THE UNIVERSITY OF MANITOBA

MOLECULAR AND CELLULAR CHARACTERIZATION OF HAMSTER AND HUMAN CELLS WITH ALTERATIONS IN RIBONUCLEOTIDE REDUCTASE.

by

AARON YEHUDA TAGGER

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

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To Mom and Dad

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ABSTRACT

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A detailed characterization of the molecular and biological properties of two hydroxyurea resistant Chinese hamster ovary cell lines was undertaken in order to (i) understand the operative mechanisms in hydroxyurea resistance (ii) provide an insight into the interrelationships of deoxyribonucleotide pool sizes, spontaneous mutation rates, and tumorigenic characteristics of cultured mammalian cells. In addition, the relationship between alterations in ribonucleotide reductase activity and transformation was examined in normal human diploid fibroblasts and their SV40 virus-transformed counterparts. These studies are important in advancing the knowledge of mechanisms involved in drug resistance and tumorigenicity, as well as providing further insight into the possible role of ribonucleotide reductase in the transformation event. Electroparamagnetic resonance spectroscopy measurements for tyrosyl free radical content and studies with M1 specific antibodies indicated that the elevation in ribonucleotide reductase activity observed in both the high (H^R-R2T) and low (HN^R-AT) hydroxyurea resistant cell lines was entirely due to an increase in protein M2. Northern blot analysis revealed increases in steady state M2 mRNA levels for both drug resistant cell lines, however Southern blots indicated increased M2 gene copy number only for the most resistant cell line (H^R-R2T). Studies with M1 cDNA showed that both drug resistant lines possessed a wild type level of M1 mRNA and a wild type M1 gene copy number. Enzyme kinetic studies performed on partially purified ribonucleotide reductase indicated increases in Ki for hydroxyurea and dATP inhibition for both mutant lines as compared to wild type. No differences in uptake of hydroxy[^{14}C]urea occured in either mutant cell line as compared to wild type cells. Taken together, these studies indicated that increased amounts of

protein M2 and alterations in sensitivity to hydroxyurea can fully account for the drug resistant phenotype of the mutant cell lines. Colony forming abilities of the H^R-R2T cell line in the presence of colchicine or puromycin indicated that hydroxyurea resistance is not associated with a multidrug resistant phenotype. Analysis of spontaneous mutation rates to ouabain and 6-thioguanine resistance, deoxyribonucleoside triphosphate (dNTP) pool sizes, growth ability in the presence of N-(phosphonacetyl)-L-aspartate (PALA), tumor growth rates and the ability to form experimental lung metastasis in nude mice for both mutant and wild type cell lines revealed the following (i) hydroxyurea resistance and alterations in ribonucleotide reductase activity need not be associated with dNTP pool imbalances and changes in spontaneous mutation rates (ii) decreases in spontaneous mutation rates can be associated with dNTP pool imbalances and (ii) the ability to form lung metastasis and generate variant subpopulations is not necessarily dependent on the type of genetic instability which leads to either increases in spontaneous mutation rates or the ability to amplify genes at elevated frequencies. The tumorigenic properties of the two CHO drug resistant cell lines are consistent with suggestions that changes in ribonucleotide reductase may accompany modifications in the malignant characteristics of cells. Support of a link between ribonucleotide reductase and the transformation event was the finding that ribonucleotide reductase activity was elevated in two SV40 virus-transformed human cell lines as compared to their parental wild type human cell strains from which they were derived. Hydroxyurea resistance accompanied the elevations of enzyme activity in one of the transformed lines examined. Furthermore, hydroxy[^{14}C]-urea uptake experiments were performed, and indicated that hydroxyurea enters human cells by mechanism of passive diffusion.

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ABBREVIATIONS

ADP	-	adenosine 5'-diphosphate
ARA-A	-	1-β-D-arabinofuranosyl adenine
ARA-C	-	1-β-D-arabinofuranosyl cytosine
ATP	-	adenosine 5'-triphosphate
bp	-	basepairs
BSA	-	bovine serum albumin
CDP	-	cytidine 5'-diphosphate
CHO	-	Chinese hamster ovary
Ci	-	Curie
CMP	-	cytidine 5'-monophosphate
CPM	-	counts per minute
CTP	-	cytidine 5'-triphosphate
CYT	-	cytidine
dA	-	2'-deoxyadenosine
dATP	-	2'-deoxyadenosine 5'-triphosphate
dCDP	-	2'-deoxycytidine 5'-diphosphate
dG	-	deoxyguanosine
dGTP	-	2'-deoxyguanosine 5'-triphosphate
dNDP	-	2'-deoxyribonucleoside 5'-diphosphate
dNTP	-	2'-deoxyribonucleoside 5'-triphosphate
DNA	-	deoxyribonucleic acid
DPM	-	disintigrations per minute
dTdR	-	2'-deoxythymidine
dTMP	-	2'-deoxythymidine 5'-monophosphate
dTTP	-	2'-deoxythymidine 5'-triphosphate

dUMP	-	2'deoxyuridine 5'-monophosphate
EPR	-	electroparamagnetic resonance
FCS	-	fetal calf serum
g	-	gram(s)
GDP	-	guanosine 5'-diphosphate
GSH	-	reduced glutathione
GSSG	-	oxidized glutathione
GTP	-	guanosine 5'-triphosphate
Hepes	-	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
hr	-	hour(s)
ITP	-	inosine 5'-triphosphate
iv	-	intravenous
K	-	Kelvin
mAMP	-	milliampere
mg	-	milligram
min	-	minute(s)
mM	-	millimolar
MMNG	-	N-methyl-N'-nitro-N-nitrosoguanidine
MOPS	-	morpholinopropanesulfonic acid
mRNA	-	messenger ribonucleic acid
MW	-	molecular weight
nm	-	nanometers
NMR	-	nuclear magnetic resonance
OU^R	-	ouabain resistance

ABBREVIATIONS (continued)

PALA	-	N-(phosphonacetyl)-L-aspartate
PBS	-	phosphate buffered saline
PEI	-	polyethyleneimine
RNA	-	ribonucleic acid
s	-	second(s)
SDS	-	sodium dodecyl sulfate
TE	-	Tris-EDTA
TBE	-	Tris-borate EDTA
uCi	-	microCurie
UDP	-	uridine 5'-diphosphate
ug	-	microgram
UTP	-	uridine 5'-triphosphate
uv	-	ultraviolet
v	-	volume
w	-	weight
WT	-	wild type
$6TG^R$	-	6-thioguanine resistance

MOLECULAR AND CELLULAR CHARACTERIZATION OF HAMSTER AND HUMAN CELLS WITH ALTERATIONS IN RIBONUCLEOTIDE REDUCTASE

INTRODUCTION

INTRODUCTION

To maintain and preserve the fidelity of DNA synthesis both a sufficient as well as a balanced supply of the precursor molecules, deoxyribonucleoside triphophates is essential. The highly allosterically regulated enzyme, ribonucleotide reductase catalyzes the rate limiting step in the formation of these molecules. The necessity of cell division and DNA replication for the survival and propagation of any species points to the key position which this enzyme holds in the biology of the cell. Because of this, the enzyme has been used as a target site for cancer chemotherapy in attempts to selectivly inhibit proliferating cells. One of the most commonly used drugs directed at this enzyme is hydroxyurea.

The work presented in this thesis was undertaken in order to advance our understanding of ribonucleotide reductase and the mechanisms involved in achieving drug resistance to hydroxyurea, a potent inhibitor of this enzyme. To this end, two drug resistant cell lines, showing relatively high and low resistance to hydroxyurea were characterized at the molecular level for possible alterations in protein, enzyme activity, message levels, gene copy number and hydroxyurea uptake. To investigate whether or not alterations in other biological properties accompanied the drug resistant phenotype, deoxyribonucleotide pool analysis, spontaneous mutation rates to ouabain and 6-thioguanine resistance, and tumorigenic properties of the mutant and wild type cell line were investigated. The differing properties of the two mutant cell lines with respect to dNTP alterations and spontaneous mutation rates provided a suitable comparative

system to investigate some of the currently postulated mechanisms of tumor progression.

Interestingly, previous studies have suggested a link between ribonucleotide reductase activity and tumorigenicity. In order to re-examine this relationship in a human cell system, the levels of ribonucleotide reductase activity were measured in intact human diploid fibroblasts and compared to enzyme levels present in SV40 virus-transformed derivatives of the primary human cell strains.

In addition, since hydroxyurea has found use in numerous biological and clinical situations, and since drug uptake is an important aspect of drug action, the uptake properties of this drug were investigated in normal human diploid fibroblast cells.

To summarize, the objectives of this study where the following (i) to understand at the molecular level, what mechanisms are operating in hydroxyurea resistant CHO cells which enable them to survive in normally cytotoxic concentrations of drug (ii) to examine and compare the interrelationships of a number of biological parameters between the drug resistant and wild type cell lines including deoxyribonucleotide pool sizes, spontaneous mutation rates, tumor growth rates, and the ability to metastasize in nude mice (iii) to re-examine the relationship between alterations in ribonucleotide reductase activity and transformation by comparing enzyme activities in transformed cells and with cell strains from which they were derived and (iv) to understand the mechanism of uptake of the antitumor agent, hydroxyurea, in human cells.

HISTORICAL REVIEW OF RIBONUCLEOTIDE REDUCTASES

1. GENERAL CHARACTERIZATION OF RIBONUCLEOTIDE REDUCTASES

1.1 Introduction

The synthesis of DNA is dependent upon the availability of the four deoxyribonucleoside triphosphates which serve as substrates for DNA polymerase. The rate limiting enzyme responsible for the de novo production of these substrates is ribonucleotide reductase, which catalyzes the reduction of ribonucleotides to 2'-deoxyribonucleotides. The enzyme, discovered in the early 1950s (Hammarsten et al., 1950; Rose and Schweigert, 1953), is present in all prokaryotic and eukaryotic cells which synthesize DNA and is tightly associated with the S phase of the cell cycle (Thelander and Reichard, 1979). There exist three distinct classes of ribonucleotide reductases (Wright et al., 1981; Lammers and Follmann, 1983; Ashley and Stubbe, 1985). One class is represented by the enzyme isolated from Lactobacillus leichmannii, has an absolute requirement for adenosylcobalamin as a cofactor and utilizes ribonucleoside triphosphates as substrates. Another class is represented by the enzyme isolated from Escherichia coli, contains two non-heme irons, a unique tyrosyl free radical and, utilizes ribonucleoside diphosophates as substrates (Thelander and Reichard, 1979; Lammers and Follmann, 1983; Wright, 1983). The third class is typified by the enzymes isolated from Brevibacterium ammoniagenes and Micrococcus luteus which are dependent on manganese for activity (Lammers and Follmann, 1983). All ribonucleotide reductases catalyze the reduction of the hydroxyl group at the 2' carbon atom on the ribose moiety of ribonucleotides, utilizing NADPH

as the ultimate hydrogen donor according to the following reaction:

$$NADPH + H^+ + \text{ribonucleotide} \text{ ------> } NADP^+ + \text{deoxyribonucleotide} + H_2O$$

1.2 Adenosylycobalamin-dependant ribonucleotide reductases

Ribonucleotide reductases which have an absolute requirement for adenosylcobalamin were first discovered in 1964 (Blakley and Barker, 1964) in the fermentative bacteria Lactobacillus leichmanni. Since that time, adenosylcobalamin dependant reductases have only been found in microorganisms (e.g. Clostridium tetanomorphaum, C. sticklandii, C. thermoaceticum (Abeles and Beck, 1967), Micrococcus denitrificans, and Sphaerophorus varius (Gleason and Hogenkamp, 1972)). Ribonucleotide reductase from L. leichmannii has been purified to homogeneity (Panagou et al., 1972: Chen et al., 1974; Hoffmann and Blakley, 1975; Singh et al., 1977; Blakley, 1982), has a molecular weight of 76,000 daltons and catalyzes the reduction of the ribonucleoside triphosphates, GTP, ITP, ATP, CTP, and UTP. The binding of the cobalamin coenzyme to the enzyme requires the binding of an effector molecule to a regulatory site (Singh et al., 1977). It has been shown that deoxyribonuleoside triphosphates as well as the ribonucleoside triphosphate substrates bind to a common regulatory region, however the binding of the ribonucleotides is two to three orders of magnitude weaker than the binding of the deoxyribonucleotides (Chen et al., 1974; Singh et al., 1977). The allosteric regulation of the enzyme appears to be base specific, thus, dATP stimulates CTP reduction, dCTP stimulates UTP reduction, dTTP stimulates GTP reduction and dGTP

stimulates the reduction of ATP (Blakley, 1966; Vitols et al., 1967; Beck, 1967; Chen et al., 1974; Ludwig and Follmann, 1978). No strong negative effector of the enzyme has yet been found.

Another type of adenosylcobalamin-dependant ribonucleotide reductase which utilizes ribonucleoside diphosphates rather than ribonucleoside triphosphates as preferred substrates has been purified from Corynebacterium nephredii (Tsai and Hogenkamp, 1980). Unlike the enzyme from L. leichmannii, the enzyme from C. nephreddii is subject to both positive and negative control; dATP and dTTP inhibit the reduction of CDP and GDP respectively and dGTP stimulates the reduction of ADP and UDP. The enzyme exists as a dimer having a molecular weight of 196,000 daltons.

Although there is no general rule as to the distribution of cobalamin-dependant reductases amongst various species, the enzyme does appear to be more prevalent amongst prokaryotes. Only two eukaryotes to date, Euglenophyta and the fungus Phitomyces chartarum have been shown to posess a cobalamin-dependant ribonucleotide reductase (Stutzenberger, 1974).

1.3 E. coli ribonucleotide reductase

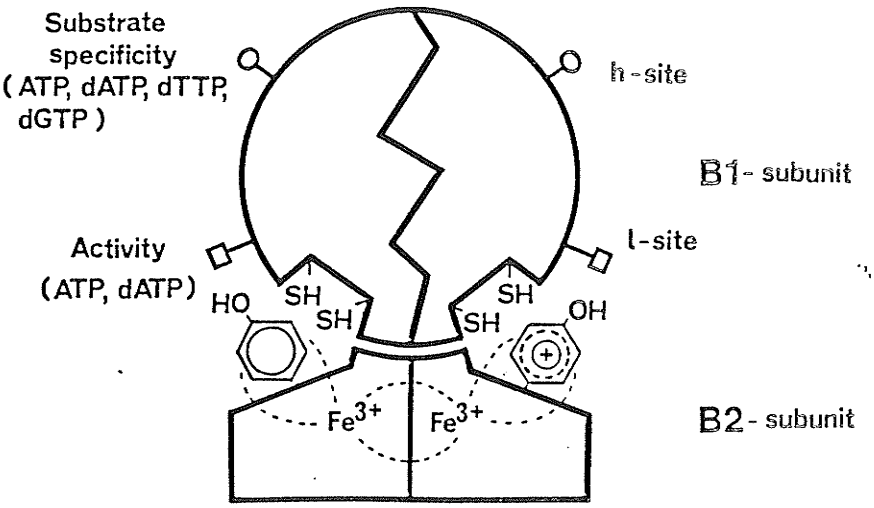
The E. coli ribonucleotide reductase is the best understood enzyme in terms of subunit interactions, allosteric regulation, and enzyme catalysis. Much of the research on this enzyme was carried out at the Karolinska Institute in Stockholm Sweden. The enzyme is

composed of two nonidentical subunits (Brown et al., 1969a) termed B1 and B2 encoded by the nrdA and nrdB genes (Bachmann et al., 1976) respectively. The purification and studies of the enzyme was greatly aided by the construction of an E. coli strain lysogenic for a defective lambda phage harbouring both the nrdA and nrdB genes (Eriksson et al., 1977). Roughly 50 times more enzyme can be produced in these strains upon induction than can be found in wild type E. coli cells (Eriksson et al., 1977).

Subunits B1 and B2 bind in a 1:1 ratio to form the active holoenzyme (Brown and Reichard, 1969a; Thelander, 1973); either subunit alone is void of activity (Brown et al., 1969a). The binding of the two subunits is believed to be weak and has an absolute requirement for magnesium ions (Brown and Reichard, 1969a). The holoenzyme is capable of reducing the ribonucleoside diphosphates CDP, ADP, UDP, and GDP at a common catalytic site (von Dobeln and Reichard, 1976); ribonucleoside triphosphates are not substrates for this enzyme. A schematic representation of the E. coli enzyme is shown in Figure 1 (from Thelander and Reichard, 1979). The B1 subunit has a molecular weight of 160,000 daltons and exists as a dimer composed of two nearly identical polypeptide chains (differing only in their amino termini) and has the general structure $\alpha\alpha'$ (Thelander, 1973). This subunit contains both substrate binding sites and effector binding sites (von Dobeln and Reichard, 1976; Brown and Reichard, 1969b). Two distinct types of effector binding sites have been revealed through binding studies, the high affinity sites (h-sites) and the low affinity sites (l-sites), defined by their ability to bind

Figure 1

Model of *E. coli* ribonucleotide reductase (taken from Thelander and Reichard, 1979).


Substrate
specificity
(ATP, dATP, dTTP,
dGTP)
h - site
B1 - subunit
Activity
(ATP, dATP)
l - site
HO
SH
SH
SH
SH
OH
Fe^{3+}
Fe^{3+}
B2 - subunit

dATP (Brown and Reichard, 1969b). The l-sites are capable of binding either dATP or ATP only; when dATP is bound, the enzyme is inactive and incapable of reducing ribonucleoside diphosphates, whereas the binding of ATP to this site acts as an overall positive allosteric effector for ribonucleotide reduction. The l-site therefore has been termed the activity site since it governs the overall activity of the enzyme. The h-sites on the other hand have been termed the substrate specificity sites since the binding of an effector nucleotide to this site results in a conformational change at the active site, in turn influencing which ribonucleoside diphosphate will preferentially be reduced (von Dobeln and Reichard, 1976; Larsson and Reichard, 1966a,b). CDP and UDP reduction are stimulated with ATP or dATP at the h-sites, GDP and ADP reduction are stimulated with dTTP at the h-sites, and ADP and GDP reduction are stimulated with dGTP at the h-sites (Brown and Reichard, 1969b). In addition, the binding of dTTP to the h-sites has a negative effect on CDP and UDP reduction. This complex allosteric regulation emphasizes the importance the cell puts on maintaining a sufficient as well as a balanced supply of deoxyribonucleoside triphosphates for DNA synthesis. Models for the relationship between the allosteric regulation of ribonucleotide reductase and the requirements of deoxyribonucleoside triphosphates for cell division will be discussed in part 2.2 of this section of this thesis.

The B2 subunit of ribonucleotide reductase has been purified to homogeneity and was found to have a molecular weight of 78,000 daltons (Thelander, 1973). It consists of two identical polypeptide chains (general structure β_2) and contains 2 molecules of iron per subunit

(Brown et al., 1969b). From Mossbauer (Atkin et al., 1973), and Raman spectroscopy studies (Petersson et al., 1980; Sjoberg et al., 1982), on ^{57}Fe-labeled B2 it was deduced that the iron atoms are present as a pair of inequivalent high spin Fe(III) ions in an antiferromagnetically coupled binuclear complex. Unique to the B2 subunit is the presence of a free radical, which from isotope substitution experiments, was assigned to a tyrosine residue in the protein (Sjoberg et al., 1977). The radical spin density is localized around the aromatic ring of the tyrosine residue (Sjoberg et al., 1978). It is believed that only one tyrosyl free radical is present per B2 subunit since (i) it has not been possible to obtain a greater radical content per two iron atoms (Eriksson et al., 1977), and (ii) more recently, it has been shown that the tyrosyl radical was generated in either one or the other, but not both, of the polypeptide chains of B2 (Sjoberg et al., 1987) . The free radical is characterized by an electoparamagnetic resonance (EPR) spectroscopy signal centered around g = 2.0047 and a sharp peak in the optical spectrum at 410 nm (Ehrenberg and Reichard, 1972). The free radical is closely linked to the presence of iron and both are essential for enzyme catalysis. The iron may be removed from the B2 subunit by dialysis against 8-hydroxy-quinoline to yield apo-B2, which then also lacks the free radical (Atkin et al., 1973; Brown et al., 1969a; Ehrenberg and Reichard, 1972). Treatment of the B2 subunit with the free radical scavanger, hydroxyurea, destroys the free radical, abolishes enzyme activity, but does not affect the iron centre (Atkin et al., 1973; Sjoberg et al., 1982). Activity can be restored following such treatment only after removal and reintroduction of the iron. The purpose of the iron

appears to be both generating the tyrosyl radical, probably by an iron-catalyzed one-electron aerobic oxidation, and to stabalize the radical by some continued interaction (Thelander and Reichard, 1979).

As mentioned previously, both the B1 and B2 subunits are required for enzyme activity. The B1 subunit in addition to containing substrate binding sites, contains oxidation-reduction active sulfhydrals capable of providing the reducing power required for catalysis (Thelander, 1974). Both the dithiols and the tyrosyl free radical have been implicated in the reaction mechanism (Reichard and Ehrenberg, 1983). Using the substrate analogue 2'azido-2'deoxycytidine 5'-diphosphate it was shown that the decay of the tyrosyl free radical occurred concomitantly with the formation of a transient nucleotide radical (Sjoberg et al., 1983) consistent with the hypothesis that ribonucleotide reduction occurs via a 3'-hydrogen abstraction and the formation of a cation radical in the substrate nucleotide (Stubbe and Ackles, 1980; Sjoberg et al., 1983).

1.4 Viral ribonucleotide reductases

Cells infected with a virus can induce the synthesis of deoxyribonucleotides required for viral replication. If the virus contains genes encoding its own ribonucleotide reductase, the viral enzyme is synthesized, if it does not encode its own ribonucleotide reductase, it utilizes deoxyribonucleotides produced via the reductase of the host. Viral ribonucleotide reductases have been difficult to identify since it is hard to distinguish them from the hosts enzyme. However,

there are two viral systems which have been shown to encode their own ribonucleotide reductase upon infection, one being bacteriophage T, and the other belonging to the herpes simplex virus group.

Bacteriophage T4, T5, and T6 induce a novel ribonucleotide reductase upon infection of E. coli. The T4 ribonucleoside diphosphate reductase has been the most extensively studied; it exists as a tetramer composed of two types of subunits, B1 and B2, and has a molecular weight of 225,000 daltons (Berglund, 1972a, 1975). The B1 subunit is composed of two polypeptide chains (general structure α_2) encoded by the nrdA gene of the virus, and the B2 subunit, also composed of two polypeptide chains (general structure β_2) is encoded by the viral nrdB gene (Yeh et al., 1969). The α and β chains have a molecular weight of 85,000 and 35,000 daltons respectively (Berglund, 1972a). The B1 subunit is very similar to the B1 subunit of E. coli, and contains binding sites for the allosteric effector, dATP (Berglund, 1972b), whereas the B2 subunit contains 2 moles of iron and contains a tyrosyl free radical which gives rise to an EPR doublet similar to, but distinct from that of the E. coli reductase (Berglund, 1975). Thus the T4 B2 subunit corresponds to the B2 subunit of E. coli. Both the B1 and the B2 subunits are required for enzyme activity and will not form active hybrids with the ribonucleotide reductase subunits from E. coli (Berglund, 1975). The allosteric regulation of the enzyme closely resembles that found in E. coli except that dATP does not act as a negative effector, instead, it stimulates the reduction of both CDP and UDP (Berglund, 1972b). It has been suggested that the phage enzyme is modulated by substrate specificity sites

only, and lacks activity sites characteristic of the E. coli enzyme (Thelander and Reichard, 1979). Unlike the enzyme from E. coli, the viral enzyme does not dissociate during purification and magnesium ions are not required for enzyme activity (Berglund, 1975). In addition, the T4 enzyme appears to be an order of magnitude more sensitive to the antitumor agent hydroxyurea (Bergland and Sjoberg, 1979).

Ribonucleotide reductase activity is dramatically increased after infection of mammalian cells with herpes simplex virus, Epstein-Barr virus or pseudorabies virus. Studies have suggested that these viruses encode their own ribonucleoside diphosphate reductase distinct from the host enzyme (Wright, 1983; Huszar and Bacchetti, 1983; Cohen et al., 1986). One of the most convincing of such studies is the induction of a novel enzyme after infection of mouse L cells with pseudorabies virus. After infection with this virus, ribonucleotide reductase activity was not affected by M1 antibody (directed against calf thymus ribonucleotide reductase), whereas the activity in an extract from uninfected cells was completely neutralized (Lankinen et al., 1982). Furthermore, virus infected cells contained an increase in the EPR signal which was different from the EPR signal characteristic of mouse cell ribonucleotide reductase. The enzyme activity from virally infected cells showed the same sensitivity to hydroxyurea as the enzyme preparation from noninfected mouse cells, however, CDP reduction in extracts prepared from infected cells was not influenced by ATP or the negative allosteric effectors dTTP and dATP. These results suggest some similarity between the pseudo-

rabies induced ribonucleotide reductase and bacteriophage T4 ribonucleotide reductase since they both appear to lack negative allosteric effectors.

1.5 Manganese-depedent ribonucleotide reductaes

That manganese was essential for cell division in some gram-positive bacteria was known for many years. Indeed, Webley (Webley, 1960; Webley et al., 1962) back in the early 1960's observed that manganese was required for optimum growth of Nocardia opaca, and later, Oka et al. (1968) and Auling et al. (1980) demonstrated that the concentration of manganese controls the overall synthesis of DNA, while having no effect on RNA, protein, or cell wall synthesis in Brevibacterium ammoniagenes. Later, it was found that cellular extracts prepared from B. ammoniagenes and Micrococcus luteus cultured in the absence of manganese contained an inactive apo-ribonucleotide reductase (Schimpff-Weiland et al., 1981) whose activity can be restored upon incubation in 10mM Mn^{++}. In addition, DNA synthesis can be resumed in manganese starved cultures if manganese is added to the medium. Thus the primary target of manganese starvation appears to be ribonucleotide reductase.

It is believed that the manganese in the Brevibacterium enzyme seves an analogous function as the iron found in the E. coli and mammalian enzymes (Lammers and Follmann, 1983). Although ferrous ions can replace manganese in the Brevibacterium apoenzyme, albeit not as effectively, cobalt or zinc ions can not. Cyanide which has no effect

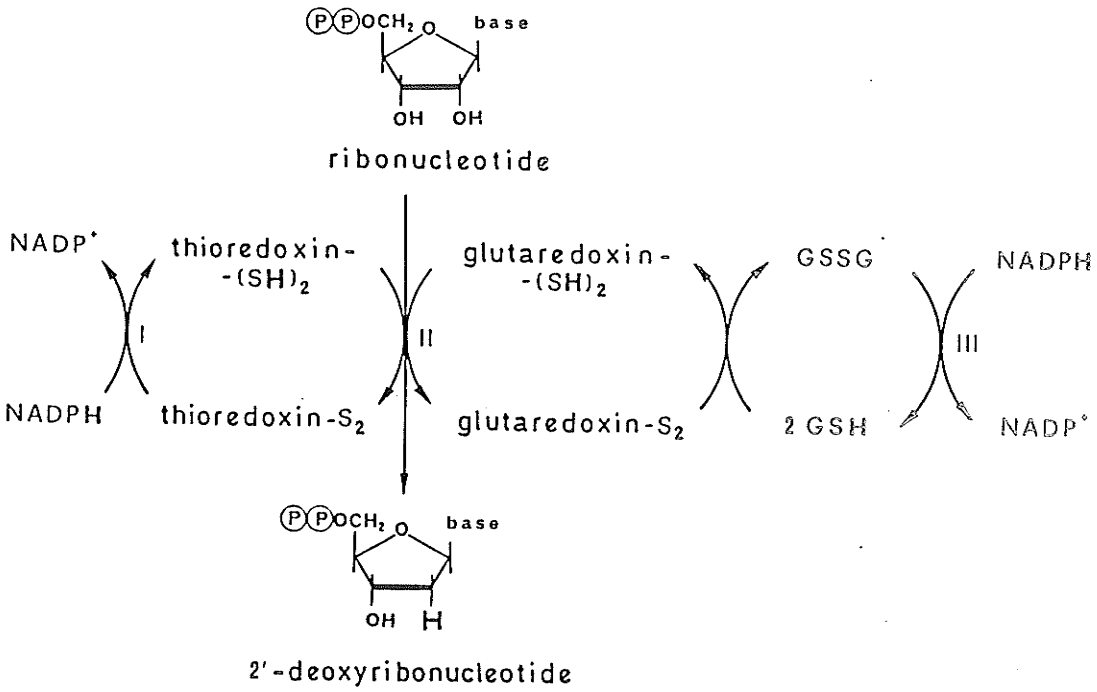
on non-heme iron containing ribonucleotide reductases, inhibits manganese containing reductases, perhaps via the formation of cyano and cyanaquo complexes with Mn^{++}. The preferred substrates for the Brevibacterium enzyme are the guanosine nucleotides, as opposed to adenine, cytidine, and uridine nucleotides which are reduced at lower rates. Both the triphosphate and diphosphate forms of the nucleotides are reduced. The allosteric regulation of the enzyme has not yet been studied in great detail, however it was found that dTTP stimulates the reduction of GTP and dATP stimulates CTP reduction (Hogenkamp, 1984).

1.6 Hydrogen transport systems

Reducing power required for the reduction of nucleotides in vitro can be provided directly by dithiols such as dithiothreitol or reduced lipoate; monothiols such as mercaptoethanol or glutathione are unable to support the reaction (Reichard, 1962). Two in vivo hydrogen donor systems capable of supplying the reducing power for the reaction have been identified (Fig. 2). The first system, discovered in E. coli (Laurent et al., 1964; Moore et al., 1964), and subsequently shown to be present in L. leichmannii (Orr and Vitols, 1966) and eucaryotes (Moore, 1967; Porque et al., 1970; Wagner and Follmann, 1977) is the thioredoxin system. The key component of this system is a small (molecular weight (MW) of 11,700 daltons) heat-stable protein, thioredoxin, containing an oxidation-reduction active disulfide (Holmgren, 1968). The reduced form of thioredoxin can serve as a direct hydrogen donor for ribonucleotide reductase. The oxidized form of the protein is in turn reduced by thioredoxin reductase, a FAD-protein containing

Figure 2

Hydrogen transport systems for ribonucleotide reduction. The enzyme reactions are I: thioredoxin reductase, II: ribonucleotide reductase, III: GSH, GSSG: reduced and oxidized glutathione; NADPH, NADP: reduced and oxidized nicotinamide adenine dinucleotide phosphate coenzymes (taken from Lammers and Follmann, 1983).


PPOCH2 O base
OH OH
ribonucleotide
NADP+
thioredoxin-(SH)2
glutaredoxin-(SH)2
GSSG
NADPH
I
II
III
NADPH
thioredoxin-S2
glutaredoxin-S2
2 GSH
NADP+
PPOCH2 O base
OH H
2'-deoxyribonucleotide

one oxidation-reduction active disulfide (Moore et al., 1964). The ultimate source of reducing power is provided by NADPH. The second system capable of providing reducing power for the ribonucleotide reduction reaction was first discovered in an E. coli mutant, tsnC 7004 (Holmgren, 1976), which is a prototrophic mutant devoid of any thioredoxin activity as a result of a nonsense or deletion mutation (Holmgren et al., 1978). The mutant, however, was unaffected in its ability to reduce ribonucleotides (Holmgren, 1976). The hydrogen transport system identified in extracts of tsnC 7004 was the glutaredoxin system, consisting of NADPH, glutathione reductase, glutathione and glutaredoxin (Fig. 2). Glutaredoxin, like thioredoxin, is a small acidic protein (about 89 amino acids), containing one oxidation-reduction active disulfide capable of providing reducing power for ribonucleotide reductase (Holmgren, 1979), likely via the active disulfides of the B1 subunit (Thelander and Reichard, 1979). Glutaredoxin is in turn reduced to a dithiol by glutathione (GSH), and NADPH-glutathione reductase. The main difference between the thioredoxin and glutaredoxin systems lies in the reduction of their sulfhydral groups by NADPH. Glutaredoxin is reduced via glutathione and glutathione reductase, and thus has the ability to utilize the monothiols of GSH, whereas thioredoxin is reduced directly by the flavoprotein thioredoxin reductase (Thelander and Reichard, 1979; Holmgren, 1981). The glutaredoxin system has also been detected in mammalian sources such as calf thymus (Luthman et al., 1979).

Although the thioredoxin system can function in vitro as a hydrogen donor system for ribonucleotide reductase, its involvement in

other physiological functions in mammalian cells at present is not clear (Thelander and Reichard, 1979; Holmgren, 1981; Hansson et al., 1986). The only established role for thioredoxin is in E. coli, where upon infection of the cell with bacteriophage T7, thioredoxin forms the active phage T7 DNA polymerase by combining with the phage-coded gene 5 protein (Mark and Richardson, 1976). In calf thymus, thioredoxin is distributed in multiple locations including the cell membrane (Holmgren and Luthman, 1978) suggesting that thioredoxin has functions in protein thiol-disulfide interchange reactions other than that involving ribonucleotide reductase (Holmgren, 1981). Other physiological roles of glutaredoxin besides its involvement in ribonucleotide reduction remain unknown.

1.7 Reaction mechanism

All three classes of ribonucleotide reductases (adenosylcobalamin dependant, manganese dependant, and the reductases characterized by an organic free radical with a binuclear Fe(III) center) catalyze the reduction of ribonucleotides to their corresponding 2'-deoxyribonucleotides with a concommitment oxidation of a protein dithiol (Thelander, 1974). In order for catalysis to proceed, the dithiol must subsequently be reduced. Despite their differences in cofactor requirement, the chemical reaction mechanism for the different classes of reductases is believed to be rather similar. In fact, ribonucleoside triphosphate reductases and ribonucleoside diphosphate reductases have many common features. Both reductases catalyze reactions in which the 2'-hydroxyl group of the substrate is replaced

by a hydrogen atom which is ultimately derived from the solvent. The steriochemistry about the 2′ carbon is retained following the reduction process. In addition, reduction of the substrate is coupled to the oxidation of a dithiol, which is subsquently reduced by an exogenous system. Moreover, both ribonucleoside triphosphate reductase and ribonucleoside diphosphate reductases can utilize the thioredoxin/thioredoxin reductase system as a source of reducing power (Thelander and Reichard, 1979). Indeed, the E. coli thioredoxin/thioredoxin reductase system can reduce Herpes Simpex virus ribonucleoside diphosphate reductase as well as the ribonucleotide triphosphate reductase from Lactobacillus.

Another feature shared by the different reductases is that they are inactivated by 2′-azido-2′-deoxynucleotides and by 2′-deoxy-2′-halonucleotides (Ashley and Stubbe, 1985). Furthermore, the fact that adenosylcobalamin can generate radical species upon homolysis of the carbon-cobalt bond (Babior and Krouwer, 1979) and, the fact that ribonucleoside diphosphate reductases contains a stable organic radical are suggestive that both reaction mechanisms involve radical chemistry.

Many models for the mechanism of reduction of ribonucleotides by ribonucleoside triphosphate reductases have been proposed (for a review see Stubbe, 1983). The observation that 2′-deoxynucleosides can be formed from ribonucleosides via free radical reactions (Robins and Wilson, 1981), together with the knowledge of the presence of a free radical in ribonucleoside diphosphate reductases, directed researchers

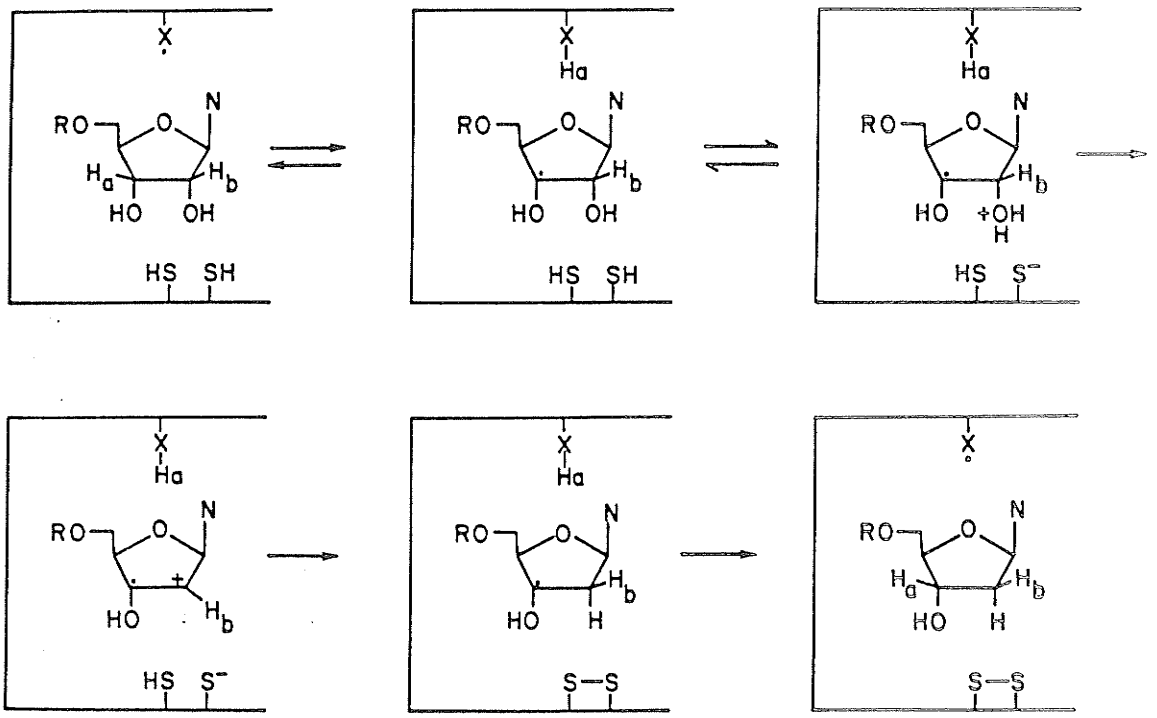
to consider free radical mechanisms for ribonucleotide reduction (Ashley aned Stubbe, 1985). In fact, it should be emphasized that enzyme activity is directly correlated to the radical content (Ehrenberg and Reichard, 1972), thereby implicating the free radical in catalysis. The reaction mechanism currently favored is presented in Figure 3 (taken from Ashley and Stubbe, 1985). This scheme assumes the presence of both a radical group (designated Enz-X·) and a dithiol at the active site of the enzyme. The reaction is envisioned to occur as follows. Enz-X· abstracts the 3′ hydrogen atom. This results in the formation of a nucleotide radical intermediate. A radical cation is then formed and a H_2O molecule is lost following protonation of the 2′-hydroxyl group via one of the active site thiols. Hybride reduction of the radical cation intermediate by the dithiol would then generate the product radical. Regeneration of the active site radical and the formation of product occurs following abstraction of the hydrogen atom from Enz-XH.

The above scheme allows for several testable predictions (Ashley and Stubbe, 1985). One is that the 3′ carbon-hydrogen bond has to be broken during the reduction of the substrate. Another prediction is that following cleavage of the 3′ carbon-hydrogen bond, the same hydrogen atom is returned to this position at the completion of the reaction. Studies using radioactive isotopes of substrates have supported both predictions. Reactions carried out using [3′-^{3}H]ADP and [3′-^{3}H]UDP by ribonucleoside diphosphate reductase and the corresponding triphosphates by ribonucleoside triphosphate reductase indicated that a small quantity of ^3H_2O was released into the solvent, demon-

Figure 3

Proposed scheme for the mechanism of ribonucleotide reductase catalysis. X = protein (taken from Ashley and Stubbe, 1985).

X= protein



strating that the hydrogen atom on the 3'-carbon is located in an exchangeable position during turnover. In addition, following reduction of the substrates [3'-^{2}H]UDP and [3'-^{2}H]UTP by ribonucleoside diphosphate and triphosphate reductases respectively, NMR spectroscopy on the products revealed that the 3'-position was completely deuterated within detectable limits. This, finding together with that showing cleavage of the 3' carbon-hydrogen bond during turnover, demonstrates that the same hydrogen atom removed from the 3' carbon is replaced following the reaction.

Another prediction which the reaction scheme poses is that the visible absorbance of the tyrosyl radical (which has an optical absorbance at 410 nm) would transiently disappear as a result of abstraction of the 3'-hydrogen atom during turnover. Although stopped-flow spectroscopy studies were not successful in demonstrating any decrease in absorbance of protein B2 during catalysis, the involvement of the tyrosyl radical in the reaction mechanism can not be ruled out. It is possible that the rate of hydrogen abstraction by the tyrosyl radical is less than the rate of return of the hydrogen atom from the tyrosine (Enz-XH) to the intermediate, thereby resulting in a very short half life and very low concentrations of the reduced tyrosine species which can not be detected. Alternatively, a steady-state between the abstraction of the hydrogen atom and its return to the product may have been reached within the dead time of the stopped-flow spectrophotometer. Either of the above possibilities are reasonable explanations for the failure to observe any decrease in absorbance of the tyrosyl radical using stopped-flow spectroscopy.

A radical mechanism has been implicated in adenosylcobalamin-dependant ribonucleotide reductase reactions (Ashley and Stubbe, 1985). Rapid-freeze EPR and stopped-flow spectroscopy have both indicated the formation of cob(II)alamin following homolytic cleavage of the carbon-cobalt bond of adenosylcobalamin. Based on the well documented chemistry of adenosylcobalamin (Orme-Johnson et al, 1974; Tamao and Blakley, 1973), it is believed that the second product of homolysis is the 5'-deoxyadenosyl radical. It is postulated that the reaction mechanism proceeds in a similar fashion to that catalyzed by tyrosyl free radical containing reductases.

2 MAMMALIAN RIBONUCLEOTIDE REDUCTASE

2.1 Structural aspects

Mammalian ribonucleotide reductase, like that of E. coli, is composed of two dissimilar subunits frequently called M1 and M2, both of which are required for enzyme activity (Hopper, 1972; Cory et al., 1978; Thelander et al., 1980). The enzyme utilizes CDP, ADP, GDP and UDP nucleoside diphosphates as substrates. With one exception, the deoxyribonucleoside diphosphates are subsequently phosphorylated by nucleoside diphosphate kinase to the triphosphates, the direct precursors of DNA synthesis. The exception is dTTP which is formed from the reduction of CDP or UDP. Mammalian ribonucleotide reductase is the sole enzyme responsible for the de novo synthesis of deoxyribonucleosides, and therefore occupies a key position in the DNA synthetic pathway. Due to the relatively low levels of ribonucleotide

reductase in mammalian cells, homogeneous preparations of the enzyme have been very difficult to obtain. Attempts to purify the enzyme have been carried out from a variety of sources including Novikoff hepatoma (Moore, 1977), rabbit bone marrow (Hopper, 1972; 1978), calf thymus (Engstrom et al. al, 1979; Thelander et al., 1980; Mattaliano et al., 1981), lamb thymus (McClarty et al., 1986a,b), human lymphoblasts (Chang and Cheng, 1979a), Ehrlich tumor cells (Cory et al., 1978; Cory and Fleischer, 1982a), hamster cells (Lewis and Wright, 1978a) and regenerating rat liver (Youdale et al., 1982); all preparations had various degrees of purity. A number of the studies reported the separation of the enzyme into two fractions, each devoid of ribonucleotide reductase activity alone, but when recombined activity was restored (Hopper, 1972; Moore, 1977; Cory et al., 1978; Chang and Cheng, 1979a; Engstrom et al., 1979; Thelander et al., 1980), suggesting that the mammalian enzyme, like that of E. coli, is composed of two types of subunits. It was not until 1980 that one of the subunits, the M1 subunit, was purified to homogeneity from calf thymus (Thelander et al., 1980). This subunit is composed of two identical polypeptide chains; each monomer (5.7 S) having a molecular weight of 84,000 daltons, and containing the binding sites for the allosteric effectors of ribnonucleotide reduction (Thelander et al., 1980).

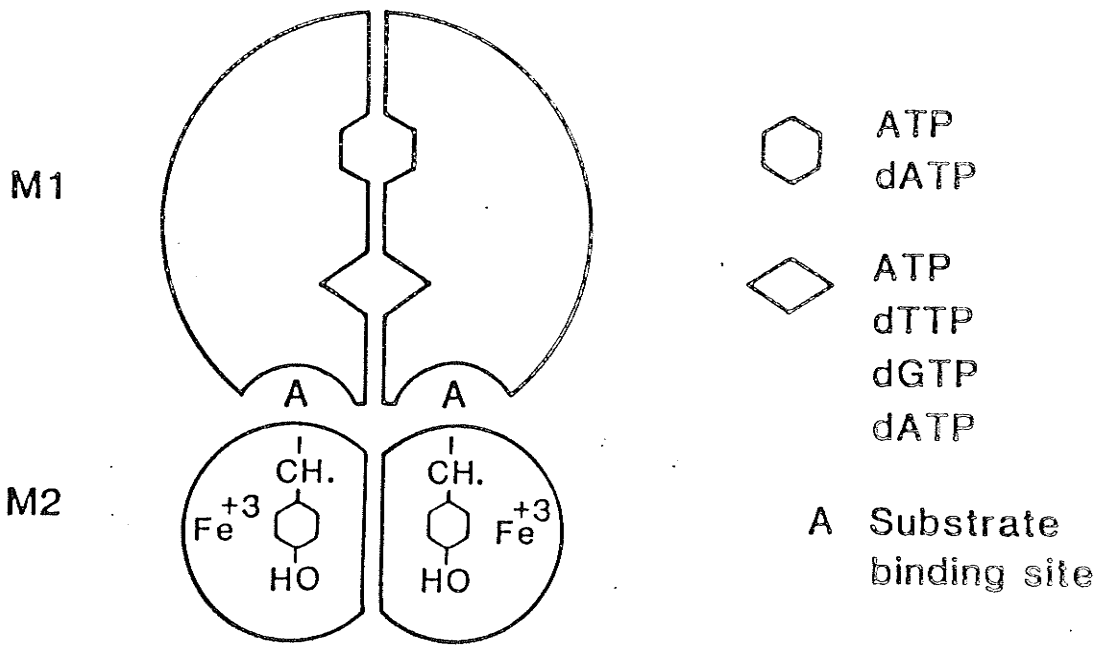
Addition of dTTP to a solution of monomeric M1 subunits lead to the formation of dimers (8.8 S), whereas tetramers (15.2 S) were formed following the addition of dATP. ATP induced the formation of both dimers and tetramers (Thelander et al., 1980). Similar observa-

tions where made on partially purified enzyme preparations from Ehrlich tumor cells (Klippenstein and Cory, 1978). These changes in sedimentation coeffecients are interpreted as reflecting conformational changes induced by the allosteric effectors. Equlibrium dialysis experiments indicated that a maximum of 1.8 mol of dATP or 0.7 mol dTTP was bound per 170,000 g of calf thymus M1 protein (Thelander et al., 1980). In addition, competition experiments indicated that ATP competed with dATP for binding at two separate sites. ATP in a molar excess of 2000 fold was required to block one of the dATP binding sites, whereas a 10,000 fold excess of ATP was required to remove dATP from the second site. dGTP and dTTP were able to block dATP binding at only one of the binding sites. dGTP was required in a 10 fold excess whereas dTTP was required in a 50 fold excess in order to remove about 30% of the bound dATP. Further increases in the concentrations of dGTP and dTTP were unable to reduce dATP binding to protein M1 by more than 40%. Taken together, the equilibrium dialysis experiments and the competition experiments indicated that the M1 subunit contains two types of binding sites for the allosteric effectors of ribonucleotide reductase, one which binds dATP and ATP only, the other in addition to binding ATP and dATP also binds dTTP and dGTP (Thelander et al., 1980). A schematic representation of the mammalian enzyme is shown in Figure 4.

Purification of the M2 subunit of mammalian ribonucleotide reductase proved to be extremely difficult. The first demonstration of a homogeneous preparation of the M2 subunit was by the Swedish group (Thelander et al., 1985), who successfully purified this component

Figure 4

A model for mammalian ribonucleotide reductase (taken from Wright, 1988).


M1
M2
A
A
CH.
CH.
Fe+3
Fe+3
HO
HO
ATP
dATP
ATP
dTTP
dGTP
dATP
A Substrate
binding site

from M2-overproducing mouse TA 3 cells. The M2 subunit, like the B2 subunit of the E. coli enzyme, is a dimer composed of two identical polypeptide chains each having a molecular weight of 44,000 daltons. This subunit contains stoichiometric amounts of a non-heme iron centre and a unique tyrosyl free radical (Thelander et al., 1985; McClarty et al., 1987a; Wright, 1983, 1988), both of which are required for enzyme activity. The mammalian M2 component shows a distinctive electronic spectrum due to the presence of the tyrosyl free radical, having a shoulder at 395 nm and a peak at 416 nm (Thelander et al., 1985) as opposed to the spectrum of the B2 subunit of E. coli which gives rise to a shoulder at 390 nm and a peak at 410 nm (Petersson et al., 1980). This difference can most likely be attributed to differences in the amino acid sequences around the tyrosyl free radical, differences which are also manifested in the low temperature EPR spectra of proteins B2 and M2 (Graslund et al., 1982). It is, however, believed that the iron centre in the mammalian enzyme is very similar to that present in E. coli (Thelander et al., 1985), [which consists of an antiferromagnetically coupled pair of high spin ferric atoms mediated by a μ-oxo bridge (Petersson et al., 1980; Sjoberg and Graslund, 1983)], since there is great similarity in the iron-related bands in the spectrum of protein M2 as compared to that of protein B2. The iron centre in protein M2 most likely is required for radical stabilization (Graslund et al., 1982). Since the tyrosyl free radical in protein M2 gives rise to an unique EPR signal it is possible to quantitate the levels of active M2 protein in whole packed cells or in cell free preparations (Graslund et al., 1982; Wright et al., 1987; McClarty et al., 1987a; Tagger and Wright, 1988).

A key difference between the mammalian M2 protein and the E. coli B2 protein is in their ability to regenerate the tyrosyl free radical structure following inactivation by hydroxyurea. In the case of the bacterial B2 protein, the iron must be removed, and reintroduced as Fe^{2+} in the presence of O_2 in order for radical regeneration to occur (Petersson et al., 1980). In contrast, protein M2 regenerates the free radical readily if incubated in the presence of iron-dithiothreitol and air (Graslund et al., 1982; Thelander et al., 1985), i.e. iron extraction and reintroduction is not required. A possible explanation for this observation would be that dithiothreitol is able to reduce the endogenous Fe^{3+} in situ in M2 but not in B2. The reaction between Fe^{2+}, tyrosine and oxygen can then give rise to both the Fe^{3+} pair and the oxidized tyrosine radical, while the oxygen is reduced (Graslund et al., 1982; Thelander et al., 1985). Consistent with such a postulation are studies using inhibitors of protein M2 which have demonstrated that the iron centre is more exposed in the mammalian protein as compared to the bacterial protein (Kjolerr Larson et al., 1982).

Another difference between the mammalian ribonucleotide reductase and that of E. coli is the requirement for magnesium ions. The bacterial enzyme has an absolute requirement for magnesium in contrast to the mammalian enzyme which does not. Enzyme activity from mammalian cells is, however, augmented in the presence of magnesium ions (Eriksson et al., 1979).

Several studies have suggested the existence of multiple forms of

mammalian ribonucleotide reductase which are specific for the reduction of each ribonucleoside diphosphate substrate (Moore and Hulbert, 1966; Collins et al., 1972; Cory and Mansell, 1975; Peterson and Moore, 1976; Blocker and Roth, 1977; Lewis et al., 1978; Youdale and MacManus, 1979; Youdale et al., 1982; Hards and Wright, 1983). It has been postulated (Whitfield and Youdale, 1985) that the M1 subunit is available in four different forms or modules ($M1^{CDP}$, $M1^{ADP}$, $M1^{GDP}$, and $M1^{UDP}$). The substrate specificity of the enzyme is then dependant on which modules (maximum of four) are bound to a common M2 component. However, the Whitfield-Youdale model is not widely accepted since purified, but not homogeneous, ribonucleotide reductases from sources such as rabbit bone marrow (Hopper, 1978), calf thymus (Eriksson et al., 1979; Engstrom et al., 1979) and human cells (Chang and Cheng, 1979a,b,c) have suggested that a single enzyme is capable of reducing all four ribonucleoside diphosphate substrates. Moreover, molecular genetic studies have not supported the concept of a family of ribonucleoside diphosphate reductase genes in mammalian cells (Engstrom and Francke, 1985; Yang-Feng et al., 1987; Tonin et al., 1987; Caras et al., 1985; Thelander and Berg, 1986). However, the possibility that posttranscriptional modifications to the M1 protein may occur leading to several different species has not been eliminated. Indeed, two-dimensional gel electrophoresis studies indicate the presence of multiple M1 proteins, detected by M1 specific antibody (Cocking et al., 1987).

2.2 Allosteric regulation

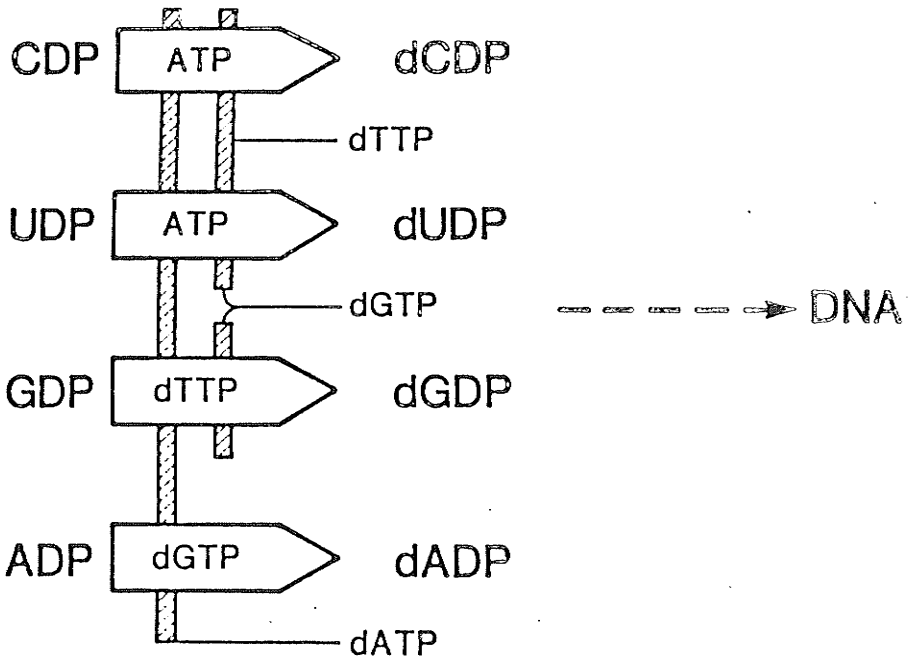
Mammalian ribonucleotide reductases are subject to complex allosteric regulation by nucleoside triphosphate effectors. The first demonstration of the allosteric nature of mammalian ribonucleotide reductase was made on enzyme present in extracts of chick embryos (Reichard et al., 1961). In most cases, enzyme kinetic analysis have been performed on partially purified preparations of the enzyme, or in some cases, enzyme kinetics were studied in vivo using a cell permeabilization assay (e.g. Lewis et al., 1978; Hards and Wright, 1984a). Kinetic analysis of ribonucleotide reductase activity have been carried out by many investigators on a variety of mammalian sources including Novikoff hepatoma (Moore and Hulbert, 1966), regenerating rat liver (Larsson, 1969), rat embryo cells (Murphree et al., 1968), mouse Ehrlich tumor cells (Cory, 1979), Chinese hamster ovary cells (Lewis et al., 1978; Wright et al., 1981; Hards and Wright, 1983, 1984a,b; Tagger and Wright, 1988), mouse L cells (Kuzik and Wright, 1979; Kucera and Paulus, 1982; McClarty et al, 1986a,b) mouse T lymphoma cells (Eriksson et al., 1981a), rat myoblasts (Creasy and Wright, 1984), human Molt-4F cells (Chang and Cheng 1979b,c), and human diploid fibroblasts (Wright et al., 1981; Dick and Wright, 1984, 1985). Studies on enzyme activity from the various sources listed above, have revealed very similar patterns of allosteric regulation (Fig. 5). In general, the reduction of CDP and UDP requires the presence of ATP, which is used as an effector and not as an energy source (Kucera and Paulus, 1982). GDP reduction requires the presence of dTTP, whereas ADP reduction requires dGTP as a positive effector.

The reduction of any of the four ribonucleoside diphosphates is strictly dependent upon the presence of a positive effector molecule. In addition, dGTP is a negative feedback inhibitor of GDP reduction, as well as a good inhibitor of pyrimidine reduction; dTTP is an inhibitor of pyrimidine reduction. Both purine and pyrimidine reduction are inhibited by dATP, an inhibition which can be reversed by ATP. The allosteric regulation of the mammalian enzyme bears some similarity to that of E. coli. It should be noted, however, that in some mammalian systems, GTP in addition to dGTP, was found to act as a positive allosteric effector of GDP reduction (Chang and Cheng, 1979c; Wright et al., 1981; Hards and Wright, 1984a,b), and that dATP at very low concentrations may in fact stimulate ribonucleotide reduction (Lewis et al., 1978).

As discussed above, the M1 component of calf thymus ribonucleotide reductase is thought to have two types of binding sites, an activity site (which binds ATP and dATP) regulating overall enzyme activity, and a specificity site (which binds ATP, dATP, dTTP and dGTP) regulating substrate specificity. A sequential regulation scheme has been postulated (Eriksson et al., 1979) in order to account for the nature of deoxyribonucleoside triphosphate production (Fig. 5). In this scheme, reduction of CDP and UDP occur first via an ATP activated enzyme, reduction of GDP follows by a dTTP regulated enzyme, and finally ADP is reduced via a dGTP activated enzyme. Accumulation of dATP (in the slowing down or absence of DNA synthesis) inhibits ribonucleotide reduction completely.

Figure 5

Schematic representation of the allosteric regulation of mammalian ribonucleotide reductase. The nucleotides shown in the arrows act as positive effectors and the bars indicate inhibitory effects (taken from Wright, 1988).


CDP
ATP
dCDP
dTTP
UDP
ATP
dUDP
dGTP
DNA
GDP
dTTP
dGDP
ADP
dGTP
dADP
dATP

The model presented above represents only one out of many which have been postulated throughout the years (Hunting and Henderson, 1982), and may in fact lead to an oversimplification of the regulation of deoxyribonucleoside pool composition in intact cells (Jackson, 1984; Fox, 1985). It should be noted that although ribonucleotide reductase occupies a key position in the DNA synthetic pathway, and probably has the greatest effects in determining the intracellular deoxyribonucleoside triphosphate pool sizes, there are other cellular enzymes such as dCMP deaminase which occupy important positions as well, and whose activities will also effect the composition of deoxyribonucleoside triphosphate pool sizes (Jackson, 1984).

In addition to the allosteric regulation of ribonucleotide reductase activity by nucleotide effectors, enzyme activity may be regulated in the cell through naturally occurring compounds. Indeed, several laboratories have reported the presence of naturally occurring inhibitors of mammalian ribonucleotide reductase. In very few cases however, was the inhibitor characterized and identified. High molecular weight heat-labile inhibitors have been detected in normal rat liver (Elford, 1972; Ikenaka et al., 1981; Cory, 1979). Levels of the inhibitor were found to be high in normal liver, which had no detectable ribonucleotide reductase activity, as compared to regenerating or neonatal liver which had lower levels of inhibitor and high levels of ribonucleotide reductase activity (Elford, 1972). Unfortunately the nature of these inhibitors remains unknown; as suggested, (Elford, 1972; Ikenaka et al., 1981) the inhibitors very well might be phosphatases which degrade the substrate CDP, or the allosteric

effector, ATP, and thus interfere with the enzymatic assay of ribonucleotide reductase. Clearly, more studies are required to determine the nature of these high molecular weight inhibitors. Low molecular weight inhibitors have been isolated from Ehrlich tumor cells (Cory and Monly, 1970). These inhibitors were not identified, but they were orcinol and diphenylamine negative and therefore probably not nucleotides. In addition, they varied in their molecular weights and their spectral properties, all having UVmax between 245 and 273 nm. A highly phosphorlyated dinucleoside, HS3, was isolated from the fungus Achlya which inhibited both fungal ribonucleotide reductase activity and ribonucleotide reductase activity from CHO cells (Lewis et al., 1976). A similar if not identical coumpound was also isolated from CHO cells undergoing glutamine starvation (Lewis et al., 1977). The coumpound inhibited partially purified CHO ribonucleotide reductase in a noncompetitive manner, having Ki values between 14 and 23 uM for CDP, ADP and GDP substrates. The chemical constituents of HS3 were determined to be uracil, adenine, mannitol, a substituent which after hydrolysis becomes glutamate, and phosphates (McNaughton et al., 1978). As might be expected, the inhibitor accumulated rapidly in CHO cells as the rate of DNA synthesis declined. The regulation of ribonucleotide reductase activity throughout the cell cycle by the HS3 coumpound is unknown, however, naturally occuring inhibitors, such as HS3, may provide yet another level at which ribonucleotide reductase activity can be regulated.

2.3 Mammalian hydrogen donor systems

Both the thioredoxin and the glutaredoxin systems discussed previously have been isolated from different mammalian sources (Luthman et al., 1979; Moore, 1967; Herrmann and Moore, 1973; Engstrom et al., 1974). Mammalian ribonucleotide reductases in addition to being able to use chemical dithiols such as dithiothreitol as a source of reducing power, are also capable of utilizing thioredoxins from a variety of sources (Moore and Reichard, 1964; Moore, 1977; Luthman et al., 1979). Homogeneous preparations of thioredoixin have been prepared from calf liver (Engstrom et al., 1974) and Novikoff hepatoma (Herrmann and Moore, 1973); both proteins having molecular weights of about 12,000 daltons. Interestingly the mammalian thioredoxins are reduced by mammmalian thioredoxin reductases and are not capable of being reduced by the enzyme from E. coli (Thelander and Reichard, 1979). Thioredoxin reductases have been purified from calf liver, calf thymus (Holmgren, 1977) and Novikoff hepatomas (Chen et al., 1977). The enzyme from Novikoff hepatomas is composed of two subunits each having a molecular weight of about 58,000 daltons, and contains one FAD molecule per subunit.

At present it is unknown whether both the gluterodoxin and the thioredoxin systems are utilized in vivo as hydrogen donor systems for mammalian ribonucleotide reduction, or whether one systems predominates over the other. Recent observations have, however, showed, that at least in adult rat tissues, there was no correlation between cells which contained thioredoxin and cells which contained the M1

subunit of ribonucleotide reductase, indicating that the thioredoxin system probably has additional physiological functions other than as a hydrogen donor system for ribonucleotide reductase (Hansson et al., 1986).

2.4 Ribonucleotide reductase and DNA synthesis

DNA synthesis requires a steady and balanced supply of the four deoxyribonucleoside triphosphates (dNTP) (Thelander and Reichard, 1979; Wright, 1983; Cory, 1983). Just prior to the onset of DNA synthesis there is a dramatic rise in the concentration of dNTPs, which in resting cells are usually in the micromolar range (Cory and Sato, 1983; Skoog et al., 1974; Skoog and Nordenskjold, 1971). This increase in dNTP pool size reaches a maximum during S phase (Skoog et al., 1974), and correlates with the S phase increase in ribonucleotide reducatse activity which is observed in many cell systems (Wright 1983, 1988; Cory and Sato, 1983; Cory, 1983). Interestingly, although DNA synthesis requires an equal supply of all four dNTPs, large differences between the four pool sizes exist during S phase. The dGTP pool is always the smallest, sufficing for possibly only 15 seconds of DNA replication. The dCTP pool on the other hand is usually the largest in size; in some instances it surpasses the size of the dGTP pool by 100-fold (Skoog et al., 1974). Although it is not an allosteric effector of mammalian ribonucleotide reductase, the variations in the dCTP pool were found to most closely reflect the rate of DNA synthesis as compared to the other dNTPs (Reichard, 1978). Indeed, it has been suggested that dCTP may be one factor involved in the

regulation of DNA synthesis (Bjursell and Reichard, 1973; Reichard, 1978), perhaps by interacting with S phase DNA synthetic enzymes such as DNA polymerase.

The key position ribonucleotide reductase occupies in DNA synthesis is evident by the tight correlation which is observed between the presence of enzyme activity and cell proliferation. Nondividing cells contain low and sometimes even undetectable levels of enzyme, whereas rapidly dividing cells and organs such as thymus, spleen and bone marrow which contain actively dividing cells show high enzyme activities (Millard, 1972; Elford, 1972; Takeda and Weber, 1981). In addition, as mentioned above, the enzyme is regulated during the cell cycle, showing greatest activity during S phase and returning to a low basal level following DNA synthesis (Peterson and Moore, 1976; Lewis et al., 1978; Lewis and Wright, 1979; Cory and Whitford, 1972; Murphree et al., 1969). Since ribonucleotide reductase appears to be a rate limiting enzyme in the synthesis of dNTPs, it is believed to play a key regulatory role in cell division (Reichard, 1978; Bjursell and Skoog, 1980; Takeda and Weber, 1981; Wright et al., 1981; Engstrom et al., 1985; Cohen et al., 1987; Wright et al., 1987, 1988).

Although the S phase specific increase in ribonucleotide reductase activity has by some investigators been attributed to changes in the allosteric regulation of the enzyme (Cory and Sato, 1983; Albert and Gudas, 1985), it is generally accepted now that the increase is in fact due to de novo synthesis of ribonucleotide reductase (Jackson, 1984; Eriksson et al., 1984). Experiments using

the protein synthesis inhibitors cycloheximide or chloramphenicol indicated that the initiation of ribonucleotide reductase activity can be blocked by these agents as the cells approached S phase. This provided support for de novo enzyme synthesis as the cell traverses through the cell cycle (Feller et al., 1980; Lowden and Vitols, 1973; Gleason, 1979; Elford, 1972). Furthermore, cyclohexamide treatment resulted in the blockage of ribonucleotide reductase activity in neonatal rat spleen and Novikoff hepatoma cells (Elford, 1972). Unfortunately, since cyclohexamide treatment may in fact lead to the inhibition of other cellular processes (e.g. posttranslational modifications) these experiments could not conclusively establish that the increases in ribonucleotide reductase activity observed was a result of de novo synthesis of the enzyme (Lammers and Follmann, 1983). Concrete evidence was, however, provided by isotope exchange studies performed by the Swedish group which showed, that in mouse TA 3 cells, the S phase correlated increase in ribonucleotide reductase activity can solely be attributed to the de novo synthesis of protein M2 (Eriksson et al., 1984). The M1 subunit on the other hand was shown to be present in about equal amounts throughout the various stages of the cell cycle (Engstrom et al., 1985). These observations have been supported by studies with mutant mammalian cell lines with elevated ribonucleotide reductase activity, which have clearly shown that M1 and M2 are regulated by different mechanisms and that the M2 protein is limiting for activity (Wright et al., 1988; Choy et al., 1988; McClarty et al, 1986a, 1987a, 1988; Tagger and Wright, 1988). This noncoordinate increase in the components of ribonucleotide reductase has also been reported for Ehrlich tumor cells and regen-

erating rat liver (Cory and Fleischer, 1982b). However, in those cases and for mouse L1210 leukemia cells (Rubin and Cory, 1986) protein M1 appeared to be the limiting subunit for enzyme activity and, was not present throughout the cell cycle, but only during S phase. This apparent discrepency between the results of different groups has not yet been resolved, but may be due to different sources of enzyme used in the studies and due to different technical approaches taken to investigate this question. Nevertheless, from the above observations, it seems likely, that the allosteric complexity of the enzyme is required to maintain a balanced supply of dNTPs, rather than to control the net activity of the enzyme, which appears to be governed by the amount of M2 or M1 protein.

The key position of ribonucleotide reductase in DNA synthesis and cell division is further illustrated by the effects observed following treatment of cells with enzyme inhibitors. Hydroxyurea, for example, a potent inhibitor of ribonucleotide reductase (Wright et al., 1988), blocks de novo synthesis of all dNTPs when added to cells in culture (Young and Hodas, 1964; Turner et al., 1966; Krakoff et al., 1968) leading to a decline in the rate of DNA synthesis (Skoog and Nordenskjold, 1971; Lewis and Wright, 1974; Nicander and Reichard, 1985). Interestingly, hydroxyurea treatment of mouse embryo cells led to the depletion of purine dNTPs but not pyrimidine dNTPs (Skoog and Nordenskjold, 1971).

Results from several laboratories have suggested that mammalian ribonucleotide reductase is associated in a multienzyme complex with

other enzymes involved in DNA synthesis. Indeed, in bacteriophage T4-infected cells, multienzyme complexes are present, which function to channel DNA precursor moleculues to replication forks. The biological significance of metabolic channeling of DNA precursors are probably to (i) efficiently synthesize dNTPs at replication forks, (ii) maintain high local concentrations of dNTPs despite their rapid turnover, and (iii) balance the synthesis of the four dNTPs at suitable rates which correspond to the nucleotide copmposition of the template DNA (Mathews and Allen, 1983). Clearly, metabolic chanelling serves a useful purpose in prokaryotes where DNA chains grow as rapidly as 1000 nucleotides per s at 37°C. In mammmalian cells however, the question as to whether a multienzyme complex in which DNA synthetic enzymes are structurally and functionally associated with each other has been harshly debated. Baril et al (1972) reported that in rat liver, enzymes involved in DNA synthesis are attached to nonnuclear membranes; in addition, an unlocalized aggregate of enzymes (8.5-12nm) including ribonucleotide reductase, DNA polymerase-alpha, thymidylate synthase, and thymidine kinase were associated with postmicrosomal membrane fragments of unknown origin in Novikoff tumor cells (Baril et al., 1973). Reddy and Pardee (1980) postulated the existence of a multienzyme complex, called replitase, capable of channeling dNTPs directly to replication forks in Chinese hamster embryo fibroblast cells. They reported that the enzyme activities of DNA polymerase, thymidine kinase, dCMP kinase, thymidylate synthetase, dihydrofolate reductase, (Reddy and Pardee, 1980) and later, ribo-nucleotide reductase (Reddy and Pardee, 1982) were located in the cytoplasm of quiscent cells, but are translocated into the nuclei of

cells that are about to replicate DNA. Futhermore, they showed that a major fraction of these enzymes from lysates of karyoplast from S phase cells, but not cells in the G1 phase, cosedimented on sucrose density gradients. The most convincing experiments, however, were the ones which demonstrated metabolic channelling of DNA precursors. These types of experiments were performed in lysolecithin-permeabilized cells, where it was found that radioactive CDP was channelled directly into newly synthesized DNA. If nonradioactive dCTP was added into the reaction mixture, it was found not to mix with the newly labelled dCTP pool formed via reduction and phosphorylation of the radioactive CDP substrate. Furthermore, addition of nonradioactive dCDP did not dilute the labelled dCDP pool formed by the reduction of radioactive CDP. These observations indicated that dCDP is probably not formed by a "free" ribonucleotide reductase and subsequently released into a noncompartmentalized general pool in the reaction mixture which are then channelled into DNA following phosphorylation by dNDP kinase, but that there exists an association between ribonucleotide reductase, dNDP kinase and DNA polymerase which prevents the free diffusion of dNDPs which are endogenously formed (Reddy and Pardee, 1982). Additional support for metabolic channelling of DNA precursors in mammmalian cells came from studies on rat thymus cells which showed that exogenously added purine deoxyribonucleosides (dG and dA) did not prevent the inhibition of DNA synthesis induced by hydroxyurea, suggesting that de novo and salvage pathways of purine deoxyribonucleotide synthesis do not converge (Forsdyke and Scott, 1980). In addition, studies measuring DNA synthesis rates using exogenously added labelled cytidine or deoxycytidine in 3T6 mouse fibroblasts,

indicated that cytidine labelled a dCTP pool directly connected with DNA synthesis whereas deoxycytidine labelled a second dCTP pool that equilibrated with the first pool slowly (Nicander and Reichard, 1983). Such studies thus provided support for compartmentalized dCTP pools, an observation in keeping with the formation of a multienzyme complex for DNA synthesis. More support for the existance of a such a complex comes from studies with a human lymphoblastoid cell line where a complex containing DNA polymerase, thymidine kinase, dTMP kinase, nucleoside diphosphokinase, and thymidylate synthetase was isolated by gel filtration (Wickremasinghe et al., 1983). The enzymes were shown to be kinetically coupled as well since distal precursors such as dTdR, dTMP or dUMP were channelled into DNA without a build up of dTTP (the immediate precursor) (Wickremasinghe et al., 1982, 1983).

Although the above studies all support the existance of a multienzyme complex for enzymes involved in mammalian DNA synthesis, the issue still remains very controversial (Spyrou and Reichard, 1983; Reddy et al., 1986; Wawra, 1988). In particular, it is very doubtful that ribonucleotide reductase is physically associated with other nuclear enzymes involved in DNA synthesis, since immunocytochemical studies with M1 specific monoclonal antibodies have shown that this subunit is exclusively located in the cytoplasm of bovine kidney MDBK cells, mouse 3T6 cells, and various rat tissues (Engstrom et al., 1984). In addition, just recently, it was shown using rat polyclonal and monoclonal antibodies directed against the M2 subunit of mouse ribonucleotide reductase, that protein M2 was located only in the cytoplasm of mouse TA 3 cells and various mouse tissues (Engstrom and

Rozell, 1988). Therefore, it appears as though ribonucleotide reduction most likely occurs in the cytoplasm, and that dNTPs are transported to the nucleus for DNA synthesis. In support of this view, was the finding that ribonucleotide reductase activity throughout the cell cycle of Chinese hamster ovary (CHO) cells increased 10 fold in extracts of S phase cells compared to G1 phase cells, but at no point throughout the cell cycle was activity detected in isolated nuclei (Leeds et al., 1985). Taken together, the above studies strongly suggest that ribonucleotide reductase activity is confined to the cytoplasm of cells, and therefore, these observations argue against ribonucleotide reductase as a component of a replitase model as proposed by Reddy and Pardee (1980, 1982).

Interestingly, from deoxyribonucleotide and DNA labelling experiments Leeds and Mathews (1987) showed that the nuclear dCTP pool equilibrated at rates identical to the whole cell dCTP pool following labelling of S or G1 phase CHO cells with either radioactive cytidine or deoxycytidine. This suggested that there is no kinetically distinct nuclear and cytoplasmic dCTP pool. These conclusions are in disagreement with those of Nicander and Reichard (1982) discussed above. In addition, studies on the distribution of dNTP pools throughout the cell cycle indicated that the cytoplasmic pool sizes were consistently greater than nuclear pool sizes, suggesting that dNTPs are present at different concentrations in the two cellular compartments (Leeds et al., 1985). From such studies it was postulated (Leeds et al., 1985) that the compartmentation of dNTPs between the nucleus and cytoplasm is possibly due to the interactions of enzymes that are differentially

distributed between the two compartments. This suggests that the compartmentation of dNTPs is a kinetic phenomenon which involves metabolic turnover as opposed to an active concentration of dNTPs into the nucleus as might be expected if a multienzyme complex existed. Clearly, more studies are required to elucidate the mechanisms whereby a balanced supply of dNTPs are made available at replication forks in mammalian cells and the involvement of ribonucleotide reductase in the distribution of dNTPs within the cell.

2.5 Biological considerations

In keeping with the importance of ribonucleotide reductase activity in the cell, it has been observed that modifications in this key enzyme activity can have a broad range of biological effects (Wright, 1983, 1988). The importance of ribonucleotide reductase in the biology of the cell is illustrated in the examples which follow.

(1) There is a tight correlation between ribonucleotide reductase activity and neoplastic proliferation. Ribonucleotide reductase activity increased in a series of rat hepatomas of increasing growth rates as measured by mean generation time (Elford et al., 1970; Takeda and Weber, 1981; Weber, 1983). In fact, in one series of rat hepatomas studied, the levels of ribonucleotide reductase activity increased to the greatest extent as compared to other key enzyme activities in purine or pyrimidine biosynthesis such as CTP synthetase, thymidine kinase and IMP dehydrogenase (Weber et al., 1980). In slow growing hepatomas, the reductase activity was increased between 7.7 to 15 fold

over that in normal liver, while in rapidly growing hepatomas reductase activity soared to values ranging from 123 to 325 fold greater than that in normal liver (Weber, 1983). An increase in dNTP pool sizes paralleled the increase in ribonucleotide reductase activity observed in the series of hepatomas studied (Weber et al., 1980). These observations suggested that an increase ability to reduce ribonucleotides is a manifestation of the biochemical commitment of the neoplatic cell to undergo replication. The link between ribonucleotide reductase activity and transformation has been extended to include human cells as well. When ribonucleotide reductase activity was compared directly between SV40 virus-transformed human cells and their normal nontransformed counterparts, from which they were derived, it was found that enzyme activity was increased by about 3 fold in the virally transformed cell lines (Tagger and Wright, 1984). In addition, a series of cultured human hematologic malignant cell lines with different cell proliferation rates showed a significant correlation between proliferation rates, as measured by the reciprocal of the doubling times, and ribonucleotide reductase activity (Takeda et al., 1984).

(2) Alterations in ribonucleotide reductase have been associated with a mutator phenotype in mammalian cells. Consistent with the role of ribonucleotide reductase in the establishment of a balanced supply of deoxyribonucleotides for DNA synthesis, it has been observed that alterations in ribonucleotide reductase activity can have a pronounced effect on the relative dNTP pool-sizes within the cell (Weinberg et al., 1981; Chan et al., 1981; Arpaia et al., 1983; Roguska and Gudas,

1984). Modifications of intracellular dNTP pool sizes have been shown to result in elevations of spontaneous mutation rates, indicating an overall decrease in the fidelity of DNA synthesis (Meuth 1981; Kunz, 1982; Trudel et al., 1984; Phear et al., 1987). Indeed, some mutants isolated having alterations in ribonucleotide reductase have been shown to exhibit increased rates of spontaneous mutation to independent genetic loci, as a direct result of deranged dNTP pool sizes (Weinberg et al., 1981; Chan et al., 1981; Arpaia et al., 1983; Roguska and Gudas, 1984). In addition, more recently, (Caras and Martin, 1988) it was observed that CHO cells, transfected with an expression plasmid containing a mutant M1 cDNA encoding a dATP feedback-resistant ribonucleotide reductase, had elevated spontaneous mutation rates to 6-thioguanine resistance even though no significant changes in deoxyribonucleotide pool sizes were detected. The mutator activity of the transfected cells might possibly be due to alterations in the specific interactions of ribonucleotide reductase with other proteins involved in DNA replication or repair. Quite clearly, the mutator gene associated with ribonucleotide reductase stresses the importance of this enzyme activity in normal cellular DNA replication.

(3) The allosteric regulation of ribonucleotide reductase may play a role in certain immunodeficiency diseases in man. The absence of either adenosine deaminase or purine nucleoside phophorylase activities in man have been associated with dysfunction of the immune system (Giblett et al., 1972, 1975). Adenosine deaminase activity is responsible for the conversion of adenosine and 2′ deoxyadenosine to inosine and 2′ deoxyinosine respectively. Patients who lack this

enzyme activity can accumulate dATP (Cohen et al., 1978a; Coleman et al., 1978) resulting from the phosphorylation of 2'deoxyadenosine. Since dATP is an allosteric inhibitor of ribonucleotide reductase, studies have suggested that the inhibition of B and T cell proliferation in adenosine deaminase deficient patients is due to a dATP mediated inhibtion of ribonucleotide reductase activity (Ullman et al., 1978; Reichard et al., 1961; Meuth and Green, 1974; Waddell and Ullman, 1983). Patients who lack purine nucleoside phosphorylase activity, an enzyme responsible for the phosphorolysis of purine nucleosides and deoxynucleosides to their respective purine bases and pentose 1-phosphates, accumulate large amounts of dGTP in their erythrocytes (Cohen et al., 1978b). Biochemical and genetic analysis of purine nucleoside phosphorylase deficient cell lines suggest that accumulation of dGTP depletes the cell of dCTP and dTTP by allosterically inhibiting the reduction of CDP and UDP respectively (Ullman et al., 1979). The unavailability of an adequate supply of dNTPs is thus postulated to cause the loss of T cell proliferative capacity required for an immune response.

(4) Drugs which selectively inhibit ribonucleotide reductase activity are being tested for their usefulness in the treatment of certain types of cancer (Elford and van't Riet, 1985; Cory and Chiba, 1985; Moore and Hulbert, 1985; McClarty et al., 1985). The only reductase inhibitor in general clinical use at present is hydroxyurea, which has been used in the treatment of a wide range of solid tumors as well as acute and chronic leukemia (Livingston and Carter, 1970; Bolin et al., 1982). Interestingly, recent studies have suggested that the chemo-

therapeutic agent, bleomycin, acts as a potent inhibitor of ribonucleotide reductase provided the enzyme had previously been exposed to hydroxyurea (McClarty et al., 1986b). The conversion of ribonucleotide reductase to a form hypersensitive to bleomycin can have important implications for combination chemotherapy directed at the reductase. Another compound, gossypol, currently in use in some countries as a male antifertility agent (Wang and Rao, 1984) was found to be a potent inhibitor of ribonucleotide reductase activity (McClarty et al., 1985). Clearly, ribonucleotide reductase has been a logical target for drugs designed to selectively inhibit proliferating cells.

(5) Evidence is accumulating that alterations in ribonucleotide reductase can result in modifications of important cellular processes such as cellular ageing and differentiation (Wright, 1983, 1988; Wright et al., 1988). For example, hydroxyurea resistant human diploid fibroblasts having alterations in ribonucleotide reductase activity and in dNTP pool sizes had replicative abilities which were signficantly reduced compared to their parental normal cell strain (Dick and Wright, 1984). Furthermore, enzyme activity declined during senescence of normal human diploid fibroblast (Dick and Wright, 1982, 1985), suggesting the involvement of ribonucleotide reductase in cellular senescence. That ribonucleotide reductase might be involved in certain aspects of cellular differentiation was suggested from studies performed on cultured rat myoblasts where it was observed that modified morphological and biochemical differentiation characteristics of the cells accompanied alterations in ribonucleotide reductase

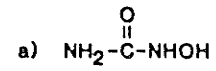
activity and dNTP pool sizes (Creasy and Wright, 1984). More testing of the above ideas is still required (Wright et al., 1988; Wright, 1988), as only a limited number of systems were thus far investigated.

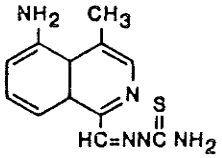
2.6 Ribonucleotide reductase mutants

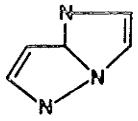
The isolation and characterization of various drug resistant mammalian cell lines over the past 15-20 years have provided valuable insight into the regulatory and structural properties of the target enzyme or protein, as well as an understanding of the cellular mechanisms involved in achieving drug resistance. Inhibitors of ribonucleotide reductase have been useful in the isolation of mammalian cells with specific alterations in the activity of this key enzyme (for reviews see Wright, 1983, 1988). The enzyme inhibitors can be classified into three broad groups based upon their specific site of action (Wright 1983, 1988)(Fig. 6). One group interacts with the M2 subunit of the enzyme, interfering with the tyrosine free radical which is essential for enzyme activity. This group includes hydroxamic acids such as hydroxyurea, thiosemicarbazones such as MAIQ-1 (4-methyl-5-amino-1-formylisoquinoline thiosemicarbazone), hydroxy and amino-substituted benzohydroxamic acids, the diazole IMPY (2,3-dihydro-1H-pyrazolo 2,3-A imidazole), the triazole gaunazole (3,5-diamino-1,2,4-triazole), and N-carbamoyloxyurea. A second group includes drugs which interact with the M1 subunit of the enzyme. This group usually inhibits enzyme activity by binding to or interfering with the effector binding or substrate binding sites of the M1 subunit. Included in this group are coumpounds such as deoxyguanosine,

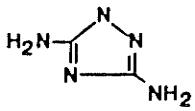
Figure 6

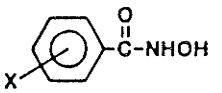
Examples of three classes of inhibitors of ribonucleotide reductase. I. M2 inhibitors; (a) hydroxyurea, (b) MIAQ-1, (c) IMPY, (d) guanazole, (e) benzohydroxamic acid (Van't Riet et al., 1979) (f) N-carbamoyloxyurea. II. M1 inhibitors (cellular phosphorylation required): (a) deoxyadenosine, (b) bromodeoxyuridine, (c) dialdehyde derivative. III. Inhibitors which require both components: 2' deoxy-2-chloro and 2' deoxy-2-azido ribonucleoside diphosphates. (taken from Wright, 1988).

I a) 

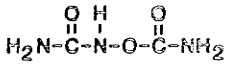
b) 

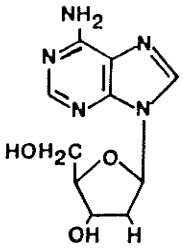
c) 

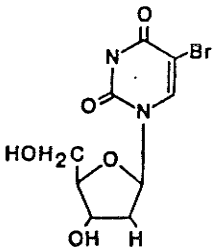
d) 

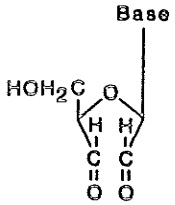
e) 

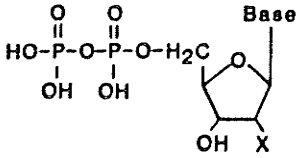
X=OH, NH_2

f) 

II a) 

b) 

c) 

III 

X=Cl, N_3

deoxyadenosine and deoxythymidine, which once incorporated into the cell are phosphorylated to their inhibitory form. Included in this group are also the periodate oxidized dialdehyde derivatives such as oxidized inosine and 5'-inosinic acid. The last group of reductase inhibitors exerts its effects on a functional holoenzyme by inactivating either the M1 or M2 subunit. Included in this group are the 2' substituted ribonucleoside diphosphates such as 2'-deoxy-2'-chlororibonucleoside diphosphate and 2'-deoxy-2'azido-ribonucleoside diphosphate.

Cell lines which are resistant to inhibitors acting at the M1 subunit of ribonucleotide reductase frequently show modifications in this subunit, whereas inhibitors which act at the M2 subunit of the enzyme usually, but not always, result in alterations in the expression and/or properties of only the M2 subunit of the reductase. Properties of some of the mutants isolated over the past few years with alterations in ribonucleotide reductase, and their contribution to the understanding of the enzyme in mammalian cells will be briefly discussed below.

Mutant cell lines with alterations in the M1 component of ribonucleotide reductase have greatly enhanced our understanding of the allosteric regulation of the mammalian enzyme. Such mutants may be selected by virtue of their ability to grow in normally cytotoxic concentrations of compounds such as deoxyguanosine, deoxyadenosine and deoxythymidine, which once converted by intracellular activities to their triphosphate form are allosteric inhibitors of ribonucleotide

reductase. Ullman et al. (1979) isolated a deoxyguanosine resistant, purine nucleoside phosphorylase (PNPase) deficient, mouse S49 T cell lymphoma line designated dGua-L following muatgenesis of the parental PNPase-deficient cell line by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), and selection in normallly cytotoxic concentrations of deoxyguanosine. Ribonucleotide reductase assays using permeabilized cells indicated that CDP reductase activity was only inhibited 50% by dGTP at concentrations which inhibited the wild type activity completely. Furthermore, CDP reductase activity was not as sensitive as wild type to inhibition by dTTP, but had the same sensitivity as the wild type activity to dATP, a general inhibitor of all four ribonucleotide reductase substrates. These observations suggested that the dGua-L cell line might be heterozygous, having one normal reductase allele and one which codes for a dGTP-resistant enzyme. By partial purification of the M1 subunit from the dGua-L cell line using dextran blue-Sepharose chromatography (Ullman et al., 1981), to which protein M1 binds, but protein M2 does not, a fraction enriched in protein M1 whose CDP reductase activity, (when reconstituted with an M2 containing fraction) totally resistant to dGTP was obtained (Eriksson et al., 1981a). Interestingly, this fraction had a markedly reduced ability to reduce ADP, an activity which is dependent on the presence of dGTP. The fact that reconstituted enzyme using protein M1 preparations from the wild type cells had a CDP reductase activity that was sensitive to dGTP and an ADP reductase activity which was dGTP-dependent might have suggested the possibility that two independent binding domains for dGTP were present on protein M1. However analysis of the mutant protein now indicates that this possibility

seems very unlikely because for the enzyme reconstituted using protein M1 from the mutant cell line, dGua-L, the dGTP-mediated inhibition of CDP reductase and the dGTP-mediated stimulation of ADP reductase were both diminished simultaneously. Thus, these studies demonstrated that a single binding site affecting substrate specificity is present on protein M1 to which dGTP (or dTTP) may bind.

Another mutant designated, dGua-L-200-1 (Ullman et al., 1980) was isolated from MNNG mutagenized S49 mouse T cell lymphoma cells, for the ability to grow in 200 uM deoxygaunosine. This mutant cell line was also cross resistant to normally cytotoxic concentrations of deoxyadenosine and thymidine. The dNTP pool sizes in this mutant were elevated 2 to 5 fold over those in the parental population, and analysis of ribonucleotide reductase activity in permeabilized dGua-L-200-1 cells indicated that CDP reduction was only 50% inhibited by dATP at concentrations which completely inhibited wild type activity. ATP is required for the reduction of all four substrates in the wild type cells whereas dATP exerts a general inhibitory effect. To gain further insight into the alterations of the reductase in the dGua-L-200-1 cell line, the M1 subunit of the enzyme was seperated from the M2 subunit by dextran blue-Sepharose (Eriksson et al., 1981b). From reconstitution experiments using purified wild type and mutant enzyme components, it was demonstrated that the dATP resistant CDP reductase activity of the mutant cell line, was due to an altered M1 component. Further purification of the protein from dGua-L-200-1 cells on dATP-Sepharose column (Eriksson et al., 1981b) revealed two types of active M1 fractions. One type eluted at low ATP concen-

trations (3-6 mM ATP), and was totally resistant to dATP inhibition and the other eluted at much higher ATP concentrations (10-20 mM ATP) and was sensitive to dATP inhibition. Protein M1 from wild type cells only eluted at the higher ATP concentrations. These observations demonstrated that the dGua-L-200-1 cells are heterozygous for protein M1 of ribonucleotide reductase, containing two types of protein M1 components, presumably, a mutant allele product and a wild type allele product. This explains the previous observation noted above, that is, only half of the CDP reductase activity in permeabilzed cells was inhibited by dATP. Detailed kinetic analysis of the mutant M1 component (Eriksson et al., 1981a) revealed that CDP reduction was sensitive to inhibition by the negative allosteric effectors, dGTP or dTTP indicating that the substrate specificity sites were unaltered. Using wild type protein M1, low concentrations of dATP stimulated CDP reduction 4 fold, but at high concentrations of dATP complete inhibition of all enzyme activity occurred. On the other hand, dATP stimulated CDP reduction 25 fold using reconstituted mutant protein M1. Therefore, dATP was able to substitute for ATP in the mutant protein M1 and hence, dGua-L-200-1 cells contained an altered binding site (activity site) in protein M1. Furthermore, in the presence of dATP, GDP reduction which requires dTTP as a positive effector, was stimulated 2 fold using protein M1 from the mutant. Unlike CDP reduction, GDP reduction using the mutant protein M1 was not stimulated by dATP alone. This raised the possibility that dATP might act as a positive effector specific for CDP reduction. To test this, GDP reduction using reconstituted protein M1 from wild type and mutant cell lines was further analyzed. dATP inhibited GDP reduction using

wild type protein M1 (ATP and dTTP being present) even if dTTP concentrations were raised 5 fold. However, increasing the dTTP concentration using mutant protein M1 significantly changed the inhibition curve. The ratio of dATP to dTTP concentrations in effect, determined the rate of GDP reduction. This indicated that dATP competes with dTTP for specifically reducing GDP. Since dTTP only binds to one site (preceeding paragraph), the specificity site, it was deduced that dATP acts as an effector at both the activity site (for all four ribonucleoside diphosphate substrates) and at the specificity site (being a positive effector specifically for CDP reduction).

The detailed biochemical genetic analysis of the mutant cell lines discussed above, have revealed the presence of two types of allosteric binding sites on protein M1, the activity site which binds ATP or dATP only, and the substrate specificity site, which binds ATP dATP, dTTP, and dGTP (see Fig. 4). These observations are in general agreement with those found by the biochemical analysis of calf thymus protein M1 discussed previously (Eriksson et al., 1979), and demonstrate the value of drug resistant cell lines in understanding the regulation of ribonucleotide reductase in normal mammalian cells.

Interestingly, cell lines selected for resistance to aphidicolin, Ara-A and Ara-C frequently possess alterations in ribonucleotide reductase. At first, this might seem unusual since the primary site of action for these drugs appears to be DNA polymerase. Although the phosphorylated forms of Ara-A and Ara-C are inhibitors of ribonucleotide reductase as well, they are much more potent inhibitors of

DNA polymerase (Ludwig and Follmann, 1978) where they resemble the polymerase substrates dCTP and dATP respectively. Aphidicolin appears to be competing with dATP (Ayusawa et al., 1981) or dCTP (Nicander and Reichard, 1981) as a regulator, substrate or cofactor of the polymerase or another protein closely associated with DNA polymerase. In fact, mammalian and Drosophila melanogaster cell lines resistant to aphidicolin due to modifications in the polymerase have been reported (Liu and Loeb, 1984; Sugimo and Nakyamo, 1980). The ribonucleotide reductase mutants resistant to Ara-A, Ara-C and aphidicolin usually contain a reductase which is less sensitive to the negative allosteric effector, dATP and consequently, these mutants usually show deranged dNTP pool sizes; in particular, dATP and dCTP pools appear to be elevated (Ayusawa et al., 1981). The mechanism of resistance of these reductase mutants to the cytotoxic effects of the polymerase inhibitors Ara-A, Ara-C and aphidicolin is by diluting the concentrations of the activated forms of these inhibitors in the vicinity of DNA polymerase through expansion of the concentration of competing deoxyribonucleotides. Thus an altered M1 component desensitized to dATP inhibition enables DNA synthesis to continue in the presence of polymerase inhibitors.

Cell lines resistant to drugs like hydroxyurea or N-carbamoyloxyurea, which act at the M2 subunit of ribonucleotide reductase, were the earliest reductase mutants to be isolated (Wright and Lewis, 1974; Lewis and Wright 1974, 1978a,b; Hards and Wright, 1981). These drug resistant cell lines commonly exhibit overproduction of ribonucleotide reductase activity (Lewis et al., 1978; Lewis and Wright, 1979;

Akerblom et al., 1981; Wright et al., 1981, 1988; Wright, 1983,1988). N-carbamoyloxyurea is an effective inhibitor of ribonucleotide reductase (Hards and Wright, 1981, 1983) and of DNA synthesis (Jacobs and Rosenkranz, 1970; Cameron and Jeeter, 1973; Hards and Wright, 1981, 1983) and therefore is cytotoxic to rapidly proliferating cells. The compound has been isolated as an oxidation product from hydroxyurea maintained at high temperatures (Jacobs and Rosenkranz, 1970). Cell lines resistant to N-Carbamoyloxyurea are cross resistant to hydroxyurea (Hards and Wright, 1981; Wright et al., 1988) and to guanazole (Wright, 1983, 1988), supporting the view that these three drugs act at a common intracellular target (Hards and Wright, 1981, 1983). Hydroxyurea is a simple analog of urea in which the hydroxyl group replaces a hydrogen atom. The drug was first synthesized in 1869 (Dresler and Stein, 1869), but it was not until 1960 when anti-neoplastic activity of the drug was first observed (Stock et al., 1960). The site of action was localized to ribonucleotide reductase in 1964 (Frenkel et al., 1964; Young and Hodas, 1964; Mohler, 1964), and later, in vitro inhibition of partially purified enzyme from mammalian sources was reported (Turner et al., 1966; Elford, 1968; Moore, 1969). The drug enters mammalian cells by a diffusion process (Morgan et al., 1986; Tagger et al., 1987) and has found use in the treatment of a wide range of solid tumors as well as acute and chronic leukemia (Krakoff et al., 1964; Bolin et al., 1982; Engstrom et al., 1984). The drug has currently found use as a cell synchronizing agent (Ashihara and Baserga, 1979). In addition, hydroxyurea has been used to isolate drug resistant cell lines from a variety of species including hamster, mouse, rat and human (e.g. Lewis et al., 1978b; Lewis and Wright,

1979; Kuzik and Wright, 1980; Hards and Wright, 1981, 1983; Akerblom et al., 1981; Lewis and Srinivason, 1983; Dick and Wright 1984; Creasy and Wright, 1984; McClarty et al., 1986a, 1987a,b; Choy et al., 1988; Wright et al., 1987, 1988; Tagger and Wright, 1988). In most cases, it is found that the hydroxyurea resistant cell lines possess elevated levels of ribonucleotide reductase activity as a result of over-production of the M2 subunit of the enzyme. This observation is in keeping with in vitro studies showing that hydroxyurea inhibits ribonucleotide reductase activity by scavenging the tyrosyl free radical in the M2 subunit of the enzyme (Graslund et al., 1982; McClarty et al., 1987a).

Interestingly, analysis of enzyme activity from some of the hydroxyurea resistant cell lines revealed that purine and pyrimidine reducing abilities were not increased to the same extent (Lewis et al., 1978; Lewis and Wright, 1979; Kuzik and Wright, 1980; Koropatnick and Wright, 1980; Hards and Wright, 1983). For instance, analysis of both CDP and ADP reductase activities in a series of hamster cell lines selected for resistance to N-carbamoyloxyurea revealed that the ratio of CDP to ADP reductase activity was not the same. Two of the drug resistant cell lines as well as the wild type population contained higher levels of purine reductase activity as compared to pyrimidine reductase activity. Five of the other drug resistant cell lines showed the opposite pattern of ribonucleoside diphosphate reduction. Unlike ADP reduction which was not increased in all of the variant cell lines in the series, CDP reduction was elevated twofold or greater in every drug resistant cell line as compared to the wild

type cell line. Similar non-coordinated changes in CDP and ADP reductase activities have also been observed following incubation of cells with N-carbamoyloxyurea prior to enzyme analysis (Hards and Wright 1983). Specifically, CDP reductase activity was substantially enhanced whereas only minor increases in ADP reductase activity were observed. Taken together, the above observations suggested that increases in CDP reduction were more important for achieving drug resistance than increases in ADP reductase activity. There does not appear to be at present any direct explanation for the noncoordinate changes between ADP and CDP reductase activities. Some investigators have taken these observations as support of the hypothesis that there exists multiple forms of M1 components each specific for the reduction of a different substrate (e.g. Cory, 1983), however, other explanations are certainly possible (Wright, 1988).

As noted above, alterations in the M1 component of ribonucleotide reductase frequently results in imbalances of dNTP pool sizes. Hydroxyurea resistant cells with alterations only in the M2 subunit of the enzyme can also exhibit pertubations in dNTP pool sizes (Akerblom et al., 1981; Dick and Wright, 1984; Tagger and Wright, 1988) possibly due to elevated levels of ribonucleotide reductase activity. However, it is worth noting that not all hydroxyurea resistant mutants contain altered dNTP pools (Tagger and Wright, 1988).

In addition to elevated levels of ribonucleotide reductase activity, some hydroxyurea resistant cell lines contain an enzyme activity exhibiting higher Ki values to hydroxyurea than the enzyme

prepared from wild type cells (Lewis and Wright, 1974, 1978b; Kuzik and Wright, 1980; Koropatnick and Wright, 1980; Hards and Wright 1983; Tagger and Wright, 1988). This suggests the presence of an altered protein less sensitive to hydroxyurea inhibition in these drug resistant cell lines. However, further studies with homogeneous preparations of protein M2 of ribonucleotide reductase are required to firmly establish the presence of any type of structural alteration. Mutants having alterations in this subunit would be very valuable since they would enhance our understanding of the mechanism of action of hydroxyurea, and of the requirments needed to maintain a functional tyrosyl free radical structure.

With the recent availability of the cDNAs encoding the two components of ribonucleotide reductase (Caras et al., 1985; Thelander and Berg, 1986) it became possible to investigate the molecular properties of ribonucleotide reductase mutants. Analysis of hydroxyurea resistant rat, mouse, hamster and human cell lines which overproduce ribonucleotide reductase activity revealed significant increases in the levels of M2 mRNA as compared to their corresponding wild type cells (Thelander and Berg, 1986; Wright et al., 1987, 1988; Cocking et al., 1987; McClarty et al., 1987a,b; Choy et al., 1987; Tagger and Wright, 1988). In most cases the increased message level was accompanied by increases in M2 gene copy number. In one study on a series of clonally related hydroxyurea resistant and revertant cell lines a strong correlation between cellular resistance to hydroxyurea, enzyme activity, M2 mRNA levels and M2 gene copy number was observed (McClarty et al., 1987b). Interestingly, M1 mRNA and gene copy number

were not elevated in any of the clonally related cell lines tested. The above studies illustrate the importance of M2 gene amplification as a mechanism for achieving cellular resistance to hydroxyurea. It should be noted that increases in M1 mRNA levels (in addition to M2 mRNA levels) have so far only been observed in cell lines selected for resistance to very high concentrations of hydroxyurea (McClarty et al., 1987a; Choy et al., 1988; Cocking et al., 1987; Wright et al., 1988). The increase in M1 mRNA can occur without amplification of the corresponding M1 gene (McClarty et al., 1987a; Choy et al., 1988). In fact, it appears that amplification of the M1 gene only occurs rarely, and is associated with the most highly resistant cells (Wright et al., 1988; Cocking et al., 1987).

Some hydroxyurea resistant cell lines showed a linear relationship between elevations in M2 mRNA levels and increases in M2 gene copy number, indicating, that at least in these cells, a single event, that of M2 gene amplification, is responsible for the increase in protein M2 and enzyme activity (Tagger and Wright, 1988). In other drug resistant cell lines, however, the M2 mRNA levels greatly exceeded the increase in M2 gene copy number (Wright et al., 1988; McClarty et al., 1987a; Choy et al., 1988). The mechanism of drug resistance in these cases is somewhat more complex and involves such events as modified rates of M2 gene transcription, alterations in M2 translational effeciency as well as increases in M1 gene transcription (McClarty et al., 1987a; Choy et al., 1988; Wright et al., 1988). It should also be noted that although amplification of the M2 gene is commonly observed in hydroxyurea resistant cells, increases in M2

message can also occur without a concomitment amplification of the M2 gene (Wright et al., 1987; Tagger and Wright, 1988).

In summary, it is apparent that drug resistant mammalian cell lines have contributed greatly to understanding structural as well as regulatory aspects of ribonucleotide reductase. These systems continue to be valuable tools in providing new information on the properties of one of the most complex enzymes in the cell.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. CELL LINES AND CULTURE CONDITIONS

1.1 CHO cell lines

The wild type Chinese hamster ovary (CHO) cells used in this study were originally established by Puck et al. (1958). As opposed to a diploid chromosome number of 22 for the Chinese hamster, the wild type cell line exhibit a stable chromosome number of 21. Although the CHO cells carry chromosomes which differ from the Chinese hamster (Kao and Puck, 1969), many of the abnormal chromosomes can be identified as rearrangements of the normal hamster chromosomes (Deaven and Peterson, 1973).

The CHO cells were routinely cultured on the surface of plastic tissue culture plates in alpha-minimal essential medium (alpha-MEM) plus 10% (v/v) fetal calf serum (FCS), supplemented with penicillin G (100 units/ml), and streptomycin sulfate (100 ug/ml). The formulation of alpha-MEM has been published by Stanners et al (1971), but contains neither ribonucleosides nor deoxyribonucleosides. Cultures were incubated at 37°C in a 5% CO_2 atmosphere in a humidity controlled incubator.

Suspension cultures of CHO cells were initiated with about 10^5 cells/ml of alpha-MEM plus 10% FCS. The cells were cultured in 500 ml medium bottles or 4 litre carboys placed in a 37°C water bath.

Continuous stirring of the cultures was achieved by Teflon coated magnetic stirring bars. Although cultures proliferated logarithmically to a density slightly greater than 10^6 cells/ml, they were routinely harvested by centrifugation for use at cell densities between 5 and 8 X 10^5 cells/ml as estimated by a particle counter (Coulter Electronics Co.).

1.2 Human cell strains and transformed human cell lines

The normal human diploid cell strains WI-38 (Hayflick and Moorhead, 1961) and WI-26 (Hayflick, 1965), are fibroblast-like cell strains derived from the normal embryonic (3-month gestation) lung tissue of a Caucasian female and male respectively. The transformed human cell lines used in this study were SV40 virus-transformed counterparts of the WI-38 and WI-26 cell strains, designated WI-38 VA13 (Girardi et al., 1966) and WI-26 VA4 (Girardi et al., 1965) respectively. Both of the transformed cell lines contain the SV40 neo (T) and transplantation antigens and appear to be capable of unlimited proliferation. All human cells were routinely maintained at 37°C on the surface of plastic tissue culture plates in alpha-MEM plus 15% FCS (v/v) supplemented with antibiotics as described for the CHO cells. Subculturing of these cells was performed using a strict protocol. At confluence, the cells were trypsinized, removed from the surface of the plate, and diluted onto fresh tissue culture plates. The dilutions were either 1:2, 1:4, or 1:8 for which 1, 2, or 3 passage (P) numbers were added to the culture respectively (Hayflick, 1965). The WI-38 cells senesced at about 50 passages whereas the WI-26 cells senesced

at about 40 passages. All experiments done with the human cells utilized cultures between the following passage numbers: WI-38, between P27 and P45; WI-38 VA13, between P274 and P314; WI-26, between P28 and P33; and WI-26 VA4, between P161 and P177.

1.3 Sources of cell lines

The wild type CHO cell line was brought from the Medical Biophysics Department, University of Toronto to the Department of Microbiology, University of Manitoba by Dr. J.A. Wright in 1971. The wild type cell line was cloned (section 2.5) prior to the selection of drug resistant mutants. The H^R-R2T drug resistant cell line was cloned from a population of wild type CHO cells selected by a two step procedure for the ability to proliferate in alpha-MEM supplemented with 0.3 mM and then 0.5 mM hydroxyurea. The HN^R-AT mutant is a cell line cloned from a population of wild type CHO cells selected for the ability to proliferate in the presence of 0.4 mM N-carbamoyloxyurea; cells resistant to N-carbamoyloxyurea are cross-resistant to hydroxyurea (Hards and Wright, 1981; Wright et al., 1988). The drug resistant cells arise at a frequency of approximately 10^{-5} to 10^{-6}.

The WI-38 and WI-26 cell strains together with their SV40 virus-transformed counterparts, WI-38 VA13 and WI-26 VA4, were purchased from the American Type Culture Collection (ATCC, Rockville, Maryland).

2. ROUTINE PROCEDURES

2.1 Cell removal with trypsin solution

Sterile 0.1% trypsin solutions were prepared in phosphate buffered saline (PBS, pH 7.3) consisting of 137 mM NaCl, 2.68 mM KCl, 1.62 mM potassium phosphate monobasic, and 8.10 mM sodium phosphate dibasic and stored at 4°C or frozen for longer term storage at -20°C. To remove cells from the surface of tissue culture plates, the growth medium was removed by aspiration, trypsin solution was added to the plates, and then immediateley aspirated off so as to allow only sufficient trypsin solution to cover the cells. Once the cells had begun to detach from the surface of the plates, an appropiate volume of growth medium was added to the plates. The cell suspension was then centrifuged gently, resuspended in growth medium, and dispensed as required.

2.2 Cell counting

Aliquots of cells removed with trypsin solution as described above or cells grown in suspension culture were diluted with saline (usually 100 ul of cell suspension was added to 40 ml saline), and counted with the aid of a Coulter Particle Counter (Coulter Electronics Ltd. Florida) in order to determine cell densities. Occasionlly, cell suspensions were counted using a hemocytometer and the results obtained were very similar to those obtained with the particle counter.

2.3 Determination of doubling times

In order to determine the growth rates of CHO cells in culture, 1.5×10^6 cells were seeded onto 150 mm plastic tissue culture plates and incubated in alpha-MEM plus 10% FCS. At various times after seeding, the cells were removed from the plates with trypsin solution and counted using the Coulter Particle Counter. Growth curves for the human cells were performed in a similar manner by seeding approximately 5×10^4 cells on 60 mm tissue culture plates and incubation in alpha-MEM plus 15% FCS. Doubling times were determined by interpolation of plots of the log of cell number versus time of incubation.

2.4 Determination of plating efficiency

In order to determine the plating efficiency of cells, 250 to 500 cells were seeded onto tissue culture plates (100 or 150 mm plates), and incubated for 7 days (CHO cells), or 10 to 14 days (human cells). Following incubation, medium was drained from the plates, and a saturated solution of methylene blue in 50% ethanol was added. After approxiametely 10 minutes, the dye was removed, the plates were rinsed with water, and the stained colonies were counted under a dissecting microscope. Only colonies containing 25 cells or more were counted. The plating effeciency was calculated by dividing the number of colonies per plate by the number of cells seeded per plate. The various CHO cell lines described in this study had plating effeciencies between 0.80 to 0.95. The human cell strain WI-26 and its SV40 virus-transformed counterpart WI-26 VA4 had plating efficiencies of

about 0.20 and 0.60 respectively.

To determine the effects of various drugs on cell viability, the colony-forming abilities of the cells in the presence of drug was assessed. Anywhere from 250 to 10^6 cells were seeded onto tissue culture plates (100 or 150 mm plates) in the presence of alpha-MEM plus 10% FCS supplemented with various concentrations of the drug to be tested. Following a suitable incubation period (usually 10 to 14 days), the medium was removed from the plates and the colonies were stained as described above. After counting the colonies, relative plating efficiencies were calculated. The relative plating efficiency is defined as the plating efficiency in the presence of drug divided by the plating efficiency in the absence of drug. Dose response curves of relative plating efficiency versus drug concentration were plotted on semi-logarithmic paper, and the drug concentration which reduces the relative plating efficiency to 0.10 (10% survival), termed the D_{10} value, was determined. The number of cells seeded at a particular drug concentration was adjusted in order to ensure the survival of no greater than 400 colonies per plate.

2.5 Cell cloning

For cloning CHO cells, cell suspensions were diluted to about 1 cell per 2 ml growth medium and dispensed at 2 ml/well onto 24-well Linbro plastic trays. After about 5 days incubation, the wells were examined for the presence of single colonies. Those wells were marked, and the trays were reincubated for an additional 3-4 days to allow for

further growth of the colonies. The colonies were then removed with trypsin solution, transferred to 60 mm tissue culture plates, and grown to confluence for subsequent use.

2.6 Long-term storage of cells

For long-term storage, wild type and mutant CHO cell lines were suspended at about 10^7 cells in 1-1.5 ml of alpha-MEM plus 10% FCS plus 7% dimethylsulfoxide. The normal human diploid fibroblast strains and their SV40 virus-transformed counterparts were suspended at approxiametly 2-3 x 10^6 cells in 1-1.5 ml of alpha-MEM plus 15% FCS plus 7% dimethylsulfoxide. The cell suspensions were placed in cryotube vials (Nunc) and frozen immediately at -76°C. To recover cells, the vial was rapidly thawed in a 37°C water bath and the suspension was placed in 10 ml of growth medium in a sterile Falcon plastic test tube, pipetted up and down and then centrifuged at 500 X g in order to pellet the cells. The cell pellet was then resuspended in normal growth medium and dispensed appropriately onto tissue culture plates.

2.7 Liquid scintillation counting

Liquid scintillation techniques were used to measure radioactiviy in studies involving radioactive compounds. Aqueous samples from ribonucleotide reductase assays and hydroxy[^{14}C]-urea uptake experiments were counted after the addition of 10 ml Scinti Verse II. Samples on membrane filters or chromatography paper were dried and placed in

vials containing a suitable amount of Aquasol II. Radioactivity was determined using a Beckman model LS 7800 liquid scintillation spectrometer with external standard corrections.

2.8 Protein determination

Protein content of samples were determined using the Bio-Rad Protein Assay (Bio Rad Bulletin 82-0275), using bovine serum albumin (BSA) as a standard. A linear relationship between absorbance and protein concentration was observed from 0 to 100 ug protein.

3. CELL PROTEIN LABELLING AND M1 IMMUNOPRECIPITATION

To determine the relative steady state levels of protein M1 in the mutant and wild type CHO cells, immunoprecipitation of ^{35}S-methionine labelled cellular proteins with rabbit M1 antiserum was performed (McClarty et al., 1986a). Subconfluent cultures of cells on 150 mm tissue culture plates were labelled with 80 uCi of ^{35}S-methionine (800 Ci/mmol/ml) for 16 hr at 37°C in methionine free alpha-MEM medium containing 10% dialyzed FCS. Following incubation the medium was aspirated and the cells were washed with ice-cold PBS, removed with trypsin solution, and washed twice with PBS. After centrifugation the cell pellet was solubilized in a buffer containing 1% Triton X-100, 0.5% sodium deoxycholate, 5 mM EDTA, 250 mM NaCl, 25 mM Tris-HCl (pH 7.5) to a final cell density of 2 X 10^7 cells/ml. The immunoprecipitation was performed according to Firestone et al. (1982) as modified by McClarty et al. (1986a). To each 200 ul of extract, 100 ul

of solubilization buffer containing 50 mg/ml BSA plus an appropriate amount of M1 antiserum or preimmune serum was added, and the sample was then incubated for 15 min at room temperature. Following incubation, 10 ul of 10% preadsorbed Staphlococcus A cells were added and the sample was further incubated for an additional 5 min. The entire sample was then carefully layered on top of 600 ul of a 1 M sucrose solution in a microfuge tube and centrifuged for 3 min in a benchtop microcentrifuge. The sample was then aspirated down to the sucrose layer and the tube was washed carefully with 2 M urea in order to remove non-specific labelled material stuck to the tube. The sucrose solution was then aspirated off and the Staphlococcus cell pellet was washed once with solubilizing buffer, centrifuged for 2 min, and then washed with 10 mM Tris-HCl (pH 7.5), plus 5 mM EDTA and finally centrifuged again for 2 min followed by aspiration of the wash solution. The cell pellet was then prepared for SDS polyacrylamide gel electrophoresis (McClarty et al., 1986a) by addition of 40 ul of sample loading buffer containing 3% (w/v) sodium dodecyl sulfate (SDS), 10% glycerol, 5% β-mercaptoethanol, 0.05% (w/v) bromophenol blue, and 625 mM Tris-HCl (pH 6.8). The sample was denatured by boiling for 2 min, followed by chilling and centrifugation. The supernatent was then loaded onto a 10% SDS polyacrylamide gel (Laemmli, 1970) composed of 1% SDS, 10% polyacrylamide, and 0.4 M Tris-HCl (pH 8.8). Following electrophoresis on a vertical slab gel (Protean, Bio-Rad Laboratories), for 4 hr at a constant current of 35 mAmp, the gel was stained with a solution containing 0.1% Coommassie Brilliant Blue R-250, 10% acetic acid, and 50% methanol. Destaining was carried out in a solution of 50% methanol, and 10% acetic acid.

The gel was then dried for 2 hr at 80°C on a Bio-Rad slab gel dryer model 1125B and autoradiographed for 7 days at -70°C using Kodak X-Omat PR film and Cronex Lightning Plus intensifying screens (Dupont). The film was developed in a Kodak PR X-Omat processor. The rabbit M1 antiserum (Caras et al., 1985) was kindly provided by B.B. Levinson and D.W. Martin, Jr. of Genentech, Inc.

4. ELECTRON PARAMAGNETIC RESONANCE (EPR) SPECTROSCOPY AND CELL CYCLE ANALYSIS

Cells in exponential growth were either removed from the surface of culture plates with a buffered trypsin solution and centrifuged, or centrifuged directly from spinner cultures, washed in ice-cold PBS, and transferred directly to EPR tubes (Wilmad Glass Co. Ltd.). The cells were packed by low speed centrifugation on a benchtop centrifuge for 10 min at 4°C. The height of the pellet was adjusted so that each tube contained greater than 170 ul of packed cells (the maximum volume measured in the EPR spectrometer). The tubes were then frozen and stored in liquid nitrogen until required.

EPR first-derivative spectra were recorded at 77K on a Bruker ER 100 Laboratory EPR spectrometer equipped with an ER 040 XR microwave bridge and a cold-finger Dewar with liquid nitrogen (Eriksson et al., 1984). Determinations of free radical content were obtained by comparing EPR signals of samples with a control signal of packed M2 overproducing mouse mammary tumor TA3 cells, which were previously shown to have a tyrosine free radical concentration of 1.4 uM (Thelan-

der and Berg, 1986) as described previously (Wright et al., 1987; Tagger and Wright, 1988). I thank Dr. Lars Thelander, University of Umea, Sweden for his help in these experiments. The proportion of cell populations in the various cell cycle phases was estimated with the kind assistance of Dr. Staffan Eriksson and Dr. Sven Skog, Karolinska Institute, Sweden, using a rapid-flow cytofluorometer ICP II (Phywe, Gottingen, West Germany; Eriksson et al., 1984). Cell cycle distribution was estimated from the area of the histograms assuming a Gaussian function of the G_1 and the G_2 + M maxima, and attributing the remaining part of the histogram to S phase cells.

5. SOUTHERN AND NORTHERN BLOT ANALYSIS

5.1 DNA isolation and Southern blot analysis

Genomic DNA was isolated from cells basically according to the procedure of Blin and Stafford (1976). Cells were harvested from 3 to 5 subconfluent 150 mm tissue culture plates with buffered trypsin solution, centrifuged, and resuspended in an ice-cold solution of 10 mM Tris-HCl (pH 8.0), plus 1 mM EDTA (TE) at a concentration of approxiametely 10^8 cells/ml. To the suspension, 10 volumes of DNA isolation buffer consisting of 0.1 M EDTA, 0.15 M NaCl, 0.5% sarcosyl and 100 ug/ml proteinase K was added. Following incubation at 50°C for 3 hr, the DNA was extracted 3 times with an equal volume of phenol. The DNA was then dialysed overnight against 4 litres of buffer containing 25 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 10 mM NaCl, allowing room in the dialysis tubing for the sample to increase about

3-fold in volume. The sample was then treated with 100 ug/ml of DNAse free RNAse at 37°C for 3 hr and then extracted twice with an equal volume of phenol/chloroform (phenol/chloroform is phenol:chloroform:isoamyl alcohol, 24:24:1), and then once with an equal volume of chloroform (chloroform is chloroform:isoamyl alcohol, 24:1). The DNA sample was then concentrated with 1-3 volumes of sec-butyl-alcohol and dialysed extensively against TE. A couple of drops of chloroform was added to preserve the DNA and the preparation was stored at 4°C.

In order to ensure that high molecular weight DNA was isolated, a sample of the preparation was electrophoresed on a 0.6% agarose minigel. Only samples which ran as one band and with a molecular weight greater than 100 Kb were used for subsequent experiments. The concentration of DNA was determined by making appropriate dilutions of the sample and measuring the absorbance at 260 nm and using the formula: 1.0 OD_{260} = 50 ug/ml DNA (Maniatis et al., 1982). Protein content in the sample was determined by measuring the absorbance at 280 nm, and only those preparations having OD_{260}/OD_{280} ratios of 1.75 to 1.80 were used.

For Southern blots, 20 ug of DNA was digested to completion with a specific restriction endonuclease (4 units of restriction endonuclease per ug DNA), and the sample was dried down to a volume of 40 ul by using a speed-vac concentrator (Savant). To the sample was added 1/5 the volume of gel loading buffer consisting of 0.25% bromophenol blue, 0.25% xylene cyanol, and 30% glycerol and the sample was loaded onto a 0.7% agarose gel and electrophoresed overnight at 30 volts in a

buffer containing 89 mM Tris-borate, 89 mM boric acid and 2 mM EDTA (TBE). DNA molecular weight markers were loaded onto a seperate lane. Gels were stained with ethidium bromide and viewed under UV light to check the digestion and to ensure that equivalent amounts of DNA were loaded from the various samples. Before blotting onto nitrocellulose membranes, the gel was treated with 0.25 M HCl for 15 min, 0.5 M NaOH plus 1.5 M NaCl twice for 15 min and finally with 0.5 M Tris-HCl (pH 7.5) plus 1.5 M NaCl for 30 to 60 min. The gel was blotted overnight in 10 X SSC (1 X SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) buffer onto nitrocellulose membranes and the membranes were then rinsed in 6 X SSC buffer, dried, and baked at 80°C for 2 hr. The blots were prehybridized overnight at 42°C in 50% (v/v) formamide, 0.1% SDS, 1.0 M NaCl, 7.5 X Denhardt's solution (1 X Denhardt's solution contains 20 mg each of Ficoll, polyvinyl pyrrolidone, and BSA in 100 ml water), 10% dextran sulfate and 100 ug/ml denatured salmon sperm. Hybridizations were performed in the same solution for 16 hr with 10^6 cpm/ml of ^{32}P-labeled probe. The blots were washed twice in 2 X SSC and 0.1% SDS at room temperature for 60 min, and then twice with 0.2 X SSC and 0.1% SDS at 57°C for 60 min. Autoradiography was performed at -70°C using Kodak X-Omat AR film and Cronex Lightning Plus intensifying screens.

5.2 RNA isolation and Northern blot analysis

Total cellular RNA was extracted from logarithmically growing cells using the guanidinium isothiocyanate/cesium chloride method (Chirqwin et al., 1979). Cells were removed from approxiametely 10

subconfluent 150 mm tissue culture plates with trypsin solution, washed once in PBS and centrifuged. For every 10^8 cells 2 ml of a solution containing 4 M guanadinium thiocyanate, 25 mM sodium citrate (pH 7.5), 0.5% N-laurylsarcosine, 0.1% antifoam A, and 0.007% β-mercaptoethanol was added. The cell pellet was resuspended in the solution by pipetting it with a syringe about 20 times. Following centrifugation on a clinical centrifuge for about 15 min, the cell lysate was layered on top a solution of 5.7 M CsCl and 0.1 M EDTA (pH 7.0) and centrifuged at 20°C in a SW41 Ti Beckman rotor at 25,000 rpm for 18 hr. The top guanadinium thiocyanate layer and the CsCl layer were removed with a pasteur pipette and the tube was cut in order to gain easier access to the RNA pellet. The sides of the centrifuge tube and the pellet were washed once with the guanidinium isothiocyanate solution, and then the pellet was gently washed twice with 70% ethanol plus 0.1% SDS. The RNA pellet was resuspended in about 300 ul sterile water, transferred to a microfuge tube and precipitated overnight at -20°C by the addition of 10% (v/v) 3 M sodium acetate (pH 5.5) and 2 volumes of absolute ethanol. The sample was then centrifuged (10 min on benchtop microcentrifuge) and the supernatent was discarded. Traces of ethanol were removed from the RNA pellet by speed vacuuming for 30 min. The pellet was resuspended in about 100 ul of sterile water and stored at -70°C.

The concentration of RNA in the sample was determined by measuring the absorbance of the sample at 260 nm and using the formula: 1 OD_{260} = 40 ug/ml RNA (Maniatis et al., 1982). The RNA was prepared for Northern blot analysis as follows: to 4.5 ul RNA sample

containing 20 ug of cellular RNA was added 3.5 ul formaldehyde, 10.0 ul formamide and 2.0 ul of 5 X gel loading buffer containing 50% glycerol, 1 mM EDTA, 0.4% bromophenol blue and 0.4% xylene cyanol. The sample was then incubated at 55°C for 15 min and loaded onto a 1% formaldehyde-agarose gel containing 18% formaldehyde, 40 mM morpholinopropanesulfonic acid (MOPS) (pH 7.0), 10 mM sodium acetate, and 1 mM EDTA and electrophoresed overnight in 40 mM MOPS (pH 7.0), 10 mM sodium acetate, and 1 mM EDTA at 30 volts. The gel was then electroblotted in a 40 mM Tris-acetate buffer containing 1 mM EDTA onto nylon membranes (Zeta-Probe) for 2 hr using a Transblot apparatus (Bio-Rad) operating at a constant voltage of 80 volts. The membranes were then baked at 80°C for 2 hr and the blots were prehybridized, hybridized, washed, and developed as outlined above for Southern blots except that prehybridization and hybridization solutions contained 750 ug/ml denatured salmon sperm DNA to allow more efficient blocking.

5.3 Labelling of cDNA probes with ^{32}P-dCTP

cDNA probes were labelled using the hexanucleotide method of Feinberg and Vogelstein (1983) to specific activities between 0.5-1.0 X 10^9 cpm/ug using ^{32}P-dCTP. Between 50 and 100 ng of an appropriate cDNA probe in a volume of 10 ul was boiled for 5 min, and then chilled. Labelling of the single stranded cDNA probe was then performed using the hexanucleotide kit supplied by Amersham, by adding 2 ul of DNA polymerase I ('Klenow' fragment: 1 unit/ul, stored in 50 mM potassium phosphate (pH 6.5), 10 mM β-mercaptoethanol, and 50% glycerol), 15 ul of multiprime buffer solution (containing dTTP, dATP,

dGTP and random hexanucleotides in a concentrated buffer solution of Tris-HCl (pH 7.8), magnesium chloride, and β-mercaptoethanol) , 18 ul water, and 5 ul ^{32}P-dCTP (specific activity 3000 Ci/mmol) to the probe. Following incubation at room temperature for a minimum of 3 hr, the reaction mixture was passed through a spin column of Sephadex G-50 (prepared in a microfuge tube) and the labelled probe was eluted with 100 ul TE. The incorporation of label into the probe was determined by counting 3 ul of the effluent using a ^{32}P Cerenkov scintillation counter program. An appropiate volume of probe was then added to the hybridization mixture to achieve 1 X 10^6 cpm/ml.

5.4 Isolation and sources of cDNA probes

E. coli MC1061 cells transformed with pcD plasmids (Appendix A) harbouring mouse M1 cDNA (clone 65) or M2 cDNA (clone 10) respectively were obtained from Dr. L. Thelander of the Karolinska Institute, Stockholm (Thelander and Berg, 1986). Large scale preparations of plasmid DNA were prepared according to the procedures of Manniatis *et al.* (1982). A single colony of *E. coli* clone 65 or clone 10 was inoculated into 5 ml LB medium (pH 7.5), (LB medium consists of 1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, and 1% (w/v) NaCl) containing 50 ug/ml ampicillin and incubated at 37°C overnight. The entire suspesnsion was then inoculated into 1 litre of M9 medium (pH 7.4), (M9 medium consists of 0.6% (w/v) sodium phosphate dibasic, 0.3% (w/v) potassium phosphate monobasic, 0.05% (w/v) NaCl, 0.01% (w/v) ammonium chloride, 0.024% (w/v) magnesium sulfate, 0.0011% (w/v) calcium chloride and 2% (w/v) glucose) containing 0.5% casamino acids

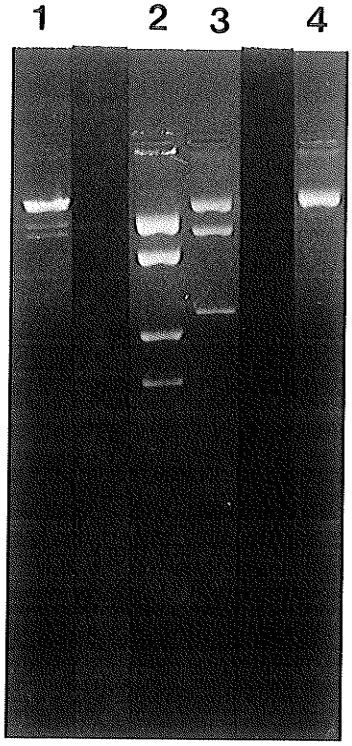
plus 2 ug/ml thiamine. The culture was incubated until an OD_{550} value of 0.1 was reached, at which time uridine was added to the culture to give a final concentration of 1 mg/ml. When the OD_{550} of the culture reached a value of 0.4, 5 ml of a 54 mg/ml solution of spectinomycin was added and the cells were reincubated overnight while being shaken at 250 rpm. The cell suspension was then centrifuged at 4,000 X g for 10 min and the supernatant was discarded while the pellet was resuspended in 25 ml ice-cold solution of 0.1 M NaCl, 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, and 0.5% (v/v) Triton X-100 (STET) and recentrifuged. The pellet was then resuspended in 10 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 10% (w/v) sucrose. To the preparation was added 20 mg lysozyme in a volume of 2 ml followed by addition of 8 ml of 0.25 M EDTA. The sample was incubated on ice for 10 min and then 4 ml of 10% SDS was added and mixed quickly in order to disperse the SDS throughout the sample. Immediately afterwards, 6 ml of 5 M NaCl was added to the sample (final concentration = 1 M) and the sample was set on ice for 1 hr. The sample was then centrifuged for 30 min at 30,000 rpm at 4°C in order to remove high molecular weight DNA and bacterial debris. The supernatant was saved and extracted twice with phenol/chloroform (phenol/chloroform is phenol:chloroform:isoamyl alcohol, 24:24:1) and then once with chloroform (chloroform is chloroform:isoamyl alcohol, 24:1). To the aqueous layer was added 0.54 volumes of isopropanol and the sample was mixed and then incubated at -20°C for a minimum of 2 hr. After centrifugation at 15,000 X g for 30 min the supernatant was discarded and the pellet was washed once with 70% ethanol, dried under vacuum and then resuspended in 7 ml TE. For each ml of the DNA solution was added 1 g of solid

cesium chloride, and the suspension was mixed gently until all the salt had dissolved. The sample was then transferred to Beckman Ti70.1 centrifuge tubes and 0.5 ml of a 10 mg/ml ethidium bromide solution was layered on top. The tube was then filled to the rim by the addition of mineral oil. The sample was then centrifuged at 42,000 rpm for 40 hr at 20°C. Two bands were visible under uv light; the lower band consisting of closed circular plasmid DNA was removed by puncturing the bottom side of the tube with a hyperdermic needle. The ethidium bromide was removed from the plasmid preparation by extracting the sample 4 to 5 times with an equal volume TE/CsCl saturated butanol. The sample was then extensively dialyzed against 3 two litre changes of TE. The concentration of plasmid DNA in the preparation was determined by measuring the absorbance of the sample at 260 nm as described previously.

The C10 plasmid harbouring the cDNA sequences encoding the M2 subunit of ribonucleotide reductase was digested first with Sal I and then with Pst I. Lane 4 of Figure 7 shows a Sal I digested C10 plasmid. As expected only one band was observed corresponding to the linearized form of the plasmid (Appendix A). The linearized DNA from clone C10 was then digested with Pst I (Fig. 7, lane 2). As expected from the DNA sequence of the clone (Thelander and Berg, 1986), 5 bands of approximately, 2483, 1487, 746, 549, and 126 basepairs (bp) were observed, with the smallest band barely being visible on the gel. All restriction enzyme digests were carried out at 37°C using 5 enzyme units per ug DNA. The 1487 bp band corresponds to the cDNA encoding the M2 subunit of ribonucleotide reductase (Thelander and Berg, 1986).

Figure 7

Photograph of an ethidiun bromide stained 2% agarose gel on which digested D65 or C10 plasmids were electrophoresed. Plasmid C10 (2 ug) was digested to completion with Sal I (lane 4) or Sal I digestion followed by digestion with Pst I (lane 2). Lane 3 shows DNA fragments obtained after digestion of plasmid D65 (2 ug) with Nco I. Molecular weight size markers (lane 1) are 9.4, 4.4, 2.0, and 0.6 Kbp.


1
2
3
4

In order to prepare large quantities of this insert, plasmid DNA was digested as described above and electrophoresed on a 1% agarose gel. Lane 2 of Figure 8 shows the digested plasmid. The 1487 bp band was removed by cutting it out of the gel with a razor (Fig. 9, lane 2) and the insert was electroeluted from the gel by placing the gel in dialysis tubing containing 0.5 X TBE buffer and passing a current of 100 volts for 2-3 hr across the sample. The plasmid insert now in 0.5 X TBE buffer was passed through a minicolumn-D (Sigma Chemical Company) in order to concentrate and clean the preparation. The DNA was then precipitated by the addition of 2 volumes of ethanol and overnight incubation at -20°C. The sample was then centrifuged, washed once with 70% ethanol, dried under vacuum and finally resuspended in about 100 ul of TE buffer.

The D65 plasmid harbouring the cDNA sequences encoding the M1 subunit of ribonucleotide reductase was digested to completion with NcoI (5 units per ug DNA). As can be seen in Figure 7, three bands corressponding to approximately 3100, 2000, and 900 basepairs were observed following electrophoresis on a 2% agarose gel (Appendix A). Figure 8, shows a 1% agarose gel on which large quantities of NcoI digested D65 plasmid were electrophoresed. The band corresponding to the cDNA sequences encoding the M1 subunit of ribonucleotide reductase (approximately 2000 bp) was removed from the gel by cutting it out with a razor (Fig. 9, lane 3) and the cDNA insert was electroeluted as described above for the M2 cDNA insert. Figure 10 shows a picture of a 1% agarose mini-gel on which the inserts from the D65 and C10 plasmid were electrophoresed. As can be seen, both of the preparations showed

Figure 8

Photograph of an ethidium bromide stained 1% agarose gel on which 75 ug of Sal I plus Pst I digested C10 plasmid (lane 2) or 75 ug of Nco I digested D65 plasmid (lane 3) was electrophoresed. Lane 1 shows DNA molecular weight markers obtained from Hinf I digested pBr322 plasmid. Fragments of the following sizes are visible on the gel: 1632, 517, 506, 396, 344, and 298 bp.

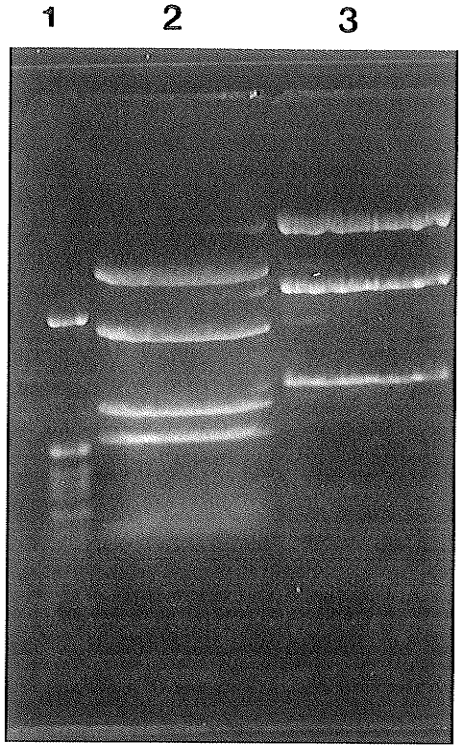

1
2
3

Figure 9

Photograph of an ethidium bromide stained 1% agarose gel on which 75 ug of Sal I plus Pst I digested C10 plasmid (lane 2) or 75 ug of Nco I digested D65 plasmid (lane 3) was electrophoresed. The figure shows the removal of the 1487 bp fragment corresponding to the cDNA encoding the M2 subunit of ribonucleotide reductase (lane 2), and the removal of the 1986 bp fragment corresponding to the cDNA encoding the M1 subunit of ribonucleotide reductase (lane 3). Lane 1 shows DNA molecular weight markers obtained from Hinf I digested pBr322 plasmid. Fragments of the following sizes are visible on the gel: 1632, 517, 506, 396, 344, and 298 bp.

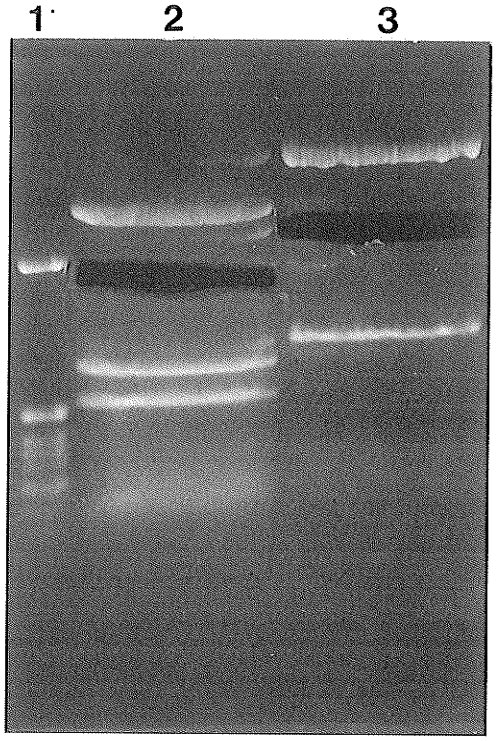
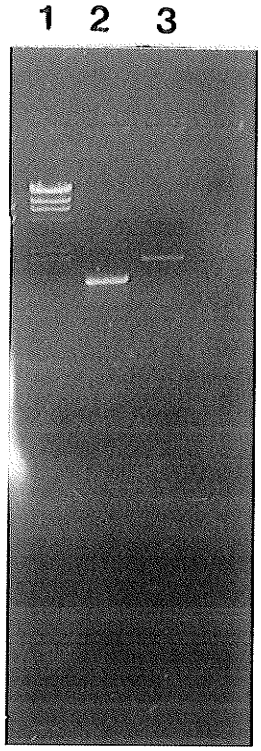

1
2
3

Figure 10

Photograph of an ethidium bromide stained 2% agarose gel on which the purified inserts of plasmid C10 and D65 corresponding to the M2 (lane 2), and the M1 (lane 3), cDNA of ribonucleotide reductase respectively were electrophoresed. DNA molecular weight markers of the following sizes are shown in lane 1: 23.6, 9.6, 6.6, 4.3, 2.3, and 2.0 Kbp.


1 2 3

only a single band, corresponding to the purified cDNA sequences of the M1 and the M2 subunits of ribonucleotide reductase.

The β-actin probe was obtained from L. Kedes.

6. PARTIAL PURIFICATION OF RIBONUCLEOTIDE REDUCTASE

A simple and practical method for partially purifying ribonucleotide reductase from cell extracts was performed according to the procedures developed by Spector (Spector and Averett, 1983; Spector, 1985). Large quantities of logarithmically growing cells were obtained by suspension culture in spinner flasks; three litre batches of cells were harvested by centrifugation at cell densities between 5 and 8 X 10^5 cells/ml as estimated with a Coulter Particle Counter. Approximately 10g of cells from each cell line was disrupted by sonication (Biosonik, Bronwill Scientific) in a buffer of 20 mM Hepes (pH 7.6), containing 10 mM $MgCl_2$, 2 mM dithiotreitol (DTT), and 0.1 mM phenylmethylsulfonyl fluoride. All procedures were carried out at 4°C. Cellular debris was removed by centrifugation at 20,000 X g for 30 min. Streptomycin sulfate was then added during a 30 min period to a final concentration of 0.65% (w/v). The sample was centrifuged at 20,000 X g for 20 min, and ammonium sulfate was added to the supernatant during a 30 min period to obtain 40% saturation. Following centrifugation (20,000 X g for 20 min) the pellet was resuspended in 1 ml of 2 mM DTT, 2 mM $MgCl_2$, 0.5 M KCl and 20 mM Tris-HCl (pH 7.6) (buffer A), and dialyzed against the same buffer for 8 hr. The extract was then diluted to 45 mg/ml with buffer A and 1.2 ml was loaded onto

a 0.7 X 26 cm ATP-agarose Type 3 (C-8 linked) column preequilibrated with the same buffer. Twenty fractions of 1 ml each were collected and assayed for ribonucleotide reductase activity and nucleoside diphosphate kinase activity. Fractions with the highest ribonucleotide reductase activity/nucleoside diphosphate kinase activity ratios were pooled and dialyzed for 8 hr against 2 mM $MgCl_2$, 2 mM DTT, and 20 mM Hepes (pH 7.6). This procedure allowed the preparation of partially purified ribonucleotide reductase that could be used for accurate kinetic analysis.

7. IN VITRO RIBONUCLEOTIDE REDUCTASE ASSAY

Enzyme activity was measured in partially purified preparations by the method of Steeper and Stuart (1970), using ^{14}C-CDP as substrate, according to the procedure of Lewis and Wright (1978a). Unless otherwise indicated, the reaction mixture contained in a final volume of 50 ul: 6 mM DTT, 4 mM $MgCl_2$, 4 mM ATP, 5 mM NaF, 100 mM Hepes buffer (pH 7.5), 50 uM ^{14}C-CDP (0.09 uCi), and an appropiate amount of partially purified enzyme preparation. Reactions were carried out for 10 or 20 min at 37°C, and terminated by boiling for 4 min. The deoxycytidine phosphates were then converted to deoxycytidine by addition of 1 mg of Crotalus atrox venom (Cory, 1973) prepared in 0.1 M Hepes (pH 8.0), plus 10 mM $MgCl_2$ (20 mg venom per ml). Following incubation for 1 hr at 37°C the reaction was terminated by boiling for 4 min and 0.5 ml distilled water was added to each assay tube. The tubes were then centrifuged in order to remove the heat precipitated material and the supernatant was loaded onto a 5 X 80 mm column of

Dowex-1-borate (Steeper and Stuart, 1970; Cory and Whitford, 1972) in order to separate the nucleosides from the deoxyribonucleosides. Briefly, the separation on Dowex-1-borate is based upon the formation of a complex between ribonucleosides and borate ions on the column resin. Since deoxynucleosides do not possess a cis-diol, they are unable to form a complex and hence are eluted from the column. Deoxycytosine was thus eluted into scintillation vials with 5 ml distilled water and the radioactivity was measured as described previously. One unit of enzyme activity was defined as the amount of enzyme that would reduce 1 nmole of nucleotide in 1 hr per mg protein.

The kinetic parameters Vmax, Km, and Ki were determined using a weighted least-squares analysis as described in Cleland (1967).

8. NUCLEOSIDE DIPHOSPHATE KINASE ASSSAY

Nucleoside diphosphate kinase activity was determined in the partially purified preparation of ribonucleotide reductase using standard CDP reductase assay conditions. Following boiling of the assay tubes for 4 min the tubes were centrifuged to remove cellular debris and 5 ul of the supernatant was applied to polyethyleneimine (PEI)-cellulose thin layer chromatography (TLC) plates prerun with water and prespotted with carrier cytosine nucleotides. The plates were developed by ascending chromatography in 0.5 M LiCl plus 0.125 M formic acid, dried, and spots were outlined under uv light and cut out. The Rf values were: cytosine, 0.80; cytidine monophosphate, 0.54; cytidine and deoxycytidine diphosphate, 0.42; cytidine triphosphate,

0.07. Radioactivity in each of the spots was determined and the percentage of total counts in each spot calculated. One unit of nucleoside diphosphate activity was defined as the amount of enzyme that would convert 1 nmol of cytidine diphosphate to cytidine triphosphate in 1 hr per mg protein.

9. ASSAYS FOR TUMOR GROWTH RATES AND EXPERIMENTAL METASTASIS

Standard assays for measuring tumor growth rates and experimental metastasis were used (e.g. Greenberg et al., 1987; Egan et al., 1987a). Clones of the three CHO cell lines (wild type, H^R-R2T, and HN^R-AT) were grown to a final population size of 10^7 cells, removed from the culture plates with trypsin solution, and washed with Hanks balanced salt solution. For tumor growth rate studies, 1 X 10^6 cells from each clone were injected subcutaneously into the lower midback of 9 to 10 week old female C57 BALB/c nudes (nu/nu). Tumor size was calculated by multiplying the dimensions of the cross section of the tumor. For the experimental metastasis assay, 5 X 10^5 cells from each clone, were injected in a volume of 0.2 ml into the tail veins of 9 to 10 week old female inbred homozygous BALB/c nudes (nu/nu). Mice were sacrificed after 3 weeks by ether anasthesia and Bouins solution (picric acid, formaldehyde, acetic acid; 15:15:1) injected intra-tracheally. Lungs were then removed, and metastatic foci were counted under a dissecting microscope. All mice were maintained in a sterile enviroment throughout these studies. Wilcoxon's two sample test was applied to determine the statistical significance of the lung metastasis data (Sokel and Rohlf, 1981).

10. DEOXYRIBONUCLEOTIDE POOL ANALYSIS

For preparation of deoxyribonucleotide extracts, cells were added to 150 mm tissue culture plates (1.5 X 10^6 cells) 40 to 50 hr prior to experimentation. About 20 plates were used for each cell line. Cells were removed from the plates with trypsin solution, centrifugred at 4°C and the cell pellet was resuspended in ice-cold PBS. The cell number was then determined with a Coulter Particle Counter as described previously. After centrifugation, deoxyribonucleotides were extracted with 10% trichloroacetic acid (TCA) containing ^{3}H-adenosine (for determining dilution) for 20 min on ice. The extract was centrifuged and the supernatant was neutralized with a 0.5 M solution of alamine 336 in Freon-TF (Khym, 1975). Neutralized extracts were stored at -20°C.

The DNA polymerase assay was used to estimate the concentrations of each deoxyribonucleoside triphosphate. The method of Hunting and Henderson (1981) was followed except for some minor modifications. For the dATP and dTTP assays the reaction mixture contained in a final volume of 180 ul: 0.02 A_{260} units poly[d(A,T)], 1.8 umol $MgCl_2$, 1.8 umol dAMP, 18 umol Hepes buffer (pH 7.4), and 0.75 Richardson units of DNA polymerase 1 (Boehringer Mannheim). In addition, the dATP assay contained 100 pmol (0.5 uCi) ^{3}H-dTTP and 0 to 75 pmol dATP standard or an appropriate amount of extract whereas, the dTTP assay contained 100 pmol (0.5 uCi) ^{3}H-dATP and 0 to 80 pmol dTTP standard or an appropriate amount of extract. The reaction mixtures for the dGTP and dATP assays contained in a final volume of 180 ul: 0.02 A_{260} units

poly[d(I,C)], 1.8 umol $MgCl_2$, 1.8 umol dAMP, and 18 umol Hepes buffer (pH 7.4). As well, the dGTP assay contained 100 pmol (2.2 uCi) ^{3}H-dCTP, 0 to 10 pmol dGTP standards or an appropriate amount of extract, and 1.9 Richardson units of DNA polymerase 1. The dCTP assay contained 240 pmol (0.5 uCi) ^{3}H-dGTP, 0 to 200 pmol dGTP standard or an appropiate amount of extract, and 3.0 Richardson units of DNA polymerase 1. Reactions were initiated by the addition of enzyme followed by incubation at 37°C. The optimum reaction time for each of the four assays was determined for each new batch of DNA polymerase 1. The reactions were terminated by the addition of 500 ul of ice-cold 5% TCA containing 1% sodium pyrophosphate to each tube. The entire contents of each tube were then placed on 3MM filters (Whatman) prewetted with 2% sodium pyrophosphate and washed twice with 10 ml of 5% TCA containing 1% sodium pyrophosphate and then twice with 10 ml of 95% ethanol. The dried filters were placed in scintillation vials, and radioactivity was determined as described in section 2.7 of this thesis. Fisher's protected LSD method (Snedecor and Cochran, 1973) was applied to test whether the means of the pool sizes from the various cell lines tested were statistically different at the 5% level of significance.

11. DETERMINATION OF SPONTANEOUS MUTATION RATES BY FLUCTUATION ANALYSIS

Fluctuation analysis (Luria and Delbruck, 1943) for determination of the rates of spontaneous mutation of the wild type, H^R-R2T, and HN^R-AT cell lines (Lewis and Wright, 1978b; Liu et al., 1982; Li et

al., 1983) to 6-thioguanine and ouabain resistance was performed. In brief, replica cultures were initiated by adding 200 cells to 60 mm tissue culture plates containing alpha-MEM plus 10% FCS. After one week incubation at 37°C, the colonies were removed with trypsin solution, cells were redispersed on to the plates, and normal growth medium was added. Three replicas were stained with a saturated solution of methylene blue in 50% ethanol, and colonies were counted to determine the initial number of viable cells plated. Once the replica cultures formed a partial monolayer on the 60 mm plates, they were again removed with trypsin solution, and transferred to 150 mm plates, where they were grown to a final cell density of 3×10^6 cells. The cells were then removed from the plates with trypsin solution and added to 150 mm tissue culture plates at 1×10^6 cells/plate containing the selective agent in alpha-MEM plus 10% dialyzed FCS. Thus, each replica was eventually divided into three. This procedure was followed to avoid possible cell density effects on colony formation that can occur in selective medium at higher cell densities. After 10 days of incubation at 37°C, plates were stained with the methylene blue solution, and the number of colonies formed in the presence of drug was counted. Concentrations of 2 uM and 1.5 mM for 6-thioguanine and ouabain, respectively, were used in these experiments. The rate of spontaneous mutation (a) was calculated using the fraction of culture plates with no resistant colonies (P_o) according to Luria and Delbruck (1943), in which $a = \ln (1/P_o)/\text{divisions}$. The standard errors of the estimated mutation rates were calculated using the formula $(1-P_o/d^2 \cdot C \cdot P_o)^{\frac{1}{2}}$, where d = number of cell divisions and C = number of parallel cultures treated with drug, as

described by Liu et al. (1982) and Li et al. (1983). Statistical analysis to test for the significance of the differences in spontaneous mutation rates observed with the various cell lines was performed as outlined by Li et al. (1983).

12. MEASUREMENT OF CELL GROWTH IN THE PRESENCE OF PALA

In order to determine the relative degree of resistance of the various CHO cell lines to N-(phosphonacetyl)-L-aspartate (PALA), cells were tested for their abilities to grow in the presence of 200 and 400 μM drug. To measure cell growth, cells were seeded at densities of 5 X 10^5 cells per 60 mm tissue culture plate in normal growth medium containing either 0, 200, or 400 μM PALA. The plates were incubated at 37°C for 72 hr after which the media was removed by aspiration and 1.5 ml of 1 M NaOH was added to each plate. The plates were then scraped with a rubber policeman to remove cellular material and the suspension was incubated at 50°C for 15 min. The extracts were diluted to an appropriate volume and the absorbance of the colorless, and clear solution at 260 nm was read as a measurement of nucleic acid content (Swyryd et al., 1974; Kempe et al., 1976). Each experimental point was performed in triplicate and the average value is expressed as a percentage taking growth in the absence of drug as 100%.

13. IN VIVO RIBONUCLEOTIDE REDUCTASE ASSAY

Ribonucleotide reductase activity in human cells was measured using a permeabilized cell assay developed by Lewis et al. (1978) as

modified by Dick and Wright (1980). Between 1.5 X 10^6 and 2.0 X 10^6 cells were added to 150 mm tissue culture plates and incubated for 40-48 hr. The logarithmically growing cells were then removed from the plates with trypsin solution, washed in alpha-MEM and centrifuged. The cell pellet was resuspended in 10 ml of growth medium and counted using a Coulter Particle Counter as described in section 2.2 of this thesis. Cells were then resuspended in permeabilizing buffer consisting of 1.0% Tween-80, 0.25 M sucrose, 0.05 M Hepes buffer (pH 7.2), and 2 mM DTT at 5 X 10^6 cells/ml. The permeabilization was carried out for 1 hr in a 30°C waterbath with gentle stirring. The cells were then resuspended in fresh permeabilizing buffer at a concentration of 1.5 x 10^7 or 2.5 X 10^7cells/ml. Aliquots of 200 ul corresponding to 3 or 5 X 10^6 cells were dispensed into reaction tubes containing 100 ul of reaction mixture. The final assay for CDP reduction consisted of 0.05 M Hepes buffer (pH 7.2), 7 mM DTT, 8 mM $MgCl_2$, 4 mM ATP, 0.4 mM ^{14}C-CDP (5,000 cpm/nmol), 0.67% Tween-80, 0.167 M sucrose, 5 mM NaF and 3 or 5 X 10^6 cells. A 2 X 7 mm magnetic stirring bar was placed in each assay tube and the tubes were incubated for 20 or 30 min in a 37°C waterbath overtop a magnetic stirrer (Bellco Glass Co.) operating at high speed. The reaction was terminated by heating the tubes in a boiling water bath for 4 min. Cytidine and deoxycytidine phosphates were hydrolyzed and deoxycytosine seperated from cytosine as described in section 7 of this thesis for the in vitro ribonucleotide reductase assay. This assay allows for the determination of in vivo ribonucleotide reductase activity in intact, nucleotide-permeable cells. Assays were performed within the linear activity region with regard to time and cell number. Activity is expressed as nmoles dCDP formed/5 X

10^6 cells/hr.

14. THYMIDINE UPTAKE

Approximately 10^6 cells were seeded onto 60 mm tissue culture plates 60 to 72 hr prior to experimentation. The growth medium was removed from the plates and 3 ml fresh growth medium containing 0.2 uM ^{3}H-thymidine (20 uCi/ml) was added to the plates and the plates were reincubated at 37°C for various lengths of time (Hards and Wright, 1983). The radioactive containing medium was then aspirated off of the plates, and 3 ml of ice-cold 10% TCA was added, and the plates were chilled on ice for 10 min. The cells were then scraped off the surface of the plates with a rubber policeman, and filtered through glass fibre filters which had been presoaked in 10% TCA. The filters were washed twice with 5% TCA, twice with PBS, and twice with 70% ethanol (all washes were with 10 ml of ice-cold solutions). The filters were then dried and radioactivity was determined as described in section 2.7 of this thesis. Additional plates were seeded with cells in order to determine the final cell number of each culture at the time nucleoside uptake was performed.

15. HYDROXY[^{14}C]-UREA UPTAKE

To study hydroxy[^{14}C]-urea uptake in human and CHO cells, the cells were seeded in normal growth medium onto six-well plates at a density of approximately 1 X 10^6 cells/well 48 hr prior to experimentation. Uptake experiments were performed using a slide warmer (Fisher

Scientific) set at 37°C upon which the multiwell plates were placed. Medium was then aspirated from the plates and labelled hydroxyurea that was prepared in PBS was added (specific activity of drug varied between 1.35×10^{10} and 1.5×10^{7} dpm/mmol, depending on the experiment). The experiment was stopped by placing the plates on ice, with immediate removal of the label, followed by three quick washes of the wells with 2 ml of ice-cold PBS (Morgan et al., 1986; Tagger et al., 1987). The cells were then solubilized by adding 1 ml of 1 N NaOH to each well. An aliquot was then removed, neutralized with HCL and counted for radioactivity. Unless otherwise specified, each experimental point was performed in duplicate or triplicate and the average value is reported. Studies involving the metabolic inhibitors NaF and dinitrophenol (DNP) were carried out following a 10 min pretreatment of the cells in the presence of the inhibitor. In all cases, background was determined by immediately placing the plates on ice after addition of labelled drug, followed by washing of the wells as described above. Background values (representing non-specifically bound label) varied very little between experiments. The cell/medium distribution ratio was determined by measuring the ratio of labelled hydroxy[^{14}C]-urea incorporated into the cell relative to the amount of labelled hydroxy[^{14}C]-urea present in an equal volume of medium surrounding the cells at the time when uptake was terminated (Lam et al., 1980). The cell volume for WI-38 cells grown under the conditions described was determined using a Coulter Particle Counter (Lam et al., 1980) and found to be 2.69 picolitres. Temperature coefficient (Q_{10}) values for hydroxyurea uptake were calculated from the ratio of hydroxy[^{14}C]-urea incorporated into the cells at temperature intervals

of 10°C.

Hydroxy[^{14}C]-urea (specific activity of 7.3 mCi/mol) was supplied by Amersham Corp. by special order and the radiochemical purity was 95-98% as determined by thin layer chromatography on cellulose, using the following three different solvent systems: (i) butan-2-one - acetone - formic acid - water (40:2:1:6), (ii) butan-2-one - diethylamine - water (921:2:77), (iii) butan-1-ol - water - acetic acid (12:5:3). The labelled drug cochromatographed with authentic hydroxyurea in each of the above solvent systems.

16. SOURCES OF MATERIALS

Most biochemicals were purchased from Sigma Chemical Co., St. Louis and unless otherwise stated, all radioactive chemicals were from Amersham/Searle Ltd., Arlington Heights. Sources of other materials are listed in Table 1.

Table 1. Sources of Materials

MATERIAL	SOURCE
acetic acid	Fisher Sci. Co. Ltd., Fairlawn
agarose	Bio-Rad Labs
Alpha-minimal essential medium	Flow Laboratories, Rockville
Aquasol II	New England Nuclear, Lachine
ATP-agarose	Pharmacia
bromophenol blue	Fisher Sci. Co. Ltd.
chloroform	Fisher Sci. Co. Ltd.
culture plates (100 mm plastic)	Falcon
culture plates (150 mm plastic)	Falcon
culture plates (60 mm plastic)	Falcon
dimethylsulfoxide	Fisher Sci. Co. Ltd.
Dowex 1-X8 anion exchange resin (200 to 400 mesh)	Bio-Rad Labs
ethanol	Fisher Sci. Co. Ltd.
ethylene diamine tetraacetic acid	Fisher Sci. Co. Ltd.
fetal calf serum	Gibco Ltd., Grand Island
formaldehyde	Fisher Sci. Co. Ltd.
formamide	Fisher Sci. Co. Ltd.
glycerol	Fisher Sci. Co. Ltd.
guanidine thiocyanate	Fisher Sci. Co. Ltd.
isoamyl-alcohol	Fisher Sci. Co. Ltd.
magnesium chloride	Fisher Sci. Co. Ltd.
methylene blue	Fisher Sci. Co. Ltd.

MATERIAL	SOURCE
nitrocellulose	Schleicher & Schuell, Inc. Keene, N.H.
phenol	Fisher Sci. Co. Ltd.
polyacrilamide	Bio-Rad Labs
polyethyleneimine cellulose	Aldrich Chemical Co., Inc.
potassium chloride	Fisher Sci. Co. Ltd.
restriction endonucleases	Boehringer Mannheim, Canada
Scinti Verse II	Fisher Sci. Co. Ltd.
sec-butyl-alcohol	Fisher Sci. Co. Ltd.
sodium chloride	Fisher Sci. Co. Ltd.
sodium citrate	Fisher Sci. Co. Ltd.
sodium dodecyl sulfate	Bio-Rad Labs
sodium flouride	Fisher Sci. Co. Ltd.
sodium hydroxide	Fisher Sci. Co. Ltd.
sodium pyrophosphate	Fisher Sci. Co. Ltd.
trichloroacetic acid	Fisher Sci. Co. Ltd.
Tween-80	J.T. Baker Co., Phillipsburg, N.J.
urea	Fisher Sci. Co. Ltd.
Zeta-probe (Zeta-probe is a registered trade mark)	Bio-Rad Labs
ficoll	Pharmacia

RESULTS

RESULTS

1. CHARACTERIZATION OF HYDROXYUREA RESISTANT CHO CELL LINES

1.1 Drug sensitivity and growth rates of wild type, H^R-R2T, and HN^R-AT cell lines

Two cell lines were selected for the ability to proliferate in the presence of normally cytotoxic concentrations of drug as described in the Materials and Methods section. The colony-forming abilities in the presence of hydroxyurea for the parental wild type CHO population and the two variant lines, H^R-R2T, and HN^R-AT are shown in Figure 11. Both H^R-R2T and HN^R-AT cell lines were clearly less sensitive than wild type to the cytotoxic effects of the drug. The H^R-R2T cell line was the most resistant line, having a D_{10} value of 1.75 mM hydroxyurea, approximately 8 fold greater than the D_{10} value obtained for the wild type (0.22 mM). The HN^R-AT population showed a D_{10} value of 0.37 mM drug, approximately 2 fold greater than wild type cells. The drug resistance characteristics of these cell lines remained stable over a period of three years of continuous cell culture in the absence of a selective agent.

The growth curves for the wild type, H^R-R2T and HN^R-AT cell lines are shown in Figure 12. The doubling times for all three cell lines under normal growth conditions were very similar and calculated to be 12.0, 10.9 and 11.8 hr for the wild type, H^R-R2T and HN^R-AT cell lines respectively. Figure 12 also illustrates that fairly large quantities

Figure 11

Relative colony-forming ability of wild type (●), H^R-R2T (▲), and HN^R-AT (■) cell lines in the presence of increasing concentrations of hydroxyurea. Error bars indicate standard errors from four independent determinations.

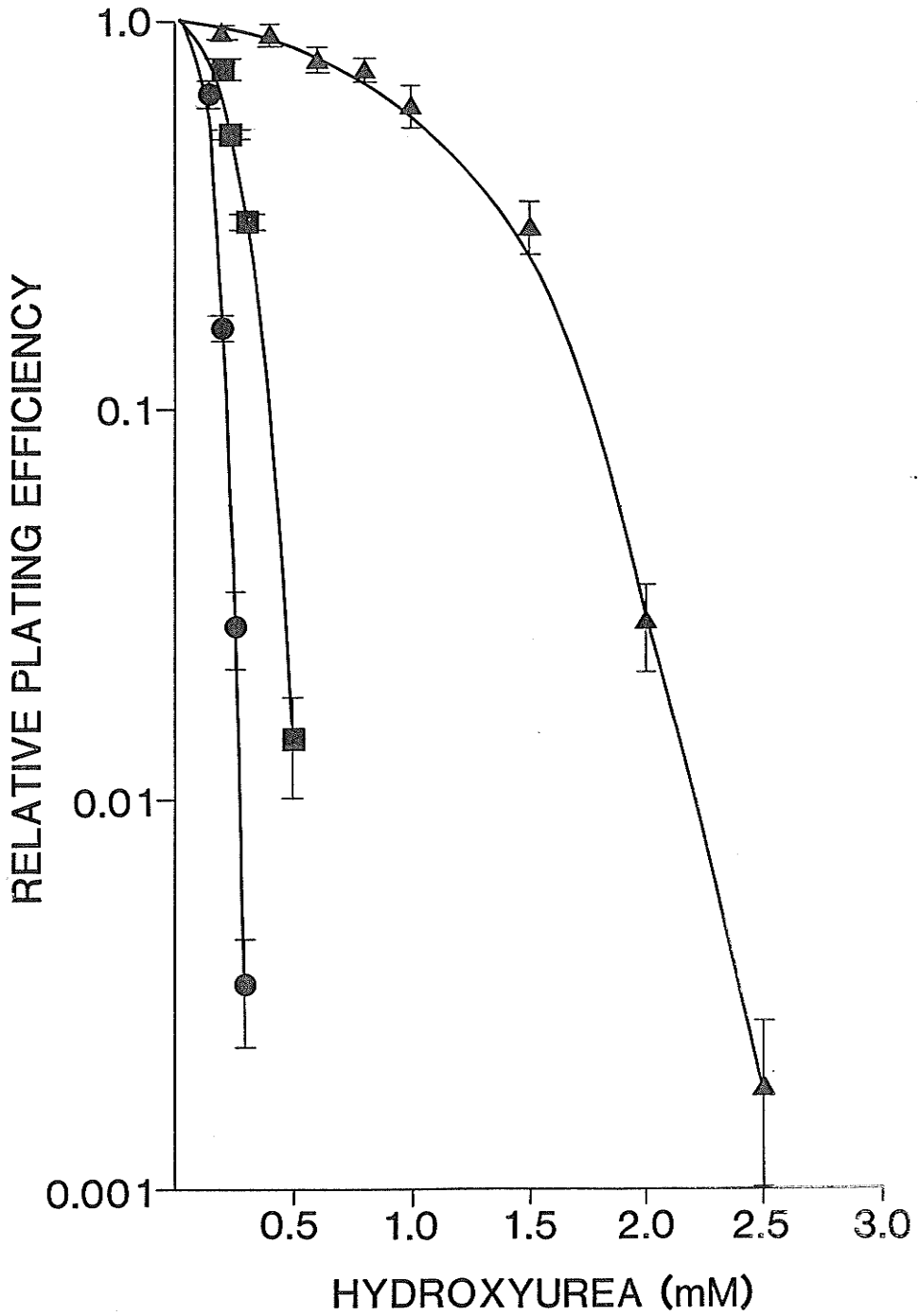
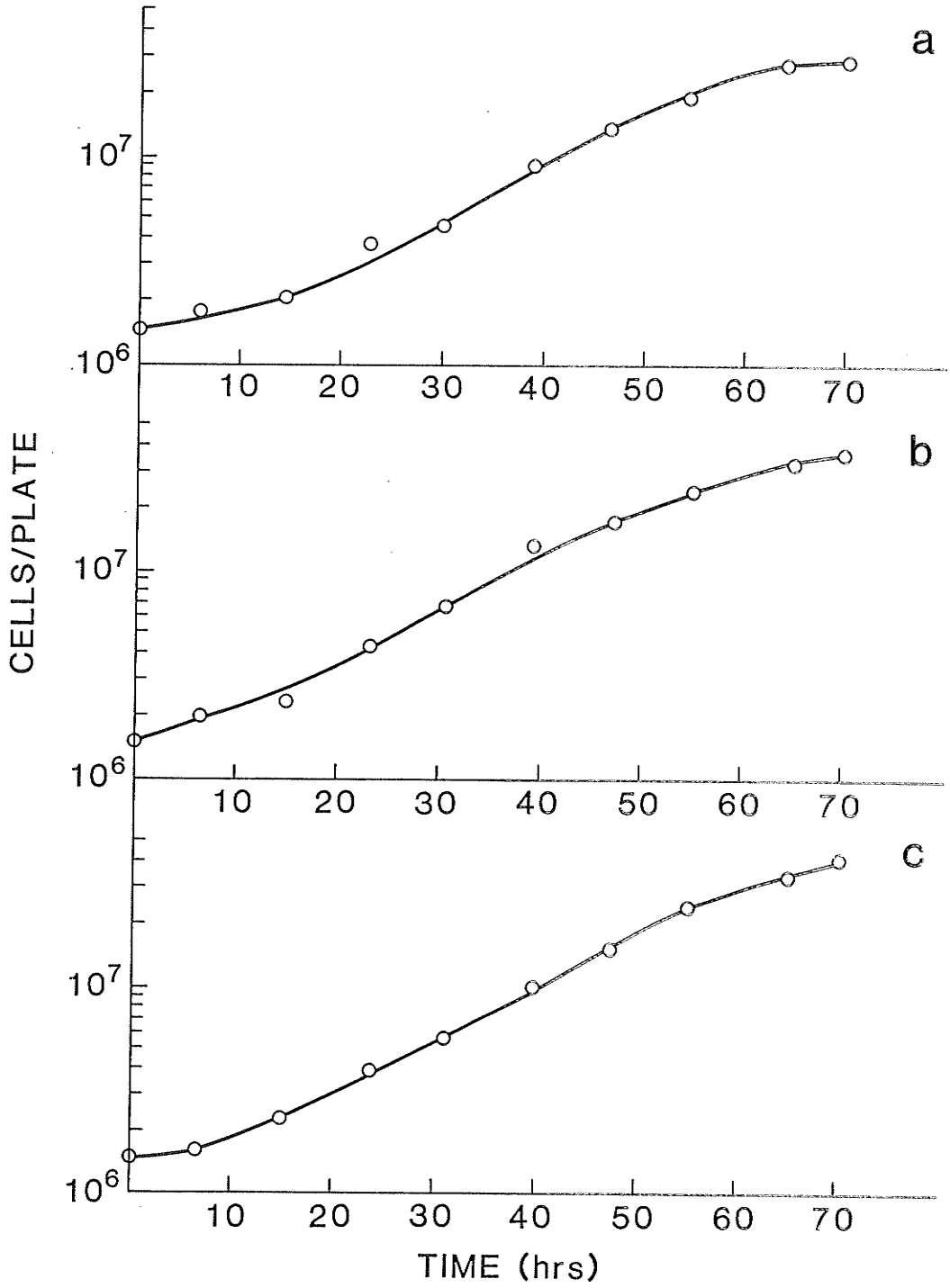

1.0
0.1
0.01
0.001
RELATIVE PLATING EFFICIENCY
0.5
1.0
1.5
2.0
2.5
3.0
HYDROXYUREA (mM)

Figure 12

Growth curves for wild type (a), H^R-R2T (b), and HN^R-AT (c) cell lines. 1.5 X 10^6 cells were seeded onto 150 mm tissue culture plates in alpha-MEM plus 10% FCS and cell numbers were determined after various lengths of incubation time as outlined in Materials and Methods.


a
b
c
10^7
10^6
10 20 30 40 50 60 70
CELLS/PLATE
TIME (hrs)

of log-phase cells can be obtained by incubating 1.5 X 10^6 cells per 150 mm tissue culture plate for 40-48 hr. Such a procedure was followed in experiments shown below for which logarithmically grown cells were required.

1.2 Mechanisms of hydroxyurea resistance in the variant CHO lines

1.2.1 Analysis of ribonucleotide reductase protein, message and DNA levels

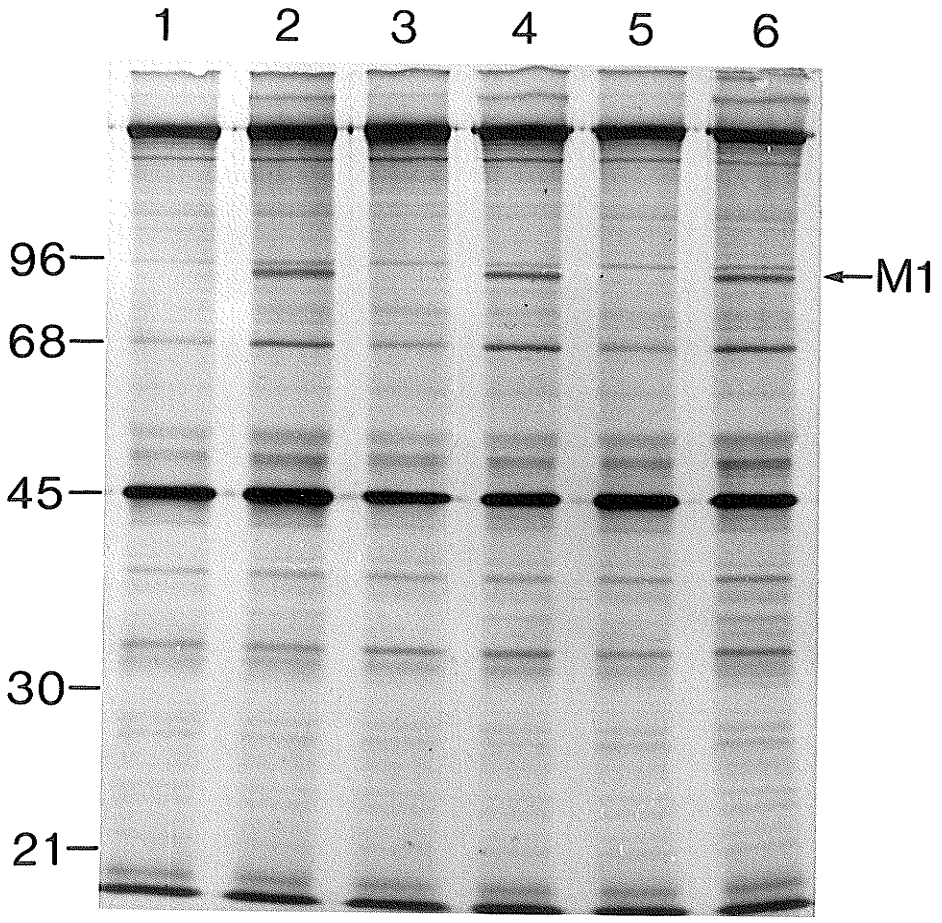
In order to understand why the CHO variant cell lines are stably resistant to the cytotoxic effects of hydroxyurea, an analysis on the levels of both components of ribonucleotide reductase was carried out. Since the primary site of hydroxyurea is at the reductase, and a common mechanism for achieving drug resistance is through elevated levels of the target protein (Stark and Wahl, 1984; Schimke, 1984a,b; Stark, 1986), it was deemed appropriate to examine this possibility first.

1.2.1.1 Analysis of the M1 and M2 proteins of ribonucleotide reductase

The relative concentrations of the M1 protein were estimated following labelling of cells with ^{35}S-methionine and immunoprecipitation with anti-M1 antiserum. The autoradiogram of the immunoprecipitated proteins is shown in Figure 13. The 88,000 dalton protein band present in extracts immunoprecipitated with anti-M1

Figure 13

Immunoprecipitation of [^{35}S] methionine-labelled cellular proteins from wild type (lanes 1 and 2), HN^{R}-AT (lanes 3 and 4), and H^{R}-R2T (lanes 5 and 6) cell lines by incubation with preimmune serum (lanes 1,3 and 5) or anti-M1 antibody (lanes 2,4, and 6) followed by adsorption to formalin fixed S. aureus A cells and analysis by SDS 10% polyacrylamide gel electrophoresis (molecular weight markers X 10^{-3}).


1
2
3
4
5
6
96
68
45
30
21
M1

antiserum (Fig. 13 lanes 2,4, and 6) and absent in extracts immunoprecipitated with preimmune serum (Fig. 13 lanes 1,3, and 5) corresponds to the M1 subunit of ribonucleotide reductase (arrowhead). There is no difference in the intensity of this band in either the H^R-R2T or HN^R-AT cell extracts as compared to the wild type cell extract, indicating that the M1 component of ribonucleotide reductase is not elevated in the drug resistant cell lines. Since ribonucleotide reductase contains a free radical necessary for activity it is possible to determine expression of M2 protein in whole cells by measuring the free radical signal characteristic of a functional M2 component (Wright et al., 1987,1988; McClarty, et al., 1987a). As indicated in Table 2 resistance to hydroxyurea was accompanied by an increase in tyrosyl free radical content when compared to wild type cells. This increase in free radical concentration and therefore M2 component, ranged from about 4 to 5 fold with H^R-R2T cells and 1.5 to 2 fold with HN^R-AT cells. Since levels of the M2 component vary during the cell cycle, being highest during S phase (Eriksson et al., 1984), cell cycle analysis was performed to determine the proportion of cells in S phase during logarithmic growth. Table 2 shows that the difference in EPR values could not be attributed to a larger proportion of drug resistant cells in the S phase of the cell cycle.

1.2.1.2 Analysis of M1 and M2 mRNA and DNA

With the recent availability of the cDNA clones encoding the two components of ribonucleotide reductase (Thelander and Berg, 1986) it became possible to determine the relative amounts of M1 and M2

TABLE 2. EPR measurements of M2 tyrosine free radical in CHO cells in exponential growth[a]

Cell Lines	EPR Signal (uM)	-fold increase	S phase (%)
WT	0.16	-	37
H^R-R2T	0.70	4.4	32
HN^R-AT	0.26	1.6	27

a Free radical concentrations were determined by comparing the EPR signals with a control signal of 1.4 uM as described in Materials and Methods.

transcripts and gene copies in mammalian cells (Thelander and Berg, 1986; Wright et al., 1987, 1988; McClarty et al., 1987a). The Northern blots shown in Figure 14 show that M1 cDNA hybridizes to a single mRNA species of about 3.1 Kb, and in keeping with M1 immunoprecipitation experiments (Fig. 13), no obvious difference in the concentration of M1 mRNA between the three cell lines was observed. The Northern blots shown in Figure 15 indicate that M2 cDNA detects two mRNA species of about 3.4 and 1.6 Kb, which is consistent with other studies of hamster cells (Wright et al., 1987). In agreement with the EPR studies (Table 2) M2 mRNA was elevated in both H^R-R2T and HN^R-AT cells. Densitometer measurements of the major M2 mRNA species estimated increases of about 5 and 2 fold in H^R-R2T and HN^R-AT cells respectively. To verify that equivalent amounts of mRNA were loaded onto the gels and blotted onto the nylon filters probed with M1 and M2, blots were probed with β-actin. As shown in Figure 16, it is evident that equal amounts of mRNA were loaded and blotted onto the filters.

To determine whether the increases in M2 mRNA, in H^R-R2T and HN^R-AT cells was due to amplification of the M2 gene, DNA from each of the cell lines was digested to completion with Eco R1 or Pst 1 endonucleases, and Southern blots of the digested mixtures were hybridized with ^{32}P-labelled M1 or M2 cDNA. Figure 17 shows pictures of ethidium bromide stained agarose gels which were blotted onto nitrocellulose and subsequently probed. As is evident by the intensity of the fragmented DNA, equivalent amounts of digested DNA from the various cell lines were loaded onto the gels. Figure 18 shows that an obvious increase in M2 gene copy number is present with DNA isloated

Figure 14

Northern blot analysis of M1 mRNA levels in wild type (lane 1), HN^R-AT (lane 2), and H^R-R2T (lane 3) cell lines. The autoradiograms were exposed for 6 hr at -70°C with intensifying screens. The arrowheads refer to the positions of 28s and 18s rRNA.

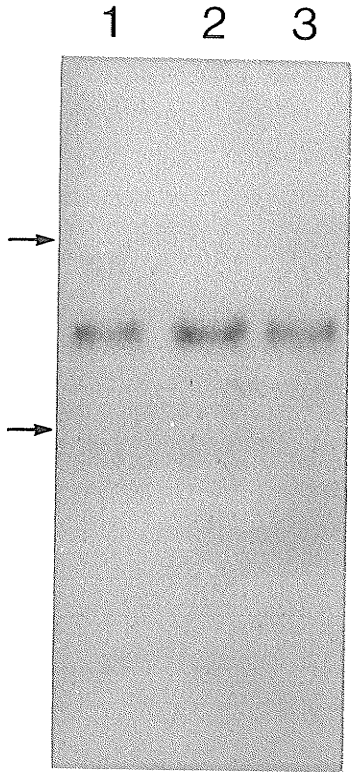

1
2
3

Figure 15

Northern blot analysis of M2 mRNA levels in wild type (lane 1), HN^R-AT (lane 2), and H^R-R2T (lane 3) cells. The autoradiograms were exposed for 6 hr at -70°C with intensifying screens. Two M2 mRNA species of approximately 3.4 Kbp and 1.6 Kbp were detected in each CHO cell line. The proportion of total M2 mRNA composing the two bands differs in different cell lines (Wright et al, 1987). The arrowheads refer to the positions of 28s and 18s rRNA.

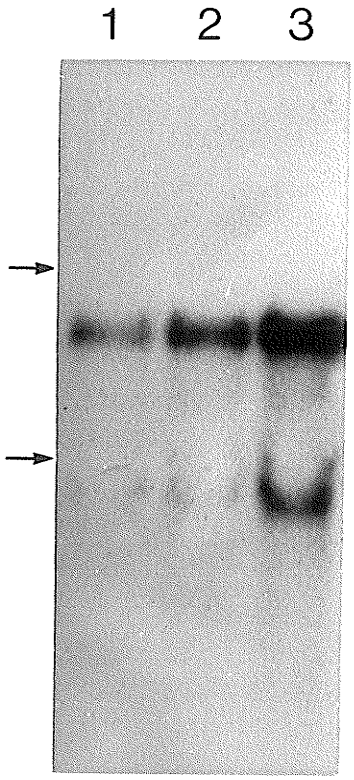

1
2
3

Figure 16

Northern blot analysis of β-actin mRNA levels in wild type (lane 1), HN^R-AT (lane 2), and H^R-R2T (lane 3) cell lines. The autoradiograms were exposed for 6 hr at -70°C with intensifying screens.

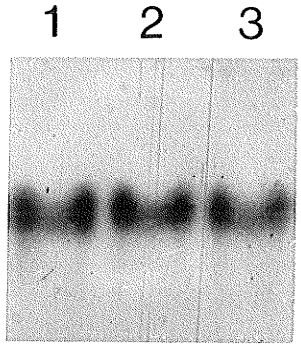

1
2
3

Figure 17

Photographs of two ethidium bromide stained agarose gels following electrophoresis of 20 ug DNA from wild type (lanes 2 and 5), HN^R-AT (lanes 3 and 6), and H^R-R2T (lanes 4 and 7) cells which have been digested to completion with Eco RI (lanes 2,3 and 4) or Pst I (lanes 5,6 and 7). Lane 1 shows Hind III digested lambda-DNA molecular weight markers of 23.6, 9.6, 6.6, 4.3, 2.3, and 2.0 kbp.

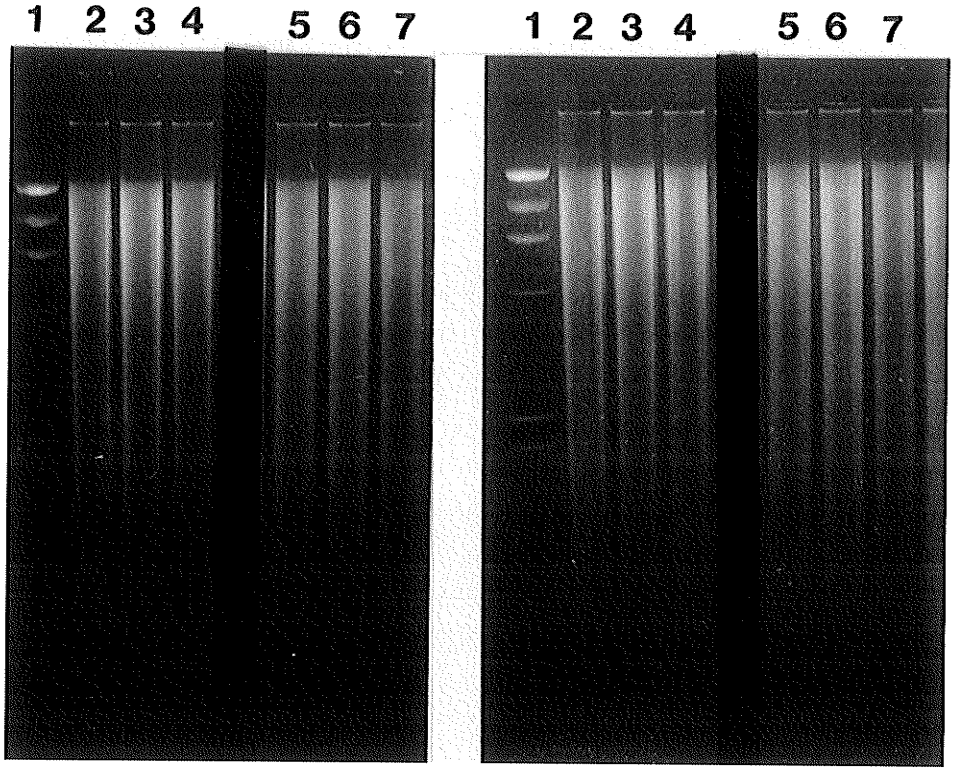
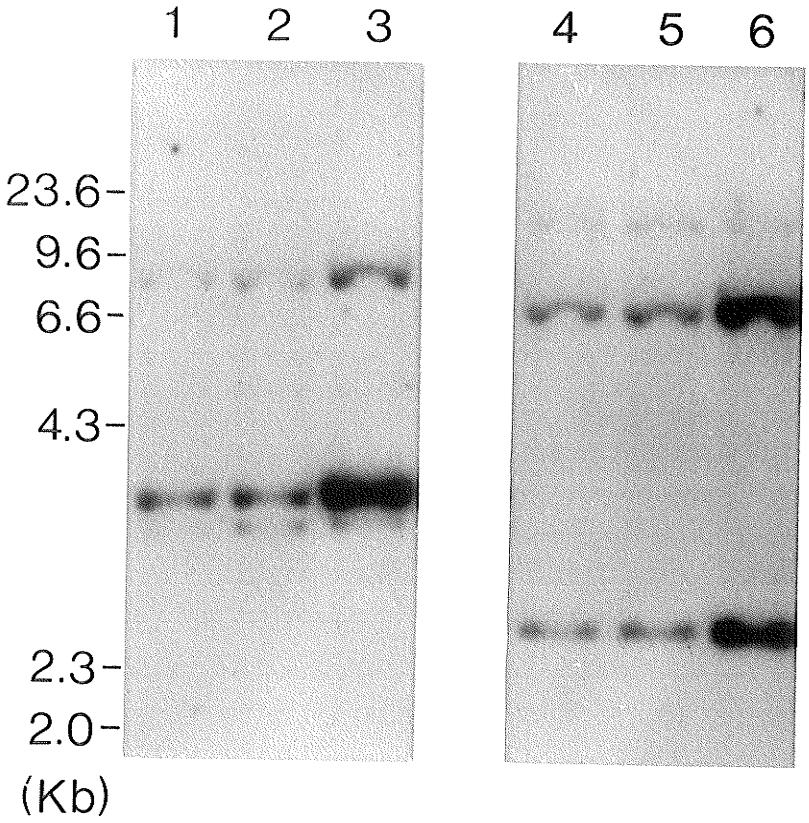

1 2 3 4 5 6 7
1 2 3 4 5 6 7

Figure 18

Southern blot analysis of M2 DNA from wild type (lanes 1 and 4), HN^R-AT (lanes 2 and 5), and H^R-R2T (lanes 3 and 6) cells. Genomic DNA (20ug) was digested to completion with Eco RI (lanes 1-3) and Pst I (lanes 4-6). The autoradiograms were exposed for 72 hr at -70°C with intensifying screens. The presence of M2 pseudogenes (Yang-Feng et al, 1987) possibly accounts for the difference in intensities observed between some bands. The DNA size markers were Hind III digested lambda-DNA.


1
2
3
4
5
6
23.6-
9.6-
6.6-
4.3-
2.3-
2.0-
(Kb)

from H^R-R2T cells (approximately a 5 fold increase as estimated by densitometry). The HN^R-AT cells, on the other hand, did not possess a M2 gene amplification (Fig. 18) even though they contained a modest elevation in M2 mRNA (Fig. 15). Neither cell line showed evidence of gross M2 gene rearrangement (Fig. 18). Presumably, other mechanisms besides gene amplification were responsible for the elevation of M2 mRNA in HN^R-AT cells. Figure 19 shows blots of wild type and variant cell DNA hybridized to M1 cDNA. As is evident from these blots, no M1 gene amplification or M1 gene rearrangements was present in the H^R-R2T or HN^R-AT cell lines. This observation is consistent with the studies of M1 protein and M1 mRNA from the three cell lines (Figs. 13 and 14).

1.2.2 Analysis of ribonucleotide reductase activity

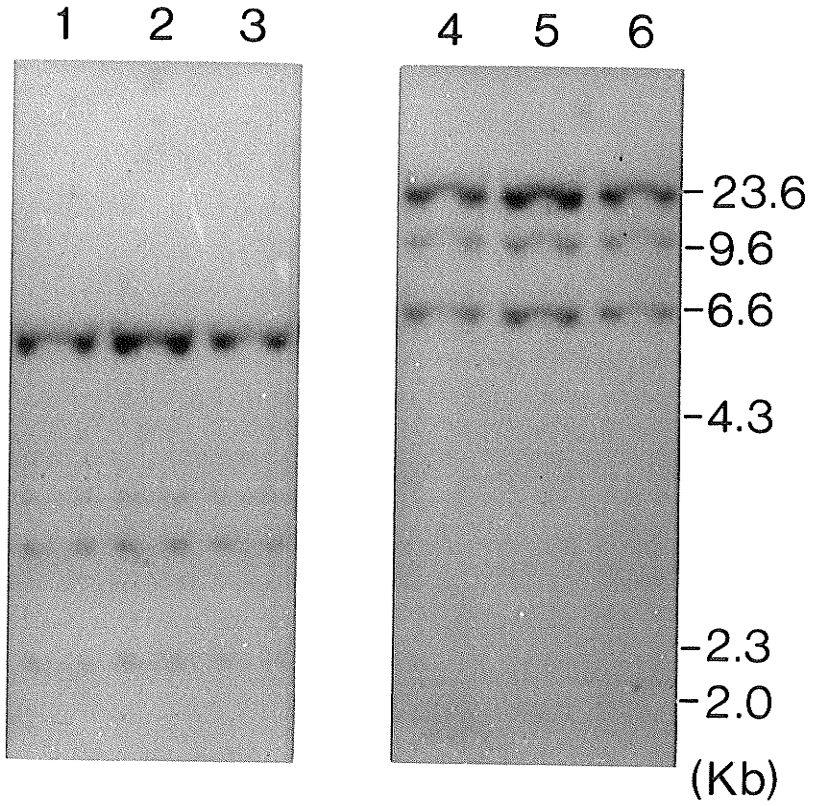
To determine if other modifications to the drug resistant cell lines occurred at the enzyme level, a detailed kinetic study of the reductase activity was carried out with partially purified preperations of ribonucleotide reductase. CDP reduction was analyzed in the partially purified preparations from the wild type and mutant cell lines.

1.2.2.1 Partial purification of ribonucleotide reductase from CHO cells

In order to obtain valid kinetic parameters for ribonucleotide reductase from the wild type and mutant cell lines, it was essential to examine an enzyme preparation which is devoid of any major

Figure 19

Southern blot analysis of M1 DNA from wild type (lanes 1 and 4), HN^R-AT (lanes 2 and 5), and H^R-R2T (lanes 3 and 6) cell lines. Genomic DNA (20ug) was digested to completion with Eco RI (lanes 1-3) and Pst I (lanes 4-6). The autoradiograms were exposed for 72 hr at -70°C with intensifying screens. The DNA size markers were Hind III digested lambda-DNA.


1
2
3
4
5
6
23.6
9.6
6.6
4.3
2.3
2.0
(Kb)

contaminants which may interfere with the assay. For this reason, a purification procedure developed by Spector's laboratory (Spector and Averett, 1983; Spector, 1985) which removes most of the nucleoside diphosphate kinase activity from cell extracts was followed. Since CDP is the substrate used here in analyzing ribonucleotide reductase activity, it was essential to remove nucleoside diphosphate kinase activity from the enzyme preparation, since its presence in the CDP reductase assay would deplete CDP by converting it to CTP, and thus make it difficult to carry out meaningful kinetic analysis of ribonucleotide reductase activity. Table 3 shows the results obtained from a typical partial purification of ribonucleotide reductase from wild type cells. Starting with about 600 mg of protein in the crude extract obtained after disruption of the cells by sonication and removal of cellular debris by centrufugation, there was a total of 25.2 ribonucleotide reductase enzyme units per ml extract. The nucleoside diphosphate kinase activity in this fraction was about 42 fold greater than the ribonucleotide reductase activity (Table 3) and resulted in approximately 72% of the ^{14}C-CDP substrate being converted to the triphosphate ^{14}C-CTP in a typical 20 min reductase assay when roughly 60 ug of protein was assayed (Table 6). The streptomycin sulfate step did not increase the purity of the enzyme preparation (Table 3); in fact it slightly lowered the specific activity of ribonucleotide reductase without significantly altering the nucleoside diphosphate activity in the fraction. This step was included in the purification since it helped remove macromolecules from the preparation which might interfere with the subsequent purification steps. The 40% ammonium sulfate cut which followed, enriched the concentra-

Table 3. Partial purification of ribonucleotide reductase and removal of NDP kinase activity from wild type cells

	Total Protein (mg)	Total RRASE[a] Activity (units)	Yield (%)	RRASE Activity (units/ml)	NDPK[b] Activity (units/ml)	RRASE/ NDPK Ratio (1/r)	RRASE Specific Activity (units/mg)	Fold Increase
Crude Extract	599	654	100	25.2	1056.6	0.024 (42)	1.1	-
Streptomycin Sulfate	383	215	32.9	9.2	1197.9	0.0077 (130)	0.56	0.51
Ammonium Sulfate	73	365	55.8	222.9	1262.2	0.18 (6)	5.0	4.5
ATP Agarose	39	505	77.2	155.9	41.1	3.8	18.2	16.5

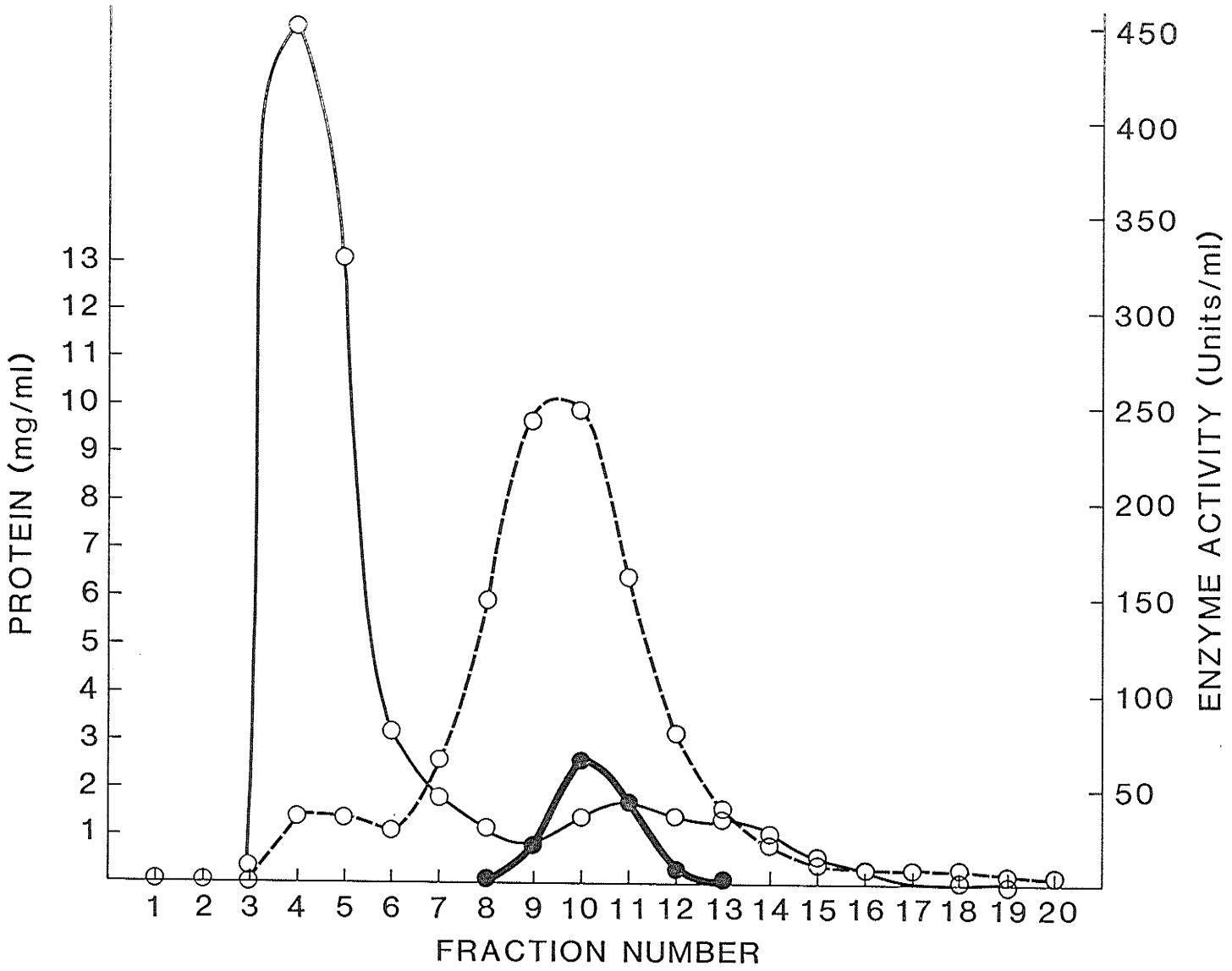
a RRASE = ribonucleotide reductase. 1 unit of ribonucleotide reductase activity is equal to the number of nmoles of CDP reduced in one hour at 37°C under standard assay conditions.

b NDPK = nucleoside diphosphate kinase. 1 unit of NDPK activity is equal to the number of nmoles of CDP converted to CTP in one hour at 37°C in the CDP reductase assay conditions.

tion of ribonucleotide reductase activity in the extract about 10 fold over that found in the crude extract, while the total amount of nucleoside diphosphate kinase activity increased moderately from 1057 units/ml in the crude extract to 1262 units/ml in the extract obtained following ammonium sulfate precipitation. An overall increase of 4.5 fold in the specific activity of ribonucleotide reductase was observed, and the ratio of nucleoside diphosphate kinase acvtivity to ribonucleotide reductase activity was reduced to 6. As is evident in Table 6, roughly 84% of the labelled CDP substrate was converted to the triphosphate form following a 20 min incubation of the extract in a standard CDP reductase assay mixture. Clearly the preparation still contained excessive amounts of nucleoside diphosphate kinase activity which would affect the interpretation of kinetic parameters obtained from ribonucleotide reductase assays. The final purification step involved the use of an ATP-agarose column. The extract was applied to the column and eluted with buffer containing 0.5 M KCl. Under such conditions, the bulk of the contaminating nucleoside diphosphate kinase remains bound to the column or elutes off in the first few fractions. Figure 20 shows a typical elution profile of a wild type extract from the ATP-agarose column. The fractions containing ribonucleotide reductase activity (fractions 9 to 12) were clearly separated from the fractions containing the bulk of the nucleoside diphosphate kinase activity. The fractions containing the greatest amount of ribonucleotide reductase activity relative to nucleoside diphosphate kinase activity were pooled and dialyzed against 2 mM $MgCl_2$, 2 mM DTT, and 20 mM Hepes (pH 7.6) to reduce the high salt concentration. These preparations from wild type cells, showed about a

Figure 20

Elution profile for protein (---), nucleoside diphosphate kinase activity (-), and ribonucleotide reductase activity (▬) from an ATP-agarose affinity column. Approximately 55 mg of ammonium sulfate precipitated protein (40% cut) was applied onto an ATP-agarose affinity column and 1 ml fractions were collected as described in Materials and Methods.


PROTEIN (mg/ml)
ENZYME ACTIVITY (Units/ml)
FRACTION NUMBER
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
1 2 3 4 5 6 7 8 9 10 11 12 13
50 100 150 200 250 300 350 400 450

17 fold increase in the purity of ribonucleotide reductase, and an increase in the yield of reductase activity compared to the previous ammonium sulfate precipitation step, and ratios of ribonucleotide reductase activity to nucleoside diphosphate activity approaching 4. Such a preparation when assayed under standard CDP reductase assay conditions for 20 min showed approximately 5% conversion of labelled CDP substrate to the triphosphate form, leaving greater than 90% of the label in the form of ^{14}C-CDP (Table 6). The apparent increase in the yield of ribonucleotide reductase in this preparation as compared to the preparation following ammonium sulfate precipitation is due to the removal of nucleoside diphosphate kinase actvity from the preparation, thus depletion of the substrate is not occuring to any great extent and the competition of the two enzyme activities for the common substrate is substantially reduced. The preparation of partially purified enzyme preparations from both the H^R-R2T and the HN^R-AT cell lines followed exactly the same protocol as that used for the wild type line and the trends observed in the results were identical as those discussed above and obtained for the wild type cells (Tables 4 and 5). The only significant quantitative difference observed was in the ratio of ribonucleotide reductase activity to nucleoside diphosphate activity, which in the mutant cell lines was consistently greater than that observed with the wild type cell line at each step throughout the purification. This was simply a result of overproduction of enzyme activity in the mutant lines as will be demonstrated later. The fold increase in the specific activity of ribonucleotide reductase ranged from about 9 to 17 fold for the various cell lines. All enzyme preparations used had ratios of ribonucleotide reductase

Table 4. Partial Purification of ribonucleotide reductase and removal of NDP kinase activity from H^R-R2T cells

	Total Protein (mg)	Total RRASE[a] Activity (units)	Yield (%)	RRASE Activity (units/ml)	NDPK Activity (units/ml)	RRASE/ NDPK Ratio (1/r)	RRASE Specific Activity (units/mg)	Fold Increase
Crude Extract	625	2500	100	89.4	1034.7	0.086 (12)	4.0	-
Streptomycin Sulfate	396	1323	52.9	54.0	1162.3	0.046 (22)	3.3	0.83
Ammonium Sulfate	93	1112	44.5	695.7	1155.4	0.602 (1.7)	12.0	3.0
ATP-Agarose	50	1278	51.1	413.5	33.2	12.4	34.6	8.7

a RRASE = ribonucleotide reductase. 1 unit of ribonucleotide reductase activity is equal to the number of nmoles of CDP reduced in one hour at 37°C under standard assay conditions.

b NDPK = nucleoside diphosphate kinase. 1 unit of NDPK activity is equal to the number of nmoles of CDP converted to CTP in one hour at 37°C in the CDP reductase assay conditions.

Table 5. Partial purification of ribonucleotide reductase and removal of NDP kinase activity from HNR^R-AT cells

	Total Protein (mg)	Total RRASE[a] Activity (units)	Yield (%)	RRASE Activity (units/ml)	NDPK[b] Activity (units/ml)	RRASE/ NDPK Ratio (1/r)	RRASE Specific Activity (units/mg)	Fold Increase
Crude Extract	604	1446	100	52.6	968.4	0.0542 (18)	2.4	-
Streptomycin Sulfate	331	724	50.1	30.8	1128.5	0.0273 (37)	2.2	0.91
Ammonium Sulfate	68	715	49.4	467.6	893.7	0.523 (1.91)	10.5	4.4
ATP-Agarose	25	623	43.1	1934.3	38.3	5.151 (0.19)	27.6	11.5

[a] RRASE = ribonucleotide reductase. 1 unit of ribonucleotide reductase activity is equal to the number of nmoles of CDP reduced in one hour at 37°C under standard assay conditions.

[b] NDPK = nucleoside diphosphate kinase. 1 unit of NDPK activity is equal to the number of nmoles of CDP converted to CTP in one hour at 37°C in the CDP reductase assay conditions.

activity to nucleoside diphosphate kinase activity greater than 3. Table 6 illustrates the distribution of ^{14}C label amongst the various cytidine phosphates following a typical ribonucleotide reductase assay on enzyme preparations from the various stages of purification of ribonucleotide reductase from wild type, H^R-R2T and HN^R-AT cells. The importance of removing nucleoside diphosphate kinase activity from the enzyme preparation is clearly evident. Up to the ATP-agarose column step, no more than 30% of the CDP substrate remained following the assay, with the majority of the substrate having been converted to CTP. However, following the ATP-agarose chromatography step, there consistently remained greater than 90% of the CDP substrate intact after a 20 min reductase assay. Although the amount of CDP converted to CTP is dependent on the amount of extract added to the assay mixture, the values shown for the preparation obtained following the ATP-agarose chromatography are for the same amount of extract used in the kinetic anlaysis of ribonucleotide reductase shown later. At no time was extract used in an amount which would result in less than 90% of the CDP remaining intact following an assay.

1.2.2.2 In vitro ribonucleotide reductase assay - optimization of assay

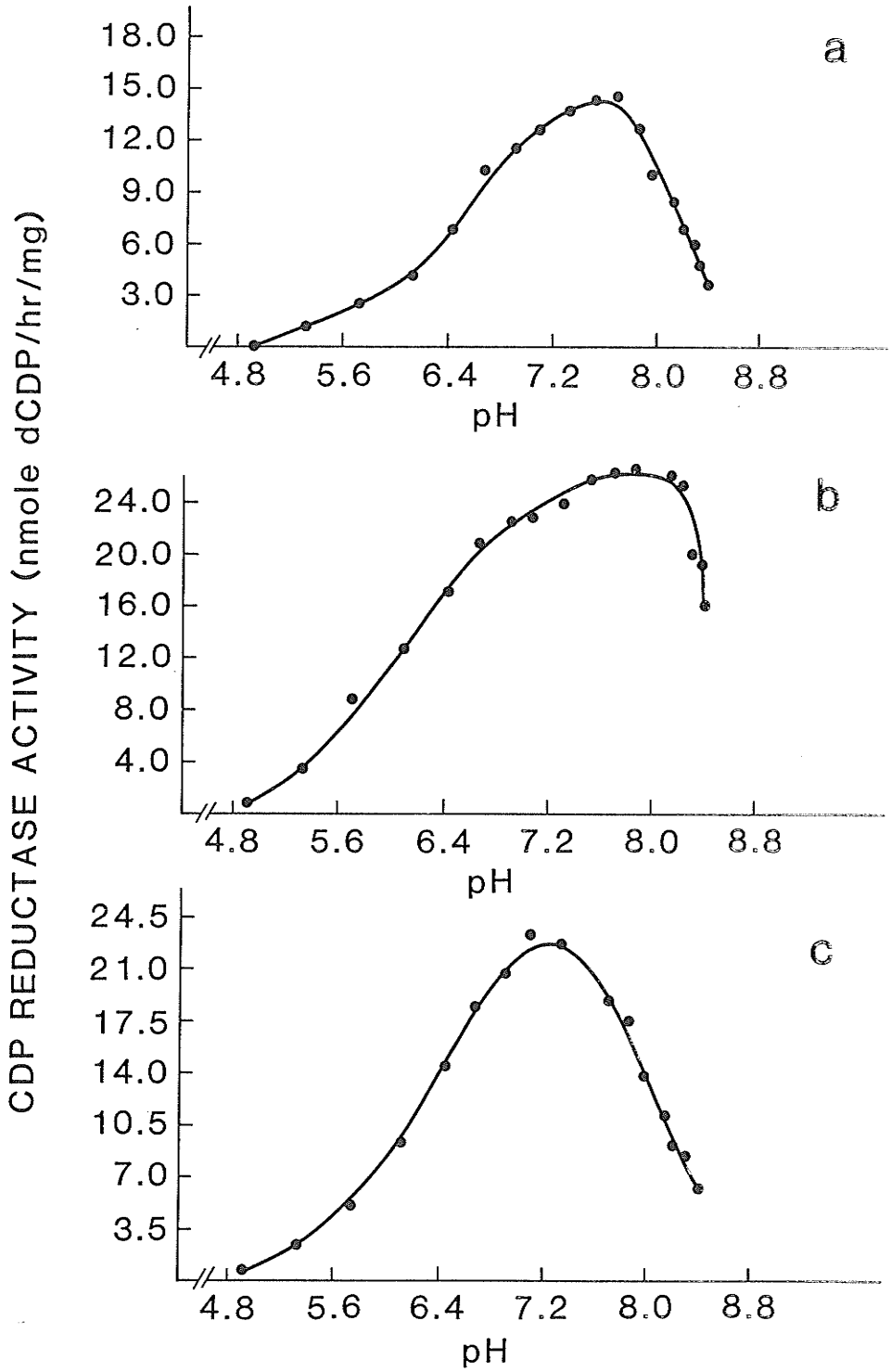
Before proceeding to examine the kinetics of CDP reduction in the wild type and mutant cell lines it was necessary to find the optimum concentrations of the various compounds in the reaction mixture, as well as the optimum pH and temperature. Figure 21 shows the effect of pH on the reaction rate for wild type and the two mutant cell lines.

Table 6. Distribution of ^{14}C-label amongst the various cytosine nucleotides during a standard ribonucleotide reductase assay (% of total)

	Wild Type C				H^R-R2T				HN^R-AT			
	CYT	CMP	CDP	CTP	CYT	CMP	CDP	CTP	CYT	CMP	CDP	CTP
Crude	3.8	4.4	20.3	71.5	4.2	5.3	21.2	69.2	4.8	8.0	22.9	64.3
Streptomycin Sulfate	4.4	1.9	13.9	79.7	3.5	2.8	16.6	77.1	4.2	2.8	17.8	75.2
Ammonium Sulfate	1.9	2.9	10.9	84.3	1.4	7.0	14.0	77.6	1.2	11.9	19.4	67.5
ATP-Agarose	0.3	4.6	91.4	3.7	0.3	4.6	91.9	3.2	0.9	4.6	90.5	4.0

Figure 21

The effect of pH on CDP reductase activity in CHO cells. Partially purified preparations of ribonucleotide reductase from wild type (a), H^R-R2T (b), and HN^R-AT (c) cell lines were incubated under standard CDP reductase assay conditions for 20 min in reaction mixtures containing Hepes buffer of varying pH.


CDP REDUCTASE ACTIVITY (nmole dCDP/hr/mg)
a
18.0
15.0
12.0
9.0
6.0
3.0
4.8
5.6
6.4
7.2
8.0
8.8
pH
b
24.0
20.0
16.0
12.0
8.0
4.0
4.8
5.6
6.4
7.2
8.0
8.8
pH
c
24.5
21.0
17.5
14.0
10.5
7.0
3.5
4.8
5.6
6.4
7.2
8.0
8.8
pH

The wild type and HN^R-AT cell lines showed a pH optimum between 7.1 and 7.7, while for the H^R-R2T line the optimum pH was between 7.4 and 7.9. Considering the error in determining the pH for the reaction mixture (about 0.1 pH units) for which the volume is a mere 50 ul, it can be assumed that there were no significant differences between the optimum pH for the reaction for the various cell lines tested. Standard reactions were routinely carried out at a pH of 7.5; shown to be an optimum value for all three CHO cell lines. The optimum temparature for the reaction was determined to be 37°C, as shown in Figure 22 for the wild type and H^R-R2T cell lines. This finding is expected for the CHO cell lines since their optimum growth is at a temperature of 37°C. Figure 23 shows a plot of reaction velocity versus magnesium chloride concentration for the three CHO cell lines. The reaction is dependent upon divalent cations which serve to stabilize the interactions between the M1 and M2 subunits of the enzyme. As can be seen, the optimum concentration of $MgCl_2$ was found to lie between 4 and 6 mM for the three CHO cell lines. A concentration of 6 mM was chosen for use in further assays. Figure 24 shows the dependance of CDP reduction on the presence of ATP. The optimum concentration of ATP for CDP reduction was determined to be about 5 mM. At higher ATP concentrations, the activity of the enzyme decreased, possibly due to the formation of enzyme aggregates incapable of reducing ribonucleotides (Eriksson et al., 1977; Klippenstein and Cory, 1978). Enzyme activity, as mentioned previously is dependent upon the presence of reducing power, which can be supplied in the in vitro assay by DTT. As can be seen in Figure 25, DTT at concentrations greater than about 2 mM are required to give maximum ribonucleotide

Figure 22

The effect of incubation temperature on CDP reductase activity in CHO cells. Partially purified preparations of ribonucleotide reductase from wild type (a) and H^R-R2T (b) cell lines were incubated under standard CDP reductase asssay conditions for 20 min at various temperatures.

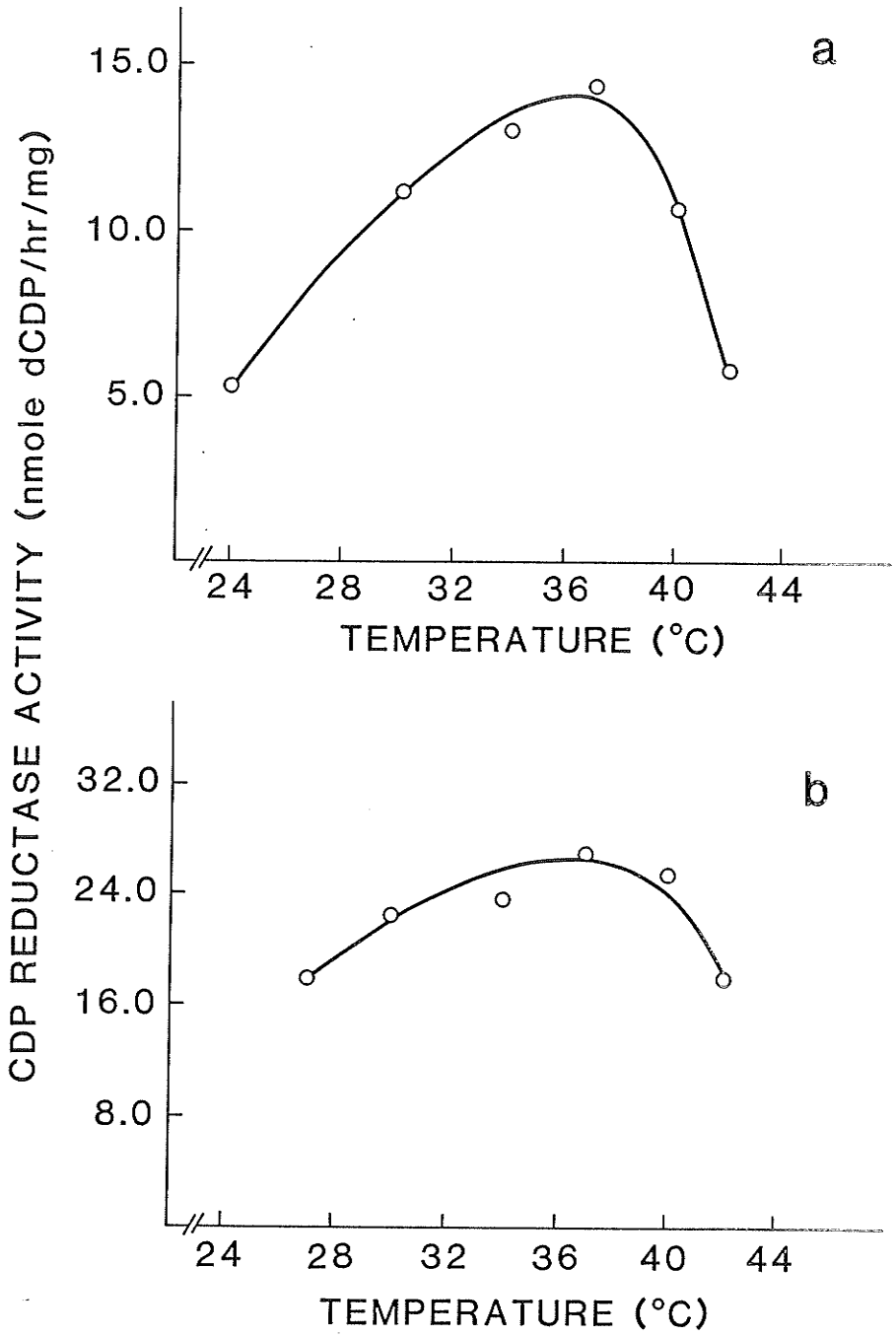

a
15.0
10.0
5.0
24
28
32
36
40
44
TEMPERATURE (°C)
CDP REDUCTASE ACTIVITY (nmole dCDP/hr/mg)
b
32.0
24.0
16.0
8.0
24
28
32
36
40
44
TEMPERATURE (°C)

Figure 23

The effect of varying concentrations of $MgCl_2$ on CDP reduction in CHO cells. Partially purified preparations of ribonucleotide reductase from wild type (a), H^R-R2T (b), and HN^R-AT (c) cell lines were incubated for 20 min under standard CDP reductase assay conditions except that the concentration of $MgCl_2$ was varied.

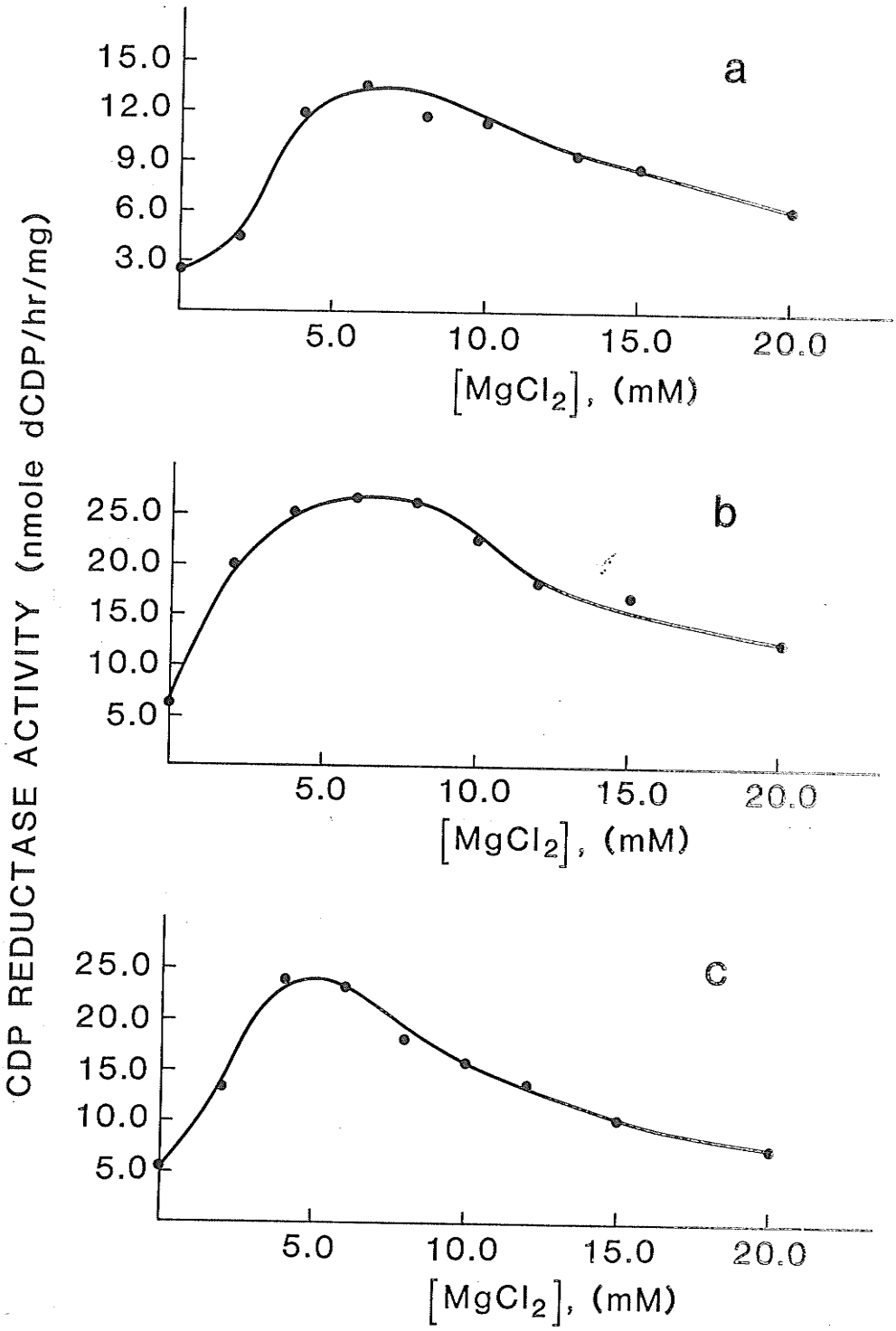

CDP REDUCTASE ACTIVITY (nmole dCDP/hr/mg)
15.0
12.0
9.0
6.0
3.0
a
5.0
10.0
15.0
20.0
[MgCl2], (mM)
25.0
20.0
15.0
10.0
5.0
b
5.0
10.0
15.0
20.0
[MgCl2], (mM)
25.0
20.0
15.0
10.0
5.0
c
5.0
10.0
15.0
20.0
[MgCl2], (mM)

Figure 24

The effect of varying concentrations of ATP on CDP reduction in CHO cells. Partially purified preparations of ribonucleotide reductase from wild type (a), H^R-R2T (b), and HN^R-AT (c) cell lines were incubated for 20 min under standard CDP reductase assay conditions except that the concentration of ATP was varied.

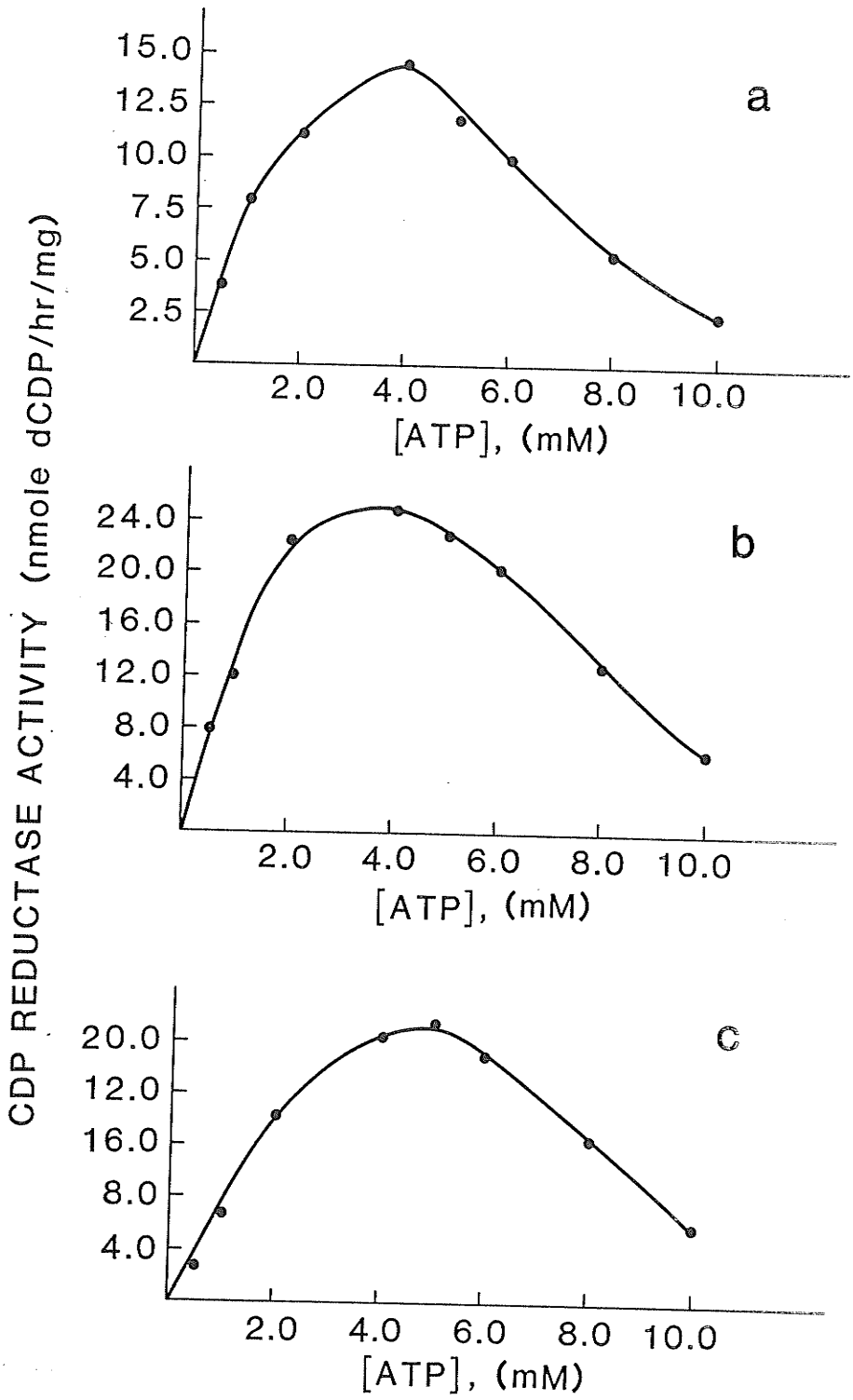
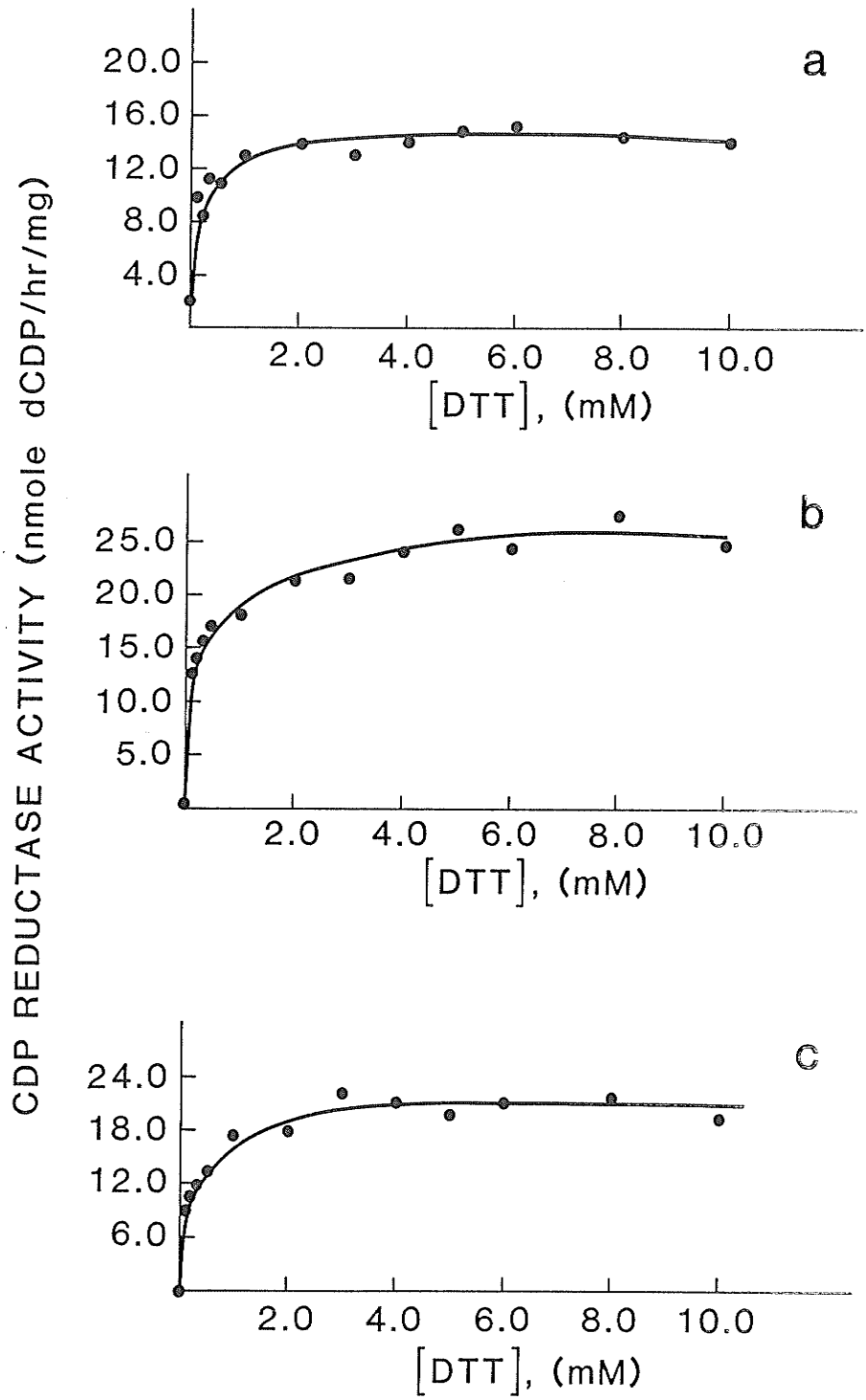

CDP REDUCTASE ACTIVITY (nmole dCDP/hr/mg)
15.0
12.5
10.0
7.5
5.0
2.5
a
2.0 4.0 6.0 8.0 10.0
[ATP], (mM)
24.0
20.0
16.0
12.0
8.0
4.0
b
2.0 4.0 6.0 8.0 10.0
[ATP], (mM)
20.0
12.0
16.0
8.0
4.0
c
2.0 4.0 6.0 8.0 10.0
[ATP], (mM)

Figure 25

The effect of varying concentrations of DTT on CDP reduction in CHO cells. Partially purified preparations of ribonucleotide reductase from wild type (a), H^R-R2T (b), and HN^R-AT (c) cell lines were incubated for 20 min under standard CDP reductase assay conditions except that the concentration of DTT was varied.


CDP REDUCTASE ACTIVITY (nmole dCDP/hr/mg)
a
20.0
16.0
12.0
8.0
4.0
2.0
4.0
6.0
8.0
10.0
[DTT], (mM)
b
25.0
20.0
15.0
10.0
5.0
2.0
4.0
6.0
8.0
10.0
[DTT], (mM)
c
24.0
18.0
12.0
6.0
2.0
4.0
6.0
8.0
10.0
[DTT], (mM)

reductase activity in wild type and mutant cell lines. A concentration of 5 mM DTT was chosen for use in the enzyme assays. Sodium fluoride, was added to the reaction mixture to a final concentration of 5 mM in order to inhibit nucleoside phosphatase activity (Eriksson and Martin, 1981; Albert and Gudas, 1985). The concentration of sodium fluoride used had no effect on ribonucleotide reductase activity.

Figure 26 shows that CDP reduction increases in a linear manner with protein concentrations above 40 ug/ 50 ul for the three CHO cell lines. At lower protein concentrations, the activity declines very rapidly. This non-linearity at low protein concentrations has been observed by others (Hopper, 1972; Kuzik and Wright, 1979; Lewis and Wright, 1979) and may be due to dissociation of the two enzyme components at low protein concentrations as has been suggested by others (Hopper, 1972; Kuzik and Wright, 1979) in studies on CDP reductase from rabbit bone marrow and mouse L cells.

As shown in Figure 27, CDP reduction increased in enzyme preparations from wild type, H^R-R2T, and HN^R-AT cells linearly with incubation time. The reaction was linear to 60 min; assays were routinely performed for either 10 or 20 min.

1.2.2.3 Kinetic analysis of CDP reduction in wild type, H^R-R2T, and HN^R-AT cells

The Km and Vmax values for CDP reduction for the wild type and mutant cell lines were determined under optimum assay conditions as

Figure 26

CDP reduction in the presence of increasing amounts of partially purified enzyme preparations. Various amounts of partially purified preparations of ribonucleotide reductase from wild type (a), H^R-R2T (b), and HN^R-AT (c) cell lines were incubated for 20 min under standard CDP reductase assay conditions and the amount of dCDP formed was measured as outlined in Materials and Methods.

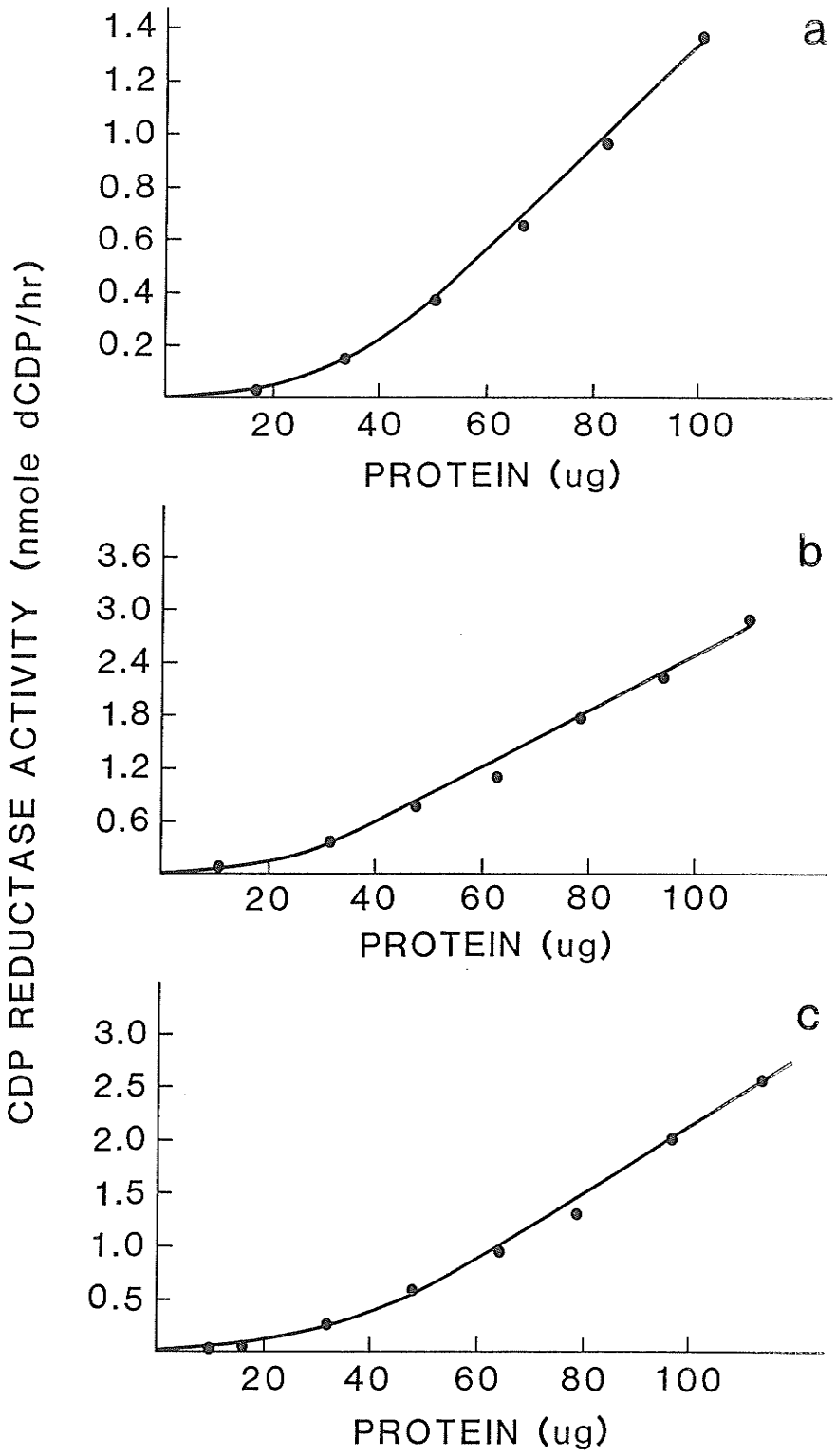
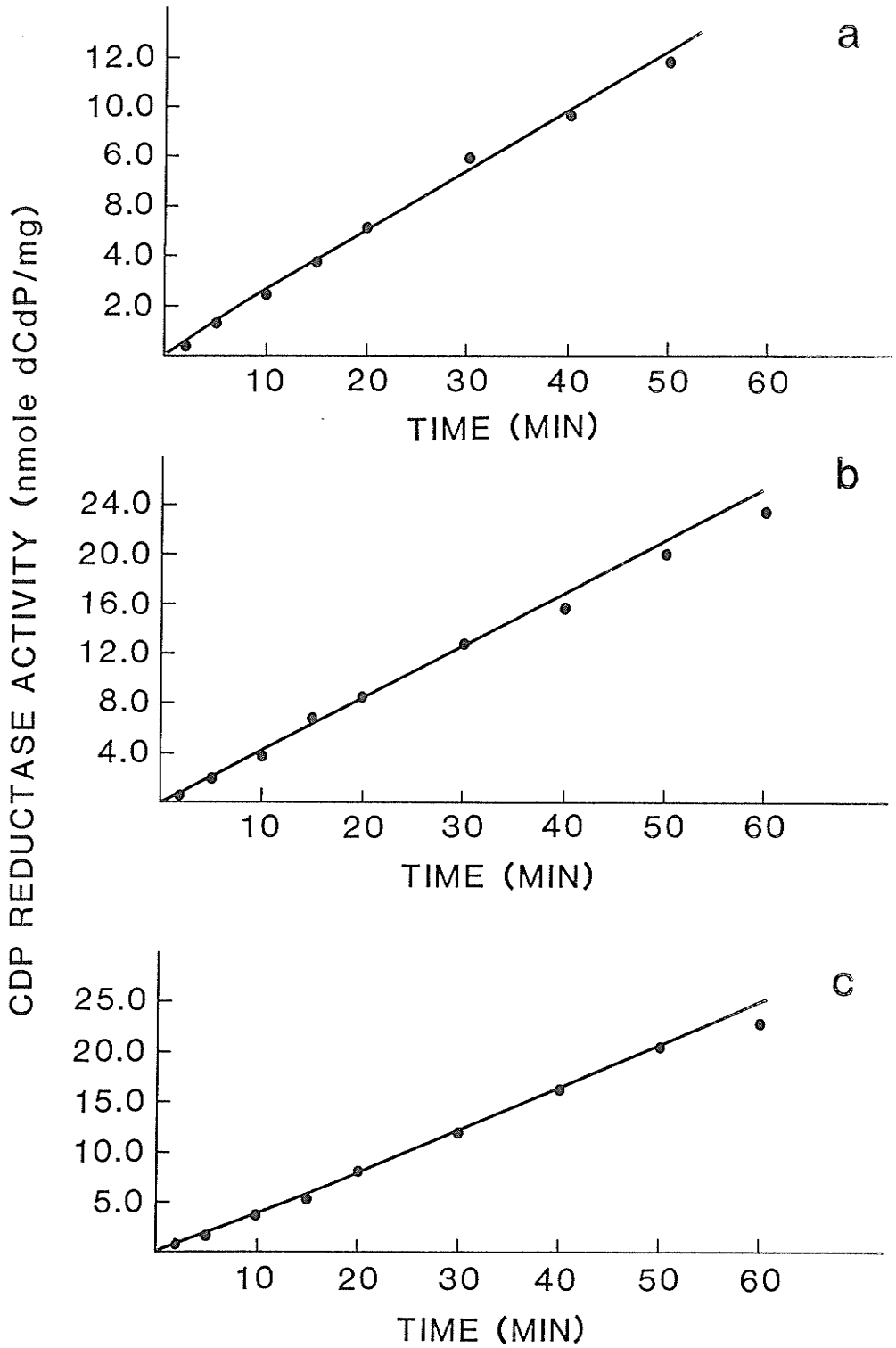

CDP REDUCTASE ACTIVITY (nmole dCDP/hr)
a
1.4
1.2
1.0
0.8
0.6
0.4
0.2
20
40
60
80
100
PROTEIN (ug)
b
3.6
3.0
2.4
1.8
1.2
0.6
20
40
60
80
100
PROTEIN (ug)
c
3.0
2.5
2.0
1.5
1.0
0.5
20
40
60
80
100
PROTEIN (ug)

Figure 27

The increase of CDP reduction with incubation time. Partially purified preparations of ribonucleotide reductase from wild type (a), H^R-R2T (b), and HN^R-AT (c) cell lines were incubated under standard CDP reductase assay conditions for varying lengths of time as indicated.


a
12.0
10.0
6.0
8.0
4.0
2.0
10
20
30
40
50
60
TIME (MIN)
b
24.0
20.0
16.0
12.0
8.0
4.0
10
20
30
40
50
60
TIME (MIN)
c
25.0
20.0
15.0
10.0
5.0
10
20
30
40
50
60
TIME (MIN)
CDP REDUCTASE ACTIVITY (nmole dCdP/mg)

described above. Figures 28, 29, and 30 show typical Lineweaver-Burk and Michaelis-Menten plots obtained from the wild type, H^R-R2T, and HN^R-AT cell populations respectively. The Km values obtained for the three cell lines were similar (Table 7) suggesting that there are no significant differences in the binding of CDP substrate to the enzyme from the mutant cell lines as compared to the wild type cells. The Vmax values indicate that the overall ribonucleotide reductase activity was increased almost twofold in both mutant cell lines compared to the wild type cells (Table 7). This consistent increase in enzyme activity is in keeping with the results shown above indicating that one of the subunits of ribonucleotide reductase is increased in the two mutant cell lines.

In order to determine if the enzyme from either mutant cell line responds differently to inhibitors of the enzyme, Ki values for hydroxyurea and dATP inhibition were determined. Figures 31, 32, and 33 show double reciprocal plots in the presence of increasing concentrations of hydroxyurea for the wild type, H^R-R2T, and the HN^R-AT cell populations respectively. For all three cell lines the kinetic pattern appeared to vary between uncompetitive inhibition at low drug concentrations and noncompetitive inhibition at higher drug concentrations. Replots of both the velocity intercepts and velocity slopes versus hydroxyurea concentration (insets to Figures 31, 32 and 33) were used to determine Ki values for hydroxyurea inhibition. Both mutant cell lines had increased Ki values for hydroxyurea inhibition (Table 7). The most pronounced increase was observed in enzyme prepared from the H^R-R2T cell line, which exhibited about a twofold

Figure 28

Double reciprocal plots of the rate of CDP reduction for partially purified enzyme preparations obtained from wild type cells. Inset shows the velocity of CDP reduction in the presence of varying concentrations of CDP. Reactions were carried out for 10 min as described in Materials and Methods.

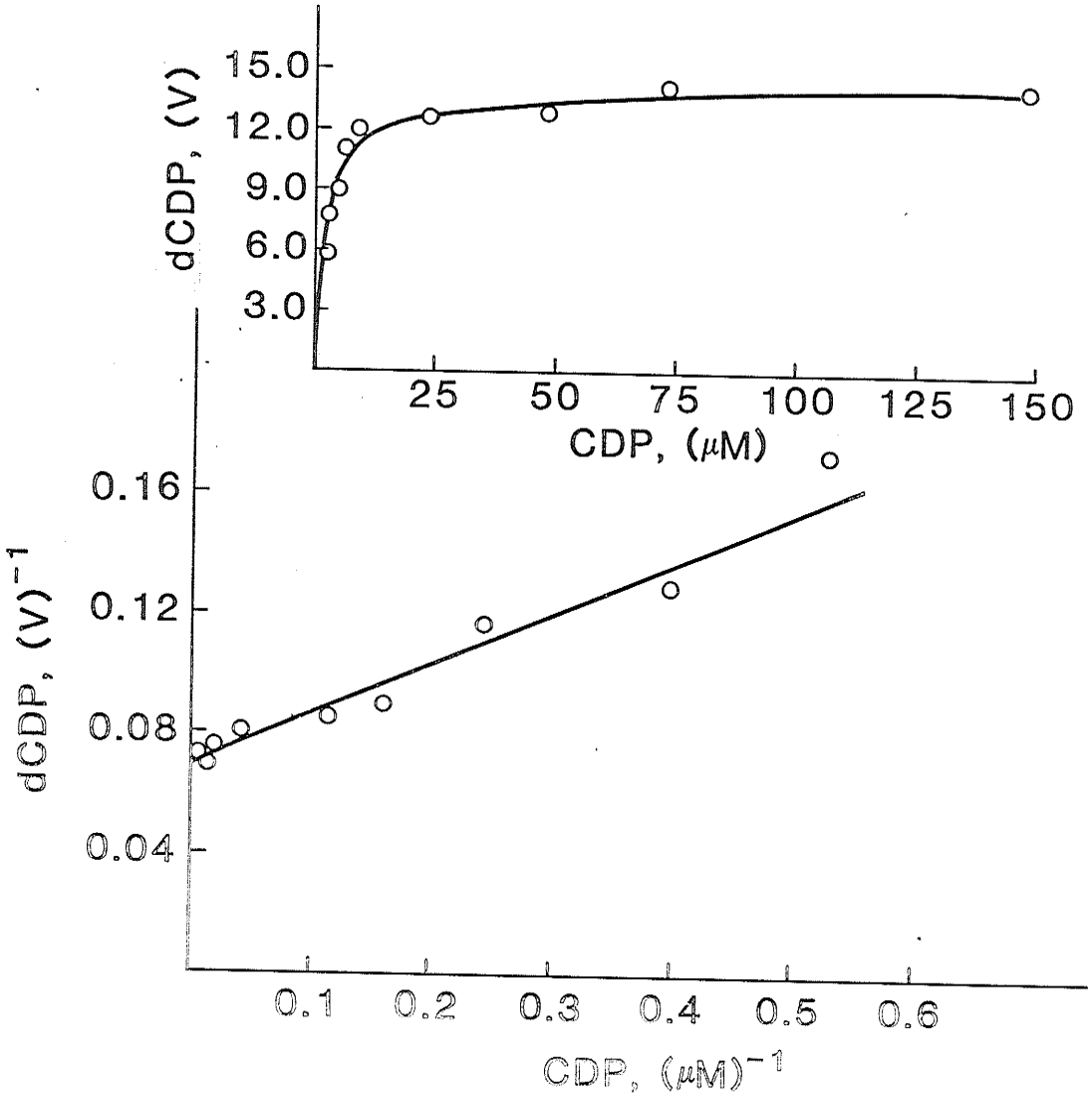

dCDP, (V)
15.0
12.0
9.0
6.0
3.0
25
50
75
100
125
150
CDP, (μM)
dCDP, (V)$^{-1}$
0.16
0.12
0.08
0.04
0.1
0.2
0.3
0.4
0.5
0.6
CDP, (μM)$^{-1}$

Figure 29

Double reciprocal plots of the rate of CDP reduction for partially purified enzyme preparations obtained from H^R-R2T cells. Inset shows the velocity of CDP reduction in the presence of varying concentrations of CDP. Reactions were carried out for 10 min as described in Materials and Methods.

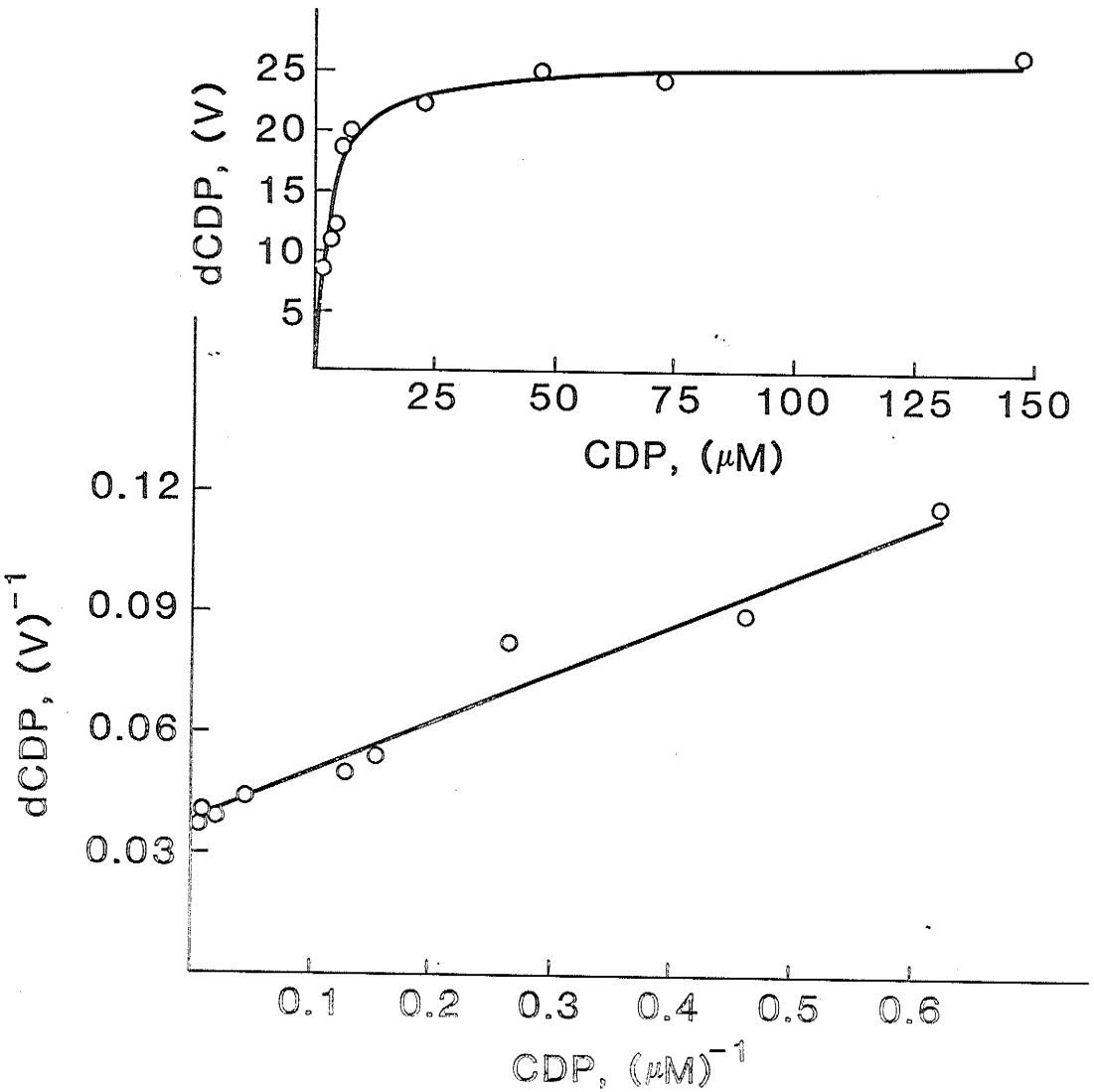

dCDP, (V)
25
20
15
10
5
25
50
75
100
125
150
CDP, (μM)
dCDP, (V)$^{-1}$
0.12
0.09
0.06
0.03
0.1
0.2
0.3
0.4
0.5
0.6
CDP, (μM)$^{-1}$

Figure 30

Double reciprocal plots of the rate of CDP reduction for partially purified enzyme preparations obtained from HN^R-AT cells. Inset shows the velocity of CDP reduction in the presence of varying concentrations of CDP. Reactions were carried out for 10 min as described in Materials and Methods.

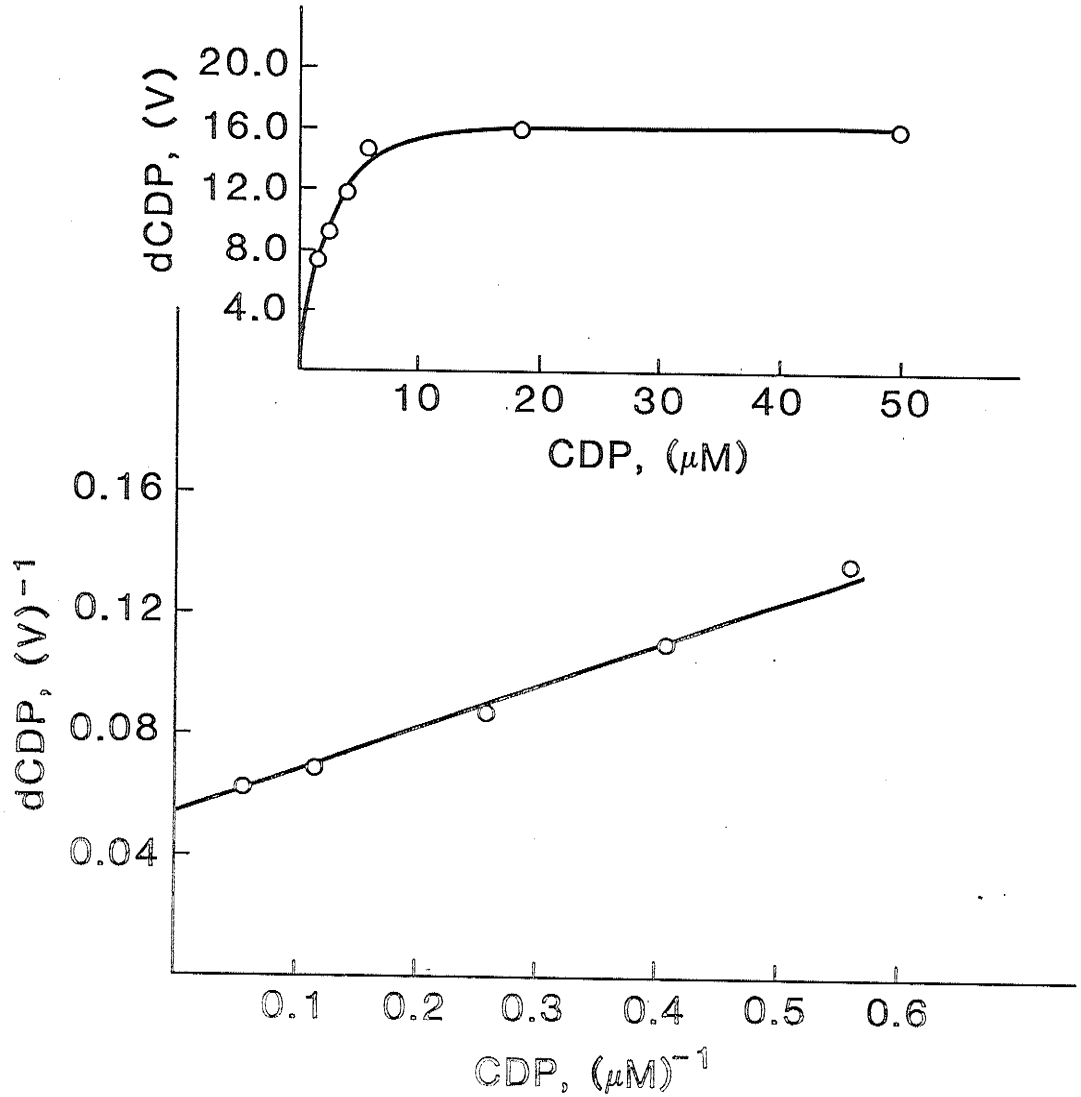

dCDP, (V)
20.0
16.0
12.0
8.0
4.0
10
20
30
40
50
CDP, (μM)
dCDP, (V)⁻¹
0.16
0.12
0.08
0.04
0.1
0.2
0.3
0.4
0.5
0.6
CDP, (μM)⁻¹

Figure 31

Double reciprocal plots of CDP reduction in the presence of various concentrations of hydroxyurea for partially purified enzyme preparations obtained from wild type cells. Reactions were carried out in the absence of hydroxyurea (●), or in the presence of 0.25 (o), 0.50 (▲), and 0.75 (□), mM hydroxyurea. Insets show replots of slope and intercept values versus drug concentration from which Ki values were determined.

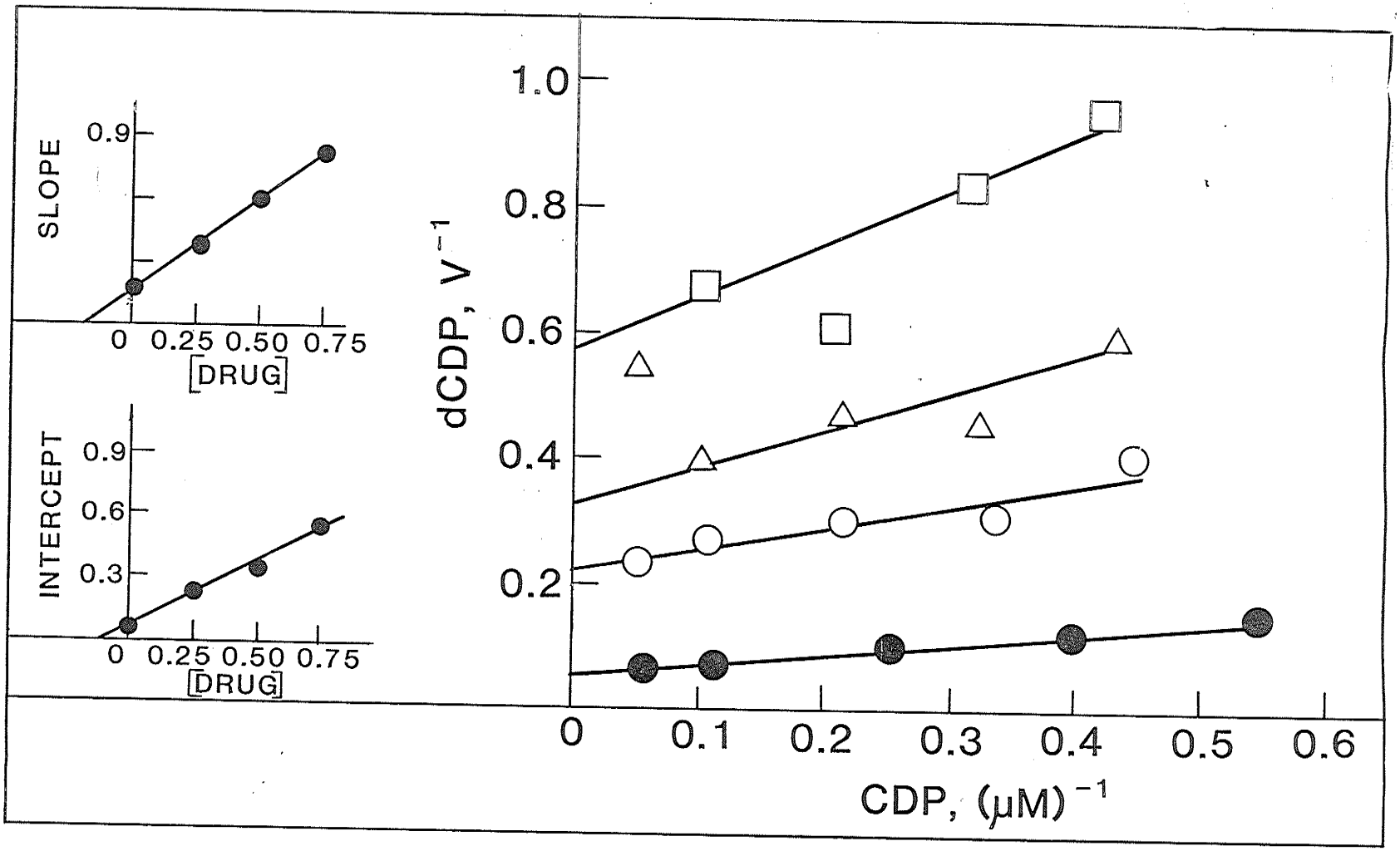

SLOPE
0.9
0 0.25 0.50 0.75
[DRUG]
INTERCEPT
0.9
0.6
0.3
0 0.25 0.50 0.75
[DRUG]
dCDP, V⁻¹
1.0
0.8
0.6
0.4
0.2
0 0.1 0.2 0.3 0.4 0.5 0.6
CDP, (μM)⁻¹

Figure 32

Double reciprocal plots of CDP reduction in the presence of various concentrations of hydroxyurea for partially purified enzyme preparations obtained from H^R-R2T cells. Reactions were carried out in the absence of hydroxyurea (●), or in the presence of 0.25 (o), 0.50 (△), and 0.75 (□), mM hydroxyurea. Insets show replots of slope and intercept values versus drug concentration from which Ki values were determined.

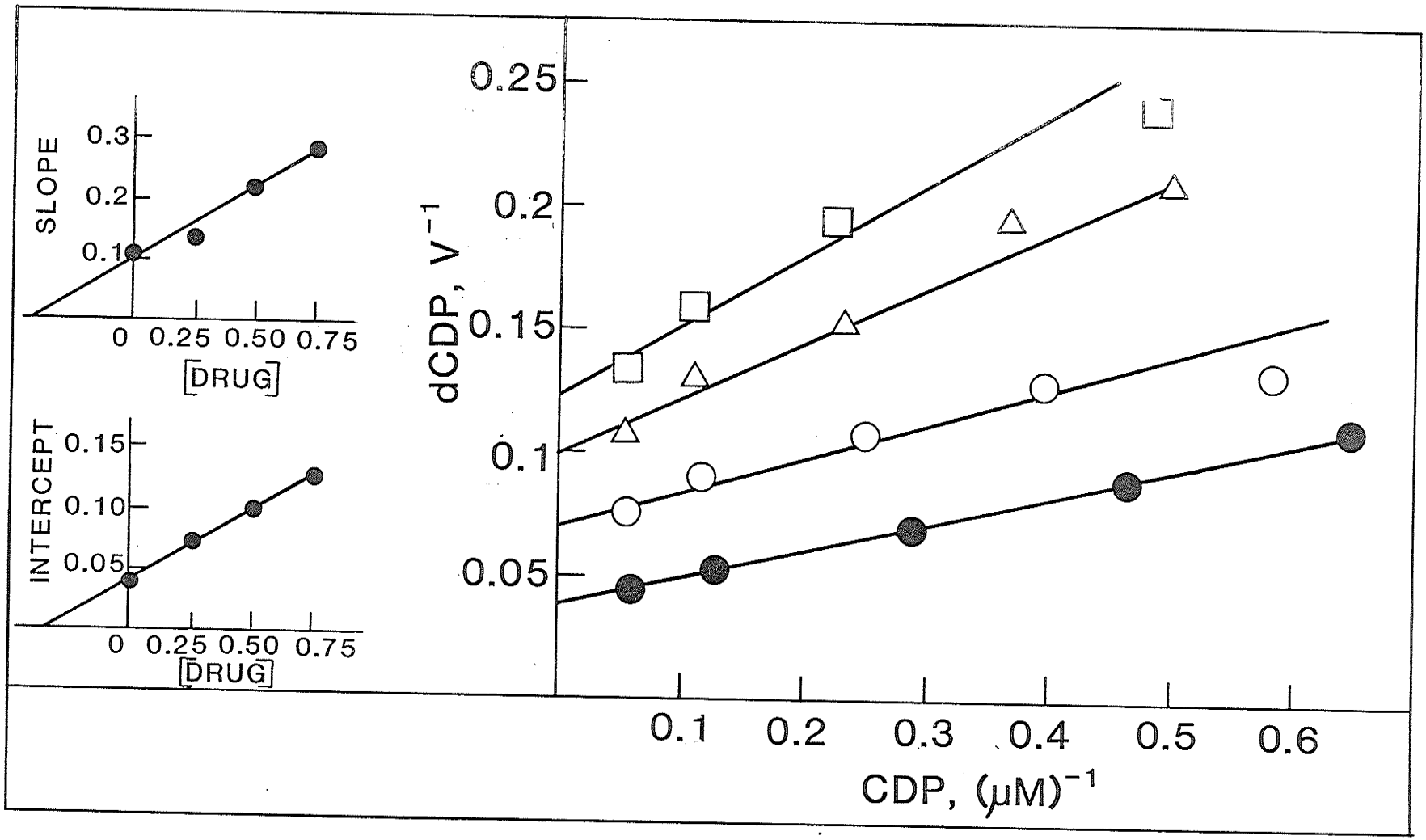
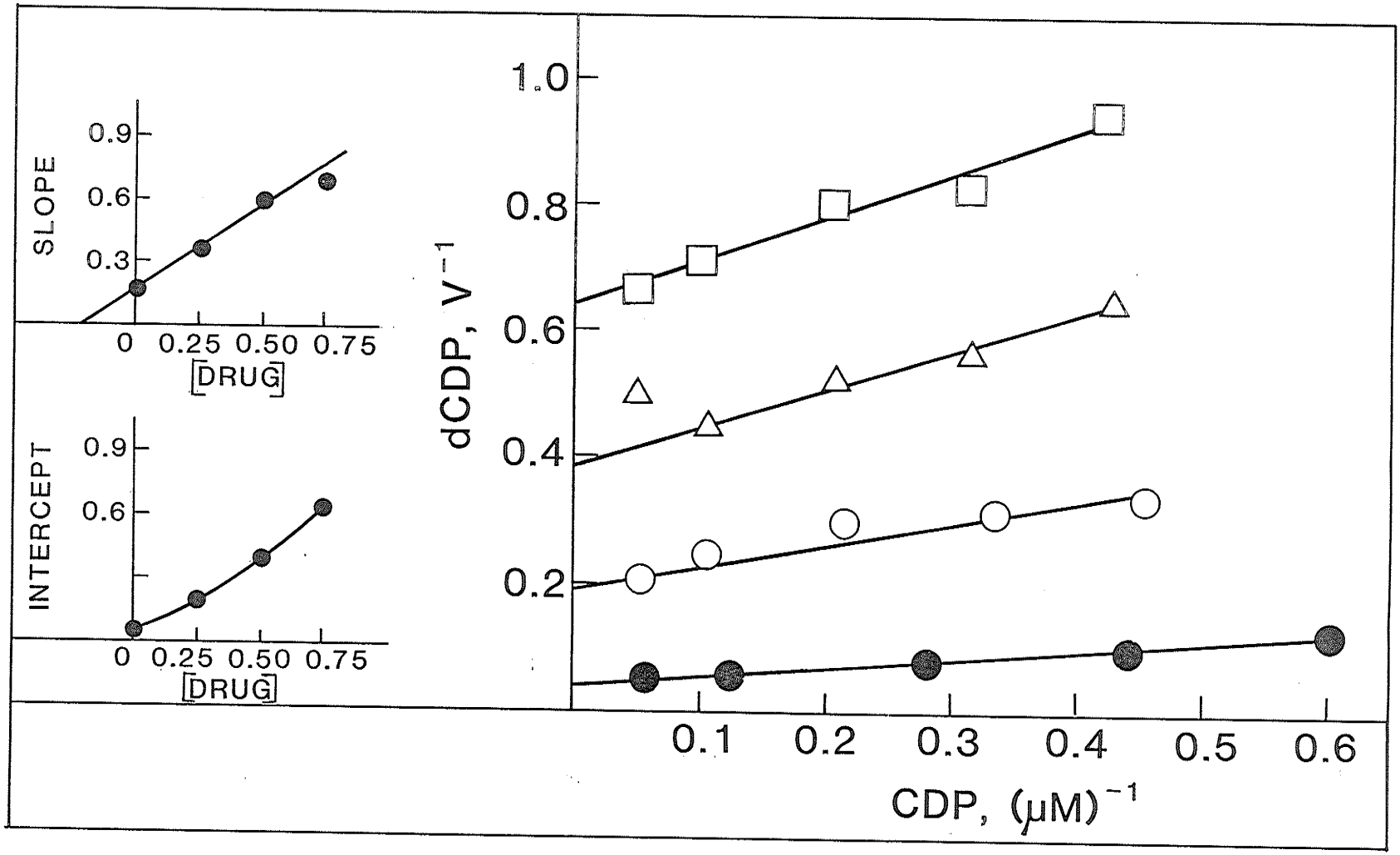

SLOPE
0.3
0.2
0.1
0 0.25 0.50 0.75
[DRUG]
INTERCEPT
0.15
0.10
0.05
0 0.25 0.50 0.75
[DRUG]
dCDP, V⁻¹
0.25
0.2
0.15
0.1
0.05
0.1 0.2 0.3 0.4 0.5 0.6
CDP, (μM)⁻¹

Figure 33

Double reciprocal plots of CDP reduction in the presence of various concentrations of hydroxyurea for partially purified enzyme preparations obtained from HN^R-AT cells. Reactions were carried out in the absence of hydroxyurea (●), or in the presence of 0.25 (o), 0.50 (Δ), and 0.75 (□), mM hydroxyurea. Insets show replots of slope and intercept values versus drug concentration from which Ki values were determined.


SLOPE
0.9
0.6
0.3
0 0.25 0.50 0.75
[DRUG]
INTERCEPT
0.9
0.6
0 0.25 0.50 0.75
[DRUG]
dCDP, V⁻¹
1.0
0.8
0.6
0.4
0.2
0.1 0.2 0.3 0.4 0.5 0.6
CDP, (μM)⁻¹

increase in Ki for hydroxyurea inhibition compared to the wild type cell line. The Ki values for the allosteric inhibitor of ribonucleotide reductase, dATP, were also determined for the various cell lines. Figures 34, 35, and 36 show double reciprocal plots in the presence of increasing concentrations of dATP for the wild type, H^R-R2T, and HN^R-AT cell lines respectively. Similar to hydroxyurea inhibition of enzyme activity the inhibition pattern observed with dATP appeared to be mixed. Table 7 shows the Ki values obtained from replots of velocity intercepts versus dATP concentration (shown in insets to Figures 34, 35, and 36). Both mutant cell lines showed increases in Ki values to dATP, the most pronounced increase shown by the H^R-R2T cell line which had a fourfold increase in Ki value compared to the wild type cells. The above results suggest that some type of structural alteration in the reductase from both mutant cell lines has occured, thus making the enzyme less refractory to inhibition by hydroxyurea or dATP. However, it is not possible to rule out that the changes observed are due to other activities in the enzyme preparations used in the study. Clearly, homogeneous preparations of enzyme components, which at present can not practically be obtained from the cell lines studied here, must be used in order to determine if the changes observed here reflect actual ribonucleotide reductase protein alterations. However, it should be noted that changes in the kinetic properties of ribonucleotide reductase in hydroxyurea resistant cells, similar to those described here, have been observed in other investigations as well (Wright, 1983; 1988).

Figure 34

Double reciprocal plots of CDP reduction in the presence of various concentrations of dATP for partially purified enzyme preparations obtained from wild type cells. Reactions were carried out in the absence of dATP (●), or in the presence of 40 (o), 80 (△), and 120 (□) uM dATP. Inset shows replots of intercept values versus dATP concentration from which Ki values were determined.

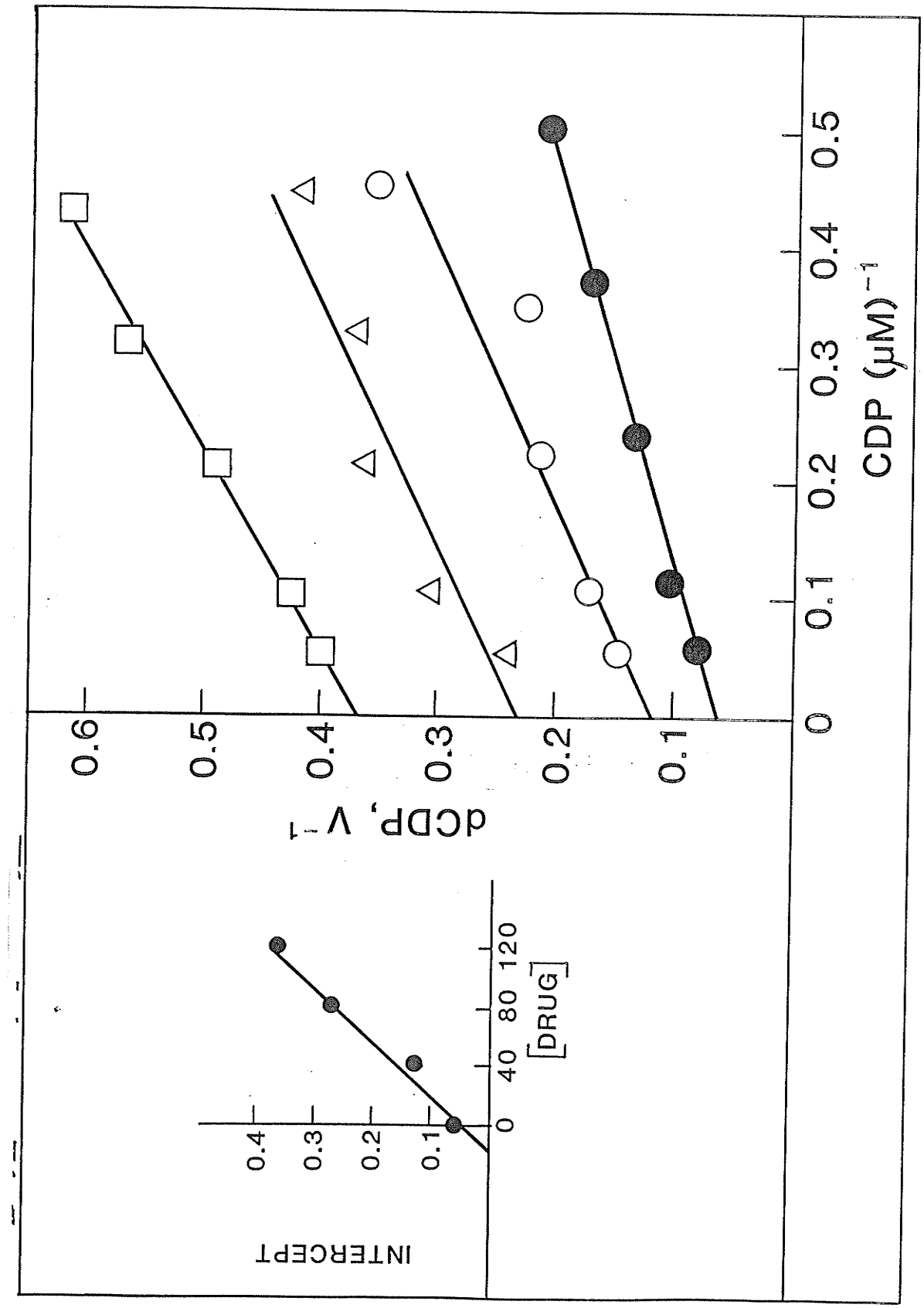

dCDP, V⁻¹
0.6
0.5
0.4
0.3
0.2
0.1
0
0.1
0.2
0.3
0.4
0.5
CDP (μM)⁻¹
INTERCEPT
0.4
0.3
0.2
0.1
0
40
80
120
[DRUG]

Figure 35

Double reciprocal plots of CDP reduction in the presence of various concentrations of dATP for partially purified enzyme preparations obtained from H^R-R2T cells. Reactions were carried out in the absence of dATP (●), or in the presence of 100 (o), 150 (Δ), and 200 (□) uM dATP. Inset shows replots of intercept values versus dATP concentration from which Ki values were determined.

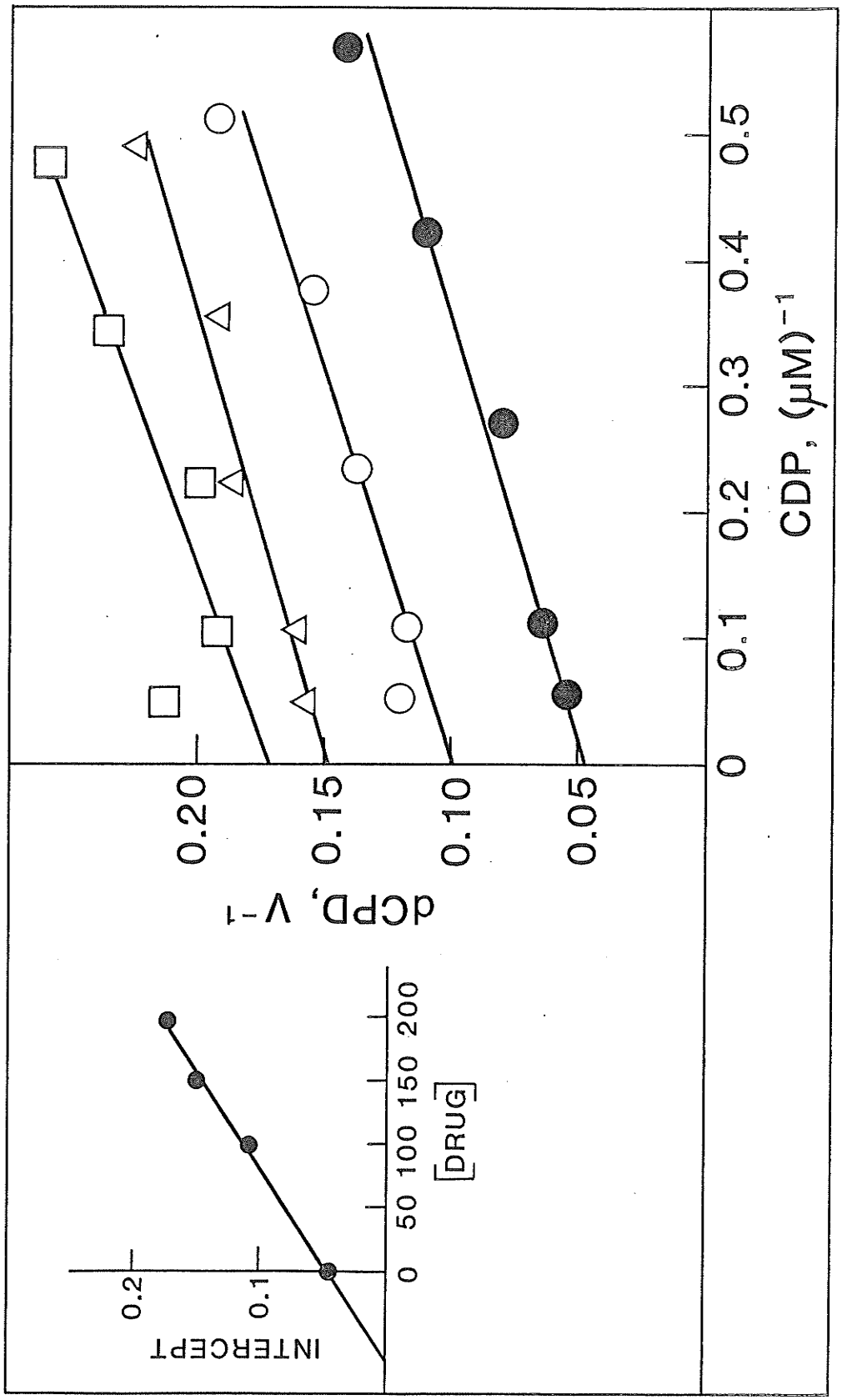

0.20
0.15
0.10
0.05
dCPD, V⁻¹
0
0.1
0.2
0.3
0.4
0.5
CDP, (μM)⁻¹
INTERCEPT
0.2
0.1
0
50
100
150
200
[DRUG]

Figure 36

Double reciprocal plots of CDP reduction in the presence of various concentrations of dATP for partially purified enzyme preparations obtained from HNR-AT cells. Reactions were carried out in the absence of dATP (●), or in the presence of 75 (o), 150 (Δ), and 200 (□) uM dATP. Inset shows replots of intercept values versus dATP concentration from which Ki values were determined.

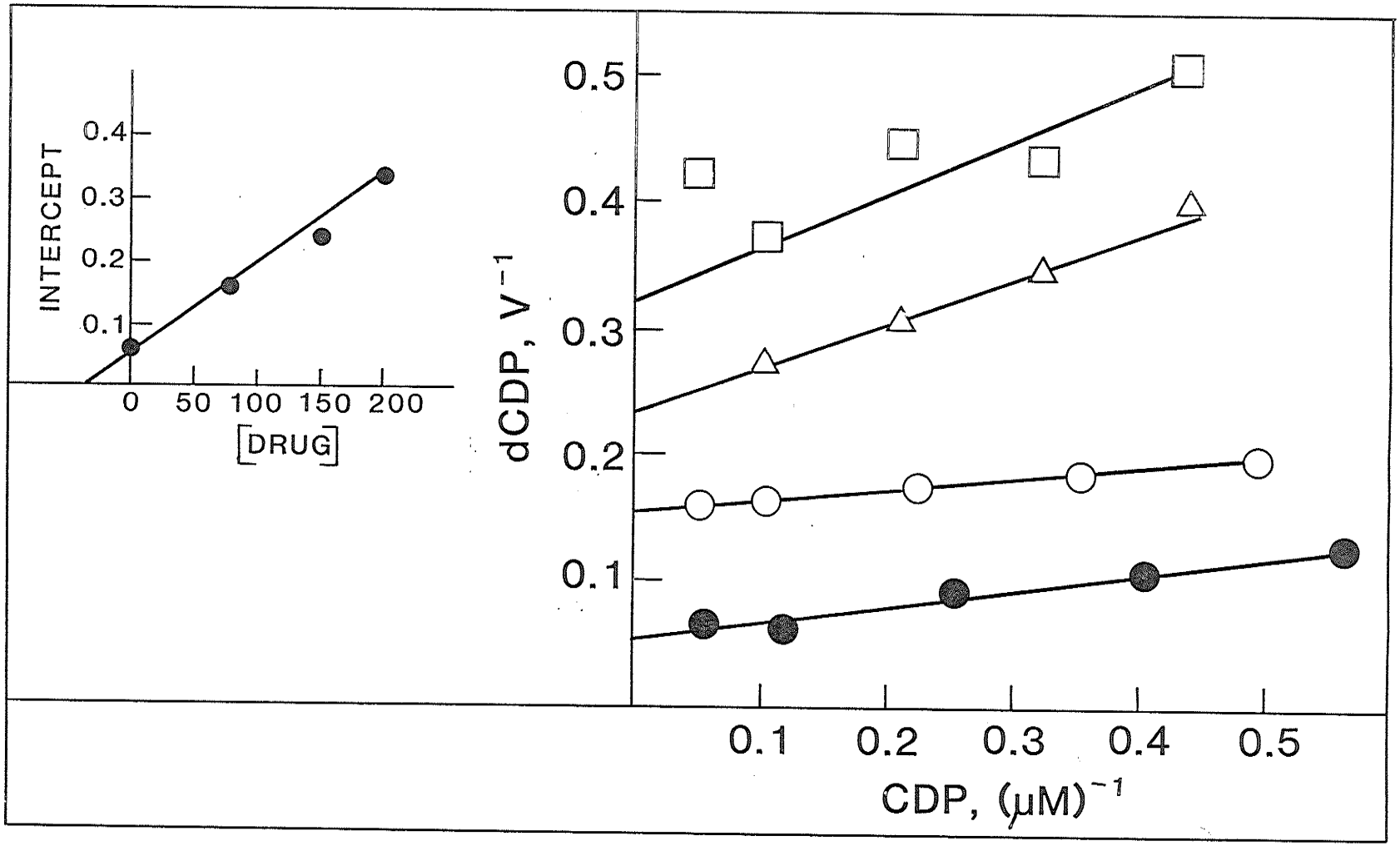

INTERCEPT
0.4
0.3
0.2
0.1
0
50
100
150
200
[DRUG]
dCDP, V⁻¹
0.5
0.4
0.3
0.2
0.1
0.1
0.2
0.3
0.4
0.5
CDP, (μM)⁻¹

TABLE 7. Kinetics of ribonucleotide reductase activity in wild type and drug resistant cell lines[a]

Cell lines	Km (CDP) (uM)	Vmax (CDP) (nmol dCDP/mg protein/hour)	Ki[d] Hydroxyurea (mM)	Ki[e] dATP (uM)
WT	2.57 ± 0.27[b]	14.53 ± 0.86[b]	0.152 ± 0.037	18.15 ± 2.63
H^R-R2T	3.27 ± 0.51[b]	25.65 ± 0.14[b]	0.380 ± 0.023	72.71 ± 2.92
HN^R-AT	3.34 ± 0.53[c]	23.14 ± 2.81[c]	0.219	35.01 ± 6.25

[a] K_M and Vmax values for CDP reduction were determined in all cases from the intercept and slope respectively of Linweaver-Burk double reciprocal plots.

[b] Values reported are means from 5 independent experiments ± standard error.

[c] Values reported are means from 4 independent experiments ± standard error.

[d] Ki values were determined from replots of both the velocity intercepts and velocity slopes versus hydroxyurea concentration, as shown in the insets to Figures 31, 32, and 33 except for the HN^R-AT cell line for which only the slope replot was used because the intercept replot was non linear, making it difficult to determine a Ki value. Values shown are the means of both determinations ± standard error.

[e] Ki values were determined from replots of velocity intercepts versus dATP concentration as shown in the insets to Figures 34, 35, and 36. Values shown are the means of two independent determinations ± standard error.

1.2.3 Hydroxy[^{14}C]-urea uptake in wild type, H^R-R2T and HN^R-AT cells

A mechanism whereby cells may become resistant to certain cytotoxic drugs is through altering the transport properties of the drug accross the plasma membrane (Flintoff et al., 1976a,b). To determine if drug uptake was altered in the hydroxyurea resistant mutants as compared to the wild type cells, the uptake of ^{14}C labelled hydroxyurea into the various cell lines was investigated. Figures 37, 38, and 39 show the uptake of increasing concentrations of hydroxy-[^{14}C]-urea into wild type, H^R-R2T, and HN^R-AT cells respectively. The uptake of hydroxyurea is linear with respect to concentration for all three cell lines. The slopes of the plots in the 0 to 1 mM range of drug were determined to be 200, 256, and 278 pmol drug incorporated/(10^6 cells · min ·mM hydroxyurea), for wild type, H^R-R2T, and HN^R-AT cells respectively. Clearly the drug resistant phenotype of the variant cell lines can not be attributed to a decreased uptake of hydroxyurea compared to the wild type cells; in fact, the wild type cell line appeared to incorporate hydroxyurea to a slightly lower extent than either of the variant lines. The insets to Figures 37, 38, and 39 show drug uptake for higher concentrations of hydroxyurea. The linearity of these plots support previous observations indicating that hydroxyurea enters CHO cells by a mechanism of simple diffusion (Morgan et al., 1986).

1.3 Cross resistance studies

The observation that the M2 components of ribonucleotide

Figure 37

Uptake of hydroxy[^{14}C]-urea into wild type cells with various concentrations of extracellular hydroxyurea. Cells were incubated in the presence of the indicated concentrations of hydroxyurea (specific activity of labelled drug varied between 7.23×10^9 and 1.46×10^7 dpm/mmol) for 1 min. Drug uptake was then determined as described in Materials and Methods. Inset shows hydroxy[^{14}C]-urea uptake at very high drug concentrations.

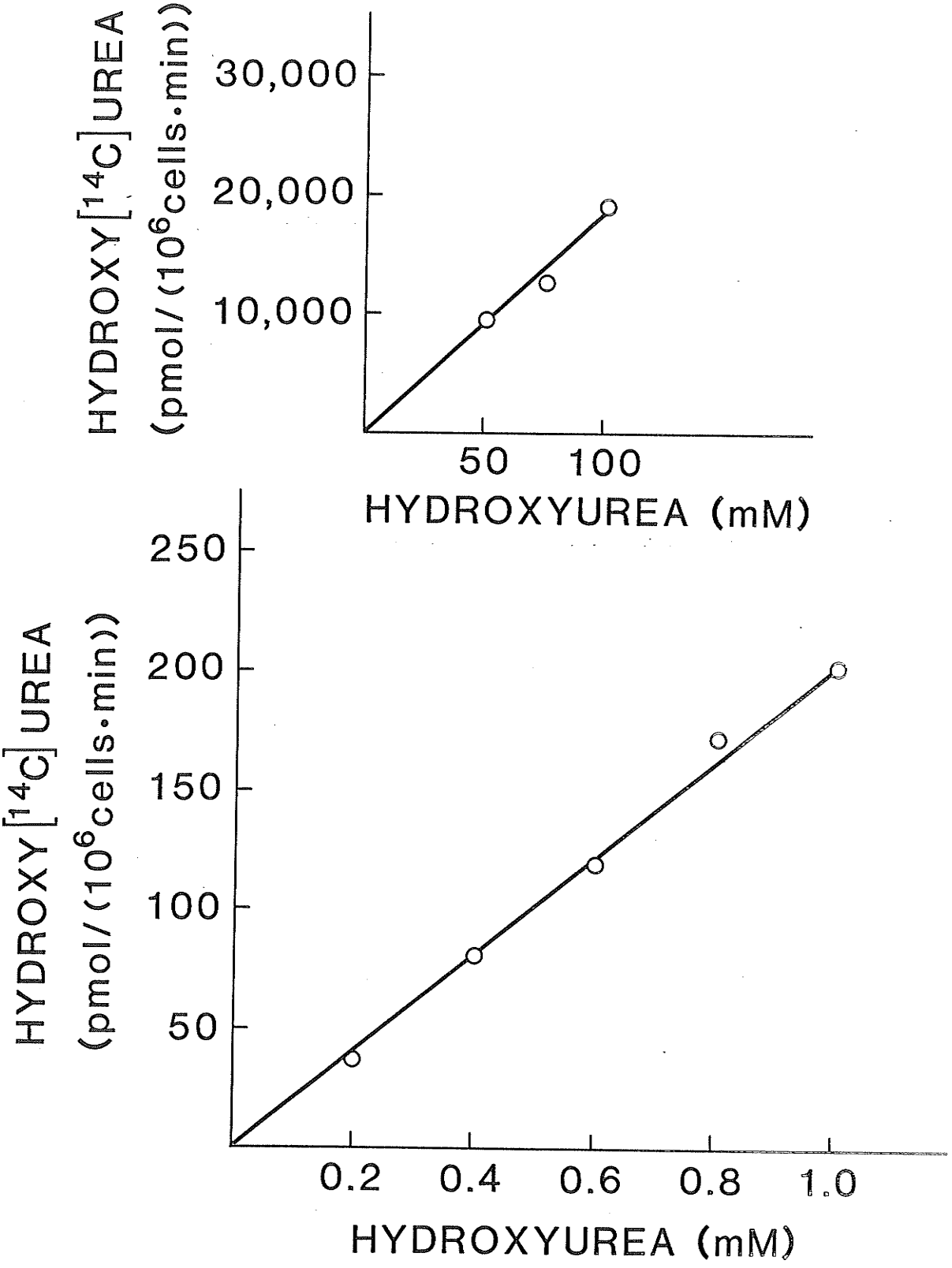

HYDROXY[^{14}C]UREA
(pmol/(10^6cells·min))
30,000
20,000
10,000
50
100
HYDROXYUREA (mM)
HYDROXY[^{14}C]UREA
(pmol/(10^6cells·min))
250
200
150
100
50
0.2
0.4
0.6
0.8
1.0
HYDROXYUREA (mM)

Figure 38

Uptake of hydroxy[^{14}C]-urea into H^R-R2T cells with various concentrations of extracellular hydroxyurea. Cells were incubated in the presence of the indicated concentrations of hydroxyurea (specific activity of labelled drug varied between 7.23×10^9 and 1.46×10^7 dpm/mmol) for 1 min. Drug uptake was then determined as described in Materials and Methods. Inset shows hydroxy[^{14}C]-urea uptake at very high drug concentrations.

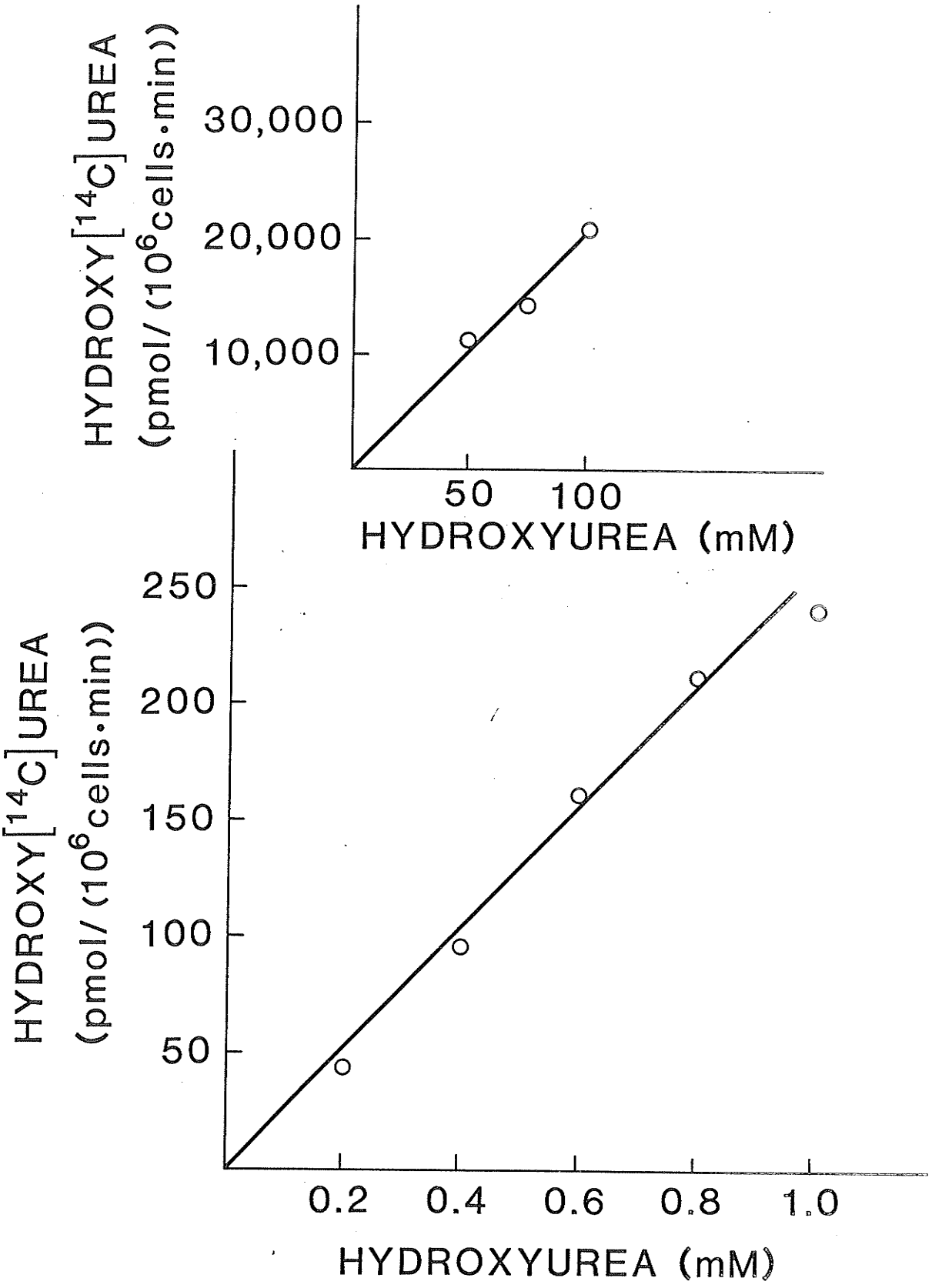
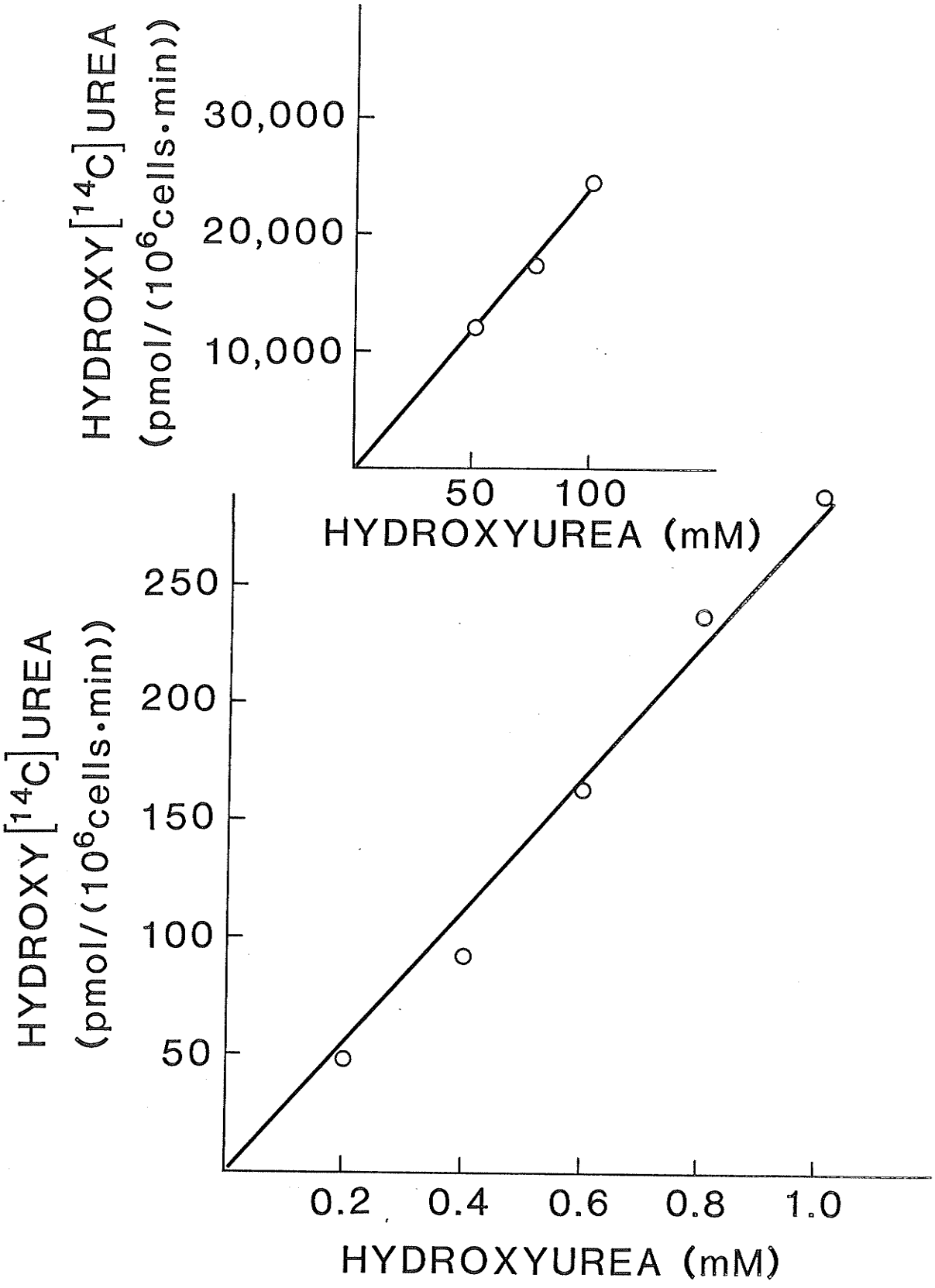

HYDROXY[14C]UREA
(pmol/(10^6cells·min))
30,000
20,000
10,000
50
100
HYDROXYUREA (mM)
HYDROXY[14C]UREA
(pmol/(10^6cells·min))
250
200
150
100
50
0.2
0.4
0.6
0.8
1.0
HYDROXYUREA (mM)

Figure 39

Uptake of hydroxy[^{14}C]-urea into HN^{R}-AT cells with various concentrations of extracellular hydroxyurea. Cells were incubated in the presence of the indicated concentrations of hydroxyurea (specific activity of labelled drug varied between 7.23×10^{9} and 1.46×10^{7} dpm/mmol) for 1 min. Drug uptake was then determined as described in Materials and Methods. Inset shows hydroxy[^{14}C]-urea uptake at very high drug concentrations.


HYDROXY[^{14}C]UREA
(pmol/(10^6cells·min))
30,000
20,000
10,000
50
100
HYDROXYUREA (mM)
HYDROXY[^{14}C]UREA
(pmol/(10^6cells·min))
250
200
150
100
50
0.2
0.4
0.6
0.8
1.0
HYDROXYUREA (mM)

reductase are elevated in the drug resistant cells predicts that the cells will be cross resistant to other drugs whose site of action is the M2 protein. The relative colony-forming abilities of wild type and H^R-R2T cell lines were examined in the presence of various concentrations of guanazole (3,5-diamino-1,2,4-triazole) and the thiosemicarbazone MAIQ (4-methyl-5amino-1-formylisoquinoline thiosemicarbazone) which have been shown to inhibit ribonucleotide reductase by interacting with the M2 component (Engstrom et al., 1979; Wright 1983, 1988; Cory and Carter, 1988). As shown in Table 8, the mutant line exhibited an increase of approximately 7 fold in cellular resistance to guanazole in keeping with previous studies (Wright and Lewis, 1974), and a modest increase in resistance to MAIQ, supporting the view that MAIQ acts at the M2 component, but inactivates the M2 protein by a different mechanism than hydroxyurea or gaunazole, and may also affect other sites besides ribonucleotide reduction. In addition, H^R-R2T and wild type cells were tested for sensitivity to puromycin and colchicine (Table 8). Resistance to these drugs frequently occurs through increased synthesis of a membrane glycoprotein, giving a multidrug resistant phenotype, and resulting in cross resistance to a structurally diverse group of compounds many of which are useful in chemotherapy (Kartner et al., 1985; Fojo et al., 1987; Arsenault et al., 1988). The drug resistant cells did not exhibit an increase in resistance to either puromycin or colchicine (Table 8), indicating that the multidrug resistant phenotype is not associated with selection to, or resistance to hydroxyurea.

TABLE 8. Relative colony forming abilities of H^R-R2T and wild type cells in the presence of various drugs

Drug	D_{10} VALUES	
	Wild Type	H^R-R2T
Gaunazole	1.63 mM	10.88 mM
MAIQ	6.9 uM	9.2 uM
Puromycin	5.0 mM	4.4 mM
Colchicine	0.21 uM	0.14 uM

2. DEOXYRIBONUCLEOTIDE POOLS, SPONTANEOUS MUTATION RATES AND TUMORIGENIC POTENTIAL OF HYDROXYUREA RESISTANT CHO CELL LINES

2.1 Tumorigenic and metastatic behavoir of the variant and wild type cell lines

The studies presented above, which showed that H^R-R2T and HN^R-AT cells contained alterations in ribonucleotide reductase, suggested that these lines would be useful for re-examining the proposed link between ribonucleotide reductase and tumorigenic behavoir (Weber, 1983). Both the wild type and the variant cell lines were highly tumorigenic when injected subcutaneously into the midback of C57 BALB/c nu/nu mice. When the tumor growth rates were measured both variant cell lines were significantly more tumorigenic than parental wild type cells (Fig. 40 and Table 9). The H^R-R2T cell line, which exhibited the largest differences in the properties of ribonucleotide reductase, also showed the greatest increase in tumor growth rate when compared to wild type cells ($p < 0.001$). Neither the wild type nor the mutant cell lines gave rise to secondary metastasis. In addition, both the wild type and the HN^R-AT cell lines were virtually non-metastatic, when injected into the tail veins of BALB/c nu/nu mice, in the experimental metastasis assay (Table 9). However, the H^R-R2T population, which was the most aggressive of the three cell lines in the tumorigenic assay, formed lung metastasis, and exhibited a metastatic phenotype significantly different from the wild type or the HN^R-AT populations ($p < 0.056$). Photographs of sample lungs from mice injected iv with either wild type, H^R-R2T, or HNR-AT cells are shown

Figure 40

Growth rates of tumors formed in mice following injection of wild type (a), H^R-R2T (b), or HN^R-AT (c), cells. 1 X 10^6 cells from each cell line were injected subcutaneously into the lower midback of C57 BALB/c nu/nu mice and tumor growth was analysed as described in Materials and Methods.

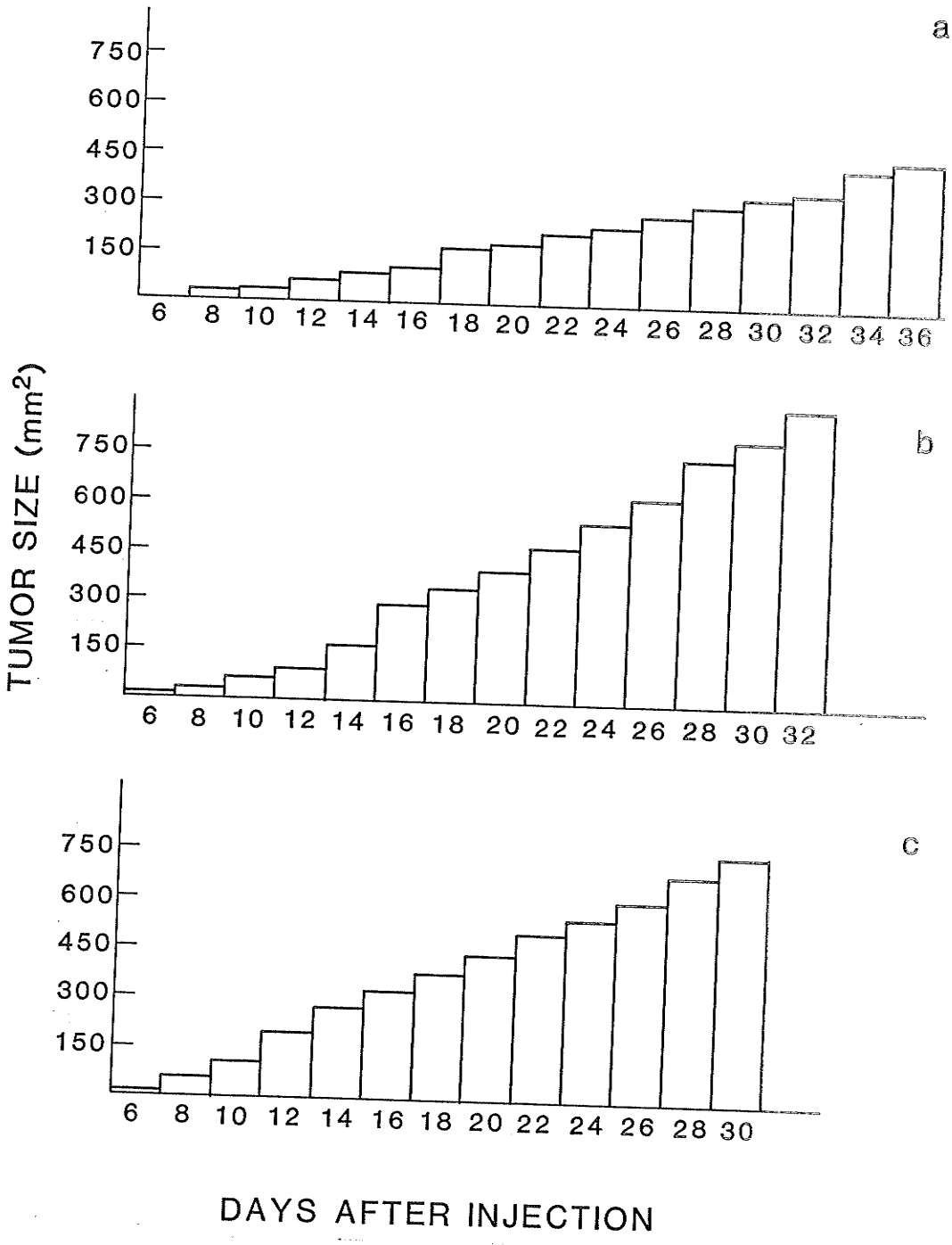

a
750
600
450
300
150
6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
TUMOR SIZE (mm²)
b
750
600
450
300
150
6 8 10 12 14 16 18 20 22 24 26 28 30 32
c
750
600
450
300
150
6 8 10 12 14 16 18 20 22 24 26 28 30
DAYS AFTER INJECTION

Table 9. Tumorigenic and metastatic behavior of the variant and wild type lines

Cell Line	Tumorigenicity[a]		Experimental Metastasis Assay[c]	
	Rate of Increase of Tumor size (mm^2/day)	Difference in Rate of Increase of Tumor Size Compared to Wild Type (mm^2/day)[b]	Mouse No.	No. Lung Metastais
WT	18.3 ± 4.0	-	1	0
			2	1
			3	0
			4	0
			5	0
H^R-R2T	39.3 ± 4.8	21.0 ± 6.2	1	100
			2	0
			3	7
			4	58
HN^R-AT	30.3 ± 2.2	12.0 ± 4.6	1	0
			2	0
			3	0
			4	0

[a] Values shown are means ± standard error of the rate of increase in tumor size from 4 mice for WT and 5 mice each for H^R-R2T and HN^R-AT.

[b] Differences in the rate of increase of tumor size for the mutant lines as compared to WT were significant with $p<0.001$ and $p<0.06$ for the H^R-R2T and HN^R-AT lines respectively.

[c] Clones from each cell line were grown to a final populaion size of 10^7 cells afterwhich $5x10^5$ cells were injected into the tail vein of each mouse. H^R-R2T formed a significantly greater amount of lung metastasis as compared to WT with $p<0.056$ as calculated by Wilcoxon's two sample test (Sokel and Rohlf, 1981). Clonal populations of WT and HN^R-AT grown to a final population size of 10^9 cells both failed to give rise to any lung metastasis upon injection of 5 mice each with $5x10^5$ cells from each cell line.

in Figure 41.

2.2 Analysis of deoxyribonucleotide pool and spontaneous mutation rates

Studies of the spontaneous mutation rates of tumor cell lines have lead to the hypothesis that elevations in spontaneous mutation rates are an important genetic mechanism for generating variant cells with increased malignancy and metastatic properties (Chambers et al., 1981; Harris et al. , 1982; Hill et al., 1984). Ribonucleotide reductase plays a critical role in maintaining a balanced supply of the four deoxyribonucleotides required for DNA synthesis. Changes in ribonucleotide reductase can lead to deoxyribonucleotide pool imbalances and alterations in spontaneous mutation rates in mammalian cells (Weinberg et al., 1981; Arpaia et al., 1983). Therefore, the hypothesis that the modified tumorigenic and metastatic properties of the H^R-R2T and HN^R-AT cell lines was due to altered mutation rates, perhaps resulting from imbalanced deoxyribonucleotide pools, was tested.

The DNA polymerase assay (Hunting and Henderson, 1981) was used to quantitate the deoxyribonucleotide pools in extracts prepared from logarithmically grown wild type, H^R-R2T, and HN^R-AT cells. As shown in Figure 42, the H^R-R2T cells showed no significant alterations in any of the four deoxyribonucleotide pools, when compared to the wild type cells . This is in contrast to the less tumorigenic HN^R-AT cell line, which showed a marked decrease in dCTP (approximately 6 fold), and an

Figure 41

Photograph of a sample of stained lungs of BALB/c nu/nu mice injected iv with wild type (a), HN^R-AT (b), and H^R-R2T (c) cells. Clonal populations of each cell line were grown to a final population size of 10^7 cells and mice were injected with 5 X 10^5 cells as outlined in Materials and Methods.

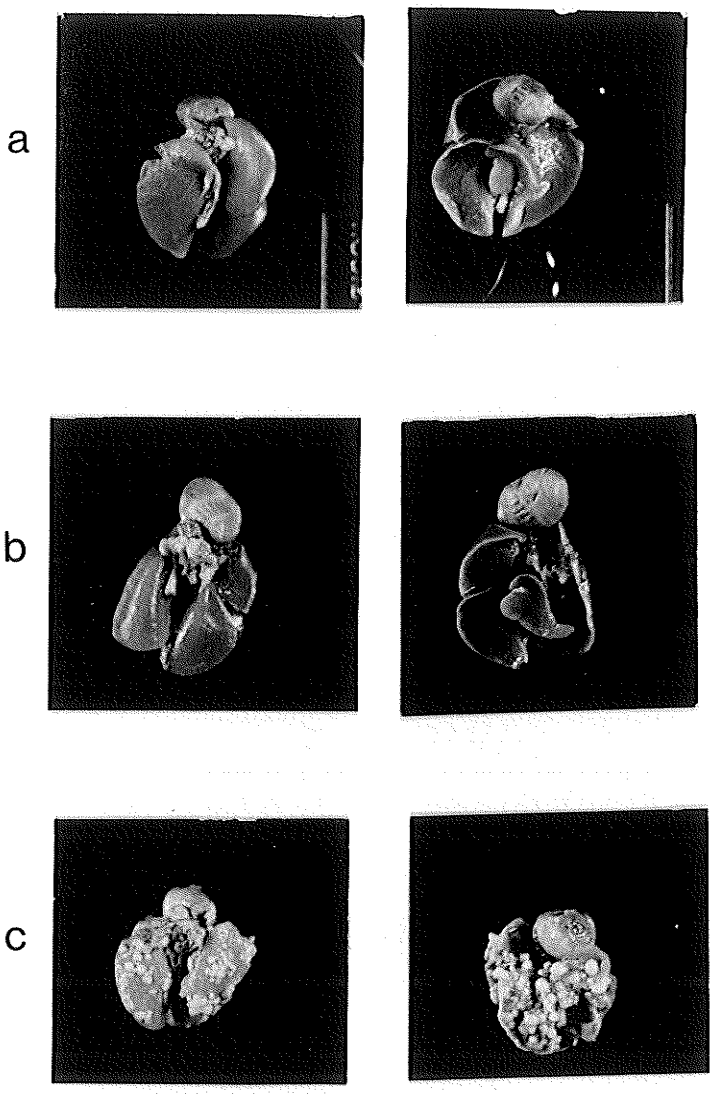
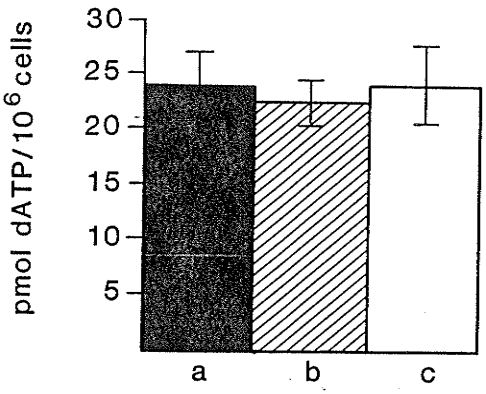
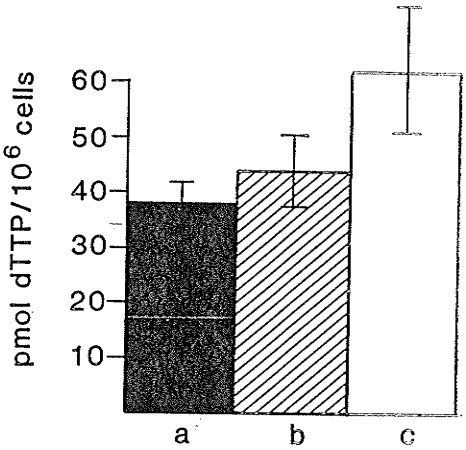
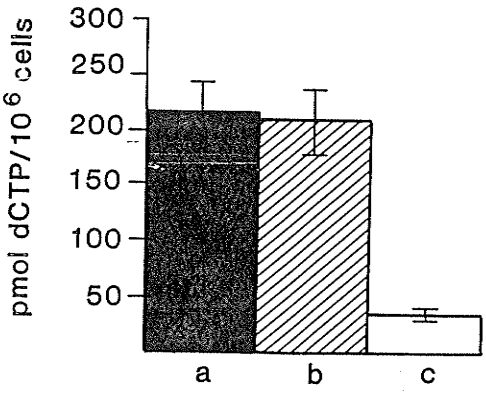

a
b
c

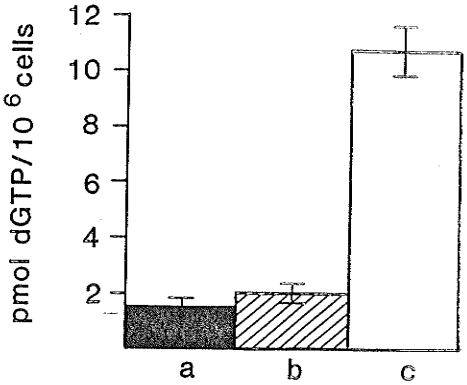
Figure 42

Deoxyribonucleotide pool sizes in wild type (a), H^R-R2T (b), and HN^R-AT (c) cells. Deoxyribonucleotides were extracted from logarithmically grown cells with 10% TCA and their concentrations were determined using the DNA polymerase assay as described in Materials and Methods. Values shown on histograms are means $\pm$ standard error from four independent determinations. Significant differences in dCTP and dGTP pool sizes were observed between wild type and HN^R-AT cells with $p < 0.05$.


pmol dATP/10^6 cells
30
25
20
15
10
5
a
b
c


pmol dTTP/10^6 cells
60
50
40
30
20
10
a
b
c


pmol dCTP/10^6 cells
300
250
200
150
100
50
a
b
c


pmol dGTP/10^6 cells
12
10
8
6
4
2
a
b
c

increase in dGTP (approximately 7 fold) concentrations as compared to the wild type cell line. The Luria and Delbruck fluctuation test was used to determine the rates of spontaneous mutation to 6-thioguanine and ouabain resistance with wild type, H^R-R2T and HN^R-AT cell lines. In order to find an appropiate drug concentration which can be used in the fluctuation analysis to detect for the presence of mutant colonies, dose response curves in the presence of 6-thioguanine or ouabain for the variant and wild type cell lines were performed. Figures 43 and 44 show dose response curves for wild type, H^R-R2T, and HN^R-AT cells in the presence of 6-thiogaunine and ouabain respectively. The curve levels off, presumably due to the formation of colonies by drug resistant cell lines (Thompson and Baker, 1973). For the three CHO cell lines studied here, this levelling off, and hence the appearance of potential mutant colonies, occurs at survival frequencies between 10^{-6} to 10^{-8} at concentrations of 1 to 3 μM for 6-thioguanine and 1 to 3 mM for ouabain. Accordingly, mutation rates to 6-thioguanine and ouabain resistance were determined at drug concentrations of 1.5 μM for 6-thioguanine and 1.5 mM for ouabain. It should be noted that the observed variation in the frequency of appearance of ouabain and 6-thioguanine resistant colonies for the three cell lines (Figs. 43 and 44) is not indicative of the rates of spontaneous mutation to these drugs since the frequency of mutants can vary significantly in parallel cultures (Thompson and Baker, 1973). Tables 10 and 11 show the results of the fluctuation analysis for the determination of spontaneous mutation rates for the variant and wild type cell lines to these two drugs. Statistical analysis indicated that there was no significant difference in the rates of spontaneous

Figure 43

Relative colony-forming ability of wild type (o), H^R-R2T (x), and HN^R-AT (●) cells in the presence of increasing concentrations of 6-thioguanine.

RELATIVE PLATING EFFICIENCIES OF CHO CELLS
IN THE PRESENCE OF 6-THIOGUANINE

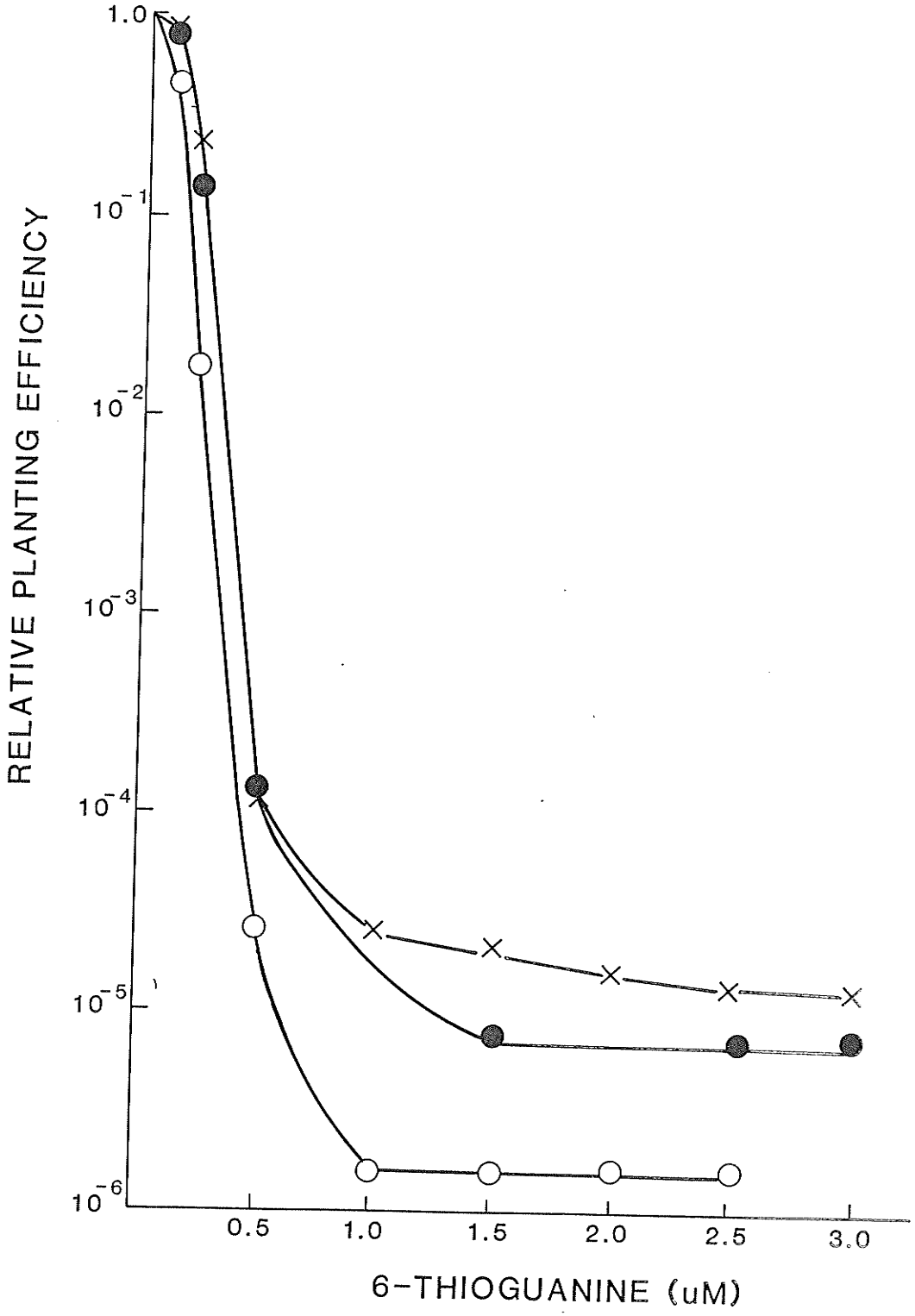


Figure 44

Relative colony-forming ability of wild type (o), H^R-R2T (x), and HN^R-AT (●) cells in the presence of increasing concentrations of ouabain.

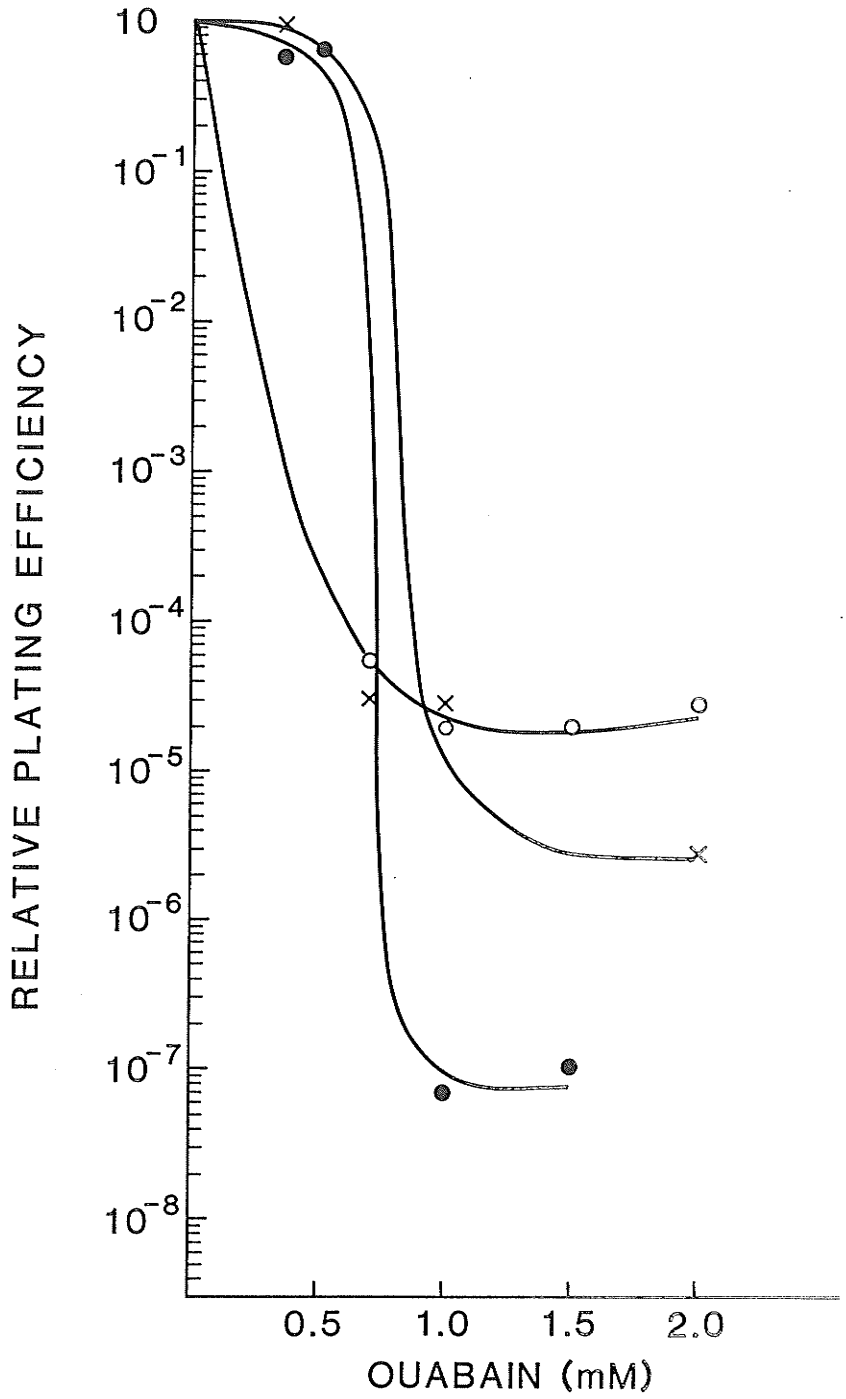

RELATIVE PLATING EFFICIENCY
10
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
10^{-8}
0.5
1.0
1.5
2.0
OUABAIN (mM)

Table 10. Determination of spontaneous forward mutation rates to 6-thioguanine resistance ($6TG^R$) by fluctuation analysis[a]

	WT	H^R-R2T	HN^R-AT
Number of Replicate Cultures Tested (C)	27	33	36
Initial Cell No.	149	180	200
Final Cell No.	2.6×10^6	2.8×10^6	3.1×10^6
Number of Resistant Mutants/Culture			
Range	O-TNTC	0-27	0-113
Variance	-	27.9	358
Mean	-	1.48	4.3
P_o	0.89	0.79	0.78
Mutation Rate (Per Cell Per Division)	3.1×10^{-8}	5.9×10^{-8}	5.5×10^{-8}
Standard Error of the Mutation Rate	1.8×10^{-8}	2.2×10^{-8}	2.0×10^{-8}

[a] The rate of spontaneous mutation (a) was calculated using the fraction of culture plates with zero resistant colonies (P_o) according to Luria and Delbruck (1943), in which $a = \ln (1/P_o)/\text{divisions}$. The standard errors of the estimated mutation rates were calculated using the formula $(1-P_o/d^2 \cdot C \cdot P_o)^{1/2}$, where d = number cell division cycles and C = number of parallel cultures treated with drug, as described by Liu et al. (1982) and Li et al. (1983). There is no significnt alteration in mutation rates to $6TG^R$ between H^R-R2T and WT cells, nor is there a significant alteration in mutation rates to $6TG^R$ between HN^R-AT and WT cells. Statistical analysis was performed as described by Li et al. (1983).

Table 11. Determination of spontaneous forward mutation rates to ouabain resistance (OU^R) by fluctuation analysis[a]

	WT	H^R-R2T	HN^R-AT
Number of Replicate Cultures Tested (C)	29	30	27
Initial Cell No.	144	192	210
Final Cell No.	2.9×10^6	3.0×10^6	3.2×10^6
Number of Resistant Mutants/Culture			
Range	0-1	0-91	0-1
Variance	0.24	273	0.07
Mean	0.36	3.77	0.08
P_o	0.66	0.50	0.93
Mutation Rate (Per Cell Per Division)	10×10^{-8}	16×10^{-8}	1.7×10^{-8}
Standard Error of the Mutation Rate	3×10^{-8}	4×10^{-8}	1.2×10^{-8}

[a] The rate of spontaneous mutation (a) was calculated using the fraction of culture plates with zero resistant colonies (P_o) according to Luria and Delbruck (1943), in which $a = \ln (1/P_o)$/divisions. The standard errors of the estimated mutation rates were calculated using the formula $(1-P_o/d^2 \cdot C \cdot P_o)^{1/2}$, where d = number cell division cycles and C = number of parallel cultures treated with drug, as described by Liu et al. (1982) and Li et al. (1983). There is no significant alteration in mutation rates to OU^R between H^R-R2T and WT cells. The spontaneous mutation rate to OU^R is significantly lower in HN^R-AT cells as compared to WT cells with $p<0.05$. Statistical analysis was performed as described by Li et al. (1983).

mutation to 6-thioguanine resistance in the three lines tested. However, when the rate of spontaneous mutation to ouabain was examined, the HN^R-AT cell line showed a decrease of 5 fold when compared to the wild type line, whereas the H^R-R2T and wild type lines were not significantly different.

In order to examine the possibility that the altered metastatic properties of the H^R-R2T cell line was associated with an enhanced ability to amplify DNA sequences, the ability of this cell line to grow in various concentrations of PALA was compared with wild type and HN^R-AT cell populations. Table 12 shows the percent of cell survival for the three cell lines in 200 and 400 μM PALA following a 72 hr incubation at 37°C. The ability to proliferate in PALA has been shown to be directly related to the degree of CAD gene amplification (Wahl et al., 1979; Cillo et al., 1987). The observation that all three of the cell lines appear to be capable of proliferating to roughly the same extent at both PALA concentrations suggests that all three cell lines have approximately the same capacity to amplify DNA sequences.

3. RIBONUCLEOTIDE REDUCTION IN NORMAL AND TRANSFORMED HUMAN DIPLOID FIBROBLASTS

Studies have shown a correlation between ribonucleotide reductase activity and the growth rates of a series of rat hepatomas as measured by mean generation time (Elford, 1972). In addition, the rise in ribonucleotide reductase activity in malignant rat hepatomas more clearly follows the S phase than any other DNA synthetic activity,

Table 12. Growth measurements of CHO cells in the presence of PALA

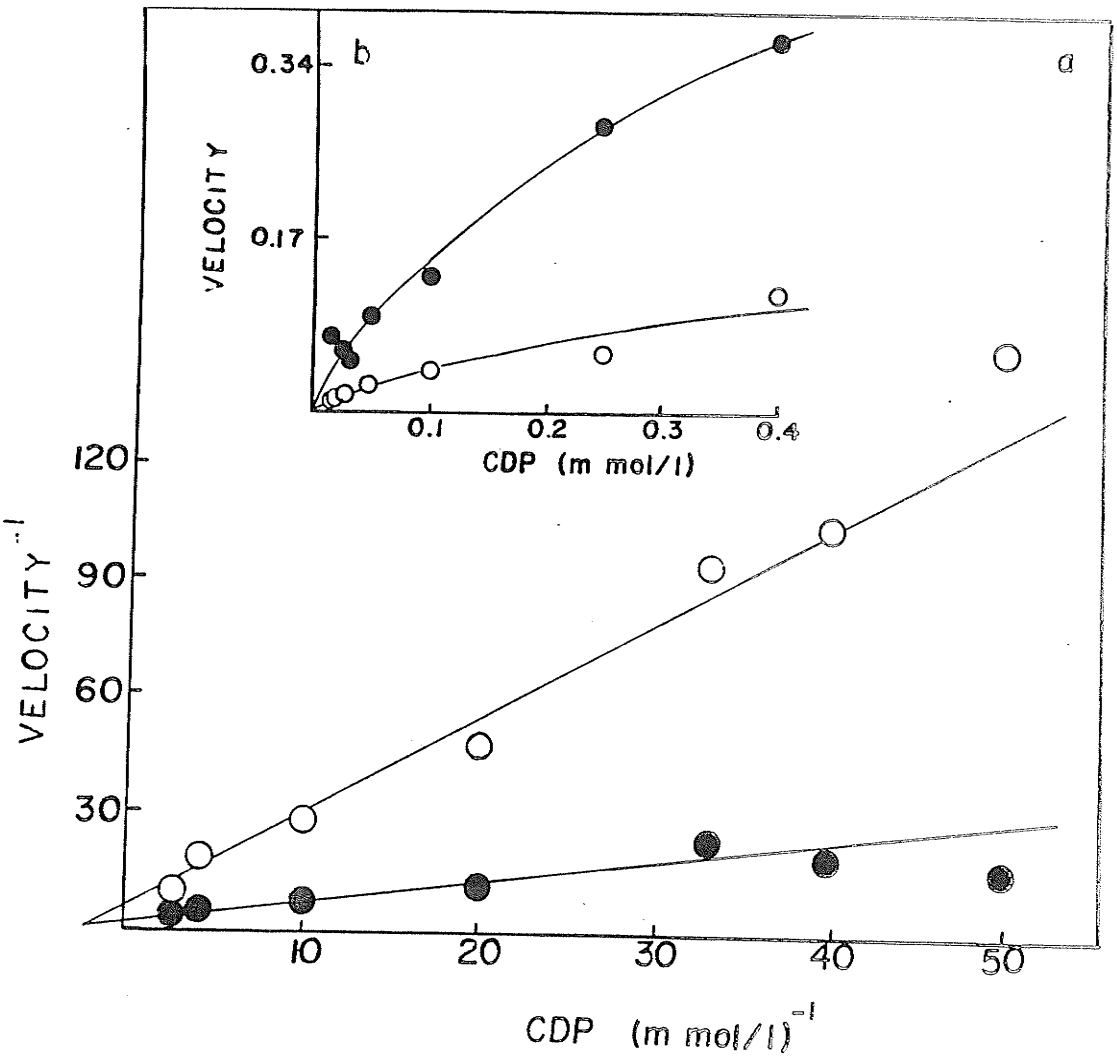
PALA (μM)	Percentage Growth*		
	Wild type	H^R-R2T	HN^R-AT
0	100	100	100
200	62	65	56
400	46	37	35

* Cells from all three lines were seeded at 5 X 10^5 cells per 60 mm tissue culture plate and growth measurements were perfomed as outlined in Materials and Methods. Growth in the absence of drug was taken to be 100%.

supporting the idea that the behavoir of the reductase is transformation linked (Takeda and Weber, 1981; Weber, 1983; Elford, 1972). The studies in parts 1 and 2 of the Results section of this thesis which showed that the H^R-R2T and HN^R-AT hydroxyurea resistant cell lines overproduced ribonucleotide reductase activity and were more tumorigenic in nude mice than the wild type cell line are in agreement with those studies. To further examine the relationship between ribonucleotide reductase and transformation, it was of interest to take an opposite approach to that discussed in the previous section, namely, to investigate whether the transformation event resulted in alterations to ribonucleotide reductase activity. Since all other studies to date which showed a correlation in the elevation of ribonucleotide reductase activity with transformation have utilized rodent cell models, it was unknown whether such a correlation existed in human cells. Furthermore these previous studies did not utilize systems which compared directly the reductase activities in transformed cells with their nontransformed parental counterparts. With the use of a permeabilized cell assay system (Dick and Wright, 1980) it was possible to measure the relatively low levels of ribonucleotide reductase activity normally present in human cells and to compare this activity directly with enzyme activity present in transformed counterparts of these cells. The activity of ribonucleotide reductase in logarithmically growing WI-38 normal human diploid fibrobalsts and their SV40 virus-transformed counterparts, WI-38 VA13 is shown in Figure 45. The Lineweaver-Burk double reciprocal plots were linear with enzyme from both the normal human fibroblasts and their transformed counterparts, and an apparent Km value of 0.37 mM CDP was

Figure 45

Double reciprocal plot of the rate of CDP reduction for WI-38 (o) and WI-38 VA13 (●) fibroblasts. Inset shows the velocity of CDP reduction in the presence of varying concentrations of CDP for WI-38 (o) and WI-38 VA13 (●) cells.

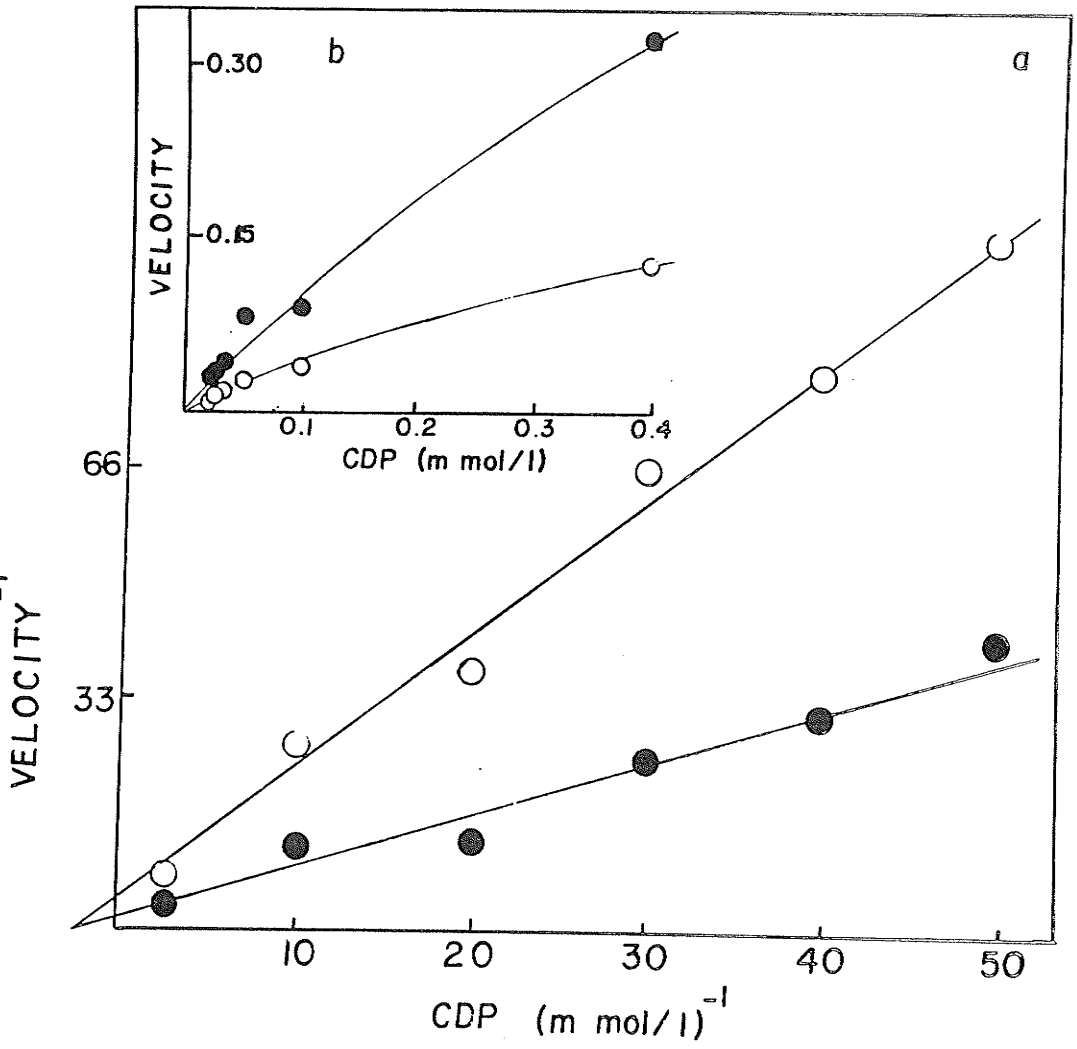

b
a
0.34
0.17
VELOCITY
0.1
0.2
0.3
0.4
CDP (m mol/l)
120
90
60
30
VELOCITY^{-1}
10
20
30
40
50
CDP (m mol/l)$^{-1}$

obtained for the enzyme from both the normal and transformed cells. It is clear, however, that there is significantly higher enzyme activity in the transformed cells as compared to the normal human fibroblasts. Analysis of the double reciprocal plots indicated that the WI-38 fibroblasts reduced a maximum of 0.15 nmol CDP/5 X 10^6 cells/hr, whereas WI-38 VA13 cells reduced a maximum of 0.52 nmol CDP/5 X 106 cells/hr. Therefore the transformed line was able to reduce approximately 3.5 times more CDP than the normal human cell strain from which the transformed line originated. Figure 46 shows a Lineweaver-Burk plot for another human diploid fibroblast strain, WI-26 and its SV40 virus-transformed counterpart, WI-26 VA4. The double reciprocal plots were linear with enzyme for both the normal and transformed cells, yielding a Km value of 0.35 mM CDP for both WI-26 and WI-26 VA4 enzyme activities. The Km values observed were nearly identical to the values obtained with WI-38 and WI-38 VA13 cells which are reasonably close to the values determined for the enzyme activity from mouse, hamster, and Novikoff hepatoma cells (Kuzik and Wright, 1980; Lewis and Wright, 1978a; Moore and Hulbert, 1966). Interestingly, the activity of ribonucleotide reductase was elevated in the transformed cells when compared to the normal human cells from which they were derived. The WI-26 fibroblasts were capable of reducing 0.19 nmol CDP/5 X 10^6 cells/hr, whereas WI-26 VA4 cells reduced 0.61 nmol of substrate/5 X 10^6 cells/hr. Ribonucleotide reductase activity in WI-26 cells was elevated by about 3.2 times after cellular transformation.

In order to rule out the possibility that the increased ribo-

Figure 46

Double reciprocal plot of the rate of CDP reduction for WI-26 (o) and WI-26 VA4 (●) fibroblasts. Inset shows the velocity of CDP reduction in the presence of varying concentrations of CDP for WI-26 (o) and WI-26 VA4 (●) cells.


b
a
0.30
0.15
VELOCITY
0.1
0.2
0.3
0.4
CDP (m mol/l)
66
33
VELOCITY^{-1}
10
20
30
40
50
CDP (m mol/l)$^{-1}$

nucleotide reductase activity observed in the virally transformed lines was not simply due to an increase in the proportion of S phase cells compared to the parental cell strains, ^{3}H-thymidine uptake experiments were performed. The extent to which ^{3}H-thymidine is incorporated into logarithmically growing cells may be used as an index for the porportion of cells in S phase (Blosmanis et al., 1987). Table 13 shows the reults for ^{3}H-thymidine uptake experiments performed with WI-38, WI-38 VA13, WI-26 and WI-26 VA4 cells. As can be judged by the ratios of the amount of label incorporated into the cells at the various time points, there is no appreciable difference between the extent of ^{3}H-thymidine incorporation into the WI-38 and WI-38 VA13 cells. This indicates that logarithmically growing WI-38 and WI-38 VA13 cells used in this study have approximtely equivalent numbers of cells undergoing DNA synthesis. On the other hand, the WI-26 VA4 cell line incorporated ^{3}H-thymidine to a slightly greater extent than the parental WI-26 cell strain. Uptake of label was increased between 1.5 to 1.7 fold for the WI-26 VA4 line as compared to the WI-26 cells at the various time points examined. The moderate increase in the porportion of cells synthesizing DNA in this transformed line may in part contribute to the increase in ribonucleotide reductase activity observed, however it can not fully account for the differences. Therefore, both the transformed counterparts of WI-38 and WI-26 human diploid fibroblast cell strains showed increases in the level of ribonucleotide reductase activity compared to their parental strains, which can not solely be attributed to differences in the amount of S phase cells within the population.

Table 13. ^{3}H-Thymidine uptake into logaritmically growing human cells

Incubation Time (min)	CPM ^{3}H-Thymidine Incorporated/10^{6} cells[a] W1-38	W1-38 VA13	Ratio[b]	CPM ^{3}H-Tymidine Incorporated/10^{6} cells[a] W1-26	W1-26 VA4	Ratio[c]
40	254,481	223,265	0.88	219,291	365,809	1.7
60	345,882	362,094	1.05	415,500	666,387	1.6
80	517,742	453,382	0.88	741,412	114,314	1.5

a Logarithmically growing cells on 60 mm tissue culture plates were incubated for the lengths of time indicated in the presence of normal growth medium containing ^{3}H-thymidine. The amount of radioactivity recovered in acid precipitated material was determined as outlined in Materials and Methods. Results shown are average values from duplicate points.

b Ratio of ^{3}H-thymidine uptake of transformed cells (W1-38 VA13) to nontransformed cells (W1-38).

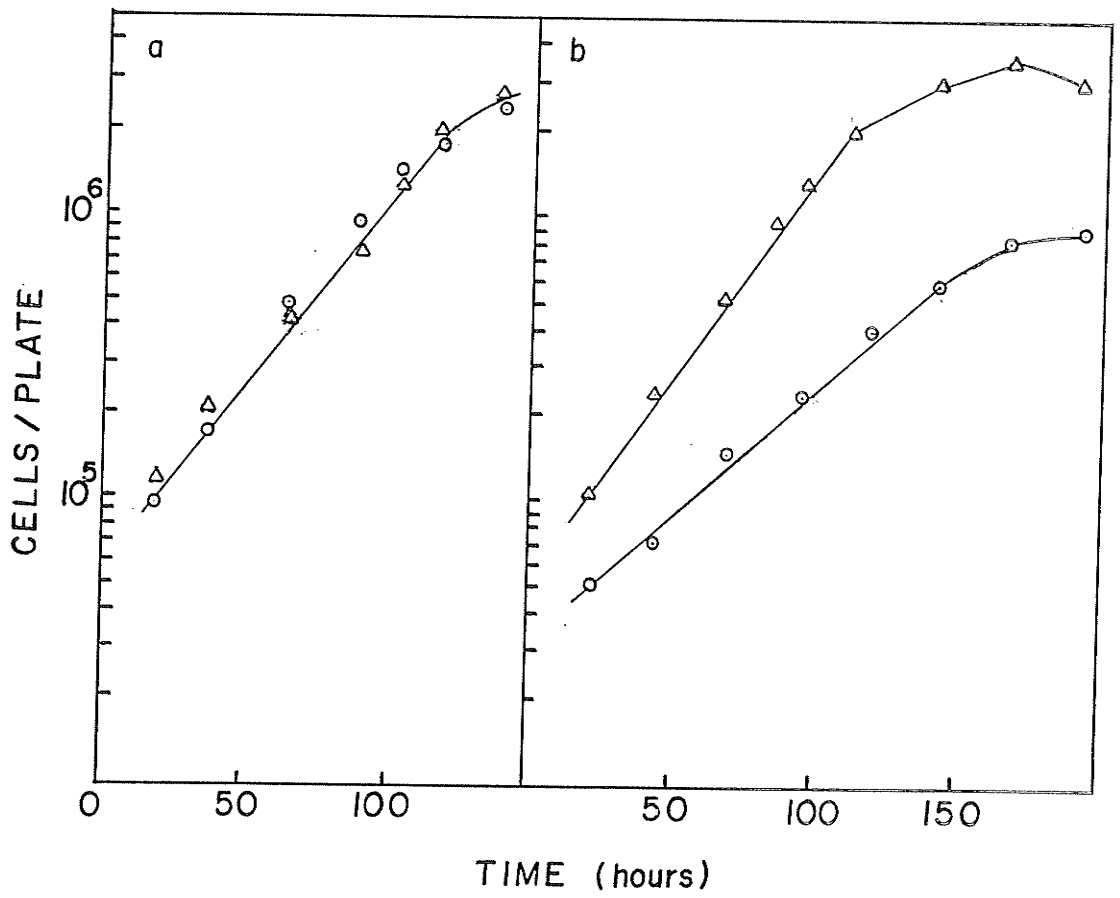
c Ratio of ^{3}H-thymidine uptake of transformed cells (W1-26 VA4) to nontransformed cells (W1-26).

In rat hepatoma cell lines, it has been observed that the increased ribonucleotide reductase activity was accompanied by increased growth rates (Takeda and Weber, 1981; Weber, 1983; Elford, 1972). To determine whether the elevation in reductase activity observed above with the transformed cell lines was also accompanied by enhanced proliferation abilities, the growth rates of the human cell strains and their transformed counterparts were compared. WI-38 and WI-38 VA13 exhibited identical doubling times of 24 hr (Figure 47a), whereas the doubling times of WI-26 and WI-26 VA4 were estimated at 30 and 21 hr. respectively (Figure 47b). Therefore there does not appear to be a general correlation between increased reductase activity and increased proliferation abilities in culture. Similar observations have been made with mutant cell lines selected for drug resistance and elevated levels of ribonucleotide reductase activity (Wright, 1983; Koropatnick and Wright, 1980), including the CHO variants characterized in the preceeding sections of this thesis; the mutant lines did not proliferate at a faster rate than their parental wild type cells (Fig. 12).

The studies presented on the hydroxyurea resistant mutants H^R-R2T and HN^R-AT have shown that hydroxyurea resistance can result in elevations in ribonucleotide reductase activity, and in addition, somatic cell genetic studies have demonstrated that mutants selected for increased levels of reductase activity exhibit resistance to hydroxyurea cytotoxicity (Wright, 1983; Koropatnick and Wright, 1980; Kuzik and Wright, 1980; Lewis and Wright, 1979). These observations suggested that the transformed human cells with elevated ribo-

Figure 47

Growth curves on 60 mm plastic tissue culture plates in alpha-MEM with 15% fetal bovine serum under culture conditions described in Materials and Methods, for (a) WI-38 (o), and WI-38 VA13 (Δ) cells, and (b) for WI-26 (o) and WI-26 VA4 (Δ) cells.


a
b
CELLS / PLATE
10^6
10^5
0
50
100
50
100
150
TIME (hours)

nucleotide reductase activity, should be more resistant than normal human cells to the cytotoxic effects of hydroxyurea. To test this point, the colony-forming abilities of WI-26 and its transformed counterpart were examined in the presence of various drug concentrations. Figure 48 shows that in keeping with the elevated levels of reductase activity observed above (Fig. 46), the transformed cells are relatively more resistant to hydroxyurea when compared to the normal nontransformed population. The D_{10} value for WI-26 fibroblasts was 0.23 mM drug and for WI-26 VA4 the D_{10} estimate was 0.37 mM hydroxyurea.

4. ON THE MECHANISM OF HYDROXY[^{14}C]-UREA UPTAKE BY NORMAL AND TRANSFORMED HUMAN CELLS

Since hydroxyurea has been used in clinical situations to treat a wide range of solid tumors, acute and chronic leukemia, as a radiation potentiator (Piver et al., 1983; Levin et al., 1977), and in conjunction with other chemotherapeutic agents in attempts to achieve a potentiated therapeutic response (Kobayashi and Hoshino, 1983; Engstrom et al., 1984; Dodion et al., 1986; Engstrom et al., 1982), and since drug uptake is an important aspect of drug action, the uptake properties of hydroxyurea in normal human diploid fibroblasts and their SV40 virus-transformed counterparts were investigated. Figure 49 shows the time course for hydroxyurea uptake into WI-38 normal human diploid fibroblast cells. Uptake was linear with time for the first two minutes after which an equilibrium between the rate of influx and the rate of efflux of drug was established. In order to

Figure 48

Relative colony-forming ability of WI-26 (o) and WI-26 VA4 (Δ) cells in the presence of increasing concentrations of hydroxyurea.

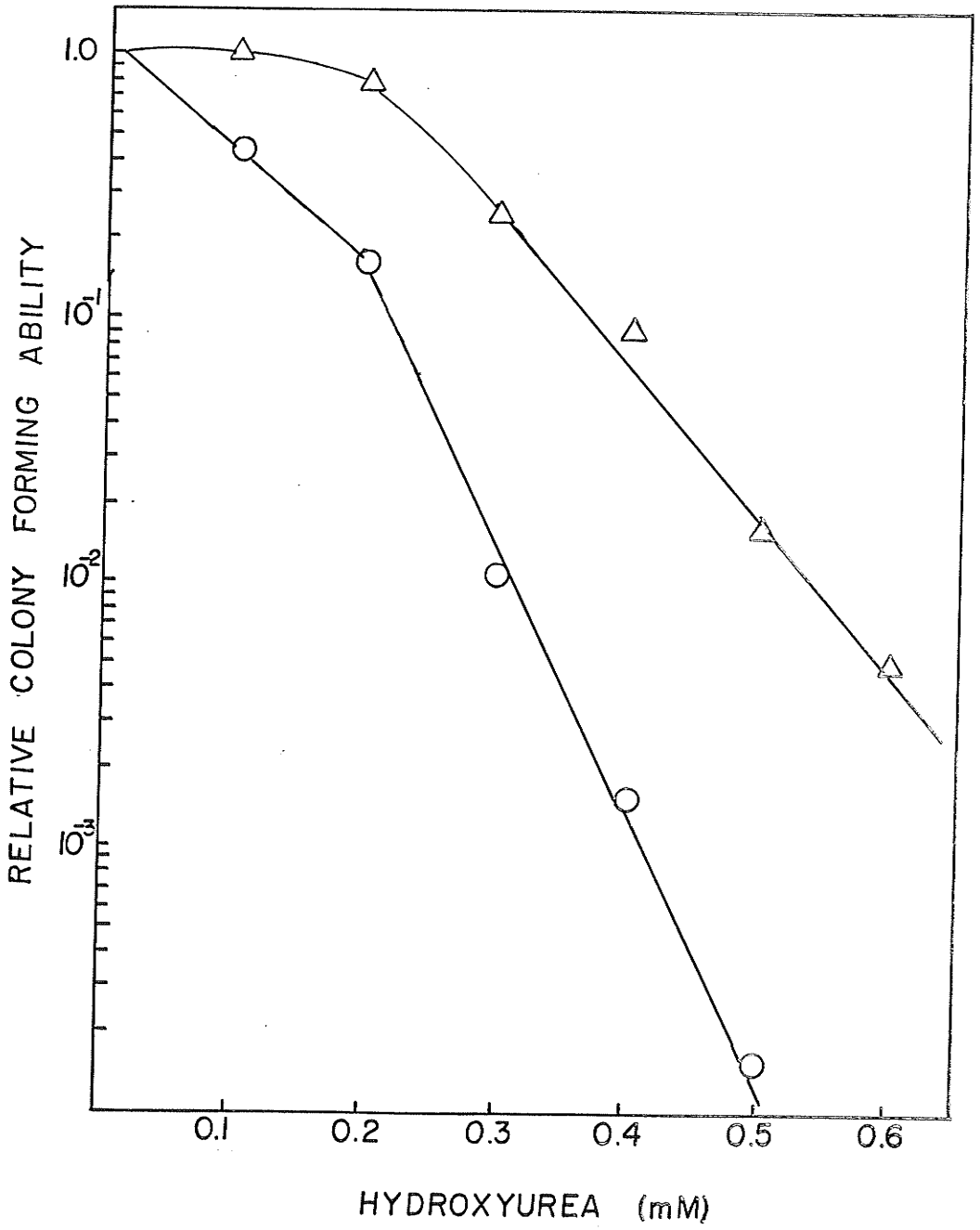
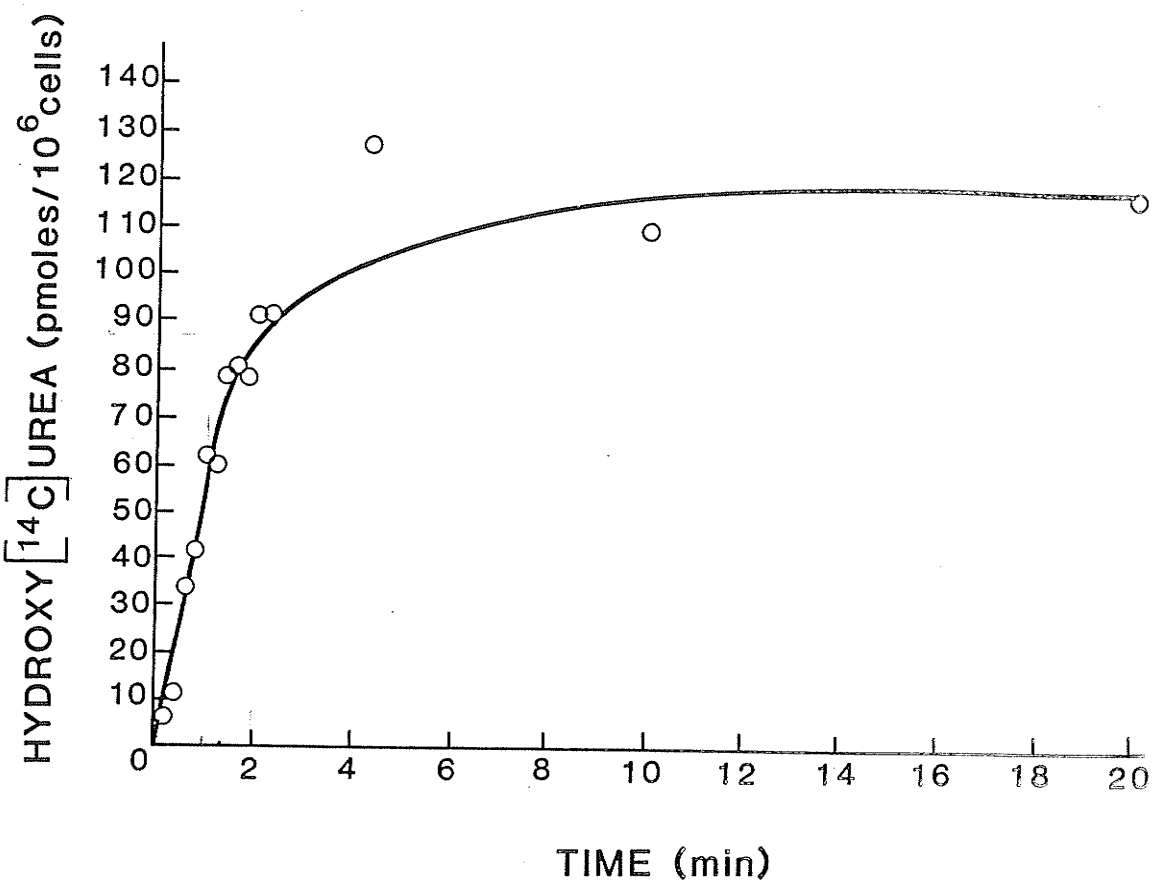

1.0
10^{-1}
10^{-2}
10^{-3}
RELATIVE COLONY FORMING ABILITY
0.1
0.2
0.3
0.4
0.5
0.6
HYDROXYUREA (mM)

Figure 49

Uptake of hydroxy[^{14}C]-urea into WI-38 cells as a function of time. WI-38 cells were incubated in the presence of 75 uM hydroxy[^{14}C]-urea for the times indicated, after which the amount of labelled hydroxy-urea incorporated into the cells was determined as described in Materials and Methods. Results shown are average values obtained from one experiment performed in duplicate. The experiment was repeated with similar results.


HYDROXY[14C]UREA (pmoles/10^6cells)
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
16
18
20
TIME (min)

study drug transport kinetics under initial rate conditions, uptake studies were carried out using a 1 min incubation time. As shown in Figure 50, both the WI-38 and their SV40 virus-transformed counterparts, WI-38 VA13 cells, showed a linear relationship between the rate of drug uptake and drug concentration between 0.1 and 1.0 mM hydroxyurea, with observed Km and Vmax values of essentialy zero and infinity, respectively. This inability to saturate the uptake which occured even between high drug concentrations of 10 and 100 mM (inset to Fig.50) is suggestive of a diffusion mechanism for hydroxyurea uptake into human cells. It should be noted that the rates of uptake were the same between normal diploid fibroblasts and their transformed counterparts, indicating that both the mechanism and kinetics of drug uptake were not altered upon transformation of these cells. As shown in Figure 51, similar results were obtained for the WI-26 cell strain and the SV40 virus-transformed counterpart WI-26 VA4 population.

A comparison of the ratio of intracellular to extracellular drug concentrations at various times of incubation in the presence of 75 uM hydroxyurea indicated that the intracellular drug concentration was consistently lower than that of the extracellular concentration, and this ratio was always less than unity (Fig. 52a). Such findings are supportive of a diffusion mechanism and argue against the involvment of an active transport system for hydroxyurea uptake by human cells. In addition, even when a much higher drug concentration was studied (100 mM hydroxyurea) the cell/medium distribution ratio was found to be less than 1 (Fig. 52b), demonstrating that hydroxyurea tends to equilibriate over the membrane.

Figure 50

Uptake of hydroxy[^{14}C]-urea into normal and transformed human cells with various concentrations of extracellular hydroxyurea. Cells were incubated in the presence of the indicated concentrations of hydroxyurea (specific activity of labelled drug varied between 7.23×10^9 and 1.46×10^7 dpm/mmol) for 1 min. Drug uptake was then determined as described in Materials and Methods. Uptake of hydroxy[^{14}C]-urea between 0.01 and 1.0 mM drug and between 10 and 100 mM drug (inset) are shown for WI-38 (o) and WI-38 VA13 (x) cells.

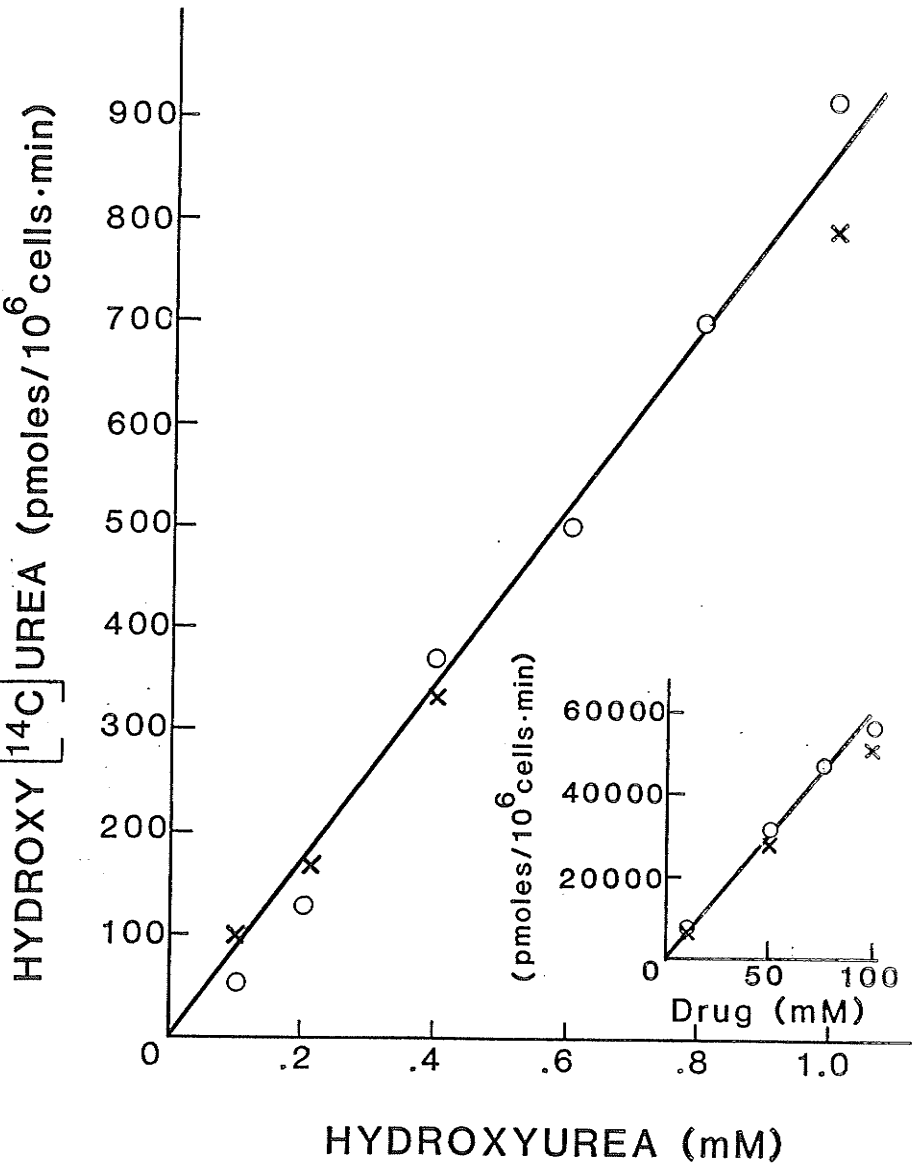

HYDROXY [14C] UREA (pmoles/10^6 cells·min)
900
800
700
600
500
400
300
200
100
0
.2
.4
.6
.8
1.0
HYDROXYUREA (mM)
(pmoles/10^6 cells·min)
60000
40000
20000
0
50
100
Drug (mM)

Figure 51

Uptake of hydroxy[^{14}C]-urea into normal and transformed human cells with various concentrations of extracellular hydroxyurea. Cells were incubated in the presence of the indicated concentrations of hydroxyurea (specific activity of labelled drug varied between 7.23 X 10^9 and 1.46 X 1097 dpm/mmol) for 1 min. Drug uptake was then determined as described in Materials and Methods. Uptake of hydroxy[^{14}C]-urea between 0.01 and 1.0 mM drug and between 10 and 100 mM drug (inset) are shown for WI-26 (o) and WI-26 VA4 (x) cells.

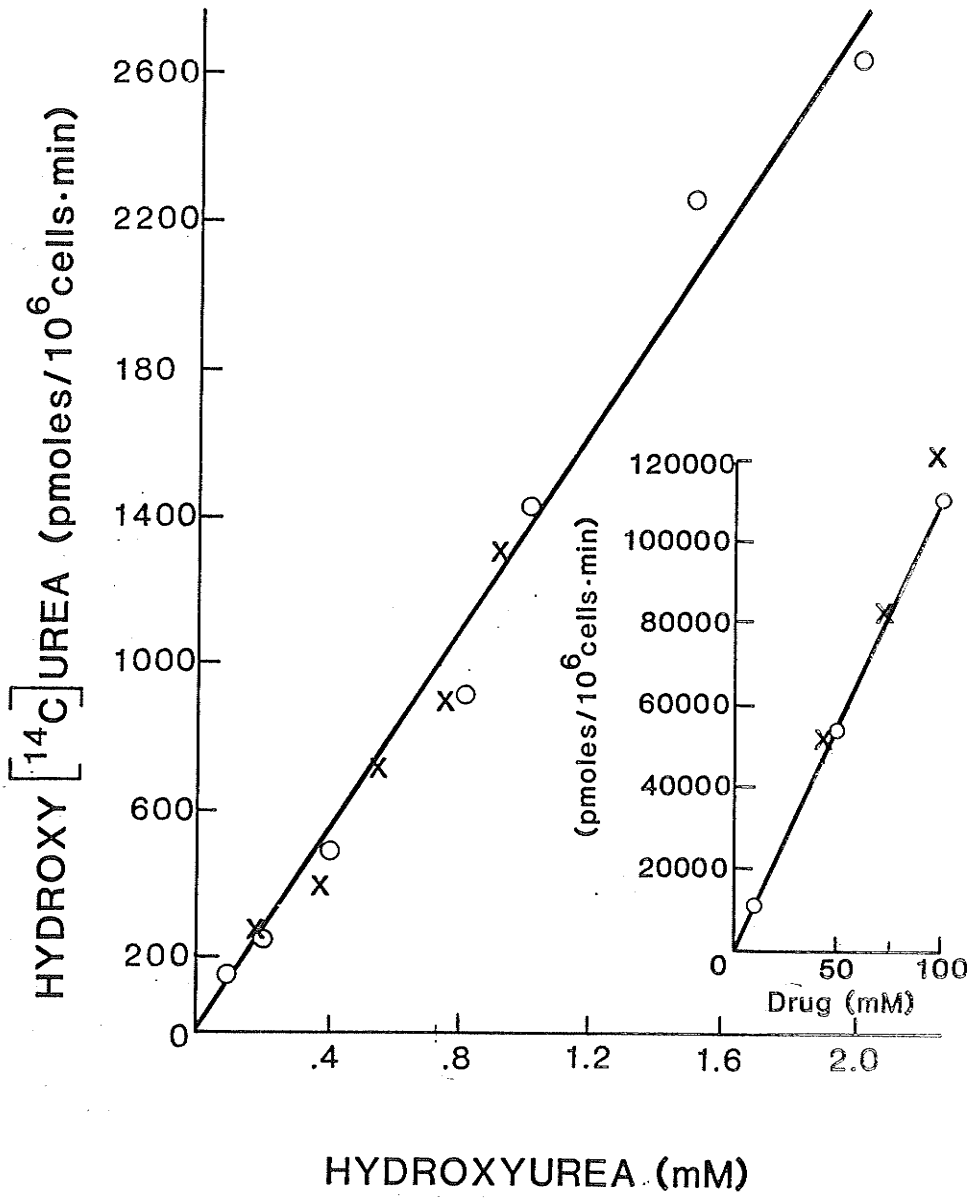
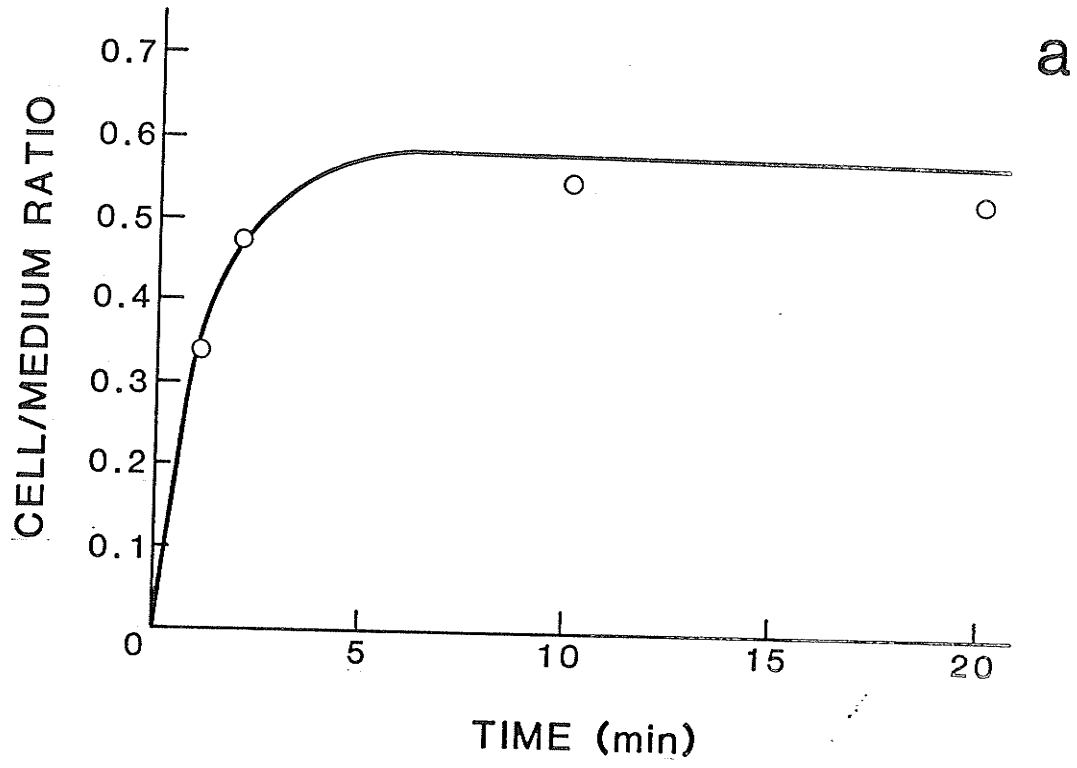
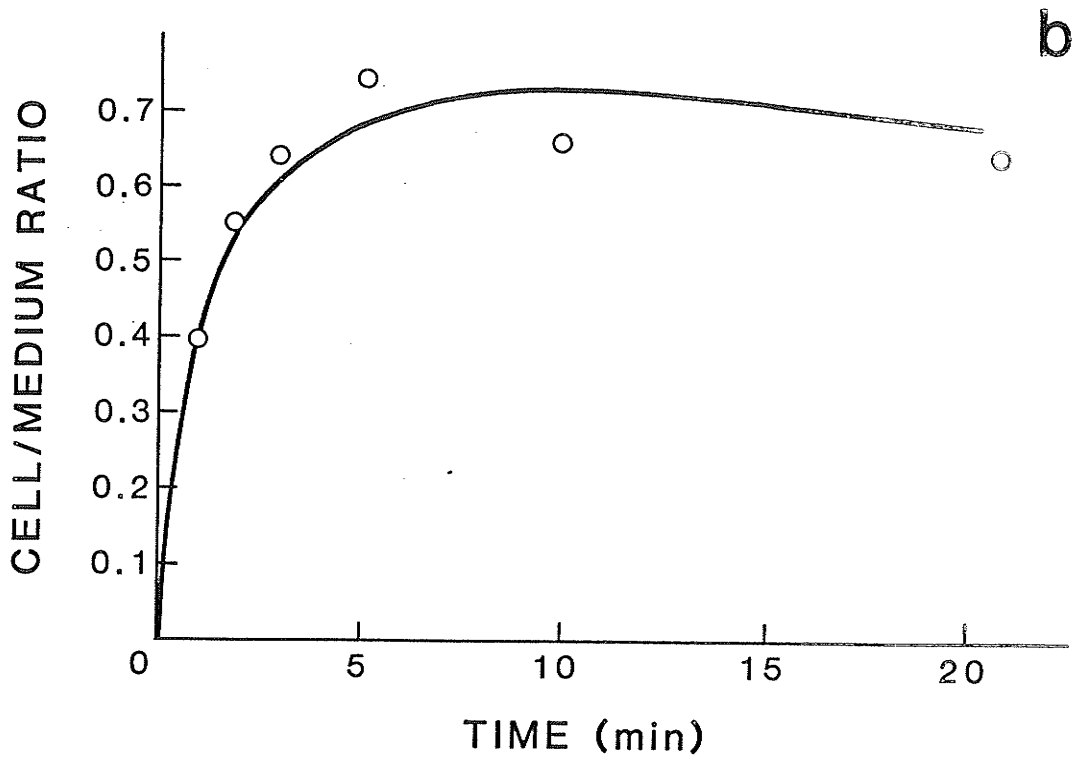

HYDROXY [14C] UREA (pmoles/10^6 cells·min)
2600
2200
180
1400
1000
600
200
0
.4
.8
1.2
1.6
2.0
HYDROXYUREA (mM)
120000
100000
80000
60000
40000
20000
0
50
100
Drug (mM)
(pmoles/10^6 cells·min)

Figure 52

Cell/medium distribution ratio of hydroxy[^{14}C]-urea for WI-38 cells. Cells were incubated in the presence of 75 uM (a) or 100 mM (b) hydroxy[^{14}C]-urea for various times, after which the amount of labelled hydroxyurea incorporated into the cells was determined, and cell/medium ratios were calculated as described in Materials and Methods.


a
CELL/MEDIUM RATIO
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
5
10
15
20
TIME (min)


b
CELL/MEDIUM RATIO
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
5
10
15
20
TIME (min)

Temperature coeffecient (Q_{10}) values for drug uptake into WI-38 cells are shown in Table 14. A Q_{10} value of close to 1 is characteristic for simple diffusion processes. However, values of 2 or more can be observed for uptake of solutes by cells, because diffusion from an aqueous environment into a nonpolar barrier most likely involves both the breaking of hydrogen bonds and thermal movement of the constituents of the barrier itself (Christensen, 1975). The results presented in Table 14 show Q_{10} values of less than 2 for drug uptake.

In order to determine whether the uptake of hydroxyurea in normal human diploid fibroblasts was dependant upon an energy source, further uptake studies were carried out in the presence of known energy inhibitors. Cells were either preincubated with an uncoupler of oxidative phosphorylation, dinitrophenol, or an inhibitor of glycolysis, sodium fluoride, at concentrations similar to those used in other studies to investigate the effects of metabolic inhibitors on drug uptake (Goldenberg et al., 1977). Table 15 shows the results of such studies. No significant decrease was observed in the ability of the cells to take up hydroxyurea in the presence of these inhibitors. Furthermore, no effect on hydroxyurea uptake was observed when cells were pretreated with both inhibitors, sodium fluoride and dinitrophenol, simultaneously. These results tend to rule out an energy requirement for hydroxyurea permeation in these cell lines.

Table 14. Temperature Coefficient (Q_{10}) for hydroxy[^{14}C]-urea uptake

Temperature (°C)	Hydroxyurea (pmol/10^6 cells)	Q_{10}
37	67.2	1.85 (37:27)
27	36.4	1.46 (27:17)
17	25.0	1.47 (17:7)
7	17.0	

NOTE: WI-38 cells were incubated at the above temperatures for 1 min in the presence of 75 μM hydroxy[^{14}C]-urea. Values shown represent the results of a typical experiment performed in duplicate. Three independent experiments were performed and, although some variation occured, Q_{10} values were consistently less than 2. Numbers in parentheses indicate the two temperatures for which Q_{10} values were calculated.

Table 15. Hydroxy[^{14}C]-urea uptake in W1-38 cells in the presence of energy inhibitors

Time (min)	Hydroxyurea (pmol/10^6 cells)			
	No drug added	+ 100 μM DNP	+ 1 mM NaF	+ 100 μM DNP + 1 mM NaF
1	101	95 (94)*	84 (83)	104 (103)
2	124	103 (83)	107 (86)	144 (116)

NOTE: The concentration of hydroxy[^{14}C]-urea in the above studies was 100 μM.

*Numbers in parentheses represent percent uptake relative to uptake in the absence of drug.

DISCUSSION

DISCUSSION

1. MOLECULAR CHARACTERIZATION OF HYDROXYUREA RESISTANT CHO CELL LINES

Mutants of mammalian cells grown in culture have been useful in studying not only the mode of action of specific drugs, but also have enhanced our understanding of the mechanisms involved in drug resistance. Mechanisms for achieving drug resistance can broadly be divided into three categories. Firstly, cells may become resistant to the cytotoxic effects of various drugs by overproducing the target protein(s) at which the drug acts, be it by gene amplification (Stark and Wahl, 1984; Schimke, 1984a,b; Stark, 1986; Wright et al., 1988) increased rates of transcription (McClarty et al., 1987a), increased rates of translation (McConlogue et al., 1986), or increases in the half life of messenger RNA or protein (McClarty et al., 1988). Secondly, the cells may be able to produce an altered protein which is less sensitive to the inhibitory effects of the drug (Flintoff et al., 1976a,b; Haber et al., 1981; Wright, 1983,1988; Bartolomei and Corden, 1987), and finally the cell may exhibit resistance to drugs by altering the transport properties of the drug accross the cell membrane (Sirotnak et al., 1981; Flintoff et al.,1976a,b). Studies on various mammalian mutants have shown that more than one of the above mechanisms may be at work in any one drug resistant cell line (e.g. Choy et al., 1988; Tagger and Wright, 1988).

It was the first objective of this study to characterize the mechanism of resistance of two variant CHO cell lines to hydroxyurea.

Hydroxyurea inhibits DNA synthesis through ribonucleotide reductase, by modifying and depleting deoxyribonucleotide concentrations essential for DNA polymerase activity and DNA synthesis (Nicander and Reichard, 1985; Moore and Hulbert, 1985; Young and Hodas, 1964; Turner et al., 1966; Krakoff et al., 1968; Skoog and Nordenskjold, 1971; Akerblom et al, 1981). The data shown in part 1.2 of the Results section of this thesis illustrate that both low and relatively high hydroxyurea resistance can occur through elevations in ribonucleotide reductase activity, which results from a specific increase in the M2 component that is usually limiting for enzyme activity (Eriksson et al. 1984), and is the precise site of action for hydroxyurea (Graslund et al., 1982; McClarty et al., 1987a). The results obtained from kinetic analysis of ribonucleotide reductase activity in partially purified preparations from both the H^R-R2T and HN^R-AT cell lines indicated that enzyme activity was increased in each mutant line by about twofold as compared to enzyme preparations from the wild type parental cell line. The EPR studies revealed that the tyrosyl free radical signal, which can be used as a quantitative measure of functional M2 components within the cells (Graslund et al., 1982; Eriksson et al., 1984; Wright et al., 1987; McClarty et al., 1987a; Choy et al., 1988; Tagger and Wright, 1988) is increased about 5 fold in the H^R-R2T cell line and about 2 fold in the HN^R-AT cell line compared to the wild type cells. In addition, Northern blot analysis revealed that the M2 mRNA is increased by roughly the same amount as the EPR signal in each of the two mutant cell lines. On the other hand, the M1 protein concentration was not elevated in either of the mutant cell lines since equivalent amounts of M1 protein was immuno-

precipitated from both the mutants and the wild type cells with anti-M1 antiserum following labelling of the cells with ^{35}S-methionine. Consistent with these findings is the observation that M1 mRNA and M1 gene copy number are not increased in either drug resistant cell line compared to the wild type cell line. Therefore, it is concluded that one mechanism whereby the hydroxyurea resistance phenotype is achieved in both mutant cell lines is through elevation of M2 message and protein levels. The modest but reproducible elevation in ribonucleotide reductase activity observed in partially purified enzyme preparations from the two mutant cell lines appears then to be entirely due to an increase in M2 protein. It is likely that the activity of the enzyme did not increase proportionately with the concentration of M2 components for the H^R-R2T cell line as a result of limiting M1 subunits which were maintained at wild type levels in this mutant. These observations are consistent with those showing that hydroxyurea inhibits ribonucleotide reductase by interacting with the M2 component of the enzyme only (Graslund, et al., 1982; Thelander et al., 1985; McClarty et al., 1987a), and with many previous studies showing that drug resistance can be achieved through elevation of the target protein (Stark and Wahl, 1984; Schimke, 1984a,b; Stark, 1986; Wright et al., 1988). Interestingly, it has been observed recently that overproduction of the M1 subunit as well as the M2 subunit can occur in cell lines selected for very high levels of resistance to hydroxyurea (Choy et al., 1988; McClarty et al., 1987; Cocking et al., 1987). In such instances the mutants studied were much more resistant to hydroxyurea than those described here. Presumably, in such cases, the M1 subunit becomes the limiting

subunit for enzyme activity when the cells are cultured in the presence of hydroxyurea, and in order for the cells to proliferate, elevated levels of M1 are required (Wright et al., 1988).

In this context, it is interesting to point out that M1 and M2 protein levels are not coordinately regulated throughout the cell cycle in mammalian cells (Engstrom et al., 1985; Eriksson and Martin, 1981; Cory and Fleischer, 1982b; Rubin and Cory, 1986). Although it is generally agreed that the two subunits of ribonucleotide reductase do not increase in a coordinate fashion, recombination experiments with partially purified enzyme components to determine which subunit is limiting for enzyme acitvity have yielded conflicting conclusions (Eriksson and Martin, 1981; Cory and Feischer, 1982b). Moreover, studies to determine the half life of protein M1 and protein M2 have also yielded conflicting results. In particular, studies on mouse leukemia L1210 cells indicated that the M1 subunit has a shorter half life than the M2 component, and that it is the M1 subunit which is the limiting subunit for enzyme activity in those cells (Rubin and Cory, 1986). On the other hand, Thelander's group using a tyrosine isotope labelling technique, elegantly showed that the S phase-correlated increase in ribonucleotide reductase activity in mouse mammary tumor TA 3 and bovine kidney MDBK cells is solely attributed to the de novo synthesis of protein M2 (Eriksson et al., 1984; Engstrom et al., 1985), which is also the limiting subunit for enzyme activity in these cells (Engstrom et al., 1985). In addition, they showed using, M1 specific antibodies, that the M1 subunit, in contrast to the M2 subunit, remains at relatively the same level throughout the cell

cycle, having a half life of about 15 hr, approximately 3 to 5 fold greater than that of the M2 subunit (Engstrom et al., 1985). Pulse chase experiments with hydroxyurea resistant mouse L cells and M2 specific monoclonal antibodies (Choy et al., 1988; McClarty et al., 1988) showed that there is a significant increase in newly synthesized M2 protein at S phase with a half life of 3 to 7 hr supporting the observations of the Swedish group. In any case, the expression of the two subunits of ribonucleotide reductase in mammalian cells is in sharp contrast to the situation in Escherichia coli where the two equivalent genes are found in one operon, and their expression is coordinately regulated (Carlson et al., 1984). The observation that only the M2 component of the enzyme is elevated in both the H^R-R2T and HN^R-AT hydroxyurea resistant cell lines are in keeping with the investigations discussed above, which show that M1 and M2 levels are controlled by different mechanisms in mammalian cells, and with studies showing that the genes for M1 and M2 are located on different chromosomes in hamster cells (Tonin et al., 1987).

Interestingly, the synthesis of ribonucleotide reductase in E. coli is controlled at the level of transcription (Hanke and Fuchs, 1983a). The bacterial genes nrdA and nrdB direct the synthesis of a 3.2 Kb polycistronic mRNA (Hanke and Fuchs, 1983b), encoding both the B1 and B2 subunits of the enzyme (Carlson et al., 1984). Whether the S phase-correlated increase in ribonucleotide reductase activity and protein M2 observed in TA 3, MDBK, and mouse L cells is controlled at the level of transcription as well is not presently known. However, it has been established that in clam oocytes where the M1 subunit is

stored as a polypeptide, the increase in ribonucleotide reductase activity following fertilization is due to the translation of prestored M2 mRNA and thus at least in this system, the control of M2 protein synthesis and ribonucleotide reducatse activity is at the level of translation (Standart et al., 1986). In addition, it has recently been observed that in mouse L cells which exhibit a stable resistance to very high concentrations of hydroxyurea, that expression of the M1 and M2 proteins can be controlled post-transcriptionally when the cells are incubated in the presence of hydroxyurea (McClarty et al., 1988). Further work is required in order to elucidate the mechanisms involved in the normal cell cycle regulation of the expression of the components of ribonucleotide reductase.

Although both drug resistant cell lines H^R-R2T, and HN^R-AT exhibited elevations in the M2 component, they accomplished this by different mechanisms. The most resistant line, H^R-R2T, contained increased copies of the M2 gene (about 5 fold), which adequately explained the elevation in M2 message and M2 protein. However, the low resistant, HN^R-AT line, utilized a process other than gene amplification. The relatively low increase of M2 message in this mutant compared to the wild type cell line makes it very difficult to determine the mechanism by which the message is increased in this mutant, however, several possibilities exist, such as an increased efficiency of M2 mRNA transcription or processing, or increased mRNA stability. Interestingly, observations made on human KB cells exhibiting low levels of resistance to colchicine also revealed the presence of increased levels of mdr1 (multidrug-resistance) mRNA

without concomitment amplification of the corresponding gene (Shen et al., 1986a). It is worth noting that there are yet other examples of variant cell lines that overproduce mRNA for aryl hydrocarbon hydroxylase (Jones et al., 1984), dihydrofolate reductase (Dedhar et al., 1984), arginosuccinate synthetase (Su et al., 1981), and ornithine decarboxylase (McConlogue et al., 1986), which do not exhibit amplification of the corresponding genes. In addition, more recently it has been shown that in a highly hydroxyurea resistant mutant which overproduces ribonucleotide reductase activity about 40 fold, there is only approximately a 6 fold increase in M2 gene copy number (McClarty et al., 1987a). This mutant cell line was shown to have increased transcriptional rates at the M2 locus compared to its parental hydroxyurea sensitive wild type cell line. In any event, the increase in M2 message observed for the HN^R-AT mutant line can fully account for the increase in the tyrosyl free radical concentration and the increase in enzyme activity in partiallly purified preparations of ribonucleotide reductase from this cell line.

The possibility that the hydroxyurea resistant phenotype of the two mutant cell lines studied here can be a result not only of altered expression of the protein as discussed above, but due to alterations in the protein itself was investigated. In order to obtain valid kinetic parameters for ribonucleotide reductase activity in the mutant and wild type cell lines it was essential to partially purify the enzyme from cell extracts from the various cell lines. Homogeneous enzyme preparations of mammalian ribonucleotide reductase are very difficult to obtain (Engstrom et al., 1979; Follmann, 1974) due to

the relatively low amounts of enzyme found in normal cells. Homogeneous preparations of the M1 subunit have been obtained from calf thymus (Thelander et al., 1980; Mattaliano et al., 1981) and lamb thymus (McClarty et al., 1986a), sources which are readily available in large quantities and which contain large amounts of dividing cells. The purification of the M2 component of the enzyme has lagged behind that of the M1 subunit. Perhaps this is due to the fact, as pointed out previously, that the M2 component, in contrast to the M1 component of the enzyme, is believed to be present only during the S phase of the cell cycle. The first demonstration of a homogeneous preparation for the M2 subunit was by Thelander's group (Thelander et al., 1985) who successfully purified the M2 component from hydroxyurea resistant mouse mammary TA 3 cells which overproduce enzyme activity by about 50 fold (Eriksson et al., 1984; Thelander et al., 1985). To date, purified active M1 and M2 components have not been purified to homogeneity from CHO cells. Indeed, the difficulty in obtaining purified M1 and M2 components from most mammalian sources, and in particular, from cells grown in tissue culture, has necessitated the analysis of enzyme activity in preparations which have only been partiallly purified. In order to analyze and compare ribonucleotide reductase activity from the mutant and wild type cell lines studied here, it was considered impractical at the present time to purify the enzyme to homogeneity from each of the cell lines (due to the very large quantities of cells which would be required), however it was desirable to partially purify the enzyme in order to remove contaminating enzyme activities which would interfere with the ribonucleotide reductase enzyme assay. As was demonstrated in the Results

section of this thesis, the major contaminating enzyme activity in cell extracts from the mutant and wild type cell lines was nucleoside diphosphate kinase activity which interfered with the ribonucleotide reductase enzyme assay by depleting the reaction mixture of the substrate ^{14}C-CDP and converting it to CTP, thus altering the concentration of the ^{14}C-CDP substrate which was used to measure and analyze ribonucleotide reductase activity. The ammonium sulfate precipitation step, which partially increased the specific activity of the reductase, and aided in concentrating the enzyme sample, did not result in the removal of nucleoside diphosphate kinase activity. The removal of the kinase activity was however, successfully achieved after passing the ammonium sulfate precipitated extract over an ATP-agarose column precisely as described by Spector (1985) and Spector and Averret (1983). The resulting enzyme preparation was substantially reduced in nucleloside diphosphate kinase activity; in fact, at least 90% of the ^{14}C-CDP substrate remained intact following a standard ribonucleotide reductase assay. The elution of the enzyme from the ATP-agarose column was carried out with a high salt (0.5 M KCl) buffer, conditions which favor the co-elution of both the M1 and M2 subunits (Spector, 1985).

In order to analyze and compare the kinetics of ribonucleotide reduction in the wild type and mutant cell lines it was essential to determine the optimum concentrations of effectors and cofactors required for enzyme activity. Enzyme preparations from all three cell lines showed similar patterns with respect to optimum concentrations of the various components of the assay mixture. Enzyme activity was

strictly dependent on the presence of ATP, which serves as a positive allosteric regulator of CDP reduction (Eriksson et al., 1979; Moore and Hulbert, 1966; Murphree et al., 1968), for which the optimum concentration was determined to be about 5 mM for all three CHO cell lines. The reducing power for the reaction was provided by DTT for which the optimum concentration was found to be above 2 mM. $MgCl_2$, which enhances mammalian ribonucleotide reductase activity (Engstrom et al., 1979), was found to yield optimum ribonucleotide reductase activity in the assay at a concentration of about 6 mM. The optimum pH and temperature for the assay were found to be very close to physiological conditions as would be expected. The optimum conditions for assaying CDP reduction in the partially purified preparations of the wild type and mutant CHO cell lines, are very similar to those reported from a variety of mammalian sources such as regenerating rat liver (Larsson, 1969), calf thymus (Eriksson et al., 1979), mouse L cells (Kuzik and Wright, 1979) and Molt-4F human lymphoblast cells (Chang and Cheng, 1979a). Interestingly, the reduction of CDP in the partially purified enzyme preparations from the wild type and mutant cell lines was linear with respect to the amount of protein assayed only when 40 ug protein or greater was assayed. The non-linearity of the reaction observed at low protein concentrations has been observed by others (Hopper, 1972; Peterson and Moore, 1976; Kuzik and Wright, 1979; Lewis and Wright, 1978a, 1979; Eriksson and Martin, 1981). The enzyme reaction from all three cell lines studied proceeded linearly for at least 60 minutes.

The enzyme preparation from all three cell lines exhibited

classical Michaelis-Menten kinetics, and analysis of Lineweaver-Burk double reciprocal plots revealed no significant differences in Km values for CDP reduction when either mutant cell line was compared to the wild type cell line. These findings indicate that CDP binding to the active site of the enzyme is not altered in the mutant cell lines. The Km values for CDP reduction determined for the three CHO cell lines, which ranged from 2.57 to 3.34 μM CDP, were slightly lower than that determined for ribonucleotide reductase from Molt-4F cells (Chang and Cheng, 1979b), and approximatly 10 fold lower than that determined for calf thymus (Eriksson et al., 1979) or Ehrlich tumor (Cory, 1979) ribonucleotide reductases. It is of interest to point out that the lowest reported Km value for CDP reduction was obtained from the herpes simplex type 1 (HSV 1) virus-induced enzyme for which the apparent Km was determined to be 0.65 μM (Averett et al., 1983). For this determination, great care was taken to remove contaminating enzyme activities from the ribonucleotide reductase preparation, in particular, an ATP-agarose column of the same type used in the partial purification of ribonucleotide reductase from the CHO cells described in this thesis was used in order to separate the HSV 1 induced-enzyme from nucleoside diphosphate kinase activity.

A detailed kinetic analysis of hydroxyurea inhibition of enzyme activity in the partially purified preparations revealed some differences in the Ki values between both mutant cell lines and the wild type cells. The difference was most pronounced for the H^R-R2T cell line which showed about a twofold increase in Ki values. Somewhat surprisingly, an increase in Ki values were also observed for dATP

inhibition in the mutant lines as compared to the wild type cells. Since dATP acts as an overall regulator of ribonucleotide reductase by binding allosterically to the M1 protein, and inhibiting the reduction of all four ribonucleoside diphosphate substrates (Thelander and Reichard, 1979; Wright, 1983, 1988), it is tempting to speculate that the increase in Ki for dATP inhibition is biologically important in order to maintain a supply of deoxyribonucleoside triphosphates available for DNA synthesis when the cells are grown in the presence of hydroxyurea. Under such conditions it is possible that inactivated M2 components are still capable of binding functional M1 proteins thus forming an inactive complex, and a decrease in available M1 components to bind functional M2 proteins. An increase in the Ki for dATP inhibition would serve the purpose of partially offsetting this apparent decrease in available M1 subunits by allowing a functional holoenzyme to increase its production of deoxyribonucleotides to a level suitable for DNA replication. In this context, it is interesting to note that the H^R-R2T cell line, which was the more resistant of the two mutant lines (and hence was able to form colonies at higher drug concentrations), also showed the greatest increase in Ki values for dATP (an increase of approximately fourfold) compared to the wild type cells.

Therefore, in addition to an increase in the M2 component of ribonucleotide reductase, and overall ribonucleotide reductase activity in the mutant cell lines, the inhibitory effects of hydroxyurea and dATP on the partially purified enzyme from both mutant cell lines appeared to be less than that observed with the wild type cells.

It is difficult, without homogeneous preparations of both the M1 and the M2 subunits of the enzyme, to prove that the differences in Ki values obtained for hydroxyurea and dATP inhibition between the mutant and wild type cell lines reflect actual changes in the ribonucleotide reductase protein itself. As previously mentioned in the Results section of this thesis, it is possible that the differences observed may be due to other activities in the enzyme preparations used in this study. However, it must also be noted that (i) changes in the kinetic properties of ribonucleotide reductase in hydroxyurea resistant cells, similar to those described here, have been observed by others (Wright, 1983; 1988), (ii) enzyme was prepared from the H^R-R2T, HN^R-AT and wild type cells using identical procedures, and (iii) the enzyme preparation from all three cell lines responded in a similar manner to the various cofactors and other effectors in the assay mixture.

Taken together, both the increase in M2 subunits and the differences observed in Ki values for hydroxyurea and dATP inhibition between the mutants and the parental line can fully account for the drug resistant phenotype of the mutant lines. The possibility that alterations in the uptake of hydroxyurea plays a role in mediating the cellular resistance of the mutants to the drug was ruled out in drug uptake studies using hydroxy[^{14}C]-urea. No decrease in hydroxyurea uptake was observed with either H^R-R2T and HN^R-AT cell lines as compared to wild type cells. Therefore, unlike some mutants isolated for resistance to methotrexate (Sirotnak et al., 1981; Flintoff et al., 1976a,b), a change in the transport properties of hydroxyurea across the plasma membrane does not appear to play a role in the

cellular resistance to hydroxyurea of the CHO mutants described in this thesis. It is interesting to note that to date, no hydroxyurea resistant mammalian cell mutants have been reported which show decreased uptake of hydroxyurea into the cell. This observation may not be surprising since it is believed that hydroxyurea uptake in mammalian cells occurs by a process of simple diffusion (Morgan et al., 1986; Tagger et al., 1987), for which no protein carriers are involved.

In summary, these studies have revealed molecular mechanisms responsible for achieving cellular resistance to low and relatively high levels of the antitumor agent, hydroxyurea, and have stressed the importance of alterations affecting the expression of the M2 protein in the developement of a hydroxyurea resistant phenotype.

The results showing that the H^R-R2T cell line has elevated levels of the M2 component of ribonucleotide reductase predicts that this cell line should be cross resistant to other drugs whose site of action is at the M2 component. Indeed, this cell line showed about a 7 fold increase in cellular resistance to guanazole and a modest increase in resistance to MAIQ, both of which inhibit ribonucleotide reductase by interacting with the M2 component (Engstrom et al., 1979; Wright et al., 1983; 1988; Cory and Carter, 1988). The lower resistance of H^R-R2T cells to MAIQ suggests that this drug inactivates the M2 component by a different mechanism than hydroxyurea and guanazole, and may also affect other sites besides ribonucleotide reductase.

The ability of cells to acquire a multidrug-resistant phenotype has become a major concern over the past few years in the treatment of human cancer (Croop et al., 1988). Cultured mammalian cell lines have provided a model system to study this problem since cell lines, although selected for resistance to a single agent, can also exhibit resistance to a wide range of chemically and structurally unrelated drugs such as colchicine, vinblastine, doxorubicin hydrochloride and actinomycin D (Beck et al., 1974; Biedler and Peterson, 1981; Ling, 1985) . Other characteristics of cultured cells exhibiting the multidrug resistant phenotype include, a decreased accumulation of these drugs due to an energy-dependent increase in drug efflux (Inaba et al., 1979; Fojo et al., 1985; Willingham et al., 1986), increased amounts of a membrane glycoprotein of MW 170,000 daltons (P-glycoprotein) (Kartner et al., 1983; Shen et al., 1986b; Ueda et al., 1986), and overexpression and amplification of the mdr 1 gene which codes for the P-glycoprotein apparently responsible for the MDR phenotype (Shen et al., 1986a; Riordan et al., 1985; Scotto et al., 1986; Van der Bliek et al., 1986). Since hydroxyurea is used in the treatment of some cancers, it was of interest to test the most resistant hydroxyurea resistant CHO cell line studied here, H^R-R2T, for resistance to a variety of structurally unrelated drugs in order to determine if selection for hydroxyurea resistant cells also results in the acquisition of a multidrug resistant phenotype. The results showed that the H^R-R2T cell line does not exhibit increased resistance to either puromycin or colchicine compared to the wild type cell line, indicating that multidrug resistance is not associated with resistance to hydroxyurea in this cell line.

2. BIOLOGICAL PROPERTIES OF HYDROXYUREA RESISTANT CHO CELL LINES

The idea that cell lines exhibiting modifications in ribonucleotide reductase may also show changes in tumorigenicity comes from studies showing a link between these two properties in at least some cell systems (for reviews see Weber, 1983; Wright et al., 1988). A remarkable correlation between the growth rates of a series of Morris hepatomas and ribonucleotide reductase activity was observed (Elford et al., 1970); in all cases examined an increase in the tumor growth rate was accompanied by an increase in ribonucleotide reductase enzyme activity. Additional studies confirmed these observations in rat hepatomas and extended the findings to include renal cell carcinoma and sarcomas in rat, where increases in ribonucleotide reductase activity of about 30 and 60 fold respectively were observed in extracts prepared from the tumor cells as compared to activity found in extracts of normal tissue (Takeda and Weber, 1981). An important contribution in the latter study was that ribonucleotide reductase activity was detected and measured in normal liver tissue thus allowing the comparison between ribonucleotide reductase activity in the tumors of varying growth rates to the activity found in normal liver tissue. The findings that all the hepatomas studied showed increased ribonucleotide reductase activity compared to normal liver tissue, and the activity of the enzyme was augmented in tumors of increased growth rates, indicated that the activity of ribonucleotide reductase is both transformation- and progression- linked according to the classifications postulated by the molecular correlation concept as outlined by Weber (Weber, 1961, 1983). Interestingly, when enzyme

activity from a rapidly growing hepatoma was compared to the activity found in normal liver tissue with the same replicative rate, such as differentiating or regenerating liver, it was found that the tumors had higher enzyme activity, indicating that the rise in ribonucleotide reductase activity might be a specific manifestation of the neoplastic state. To re-examine this proposal of a link between tumor growth rate and ribonucleotide reductase activity, it was of interest to study the tumorigenic properties of the two hydroxyurea resistant cell lines which overproduce ribonucleotide reductase activity. Interestingly, both H^R-R2T and HN^R-AT populations exhibited increased tumor growth rates in vivo compared to the wild type population. In addition, the H^R-R2T cells behaved significantly different from wild type or HN^R-AT cells in the spontaneous metastasis assay.

Although these results are consistent with the view that alterations in ribonucleotide reductase accompany tumorigenic and metastatic properties of cells, the results must be interpreted with caution (Wright et al., 1988). Although ribonucleotide reductase is the main site of action for hydroxyurea, and the drug resistant characteristics of the lines have remained stable for several years of continuous cell culture, one can not rule out the possibility that the original treatment with hydroxyurea or N-carbamoyloxyurea, in the selection of H^R-R2T or HN^R-AT cell lines may have affected other cellular activities in addition to ribonucleotide reductase (Platt et al., 1984; Frost et al., 1987; Wright et al., 1988). In a study conducted recently it was observed that brief treatment of nonmetastatic mouse mammary adenocarcinoma cells with hydroxyurea resulted in

the formation of secondary lung metastasis in mice which had been injected subcutaneously in contrast to nontreated cells for which no secondary metastasis occurred (Frost et al., 1987). In an additional study (McMillan et al., 1986), three murine melanoma cell lines treated with hydroxyurea concentrations, which markedly reduce long term survival of the cells, enhanced the ability of the tumor cells to form experimental lung metastasis following iv injection. Interestingly, the effect was most pronounced after the cells were allowed to recover for 6 hr in the absence of drug prior to injection. A 24 hr recovery period reduced the amount of lung metastasis formed. Such studies raise questions regarding the treatment of cancer patients with anticancer therapy such as hydroxyurea administration which may in fact lead to the facilitation of tumor progression. At any rate, the mechanism of enhancement of lung metastasis following brief exposure of cells to hydroxyurea is unclear at present, but may in fact be due to the transient inhibition of DNA synthesis caused by the drug, which may facilitate the process of gene amplification (Brown et al., 1983; Mariani and Schimke, 1984; Hoy et al., 1987). The amplification of specific genes has been observed in many neoplasms and it has been demonstrated that increased copies of specific genes in cells can result in increased metastatic potential (Schimke, 1984a,b; Egan et al., 1987a,b). Although one can not rule out the possibility that such a mechanism had occured in the H^R-R2T mutant cell line, during drug selection, a molecular approach to test the relationship between increased ribonucleotide reductase activity and tumorigenic characteristics would be worthwhile. For example, it may be possible to directly isolate cell lines expressing the altered M2 properties of

the H^R-R2T population in transfection experiments using full-length M2 expressing cDNA linked to a strong promoter. In addition, it should be noted that it is unlikely that the brief exposure of cells to hydroxyurea in the aforementioned studies (McMillan et al., 1986; Frost et al., 1987) resulted in enhancement of lung metastasis due to the selection of mutants with altered ribonucleotide reductase activity, since the exposure of the cells to the drug was very short. However, since recent observations have shown that at least in some cell systems, hydroxyurea can modulate the expression of ribonucleotide reductase activity by post-transcriptional mechanisms (McClarty et al., 1988) one can not entirely rule out the possibility that the effect of hydroxyurea exposure on metastasis formation is not due in part to changes in the expression of ribonucleotide reductase. Nevertheless, the altered molecular and cellular properties of H^R-R2T and HN^R-AT cell lines have made them interesting biological tools for further investigations, and have provided additional support for the involvement of ribonucleotide reductase in the progression of tumors to an enhanced malignant phenotype. In addition, as discussed below, the properties of the two mutant cell lines have shed light on an important tumor biology question, namely, they have provided further information on the relationship between altered spontaneous mutation rates and tumorigenic characteristics of cells.

Many studies have revealed that certain types of drug resistant mammalian cell lines exhibit elevated rates of spontaneous mutation. In cases where the biochemical lesion responsible for drug resistance was identified it was found that alterations in enzymes directly

involved in DNA synthesis such as DNA polymerase alpha (Liu et al., 1983), or enzymes involved in DNA precursor metabolism such as ribonucleotide reductase (Weinberg et al., 1981; Ayusawa et al., 1983) or CTP synthetase (Trudel et al. 1984) had occured. These types of alterations probably reduce the fidelity of DNA replication by changes in substrate selectivity of the polymerizing enzyme, or by alterations in the regulation of enzymes providing the precursors of DNA synthesis. In the latter case, it is believed that the enzyme alterations result in deoxyribonucleotide pool imbalances that affect the fidelity of DNA replication. That pertubations in these pools can reduce the accuracy of replication and cause increases in spontaneous mutation rates has been demonstrated in vivo by pharmacological manipulation of precursor pools, through the addition of various deoxyribonucleosides to the culture medium (Weinberg et al., 1981; Meuth et al., 1979; Aizawa et al., 1985).

Several interesting observations were made concerning the relationship between deoxyribonucleotide pools and spontaneous mutation rates. For example, the H^R-R2T cell line did not show significant alterations in deoxyribonucleotide pool sizes, and in this regard was clearly different from other hydroxyurea resistant lines that have shown abnormalities in these pools when examined (Akerblom et al., 1981; Creasy and Wright, 1984; Dick and Wright, 1984; Albert et al., 1987). These results show for the first time that an increase in the M2 subunit of ribonucleotide reductase, and an increase in enzyme activity, as is observed in the H^R-R2T cells, does not necessarily lead to changes in deoxyribonucleotide pool sizes,

suggesting that there must be a mechanism working to maintain wild type pool levels in this mutant cell line. The finding that the spontaneous mutation rates for 6-thioguanine and ouabain resistance were not significantly different when H^R-R2T and wild type cells were compared is consistent with the deoxyribonucleotide pool analysis, and indicates that an excess of M2 subunit of ribonucleotide reductase is not necessarily mutagenic to cells.

It should be noted that the mutation rates which have been determined from the fluctuation experiments for the wild type CHO cells, used in this study, are very close to the mutation rates reported for 6-thioguanine resistance (Meuth et al., 1979; Caskey and Kruh, 1979) and ouabain resistance (Meuth et al., 1979; Baker et al., 1974) in other CHO cell lines. 6-thioguanine resistance and ouabain resistance are believed to occur through point mutations or deletions in the hypoxanthine-guanine phosphoribosyl transferase (hgprt) and Na^+/K^+ ATPase genes respectively (Ling, 1982; Baker et al., 1974). The high variance observed is in keeping with the random appearance of drug resistant cells harboring mutations as described in the classic study by Luria and Delbruck (1943).

Measurements of the four deoxyribonucleotide pools in the HN^R-AT line revealed gross alterations in both dCTP and dGTP concentrations. Although a likely candidate, there is no evidence to support the view that the altered pool sizes observed in this line are a direct result of alterations in ribonucleotide reductase activity. Since the HN^R-AT cell line was selected for resistance to N-carbamoyloxyurea, which

like hydroxyurea appears to inhibit ribonucleotide reductase by scavenging the tyrosyl free radical in the M2 subunit of the enzyme, other alterations might have occurred in this line as well because there is evidence to suggest additional sites of action of N-carbamoyloxyurea (Hards and Wright, 1981).

As compared to the wild type population, the HN^R-AT cell line showed no alteration in the rate of spontaneous mutation to 6-thioguanine resistance, but the rate of mutation to ouabain resistance was significantly decreased by about 5 fold. This observation is interesting since not only did the mutation rates to the two markers behave differently, but the rate in one case actually decreased. Other examples of cases where a cell line showed altered mutation rates to one marker but not to another are known (e.g. Meuth et al., 1979; Arpaia et al., 1983; Drobetsky and Meuth, 1983). In one study, CHO cells auxotrophic for proline that were selected for resistance to arabinosylcytosine exhibited elevated levels of dCTP due to a mutation in CTP synthetase. The cells showed an increase in the rate of spontaneous mutation to 6-thioguanine and ouabain resistance, however, the rate of spontaneous mutation to emetine resistance and reversion to proline prototrophy were not significantly different from the wild type parental cell line (Meuth et al., 1979). In another study, CHO cell mutants resistant to the drug 6(p-hydroxyphenylazo)-uracil exhibited alterations in ribonucleotide reductase activity and showed changes in dCTP and TTP pool sizes. This mutant, while having increased rates of spontaneous mutation to 6-thioguanine resistance, did not show a change in the rate of spontaneous mutation to emetine

resistance as compared to the wild type cell line. In addition, Drobetsky and Meuth (1983) isolated a series of CHO cells having mutator phenotypes by exposing the cells to prolonged serial cultivation in cytotoxic drugs. Although the exact biochemical lesions in these cells are unknown, the rates of spontaneous mutation to ouabain resistance for two of the mutants isolated were increased, whereas the rate of spontaneous mutation to 6-thioguanine resistance in these mutants was not different from that determined for the wild type cells. Thus it has not been uncommon to observe changes in spontaneous mutation rates to some markers and yet not to others in various types of mutant cell lines. The reason why mutation rates at some loci are altered but not at others in mutant cell lines is not entirely understood, but is probably dependent upon several different factors, including the specific type of mutations required to achieve resistance, the precise DNA sites at which the mutations occur, and the type of deoxyribonucleotide pool imbalances that are produced (Phear et al., 1987).

Unique to the HN^R-AT cell line is the observation that it appears to be the first drug resistant cell line to show an actual decrease in mutation rate, probably as a consequence of altered deoxyribonucleotide pool levels. A decrease in the rate of spontaneous mutation to ouabain resistance may be interpreted to mean that mutations are occurring in HN^R-AT cells either at the Na^+/K^+ locus or at another locus affecting ouabain resistance (Schulz and Cantley, 1988), at a greater rate than those occurring in wild type cells, but the mutations are occuring in the reverse direction; i.e. mutations

leading to increased sensitivity as opposed to resistance to ouabain. Such mutations may affect either the expression of a gene product or result in structural alterations in a gene product which render the cell hypersensitive to ouabain. In this context it is worthwhile reviewing the recent work of Phear et al., (1987) who report the types of mutations induced by dNTP pool imbalances at the adenosine phosphoribosyl transferase locus in CHO cells. In most of the mutants studied, base pair substitutions had occurred and it was concluded that the local sequence context will affect the position at which base pair substitutions occur; in particular, the nucleotide in excess was present 3' to the misincorporated nucleotide in the majority of transitions which were characterized. Next-nucleotide effects seem, therefore, to play a critical role in determining the sites at which mutations caused by specific dNTP imbalances occur, and hence the observation that the rate of mutation to ouabain resistance in HN^R-AT cells had decreased might be explained in terms of the specific nucleotide sequence of either the Na^+/K^+ ATPase locus or another locus affecting ouabain resistance, which may be more susceptable to base pair transitions induced by the dNTP pools in the HN^R-AT line. It is tempting to speculate that such sites, if mutated, lead to an altered gene product which cause the cell to be hypersensitive to ouabain, thereby resulting in a lower rate of spontaneous mutation to ouabain resistance. It follows that such sites are not as predominant or that mutations at them have no effect on the hgprt locus, thereby resulting in spontaneous mutation rates to 6-thioguanine resistance in HN^R-AT cells similar to that observed in wild type cells. Further studies at the DNA sequence level to determine the nature of the mutations which

are induced by the types of nucleotide pool imbalances observed with the HN^R-AT cells would be worthwhile.

Understanding the mechanisms involved in the generation of metastatic variants from the primary tumor is crucial in order to develop effective treatments for the metastatic disease. Basically, two different theories have emerged to explain the development of the malignant phenotype. One, proposed by Fidler (Fidler and Kripke, 1977; Poste and Fidler, 1980), states that metastasis is not formed from the primary tumor through random events, but rather is due to the presence of preexisting specialized cells endued with properties enabling them to execute each step along the metastatic cascade. This implies that the tumor is heterogeneous in nature, containing stable subpopulations of both metastatic and nonmetastatic cancer cells. Another view is taken by Weiss (1983) and Harris et al (1982), who both propose that the metastatic process is a random event, and have postulated similar theories termed the "transient compartment" and the "dynamic heterogeneity" models respectively. These models propose that the metastatic phenotype is expressed transiently in the primary tumor and can be rapidly lost under non-selecting conditions. Indeed, using Luria and Delbruck fluctuation analysis, Harris et al (1982) demonstrated that metastatic variants of a mouse KHT sarcoma cell line arise spontaneously during the expansion of clonal populations at a rate of about 10^{-5} per cell per generation. Still, very little is known about either the way in which tumors are able to produce altered subpopulations, or the selection pressures conferred by the host. The finding that there are multiple clonal subpopulations within tumors of

a single clonal origin suggests that cellular mechanisms function to generate genetic diversity within a tumor population (Nowell, 1976). Some studies have suggested that increased spontaneous mutation rates are an important genetic mechanism for producing variants with increased malignant and metastatic potential (e.g. Chambers et al., 1981; Harris et al., 1982; Hill et al., 1984). A key aspect of this work was the demonstration of genetic instability of tumor cells associated with the metastatic phenotype, by observing increased spontaneous mutation rates at independent genetic loci in the most aggressive variant populations. For example, Cifone and Fidler (1981) who examined spontaneous mutation rates to both 6-thioguanine and ouabain resistance in low and high metastatic mouse UV- 2237 fibrosarcoma clones, found the high metastatic clones to have a 3-7 fold increase in the rate of spontaneous mutation at both genetic loci, as compared to the low metastatic clone. Cifone and Fisher (1980) and Fidler (1978) obtained similar results in their studies using mouse SF-19 fibrosarcoma cells and mouse K-1735 melanoma cells respectively. However, there has been considerable debate about the general significance of these findings. For instance, when chemically transformed skin fibroblasts were compared to their parental normal skin fibroblasts, no differences in the rates of spontaneous mutation to 6-thioguanine resistance or ouabain resistance were detected (Elmore et al., 1983). Furthermore, the rates of spontaneous mutation showed no correlation with the degree of metastatic ability in three mouse mammary tumor cell lines (Yamashina and Heppner, 1985), and in metastatic variants of NIH 3T3 and CBA SP-1 cells (Frost et al., 1987). The observations concerning the spontaneous mutation rates to

6-thioguanine and ouabain resistance, and the tumorigenic and metastatic properties of H^R-R2T and HN^R-AT cell populations provide further information on this subject. Although an altered rate of spontaneous mutation to ouabain resistance was observed with the HN^R-AT cell line, it did not exhibit an increase in the generation of metastatic variants as compared to the wild type cells. Furthermore, the H^R-R2T cell line, which was the more aggressive of the two lines as judged by tumor growth rates in vivo, and by the ability to form lung colonies in the experimental metastasis assay, did not exhibit altered spontaneous mutation rates characteristic of genetic instability. These results suggested that the rates of spontaneous mutation were unrelated to increased tumorigenicity and that the cells have undergone other genetic or epigenetic events (Chow and Greenberg, 1980; Chow et al., 1983) responsible for progression to the more tumorigenic and metastatic phenotypes. Therefore, this study questions the importance of altered spontaneous mutation rates as a mechanism for generating variants with increased metastatic potential. The results, however, are consistent with suggestions that changes in ribonucleotide reductase may accompany modifications in the malignant characteristics of cells as previously discussed.

A recent study utilizing high and low metastatic mouse melanoma cell lines, B16F10 and B16F1 indicated that the cells with a higher rate of generation of metastatic variants (B16F10) also show a higher rate of generation of PALA and methotrexate resistant variants (Cillo et al., 1987). The interesting aspect of this work was that cells were resistant to these drugs due to amplification of carbamyl phosphate

synthetase-aspartate transcarbamylase-dihydroorotase (CAD) or dihydrofolate reductase genes. The studies had suggested that a gene amplification mechanism may be involved in the generation of metastatic variants, a process which occurs at rates as high as 10^{-3} - 10^{-4}/cell/generation. In order to test the possibility that the H^R-R2T cell line showed a higher frequency of metastatic variants compared to the parental wild type cell line and the HN^R-AT cell line, due to an increased ability to amplify DNA sequences, the H^R-R2T, HN^R-AT and wild type cell lines were grown in the presence of varying concentrations of PALA. The only mechanism of resistance to PALA that has been observed is amplification of the CAD gene (Wahl et al., 1979). The results indicated that the hydroxyurea resistant mutant is not capable of proliferating at greater rates than the HN^R-AT or wild type cell line, at any of the PALA concentrations tested, suggesting that an enhanced ability to amplify DNA sequences is not characteristic of the H^R-R2T cell line, and thus it is unlikely that a mechanism of increased rates of gene amplification can account for the elevated metastatic potential of H^R-R2T cell line compared to either HN^R-AT or the wild type cell lines.

3. RIBONUCLEOTIDE REDUCTASE ACTIVITY IN NORMAL AND TRANSFORMED HUMAN CELLS

In order to further examine the relationship between ribonucleotide reductase activity and tumorigenicity, a comparison between ribonucleotide reductase activity in transformed human cells versus their nontransformed parental human cell strains was conducted. This

system was chosen because it allowed a direct comparison of enzyme activity between transformed human cell lines and genetically matched cell strains; unlike other studies performed by others (Elford et al., 1970; Takeda and Weber, 1981) for which no parental cell strain was examined. The results indicated that ribonucleotide reductase activity was elevated 3.2 fold in WI-26 VA4 cells and 3.5 fold in WI-38 VA13 cells as compared to their respective parental cell strains. The rise in ribonucleotide reductase activity following transformation of WI-38 cells can not be attributed to increases in the proportion of cells going under DNA synthesis, since ^{3}H-thymidine uptake experiments for WI-38 and WI-38 VA13 cell lines indicated that there was no significant difference between transformed and parental populations, in the proportion of cells in S phase of the cell cycle. In addition, although the WI-26 VA4 cells appear to have an increased proportion of S phase cells as judged by ^{3}H-thymidine uptake experiments, the increase does not fully account for the enhanced ribonucleotide reductase activity observed in this line compared to the WI-26 cell strain. In contrast with studies of rat hepatoma lines (Takeda and Weber, 1981; Elford, 1972) the rise in reductase levels did not positively correlate with the growth rates of the human transformed cells, since one cell line, WI-38 VA13, had the same growth rate as its normal counterpart. It seems likely that other changes (De Larco and Todaro, 1978) in the proliferation pathway besides those affecting ribonucleotide reductase are required to bring about altered growth abilties. This observation agrees with the studies presented in this thesis on the H^{R}-R2T and the HN^{R}-AT hydroxyurea resistant cell lines, and with previous studies showing that increased levels of ribo-

nucleotide reductase in mutant cell lines does not lead to shorter doubling times (Wright, 1983; Koropatnick and Wright, 1980; Kuzik and Wright, 1980; Lewis and Wright, 1979). Presumably, additional changes in the proliferation pathway have occurred in the rat hepatoma cells (Takeda and Weber, 1981; Elford, 1972), and in the WI-26 VA4 human cells examined in this study, since they exhibited increased growth rates as well as changes in the reductase activity. The results suggest, however, that regulation of ribonucleotide reductase activity is less stringently controlled in transformed cells. In addition, the observation that normal human cells are more sensitive than transformed cells to the cytotoxic effects of the antitumor agent, hydroxyurea, indicates that an important point to be considered during drug therapy targeted to the reductase, is the greater sensitivity of normal compared to transformed human cells. This phenomenon is most likely due to the lower levels of enzyme activity in the non-transformed cells in comparison to the transformed lines. In summary, the results presented here extend the findings linking increases in ribonucleotide reductase activity to a mechanism of oncogenesis (Takeda and Weber, 1983; Weber, 1983; Elford, 1972), to include the transformation of human fibroblasts. An increased ability to reduce ribonucleotides may be a key step towards the development of a neoplastic program in both rodent and human cells (Tagger and Wright, 1984).

4. HYDROXY[^{14}C]-UREA UPTAKE BY HUMAN CELLS

Previous studies on the transport of hydroxyurea across the

mucosa of the rat small intestine and by Chinese hamster ovary cells in culture (Evered and Selhi, 1971; Morgan et al., 1986) have implicated that hydroxyurea uptake occurs by a diffusion process. Since hydroxyurea has been used clinically in the treatment of a wide range of solid human tumors as well as acute and chronic leukemia (Krakoff et al., 1964; Bolin et al., 1982; Engstrom et al., 1984), and since drug intake is an important aspect of drug action, the uptake properties of hydroxyurea in normal human diploid fibroblasts and their SV40 virus-transformed counterparts were investigated. In order to study the uptake properties of hydroxyurea it was essential to conduct drug transport kinetics under initial rate conditions. Therefore, the uptake of hydroxyurea into WI-38 cells as a function of time was determined. Drug uptake was found to proceed in a linear manner for the first 2 min after which drug uptake did not increase further, indicating that an equilibrium had been established between the rate of influx and the rate of efflux of drug. Further experiments to elucidate the mechanism of drug uptake in human cells were conducted using 1 min incubation times. Both WI-38 and WI-26 populations as well as their SV40 virus-transformed counterparts WI-38 VA13 and WI-26 VA4 lines respectively, showed a linear relationship between the rate of drug uptake and drug concentration between 0.1 and 1.0 mM hydroxyurea, as well as between much higher drug concentrations of 10 and 100 mM. Such results are consistent with a mechanism of diffusion for hydroxyurea uptake into human cells. When the ratio of intracellular to extracellular drug concentrations at various times of incubation in the presence of hydroxy[^{14}C]urea were examined, the ratio was always less than unity, supporting a diffusion mechanism for

hydroxyurea uptake. In addition, the temperature coefficient values (Q_{10}) for drug uptake in WI-38 cells was consistently less than 2, providing further support of a diffusion mechanism for hydroxyurea uptake in human cells. Finally, in order to rule out an energy requirement for hydroxyurea permeation, drug uptake was studied in the presence of an oxidative phosphorylation inhibitor, dinitrophenol, or a glycolysis inhibitor, sodium fluoride, or in the presence of both inhibitors. The results indicated that drug uptake was unaffected by any such treatments, hence ruling out an energy requirement for hydroxyurea permeation in WI-38 cells.

Taken together, the findings that (i) hydroxyurea uptake was not saturable even at high drug concentrations, (ii) the cell/medium distribution ratio was consistently less than 1, and (iii) known metabolic energy inhibitors do not affect drug uptake are all supportive of a mechanism of simple diffusion for hydroxyurea uptake in both normal and transformed human diploid fibroblasts. In addition, the Q_{10} values determined are consistent with such a mechanism.

It is interesting to note that the drug uptake did not differ between the normal cells and their transformed counterparts. This also indicates that the decreased sensitivity of the SV40 virus-transformed line WI-26 VA4 to hydroxyurea (Tagger and Wright, 1984), is not a result of a decreased inability to incorporate the drug.

The findings discussed above are the first to describe the characteristics of hydroxyurea uptake by human diploid fibroblasts and

helps to provide a more complete understanding of the mode of action of hydroxyurea in both normal and transformed human cells (Tagger et al., 1987).

SUMMARY

SUMMARY

Ribonucleotide reductase catalyzes the conversion of ribonucleotides to deoxyribonucleotides, the first unique step leading to DNA synthesis. The mammalian enzyme consists of two protein components frequently called M1 and M2, both of which are required for enzyme activity. Enzyme levels are high in rapidly proliferating cells compared to nonproliferating cells which contain undetectable levels. Enzyme activity is further augmented in transformed cells suggesting a link between tumorigenic properties of cells and an enhanced ability to reduce ribonucleotides. The antitumor agent, hydroxyurea inhibits DNA synthesis by interacting with the M2 protein and destroying a unique tyrosyl free radical which is an intrinsic part of its structure and essential for enzyme catalysis. In order to gain further insight into the mechanism of hydroxyurea resistance and the accompanying modifications of ribonucleotide reductase a detailed molecular and cellular characterization of two hydroxyurea resistant Chinese hamster ovary cells exhibiting either low or relatively high resistance to hydroxyurea was carried out. In addition to illustrating the molecular mechanisms involved in cellular resistance to hydroxyurea, the work presented in this thesis shows biological and tumorigenic characteristics of the drug resistant cells, and relates these properties to possible mechanisms such as alterations in dexyribonucleotide pools and spontaneous mutation rates. In addition, the relationship between ribonucleotide reductase activity and transformation was examined in cultured human diploid fibroblast cells; enzyme levels were found to be increased following transformation of the fibroblasts

with SV40 virus. In order to understand more about the mechanism of hydroxyurea action, uptake studies using hydroxy[^{14}C]-urea were performed in normal and SV40 virus transformed human fibroblasts.

The two hydroxyurea resistant cell lines had increased levels of ribonucleotide reductase activity. This increase was due entirely to an increase in the M2 component of the enzyme since studies with M1 specific antibodies indicated that both mutants contained wild type levels of protein M1 whereas EPR measurements for tyrosyl free radical content showed a 4.4 and 1.6 fold increase for H^R-R2T and HN^R-AT cells respectively. Hybridization studies revealed that both drug resistant cell lines contained a wild type level of M1 mRNA and a wild type M1 gene copy number. On the other hand, studies with M2 cDNA indicated that the two drug resistant cell lines had increased levels of M2 message that could adequately explain the observed elevations in the M2 component. Elevated M2 mRNA in the most resistant cell line, H^R-R2T, was a result of increased M2 gene copy number. Interestingly the low resistant cell line, HN^R-AT, had wild type levels of M2 gene copy number and therefore the increase in M2 message occurred through a process other than gene amplification. In order to explore the possibility that the kinetic properties of the enzyme were altered in the drug resistant cell lines as compared to the wild type population, the enzyme was partially purified from the three cell lines. Using ATP-agarose chromatography it was possible to obtain a partially purified preparation of ribonucleotide reductase mostly devoid of contaminating nucleoside diphosphate kinase activity which severely affects the enzyme assay by depleting the substrate. Kinetic studies

with the partially purified preparations showed no significant alterations in Km values for CDP reductase between the variant cell lines and the wild type line. However, enzyme prepared from both drug resistant cell lines showed reduced sensitivity to hydroxyurea and the negative allosteric effector, dATP. The changes in enzyme drug sensitivity was most pronounced with preparations prepared from H^R-R2T cells. The changes in these kinetic parameters possibly plays a role in mediating the drug resistant phenotype in the variant cell lines. Hydroxyurea uptake into the two mutant cell lines was not decreased compared to the wild type population, indicating that alterations in drug uptake can not account for the hydroxyurea resistant phenotype.

Consistent with the observed alteration in ribonucleotide reductase in the H^R-R2T cell line are colony forming experiments showing that the cells are not only resistant to the selective agent hydroxyurea, but are also cross resistant to several other drugs whose site of action is the M2 component. Furthermore, the H^R-R2T cells were not cross resistant to colchicine or puromycin suggesting that hydroxyurea resistant cells do not share the multidrug resistant phenotype which is frequently associated with cross resistance to these drugs.

The mutant cell lines were also examined for tumor growth rates, metastatic potential, deoxyribonucleoside triphosphate pool sizes and spontaneous mutation rates to 6-thioguanine and ouabain resistance. Compared to the parental wild type cell line, both mutant lines showed

an increase in tumor growth rates in BALB/c (nu/nu) mice. The H^R-2RT cell lined which contained the greatest changes in ribonucleotide reductase and was the most aggressive line in the tumorigenic assay, formed lung metastasis in experimental metastais assays with BALB/c (nu/nu) mice, and exhibited a metastatic phenotype significantly different from the wild type or the HN^R-AT populations. Compared to the wild type line, the H^R-R2T cell line showed no changes in deoxyribonucleotide pool sizes or in the rates of spontaneous mutation to the two markers examined indicating, i) that alterations in ribonucleotide reductase activity need not be associated with imbalances in pools and changes in spontaneous mutation rates and ii) that the ability to form lung metastasis and generate variant subpopulations is not necessarily dependant on the type of genetic instability which leads to increases in spontaneous mutation rates. In addition, growth studies in the presence of PALA also suggested that an increase ability to amplify DNA sequences can not explain the enhanced metastatic phenotype observed with the H^R-R2T cell line. The HN^R-AT cell line had gross alterations in dCTP and dGTP pools and showed a decrease in the rate of spontaneous mutation to ouabain resistance compared to the wild type line. This cell line is the first reported to show an actual decrease in spontaneous mutation rate, probably as a result of alterations in deoxyribonucleotide pools, and since it did not form any lung metastasis, it supports the observation that altered spontaneous mutation rates are not an important mechanism in these cells for producing the observed increased malignant phenotype. Taken together, the results obtained with H^R-R2T and HN^R-AT question the general significance of altered mutation rates as a

mechanism for generating variants with increased metastatic potential. The results are, however, consistent with earlier suggestions that changes in ribonucleotide reductase may accompany modifications in the malignant characteristics of cells.

To investigate the relationship between ribonucleotide reductase activity and the transformation event, two human diploid fibroblasts WI-26 and WI38 and their SV40 virus-transformed counterparts WI26-VA4 and WI38-VA13 were examined for their levels of ribonucleotide reductase activity using a permeabalized cell assay. Elevated levels of enzyme activity were detected in both transformed cell lines as compared to their respective parental cell strains. Furthermore, in keeping with increased levels of enzyme, the WI-26 VA4 cell line was more resistant to hydroxyurea than the parental WI-26 cells.

Finally, in order to establish the mechanism of hydroxyurea uptake, various drug uptake experiments using hydroxy[^{14}C]urea were performed on human cell strains. The results indicated that hydroxyurea enters human cells by a mechanism of passive diffusion.

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APPENDIX

APPENDIX A

Diagram of plasmid D65 (pcD-M1) and plasmid C10 (pcD-M2) showing a number of restriction endonuclease sites (after Thelander, L. and Berg, P., Mol. Cell. Biol., 6, 3433-3442 (1986) and Okayami, H. and Berg, P., Mol. Cell. Biol., 3, 280-289 (1983)).