THE UNIVERSITY OF MANITOBA

MAMMALIAN RIBONUCLEOTIDE REDUCTASE: THE REGULATION OF ENZYME ACTIVITY IN WILD-TYPE AND N-CARBAMOYLOXYUREA-RESISTANT MUTANTS OF CHINESE HAMSTER OVARY CELLS.

by

ROBERT GEORGE HARDS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF MICROBIOLOGY

WINNIPEG, MANITOBA

SEPTEMBER 1982

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To my parents, without whose love and guidance none of this would have been possible.

May God bless and keep you always.

May your wishes all come true.

May you always do for others

And let others do for you.

May you build a ladder to the stars

And climb on every rung.

And may you stay forever young.

Bob Dylan

You have to be smart enough to play the game.

And dumb enough to think it matters.

Joe Paterno

ACKNOWLEDGEMENTS

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I would like to thank my advisor Jim Wright, for his efforts during the course of these studies. To John Dick, Thor Borgford, Craig Parfett and John Langstaff, my thanks for their help and more importantly their friendship. I would also like to thank Sharon McKinley for her perserverance in the typing of this thesis.

The financial support of the Natural Sciences and Engineering Research Council and the University of Manitoba is acknowledged.

Finally, to Wayne Rose, who has the ability to see humour in the world when things look worse than bleak, my most grateful thanks.

ABSTRACT

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An intact cell assay system based on Tween 80 permeabilization was used to investigate ribonucleotide reductase activity in CHO cells. The method was found to provide access to the intracellular enzyme without undue disruption of cellular structure and function. Other nucleotide metabolizing enzymes, whose presence were found to influence the estimates of ribonucleotide reductase, were also active in intact hamster cells. The incorporation of ribonucleotide substrates and deoxyribonucleotide products into RNA and DNA was found to occur at very low levels and to have no detectable effect on reductase activity estimates. Of major concern was the observation of various kinases and phosphatases which caused the redistribution of the phosphates of the ribonucleoside diphosphate substrates and the nucleoside triphosphate effectors. The base composition of the nucleotides added to the assay systems were not altered. Although movement of phosphates occurred during the assays, the concentrations of substrates and effectors quickly reached equilibrium with their respective nucleosides and nucleotides, resulting in relatively constant although reduced levels of the various nucleotides being present during the assays. Precise determinations of ribonucleotide reductase activity can be calculated when these alterations in nucleotide concentration are taken into consideration.

The Tween 80 permeabilization technique was used to study the properties of ribonucleotide reductase from a wild type CHO cell line. The optimal assay conditions and kinetic characteristics for the reduction of CDP and ADP were found to be very similar to those of the enzyme from a variety of mammalian cell extracts, including the most

highly purified preparations.

The usual model cited in the description of the regulation of reductase activity is that proposed by Thelander and Reichard, which is based on studies with purified enzyme. In order to assess the applicability of this model in a situation more relevant to the in vivo state, the allosteric regulation of mammalian ribonucleotide reductase was investigated using intact CHO cells. While certain aspects of the Thelander-Reichard model were applicable to the enzyme within the intact cell, major tenets did not seem to apply. Specifically the model proposes that reduction is carried out by a single enzyme with specificity of reduction determined by the binding of various nucleoside triphosphate effectors to an allosteric regulatory site. According to this theory, activators specific for the reduction of the various substrates should compete for the regulatory site and thus the binding of one nucleoside triphosphate should exclude the binding of all others to this site. Within intact CHO cells this did not seem to be the case, as it was found that various effectors could bind to the enzyme simultaneously. The regulatory pattern observed within intact CHO cells could be most readily explained if it is assumed that four distinct proteins are involved in the reduction of the four ribonucleotide substrates. Such a model has previously been proposed and there is evidence to support its existence.

N-carbamoyloxyurea was cytotoxic for cells in culture and, like hydroxyurea and guanazole, was an effective inhibitor of mammalian ribonucleotide reductase and DNA synthesis. The drug proved to be a good selective agent for the isolation of phenotypically stable drug-resistant mutants. Using the mammalian cell permeabilization assay, a detailed analysis of the target enzyme, ribonucleotide reductase, from a drug resistant cell line was carried out. Both the mutant and

wild type cells required the same optimum conditions for enzyme activity. Moreover, the enzyme from the two cell lines displayed equal sensitivity to inhibition by N-carbamoyloxyurea. The sole difference in reductase activities between the WT and NC^R-30A cells was an approximate two-fold increase in level observed in the mutant cell line. This increase in reductase activity at least partially accounted for the 2.6 fold increase in cellular resistance to N-carbamoyloxyurea exhibited by NC^R-30A cells. The mutant cell line was also cross-resistant to the antitumor agents, hydroxyurea and guanazole. No differences in Ki values for inhibition of CDP and ADP reduction by these two drugs were detected and cellular resistance could be entirely accounted for by the elevation in activity of the reductase in the NC^R-30A cell line. Preliminary investigations into the mechanism by which the NC^R-30A cells produced elevated levels of reductase activity indicated an alteration in the metabolism of the mRNA specific for the enzyme.

To further study the effects of N-carbamoyloxyurea, a series of resistant CHO cells were selected in the presence of increasing concentrations of drug. Investigation of the CDP and ADP reductase activities in these drug resistant cell lines showed them to possess two types of alterations; elevated levels of enzyme with wild type sensitivity to drug, and altered levels of reductase with reduced drug sensitivity. Furthermore, N-carbamoyloxyurea-resistant lines contained another alteration as well, presumably at a second site of drug action. This second site is apprently involved in the synthesis of DNA but is distinct from ribonucleotide reductase. The drug resistant lines were also cross-resistant to hydroxyurea and guanazole and studies on enzyme activity levels support previous findings with cells selected for resistance to hydroxyurea, which showed changes in CDP reductase activity are not

always coordinated with changes in ADP reductase. Possible explanations for these observations are provided. Increases in cellular resistance were accompanied by significant increases in CDP but not ADP reductase activity, suggesting that an ability to maintain an adequate level of dCTP (and therefore CDP reductase activity) is especially important to achieve resistance to DNA synthesis inhibitors like N-carbamoyloxyurea, hydroxyurea and guanazole. These observations also fit with indications from other studies that dCTP may play a special role in the regulation of mammalian DNA synthesis.

There is an iron-dependent activity in the venom of Crotalus atrox which appears to hydrolyze the N-glycosidic sugar-base bond of pyrimidine and purine nucleotides. Maximal activation of this activity occurred at 1 mM and 0.8 mM $FeCl_3$ for CDP and ADP substrates respectively. The release of free base affects the background values of control experiments carried out during nucleotide labelled assays of ribonucleotide reductase activity which require the conversion of nucleotides to nucleosides by treatment with the venom. When the reductase was examined in intact cells, $FeCl_3$ was found to be an inhibitor of ADP reduction and varied from a mild stimulator to a significant inhibitor of CDP reductions depending upon $FeCl_3$ concentration.

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ABBREVIATIONS

α-MEM	- alpha minimal essential medium
ACS	- aqueous counting scintillant
ADP	- adenosine 5'-diphosphate
AMP	- adenosine 5'-monophosphate
araA	- 1-β-D-arabinofuranosyl adenine
araATP	- 1-β-D-arabinofuranosyl adenosine 5'-triphosphate
araC	- 1-β-D-arabinofuranosyl cytosine
ATP	- adenosine 5'-triphosphate
Bt_2cAMP	- N^6,O^2-dibutyryl adenosine 3':5'-monophosphate
CDP	- cytidine 5'-diphosphate
CHO	- Chinese hamster ovary
CMP	- cytidine 5'-monophosphate
cpm	- counts per minute
CTP	-cytidine 5'-triphosphate
D_{10}	- concentration of drug which reduces RPE to 10%
dADP	- 2'-deoxyadenosine 5'-diphosphate
dAMP	- 2'-deoxyadenosine 5'-monophosphate
dATP	- 2'-deoxyadenosine 5'-triphosphate
dCDP	- 2'-deoxycytidine 5'-diphosphate
dCMP	- 2'-deoxycytidine 5'-monophosphate
dCTP	- 2'-deoxycytidine 5'-triphosphate
DEAE	- diethylamino ethyl
dFCS	-dialyzed fetal calf serum
dGDP	- 2'-deoxyguanosine 5'-diphosphate
dGMP	- 2'-deoxyguanosine 5'-monophosphate
dGTP	- 2'-deoxyguanosine 5'-triphosphate
DMSO	- dimethyl sulfoxide

DNA	- deoxyribonucleic acid
dTDP	- 2'-deoxythymidine 5'-diphosphate
dTMP	- 2'-deoxythymidine 5'-monophosphate
DTT	- dithiothreitol
dTTP	- 2'-deoxythymidine 5'-triphosphate
dUDP	- 2'-deoxyuridine 5'-diphosphate
dUMP	- 2'-deoxyuridine 5'-monophosphate
dUTP	- 2'-deoxyuridine 5'-triphosphate
EDTA	- ethylenediamine tetraacetic acid
EPR	- electron paramagnetic resonance
FAD	- flavin adenine dinucleotide
FCS	- fetal calf serum
GAT	- glycine, adenosine, thymidine
GDP	- guanosine 5'-diphosphate
glutaredoxin-$(SH)_2$	- reduced glutaredoxin
glutaredoxin-S_2	- oxidized glutaredoxin
GMP	- guanosine 5'-monophosphate
GSH	- reduced glutathione
GSSG	- oxidized glutathione
GTP	- guanosine 5'-triphosphate
HEPES	- N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
h-sites	- high affinity binding sites
HMGCoA	- hydroxymethylglutaryl-coenzyme A
HPLC	- high performance liquid chromatography
HS	- horse serum
IMP	- inosine 5'-monophosphate
K_i	- concentration of drug which reduces enzyme activity by 50%

L_1	- effector binding subunit of rat liver ribonucleotide reductase
L_2	- iron containing subunit of rat liver ribonucleotide reductase
1-sites	- low affinity binding sites
M_1	- effector binding subunit of calf thymus ribonucleotide reductase
M_2	- iron containing subunit of calf thymus ribonucleotide reductase
mRNA	- messenger ribonucleic acid
$NADP^+$	- reduced nicotinamide adenine dinucleotide phosphate
NADPH	- oxidized nicotinamide adenine dinucleotide phosphate
NC^R	- N-carbamoyloxyurea-resistant
PBS	- phosphate buffered saline
PE	- plating efficiency
PIPES	- piperazine-N,N'-bis(2-ethanesulfonic acid)
POPOP	- 1,4-bis[2(5-phenyloxazolyl)]benzene
PPO	- 2,5-diphenyloxazole
RPE	- relative plating efficiency
rpm	- revolutions per minute
RNA	- ribonucleic acid
$t_{1/2}$	- half-life
TCA	- trichloroacetic acid
thioredoxin-$(SH)_2$	- reduced thioredoxin
thioredoxin-S_2	- oxidized thioredoxin
UDP	- uridine 5'-diphosphate
UMP	- uridine 5'-monophosphate
UTP	- uridine 5'-triphosphate
UV	- ultraviolet
WT	- wild-type

INTRODUCTION

INTRODUCTION

To understand how an enzyme functions in its physiological environment, it is necessary to characterize its properties. This usually entails extraction and purification of the enzyme of interest. However, there is a growing amount of evidence that the properties of many enzymes determined *in vitro* do not reflect the true *in vivo* situation. To overcome this problem intact cell assay systems have been developed. The intact cell assay system based on Tween 80 permeabilization has previously been used to study ribonucleotide reductase activity in mammalian cells. Because intact cells are the ultimate in crude enzyme preparation, the possibility that other enzymes, active within the permeabilized cells, interfered with the estimation of ribonucleotide reductase activity was suspected. This study was the first to examine this question in detail. It was found that other nucleotide metabolizing enzymes were indeed active within the intact cells. However, their effects could be accounted for and precise determinations of reductase activity were possible.

During the investigation of the properties of ribonucleotide reductase, a previously undescribed iron-dependent 5'-nucleoside phosphohydrolase activity in the venom of *Crotalus atrox* was detected.

Using the intact cell assay technique, the optimal assay conditions and kinetic characteristics for the reductase from wild-type CHO cell line were determined. Particular attention was paid to the manner in which nucleotide effectors, either singly or in pairs, interacted with the enzyme. The results of these studies were used to evaluate the validity of the *in vitro* models most frequently used to explain the allosteric regulation of ribonucleotide reductase. It was hoped that the use of intact cells would more closely resemble the true *in vivo* conditions. The results obtained suggested that while certain aspects of the *in vitro* models are functional in the intact cells, discrepancies do exist.

Nucleotide pool sizes in exponentially growing CHO cells were measured.

These levels were compared to the concentrations of substrates and effectors found to be effective in the regulation of mammalian ribonucleotide reductase in intact permeabilized cells to determine if the measured values made sense in regards to the *in vivo* pool sizes.

The selection and characterization of mammalian somatic cell mutants has proved valuable in the study of metabolic pathways, mechanisms of enzymatic and cellular regulation and the site and manner of interaction between organisms and various chemical agents. Although known to be cytotoxic and postulated to inhibit ribonucleotide reductase, the exact mechanism of action for the potential chemotherapeutic agent N-carbamoyloxyurea was uncertain. In order to study this question, N-carbamoyloxyurea was used to select a series of drug-resistant mutants. Biochemical analysis of the mutants indicated that while the drug does inhibit ribonucleotide reductase, there is a second, more sensitive, site of action. The second site would seem to involve the synthesis of DNA but be distinct from ribonucleotide reductase. In addition to the mechanisms by which N-carbamoyloxyurea inhibits the growth of mammalian cells, the mutants have given some insights into the manner in which the reduction of ribonucleotides are involved in DNA synthesis. Of particular interest was the suggestion that intracellular levels of dCTP may play a specific role in the regulation of DNA replication.

In summary, the objective of this study were: (1) to critically evaluate, in detail, the intact cell permeabilization technique used in the assay of mammalian ribonucleotide reductase; (2) to utilize this intact cell assay system to study the properties of ribonucleotide reductase in order to evaluate models which describe the regulation of the enzyme; (3) to use the isolation of somatic cell mutants to study the interaction of N-carbamoyloxyurea with mammalian cells in order to evaluate its potential as a chemotherapeutic agent.

HISTORICAL REVIEW OF MAMMALIAN RIBONUCLEOTIDE REDUCTASE

HISTORICAL REVIEW OF MAMMALIAN RIBONUCLEOTIDE REDUCTASE

1. INTRODUCTION

1.1 Importance of Ribonucleotide Reductase to Mammalian Cells

Many studies have indicated the key role ribonucleotide reductase (EC 1.17.4.1) plays in cell growth. While cells do possess measurable levels of deoxynucleoside triphosphates, those in S phase have sufficient levels to maintain DNA synthesis for only a very short time. The smallest of the deoxynucleoside triphosphate pools, dGTP, was estimated to be present in amounts capable of sustaining DNA replication for only 15 to 30 seconds (Skoog and Nordenskjöld 1971). Thus cells in the S phase of growth must be constantly supplied with deoxynucleoside triphosphates in order for growth to continue. To date, the only enzyme found to be capable of generating the required deoxynucleotides in sufficient quantities has been ribonucleotide reductase. The essential nature of the enzyme, at least in bacterial cells, was conclusively proven by the isolation of a temperature sensitive mutant of DNA synthesis (DNAF). It was found that the lesion occurred in the gene coding for ribonucleotide reductase (Fuchs et al 1972).

The critical association of ribonucleotide reductase activity and cell growth has been illustrated by synchronization studies involving mammalian cells. The pool sizes of all four deoxyribonucleoside triphosphates increased immediately prior to the initiation of DNA synthesis and remained elevated during S phase after which there was a decrease to the low basal levels found in G1 (Walters et al 1973, Bray and Brent 1972). Measurement of ribonucleotide reductase activity in synchronized cells showed the same dramatic increase just prior to DNA replication and then a return to low basal levels near the end of S phase which

continued through G1 (Murphree et al 1969, Peterson and Moore 1976, Lewis et al 1978).

Enhanced levels of ribonucleotide reductase are characteristic of rapidly proliferating tissues such as regenerating liver (Larsson 1969) and embryonic organs such as liver (Elford 1972) and brain (Millard 1972). Newly born (5 to 6 days old) rats showed a peak of reductase activity in the spleen, thymus (Elford 1972) and brain (Eels and Spector 1982) which declined as the animals matured. In all of these organs the peak of enzyme activity corresponded to a time of high mitotic activity within the particular organ, be it production of white blood cells in the spleen or neuronal proliferation in the brain.

Ribonucleotide reductase also seems to be intimately involved in neoplastic transformation of cells. A direct relationship between the level of ribonucleotide reductase and the growth rates of a series of rat hepatomas has been shown to exist (Elford 1972). This correlation between ribonucleotide reductase and cancer is further supported by the use of hydroxyurea in the treatment of malignancies. In 1960, animal screening studies indicated that hydroxyurea possessed anti-tumor activity against Sarcoma 180 (Stock et al 1960). Since then, the drug has been shown to be selectively toxic for rapidly proliferating mammalian tissues and has been used clinically to treat a wide variety of solid tumors as well as acute and chronic leukemia (Bergsagel et al 1964, Bolton et al 1964, Fishbein et al 1964). As the sole mode of hydroxyurea action appears to be inactivation of ribonucleotide reductase (Adams and Lindsay 1967, Elford 1968, Krakoff et al 1968, Lewis and Wright 1974), the above studies point out the intimate association between ribonucleotide reductase and neoplastic cells.

In addition to cancer, ribonucleotide reductase may also play an active role in other metabolic disorders. Immunodeficiency diseases in humans has been associated with decreased levels of purine-nucleoside phosphorylase (Ullmann et al 1979) or adenosine deaminase (Lin and Elford 1980). It has been suggested that cells lacking purine-nucleoside phosphorylase accumulate high levels of GTP and dGTP while those lacking adenosine deaminase accumulated dATP. In both cases the accumulated nucleotides would inhibit ribonucleotide reductase, especially the production of dCDP and hence dCTP. This depletion would prevent DNA synthesis, a process necessary for the proliferation-dependent function of T cells.

Recent studies (Meuth 1981, Bradley and Sharkey 1978, Ashman et al 1981) have indicated that an imbalance of deoxyribonucleoside triphosphates can lead to mutagenesis, possibly via base substitution. Ribonucleotide reductase may play a key role in the generation of the umbalanced state. Of particular interest in this regard was the isolation of two mutator genes in mammalian cells. The first, designated thy$^-$ (Meuth et al 1979) appeared to be a single mutation that both increased the reduction of CDP and decreased the reduction of UDP. These mutants were selected on the basis of their resistance to arabinosylcytosine and auxotrophy towards thymidine. The second class of mutants (Chan et al 1981) were selected for resistance to arabinosyladenosine and like the first class they seemed to possess an altered ribonucleotide reductase that caused alterations in the pool sizes of the deoxynucleoside triphosphates.

There are indications that ribonucleotide reductase plays a key role in other metabolic processes. As normal human diploid fibroblast grown in culture age, their growth rates decrease until finally

the cells die. This senescence was found to be accompanied by a decrease in ribonucleotide reductase levels (Dick and Wright 1982). Recently, there has been some evidence presented that ribonucleotide reductase may be involved in cellular differentiation. L6 myoblasts grown in culture are capable of undergoing myogenesis during which fusion of the mononucleated myoblasts leads to formation of multinucleated muscle fiber (Parfett et al 1981). Variant cell lines selected for resistance to hydroxyurea (Creasey et al 1982) were found to be unable to undergo this differentiation. This would seem to indicate that ribonucleotide reductase may play a significant role in cellular differentiation.

Finally, there are indications that ribonucleotide reductase may be a possible site of action for certain commonly used pesticides. 3-methyl-4-nitrophenol, a major breakdown product of the insecticide fenitrothion, has been shown to inhibit CDP and ADP reduction at concentrations very similar to those reported for various antitumor agents (Wright et al 1982). Thus the possibility of inhibiton of ribonucleotide reductase should be taken into account when the overall biological activity of this organophosphate insecticide are considered.

The results discussed above all serve to illustrate the key role ribonucleotide reductase plays in several cellular processes and how alterations in this activity can have drastic and damaging effects.

1.2 Non-mammalian Ribonucleotide Reductase Activities

The first suggestion that doexyribonucleotides are formed by the direct reduction of ribonucleotides was supplied by Hammarsten et al (1950) who, using in vivo experiments with rats, showed that pyrimidine ribonucleotides were efficiently incorporated into DNA. Within short order, Rose and Schweigert (1953) were able to show that conversion of

ribonucleotides to deoxyribonucleotides occurred without cleavage of the N-glycosidic bond. Furthermore, it was shown that when ^{32}P-phosphate and uniformly labelled ^{14}C-cytidine were administered to regenerating rat liver, the cytidylate from RNA and the deoxycytidylate from DNA had the same ^{32}P:^{14}C ratio (Larsson and Neilands 1966). This demonstrated that both compounds were derived from a common precursor, presumably a ribonucleotide.

The enzyme responsible for the conversion of ribonucleotides to deoxyribonucleotides was first directly detected in chick embryo extracts as early as 1958 (Reichard 1958, Reichard 1960). In a very short time the presence of ribonucleotide reductase in mammalian cell extracts was also shown (Moore and Hurlbert 1960, Abrams et al 1960). However, the mammalian enzyme proved to be very difficult to purify and until very recently much of the physicochemical characterization of the enzyme was carried out using ribonucleotide reductase obtained from E. coli. As the mammalian enzyme and that from E. coli bear many similarities a brief review of the bacterial system will be presented.

The enzyme from E. coli is composed of two distinct subunits B1 and B2 (Brown et al 1967) and is capable of reducing all four ribonucleotides (Larsson and Reichard 1968). Each of the subunits was found to be made up of two polypeptide chains. The B1 subunit contains two similar chains of MW 82,000 (Thelander 1973) while the B2 subunit has two identical chains of MW 43,000 (Thelander 1973). Alone neither B1 nor B2 was found to possess catalytic activity but in the presence of magnesium ions the two subunits became associated in a one-to-one ratio to form an active enzyme of MW 240,000 (Brown and Reichard 1969a). Two non-heme iron atoms and one or possibly two tyrosine free radicals were found to exist per molecule of protein B2 (Petersson et al 1980).

The free radical has been implicated in the reduction mechanism (Stubbe and Ackles 1980) while the iron center is thought to be involved in the generation of the free radical (Sjöberg et al 1982). Protein B1 was shown to contain two substrate binding sites (von Döbeln and Reichard 1976). The enzyme extracted from E. coli cells reduces ribonucleotides at the diphosphate level (Reichard 1967). In addition, each B1 subunit has been shown to possess four nucleoside triphosphate effector binding sites distinct from the substrate binding sites. These can be divided into two classes each containing two sites (Brown and Reichard 1969b). The first class (h-sites) have a high affinity for dATP and also bind ATP, dGTP and dTTP while the second class (l-sites) have a lower affinity for dATP and in addition bind only ATP. The binding of one of ATP, dATP, dGTP or dTTP at the h-sites results in a conformation change in the enzyme which induces preferential binding and hence reduction of a specific substrate. ATP and dATP activate the reduction of pyrimidine nucleotides while dGTP and dTTP activate the reduction of ADP and GDP respectively. The l-sites regulate the overall activity of the enzyme with dATP acting as the negative effector and ATP as the positive. With dATP bound to the l-site, it was found there was an increase in the molecular weight of the inactive enzyme suggestive of a dimerization (Brown and Reichard 1969a).

In addition to allosteric regulation, ribonucleotide reductase activity is subject to control via synthesis and degradation of the enzyme. Blockage of DNA synthesis by addition of chemical inhibitors (Filpula and Fuchs 1978, 1979), the use of mutants defective in DNA initiation or elongation (Filpula and Fuchs 1978, 1979) or thymine starvation (Fuchs 1977b, Ericksson et al 1977b) resulted in increased synthesis of ribonucleotide reductase. As measured by either enzymatic (Fuchs 1977b) or immunological (Eriksson et al 1977b) assays, the elevation of

the enzyme levels in thymine starved E. coli cells was found to be due to increased production of both subunits B1 and B2. Induction of ribonucleotide reductase activity by inhibition of DNA synthesis is accompanied by an increase in the levels of mRNA coding for the enzyme (Hanke and Fuchs 1980).

The E. coli type of ribonucleotide reductase, representative of many bacterial as well as some fungal (Lowdon and Vitols 1973, Lewis et al 1976), plant (Hovemann and Follmann 1977) and algal (Feller et al 1980) enzymes is schematically represented in figure 1. The ribonucleotide reductase of mammalian cells also shares many characteristics with the E. coli enzyme.

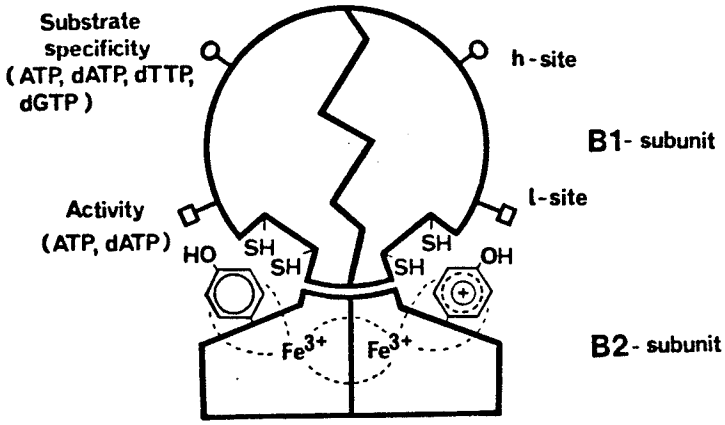
In addition to ribonucleotide reductase, the reduction of ribonucleoside diphosphates to deoxyribonucleoside diphosphates by E. coli required the presence of reducing power. With cell extracts this power could be supplied by dithiols such as dithiothreitol, dithioerythritol or reduced lipoic acid (Larsson and Reichard 1968). In vivo, E. coli would seem to use one of two systems. The thioredoxin system, consisting of NADPH, thioredoxin and thioredoxin reductase (Laurent et al 1964, Moore et al 1964), operates according to reactions (1) and (2).

(1) $$\text{thioredoxin-}S_2 + \text{NADPH} + H^+ \xrightarrow[\text{reductase}]{\text{thioredoxin}} \text{theoredoxin-}(SH_2) + \text{NADP}^+$$

(2) $$\text{thioredoxin-}(SH_2) + \text{rNDP} \xrightarrow[\text{reductase}]{\text{ribonucleotide}} \text{thioredoxin-}S_2 + \text{dNDP} + H_2O$$

Thioredoxin is not directly involved in the reduction of the ribonucleotides. It has been discovered that thioredoxin-$(SH)_2$ reduces a pair of disulfides of protein B1 and that these sulfhydryls in turn reduce the ribonucleotides (Thelander 1974).

Fig. 1 Model of ribonucleotide reductase from E. coli (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
Fe3+
Fe3+
B2 - subunit

Initially it was supposed that thioredoxin was the sole source of reducing power available to ribonucleotide reductase. However, isolation of mutant E. coli strains lacking detectable levels of thioredoxin (Holmgren 1976) or thioredoxin reductase (Fuchs 1977a) led to the discovery of the glutaredoxin system. This sytem which consists of glutaredoxin, glutathione, glutathione reductase and NADPH (Holmgren 1979a,b) is summarized in reactions (3) to (5).

(3) $NADPH + H^+ + \text{oxidized glutathione} \xrightarrow[\text{reductase}]{\text{glutathione}} NADP^+ + 2 \text{ reduced glutathione}$

(4) $2 \text{ reduced glutathione} + \text{glutaredoxin-}S_2 \rightarrow \text{oxidized glutathione} + \text{glutaredoxin-}(SH)_2$

(5) $\text{glutaredoxin - }(SH)_2 + rNDP \xrightarrow[\text{reductase}]{\text{ribonucleotide}} \text{glutaredoxin - } S_2 + dNDP + H_2O$

While both systems can provide reducing power for ribonucleotide reductase, the 10-fold higher activity found with glutaredoxin over thioredoxin is suggestive of the glutaredoxin system being the natural hydrogen source. Moreover, E. coli mutants with lesions in the biosynthetic pathway of glutathione have been isolated (Apontoweil and Berends 1975, Fuchs and Warner 1975). These mutants, which possessed decreased levels of glutathione, had derepressed and increased levels of ribonucleotide reductase. Possibly this derepression occurred as a consequence of the lack of reducing power available in the mutant cells. There also exists certain evidence that glutaredoxin and ribonucleotide reductase may be components of a multienzyme complex involved in the synthesis of DNA (Holmgren 1979b). These results would all seem to indicate that glutaredoxin and not thioredoxin is the preferred reducing agent for ribonucleotide reductase from E. coli.

The glutaredoxin hydrogen transport system may be involved in the control of ribonucleotide reductase activity. In the presence of glutaredoxin and ribonucleotide reductase, the reduction of CDP to dCDP was shown to be greatly influenced by the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) with even small amounts of GSSG being inhibitory (Thelander et al 1979b). The mechanism behind this regulation of reductase activity is likely a strong product inhibition of reaction (4) above by GSSG which blocks the formation of glutaredoxin-$(SH)_2$. Thus glutathione reductase may play a pivotal role in the reduction of ribonucleotides by controlling the GSH to GSSG ratio. Also, the rate of GSH formation by glutathione synthetase and its subsequent utilization in other reactions will influence the GSH to GSSG ratio and hence the activity of ribonucleotide reductase.

The second major type of ribonucleotide reductase (EC1.17.4.2) is found in bacteria such as Lactobacillus leichmannii (Panagou et al 1972, Chen et al 1974) Bacillus megaterium (Yan and Wachsman 1973), Miccrococcus denitrificans (Gleason and Hogenkamp 1972), Pseudomonas stutzeri (Gleason and Hogenkamp 1972 , Hamilton and Easley 1980), Clostridium sticklandii (Abeles and Beck 1967), Rhizobium meliloti (Cowles et al 1969), cyanobacteria (Gleason 1978, Gleason 1979, Gleason and Frick 1980) and the thermophilic Thermus X1 (Sando and Hogenkamp 1973). The only eucaryote known to possess this second type of ribonucleotide reductase is the euglenoid flagellate Euglena gracilis (Gleason and Hogenkamp 1970, Hamilton 1974, Carell and Seeger 1980).

The enzyme from Lactobacillus leichmannii has been purified to homogeneity and found to consist of a single polypeptide chain (MW 76,000), capable of reducing all four ribonucleotides (Panagou et al 1972). Unlike the E. coli type of enzyme, the reductase of L.

leichmannii reduces ribonucleoside triphosphates but not diphosphates (Blakely et al 1965). Activity was found to be totally dependent upon the presence of the vitamin B12 coenzyme, 5'-deoxy-5'-adenosylcobalamin (Vitols et al 1967). This coenzyme is involved in the generation of a free radical and is thought to function in a manner analogous to the B2 subunit of the E. coli enzyme. Like the E. coli type of enzyme, the cobalamin-dependent reductase contains a disulfide that is reduced by Thioredoxin-$(SH)_2$ or dithiothreitol. The resulting sulfydryls are involved in the reduction of the ribonucleotides. Substrate specificity of this second type of ribonucleotide reductase is under the allosteric control of deoxynucleoside triphosphates. DeoxyATP activates the reduction of CTP, dCTP the reduction of UTP, dTTP the reduction of GTP and dGTP the reduction of ATP (Vitols et al 1967). Even though both substrate and regulatory sites bind nucleoside triphosphates, the structural difference between ribose and deoxyribose sugars causes the regulatory site to bind the ribonucleotide substrates with an affinity 100 to 1000-fold lower than that exhibited towards the deoxynucleotide effectors. In contrast to the enzyme isolated from E. coli, no overall negative effector is known to exist for the reductase from L. leichmannii.

Cobalamin-dependent reductases utilize a thioredoxin system to supply the hydrogen atoms required for the reduction of ribonucleotides. A thioredoxin from the cyanobacterium Anabaena sp., an organism that possesses an adenosylcobalamin-dependent reductase, has been isolated and characterized (Gleason and Holmgren 1981). Similar to the molecule from E. coli, the cyanobacterium thioredoxin has a molecular weight of 12,000 and contains a single oxidation-reduction active disulfide bridge with the amino acid sequence: -cys-gly-pro-cys-lys-. In addition, there

is immunochemical cross reactivity between the two molecules, the thioredoxin from Anabaena sp. can act as a hydrogen donor for E. coli ribonucleotide reductase and E. coli thioredoxin reductase can reduce Anabaena sp. thioredoxin-S_2.

Corynebacterium nephridii (Tsai and Hogenkamp 1979) and Rhizobium melitoti (Cowles and Evans 1968) were found to possess unusual ribonucleotide reductase activities. The enzymes were adenosylcobalamin-dependent and yet reduced ribonucleoside diphosphates preferentially over the triphosphates. Another unusual aspect of the reductase from these two sources was the apparent ineffectiveness of nucleoside triphosphates on substrate specificity or activity levels (Tsai and Hogenkamp 1980, Cowles et al 1969). It would seem that the ribonucleotide reductase from these two bacteria exist in a single conformation that allows for the reduction of all four ribonucleotides.

Another unusual ribonucleotide reductase has recently been detected in Brevibacterium ammoniagenes and Miccrococcus luteus (Schimpff-Weiland et al 1981). The enzymes from these two organisms required neither adenosylcobalamin nor iron but were dependent upon manganese for activity. The reductases exhibited a strange mix of E. coli and L. leichmannii type properties. While allosteric effectors behaved in a manner similar to the situation found with the adenosylcocalamin-dependent enzyme, hydroxyurea inhibited the reaction as it does for the iron-dependent enzyme. To further confuse the picture, it would seem that both ribonucleoside di- and triphosphates were reduced at comparable rates.

Several bacteriophages have been found to contain genes that code for viral specific ribonucleotide reductase activities (Berglund 1972a,b, Langelier and Buttin 1981, Lankinen et al 1982). Viral ribonucleotide

reductases have many properties similar to but not identical with those found in the E. coli enzyme. The reductase from T4 phage has been purified (Berglund 1972a, Sahlin et al 1982) and shown to consist of two non-identical subunits, α_2 and β_2, both of which are required for enzyme activity. Each subunit is made up of two identical polypeptides coded for by the viral genes nrdA and nrdB (Yeh et al 1969). The α_2 subunit has a molecular weight of 160,000, contains binding sites for ribonucleoside diphosphate substrates and nucleoside triphosphate effectors and possesses thiol groups at the active center that are essential for activity. The β_2 subunit contains two moles of non-heme iron plus a tyrosine free radical and thus is thought to function in an analogous manner to the B2 subunit of the E. coli enzyme. The viral enzyme differs from that of E. coli in that the association of the subunits is much tighter and no magnesium ion is required. The T6-induced ribonucleotide reductase has many characteristics of the T4 enzyme and there is immunological cross-reactivity between the two (Eriksson and Berglund 1974). Although not yet purified, the ribonucleotide reductase from T5 bacteriophage appears to differ from the other viral reductases in some properties including the use of ribonucleoside triphosphates as substrates (Eriksson and Berglund 1974).

The allosteric regulation of T4 ribonucleotide reductase is similar to that of the E. coli enzyme except for the absence of inhibition by dATP (Berglund 1972b). This nucleotide acts only as a positive effector for the reduction of CDP and UDP. It would thus seem that the T4 enzyme lacks the activity or l-sites of the E. coli ribonucleotide reductase and its activity is modulated only by substrate specificity. It would appear that bacteriophage and mammalian virus enzymes regulated in a different manner.. Whereas ATP and dATP are stimulatory for the

reduction of CDP by the T4 reductase, they have no effect on the enzyme isolated from the herpes simplex virus (Langelier and Buttin 1981, Lankineu et al 1982). Other studies (Huszar and Bacchetti 1981) indicate that not only does ATP not stimulate the reduction of CDP by the herpes ribonucleotide reductase but that it is actually inhibitory.

The bacteriophage and mammalian viruses also differ in their sensitivity to hydroxyurea, a drug which inhibits ribonucleotide reductase via destruction of the tyrosine free radical (Åkerblöm et al 1981, Atkin et al 1973, Sjöberg et al 1977). While the enzyme activity of T4 phage is inhibited 50% by hydroxyurea concentrations of 10 to 30 μM, the herpes simplex enzyme activity requires 0.45 mM to achieve the same level of inhibition. There is also a report that the Epstein-Barr virus, a member of the herpes virus group, is resistant to the effects of hydroxyurea (Henry et al 1978). Whether this means the Epstein-Barr virus enzyme does not possess a free radical site of action for hydroxyurea is not yet known.

The above results suggest that the viruses which infect bacterial and mammalian cells should be grouped separately as to the type of ribonucleotide reductase each possesses.

2. STRUCTURE OF MAMMALIAN RIBONUCLEOTIDE REDUCTASE

Although ribonucleotide reductase activity was demonstrated in mammalian cells as early as 1960 (Moore and Hurlbert 1960, Abrams et al 1960), the purification of the enzyme and hence the elucidation of its physicochemical properties proved to be most difficult. Much of the early work using impure mammalian enzyme suggested a great deal of similarity between it and the enzyme from E. coli. Recent studies with more homogeneous preparations have shown that while the two enzymes do possess

some common features, the mammalian ribonucleotide reductase is a more complex entity.

Like the E. coli enzyme, mammalian ribonucleotide reductase reduces ribonucleoside diphosphates under the allosteric regulation of nucleoside triphosphates (Larsson 1973, Thelander and Reichard 1979). The substrate and effector binding sites are distinct from one another and as will be discussed below there are apparently two classes of binding sites for the allosteric effecters (Eriksson et al 1981). As with the E. coli enzyme, mammalian ribonucleotide reductase requires iron (Engström et al 1979, Youdale et al 1982) but not 5'-deoxyadenosyl B12 (Fujioka and Silber 1969) for activity. Åkerblom et al (1981) were able to generate a mutant mouse fibroblast 3T6 cell line that was resistant to hydroxyurea as previously described by this laboratory (Wright et al 1980, Wright et al 1981). This mutant which exhibited increased levels of ribonucleotide reductase activity had an electron paramagnetic resonance (EPR) spectrum very similar to that of the tyrosine radical of the bacterial enzyme. Thus like the E. coli ribonucleotide reductase, the mammalian enzyme's reaction mechanism would appear to involve a tyrosine free radical stabilized by the presence of the non-heme iron.

Many of the properties of mammalian ribonucleotide reductase are very similar to those seen in the E. coli enzyme and until very recently, it was assumed that the enzymes from the two sources were virtually identical. Mammalian ribonucleotide reductase was first purified to a high degree from calf thymus (Eriksson et al 1977a, Engström et al 1979) according to the method summarized in Table 1. When samples were analysed by electrophoresis on polyacrylamide gels containing glycerol a single assymetric band, indicative of a highly purified preparation, was observed. Using a method patterned on that depicted in Table 1 the

Table 1. Purification of calf thymus ribonucleotide reductase from 2.7 kg of thymus.

	protein (mg)	total act. (units)	sp act. (units/mg)
crude extract	115,500	800[a]	0.007[a]
streptomycin supernatant	90,000	900[a]	0.01[a]
ammonium sulfate	26,550	800	0.03
DEAE-cellulose	8,010	640	0.08
hydroxylapatite	1,100	429	0.39
dATP-Sepharose	10	237	23.7

[a]Approximate figures since the extracts at this level of purity contain inhibitory factors. The assays were performed at 25°C, and the values obtained were doubled.

(taken from Engström et al 1979)

purification to homogeneity of two non-identical subunits of calf thymus ribonucleotide reductase was achieved (Mattaliano et al 1981). Other methods including chromatography on deoxyguanosine triphosphate Sepharose (Chang and Cheng 1979c) or blue dextran Sepharose (Cory et al 1978) have proven successful in the separation of ribonucleotide reductase into two protein fractions, each of which is required for activity. One of the subunits, designated M1, was shown to have a molecular weight of 84,000 and to possess the binding sites for the nucleoside triphosphate allosteric effectors (Eriksson et al 1982). This subunit would seem to correspond to the B1 subunit of the E. coli enzyme. The second subunit isolated was found to have a molecular weight of 55,000 and to contain non-heme iron. It has also been established that this iron-containing subunit, designated M2, possesses the tyrosine free radical found in the enzyme (Åkerblom et al 1981). Thus the M2 protein bears a very strong resemblence to the B2 subunit of the E. coli enzyme. Subsequent analysis of the M2 subunit on the nondenaturing gel, Ultragel AcA34, gave a molecular weight of 110,000 (Thelander et al 1980). Thus it would seem that in its active form M2 exists as a dimer. Active ribonucleotide reductase was found to consist of a one to one ratio of the M1 and M2 subunits. However, with homogeneous preparations to date, it would seem that subunit M2 is present in non-stoichiometric amounts within mammalian cells (Engström et al 1979, Mattaliano et al 1981). Magnesium ions stimulate the mammalian reductase activity but unlike the situation with the bacterial enzyme, they are not absolutely essential for the association of the two protein subunits.

Data from the calf thymus enzyme indicated that a single enzyme catalyzes the reduction of all four ribonucleoside diphosphates and that, like the E. coli enzyme, substrate specificity and activity are

controlled by allosteric effectors (Eriksson et al 1979). These conclusions were based on the following evidence. During purification of the ribonucleotide reductase, measurements of GDP and CDP reduction at different stages showed complete co-purification of the two activities. Furthermore, all four ribonucleoside diphosphate substrates were reduced at comparable rates by the purified enzyme and there was shown to exist a direct competition between CDP and GDP for the same catalytic site. Human diploid fibroblasts have been shown to reduce CDP and ADP at comparable rates (Dick and Wright 1981) while competition between ribonucleoside diphosphates has also been observed with the enzyme from human Molt-4F cells (Chang and Cheng 1979a). Additional proof was supplied by analytical glycerol gradient centrifugation experiments (Engström et al 1979). On these non-denaturing gels, the highly purified enzyme showed only one slightly assymetric band and one major protein peak at about 9S. While the above results support the idea of one enzyme reducing all four ribonucleotides, other investigators have found evidence which suggests the existence of more than one enzyme or at least variable associations of a multisubunit enzyme.

During the purification of ribonucleotide reductase from Ehrlich tumor cells it was observed that the various steps did not always result in enzyme fractions that had a constant ratio of CDP to ADP reductase activities (Cory and Mansell 1975a). Moreover, the tumor cells reduced the four ribonucleoside diphosphate substrates at different rates varying over a 4-fold range. Other cell lines including Chinese hamster ovary (Lewis et al 1978, Hards and Wright 1981), mouse L (Kuzik and Wright 1979) and rabbit bone marrow (Hopper 1978) also have been shown to exhibit differences in the rate of reduction of the various substrates. More significantly, UDP did not inhibit the reduction of CDP in Ehrlich

cell extracts even when present in a 25-fold excess (Cory and Mansell 1975a). The reduction of the four ribonucleotides by partially purified extracts of Ehrlich cells showed vastly different sensitivities to inhibition by pyridoxal phosphate, Triton X-100 and blue dextran (Cory 1979). According to the investigator who performed the inhibition studies "the data suggest that the mammalian enzyme system consists of multiple, independent substrate binding sites (or proteins)." Other pieces of information also suggest that a single enzyme may not be responsible for the reduction of all four ribonucleotides. In regenerating rat liver actinomycin D was reported to only partly interfere with the appearance of ADP reductase while completely preventing that of CDP reductase (Collins et al 1972). In the same study, after acidification of the extract to pH 5 most of the CDP reductase activity remained soluble while the precipitate contained most of the ADP reductase activity. The progression of synchronized mammalian cells through the cell cycle gives rise to some interesting observations concerning the possibility of there existing more than a single ribonucleotide reductase. Using cultured Chinese hamster fibroblasts it was shown that CDP and ADP reductase levels varied independently throughout the cell cycle (Peterson and Moore 1974,1976). The ratio of CDP to ADP reductase activity varied from 0.26 in G1 and early S phase to 0.83 in late S and G2 phase while exponentially growing cells exhibited a ratio of 2.39. Chinese hamster ovary cells released from isoleucine starvation showed a peak of GDP reductase activity 14 hours after release while the peak of CDP reductase activity did not occur until 16 hours (Lewis et al 1978). Because both the relative activity levels and the time of maximum activities for the reduction of various substrates differ in synchronized cells it would seem a single enzyme could not be

responsible for the reduction of all four substrates.

The selection of somatic cell mutants possessing altered ribonucleotide reductase activities have also been supportive of the existence of more than one enzyme or multiple substrate binding subunits. Hydroxyurea, an inhibitor of ribonucleotide reductase activity, was used to select drug-resistant mutants with altered levels of enzyme activity (Wright et al 1980, Wright et al 1981). Several of the mutants isolated showed large increases in CDP reductase levels with smaller increases observed for ADP reductase levels (Koropatnick and Wright 1980). Instances also occurred where CDP reductase levels increased while those of ADP remained near wild-type levels. It is interesting to note that while CDP and ADP reductase levels did sometimes increase in a co-ordinated manner, never was a greater increase in ADP reductase levels over those of CDP reductase observed. These results again seem to argue against a single enzyme, similar to the E. coli reductase, being responsibile for the reduction of all four ribonucleotides.

While the various studies cited above indicated that multiple forms of ribonucleotide reductase might exist in at least some mammalian cells, it has only been very recently that highly purified protein fractions involved in the reduction of single ribonucleoside diphosphates have been isolated. Using regenerating rat liver as the enzyme source, Youdale and MacManus (1979) were able to isolate a protein fraction specific for the reduction of CDP. The critical separation of reductase activities occurred when partially purified extract was applied to a dATP Sepharose column. Washing the column with buffer (100 mM Tris HCl, 10 mM $MgCl_2$ and 2 mM DTT pH 7.6) eluted a fraction able to reduce ADP, UDP and GDP but not CDP. Reduction of CDP required two protein fractions, one which eluted with the buffer plus a fraction eluted with buffer containing

50 mM ATP. Further work lead to the purification to homogeneity of the two non-identical fractions (Youdale and MacManus 1981). The L1 subunit, which bound to the dATP column and was eluted with ATP, had an apparent molecular weight of 45,000, bound the substrate and exhibited a Km of 0.09 mM CDP. Subunit L2, which did not bind to the dATP Sepharose column had an apparent molecular weight of 100,000 and was shown to contain iron. Thus the L2 subunit of the rat liver system would seem to be analogous to the M2 subunit isolated from calf thymus. However, analysis of the L2 subunit by sodium dodecyl sulfate electrophoresis indicated the presence of two non-identical components of molecular weight 45,000 and 75,000 (Youdale and MacManus 1982) and not the dimer seen with the M2 subunit (Mattaliano et al 1981).

Youdale et al (1982) presented a table (Table 2) which listed the relative molecular weights of the subunits of ribonucleotide reductase isolated from various mammalian sources and suggested a model to account for the differences observed. This model assumes that there exists a single L2 subunit but four distinct L1 subunits, one specific for the reduction of each ribonucleoside diphosphate. Each of these L1 subunits is proposed to have a molecular weight of 45,000. In regards to their data the authors believe they have "some how specifically isolated an L1 subunit which, when combined with the L2 subunit, forms an exclusively CDP reducing holoenzyme" and that other investigators have isolated protein complexes of various numbers and types of L1 subunits. According to this model the complete multisubunit holoenzyme should have a relative molecular weight of 280,000, the value obtained for regenerating rat liver. The average relative molecular weight value of 90,000 reported for the L1 equivalent of calf thymus ribonucleotide reductase (M1) can be explained by this model if it is assumed that the

Table 2. Relative molecular weights of mammalian subunits of ribonucleotide reductase from different sources.

Source	Method of M_r determination[a]	dATP-binding subunit (L_1 equivalent)	Iron-containing subunit (L_2 equivalent)	Holoenzyme
Regenerating rat liver	A	45,000 (reduces only CDP)	120,000	280,000
	B	45,000 (reduces only CDP)		
Novikoff ascites	C	180,000	NA[b]	250,000
	B	90,000		
Calf thymus	C	98,000 (reduces all four ribo-nucleotides)	110,000	
	B	84,000 (reduces all four ribo-nucleotides)		
Human lymphoblast	E	100,000 (reduces all four ribo-nucleotides)	100,000	210,000
Erlich tumour cells	D	127,000	75,000	313,000

[a]Method of M_r determination by molecular exclusion HPLC (A), SDS-polyacrylamide gel electrophoresis (B), gel filtration (C), sedimentation equilibrium (D), or sedimentation velocity (E).

[b]Data not available

(taken from Youdale et al 1982)

species isolated was made up of two L1 proteins. The ability of the calf thymus preparation to reduce all four ribonucleotides can be accounted for as the L1 (M1) isolate would have various pairings of each substrate specific subunit. Thus the 90,000 molecular weight species isolated would contain a mixture of ($L1^{CDP} \cdot L1^{UDP}$), ($L1^{ADP} \cdot L1^{GDP}$), ($L1^{CDP} \cdot L1^{ADP}$), etc., all of which presumably would have similar physical characteristics, and hence would co-purify with each other to give the impression of there being a single molecule capable of reducing all four ribonucleotides.

Whether or not multiple L1 subunits, specific for single ribonucleoside diphosphate substrates, do exist still remains largely unresolved. To date, the only studies involving purified ribonucleotide reductase have used calf thymus and regenerating rat liver as the enzyme source. Although it is unlikely, it may be that the observed differences reflect the existence of two distinct ribonucleotide reductase activities within the two systems studied. More work on the enzyme from calf thymus and rat liver as well as from other sources is required before a final judgement can be made.

3. HYDROGEN DONOR SYSTEM OF MAMMALIAN RIBONUCLEOTIDE REDUCTASE

The reduction of ribonucleotides to deoxyribonucleotides in mammalian cells requires the presence of a hydrogen donor system. As is the case for *E. coli* cells, mammalian cells possess a thioredoxin system consisting of thioredoxin, thioredoxin reductase and NADPH (Moore 1967, Proqué *et al* 1970, Wagner and Follman 1977) that functions in the reduction of ribonucleotides as depicted for the *E. coli* system previously. Thioredoxin has been purified to homogeneity from rat Novikoff ascites hepatoma (Herrmann and Moore 1973) and calf liver (Engström *et al* 1974) cells. In both cases, the molecule was

found to be heat-resistant, had a molecular weight of between 11,400 and 12,100 and had valine as the sole amino-terminal amino acid. Amino acid analysis of calf liver thioredoxin showed it to contain 103 residues, including four half-cystines. In vitro oxidation of the reduced form of thioredoxin indicated that only two of the half-cystine residues formed an oxidation-reduction active disulfide bridge. The active center sequence of mammalian thioredoxin, -cys-gly-pro-cys-, was found to be identical to that of E. coli thioredoxin. In fact, the two molecules are similar in many ways including size (108 amino acid residues for E. coli versus 103 for mammals), heat-stability and a general cross-reactivity between bacterial and mammalian thioredoxin $-(SH)_2$ and ribonucleotide reductase (Luthman et al 1979). Thus it would seem that thioredoxin structure has been highly conserved during evolution.

Thioredoxin reductase has been isolated and purified from calf thymus and liver (Holmgren 1977) and Novikoff rat tumors (Chen et al 1977). The purest preparation, that from rat tumor cells, contained two subunits of molecular weight 58,000 with one FAD molecule per subunit. Calf liver thioredoxin reductase had an apparent Km value of 5 μM for thioredoxin. This value corresponds well with the thioredoxin concentration of 5 to 10 μM determined for calf liver.

While the thioredoxin system can act as the hydrogen donor for mammalian ribonucleotide reduction in vitro, its in vivo role has been questioned. The subcellular distribution of calf thymus thioredoxin, which shows multiple locations including membrane associations (Holmgren and Luthman 1978) suggests that the majority of thioredoxin in mammalian cells functions in protein thiol-disulfide interchange reactions unrelated to ribonucleotide reduction (Holmgren 1980). One

of these reactions may be to catalyze the reduction of insulin. The first step in the physiological degradation of insulin is the enzymatic reduction of its disulfide bonds followed by proteolytic degradation of free A and B chains by "insulinase" (Czech 1977, Varandani et al 1972, Freedman 1979). The exceptional reactivity of thioredoxin $-(SH)_2$ with insulin in vitro is suggestive of a physiological role for mammalian thioredoxin in the degradation of insulin (Holmgren 1979c, 1979d). The reduction of insulin yields thioredoxin $-S_2$ and there has been speculation that the oxidized molecule may generate a signal in the cell via oxidation of certain dithiols to disulfides in key enzymes, with a concomitant change in enzyme activity. As thioredoxin has been associated with cell membranes it may be that the molecule is also associated with insulin receptors and thus the degradation of insulin may be coupled to its hormonal effect through thioredoxin (Holmgren 1979c).

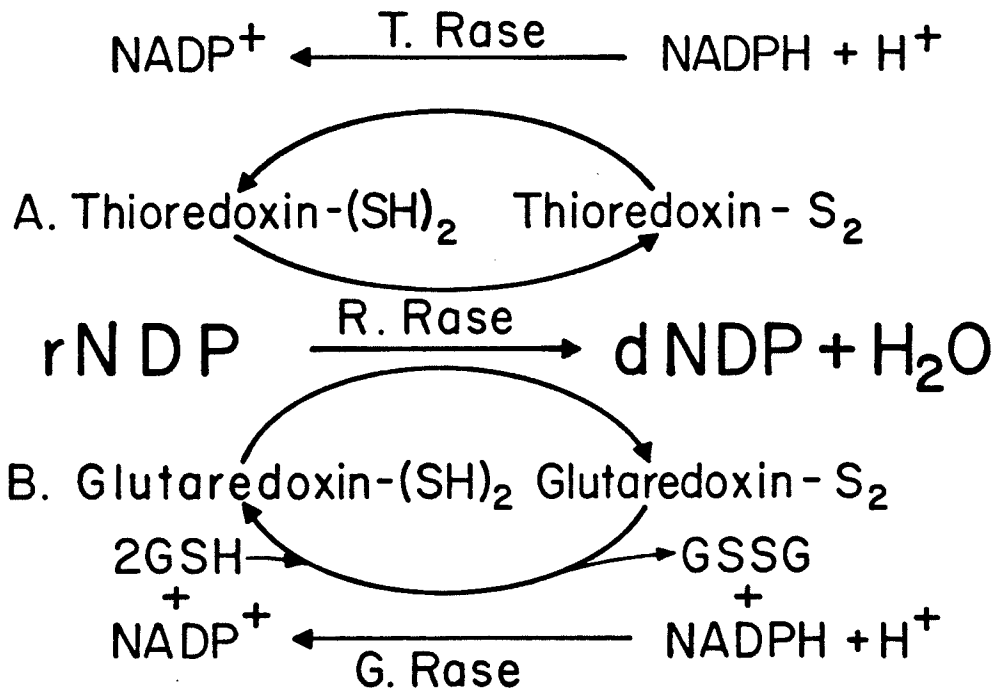
Further evidence that thioredoxin does not supply ribonucleotide reductase with hydrogen atoms in vivo was supplied from work with thioredoxin and thioredoxin reductase of rabbit bone marrow. Thioredoxin purified more than 500- fold was found to catalyze the reduction of insulin by dithiothreitol (Hopper and Iurlano 1980) and to serve as a hydrogen donor for the reduction of methionine sulfoxide by methionine sulfoxide reductase of yeast (Hopper and Iurlano 1982). However, the bone marrow thioredoxin failed to act as a hydrogen donor for the homologous ribonucleotide reductase (Hopper and Iurlano 1982).

There is growing evidence that another small molecule, glutaredoxin, may be the physiological hydrogen donor for mammalian ribonucleotide reductase just as it is thought to be in E. coli cells (Holmgren 1979a,b). Glutaredoxin from calf thymus has been isolated and partially purified (Luthman et al 1979). Glutaredoxin was found to have a molecular

weight of 12,000 as determined by gel chromatography, to be heat-stable, to be active with ribonucleotide reductase in the presence of reduced glutathione, NADPH and glutathione reductase but to have no activity with NADPH and thioredoxin reductase and to be immunologically different from thioredoxin as it did not bind to antithioredoxin immunoadsorbants. Glutaredoxin was also found to be unlike thioredoxin in that it exhibited a high degree of species specificity. Whereas the ribonucleotide reductases and thioredoxins of calf thymus and E. coli cells were cross-reactive, the glutaredoxin molecules of the two sources did not exhibit such a pattern.

The results discussed above have led to the proposal that glutaredoxin is the major supplier of hydrogens to mammalian ribonucleotide reductase while thioredoxin functions as a general carrier of hydrogens and is not specific for ribonucleotide reductase. One hypothesis suggests that both glutaredoxin and theoredoxin are involved in the reduction of ribonucleotides (Holmgren 1981). According to this model, the glutaredoxin system is involved in DNA replication as one component of a multienzyme complex which includes ribonucleotide reductase. Thioredoxin is proposed to function with "free" ribonucleotide reductase to supply deoxyribonucleotides for DNA repair and error prone DNA replication. At this time the hypothesis cannot be critically evaluated as no mutants with lesions in the gene for glutaredoxin are available. A summary of the thioredoxin and glutaredoxin systems and their interaction with ribonucleotide reductase is presented in figure 2.

Fig. 2. Sources of reducing power for mammalian ribonucleotide reductase. (T. Rase = thioredoxin reductase, R. Rase = ribonucleotide reductase, G. Rase = glutathione reductase.)


NADP+
T. Rase
NADPH + H+
A. Thioredoxin-(SH)2
Thioredoxin-S2
rNDP
R. Rase
dNDP + H2O
B. Glutaredoxin-(SH)2
Glutaredoxin-S2
2GSH
+
NADP+
GSSG
+
NADPH + H+
G. Rase

4. REGULATION OF MAMMALIAN RIBONUCLEOTIDE REDUCTASE

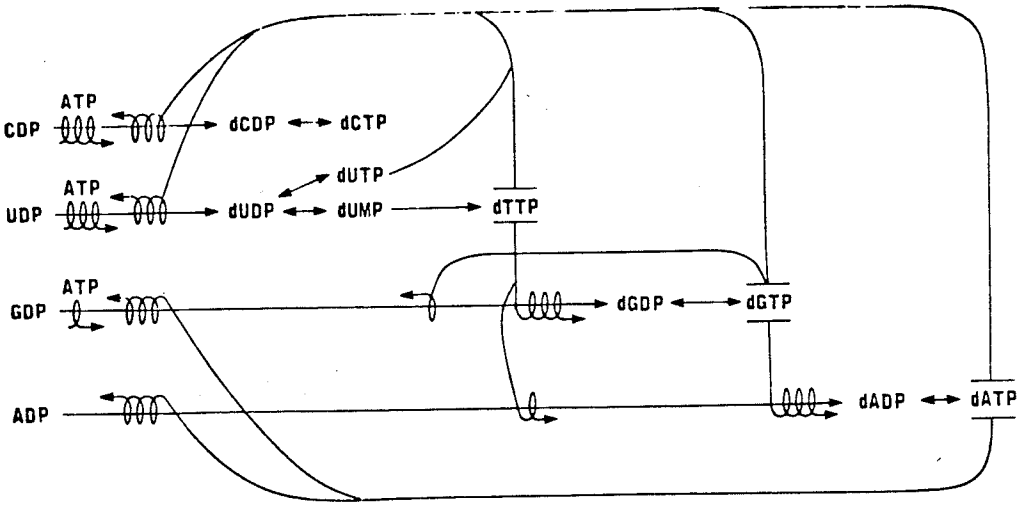
Because of ribonucleotide reductase's key role in the replication of DNA, stringent regulation of its activity is expected. This is indeed the case as enzyme activity is regulated in an allosteric manner and by de novo synthesis.

4.1 Allosteric Regulation

Early studies using partially purified ribonucleotide reductase preparations from Novikoff rat tumors were able to show the enzyme responded to several nucleoside triphosphates (Moore and Hurlbert 1966). The reduction of CDP and UDP to dCDP and dUDP required activation of the enzyme by ATP. Reduction of GDP required the presence of dTTP while the reduction of ADP was activated by dGTP or dTTP. Further stimulation of GDP and ADP reduction could be achieved if in addition to dGTP and dTTP, ATP was also present. The reduction of all four ribonucleotides was inhibited by the presence of dATP. The reduction of CDP and UDP was also inhibited by dTTP and dGTP while the reduction of GDP was also inhibited by dGTP. A virtually identical pattern of regulation seems to exist in all mammalian cells including mouse L (Kuzik and Wright 1979, Kucera and Paulus 1982b), Chinese hamster ovary (Lewis et al 1978, Wright et al 1981, Hards and Wright 1981), rat embryo (Murphree et al 1968), calf thymus (Eriksson et al 1979), human Molt-4F (Chang and Cheng 1979b) and human diploid fibroblast (Dick and Wright 1980) cells. A summary of the regulatory pattern is given in figure 3.

Minor variations within the pattern have been noted for ribonucleotide reductase isolated from different sources. For example, GTP was found to significantly activate ADP reduction in human Molt-4F (Chang and Cheng 1979b) and Chinese hamster ovary (Hards and Wright

Fig. 3. Sequence of stimulations and inhibitions by nucleotides in ribonucleotide reductase of rat hepatoma cells (taken from Moore and Hurlbert 1966). Spirals and arrows pointing to right symbolize activation; those to left symbolize inhibition.


ATP
CDP
dCDP
dCTP
dUTP
ATP
UDP
dUDP
dUMP
dTTP
ATP
GDP
dGDP
dGTP
ADP
dADP
dATP

1981) cells. While dATP has been found to inhibit the reduction of all ribonucleotides when present in high concentrations, the situation with low concentrations is less clear. In the presence of optimal activator concentrations, dATP was found to inhibit the reduction of both purines and pyrimidines by mouse L cells (Kucera and Paulus 1982b) with 1 mM being the lowest concentration tested while with Chinese hamster ovary cells tested in the same way (Lewis et al 1978) 2.5 μM dATP was found to further stimulate CDP reduction by 15% and 40 μM dATP was found to cause a 10% increase in GDP reductase activity. To further complicate the issue dATP was found to inhibit the reduction of ADP in human diploid fibroblasts (Dick and Wright 1980) at all concentrations tested while 5 μM dATP was found to stimulate CDP reduction.

As the pattern of allosteric regulation of mammalian ribonucleotide reductase was very similar to that observed with the E. coli enzyme (Larsson and Reichard 1966a,b) it was assumed that like the E. coli enzyme (Brown and Reichard 1969b), mammalian ribonucleotide reductase had two classes of allosteric binding sites. One class was thought to bind dATP and ATP and to control overall enzyme activity while the other was believed to bind ATP, dATP, dGTP and dTTP and to control substrate specificity of the enzyme. The allosteric effectors were thought to control specificity by inducing conformations of the catalytic site that fit only one specific substrate. By inducing a conformation of the enzyme that was functional for only one substrate the allosteric effectors had the additional property of inhibiting the reduction of the other three substrates (Eriksson et al 1979). DeoxyATP bound to the activity site was believed to block the reduction of all ribonucleotides by causing a conformational change which led to the aggregation of the enzyme (Thelander et al 1980). This inhibition could

be overcome by ATP which also was believed to bind to the activity site (Eriksson et al 1979). The ability of ATP to further stimulate purine reduction already activated by dGTP, GTP or dTTP was postulated to be due to its binding at the activity site. It was supposed that the conformation of the enzyme with ATP bound at the activity site had a higher turnover rate than the conformation of the enzyme with its activity site empty. As shown by studies using ribonucleotide reductase isolated from human Molt-4F cells not only did ATP increase the velocity of ADP and GDP reduction, it also decreased the apparent Ka values for the respective allosteric effectors GTP and dTTP.

Because of the difficulty encountered in the purification of ribonucleotide reductase, it has only been very recently that binding studies with purified calf thymus extract have been undertaken (Thelander et al 1980). As discussed earlier, the M1 subunit of calf thymus ribonucleotide reductase was found to have a molecular weight of 84,000 when analysed using soidum dodecyl sulfate electrophoresis. However analysis under non-denaturing conditions (glycerol gradient centrifugation) in the absence of effectors revealed the subunit to exist in a monomer-dimer equilibrium. Addition of dTTP caused a shift in the equilibrium such that the dimer form of molecular weight 170,000 was found exclusively. Aggregation of the nucleotide-binding subunit of ribonucleotide reductase extracted from Ehrlich tumor cells had been reported earlier (Klippenstein and Cory 1978). In this instance, ATP and dATP were found to induce association of nucleoside triphosphate binding subunits while at the same time causing a dissociation of the iron containing subunit. This dissociation led to inactivation of the enzyme. DeoxyGTP had little if any effect on the sedimentation pattern of ribonucleotide reductase isolated from

Ehrlich tumor cells. It is unclear as to whether the differences observed for calf thymus and Ehrlich tumor cells were due to variations in experimental procedures used or to fundamental differences within the two systems under study. Preliminary results using ribonucleotide reductase isolated from human cells also indicated that the sedimentation rates of the enzyme were greater in the presence of regulators such as ATP or dGTP (Chang and Cheng 1976). An interesting aspect of the association of the M1 subunits of calf thymus enzyme induced by the allosteric effectors is the similarity in molecular weights between the resulting dimer (Thelander et al 1980) and the multisubunit complex proposed by Youdale et al (1982). It is possible that the presence of the allosteric effectors induces the association of the four specific L1 subunits proposed by Youdale et al (1982) to exist.

Equilibrium dialysis binding experiments were carried out using the purified calf thymus M1 subunit (Thelander et al 1980). Using the dimer molecular weight of 170,000 to calculate the number of binding sites per M1 molecule, it was found that dATP bound to two sites while dTTP bound to only one per dimer. In both cases, cooperativity of binding was observed with final dissociation constants of 3×10^{-7} M and 2×10^{-6} M for dATP and dTTP, respectively. Competition studies showed that dGTP and dTTP could block the binding of dATP to one of the sites while ATP could block the binding at both sites. The inhibition of dATP binding by ATP seemed to occur in two phases with a 2000-fold excess of ATP over dATP sufficient to remove dATP from one site while a 10,000-fold excess was required to remove 90% of the bound dATP. The competition studies also revealed that both dATP and ATP could displace dTTP bound to the M1 subunit. Taken together the results indicated that protein M1 contains two types of effector binding

sites, one specific for ATP and dATP and the other capable of binding ATP, dATP, dTTP and dGTP. This situation is very similar to that encountered with subunit B1 of the E. coli enzyme and tends to support the contention that allosteric regulation in the two systems is very similar. There do however remain certain discrepancies which for the time being remain unexplanable. It would seem likely that the number of binding sites per dimer should be some multiple of two, but results indicated a single dTTP site per dimer. Also, the two sites do not appear to possess different affinities for dATP as observed with the E. coli enzyme.

The presence of the two different classes of allosteric binding sites has been confirmed genetically through the selection of mutant T lymphoma cells which possessed ribonucleotide reductase activities with altered regulation patterns (Ullman et al 1979, 1980, 1981, Eriksson et al 1981a,b). From a mutagenized population of lymphoma cells were selected six colonies resistant to the cytotoxic effects of 200 μM deoxyguanosine. One of the colonies, dGuo-200-1, also proved to be less sensitive to inhibition by deoxyadenosine and thymidine (Ullman et al 1980). Analysis of the ribonucleotide reductase of dGuo-200-1 cells revealed it to possess two types of M1 subunit based upon separation on a dATP-Sepharose column (Eriksson et al 1981a). With one subunit, CDP reduction was stimulated and not inhibited by dATP while with the other, normal regulation was exhibited. GDP reduction using the mutant M1 subunit from dGuo-200-1 cells required dTTP (the specificity effector) at the same concentration as did wild type protein M1, but unexpectedly, GDP reduction was stimulated 2-fold when dATP and dTTP were combine. The mutant thus seemed to have lost the ability to distinguish between dATP and ATP while retaining normal sensitivity to dTTP and dGTP. From the results it was concluded that

one of the M1 subunits produced by dGuo-200-1 cells was altered at the activity site. A second mutant selected for resistance to 200 μM deoxyguanosine but with normal sensitivity to deoxyadenosine was selected and designated dGuo-L (Ullman et al 1979). Analysis of the CDP reductase activity of this mutant showed it to be resistant to the inhibitory effects of dGTP and dTTP while retaining normal sensitivity to dATP (Ullman et al 1981). The dGuo-L protein also showed a decreased capacity to use ADP as a substrate. As was the case with the dGuo-200-1 cell line, the dGuo-L cells were found to possess equal amounts of normal and altered M1 subunit. It would thus appear that the cultured mouse T-lymphoma cells are functionally diploid for the gene coding for the M1 subunit of ribonucleotide reductase and that in the two mutants described one gene is altered in each case. A third mutant isolated from a mutagenized population of wild type cells by selection in medium containing 0.5 mM hypoxanthine, 0.4 μM amethopterin and 1.5 mM thymidine, and designated HAT-1.5A was found to possess a CDP reductase kinetically indistinguishable from that of dGuo-L cells. The results indicated the dGuo-L and HAT-1.5A cells had a mutant protein M1 altered in the regulation of substrate specificity.

From a mutagenized population of dGuo-L cells, aphidicolin was used to select a cell line, dGuo-L-Aphid-G5, whose CDP reductase activity was found to be resistant to dATP as well as dGTP inhibition (Ullman et al 1981). Chromatography of the protein M1 subunits on dATP-Sepharose resolved two kinetically distinct components. With the first, CDP reduction was resistant to inhibition by dGTP but normally sensitive to dATP while with the second dGTP caused normal inhibition but dATP was stimulatory not inhibitory. Thus the double mutants would seem to possess one M1 subunit like that isolated from

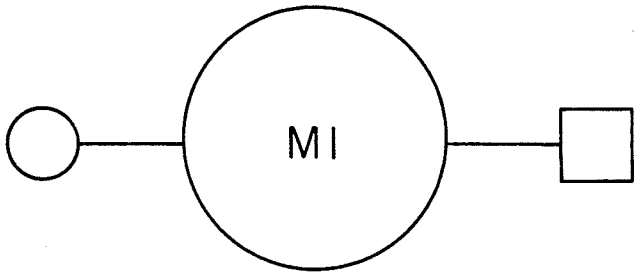
dGuo-200-1 and a second like that isolated from dGuo-L and HAT-1.5A. The existence of this mutant confirmed that the dGuo-L parent was heterozygous for protein M1, containing one wild type and one mutant allele.

The data obtained from analysis of the M1 proteins isolated from the various mutant lymphoma cells support the binding studies previously described above. According to the proposed model there exists two classes of allosteric binding sites. One site binds ATP and dATP and controls overall enzyme activity with ATP being the positive effector and dATP the negative. The second class of site binds ATP, dATP, dTTP and dGTP and determines the substrate specificity of the enzyme. A summary of the model and the various regulatory mutants discussed is presented in figure 4.

Based on current knowledge, the physiological allosteric regulation of mammalian ribonucleotide reductase may be visualized as follows. Binding of ATP to the M1 subunits leads to a dimerization or possibly tetramerization of the protein. As discussed above, the polymerized entity contains two classes of allosteric binding sites. Where ATP is bound to both sites, the reduction of CDP and UDP to dCDP and dUDP results. DeoxyUDP is then further metabolized to dTTP which displaces ATP from the specificity site and induces the reduction of GDP to dGDP. Phosphorylation of this product produces dGTP which displaces dTTP to cause conformational changes which result in the reduction of ADP. This causes a subsequent increase in dATP levels within a cell. DeoxyATP then displaces ATP from the activity site and inactivation of the enzyme occurs. This inactivation would seem to be accompanied by aggregation of ribonucleotide reductase molecules.

Discussion concerning the allosteric regulation of mammalian

Fig. 4. Schematic model for the allosteric regulation of protein M1 of ribonucleotide reductase in S49 wild-type and mutant cells (taken from Eriksson et al 1981b). The actual number of binding sites per protein M1 molecule has not been determined but is represented as one activity site and one specificity site. UDP reduction has not been measured with the S49 ribonucleotide reductase but by analogy to other known mammalian enzymes (Thelander and Reichard 1979, Eriksson et al 1979) it has been included, in parentheses.



Activity site		Specificity site	
Binds:	Effects:	Binds:	Substrate reduced:
ATP	activation	ATP, dATP	CDP (UDP)
dATP†	inhibition†	dTTP*	GDP
		dGTP*	ADP*

†Altered in protein M1 from dGuo-200-1 and dGuo-L-Aphid-G5

*Altered in protein M1 from dGuo-L, HAT-1.5 and dGuo-L-Aphid-G5

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ribonucleotide reductase has thus far assumed the existence of a single enzyme capable of reducing all four ribonucleotides. The systems in which the regulation has been studied, calf thymus (Eriksson et al 1979) and mouse lymphoma cells (Eriksson et al 1981b, 1982), indicated the existence of a single enzyme and thus the results have been interpreted on this basis. Other systems, notably that of rat liver (Youdale et al 1981, 1982) suggested the reduction of the various ribonucleoside diphosphates required separate and distinct proteins. As these discoveries are very recent, no work concerning the allosteric regulation of reduction in this system has yet been reported.

While the majority of studies reported to date support the general model of ribonucleotide reductase regulation depicted in figure 3, there are a couple which do not. By manipulating ribo- and deoxyribonucleoside triphosphate concentrations through the use of drugs or naturally occurring deoxyribonucleosides, an attempt was made to study the in vivo regulation of the enzyme found in cultured Chinese hamster ovary cells (Hunting and Henderson 1981a,b, Hunting et al 1981). While portions of the model were found to be applicable to the in vivo situation, it was found that increased dGTP levels failed to increase ADP reduction or inhibit CDP reduction. Furthermore, increased dATP levels seemed to have no effect on CDP reduction. Conclusions drawn from these results however must be viewed with some caution as they are based on the assumption that changes in measured deoxyribonucleoside triphosphate levels correlate with intracellular changes in the rate of ribonucleotide reduction. There is some evidence that this may not always be so. Studies with somatic cell mutants possessing elevated levels of ribonucleotide reductase activity have shown a concomitant increase in deoxynucleoside triphosphate levels does not always occur

(Åkerblom et al 1981, Ullman et al 1979, Ullman et al 1980). Moreover, studies with intact permeabilized Chinese hamster ovary cells did show that dGTP does inhibit CDP reduction and activate ADP reduction and that dATP does inhibit CDP reduction (Hards and Wright 1981, Wright et al 1981).

4.2 Regulation of Enzyme Synthesis

As discussed above, ribonucleotide reductase activity levels are a function of the cell cycle with activity peaking in S phase and then declining to a low in G1 cells (Murphree et al 1969, Peterson and Moore 1976, Lewis et al 1978). It would seem that the observed increases were due to de novo synthesis of ribonucleotide reductase. When cycloheximide, an inhibitor of protein synthesis in mammalian cells, was added to either logarithmically growing or synchronized mouse L cells in S phase, at a concentration of 1 µg per ml of growth medium, ribonucleotide reductase activity was found to decay exponentially with a half-life of approximately 2 hours (Turner et al 1968). This half-life represented one of the shortest measured in mammalian tissue (Schimke and Doyle 1970). Untreated exponentially growing cells maintained an unaltered level of activity. Cycloheximide did not act directly upon the reductase as cell free extracts assayed in the presence of up to 100 µg of drug/ml showed no inhibition. These results suggested that in vivo ribonucleotide reductase was labile. The addition of actinomycin D, an inhibitor of RNA synthesis, at concentrations of either 1 or 10 µg per ml of culture media to synchronized Chinese hamster fibroblasts during the G1 phase, blocked the increase in ribonucleotide reductase activity usually observed as the cells passed through S phase (Murphree et al 1969). These results suggest that both RNA and

protein synthesis are required for the cell cycle dependent increase of ribonucleotide reductase.

To preclude the possibility that low periods of reductase activity were due to the presence of soluble inhibitors, mixing experiments with G1 and S phase cells were conducted. The results failed to indicate the presence of an inhibitor.

Ribonucleotide reductase shows characteristic patterns of activity in the developing spleen and thymus of rats (Elford 1971). Intraperetoneal administration of 2.5 µg of cycloheximide, 24 hours prior to analysis, limited the normal increase of the enzyme activity to 40% in the spleen and 10% in the thymus. Injection of cycloheximide at 48 and then 24 hours prior to sacrifice resulted in only 20% of usual levels in spleen and 10% in thymus (Elford 1971). Fifty micrograms of actinomycin D administered 24 hours prior to sacrifice and analysis resulted in a 90% decrease of the enzyme activity in spleen as compared to control values. When levels of ribonucleotide reductase activity were monitored after intraperetoneal injection of either 2.5 µg of cycloheximide or 2 µg of actinomycin D, it was found that after 3 hours the cycloheximide caused a decrease of 50% and actinomycin D one of 60%. Thus the half-life of the enzyme appeared to be 3 hours and that of its mRNA slightly less (Elford 1971). No ribonucleotide reductase activity could be demonstrated in normal adult rat liver (Elford 1972, King and Van Lancker 1969) but 24 hours after partial hepatectomy, the activity was well developed (King and Van Lancker 1969). When 0.2 mg of actinomycin D per kg of body weight was administered intraperetoneally 3 hours after hepatectomy, no increase in reductase activity was observed. The results cited above support the contention that within mammalian cells, increased ribonucleotide reductase activity is dependent upon de novo

synthesis of the enzyme. Moreover, the enzyme would seem to have a rapid turnover rate and its messenger RNA a short half-life.

De novo synthesis of ribonucleotide reductase was studied in tumor cells and found to differ from the situation in non-tumor cells. When Erhlich ascites tumor cells were taken from mice and incubated in culture medium at 37°C for one hour, CDP reductase activity rose to 202% of the initial levels. When 0.01 mM cycloheximide or 25 µg actinomycin D per ml of culture medium was added with the cells, no reduction of CDP reductase activity was observed (Cory et al 1976) even though the drug levels used reduced general protein and RNA synthesis by 87% and 97%, respectively as measured by ^{3}H-leucine and ^{3}H-thymidine incorporation. The ineffectiveness of cycloheximide and actinomycin D to block increases in ribonucleotide reductase activity were also observed with Novikoff hepatomas (Elford 1972). When cycloheximide (1.5 mg per kg of body weight) was administered to tumor-bearing mice a rapid decrease in reductase levels was observed. This decrease reached a maximum 2 hours after drug administration with 40% of the activity of untreated cells remaining. Beyond this point, activity increased until at 24 hours after drug injection the levels had returned to those found in untreated cells. This behaviour contrasts with that described for normal tissue. Two 100 µg doses of actinomycin D intraperetoneally injected into tumor-bearing mice 7 and 4 hours prior to sacrifice decreased ^{3}H-uridine incorporation into RNA by 70% but caused a slight increase in reductase activity. Thus it would seem that de novo synthesis of ribonucleotide reductase in malignant cells has in some way been altered. The increased stability of the mRNA fits with the theory of altered template stability as being the

explanation for the defective mechanisms regulating the control of genetic expression in neoplastic tissue (Pitot 1964). Presumably, malignant cells with templates for enzymes required for the synthesis of DNA more stable than those found in normal cells would have a distinct growth advantage.

De novo synthesis of mammalian ribonucleotide reductase subunits seems to occur in a non-coordinated manner. Treatment of mouse S49 T-lymphoma cells with N^6,O^2-dibutyryl adenosine 3':5'-monophosphate (Bt_2cAMP) resulted in the protein kinase-dependent arrest of the cells in the G1 phase of the cell cycle. Cells treated for more than 16 hours showed a decrease in ribonucleotide reductase activity. This decrease in overall activity was paralleled by a decrease in the activity of the M2 protein subunit with the activity of the M1 protein subunit being altered to a significantly lower degree. Removal of Bt_2cAMP induced an increase in holoenzyme and protein M2 activities but had little effect on protein M1 activity (Eriksson and Martin 1981). The results indicate that fluctuations of ribonucleotide reductase activity during the cell cycle are due to variation in the levels of subunit protein M2 and that subunit protein M1 varies less dramatically and in a manner not coordinated with overall activity.

Centrifugal elutriation of exponentially growing S49 cells could separate them into a 90% pure G1 population, a mixture of G1 and early S phase cells and a 95% pure S phase/G2 population. Whereas the specific catalytic activity of protein subunit M1 was the same in all fractions, that of protein M2 was decreased 60% in the G1 cell population (Eriksson and Martin 1981). The ratio of protein M2 to protein M1 increased from 0.4 in G1 cells to 1.0 in the G2/S cells. These

results support the proposal that cell cycle variation of ribonucleotide reductase activity depends upon the protein M2 subunits.

The ribonucleotide reductase from Ehrlich tumor cells can be separated into two non-identical subunits on a blue dextran Sepharose column (Cory and Fleischer 1979). Both subunits are required for enzyme activity. The effector binding subunit of this study was retained by the column and seemed to correlate with the M1 subunit of calf thymus enzyme. The non-heme iron containing component did not bid to the column and was judged to be analogous to the M2 subunit. When Ehrlich tumor cells were grown in mice there was a steady decrease in ribonucleotide reductase activity in the days following transplantation. It was found that addition of exogeneous effector-binding subunit but not exogeneous non-heme iron subunit would stimualte the reductase activity (Cory and Fleischer 1982). Thus the decrease in activity would seem to be due to a decrease in the effector-binding or M1 subunit.

As discussed above, partial hepatectomy induced increased CDP reductase activity (King and Van Lancker 1969). Maximum activity was found to occur 36 hours after the removal of the tissue (Cory and Fleischer 1982). The enzyme activity could be separated into effector-binding and non-hem iron subunits. When exogeneous non-heme iron component was added to cell-free extracts of the regenerating liver, a slight decrease in activity was detected. On the other hand, addition of exogeneous effector-binding component resulted in a statisically significant increase in activity levels (Cory and Fleischer 1982). While the levels of the non-heme iron subunit did fluctuate in regenerating rat liver, these variations were not

coordinated with those of overall enzyme activity. Maximum levels of the non-heme iron subunit occurred 24 hours after hepatectomy while overall activity peaked at 36 hours. In addition, 48 to 72 hours after hepatectomy there was a 55% decrease in overall enzyme activity but only a 25% decrease in the activity of the non-heme iron component. These results showed that the two components making up active ribonucleotide reductase in regenerating rat liver were not coordinately regulated. The non-heme iron or M2 subunit increased rapidly after hepatectomy and remained elevated for an extended period of time whereas the effector-binding or M1 subunit increased more slowly and decreased more rapidly.

It is interesting to note that while overall ribonucleotide reductase activity seemed to be dependent upon M2 subunit levels in mouse lymphoma cells, the variation in M1 subunit levels seemed to be responsible for the fluctuations of enzyme activity in Ehrlich tumor cells and regenerating rat liver. These differences may represent the existence of fundamentally different methods for the regulation of ribonucleotide reductase synthesis in mammalian cells of differing origins.

The ratio of the two components of the active enzyme present in a cell at any one time may affect the allosteric regulation of the enzyme. Varying the ratio of the two components in vitro generated forms of CDP reductase with differing sensitivities to dATP, dGTP and dTTP (Sato and Cory 1981a).

The interaction of allosteric regulation and de novo synthesis in the control of ribonucleotide reductase activity remains largely unstudied. It would seem that synthesis and subsequent degradation

of the enzyme are responsible for the large changes in activity observed during the cell cycle or periods of intense cellular division. Malignant cells would appear to have lost this ability to regulate the synthesis of ribonucleotide reductase. Allosteric control would seem to function as a fine tuning mechanism responsible for the production of a balanced supply of the four deoxyribonucleotides required for DNA synthesis. DeoxyATP inhibition of the enzyme likely serves to shut down the production of deoxyribonucleotides at the end of DNA replication when they are no longer required in such large amounts. This allosteric inhibition would allow normal cell cycle degradation of the enzyme activity to occur without production of unnecessary deoxyribonucleotides, an energetically wasteful situation.

5. MULTIENZYME COMPLEX AND METABOLIC CHANNELLING

Ribonucleotide reductase activity isolated from Novikoff hepatoma and regenerating rat liver tissue was found to be associated with a membrane fraction derived from the postmicrosomal supernatant (Elford 1972, 1974) after centrifugation in a discontinuous sucrose gradient. This membrane associated ribonucleotide reductase activity had properties similar to those found in other preparations of the enzyme (Elford 1974). Also associated with this particular membrane was much of the cellular content of thymidine kinase (Elford 1972, Baril *et al* 1972), thymidylate synthetase (Baril *et al* 1972), deoxycytidylate deaminase (Elford *et al* 1973) and DNA polymerase (Baril *et al* 1970, 1971). These findings strongly suggested the existence of a multi-enzyme complex, of which ribonucleotide reductase was a component, being involved in the replication of DNA.

The study of Chinese hamster embryo fibroblast extracts also

indicated the existence of a multienzyme complex containing DNA polymerase, thymidine kinase, dihydrofolate reductase, nucleoside diphosphate kinase, thymidylate synthetase, ribonucleotide reductase, topoisomerase, template and nascant DNA (Reddy and Pardee 1980, Reddy et al 1982). Whereas previous work had indicated that ribonucleotide reductase, and hence the multienzyme complex of which it appears to be a component of, was located almost entirely in the cellsap (Elford 1974, Larsson 1969), the studies with the fibroblast cells indicated the activities associated with the complex to be localized in the nuclear region during S phase and in the cytoplasm in quiescent and G1 phase cells (Reddy and Pardee 1980). Analysis of the various enzyme activities on sucrose density gradients showed that the nuclear or S phase enzyme activities all cosedimented while the cyotplasmic or G1 or Go activities did not. As nuclear de novo synthesis of at least nucleoside diphosphate kinase is known not to occur, its increased levels in the nucleus and decreased levels in the cytoplasm as cells progressed from Go to S phase would seem to be due to its translocation into the nucleus. Presumably, the other enzyme activities involved in the multienzyme complex are also translocated from the cytoplasm to the nucleus. Once in the nuclear region, the various enzymes are arranged into the multienzyme complex, which exists for the duration of S phase. The translocation of these enzymes is supported by analysis of the nuclear and cytoplasmic deoxynucleoside triphosphate pools of Chinese hamster ovary cells (Skoog and Bjursell 1974). As cells passed from G1 to S phase there was a 2 to 9-fold concentration of the various nucleotides within the nuclear membrane. Particularly interesting was the observation that S phase cells had their entire dTTP

pool located within the nucleus.

The complex described by Reddy and Pardee (1980) seemed to selectively channel ribonucleoside diphosphates into DNA. When L-α-lysophosphatidylcholine permeabilized fibroblast cells were supplied with dithiothreitol, required for ribonucleotide reductase activity, and formaldehyde plus dl-L-tetrahydrofolate, which generates methylenetetrahydrofolate required for thymidylate synthetase activity, ribonucleoside diphosphates were incorporated into DNA more efficiently than were deoxyribonucleoside triphosphates. There appears to be a very intricate association of the activities within the complex. Inhibition of one activity by a specific inhibitor (eg. hydroxyurea for ribonucleotide reductase, aphidicolin for DNA polymerase or novobiosin for topoisomerase) also inhibited *in vivo* thymidylate synthetase activity measured in S phase cells. *In vitro* none of these agents had an inhibitory effect on thymidylate synthetase activity (Reddy *et al* 1982). The term "replitase" has been proposed for this complex which contains the enzymes required for the synthesis of DNA from ribonucleoside diphosphates and channels the metabolites to the final product.

Studies investigating the effect of purine deoxyribonucleosides on the prevention of hydroxyurea induced inhibition of DNA synthesis indicated that the *de novo* and salvage pathways of purine deoxyribonucleotide synthesis do not converge (Forsdyke and Scott 1980, Scott and Forsdyke 1980). The results were interpreted in terms of a multienzyme complex which among other activities contained ribonucleotide reductase and DNA polymerase. According to the proposed model only those deoxyribonucleotides generated by the reductase within the complex can be used in the synthesis of DNA. Deoxyribonucleotide

triphosphates generated by the salvage pathways are unable to enter the complex for use in the synthesis of DNA. However, deoxyribonucleoside triphosphates generated via the salvage pathways may still regulate ribonucleotide reductase activity by acting as allosteric effectors. Thus according to the model proposed, the regulatory sites of ribonucleotide reductase are accessible to nucleotides outside of the multienzyme complex. Under conditions which slow DNA synthesis, either an imbalance in deoxynucleoside triphosphate levels or the need for DNA repair, it is proposed that the multienzyme complex does not channel deoxyribonucleoside triphosphates towards DNA replication as usual, but instead releases these nucleotides into the intracellular environment. These free nucleotides may then act to regulate ribonucleotide reductase to correct a deoxyribonucleotide imbalance or may be utilized in the repair of DNA. They cannot however be normally used as substrates for the multienzyme complex bound DNA polymerase involved in DNA replication.

The existence of a multienzyme complex capable of channelling ribonucleoside diphosphates to DNA is an attractive hypothesis for several reasons (Holmgren 1981). First, deoxyribonucleoside diphosphates are highly specialized metabolites and their sequestration within the complex means they need not be distributed throughout the cell. Second, DNA chains grow rapidly and at a limited number of intracellular sites. Third, most studies of DNA synthesis _in vitro_ indicate that much higher deoxyribonucleoside triphosphate concentrations than the average intracellular deoxyribonucleoside triphosphate levels measured _in vivo_ are required to maintain maximal incorporation rates. The channelling of ribonucleoside diphosphates to DNA

allows for concentration of the required components within the region they are utilized.

The association of ribonucleotide reductase with other enzymes involved in the synthesis of DNA in a multienzyme complex has also been suggested in studies with E. coli (Lunn and Pigiet 1979) and phage infected bacteria (Allen et al 1980, Allen et al 1981, Cook and Greenberg 1981, Wirak 1981).

6. RIBONUCLEOTIDE REDUCTASE AS A SITE OF DRUG ACTION

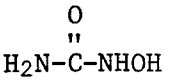
Because of the key role ribonucleotide reductase plays in the process of cell growth, there is a great deal of interest in drugs that can control its intracellular activity. Such drugs are of particular interest in the field of cancer chemotherapy as there seems to be a direct correlation between levels of the enzyme and rate of malignant cell growth (Elford 1972, Weber 1981). Drugs effective in controlling ribonucleotide reductase activity are depicted in Table 3.

6.1 Hydroxyurea

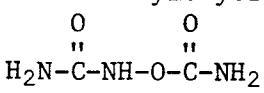
To date, the drug most widely used in the control of ribonucleotide reductase activity has been hydroxyurea. First synthesized in 1869 (Dresler and Stein 1869), current interest in the drug arose from a 1960 screening study which showed it to have antitumor activity against Sarcoma 180 (Stock et al 1960). Subsequently, hydroxyurea was shown to be effective against a wide range of solid tumors, acute and chronic leukemias and melanomas (Bergsagel et al 1964, Bolton et al 1964, Fishbein et al 1964, Squibb Institute for Medical Research 1968, Kennedy et al 1969). The first clinical trials of hydroxyurea

Table 3. Inhibitors of Ribonucleotide Reductase

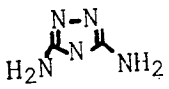
(i) hydroxyurea



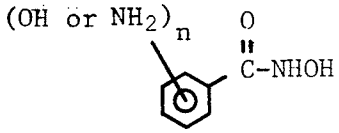
(ii) N-carbamoyloxyurea



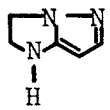
(iii) guanazole



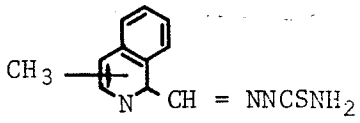
(iv) benzohydroxamic acids



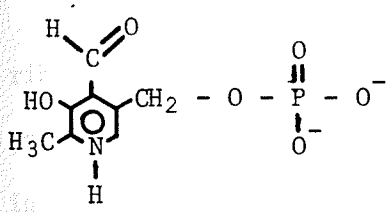
(v) 2,3-dihydro-1H-pyrazole [2,3-A]imidazole



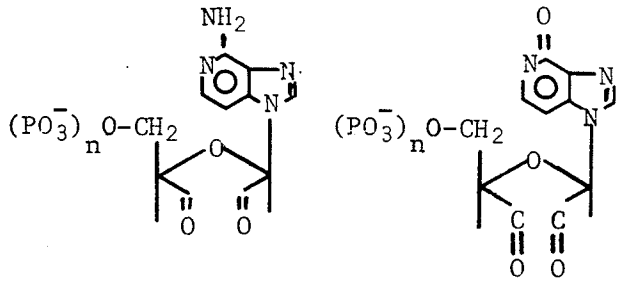
(vi) thiosemicarbazones



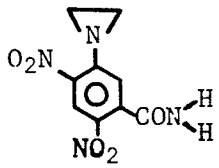
(vii) pyridoxal phosphate



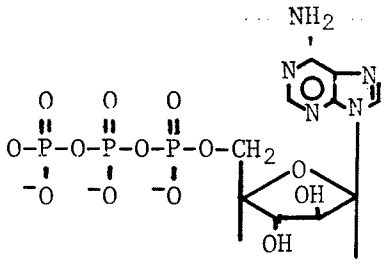
(viii) periodate oxidized derivations of adenosine and inosine



(ix) 5(1-aziridinyl)-2,4-dinitrobenzamide



(x) araATP



suggested that it interfered with DNA synthesis (Bergsagel et al 1964) and subsequent studies confirmed this to be so in a variety of organisms including bacteria (Rosenkranz and Levy 1965, Gale et al 1964), HeLa cells (Young and Hodas 1964), ascites tumor cells (Gale 1964, Yarbro et al 1965a), regenerating liver (Schwartz et al 1965, Yarbro et al 1965b), hamster cells (Mohler 1964, Lewis and Wright 1974) and mouse jejunal crypts (Allison and Wright 1979). Ribonucleotide reductase was identified as a site of hydroxyurea action in E. coli (Krakoff et al 1968, Elford 1968), bacteriophage (Yeh and Tessman 1978, Berglund and Sjöberg 1979) and mammalian cells including Novikoff hepatoma rat tumor (Elford 1968), mouse L cells (Adams and Lindsay 1967, Kuzik and Wright 1980, Koropatnick and Wright 1980), Ehrlich ascites cells (Turner et al 1966), human bone marrow cells (Frenkel et al 1964), normal human diploid fibroblasts (Dick and Wright 1980) and Chinese hamster ovary cells (Lewis and Wright 1974,1979, Hards and Wright 1981).

Studies of synchronized mammalian cells have shown that the deoxyribonucleoside triphosphate pool sizes increase during S phase (Bray and Brent 1972, Skoog et al 1974). In confirmation of the observed in vitro effect of hydroxyurea upon ribonucleotide reduction, it has been found that addition of the drug to S phase cells caused an immediate decrease in deoxyribonucleoside triphosphate levels (Walters et al 1973, Skoog and Nordenskjöld 1971, Skoog and Bjursell 1974). Even though hydroxyurea has been shown to inhibit the reduction of both purine and pyrimidine nucleotides in vitro (Elford 1968, Krakoff et al 1968, Moore 1969, Lewis and Wright 1974, Lewis and Wright 1978b, Yeh and Tessman 1978, Berglund and Sjöberg 1979) it is still unclear as to whether this inhibition is operative in vivo.

Addition of the drug to the growth media of cells in culture has been reported to reduce the dGTP, dATP and dCTP pool sizes found in S phase Chinese hamster ovary cells (Skoog and Bjursell 1974), the dATP and dGTP levels in mouse (Skoog and Nordenskjöld 1971, Adams et al 1972) and hepatoma (Plagemann and Erbe 1974) and only dATP levels in hamster cells (Walters et al 1973). These results have lead to speculation that hydroxyurea induces the inhibition of purine ribonucleotides without affecting the reduction of pyrimidine nucleotides. According to this idea, purine nucleotide reductase is much more sensitive to the action of hydroxyurea and thus intracellular levels of dATP and dGTP are decreased. This decrease removes inhibitors of CDP and UDP reductase and hence an increase in dCTP and dTTP levels might be expected. Such increases have indeed been noted (Skoog and Nordenskjöld 1971, Adams et al 1972, Walters et al 1973, Plagemann and Erbe 1974). Other explanations, including more efficient salvage pathways for dCTP and dTTP as compared to those for dATP and dGTP, can be used to account for the obtained results and a more definitive answer must await futher study.

It is now generally accepted that the sole intracellular site of hydroxyurea action is ribonucleotide reductase. Selection of somatic cell mutants resistant to the drug (for reviews on the subject see Wright et al 1980 and Wright et al 1981) confirm this, as in all resistant cell lines isolated to date the ability to survive in the drug can be traced to alterations in the activity of this enzyme (Lewis and Wright 1974, Lewis and Wright 1979, Kuzik and Wright 1980, Lankinen 1980, Koropatnick and Wright 1980, Åkerblom et al 1981, Hards and Wright 1981). At one time or another, hydroxyurea has been

reported to have other modes of action. These have included direct degradation of cellular DNA (Bendich et al 1964, Rosenkranz et al 1968, Jacobs and Rosenkranz 1970, Walker et al 1977), inhibition of orotic acid synthesis (Vogler et al 1966), inhibition of histone synthesis (Yarbro et al 1965b, Yarbro 1967), inhibition of cell surface glycosyl transferases (Hawtrey et al 1974), inhibition of thymidylate synthase (Boehm et al 1982) and inhibition of L-ornithine decarboxylase (Cress and Gerner 1979). However, hydroxyurea would seem to carry out the effects described above via indirect action. Alterations in the deoxynucleotide pool levels induced by hydroxyurea inhibition of ribonucleotide reductase were the likely cause of the degradation of cellular DNA, the inhibition of orotic acid biosynthesis and the inhibition of L-ornithine decarboxylase activity. The inhibition of glycosyl transferase activities was reported to occur at 10 mM hydroxyurea in hamster embryo fibroblasts (Hawtrey et al 1974). This represents a concentration 30 to 100 times greater than that required to inhibit DNA synthesis in hamster cells (Lewis and Wright 1974). Similarly, while 15 mM hydroxyurea reduced DNA synthesis to unmeasurably low levels in mastocytoma cells, thymidylate synthase was inhibited only 3.3% at the same concentration (Boehm et al 1982). Thus the effect of hydroxyurea on the glycosyl transferase and thymidylate synthase activities occurred at concentration levels much too high to be of significance to the cells involved. Inhibition of histone synthesis was likely a side effect of hydroxyurea's inhibition of DNA synthesis, as synthesis of the two compounds are closely coupled (Balhorn et al 1972) and inhibition of DNA synthesis by other compounds also causes

inhibition of histone synthesis (Sadgopol and Banner 1969, Chae et al 1970). Moreover, 1 mM hydroxyurea has been shown to block DNA synthesis in HTC cells while having little effect on histone synthesis (Balhorn et al 1972). Thus to the best of present knowledge, hydroxyurea is a potent and specific inhibitor of ribonucleotide reductase.

Hydroxyurea inhibits ribonucleotide reductase by acting as a scavenger of the tyrosine free radical found in both the E. coli and mammalian enzymes. In each case, the iron containing subunit, M2 or L2 in the mammalian cells and B2 in the bacterial cells, possesses a tyrosine free radical which generates a characteristic electron paramagnetic resonance (EPR) signal (Ehrenberg and Reichard 1972, Sjöberg et al 1978, Åkerblom et al 1981, Gräslund et al 1982). Addition of hydroxyurea to preparations of either protein subunit M2 (Åkerblom et al 1981, Gräslund et al 1982) or B2 (Atkin et al 1973, Sjöberg et al 1977) destroyed the characteristic signals along with enzyme activity. The treatment of protein B2 with hydroxyurea did not remove or in anyway affect the non-heme iron of the subunit. Similar studies have yet to be carried out with the M2 protein.

Because hydroxyurea contains an oxidized peptide bond, it is classified as a hydroxamic acid. The study of structurally related compounds suggested that it is this bond which contributes most to the drug's biological activity. The hydroxamic acid group would seem to be necessary for antitumor activity as compounds such as isohydroxyurea have been shown to be ineffective (Adamson 1965). One study indicated that the presence of the hydroxylamine group to be most important (Young et al 1967). While the carboxyl group did not seem to be required for activity, the most potent inhibitors were shown to

possess such a group. While both the mammalian and E. coli enzymes possess tyrosine-free radicals which generate similar EPR signals and are inactivated by hydroxyurea, differences in the two systems do exist. Small differences in the low temperature EPR signal for the two enzymes are likely due to slightly different angles of the β-methylene group in relation to the place of the aromatic ring of the tyrosine. This difference is likely the result of the polypeptide chains surrounding the two tyrosines being in different conformations.

The action of hydroxyurea on mammalian cells is reversible and removal of the drug will allow the resumption of normal growth (Skoog and Nordenskjöld 1971, Engström et al 1979). In fact the decrease in deoxynucleotide pools induced by hydroxyurea can be used to synchronize mammalian cells (Adams and Lindsay 1967). The exact location of the block is in some doubt and various investigators have placed it in late G1 just before S phase (Tobey and Crissman 1972), early S phase (Walters et al 1976) or right at the G1/S boundary (Elford et al 1981).

Hydroxyurea is sometimes used to enhance the effect of other anti-neoplastic agents such as cyclophosphamide, platinum compounds or cytosine arabinoside (Gale et al 1979, Moran and Straus 1980, Walsh et al 1980). Mechanisms behind this enhancement are not clearly understood but have been postulated to involve synchronization of the cells at the beginning of S phase to accentuate the killing power of S phase specific cytotoxic agents (Moran and Straus 1980) or alterations in deoxyribonucleoside triphosphate pools that act to regulate the conversion of the second prodrug to its active form (Walsh et al 1980). Interesting results have recently been obtained using combinations of agents directed against the two subunits of ribonucleotide reductase

(Sato and Cory 1981b, Cory and Fleischer 1979, Cory et al 1981b). When hydroxyurea was used in combination with deoxyribonucleoside triphosphates and other M1 subunit specific inhibitors (see section 6.5), it was found that the inhibition produced was additive. Clinical trials utilizing this approach should be most interesting.

Hydroxyurea inhibition of ribonucleotide reductase activity is limited to enzymes possessing an iron containing subunit. The enzyme isolated from Lactobacillus leichmannii which does not have a non-heme iron center is not inhibited by hydroxyurea (Elford 1968).

6.2 N-carbamoyloxyurea

In addition to hydroxyurea, several other drugs have been found to be active in the inhibition of mammalian ribonucleotide reductase. N-carbamoyloxyurea, an oxidation product of hydroxyurea, is produced when hydroxyurea is incubated at 56^{o}C for 72 hours (Jacobs and Rosenkranz 1970). Conversion of hydroxyurea to N-carbamoyloxyurea was found to occur much more rapidly at alkaline pH values (Jacobs and Rosenkranz 1970). At one time it was thought that hydroxyurea owed its cytotoxicity to an intracellular oxidation to N-carbamoyloxyurea (Rosenkranz and Rosenkranz 1969, Jacobs and Rosenkranz 1970). However the intracellular environment would not seem to provide suitable conditions for the conversion of hydroxyurea to N-carbamoyloxyurea and indeed there now exists evidence which suggests that the intracellular conversion does not occur (Hards and Wright 1981).

However, N-carbamoyloxyurea is a potent inhibitor of DNA synthesis and cell division (Rosenkranz 1970, Lewis and Wright 1978a, Hards and Wright 1981). In a study of the action of N-carbamoyloxyurea on the cell cycle of Tetrahymena (Cameron and Jeeter 1973), it was suggested

that N-carbamoyloxyurea inhibits ribonucleotide reductase activity but no direct proof was supplied. Subsequently, it was shown that cell lines selected for resistance to hydroxyurea and thus possessing altered ribonucleotide reductase activity were also cross-resistant to N-carbamoyloxyurea (Lewis and Wright 1978a). Recently, direct enzymatic evidence has shown that N-carbamoyloxyurea does act on ribonucleotide reductase (Hards and Wright 1981) probably via interference with the tyrosine-free radical of the M2 subunit.

6.3 Guanazole

Guanazole (3,5-diamino-1,2,4-triazole), first synthesized in 1893 (Pellizarri 1893), has been shown to have anti-tumor activity against various animal leukemias and tumors (Hahn and Adamson 1972). Clinical use has resulted in remissions in some patients with various neoplastic diseases (Yakar et al 1973, Hewlett et al 1973, Land et al 1974, Caoili et al 1974). Guanazole has been shown to effect DNA synthesis and ribonucleotide reductase in a manner similar to that of hydroxyurea (Brockman et al 1970). Selection of hydroxyurea-resistant Chinese hamster ovary cells which exhibited cross-resistance to guanazole and vice versa (Wright and Lewis 1974) indicated that like hydroxyurea and N-carbamoyloxyurea, guanazole likely interfers with the tyrosine-free radical of the M2 subunit. These results were further supported when partially purified ribonucleotide reductase from a hydroxyurea-resistant cell line (Lewis and Wright 1978b) was shown to have a similar increase in K_i values for both hydroxyurea and guanazole. Structure-activity relationships determined for derivatives of guanazole have established the importance of both amide groups for the antineoplastic activity of the drug (Brockman et al 1970, Hahn and

Adamson 1972).

There is interest in guanazole as a clinical agent because of its dissimilarity to hydroxyurea. However, due to its high polarity and water solubility, low molecular weight and relatively low potency, continuous intravenous infusion at frequent intervals are required for effective clinical use (Hewlett et al 1973). This factor has limited extensive clinical use of the drug. In an effort to overcome this problem, amide derivatives of the drug have been synthesized and screened for antineoplastic activity versus L1210 cells in vitro (Selassie et al 1981). One of these drugs, 3,5-dibenzamide-1,2,4-triazole, was substantially more active than guanazole. This increased activity could be due to increased interaction with ribonucleotide reductase or to increased lipophilicity which facilitates transport into the cell where subsequent hydrolysis to form guanazole could take place.

6.4 Benzohydroxamic acids

As clinical use of hydroxyurea requires frequent and high doses of the drug to maintain effective levels (van't Riet et al 1979, Elford et al 1979, Elford et al 1981), there has been a search for more advantageous compounds. A series of hydroxy- and amino substituted benzohydroxamic acids have shown promise (van't Riet et al 1980). A majority of the compounds increased the life span of mice bearing L1210 tumors, at concentrations considerably below those required for hydroxyurea (van't Riet 1979, Elford et al 1979). The benzohydroxamic acids have also been shown to inhibit ribonucleotide reductase activity in vitro. The ID_{50} values obtained were generally lower than that exhibited by hydroxyurea (Elford et al, 1979, van't Riet et al 1979).

Potency was found to increase with the number of adjacent hydroxy and amino groups present on the benzene ring. 2,3,4-Trihydroxybenzohydroxamic acid has been found to be 150 times more potent than hydroxyurea in the inhibition of ribonucleotide reductase (Elford et al 1979, Elford et al 1981).

The increased potency of the substituted benzohydroxamic acids may be due to the simultaneous presence of two centers active against ribonucleotide reductase. The benzohydroxamic acids were shown to act as free radical scavengers by their ability to destroy the stable free radical, diphenylpicrylhydrazyl (Elford et al 1981). In acetone, this compound has an absorbance at 518 nm that is dependent upon the presence of the free radical and thus descruction of the radical may be followed spectrophotometrically. Compounds with the highest potency for the inhibition of ribonucleotide reductase were also found to have the greatest ability to scavenge free radicals. The ability to act on the radical was shown to be largely dependent on the presence of the hydroxamic acid moiety as 2,3,4-trihydroxybenzohydroxamic acid was a much better scavenger than 2,3,4-trihydroxybenzoic acid. However, the presence of the hydroxamic portion of the compounds was not essential for biological activity. Several hydroxy or amino substituted phenyl compounds were found to both increase the life span of tumor bearing mice and inhibit ribonucleotide reductase activity independent of the hydroxamic acid function (Elford et al 1981). As these substituted phenyl compounds failed to destroy the diphenylpicrylhydrazyl radical when tested, it would seem unlikely that they inhibit ribonucleotide reductase activity via interference with the tyrosine free radical. Thus benzohydroxamic acids would appear to

inhibit the reductase in two ways. The first which requires the hydroxamic acid portion of the molecule involves destruction of the tyrosine free radical. While the second mechanism remains unknown it seems to require the aromatic ring protion of the compounds.

7.5 Inhibitors Which Do Not Scavenge at the Tyrosine Free Radical

In addition to the drugs discussed above, there do exist others whose mode of action do not involve destruction of the tyrosine free radical. 2,3-Dihydro-1H-pyrazole[2,3-A]imidazole (NSC 51143) has been shown to inhibit partially purified ribonucleotide reductase from Ehrlich tumor cells (Cory and Fleischer 1980). The drug interacts with the non-heme iron subunit M2 but it is not yet known whether inhibition is due to binding of the imidazole to the enzyme or to removal of the iron from the enzyme by the compound. Phase I clinical trials with the drug showed that its disappearance from plasma after intravenous infusion was biphasic with half-lives of less than 10 minutes for the first 25 minutes and then 4 hours for the remainder. The drug was detectable in gliablastoma, brain, temparalis muscle, kidney and liver tissue within 4 to 5 hours of infusion (Fong et al 1980). In light of the effect of 2,3-dihydro-1H-pyrazole[2,3-A]imidazole on ribonucleotide reduction it is interesting to note that the naturally occurring metal-chelator picolinic acid has many of the same effects on cultured cells as reported for hydroxyurea (Gurley and Jett 1980). It has been suggested that picolinic acid may regulate DNA synthesis by interfering with the iron-dependent production of the tyrosine free radical.

Another group of compounds that inhibit ribonucleotide reductase are the α(N)-heterocyclic carboxaldehyde thiosemicarbazones

(Sartorelli et al 1971). These compounds are very potent with only 0.6 μM of 4-methyl-5-amino-1-formylisoquinoline thiosemicarbazone required to inhibit enzyme activity by 50% (Agrawal et al 1977). These compounds are strong iron chelators but unlike picolinic acid or 2,3-dihydro-1H-pyrazolo[2,3-A]imidazole they do not interact with the non-heme iron of protein subunit M2. Instead, the active form of the inhibitor is thought to be a ferrous chelate (Cory et al 1981a, Preidecker et al 1980) which interacts with the enzyme at or near the site occupied by the dithiol substrate (Sartorelli et al 1977). There is however some controversy as to which subunit the ferrous chelate interacts with as Cory and his associates have published results which indicate that the complex does inactivate the non-heme iron containing subunit (Cory and Fleischer 1979, Cory et al 1981b).

Pyridoxal phosphate and the periodate oxidized dialdehyde derivatives of adenosine, AMP, ATP, inosine 5'-deoxyinosine and IMP were found to inhibit ribonucleotide reductase activity in a partially purified enzyme preparation from Ehrlich tumor cells (Cory and Mansell 1975b, Cory et al 1976b, Ohmstede and Cory 1981). These compounds were shown to specifically inhibit the effector-binding or M1 subunit of mammalian ribonucleotide reductase (Cory and Fleischer 1979, Cory et al 1981b). Inhibition is thought to be due to the formation of a Schiff base between the dialdehyde derivatives and the ε-amino group of a lysine residue (Cory and Mansell 1975b). Pyridoxal phosphate inhibition would seem to be due to interaction with a lysine residue of the allosteric site(s) as inhibition was not influenced by increasing the substrate (CDP or ADP) concentration but was diminished by increasing the ratio of allosteric effector to pyridoxal phosphate

concentrations. Conversely, the dialdehyde derivatives of adenosine, AMP and ATP would seem to interact with residues of the catalytic site as in this case inhibition could be reduced by increasing substrate concentrations (Cory and Mansell 1975b). Inactivation of ribonucleotide reductase from Corynebacterium nephridii with the 2',3'-dialdehyde derivatives of ADP or CDP was shown to involve the binding of the base and sugar moieties but not the pyrophosphate group of the nucleotide to the enzyme (Tsai 1981). Thus the inactivation would seem to involve covalent modification of the enzyme by the nucleoside dialdehydes with the concomitant of pyrophosphate.

The monofunctional alkylating agent 5-(1-aziridinyl)-2,4-denitrobenzamide (CB 1954) has been found to be a selective inhibitor of ribonucleotide reductase isolated from Walker carcinoma, human bladder carcinoma and TLX5 lymphoma (Tisdale and Habberfield 1980). Inhibition of 50% of enzyme activity required a drug concentration of between 0.38 to 0.92 mM depending upon the source of the enzyme. The inhibition of ribonucleotide reductase by CB 1954 was found to be irreversible and was thought to likely involve the binding of the amide group of the inhibitor to the enzyme, possibly by chelation of the non-heme iron. Once bound to the enzyme, it is supposed that the aziridine ring could alkylate appropriate nucleophilic centers such as the sulphydryl groups which are necessary for the activity of the enzyme (Moore 1977).

Arabinonucleotides have been shown to inhibit ribonucleotide reductase (Moore and Cohen 1967, Chang and Cheng 1980) however, the mechanism(s) responsible for this inhibition remains unclear. With partially purified enzyme from rat tumor, Moore and Cohen (1967)

found that the effects of araATP were similar to those of dATP except that dATP was 5 to 8 times more active on a concentration basis. From these results it could be concluded that araATP mimiced the structure of dATP and bound at the allosteric regulatory sites. Using partially purified enzyme from human Molt-4F cells, Chang and Cheng (1980) found that dATP acted as a non-competitive inhibitor for both ADP and CDP reduction with respect to their nucleoside triphosphate activators while araATP exhibited a competitive pattern for both. From these results they concluded that araATP and dATP did not exert their influence via the same mechanism. However, no explaination as to what this second mechanism might be was provided.

MATERIALS AND METHODS

1. CELL LINES AND CULTURE CONDITIONS

1.1 CHO Cell Lines

The Chinese hamster ovary (CHO) cell line chosen as the starting material for the studies reported here was first established by Puck et al (1958). These cells exhibit a stable chromosome number of 21 as compared to the diploid number of 22 for the Chinese hamster. Although CHO cells do contain many chromosomes that differ from those of the Chinese hamster karyotype (Kao and Puck 1969), rearrangements of the normal chromosome can account for many of the abnormal chromosomes (Deaven and Peterson 1973, Worton et al 1977).

CHO cells grew rapidly and to a high cell density in either monolayer or suspension culture. In addition the cells have a high plating efficiency (Kao and Puck 1967). In monolayer culture the cells were grown in 16 oz Brockway bottles or on 30, 60, 100 or 150 mm plastic tissue culture plates. The culture medium routinely used was alpha minimal essential medium (α-MEM) plus 10% (v/v) fetal calf serum (FCS) supplemented with penicillin G (100 units/ml) and streptomycin sulfate (100 µg/ml). The formulation of α-MEM was as published by Stanners et al (1971) and contained no ribonucleotides or deoxyribonucleotides. In some experiments 8% horse serum (HS) plus 2% FCS was substituted for 10% FCS. The Brockway bottles or culture plates were incubated at 37°C in a 5% CO_2 atmosphere in a humidity controlled incubator. The doubling times of the various cell lines used in this study were as follows: WTC125, 16 hours; NC^R-30A, 12 hours; NC^R-30A0.7, NC^R-30A1, NC^R-30A1.5, NC^R-30A2, NC^R-30A3 and NC^R-30A5, 14 hours.

MATERIALS AND METHODS

Suspension cultures of CHO cells were started with approximately 10^5 cells/ml of α-MEM plus 10% FCS. The cells were grown in either 500 ml medium bottles or in 4 l glass carboys through which 5% CO_2 was bubbled at a slow rate to maintain pH and oxygen tension. The cultures were placed in a 37°C water bath and vigorous agitation was provided by Teflon coated magnetic stirring bars. Cultures maintained logarithmic growth up to a density of 10^6 cells/ml but cells were routinely harvested at 700,000 cells/ml. In suspension culture wild-type cells exhibited a doubling time of 16 hours.

1.2 Cell Line Sources

CHO cells were brought to the Department of Microbiology, University of Manitoba from the Medical Biophysics Department, University of Toronto by Dr. J.A. Wright in 1971. The WTC125 was cloned (see section 2.5 for procedure) from these cells and used in the selection of drug and lectin-resistant variants (Lewis and Wright 1978a,b, Wright and Ceri 1977, Hards and Wright 1981).

The N-carbamoyloxyurea-resistant line designated NC^R30A was selected according to a procedure outlined by Lewis and Wright (1978a). An aliquot of 5 x 10^5 WTC125 cells was added to a 100 mm tissue culture plate containing growth medium supplemented with 0.4 mM N-carbamoyloxyurea. After a 16 day incubation, many small (<20 cells) colonies and several large (>100 cells) were observed. The plate was washed with sterile PBS to remove any loosely attached or floating cells, a buffered solution of 0.05% trypsin was gently added and after several minutes a sterile Pasteur pipette was lowered over the desired colony. Cells were drawn up and retested for the ability to proliferate in the presence of drug by dispensing the cells directly into another plate containing growth

medium supplemented with 0.4 mM N-carbamoyloxyurea. These cells grew to a partial monolayer and were then cloned to insure purity (for cloning procedure see below). One such clone was designated NC^R-30A.

Starting with a non-mutagenized population of NC^R-30A cells, a series of cell lines resistant to the cytotoxic effects of N-carbamoyloxyurea were sequentially selected in single steps in the following manner: NC^R-30A $\xrightarrow{1}$ NC^R-30A0.7 $\xrightarrow{2}$ NC^R-30A1 $\xrightarrow{3}$ NC^R-30A1.5 $\xrightarrow{4}$ NC^R-30A2 $\xrightarrow{5}$ NC^R-30A3 $\xrightarrow{6}$ NC^R-30A5. The following concentrations of N-carbamoyloxyurea were used at each selective step: 1=0.7 mM, 2=1.0 mM, 3=1.5 mM, 4=2.0 mM, 5=3.0 mM, 6=5.0 mM. For each selection approximately 10^6 cells were added to a 16 oz Brockway bottle containing α-MEM plus 10% FCS supplemented with the appropriate concentration of N-carbamoyloxyurea. When the cells reached a partial monolayer the drug supplemented medium was replaced with normal growth medium and the culture incubated until a full monolayer was obtained. An aliquot (10^6 cells) was then used in the next selective step.

2. ROUTINE PROCEDURES

2.1 Trypsinization

Sterile 0.05% trypsin solutions were prepared in PBS and stored at 4^oC. Cells were removed from the surface of bottles of plates as follows. The growth medium was removed and sufficient trypsin solution added to cover the cells. After 5 to 10 minutes at room temperature the cells were detached from the growth surface by gentle agitation. The cell suspension was then transferred to a sterile plastic tube and centrifuged. The resulting pellet was resuspended in an appropriate volume of α-MEM plus 10% FCS and aliquots dispensed as required.

2.2 Cell Counting

Aliquots of suspension grown or freshly trypsinized cells varying from 0.5 to 5 ml were diluted to 40 ml with saline. The density of this cell suspension was determined with a Coulter Particle Counter (Coulter Electronics Ltd., Florida). As a verification of the accuracy of the particle counter cell suspensions were occasionally also counted with the aid of a hemocytometer.

2.3 Determination of Doubling Times

Exponentially growing cells were plated at a density of between 4×10^4 and 5×10^5 cells/60 or 100 mm culture plate in prewarmed α-MEM plus 10% FCS or in some cases 8% HS and 2% FCS. At various times after innoculation the medium was removed from the plates and the cells trypsinized and counted using the Coulter Particle Counter. Plots of the log of cell number versus time of incubation were constructed and the doubling times determined by interpolation. For cells grown in suspension appropriate aliquots were counted at various times after innoculation.

2.4 Determination of Plating Efficiency

To determine plating efficiency (PE) cells were added to culture plates at numbers ranging from 10^2 to 10^6 per 100 mm plate. After a suitable period of incubation the plates were drained of medium and a saturated solution of methylene blue in 50% ethanol was added to the plates. After approximately 10 minutes the plates were rinsed in water and the stained colonies on each plate counted. Only colonies containing 20 or more cells were counted. Plating efficiency was calculated by dividing the number of colonies counted by the number of

cells plated. For the cell lines used in this study the plating efficiencies varied between 0.66 and 0.90.

To determine the effect of a drug on cellular viability, relative plating efficiency (RPE) was calculated. The RPE is defined as the plating efficiency in the presence of drug divided by that in the absence of drug. The effectiveness of a drug on a particular cell line is usually measured by its D_{10} value. This value is defined as the concentration of drug that reduces the RPE to 10%. The number of cells plated at a particular concentration of drug was adjusted to insure the survival of between 20 and 300 colonies per 100 mm plate. RPE data was presented by plotting the log of RPE versus drug concentration (see Thompson and Baker 1973).

2.5 Cell Cloning

Two methods were used to obtain clones of CHO cells. The first involved dilution of a cell suspension to 5 cells/ml of α-MEM plus 10% FCS. This diluted suspension was then dispensed at 0.2 ml/well into a 96-well Linbro plastic tray. After about 10 days at 37°C, those cells showing development of a single colony were flooded with trypsin solution. The cells were removed with a sterile Pasteur pipette, added to a culture plate containing normal growth medium and incubated at 37°C. This was the method used in the isolation of the WTC125 and NC^R-30A cell lines.

The second method involved the use of glass cloning discs. These cylindrically shaped discs had a diameter of 5 mm and a depth of 8 mm. In addition they possessed a wider flange at one end. Plates containing α-MEM plus 10% FCS were seeded at low cell concentrations and incubated at 37°C for approximately 7 days. Colonies containing 20 to 30 cells

were identified, the plates drained of medium and washed once with sterile PBS. Previously sterilized cloning discs were placed over the desired colonies and tightly affixed to the surface of the culture plate by coating the flange portion of the disc with sterile vacuum grease and applying a small amount of pressure. Thus a well containing a single colony was constrcutred and to this well was added a small volume of trypsin solution. After 5 to 10 minutes the solution was agitated with a sterile Pasteur pipette and the detached cells transferred to a new plate containing α-MEM plus 10% FCS.

2.6 Long-term Storage of Cell Lines

Wild-type and mutant CHO cell lines were stored as a suspension of 0.5 to 1 x 10^7 cells per 2 ml of α-MEM plus 10% FCS plus 7% dimethyl sulfoxide (DMSO). The cell suspension was placed in a small (12 x 75 mm), sterile plastic tube which was tightly capped and placed directly at -76°C. For recovery the suspensions were rapidly thawed at 37°C, the cells pelleted by centrifugation, and the supernatant discarded. The pellet was then resuspended in α-MEM plus 10% FCS or 8% HS and 2% FCS in some cases and dispensed to bottles or plates containing normal growth medium. After allowing sufficient time for the cells to adhere to the culture surface (approximately 12 hours) the medium was drained and replaced with fresh.

2.7 Incorporation of Radioactive Precursors

A modification of the methods outlined by Lewis and Wright (1974) and Ceri and Wright (1977) was used. Exponentially growing cells were plated at a density of 2 x 10^6/100 mm or 7.2 x 10^5/60 mm tissue culture plate in α-MEM plus 10% FCS. After incubation at 37°C for 36 hours

the medium was aspirated, the plates washed with sterile PBS and α-MEM plus 10% dialysed fetal calf serum (dFCS) plus precursor, both labelled and unlabelled, and relevant drugs added. The plates were incubated at 37°C for specific time periods after which the tritiated medium was aspirated and 10% ice-cold trichloroacetic acid (TCA) was added. The plates were kept at 2 to 4°C for at least 30 minutes after which time the cells were scraped from the plates and filtered through glass fiber filters which had been presoaked in ice-cold 10% TCA. The filters were then washed twice with 10 ml aliquots of 5% TCA, PBS and 70% ethanol (all ice-cold). Next the filters were placed in scintillation vials, 1 ml of tissue solubilizer added, the caps placed tightly on and the vials heated for 1 hour at 60°C. After heating either 10 ml of ACS plus 75 µl of glacial acetic acid or 10 ml of a toluene-PPO-POPOP sctinillation cocktail was added to the vials. Radioactive counting was carried out using a Beckman model LS230 or a LKB model 1215 Rack beta liquid scintillation spectrometer.

For DNA synthesis 1 µCi ^{3}H-thymidine and 0.5 µg thymidine/ml of α-MEM plus 10% dFCS was used; for RNA synthesis 1 µCi ^{3}H-uridine and 0.5 µg uridine/ml of α-MEM plus 10% dFCS; and for protein synthesis 1 µCi ^{3}H-leucine and 52.5 µg leucine/ml of α-MEM plus 10% FCS was used.

Incorporation of label from ribonucleotide substrates into RNA or DNA during the assay of ribonucleotide reductase was monitored as follows. Enzyme reactions were stopped by the addition of 5 ml of ice-cold 10% TCA and then kept on ice for 30 minutes. The suspension was filtered through glass fiber filters which had been pre-soaked in ice cold 10% TCA. The filters were then washed twice with 10 ml

aliquots of 5% TCA, PBS and finally 70% ethanol. The filters were placed in scintillation vials, 1 ml of tissue solubilizer was added, the caps were tightened and the vials were heated at 60°C for 1 hour. After heating, 10 ml of ACS and 75 µl of glacial acetic acid were added to each vial and the radioactivity was determined using a LKB model 1215 Rackbeta liquid scintillation spectrometer. This method prepares RNA plus DNA. To determine the DNA portion, the TCA precipitated material obtained after termination of the enzyme reaction was removed by centrifugation, resuspended in 1 ml of 1.0 M KOH and incubated at 37° for 16 hours. The suspension was reprecipitated with ice-cold 10% TCA and treated as descirbed above. The radioactivity present at the end of this procedure is in DNA. The amount of radioactivity in RNA can be estimated by subtraction of the value determined in DNA from that found in the sample of RNA plus DNA.

2.8 Liquid Scintillation Counting

In studies involving radioactive compounds, liquid scintillation techniques were used to measure ratioactivity. Aqueous samples from the assay of ribonucleotide reductase activity or HPLC nucleotide analysis were counted after the addition of 16 ml of Scintiverse or 10 ml of ACS. Samples of membrane filters were placed in vials containing either 10 ml of ACS plus 75 µl of glacial acetic acid or 10 ml of toluene-PPO-POPOP cocktail consisting of 4 g PPO and 0.2 g POPOP per liter of scintillized toluene. Radioactivity was determined using a Beckman model LS230 or an LKB model 1215 Rackbeta liquid scintillation spectrometer with external standard corrections.

2.9 Protein Determination

Protein content of cell extracts was determined by the method of Lowry et al (1951) using bovine serum albumin as a standard. Using this procedure a linear relationship between absorbance and protein content was observed from 0 to 100 μg.

3. RIBONUCLEOTIDE REDUCTASE IN VITRO ASSAY

3.1 Preparation of Cell Extracts

CHO cells used in the preparation of extracts were grown in 4 l carboys as described in section 1.1 and harvested by centrifugation when they were in the log phase of growth at a density of approximately 700,000 cells/ml. The resulting pellet was washed once with PBS and then suspended in an equal volume of 0.05 M N-2-hydroxyethyl-piperazine N'-2-ethanesulfonic acid (Hepes) buffer, pH 7.2 and 5 mM dithiothreitol (DTT). This suspension was homogenized using a Tri-R tissue homogenizer fitted with a glass-hardened Teflon pestle and a glass tube. Ten strokes at a setting of 4 resulted in the disruption of greater than 95% of the cells. The homogenate was then centrifuged at 100,000 x g for 60 minutes. A dense thin layer of lipid which formed at the supernatant surface was removed. The remaining supernatant was applied to a Sephadex G-25 column previously equilibrated with 0.05 M Hepes, pH 7.2 and 5 mM DTT. Elution with the same buffer removed endogeneous nucleotides from the preparation. The ribonucleotide reductase activity was found to elute immediately after the void volume. The enzyme preparation was routinely found to contain 15 mg protein/ml.

Whole cells and crude or desalted extract were all stored at -76°C for up to several months with little apparent decrease in activity.

During purification and handling the cell extracts were maintained at 2 to 4°C.

3.2 Standard CDP Reductase Assay

The reduction of CDP was monitored according to the procedure described by Lewis et al (1977). The standard reaction mixutre of 150 µl contained 2 mM ATP, 8 mM $MgCl_2$, 6 mM DTT, 50 mM Hepes pH 7.2 and 0.4 mM ^{14}C-CDP (5000 cpm/nmole). Of the total volume, 100 µl was supplied by the cell extract which contained approximately 1.5 mg of protein. After incubation at 37°C for between 30 to 45 minutes the reaction was terminated by heating in a boiling water bath for 2 minutes. The deoxycytidine phosphates were converted to deoxycytidine by the action of Crotalus atrox venom as described by Cory (1973). The reaction mixture was incubated with 1 mg of snake venom suspended in 0.1 M Hepes pH 8.0 plus 10 mM $MgCl_2$ (20 mg venom per ml) for 2 hours at 37°C. The reaction was again terminated by boiling for 2 minutes and 0.8 ml of distilled water (dH_2O) was added to each assay tube. The heat precipitated material was removed by centrifugation. The supernatant was kept and the deoxycytidine separated from the cytidine on a 5 x 80 column of Dowex-1-borate or AG1-X8-borate according to the method of Steeper and Stewart (1970) as modified by Cory and Whitford (1972). Briefly, the separation depends upon the formation of a complex between ribonucleosides and borate ions on the column resin. Deoxynucleosides do not possess a cis-diol and hence cannot form a complex. Deoxycytidine was eluted from the column into scintillation vials with 4 ml of dH_2O. Ten milliliters of ACS or 16 ml of Scintiverse was then added for liquid scintillation counting and the mixture shaken until a single translucent gel phase formed.

3.3 Standard ADP Reductase Assay

ADP reduction was measured in a standard reaction mixture of 50 mM piperazine-N,N'-bis[2-ethane-sulfonic acid] (Pipes) pH 6.8, 6 mM DTT, 0.5 mM dGTP and 0.4 mM ^{14}C-ADP (5000 cpm/nmole) in a total volume of 150 µl according to procedure outlined by Lewis et al (1977). One hundred microliters of the reaction mixture was made up of cell extract, corresponding to approximately 1.5 mg of protein. After incubation at 37^{o} for between 20 and 30 minutes the reaction was terminated by heating in a boiling water bath for 2 minutes. To each reaction mixture was added 0.3 µmole of carrier dAMP and 50 µl of Crotalus atrox venom (20 mg/ml of 0.1 M Hepes pH 8.0, 10 mM $MgCl_2$). After incubation at 37^{o}C for 2 hours to dephosphorylate the nucleotides, the reaction was terminated by boiling for 2 minutes. Deoxyadenosine was separated from adenosine according to the method of Cory et al (1973). To the boiled reaction mixture was added 0.8 ml of 1 mM sodium borate. The assay tubes were centrifuged to remove the heat precipitated material and the supernatant was applied to a 5 x 80 mm Dowex-1-borate or AG1-X8-borate previously equilibrated with 10 ml of 1 mM sodium borate. The deoxyadenosine was eluted with 20 ml of 1 mM sodium borate. The first 4 ml were discarded and of the remaining 16 ml, which contained the deoxyadenosine, 4 ml were transferred to a scintillation vial. Sixteen ml of Scintiverse or 10 ml of ACS was added and the vials shaken until a single translucent phase formed.

4. ASSAY OF RIBONUCLEOTIDE REDUCTASE IN INTACT, PERMEABILIZED CELLS

4.1 Permeabilization of CHO Cells

CHO cells were made permeable to nucleotides using the Tween 80 method developed in this laboratory (Lewis et al 1978, Koropatnick and Wright 1980, Dick and Wright 1980, Hards and Wright 1981, Wright et al 1981). Exponentially growing cells were plated at a density of 2 x 10^6/100 mm or 5 x 10^6/150 mm plastic tissue culture plate containing α-MEM plus 10% FCS or 8% HS and 2% FCS and incubated at 37°C. After 40 hours for wild-type CHO cells, 36 hours for NC^R-30A cells and 38 hours for all other cell lines, the cells were removed from the surface of the plates with trypsin solution, centrifuged, washed once with α-MEM plus 10% FCS or 8% HS and 2% FCS and counted by means of a Coulter particle counter (Coulter Electronics Ltd., Florida). The cells were then resuspended at 10^7 cells/ml of permeabilizing buffer consisting of 1% Tween 80, 0.25 M sucrose, 50 mM Hepes pH 7.2 and 2 mM DTT. The cells were incubated at 22°C for 30 minutes with occasional agitation and then centrifuged to remove endogeneous inhibitors of the reaction. The cell pellet was resuspended in fresh permeabilizing buffer at a concentration of 2.5 x 10^7 cells/ml. Aliquots of 200 µl, corresponding to 5 x 10^6 cells, were then added to assay tubes.

4.2 Standard CDP Reductase Assay

For CDP reduction, the cell pellet was resuspended in the above specified permeabilizing buffer and made up to a final volume of 300 µl. The standard reaction mixture contained 50 mM Hepes pH 7.2, 2 mM ATP, 6 mM DTT, 8 mM $MgCl_2$, 0.4 mM ^{14}C-CDP (5000 cpm/nmole), 0.67% Tween 80 and 0.167 M sucrose. The assays were then agitated with either a Dubenoff shaking water bath (Precision Scientific) or 2 x 7

mm stirring bars for 20 to 25 minutes at 37°C. The reaction was terminated by heating in a boiling water bath for 4 minutes.

Nucleotides were converted to nucleosides and deoxycytidine separated from cytidine using the methods outlined for the *in vitro* assay system except that 100 µl (2 mg) of *Crotalus atrox* venom was used per assay and 0.5 ml of dH_2O was added to each tube prior to centrifugation of the heat precipitated material (Hards and Wright 1981).

4.3 Standard ADP Reductase Assay

To assay the reduction of ADP, the cell pellet was resuspended in permeabilizing buffer consisting of 1% Tween 80, 0.25 M sucrose, 50 mM Pipes pH 6.8 and 2 mM DTT. The final reaction volume of 300 µl standardly contained 50 mM Pipes pH 6.8, 0.5 mM dGTP, 6 mM DTT, 0.4 mM ^{14}C-ADP (5000 cpm/nmole), 0.67% Tween 80 and 0.167 M sucrose. The assay tubes were then agitated with a shaking water bath or 2 x 7 mm stirring bars, for 15 to 25 minutes at 37°C after which the reaction was terminated by heating in a boiling water bath for 4 minutes.

Nucleotides were converted to nucleosides and deoxyadenosine separated from adenosine by the methods outlined for the *in vitro* assay system except that 100 µl of snake venom (2 mg) was used per assay and 0.5 ml of 1 mM sodium borate was added to each tube prior to centrifugation of the heat precipitated material (Hards and Wright 1981).

One unit of enzyme activity was defined as the amount of enzyme that reduces one nmole of nucleotide per 5 x 10^6 permeabilized cells in one hour.

5. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY SEPARATION OF NUCLEOTIDES, NUCLEOSIDES AND PURINE AND PYRIMIDINE BASES

5.1 Nucleoside and Base Analysis

To ascertain the Dowex-1-borate or AG1-X8-borate effluent composition the following procedure was used. The 4 ml solution for a CDP reductase assay or the 16 ml of an ADP reductase assay were evaporated to dryness by warming the solutions to 37°C and passing air over the liquid surface. After complete evaporation 5 nmoles of cytosine, deoxycytidine and cytidine or adenine, deoxyadenosine and adenosine were added and the volume brought up to 100 µl with dH_2O. Twenty to 25 µl of these solutions were analysed on a C-18 reverse phase high performance liquid chromatography (HPLC) column. Pressure was applied with a Waters 4500 HPLC pump and nucleosides and bases were detected with a Beckman double beam ultraviolet light detector operating at a wavelength of 254 mm. The nucleosides and bases found in CDP reductase experiments were eluted with dH_2O while those formed in ADP reductase assays were eluted with CH_3OH:dH_2O (7:93). Elution buffers were filtered through 0.22 µ (dH_2O) or 0.5 µ (CH_3OH) filters and degassed immediately prior to use.

As the nucleosides and bases were eluted from the column, 1 ml fractions were collected, transferred to scintillation vials, 10 ml of ACS added and the vials counted in a Beckman model LS230 liquid scintillation spectrometer.

5.2 Separation of Nucleotides

The separation of the mono-, di- and triphosphate 5'-ribonucleosides of adenine, guanine, cytosine, uracil and thymine was achieved using a modification of the method outlined by Hartwick and Brown (1975) and

Van Haverbeke and Brown (1978).

A Beckman model 332 gradient liquid chromatograph (Altex: Beckman, Berkeley, Calif.) consisting of two model 110A solvent metering pumps co-ordinated by a model 420 system controller programmer was used. The nucleotides were detected with a Beckman model 153 analytical detector at a wavelength of 254 nm and the peaks were recorded on a Zipp and Zonen BD 40 recorder (Delft: Holland). Compounds were identified by retention time as compared to prepared samples. Nucleotides were quantitated by measuring the peaks heights as there existed a linear relationship between peak height and sample size.

In certain experiments the fates of radioactively labelled nucleotides were examined. In such experiments an LKB fraction collector was used to collect 4 ml (2 minute) fractions during the course of separation. The fractions were transferred to scintillation vials and counted.

Separation was achieved using a Partisil PXS 10/25-SAX strong anion exchange column (Whatman: Clifton, N.J.). The resin came prepackaged in a 25 cm x 4.6 mm I.D. stainless steel column. The ion-exchange moiety was a quaternary ammonium ion bound to the silica base through a Si-O-Si band. To prolong the lifetime of the column a 5 cm x 4.6 mm hand packed column of Partisil PXS/10-SAX was coupled to the inlet of the 25 cm column. This precolumn was routinely cleaned and repacked as required during the analysis of nucleotide levels.

The separation of nucleotides was achieved using the following protocol. The initial eluent, 0.007 M KH_2PO_4 and 0.007 M KCl, was adjusted to pH 4.0 with HCl. The final eluent, 0.25 M KH_2PO_4 and 0.5 M KCl, was adjusted to pH 5.0 with NaOH. An isocratic elution on the initial eluent lasting 20 minutes was followed by a linear gradient

from 0% of the final eluent to 100% of the final eluent run in 35 minutes. Finally, an isocratic elution on the final eluent was run for 15 minutes. The flow rate throughout the separation was 2.0 ml per minute. All buffers used were filtered through 0.22 μ Millipore membrane filters and degassed prior to use.

5.3 Extraction of Nucleotides from CHO Cells

Nucleotides were extracted from CHO cells using the method of Garrett and Santi (1979). The cells were grown to a density of 2.2 $\pm$ 0.2 x 10^7 cells/150 mm plastic tissue culture plate in α-MEM plus 10% FCS. For each extraction the cells from five such plates were removed with trypsin solution, washed twice with ice-cold PBS, counted with a Coulter particle counter (Coulter Electronics Ltd., Florida) and resuspended in PBS at a concentration of 0.5 to 3.0 x 10^8 cells/ml. The PBS into which the cells were resuspended contained ^{3}H-uridine (130,000 cpm/ml) as a concentration marker and aliquots of the PBS were removed for counting. Seventy percent perchloric acid was then added to the suspension to give a final concentration of 0.5M. After 30 minutes on ice the precipitate was removed by centrifugation in an Eppendorf centrifuge, 10 μl of bromocresol was added and the supernatant was neutralized with 4 M KOH containing 0.4 M KH_2PO_4. The neutralized extract was kept on ice for at least 10 minutes to allow for the precipitation of potassium perchlorate. The solution was again cleared by centrifugation and an aliquot of the supernatant analysed for radioactivity in order to determine dilution factors.

5.4 Analysis of Deoxyribonucleotides

As deoxyribonucleotides are often obscurred by the presence of ribonucleotides, analysis of each required the separation of the two.

The method of Garrett and Santi (1979) which involved destruction of the ribonucleotides to allow for detection and quantitation of the deoxyribonucleotides was utilized with slight modifications. Forty microliters of 0.5 M sodium periodate was added to one ml of neutralized cell extract or nucleotide solution. After 5 minutes, 50 µl of 4 M methylamine, which had been slowly brought to pH 7.5 with phosphoric acid, was added. The reactants were mixed and incubated at 37°C for 60 minutes. Ten microliters of 1 M rhamnose was then added to destroy any remaining periodate and the samples immediately put on ice. The solutions were cleared by centrifugation and an aliquot was counted in a scintillation counter to determine dilution factors.

5.5 Analysis of Nucleotides Present During the Assay of Ribonucleotide Reductase using Permeabilized Cells

Since permeabilized cells still contained enzymes capable of metabolizing nucleotide substrates and effectors added during the assay of ribonucleotide reductase, it was deemed necessary to determine the extent of this metabolization. Assays were set up and carried out as described previously in section 4 except that they were terminated by the addition of sufficient 70% perchloric acid to give the mixture a final concentration of 0.5 M. The assay tubes were placed on ice for 30 minutes after which time 10 µl of bromocresol blue was added and the solution neutralized with 4 M KOH containing 0.4 M KH_2PO_4. The mixtures were transferred to Eppendorf tubes and cleared by centrifugation. Analysis of the nucleotides was carried out on a Partisil PXS 10/25-SAX column as described above. Because the substrates were radioactive their fates were determined by collecting and counting 4 ml (2 minute) fractions. The fates of the various effectors were followed by monitoring the appropriate peak heights and comparing them

to prepared calibration curves. The validity of using peak height to quantitate the amount of nucleotide present was verified by the observation that in cases of labelled compounds, the amounts calculated to be present using peak height and radioactivity matched almost exactly.

During the course of these particular analyses, the separation of ribonucleotides from deoxyribonucleotides was not carried out. As the reduction of substrates was occurring during the incubation period some deoxyribonucleotides were generated. However, the degree of conversion from ribo- to deoxyribonucleotide would at most be 0.30% of the added substrate, an insignificant value. In cases where deoxyribonucleotides were added to assays as allosteric effectors, there was no feasible route by which they could be converted to ribonucleotides. Thus in these instances peaks were identified as being either ribo- or deoxyribonucleotide on the basis of the assay being analysed.

5.6 Cleaning of the Partisil PXS 10/25-SAX Column

It was found that during separation of the nucleotides an unknown impurity, apparently from the potassium phosphate buffer used in the elution, collected on the column. After a time this impurity accumulated to such a degree that it was eluted by the final eluent. As the impurity absorbed light at 254 nm, this gave rise to a drifting baseline which caused problems in the determination of peak heights. A simple technique was devised to remove this impurity when it began to cause problems (Rabel 1980). One hundred microliters of 0.1 M EDTA was injected into the column to loosen the impurity. The impurity was then slowly eluted by washing the column with 0.007 M KH_2PO_4 pH 4.0 at a flow rate of 0.5 ml/min until a stable baseline resulted. The

washing procedure was found to last from 10 to 24 hours.

6. SOURCE OF MATERIALS

Most biochemicals were purchased from Sigma Chemical Co., St. Louis and all radioactive chemicals were from Amersham/Searle Ltd., Arlington Heights. Sources of all other materials are listed in Table 4.

Table 4. Source of materials.

Material	Source
ACS (aqueous counting scintillant)	Amersham/Searle, Arlington Heights
AG1-X8 anion exchange resin (200-400 mesh)	Bio-Rad Labs, Richmond
Alpha-minimal essential medium	Flow Laboratories, Rockville
bromocresol blue	Fisher Sci. Co. Ltd., Fairlawn
C-18 reverse phase high performance liquid chromatography column	Dr. F.E. Hruska, Department of Chemistry, Univ. of Manitoba
N-carbamoyloxyurea SQ #10,726	Ms. B. Stearn and Mr. S.I. Lucania, Squibb Research Foundation, Princeton, N.J.
Crotalus atrox venom	Sigma Chemical Co.
culture plates (60 mm plastic)	Lux Sci. Corp, Newburg
culture plates (100 mm plastic)	Lux Sci. Corp.
culture plates (150 mm plastic)	Lux Sci. Corp.
culture bottles (16 oz. glass)	Brockway Glass Co.
Dowex 1-X8 anion exchange resin (200-400 mesh)	Bio-Rad Labs
fetal calf serum	Gibco Ltd., Grand Island
fetal calf serum (dialysed)	Gibco Ltd.
glacial acetic acid	Fisher Sci. Co. Ltd.
guanazole NSC #1895	B. Wood, Jr. Drug Development Branch, National Cancer Institute, Bethesda Maryland & Leonard H. Kedda, Drug Synthesis and Chemistry Branch, National Cancer Institute, Silver Springs, Maryland
horse serum	Gibco Ltd.
filters, 2.5 cm glass fiber #9-730-62	Fisher Sci. Co. Ltd.
Linbro cloning dishes #IS-FS-96-7C	Linbro Chemical Co.
methanol (HPLC grade)	Fisher Sci. Co. Ltd.
methylamine	Fisher Sci. Co. Ltd.
methylene blue	Fisher Sci. Co. Ltd.
NCS tissue solubilizer	Amersham/Searle Ltd.

...continued...

Table 4. (continued)

Material	Source
Partisil PXS 10/25-SAX anion exchange column	Whatman, Clifton, N.J.
penicillin G	Gibco Ltd.
perchloric acid	Fisher Sci. Co. Ltd.
POP	Fisher Sci. Co. Ltd.
POPOP	Fisher Sci. Co. Ltd.
potassium chloride (ACS grade)	J.T. Baker Co.
potassium dihydrogen phosphate (HPLC grade)	Fisher Sci. Co. Ltd.
protosol tissue solubilizer	New England Nuclear Ltd., Boston, Mass.
rhamnose	Nutritional Biochem. Corp., Cleaveland, Ohio
scintiverse	Fisher Sci. Co. Ltd.
sodium borate	Fisher Sci. Co. Ltd.
sodium periodate	Fisher Sci. Co. Ltd.
spinner bottles (suspension cultures)	Gibco Ltd.
streptomycin	Gibco Ltd.
sucrose	Fisher Sci. Co. Ltd.
toluene (scintanalyzed)	Fisher Sci. Co. Ltd.
trypsin, Bacto #0153-61	Difco Labs, Detroit
Tween 80 (polyethylene [20] sorbitan monooleate)	J.T. Baker Co., Phillipsburg, N.J.

RESULTS

RESULTS

1. ANALYSIS OF THE PERMEABILIZATION TECHNIQUE USED IN THE ASSAY OF RIBONUCLEOTIDE REDUCTASE.

The study of various parameters of enzymatic reactions in cell-free extracts or purified preparations may not reflect true physiological properties in some cases. There is increasing evidence that the properties of an enzyme may be affected by its intracellular environment (Aragon et al 1980). The large dilution that accompanies cell breakage can have drastic effects on the properties of an enzyme. The association of enzyme subunits or of different enzymes to form multienzyme complexes often have far reaching effects on catalytic and regulatory properties of such enzymes. Extraction procedures, which may disrupt these associations, could profoundly alter the properties as measured in vitro. Ribonucleotide reductase would seem to be an enzyme whose properties could be greatly affected by extraction from the intracellular environment. Not only is the enzyme made up of a minimum of two protein subunits (Cory et al 1978, Engström et al 1979, Chang and Cheng 1979c, Mattaliano et al 1981, Youdale et al 1982), it would also seem to be a component of a large multienzyme complex involved in the synthesis of DNA (Elford 1974, Reddy and Pardee 1980, Forsdyke and Scott 1980).

In order to study the properties of mammalian ribonucleotide reductase in a situation as close to the normal in vivo environment as possible, this laboratory has developed a Tween 80 permeabilization technique (Lewis et al 1978, Wright et al 1981) based on the method of Billen and Olson (1976). To date the technique has successfully been used to study the enzyme from Chinese hamster ovary (Lewis et al 1978, Hards and Wright 1981), human diploid fibroblast (Dick and Wright 1980,

1982), mouse L (Koropatnick and Wright 1980), T-lymphoma (Ullman et al 1980) and rat myoblast (Wright et al 1982b, Creasey et al 1982) cells.

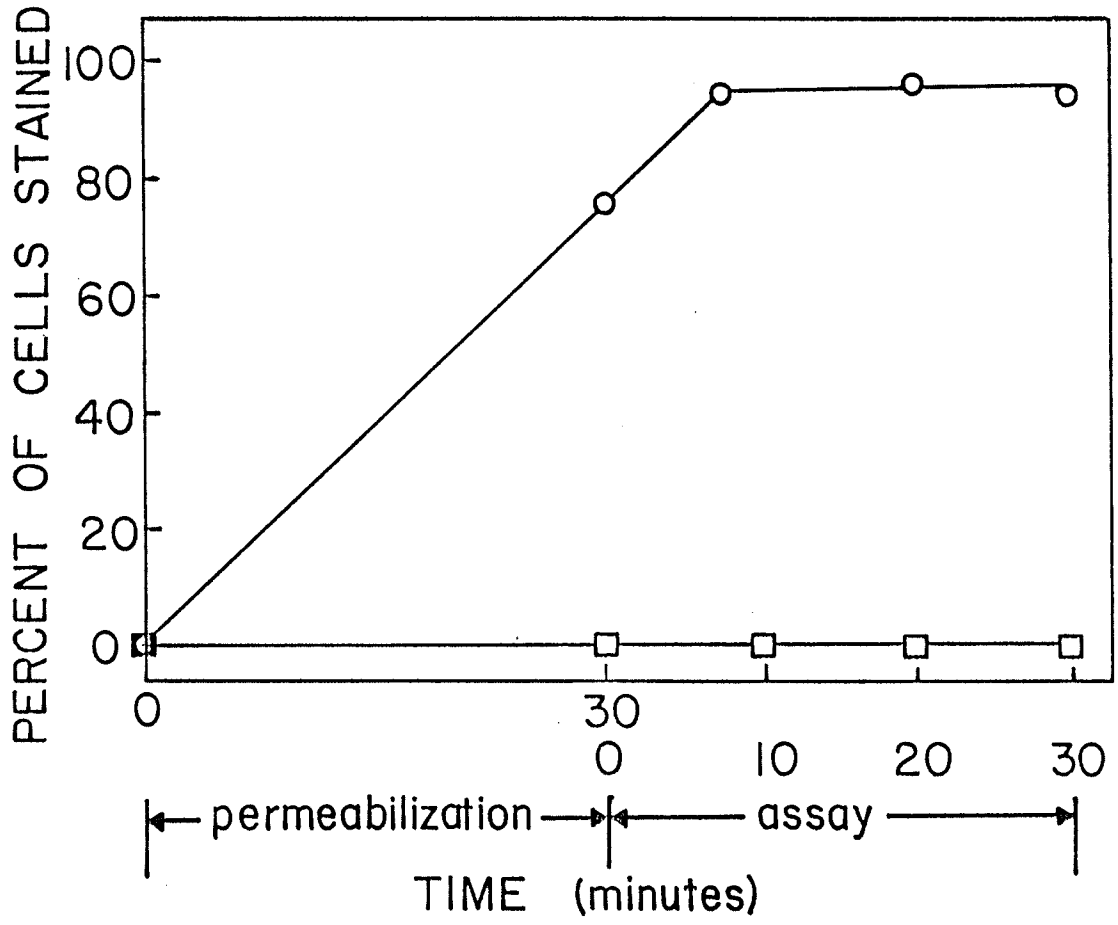
1.1 Verification of Permeabilization Induced by Tween 80 Treatment

It would seem that treatment of CHO cells with permeabilizing buffer as described in Materials and Methods does allow for passage of nucleotides through the normally impermeable cellular membrane as the compounds are reduced by the intact cells (Wright et al 1981). Cells not treated with Tween 80 have been shown to reduce ribonucleoside diphosphates at a rate of approximately 10% of that observed in the presence of the detergent (Lewis et al 1978). In order to confirm that Tween 80 did indeed act as a permeabilizing agent, cells were treated with the standard permeabilization buffer for 30 minutes at 22°C, centrifuged, resuspended in fresh permeabilizing buffer, dispensed into standard reaction mixtures, as described in Materials and Methods, and then incubated at 37°C. At various times during the assay, aliquots of cells were removed and added to a 0.4% solution of trypan blue, a compound excluded from physically intact cells (Nardone 1977), made up in PBS. After 10 to 15 minutes incubation at room temperature cells having taken up the dye were determined with the aid of a hemocytometer and a microscope. A parallel experiment was run in an identical manner except that Tween 80 was omitted from all buffers. As can be seen in figure 5, cells treated with Tween 80 took up the trypan blue while unpermeabilized cells actively excluded it.

1.2 Reversibility of Tween 80 Treatment

One of the main reasons for using the Tween 80 permeabilization technique to measure ribonucleotide reductase activity was the hope that much of the intracellular environment would be retained during the assay. As treatment of mammalian cells with Tween 80 is known to

Fig. 5. Uptake of trypan blue by Tween 80 permeabilized (O) and non-permeabilized (□) wild-type CHO cells. Cells were treated with complete permeabilzing buffer (O) or buffer lacking Tween 80 (□) using standard procedure and then suspended in a 0.40% solution of trypan blue for 10 to 15 minutes.


PERCENT OF CELLS STAINED
100
80
60
40
20
0
0
30
0
10
20
30
permeabilization
assay
TIME (minutes)

disrupt membrane structure (Kay 1965) and to affect other cellular structures such as mitochondria (Heppel and Makan 1977), the ability of permeabilized cells to recover from the treatment and grow normally was used to measure the degree to which cells were damaged.

CHO cells were subjected to standard permeabilization and assay techniques as described in Materials and Methods. At various times aliquots were removed and dispersed to tissue culture plates containing α(MEM) plus 10% FCS. After a suitable period of incubation, the medium was drained and the colonies stained with methylene blue. A parallel experiment in which cells were treated in an identical manner except that Tween 80 was omitted from all buffers was also run. After the 30 minute incubation with permeabilizing buffer the plating efficiency was 0.502 ± 0.046 while at the end of a 30 minute assay period the value was 0.505 ± 0.039. With cells treated with buffers lacking Tween 80 the plating efficiencies were 0.705 ± 0.083 after 30 minutes and 0.720 ± 0.031 at the end of the assay period. In each case, the result is an average of ten trials. It would thus seem that 70% of CHO cells permeabilized with Tween 80 had retained sufficient cellular integrity to allow for resealing and resumption of growth.

1.3 Incorporation of ADP and CDP into Macromolecules

Since most permeabilized cells are capable of forming colonies when placed in growth medium, their abilities to synthesize macromolecules have not been permanently damaged by the procedure. In fact, various permeabilization techniques have been used to study the ability of cells to synthesize protein, RNA and DNA when supplied with the appropriate substrates and cofactors (Burgoyne 1972, Castellot et al 1978, Kitagawa 1980). Significant nucleotide incorporation into RNA and DNA during ribonucleotide reductase assays could lead to incorrect estimates of enzyme activity if proper correction

factors were not applied. Therefore, studies on the incorporation of enzyme substrates and products into these nucleic acids were carried out. Ribonucleotide reductase catalyzes the reduction of both pyrimidine and purine ribonucleoside diphosphates required for the synthesis of DNA. Therefore, the incorporation of label from pyrimidine (CDP) and purine (ADP) substrates into RNA and DNA was analysed in permeabilized cells, under conditions routinely used to assay enzyme activity.

In four independent experiments it was found that label from ^{14}C-CDP was incorporated into RNA at a level of 0.300 $\pm$ 0.122 nmoles of nucleotide. As this represents only 0.25% of the total substrate used in a standard CDP reductase assay (120 nmoles), the relatively small amount of substrate found associated with RNA should not significantly affect enzyme activity determinations. In three independent experiments, no label from ^{14}C-CDP was incorporated into DNA. In a fourth experiment, 0.036 nmoles of labelled nucleotide were found associated with DNA. While this amount was only 0.03% of the total substrate normally included in an enzyme assay, it also represented lost dCDP formed by ribonucleotide reductase. Enzyme activity determinations with these CHO cells showed that the amount lost to DNA in this experiment was 15.2% of 0.197 nmoles dCDP formed/5 x 10^6 cells/20 minutes. The permeabilization step not only allows the entry of nucleotide substrates and effectors into the cell but also reduces intracellular nucleotide concentrations. For example, HPLC analysis of nucleotide pools of the CHO cells showed permeabilized cells to contain 0.16 nmoles of ATP/10^6 cells while in untreated cells the concentration was found to be 1.589 nmoles of ATP/10^6 cells. Thus permeabilization of the cells decreases intracellular ATP levels by 90%. Indeed, for several

ribo- and deoxyribonucleoside triphosphates, permeabilization reduced intracellular levels to unmeasurably low values. Since all four deoxyribonucleotide substrates are required for DNA polymerase activity (Billen and Olson 1976) and only one is supplied via the ribonucleotide reductase assay, it is likely that the amount of label incorporated into DNA in these experiments is dependent upon the effectiveness of the permeabilization step.

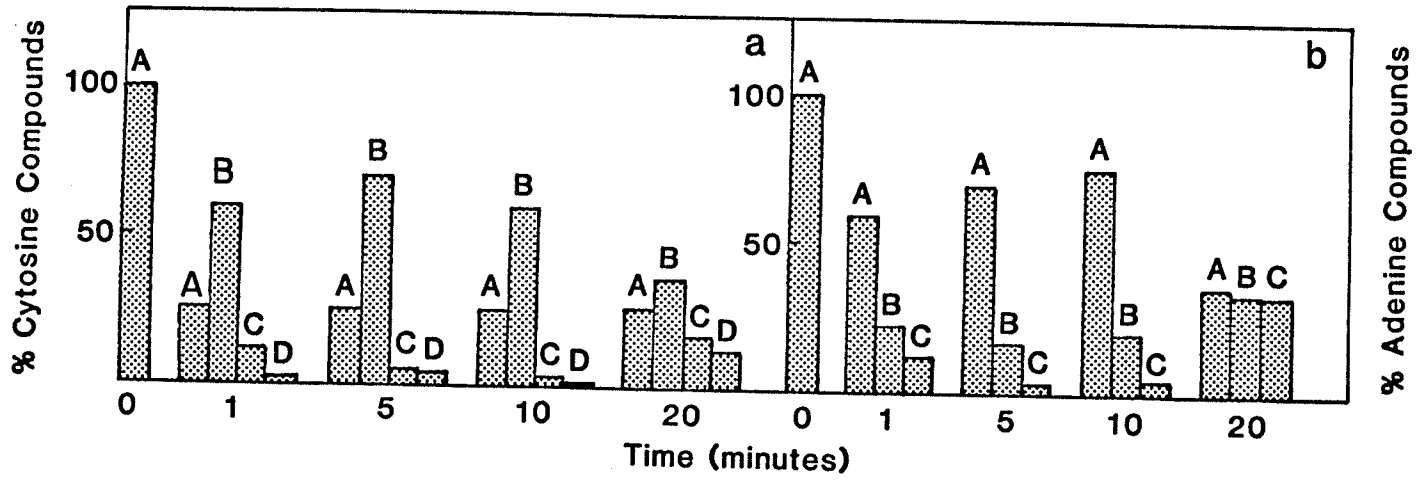
Similar experiments were performed on the incorporation of ^{14}C-ADP into RNA and DNA. In four separate tests 0.115 $\pm$ 0.046 nmoles of labelled substrate were found in RNA. This represented 0.10% of the ADP concentration (120 nmoles) routinely used in enzyme assays. As with CDP incorporation studies, the amount of ADP substrate lost to RNA should not significantly affect estimates of intracellular reductase activity. The amount of labelled nucleotide found in DNA ranged from 0 to 0.068 nmoles with an average of 0.025 nmoles. Enzyme activity estimates indicated that an average of 9.3% of the dADP formed by ribonucleotide reductase during a 20 minute assay was eventually incorporated into DNA.

1.4 Activity of Nucleotide Metabolizing Enzymes in Intact Permeabilized Cells.

Since the CHO cells remain intact during the enzyme assay, it seemed likely that there would be some interconversion of nucleotide substrates and effectors during the normal 20 minute assay period. An analysis by HPLC of the nucleotides present at the end of both CDP and ADP reductase determinations indicated that various kinase and phosphatase activities were present in permeabilized cells, as all levels of phosphorylation of substrates and effectors were detected. However,

the base composition of the nucleotides added to the assay systems were not altered. For example, the formation of uridine or inosine containing nucleotides was not detected in the assays. This indicates that activities such as cytidine deaminase and adenosine deaminase were not active in the system. Since there was movement of phosphates during the assays, nucleotide distributions were examined at various times. Replicate determinations were performed at 0, 1, 5, 10 and 20 minutes, after which the reactions were terminated with perchloric acid and analyzed by HPLC as described in Materials and Methods. As shown in figure 6a, during the CDP reductase reaction, the intracellular concentration of substrate reached equilibrium (25 to 27%) with the other cytosine containing compounds within the first minute of the 20 minute assay. The concentrations of cytidine, CMP and CTP changed relative to one another during the assay, with CTP levels declining from about 60% within the first minute to approximately 38% after 20 minutes and cytosince and CMP levels increasing from 3% to 15% and 12% to 23% respectively. Figure 6b shows that the level of the allosteric activator of CDP reductase, ATP, declined to between 63% and 84% of the added amount within the first 10 minutes of the reaction and to 32% after 20 minutes. The decrease in ATP level at 20 minutes was matched by a corresponding increase in AMP and ADP concentrations, so that the intracellular levels of the three purine nucleotides are approximately equal. Nucleotide distributions during the ADP reductase assay are shown in figure 7. As with the CDP reductase assay, the substrate concentration in the ADP reductase system reached equilibrium with the nucleoside and the other two nucleotides very quickly (figure 7a) and there was little fluctuation in the data for ADP concentrations (37 to 45%) at

Fig. 6. (a) Relative concentrations of cytosine containing compounds: (A) CDP, (B) CTP, (C) CMP, and (D) cytidine; (b) relative concentrations of adenine containing compounds: (A) ATP, (B) ADP and (C) AMP at various times during the CDP reductase assay as determined by HPLC proceudres. The reactions contained 120 nmoles CDP and 600 nmoles ATP at the start.


% Cytosine Compounds
100
50
A
B
C
D
a
b
0
1
5
10
20
Time (minutes)
% Adenine Compounds

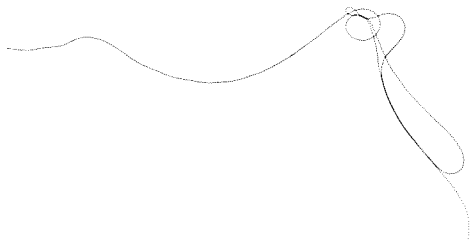
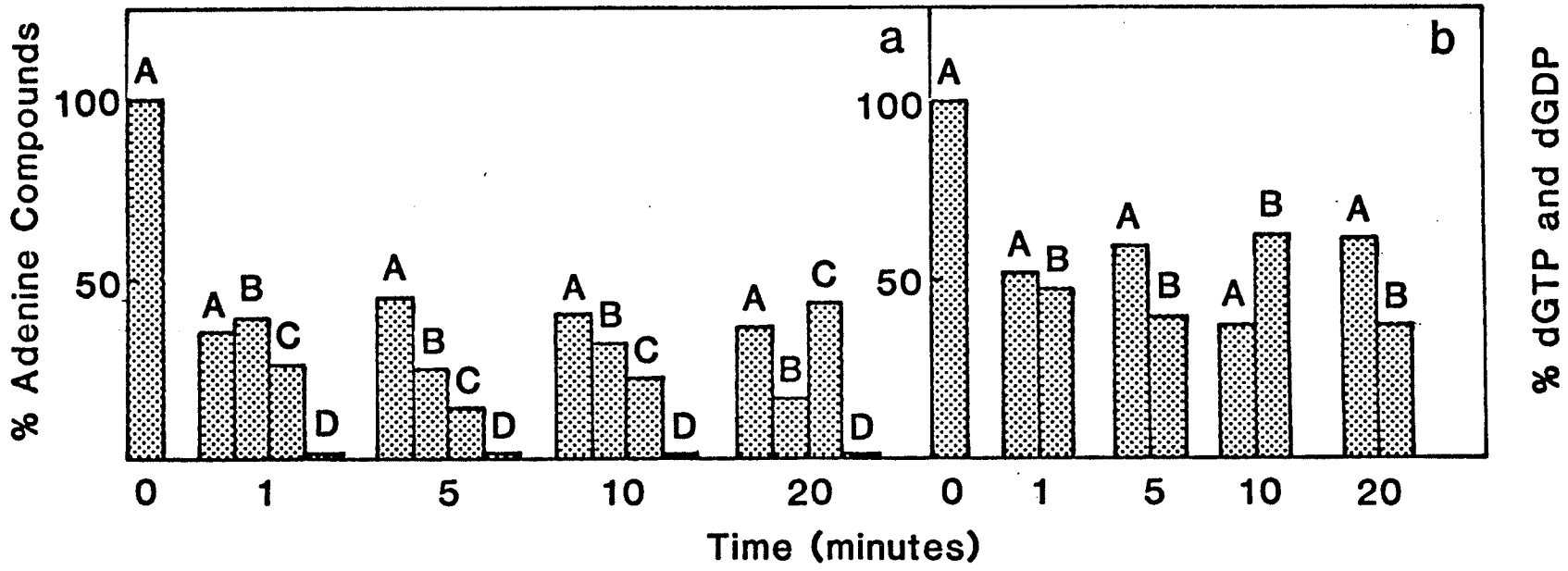


Fig. 7. (a) Relative concentrations of adenine containing compounds: (A) ADP, (B) ATP, (C) AMP and (D) adenosine; (b) Relative concentrations of A(dGTP) and B(dGDP) at various times during the ADP reductase assay as determined by HPLC procedures. The reactions contained 120 nmoles ADP and 150 nmoles dGTP at the start.


% Adenine Compounds
100
50
A
A B C D
A B C D
A B C D
A B C D
0
1
5
10
20
a
b
A
100
50
A B
A B
A B
0
1
5
10
20
Time (minutes)
% dGTP and dGDP

all times tested between one and twenty minutes. The activity of ADP reductase is dependent upon the presence of the allosteric activator, dGTP (Wright et al 1981). Figure 7b shows that no dGMP was detected during the assay and that the concentration of dGTP relative to dGDP fluctuated during the 20 minute assay between 38% (at 10 min) and 62% (at 20 min). Apparently there is a continuous interconversion between these two nucleotides throughout the ADP reductase assay period.

Because much of the work connected with this investigation involved kinetic analysis at various CDP and ADP concentrations, the effect of substrate concentration on the distribution of the various nucleotides was investigated. As can be seen from Table 5, variation in CDP concentration had no effect on any of the nucleoside or nucleotide levels found in the system at the end of a standard 20 minute assay. Within the ADP reductase system, substrate concentrations of 2.6 mM were found to increase the relative ADP levels at the expense of both AMP and ATP. In addition the presence of 2.6 mM ADP stimulated the conversion of dGTP to dGDP.

1.5 Evaluation of Permeabilization Assay

Since the reductase activity is determined in the physiologically relevant environment of the intact cell, it is not surprising that other nucleotide metabolizing enzymes are also observed. Studies on the incorporation of CDP and ADP into RNA indicated that a very small proporation of these reductase substrates were eventually incorporated into this macromolecule during a routine enzyme assay. The small decrease in substrate concentrations, 0.25% and 0.10% of added CDP and ADP respectively, would have an undetectable effect on the estimates of ribonucleotide reductase activity. The possibility that the products

Table 5. Effect of substrate concentration on the distribution of nucleosides and nucleotides in standard CDP and ADP reductase assay systems using permeabilized hamster cells[1,2]

Nucleoside or Nucleotide	CDP concentration (mM) added to assay		
	0.02	0.4	1.2
cytidine[3]	7	15	6
CMP[3]	28	22	26
CDP[3]	28	27	32
CTP[3]	37	36	36
AMP[4]	34	34	36
ADP[4]	39	34	36
ATP[4]	28	32	28

	ADP concentration (mM) added to assay		
	0.02	0.4	2.6
adenosine[3]	1	1	1
AMP[3]	44	43	23
ADP[3]	38	41	67
ATP[3]	17	14	9
dGDP[4]	54	54	20
dGTP[4]	47	46	20

[1]data expressed as percentage of total amount of each base moiety present.

[2]distribution after standard 20 minute assay.

[3]levels determined by radioactivity.

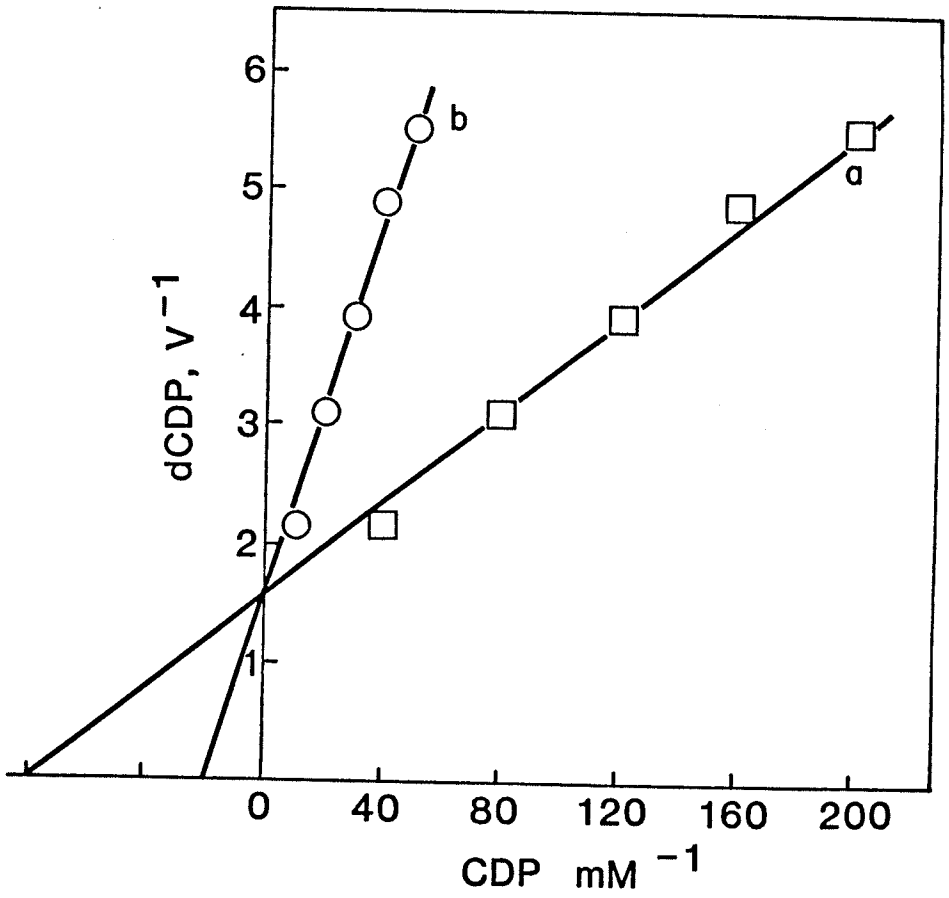
[4]levels determined from peak height of chromatogram.

of the enzyme reaction, dCDP and dADP, were phosphorylated to dCTP and dATP and then incorporated into DNA was also examined. Since all four deoxyribonucleotide substrates are required for DNA polymerase activity, proper permeabilization of the cells, which dilutes intracellular deoxyribonucleoside triphosphate levels, should eliminate or at least greatly reduce loss of deoxyribonucleotide products through incorporation of DNA. Studies showed that in four independent determinations an average of 3.8% of the dCDP and 9.3% of the dADP produced during a standard 20 minute assay was eventually incorporated into DNA. This small loss of reductase products is tolerable and would not seriously affect calculated enzyme activities. However, if desired, it would be possible to make corrections for incorporation into DNA by performing the appropriate incorporation experiments with the permeabilized cells used in enzyme assays.

While the incorporation of substrates or products into nucleic acids was not a significant problem in the assay systems, the presence of intracellular kinase and phosphatase activities was important. When the concentrations of nucleosides derived from substrates of the reaction were analyzed, nucleosides and nucleoside mono-, di- and triphosphates were found. The compounds derived from CDP , cytosine, CMP and CTP , have no effect on CDP reductase assays as they do not act as substrates or effectors of pyrimidine ribonucleotide reduction. In ADP reductase assays the presence of adenosine and AMP do not affect the reaction, but ATP is a positive effector for CDP reduction and therefore has an inhibitory effect upon ADP reduction (Chang and Cheng 1979b, Wright et al 1981). Interestingly, ATP concentration slowly falls during the assay between one minute and

20 minutes. It is important to note that although movement of phosphates occurred during the 20 minute assay of CDP and ADP reductase, the concentrations of the substrates quickly reached an equilibrium (within one minute) with their respective nucleosides and nucleotides which was maintained for the duration of the assay. Therefore, during most of the assay there is a relatively constant, although reduced, concentration of CDP or ADP substrate present. In the case of CDP, 25% to 27% of the initial concentration remains while for ADP, 35% to 45% of the initial remains. This approximately fixed level of substrate during the assay is reflected in the linearity of the plot of enzyme activity against time for at least 20 minutes for CDP reductase (figure 11b) and about 40 minutes with ADP reductase (figure 19a). Although useful approximations of enzyme activity, such as those comparing mutants to their parental wild types (Wright et al 1981, Ullman et al 1980), can be obtained without correcting for reduced substrate concentrations, more precise determinations can be calculated if the altered substrate values are taken into consideration. For example figure 8 shows double reciprocal plots of the variation in the rate of CDP reduction with CDP concentration in intact cells. In one case (a) the plot has been corrected for decreased substrate level while in the other (b) the plot remains uncorrected. The uncorrected analysis led to the calculation of a V_{max} value of 0.65 nmoles dCDP formed/5 x 10^6 cells/hour and a Km value of 0.05 mM CDP. Using the correction factor for CDP concentration, the V_{max} value remained unaltered but the Km value was 0.0125 mM CDP. These estimates are very similar to the values determined with highly purified mammalian ribonucleotide reductase (Eriksson et al 1979, Chang and Cheng 1979a, Youdale et al

Fig. 8. Double reciprocal plots of variation in rate of CDP reduction with CDP concentration using permeabilized wild type CHO cells, corrected (a) and uncorrected (b) for the substrate level change that occurs during the assay procedure. Activity expressed as nmoles dCDP formed /5 x 10^6 cells/hour.


6
5
4
3
2
1
dCDP, V^{-1}
b
a
0
40
80
120
160
200
CDP mM $^{-1}$

1982).

Since CDP and ADP reductase activities are dependent upon the presence of allosteric nucleotide effectors, their concentrations during enzyme assays were also determined. The concentration of ATP in the CDP reductase assay remained fairly constant (between 60% and 75% of the initial concentration) for the first 10 minutes of the assay, and then declined to 32% at 20 minutes. As ATP is the positive effector for the reduction of CDP, a significant decline in its concentration should result in a decrease in enzyme activity 20 minutes after the start of the assay. As shown in figure 11b, the reduction of CDP does deviate from linearity at about 20 minutes. Thus it would seem that the initiation of the assay causes a rearrangement in the level of phosphorylation of the adenine containing nucleotides which reaches an equilibrium within the first minute that is maintained for much of the assay time. Since the nucleotide levels are relatively constant during much of the assay time, a linear relationship between time and product formation is observed. However by 20 minutes, several changes have occurred, the most important being a decrease in ATP concentration, which is probably the major reason for the observed deviation from linearity. There is also an increase in ADP concentration within the CDP reductase assay time, but this is unlikely to be important since its interaction with the enzyme requires dGTP, GTP or dTTP (Wright et al 1981, Eriksson et al 1979, Murphree et al 1968, Peterson and Moore 1966) none of which are present in this assay system. The concentration of dGTP in the ADP reductase assay was observed to continually fluctuate with the concentration of dGDP during the course of the reaction. Why this should occur is not immediately obvious.

However, a major difference between the ADP and CDP reductase reactions is the lack of a sudden drop in activator concentration at 20 minutes in the ADP reductase system. This implies that the ADP reductase reaction should be linear for a longer time than was observed with CDP reductase. This is indeed what is observed as ADP reduction was linear with time to about 40 minutes (figure 19a).

The correction factors determined for the standard CDP and ADP reductase assays may also be applied to assays involving variable substrate concentration. As shown in Table 5, variation in CDP concentration from 0.02 to 1.2 mM (the maximum range used in this study) had no effect on the distribution of adenosine or cytidine compounds. The distribution of adenosine and deoxyguanosine compounds within ADP reductase assays was unaffected by substrate concentration variation ranging from 0.02 to 0.4 mM. At the highest ADP concentration tested (2.6 mM) there was a relative increase in dGDP and ADP levels but these effects can be corrected for.

Where feasible and relevant, nucleotide concentrations within the various ribonucleotide reductase assays reported in this study have been corrected for the kinase and phosphatase activities described.

2. CHARACTERIZATION OF RIBONUCLEOTIDE REDUCTASE ACTIVITY IN PERMEABILIZED WILD TYPE CHO CELLS.

Having determined that treatment of CHO cells with permeabilizing buffer containing Tween 80 does indeed provide access to intracellular enzymes without extensive disruption of cellular structure or function, the characterization of ribonucleotide reductase from a cloned wild type population was carried out. The clone slected for analysis had been designated WTC125 (Lewis and Wright 1978a). Cells were grown and assayed as described in Materials and Methods unless otherwise stated.

As ribonucleotide reductase is capable of reducing all four ribonucleotides, the properties of the enzyme were studied using a pyrimidine (CDP) and a purine (ADP) substrate.

2.1 CDP Reductase Activity.

Studies of ribonucleotide reductase activity using extracts of mammalian cells indicated that the reduction of CDP required the presence of the allosteric activator ATP, a dithiol reducing agent and magnesium ions (Moore and Reichard 1964, Larsson 1969, Fujioka and Silber 1970). Some studies indicated that while magnesium ions did stimulate the reduction, their presence was not essential for activity (Tyrsted and Gamulin 1979, Engström et al 1979). CDP reduction in permeabilized CHO cells showed an absolute requirement for ATP (figure 9b). While not essential, $MgCl_2$ (figure 9a) and DTT (figure 9c) were found to stimulate activity 7 and 2.4 fold respectively. Optimal activity occurred with 1.5 mM ATP, 7 mM $MgCl_2$ and 6 mM DTT. The effects of pH and temperature on the reduction of CDP are illustrated in figure 10. The optimal temperature was found to be $37^{o}C$, the same as the optimum for cell growth. Due to the method employed to measure the effect of

Fig. 9a. Response of CDP reductase activity in permeabilized WT cells to $MgCl_2$ concentration. Standard CDP reductase assay conditions were used except for the presence of varying concentrations of $MgCl_2$.

Fig. 9b. Response of CDP reductase activity in permeabilized WT cells to ATP concentration. Standard CDP reductase assay conditions were used except for the presence of varying concentrations of ATP. Results corrected for presence of kinase and phosphatase activities in intact cells.

Fig. 9c. Response of CDP reductase activity in permeabilized WT cells to DTT concentration. Standard CDP reductase assay conditions were used except for the presence of varying concentrations of DTT.

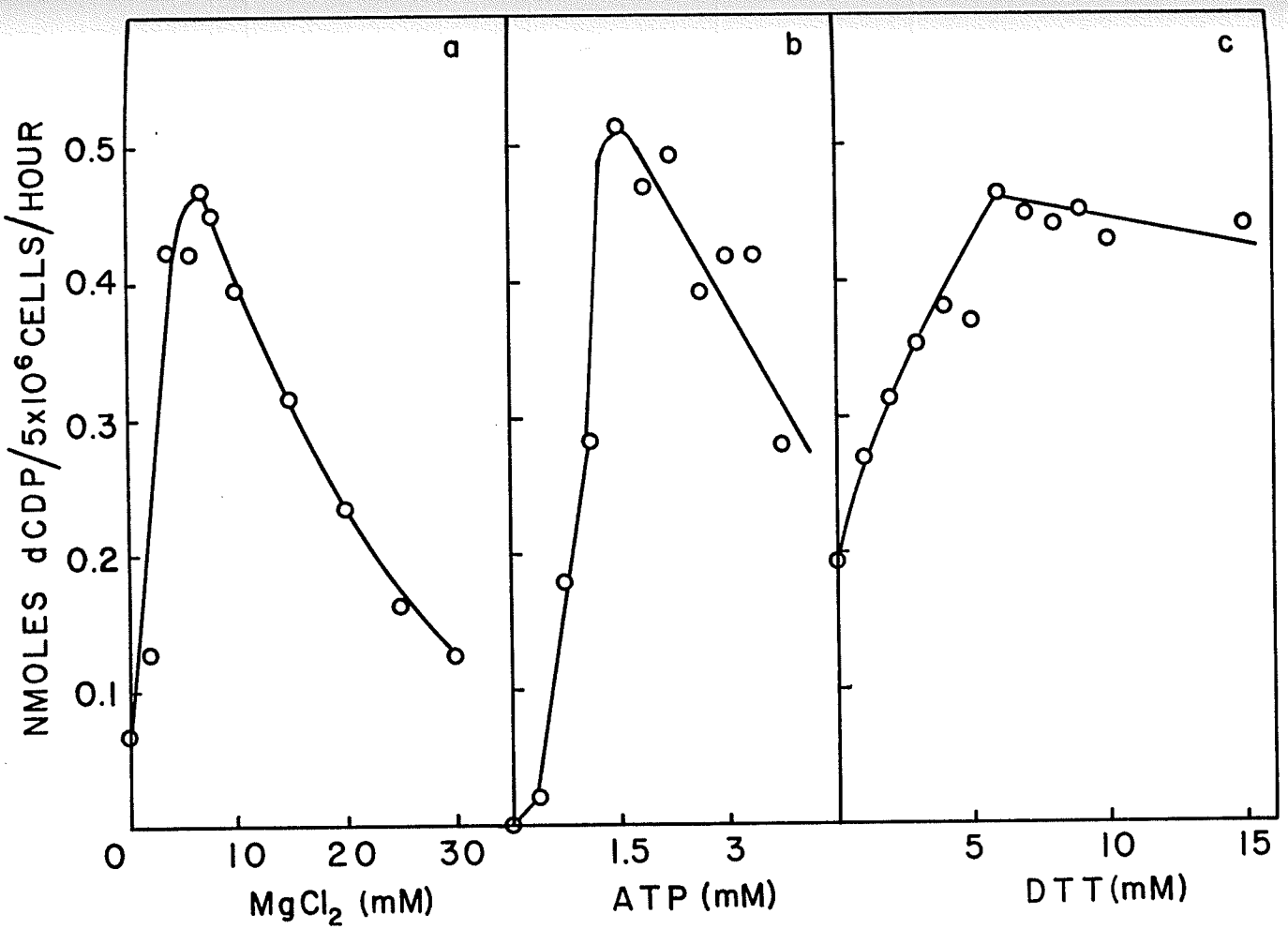
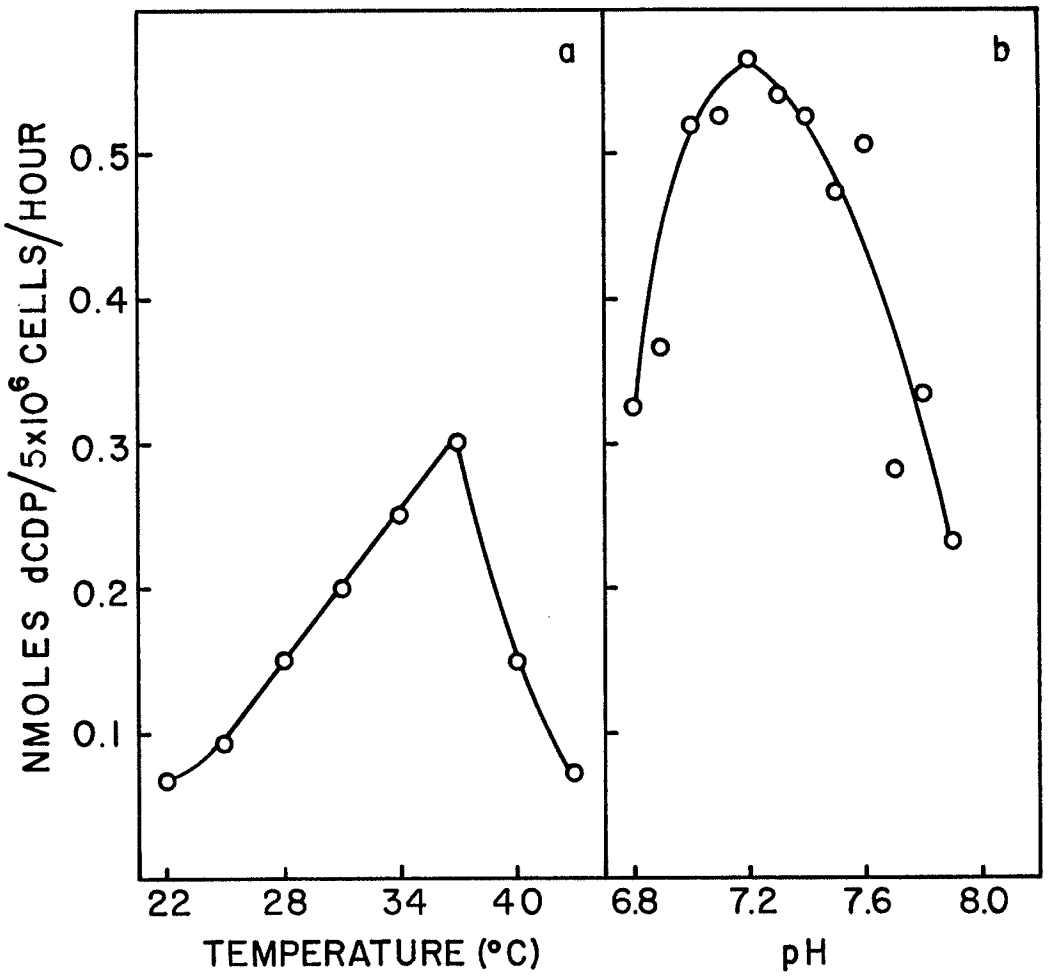

a
b
c
NMOLES dCDP/5x10⁶CELLS/HOUR
0.5
0.4
0.3
0.2
0.1
0
10
20
30
MgCl₂ (mM)
1.5
3
ATP (mM)
5
10
15
DTT(mM)

Fig. 10a. Response of CDP reductase activity in permeabilized WT cells to temperature. Standard CDP reductase assay conditions were used except for the variation in temperature indicated.

Fig. 10b. Response of CDP reductase activity in permeabilized WT cells to pH. Standard CDP reductase assay conditions were used except for the variation in pH indicated.

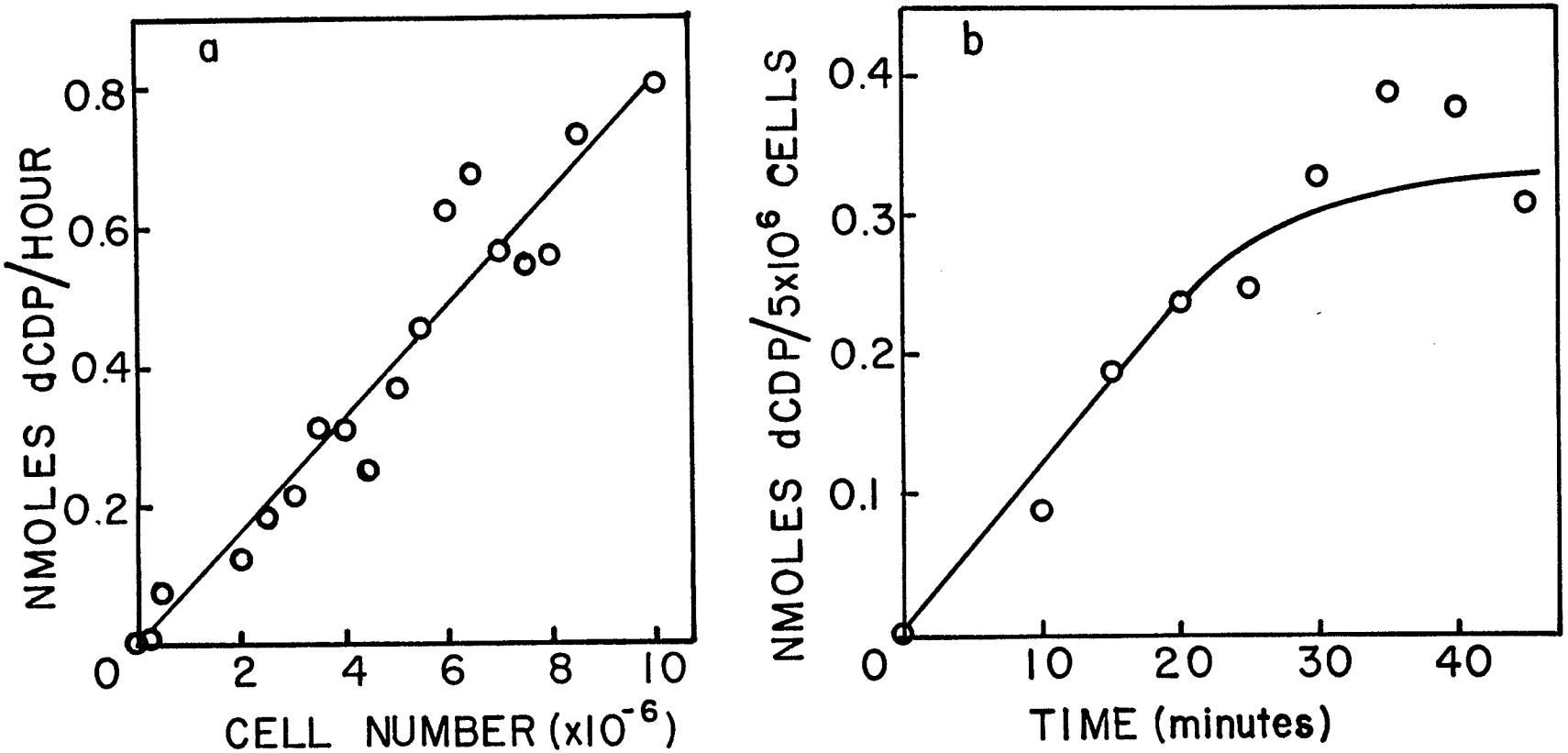

a
b
NMOLES dCDP/5x10^6 CELLS/HOUR
0.5
0.4
0.3
0.2
0.1
22
28
34
40
TEMPERATURE (°C)
6.8
7.2
7.6
8.0
pH

temperature on the reaction, the assays could not be agitated as usual. This resulted in the decreased levels of activity observed for this particular experiment. These values are similar to those reported for CDP reduction by various mammalian cell extracts (Moore and Reichard 1964, Larsson 1969, Fujioka and Silber 1970, Lewis and Wright 1978b, Kuzik and Wright 1979). The reduction of CDP by permeabilized CHO cells exhibited a pH optimum of 7.2 with greater than 90% of maximum activity occurring over a range from pH 7.0 to 7.4. This range is narrower than that reported for the enzyme extracted from regenerating rat liver (Larsson 1969) or leukemic mouse spleen (Fujioka and Silber 1970).

Having ascertained the optimal conditions for the assay of CDP reduction in permeabilized CHO cells, the linearity of the reaction with time and cell number was investigated (figure 11). Reduction was linear with time for 20 minutes and with cell number for up to 10^7 cells/assay. Of greater interest was the linearity of response at low cell number. Studies with enzyme extracted from a variety of sources have shown that at low protein concentrations CDP reductase activity falls off rapidly in a non-linear manner (Larsson 1969, Hopper 1972, Engström et al 1979, Kuzik and Wright 1979). This decrease in activity at low protein concentrations is thought likely to be due to the dissociation of enzyme subunits (Hopper 1972, Kuzik and Wright 1979). With the permeabilized cells, linearity was maintained at all cell numbers tested. To ensure that the observed linearity was due to the use of permeabilized cells and not to some property of the ribonucleotide reductase from CHO cells, the enzyme was extracted from the cells and a crude extract prepared as described in Materials and Methods.

Fig. 11a. Response of CDP reductase activity in permeabilized WT cells to cell number. Standard CDP reductase assay conditions were used except for the variation in cell number indicated.

Fig. 11b. Response of CDP reductase activity in permeabilized WT cells to incubation time. Standard CDP reductase assay conditions were used except for the variation in incubation time indicated.


a
NMOLES dCDP/HOUR
0.8
0.6
0.4
0.2
0
2
4
6
8
10
CELL NUMBER (x10^{-6})
b
NMOLES dCDP/5x10^6 CELLS
0.4
0.3
0.2
0.1
0
10
20
30
40
TIME (minutes)

Using the optimum assay conditions determined for the enzyme in permeabilized cells, the linearity of CDP reduction with protein concentration was determined (figure 12). As can be seen, at protein concentrations below 0.7 mg/assay there was the expected deviation from linearity. Using the procedures described in Materials and Methods, one milligram of crude enzyme preparation was extracted from approximately 10^7 cells. Thus with enzyme extracts, deviation from linearity with protein concentration occurred when the amount of ribonucleotide reductase present fell below the levels normally found within 7×10^6 cells. As can be seen from figure 11a reduction with intact cells was linear to values much lower than 7×10^6 cells.

A double reciprocal plot of the variation in CDP reduction with CDP concentration is shown in figure 13. The plot is the average of ten trials conducted over the course of approximately one year with similar results obtained each time. The V_{max} exhibited was 0.49 ± 0.17 nmoles dCDP formed/5×10^6 cells/hour. The Km value of 0.021 ± 0.005 mM CDP compares favourably with those determined with partially purified and highly purified enzyme from Novikoff ascites rat tumor (Moore and Hurlbert 1976), human Molt-4F cells (Chang and Cheng 1979a) and calf thymus (Eriksson et al 1979).

The CDP reductase activity of CHO cells grown in suspension showed the same kinetic properties as that from cells grown on solid surfaces. A double reciprocal plot of velocity of CDP reduction versus CDP concentration for cells grown in suspension (figure 14) gives a Km value 0.023 mM CDP and a V_{max} value of 0.43 nmoles dCDP formed/5×10^6 cells/hour. Thus the kinetic properties of CDP reductase from cells grown in suspension and on solid surfaces are virtually identical.

Fig. 12. Response of CDP reductase activity in extracts of WT cells to protein concentration. Varying amounts of extract were added to standard reaction mixtures and incubated for 30 minutes at 37°C.

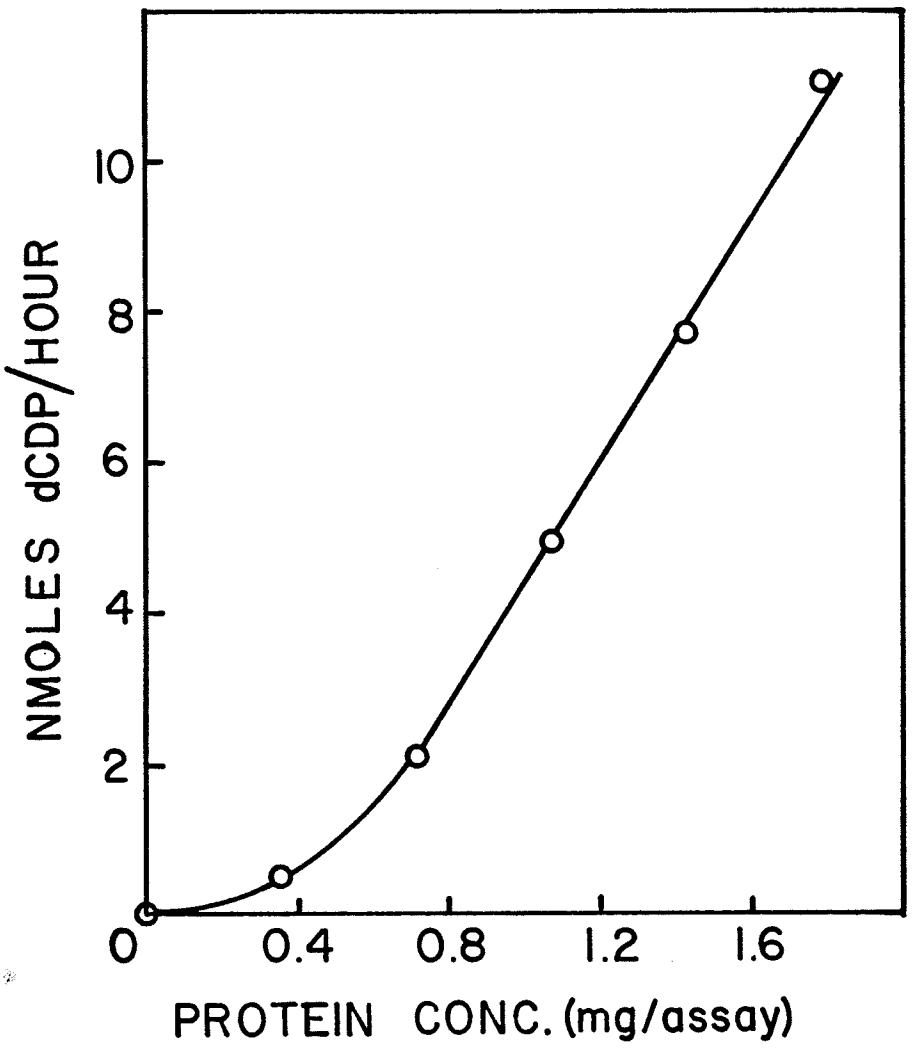

NMOLES dCDP/HOUR
0
2
4
6
8
10
0.4
0.8
1.2
1.6
PROTEIN CONC. (mg/assay)

Fig. 13. Double reciprocal plot of the variation in rate of CDP reduction with CDP concentration using permeabilized wild type CHO cells. Activity expressed as nmoles dCDP formed/5 x 10^6 cells/hour. Inset: velocity of CDP reduction in the presence of varying concentrations of CDP. Results corrected for the presence of kinase and phosphatase activities in intact cells.

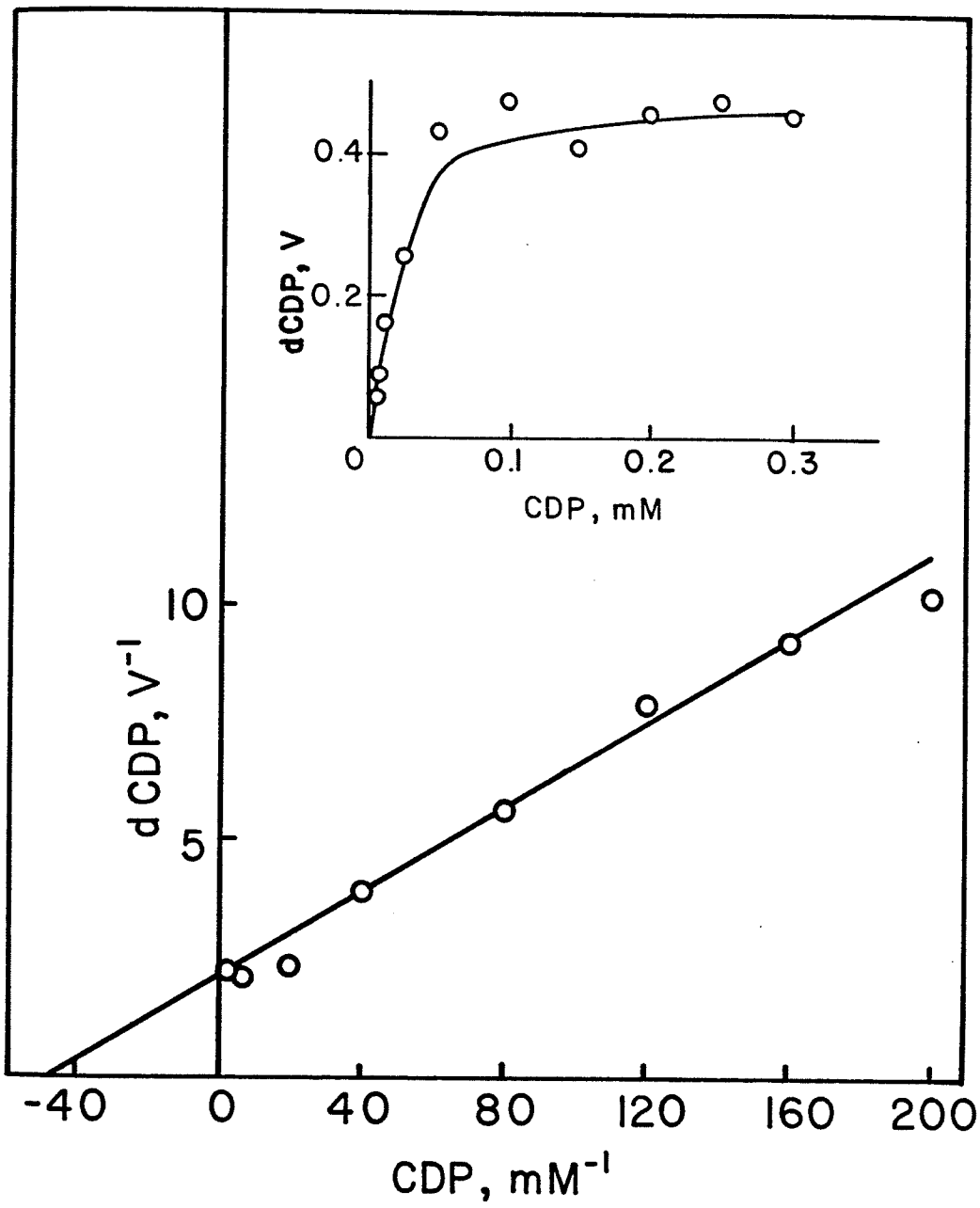
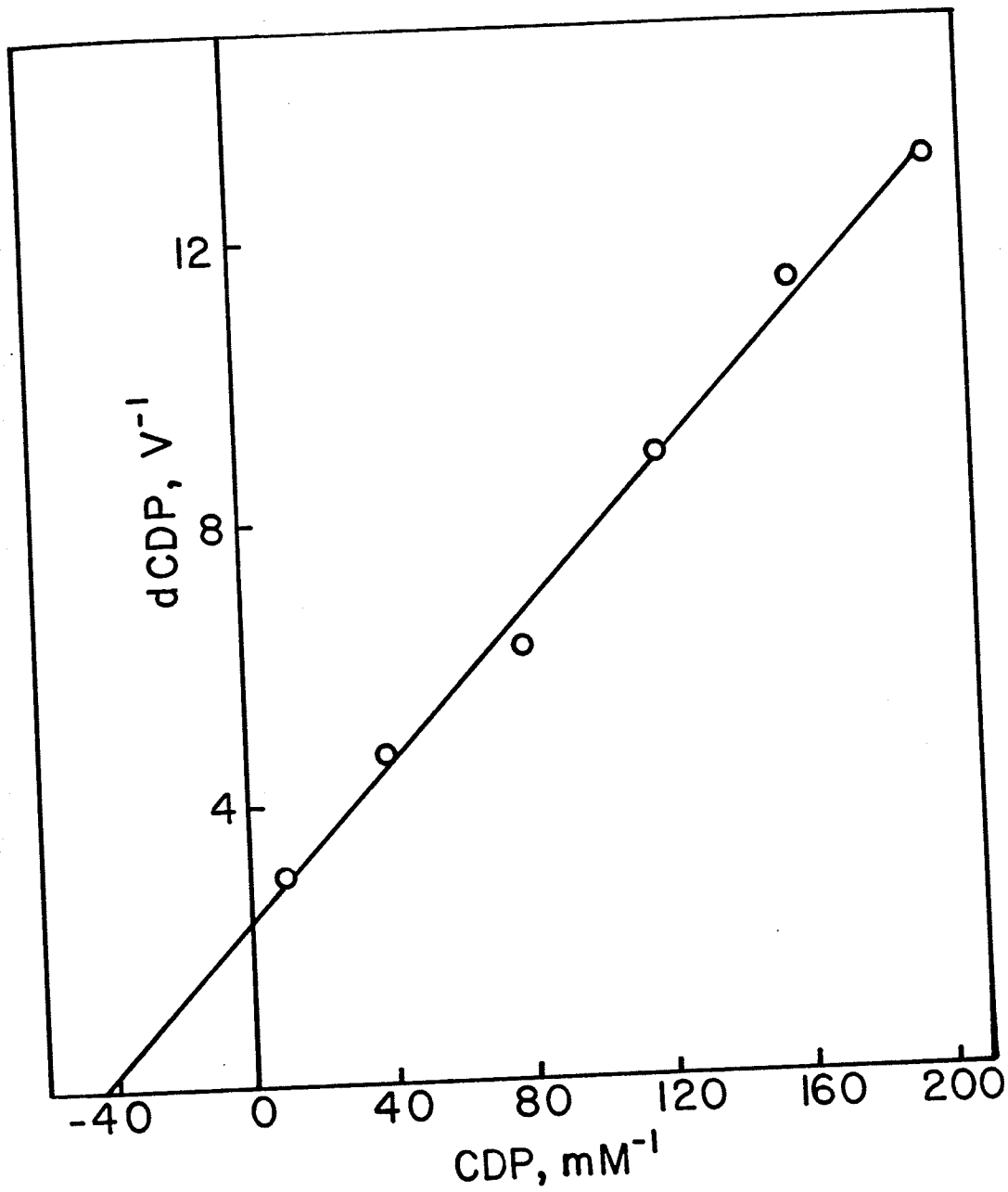

0.4
0.2
dCDP, V
0
0.1
0.2
0.3
CDP, mM
10
5
dCDP, V⁻¹
-40
0
40
80
120
160
200
CDP, mM⁻¹

Fig. 14. Double reciprocal plot of the variation in rate of CDP reduction with CDP concentration using permeabilized wild type CHO cells grown in suspension culture. Activity expressed as nmoles dCDP formed/5 x 10^6 cells/hour. Results corrected for the presence of kinase and phosphatase activities in intact cells.


12
8
4
dCDP, V⁻¹
-40
0
40
80
120
160
200
CDP, mM⁻¹

2.2 ADP Reductase Activity

The reduction of ADP by partially purified ribonucleotide reductase has not been as well studied as CDP reduction. However, it has been found that the reaction is stimulated by the presence of a dithiol reducing agent and requires the allosteric activator dGTP (Moore and Hurlbert 1966, Eriksson et al 1979, Cory 1979, Eriksson et al 1981b). With permeabilized CHO cells, it was found that DTT was not essential for activity but that a two-fold increase in activity was induced by 6 mM DTT (figure 15). ADP reduction was dependent upon the presence of dGTP with maximum activity requiring 0.04 to 0.2 mM dGTP (figure 16). These optimum values are similar to those reported for highly purified enzyme from regenerating rat liver (Youdale and MacManus 1979), and partially purified enzyme from rat embryos (Murphree et al 1968) and Novikoff ascites rat tumor (Moore and Hurlbert 1966). There is some question as to the magnesium ion requirement in the reduction of ADP as in some cases it has been added to the assay (Chang and Cheng 1979a, 1979b, Eriksson et al 1979) while in other investigations it has been omitted (Eriksson et al 1981b, Cory 1979, Cory and Mansell 1975a, Lewis and Wright 1978b, Kuzik and Wright 1979). With intact permeabilized CHO cells it was found that all concentrations of $MgCl_2$ tested were inhibitory (figure 17) with 50% inhibition occurring at 3.2 mM. Thus a distinct difference in response to $MgCl_2$ is exhibited by CDP and ADP reduction within intact permeabilized CHO cells. ADP reduction reached a maximum at a temperature of $37^{o}C$ and a pH range of 6.9 to 7.1 (figure 18). These values also represent the optimum conditions for cell growth. Having determined the optimal conditions for the assay of ADP reduction, the linearity of the reaction with time and

Fig. 15. Response of ADP reductase activity in permeabilized WT cells to DTT concentration. Standard ADP reductase assay conditions were used except for the presence of varying concentrations of DTT.

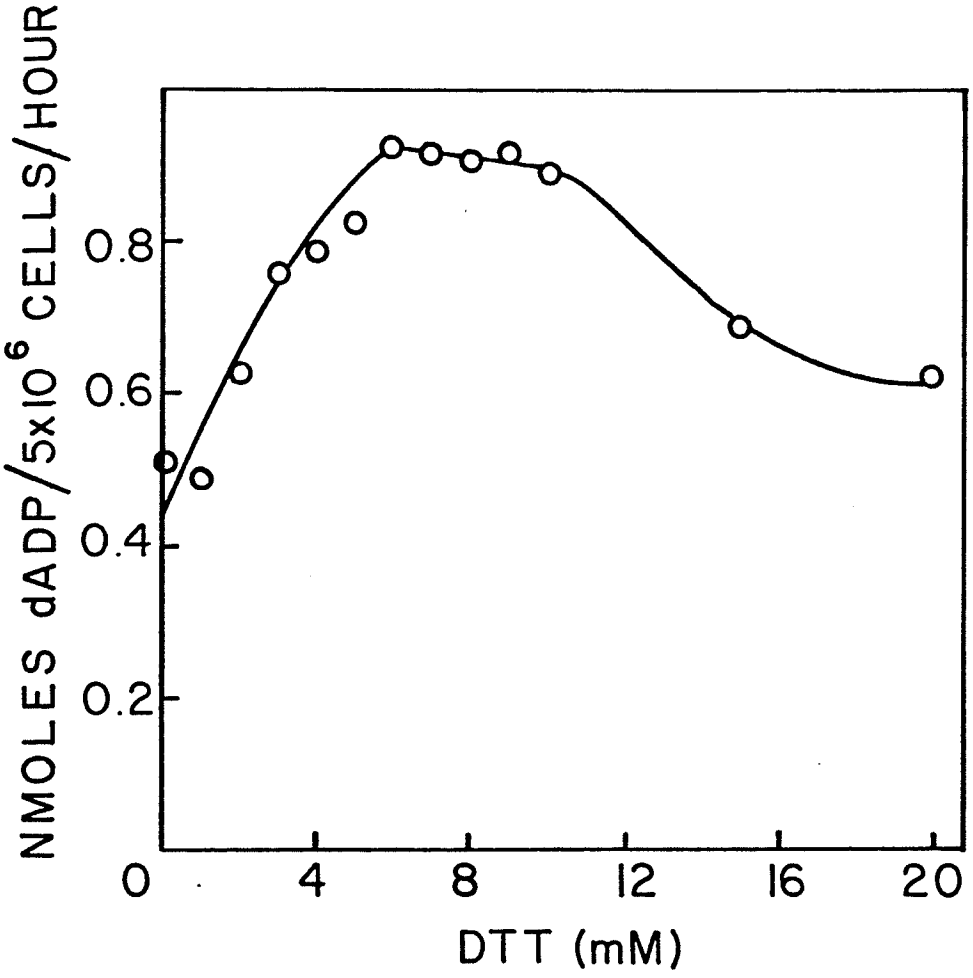

NMOLES dADP/5x10^6 CELLS/HOUR
0.8
0.6
0.4
0.2
0
4
8
12
16
20
DTT (mM)

Fig. 16. Response of ADP reductase activity in permeabilized WT cells to dGTP concentration. Standard ADP reductase assay conditions were used except for the presence of varying concentrations of dGTP. Results corrected for the presence of kinase and phosphatase activities in intact cells.

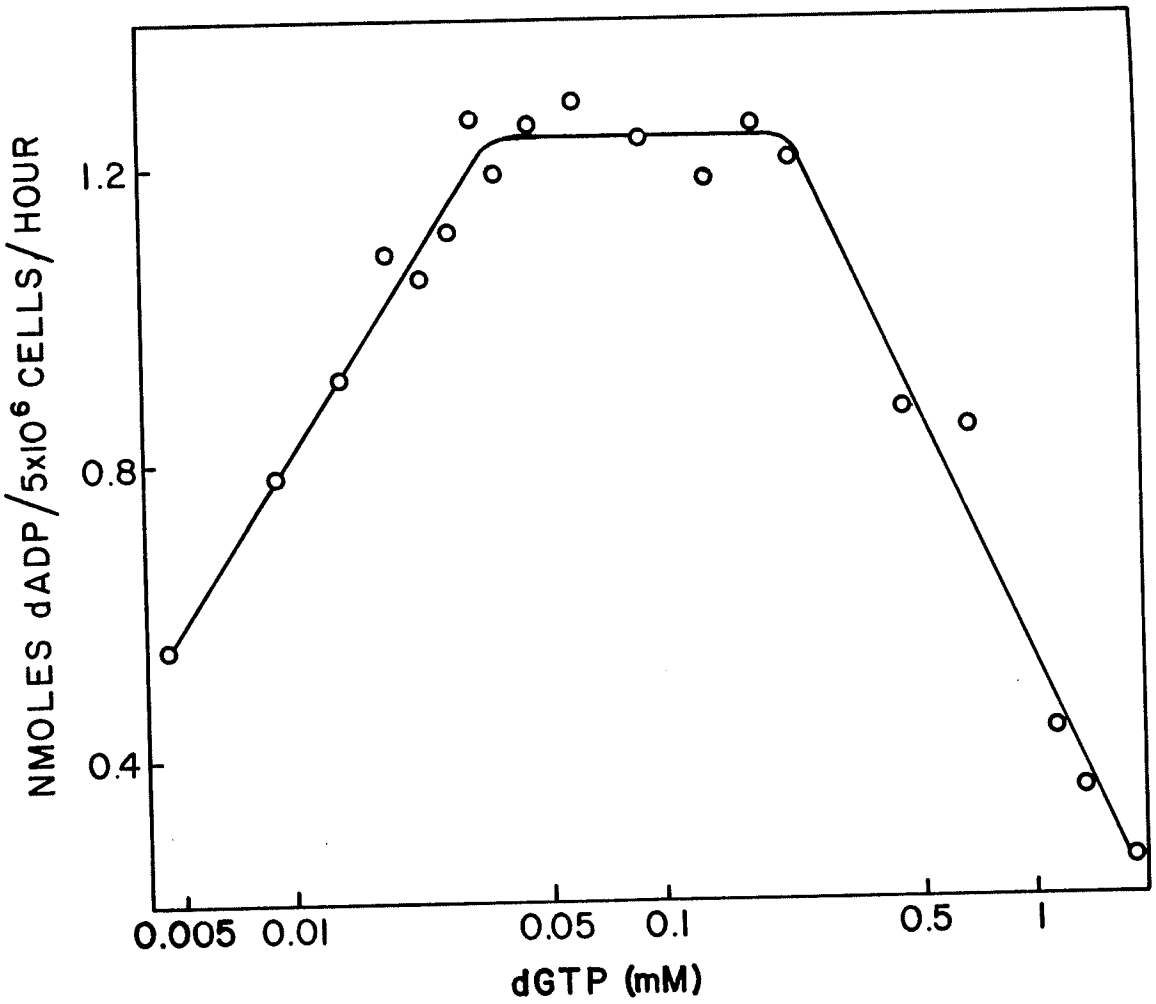

NMOLES dADP/5x10^6 CELLS/HOUR
1.2
0.8
0.4
0.005
0.01
0.05
0.1
0.5
1
dGTP (mM)

Fig. 17. Response of ADP reductase activity in permeabilized WT cells to $MgCl_2$ concentration. Standard ADP reductase assay conditions were used except for the presence of varying concentrations of $MgCl_2$.

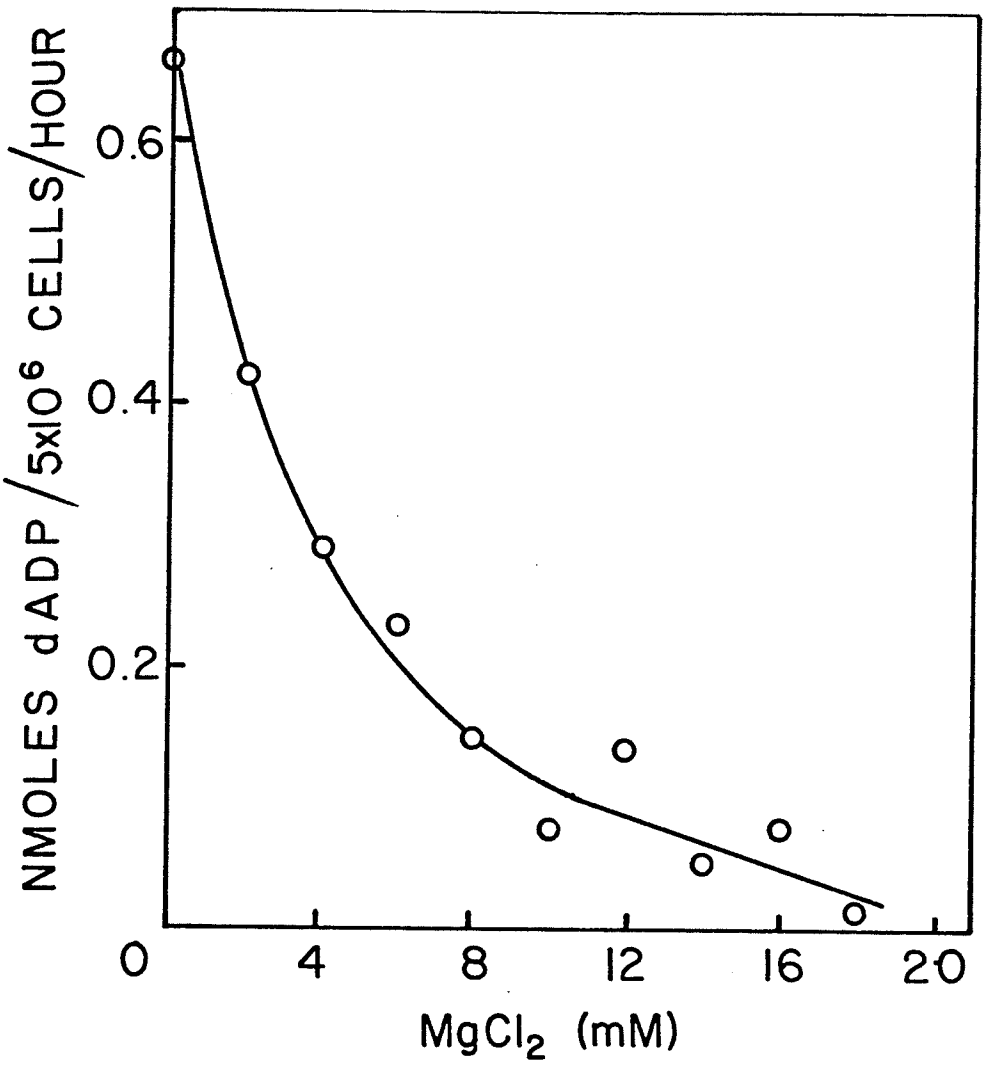
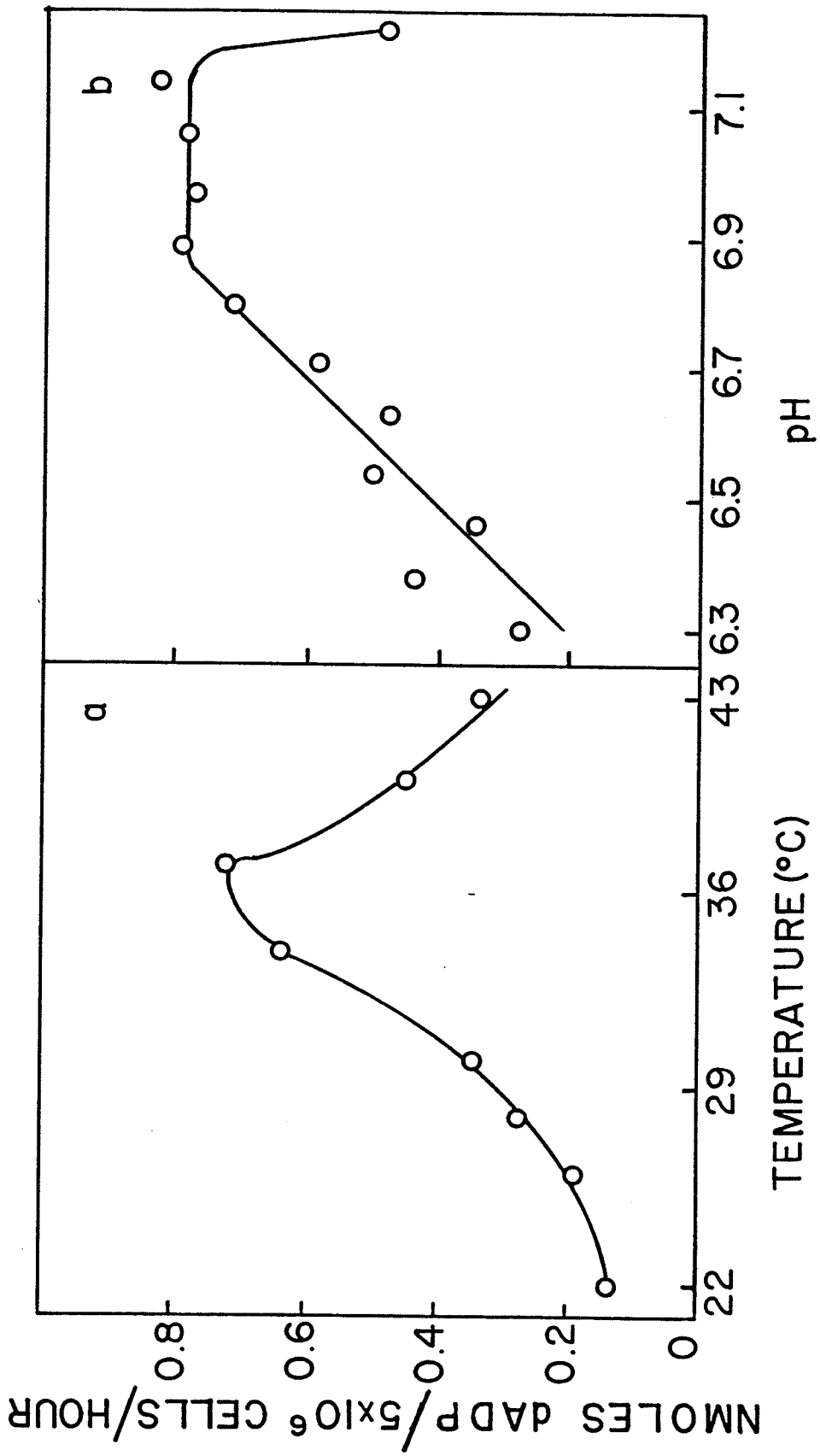

NMOLES dADP /5x10⁶ CELLS/HOUR
0.6
0.4
0.2
0
4
8
12
16
20
MgCl₂ (mM)

Fig. 18a. Response of ADP reductase activity in permeabilized WT cells to temperature. Standard ADP reductase assay conditions were used except for the variation in temperature indicated.

Fig. 18b. Response of ADP reductase activity in permeabilized WT cells to pH. Standard ADP reductase assay conditions were used except for the variation in pH indicated.


NMOLES dADP/5x10⁶ CELLS/HOUR
0
0.2
0.4
0.6
0.8
a
22
29
36
43
TEMPERATURE (°C)
b
6.3
6.5
6.7
6.9
7.1
pH

cell number was investigated (figure 19). It was found that reduction was linear with time for 40 minutes and with cell number up to and including 10^7 cells/assay.

A double reciprocal plot of the variation in rate of ADP reduction with ADP concentration is shown in figure 20. As with the kinetic values for CDP reduction, the reported results are the average of ten independent trials conducted over the course of approximately one year. The V_{max} determined was 1.00 $\pm$ 0.13 nmoles dADP formed/5 x 10^6 cells/hour while the Km value was 0.095 $\pm$ 0.017 mM ADP. This Km value is virtually identical to that reported for the enzyme from human Molt-4F cells (Chang and Cheng, 1979a) and Novikoff ascites rat tumor (Moore and Hurlbert 1966) and close to the value obtained with calf thymus (Eriksson et al 1979).

The kinetic properties of ADP reduction in permeabilized CHO cells were also determined in cells grown in suspension (figure 21). The V_{max} value was 0.91 nmoles dADP formed/5 x 10^6 cells/hour and the Km value was 0.14 mM ADP. These values are sufficiently similar to those obtained from cells grown on solid surfaces to allow for the conclusion that ADP reduction parameters do not vary under the different growth conditions.

A summary of the general properties of CDP and ADP reduction in permeabilized cells is presented in Table 6.

2.3 Secondary Activities of CDP and ADP Reduction

Reports that CDP and ADP reduction could be activated by nucleotides other than ATP and dGTP (Murphree et al 1968, Fujioka and Silber 1970, Peterson and Moore 1976, Dick and Wright 1980) led to the study of the effects of various nucleoside triphosphates on the reduction of

Fig. 19a. Response of ADP reductase activity in permeabilized WT cells to incubation time. Standard ADP reductase assay conditions were used except for the variation in incubation time indicated.

Fig. 19b. Response of ADP reductase activity in permeabilized WT cells to cell number. Standard ADP reductase assay conditions were used except for the variation in cell number indicated.

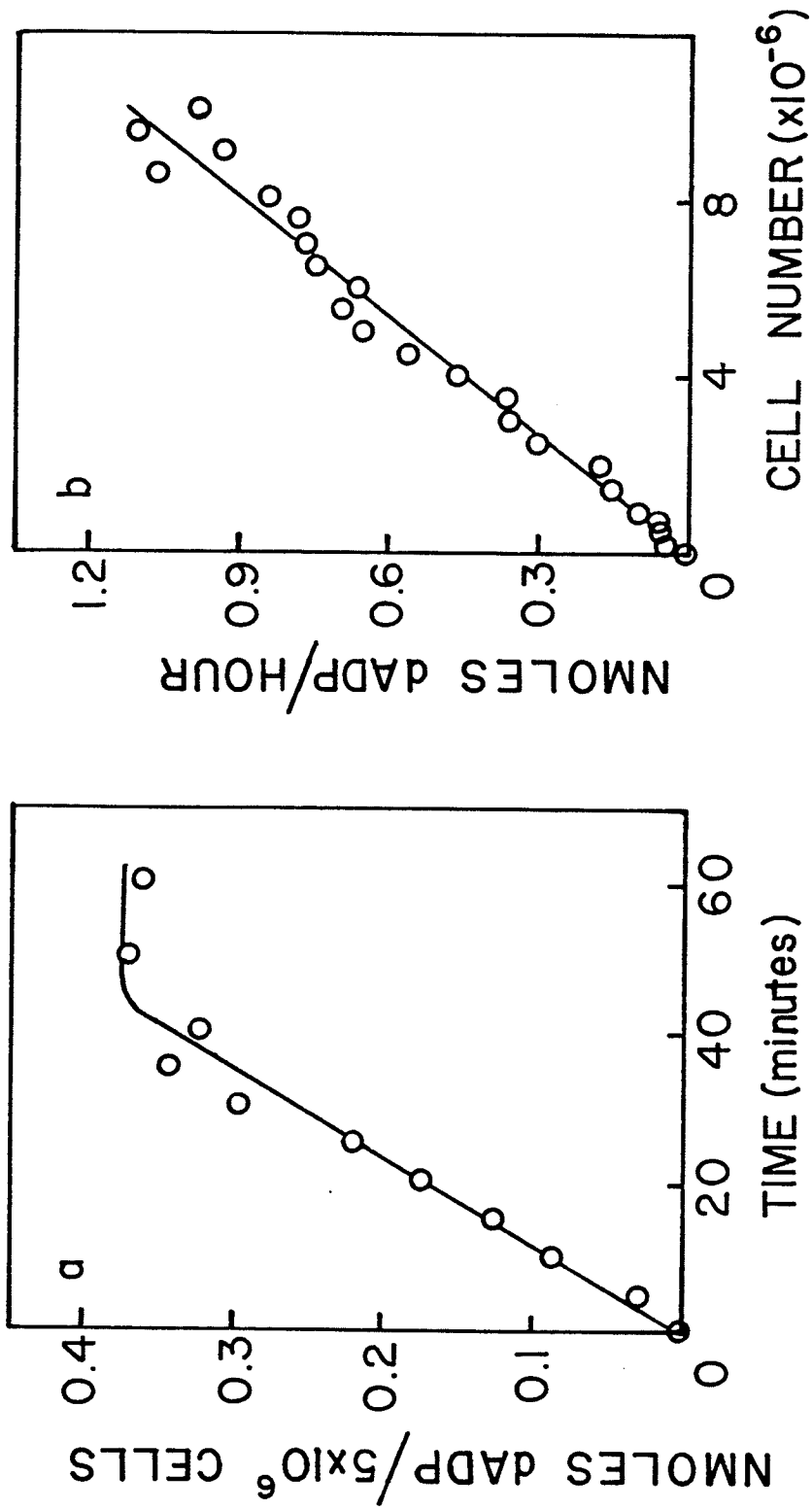

a
b
NMOLES dADP/5x10⁶ CELLS
NMOLES dADP/HOUR
TIME (minutes)
CELL NUMBER (x10⁻⁶)
0
0.1
0.2
0.3
0.4
0.3
0.6
0.9
1.2
20
40
60
4
8

Fig. 20. Double reciprocal plot of the variation in rate of ADP reduction with ADP concentration using permeabilized WT cells. Activity expressed as nmoles dADP formed/ 5×10^6 cells/hour. Inset: velocity of ADP reduction in the presence of varying concentrations of ADP. Results corrected for the presence of kinase and phosphatase activities in intact cells.

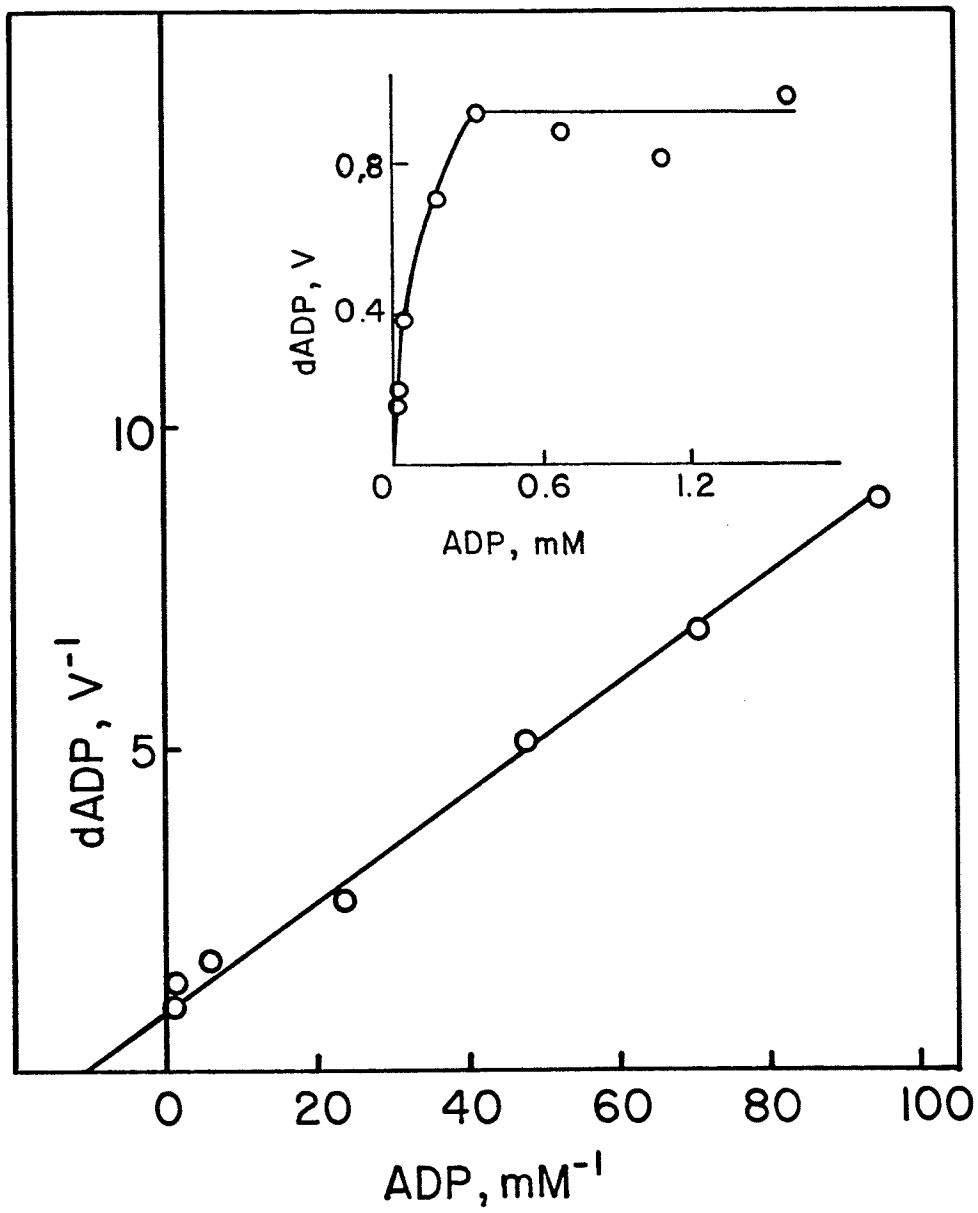

dADP, V
0.8
0.4
0
0.6
1.2
ADP, mM
dADP, V⁻¹
10
5
0
20
40
60
80
100
ADP, mM⁻¹

Fig. 21. Double reciprocal plot of the variation in rate of ADP reduction with ADP concentration using permeabilized WT cells grown in suspension culture. Activity expressed as nmoles dADP formed/5 x 10^6 cells/hour. Results corrected for the presence of kinase and phosphatase activities in intact cells.

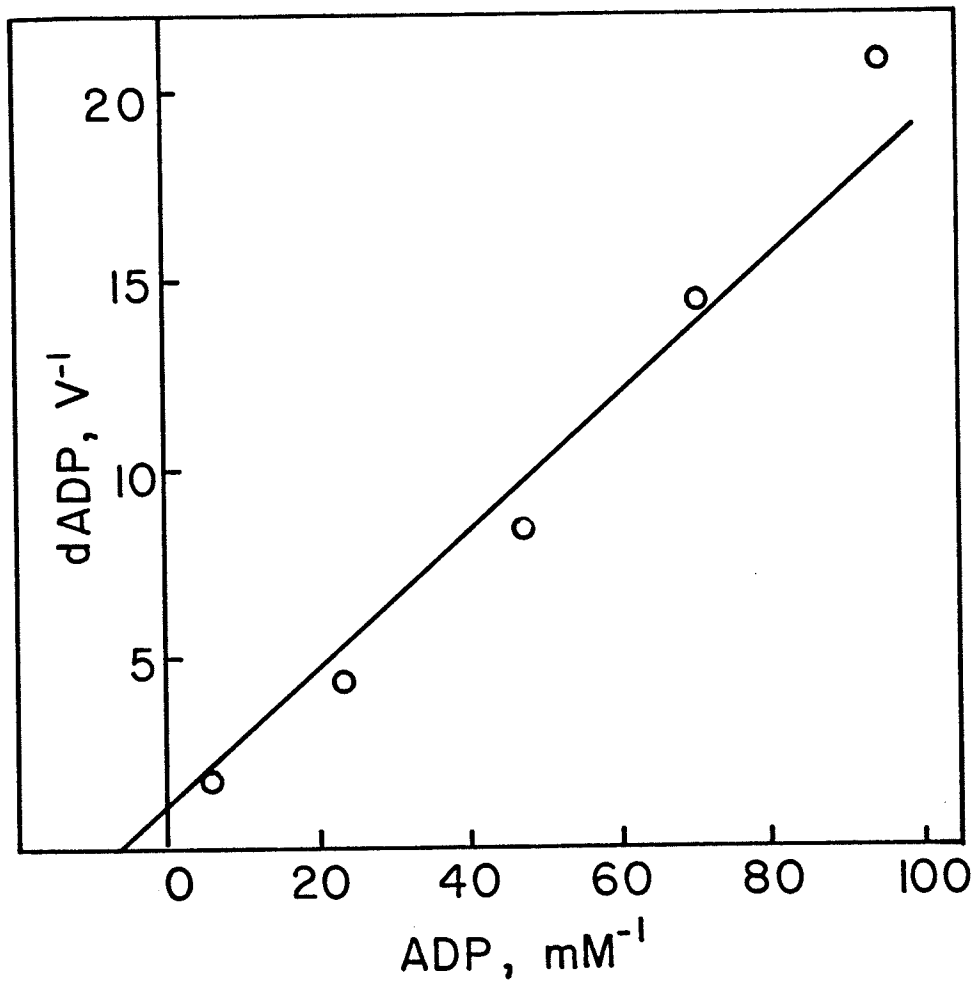

20
15
10
5
dADP, V⁻¹
0
20
40
60
80
100
ADP, mM⁻¹

Table 6. General properties of CDP and ADP reduction in permeabilized WTC125 CHO cells.

Addition	Requirement for optimum activity	
	CDP Reduction	ADP Reduction
DTT	6 mM	6 mM
ATP	1.5 mM	-
dGTP	-	0.04 to 0.2 mM
$MgCl_2$	7 mM	-
pH	7.2	6.9 to 7.1
Temperature	37°C	37°C

Omission	Percentage activity remaining	
	CDP Reduction	ADP Reduction
none	100%	100%
DTT	42	42
ATP	0	-
dGTP	-	0
$MgCl_2$	14	-

Parameter	Kinetic parameters	
	CDP Reduction	ADP Reduction
Km	0.021 ± 0.005 mM CDP	0.095 ± 0.017 mM ADP
V_{max}	0.49 ± 0.17 nmoles dCDP formed/$5x10^6$ cells/hour	1.00 ± 0.013 nmoles dADP formed/$5x10^6$ cells/hour

the two substrates within intact permeabilized CHO cells. ATP and dGTP were added at concentrations found to be optimum for the reduction of CDP and ADP, respectively, while dATP, CTP, dCTP, dTTP and UTP were added at concentrations found to be effective in other systems (Murphree et al 1968, Moore and Hurlbert 1966). The results shown in Table 7 indicate that under physiologically relevant conditions, dCTP may act as an activator of CDP reduction and GTP and dTTP may induce significant reduction of ADP. It is interesting to note that the stimulation of ADP reduction by GTP (Peterson and Moore 1976, Chang and Cheng 1979b) and dTTP (Moore and Hurlbert 1966, Murphree et al 1968) has been previously reported. There also exists a report which indicates that dCTP can stimulate CDP reduction in vitro (Fujioka and Silber 1970).

The stimulatory effects of GTP, dTTP and dCTP were further studied in order to determine the optimum concentration at which each activates reduction. Unlike the nucleotide levels reported in Table 6, those discussed here have been corrected for the presence of kinase and phosphatase activities during the assays. Reduction of ADP reached a maximum at 0.2 mM GTP (figure 22a) or 0.065 mM dTTP (figure 22b). As compared to the rate of reduction with the optimum levels of dGTP, that achieved by 0.2 mM GTP and 0.065 mM dTTP was 45% and 37%, respectively. DeoxyCTP was found to maximally stimulate CDP reduction at a concentration of 3.6 mM (figure 22c). The rate of reduction reached was 29% of that obtained when 1.5 mM ATP was used as the activator.

In order to determine the kinetic parameters of the GTP and dTTP stimulated reduction of ADP and the dCTP stimulated reduction of CDP, double reciprocal plots of the variation in the rate of reduction with varying substrate concentrations were constructed. At 0.2 mM GTP,

Table 7. Effects of various nucleoside triphosphates as activators of CDP and ADP reduction in intact WTC125 CHO cells.

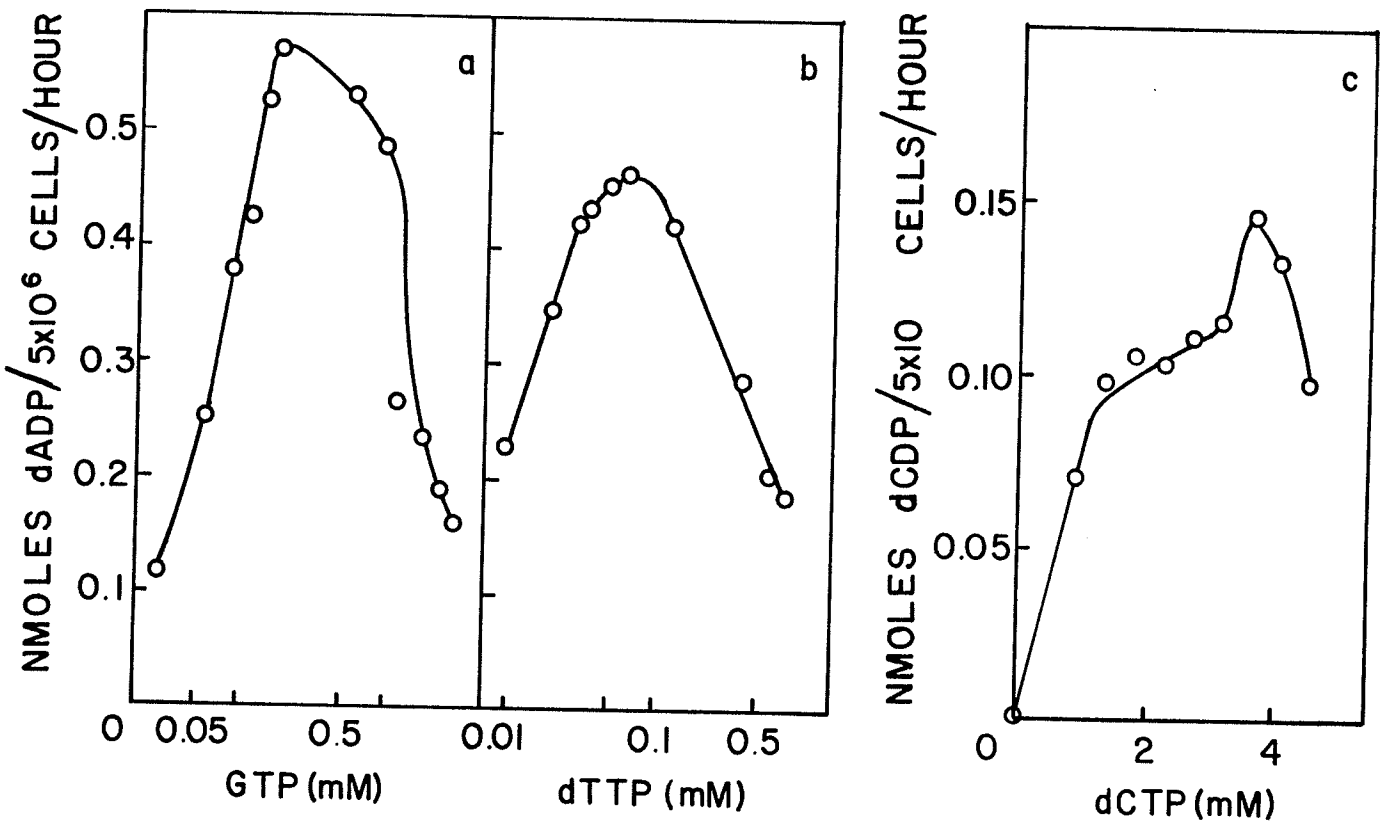
Nucleoside triphosphate	Concentration† (mM)	% activity* CDP reduction	% activity* ADP reduction
ATP	2.0	100	9
dATP	0.05	7	0
GTP	1.5	12	28
dGTP	0.5	11	100
CTP	1.0	7	0
dCTP	1.0	22	3
UTP	1.0	5	8
dTTP	1.0	7	14
none	-	2	0

†Nucleotide concentrations uncorrected for the presence of kinase and phosphatase activities in intact cells.
*% activity as compared to CDP reduction in presence of ATP and ADP reduction in presence of dGTP.

Fig. 22a. Response of ADP reductase activity in permeabilized WT cells to GTP concentration. Standard ADP reductase assay conditions were used except for the omission of dGTP and the presence of varying concentrations of GTP. Results corrected for the presence of kinase and phosphatase activities in intact cells.

Fig. 22b. Response of ADP reductase activity in permeabilized WT cells to dTTP concentration. Standard ADP reductase assay conditions were used except for the omission of dGTP and the presence of varying concentrations of dTTP. Results corrected for the presence of kinase and phosphatase activities in intact cells.

Fig. 22c. Response of CDP reductase activity in permeabilized WT cells to dCTP concentration. Standard CDP reductase assay conditions were used except for the omission of ADP and the presence of varying concentrations of dCTP. Results corrected for the presence of kinase and phosphatase activities in intact cells.


NMOLES dADP/5x10⁶ CELLS/HOUR
0.5
0.4
0.3
0.2
0.1
a
b
0
0.05
0.5
0.01
0.1
0.5
GTP (mM)
dTTP (mM)
NMOLES dCDP/5x10 CELLS/HOUR
0.15
0.10
0.05
c
0
2
4
dCTP (mM)

the reduction of ADP exhibited a Km value of 0.20 mM ADP and a V_{max} value of 0.667 nmoles dADP formed/5 x 10^6 cells/hour (figure 23a). For the reaction activated by 0.065 mM dTTP, the calculated Km value was 0.333 mM ADP and the V_{max} value was 0.384 nmoles of dADP formed/ 5 x 10^6 cells/hour (figure 23b). The double reciprocal plot of the variation in CDP reduction with CDP concentration in the presence of 3.6 mM dCTP (figure 24) indicates that the Km value was 0.033 mM CDP while the V_{max} value was 0.158 nmoles dCDP formed/5 x 10^6 cells/hour.

In order to confirm that the activation of ADP reduction by GTP and dTTP and CDP reduction by dCTP was not due to indirect effects (eg. dTTP stimulates formation of dGTP which in turn leads to reduction of ADP) an HPLC analysis of the nucleotides present at the end of each reaction was carried out. The results of the analyses showed that in each case the distribution of nucleotides observed could be explained on the basis of the kinase and phosphatase activities known to be present in the cells. Moreover, in none of the assays was there detected any nucleotide which did not have the base component of the added substrate or effector. Thus it would seem that the activations observed with dCTP, GTP and dTTP are due to their direct interaction with ribonucleotide reductase and not to their conversion to another nucleotide or to their activating the reduction of a second substrate.

Analyzing the nucleotide distributions of the assays also allowed for the determination of correction factors used in the preceeding studies with dTTP, GTP and dCTP. For the dCTP activation of CDP reduction it was found that 25% of the CDP remained as such while 90% of the dCTP remained during the assay. With GTP and dTTP activated ADP reduction, 59% and 39% respectively of each activator remained

Fig. 23a. Double reciprocal plot of the variation in rate of ADP reduction with ADP concentration using permeabilized WT cells. Reduction activated by 0.2 mM GTP. Activity expressed as nmoles dADP formed/5 x 10^6 cells/hour. Results corrected for the presence of kinase and phosphatase activities in intact cells.

Fig. 23b. Double reciprocal plot of the variation in rate of ADP reduction with ADP concentration using permeabilized WT cells. Reduction activated by 0.065 mM dTTP. Activity expressed as nmoles dADP formed/5 x 10^6 cells/hour. Results corrected for the presence of kinase and phosphatase activities in intact cells.

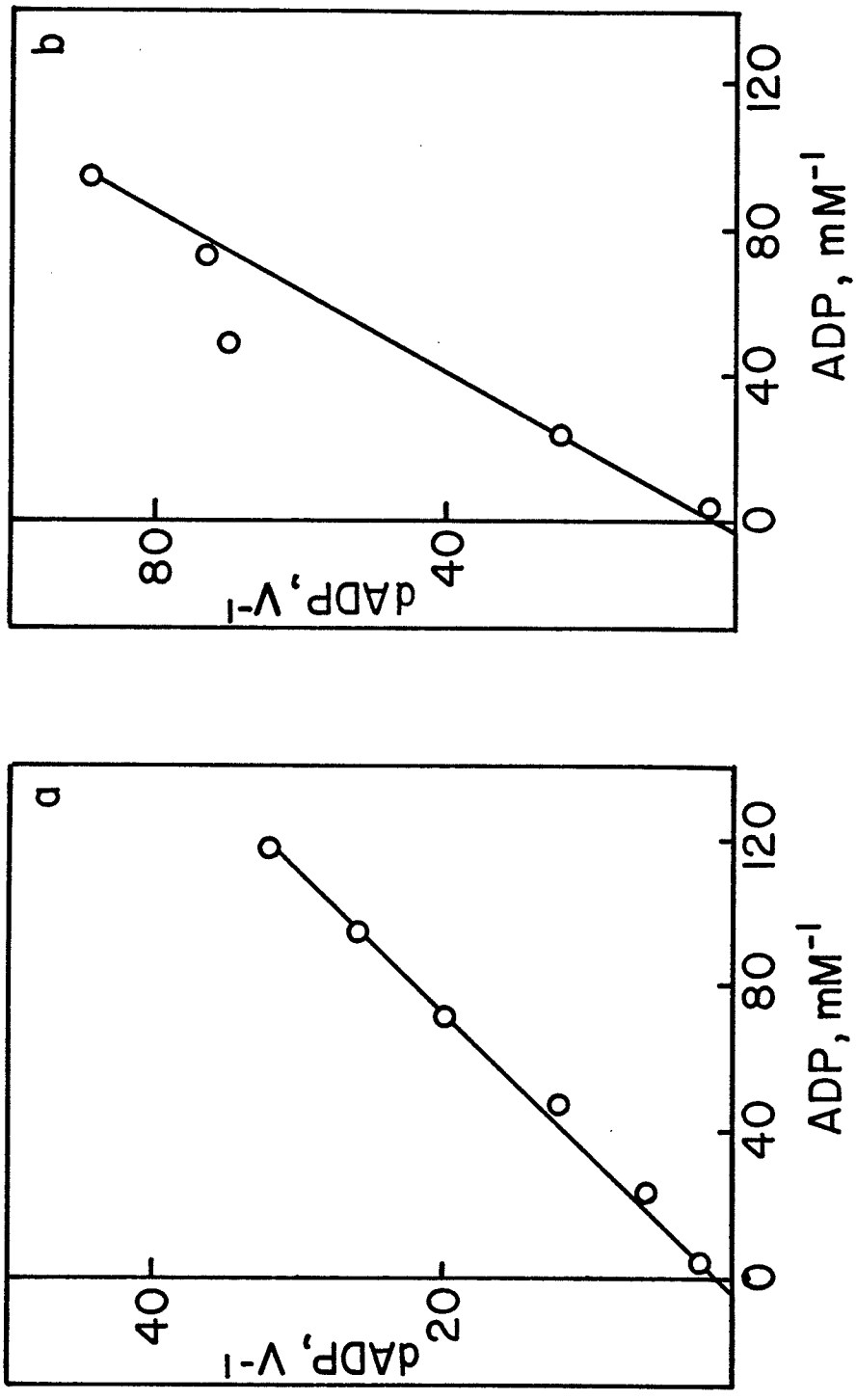
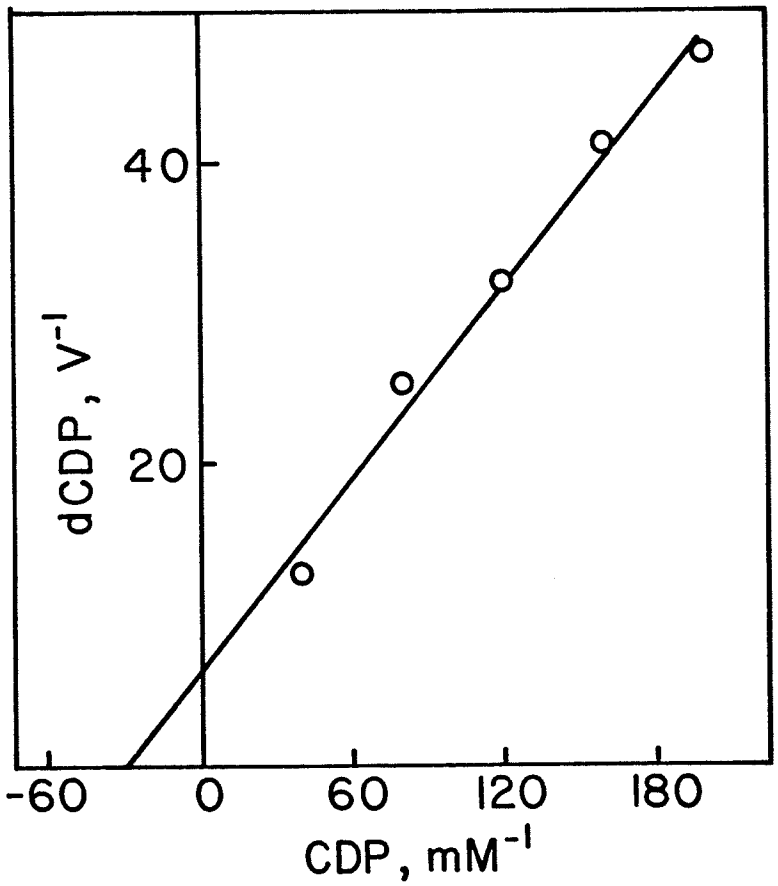

b
dADP, V-1
80
40
0
40
80
120
ADP, mM-1
a
dADP, V-1
40
20
0
40
80
120
ADP, mM-1

Fig. 24. Double reciprocal plot of the variation in rate of CDP reduction with CDP concentration using permeabilized cells. Reduction activated by 3.6 mM dCTP. Activity expressed as nmoles dCDP formed/5 x 10^6 cells/hour. Results corrected for the presence of kinase and phosphatase activities in intact cells.


40
20
dCDP, V⁻¹
-60
0
60
120
180
CDP, mM⁻¹

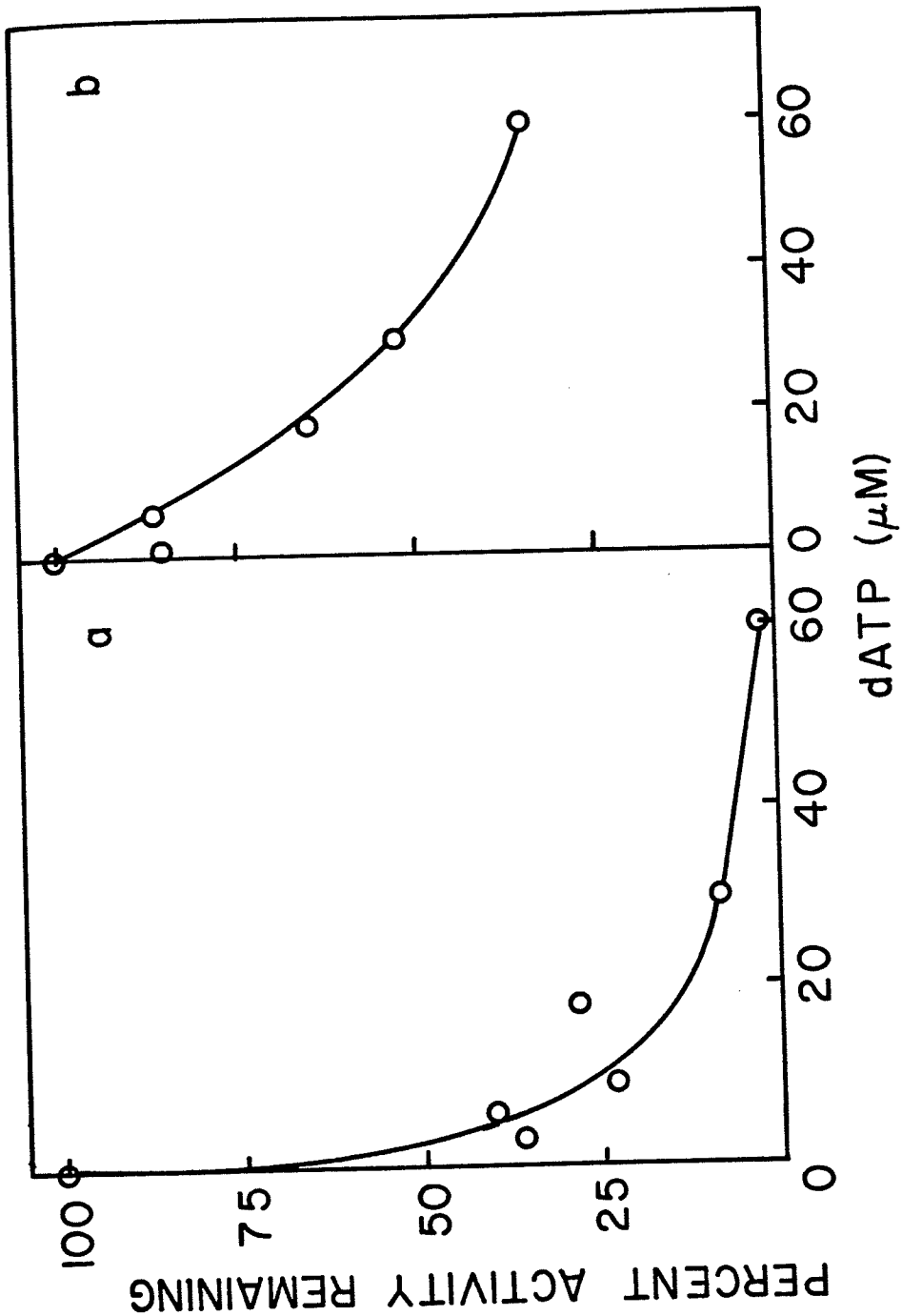
during the assay period. In each case 40% to 42% of the added substrate remained as ADP. It is interesting to note that no matter which activator was used, the levels of substrate present during the assay were the same. For CDP reduction 25% to 27% of added CDP remained as such with ATP or dCTP present. For ADP reduction 37% to 42% of the added substrate remained as ADP regardless of whether dGTP, GTP or dTTP was used as the activator.

2.4 Inhibition of Ribonucleotide Reductase by Nucleoside Triphosphates.

DeoxyATP is known to inhibit the reduction of all four ribonucleoside diphosphates when extracts of mammalian cells are used as the enzyme source (Murphree et al 1968, Larsson 1973, Chang and Cheng 1979b, Eriksson et al 1979). The effect of dATP on the reduction of CDP and ADP in permeabilized CHO cells is shown in figure 25. Optimal concentrations of ATP and dGTP were present in the assay mixtures. For both CDP and ADP reduction approximately 60% of the dATP added to each assay remained as such. DeoxyATP inhibited the reduction of both CDP and ADP. The concentrations required to reduce activity by 50% were 2.7 μM for CDP reductase and 32 μM for ADP reductase. The value determined for the inhibition of ADP reductase was similar to that reported with enzyme extracted from mammalian cells (Eriksson et al 1979, Murphree et al 1968). The concentration of dATP that reduced CDP reductase activity by 50% in the intact cells was found to be considerably less than that required with cell extracts (Murphree et al 1968, Larsson 1973, Chang and Cheng 1979b, Eriksson et al 1979). Moreover, whereas all extract studies except one indicated that dATP was equally effective against CDP and ADP reduction, the results with intact CHO cells indicated CDP reductase was more sensitive by a factor

Fig. 25a. Response of CDP reductase activity in permeabilized WT cells to dATP concentration. Standard CDP reductase assay conditions were used except for the presence of varying concentrations of dATP. Results corrected for the presence of kinase and phosphatase activities in intact cells.

Fig. 25b. Response of ADP reductase activity in permeabilized WT cells to dATP concentration. Standard ADP reductase assay conditions were used except for the presence of varying concentrations of dATP. Results corrected for the presence of kinase and phosphatase activities in intact cells.


PERCENT ACTIVITY REMAINING
100
75
50
25
0
a
b
0
20
40
60
0
20
40
60
dATP (μM)

of 12. Interestingly, in the one study with cell extracts where sensitivity of CDP and ADP reduction to inhibition by dATP was not equal, it was the reduction of ADP which was more sensitive (Chang and Cheng 1979b).

Nucleoside triphosphates that activate the reduction of one substrate have been shown to inhibit the reduction of the other substrates during in vitro studies of ribonucleotide reductase (Moore and Hurlbert 1966, Murphree et al 1968, Chang and Cheng 1979b). This effect was studied using permeabilized CHO cells. Experiments involving CDP reductase were conducted in the presence of optimal ATP concentration while those involving ADP reduction were carried out in the presence of the optimal level of dGTP. As can be seen from figure 26, all three activators of ADP reduction inhibited CDP reductase activity with 0.125 mM dGTP, 1.85 mM GTP and 0.32 mM dTTP reducing the reduction rate by 50%. In a similar manner, ATP and dCTP inhibited the reduction of ADP reduction (figure 27). Activity was inhibited 50% by 0.9 mM ATP or 1.5 mM dCTP.

3. REGULATION OF RIBONUCLEOTIDE REDUCTASE ACTIVITY

Having determined the various compounds which interact with ribonucleotide reductase to stimulate or inhibit its activity, it was of interest to determine exactly how they mediate their effects. This study involved three phases. The first investigated how the various effectors altered the rate of reduction and/or the affinity of the enzyme for the substrates, the second studied the interaction of various allosteric effectors and the third attempted to correlate the kinetic values determined with the in vivo nucleotide pools of CHO cells. Again it was hoped that use of permeabilized mammalian

Fig. 26a. Response of CDP reductase activity in permeabilized WT cells to dGTP concentration. Standard CDP reductase assay conditions were used except for the presence of varying concentrations of dGTP. Results not corrected for the presence of kinase and phosphatase activities in intact cells.

Fig. 26b. Response of CDP reductase activity in permeabilized WT cells to GTP concentration. Standard CDP reductase assay conditions were used except for the presence of varying concentrations of GTP. Results not corrected for the presence of kinase and phosphatase activities in intact cells.

Fig. 26c. Response of CDP reductase activity in permeabilized WT cells to dTTP concentration. Standard CDP reductase assay conditions were used except for the presence of varying concentrations of dTTP. Results not corrected for the presence of kinase and phosphatase activities in intact cells.

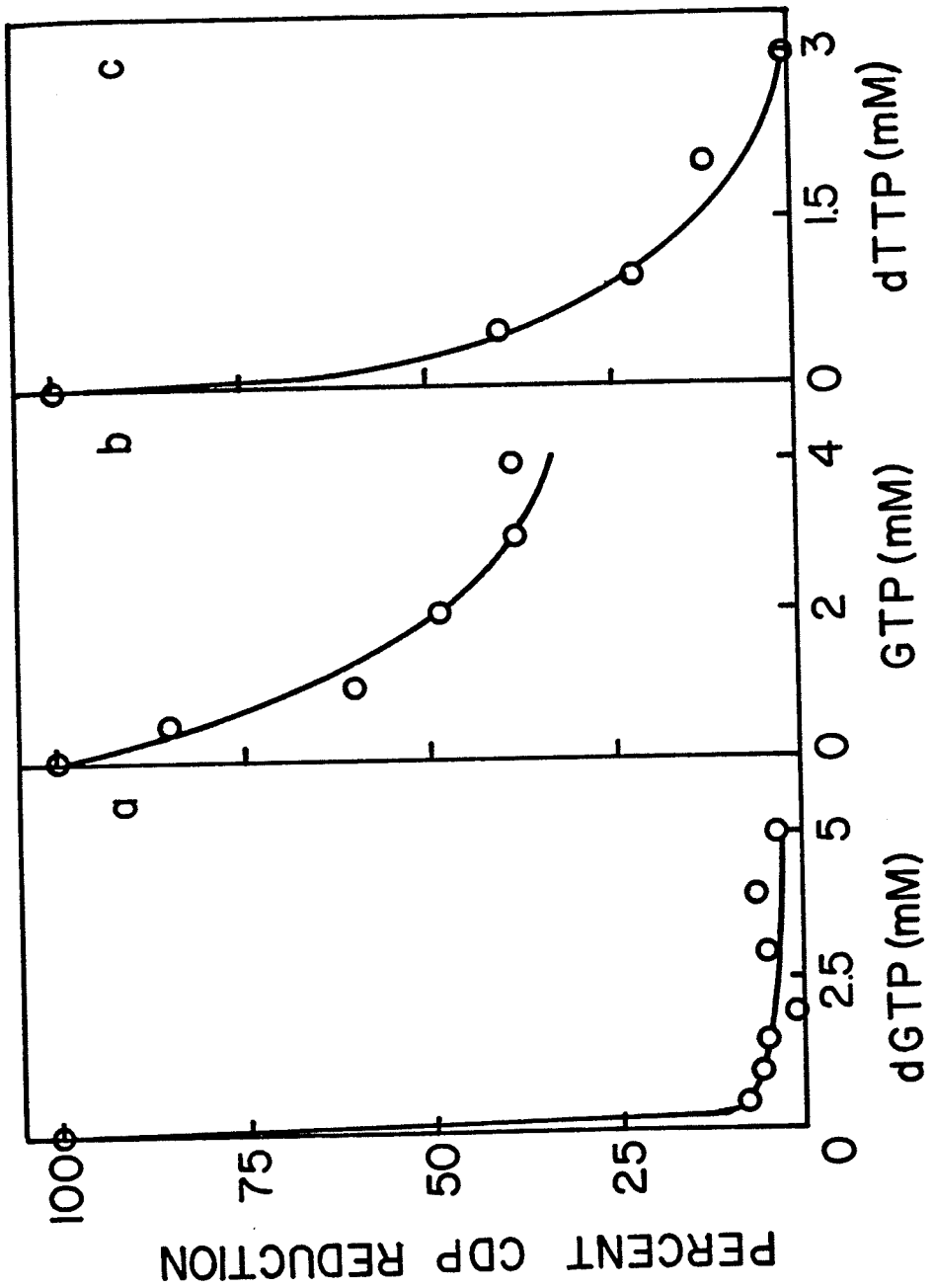
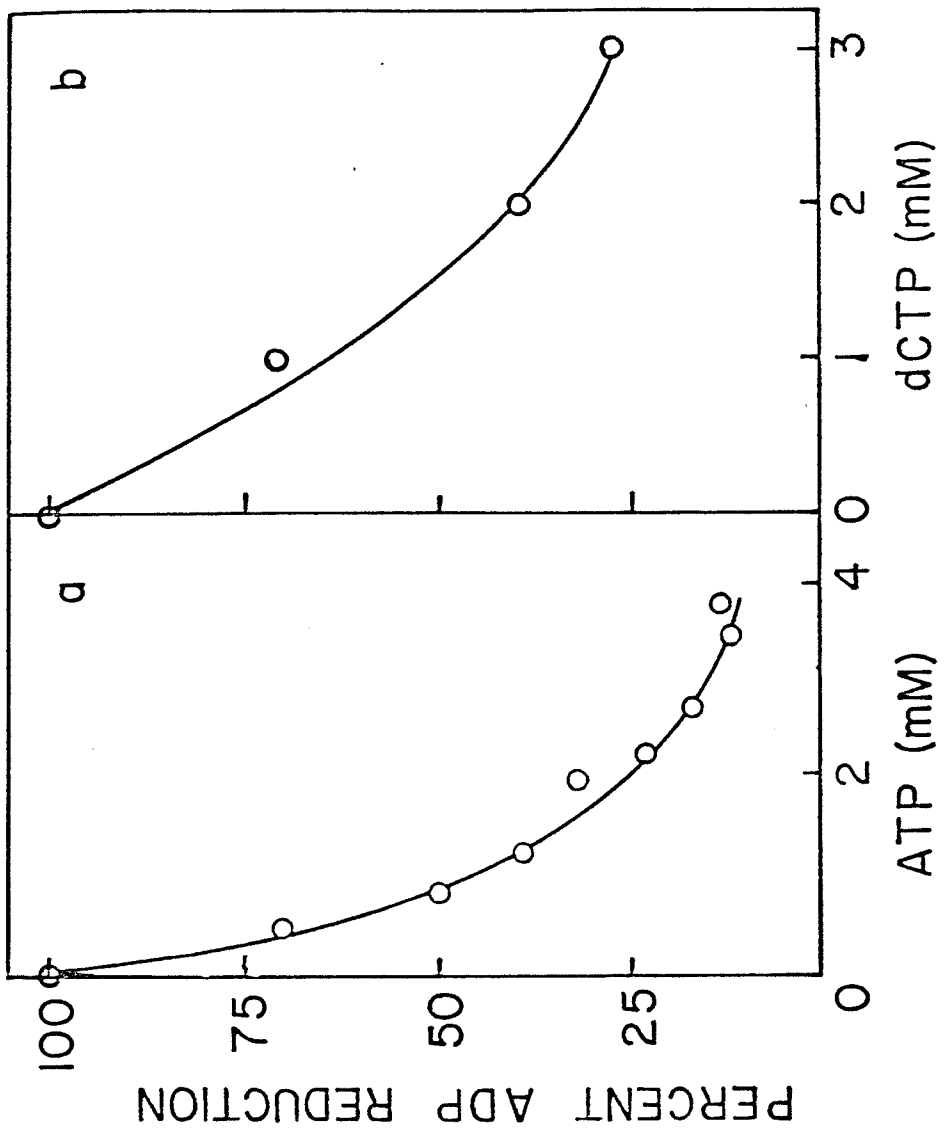

PERCENT CDP REDUCTION
100
75
50
25
0
a
b
c
dGTP (mM)
0
2.5
5
GTP (mM)
0
2
4
dTTP (mM)
0
1.5
3

Fig. 27a. Response of ADP reductase activity in permeabilized WT cells to ATP concentration. Standard ADP reductase assay conditions were used except for the presence of varying concentrations of ATP. Results corrected for the presence of kinase and phosphatase activities in intact cells.

Fig. 27b. Response of ADP reductase activity in permeabilized WT cells to dCTP concentration. Standard ADP reductase assays conditions were used except for the presence of varying concentrations of dCTP. Results not corrected for the presence of kinase and phosphatase activities in intact cells.


PERCENT ADP REDUCTION
100
75
50
25
0
a
b
0
2
4
ATP (mM)
0
1
2
3
dCTP (mM)

cells would more closely represent the true *in vivo* regulation of ribonucleotide reductase.

3.1 Effect of Non-nucleotide Compounds

While $MgCl_2$ and DTT are not essential for the reduction of CDP in intact CHO cells, they do significantly enhance the activity (figure 9). In order to determine how these two compounds stimulate reduction, double reciprocal plots of velocity against CDP concentration at several fixed levels of $MgCl_2$ and DTT were constructed (figure 28). Magnesium ions increased both the rate of reduction and the affinity of the enzyme for the substrate. As expected, DTT, which acts as a hydrogen donor for the reaction, increased only the velocity of the reaction and did not effect the binding of the CDP. Double reciprocal plots of the variation in velocity of CDP reduction at varying $MgCl_2$ or DTT concentrations at several fixed levels of CDP (data not shown) indicated that in neither case did the substrate level affect the affinity of the enzyme for the $MgCl_2$ or DTT. The Ka values determined for the two compounds were 2.4 mM $MgCl_2$ and 2.8 mM DTT.

Dithiothreitol stimulated the reduction of ADP by increasing the velocity of the reaction (figure 29). As was the case with CDP, DTT did not affect the enzyme's affinity for ADP. Rearranging the data to give the double reciprocal plot of velocity against DTT concentration at several fixed levels of ADP (data not shown) illustrated that as expected ADP had no effect on the interaction of ribonucleotide reductase and DTT. The Ka value determined from the graph was 2.7 mM DTT. Thus in regards to the reducing agent DTT, CDP and ADP reductase activities would seem to respond in an identical manner.

Fig. 28a. Double reciprocal plot of the variation in rate of CDP reduction with CDP concentration at several fixed levels of $MgCl_2$ using permeabilized WT cells. Assays were performed in the absence (O) or presence of 2 mM (Δ), 2.5 mM (□) and 6 mM (⬡) MgCl . Activity expressed as nmoles dCDP formed/5 x 10^6 cells/hour. Results corrected for the presence of kinase and phosphatase activities in intact cells.

Fig. 28b. Double reciprocal plot of the variation in rate of CDP reduction with CDP concentration at several fixed levels of DTT using permeabilized WT cells. Assays were performed in absence (O) or presence of 1.5 mM (Δ), 3 mM (⬡) and 6 mM (□) DTT. Activity expressed as nmoles of dCDP formed/5 x 10^6 cells/hour. Results corrected for the presence of kinase and phosphatase activities in intact cells.

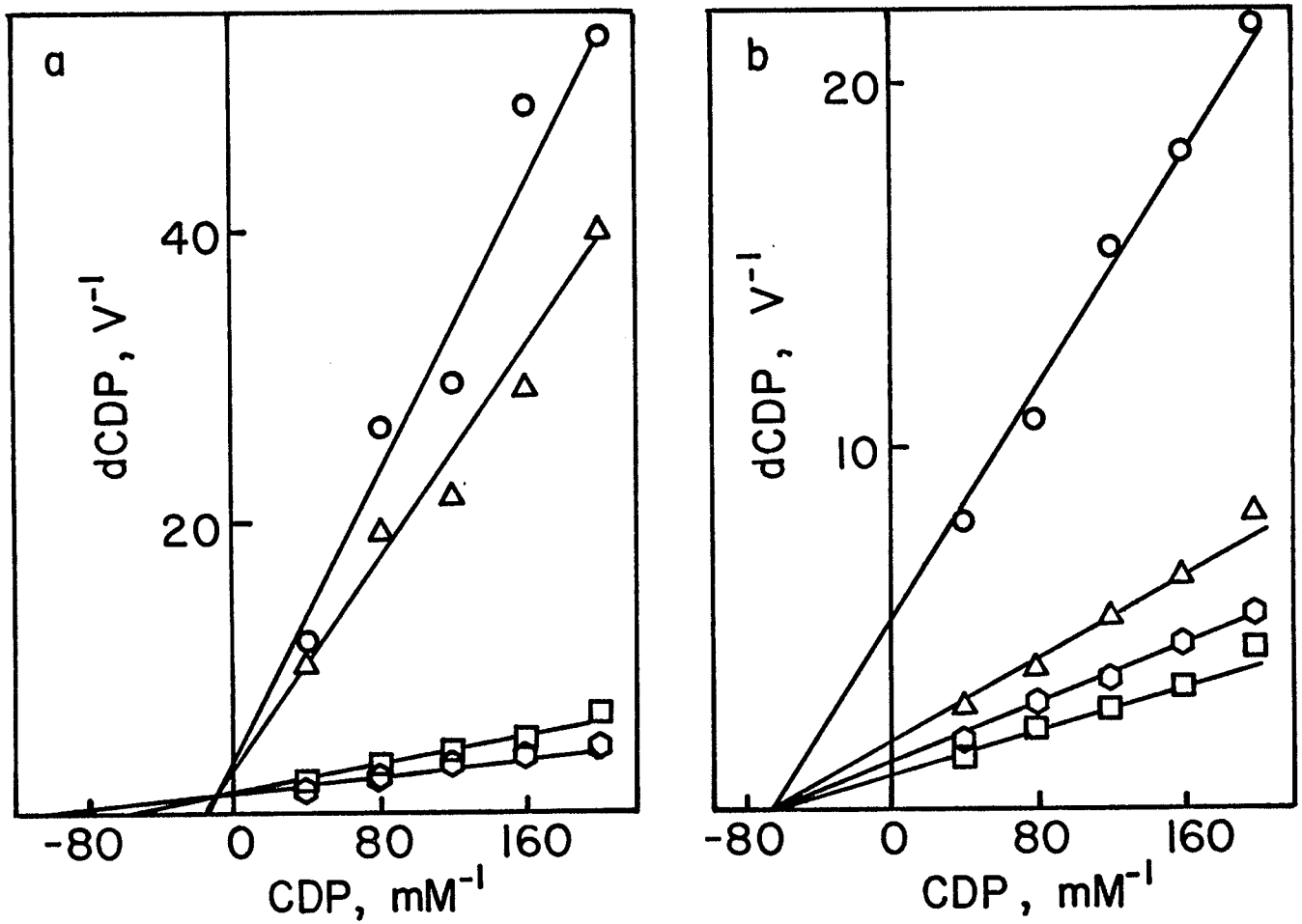
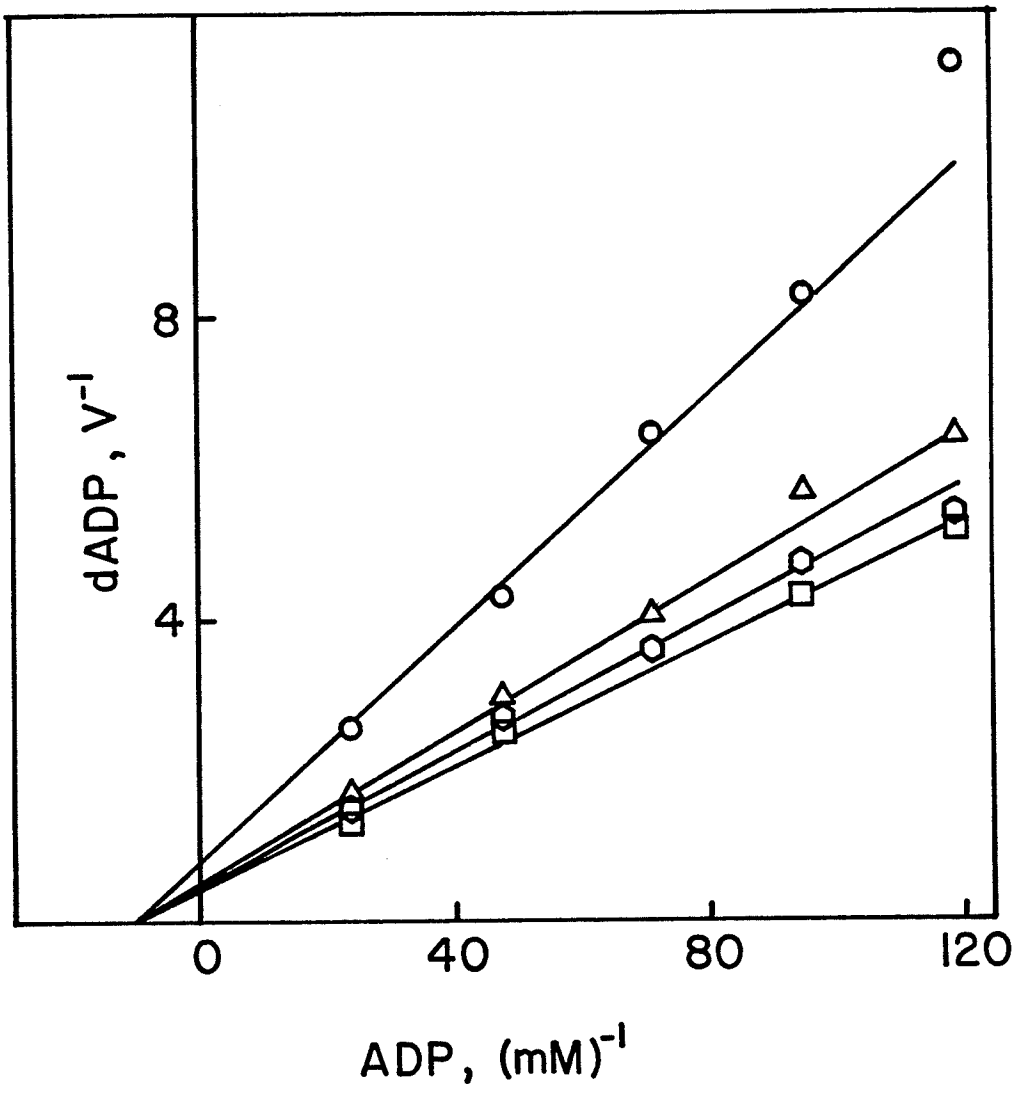

a
dCDP, V⁻¹
40
20
-80
0
80
160
CDP, mM⁻¹
b
dCDP, V⁻¹
20
10
-80
0
80
160
CDP, mM⁻¹

Fig. 29. Double reciprocal plot of the variation in rate of ADP reduction with ADP concentration at several fixed levels of DTT using permeabilized WT cells. Assays were performed in the presence of 1.5 mM (O), 3 mM (Δ), 4.5 mM (○) and 6 mM (□) DTT. Activity expressed as nmoles dADP formed/5 x 10^6 cells/hour. Results corrected for the presence of kinase and phosphatase activities in intact cells.


dADP, V⁻¹
8
4
0
40
80
120
ADP, (mM)⁻¹

3.2 Effect of Nucleotides

The effect of the activators of CDP reduction, ATP and dCTP, on the kinetic properties of ribonucleotide reductase were determined (figure 30). It was found that ATP increased both the rate of reduction and the affinity of the enzyme for CDP. A similar effect for ATP has been reported with the enzyme extracted from Novikoff ascites rat tumor (Moore and Hurlbert 1966). Though more difficult to interpret, it would seem that dCTP exerted its influence over CDP reduction solely by increasing the affinity of the enzyme for CDP.

The effects of dGTP and GTP upon the reduction of ADP were investigated (figure 31). Due to the nature of the reactions studied - high Km values, low V_{max} values and a limited range of activator concentrations which significantly stimulate the reductions - the results obtained were somewhat difficult to interpret. However, it would seem that like the ATP activation of CDP reduction, both dGTP and GTP increased the rate of ADP reduction and the affinity of the enzyme for ADP. This contrasts with the situation observed with the enzyme extracted from rat tumors (Moore and Hurlbert 1966) which suggested that dGTP activated ADP reduction solely via increasing the affinity for the substrate. Due to the problems mentioned directly above, the effect of dTTP on the kinetic parameters of ADP reduction could not be investigated.

The effect of dATP, a known inhibitor of the reduction of all four ribonucleotides (Murphree et al 1968, Larsson 1969, Chang and Cheng 1979b, Eriksson et al 1979, Dick and Wright 1980), on the velocity and substrate binding in the reduction of CDP and ADP was studied. The experiments were carried out under standard conditions

Fig. 30a. Double reciprocal plot of the variation in rate of CDP reduction with CDP concentration at several fixed levels of ATP using permeabilized WT cells. Assays were performed in the presence of 0.6 mM (O), 0.9 mM (Δ), 1.2 mM (□) and 1.5 mM (⬡) ATP. Activity expressed as nmoles dCDP formed/5 x 10^6 cells/hour. Results corrected for the presence of kinase and phosphatase activities in intact cells.

Fig. 30b. Double reciprocal plot of the variation in rate of CDP reduction with CDP concentration as several fixed levels of dCTP using permeabilized WT cells. Assays were performed in the presence of 0.9 mM (O), 1.35 mM (□) and 3.6 mM (⬡) dCTP. Activity expressed as nmoles dCDP formed/5 x 10^6 cells/hour. Results corrected for the presence of kinase and phosphatase activities in intact cells.

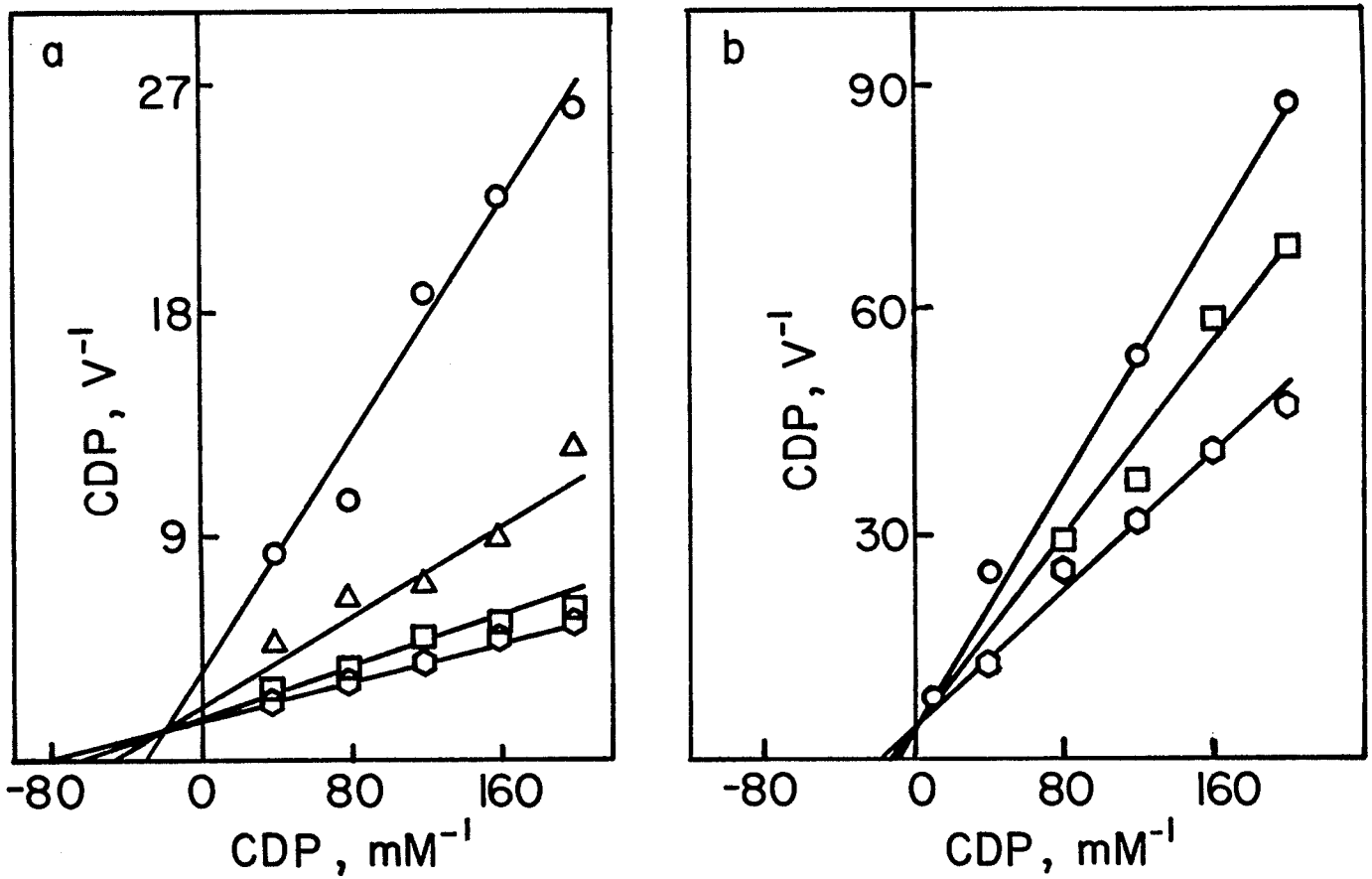
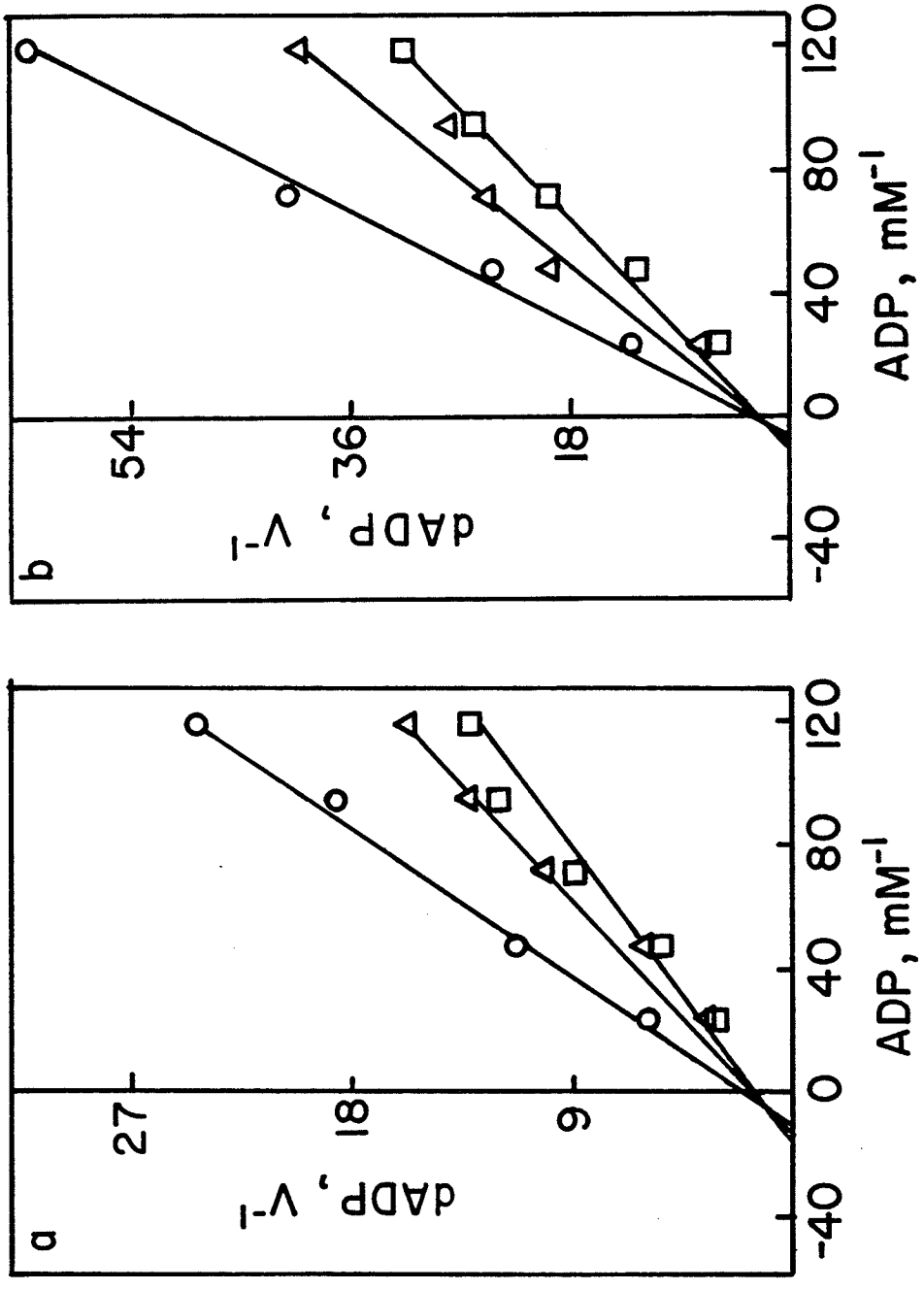

a
27
18
9
CDP, V⁻¹
-80
0
80
160
CDP, mM⁻¹
b
90
60
30
CDP, V⁻¹
-80
0
80
160
CDP, mM⁻¹

Fig. 31a. Double reciprocal plot of the variation in rate of ADP reduction with ADP concentration at several fixed levels of dGTP using permeabilized WT cells. Assays were performed in the presence of 0.046 mM (O), 0.092 mM (Δ) and 0.184 mM (□) dGTP. Activity expressed as nmoles dADP formed/5 x 10^6 cells/hour. Results corrected for the presence of kinase and phosphatase activities in intact cells.

Fig. 31b. Double reciprocal plot of the variation in rate of ADP reduction with ADP concentration at several fixed levels of GTP using permeabilized WT cells. Assays were performed in the presence of 0.18 mM (O), 0.36 mM (Δ) and 0.54 mM (□) GTP. Activity expressed as nmoles dADP formed/ 5 x 10^6 cells/hour. Results corrected for the presence of kinase and phosphatase activities in intact cells.


a
b
dADP, V⁻¹
ADP, mM⁻¹
-40
0
40
80
120
6
18
27
36
54

with the optimal concentrations of either ATP or dGTP. As shown in figures 32 and 33, dATP inhibited both CDP and ADP reduction by decreasing the apparent V_{max} values. In neither case was the affinity of the enzyme for the substrate affected. This is in contrast to the situation observed with ribonucleotide reductase from human Molt-4F cells, where dATP was shown to alter both velocity and substrate binding (Chang and Cheng 1979b). Replots of the velocity intercepts versus dATP concentrations indicated the K_i values to be 11 μM dATP for CDP reduction (inset figure 32) and 22 μM dATP for ADP reduction (inset figure 33). These values are within the range reported to reduce activity by 50% with enzyme from a variety of sources (Murphree et al 1968, Moore and Hurlbert 1966, Eriksson et al 1979, Chang and Cheng 1979b, Dick and Wright 1980).

As previously shown, CDP reduction is also inhibited by dGTP (figure 26a) and ADP reduction by ATP (figure 27a). Figures 34 and 35 show that in each case inhibition was competitive with regards to the respective substrates. The K_i values calculated were 0.0083 mM dGTP for CDP reduction (inset figure 34) and 0.20 mM ATP for ADP reduction (inset figure 35). These results do not agree with those obtained using enzyme extracted from human Molt-4F cells (Chang and Cheng 1979b). In that system both ATP and dGTP were found to be non-competitive inhibitors in regards to ADP and CDP respectively.

The second phase in the study of the regulation of ribonucleotide reductase in intact permeabilized CHO cells investigated the interaction of various allosteric effectors. For the most part, the study involved construction of double reciprocal plots of velocity against activator concentration at several fixed levels of inhibitor. From these plots

Fig. 32. Double reciprocal plot of the variation in rate of CDP reduction with CDP concentration at several fixed levels of dATP using permeabilized WT cells. Assays were performed in the absence (O) or presence of 7.5 μM (□), 15 μM (○), 22.5 μM (Δ) and 30 μM (●) dATP. Activity exrpessed as nmoles dCDP formed/5 x 10^6 cells/hour. Inset: replot of velocity intercepts against dATP concentrations. Results corrected for presence of kinase and phosphatase activities in intact cells.

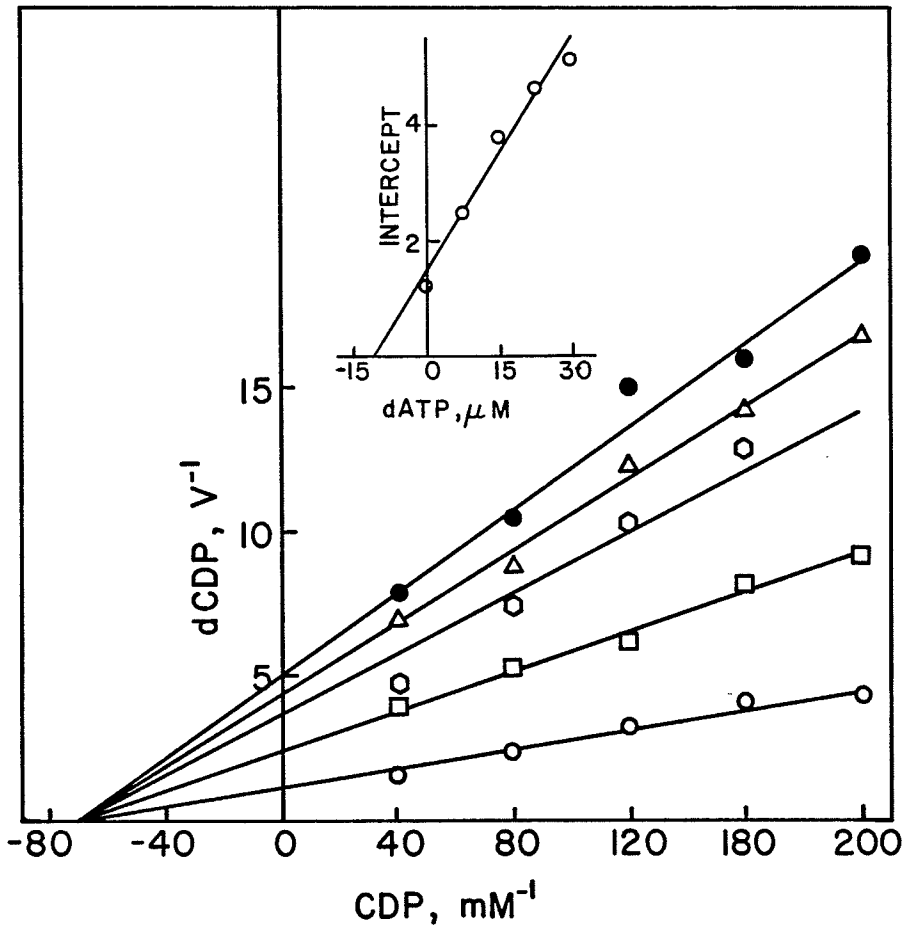

INTERCEPT
4
2
-15
0
15
30
dATP, μM
15
10
5
dCDP, V⁻¹
-80
-40
0
40
80
120
180
200
CDP, mM⁻¹

Fig. 33. Double reciprocal plot of the variation in rate of ADP reduction with ADP concentration at several fixed levels of dATP using permeabilized WT cells. Assays were performed in the absence (O) or presence of 7.5 μM (□), 15 μM (○), 30 μM (Δ) and 60 μM (●) dATP. Activity expressed as nmoles dADP formed/5 x 10^6 cells/hour. Inset: replot of velocity intercepts against dATP concentrations. Results corrected for presence of kinase and phosphatase activities in intact cells.

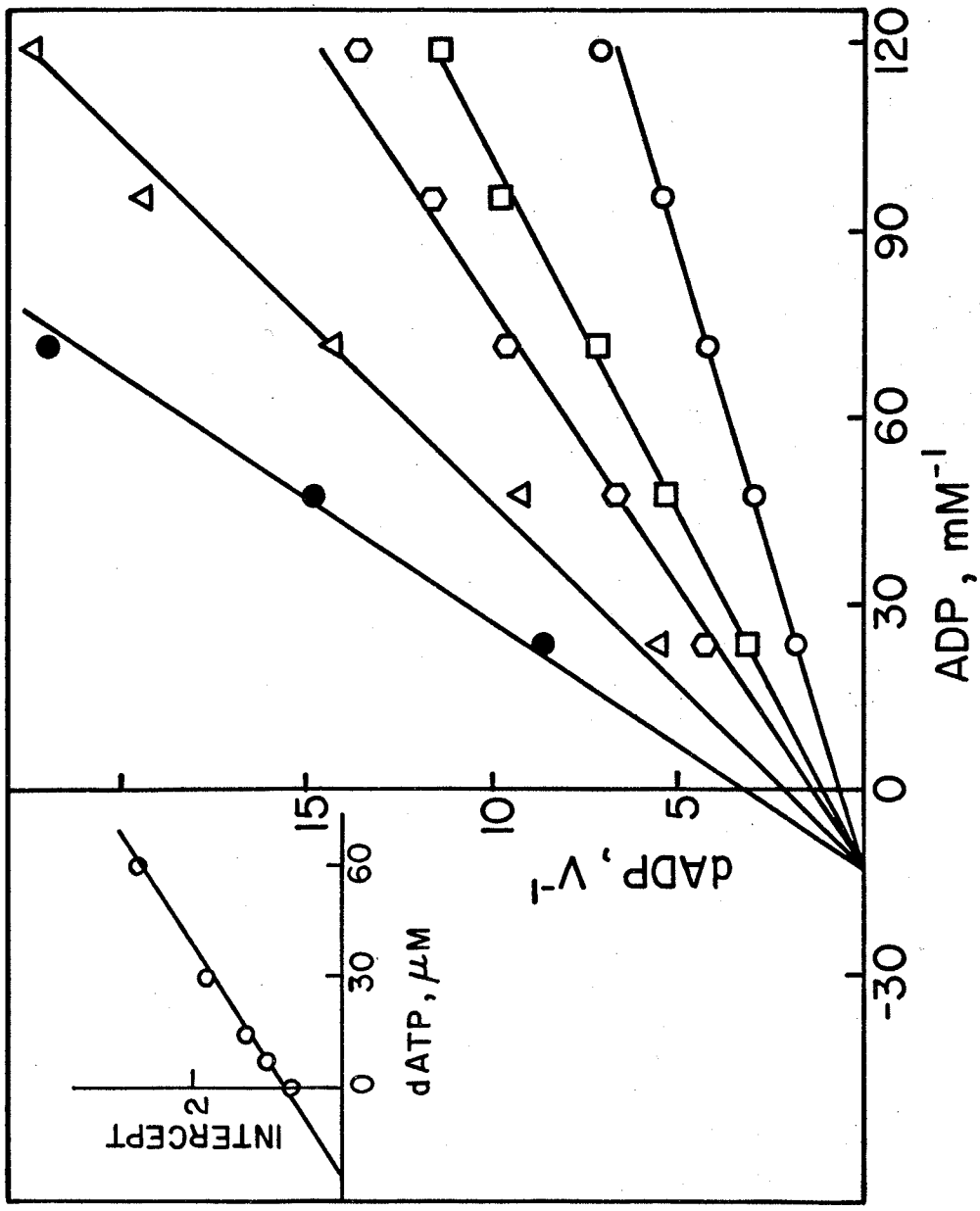

ADP, mM⁻¹
120
90
60
30
0
-30
dADP, V⁻¹
5
10
15
dATP, μM
0
30
60
INTERCEPT
2

Fig. 34. Double reciprocal plot of the variation in rate of CDP reduction with CDP concentration at several fixed levels of dGTP using permeabilized WT cells. Assays were performed in the absence (O) or presence of 0.0092 mM (□), 0.046 mM (Δ) and 0.184 mM (⬡) dGTP. Activity expressed as nmoles dCDP formed/5 x 10^6 cells/hour. Inset: replot of apparent Km values against dGTP concentration. Results corrected for presence of kinase and phosphatase activities in intact cells.

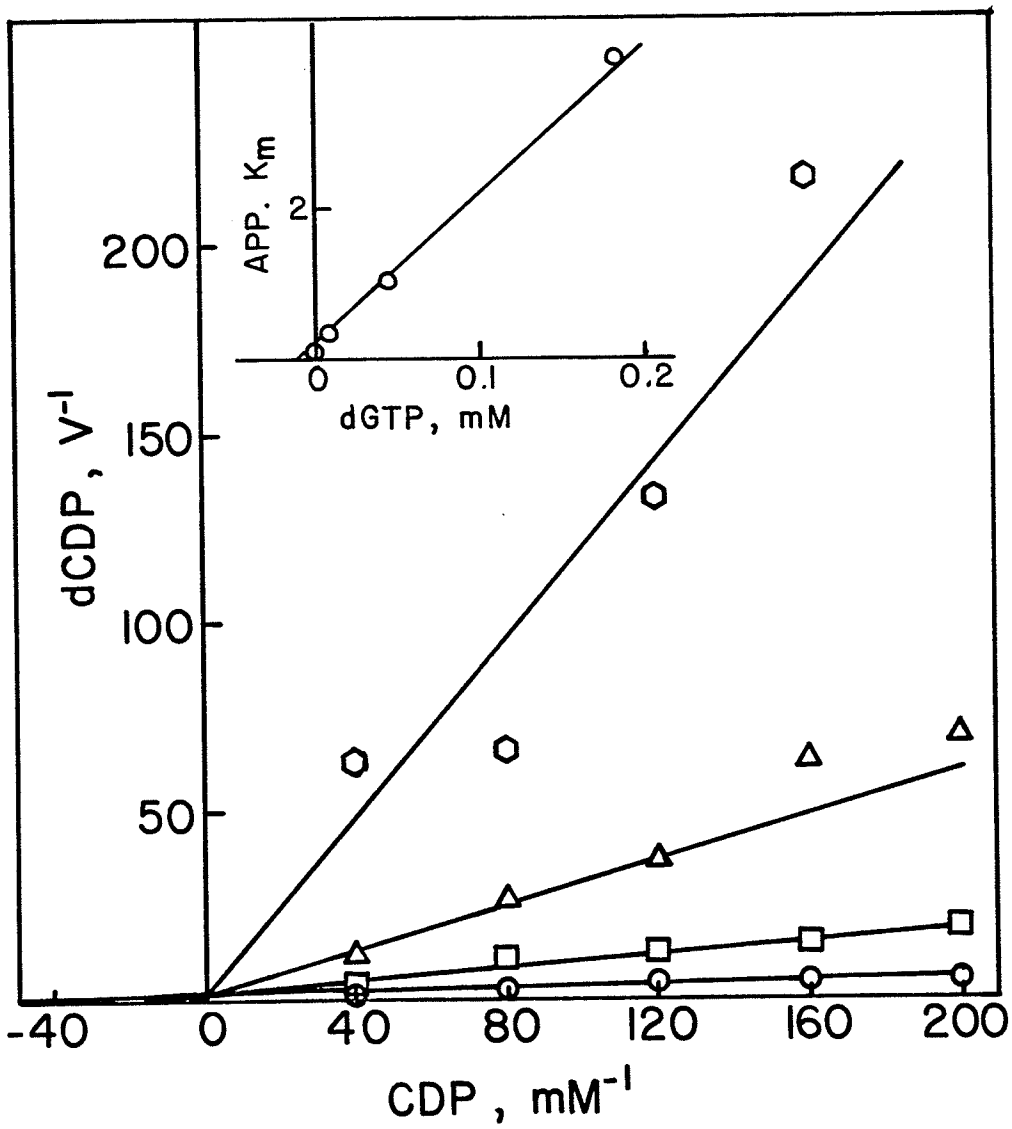
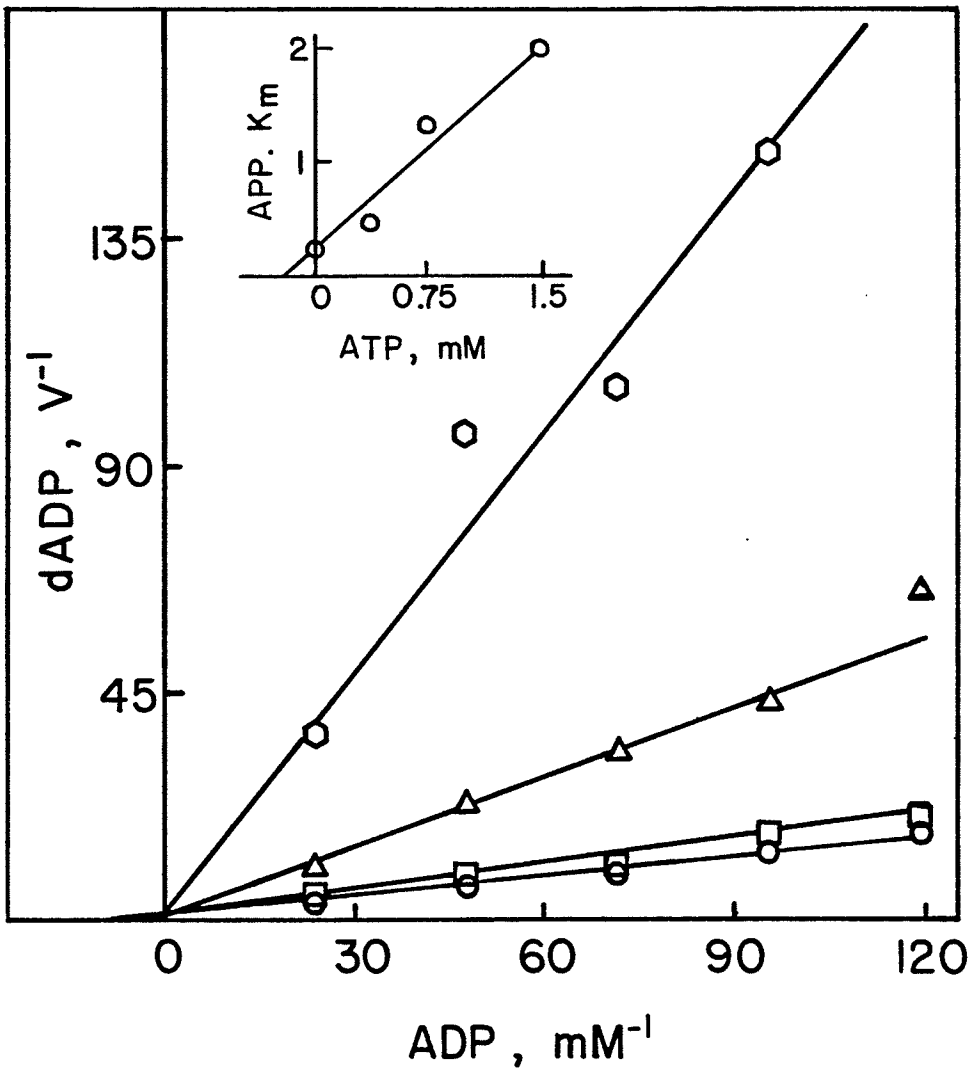

APP. Km
2
0
0.1
0.2
dGTP, mM
200
150
100
50
dCDP, V-1
-40
0
40
80
120
160
200
CDP, mM-1

Fig. 35. Double reciprocal plot of the variation in rate of ADP reduction with ADP concentration at several fixed levels of ATP using permeabilized WT cells. Assays were performed in the absence (O) or presence of 0.375 mM (□), 0.75 mM (Δ) and 1.5 mM (⬡) ATP. Activity expressed as nmoles dADP formed/ 5 x 10^6 cells/hour. Inset: replot of apparent Km values against ATP concentrations. Results corrected for presence of kinase and phosphatase activities in intact cells.


APP. Km
2
1
0
0.75
1.5
ATP, mM
135
90
45
dADP, V⁻¹
0
30
60
90
120
ADP, mM⁻¹

the mode of inhibition and the K_i values could be determined.

The effect of dATP on the reduction of CDP and ADP with respect to the various activators of the reactions was studied. DeoxyATP inhibited the reduction of ADP in a noncompetitive manner with respect to dGTP (Figure 36). With respect to GTP, dATP was a noncompetitive inhibitor at low concentrations but a competitive inhibitor at high concentrations (figure 37). Also, the K_i values differ in each case. For dGTP stimulated reduction of ADP the value was 75 μM while for the GTP stimulated reaction the value was 21 μM. Thus it may be that while both GTP and dGTP activate the reduction of ADP, the exact manner by which they do so may differ.

The effects of ATP and dATP on the reduction of CDP were most unusual (figure 38). First of all, in the absence of dATP, the double reciprocal plot of velocity versus ATP concentration gave a curved plot indicative of co-operative ATP finding. A double reciprocal plot of velocity versus the square of ATP concentration (inset figure 38) was linear suggesting that ATP binds to two sites on the enzyme and does so in a highly cooperative manner. The presence of two classes of ATP binding sites per ribonucleotide reductase molecule has been proposed for the enzyme isolated from calf thymus (Eriksson et al 1979) and cultured mouse lymphoma cells (Eriksson et al 1981b). Cooperatively in ATP binding has not previously been reported. In the presence of dATP, the binding of ATP does not appear to be cooperative except perhaps in the case of low ATP (<0.6 mM) and low dATP (16.7 μM) concentrations. Instead, the double reciprocal plots of velocity versus ATP concentration are linear at high ATP concentrations when dATP is present. At low ATP concentrations, dATP would seem to be

Fig. 36. Double reciprocal plot of the variation in rate of ADP reduction with dGTP concentration at several fixed levels of dATP using permeabilized WT cells. Assays were performed in the absence (O) or presence of 20 μM (Δ), 50 μM (□) and 100 μM (⬡) dATP. Activity expressed as nmoles dADP formed/5 x 10^6 cells/hour. Inset: replot of velocity intercepts against dADP concentrations. Result not corrected for the presence of kinase and phosphatase activities in intact cells.

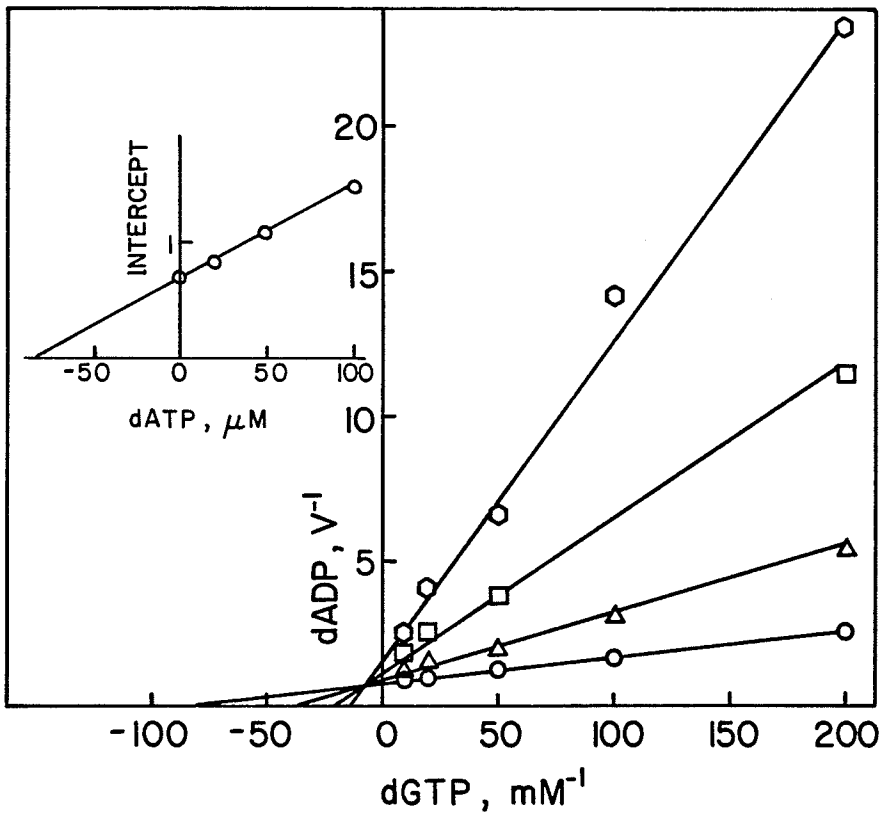

INTERCEPT
1
-50
0
50
100
dATP, μM
20
15
10
5
dADP, V⁻¹
-100
-50
0
50
100
150
200
dGTP, mM⁻¹

Fig. 37. Double reciprocal plot of the variation in rate of ADP reduction with GTP concentration at several fixed levels of dATP using permeabilized WT cells. Assays were performed in the absence (O) or presence of 20 μM (□), 37.5 μM (Δ) and 50 μM (⬡) dATP. Activity expressed as nmoles dADP formed/5 x 10^6 cells/hour. Inset: replot of velocity intercepts against dATP concentrations. Results not corrected for the presence of kinase and phosphatase activities in intact cells.

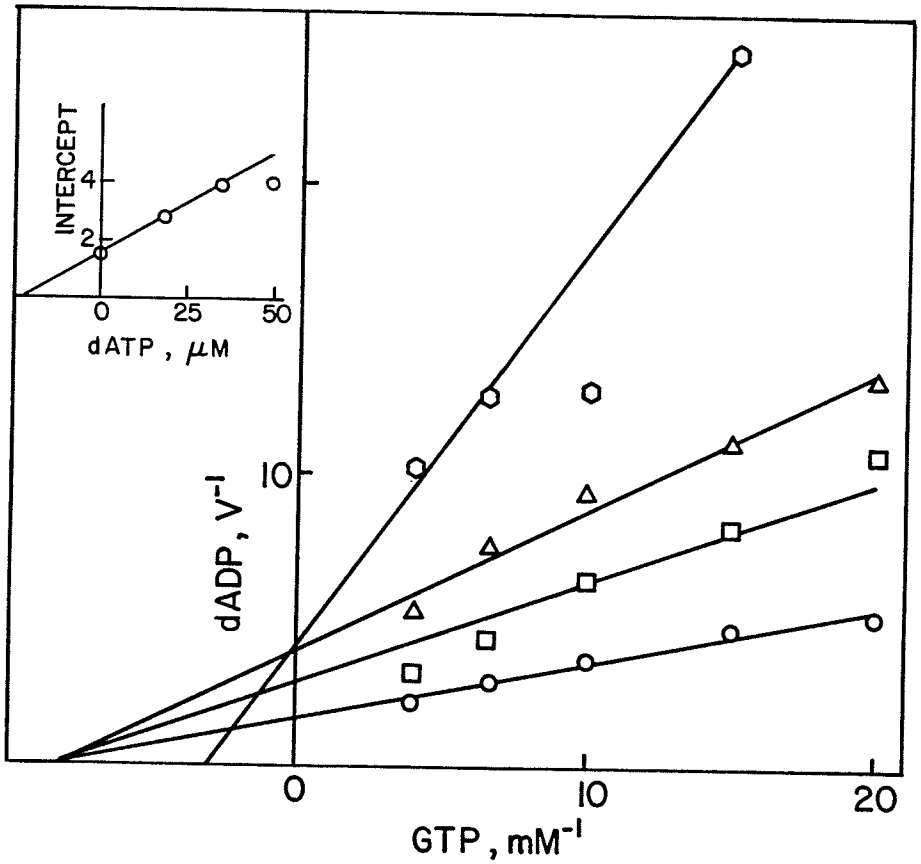
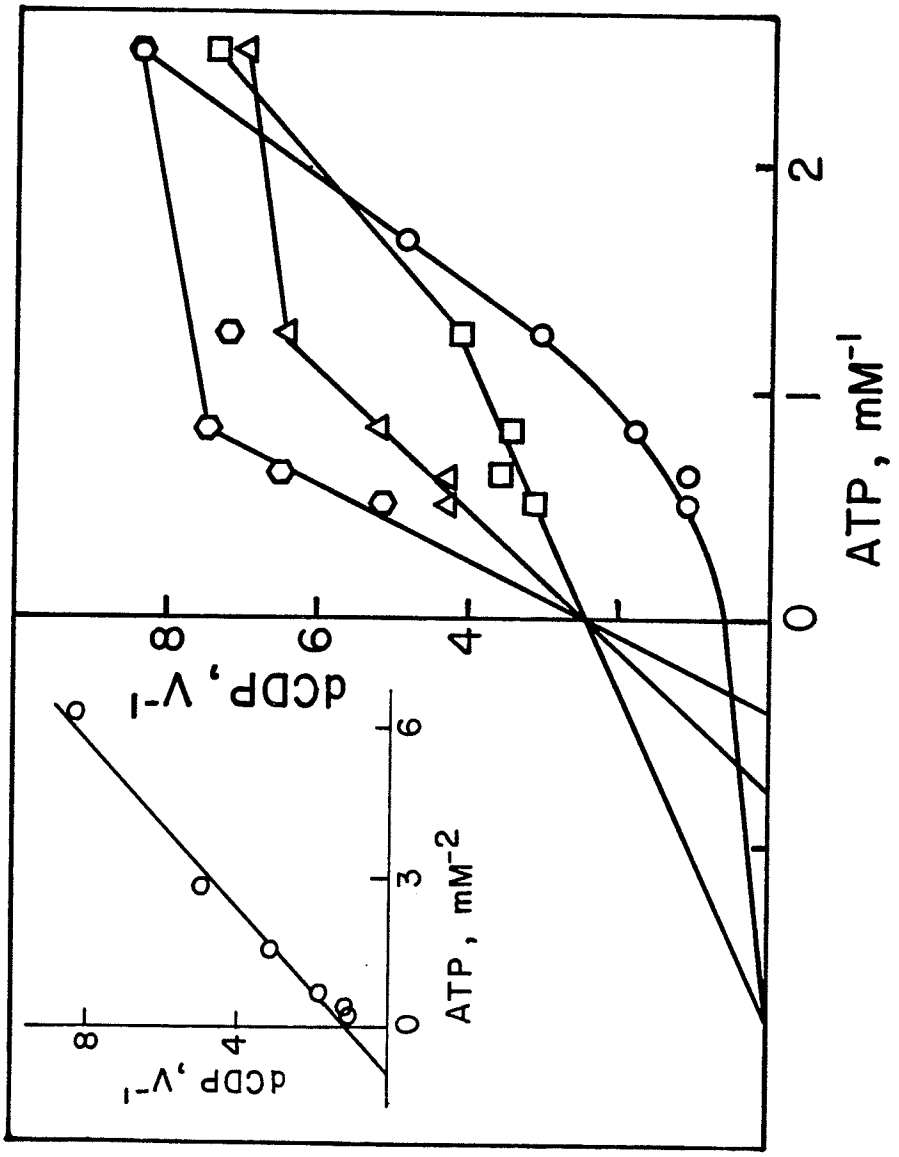

INTERCEPT
4
2
0
25
50
dATP , μM
dADP, V⁻¹
10
0
10
20
GTP , mM⁻¹

Fig. 38. Double reciprocal plot of the variation in rate of CDP reduction with ATP concentration at several fixed levels of dATP using permeabilized WT cells. Assays were performed in the absence (O) or presence of 16.7 μM (□), 33.3 μM (Δ) and 50 μM (⬡) dATP. Activity expressed as nmoles dCDP formed/5 x 10^6 cells/hour. Inset: plot of reciprocal of variation in rate of CDP reduction with reciprocal of the square of ATP concentration in the absence of dATP. Results not corrected for the presence of kinase and phosphatase activities in intact cells.


dCDP, V-1
8
9
4
0
1
2
ATP, mM-1
dCDP, V-1
8
4
0
3
6
ATP, mM-2

slightly stimulatory up to a concentration of 50 μM. This stimulation results in the non-linearity of the double reciprocal plot observed at high 1/ATP values when dATP was present. The inhibition pattern exhibited by dATP with respect to ATP is also unusual. Whereas dATP seemed to initially inhibit the reduction of CDP by greatly reducing the maximum velocity of the reaction, higher levels of the inhibitor (>16.7 μM) seemed to function by decreasing the affinity of the enzyme for CDP.

As shown previously, the activators of CDP reduction are also inhibitors of ADP reduction (figure 27) and vice versa (figure 26). With respect to dGTP, ATP inhibited ADP reduction in an uncompetitive manner at low concentrations and in a noncompetitive manner at higher ATP levels (figure 39). DeoxyCTP on the other hand was a noncompetitive inhibitor of ADP reduction with respect to dGTP at all concentrations tested (figure 40). The K_i values determined for the inhibition of ADP reduction were 0.226 mM ATP and 1.38 mM dCTP.

The GTP stimulated reduction of ADP was also inhibited by ATP and dCTP. ATP was an uncompetitive inhibitor of ADP reduction with respect to GTP (figure 41). At high concentrations, dCTP inhibited ADP reduction in a noncompetitive manner with respect to GTP, while at lower concentrations of dCTP the inhibition was uncompetitive (figure 42). It would thus seem that although both GTP and dGTP activate the reduction of ADP, the manner in which they interact with ribonucleotide reductase must differ. The K_i values determined for ATP and dCTP inhibition of GTP stimulated ADP reduction were 0.295 mM and 1.1 mM respectively. These values are very similar to those determined for the dGTP activated reduction of ADP.

Fig. 39. Double reciprocal plot of the variation in rate of ADP reduction with dGTP concentration at several fixed levels of ATP using permeabilized WT cells. Assays were performed in the absence (O) or presence of 0.5 mM (□), 1 mM (Δ) and 2 mM (○) ATP. Activity expressed as nmoles dADP formed/5 x 10^6 cells/hour. Inset: replot of velocity intercepts against ATP concentrations. Results not corrected for the presence of kinase and phosphatase activities in intact cells.

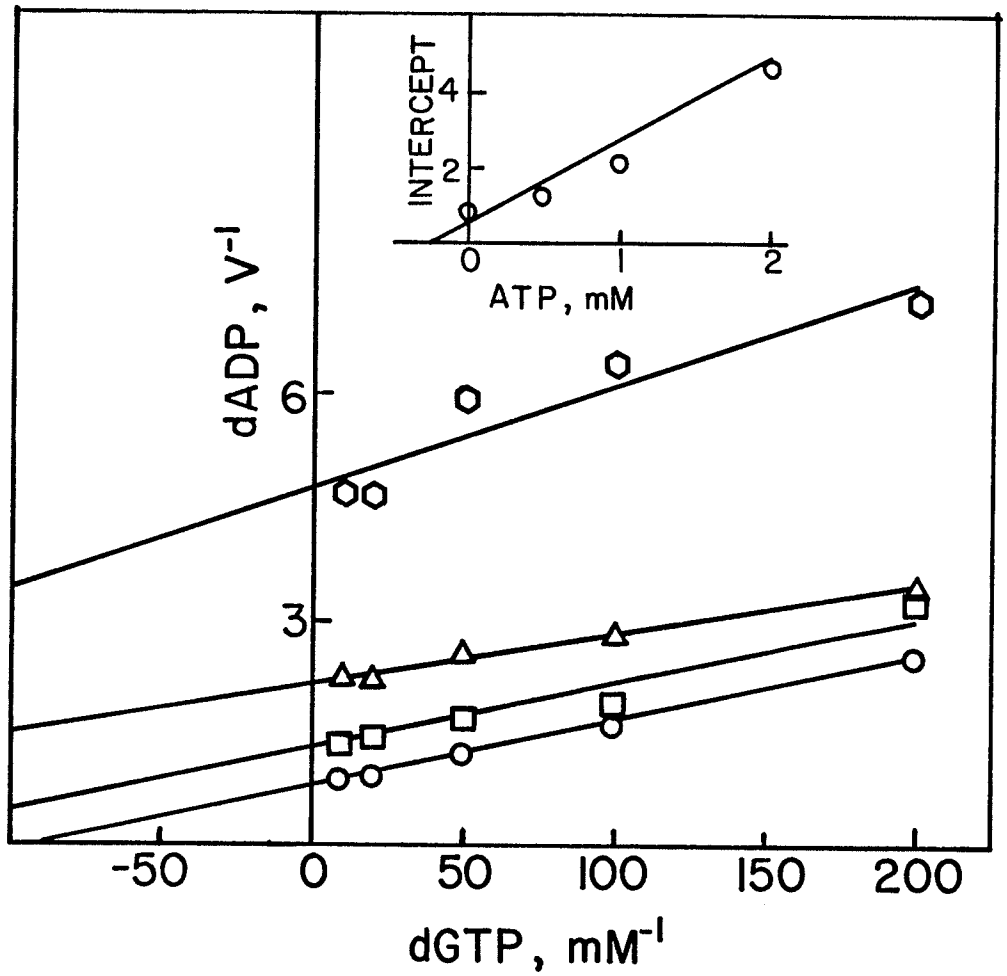

INTERCEPT
4
2
0
1
2
ATP, mM
dADP, V⁻¹
6
3
-50
0
50
100
150
200
dGTP, mM⁻¹

Fig. 40. Double reciprocal plot of the variation in rate of ADP reduction with dGTP concentration at several fixed levels of dCTP using permeabilized WT cells. Assays were performed in the absence (O) or presence of 1 mM (□), 2 mM (Δ) and 3 mM (⬡) dCTP. Activity expressed as nmoles dADP formed/5 x 10^6 cells/hour. Inset: replot of velocity intercepts against dCTP concentrations. Results not corrected for the presence of kinase and phosphatase activities in intact cells.

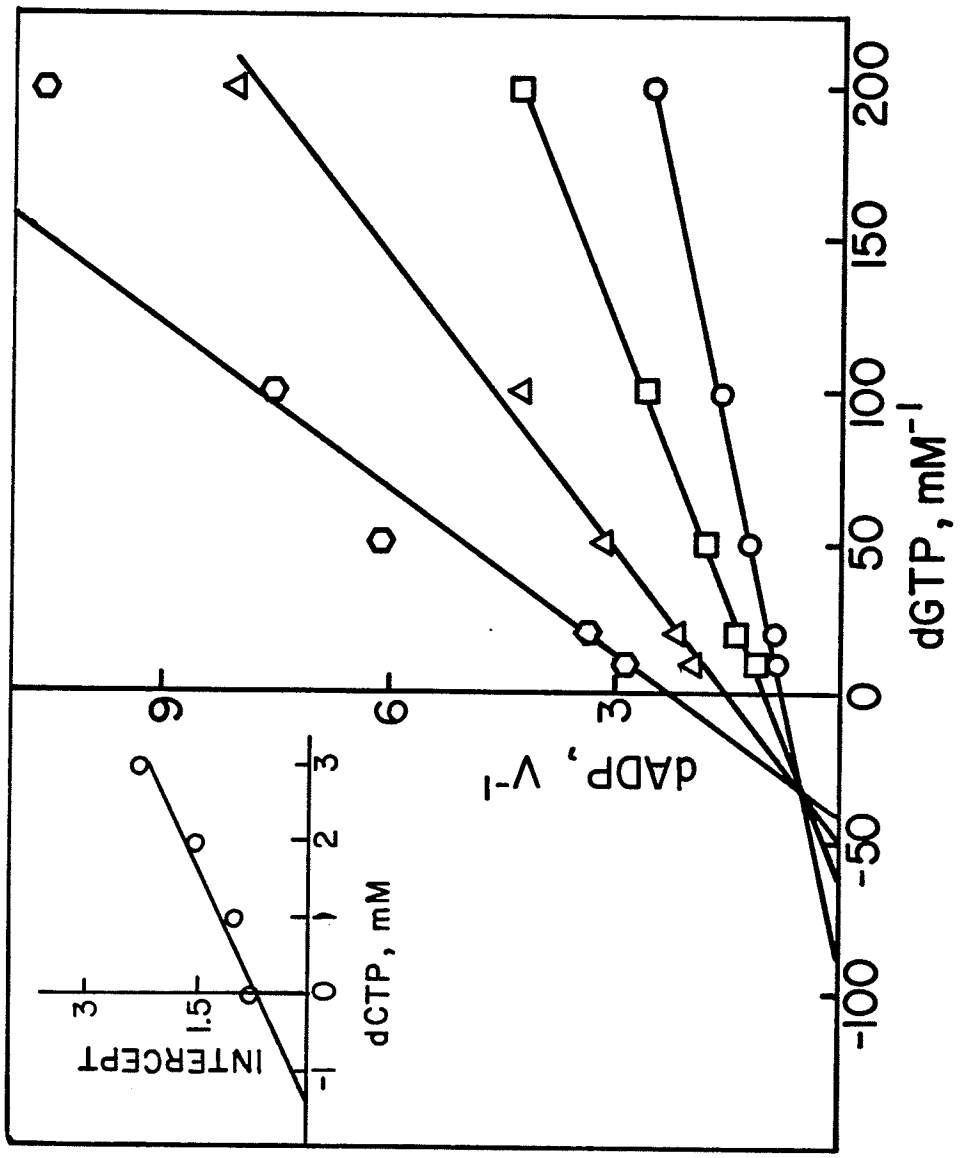

dADP, V-1
dGTP, mM-1
-100
-50
0
50
100
150
200
3
6
9
INTERCEPT
3
1.5
dCTP, mM
-1
0
1
2
3

Fig. 41. Double reciprocal plot of the variation in rate of ADP reduction with GTP concentration at several fixed levels of ATP using permeabilized WT cells. Assays were performed in the absence (O) or presence of 0.5 mM (□), 1 mM (Δ) and 2 mM (⬡) ATP. Activity expressed as nmoles dADP formed/5 x 10^6 cells/hour. Inset: replot of velocity intercepts against ATP concentrations. Results not corrected for the presence of kinase and phosphatase activities in intact cells.

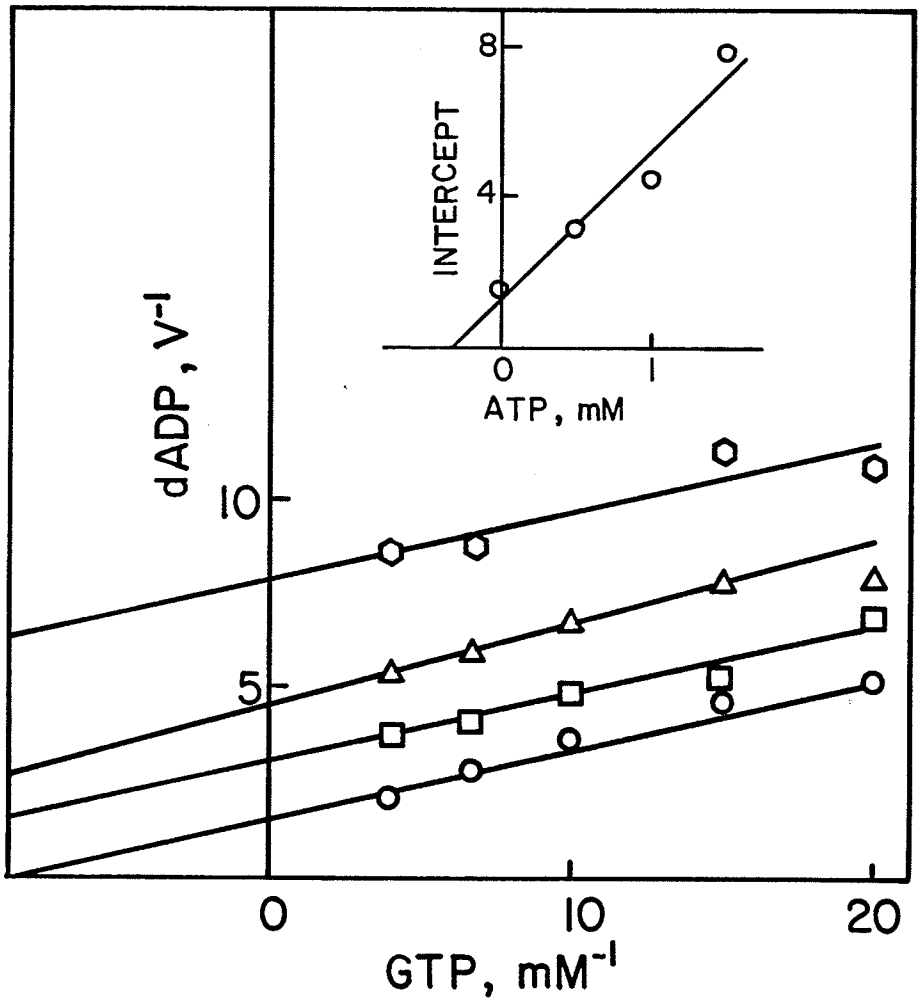
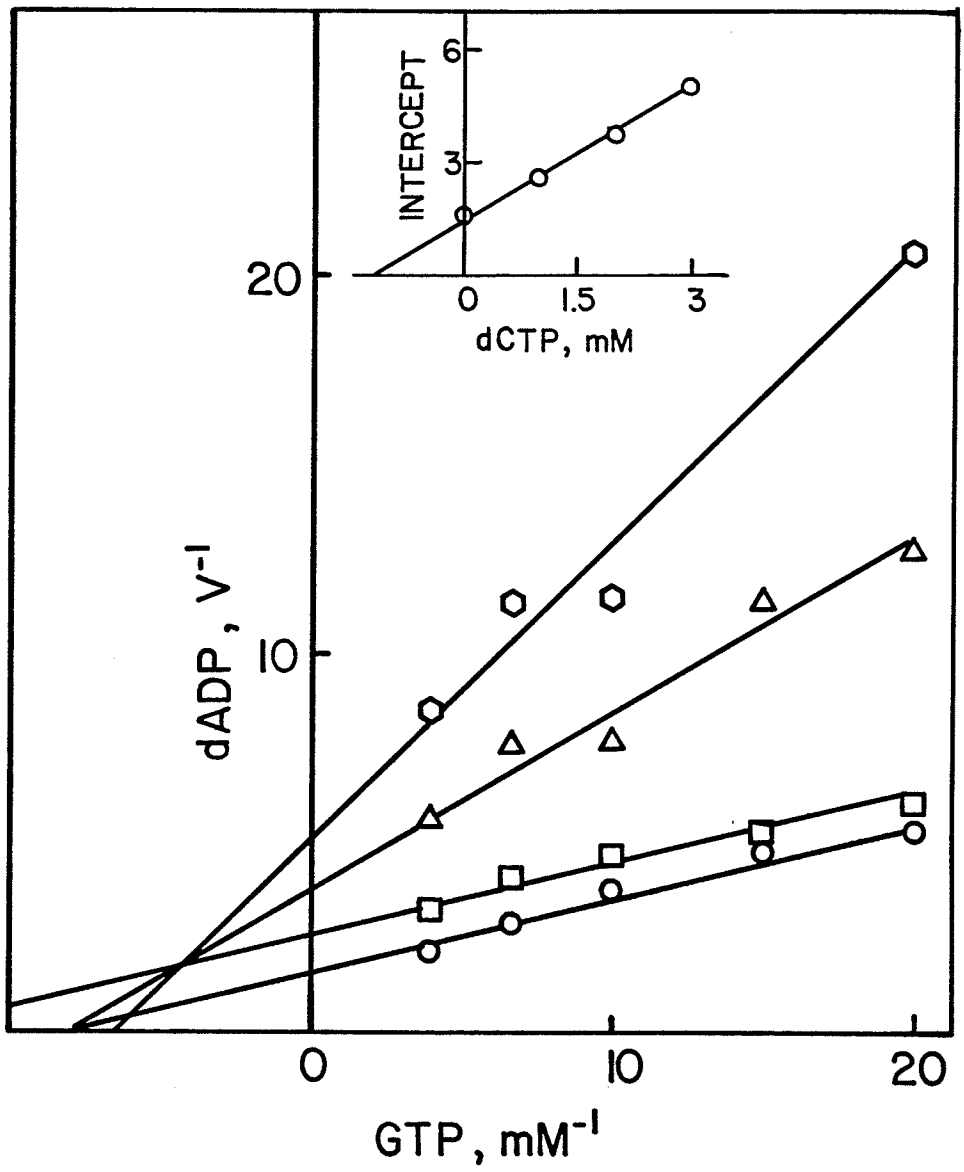

8
4
INTERCEPT
0
1
ATP, mM
dADP, V-1
10
5
0
10
20
GTP, mM-1

Fig. 42. Double reciprocal plot of the variation in rate of ADP reduction with GTP concentration at several fixed levels of dCTP using permeabilized WT cells. Assays were performed in the absence (O) or presence of 1 mM (□), 2 mM (Δ) and 3 mM (⬡) dCTP. Activity expressed a nmoles dADP formed/5 x 10^6 cells/hour. Inset: replot of velcoity intercepts against dCTP concentration. Results not corrected for the presence of kinase and phosphatase activities in intact cells.


INTERCEPT
6
3
0
1.5
3
dCTP, mM
20
10
dADP, V⁻¹
0
10
20
GTP, mM⁻¹

In addition to dATP, CDP reduction was inhibited by the presence of dGTP, dTTP or GTP, the activators of ADP reduction (figure 26). With respect to ATP, dGTP inhibited the reduction of CDP in a non-competitive manner such that both the velocity of the reaction and the affinity of the enzyme for ATP were decreased (figure 43). DeoxyTTP inhibited CDP reduction in an uncompetitive manner with respect to ATP (figure 44). As was evident in other results (figure 38), the double reciprocal plot of velocity versus ATP concentration was non-linear in a fashion suggestive of cooperative binding of the effector to the enzyme. The non-linear kinetics of ATP activation were abolished by the presence of either dGTP or dTTP. The K_i values determined for the inhibition of CDP reduction were 0.04 mM dGTP and 0.27 mM dTTP.

When the inhibition of ATP activated CDP reduction by GTP was studied an unexpected observation was made. As GTP is generally believed to act in a manner analogous to dGTP (Chang and Cheng 1979a,b, Wright et al 1981), it was expected that the nucleotide would inhibit the reduction of CDP. As shown in figure 26b this did occur when optimal levels of ATP were present. However, it was found that at suboptimal concentrations of ATP, GTP stimulated the reduction of CDP (Table 8). Moreover the degree of stimulation by GTP increased as the levels of ATP decreased. For example in the presence of 0.4 mM ATP, the reduction of CDP was increased by 70% upon the addition of 1 mM GTP while in the presence of 0.8 mM ATP the increase was limited to 35%. With 0.4 mM ATP present, all levels of GTP tested gave increasing activation of CDP reduction while with 0.8 mM ATP, activity levels increased as up to 1 mM GTP was added to the assays but beyond this point activity decreased. With 2 mM ATP, the optimal concentration

Fig. 43. Double reciprocal plot of the variation in rate of CDP reduction with ATP concentration at several fixed levels of dGTP using permeabilized WT cells. Assays were performed in the absence (O) or presence of 0.05 mM (□) and 0.133 mM (Δ) dGTP. Activity expressed as nmoles dCDP formed/5 x 10^6 cells/hour. Inset: replot of velocity intercepts against dGTP concentrations. Results not corrected for the presence of kinase and phosphatase activities in intact cells.

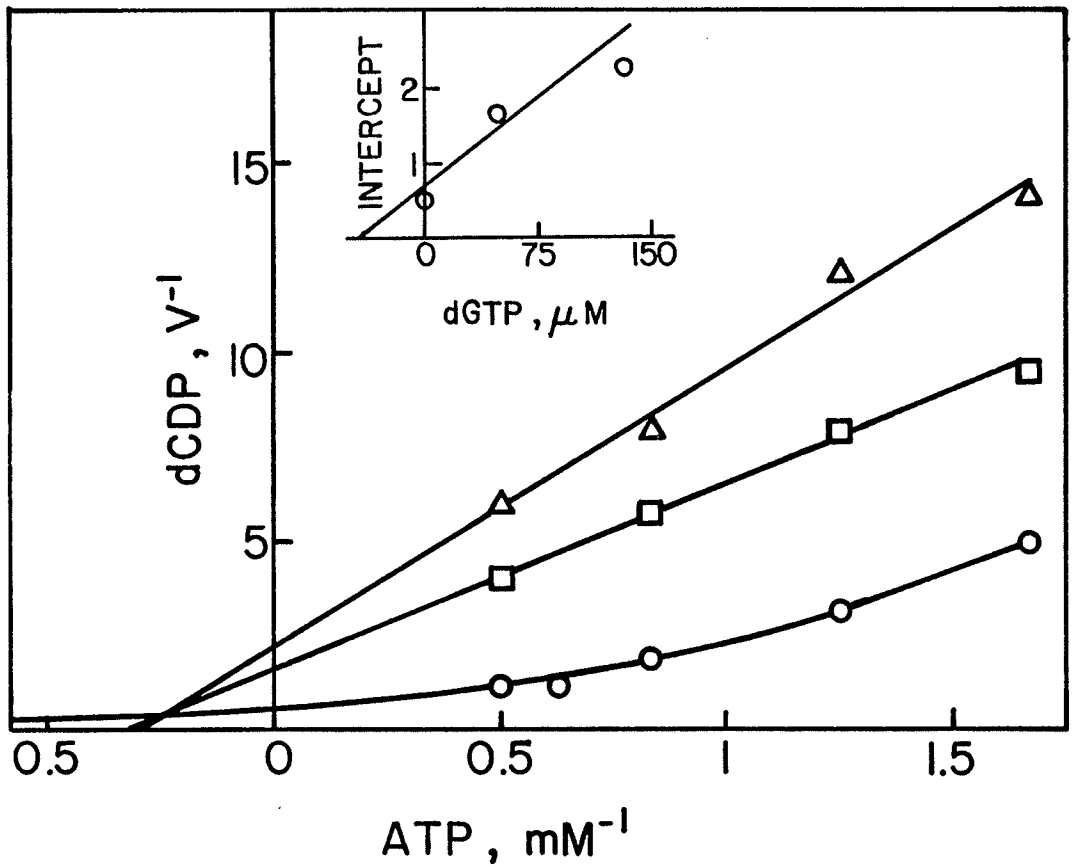

INTERCEPT
2
1
0
75
150
dGTP, μM
15
10
5
dCDP, V⁻¹
0.5
0
0.5
1
1.5
ATP, mM⁻¹

Fig. 44. Double reciprocal plot of the variation in rate of CDP reduction with ATP concentration at several fixed levels of dTTP using permeabilized WT cells. Assays were performed in the absence (O) or presence of 0.5 mM (□), 1 mM (Δ) and 2 mM (⬡) dTTP. Activity expressed as nmoles of dCDP formed/5 x 10^6 cells/hour. Inset: replot of velocity intercepts against dTTP concentrations. Results not corrected for the presence of kinase and phosphatase activities in intact cells.

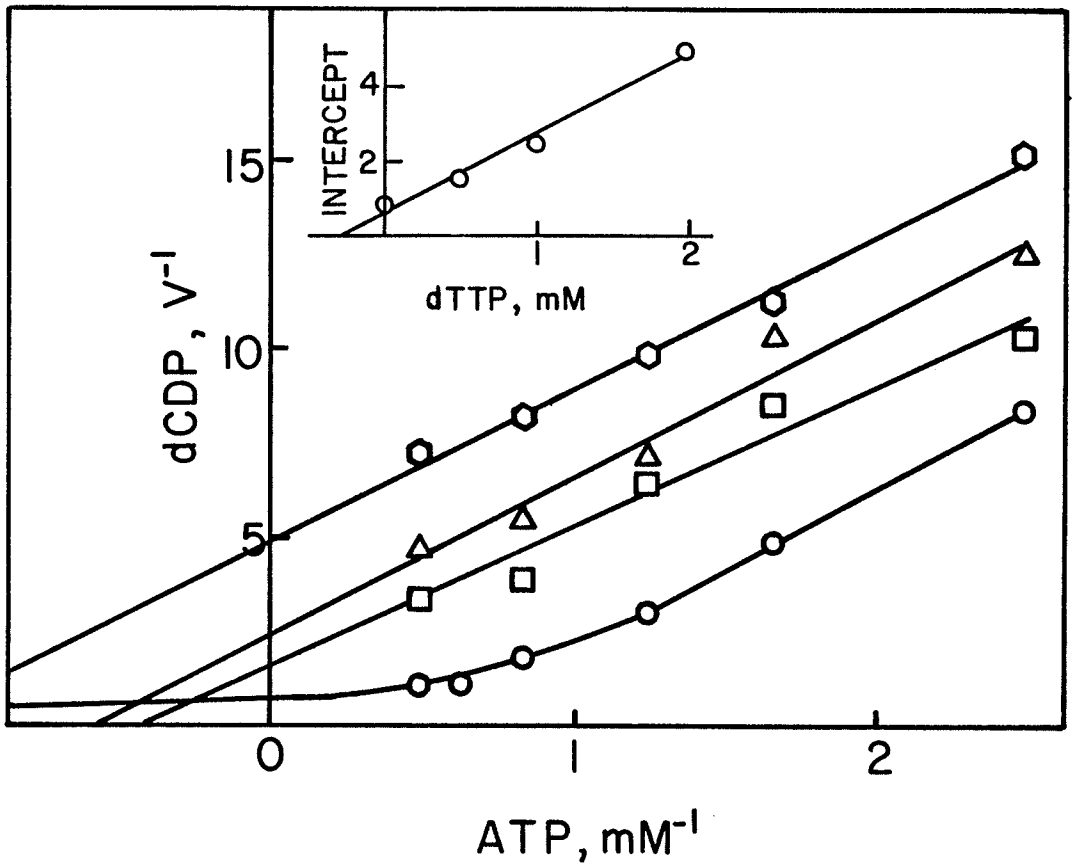

INTERCEPT
4
2
1
2
dTTP, mM
dCDP, V⁻¹
15
10
5
0
1
2
ATP, mM⁻¹

Table 8. Effect of various combinations of GTP and ATP on the reduction of CDP in permeabilized WTC125 CHO cells.

[ATP] (mM)†	[GTP] (mM)†	Activity*
0.4	0	0.159
0.8	0	0.359
2.0	0	0.957
0.4	0.5	0.266
0.8	0.5	0.467
2.0	0.5	0.567
0.4	1	0.270
0.8	1	0.483
2.0	1	0.455
0.4	1.5	0.281
0.8	1.5	0.437
2.0	1.5	0.325

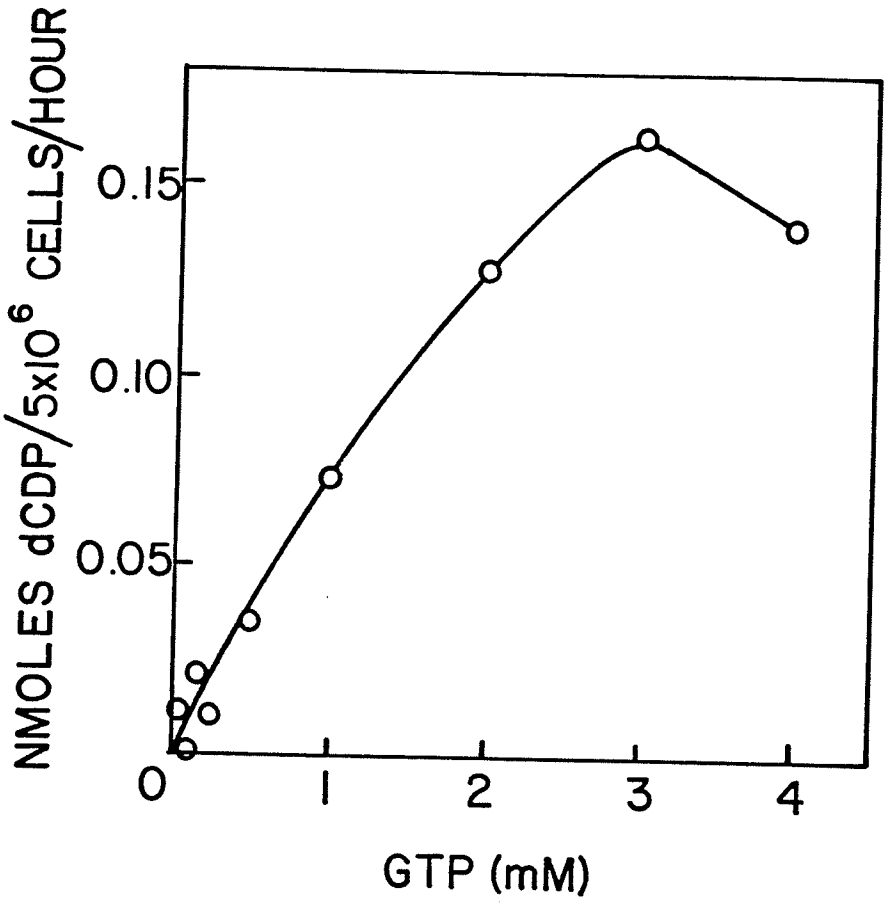
*Activity expressed as nmoles dCDP formed/5 x 10^6 cells/hour. Results are the average of two independent experiments. Average uncertainty is ±18% of reported value.

†Nucleotide levels uncorrected for presence of kinase and phosphatase activities in intact cells.

for CDP reduction, all GTP concentrations tested were found to be inhibitory. To further study this unusual phenomena, the effect of GTP upon the reduction of CDP in the absence of ATP but in the presence of optimal amounts of $MgCl_2$ and DTT was studied. As can be seen in figure 45, GTP did stimulate the reaction with maximal activity occurring when 3 mM GTP was added. The maximum rate of reduction was only 18% of that achieved when 2 mM ATP was added to the assays. Of greater interest is the observation that by itself 1 mM GTP induced the formation of only 0.073 nmoles dCDP/5 x 10^6 cells/hour whereas in the presence of 0.4 to 0.8 mM ATP the same level of GTP resulted in the formation of an extra 0.111 to 0.124 nmoles of dCDP/ 5 x 10^6 cells/hour, thus bringing the activity levels to 0.270 and 0.483 nmoles of dCDP formed/5 x 10^6 cells/hour, respectively. This synergistic effect was even more dramatic with 0.5 mM GTP. Alone this level of GTP stimulated the formation of only 0.035 nmoles dCDP/5 x 10^6 cells/hour while its addition to reaction mixtures containing low levels of ATP (0.4 to 0.8 mM) caused an increase in activity of 0.105 to 0.107 nmoles dCDP formed/5 x 10^6 cells/hour. Thus it would seem that while GTP can stimulate the reduction of CDP to some extent by itself, the combined effect of GTP and ATP must involve more than a simple addition of the activities induced by each separately.

As the manner by which GTP and dGTP activate ADP reduction was found to be different (figure 31) and because ADP reduction activated by the two nucleotides responded in a different manner to inhibition by dATP (figures 36 and 37), ATP (figures 39 and 41) and dCTP (figures 40 and 42) it was decided to observe how these two nucleotides behaved when various combinations were used to activate the reduction. As

Fig. 45. Response of CDP reductase activity in permeabilized WT cells to GTP concentration. Standard CDP reductase assay conditions were used except for the omission of ATP and the presence of varying concentrations of GTP. Results not corrected for the presence of kinase and phosphatase activities in intact cells.


NMOLES dCDP/5x10^6 CELLS/HOUR
0.15
0.10
0.05
0
1
2
3
4
GTP (mM)

dTTP also activated ADP reduction and because the manner by which ATP inhibited the reduction of ADP differed when the reaction was activated by dTTP (figure 44) as compared to dGTP (figure 43) or GTP (table 8), this nucleotide was also tested with various combinations of dGTP and GTP. It was found that at suboptimal levels of GTP and dGTP, the rates of reduction induced by various combinations of the two were equal to the sums of the individual activities (table 9). When the optimal concentration of dGTP was present, the rate of ADP reduction was unaffected by GTP until a GTP to dGTP ratio of approximately ten was reached. At this point and beyond GTP caused a decrease in ADP reductase activity. Conversely, when the optimal concentration of GTP was present increasing dGTP levels caused an elevation in activity levels. At the concentrations of dGTP and dTTP tested (table 10) it was found that the level of reductase activity was essentially determined by the concentration of dTTP present. While increasing dGTP levels in the presence of dTTP did cause an increase in activity levels over those observed with just dTTP these increases were less than the additive effects of the two nucleotides. Moreover, except when 0.5 mM dTTP and 0.005 mM dGTP were present simultaneously, the activity levels for all combinations of the two nucleotides were less than those observed when dGTP was present alone.

The effect of various combinations of GTP and dTTP on ADP reductase activity is shown in table 11. At suboptimal concentrations of the two activators, the rate of reduction induced by various combinations was equal to the sum of the individual activities. This situation was similar to that previously encountered with suboptimal levels of GTP and dGTP (table 9). However, unlike the two situations

Table 9. Effect of various combinations of dGTP and GTP on the reduction of ADP in permeabilized WTC125 CHO cells.

[dGTP] (mM)†	[GTP] (mM)†	Activity*
0.005	0	0.276
0.010	0	0.391
0.100	0	1.014
0.005	0.125	0.517
0.010	0.125	0.645
0.100	0.125	0.947
0.005	0.250	0.699
0.010	0.250	0.790
0.100	0.250	0.977
0.005	1.000	0.598
0.010	1.000	0.617
0.100	1.000	0.679
0	0.125	0.235
0	0.250	0.428
0	1.000	0.435
0.005	0.125	0.517
0.005	0.250	0.699
0.005	1.000	0.598
0.010	0.125	0.645
0.010	0.250	0.790
0.010	1.000	0.617
0.100	0.125	0.947
0.100	0.250	0.977
0.100	1.000	0.679

*Activity expressed as nmoles dADP formed/5 x 10^6 cells/hour. Results are average of two independent experiments. Average uncertainty is ±17% of reported value.

†Nucleotide levels uncorrected for presence of kinase and phosphatase activities in intact cells.

Table 10. Effect of various combinations of dTTP and dGTP on the reduction of ADP in permeabilized WTC125 CHO cells.

[dGTP] (mM)†	[dTTP] (mM)†	Activity*
0.005	0	0.345
0.020	0	0.685
0.100	0	1.135
0.005	0.5	0.422
0.020	0.5	0.433
0.100	0.5	0.586
0.005	1	0.337
0.020	1	0.361
0.100	1	0.370
0.005	2	0.233
0.020	2	0.246
0.100	2	0.205
0	0.5	0.362
0	1	0.284
0	2	0.184
0.005	0.5	0.442
0.005	1	0.337
0.005	2	0.233
0.020	0.5	0.433
0.020	1	0.361
0.020	2	0.246
0.100	0.5	0.586
0.100	1	0.370
0.100	2	0.205

*Activity expressed as nmoles dADP formed/5 x 10^6 cells/hour. Results are the average of two independent experiments. Average uncertainty is ±13% of reported value.

†Nucleotide levels uncorrected for presence of kinase and phosphatase activities in intact cells.

Table 11. Effect of various combinations of GTP and dTTP on the reduction of ADP in permeabilized WTC125 CHO cells.

[GTP] (mM)†	[dTTP] (mM)†	Activity*
0.050	0	0.192
0.100	0	0.272
0.250	0	0.468
0.050	0.025	0.327
0.100	0.025	0.428
0.250	0.025	0.648
0.050	0.050	0.452
0.100	0.050	0.529
0.250	0.050	0.657
0.050	0.167	0.430
0.100	0.167	0.456
0.250	0.167	0.444
0	0.025	0.130
0	0.050	0.284
0	0.167	0.463
0.050	0.025	0.327
0.050	0.050	0.452
0.050	0.167	0.430
0.100	0.025	0.428
0.100	0.050	0.529
0.100	0.167	0.456
0.250	0.025	0.648
0.250	0.050	0.657
0.250	0.167	0.444

*Activity expressed as nmoles dADP formed/5 x 10^6 cells/hour. Results are the average of two independent experiments. Average uncertainty is ±15% of reported value.

†Nucleotide levels uncorrected for the presence of kinase and phosphatase activities in intact cells.

described directly above, where in no case could any combination of activators induce a rate of ADP reduction greater than that observed with one of the nucleotides alone, it would seem that certain combinations of GTP and dTTP were able to stimulate the reduction of ADP at a rate higher than that obtainable with a single activator. For example, the maximum rates of ADP reduction induced by the two nucleotides were 0.468 nmoles/5 x 10^6 cells/hour at 0.25 mM GTP and 0.463 nmoles/5 x 10^6 cells/hour at 0.167 mM dTTP but in the presence of 0.25 mM GTP it was found that 0.025 mM dTTP could increase ADP reductase activity to a maximum of 0.684 nmoles/5 x 10^6 cells/hour. This activity represents a 46% increase over that obtainable with either of the nucleotide activators separately. When 0.25 mM GTP and 0.05 mM dTTP were both present the activity levels achieved, while still in excess of those obtained with the optimal levels of either GTP or dTTP alone, were now less than the sum of the individual activities. This situation contrasts with that observed with combinaitons of dGTP and GTP or dTTP and dGTP. In each of these cases the highest rate of reduction was achieved with 0.1 mM dGTP as the activator and no combinations of nucleotides could induce a greater rate. It should be noted that when optimal levels of both GTP (0.25 mM) and dTTP (0.167 mM) were present in the assay the level of ADP reductase activity (0.444 nmoles ADP formed/5 x 10^6 cells/hour) was approximately the same as that obtained with each nucleotide individually.

As both ATP and dCTP activate the reduction of CDP, the effect of various combinations of these two nucleotides on enzyme activity was studied (table 12). From the results it can be seen that at

Table 12. Effect of various combinations of ATP and dCTP on the reduction of CDP in permeabilized WTC125 CHO cells.

[ATP] (mM)†	[dCTP] (mM)†	Activity*
0.4	0	0.119
0.8	0	0.328
2.0	0	0.991
0.4	1.0	0.326
0.8	1.0	0.798
2.0	1.0	1.096
0.4	2.0	0.548
0.8	2.0	0.834
2.0	2.0	0.976
0.4	3.0	0.490
0.8	3.0	0.816
2.0	3.0	0.852
0	1.0	0.144
0	2.0	0.211
0	3.0	0.218
0.4	1.0	0.326
0.4	2.0	0.548
0.4	3.0	0.490
0.8	1.0	0.798
0.8	2.0	0.834
0.8	3.0	0.816
2.0	1.0	1.096
2.0	2.0	0.976
2.0	3.0	0.852

*Activity was expressed as nmoles dCDP formed/5 x 10^6 cells/hour.

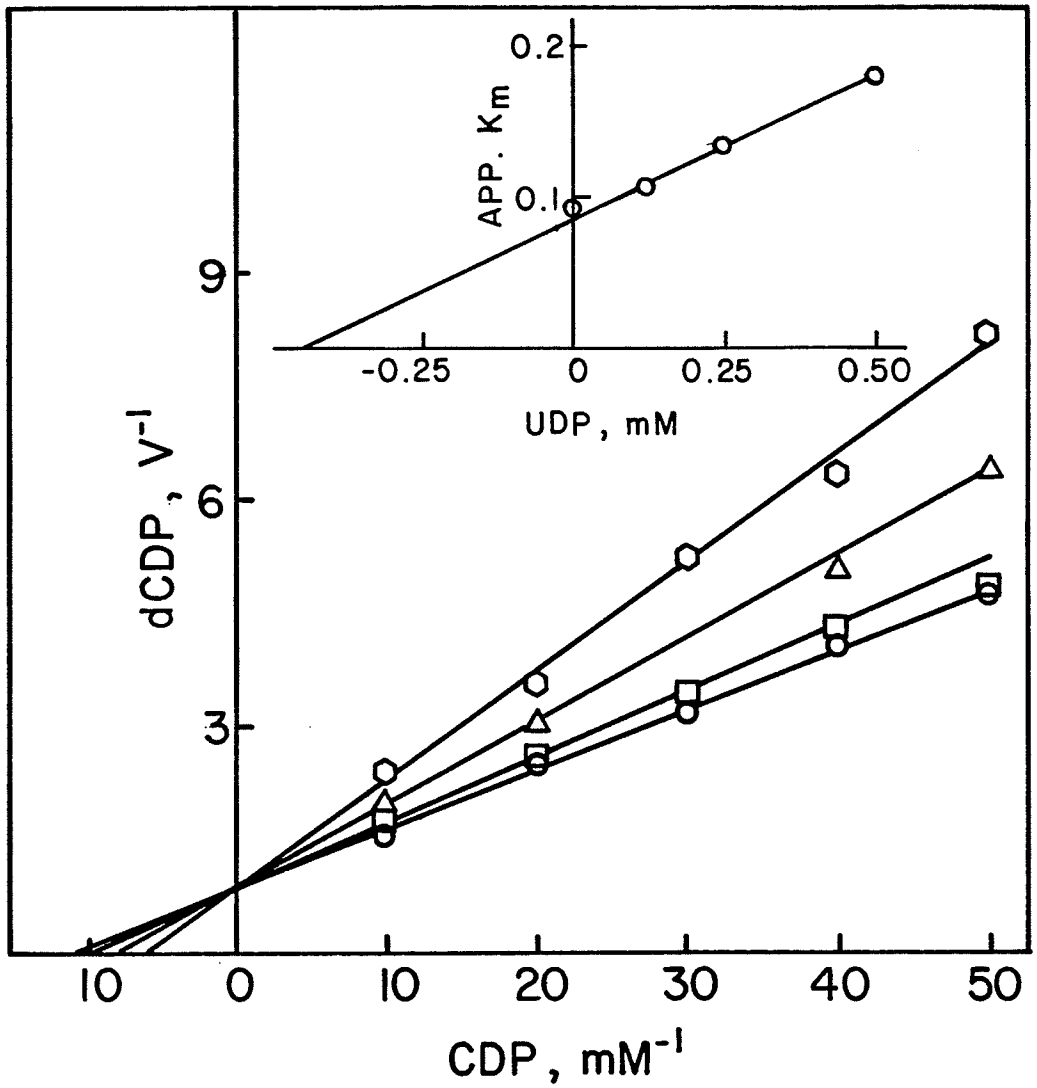
†Nucleotide levels uncorrected for presence of kinase and phosphatase activities in intact cells.

suboptimal concentrations of ATP, dCTP further stimulated reduction and vice versa. Moreover, the levels of activity achieved in the presence of the two activators were not simply the sum of the individual activities. For example, 0.8 mM ATP induced the reduction of 0.328 nmoles of CDP/5 x 10^6 cells/hour while 1 mM dCTP induced the reduction of 0.144 nmoles of CDP/5 x 10^6 cells/hour. If the two activators were merely additive, a reduction rate of 0.472 nmoles dCDP formed/5 x 10^6 cells/hour would be expected when both activators were present at the above concentrations. However, a rate of 0.798 nmoles dCDP formed/5 x 10^6 cells/hour, 170% of the expected rate, was achieved. While this was the greatest synergistic effect observed, similar results were found for all assays in which dCTP was present with sub-optimal levels of ATP (table 12).

3.3 Substrate competition

If the reduction of all four ribonucleotides is carried out by a single enzyme as suggested by several laboratories (Eriksson et al 1979, Engström et al 1979, Ullman et al 1981, Chang and Cheng 1979a,b), there should be a competition between the various ribonucleoside diphosphate substrates for the enzyme. Since both require ATP as the allosteric activator, the easiest system to investigate substrate competition involved CDP and UDP (Moore and Hurlbert 1966, Murphree et al 1968, Eriksson et al 1979, Robert de Saint Vincent et al 1980). As shown in figure 46, with permeabilized intact CHO cells UDP inhibited CDP reduction in a competitive manner. The apparent K_i determined was 0.44 mM UDP.

Fig. 46. Double reciprocal plot of the variation in rate of CDP reduction with CDP concentration at several fixed levels of UDP using permeabilized WT cells. Assays were performed in the absence (O) or presence of 0.125 mM (□), 0.25 mM (Δ) and 0.5 mM (⬡) UDP. Activity expressed as nmoles dCDP formed/5 x 10^6 cells/hour. Inset: replot of apparent Km values against UDP concentrations. Results not corrected for the presence of kinase and phosphatase activities in intact cells.


0.2
APP. Km
0.1
-0.25
0
0.25
0.50
UDP, mM
9
6
3
dCDP, V⁻¹
10
0
10
20
30
40
50
CDP, mM⁻¹

3.4 Relationship of Kinetic Constants to Nucleotide Pool Sizes of CHO cells

In order to determine whether the kinetic constants determined for the reduction of ribonucleotides in permeabilized intact CHO cells were feasible with regards to the *in vivo* situation, the ribo- and deoxyribonucleoside di- and triphosphate pool sizes were determined. The pool sizes were determined in cells grown in a manner identical to those used in the enzyme assays. As such they were an unsynchronized population in the exponential stage of growth. The nucleotides were extracted and analysed as described in Materials and Methods. The relevant results obtained are presented in table 13. The deoxyribonucleoside triphosphate levels determined for CHO cells were well within the range of values obtained with many mammalian cell types (Weber and Edlin 1971, Lowe and Grindey 1976, Garrett and Santi 1979, Cohen *et al* 1980, Robert de Saint Vincent *et al* 1980, Fox *et al* 1980, Zannis-Hadjopoulous *et al* 1980, Nilsson *et al* 1980, Kinahan *et al* 1980, Cohen *et al* 1981, Ross *et al* 1981, Hunting and Henderson 1981a,b Hunting *et al* 1981). The ribonucleoside triphosphate pool sizes determined for CHO cells were also within the range of values obtained for other mammalian cells except that in this case they were well towards the lower end of the range (Weber and Edlin 1971, Garrett and Santi 1979, Maybaum *et al* 1980, Cohen *et al* 1980, Kinahan *et al* 1980, Cohen *et al* 1981, Hunting and Henderson 1981a, Hunting *et al* 1981). Though relatively few studies of the intracellular ribonucleoside diphosphate pool sizes have been conducted, those that have been carried out agree reasonably well with the values determined for CDP and UDP whereas the purine ribonucleoside diphosphates, ADP and GDP, were found in slightly higher levels in the CHO cells as compared to

Table 13. Nucleotide pool sizes of exponentially growing WTC125 CHO cells

Nucleotide	Intracellular Concentration	
	nmoles/10^6 cells	mM
GTP	0.334 ± 0.038	0.152 - 0.223
ATP	1.589 ± 0.258	0.682 - 1.059
CTP	0.604 ± 0.379	0.259 - 0.403
UTP	0.387 ± 0.030	0.153 - 0.238
GDP	0.157 ± 0.025	0.067 - 0.106
ADP	1.060	0.379 - 0.558
CDP	0.048 ± 0.009	0.017 - 0.026
UDP	0.121 ± 0.011	0.049 - 0.075
dGTP	ND[1]	-
dATP	0.028 ± 0.007	0.013 - 0.019
dCTP	0.105 ± 0.065	0.045 - 0.070
dTTP	0.053 ± 0.035	0.023 - 0.035

Results are the average of two independent trials.

[1]not detected

most other mammalian sources (Weber and Edlin 1971, Maybaum et al 1980, Cohen et al 1980).

In order to determine the molar concentrations of the various nucleotides within the cell, an estimate of the volume of a CHO cell was required. Various reports with different lines of CHO cells have estimated this volume to range from 1.5 pl (Fox and Pardee 1970) to 2.33 pl (Ceri and Wright 1977, Wright and Ceri 1977). The conversion to molar concentration was calculated using both values and a range given.

The reduction of CDP can be activated by either ATP or dCTP. The optimal concentration of ATP was found to be 1.5 mM (figure 9b), a value 1.5 to 2 fold higher than the intracellular concentration. The concentration of dCTP determined to induce the greatest rate of CDP reduction was 3.6 mM, a level some 35 fold greater than the intracellular concentration of the nucleotide. The Km values exhibited by the reactions were 0.021 mM CDP and 0.033 mM CDP for the ATP and dCTP activated reduction respectively. The lower value is within the intracellular range while the higher value is just above the determined range.

The reduction of ADP required the presence of dGTP, GTP or dTTP as the allosteric activator. The optimal levels for each nucleotide were determined to be 0.04 to 0.20 mM dGTP (figure 16), 0.2 mM GTP (figure 22a) and 0.065 mM dTTP (figure 22b). Within the CHO cells, only GTP would seem to have a concentration sufficient to induce its optimal effect. DeoxyTTP was found to be present at approximately one-half the level required for maximum activity, while dGTP was not detected at all. The Km values determined for the various activators

were 0.095 mM (figure 21), 0.2 mM (figure 23a) and 0.333 mM ADP (figure 23b) for the dGTP, GTP and dTTP stimulated reductions respectively. All of these values are lower than the intracellular concentration of ADP measured.

4. CHARACTERIZATION OF A LOW LEVEL N-CARBAMOYLOXYUREA-RESISTANT CHO CELL LINE.

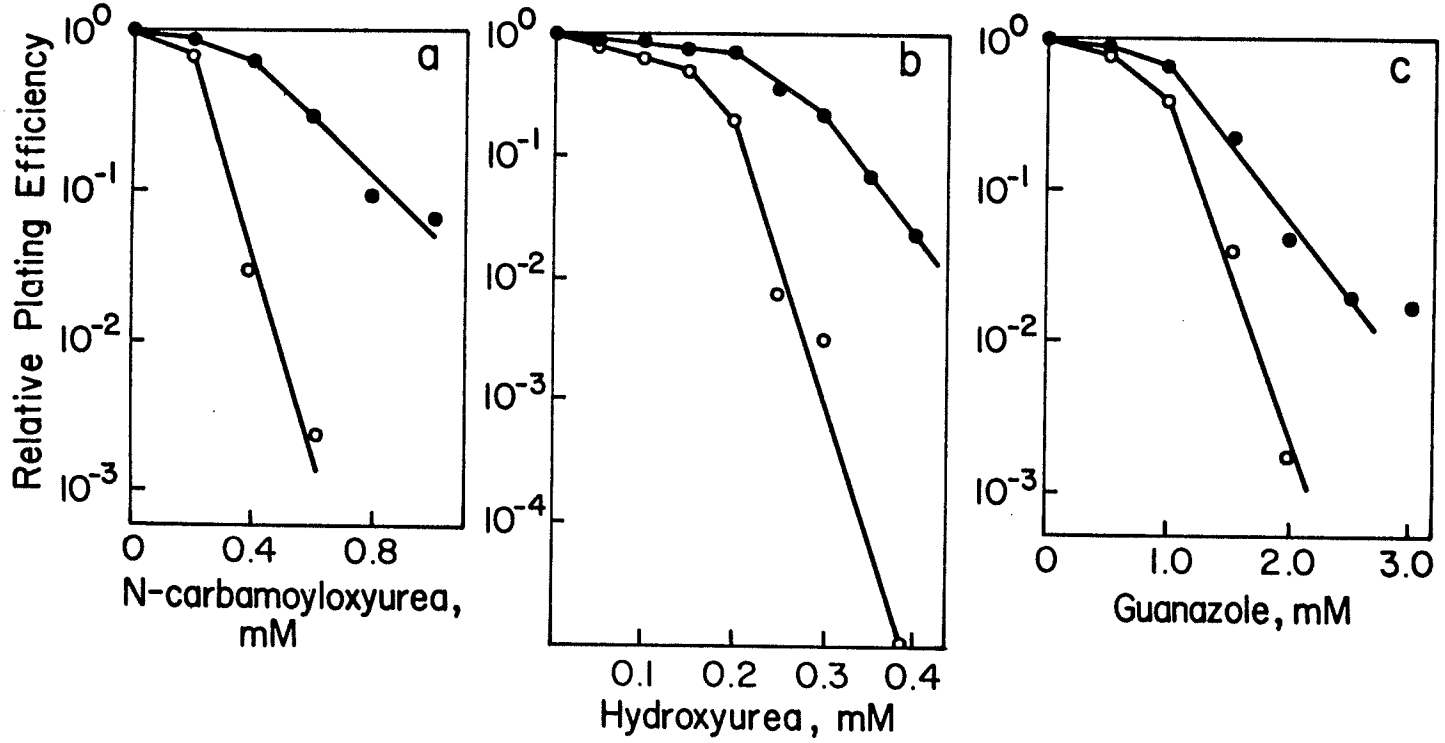
Since the selection of the first somatic cell mutant 26 years ago (Puck and Fisher 1956), the technique has gained wide spread applicability in the study of many aspects of cell biology (for reviews see Wright et al 1980,1981 and Siminovitch 1976). The selection of drug-resistant mutants has progressed particularly quickly because of the availability of a wide range of cytotoxic drugs which inhibit specific cellular functions. In addition, the wide variety of drug resistant mutants generated are usually easy to isolate.

A mutant resistant to the drug N-carbamoyloxyurea was selcted for two reasons. The first was to demonstrate that the drug did act at the site of ribonucleotide reductase while the second was to study the properties of the enzyme in a drug-resistant cell line.

4.1 Drug Sensitivity of NC^R-30A and Parental WT Cells.

The cloned cell line, designated NC^R-30A, was selected from the parental line, WTC125, by its ability to grow in the presence of normally cytotoxic concentrations of N-carbamoyloxyurea (see Materials and Methods for selection procedure). Figure 47a shows the ability of the wild type and mutant cells to form colonies in the presence of varying concentrations of N-carbamoyloxyurea. The NC^R-30A cells were clearly more resistant to the cytotoxic effects of the drug. During 2 years of continuous culture in the absence of selective agent the

Fig. 47. Relative plating efficiencies of WTC125 (O) and NC^R-30A (●) cells in increasing concentrations of N-carbamoyloxyurea (a), hydroxyurea (b) and guanazole (c). From 250 to 1,000,000 cells per 100 mm plastic culture plate were incubated at 37°C for 7 to 14 days in standard growth medium containing varying concentrations of N-carbamoyloxyurea, hydroxyurea or guanazole.


Relative Plating Efficiency
10^0
10^{-1}
10^{-2}
10^{-3}
a
0
0.4
0.8
N-carbamoyloxyurea, mM
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
b
0.1
0.2
0.3
0.4
Hydroxyurea, mM
10^0
10^{-1}
10^{-2}
10^{-3}
c
0
1.0
2.0
3.0
Guanazole, mM

D_{10} values for the WT and NC^R-30A cells remained essentially unchanged at 0.32 ± 0.02 and 0.84 ± 0.08 mM N-carbamoyloxyurea respectively. Each value was determined from a minimum of ten independent tests.

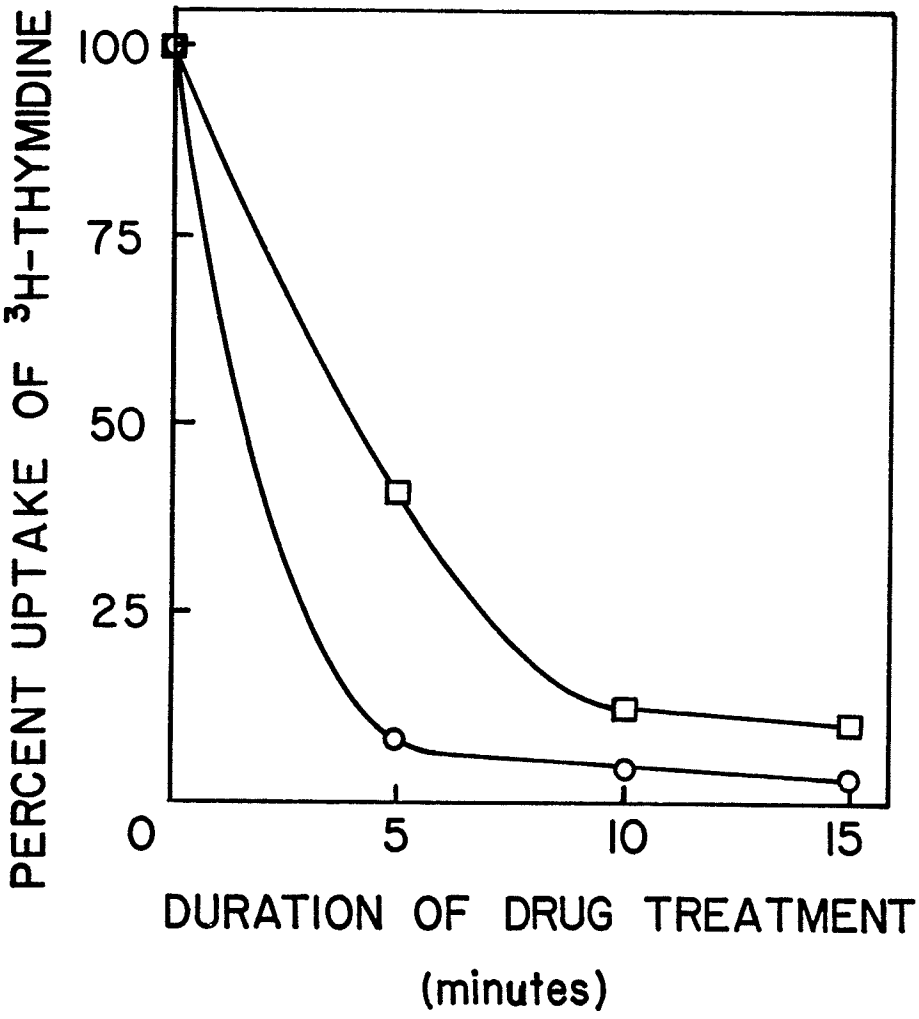
Chinese hamster ovary cells resistant to hydroxyurea have been shown to be cross-resistant to a number of other drugs including guanazole and N-carbamoyloxyurea (Wright and Lewis 1974, Lewis and Wright 1978a). Therefore the effects of hydroxyurea and guanazole on the colony-forming ability of WT and NC^R-30A cells were examined. As can be seen from figure 47b and c, NC^R-30A cells were also more resistant than WT cells to hydroxyurea and guanazole. The D_{10} values determined from a minimum of four separate experiments with each drug were 1.77 ± 0.05 mM guanazole and 0.35 ± 0.02 mM hydroxyurea for NC^R-30A cells and 1.21 ± 0.05 mM guanazole and 0.21 ± 0.02 mM hydroxyurea for parental wild type cells.

In the absence of any drug, both cell lines consistently exhibited a plating efficiency of between 0.80 and 0.90 when grown in α(MEM) plus 10% FCS at 37°C.

4.2 DNA Synthesis

As the NC^R-30A cells were cross-resistant to hydroxyurea and guanazole (figure 47) and these two drugs are known to inhibit DNA synthesis (Young and Hodas 1964, Sinclair 1967, Bacchetti and Whitmore 1969, Brockman et al 1970, Lewis and Wright 1974), the effect of N-carbamoyloxyurea on ^{3}H-thymidine incorporation into acid precipitable material was studied. As can be seen from figure 48, N-carbamoyloxyurea induced an immediate slow down in DNA synthesis in both WTC125 and NC^R-30A cells. As expected the mutant cell line showed a greater resistance to the effect of the drug. Studies with 0.18 mM hydroxyurea

Fig. 48. Thymidine uptake into acid precipitable material of WTC125 (O) and NC^R-30A (□) cells in the presence of 0.3 mM N-carbamoyloxyurea. To cells growing exponentially on the surface of plastic tissue culture plates was added sufficient amounts of concentrated N-carbamoyloxyurea to result in the final level of 0.3 mM. After 5, 10 or 15 minutes of incubation at 37°C the drug containing medium was aspirated, the plates washed with α-MEM + 10% FCS + 0.3 mM N-carbamoyloxyurea and then 5 ml of α-MEM + 10% FCS + ^{3}H-thymidine (1 μCi/ml, 0.5 μg/ml) added and the plates incubated for 10 minutes at 37°C. The amount of ^{3}H-thymidine uptake into acid precipitable material was measured as described in Materials and Methods.


PERCENT UPTAKE OF ^{3}H-THYMIDINE
100
75
50
25
0
5
10
15
DURATION OF DRUG TREATMENT
(minutes)

and 1.2 mM guanazole produced similar inhibition of DNA synthesis with the mutant cells again being more resistant to the effects of the drugs as compared to the wild type parental cells (data not shown).

4.3 Alterations in Activity of Ribonucleotide Reductase

Because the N-carbamoyloxyurea-resistant cell line was cross-resistant to hydroxyurea and guanazole, two drugs whose intracellular site of action was known to be ribonucleotide reductase (Elford 1968, Brockman et al 1970, Lewis and Wright 1974), it seemed highly likely that the NC^R-30A cells possessed an alteration in this activity. Thus the reduction of ribonucleotides by permeabilized NC^R-30A cells was characterized and compared to that determined for WTC125 cells.

The optimal conditions for the reduction of CDP and ADP by permeabilized NC^R-30A cells were determined in the same manner as those previously described for WTC125 cells. A comparison of the two sets of conditions is presented in Table 14. As can be seen the optimal conditions for the reduction of both substrates were virtually identical for both cell lines. Moreover, the omission of various components from the reaction mixtures had approximately the same effect with both cell lines. Using the optimal assay conditions determined for the reduction of both CDP and ADP in NC^R-30A cells, the linearity of reduction versus cell number and time was investigated (figure 49). As can be seen the reduction of both substrates was linear with cell number up to and including 7×10^6 cells per assay and with time up to 20 minutes.

With the studies involving permeabilized NC^R-30A cells, it was assumed that kinase and phospatase activities found in permeabilized wild type cells were present at the same levels in the mutant cells.

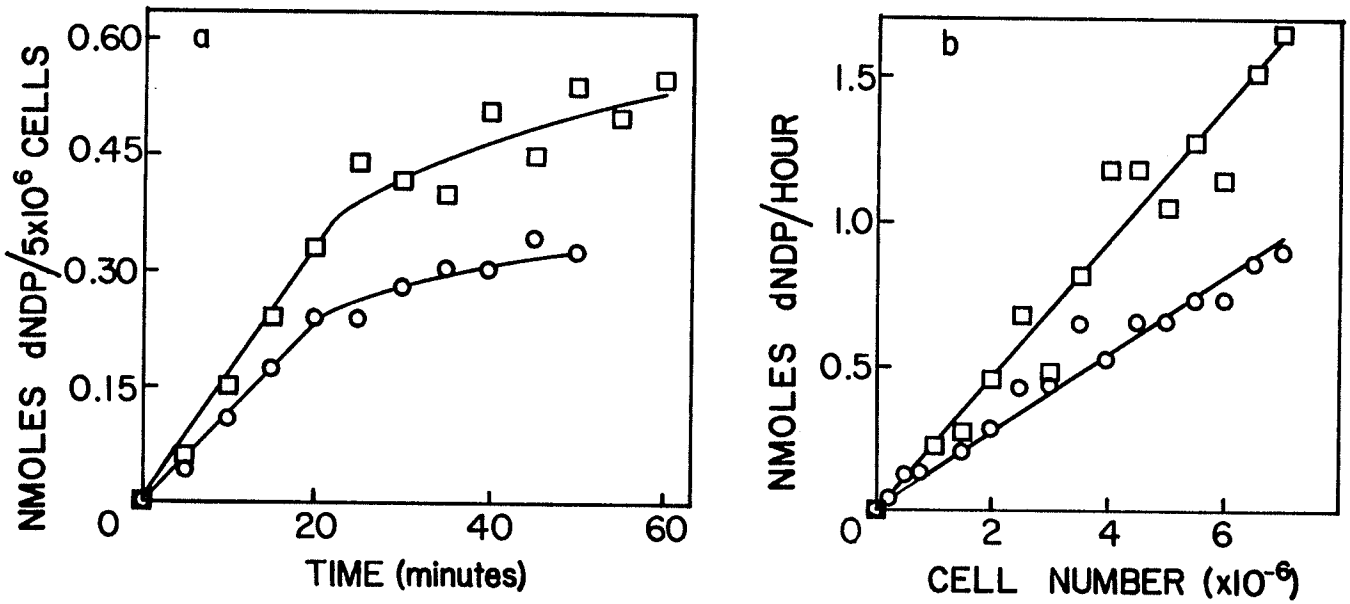
Table 14. General properties of ribonucleotide reduction in permeabilized WTC125 and NC^R-30A cells.

Addition	Requirement for optimum activity			
	CDP Reduction		ADP Reduction	
	WTC125	NC^R-30A	WTC125	NC^R-30A
DTT	6 mM	6 mM	6 mM	6 mM
ATP	1.5 mM	1.5 mM	-	-
dGTP	-	-	0.04-0.2	0.2
$MgCl_2$	7 mM	7 mM	-	-
pH	7.2	7.4	6.9-7.1	6.9
temperature	37°C	37°C	37°C	37°C

Omission	Percentage activity remaining			
	CDP Reduction		ADP Reduction	
	WTC125	NC^R-30A	WTC125	NC^R-30A
none	100%	100%	100%	100%
DTT	42	26	42	55
ATP	0	1	-	-
dGTP	-	-	0	1
$MgCl_2$	14	23	-	-

Fig. 49a. Response of CDP (□) and ADP (○) reductase activity in permeabilized NC^R-30A cells to incubation time. Standard CDP and ADP reductase assay conditions were used except for the variation in incubation time indicated.

Fig. 49b. Response of CDP (□) and ADP (○) reductase activity in permeabilized NC^R-30A cells to cell number. Standard CDP and ADP reductase assay conditions were used except for the variation in cell number indicated.


a
NMOLES dNDP/5x10^6 CELLS
0.60
0.45
0.30
0.15
0
20
40
60
TIME (minutes)
b
NMOLES dNDP/HOUR
1.5
1.0
0.5
0
2
4
6
CELL NUMBER (x10^-6)

As ribonucleotide reductase is likely the only nucleotide metabolizing enzyme altered in the mutant cell line (see results presented below), the distribution of phosphates in the CDP and ADP reductase assays should be the same. Moreover, as ribonucleotide reductase from NC^R-30A cells responded to nucleotides in a manner identical to that observed with WTC125 cells (table 14 and below) it seems probable that no differences in nucleotide phosphate metabolism exists between the two cell lines.

In addition to optimum assay conditions, the enzyme activity from the mutant cell line was tested for response to inhibitory nucleoside triphosphates. As was the case for the wild type cells, CDP reduction was inhibited by dATP, dGTP and GTP (figure 50) while ADP reduction was inhibited by dATP and ATP (figure 51). CDP reduction was reduced 50% by 4.5 μM dATP, 0.08 mM dGTP and 1.45 mM GTP while ADP reduction was reduced by one-half by 25 μM dATP and 1.1 mM ATP. These values are very similar to those obtained with the wild type cells (figures 25, 26 and 27).

The effect of various nucleoside triphosphates as activators of CDP and ADP reduction in the absence of ATP and dGTP was also tested. As can be seen from table 15, CDP reduction was activated by dCTP as well as ATP and ADP reduction was activated by GTP and dTTP in addition to dGTP. These nucleotides also activated reductase activity in the wild type cells (table 7). Moreover, the various nucleotides tested were found to induce similar levels of reductase activity in the two cell lines. For example, in comparison to the levels achieved with the optimal concentration of ATP, 1 mM dCTP induced activity levels of 22% in WTC125 cells and 20% in NC^R-30A cells.

Fig. 50a. Response of CDP reductase activity in permeabilized NC^R-30A cells to dATP concentration. Standard CDP reductase assay conditions were used except for the presence of varying concentrations of dATP. Results corrected for the presence of kinase and phosphatase activities in intact cells.

Fig. 50b. Response of CDP reductase activity in permeabilized NC^R-30A cells to dGTP concentration. Standard CDP reductase assay conditions were used except for the presence of varying concentrations of dGTP. Results not corrected for the presence of kinase and phosphatase activities in intact cells.

Fig. 50c. Response of CDP reductase activity in permeabilized NC^R-30A cells to GTP concentration. Standard CDP reductase assay conditions were used except for the presence of varying concentrations of GTP. Results not corrected for the presence of kinase and phosphatase activities in intact cells.

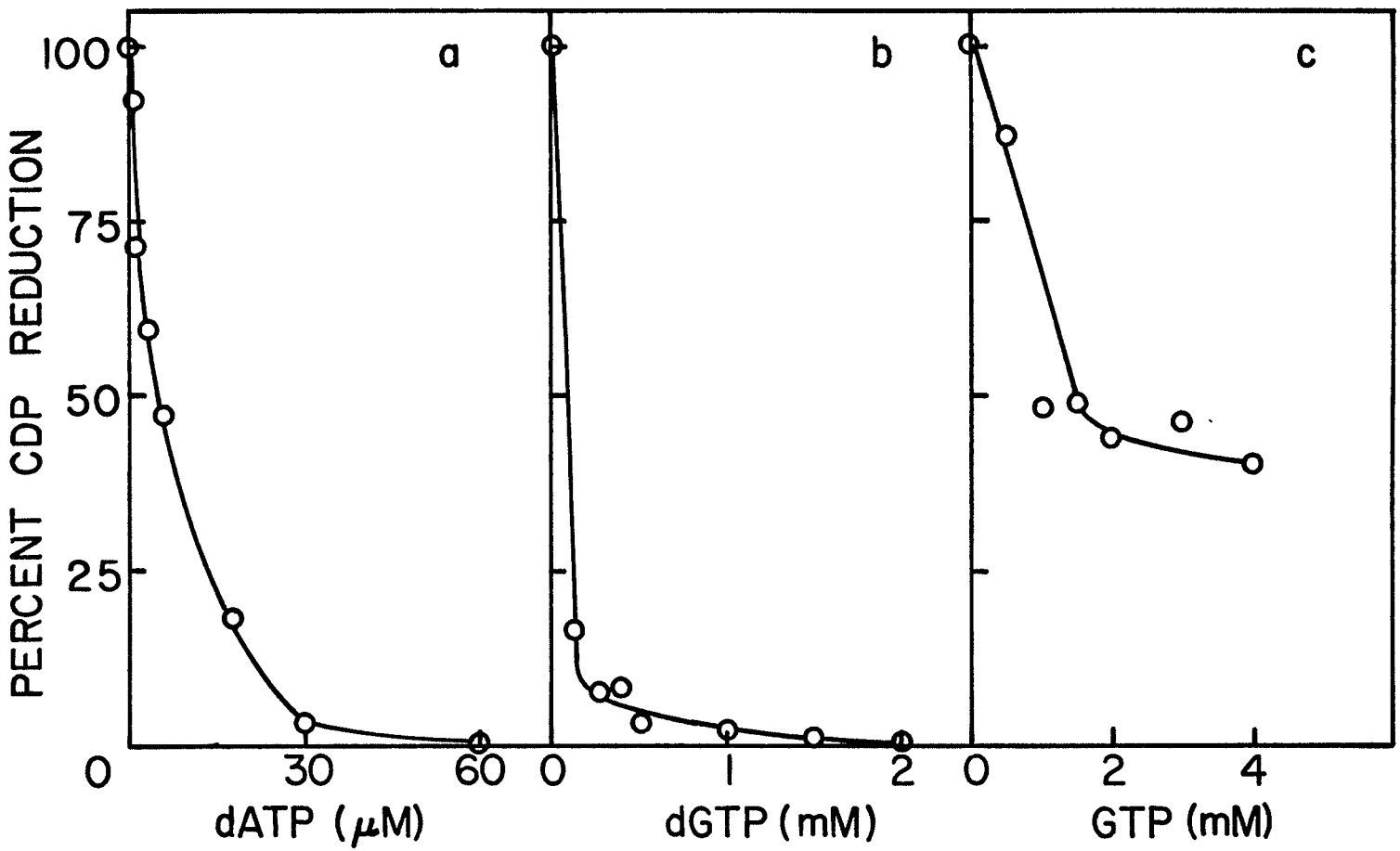

PERCENT CDP REDUCTION
100
75
50
25
0
a
b
c
0
30
60
0
1
2
0
2
4
dATP (μM)
dGTP (mM)
GTP (mM)

Fig. 51a. Response of ADP reductase activity in permeabilized NC^R-30A cells to dATP concentration. Standard ADP reductase assay conditions were used except for the presence of varying concentrations of dATP. Results corrected for the presence of kinase and phosphatase activities in intact cells.

Fig. 51b. Response of ADP reductase activity in permeabilized NC^R-30A cells to ATP concentration. Standard ADP reductase assay conditions were used except for the presence of varying concentrations of ATP. Results corrected for the presence of kinase and phosphatase activities in intact cells.

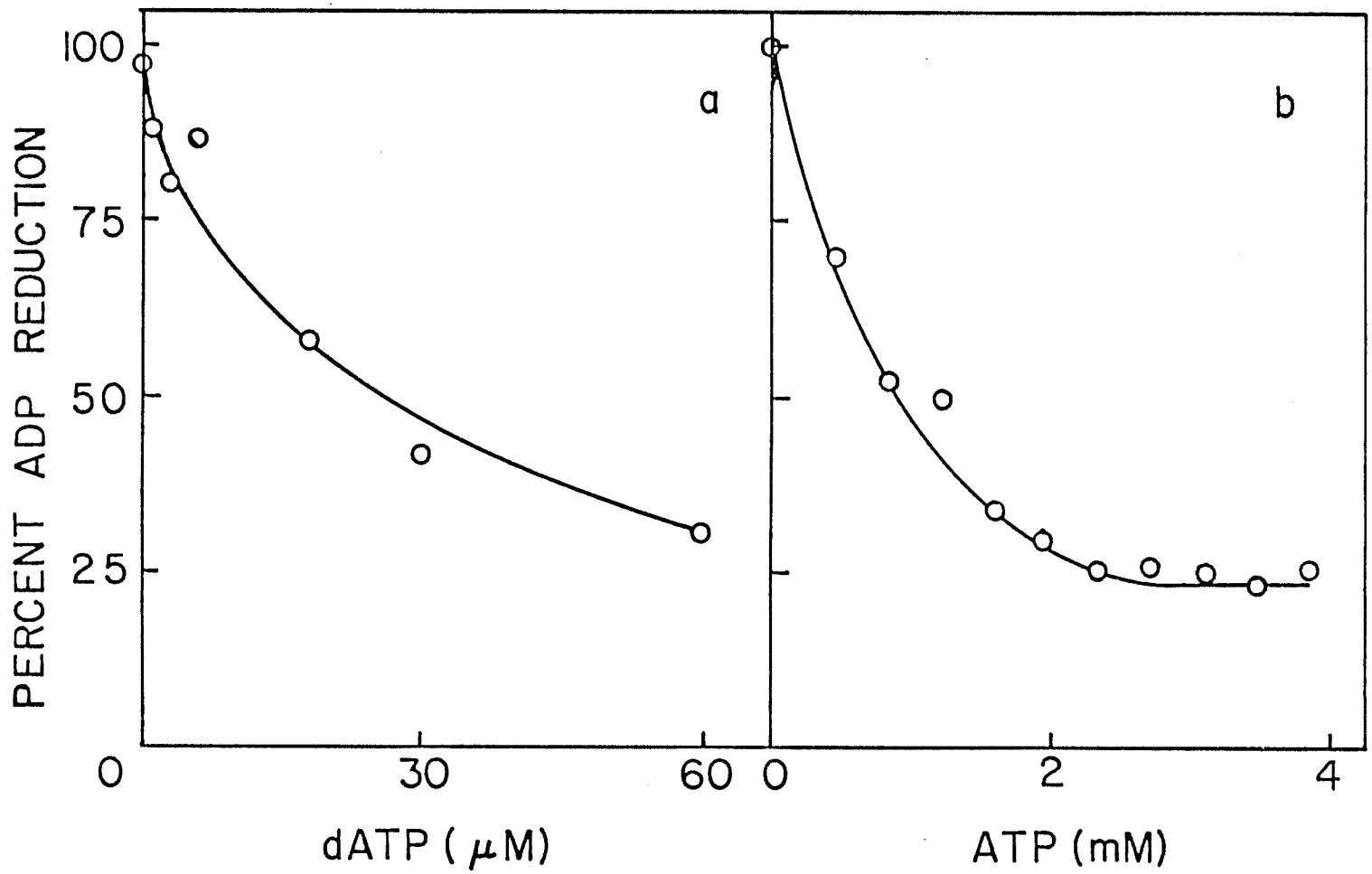

PERCENT ADP REDUCTION
100
75
50
25
0
a
b
30
60
0
2
4
dATP (μM)
ATP (mM)

Table 15. Effect of various nucleoside triphosphates as activators of CDP and ADP reduction in intact NC^R-30A cells.

Nucleoside triphosphate	Concentration† (mM)	% activity* CDP reduction	% activity* ADP reduction
ATP	2	100	6
dATP	0.05	4	2
GTP	1.5	12	40
dGTP	0.5	10	100
CTP	1	6	6
dCTP	1	20	10
UTP	1	0	11
dTTP	1	1	19
None	-	8	1

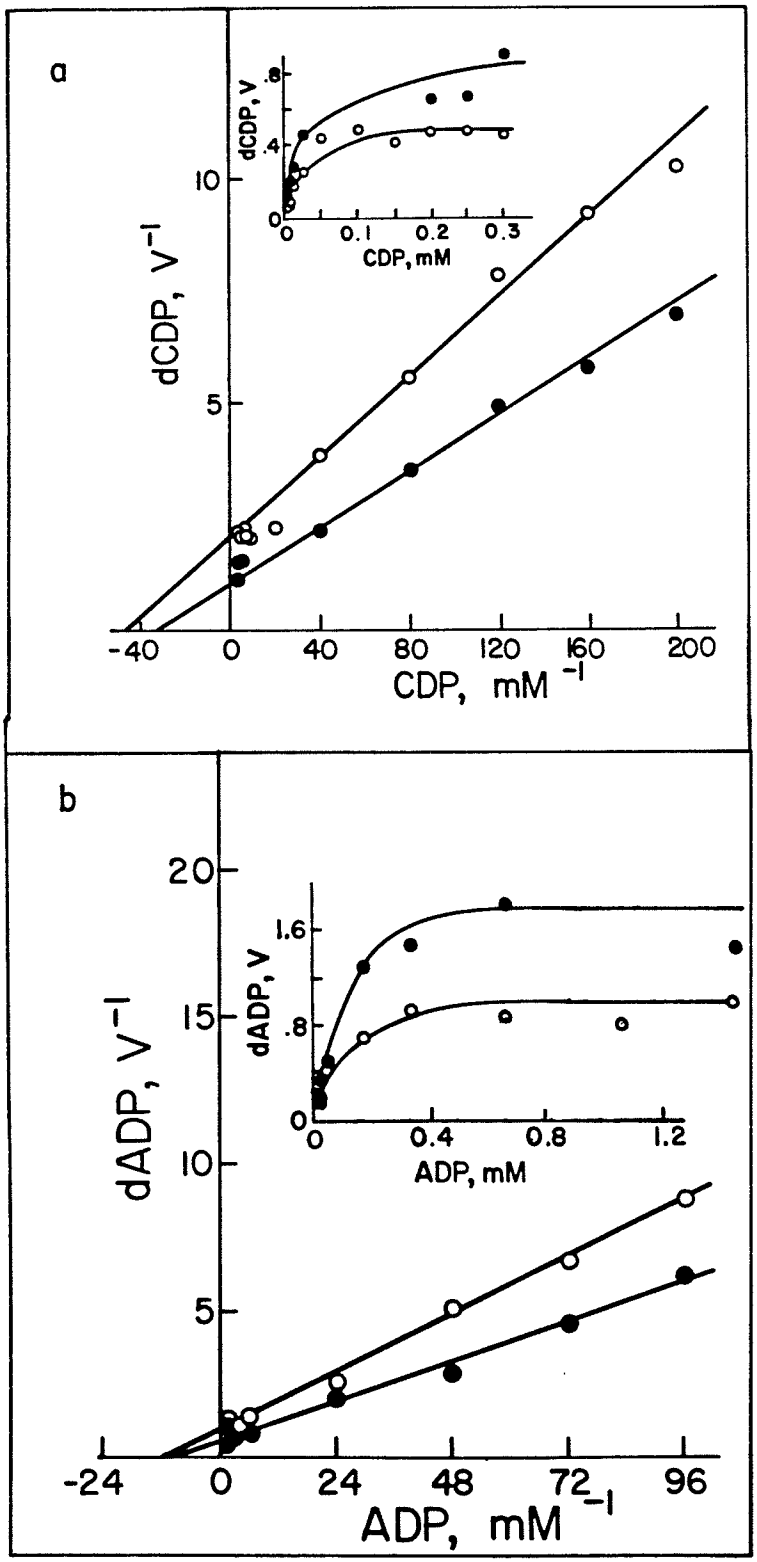
†Nucleotide concentrations uncorrected for the presence of kinase and phosphatase activities in intact cells.
*% activity when compared to CDP reduction in presence of ATP and ADP reduction in presence of dGTP.

In comparing the properties of ribonucleotide reductase from WTC125 and NC^R-30A cells, the sole difference between the two cell lines seemed to be that the mutant possessed enhanced intracellular levels of activity. For example under standard assay conditions 5 x 10^6 WTC125 cells were found to reduce 0.24 nmoles of CDP (figure 11b) and 0.175 nmoles of ADP (figure 19a) in 20 minutes while the same number of NC^R-30A cells were found to reduce 0.33 nmoles of CDP and 0.24 nmoles of ADP in 20 minutes (figure 49). To investigate this increased activity more fully, double reciprocal plots of activity versus substrate concentration were constructed (figure 52). The plots were linear for both nucleoside diphosphate substrates. For the reduction of CDP the data yielded a Km value of 0.033 $\pm$ 0.005 mM CDP and a V_{max} of 1.01 $\pm$ 0.14 nmoles dCDP formed/5 x 10^6 cells/hour. Experiments with ADP as the substrate yielded an apparent Km of 0.095 $\pm$ 0.017 mM ADP and a V_{max} of 1.83 $\pm$ 0.30 nmoles dADP formed/5 x 10^6 cells/hour. The values determined with permeabilized WTC125 cells were Km values of 0.021 $\pm$ 0.005 mM CDP and 0.095 $\pm$ 0.017 mM ADP and V_{max} values of 0.49 $\pm$ 0.17 nmoles dCDP and 1.00 $\pm$ 0.13 nmoles dADP formed/5 x 10^6 cells/hour. Each of the experiments was performed at least ten times over the course of approximately one year with similar results being obtained in each case. From the results it would appear that the N-carbamoyloxyurea-resistant cells contained approximately twice the wild type intracellular level of ribonucleotide reductase activity.

Studies with hydroxyurea-resistant mammalian cells have identified three classes of drug resistant mutants (Wright et al 1981). Mutants of the first class contain an apparent structural alteration to the

Fig. 52a. Double reciprocal plots of the variation in the rate of CDP reduction with CDP concentration for WT (O) and NC^R-30A (●) cells. Activity was expressed as nmoles dCDP formed/5 x 10^6 cells/hour. Inset: the velocity of CDP reduction in the presence of varying concentrations of CDP for WT (O) and NC^R-30A (●) cells.

Fig. 52b. Double reciprocal plots of the variation in the rate of ADP reduction with ADP concentration for WT (O) and NC^R-30A (●) cells. Activity was expressed as nmoles dADP formed/5 x 10^6 cells/hour. Insets: The velocity of ADP reduction in the presence of varying concentrations of ADP for WT (O) and NC^R-30A (●) cells.

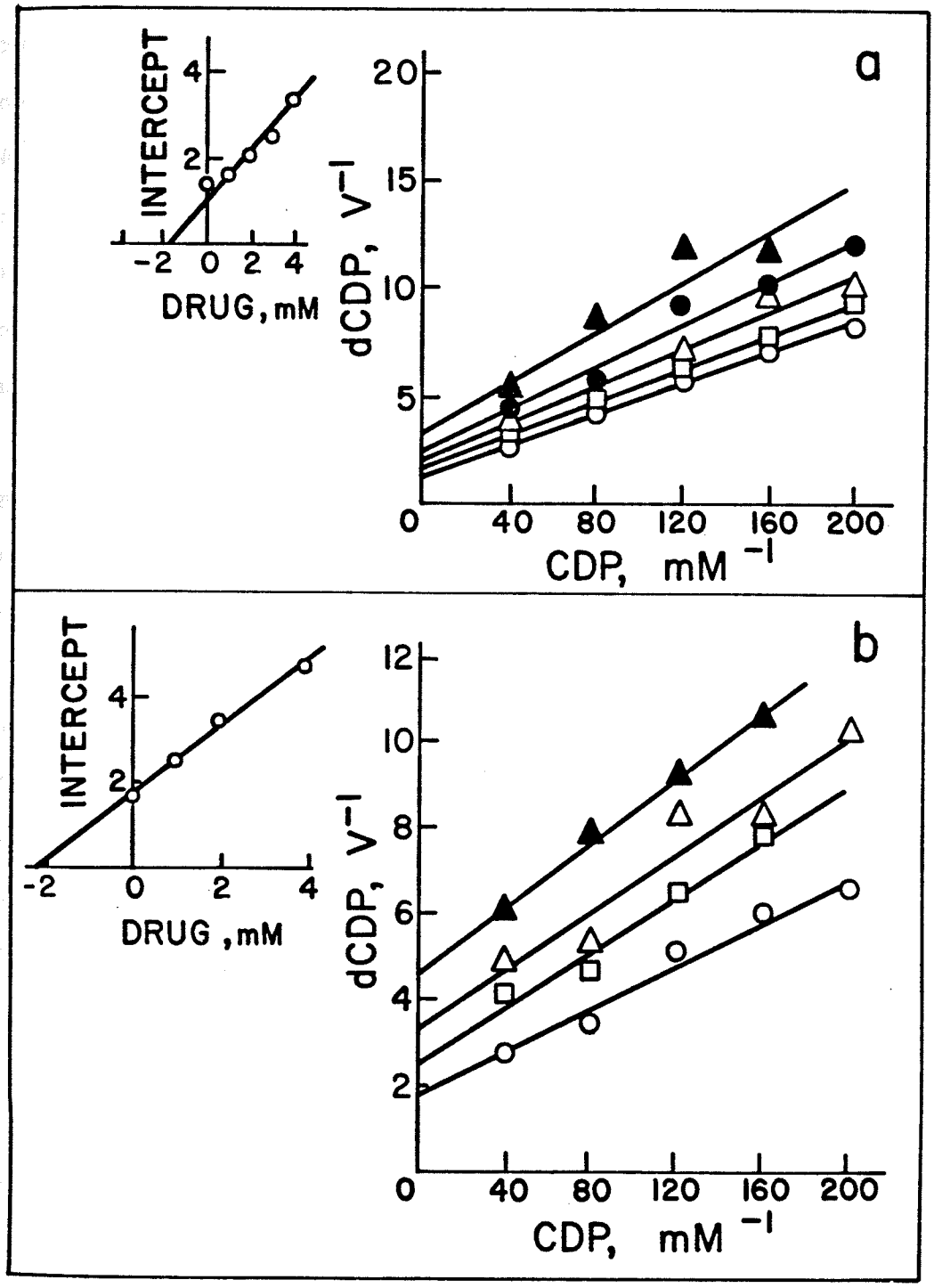

a
dCDP, V
dCDP, V
CDP, mM
CDP, mM
b
dADP, V
dADP, V
ADP, mM
ADP, mM

enzyme which renders the reductase activity of these cells less sensitive to drug inhibition (Wright et al 1980, Lewis and Wright 1974, Lewis and Wright 1978b). These cell lines frequently exhibit alterations in other kinetic parameters such as substrate Km values. The second class of drug-resistant mutants possess a reductase activity with wild type sensitivity to drug inhibition but with enhanced intracellular levels of enzyme activity (Lewis et al 1978, Wright et al 1980, Koropatnick and Wright 1980, Lewis and Wright 1979). Drug sensitive revertants of this class have been isolated and found to possess reduced enzyme activity levels (Lewis and Wright 1979). The third class of drug resistant mutants contain a combination of the first two alterations described. These mutants appear to contain elevated levels of a ribonucleotide reductase enzyme which has a structural alteration making it less sensitive to drug inhibition (Kuzik and Wright 1980, Koropatnick and Wright 1980).

From the data presented thus far it would seem that the NC^{R}-30A cell line was a class two mutant (ie. it possessed an elevated level of normally sensitive ribonucleotide reductase activity). To examine this possibility, a series of kinetic studies were performed with the reductase activity in NC^{R}-30A and WTC125 cells in the presence of N-carbamoyloxyurea, hydroxyurea and guanazole.

Figure 53 shows the double reciprocal plots of velocity against CDP concentrations at several fixed levels of N-carbamoyloxyurea for the drug resistant and wild type cells. Clearly N-carbamoyloxyurea was an inhibitor of ribonucleotide reduction in both cell lines. The pattern of inhibition appeared to be a mixture of noncompetitive and uncompetitive inhibition and a replot of velocity intercepts against

Fig. 53. Double reciprocal plots of the variation in rate of CDP reduction with CDP concentration at several fixed levels of N-carbamoyloxyurea using permeabilized NC^R-30A (a) and WTC125 (b) cells. Assays were performed in the absence (O) or presence of 1 mM (□), 2 mM (Δ), 3 mM (●) and 4 mM (▲) N-carbamoyloxyurea. Activity expressed as nmoles dCDP formed/5 x 10^6 cells/hour. Insets: replots of velocity intercepts against N-carbamoyloxyurea concentrations. Results corrected for the presence of kinase and phosphatase activities in intact cells.


a
INTERCEPT
4
2
-2 0 2 4
DRUG, mM
dCDP, V⁻¹
20
15
10
5
0 40 80 120 160 200
CDP, mM⁻¹
b
INTERCEPT
4
2
-2 0 2 4
DRUG, mM
dCDP, V⁻¹
12
10
8
6
4
2
0 40 80 120 160 200
CDP, mM⁻¹

drug concentrations showed a K_i value of 2.0 mM drug (inset, fig. 53a) for NC^R-30A activity and 2.3 mM (inset, fig. 53b) for WTC125 activity. Figure 54 shows the double reciprocal plots of velocity against ADP concentrations at several fixed levels of N-carbamoyloxyurea. The reduction of ADP was inhibited in a non-competitive fashion in both NC^R-30A and WTC125 cells. Replots of velocity intercepts versus N-carbamoyloxyurea concentrations yielded K_i values of 2.3 mM drug for both cell lines. From these results it is apparent that the NC^R-30A cells contain a reductase which is as sensitive to N-carbamoyloxyurea as that of the WTC125 cells.

As the NC^R-30A cells were cross-resistant to hydroxyurea and guanazole, the inhibition of CDP and ADP reduction in these and the parental cells by the two drugs was also analyzed in double reciprocal plots of velocity against varying pyrimidine and purine concentrations. Figure 55 shows the double reciprocal plots of velocity against CDP concentration at several fixed levels of hydroxyurea for the NC^R-30A and WTC125 cells. In both cases hydroxyurea inhibited the reduction in a uncompetitive manner. A replot of velocity intercepts against drug concentrations exhibited a K_i value of 0.24 mM drug (inset, fig. 55a) for NC^R-30A activity and 0.20 mM (inset, fig. 55b) for WTC125 activity. Figure 56 shows double reciprocal plots of velocity against ADP at various constant levels of hydroxyurea. The pattern of inhibition was a mixture of noncompetitive and uncompetitive and a replot of velocity intercepts against drug concentrations showed K_i values of 0.17 mM (inset, fig. 56a) and 0.16 mM (inset, fig. 56b) hydroxyurea for NC^R-30A and WTC125 cells respectively. Guanazole was found to inhibit the reduction of CDP in an uncompetitive manner in both NC^R-30A and WTC125

Fig. 54. Double reciprocal plots of the variation in rate of ADP reduction with ADP concentration at several fixed levels of N-carbamoyloxyurea using permeabilized NC^R-30A (a) and WTC125 (b) cells. Assays were performed in the absence (O) or presence of 2.5 mM (□), 5 mM (Δ), 7.5 mM (▲) and 10 mM (●) N-carbamoyloxyurea. Activity expressed as nmoles dADP formed/5 x 10^6 cells/hour. Insets: replots of velocity intercepts against N-carbamoyloxyurea concentrations. Results corrected for presence of kinase and phosphatase activities in intact cells.

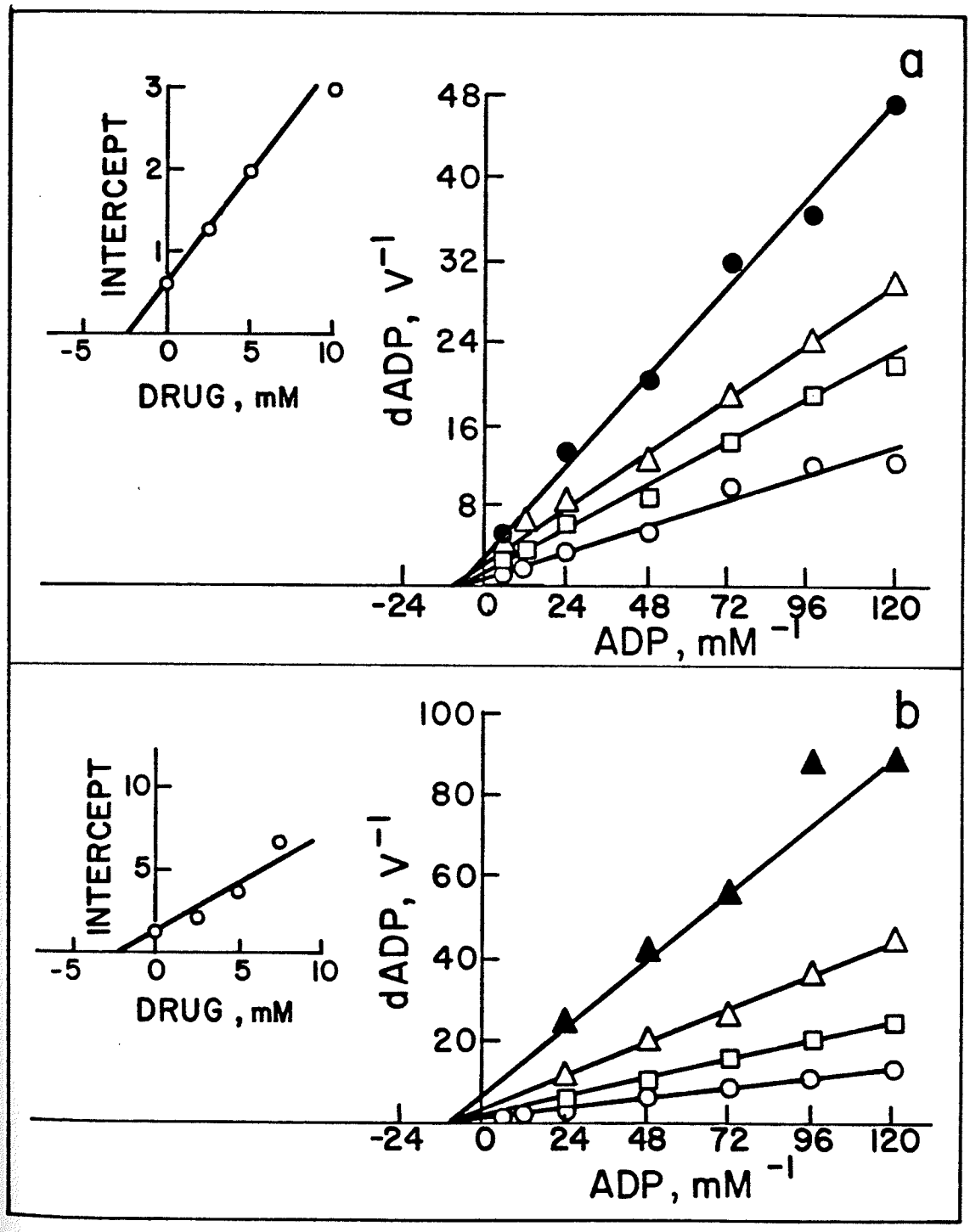

a
INTERCEPT
3
2
1
-5
0
5
10
DRUG, mM
dADP, V⁻¹
48
40
32
24
16
8
-24
0
24
48
72
96
120
ADP, mM⁻¹
b
INTERCEPT
10
5
-5
0
5
10
DRUG, mM
dADP, V⁻¹
100
80
60
40
20
-24
0
24
48
72
96
120
ADP, mM⁻¹

Fig. 55. Double reciprocal plots of the variation in rate of CDP reduction with CDP concentration at several fixed levels of hydroxyurea using permeabilized NC^R-30A (a) and WTC125 (b) cells. Assays were performed in the absence (O) or presence of 0.1 mM (□), 0.2 mM (Δ), 0.3 mM (○) and 0.4 mM (●) hydroxyurea. Activity expressed as nmoles of dCDP formed/5 x 10^6 cells/hour. Insets: replots of velocity intercepts against hydroxyurea concentration. Results corrected for presence of kinase and phosphatase activities in intact cells.

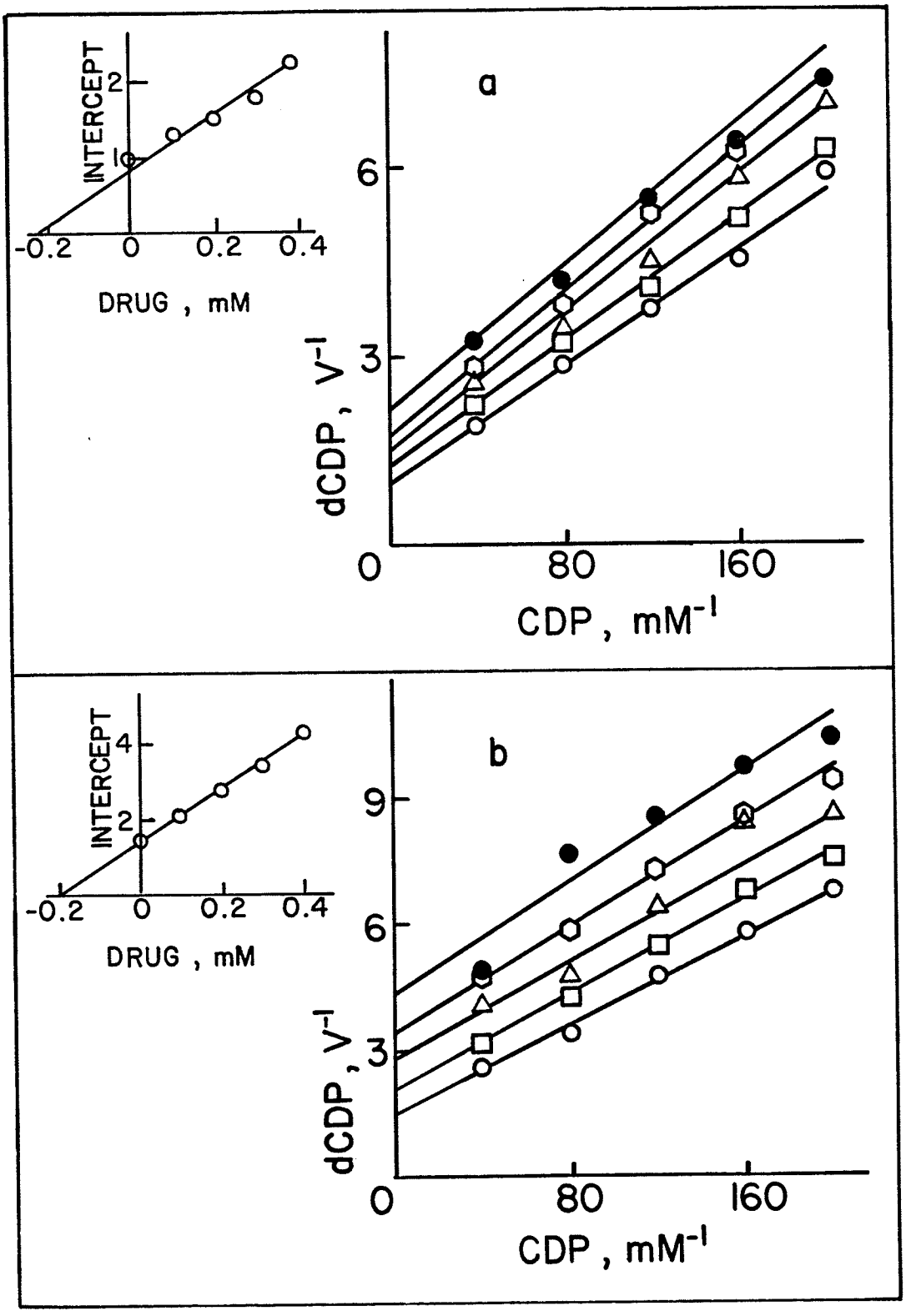
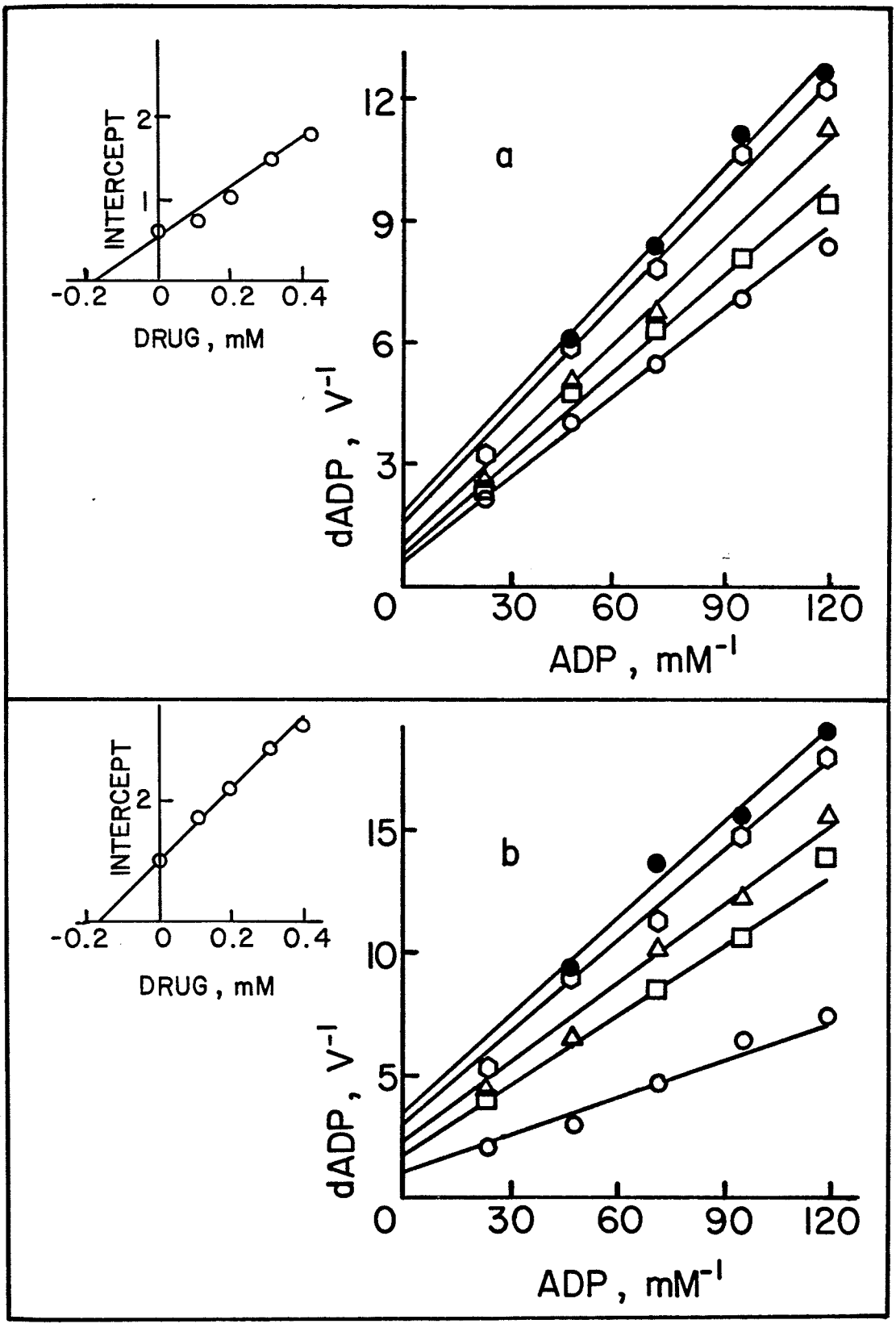

a
INTERCEPT
2
1
-0.2
0
0.2
0.4
DRUG , mM
6
3
0
80
160
dCDP, V⁻¹
CDP , mM⁻¹
b
INTERCEPT
4
2
-0.2
0
0.2
0.4
DRUG , mM
9
6
3
0
80
160
dCDP, V⁻¹
CDP , mM⁻¹

Fig. 56. Double reciprocal plots of the variation in rate of ADP reduction with ADP concentration at several fixed levels of hydroxyurea using permeabilized NC^R-30A (a) and WTC125 (b) cells. Assays were performed in the absence (O) or presence of 0.1 mM (□), 0.2 mM (Δ), 0.3 mM (⬡) and 0.4 mM (●) hydroxyurea. Activity expressed as nmoles dADP formed/5 x 10^6 cells/hour. Insets: replots of velocity intercepts against hydroxyurea concentrations. Results corrected for presence of kinase and phosphatase activities in intact cells.


INTERCEPT
2
1
-0.2
0
0.2
0.4
DRUG, mM
a
12
9
6
3
0
30
60
90
120
dADP, V⁻¹
ADP, mM⁻¹
INTERCEPT
2
-0.2
0
0.2
0.4
DRUG, mM
b
15
10
5
0
30
60
90
120
dADP, V⁻¹
ADP, mM⁻¹

cells (figure 57). Replots of velocity intercepts against drug concentrations indicated K_i values of 1.25 mM (inset, fig. 57a) and 1.15 mM (inset, fig. 57b) for the mutant and parental cell lines respectively. In regards to the reduction of ADP, the pattern of inhibition induced by guanazole seemed to be a mixture of non-competitive and uncompetitive for both cell lines (figure 58). The K_i values determined were 1.10 mM for NC^R-30A cells (inset, fig. 58a) and 1.15 mM for WTC125 cells (fig. 58b). As was the case with N-carbamoyloxyurea, no significant differences in the sensitivity to hydroxyurea and guanazole between the ribonucleotide reductase activity from NC^R-30A and WTC125 cells were detected. Thus it would appear that the NC^R-30A cell line is a true representative of the class II group of mutants in that it possesses a reductase activity with wild type sensitivity to drug inhibition but with enhanced intracellular levels of enzyme activity. In this particular case the CDP reductase activity has increased two-fold while that of ADP reductase has increased 1.8 fold.

In order to ensure the variation in substrate concentration or the presence of the various drugs did not adversely affect the reactions, the linearity of reduction with time was determined in the presence of 0.4 mM hydroxyurea, 2 mM guanazole and 4 mM N-carbamoyloxyurea. For both CDP and ADP reduction, these drugs had no adverse effects on the linearity of the reductions (data not shown). The presence of 0.005 to 0.3 mM CDP or 0.0084 to 1.74 mM ADP did not decrease the length of time the various assays remained linear with time (data not shown).

Fig. 57. Double reciprocal plots of the variation in rate of CDP reduction with CDP concentration at several fixed levels of guanazole using permeabilized NC^R-30A (a) and WTC125 (b) cells. Assays were performed in the absence (O) or presence of 0.5 mM (□), 1 mM (Δ), 1.5 mM (○) and 2 mM (●) guanazole. Activity expressed as nmoles dCDP formed/ 5 x 10^6 cells/hour. Insets: replots of velocity intercepts against guanazole concentrations. Results corrected for presence of kinase and phosphatase activities in intact cells.

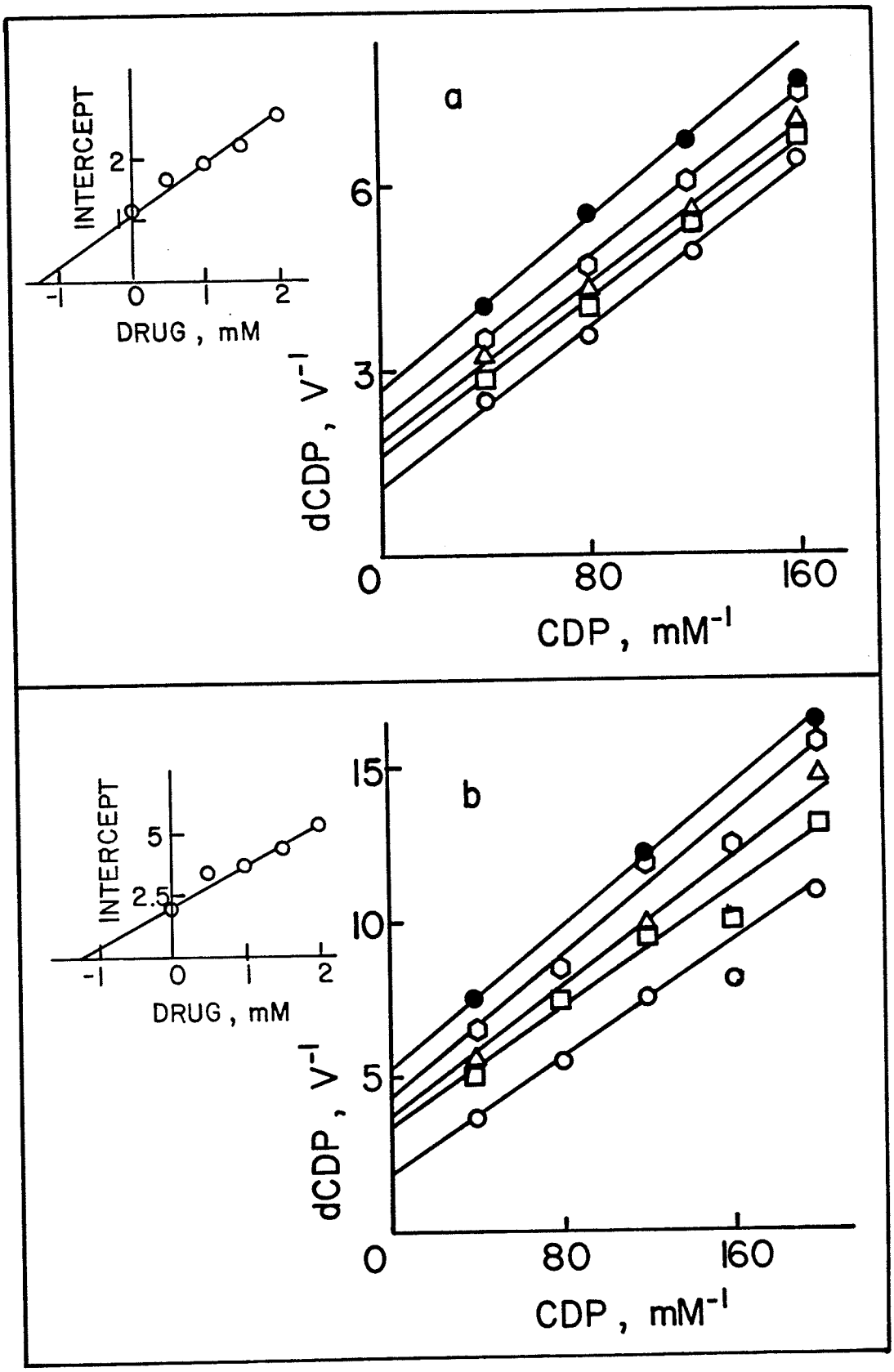
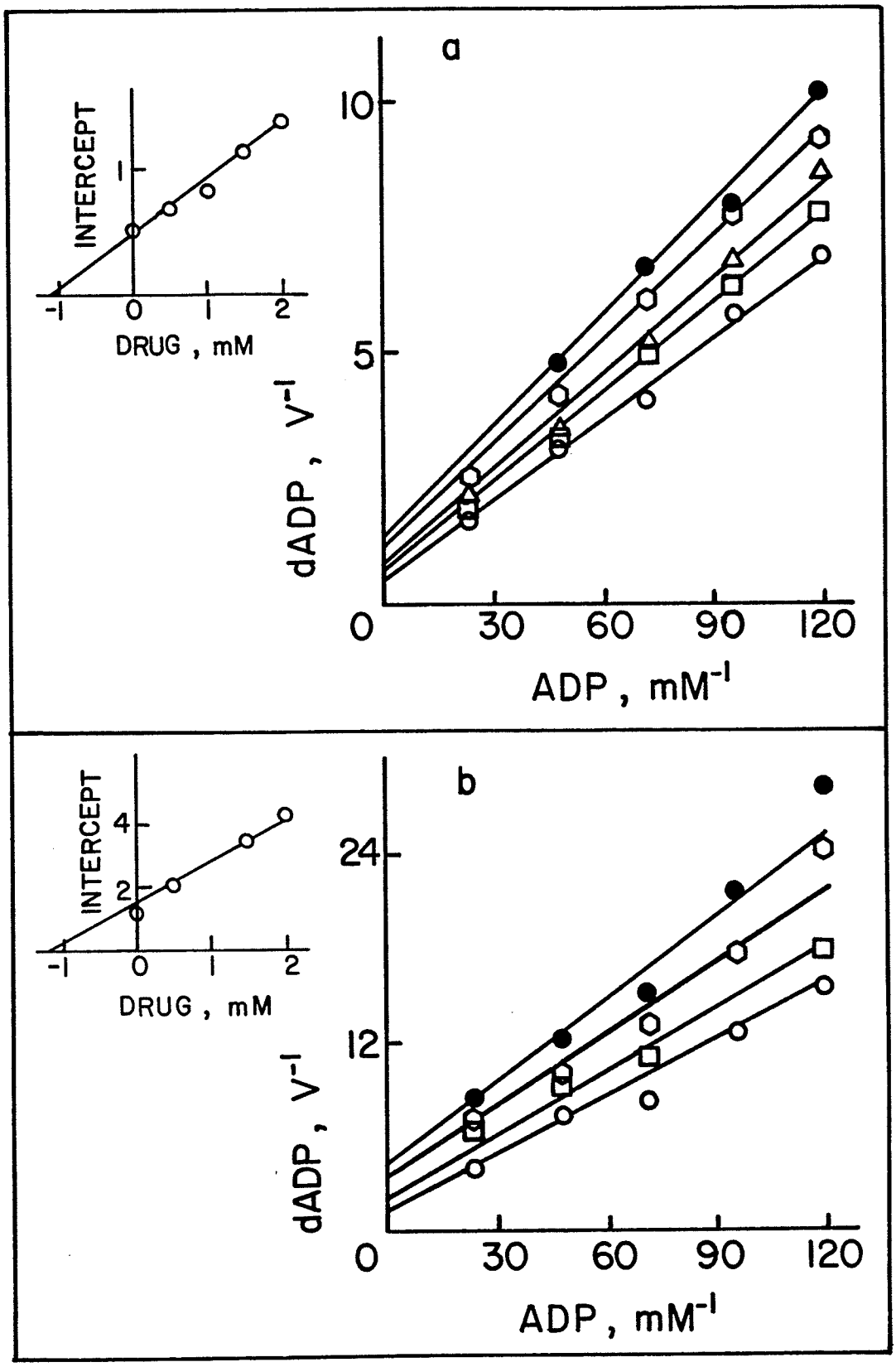

a
INTERCEPT
2
1
-1
0
1
2
DRUG , mM
6
3
0
80
160
dCDP , V⁻¹
CDP , mM⁻¹
b
INTERCEPT
5
2.5
-1
0
1
2
DRUG , mM
15
10
5
0
80
160
dCDP , V⁻¹
CDP , mM⁻¹

Fig. 58. Double reciprocal plots of the variation in rate of ADP reduction with ADP concentration at several fixed levels of guanazole using permeabilized NC^R-30A (a) and WTC125 (b) cells. Assays were performed in the absence (O) or presence of 0.5 mM (□), 1 mM (Δ), 1.5 mM (⬡) and 2 mM (●) guanazole. Activity expressed as nmoles dADP formed/ 5 x 10^6 cells/hour. Insets: replots of velocity intercepts against guanazole concentrations. Results corrected for presence of kinase and phosphatase activities in intact cells.


a
INTERCEPT
DRUG , mM
dADP , V⁻¹
ADP , mM⁻¹
b
INTERCEPT
DRUG , mM
dADP , V⁻¹
ADP , mM⁻¹

4.4 Altered mRNA Metabolism in NC^R-30A Cells

In order to elucidate the possible mechanism(s) by which NC^R-30A cells overproduce ribonucleotide reductase activity, the effect of cycloheximide and actinomycin D on the CDP and ADP reductase activities of the two cell lines was studied.

Cycloheximide inhibits protein synthesis in eucaryotes by blocking the formation of peptide bonds. It has been found that in L cells 1 µg of the drug per ml of growth medium will inhibit protein synthesis by 85% to 90% as judged by the incorporation of ^{14}C-lysine into acid precipitated material (Turner et al 1968). The same study also showed that cycloheximide had no direct effect on ribonucleotide reductase activity. When cycloheximide at a concentration of 1 µg/ml of growth medium was added to exponentially growing cultures of WTC125 and NC^R-30A cells there was an immediate cessation of growth in both cases (data not shown). To discover what effect the drug had on the reductase activity of the two cell lines, cycloheximide was added to exponentially growing cells and CDP and ADP reduction in permeabilized cells was determined at various times after the addition. In both cases the levels of activity fell and when the log of the activity levels was plotted against time of exposure to cycloheximide (figures 59 and 60) a linear relationship was found in each case. This indicated that the degradation of CDP and ADP reductase activity was a first order process in both cell lines. Within WTC125 cells CDP reductase was found to have a half-life of 1.7 hours while for ADP reductase the $t_{\frac{1}{2}}$ value was found to be 2.8 hours. For NC^R-30A cells the half-life values were found to be 1.7 and 3.3 hours for CDP and ADP reductase respectively. These results indicate that the enhanced levels of ribonucleotide

Fig. 59. Effect of cycloheximide on the levels of CDP reductase activity in WTC125 (O) and NC^R-30A (□) cells. Cycloheximide (1 μg/ml) was added to cells growing logarithmically on plastic culture plates at 37°C. After the indicated length of treatment, the cells were permeabilized and assayed using standard CDP reductase assay conditions.

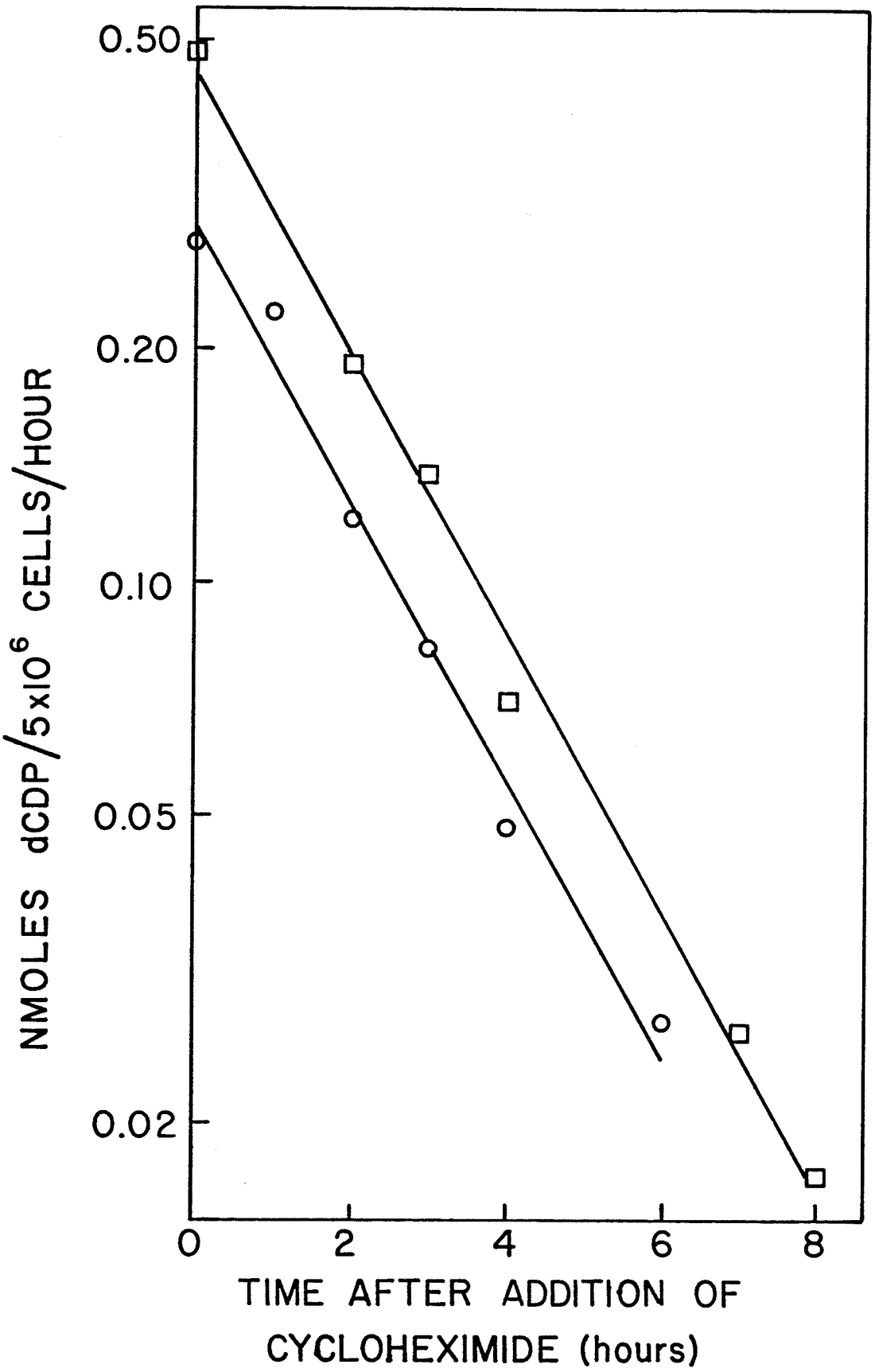
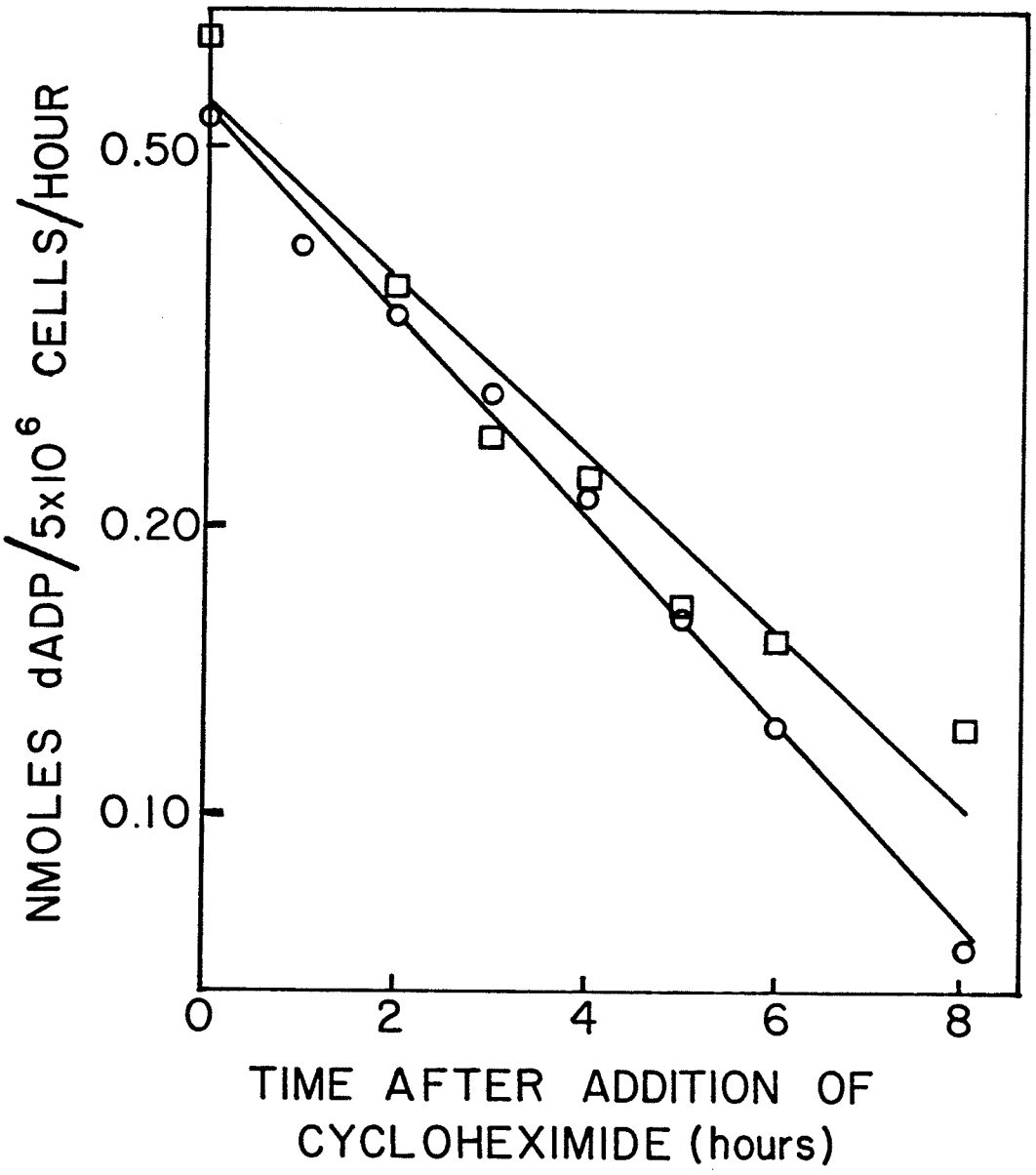

0.50
0.20
0.10
0.05
0.02
NMOLES dCDP/5x10^6 CELLS/HOUR
0
2
4
6
8
TIME AFTER ADDITION OF
CYCLOHEXIMIDE (hours)

Fig. 60. Effect of cycloheximide on the levels of ADP reductase activity in WTC1 25 (O) and NC^R-30A (□) cells. Cycloheximide (1 μg/ml) was added to cells growing logarithmically on plastic culture plates at 37°C. After the indicated length of treatment, the cells were permeabilized and assayed using standard ADP reductase assay conditions.


NMOLES dADP/5x10^6 CELLS/HOUR
0.50
0.20
0.10
0
2
4
6
8
TIME AFTER ADDITION OF
CYCLOHEXIMIDE (hours)

reductase in the NC^R-30A cells were not likely to be due to alterations in protein synthesizing or degrading mechanisms of the mutant. Moreover the half-lives determined for CDP reductase were very similar to that reported for the activity in L cells (Turner et al 1968).

Actinomycin D blocks the transcription of DNA to RNA by forming a non-covalent complex with DNA which impairs its template function. The drug intercalates between two G-C base pairs to inhibit chain elongation. In order to determine whether the mutant possessed an alteration in the processing of RNA involved in the production of ribonucleotide reductase, actinomycin D was added to exponentially growing cells and the activities of CDP and ADP reductase were followed (figures 61 and 62). The presence of either 1 μg or 10 μg of drug per ml of growth medium induced the same pattern and the results shown are the average of trials conducted at both concentrations. In WTC125 cells the decrease in both CDP and ADP reductase activity induced by actinomycin D was a first order process with a half-life of 4.6 to 4.8 hours. However the situation in NC^R-30A cells was much more complex. The addition of actinomycin D to NC^R-30A cells led to a rapid decrease in both CDP and ADP reductase levels. This decrease persisted for only one hour and there followed an increase in enzyme activities which lasted for 2 hours. Another decrease followed which persisted for 1 hour for CDP reduction and 2 hours for ADP reduction. The activity then increased again for a duration of 1 hour in both cases and then decreased to the end of the period observed. It would thus seem that the RNA metabolism of ribonucleotide reductase from the NC^R-30A cells has in some way been altered and that this alteration affects the reduction of CDP and ADP in a virtually identical manner. It should be

Fig. 61. Effect of actinomycin D on the levels of CDP reductase activity in WTC125 (O) and NC^R-30A (□) cells. Actinomycin D (1 or 10 μg/ml) was added to cells growing logarithmically on plastic culture plates at 37°C. After the indicated length of treatment, the cells were permeabilized and assayed using standard CDP reductase assay conditions.

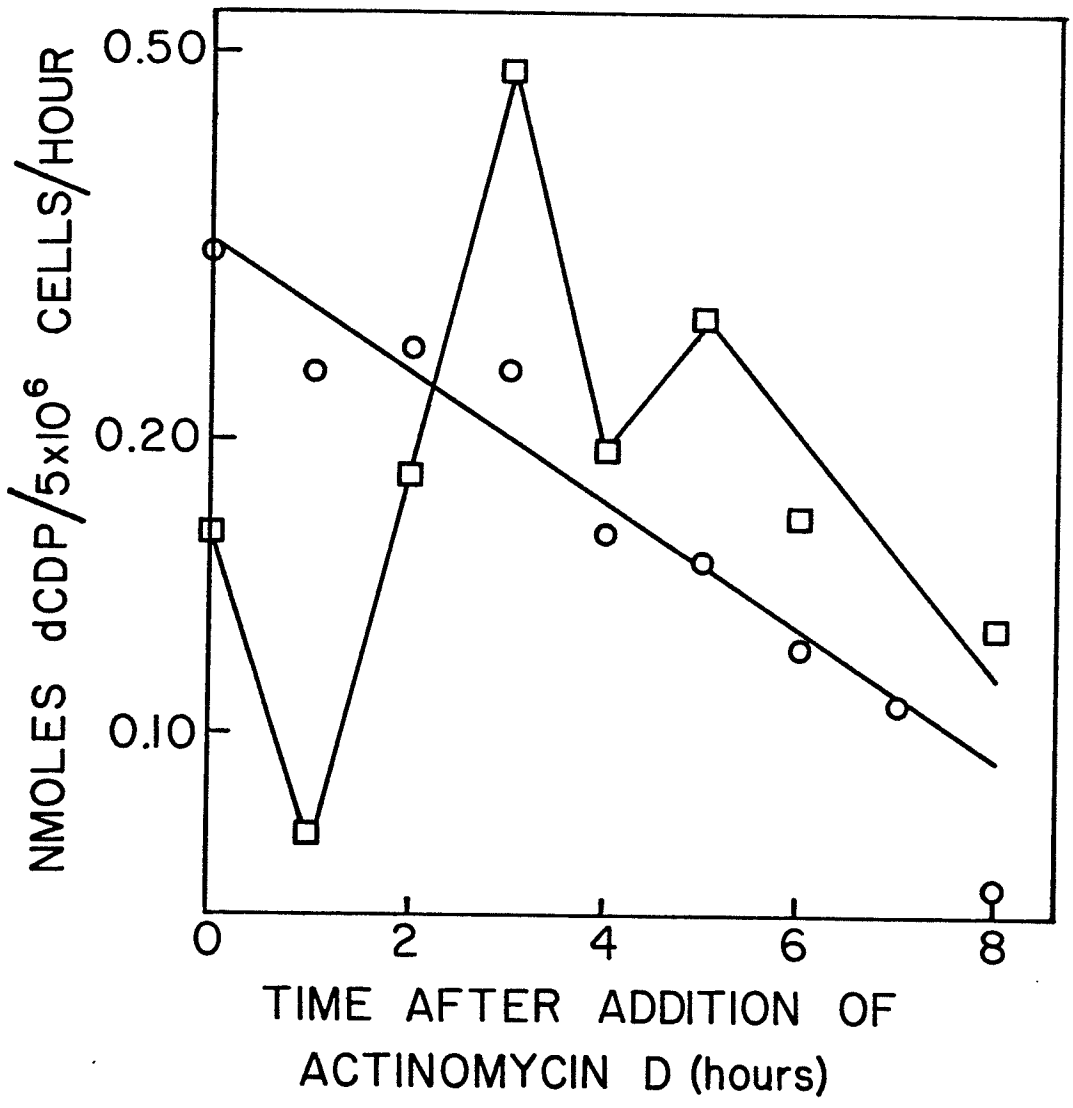
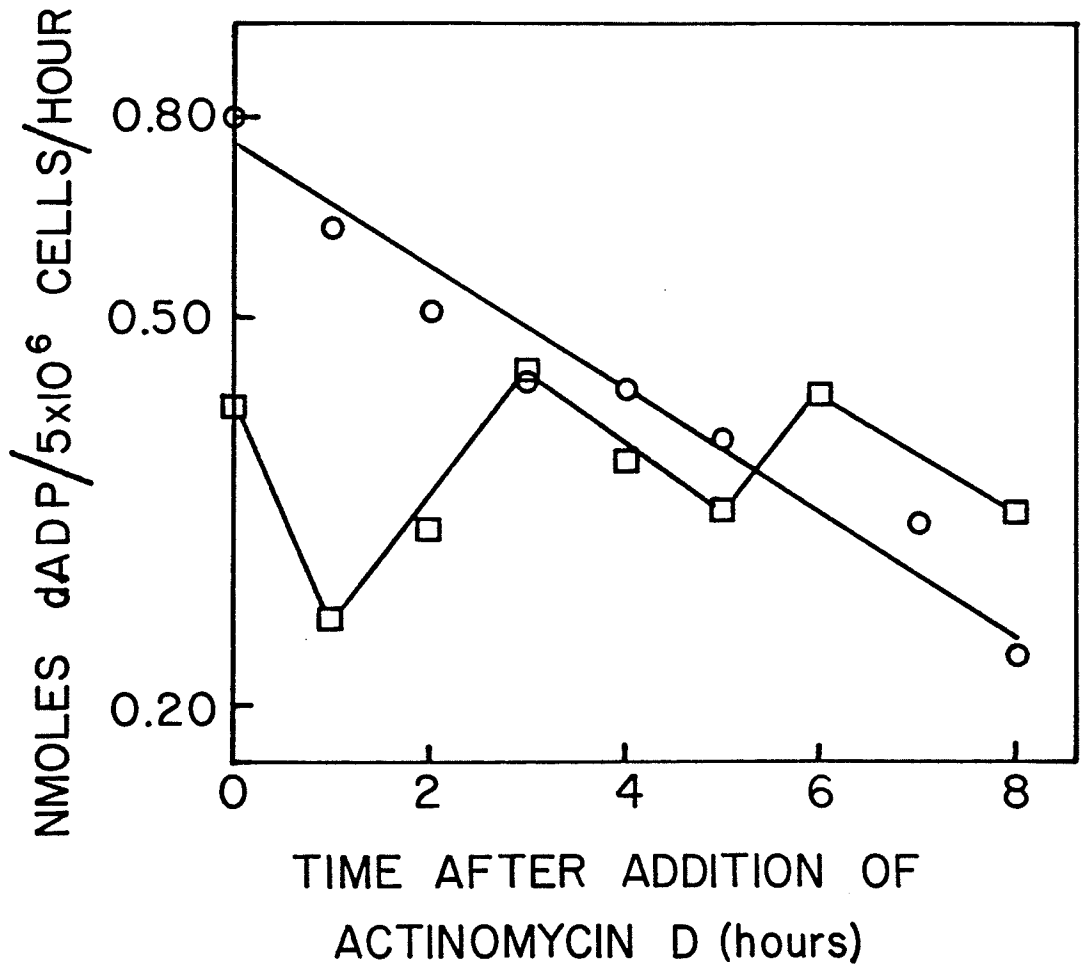

0.50
0.20
0.10
NMOLES dCDP/5x10⁶ CELLS/HOUR
0
2
4
6
8
TIME AFTER ADDITION OF
ACTINOMYCIN D (hours)

Fig. 62. Effect of actinomycin D on the levels of ADP reductase activity in WTC125 (O) and NC^R-30A (□) cells. Actinomycin D (1 or 10 µg/ml) was added to cells growing logarithmically on plastic culture plates at 37°C. After the indicated length of treatment, the cells were permeabilized and assayed using standard ADP reductase assay conditions.


NMOLES dADP/5x10⁶ CELLS/HOUR
0.80
0.50
0.20
0
2
4
6
8
TIME AFTER ADDITION OF
ACTINOMYCIN D (hours)

noted that the presence of either 1 or 10 μg of actinomycin D per ml of growth medium caused an immediate halt to the growth of the CHO cells used in this study (data not shown).

In order to confirm that the changes in CDP and ADP reductase levels that occurred upon addition of cycloheximide and actinomycin D were due to the presence of the drugs and not a property of the culture conditions, the exponentially growing WTC125 and NC^R-30A cells were treated in a manner identical to that used in the drug studies except that the medium added contained no cycloheximide or actinomycin D. Under these conditions it was found that the levels of both CDP and ADP reductase increased to some extent after the addition of fresh medium lacking drug. Therefore it can be concluded that the changes in activity observed were due to the presence of cycloheximide or actinomycin D.

5. CHARACTERIZATION OF A SERIES OF N-CARBAMOYLOXYUREA-RESISTANT CELL LINES

While studies with the WTC125 and NC^R-30A cell lines showed that N-carbamoyloxyurea was an inhibitor of ribonucleotide reductase there were indications that the drug may have a second site of action in mammalian cells. The mutant cell line was found to exhibit a 1.6 fold increase in cellular resistance to hydroxyurea over that of the parental cell line. A similar increase was found for guanazole. The increase in cellular resistance to these drugs could be explained by the two-fold increase in CDP reduction and 1.8 fold increase in ADP reduction seen in NC^R-30A cells over that of WTC125 cells. However, the increase in reductase activity could not totally account for the 2.6 fold increase in cellular resistance observed for N-carbamoyloxyurea.

A second piece of evidence for the existance of a second site of action for N-carbamoyloxyurea involves the ratio of the K_i and D_{10} values for WTC125 cells. For guanazole and hydroxyurea the K_i/D_{10} ratio is approximately one. This indicates that inhibition of 50% of the enzyme activity reduced plating efficiency by 90%. For N-carbamoyloxyurea the K_i/D_{10} ratio was found to be 7.2. Thus at levels of N-carbamoyloxyurea which reduce ribonucleotide reductase by one-half, the wild type cells would fail to grow at all. Thus it appeared that N-carbamoyloxyurea does possess a second site of action. Moreover this second site was likely to be more sensitive to the drug than the ribonucleotide reductase site. In order to gain a greater understanding of the mode of action of N-carbamoyloxyurea, a series of CHO cell lines capable of proliferating in the presence of normally cytotoxic levels of N-carbamoyloxyurea were sequentially selected with increasing concentrations of drug as described in Methods and Materials.

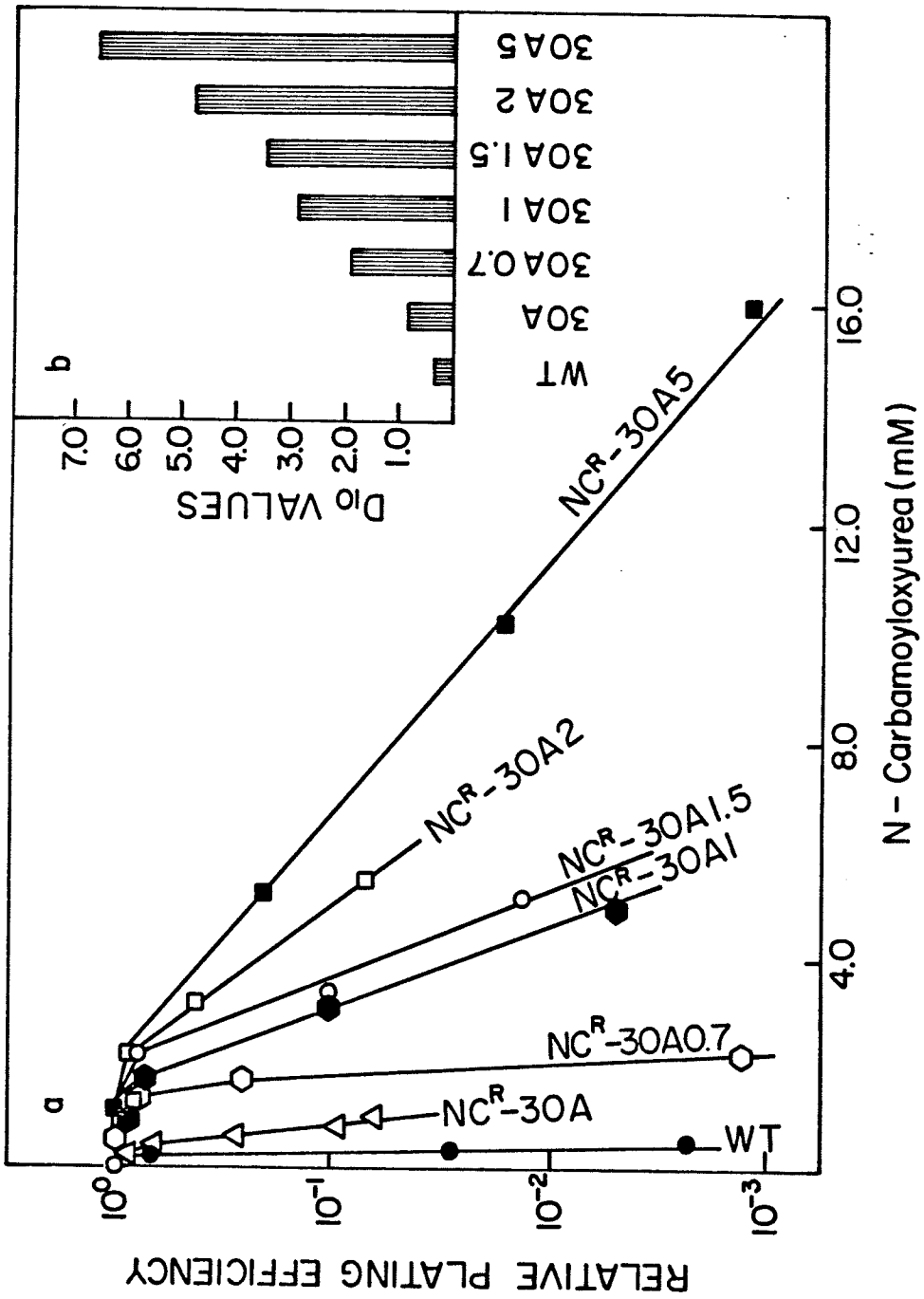
5.1 Cellular Resistance

The effect of various concentrations of N-carbamoyloxyurea on the relative plating efficiencies of wild-type and drug-resistant lines is shown in figure 63a. Clearly all cell lines were able to form colonies at a higher efficiency than wild type cells in the presence of the selective agent. When the D_{10} values of the various lines were examined it was clear that a gradual increase in resistance to N-carbamoyloxyurea existed within the series from NC^R-30A to NC^R-30A5 (figure 63b).

It has been shown above that cells selected for resistance to N-carbamoyloxyurea are cross resistant to the antitumor agents hydroxyurea and guanazole (section 4.1). Moreover, cells selected for resistance to

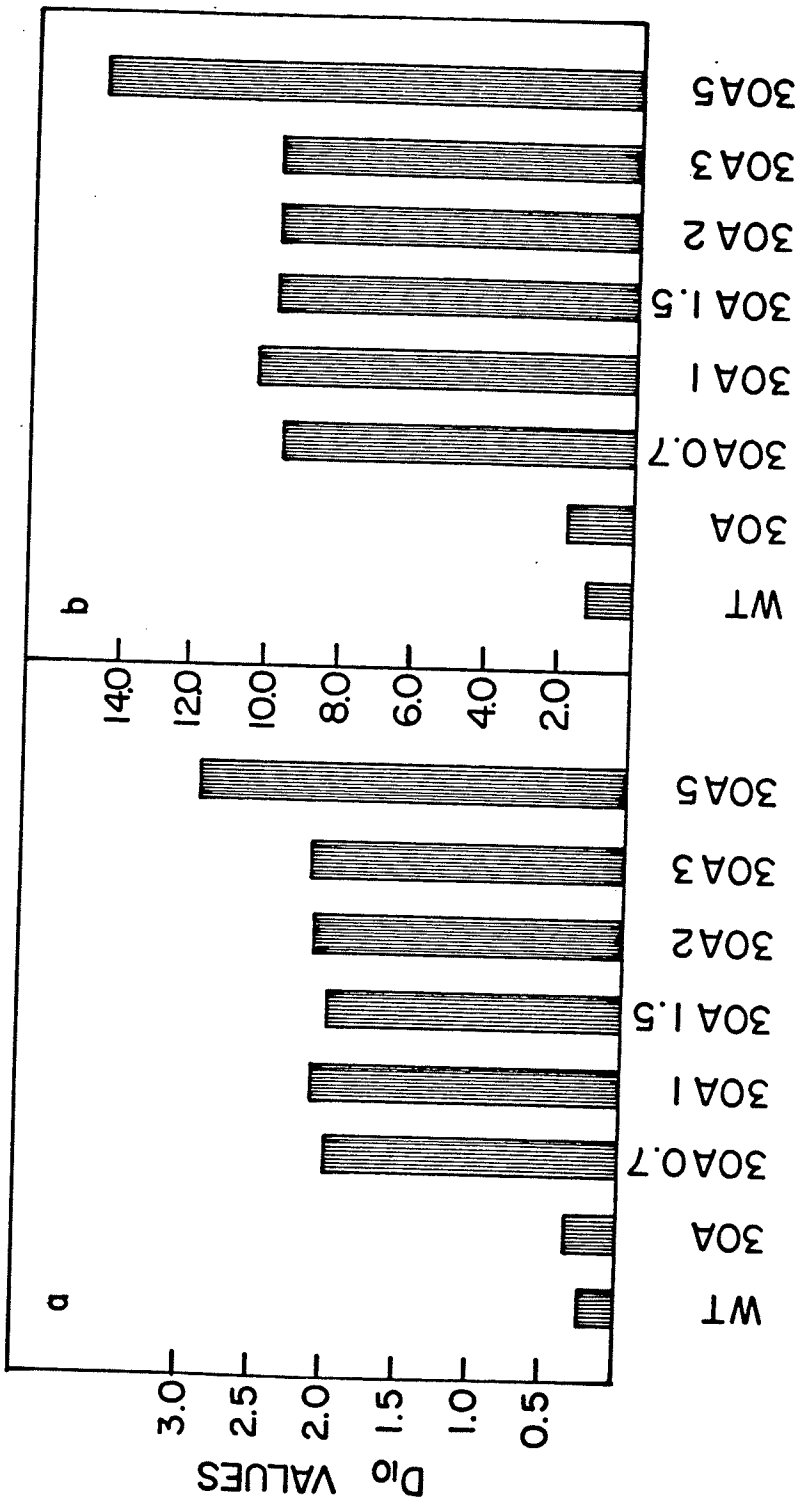
Fig. 63a. Relative plating efficiencies of the series of cell lines selected for resistance to the cytotoxic effects of N-carbamoyloxyurea in increasing concentrations of N-carbamoyloxyurea. From 250 to 100,000 cells per 100 mm plastic tissue culture plate were incubated at 37°C for 7 to 14 days in standard growth medium containing varying concentrations of N-carbamoyloxyurea.

Fig. 63b. The D_{10} values for the series of cell lines selected for resistance to the cytotoxic effects of N-carbamoyloxyurea determined in the presence of N-carbamoyloxyurea. Values determined from releative plating efficiency experiments.


a
b
RELATIVE PLATING EFFICIENCY
10^{0}
10^{-1}
10^{-2}
10^{-3}
WT
NC^{R}-30A
NC^{R}-30A0.7
NC^{R}-30A1
NC^{R}-30A1.5
NC^{R}-30A2
NC^{R}-30A5
4.0
8.0
12.0
16.0
N - Carbamoyloxyurea (mM)
D_{10} VALUES
1.0
2.0
3.0
4.0
5.0
6.0
7.0
WT
30A
30A0.7
30A1
30A1.5
30A2
30A5

hydroxyurea and guanazole are cross-resistant to N-carbamoyloxyurea (Wright and Lewis 1974, Wright et al 1981). It is also known that the primary and probably only intracellular target for hydroxyurea and guanazole is ribonucleotide reductase (Wright et al 1981). If a second site of N-carbamoyloxyurea action exists within mammalian cells it was thought likely this second target could be modified in the N-carbamoyloxyurea-resistant cell lines. If this were correct, one might expect to find differences in the increase in cellular resistance to hydroxyurea and guanazole within the series of drug resistant lines, as compared with the results obtained with the selective agent, N-carbamoyloxyurea. Indeed when the D_{10} values from plating efficiency experiments with hydroxyurea (figure 64a) and guanazole (figure 64b) were examined, the smooth progressive increase in drug resistance observed with N-carbamoyloxyurea was not found. Instead, there was an increase in cellular resistance towards both hydroxyurea and guanazole from wild type to NC^R-30A cells and then a further increase from NC^R-30A to NC^R-30A0.7 cells. However, the next four lines (NC^R-30A1, NC^R-30A1.5, NC^R-30A2 and NC^R-30A3) showed approximately the same drug resistance properties exhibited by NC^R-30A0.7 cells. The final cell line in the series, NC^R-30A5, showed another significant increase in drug resistance when compared to the five middle cell lines. These results, which indicated that for the five intermediately resistant cell lines N-carbamoyloxyurea resistance increased while hydroxyurea and guanazole resistance remained unchanged, suggested that resistance to N-carbamoyloxyurea may arise via an alteration in a minimum of two distinct sites of action, one of which is also affected by hydroxyurea and guanazole.

Fig. 64. The D_{10} values for series of cell lines selected for resistance to the cytotoxic effects of N-carbamoyloxyurea determined in the presence of hydroxyurea (a) and guanazole (b). Values determined from relative plating efficiency experiments.


D_{10} VALUES
3.0
2.5
2.0
1.5
1.0
0.5
a
WT
30A
30A0.7
30A1
30A1.5
30A2
30A3
30A5
14.0
12.0
10.0
8.0
6.0
4.0
2.0
b
WT
30A
30A0.7
30A1
30A1.5
30A2
30A3
30A5

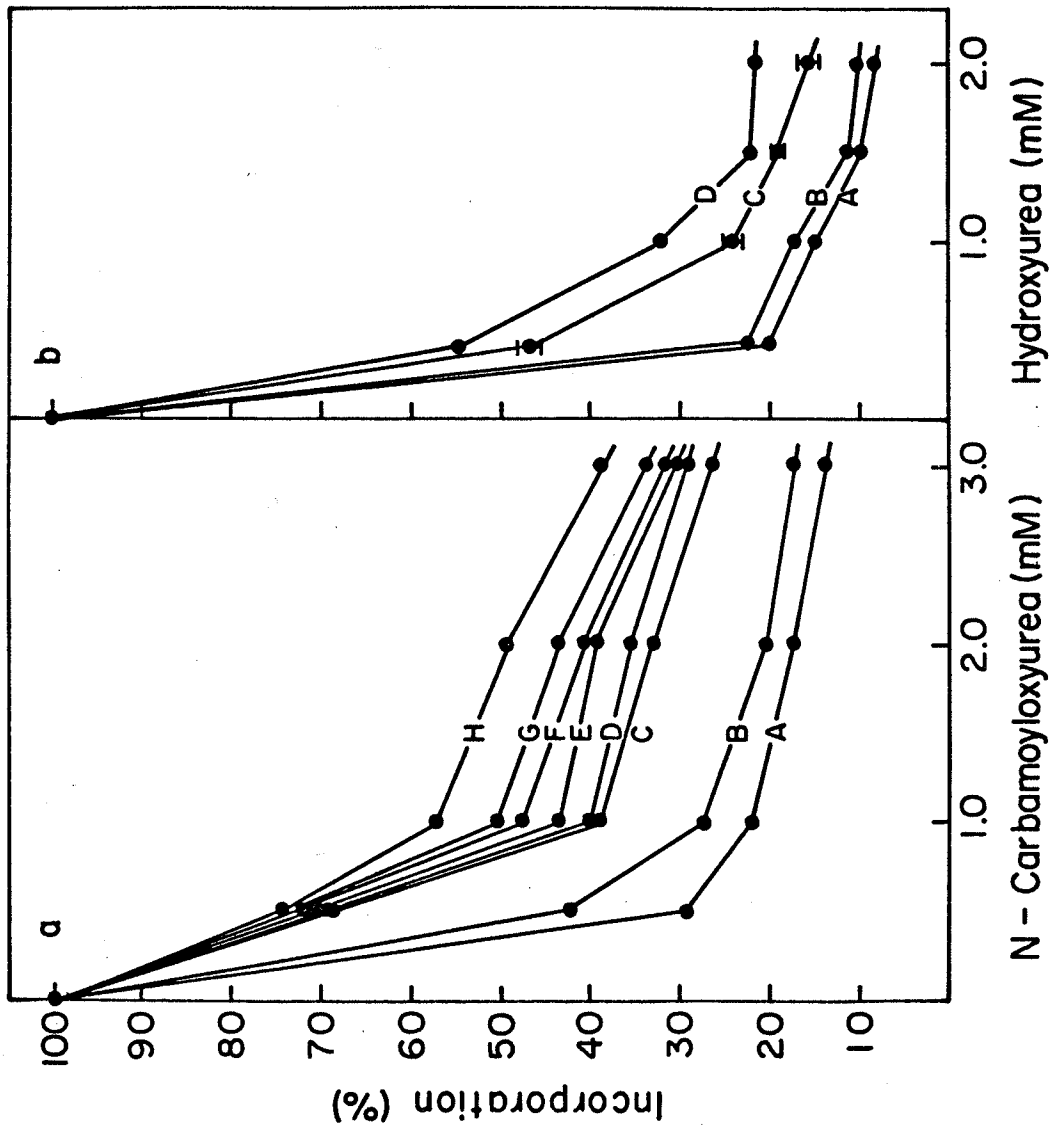
5.2 DNA Synthesis

Plating efficiency results are obtained from long term experiments in which cells are incubated in the presence of drug for many days. As N-carbamoyloxyurea and hydroxyurea are effective inhibitors of DNA synthesis (Young et al 1967, figure 49), it was possible to investigate cellular resistance by examining incorporation of ^{3}H-thymidine into DNA soon after addition of the drugs to the various cultures. The resistance properties of the various cultures as determined by ^{3}H-thymidine incorporation experiments are shown in figure 65. The results confirm the previous finding that resistance to N-carbamoyloxyurea increased progressively through the entire series of cell lines whereas resistance to hydroxyurea increased significantly at only three points: wild type to NC^{R}-30A, NC^{R}-30A to NC^{R}-30A0.7 and NC^{R}-30A3 to NC^{R}-30A5. These observations supported the view that at least two sites of action exist for N-carbamoyloxyurea, one of which is shared by hydroxyurea.

5.3 Effect of N-carbamoyloxyurea on Macromolecular Synthesis

In an effort to elucidate the location of the second site(s) of N-carbamoyloxyurea action in CHO cells, the effect of the drug on protein and RNA as well as DNA synthesis was studied. N-carbamoyloxyurea, like hydroxyurea (Young et al 1967), had very little effect upon protein or RNA synthesis during short term (15 minute) precursor incorporation studies. For WTC125 cells it was found that 0.4 mM N-carbamoyloxyurea (ie. the concentration used to select NC^{R}-30A cells) inhibited DNA synthesis by 62% but that protein and RNA synthesis exhibited 102% and 100% of control values for the incorporation of ^{3}H-leucine and ^{3}H-uridine respectively (data not shown).

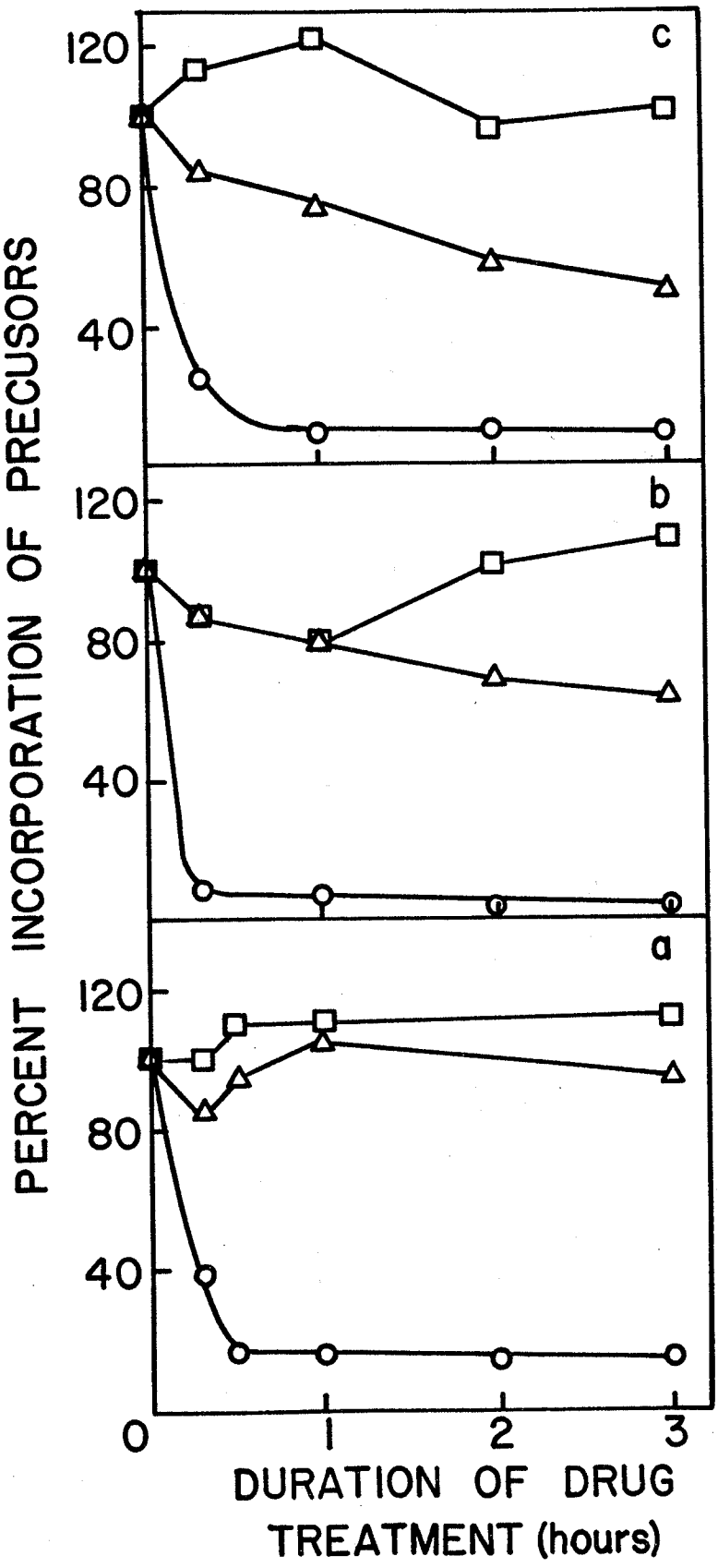
Fig. 65. Effect of N-carbamoyloxyurea and hydroxyurea on DNA synthesis in the series of cell lines selected for resistance to cytotoxic effects of N-carbamoyloxyurea. Percent of ^{3}H-thymidine incorporated into acid precipitable material during a 15 minute treatment with N-carbamoyloxyurea (a) or hydroxyurea (b) as compared to untreated control cultures. The cells in (a) were A,WTC125; B, NC^{R}-30A; C, NC^{R}-30A0.7; D, NC^{R}-30A1; E, NC^{R}-30A1.5; F, NC^{R}-30A2; G, NC^{R}-30A3; H, NC^{R}-30A5. The cells in (b) were A, WTC125; B, NC^{R}-30A; C, NC^{R}-30A0.7, NC^{R}-30A1, NC^{R}-30A1.5, NC^{R}-30A2, NC^{R}-30A3; D, NC^{R}-30A5. The bars indicate the spread in data points for the five intermediately resistant lines.


Incorporation (%)
100
90
80
70
60
50
40
30
20
10
a
b
H
G
F
E
D
C
B
A
1.0
2.0
3.0
N - Carbamoyloxyurea (mM)
Hydroxyurea (mM)

The possibility that longer drug treatment times and higher N-carbamoyloxyurea concentrations would effect precursor incorporation into protein or RNA, as well as DNA was investigated in an attempt to obtain more information on a possible second target for N-carbamoyloxyurea. Treatment of WTC125 cells with 0.4 mM N-carbamoyloxyurea for up to three hours had no significant effect on RNA or protein synthesis (figure 66a). However incubation with 8.7 mM N-carbamoyloxyurea (figure 66b) did cause a decrease in protein synthesis of 36% while RNA synthesis remained largely unaffected. Protein synthesis, however, does not appear to be the second target for N-carbamoyloxyurea inhibition. When the effect of 8.7 mM N-carbamoyloxyurea on protein synthesis in a drug resistant cell line, NC^{R}-30A5, was tested (figure 66c) it was found that the inhibition pattern was virtually identical to that observed with the wild type cells. These experiments seemed to rule out the possibility of the second site of N-carbamoyloxyurea action involving protein or RNA synthesis. Furthermore, they seemed to indicate that the second site of action like the first existed in the pathway of DNA synthesis.

As the K_i values for N-carbamoyloxyurea (2.3 mM) and hydroxyurea (0.2 mM) inhibition of ribonucleotide reductase from WTC125 cells have been determined (section 4.3) it was possible to test this idea further. Incorporation of ^{3}H-thymidine into DNA was measured in the presence of drug concentrations (K_i values) which would inhibit reductase activity to the same extent. It was found that 2.3 mM N-carbamoyloxyurea consistently reduced the incorporation of ^{3}H-thymidine to a greater extent than 0.2 mM hydroxyurea. For example after a 15 minute treatment with N-carbamoyloxyurea and hydroxyurea

Fig. 66. Effect of the duration of N-carbamoyloxyurea treatment on incorporation of ^{3}H-thymidine into DNA (O), ^{3}H-uridine into RNA (□) and ^{3}H-leucine into protein (△) for WTC125 cells in 0.4 mM N-carbamoyloxyurea (a), WTC125 cells in 8.7 mM N-carbamoyloxyurea (b) and NC^{R}-30A5 cells in 8.7 mM N-carbamoyloxyurea (c).

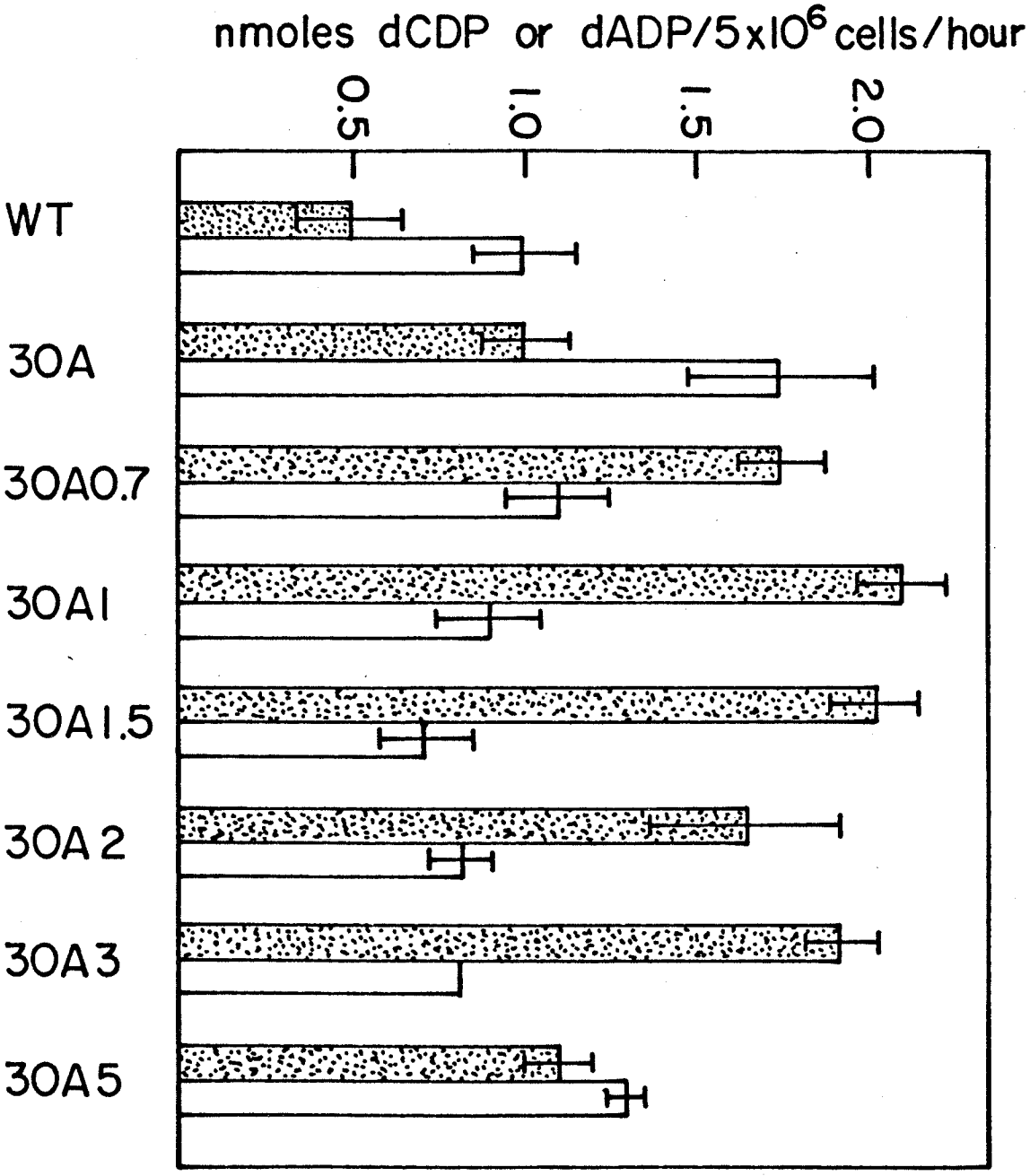

c
b
a
120
80
40
0
1
2
3
PERCENT INCORPORATION OF PRECUSORS
DURATION OF DRUG TREATMENT (hours)

respectively, 12.0 ± 0.5% and 30.7 ± 2.6% DNA synthesis remained as compared to untreated control cultures. These results which show that N-carbamoyloxyurea was more inhibitory than hydroxyurea, at concentrations of the two drugs that have the same effect on the reductase, support the proposal that the second site involves DNA synthesis and is distinct from ribonucleotide reductase.

5.4 Ribonucleotide Reductase Activity in Resistant and Wild Type Cells

Because the N-carbamoyloxyurea-resistant cell lines also showed an increase in resistance to hydroxyurea and guanazole, two drugs whose sole site of action appears to be ribonucleotide reductase, this activity was examined in the various lines. As already discussed, the NC^R-30A cell line possessed elevated levels of drug sensitive reductase activity and thus resembled class 2 hydroxyurea-resistant cell lines (Wright et al 1980, Wright et al 1981, section 4.3). The intracellular levels of ribonucleotide reductase activity in the series of cell lines selected from NC^R-30A were examined (figure 67). Since the enzyme reduces both pyrimidine and purine nucleotides (Wright et al 1981), one pyrimidine (CDP) and one purine (ADP) substrate were used in the investigation. All drug resistant cell lines showed an increase in total intracellular ribonucleotide reductase activity (the sum of CDP and ADP reductions) when compared to wild type cells. Interestingly, the ratio of CDP to ADP reductase activities in the various cell lines was not the same. WTC125, NC^R-30A and NC^R-30A5 cells contained higher intracellular levels of purine compared to pyrimidine reductase activity, while the five remaining cell lines showed the opposite pattern. Drug resistance in all the variant cell lines was accompanied by significant increases in CDP reductase

Fig. 67. Levels of CDP (■) and ADP (□) reductase activity in permeabilized wild-type and N-carbamoyloxyurea-resistant cell lines. Standard reductase assay conditions were used except for the presence of 0.3 mM CDP and 1.75 mM ADP. Variation in data for multiple determinations is shown by bars.


nmoles dCDP or dADP/5x10^6 cells/hour
0.5
1.0
1.5
2.0
WT
30A
30A0.7
30A1
30A1.5
30A2
30A3
30A5

activity, which ranged from approximately 2-fold above wild type levels, with NC^R-30A and NC^R-30A5 cells, to an average of 3.8 $\pm$ 0.4 fold above wild type for the five intermediately resistant cell lines. This is in contrast to the results obtained for ADP reductase activity, which showed no obvious pattern, and ranged from an increase in activity (NC^R-30A and NC^R-30A5) to approximately wild type or slightly lower levels in the five intermediate cell lines. These results, which suggest that an elevation in CDP reductase levels is more important for drug resistance than an increase in ADP reductase activity, are in keeping with the view that dCTP pools may play a special role in the regulation of mammalian DNA synthesis (Nicander and Reichard 1981, Davidson and Kaufman 1979). The uncoupling between changes in pyrimidine and purine reductase activities for the N-carbamoyloxyurea-resistant cell lines is similar to that observed with hydroxyurea-resistant rodent lines (Koropatnick and Wright 1980, Wright et al 1981).

Cells resistant to drugs which specifically inhibit ribonucleotide reductase may contain a structurally altered enzyme which is less sensitive to drug inhibition (Wright et al 1980, Wright et al 1981). The possibility that the N-carbamoyloxyurea-resistant cell lines possessed enzyme activities less sensitive to the drug was investigated. The effect of 0.18 mM hydroxyurea, 1.2 mM guanazole and 2 mM N-carbamoyloxyurea on the reductase activity of the various cell lines was studied. As expected from previous results (section 4.3) these concentrations of drug reduced activity by approximately one-half in WTC125 and NC^R-30A cells. However, the decreases in activity caused by these concentrations of drugs in the more resistant cell lines were

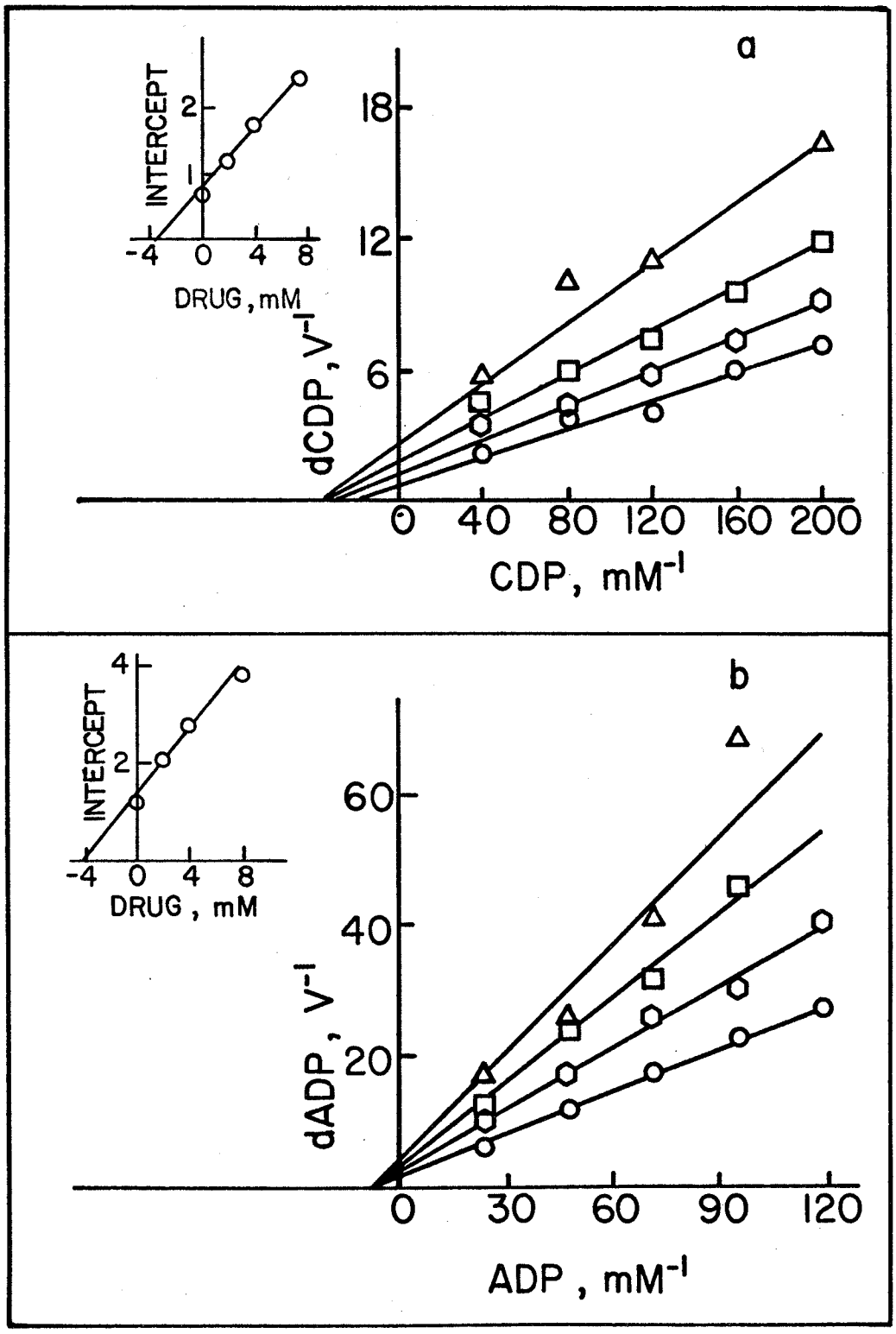
less dramatic. For the reduction of both CDP and ADP, the cell lines NC^R-30A0.7 through NC^R-30A5.0 were found to retain 80% to 85% of their reductase activity in the presence of the drugs used (data not shown). From the enzyme level and drug resistance studies it would seem that changes in ribonucleotide reductase activity occurred from WTC125 to NC^R-30A, from NC^R-30A to NC^R-30A0.7 and from NC^R-30A3 to NC^R-30A5. In comparison to NC^R-30A0.7 cells, the CDP and ADP reductase activities of the cell lines NC^R-30A1 through NC^R-30A3 apparently remain unaltered. These results resemble the earlier cellular resistance (figure 64) and DNA synthesis studies (figure 65) which suggested that reductase alterations occurred at these three steps in the selection process.

The first step, between wild type and NC^R-30A, has been studied in detail (section 4.3) and showed that while NC^R-30A cells contained an approximate 2-fold increase in enzyme activity (figure 52), ribonucleotide reductase exhibited a wild type sensitivity to drug inhibition. Except for an elevation in enzyme level, this cell line contained a reductase with normal catalytic properties. Enzyme activities in NC^R30A2 cells, taken as being respresentative of the intermediately resistant lines, and NC^R-30A5 cells, the most resistant population in the series, were investigated further.

Figure 68 shows double reciprocal plots of velocity against CDP and ADP concentration at various fixed levels of N-carbamoyloxyurea with the enzyme in NC^R-30A2 cells. N-carbamoyloxyurea inhibited both pyrimidine and purine reduction in an apparently noncompetitive fashion and a replot of velocity intercepts against drug concentrations (insets, figures 68a and 68b) gave a K_i value of 3.4 mM for CDP reduction and 4.0 mM for ADP reduction. The data indicated maximum velocities of

Fig. 68a. Double reciprocal plot of the variation in rate of CDP reduction with CDP concentration at several fixed levels of N-carbamoyloxyurea using permeabilized NC^R-30A2 cells. Assays were performed in the absence (O) or presence of 2 mM (⬡), 4 mM (□) and 8 mM (Δ) N-carbamoyloxyurea. Activity expressed as nmoles dCDP formed/ 5 x 10^6 cells/hour. Inset: replot of velocity intercepts against N-carbamoyloxyurea concentrations. Results corrected for presence of kinase and phosphatase activities in intact cells.

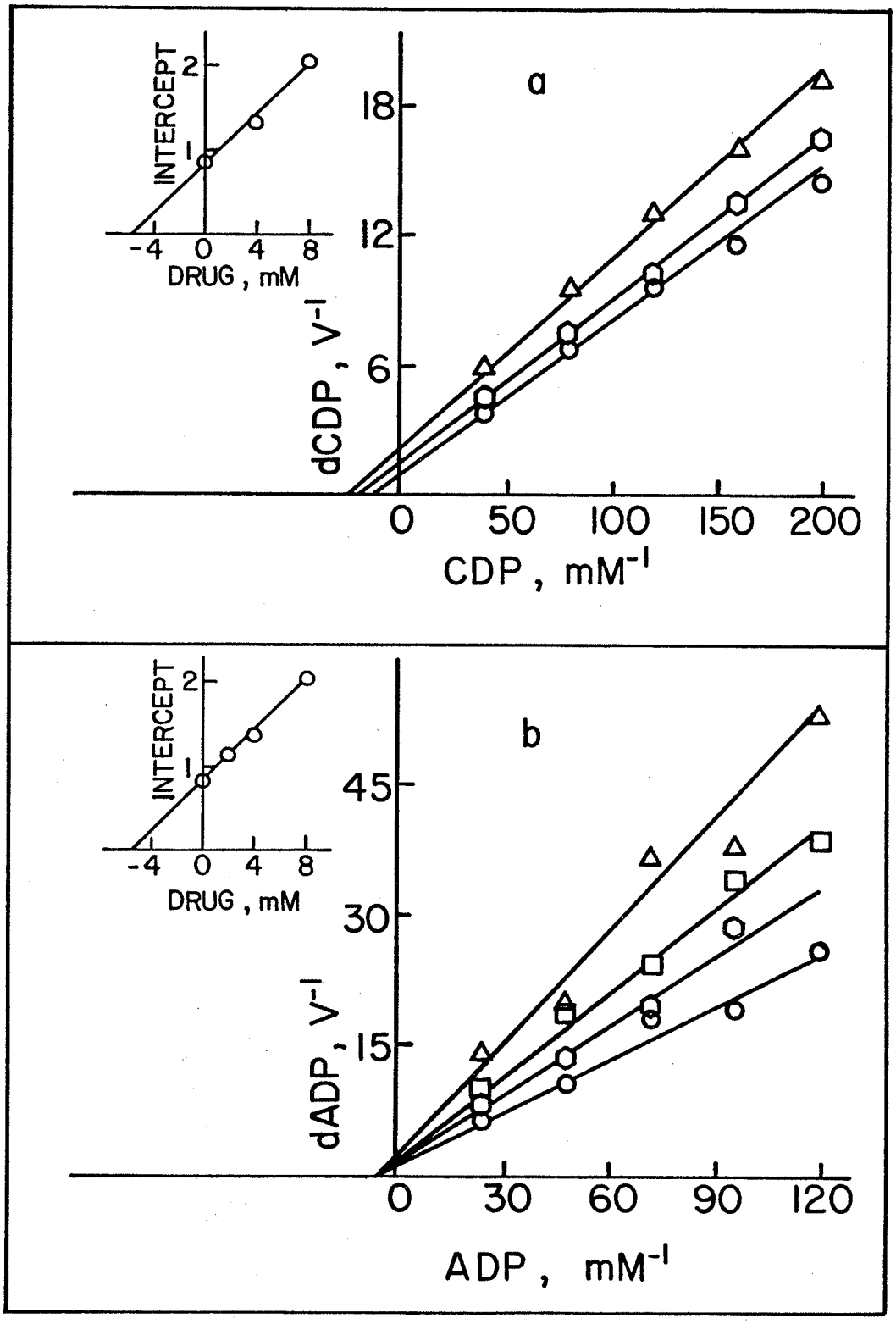
Fig. 68b. Double reciprocal plot of the variation in rate of ADP reduction with ADP concentration at several fixed levels of N-carbamoyloxyurea using permeabilized NC^R-30A2 cells. Assays were performed in the absence (O) or presence of 2 mM (⬡), 4 mM (□) and 8 mM (Δ) N-carbamoyloxyurea. Activity expressed as nmoles dADP formed/5 x 10^6 cells/ hour. Inset: replot of velocity intercepts against N-carbamoyloxyurea concentrations. Results corrected for presence of kinase and phosphatase activities in intact cells.


a
INTERCEPT
2
1
-4
0
4
8
DRUG, mM
18
12
6
dCDP, V⁻¹
0
40
80
120
160
200
CDP, mM⁻¹
b
INTERCEPT
4
2
-4
0
4
8
DRUG, mM
60
40
20
dADP, V⁻¹
0
30
60
90
120
ADP, mM⁻¹

1.56 nmoles dCDP and 0.86 nmoles dADP formed/5 x 10^6 cells/hour. Similar studies carried out with the enzyme activity in NC^R-30A5 cells indicated noncompetitive inhibition of both CDP and ADP reductions by N-carbamoyloxyurea (figure 69). Replots of velocity intercepts against drug concentrations (insets, figure 69) gave K_i estimates of 5.9 mM for CDP reduction and 5.2 mM for ADP reduction. Velocity intercepts gave V_{max} values of 1.16 nmoles dCDP and 1.25 nmoles of dADP formed/ 5 x 10^6 cells/hour for the NC^R-30A5 cells. Comparison of the drug sensitivities of the reductase from these two cell lines with enzyme activity from wild type cells showed that NC^R-30A2 cells have a CDP and ADP reductase 1.5 and 1.7 times more resistant to inhibition by N-carbamoyl-oxyurea, while the NC^R-30A5 cells contained CDP and ADP reductase activities 2.3 and 2.6-fold more resistant. The enzyme activity levels in the NC^R-30A2 cells showed a 3.2-fold increase in CDP reduction and a 14% decrease in ADP reduction over those found in wild type cells. NC^R-30A5 cells showed a 2.4 fold increase in CDP reductase activity and a 1.25 fold increase in ADP reductase activity in comparison to the wild type cells. These results, which suggested structural as well as enzyme activity level changes were supported by observations that enzyme substrate Km values in resistant cells differed from those found in wild type and NC^R-30A cells. The CDP Km values for the reductase in wild type and NC^R-30A cells were 0.021 and 0.033 mM respectively, whereas NC^R-30A2 contained an activity with a CDP Km value of 0.068 mM and NC^R-30A5 contained an activity with a CDP Km value of 0.073 mM. Wild type and NC^R-30A cells both possessed a reductase that exhibited an ADP Km value of 0.095 mM. Enzyme activities from NC^R-30A2 and NC^R-30A5 gave values of 0.155 and 0.228 mM ADP respectively. The

Fig. 69a. Double reciprocal plot of the variation in rate of CDP reduction with CDP concentration at several fixed levels of N-carbamoyloxyurea using permeabilized NC^R-30A5 cells. Assays were performed in the absence (O) or presence of 4 mM (□) and 8 mM (Δ) N-carbamoyloxyurea. Activity expressed as nmoles dCDP formed/5 x 10^6 cells/hour. Inset: replot of velocity intercepts against N-carbamoyloxyurea concentrations. Results corrected for presence of kinase and phosphatase activities in intact cells.

Fig. 69b. Double reciprocal plot of the variation in rate of ADP reduction with ADP concentration at several fixed levels of N-carbamoyloxyurea using permeabilized NC^R-30A5 cells. Assays were performed in the absence (O) or presence of 2 mM (○), 4 mM (□) and 8 mM (Δ) N-carbamoyloxyurea. Activity expressed as nmoles dADP formed/5 x 10^6 cells/hour. Inset: replot of velocity intercepts against N-carbamoyloxyurea concentrations. Results corrected for presence of kinase and phosphatase activities in intact cells.


a
INTERCEPT
2
1
-4 0 4 8
DRUG , mM
18
12
6
dCDP , V⁻¹
0 50 100 150 200
CDP , mM⁻¹
b
INTERCEPT
2
1
-4 0 4 8
DRUG , mM
45
30
15
dADP , V⁻¹
0 30 60 90 120
ADP , mM⁻¹

NC^R-30A2 and NC^R-30A5 cell lines may thus be grouped with class 3 hydroxyurea-resistant cell lines as they appear to possess altered levels of drug resistant ribonucleotide reductase activity.

Inhibition of CDP and ADP reductions in intact NC^R-30A2 and NC^R-30A5 cells by hydroxyurea and guanazole were also analysed by double reciprocal plots of velocity against varying CDP and ADP concentrations. In all cases the inhibition appeared to be noncompetitive. The essential results of these detailed kinetic experiments have been summarized and presented in Table 16. The relative increases in enzyme resistance towards hydroxyurea and guanazole closely matched those found for N-carbamoyloxyurea. This confirms that the three drugs share a common site of intracellular action in mammalian cells.

It is interesting to note that whereas the activity levels of CDP and ADP reductase were altered in an uncoordinated manner within the series of N-carbamoyloxyurea resistant cell lines, the two activities exhibited equal changes in drug sensitivity throughout the various cell lines. For example, the NC^R-30A2 cell line was found to possess an approximate 3.5 fold increase in CDP reductase activity over that observed in WT cells but the levels of ADP reductase activity in the two cell lines was approximately equal. Yet both CDP and ADP reduction show about a 1.8 fold decrease in sensitivity to N-carbamoyloxyurea, hydroxyurea or guanazole. Possible implications of this finding in regards to a proposed structure for ribonucleotide reductase (Youdale and MacManus 1981, Youdale et al 1982) will be discussed later.

No changes in the kinetic properties of the enzyme in NC^R-30A2 and NC^R-30A5 cells were detected in experiments performed with these cell lines during a period of about six months of continuous culture.

Table 16. Hydroxyurea and guanazole inhibition of NC^R-30A2 and NC^R-30A5 CDP and ADP Reduction.

Inhibitor	Cell Line	Varied substrate	Ki values[a]	Increase in Ki
hydroxyurea	NC^R-30A2	CDP	0.38 mM	1.9
hydroxyurea	NC^R-30A2	ADP	0.33 mM	2.1
guanazole	NC^R-30A2	CDP	2.0 mM	1.8
guanazole	NC^R-30A2	ADP	2.6 mM	2.2
hydroxyurea	NC^R-30A5	CDP	0.46 mM	2.3
hydroxyurea	NC^R-30A5	ADP	0.52 mM	2.6
guanazole	NC^R-30A5	CDP	2.75 mM	2.4
guanazole	NC^R-30A5	ADP	2.5 mM	2.2

[a]Determined from examining velocity intercepts versus drug concentrations used in double reciprocal plots.

Moreover, ten subclones were isolated from cultures of NC^R-30A2 and NC^R-30A5 and each was examined for intracellular CDP and ADP reductase levels and for drug (N-carbamoyloxyurea, hydroxyurea and guanazole) inhibition of CDP and ADP reductase activities. The subclones showed enzyme levels and inhibition parameters essentially identical to the cell line from which they were isolated, indicating that individual cells of each line contained enzymes with very similar properties.

It should be noted that some of the experiments conducted above used cells grown in the presence of 8% HS and 2% FCS while other used cells grown in the presence of 10% FCS. In order to ensure that the results observed were not affected by the different types of sera used in the growth medium, kinetic analyses of the four cell lines studied most thoroughly (WTC125, NC^R-30A, NC^R-30A2 and NC^R-30A5) were carried out with cells grown under both conditions. In all cases the results obtained with cells grown in 10% FCS matched those with the cells grown in 8% HS and 2% FCS (data not shown).

5.5 Correlation of CDP and ADP Reductase Activities with Cellular Resistance to Hydroxyurea and Guanazole

Previous studies with hydroxyurea-resistant cell lines have shown that increases in cellular resistance could be totally accounted for by one of three mechanisms: a reductase with a reduced sensitivity to drug inhibition (Lewis and Wright 1978a), elevated levels of drug sensitive enzyme activity (Lewis and Wright 1979) or the production of increased levels of ribonucleotide reductase with decreased sensitivity to drug inhibition (Koropatnick and Wright 1980, Kuzik and Wright 1980).

In this investigation the 1.6 fold increase in cellular resistance exhibited by NC^R-30A cells in the presence of hydroxyurea or guanazole

could be fully explained by the 1.8-fold enhancement of ADP reductase activity and the 2-fold elevation in CDP reductase observed with the cells. The five intermediately resistant cell lines were 8-fold more resistant to hydroxyurea and guanazole than wild type cells while NC^R-30A5 cells showed an increase in cellular resistance of 11 to 12-fold. As the only intracellular target for these drugs was probably ribonucleotide reductase, the increase in cellular resistance exhibited by NC^R-30A2, representative of the five intermediate cell lines, and NC^R-30A5 should have been totally accountable via observed changes in their reductase activities. This accountability assumes that drug permeability properties remain unchanged in the drug resistant series of cell lines. When enzyme level changes and the decrease in enzyme sensitivities to the two drugs (table 16) were considered for each of the cell lines it appeared at first that they could not totally account for the observed increases in cellular resistance to hydroxyurea and guanazole. For example with NC^R-30A2 cells, the 3.2-fold increase in CDP reductase levels coupled with the 1.8 to 1.9-fold increase in K_i values resulted in an activity 5.8 to 6.1-fold more resistant to the effects of the drugs whereas cellular resistance increased 8-fold. For ADP reduction in NC^R-30A2 cells, the combination of enhanced levels and decreased drug sensitivity showed only a 1.8 to 1.9-fold elevation in activity over that found in wild type cells. A similar situation existed for NC^R-30A5 cells which showed an increase in cellular resistance of 11 to 12-fold towards hydroxyurea and guanazole but possessed CDP and ADP reductase activities which were only 2.7 to 2.8-fold and 2.8 to 3.3-fold more resistant to the drugs respectively. However, it has been previously shown that hydroxyurea-resistant cells

were capable of further increasing their intracellular levels of enzyme activity when they were cultured in the presence of drug concentrations normally cytoxic to wild type cells (Wright et al 1981, Lewis and Wright 1979). Evidence that the same mechanism operated in the N-carbamoyloxyurea resistant cells came from studies in which NC^R-30A2 and NC^R-30A5 cells were first cultured for 36 hours in the presence of 0.3 mM and 0.4 mM N-carbamoyloxyurea, respectively, before enzyme levels were determined. For NC^R-30A2 cells the treatment produced intracellular activity levels of 2.49 nmoles dCDP and 1.88 nmoles dADP formed/5 x 10^6 cells/hour. NC^R-30A5 cells pretreated with drug were found to have activity levels of 3.64 nmoles dCDP and 1.48 nmoles of dADP formed/5 x 10^6 cells/hour. The levels of CDP reductase were enhanced 5-fold and 7.3-fold over wild type in NC^R-30A2 and NC^R-30A5 populations respectively. By combining these values with those for increased resistance at the enzyme level, 1.8 fold for NC^R-30A2 and 2.2 fold for NC^R-30A5 (Table 16), the entire cellular resistance to hydroxyurea and guanazole could be explained. The situation with the ADP reductase data is not as straightforward. Growth in N-carbamoyloxyurea supplemented media enhanced ADP reductase levels by only 2-fold in NC^R-30A2 cells and 1.5-fold in NC^R-30A5 cells over that found in wild type cells. When these increases were combined with the increased K_i values (Table 16) for drug inhibition of ADP reduction, the results fell short of accounting for the observed increase in cellular resistance. These results support the view noted earlier, that an increase in CDP reductase activity seemed to be more important for drug resistance than an increase in ADP reductase. These findings are in keeping with the idea that dCDP, perhaps in the form of dCTP, may have a special part to play in the regulation of mammalian DNA synthesis (Meuth et al 1976, Nicander and Reichard 1981).

The justification for culturing the drug resistant cells in the presence of N-carbamoyloxyurea to induce an even greater elevation in their reductase levels was as follows. After CHO cells containing altered ribonucleotide reductase activities had been selected in the presence of N-carbamoyloxyurea, they continued to be cultured in the presence of drug. As was observed with hydroxyurea-resistant mutants (Wright et al 1981) the continued presence of the drug likely induced an elevation in enzyme levels. Thus resistance to N-carbamoyloxyurea was due mainly to the genetically altered reductase activity but also to some extent to elevation of activity levels induced by the continued presence of the drug. Thus, preincubation of the cells with N-carbamoyloxyurea immediately prior to enzymatic assay likely induced enzyme levels similar to those which were operative during the selection procedure. The drug concentrations used in the study were chosen because they had no effect on plating efficiency and did not reduce growth rates by more than 50%. These considerations were important in obtaining cells that had optimum levels of reductase activity.

6. AN IRON DEPENDENT 5'-NUCLEOTIDE PHOSPHORIBOHYDROLASE ACTIVITY IN THE VENOM OF CROTALUS ATROX AND ITS EFFECT UPON THE DETERMINATION OF MAMMALIAN RIBONUCLEOTIDE REDUCTASE

Venoms from snakes of various genera of widely different species contain many enzymes (Devi 1971, Russell 1980). The activities of these enzymes vary from venom to venom and the toxicity may change with the season (Devi 1971). Venoms have proved to be a useful source of enzymes for biochemical procedures. For example, the assay of ribonucleotide reductase activity in this investigation employed a commonly used method which involved the conversion of labelled

nucleotides to nucleosides by snake venom activities followed by separation of deoxyribonucleosides from ribonucleosides on Dowex-1-borate or AG1-X8-borate columns (see Materials and Methods). Various laboratories, including this one, have observed that the addition of iron to the assay of ribonucleotide reductase (an iron containing enzyme) influences reductase activity. The iron effect varied from considerable stimulation to inhibition (Moore 1969, Larsson 1969, Hopper 1978, Kuzik and Wright 1979). The possibility that the venom of Crotalus atrox contained interfering enzyme activities that could help to explain the variable effects of iron was examined.

When ribonucleotide reductase activity was measured in permeabilized cells or cell-free extracts, a control experiment was performed. This control involved inactivation of the reaction by immediate boiling upon addition of the enzyme source. These controls were assayed in a manner identical to that routinely used and thus involved conversion of nucleotides to nucleosides by enzymes of snake venom and subsequent separation of deoxyribonucleosides from ribonucleosides on Dowex-1-borate or AG1-X8-borate columns as described in Materials and Methods. Counts obtained in these control experiments (background) were subtracted from the counts obtained in assays containing active reductase. Obviously background levels are important for establishing the actual activity of ribonucleotide reductase and hence must be precisely determined. The effects of $FeCl_3$, a variable activator of reductase activity, on background levels was examined. Since both purine and pyrimidine nucleotides are substrates for ribonucleotide reductase, the tests were carried out with one pyrimidine (CDP) and one purine (ADP). These studies will henceforth simply be referred to as CDP

and ADP experiments.

Figure 70 shows the effect various concentrations of $FeCl_3$ had on the background counts in CDP and ADP experiments. In both cases the addition of $FeCl_3$ significantly elevated background counts. With CDP experiments an approximately bell-shaped curve was observed with 1.0 mM $FeCl_3$ inducing the highest background levels. ADP experiments showed an enhancement to about 0.8 mM $FeCl_3$ without a further change beyond this point. With snake venom used in these experiments, the addition of $FeCl_3$ increased background levels by a maximum of 55% and 30% in CDP and ADP reductase experiments, respectively. Actual enhancement values were found to be dependent upon the batch of snake venom used in the experiments. Although the results were reproducible with each batch, they varied significantly from one batch of venom to another. For example, enhancement in CDP experiments were observed as high as 220% and as low as 30% at 1 mM $FeCl_3$ depending upon the batch of venom used.

The enhancement of background levels would seem to be a phenomena unique to $FeCl_3$, at least among the usual components of the CDP and ADP reductase assay systems. When the various compounds in the reaction systems (see Materials and Methods) were omitted singly or in combination it was found that enhancement of background levels occurred only in the presence of $FeCl_3$ and was dependent upon $FeCl_3$.

When the elevated background counts were taken into consideration during estimation of ribonucleotide reductase activity in the presence of various $FeCl_3$ concentrations (figure 71), it was found that $FeCl_3$ inhibited CDP reduction at concentrations greater than 0.4 mM with 50% inhibition occurring at 1.4 mM. Slight stimulation occurred at lower concentrations with 0.2 mM enhancing activity by about 20%. $FeCl_3$ inhibited ADP reduction at all concentrations tested with 0.8 mM reducing activity by 50%.

Fig. 70. Percent increase in radioactive counts found in CDP (O) and ADP (X) control experiments in the presence of various concentrations of $FeCl_3$. Values for CDP and ADP experiments in the absence of $FeCl_3$ were 1050 ± 157 cpm and 165 ± 17 cpm, respectively. Each experiment was performed a minimum of three times with similar results obtained.

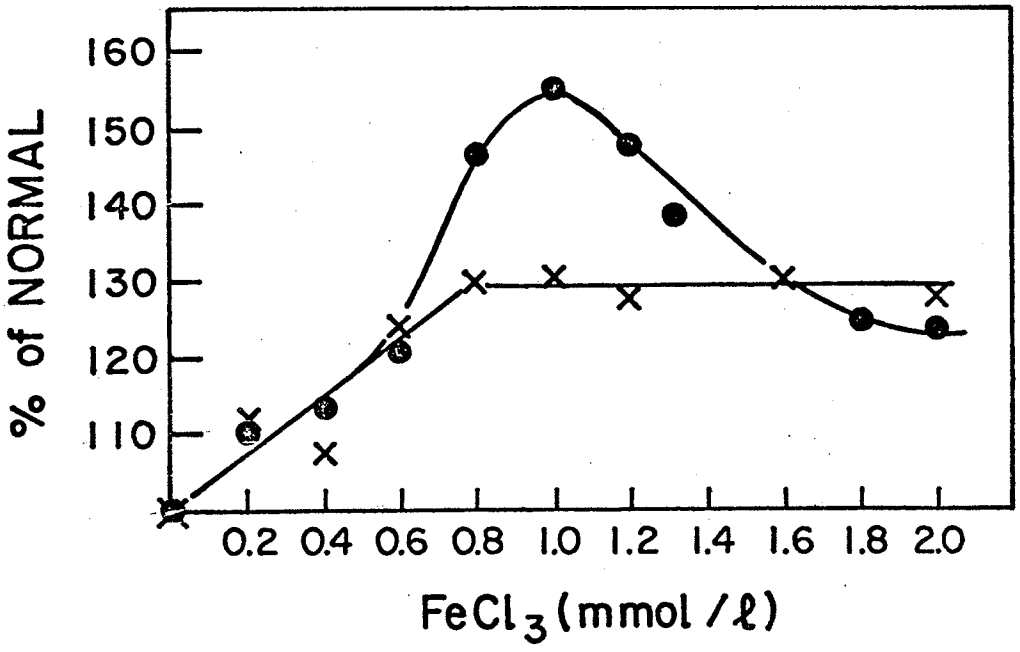
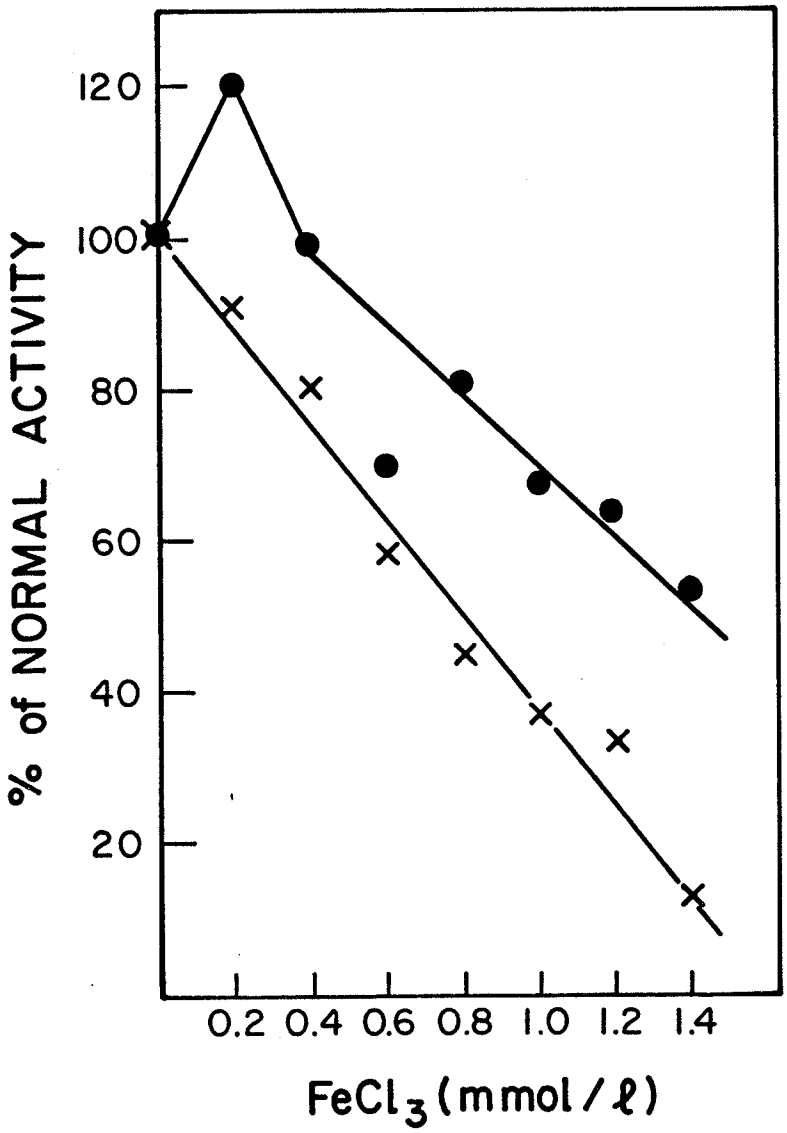

160
150
140
130
120
110
% of NORMAL
0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0
FeCl3 (mmol /ℓ)

Fig. 71. Response of CDP (O) and ADP (X) reductase activity in permeabilized WT cells to $FeCl_3$ concentration, expressed as a percent of activity observed in the absence of $FeCl_3$. Standard reductase assay conditions were used except for the presence of varying concentrations of $FeCl_3$. Similar results have been obtained in three separate experiments. In the absence of $FeCl_3$ CDP reductase activity was 0.15 nmoles dCDP formed/5 x 10^6 cells/ 20 minutes and ADP reductase activity was 0.23 nmoles dADP formed/5 x 10^6 cells/20 minutes.


120
100
80
60
40
20
% of NORMAL ACTIVITY
0.2 0.4 0.6 0.8 1.0 1.2 1.4
FeCl3 (mmol/ℓ)

The changes in background levels brought about by $FeCl_3$ (figure 70) were examined further. $FeCl_3$ did not effect the performance of the column as judged by experiments in which ^{14}C-cytidine and ^{14}C-adenosine were mixed with $FeCl_3$, in the presence or absence of the other components in the reaction systems, and then the mixtures eluted from the columns as usual. As shown in figure 72, addition of various concentrations of $FeCl_3$ did not affect the retention of labelled cytidine or adenosine by the column. Incubating cytidine/$FeCl_3$ mixtures for as long as 3.5 hours had no discernable effect on the amount of radioactivity released. The possibility that the CHO cells contained a heat-resistant iron-dependent enzyme activity was eliminated by the observation that the iron effect did not require the presence of cells, since background values for CDP and ADP experiments were not affected if cells were left out of the tests (data not shown).

The above results strongly suggested that $FeCl_3$ affected background values in reductase assays by influencing an activity found in snake venom. To study this possibility, the effluent of the columns was analyzed by reverse phase HPLC as described in Materials and Methods. The elution times for cytosine, cytidine, deoxycytidine, adenine, adenosine and deoxyadenosine were determined. To examine the $FeCl_3$ effect, the CDP and ADP reductase control experiments were performed in the presence and absence of 1.3 mM $FeCl_3$ and then analyzed with reverse phase HPLC (figure 73). For CDP and ADP experiments with $FeCl_3$ an increase in the amount of cytosine and adenine, respectively, was found which correlated with the increase in background levels previously observed.

The results support the idea that the increase in background due to the presence of $FeCl_3$ resulted from the cleavage of a sugar-base N-glycosidic bond. The purine and pyrimidine base released from such a

Fig. 72. Effect of $FeCl_3$ on the elution of ^{14}C-cytidine (589 cpm/nmole) (O) and ^{14}C-adenosine (313 cpm/nmole) (X) from Dowex-1-borate columns. 120 nmoles of CDP or ADP was mixed with various $FeCl_3$ concentrations, applied to Dowex-1-borate comumns and eluted as described in Materials and Methods.

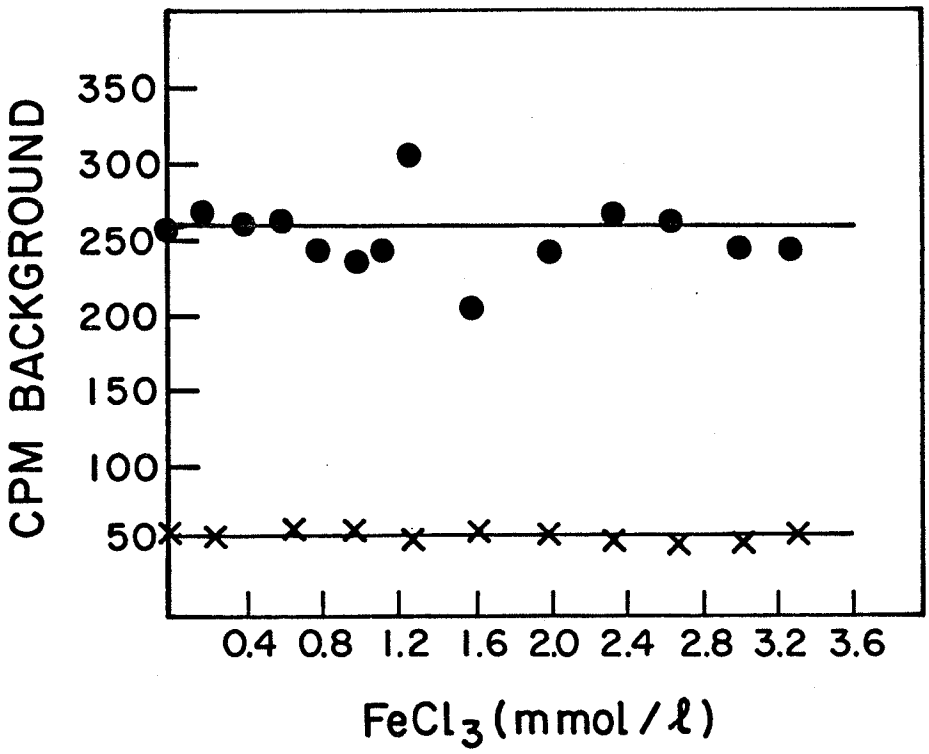
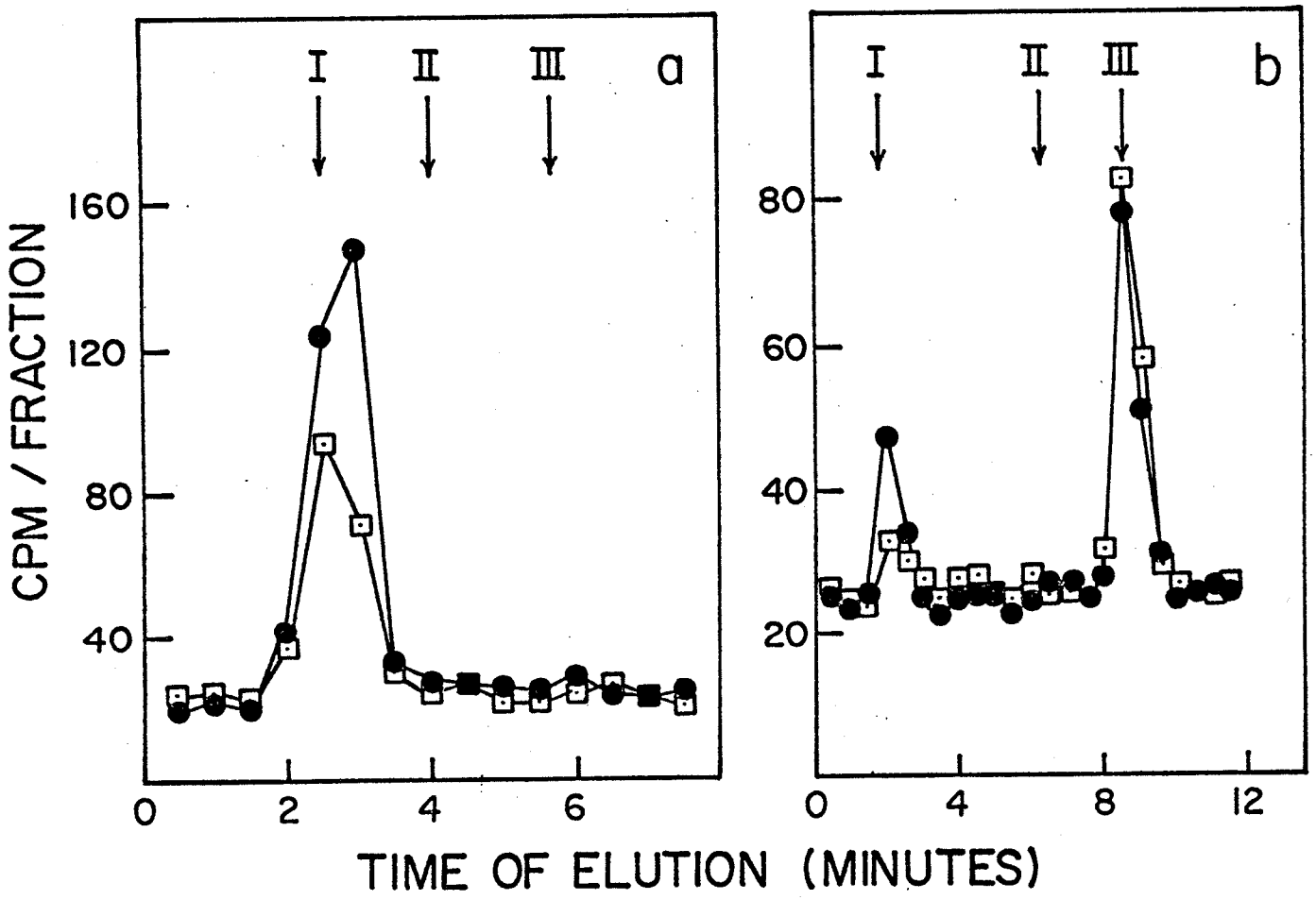

CPM BACKGROUND
350
300
250
200
150
100
50
0.4 0.8 1.2 1.6 2.0 2.4 2.8 3.2 3.6
FeCl3 (mmol/l)

Fig. 73. Chromatographic separation of the compounds eluted from Dowex-1-borate columns using C-18 reserve phase HPLC columns. Location of (a) cytosine (I), cytidine (II) and deoxycytidine (III) or (b) adenine (I), adenosine (II) and deoxyadenosine (III) on C-18 reserve phase columns. Samples were prepared, applied, eluted and collected as described in Materials and Methods, in the absence (□) or presence (O) of 1.3 mM $FeCl_3$. Each experiment was performed a minimum of three times with similar results obtained. The results shown in figures 70 and 73 were obtained with different batches of snake venom.


I
II
III
a
b
160
120
80
40
80
60
40
20
CPM / FRACTION
0
2
4
6
0
4
8
12
TIME OF ELUTION (MINUTES)

cleavage would not be bound to the Dowex-1-borate column as it lacks a cis-diol (Khym and Zill 1952, Khym 1966). A logical assumption from the data was that phosphodiesterase and 5'-nucleotidase activities of snake venom produced ribonucleosides which served as substrates for an iron-dependent activity. To test this hypothesis, complete reaction mixtures for both CDP and ADP reduction assays with ^{14}C-cytidine substituted for ^{14}C-CDP and ^{14}C-adenosine substituted for ^{14}C-ADP were subjected to snake venom treatment both in the presence and absence of 1.3 mM $FeCl_3$ and then passed over Dowex-1-borate columns. Interestingly, $FeCl_3$ did not increase the background counts in experiments with either cytidine or adenosine. Therefore, the increase in background levels was not due to the presence of an iron-dependent nucleosidase. Instead the activity in snake venom, which is known to contain many nucleotide metabolizing enzymes (Devi 1971, Russell 1980), appeared to require a nucleotide phosphate for activity. This suggested that hydrolysis of the N-glycosidic linkage involved a pyrimidine/purine 5'-nucleotide phosphoribohydralase activity activated by $FeCl_3$.

DISCUSSION

DISCUSSION

1. ASSAY OF RIBONUCLEOTIDE REDUCTASE ACTIVITY USING PERMEABILIZED CHO CELLS.

One of the disadvantages of using cell free preparations to study the properties of an enzyme is that the extraction procedure introduces a large dilution factor into the system. This dilution may produce major alterations in the properties of an enzyme assayed *in vitro* as compared to its *in vivo* situation. This is especially true for an enzyme such as ribonucleotide reductase which possesses dissociable subunits (Cory *et al* 1978, Chang and Cheng 1979c, Mattaliano *et al* 1981, Youdale and MacManus 1981, Youdale *et al* 1982) and appears to be part of a large multienzyme complex involved in the replication of DNA (Elford 1974, Reddy and Pardee 1980, Forsdyke and Scott 1980, Scott and Forsdyke 1980, Reddy *et al* 1982).

To make intracellular enzymes accessible to exogenously added substrates and effectors without unduly disrupting cell organization, many investigators have successfully employed various cell permeabilization techniques. The agents commonly used to permeabilize mammalian cells include toluene (Hilderman and Deutscher 1976, Dohadwata and Chaudhuri 1979), lysolecithin (Miller *et al* 1978, Miller *et al* 1979, Wydro *et al* 1980), Ficoll (Burgoyne 1972), Nonidet-P40 (Chen *et al* 1980), Triton X-100 (Hyodo and Suzuki 1980), the ionophore nigericin (Alonso and Carvasco 1980), dextran sulfate (McCoy and Racker 1976, Kucera and Paulus 1982a), Tween 60 (Malenkov *et al* 1967), Brij 58 and polyethylene glycol (Cande 1980, Cande *et al* 1981, Yarosh and Settlow 1981). Adenosine triphosphate has been found to be useful in the permeabilization of transformed cells but ineffective with untransformed cells (Rozengurt *et al* 1977, Kitagawa 1980). Hypotonic (Berger and Johnson

1976) and hypertonic (Castelott et al 1978) treatment has also been found to render animal cells permeable to normally excluded molecules. A procedure has also been developed in which treatment of the cells with bifunctional reagents to crosslink proteins creates a network that allows for permeabilization by delipidation (Aragon et al 1980). Dimethyl suberimidate, dimethyl 3,3'-dithiobis-propionimidate and toluene-2,4-diisocyanate have been used as cross-linking agents while digetonin has been used for delipidation. In order to study the properties of mammalian ribonucleotide reductase in a situation as similar to the normal in vivo environment as possible, a Tween 80 permeabilizing technique (Lewis et al 1978, Wright et al 1981) based on the method of Billen and Olsen (1976) has been developed.

In using permeabilized CHO cells for assaying ribonucleotide reductase activity, two critical requirements must be met. First, the cellular membrane must be altered to allow the free movement of enzyme substrates and effectors into and products out of the cell. Secondly, the treatment must be sufficiently mild to ensure that important internal cellular structures are not disrupted. On both counts the assay for ribonucleotide reductase using Tween-80 permeabilized CHO cells is successful. Normally excluded molecules, such as trypan blue, were shown to be taken up by permeabilized cells (figure 5). This indicates that the treatment does alter the membrane properties in such a way as to remove the barrier to free movement of previously excluded molecules. In the particular case of ribonucleotide reductase the Tween-80 treatment allows for free movement of substrates, products and effectors into and out of the cell as evidenced by the fact that the level of reduction was found to vary in a linear manner with both time and cell number (figures 11, 19 and 51). When permeabilized CHO

cells were removed from the Tween-80 permeabilizing buffer and resuspended in α-MEM + 10% FCS, it was found that the cells resealed themselves and grew in culture with a plating efficiency of 63% as compared to cells that had not been treated with the detergent. This deomonstrates that, in most cells, much of the machinery necessary for the cells to survive and grow was left unaltered by treatment with the permeabilzing buffer.

Since a great deal of the cellular machinery remains unaltered in permeabilized CHO cells, it is not surprising that other enzymes in addition to ribonucleotide reductase were active during the assays. Indeed, activities including tRNA nucleotidyl transferase, amino acyl-tRNA synthetase (Hilderman and Deutscher 1974, Hilderman et al 1975), DNA polymerase (Burgoyne 1972), AMP deaminase (Murakami et al 1980), the enzymes of the glycolytic pathway (Heppel and Makan 1977, Aragon et al 1980) and protein synthesis (Kitagawa 1980, Castellot et al 1978) have been measured in eucaryotic cells permeabilized with various agents. Of particular interest, in the assay of ribonucleotide reductase, was the fate of the nucleotide substrates and effectors used in the various reaction mixtures.

As shown and discussed previously (Results section 1.5) permeabilized CHO cells retain several nucleotide metabolizing activities. Although uptake of substrate into RNA and of product into DNA did occur, the level of incorporation was of such a low magnitude as to not significantly affect the determination of ribonucleotide reductase activity. Kinases and phosphatases involved in the phosphate metabolism of nucleotides were sufficiently active in the permeabilized cells to alter the results obtained with standard reductase assays. However, the effect of these activities on the substrate and effector concentrations may be corrected (figure 8). Where ever possible the effects

of these phosphate metabolizing enzymes have been taken into account in the reporting of results in this thesis.

A major reason for using intact permeabilized CHO cells to study ribonucleotide reductase activity was the hope that in vivo properties of the enzyme would be retained. One area where this definitely seemed to be the case was the linearity of CDP reduction with cell number. As shown in figure 12 and by other investigators (Larsson 1969, Lewis and Wright 1978b, Kuzik and Wright 1979, Engström et al 1979) CDP reduction was not linear with low concentrations of protein. Presumably, this deviation occurs as a result of the dissociation of the enzyme's subunits. When CDP reductase levels were measured as a function of cell number, linearity was observed at all values tested (figure 11a). Thus it would seem that the intact permeabilized cells maintain a structural feature (possibly a multienzyme complex) that is lost when ribonucleotide reductase is extracted from the cells. Disruption of this structure may cause the dissociation of the enzyme subunits observed at low protein concentrations and possibly other undetected alterations in the properties of the enzyme as well.

In addition to maintaining enzymes in a more natural environment, the permeabilization assay has several other advantages. Enzyme assays can be carried out on very low cell numbers. Although a concentration of 5×10^6 cells per assay was routinely used, reproducible activity levels were obtainable with significantly fewer cells per assay (figures 11a, 19b and 51). In the selection of somatic cell mutants, this allows for the screening of potential variants without the need to grow the large amounts of cells required for preparing cell free extracts. This method is also faster than extracting the enzyme from cells, as permeabilized cells are ready for analysis 30 minutes

after they have been harvested. The permeabilization technique developed is also successful with cells grown under different conditions. As shown by figures 14 and 21, the kinetic parameters of ribonucleotide reductase from cells grown in suspension were identical to those for cells grown on solid surfaces (figures 13 and 20). Thus the environment in which the cells were grown did not affect the permeabilization process. The similarity of the results from the cells grown in suspension and on solid surfaces also eliminated the possibility that the removal of cells from solid surfaces with trypsin in some way affected the permeabilization of the cells. As trypsin does act on proteins and glycoproteins of the cell surface, it seemed possible that it could affect the interaction of Tween 80 with the cells. However, the finding that the ribonucleotide reductase of cells grown in suspension, and thus harvested without the aid of trypsin, exhibited the same properties as that from cells grown on solid surfaces excluded this possibility. As further confirmation of the non-involvement of trypsin in the permeabilization process, it was found that detachment of cells grown on solid surfaces with EDTA did not affect the measured properties of ribonucleotide reductase (data not shown).

To date, the Tween 80 permeabilization technique has been used to study ribonucleotide reductase from CHO (Lewis et al 1978, Hards and Wright 1981), human diploid fibroblast (Dick and Wright 1980, 1982) mouse L (Koropatnick and Wright 1980, Kuzik and Wright 1980), T-lymphoma (Ullman et al 1979, 1980) and rat myoblast (Creasey et al 1982, Wright et al 1982b) cells.

In summary, the intact cell assay system, based upon Tween 80 permeabilization, provides useful estimates of pyrimidine and purine reductase activities in mammalian cells in a physiologically relevant

environment for the enzyme. Good approximations of enzyme kinetic data can be obtained quickly from relatively few cells from many different sources grown on the surface of tissue culture plates or in suspension culture. Although a number of nucleotide metabolizing enzymes are active in intact cells, their effects on substrate and effector concentrations can be estimated and taken into account if necessary, to obtain corrected determinations of ribonucleotide reductase activity. Hamster cells were used in this study but it is reasonable to assume that, in general, similar observations would also be made with other mammalian cells.

2. GENERAL CHARACTERIZATION OF RIBONUCLEOTIDE REDUCTASE ACTIVITY IN INTACT CHO CELLS

As ribonucleotide reductase catalyzes the reduction of both purine and pyrimidine ribonucleotides required for DNA synthesis, the properties of the enzyme were examined in the presence of a purine (ADP) and a pyrimidine (CDP) substrate. The optimum conditions determined for the reduction of the two substrates were very similar to those observed with cell free preparations from a variety of mammalian sources including Chinese hamster fibroblasts (Peterson and Moore 1976), regenerating rat liver (Larsson 1969), CHO cells (Lewis and Wright 1978b), calf thymus (Eriksson et al 1979) and mouse L cells (Kuzik and Wright 1979). Conditions used to measure ribonucletide reduction in permeabilized CHO cells closely resemble those subsequently worked out for the enzyme from intact normal human diploid fibroblasts (Dick and Wright 1980). In addition, conditions used to measure CDP reduction were very similar to those determined for the enzyme in intact hamster cells auxotrophic for glycine, adenine and thymidine (Lewis et al 1978). The most favourable conditions for

assaying CDP and ADP reductase activities are listed in Table 6 and may be summarized as follows. Enzyme activity was largely dependent upon the exogenous reducing agent DTT, with maximum activity with both substrates occurring at 6 mM (figure 9c and 15) followed by a slow decline in activity measured up to 15 mM for CDP reductase and to 20 mM with ADP reductase. CDP reduction was greatly stimulated by 7 mM $MgCl_2$ (figure 9a) and showed an absolute requirement for ATP with the optimum concentration being 1.5 mM (figure 9b). ADP reduction did not require the presence of $MgCl_2$ and was in fact inhibited at all concentrations of $MgCl_2$ tested (figure 17). ADP reductase activity exhibited an absolute requirement for dGTP with maximum activity taking place between 0.04 and 0.20 mM (figure 16). Both CDP and ADP reductase were active over a broad pH range with optimums of pH 7.2 for CDP reduction (figure 10b) and pH 6.9 to 7.1 for ADP reduction (figure 18b). A temperature of $37^{o}C$ was found to be most favourable for the reduction of both CDP (figure 10a) and ADP (figure 18a). Using the optimum assay conditions described, reduction was found to be linear with cell number up to and including 10^7 cells/assay for both the pyrimidine (figure 11a) and the purine (figure 19b) substrate. Reduction was determined to be linear with time for up to 20 minutes with CDP (figure 11b) and for up to 40 minutes with ADP (figure 19a).

As there are reports that other nucleoside triphosphates, in addition to ATP and dGTP, may stimulate CDP and ADP reduction, respectively, (Murphree et al 1968, Fujioka and Silber 1970, Peterson and Moore 1976), the ability of various nucleoside triphosphates to act as activators of CDP and ADP reduction was determined as described in Table 7. In addition to ATP, it was found that dCTP stimulated significant reduction of CDP while dTTP and GTP, as well as dGTP were

found to activate ADP reduction. On closer inspection it was found that maximum reduction of CDP occurred at 3.6 mM dCTP (figure 22c) and that the rate of reduction induced by this activator was 29% of that obtained with 1.5 mM ATP. The concentrations of GTP and dTTP which were most effective in stimulating the reduction of ADP were 0.2 mM (figure 22a) and 0.065 mM (figure 22b), respectively. As compared to the dGTP activated reduction of ADP, optimum concentrations of GTP achieved a reduction rate of 45% while optimum concentrations of dTTP achieved a rate of 37%.

The addition of iron to the assay of ribonucleotide reductase (an iron containing enzyme) has been found to have varied effects ranging from considerable stimulation to inhibition of enzyme activity (Moore 1969, Larsson 1969, Hopper 1978, Kuzik and Wright 1979). In the present study using permeabilized CHO cells, $FeCl_3$ at a concentration of 0.2 mM was routinely found to stimulate CDP reduction by approximately 20% (figure 73) while at concentrations greater than 0.4 mM, inhibition was observed. ADP reduction was inhibited at all concentrations of $FeCl_3$ tested (figure 73). Therefore, in intact cells, a situation resembling physiological conditions, $FeCl_3$ acts as an inhibitor of ADP reductase and ranged from a modest stimulator of CDP reductase at low concentration to an effective inhibitor at higher concentrations.

Double reciprocal plots of CDP (figure 13) and ADP (figure 20) reduction, activated by ATP and dGTP respectively, at varying substrate concentrations were linear for both ribonucleoside diphosphate substrates. For pyrimidine reduction the data yielded an apparent Km value of 0.021 ± 0.005 mM CDP and a V_{max} value of 0.49 ± 0.17 nmoles dCDP formed/5 x 10^6 cells/hour. Experiments with the purine substrate yielded an apparent Km estimate of 0.095 ± 0.017 mM ADP and a V_{max}

value of 1.00 ± 0.13 nmoles dADP formed/5 x 10^6 cells/hour. Each of these experiments were performed at least 10 times over the course of more than one year with similar results being obtained each time. Although estimates of substrate Km values vary from one laboratory to another depending upon assay conditions and enzyme source, it is interesting to note that the values obtained with intact hamster cells are similar to many of those reported for enzyme prepared from a variety of sources (Peterson and Moore 1976, Lewis and Wright 1978b, Eriksson et al 1979, Kuzik and Wright 1979, Chang and Cheng 1979a, Youdale et al 1982) and resemble the apparent Km estimates of 0.04 mM CDP and 0.1 mM ADP reported for the enzyme from intact human diploid fibroblasts (Dick and Wright 1980). When ribonucleotide reductase was extracted from the CHO cells used in this study and double reciprocal plots of activity versus CDP concentration constructed, a Km value of 0.051 ± 0.004 mM CDP was obtained (data not shown). This value is very similar to the one obtained with the intact cells. The similarity of the CDP Km values for ribonucleotide reductase assayed in intact cells and in cell extracts would seem to verify the usefulness of the permeabilization assay.

Double reciprocal plots of velocity against substrate concentration for the GTP and dTTP stimulated reduction of ADP and the dCTP stimulated reduction of CDP yielded the following data. For the GTP stimulated reduction of ADP, a Km value of 0.20 mM ADP and a V_{max} value of 0.667 nmoles dADP formed/5 x 10^6 cells/hour were obtained (figure 23a). This Km value is similar to that obtained with purified enzyme from human Molt-4F cells where GTP was used to activate the reaction (Chang and Cheng 1979a). For the dTTP stimulated reaction the Km value was 0.333 mM ADP and the V_{max} value was 0.384 nmoles dADP

formed/5 x 10^6 cells/hour (figure 23b). The dCTP activated reduction of CDP had a Km value of 0.033 mM CDP and a V_{max} value of 0.158 nmoles dCDP formed/5 x 10^6 cells/hour (figure 24). No Km values for the dTTP and dCTP activated reactions have previously been published.

Substrate specificity and activity of ribonucleotide reductase are controlled by allosteric mechanisms involving nucleotide effectors, with dATP acting as an overall negative effector in the reduction of all four ribonucleotide substrates (Thelander and Reichard 1979, Holmgren 1981). Figure 25 shows purine and pyrimidine reduction in intact CHO cells in the presence of varying concentrations of dATP. As with studies using cell-free preparations from a variety of mammalian sources (Larsson 1969, Eriksson et al 1979, Murphree et al 1968, Chang and Cheng 1979b, Moore and Hurlbert 1966, Eriksson et al 1981a,b), dATP acted as a very potent inhibitor of both CDP and ADP reduction in intact CHO cells. A 50% inhibition of CDP and ADP reduction was observed at approximately 2.7 and 32 μM dATP, respectively. The value determined for the inhibition of 50% of ADP reductase activity was similar to those reported with the enzyme extracted from mammalian cells (Eriksson et al 1979, Murphree et al 1968) and, to that reported for the reduction of ADP in intact human diploid fibroblasts (Dick and Wright 1980). The concentration of dATP that reduced CDP reductase activity by one-half in intact CHO cells was found to be considerably less than that required with cell extracts of rat embryos (Murphree et al 1968), regenerating rat liver (Larsson 1969), human Molt-4F cells (Chang and Cheng 1979b) and calf thymus (Eriksson et al 1979) or with other intact mammalian cells (Dick and Wright 1980, Ullman et al 1980). Moreover, whereas all extract studies except one indicated

that dATP was equally effective against CDP and ADP reduction, the results with intact CHO cells showed CDP reductase was more sensitive by a factor of 12. Interestingly, in the one study with cell extracts in which sensitivity of CDP and ADP reduction to dATP was not equal, it was the reduction of ADP which was more sensitive (Chang and Cheng 1979b). Though not displaying as large a difference, CDP reductase has also been found to be more sensitive than ADP reductase to dATP inhibition when assayed in intact human diploid fibroblasts (Dick and Wright 1980). Whether the observed differences between cell extracts and intact cells are due to differences in environment or to differing sources of enzyme remains unclear. As ADP reductase generates the immediate precursor of the dATP, it would be advantageous for the cell if this activity was more resistant to the effects of dATP than the reduction of other ribonucleotides. If the utilization of deoxyribonucleotides for DNA synthesis became unbalanced and dATP accumulated, the relative resistance of ADP reduction to the inhibitory effect would ensure that sufficient dATP would be produced to block the reduction of other ribonucleotides prior to the shut down of ADP reduction. Such a system would be energetically conservative.

In addition to activating CDP reduction, ATP and dCTP were found to inhibit the reduction of ADP. Similarly, dGTP, GTP and dTTP not only activated ADP reduction but also inhibited CDP reduction. This two edged response to the allosteric effectors is probably a consequence of the specificity sites (Thelander et al 1980, Eriksson et al 1981b). When the enzyme is directed towards the reduction of one nucleoside diphosphate there is an exclusion of the other substrates. As shown in figure 27, 0.9 mM ATP and 1.5 mM dCDP inhibited ADP

reduction by one-half. The reduction of CDP was reduced 50% by 0.32 mM dTTP, 1.8 mM GTP or 0.125 mM dGTP (figure 26). Results with very similar numerical values have been obtained with cell free extracts from rat embryos (Murphree et al 1966), mouse spleen (Fujioka and Silber 1970), calf thymus (Ericksson et al 1979), human Molt-4F cells (Chang and Cheng 1979b) and mouse T-lymphoma cells (Eriksson et al 1981a). Interestingly, CDP reduction in permeabilized T-lymphoma cells has been shown to be reduced by one-half by 5 μM dGTP and 78 μM dTTP (Ullman et al 1980). These results indicate that CDP reductase activity from this source is much more sensitive to inhibitory effects of dGTP and dTTP than the enzyme in intact permeabilized cells.

While for the most part, the results using intact CHO cells confirm earlier conclusions concerning the properties of ribonucleotide reductase that were drawn from studies using cell free extracts, there is one situation where a discrepancy does exist. Whereas studies with cell free extracts have shown that ADP reduction maximally stimulated by dGTP can be induced to even higher rates by the simultaneous presence of ATP (Chang and Cheng 1979b, Eriksson et al 1979, Thelander and Reichard 1979, Holmgren 1981), in this study with permeabilized CHO cells, all concentrations of ATP tested were found to be inhibitory for the reduction of ADP (figures 27 and 39). Why this is so remains unclear, but it is possible that within the intact cell ATP does not bind to a regulatory site to increase the reduction rates as is proposed to occur in the cell free extracts (Eriksson et al 1979) or it may be that in the intact cells ATP does bind to the regulatory site but does not induce a conformation more favourable towards the reduction of ADP. Further work is required to resolve this question.

3. REGULATION OF RIBONUCLEOTIDE REDUCTASE ACTIVITY IN INTACT CHO CELLS

Having determined the general properties of ribonucleotide reductase, attention was directed towards studying how the various components within the assay systems interacted with the enzyme. In general, double reciprocal plots of velocity against the concentration of a specific nucleotide were used to determine whether a component affected the affinity of the enzyme for a substrate or effector, the velocity of the reaction, or both. By using intact CHO cells it was hoped that a situation more relevant to the *in vivo* state would exist.

In addition to the various nucleoside triphosphates, which directed enzyme activity and specificity, the reduction of CDP required DTT and $MgCl_2$ (Figure 9a and c) while ADP reduction required DTT (figure 15). Dithiothreitol stimulated both CDP and ADP reduction by increasing the velocity of the reaction (figures 28b and 29). In neither case did the dithiol affect the affinity of the enzyme for the substrates. These findings are consistent with the proposed role of DTT in the reaction. The dithiol apparently reduces a disulfide of the active site which in turn reduces the ribonucleotide substrate (Thelander 1974). The reduction of the disulfide of the active site would thus seem to be required for enzyme activity but not for substrate binding.

Magnesium chloride had a dual effect on CDP reduction as it increased both the velocity of the reaction and the affinity of the enzyme for CDP (figure 28a). Magnesium ions are thought to promote the association of the two non-identical subunits of ribonucleotide reductase to form the holoenzyme capable of reducing CDP (Hopper 1972, Mattaliano *et al* 1981). This assocation would explain the observed increase in velocity caused by $MgCl_2$. The increased affinity of the enzyme for CDP is likely due to the fact that the active site of the enzyme is made up of both

subunits and that their association affects the binding of CDP in a positive way.

The most widely cited model for the allosteric regulation of mammalian ribonucleotide reductase is that proposed by Thelander and Reichard (1979). The nucleoside triphosphate allosteric regulators bind to the M1 subunit (Eriksson et al 1982) at two different types of sites. One site is proposed to bind ATP, dGTP and dTTP and direct the specificity of the enzyme while the other is thought to bind ATP or dATP and control the overall activity of the enzyme (Eriksson et al 1979, 1981b). The regulation of enzyme activity is proposed to occur as follows. In the absence of positive effectors the enzyme is inactive with all ribonucleoside diphosphate substrates. The reduction of CDP and UDP is stimulated by ATP, which presumably binds to the specificity site. Similarly, the reduction of GDP and ADP is stimulated by the binding of dTTP and dGTP, respectively, to the specificity site of the M1 subunit. Reduction of the purines can be further stimulated by the simultaneous presence of ATP with dTTP or dGTP. Presumably ATP binds to the activity site to induce a conformational change that increases the rate of ADP and GDP reduction. DeoxyATP serves as a general inhibitor whose negative effects can be overcome by high concentrations of ATP.

The model was constructed using data obtained with cell free extracts, and there have been suggestions that in vivo regulation of the enzyme may not coincide with the model (Hunting et al 1981, Hunting and Henderson 1981a,b). However the in vivo studies used indirect methods to both manipulate and measure enzyme activity. In order to directly study the regulation of ribonucleotide reductase in a situation as close to the in vivo state as possible, permeabilized CHO cells were used.

As discussed previously, some aspects of the model are clearly different from what is observed in intact CHO cells. Whereas the model predicts that ADP reduction is stimulated by only dGTP, within CHO cells significant activity levels were also observed with GTP and dTTP (figure 22a and b). Similarly, in addition to ATP, the reduction of CDP could be stimulated by dCTP (figure 22c). While the model proposes that the reduction of ADP can be further stimulated by ATP, with intact CHO cells ATP was found to inhibit the reaction at all concentrations tested (figure 27a).

The initial phase in the study of the allosteric regulation of ribonucleotide reductase involved determining how the various positive effectors mediated their response. Activation of CDP reduction by ATP and dCTP seemed to involve two different processes. While ATP was found to increase the affinity of the enzyme for CDP and also increase the rate of reduction (figure 30a), the sole effect of dCTP was to increase the affinity of the enzyme for CDP (figure 30b). Thus it would seem that in its native conformation ribonucleotide reductase is capable of reducing CDP but that the Km value for CDP is prohibitively high. Binding of dCTP to the regulatory site induces a conformational change which facilitates binding of the substrate but does not alter the maximum velocity obtainable. Binding of ATP to the regulatory site induces a conformational change which not only facilitates binding of CDP but also increases the maximum velocity which can be reached. The pattern of ATP activation was similar to that seen with cell free preparations from rat tumor (Moore and Hurlbert 1966). In regards to the reduction of ADP, the nucleotides GTP and dGTP both activated the reaction by increasing both the rate of reduction and the affinity of the enzyme for the substrate (figure 31). Thus like ATP in the

reduction of CDP, the activators of ADP reduction would seem to induce a conformational change which has a dual effect.

The second phase of the study involved determining how two different nucleotide effectors interact with the enzyme when both are present at the same time. These interactions are discussed in terms of their applicability to the proposed Thelander-Reichard model.

Much of the kinetic data obtained with intact CHO cells agreed with the proposals of the model. The competitive inhibition of CDP reduction by UDP (figure 46) strongly suggested the existance of a single enzyme, at least for these two substrates. With respect to CDP, dGTP was found to be a competitive inhibitor of CDP reduction (figure 34). As optimal levels of ATP are present in each assay, it is likely that dGTP competes with ATP for the specificity site and that where dGTP is bound, CDP is excluded from the active site. Thus the observed competition between CDP and dGTP is not for a binding site but rather for the enzyme. A similar competitive inhibition of ADP reduction by ATP with respect to ADP concentration (figure 34) would seem to confirm that as proposed by the model, ATP and dGTP compete for an effector site which controls the specificity of the enzyme.

The kinetic studies also support the proposal that dATP inhibits ribonucleotide reductase by binding to a site distinct from the specificity site. With respect to dGTP, dATP was a non-competitive inhibitor of ADP reduction (figure 36). This pattern of inhibition results when dGTP and dATP bind reversibly, randomly and independently at different sites. With respect to GTP, low concentrations of dATP inhibited the reduction of ADP in a noncompetitive manner, while higher concentrations were found to inhibit the reduction in a competitive manner (figure 37). With respect to ATP, dATP inhibited the reduction

of CDP in a similar manner (figure 38). Low concentrations of dATP did not affect the binding of ATP while high concentrations of dATP were found to compete with ATP for the enzyme. In terms of the proposed model it would seem that at low concentrations, dATP binds to a site distinct from the specificity site, while at high concentrations it can inhibit the reduction of CDP and ADP by displacing ATP and GTP from the specificity site. The binding of dATP to the specificity site has been noted for the reductase enzyme from E. coli (Brown and Reichard 1969b) and calf thymus (Eriksson et al 1981b). However in these systems dATP bound to the specificity site was found to induce the reduction of purines while with intact CHO cells dATP bound to either the specificity or regulatory sites caused inhibition of reductase activity. Moreover, it has been shown with the E. coli enzyme, and implied with the calf thymus enzyme, that the specificity sites have a higher affinity for dATP than the regulatory sites. With intact CHO cells, the opposite would seem to be true, dATP binds first to the regulatory site and then to the specificity site.

While the results discussed above are supportive of the proposed in vitro model, other kinetic studies were not. With respect to dGTP or GTP, the reduction of ADP was inhibited by ATP and dCTP in a non-competitive manner (dGTP and dCTP, figure 40), an uncompetitive manner (GTP and ATP, figure 41) or a mixture of the two (dGTP and ATP, figure 39; GTP and dCTP, figure 42). Each of these inhibition patterns indicates that the activator, GTP or dGTP, can be bound to the enzyme at the same time as the inhibitor, ATP or dCTP. Such results are inconsistent with the model which requires the specificity sites to bind only one nucleotide at a time. The inhibition of CDP reduction by dTTP was found to be uncompetitive with respect to ATP (figure 44).

Such a pattern means that ATP must be bound to the enzyme in order for dTTP to bind. DeoxyGTP was found to inhibit CDP reduction in a non-competitive manner (figure 43) indicating the inhibitor can bind to either the few enzyme or the enzyme-activator complex. Thus the inhibition of CDP reduction by the activators of ADP reductase activity again indicates that two nucleotides which theoretically should compete for the same site can in actuality both be bound to the enzyme at the same time.

These results suggest that the in vivo regulation of ribonucleotide reductase is more complex than indicated by the Thelander-Reichard model. However, using a model based on that proposed by Youdale et al (1982), many of the results observed with intact CHO cells can be explained. According to this model four distinct substrate binding or L1 subunits plus one iron containing subunit (L2) are proposed to exist. The L1 subunits bear many of the properties of the M1 subunit described for calf thymus ribonucleotide reductase, so it is likely that allosteric effectors bind to this subunit. The observed kinetic responses can be explained if it is assumed that each of the L1 subunits has two classes of allosteric binding sites. All four subunits possess a similar regulatory site to which dATP binds to inhibit enzyme activity. Each subunit also possesses a specificity site but unlike the in vitro model, it is proposed that each L1 subunit can bind only those nucleotides which activate the particular substrate it binds. For example, the L1 subunit specific for the reduction of CDP would bind ATP and dCTP to its specificity site while the ADP specific L1 subunit would bind GTP, dGTP and dTTP. If activators specific for each of the substrates bind to different proteins, how then can the activators of ADP reduction inhibit CDP reductase activity and vice versa? The various L1 subunits

are postulated to associate with one another (Youdale et al 1982) and within the in vivo environment, it is likely that the holoenzyme possesses the four separate L1 subunits plus the common L2 subunit. These subunits would presumably have points of interaction with each other, and thus it is conceivable that, for example, the binding of dGTP to the L1·ADP subunit could cause a conformational change that would inhibit the activity of the L1·CDP subunit without requiring the displacement of ATP or dCTP from the L1·CDP specificity site. This proposed model would allow for the control of enzyme specificity required but not disallow simultaneous binding of two different activators. While the model is plausible, purification of the various L1 subunits and exhaustive binding studies are required to prove its validity.

While the model proposed immediately above would seem to account for the inhibition patters observed for ribonucleotide reductase, the synergistic activation of ADP and CDP by various combinations of nucleotides would seem to require a different explanation. Suboptimal levels of ATP plus dCTP were found to induce the reduction of CDP at rates greater than the sum of the two individual activities (Table 12). As both ATP and dCTP are thought to bind to the same allosteric specificity site it is initially difficult to see how the synergism arises. However, if each L1 subunit contained more than one specificity site, a relatively straight forward explanation is possible. If, as is known to occur in the E. coli B1 subunit (Thelander 1973, Thelander and Reichard 1979), the various L1 subunits are composed of two polypeptide chains, it is possible that there are two specificity sites per L1 molecule. Under such a situation the binding of ATP to one of the sites and dCTP to the other might induce a comformation that has a higher rate of activity than the enzyme to which only one type of nucleotide is bound to both sites.

The existence of two specificity sites per L1 subunit would also explain the cooperative binding of ATP observed during the reduction of CDP (figure 38). A similar explanation can be invoked to explain the ability of GTP plus dTTP to induce a rate of ADP reduction greater than that possible with either nucleotide separately (Table 11). In this case the binding of GTP to one specificity site and dTTP to the other is proposed to induce a conformation that reduces ADP at a greater rate than the conformations induced when only one type of effector is bound to both specificity sites. As dGTP induces the greatest rate of ADP reduction (compare figure 22a and b to figure 16), it seems likely that the simultaneous binding of GTP and dTTP induces a conformation more closely resembling that induced by dGTP.

The final case of synergistic activation observed is more difficult to explain. At suboptimal levels of ATP, GTP was found to be an activator of CDP reduction (Table 8). In the model containing four separate L1 subunits, GTP is proposed to bind to a subunit not involved in the reduction of CDP. However, as discussed above, the binding of an effector to one subunit may affect the activity of another subunit if they are associated with one another. Whereas in the other cases an inhibitory effect were observed, it would seem that the conformation of the $L1_{ADP}$ subunit induced by GTP could interact with the $L1_{CDP}$ subunit to enhance the reduction of CDP. As was stated earlier, a great deal of work involving purification and binding studies must be carried out to test the model proposed from the results of this study.

The final phase in the study of the regulation of ribonucleotide reductase in intact CHO cells involved measuring the intracellular pool sizes and comparing them to the kinetic constants determined. The intracellular nucleotide pools of the wild type CHO cells used in this study

are listed in Table 13. In regards to the reduction of CDP, the intracellular nucleotide levels would seem to indicate that only ATP acts as an activator in vivo. The intracellular concentration of CDP (0.017 to 0.026 mM) was similar to the Km value determined for the ATP stimulated reduction (0.021 mM; figure 13) but lower than that of the dCTP stimulated reduction (0.033 mM; figure 24). Moreover, the ATP level within a cell was found to be 40 to 70% of that required for maximum activation while the dCTP level present represented only 1 to 2% of that required for maximum activation. In addition, the reduction of CDP by the suboptimum levels of ATP may be aided by the presence of GTP (see Table 8 and discussion above). These findings coupled with the fact that ATP induced a greater rate of CDP reduction than dCTP would seem to eliminate dCTP as a physiological activator of CDP reduction.

The situation is not as clear in regards to the reduction of ADP. The intracellular concentration of ADP (0.379 to 0.558 mM) indicated that the substrate was saturating for the dGTP (Km = 0.014 mM; figure 20) and GTP (Km = 0.20 mM; figure 23a) activated reductions and near saturating for the dTTP activated reduction (Km = 0.333; figure 23b). At optimum concentrations of the three activators, dGTP induced the greatest rate of ADP reduction with GTP and dTTP exhibiting 65% and 40% of this rate, respectively. However, the intracellular pool of dGTP was undetectable while dTTP and GTP were present at 35% to 55% and 75% to 110%, respectively, of the levels required to induce their maximum effect. Thus dGTP may be the most potent activator of ADP reduction but its low intracellular level may mean that GTP is the major in vivo activator of ADP reductase.

One important consideration must be paid to the interpretation of

the intracellular nucleotide pool levels discussed above. Whereas the values presented represent the average concentration throughout a cell, there is a great deal of evidence that intracellular nucleotide pools are compartmentalized (Skoog and Bjursell 1974, Losman and Harley 1978, Kleineke et al 1979a, Akerboom et al 1979, Kleineke et al 1979b, Genchev 1980, Genchev et al 1980, Böck 1980, Piper 1981) in mammalian cells. If as proposed (Reddy and Pardee 1980, Forsdyke and Scott 1980, Scott and Forsdyke 1980) ribonucleotide reductase is a component of a large multienzyme complex which channels ribonucleoside diphosphates to DNA, the deoxynucleotide levels within this complex are very likely much higher than the average intracellular levels. It would be these locally concentrated levels which would regulate ribonucleotide reductase activity. Thus even though dGTP and dCTP levels are low within the entire cell they may be present within the complex in sufficient concentrations to effect the reduction of ribonucleotides. In order to study this possibility, nuclear nucleotide pool levels should be investigated as they are likely to be much more representative of the nucleotide concentrations encountered by the enzyme.

4. CHARACTERIZATION OF A LOW LEVEL N-CARBAMOYLOXYUREA-RESISTANT CELL LINE.

Initially, it was suggested that the N-carbamoyloxyurea inhibited RNA and protein synthesis via the degradation of cellular DNA (Rosenkranz 1970). Though lacking enzymatic data, Cameron and Jeeter (1973) subsequently suggested that the drug may inhibit ribonucleotide reductase activity. The selection of hydroxyurea-resistant CHO cell lines which exhibited cross resistance to N-carbamoyloxyurea (Lewis and Wright 1978a) gave further support to the suggestion that the drug interacted with ribonucleotide reductase. To resolve the question of the intracellular site(s) of N-carbamoyloxyurea action, a CHO cell line resistant to the effect(s) of the drug was selected as described in Materials and Methods and designated NC^R-30A.

As shown in figure 48a, N-carbamoyloxyurea was cytotoxic for CHO cells in culture and could be used as a selective agent for the isolation of drug-resistant cell lines. The cell line described in this report was phenotypically stable as it was maintained in continuous culture, in the absence of selective agent, for approximately two years without showing a change in cellular resistance. As expected from previous results (Lewis and Wright 1978a), the NC^R-30A cells were cross-resistant to hydroxyurea and guanazole (figure 47b and c). As these drugs are thought to be specific for the inhibition of ribonucleotide reductase (Adams and Lindsay 1967, Turner et al 1966, Frenkel et al 1964, Brockman et al 1970, Lewis and Wright 1974), these findings support the contention that N-carbamoyloxyurea also acts on this enzyme. Also consistent with this hypothesis was the finding that N-carbamoyloxyurea blocked the synthesis of DNA (figure 48), which requires the deoxynucleotides generated by ribonucleotide reductase. Moreover, the drug-resistant cell line showed greater incorporation of thymidine into

DNA than did the parental cells in the presence of the same concentrations of N-carbamoyloxyurea.

To test the idea that ribonucleotide reductase was an intracellular site of N-carbamoyloxyurea action, the properties of the enzyme were investigated in intact NC^R-30A cells using the permeabilization assay described. In regards to optimum assay conditions, the mutant and wild type cell lines were virtually identical (Table 14). The response of CDP and ADP reduction to activation by various nucleoside triphosphates was also very similar for the two cell lines (compare Table 15 to Table 7). In addition, dATP was found to inhibit CDP and ADP reduction in NC^R-30A cells (figures 50a and 51a) to the same extent as was observed with parental wild type cells (figure 25). The inhibition of CDP reduction by dGTP (figure 50b and 26a) and GTP (figures 52c and 26b) and of ADP reduction by ATP (figures 51 and 27a) were also the same in both cell lines.

From the results discussed above, it was concluded that the mutant enzyme was not structurally altered from that which exists in the parental wild type cell. The only observed difference in ribonucleotide reductase activities between the two cell lines was an apparent increase in the levels of both CDP and ADP reduction found with the NC^R-30A cells (compare figures 49 to figures 11 and 19). Analysis of CDP and ADP reduction in the parental and drug resistant cell lines using double reciprocal plots of velocity against substrate competition confirmed the increased activity levels for the NC^R-30A cells (figure 52). For both the purine and pyrimidine ribonucleoside diphosphates, the plots were linear and showed apparent Km values of 0.095 mM ADP (both cell lines) and 0.033 mM CDP (NC^R-30A cells) or 0.023 mM CDP (WTC125 cells). While the Km values showed little or no change for the

mutant enzyme, there was a significant increase in both CDP and ADP reductase activity levels. A two-fold increase (0.49 to 1.01 nmoles dCDP formed/5 x 10^6 cells/hour) was observed for CDP reductase levels and a 1.8 fold increase (1.00 to 1.83 nmoles dADP formed/5 x 10^6 cells/hour) observed for ADP reductase levels.

When the pattern of N-carbamoyloxyurea inhibition of ribonucleotide reductase was examined in double reciprocal plots, enzyme activity in both cell lines was inhibited in a mixed fashion with CDP as the substrate (figure 53) and in a non-competative manner with ADP (figure 54). Intercept plots were linear and yielded K_i values for N-carbamoyloxyurea of 2.0 mM (NC^R-30A cells) and 2.3 mM (WTC125 cells) for CDP reduction and 2.3 mM (both cell lines) for ADP reduction. These results confirm the earlier suggestion (Cameron and Jeeter 1973, Lewis and Wright 1978a) that N-carbamoyloxyurea does indeed inhibit ribonucleotide reductase activity. Moreover, the unchanging K_i values of the wild type and mutant enzymes confirm that in NC^R-30A cells, resistance to N-carbamoyloxyurea was accompanied solely by the overproduction of the reductase activity. In both WTC125 and NC^R-30A cells, hydroxyurea and guanazole inhibited CDP reduction in an uncompetitive manner (figures 55 and 57) but showed a mixture of uncompetitive and non-competitive inhibition in the reduction of ADP (figures 56 and 58). Once again intercept plots were linear and yielded K_i values of 0.20 mM and 0.24 mM hydroxyurea for CDP reduction and 0.16 mM and 0.17 mM hydroxyurea for ADP reduction with WTC125 and NC^R-30A cells, respectively. The K_i values determined for guanazole were 1.15 mM for the reduction of both substrates by WTC125 cells and 1.25 mM and 1.10 mM for the reduction of CDP and ADP, respectively, by NC^R-30A cells. As expected, ribonucleotide reductase from the mutant cells was as sensitive to

hydroxyurea and guanazole as the wild type enzyme. Cellular resistance of the NC^R-30A cells to the drugs was due solely to an increase in ribonucleotide reductase levels. The data strongly suggests that like hydroxyurea and guanazole (Åkerblom et al 1981, Wright and Lewis 1974), N-carbamoyloxyurea acts as a scavenger of the tyrosine free radical of the enzyme. However, as the inhibition patterns observed with hydroxyurea and guanazole differ from those seen with N-carbamoyloxyurea, it is possible that differences exist in the way N-carbamoyloxyurea interacts with the enzyme as compared to the other two drugs.

All three drugs, N-carbamoyloxyurea, guanazole and hydroxyurea, were shown to be equally effective in the inhibition of both purine and pyrimidine reduction. This disagrees with some in vivo studies which suggest hydroxyurea is selectively toxic for purine reduction (Skoog and Nordenskjöld 1971, Walters et al 1973). However the results do agree with in vitro enzyme studies which indicate CDP, ADP and GDP reductase activities are all equally sensitive to hydroxyurea (Lewis and Wright 1978b, Koropatnick and Wright 1980, Dick and Wright 1980, Kuzik and Wright 1980). The discrepancy between the in vivo and in vitro results may be due to the disruption of some aspect of the multienzyme complex, of which ribonucleotide reductase is postualted to be a component (Reddy and Pardee 1980, Scott and Forsdyke 1980, Forsdyke and Scott 1980, Elford 1974), by the extraction procedure or even the relatively gentle permeabilization treatment. Another possibility is that dilution of the endogeneous nucleotide pools, by the extraction or permeabilization procedures, in some way affects the response of ribonucleotide reductase to inhibition by hydroxyurea.

The increase in ribonucleotide reductase activity levels exhibited by NC^R-30A cells was accompanied by a decrease in the doubling time of

the cells to 12 hours from 16 hours seen with WTC125 cells. This situation was reminiscent of the correlation observed between ribonucleotide reductase levels and the growth rates of a series of hepatomas (Elford 1972). This reciprocal relationship between enzyme level and growth rate is supportive of the proposed key role of ribonucleotide reductase in cell growth.

Studies of ribonucleotide reductase activity in cell lines resistant to hydroxyurea and guanazole have identified three classes of drug resistant mutants. Mutants of the first class contain an apparent structural alteration to the enzyme which results in the reductase activity of these cells being less sensitive to drug inhibition (Wright et al 1980, Lewis and Wright 1974, Lewis and Wright 1978b). In addition to K_i value changes, these mutants frequently exhibit alterations in other kinetic parameters, such as substrate Km values. The most logical explanation for results obtained with these cells is that they contain a structural gene mutation. A second class of drug resistant mutants possesses reductase activity with a wild type sensitivity to drug inhibition but with enhanced intracellular levels of enzyme activity (Lewis et al 1978, Wright et al 1980, Koropatnick and Wright 1980, Lewis and Wright 1979, Lankinen 1980, Åkerblom et al 1981). The third class of drug resistant mutants contain a combination of the first two alterations described. These mutants appear to contain elevated levels of a ribonucleotide reductase enzyme which has a structural alteration rendering it less sensitive to inhibition by drug (Kuzik and Wright 1980, Koropatnick and Wright 1980). The NC^R-30A cell line is clearly a class two type of mutant.

Mutants of the class two type should be extremely useful for investigating regulatory mechanisms of gene expression in mammalian cells.

Several mechanisms by which a mutant cell line might increase its intracellular level of a specific enzyme above that of its parental line are known to exist. Sinensky et al (1979) have isolated a regulatory mutant defective in the response of synthesis of a specific protein, 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, to normal environmental regulators. The mutant was shown to possess normal levels of HMG CoA reductase which were degraded at the same rate as in the wild type cells. However, the activity was not repressed by 25-hydroxycholesterol as in the wild type cells. The same laboratory has also isolated a CHO mutant which maintains high levels of HMG CoA reductase because of defective degradation of the enzyme (Sinensky et al 1980). Increased stability of mRNA has been implicated in the enhancement of dihydrofolate reductase levels in mouse cells (Leys and Kellems 1981) and it may be that in certain cases mutants acquire resistance to a drug through altered mRNA metabolism. Resistance to methotrexate, N-(phosphonacetyl)-L-aspartate and cadmium may be achieved via amplification of the genes coding for dihydrofolate reductase (Schimke et al 1978), the multifunctional protein containing the enzymatic activities carbamyl phosphate synthetase, aspartate transcarbamylase and dihydroorotase (Wahl et al 1979) and metallothionein (Beach and Palmiter 1981), respectively. The amplification of the genes is accompanied by an elevation in level of the corresponding protein. For methotrexate resistant cells which have amplified their number of dihydrofolate reductase genes, proportional increases in the specific mRNA and enzyme levels have been reported (Melera et al 1980). The gene amplification model for the overproduction of enzyme activities is of particular interest as there is evidence for the occurrence of this process during cancer transformation (Pall 1981, Lavi 1982).

Although a number of different mechanisms for increased enzyme activity in NC^R-30A cells are possible, examination of reductase activity in NC^R-30A and WTC125 cells in the presence of actinomycin D suggested that the overproduction in the resistant cells could involve mRNA metabolism. When WTC125 cells were treated with actinomycin D (either 1 or 10 µg/ml of culture medium) both CDP and ADP reductase levels decreased in a first-order manner with a half life of 4.6 to 4.8 hours (figure 63 and 64). Such was not the case with NC^R-30A cells. In regards to both CDP and ADP reduction, the drug-resistant cell line showed fluctuating levels of activity after the addition of actinomycin D (figures 63 and 64). After an initial rapid decrease ($t_{\frac{1}{2}}$ = 1.4 to 1.9 hours), there was an increase in both CDP and ADP reductase activities which peaked 3 hours after addition of actinomycin D. The peaks of activity were followed by a second decrease which reached a minimum 4 hours after the addition of drug for CDP reduction and at 5 hours for ADP reduction. There was a small rise in each activity which lasted for one hour followed by a decrease which persisted for the duration of the experiment. These final decreases in activity had $t_{\frac{1}{2}}$ values similar to those observed for WT enzyme activities. It is interesting to note that whereas in the WTC125 cells CDP and ADP reductase levels had decreased by 70% 8 hours after the addition of actinomycin D, the decrease in NC^R-30A cells was only 20% for both activities. Thus overall, actinomycin D decreased ribonucleotide reductase activity to a much greater extent in WTC125 cells than in NC^R-30A cells.

Alterations in the metabolism of NC^R-30A cells would seem to be limited to mRNA as cycloheximide, an inhibitor of protein synthesis, induced similar effects in both WTC125 and NC^R-30A cells (figures 61 and 62). Addition of the drug to both cell lines caused a first order

decrease in the levels of CDP and ADP reductase activity. For CDP reductase the half life was 1.7 hours while for ADP reductase the value was between 2.8 and 3.3 hours.

While it is presently unclear as to why the levels of CDP and ADP reduction in NC^R-30A cells fluctuate upon addition of actinomycin D to the cells, it is interesting to note that other investigations have found mRNA involved in ribonucleotide reductase synthesis to possess unusual characteristics. The observation that actinomycin D decreased but did not eliminate the rise of reductase levels observed during the cell cycle (Murphree et al 1969) has led to speculation that there exists carryover of mRNA from one cycle to another (Moore 1976). This theory would require that the translation of the pre-existing mRNA be turned on by some signal, and thus it is possible that the signal generation or reception has been altered in NC^R-30A cells. However, further work is obviously required to test this point.

5. SELECTION OF A SERIES OF CHO CELL LINES WITH INCREASING RESISTANCE TO N-CARBAMOYLOXYUREA

While the work with the WTC125 and NC^R-30A cell lines confirmed that N-carbamoyloxyurea does inhibit ribonucleotide reductase in a manner very similar to hydroxyurea and guanazole, certain differences between the effects of N-carbamoyloxyurea and the other two drugs in CHO cells and the reductase were also noticed. Examination of the WT CHO cells showed that the ratio of the concentration of hydroxyurea required to inhibit 50% of the enzyme activity (0.16 to 0.20 mM) to the concentration of hydroxyurea that reduced the relative plating efficiency to 10% was on the average 0.86. Similarly the K_i (1.15 mM) to D_{10} (1.21 mM) ratio for guanzole inhibition was 0.95. Thus for hydroxyurea and guanazole it would seem that inhibition of 50% of the

reductase activity decreases the plating efficiency to approximately 10%. This correlation was not found with N-carbamoyloxyurea. With this drug, the ratio of K_i (2.0 to 2.3 mM) to D_{10} (0.32 mM) values was seven. These results suggest that while ribonucleotide reductase is one site of action for N-carbamoyloxyurea, the drug may have other site(s) of action. Further support for this idea comes from the cellular resistance data shown in figure 47 and the enzyme activity levels shown in figure 54. The two fold increase in CDP reductase activity and the 1.8 fold increase in ADP reductase activity observed in NC^R-30A cells, as compared to WTC125 cells, was sufficient to account for the 1.5 to 1.6 fold increase in cellular resistance to hydroxyurea and guanazole observed for the mutant cell line. However, the enhanced enzyme level count not totally account for the 2.6 fold increase observed with N-carbamoyloxyurea. To further clarify the effect of N-carbamoyloxyurea on mammalian cells, a series of CHO cell lines selected for increasing resistance to the drug were isolated by the procedure described in Materials and Methods.

The increases in cellular resistance to N-carbamoyloxyurea exhibited by the cell lines in the series did not match the increases observed for hydroxyurea and guanazole (figures 63 and 64). Whereas the cells showed a steadily increasing resistance to N-carbamoyloxyurea, resistance to hydroxyurea and guanazole occurred at only three points; WTC125 to NC^R-30A, NC^R-30A to NC^R-30A0.7 and NC^R-30A3 to NC^R-30A5. These results indicated that while N-carbamoyloxyurea does act at ribonucleotide reductase, thus inducing cross-resistance to hydroxyurea and guanazole, at least one other site of action does exist. This view was also supported by the observation that, in general, each cell line in the series was more resistant to the selective agent than to

hydroxyurea or guanazole. For example, NC^R-30A5, the most resistant line was 21 fold more resistant to N-carbamoyloxyurea than the wild type population, while resistance to hydroxyurea and guanazole increased by only 11 to 12-fold. As hydroxyurea and guanazole have only one confirmed intracellular site of action, ribonucleotide reductase, a site shared by N-carbamoyloxyurea (see above), it would seem that the alterations in reductase activity increase cellular resistance 11 to 12-fold while a further approximately 2-fold increase is due to modifications involving a second site(s) of action.

The differing effects of N-carbamoyloxyurea and hydroxyurea upon the uptake of thymidine into DNA for the series of cell lines (figure 65) confirmed the cellular resistance data. Again the presence of a second site(s) of N-carbamoyloxyurea action was most evident in the NC^R-30A0.7 through NC^R-30A3 cell lines. For these cell lines resistance to hydroxyurea, and thus the activity of ribonucleotide reductase, did not change while resistance to N-carbamoyloxyurea continued to increase.

To further investigate the second site(s) of N-carbamoyloxyurea action, the effect of the drug on protein and RNA as well as DNA synthesis was studied. In contrast to reports with E. coli cells (Rosenkranz 1970) which indicated RNA and protein synthesis to be more sensitive than DNA synthesis to the effects of the drug, N-carbamoyloxyurea was found to have essentially no inhibitory effect on RNA synthesis and to block protein synthesis at only very high concentrations (figure 66) in CHO cells. Even though N-carbamoyloxyurea could inhibit protein synthesis if present at high enough levels, the second altered site(s) in the series of mutants would not seem to involve this process for two reasons. First protein synthesis in NC^R-30A5 cells was as sensitive if not more so to the drug than in WTC125 cells

(figure 66b and c). If the second altered site(s) involved protein synthesis, the uptake of leucine into protein would be less affected by N-carbamoyloxyurea in the NC^R-30A5 cells. Secondly when WTC125 cells were treated with 0.4 mM N-carbamoyloxyurea (the concentration used in the section of the NC^R-30A cell line) protein synthesis seemed unaffected (figure 66a) making it unlikely this cellular process was altered in the mutant. It would thus seem that the second site of action for N-carbamoyloxyurea involves the synthesis of DNA.

The incorporation of thymidine into the DNA of WTC125 cells, measured in the presence of N-carbamoyloxyurea or hydroxyurea at concentrations which inhibited the reductase activity to the same extent (the K_i values for each drug), was found to be significantly more sensitive to N-carbamoyloxyurea. This result supports the proposal that the second site(s) of N-carbamoyloxyurea involves the synthesis of DNA. It has been suggested that in E. coli (Rosenkranz 1970), calf thymus (Rosenkranz and Rosenkranz 1969) and Tetrahymena (Cameron and Jeeter 1973) N-carbamoyloxyurea may function in the degradation of cellular DNA. It is therefore possible that the second site(s) of drug action may involve a DNA repair mechanism. Further work with the drug resistant lines could provide more information on this subject.

The existence of a second site of action for N-carbamoyloxyurea in mammalian cells may have ramifications in the field of chemotherapy. Even though N-carbamoyloxyurea is some 12-fold less potent than hydroxyurea as an inhibitor of ribonucleotide reductase (WT K_i values of 2.3 mM N-carbamoyloxyurea versus 0.18 mM hydroxyurea), it is only slightly less cytotoxic (WT D_{10} values of 0.32 mM N-carbamoyloxyurea and 0.21 mM hydroxyurea). If N-carbamoyloxyurea, like hydroxyurea and guanazole, acted solely to inhibit ribonucleotide reductase activity,

the D_{10} value for the drug, based on a K_i value of 2.3 mM (figures 53 and 54) would be 2.56 mM N-carbamoyloxyurea. As the true D_{10} value is 0.32 mM, this indicates that N-carbamoyloxyurea is some 8-fold more toxic for its second site of action. These properties suggest that although N-carbamoyloxyurea has not been tested as to antineoplastic activity such tests may be worthwhile.

Because the series of N-carbamoyloxyurea-resistant cell lines were cross-resistant to hydroxyurea and guanazole, ribonucleotide reductase activities of the various cell lines were investigated. The results support the previous suggestion (see above) that N-carbamoyloxyurea, hydroxyurea and guanazole have a similar mechanism of ribonucleotide reduction inactivation. As shown by figures 68 and 69 and Table 16, the enzyme activities from NC^R-30A2 and NC^R-30A5 cells, each exhibit an equal decrease in sensitivity to all three drugs. It is very likely that similar to hydroxyurea (Åkerblom et al 1981), N-carbamoyloxyurea inhibits the reductase by interacting with one of the two enzyme subunits (M2) and inactivating a tyrosine free radical required for the reductive process. However, as noted earlier, some differences in the precise manner in which N-carbamoyloxyurea and hydroxyurea or guanazole inhibit enzyme activity may exist, since differences in enzyme inhibition patterns between N-carbamoyloxyurea and the other two drugs in kinetic experiments were observed.

The studies reported here support previous observations with hydroxyurea-resistant mouse lines (Wright et al 1981, Koropatnick and Wright 1980) which showed changes in CDP reductase activity are not always coordinated with changes in ADP reduction. Although the selection of the NC^R-30A line was accompanied by a similar increase for both CDP and ADP reductase activity (about two-fold), this relationship was not

found for enzyme activity in the other resistant cell lines (figure 67). CDP reductase activity was consistently higher than the wild type levels in the cell lines NC^R-30A 0.7 through to NC^R-30A5, whereas ADP reductase activity was found at wild type, slightly lower than wild type or slightly higher than wild type levels in these lines. Even the induction of enzyme activity in drug-resistant cells by culturing them in the presence of N-carbamoyloxyurea, showed a lack of coordination in pyrimidine and purine reductase increases, since significant elevations in CDP reductase activity were observed but only modest increases in ADP reductase.

The lack of coordination in pyrimidine and purine reductase activity changes in resistant lines could be explained if, as suggested (Cory and Mansell 1975a, Lewis et al 1978, Kuzik and Wright 1979, Hopper 1978, Collins et al 1972, Peterson and Moore 1974, 1976), more than one type of ribonucleotide reductase exists in mammalian cells, or if the mammalian ribonucleotide reductase system consists of multiple, independent substrate binding sites or proteins (Cory 1979, Youdale et al 1982). Other findings presented in this report would also seem to suggest that CDP and ADP reduction are not carried by the same enzyme in permeabilized CHO cells. Whereas CDP reduction was greatly stimulated by magnesium ions and reached optimum activity in the presence of 7 mM $MgCl_2$ (figure 9a), ADP reduction was inhibited at all concentrations of $MgCl_2$ tested (figure 17). When cycloheximide was added to WTC125 or NC^R-30A cells both CDP and ADP reductase levels decreased in a first order process. However, the half lives of the activities were found to be 1.7 hours for CDP reductase (figure 59) and approximately 3 hours for ADP reductase (figure 60). The response of ADP and CDP reduction to $FeCl_3$ (figure 71) also differed. Whereas CDP reduction

could be stimulated by 0.2 mM $FeCl_3$, ADP reduction was inhibited at all concentrations tested. Even the inhibition of CDP and ADP reduction by N-carbamoyloxyurea, hydroxyurea and guanazole would seem to support the existence of different enzymes. While both activities exhibited the same K_i values for each of the three drugs, the pattern of inhibition induced by the drugs differed for CDP reduction as compared to ADP reduction (figures 53 to 58). Whereas N-carbamoyloxyurea inhibited CDP reduction in a mixed manner, it inhibited ADP reduction in a non-competitive fashion. While hydroxyurea and guanazole inhibited CDP reduction in an uncompetitive manner, the pattern of inhibition appeared to be a mixture of noncompetitive and uncompetitive inhibition for ADP reduction. These results not only suggest an apparent non-identity of the CDP and ADP reductase enzymes but, as noted previously, also indicate differences in the way N-carbamoyloxyurea interacts with the enzymes when compared with hydroxyurea and guanazole.

Recently, Youdale et al (1982) have succeeded in purifying to homogeneity a protein subunit specific for the reduction of CDP, as well as the iron containing subunit (L2) from regnerating rat liver. Both subunits were required for activity and would reduce CDP but not the other three ribonucleotide substrates. Molecular weight studies of the holoenzyme and the subunit proteins have suggested the existence of four different L1 subunits, each specific for the reduction of a single ribonucleotide, bound to a common L2 subunit (Youdale and MacManus 1979, Youdale et al 1972). The results obtained with the series of N-carbamoyloxyurea resistant cell lines would seem to fit nicely with this proposal. Resistance of enzyme activity to inhibition by hydroxyurea, guanazole and N-carbamoyloxyurea, a property of the L2 subunit (Åkerblom et al 1981), was the same for ADP and CDP reduction in all

cell lines tested. However, the levels of reductase activity, which are dependent on both the L2 and L1 subunits, were different with CDP and ADP as substrates. These results support the proposal that CDP and ADP reductase share a common L2 subunit but possess different L1 subunits. The effect of dATP on CDP and ADP reduction would also seem to support the model proposed by Youdale et al. As an allosteric effector of the reductase activity, dATP binds to the M1 (or L1) subunit (Eriksson et al 1982). The differing sensitivities of ADP and CDP reduction (figures 25, 32, 33, 36, 38, 50 and 51) to dATP inhibition would seem to indicate that different M1 subunits exist for the two substrates. Also, as discussed earlier (section 3), the regulation of ribonucleotide reductase activity in intact CHO cells would seem to be more easily explained by a model that possesses four separate substrate binding subunits as opposed to the single enzyme concept.

While there does exist a great deal of evidence supporting the existence of multiple, independent L1 (M1) proteins, the possibility that a single enzyme catalyzes the reduction of all four ribonucleotides cannot be ignored. Enzymatic studies with purified preparations of ribonucleotide reductase from calf thymus (Eriksson et al 1979, Engström et al 1979) as well as genetic studies with mouse T-lymphoma cells (Eriksson et al 1981b, 1982) are supportive of the idea of a single mammalian ribonucleotide reductase which reduces all four ribonucleotides. Indeed some results obtained with intact CHO cells in the present study did seem to indicate the existence of a single enzyme. As shown by figure 46, UDP did inhibit the reduction of CDP in a competitive manner. This would seem to indicate the two substrates were competing for the same active site. Treatment of WTC125 cells with actinomycin

D induced a first order decrease in both CDP and ADP reductase activities and in each case the half life determined was 4.6 to 4.8 hours (figures 61 and 62). In NC^R-30A cells, both CDP and ADP reductase reacted to the presence of actinomycin D in a most unusual way but significantly, the two activities were virtually identical in their pattern of response to the drug (figures 61 and 62). Finally both CDP and ADP reduction responded to DTT in a virtually identical manner. Maximum activation with both substrates occurred at 6 mM DTT (figure 9c and 15) while Ka values of 2.7 and 2.8 mM DTT were determined for ADP and CDP reduction, respectively.

If as suggested (Eriksson et al 1979, Engström et al 1979, Eriksson et al 1981b) a single enzyme does catalyze the reduction of both purine and pyrimidine substrates, how can the non-coordination of CDP and ADP reductase levels be explained. One possibility involves the nature of the proposed structural alterations in the reductase in cell lines NC^R-30A0.7 through to NC^R-30A5. It has recently been suggested that different mechanisms regulate the levels of M1 and M2, and that fluctuations in enzyme activity are primarily dependent upon the level of the M2 subunit (Eriksson and Martin 1981). As N-carbamoyloxyurea as well as hydroxyurea and guanazole likely act at the M2 subunit, interesting possibilities arise. Enzyme activity must depend upon a precise interaction between the two subunits and it is reasonable to assume that alterations in the structure of M2 might affect its interaction with M1, thus modifying enzyme characteristics. The alterations in substrate Km values obtained with enzyme from

resistant cells support this idea. The critical association of the subunits in regards to the characteristics of the enzyme is also shown in figure 28a. Here, $MgCl_2$ which presumably promotes the association of the two subunits (Hopper 1972, Mattaliano et al 1981) was found to enhance not only the rate of reduction but also the affinity of the enzyme for CDP. If M1 binds all pyrimidine and purine substrates, as B1 does in E. coli, and the M2 subunit is structurally altered in resistant cells it could be argued that changes in Km values reflect a modification in the interaction between the two subunits. Since enzyme activity and substrate specificity are dependent upon binding of nucleotide substrates and effectors (Wright et al 1981), it is possible to derive models based upon modified subunit interaction and altered nucleotide affinities which could result in changes of pyrimidine relative to purine reductase activities. In support of this view it is worth noting that the only cell line which exhibited coordinated changes in CDP and ADP reductase was NC^R-30A, which contained enzyme that was apparently not structurally altered. Further studies with purified enzyme components from resistant and sensitive cell lines are required to determine whether reduction of ribonucleotides is carried out by a single enzyme or not.

The non-coordination of CDP and ADP reductase activity also has a bearing upon cellular resistance of the N-carbamoyloxyurea-resistant cells to hydroxyurea and guanazole. In previous studies with hydroxyurea-resistant cell lines, increased cellular resistance could be totally accounted for by increased levels of enzyme (Lewis and Wright 1979), decreased sensitivity of the enzyme to drug inhibition (Lewis and Wright 1978a) or to a combination of these two effects (Kuzik and Wright 1980, Koropatnick and Wright 1980). In the NC^R-30A2 and NC^R-30A5 cell

lines, the increases in cellular resistance to hydroxyurea and guanazole observed could be explained in terms of altered CDP reductase activity but not in terms of ADP reductase activity. Thus cellular resistance appears to require significant elevations in CDP but not ADP reductase activity. Such findings fit in with the results of those studies (Bjursell and Reichard 1973, Bjursell et al 1972, Meuth et al 1976, Reichard 1978, Walters et al 1973) which indicate dCTP pool levels, and therefore CDP reductase activity, in mammalian cells correlate with DNA synthesis more closely than any of the other deoxyribonucleoside triphosphate pools. Recently (Nicander and Reichard 1981), the hypothesis that dCTP binds at an allosteric site of a multienzyme complex (Reddy and Pardee 1980) involved in the replication of DNA, to increase the affinity of the system for deoxynucleoside triphosphate substrates, has been advanced. It appears to be especially critical for the cells to maintain adequate levels of dCTP, and therefore CDP reductase activity, if resistance to DNA synthesis inhibitors like hydroxyurea, guanazole and N-carbamoyloxyurea is to occur.

6. NUCLEOTIDE PHOSPHOHYDROLASE ACTIVITY IN CROTALUS ATROX VENOM

During the investigation of the effect of $FeCl_3$ on ribonucleotide reductase activity, it was discovered that the venom of Crotalus atrox contained an iron stimulated activity that increased background measurements of the reductase assays. Unless taken into consideration, the effect of this iron stimulated activity would lead to inaccurate estimations of the reductase activity. The $FeCl_3$ was shown to have no effect on the operation of the Dowex-1-borate columns. Nor was the increase in background levels due to a heat resistant $FeCl_3$ activated activity of the CHO cells. Reverse phase HPLC analysis of the effluent of

Dowex-1-borate columns (figure 73) revealed that $FeCl_3$ caused an increase in the amount of cytosine or adenine present in the effluent. Moreover, the increase in the levels of the pyrimidine and purine bases correlated with the increased background levels induced by $FeCl_3$. The increase in background levels required only the presence of $FeCl_3$ and the substrate, either CDP or ADP. All other components of the reaction mixture could be eliminated without altering the $FeCl_3$ induced elevation in background levels. It appears that the Crotalus atrox venom contained an $FeCl_3$ stimulated activity capable of cleaving sugar-base N-glycosidic bonds. When cytidine or adenosine was incubated with $FeCl_3$ under conditions identical to those which induced cleavage of the N-glycosidic bonds in CDP or ADP, no release of base moieties was observed (figure 72). Therefore the increase in adenine and cytosine levels was not due to the presence of an iron-dependent nucleosidase. Instead, the activity in snake venom, which is known to contain many nucleotide metabolizing enzymes (Devi 1971, Russell 1980), appears to require a nucleoside phosphate for activity. This suggests that hydrolysis of the N-glycosidic linkage involves a pyrimidine/purine 5'-nucleotide phosphoribohydrolase activity (Imada et al 1967) activated by $FeCl_3$. Even though the activity can use either pyrimidine or purine substrates, at $FeCl_3$ concentrations which induced maximum activity the cleavage of CDP occurred at approximately twice the rate observed with ADP (figure 70).

In regards to the measurement of ribonucleotide reductase activity, these observations indicate that false estimates can occur if proper control experiments are not performed for each assay carried out in the presence of iron. Using carefully determined background levels it was found that in CHO cells $FeCl_3$ was inhibitory for ADP reductions and varied from a mild stimulator to a significant inhibitor of CDP

reductions depending upon the $FeCl_3$ concentration present.

In general, enzymes associated with synthetic reactions or energy production are not present in snake venoms (Devi 1971). Instead, the large numbers of enzymes found usually exhibit hydrolytic activities. This fits in with the observation that Crotalus atrox venom contains a $FeCl_3$ dependent activity involved in cleaving sugar-base bonds of nucleotides. This interesting activity deserves further investigation to determine, for example, whether it may be involved in a toxic mechanism.

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