to keep up with Site A, however, dimerization of NO ($+O_2$) is becoming more significant (path b). (The hydroxylamine concentration is becoming high enough to allow a second hydroxylamine molecule to bind to P-460 while the other heme is still occupied.) At still higher hydroxylamine concentrations, (≥50 μM) Site B is no longer kinetically competent to keep up with Site A, and the hydroxylamine concentration is high enough to overcome the partial competitive inhibition by oxidized intermediates. Thus path a is the predominant reaction.

Cyanide affected the NO_2^- yield at all hydroxylamine concentrations; this is consistent with binding to the other heme and inhibiting HNO oxidation. By acting as a partial competitive inhibitor, the initial rate of oxidation is inhibited at lower hydroxylamine concentrations. As the hydroxylamine concentration is raised (≥50 μM), it becomes appreciably higher than the CN^- inhibited Km for hydroxylamine, so the dimerization

reaction to N_2O is promoted (path a). Since this involves P-460 and Site A with its high Vmax, the activation observed is understandable.

Mn (II) and reduced cytochrome c (as with hydrazine) function by blocking Site B. In addition to the previously mentioned activation of the rates of hydrazine and hydroxylamine oxidation, Hooper and Terry (1979) also observed a great increase in the amount of N_2O formed in the presence of Mn (II).

The behaviour of hydrazine and hydroxylamine is believed to be different. The stability of N_2, the product of hydrazine oxidation, drives the reaction to completion, "forcing" the previously suggested short-circuiting to occur. The lability of HNO, however, allows the dimerization reaction (path a) to occur.

Potassium nitrite has a very high Ki, if it is capable of inhibiting the reaction at all. If the end product of hydroxylamine oxidation is $N\equiv O^+$, this is understandable. Under aqueous conditions, $N\equiv O^+$ is totally hydrolyzed to NO_2^-, which will have a different affinity and/or may be too large to effectively access the active site.

Yamanaka and Sakano (1980) reported essentially stoichiometric production of NO_2^- from hydroxylamine; optimal conditions were in 50 mM potassium phosphate buffer pH 8.0, and when the oxidant:hydroxylamine ratio exceeded 4. It is notable that in their results, only relatively

low (10 μM) concentrations of hydroxylamine gave stoichiometric yields. Other than cytochrome c-554 of the organism, they also used horse heart cytochrome c and potassium ferricyanide as oxidants. It is suggested that the presence of the high oxidant concentration may be to provide a high reductant concentration after the initial oxidation to NO occurs, favoring path c over path b (figure 12). Anaerobic formation of NO_2^- is interpreted as being due to path d (figure 12).

If the overall model is essentially correct, the relevance to the behaviour of HAO and the overall mechanism of ammonia oxidation *in vivo* is not certain:

(i) Horse heart cytochrome c is not the natural oxidant for HAO (cytochrome c-554 of the organism seems to be the natural oxidant (Yamanaka and Shinra, 1974; Tsang and Suzuki, 1982).

(ii) Ammonia, not hydroxylamine is the natural substrate for the organism.

(iii) It seems likely that HAO is closely associated with the membrane in whole cells or membrane preparations with ammonia oxidizing activity.

The model is capable of providing a consistent explanation for the *in vitro* behaviour of HAO; however, the results presented here are not conclusive. Further study, to confirm or reject this model and to elucidate the behaviour and role of HAO in ammonia oxidation is necessary.

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