THE UNIVERSITY OF MANITOBA

CHARACTERIZATION OF CYTIDINE 5'-DIPHOSPHATE REDUCTASE ACTIVITY FROM THE FUNGUS *ACHLYA KLEBSIANA*

BY

JOHN WILLIAM HICKERSON

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To my darling wife, Alison, who loved and supported me throughout this whole ordeal.

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TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS.....ii

TABLE OF CONTENTS.....iii

LIST OF TABLES.....v

LIST OF FIGURES.....vi

ABBREVIATIONS.....vii

INTRODUCTION.....1

HISTORICAL.....4

Ribonucleotide Reductase.....5

The Mechanism of Reduction.....7

Types of Ribonucleotide Reductase.....8

Drugs that Act on Ribonucleotide Reductase.....16

Achlya klebsiana.....17

MATERIALS AND METHODS.....19

Organism.....20

Growth Media.....20

Preparation of Cultures.....20

Purification of Ribonucleotide Reductase.....22

Standard CDP Reduction Assay.....23

Standard Protein Assay.....24

Molecular Ultrafilters.....25

Tritiated Thymidine Uptake.....26

Sources of Material.....28

RESULTS.....29

Purification of CDP Reductase.....30

Characteristics of the Reductase Assay.....37

Kinetics of CDP Reductase.....54

Summary of Effectors.....85

DISCUSSION.....87

CONCLUSION.....99

REFERENCES.....101

LIST OF TABLES

Table		Page
1	Effect of magnesium on CDP reductase activity	47
2	Standard CDP reduction assay conditions	52
3	Properties of CDP reductase from *Achlya*	53
4	Effect of gossypol on CDP reductase	80
5	Effect of guanazole on CDP reductase	82
6	Effect of MAIQ on CDP reductase	83
7	Effect of bleomycin on CDP reductase	84
8	Comparison of the effectors of CDP reductase from various sources	86

LIST OF FIGURES

Figure		Page
1	Thymidine uptake by germinating spores of *Achlya klebsiana*	32
2	Purification scheme for ribonucleotide reductase from *A. klebsiana*	34
3	The effect of pH on the CDP reductase assay	39
4	The response of CDP reductase to changes in temperature	41
5	The effect of increasing concentrations of dithiothreitol on CDP reductase	44
6	The response of CDP reductase to increasing concentrations of ATP	46
7	The effect of protein concentration on the rate of CDP reduction	50
8	Determination of Km for CDP with ATP as an activator	56
9	The response of CDP reductase to dATP	58
10	The effect of altering the dATP/ATP ratio on the rate of CDP reduction	60
11	Activation of CDP reductase by dGTP	63
12	Inhibition of CDP reductase by dTTP	65
13	Response of CDP reductase to the addition of dCTP	67
14	The response of CDP reductase to the addition of CTP	69
15	GTP as a replacement of ATP as the activator of CDP reductase	72
16	Determination of Km for CDP with GTP as the activator	74
17	The response of CDP reductase to the addition of UDP	76
18	CDP reductase in the presence of increasing concentrations of hydroxyurea	79

ABBREVIATIONS

ADP	- adenosine 5'-diphosphate
ATP	- adenosine 5'-triphosphate
B1	- effector-binding subunit of Escherichia coli ribonucleotide reductase
B2	- iron-containing subunit of Escherichia coli ribonucleotide reductase
BSA	- bovine serum albumin
CDP	- cytidine 5'-diphosphate
CTP	- cytidine 5'-triphosphate
dATP	- 2'-deoxyadenosine 5'-triphosphate
dCTP	- 2'-deoxycytidine 5'-triphosphate
dGTP	- 2'-deoxyguanosine 5'-triphosphate
DNA	- deoxyribonucleic acid
dNTP's	- 2'-deoxyribonucleoside 5'-triphosphates
dpm	- decays per minute
DTT	- dithiothreitol
dTTP	- 2'-deoxythymidine 5'-triphosphate
EPR	- electron paramagnetic resonance
GDP	- guanosine 5'-diphosphate
GTP	- guanosine 5'-triphosphate
G Y 2	- 5g glucose, 0.5g yeast extract/L medium
HCl	- hydrochloric acid
HEPES	- N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid
Hu	- hydroxyurea
KCl	- potassium chloride
L1	- effector-binding subunit of rat liver ribonucleotide reductase

L2	- iron-containing subunit of rat liver ribonucleotide reductase
M1	- effector-binding subunit of mammalian ribonucleotide reductase
M2	- iron-containing subunit of mammalian ribonucleotide reductase
MAIQ	- 4-methyl-5-amino-1-formylisoquinoline thiosemicarbazone
MES	- 2-[N-morpholinolethanesulfonic acid
$MgCl_2$	- magnesium chloride
MW	- molecular weight
NADPH	- reduced nicotinamide adenine dinucleotide phosphate
PBS	- phosphate buffered saline
PEG	- polyethylene glycol
PIPES	- piperazine-N,N'-bis[2-ethanesulfonic acid
psi	- pounds per square inch
PYG	- 3g glucose, 1g peptone, 1g yeast extract per liter medium
rpm	- revolutions per minute
TCA	- trichloroacetic acid
Tris	- tris(hydroxymethyl)aminomethane
UDP	- uridine 5'-diphosphate

INTRODUCTION

Ribonucleotide reductase is responsible for the production of deoxyribonucleotides , a rate-limiting step in DNA synthesis. It is a highly regulated allosteric enzyme and, in mammalian cells, it consists of two non-identical proteins M1 and M2. A variety of positive and negative effectors and several uncharacterized endogenous molecules control the specificity of the enzyme and ensure a balanced supply of deoxyribonucleotides necessary to preserve the fidelity of DNA synthesis.

Ribonucleotide reductase activity is tightly correlated with the rate of mammalian cell proliferation, and several studies have shown that it increases with the expression of neoplasia. Alterations in the activity of the mammalian enzyme appears to affect many biological properties including the senescence of human diploid fibroblasts, the cellular differentiation of rat myoblasts, and may play a role in certain immunodeficiency syndromes. Since very little information is currently available about ribonucleotide reductase in fungal cells, a study was initiated to examine the general properties of this enzyme in Achlya klebsiana. This fungus belongs to a group of eukaryotic microbes commonly known as water moulds. Achlya has one of the fastest growth rates for a eukaryote and grows synchronously from spores, making it a good source of eukaryotic ribonucleotide

reductase.

The enzyme has been partially purified from the fungal mycelia 12 hours after germination. The purification protocol was based on the facts that the enzyme aggregates when in the presence of a great excess of ATP and will be retained by a molecular ultrafilter. When potassium chloride was added, the aggregates broke up and the subunits of the enzyme dissociated and passed through the membrane. After dialysis, the subunits recombined to form a functional enzyme. A 150 fold purification of ribonucleotide reductase was accomplished using this method.

Many of the kinetic properties of the enzyme differed significantly from those previously described from mammalian sources. For example, there was a relatively high Km value of 16 mM for CDP and a lack of inhibition of activity with UDP, a second enzyme substrate. Also, in contrast to the mammalian enzyme, GTP was as good an activator of CDP reduction as ATP, dATP did not appear to inhibit enzyme activity even at high concentrations, $MgCl_2$ was not required for the reaction, and the anti-proliferation drugs hydroxyurea and gossypol were either poor or ineffective as enzyme inhibitors.

In this thesis, data is discussed in terms of allosteric regulation of ribonucleotide reductase and in terms of usefulness as a source of ribonucleotide reductase for future investigations.

HISTORICAL

Ribonucleotide Reductase

Ribonucleotide reductase (EC 1.17.4.1) is responsible for the direct conversion of ribonucleotides to deoxyribonucleotides necessary for the fidelity of DNA synthesis (Blakley and Vitols,1968; Reichard,1968). It is a highly regulated allosteric enzyme, controlled by a variety of positive and negative effectors. There is a very tight correlation between the activity of this key enzyme and the rate of cell proliferation. Mammalian organs such as thymus, bone marrow, spleen and intestine (all sites of very active cell renewal) have high ribonucleotide reductase activity while non-dividing cells have low or even undetectable levels (Elford ,1972 ; Takeda and Weber,1981). Elevated ribonucleotide reductase levels are concommitant with the expression of neoplasia in rat hepatoma cells and, in fact, are more tightly correlated with tumor growth rates than any other enzyme active in DNA synthesis (Elford,1972; Takeda and Weber,1981; Elford et al, 1970; Weber et al, 1981). The concentrations of all four deoxyribonucleotides are much higher in hepatomas than in newborn and regenerating rat liver (Takeda and Weber,1981; Weber et al, 1981). Several antitumor drugs have been synthesized that are aimed at ribonucleotide reductase in an attempt to arrest rampant neoplastic proliferation. Although hydroxyurea has found use in

the clinic (Krakoff et al,1964; Bolin et al,1982; Piver et al, 1983; Donovan et al,1984; McDonald,1981) few other drugs have proven to be clinically satisfactory (Elford et al, 1981).

The relative concentrations of the nucleotides contribute to the characteristic mutation rate of an organism. In the mammalian system, a mutator gene is associated with ribonucleotide reductase which causes increased rates of spontaneous mutation if there exists an imbalance of endogenous deoxyribonucleoside triphosphate pools (Weinberg et al, 1981). When the dCTP/TTP ratio is disturbed, the fidelity of DNA synthesis is most profoundly affected (Meuth et al,1979; Ashman and Davidson,1981; Ashman et al,1981).

Changes in the activity of ribonucleotide reductase have been implicated in the senescence of normal human diploid fibroblasts in culture and the cytotoxic action of chemotherapeutic agents directed towards this enzyme is affected by such changes (Dick and Wright,1982;1985).

There is also a close relationship between ribonucleotide reductase activity and the initiation of myoblast differentiation. Drug-resistant myoblasts that had elevated levels of ribonucleotide reductase activity and had altered pools of deoxyribonucleotides were unable to differentiate into multinucleated myotubes (Creasey and Wright, 1983).

Certain human immunodeficiency diseases involve a combination of abnormal intracellular deoxyribonucleotide concentrations and the allosteric properties of ribonucleotide reductase (Ullman et al, 1976). Deficiencies of purine nucleoside phosphorylase and adenosine deaminase lead to a build up of dGTP and dATP (Ullman et al,1976; Chan,1978). CDP reductase is inhibited by the nucleotides causing a depletion of the dCTP pool which arrests DNA synthesis and cell division (Cohen et al,1983).

The Mechanism of Reduction

Ribonucleotide reduction in prokaryotes and eukaryotes involve substrate-radical intermediates and the C-(3) hydrogen of ribose (Reichard and Ehrenberg,1983). Also involved is a tyrosine free radical (Sjöberg et al,1982) and a non-heme iron (Atkin et al,1973) in the B2 subunit of E. coli/M2 subunit of mammalian ribonucleotide reductase. The tyrosyl radical removes the C(3′) hydrogen of the ribose moiety which activates a dithiol-mediated release of the C(2′) hydroxyl group (Reichard and Ehrenberg,1983). The iron group is involved in the stabilization and regeneration of the tyrosyl radical (Sjöberg et al,1982). The tyrosyl radical of mammalian ribonucleotide reductase is regenerated in a regulated mechanism requiring iron, oxygen and dithiothreitol (Thelander et al,1983). The hydrogen donor substrate (to maintain a reduced

dithiol group on the enzyme) is most likely reduced thioredoxin and/or glutaredoxin (Holmgren,1981). NADPH is the ultimate hydrogen donor, reducing the two as reducing power carriers.

Types of Ribonucleotide Reductase

There are several different types of ribonucleotide reductase. These criteria were used in classification:

1) ribonucleoside di- or triphosphate as substrate?
2) require 5'-deoxyadenosylcobalamin(Co B$_{12}$)?
3) contain non-heme iron?
4) specific nucleotide activators/inhibitors?
5) enzyme subunit composition?

(I) Lactobacillus leichmannii (single peptide enzyme) and Euglena gracillus (tetrameric enzyme) are in this group. Both of these enzymes use nucleoside triphosphates as substrates and require Coenzyme B$_{12}$ as a hydrogen carrier between thioredoxin and the reductase. Neither enzyme contains non-heme iron. There is a certain amount of enzyme regulation by nucleotide activators (eg. dATP stimulates CTP reduction by L. leichmannii) but there is relatively little product inhibition by deoxyribonucleotide triphosphates (dNTP's). These enzymes are resistant to inhibition by the chemotherapeutic agent, hydroxyurea (Beck et al,1966; Panagou et al,1972; Hamilton,1974)

(II) Rhizobium meliloti and Bacillus megaterium (both tetrameric enzymes) as well as Corynebacterium

nephridii (a dimeric enzyme) represent this group. All of these bacteria use nucleoside diphosphates as the substrates and require Coenzyme B_{12}. None of these enzymes contain non-heme iron and are not regulated by nucleotide effectors to any great extent; dATP inhibits CDP reduction by C. nephridii. Tsai and Hogenkamp (1980) suggested "in evolutionary terms the ribonucleotide reductase from C. nephridii is intermediate between the enzyme from E. coli and L. leichmannii." (Cowles et al, 1969; Yau and Wachsman, 1973; Hogenkamp,1984)

(III) Nocardia opaca , Brevibacterium ammoniagenes and Micrococcus luteus have a unique class of ribonucleotide reductase. This enzyme can use both ribonucleoside diphosphates and triphosphates as substrates but does not require Coenzyme B_{12} or iron. Instead, this mettaloenzyme requires manganese ions for maximum activity (some activity with magnesium ions). The only reported influence of nucleotide effectors are the stimulation of GTP reduction by dTTP and CTP reduction by dATP in B. ammoniagenes. (Auling et al, 1980; Schimpff-Weiland and Follmann, 1981; Hogenkamp, 1984)

(IV) Escherichia coli and all eukaryotes (except Euglena gracillus and Phytomyces chartarum; Type I) comprise this last class of ribonucleotide reductase. All of these enzymes use ribonucleoside diphosphates as

substrates and do not require Coenzyme B[12]. These enzymes are composed of dimers of two non-identical subunits: the B1 (E. coli) or M1 (mammalian) subunit contains the binding sites for the nucleotide effectors and substrates as well as the dithiols involved in the reduction. The B2/M2 subunits contain the non-heme iron and the tyrosyl radical. These enzymes are strictly regulated; specific nucleotides activate and inhibit the reduction of all substrates. (Brown et al,1969; Larsson and Reichard,1966; Hopper,1972; Moore,1977; Cory et al,1978; Chang and Cheng,1979b; Thelander et al,1980; Mattaliano et al,1981; Stutzenberger,1974)

The B1 subunit of the E. coli enzyme is composed of a single polypeptide chain of MW 82,000 while the B2 contains a chain of MW 43,000 (Thelander,1973). In the presence of magnesium ions, two B1 and two B2 combine to form an active enzyme of MW 240,000 (Brown and Reichard,1969). Each B1 subunit contains two substrate binding sites (von Döbeln and Reichard,1976) and four nucleoside triphosphate effector binding sites (Brown and Reichard,1969). These effector binding sites are classified as "h"-sites (high affinity for dATP and also bind ATP,dGTP and dTTP) and "l"-sites (low affinity for dATP but can only bind ATP in addition). The "l"-site seems to determine the overall activity of the enzyme; if dATP is bound, the enzymes dimerize and are rendered inactive (conversely, ATP activates the enzyme). The binding of ATP or dATP by the "h"-sites

causes a conformational change in the enzyme which favors the reduction of pyrimidine nucleotides. The binding of dGTP stimulates the reduction of ADP and dTTP binding stimulates GDP reduction (Brown and Reichard,1969).

This "E. coli"-type of ribonucleotide reductase has been found in fungi (Vitols et al,1970; Lewis et al,1976), algae (Feller et al,1980), plants (Hovemann and Follmann,1977), and animal cells (Hards and Wright,1984a,b).

The ribonucleotide reductase of Saccharomyces cerevisiae shares qualities of the enzyme from E. coli and mammalian reductase. It is a very unstable enzyme that requires the binding of ATP and magnesium for CDP reduction (Vitols et al,1970). In vitro assays require thioredoxin and thioredoxin reductase for the production of reducing power (dithiothreitol and dithioerythritol were not sufficient). The enzyme did not use Coenzyme B[12] nor did it require the addition of iron salts. This reductase was inhibited by dATP (50% activity at 0.5 mM) and by hydroxyurea (50% activity at 5 mM hydroxyurea) as well as by moderately high salt concentrations (0.2 M ammonium sulfate completely destroyed activity while 0.2 M sodium chloride caused a 90% drop in activity). The enzyme is composed of two non-identical subunits and has a relative molecular weight of 250,000 as a holoenzyme. The reduction of GDP

requires dTTP as an activator while ADP reduction requires dGTP. As aforementioned, pyrimidine nucleotide reduction is stimulated by any deoxyribonucleotide as long as ATP is also present (Lammers and Follmann,1984).

Lewis et al, (1976) described the ribonucleotide reductase from the water mould Achlya. The specific activity of the enzyme was found to peak at the same time as the peak rate of tritiated thymidine incorporation during the asexual growth cycle. This time corresponds with the point where the cells are entering the S-phase of cell division. Using a partially purified enzyme preparation, they found that CDP reduction proceeded (linearly with time for 60 minutes) at an optimum temperature of 22 ^{o}C as long as at least 0.75 mg protein was present per assay. The CDP reductase was sensitive to dATP (1.0 mM caused 89% inhibition) and hydroxyurea (1.0 mM caused 88% inhibition of specific activity). They included 10 mM $MgCl_2$, 10 mM dithiothreitol, 10 mM ATP and 0.4 mM CDP in their CDP reductase assay. The addition of ribonucleosides or their di- and triphosphates did not significantly inhibit the reduction of CDP.

Studies with mammalian ribonucleotide reductase have shown it to be similar in many regards to the enzyme of E. coli. The reduction of ribonucleoside diphosphates is regulated by nucleoside triphosphates (Thelander and Reichard,1979; Hards and Wright,

1984a,b). There are distinct binding sites for substrates and two types of sites for the allosteric effectors (Eriksson et al,1981). The enzyme requires iron but does not require Coenzyme B12 (Youdale et al,1982; Fujioka and Silber,1969). The enzyme reaction involves a tyrosine free radical and this radical was shown to be similar to that of the E. coli enzyme by electron paramagnetic resonance (EPR) spectroscopy (Åkerblom et al,1981).

The purification of the mammalian enzyme by various researchers has yielded a range of molecular weights for the two subunits. Moore (1977) determined the molecular weight of M1 from rat Novikoff ascites as 90,000 using SDS-polyacrylamide gel electrophoresis (holoenzyme weight of 200,000 - 250,000). Youdale et al (1982) described an M1 from regenerating rat liver that only reduced CDP as being 45,000 MW (by electrophoresis and molecular exclusion high performance liquid chromatography). The M2 dimer was measured in the same manner and found to have a molecular weight of 120,000 making the holoenzyme 280,000 MW. Chang and Cheng (1979a) measured both the M1 and the M2 dimers at 100,000 MW each, giving the holoenzyme from human lymphoblast cells a molecular weight of 210,000 (using sedimentation velocities). Several groups have researched the enzyme from calf thymus. Engström et al (1979), using SDS-polyacrylamide gel electrophoresis,

reported weights of 84,000 for M1 and 55,000 for M2. Thelander et al (1980) found M1 to be 98,000 MW and the M2 dimer at 110,000 MW using the sedimentation velocities. Mattaliano et al (1981) reported the molecular weight of M1 as 84,000 and M2 as 58,000 using the electrophoresis method. Cory and Fleischer (1982) using sedimentation equilibrium produced different values for the enzyme depending on the substate being reduced. When CDP reduction was proceeding, they found weights of 127,000 for M1 and 81,000 for M2 and a holoenzyme of 304,000 MW. The enzyme that reduced ADP was found to have a 95,000 MW M1 (same M2) making a holoenzyme weight of 254,000.

The allosteric regulation of mammalian ribonucleotide reductase by nucleoside diphosphates is quite similar for most sources tested. ATP is the positive effector for CDP and UDP reduction. The reduction of GDP is stimulated by dTTP and ADP reduction stimulated by dGTP. The presence of dATP inhibited the reduction of all four deoxyribonucleosides. Inhibition of CDP and UDP reduction was mediated by dTTP and dGTP. The reduction of GDP was also inhibited by dGTP. This regulation pattern was found in human diploid fibroblast (Dick and Wright,1980), human Molt-4f (Chang and Cheng,1979b), calf thymus (Eriksson et al,1979), Chinese hamster ovary (Hards and Wright,1981), mouse L-cells (Kuzik and Wright,1979) and rat embryo (Murphree et al,1968).

Chang and Cheng (1979b) reported a slight variation in this scheme; GTP was found to be as effective an activator of ADP reduction by Molt-4F cells as was dGTP. Hards and Wright (1981) reported this same finding in Chinese hamster ovary cells. Lewis et al (1978) found that 2.5 μM dATP stimulated CDP reduction by 15% in Chinese hamster ovary cells. They also reported that 40 μM dATP stimulated GDP reduction by 10%.

Cells infected by some viruses produce altered forms of ribonucleotide reductase (Wright,1983). Infection by equine herpes virus or Epstein-Barr virus results in an observed increase in resistance to hydroxyurea and guanazole (Hampar et al,1972; Mele et al,1974; Cohen et al,1977; Allen et al,1978). A virus-encoded M2 or a modified cellular M2 is suspected because the free radical is the target of these drugs. The enzyme in cells infected with herpes simplex and pseudorabies is not resistant to hydroxyurea and EPR studies of M2 tyrosine free radical indicated that it was similar (though slightly different) to the host enzyme (Langelier and Buttin,1981; Lankinen et al,1982). The CDP reductase of Herpes simplex does not require ATP and is not inhibited by dTTP or dATP (Langelier and Buttin,1981; Huszar and Bacchetti,1981; Lankinen et al,1982).

The allosteric regulation of the T4 bacteriophage ribonucleotide reductase is similar to that of its

host, E. coli, except that dATP activates the reduction of CDP and UDP rather than inhibiting all reductions (Berglund,1972). In fact, CDP reduction is stimulated by dTTP, dCTP, dATP and ATP.

Drugs that Act on Ribonucleotide Reductase

There are several drugs that are known to have ribonucleotide reductase as their site of action. Hydroxyurea is by far the most widely used chemotherapeutic agent that acts on the enzyme. It has been used to treat a variety of solid tumors, acute and chronic leukemias and melanomas (Bergsagel et al,1964, Bolton et al,1964, Fishbein et al,1964; Kennedy,1969). Its chemotherapeutic action was shown to be manifested in the inhibition of ribonucleotide reductase (Mohler,1964; Young and Hodas,1964). Hydroxyurea scavenges the tyrosine free radical, an action that can be followed with EPR spectroscopy (Ehrenberg and Reichard,1972; Åkerblom et al,1981).

Guanazole (3,5-diamino-1,2,4-triazole) has some anti-tumor activity (Hahn and Adamson,1972; Yakar et al,1973). Brockman et al (1970) showed that it affected ribonucleotide reductase in the same manner as hydroxyurea. In keeping with this observation, Wright and Lewis (1974) showed that cells selected for resistance to hydroxyurea or guanazole were resistant

to both hydroxyurea and guanazole, indicating that the two drugs shared a common intracellular site of action.

An extremely potent inhibitor of ribonucleotide reductase is 4-methyl-5-amino-1-formylisoquinoline thiosemicarbazone (MAIQ). This is an iron chelator but is not believed to interfere with the non-heme iron of M2. It appears to interact with the enzyme at or near the site where the dithiol substrate binds (Sartorelli et al,1977).

Gossypol, a male infertility agent, is another drug that targets ribonucleotide reductase (McClarty et al,1985). Furthermore, a mouse L cell line that had been selected for resistance to hydroxyurea was found to be also resistant to the action of gossypol.

Bleomycin, a 1500 MW glycopeptide antibiotic, was found to inhibit ribonucleotide reductase of mouse L-cells, presumably by removing the iron from the M2 subunit (McClarty et al,1986). Prior exposure to hydroxyurea made the enzyme extremely sensitive to inhibition by bleomycin , probably by causing a conformational change in the enzyme and exposing the iron center.

Achlya klebsiana

This water mould belongs to the class, Oomycetes; order, Saprolegniales; and family, Saprolegniaceae (Alexopoulos,1962). At the end of its vegetative cycle,

the organism forms asexual sporangia and sexual oogonia and antheridia. Its thallus is filamentous and contains a multinucleate mass of protoplasm formed by the division of nucleus but not of cytoplasm of an original zoospore. It produces nonseptate mycelia and very stout hyphae. It is a common saprophyte in moist soil and in waterlogged plant and animal debris.

The growth and other characteristics of Achlya klebsiana have been reported by Cameron and LéJohn (1972). In liquid media with limited nitrogen, phosphate and glucose, the organism enters the asexual growth cycle. Cytoplasm streams to the tip of the hypha and is sequestered by the formation of a basal septum. Sporangiospores are formed from individual nuclei and released. Primary zoospores are encysted and rarely motile while the secondary zoospores are not encysted and have two flagella. During germination, a thin germ tube is produced which elongates and widens to form a hypha. A multinucleate hyphal tube grows and branches without cross septae. This whole process is completed in 40 hours at 24 ^{o}C and proceeds in a synchronous manner.

MATERIALS AND METHODS

(I) Organism

The organism used in this study was the coenocytic filamentous fresh water mould Achlya klebsiana which was obtained from Dr. Glen Klassen of the Department of Microbiology, University of Manitoba. The life cycle of this fungus was monitored microscopically (Cameron and LéJohn, 1972) as it grew in liquid medium.

(II) Growth Media

(a) G Y medium
2

Achlya was maintained as mats of sporulating mycelia in petri plates or Roux bottles containing G Y medium (Cameron and LéJohn, 1972). It consisted of 5.0 g glucose and 0.5 g Bacto yeast extract per L of distilled water. The solution was made 50 μM $CaCl_2$ and 50 μM $MgCl_2$ and sterilized by autoclaving.

(b) PYG medium

Achlya spores were allowed to germinate in PYG medium,(Cantino and Lovett, 1960) and the undifferentiated hyphae were harvested for the purification of ribonucleotide reductase. PYG consisted of 3.0 g glucose, 1.0 g Bacto peptone and 1.0 g Bacto yeast extract per L of distilled water. It was made 500 μM $CaCl_2$ and 500 μM $MgCl_2$ and sterilized by autoclaving.

(III) Preparation of Achlya klebsiana cultures

(a) Maintenance culture

Viable cultures of Achlya klebsiana were maintained in 100 mm petri plates containing 20 mL of G Y medium for up to 6 weeks at room temperature. Heavy mats of mycelia developed in 3 or 4 days.

(b) Culture for spore harvest

The mycelial mats from 5 petri plate cultures were aseptically removed and combined in 600 mL of G Y medium in a sterile 1 L Erlenmyer flask. After vigorous agitation, 100 mL was removed and dispensed into 5 100 mm petri plates to ensure culture maintenance. The remaining suspension was dispensed in 5 mL aliquots into 84 sterile 1 L Roux bottles containing 80 mL G Y medium. The bottles were stoppered with cotton plugs and stacked on their sides. These cultures were incubated for 5 or 6 days at room temperature until a thick mycelial mat developed and sporangia appeared to be plentiful upon microscopic examination.

(c) Germling preparation

Each of the 84 Roux bottles was shaken vigorously and filtered through a double layer of cheesecloth into sterile 2 L Erlenmyer flasks. About 600 mL of this spore suspension was dispensed into each of 12 sterile 2 L Erlenmyer flasks containing 500 mL PYG medium. These cultures were incubated at 28 C with constant shaking for 12 hours (hr). The cells were harvested by suction filter on a Whatman #1 filter in a plastic

funnel and washed twice with 200 mL distilled water. The fungal cake was diced and immediately frozen in liquid nitrogen. The fungus was either stored at this stage (-70 °C) or used for the purification of ribonucleotide reductase.

(IV) Purification of ribonucleotide reductase

Frozen Achlya klebsiana cubes (100-150g) were ground to a fine powder in liquid nitrogen with a pre-cooled mortar and pestle and mixed with an equal volume/weight of isolation buffer (50 mM Hepes-pH 7.6, 5 mM dithiothreitol and 250 mM sucrose in distilled water). The mixture was further ground with a Beckman polytron for 10 minutes (min) on ice. The mixture was then centrifuged at 15,000xg for 20 min at 4 °C. The supernatant was made 0.5 % protamine sulphate over a 10 min period with stirring on ice. This mixture was then centrifuged at 15,000xg for 15 min at 4 °C. The pellet was redissolved in isolation buffer containing 6 mM ATP (adenosine triphosphate) and centrifuged at 15,000xg for 15 min. The pellet was redissolved in isolation buffer (without added ATP) and centrifuged again at 15,000xg for 15 min at 4 °C. The resulting supernatant showed an approximately 20 fold purification of ribonucleotide reductase. This supernatant was made 6 mM ATP and was placed on a XM100A ultrafilter (Amicon Diaflo filter that retains molecules greater than 100,000 molecular weight). After several hours at 60

psi of nitrogen, the retentate (about 10 mL) was removed and made 100 mM potassium chloride. This solution was subjected to the same ultrafiltration and the final filtrate was made 0.65 % streptomycin sulphate. This mixture was centrifuged at 15,000xg for 15 minutes at 4 ^{o}C and the supernatant was dialyzed for 4 hours against 2 changes of 4 L of 20 mM Hepes-pH 7.8, 5mM dithiothreitol. As presented in the Results section (Figure 2), the ribonucleotide reductase in this fraction proved to be approximately 150 fold purified.

(V) Standard ribonucleotide reductase assay

The assay for the reduction of CDP (cytidine diphosphate) was a modified version of the reaction used by Lewis et al (1976;1977). A total reaction mixture of 150 μL contained 32 mM cold CDP, 0.33 nmole ^{14}C-CDP, 6 mM dithiothreitol, 6 mM ATP, 50 mM Hepes-pH 7.6 and up to 100 μL of cell extract. The reaction was incubated at 37 ^{o}C for 30 min and terminated by heating in a boiling water bath for 4 minutes. 1 mg Crotalus atrox venom was added to convert the deoxycytidine phosphates to deoxycytidine (Cory,1973). The reaction was incubated for 1 hr at 37 ^{o}C and terminated by heating in a boiling water bath for 4 min. The reaction mixture was then passed through a column of Dowex 1-borate (Steeper & Steuart, 1970). The column was washed with 5 mL of distilled water and the eluant containing

the deoxycytidine was collected. 10 mL of Fisher Scintiverse II was added to each sample for liquid scintillation counting. Enzyme activity was expressed as nanomoles CDP reduced per milligram protein per hour.

The ion exchange resin (Dowex 1-Borate) was prepared by converting Dowex 1-chloride x 8 (200 - 400 mesh) to the borate form. This was accomplished by resuspending 500 g of the resin in 6 L of saturated sodium borate with stirring overnight at room temperature. The resin was then filtered on a large Buchner funnel and resuspended in another 6 L of saturated sodium borate. The resin was filtered in the same manner after it had stirred overnight. After washing with 16 L of deionized water, the Dowex 1-Borate was made into a thick slurry with the addition of a small amount of deionized water and stored at 4 °C.

(VI) Standard Protein Assay

The standard assay procedure used for protein determination was the "Bio-Rad" method using the Bio-Rad protein determination kit (Technical Bulletin 1051). A series of standards (100-800 μg bovine serum albumin/mL) were first prepared using isolation buffer (50 mM Hepes-pH 7.6, 5 mM dithiothreitol and 250 mM sucrose in distilled water). 0.1 mL of these standards and diluted samples of the extracts were placed in test tubes. The Bio-Rad reagent was diluted 1:5 with deionized water and a 5 mL aliquot added to each

sample. The tubes were mixed by gentle inversion and , after 5 min , the absorbance at 595 nanometers was recorded. After plotting the standards on an absorbance versus concentration curve, the protein content of the extracts was determined.

(VII) Preparation and Storage of Molecular Ultrafilters

Two different Amicon Diaflo ultrafilters were used in the purification process. The XM100A membrane was used to separate molecules greater than 100,000 molecular weight from the smaller molecules in the extracts. The UM10 membrane was used to both concentrate and desalt some of the large volumes of extracts.

The XM100A membranes were soaked in distilled water prior to use. The filtration took place at 4 ^{o}C under pressures not exceeding 60 psi. After use, the filters were washed with 1 M sodium chloride followed by distilled water and stored wet in 10 % ethanol/water at 4 ^{o}C.

The UM10 membranes were soaked in 25 % glycerin/water for at least 10 min followed by a 1 hr rinse in distilled water (several changes). The filtration took place at 4 ^{o}C at pressures as high as 90 psi. The membranes were cleaned by washes with 1 M sodium chloride and distilled water and stored in the same manner as the XM100A membranes.

(VIII) Tritiated Thymidine Uptake

The uptake of tritiated thymidine by Achlya mycelia was monitored to determine the optimum time after germination for the harvest of the mycelia for the purification of ribonucleotide reductase. Lewis et al (1976) showed that the specific activity of ribonucleotide reductase peaked at the same time as the peak rate of thymidine incorporation into acid precipitable matter was observed.

Seven six-day Roux bottles of Achlya mycelia were added to 500 mL of PYG medium in a 2 L shaker flask and incubated in an orbital shaker (170 rpm) at 28 °C. After 2 hr, 100 µL of [6-^{3}H]-thymidine (1 mCi/mL stock concentration) was added to the culture making it 0.1µ Ci/mL. After another 2 hr, a 25 mL aliquot of the culture was removed, mixed with 25 mL of cold 50% trichloroacetic acid (TCA) and allowed to sit for 10 min at 4 °C. The mixture was then filtered through a glass fibre filter (prewashed with 10% TCA). The filter was then washed twice with each of cold 5% TCA and cold phosphate-buffered saline (PBS), followed by drying in a 200 °C oven for 5 min. The filter was then placed in a scintillation vial with 1 mL Amersham NCS tissue solubilizer and incubated for 1 hr at 60 °C. After the vial had cooled, 65 µL of concentrated hydrochloric acid (HCl) was added as well as 5 mL of Scintiverse II(Fisher). Time-course aliquots were taken hourly

until 16 hours after germination and were treated exactly as described above.

SOURCES OF MATERIAL

Most biochemicals were obtained from Sigma Chemical Company, St. Louis,MO or from Fisher Scientific Company Ltd., 18 Plymouth St., Winnipeg,MB. Materials that were purchased from other sources are listed below.

Ammonium sulfate	Aldrich Chemical Co., P.O.Box 2060, Milwaukee, Wisconsin, USA
Bleomycin	Bristol Laboratories of Canada Belleville, Ontario
^{14}C-CDP	Amersham Corp., 505 Iroquois Rd. Oakville, Ontario
Dowex AG1-X8 exchange resin	Bio-Rad Labs, 3140 Universal Dr. Mississauga, Ontario
Filters: XM100A,UM10	Amicon Corp., 1226 White Oaks Blvd., Oakville, Ontario
glass fibre	Gelman Sciences,Inc., 2906 Diab St., Montreal, Quebec
cellulose	Whatman Inc.,9 Bridewell Place, Clifton, New Jersey, USA
Guanazole	B. Woods,Jr.& L.H.Kedda U.S. National Cancer Institute Silver Springs, MD, USA
MAIQ	B. Woods,Jr.& L.H.Kedda (see above)
NCS tissue solubilizer	Amersham Corp. (see above)
Peptone	Difco Laboratories Detroit, Michigan, USA
Protein Assay	Bio-Rad Labs (see above)
^{3}H-thymidine	Amersham Corp. (see above)
Yeast Extract	Difco Laboratories (see above)

RESULTS

(I) Purification of Ribonucleotide Reductase

(a) Growth and harvest of mycelia

Cultures of Achlya klebsiana were maintained as described in the Materials and Methods. In order to determine the optimum time to harvest the germinating spores for the purification of ribonucleotide reductase, their uptake of tritiated thymidine was monitored. Lewis et al (1976) showed that the specific activity of ribonucleotide reductase peaked at the same time as the peak rate of thymidine incorporation into acid precipitable matter was observed. Figure 1 illustrates the incorporation of tritiated thymidine by germinating spores of Achlya klebsiana. There was no appreciable amount of incorporation until 9 hr after germination when a great burst of thymidine uptake was observed. The peak in the rate of uptake occurred at 12 hr after germination, showing a seven fold increase over the rate at 9 hr. Over the next 4 hr, the rate dropped gradually to about 2/3 of its peak value. Achlya mycelia were harvested at 12 hr after spore germination for the purification of ribonucleotide reductase.

(b) Purification protocol

A scheme of the method of enzyme purification which yielded the best results is presented in Figure 2. This method exploited the characteristic aggregation of the enzyme when in the presence of 6 mM ATP. The

Figure 1: Thymidine uptake by germinating spores of *Achlya klebsiana*.

A 2 L flask with 500 mL PYG medium and 500 mL germinating spores was made 0.1 μCi/mL with ^{3}H-thymidine. 25 mL samples were taken hourly and treated as described in Materials and Methods.

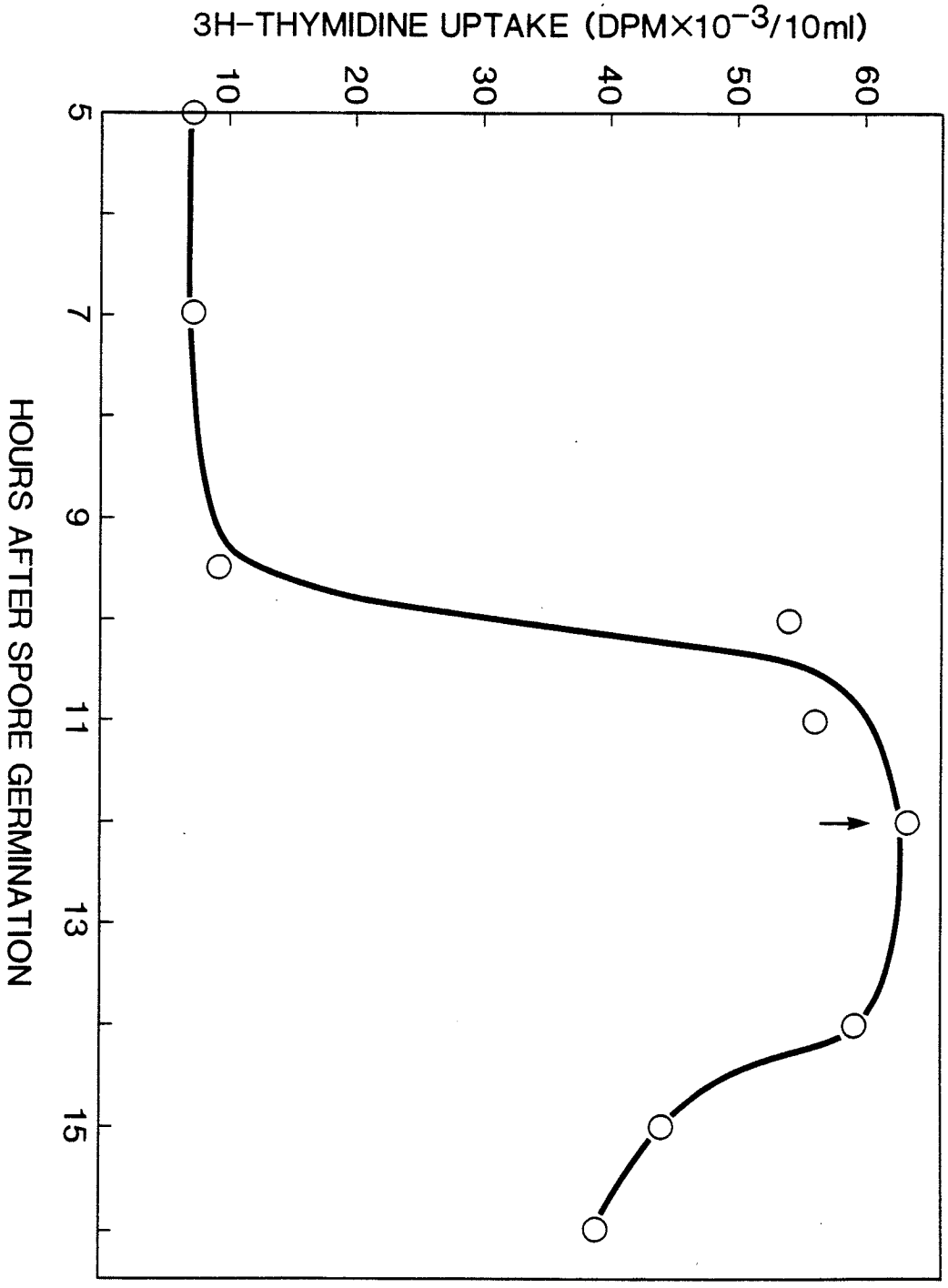
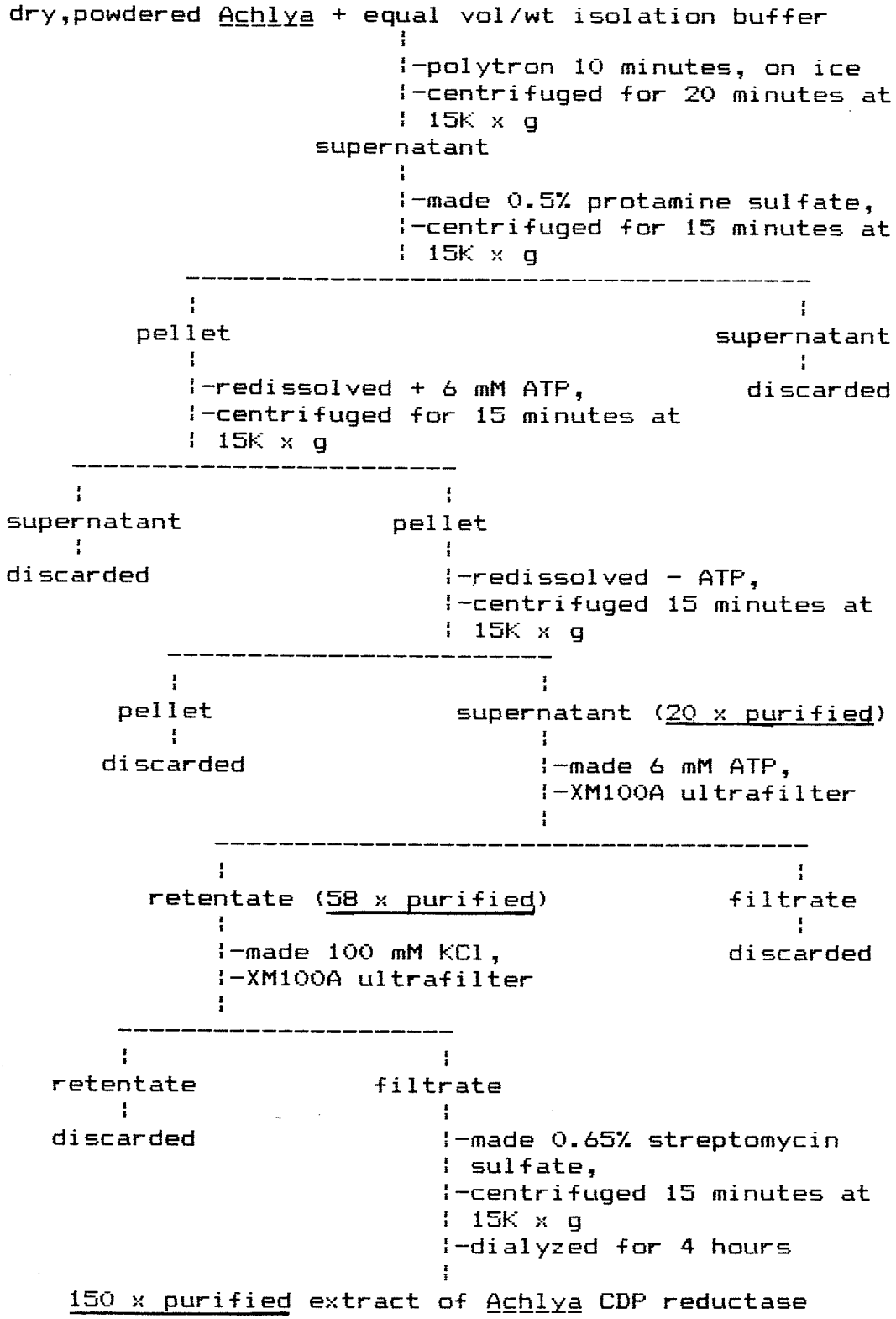

3H-THYMIDINE UPTAKE (DPM×10⁻³/10ml)
10
20
30
40
50
60
5
7
9
11
13
15
HOURS AFTER SPORE GERMINATION

Figure 2: Purification scheme for ribonucleotide reductase from Achlya klebsiana.

The protocol is described in detail in Materials and Methods. The extract applied to the XM100A ultrafilter was already purified 20 fold. The result of the first ultrafiltration was a 58 fold purification. The second ultrafiltration step resulted in a 150 fold purification.


dry,powdered Achlya + equal vol/wt isolation buffer
-polytron 10 minutes, on ice
-centrifuged for 20 minutes at 15K x g
supernatant
-made 0.5% protamine sulfate,
-centrifuged for 15 minutes at 15K x g
pellet
supernatant
discarded
-redissolved + 6 mM ATP,
-centrifuged for 15 minutes at 15K x g
supernatant
discarded
pellet
-redissolved - ATP,
-centrifuged 15 minutes at 15K x g
pellet
discarded
supernatant (20 x purified)
-made 6 mM ATP,
-XM100A ultrafilter
retentate (58 x purified)
filtrate
discarded
-made 100 mM KCl,
-XM100A ultrafilter
retentate
discarded
filtrate
-made 0.65% streptomycin sulfate,
-centrifuged 15 minutes at 15K x g
-dialyzed for 4 hours
150 x purified extract of Achlya CDP reductase

aggregate precipitated when mixed with 0.5% protamine sulfate but dissociated when the ATP and protamine sulfate were removed, remaining as a functional enzyme. The 6 mM ATP caused the enzyme to aggregate and be retained by the XM100A membrane (100,000 MW ultrafilter). When 100 mM KCl was added, the aggregate dissociated and was able to pass through that same membrane. This method yielded a fairly high degree of purification (approximately 150 fold) and proved to be the most gentle approach.

The ribonucleotide reductase from Achlya was very sensitive to dilution. Any purification step that resulted in low protein concentrations resulted in huge losses of enzyme activity. Lewis and Wright (1978) showed that the activity of ribonucleotide reductase decreases logarithmically with decreasing protein concentration. For this reason, ultracentrifugation was an unsuccessful step as was any chromatography that greatly increased sample volume. Lost activity was not restored by concentration so steps were taken to ensure such dilution did not take place. When grinding the frozen mycelia mats, no more than an equal volume/weight of isolation buffer was used (1 mL per g cells). The smallest possible volumes were used to resuspend the various pellets. Gel filtration steps for desalting were replaced by dialysis. Concentrating the sample extracts also proved difficult as the enzyme seemed to be extremely sensitive to destruction by

ammonium sulfate. Cooper (1977) suggested that the salt should be added as a solution in these cases and that the duration of this exposure be minimized. These suggestions did not help as it caused the volume to be increased and exposure was lengthy during dialysis. Even the use of 99.9999% pure ammonium sulfate (Aldrich Chemical Co.-"Gold Label"), ground to a fine powder was too destructive. Experiments with polyethylene glycol (PEG) showed that the enzyme was precipitated by 15% PEG but at an unacceptable loss of specific activity.

A hollow fibre concentrator was also employed, but with limited success. A large proportion of the protein was lost in the concentrator and the protein that was concentrated had quite low specific activity. The Amicon Diaflo UM10 ultrafilters gave the best results in both the amount of protein recovered and in maintaining high specific activities of the extracts after concentration. The drawback with both of these mechanical concentrators was the amount of time required for the process (2 - 5 hours).

Another step that was employed to keep protein concentrations above a critical level was the addition of bovine serum albumin (BSA) to the extracts. Most assays were performed on extracts that were at least 0.5 mg/mL (if they were less concentrated than this, sufficient BSA was added). BSA was often added in concentrations as high as 5 mg/mL to improve activity.

(II) Characteristics of the Reductase Assay

(a) Effect of pH

The effect of pH on the specific activity of ribonucleotide reductase was determined by making a series of buffers spanning the range of pH 5.6 - 8.8. The first subset of that range (pH 5.6 - 6.4) was made using 2-[N-Morpholino]ethanesulfonic acid (MES). Piperazine-N,N'-bis[2-ethanesulfonic acid] (PIPES) was used for the range of pH 6.4 - 7.4. N-2-Hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES) was used for pH 6.8 - 8.0. Tris(hydroxymethyl)aminomethane (TRIS) was used for the range of pH 7.0 - 8.8. Each assay tube contained the same enzyme preparation (11.8 mg/mL). Figure 3 indicates a rather narrow pH range for effective reduction of CDP (pH 7.2 - 8.5), with optimum pH being 7.8. HEPES at pH 7.8 was chosen as the standard CDP reaction buffer.

(b) Effect of Temperature

The effect of temperature on CDP reduction was determined by dispensing 250 μg of an enzyme preparation into duplicate reaction tubes in each of four different water baths (23, 28, 37 and 45 °C). Each temperature had a separate control tube as well, with BSA replacing the enzyme. Figure 4 shows a rather narrow temperature preference for the enzyme, with the optimum temperature being 37 °C even though the fungus grows optimally at 28 °C. The standard CDP reductase

Figure 3: The effect of pH on the CDP reductase assay. As described in the Materials and Methods, reaction mixtures were prepared using different buffer solutions (MES, PIPES, HEPES and Tris) covering a pH range of 5.6 - 8.8. The assay was performed in the usual manner.

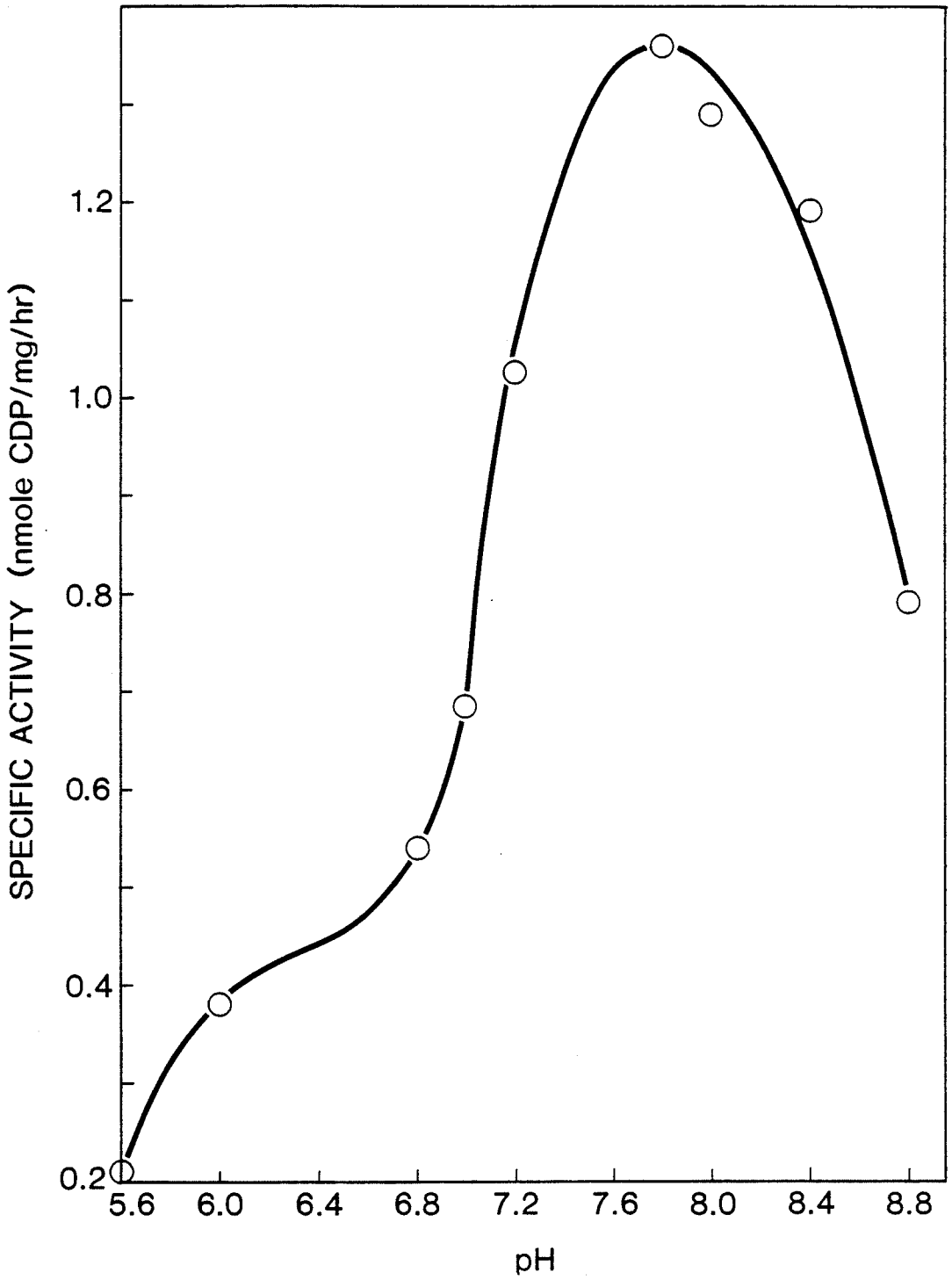
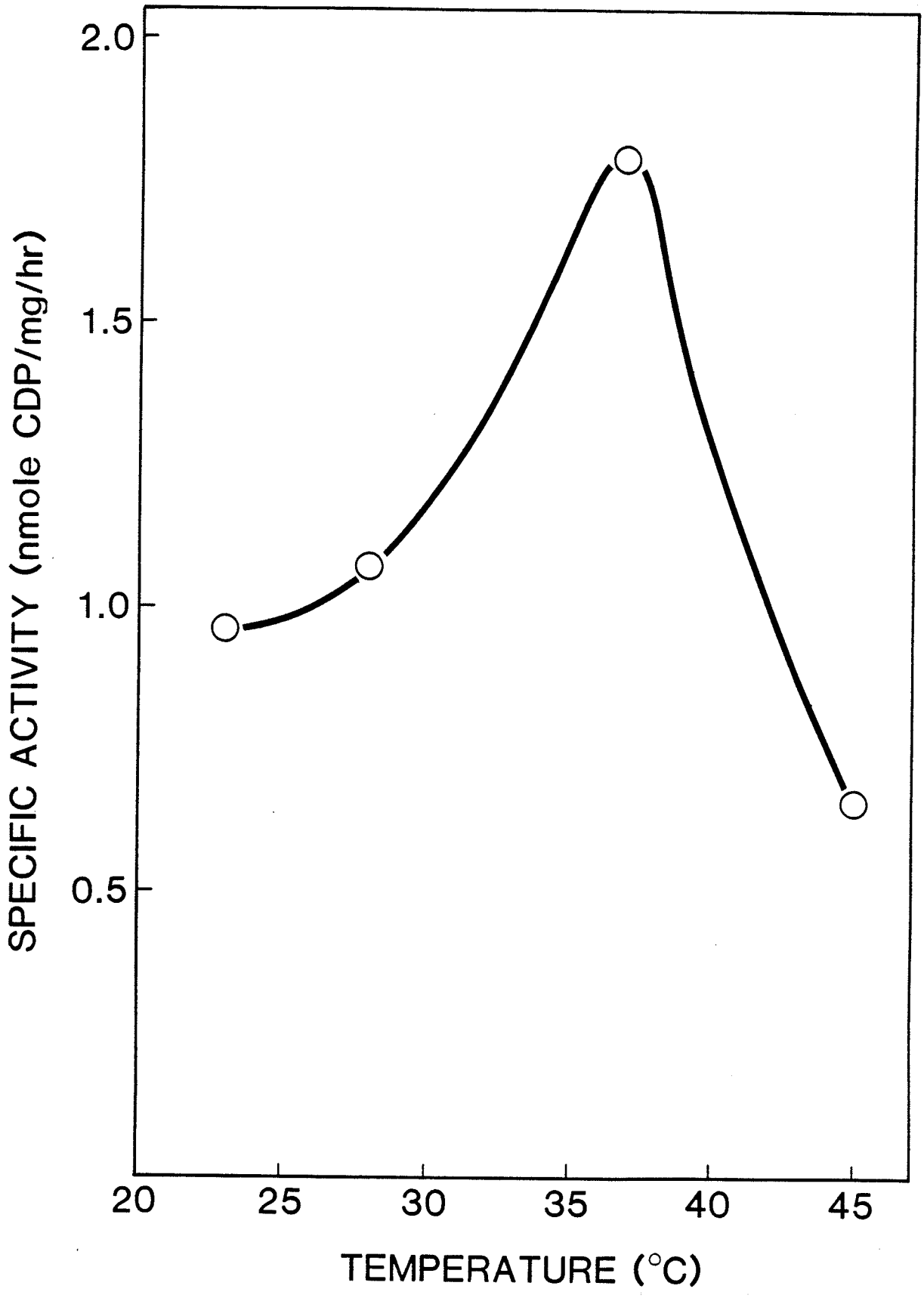

SPECIFIC ACTIVITY (nmole CDP/mg/hr)
0.2
0.4
0.6
0.8
1.0
1.2
5.6
6.0
6.4
6.8
7.2
7.6
8.0
8.4
8.8
pH

Figure 4: The response of CDP reductase to changes in temperature.

The assay was performed as described in the Materials and Methods except that 4 different incubation temperatures were used concurrently.


2.0
1.5
1.0
0.5
SPECIFIC ACTIVITY (nmole CDP/mg/hr)
20
25
30
35
40
45
TEMPERATURE (°C)

assay was performed at 37°C.

(c) Effect of Dithiothreitol on CDP Reduction

A range of dithiothreitol (DTT) concentrations (0 - 12 mM) was tested for their effect on CDP reduction. Each tube received 295 μg of an enzyme preparation that was dialysed against a buffer containing no DTT. Figure 5 indicates that some enzyme activity remained even in the absence of DTT. Specific activity increased steadily to a peak at 6 mM DTT followed by a gradual drop in activity from 6 mM to 12 mM. The standard assay conditions for CDP reduction included 6 mM DTT.

(d) Effect of Adenosine Triphosphate Concentration

Figure 6 shows the effect of increasing concentrations of adenosine triphosphate (ATP) on the rate of CDP reduction. There was quite a bit of enzyme activity even in the absence of ATP. Specific activity peaked at 6 mM ATP and decreased until a concentration of 14 mM ATP was reached. Subsequent additions of ATP (14 - 20 mM) had no effect on the specific activity. This finding was consistent with those of Moore and Hurlbert (1966) in mammalian systems in which it was found that ATP was required for maximum CDP reduction. The standard assay of CDP reduction by Achlya preparations contained 6 mM ATP.

(e) Requirement of Magnesium Ions

The addition of magnesium ions ($MgCl_2$) to the CDP reductase assay mixture did not enhance specific activity at all. The addition of magnesium has been

Figure 5: The effect of increasing concentrations of dithiothreitol on CDP reductase.

The standard CDP reduction assay was performed except that increasing concentrations of DTT were added to the assay tubes.

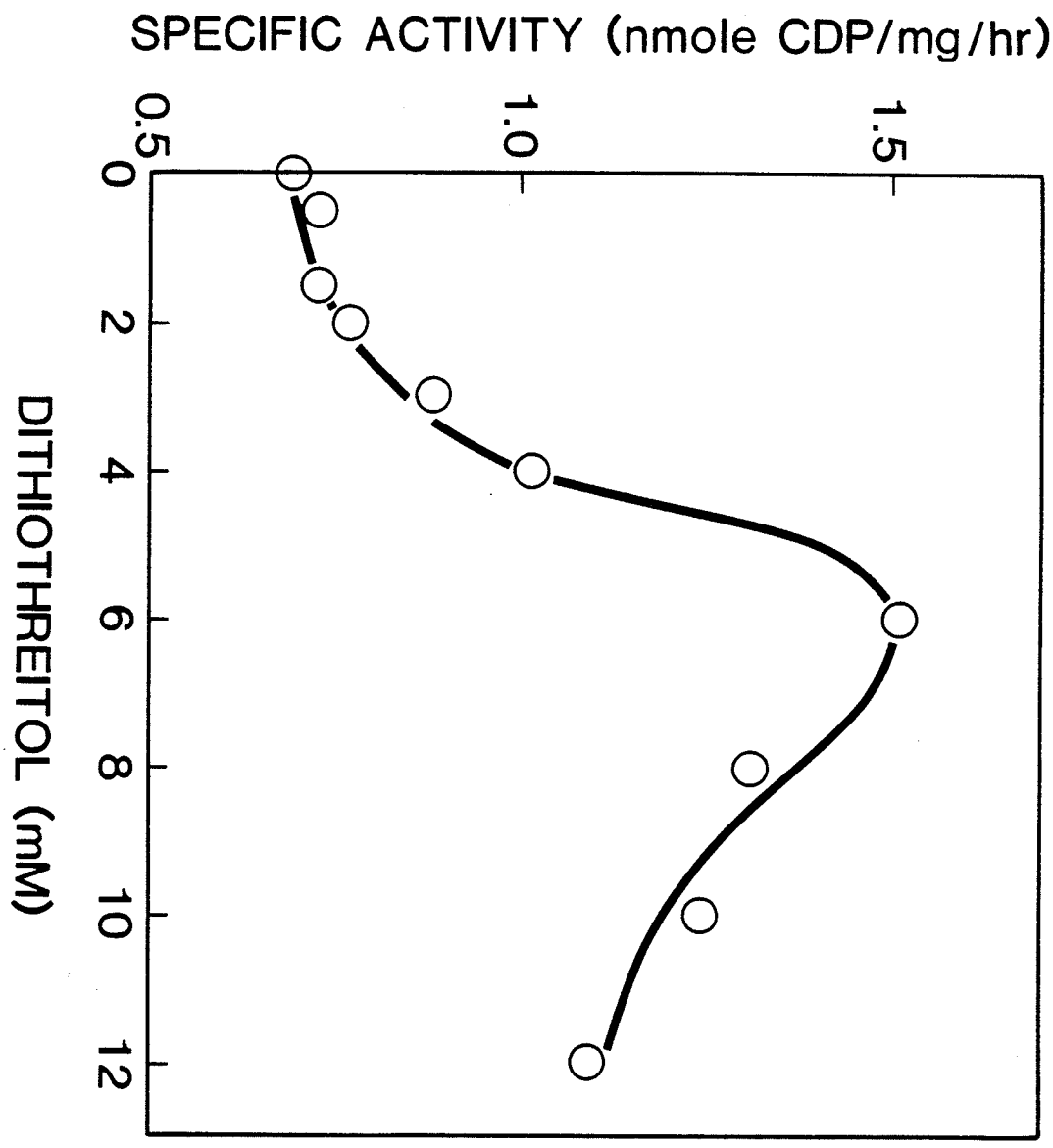

SPECIFIC ACTIVITY (nmole CDP/mg/hr)
0.5
1.0
1.5
0
2
4
6
8
10
12
DITHIOTHREITOL (mM)

Figure 6: The response of CDP reductase to increasing concentrations of ATP.

The assay was performed as before except that the concentration of ATP was varied from 0 - 20 mM.

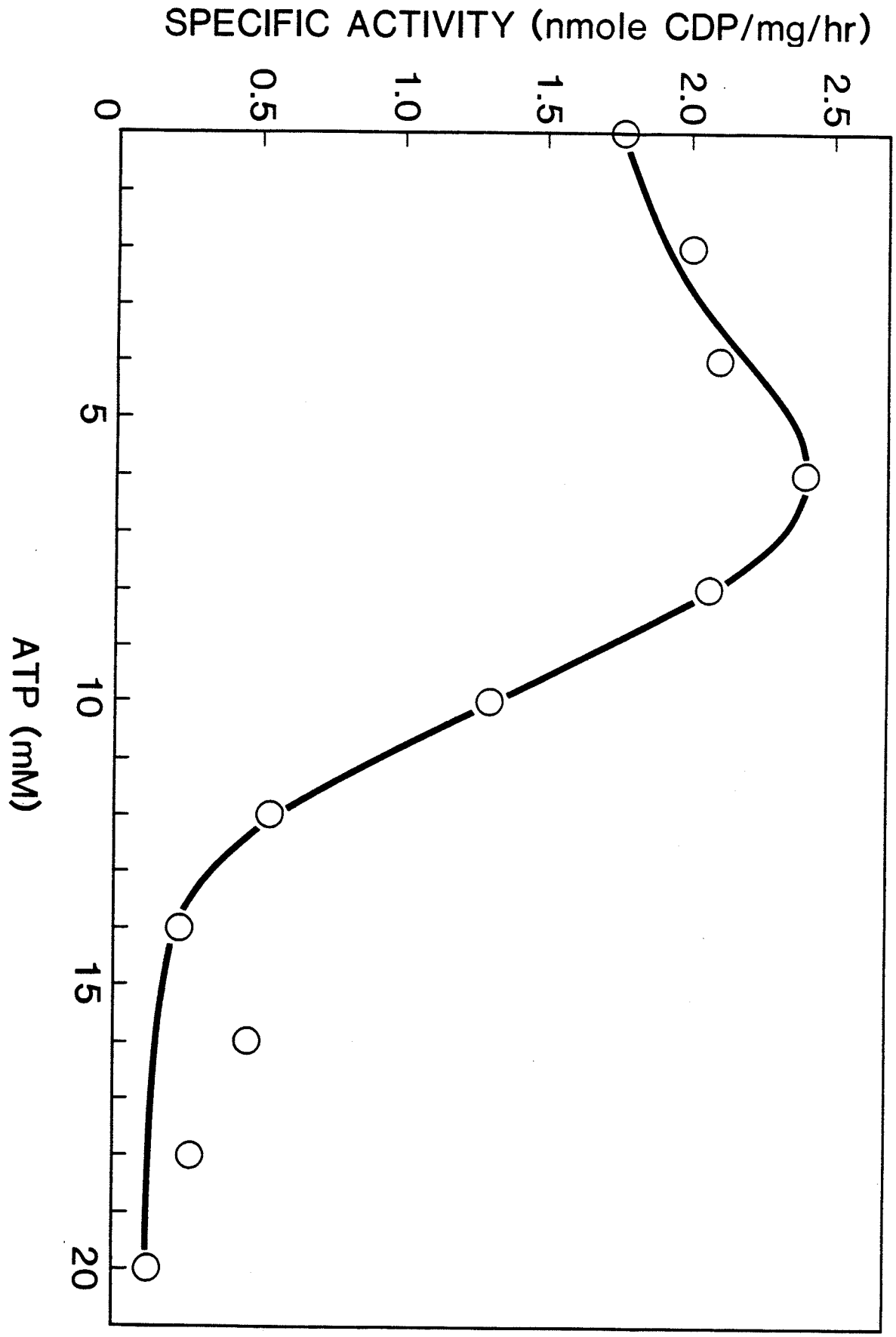

SPECIFIC ACTIVITY (nmole CDP/mg/hr)
0
0.5
1.0
1.5
2.0
2.5
ATP (mM)
5
10
15
20

Table 1. Effect of $MgCl_2$ on CDP reductase activity

[$MgCl_2$] (mM)	% Specific Activity
0	100%
2	58.9
4	45.0
8	53.6
10	60.9
12	66.9
14	67.5
18	56.3
20	78.8

100% activity = 1.51 nmole CDP/mg/hr

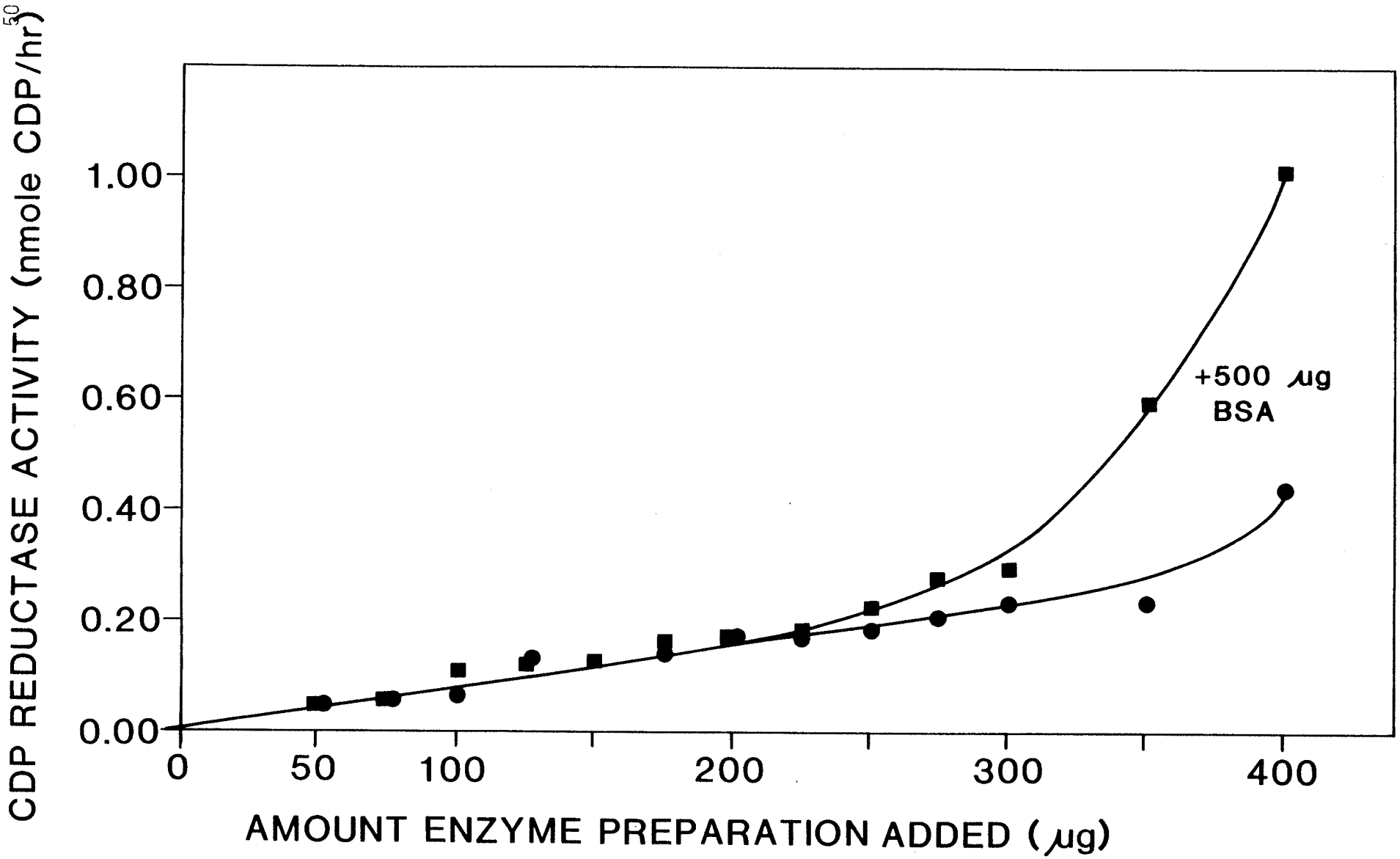
shown to enhance CDP reductase in E. coli (Engström et al,1979), S. cerevisiae (Vitols et al,1970), rabbit bone marrow (Hopper,1972), calf thymus (Mattaliano et al,1981), and Chinese hamster ovary cells (Hards and Wright,1984a). Hopper (1972) and Mattaliano et al (1981) suggested that magnesium ions promoted the binding together of the two non-identical subunits of ribonucleotide reductase. Table 1 indicates that this addition may actually inhibit slightly the reduction of CDP. Tyrsted and Gamulin (1979) found that the presence of magnesium ions was not essential for CDP reduction in phytohemagglutinin stimulated human lymphocytes. Magnesium ions were not included in the standard assay for CDP reduction.

(f) Response to Increasing Protein Concentration

Figure 7 shows the effect that increasing amounts of protein had on CDP reductase activity. Up to 225 μg of extract, the effect of added BSA was not detected. At this point, the two curves diverge and the curve with added BSA rose at much faster rate. This was consistent with the findings of Lewis and Wright (1978) and Kuzik and Wright (1980) and suggests that the enzyme may be dissociating at low protein concentrations (Hopper,1972). On the basis of these findings, the standard CDP reduction assay was performed using at least 0.5 mg of protein (dilute samples supplemented with BSA).

Figure 7: The effect of protein concentration on the rate of CDP reduction.

The amount of protein extract added to the assay tubes was varied from 50 - 400 µg. The curve for enzyme extract alone is plotted with (●) while the tubes that received 0.5 mg BSA are plotted with (■). The assay was performed in manner described in the Materials and Methods.


CDP REDUCTASE ACTIVITY (nmole CDP/hr)50
1.00
0.80
0.60
0.40
0.20
0.00
0
50
100
200
300
400
AMOUNT ENZYME PREPARATION ADDED (µg)
+500 µg
BSA

(g) Standard CDP Reductase Assay Conditions

The standard conditions for the assay of CDP reduction are presented in Table 2. The assay was carried out in a total volume of 150 μL, so the volume of 50 mM HEPES pH 7.8 was altered to accommodate the various additives being tested.

The properties of CDP Reductase from Achlya are presented in Table 3. The effects of various additions or omissions on the activity of the enzyme are expressed.

Table 2. Standard CDP reduction assay conditions

- 32 mM cold CDP
- 0.33 nmole ^{14}C-CDP
- 6 mM dithiothreitol
- 6 mM ATP
- 50 mM HEPES buffer pH 7.8
- at least 0.5 mg total protein/assay point
- reaction was carried out at 37 °C for 30 minutes and was terminated by boiling for 4 minutes.

Table 3. Properties of CDP reductase from Achlya

Omission or Addition	% Activity
complete	100%
- ATP	77.5
- DTT	46.7
- $MgCl_2$	100
+ 4 mM dGTP	380
+ 10 mM CTP	256
+ 10 mM dCTP	81
+ 10 mM dTTP	15
+ 200 mM hydroxyurea	20

(III) Kinetics of CDP Reductase from Achlya

(a) Km for CDP

Figure 8 is a Lineweaver-Burk double reciprocal plot showing the dependence of reaction rate upon CDP concentration with 6 mM ATP as the positive effector. The curve is linear and gives an apparent Km value of 20 mM CDP and a Vmax value of 13.3 nmoles CDP/mg/hr.

(b) Effect of dATP on CDP reductase

Figure 9 indicates that dATP had no inhibitory effect on the CDP reductase of Achlya klebsiana. Vitols et al (1970) showed that dATP was inhibitory to CDP reduction by Saccharomyces cerevisiae. Thelander et al (1979) demonstrated dATP inhibition of ribonucleotide reductase in Escherichia coli. Wright (1983) reported that the CDP reductase of Chinese hamster ovary cells was inhibited by dATP.

Figure 10 shows that the CDP reductase of Achlya was actually stimulated (up to 3 fold) by addition of small amounts of dATP. This experiment was performed by altering both the concentration of dATP and ATP (ie. the ratio of dATP/ATP was altered). The addition of 0.5 mM dATP caused an increase in activity of about 160% over the range of 1-5 mM ATP. 1 mM dATP caused more than 300% stimulation of activity over the same range of ATP concentrations. The addition of 2 mM dATP caused a 255% stimulation with 2.5 mM ATP present but this stimulation dropped to 160% at 5 mM ATP.

Figure 8: Determination of Km for CDP with ATP as an activator.

The standard CDP assay was performed except that the concentration of CDP was varied from 0.2 -1 mM. A Lineweaver-Burk double reciprocal plot was generated and the Km found to be 20 mM.

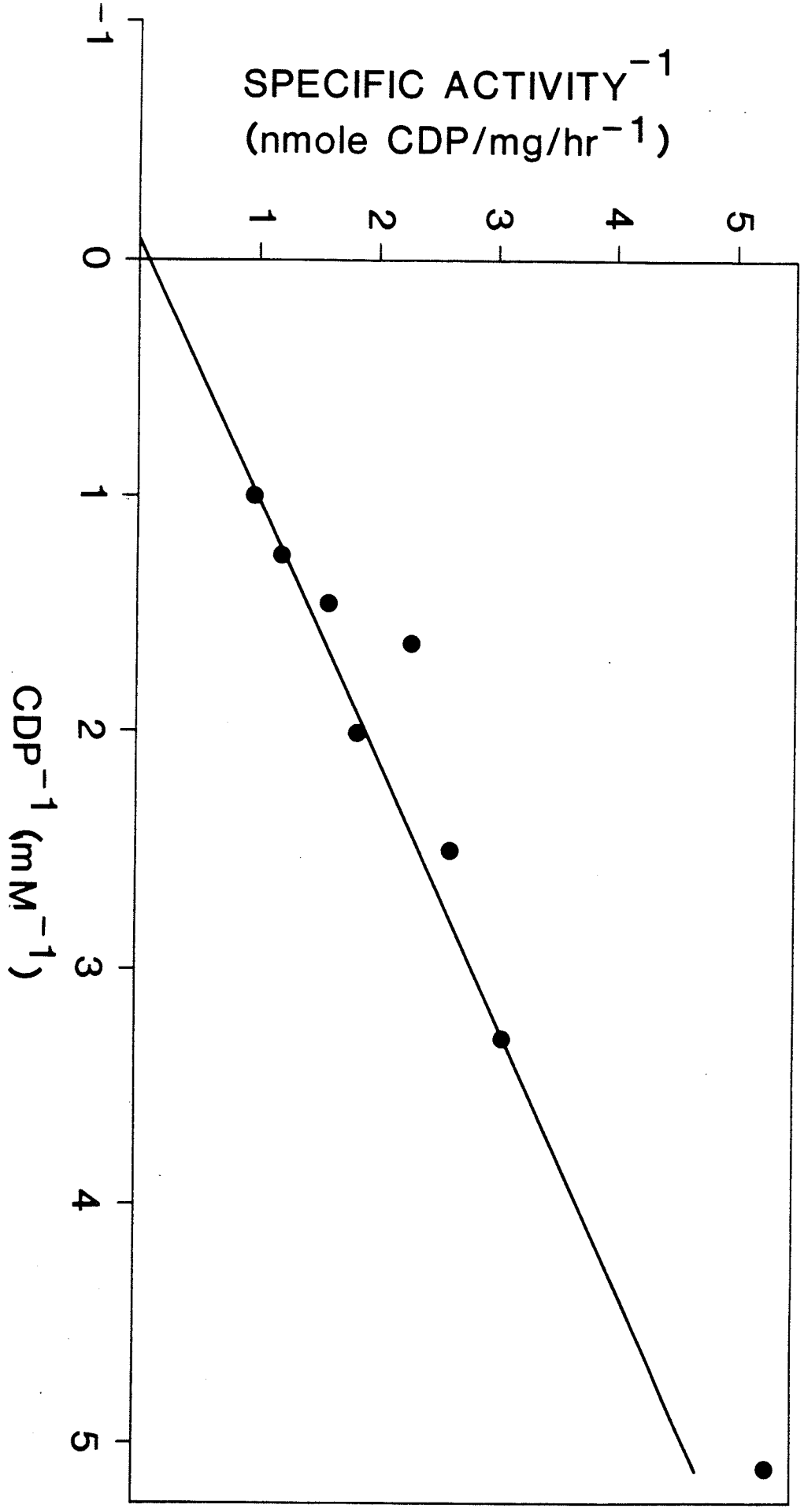

SPECIFIC ACTIVITY^{-1}
(nmole CDP/mg/hr^{-1})
1
2
3
4
5
−1
0
1
2
3
4
5
CDP^{-1} (mM^{-1})

Figure 9: The response of CDP reductase to dATP. The assay was performed in the manner described in the Materials and Methods. The tubes received from 0 - 10 mM dATP.

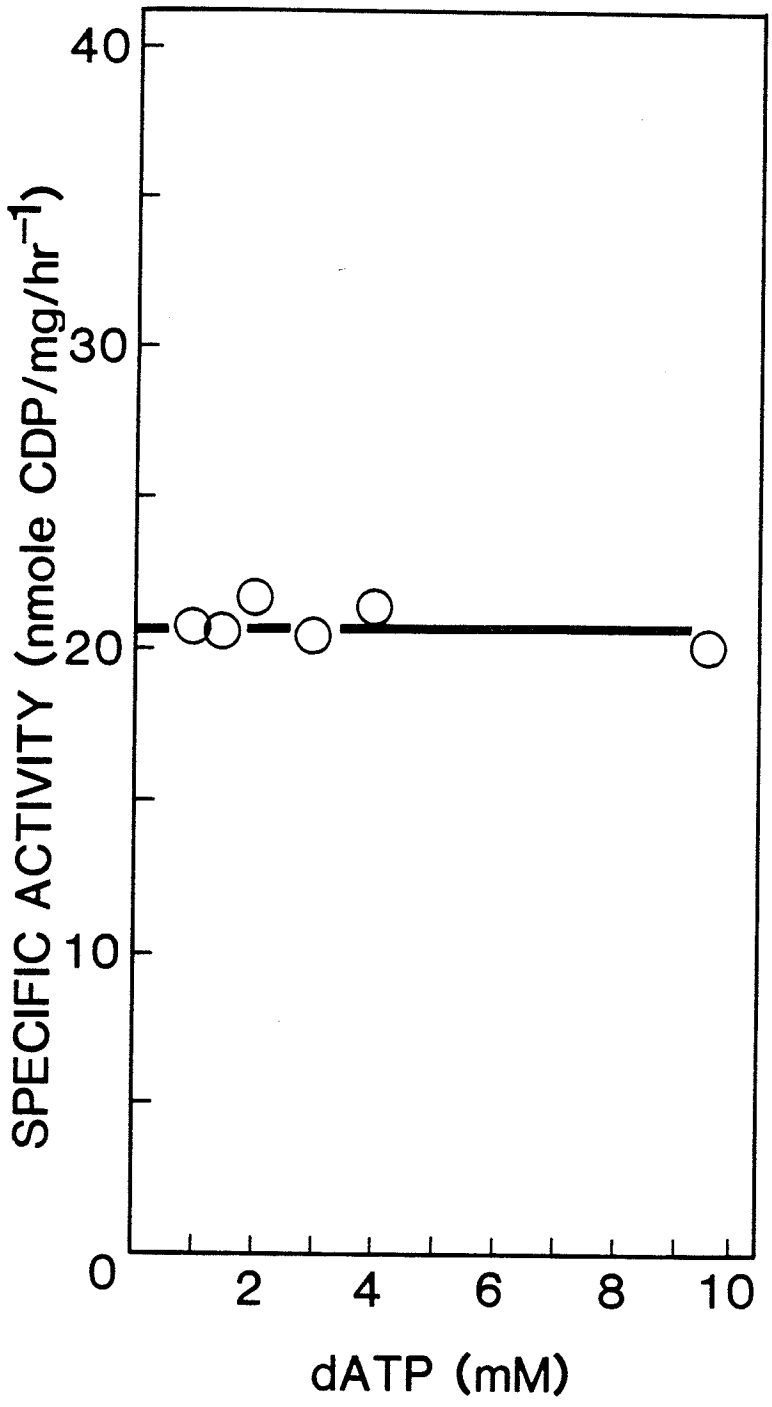
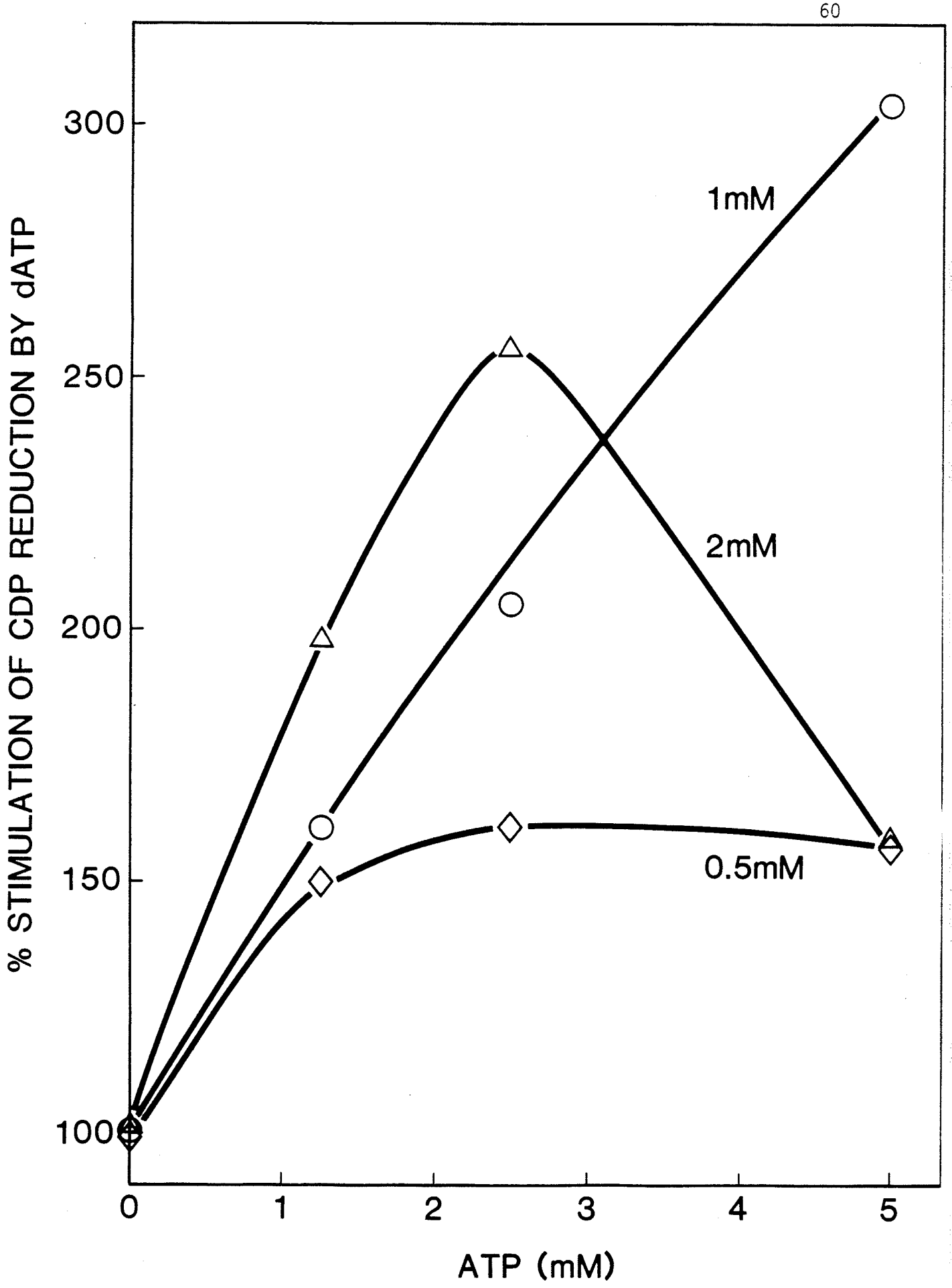

SPECIFIC ACTIVITY (nmole CDP/mg/hr^{-1})
40
30
20
10
0
2
4
6
8
10
dATP (mM)

Figure 10: The effect of altering the dATP/ATP ratio on the rate of CDP reduction.

Each assay tube received a range of 1 - 5 mM ATP. The tubes in the (◆) plot also received 0.5 mM dATP. Those in the (O) plot received 1.0 mM dATP and in the (▲) plot , 2 mM dATP.


% STIMULATION OF CDP REDUCTION BY dATP
300
250
200
150
100
0
1
2
3
4
5
ATP (mM)
1mM
2mM
0.5mM

(c) Effect of dGTP on CDP reduction

Figure 11 shows that CDP reductase activity was enhanced by the addition of dGTP. With 3 mM dGTP, activity was elevated 3.8 fold. Further additions elicited a lesser response but at 10 mM dGTP stimulation was still 2.5 fold. Thelander et al (1979) showed that dGTP was inhibitory to CDP reduction in Escherichia coli. Wright (1983) reported that dGTP was inhibitory to the CDP reductase of Chinese hamster ovary cells.

(d) Effect of dTTP on CDP reductase

The addition of dTTP caused substantial inhibition of CDP reduction. Figure 12 shows a marked decrease in activity when 1 mM dTTP was added (about 50% activity). When 5 mM dTTP was added, CDP reductase activity was reduced to just 14%.

(e) Response of CDP reductase to dCTP addition

The addition of dCTP had a minimal effect on CDP reductase. This is demonstrated in Figure 13. There is a slight increase in activity (13%) when 2 mM dCTP was added. An addition of 10 mM dCTP caused a 19% decrease in CDP reduction. Hards and Wright (1984b) reported a more dramatic stimulation (about 15 fold at 4 mM dCTP) of the CDP reductase in Chinese hamster ovary cells.

(f) Effect of CTP on CDP reductase

The addition of CTP to the reaction mixture of the CDP reductase assay caused an increase in specific activity. Figure 14 shows a gradual increase in

Figure 11: Activation of CDP reductase by dGTP.

A range of 0 - 10 mM dGTP was added to the assay tubes and the CDP reduction assay was performed as previously described.

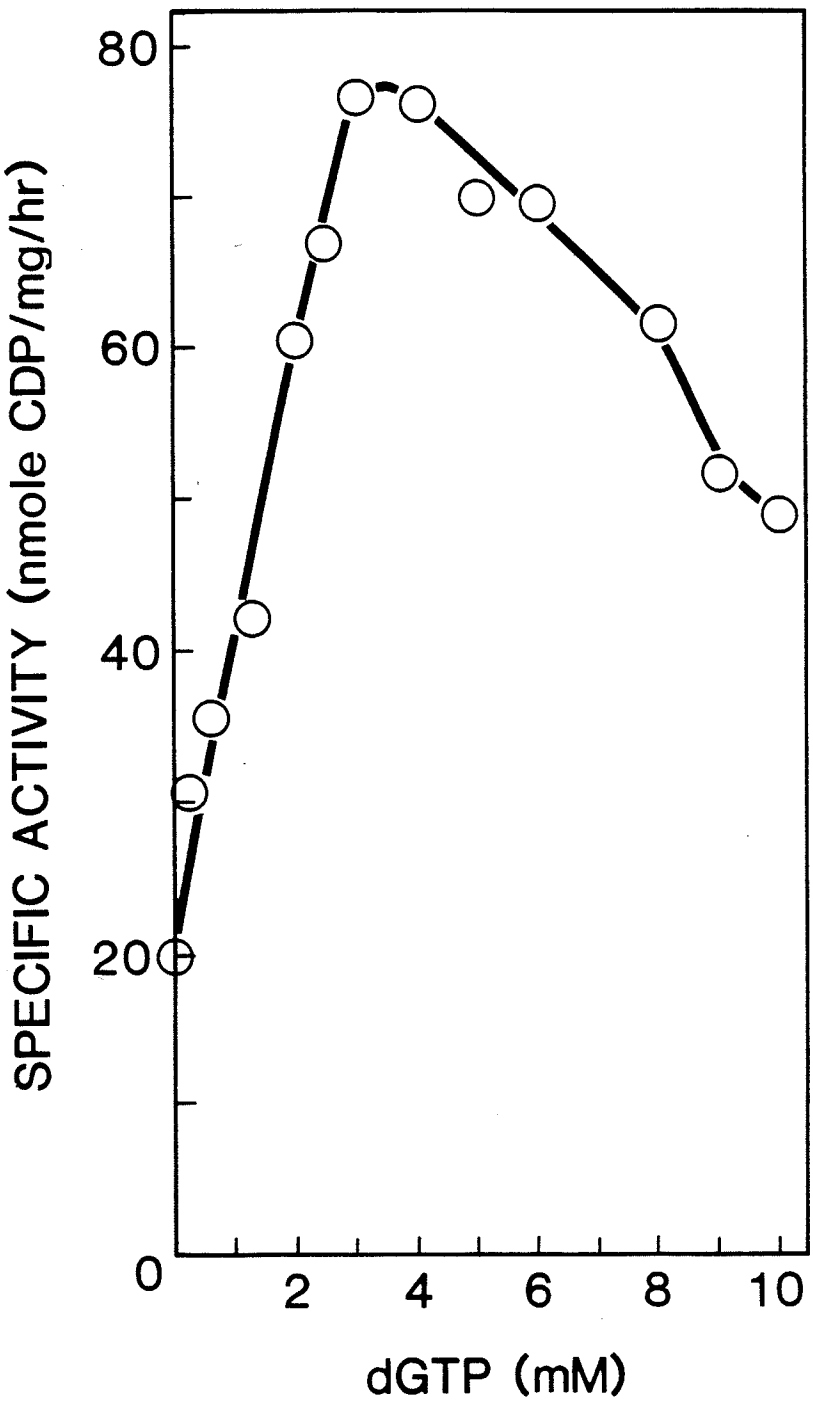

SPECIFIC ACTIVITY (nmole CDP/mg/hr)
80
60
40
20
0
2
4
6
8
10
dGTP (mM)

Figure 12: Inhibition of CDP reductase by dTTP.

The CDP reductase assay was performed as before with the addition of 0 - 10 mM dTTP.

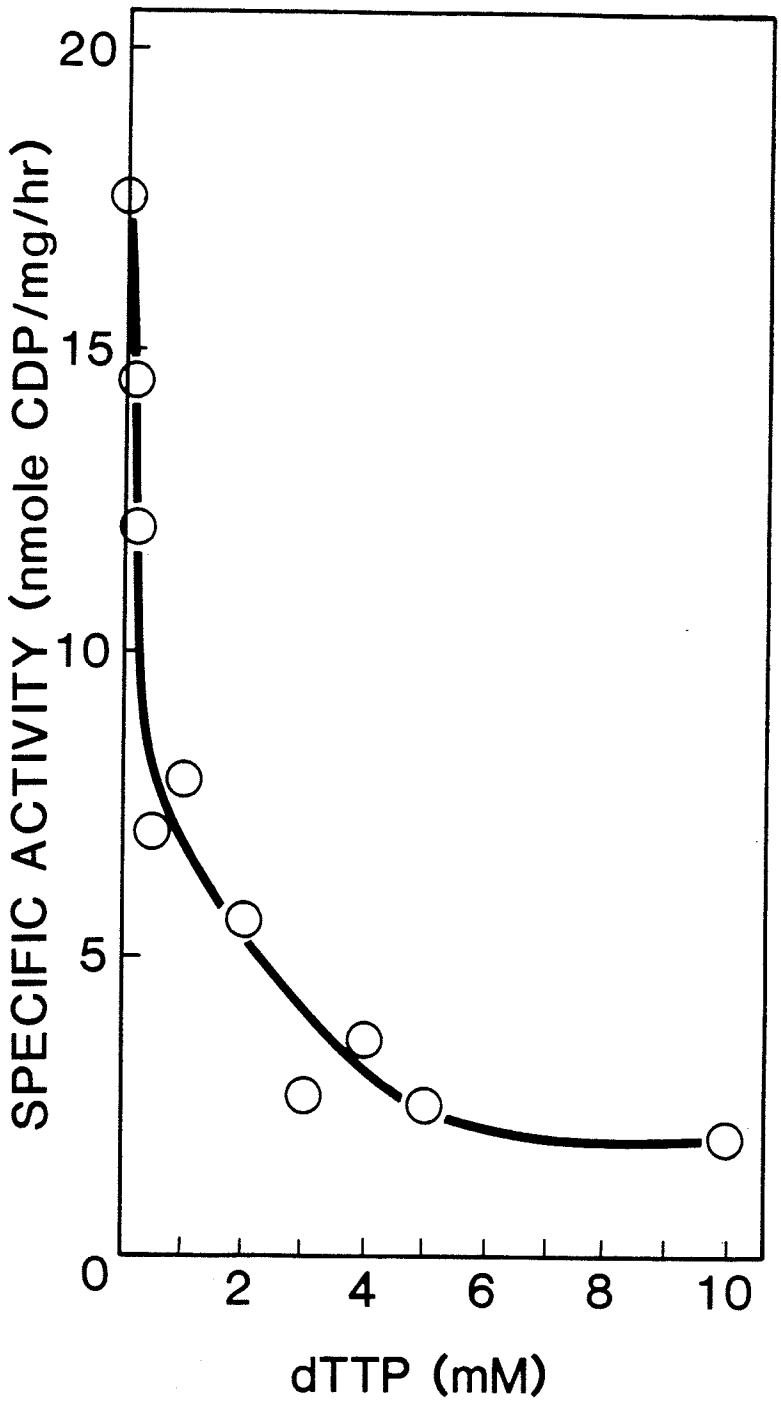

SPECIFIC ACTIVITY (nmole CDP/mg/hr)
20
15
10
5
0
2
4
6
8
10
dTTP (mM)

Figure 13: Response of CDP reductase to the addition of dCTP.

The CDP reductase assay was carried out as described in the Materials and Methods with the addition of 1 - 10 mM dCTP to the assay tubes.

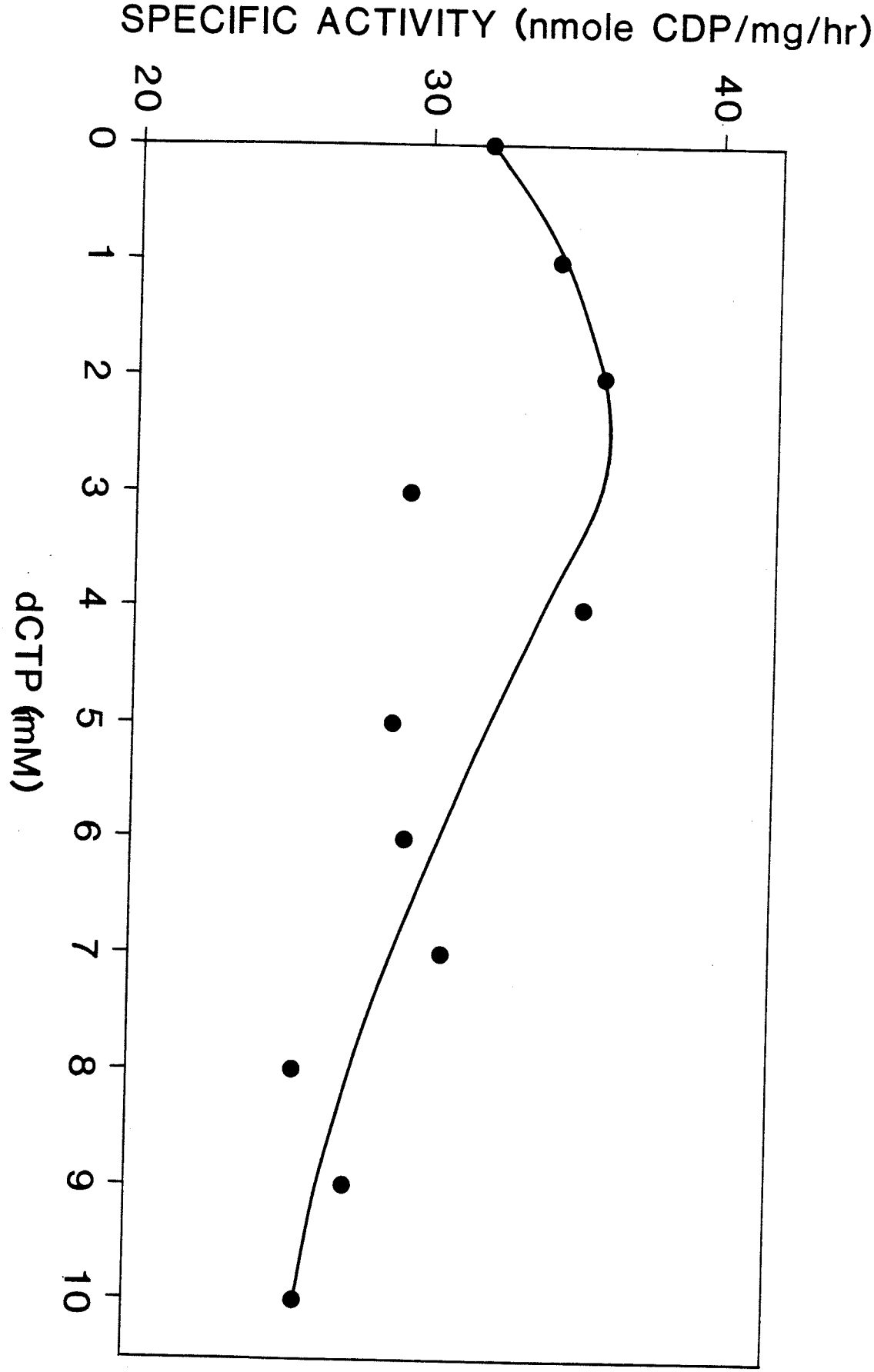
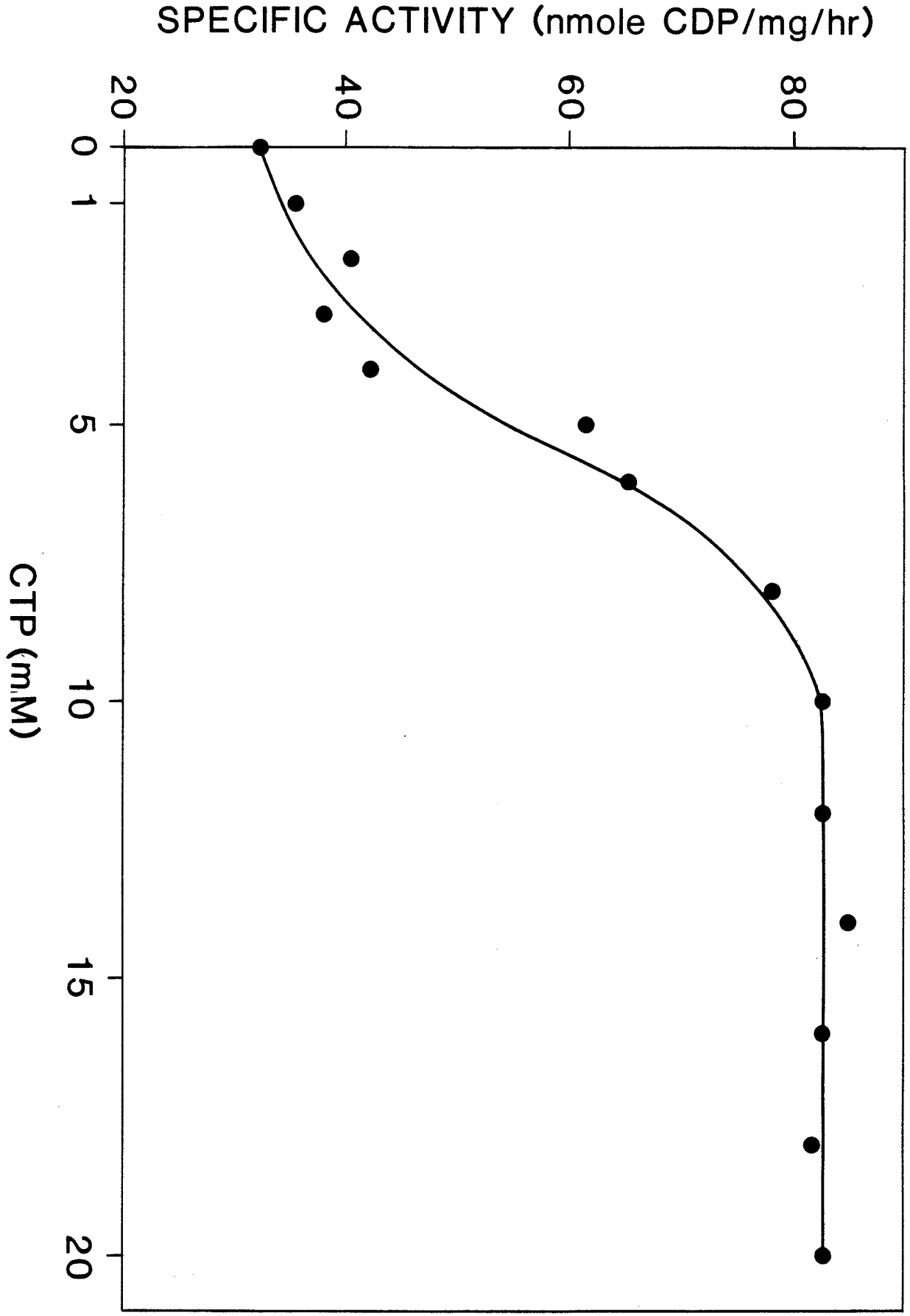

SPECIFIC ACTIVITY (nmole CDP/mg/hr)
20
30
40
0
1
2
3
4
5
6
7
8
9
10
dCTP (mM)

Figure 14: The response of CDP reductase to the addition of CTP.

The CDP reductase assay was performed in the manner previously described. The assay tubes also received 1 - 20 mM CTP.


SPECIFIC ACTIVITY (nmole CDP/mg/hr)
20
40
60
80
0
1
5
10
15
20
CTP (mM)

activity until 10 mM CTP when a 2.6 fold stimulation was produced. Further additions (up to 20 mM CTP) did not affect this level of stimulation.

(g) Response of CDP reductase to GTP

Figure 15 illustrates the response of CDP reductase to the presence of GTP as a positive effector in the absence of ATP. The curve is similar to Figure 6 (Effect of ATP) in that it shows that there is some activity in the absence of any activator (GTP or ATP) but that specific activity increased to a maximum with the addition of the activator (in both cases 6 mM). Figure 16 is the Lineweaver-Burk plot of initial reaction rates using 6 mM GTP as the positive effector. This curve is linear and yields an apparent Km value of 16 mM CDP and a Vmax value of 67.0 nmoles CDP/mg/hr. Hards and Wright (1984b) reported this same effect in the Chinese hamster ovary system, where GTP could replace ATP as the positive effector.

(h) Effect of UDP on CDP reductase

Figure 17 indicates that the addition of UDP did not inhibit the reduction of CDP. Cory (1979) had postulated that UDP would act as a competitive inhibitor of CDP reduction in Ehrlich Tumor cells because both CDP and UDP reduction require ATP as an activator. Also, the two substrates apparently bind at the same catalytic site in mammalian cells and should compete for this site, however he did not find this

Figure 15: GTP as a replacement of ATP as the activator of CDP reductase.

The standard CDP reductase was carried out without any ATP in the reaction mixture. This was replaced by 0 - 10 mM GTP.

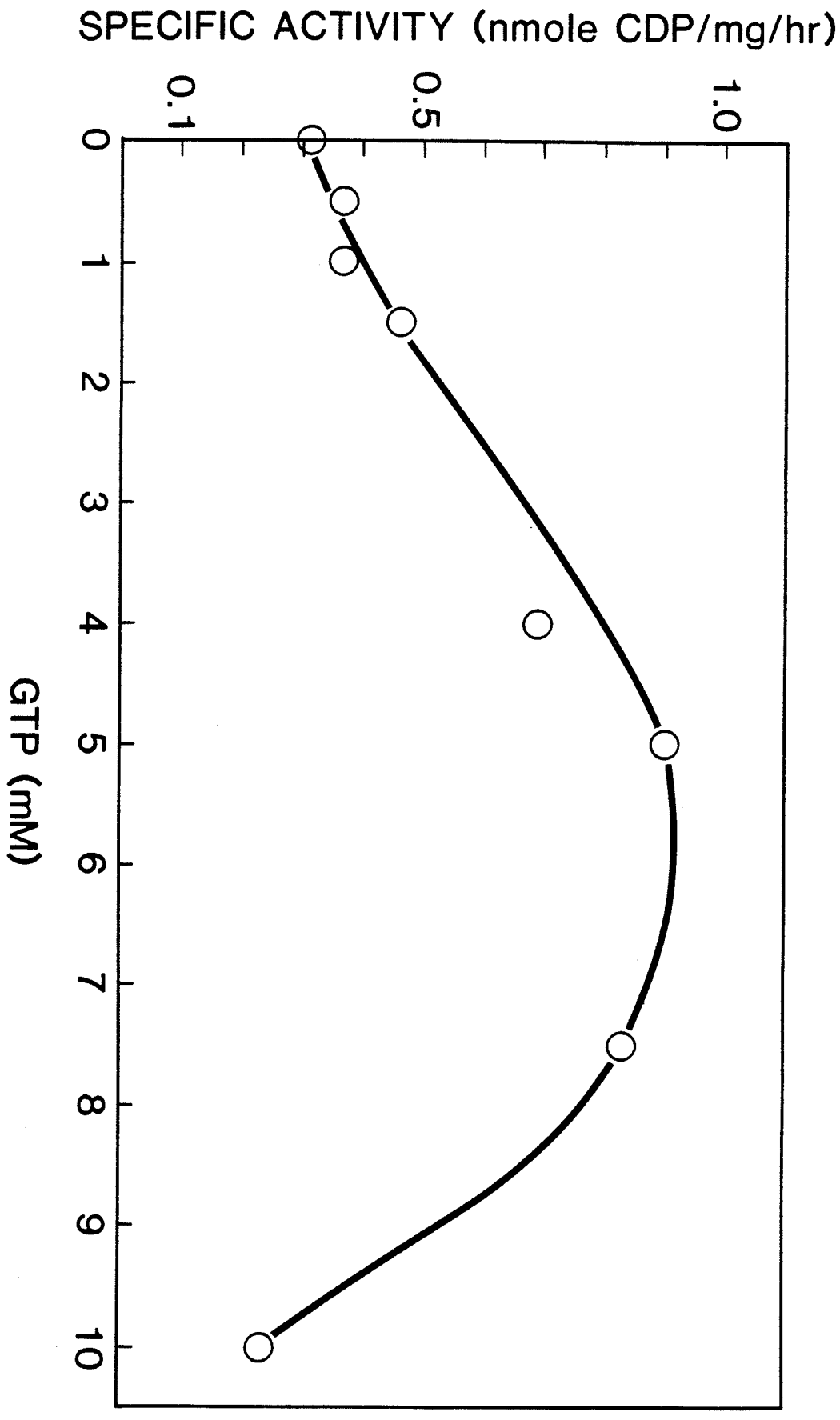

SPECIFIC ACTIVITY (nmole CDP/mg/hr)
0.1
0.5
1.0
0
1
2
3
4
5
6
7
8
9
10
GTP (mM)

Figure 16: Determination Km for CDP with GTP as the activator.

This assay was done in the same manner as in Figure 8 except that [CDP] ranged from 4 - 40 mM and 6 mM GTP was used as the activator. The Km was found to be 16 mM CDP.

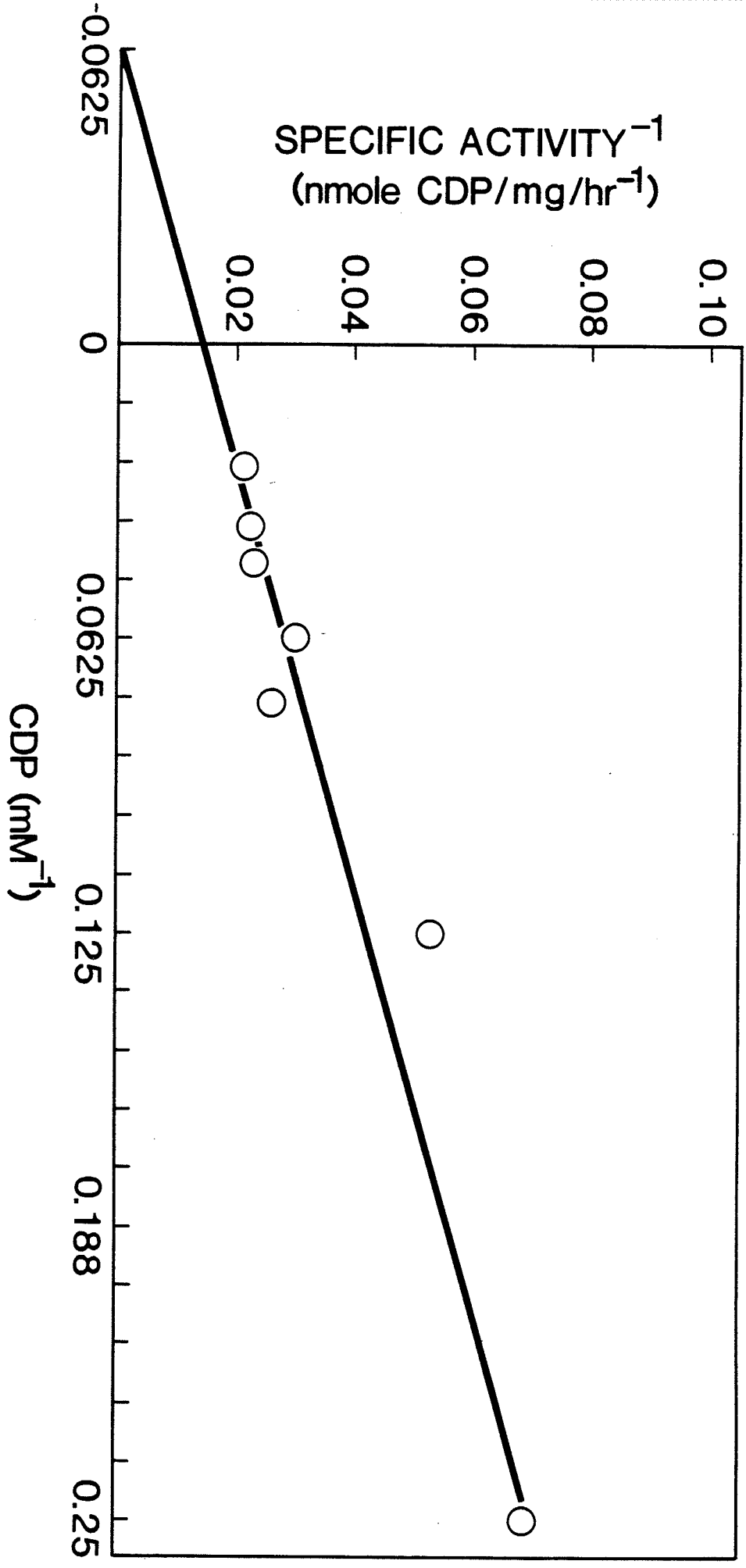
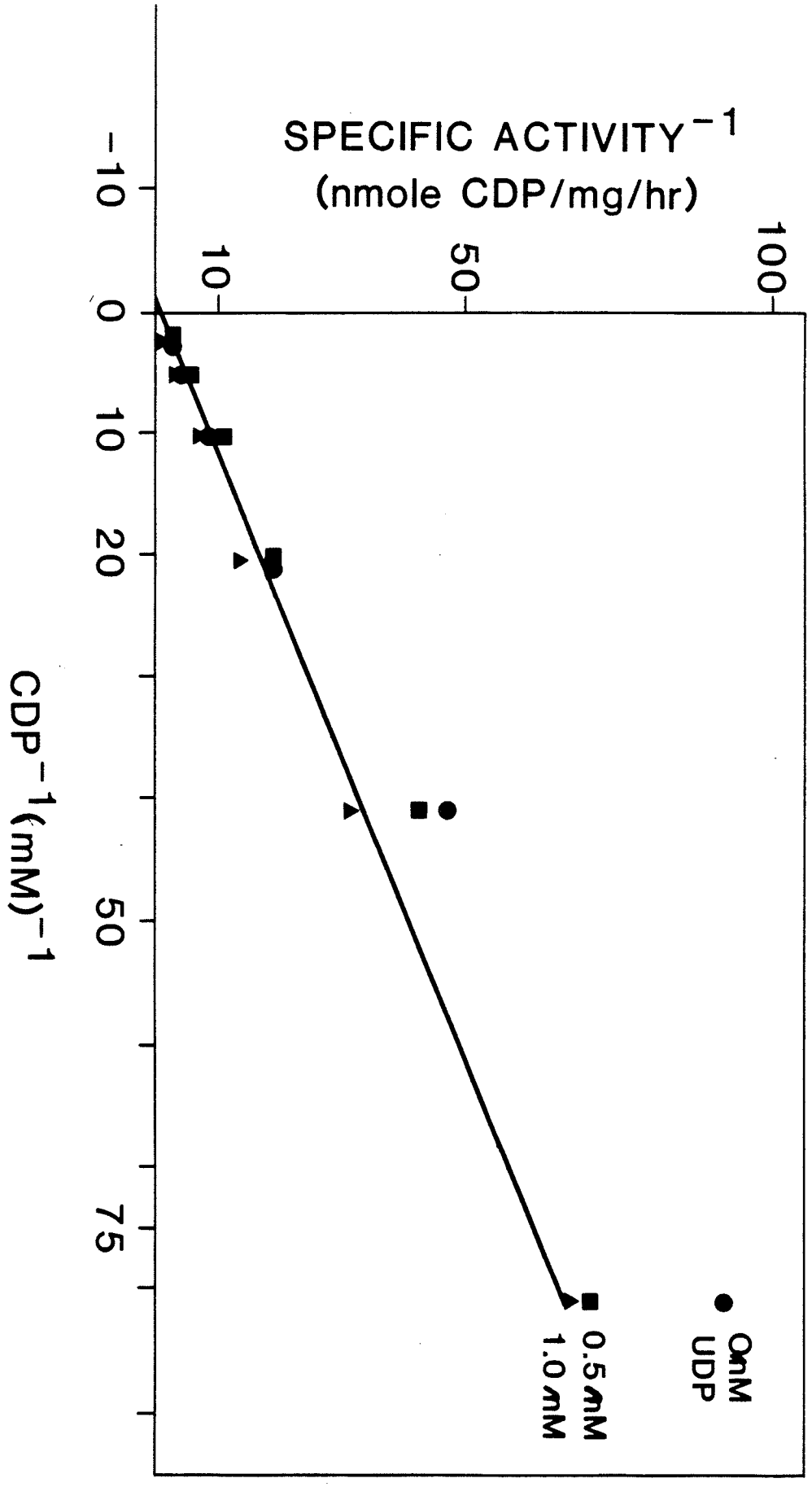

SPECIFIC ACTIVITY^{-1}
(nmole CDP/mg/hr^{-1})
0.02
0.04
0.06
0.08
0.10
-0.0625
0
0.0625
0.125
0.188
0.25
CDP (mM^{-1})

Figure 17: The response of CDP reductase to the addition of UDP.

The CDP reductase assay was performed as described in the Materials and Methods with a range of CDP concentrations of 0.01 to 0.4 mM. One set of assay tubes didn't receive any UDP (●). The tubes that form the (■) plot received 0.5 mM UDP. The (▲) set received 1.0 mM UDP. A double reciprocal plot was made with a single line that represents all the points.


SPECIFIC ACTIVITY^{-1}
(nmole CDP/mg/hr)
100
50
10
0
10
20
50
75
-10
CDP^{-1}(mM)$^{-1}$
0 mM UDP
0.5 mM
1.0 mM

with enzyme prepared from Ehrlich Tumor cells. Therefore, the results obtained with CDP reductase prepared from Achlya resemble those observed by Cory (1979).

(i) Inhibition of CDP reductase by hydroxyurea

The introduction of hydroxyurea (Hu) to the optimized assay conditions caused inhibition of CDP reduction. Figure 18 illustrates this effect. The addition of 24 mM hydroxyurea caused a 50% reduction in CDP reductase activity while only 20% activity remained after the addition of 200 mM hydroxyurea. These were extremely high doses of drug to illicit this degree of inhibition. Vitols et al (1970) added 5 mM hydroxyurea to extracts of Saccharomyces cerevisiae and found 54% of the CDP reductase activity remained. Wright (1983) reported that the addition of 0.7 mM hydroxyurea caused an 80% decrease in the CDP reductase activity of Chinese hamster ovary cell extracts.

(j) Response of CDP reductase to gossypol

The addition of gossypol (an antiproliferative, male antifertility agent - Chang et al,1980) had no effect on the CDP reductase of Achlya klebsiana. Table 4 indicates that there was no apparent inhibition of specific activity caused by this drug. McClarty et al (1985) demonstrated that the ribonucleotide reductase of mouse L cells was very sensitive to inhibition by gossypol. At a drug concentration of just 50 μM, enzyme activity was reduced to less than 10%. When

Figure 18: CDP reductase activity in the presence of increasing concentrations of hydroxyurea. The standard CDP reductase assay was carried out. Concentrations of hydroxyurea from 0 - 200 mM were added to the assay tubes.

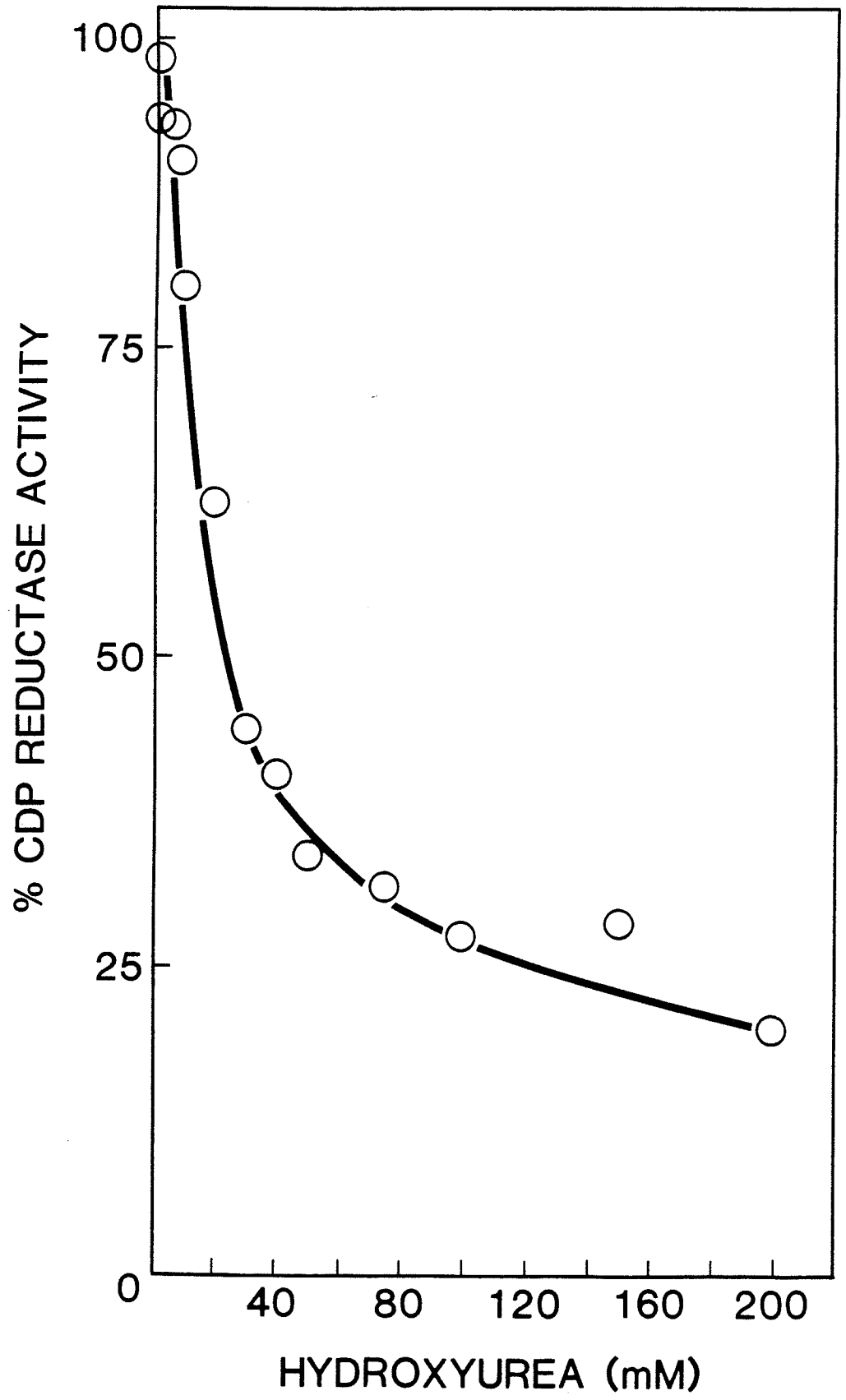

100
75
50
25
0
% CDP REDUCTASE ACTIVITY
40
80
120
160
200
HYDROXYUREA (mM)

Table 4. Effect of gossypol on CDP reductase

[Gossypol] (μM)	% Specific Activity
0	100%
10	86.1
20	113.9
40	83.3
60	83.3
80	86.1
100	119.4
300	100.0
400	97.2

100% activity = 0.36 nmole CDP/mg/hr

8 times as much gossypol (0.4 mM) was added to Achlya CDP reductase, no effect was detected.

(k) Response of CDP reductase to guanazole

Table 5 shows that the addition of very large amounts of guanazole had no inhibitory effect on the CDP reductase of Achlya klebsiana. Lewis and Wright (1978) found that the CDP reductase prepared from Chinese hamster ovary cells was sensitive to guanazole; the addition of 2 mM guanazole caused a 65% decrease in CDP reduction. The addition of as much as 50 mM guanazole did not inhibit the CDP reductase of Achlya(see Table 5).

(l) Effect of MAIQ on CDP reductase

Table 6 shows that MAIQ (4-Methyl-5-Amino-1-Formyl-isoquinoline Thiosemicarbazone) had no inhibitory effect on CDP reductase from Achlya. In fact, it seemed to enhance specific activity by as much as 66% (at 0.4 mM). Preidecker et al (1980) reported that partially purified extracts from rat Novikoff Tumor cells subjected to 25 μM MAIQ showed 83% inhibition of CDP reduction.

(m) Response of CDP reductase to bleomycin

The introduction of bleomycin to the assay for CDP reductase with Achlya enzyme preparations did not cause any inhibition of specific activity. Table 7 indicates that high levels of the drug actually enhanced CDP reduction (707 μM bleomycin caused an increase to 222.9% of control activity. McClarty et al (1986)

Table 5. Effect of guanazole on CDP reductase

[Guanazole] (mM)	% Specific Activity
0	100%
1	76.8
5	83.7
10	95.2
15	77.5
20	85.6
25	94.0
30	64.4
35	117.0
40	85.6
45	80.9
50	108.6

100% Activity = 41.8 nmole CDP/mg/hr

Table 6. Effect of MAIQ on CDP reductase.

[MAIQ] (μM)	% Specific Activity
0	100%
10	164.5
50	147.4
100	149.3
150	148.1
200	160.8
400	166.0

100% Activity = 41.8 nmole CDP/mg/hr

MAIQ = 4-Methyl-5-Amino-1-Formylisoquinoline Thiosemicarbazone

Table 7. Effect of bleomycin on CDP reductase

[Bleomycin] (μM)	% Specific Activity
0	100%
20	81.0
50.7	79.9
100	81.7
150.7	135.9
200	100.3
307	106.7
400	94.7
507	141.5
600	146.5
707	222.9
800	220.4
907	200.4
1.0 mM	166.2

100% Activity = 28.4 nmole CDP/mg/hr

described 15 - 20% inhibition of CDP reduction by extracts of mouse L cells when exposed to 6 μM bleomycin.

(IV) Summary of the Effectors of CDP reductase

Some of characteristic responses of CDP reductase from Achlya klebsiana to a variety of positive and negative effectors and antiproliferative drugs are presented in Table 8. Vitols et al (1970) was used as the source of the data on Saccharomyces cerevisiae. The characteristics of the Escherichia coli enzyme were found in the work of Thelander et al (1979), Hogenkamp (1984) and Cohen et al (1985). The information about the CDP reductase from Chinese hamster ovary cells was taken from Wright (1983) and Hards and Wright (1984b). The data about the Herpes simplex II CDP reductase was supplied by Wright (1983), Huszar and Bacchetti (1981) and Cohen et al (1985). The characteristics of the CDP reductase of T4 phage was taken from the work of Hogenkamp (1984), Follman (1974), Cohen et al (1985) and Berglund (1972).

Table 8. Comparison of the effectors of CDP reductase from various sources

\	dATP	dGTP	dTTP	UDP	ATP	GTP	MgCl2	DTT	Hu	MAIQ	Guan
A.k.	+	+	−	0	+	+	0	+	−*	0*	0*
S.c.	−				+		+	+	−		
E.c.	−	−	+	−	+		+	+	−	−	−
CHO	−	−	−	−	+	+	+	+	−	−	−
HsII	0	0	0		−		0	+	−	−	−
T4	+	0	+		+	+	0	+	−	−	−

* = extremely high level of drug used

+ = activation

− = inhibition

0 = no effect

A.k. = Achlya klebsiana

S.c. = Saccharomyces cerevisiae

E.c. = Escherichia coli

CHO = Chinese hamster ovary cells

HsII = Herpes simplex II

T4 = T4 bacteriophage

DISCUSSION

Ribonucleotide reductase was partially purified from Achlya klebsiana, a common water mould. The onset of S-phase was determined by monitoring the rate at which tritiated thymidine was incorporated into acid precipitable material (Lewis et al, 1976). This indicated that 12 hours after spore germination was the time to harvest the fungal mycelia to obtain the maximal amount of ribonucleotide reductase (Figure 1). Lewis et al (1976) reported that the peak of thymidine uptake and maximal CDP reductase specific activity occurred at 9 hours after spore germination but chose to routinely harvest the cells after 10 hours. This time difference was most likely due to differences in the growth conditions. Lewis et al (1976) used G Y media in 15 L carboys incubated at 28 C with aeration. This study found that PYG medium in 2 L flasks in an orbital shaker provided better growing conditions, was more easily managed, and yielded higher CDP reductase specific activities.

The enzyme purification was designed to exploit the characteristic aggregation of ribonucleotide reductase when in the presence of ATP. Youdale et al (1982) reported that ATP caused the aggregation of the L1 (M1) subunits of regenerating rat liver ribonucleotide reductase. They had reasoned that because the optimum effector concentration of ATP was 3.3 mM, L1 purified in the presence of ATP would yield an enzyme activated to reduce CDP (when recombined with L2/M2). They found

that aggregates resulted, increasing the apparent molecular weight by roughly 10 fold (measured by molecular exclusion high performance liquid chromatography). These aggregates dissociated when the sample was made 0.1 M potassium chloride.

Thelander et al (1980) found that dTTP caused M1 of calf thymus to form dimers, dATP caused tetramers and ATP produced both dimers and tetramers. They suspected that these nucleotide effectors manifested their activation or inhibition by inducing conformational changes in the enzyme that resulted in aggregation.

Brown and Reichard (1969) reported that dATP caused the aggregation of both B1 and B2 of the E. coli enzyme. The addition of dATP or ATP resulted in the formation of aggregates of M1("dye fraction") of ribonucleotide reductase from Ehrlich tumor cells (Klippenstein and Cory, 1978).

This study showed that 6 mM ATP resulted in more CDP reductase retained by the XM100A ultrafilter (ie. more aggregation) than did 3.3 mM. Further increases in the ATP concentration did not improve the purification. It should be noted that 6 mM ATP was the optimum concentration for activating CDP reduction (Figure 6).

These aggregates precipitated when mixed in 0.5% protamine sulfate and centrifuged but when the resulting pellet was redissolved in isolation buffer without ATP, subsequent centrifugation left the CDP

reductase activity in the supernatant (Figure 2).

The addition of 100 mM KCl was sufficient to dissociate the aggregates retained by the XM100A ultrafilter and did not destroy enzyme activity (assayed after dialysis). This concurs with the findings of Youdale et al (1982) and Mattaliano et al (1981).

The ribonucleotide reductase from Achlya klebsiana was found to be extremely sensitive to dilution. Concentration of dilute extracts did not restore CDP reductase activity. Lewis and Wright (1978) reported a logarithmic decrease in enzyme activity with decreasing protein concentration with Chinese hamster ovary cells, in agreement with the studies of Peterson and Moore (1976). Similar non-linear response to protein concentration was reported by Hopper (1972) for ribonucleotide reductase from rabbit bone marrow and in mouse L-cells by Kuzik and Wright (1980).

As a result of this sensitivity, purification steps that might increase sample volume profoundly were not successful. Ultracentrifugation was not used and gel filtration was replaced by dialysis. Bovine serum albumin was added to extracts as a protein carrier to protect the enzyme from this dilution effect. Lewis (1978) found that the addition of BSA did not prevent the inactivation of ribonucleotide reductase from Chinese hamster ovary cells. Enzyme assays were performed on extracts from Achlya that were at least

0.5 mg/mL protein (sufficient BSA was added to more dilute samples).

The CDP reductase from Achlya was also very easily destroyed by ammonium sulfate and by polyethylene glycol so these could not be used to concentrate the enzyme. Vitols et al (1970) encountered the same problem in the purification of ribonucleotide reductase from Saccharomyces cerevisiae. No CDP reduction activity was detected in extracts that had been exposed to 0.2 mM ammonium sulfate. Similarly, 0.2 M sodium chloride left only about 10% of control activity and 0.2 M potassium phophate left about 20% activity. They suggested that this inhibition was most likely a result of increased ionic strength (not caused by the species of ion itself). The yeast ribonucleotide reductase was found to be very unstable (Vitols et al,1970; Lammers and Follmann,1984). The enzyme dissociated in solution into its component subunits which were subsequently irreversibly damaged.

Lewis et al (1976) assayed the activity of CDP reductase from Achlya in a manner that differed in several ways from that found in the Materials and Methods. They included 10 mM $MgCl_2$, 10 mM dithiothreitol, 10 mM ATP and 0.4 mM CDP in 50 mM Hepes at pH 7.5 and ran their assays for 60 minutes at 22°. This study indicated that some changes to these parameters would be beneficial.

The addition of magnesium ions had no positive

effect on CDP reduction. In fact, they may have caused a slight inhibition of the reaction (Table 1). Magnesium was omitted from the standard CDP reductase assay. Tyrsted and Gamulin (1979) reported that the enzyme from phytohemagluttinin-stimulated human lymphocytes did not require magnesium ions for CDP reduction. Engström et al (1979) found that they did not have to include magnesium in their purification of the calf thymus ribonucleotide reductase in contrast to their studies on the E. coli enzyme. They also reported that the calf thymus enzyme showed approximately 50% maximal activity when magnesium was omitted from the assay.

Magnesium ions are not required by the enzyme from bacteriophage T4 (Berglund,1972). The subunits of this enzyme are bound together much more tightly and do not dissociate upon purification. Huszar and Bacchetti (1981) found that the ribonucleotide reductase induced by Herpes simplex virus did not require magnesium ions for activity.

The optimum concentration of dithiothreitol in the CDP reductase assay was determined to be 6 mM (Figure 5). This seems to be a standard value for ribonucleotide reductase assays. Huszar and Bacchetti (1981) used 6.2 mM dithioerythritol (an analog of DTT) in the assay of activity in extracts of Herpes simplex virus-infected baby hamster kidney cells. Hards and Wright (1984a) used 6 mM DTT in the assay for reductase

activity in intact permeabilized Chinese hamster ovary cells. Mattaliano et al (1981) used 6.2 mM DTT in the assays of activity in extracts of calf thymus.

The concentration of ATP used in the standard CDP reductase assay with extracts from Achlya klebsiana was 6 mM (Figure 6). When ATP was omitted, 77.5% of control activity was detected. This is consistent with the findings of Moore and Hurlbert (1966); that ATP was required for maximal CDP reduction.

The decision to include 32 mM CDP in the assay was based on the determination of the Km for CDP. Figure 16 showed this determination to be 16 mM CDP. This is relatively high; Moore and Hurlbert (1966) report a Km for CDP of 15 - 47 μM for the enzyme from Novikoff hepatoma. Larsson (1969) reported a Km for CDP of 10 μM in regenerating rat liver. Cory (1979) found the Km for CDP to be 26.6 μM for the enzyme from Ehrlich tumor cells. The enzyme from calf thymus had a Km for CDP of 30 - 32 μM (Eriksson et al,1979).

The pH at which the CDP reductase assay was performed with Achlya extracts was pH 7.8 (Figure 3). Greater than 90% of maximal activity occurred over a range of pH 7.4 - 8.3. This is a narrow range when compared to that reported by Larsson (1969) for the enzyme from regenerating rat liver. Lewis (1978) also found a large pH range for the CDP reductase assay on extracts of Chinese hamster ovary cells.

Similarly, the temperature range for maximal CDP

reductase activity was relatively narrow (Figure 4). Lewis et al (1976) performed CDP reductase assays with Achlya extracts at 22 °C, the temperature usually used to grow the fungus. Interestingly, this study with partially purified Achlya enzyme preparations showed only 53% of maximal specific activity occurred at 22 °C, when compared to an optimum temperature of 37 °C. Greater than 90% of maximal activity occurred over a temperature range of about 34 °C - 39 °C. Lewis (1978) found a much larger temperature range for the enzyme isolated from Chinese hamster ovary cells.

The addition of dATP had no inhibitory effect on CDP reduction by Achlya (Figure 9). Figure 10 shows that, at low levels of ATP, dATP caused a stimulation of CDP reductase activity of up to 300%. Ribonucleotide reductase from mammalian sources is generally inhibited by the addition of dATP (Murphree et al,1968; Larsson,1973; Chang and Cheng,1979b; Eriksson,1979; Hards and Wright,1984b). Vitols et al (1970) showed that dATP was inhibitory to CDP reduction by Saccharomyces cerevisiae. The enzyme from E. coli was inhibited by dATP (Thelander,1979). Huszar and Bacchetti (1981) showed that the enzyme induced by Herpes simplex II was much more resistant to inhibition by dATP than the cellular (baby hamster kidney cell) enzyme (the viral enzyme was strongly inhibited by ATP though). Berglund (1972) found that dATP was a prime activator of CDP reduction by the enzyme induced by

bacteriophage T4.

CDP reductase activity in Achlya was activated by the addition dGTP (Figure 11). An increase in activity of 3.8 fold was observed with the addition of 3 mM dGTP. The E. coli enzyme was inhibited by dGTP (Thelander,1979). Hards and Wright (1984b) reported that dGTP inhibited CDP reductase from Chinese hamster ovary cells.

The CDP reductase from Achlya was markedly inhibited by the addition of dTTP (Figure 12). Inhibition of CDP reductase by dTTP is a widely observed phenomenon (rat embryo-Murphree et al,1968; Novikoff hepatoma-Moore and Hurlbert,1966; calf thymus-Eriksson et al,1979; human Molt-4F-Chang and Cheng,1979b; Chinese hamster ovary cell-Hards and Wright,1984b). The addition of dTTP stimulated the reduction of CDP in E. coli (Larsson and Reichard,1966) and in bacteriophage T4 (Berglund,1972).

The addition of dCTP had very little effect on the reduction of CDP by Achlya (Figure 13). A slight enhancement (13%) was seen at 2 mM dCTP. Hards and Wright (1984a) reported a more dramatic stimulation (about 15 fold at 4 mM dCTP) of the CDP reductase from Chinese hamster ovary cells. The prime activator of CDP reductase from bacteriophage T4 was found to be dCTP (Berglund,1972).

The addition of CTP to the CDP reductase of Achlya

resulted in a 2.6 fold stimulation of specific activity (Figure 14). Wright et al (1981) reported that at 1 mM CTP, CDP reduction in hamster cells was only 1-2% of control activity.

This study indicated that GTP could replace ATP as the prime activator of CDP reduction in Achlya (Figure 15). The Km for CDP was lower when GTP was the activator (16 mM CDP-Figure 16) than when ATP was used (20 mM CDP-Figure 8). Hards and Wright (1984a) reported this same phenomenon in Chinese hamster ovary cells with maximal enzyme activity at 0.2 mM GTP. This suggests that models developed to explain the allosteric properties of ribonucleotide reductase should give more serious consideration to GTP as a positive effector of enzyme activity than has occurred in many cases (e.g. Thelander and Reichard,1979).

The addition of UDP to the CDP reductase of Achlya did not result in any observed inhibition (Figure 17). Cory (1979) had postulated (but did not observe in the Ehrlich tumor system) that there would be competitive inhibition of CDP reduction by UDP because ATP is the prime activator for both reductions. Larsson and Reichard (1966) reported competitive inhibition between CDP and UDP with the enzyme from Escherichia coli. Hards and Wright (1984b) found that there was competitive inhibition between CDP and UDP with ribonucleotide reductase in intact permeabilized Chinese hamster ovary cells.

The introduction of hydroxyurea to the assay of CDP reductase from Achlya caused substantial inhibition when present in very high levels (Figure 18). Lewis et al (1976) reported that 1.0 mM hydroxyurea caused 88% inhibition of maximal CDP reduction by the Achlya enzyme. Vitols et al (1970) found that 5 mM hydroxyurea left 54% control activity with the Saccharomyces cerevisiae enzyme. Wright (1983) reported that 0.7 mM hydroxyurea caused an 80% decrease in the CDP reduction by Chinese hamster ovary cells. The E. coli enzyme showed about the same sensitivity as the mammalian enzyme (Cohen et al,1985). The ribonucleotide reductase from bacteriophage T4 showed 70% inhibition by 0.5 mM hydroxyurea. Cohen et al (1985) used 0.65 mM to reduce the activity of the Herpes simplex induced enzyme by 50%.

Gossypol had no effect on the CDP reductase from Achlya (Table 4). McClarty et al (1985) demonstrated that the ribonucleotide reductase of mouse L-cells was very sensitive to inhibition by gossypol (less than 10% activity remained at 50 μM gossypol). No effect was observed with the addition of 0.4 mM gossypol to the Achlya enzyme.

The additions of very large amounts of guanazole had no inhibitory effect on the CDP reductase (Table 5). Guanazole concentrations to yield 50% inhibition are usually about 10 times higher than that of

hydroxyurea (Moore and Hurlbert,1985). Lewis and Wright (1978) found that 2 mM guanazole caused a 65% decrease in CDP reduction by Chinese hamster ovary cells. The enzyme from Herpes simplex was inhibited by 50% with 4 mM guanazole (Cohen et al, 1985).

Table 6 shows that MAIQ had no inhibitory effect on the CDP reductase from Achlya. The apparent enhancement of activity (66% at 0.4 mM) was most likely due to the inhibition of some other enzyme catalyzing a side reaction (eg. phosphatase). Preidecker et al (1980) reported that 33 ng/mL (0.13 μM) MAIQ caused 92% inhibition of the CDP reductase from rat Novikoff tumor. They also found that 5000 ng/mL (19.2 μM) MAIQ caused 46% inhibition of the E. coli enzyme (MW of MAIQ = 259.36). Cohen et al (1985) found that 0.3 μM MAIQ caused 50% inhibition of the Herpes simplex-induced enzyme while the cellular (baby hamster kidney cells) enzyme was inhibited to the same extent by just 0.1 μM MAIQ.

The CDP reductase from Achlya was not inhibited by bleomycin (Table 7). As in the case with MAIQ, the enhancement of activity (222.9% at 0.707 mM) was most likely due the inhibition of another enzyme catalyzing a side reaction. McClarty et al (1986) reported that mutants of mouse L-cells with elevated levels of ribonucleotide reductase were hypersensitive to bleomycin (30% activity remained at 8 μM after treatment with hydroxyurea). They also found that the enzyme was made

hypersensitive by pretreatment with hydroxyurea, guanazole or MAIQ and suggested that these drugs caused a conformational change in the M2 subunit exposing the iron group to chelation by bleomycin.

Conclusion

Table 8 presents a comparison of the effectors of CDP reductase from a variety of sources. While some molecules had a similar effect on all these forms of the enzyme (DTT,hydroxyurea), others provoked a range of effects. ATP is an activator of CDP reductase in all but Herpes virus. The insensitivity to (and activation by) dATP of the Achlya enzyme and its lack of a requirement for magnesium ions suggest that this enzyme is more akin to the virally-induced enzymes. It has more in common with these forms than the enzyme from Saccharomyces cerevisiae, which requires magnesium ions as well as thioredoxin/thioredoxin reductase for activity and is strongly inhibited by dATP. The enzyme is distinct from the Chinese hamster ovary ribonucleotide reductase in its response to dATP, dGTP, UDP and magnesium ions.

The relative insensitivity to the anti-proliferative drugs tested on the Achlya enzyme makes it distinct from all these forms of ribonucleotide reductase. The response to hydroxyurea and guanazole suggest that the free radical of the Achlya enzyme must be somehow shielded from these drugs by the protein

domains around the radical. Alternatively, there may be a large excess of the radical-containing subunit in these cells. The resistance to the effects of bleomycin and MAIQ suggest that the iron group may be protected by a protein conformation that differs from sensitive enzymes. An excess of the iron-containing subunit in Achlya would also explain this observation.

The work presented in this thesis indicates that the ribonucleotide reductase from Achlya differs in several interesting ways from the enzyme isolated from other sources. However, further work with homogeneous enzyme preparations is required to analyze these differences in detail. Clearly, Achlya is a good source of fungal enzyme for future studies of this key activity of DNA synthesis.

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