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# THE CATALYTIC ROLE OF SULFHYDRYL GROUP(S) IN THE MECHANISM OF NUCLEOSIDE TRIPHOSPHATE PYROPHOSPHOHYDROLASE ACTIVITY

By

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ABSTRACT

THE CATALYTIC ROLE OF SULFHYDRYL GROUP(S) IN THE MECHANISM OF NUCLEOSIDE TRIPHOSPHATE PYROPHOSPHOHYDROLASE ACTIVITY

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The sulfhydryl requirement of the enzyme nucleoside triphosphate pyrophosphohydrolase was investigated. Dithiothreitol and EDTA enhanced enzyme activity 2.3 fold and 1.5 fold, respectively. N-ethylmaleimide completely inactivates the enzyme, demonstrating pseudo 1st-order rate constants for the inhibition reaction. A second order rate constant of $9.08 \times 10^{-5}$ $M^{-1}$ $min^{-1}$ for the overall process of sulfhydryl derivitization was obtained. The presence of either $MgCl_2$ or ITP enhances the NEM reaction, while both $MgCl_2$ and ITP together decrease the rate of enzyme inactivation by NEM. Computer analysis of the concentrations of the various metal-nucleotide complexes present during the inhibition reaction revealed that

$MgITP^{2-}$ effects up to 70% protection from the effects of N-ethyl-maleimide. These results are consistent with our previous work, and indicate that the thiols are located at or near the active site of this enzyme.

To Eddie C., Jeanne, Chris, Becky, Jan,

and my good friend, Jefalan

"Nothing great was ever achieved without enthusiasm."

- R.W. Emerson

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| DTT | Dithiothreitol |
| EDTA | Ethylindiamine Tetraacetic Acid |
| pHMB | p-Hydroxymercuribenzoate |
| IAA | Iodoacetamide |
| ITP | Inosine-5'-Triphosphate |
| NEM | N-ethylmaleimide |
| NMP | Nucleoside Monophosphate |
| NTP | Nucleoside Triphosphate |
| NTPH | Nucleoside Triphosphate Pyrophosphohydrolase |
| Km | Michaelis Consant for the Binding of Substrate to Enzyme |
| Ks | Dissociation Constant for the Binding of Substrate to Enzyme |

For the Reaction:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k} EP \overset{k_2}{\rightleftharpoons} E + P$$

$$Km = \frac{k_2 + k_{-1}}{k_1} = \frac{k_2}{k_1} + \frac{k_{-1}}{k_1} = K_D + \frac{k2}{k_1}.$$

Under the condition that $k_2 \lll k_{-1}$, Km = Ks.

## INTRODUCTION

Prior investigations in this laboratory of *in vitro* globin synthesis by rabbit reticulocytes led to the observation that a GTP hydrolase activity was present in these lysates. This activity was subsequently characterized as being a nucleoside triphosphate pyrophosphohydrolase (NTPH). This enzyme catalyzes the following reaction:

$$NTP + H_2O \rightleftharpoons NMP + PP_i$$

where NTP and NMP refer to nucleoside triphosphate and monophosphate, respectively (1).

Since its initial discovery, the enzyme has been partially purified from the rabbit reticulocyte and liver, and from human erythrocytes. All three forms have been extensively characterized, showing the same basic properties (1,2,3). The best substrates for NTPH are ITP, dITP, and XTP. GTP was hydrolyzed at 3% of the rate of ITP, with ATP, GDP, and IMP not being hydrolyzed at all. The enzyme requires 10 mM $MgCl_2$, a pH of 9.75, and mM concentrations of a sulfhydryl reducing reagent for maximum activity. Its molecular weight has been estimated to be 37,000 daltons by sucrose density gradient sedimentation. NTPH from human erythrocytes exhibits an apparent Km value of $3.82 \times 10^{-3}$ M for GTP and $2.3 \times 10^{-5}$ M for ITP (3).

The enzyme from human red cells also exhibits a lower degree of stability than do other forms, a problem which has made its purification to homogeneity extremely difficult. Various studies have yielded conflicting reports of its requirement for a reduced thiol(s) (1,2,3,4,5).

In recent work by B.S. Vanderheiden, 2000 fold purified NTPH did not require sulfhydryl compounds for activity or stability (4). Studies by Holmes et al., however, have shown the opposite to be true (5). All work done to date in the laboratory of Dr. Morris has shown that NTPH in crude erythrocyte lysates or in partially purified form does have a sulfhydryl requirement for stability and activity. This was best demonstrated by S. Fuller, who recently conducted his Ph.D. thesis research in this laboratory. His work confirmed that sulfhydryl compounds, particularly DTT, were required for maximal NTPH activity and for stability in purified enzyme preparations (55). Moreover, N-ethylmaleimide (NEM), iodoacetamide (IAA), and p-hydroxymercuribenzoate (pHMB), were shown to inhibit NTPH activity in lysates and in purified enzyme preparations. NEM and pHMB, at 50 mM and 10 mM concentrations, respectively, resulted in complete loss of enzyme activity, while the presence of 50 mM IAA resulted in 71% inhibition. Work by L. McKenzie, also in this laboratory, has further demonstrated that EDTA decreased the rate of inactivation of NTPH in the absence of sulfhydryl compounds. These results, and the fact that instability had impeded previous efforts to purify this enzyme to homogeneity, led us to reexamine the effects, upon NTPH, of DTT, EDTA, and N-ethylmaleimide.

Reactivation studies were performed, using an enzyme preparation which had been desalted on Sephadex G-25 to completely remove DTT

present in the storage buffer. Subsequent assay in the presence of either DTT, or EDTA, or both reagents together, showed DTT to effect a 2.3 fold increase in enzyme stability over that exhibited in the absence of DTT. Moreover, assays conducted in EDTA alone enhanced the enzyme stability by 1.5 fold. Similar studies of enzyme which has been both desalted and assayed in the presence of EDTA show the enzyme to exhibit a 2.5 fold increase in stability over that seen by NTPH preparations which have been both desalted and assayed in the absence of either DTT or EDTA.

The sulfhydryl requirement(s) of NTPH was further investigated by incubation with N-ethylmaleimide which, at greater than 0.5 mM concentrations, completely inactivates the enzyme. Pseudo 1st order rate constants were obtained for this inhibition reaction, and yielded a 2nd order rate constant of $9.08 \times 10^{-5}$ % $M^{-1}$ $min^{-1}$ for the overall process of sulfhydryl derivatization.

The effects of magnesium, ITP, and their metal-nucleotide complexes, upon the reactivity of the sulfhydryl groups in NTPH was also investigated. In the presence of 1 and 10 mM $MgCl_2$, the rate of the reaction between NTPH and NEM is enhanced 10% and 60%, respectively. A similar effect is observed in the presence of ITP, with 0.5 mM concentrations of nucleotide demonstrating a 3-fold enhancement of enzyme inhibition. The presence of both $MgCl_2$ and ITP, conversely, results in a 5-fold decrease in the velocity of the inhibition reaction. In the latter instance, computer calculation of the concentrations of the various metal and nucleotide species present during the inhibition reaction revealed that $MgITP^{2-}$ effects up to 70% protection from the effects of N-ethylmaleimide. At 5 and 10 mM

$MgCl_2$, this protection decreases, as an apparent function of both decreasing concentrations of $MgITP^{2-}$ and increasing concentrations of free $Mg^{++}$.

These results are consistent with our previous work, which showed that NTPH does, indeed, require one or more reduced thiols for maximum stability and activity. Metal catalyzed oxidation of the(se) sulfhydryl group(s) may occur, since EDTA prevents inactivation of the enzyme. While the number and location of these groups is, as yet, undefined, the protection shown by substrate $MgITP^{2-}$ implies that they are located at or near the active site.

## LITERATURE REVIEW

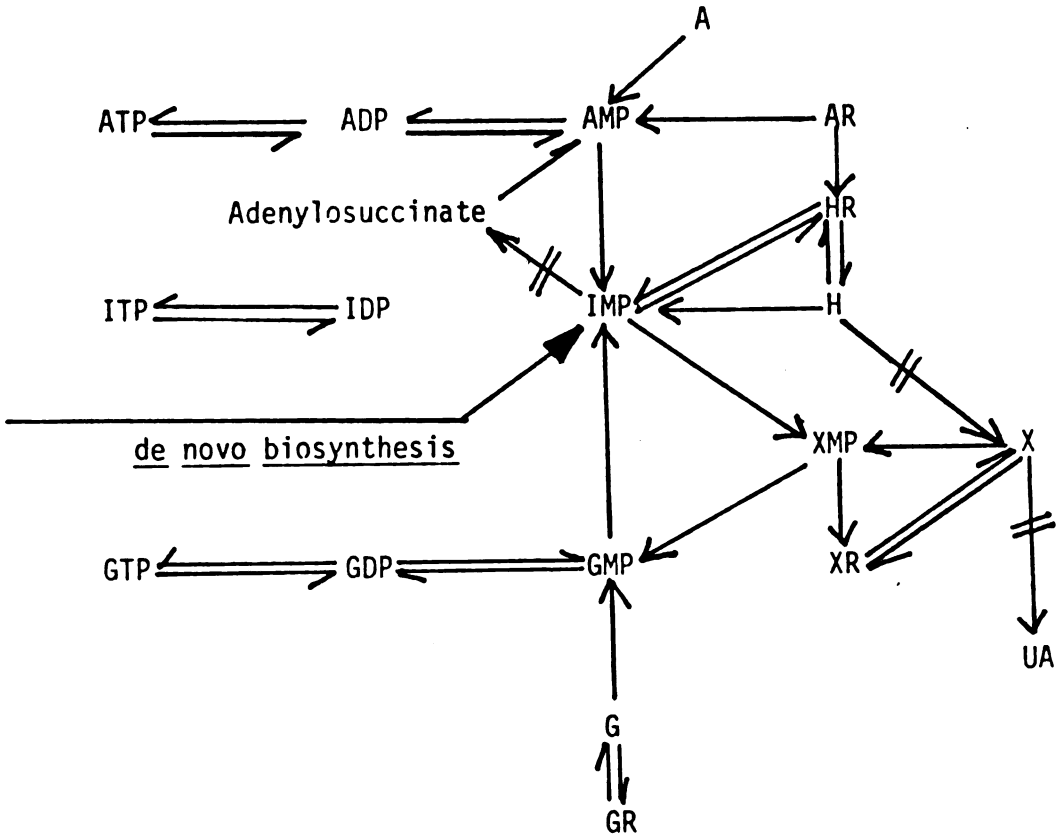
### A. Metabolic Turnover of Preformed Purines in the Red Blood Cell

Human erythrocytes lack three enzymes of purine metabolism usually found in other tissues: phosphoribosylpyrophosphate amidotransferase, adenylosuccinate synthetase and xanthine oxidase (6,7,8,9). Therefore, erythrocytes cannot synthesize purines *de novo*, AMP from IMP, or oxidize hypoxanthine to xanthine or uric acid, and the red cells must depend on salvage and transport mechanisms to maintain their proper levels of nucleotides. Two reviews by Murray give a good background to these mechanisms by emphasizing the significance of the salvage pathways and the regulatory controls of nucleotide biosynthesis from preformed purines (10,11).

Free purines or nucleosides normally are not found in the cell but exist in the form of nucleotides. Anion exchange chromatography of acid extracts of fresh erythrocytes indicates that adenine is present as AMP (13-60 μM), ADP (100-270 μM) or ADP (0.85-1.7 μM) while guanine exists as GDP (20 μM) or GTP (10-35 μM) and hypoxanthine as IMP (0-50 μM) and ITP (0-183 μM) (12,13,14,15,16,17).

*In vitro* experiments with rabbit erythrocytes indicates that adenine is primarily used for adenine nucleotide synthesis, while xanthine, guanine and guanosine are precursors of guanine nucleotides,

Figure 1. Interconversions of Purine Nucleotides in the Red Blood Cell. The following abbreviations are used in this figure: A, AR, AMP, ADP and ATP refer to adenine, adenosine, adenosine mono-, di-, and triphosphate, respectively; H, HR, IMP, IDP and ITP refer to hypoxanthine, inosine, inosine mono-, di-, and triphosphate, respectively; UA, X, XR and XMP refer to uric acid, xanthine, xanthosine and xanthosine monphosphate, respectively; and G, GR, GMP, GDP and GTP refer to guanine, guanosine, guanosine mono-, di-, and triphosphate, respectively.


A
ATP
ADP
AMP
AR
Adenylosuccinate
HR
ITP
IDP
IMP
H
de novo biosynthesis
XMP
X
GTP
GDP
GMP
XR
UA
G
GR

and, unlike human erythrocytes, hypoxanthine, inosine and adenosine can be converted to both guanine and adenine nucleotides (18). Combining the evidence derived from both rabbit and human erythrocytes and taking into account the enzyme deficiencies mentioned above, Figure 1 describes the interconversions of purines in the human red blood cell (19). As may be seen in the figure, purines synthesized de novo enter the scheme at the level of IMP (20). Thus, biosynthesis and catabolism of purines is accomplished by controlled and defined pathways with IMP playing a key intermediary role.

B. ITP Metabolism

1. Synthesis and Natural Occurance of ITP

The synthesis and role of ITP in the cell have not been clearly defined and remain the subject of investigation. E. coli, pig kidney extracts, and calf-liver extracts have all been found to lack IMP kinase activity (21,22,23,24). The natural occurance of inosine polyphosphates also remains an open question. The early literature has reports of ITP being present in extracts of frog muscle (25), rabbit muscle (26), guinea pig liver and skeletal muscle (27), and rat liver mitochondria (28). Vanderheiden has presented evidence that ITP may normally be present in human red cells (16,17). In the latter case, however, the quantitative analysis performed may not have given accurate estimates of endogenous ITP concentrations in red cells. This in turn yielded considerable variation in his results.

2. Accumulation of $^{14}C$ ITP in Erythrocytes

While the synthesis and intracellular concentrations of ITP remain, as stated, open questions, studies of the long and short-term effects of whole blood storage and incubation have shown that, under

certain incubation conditions, erythrocytes can accumulate high levels of ITP (29,30,31,32,33). This phenomenon has also been examined in fresh erythrocytes. Of particular note are those studies investigating the possible relationship(s) between $^{14}C$ ITP accumulation and various disease conditions, levels of NTPH activity, or conditions of incubation.

In 1975, investigators in J.F. Hendersons' laboratory studied the variation in $^{14}C$-ITP accumulation from $^{14}C$-hypoxanthine in a control and mentally retarded population (34). They suggested that there are two classes of individuals, those whose red cells could accumulate 0 to 70 nmoles $^{14}C$-ITP/$10^{10}$ cells, and those whose cells could accumulate 70-240 nmoles $^{14}C$-ITP/$10^{10}$ cells during two hours of incubation. The data show that 5% of the control population (80 individuals) compared to 15% of a mentally retarded population (100 individuals) could be classified in the high category. Later work by Henderson and Morris revealed that several factors can increase the accumulation of inosine nucleotides, including levels of PRPP, type of incubation medium, and period of exposure to the medium (35).

In a collaborative effort between our laboratory and that of Hendersons' laboratory, an investigation was made of a possible relationship between $^{14}C$-ITP accumulation from $^{14}C$-hypoxanthine and NTPH activity (36). This study revealed that there did, indeed, appear to be a relationship between NTPH activity and $^{14}C$-ITP accumulation and that this relationship is comprised of a continuum of values. It was further shown that when this accumulation is defined as a function of NTPH activity the results follow closely the theoretical relationship between enzyme activity and steady-state substrate

concentration as calculated from the Michaelis-Menton equation. The fact that the relationship between NTPH activity and $^{14}$C-ITP accumulation from $^{14}$C-hypoxanthine is characterized by a continuum of values was in apparent contradiction to earlier work by B.S. Vanderheiden (37). He suggested that the relationship between "ITP-ase" activity and accumulation of $^{14}$C-ITP from $^{14}$C-IMP shows 3 predominant groups of people. In this work, however, Vanderheiden was measuring a coupled reaction between NTPH and endogenous red cell inorganic pyrophosphatase activity; hence, variations in either NTPH or red cell inorganic pyrophosphatase activity could have accounted for his observations.

In another report of ITP accumulation, Nelson et al. observed that cells incubated for 24 hrs in the presence of 0.5 mM hypoxanthine or inosine, 25 mM glucose, and 50 mM phosphate accumulated 0.4 mM ITP (38). However, some kinetic differences existed between the accumulation of ITP from hypoxanthine and the accumulation from inosine. In the presence of hypoxanthine, the intracellular level of IMP had stabilized at 12 hours, but ITP accumulation was linear through 24 hours. On the other hand, the presence of inosine caused an initial burst of both IMP and ITP synthesis, followed by linear accumulation of each. These authors do not offer any explanation for the kinetic differences exhibited by these two precursors, but suggest that their results provide evidence that ITP is synthesized by an "IMP kinase" coupled to NDP kinase.

Thus, it can be seen that, under the proper conditions, red cells have mechanisms with which to accumulate $^{14}$C-ITP. An apparent relationship also exists between this ability to accumulate $^{14}$C-ITP

and the levels of its degradative enzyme, NTPH.

3. Degradation of ITP

ITP may be substituted for ATP in reactions catalyzed by a wide variety of enzymes. For example, ITP can substitute for ATP as phosphoryl donor in many NMP and NDP kinase reactions (39,40), and may be used by phosphofructokinase (41), phosphoenolpyruvate carboxykinase (42), succinate thiokinase (43), pyruvate kinase (44), and glucose-6--phosphatase (45). Yeast inorganic pyrophosphatase will also hydrolyze ITP in the presence of $Zn^{++}$ (46). ITP hydrolysis in red cells occurs by means of the pyrophosphohydrolase, NTPH, yielding IMP and pyrophosphate (1,2). Because this thesis deals with the properties of NTPH, they will now be discussed in some detail.

NTPH was originally purified from rabbit reticulocytes and extensively characterized by Chern, MacDonald and Morris in 1969 (1). They achieved a 2000 fold level of purification from rabbit reticulocyte lysates, and demonstrated that the enzyme catalyzed a pyrophosphate hydrolysis, had a pH optimum of 9.75, and required both 10 mM $MgCl_2$ and 1 mM sulfhydryl reagent for maximum activity. High concentrations of either ITP (1 mM), the monovalent cations KCl, NACl, and $NH_4Cl$ (70 mM), or DTT (6 mM) were found to inhibit the enzyme in varying degrees. Of the divalent cations tested ($CaCl_2$, $FeCl_2$, $ZnCl_2$, $CuCl_2$ and $MnCl_2$), $Mn^{++}$ alone resulted in small amounts of activity.

Studies of the substrate specificity revealed that ITP and dITP are most effectively degraded, with XTP being degraded at 76% of the rate shown for ITP. GTP, dGTP, UTP and dUTP were hydrolyzed at only 10% of the rate shown for ITP, while ATP, dATP, CTP and dCTP did not serve as substrates. Nucleoside mono- or diphosphates were not

hydrolyzed by the pyrophosphohydrolase. These and other studies showed NTPH activity to be the result of a single enzyme molecule with broad specificity.

Inhibition studies which investigated the effect of nucleotide derivatives upon NTPH revealed that IDP exerted the most potent inhibition. Other nucleotide derivatives exerted levels of inhibition ranging from 1% ($10^{-4}$ M adenosine) to 25% ($10^{-3}$ M ATP) (1).

These same investigators determined that the apparent Michaelis constants (Km) were $3.37 \times 10^{-5}$ M for ITP and $4 \times 10^{-4}$ M for GTP. Molecular weight determination using sucrose density gradient centrifugation yielded a value of 37,000 daltons. The pI of NTPH was determined to be 4.3.

Wang and Morris subsequently purified NTPH from rabbit liver (2). The substrate specificity, pH optimum, electrophoretic mobility in polyacrylamid gels, and apparent molecular weight of this enzyme were essentially identical to those of rabbit reticulocyte NTPH. These workers also showed the enzyme to be located in the cytosol, results have since been confirmed by Hirschhorn, et al., in studies on human erythrocyte NTPH (5).

Subsequent 1200-fold purification of human erythrocyte NTPH (3) revealed its properties to be quite similar to those of the rabbit. Certain differences do exist, however, including an apparent Km value of $3.82 \times 10^{-3}$ M for GTP; that for ITP was relatively unchanged at $2.3 \times 10^{-5}$ M. The human form of NTPH also exhibits an apparently lower degree of stability than do other forms, a problem which has made purification to homogeneity (and beyond a 2500-fold level of purification) extremely difficult (unpublished results). A recent

report by Vanderheiden has stated that NTPH which was purified 2000-fold from human red cells exhibited no sulfhydryl requirement for enzyme activity or stability (4). The purification steps, however, were all carried out in the presence of mM 2-mercaptoethanol. Studies by Hirschhorn, et al., have, however, shown the opposite to be true (5). Stephen Fuller, who recently conducted his Ph.D. thesis research in this laboratory, clearly demonstrated that sulfhydryl compounds are required for stability and maximal activity of NTPH (55). Evidence to be presented here further confirms his work, and provides, as well, some additional information concerning the nature of this sulfhydryl requirement.

NTPH analysis has been performed by a coupled enzyme assay which includes yeast inorganic pyrophosphatase to hydrolyze the pyrophosphate produced by NTPH followed by colorimetric analysis of the orthophosphate (1,47,48). Vanderheiden has described a microassay of NTPH by liquid scintillation which appears to be a valid but tedious method (49). The high pH optimum exhibited by NTPH makes other assays, such as that using UDP-glucose pyrophosphorylase (50) very difficult to perform (unpublished communication). Past attempts to purify the enzyme IMP dehydrogenase have also proven difficult (unpublished communication). In the latter case, however, recent work has indicated that reexamination of the purification of this enzyme could prove worthwhile (51).

Two tissue distribution studies of NTPH can be found in the literature. Wang and Morris have shown that the activity per cell is highest in the brain, liver and kidney of the rabbit but lowest in its red cells (2). Moreover, NTPH activity was detected in all 13 tissues examined, suggesting that the enzyme plays a significant metabolic

role in the body. They suggested that the biological role of NTPH may be to prevent insertion of ITP or XTP into RNA, or dITP into DNA.

A study of both ITP pyrophosphohydrolase and IDP phosphohydrolase activity in rat tissues has been reported by Vanderheiden (52). He attempted to distinguish the NTPH activity from non-specific phosphatases by denaturation through heat treatment at 57°C for 5 minutes. It was pointed out by V. Verhoef, in this laboratory (53), that although NTPH activity of red cells and liver has been shown to be comparatively stable to heat treatment (2,47), this treatment may cause erroneous estimates of activity per mg tissue. Partial degradation of the enzyme, variation in enzyme stability between tissues, and alteration of the kinetic parameters are all examples of ways in which heat treatment may cause erroneous estimates of activity. One interesting observation made by Vanderheiden is that the relatively low NTPH activity in the erythrocytes of one rat was also reflected in its other tissues. He suggested that NTPH plays a role in an ITP-IMP cycle which regulates the level of ATP.

In studies previously mentioned, it was demonstrated that individuals show large variations in the specific activity of their red cell NTPH (36,39). At least two population surveys have been conducted in an attempt to characterize this variation. The first of these occurred in 1969, when B.S. Vanderheiden proposed that the distribution of "ITP-ase" activity was bimodal, with approximately 20% of the screened individuals containing lower levels of activity, and the other 80% exhibiting higher levels of specific activity (37). Due to uncertainties produced by his previously-discussed technique of coupling assays of NTPH activity to unspecified concentrations of endogenous red-cell

inorganic pyrophosphatase, another population survey was conducted in 1978 by Morris and co-workers in this laboratory (54). Their findings were in agreement with those of Vanderheiden, and showed the existence of a bimodal distribution of NTPH activity within a randomly-selected caucasion population. This bimodality consisted of a low NTPH group, in which 18% of the population demonstrated specific activities which ranged from 0 to 27.5 nmoles of ITP cleaved/20 min/mg hemoglobin, and a high NTPH group, in which 82% of the population demonstrated specific activities which ranged from 27.5-125 nmoles ITP cleaved/20 minutes/mg hemoglobin. Moreover, this work also demonstrated that a direct correlation exists between the specific activity of NTPH found in human red cells and that found in the platelets, granulocytes, and lymphocytes of the same individual.

Studies of the biochemical basis of the variability of NTPH activity in red cells of the human population were carried out in several ways. Previous data from this laboratory (2,55) showed no evidence for NTPH electrophoretic variants in human red cells, rabbit red cells, and rabbit liver NTPH preparations. Similar results (one such variant in a "large number" of red cell lysates from individuals of diverse racial origins) were obtained by Harris and Hopkinson (56).

The substrate specificities of NTPH from high and low activity individuals was investigated, using XTP, dITP and GTP (55). All individuals used these alternate substrates in the same proportion when ITP hydrolysis was taken as the reference for comparison.

The <u>in vivo</u> stability of NTPH was examined by the separation of red cell populations according to their density (age). A comparison

was then made between the activity present in the old and young cells. While NTPH activity declines with the age of the cells, no correlation could be made between the distribution of NTPH activity and the age-associated loss of enzyme activity (55).

Mixing studies were performed, in which hemolysates from individuals with low and high levels of activity were added to partially purified NTPH. The activity thus obtained was strictly additive in nature, demonstrating an apparent lack of cytoplasmic activators or inhibitors of NTPH (36). Moreover, the Km of NTPH for ITP does not vary significantly among individuals whose specific activities range from 12-124 (36).

Examination of the thermostability of NTPH in lysates of individuals with a wide range of specific activities revealed the first indication of biochemical differences in NTPH (67). Individuals with high specific activity (i.e., greater than 25 units) had enzyme which was more heat stable than that obtained from individuals whose specific activity ranged between 5 and 25 units. Individuals with NTPH specific activity below 5 have enzyme which is significantly more thermolabile than enzyme in either of the other two groups. These results have been interpreted to indicate that differences in the specific activity of NTPH from human red cells occur as a result of differences in the amino acid sequence of this enzyme.

Interest in the inheritance of NTPH activity and its genetic characteristics was stimulated when Vanderheiden, in 1969, suggested that an "ITPase" activity seemed to be limiting the amount of ITP which normally exists in the red cell, as well as limiting the ability of

these cells to accumulate ITP from radioactive inosine (37). He further suggested that the "ITPase" specific activity is a genetically inherited trait. The basis for this last proposal was not well documented, however, and consists of the detection of high levels of "ITPase" activity within the members of a single family. The fact that the activity assays were conducted under sub-optimum conditions adds uncertainities to his data. More recent studies by Morris and co-workers have investigated the proposal that NTPH variation in human red cells is an inherited trait. The results of surveying the pedigrees of 57 families have indicated that the mode of NTPH specific activity inheritance conforms well to a hypotheses of three alleles on one NTPH locus (57).

Two reports suggest that NTPH is located on chromosome 20 in humans, as based upon evidence of its synteny with adenosine deaminase in human-chinese hamster somatic cell hybrids (58,59). The pooled data from these reports, however, cannot exclude chromosomes 7 and 22 as being the locus for NTPH activity.

At present, there are no known clinical effects resulting from low or absent NTPH activity. Vanderheiden has presented data concerning a higher proportion of NTPH-deficient individuals among paranoid schizophrenics than among non-schizophrenic populations (83). However, Hirschhorn, et al., found no differences in the red cell NTPH activity of schizophrenic and non-schizophrenic populations (5).

As previously mentioned, Wang and Morris have proposed that the metabolic role of NTPH may be to prevent incorporation of ITP into RNA and dITP into DNA by keeping levels of these nucleotides low in cells (3).

This proposal was subsequently discussed in relation to incorporation and excision of dITP in E. coli DNA (54).

It can thus be seen that further work is required to define both the function of this enzyme and the cause of its distribution characteristics in humans.

C. Interactions Between Metal Ions and Enzymes

Portions of this thesis investigate the effects of Magnesium, ITP, and their metal-nucleotide complexes, upon the reactivity of the sulfhydryl group(s) in NTPH. This in turn has led to a general consideration of the nature of the scheme which describes, for NTPH, the catalytically active enzyme complex. Therefore, this literature review includes a brief review of the research which has led to an understanding of the nature of the catalytically active complex of metal, enzyme, and substrate.

The interactions between metal ions and enzymes have been extensively reviewed (68,69,70). Research which has been conducted since the 1950's has made it possible to distinguish between metalloenzymes (those which have built-in metals) and metal-activated enzymes (to which metals must be added for activity (68). The use of direct binding studies of metals to enzymes and to substrates has revealed that metalloenzymes have a higher affinity for a metal than do metal-activated enzymes. Mildvan has stated (68) that a stability constant of $10^8$ $M^{-1}$ may serve as a rough dividing line between metalloenzymes and metal-activated enzymes. Borderline instances do exist, however, in which the partial purification of an enzyme results in the retention of small amounts of metal activators. Subsequent addition of metal ions may enhance the residual activity, yielding misleading results concerning the affinity of the active enzyme for

the metal. Further purification in the presence of chelating agents may markedly decrease this residual activity, and demonstrate an absolute dependence upon added metal ions (68).

Correlations between enzyme mechanism, substrate binding and reaction kinetics have been performed by various workers. Reynard, et al., have combined kinetics and binding studies to propose that the catalytic activity of pyruvate kinase involves direct transfer of the phosphoryl group from donor to acceptor (71). Further work elucidating the roles of monovalent and divalent cations in this phosphoryl transfer has been performed by Mildvan and Cohn (72,73). In the latter instance, paramagnetic resonance methods were combined with kinetic studies to provide a detailed analysis of the catalytic role of metal cations. Suelter et al. made use of difference spectroscopy in similar studies of the interactions between substrate and metal cations with pyruvate kinase (74). This and other work has indicated that the functional ternary complexes of metal-activated enzymes have a simple 1:2:1 stoichiometric relationship among enzyme sites, bound metal, and bound substrate. Three possible coordination schemes for such enzymes are possible; they have been summarized by Mildvan and are shown below (68).

Substrate Bridge Complex

E-S-M

Enzyme Bridge Complex

M-E-S

Metal Bridge Complexes

E-M-S (simple)


(cyclic)

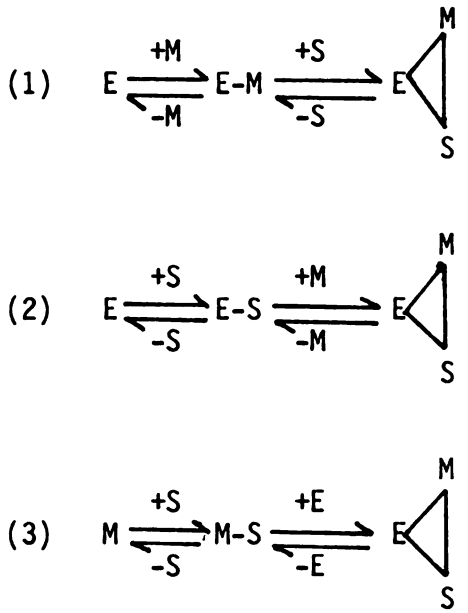
Various methods are available for defining the coordination scheme of a ternary enzyme complex. Some of the more commonly used techniques include gel filtration (75) and equilibrium dialysis (77). Suelter and Milander applied the technique of protein difference spectroscopy to the determination of the dissociation constants for enzyme-metal complexes (76,74). These, and other generally applicable and convenient methods, are currently used in investigations of protein binding phenomena.

Of the four possible coordination schemes for enzyme, metal and substrate complexes, perhaps the least understood is the enzyme bridge complex (M-E-S). The binding of the metal may occur to varying extents, and in a variety of locations upon the enzyme. Its function is thought to be that of stabilizing a catalytically active protein conformation (68).

The ternary complexes of nucleoside di- and triphosphates appear to be universally characterized by substrate-bridge complexes (E-S-M) (68). The mechanism for formation of such complexes has been discussed by Cleland (78). He pointed out that the predominant reactions consist of the initial formation of a metal-nucleotide complex, and its subsequent binding to the enzyme. Later work by Hammes and Hurst, indicated that initial addition of free nucleotide to the enzyme can and does occur (particularly at high enzyme concentrations); this process is then followed by addition of the metal to the substrate (79).

Metal bridge complexes (E-M-S) have been suggested as functional intermediates in a wide variety of both metal-activated and

metallo-enzymes. The formation of such bridges with metallo-enzymes may involve one or more of the following 3 pathways (68).




Kinetic and binding measurements are used to demonstrate which, if any, of these paths predominate for a given enzyme. One such example is pyruvate kinase, which, under various conditions, has been shown to proceed through all three pathways (73). Comparisons between the substrate kinetics and binding data of the allosteric and non-allosteric forms of this enzyme in yeast and muscle was performed by Mildvan, Hunsley, and Suelter (80). Their work suggested that muscle pyruvate kinase forms an active, quaternary, E-M-$S_1$-$S_2$ complex by multiple pathways. It was further suggested that yeast pyruvate kinase forms ternary E-M-S complexes which are structurally similar to the muscle enzyme.

Thus, it can be seen that for metal-activated enzymes, a complex relationship exists between the enzyme binding sites, the metal, and the substrate. Much detailed investigation is therefore required before a clear understanding of this relationship, and its role in the catalytic mechanism, is obtained.

## III. Reagents, Materials, and Methods

### A. Reagents

#### 1. Commercial

Sigma Chemical Company, St. Louis, Missouri, supplied the following: dithiothreitol, bovine serum albumin, yeast inorganic pyrophosphatase (600-800 μ/mg), CM-Sephadex (C-50-120), Sephadex G-25-80, N-ethylmaleimide, imidazole, tris base, and N;N,N'N'-tetramethylethylenediamine. The sodium salt of inosine-5'-triphosphate was also purchased from Sigma (Sigma Grade I, 95-97%, shipped on dry ice), as well as from PL Biochemicals, Milwaukee, Wis.

Aldrich Chemical Corporation, Inc., Milwaukee, Wis., supplied N,N'-methylenebisacrylamide, electrophoresis grade. Ammonium persulfate, was purchased from Miles Laboratories, Elkhardt, Ind. Sodium dodecyl sulfate was obtained from Fisher Scientific Company, Pittsburgh, Penn. Acrylamide, gel electrophoresis grade, was obtained from Bethesda Research Labs, Inc., Rockville, Md. 2-Mercaptoethanol was purchased from Mallinckrodt Chemical Works, St. Louis, Missouri. Hycel cyanmethemoglobin reagent (Mo.116E) was obtained from Hycel, Inc., Houston, Texas. Analytical reagent grade ammonium sulfate, from Mallinkrodt Chemical Works, St. Louis, Missouri, was ground in a mortar to a fine powder before use.

Tris·Cl buffer was prepared from the free base by titration with HCl, and titrated to the pH indicated at the temperature at which it was to be used. β-Alanine buffer was prepared by titration of β-alanine (U.S. Biochemical Corporation, Cleveland, Ohio) with sodium hydroxide to pH 9.5 at 37°C. Imidazole-acetate buffer was prepared by titrating 1 M acetic acid with 1.0 M imidazole to pH 6.25 at 4°C.

2. Special Reagents

a. Water

The water which was used for all the NTPH purification and analytical work was glass-distilled, and kindly donated by Dr. Wilson's laboratory, Department of Biochemistry, Michigan State University. It was stored in containers which had been thoroughly leached.

The water which was used for HPLC analysis was kindly donated by the Department of Dairy Science at Michigan State University. Following steam distillation, the water is passed through two mixed-bed resins, one heavy-metal resin, and one charcoal filter. It is then glass-distilled using a Corning Megapure water still, and stored in glass containers.

b. Preparation of $CaPO_4$ Gel

Calcium phosphate gel was prepared by the method of Tsuboi and Hudson (60), which is described briefly as follows. To 400 ml of 0.5 M $NA_2HPO_4$ (anhydrous) is added 12 ml of concentrated $NH_4OH$, followed immediately by 300 ml of 0.1 M $CaCl_2$ solution. The mixture is stirred gently with continual monitoring of the pH. The calcium phosphate thus formed is then washed at 4°C by suspension and resedimentation. When conductivity measurements of the wash water are found to be constant (usually after 10 washes), as much $H_2O$ as possible is siphoned off the sedimented gel, and it is stored at 4°C. The concentration of the $CaPO_4$, determined via gravimetric analysis, is approximately 13 mg/ml.

B. Materials

1. Commercial

Vacutainers and needles were obtained from Becton-Dickinson, Rutherford, NJ. PM-10 Diaflo ultrafiltration membranes were purchased from Amicon Corp., Lexington, Mass. Prepacked Partisil PXS-10/25 SAX columns for HPLC analysis were purchased from Whatman, Inc., Clifton, NJ.

2. Preparation of Dialysis Tubing

Dialysis tubing was obtained from Union Carbide, Chicago, Ill. It was prepared for use by boiling in a minimal amount of EDTA (di-Na salt). The tubing was then rinsed several times and stored in glass-distilled water.

3. Preparation of Glass Tubes for SDS-Gel Electrophoresis

Glass tubes of dimensions 0.5 x 14 cm were acid-washed overnight and rinsed with distilled water. They were then dipped in a 0.1% solution of Prosil-28 for 1-2 minutes, rinsed with distilled water, and air-dried overnight.

C. Methods

1. Analytical

a. NTPH and Phosphate Analysis

NTPH catalyzes the general reaction:

$$NTP + H_2O \quad NMP + PP_i \cdot$$

According to the method of Chern et al., the pyrophosphate produced during the incubation of NTPH with ITP may be hydrolyzed by yeast inorganic pyrophosphatase (1). The inorganic phosphate thus formed may then be analyzed colorimetrically by the method of Rathbun and Betlach using $K_2HPO_4$ as a reference standard (61).

Unless otherwise noted, the assay mixtures contained 50 mM β-alanine buffer (pH 9.5), 10 mM $MgCl_2$, 2 mM DTT, 1 unit yeast inorganic pyrophosphatase, 0.5 mM ITP and the NTPH-containing solution. Appropriate control mixtures were incubated concurrently to account for trace phosphate contamination present in ITP and NTPH-containing solutions and others, as necessary.

0.5 mM ATP was sometimes substituted for ITP to test for the presence of non-specific phosphatases. Following the incubation of these mixes for 20 minutes at 37°C, the reactions were terminated by the addition of 2.2 ml of 7.27% trichloroacetic acid. The precipitated protein was removed by centrifugation (1000 x g for 10 minutes at 4°C) and the supernatants were decanted into phosphate-free test tubes for inorganic phosphate determination. Phosphate analysis was accomplished by adjusting the pH to ca. 4.5 with 1.88 ml of 3 M acetate buffer (1:1 mixture of 3.0 M sodium acetate and 3.0 M acetic acid) to which 1/10 part 37% formaldehyde (10 ml formaldehyde for every 90 ml acetate buffer) had been added to diminish the interference of sulfhydryl reagents in the color development step. Then 2.0 ml 2% ammonium molybdate, followed immediately by 0.4 ml 6.75 mM stannous chloride were added to each tube. After 15 minutes, the absorbance at 700 nm of each of the solutions was determined with a Gilford 300 micro-sample spectrophotometer.

A unit of NTPH activity is defined as that amount of enzyme which hydrolyzes one nmole of ITP at 37°C in the standard 20 minute incubation. The specific activity may be expressed as units per mg protein or units per mg hemoglobin.

b. Hemoglobin and Protein Analyses

The hemoglobin concentration in hemolysates was determined by the method of Austin and Drabkin (62). The determinations were facilitated by the use of Hycel cyanmethemoglobin reagent. The absorbance at 540 nm of a 1 mg/ml cyanmethemoglobin solution has been determined to the 0.718. Other protein analyses were carried out by the method of Lowry et al. with bovine serum albumin as the reference standard (63).

c. High Pressure Liquid Chromatography

HPLC analysis was performed on various commercial preparations of ITP. The system used was that established by Vernon Verhoef, working in this laboratory (53).

Analyses were performed at ambient temperature with a Perkin-Elmer 1250 HPLC equipped with a prepacked Partisil-10 SAX column and UV detector. The pump was routinely operated at 35% capacity, giving a flow rate of 180 ml/hr, with 600 psi. operating pressure. $KHPO_4$ buffer (0.45 M, pH 3.4 at 25°C) was prepared with $H_2O$ kindly donated from the Department of Dairy Science, Michigan State University, as previously described.

The buffer was stored in glass containers, filtered through a 0.45 micron filter and degassed immediately prior to use. Samples were injected onto the column using an automated injector system. Quantitative analysis was performed by standardizing the peak height of a particular nucleotide of known concentrations. The purity of standards or other samples was determined by estimating the peak areas of the mono- di and triphosphate peaks.

d. SDS Polyacrylamide Gel Electrophoresis of Partially-Purified NTPH

SDS polyacrylamide gel electrophoresis was performed following the method of Weber and Osborn (64). The 7.5% acrylamide gels were prepared as follows: one part solution A (1 M $NaH_2PO_4$, pH 7.1, 40 mL; 10% sodium dodecyl sulfate, 4 ml; 0.12 ml TEMED; water to 100 ml), one part solution B (acrylamide, 30 gm; BIS, 0.65 gm; water to 100 mL total volume), and one part water were combined. The polymerization reaction was initiated by the addition of one part solution C (ammonium persulfate, 0.28 gm; water to 100 mL). Without delay, the mixture was added to gel tubes (0.5 cm diameter) to a depth of about 10 cm, and 0.1 mL of water was carefully layered on top of the gel solution. Polymerization was allowed to proceed for 1 hr. The protein solutions consisted of 0.2% 2-mercaptoethanol, 0.2 M $NaH_2PO_4$, glycerol, 20%, and protein in a concentration of 1 mg/ml. 5 µL of a 0.15% solution of bromophenol blue was added as tracking dye. The samples were treated to boiling in a water bath for 1 minute. The gels were subjected to a current of 8 ma per gel cyclinder, in 0.1 M $NaH_2PO_4$ buffer, pH 7.1 containing 0.2% sodium dodecyl sulfate and 0.2% 2-mercaptoethanol as electrode buffer.

After electrophoresis, the gels were stained overnite in a solution containing methanol, 227 ml, acetic acid, 46 ml, Coomassie Brilliant Blue R., 1.25 gm., and water to 500 ml. Destaining was effected in a solution of 30% ethanol and 7.5% acetic acid. Gels were stored in 7% acetic acid.

2. General Procedures

a. Partial Purification of Human Erythrocyte NTPH

The purification procedure described below is that established by A.J. Morris (3). As has previously been stated, the NTPH activity of human erythrocytes varies widely between individuals. Therefore, a preliminary analysis of NTPH activity is routinely performed on each unit of outdated Red Cross blood. Red cell lysates with the highest specific activity available were utilized in the purification procedure.

1. Preparation of Crude Lysate and Lysate

The outdated human blood (CA 300 ml) is combined with equal volumes of 0.9% NaCl and mixed gently by inversion. Following centrifugation at 4000 G for 10 minutes at 4°C, the plasma and buffy coat are removed by gentle aspiration. This process of washing the cells is repeated twice as described. The washed cells (180 ml packed cell volume) are lysed in 4 volumes of 1 mM dithiothreitol, 0.25 mM EDTA, with gently stirring for 60 minutes (crude lysate). The crude lysate is centrifuged at 10,000 G for 20 minutes, at 4°C. The supernatant is removed, and the pellet is further centrifuged at 27,000 G for 20 min at 4°C. This supernatant is added to that obtained from the previous spin (680 ml, lysate). The absorbance at 280 nm of an appropriate dilution of the lysate is determined.

2. Adsorption onto Calcium Phosphate Gel

Calcium phosphate gel is prepared, and the concentration determined, as previously described. The gel is added to the lysate in an amount equalling 0.11 mg dry weight of gel per mL of

lysate for each unit of absorbance at 280 nm. The mixture is stirred for 1 hr and centrifuged at 10,000 G for 20 minutes. The supernatant fraction is discarded. The gel pellet is washed by resuspension in 1 mM DTT, 0.25 mM EDTA (300 ml per unit of blood). This mixture is stirred for 30 minutes at 4°C, and centrifuged at 1500 G for 5 min at 4°C. A total of 3 such washings are performed. NTPH is eluted from the calcium phosphate by resuspension in a solution which is 10% ammonium sulfate, 1 mM dithiothreitol, and 0.25 mM EDTA (300 ml per unit of blood). This mixture is stirred for 1 hour at 4°C, and centrifuged at 10,000 G for 20 minutes, also at 4°C. The pellet is discarded, and the supernatant (380 ml, gel eluate) subjected to ammonium sulfate fractionation.

3. Ammonium Sulfate Fractionation

Ammonium sulfate is added to the gel eluate so as to achieve a final salt concentration of 50% saturation (23.3 gm ammonium sulfate per 100 mL gel eluate). This addition is performed over a period of 30 minutes at 4°C with gentle stirring. The mixture is then stirred for another 30 minutes, and centrifuged at 10,000 G, 4°C, for 20 minutes. The protein pellet is discarded. Ammonium sulfate is added to the supernatant (420 ml) so as to achieve a final concentration of 70% salt (12.5 gm/100 mL supernatant). This addition is performed as previously described. Following centrifugation at 10,000 G, 4°C, for 30 minutes, the protein pellet is resuspended in a minimal volume (11 ml) of imidazole-acetate buffer (.01 M, pH 6.25 at 4°C) which is 1 mM DTT, 0.25 mM EDTA (ammonium sulfate fraction). The sample is then dialyzed against two-2 L portions of the same imidazole-acetate buffer.

4. Chromatography on C-50-120 Sephadex

The CM sephadex column is prepared by gently stirring C-50-120 Sephadex (4.5 ± 0.5 meq./G) in a 1:10 dilution of stock 1 M imidazole-acetate buffer, pH 6.25 at 4°C. This is allowed to swell overnite at 4°C. The CM sephadex is then washed twice by resuspension and sedimentation with a 1:100 dilution of the stock imidazole-acetate buffer.

The pH of the gel is then brought to 6.25 using a 0.01 M concentration of the appropriate buffer component. The resulting column bed is typically 3 x 40 cm. It is washed with a 1:100 dilution of the stock buffer, which is also 1 mM DTT, 0.25 mM EDTA, until the pH of the eluate is identical to that of the wash buffer. The ammonium sulfate fraction (13.6 mL after dialysis) is carefully added to the column bed. NTPH activity is eluted at the void volume of the column, while many contaminating proteins are retained on the column. The NTPH-containing fractions are pooled and concentrated to approximately 10 mL by ultrafiltration. The final sample (C-50 eluate fraction) is dialyzed against two 2 L portions of Tris·HCl, 50 mM, Ph 8.0 at 4°C, 1 mM DTT, 0.25 mM EDTA. It is then aliquoted into glass vials (0.5 dram) and frozen in liquid nitrogen for future use. Unless otherwise stated, all enzyme studies presented in this thesis were performed on enzyme which had undergone the above purification procedure. This NTPH preparation will be referred to as C-50 purified NTPH.

b. Analysis of the Sulfhydryl Requirement of NTPH

1. Effect of DTT and EDTA upon NTPH Activity

Analysis of the sulfhydryl requirement of NTPH involved desalting on Sephadex G-25 in order to effect rapid and

efficient removal of DTT (present in the enzyme storage buffer) from NTPH. For these experiments, 5.45 units of C-50 purified NTPH was desalted on a column of Sephadex G-25 (1 x 25 cm) equilibrated and eluted in 10 mM Tris·Hcl, pH 8.0, 4°C, 50 mM NaCl. The enzyme was then added to each of 4 mixes containing the standard assay components plus either 2 mM DTT or 0.25 mM EDTA or both. The control mix contained NTPH without DTT or EDTA. The reaction was initiated by the addition of the enzyme to the substrate and immediately placed in a 37°C water bath. At 5 minute intervals, 1 ml aliquots were removed from each mix and added to 2.2 ml of 7.27% cold TCA, which was then assayed for inorganic phosphate. The same experiment was also performed using NTPH which had been desalted on Sephadex G-25 equilibrated and eluted in buffer containing 0.25 mM EDTA. In all the experiments to be described, NTPH was desalted on a Sephadex G-25 column in 10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.25 mM EDTA at 4°C and used immediately.

2. The Effect of N-Ethylmaleimide Upon NTPH Activity

C-50 purified NTPH was incubated at 4°C for 15 minutes with various concentrations of NEM and the standard assay components of β-ala buffer, yeast inorganic pyrophosphatase, and water to a final volume of 0.80 mL. The reaction was terminated by the addition of DTT which was 2 mM in excess of the NEM concentration. ITP, $MgCl_2$, and $H_2O$ were then added to a final volume of 1 ml, so as to achieve standard assay conditions, and NTPH activity was determined for each sample. Each assay was done in triplicate.

Control experiments demonstrating complete inactivation of the effects of NEM by DTT were performed by incubating DTT with NTPH and NEM under the above described conditions.

The effect of NEM upon yeast inorganic pyrophosphatase was also determined. The experiment was of the same design as just described, with the exception that NTPH was omitted, and 0.1 mM inorganic pyrophosphate was used as the substrate.

3. Effects of Incubating NTPH with ITP and/or $MgCl_2$

a. Incubation with ITP and/or $MgCl_2$

C-50 purified NTPH was added to a series of test tubes containing β-alanine buffer, and yeast inorganic pyrophosphatase (in the standard assay concentrations) at 4°C in a total volume of 0.80 mL. Also present were either 0.5 mM ITP, or various concentrations of $MgCl_2$ ranging from 0 to 10 mM, or both ITP and $MgCl_2$. Control samples did not contain either $MgCl_2$ or ITP. Following a 15 minute incubation period, 2 mM DTT was added to the reaction. $MgCl_2$, ITP, and $H_2O$ to a final volume of 1 mL were then added so as to obtain the standard assay conditions, and NTPH activity was determined. All samples were performed in triplicate.

b. Destabilization by $MgCl_2$: Effects of Incubation Time

C-50 purified NTPH was added to an assay mixutre containing, at 4°C, 0 and 10 mM $MgCl_2$, β-alanine buffer, and yeast inorganic pyrophosphatase (in the standard assay concentration) in a total volume of 0.80 mL. At timed intervals, 2 mM DTT was added to the reaction. ITP, $MgCl_2$, where necessary, and $H_2O$ to a final volume of 1 ml were then added so as to obtain the standard assay conditions, and NTPH activity was determined.

4. Effect of $MgCl_2$ and/or ITP Upon NTPH Inactivation by N-ethylmaleimide

C-50 purified NTPH was added to a series of test tubes containing, at 4°C, the standard assay components of β-alanine buffer, yeast inorganic pyrophosphatase, and $H_2O$ to a final volume of 0.80 mL. Also present was 0.5 mM ITP, both alone and in the presence of various concentrations of $MgCl_2$ ranging from 0 to 10 mM. NEM, 3.5 mM, was added to initiate each reaction. Following a 5 minute incubation, DTT was added to a concentration which was 2 mM in excess of the NEM concentration. $MgCl_2$, ITP, and $H_2O$ to a final volume of 1 ml were then added so as to achieve standard assay conditions, and NTPH activity was determined for each sample. Control samples were performed which measured activity seen with and without NEM present (in the absence of ITP and $MgCl_2$). A separate experiment was conducted under exactly the same conditions, with the exception that $MgCl_2$ alone, without ITP, was present during the reaction with NEM.

In a later experiment, this work was repeated exactly as described above, with the exception that 0.25 mM EDTA was included in each incubation reaction.

5. Rate Analysis of NTPH Inactivation by N-ethylmaleimide

The time course of NTPH inactivation by NEM was documented as follows. C-50 purified NTPH was added to a master mix containing, at 4°C, 0.25 mM EDTA, various concentrations of NEM, the standard assay components of β-ala buffer, yeast inorganic pyrophosphatase, and $H_2O$ to a final unit volume of 0.80 ml. At 2 minute intervals, for a period of 60 minutes, 0.80 ml of this mix was removed and added to a DTT concentration which was 2 mM in excess of the NEM

concentration. ITP, $MgCl_2$ and $H_2O$ were then added to a final volume of 1 mL so as to yield standard assay conditions, and NTPH activity was determined in each sample. All of these experiments were done in duplicate.

6. Effect of $MgCl_2$ and/or ITP Upon the Time Course of NTPH Inactivation by N-ethylmaleimide

These experiments were performed as described in the previous reaction, wherein the time course of NEM inactivation was assessed. In this case, however, concentrations of $MgCl_2$ ranging from 1 mM to 10 mM, or 0.5 mM concentrations of ITP were present during the derivitization reaction. Following the addition of DTT, ITP, $MgCl_2$ and $H_2O$ were again added to a final volume of 1 ml, and so as to yield standard assay conditions, and NTPH activity was determined in each sample. All of these experiments were done in duplicate. In each instance, control experiments demonstrated that the incubation conditions themselves had no effect upon NTPH activity. In a similar experiment, 0.5 mM concentrations of both $MgCl_2$ and ITP were present during the inhibition reaction, with all other conditions being as described above.

7. Computer Calculation of Metal-Nucleotide Complexes

Computer calculation of the concentrations of metal-nucleotide complexes was based upon a program designed by Cornish-Bowden (81). A complete description of this method is provided in Appendix A. The CYBER-750 computer, located at the Michigan State University Computer Laboratory, was used for this work.

## IV. Results

### a. HPLC Analysis of Commercially Available Inosine 5'-Triphosphate

The work of Chern et al. (1) has shown that the nucleotides IMP and IDP inhibit NTPH activity 23% and 57%, respectively, at 1 mM concentrations. Moreover, investigations performed on yeast inorganic pyrophosphatase have shown this enzyme to be strongly inhibited by inorganic phosphate (65). This information, combined with the fact that phosphate analysis forms the basis for determinations of NTPH activity, dictated the need of a highly purified form of ITP.

Initial studies of NTPH activity used substrate ITP purchased from PL Biochemicals (Milwaukee, Wisconsin). HPLC analysis of this ITP (performed as described in Methods, Section 1c) showed it to contain up to 20% contaminating IDP and 2% IMP, as shown in Figure 2. Similar analysis of ITP obtained from Sigma, however, showed the presence of less than 3% IDP, and negligibly small amounts of IMP, also as shown in Figure 2. In all the studies performed in this thesis, therefore, the Sigma brand of ITP was used.

### b. Partial Purification of Human Erythrocyte NTPH

The partial purification of NTPH from human erythrocytes was performed as described in Methods (section 2a). Fractions obtained from CM-Sephadex chromatography were analyzed both for absorbance at 280 nm, and for pyrophosphohydrolase activity. The resulting chromatogram in given in Figure 3. Fractions containing pyrophosphohydrolase activity were pooled, concentrated, and stored in liquid nitrogen as previously described. A summary of the purification process is provided in Table 1. A final specific activity of 47,700 units/mg

Figure 2. HPLC Analysis of Commercially Available ITP. Inosine 5'-triphosphate was purchased from PL Biochemicals (Milwaukee, Wisc.) and from Sigma Chemical Company (St. Louis, Missouri). HPLC analysis of these nucleotides was performed at ambient temperature with a Perkin-Elmer 1250 HPLC equipped with a prepacked Partisil-10 SAX column and UV detector. The pump was routinely operated at 35% capacity, giving a flow rate of 180 ml/hr with 600 psi operating pressure. $KHPO_4$ buffer (0.45 M, pH 3.4 at 25°C) was prepared as described in Methods. Quantitative analysis was performed by standardizing the peak height of a particular nucleotide of known concentrations. The purity of standards or other samples was determined by estimating the peak areas of the mono-, di- and triphosphate peaks.

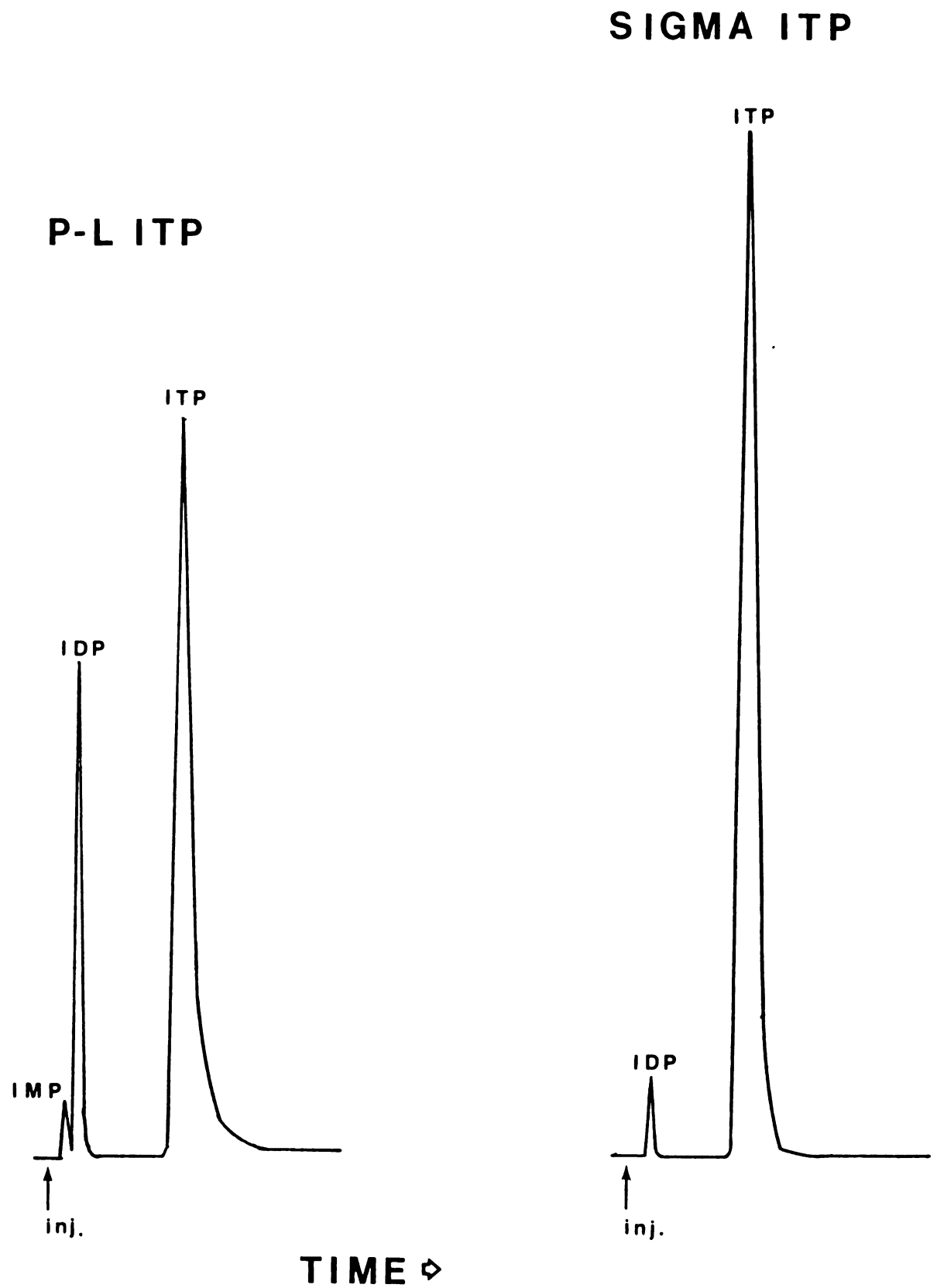

SIGMA ITP
ITP
P-L ITP
ITP
IDP
IMP
inj.
IDP
inj.
TIME

Figure 3. CM Sephadex C-50-120 Column Chromatography of NTPH. The sample (13.6 ml) was layered onto a column bed (3 x 40 cm) equilibrated in imidazole-acetate buffer, pH 6.25, 1 mM DTT, 0.25 mM EDTA at 4°C. The fractions are analyzed for NTPH activity (absorbance at 700 nm) and absorbance at 280 nm. NTPH activity is eluted at the void volume of the column, while many contaminating proteins are retained on the column.

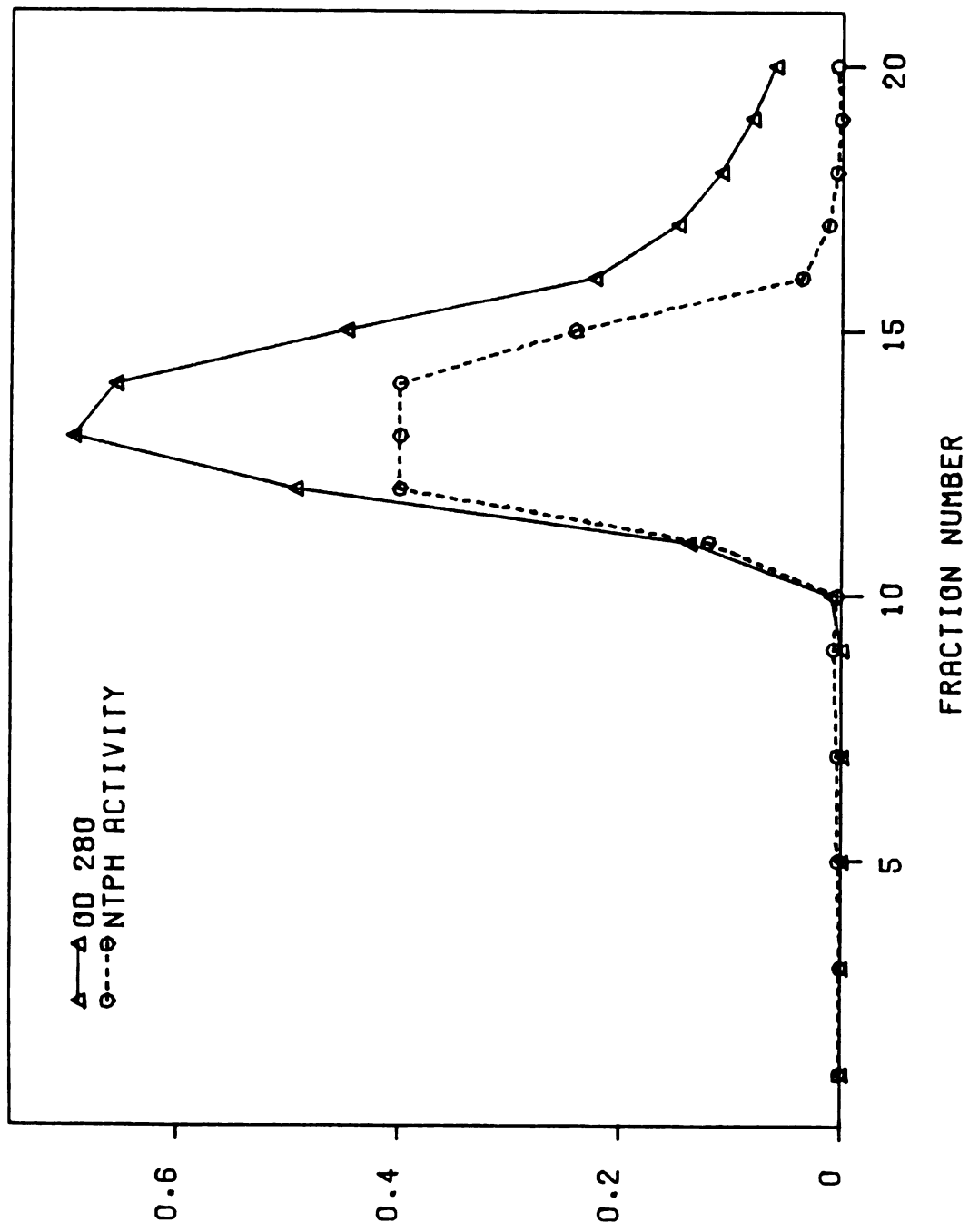

OD 280
NTPH ACTIVITY
0
0.2
0.4
0.6
5
10
15
20
FRACTION NUMBER

Table 1. Purification of Nucleoside Triphosphate Pyrophosphohydrolase.

| Procedure | Volume (ml) | Units per ml* | Total Units | % Recovery | Protein mg/ml | S.A.** | Fold Purification |
|---|---|---|---|---|---|---|---|
| Crude Lysate | 860 | 3.61 | 3102 | 100 | 62 | 58 | -- |
| Lysate | 680 | 3.49 | 2373 | 77 | 75 | 47 | .8 |
| Gel Eluate | 382 | 5.63 | 2150 | 69 | 11 | 530 | 9 |
| Ammonium Sulfate Fractionation | 14 | 106 | 1437 | 47 | 10 | 11,033 | 190 |
| CM Sephadex Chromatography | 9 | 97 | 872 | 29 | | 47,680 | 822 |

*One unit of activity is expressed as that amount of enzyme which hydrolyzes one nmole of ITP at 37°C in the standard 20 minute incubation.

**S.A.; Specific activity is expressed as units per mg protein.

protein was routinely obtained, with an 800 fold level of purification achieved from the crude extract. Electrophoresis of this preparation upon SDS gels produces at 15 protein bands, as shown in Figure 4.

c. Analysis of the Sulfhydryl Requirement of NTPH

1. Effect of DTT and EDTA Upon NTPH Activity

Discussion has been made of previous work in this area (see INTRODUCTION). In order to advance these studies, it was first necessary to remove any sulfhydryl reagents present in the enzyme storage buffer. Previously, this was accomplished by dialyzing the enzyme against an appropriate buffer for as much as 18 hrs. The current studies, however, replaced dialysis with gel filtration on Sephadex G-25, and yielded an active enzyme preparation which contained no sulfhydryl reagents.

Following methods previously described (see METHODS section), NTPH was desalted on a column of Sephadex G-25, equilibrated and eluted in 10 mM Tris·HCl, 50 mM NACl, pH 8.0 at 4°C. The enzyme was then assayed for activity in the presence of either 2 mM DTT, or 0.25 mM EDTA, or both DTT and EDTA together. The control experiment contained enzyme which was assayed in the absence of either of these reagents. Figure 5 shows the results of this work. As shown in Table 2, the presence of 0.25 mM EDTA alone enhances NTPH stability 1.5 fold, while 2 mM DTT, with or without EDTA, enhances stability by 2.3 fold.

The same experiment was repeated with NTPH which was desalted on Sephadex G-25 equilibrated and eluted in buffer containing 0.25 mM EDTA. These results are shown in Figure 6. Enzyme which has been both desalted and assayed in the presence of EDTA demonstrates a 2.5 fold increase in stability over enzyme which has been desalted and assayed

Figure 4. SDS Gel Electrophoresis of 800-Fold Purified NTPH. Electrophoresis of partially purified NTPH was performed as described in Methods. The protein bands were visualized with Coomassie brillian blue.

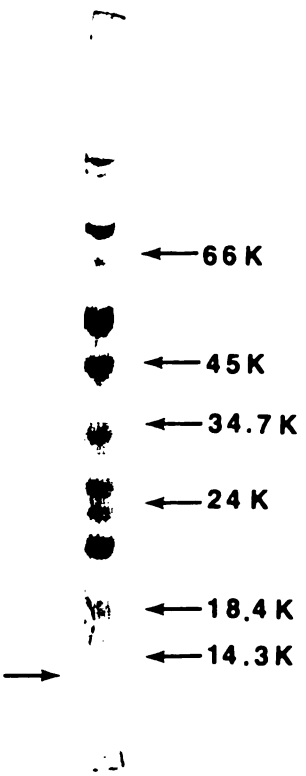

66K
45K
34.7K
24K
18.4K
14.3K

Figure 5. The Effect of DTT and EDTA Upon NTPH Activity. 5.45 Units (.114 mg protein) of C-50 purified NTPH was desalted on a column (1 x 25 cm) of Sephadex G-25 equilibrated and eluted in 10 mM Tris·HCL, 50 mM NACL, pH 8.0 at 4°C. The enzyme was then assayed for activity in the presence of either 2 mM DTT, or 0.25 mM EDTA, or both. The control assay contained NTPH without DTT or EDTA (see Methods).

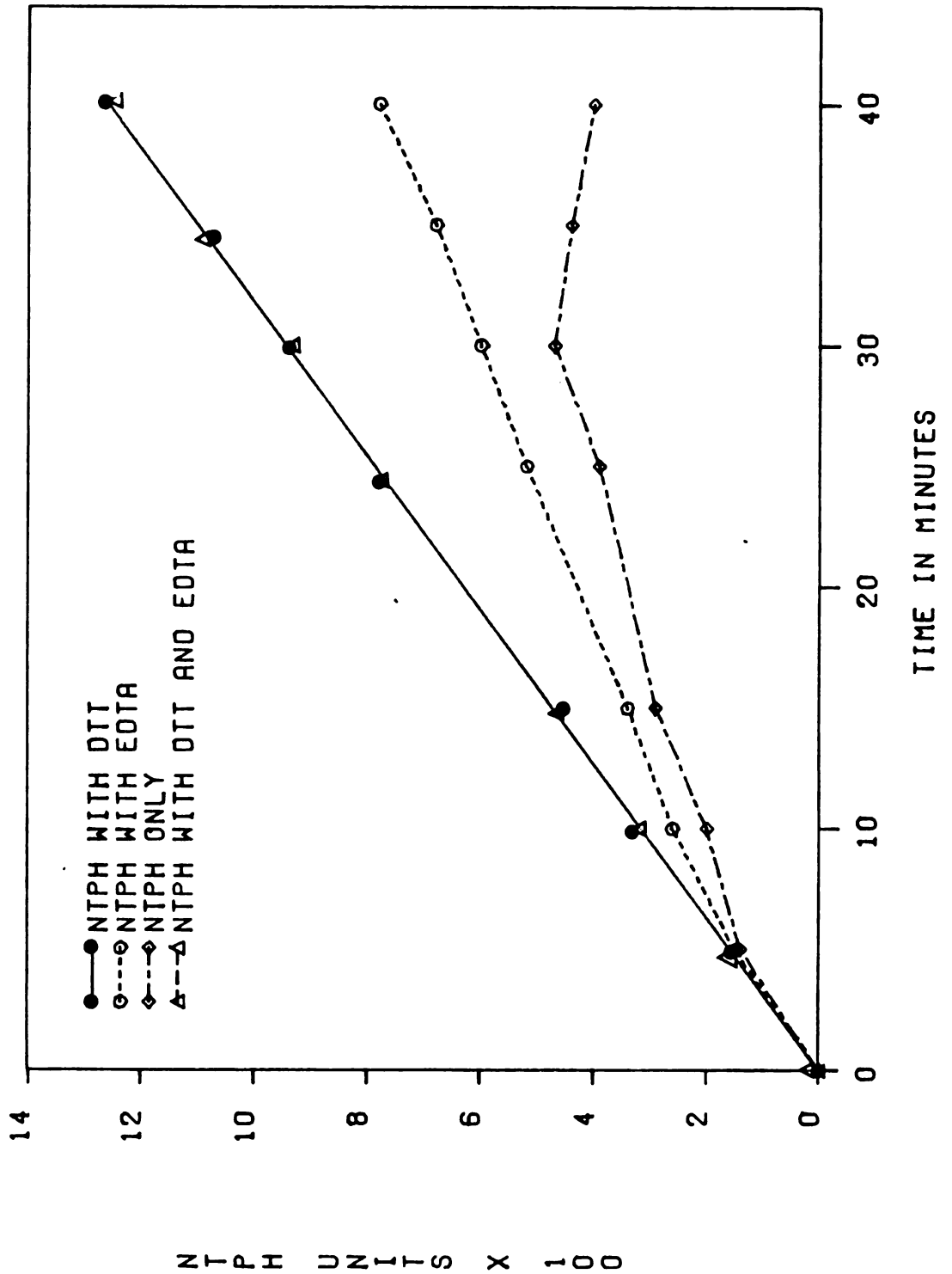
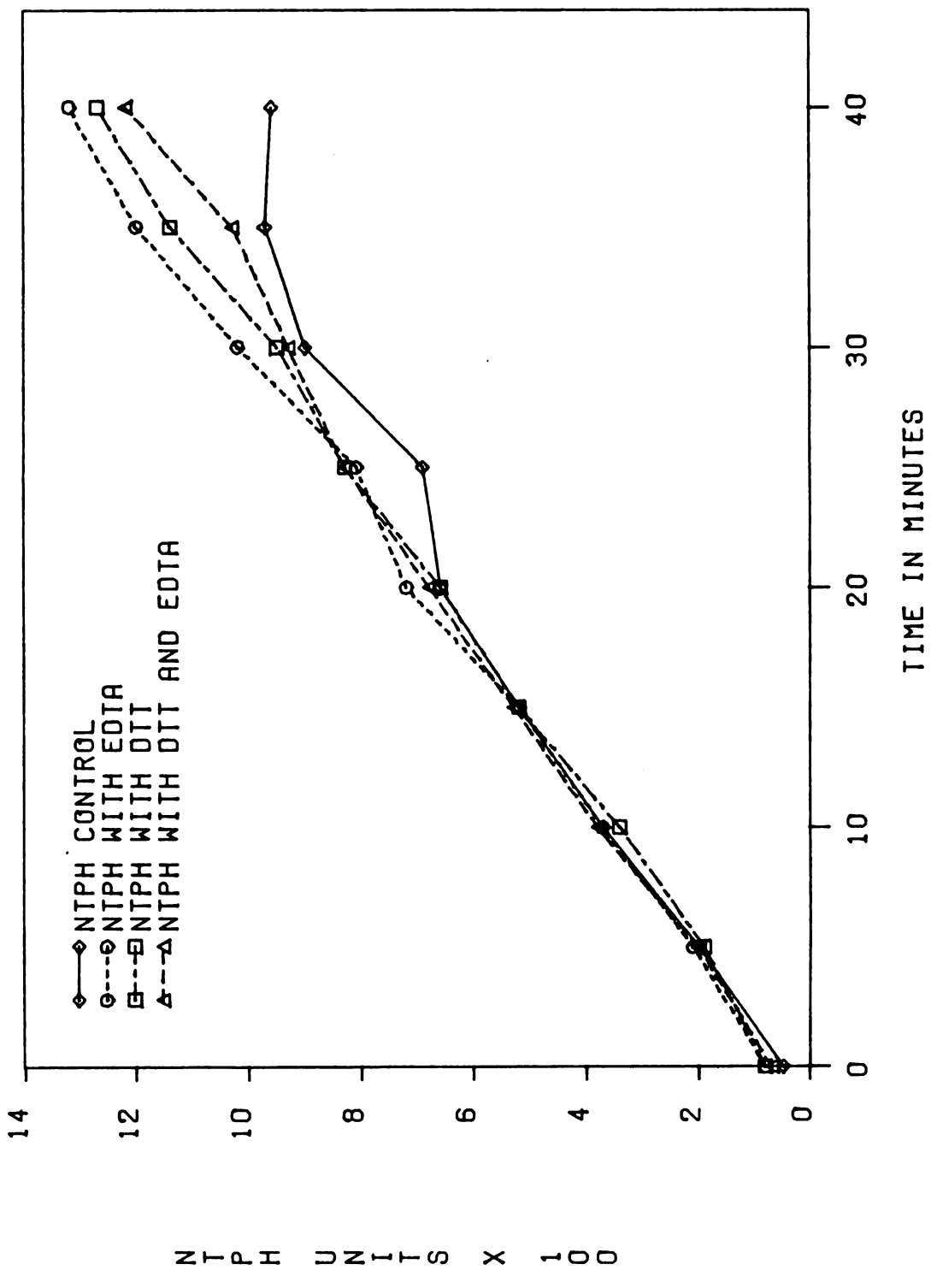

NTPH WITH DTT
NTPH WITH EDTA
NTPH ONLY
NTPH WITH DTT AND EDTA
0
2
4
6
8
10
12
14
0
10
20
30
40
TIME IN MINUTES
NTPH UNITS X 100

Figure 6. The Effect of DTT and EDTA Upon NTPH Activity. 5.45 units (.114 mg protein) C-50 purified NTPH was desalted on a column of Sephadex G-25 equilibrated and eluted in 10 mM Tris·HCL, 50 mM NACL, 0.25 mM EDTA, pH 8.0 at 4°C. The enzyme was assayed for activity in the presence of either 2 mM DTT, or 0.25 mM EDTA, or both. The control assay contained NTPH without DTT or EDTA.


NTPH CONTROL
NTPH WITH EDTA
NTPH WITH DTT
NTPH WITH DTT AND EDTA
NTPH UNITS X 100
TIME IN MINUTES
0
2
4
6
8
10
12
14
0
10
20
30
40

in the absence of either DTT or EDTA. Moreover, this increase is of the same magnitude as that previously seen when 2 mM DTT was present in the assay mixture. A summary of these experiments is provided in Table 2. In all future experiments, NTPH was used immediately after desalting on a Sephadex G-25 column equilibrated and eluted in 10 mM Tris·HCl, pH 8.0 at 4°C, 50 mM NaCl, 0.25 mM EDTA.

2. The Effect of N-ethylmaleimide Upon NTPH Activity

By avoiding dialysis, and including 0.25 mM EDTA in the gel filtration buffer, an active enzyme preparation had been produced which was devoid of sulfhydryl compounds. It was now possible to critically examine the reaction between N-ethylmaleimide and NTPH. As previously described (see METHODS), NTPH was incubated for 15 minutes at 4°C, with various concentrations of NEM. As shown in Figure 7, NEM inhibits NTPH activity, with an effect ranging from 14% inhibition in the presence of 1 μM, to 95% inhibition at 5 mM NEM.

Control experiments demonstrated that DTT, in concentrations equivalent to those of NEM, completely protects NTPH from inhibition by N-ethylmaleimide. It was also determined that, in the absence of NEM, the incubation conditions themselves had little effect upon NTPH activity. Moreover, under these conditions, NTPH was unreactive toward the substrate form of ITP. The effects of NEM and the incubation conditions upon yeast inorganic pyrophosphatase were determined as described in Methods. As shown in Figure 8, there was no measurable effect upon this enzyme.

Chern, et al., (1) and, subsequently, Wang and Morris (2), have shown that the small variations in DTT concentration which occur after

Table 2. Effect of DTT and EDTA Upon NTPH Activity

| Assay Description | Desalted Without EDTA | | Desalted With EDTA | |
|---|---|---|---|---|
| | Reaction Velocity* | Fold Increase Over Control | Reaction Velocity | Fold Increase Over Control |
| Control | .126 | | .308 | 2.44 |
| 0.25 mM EDTA | .196 | 1.55 | .318 | 2.52 |
| 2 mM DTT | .290 | 2.30 | .300 | 2.38 |
| 0.25 mM EDTA + 2 mM DTT | .272 | 2.16 | .310 | 2.46 |

*Reaction velocity is defined as μmoles $PP_i$ produced x $10^2$ per minutes over a period of 30 minutes.

Figure 7. The Effect of N-ethylmaleimide Upon NTPH Activity. C-50 purified NTPH was incubated with various concentrations of N-ethylmaleimide for 15 minutes, at 4°C, as described in Methods. In the results shown here the data is presented as % inhibition plotted as a function of the M concentration of NEM. Note the logarithmic scale.

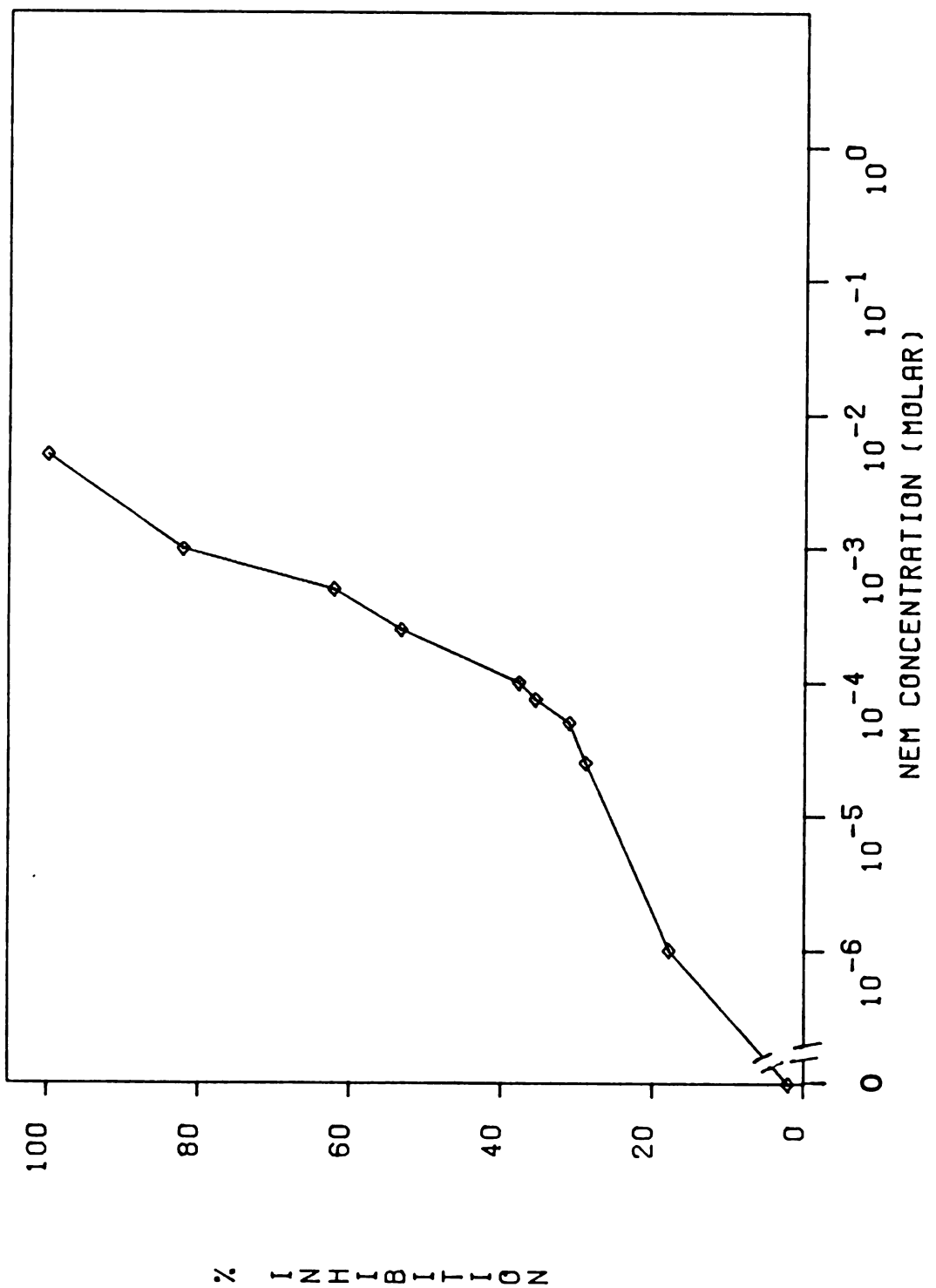
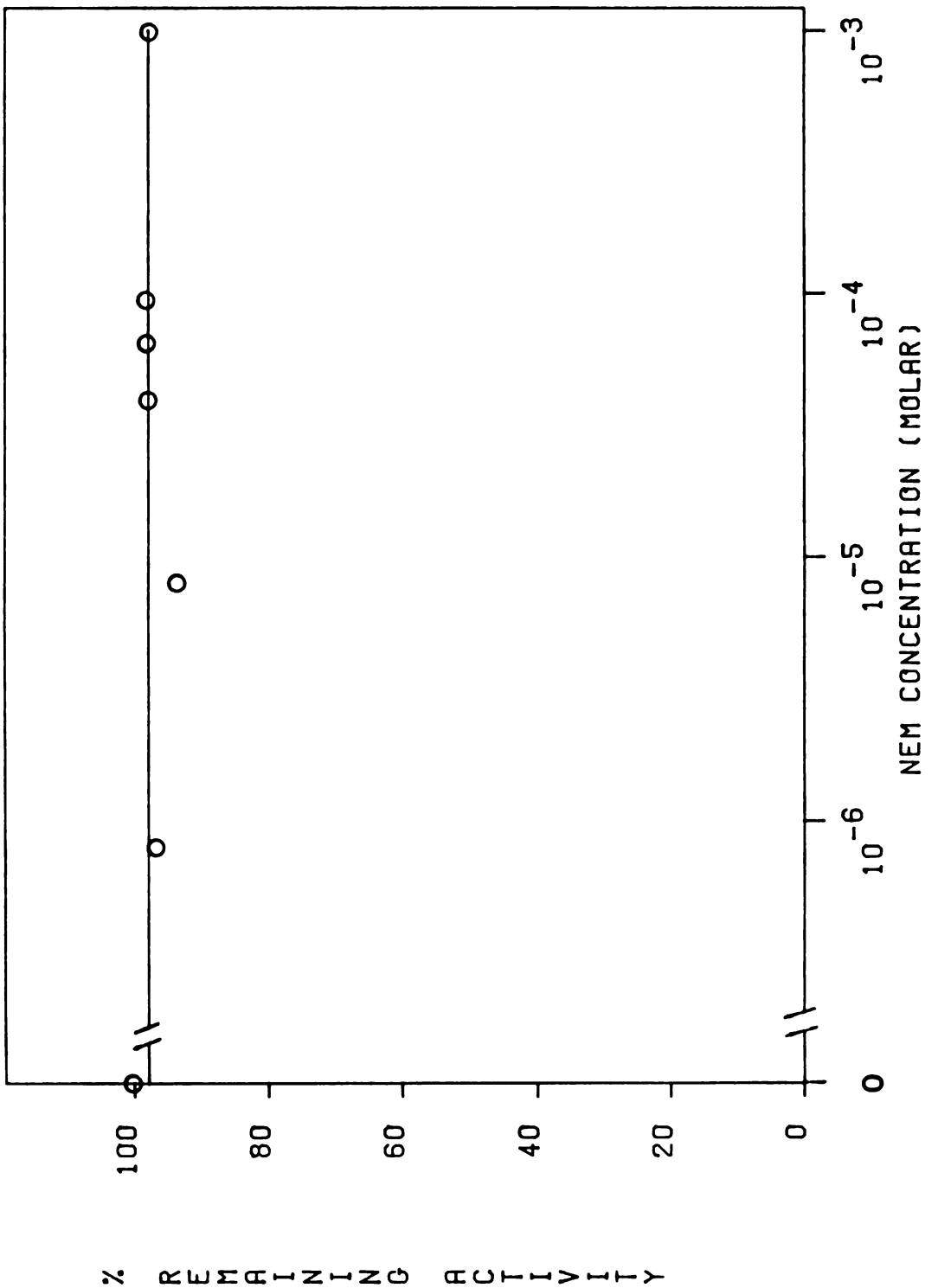

% INHIBITION
100
80
60
40
20
0
0
$10^{-6}$
$10^{-5}$
$10^{-4}$
$10^{-3}$
$10^{-2}$
$10^{-1}$
$10^{0}$
NEM CONCENTRATION (MOLAR)

Figure 8. The Effect of N-ethylmaleimide Upon Yeast Inorganic Pyrophosphatase Activity. The effects of NEM upon yeast inorganic pyrophosphatase were determined as described in Methods. NTPH was omitted from this experiment and 0.1 mM inorganic pyrophosphate was used as the substrate.


% REMAINING ACTIVITY
100
80
60
40
20
0
0
$10^{-6}$
$10^{-5}$
$10^{-4}$
$10^{-3}$
NEM CONCENTRATION (MOLAR)

terminating the NEM reaction at varying levels of inhibition have no effect upon NTPH activity determinations.

3. Effect of Incubation Time Upon $MgCl_2$ Destabilization of NTPH

In order to gain further insight into the role of sulfhydryl groups in the catalytic activity of NTPH, we decided to determine whether the substrate, in one of several possible forms (ITP, MgITP or $Mg_2ITP$), protected against inactivation by NEM.

Control experiments were first performed in which NTPH, in the absence of NEM, was incubated with 0.5 mM ITP and/or $MgCl_2$ in concentrations ranging from 0 to 10 mM (see Methods, section 2.b.3a.). The enzyme was stable under these conditions, and unreactive toward either ITP or ITP complexed with $MgCl_2$. Incubation with $MgCl_2$ alone, however, destabilized NTPH, as shown in Figure 9. The time course of this process was investigated as described in Methods. The results, given in Figure 10, indicate that incubation of the enzyme for 120 minutes, and in the absence of $MgCl_2$, results in loss of up to 18.5% of its activity. Such an incubation, when performed in the presence of $MgCl_2$, on the other hand, results in loss of up to 33% of the enzyme activity. These losses of activity become negligible when the incubation time is shortened to only 5 minutes. It is of further note that prolonged incubations appear to destabilize the enzyme, resulting in erratic activity determinations. This effect is much more pronounced in the presence of $MgCl_2$.

Figure 9. The Effect of Incubating NTPH with $MgCl_2$. C-50 purified NTPH was incubated with concentrations of $MgCl_2$ ranging from 0 to 10 mM as described in Methods. Following a 15 minute incubation period, 2 mM DTT was added to the reaction. NTPH activity was then determined under the standard assay conditions.

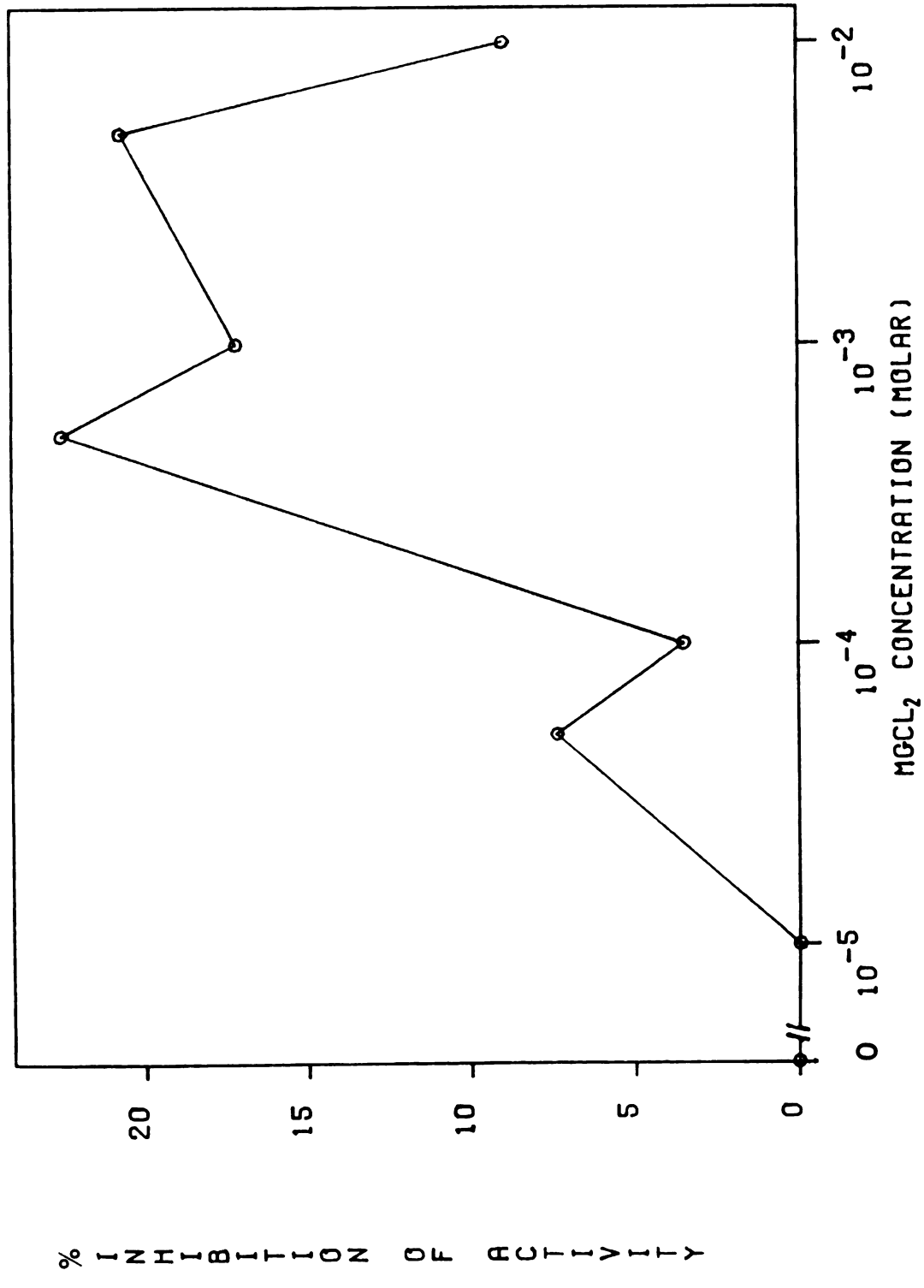
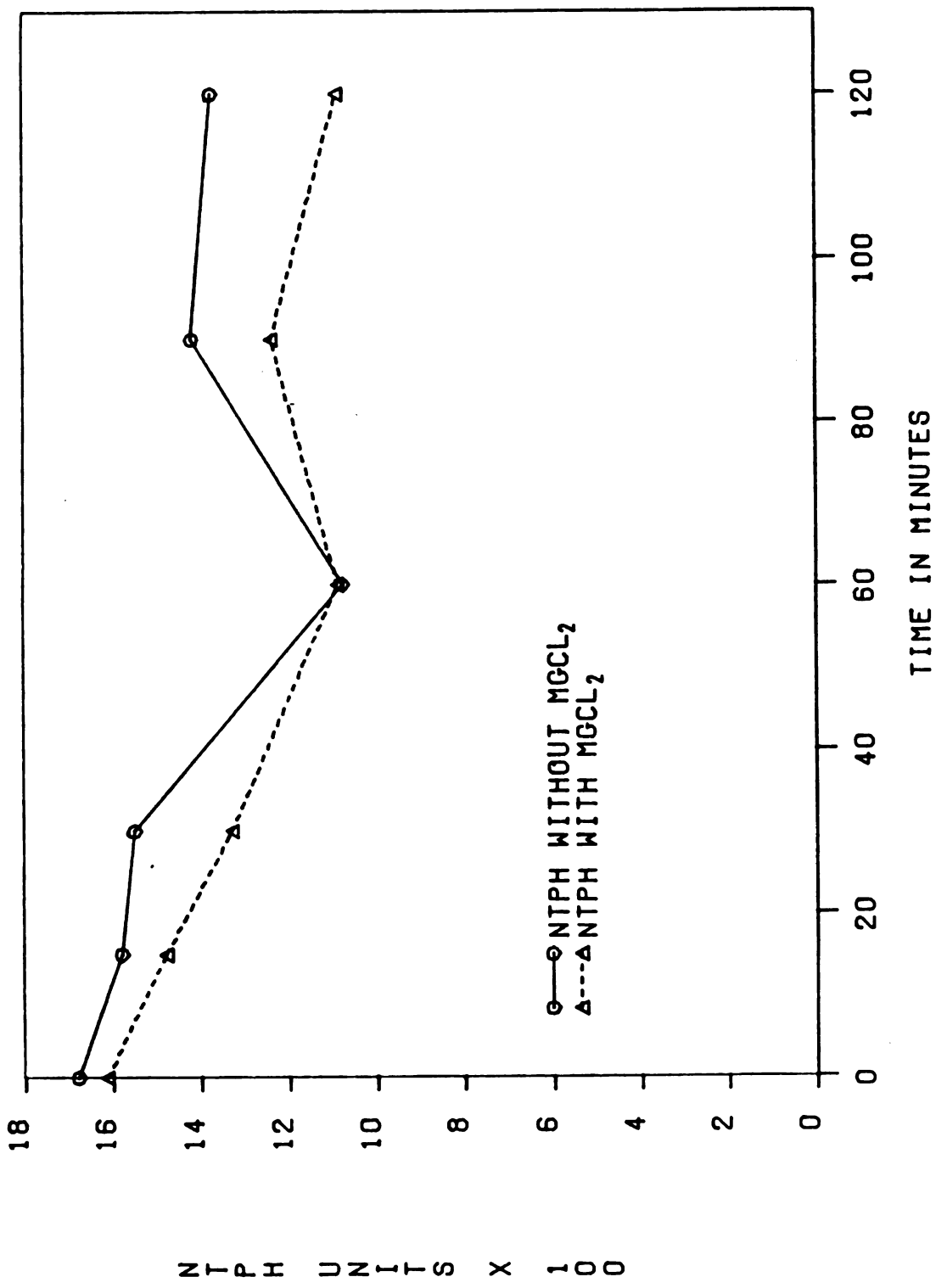

% INHIBITION OF ACTIVITY
0
5
10
15
20
0
$10^{-5}$
$10^{-4}$
$10^{-3}$
$10^{-2}$
$MGCL_2$ CONCENTRATION (MOLAR)

Figure 10. Destabilization of NTPH by 10 mM $MgCl_2$: Effects of Incubation Time. C-50 purified NTPH was incubated, at 4°C, with 0 and 10 $MgCl_2$, as described in Methods. At timed intervals, 2 mM DTT was added to the reaction. NTPH activity was then determined under the standard assay conditions.

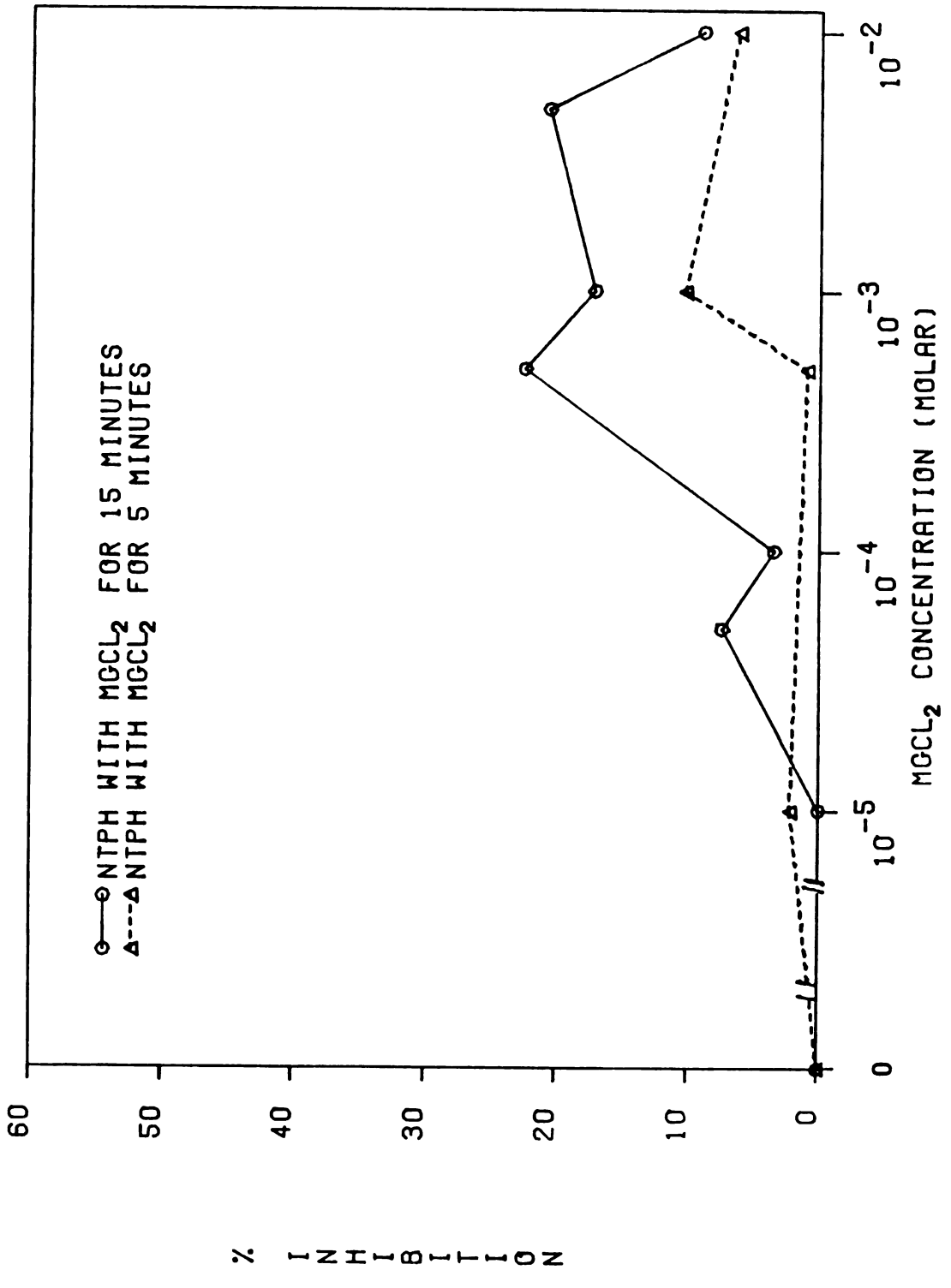

NTPH UNITS X 100
18
16
14
12
10
8
6
4
2
0
0
20
40
60
80
100
120
TIME IN MINUTES
NTPH WITHOUT MGCL2
NTPH WITH MGCL2

On the basis of these results, the effects upon NTPH of a 5 minute incubation with various concentrations of $MgCl_2$ were investigated. As shown in Figure 11 (open triangles), much of the enzyme instability has been removed by decreasing the incubation time from 15 minutes to 5 minutes. The open circles in this figure repeat the results given in Figure 9, and are presented for comparison.

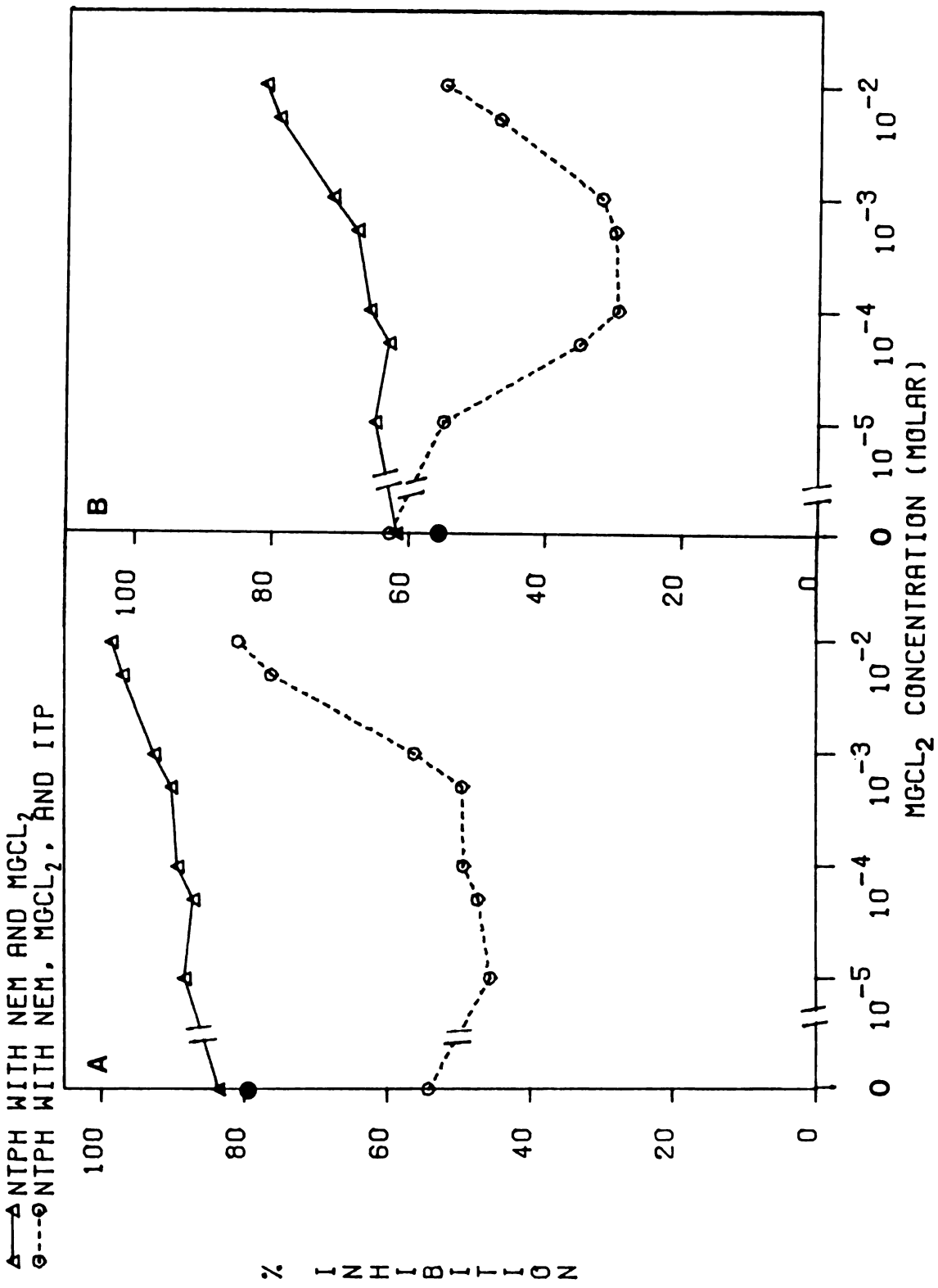
4. Effects of $MgCl_2$ and ITP Upon NTPH Inactivation by N-ethylmaleimide

It was now considered possible to pursue the original goal of investigating the effects of ITP and/or $MgCl_2$ upon NEM inhibition of NTPH. Basically, these experiments (described in Methods, section 2.b.4.) involved incubating NTPH with NEM plus 0.5 mM ITP and/or $MgCl_2$ concentrations ranging from 0 to 10 mM. An additional experiment consisted of incubating NTPH with NEM and the various concentrations of $MgCl_2$; ITP was not present. The results are given in Figures 12A and 12B, and represent 2 sets of incubations using 2 different concentrations of NEM. The effects of $MgCl_2$ upon the inhibition reaction seem fairly consistent between the 2 experiments, revealing that the high concentrations of $MgCl_2$ enhance the reaction by approximately 20%. The effects of ITP upon NEM inhibition, however, are very ambiguous. In one instance, ITP confers up to 30% protection of the enzyme, and in another instance, it enhances the inhibition nearly 10%. The presence of both ITP and $MgCl_2$ result in protection of the enzyme from inhibition, but again, the exact form of this protection is not consistent between the two experiments.

Figure 11. Destabilization of NTPH by Various Concentrations of $MgCl_2$. C-50 purified NTPH was incubated, at 4°C, with concentrations of $MgCl_2$ ranging from 0 to 10 mM. Following a 5 minute incubation period, 2 mM DTT was added to the reaction. NTPH activity was then determined under the standard assay conditions. The results are represented by the open triangles; the open circles represent the results shown in Figure 9, and are presented for comparison.


NTPH WITH MGCL2 FOR 15 MINUTES
NTPH WITH MGCL2 FOR 5 MINUTES
% INHIBITION
60
50
40
30
20
10
0
0
10-5
10-4
10-3
10-2
MGCL2 CONCENTRATION (MOLAR)

Figures 12A and B. The Effect of $MgCl_2$ and ITP Upon N-ethylmaleimide Inhibition of NTPH. C-50 purified NTPH was incubated with NEM, ITP and $MgCl_2$ as described in Methods. Figures A and B indicate that two different concentrations of N-ethylmaleimide were used in these studies: in Figure A, 3.5 mM NEM wa used, and in Figure B, 1.5 mM NEM was used. All other conditions were constant between the two experiments. The open circles represent incubation of NTPH with NEM, 0.5 mM ITP, and varying amounts of $MgCl_2$; the open triangles represent incubation of NTPH with NEM and varying amounts of $MgCl_2$ (no ITP is present). The solid circle/triangle represents the level of inhibition caused by incubation of NTPH with only NEM, no ITP or $MgCl_2$ present.


NTPH WITH NEM AND MGCL2
NTPH WITH NEM, MGCL2, AND ITP
% INHIBITION
A
B
100
80
60
40
20
0
0
10-5
10-4
10-3
10-2
MGCL2 CONCENTRATION (MOLAR)

The work thus far accomplished had demonstrated that NTPH is unstable in the absence of sulfhydryl reducing agents, and that EDTA can function as a substitute for such reagents to maintain enzyme stability for up to 40 minutes. Analysis of the reaction between N-ethylmaleimide had led to the combined problems of ambiguous results and enzyme instability. Therefore, it was decided to analyze the complete time course of NTPH inactivation by NEM, and to include 0.25 mM EDTA in all such studies.

5. Rate Analysis of the Reaction Between NTPH and N-ethylmaleimide

The reaction between NTPH and various concentrations of NEM was documented as a function of time, and yielded the results given in Figure 13. It can be seen that 0.5 mM concentrations of NEM do not completely inactivate the enzyme within 60 minutes. 100% Inhibition does occur, however, in the presence of 1.0 and 2.0 mM NEM. If the change in activity is plotted logarithmically as a function of time, as in Figure 14, the results yield a series of pseudo first order rate constants, k'. The values for these constants increase linearly as a function of their corresponding N-ethylmaleimide concentrations (see inset to Figure 14) and yield a second order rate constant of $9.08 \times 10^{-5}$ $M^{-1}$ $min^{-1}$ for the overall process of sulfhydryl derivatization.

6. Effect of $MgCl_2$ and/or ITP Upon the Time Course of NTPH Inactivation by N-ethylmaleimide

The effects of $MgCl_2$ and ITP upon the rate of inhibition were investigated as described in methods. The effects of various concentrations of $MgCl_2$ are as shown in Figure 15. While in

Figure 13. The Time Course of NTPH Inactivation by N-ethylmaleimide. The time course of NTPH inactivation by NEM was documented as described in Methods. The reaction rate was measured for 3 different concentrations of N-ethylmaleimide, as indicated in the figure.

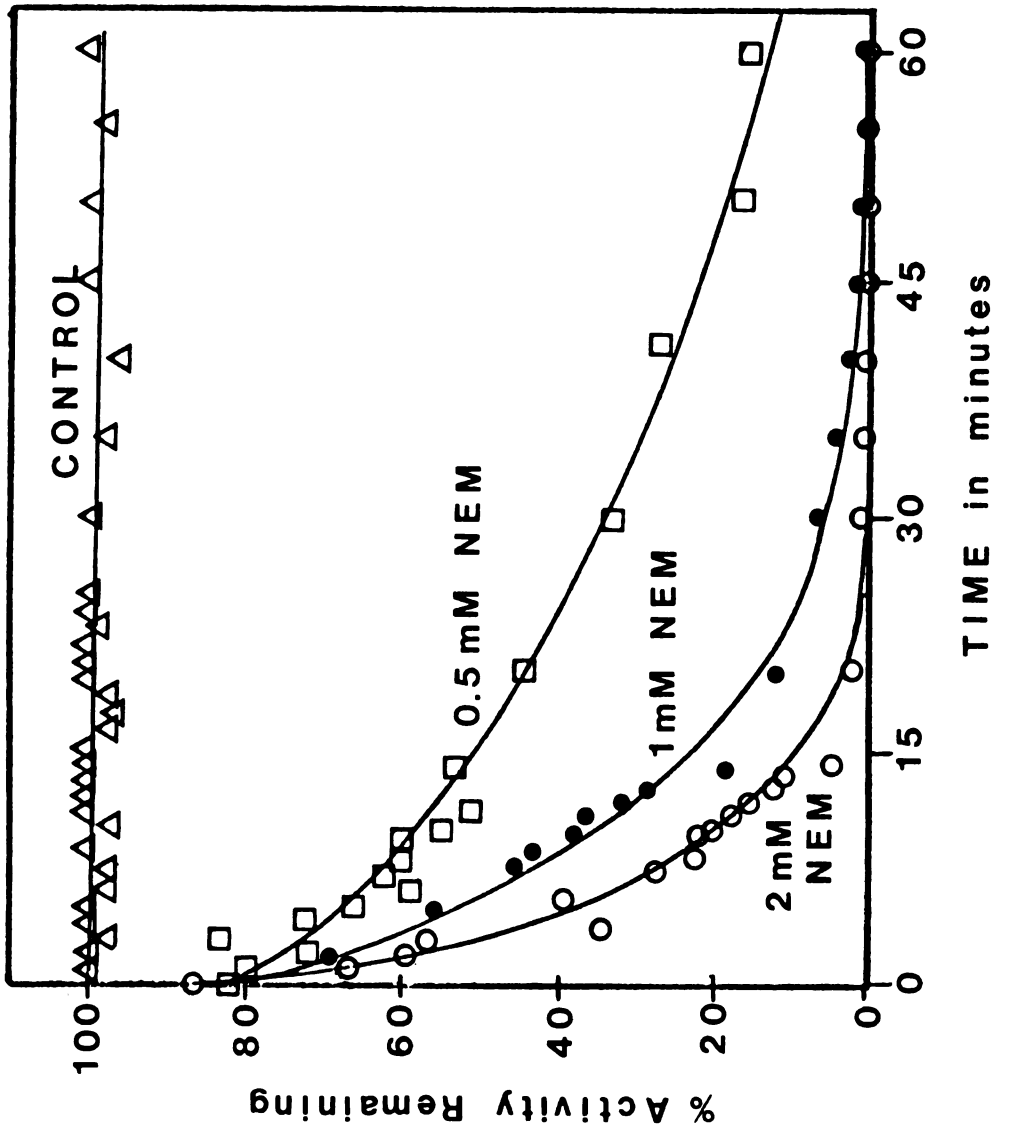

CONTROL
0.5 mM NEM
1 mM NEM
2 mM NEM
% Activity Remaining
TIME in minutes
0
20
40
60
80
100
0
15
30
45
60

Figure 14. Rate Analysis of NTPH Inactivation by N-ethylmaleimide. The results shown in Figure 13 are shown here to yield pseudo first-order rate constants for the inhibition reaction. The inset shows these pseudo first-order rate constants as a function of the N-ethylmaleimide concentration.

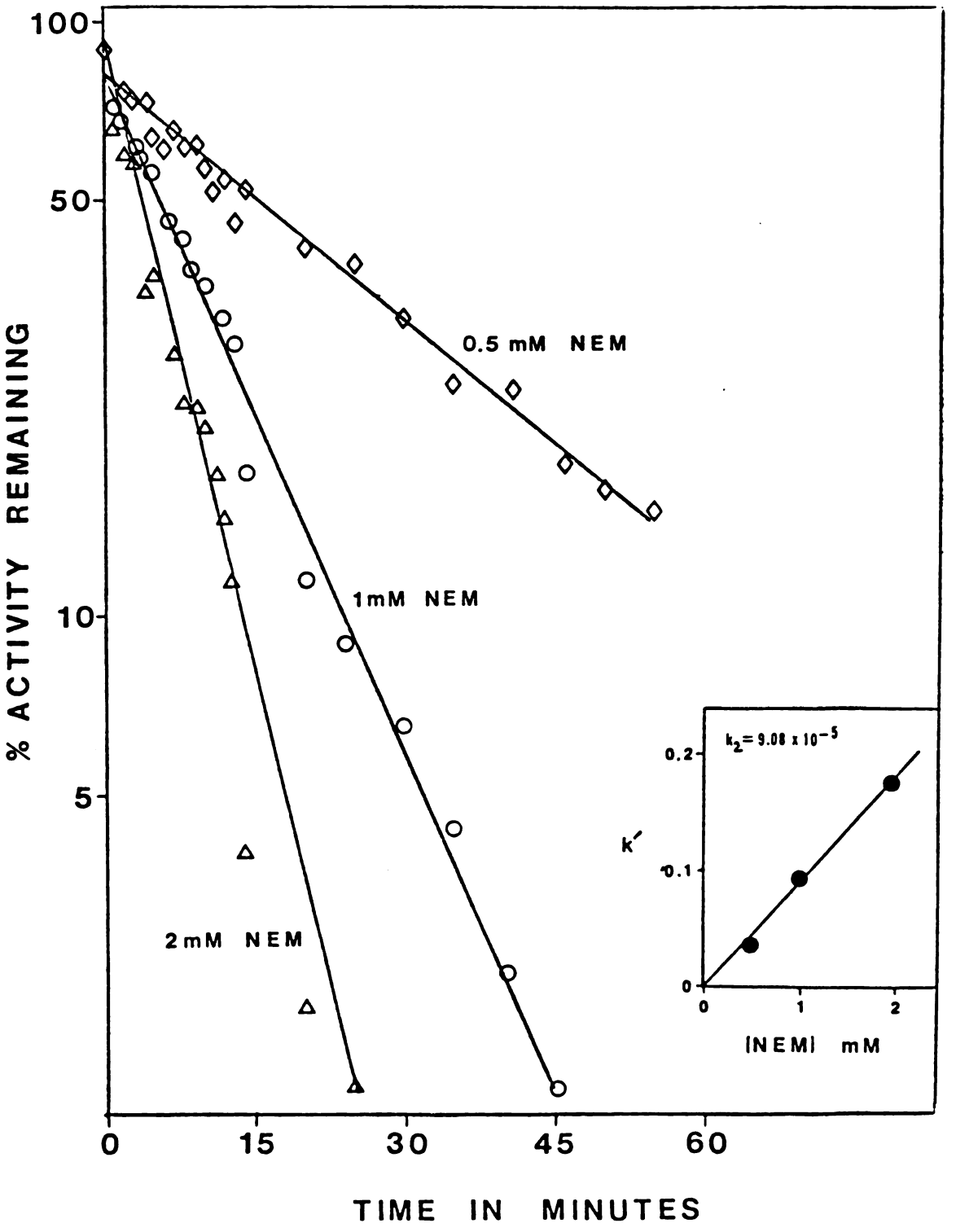
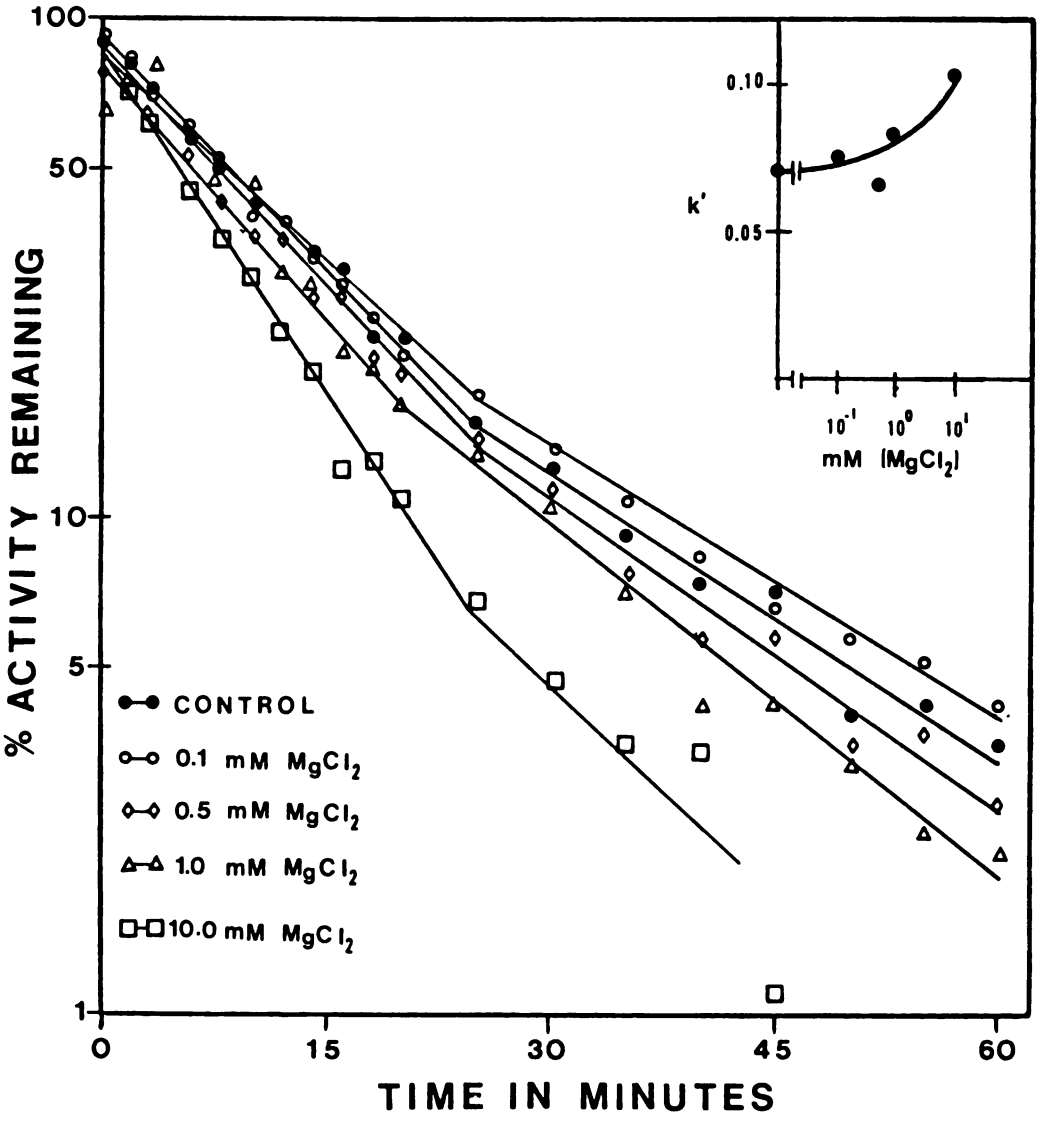

100
50
10
5
% ACTIVITY REMAINING
0.5 mM NEM
1mM NEM
2mM NEM
0
15
30
45
60
TIME IN MINUTES
$k_2 = 9.08 \times 10^{-5}$
k'
0.2
0.1
0
0
1
2
[NEM] mM

Figure 15. The Effect of $MgCl_2$ Upon the Time Course of NTPH Inactivation by 1.0 mM N-ethylmaleimide. The effect of $MgCl_2$ concentration upon the inactivation reaction is shown to yield pseudo first-order rate constants for the reaction which results in up to 85% inhibition of NTPH activity. The inset shows these pseudo first-order rate constants as a function of $MgCl_2$ concentration.


100
50
10
5
1
% ACTIVITY REMAINING
0
15
30
45
60
TIME IN MINUTES
CONTROL
0.1 mM MgCl2
0.5 mM MgCl2
1.0 mM MgCl2
10.0 mM MgCl2
0.10
0.05
k'
10-1
100
101
mM (MgCl2)

each case examined, 100% inhibition is achieved, the lower concentrations of $MgCl_2$ show only small variations (± 10%) in the rate at which this process occurs. Conversely, 10 mM $MgCl_2$ increases the reaction rate by 58%. This confirms our earlier results, in which enhanced inhibition occurred only at the higher (i.e., 5 and 10 mM) concentrations of $MgCl_2$.

Figure 15 also demonstrates that, at the higher levels of inhibition, and in the presence of $MgCl_2$, the reaction appears to become biphasic, with a rate which is slower than that seen at the lower levels of inhibition. Unfortunately, however, the limits of phosphate detection in our assay make these correspondingly low values for enzyme activity questionable. Therefore, rate constants were obtained for the reaction which effected up to 85% inhibition. These pseudo first order rate constants are included in Table 3, which summarizes the results of this experiment.

The effect of ITP upon the reaction between NEM and NTPH is as shown in Figure 16. The velocity determinations were made by measuring the levels of activity which were present following 0, 5, and 10 minutes of reaction time. The effects of 0 and 0.5 mM ITP upon three different concentrations of NEM were examined. These data again yield pseudo first-order rate constants for the inhibition reaction. In the presence of 0.5 mM ITP, these rate constants are consistently of a higher value. Table 4 summarizes these results, and demonstrates the effects of ITP to be inversely proportional to the concentration of NEM.

In a similar experiment to the two described above, 0.5 mM ITP and 0.5 mM $MgCl_2$ were added to an NEM reaction mixture, and the time

Table 3. Summary of the Effects of $MgCl_2$ Upon the Reaction Between NTPH and N-Ethylmaleimide

| mM[$MgCl_2$] | k' | % Control |
|---|---|---|
| 0 | .0709 | 100 |
| 0.1 | .0744 | +4.8 |
| 0.5 | .0654 | -7.9 |
| 1.0 | .0815 | +10.0 |
| 10.0 | .1124 | +58.53 |

Figure 16. The Effect of ITP Upon the Time Course of NTPH Inactivation by N-ethylmaleimide. ITP is shown to yield pseudo first-order rate constants for the inhibition reaction. The effects of 0.5 mM ITP was determined for 3 different NEM concentrations, as is shown in the figure. Open circles represent reactions which were conducted in the absence of ITP, and closed circles represent reactions conducted in the presence of 0.5 mM ITP.

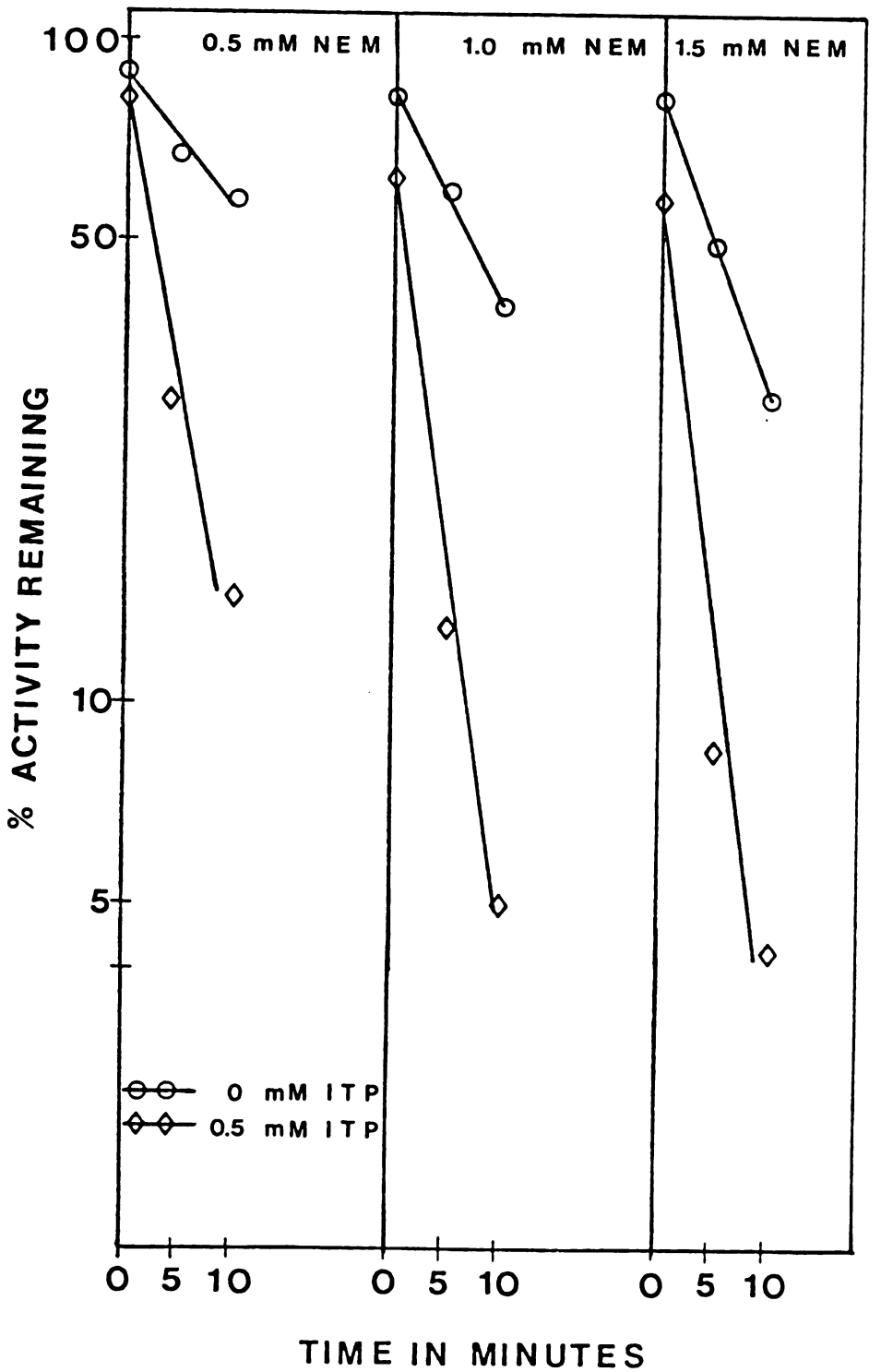

100
50
10
5
0.5 mM NEM
1.0 mM NEM
1.5 mM NEM
% ACTIVITY REMAINING
0 mM ITP
0.5 mM ITP
0 5 10
0 5 10
0 5 10
TIME IN MINUTES

Table 4. Summary of the Effects of ITP Upon the Reaction Between NTPH and N-Ethylmaleimide

| mM[NEM] | mM[ITP] | k | Fold Increase Over Control |
|---|---|---|---|
| 0.5 | 0 | .044 | |
| | 0.5 | .1743 | 3.97 |
| 1.0 | 0 | .0725 | |
| | 0.5 | .2460 | 3.39 |
| 1.5 | 0 | .1040 | |
| | 0.5 | .2522 | 2.52 |

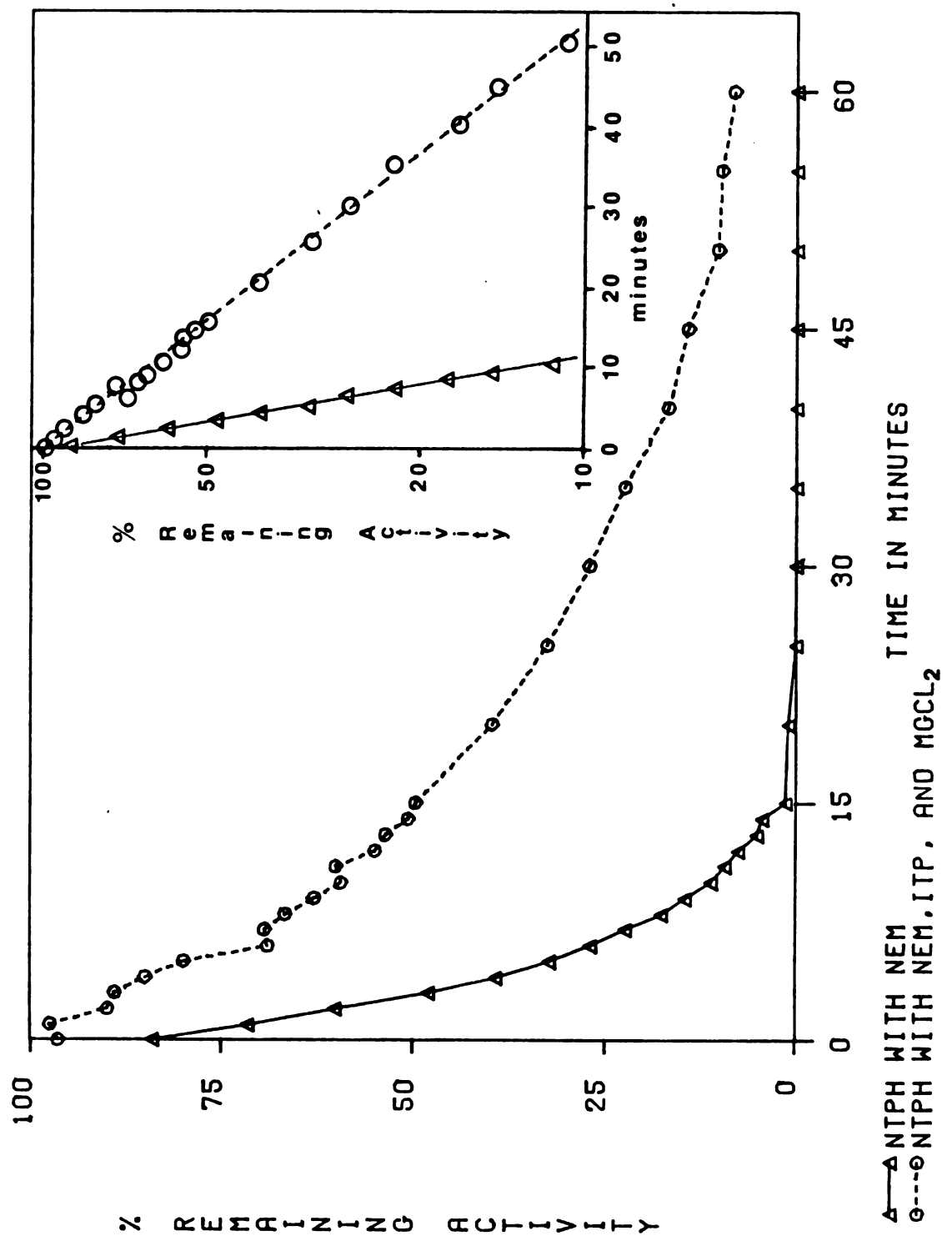
course of the reaction was followed. These results are given in Figure 17. The inset to this figure shows the data to again yield pseudo first-order rate constants, with ITP and $MgCl_2$ effecting a 5-fold decrease in the rate of the inhibition reaction.

These results, therefore, show that N-ethylmaleimide can completely inhibit NTPH. It has further been demonstrated that 0.5 mM ITP or concentrations of $MgCl_2$ in excess of 1.0 mM will measurably enhance the inactivation, while 0.5 mM concentrations of both ITP and $MgCl_2$ together will substantially protect NTPH from inactivation. Both the time course of the inhibition reaction and its experimental reproducibility have, therefore, been established. It was now considered possible to investigate, again, the role assumed by ITP (in one of several possible forms) during the process of sulfhydryl derivatization.

7. Effect of ITP and/or $MgCl_2$ Upon N-ethylmaleimide Inhibition of NTPH

Basically, the method which was followed in these experiments consisted of incubating NTPH, in the presence of 0.25 mM EDTA, with 3.5 mM NEM and 0.5 mM ITP, concentrations which effected loss of 100% of the enzyme activity. $MgCl_2$ was present in these incubations, in concentrations ranging from 0 to 10 mM. An additional experiment was conducted in a manner identical to that just described, with the exception that ITP was not present. The results of these experiments are given in Figure 18, where the % inhibition is plotted as a function of the $MgCl_2$ concentrations. It can again been seen that both 0.5 mM ITP and $MgCl_2$, in concentrations exceeding 1 mM, enhance the

Figure 17. The Effect of ITP and $MgCl_2$ Upon the Time Course of NTPH Inactivation by N-ethylmaleimide. The combined effects, upon the N-ethylmaleimide reaction, of 0.5 mM ITP and 0.5 mM $MgCl_2$ were documented as described in Methods. The inset shows these reactions to yield pseudo first-order rate constants.

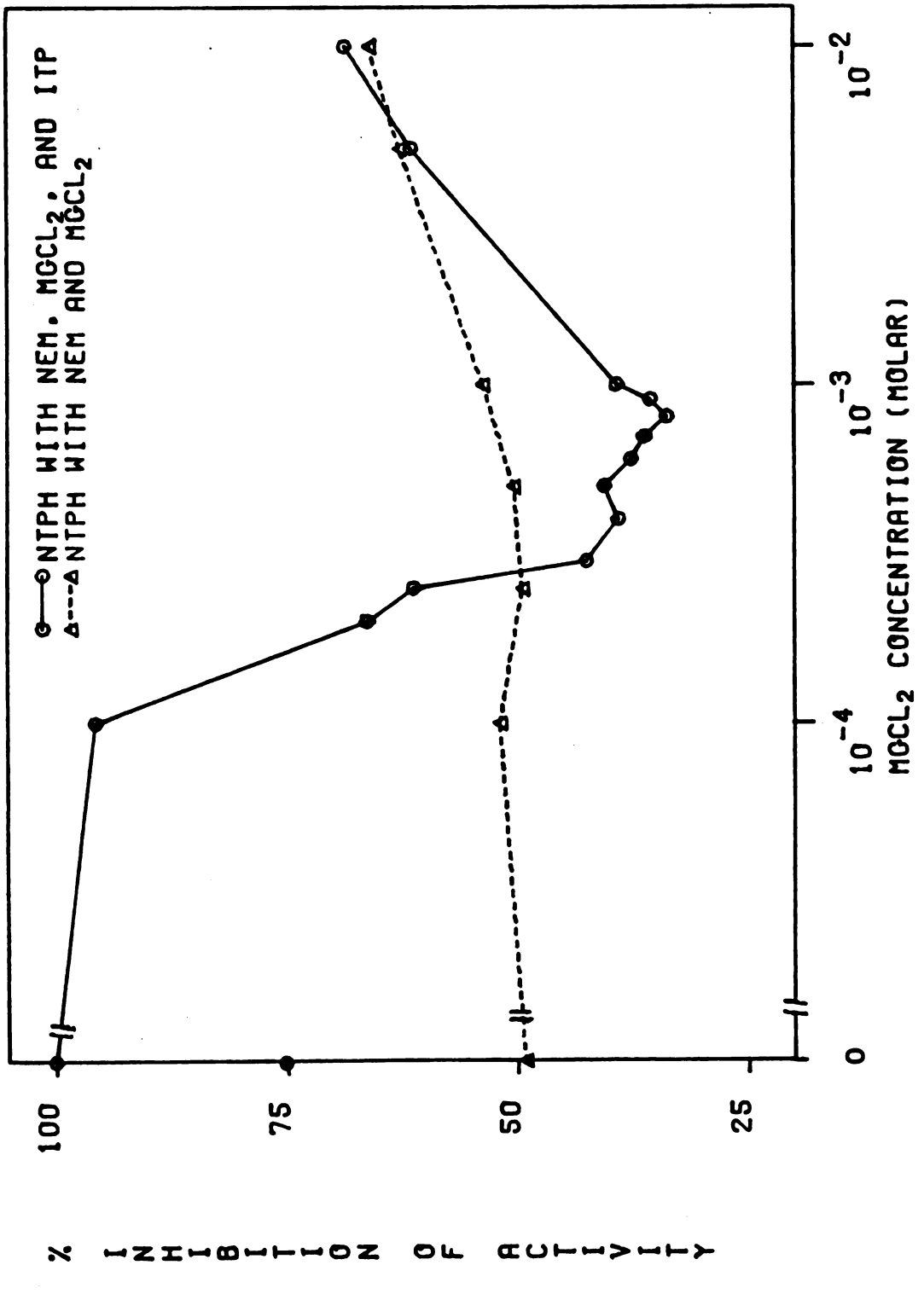

% REMAINING ACTIVITY
100
75
50
25
0
0
15
30
45
60
TIME IN MINUTES
NTPH WITH NEM
NTPH WITH NEM, ITP, AND MGCL2
% Remaining Activity
100
50
20
10
0
10
20
30
40
50
minutes

inhibition reaction. The presence of both reagents together, however, confers up to 70% protection from the effects of N-ethylmaleimide.

It will be recalled that the results of this same experiment, performed in the absence of EDTA, were presented earlier and shown to be ambiguous (see Figures 12A and 12B). By comparison, the results of the present work have proven to be exactly reproducible, and indicate that a metal contaminant, present in the earlier experiments, has been removed by chelation with EDTA. The exact nature and concentration of this contaminant are not known at this time. Nonetheless, it was now considered possible to examine in detail the roles assumed by the various species present during the course of this inhibition study.

A computer analysis was used to calculate the concentrations of the various species present in this system (see Methods). When considering the information thus obtained, it must be remembered that a lack of knowledge concerning the concentration of the contaminant dictates also a lack of knowledge concerning the exact concentration of uncomplexed EDTA. The latter will be available for binding of the free $Mg^{++}$ which is introduced into the system in the form of $MgCl_2$. The assumption was made that the contaminant was present in very small amounts by comparison with the total concentration of EDTA. Therefore, the calculated concentrations of $Mg^{++}$, $ITP^{4-}$, $MgITP^{2-}$ and $Mg_2ITP$ were determined by assuming <u>all</u> the EDTA to be available for the binding of Magnesium. This, in turn, defines the calculated concentrations of the above-mentioned components to be the lower limits upon their actual concentrations. It should be pointed out, however, that the concentration of the contaminant is <u>not</u> small in comparison to the calculated or actual concentrations of these species. This being

Figure 18. The Effect of ITP and/or Various Concentrations of $MgCl_2$ Upon N-ethylmaleimide Inhibition of NTPH. The effects of 0.5 mM ITP and various concentrations of $MgCl_2$ upon NEM inhibition of NTPH (see Methods) are depicted by the open circles in the figure. The closed circle indicates NTPH activity in the presence of N-ethylmaleimide alone, without ITP or $MgCl_2$. The effects of $MgCl_2$ (no ITP present) upon NEM inhibition of NTPH are shown with the open triangles the closed triangle represents the reaction between NTPH and N-ethylmaleimide alone, no $MgCl_2$ being present.


NTPH WITH NEM. $MGCL_2$. AND ITP
NTPH WITH NEM AND $MGCL_2$
% INHIBITION OF ACTIVITY
100
75
50
25
0
$10^{-4}$
$10^{-3}$
$10^{-2}$
$MGCL_2$ CONCENTRATION (MOLAR)

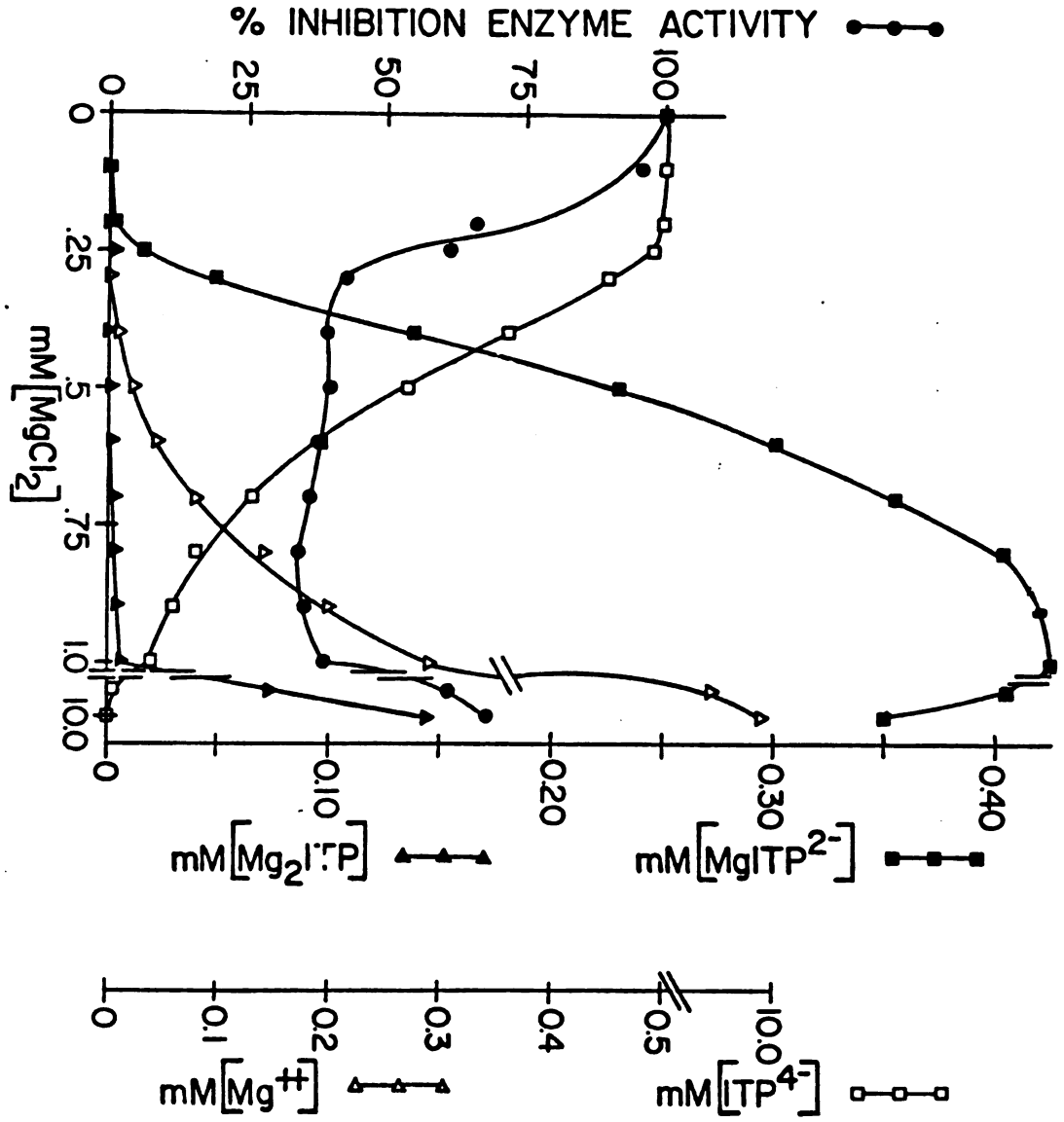
the case, even small variations in the contaminant concentration will result in very large variations in the concentrations of metal, nucleotide, and their associated complexes. This fact should be borne in mind throughout the remainder of this thesis.

The combined results of the inhibition study and of the computer analysis are summarized in Figure 19. At 100% enzyme inactivation, and in the absence of $MgCl_2$, the predominant species present is $ITP^{4-}$, as would be expected. At increasing concentrations of $MgCl_2$, the concentration of $ITP^{4-}$ decreases, while increasing amounts of $MgITP^{2-}$ are to be found. In spite of the fact that $ITP^{4-}$ remains the predominant species, the presence of even small amounts of MgITP coincides with a marked decrease in enzyme inhibition. Upon the addition of 0.3 mM $MgCl_2$, the inhibition from NEM has decreased from 100% to 42%, while the concentration of $MgITP^{2-}$ has become 0.048 mM. Between 0.3 and 1.0 mM concentrations of $MgCl_2$, this maximum level of protection remains relatively constant, in spite of increasing concentrations of $MgITP^{2-}$, $Mg_2ITP$ and $Mg^{++}$.

In the presence of higher (i.e., 5 and 10 mM) concentrations of $MgCl_2$, the % inhibition exhibits a dramatic 30% increase. Although the $MgITP^{2-}$ concentration is decreasing at this point (with a comensurate increase in $Mg_2ITP$) the amount present remains equal to that which earlier provided c.a. 65% protection from inhibition. Clearly, another factor is influencing the process of enzyme inactivation.

Previous work has shown that greater than 1 mM concentrations of $MgCl_2$ will enhance the inhibitory effects of N-ethylmaleimide upon NTPH. That evidence, and the results presented in Figure 17, define a

Figure 19. Relationships Between N-ethylmaleimide Inhibition of NTPH and the Calculated Concentrations of Metal, Nucleotide, and Metal-Nucleotide Complexes. The results shown in Figure 18 were combined with the results of a computer analysis of the various species ($ITP^{4-}$, $MgITP^{2-}$, MgITP, and $Mg^{++}$) present during the inhibition reaction. The % inhibition and the mM concentrations of these species are plotted as a function of the $MgCl_2$ concentration which was used in the inhibition experiment: closed circles represent the per cent inhibition of enzyme activity; close squares represent the $[MgITP^{2-}]$; closed triangles indicate the [MgITP]; open circles represent the $[ITP^{4-}]$; open squares represent the $[Mg^{++}]$.


% INHIBITION ENZYME ACTIVITY
0
25
50
75
100
mM[MgCl2]
0
.25
.5
.75
1.0
10.0
mM[Mg2ITP]
0
0.10
0.20
0.30
0.40
mM[MgITP2-]
mM[Mg+]
0
0.1
0.2
0.3
0.4
0.5
10.0
mM[ITP4-]

situation in which high $Mg^{++}$ concentrations both promote the formation of $Mg_2ITP$ (thus effectively removing some of the MgITP) and interact with NTPH itself so as to enhance the inhibitory effects of NEM.

It has thus been shown that both $ITP^{4-}$ and $Mg^{++}$ enhance the inhibition of NTPH by N-ethylmaleimide, while $MgITP^{2-}$ protects the enzyme from this inhibition. It has further been demonstrated that high $Mg^{++}$ concentrations promote the formation of $Mg_2ITP^{2-}$, while decreasing the $MgITP^{2-}$ concentration. The presence and nature of any interactions between $Mg_2ITP$ and NTPH have not been clearly demonstrated.

## DISCUSSION

Nucleoside triphosphate pyrophosphohydrolase is a unique enzyme having the ability to catalyze the hydrolysis of pyrophosphate from several nucleotide triphosphates. Since its discovery, the enzyme has been partially purified from several sources (1,2,3). Purification to homogeneity and delineation of its function, however, have proven difficult, particularly in the case of human erythrocyte NTPH. Recent reports have been ambiguous as to this enzyme's requirement for a reduced thiol(s), a factor which could be crucial in future attempts to purify and more clearly define the properties of this enzyme. All of the above dictated the need of a general review of those factors which are involved in maintaining optimum stability and activity in NTPH.

The requirement of this enzyme for an active - i.e., reduced - thiol group(s) was demonstrated in a variety of ways. Initially, this consisted of reactivating with DTT NTPH which had been freed of sulfhydryl-reducing reagents present in the storage buffer. EDTA under certain prescribed conditions quantitatively reproduced the effects demonstrated by DTT, implying that metal-catalyzed oxidation of these sulfhydryl groups may be occurring. Later studies demonstrated that NTPH is both unstable and, over a period of 1-2 hours, capable of losing at least 20% of its activity in the absence of DTT. $MgCl_2$

markedly accentuates this process of destabilization and inactivation. The presence of a contaminant in our system compounded the above problems by creating ambiguous results in the early investigations of the reaction between N-ethylmaleimide and NTPH. The addition of EDTA to the reaction mixture, however, yielded both reproducible results and enzyme which was stable for up to one hour in the absence of DTT and under a variety of conditions. This, in turn, made possible a more detailed analysis of the role played by thiol(s) in the catalytic mechanism of NTPH activity.

Before discussing the results of such work, it is important to emphasize that thiol groups which are required for catalysis may function from a variety of locations upon the enzyme. It follows, therefore, that derivatization of such groups may inactivate an enzyme in several ways. Only one of these will be as a result of derivatizing a thiol located at the active site. Stearic hindrance of a substrates' binding onto the enzyme frequently occurs when bulky reagents interact with sulfhydryl groups located near, but not at, the active site (66). Derivatization of thiols which are located some distance from the active site may either cause, or prevent from occuring, a conformational change in the enzyme. In both these cases, loss of sensitivity occured through loss of sulfhydryl groups which are not located at the active site.

Classically, such difficulties have been overcome by making use of the phenomenon of protection by competition with the substrate (66). If the enzyme is saturated with substrate before treatment with the derivatizing reagent, the groups at the substrate-binding site will be combined with substrate and masked against attack by the reagent. Even

under these circumstances, however, and in the absence of additional data, it remains conceivable that association of a substrate with its binding site can effect a conformational change which, in turn, protects from derivatization sulfhydryls which are located adjacent to, but not at, this site on the enzyme.

Rate analysis of the reaction between N-ethylmaleimide and NTPH demonstrates that NTPH requires one or more reduced thiols for maximum activity. The exact number and location of such thiols has not been determined, however. Several equally accessible sulfhydryls may interact with a reagent at the same rate to yield pseudo first-order rate constants. The fact that the inhibition reaction yields such constants in both the presence and absence of $MgCl_2$ and ITP certainly implies that one thiol is required for maximum enzyme activity. The fact that NTPH, in the presence of $MgCl_2$ plus ITP, is protected from inhibition by N-ethylmaleimide indicates that this sulfhydryl group is located in, or adjacent to, the active site.

It was earlier shown, in Figure 14, that a second order rate constant of $9.08 \times 10^{-5}$ $M^{-1}$-$min^{-1}$ was obtained for the overall process of sulfhydryl derivatization. Given the presence of several proteins of indeterminate concentrations, and considering that the number of essential sulfhydryl groups required by NTPH is not known, it is difficult to comment upon the significance of this value.

All of the above work demonstrates that reduced sulfhydryl groups assume a complex rate in the mechanism of NTPH activity. The data given in Figures 18 and 19 suggest that free $Mg^{++}$, at low concentrations, does not bind to the enzyme so as to measurably affect its sulfhydryl groups. $ITP^{4-}$ on the other hand, does bind to the

enzyme in a way which markedly affects the availability of its thiol groups. $MgITP^{2-}$ binds to NTPH so as to confer partial protection against N-ethylmaleimide, an effect which is overcome by the presence of high concentrations of $MgCl_2$.

In assessing the relative importance of $MgITP^{2-}$ in protecting thiol(s) from reaction with NEM, it must be stressed that, even in the presence of increasing concentrations of $MgITP^{2-}$, NTPH demonstrated loss of up to 35% of its normal activity. One possible cause of such incomplete protection is the presence of competition reactions between $MgITP^{2-}$ and N-ethylmaleimide for thiols which are located at (or near) the substrate binding site. Another possibility includes the derivitization of sulfhydryls which are not at the binding site but which are none-the-less necessary for maximum catalytic activity. In either, or both of these situations, the binding of $MgITP^{2-}$ to NTPH may confer incomplete protection from inhibition by N-ethylmaleimide.

The coordination schemes which characterize metal-activated enzymes were discussed earlier (see Literature Review, part C). The observation that NTPH requires 10 mM $MgCl_2$ for optimum activity suggests that $Mg^{++}$ plays a role which is more complex that simple 1:2:1 stoichiometric addition to the enzyme and nucleotide. The coordination scheme best represented by the data is one which includes the possibility of $ITP^{4-}$ binding to the enzyme in the absence of free $Mg^{++}$. Were such an event to occur, NTPH would assume a new conformation, exposing one or more thiols which would otherwise be covered upon binding of the true substrate, $MgITP^{2-}$. The model must also include the interaction of mM $Mg^{++}$ so as to confer upon NTPH that conformation which is optimal for catalysis. Without

$Mg^{++}$, and in the presence of $MgITP^{2-}$, other groups may not be in the positions required for maximum activity. In the absence of further data, it is impossible to make a definitive statement as to the nature of the coordination scheme which accurately depicts the catalytic unit. The general indications are that different conformations exist for the native-enzyme, the ITP-enzyme complex, the $MgITP^{2-}$-enzyme complex, and the Mg-enzyme complex. How these conformations affect the rate and location of substrate and ligand binding is not presently known.

It has previously been mentioned that the Km of human erythrocyte NTPH for ITP is $2.21 \times 10^{-5}$ M (3). The present work, however, provides strong evidence that $MgITP^{2-}$, not $ITP^{4-}$, is the true substrate for NTPH. This being the case, the kinetic data which originally defined this Km, in conjunction with the computer program, was used to calculate the corresponding concentrations of $MgITP^{2-}$. This information indicated that the Km of human erythrocyte NTPH for $MgITP^{2-}$ is $1.1 \times 10^{-5}$ M. Work presented in this thesis has demonstrated that the presence of only $1 \times 10^{-6}$ M $MgITP^{2-}$ confers upon NTPH approximately 50% of the total protection of the enzyme from N-ethylmaleimide. If one thiol is necessary for optimum enzyme activity, and if the addition of substrate $MgITP^{2-}$ protects this thiol from interaction with NEM, one would expect 50% protection to be achieved in the presence of substrate concentrations equivalent to the $K_m$ of NTPH for $MgITP^{2-}$. Several explanations for why this does not occur are possible. The original value for the $K_m$ was determined at the lower limits of the standard, end-point phosphate analysis, and this may have been inaccurate. The calculated

concentrations of the various metal-nucleotide complexes may also have been inaccurate. As is evident in Appendix A, the computer program which was used to calculate these concentrations utilized published stability constants which had been determined under a variety of conditions. These constants are not automatically applicable to the case in point, particularly when one considers that all of the work presented in this thesis was performed at pH 9.5. Previous mention has been made (see Results) of the presence of an unknown contaminant in our system, and the assumptions which were made about its concentration when utilizing the computer program. It is important to stress that even small variations in the concentration of this unknown metal will lead to large variations in the concentrations of the metal, nucleotide, and metal-nucleotide complexes present in our system. This uncertainty will persist until such time as the exact identity and amount of contaminating metal are known. Finally, it is important to note that protection was achieved in the presence of relatively high (3.5 mM) concentrations of N-ethylmaleimide. Lower inhibitor concentrations or prolonged incubation times, may indeed, vary the pattern of protection shown in these experiments. This in turn would imply that the catalytically active form of the coordination complex has other requirements than substrate binding alone. In vitro, this is perhaps reflected by the high $Mg^{++}$ and pH requirements of this enzyme. In vivo, this requirement would relate to the function of the enzyme within the cell. An example of such a situation concerns the proposal by Morris and Wang (2) that the function of NTPH is to prevent the incorporation of ITP into RNA or dITP into DNA. If this were the true role of NTPH, it follows that fluctuations in the level

of nucleic acid synthesis could markedly affect the relationship between binding of substrate and release of product. During periods of increased nucleic acid synthesis, the Ks for substrate binding would more closely approximate the Km. Conversely, during periods of decreased nucleic acid synthesis, actual catalysis, with subsequent product release, may be measurably less than the Ks for binding of the substrate to the enzyme.

When considering these proposals, it is important to stress that conclusive evidence would only be provided with a more detailed analysis than is presented here. This would include performing binding studies, reacting NTPH with a variety of sulfhydryl reagents, and following the proposed conformational changes with a technique such as circular dichroism or protein difference spectrophotometry. Reexamination of this enzyme's activity in the presence of manganese would indicate the feasibility of performing NMR and/or EPR analysis of the various possible coordination schemes. Also indicated are experiments which would more clearly define the function of this enzyme; this discussion encompasses only one of several possibilities. In all of these proposals, however, first consideration must be given to the difficulties entailed in obtaining a homogeneous enzyme preparation.

# APPENDICES

## APPENDIX A

The method of Cornish-Bowden (81) was used to calculate the true concentrations of species present in mixtures of associating ions. The procedure which is followed in this method is outlined below.

1. Conservation of Mass

The well known statement of the conservation of mass defines the concentration of a given component within a system as being the sum of its free and complexed concentrations. Thus, in a mixture of m different complexes formed by n different components, the total concentration of $A_i$ of the ith component is defined by the following equation:

$$A_i = a_i + \sum_{j=1}^{M} \alpha_{ij} x_j \qquad \text{Equation A}$$

where $a_i$ is the free concentration of the ith component and $x_j$ is the concentration of all the m complexes, with each of the compexes being given a weight of $\alpha_{ij}$ equal to the number of molecules or ions of the ith component contained in one molecule or ion of complex. consider the total concentration of $ITP^{4-}$, which would be given as*

$$A_{ITP} = [ITP^{4-}] + 1 \cdot [MgITP^{2-}] + 1 \cdot [Mg_2ITP].$$

2. Definition of Equilibrium Constant

In general, this definition can be written as follows:

$$x_j = K_j \prod_{k=1}^{n} a_k^{\alpha kj} \qquad \text{Equation B}$$

where the exponent $\alpha_{kj}$ is the number of molecules of component k in the complex j. For example, given the reaction:

$$Mg^{++} + ITP^{4-} \rightleftharpoons MgITP^{2-}$$

$$K_{eq} = \frac{[MgITP^{2-}]}{[Mg^{++}][ITP^{4-}]}$$

$$[MgITP^{2-}] = K_{eq}[Mg^{++}][ITP^{4-}]$$

3. Substitution of Equation B into Equation A yields the following:

$$A_i = a_i + \sum_{j=1}^{m} \left(\alpha_{ij} K_j \sum_{k=1}^{n} a_k^{\alpha kj}\right)$$

Dividing both sides by the right hand side of the equation, and multiplying by $A_i$ yields Equation C:

$$A_i = A_i a_i / \left[a_i + \sum_{j=1}^{m} \left(\alpha_{ij} K_j \prod_{k=1}^{n} a_k^{\alpha kj}\right)\right]$$

4. Equation C above is useful for computer iterations. Each free concentration $a_i$ is initially set to the corresponding total concentration $A_i$. This new value of $A_i$ is used in the right hand side of equation C to generate improved estimates for $A_i$. Providing that

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*The various protonated forms not included for the sake of simplicity.

the series of $A_i$'s converges, there are generated successive values for $A_i$ which are closer and closer to the real value. In this way, improved estaimates of all the $A_i$ up through $A_n$ are obtained so that there is one Equation C for each component considered. Because these values are likely to be very poor approximations to the true values, the entire process is repeated several times until 2 iterations are sufficiently close together - i.e., agree to within 0.01% for all n components.

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