A STUDY OF RIBONUCLEOTIDE REDUCTASE FROM WILD-TYPE AND HYDROXYUREA RESISTANT MOUSE L CELLS IN CULTURE

by

BRIAN ANDREW KUZIK

A thesis submitted to the Faculty of Graduate Studies in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE

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This thesis is dedicated to the unyielding love, tolerance, and understanding of my parents.

ABSTRACT

The antitumor agent hydroxyurea is cytotoxic to mouse L cells in culture. At a concentration of 0.35 mM, hydroxyurea stops cellular growth and reduces plating efficiency to 6 X 10^{-6} in wild-type cultures. Ribonucleotide reductase activity was partially purified from wild-type L cells, and the optimum assay conditions and kinetic characteristics were determined for the substrates CDP and GDP. Enzyme activity from wild-type L cells was found to be similar to enzyme activity isolated from other mammalian sources. In agreement with other studies, both CDP and GDP reductase activities were potently inhibited by hydroxyurea, suggesting that the cytotoxic effects of this drug are due to its inhibition of ribonucleotide reductase.

A somatic cell mutant was isolated which exhibits a pleiotropic phenotype rendering it resistant to normally highly cytotoxic concentrations of hydroxyurea and guanazole (3,5-diamino-1,2,4-triazole, NSC 1895). The drug resistant phenotype was stable during long periods of non-selective cultivation and appears to have arisen by mutational rather than epigenetic means. Ribonucleotide reductase activity was also studied in detail from partially purified extracts of the drug resistant cell line designated L_2Cl_3. The optimum assay conditions for CDP and GDP reduction in L_2Cl_3 extracts resembled the conditions employed with wild-type extracts. However, ribonucleotide reductase activity from L_2Cl_3 extracts exhibited some significant kinetic differences when compared with the wild-type enzyme. For example, the Km value for

CDP with the L_2Cl_3 enzyme preparations was 3-fold higher than the value for this substrate with the wild-type enzyme. Ribonucleotide reductase activity from L_2Cl_3 extracts was also less sensitive to inhibition by hydroxyurea, with both CDP and GDP reductase activities showing increased Ki values for hydroxyurea. These findings are consistant with the presence of a structurally altered ribonucleotide reductase enzyme in L_2Cl_3 cells.

A technique is described for measuring ribonucleotide reduction in small numbers of whole cells made permeable to nucleotides by treatment with the detergent Tween-80. This procedure produced high enzyme activity per cell and a linear response to enzyme concentration which did not occur in cell-free extracts. The in vivo measurement of CDP and GDP reductase activity also revealed a difference between wild-type and L_2Cl_3 cells in their response to assay temperature, which supports the presence of an altered enzyme in L_2Cl_3 cells.

The in vivo assay system was also used to measure levels of CDP and GDP reductase activity in nucleotide-permeable wild-type and L_2Cl_3 cells. It was discovered that L_2Cl_3 cells contained approximately 3 times the wild-type level of CDP reductase, and twice the wild-type level of GDP reductase activity. These enhanced enzyme levels complemented by a decreased sensitivity of the L_2Cl_3 enzyme to inhibition by hydroxyurea can account for the cellular resistance of L_2Cl_3 cells to the drug.

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INTRODUCTION

A genetic approach to the study of the cell biology of bacteria and lower eukaryotes has been instrumental to the understanding of many aspects of the biochemistry and molecular processes present in these organisms. A similar approach in the study of multicellular animals is severly hindered by their very complex phenotypes. However, with the development of techniques for cell culture, biologists are now able to cultivate pure lines of higher eukaryotic cells as if they were microorganisms. This has allowed mammalian cell biologists to complement biochemical studies with genetic studies in attempts to gain further insight into the nature of biochemical pathways and molecular controls in mammalian cells.

The field of somatic cell genetics has expanded enormously within the last decade, with much of the research involving the isolation and characterization of cultured somatic cells with variant phenotypes. The exact genetic nature of the hereditable variation of cells in culture has not often been established. However, many well characterized systems are now available in which the variation in phenotype can be attributed to mutational rather than epigenetic events.

The work reported in this thesis involves the isolation and characterization of a variant mouse L cell line which is resistant to the cytotoxic effects of the anti-tumor agent hydroxyurea. This study involves the determination of the nature of the hydroxyurea resistant phenotype in this variant of an aneuploid permanent cell line.

Evidence exists indicating that hydroxyurea exerts its cytotoxic effects through inhibition of ribonucleotide reductase, an enzyme which plays a very important role in the synthesis of DNA. Therefore, ribonucleotide reductase activity was partially purified and characterized from extracts of wild-type and hydroxyurea resistant L cells. Optimal assay conditions and kinetic characteristics of the enzyme were compared to determine if the drug resistant cell line contained a ribonucleotide reductase enzyme which was structurally altered from the wild-type enzyme.

The study of ribonucleotide reductase activity in cell-free extracts presents a number of difficult problems. In an attempt to minimize these problems, a method was developed to measure enzyme activity in small numbers of whole cells made permeable to nucleotides by treatment with the detergent Tween-80. This procedure allowed the measurement of *in vivo* levels of ribonucleotide reductase in mouse cells. Ribonucleotide reductase levels were compared in wild-type and drug resistant L cells to determine if enhanced enzyme levels played a role in resistance to hydroxyurea.

HISTORY

MUTANTS OF SOMATIC CELLS IN CULTURE

The complete understanding of cellular processes in any living ststem is dependent upon our ability to understand the genetic basis of these functions. For many years, the study of basic cell biology in bacteria has been complemented by the isolation and characterization of mutant cells (for review see Harris, 1964). Such cells, which can show a variety of alterations or defects in phenotype, have contributed greatly to the elucidation of biochemical pathways and molecular controls.

Attempts have been made to apply the same methodology of genetic analysis used with bacteria to similar studies in multicellular organisms (eg. Simmons and Crow, 1977; Ward, 1977). These studies are severely hindered, however, by the immense complexity of the phenotypes of these organisms.

The development of cell culture techniques was an enormous breakthrough in allowing a genetic approach to studying the biology of multicellular organisms. Pure lines of mammalian cells that would grow in semi-defined medium provided cell biologists with a valuable tool for qualitative and quantitative analysis of genetic and biochemical properties of the multicellular animal. It

is quite natural that a great deal of the recent work being done on somatic cells in culture has revolved around the selection, isolation, and characterization of cells with stable alterations in their phenotype.

Clements (1975) has emphasized the importance of considering cultured mammalian cells as a population of individuals capable of expressing subtle differences not readily detectable under standard growth conditions. It is this background variation which produced the first reported variant cell lines in culture. Puck and Fischer (1956) observed that cloning of a HeLa cell population would isolate variant cell lines with altered growth requirements. A short time later, the first drug-resistant variants were isolated in culture; among a variety of cell types, variants were isolated with resistance to folic acid antagonists (Vogt, 1959; Szybalski, 1959), antipurines (Lieberman and Ove, 1959), and antipyrimidines (Morris and Fischer, 1960).

Since that time, the field of somatic cell genetics has expanded rapidly. Many reviews have been published (eg. Clements, 1975; Siminovitch, 1976; Basilico, 1977; Wasmuth and Caskey, 1978), all of which emphasize the immense variety of mutant phenotypes which have been isolated from somatic cells in culture.

Perhaps the major controversy in the study of somatic cell genetics has to do with the origin of the observed

genetic variations of cells in culture. Stable alterations in cellular phenotype may arise at different levels of organization in the cell, and hence may have a genetic or epigenetic origin (Harris, 1971). A true genetic mutation has been defined as a genetically based alteration in phenotype which has resulted from any hereditable change in the DNA; this includes changes in primary structure due to deletions, point mutations, etc., as well as chromosome rearrangement and/or loss (DeMars, 1974; Siminovitch, 1976). Epigenetic changes may also arise. These have been described (Nanney, 1968) as being the result of directed shifts in phenotypic expression which do not involve alterations in the DNA per se, but nevertheless result in hereditable variations.

Epigenetic changes on a programmed basis are a familiar part of embryonic development and cellular differentiation (Coon, 1966, Gehring, 1968). Similarily, Harris (1971) has suggested that permanent cell lines which are grown in a highly artificial environment may exhibit unstable mechanisms of genetic expression; and may exhibit stable phenotypic changes during cell culture which are brought about by epigenetic mechanisms rather than actual mutational events. It has been suggested, in fact, that most variant somatic cells in culture have arisen by epigenetic means (Harris 1971,1973,1975; Mezger-Freed 1971, 1972). The support for this statement comes from many different

investigators who provide evidence contraindicating a mutational basis to a variety of observed variant phenotypes. For example, many variant phenotypes are reported to be unstable when grown for long periods under non-selective conditions (Littlefield, 1965; Morrow,1970; Mezger-Freed, 1971; Alt et al, 1978). Also, cultured somatic cells had often shown mutation rates which were much higher than the accepted rates in bacteria, and hence were thought to arise by mechanisms other than gene mutation (Breslow and Goldsby, 1969; Harris,1971, 1973; Mezger-Freed, 1972). In addition, the mutation rates in these cultured somatic cells did not always increase when the cells were exposed to agents known to be mutagenic to bacterial cultures (Mezger-Freed, 1972, Szybalski et al, 1964). Examples were also uncovered where the frequency of variants observed did not appear to depend on cell ploidy. Harris (1971), for instance, examined the frequency of occurence of variant Chinese Hamster cells which were resistant to a purine analogue or thermal shock. It was observed that the rates of occurence of these markers was approximately the same in cultures of diploid, tetraploid, or octaploid Hamster cells; and therefore was not in support of the assumption that the variant phenotype was the result of a dominant, co-dominant, or recessive change at the gene level. This was supported by Mezger-Freed (1972) who observed that the rate of occurrence of

bromodeoxyuridine-resistant frog embryo cells did not appear to depend on cell ploidy, as it should if the alteration in phenotype was due to a gene mutation.

A controversy exists, however, and many of the observations of Harris and Mezger-Freed have been severely challenged by DeMars (1974), who also points out that mutation rates for drug resistance in cultured somatic cells are in accordance with rough estimates of germinal mutation rates in humans. A great deal of data has since accumulated to support a genetic basis of hereditable mutation in cultured somatic cells. The enhancing effect of mutagens in increasing the frequency of occurence of variants in many genetic systems is now widely recognized (see reviews by Chu, 1974; Siminovitch, 1976) and has been carefully quantitated (eg. Hsie et al, 1975a, 1975b; Friedrich and Coffino, 1977). Also, many investigators have shown that mutation rates in cultured somatic cells respond to cell ploidy in a manner consistant with a mutational basis for the genetic variation (eg. Chasin, 1972, 1973; Chasin and Urlaub, 1975; McBurney and Whitmore, 1974; Jones and Sargeant, 1974; Raskind and Gartley, 1978).

It seems certain that many isolated variants have a stably altered phenotype due to an actual alteration in their DNA. Such variants would therefore be classed as true mutants. The ultimate evidence indicating the presence of a true mutation, however, would involve sequenc-

ing the DNA of the wild-type parent and observing alterations in the mutant. Failing this, somatic cell geneticists must rely on evidence which will infer that an observed variation is mutationally based. Towards this end, a number of criteria have been established which, ideally, should all be satisfied when defining a mutant somatic cell line. The major requirements include (Chu, 1974; Clements, 1975):

1. Spontaneous random occurence at a low frequency
2. Retention of stable phenotype in the absence of selection
3. Increased frequency of appearance of mutants after exposure to mutagens
4. Production of an altered gene product
5. Interallelic complementation
6. Localization of the lesion on a specific chromosome
7. Expression in somatic hybrids in a recessive, dominant, or co-dominant fashion

Following is a discussion of some of the better documented examples of hereditable variations of apparent mutational origin.

A. Drug Resistance

Drug resistance is perhaps the most studied mutant phenotype of somatic cells in culture. Recent reviews on the subject (Clements, 1975; Siminovitch, 1976; Wasmuth and Caskey, 1978) exemplify the wide variety of drug resistant mutants available. Also, Clements (1975) has summarized the many adaptations which alone or in combination may result in drug resistance in cultured somatic cells.

Purine analogue resistance: The purine analogues 6-thioguanine, 8-azahypoxanthine, 8-azaguanine, and 6-mercaptopurine are toxic to mammalian cells after being processed by the enzyme hypoxanthineguanine phosphoribosyltransferase (HPRT), an enzyme which is non-essential to the cell under certain culture conditions. These analogues have therefore been used to select for cultured somatic cells deficient or lacking HPRT enzyme activity (phenotype termed $hprt^-$) (eg. Lieberman and Ove, 1959; Littlefield, 1963; Fujimoto et al, 1971, DeMars, 1974). The resistant phenotype is stable for long periods in the absence of drug and the frequency of mutation is increased by mutagenesis (Chu and Malling, 1968; Hsie et al, 1975). These mutants also appear phenotypically identical to cells obtained from human males with the Lesch-Nyan syndrome where the defect has been located on the X chromosome (Seegmiller et al, 1967; Fujimoto et al, 1971)

There is a great deal of evidence for the production of an altered gene product in $hprt^-$ mutants. Cell lines with the $hprt^-$ phenotype have been isolated which produce an enzymatically inactive protein which cross reacts immunologically (CRM^+) with antibodies to purified HPRT enzyme (Beudet et al, 1973; Wahl et al, 1975). Recently, Capecchi et al (1977) reported the presence of a CRM^+ protein in $hprt^-$ L cells which was found to have an altered carboxy-terminal peptide. Chromatographic analysis

strongly suggested that the mutant peptide was shorter than the HPRT wild-type peptide. It was then postulated that the hprt$^-$ phenotype resulted from a nonsense mutation in the HPRT gene. This was strongly supported by the finding that the mutant could be "phenotypically corrected" by microinjection of bacterial or yeast ochre-suppressor tRNA, but not wild-type tRNA. It was then claimed that this mouse L cell hprt$^-$ variant was a genetically based suppressible nonsense mutant.

Resistance to purine analogues via loss of HPRT activity may, of course, arise by means other than nonsense nutation. For example, Fenwick et al (1977) reported the isolation of Chinese hamster hprt$^-$ variants with an altered enzyme of the same molecular weight as the wild-type enzyme, but with different substrate binding properties. It was suggested that the hprt$^-$ phenotype was the result of a missense mutation in the HPRT gene. Further evidence for the production of an altered enzyme in hprt$^-$ variants has been obtained from a Chinese Hamster Ovary (CHO) cell mutant which shows temperature-sensitivity for both HPRT activity and purine analogue resistance (Fenwick and Caskey, 1975).

In conclusion, it appears that at least some of the variant cells resistant to purine analogues satisfy most of the major criteria for classification as a genetically based mutant cell line. The altered phenotype is stable;

its occurence is increased by mutagenesis, and the lesion has been localized on the X chromosome. In addition, the evidence of the production of an altered gene product is strong.

Hydroxyurea resistance: Stable resistance to the cytotoxic effects of the drug hydroxyurea can be obtained from CHO cells (Wright and Lewis, 1974; Lewis and Wright, 1978b). The variant phenotype is stable during extended cultivation under non-selective conditions, and its occurence in the wild-type population is enhanced by mutagenesis (Lewis and Wright, 1978b). Hydroxyurea-resistant cell lines have been isolated which produce an altered target enzyme, ribonucleotide reductase (Lewis and Wright, 1978a). The altered enzyme showed a several fold higher resistance to hydroxyurea in vitro when compared to the wild-type enzyme. Other variants isolated owed their resistance to the production of enhanced levels of drug-sensitive ribonucleotide reductase (Lewis and Wright, 1978c; Lewis, Kuzik, and Wright, 1978).

α-amanitin resistance: α-amanitin inhibits the enzyme RNA polymerase II , and is therefore cytotoxic to cultured mammalian cells. Many cell lines have been isolated, however, which are resistant to normally toxic levels of α-amanitin. For example, α-amanitin-resistant CHO cells have been isolated which produce an altered RNA polymerase II enzyme, which can account for the drug resistant pheno-

type (Lobban et al, 1976; Ingles et al, 1976).

Emetine resistance: Gupta and Siminovitch (1976) have isolated mutant CHO cells which are resistant to the protein synthesis inhibitor emetine. Drug resistance in these cells has been attributed to the production of an altered 60S ribosomal subunit which is less sensitive to inhibition by emetine than is the wild-type 60S subunit ((Gupta and Siminovitch, 1978).

cAMP resistance: Daniels et al (1973) reported that S49 mouse lymphoma cells would lyse in the presence of elevated levels of cAMP in the culture medium, but variants could also be isolated which were cAMP-resistant. It has since been shown that many cAMP-resistant phenotypes are the result of a mutational event which has produced an altered regulatory subunit of a cAMP protein kinase enzyme (Hochman et al, 1975; Steinberg et al, 1977).

Oubain resistance: Oubain is normally cytotoxic to cultured mammalian cells via its inhibition of Na^+/K^+ ATPase (Baker et al, 1974). Oubain-resistant variants have been isolated, however, and appear to have resulted from a mutational event which has led to the production of an altered Na^+/K^+ ATPase enzyme which is resistant to inhibition by oubain (Baker et al, 1974, Robbins and Baker 1977)

B. OTHER GENETIC SYSTEMS

Immunoglobulin variants: One of the most intensively studied genetic systems is that of the spontaneously arising clonal variants of the mouse myeloma MOPC-21 cell line (for reviews see Adetugbo et al, 1977; Cramer 1978). To date, four spontaneous structural mutants have been studied in detail. These mutants secrete an altered gamma immunoglobulin heavy chain which has been systematically compared to the wild-type chain on the basis of fingerprint analysis of the proteins and mRNA's, cell free translation of the isolated mRNA's, and by cell fusion complementation analysis. The results have shown that three of the four variants are deletion mutants, whereas the fourth variant is the result of a single missense mutation. This somatic cell system, therefore, has provided the most definitive evidence for a mutational basis to somatic cell variation in culture.

Auxotrophy: Many nutritional auxotrophs have been isolated from cultured somatic cells, and in many cases, the evidence for a mutational basis for the altered phenotype is strong. For example, glycine requiring CHO cells have been isolated which have a defect in serine hydroxymethyl transferase activity (Kao et al, 1969), and a revertant of one such mutant was shown to regain serine hydroxymethyl transferase activity which was more thermolabile than the

wild-type enzyme (Chasin et al, 1974). Also, mutant CHO cells have been isolated which are auxotrophic for unsaturated fatty acids (Chang and Vagelos, 1976). This particular variant phenotype is reported to be stable, and has been attributed to the presence in these cells of an altered stearoyl-CoA desaturase enzyme.

Temperature-sensitive conditional lethality: The isolation of temperature-sensitive (ts) conditionally lethal mutants offers evidence strongly indicating the occurence of missense mutations in cultered somatic cells. The configuration and/or stability of a polypeptide may be critically temperature-dependant. A missense mutation might therefore modify a protein so that it functions normally at one temperature (permissive temperature) but abnormally at a different (non-permissive) temperature. Conditional lethality would then result if normal functioning of the affected protein was crucial to the cell.

Many stable ts cell lines have been isolated (see review by Basilico, 1977); and in many cases, the affected protein or process has been identified (eg. Ceri and Wright, 1977; Thompson et al, 1971, 1975, 1977). Future work in firmly establishing the genetic basis of ts variants and selecting for an increasing number of ts cell lines will prove very beneficial to the study of somatic cells in culture. Since cells can be studied under permissive and non-permissive conditions, proteins carrying

out essential processes can be investigated.

Reviews by Clements (1975) and Siminovitch (1976) offer many more examples of other isolated variants of somatic cells in culture, and in most cases, the evidence for a mutational basis for the altered phenotype is strong.

RIBONUCLEOTIDE REDUCTASE

In 1953, Rose and Schweigert reported that cytidine could be incorporated into the DNA of rats without cleavage of the sugar-phosphate backbone. This was the first indication of the existence of an enzyme which could convert nucleotides to deoxynucleotides by simple reduction. It was not until 1960, however, that conversion of cytidine to deoxycytidine could be routinely measured in mammalian cell extracts (Moore and Hurlbert, 1960). Since that time, a great deal of research has been done on the ribonucleotide reductase enzyme, and many investigators have uncovered a close association between this enzyme and the DNA synthesis machinery.

Ribonucleotide reductase (EC 1.17.4.1) is the enzyme solely responsible for the conversion of the four ribo-

nucleotides to the corresponding deoxyribonucleotides required as the building blocks for DNA synthesis (Reichard 1968; Larsson, 1969). The study of a variety of organisms has uncovered two basic forms of the enzyme. One form is characterized by the enzyme found in Lactobacillus (Goulian and Beck, 1966; Panagou et al, 1972), the other form as found in E. coli (Reichard, 1968). The Lactobacillus enzyme is a monomeric protein which reduces ribonucleotide triphosphates and requires the cofactor 5'-deoxyadenosyl-cobalamin. The reductase enzyme in E. coli is composed of two non-identical subunits which reduce nucleotides at the diphosphate level.

Most of the ribonucleotide reductase enzymes studied appear to be of the E. coli type. This includes all of the mammalian enzymes studied to date (Moore, 1967, 1976; Cory et al, 1975; Wright and Lewis, 1974; Lewis and Wright, 1978a) as well as some fungi (Lowdan and Vitols 1973; Lewis et al, 1976) and plant (Hovemann and Follmann, 1977) enzymes.

The enzyme has been purified to homogeneity from Escherichia coli (Thelander, 1973). Purification of eukaryotic ribonucleotide reductase has met with only limited success (Moore, 1967; Larsson, 1973; Lewis and Wright, 1978a). For this reason, much of the data concerning the biochemical nature of the enzyme has been obtained from E. coli and tentatively extrapolated to mammalian systems on the

basis of known similarities.

The current knowledge regarding the nature of the ribonucleotide reductase system from E. coli may be summarized a follows. The enzymatic reduction of the four common ribonucleoside diphosphates is schematically represented in Figure (i). Reduction occurs at the 2' position of the ribose (Thelander, 1974) without cleavage of the sugar-base backbone (Rose and Schweigert, 1953). The ultimate hydrogen donor is NADPH which primarily reduces a disulfide bond within a single cystine residue in a small protein, thioredoxin (Thelander, 1967, Reichard, 1971). Recently, however, mutant E. coli cells have been isolated which lack significant levels of thioredoxin reductase activity (Fuchs, 1977), and yet have no apparent defect in in vivo ribonucleotide reductase activity. In an attempt to explain this, it has been suggested that glutathione may be of critical importance in the coupling of NADPH oxidation to ribonucleotide reduction, (Fuchs and Warner, 1975; Fuchs, 1977), although this hypothesis needs to be studied further.

Ribonucleotide reductase in E. coli consists of two protein subunits, B_1 and B_2 (Holmgren, Reichard and Thelander, 1965). Separately, these subunits are catalytically inactive; however, in the presence of Mg^{+2} they combine to form the enzymatically active complex (Brown, Larsson and Reichard, 1967). Protein B_2 contains two

non-haem irons and varying amounts of an organic free radical required for enzyme activity (Atkin et al, 1973). The benzyl carbon of a tyrosine residue in the B_2 subunit has been identified as the location of the radical (Sjoberg et al, 1977). The B_1 subunit contains the substrate binding sites as well as all four binding sites for the allsoteric effectors (vonDobeln and Reichard, 1976; Brown and Reichard, 1969b). The structural genes coding for the B_1 and B_2 subunits have been mapped in E. coli and are located at approximately 48 minutes (Fuchs and Karlstrom, 1976).

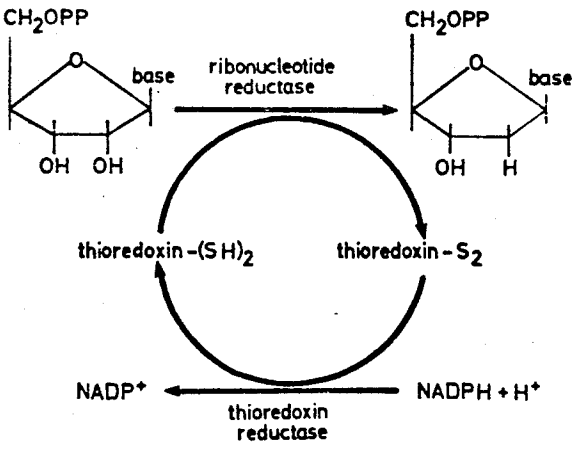


FIGURE (i): The ribonucleotide reductase system.

The substrate specificity as well as the reaction rates of the enzyme are strictly controlled in a complex, allosteric fashion by several nucleoside triphosphates

(Brown and Reichard, 1969b; Moore and Hurlbert, 1966; Elford, 1971). Brown and Reichard (1969a) have also proposed the presence of high and low affinity effector binding sites; low affinity sites involved in regulation of enzyme activity, and high affinity sites determining substrate specificity. In the absence of the positive nucleotide effectors, ribonucleotide reductase shows little activity towards any of the four substrates. In the presence of ATP, such as when the cell has sufficient energy available to replicate its DNA, the enzyme is stimulated towards reduction to the pyrimidines CDP and UDP. The resulting accumulation of dTTP (from UDP) stimulates purine reduction, especially toward GDP. dGTP, in turn, stimulates ADP reduction. The final product, dATP, serves as a master regulator; if it is not immediately utilized for DNA synthesis, it will shut down the synthesis of all the deoxyribonucleotides. This is accomplished through dimerization of the B_1-B_2 protein complex in the presence of dATP (Brown and Reichard, 1969a). The inhibition by dATP is counteracted by ATP and the overall activity of the enzyme is thus determined by the ratio of these two nucleotides. A second type of negative effect is exerted by dTTP in the presence of ATP. Under the proper conditions, these two nucleotides inhibit the reduction of pyrimidines only. This results in turning the substrate specificity of the enzyme from pyrimidine to purine ribo-

nucleotides (Brown and Reichard, 1969a).

The overall pattern of allosteric control of the E. coli enzyme is almost identical to that observed in Novikoff ascites rat tumor cells (Moore and Hurlbert, 1966; Murphee et al, 1968). The pattern determined for the enzyme present in these mammalian cells is schematically represented in Figure (ii). It can be seen that the net effect of these interactions is to finely regulate the activity of the enzyme in order to ensure a balanced supply of the nucleotide precursors for DNA synthesis.

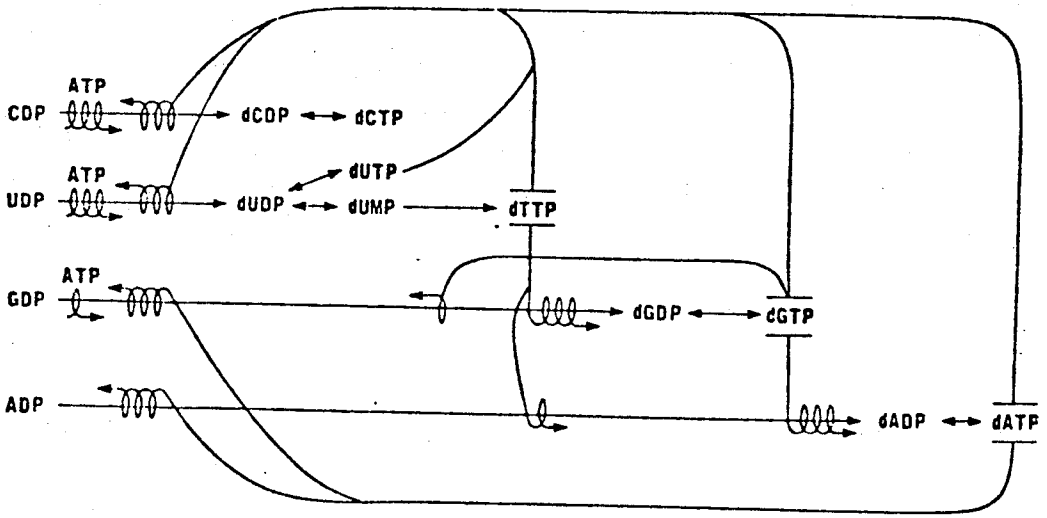


Figure (ii): Allosteric regulation of mammalian ribonucleotide reductase by nucleoside triphosphate effectors. Swirled arrows to right signify activation, to left signify inhibition. (from Moore and Hurlbert, 1966).

It is reasonable to apply the above information obtained from the E. coli system to the mammalian enzyme as there are many parameters which suggest that the two enzyme systems are very similar. The most important example is the similarity in the mechanism of allosteric control by nucleo ide triphosphates (mentioned above). Also, a thioredoxin/thioredoxin reductase system similar to that in E. coli has been purified from regenerating rat liver (Larson and Larsson, 1972) and ascites hepatoma cells (Herrmann and Moore, 1973). Mammalian ribonucleotide reductase has also been separated into two separate components. Partially purified preparations from rabbit bone marrow (Hopper, 1972) and Novikoff tumors in rats (Moore, 1967, 1976) indicate that both fractions are required for activity. The mammalian enzyme is also stimulated by added iron (Moore, 1969; Larsson, 1969), indicating a possible similarity with the iron content of the E. coli enzyme.

It has been suggested that mammalian ribonucleotide reductase may not be a single enzyme as in E. coli; rather, at least two separate enzymes may exist which are independantly responsible for purine and pyrimidine reduction (Cory and Mansell, 1975; Cory, Mansell and Whitford, 1975; Peterson and Moore, 1976; Lewis, Kuzik, and Wright, 1978). These suggestions are based, for example, on the lack of synchrony in the initiation of purine and pyrimi-

dine ribonucleotide reduction during the cell cycle; as well as on the failure of the ratio of purine to pyrimidine reductase activities to remain constant during purification procedures. The resolution of this dispute, however, is obviously dependent upon the final purification of the mammalian enzyme system, as well as a clear understanding of the mechanism(s) responsible for the cell cycle-dependent fluctuation of enzyme activity.

Ribonucleotide reductase activity and mammalian cell proliferation rates: The increased cell proliferation rates seen in rapidly growing tissues such as tumors, regenerating liver, and developing embryos are clearly dependent on the ability of these cells to replicate their DNA quickly. Ribonulceotide reductase activity has been studied in relation to the growth rate of many types of tissues. The close correlation observed suggests a critical role of this enzyme in DNA synthesis and cell division.

Elford et al (1970) has observed the variations of ribonucleotide reductase activity with tumor growth rate in a series of rat hepatomas. Enzyme activity was found to increase in nearly a linear fashion with increasing growth rate of the tumors. Other enzymes involved in DNA synthesis were also observed. Thymidine kinase and thymidylate synthetase activities, however,

showed only a poor and inconsistent correlation with tumor growth rate.

Ribonucleotide reductase levels are also closely associated with the increased rates of cell division seen in regenerating rat liver. After partial hepatectomy in rats, the remaining liver tissue rapidly regenerates; also, the rate of DNA synthesis in the regenerating tissue is dramatically increased over the levels seen in normal rat liver (Hecht and Potter, 1956). Ribonucleotide reductase activity has been observed to increase dramatically in association with this state of rapid cell proliferation (King and vanLanker, 1969, Larsson, 1969). It has been suggested that the biochemical background for this transition might be the increase in the level of one or several critical enzymes involved in DNA synthesis, including ribonucleotide reductase (Larsson, 1969)

The profile of ribonucleotide reductase activity during neonatal development in several rat organs was studied by Elford (1971). In each case, enzyme activity varied greatly during organ development, with maximum activity occurring at the same time as periods of rapid cell proliferation and DNA synthesis.

Ribonucleotide reductase activity has also been measured as a function of gestational age in the brains

of the mouse, rat, chicken and human and in the cerebellum of rats during the first two weeks of birth (Millard, 1972). In each case studied, enzyme activity fluctuated in accordance with periods of neuronal proliferation. For example, the maximum ribonucleotide reductase activity in the brains of chick embryos occurred at the early part of the fifth day of incubation. This compares with extensive neuronal multiplication and migration which occurs in the chick brain at the end of the fifth and beginning of the sixth day of incubation (Levi-Montalcini, 1964)

The close association of ribonucleotide reductase activity with periods of rapid cell growth and hence DNA synthesis, can also be observed in non-mammalian sources. Enzyme activity has been reported to rise and fall in concert with very early development of fertilized sea urchin (Arbacia punctulata) eggs (Norona, Sheys, and Buchanan, 1972) and fertilized Xenopus sp. eggs (Tondeur-six et al, 1975), as well as during differentiation of the water-mould Achlya (Lewis et al, 1976). In the case of the sea urchin eggs, ribonucleotide reductase activity could not be detected in unfertilized eggs; although these eggs did show DNA polymerase, thymidine and thymidylate kinase, thymidylate synthetase, and dCMP deaminase activities.

Ribonucleotide reductase profile during the mammalian cell cycle: DNA synthesis is an absolute prerequisite for cell division, and the DNA is synthesized only during a clearly distinguished phase of the cell cycle. Ribonucleotide reductase activity, as well as the activity of other DNA enzymes, has been studied during the replication cycle of synchronized mammalian cells in culture. The consistently close correlation between ribonucleotide reductase activity and the occurrence of DNA synthesis in the single cell complements the parallelism found between enzyme activity and mammalian cell proliferation rates.

Turner, Abrams, and Lieberman (1968) studied the fluctuation in ribonucleotide reductase activity during the division cycle of the L cell. A constant ratio was observed between enzyme activity and the fraction of a synchronized population that was replicating DNA. Ribonucleotide reductase activity was also observed to decay rapidly after completion of DNA synthesis. This rapid degradation and the tight correlation of the reductase activity with DNA synthesis is in contrast to the properties of other enzymes associated with DNA synthesis. DNA polymerase activity remains relatively constant during the course of the cell cycle (Turner et al, 1968) in L cells. Thymidine kinase activity does increase in cells that are synthesizing DNA, but

there is only a limited correlation between the levels of the enzyme and the replicative period (Littlefield, 1966).

The tight correlation between maximum ribonucleotide reductase activity and periods of DNA synthesis has also been observed in Chinese hamster fibroblasts (Murphee et al, 1969; Peterson and Moore, 1976) and nucleotide-permeable Chinese hamster ovary cells (Lewis et al, 1978).

Role of de novo enzyme synthesis: The large increase seen in ribonucleotide reductase activity in rapidly proliferating tissues has often been attributed to de novo synthesis of the enzyme. This has been based on the finding that protein synthesis inhibitors prevent the expected rise in enzyme activity in developing rat organs (Elford, 1971), regenerating rat liver (King and vanLanker, 1969), and fertilized eggs of sea urchins (Noronha et al, 1972) and Xenopus (Tondeur-six et al, 1975). Similarily, the increase in ribonucleotide reductase activity during the S phase of the cell cycle can also be prevented by the presence of protein synthesis inhibitors (Turner et al, 1968; Murphee et al, 1969; Peterson and Moore, 1976).

Some investigators, however, have observed simultaneous peaks of DNA synthesis and ribonucleotide reductase

activity which is not effected by protein synthesis inhibitors, although the reason for this is not known (Cory, Mansell and Whitford, 1975).

Recently, the role of de novo protein synthesis during S phase in the replication of the mammalian genome has been studied in a Chinese Hamster Lung cell mutant with thermolabile ribosomes making it temperature sensitive for protein synthesis (Haralson and Roufa, 1975; Roufa, 1978). It was suggested (Roufa, 1978) that de novo protein synthesis is not required after late G_1 phase to maintain a single round of DNA replication. In other words, de novo synthesis of ribonucleotide reductase may not be responsible for the large increase in activity seen during the S phase of the cell cycle.

Alternatively, it may be suggested that the rise in ribonucleotide reductase activity in concert with DNA synthesis may be due to allosteric activiation of pre-existing enzyme. Enzyme activity in vitro (Moore and Hurlbert, 1966) and in vivo (Lewis, Kuzik, and Wright, 1978) can be stimulated several fold by the presence of optimum concentrations of positive nucleotide effectors. Further work on the size and intracellular distribution of nucleoside triphosphate pools should help resolve these questions. It seems unlikely however, that either de novo enzyme

synthesis or allosteric activation of pre-existing enzyme is solely responsible for the profile of ribonucleotide reductase activity during the cell cycle. Although evidence is available to support each contention, it seems more likely that both systems contribute to the increase in enzyme activity observed during DNA synthesis.

Deoxyribonucleoside Triphosphate pools and DNA synthesis: An observation of the cellular pool sizes of the ultimate products of ribonucleotide reductase, the deoxyribonucleoside triphosphates (dNTP), has helped to elucidate the relationship between the enzyme and the control of DNA synthesis. The profile of fluctuating pool sizes during the cell cycle complements the information available on the variations of reductase levels. This data has also fostered some hypotheses regarding the control of the synthesis of ribonucleotide reductase and DNA.

The cell cycle dependent variations of dNTP pools has been studied in many different permanent mammalian cell lines, including Chinese Hamster cells (Skoog et al, 1973; Walters et al, 1973; Walters and Ratliff, 1975; Bjursell and Reichard, 1973) and Baby Hamster Kidney and Mouse Embryo cells (Reichard, 1971). In each case, the levels of the dNTPs in cells not involved in DNA synthesis is very low; in fact, Walters et al (1973) failed to find detectable levels of dATP, dGTP, or dCTP in synchronized Hamster cells in G_1 phase. Generally, pool sizes are lowest in G_1 but increase just prior to the initiation of DNA synthesis. Maximum levels are reached in late S/early G_2, and degradation

begins in G_2. Although all four dNTP pools are near to or at their maximum levels during S phase, they can only maintain DNA synthesis for a very short period of time. Mid-S phase pools of dGTP will only support DNA synthesis for one minute or less (Walters et al, 1973).

The dNTP pool sizes correlate well with periods of high and low ribonucleotide reductase activity during the cell cycle; with high enzyme activity associated with maximum pool sizes. The presence of only very low levels of DNA precursors in G_1, and limiting levels during S phase verify that reductase activity is required during these periods. This fact, plus the correlation previously mentioned between enzyme levels and cell proliferation rates, has led to the suggestion that ribonucleotide reduction is the rate limiting step in DNA synthesis (Elford et al, 1970; Cory and Whitford, 1972). However, the presence of detectable amounts of all four dNTPs in some of the Chinese Hamster cell systems (Skoog et al, 1973) during periods when DNA was not being synthesized appears to exclude the possibility that the absence of DNA synthesis in these cells is the mere result of a general limitation of the supply of DNA precursors.

The variations seen in the pool sizes of dCTP during the cell cycle are of particular interest. Although all four dNTP levels rise and fall in association with DNA synthesis, the levels of dCTP are the most tightly synchronized with the rate and occurrence of DNA synthesis (Bjursell and Reichard, 1973; Skoog

et al, 1973). Also, the dNTPs are not present in equimolar concentrations; with the dCTP levels always being the highest and often one to two orders of magnitude larger than the smallest pool (dGTP) (Walters et al, 1973; Skoog et al,1973). This has led to the hypothesis that aside from supplying DNA precursors, the dCTP pool may have some special regulatory role connected with the rate of DNA synthesis (Bjursell and Reichard, 1973; Skoog et al, 1973). This has been challenged, however, by Walters and Ratliff (1975) who observed that dCTP levels could be increased in Chinese Hamster cells without a concurrent increase in DNA synthesis. Nevertheless, Reichard (1978) has recently proposed that dCTP or a derivative thereof may have a special function in the initiation of new rounds of replication.

Inhibition of Ribonucleotide Reductase in vivo: Studies involving the inhibition of ribonucleotide reductase activity in the intact cell have emphasized the critical role of this enzyme in DNA synthesis. As would be expected, the enzyme is essential for DNA replication and cell division. E. coli dna F mutants are temperature sensitive for ribonucleotide reductase activity, and are hence also temperature sensitive for DNA synthesis and growth (Fuchs et al, 1972).

It has been known for over a decade that the addition of thymidine to mammalian cells in culture causes a rapid inhibition of DNA synthesis (Moore and Hurlbert, 1966). It has since been shown that this inhibition is a result of direct and/or indirect effects on ribonucleotide reductase.

The addition of thymidine to the culture medium causes a rapid increase in intracellular levels of dTTP (Bjursell and Reichard, 1973; Skoog and Bjursell, 1974). The excess levels of dTTP would then exert a strong negative allosteric effect on the reduction of CDP (figure ii), leading to a general decrease in the supply of dCTP. Skoog and Bjursell (1974) reported that the addition of thymidine to the medium caused a complete depletion of nuclear pools of dCTP. Therefore, the lack of this precursor could easily explain the inhibition of DNA synthesis. This is supported by the finding that thymidine inhibition of DNA synthesis can be reversed by the addition of deoxycytidine (Bjursell and Reichard, 1973). Recently, however, Reichard (1978) has postulated that thymidine induced inhibition of DNA synthesis is not due to the depletion of the dCTP pools needed as precursors of DNA, but rather is due to the elimination of dCTP acting in some unknown regulatory capacity.

Thymidine inhibition studies have also led to the finding that a phosphorylated derivative of thymidine, presumably dTTP, may also act as a repressor or corepressor of the synthesis of ribonucleotide reductase in E. coli (Biswas, Hardy and Beck, 1965). This has also been suggested to occur in mammalian cells (Elford et al, 1977); although Moore (1976) suggests the enzyme is synthesized normally in the presence of excess dTTP. Nevertheless, it would appear that dTTP may play a very important role in cell growth if indeed the dramatic increases in ribonucleotide reductase activity in rapidly proliferating tissues and during the cell

cycle are due to de novo enzyme synthesis.

Hydroxyurea is a potent inhibitor of mammalian ribonucleotide reductase (Turner et al, 1966; Elford, 1968; Moore, 1969). In E. coli, hydroxyurea is believed to interact with the free radical in the B_2 subunit which is required for activity (Ehrenberg and Reichard, 1972). The drug will quickly stop DNA synthesis in cultured mammalian cells; the loss in enzyme activity allowing a rapid depletion of dGTP and dATP pools (Adams et al, 1971; Skoog and Nordendkjold, 1971; Reichard, 1971). This would be expected from the known pool sizes of dGTP and dATP in cells synthesizing DNA (Walters et al, 1973) which indicate that continued enzyme activity is required during S phase.

A final source of control of ribonucleotide reductase activity in mammalian cells may involve the activity of presently unidentified endogenous inhibitors. Such compounds have been studied in Ehrlich ascites cells (Cory and Monley, 1970; Cory, 1973), rat liver (Elford, 1972) and the water mould Achlya which produces highly phosphorylated compounds inhibitory to partially purified ribonulceotide reductase from Chinese Hamster Ovary cells (Lewis et al, 1976).

Ribonucleotide reductase obviously occupies a critical position in the DNA synthesis machinery of the cell. Its close association with cell growth rates, periods of DNA synthesis, plus the many complex controls on the enzyme, as well as the results when these controls are interfered with, all support the contention

that the enzyme may be closely involved in the regulation of DNA synthesis. However, one cannot conclude on the basis of present knowledge that the enzyme is the critical regulatory agent. It seems unlikely that the total cellular activity of any single enzyme can be solely responsible for the quantitatively exact duplication of DNA. Nevertheless, it is not unreasonable to postulate that the cellular levels/activities of ribonulceotide reductase constitute one very important element of a complex control system.

MATERIALS AND METHODS

1. CELL LINES AND CULTURE CONDITIONS

a) mouse L cells: The Mouse L cell line originally isolated by Earle (1943) was used in this study. Permanent mouse cell lines exhibit an unstable karyotype in culture, showing many heteroploid shifts and formation of metacentric chromosomes (Hsu and Klatt, 1958; Rothfels and Parker, 1959). This has been recognized as a general phenomenon, however, and is characteristic of many permanent cell lines (Hsu, 1961; Harris, 1964). Nevertheless, L cells have been successfully used to isolate a variety of mutant phenotypes (eg. Thompson et al, 1970, 1971; Dubbs and Kit, 1964; Lewis and Wright, 1974; Capecchi et al, 1977).

In our hands, L cells had a doubling time of approximately 18 hours at 37°, and grew rapidly to monolayer on the surface of 16 ounce Brockway bottles or on 60 or 100 mm plastic tissue culture plates. The medium used was alpha minimal essential medium (αMEM) plus 10% foetal calf serum (FCS) supplemented with penicillin G (100 units/ml) and streptomycin sulfate (100 μg/ml). The formulation of αMEM has been published by Stanners et al (1971) and did not contain ribonucleosides or deoxyribonucleosides. Cells were incubated at 37°in a humidity-controlled incubator containing a 5% CO_2 atmosphere (Lewis and Wright, 1978b).

Mouse L cells were also routinely grown in suspension culture. From 200 to 400 ml of αMEM plus serum was placed in a 500 ml medium bottle (GIBCO Ltd.) containing a Teflon-coated stirring bar. The bottle was seeded with at least 3 X 10^4 cells/ml, tightly

capped, and placed in a 37^{o} water bath. The culture was stirred continuously with the aid of a magnetic stirrer. The cell suspension was occasionally diluted with fresh complete medium to maintain a density of 4 to 8 X 10^{5} cells/ml.

The wild-type mouse L cell line used in this study was generously provided by Drs. D. Cormack and A. Holloway of the Manitoba Cancer Treatment and Research Foundation.

b) Chinese Hamster Ovary Cells: The Chinese Hamster Ovary (CHO) cell line established by Puck et al (1958) was also used for part of this study, and was obtained from the University of Toronto, Department of Medical Biophysics. The CHO cells were cultivated in the same way as described for the L cells, and displayed a doubling time of approximately 14 hours at 37^{o}.

2. STANDARD PROCEDURES

a) trypsinization: Trypsin solutions, consisting of 0.05% trypsin in sterile phosphate-buffered saline, were stored at 4^{o}C and pre-warmed to approximately room temperature before use. Cells to be removed from the surface of a culture vessel were treated as follows. The growth medium was poured off and asceptically replaced with a sufficient volume of trypsin solution to cover the cells. After 10 or 15 minutes at room temperature, the cells were detached from the growth surface with gentle agitation. The cell suspension was then centrifuged, and the cell pellet was resuspended in a convienient volume of αMEM plus 10% FCS.

b) cell counting: Aliquots of 0.5 to 2.0 ml of suspension grown cells or cells freshly trypsinized from the surface of a culture vessel were diluted in an appropriate volume of saline. The cell density of this suspension was determined with the aid of a Coulter Particle Counter (Coulter Electronics Ltd., Florida).

c) plating efficiency (PE): Exponentially growing cells were trypsinized off the surface of Brockway bottles and counted. A known number of cells was then added to a plastic tissue culture plate or glass Brockway bottle containing complete medium plus serum. After incubation at 37^{o} for approximately 10 days, the plates or bottles were drained and a saturated solution of methylene blue in 50% ethanol was added. After 10 minutes at room temperature, the dye was poured off, and the plates or bottles were rinsed gently with cool water. Colonies consisting of at least 30 cells were scored. Plating efficiency was determined by dividing the number of colonies scored by the number of cells plated, The PE of wild-type (WT) Mouse L cells ranged from 0.6 to 0.8.

The relative plating efficiency (RPE) values reported in drug inhibition studies is defined as the PE of the cells in the presence of drug divided by the PE of the same cell line in the absence of drug. For example, a cell line exhibiting a PE of 0.80 in the absence of drug, and 0.40 in the presence of a given concentration of drug, is defined as having a RPE of 0.5 at that drug concentration. When routine tests of the drug sensitivity

of different cell lines were carried out, the number of cells plated depended upon the concentration of drug tested, as well as the previously determined PE for the cell line at that particular drug concentration. For example, enough cells would be plated onto a 100 mm culture plate so that approximately 30 to 300 surviving colonies could be expected.

d) cloning: At the initial stages of this study, the WT cell line was cloned according to the following procedure. Cells growing exponentially on bottles or plates were tyrpsinized, counted, and suspended in αMEM plus serum at approximately 5 cells/ml. The suspension was dispensed into a 96-well Linbro dish at 0.2 ml per well. After 10 days at 37^{o}, wells containing single colonies were identified with the aid of a microscope. These colonies were individually trypsinized from the wells and transfered to plates containing complete medium. One such WT clone, designated Ll.1, was used in all the experiments reported.

In some cases, a clone was isolated from a culture plate containing several colonies as follows. The growth medium was first removed, and the plate rinsed with warm medium to wash off loose cells. The plate was then gently flooded with a trypsin solution. After 10 to 15 minutes at room temperature, each colony could be separately removed from the surface of the plate with a sterile pasteur pipette and transfered to a fresh plate containing medium. However, these cloned lines were then recloned in a Linbro dish as described above before being used in the experiments described in this thesis.

3. RIBONUCLEOTIDE REDUCTASE in vitro ASSAY

a) preparation of cell extracts: The preparation of mouse L cell extracts required a large number of cells; approximately 2.5 X 10^8 cells were needed per ml of final extract. It was therefore necessary to grow the cells in suspension in large culture flasks. Routinely, 4 litre carboys of αMEM plus 10% FCS were inoculated with approximately 4 X 10^4 cells/ml. The cells were maintained in suspension by constant stirring with the aid of a magnetic stirrer and a teflon-coated stirring bar. The carboy was placed in a 37° water bath, and the contents were constantly bubbled with sterile 5% CO_2/95% air. Under these conditions, the cells would grow exponentially to at least 7 X 10^5 cells/ml, at which point they were harvested by centrifugation. The cell pellet was gently washed once in phosphate-buffered saline before being stored at -76°C. When a sufficient volume of cells had been obtained, they were thawed and suspended in two volumes of ice-cold 0.5M HEPES buffer pH 7.2 plus 2mM dithiothreitol (DTT). The suspension was then held in ice and homogenized with 10 strokes of a TRI-R power driven tissue homgenizer fitted with a glass hardened teflon pestle and tube. The resulting homogenate was centrifuged at 100,000g for 1 hour. This produced a pellet of cellular debris and a layer of lipid floating above the cell extract. The lipid layer was removed with a pipette and discarded. The remaining extract was added to a G-25 (medium) Sephadex column (1.5 X 45 cm) which was previously equilibrated with 0.05 M HEPES buffer pH 7.2 plus 2 mM DTT. The enzyme activity was eluted from the column with

the equilibration buffer. The final eluant usually contained from 10 to 15 mg protein/ml as determined by the method of Lowry et al (1951). This enzyme preparation was diluted to 10 mg protein per ml with 0.05 M HEPES buffer pH 7.2 plus 2 mM DTT. The extract was then distributed into 1 ml aliquots for storage at -76°C until used.

b) standard CDP reductase assay: CDP reduction was measured in a reaction mixture containing 100µl extract (corresponding to 1 mg protein) and 50 µl concentrated reaction buffer. The final assay volume of 150 µl contained 1 mg protein, 0.05 M HEPES pH 7.2, 5 mM $MgCl_2$, 6 mM DTT, and 0.4 mM ^{14}C-CDP (4800 cpm/nmole). The assay also included ATP at a final concentration of 4 mM for the WT enzyme, and 6mM when the L_2Cl_3 enzyme was studied. $FeCl_3$ was prepared fresh for each experiment. A 5 µl aliquot of anhydrous $FeCl_3$ in distilled water was added per assay to achieve a final concentration of from 0.5 to 1.5 mM $FeCl_3$. The reaction was allowed to proceed for 40 minutes at 37°, after which time the reaction was terminated by placing the assay tubes in a boiling water bath for 2 minutes. The deoxycytidine phosphates were then converted to deoxycytidine by treatment for 2 hours at 37° with 1 mg/assay Crotalus atrox venom dissolved in 0.1 M HEPES pH 8.0 plus 10 mM $MgCl_2$. The reaction was again terminated by boiling for 2 minutes. The reaction mixture was centrifuged to remove the heat-precipitated material. The supernatant was removed and the deoxycytidine was separated from the cytidine compounds on a Dowex-1-Borate collumn (5 X 80 mm) according to the method of

Steeper and Steuart (1970) and as modified by Cory and Whitford (1972). Briefly, the separation depends upon the formation of a complex between ribonucleosides and the borate ion in the column resin. Deoxycytidine does not complex with the borate ion, and is eluted from the column with 4 ml of distilled water. The eluant is then added to 16 ml of Scintiverse and shaken vigouously. Radioactivity was determined in a Beckman model LS230 liquid scintilation spectrophotometer. The spectrophotometer contained a ^{14}C channel set at a pulse height of 100-1000 divisions (4.5% gain) and counted a ^{14}C standard with an efficiency of 60%.

One unit of enzyme activity was defined as the amount of enzyme that would reduce 1 nmole of nucleotide in 1 hour.

c) standard GDP reductase assay: GDP reductase activity in both WT and L_2Cl_3 extracts was measured in a reaction mixture of 0.05 M HEPES pH 7.0 buffer, 2 mM dTTP, 6mM DTT, and 0.022mM ^{14}C-GDP (96,000 cpm/nmole) in a total volume of 150 µl. The reaction was allowed to proceed for 40 min at 37^{o} before it was terminated by boiling for 2 minutes. Each assay tube then received 1 mg Crotalus atrox venom dissolved in 0.1 M HEPES pH 8.0, plus 10 mM $MgCl_2$. After 2 hours at 37^{o}, the reaction was again terminated by boiling for 2 minutes. A small amount of unlabelled carrier guanosine and 2'- deoxyguanosine was then added to each assay. After centrifugation, 20 µl of the supernatant was spotted on a polyethyleneimine - cellulose (PEI) thin layer chromatography plate. The chromatogram was then developed for 2.5 hours at room temperature in a solvent consisting of

110 ml ethanol, 40 ml of saturated sodium borate solution, 10 ml of 5 M ammonium acetate pH 9.5, 0.25 ml of 0.5 M ethylenediamine tetraacetic acid (Reichard, 1958). The location of the spots corresponding to guanosine and deoxyguanosine were identified by means of a UV scanning lamp. Each spot was carefully cut out and immersed in a glass scintillation vial containing a toluene - PPO - POPOP scintillation cocktail. The amount of radioactivity associated with the deoxyguanosine spot was expressed as a per cent of the total radioactivity in the two spots and was used to calculate the amount of GDP reduced.

4. RIBONUCLEOTIDE REDUCTASE in vivo ASSAY

a) Tween-80 permeabilization of CHO and L cells: Both CHO and L cells were made permeable to nucleotides by a slight modification (Lewis, Kuzik, and Wright, 1978) of the Tween-80 treatment of Billen and Olsen (1976). Exponentially growing cells were plated at a density of 2 X 10^6 cells/100 mm plastic tissue culture plate in αMEM plus 10% serum. CHO cells were then incubated at 37^o for approximately 40 hours, while L cells were routinely grown for 48 hours as they have a slightly longer doubling time. Cells were harvested by trypsinization, washed once in medium plus serum and counted. The cells were then resuspended at 10^7 cells/ml in permeabilizing buffer consisting of 1% Tween-80, 0.25 M sucrose, 0.01 M HEPES pH7.2, 2 mM DTT. After 30 minutes at 24^{o}C, the cells were centrifuged and resuspended in fresh permeabilizing buffer at a concentration of 2.5 X 10^7 cells/ml. Aliquots of 200 µl, corresponding to 5 X 10^6 cells, were then dispensed into assay tubes.

For CDP reduction, 200 µl of the above cell suspension was made up to 300 µl with the addition of concentrated reaction buffer. For CHO cells, the final assay mix contained 0.05 M HEPES pH 7.2 buffer, 6 mM ATP, 6 mM DTT, 10 mM $MgCl_2$, 0.037 mM ^{14}C-CDP (55,000 cpm/nmole), 0.67% Tween-80, and 0.167 M sucrose. The reaction was allowed to proceed for 1 hour at 37° before being terminated by boiling for 4 minutes. The amount of CDP reduced was determined as described previously for the in vitro assay. The final conditions for measuring CDP reductase in L cells consisted of 0.05 M HEPES pH 7.2 buffer, 5 mM $MgCl_2$, 6 mM DTT, 0.4 mM ^{14}C-CDP (4800 cpm/nmole), 0.67% Tween-80, and 0.167 M sucrose. A final concentration of 4 mM ATP was included for the WT L cells, and 6 mM ATP for L_2Cl_3 cells. The reaction was terminated after 60 minutes incubation at 34° for WT cells, and 37° for L_2Cl_3 cells. The amount of product formed was determined as described above.

For GDP reduction in WT and drug resistant L cells, 200 µl of the above cell suspension was made up to 300 µl final volume containing 0.05 M HEPES pH 7.0, 2 mM dTTP, 6 mM DTT, 0.33 mM ^{14}C-GDP (6400 cpm/nmole), 0.67% Tween-80, and 0.167 M sucrose. Each assay mixture was incubated for 60 minutes at 34° (WT cells) or 37° (L_2Cl_3 cells) before being terminated by boiling for 4 minutes. The amount of GDP reduced was determined as described for the measurement of GDP reduction in vitro.

For the measurement of GDP reduction in CHO cells, the cells were resuspended after the 30 minute Tween-80 treatment in fresh permeabilizing buffer in which the HEPES pH 7.2 buffer had been

replaced by 0.075 M PIPES pH 7.0 buffer. Aliquots of 5 X 10^6 cells in 200 µl were transfered to assay tubes and made up to a final volume of 300 µl containing 0.05 M PIPES pH 7.0 buffer, 6 mM DTT, 1 mM dTTP, 0.1 mM $FeCl_3$, 0.022 mM ^{14}C-GDP (96,000 cpm/nmole), 0.67% Tween-80, and 0.167 M sucrose. The reaction was allowed to proceed for 60 minutes at 37^{o} before being terminated by boiling for 4 minutes. The amount of product formed was determined as described above.

5. SOURCE OF MATERIALS

Most biochemicals were from Sigma Chemical Co., St. Louis and all radiochemicals were from Amersham Corp., Illinois. All other materials were obtained from sources listed in Table 1.

TABLE 1: Source of Materials

MATERIAL	SOURCE
culture plates (60 mm plastic)	Lux Scientific Corp., Newbury
culture plates (100 mm plastic)	"
culture bottles (16 oz. glass)	Brockway Glass Co., New York
Dowex 1-X8 anion exchange resin (200-400 mesh)	Bio-Rad laboratories, Richmond
foetal calf serum	GIBCO Ltd, Grand Island
Guanazole	Dr. B. Wood Jr. Drug Development Branch National Cancer Institute Bethesda, Maryland
Linbro cloning dish (#1S-FS-96-TC)	Linbro Scientific Inc., Connecticut
PEI-cellulose Thin-layer chromatography plates	Brinkman Inst., Westbury
Penicillin G	GIBCO Ltd., Detroit
POP, POPOP	Fischer Scientific Co. Ltd., New Jersey
Scintiverse	"
spinner bottles (suspension culture)	GIBCO Ltd., Detroit
Streptomycin	"
toluene (scintilized)	Fischer Scientific Co.
trypsin (Bacto #0153-61)	Difco Laboratories, Detroit
Tween-80	J.T. Baker Chemical Co., Phillipsberg

RESULTS

1. RESPONSE OF WILD-TYPE L CELLS TO HYDROXYUREA AND GUANAZOLE

a) growth rate in the presence of hydroxyurea: Figure 1 shows the growth rate of WT L cells under routine culture conditions both in the presence and absence of hydroxyurea. WT cells on plastic tissue culture plates in αMEM supplemented with 10% FCS will grow exponentially at 37^{o} to at least 4 X 10^{6} cells/60 mm plate. This cell line exhibited a doubling time of approximately 18 hours under standard conditions in the absence of drug. Figure 1 shows that when exponentially growing WT cells were exposed to 0.35 mM hydroxyurea, the cellular growth rate was severely inhibited. Exposure to drug allowed only a very slight initial increase in cell number before growth was completely halted within 30 hours.

b) plating efficiency in the presence of hydroxyurea: The ability of WT cells to produce colonies of at least 30 cells on the surface of Brockway bottles was observed in the presence of increasing concentrations of hydroxyurea. Figure 2 shows that the presence of less than 0.1 mM hydroxyurea caused only a slight decrease in the relative plating efficiency (RPE) of the WT cells. Concentrations of drug greater than 0.1 mM caused an exponential decrease in plating efficiency, with 0.35 mM hydroxyurea reducing the RPE to 6 X 10^{-6}.

c) growth and plating efficiency in the presence of guanazole: Guanazole (3,5-diamino-1,2,4-triazole, NSC 1895) is a cytotoxic drug which has been investigated as a therapeutic agent in the treatment of some neoplasms. It has been shown, for example, to be effective against L1210 leukemia in mice and the P815 mast cell

FIGURE 1: Effect of 0.35 mM hydroxyurea (▽) and 2.0 mM guanazole (△) on the normal growth rate (○) of WT L cells. Exponentially growing L cells were removed from the surface of a Brockway bottle with a sterile trypsin solution. Aliquots of 4 X 10^4 cells were then plated onto 60 mm plastic culture plates containing complete medium plus serum. At various time intervals, cells were removed from the surface of the plates and counted. The arrow indicates the time of addition of hydroxyurea and guanazole.

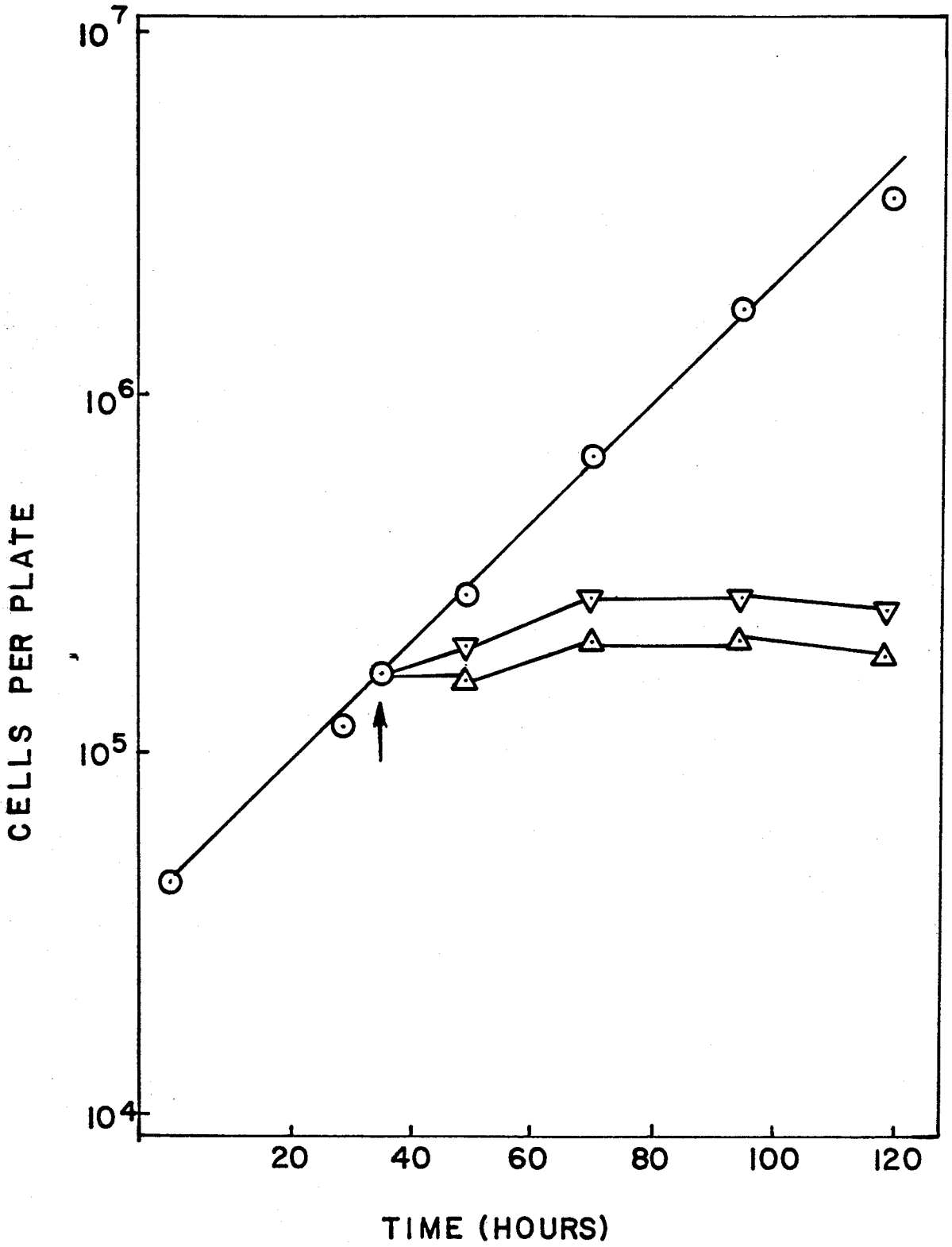
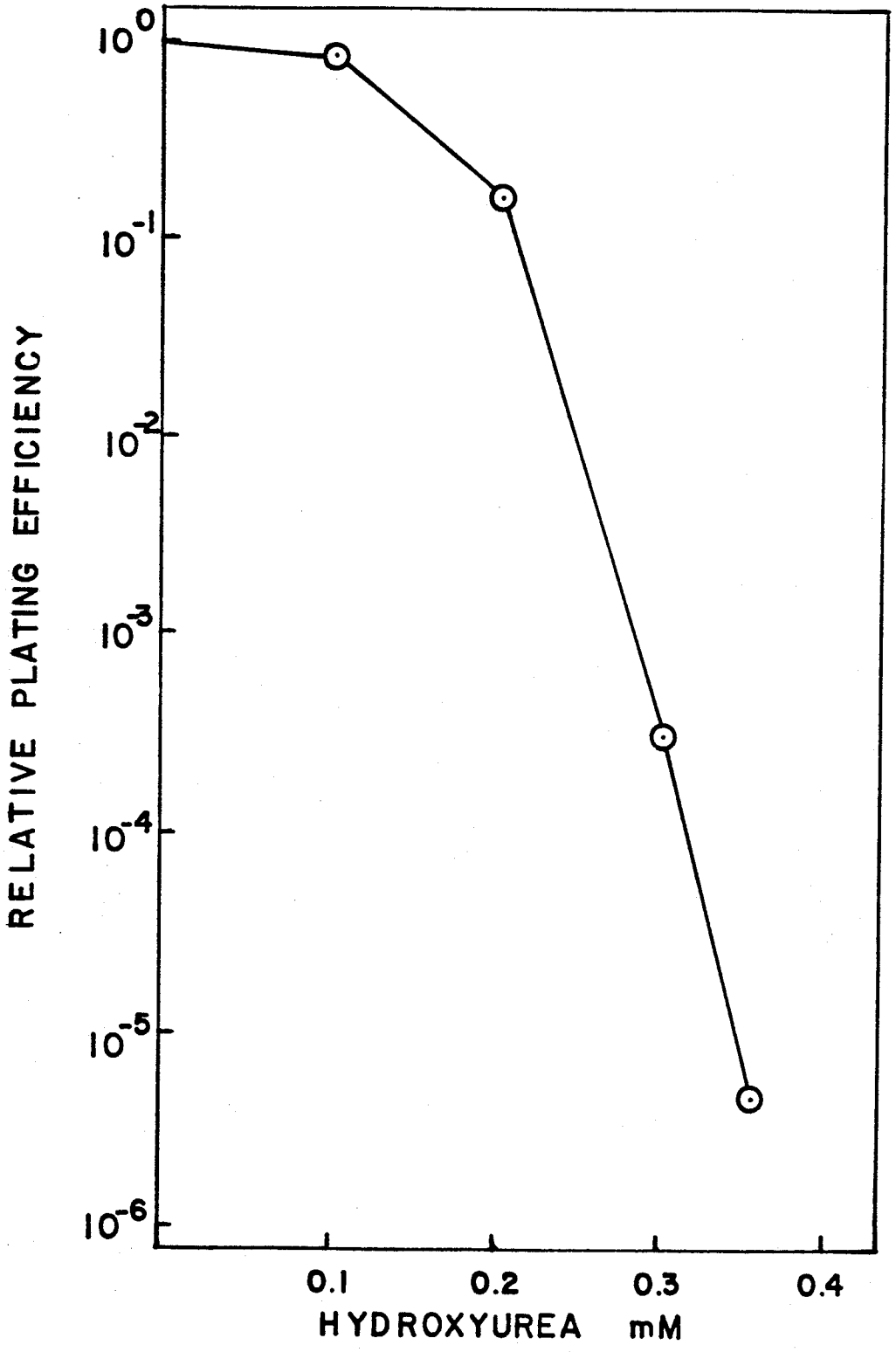

10^7
10^6
10^5
10^4
CELLS PER PLATE
20
40
60
80
100
120
TIME (HOURS)

FIGURE 2: Effect of hydroxyurea on the relative plating efficiency of WT L cells. From 500 to 10^6 cells were seeded onto 16 oz. Brockway bottles containing standard medium plus various concentrations of hydroxyurea. After 10 days at 37°, the medium was poured off, and the colonies were stained with methylene blue and counted.


RELATIVE PLATING EFFICIENCY
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
0.1
0.2
0.3
0.4
HYDROXYUREA mM

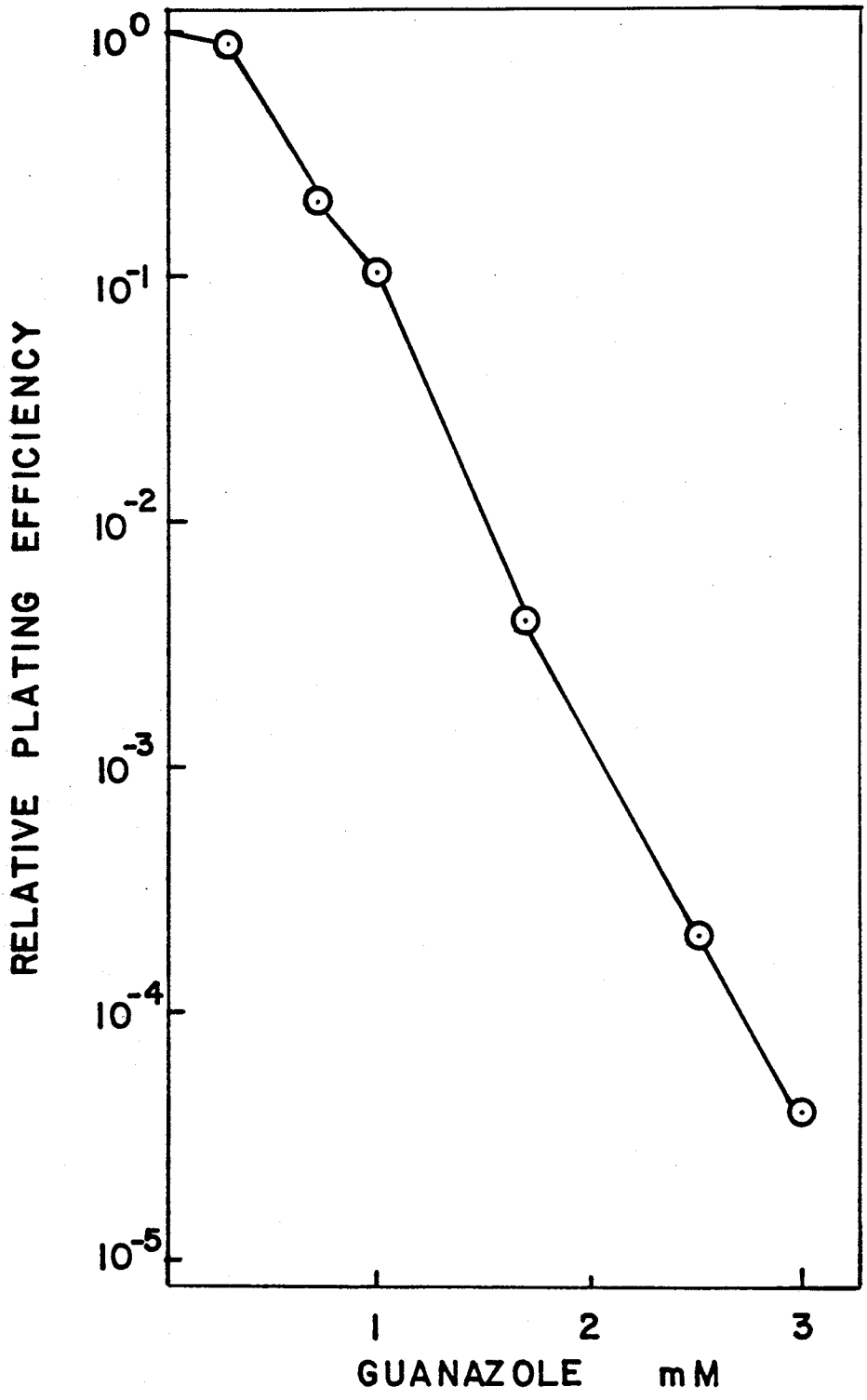
tumor (Dave et al, 1978; Hahn and Adamson, 1970). Similar results have also been reported for hydroxyurea (eg. Stearns, Losee and Berstein, 1963; Wilkoff et al, 1970). Brockman et al (1970) suggested that hydroxyurea and guanazole may act at a common biochemical site. This is supported by the finding that mutant mammalian cells in culture which are selected for resistance to one of the drugs, are also resistant to the other non-selective drug (Wright and Lewis, 1974). It was decided, therefore, to test the effect of guanazole on WT mouse L cells.

Figure 1 shows that 2 mM guanazole was able to stop growth of an exponentially growing WT cell population within 30 hours. As expected, guanazole also reduced the plating efficiency of WT cells in a fashion similar to that of hydroxyurea (figure 3). Guanazole, however, was much less potent than hydroxyurea on a molar basis. Figure 3 shows that 3 mM guanazole was required to reduce the RPE of WT cells to 10^{-4}, whereas this same effect could be achieved with only 0.3 mM hydroxyurea (figure 2).

2. ISOLATION OF L CELL LINES HIGHLY RESISTANT TO HYDROXYUREA

Cells selected from colonies formed in the presence of normally very cytotoxic concentrations of drug are often genetically stable highly drug resistant variants (Siminovitch et al, 1972). With this view in mind, attempts were made to select for L cells that were capable of forming colonies in the presence of highly toxic concentrations of hydroxyurea (eg. $\geq$ 1 mM drug). The plating efficiency of WT L cells at concentrations of 1 mM hydroxyurea or higher is below 10^{-7} (figure 2). Therefore, the appearance within the WT

FIGURE 3: Effect of guanazole on the relative plating efficiency of WT L cells. From 600 to 5 X 10^5 cells were plated onto 100 mm plastic culture plates containing standard medium plus various concentrations of guanazole. After approximately 10 days at 37°, the medium was poured off and the colonies were stained with methylene blue and counted.


RELATIVE PLATING EFFICIENCY
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
1
2
3
GUANAZOLE mM

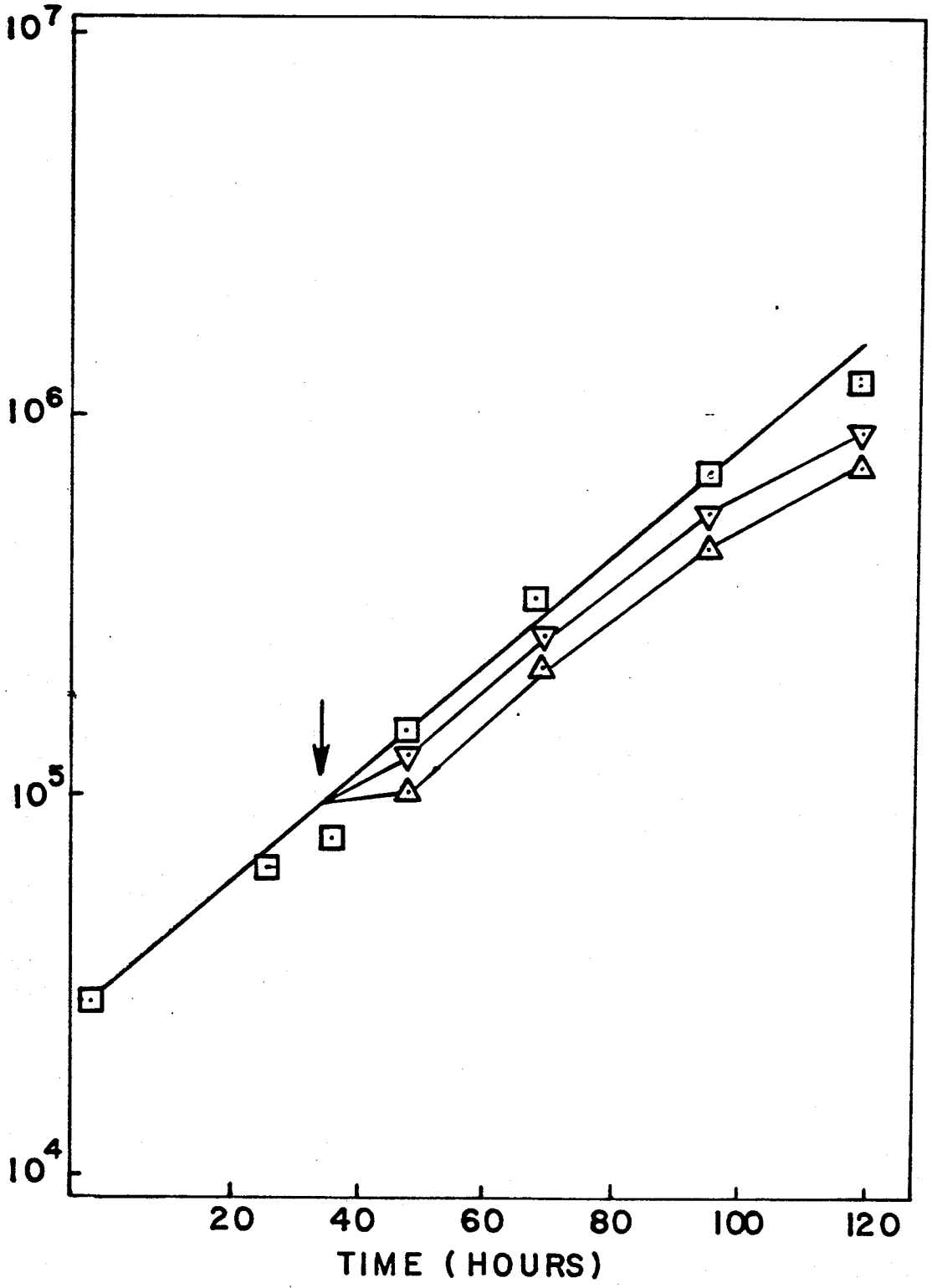
population of a variant which could form colonies at this concentration would be a rare event. Other investigators have isolated mutant mammalian cells resistant to very highly cytotoxic concentrations of drug by employing a multiple step selection procedure (eg. Bunn and Eisenstadt, 1977; Gupta and Siminovitch, 1978; Lewis and Wright, 1978b). A similar approach was therefore used to select L cells resistant to very high concentrations of hydroxyurea.

Wild-type L cells were placed onto plastic tissue culture plates at a concentration of 5×10^5 cells/100mm plate. The cells were incubated in complete medium plus 0.35 mM hydroxyurea, a concentration of drug which reduces WT PE to 6×10^{-6} (figure 2). After 10 days at 37°, the surviving colonies were transfered to medium lacking hydroxyurea and allowed to grow to a monolayer of cells. These cells were then trypsinized, and 5×10^5 cells were placed onto a 100 mm plastic plate containing complete medium plus 1.3 mM hydroxyurea. After 14 days at 37°, the cells which survived in this concentration of drug were again trypsinized and cloned in a 96 well Linbro dish (see Materials and Methods). One clone isolated in this way, was designated L_2Cl_3 and used for further study.

3. RESPONSE OF HYDROXYUREA-RESISTANT L CELLS TO HYDROXYUREA AND GUANAZOLE

a) growth rate: Figure 4 indicates the growth rate of L_2Cl_3 cells under routine culture conditions, both in the presence and absence of hydroxyurea and guanazole. L_2Cl_3 cells grown on 60 mm plastic culture plates in αMEM plus 10% FCS increased exponentially in cell

FIGURE 4: Effect of 0.35 mM hydroxyurea (▽) and 2.0 mM guanazole (Δ) on the normal growth rate (⊙) of L_2Cl_3 cells. Exponentially growing L_2Cl_3 cells were removed from the surface of a Brockway bottle with a sterile trypsin solution. Aliquots of 4 X 10^4 cells were then plated onto 60 mm plastic culture plates containing complete medium plus serum. At various times, cells were removed from the surface of the plate with trypsin and counted. The arrow indicates the time of addition of hydroxyurea and guanazole.


10^7
10^6
10^5
10^4
20
40
60
80
100
120
TIME (HOURS)

number to over 10^6 cells/plate. The doubling time at 37^o under routine conditions and in the absence of drug was approximately 21 hours. When exponentially growing L_2Cl_3 cells were exposed to 0.35 mM hydroxyurea, the cells continued to grow with a slightly extended doubling time of approximately 24 hours. This is the same concentration of hydroxyurea which was shown to cause a complete halt in cell division in WT cells within 30 hours of exposure (figure 1).

Figure 4 also shows that L_2Cl_3 cells are able to grow in normally cytotoxic concentrations of guanazole. In the presence of 2 mM guanazole, which completely stopped cell division in WT cells (figure 1), the L_2Cl_3 cells continued to grow with a slightly lengthened doubling time of approximately 24 hours.

b) plating efficiency: The high level of resistance of L_2Cl_3 cells to the cytotoxic effects of hydroxyurea was also shown by their ability to form colonies in the presence of increasing concentrations of drug (figure 5). In the presence of less than 0.1 mM hydroxyurea, neither the WT nor the L_2Cl_3 line showed a large decrease in RPE. At higher drug concentrations, however, the resistance of L_2Cl_3 cells to the toxic effects of hydroxyurea became more obvious. At 0.35 mM hydroxyurea, for example, the RPE of the WT cells dropped to 6×10^{-6}, while the L_2Cl_3 cell line retained a RPE of approximately 0.75. This was a stable characteristic of the L_2Cl_3 line. After 11 months of continuous subculture in the absence of drug, the RPE of L_2Cl_3 cells was again tested in the presence of 0.35 mM hydroxyurea and was found to be 0.80.

FIGURE 5: Effect of hydroxyurea on the relative plating efficiency of L_2Cl_3 cells (□). 400 cells were plated onto 100 mm plastic culture plates containing standard medium plus various concentrations of hydroxyurea. After approximately 10 days at 37 °, the medium was poured off and the colonies were stained and counted. Dashed line represents the RPE of WT cells as obtained from FIGURE 2.

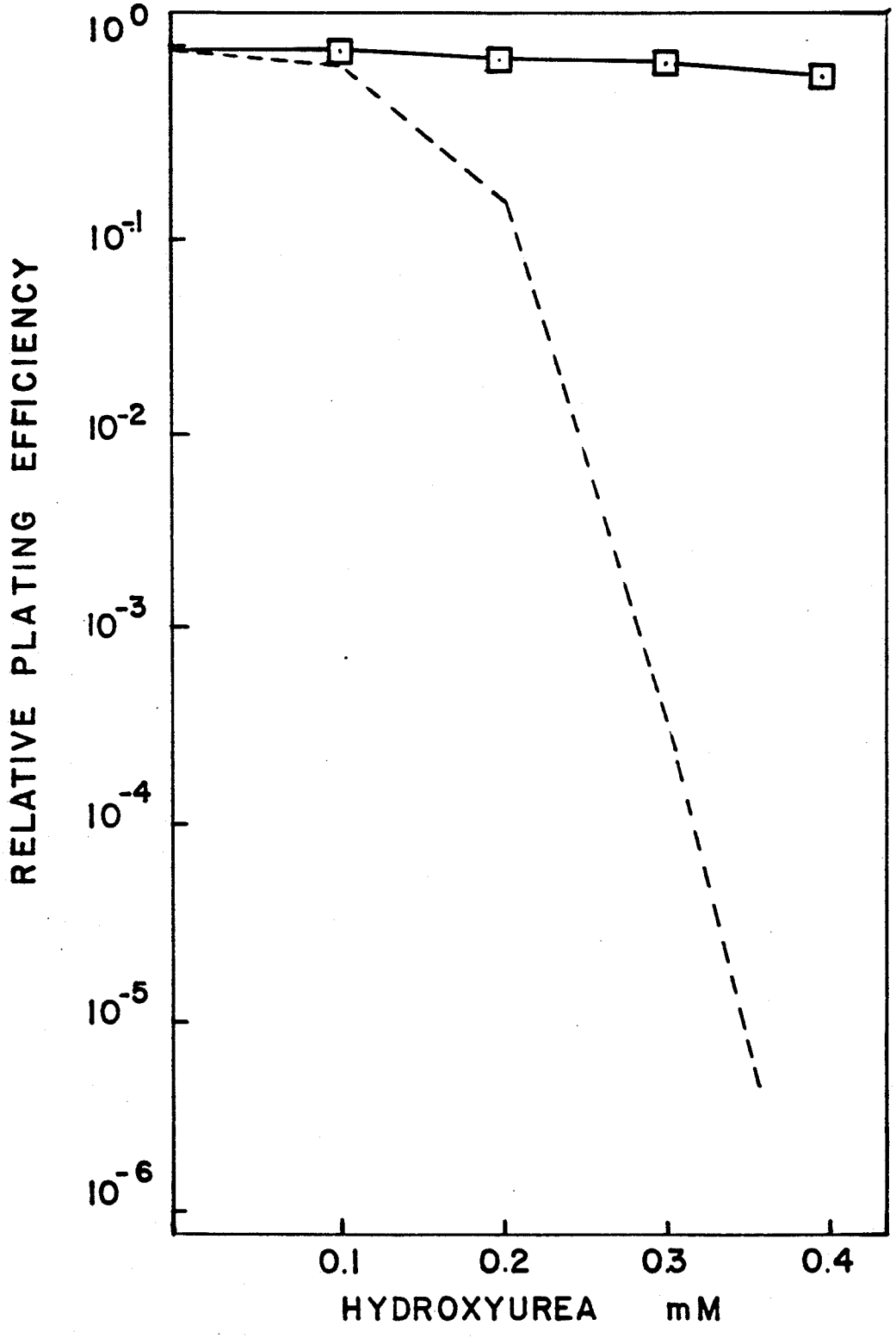

RELATIVE PLATING EFFICIENCY
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
0.1
0.2
0.3
0.4
HYDROXYUREA mM

FIGURE 6: Effect of guanazole on the relative plating efficiency of L_2Cl_3 cells (□). 400 cells were plated onto 100 mm plastic culture plates containing standard medium plus various concentrations of guanazole. After approximately 10 days at 37^{o}, the medium was poured off and the colonies were stained and counted. Dashed line represents the RPE of WT cells obtained from FIGURE 3.

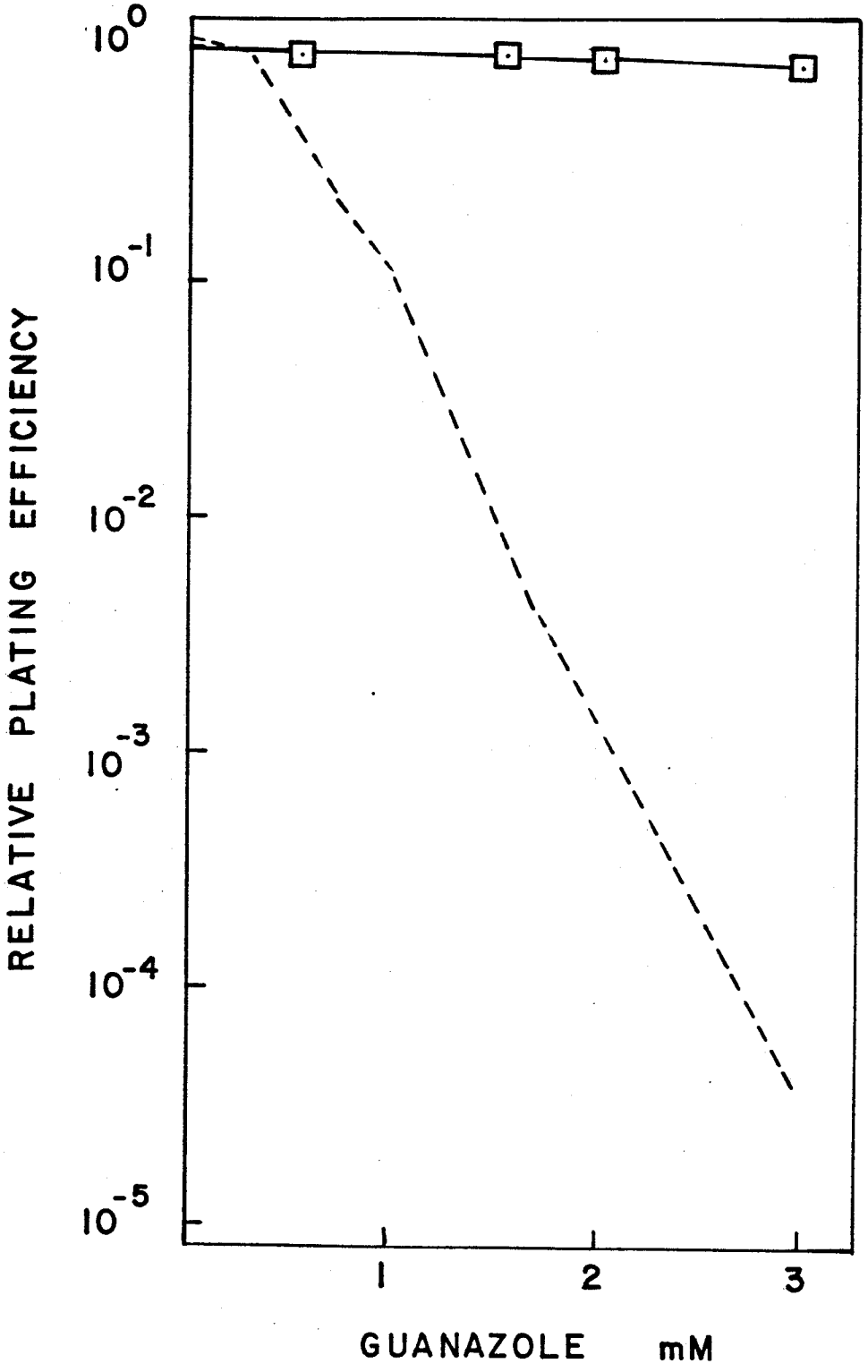

RELATIVE PLATING EFFICIENCY
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
1
2
3
GUANAZOLE mM

Figure 6 shows that the RPE of the L_2Cl_3 cell line is nearly unaffected by the presence of increasing amounts of guanazole in a manner similar to that shown for hydroxyurea. For example, 3 mM guanazole reduced the RPE of WT cells to 10^{-4}, whereas this concentration of drug only reduced the RPE of L_2Cl_3 cells by less than 20%.

4. RIBONUCLEOTIDE REDUCTASE

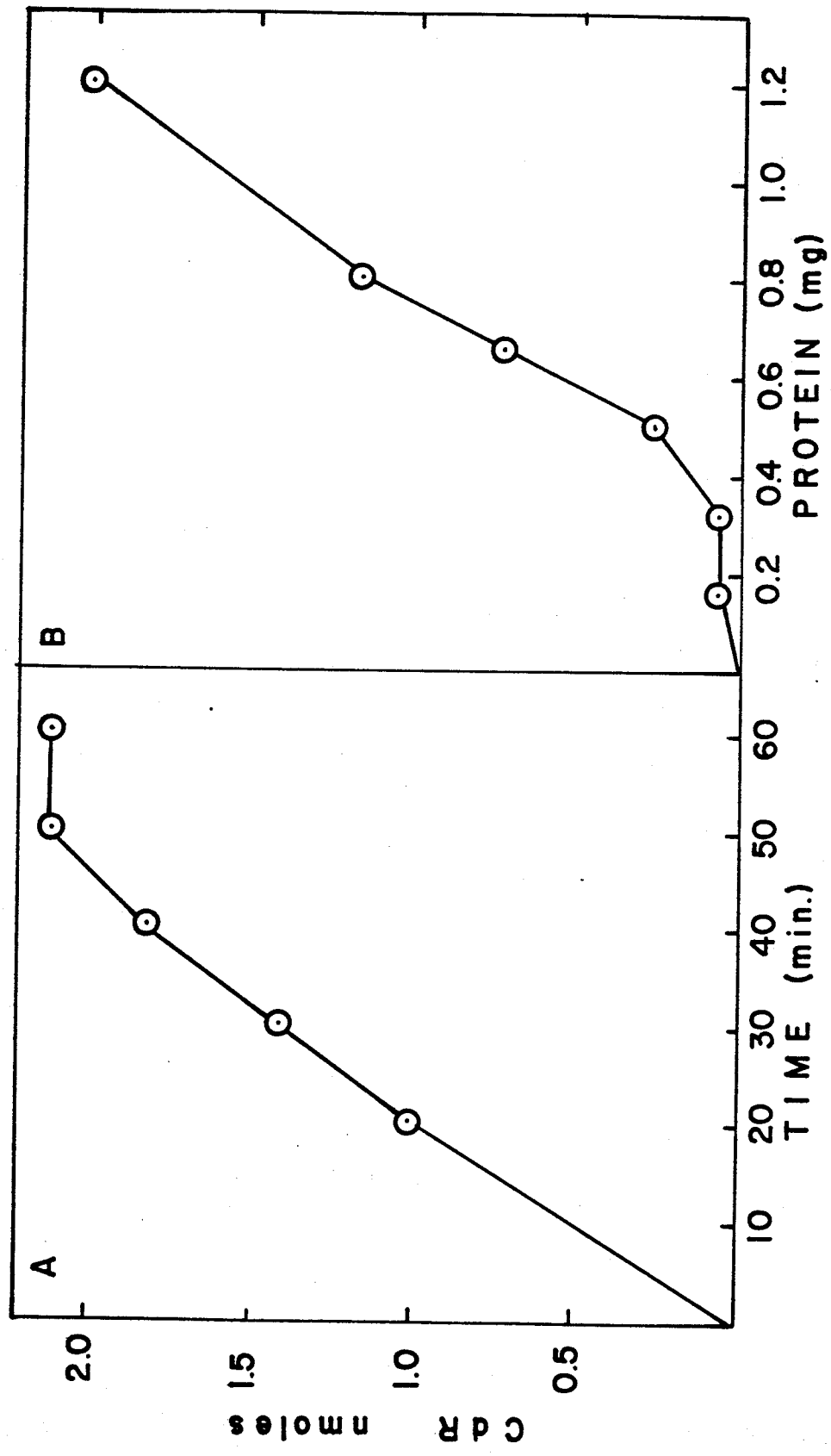
a) characterization of ribonucleotide reductase from WT L cells: Ribonucleotide reductase activity was studied with WT L cell extracts which were partially purified as described in Materials and Methods. The extracts were routinely prepared so that the standard aliquot of 100 µl used per assay contained approximately 1 mg of protein. Normally, these extracts contained 1.5 to 2.0 CDP reductase units per mg of protein.

(i) CDP reduction: CDP reductase activity was determined by measuring the conversion of labelled CDP to dCDP. Figure 7a shows that the reaction proceeded linearly with time for at least 40 minutes. Therefore a time of 40 minutes was chosen as the standard incubation time in future work.

Enzyme activity did not increase linearly with increasing protein concentration. Figure 7b shows that CDP reductase activity decreased in a logarithmic fashion when less than 400 µg of protein was included in the standard assay volume. Similar results have also been reported in other mammalian systems such as CHO cells (Lewis and Wright, 1978) and rabbit bone marrow (Hopper, 1972). Moore (1967, 1972) has suggested that this phenomenon may be due to

FIGURE 7a: Response of CDP reduction assay in extracts of WT L cells to time. Standard aliquots of WT cell extract (corresponding to 1.0 mg protein) were added to complete reaction mixtures and incubated at 37°. At the times indicated, the reaction was terminated by boiling, and the amount of CDP reduced was determined as described in Materials and Methods.

FIGURE 7b: Effect of increasing protein content on WT CDP reduction. Various amounts of WT cell extract (corresponding to 0.0 to 1.2 mg protein) were inclcuded in a standard CDP reductase assay. After incubation at 37° for 40 minutes, the amount of CDP reduced was determined.


A
B
C d R nmoles
2.0
1.5
1.0
0.5
10
20
30
40
50
60
TIME (min.)
0.2
0.4
0.6
0.8
1.0
1.2
PROTEIN (mg)

the existence of the enzyme as 2 separate subunits. Enzyme activity would then be lost as the subunits dissociate at low portein concentration. All of the subsequent work done with L cell CDP reductase was carried out with protein levels in the linear range of figure 7b.

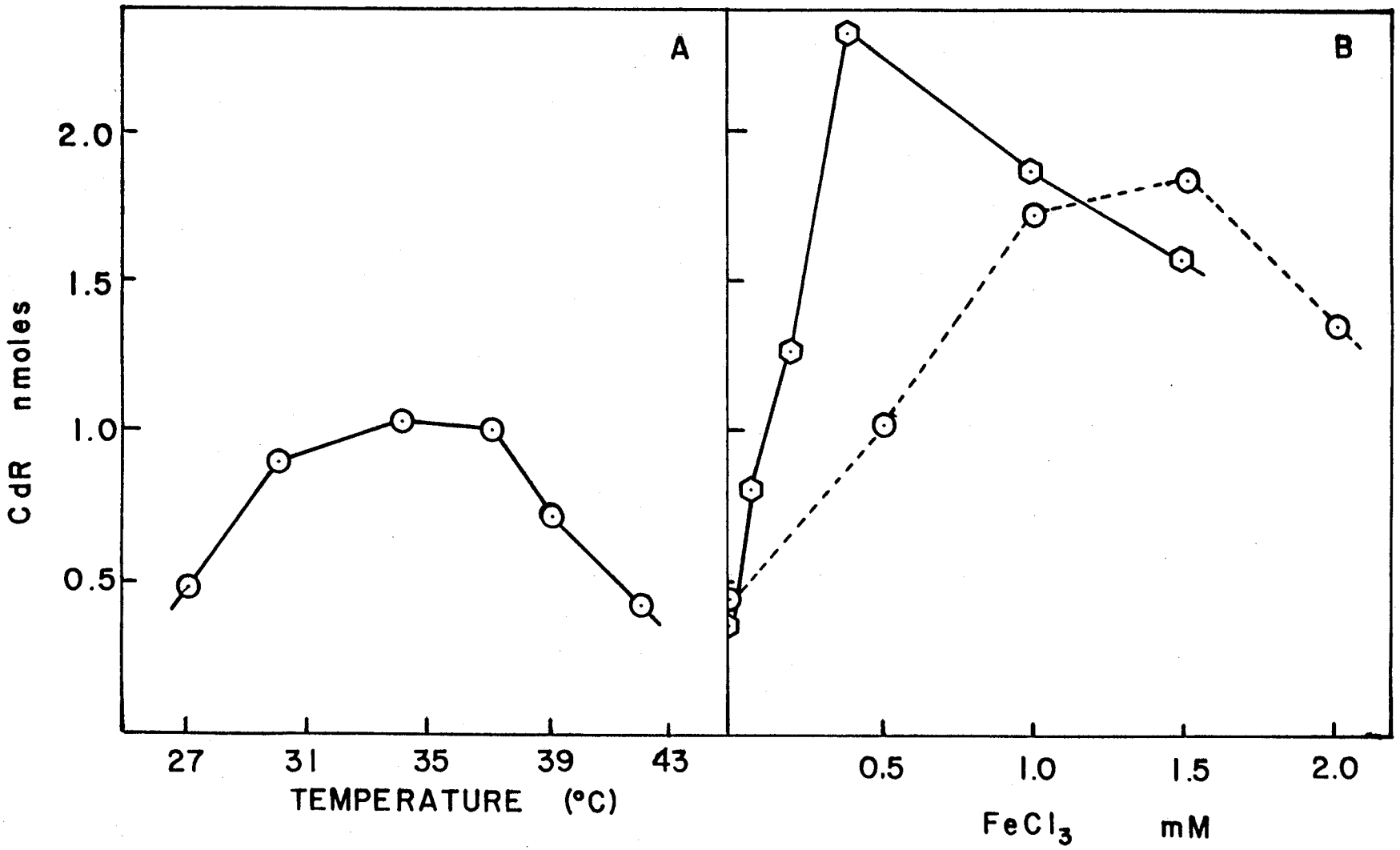
Figure 8a shows that the optimum assay temperature for the reaction was between 35^{o} and 37^{o}, which approximately corresponds to the normal growth temperature for L cells.

Similar to other mammalian systems (Moore 1969), CDP reduction in L cell extracts was stimulated several fold by the addition of $FeCl_3$ (figure 8b). However, the concentration of $FeCl_3$ required for maximum activity varied from 0.5 to 1.5 mM, depending on the batch of enzyme tested. A similar phenomenon was reported in partially purified extracts of CHO cells (Lewis and Wright, 1978a). Maximum stimulation of CDP reduction in L cells required abnormally high concentrations of $FeCl_3$; 1.5 mM $FeCl_3$ is 10 times the level found to be optimal in CHO extracts (Lewis and Wright, 1974) and 75 times the level of $FeCl_3$ used to assay the enzyme in regenerating rat liver (Larsson, 1969).

ATP is an important allsoteric activator of CDP reductase activity in E. coli (Brown and Reichard, 1969b) and in the mammalian systems examined so far (Moore and Hurlbert, 1966). CDP reductase activity in L cells was also stimulated by ATP; with an optimum concentration of 4 mM ATP resulting in a several fold increase in enzyme activity (figure 9a). Figure 9a also shows that activity declined sharply when more than 4 mM ATP was added. Similar results have been reported for CDP reduction in regenerating rat liver (Larsson,

FIGURE 8a: Effect of assay temperature on CDP reductase activity in WT extracts. Standard assay mixtures were incubated for 40 minutes at the temperatures indicated, and the amount of CDP reduced was determined as described previously.

FIGURE 8b: Effect of addition of $FeCl_3$ to the assay mixture for the measurement of CDP reduction in 2 different (◇ and ⊙) batches of WT extracts. A concentrated 5 µl aliquot of $FeCl_3$ in distilled water was added to each assay to achieve the final concentrations of $FeCl_3$ indicated. The reaction was then allowed to proceed under standard conditions.


A
B
2.0
1.5
1.0
0.5
CdR nmoles
27
31
35
39
43
TEMPERATURE (°C)
0.5
1.0
1.5
2.0
FeCl3 mM

1969) and CHO cells (Lewis and Wright, 1978a), although the reason for this effect is unknown.

Dithiothreitol (DTT) is a reducing agent which can replace thioredoxin (Moore, 1969) which is thought to be the endogenous source of reducing power for ribonucleotide reductase in the cell (Thelander, 1967; Laurent et al, 1964). Figure 9b shows that CDP reductase activity in extracts of WT L cells was dependent upon the addition of exogenous DTT, as there was little measurable activity in the absence of added DTT. A concentration of 6 mM DTT was chosen for future work as the addition of more DTT did not appreciably increase enzyme activity.

CDP reduction was slightly stimulated by magnesium ion added as $MgCl_2$ (figure 10a). The optimum $MgCl_2$ concentration occurred between 5 and 10 mM; 5 mM $MgCl_2$ was chosen for future work. This compares with 4 mM $MgCl_2$ used to measure CDP reduction in Ehrlich tumor cells (Cory et al, 1975).

Figure 10b indicates a pH optimum of 7.0 for CDP reduction. $FeCl_3$ was not added during this experiment as problems were encountered with the solubility of the iron in the pH range tested. It was observed however, that these problems were minimized, and the results were reproducible at a pH of 7.2. Therefore, HEPES buffer at pH 7.2 was used for all subsequent work.

The initial rate of reaction of CDP reductase in WT cells was determined as a function of increasing concentration of substrate (figure 11). A Lineweaver-Burk double reciprocal plot of this data

FIGURE 9a: Effect of ATP on CDP reductase activity in WT cell extracts. The standard aliquot of 1.0 mg of protein from a partially purified WT extract was added to CDP reductase assay mixtures containing various amounts of ATP. The reaction was allowed to proceed under standard conditions.

FIGURE 9b: Response of CDP reductase activity in WT cell extracts to the presence of a reducing agent. Standard CDP reductase assay conditions were used except for the presence of varied concentrations of DTT.

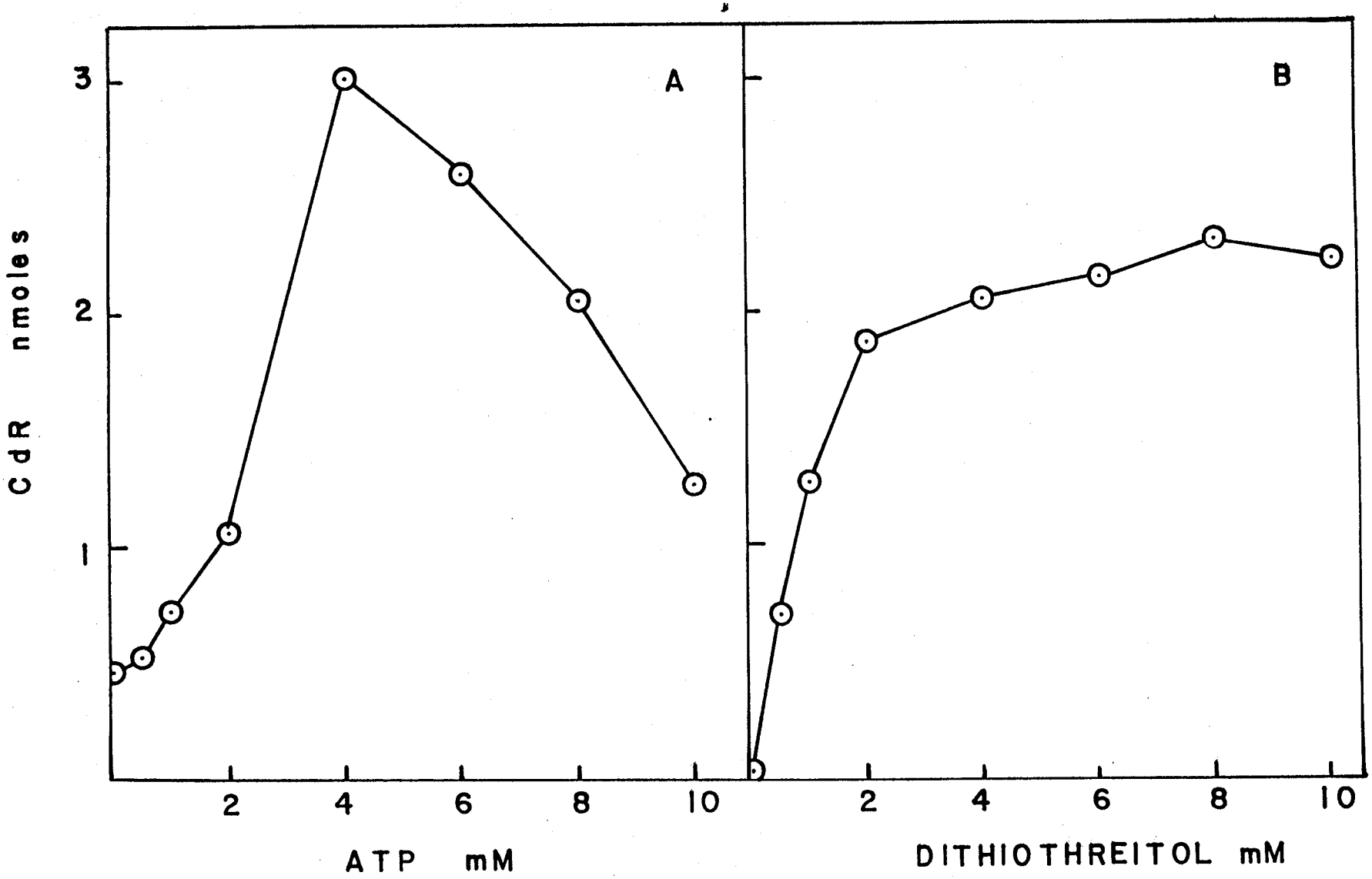

A
B
CdR nmoles
3
2
1
2
4
6
8
10
ATP mM
DITHIOTHREITOL mM

FIGURE 10a: Effect of magnesium ion on CDP reduction. CDP reductase activity was determined in partially purified WT extracts in the presence of varied concentrations of $MgCl_2$. Except for this variable, standard assay conditions were used.

FIGURE 10b: Effect of pH on WT CDP reductase activity in the absence of $FeCl_3$. Aliquots of partially purified WT extracts (corresponding to 1.0 mg protein) were assayed for CDP reduction in reaction mixtures lacking $FeCl_3$ and containing 0.05 M HEPES buffer at various pH's.

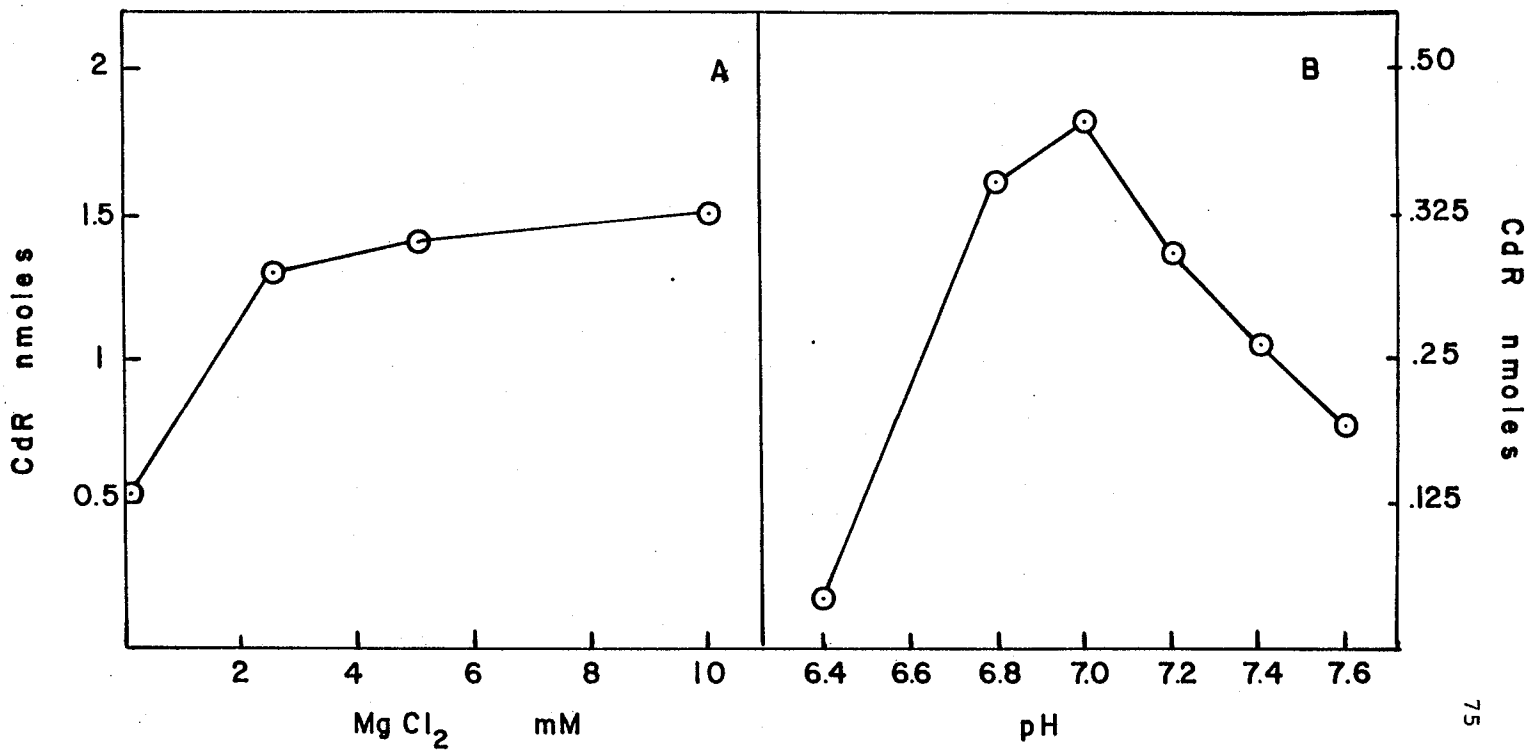
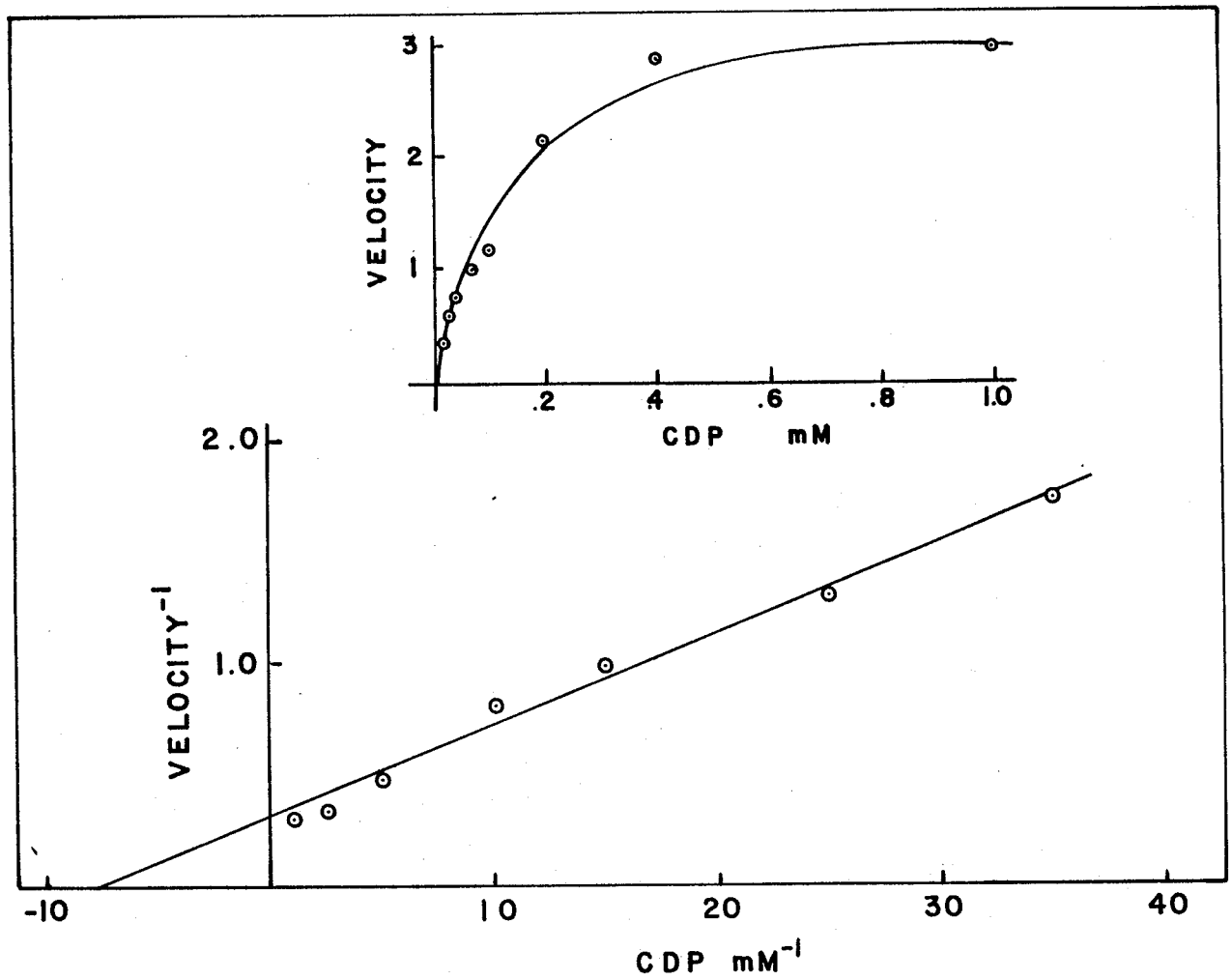

A
B
CdR nmoles
2
1.5
1
0.5
2
4
6
8
10
Mg Cl2 mM
.50
.325
.25
.125
CdR nmoles
6.4
6.6
6.8
7.0
7.2
7.4
7.6
pH

FIGURE 11: Double reciprocal plot of the variation in rate of CDP reduction with CDP concentration. A standard aliquot (1.0 mg protein) of partially purified WT extract was incubated under standard CDP reductase assay conditions, except that the concentration of CDP was varied.

Insert: Response of initial velocity of CDP reduction to various concentrations of CDP.


VELOCITY
3
2
1
.2
.4
.6
.8
1.0
CDP mM
2.0
1.0
VELOCITY^{-1}
-10
10
20
30
40
CDP mM^{-1}

was linear and yielded an apparent Km value for CDP of 0.14 mM. This is very close to the values of 0.13 mM reported in CHO cells (Lewis and Wright, 1978a), and 0.12 mM reported for Novikoff hepatoma cells (Moore and Hurlbert, 1966).

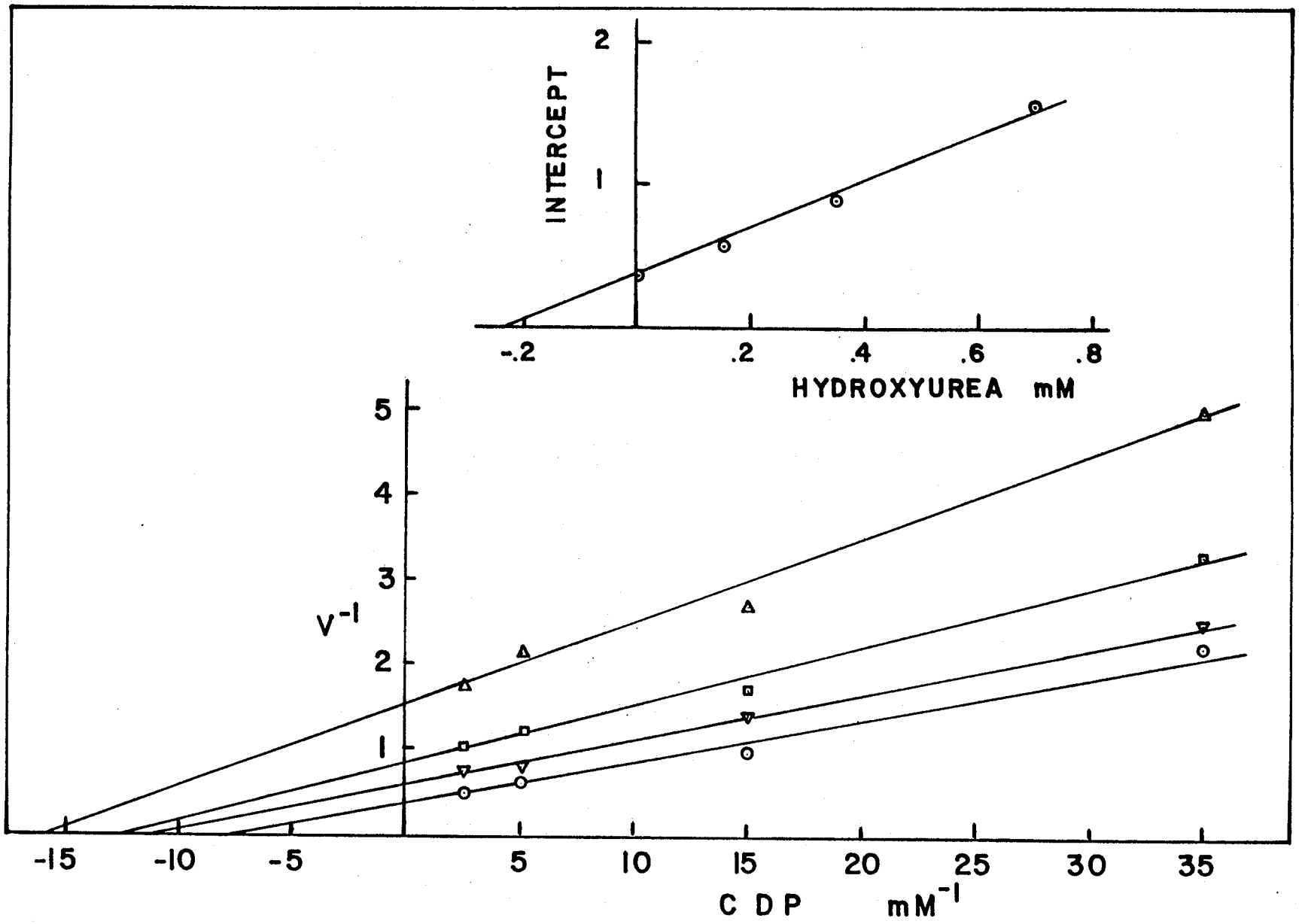
Attempts were made to test the effects of different concentrations of hydroxyurea on CDP reduction over a range of substrate concentrations. Simple addition of hydroxyurea to the optimized assay system often produced unreliable results. Rather, it was necessary to pre-incubate the extract with the desired amount of hydroxyurea for 30 minutes at room temperature. After the 30 minute equilibration, an aliquot of the extract/drug mixture was assayed for CDP reduction under standard conditions.

Figure 12 shows the inhibition by hydroxyurea of WT CDP reductase over a range of substrate concentrations. Optimized assay conditions were used, which in figure 12, included the presence of 1.5 mM $FeCl_3$. Hydroxyurea appeared to be an uncompetitive inhibitor at a concentration of 0.15 mM. However, inhibition appeared noncompetitive at higher concentrations of drug. This mixed pattern of inhibition has also been observed with CDP reductase activity isolated from extracts of CHO cells (Lewis and Wright, 1978a). The inset of figure 12 shows a linear replot of intercept values versus hydroxyurea concentration. This graph yielded a Ki value of 0.24 mM hydroxyurea for WT CDP reduction.

Hydroxyurea has been shown to specifically inactivate the iron containing subunit of ribonucleotide reductase from E. coli

FIGURE 12: Double reciprocal plot of the effect of hydroxyurea on the rate of CDP reduction in the presence of various substrate concentrations and in the presence of 1.5 mM $FeCl_3$. A standard aliquot (1.0 mg protein) of partially purified WT extract was incubated under routine CDP assay conditions with various concentrations of CDP and in the presence of 0 mM (⊙), 0.15 mM (▽), 0.35 mM (□), and 0.70 mM (△) hydroxyurea.

Insert: Replot of the velocity intercepts versus concentration of hydroxyurea.


INTERCEPT
2
1
-.2
.2
.4
.6
.8
HYDROXYUREA mM
V⁻¹
5
4
3
2
1
-15
-10
-5
5
10
15
20
25
30
35
C D P mM⁻¹

(Brown et al, 1969). Also, Moore (1969) has shown that the inhibition by hydroxyurea of mammalian ribonucleotide reductase in vitro can be reduced by the addition of iron. It has already been mentioned that different batches of L cell extract required from 0.5 to 1.5 mM $FeCl_3$ for optimal activity. The decision was made to test if this variability in iron requirement might also reflect a variability in sensitivity to hydroxyurea. Figure 13 shows the pattern of inhibition by hydroxyurea in an enzyme preparation requiring 0.5 mM $FeCl_3$ for maximum activity. It can be seen that the pattern of inhibition as well as the Ki value obtained from figure 13 is nearly identical to that observed in an extract requiring 1.5 mM $FeCl_3$ (figure 12) for maximum activity.

Guanazole was found to inhibit CDP reductase from WT extracts in a manner very similar to hydroxyurea (figure 14). Guanazole appeared to inhibit CDP reduction uncompetitively at the lowest drug concentration tested (0.75 mM). The higher concentrations of guanazole tested, however, resembled a noncompetitive type of inhibition as was seen with high concentrations of hydroxyurea (figure 12). The inset of figure 14 shows a Ki value of 1.5 mM guanazole; this is over 6 times greater than the Ki value for hydroxyurea (figure 12). This is not unexpected, however. It has already been shown that much less hydroxyurea is required to inhibit cell division (figure 1) and colony formation (figures 2 and 3) to the same extent as any given amount of guanazole. In fact, there is a direct correlation between the Ki values found in vitro and the growth inhibition that these concentrations of drug cause in vivo. Figures 2 and 3 show

FIGURE 13: Double reciprocal plot of the effect of hydroxyurea on the rate of CDP reduction in the presence of various substrate concentrations and in the presence of 0.5 mM $FeCl_3$. A standard aliquot (1.0 mg protein) of partially purified WT extract was incubated under routine CDP assay conditions with various concentrations of CDP and in the presence of 0 mM (⊙), 0.15 mM (▽), and 0.35 mM (▣) hydroxyurea.

Insert: Replot of the velocity intercept versus concentration of hydroxyurea.

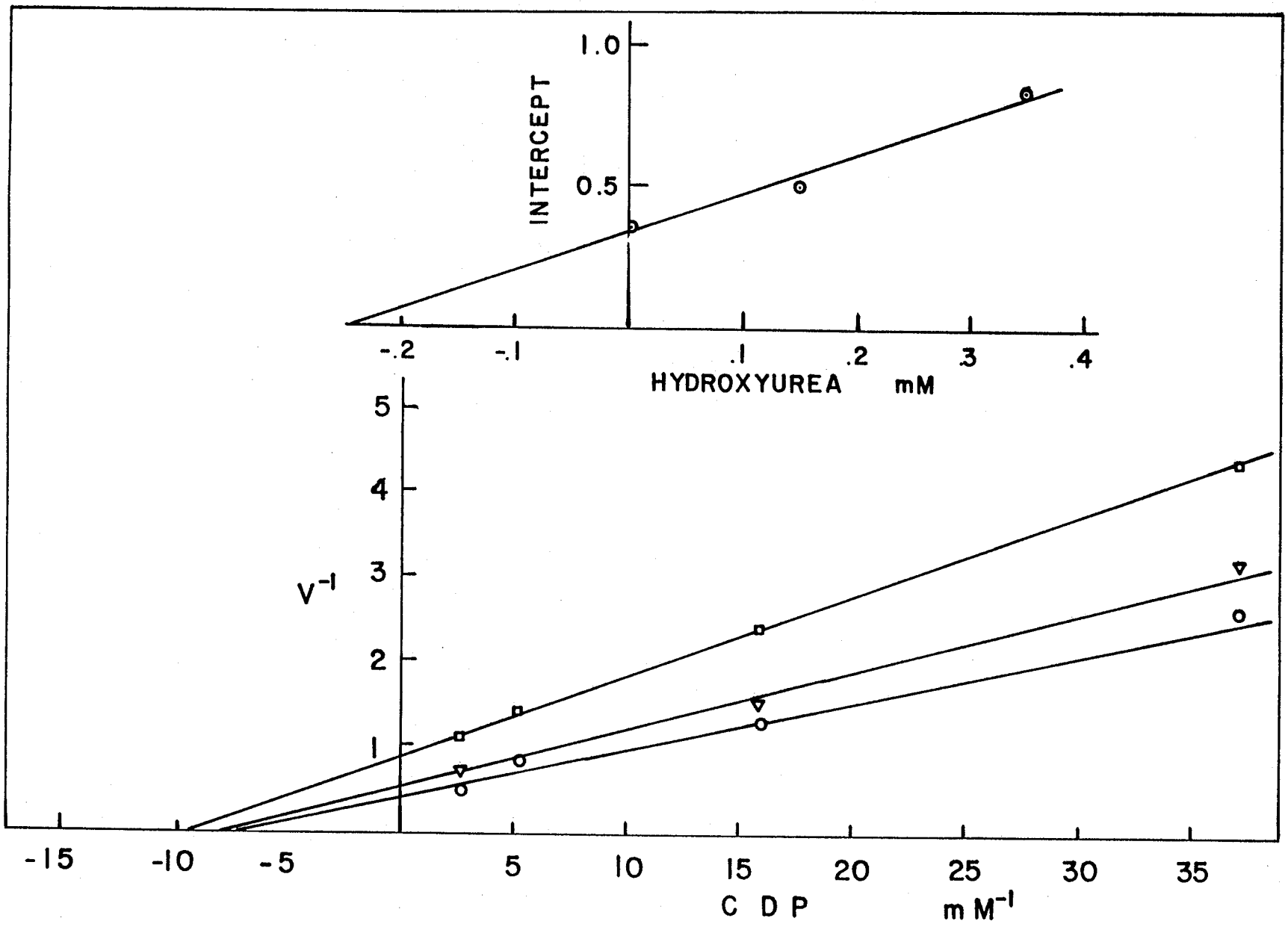
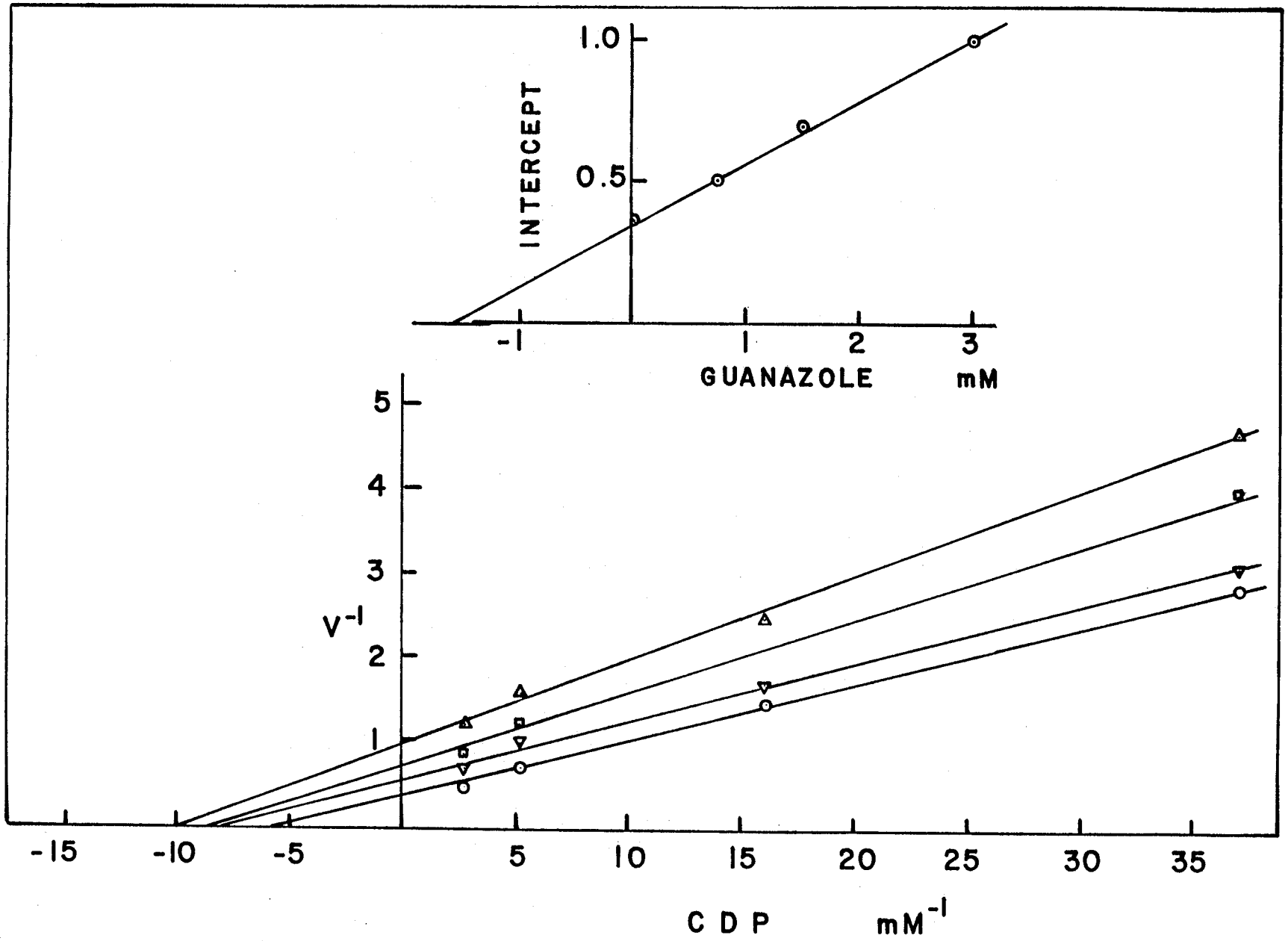

1.0
0.5
INTERCEPT
-.2
-.1
.1
.2
.3
.4
HYDROXYUREA mM
5
4
3
2
1
V⁻¹
-15
-10
-5
5
10
15
20
25
30
35
C D P
mM⁻¹

FIGURE 14: Double reciprocal plot of the effect of guanazole on the rate of CDP reduction in the presence of various substrate concentrations. A standard aliquot (1.0 mg protein) of partially purified WT extract was incubated under routine CDP assay conditions with various concentrations of CDP and in the presence of 0 mM (⊙), 0.75 mM (▽), 1.5 mM (⧈), and 3.0 mM (△) guanazole.

Insert: Replot of the velocity intercept versus concentration of guanazole.


1.0
0.5
INTERCEPT
-1
1
2
3
GUANAZOLE
mM
5
4
3
2
1
V-1
-15
-10
-5
5
10
15
20
25
30
35
C D P
mM-1

that the Ki values of 0.24 mM hydroxyurea and 1.5 mM guanazole both reduce the RPE of WT cells to 1×10^{-2}.

(ii) GDP reduction: GDP reductase activity was determined by measuring the amount of conversion of radioactively labeled GDP to dGDP as described in Materials and Methods. Figure 15 shows the response of enzyme activity to assay temperature and protein concentration. Maximum GDP reductase activity occurred at 30° (figure 15a). When assayed at the optimal cellular growth temperature of 37°, enzyme activity was nearly 70% lower than at 30°. The reason for this is not understood, but may be due to the activity of unknown enzymes or inhibitors which interfere with the in vitro assay of GDP reduction to a greater extent at 37° than at 30°. Similarily, other investigators have also assayed ribonucleotide reductase activity at a temperature well below the normal growth temperature of the organism from which the enzyme was obtained (Elford, 1974; Lewis et al, 1976).

Similar to CDP reductase, GDP reductase activity did not respond in a linear fashion to protein concentration (figure 15b). Enzyme activity fell off rapidly when less than approximately 300 µg of protein were included in the assay mixture. All subsequent work was done using 1 mg of protein per assay.

Under the optimized assay conditions, GDP reductase activity increased linearly with time for only 40 minutes, the standard assay time (figure 16a). Figure 16b shows that unlike CDP reductase, GDP reductase activity was not stimulated by added iron. In fact, activity decreased approximately 50% at 0.5 mM $FeCl_3$, a concentration

FIGURE 15a: Effect of assay temperature on GDP reduction in WT extracts. Standard assay mixtures were incubated for 40 minutes at the temperatures indicated, and the amount of GDP reduced was determined as described in Materials and Methods.

FIGURE 15b: Effect of increasing protein content on WT GDP reduction. Various amounts of partially purified WT cell extract (corresponding to from 0 to 1.0 mg protein) were added to a standard GDP reductase assay. After incubation at 30° for 40 minutes, the amount of GDP reduced was determined.

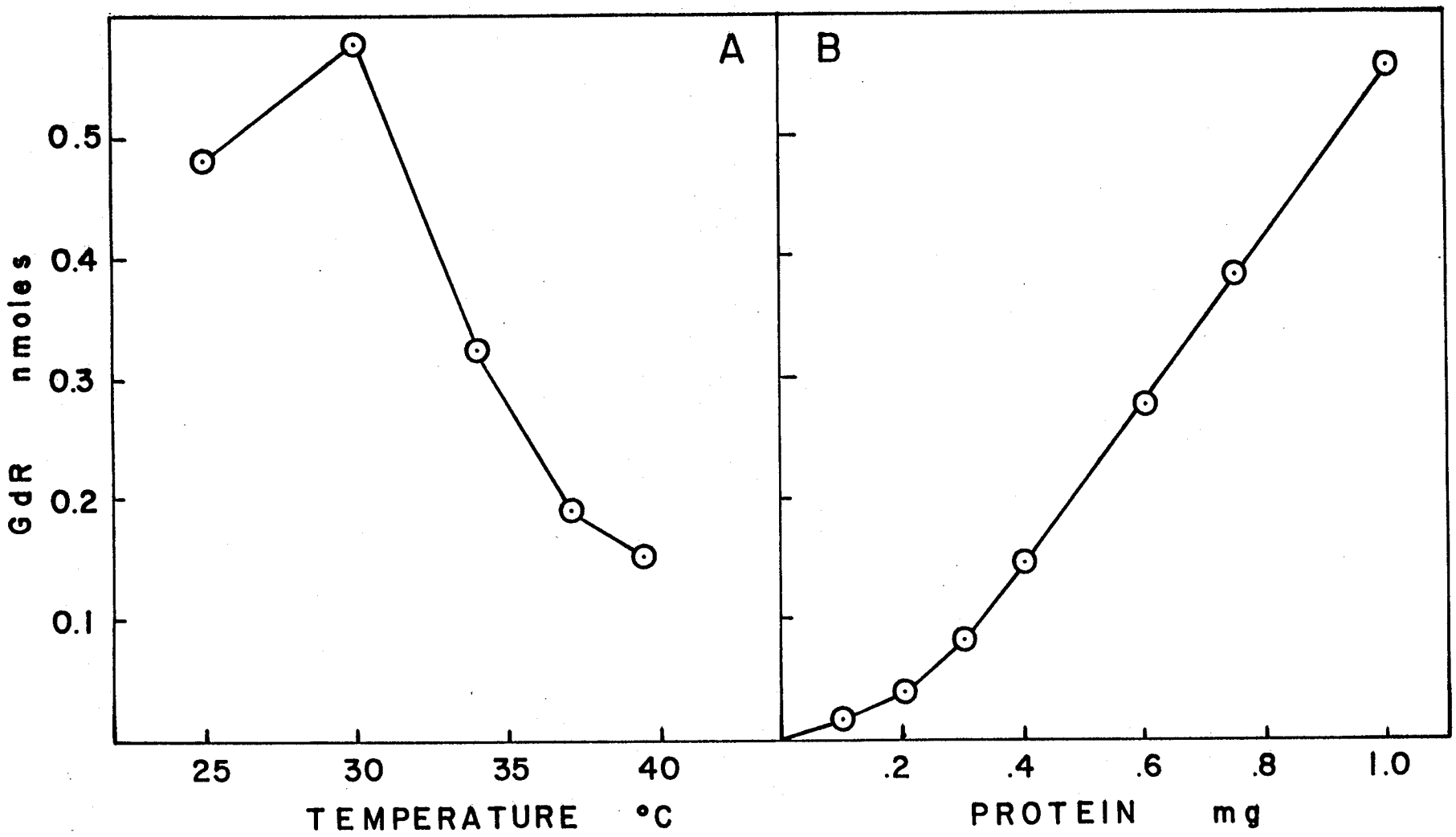
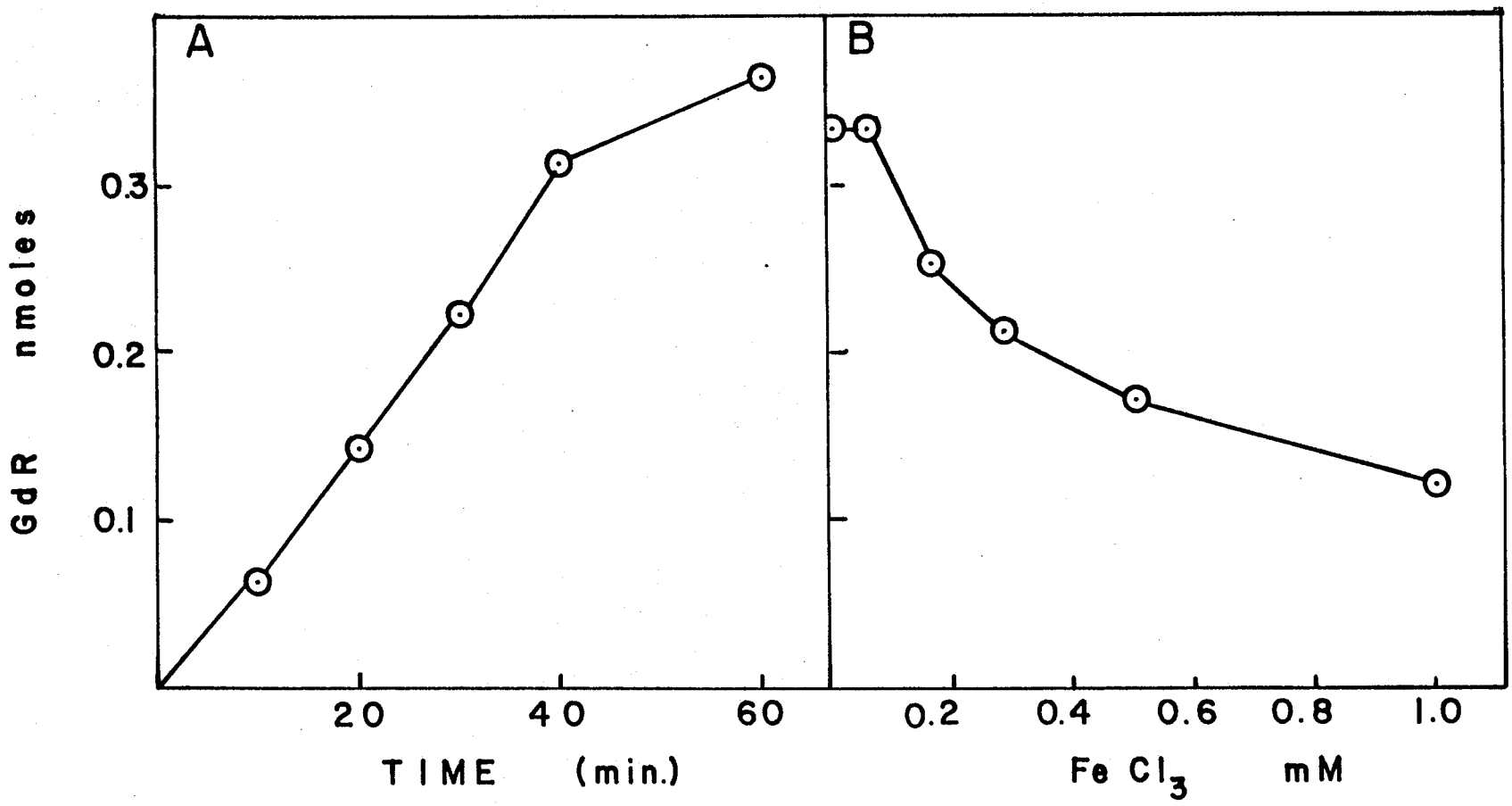

A
B
GdR nmoles
0.5
0.4
0.3
0.2
0.1
25
30
35
40
TEMPERATURE °C
.2
.4
.6
.8
1.0
PROTEIN mg

FIGURE 16a: Response of GDP reduction assay in extracts of WT L cells to time. Standard aliquots of WT cell extract (1.0 mg protein) were added to complete GDP reductase assay mixtures and incubated at 30^{o} for the times indicated. The amount of GdR formed was determined as described previously.

FIGURE 16b: Effect of the presence of $FeCl_3$ on GDP reductase activity in WT cell extracts. Standard aliquots of WT cell extract (1.0 mg protein) were added to complete GDP reductase assay mixtures to which various amounts of $FeCl_3$ had been added. The reaction was then allowed to proceed under standard conditions.


A
B
GdR nmoles
0.3
0.2
0.1
20
40
60
TIME (min.)
0.2
0.4
0.6
0.8
1.0
Fe Cl3 mM

of iron which stimulated CDP reductase activity several fold (figure 8b).

The optimim pH for GDP reductase in HEPES buffer was found to be 7.0 (figure 17a). This was identical to the value for CDP reduction in WT extracts (figure 10b). GDP reductase activity fell off rapidly at pH values above or below 7.0. Activity at pH 6.6 and 7.4 was approximately one-third less than the activity observed at pH 7.0.

Figure 17b indicates that dTTP greatly stimulated GDP reductase activity. In the absence of added dTTP, enzyme activity was only 4% of the activity found at the optimal concentration of 2 mM dTTP. This agrees with other ribonucleotide reductase systems where dTTP has been shown to be an important allosteric activator of GDP reductase (Moore and Hurlbert, 1966; Brown and Reichard, 1969b, Lewis and Wright, 1978a).

Figure 18 shows the dependence of enzyme activity on the addition of DTT. Maximum activity was achieved with approximately 6 mM DTT, which is the same amount needed for optimal CDP reductase activity. However, unlike CDP reductase, there was almost 25% residual activity in the absence of added DTT.

The initial rate of reaction of GDP reductase activity was determined over a range of substrate concentrations (figure 19). A Lineweaver-Burk double reciprocal plot was linear and gave an apparent Km value of 0.05 mM GDP for WT L cells. This value is similar to the Km's obtained with other mammalian systems (Moore and Hurlbert, 1966; Lewis and Wright, 1978a).

FIGURE 17a: Effect of pH on WT GDP reductase activity. Aliquots of partially purified WT extracts (corresponding to 1.0 mg protein) were assayed for GDP reduction in reaction mixtures containing 0.05 M HEPES buffer at various pH's.

FIGURE 17b: Response of WT GDP reductase activity to the presence of dTTP. The standard aliquot of 1.0 mg of protein from a partially purified WT extract was added to GDP reductase assay mixtures containing various concentrations of dTTP. The reaction was then allowed to proceed under standard assay conditions.

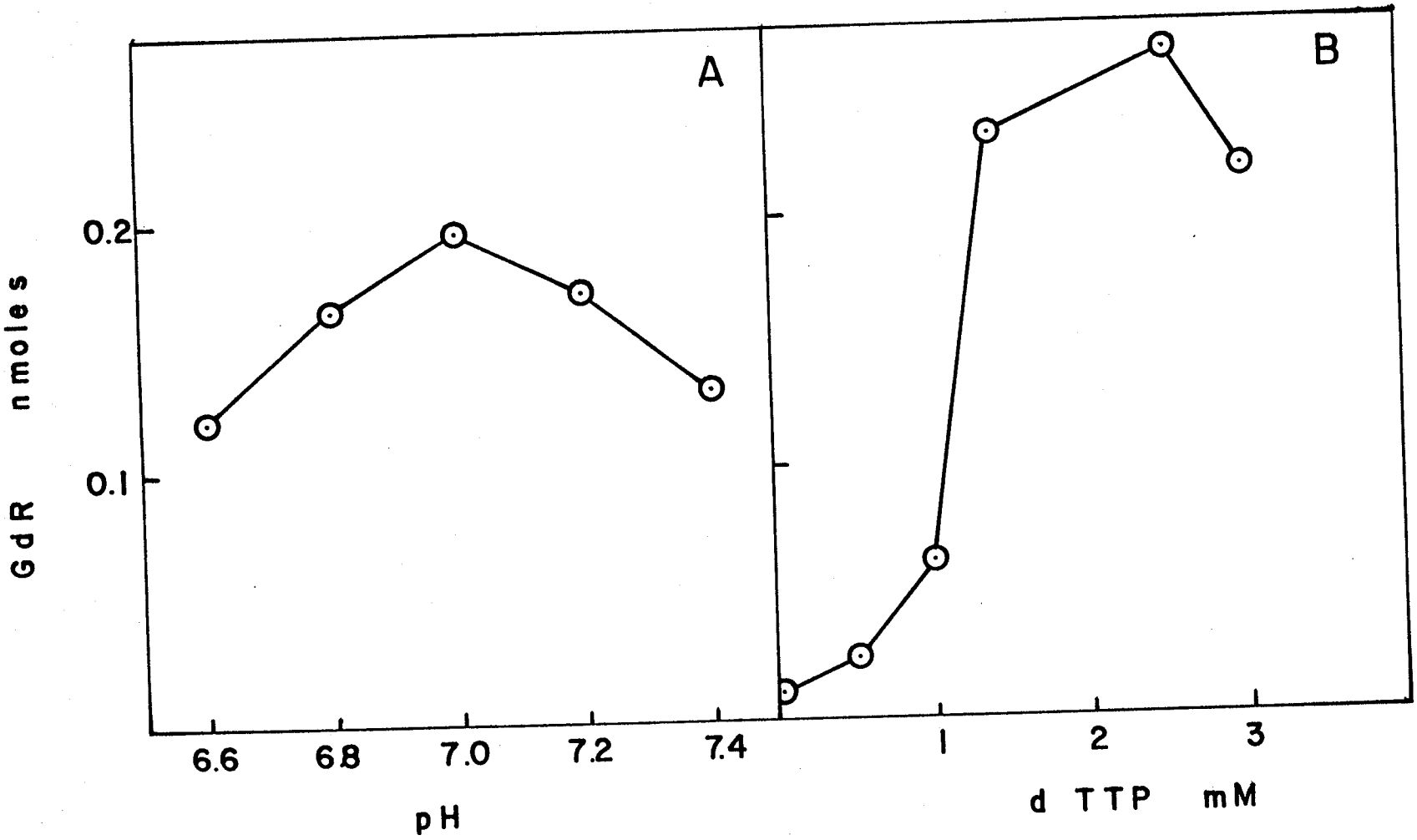

A
B
0.2
0.1
GdR nmoles
6.6
6.8
7.0
7.2
7.4
pH
1
2
3
d TTP mM

FIGURE 18: Response of GDP reductase activity in partially purified WT cell extracts to the presence of reducing agent. Standard GDP reductase assay conditions were used, except for the presence of varied concentrations of DTT.

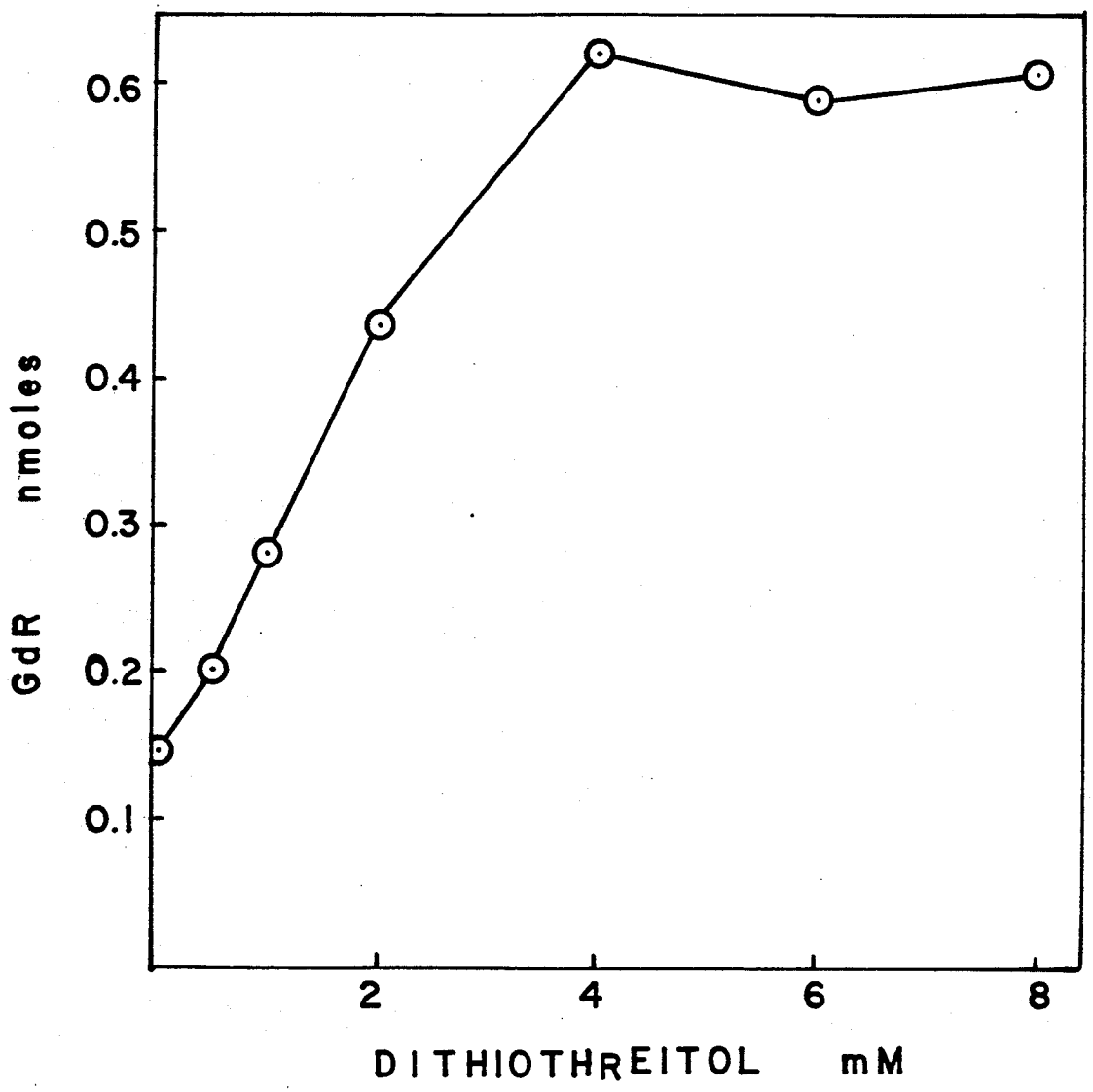

0.6
0.5
0.4
0.3
0.2
0.1
GdR nmoles
2
4
6
8
DITHIOTHREITOL mM

FIGURE 19: Double reciprocal plot of the variation in rate of GDP reduction with concentration of substrate. A standard aliquot (1.0 mg protein) of partially purified WT extract was incubated under standard GDP reductase assay conditions, except that the concentration of GDP was varied.

Insert: Response of initial velocity of GDP reduction to various concentrations of GDP.

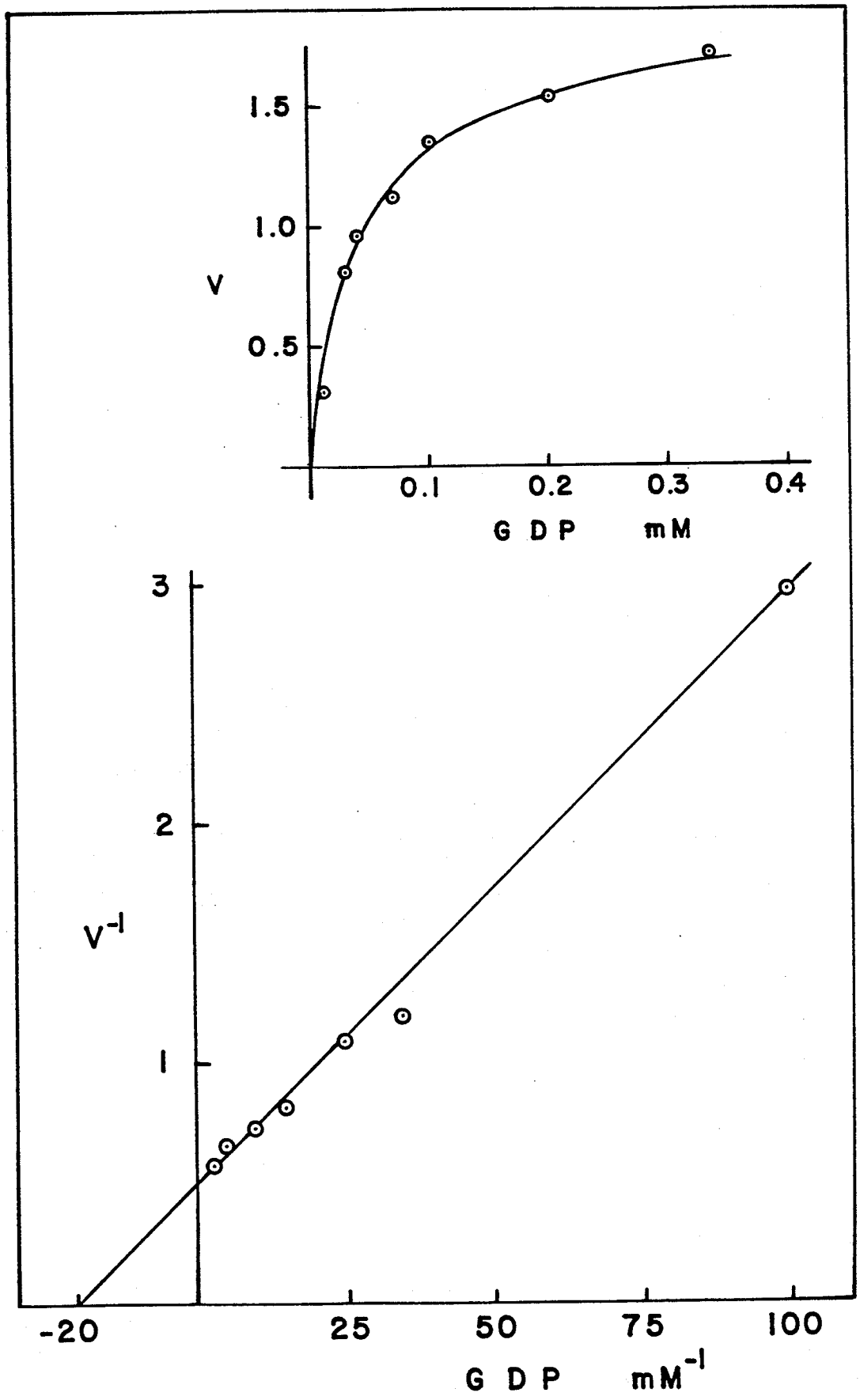

1.5
1.0
V
0.5
0.1
0.2
0.3
0.4
G D P mM
3
2
V-1
1
-20
25
50
75
100
G D P mM-1

Figure 20 shows the effect of different concentrations of hydroxyurea on GDP reductase activity over a range of substrate concentrations. As was the case with CDP reductase, the extract was equilibrated with drug for 30 minutes at room temperature before the enzyme activity was determined. Unlike the inhibition by hydroxyurea of CDP reduction which resembled an uncompetitive pattern at 0.15 mM drug (figure 12), GDP reduction was inhibited noncompetitively by all concentrations of hydroxyurea tested (figure 20). The insert of figure 20 provided a Ki value of 0.15 mM hydroxyurea for GDP reduction, which is twice the value obtained from CHO cells (Lewis and Wright, 1978a).

As expected, the response of GDP reduction to the presence of guanazole resembled the pattern found with hydroxyurea. Figure 21 shows that guanazole inhibited enzyme activity noncompetitively with a Ki value of 0.50 mM guanazole for GDP reduction. As was found with CDP reductase, the Ki value for guanazole was much higher than the Ki value for hydroxyurea. This correlated well, however, with the cellular response to these two drugs. Figures 2 and 3 show that the Ki values for GDP reductase of 0.15 mM hydroxyurea and 0.50 mM guanazole both reduced the RPE of WT cells to approximately 0.5.

b) Characterization of ribonucleotide reductase from hydroxyurea resistant L cells: Ribonucleotide reductase was studied in extracts of the drug resistant L_2Cl_3 line. Partially purified extracts were prepared as described in Materials and Methods. Both CDP and GDP reductions were characterized in an attempt to determine if the

FIGURE 20: Double reciprocal plot of the effect of hydroxyurea on the rate of GDP reduction in the presence of various substrate concentrations. A standard aliquot (1.0 mg protein) of partially purified WT extract was incubated under routine GDP assay conditions with various concentrations of GDP and in the presence of 0 mM (⊙), 0.15 mM (▽), 0.35 mM (▫), and 0.70 mM hydroxyurea.(△)

Insert: Replot of the velocity intercepts versus concentration of hydroxyurea.

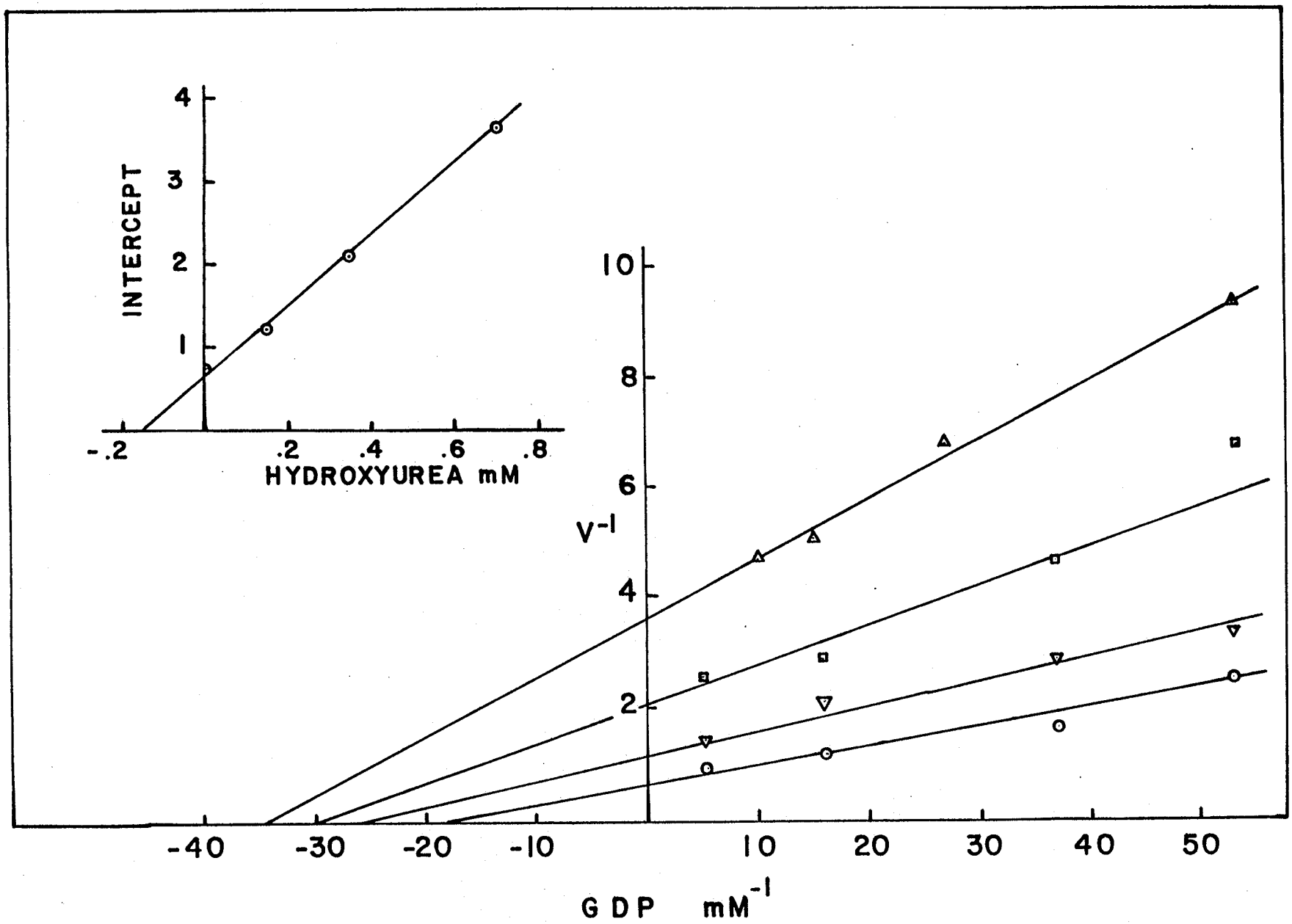
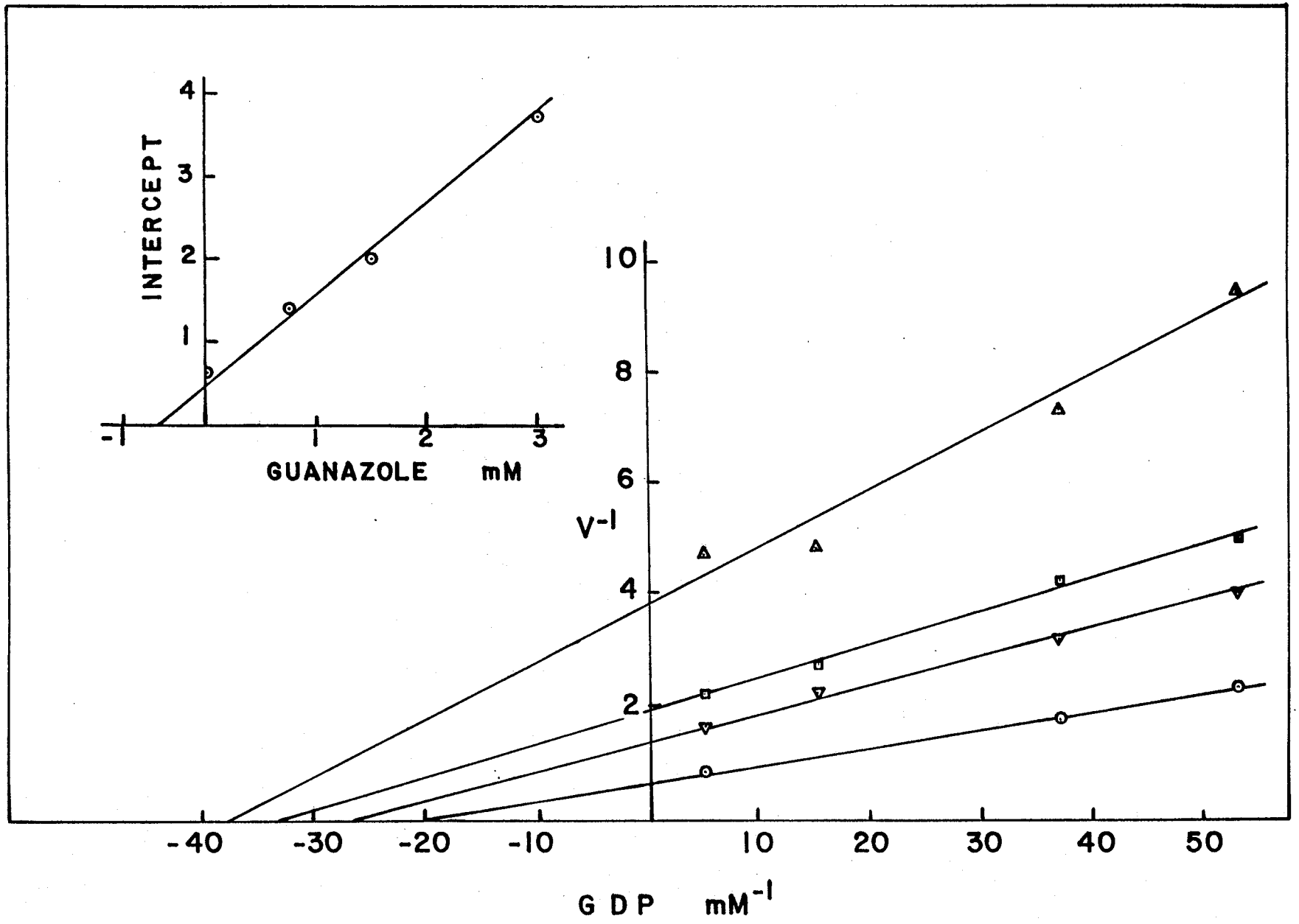

INTERCEPT
4
3
2
1
-.2
.2
.4
.6
.8
HYDROXYUREA mM
10
8
6
4
2
V-1
-40
-30
-20
-10
10
20
30
40
50
GDP mM-1

FIGURE 21: Double reciprocal plot of the effect of guanazole on the rate of GDP reduction in the presence of various substrate concentrations. A standard aliquot (1.0 mg protein) of partially purified WT extract was incubated under routine GDP assay conditions with various concentrations of GDP and in the presence of 0 mM (O), 0.75 mM (▽), 1.5 mM (□), and 3.0 mM (△) guanazole.

Insert: Replot of the velocity intercepts versus concentration of guanazole.


INTERCEPT
4
3
2
1
-1
1
2
3
GUANAZOLE mM
10
8
6
4
2
V-1
-40
-30
-20
-10
10
20
30
40
50
G D P mM-1

enzyme in L_2Cl_3 cells had significantly different properties from the WT enzyme. The specific activity of ribonucleotide reductase in L_2Cl_3 extracts was routinely 3 to 4 times higher than in WT extracts, and usually contained 7to 8 units of CDP reductase activity per mg of protein.

(i) CDP reduction: CDP reductase activity was measured using the same techniques employed in the study of the WT enzyme, and as described in Materials and Methods. Figure 22a shows that the L_2Cl_3 enzyme, similar to the WT enzyme, had an optimum assay temperature of 37^{o}; which corresponds to the standard growth temperature for this cell line. Also like the WT enzyme, activity did not respond linearly to changing protein concentration (figure 22b). Enzyme activity fell off rapidly when less than approximately 400 µg of protein were included in the standard assay. Therefore, all future work was carried out in the linear range of protein concentrations (figure 22b)

Under optimal assay conditions, enzyme activity was approximately linear with time for at least the standard incubation time of 40 minutes (figure 23a).

Figure 23b shows that CDP reductase activity in L_2Cl_3 extracts was stimulated by the presence of magnesium ion. This corresponds to the finding in WT extracts (figure 10a), as well as other mammalian cell systems (eg. Larsson, 1969; Elford, 1974). Similar to the study of the enzyme in WT extracts, a concentration of 5 mM $MgCl_2$ was chosen for use in all subsequent work with the enzyme in extracts of L_2Cl_3 cells.

FIGURE 22a: Effect of assay temperature on CDP reductase activity in L_2Cl_3 extracts. Standard assay mixtures were incubated for 40 minutes at the temperatures indicated, and the amount of CDP reduced was determined as described previously.

FIGURE 22b: Effect of increasing protein content on L_2Cl_3 CDP reduction. Various amounts of L_2Cl_3 cell extract (corresponding to from 0 to 1.0 mg protein) were added to a standard CDP reductase assay. After incubation at 37^{o} for 40 minutes, the amount of CDP reduced was determined.

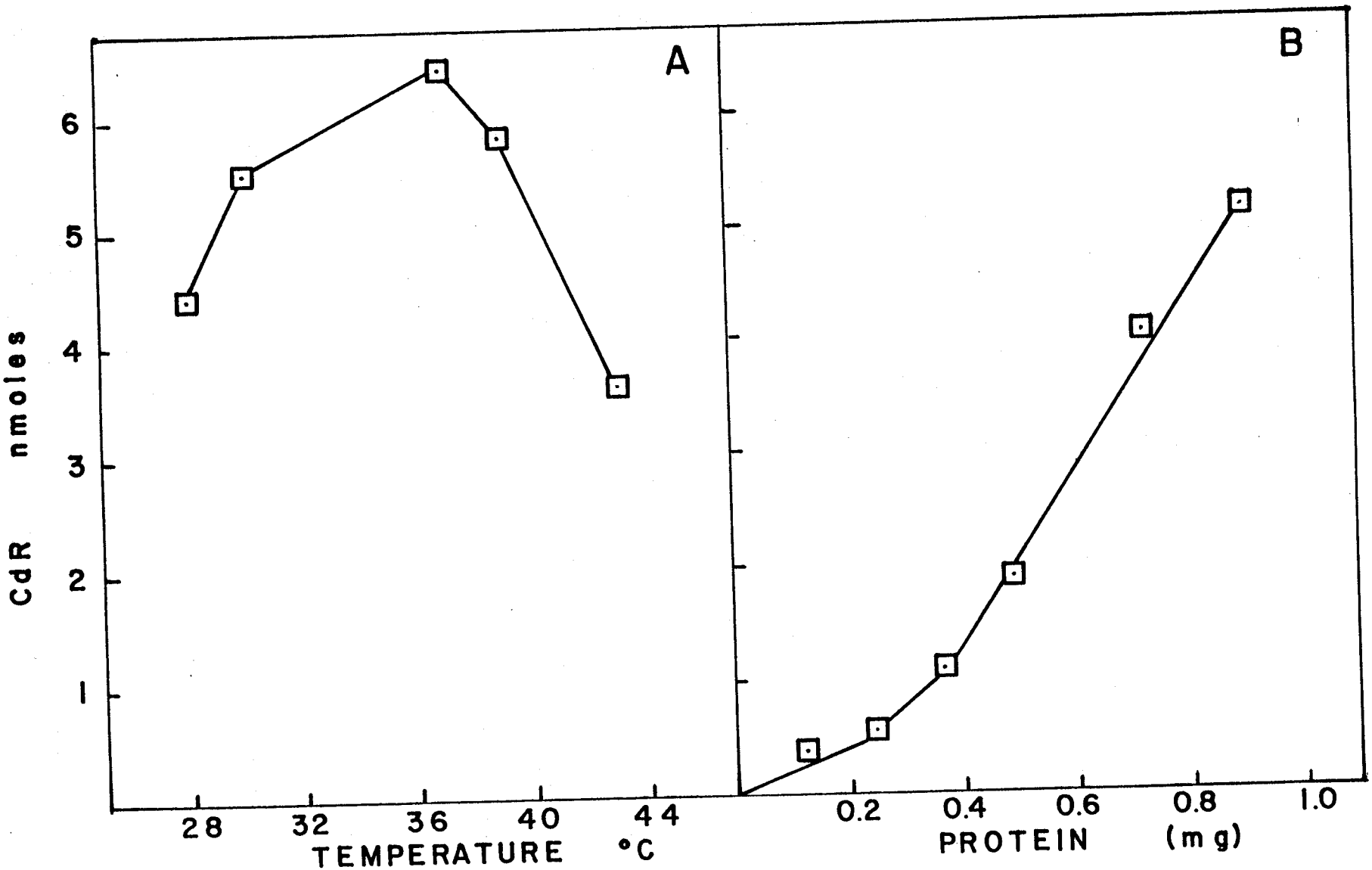
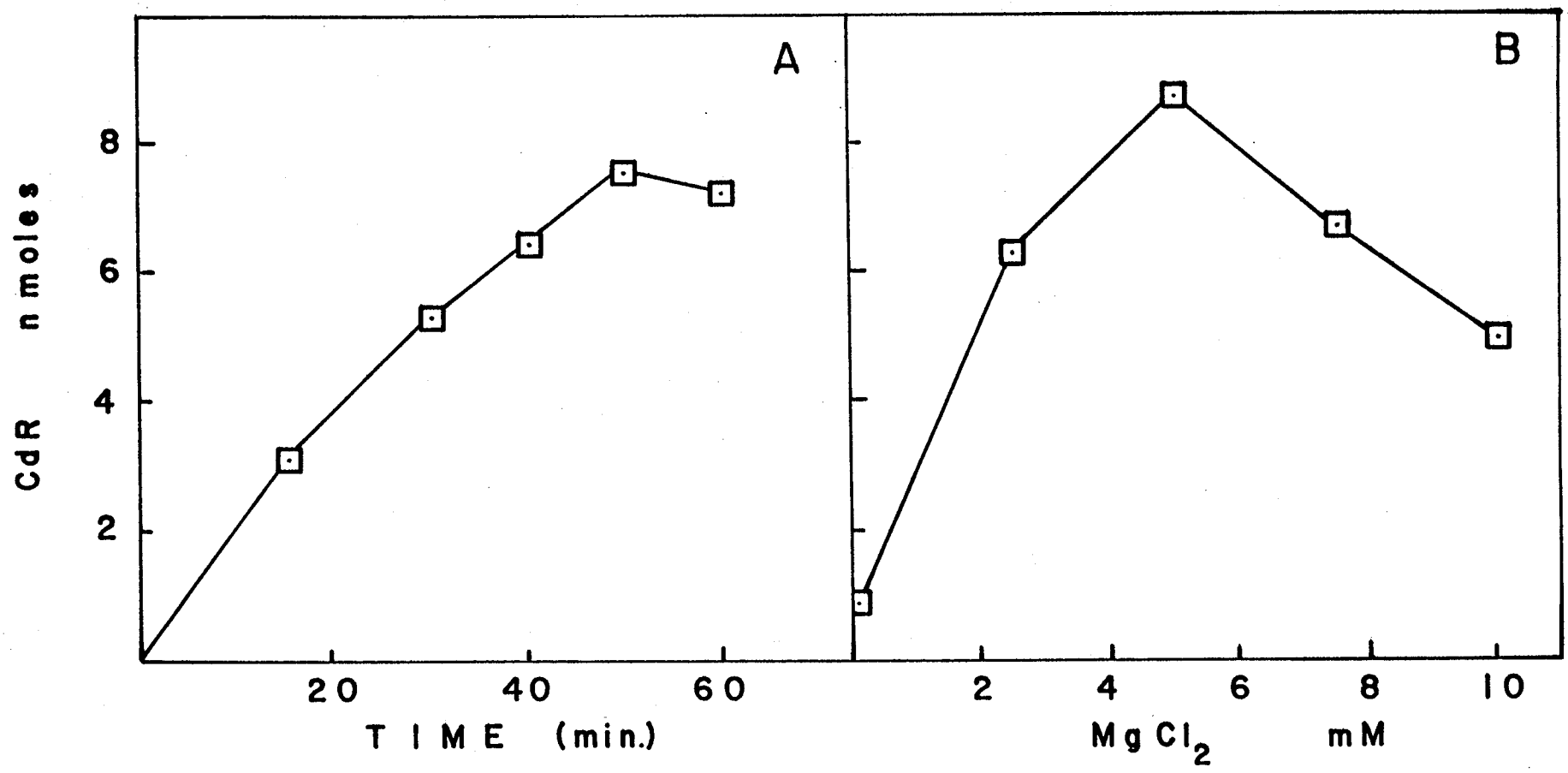

A
B
CdR nmoles
1
2
3
4
5
6
28
32
36
40
44
TEMPERATURE °C
0.2
0.4
0.6
0.8
1.0
PROTEIN (mg)

FIGURE 23a: Response of CDP reduction assay in extracts of L_2Cl_3 cells to time. Standard aliquots of L_2Cl_3 cell extract (corresponding to 1.0 mg protein) were added to complete reaction mixtures and incubated at 37^{o}. At the times indicated, the reaction was terminated by boiling, and the amount of CDP reduced was determined as described in Materials and Methods.

FIGURE 23b: Effect of magnesium ion on CDP reduction. CDP reductase activity was determined in partially purified L_2Cl_3 extracts in the presence of varied concentrations of $MgCl_2$. Except for this variable, standard assay conditions were used.


A
B
CdR nmoles
8
6
4
2
20
40
60
TIME (min.)
2
4
6
8
10
MgCl2 mM

The response of CDP reductase activity to pH in the absence of $FeCl_3$ is shown in figure 24a. The enzyme from L_2Cl_3 cells showed a pH optimim of 7.0, which was identical to the WT enzyme (figure 10b). The L_2Cl_3 enzyme, however, showed more residual activity at pH's above 7.0. A pH of 7.2 was chosen for future work to minimize problems encountered when $FeCl_3$ was added to the assay at pH 7.0. A similar decision had been made for studying the WT enzyme (see page 71).

CDP reductase activity was greatly stimulated by the addition of the reducing agent DTT (figure 24b). The response of enzyme activity in L_2Cl_3 cell extracts to DTT was very similar to the response seen in WT extracts (figure 9b). Both the WT and L_2Cl_3 enzyme showed little activity in the absence of added DTT; and maximim activity occurred in the presence of approximately 6 mM DTT, whereas the addition of more DTT did not appreciably increase activity. Therefore, a concentration of 6 mM DTT was used when studying both the mutant and the WT enzyme.

Figure 25a shows that ATP is a strong activator of CDP reductase in L_2Cl_3 cells. Optimim activity occurred at 6 mM ATP, which is very close to the ATP concentration used for the WT enzyme (figure 9a) as well as in other mammalian systems (eg. Peterson and Moore, 1967; Moore, 1967). The presence of more than 6 mM ATP inhibited L_2Cl_3 enzyme activity in a manner similar to what was observed in WT extracts (figure 9a).

The effect of iron on enzyme activity in L_2Cl_3 extracts is seen in figure 25b. As was observed in WT extracts (figure 8b),

FIGURE 24a: Effect of pH on L_2Cl_3 CDP reductase activity in the absence of $FeCl_3$. Aliquots of partially purified L_2Cl_3 extracts (corresponding to 1.0 mg protein) were assayed for CDP reduction in reaction mixtures lacking $FeCl_3$ and containing 0.05 M HEPES buffer at various pH's.

FIGURE 24b: Response of CDP reductase activity in L_2Cl_3 cell extracts to the presence of a reducing agent. Standard CDP reductase assay conditions were used except for the presence of varied concentrations of DTT.

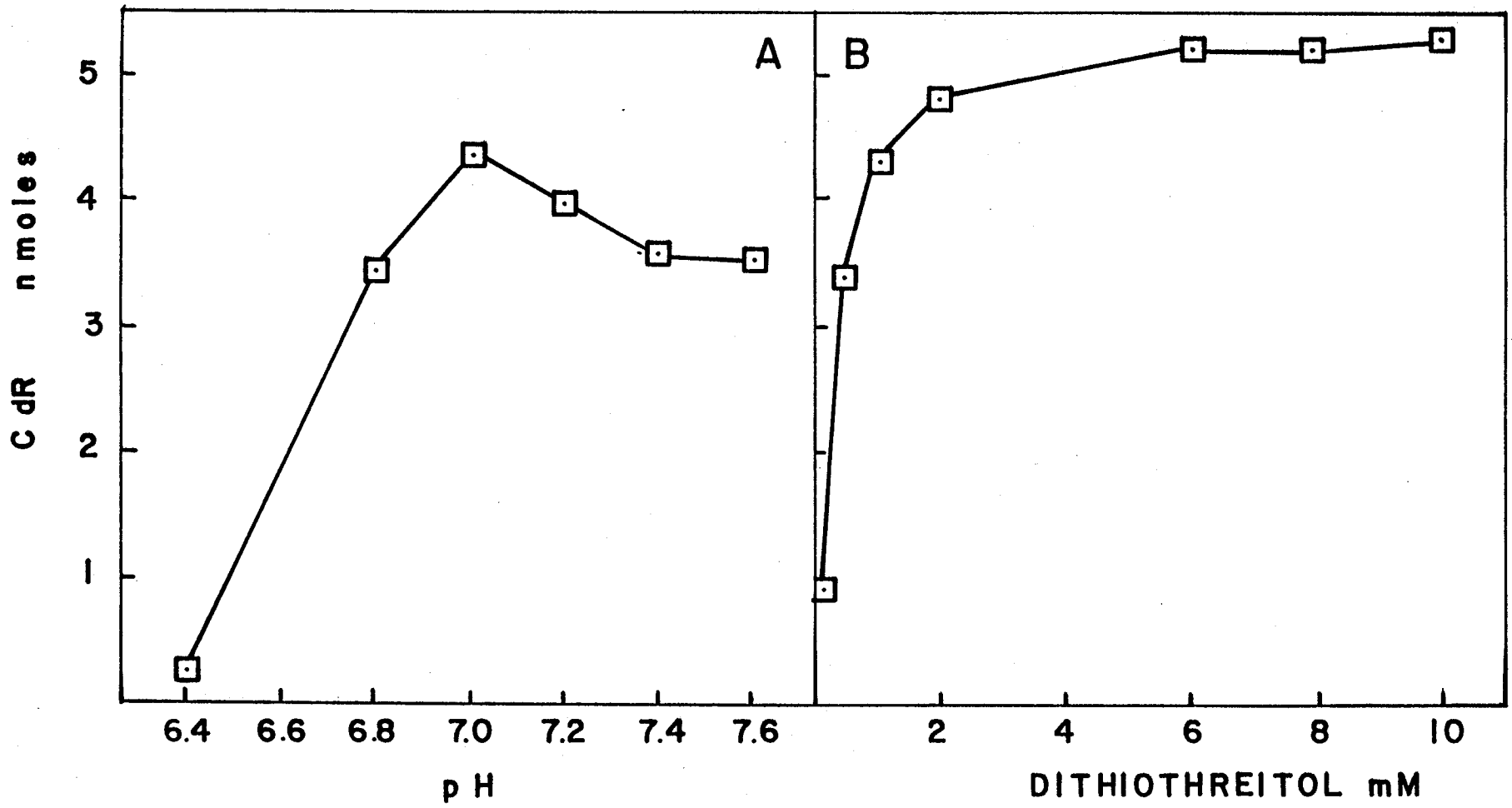
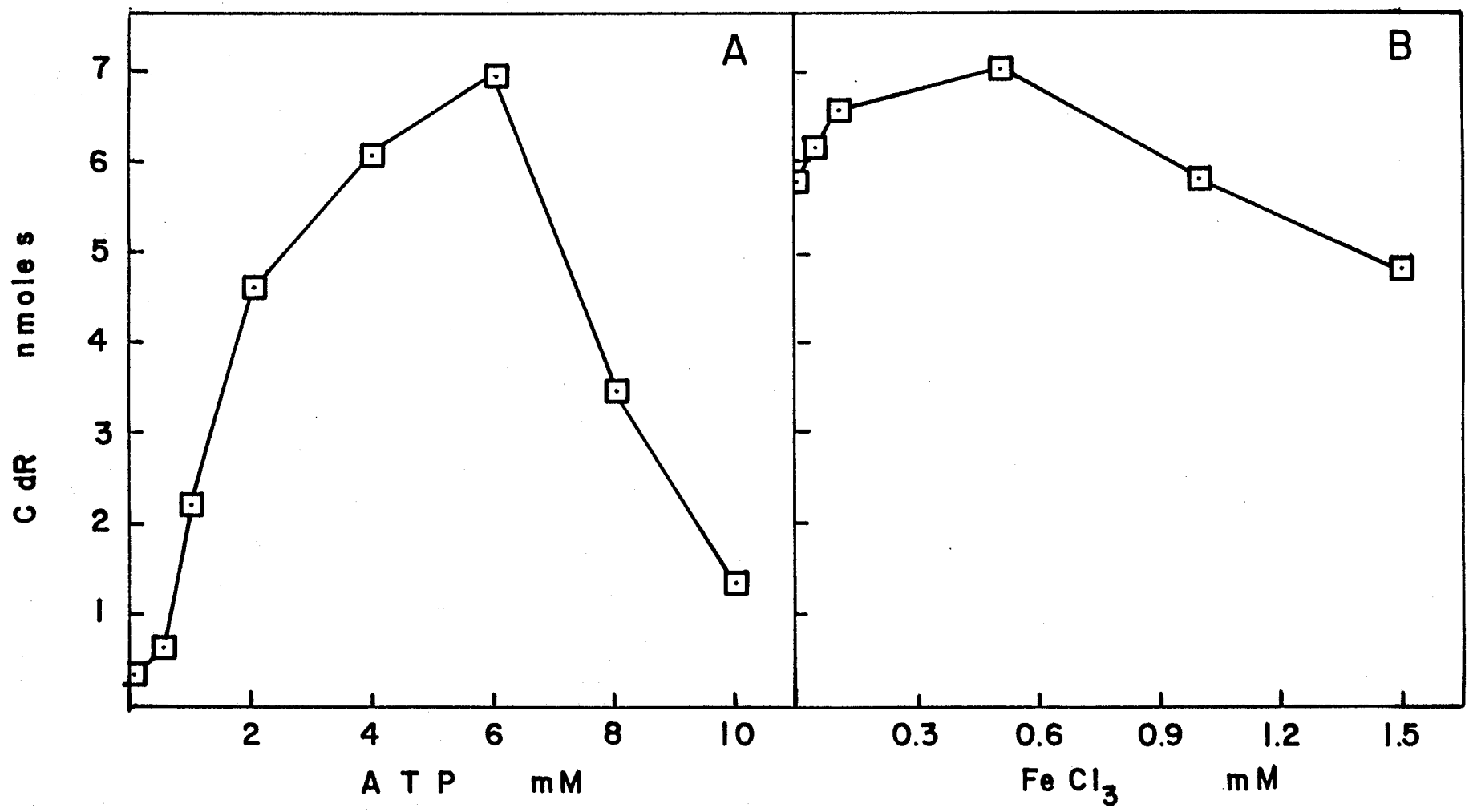

A
B
C dR nmoles
1
2
3
4
5
6.4
6.6
6.8
7.0
7.2
7.4
7.6
pH
2
4
6
8
10
DITHIOTHREITOL mM

FIGURE 25a: Effect of ATP on CDP reductase activity in L_2Cl_3 extracts. The standard aliquot of 1.0 mg of protein from a partially purified L_2Cl_3 extract was added to CDP reductase assay mixtures containing various amounts of ATP. The reaction was allowed to proceed under standard conditions.

FIGURE 25b: Effect of addition of $FeCl_3$ to the assay mixture for the measurement of CDP reduction in extracts of L_2Cl_3 cells. A concentrated 5 µl aliquot of $FeCl_3$ in distilled water was added to each assay to achieve the final concentrations of $FeCl_3$ indicated. The reaction was then allowed to proceed under standard conditions.


A
B
C dR nmoles
7
6
5
4
3
2
1
2
4
6
8
10
A T P mM
0.3
0.6
0.9
1.2
1.5
Fe Cl3 mM

enzyme activity was stimulated by added $FeCl_3$. Two separate batches of L_2Cl_3 enzyme were used during this study, and both displayed maximum activity at 0.5 mM $FeCl_3$. This is within the range of $FeCl_3$ concentrations used when sutdying the WT enzyme.

The response of CDP reductase activity to different substrate concentrations is shown in figure 26. The Lineweaver-Burk plot was linear and yielded an apparent Km value of 0.41 mM CDP. This value is over 3 times greater than the WT value of 0.13 mM CDP (figure 11), and is approximately equal to the substrate concentration of 0.4 mM CDP which was used to study the characteristics of CDP reduction in L_2Cl_3 extracts (see Materials and Methods). Because of the use of radioactively labeled substrate in the enzyme assay, it was impractical to use CDP concentrations above 0.4 mM and still maintain a reasonable level of radioactivity. It is recognized that this concentration of CDP is non-saturating for the L_2Cl_3 enzyme (figure 26). The comparison of the WT and L_2Cl_3 enzymes is still valid however, as the presence of higher substrate concentrations would only serve to enhance the subtle differences noted between these two enzymes.

Hydroxyurea was also able to inhibit CDP reduction in L_2Cl_3 extracts. As was done with the WT enzyme, L_2Cl_3 enzyme preparations were equilibrated for 30 minutes at room temperature with different concentrations of hydroxyurea before activity was determined at various substrate concentrations. Figure 27 shows that concentrations as high as 0.9 mM hydroxyurea continued to exert an uncompetitive type of inhibition on CDP reductase activity in L_2Cl_3 extracts.

This is different from the WT enzyme (figures 12 and 13) which appeared to be inhibited uncompetitively only at the lowest concentration of hydroxyurea tested (0.15 mM drug), whereas 0.35 mM and 0.70 mM hydroxyurea seemed to exert more of a noncompetitive form of inhibition. This difference in hydroxyurea inhibition patterns between the WT and L_2Cl_3 enzyme is tentative however, and requires further experimentation to solidify its importance. Nevertheless, the inset of figure 27 shows a Ki for L_2Cl_3 CDP reductase of 0.31 mM hydroxyurea, a value which is different from the WT Ki value of 0.24 mM hydroxyurea (figures 12 and 13). Similar kinetics could also be obtained in other independent experiments with enzyme activity from L_2Cl_3 extracts. One such experiment yielded an L_2Cl_3 Ki value of 0.39 mM hydroxyurea. Thus the enzyme from L_2Cl_3 cells exhibited an <u>in vitro</u> Ki for hydroxyurea which was approximately 1.5 times greater than the WT Ki of 0.24 mM.

Similar to hydroxyurea, guanazole appeared to inhibit CDP reduction in L_2Cl_3 extracts in an uncompetitive fashion at all drug concentrations tested (figure 28). This is different from the inhibition by guanazole of WT CDP reductase activity, which appeared to show an uncompetitive pattern only at 0.75 mM guanazole, whereas inhibition by 1.5 mM and 3.0 mM drug was noncompetitive. Once again, however, this difference in patterns of guanazole inhibition of WT and L_2Cl_3 CDP reduction requires further work for confirmation. The inset of figure 28 yielded an L_2Cl_3 Ki value of 1.5 mM guanazole, which is the same as the value obtained

FIGURE 26: Double reciprocal plot of the variation in rate of CDP reduction with CDP concentration. A standard aliquot (1.0 mg protein) of partially purified L_2Cl_3 extract was incubated under standard CDP reductase assay conditions, except that the concentration of CDP was varied.

Insert: Response of initial velocity of CDP reduction to various concentrations of CDP.

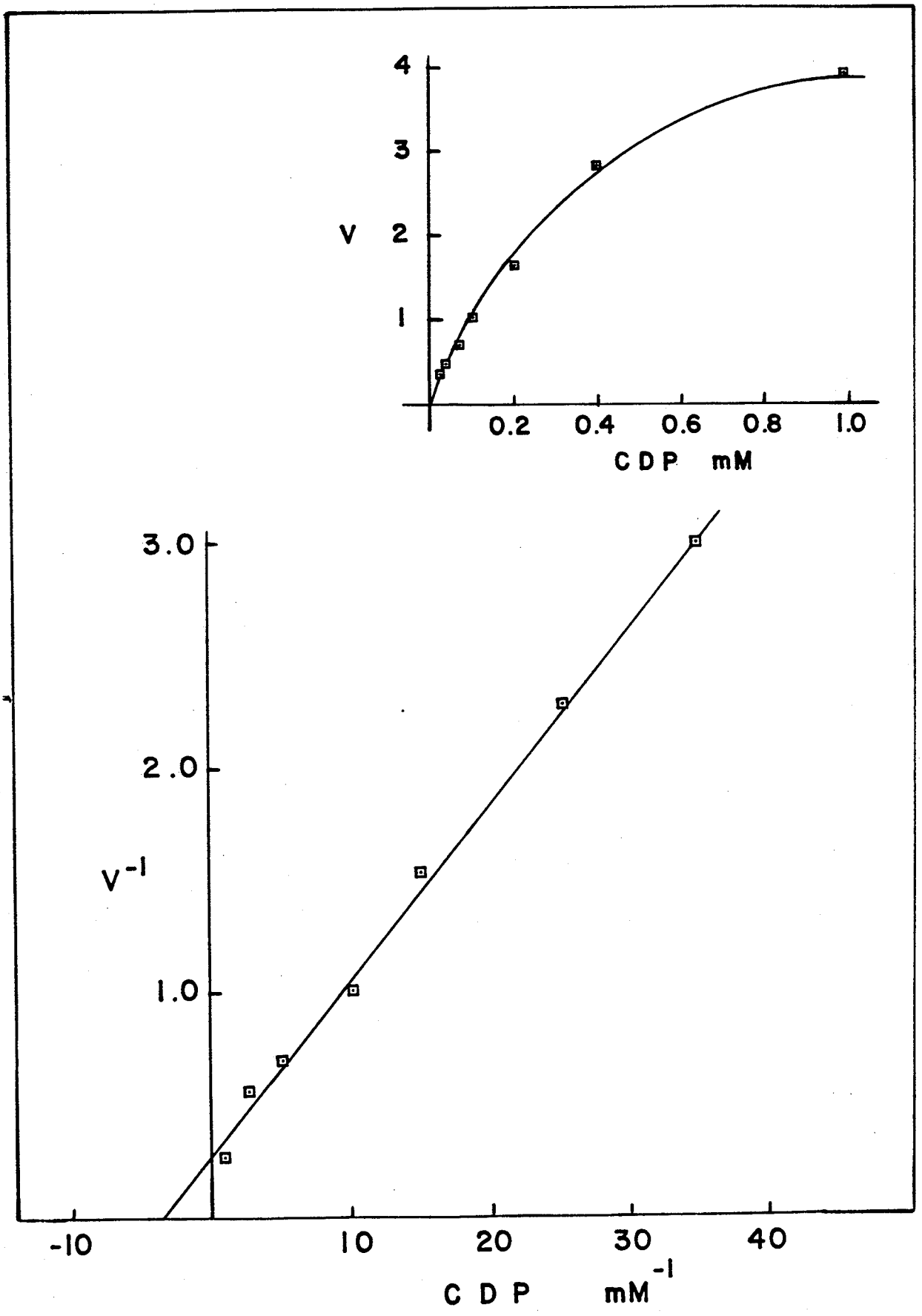

4
3
V 2
1
0.2
0.4
0.6
0.8
1.0
CDP mM
3.0
2.0
V-1
1.0
-10
10
20
30
40
CDP mM-1

FIGURE 27: Double reciprocal plot of the effect of hydroxyurea on the rate of CDP reduction in the presence of various substrate concentrations and in the presence of 0.5 mM $FeCl_3$. A standard aliquot (1.0 mg protein) of partially purified L_2Cl_3 extract was incubated under routine CDP assay conditions with various concentrations of CDP and in the presence of 0 mM (⊙), 0.3 mM (▽), 0.6 mM (▫), and 0.9 mM (△) hydroxyurea.

Insert: Replot of the velocity intercept versus concentration of hydroxyurea.

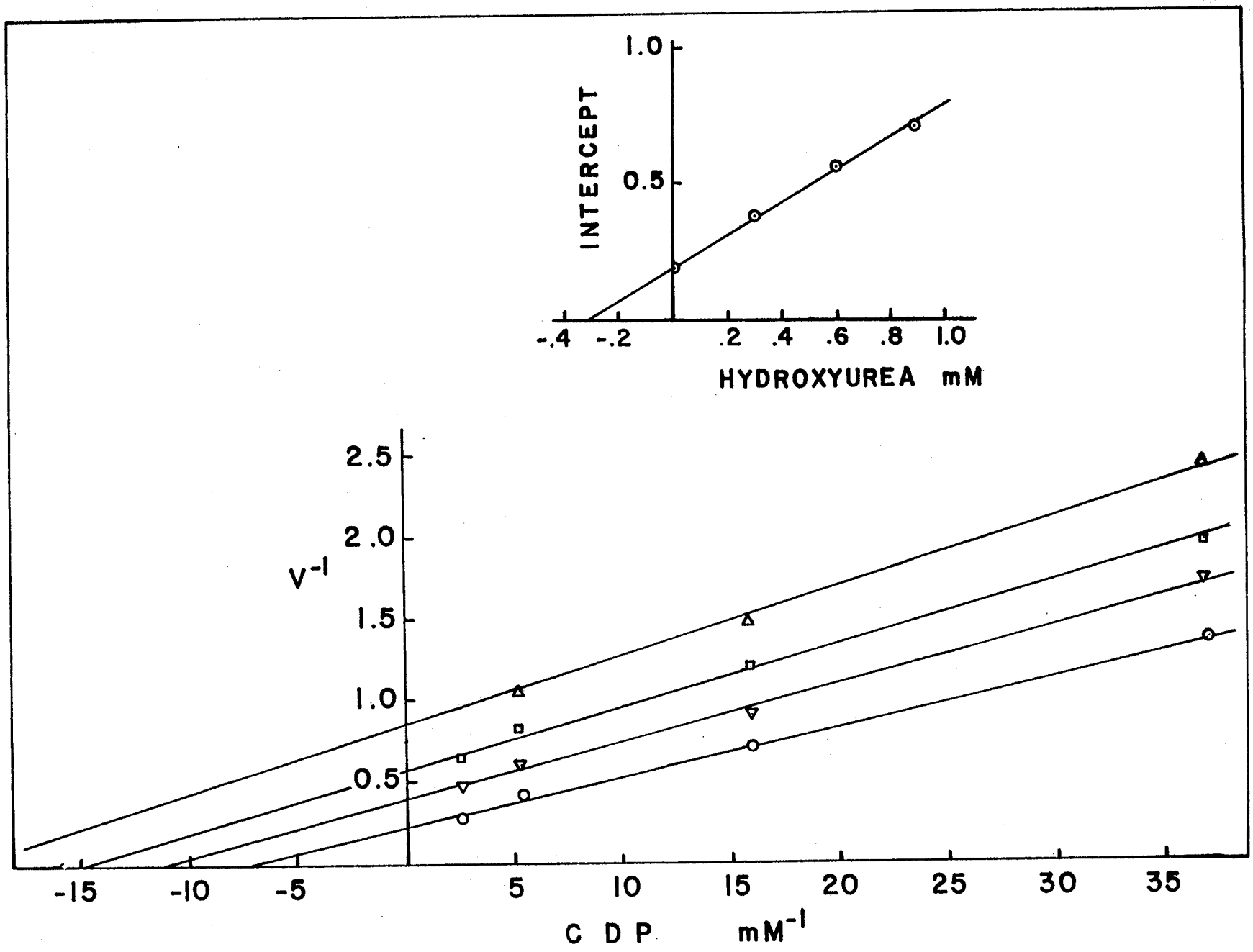
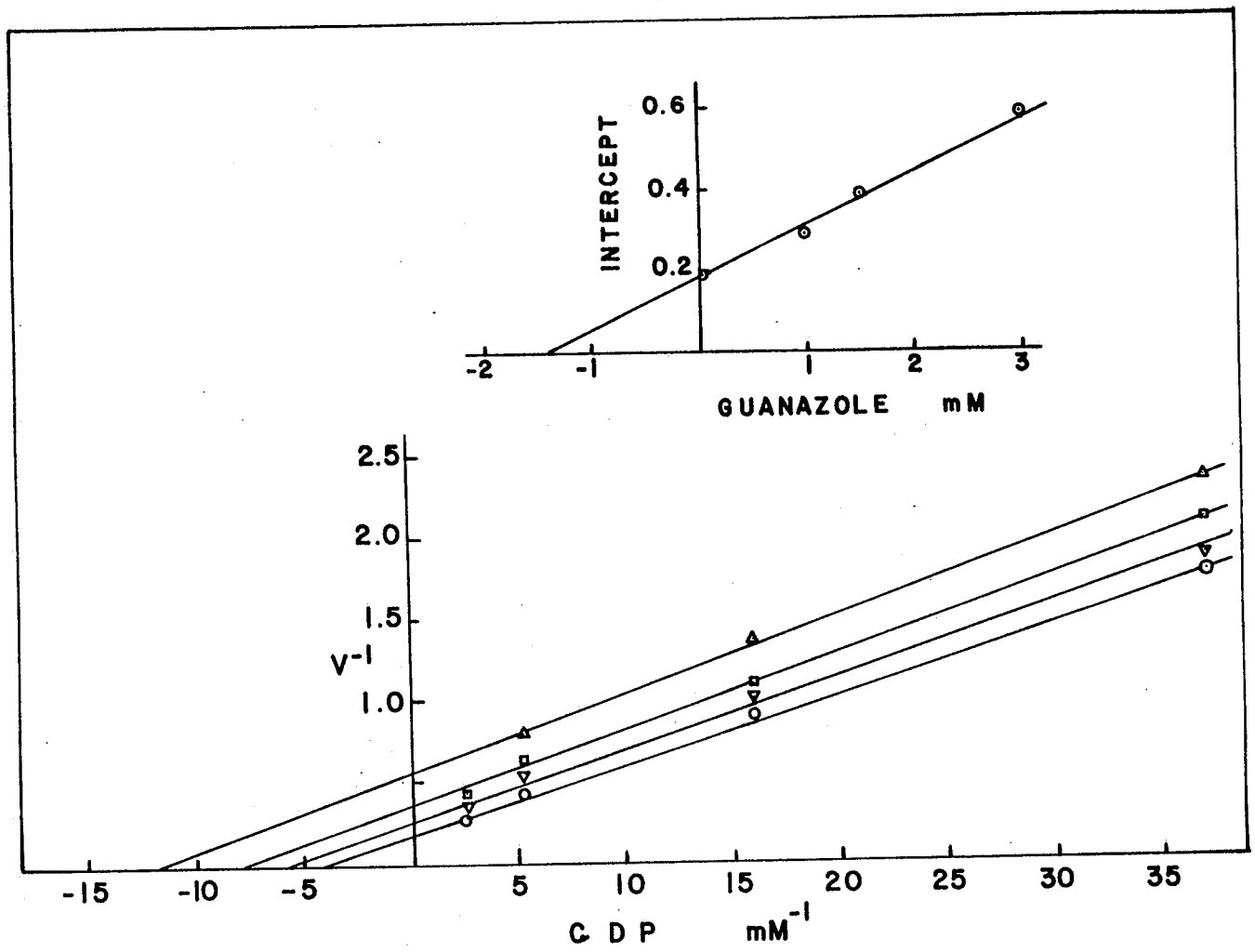

1.0
0.5
INTERCEPT
-.4
-.2
.2
.4
.6
.8
1.0
HYDROXYUREA mM
2.5
2.0
1.5
1.0
0.5
V-1
-15
-10
-5
5
10
15
20
25
30
35
C D P mM-1

FIGURE 28: Double reciprocal plot of the effect of guanazole on the rate of CDP reduction in the presence of various substrate concentrations. A standard aliquot (1.0 mg protein) of partially purified L_2Cl_3 extract was incubated under routine CDP assay conditions with various concentrations of CDP and in the presence of 0 mM (⊙), 0.75 mM (▽), 1.5 mM (◘), and 3.0 mM (△) guanazole.

Insert: Replot of the velocity intercept versus concentration of guanazole.


INTERCEPT
0.6
0.4
0.2
-2
-1
1
2
3
GUANAZOLE mM
2.5
2.0
1.5
V-1
1.0
-15
-10
-5
5
10
15
20
25
30
35
C D P mM-1

from WT extracts (figure 14).

(ii) GDP reduction: GDP reductase activity was determined in the same manner as was described for the WT enzyme. Figure 29 shows that enzyme activity responded to temperature and iron content in a similar manner to the WT enzyme. Maximum enzyme activity occurred at 30° (figure 29a), even though the cells were routinely cultured at 37°. The addition of $FeCl_3$ inhibited GDP reductase activity in extracts of L_2Cl_3 cells, with activity decreased 50% at a concentration of 0.5 mM $FeCl_3$ (figure 29b). This was very similar to the effects of $FeCl_3$ on GDP reduction in WT extracts (figure 16b).

Under optimal assay conditions, the reaction proceeded linearly with time for at least 60 minutes (figure 30a), which was chosen as the standard incubation time. Activity was also dependent on the addition of DTT (figure 30b). Similar to the WT enzyme (figure 18), activity in L_2Cl_3 extracts did not increase in the presence of concentrations greater than 6 mM DTT. Therefore, this concentration was selected for all subsequent work

GDP reductase activity in L_2Cl_3 extracts appeared to respond in a linear fashion to increased protein concentrations from 0.2 mg to 1.0 mg protein per assay, but was not linear at very low protein concentrations (below approximately 0.1 mg protein per assay) (figure 31). GDP reductase activity from WT cells was also nonlinear at low protein concentrations (figure 15b).

FIGURE 29a: Effect of assay temperature on GDP reduction in L_2Cl_3 extracts. Standard assay mixtures were incubated for 40 minutes at the temperatures indicated, and the amount of GDP reduced was determined as described in Materials and Methods.

FIGURE 29b: Effect of the presence of $FeCl_3$ on GDP reductase activity in L_2Cl_3 extracts. Standard aliquots of L_2Cl_3 cell extract (1.0 mg protein) were added to complete GDP reductase assay mixtures to which various amounts of $FeCl_3$ had been added. The reaction was then allowed to proceed under standard conditions.

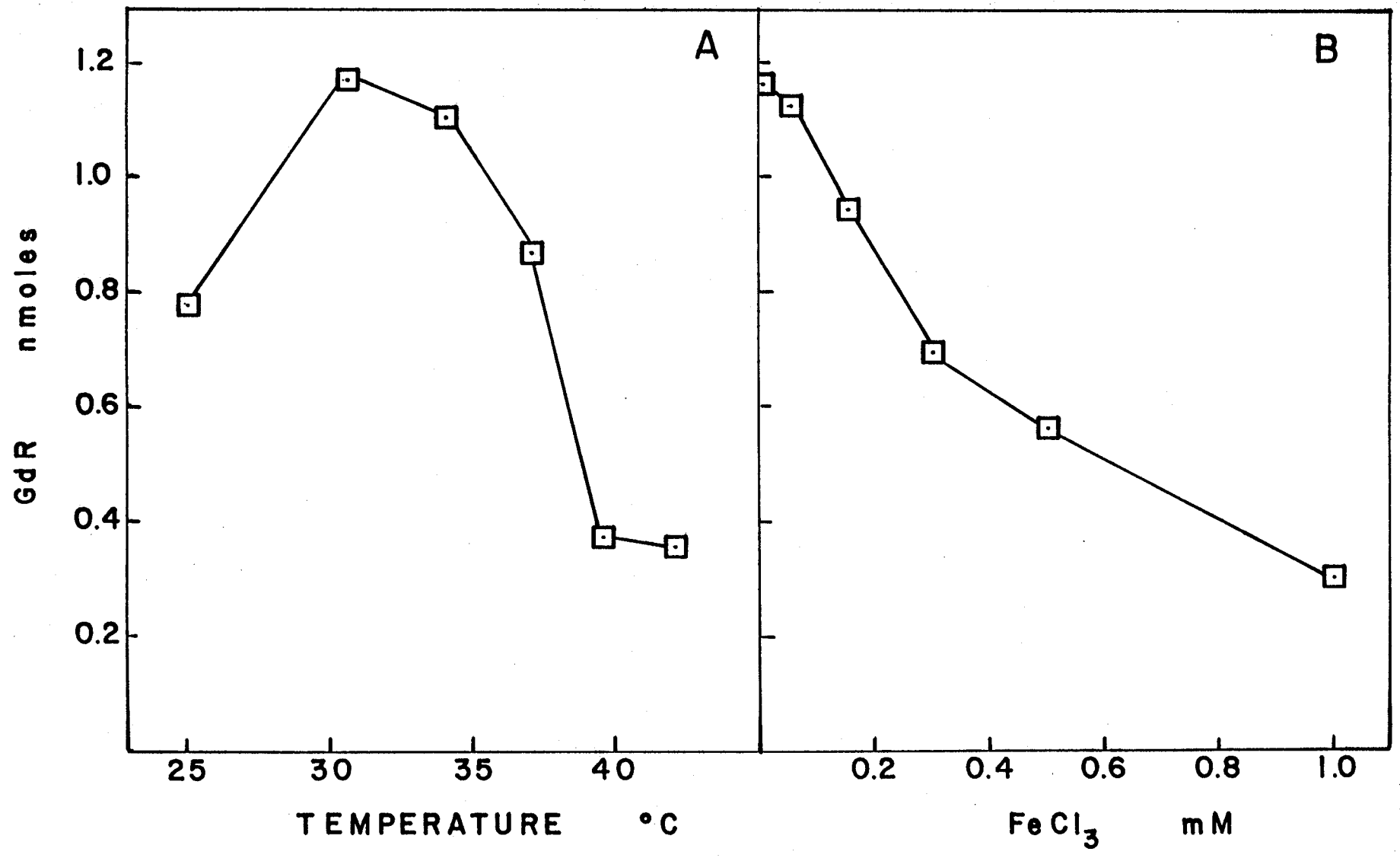

A
B
1.2
1.0
0.8
0.6
0.4
0.2
GdR nmoles
25
30
35
40
TEMPERATURE °C
0.2
0.4
0.6
0.8
1.0
FeCl3 mM

FIGURE 30a: Response of GDP reduction assay in extracts of L_2Cl_3 cells to time. Standard aliquots of L_2Cl_3 cell extracts (1.0 mg protein) were added to complete GDP reductase assay mixtures and incubated at 30° for the times indicated. The amount of GdR formed was determined as described previously.

FIGURE 30b: Response of GDP reductase activity in partially purified L_2Cl_3 cell extracts to the presence of reducing agent. Standard GDP reductase assay conditions were used, except for the presence of varied concentrations of DTT.

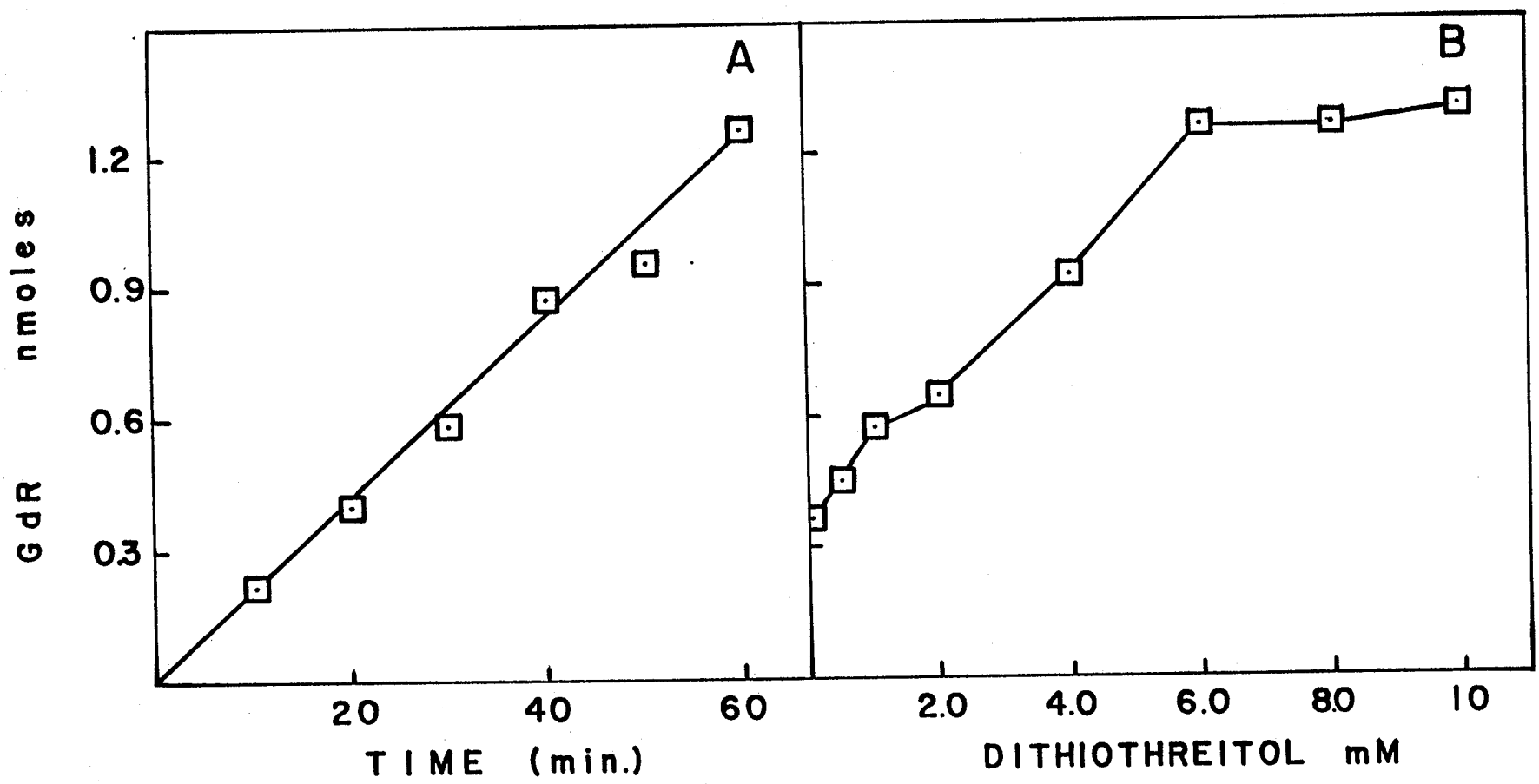

A
B
GdR nmoles
1.2
0.9
0.6
0.3
20
40
60
TIME (min.)
2.0
4.0
6.0
8.0
10
DITHIOTHREITOL mM

FIGURE 31: Effect of increasing protein content on GDP reduction in extracts of L_2Cl_3 cells. Various amounts of partially purified L_2Cl_3 cell extract (corresponding to from 0 to 1.0 mg protein) were added to a standard GDP reductase assay. After incubation at 30° for 40 minutes, the amount of GDP reduced was determined.

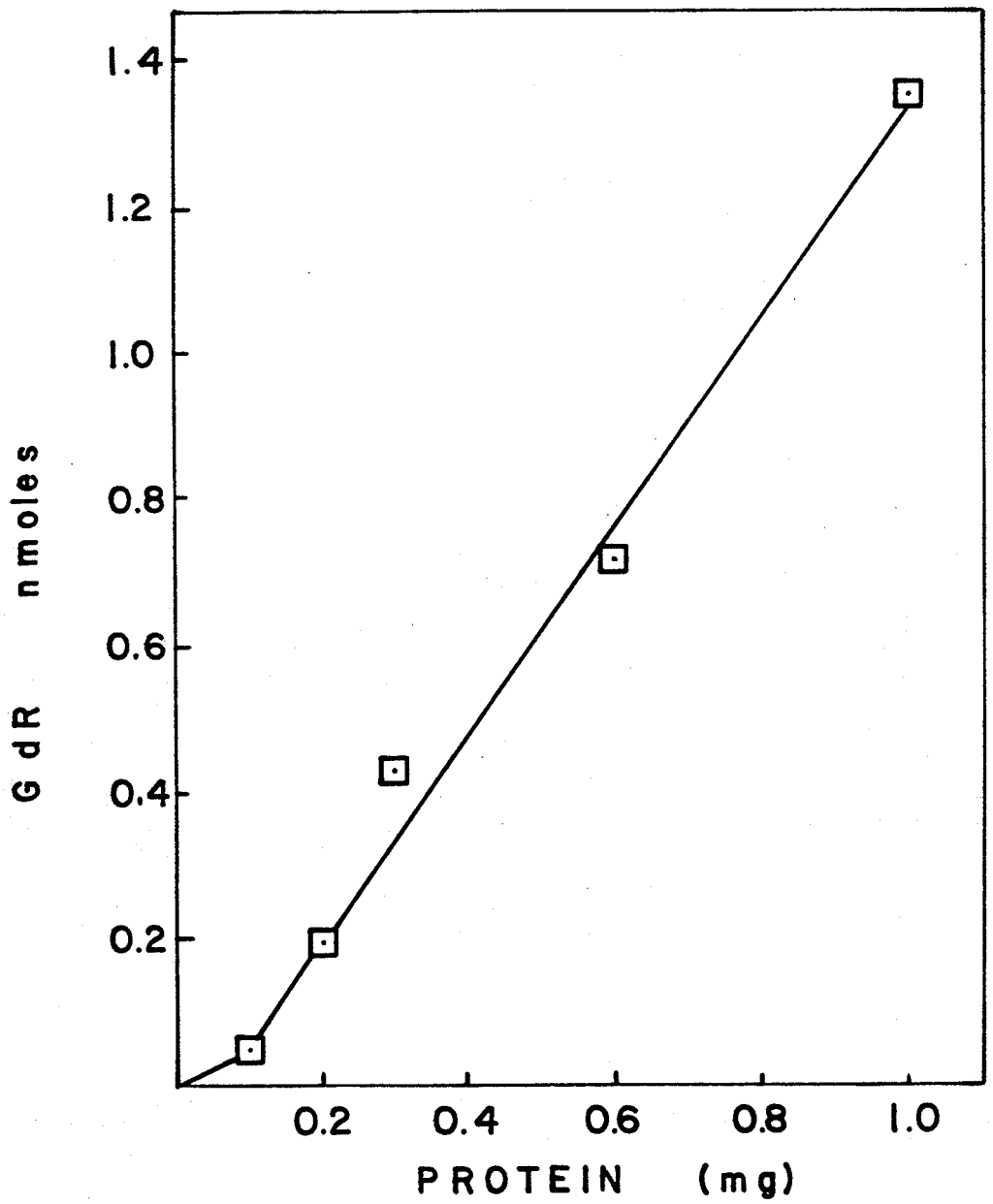

1.4
1.2
1.0
0.8
0.6
0.4
0.2
G dR nmoles
0.2
0.4
0.6
0.8
1.0
PROTEIN (mg)

Figure 32 shows the response of enzyme activity to pH and to the presence of various dTTP concdntrations; in each case the response was nearly identical to the results that were observed with the WT enzyme activity. Maximum GDP reductase activity in L_2Cl_3 extracts occurred at a pH of 7.0 (figure 32a) and in the presence of 2 mM dTTP (figure 32b).

The initial reaction rate under optimal conditions was determined over a range of substrate concentrations (figure 33). A linear Lineweaver-Burk double reciprocal replot of the data showed an apparent Km of 0.06 mM GDP. This is very close to the WT enzyme Km value of 0.05 mM GDP (figure 19).

GDP reductase activity partially purified from L_2Cl_3 cells was much more resistant in vitro to inhibition by hydroxyurea than was GDP reductase activity isolated from WT cells. The effect of hydroxyurea on GDP reduction in L_2Cl_3 extracts is shown in figure 34. Hydroxyurea showed a noncompetitive pattern of inhibition at all drug concentrations tested. This is similar to the inhibition pattern that hydroxyurea showed with GDP reductase activity from WT cells (figure 20). However, the insert of figure 34 yielded an L_2Cl_3 Ki value of 0.39 mM hydroxyurea, a value nearly 3 times greater than the WT Ki value of 0.15 mM hydroxyurea (figure 20).

The L_2Cl_3 enzyme also showed an increased resistance to in vitro inhibition by guanazole. Figure 35 shows that guanazole

FIGURE 32a: Effect of pH on L_2Cl_3 GDP reductase activity. Aliquots of partially purified L_2Cl_3 extracts (corresponding to 1.0 mg protein) were assayed for GDP reduction in reaction mixtures containing 0.05 M HEPES buffer at various pH's.

FIGURE 32b: Response of L_2Cl_3 GDP reductase activity to the presence of dTTP. The standard aliquot of 1.0 mg of protein from a partially purified L_2Cl_3 cell extract was added to GDP reductase assay mixtures containing various concentrations of dTTP. The reaction was then allowed to proceed under standard assay conditions.

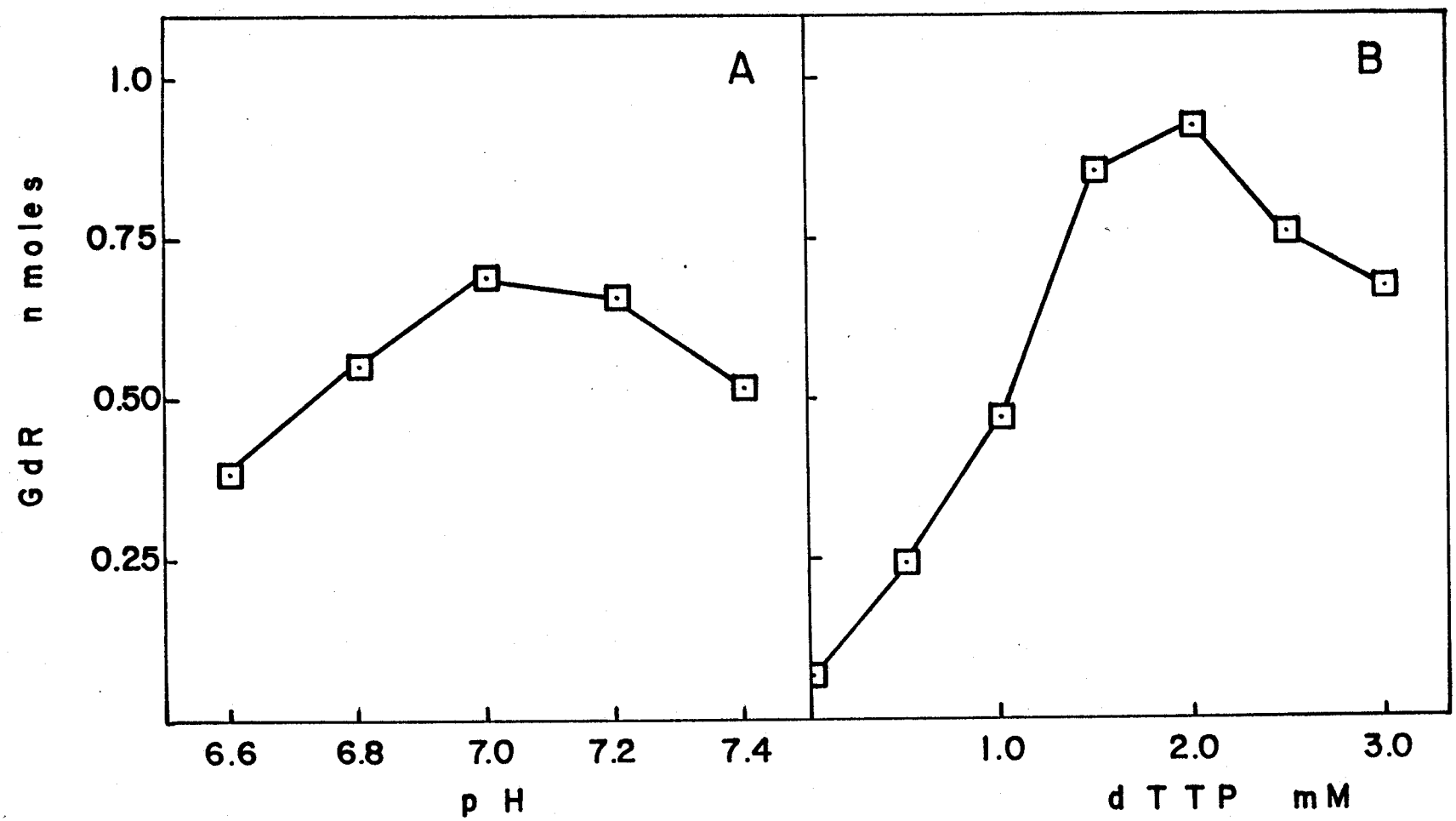

A
B
1.0
0.75
0.50
0.25
GdR n moles
6.6
6.8
7.0
7.2
7.4
p H
1.0
2.0
3.0
d T T P mM

FIGURE 33: Double reciprocal plot of the variation in rate of GDP reduction with concentration of substrate. A standard aliquot (1.0 mg protein) of partially purified L_2Cl_3 extract was incubated under standard GDP reductase assay conditions, except that the concentration of GDP was varied.

Insert: Response of initial velocity of GDP reduction to various concentrations of GDP.

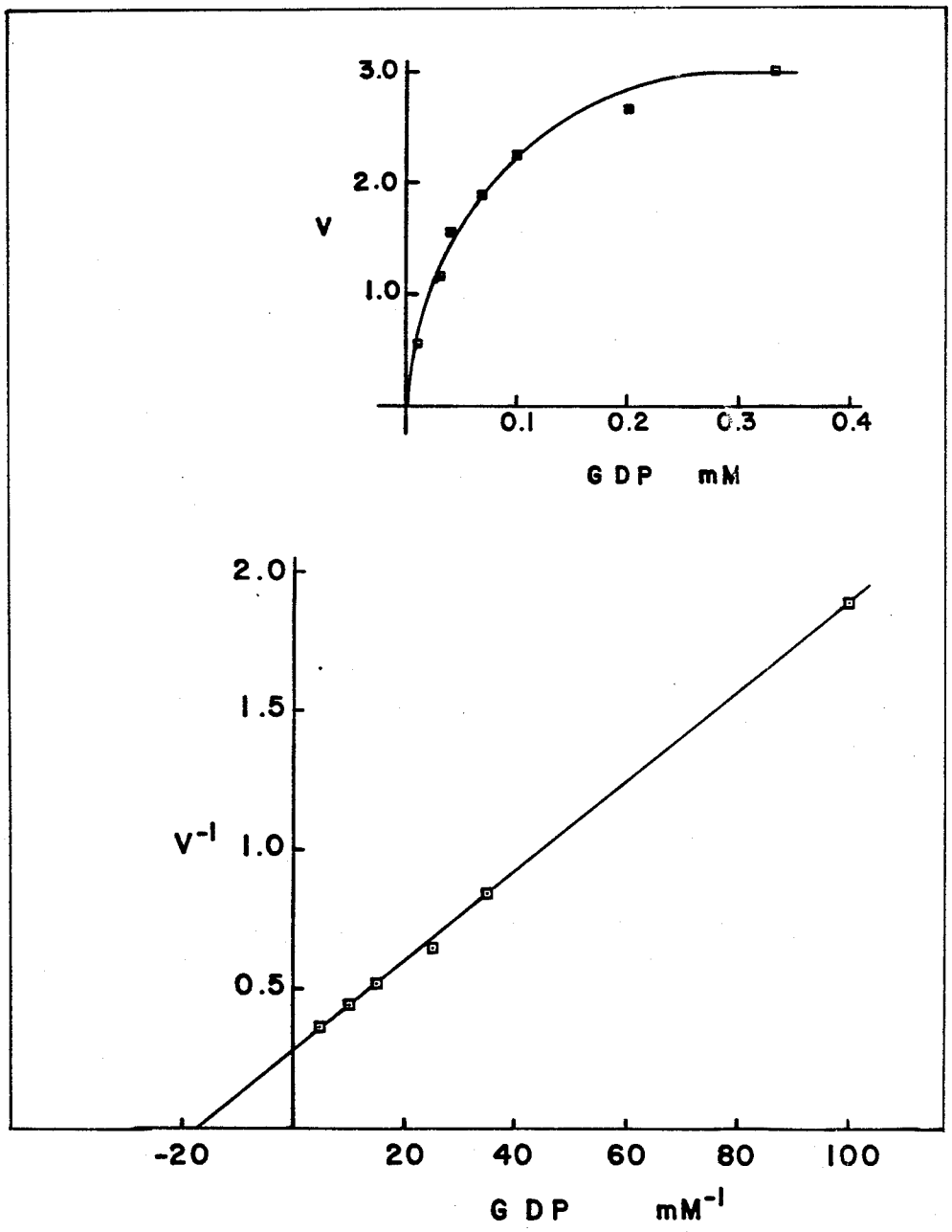
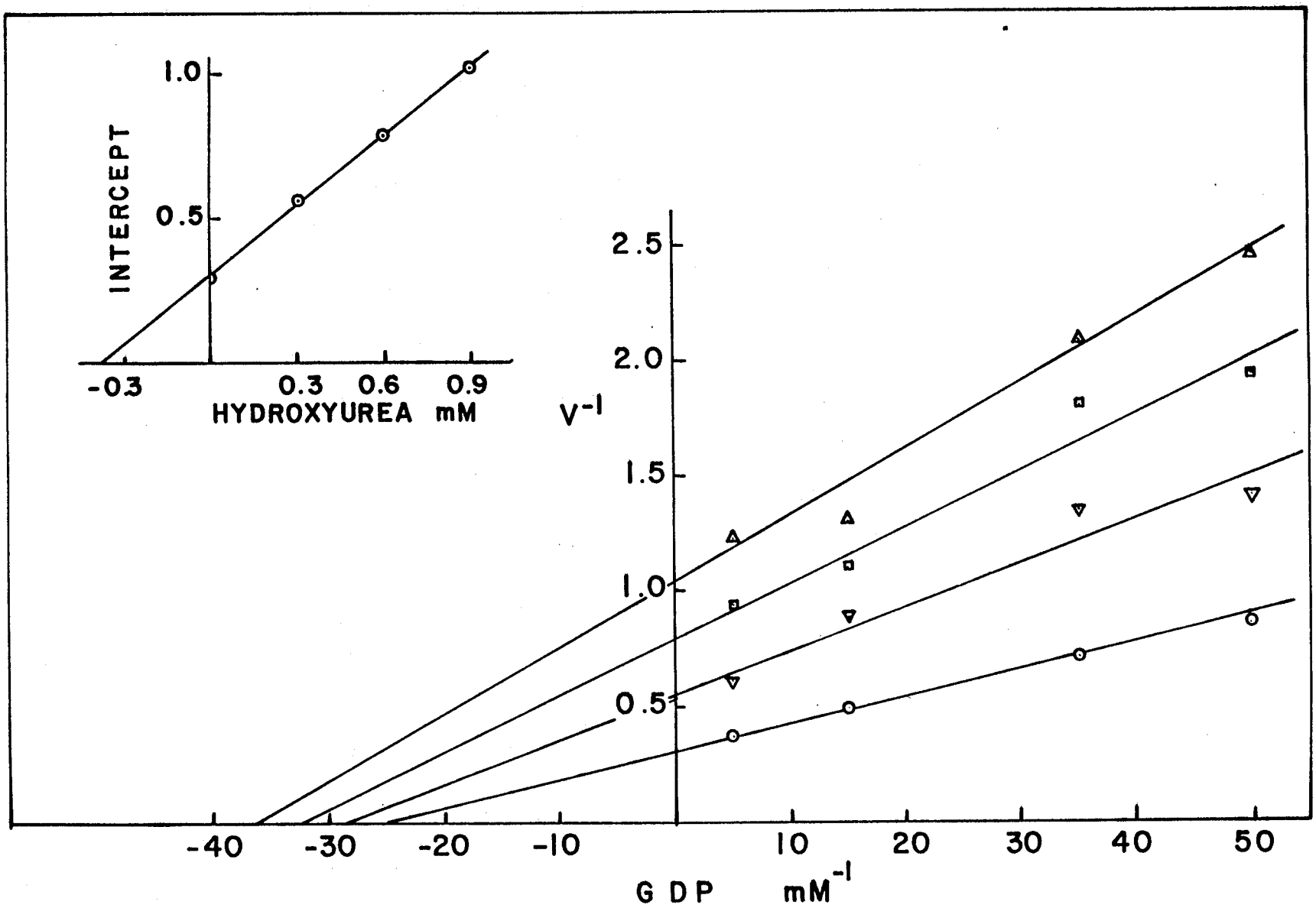

3.0
2.0
1.0
V
0.1
0.2
0.3
0.4
GDP mM
2.0
1.5
1.0
0.5
V-1
-20
20
40
60
80
100
GDP mM-1

FIGURE 34: Double reciprocal plot of the effect of hydroxyurea on the rate of GDP reduction in the presence of various substrate concentrations. A standard aliquot (1.0 mg protein) of partially purified L_2Cl_3 cell extract was incubated under routine GDP assay conditions with various concentrations of GDP and in the presence of 0 mM (⊙), 0.3 mM (▽), 0.6 mM (⊡), and 0.9 mM (△) hydroxyurea.

Insert: Replot of the velocity intercepts versus concentration of hydroxyurea.


1.0
0.5
INTERCEPT
-0.3
0.3
0.6
0.9
HYDROXYUREA mM
2.5
2.0
1.5
1.0
0.5
V-1
-40
-30
-20
-10
10
20
30
40
50
GDP mM-1

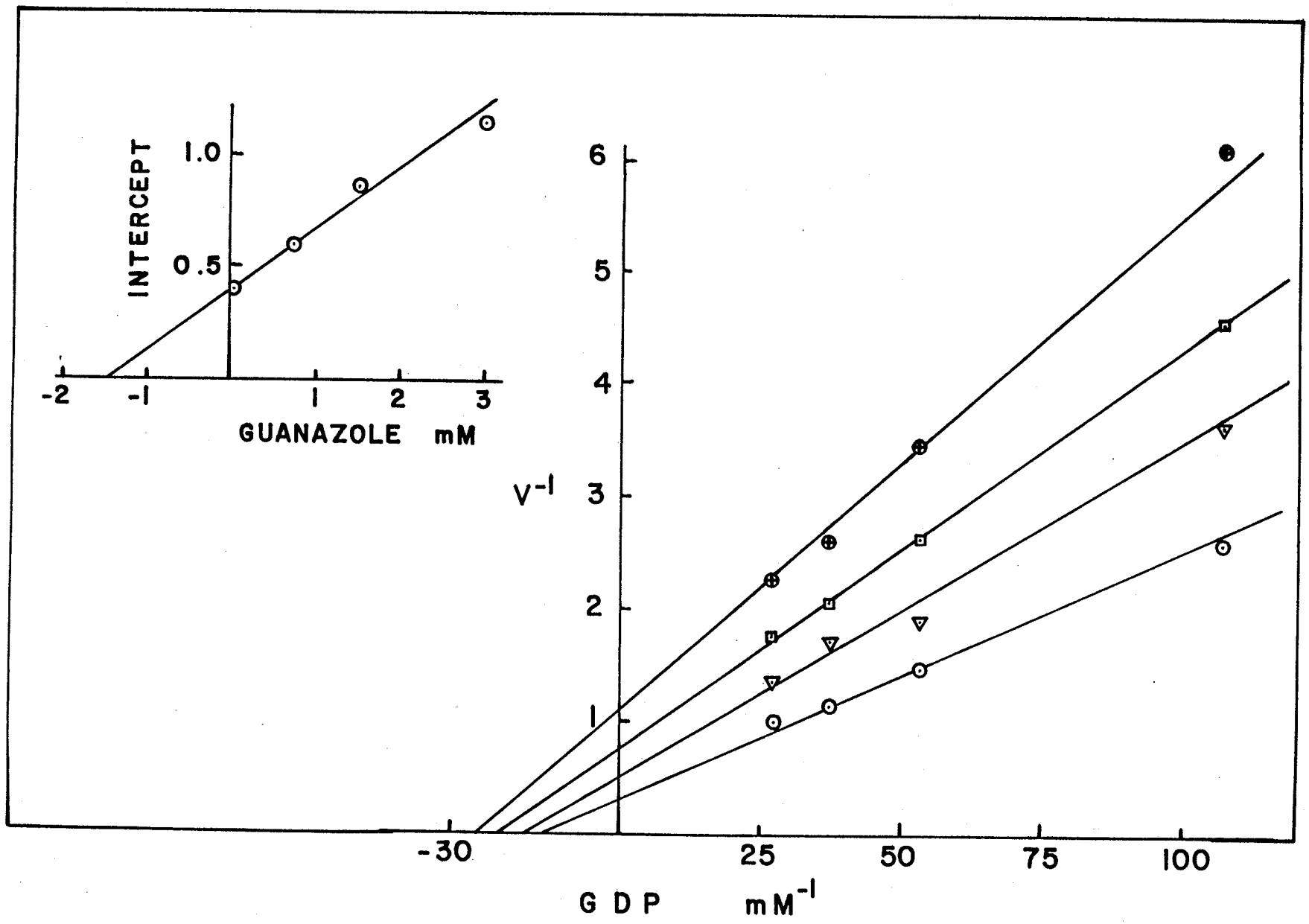
inhibited GDP reduction from L_2Cl_3 extracts in a noncompetitive manner and exhibited a Ki value of 1.55 mM guanazole (insert, figure 35). The concentrations of guanazole tested also showed a noncompetitive pattern of inhibition on GDP reduction from WT cells (figure 21). However, the L_2Cl_3 Ki value of 1.55 mM guanazole is over 3 times greater than the Ki value of 0.05 mM obtained from WT preparations of GDP reductase activity.

5. ASSAY OF RIBONUCLEOTIDE REDUCTION IN NUCLEOTIDE-PERMEABLE CELLS

There are many difficulties encountered when studying ribonulceotide reduction in cell-free extracts. Some of the major disadvantages include the nonlinear response of enzyme activity to protein content (e.g. figure 7b and 15b). An additional complication is the steady loss of activity sometimes encountered with extracts stored at -76°C for extended periods of time. It is also often difficult and laborious to prepare the large volumes of suspension grown cells required to prepare an extract of reasonable volume and protein content. Crude cell extracts also contain significant amounts of dATP and other naturally occurring inhibitors of ribonucleotide reduction (Cory and Monley, 1970; Lewis et al 1976). These compounds must first be removed by methods such as gel filtration (Tondeur-six et al, 1975) or Dowex ion exchange resin treatment (Peterson and Moore, 1976).

FIGURE 35: Double reciprocal plot of the effect of guanazole on the rate of GDP reduction in the presence of various substrate concentrations. A standard aliquot (1.0 mg protein) of partially purified L_2Cl_3 extract was incubated under routine GDP assay conditions with various concentrations of GDP and in the presence of 0 mM (⊙), 0.75 mM (▽), 1.5 mM (⧈), and 3.0 mM (△) guanazole.

Insert: Replot of the velocity intercepts versus concentration of guanazole.


INTERCEPT
1.0
0.5
-2
-1
1
2
3
GUANAZOLE mM
V-1
6
5
4
3
2
1
-30
25
50
75
100
G D P mM-1

Similar problems in studying the enzyme in E. coli extracts were overcome by developing an in vivo assay for ribonucleotide reductase in bacterial cells made permeable to nucleotides by treatment with toluene (Warner, 1973). The advantages of this method included a high enzyme activity per cell number, as well as a linear response between enzyme activity and protein concentration.

Some accomplishments have been made in altering the permeability of mammalian cells in culture (for review see Heppel and Makan, 1977). Recent progress in this field (Billen and Olson, 1976; Berger and Johnson, 1976) prompted the investigation of whether or not a system could be developed to assay ribonucleotide reduction in small numbers of nucleotide-permeable mammalian cells.

A novel in vivo assay for ribonucleotide reductase in nucleotide-permeable mammalian cells has been developed in this laboratory (Lewis, Kuzik and Wright, 1978). The original work was carried out using Chinese Hamster Ovary (CHO) cells, and has since been extended to work with mouse L cells.

a) Tween-80 permeabilization of CHO cells: Several methods are available to make mammalian cells permeable to nucleotides (Kaltenbach 1966; Sedi et al, 1975; Berger and Johnson, 1976; Billen and Olson, 1976; Castellot, Miller and Pardee, 1978). A slight variation of the Tween-80 method of Billen and Olson (1976) was chosen as it is rapid, simple, and only marginally reduces cell viability. Table 2 shows the effect of a 30 minute treatment with 1.0% Tween-80 on the ability of CHO cells to reduce CDP or GDP to their respective

TABLE 2

Effect of Tween-80 permeabilization on ribonucleotide reduction by hamster cells

Treatment	pmoles Cdr	pmoles GdR
None	<10	90
1.0% Tween-80 at $4^{o}C$	115	580
1.0% Tween-80 at $22^{0}C$	190	640

deoxynucleotides. With no treatment, very little GDP reductase and almost no CDP reductase activity could be detected. However, after Tween-80 treatment, the reduction of both CDP and GDP is dramatically increased. Presumably, the detergent alters the cell membrane and allows passage of the nucleotide substrates and effectors.

b) characterization of CDP reduction in permeabilized CHO cells: CDP reductase activity in whole CHO cells was dependent on the presence of a reducing agent (DTT), $MgCl_2$, and a nucleotide effector (ATP) (Table 3). Also, optimal activity occurred at a pH of 7.2, and a Km value of 0.13 mM CDP was obtained (Lewis, Kuzik and Wright, 1978). These values are all very close or identical to what has been observed with CDP reductase from partially purified CHO extracts (Lewis and Wright, 1978a), as well as what has been reported in this thesis regarding L cell extracts. Table 3 also shows that hydroxyurea is a potent inhibitor of ribonucleotide reductase when assayed in vivo. In addition, the presence of added $FeCl_3$ inhibits CDP reductase activity measured in vivo in CHO cells (Table 3). This result is unlike observations reported for other mammalian enzyme systems (eg.Moore, 1969), including CHO cells (Lewis and Wright, 1978a) and mouse L cells (figure 8b) assayed in vitro.

c) characterization of GDP reduction in permeabilized CHO cells: Optimal GDP reduction in whole CHO cells required assay conditions similar to those used in measuring in vitro GDP reductase activity in both CHO cell extracts (Lewis and Wright, 1978a), and L cell extracts (this thesis). Table 3 shows that in vivo enzyme activity was stimulated by the presence of reducing agent (DTT) and a nucleotide effector (dTTP), and was inhibited by hydroxyurea. Table 3 shows

TABLE 3

Properties of ribonucleotide reductase in permeabilized hamster cells

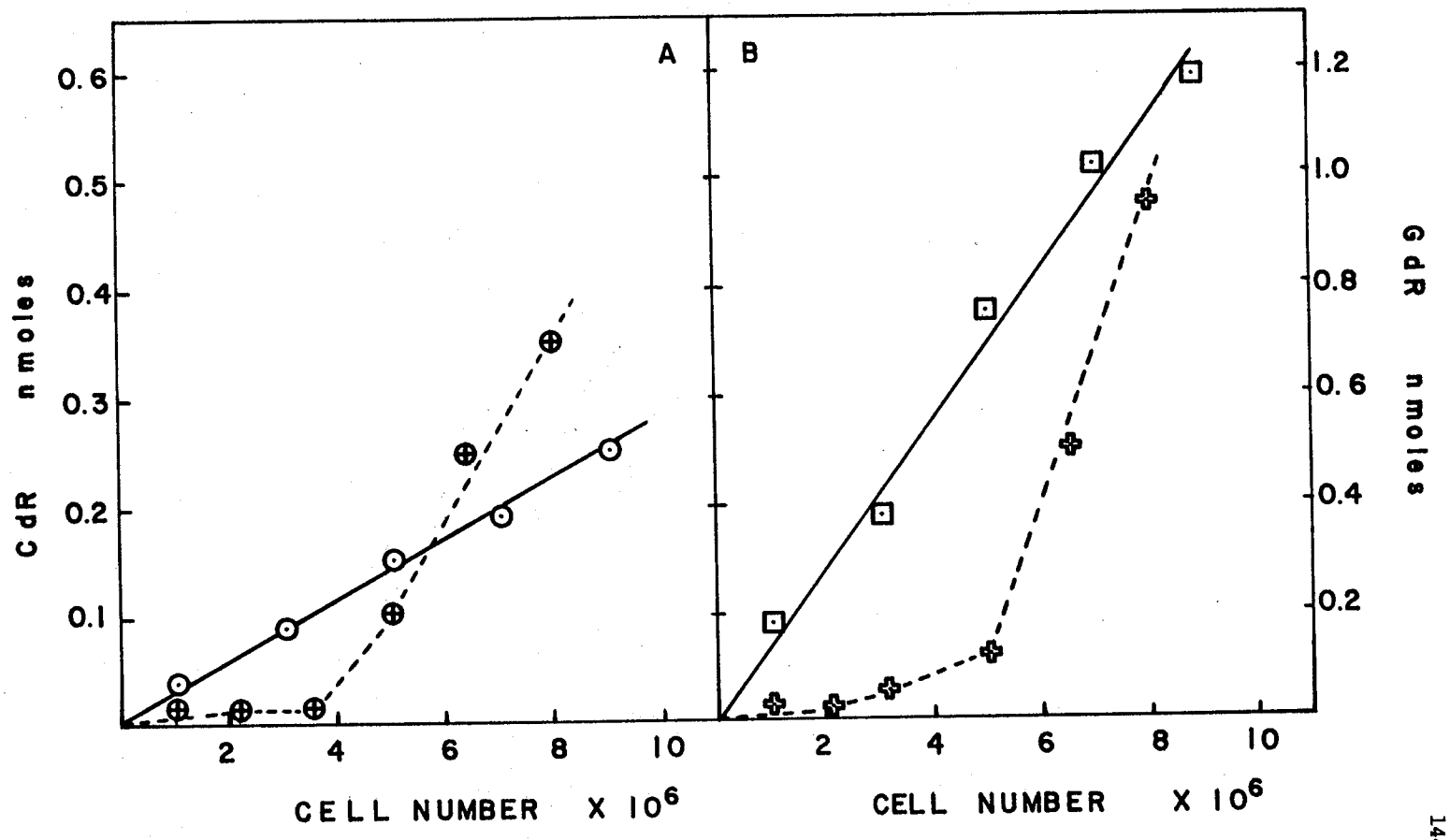
Omission or Addition	%CDP reduction	%GDP reduction
complete*	100	100
- DTT	11	13
- $MgCl_2$	24	-
+ 1.0 mM $MgCl_2$	-	35
- $FeCl_3$	-	35
+ 0.1 mM $FeCl_3$	61	-
+ 1.0 mM hydroxyurea	45	21
- ATP	10	-
- dTTP	-	8

*complete reaction mixtures for CDP and GDP reduction are not identical, and are as described in Materials and Methods

that GDP reductase activity was stimulated nearly 3 fold in the presence of 0.1 mM $FeCl_3$. This in unlike the response seen in extracts of CHO cells (Lewis and Wright, 1978a) or mouse L cells (figure 16b). Enzyme activity in whole CHO cells had a pH optimum very close to that observed in CHO (Lewis and Wright, 1978a) and L cell extracts (figure 17a). However, GDP reductase activity in permeabilized CHO cells had an apparent Km of 0.5 mM GDP (Lewis, Kuzik and Wright, 1978), a value which is 10 fold greater than the Km values found in extracts of CHO cells (Lewis and Wright, 1978a) and L cells (figure 19).

d) effect of protein concentration: As was previously mentioned, ribonucleotide reductase activity in L cells does not respond in a linear fashion to very low protein concentrations (figures 7b, 15b, 22b, 31). A similar phenomenon is reported in CHO (Lewis and Wright, 1978a) and E. coli (Larsson and Reichard, 1966) extracts. Warner (1973), however, found that enzyme activity measured in nucleotide-permeable E. coli cells maintained a linear response to protein content (cell number) even at low protein concentrations. To determine whether a similar result could be obtained with permeabilized mammalian cells, ribonucleotide reductase activity was measured in both whole cells and corresponding extracts. Figure 36 shows that both CDP and GDP reduction in nucleotide-permeable CHO cells increased linearly with cell number (protein content) to at least 9×10^6 cells per assay. This also served to show that enzyme activity could easily be measured using a quantity of cells that is routinely grown on a single 100 mm tissue culture plate.

FIGURE 36: Dependence of the rate of ribonucleotide reduction on enzyme concentration. CDP reduction (fig.36a) was measured for 60 minutes with the standard reaction mixture containing varying numbers of permeabilized cells (⊙) or varying amounts of cell-free extract (⊕). GDP reduction (fig.36b) was measured for 60 minutes with the standard reaction mixture containing varying numbers of permeabilized cells (⧈) or varying amounts of cell-free extract (✤). (From Lewis, Kuzik, and Wright, 1978 J. Cell Physiol. 94: 287-298.)


A
B
0.6
0.5
0.4
0.3
0.2
0.1
CdR nmoles
1.2
1.0
0.8
0.6
0.4
0.2
GdR nmoles
2
4
6
8
10
CELL NUMBER X 10^6
CELL NUMBER X 10^6

6. MEASUREMENT OF RIBONUCLEOTIDE REDUCTASE ACTIVITY IN NUCLEOTIDE-PERMEABLE L CELLS

Clements (1975) has summarized the major adaptations which may result in a drug resistant phenotype in cultured somatic cells. One way in which a cell could become drug resistant is by producing a structurally altered target enzyme which is less sensitive to the drug. For example, the resistance of the L_2Cl_3 line to growth inhibition by hydroxyurea and guanazole (figures 4, 5, and 6) may be due, at least in part, to the presence of GDP reductase activity which has a Ki value for both drugs that is approximately 3 fold higher than in WT cell extracts.

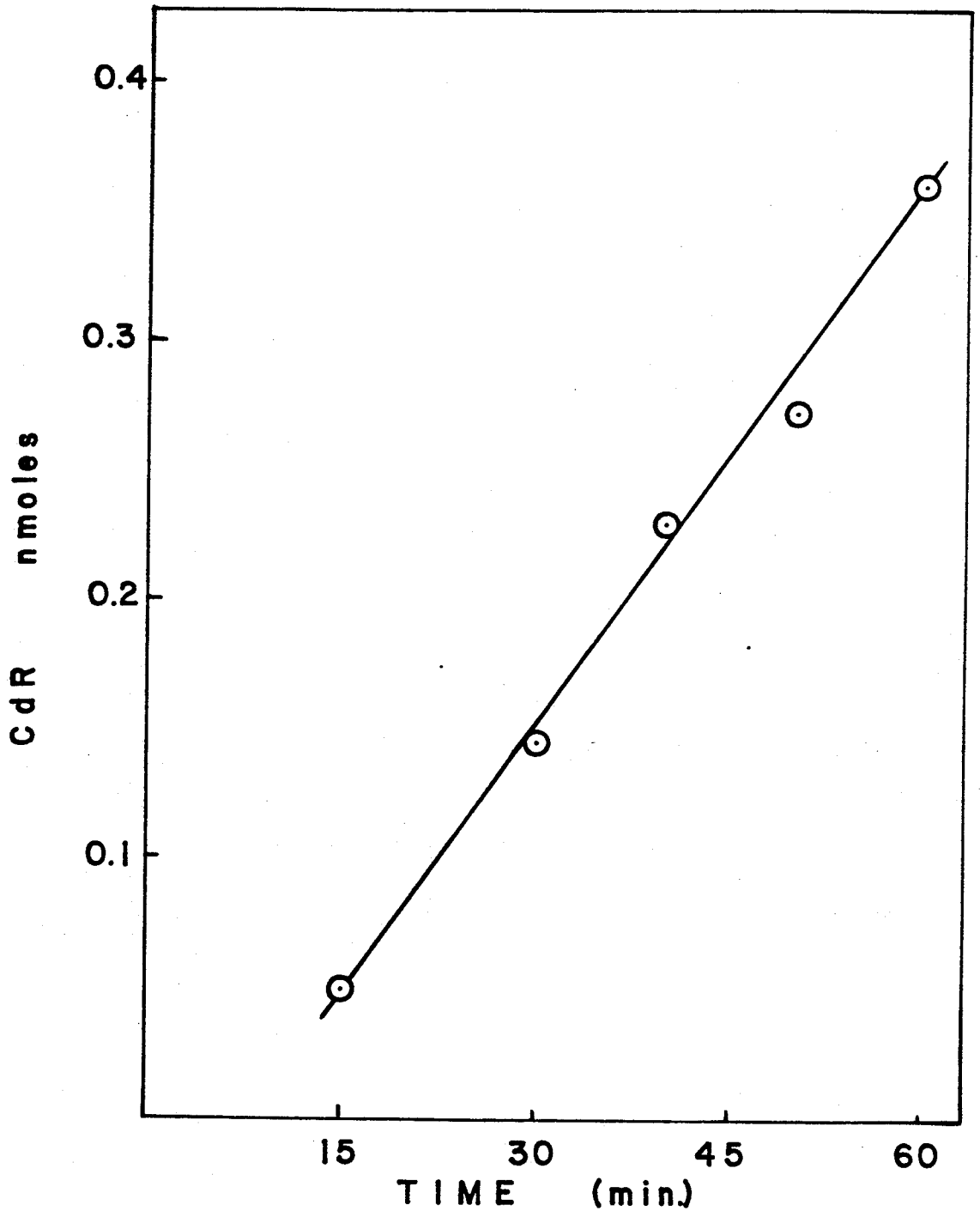
Drug resistance can also result from the production of enhanced amounts of drug sensitive enzyme. In this case, cell growth is maintained by residual enzyme activity in the presence of normally toxic levels of drug. For example, cell lines have been isolated which are resistant to the folic acid analogue methotrexate; and such resistance has been attributed to the presence of greatly increased levels of the target enzyme dihydrofolate reductase (Chang and Littlefield, 1976). Similarily, a hydroxyurea resistant CHO cell line has been isolated which appears to owe its resistance solely to the production of 5 to 7 times the WT amount of drug sensitive ribonucleotide reductase (Lewis and Wright, 1978c). Ribonucleotide reduction was measured in nucleotide-permeable L cells to determine if exponentially growing drug resistant cells would routinely show higher enzyme levels than WT cells tested under identical growth conditions.

a) CDP reduction in nucleotide-permeable L cells: CDP reductase activity was assayed in L cells that were treated with Tween-80 according to the procedure used with CHO cells and as described in Materials and Methods. Briefly, cells were permeabilized with Tween-80 for 30 minutes at 24°C, centrifuged, and resuspended in fresh permeabilizing buffer containing all of the reaction ingredients used when assaying CDP reduction in cell-free extracts. It was observed, however, that the addition of $FeCl_3$ to the in vivo assay had variable effects, and often was associated with clumping of the cells. Therefore $FeCl_3$ was routinely omitted from the reaction mixture.

Mouse L cells were routinely permeabilized for 30 minutes at 24°. Permeabilizing for longer periods of time, or at temperatures above 24° usually resulted in higher but less reproducible activity (data not shown). Therefore, a 30 minute treatment at 24° was chosen for future work. Figure 37 shows that after a short lag period, reduction of CDP proceeded linearly with time from 15 minutes to at least the standard incubation time of 60 minutes.

The measurement of in vivo CDP reductase activity in nucleotide-permeable L cells uncovered a difference in temperature profile between WT and L_2Cl_3 cells which could not be detected by observing enzyme activity in cell-free extracts. Maximum in vitro CDP reductase activity in both L_2Cl_3 and WT cell extracts occurred at an assay temperature of 37° (figures 8a and 22a), which also corresponds to the normal growth temperature for these cell lines. Figure 38, however, shows that maximum in vivo CDP reductase activity

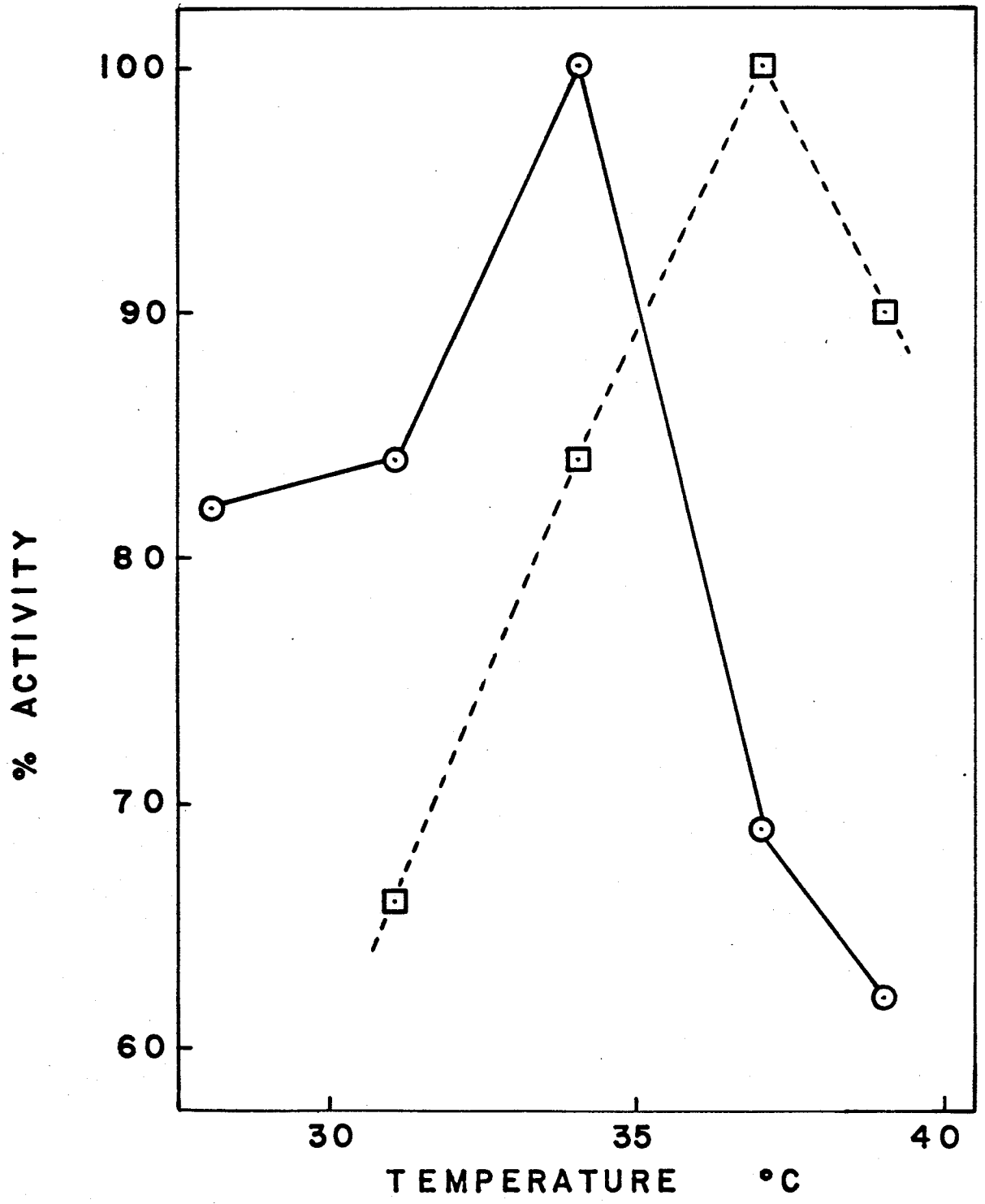
FIGURE 37: Progress of CDP reduction in nucleotide-permeable WT L cells with time. Samples of 5 X 10^6 nucleotide-permeable WT cells were added to standard in vivo CDP reductase assay mixtures and incubated at 37°. After various time intervals, the reactions were terminated by boiling and the amount of CdR formed was determined as described in Materials and Methods. A similar result could be obtained with L_2Cl_3 cells (data not shown).


0.4
0.3
0.2
0.1
CdR nmoles
15
30
45
60
TIME (min.)

in nucleotide-permeable WT and L_2Cl_3 cells occurred at different temperatures. The optimum temperature for CDP reduction in L_2Cl_3 cells was 37^{o}, whereas the optimum assay temperature for WT cells was 34^{o} (figure 38).

Using the standard permeabilizing and assay conditions, CDP reductase activity could be reproducibly measured in WT and L_2Cl_3 cells. Six independant batches of WT cells were assayed for levels of CDP reductase activity in the presence of 0.4 mM CDP, a concentration which is near saturating for in vivo CDP reduction (S. Koropatnick, unpublished observations). It was discovered that WT cells reduced an average of 375±60 pmoles of CDP/5 X 10^6 cells/hour. L_2Cl_3 cells were also measured for CDP reductase levels in the presence of 0.4 mM CDP, a substrate concentration which is probably non-saturating for these cells (figure 26), but was among the highest concentrations of radioactively labelled substrate that was found to be optimal for this assay system (see page 113). Nevertheless, 3 separate batches of L_2Cl_3 cells still reduced an average of 1144 ± 43 pmoles CDP/5 X 10^6 cells/hour, which is from 2.6 to 3.8 times higher than in permeabilized WT cells. This confirms the information obtained in vitro which showed that the specific activity of L_2Cl_3 extracts averaged 3 to 4 times higher than the specific activity of WT extracts (see page , also figures 7b & 22b, and figures 15b & 31). These differences in enzyme levels between WT and L_2Cl_3 cells would perhaps be enhanced further if CDP concentrations closer to saturation could have been used.

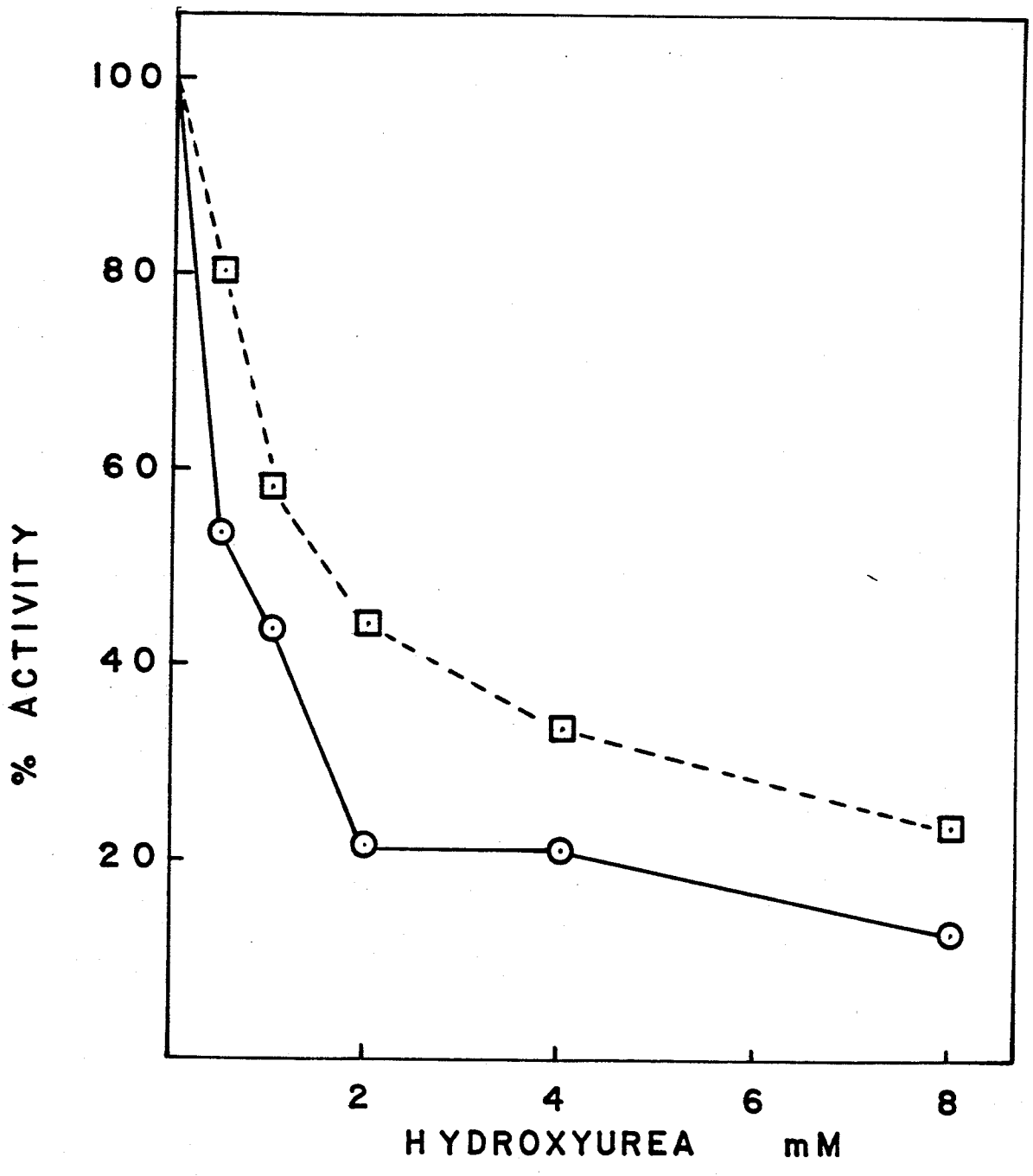
FIGURE 38: Effect of assay temperature on *in vivo* CDP reduction in nucleotide-permeable WT (⊙) and L_2Cl_3 (⊡) cells. Standard aliquots of 5 X 10^6 permeabilized WT and L_2Cl_3 cells were added to complete CDP reductase assay mixtures and incubated for 60 minutes at the temperatures indicated. The amount of CDP reduced was then determined as previously described. Identical results were also obtained from independent experiments using different batches of L cells.


100
90
80
70
60
% ACTIVITY
30
35
40
TEMPERATURE °C

The response of CDP reductase activity in permeabilized L cells to the presence of increasing concentrations of hydroxyurea is shown in figure 39. At all drug concentrations tested, enzyme activity in L_2Cl_3 cells was more resistant to hydroxyurea inhibition than was the activity in WT cells. In the presence of up to 8 mM hydroxyurea, the percent residual enzyme activity in L_2Cl_3 cells averaged 1.7 times higher than in WT cells. This correlates well with the *in vitro* L_2Cl_3 Ki value for inhibition of CDP reeuctase by hydroxyurea which averaged 1.5 times higher than the WT value.

In the original selection of drug resistant L cells, the cells destined to become the L_2Cl_3 line were selected by their ability to grow in the presence of 1.3 mM hydroxyurea. Figure 39 shows that at 1.3 mM drug, L_2Cl_3 cells still showed approximately 54% residual CDP reductase activity. Considering that L_2Cl_3 cells reduced an average of 1144 ± 43 pmoles CDP/5 X 10^6 cells/hour, 54% residual activity corresponds to approximately 615 pmoles CDP reduced/ 5 X 10^6 cells/hour. This value is well above the average amount of enzyme activity reported for WT cells in the absence of drug (375 ± 60 pmoles CdR/5 X 10^6 cells/hour), and therefore can satisfactorily account for the survival of L_2Cl_3 cells in the presence of 1.3 mM hydroxyurea. Wild-type cells, however, showed only approximately 40% residual activity in the presence of 1.3 mM hydroxyurea (figure 39). In the absence of drug, WT cells reduced an average of 375 ± 60 pmoles/5 X 10^6 cells/hour. Therefore, 40% of this value, or approximately 150 pmoles CDP/5 X 10^6 cells/ hour, presumably would be insufficient to sustain cell growth.

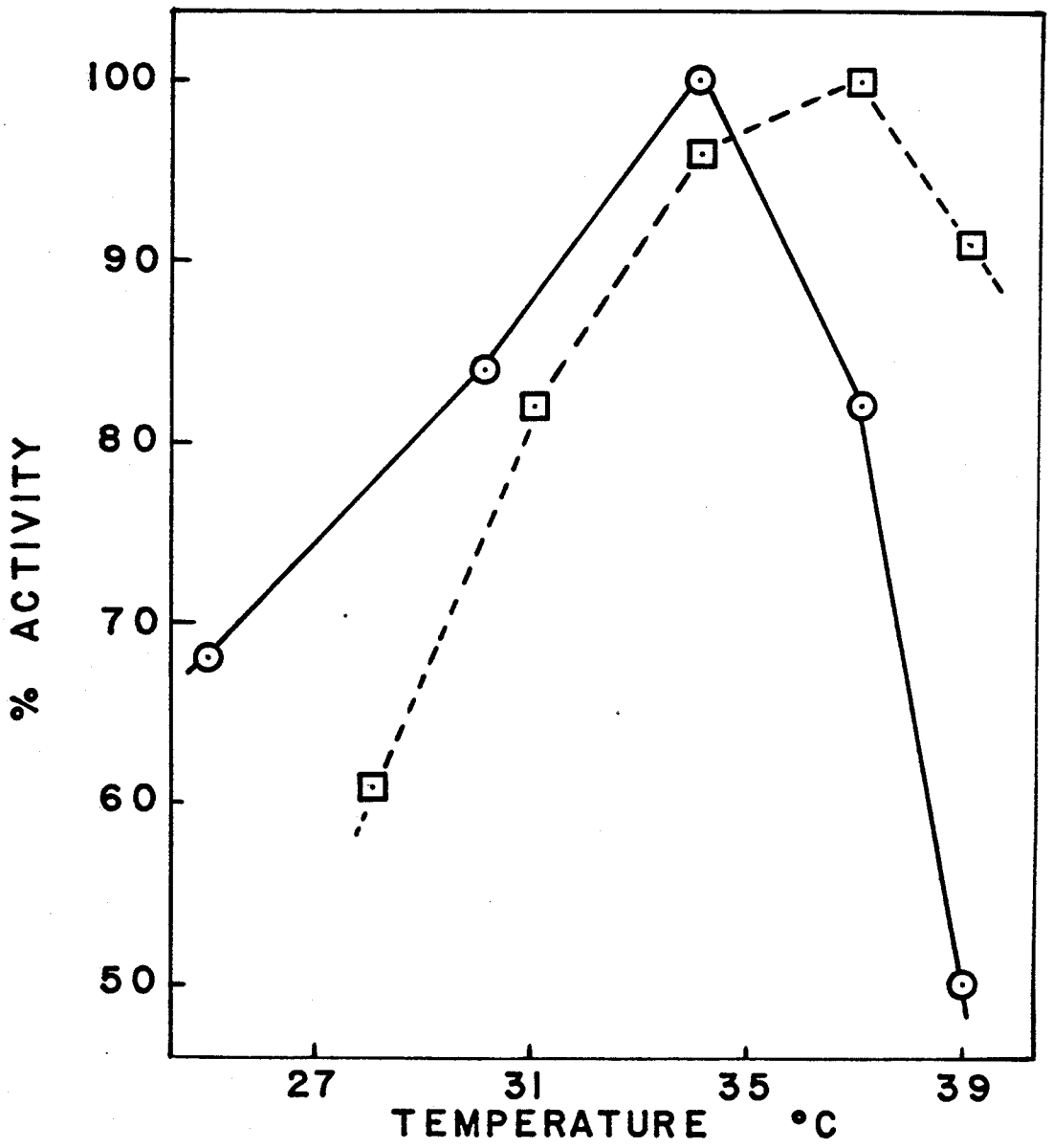
FIGURE 39: Inhibition of in vivo CDP reductase activity in nucleotide-permeable WT (⊙) and L_2Cl_3 (⊡) cells by hydroxyurea. Samples of 5 X 10^6 permeabilized WT and L_2Cl_3 cells were added to standard in vivo CDP reductase assay mixtures containing various concentrations of hydroxyurea. After incubation under routine assay conditions, the amount of CDP reduced was determined as described in Materials and Methods.


100
80
60
40
20
% ACTIVITY
2
4
6
8
HYDROXYUREA mM

b) GDP reduction in nucleotide-permeable L cells: GDP reduction was measured in WT and drug resistant L cells treated with Tween-80 by the same procedure used in measuring CDP reductase levels, and as described in Materials and Methods. Assay conditions found to be optimal for in vitro measurement of GDP reduction were employed. Figure 40 shows that in vivo GDP reductase activity in nucleotide-permeable WT and L_2Cl_3 cells responded differently to assay temperature. As was observed with in vivo CDP reduction (figure 38), maximum in vivo GDP reduction occurred at 37° in L_2Cl_3 cells and at 34° in WT L cells. This compares with the in vitro GDP reductase assay temperature of 30° used for extracts of both cell lines (figures 15a and 29a).

Under optimal assay conditions, 2 separate batches of L_2Cl_3 cells reduced GDP at an average rate of 3.1 nmoles/5 X 10^6 cells/hour. Under identical conditions, 3 separate measurements in WT cells yielded a value of only 1.6 ±0.2 nmoles GDP reduced/5 X 10^6 cells/hour. It appears, therefore, that the drug resistant L_2Cl_3 line has approximately twice the WT level of GDP reductase activity, as well as enhanced CDP reductase levels.

FIGURE 40: Effect of assay temperature on *in vivo* GDP reduction in nucleotide-permeable WT (⊙) and L_2Cl_3 (⊡) cells. Standard aliquots of 5 X 10^6 permeabilized WT and L_2Cl_3 cells were added to complete GDP reductase assay mixtures and incubated for 60 minutes at the temperatures indicated. The amount of GDP reduced was then determined as previously described. Identical results were also obtained in independent experiments using different batches of L cells.


100
90
80
70
60
50
% ACTIVITY
27
31
35
39
TEMPERATURE °C

DISCUSSION AND CONCLUSIONS

a) effect of hydroxyurea and guanazole on Wild Type L cells:

Hydroxyurea has a very potent cytoxic effect on WT L cells grown in culture. A concentration of 0.35 mM hydroxyurea is sufficient to completely stop growth of WT L cells (figure 1), and reduce plating efficiency to 6×10^{-6} (figure 2). Similar results have also been reported by other investigators using mouse cell lines (Bacchetti and Whitmore, 1969; Skoog and Nordenskjold, 1971) as well as with other mammalian systems (eg. Lewis and Wright, 1978b).

Guanazole has been reported to be cytotoxic to mammalian cells in a manner analagous to hydroxyurea (Brockman et al 1970; Wright and Lewis, 1974; Lewis and Wright, 1978b). This was also found to occur in WT L cells. A concentration of 2 mM guanazole was sufficient to completely stop growth of a WT cell population (figure 1), whereas 3 mM drug could reduce WT plating efficiency to 5×10^{-4} (figure 3).

Similar to other mammalian systems (eg. Turner et al, 1966; Elford, 1968; Lewis and Wright, 1978a),ribonucleotide reductase activity in extracts of L cells is sensitive to inhibition by hydroxyurea. Hydroxyurea exhibited an uncompetitive pattern of inhibition with WT CDP reductase activity at low drug concentrations only, whereas inhibition became noncompetitiveat higher drug levels (figures 12 and 13). A similar type of mixed inhibition of ribonucleotide reductase by hydroxyurea has also been reported in extracts of CHO cells (Lewis and Wright, 1978a). A Ki value for WT L cell CDP reduction of 0.24 mM hydroxyurea was obtained. Similarily, guanazole also showed a mixed pattern of inhibition with in vitro WT CDP reductase activity, yield-

ing a Ki value of 1.5 mM (figure 14). Although the Ki values for hydroxyurea and guanazole are very different, these drug concentrations have similar effects at the cellular level. Addition of either 0.24 mM hydroxyurea or 1.5 mM guanazole to the growth medium of WT L cells reduces the RPE by the same amount (figures 2 and 3). These observations support the suggestion that hydroxyurea and guanazole exert their cytotoxic effects through an inhibition of mammalian ribonucleotide reductase (Wright and Lewis, 1974).

Hydroxyurea and guanazole are also potent inhibitors of GDP reduction in extracts of WT L cells. Both drugs show noncompet patterns of inhibition, with Ki values of 0.15 mM hydroxyurea (figure 20) and 0.50 mM guanazole (figure 21). Figures 2 and 3 show that these concentrations of drug both reduce the RPE of WT cells by approximately 50%. It appears, therefore, that the inhibition of the target enzyme, GDP reductase, in vitro correlates well with the killing effect of the drugs at the cellular level.

It is obvious from the data presented that hydroxyurea and guanazole exert a potent physiological effect on WT L cells in culture. These drugs are capable of quickly halting cell division, dramatically decreasing plating efficiency, and inhibiting a key enzyme activity required for DNA synthesis. These properties of hydroxyurea and guanazole suggested that they could serve as effective agents in the selection of drug-resistant mouse cell mutants in cell culture. Since hydroxyurea is much more potent than guanazole on a molar basis, hydroxyurea was chosen as the selective agent for the isolation of a highly drug resistant mutant cell line from mouse L cells in culture.

b) drug resistant L cell line: selection and cellular characteristics

Many investigators have employed a multiple-step selection procedure to isolate highly drug resistant mutant cell lines (eg. Bunn and Eisenstadt 1977; Gupta and Siminovitch, 1978). Similarily, the mouse L cell line designated L_2Cl_3 was selected for resistance to highly cytotoxic levels of hydroxyurea by a two step selection procedure. The results obtained indicate that the L_2Cl_3 cell line is obviously very resistant to hydroxyurea. In addition, L_2Cl_3 cells exhibited a pleiotropic phenotype since they were also cross-resistant to guanazole, a drug which has a molecular configuration that is quite different from hydroxyurea (Brockman et al, 1970). This occurred even though the L_2Cl_3 cell line was originally selected by its ability to grow in cytotoxic levels of hydroxyurea only. The phenomenon of cross-resistance with hydroxyurea and guanazole has also been observed in drug resistant CHO cells (Wright and Lewis, 1974). Pleiotropic phenotypes have also been observed in many other different types of drug resistant cell lines in culture (eg. Bech-Hansen, Till and Ling, 1976; Hidaka et al, 1978; Gupta and Siminovitch, 1978b; Ceri and Wright, 1978; Epstein et al, 1978).

L_2Cl_3 cells maintain an exponential growth rate (figure 4) and show only marginally reduced plating efficiencies (figure 5) in the presence of highly cytotoxic concentrations of hydroxyurea. hydroxyurea resistant phenotype exhibited by L_2Cl_3 cells was stable since the RPE in the presence of hydroxyurea remained constant when cells were cultured under non-selective conditions for 11 months. It was obviously important that the drug resistant phenotype was stable in order for these cells to be useful in future genetic and biochemical

studies. Also, the stable phenotype suggests that mutational rather than epigenetic events resulted in the hydroxyurea resistant phenotype. In contrast, many other drug resistant cell lines do not retain the drug resistant property when cultivated for long periods of time under non-selective conditions (eg. Mezger-Freed, 1971; Littlefield, 1965). The original drug resistant phenotypes in these cell lines is probably due to epigenetic rather than mutational events and have been called phenotypic variants (Harris, 1971).

c) Ribonucleotide reductase in mouse L cells:

Wild-type cells - WT mouse L cells grown in suspension culture were found to be an excellent source of ribonucleotide reductase activity. Crude extracts prepared as described in Materials and Methods contained from 1.5 to 2.0 CDP reductase units per mg of protein. This is very similar to the specific activity found in crude extracts of CHO cells (Lewis and Wright, 1978a), and is much higher than the specific activity from any other eukaryotic sources reported (eg. Cory and Mansell, 1975; King and VanLancker, 1969; Moore, 1967).

The properties of CDP reductase activity in WT L cells is very similar to those described for other mammalian systems (eg. Larsson, 1969; Moore, 1967, 1976). Maximum CDP reduction occurred at a pH of 7.0 (figure 10b) and at an assay temperature of 37^{o} (figure 8a). Maximum activity also required the presence of 5 mM $MgCl_2$ (figure 10a) and 6 mM DTT (figure 9b). ATP is a potent activator of CDP reduction in L cells as it is in other mammalian cell systems, (eg. Moore and Hurlbert. 1966); activity was stimulated several fold by the presence

of 4mM ATP (fiugre 9a). Activity was also stimulated several fold in the presence of added $FeCl_3$ (figure 8b). The level of iron required, however, was much higher than the concentrations used in other mammalian cell systems (Lewis and Wright, 1974; Larsson, 1969). CDP reductase activity does not respond in a linear fashion to low protein concentration (figure 7b). A similar phenomenon has been observed with CDP reductase activity isolated from other mammalian cells (eg. Hopper 1972). The apparent Km for CDP reduction in extracts of WT L cells was found to be 0.13 mM (figure 11), which is identical to the value found in CHO cells (Lewis and Wright, 1978a) and very close to the value of 0.12 mM found in Novikoff rat hepatoma cells (Moore and Hurlbert, 1966).

GDP reductase activity, partially purified from WT L cells, resembled the enzyme activity reported for other cultured mammalian cell lines (Moore and Hurlbert, 1966; Moore, 1967; Lewis, Kuzik, and Wright, 1978). Similar to CDP reductase activity in WT L cells, GDP reduction does not respond in a linear fashion at low protein concentration (figure 15b). Optimum assay conditions included a pH of 7.0 (figure 17a), and the presence of 6mM DTT (figure 18). As in other mammalian systems (Moore and Hurlbert, 1966; Brown and Reichard, 1969b), dTTP is an important activator of GDP reduction in L cells (figure 17b). The addition of $FeCl_3$ to the reaction mixture inhibited the activity of GDP reductase (figure 16b). The apparent Km value was found to be 0.05 mM GDP, which is similar to the Km value of 0.03 mM in CHO cells (Lewis and Wright, 1978a).

Maximum GDP reductase activity in WT L cell extracts

occurred at 30°, instead of 37° which is the normal temperature at which L cells were cultured (figure 15a). The reason for this is not known, but may be due to the presence of unidentified inhibitors which exert more of an effect on the in vitro measurement of GDP reduction at 37° than at 30°. Similarly, other investigators have also assayed ribonucleotide reductase activity at a temperature well below the normal growth temperature of the organism from which the enzyme was obtained (Lewis et al, 1976; Elford, 1974).

Drug resistant L cells - The properties of CDP reductase activity in partially purified extracts of L_2Cl_3 cells were nearly identical to enzyme activity from WT cells. Optimal assay conditions included a pH of 7.0 (figure 24a), an assay temperature of 37° (figure 22a), and the presence of 5 mM $MgCl_2$ (figure 23b), 6 mM DTT (figure 24b), 6 mM ATP (figure 25a), and 0.5 mM $FeCl_3$ (figure 25b). Also similar to the WT enzyme, activity did not respond linearly to protein concentrations below approximately 400 µg protein per assay volume (figure 22b). The apparent Km for CDP reductase activity in L_2Cl_3 cell extracts was found to be 0.41 mM (figure 26); a value over 3-fold greater than the WT Km of 0.13 mM CDP.

GDP reductase activity in extracts of L_2Cl_3 cells resembled activity found in extracts to WT cells. Maximum GDP reduction in L_2Cl_3 extracts required a pH of 7.0 (figure 32a), an assay temperature of 30° (figure 29a), and the presence of 2 mM dTTP (figure 32b) and 6 mM DTT (figure 30b). Also similar to the WT enzyme, activity was inhibited by added $FeCl_3$ (figure 29b), and the activity did not respond linearly to low protein concentrations (figure 31). The

apparent Km for GDP reductase activity in L_2Cl_3 cells extracts was found to be 0.06 mM (figure 33); a value nearly identical to the Km of 0.05 mM GDP found with WT enzyme preparations.

d) Ribonucleotide reduction in nucleotide-permeable cells: Ribonucleotide reductase activity can be easily measured in intact mammalian cells made permeable to nucleotides by treatment with the detergent Tween-80 (Table 2). Both CDP and GDP reductase activities could routinely be measured in a small number of cells which were grown on the surface of a single plate. In contrast, the measurment of ribonucleotide reductase activity in cell-free extracts requires the preparation of large volumes of suspension grown cells. In addition, unlike activity in cell extracts, enzyme activity in nucleotide-permeable cells follows a linear response to protein concentration, even at very low cell numbers (figure 36).

The various characteristics of CDP and GDP reduction in nucleotide-permeable CHO cells are shown in Table 3. Ribonucleotide reduction as measured *in vivo* responded to pH, temperature, DTT, and nucleotide effectors in essentially the same way as ribonucleotide reduction as measured *in vitro*. Therefore the investigation of enzyme activity in nucleotide-permeable L cells was performed under the same assay conditions which were optimal for the measurement of enzyme activity in cell extracts.

The novel *in vivo* assay procedure revealed a difference in temperature profile between ribonucleotide reductase activity in nucleotide-permeable WT and L_2Cl_3 cells, Maximum *in vivo* CDP and GDP

reduction in WT cells occurred at 34°, whereas maximum activity in L_2Cl_3 cells occurred at 37° (figures 38 and 40). The standard *in vitro* assay of CDP and GDP reduction in cell-free extracts was unable to detect any similar difference between these two cell lines (figures 8a, 15a, 22a, 29a). The reason for the different temperature responses in permeabilized WT and L_2Cl_3 cells is unknown. However, Elford (1974) reported that ribonucleotide reductase in Novikoff hepatoma and regenerating rat liver is associated with a membrane fragment isolated from the postmicrosomal supernatant. It may be possible, therefore, that ribonucleotide reductase in L_2Cl_3 cells is somehow altered in its association with this membrane fragment; and this alteration is reflected in a response to assay temperature which is different from WT cells. Presumably, this enzyme/membrane association would be disrupted during the preparation of cell-free extracts. This would then explain why the difference in temperature profile between WT and L_2Cl_3 cells was not detected using *in vitro* assay conditions. Other explanations are possible, however, and the resolution of this question may be dependent on further purification of the mammalian enzyme and a better understanding of its association with other cellular components.

CDP reduction in nucleotide-permeable WT and L_2Cl_3 cells is sensitive to inhibition by hydroxyurea (figure 39). However, at all drug concentrations tested, L_2Cl_3 cells show more residual enzyme activity than do WT cells. Also, this increased residual activity can be accounted for by the increased *in vitro* Ki value for hydroxyurea which was observed in extracts of L_2Cl_3 cells (figure 27).

Many separate batches of L cells were used to determine the mean levels of CDP and GDP reductase activity in exponentially growing WT and drug resistant cell lines, It was discovered that L_2Cl_3 cells routinely reduced 1144 ± 43 pmoles CDP/5 X 10^6 cells/hour, and 3100 pmoles of GDP/5 X 10^6 cells/hour. Wild-type cells, however, reduced only 375 ± 60 pmoles CDP and 1600 pmoles GDP/5 X 10^6 cells/hour. The drug resistant L_2Cl_3 cell line, therefore, produces enhanced levels of ribonucleotide reductase which are approximately 2 to 3 fold higher than WT levels.

e) Mechanism of resistance to hydroxyurea and guanazole by the L_2Cl_3 cell line: The data presented strongly suggests that the ribonucleotide reductase enzyme present in L_2Cl_3 cells may be structurally altered. GDP reductase activity from extracts of L_2Cl_3 cells was highly resistant to inhibition by hydroxyurea and guanazole. The L_2Cl_3 enzyme showed in vitro Ki values of 0.39 mM hydroxyurea (figure 27) and 1.55 mM guanazole (figure 28). These values are both approximately 3-fold greater than the WT Ki values for GDP reduction of 0.15 mM hydroxyurea (figure 20) and 0.50 mM guanazole (figure 21). CDP reductase activity partially purified from L_2Cl_3 extracts also appears to be modified. The L_2Cl_3 CDP reductase enzyme has a Km value of 0.41 mM CDP (figure 26), which is over 3 times greater than the WT Km of 0.13 mM CDP (figure 11). In addition, the in vitro inhibition by hydroxyurea appears different between CDP reductase activities from WT and L_2Cl_3 extracts. Hydroxyurea inhibits L_2Cl_3 CDP reductase activity uncompetitively at all drug concentrations

tested (figure 27), whereas inhibition of WT CDP reduction is mixed (figures 12 and 13). Also, the L_2Cl_3 Ki value obtained for CDP reduction varied from 0.35 mM to 0.39 mM hydroxyurea (figure 27), which was about 1.5 times greater than the WT Ki of 0.24 mM drug (figures 12 and 13). The contention that ribonucleotide reductase in L_2Cl_3 cells may contain subtle structural alteration(s) which distinguish it from the WT enzyme is further supported by the finding that the optimim assay temperature for in vivo CDP and GDP reduction in nucleotide-permeable L_2Cl_3 cells is quite different than the optimim assay temperature with permeabilized WT cells (figures 38 and 40).

Many cultured somatic cell mutants have been isolated in which production of an altered target enzyme has been offered as at least a partial explanation for drug resistance. Examples include mammalian cells resistant to emetine (Gupta and Siminovitch, 1978), methotrexate (Flintoff et al, 1976), hydroxyurea (Lewis and Wright, 1978a), αamanitin (Lobban, Siminovitch and Ingles, 1976), oubain (Robbins and Baker, 1977) and fluorocitrate (Wright, 1975). Similarly it is reasonable to conclude that the production of a structurally altered ribonucleotide reductase enzyme in L_2Cl_3 cells can at least partially explain the drug resistant phenotype exhibited by these cells. Other explanations are still possible, however, and definitive evidence must await the final purification of the enzyme.

Drug resistance in cultured somatic cells can also arise from the production of enhanced levels of a target enzyme (Clements, 1975). For example, methotrexate resistance in Baby Hamster Kidney

cells (Chang and Littlefield, 1976) and CHO cells (Flintoff et al, 1976) is often accompanied by enhanced levels of dihydrofolate reductase; and a hydroxyurea resistant CHO cell line has recently been found to have elevated levels of ribonucleotide reductase (Lewis and Wright, 1978c). Therefore, levels of ribonucleotide reductase activity were compared in nucleotide-permeable WT and hydroxyurea resistant L cells. It was discovered that L_2Cl_3 cells contained from 2.6 to 3.8 times the level of CDP reductase that was found in WT cells. Similarly, the levels of GDP reductase activity in L_2Cl_3 cells was found to average twice the level found in WT cells.

The drug resistant phenotype of the L_2Cl_3 cell line can now be fully accounted for by the presence in these cells of enhanced levels of a structurally altered ribonucleotide reductase enzyme which exhibits a decreased sensitivity to hydroxyurea and guanazole. The L_2Cl_3 cell line was originally selected by its ability to grow in the presence of 1.3 mM hydroxyurea. At this concentration of drug, L_2Cl_3 cells show approximately 17% more residual CDP reductase activity than do WT cells (figure 39). Also, the mean level of CDP reductase activity in L_2Cl_3 cells averages approximately 3-fold greater than the mean level in WT cells. This results in a residual enzyme activity in L_2Cl_3 cells at 1.3 mM hydroxyurea which is still well above the level of activity observed in WT cells in the absence of drug, and therefore is more than sufficient to sustain growth. For example, at 1.3 mM hydroxyurea, the L_2Cl_3 cells would be expected to contain 615 pmoles CDP reductase activity whereas the parental wild-type cells would exhibit only 150 pmoles CDP reductase activity.

In other words, the activity remaining in L_2Cl_3 cells is actually greater than the activity observed in WT cells growing in the absence of drug (375 pmoles/5 X 10^6 cells/hour), and would therefore easily support the growth of the mutant cells in the presence of normally cytotoxic concentrations of hydroxyurea.

The L_2Cl_3 line also contains elevated levels of GDP reductase activity which exhibits Ki values for hydroxyurea and guanazole that are approximately 3 times greater than the WT Ki values. It appears, therefore, that the presence of elevated CDP and GDP reductase levels, complemented by a decreased enzyme sensitivity to drug inhibition, allows the L_2Cl_3 cell line to grow normally in the presence of highly cytoxic concentrations of hydroxyurea and guanazole.

Conclusions: Hydroxyurea is an excellent selective agent for obtaining genetically stable drug resistant L cell mutants in culture. The hydroxyurea resistant trait of L_2Cl_3 cells was stable after long periods of cultivation under non-selective conditions. This supports (Clements, 1975), but does not prove the contention that the drug resistant phenotype has arisen by mutational rather than epigenetic means. It also emphasizes that stable genetic variants can be isolated from aneuploid cell lines in culture (i.e. mouse L cells).

Studies with CHO cells have indicated that there are at least two classes of hydroxyurea resistance. Class I cells contain altered ribonucleotide reductase activity which is less sensitive to inhibition by the drug (Lewis and Wright, 1978a). Class II cells contain elevated levels of drug sensitive enzyme (Lewis and Wright,

1978c). The L_2Cl_3 cell line described in this thesis represents a new class of hydroxyurea resistant mutant: cells with enhanced levels of a structurally altered ribonucleotide reductase which is less sensitive to inhibition by the drug. It is expected that the hydroxyurea resistant trait in L_2Cl_3 cells may behave dominantly or codominantly in cell-cell hybrids since the drug resistant phenotype in both Class I and II hydroxyurea resistant CHO cells is expressed codominantly (Lewis and Wright, 1978 a,b,c). If this is the case, then a hydroxyurea resistance marker in mouse cells should prove to be very useful in future somatic cell genetic studies. For example, the hydroxyurea resistant property can be used for the selection of hybrids of hydroxyurea sensitive and hydroxyurea resistant cells. For example, a cell line containing both a recessive marker and the hydroxyurea resistance trait could be used in hybridization experiments with other cell lines which do not carry any selective markers.

Hydroxyurea resistance of L_2Cl_3 cells at the cellular level is accompanied by an increased resistance of ribonucleotide reductase to hydroxyurea inhibition. This is strong evidence that the primary site of action of this drug is the inhibition of ribonucleotide reductase. Similarly, the cross-resistance observed at both the cellular and enzyme levels to the inhibitory effects of guanazole indicates that this drug also owes its cytotoxicity to an inhibition of ribonucleotide reductase.

The L_2Cl_3 cell line was selected for resistance to highly cytotoxic concentrations of hydroxyurea by a two step selection procedure. The resultant drug resistant phenotype of L_2Cl_3 cells

includes elevated levels of ribonucleotide reductase, as well as the production of a structurally altered ribonucleotide reductase enzyme. It appears, therefore, that the high level of drug resistance in the L_2Cl_3 cell line may have arisen from two separate mutations; one mutation causing a structural alteration in the ribonucleotide reductase enzyme, the other mutation possibly causing an alteration in the regulation of ribonucleotide reductase synthesis. Other explanations are still possible, however, including the possibility that a single mutation may have given rise to both changes. Work is currently under way in this laboratory to develop a clearer understanding of the mutations which may result in hydroxyurea resistance in mammalian cells in culture.

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