THE UNIVERSITY OF MANITOBA

ISOLATION AND CHARACTERIZATION OF HYDROXYUREA-RESISTANT MUTANTS OF CHINESE HAMSTER OVARY CELLS, AND STUDIES ON EUCARYOTIC RIBONUCLEOTIDE REDUCTASE

BY

WILLIAM HARRY LEWIS

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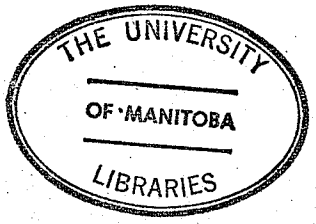

TO MAZ

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Scientific discovery, homosexual liberation,
emotional and sexual intimacy,
ultimately I experience all
for my own pleasure and future security.

ABSTRACT

Somatic cell mutants resistant to the antitumor drug hydroxyurea have been isolated from Chinese hamster ovary (CHO) cells. Hydroxyurea, at concentrations of 0.33 mM, stops DNA synthesis and cell division, reduces plating efficiency to 10^{-5} and inhibits the activity of the enzyme ribonucleotide reductase in wild-type CHO cells. Clones which are 6- to 19-fold more resistant to hydroxyurea than wild-type can be obtained from a CHO cell population by a single step selection in the presence of the drug. The phenotypes of the hydroxyurea-resistant clones were reproducible with high fidelity and stable over long intervals of growth in the absence of selecting drug. The spontaneous mutation rate of resistance to 0.33 mM hydroxyurea was estimated by Luria-Delbrück fluctuation analysis to be 5×10^{-6} per cell per generation. Cell-cell hybridization experiments using hydroxyurea-resistant clones and wild-type clones indicated that resistance to hydroxyurea behaves as a codominant trait and that the marker can be useful for selection of somatic cell hybrids.

Ribonucleotide reductase was partially purified from wild-type CHO cells by novel techniques of sequential affinity chromatography on ATP-agarose, blue dextran-Sepharose and GTP-agarose. The final specific activity achieved was higher than that reported for any other eucaryotic ribonucleo-

tide reductase. The optimal assay conditions and kinetic characteristics of the partially purified enzyme from CHO cells were similar to those of the enzyme from other mammalian sources.

Comparative studies with partially purified ribonucleotide reductase preparations from wild-type and one hydroxyurea-resistant clone indicated that the enzyme activity from the resistant cells was significantly less sensitive to hydroxyurea than the wild-type enzyme. Studies with three of the ribonucleotide substrates indicated that the altered enzyme had 3- to 6-fold higher Ki values for hydroxyurea inhibition. The results are consistent with mutation to hydroxyurea-resistance involving a change in a structural gene for ribonucleotide reductase. The genetic characterization data strongly suggest that hydroxyurea-resistance is due to mutation of cellular DNA sequences as opposed to epigenetic changes.

A technique was developed to measure ribonucleotide reductase levels in whole cells made permeable to nucleotides by treatment with the detergent Tween-80. The permeabilizing treatment resulted in the removal of low molecular weight inhibitors of ribonucleotide reduction while preserving high enzyme activity and a linear response to enzyme concentration. The permeabilized cell assay was used to measure CDP and GDP reductase activities in CHO cells synchronized by isoleucine starvation. CDP reductase

activity was low in G1-arrested cells, but increased ten-fold by 16 hours after the readdition of isoleucine to the growth medium. GDP reductase which is present at much higher levels is similarly induced after isoleucine addition, but only by two-fold. The maximum activity of both CDP and GDP reductases occurred from 14 to 16 hours after isoleucine addition. This corresponded to the period of maximum DNA synthesis.

When ribonucleotide reductase activity from one hydroxyurea-resistant clone was examined for sensitivity to the drug, it exhibited a sensitivity indistinguishable from wild-type enzyme activity. The hydroxyurea-resistant clone, however, was found to contain four- to seven-fold higher levels of CDP and GDP reductase activities when compared to the parental or revertant cell lines. The increased ribonucleotide reductase levels in this clone only occurred during the DNA synthetic or S phase of the cell cycle.

The hydroxyurea-resistant CHO clones showed cross-resistance to the antitumor drug guanazole as well as to the hydroxyurea structural analogs, N-carbamoyloxyurea, formamidoxime and hydroxyurethane. Studies carried out on cellular plating efficiencies and ribonucleotide reductase activities indicated that all these compounds owe their primary cytotoxicity to an inhibition of ribonucleotide reductase.

HS3, a highly phosphorylated dinucleoside isolated from the fungus Achlya and CHO cells starved of glutamine, was shown to inhibit ribonucleotide reduction in enzyme preparations from both the fungal and mammalian cells. Studies with HS3 and partially purified mammalian ribonucleotide reductase indicated that the compound non-competitively inhibited the reduction of varying concentrations of the substrates CDP, ADP and GDP with Ki values of 23 μM, 14 μM and 16 μM respectively. Increased biosynthesis of HS3 was correlated with a decline in the rate of DNA synthesis and ribonucleotide reductase activity in both Achlya and CHO cell systems.

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ABBREVIATIONS

α-MEM	- alpha minimal essential medium
ADP	- adenosine 5'-diphosphate
AdR	- deoxyadenosine
AG	- 8-azaguanine
AMP	- adenosine 5'-monophosphate
AR	- adenosine
ara-C	- 1-β-D-arabinofuranosyl cytosine
ATP	- adenosine 5'-triphosphate
CDP	- cytidine 5'-diphosphate
CdR	- deoxycytidine
CHO	- Chinese hamster ovary
CMP	- cytidine 5'-monophosphate
cpm	- counts per minute
CTP	- cytidine 5'-triphosphate
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
DTE	- dithioerythritol
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
EMS - ethylmethane sulfonate
EPR - electron paramagnetic resonance
FAD - flavin adenine dinucleotide
FCS - fetal calf serum
GAT - glycine, adenosine, thymidine
GDP - guanosine 5'-diphosphate
GdR - 2'-deoxyguanosine
GMP - guanosine 5'-monophosphate
GR - guanosine
GTP - guanosine 5'-triphosphate
HAU - haemagglutination unit
HEPES - N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HU^R - hydroxyurea-resistant
MNNG - N-methyl-N'-nitro-N-nitrosoguanidine
NADH - reduced nicotinamide adenine dinucleotide
NADPH - reduced nicotinamide adenine dinucleotide phosphate
O^R - ouabain-resistant
PE - plating efficiency
PEG - polyethylene glycol 6000
PEI - polyethyleneimine
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
SC - subclone
TCA - trichloroacetic acid
tRNA - transfer ribonucleic acid
ts - temperature-sensitive
UDP - uridine 5'-diphosphate
UdR - 2'-deoxyuridine
UMP - uridine 5'-monophosphate

UR	- uridine
UTP	- uridine 5'-triphosphate
UV	- ultraviolet
TDP	- thymidine 5'-diphosphate
TdR	- 2'-deoxythymidine
TMP	- thymidine 5'-monophosphate
TR	- thymidine
TTP	- thymidine 5'-triphosphate

INTRODUCTION

INTRODUCTION

A cursory review of current research literature on bacteria, viruses and the lower eucaryotes reveals the tremendous usefulness of a genetic approach to the study of fundamental biological processes. In bacteria and viruses the isolation of progeny with mutations or stable alterations in DNA has proven invaluable in elucidating metabolic pathways, mechanisms of control and morphogenesis, and sites and modes of interaction between the organisms and environmentally encountered agents.

The development of techniques for culturing somatic mammalian cells *in vitro* has allowed the application of a similar genetic approach to the study of higher eucaryotic organisms. Thus, it has become possible to treat mammalian cells as microorganisms and to investigate mammalian cellular physiology and organization in a manner analogous to the molecular genetic study of *Escherichia coli*.

A wide range of mammalian cell lines with variant phenotypes have recently been isolated from somatic cells in culture. These have included drug-resistant, nutritional auxotrophic and conditional lethal mutants. However, few of the systems have been well defined genetically and it has been questioned whether the variants produced in cell culture

are due to a genetic alteration in cellular DNA, or whether in fact the cell lines result from epigenetic events and thus represent stable phenotypic variants. The issue becomes a crucial one when interpretations obtained from clonal cell lines in culture are extrapolated to cells as they function *in vivo*.

The work reported in this thesis is an attempt to develop a genetically well-defined mutant type of a mammalian cell line in order to shed light on the origins of hereditable variations in cultured somatic cells.

The antitumor agent hydroxyurea was used to select for drug-resistant cell lines from Chinese hamster ovary (CHO) cells in culture. Hydroxyurea is a small molecule which potently and specifically inhibits mammalian DNA synthesis. It was hoped that the hydroxyurea-resistant CHO cell lines would be useful in confirming the mode of action of this chemotherapeutic agent as well as providing an additional genetic marker for such studies as somatic cell hybridization, mutagenicity testing *in vitro*, and gene expression.

The hydroxyurea-resistant cell lines obtained were characterized for phenotypic stability, mutation rates, response of mutation frequency to mutagenesis, cross-resistance to related cytotoxic compounds and phenotypic behaviour of hybrid cell lines containing genetic complements from both wild-type and hydroxyurea-resistant cells.

Evidence existed to suggest that hydroxyurea owed its cytotoxicity to an inhibition of ribonucleotide reductase, the enzyme responsible for the biosynthesis of the four deoxyribonucleotides required for DNA synthesis. Therefore, the hydroxyurea-resistant cell lines were examined for alterations in ribonucleotide reductase activity. Ribonucleotide reductase is a complex, allosteric enzyme and has not been extensively purified from any eucaryotic source. Thus, an attempt was made to purify the enzyme from wild-type and hydroxyurea-resistant CHO cell lines, and to examine the kinetic properties of enzyme from both sources.

Since the measurement of ribonucleotide reductase levels in cell extracts is difficult, a method was developed to measure enzyme levels in intact CHO cells made permeable to nucleotides by treatment with the detergent Tween-80. This technique facilitated the study of ribonucleotide reductase levels in hydroxyurea-resistant cell lines, as well as the study of fluctuations in enzyme levels during the normal course of the CHO cell cycle.

While studying ribonucleotide reductase, it was observed that the enzyme is inhibited by an unusual dinucleotide compound produced in both the fungus Achlya and in the CHO cell line. The interaction of the purified dinucleotide with ribonucleotide reductase from Achlya and CHO cells was investigated and an attempt was made to correlate cellular biosynthesis of the dinucleotide with regulation of ribo-

nucleotide reduction and the concomitant inhibition of DNA synthesis.

HISTORICAL

HISTORICAL

A. Mutants of Somatic Cells in Culture

The study of basic cell biology of a wide variety of microorganisms has depended extensively on the large body of knowledge concerning the genetics of these organisms. Thus, the progress of microbial molecular biology has been firmly rooted in the technology for isolating mutations affecting a wide range of biological functions and for manipulating the mutant genes in order to study consequent changes.

The genetic technology developed with microbial systems has only recently been applied to the study of higher eucaryotic organisms. Problems are inherent in obtaining mutations in complex diploid organisms with long generation times. Furthermore, where mutants can be isolated, as in Drosophila (Suzuki, 1970), their biochemical and genetic characterization has most often proven extremely difficult due to the diverse cellular phenotypes present in such multicellular organisms. Similar problems exist in the study of mutations which occur within natural mammalian populations, as with certain human genetic diseases (Krooth and Sell 1970, Krooth et al. 1972), and until recently the molecular basis of most have remained unknown.

The establishment of in vitro cultures of mammalian

somatic cells provided the starting materials for a genetic approach to the study of fundamental biological processes in these organisms. Permanent cell lines were developed from a variety of mammalian sources including mouse (L) (Earle 1943), human (HeLa) (Gey et al 1952), and hamster (CHO) (Puck et al 1958) and provided, for the first time, mammalian cells which could be treated as microorganisms.

In 1956, Puck and Fisher reported the first selection of variant cell lines established in cell culture. They showed by cloning that within the HeLa cell population there were spontaneous variants with different growth requirements. The first drug-resistant cell variants were reported in 1959, when Lieberman and Ove (1959) reported the selection of puromycin-resistant L cells and Szybalski (1959) reported the selection of 8-aguanosine-resistant HeLa cells.

Since that time, progress has been rapid in both the detection and isolation of clones of mammalian cell lines with stably altered phenotypes. The observed hereditary changes have been of almost every conceivable variety and have included mutations to auxotrophy, drug-resistance, temperature sensitive conditional lethality and alterations in products of differentiated cells (see reviews by Clements 1975, Siminovitch 1976, Basilico 1977).

Despite the isolation of such a wide variety of

biochemically variant cell lines, few of the systems have been well defined genetically and this has resulted in controversy over whether the origins of the observed variations are genetic or epigenetic events. Genetic variants have been defined (Siminovitch 1976) as being due to a mutational event involving a hereditable nucleotide base change, deletion or rearrangement in the primary structure of DNA. Epigenetic variants have been defined (Nanney 1968) as being due to directed alterations in phenotypic expression such as are encountered in the inheritance of cellular differentiation (Cahn & Cahn 1966, Coon 1966, Gehring 1968). It becomes important to distinguish between the two events when experimental results obtained with cell lines in culture are extrapolated to cells as they behave in vivo. For example, the development of in vitro mutagenesis detection systems with mammalian cells must depend upon the knowledge of the genetic basis of the variant phenotype measured (DeMars 1974).

Harris (1971, 1973, 1974) and Mezger-Freed (1972) have suggested that most spontaneous and induced variation in cultured somatic cells result from epigenetic changes. Since mechanisms of epigenetic change are largely unknown, the evidence for such changes rests indirectly on observations which contradict a genetic mechanism for the variance. Several investigators, for example, have reported that some cell lines isolated in culture do not retain the variant

phenotype with cultivation (Morrow 1970, Mezger-Freed 1971, Orkin & Littlefield 1971) and somatic cell variants have had a reputation in the past for being "notoriously unstable". (Nature (London), New Biol. 237:98 (1972)). Furthermore, in some early studies it was not possible to increase the frequency of drug-resistance by treatment with compounds known to be potently mutagenic in bacteria (Szybalski et al 1964). Finally, one of the main arguments for epigenetic changes came from studies of mutation rates in cultured somatic cells. In some cases the mutation rates were higher than predicted by analogous microbial studies (Breslow & Goldsby 1969, Harris 1971, 1973, Mezger-Freed 1972). In particular the work of Harris and Mezger-Freed indicated that the frequency of cells resistant to certain agents did not vary as expected with cell ploidy or mutagen treatment. However their data and interpretations have been severely criticized by DeMars (1974), who has also argued that mutation rates for drug-resistance in cultured mammalian cells are similar to germinal mutation rates estimated for humans (DeMars 1974). Furthermore, studies in some laboratories have since provided evidence that mutation rates for both dominant and recessive events do respond to cell ploidy in a manner consistent with a genetic or chromosomal basis (Chasin 1973, Chasin & Urlaub 1975, McBurney & Whitmore 1974).

It is likely, however, that epigenetic changes do result in some hereditable somatic cell variation observed in

vitro. Recently, Meins and Binn (1977) presented evidence for an epigenetic change in cultured totipotent plant cells. They described the hereditable change involving loss of auxotrophy for the growth hormone cytokinin by tobacco cells in culture. This process, called cytokinin habituation, was gradual rather than all-or-none, and the cells in culture showed reversible shifts among a range of habituated states while remaining totipotent (Meins & Binn 1977). Similarly, DeMars (1974) suggested a number of experimental systems with mammalian cells, which if investigated could provide direct evidence for epigenetic variation.

To rule out epigenetic causes, it is necessary to test rigorously isolated somatic cell variants to determine if the observed variation is genetic in origin. Four criteria have been proposed (Thompson & Baker 1973, DeMars 1974) for the classification of a variant as a genetic mutant:

i) That the altered phenotype breeds true in the sense that it is stably transmitted through consecutive generations.

ii) That the frequency of occurence of the phenotype can be enhanced by the application of mutagenic agents

iii) That the phenotype can ordinarily be associated with an altered gene product (usually a protein)

iv) That the phenotype can be attributed to a specific region of the genome, ie that it can be mapped in a linkage group which behaves in a Mendelian manner.

Fortunately, during the past five years, evidence has rapidly accumulated indicating that many somatic cell variants exhibit most of the above traits and probably represent true mutants.

Recently, Siminovitch (1976) has reviewed the literature on a broad range of phenotypic variants which fulfill at least some of the above criteria and thus represent the best evidence of the genetic origin of such variants. Since major additional contributions have been made since his review, a few examples of well-characterized systems for several categories of mammalian somatic cell mutants are described below.

1. Drug-resistant mutants.

Variant mammalian cell lines have been isolated for resistance to a multitude of cytotoxic compounds. The isolation and characterization of such variants have been reviewed recently (Clements 1975). As a class, the drug-resistant cell lines have been more thoroughly studied than any other variant type, and it is not surprising that several systems have yielded some of the most convincing evidence of a genetic basis for the altered phenotype.

Purine analog resistance: One of the oldest and most widely studied systems of drug-resistant somatic cells is the development of resistance to purine analogs. 8-Azaguanine (AG)

or several similar purine analogs (eg 6-thioguanine, 6-mercaptopurine, 8-azahypoxanthine) become toxic for mammalian cells after being "salvaged" by the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT). Resistant cell lines often completely lack, or have very low levels of this salvage activity (designated $HGPRT^-$) (Szybalski et al 1962, Littlefield 1963, Chu et al 1969, Gillen et al 1972, DeMars & Held 1972). It has been possible to isolate AG-resistant mutants in a single selection step resulting in a 10 to 100 fold increased resistance to the drug from a wide variety of mammalian cells in culture, including diploid human fibroblasts (DeMars & Held, 1972). The resistant phenotype is stable with cultivation in the absence of AG and the frequency of mutation is increased by mutagenesis (Chu & Malling 1968, Bridges et al 1970, Hsie et al 1975).

Evidence that the cell lines lacking HGPRT activity have a structurally altered enzyme molecule comes from a variety of sources. In 1973, Beaudet et al demonstrated in cells lacking HGPRT activity, the presence of material which cross reacts immunologically with antibody to purified HGPRT enzyme. This result was duplicated by Wahl et al (1974) with mouse L cells. Capecchi et al (1974) found that revertants which regain HGPRT activity often have a more thermolabile enzyme activity in vitro when compared to the original wild-type enzyme. Two separate investigators working with cell clones showing altered HGPRT activities have now demonstrated

that the enzyme is also electrophoretically altered (Chasin & Urlaub 1976, Milman et al 1976). Such an alteration would be expected with certain amino acid substitutions or deletions arising from changes in the nucleotide sequence of the cellular DNA. Also consistent with a structurally altered HGPRT protein are the results obtained from a CHO cell mutant which shows temperature sensitivity for both resistance to 6-thioguanine and HGPRT activity (Fenwick & Caskey 1975). Finally, Fenwick et al (1977) have also been able to isolate revertants from $HGPRT^-$ cells which have altered antigenic properties when compared to the wild-type enzyme (Fenwick et al 1977).

The observations with purine analog-resistant mammalian cells in culture have been duplicated in male human patients with the Lesch-Nyhan Syndrome (Seegmiller et al 1967). This genetic defect is known to involve structural alterations in the HGPRT protein and behaves in an X linked Mendelian fashion (McDonald & Kelley 1971, Kelley & Meade 1971, Arnold et al 1972).

In summary, at least some of the purine analog resistant variants isolated in mammalian cell culture have fulfilled all the criteria of a genetic basis for the altered phenotype. The phenotype is stable, mutation frequency is enhanced by mutagenesis, the HGPRT protein in such mutants has been shown to be altered in thermostability, antigenicity or electrophoretic behaviour and finally the genetic determinant for the HGPRT

enzyme has been located on the X chromosome.

α-amanitin resistance: α-Amanitin is a bicyclic octapeptide cytotoxic for a variety of mammalian cells because it inhibits the enzyme RNA polymerase II (Lindell et al 1970). Variant cell lines resistant to the drug have been isolated from CHO cells (Chan et al 1972), rat myoblast cells (Somers et al 1975), BHK cells (Amati et al 1975) and human diploid fibroblasts (Buchwald & Ingles 1976). The α-amanitin resistant phenotype has been found to be stable and the frequency of mutation is increased by mutagenesis. The mutant cell lines have been shown to contain RNA polymerase II activity less sensitive to α-amanitin inhibition. Lobban et al (1976) reported that the purified enzyme from one α-amanitin resistant CHO cell line was 600 times less sensitive to inhibition by the drug. In addition, resistant RNA ploymerase was found to be more thermolabile than wild-type enzyme. The altered enzyme has been shown to bind less α-amanitin than the wild-type (Ingles et al 1976).

Cyclic AMP resistance: Cultured S49 mouse lymphoma cells are lysed by the presence of high levels of cAMP in the external culture medium. Stable mutants resistant to cAMP can be isolated after mutagenesis (Coffino et al 1975). Hochman et al (1975) have purified the regulatory subunit of cAMP-dependent protein kinase in one such mutant. The mutant protein was shown to have altered temperature sensitive and enzymatic

properties. Recently, Steinberg et al (1977) have reported that a large proportion of the cAMP-resistant mutants isolated contain a cAMP-dependent protein kinase with an electrophoretically altered regulatory subunit. The charge alterations observed were consistent with single amino acid substitutions in the protein molecule.

2. Auxotrophs:

A large number of nutritional auxotrophs of CHO cells have been isolated by Puck and his co-workers. By use of BUdR incorporation plus exposure to visible light they were able to select for those cells unable to grow in the absence of a particular nutrient. A large number of glycine requiring clones of CHO cells were isolated. The auxotrophy was stable and the frequency of occurence could be increased by mutagenesis (Kao & Puck 1968, Kao et al 1969). The glycine requiring mutants could be divided into four classes by complementation analysis utilizing cell hybridization. Mutants of the 'A' complementation group were shown to have low levels of the enzyme serine hydroxymethylase (Kao et al 1969), and a revertant of one such mutant was shown to regain a serine hydroxymethylase activity more thermolabile than the original wild-type enzyme (Chasin et al 1974).

3. Temperature-sensitive conditional lethality:

Many variant mammalian cell lines have been isolated which exhibit temperature-sensitive conditional phenotypes

(see review by Basilico 1977). These have included variants which cannot grow at high temperatures (eg Toniolo et al 1973) and those which are cold-sensitive (Farber & Unrau 1975). The best characterized temperature-sensitive mutants are those which have defects in protein synthesis. Thompson et al (1973) first reported the characterization of a CHO cell line (designated tsH1) temperature-sensitive for growth. The tsH1 cell line rapidly ceased protein synthesis when shifted to 39°C, the non-permissive temperature. The cells were shown to have a temperature-sensitive ability to charge leucyl-t-RNA and the leucyl-t RNA synthetase activity was reduced in cell extracts. Both results suggested that tsH1 had a defect in the leucyl-t RNA synthetase molecule which conferred temperature-sensitivity on the leucine charging activity. The authors have since devised specific selection procedures for isolating protein synthesis mutants (Thompson et al 1975) and there are now available temperature-sensitive CHO cell lines for at least 6 different amino acyl t RNA synthetases (Wasmuth & Caskey 1976, Thompson et al 1977).

4. Spontaneous variants:

A number of systems have been exploited to yield mammalian cell variant phenotypes without using selective pressures such as drug toxicity.

Glucose-6-phosphate dehydrogenase (G6PD) deficient mutants: Stable variants lacking the enzyme glucose-6-phosphate dehydrogenase have been isolated from mutagenized CHO cells

in culture. A cyclical sib-selection technique combined with a histochemical stain for colonies containing G6PD activity was utilized to yield CHO clones lacking G6PD (Rosenstraus & Chasin 1975). One such variant line did produce some G6PD activity (11% of wild-type activity) but this residual activity was more thermolabile than the wild-type activity. Similarly, a revertant of a separate mutant lacking any enzyme activity exhibited partial restoration of G6PD activity which again was more heat-sensitive than wild-type enzyme (Rosenstraus & Chasin 1977). Both results strongly suggest that there are structural alterations in the G6PD protein. Finally, the authors were also able to show that the altered G6PD activity was linked to the HGPRT determinant on the X chromosome.

Immunoglobulin variants: The evidence for structural changes in proteins cited in the examples so far are all consistent with mutations in structural genes. However, the methods of protein analyses utilized (enzyme activity, immunoreactivity, temperature-sensitivity, electrophoretic properties) do not rule out post-translational protein modifications such as carbohydrate addition, phosphorylation, processing of precursor molecules etc., any of which may alter physiochemical and biological properties of the protein molecule. Recently a direct determination of amino acid sequences of an altered protein has been reported. Thus, spontaneous variants of a permanent cell line of mouse plasmacytoma (P3) have provided the most definitive evidence for the genetic basis of somatic

cell variation. The P3 cell line in culture secretes an immunoglobulin consisting of a light and heavy protein subunit. The amino acid sequences of both chains have been determined (Horibata & Harris 1970). Mutants of P3 cells have been isolated which produce variant immunoglobulin molecules. Such mutants are stable and their frequency is increased by mutagenesis (Birshstein et al 1974, Secher et al 1974). In a recent article, Adetugba et al (1977) reported screening 7000 random subclones of non-mutagenized P3 cells for the presence of spontaneous isoelectric focusing variants of the secreted immunoglobulin. They characterized four variants (IF1 to IF4) for immunoglobulin amino acid sequence, and the size of the immunoglobulin mRNA and its in vitro translational product.

Briefly, they were able to show that variant IF1 was due to a nonsense point mutation resulting in a deletion of 82 residues of the heavy chain. IF2 was due to an intracistronic deletion and IF3 was due to the deletion of 2 bases resulting in a frameshift and subsequent premature chain termination. Finally, IF4 was due to a missence mutation presumably resulting from an adenine to guanine transition in the DNA sequence (Adetugbo et al 1977).

In summary, compelling evidence has accumulated during recent years suggesting that the majority of phenotypic variants isolated from mammalian somatic cells in culture are due to

classical genetic changes occurring at the DNA level. This augurs well for the potential development of a broad range of mutant cell lines which will prove immensely useful in the study of mammalian cell biology.

B. HYDROXYUREA

Hydroxyurea is a derivative of urea in which one of the hydrogen atoms is replaced by a hydroxyl group: $NH_2-\overset{O}{\overset{\|}{C}}-NHOH$. The compound was synthesized in 1869 by Dresler and Stein, and was first employed in biological studies in 1928 when it was shown that hydroxyurea induced a pernicious anemia-like state and a depression of leucocyte formation in animals (Rosenthal et al 1928). Current interest in hydroxyurea stems from a 1960 screening study showing that the drug had antitumor activity against Sarcoma 180 (Stock et al 1960). In preliminary clinical trials hydroxyurea was reported effective against a wide range of solid tumors as well as acute and chronic leukemia (Bersagel et al 1964, Bolton et al 1964, Fishbein et al 1964). In 1967, The Squibb Institute was allowed to release hydroxyurea commercially as a drug effective in the treatment of both chronic myeloid leukemia and malignant melanoma (Squibb Institute for Medical Research 1968). Hydroxyurea has subsequently proven most useful in the treatment of chronic myelogenous leukemia (Kennedy 1969) although its antitumor activity with a variety of carcinomas is still under investigation (Talley 1973, Johnson et al 1975). Hydroxyurea has also been used in combination with other cancer chemotherapeutic agents to achieve a potentiated therapeutic response (Johnson et al 1975, Gale et al 1977).

The first clinical trials of hydroxurea suggested that the drug interfered with DNA synthesis (Bergsagel 1964). Subsequently, hydroxyurea was indeed found to inhibit DNA synthesis potently and specifically in a variety of systems including bacteria (Rosenkranz et al 1965, Gale et al 1964), HeLa cells (Young et al 1964), ascites tumor cells (Gale 1964, Yarbro et al 1965a), regenerating liver (Schwartz et al 1965, Yarbro et al 1965b) and hamster cells (Mohler 1964).

Hydroxyurea contains an oxidized amide bond and is therefore classified as an hydroxamic acid. A number of naturally occurring compounds which have potent biological activities also contain the oxidized amide bond (Neilands 1967) and several have been shown to inhibit DNA synthesis (Young et al 1967, Gale 1966). Despite intensive study on the hydroxamic acids, their mechanism of action and the structure/activity relationship of the oxidized amide bond have not been elucidated. However, several studies done with compounds structurally related to hydroxyurea have suggested that it is this bond which contributes most to its biological activity. Adamson (1965) suggested that the hydroxamic group is required for antitumor activity, since compounds like isohydroxyurea ($NH_2-\overset{O}{\overset{\|}{C}}-O-NH_2$) were devoid of activity. Young et al (1967), studying a series of

similar compounds, showed the -NOH group to be the most important. The carbonyl group seemed not to be a prerequisite for antitumor activity, since formamidoxime ($HO-N=CH-NH_2$) was also inhibitory. However the most potent inhibitors of cellular proliferation do contain a carbonyl group (eg. hydroxyurea, dihydroxyurea and hydroxyguanidine). Young also showed that all the compounds tested which could be shown to inhibit DNA synthesis also formed complexes with metal ions. A more recent study of the metal binding properties and antitumor activity of several 1-substituted 3-hydroxyureas revealed that only those compounds capable of forming complexes with Fe^{+3} possed antitumor activity (Harmon et al 1970).

Physiological studies suggested that hydroxyurea did not block DNA synthesis by inhibition of the enzyme DNA polymerase. Young and Hodas (1964) showed hydroxyurea to be without effect on thymidine incorporation into DNA in a cell-free system containing the four deoxyribonucleoside triphosphates and a DNA primer. Similarly hydroxyurea did not inhibit the conversion of deoxyribonucleoside diphosphates to the triphosphate level by cellular nucleotide kinases (Adams & Lindsay 1967).

In 1964 several authors suggested that hydroxyurea blocked the conversion of ribonucleotides to deoxyribo-

nucleotides by inhibiting the enzyme ribonucleotide reductase (EC 1.17.4.1) (Frenkel et al 1964, Mohler 1964, Young & Hodas 1964). Ribonucleotide reductase is the enzyme responsible for the reduction of the four ribonucleoside diphosphates to the corresponding deoxyribonucleoside diphosphates required for DNA synthesis (Reichard 1968). The enzyme is essential for the replication of DNA and cell division. In E. coli mutants at the dnaF locus which are temperature-sensitive for ribonucleotide reductase activity are also temperature-sensitive for DNA synthesis and growth (Fuchs et al 1972).

Much evidence has accumulated supporting the belief that ribonucleotide reduction is the primary target for hydroxyurea. Hydroxyurea was shown to inhibit purified E. coli ribonucleotide reductase activity in vitro (Krakoff et al 1968). The purified bacterial enzyme is composed of two non-identical subunits, one of which contains two atoms of inorganic iron per molecule of protein (Brown et al 1969a). Hydroxyurea was shown to inactivate specifically the iron containing subunit (Brown et al 1969b). Similarly, hydroxyurea inhibits the activity of ribonucleotide reductase in a variety of partially purified mammalian cell preparations (Turner et al 1966, Elford 1968, Moore 1969).

After exposure of E. coli cells to hydroxyurea, their is a concentration-dependent decrease in acid soluble deoxy-

ribonucleoside triphosphate pools (Neuhard 1967, Neuhard & Thomassen 1971). More detailed studies with E. coli showed that hydroxyurea caused a decrease in the concentration of both deoxyribonucleoside diphosphates and deoxyribonucleoside triphosphates and a corresponding increase in the total concentration of ribonucleotides (Sinha & Snustad 1972). These results strongly suggest that inhibition of ribonucleotide reduction is the basis of hydroxyurea action.

In mammalian systems the presence of hydroxyurea results in a rapid depletion of the dATP and dGTP pools (Skoog & Nordenskjold 1971, Adams et al 1971) or of the dATP pools alone (Walters et al 1973). Furthermore, the block of DNA synthesis by hydroxyurea can be partially or completely reversed by the addition of deoxyribonucleosides to inhibited cells (Mohler 1964, Adams & Lindsay 1967, Young et al 1967, Plagemann & Erbe 1974).

In addition to the inhibition of ribonucleotide reductase, many other modes of action have been suggested for hydroxyurea. These have included a direct degradation of cellular DNA; an inhibition of pyrimidine synthesis; an inhibition of histone synthesis; and an inhibition of cell surface glycosyl transferases.

Many workers have observed the degradation of cellular DNA after hydroxyurea treatment (Bendich et al 1964, Rosenkranz et al 1968, Jacobs & Rosenkranz 1970). Rosenkranz and his

co-workers have proposed that hydroxyurea owes its bactericidal action upon E. coli to a modification of cellular DNA. In vitro studies showed that whereas fresh solutions of hydroxyurea were without effect on purified DNA, aged or heated solutions of the drug were capable of causing alterations in DNA structure (Rosenkranz & Jacobs 1968). Rosenkranz has suggested that N-carbamoyloxyurea, an oxidation product of hydroxyurea, is the reactive intermediate responsible for the degradation of DNA (Rosenkranz 1970, Jacobs & Rosenkranz 1970). In all of these studies, however, very high concentrations of hydroxyurea (0.2 M) were used and significant N-carbamoyloxyurea formation occurred only when solutions of hydroxyurea were incubated at 56^{o}C for extended periods (Jacobs & Rosenkranz 1970). Sinha & Snustad (1972) showed that much lower concentrations of hydroxyurea (0.03 M) still potently inhibited E. coli DNA synthesis and ribonucleotide reductase activity, and that no single or double stranded breaks in cellular DNA could be detected at these concentrations.

In mammalian systems chromosomal abnormalities have been reported as a consequence of hydroxyurea treatment and these have included DNA strand breaks (Coyle & Straus 1970, Walker et al 1977); chromatid aberrations (Oppenheim & Fishbein 1965, Yu & Sinclair 1968, Borst 1972); abnormally small newly synthesized DNA (Coyle & Straus 1970); and a peripheral distribution within the nucleus of newly synthesized

DNA (Ockey & Allen 1975). Hydroxyurea is known to be selectively toxic for mammalian cells in the S or DNA synthetic phase of the cell cycle (Sinclair 1967, Kim et al 1967, Bacchetti & Whitmore 1969). This S phase specific toxicity and the chromosomal damage observed has suggested that hydroxyurea has a direct degradative effect on DNA molecules during the S phase (Bacchetti & Whitmore 1969, Cameron & Jeeter 1973). Countering this suggestion is strong evidence showing that a variety of compounds known to inhibit DNA synthesis cause similar chromosomal abnormalities and S phase toxicity (Karon & Benedict 1972, Oppenheim & Fishbein 1965, Ramseier et al 1977). Thus the primary event would be the interruption of normal DNA synthesis and, in the case of hydroxyurea, this is probably due to a depletion of deoxyribonucleoside triphosphate pools.

Early studies by Yarbro suggested that histone synthesis was dramaticallydecreased in the presence of hydroxyurea (Yarbro et al 1965b, Yarbro 1967). The lysine rich fraction of the histones seemed to be especially sensitive to drug treatment. However, it is known that histone synthesis is coupled to DNA synthesis (Balhorn et al 1972) and inhibition of DNA synthesis with a variety of compounds also results in a subsequent inhibition of histone synthesis (Sadgopal & Bonner 1969, Chae et al 1970). More recently, Balhorn et al (1973) have shown that in HTC cells , 1 mM

hydroxyurea causes a rapid and efficient cessation of DNA synthesis while exerting little immediate effect on histone synthesis, suggesting that the effect of hydroxyurea on histone production is a consequence of DNA synthesis inhibition.

One of the early observations on the mechanism of action of hydroxyurea was that it seemed to block some step in the biosynthesis of orotic acid. When patients were given 6-azauridine, which is known to inhibit the enzyme orotidylic decarboxylase, they excreted large amounts of orotic acid and orotidine into the urine (Bono et al 1964). If the patients were also given hydroxyurea, however, this excretion was significantly reduced. Therefore, it was concluded that hydroxyurea was blocking a biosynthetic step prior to orotic acid synthesis (Vogler et al 1966). However, the reduction in excretion of these products could also be explained by a feedback inhibition of the pyrimidine biosynthetic enzymes by high levels of UTP and CTP which are known to accumulate if the action of ribonucleotide reductase is inhibited by hydroxyurea (Sinha & Snustad 1972).

Finally, hydroxyurea has been reported to inhibit the incorporation of D-glucosamine into glycoproteins and glycolipids of hamster embryo fibroblasts (Hawtrey et al 1974). The authors suggested that hydroxyurea directly inhibited a number of glycosyl transferases but presented no direct evidence of such an inhibition. Furthermore, the concentra-

tion (10 mM) of hydroxyurea they employed to inhibit D-glucosamine uptake was some 30 to 100 fold greater than the concentration required to inhibit DNA synthesis and hamster cell division strongly (Lewis & Wright 1974). The inhibition of D-glucosamine incorporation which was observed in the presence of hydroxyurea may be a consequence of the inhibition of DNA synthesis, for ara-C, also an inhibitor of mammalian DNA synthesis, caused similar effects (Hawtrey et al 1974).

Even though the exact mechanism of hydroxyurea action remains in dispute, it is still a very specific inhibitor of DNA synthesis and as such is finding a wide application in physiological studies. Highly specific inhibitors (eg hydroxyurea, chloramphenicol, actinomycin D) have become standard tools in the study of macromolecular biosynthesis and physiology. One application of hydroxyurea is in the study of the cell cycle of mammalian cells in culture. Low concentrations of hydroxyurea inhibit only those cells engaging in DNA synthesis, and therefore exponentially growing cells exposed to a hydroxyurea block gradually halt at the G1/S boundary (Adams & Lindsay 1967, Hamlin & Pardee 1976). If the drug is removed within 24 hours of addition, the cells will then proceed synchronously through the S phase with little apparent damage. Hydroxyurea has also been used to synchronize cells in vivo (Rajewsky 1970) and in combination with mitotic detachment to produce highly syn-

chronized Chinese hamster cell populations (Walters et al 1976).

Hydroxyurea has also been useful as an aid in studying mammalian DNA repair synthesis. The drug has frequently been used to inhibit preferentially semiconservative replication, thereby improving the resolution of repair replication (Cleaver 1969, Brandt et al 1972, Cleaver 1974). Since it is known that mammalian cells possess at least three different DNA polymerase activities (Parker & Leiberman 1977, Weissbach 1977) it has been suggested that separate DNA polymerases are responsible for semiconservative replication and gap-filling or repair synthesis (Martin et al 1977, Laipis & Levine 1973), but the different polymerases would differ in their utilization or affinity for the deoxyribonucleoside triphosphates. The DNA polymerase responsible for semiconservative replication would presumably be more sensitive to a drop in deoxyribonucleotide pools caused by hydroxyurea. With prolonged exposure hydroxyurea has been shown to inhibit repair synthesis eventually, and this inhibition can be reversed by addition of the four deoxyribonucleosides at concentrations which do not reverse the inhibition of total DNA synthesis (Ben-Hur & Ben-Ishai 1971). These results are all consistent with the belief that the primary effect of hydroxyurea is a depletion of deoxyribonucleotide pools by inhibition of ribonucleotide reductase.

Guanazole

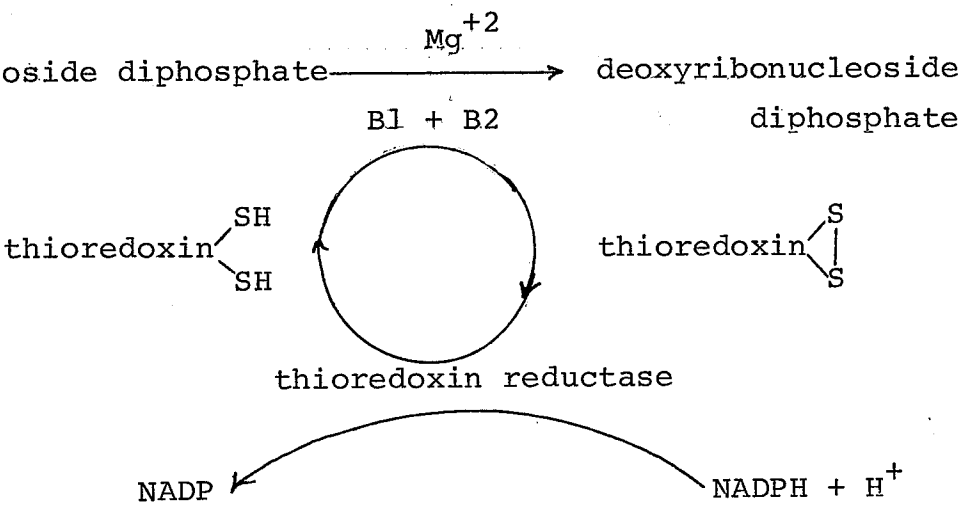
Although it was synthesized some time ago (Hoffman & Ehrhart 1912) guanazole (3,5-diamino-1,2,4-triazole) has only recently been shown to have antitumor activity against murine leukemia L1210, a mast cell tumor, a carcinosarcoma and a reticulum cell sarcoma (Hahn & Adamson 1972). Guanazole has been shown to inhibit DNA synthesis specifically and to act on ribonucleotide reductase in a manner analagous to hydroxyurea (Brockman et al 1970). The drug is highly soluble in water and is stable for at least two weeks at room temperature (Livingston & Carter 1969), and as well appears to be remarkably stable upon administration to animals (Gerber et al 1973). Promising clinical trials have recently been conducted on patients with various neoplastic deseases (Yakar et al 1973, Hewlett et al 1973, Land et al 1974, Caoili et al 1975). Almost no physiological studies have been done with guanazole, but because of its structural dissimilarity to hydroxyurea and its apparent stability, guanazole is useful in studies carried out in parallel with hydroxyurea (Wright & Lewis 1974).

C RIBONUCLEOTIDE REDUCTASE

Ribonucleotide reductase (EC 1.17.4.1) is the enzyme responsible for the conversion of the four ribonucleotides to the deoxyribonucleotides required for DNA synthesis (Reichard 1968, Blakely & Vitols 1968, Larsson 1969). Both the substrate specificity and rate of reaction of this enzyme are strictly regulated in a complex, allosteric fashion by the cellular content of ATP and several deoxyribonucleoside triphosphates (Reichard 1972, Elford 1972). Two forms of ribonucleotide reductase have been found in the variety of organisms examined. The E. coli type enzyme is composed of two non-identical subunits, one of which contains iron. The ribonucleotide substrates are reduced at the diphosphate level and the reaction is inhibited by hydroxyurea (Brown et al 1969a, 1969b). The Lactobacillus type enzyme is a monomeric protein which reduces the ribonucleoside triphosphates (Panagou et al 1972). This enzyme requires the cofactor 5'-deoxyadenosylcobalamin for activity and is not inhibited by hydroxyurea (Elford 1968).

All mammalian systems examined to date (eg Moore 1967, Hopper 1972, Larsson 1973a, Cory & Mansell 1975, Lewis & Wright 1974) as well as several fungi (Lowdon & Vitols 1970, Lewis et al 1976) seem to contain a ribonucleotide reductase activity of the same type as the E. coli enzyme. However,

purification of the eucaryotic enzyme has proven extremely difficult and purification to homogeneity has only been achieved with E. coli (Thelander 1973). The E. coli enzyme consists of two subunits, B1 and B2, each alone completely devoid of enzyme activity (Brown et al 1969a). Protein B1 binds the ribonucleotide substrates and effectors (Brown & Reichard 1969b, v.Döbeln 1976), and contains active thiol groups which are directly involved in the replacement of an -OH group with a hydrogen at the 2' position of the ribonucleotide ribose (Thelander 1974). The reducing agent for this reaction is a low molecular weight protein called thioredoxin (Laurent et al 1964). Thioredoxin in the oxidized form contains a disulfide bridge that arises from a single cystine residue in the molecule and which can be reduced by the FAD containing enzyme thioredoxin reductase (Thelander 1967). The hydrogen donor for this reaction is NADPH. The complete ribonucleotide reduction reaction can be summarized:



Protein B2 contains a free radical dependent upon the presence of 2 atoms of non-haem iron. This free radical, which can be recognized by an optical absorption peak at 410 nm and a characteristic EPR signal (Ehrenberg & Reichard 1972), is thought to arise at the β position of a tyrosine residue in the protein (Sjöberg et al 1977). It can be destroyed by treatment with hydroxyurea which results in a time-dependent inactivation of the enzyme parallel to loss of absorption at 410 nm and loss of the EPR signal (Brown et al 1969b, Ehrenberg & Reichard 1972).

In the absence of certain nucleotide allosteric effectors, ribonucleotide reductase shows little enzymatic activity with any of the four ribonucleoside diphosphate substrates. However, in the presence of ATP the reduction of both pyrimidine ribonucleotides is stimulated. Similarly, dTTP stimulates GDP reduction and dGTP stimulates ADP reduction. dATP, which can be considered the end product of the reaction series, acts as a general feedback inhibitor of all four reductions (Larsson & Reichard 1966a, 1966b). The net result of these allosteric interactions is to produce a balanced supply of the four deoxyribonucleotides required for DNA synthesis.

Brown and Reichard (1969a) showed that in the presence of dATP, the (B1-B2) protein complex formed dimers which were enzymatically inactive. They also reported the presence

of high and low affinity binding sites for the nucleotide effectors and proposed a model whereby the low affinity binding sites were responsible for regulation of over-all activity of the enzyme while the high affinity sites determined substrate specificity (Brown & Reichard 1969b).

Although ribonucleotide reductase activity was first reported in mammalian extracts as early as 1960 (Moore & Hurlbert 1960), purification has proven extremely difficult, and the subunit composition and physical chemistry of the mammalian enzyme remain unknown. To date, Larsson (1973a) has achieved the highest specific activity for ribonucleotide reductase. Working with the enzyme from regenerating rat liver, he was able to confirm that the ribonucleotide substrates were reduced at the diphosphate level and that the presence of dATP caused an aggregation of the enzyme accompanied by loss of enzyme activity. Hopper (1972), working with rabbit bone marrow, was able to separate a partially purified preparation into two inactive fractions which when combined restored ribonucleotide reducing activity. Thioredoxin has been identified and purified in both rat liver (Larsson & Larsson 1972) and in Novikoff ascites tumor cells (Hermann & Moore 1973), and it appears that it functions in conjunction with thioredoxin reductase in a manner analagous to the E. coli system (Larsson 1973b).

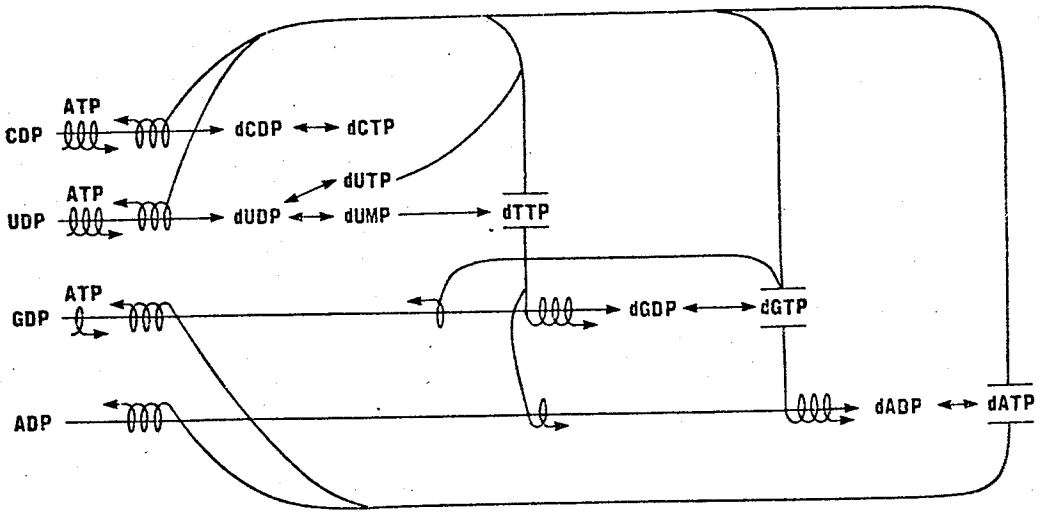
When ribonucleotide reductase preparations from Novikoff ascites rat tumor (Moore & Hurlbert 1966) or from rat embryo

(Murphee et al 1968) were tested for activation and inhibition by a variety of nucleotide effectors, the patterns of substrate specificity and total enzyme activity were very similar to those of the E. coli system. Figure 1 shows the sequence of stimulations and inhibitions of Novikoff ascites ribonucleotide reductase (from Moore & Hurlbert 1966).

The levels of ribonucleotide reductase are known to fluctuate during the course of the mammalian cell cycle. The levels of CDP reductase, for example, are extremely low in the G1 phase but increase rapidly as the cells progress into the DNA synthetic or S phase (Turner et al 1968, Murphee et al 1969). Similarly, during periods of cellular differentiation, when DNA synthesis occurs, there are greatly increased levels of ribonucleotide reductase which subsequently decline in concert with DNA synthesis (Millard 1972, Lewis et al 1976).

In a variety of mammalian cells the pool sizes of the DNA precursors dATP, dCTP, dTTP and dGTP have been shown to be extremely small and are sufficient to maintain DNA synthesis for only a short period (Reichard 1972, Walters et al 1973). This fact, combined with the tremendous increase in ribonucleotide reductase activity during periods of rapid cell division, has led to the suggestion that ribonucleotide reduction is the rate-limiting step in DNA synthesis and cell division (Elford et al 1970, Cory & Whitford 1972). If so,

Figure 1. Sequences of stimulation and inhibitions by nucleotides in ribonucleotide reduction of rat hepatoma cells (taken from Moore & Hurlbert 1966). Spirals and arrows pointing to right symbolize activation; those to the left symbolize inhibition.


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

the enzyme may be an important point for the control of DNA synthesis. A number of naturally occurring inhibitors of ribonucleotide reduction have been observed (Cory & Monley 1970, Vitols et al 1970, Elford 1972, Cory 1973, Lewis et al 1976). However the identification and physiology of these compounds have yet to be reported.

Finally, it has been suggested that, unlike E. coli, mammalian cells possess at least two separate ribonucleotide reductase proteins; one specific for pyrimidine ribonucleotides and one for purine ribonucleotides (Cory & Mansell 1975, Peterson & Moore 1976). The evidence for this suggestion is indirect, however, and resolution of the various ribonucleotide reductase activities must await the final purification of the mammalian enzyme(s).

MATERIALS AND METHODS

MATERIALS AND METHODS

A. ORGANISMS AND CULTURE CONDITIONS

CHO Cell Line: The Chinese hamster ovary (CHO) cell line first established by Puck et al (1958) was chosen as the starting material for the studies reported here. This cell line has several advantages over other permanent mammalian cell lines. The CHO clone contains a stable modal chromosome number of 21 compared to a diploid number of 22 for the Chinese hamster. The CHO cells do carry many chromosomes which differ from those of the Chinese hamster karyotype (Kao & Puck 1969), but, many of the abnormal chromosomes can be identified as rearrangement products of the normal hamster chromosomes (Deaven & Peterson 1973). Recently Worton et al (1977) studied the banded karyotype of a large number of CHO wild-type (WT) and mutant clones. They found the CHO cell line to have a relatively stable karyotype, the same basic karyotype being found in a majority of the clones examined. Thus, the low chromosome number and relative stability greatly facilitate cytogenetic analysis in the CHO cell line.

CHO cells grow rapidly in monolayer or suspension culture and have a high plating efficiency (Kao & Puck 1967). Finally, the CHO cell line is increasing being used by other investigators to isolate a wide variety of mutant types (Kao & Puck 1968, Rosenstraus & Chasin 1975, Stanley et al 1975, Hankinson

1976, Patterson 1976, Gupta & Siminovitch 1977, Thompson et al 1977, Ceri & Wright 1977).

CHO cells were grown in monolayer culture in 16 oz Brockway bottles or on 60 or 100 mm plastic tissue culture plates. The culture medium routinely utilized was alpha minimal essential medium (α-MEM) plus 10% fetal calf serum (FCS) which was 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 ribonucleosides or deoxyribonucleosides. The Brockway bottles or culture plates containing cells were incubated at 37°C (unless otherwise noted) in a 5% CO_2 atmosphere in a humidity controlled incubator.

Suspension cultures of CHO cells were started with approximately 5 X 10^4 cells/ml in 250 ml of α-MEM + 10% FCS in a 500 ml medium bottle (GIBCO Ltd.) containing a 2.5 cm Teflon coated magnetic stirring bar. The culture was placed in a temperature controlled (37°C) water bath and stirred continuously. The culture was periodically diluted with fresh complete medium to maintain a cell density of 1 to 3 X 10^5 cells/ml.

Cell Lines Sources: The WT CHO cell line was obtained from the University of Toronto, Department of Medical Biophysics. GAT^-CHO is an auxotroph requiring glycine, adenosine and thymidine because of a defect in folic acid metabolism (McBurney & Whitmore 1974). The GAT^-CHO cell line was pro-

vided by Drs. P. Stanley anf L. Siminovitch, Department of Medical Genetics, University of Toronto. GAT^-CHO was routinely grown in α-MEM plus 10% FCS supplemented with thymidine (10 μg/ml) and adenosine (10 μg/ml). Since glycine is a basic component of α-MEM it was not necessary to add additional amounts of this amino acid.

The tsH1 CHO cell line is temperature-sensitive and is unable to growth at 39.5°C because of a defect in leucyl-tRNA synthetase (Thompson et al 1973). The tsH1 CHO cell line was provided by Drs. W. Flintoff and L. Siminovitch at the Department of Medical Genetics, University of Toronto. It was routinely grown in α-MEM plus 10% FCS in an incubator maintained at 34°C.

The mouse L cell line originally isolated by Earle (1943) was provided by Drs. D. Cormack and A. Holloway of The Manitoba Cancer Foundation. The L cells were cultivated in the same way as the WT CHO cell line.

The hydroxyurea-resistant cell line designated GAT^-HU^R-1 was selected from GAT^-CHO cells in two steps. In the first step, 5×10^5 GAT^-CHO cells were plated in a 100 mm culture dish containing medium and 0.33 mM hydroxyurea. After 16 days incubation at 37°C, the surviving cells were trypsinized and replated in the absence of drug. After an additional 8 days incubation, the cells were trypsinized, and 5×10^5 cells were plated in a 100 mm culture dish containing medium with 1.33 mM hydroxyurea. After 16 days further incubation, a surviving colony was picked, and cloned in a Linbro dish con-

taining medium plus 0.33 mM hydroxyurea. One independent clone was picked and designated $GAT^{-}HU^{R}$-1.

B. ROUTINE PROCEDURES

Trypsinization: Sterile solutions of 0.05% trypsin in Dulbecco's phosphate buffered saline (PBS) (Dulbecco & Vogt 1954) were prepared and stored at $4^{o}C$. The medium was removed from cells growing on culture plates or Brockway bottles and sufficient trypsin was added to cover the cell monolayer. After incubation at room temperature for 5 to 10 min, the cells were detached from the surface and the cell suspension centrifuged. The cell pellet was then resuspended in an appropriate volume of α-MEM plus 10% FCS.

Cell Counting: Suspension grown or freshly trypsinized cells were counted after dilution in PBS by means of an electronic particle counter (Coulter Electronics Ltd.). The accuracy of the electronic count was occasionally checked by means of a microscopic cell count utilizing a haemocytometer.

Determination of Doubling Times: Exponentially growing cells were dispensed at 5×10^{4} cells/60 mm culture plate containing 5 ml of α-MEM plus 10% FCS. After 24 hours at $37^{o}C$, the plates were removed at specific times, the cells trypsinized and counted by means of a Coulter Counter. During drug inhibition studies, the drug was added after the initial 24 hour adaptation period. The log of the cell number per plate was plotted against time, and the number of hours for the cell number to double was interpolated from the graph.

Determination of Plating Efficiency: Cells grown at a density of approximately 5 X 10^6 cells/100 mm culture plate were trypsinized and counted. To determine plating efficiency (PE) in the absence of the drug, 300 cells were added to a 60 mm culture plate containing 5 ml of standard medium. After 8 days incubation at 37°C, the plates were drained and a saturated solution of methylene blue in 50% ethanol was gently added to the plates. After 10 min the plates were rinsed carefully in room temperature water and the stained colonies on each plate were counted. Only colonies containing greater than 25 cells were scored. The PE was calculated by dividing the number of colonies by the number of cells plated. For the CHO WT the PE ranged from 0.8 to almost 1.0.

In drug inhibition studies, relative plating efficiency (RPE) was determined. The RPE is defined as the PE in the presence of a concentration of drug divided by the PE in the absence of drug. If the drug had no effect on PE than the RPE would equal 1.0 by definition. The number of cells used and the size of plate depended upon the level of drug being tested. For instance, at concentrations of drug which reduced the RPE only by 10^{-2}, 500 cells were laid down in 60 mm plates containing 5 ml of medium plus drug. At higher concentrations of drug, known to reduce the RPE to a greater extent, cell numbers up to 5 X 10^5 were laid down in 100 mm culture plates containing 20 ml of medium plus drug.

RPE data was represented graphically by plotting the log of RPE against drug concentration (see Thompson & Baker

1973 for a more detailed discussion).

Cell Cloning: Two methods were utilized to obtain clones of CHO cells. The routine cloning procedure consisted of diluting out a suspension of cells to 5 cells/ml. The suspension was then dispensed at approximately 0.2 ml per well of a 96 well Linbro dish. After 8 to 10 days at 37°C, those wells showing the development of a single colony were flooded with trypsin solution and the cells removed with a Pasteur pipette and redispensed into a culture plate or bottle containing fresh medium. When a number of colonies had developed on a single plate as in drug-resistance selection experiments, the plate was first rinsed several times with sterile PBS to remove loosely attached or floating cells. Trypsin solution was added gently and allowed sufficient time to detach cells. Without agitating the plate, a sterile Pasteur pipette was lowered over the desired colony and the colony drawn up and dispensed into another plate containing medium. Clones obtained in this fashion were always recloned using Linbro dishes to ensure clonal purity.

Karyotype Analysis: Karyotype analyses were performed with CHO cells exponentially growing on 100 mm culture plates. Colchicine (0.5 µg/ml) was added to the medium and the culture plate incubated at 37°C for 1 to 2 hours. The cells were then typsinized, centrifuged and washed once with PBS. The cells were suspended in hypotonic sodium citrate (Rothfels & Siminovitch 1958) for 10 min before pelleting in a conical

centrifuge tube. The cell pellet was rinsed, without disturbing, with 1 ml of 50% acetic acid and finally a small volume of 1% orcein in 50% acetic acid (aceto-orcein stain) was used to resuspend the cells. A drop of cell suspension was placed on a slide and a glass cover-slip placed over it. Even, but firm, pressure was applied to the cover-slip to break the cells and provide a suitable chromosome spread. The slide was examined microscopically and photos were taken of at least 50 metaphase cells. Enlarged prints of each metaphase cell examined were made and the number of chromosomes per cell counted.

Cell Hybridization: Hybrid CHO clones were obtained by Sendai virus-mediated cell fusion (Okada et al 1961, Yerganian & Nell 1966). The method used by Sun et al (1974) was modified as described below. The cell lines to be used were grown to stationary phase on 100 mm culture plates and removed by trypsinization. The cells were washed once with PBS and resuspended in chilled (4^{o}C) α-MEM lacking serum. Five million cells of each parental line were mixed together and pelleted in a small tube by centrifugation. The cell pellet was resuspended in 0.75 ml cold α-MEM and 20 µl of UV inactivated Sendai virus containing 1000 to 4000 HAU was added. The cells were incubated for 10 min on ice and then 20 min at 37^{o}C, at which time 10 ml of α-MEM plus 10% FCS at 37^{o}C was gently added and the suspension centrifuged. The cell pellet was gently resuspended in medium and the cells

dispensed at 1 X 10^5 cells/100 mm plate or 1 X 10^4 cells/plate in the appropriate selective medium.

The colonies which developed after 10 to 14 days were examined and several composed of cells of large volume were trypsinized and recloned in Linbro dishes under selective conditions. The resulting hybrid cells were stained and their near tetraploid karyotype confirmed by microscopic examination.

The conditions used to select against each parental cell line can be summarized:

WT CHO	α-MEM + 10% FCS + 1.0 mM ouabain
HU^R-2	α-MEM + 10% FCS + 1.0 mM ouabain
HU^R-4	α-MEM + 10% FCS + 1.0 mM ouabain
tsH1 O^R-2A	α-MEM + 10% FCS incubated at 39.5°C
GAT^-O^R-1	α-MEM + 10% dFCS (lacks adenosine & thymidine)
GAT^-HU^R-12SC8	α-MEM + 10% dFCS (lacks adenosine & thymidine)

In subsequent experiments, the polyethylene glycol (PEG)-mediated fusion method of Davidson and Gerald (1976) was used. The method was improved by the inclusion of dimethyl sulfoxide (DMSO) in the PEG solution (Norwood et al 1976). Briefly, the two parental lines (for example tsH1 and GAT^- HU^R-12SC8) were grown exponentially in 100 mm culture plates. The cells were trypsinized, washed once with PBS and 1 X 10^5 GAT^-HU^R-12SC8 cells were mixed with 1 X 10^6 tsH1 cells in a 60 mm culture plate containing 5 ml of α-MEM plus 10% FCS

supplemented with thymidine and adenosine. After 24 hours incubation under non-selective conditions (34°C), the medium was aspirated and 3 ml of 47% PEG, 5% DMSO in α-MEM at room temperature was added to the plate. Sixty seconds later the PEG-DMSO solution was aspirated and the plate immediately washed three times with α-MEM plus 10% DMSO, followed by two washings with α-MEM alone. The cells were then covered with non-selective medium (α-MEM + 10% FCS + 10 μg/ml adenosine + 10 μg/ml thymidine) and incubated at the non-selective temperature (34°C) for 24 hours. The cells were then trypsinized, washed with PBS and resuspended in 10 ml of α-MEM + 10% dFCS. Half a millilitre of this cell suspension was placed in a 100 mm culture plate containing 20 ml of selective medium (α-MEM + 10% dFCS) and incubated at 39.5°C. After 8 to 10 days any colonies developing were typsinized, cloned and tested for hybrid karyotype. The PEG-DMSO method of cell fusion was found to be easier, more reliable, and more reproducible than the inactivated Sendai virus-mediated fusion method.

Long-term Storage of Cell Lines: WT and mutant clones of CHO were stored by suspending approximately 1 X 10^7 cells in 1 ml of α-MEM plus 10% FCS plus 7% DMSO. The cell suspension was placed in a small plastic tube (12 X 75 mm), tightly capped and placed directly at -76°C. For recovery, the tubes were thawed rapidly at 37°C and the cells pelleted by centrifugation and the supernatant discarded. The cells were then

resuspended in α-MEM plus 10% FCS and placed in a Brockway bottle containing medium.

In order to maintain culture uniformity, a large number of aliquots of all cell lines were frozen away after their isolation or arrival in the laboratory. Fresh cultures of each cell line were started from the frozen stocks at least every two months.

Contamination Checks: Shortly before its arrival in our labs, the WT CHO cell line had been found negative for mycoplasma comtamination by autoradiography of the cells after ^{3}H-thymidine labelling (Levine et al 1968). Subsequently all WT and drug-resistant cell lines were periodically tested for mycoplasma by routine plating techniques (Hayflick 1965, Crawford 1968). Although no cultures were confirmed to be positive for mycoplasma contaminatiom, samples which yielded mycoplasma-like colonies on cultivation were discarded and fresh cultures started from uncontaminated stocks. Cultures were also occasionally tested for mycoplasma by determination of uridine phosphorylase activity (Levine 1972). The amount of ^{14}C-uridine converted to ^{14}C-uracil never exceeded the criteria for presence of mycoplasma as set out in Levine's method.

Samples of cells and culture fluid were occasionally streaked on eugonagar, mycophil agar or inoculated into thioglycollate anaerobic medium and incubated at 37^{o}C to

check for bacterial and fungal contamination. Any cell culture yielding growth was subsequently discarded.

Mutagenesis: CHO cells were grown to 5 X 10^6 cells per 100 mm culture plate at 37°C. The medium was removed and replaced with complete prewarmed medium containing 300 µg/ml EMS. After 16 hours at 37°C, the plate was washed two times with PBS and the cells trypsinized, centrifuged and washed once more with PBS. After centrifugation, the cells were resuspended in complete medium and an aliquot taken to determine PE. The remaining cells were dispensed into three 100 mm culture plates. This procedure resulted in a 50 to 90% reduction in PE. The surviving cells were subcultured for approximately 10 days until the PE had returned to normal for the culture. The cells were then used in drug-resistant mutant selection.

Thymidine Uptake: Thymidine uptake was performed both with cells growing in suspension and with cells growing in monolayer culture on 60 mm plates. In either case the cells were washed once with α-MEM plus 10% dFCS and then incubated in the same medium containing ^{3}H-thymidine (1 µCi/ml,0.5 µg/ml) at 37°C for specific times. In the plate cultures the radioactive containing medium was then aspirated, 10% trichloroacetic acid (TCA) was added, and then the plates were chilled on ice for at least 10 min. The cells were then scaped off the plate and filtered through glass fibre filters which had been prewashed with cold 10% TCA. In the suspen-

sion culture experiments, the radioactive cell suspension was filtered directly through the glass fibre filter and immediately washed with 10 ml ice-cold TCA. In routine experiments 2 ml of cell suspension containing 1 X 10^5 cells/ml was filtered per point. The filters were exposed to 10% ice-cold TCA for at least 10 min before subsequent washing. The filters from either plate or suspension cell experiments were then washed two times with 5% TCA, two times with PBS and two times with 70% ethanol (all solutions at 4^oC). The filters were then placed in scintillation vials and 1 ml of NCS tissue solubilizer was added. The vials were tightly capped and incubated at 60^oC for 1 hour before addition of 10 ml of a toluene-PPO-POPOP scintillation fluid and counting in a Beckman model LS230 liquid scintillation spectrometer.

Liquid Scintillation Counting: In the studies involving radioactive compounds, liquid scintillation techniques were used to measure radioactivity. Aqueous samples were counted after addition of at least two volumes of Aquasol scintillation fluid. Samples on membrane filters, chromatography paper, plates or other solid supports were dried and placed in a standard solution of toluene containing 4 g PPO/l and 0.2 g POPOP/l (Amersham 1977). Radioactivity was determined with the use of a Beckman model LS230 liquid scintillation spectrometer, with external standard corrections.

Protein Determination: Protein content of cell extracts was determined by the method of Lowry et al (1951) using bovine serum albumin as a standard. With purified cell extracts, the protein was first TCA-precipitated onto Millipore filters before colorimetric determination (Bennett 1967). This prevented interference by the HEPES and dithiothreitol content of the buffer when large volumes had to be used.

Hydroxyurea Determination: Hydroxyurea behaves chemically like urea and can be detected by colorimetric methods used for detecting urea or hydroxamic acids (Davidson & Winter 1963). Hydroxyurea gives a positive colour development in the Fearon carbamido diacetyl reaction (Fearon 1939, Hunninghake & Grisolia 1966). In 1971, Levine and Kretchmer reported a modification of this reaction, developing a specific and sensitive colorimetric method for the determination of hydroxyurea. Briefly, 1 ml of sample containing hydroxyurea was mixed with 0.5 ml of 3% diacetyl monoxime (Eastman Organic Chemicals). Then 0.5 ml of concentrated H_2SO_4 was added and the sample vortexed. This was followed by the addition of 0.5 ml of a 0.2% solution of N-phenyl-p-phenyldiamine·HCl. After vortexing, the samples were heated in a boiling water bath for 10 min, cooled for 10 min at room temperature and the absorbance at 540 nm was determined for each sample.

C. RIBONUCLEOTIDE REDUCTASE IN VITRO ASSAY

Preparation of Cell Extracts:

CHO: CHO cells used for routine purification were grown in 4 l spinner cultures. Four litres of α-MEM plus 10% FCS were inoculated with approximately 1 X 10^5 cells/ml and the culture placed in a 37°C water bath and stirred rapidly by means of a Teflon-coated magnetic stirring bar (5 cm). Sterile, 5% CO_2 - 95% air was bubbled through the culture vessel to maintain pH and oxygen tension. Under these conditions, the cells would grow exponentially to approximately 1 X 10^6 cells/ml. Cells were routinely harvested by centrifugation at approximately 8 X 10^5 cells/ml. The cells were washed once with PBS and the pellet resuspended in two volumes of 0.02 M HEPES pH 7.2, 1 mM DTT. The cell suspension was homogenized with the aid of a Tri-R tissue homogenizer fitted with a glass-hardened Teflon pestle and a glass tube. Usually 10 strokes at a setting of 4 was sufficient to disrupt more than 95% of the cells. The homogenate was then centrifuged at 100,000g for 60 min, after which time a dense thin layer of lipid formed at the supernatant surface. This layer was pipetted off and discarded. The remaining supernatant was removed and placed in a small beaker with a magnetic stirring bar. While monitoring the pH of the extract, I added 1.0 M acetic acid to lower the pH to 5.0 - 5.2. The extract was immediately

transferred to a Corex centrifuge tube and accelerated to 10,000 rpm in a Sorval RC2B centrifuge. The centrifugation was then terminated and the rotor allowed to decelerate to 6,000rpm under normal braking conditions. The rotor was then manually braked from 6,000rpm to a stop, the extract removed and the supernatant discarded. The pellet was immediately resuspended in 0.05 M HEPES pH 8.0, 1 mM DTT. After resuspension the extract was frozen in liquid nitrogen and stored at -76^{o}C.

CHO cell ribonucleotide reductase preparation was routinely purified approximately 5 fold further by ATP-agarose affinity chromatography. The resuspended pH 5.2 step extract was centrifuged to remove a precipitate which developed upon storage. A 4 millilitre sample of the extract was then passed through a 5 ml column of ATP-agarose. This was followed by 1 ml of 50 mM HEPES, 1 mM DTT pH 7.2, and then 10 ml of the same buffer containing 10 mM ATP. The first 1.5 ml of ATP-containing eluant was collected and desalted on Sephadex G25 equilibrated with 50 mM HEPES pH 7.2, 1 mM DTT. The resulting enzyme preparation was stored in small aliquots at -76^{o}C. During purification and handling the cell extracts were maintained at approximately 4^{o}C.

Achlya: The conditions for growing the water-mold Achlya have been described (LéJohn et al 1970, LéJohn & Stevenson 1970). Routinely, 1 X 10^{5} Achlya spores were inoculated into a 60 mm bacterilogical petri dish containing 20 ml of a

medium consisting of 5 g/l glucose and 0.5 g/l yeast extract. After 48 hours incubation at 28°C, the fungal cell mat was agitated in the culture medium and filtered through a double layer of cheesecloth. The filtrate contained mainly spores with only minimal hyphal contamination. The spores obtained in this manner were used to inoculate large carboys of the above medium (6×10^7 spores/15l medium) and the carboys were aerated vigorously at 28°C. After 9 to 10 hours incubation, the fungal mat was retrieved by filtration and washed once with distilled water. The cells were then ground to a fine powder in liquid nitrogen with the use of a precooled motor and pestle. One half millilitr of buffer (0.1 M HEPES pH 8.0, 5 mM DTT) was added for every gram of cell powder. The liquified cell extract was then centrifuged at 50,000g for 20 min and the supernatant passed through a column of medium grade Sephadex G25 (1 X 16 cm, equilibrated with 50 mM HEPES pH 7.5, 5 mM DTT). The extract was frozen in liquid nitrogen and stored at -76°C.

E. coli: E. coli KL161, a thymine requiring strain, was obtained from Dr. B. Bachmann of the E. coli Genetic Stock Centre at Yale University School of Medicine. E.coli was grown and crude extract prepared as described (Biswas et al 1965). The extract was dialysed 4 hours against 50 mM HEPES pH 7.5, 5 mM DTT. After freezing in liquid nitrogen the extract was stored at -76°C.

Affinity Chromatography Materials: ATP and GTP covalently attached to agarose were obtained commercially from P-L Biochemicals Ltd. Blue dextran-Sepharose was prepared by coupling blue dextran to cyanogen bromide (CNBr) activated Sepharose 4B. Two grams of CNBr-Sepharose were washed with 200 ml of 1 mM HCl and resuspended in 10 ml of 0.4 M sodium carbonate pH 10 containing 0.2 g blue dextran. The suspension was mixed gently on a rotary mixer for 16.5 hr at $4^{o}C$. The Sepharose was then washed with 500 ml of 0.4 M sodium carbonate pH 10. Fifty millilitre of ethanolamine pH 8.0 was incubated with the Sepharose for 1.5 hours before a final washing with 2 l of 1 M KCl.

Standard CDP Reductase Assay: CDP reductase was initially measured in a reaction mixture modified from that of Moore (1967). A total reaction mixture of 150 µl contained 4.4 mM ATP, 6.2 mM DTE, 2.7 mM magnesium acetate, 0.12 mM $FeCl_3$, 8.3 mM potassium phosphate buffer pH 7.0, and 0.40 mM ^{14}C-CDP (5000cpm/nmole) and up to 100 µl of cell extract. Subsequently, an optimized CDP reductase assay mixture was developed for the CHO enzyme. This standard assay mixture consisted of 6 mM ATP, 50 mM HEPES pH 7.2, 10 mM $MgCl_2$, 6 mM DTT and 0.4 mM ^{14}C-CDP (4800cpm/nmole) in a total volume of 150 µl. After incubation at $37^{o}C$ for 60 min, the reaction was terminated by heating in a boiling water bath for 4 min. Carrier dCMP (0.3 µmole) was added to each tube and the deoxycytidine phosphates were converted to deoxycytidine by the action of

Crotalus adamanteus or atrox venom as described by Cory (1973). Briefly, the reaction mixture was incubated with 1 mg venom dissolved in 0.1 M HEPES pH 8.0, 10 mM $MgCl_2$ (20 mg venom per ml) for two hours at 37°C. After incubation, the reaction was terminated by boiling for 4 min and the supernatant passed through a column Dowex 1-borate (Steeper & Steuart 1970). The column was washed with 4 ml of distilled water and the eluant containing the deoxycytidine was collected. Absorption at 270 nm was determined to calculate percent recovery, which usually averaged between 80 and 90%. The eluant was then added to 15 ml of Aquasol for liquid scintillation counting.

Standard ADP Reductase Assay: ADP reductase was measured in the standard reaction mixture of 50 mM PIPES pH 6.8, 6 mM DTT, 1 mM dGTP and 0.4 mM ^{14}C-ADP (4300cpm/nmole) in a total volume of 150 µl. The deoxyadenosine formed after 1 hour at 37°C was measured as described by Cory et al (1973). Briefly, the reaction was terminated by boiling for 4 min and 0.3 µmole of carrier dAMP added to each assay. Fifty µl of Crotalus snake venom (20 mg/ml 0.1 M HEPES pH 8, 10 $MgCl_2$) was added and the reaction incubated at 37°C for 2 hours. After termination by boiling, the reaction mixture was centrifuged and the supernatant passed through a column of Dowex 1-borate equilibrated with 1 mM sodium borate. The column was then eluted with a solution of 1 mM sodium borate. The first 4 ml of eluant were discarded and the next 16 ml containing deoxyadenosine

were collected. Aliquots of this eluant were checked for absorption at 270 nm and for radioactivity.

Standard GDP Reductase Assay: GDP reductase was routinely measured in a reaction mixture of 50 mM PIPES pH 6.8, 6 mM DTT, 2 mM dTTP and 0.4 mM ^{14}C-GDP (4500 cpm/nmole) in a total volume of 150 µl. After incubation at 37°C for 1 hr, the reaction was terminated by boiling and 1 mg Crotalus venom in 50 µl of 0.1 M HEPES pH 8, 10 mM $MgCl_2$ was added. After incubation at 37°C for two hours the reaction was again terminated by boiling. After centrifugation, 10 µl of the supernatant was spotted on a polyethyleneimine-cellulose (PEI-cellulose) thin layer plate and developed for 3 hours at room temperature in a solvent of the following composition: 10 ml 5 M ammonium acetate pH 9.5, 40 ml saturated sodium borate, 110 ml ethanol and 0.25 ml 0.5 M ethylenediamine tetraacetic acid (Reichard 1958). After scanning the plates with a UV light monitor, the areas corresponding to guanosine (GR) and deoxyguanosine (GdR) were cut out and their radioactivity determine by liquid scintillation counting after immersion in a toluene-PPO-POPOP scintillation cocktail. The amount of radioactivity associated with GdR was expressed as a percent of the total radioactivity in the two spots and was used to calculate the rate of GDP reduction.

Unlike the E. coli enzyme, mammalian ribonucleotide reductase was sometimes stimulated by added ferrous or ferric ion (Moore & Hurlbert 1960, Moore & Reichard 1964, Moore

1969). The standard assay mixture frequently used to measure mammalian ribonucleotide reductase contains 0.08 mM $FeCl_3$ (Moore 1967). The amount of stimulation has been reported to vary greatly, however, depending upon the buffer used, the dithiothreitol concentration and the degree of enzyme purity (Moore 1969, Hopper 1974). More recently, many investigators have chosen to omit iron from the ribonucleotide reductase assay mixtures (Noronha et al 1972, Meuth & Green 1974, Cory & Mansell 1975). In the studies reported here, ferrous and ferric ions were omitted from the standard CHO cell ribonucleotide reductase assay mixtures for the following reasons. a) The amount of stimulation of CDP reduction by $FeCl_3$ varied from no stimulation to 100% depending upon the batch of enzyme and degree of purity. b) The purer the enzyme preparation, the less stimulation was observed. Most enzyme preparations purified beyond the ATP affinity chromatography step showed no stimulation of CDP or ADP reduction, and little stimulation of GDP reduction by ferrous or ferric ion. c) Adding ferric or ferrous ion often caused precipitation problems with the other reaction components. This sometimes resulted in an inhibition of ribonucleotide reductase activity. In some trial experiments, low levels of $FeCl_3$ were included in buffers used during enzyme purification in the hope of stabilizing the enzyme. However, the presence of iron had no effect on enzyme stability in any of the purification studies.

Ribonucleotide Reductase Assay in Achlya: The same general procedures were used for CDP and ADP reduction assays in fungal extracts as for CHO preparations described above. However, the following conditions were found to be optimal for the Achlya system: CDP reduction; 50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 10 mM ATP and 0.4 mM ^{14}C-CDP (4000cpm/nmole), ADP reduction; 50 mM HEPES pH 7.5, 10 mM DTT, 1 mM dGTP and 0.4 mM ^{14}C-ADP (4300cpm/nmole). The reactions were carried out at 28^{o}C for 60 min before termination by boiling. The determination of CdR and AdR was the same as described for the CHO system.

D. PROCEDURES FOR TWEEN-80 PERMEABILIZED CHO CELLS:

Cell Permeabilization and Assay of Ribonucleotide Reduction: CHO cells were made permeable to nucleotides with only slight modifications of the method of Billen and Olson (1976). Exponentially growingCHO cells were plated at a cell density of 2 X 10^6 cells/100 mm culture plate in α-MEM plus 10% FCS. After incubation at 37°C for approximately 40 hr, the cells were trypsinized, centrifuged and washed once with α-MEM plus serum. The cells were then resuspended at 1 X 10^7 cells/ml of permeabilizing buffer consisting of 1% Tween-80, 0.25 M sucrose, 10 mM HEPES buffer pH 7.2, 2 mM DTT and incubated for 30 min at 22°C. The cells were dispensed into assay tubes (0.5 ml/tube) and centrifuged.

For CDP reduction, the cell pellet was gently resuspended in 200 µl of the above permeabilizing buffer and made up to a final volume of 300 µl containing 50 mM HEPES pH 7.2, 6 mM ATP, 6 mM DTT, 10 mM $MgCl_2$, 0.037 mM ^{14}C-CDP (55,000cpm/nmole), 0.67% Tween-80 and 0.167 M sucrose.

For GDP reduction, the cell pellet was gently resuspended in 200 µl of 1% Tween-80, 75 mM PIPES pH 7.0, 2 mM DTT, 0.25 M sucrose and made up to 300 µl final volume containing 50 mM PIPES pH 7.0, 6 mM DTT, 1 mM dTTP, 0.1 mM $FeCl_3$, 0.022 mM ^{14}C-GDP (96,000cpm/nmole), 0.67% Tween-80 and 0.167 M sucrose.

Both CDP and GDP reductase assays were then agitated for 60 min at 37°C, after which time the reaction was terminated by boiling for 4 min. The amount of CdR or GdR formed was measured after treatment with snake venom as described for the CHO system.

CHO Cell Synchronization: CHO cells were synchronized by isoleucine deprivation (Ley & Tobey 1970). Cells were plated at densities of 2 X 10^6 cells/100 mm culture plate in α-MEM plus 10% FCS. After incubation at 37°C for 24 hours, the cells were washed once with PBS and α-MEM minus isoleucine plus 10% dFCS was added to each plate. After 40 hr, the deficient medium was replaced with complete α-MEM containing isoleucine and 10% dFCS.

Assay of Thymidine Kinase: CHO cells were grown and permeabilized by Tween-80 treatment as described above. Five million permeabilized CHO cells were resuspended in a volume of 300 µl containing 0.67% Tween-80, 0.167 M sucrose, 33 mM HEPES pH 8, 5 mM ATP, 5 mM $MgCl_2$ and 30 µM ^{3}H-thymidine (3 µCi/ml). The samples were agitated at 37°C and at intervals the reaction was terminated by boiling for 4 min. After centrifugation, 20 µl of supernatant was spotted on a 2 cm^2 piece of DEAE-cellulose paper filter (Breitman 1963). The filter was washed by suction with 40 ml of 1 mM ammonium formate, and 10 ml acetone. It was then placed in a scintillation vial and 0.1 ml 70% ethanol and 1 ml NCS solubilized added. The vials were tightly capped and incubated at 60°C

for 1 hr, before the addition of 10 ml toluene-PPO-POPOP scintillation fluid and determination of radioactivity.

E. ISOLATION AND PURIFICATION OF HS COMPOUNDS:

Achlya: HS2 and HS3 were isolated and purified from formic acid extracts of Achlya cells by D. McNaughton and G. Klassen as described (LéJohn et al 1975, Lewis et al 1976). HS3 is a dinucleotide composed of equimolar amounts of uridine and adenosine. Recent work indicates a possible structure of glutamyl-ADP ribitol:UDP mannitol tetraphosphate (H. LéJohn, personal communication). The concentration of HS3 was determined by absorption at 260 nm using an extinction coefficient of 23,200. Similarly, HS2, which consists of two uridine moeities per molecule, was determined using an extinction coefficient of 19,800 (H. LéJohn, personal communication).

CHO: HS3 was isolated from formic acid extracts of CHO cells starved of glutamine by S.Goh and D. McNaughton as described (Goh & LéJohn 1977, Goh et al 1977, Lewis et al 1977). The HS3 from CHO cells was purified and quantitated in a manner identical to the fungal compound.

HS3 Biosynthesis and Pool Sizes: The rate of synthesis and pool sizes of HS3 were measured by ^{32}P-orthophosphate labelling of Achlya or CHO cells. Uptake of ^{32}P-orthophosphate into formic acid cellular extracts and determination of the amount of radioactivity were performed by D. McNaughton and S. Goh as described (Goh & LéJohn 1977).

F. SOURCE OF MATERIALS

Most biochemicals were purchased from Sigma Chemical Co., St. Louis and all radiochemicals were from Amersham/ Searle Ltd. Sources of all other materials are listed in Table 1.

Table 1. Source of materials.

Material	Source
agarose-hexane-adenosine-5'-triphosphate	P-L Biochemicals, Milwaukee
agarose-hexane-guanosine-5'-triphosphate	P-L Biochemicals
alpha-minimal essential medium	Flow Labs, Rockville
aquasol	NEN Ltd.
N-carbamoyloxyurea SQ #10,726	Ms. B. Stearn Research Evaluation Squibb Institute For Medical Research Princeton, N.J.
colchicine	BDH Labs, Toronto
Crotalus adamanteus venom	Sigma Chemical Co.
Crotalus atrox venom	Sigma Chemical Co.
culture plates (60 mm plastic)	Lux Sci. Corp., Newbury
culture plates (100 mm plastic)	Lux Sci. Corp.
culture bottles (16 oz glass)	Brockway Glass Co.
Dowex 1-X8 anion exchange resin 200-400	Bio-Rad Labs, Richmond
ethyl methane sulfonate	Eastman Kodak, Rochester
fetal calf serum	GIBCO Ltd., Grand Island
fetal calf serum (dialysed)	GIBCO Ltd.
formamidoxime	Aldrich Chemical Co.
guanazole NSC #1895	B. Wood, Jr. Drug Development Branch National Cancer Institute Bethesda, Maryland

Table 1. Continued.

Material	Source
hydroxylamine	Matheson, Cole & Bell Ltd.
hydroxyurethane	J.T. Baker, Phillipsburg
filters, 2.5 cm Millipore	Millipore Corporation
filters, 2.5 cm glass fibre #9-730-62	Fisher Chemical Co., Fair Lawn
Linbro cloning dishes #IS-FS-96-TC	Linbro Chemical Co.
methylene blue	Fisher Sci. Co. Ltd.
methylhydroxylamine	Aldrich Chem., Milwaukee
NCS tissue solubilizer	Amersham/Searle, Arlington Heights
PEI-cellulose thin layer plates	Brinkmann Inst., Westbury
penicillin G	GIBCO
polyethylene glycol 6000	J.T. Baker
POP	Fisher Sci. Co. Ltd.
POPOP	Fisher Sci. Co. Ltd.
Sendai virus Lot #134-1	Connaught Labs, Toronto
spinner bottles (suspension culture)	GIBCO
streptomycin	GIBCO
toluene (scintanalyzed)	Fisher Sci. Co. Ltd.
trypsin Bacto #0153-61	Difco Labs, Detroit
Tween-80 (polyoxyethylene(20)sorbitan monooleate)	JT. Baker
urea	GIBCO

RESULTS

RESULTS

A. RESPONSE OF WT CHO CELLS TO HYDROXYUREA AND GUANAZOLE

1. Growth rate: Figure 2 shows the growth of WT CHO cells on plastic tissue culture plates in α-minimal essential medium containing 10% fetal calf serum. The cell number increased exponentially to at least 1 X 10^7 cells/60 mm plate. At 37°C, the doubling time of WT CHO in plate culture was approximately 12 to 14 hours depending upon the particular batch of medium or serum. Figure 2 also shows the effect of hydroxyurea and guanazole on cellular growth rate. Upon addition of 0.33 mM hydroxyurea, cell division was almost completely halted, there being only a slight increase in cell number before all cell division ceased 40 hours after drug addition. In a similar manner 2 mM guanazole stopped all WT CHO cell division within 40 hours.

2. DNA synthesis: Hydroxyurea has been reported to be a specific inhibitor of DNA synthesis in a variety of mammalian cells (Young & Hodas 1964, Sinclair 1967, Bacchetti & Whitmore 1969). Therefore the effect of hydroxyurea on ^{3}H-thymidine incorporation into acid precipitable, CHO cellular material was measured. Figure 3 shows that in the absence of drug, ^{3}H-thymidine incorporation increases linearly with time for at least 50 min. However, within 10 min of the addition of 0.26 mM hydroxyurea, further thymidine incorpora-

Figure 2: Effect of hydroxyurea (⊡) and guanazole (⊙) on the normal growth rate (△) of WT CHO cells. CHO cells growing exponentially on plates were removed with phosphate buffered saline containing trypsin and replated at 4 X 10^4 cells per 60 mm plate in standard growth medium. After incubation at 37^{o}C for specific times, cells were trypsinized from the plates and counted. The arrow indicates addition of 0.33 mM hydrozyurea or 2.0 mM guanazole.

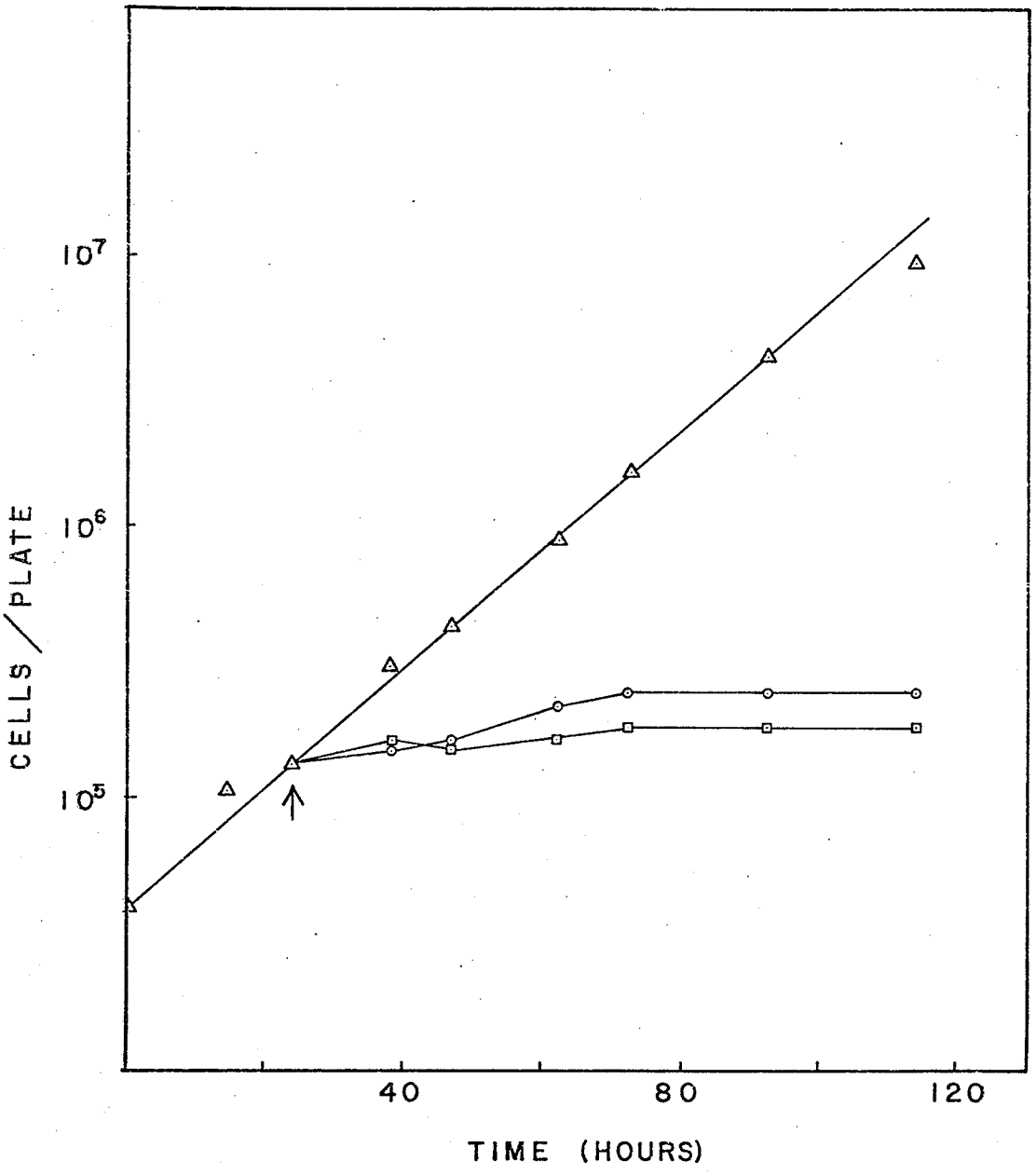
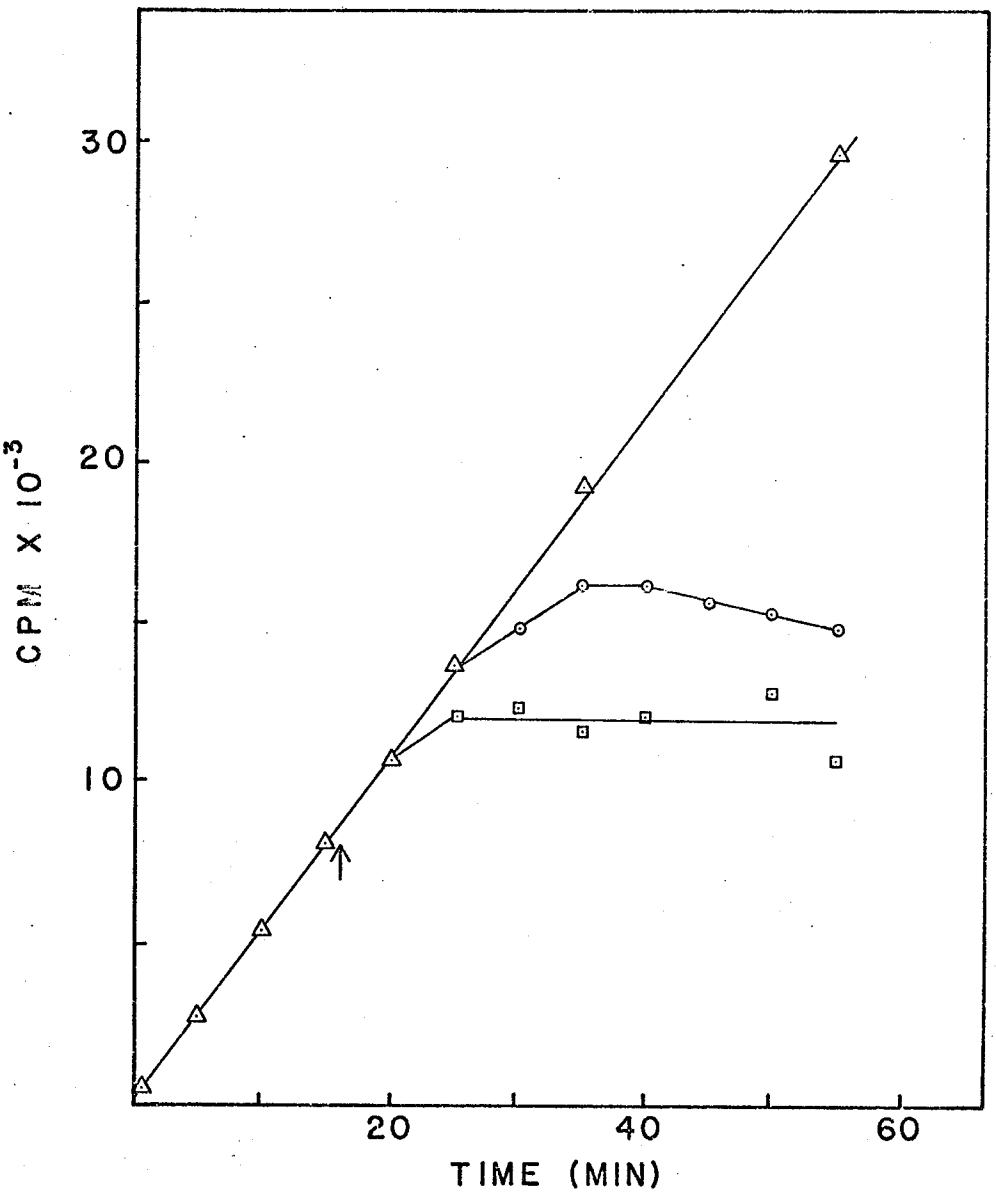

10^7
10^6
10^5
CELLS / PLATE
40
80
120
TIME (HOURS)

Figure 3: Thymidine uptake into WT CHO acid precipitable material in the absence (Δ) and presence of 0.26 mM hydroxyurea (⊡) or 2.0 mM guanazole (⊙). Three separate suspension cultures of WT CHO cells were incubated at 37°C under 5% CO_2 in the presence of standard medium containing 1 μCi/ml, 0.5 μg/ml ^{3}H-thymidine. The arrow indicates the addition of hydroxyurea or guanazole to the reaction vessels. At intervals, 2 ml (2 X 10^5 cells) were filtered onto glass fibre filters, rinsed with 10% TCA and radioactivity determined as described in Materials And Methods.


30
20
10
CPM X 10^{-3}
20
40
60
TIME (MIN)

tion was prevented. Guanazole at 2 mM had a similar effect although at this concentration the drug took somewhat longer than hydroxyurea to halt thymidine incorporation completely.

3. Plating efficiency: When plated at low cell numbers into plastic tissue culture plates WT CHO cells formed macroscopic colonies within 8 days at 37°C. Figure 4 shows that the number of colonies formed is proportional to the number of CHO cells plated. In this experiment, approximately 80% of cells plated formed colonies; ie the plating efficiency (PE) equaled 0.80.

Figure 5 shows the reduction in relative plating efficiency (RPE) of the WT CHO when plated in the presence of increasing amounts of hydroxyurea. Hydroxyurea concentrations below 0.05 mM have little or no effect on RPE. As the drug concentration increased the RPE fell logarithmically. By 0.3 mM hydroxyurea, the PE had been reduced to 1 colony per 10^5 cells or less. Plating efficiencies below 10^{-5} were difficult to measure because of a cell density effect which decreased mutant survival at high cell densities. This phenomenon will be discussed in the next section.

Guanazole reduced the RPE of WT CHO as hydroxyurea did, although hydroxyurea was far more potent on a molar basis. It took almost 2 mM guanazole to reduce the RPE below 10^{-5} (Fig 6).

Figure 4: The effect of number of cells plated on the colony forming ability of WT CHO cells. Plastic plates (60 mm) were inoculated with increasing numbers of cells and after incubation at 37°C for 8 days, the colonies on each plate were stained and counted. The slope of the line is 0.80, and is equal to the plating efficiency.

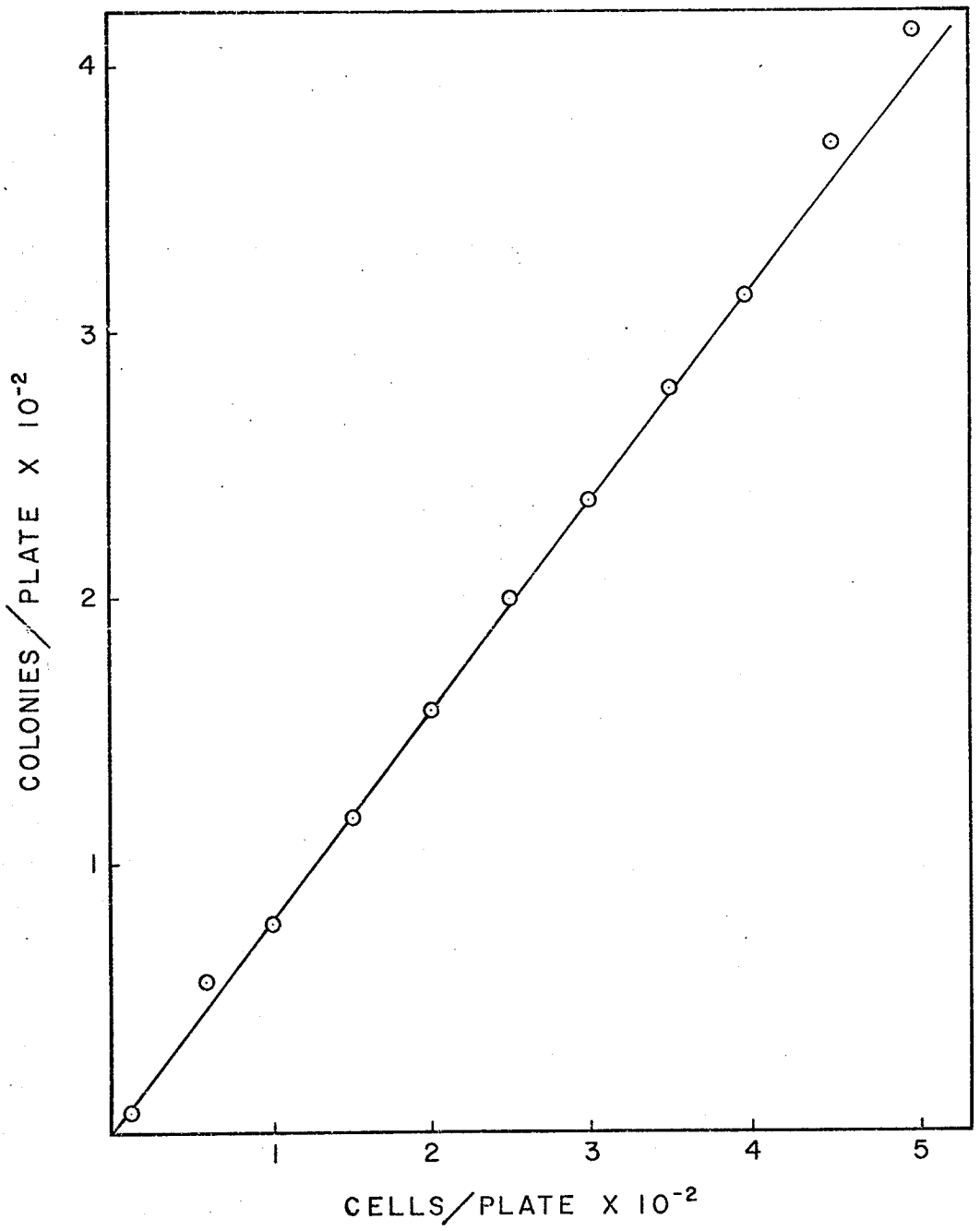

COLONIES/PLATE X 10^{-2}
CELLS/PLATE X 10^{-2}
1
2
3
4
5

Figure 5: Relative plating efficiencies of WT CHO cells plated in the presence of increasing hydroxyurea concentrations. From 3 X 10^2 to 5 X 10^5 cells were plated in 100 mm plastic plates containing standard medium plus varying concentrations of hydroxyurea. After 8 to 16 days incubation at 37^{o}C, the plates were rinsed, stained and surviving colonies counted.

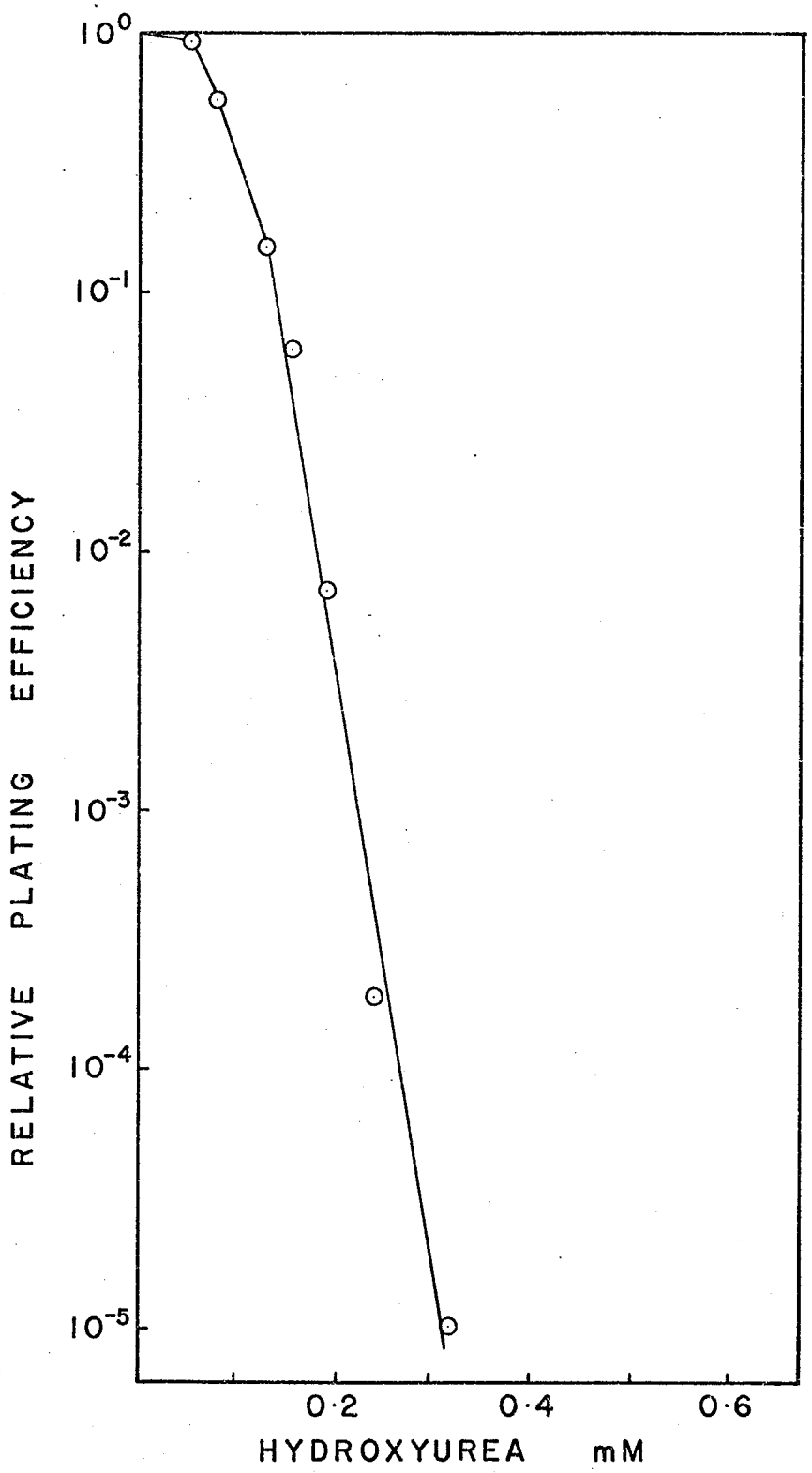
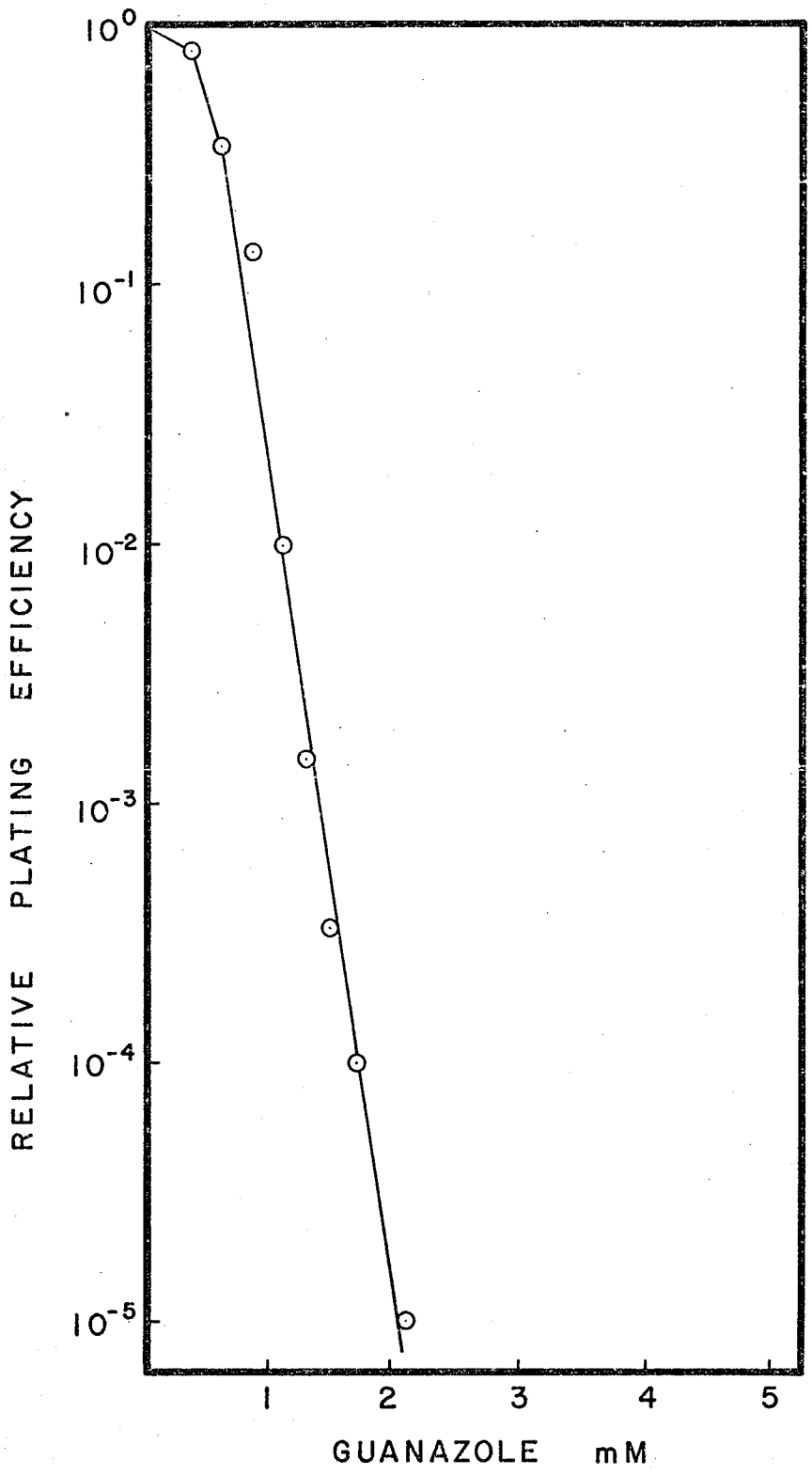

RELATIVE PLATING EFFICIENCY
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
0·2
0·4
0·6
HYDROXYUREA mM

Figure 6: Relative plating efficiencies of WT CHO cells plated in the presence of increasing guanazole concentrations. From 3 X 10^2 to 5 X 10^5 cells were plated in 100 mm plastic plates containing standard medium plus varying concentrations of guanazole. After 8 to 16 days incubation at 37°C, the plates were rinsed, stained and the surviving colonies counted.

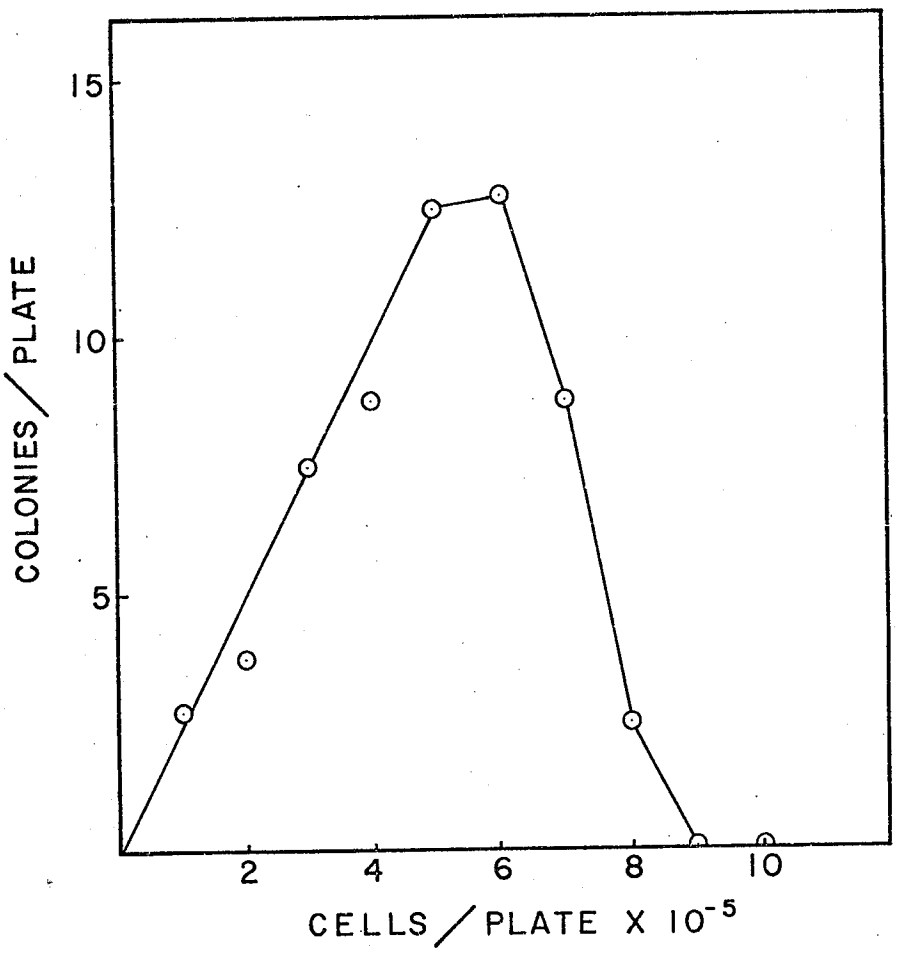

RELATIVE PLATING EFFICIENCY
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
1
2
3
4
5
GUANAZOLE mM

B. ISOLATION OF HYDROXYUREA-RESISTANT CELL LINES

1. Cell density effect: It was decided to select those cells which were capable of forming colonies in 0.33 mM hydroxyurea as likely candidates for mutant cell lines resistant to the drug. The PE of WT CHO at this concentration varied from 5×10^{-5} to 5×10^{-6} when the line was plated at 5×10^{5} cells per 100 mm culture plate. At cell densities approaching 10^{6} cells/plate, however, the number of surviving colonies fell drastically with increasing cell number (Fig 7). In this particular experiment the number of colonies surviving in 0.33 mM hydroxyurea increased linearly with increasing number of cells plated up to approximately 6×10^{5} cells/plate. Above this cell density the number of surviving colonies decreased and eventually fell to zero by 9×10^{5} cells/plate. In similar experiments the cell density was occasionally higher before the number of surviving colonies declined, but in all experiments cell densities over 10^{6} cells/plate resulted in no colony formation. For this reason it was decided not to exceed 5×10^{5} cells/100 mm plate when selecting resistant cell lines.

The phenomenon of decreased mutant survival at high cell densities has been encountered in many drug-resistant selection systems. For example, Fujimoto et al (1971) and Clements (1972) showed that metabolite exchange between

Figure 7: Effect of cell density upon colony survival in the presence of 0.33 mM hydroxyurea. Increasing numbers of WT CHO cells were plated in 100 mm plates containing standard medium plus 0.33 mM hydroxyurea. After 16 days at 37^{o}C, the plates were stained and the colonies counted. Each point represents the average result obtained from 4 individual plates.


15
10
5
COLONIES / PLATE
2
4
6
8
10
CELLS / PLATE X 10^{-5}

sensitive and resistant cells prevents the recovery of thioguanine-resistant variants from mixtures of cells grown at high cell densities under selective conditions. Similarly, Shapiro et al (1972) found the yield of 6- mercaptopurine-resistant cells decreased rapidly at cell densities above 1.8×10^3 cells per cm^2. The reason for the reduced yield of mutant colonies at high cell densities is not known, but may involve such factors as metabolic cooperation, depletion of medium components or the accumulation of unidentified toxic substances originating from the death of sensitive cells.

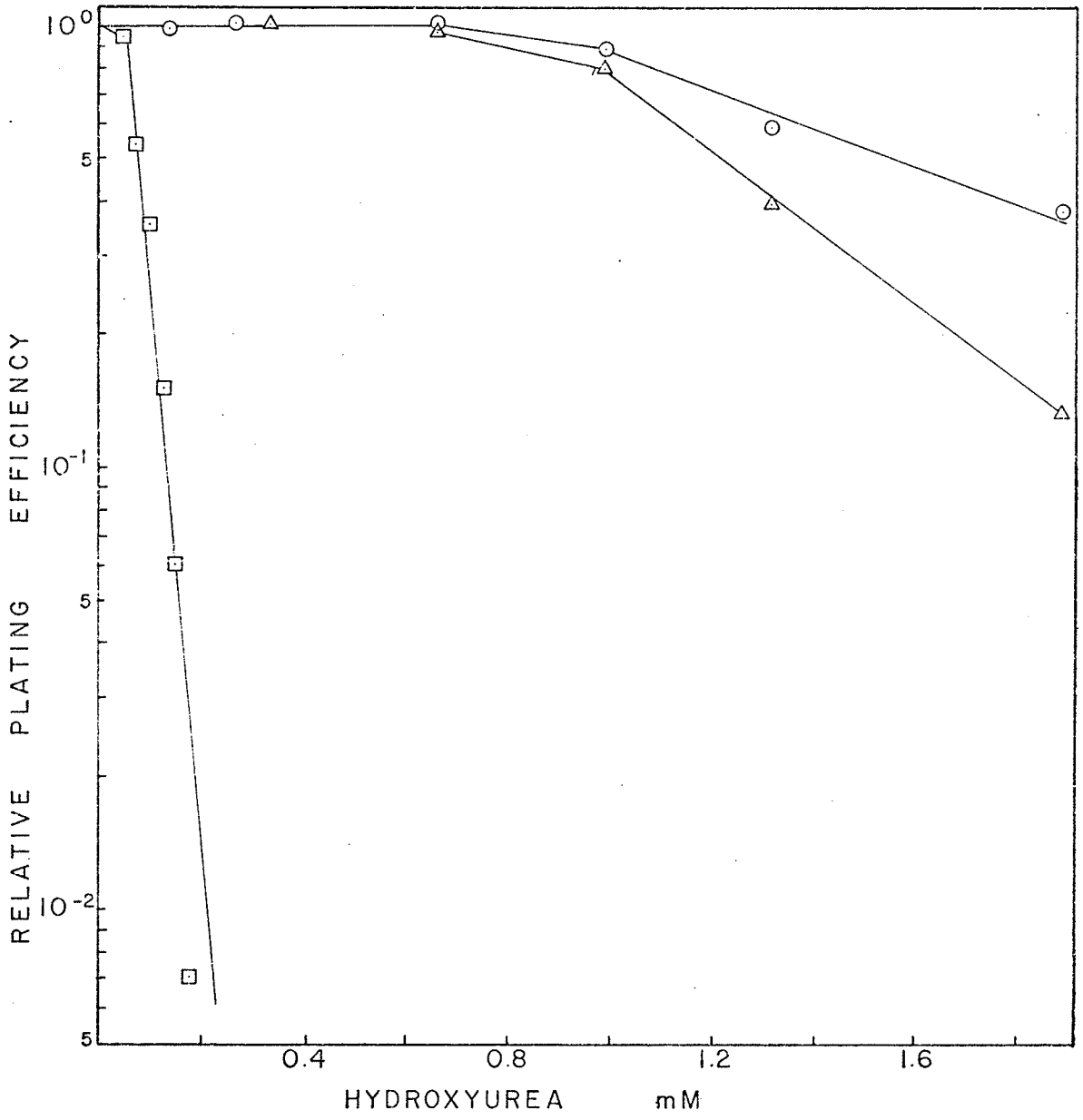
2. Isolation: Cells from two independent clones of WT CHO cells were plated at 5×10^5 cells per 100 mm plastic tissue culture plate in the presence of standard medium containing 0.33 mM hydroxyurea. The plate with cells from the recently cloned WT CHO culture contained a single surviving colony after 16 days incubation at 37^oC. The colony was trypsinized, picked with a sterile Pasteur pipette and transferred to medium lacking hydroxyurea. After 8 days the cells were again typsinized, diluted and dispensed into Linbro cloning wells at approximately 1 cell per well. After an additional 8 days, several independent clones were isolated and tested for hydroxyurea sensitivity. One clone, designated HU^R-4, was chosen for further study. Another cell line, designated HU^R-2, was isolated from the second independent WT CHO clone which had been maintained in serial

culture for several months before the selection took place. Again, 5 X 10^5 cells were plated with standard medium containing 0.33 mM hydroxyurea. After 16 days incubation about 25 large colonies had developed on the plate. All cells on the plate were trypsinized and transferred to another plate containing 0.46 mM hydroxyurea. The cells quickly grew to a monolayer with apparently very little cell death occurring. The cells were then cloned in the presence of 0.33 mM hydroxyurea and one clone, HU^R-2, was chosen for further study.

3. Plating efficiencies of HU^R-2 and HU^R-4: Figure 8 shows the response to hydroxyurea of the two cell lines which survived the selection procedure described above. Both HU^R-2 and HU^R-4 are obviously far more resistant to the cytotoxic effects of hydroxyurea than the parental cell line. For example, at 0.33 mM hydroxyurea WT CHO cells have a RPE of approximately 10^{-5} (Fig 5), whereas the RPE values of the two resistant cell lines are unaffected by the presence of this concentration of drug (Fig 8). The RPE of the parental cell line is reduced by 50% at 0.07 mM hydroxyurea, while it requires 15-fold and 19-fold more hydroxyurea to reduce the RPE of HU^R-4 and HU^R-2 respectively by 50% (Fig 8).

4. Growth rates of HU^R-2 and HU^R-4: Although both HU^R-2 and HU^R-4 are capable of 100% relative colony forming ability in concentrations of hydroxyurea which reduce the ability of

Figure 8: Relative plating efficiencies of WT CHO (⊡), HU^R-2 (⊙) and HU^R-4 (⍋) in increasing concentrations of hydroxyurea. Cells (350) were incubated at 37°C for 8 days in standard medium containing varying amounts of hydroxyurea.


RELATIVE PLATING EFFICIENCY
10^0
5
10^-1
5
10^-2
5
0.4
0.8
1.2
1.6
HYDROXYUREA mM

WT CHO cells to form colonies by more than 10^{-5}, the resistant colonies which eventually develop, especially at higher concentrations of the drug, are often reduced in size. Most likely this is due to a reduced growth rate in the presence of hydroxyurea. Figures 9 and 10 show growth curves for HU^R-2 and HU^R-4 in the presence and absence of hydroxyurea. In the absence of the drug both cell lines exhibited doubling times of approximately 14 hours, a value indistinguishable from the WT CHO cell doubling time under these conditions. However, after the addition of 0.33 mM hydroxyurea, a concentration which reduces the RPE of WT CHO cells by approximately 10^{-5}, both HU^R-2 and HU^R-4 were capable of fairly high but significantly reduced growth rates. Figure 9 shows that after a 24 hour lag HU^R-2 had a doubling time of 19 hours in the presence of 0.33 mM hydroxyurea. Under slightly different experimental conditions, HU^R-4 showed a constant doubling time of 22 hours in the presence of the same concentration of drug (Fig 10).

5. Stability of hydroxyurea-resistant phenotype: In order to determine whether, upon cultivation in the absence of hydroxyurea, the resistant cell lines would maintain their resistant phenotype, RPE values were dertermined on HU^R-2 and HU^R-4 over a period of 27 months of serial culture in the absence of the drug. Table 2 shows that both cell lines maintained a reproducibly high level of resistance to 1 mM hydroxyurea. This concentration of hydroxyurea is three-fold

Figure 9: Growth rate of HU^R-2 in the presence of 0.33 mM hydroxyurea. Plates (60 mm) containing 1 X 10^5 cells each were incubated at 37^oC in standard medium in the absence of hydroxyurea. Twenty four hours later, 0.33 mM drug was added to half the plates. At specific time intervals the plates were drained and the cells trypsinized and counted. (⊙) 0 mM hydroxyurea, (●) 0.33 mM hydroxyurea.

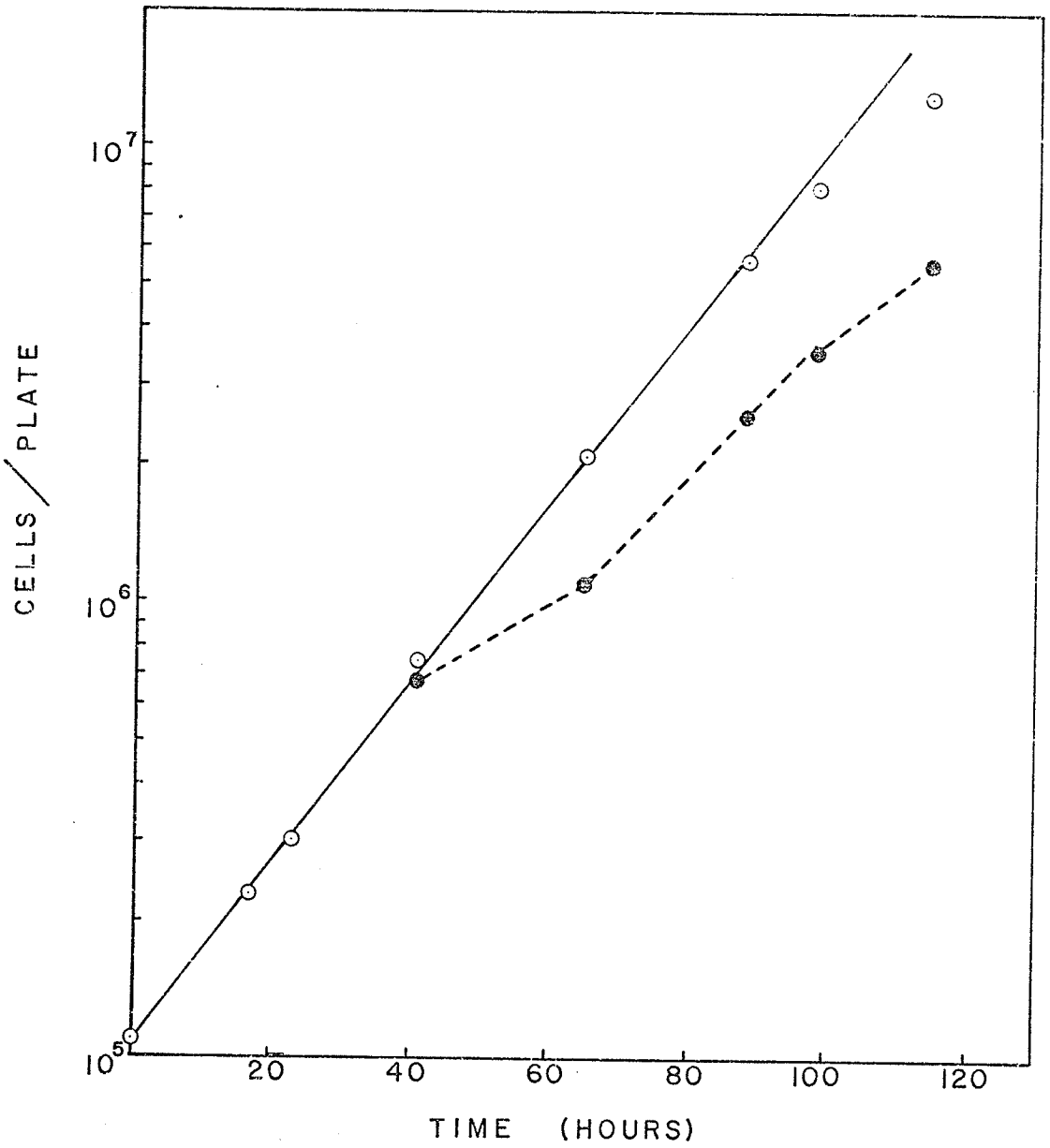

CELLS / PLATE
10^7
10^6
10^5
20
40
60
80
100
120
TIME (HOURS)

Figure 10: Growth rate of HU^R-4 in the presence of 0.33 mM hydroxyurea. Plates (60 mm) containing 5 X 10^4 cells each were incubated at 37°C in standard medium in the absence (⊙) or presence (●) of 0.33 mM hydroxyurea. At specific time intervals the plates were drained and the cells trypsinized and counted.

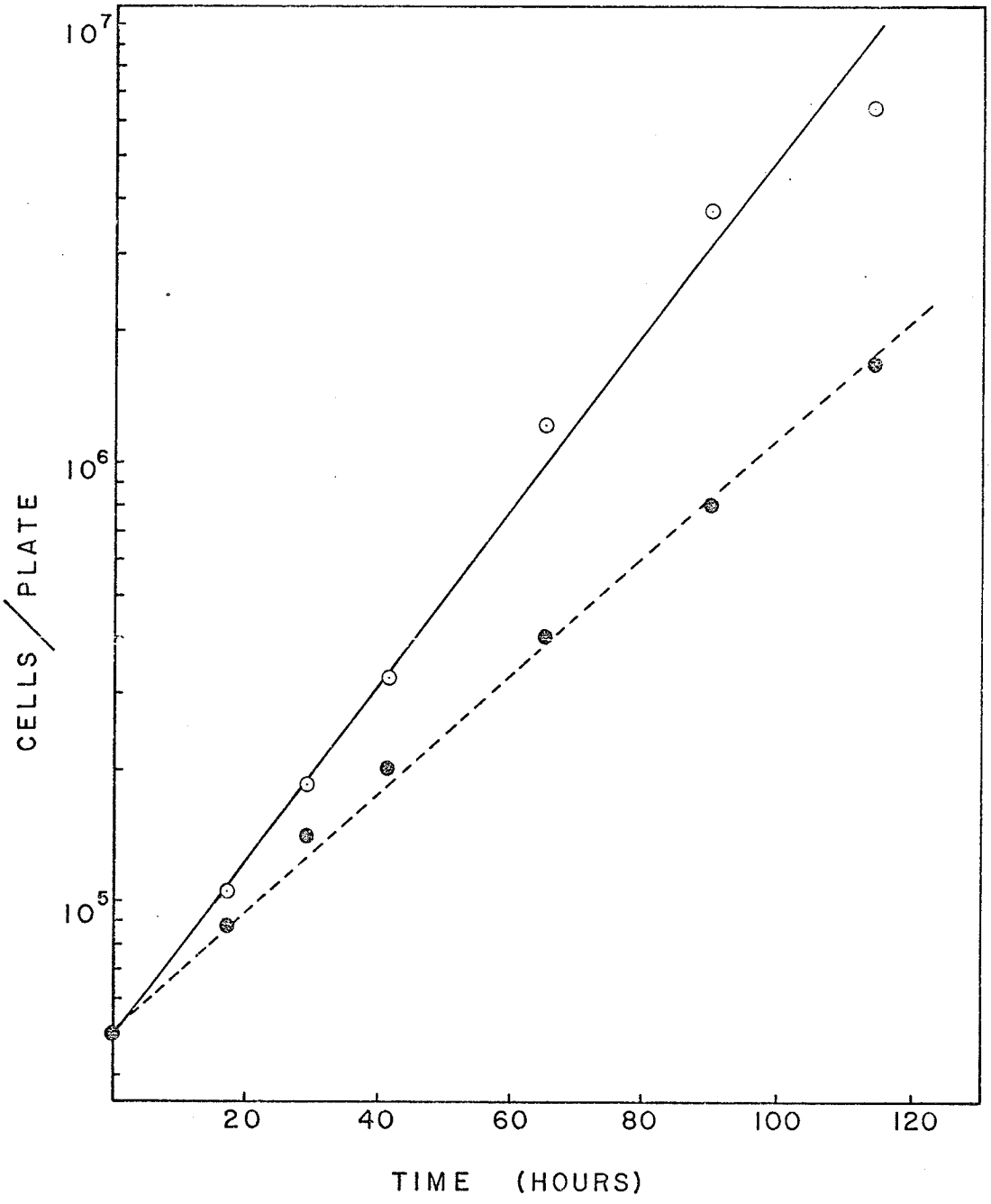

10^7
10^6
10^5
CELLS / PLATE
20
40
60
80
100
120
TIME (HOURS)

Table 2. Relative plating efficiency (RPE) of HU^R-2 and HU^R-4 with serial cultivation.

Culture Time (months)	RPE at 1 mM Hydroxyurea	
	HU^R-2	HU^R-4
0	0.92	0.76
5	0.90	0.81
15	0.84	0.74
27	0.93	0.80

higher than the concentration required to reduce the WT CHO cell RPE to 10^{-5} (Fig 5).

6. Reconstruction experiments: Table 3 shows the results of an experiment testing the expression of the hydroxyurea-resistant trait of HU^R-2 in the absence and presence of WT CHO cells. HU^R-2 shows almost identical RPE in the absence or presence of 0.40 mM hydroxyurea, although the colonies in the presence of the drug were smaller than in its absence. Under the same conditions, out of 5 X 10^5 WT cells plated, only one colony developed. The addition of 5 X 10^5 WT CHO cells to the plate containing 400 HU^R-2 cells and 0.40 mM hydroxyurea, only marginally increased the number of colonies developing. The 359 colonies which did develop were generally larger than those of HU^R-2 plated alone with hydroxyurea. This may be because of a feeding effect of the excess numbers of inhibited WT CHO cells. It is clear from this experiment that HU^R-2 does not owe its resistance to a destruction of hydroxyurea in the external growth medium. Such a destruction would have allowed growth of the WT CHO cells during the mixing experiment.

7. Fluctuation analyses and mutation rates: Fluctuation analyses (Luria & Delbrück 1943) were performed to determine the pattern and rate of appearance of hydroxyurea-resistant cells in the WT CHO population.

Table 4 shows the result of a fluctuation analysis with 38 replicate cultures compared to a control experiment

Table 3. Response of mixtures of hydroxyurea-resistant and wild-type CHO cells to hydroxyurea.

Cell Line	Cells Plated	Hydroxyurea	Colonies
1. HU^R-2	400	0 mM	335
2. HU^R-2	400	0.4 mM	325
3. WT CHO	400	0 mM	370
4. WT CHO	5×10^5	0.4 mM	1
5. HU^R-2 + WT CHO	400 + 5×10^5	0.4 mM	359

Cells were plated in 100 mm plastic plates containing α-MEM plus 10% FCS in the presence or absence of 0.4 mM hydroxyurea. After 10 days at 37°C, the plates were rinsed, stained and the visible colonies counted.

Table 4. CHO cell fluctuation test for hydroxyurea-resistance.

	Experiment A	Replicate Sample Control
No. replicate cultures	38	1
No. samplings per culture	1	30
Initial no. cells per replicate	100	-
Final no. cells per replicate	$1.4 \pm 0.2 \times 10^7$	1.5×10^7
Final no. cells per selection	5.0×10^5	5.0×10^5
Hydroxyurea concentration	0.26 mM	0.26 mM
No. of replicates with N hydroxyurea-resistant colonies		
N = 0 - 20	6	0
21 - 30	9	23
31 - 40	7	7
41 - 80	10	0
81 - 120	2	0
121 - 200	2	0
201 - 300	1	0
320	1	0
Range	17 - 320	20 - 35
Median	36	25
Mean	59	27
Variance	3674	20
Ratio of $\frac{\text{variance}}{\text{mean}}$	62	0.74
Mutation rate per cell per generation *	2.0×10^{-5}	

*Mutation rate calculated from the median number of mutants as described by Lea and Coulson (1949).

consisting of 30 replicate samples of a single culture. In the fluctuation test the 38 replicate cultures of WT CHO cells were grown from initial inocula of 100 cells each to final populations of 1.4×10^7 cells. A sample of 5×10^5 cells from each replicate was then exposed to 0.26 mM hydroxyurea for 16 days before counting the number of resistant colonies developing per plate. In the control experiment 30 replicate samples of 5×10^5 cells each were taken from the stock WT CHO culture and exposed to 0.26 mM hydroxyurea as above.

The data in Table 4 show that the variance in the number of resistant colonies observed among replicate cultures in the fluctuation experiment is much greater than that found for replicate platings from a single culture (3674 versus 20) and is clearly skewed toward higher frequencies of resistant clones. This indicates random generation of the hydroxyurea-resistant phenotype during proliferation of the WT CHO population, as would be expected if it represents a mutant genotype. With these data a mutation rate of 2×10^{-5} per cell per generation can be estimated from the median frequency of resistant cells per independent replicate culture.

In order to confirm the fluctuation analysis results, the independent replicate which yielded the highest frequency of hydroxyurea-resistant clones in the previous experiment was used to repeat a fluctuation test. The culture was divided into 30 replicate cultures each containing 100

cells, and each was allowed to grow to a final cell population of approximately 2.1×10^7 cells. A sample of 5×10^5 cells from each of the new replicates was then plated with two concentrations of hydroxyurea (0.26 mM, 0.33 mM). Table 5 shows that the variances at both selecting concentrations of hydroxyurea were far greater than the variance of the replicate control, an observation that indicates the spontaneous nature of hydroxyurea-resistance. Using the median frequency of hydroxyurea-resistant colonies the mutation rate for each level of drug was calculated to be: 0.26 mM hydroxyurea: 3×10^{-5} per cell per generation; 0.33 mM hydroxyurea: 0.48×10^{-5} per cell per generation.

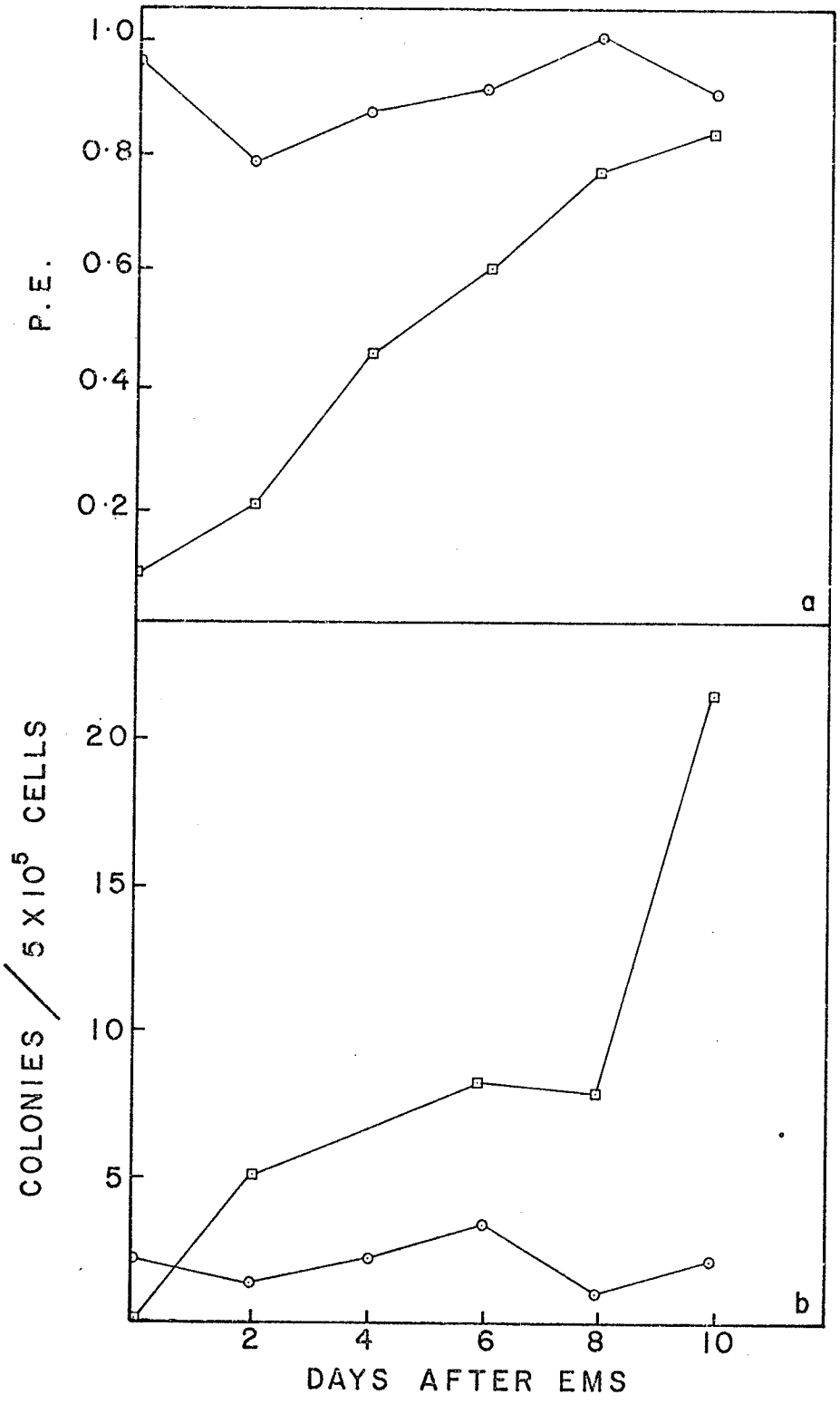
8. Effect of mutagens on hydroxyurea-resistance frequency: In a series of pilot experiments WT CHO cells were exposed to various concentrations of the mutagens ethyl methane sulfonate (EMS) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). MNNG seemed to have little, if any, effect on the frequency of surviving colonies. EMS, on the other hand, increased the frequency of hydroxyurea-resistant colonies from 2- to 10-fold depending upon the extent of treatment and recovery time allowed before exposure to the selective agent. Figure 11a shows that upon exposure for 16 hr to 300 µg/ml EMS, the PE of CHO cells was reduced to about 0.10. It required more than 10 days for the culture to recover the normal PE. Figure 11b shows that the number of cells resistant to hydroxyurea in the EMS-treated population, from 2 days on,

Table 5. CHO cell fluctuation test for hydroxyurea-resistance.

	Exp. A	Exp. B	Replicate Control
No. of replicate cultures	30	30	1
No. of samplings per culture	1	1	30
Initial no. cells/replicate	100	100	-
Final no. cells per replicate	$2.1\pm0.4 \times 10^7$	$1.9\pm0.2 \times 10^7$	2.0×10^7
Final no. cells/selection	5.0×10^5	5.0×10^5	5.0×10^5
Hydroxyurea concentration	0.26 mM	0.33 mM	0.33 mM
No. of replicates with N hydroxyurea-resistant colonies			
N = 0	0	1	0
1 - 2	0	6	0
3 - 5	0	10	4
6 - 10	0	8	19
11 - 15	1	1	7
16 - 20	0	2	0
21 - 30	1	0	0
31 - 50	10	1	0
51 - 100	9	1	0
101 - 200	3	0	0
201 - 300	5	0	0
340	1	0	0
Range	22 - 340	0 - 79	4 - 13
Median	61	5	9
Variance	9203	221	5.2
Ratio of $\frac{\text{variance}}{\text{mean}}$	88	24	0.6
Mutation rate/cell/generation*	3.0×10^{-5}	4.8×10^{-6}	

* Calculated from the median number of mutants (Lea & Coulson 1949).

Figure 11: Response of WT CHO cells to treatment with EMS. WT CHO cells (5×10^6) exponentially growing in 100 mm plates were treated with 300 µg EMS/ml standard medium for 16 hours at 37°C. The cells were then trypsinized, washed and redispensed at 5×10^5 cells per plate. At two day intervals the cells were trypsinized and tested for PE (a) (⊡) or for the number of cells capable of forming colonies in 0.33 mM hydroxyurea within 16 days (b) (⊡). Control cells from non-EMS exposed cultures were also tested for PE (a) (⊙) or for the number of colonies formed in the presence of 0.33 mM hydroxyurea (b) (⊙).


1·0
0·8
0·6
0·4
0·2
P.E.
a
20
15
10
5
COLONIES / 5 X 10⁵ CELLS
b
2
4
6
8
10
DAYS AFTER EMS

is greater than the number found in control cultures. In a separate experiment cells which had been allowed 10 days for expression of EMS-induced mutations and control cells were plated with increasing concentrations of hydroxyurea and the RPE was determined. Figure 12 shows that at almost all concentrations tested, the EMS-treated population had an increased frequency of hydroxyurea-resistant cells. At 0.33 mM hydroxyurea, the EMS-treated population had an approximately 10-fold increase in RPE when compared to the non-mutagenized population.

9. Cross-resistance to other compounds: Cell lines selected for resistance to one cytotoxic chemical sometimes exhibit an altered response to other agents. For example, resistance to colchicine (Ling & Thompson 1974) confers cross-resistance to other unrelated drugs, whereas resistance to the membrane active agent, concanavalin-A, results in collateral-sensitivity to a variety of unrelated agents (Wright 1973). To test whether similar phenomena could be demonstrated with the hydroxyurea-resistant cell lines, the plating response of HU^R-2 was compared to WT CHO cells with a variety of compounds (Table 6).

Five compounds which are structurally unrelated to hydroxyurea were tested for their ability to reduce the PE of HU^R-2 and WT CHO cells. All of α'α'dipyridyl, phenethyl alcohol, digitonin, ouabain and 1-β-D-arabinofuranosylcytosine (ara-C) were cytotoxic for both lines. No differential

Figure 12: Relative plating efficiencies of untreated cells (⊙) and cells treated with EMS (△) as described in Materials & Methods. The treatment reduced the PE of the culture by 80%. After 10 days of recovery, the cells were trypsinized and replated in standard medium containing increasing concentrations of hydroxyurea.

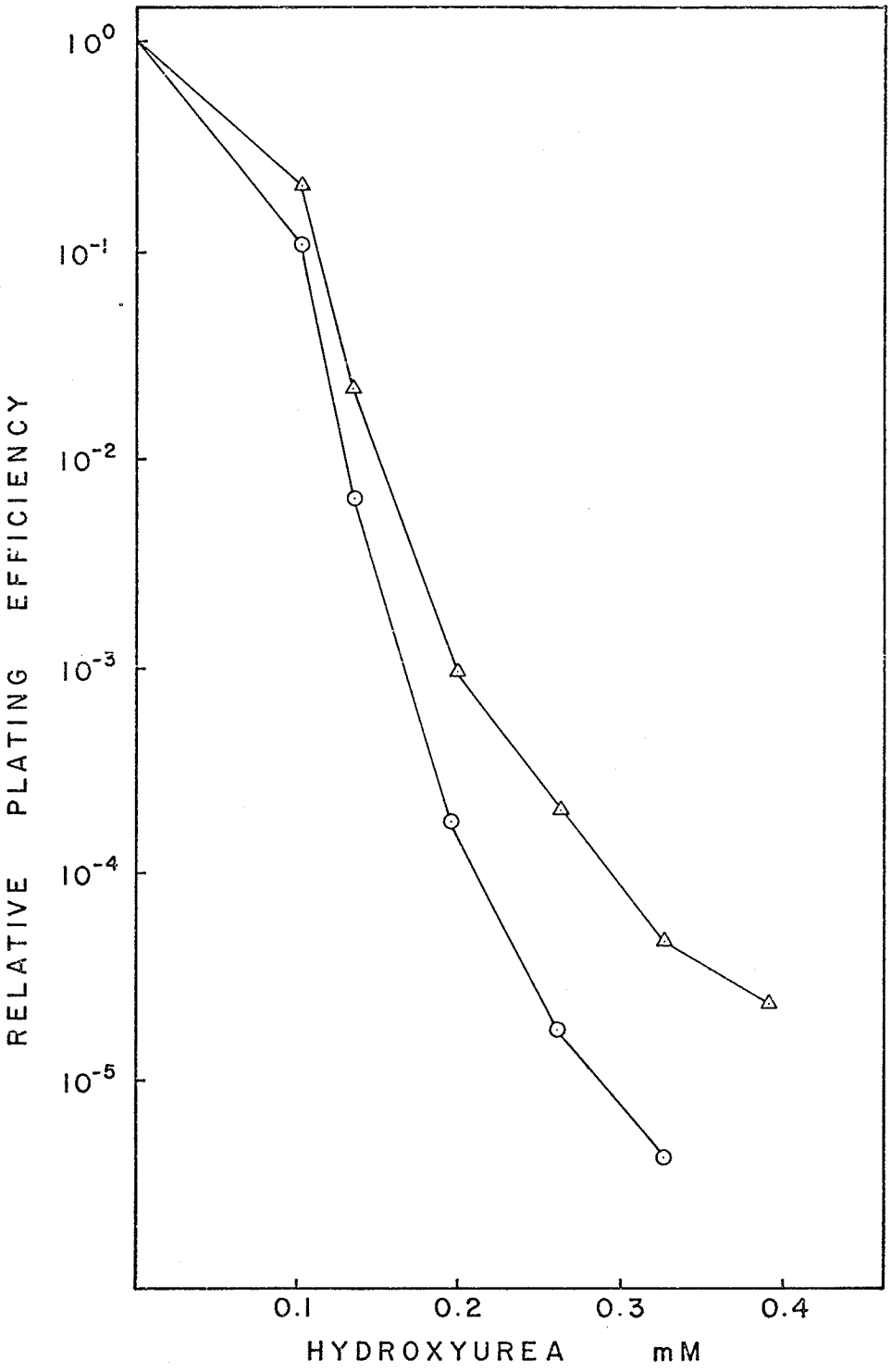

RELATIVE PLATING EFFICIENCY
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
0.1
0.2
0.3
0.4
HYDROXYUREA mM

Table 6. Plating response of WT CHO and HU^R-2 cells to various chemicals.

Compound	Concentration	WT CHO	HU^R-2
urea	33.3 mM	+	+
urethane	22.0 mM	+	+
hydroxyurea	0.66 mM	-	+
guanazole	5.26 mM	-	+
N-carbamoyloxyurea	0.49 mM	-	+
formamidoxime	1.00 mM	-	+
hydroxyurethane	0.57 mM	-	+
thiourea	2.63 mM	-	-
hydroxylamine	3.03 mM	-	-
semicarbazide	13.3 mM	-	-
methylhydroxylamine	10.7 mM	-	-
α'α' dipyridyl	0.01 mM	-	-
phenethyl alcohol	1.00 mM	-	-
digitonin	5.00 μM	-	-
ouabain	1.00 mM	-	-
ara-C	0.21 μM	-	-

+ = plating efficiency of 80 to 100%

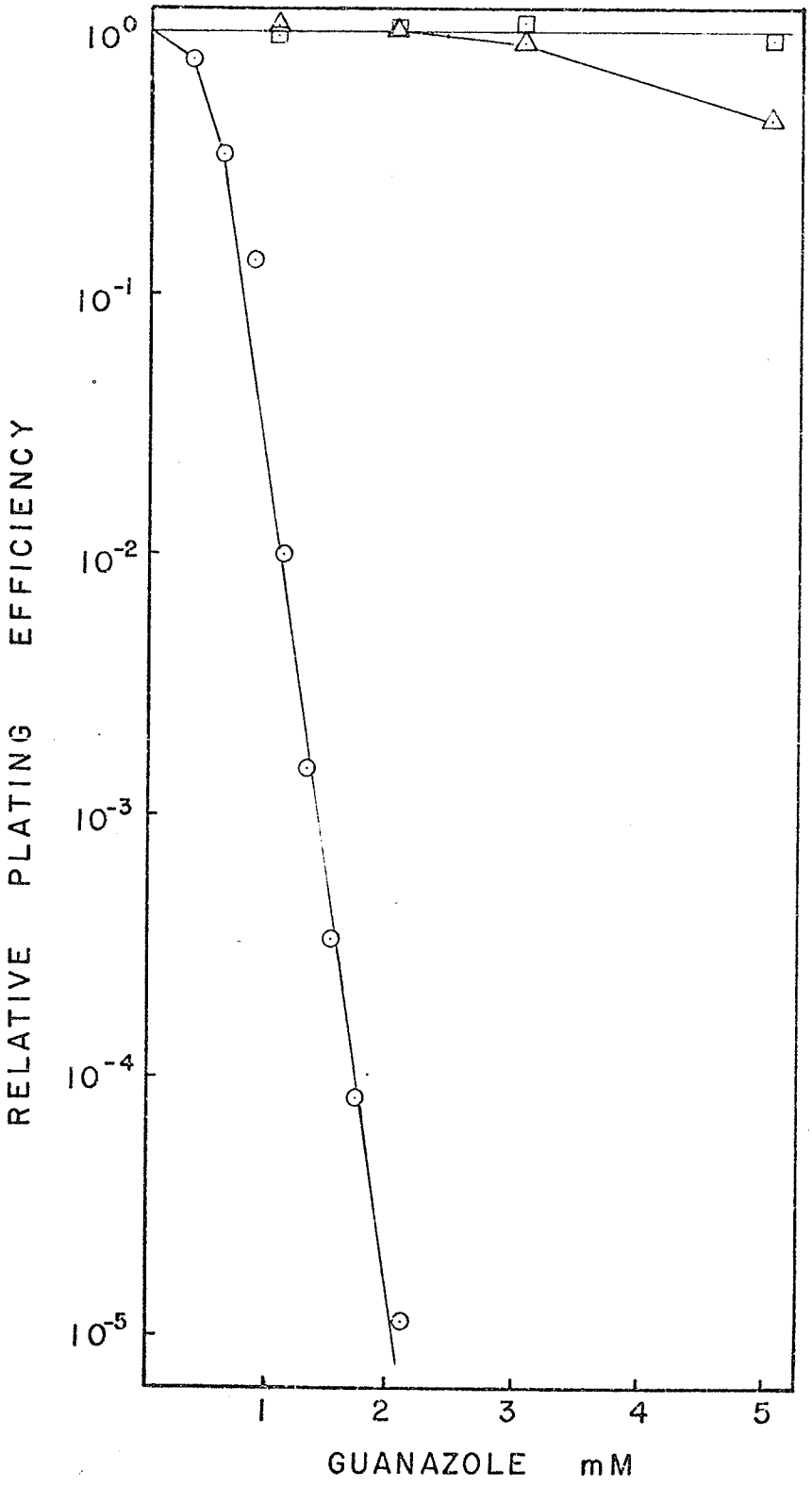
- = plating efficiency of less than 1%

sensitivity towards these drugs could be demonstrated with HU^R-2 and WT CHO cells, even at concentrations which reduced the RPE of both lines by only 50%.

Next, 10 compounds bearing some structural similarity to hydroxyurea were tested. On the basis of their effect on PE these compounds could be divided into three groups. The first group consisted of urea and urethane, both of which did not significantly reduce the PE of either HU^R-2 or WT CHO. The next group consisted of thiourea, hydroxylamine, semicarbazide and methylhydroxylamine were all capable of dramatically decreasing the PE of both WT CHO and HU^R-2. However, once again no diiferential sensitivity could be demonstrated (Table 6). The last group consisting of hydroxyurea, guanazole, N-carbamoyloxyurea, formamidoxime and hydroxyurethane were effective in reducing the PE of the WT CHO cells, but all showed a marked decrease in their cytoxicity with the hydroxyurea-resistant HU^R-2 cell line. This suggestes that, unlike the other groups of compounds, these have their primary lethal sites of action at the same site as hydroxyurea. Cellular changes resulting in resistance to hydroxyurea would then automatically result in resistance to the other four drugs.

Figure 13 shows the RPE response of HU^R-2 and HU^R-4 to the cytotoxic effects of guanazole. As predicted, both hydroxyurea-resistant cell lines are dramatically more resistant

Figure 13: Relative plating efficiency of WT CHO (⊙), HU^R-2 (⊡) and HU^R-4 (△) with increasing concentrations of guanazole.


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

to guanazole than the parental WT CHO.

To investigate the cross-resistance phenomenon further, cell lines were selected separately from the WT CHO cell population for resistance to each of the four compounds showing cross-resistance. Briefly, 5 X 10^5 WT CHO cells were laid down in 100 mm culture plates containing standard medium plus a concentration of drug found in preliminary experiments to reduce the RPE to approximately 10^{-5}. After 16 days incubation, surviving colonies were picked and recloned in the presence of the appropriate drug. The cell lines were designated NC^R-1, G^R-3, U^R-1 and F^R-1 and when tested were found to be resistant to 0.4 mM N-carbamoyloxyurea, 2.6 mM guanazole, 1.1 mM hydroxyurethane and 1.2 mM formamidoxime respectively. These concentrations reduced the WT CHO cell RPE to approximately 10^{-5} for each drug. The cell lines were then tested for resistance to the four compounds plus hydroxyurea. Table 7 summarizes the key results obtained from detailed studies of colony-forming ability of the cell lines in the presence of various concentrations of the five drugs. The concentration of drug required to reduce the RPE to 0.5 for WT CHO cells was compared to the concentration required for the resistant cell lines. All the resistant cell lines exhibited some degree of cross-resistance to all five compounds (Table 7). However, the degree of resistance to the isolation drug and cross-resistance to the other compounds varied depending upon

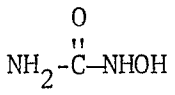
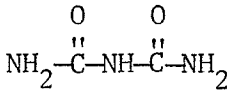
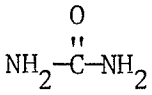
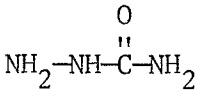
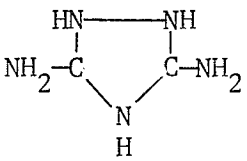
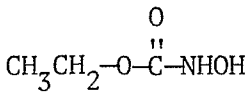
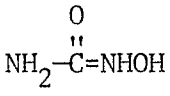
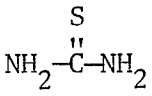
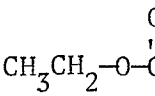
Table 7. Fold increase in resistance over WT CHO at 0.5 RPE.

Drug	Cell Line				
	HU^R-2	NC^R-1	G^R-3	U^R-1	F^R-1
hydroxyurea	15.0	8.1	3.2	11.0	3.0
guanazole	10.8	5.0	4.0	8.5	2.5
N-carbamoyloxyurea	9.6	4.0	2.4	6.7	2.1
formamidoxime	15.0	5.5	2.9	10.0	2.6
hydroxyurethane	15.6	6.0	3.5	9.6	1.8

the cell line. HU^R-2, NC^R-1 and U^R-1 were all very resistant to their selecting drug as well as to the alternate drugs. For example, it took 15 times more hydroxyurea and 11 times more guanazole to reduce the RPE of HU^R-2 to 50% than it did with WT CHO cells. Two of the cell lines, G^R-3 and F^R-1 showed only marginal resistance to the selecting drug and, as expected, showed only moderate cross-resistance to the other compounds (Table 7).

In 1971, Levine and Kretchmer reported the development of a sensitive colorimetric assay which could differentiate hydroxyurea from hydroxylamine. In the hope that the structural similarities or cellular inhibition patterns could be correlated with reactivity in this colorimetric assay, the method was used to assay each compound as detailed in Materials And Methods. If it existed, such a correlation could yield information on the structure-activity relationship of inhibition by these compounds, and prove useful in the screening of additional compounds. However, Table 8 shows that only hydroxyurea and N-carbamoyloxyurea showed significant colour development at 0.2 μmoles, and even then N-carbamoyoxyurea yielded only half the absorbance at 540 nm when compared on an equimolar basis to hydroxyurea. Both urea and semicarbazide gave only marginal colour development at this concentration. The remaining seven compounds, when tested at 0.20 μmoles, developed no absorbance at 540 nm above blank.

Table 8. Structure and colorimetric reactivity of hydroxyurea-related compounds.

Compound	Structure	Ab_{540nm}*
hydroxyurea		0.90
N-carbamoyloxyurea		0.41
urea		0.04
semicarbazide		0.02
guanazole		0.00
hydroxyurethane		0.00
formamidoxime		0.00
thiourea		0.00
hydroxylamine	NH_2OH	0.00
methylhydroxylamine	CH_3-NHOH	0.00
urethane		0.00

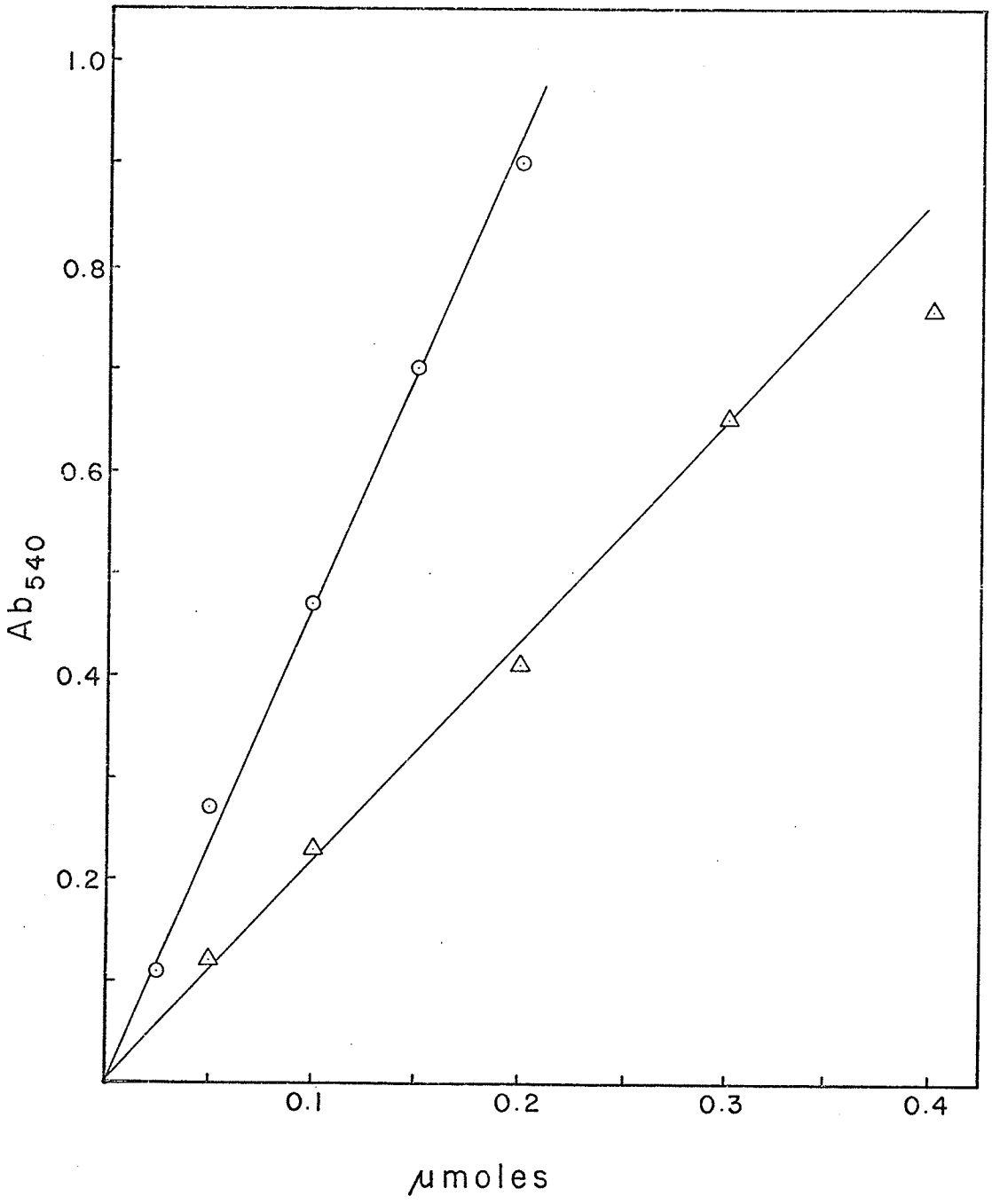
* The colorimetric method of Levine and Kretchmer (1971) was used to measure the absorbance at 540 nm for 0.2 µmoles of each compound.

The colorimetric test seems to be remarkably specific for hydroxyurea and N-carbamoyloxyurea. The basis for this specificity is unknown and an examination of Table 8 shows that it cannot be correlated with any specific chemical structure. Also disappointing is that the colorimetric assay does not correlate in any way with the cellular cross-resistant inhibitory pattern of the compounds. Hydroxyurea, for example, is highly reactive while guanazole shows no reactivity even at concentrations as high as 5.0 µmoles.

Figure 14 shows that the colorimetric reaction obeys Beer's law and both hydroxyurea and N-carbamoyloxyurea can be accurately and sensitively quantitized with as little as 0.025 µmoles of the compounds.

10. Cell hybridization studies: In order to determine the behaviour of the hydroxyurea-resistant trait in the presence of a WT genome, the hydroxyurea-resistant cell lines were hybridized with WT CHO lines by means of Sendai virus-mediated fusion (Kao et al 1969). With this method, mixed populations of WT CHO and drug-resistant cell lines are treated with inactivated Sendai virus and the cells are then plated out under conditions which select against genetic markers present on the parental cell lines, thus allowing growth of only hybrid cells. Unfortunately, the hydroxyurea-resistant cell lines HU^R-2 and HU^R-4 carry no additional markers which can be used in hybrid selection. Therefore,

Figure 14: Colorimetric determination of hydroxyurea (⊙) and N-carbamoyloxyurea (△) by the method of Levine and Kretchmer (1971).


1.0
0.8
0.6
0.4
0.2
Ab_{540}
0.1
0.2
0.3
0.4
μmoles

it was necessary to construct a non-hydroxyurea resistant cell line carrying two markers which could be selected against. Two such lines were developed. It was decided to start with cell lines already carrying well defined recessive markers. GAT^-CHO is a cell line auxotrophic for glycine, adenine and thymidine because of a defect in folic acid metabolism (McBurney & Whitmore 1974), and tsH1 CHO carries a temperature-sensitive lesion in its leucyl-tRNA synthetase (Thompson et al 1975). Both traits have been shown to behave recessively in hybrid cells. These two lines were then used to select cells resistant to the cytotoxic effects of ouabain. Ouabain resistance is known to behave codominantly in cell hybrids (Baker et al 1974). Thus a hybrid consisting of a parental line resistant to ouabain and another line sensitive to the drug, will display resistance to ouabain approaching that of the ouabain-resistant parental line.

Briefly, 10^6 cells from either tsH1 or GAT^-CHO were plated in standard medium containing 2 mM ouabain. In the case of GAT^-CHO, the standard medium of α-MEM plus 10% FCS was supplemented with adenosine and thymidine as detailed in Materials and Methods. After 14 days large surviving colonies were picked from each cell line, recloned and tested for ouabain sensitivity. Two clones, designated tsH1 O^R-2A and GAT^-O^R-1, showed close to 100% RPE at 2 mM ouabain, a concentration of drug which reduced the RPE of WT CHO cells by at least 10^{-5} under our growth conditions.

When tested for hydroxyurea sensitivity, both ouabain-resistant cell lines exhibited the same sensitivity as the WT CHO cell line.

The two ouabain-resistant cell lines were then used to hybridize with HU^R-2 or HU^R-4. Mixtures of tsH1 O^R-2A or GAT^-O^R-1 with either HU^R-2 or HU^R-4 were treated with inactivated Sendai virus as described in Materials and Methods. The following plating conditions were then used to select hybrid cells containing one genome from each parental cell line:

tsH1 O^R-2A X HU^R-2 — plated in α-MEM + 10% dFCS + 1 mM ouabain incubated at 39.5°C;

GAT^-O^R-1 X HU^R-2 — plated in α-MEM + 10% dFCS + 1 mM ouabain, incubated at 37°C (medium lacks TdR and AdR);

GAT^-O^R-1 X HU^R-4 — plated in α-MEM + 10% dFCS + 1 mM ouabain, incubated at 37°C (medium lacks TdR and AdR).

After 8 to 16 days incubation under the selective conditions, hybrid colonies were picked and recloned in the same selective medium. The resulting clones were tested for chromosome complement by standard karyotype analyses as described in Materials and Methods.

Table 9 summarizes the various hybrid cell lines produced, listing also the parental cell lines and selecting

Table 9. Cataloging of hybrid cell lines produced.

Hybrid acronym	Parental lines	Markers selected
(GAT X H2)	$GAT^{-}O^{R}$-1 HU^{R}-2	* GAT^{-} & ouabain †
(GAT X H4)	$GAT^{-}O^{R}$-1 HU^{R}-4	GAT^{-} & ouabain
(ts X H2)	tsH1 O^{R}-2A HU^{R}-2	39.5°C & ouabain
(GAT X WT)	$GAT^{-}O^{R}$-1 WT CHO	GAT^{-} & ouabain
(ts X WT)	tsH1 O^{R}-2A WT CHO	39.5°C & ouabain

*The GAT^{-}CHO cell auxotroph was selected against by omission of thymidine (TdR) and adenosine (AdR) from the culture medium.

†The ouabain-sensitive cell lines were selected against by the inclusion of 1 mM ouabain in the culture medium.

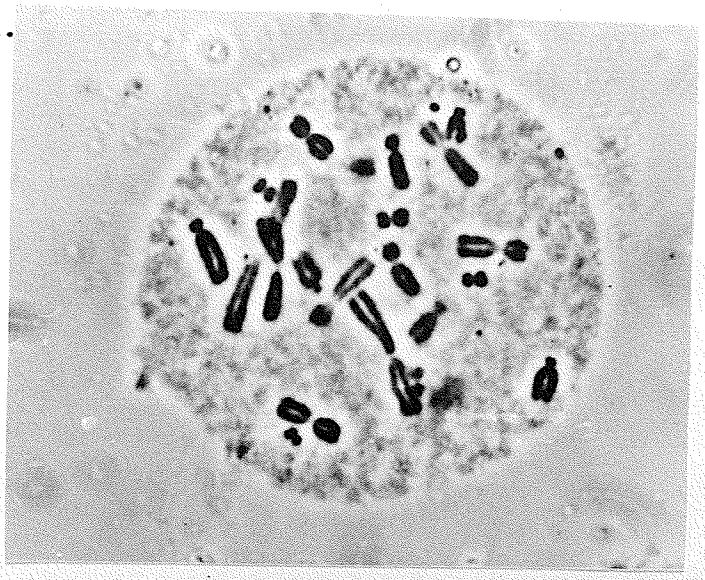
conditions. Control hybrids were also produced between the non-hydroxyurea-resistant parental cell lines (eg WT X ts, WT X GAT).

Plates 1,2,and 3 show representative chromosome complements of three of the hybrid cell lines and their parental cell lines. In all cases at least 50 of the metaphase figures were photographed, enlarged and the number of chromosomes scored. The results are expressed in the bar graphs of Figures 15, 16 and 17. In all cases of the parental lines (HU^R-2, WT CHO, tsH1 O^R-2A and GAT^-O^R-1) 60 to 70% of the metaphase cells had 21 chromosomes. Thus the modal chromosome number for these lines was found to be 21, the normal pseudodiploid complement for WT CHO (Kao & Puck 1967).

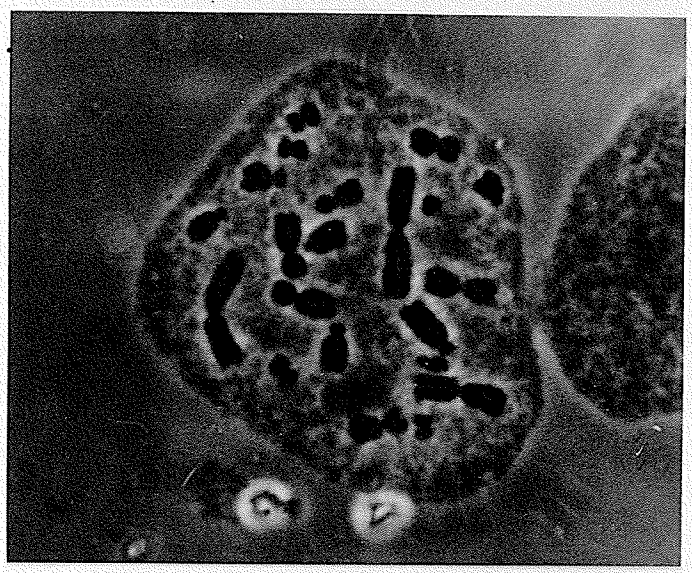
The chromosome numbers in the hybrid cells showed more variability. The chromosome number of all the hybrids scored, however, centred around 40 or 42 — as expected, approximately double the normal CHO cell chromosome complement. For example, with (GAT X H2) the highest percentage (30%) of metaphase cells had 40 chromosomes. Similarly, 37% of (ts X H2) had 40 chromosomes and 28% of (ts X WT) had 42 chromosomes. These results are consistent with the reported decrease in chromosome stability in hybrid CHO clones (Worton et al 1977), and are close to the results reported by Baker et al (1974) for hybrid cell lines formed with ouabain sensitive and resistant cell lines.

Plate 1: Aceto-orcein stained metaphase cells of A) HU^{R}-2, B) $GAT^{-}O^{R}$-1 and C) (GAT X H2).

A.



B.



C.

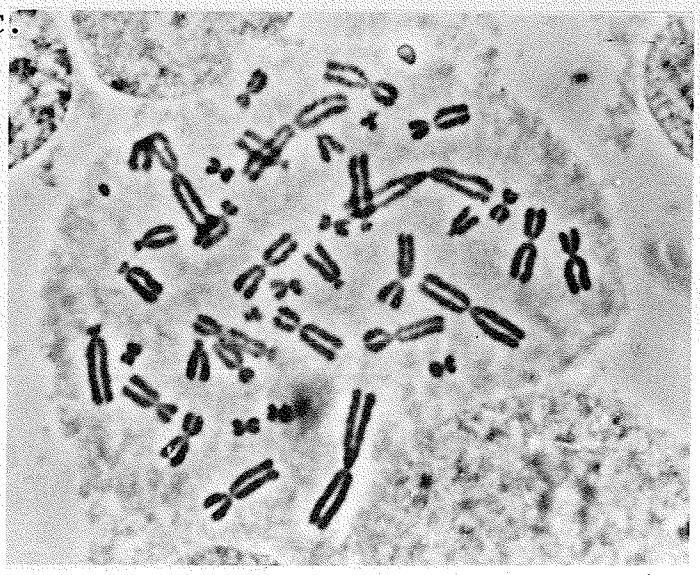


Figure 15: Chromosome number distribution from 50 to 100 metaphase cells of A) HU^{R}-2, B) $GAT^{-}O^{R}$-1 and C) (GAT X H2).

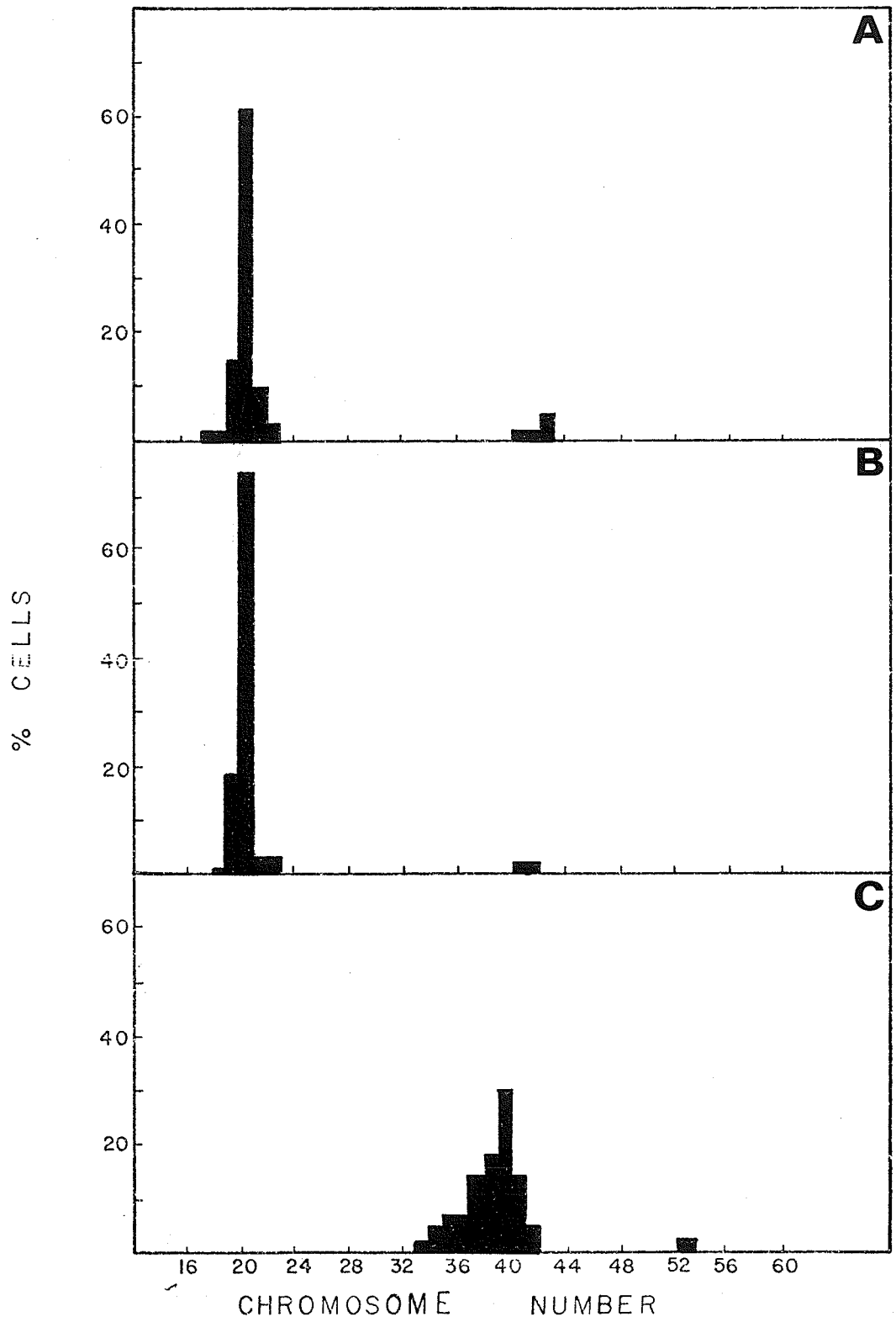
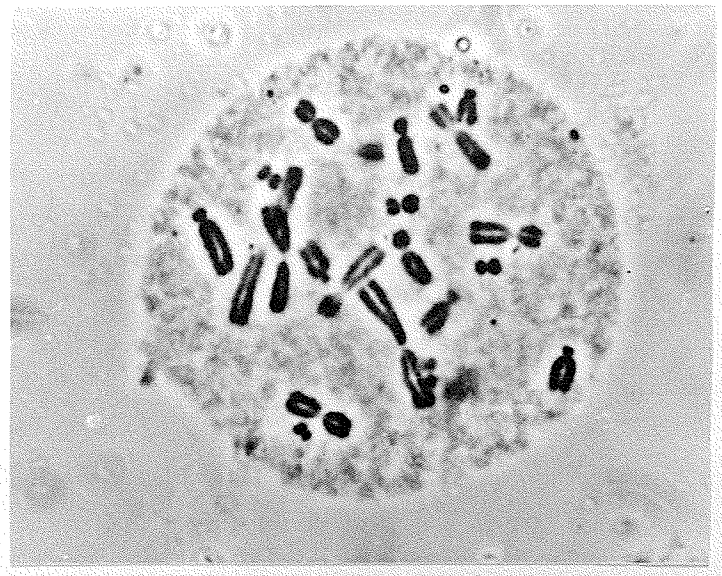
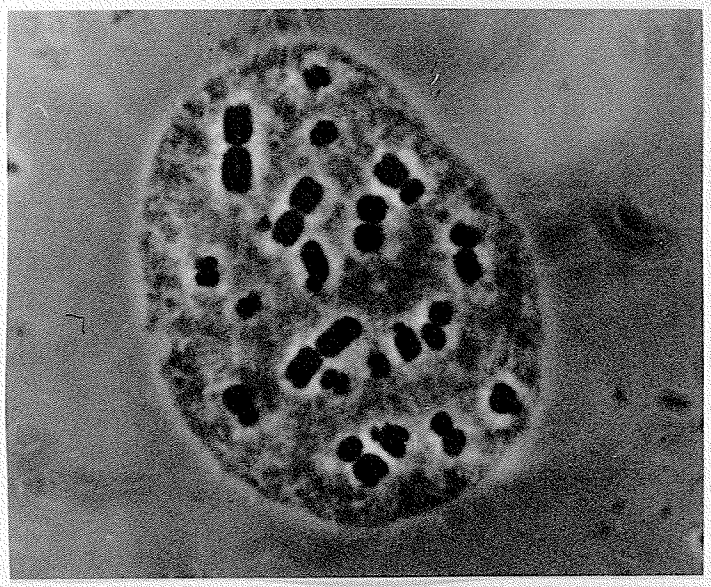

A
B
C
60
40
20
% CELLS
16 20 24 28 32 36 40 44 48 52 56 60
CHROMOSOME NUMBER

Plate 2: Aceto-orcein stained metaphase cells of A) HU^{R}-2, B) tsH1 O^{R}-2A and C) (ts X H2).

A.



B.



C.

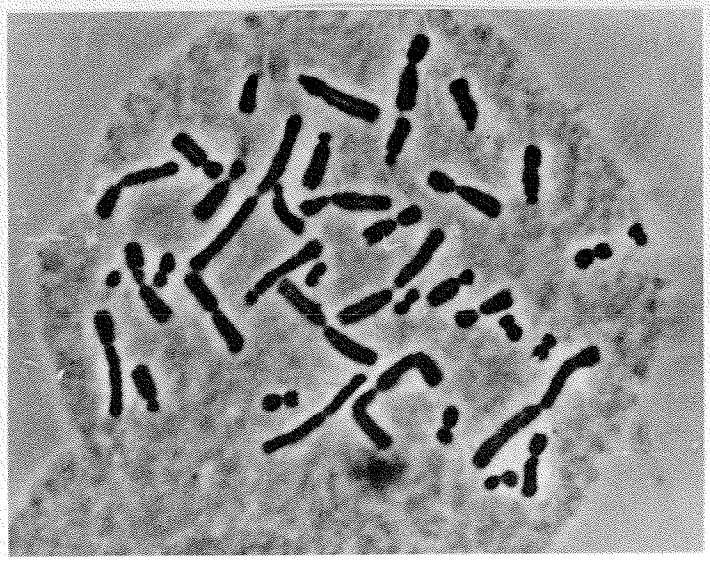


Figure 16: Chromosome number distribution from 50 to 100 metaphase cells of A) HU^R-2, B) tsH1 O^R-2A and C) (ts X H2).

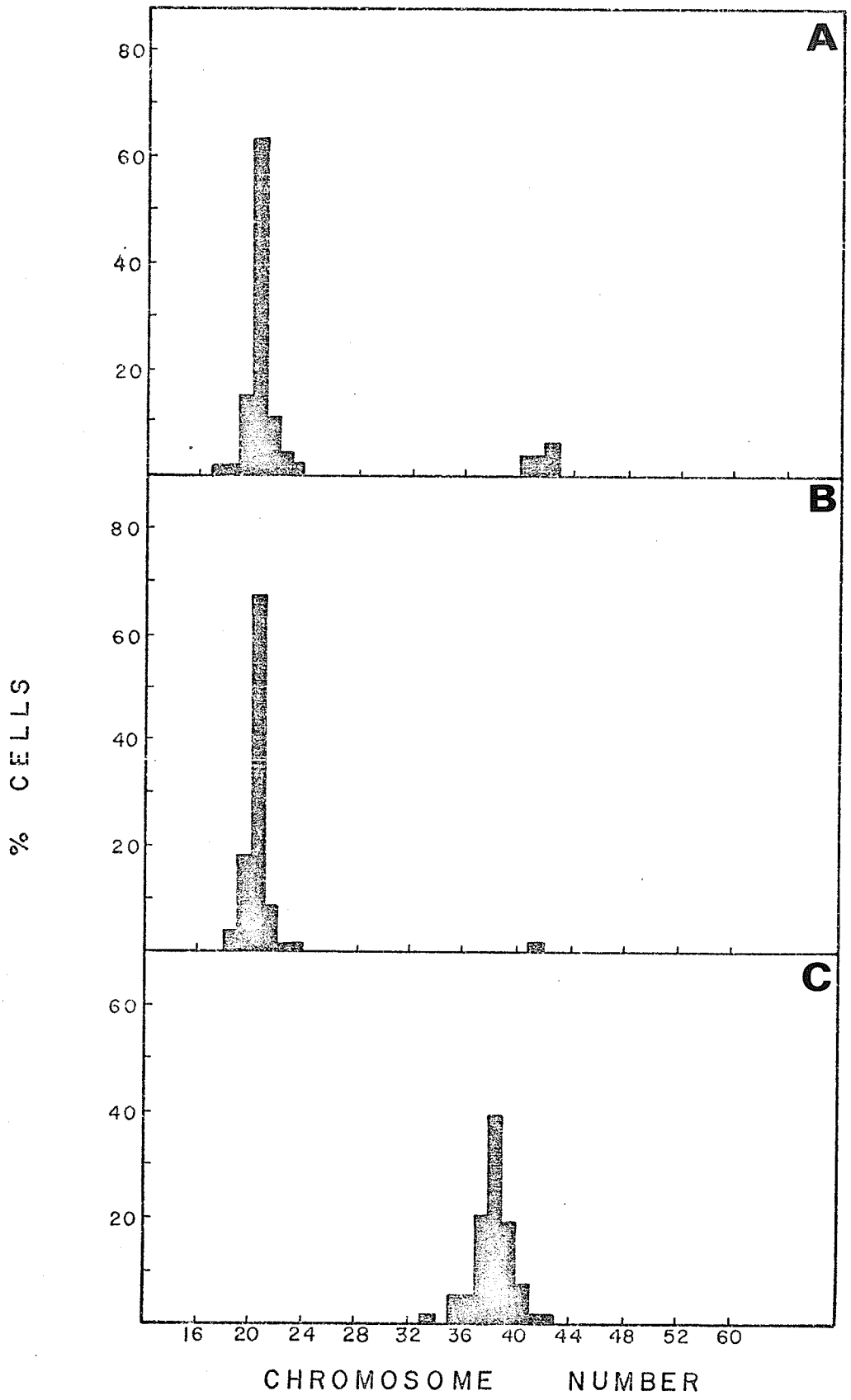
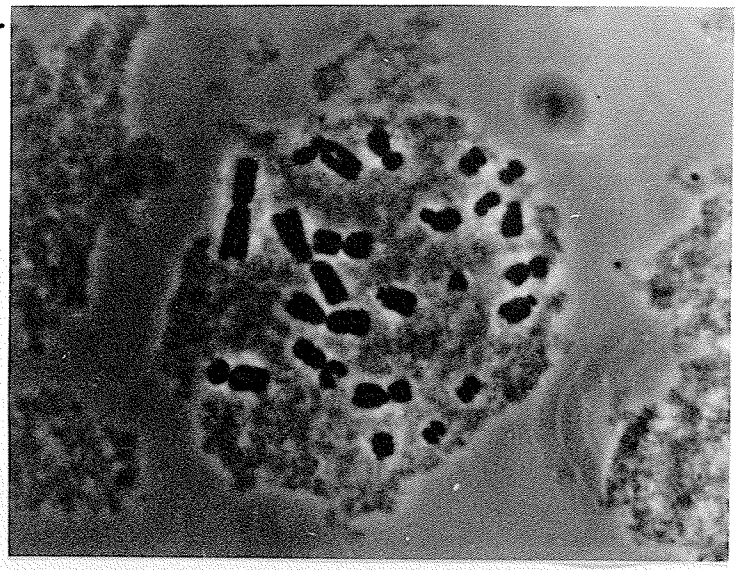
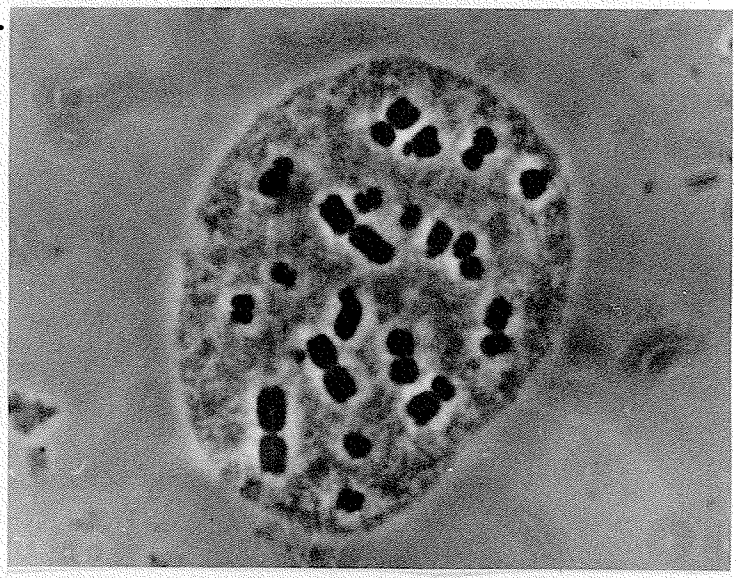

A
B
C
80
60
40
20
% CELLS
16
20
24
28
32
36
40
44
48
52
60
CHROMOSOME NUMBER

Plate 3: Aceto-orcein stained metaphase cells of A) WT CHO, B) tsH1 O^R-2A and C) (ts X WT).

A.



B.



C.

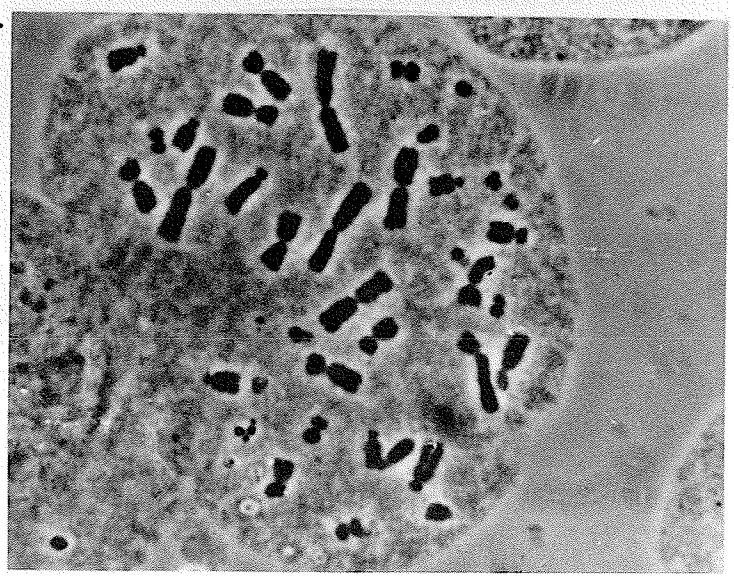


Figure 17: Chromosome number distribution from 50 to 100 metaphase cells of A) WT CHO, B) tsH1 O^{R}-2A and C) (ts X WT).

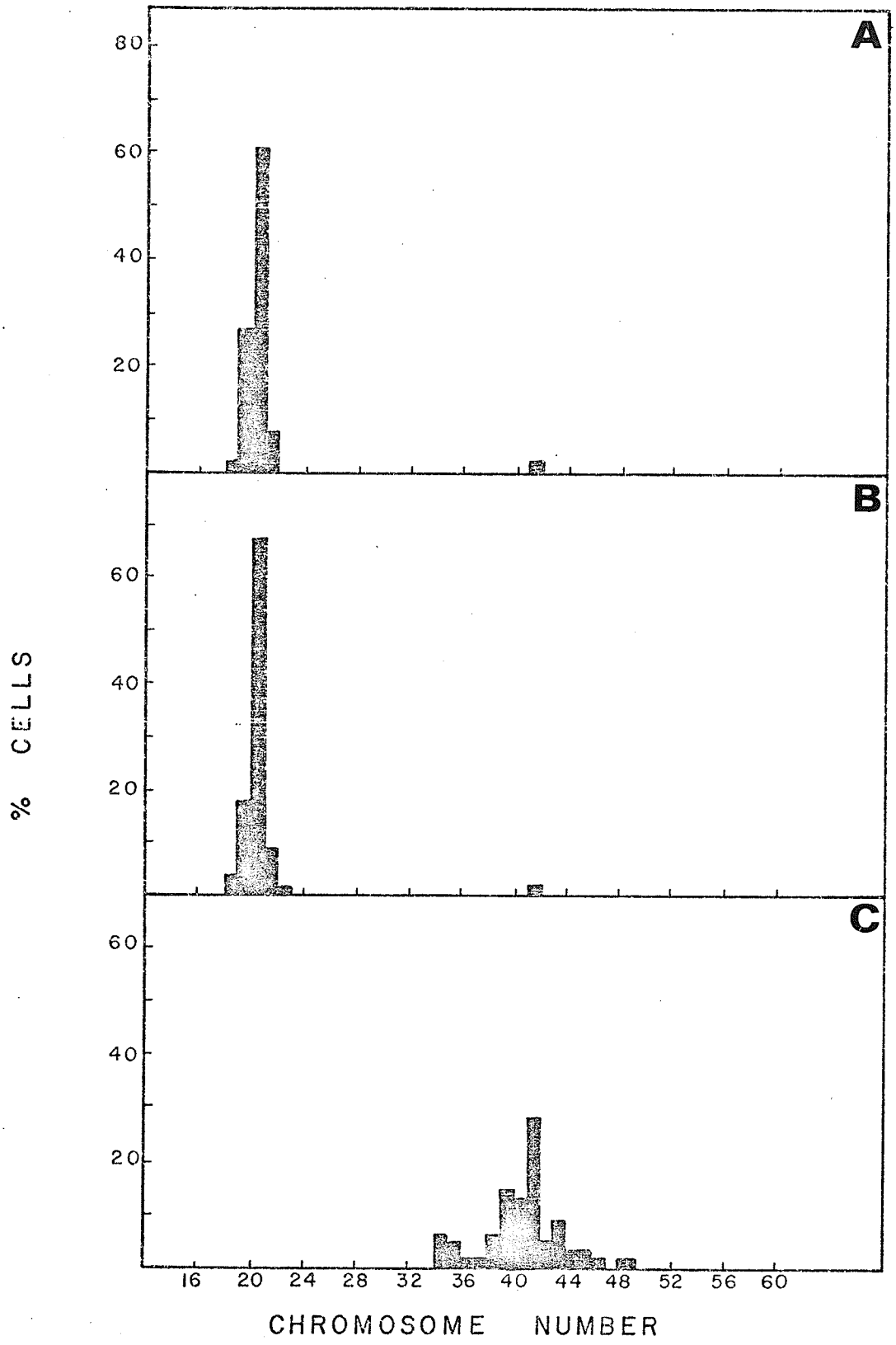

A
B
C
80
60
40
20
60
40
20
60
40
20
% CELLS
16 20 24 28 32 36 40 44 48 52 56 60
CHROMOSOME NUMBER

Figure 18 shows the RPE in the presence of hydroxyurea of the hybrid (GAT X H2) compared to the parental cell lines and the control (GAT X WT) hybrid. It can be seen that (GAT X H2) exhibits nearly the same resistance to hydroxyurea as the original drug-resistant HU^R-2. Thus the hydroxyurea-resistant phenotype behaves dominantly or codominantly with this cell line. As expected, the tetraploid (GAT X WT) control showed hydroxyurea-sensitivity close to the pseudodiploid GAT^-O^R-1 parent. There is consistently a slight increase in hydroxyurea-resistance with the change from diploid to tetraploid chromosome complement. The reason for this is not known, but may have to do with gene dosage effects.

The same hybrid line (GAT X H2) was also tested for its sensitivity to guanazole. As predicted, the hybrid showed a high level of resistance to guanazole, approaching the resistance of the HU^R-2 parental line (Fig 19).

To test whether hybrids of HU^R-2 with other cell lines would also exhibit codominance for the hydroxyurea-resistant trait, the hybrid between tsH1 O^R-2A and HU^R-2, designated (ts X H2), was tested for resistance to hydroxyurea. Figure 20 shows that in this hybrid,as well,hydroxyurea-resistance behaves codominantly.

Figure 21 shows the RPE of (GAT X H4), a hybrid between HU^R-4 and GAT^-O^R-1. Once again the hydroxyurea-resistance of

Figure 18: Relative plating efficiency of $GAT^{-}O^{R}$-1 (✣), (GAT X WT) (△), HU^{R}-2 (⊙) and (GAT X H2) (⊡) with increasing concentrations of hydroxyurea.

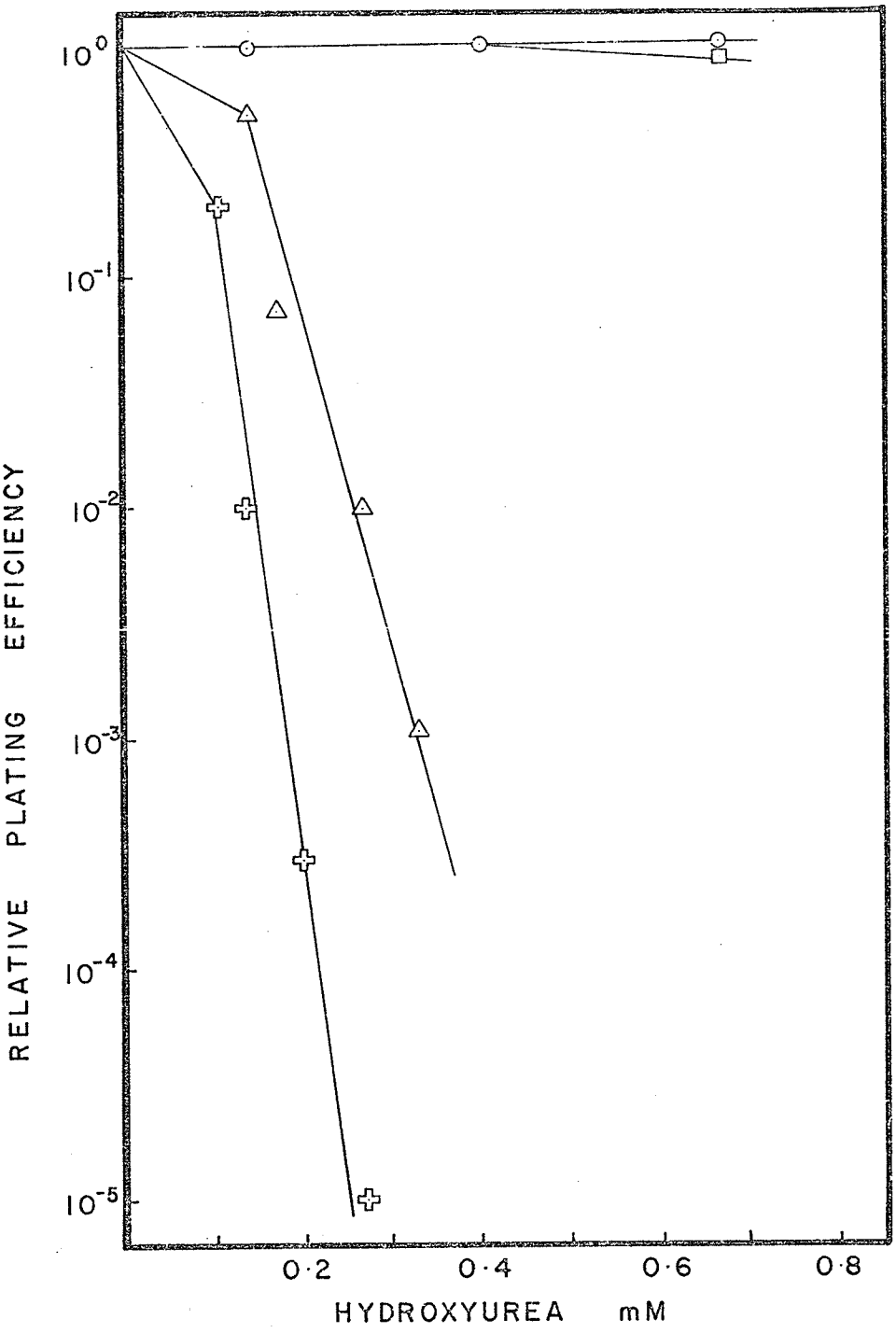

RELATIVE PLATING EFFICIENCY
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
0·2
0·4
0·6
0·8
HYDROXYUREA mM

Figure 19: Relative plating efficiency of $GAT^{-}O^{R}$-1 (✣), (GAT X WT) (△), HU^{R}-2 (⊙) and (GAT X H2) (⊡) with increasing concentrations of guanazole.

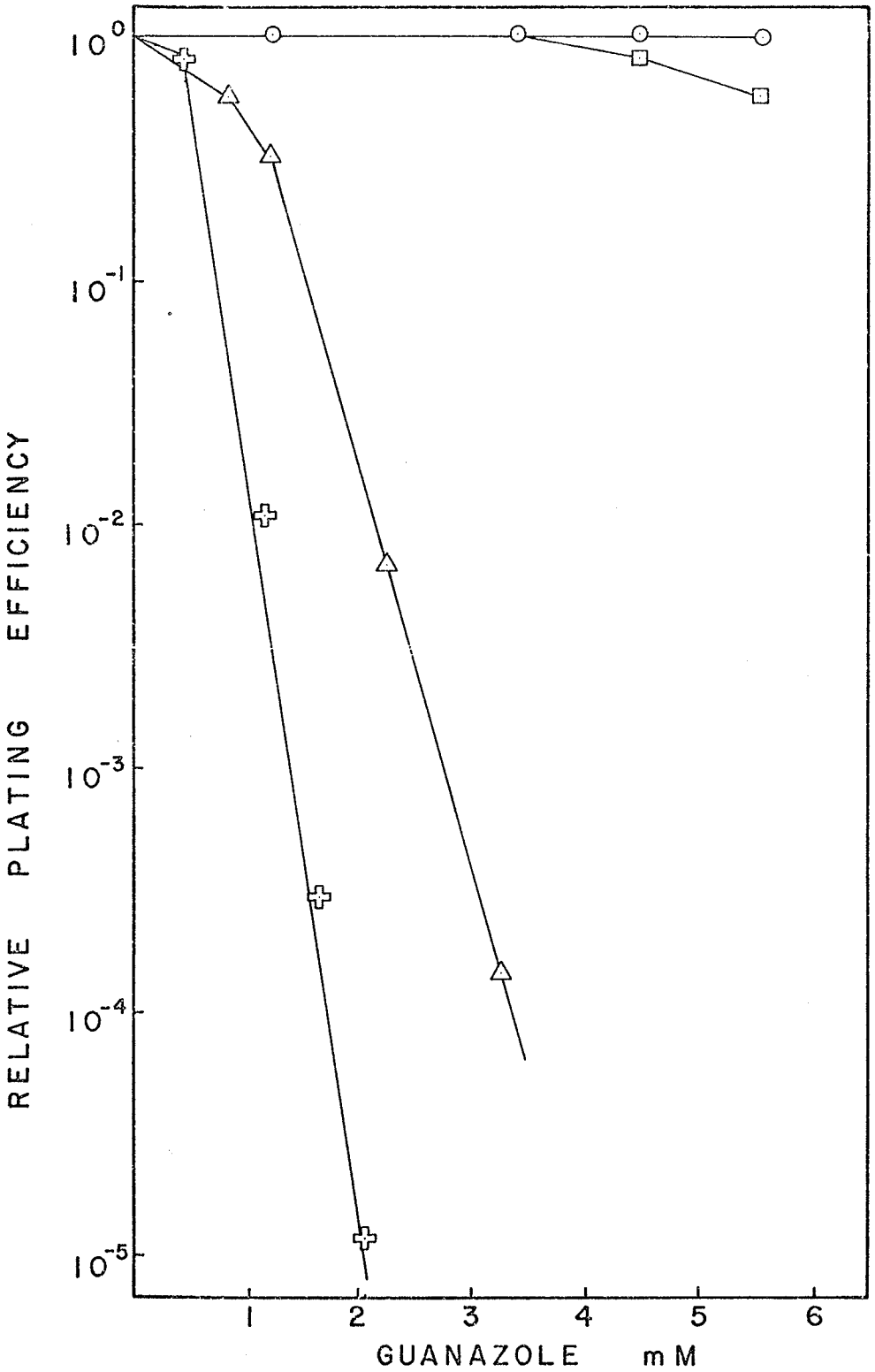

RELATIVE PLATING EFFICIENCY
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
1
2
3
4
5
6
GUANAZOLE mM

Figure 20: Relative plating efficiency of (ts X WT) (⊡), (ts X H2) (△), and HU^R-2 (⊙) with increasing concentration of hydroxyurea.

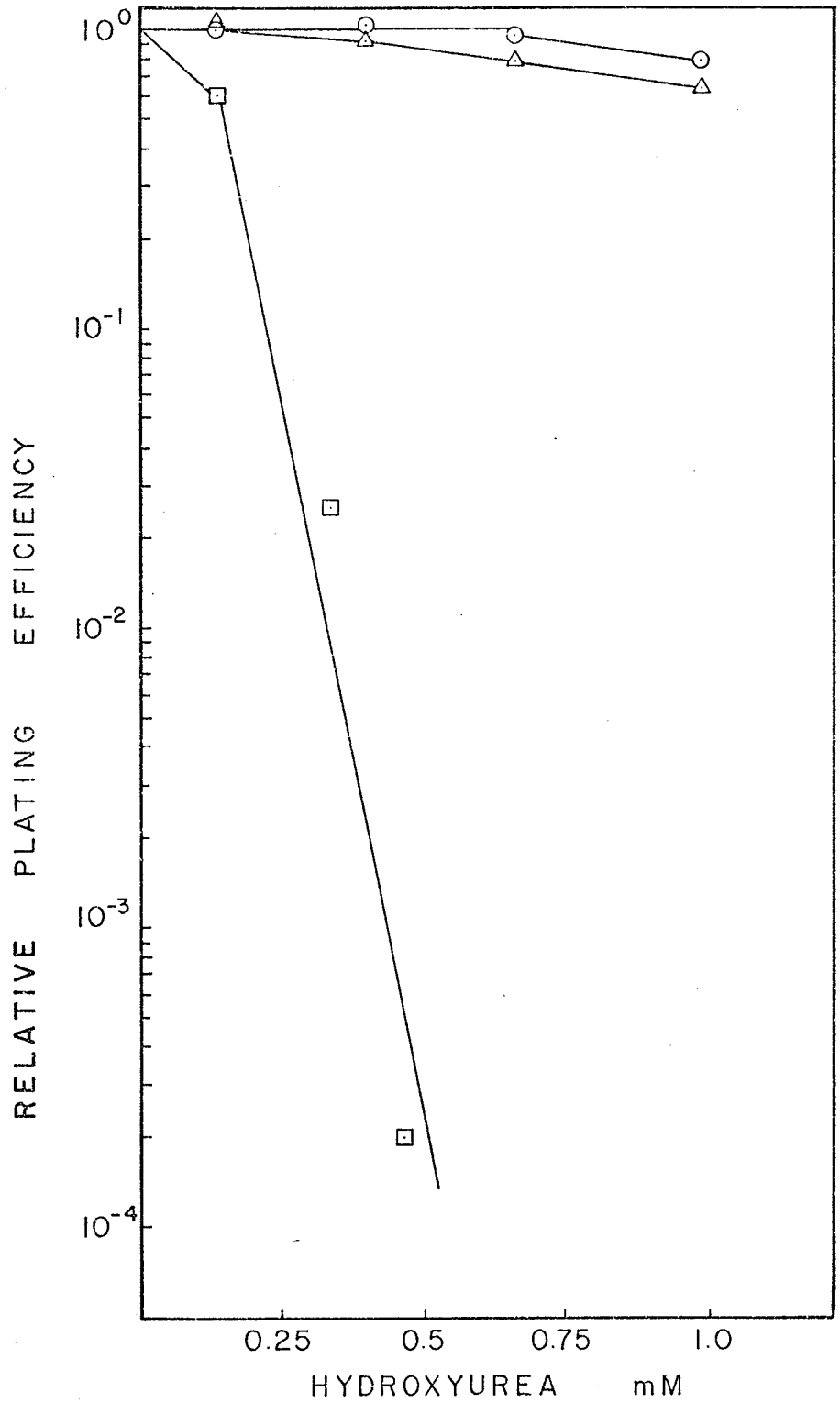
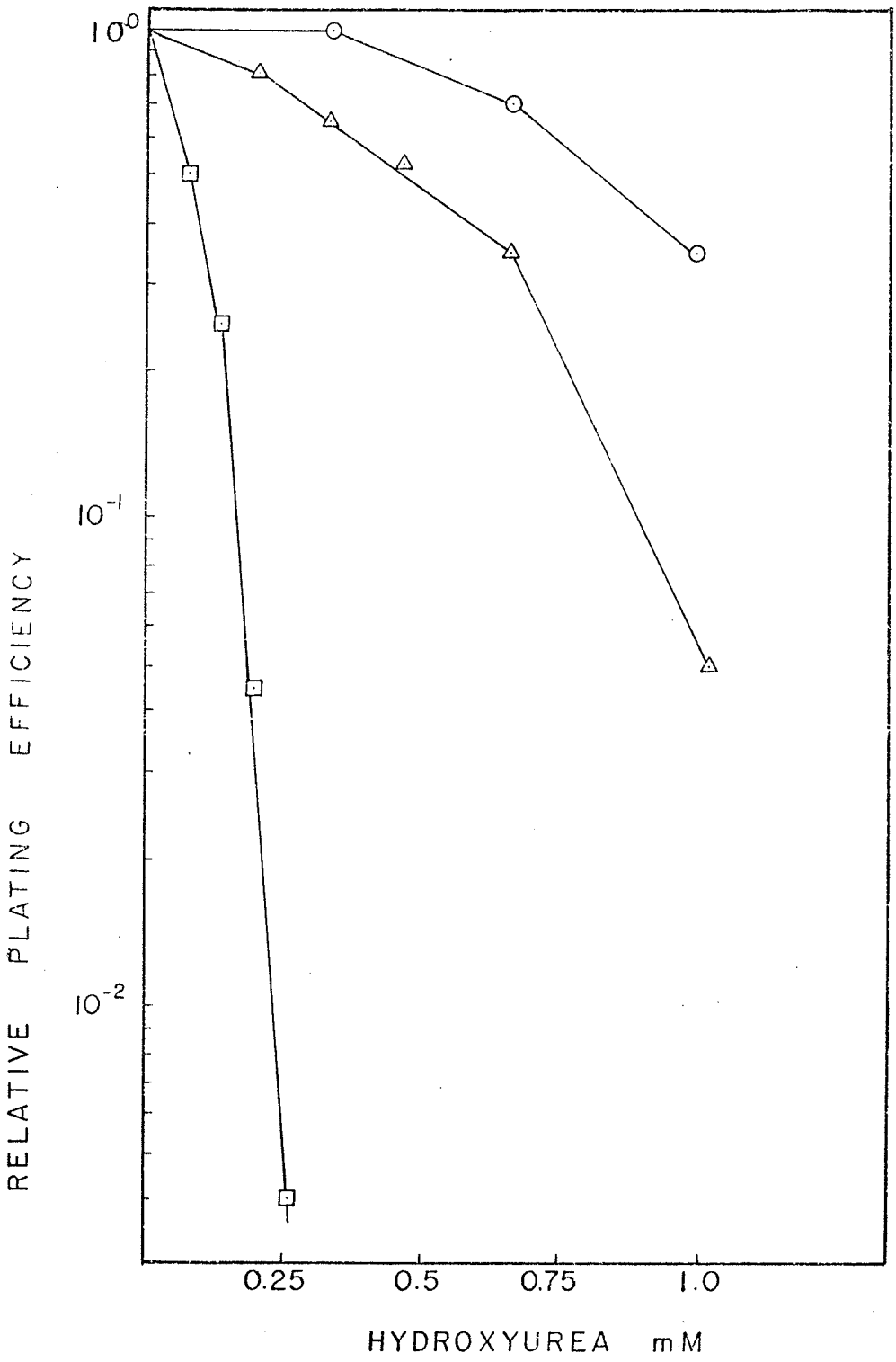

RELATIVE PLATING EFFICIENCY
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
0.25
0.5
0.75
1.0
HYDROXYUREA mM

Figure 21: Relative plating efficiency of (GAT X WT) (⊡), (GAT X H4) (△), and HU^R-4 (⊙) with increasing concentration of hydroxyurea.


10^0
10^{-1}
10^{-2}
RELATIVE PLATING EFFICIENCY
0.25
0.5
0.75
1.0
HYDROXYUREA mM

the hybrid is closer to the HU^R-4 parental line than to the sensitive WT CHO or (GAT X WT) control tetraploid hybrid. However,(GAT X H4) is clearly much less resistant than the hydroxyurea-resistant HU^R-4.

Because the hydroxyurea-resistant phenotype apparently behaved in a codominant fashion in cell hybrids, it was tested whether hydroxyurea-resistance could be used as a selective marker in cell hybridization studies. Table 10 shows the results of a hybrid recovery experiment using two independent GAT^- hydroxyurea-resistant cell lines to achieve hybridization with the tsH1 cell line. A treatment of polyethylene glycol plus dimethylsulfoxide (PEG + DMSO)—instead of inactivated Sendai virus—was used to promote cell fusion (Norwood et al 1976). As detailed in Materials and Methods GAT^-HU^R-1 is a double step mutant of GAT^- CHO cells resistant to 1.33 mM hydroxyurea, and GAT^-HU^R-12SC8 is a single step mutant of GAT^-CHO resistant to 0.33 mM hydroxyurea and will be described below.

In the first experiment of Table 10, PEG treatment was used to dramatically increase the frequency of hybrids between tsH1 and GAT^-HU^R-1. Without the combined PEG and DMSO treatment, no hybrid colonies developed, while with the fusion treatment 96 colonies developed under the selective conditions of 39.5°C and GAT^-. When hydroxyurea was used instead of temperature-sensitivity to select against the tsH1 parental line, 86 hybrid colonies were recovered, a number

Table 10. Use of hydroxyurea-resistance as a marker in cell hybridization studies.

Cell Lines treated	Treatment	Markers selected	Cells plated	No. Hybrid colonies
1. tsH1 +				
$GAT^{-}HU^{R}$-1	none	39.5^{o},GAT*	1.6×10^{5}	0
	PEG + DMSO	39.5^{o},GAT	1.1×10^{5}	96
	none	HU†, GAT	1.6×10^{5}	0
	PEG + DMSO	HU†, GAT	1.1×10^{5}	89
2. tsH1 +				
$GAT^{-}HU^{R}$-12SC8	none	39.5^{o},GAT	4.0×10^{5}	0
	PEG + DMSO	39.5^{o},GAT	1.9×10^{5}	24
	none	HU§, GAT	4.0×10^{5}	2
	PEG + DMSO	HU§, GAT	1.9×10^{5}	44
3. tsH1	PEG + DMSO	39.5^{o}	2.0×10^{4}	0
4. tsH1	PEG + DMSO	HU†	2.0×10^{4}	0
5. $GAT^{-}HU^{R}$-1	PEG + DMSO	GAT	1.8×10^{5}	0
6. $GAT^{-}HU^{R}$-12SC8	PEG + DMSO	GAT	1.6×10^{5}	0

*GAT selective medium consisted of α-MEM + 10% dFCS and lacked TdR and AdR.

†Hydroxyurea selective medium consisted of α-MEM + 10% dFCS and 0.5 mM hydroxyurea.

§Hydroxyurea selective medium consisted of α-MEM + 10% dFCS and 0.26 mM hydroxyurea.

almost equal to that obtained with the temperature-sensitivity selection. In the second experiment $GAT^{-}HU^{R}$-12SC8 was used to obtain hybrids with tsH1. Once again hybrid colonies appeared only after PEG + DMSO treatment and, in this case, the hybrid frequency was greater when hydroxyurea sensitivity was used instead of temperature-sensitivity to select against the tsH1 parent. Because $GAT^{-}HU^{R}$-12SC8 is not nearly as resistant as $GAT^{-}HU^{R}$-1, however, a much lower concentration of hydroxyurea had to be used to prevent inhibition of hybrid growth. Thus the selection with $GAT^{-}HU^{R}$-12SC8 was not as stringent as with $GAT^{-}HU^{R}$-1 and some diploid parental colonies developed even in the presence of hydroxyurea (Table 10). Table 10 also shows that PEG treatment of any of the parental cells alone results, as expected, in no hybrids capable of surviving the selection conditions.

C. ISOLATION OF REVERTANTS OF HYDROXYUREA-RESISTANT MUTANTS

In an experiment to determine the phenotypic stability of a large number of independently isolated hydroxyurea-resistant CHO clones, 10 drug-resistant cell lines were isolated from the auxotrophic GAT^-CHO cell line and tested for their plating response to hydroxyurea at intervals during four months of serial cultivation in the absence of the drug. Five of the clones (#1,4,7,8,10) were originally isolated from an EMS-mutagenized population of GAT^-CHO cells. Five hundred thousand of the mutagenized cells had been placed in 100 mm culture plates containing standard medium having the required adenosine and thymidine plus 0.33 mM hydroxyurea. After 16 days incubation, the five surviving colonies referred to above were picked and recloned in the absence of the drug. Similarly, five additional clones (#12,13,14,15,19) were each isolated from non-mutagenized cells. These clones were derived from five independent GAT^- CHO clones exposed individually to medium containing 0.33 mM hydroxyurea as outlined above. One surviving colony from each culture was picked and recloned in the absence of hydroxyurea.

Table 11 shows that all of the cell lines isolated were significantly more resistant to the cytotoxic effects of hydroxyurea than the parental GAT^-CHO population at the beginning of the serial cultivation. Seven of the lines maintained a fairly constant level of resistance throughout

Table 11. Stability of hydroxyurea-resistant phenotype.

Clone #	RPE at 0.26 mM hydroxyurea				
	Day 0	Day 13	Day 57	Day 87	Day 117
1	0.46	0.26	0.48	0.48	0.37
4	0.92	0.70	0.89	0.65	0.72
7	0.38	0.55	0.58	0.15	0.05
8	0.36	0.86	0.89	0.89	0.81
10	0.79	0.57	0.59	0.57	0.54
12	0.92	0.69	0.36	0.26	0.22
13	0.62	0.71	0.62	0.53	0.59
14	0.33	0.54	0.59	0.84	0.80
15	0.82	0.68	0.37	0.47	0.39
19	0.67	0.57	0.24	0.20	0.05
GAT^- WT CHO	5.0×10^{-5}	3.0×10^{-5}	1.5×10^{-5}	1.0×10^{-5}	3.0×10^{-5}

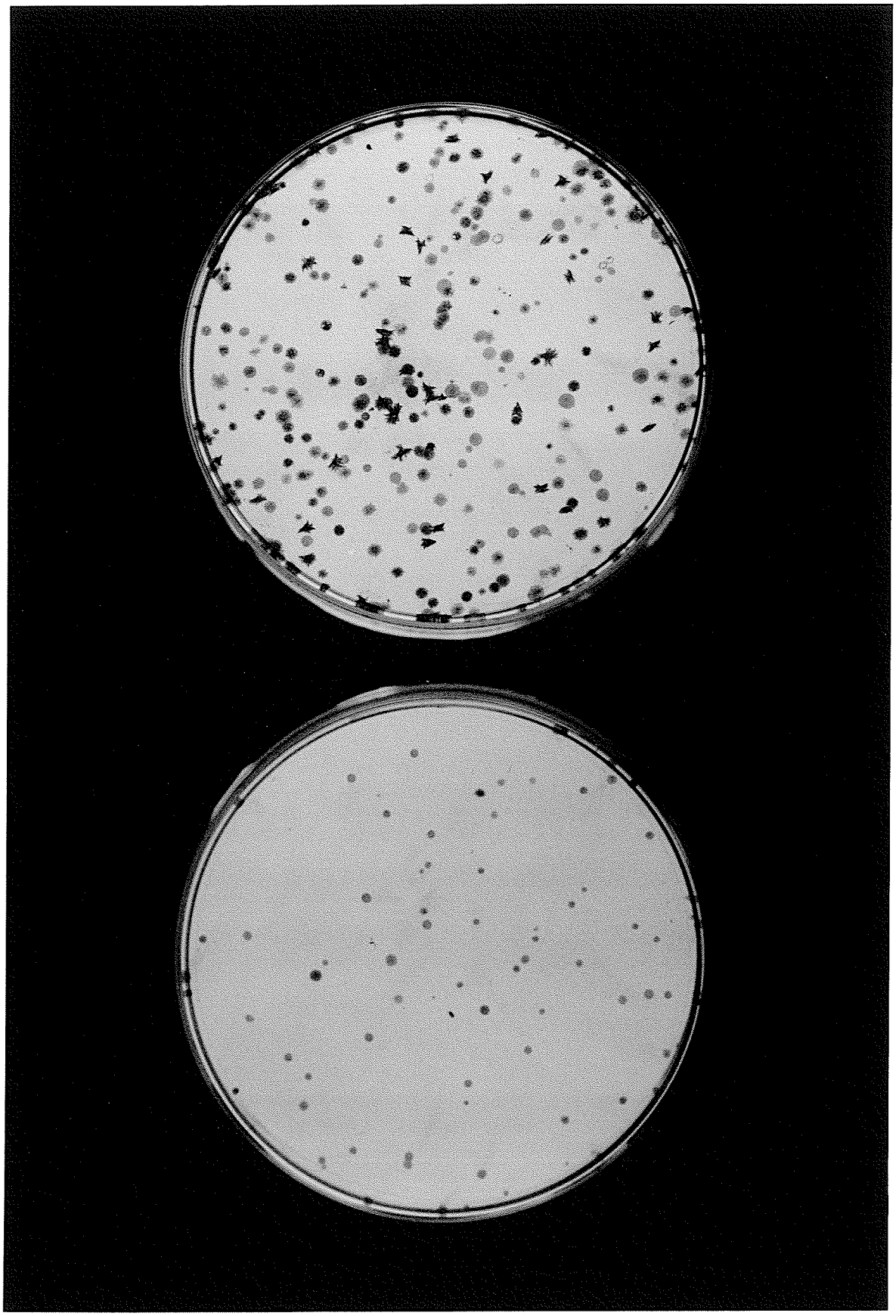
Relative plating efficiencies were determined by plating 300 cells in the absence and presence of 0.26 mM hydroxyurea. Plates were incubated for 8 days and the resulting colonies stained and counted.

the 117 day serial cultivation period. Three of the cell lines (#7,12,19), however, showed a significant reduction in RPE during this time in culture. In order to investigate this apparent decrease in resistance to hydroxyurea, one of the cultures (#12),designated $GAT^{-}HU^{R}$-12, was chosen for further study.

Table 11 shows that the RPE for $GAT^{-}HU^{R}$-12 in the presence of 0.26 mM hydroxyurea, gradually declined from 0.92 immediately after isolation to approximately 0.20 after 117 days in culture.

Plate 4 shows colony development from the 117 day cultivated HU^{R}-12 cell population in the absence (top plate) or presence (bottom plate) of 0.26 mM hydroxyurea. As can be seen, in the absence of the drug, the colonies exhibited a wide range of sizes and morphologies: from large and feathery to small and dense. This contrasts to the single colony morphology type observed shortly after HU^{R}-12 had been isolated and cloned. Furthermore, the different morphological colony types now present in the population were inherited. That is, when cells within a feathery colony were cloned and cultivated they always gave rise to more feathery colonies. Plate 4 (bottom figure) shows the same population of HU^{R}-12 exposed to 0.26 mM hydroxyurea in the growth medium. In this case there was a reduction of about 80% in RPE. However, the colonies which did survive in the presence of the drug were uniform in size and morphology. This suggested that a portion

Plate 4: Plating response of $GAT^{-}HU^{R}$-12 to 0.26 mM hydroxyurea after cultivation in hydroxyurea-free medium for 4 months. A) 60 mm plate, 8 days after incubation of $GAT^{-}HU^{R}$-12 in hydroxyurea-free medium. B) 60 mm plate, 8 days after incubation of $GAT^{-}HU^{R}$-12 in medium containing 0.26 mM hydroxyurea.



A.

B.

of the original HU^R-12 population had retained the hydroxyurea-resistant phenotype, but that these resistant cells were gradually being overgrown by a variety of revertants less resistant to the drug.

To test whether, in fact, subpopulations with differing sensitivities to hydroxyurea existed within the HU^R-12 culture, the population was diluted out and single cells plated into Linbro cloning wells. After 8 days incubation, 23 independent clones were picked and dispensed into three 60 mm culture plates containing medium plus 0 mM. 0.2 mM and 0.33 mM hydroxyurea respectively. After a further 8 days incubation the plates were observed with a microscope and the number of cells per colony estimated in the control and drug-containing plates. Table 12 shows that only 6 of the clones were able to form colonies in the presence of 0.2 mM hydroxyurea and that of these only 3 were also able to form colonies in 0.33 mM drug. The remaining clones showed no growth except on the control plates. Three of the clones representing each level of hydroxyurea-resistance were chosen for further study: the fully resistant subclone #8 (SC8), the partially resistant subclone #13 (SC13) and the sensitive subclone #17 (SC17). Each of these clones was recovered from the control plate containing medium with no hydroxyurea. Figure 22 shows the RPE of the three subclones in various concentrations of hydroxyurea. As expected, SC8 was as resistant as the original HU^R-12 isolate, showing a RPE of 0.87 at 0.26 mM hydroxyurea. The putative revertants

Table 12. Growth of subclones of $GAT^{-}HU^{R}$-12 in the presence of hydroxyurea.

Subclone	0 mM	0.20 mM	0.33 mM
1	++	-	-
2	++	-	-
3	++	-	-
4	++	-	-
5	++	-	-
6	++	-	-
7	++	-	-
8*	++	++	++
9	++	-	-
10	++	-	-
11	++	+	-
12	++	-	-
13†	++	+	-
14	++	-	-
15	++	+	-
16	++	++	++
17§	++	-	-
18	++	-	-
19	++	++	++
20	++	-	-
21	++	-	-
22	++	-	-
23	++	-	-

++ = >100 cells/colony after 8 days incubation at 37°C

\+ = > 10 cells/colony after 8 days incubation at 37°C

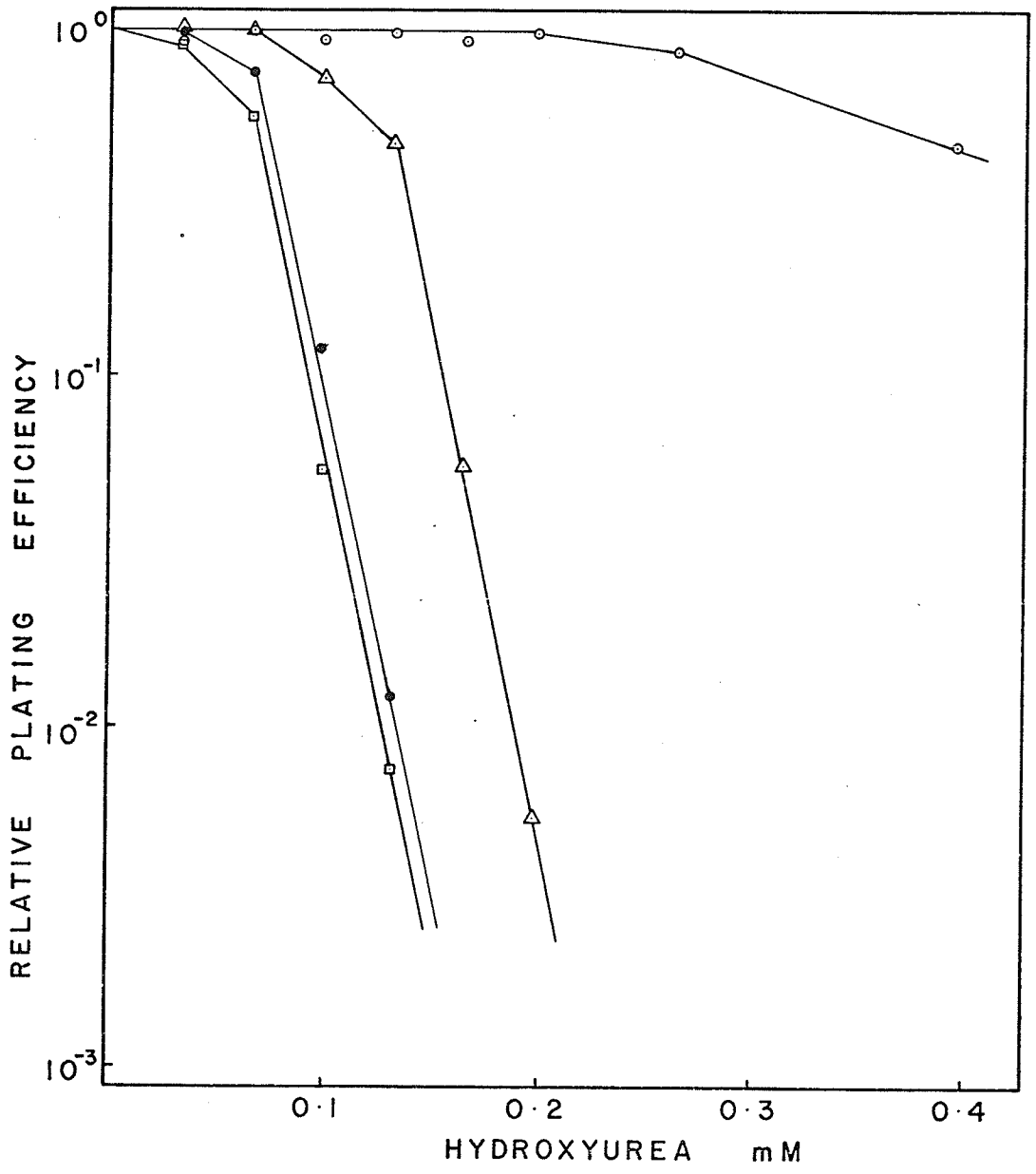
\- = < 10 cells/colony after 8 days incubation at 37°C

*Stable resistant cell line designated $GAT^{-}HU^{R}$-12 SC8

†Partially revertant cell line designated $GAT^{-}HU^{R}$-12 SC13

§Totally revertant cell line designated $GAT^{-}HU^{R}$-12 SC17

Figure 22: Relative plating efficiency of $GAT^{-}HU^{R}$-12 subclones SC17 (⊡), SC13 (△), SC8 (⊙) and $GAT^{-}WT$ (●) with increasing concentration of hydroxyurea.


RELATIVE PLATING EFFICIENCY
10^{0}
10^{-1}
10^{-2}
10^{-3}
0·1
0·2
0·3
0·4
HYDROXYUREA mM

SC13 and SC17, showed hydroxyurea sensitivities closer to the parental $GAT^{-}CHO$ cell line. However, SC13 was significantly more resistant to hydroxyurea than either SC17 or $GAT^{-}CHO$ cells and thus may represent a partial revertant. Plate 5 shows stained culture dishes of the three subclones in the absence and presence of hydroxyurea. It is clear that SC8 is much more resistant to hydroxyurea than SC13 which in turn is more resistant than SC17. The parental $GAT^{-}CHO$ cells showed a sensitivity to hydroxyurea indistinguishable from SC17.

From the growth curve in Figure 23 it can be seen that the growth of SC8 is almost unaffected by the presence of 0.26 mM hydroxyurea in the growth medium. In the presence or absence of this concentration of hydroxyurea, SC8 showed a doubling time of approximately 14 to 15 hours.

To test whether the newly cloned SC8 was stable in its resistance to hydroxyurea, 20 subclones of the SC8 population were isolated and tested for their RPE in the presence of 0.26 mM hydroxyurea. Table 13 shows that all the subclones tested show a high RPE in the presence of the drug. The average RPE in 0.26 mM hydroxyurea was 0.91, a value very close to that obtained with the original HU^{R}-12 isolate and SC8. It should be noted that these fresh subclones and the parental SC8 had not been exposed to hydroxyurea prior to their testing for RPE in the presence of the drug. The history of the subclones can be represented

Plate 5: Response of $GAT^{-}HU^{R}$-12 subclones to growth in medium containing hydroxyurea.

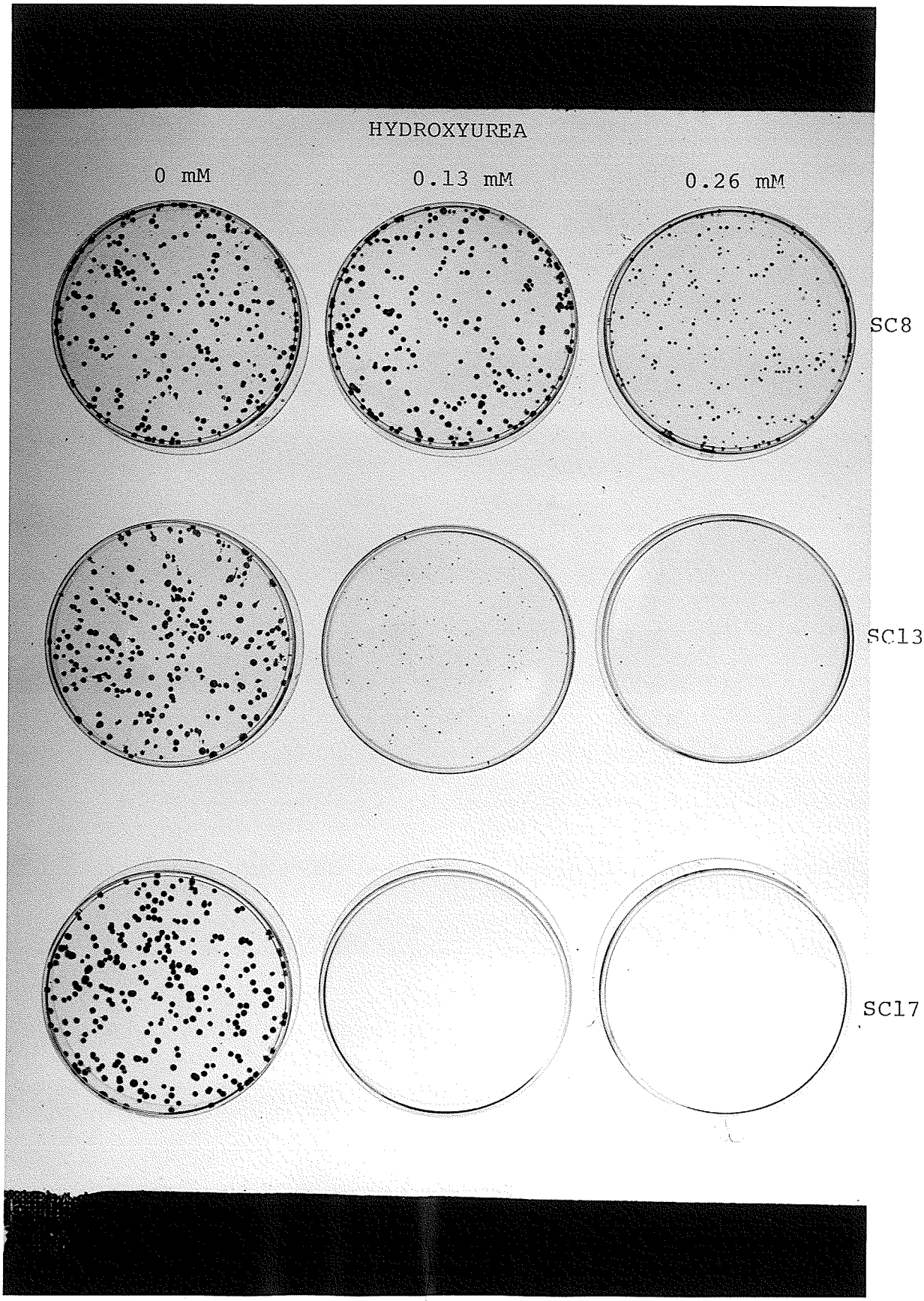

HYDROXYUREA
0 mM
0.13 mM
0.26 mM
SC8
SC13
SC17

Figure 23: Growth curve of $GAT^{-}HU^{R}$-12 SC8 in the absence (⊙) and presence (●) of 0.26 mM hydroxyurea.

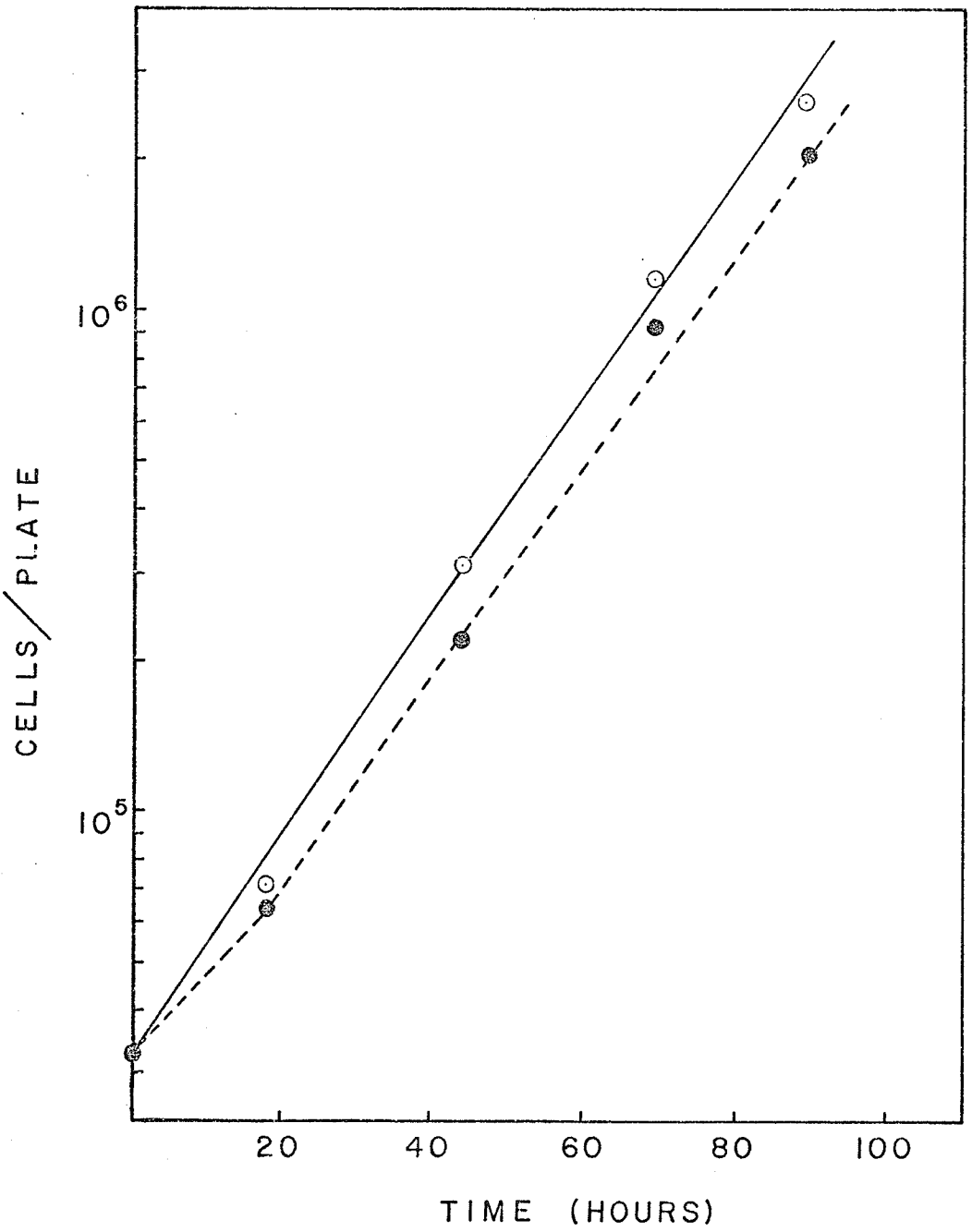

CELLS / PLATE
10^6
10^5
20
40
60
80
100
TIME (HOURS)

Table 13. Relative plating efficiencies of subclones of $GAT^{-}HU^{R}$-12 SC8

Subclone	Number of Colonies		RPE
	0 mM	0.26 mM hydroxyurea	
1	604	524	0.87
2	342	315	0.92
3	526	447	0.85
4	688	640	0.93
5	385	349	0.91
6	620	546	0.88
7	693	658	0.95
8	744	751	1.00
9	TNTC	TNTC	-
10	710	618	0.87
11	461	415	0.90
12	NG	NG	-
13	684	636	0.93
14	543	463	0.85
15	488	464	0.95
16	639	620	0.97
17	392	337	0.86
18	473	397	0.84
19	449	413	0.92
20	contaminated	521	-
SC8	250	218	0.87

TNTC = too numerous too count

NG = no growth

The average RPE of all subclones of SC8 = 0.91

more clearly schematically:

HU^R-12 $\xrightarrow{117\ \text{days}}$ 23 subclones

SC8 $\xrightarrow{8\ \text{days}}$ 20 subclones

The RPE of HU^R-12, SC8 and the average RPE of the final 20 subclones were 0.92, 0.87 and 0.91 respectively. Thus these cells had retained the resistant phenotype for 4 months in serial culture and through an additional 2 cloning procedures all in the absence of hydroxyurea. This demonstrates the stability of the hydroxyurea-resistant trait in these cells.

In subsequent studies it was found that the SC8 population could be maintained in serial culture without significant reversion if fewer than 10^4 cells were used as an inoculum at each transfer of cells. The frequency of reversion to hydroxyurea-sensitivity is apparently less than 10^{-4}. Thus the decrease in RPE during serial cultivation of HU^R-12 can best be explained by the appearance of revertant cells which gradually overgrew the resistant population. To test whether the revertant cells would have any growth advantage in a mixed population, the growth rates of the various subclones were tested in the presence and absence of hydroxyurea. Table 14 shows that the parental GAT^-CHO population and revertant SC17 line had significantly faster doubling times than the resistant SC8 and SC13. The growth rate determinations were carried out with the same batch of medium and serum and at the same time for all 4 cell

Table 14. Doubling times of $GAT^{-}HU^{R}$-12 subclones.

Subclone	Hydroxyurea		
	0 mM	0.13 mM	0.26 mM
HU^{R}-12 SC8	14	14	14
HU^{R}-12 SC13	14	15	54
HU^{R}-12 SC17	12	42	NG
GAT^{-}WT CHO	12	50	NG

lines tested. In a similar experiment, cells from the 3 subclones and the parental GAT$^-$CHO cell line were inoculated at 10^5 cells per 16 oz Brockway bottle. After 4 days incubation at 37^{o}C in the absence of hydroxyurea both SC8 and SC13 contained significantly fewer cells per bottle compared to GAT$^-$CHO or revertant SC17 population. From these growth rate results it is predictable that in the absence of hydroxyurea, cells which revert to the WT phenotype, as SC17 did, will eventually overgrow the remaining resistant cells. On the other hand, Table 14 also shows that both SC8 and to a lesser extent SC13 have definite growth advantages in the presence of hydroxyurea.

Plate 6 shows the results of a reconstruction experiment where a small number of resistant SC8 cells were mixed with a large excess of revertant SC17 cells and incubated in medium containing hydroxyurea. The same number of colonies developed with the mixture of cells as did SC8 when it was plated alone. The colonies on the culture plate with the excess of SC17 cells were somewhat larger than the SC8 colonies which developed in the absence of SC17. This may be due to a cross-feeding effect from the excess of inhibited revertant SC17 cells.

In order to determine whether the hydroxyurea-resistance of SC8 would behave codominantly and be expressed in cell hybrids with the WT genome, a mixture of SC8 and tsH1 cells were exposed to standard PEG fusing conditions as

Plate 6: Reconstruction experiments with $GAT^{-}HU^{R}$-12SC8 and SC17.

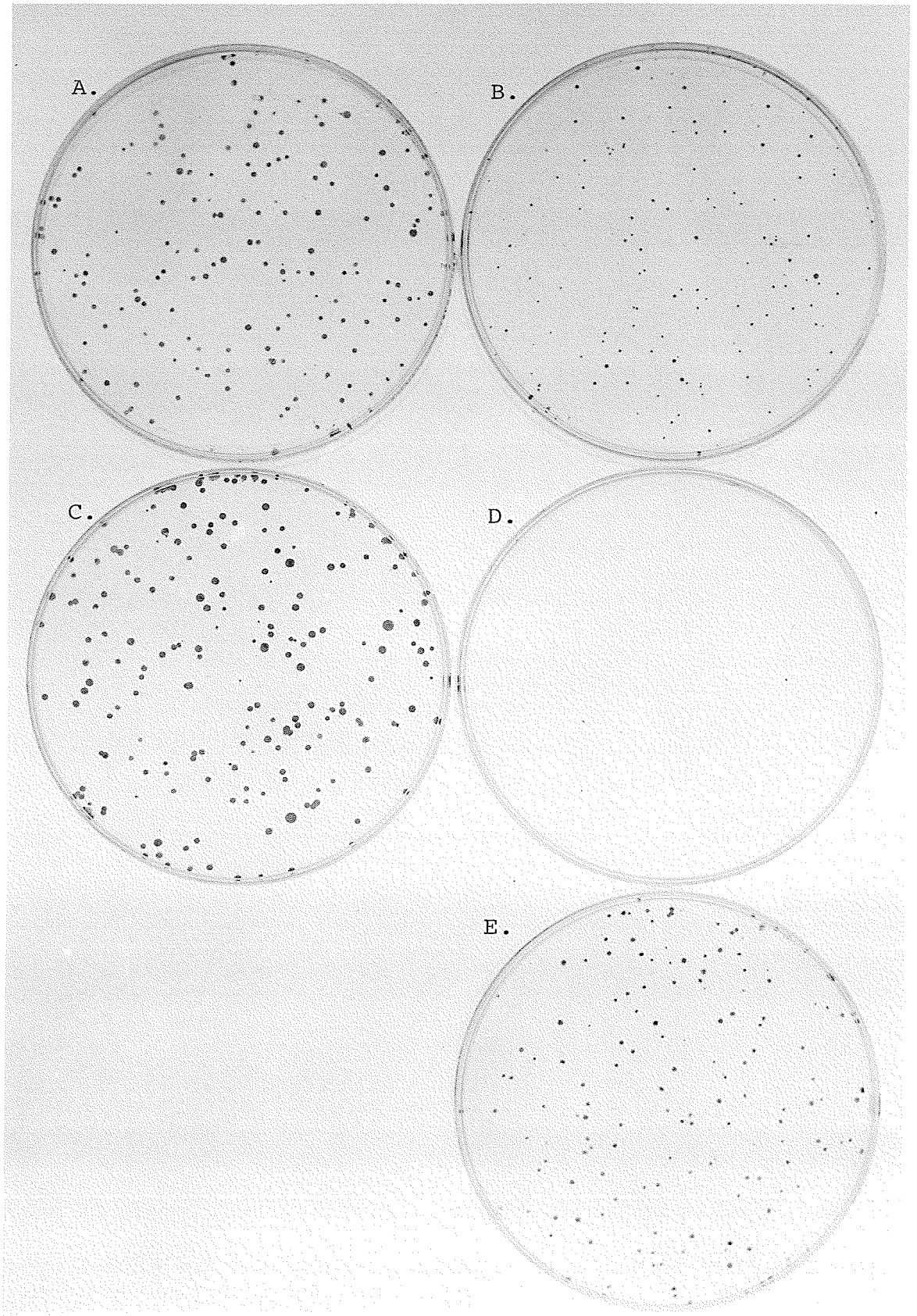
A: 200 cells GAT^{-}SC8

B: 200 cells GAT^{-}SC8 + 0.26 mM hydroxyurea

C: 200 cells GAT^{-}SC17

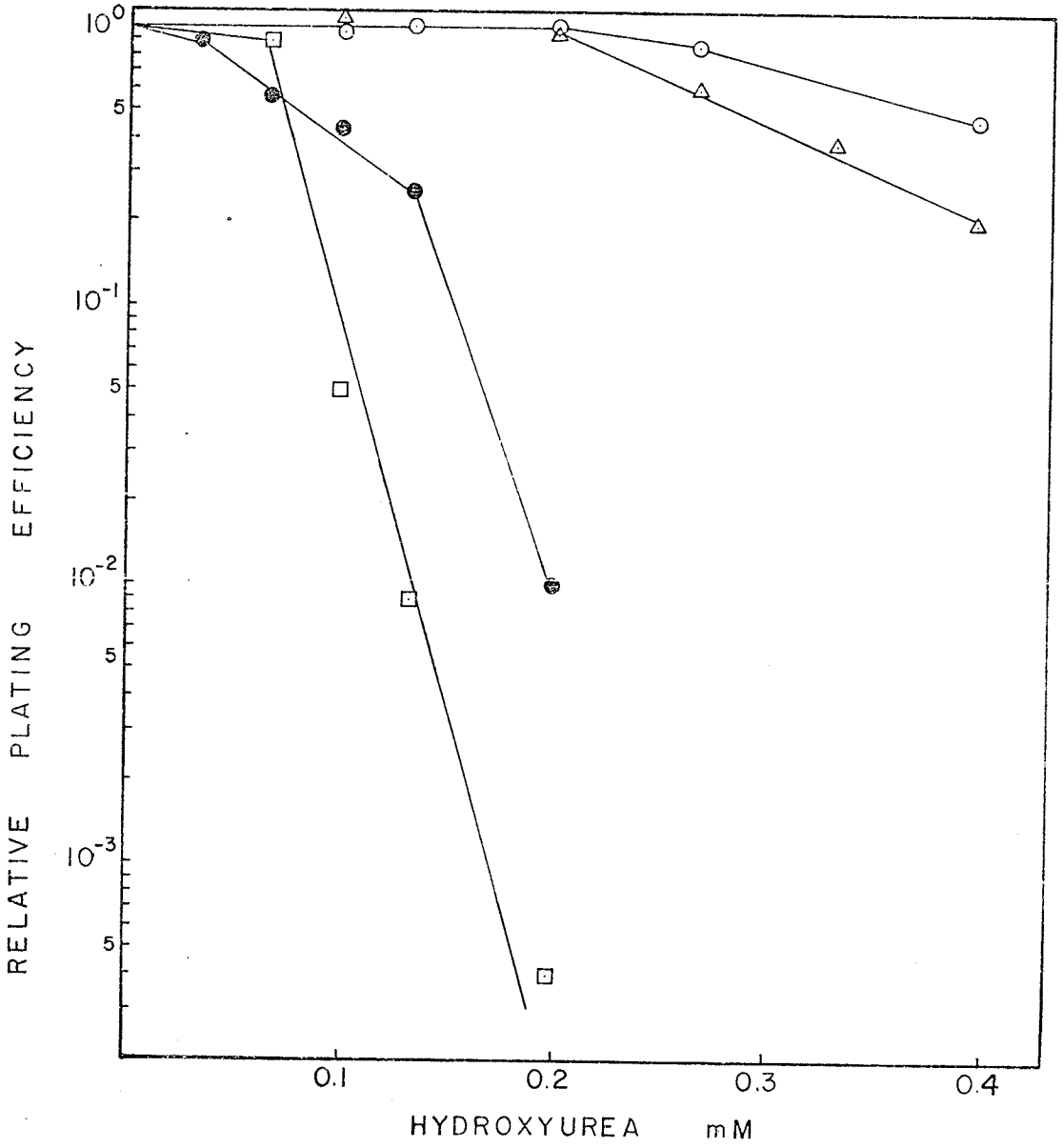
D: 1×10^{5} cells GAT^{-}SC17 + 0.26 mM hydroxyurea

E: 200 cells GAT^{-}SC8 + 1×10^{5} cells GAT^{-}SC17 + 0.26 mM hydroxyurea


A.
B.
C.
D.
E.

detailed in Materials and Methods. The resulting hybrid cells were selected by incubation at 39.5°C in α-MEM plus 10% dFCS with no added thymidine or adenosine. Since SC8 is descendent from the auxotrophic GAT⁻CHO cell line it requires thymidine and adenosine for growth. After 14 days one surviving colony was picked, recloned under the selective conditions and the hybrid nature of the resulting clone confirmed by karyotype analysis. Figure 24 shows the RPE of the hybrid (ts X SC8) compared to the parental and control hybrid cell lines. Once again hydroxyurea-resistance is retained in the hybrid and the RPE for (ts X SC8) approached that of the resistant SC8 line. As before, the pseudotetraploid control line (ts X GAT) showed a slight increase in RPE when exposed to hydroxyurea as compared to the parental cell lines. The reason for this is not known but the phenomenon has been observed in other cell hybrids (Baker et al 1974).

Figure 24: Relative plating efficiency of $GAT^{-}CHO$ (⊡), (ts X GAT) (●), (ts X SC8) (△) and $GAT^{-}HU^{R}$-12 SC8 (⊙) in the presence of increasing concentrations of hydroxyurea.


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

D. RIBONUCLEOTIDE REDUCTASE

1. Assay procedures: Assays of ribonucleotide reductase in mammalian extracts are difficult to perform. The enzyme is present in only very low amounts and often contaminating enzymes are present which compete for nucleotide substrates and effectors. Because of the low enzyme activity ribonucleoside diphosphate substrates radioactively labelled at high specific activities have been used. After incubation, the ribonucleotide substrate must then be separated from the deoxyribonucleotide product by chromatographic separation techniques.

For CDP and ADP reductase assays we chose the Dowex 1-borate column separation technique of Steeper and Steuart (1970) as modified by Cory et al (1972,1973). Briefly, the method depends upon the affinity of ribose-containing nucleosides for the borate ion. After the cell extract is incubated with the appropriate substrate and reaction mixture, the reaction is terminated by boiling and Crotalus adamanteus or Crotalus atrox snake venom is added to dephosphorylate the ribonucleotides and deoxyribonucleotides. The assay mixture is then run through a column of Dowex 1-borate. The ribonucleosides form a complex with the borate ion and are retained while the deoxyribonucleosides pass through the column unimpeded. If necessary the ribonucleosides can be subsequently eluted with concentrated solutions of sodium borate.

Figure 25 shows the separation of cytidine (CR) from deoxycytidine (CdR) by Dowex 1-borate chromatography. In Figure 25A, unlabelled CdR was passed through a column of Dowex 1-borate as described in Materials and Methods. It can be seen that all the CdR eluted freely from the column. Saturated sodium borate released no further nucleoside from the column. On the other hand, when CR was passed through the column, no nucleoside was eluted until the addition of saturated sodium borate (Fig 25B).

Figure 26 shows the results of experiments utilizing cell extracts from CHO cells. In Figure 26A, CDP labelled with ^{14}C was incubated with the standard reaction mixture containing 1 mg of boiled CHO cell extract. As expected, all of the label eluted with the CR peak. When ^{14}C-CDP was incubated with active CHO cell extract (Fig 26B), however, a significant proportion of the label now migrated with the CdR peak.

The standard assay procedure adopted for measuring CDP reduction was the passage of snake venom-treated extract through a column of Dowex 1-borate, collecting the first 4 ml of eluant and determining its radioactivity. The efficiency of recovery could be determined by adding known amounts of unlabelled dCMP before snake venom treatment. The total Absorbance at 270 nm of the final Dowex 1-borate eluant was measured and the percent recovery of CdR could be determined. The recovery routinely varied from 80% to 90%.

Figure 25: Separation of cytidine from deoxycytidine by chromatography on Dowex 1-borate.
a) CdR (0.3 µmoles in 1 ml) was added to a column (0.5 X 8 cm) of Dowex 1-borate prepared as described in Materials & Methods. The column was washed with distilled water and 0.5 ml fractions collected and the absorbance at 270 nm determined for each fraction. After Fraction #13 (indicated by arrow) the elution solvent was changed to saturated sodium borate.
b) CR (0.3 µmoles in 1 ml) was added to a 0.5 X 8 cm column of Dowex 1-borate and eluted and monitored at 270 nm as described above.

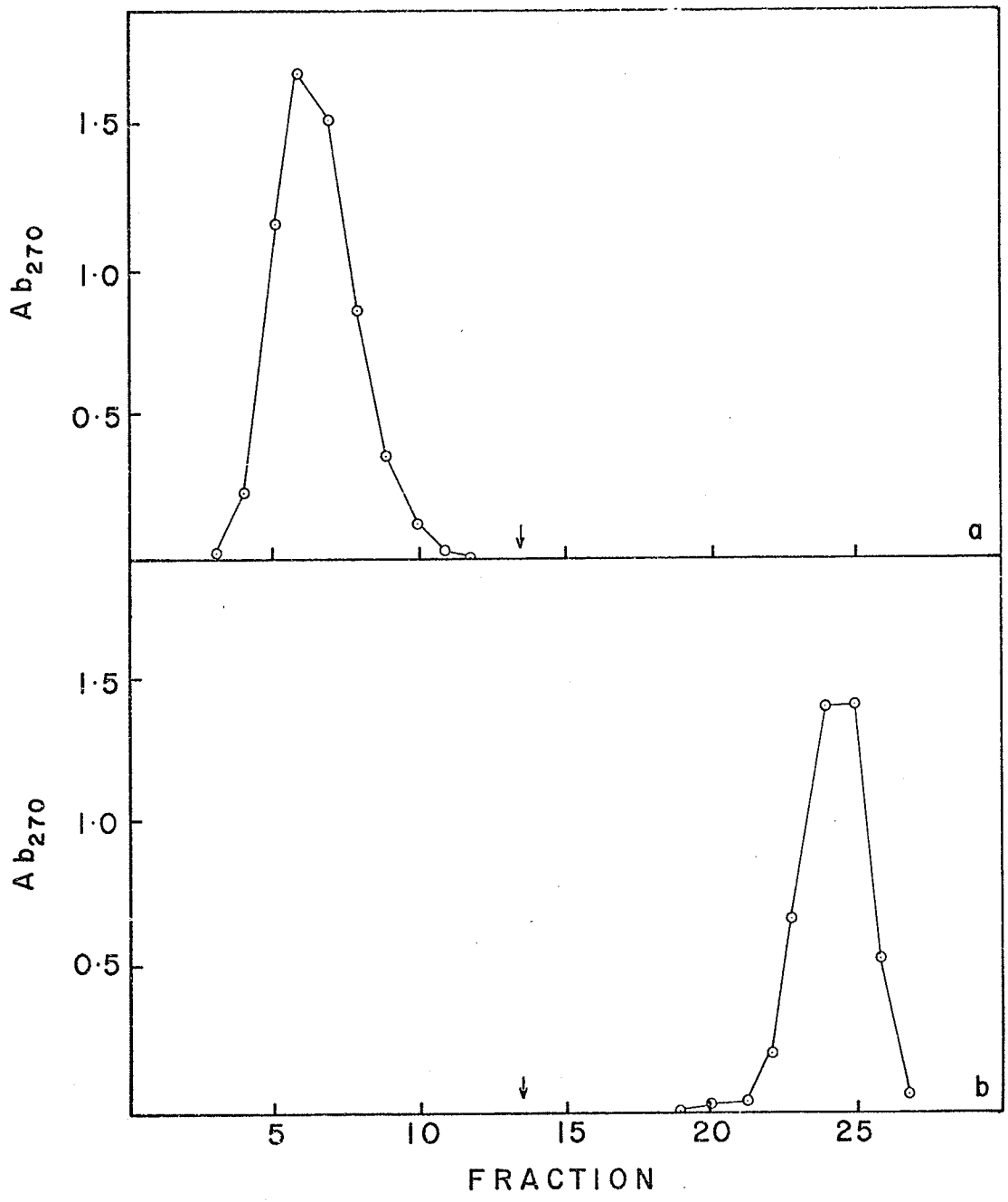
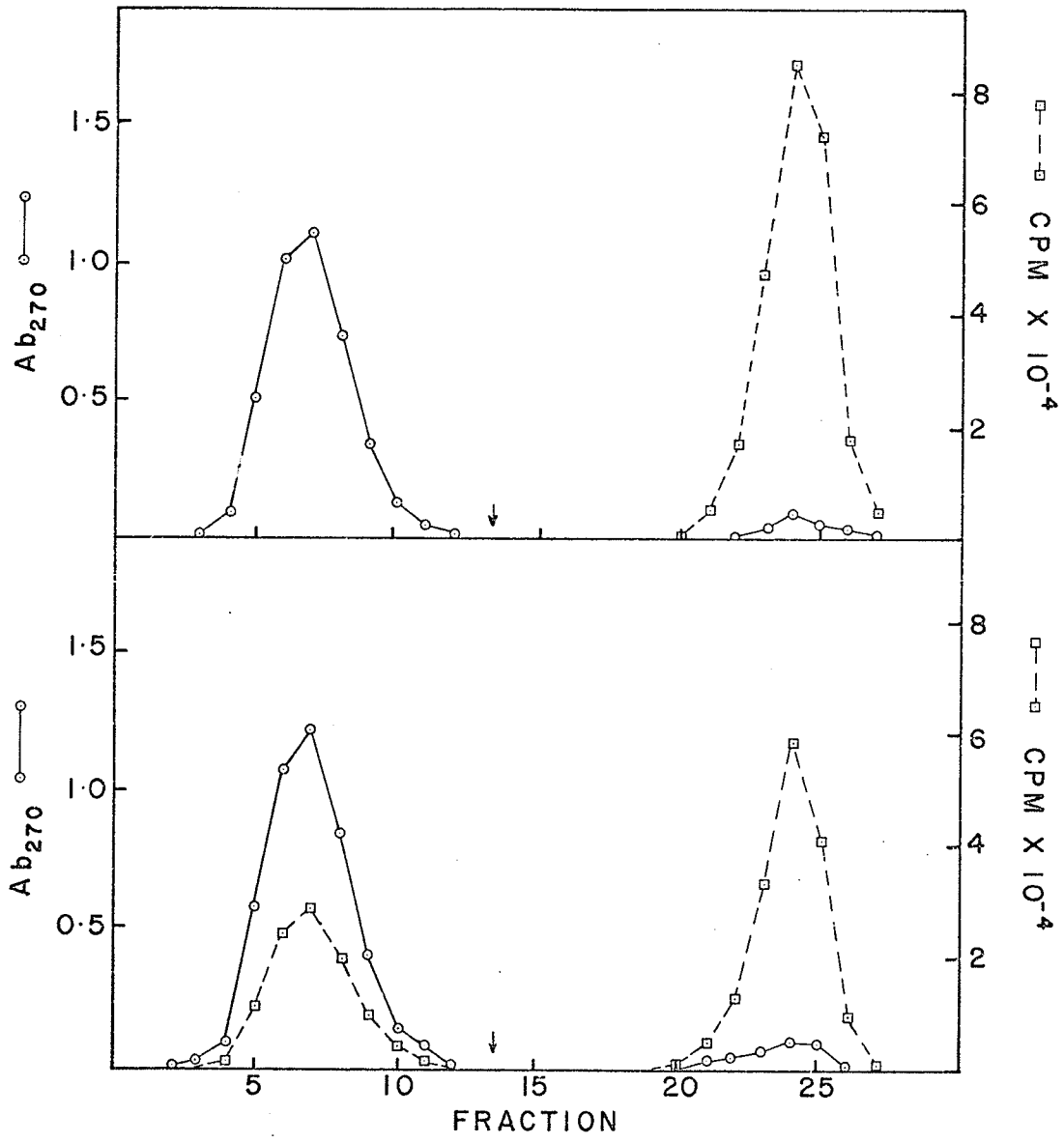

1·5
1·0
0·5
Ab270
a
1·5
1·0
0·5
Ab270
b
5
10
15
20
25
FRACTION

Figure 26: Chromatographic separation of cytidine and deoxycytidine after incubation with CHO cell extracts. ^{14}C-CDP (300,000 cpm, 0.4 mM) was incubated with the standard CDP reductase reaction mixture containing 1 mg boiled CHO cell extract (A) or 1 mg unheated cell extracted (B). After incubation for 40 min at 37^{o}C, the reactions were terminated by boiling and 0.3 µmoles carrier dCMP was added to each sample. The samples were then incubated with Crotalus atrox snake venom as described in Materials and Methods, and then made up to 1 ml with distilled water and centrifuged to remove precipitated protein. The supernatant was added to a 0.5 X 8 cm column of Dowex 1-borate and eluted with water and then saturated sodium borate (arrow indicates addition of borate). As in Figure 25, 0.5 ml fractions were collected and their absorbances at 270 nm (⊙) measured before determination of radioactivity of each fraction (⊡).


1·5
1·0
0·5
Ab270
8
6
4
2
CPM X 10-4
5
10
15
20
25
FRACTION

ADP reduction was measured with Dowex 1-borate columns in a similar manner to CDP reduction. Once again, after the enzyme reaction had been terminated by boiling, snake venom was used to convert all nucleoside phosphates to nucleosides. The mixture was then passed through a column of Dowex 1-borate equilibrated with 1 mM sodium borate. The low concentration of borate was sufficient to elute the deoxyadenosine (AdR) while leaving the adenosine (AR) retained on the Dowex 1-borate (Cory et al 1973).

Figure 27 shows the separation of AR from AdR by this technique. In Figure 27A unlabelled AdR was passed through the Dowex 1-borate column which was then eluted with 1 mM borate. The AdR did not elute as a sharp peak, but after 16 ml of 1 mM borate no further AdR eluted. The addition of saturated sodium borate also did not result in the elution of any further UV absorbing material.

In Figure 27B, unlabelled AR was added to the column and elution conditions identical to Figure 27A were utilized. No AR eluted in the first twenty 1 ml fractions. However, after the addition of saturated sodium borate, the AR began to elute.

Figure 28 shows the results using CHO cell extracts and standard ADP reduction conditions. In Figure 28A, ^{14}C-ADP was incubated with boiled CHO cell extract and treated as described in Materials and Methods. As can be seen very little of the label eluted with AdR, while the majority

Figure 27: Separation of adenosine from deoxyadenosine by chromatography on Dowex 1-borate.
A) AdR (0.3 μmoles in 1 ml) was added to a 0.5 X 8 cm column of Dowex 1-borate equilibrated with 1 mM sodium borate. The column was eluted with 1 mM sodium borate and 1 ml fractions collected and the absorbance at 270 nm determined for each fraction. At the arrow, the elution solvent was changed to saturated sodium borate.
B) AR (0.3 μmoles in 1 ml) was added to a 0.5 X 8 cm Dowex 1-borate column and eluted and monitored at 270 nm as described above.

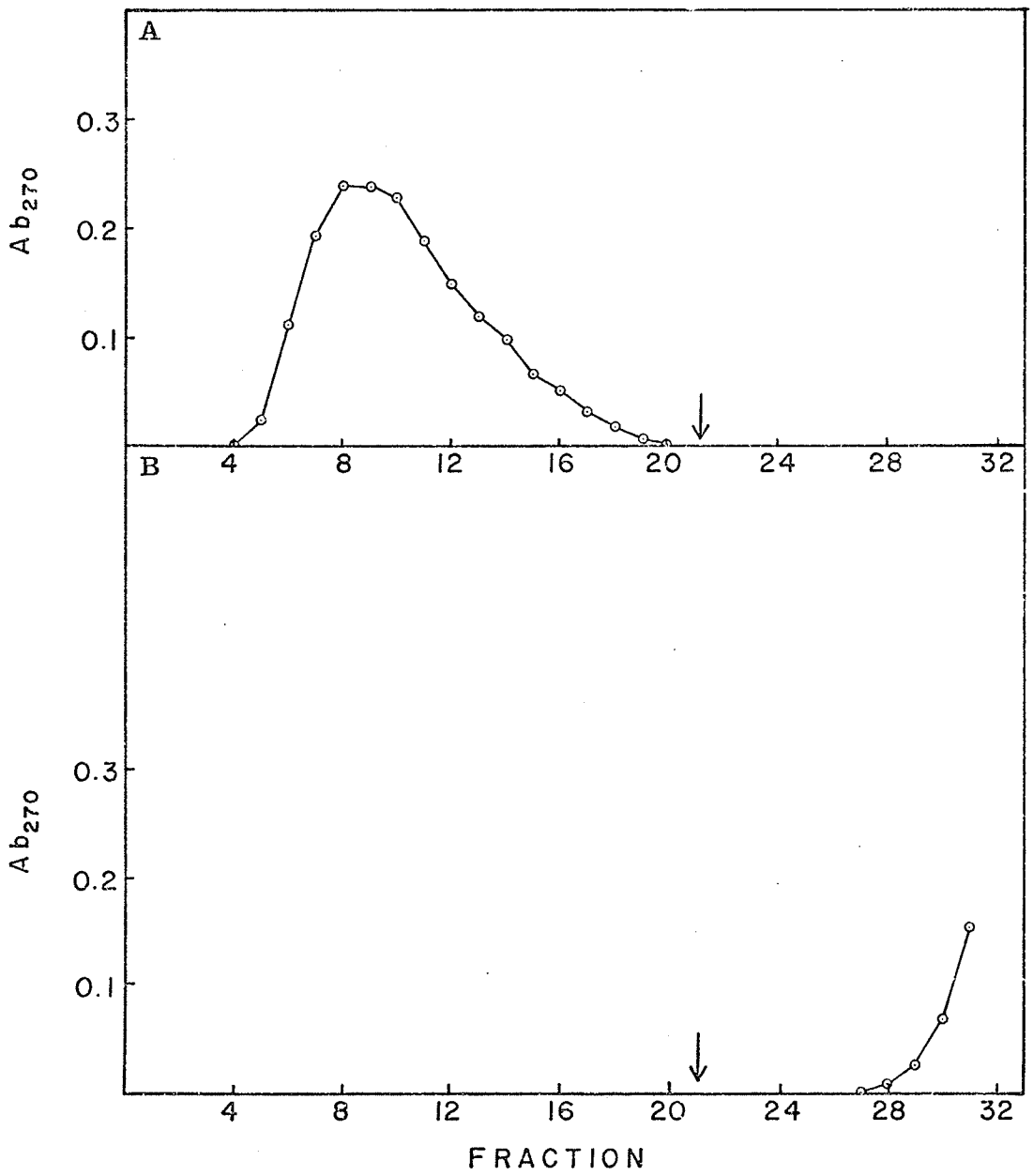
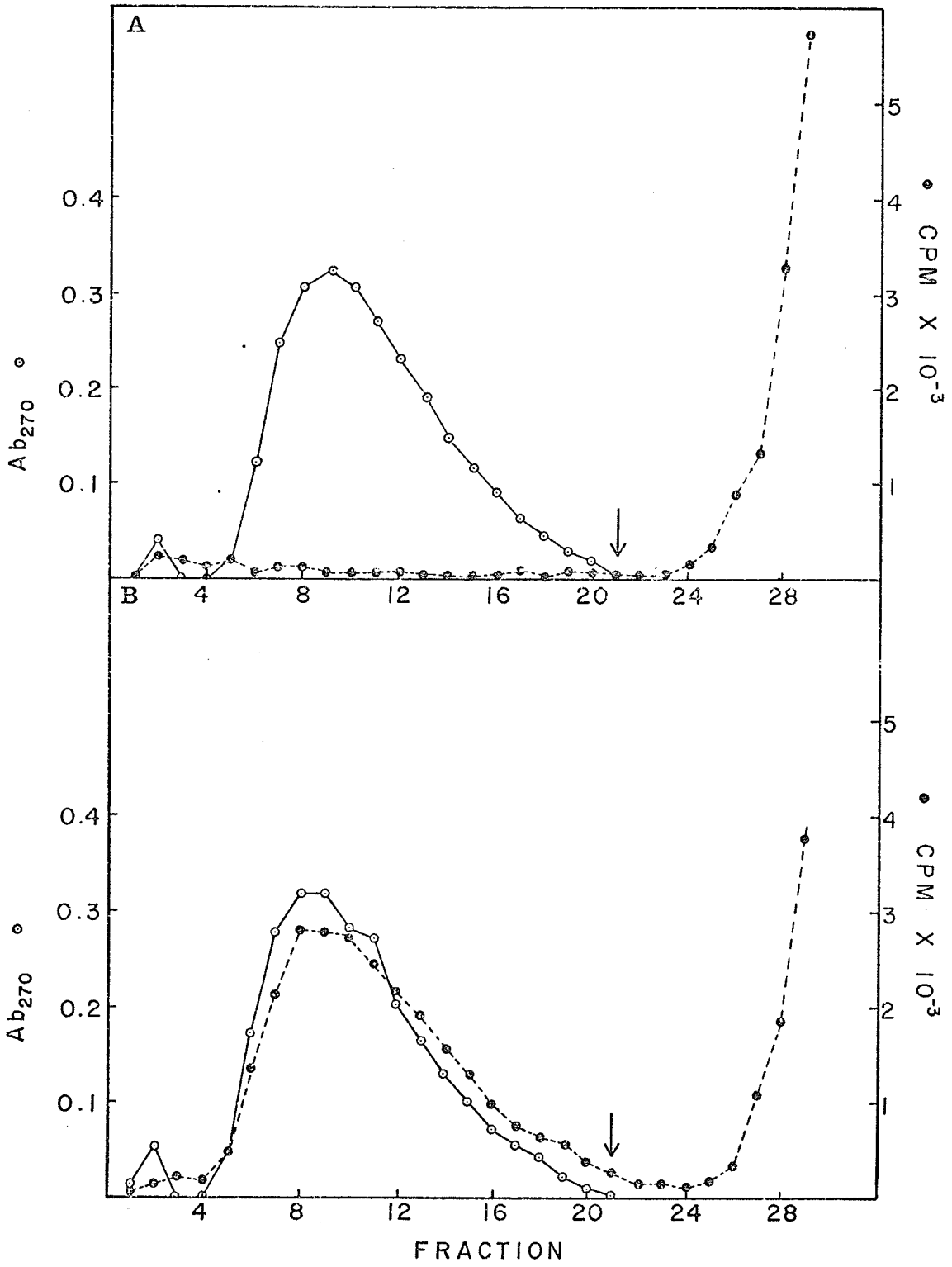

A
B
Ab_{270}
0.3
0.2
0.1
4
8
12
16
20
24
28
32
FRACTION

Figure 28: Chromatographic separation of adenosine and deoxyadenosine after incubation with CHO cell extracts. ^{14}C-ADP (300,000 cpm, 0.4 mM) was incubated with the standard ADP reductase reaction mixture containing 1 mg boiled CHO cell extract (A) or 1 mg unheated cell extract (B). After incubation for 60 min at 37°C, the reactions were terminated by boiling and 0.3 µmoles carrier dAMP was added to each sample. The samples were then incubated with Crotalus atrox snake venom as described in Materials & Methods, made up to 1 ml with distilled water and centrifuged to remove precipitated protein. The supernatant was added to a 0.5 X 8 cm column of Dowex 1-borate equilibrated with 1 ml sodium borate. The columns were eluted with 1 mM sodium borate and then saturated sodium borate (arrow indicates the addition of saturated sodium borate). As in Figure 27, 1 ml fractions were collected and their absorbances at 270 nm (⊙) measured before determination of the radioactivity of each fraction (●).


A
B
Ab_{270} ●
● CPM X 10^{-3}
0.4
0.3
0.2
0.1
5
4
3
2
1
4
8
12
16
20
24
28
FRACTION

remained with the AR elution position. In Figure 28B, the same experiment was performed with fresh, non-boiled CHO cell extract. In this case a significant amount of the label co-chromatographed with the AdR peak.

The standard assay procedure adopted for ADP reductase was passage of the snake venom-treated extract through a Dowex 1-borate column followed by elution with 1 mM sodium borate. The first 4 ml of eluant was discarded and the next 16 ml collected and the radioactivity determined. As with the CDP reductase assay, a known amount of unlabelled dAMP was added at the termination of the enzyme reaction and by measuring the absorbance at 270 nm of the final 16 ml collected, the efficiency of product recovery was calculated. Recovery varied from 80% to 90%.

The Dowex 1-borate columns could not be used for either GDP or UDP reductase measurements as the Dowex 1-borate nonpreferentially retained both these ribonucleosides and their deoxyribonucleosides. Therefore, it was necessary to separate the substrates and products by thin layer chromatography. Aliquots of snake venom-treated incubation mixtures were spotted on PEI-cellulose thin layer plates and developed by ascending chromatography at room temperature in a solvent system originally developedby Reichard (1958) for separation of cytosine containing compounds. The composition of the solvent system is given in Materials and Methods. Under these

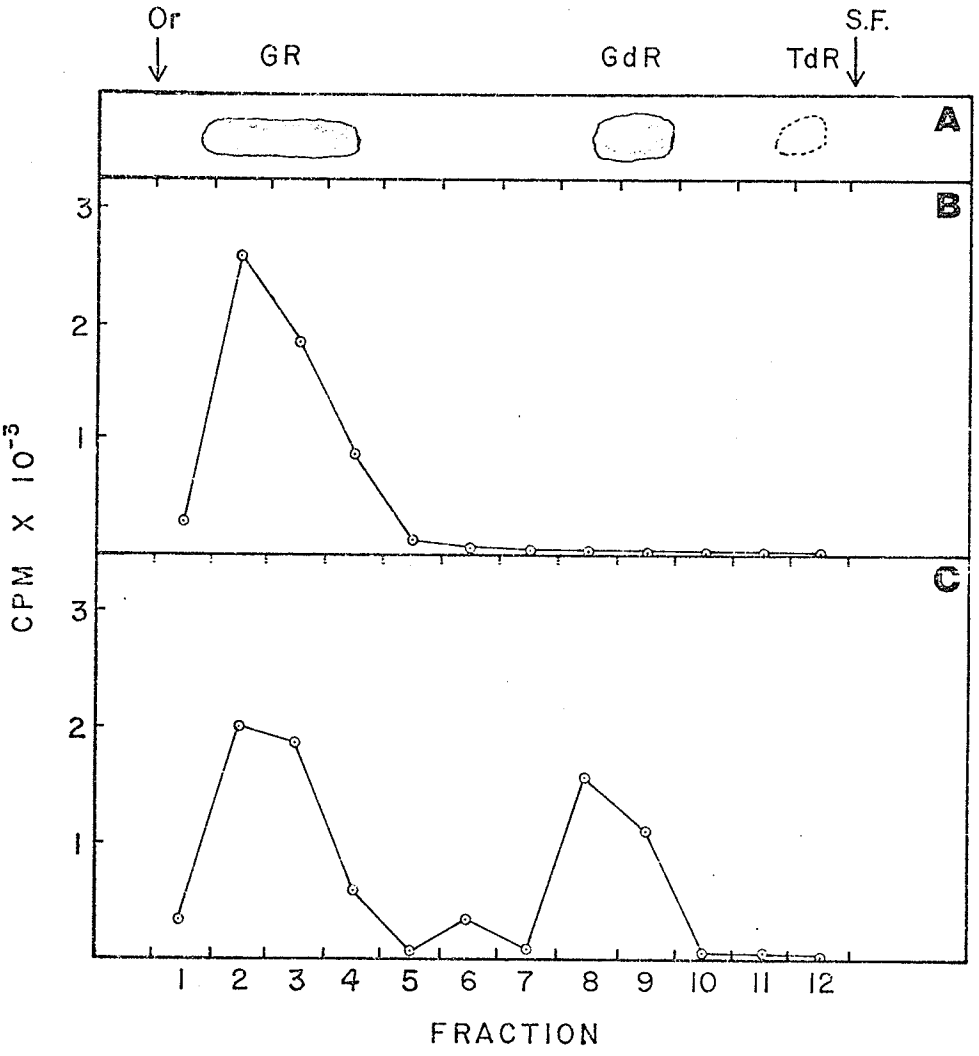
conditions the following R_f values were obtained:

guanosine	0.16	uridine	0.23
deoxyguanosine	0.76	deoxyuridine	0.72

Figure 29A is a replica sketch of a typical thin layer chromatographic separation of guanosine (GR) and deoxyguanosine (GdR). The faint UV absorbing spot to the top of the chromatogram represents deoxythymidine (TdR) which is derived from the dTTP added to the reaction mixture as an activator of GDP reduction. In Figure 29B boiled CHO cell extract has been incubated with the standard reaction mixture containing ^{14}C-GDP. As can be seen, the majority of the label co-chromatographs with GR and only a very small proportion is found associated with the GdR position. However, when non-boiled extract is used, a significant proportion is found associated with the GdR (Fig 29C).

The standard assay procedure developed for the assay of GDP reductase was the spotting of 10 µl of snake venom-treated reaction mixture on a PEI-cellulose thin layer plate. Carrier unlabelled GR and GdR were also spotted with the sample. The plate was then developed for 2.5 hours at room temperature using Reichard's solvent system (Reichard 1958). After locating the GR and GdR spots with a UV light scanner, the spots were cut out, independently immersed in toluene-PPO-POPOP scintillation fluid and counted for radioactivity. The amount of radioactivity associated with GdR was expressed as a percentage of the total radioactivity in the two spots

Figure 29: Chromatographic separation of guanosine and deoxyguanosine after incubation with CHO cell extracts. ^{14}C-GDP (300,000 cpm, 0.02 mM) was incubated with the standard GDP reductase assay mixture containing 1 mg boiled CHO extract (B) or 1 mg unheated extract (C). After incubation for 60 min at 37^{o}C the reactions were terminated by boiling for 4 min. The samples were treated with Crotalus atrox venom as described in Materials & Methods. After snake venom digestion, the samples were centrifuged and 10 µl of of boiled extract assay (B) or unheated extract assay (C) were spotted on a PEI-cellulose thin layer plate. Carrier unlabelled GR and GdR were also spotted at the same locations. The plate was developed by ascending chromatography at room temperature in Reichard's solvent (1958). After 2.5 hr, the plate was dried and the UV absorbing spots located and marked. A replica sketch noting the origin (Or.) and solvent fron (S.F.) is given in A. The plate was cut into 1 cm strips and each section assayed for radioactivity after immersion in toluene-PPO-POPOP scintillation fluid.


Or
GR
GdR
TdR
S.F.
A
B
C
CPM X 10^{-3}
3
2
1
3
2
1
1 2 3 4 5 6 7 8 9 10 11 12
FRACTION

and thus was used to calculate the rate of GDP reduction.

Major problems were encountered in the assay of UDP reductase. Uridine (UR) could easily be separated from deoxyuridine (UdR) by thin layer chromatography as described above. However, after incubation of cell extracts with ^{14}C-UDP in a standard reaction mixture (Moore 1967), followed by routine snake venom treatment, the label was found spread throughout the PEI-cellulose chromatogram unassociated with any one peak. Likely, contaminating enzymes present in the CHO cell extract were converting either the UDP substrate or dUDP product or both into other compounds which migrated with a variety of R_f values. An attempt was made to assay UDP reductase activity in reaction mixtures which had not been treated with snake venom. In this method, UDP reduction assays were terminated by boiling for 4 min, a length of time sufficient to convert all nucleoside di or triphosphates to the nucleoside monophosphate. A 10 µl sample was then spotted on a PEI-cellulose thin layer plate and developed for 90 min at room temperature in a solvent of the following composition: 40 ml saturated sodium borate, 40 ml water and 80 ml 1.2 M LiCl. Under these conditions UMP migrated with a R_f=0.25 and dUMP migrated with a R_f=0.54. In control experiments with boiled cell extract, over 90% of the label of added ^{14}C-UDP migrated as expected with the UMP spot and less than 0.1% was associated with dUMP. However, upon incubation with non-boiled extract the label

was once again found smeared throughout the chromatogram with no discernible peaks. Because of these problems, UDP reduction could not be measured in CHO cell extracts.

2. Inhibition of CDP reductase by hydroxyurea and guanazole: In order to determine whether ribonucleotide reductase from CHO cells was inhibited by hydroxyurea or guanazole, dialysed crude extracts of CHO cells were tested for their ability to reduce CDP in the presence of hydroxyurea or guanazole, using the standard reaction conditions of Moore (1967). Figure 30 shows a time course of CDP reduction with a dialysed preparation of WT CHO extract. In the absence of drug the reaction proceeds linearly for at least 60 min. Similarly, in the presence of 3 mM guanazole or 0.66 mM hydroxyurea CDP reduction proceeds linearly although at a much reduced rate. From Figure 30 it can be seen that 3 mM guanazole reduces CDP reduction by 73%, while 0.66 mM hydroxyurea reduces it by 80%. The linear response during the incubation suggests that neither drug is being converted to a more or less inhibitory form during the incubation.

Figure 31 shows the inhibition of CDP reduction by increasing concentrations of hydroxyurea, again using the reaction assay conditions developed by Moore (1967). Significant inhibition of CDP reduction can be seen below 0.1 mM hydroxyurea and the inhibition rapidly increases until by 1 mM drug only 10% of CDP reductase activity remains.

Figure 30: CDP reduction with time in the absence (△) or presence of 3 mM guanazole (⊡) or 0.66 mM hydroxyurea (⊙). Dialysed WT CHO cell extract (1 mg protein) was incubated in the reaction mixture. At specific times, the reactions were terminated and the amount of CDP reduced was measured.

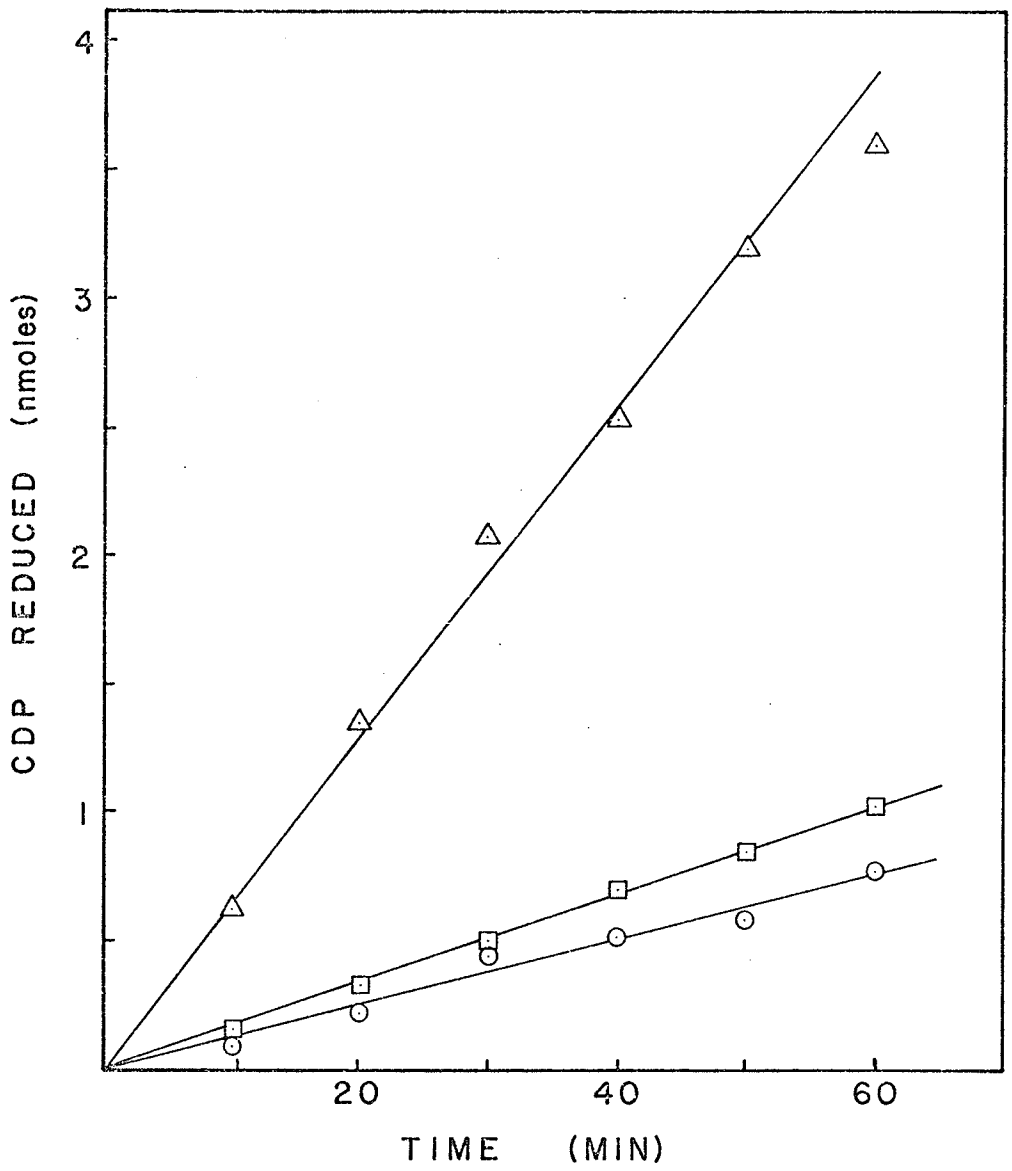
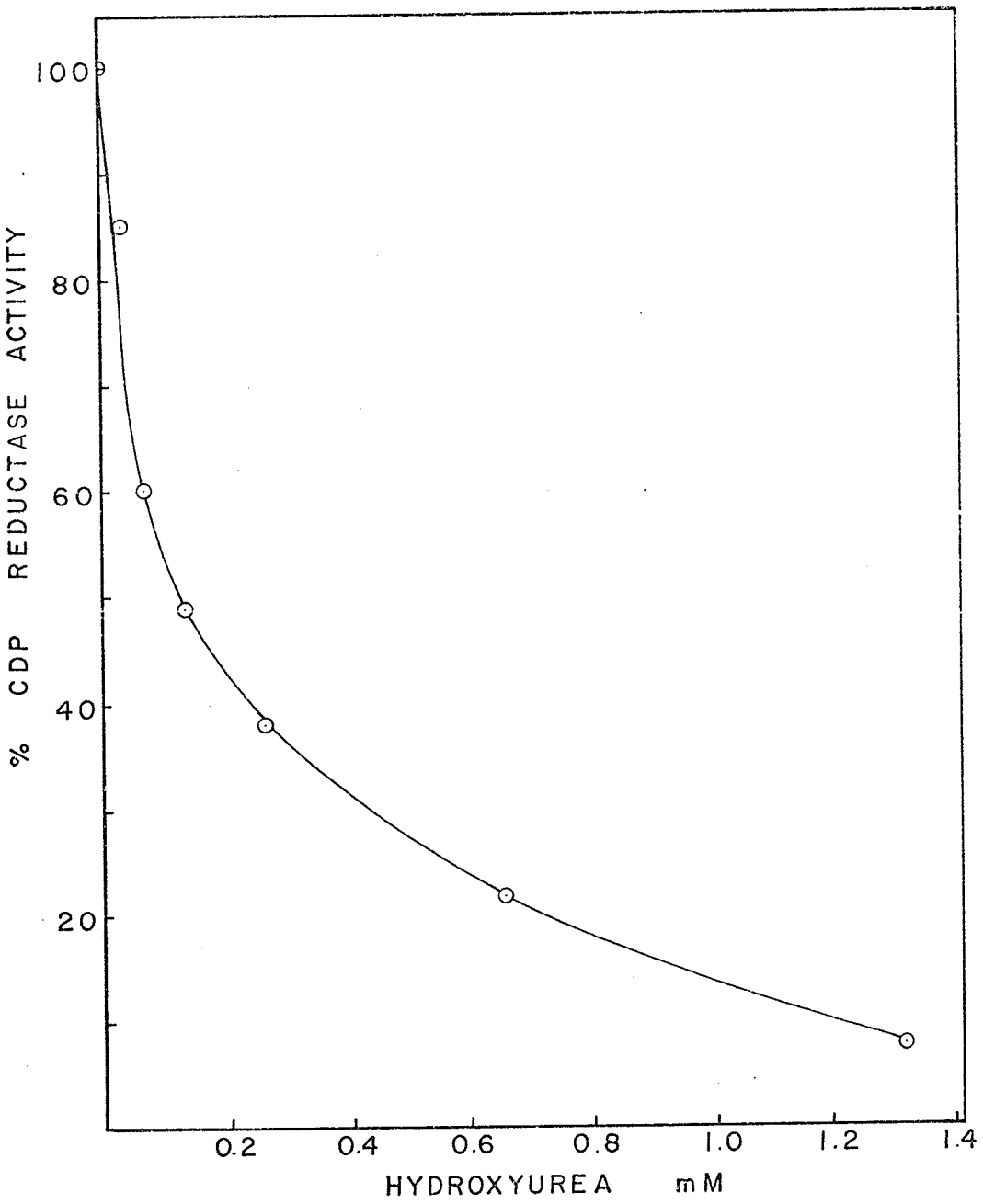

CDP REDUCED (nmoles)
4
3
2
1
20
40
60
TIME (MIN)

Figure 31: Inhibition of CDP reductase by hydroxyurea. Reaction mixtures containing varying concentrations of hydroxyurea were incubated with 1 mg protein of a dialysed WT CHO cell extract (specific activity of 2 nmoles CDP reduced/hr/mg protein). After 40 min the reactions were terminated and the amount of CDP reduced was determined.


100
80
60
40
20
% CDP REDUCTASE ACTIVITY
0.2
0.4
0.6
0.8
1.0
1.2
1.4
HYDROXYUREA mM

In order to determine whether the highly hydroxyurea-resistant cell lines HU^R-2 and HU^R-4 contain ribonucleotide reductase activity which is less sensitive to the drug, preliminary inhibition studies were carried out with the dialysed crude extracts again using Moore's reaction conditions.

Figure 32 shows the response of CDP reductases from HU^R-2 and HU^R-4 to increasing concentrations of hydroxyurea. As can be seen, at all concentrations tested, both mutant enzymes were significantly less inhibited by hydroxyurea than enzyme from either WT CHO or PHA^R-3 CHO cell lines. PHA^R-3 is a variant CHO cell line selected for resistance to the lectin phytohemagglutinin-P (Wright 1973) and has had no exposure to hydroxyurea or related compounds. From Figure 32 it can be seen that at 0.2 mM hydroxyurea the CDP reductase activity of the control lines have been reduced to approximately 50%. HU^R-2 on the other hand shows 98% activity and HU^R-4 an intermediate 75% CDP reductase activity.

Similarly, when guanazole was tested for its effect on CDP reduction in WT CHO cell extracts, the drug was capable of reducing CDP reduction by close to 80% at a concentration of 3 mM. As expected from the cellular responses, both HU^R-2 and HU^R-4 CDP reductase activities were very much less sensitive to the inhibitory action of this drug. Figure 33 shows that at 1 mM guanazole, the WT CHO and PHA^R-3 CDP reductase activities were reduced to about 55% of control values.

Figure 32: Hydroxyurea inhibition of CDP reductase from hydroxyurea-resistant CHO cell lines. Dialysed extract was prepared from WT CHO (△), PHA^R-3 (▲), HU^R-4 (⊡), and HU^R-2 (⊙). The specific activities of the various enzyme preparations varied from 2 to 6 nmoles CDP reduced per 40 min. The points for WT CHO are the average of duplicate assays from three different experiments utilizing separate enzyme preparations. The points for the remaining cell lines are the average of duplicate assays obtained from a single enzyme preparation of each cell line. One mg of extract protein was incubated for 40 min in the reaction mixture containing various concentrations of hydroxyurea.

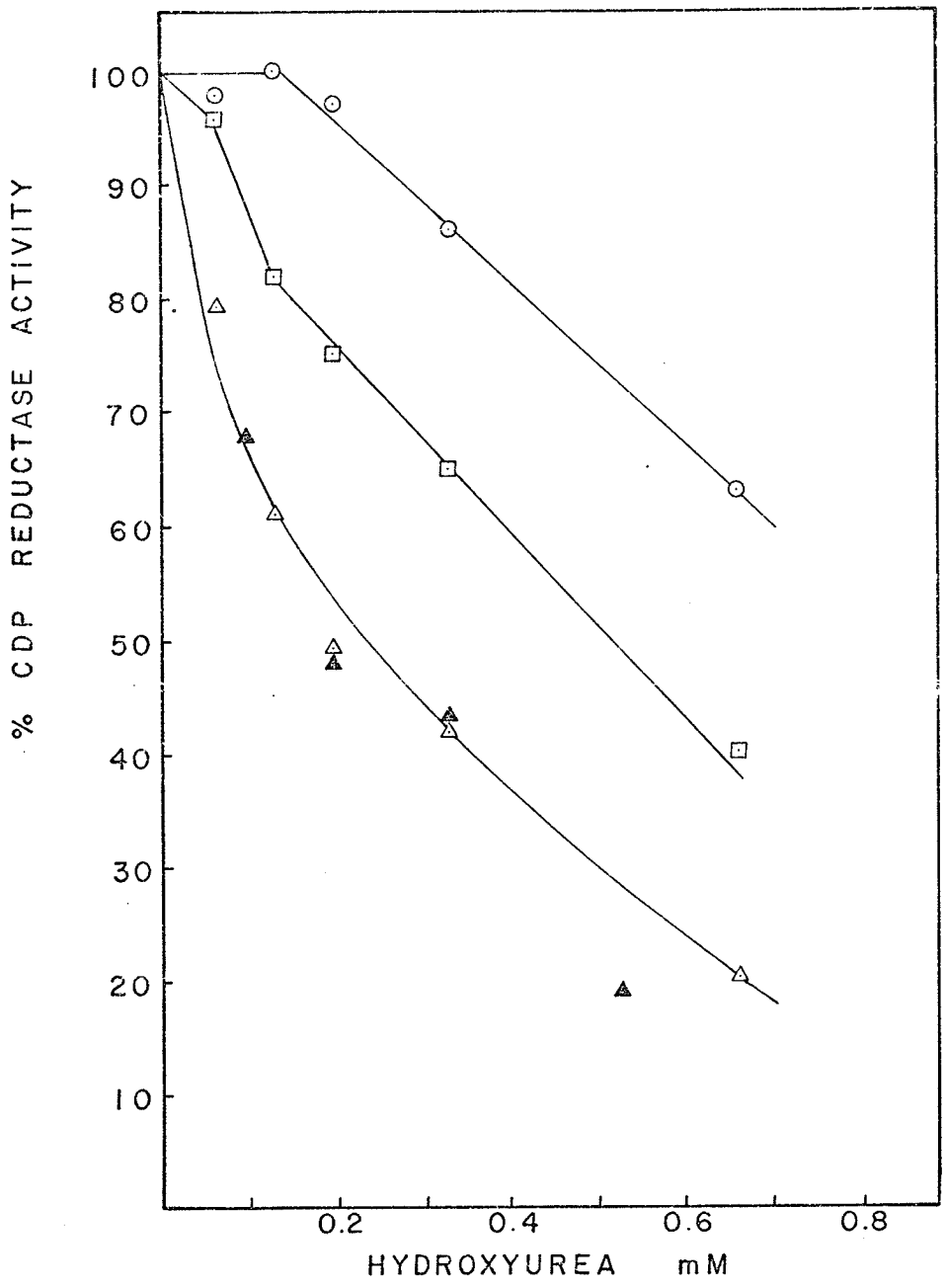
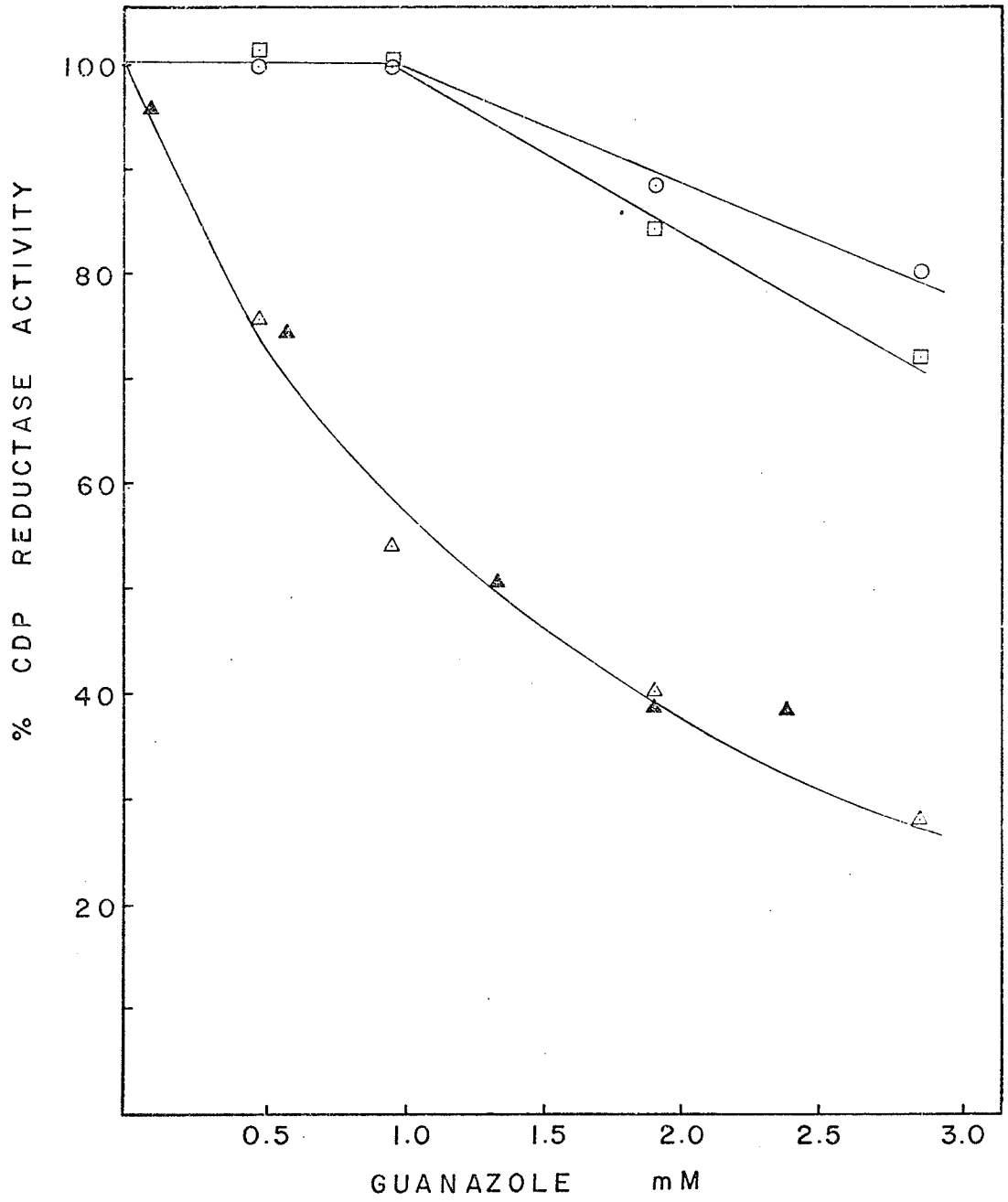

% CDP REDUCTASE ACTIVITY
100
90
80
70
60
50
40
30
20
10
0.2
0.4
0.6
0.8
HYDROXYUREA mM

Figure 33: Guanazole inhibition of CDP reductase from hydroxyurea-resistant cell lines. The cell lines and extracts were the same as described in Figure 32 except guanazole was included in the reaction mixtures instead of hydroxyurea.


% CDP REDUCTASE ACTIVITY
100
80
60
40
20
0.5
1.0
1.5
2.0
2.5
3.0
GUANAZOLE mM

On the other hand, both HU^R-2 and HU^R-4 CDP reductase activities were unaffected by the same concentration of guanazole (Fig 33).

In order to determine whether hydroxyurea-resistant cell lines could be obtained from other mammalian cells in culture, mouse L cells were used to select an additional hydroxyurea-resistant cell line. L cells were found to have the same sensitivity as WT CHO cells to hydroxyurea. Therefore, 5×10^5 L cells were plated in a 100 mm culture plate containing standard medium plus 0.33 mM hydroxyurea. After 16 days incubation, the surviving colonies were transferred to medium lacking hydroxyurea and allowed to grow to a monolayer of cells. These cells (5×10^5) were then plated in another 100 mm culture plate containing standard medium plus 1.33 mM hydroxyurea. After an additional 16 days incubation, the cells which survived in this concentration of drug were trypsinized and recloned in a Linbro dish. Several clones were isolated and one, designated L HU^R-2, was chosen for further study. This cell line exhibited an increased RPE in the presence of hydroxyurea when compared to the wild-type L cells. For example, concentrations of hydroxyurea and guanazole which reduced the RPE of WT L cells to 10^{-5} resulted in little, if any, effect on the RPE of L HU^R-2 cells (Table 15). When the enzyme preparation from the hydroxyurea-resistant L cell line was assayed for CDP reductase activity, it showed a 40% increase in activity when

Table 15. Hydroxyurea-resistant Mouse L cell line.

	Relative Plating Efficiency		CDP Reductase Activity	
Cell Line	hydroxyurea	guanazole	hydroxyurea	guanazole
WT L Cell	10^{-5}	10^{-5}	29% ± 7%	47% ± 3%
L HU^{R}-2	1	1	69% ± 5%	83% ± 5%

The concentrations of the drugs in both sets of experiments were 0.26 mM hydroxyurea and 2.0 mM guanazole.

The CDP reductase activity figures are given as a percent of the activity in the absence of drug and are the average of duplicate samples of three separate experiments.

compared to the parental L cell activity at a concentration of 0.33 mM hydroxyurea. As expected, the CDP reductase activity of L HU^R-2 also exhibited increased resistance to the inhibitory effect of guanazole as shown in Table 15.

3. Determination of hydroxyurea after incubation with cell extracts: One mechanism by which cells may become resistant to a drug is to induce a new enzyme activity or modify a preexisting one such that the drug is metabolized to a non-inhibitory form. In other words the resistant cell lines may contain enzymes with altered substrate specificities or with increased activity levels, which are capable of detoxifying hydroxyurea by chemical modification. Several lines of reasoning argue against this possibility. First, the hydroxyurea-resistant cell lines exhibit cross-resistance to a large number of compounds, some of which are structurally quite unlike hydroxyurea (eg guanazole). Although it is possible that a detoxifying or degradative enzyme would act upon all these compounds, this is not likely. Furthermore, from the cellular mixing experiments reported in Table 3 and Plate 6, it is known that the extracellular concentration of hydroxyurea does not dramatically change in the presence of hydroxyurea-resistant cells.

The most conclusive evidence, however, is a series of experiments presented in Table 16. In the first experiment extracts from WT CHO and HU^R-2 cells were incubated under standard assay conditions with 0.66 mM hydroxyurea. After

Table 16. Measurement of hydroxyurea after incubation with cell extracts.

Cell Extract	Hydroxyurea Added	% Inhibition CDP Reduction	Hydroxyurea[§] Assayed
1. WT CHO	0.66 mM	80 %	0.66 mM
2. HU^R-2	0.66 mM	38 %	0.66 mM
3. WT CHO	0.13 mM*	38 %	0.12 mM
4. HU^R-2	0.13 mM*	2 %	0.14 mM
5. WT CHO	0.33 mM	70 %	0.33 mM
6. WT CHO	2 mM guanazole	65 %	0.0 mM

[§]hydroxyurea determined colorimetrically after enzyme incubation

*hydroxyurea dialysed from #2 HU^R-2 after enzyme incubation

40 min incubation, aliquots of the reaction mixture were analyzed for hydroxyurea by the colorimetric method of Levine and Kretchmer (1971). Using this chemical assay and the standard curve of Figure 14, the reaction mixtures were calculated still to contain 0.66 mM hydroxyurea. As noted previously, this colorimetric method is remarkably specific for hydroxyurea and it is possible to differentiate hydroxyurea from a number of structural analogs as well as from such possible degradative products as urea and hydroxylamine.

In an additional experiment, HU^R-2 extract was again incubated with the complete reaction mixture containing 0.66 mM hydroxyurea. After 40 min incubation at 37°C, the reaction mixture was dialysed overnight at 4°C against an equal volume of distilled water. Various aliquots of the dialyzed water sample were then added to standard reaction mixtures and incubated at 37°C with extract from WT CHO or HU^R-2 cells. In each case CDP reduction was inhibited by the amount predicted by the theoretical final concentration of hydroxyurea. For example, Table 16 shows that an aliquot representing a 1/5 dilution inhibited WT CHO CDP reduction by 38% while HU^R-2 CDP reduction was only inhibited 2%. Furthermore, after incubation, the reaction mixtures were determined colorimetrically to have hydroxyurea concentrations close to the expected 0.13 mM. These experiments would seem to rule out the possibility that hydroxyurea is

being degraded to a less inhibitory form by enzymes in the extracts of the hydroxyurea-resistant cell lines.

To determine if the cross-reacting compounds like guanazole are being converted to hydroxyurea by an intracellular enzyme system, hydroxyurea and guanazole were incubated with enzyme preparation under standard assay conditions. The reactions were terminated and the amount of hydroxyurea present was assayed by the colorimetric method. Table 16, Experiment 5, shows that as before, hydroxyurea incubated under standard assay conditions still gives the expected colorimetric reaction indicating that no degradation has occurred. In contrast, the amount of guanazole causing a similar inhibition of CDP reduction gives no colour reaction with the colorimetric assay and therefore contained no detectable hydroxyurea (Table 16, Experiment 6). This rules out the possibility that guanazole owes its inhibition of ribonucleotide reductase to a conversion to hydroxyurea.

4. Purification of ribonucleotide reductase: The hydroxyurea-resistant cell lines may contain structural alterations in the ribonucleotide reductase enzyme itself, rendering it less sensitive to inhibition by hydroxyurea. Alternatively, but less likely, a large molecular weight component which interacts with ribonucleotide reduction could be altered in structure or quantity. The only way to examine whether a structural alteration is indeed present in the ribonucleotide reductase molecule is to purify the enzyme to homogeneity.

Purification of eucaryotic ribonucleotide reductase has proven to be a difficult task. The enzyme activity can be demonstrated in a wide variety of cell types, although in some cases with much difficulty. Table 17 lists the purification attempts reported to date. The table indicates enzyme sources, purification techniques and final specific activity achieved by each investigator. Considering that ribonucleotide reductase was first reported in mammalian cells 17 years ago (Moore & Hurlbert 1960) the degree of purification obtained is not impressive. Three major problems have been encountered when attempting to purify ribonucleotide reductase from mammalian sources:

i) The enzyme is present in only very low amounts and then only during periods of rapid DNA synthesis. For example, with our CHO cells, 10 times more CDP reductase activity exists in S phase cells than in G1 phase cells (Section E). Therefore cell sources must be used which have optimal proportions of the cell population synthesizing DNA. Because of the low enzyme activity, highly labelled ribonucleoside diphosphate substrates must be used. Two important limitations on our purification attempts were the cost of cultivating large quantities of exponentially growing CHO cells and the cost of the labelled ribonucleoside diphosphate substrates. In passing it can be noted that during typical purification runs in E.coli, which contains a more active form of ribonucleotide reductase, it took 1500 litre batches of cells to purify the enzyme (Brown et al 1969).

Table 17. Purification attempts with eucaryotic ribonucleotide reductase.

Reference	Source	Starting Spec. Act.*	Techniques	Final Spec. Act.
Moore (1967)	rat hepatoma	0.65	100,000g pH 5.2 DEAE cellulose	24.0
King & Lancker (1969)	rat liver	0.60	no purification	0.6
Elford et al (1970)	rat hepatoma	0.76	no purification	0.76
Lowdon & Vitols (1973)	Saccharomyces	0.00†	streptomycin sulfate ammonium sulfate DEAE cellulose	12.0
Hopper (1972)	rabbit bone marrow	0.30	ammonium sulfate 2 X Sephadex G200 hydroxylapatite	17.0
Larsson (1973a)	rat liver	0.10	100,000g pH 5.2 ammonium sulfate Cγ-alumina DEAE cellulose	30.0
Cory (1975)	Ehrlich Ascites cells	0.15	100,000g pH 5.2 protamine sulfate ammonium sulfate hydroxylapatite	11.6
Meuth & Green (1974)	mouse 3T6	0.40	Mutant cell line 3T6 CA/dA14.4	2.7

*Specific activity of CDP reductase in nmoles per hour per mg protein.

†No enzyme activity detectable in crude extracts.

ii) At low protein concentrations the activity of ribonucleotide reductase declines logarithmically with decreasing protein concentration. In our hands this decrease in enzyme activity could not be recovered by concentrating the diluted extracts. Thus any purification steps which resulted in significant dilution of the cell extract resulted in irreversible loss of ribonucleotide reductase activity. Thus it was necessary to start with extracts containing high concentrations of protein (>10 mg/ml) and to minimize extract dilution during purification. Addition of protein carriers, such as bovine serum albumin, did not protect ribonucleotide reductase from inactivation. Neither did the addition of a variety of nucleotide substrates and effectors or compounds such as protamine or spermidine have any effect on enzyme stability during purification.

iii) The enzyme activity from CHO cells often behaved as if the enzyme protein was complexed to a variety of other proteins. For example, CDP reductase activity would precipitate over a broad range of ammonium sulfate concentrations and during DEAE cellulose chromatography trials, the activity once again eluted over a broad range of ionic strengths. Both results suggest that the enzyme may be aggregated with other proteins. Elford (1974) has shown that in rat hepatoma cells, greater than 90% of the ribonucleotide reductase activity is bound to small membrane fragments. Such results with the CHO cell system frustrated purification at-

tempts with procedures which have been marginally successful for other systems (Table 17).

In an attempt to achieve some degree of purification, we turned to affinity chromatography. This separation technique depends upon the specific affinity of an enzyme for certain ligands rather than gross physical properties of the individual protein.

The following purification steps are ones which we have found useful in partially purifying the CHO cell enzyme.

a. Centrifugation at 100,000g for 60 min.

This is a standard procedure employed by most workers attempting to purify the enzyme (Moore 1967). It results in a slight increase in CDP reductase activity over lower speed centrifugations while ridding the extract of membranous or large protein aggregate material. It also has the advantage that after centrifugation, a dense layer of lipid is formed on the top of the extract. This material must be discarded as it tends to clog the affinity agarose columns employed subsequently.

b. Precipitation at pH 5.0 to 5.2 by acetic acid.

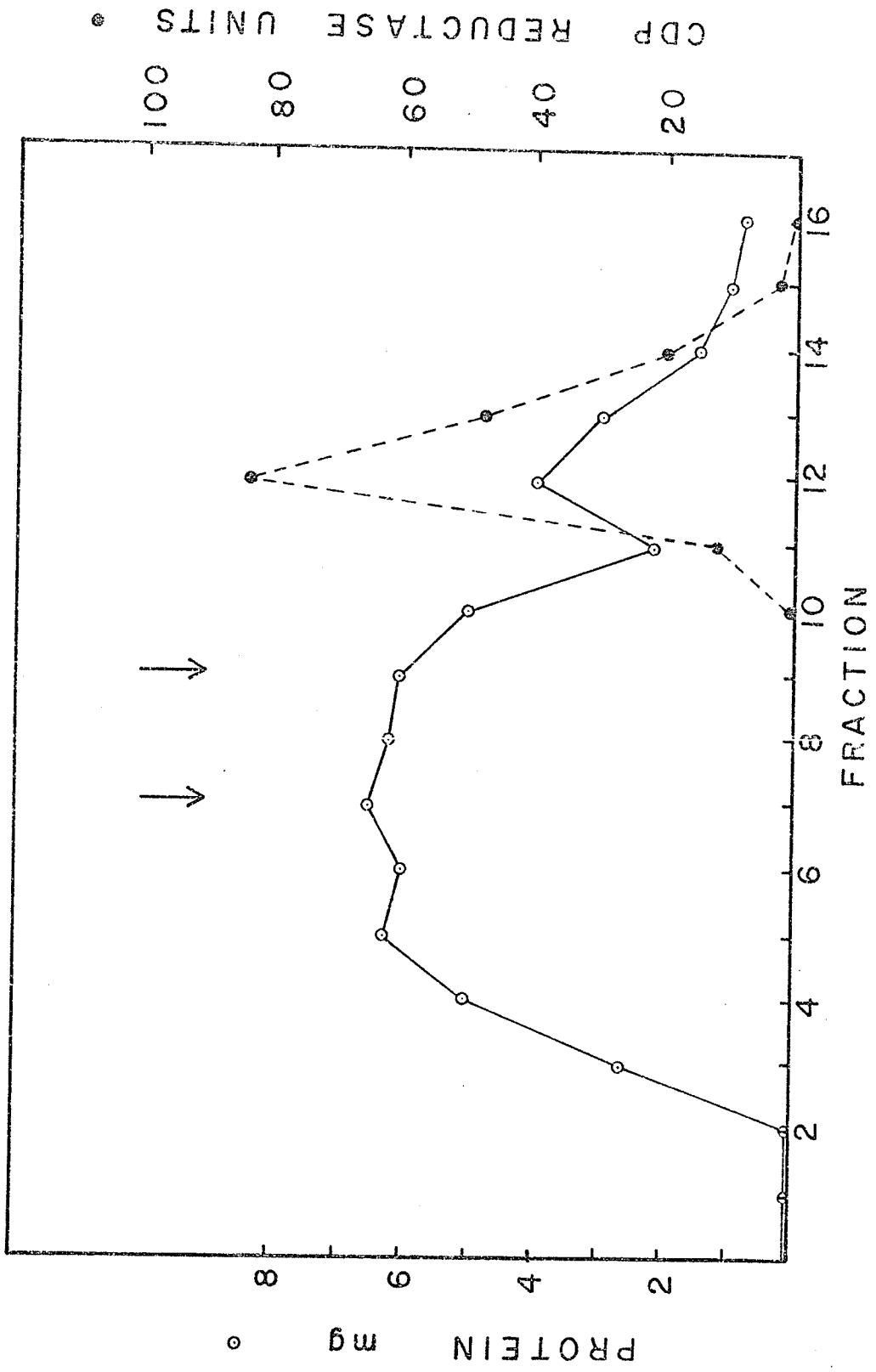
Once again this is a routinely employed step which was originally developed for the E. coli system (Brown et al 1969) to separate ribonucleotide reductase from thioredoxin which remains in the supernatant; the technique has proven useful in mammalian systems as well (Moore 1967). If suffi-

cient volume of CHO cell extract is available, it is an easily performed step which results in a 2- to 3-fold increase in specific activity. Briefly, the pH of the extract is lowered to pH 5.0 with acetic acid, the extract is immediately centrifuged and the pellet containing the ribonucleotide reductase activity is redissolved in pH 8 HEPES buffer. We routinely reduce the volume of the extract by half which is more convenient when working with the affinity columns.

c. ATP-agarose affinity chromatography.

Recently a large number of nucleotides immobilized on agarose polysaccharide supports have become commercially available. We tested a number of these substituted agaroses (obtained from P-L Biochemicals Inc.) and found that both ATP-agarose and GTP-agarose would retain ribonucleotide reductase activity when crude CHO cell extracts were passed through small columns containing 1 to 5 ml of the solid nucleotide containing support. Figure 34 shows the results of passing 4 ml of CHO cell extract through a 1 X 2cm high column of ATP-agarose. The extract contained 14 mg protein per ml and a total of 250 units of ribonucleotide reductase activity. After passage of the 4 ml of extract, the column was washed with 1 ml of HEPES buffer followed by the same buffer containing 10 mM ATP as described in Materials and Methods. As can be seen no ribonucleotide reductase eluted with the majority of the extract protein. However, upon the addition of 10 mM ATP approximately 66% of the added CDP reductase

Figure 34: Affinity chromatography of ribonucleotide reductase on ATP-Sepharose. Four ml of WT CHO cell extract containing 14 mg protein per ml and 250 units of CDP reductase was passed through a 1 X 2 cm high column of ATP-agarose equilibrated with 50 mM HEPES pH 7.2 containing 1 mM DTT. The column was then washed with 1 ml of the same HEPES buffer (first arrow) before addition of the buffer containing 10 mM ATP (second arrow). Eluant fractions (0.5 ml) were collected and assayed for CDP reductase activity (●) or protein content (⊙).


CDP REDUCTASE UNITS
100
80
60
40
20
PROTEIN mg
8
6
4
2
FRACTION
2
4
6
8
10
12
14
16

activity added was eluted in the first 4 ATP containing fractions. When pooled, these fractions contained 9.6 mg protein, having a final specific activity of approximately 17 units/mg. The ATP-agarose affinity chromatography reproducibly resulted in a 4-to 6-fold purification. This was achieved rapidly and with a minimum of materials.

Attempts were made to improve the purification achieved with the ATP-agarose affinity technique. The major problem was that if increased time elapsed before elution with ATP, the yield of ribonucleotide reductase activity rapidly fell to zero. This of course prevented extensive removal of extraneous protein not firmly bound to the column. Even with the 1 ml wash utilized, the recovery of ribonucleotide reductase was occassionally as low as 30%. Recovery could be as high as 80%, however, with the typical recovery being approximately 60%. It is not known whether the decrease in enzyme yield upon washing is because of a tighter irreversible binding of the enzyme to the column, or whether as the protein concentration falls the immobilized enzyme is inactivated. Undoubtedly other explanations are possible. Various elution conditions were tried in an attempt to elute the enzyme after more extensive washing. These included varying the pH from 5 to 9, increasing ionic strength to 0.1 M HEPES or 1 M NaCl, and eluting with a variety of ribonucleotide substrates and effectors, both alone and in combination. No ribonucleotide reductase activity was recovered from the column under any of these

conditions.

d. Blue dextran-Sepharose affinity chromatography:

It has been reported that blue dextran immobilized on Sepharose would bind a variety of proteins whose ligand binding sites contain the supersecondary structure called the dinucleotide fold (Thompson et al 1975). Blue dextran-Sepharose will bind a variety of proteins containing NAD or ATP binding sites. However, some proteins with ATP binding sites will not bind to blue dextran and presumably do not contain the dinucleotide fold structure (Thompson et al 1975). Blue dextran-Sepharose was prepared by coupling blue dextran dye to cyanogen bromide activated Sepharose (Pharmacia). When CHO cell extract containing ribonucleotide reductase was passed through the blue dextran-Sepharose, the enzyme activity passed freely through the column while a small but significant proportion of the protein extract was retained or retarded. Thus even though the CHO ribonucleotide reductase was capable of binding strongly to immobilized ATP it showed little if any affinity for blue dextran, suggesting the lack of a dinucleotide fold structure. The blue dextran-Sepharose was used to adsorb out those proteins with affinity for the dye. The majority of these proteins would probably also have bound to ATP or GTP-agarose and would have eluted from the column with ribonucleotide reductase activity, so their elimination improved the overall purification. There has been some variability with blue dextran-Sepharose chromatography results. Several batches of CBBr-activated Sepharose have yielded preparations of blue dextran-Sepharose which

have retarded or inactivated some of the ribonucleotide reductase activity added. Although this problem has not been resolved, additional polysaccharide support matrices are now becoming available (eg P-L Biochemicals Inc.) and may prove more reliable than the Pharmacia product.

e. GTP-agarose affinity chromatography:

When passed through columns of GTP immobilized on agarose, ribonucleotide reductase was retained in an identical manner as with ATP-agarose. In fact the enzyme could be eluted with either ATP or GTP. The GTP-agarose chromatography was used several times to achieve high purifications of ribonucleotide reductase after ATP-agarose and blue dextran-Sepharose chromatography. However, the protein preparations by this stage were very dilute and the enzyme was extremely sensitive to handling. At times recovery from the GTP-agarose columns approached 80% but more often was less than 20% and hence resulted in no net purification. Because of these difficulties the GTP-agarose step was not routinely used for ribonucleotide reductase purification in the subsequent studies. However, the technique shows great promise providing that higher specific activities and more concentrated samples of the enzyme are obtained for starting material.

Table 18 shows the results of a purification attempt using the procedures described above. Passage of the extract through Sephadex G25 was necessary after ATP-agarose chromatography in order to remove ATP which would reduce

Table 18. Purification of ribonucleotide reductase from WT CHO cells.

Step	Volume	Protein	Activity	Recovery	Specific Act.
crude	14 ml	274 mg	618 units*	100%	2.3
100,000g	12	180	545	88	3.0
pH 5.2	6	72	489	79	6.8
ATP-agarose	3	6.9	153	25	22.2
G25	5.5	6.9	102	17	14.8
Blue dextran-Sepharose	5.5	3.5	69	9	19.7
GTP-agarose	2.0	0.8	81	13	101.3

* 1 unit = 1 nmole deoxycytidine formed per hour

protein binding in the subsequent blue dextran-Sepharose and GTP-agarose steps. The final specific activity of 101 units/mg was greater than those reported to date by other workers. However, this preparation was far from pure. Polyacrylamide gels of the final sample showed some 10 to 15 major protein bands and a number of minor bands.

The purification scheme presented in Table 18 has varied greatly with each purification attempt. This is largely because of the low concentrations of enzyme in the mammalian starting material and the resulting loss of activity upon purification and dilution. One of the hydroxyurea-resistant CHO cell mutants ($GAT^{-}HU^{R}$-12 SC8) which was recently isolated has now been shown to contain far greater levels of ribonucleotide reductase than the WT CHO cells (see Section E). This cell line may prove to be invaluable in future attempts to achieve purification of mammalian ribonucleotide reductase.

5. Characterization of ribonucleotide reductase from WT CHO cells: Most likely because of their rapid growth rate and hence heavy demand for deoxyribonucleotides, CHO cells contain higher levels of ribonucleotide reductase than most eucaryotic sources reported to date (see Table 17). Routinely, crude CHO cell extracts contain 1.0 to 2.5 CDP reductase units per mg of protein.

In order to characterize the reduction of ribonucleo-

tides by the CHO cell system, the following purification procedure, which rapidly and reproducibly yielded 10- to 15-fold purified preparations, was used. Briefly, CHO cells exponentially growing in suspension culture were harvested, HEPES buffer added, the cell suspension was homogenized, centrifuged at 100,000g,the upper lipid layer removed, the enzyme precipitated at pH 5 with acetic acid, redissolved in HEPES buffer, chromatographed on ATP-agarose and finally passed through Sephadex G25 to remove low molecular weight compounds. The standard purification is described in detail in the Materials and Methods section.

CDP Reduction: The reduction of CDP to dCDP was assayed as described in Materials and Methods. Figure 35 shows the response of enzyme activity to incubation time and protein concentration. The reaction proceeded linearly for at least 60 min, the standard incubation period (Fig 35A). CDP reductase activity response to protein concentration was not linear. Figure 35B shows that at protein concentrations below 300 µg/ml CDP reductase activity fell off rapidly. The inset of Figure 35B suggests that if the squares of the lower protein concentrations are plotted against CDP reductase activity, the plot appears to be linear. Hopper (1972) reported similar results with CDP reductase from rabbit bone marrow and suggested the enzyme consists of two components which dissociate at low protein concentrations. For the following work with CHO cell CDP reductase, the routine kinetic studies were done in the linear range of Figure 35B.

Figure 35A: Response of CDP reduction to incubation time. A partially purified WT CHO cell extract (500 µg protein) was added to standard CDP reductase assay mixtures and after incubation at 37°C for for specific times, the reactions were terminated by boiling and the amount of CdR formed was measured as described in Materials & Methods.

35B: Response of CDP reduction to increasing protein concentration. Varying amounts of partially purified WT CHO cell extract were added to standard reaction mixtures and incubated at 37°C for 60 min, at which time the reactions were terminated by boiling and the amount of CdR formed was measured.

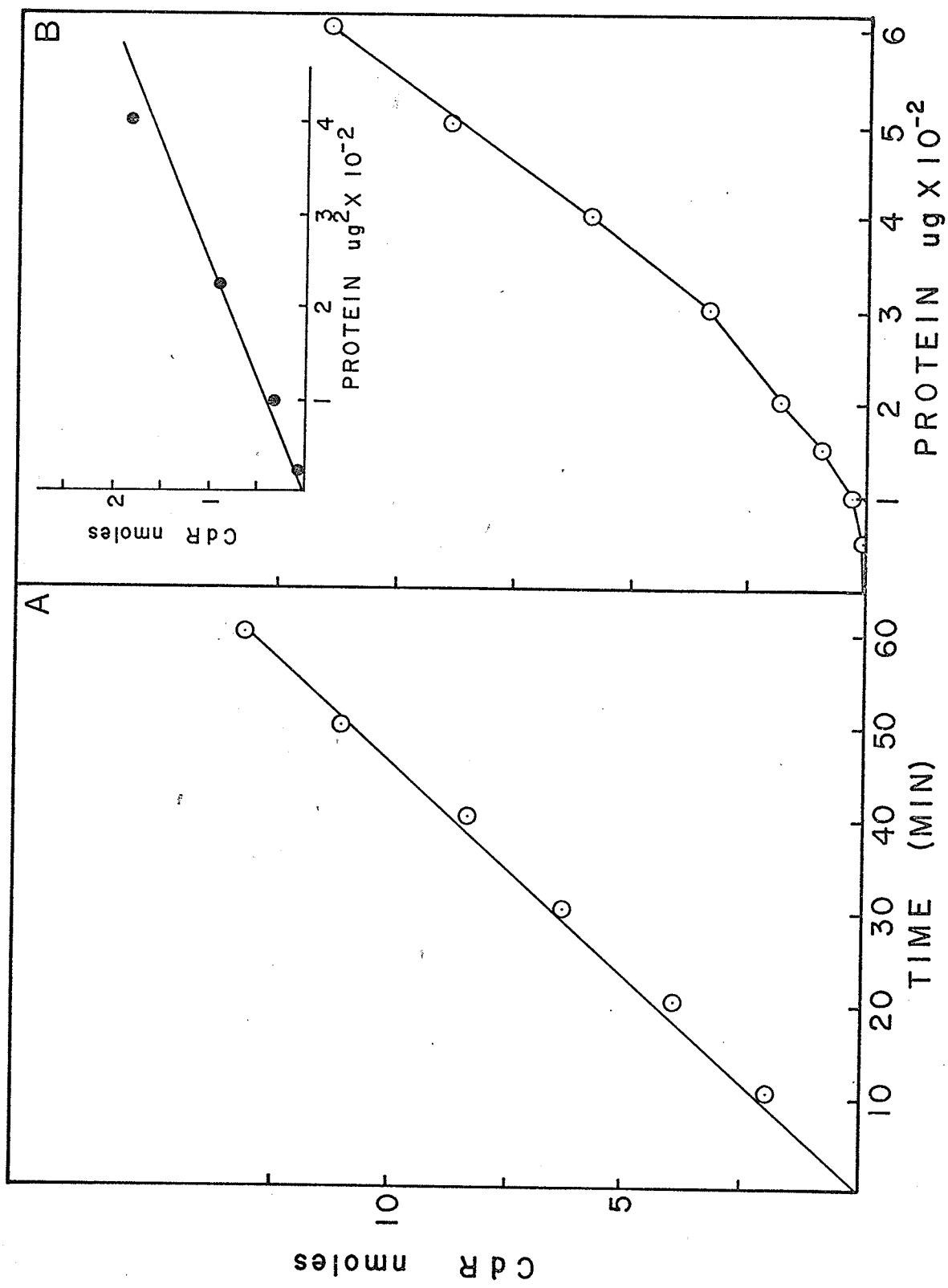

A
B
cdR nmoles
TIME (MIN)
PROTEIN ug X 10⁻²
PROTEIN ug X 10⁻²
cdR nmoles

Figure 36A shows the temperature optimum of CDP reduction to be 37^{o}C, which corresponds to the cellular growth temperature optimum of the CHO cell line. As can be seen in Figure 36B, the reaction showed a broad pH optimum ranging from about pH 6.8 to 7.4, both with PIPES and HEPES buffers. HEPES at pH 7.2 was chosen as the standard reaction buffer.

Figure 37A shows that the reduction of CDP is markedly stimulated by the reducing agent dithiothreitol (DTT). Dithioerythritol (DTE) was almost as effective as DTT, but other compounds such as reduced lipoic acid, NADH or NADPH were far less effective. In the absence of DTT there remained some enzyme activity. This may be because of the incomplete removal of DTT from the buffering system used for stabilization during purification, the presence of high molecular weight hydrogen donors (eg reduced thioredoxin) present in the extract or a self-reduction of the enzyme as occurs in E. coli (thelander 1974). Since concentrations of DTT above 6 mM did not stimulate CDP reduction further, this concentration of reducing agent was chosen for the standard assay.

Similarly, CDP reduction was marked stimulated by magnesium ion (Figure 37B). The optimal concentration of Mg^{+2} as $MgCl_2$ was found to be 10 mM. Once again there was some CDP reductase activity in the absence of added $MgCl_2$ and may be due to residual low amounts in the cell extract,

Figure 36A: Response of CDP reduction to incubation temperature. A partially purified WT CHO cell extract (500 µg protein) was incubated with the standard CDP reductase assay mixture for 1 hour at varying temperatures, and the amount of CdR formed was measured.

36B: Response of CDP reduction to varying pH of reaction mixture. A partially purified CHO cell extract (500 µg protein) was incubated under standard CDP reductase assay conditions except that 50 mM HEPES buffer (⊙) or 50 mM PIPES buffer (●) was used to vary the pH of the reaction mixture.

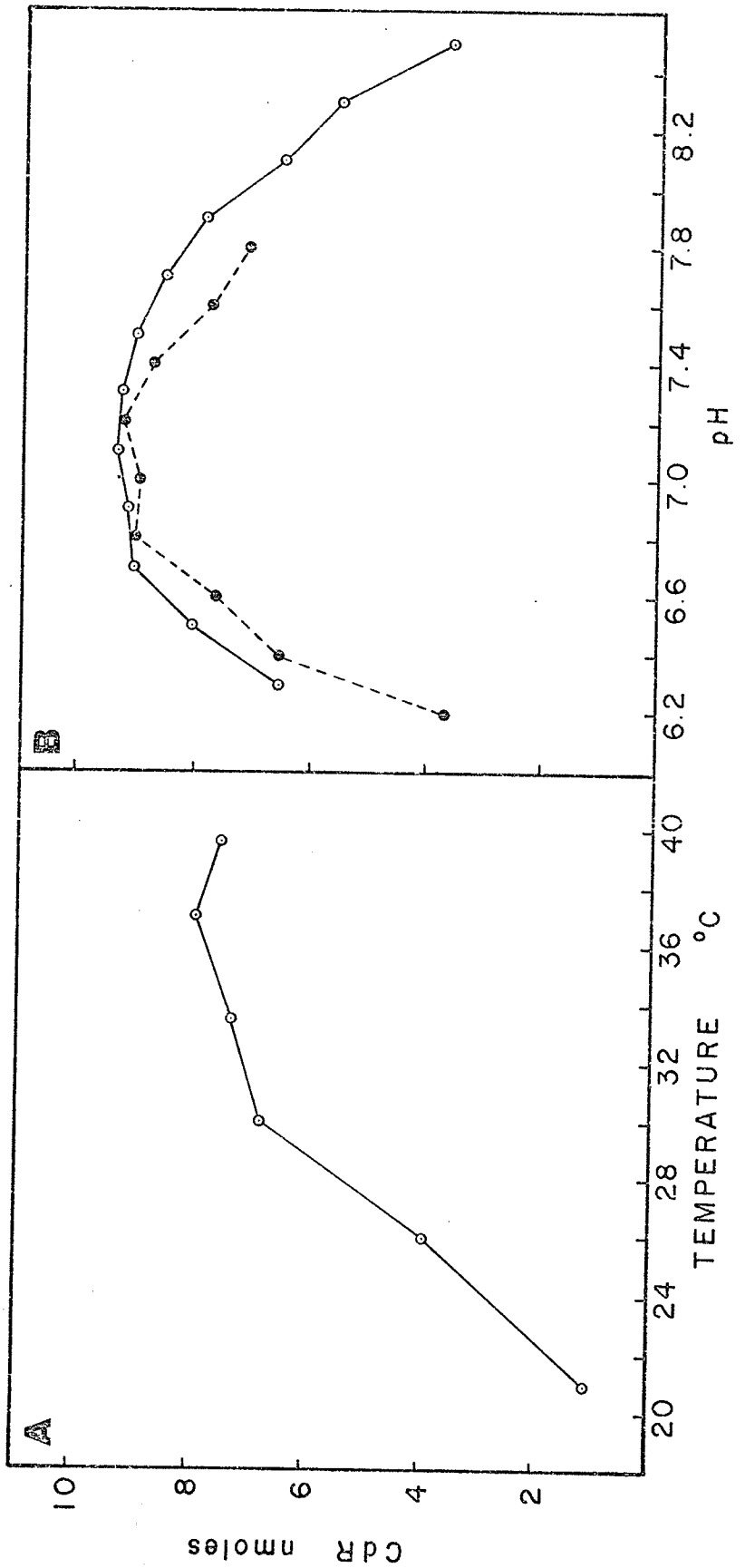
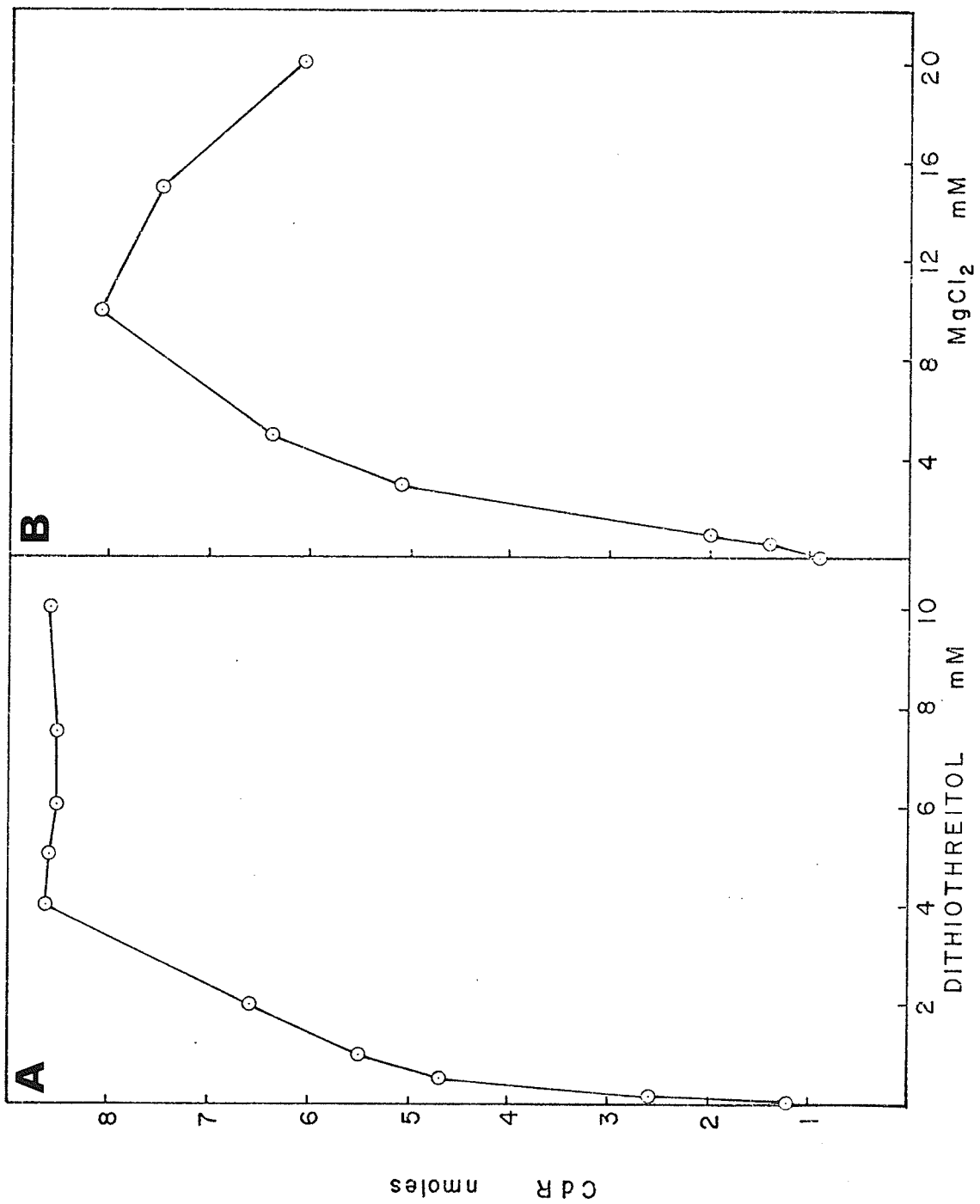

A
B
CdR nmoles
2
4
6
8
10
20
24
28
32
36
40
TEMPERATURE °C
6.2
6.6
7.0
7.4
7.8
8.2
pH

Figure 37A: Response of CDP reduction to varying concentration of dithiothreitol. A partially purified CHO cell extract (500 µg protein) was incubated under standard CDP reductase assay conditions except the concentration of DTT was varied.

37B: Response of CDP reduction to varying concentrations of $MgCl_2$. A partially purified CHO cell extract (500 µg protein) was incubated under standard CDP reductase assay conditions except that the concentration of $MgCl_2$ was varied.


A
B
CdR nmoles
1
2
3
4
5
6
7
8
2
4
6
8
10
DITHIOTHREITOL mM
4
8
12
16
20
$MgCl_2$ mM

contaminants in the reaction components, or the requirement for Mg^{+2} may not be absolute.

As with other mammalian systems (Moore & Hurlbert 1966), Figure 38 confirms that ATP is required for maximum CDP reduction, although about 4% activity is found even in the absence of ATP. This is very similar to the purified enzyme from E.coli, where ATP is known to stimulate allosterically the low amount of CDP reductase activity (Larsson & Reichard 1966). Figure 38 indicates that for the CHO cell CDP reductase, 6 mM ATP results in optimal CDP reduction, higher concentrations resulted in decreased amounts of CDP reduced.

The dependence of the reaction rate upon CDP concentration is shown in Figure 39. The Lineweaver-Burk double reciprocal plot of initial reaction rates was linear and yielded an apparent Km value of 0.13 mM for CDP.

The response of CDP reduction to the presence of hydroxyurea under the optimized assay conditions was investigated. Figure 40 shows the inhibition of CDP reductase by hydroxyurea over a range of CDP concentrations. Hydroxyurea significantly inhibited the reaction at the drug concentrations tested. The pattern of inhibition appeared uncompetitive at lower concentrations of hydroxyurea, but at the highest concentration of drug tested, the inhibition pattern appeared mixed. A replot of the intercepts, shown

Figure 38: Response of CDP reduction to varying concentrations of ATP. A partially purified CHO cell extract (500 µg protein) was incubated under standard CDP reductase assay conditions except that the concentration of ATP was varied.

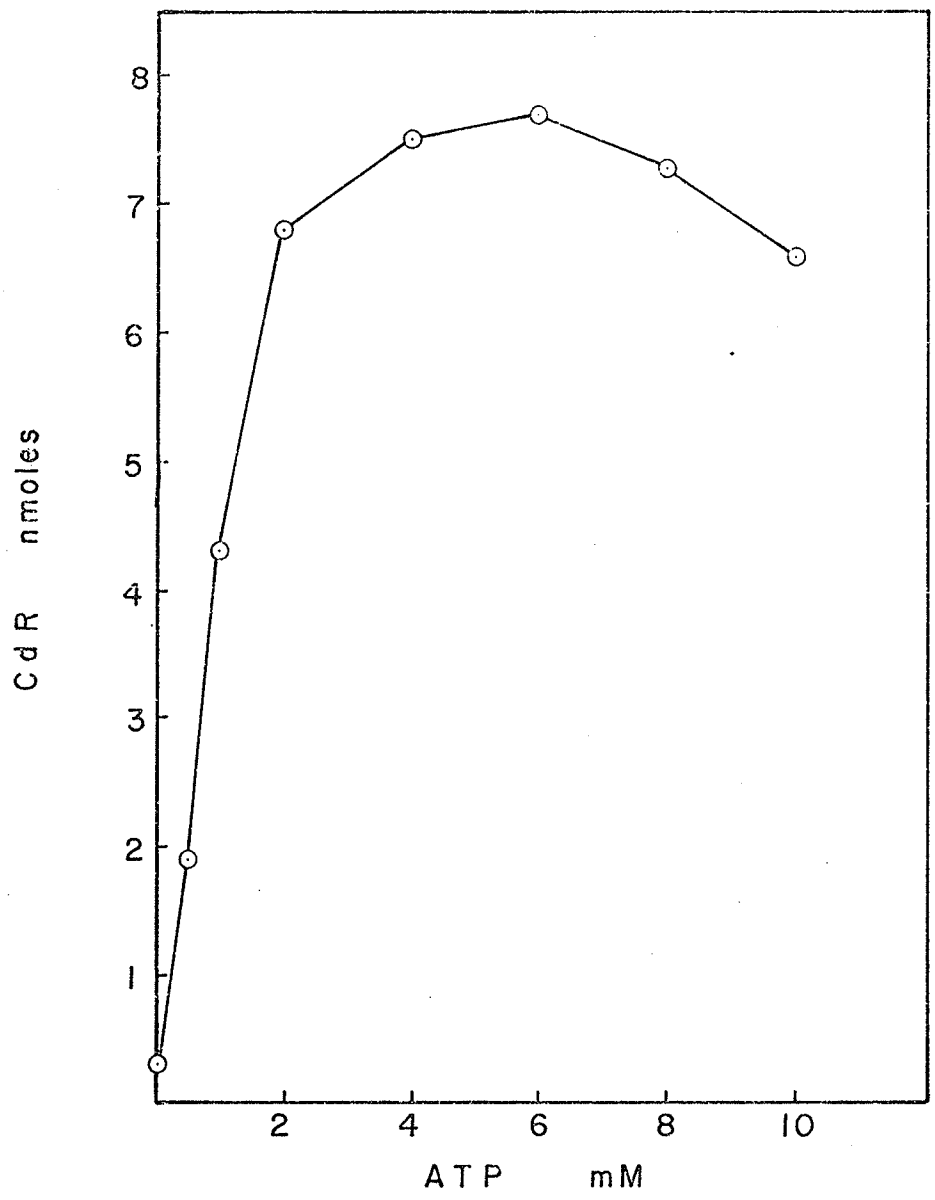

8
7
6
5
4
3
2
1
CdR nmoles
2
4
6
8
10
ATP mM

Figure 39: Double reciprocal plot of the variation in rate of CDP reduction with CDP concentration. A partially purified CHO cell extract (250 µg protein) was incubated under standard CDP reductase assay conditions except that the concentration of CDP was varied.
Inset: Response of the initial velocity of CDP reduction with varying concentration of CDP.

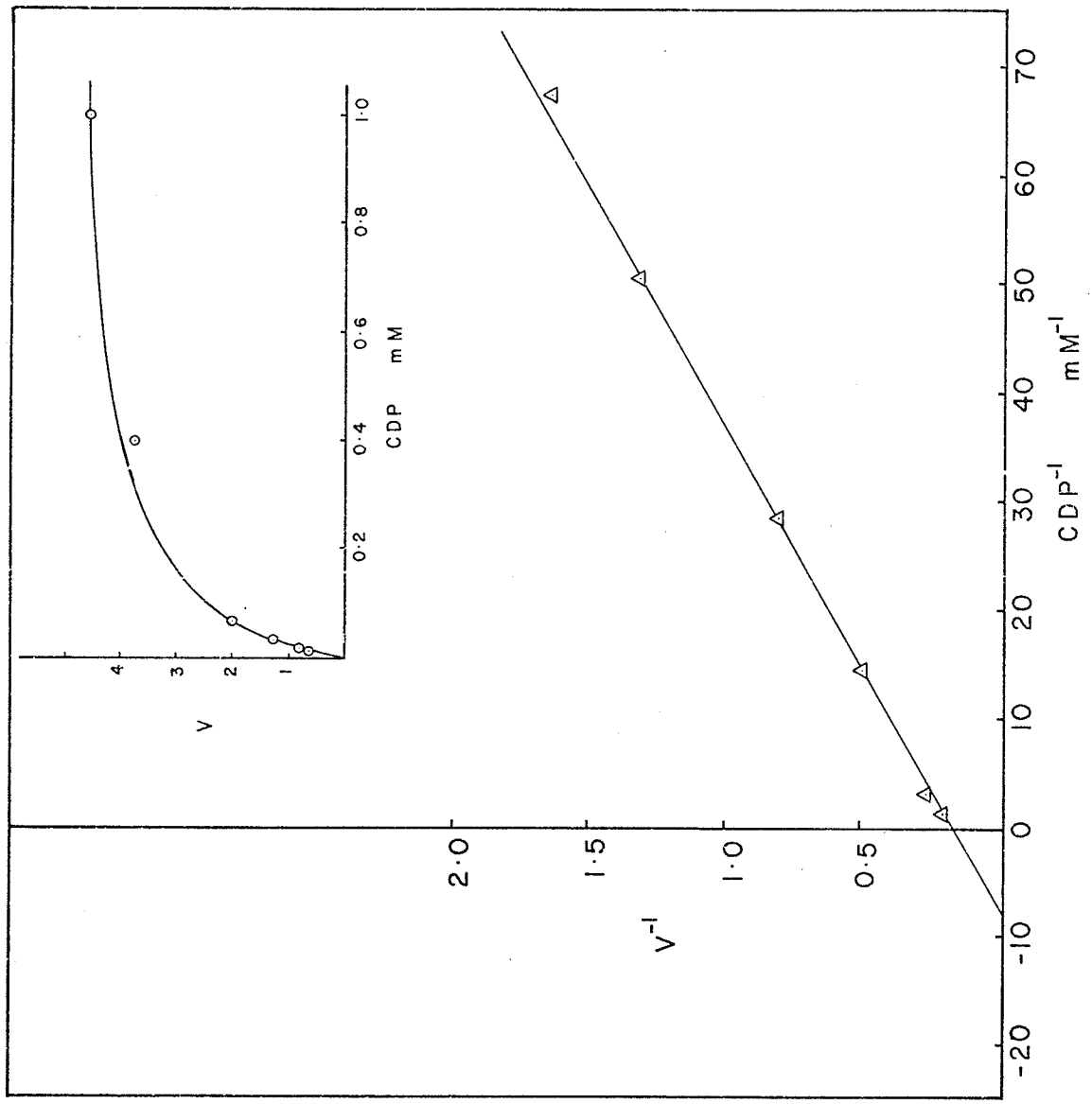
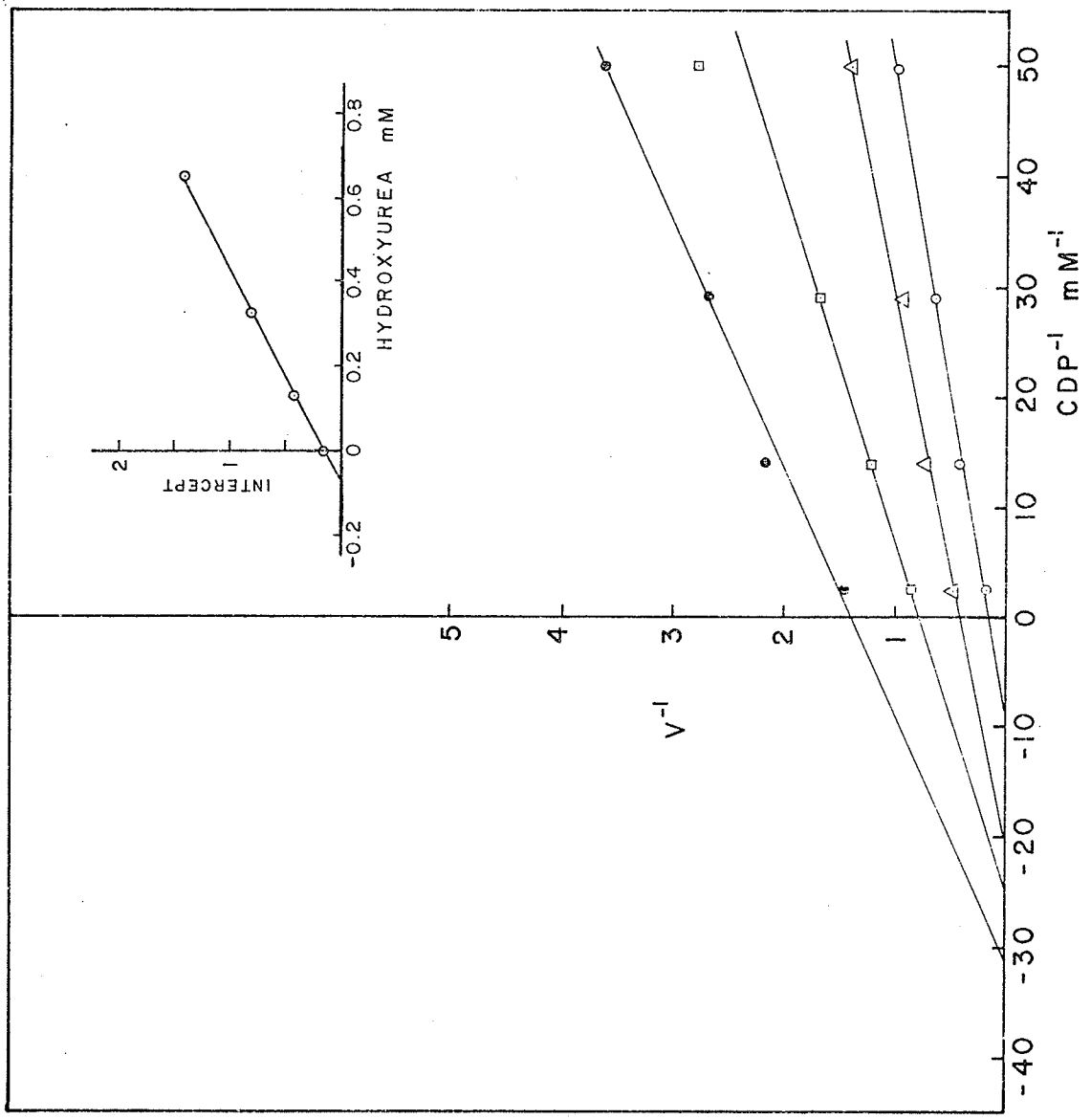

V
CDP mM
0·2
0·4
0·6
0·8
1·0
1
2
3
4
V^{-1}
2·0
1·5
1·0
0·5
CDP^{-1} mM^{-1}
-20
-10
0
10
20
30
40
50
60
70

Figure 40: Double reciprocal plot of the effect of hydroxyurea on CDP reduction with varying CDP concentration. A partially purified WT CHO cell extract (250 µg protein) was incubated with standard CDP reductase assay mixtures containing varying concentrations of CDP and 0 mM hydroxyurea (⊙), 0.17 mM hydroxyurea (△), 0.33 mM hydroxyurea (⊡) or 0.66 mM hydroxyurea (●).
Inset: Replot of the velocity intercepts versus hydroxyurea concentration.


V⁻¹
CDP⁻¹ mM⁻¹
HYDROXYUREA mM
INTERCEPT

in the inset of Figure 40, yielded a Ki value of 0.08 mM for hydroxyurea inhibition of CDP reduction.

Figure 41 shows that, as expected, guanazole inhibits CDP reduction in a manner almost identical to hydroxyurea. As indicated in previous experiments, guanazole is a much less effective inhibitor than hydroxyurea on a molar basis. The inset of Figure 41 shows that the Ki value for guanazole is 0.60 mM, almost 8-fold higher than the Ki value for hydroxyurea. These Ki values correlate well with the CHO cellular response to the two drugs. For example, Figures 5 and 6 show that 0.08 mM hydroxyurea and 0.60 mM guanazole each reduce the RPE of WT CHO by approximately 50%.

ADP Reduction: The reduction of ADP to dADP was assayed as described in Materials and Methods. Figure 42 shows the response of enzyme activity to incubation time and protein concentration. The reduction of ADP proceeded linearly for at least 60 min, the standard incubation period (Fig 42A). Unlike CDP reduction, the amount of ADP reduced responded linearly to protein concentration (Fig 42B). This confirms the finding of Peterson and Moore (1976).

As can be seen from Figure 43A, the pH optimum for ADP reduction was pH 6.8 with either HEPES or PIPES buffer, although the activity was significantly higher with PIPES. PIPES at 50 mM was chosen as the buffer for the stan-

Figure 41: Double reciprocal plot of the effect of guanazole on CDP reduction with varying CDP concentration. A partially purified WT CHO cell extract (250 µg protein) was incubated with standard CDP reductase assay mixtures containing varying concentrations of CDP and 0 mM guanazole (○), 0.6 mM guanazole (△), 1.5 mM guanazole (□), or 3.0 mM guanazole (●). Inset: Replot of the velocity intercepts versus guanazole concentration.

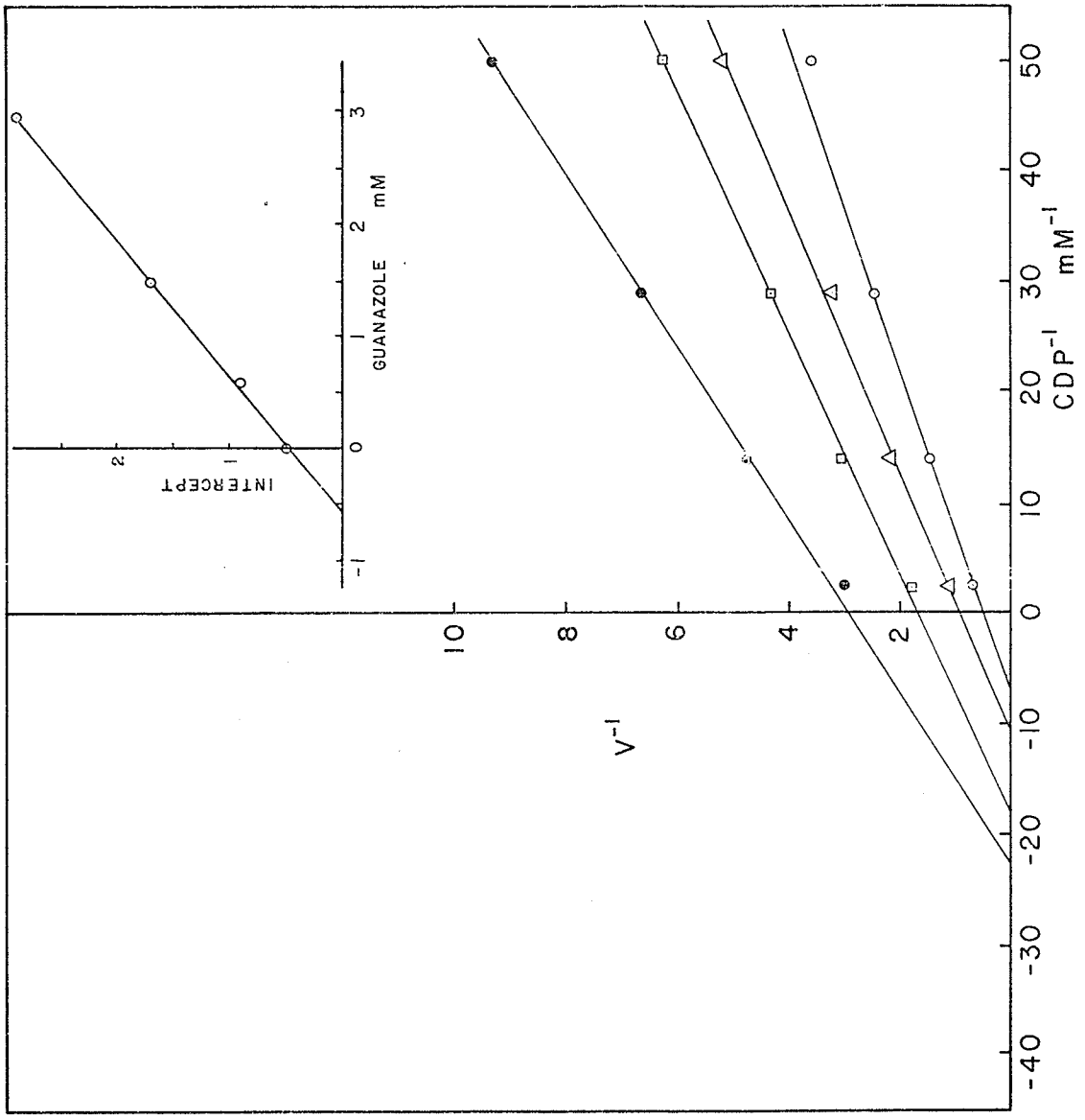

V^{-1}
CDP^{-1} mM^{-1}
GUANAZOLE mM
INTERCEPT

Figure 42A: Response of ADP reduction to incubation time. A partially purified WT CHO cell extract (500 µg protein) was added to standard ADP reductase assay mixtures and after incubation at 37°C for specific times, the reactions were terminated by boiling and the amount of AdR formed was measured as described in Materials & Methods.

42B: Response of ADP reduction to increasing extract protein concentration. Varying amounts of partially purified WT CHO cell extract were added to standard ADP reaction mixtures and incubated at 37°C for 1 hour, at which time the reaction was terminated by boiling and the amount of AdR formed was measured.

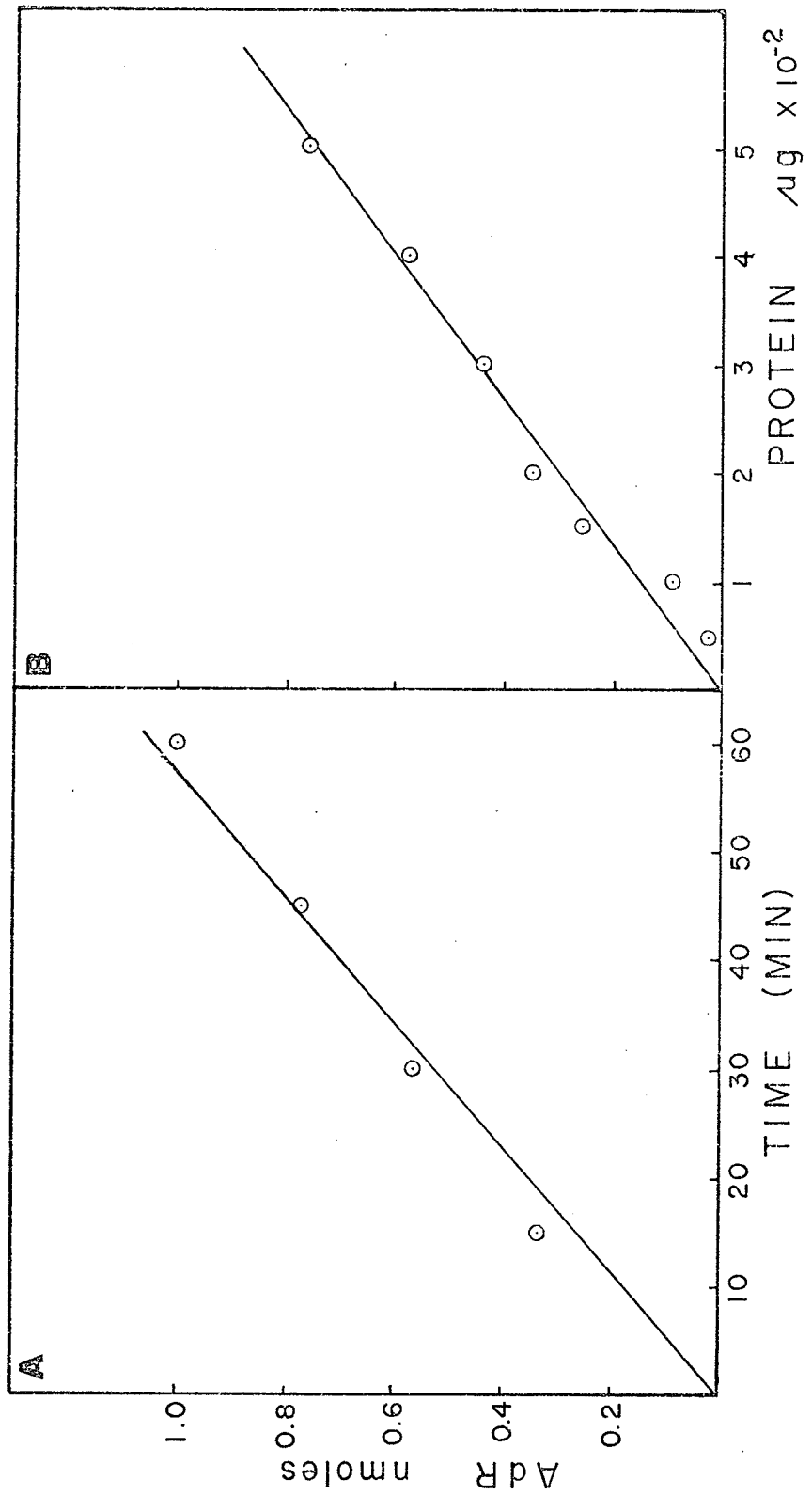
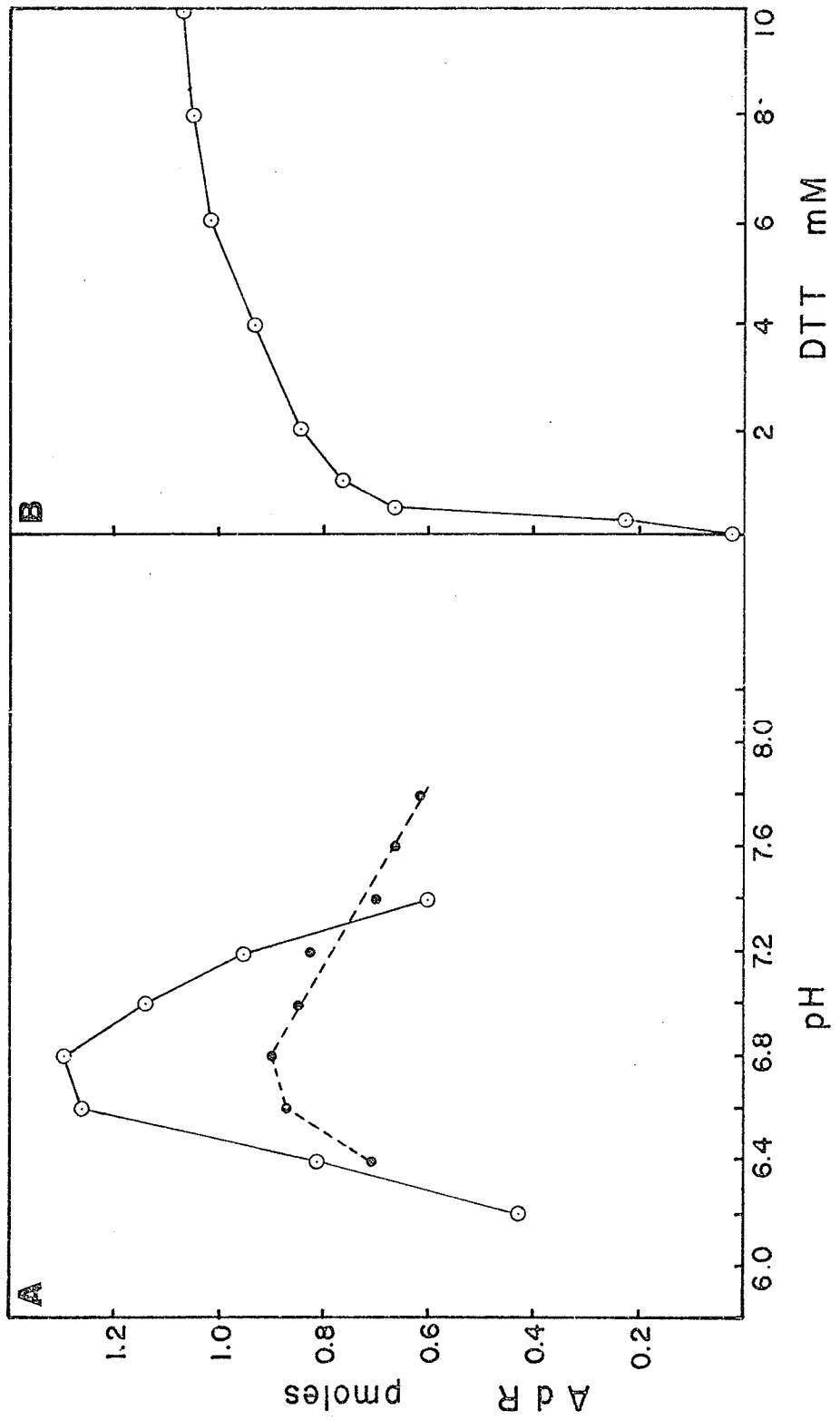

A
B
AdR nmoles
1.0
0.8
0.6
0.4
0.2
10
20
30
40
50
60
TIME (MIN)
1
2
3
4
5
PROTEIN µg x10-2

Figure 43A: Response of ADP reduction to varying pH of the reaction mixture. A partially purified CHO cell extract (500 µg protein) was incubated under standard ADP reductase assay conditions except that 50 mM HEPES buffer (●) or 50 mM PIPES buffer (⊙) was used to vary the pH of the reaction mixture.

43B: Response of ADP reduction to varying concentrations of dithiothreitol. A partially purified CHO cell extract (500 µg protein) was incubated under standard ADP reductase assay conditions except that the concentration of DTT was varied.


A
B
A d R
pmoles
1.2
1.0
0.8
0.6
0.4
0.2
6.0
6.4
6.8
7.2
7.6
8.0
pH
2
4
6
8
10
DTT mM

dard ADP reduction assay, both because of its pKa value of 6.8 and because ADP reduction was higher in this buffer than with either HEPES or phosphate buffer (data not shown).

Figure 43B shows that DTT is an effective hydrogen donor for ADP reduction. As with CDP reduction, there was a small, but significant, amount of ADP reduced in the absence of DTT. The optimal DTT concentration for ADP reduction was approximately 6 mM and this concentration was chosen for the standard assay mixture.

As with other mammalian systems (Moore & Hurlbert 1966) dGTP is a potent activator of ADP reduction. Figure 44 shows that only 0.18 nmoles of ADP is reduced in the absence of dGTP, but that this increases almost 7-fold to 1.2 nmoles at 1 mM dGTP. Half maximal activation of ADP reduction occurs at approximately 0.1 mM dGTP. Concentrations of dGTP above 1 mM resulted in less ADP reduction, and therefore 1 mM dGTP was adopted for the standard assay mixture.

The dependence of the initial velocity of ADP reduction upon substrate concentration is shown in Figure 45. The Lineweaver-Burk double reciprocal plot was linear and yielded an apparent Km value of 0.13 mM for ADP.

Some investigators have reported that Mg^{+2} stimulates ADP reduction (Cory & Mansell 1975). In contrast, concentrations of Mg^{+2} from 0.1 to 10 mM either had no effect or

Figure 44: Response of ADP reduction to varying concentrations of dGTP. A partially purified CHO cell extract (500 µg protein) was incubated under standard ADP reductase assay conditions except that the concentration of dGTP was varied.

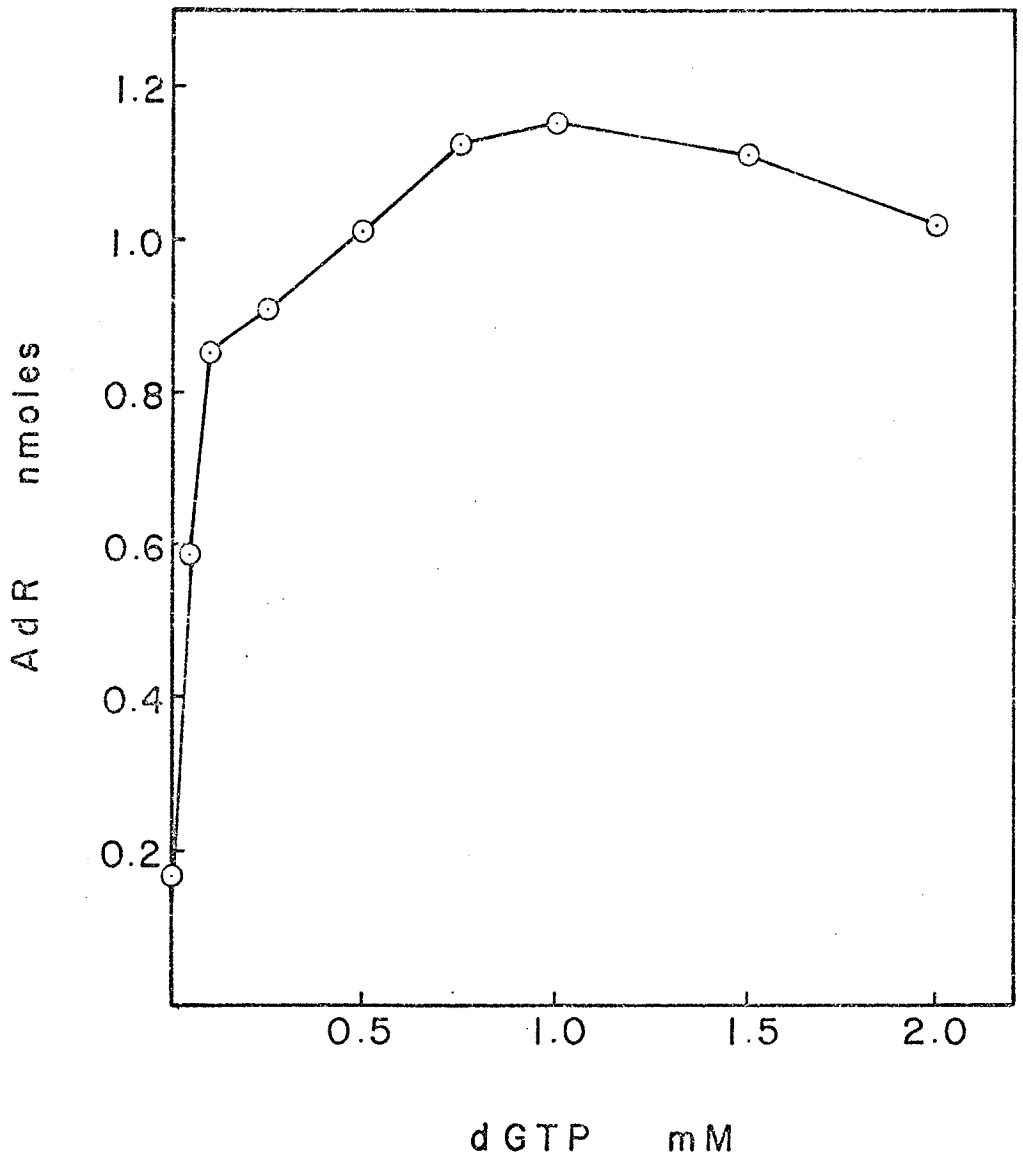
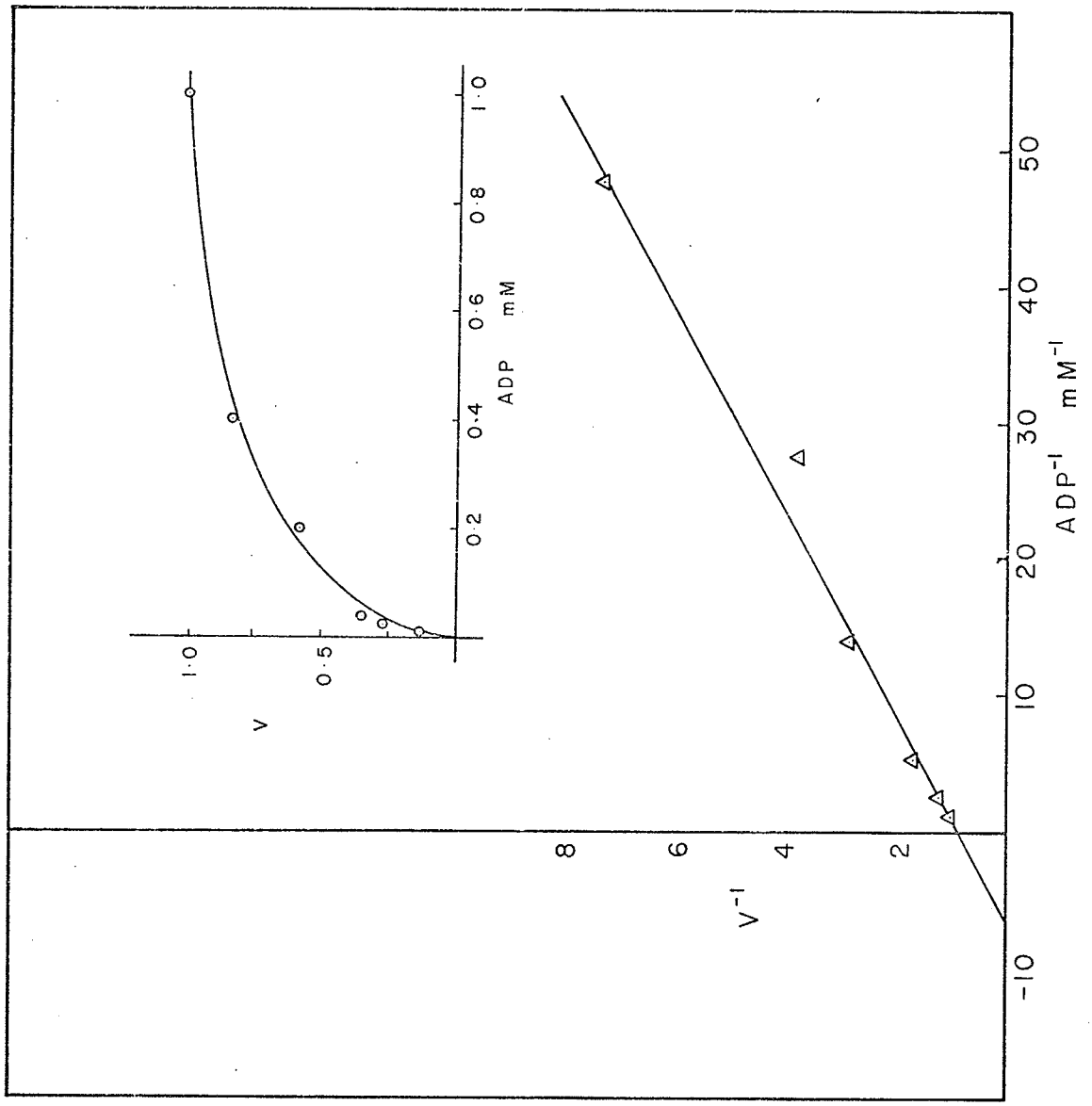

1.2
1.0
0.8
0.6
0.4
0.2
AdR nmoles
0.5
1.0
1.5
2.0
dGTP mM

Figure 45: Double reciprocal plot of the variation in rate of ADP reduction with ADP concentration. A partially purified CHO cell extract (500 µg protein) was incubated under standard ADP reductase assay conditions except that the concentration of ADP was varied.
Inset: Response of the initial velocity of ADP reduction with varying ADP concentration.


v
1·0
0·5
0·2
0·4
0·6
0·8
1·0
ADP mM
V^{-1}
8
6
4
2
-10
10
20
30
40
50
ADP^{-1} mM^{-1}

inhibited the reaction (Table 19) in the CHO cell system. Therefore, $MgCl_2$ was omitted from the standard ADP reductase assay mixture.

The response of ADP reduction to the presence of hydroxyurea under the optimized assay conditions was investigated. Figure 46 shows that hydroxyurea significantly inhibited the reaction at all concentrations of ADP tested. As with hydroxyurea inhibition of CDP reduction, the pattern of inhibition was uncompetitive at low hydroxyurea concentrations, but became clearly a mixed type of inhibition at higher drug levels. The inset of Figure 46 shows a replot of the intercepts versus concentration of hydroxyurea. The replot is linear and yields a Ki value of 0.13 mM hydroxyurea for ADP reduction.

GDP Reduction: The reduction of GDP was assayed as described in Materials and Methods. Figure 47 shows the response of enzyme activity to incubation time and protein concentration. The reaction proceeded linearly for at least 60 min, the standard incubation period (Fig 47A). GDP reductase activity response to protein concentration was not linear. Figure 47B shows that at protein concentrations below 300 µg/ml GDP reductase activity fell off rapidly. The inset of Figure 47B suggests that if the squares of the lower protein concentrations are plotted against GDP reduced the plot is linear. This is similar to the results with CDP reduction (Figure 35B). Routine kinetic studies

Table 19. Effect of $MgCl_2$ on purine ribonucleotide reduction.

$MgCl_2$	nmoles deoxyguanosine	nmoles deoxyadenosine
0.0 mM	1.17	2.40
0.1 mM	1.10	3.38
1.0 mM	1.08	2.35
10 mM	0.98	1.95

Standard reaction conditions (see Materials & Methods) with 0.4 mM ADP or 0.05 mM GDP.

Figure 46: Double reciprocal plot of the effect of hydroxyurea on ADP reduction with varying ADP concentrations. A partially purified WT CHO cell extract (500 µg protein) was incubated with standard ADP reductase assay mixtures containing varying concentrations of ADP and 0 mM hydroxyurea (○), 0.17 mM hydroxyurea (△), 0.33 mM hydroxyurea (⊡) or 0.66 mM hydroxyurea (●). Inset: Replot of the velocity intercepts versus hydroxyurea concentration.

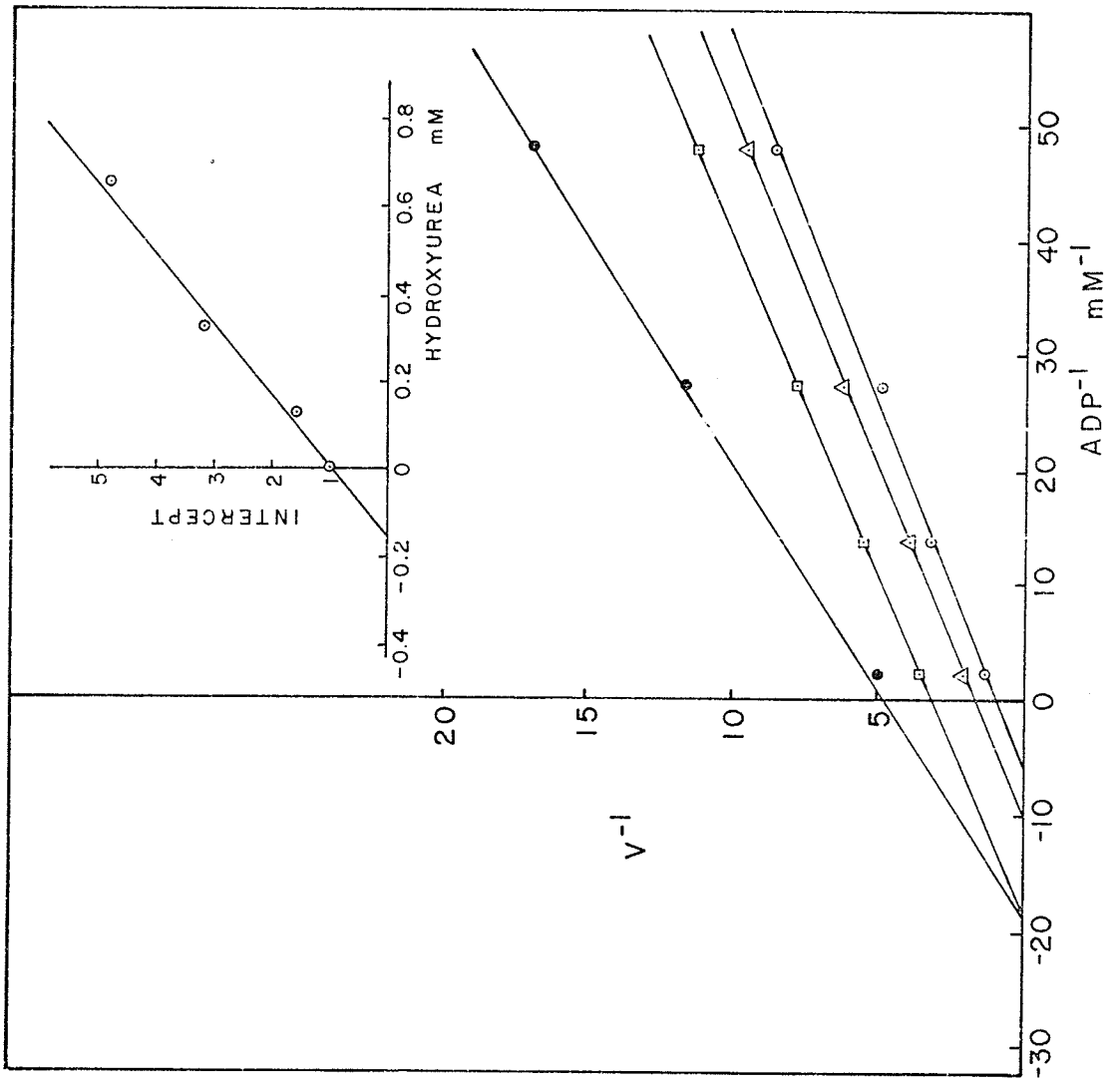
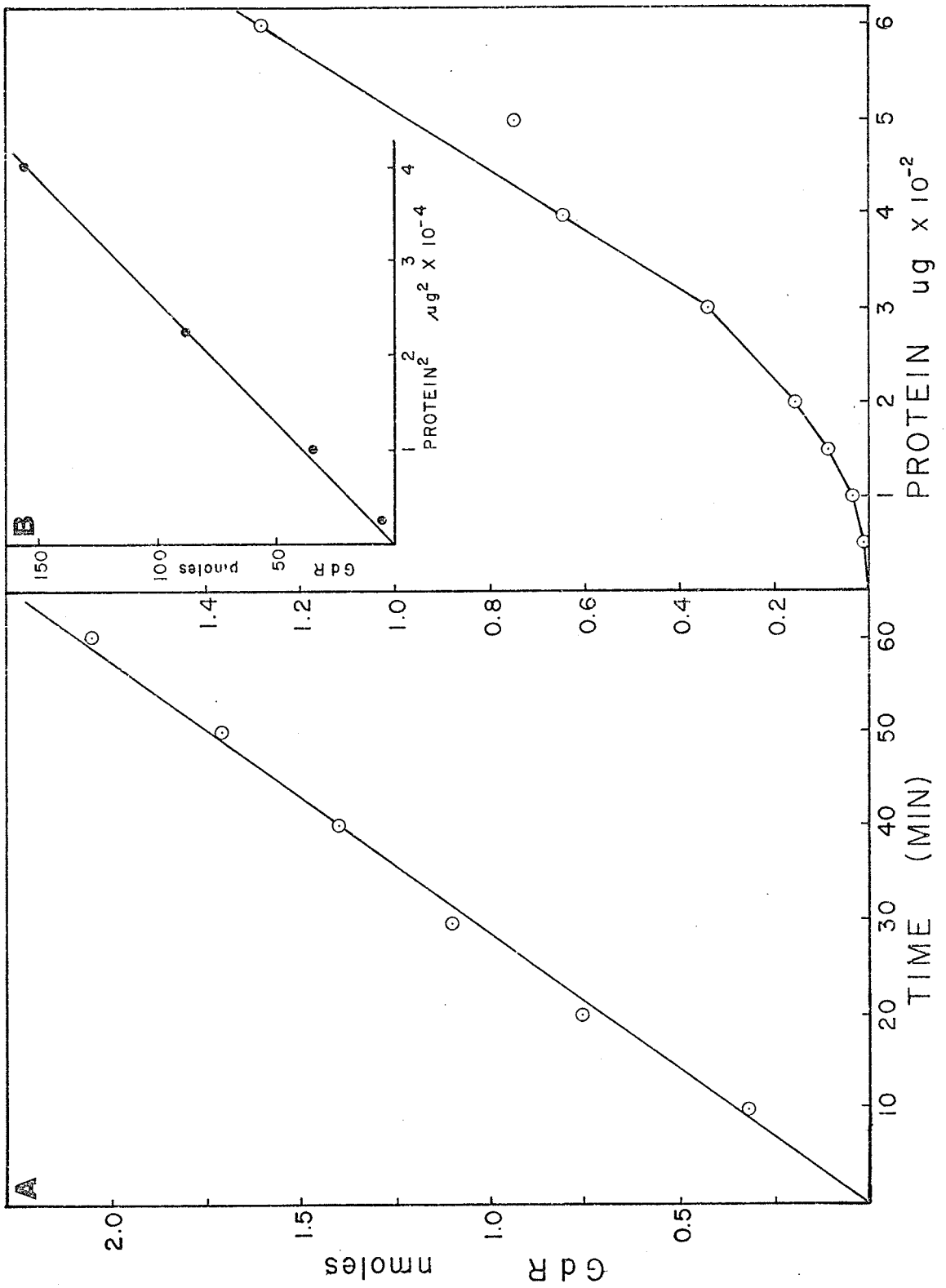

V⁻¹
ADP⁻¹ mM⁻¹
HYDROXYUREA mM
INTERCEPT

Figure 47A: Response of GDP reduction to incubation time. A partially purified WT CHO cell extract (500 µg protein) was added to standard GDP reductase assay mixtures and after incubation at 37°C for specific times, the reactions were terminated by boiling and the amount of GdR formed was measured as described in Materials & Methods.

47B: Response of GDP reduction to increasing protein concentration. Varying amounts of partially purified WT CHO cell extract were added to standard GDP reductase reaction mixtures and incubated at 37°C for 1 hour, at which time the reactions were terminated by boiling and the amount of GdR formed was measured.


A
B
GdR nmoles
TIME (MIN)
PROTEIN ug x 10^-2
PROTEIN^2 ug^2 X 10^-4
GdR pmoles

done with the CHO cell GDP reductase were always performed in the linear portion of Figure 47B.

Figure 48A shows the response of GDP reduction to varying pH with 50 mM PIPES buffer. The pH optimum of the reaction was found to be pH 6.8, a value similar to the pH optimum for ADP reduction (Fig 43A). Figure 48B indicates that DTT is an effective hydrogen donor for GDP reduction. As with both CDP and ADP reduction concentrations of DTT above 6 mM resulted in little increase in the amount of GDP reduced and therefore this concentration was chosen for the standard GDP reductase assay.

As with other mammalian systems and the purified E.coli enzyme (Moore & Hurlbert 1966, Larsson & Reichard 1966b), Figure 49 confirms that dTTP is a potent activator of CHO GDP reduction. In the absence of dTTP only 0.08 nmoles of deoxyguanosine were formed, while in the presence of 2 mM dTTP approximately 1.5 nmoles were formed, and this latter concentration was chosen for the standard GDP reductase assay. Half maximal activation of GDP reduction occurred at approximately 0.8 mM.

Table 19 shows that low concentrations of $MgCl_2$ resulted in a stimulation of GDP reduction, but an inhibitory effect developed as the concentration of Mg^{+2} increased beyond 1 mM. However, the effect of Mg^{+2} on GDP reduction varied with different batches of enzyme; at times even 0.1 mM $MgCl_2$ was

Figure 48A: Response of GDP reduction to varying pH of the reaction mixture. A partially purified CHO cell extract (500 µg protein) was incubated under standard GDP reductase assay conditions except that 50 mM PIPES buffer was used to vary the pH of the reaction mixture.

48B: Response of GDP reduction to varying concentrations of dithiothreitol. A partially purified CHO cell extract (500 µg protein) was incubated under standard GDP reductase assay conditions except that the concentration of DTT was varied.

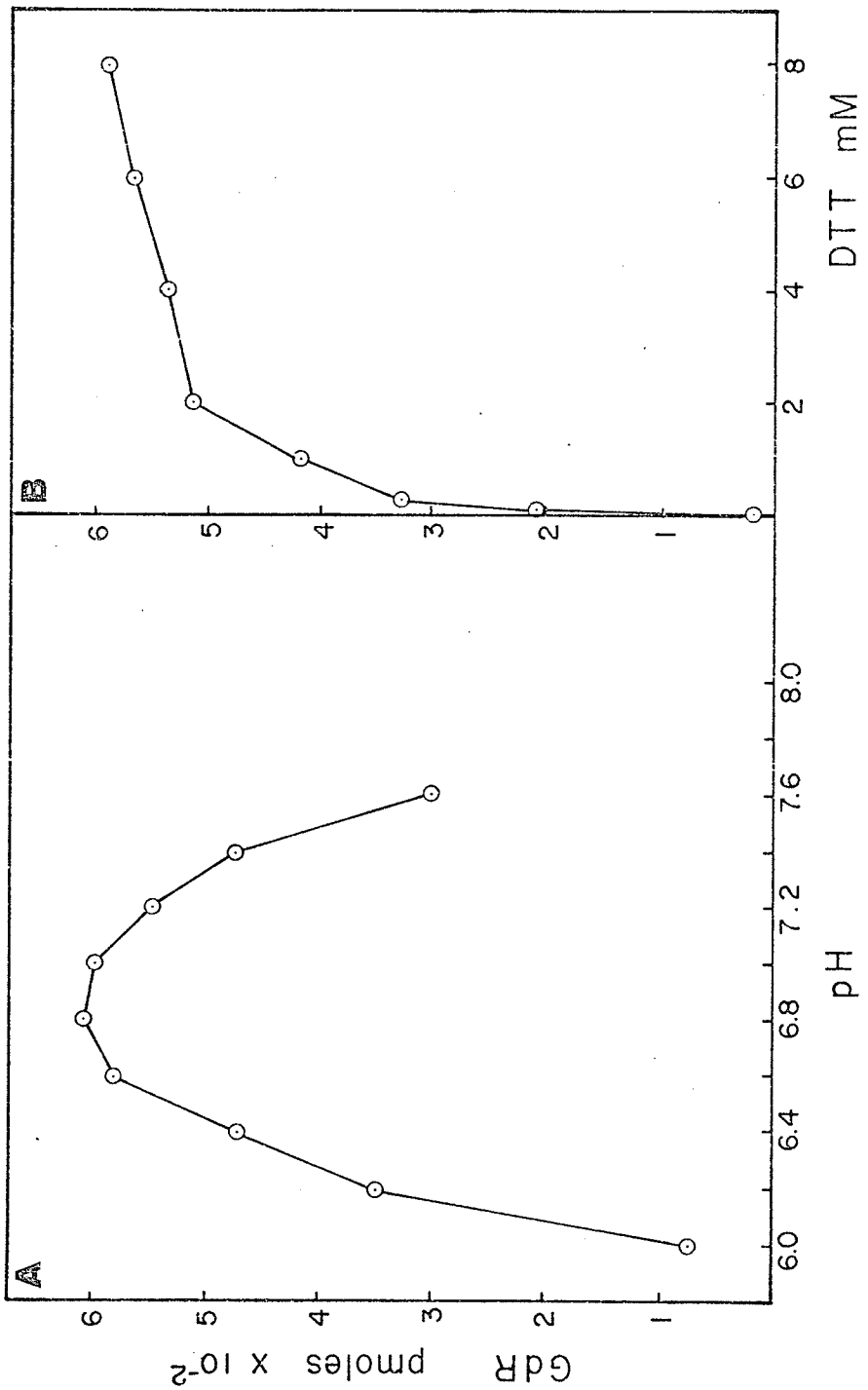
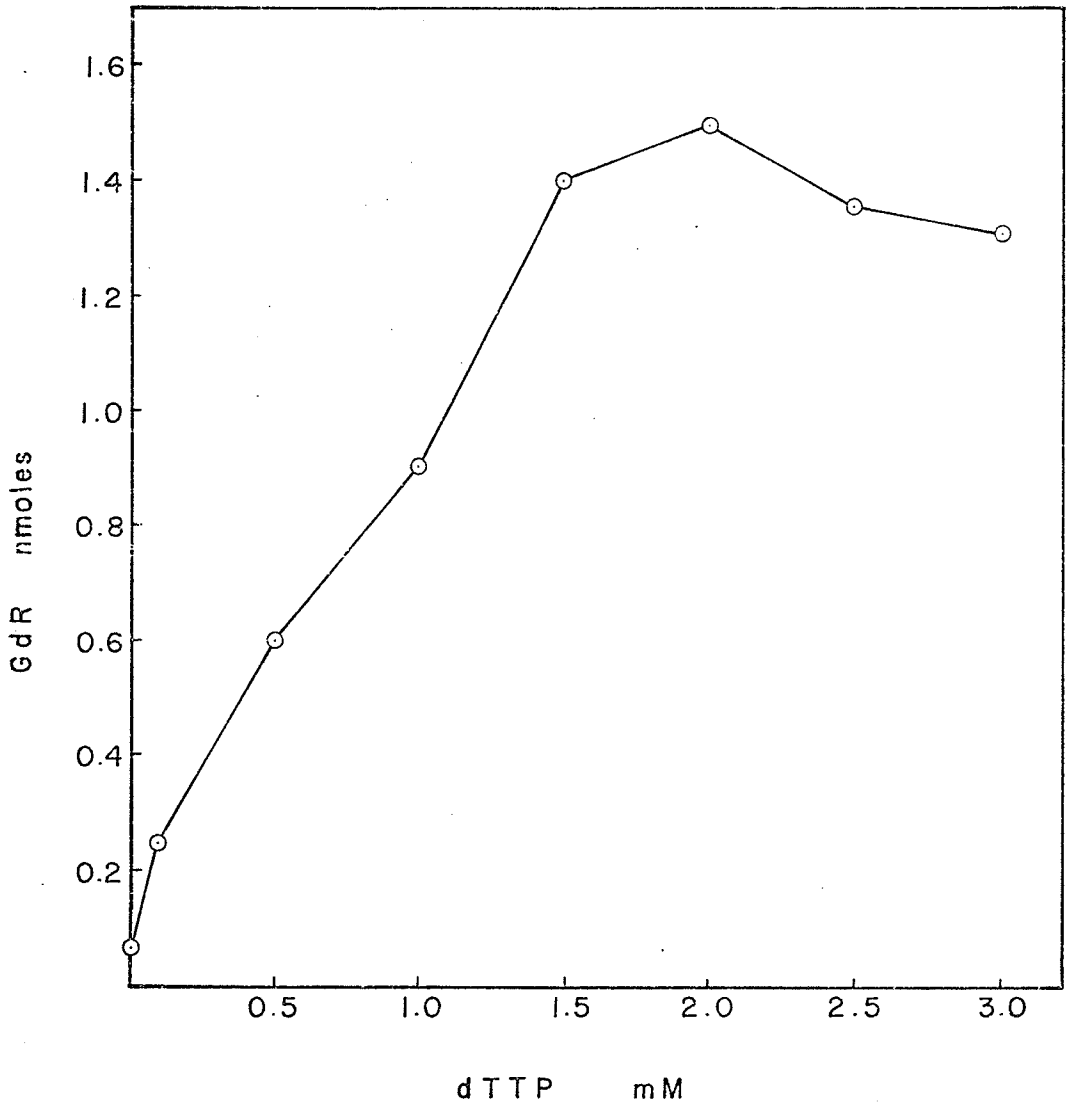

A
B
GdR pmoles x 10^{-2}
pH
DTT mM
6.0
6.4
6.8
7.2
7.6
8.0
2
4
6
8

Figure 49: Response of GDP reduction to varying concentrations of dTTP. A partially purified CHO cell extract (500 µg protein) was incubated under standard GDP reductase assay conditions except that the concentration of dTTP was varied.


1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
GdR nmoles
0.5
1.0
1.5
2.0
2.5
3.0
dTTP mM

slightly inhibitory. Therefore, it was decided to omit $MgCl_2$ from the standard reaction mixture.

The dependence of the reaction rate upon substrate concentration is shown in Figure 50. The Lineweaver-Burk double reciprocal plot of initial reaction rates versus GDP concentration was linear and yielded an apparent Km of 0.03 mM for GDP.

The response of GDP reduction to the presence of hydroxyurea under the optimized assay conditions was investigated. Figure 51 shows the inhibition by hydroxyurea of GDP reduction over a range of GDP concentrations. Hydroxyurea inhibited the reaction at all substrate concentrations tested. As with CDP and ADP reduction, the pattern of inhibition was uncompetitive at the lower concentrations of drug, but appeared mixed as the concentration of hydroxyurea was raised to 0.66 mM. The inset of Figure 51 shows that a replot of intercepts versus hydroxyurea concentration yields a Ki of 0.07 mM for hydroxyurea.

6. Inhibition by hydroxyurea and related compounds of ribonucleotide reductase from drug-resistant cell lines: HU^R-2, the cell line exhibiting the greatest resistance to hydroxyurea inhibition, was grown in suspension culture and harvested. The ribonucleotide reductase present in the cell extract was partially purified in an identical manner to that described for WT CHO cells (see Materials & Methods).

Figure 50: Double reciprocal plot of the variation in rate of GDP reduction with GDP concentration.
A partially purified CHO cell extract (500 µg protein) was incubated under standard GDP reductase assay conditions except that the concentration of GDP was varied.
Inset: Response of the initial velocity of GDP reduction with varying concentrations of GDP.

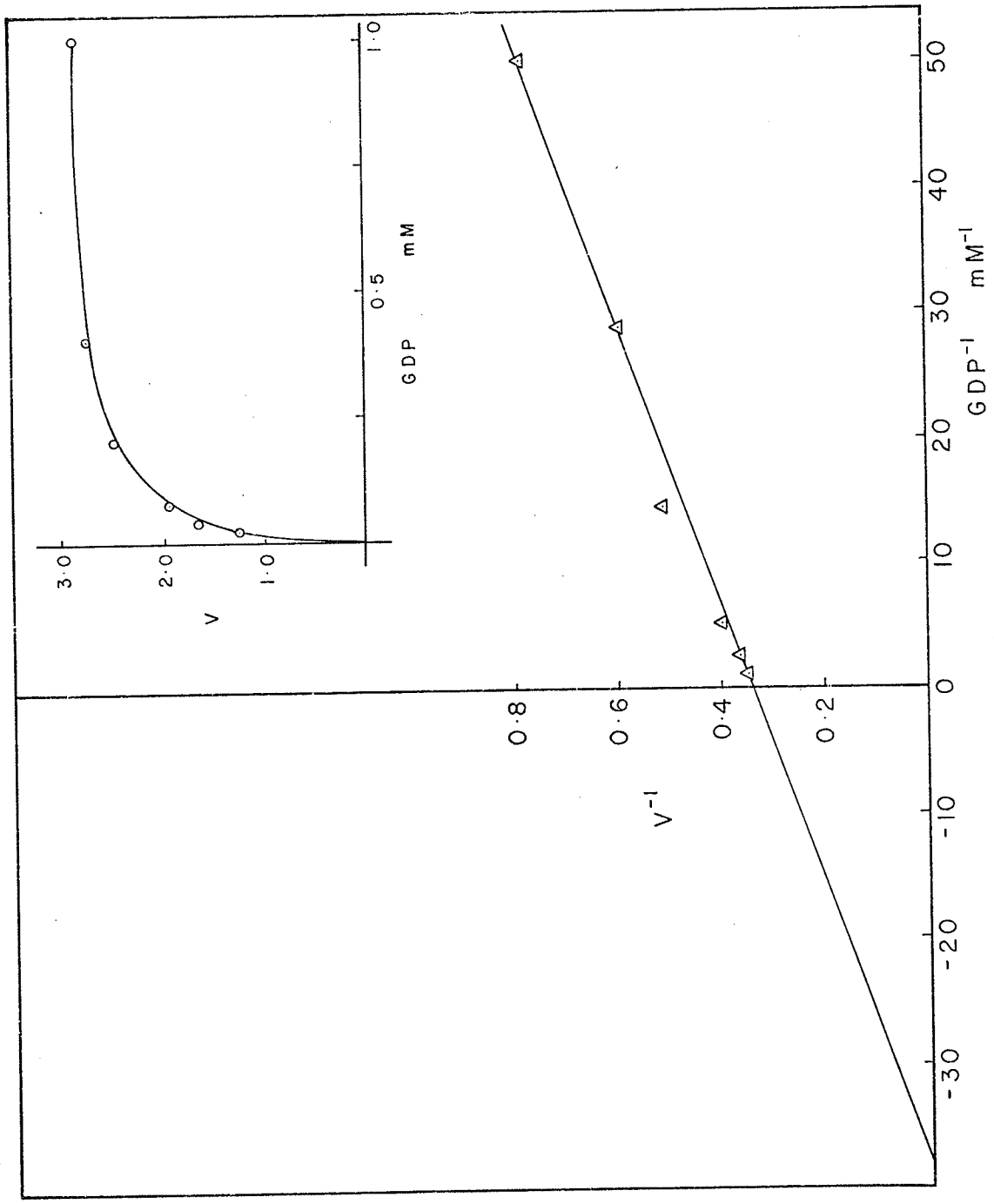
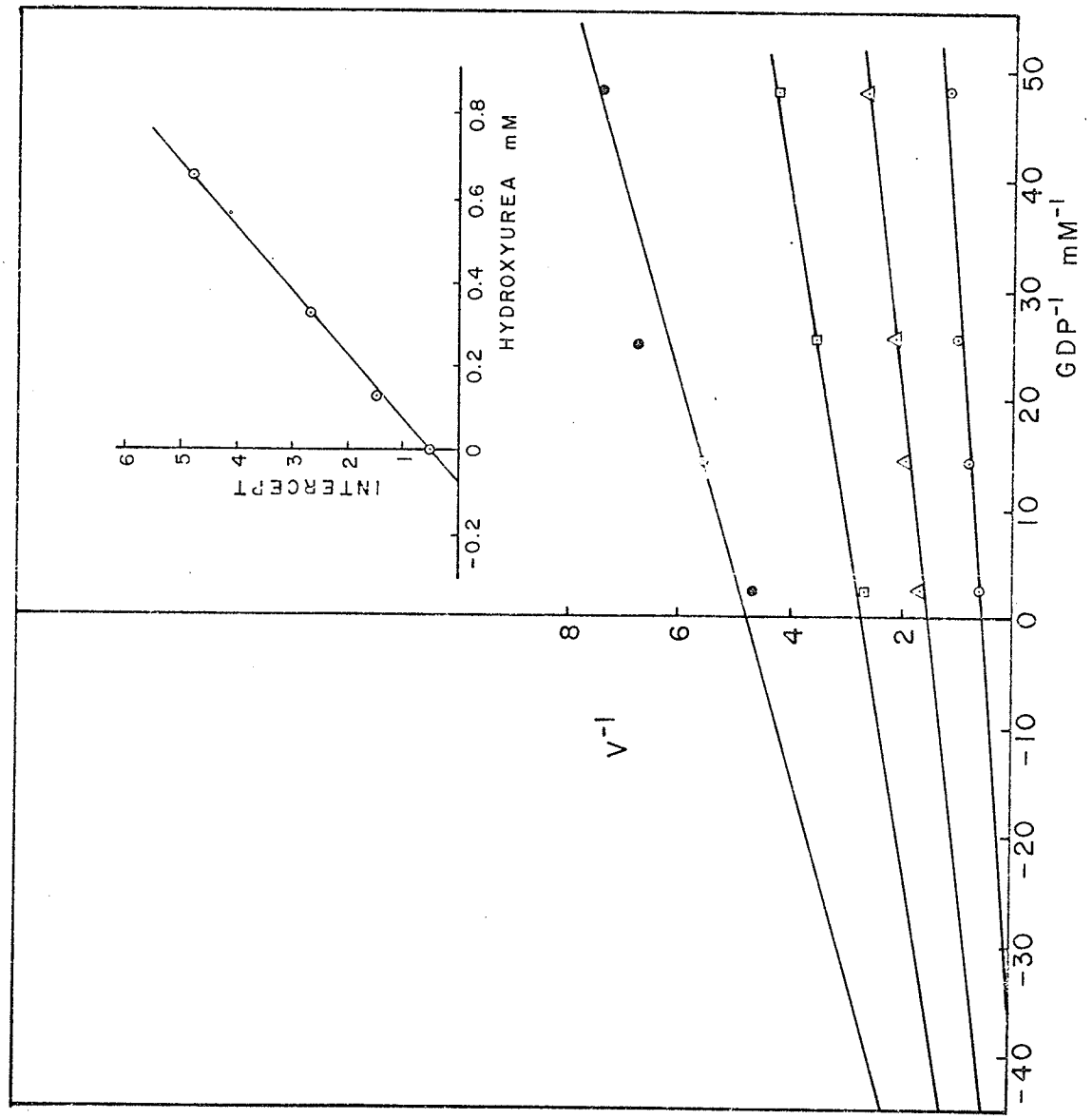

V
3·0
2·0
1·0
GDP mM
0·5
1·0
V⁻¹
0·8
0·6
0·4
0·2
0
-10
-20
-30
10
20
30
40
50
GDP⁻¹ mM⁻¹

Figure 51: Double reciprocal plot of the effect of hydroxyurea on GDP reduction with varying GDP concentration. A partially purified WT CHO cell extract (500 µg protein) was incubated with standard GDP reductase assay mixtures containing varying concentrations of GDP and 0 mM hydroxyurea (⊙), 0.17 mM hydroxyurea (△), 0.33 mM hydroxyurea (⊡) or 0.66 mM hydroxyurea (●).
Inset: Replot of the velocity intercepts versus hydroxyurea concentration.


V⁻¹
GDP⁻¹ mM⁻¹
HYDROXYUREA mM
INTERCEPT

Using the optimized CDP reductase assay conditions, the effect of hydroxyurea on CDP reduction was measured at various CDP concentrations. Figure 52 shows that, as with WT CHO cell enzyme preparations, hydroxyurea uncompetitively inhibited CDP reduction at the lower drug concentrations tested, with the inhibition pattern appearing to be mixed at the highest hydroxyurea concentration. When the intercepts wereplotted against hydroxyurea concentration a Ki value of 0.4 mM drug was obtained. This value is 5-fold higher than the Ki value of 0.08 mM obtained with the WT CHO cell enzyme preparation (Fig 40).

As expected, guanazole inhibited CDP reduction with HU^R-2 preparations in a similar fashion as hydroxyurea. Figure 53 shows the typical uncompetitive inhibition pattern and the replot of the intercepts versus guanazole concentration yielded a Ki value of 3.6 mM guanazole. This compares to a Ki value of 0.57 mM for WT CHO CDP reduction.

Using the optimized ADP reduction assay conditions, the inhibition by hydroxyurea of HU^R-2 ADP reduction was measured at various concentrations of ADP. Figure 54 shows that hydroxyurea inhibited the reaction in the usual uncompetitive fashion. Intercept replots of Figure 54 (inset) yielded a Ki value of 0.42 mM hydroxyurea. This compares to the Ki value of 0.13 mM found for WT CHO cell ADP reductase (Fig 46).

Figure 52: Double reciprocal plot of the effect of hydroxyurea on HU^R-2 CDP reduction with varying concentration of CDP. A partially purified HU^R-2 cell extract (250 µg protein) was incubated with standard CDP reductase assay mixtures containing varying concentrations of CDP and 0 mM hydroxyurea (⊙), 0.17 mM hydroxyurea (△), 0.33 mM hydroxyurea (⊡) or 0.66 mM hydroxyurea (●).
Inset: Replot of the velocity intercepts versus hydroxyurea concentration.

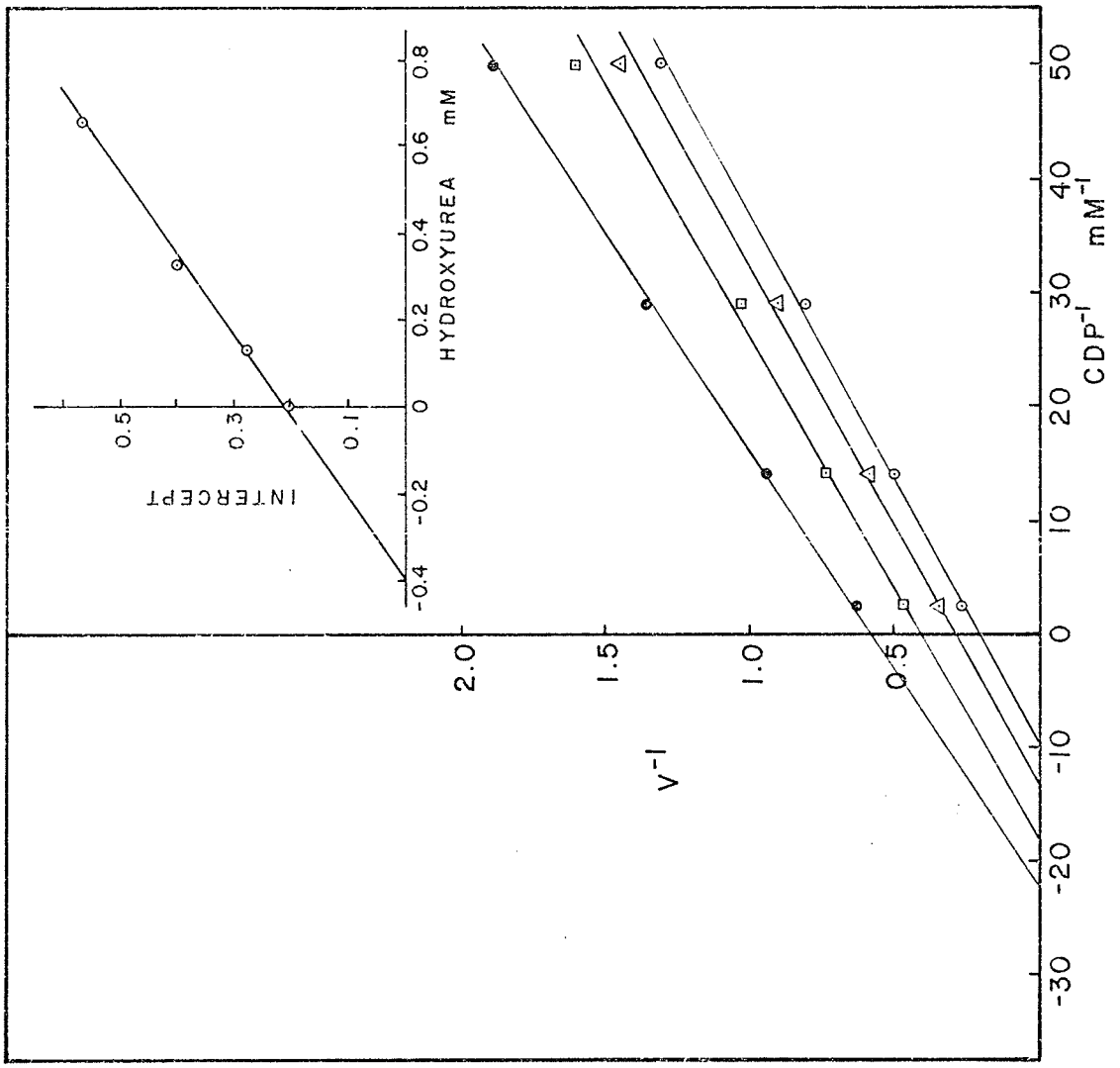

V⁻¹
CDP⁻¹ mM⁻¹
2.0
1.5
1.0
0.5
0
-10
-20
-30
10
20
30
40
50
HYDROXYUREA mM
0.2
0.4
0.6
0.8
-0.2
-0.4
INTERCEPT
0.5
0.3
0.1

Figure 53: Double reciprocal plot of the effect of guanazole on HU^R-2 CDP reduction with varying concentrations of CDP. A partially purified HU^R-2 cell extract (250 µg protein) was incubated with standard CDP reductase assay mixtures containing varying concentrations of CDP and 0 mM guanazole (⊙), 0.6 mM guanazole (△), 1.5 mM guanazole (⊡) or 3.0 mM guanazole (●).

Inset: Replot of the velocity intercepts versus guanazole concentrations.

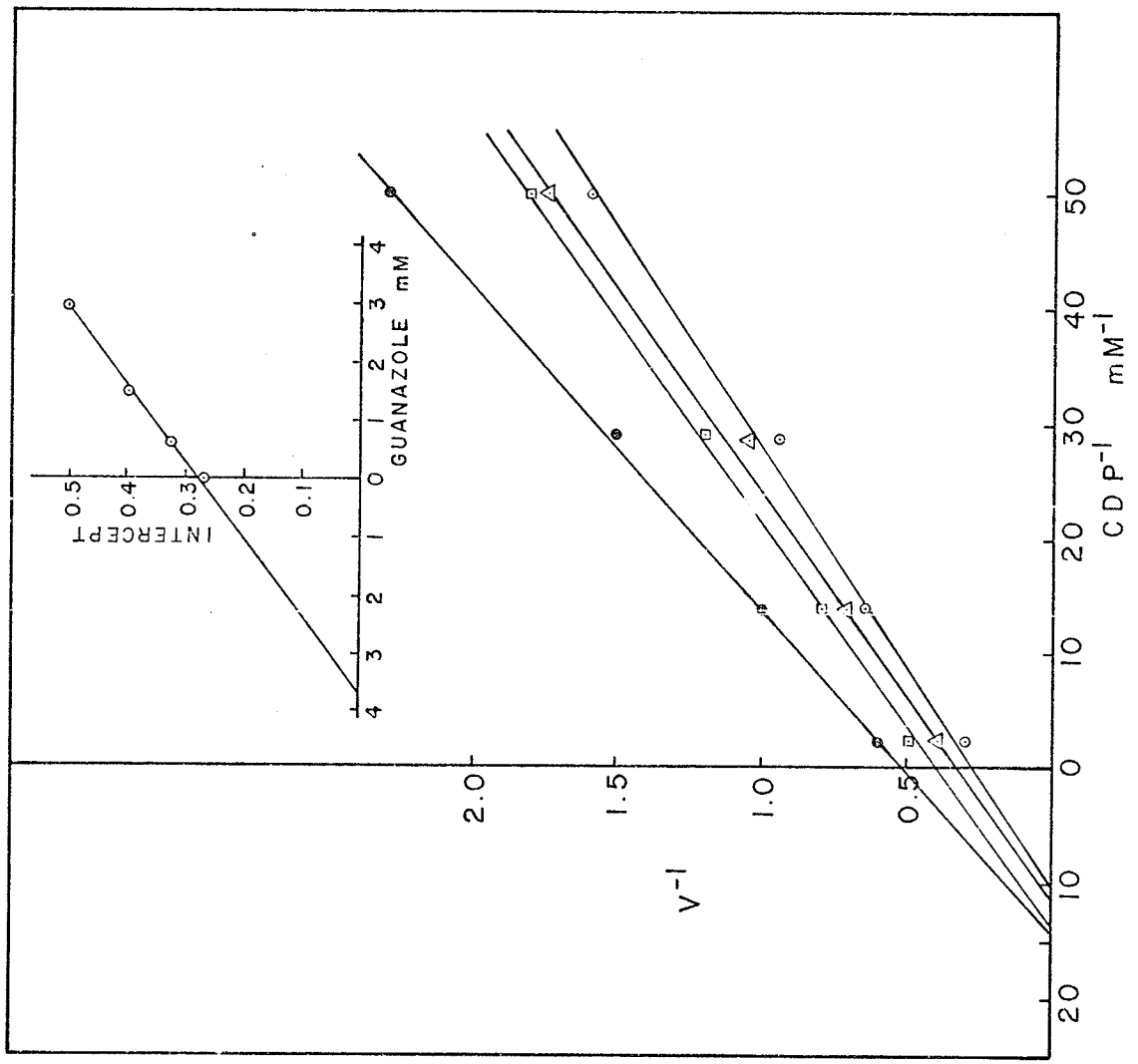

2.0
1.5
1.0
0.5
V^{-1}
0
10
20
30
40
50
CDP^{-1} mM^{-1}
INTERCEPT
0.5
0.4
0.3
0.2
0.1
GUANAZOLE mM
4
3
2
1
0
1
2
3
4

Figure 54: Double reciprocal plot of the effect of hydroxyurea on HU^R-2 ADP reduction with varying concentrations of ADP. A partially purified HU^R-2 cell extract (250 µg protein) was incubated with standard ADP reductase assay mixtures containing varying ADP concentrations and 0 mM hydroxyurea (○), 0.17 mM hydroxyurea (△), 0.33 mM hydroxyurea (⊡) or 0.66 mM hydroxyurea (●). Inset: Replot of the velocity intercepts versus hydroxyurea concentration.

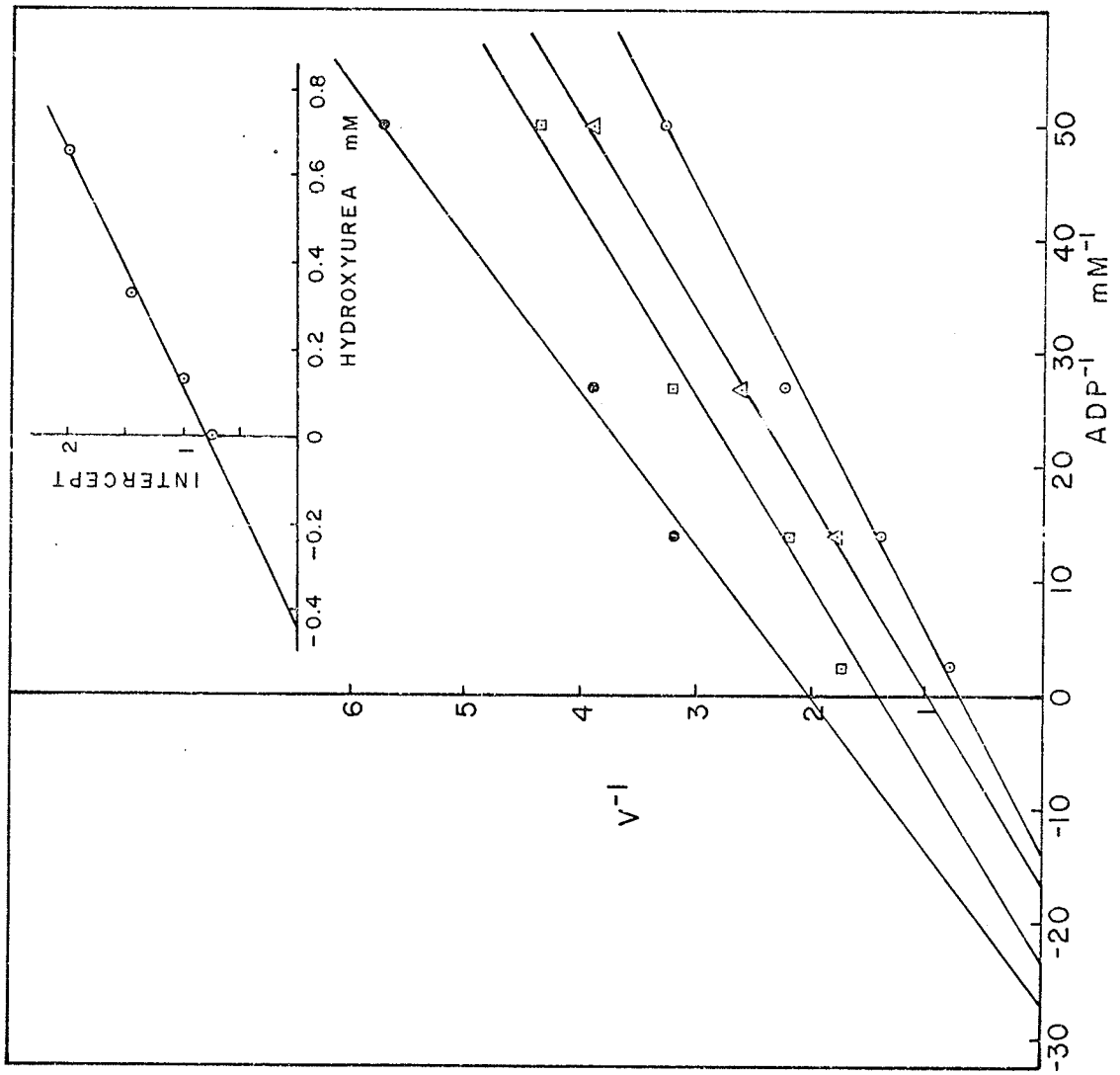

V⁻¹
ADP⁻¹ mM⁻¹
HYDROXYUREA mM
INTERCEPT

Figure 55 shows the inhibition of HU^R-2 GDP reduction by hydroxyurea. Again the inhibition pattern is near uncompetitive, and the intercept replot (Fig 55, inset) yielded 0.45 mM as the Ki value for hydroxyurea inhibition of GDP reduction. This value is 6-fold higher than that obtained for hydroxyurea inhibition of WT CHO GDP reduction.

Finally, the hybrid cell line (GAT X H2) described in Section B10 was cultivated, harvested and the ribonucleotide reductase activity partially purified as described for WT CHO and HU^R-2. Figure 56 shows the effect of hydroxyurea on CDP reduction by the hybrid cell preparation. The pattern of inhibition was the same as for the previously described cell lines. This time, however, the intercept replot (Fig 56, inset) yielded a Ki value of 0.24 mM hydroxyurea, a value almost half way between the WT CHO Ki value of 0.08 mM and the hydroxyurea-resistant HU^R-2 Ki value of 0.40 mM. This would be expected if the expression of CDP reductase by WT CHO cells and HU^R-2 cells remained the same in the hybrid line containing both parental genomes.

Table 20 summarizes the Ki values obtained for hydroxyurea and guanazole with the various cell lines tested. Also included are data obtained from the GAT^-CHO cell line, which is also wild-type for hydroxyurea resistance. It can be seen that with all substrates tested, the ribonucleotide reductase activity from the hydroxyurea-resistant HU^R-2 is far less sensitive to inhibition by either hydroxyurea or

Figure 55: Double reciprocal plot of the effect of hydroxyurea on HU^R-2 GDP reduction with varying concentration of GDP. A partially purified HU^R-2 cell extract (250 µg protein) was incubated with standard GDP reductase assay mixtures containing varying GDP concentrations and 0 mM hydroxyurea (⊙), 0.17 mM hydroxyurea (△), 0.33 mM hydroxyurea (⊡) or 0.66 mM hydroxyurea (●). Inset: Replot of the velocity intercepts versus hydroxyurea concentration.

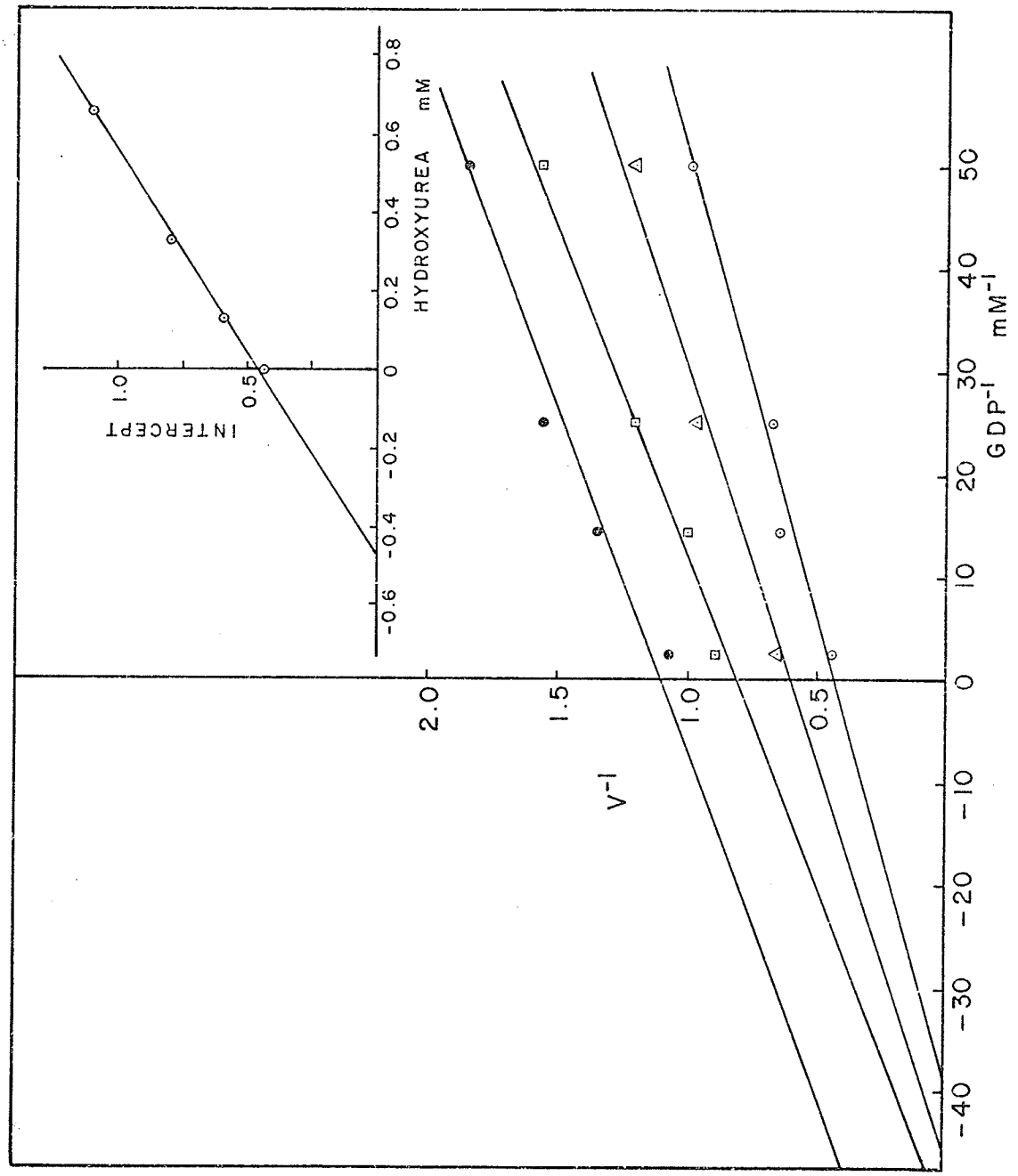

V⁻¹
GDP⁻¹ mM⁻¹
HYDROXYUREA mM
INTERCEPT

Figure 56: Double reciprocal plot of the effect of hydroxyurea on the hybrid (GAT X H2) CDP reduction with varying CDP concentration. A partially purified (GAT X H2) cell extract (500 µg protein) was incubated with standard CDP reductase assay mixtures containing varying CDP concentrations and 0 mM hydroxyurea(○), 0.17 mM hydroxyurea (△), 0.33 mM hydroxyurea (⊡) or 0.66 mM hydroxyurea (●).

Inset: Replot of the velocity intercepts versus hydroxyurea concentration.

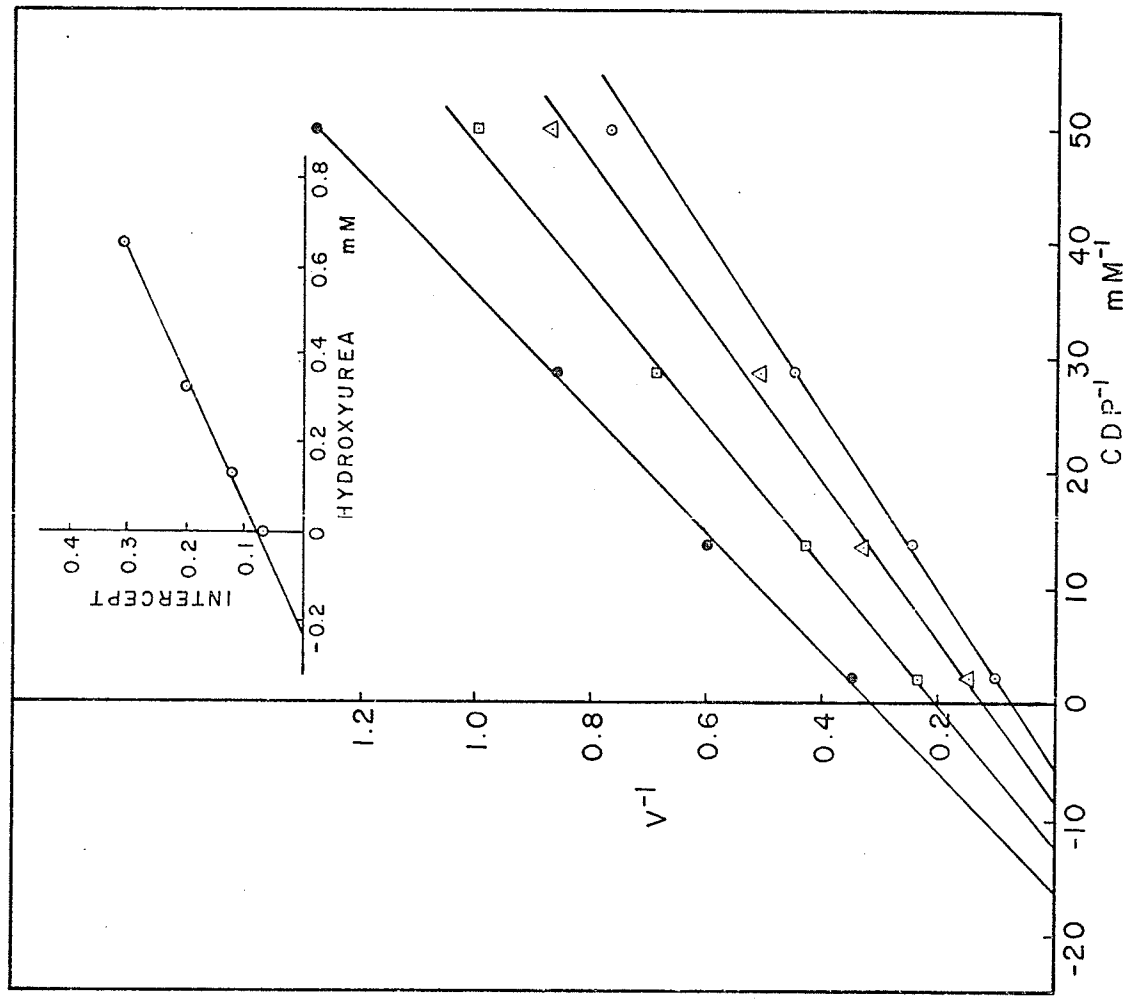

V⁻¹
CDP⁻¹ mM⁻¹
HYDROXYUREA mM
INTERCEPT

Table 20. Ki values for inhibition of ribonucleotide reductase by hydroxyurea and guanazole.

Cell Line	CDP Reduction		ADP Reduction	GDP Reduction
	hydroxyurea	guanazole	hydroxyurea	hydroxyurea
WT CHO	0.08 mM	0.57	0.13	0.07
GAT$^-$CHO*	0.09	0.57		
HU^R-2	0.40	3.62	0.42	0.45
(GAT X H2)	0.24			

*Ki values for GAT$^-$CHO cell purified CDP reductase were calculated from double reciprocal velocity plots in the same way as the value for WT CHO cell enzyme.

Table 21. Apparent Km values for ribonucleotide reductase substrates during hydroxyurea inhibition studies.

Cell Line	CDP	ADP	GDP
WT CHO	0.12 mM	0.14 mM	0.026 mM
GAT$^-$CHO	0.17	0.15	0.030
HU^R-2	0.11	0.09	0.026
(GAT X H2)	0.18		

guanazole than the two wild-type cell lines. As predicted, the hybrid between resistant and non-resistant cell lines shows an intermediate sensitivity to hydroxyurea.

Table 21 shows the apparent Km values for the ribonucleotide substrates with the various cell lines. Since the standard deviation calculated for CDP Km values was approximately 0.05 mM for many WT cell enzyme preparations, there appears to be no significant difference among the apparent Km values for all the CHO lines tested.

To test whether CDP reductase from other drug-resistant cell lines would show the same pattern of cross-resistance to compounds as the whole cells did in PE experiments (Section B9, Table 7), inhibition of CDP reduction was measured in a number of cell lines. Cell extracts were prepared by homogenization, centrifugation at 100,000g and passage through Sephadex G25 equilibrated with standard HEPES buffer. The CDP reductase activity from two wild-type cell lines, WT CHO and $GAT^{-}CHO$; one hydroxyurea-resistant cell line, HU^{R}-4; a guanazole-resistant cell line, G^{R}-3; and finally a cell line resistant to N-carbamoyloxyurea, NC^{R}-1, were all tested for sensitivity to the inhibitory compounds. Four concentrations of each compound were used to inhibit the CDP reductase activity from each cell line at a fixed substrate concentration of 0.40 mM CDP. The reciprocal of the rate of CDP reduction at each concentration of drug was plotted against the corresponding drug concentration. In

all cases the plots were linear and yielded the apparent Ki values listed in Table 22.

Three compounds (hydroxyurea, guanazole and N-carbamoyloxyurea) which exhibited cross-resistance at the cellular level, dATP an allosteric inhibitor of ribonucleotide reduction and the non-inhibitory compound urea were all tested for inhibition of CDP reductase from the various cell lines. Table 22 lists the results obtained. As expected, the two wild-type lines responded almost identically to the five compounds. However, HU^R-4 CDP reductase, which exhibited resistance to hydroxyurea in preliminary experiments (Section C2, Fig. 32) also did so in this experiment. The apparent Ki value for HU^R-4 CDP reduction was 3.3-fold higher for hydroxyurea and 2.0-fold higher for guanazole. The response of HU^R-4 CDP reductase to inhibition by dATP was indistinguishable from the wild-type response. Table 22 also shows that NC^R-1, selected for resistance to N-carbamoyloxyurea, has a CDP reductase activity less sensitive to inhibition by this drug, as well as being less sensitive to inhibition by guanazole and hydroxyurea. G^R-3, a cell line selected for resistance to guanazole, showed only marginal resistance and cross-resistance at the cellular level (Section B9, Table 7). Correspondingly, the CDP reductase from this line showed only marginal cross-resistance to the inhibitory action of either hydroxyurea or guanazole (Table 22). As expected, dATP inhibited CDP reduction by NC^R-1 or G^R-3 to the same

Table 22. Apparent inhibition constants for hydroxyurea inhibition of CDP reduction for several drug-resistant cell lines[†].

Compound	Apparent Ki Value (mM)[*]				
	WT CHO	GAT$^-$CHO	HU^R-4	NC^R-1	G^R-3
hydroxyurea	0.16	0.16	0.53	0.46	0.28
guanazole	0.95	1.01	1.90	2.67	1.90
N-carbamoyloxyurea	0.97	0.78	NT[§]	1.75	NT
dATP	0.025	0.017	0.025	0.030	0.026
urea	>10	>10	>10	>10	>10

[†]Four concentrations of each compound were used to inhibit CDP reduction under standard CDP reductase assay conditions with a fixed CDP concentration of 0.4 mM.

[*]The reciprocal of the rate of CDP reduction at each concentration of drug was plotted against the corresponding concentration of drug and the drug concentration intercept value was expressed as an apparent Ki value in mM.

[§]Not tested.

extent as wild-type enzyme activity, and concentrations of urea below 10 mM had no effect on CDP reduction of any of the cell lines tested.

E. ASSAY OF RIBONUCLEOTIDE REDUCTASE LEVELS IN NUCLEOTIDE-PERMEABLE CHO CELLS

One way that variant cell lines could become resistant to hydroxyurea would be via an increase in the level of the target molecule. For example, cell lines resistant to the anti-folate drug, methotrexate, often exhibit 10 to 140 times higher levels of dihydrofolate reductase than parental lines (Chang & Littlefield 1976, Flintoff et al 1976a). If hydroxyurea-resistant cells contained elevated levels of ribonucleotide reductase, then at a particular concentration of hydroxyurea enough residual ribonucleotide reducing activity might remain to enable the cells to produce an adequate supply of deoxyribonucleotides necessary for DNA replication and cell division.

When partially purified extracts were prepared from the hydroxyurea-resistant HU^R-2 and HU^R-4 cell lines, it was often observed that the ribonucleotide reductase levels were as much as 2-fold higher than corresponding levels in WT CHO cell extracts. However, the levels of ribonucleotide reductase in extracts from any of the cell lines tested varied from batch to batch. In addition, when CDP reductase from the HU^R-12 SC8 cell line was examined no increased resistance of the enzyme to inhibition by hydroxyurea could be demonstrated. The levels of CDP reductase in extracts of this cell line, however, were dramatically higher than the levels

found in extracts of the parental GAT$^-$CHO or revertant subclones. Figure 57 shows that when cultivated under identical conditions, SC8 extracts had approximately 10-fold higher levels of CDP reductase than the WT GAT$^-$CHO, 7-fold higher enzyme levels than the revertant SC17 and only 4-fold higher levels than the partial revertant SC13 (all levels compared at 1 mg extract protein per assay).

Thus it appeared that the HU^R-12 SC8 line may owe its resistance to hydroxyurea solely to increased ribonucleotide reductase levels. In addition to altered enzyme resistance to hydroxyurea, both HU^R-2 and HU^R-4 cell lines may also have elevated enzyme levels. It was decided to develop a more accurate and sensitive technique for measuring levels of ribonucleotide reductase in CHO cells.

Studies of the variation in ribonucleotide reductase levels are difficult to carry out because the activity present in cell extracts is low, and it is difficult to partially purify small quantities of the enzyme. In order to obtain sufficient cells to measure ribonucleotide reductase levels in cell extracts, it had been necessary to cultivate the cells in suspension cultures of large volume. Since ribonucleotide reductase is induced during only a fraction of the hamster cell cycle (Murphee et al 1969), it was necessary to grow and harvest exponentially growing cells under strictly controlled and reproducible conditions. Suspension cultures were unsatisfactory for this purpose

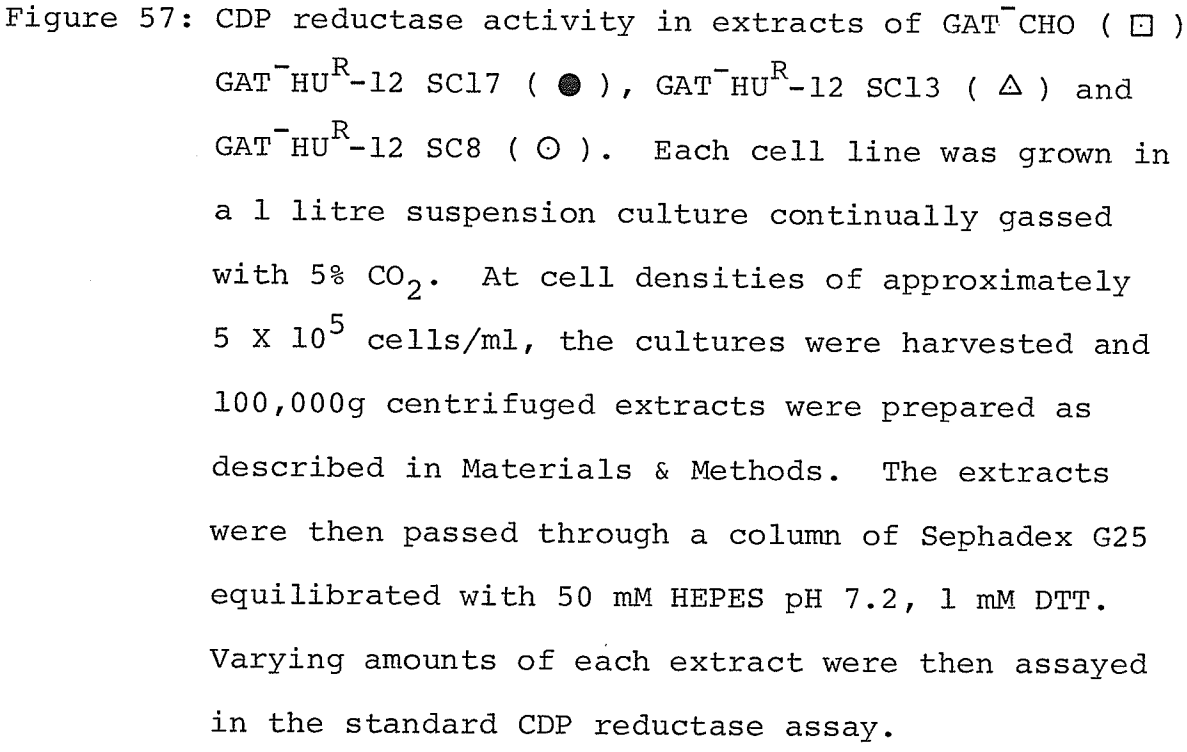
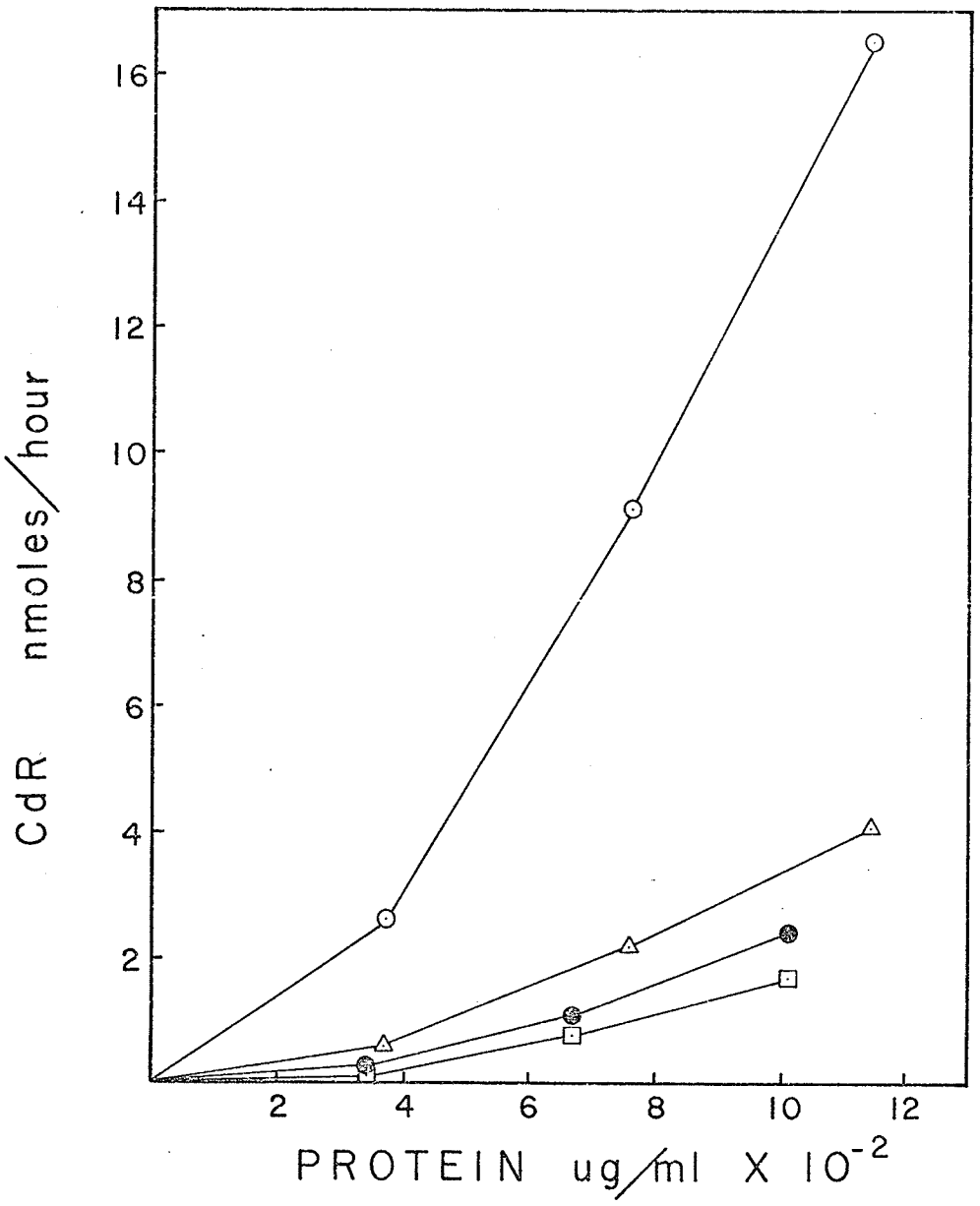


Figure 57: CDP reductase activity in extracts of $GAT^{-}CHO$ (⊡), $GAT^{-}HU^{R}$-12 SC17 (●), $GAT^{-}HU^{R}$-12 SC13 (△) and $GAT^{-}HU^{R}$-12 SC8 (⊙). Each cell line was grown in a 1 litre suspension culture continually gassed with 5% CO_2. At cell densities of approximately 5 X 10^5 cells/ml, the cultures were harvested and 100,000g centrifuged extracts were prepared as described in Materials & Methods. The extracts were then passed through a column of Sephadex G25 equilibrated with 50 mM HEPES pH 7.2, 1 mM DTT. Varying amounts of each extract were then assayed in the standard CDP reductase assay.


CdR nmoles/hour
16
14
12
10
8
6
4
2
2
4
6
8
10
12
PROTEIN ug/ml X 10⁻²

because of variability and low growth rates as compared to cultures on plastic tissue culture plates. Thus it was necessary to develop a method of assaying ribonucleotide reductase levels which would be sensitive enough to measure levels of the enzyme accurately in the low numbers of cells easily obtainable from plate culture.

Another problem with measurement of ribonucleotide reductase levels is that crude extracts contain significant and varying levels of dATP and other naturally occurring inhibitors of ribonucleotide reduction (Cory & Monley 1970, Lewis et al 1976, Lewis et al 1977). These compounds must first be removed by methods such as gel filtration (Tondeur-Six et al 1975) or Dowex ion exchange resin treatment (Peterson & Moore 1976). An additional complication is that at low enzyme levels the activity does not increase linearly, but rather exponentially, with increasing protein concentration (Figs. 35B,44B). Similar problems have been encountered with the enzyme from E. coli, where in order to facilitate the study of the bacterial ribonucleotide reductase, Warner (1973) developed an assay for CDP reduction in toluene-permeabilized cells. With this method the activity of CDP reductase was far greater than that in comparable bacterial cell extracts and was found to increase linearly with protein concentration. Recent progress on the permeabilization of mammalian cells (Seki et al 1975, Billen & Olson 1976, Berger & Johnson 1976) prompted the investigation

of whether a similar system could be developed for the assay of ribonucleotide reductase in mammalian cells. Such a technique would be useful in examining the mutant CHO cell lines containing altered ribonucleotide reductase levels, as well as in examining fluctuations in the enzyme level during the course of the regular hamster cell cycle.

1. Tween-80 Permeabilization: Of the several methods developed recently for rendering mammalian cells permeable to nucleotides, the Tween-80 treatment of Billen and Olson (1976) was chosen. This method is not only rapid and simple, but also only marginally reduces cell viability. Since mammalian ribonucleotide reductase is capable of reducing all four ribonucleoside diphosphates, one purine (GDP) and one pyrimidine (CDP) were chosen as substrates for permeabilized CHO cells. CDP reduction was chosen because most work in mammalian systems has been done with this substrate, while on the other hand GDP reduction was measured because almost no work has been reported on this substrate since the original study of Moore and Hurlbert (1966).

Table 23 shows the effect of a 30 min treatment with 1 % Tween-80 on the ability of CHO cells to reduce CDP or GDP to their corresponding deoxyribonucleotides. With no treatment, almost no CdR was formed while only low amounts of GdR were formed. The presence of the detergent Tween-80 dramatically increased the reduction of both CDP and GDP, presumably because of an alteration of the cell membrane which

Table 23. Effect of Tween-80 permeabilization on ribonucleotide reduction by CHO cells.

Treatment	pmoles CdR	pmoles GdR
none	< 10	45
1 % Tween-80 at 4°C	115	290
1% Tween-80 at 20°C	190	320

Table 24. Properties of ribonucleotide reductase in permeabilized WT CHO cells.

Omission or Addition	% CDP Reductase	% GDP Reductase
complete	100 %	100 %
- dithiothreitol	11	13
- $MgCl_2$	24	-
+ 1 mM $MgCl_2$	-	35
- $FeCl_3$	-	35
+ 0.1 mM $FeCl_3$	61	-
+ 1 mM hydroxyurea	45	21

The control activities were 170 pmoles CdR formed per hour per 5 X 10^6 cells and 700 pmoles GdR formed per hour per 5 X 10^6 cells.

allows entry of the nucleotide substrates and effectors. Permeabilizing at 22°C rather than 4°C significantly increased ribonucleotide reduction especially in the case of CDP reduction (Table 23). Additional washes with permeabilizing buffer did not increase CDP or GDP reductase activity. In all cases the activity per cell of GDP reductase was far greater than CDP reductase. At saturating levels of both CDP and GDP, the specific activity of GDP reductase was approximately 14-fold higher than that of CDP reductase.

Using the standard permeabilizing and assay procedures, ribonucleotide reductase levels could be measured reproducibly in CHO cells. For example, assays of CDP reductase levels in 10 separate batches of WT CHO cells yielded an average value of 195 ± 16 pmoles CdR formed per hour per 5×10^6 cells.

2. Characterization of CDP reduction in whole cells: Optimal activity of CDP reductase in the permeabilized CHO cells required the presence of ATP, a reducing agent (DTT) and $MgCl_2$ (Table 24, Fig. 59). Under the standard assay conditions, the permeabilized cells would reduce CDP at a linear rate for approximately 60 min (Fig. 58). Table 24 also shows that unlike the case with some mammalian systems (Moore 1969) $FeCl_3$ did not stimulate the reaction. On the other hand, hydroxyurea which is known to be a specific and potent inhibitor of mammalian ribonucleotide reductase in vitro (Fig. 31), inhibited CDP reduction by 55% at a concen-

Figure 58: Effect of incubation time on CDP reduction (A) and GDP reduction (B) in permeabilized CHO cells. Permeabilized WT CHO cells (5×10^6) were incubated with standard CDP or GDP reduction mixtures at $37^{\circ}C$ as described in Materials & Methods. At intervals, the reactions were terminated by boiling and the amount of GdR or CdR formed was measured.

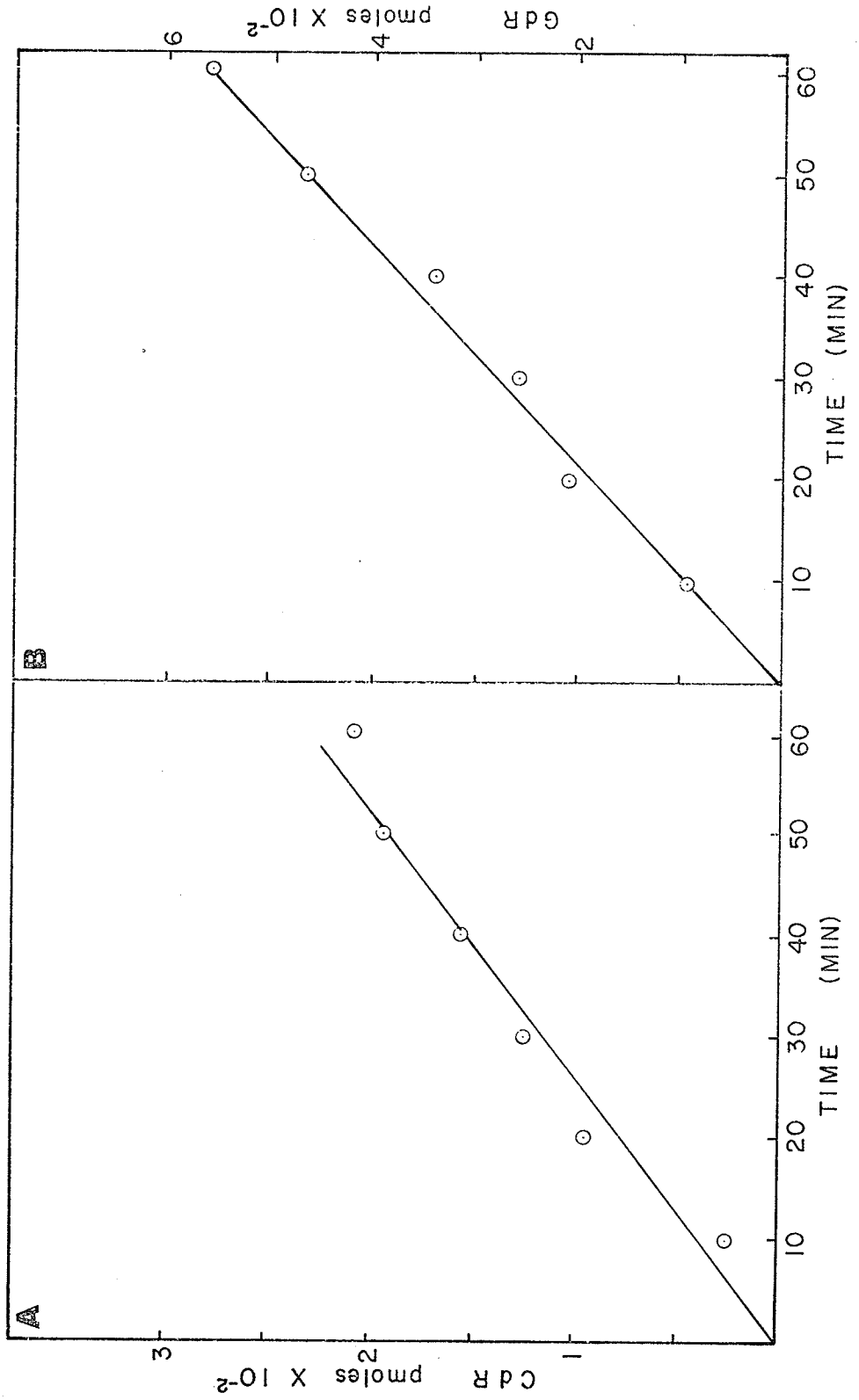
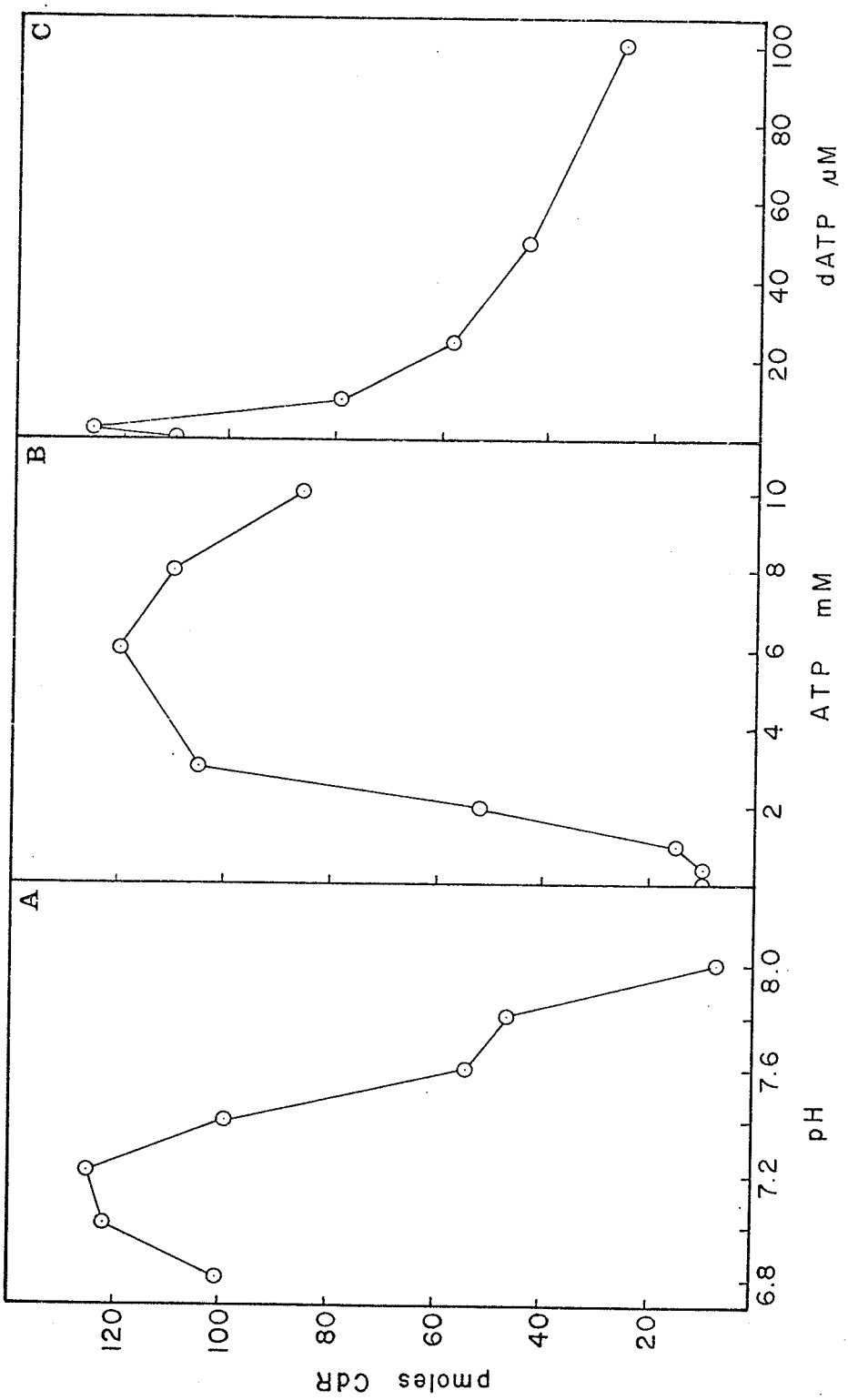

A
B
CdR pmoles X 10-2
GdR pmoles X 10-2
TIME (MIN)
TIME (MIN)

Figure 59: CDP reduction in permeabilized CHO cells. Effect of pH (A), ATP concentration (B) and dATP concentration (C) on reaction rate. CDP reduction was measured with the standard procedure using 5×10^6 permeabilized cells incubated with the reaction mixture for 40 min at 37° as described in Materials & Methods.


A
B
C
pmoles cdR
120
100
80
60
40
20
6.8
7.2
7.6
8.0
pH
2
4
6
8
10
ATP mM
20
40
60
80
100
dATP μM

tration of 1 mM.

Figure 59A shows the pH optimum of the reaction to be 7.2, a value identical to that found with both cell extracts and partially purified enzyme (Fig 36A). Similarly, 6 mM ATP maximally activated the reaction with whole cells or cell extracts, and activity in the absence of ATP was only about 10% of the activity in the presence of ATP (Fig 59B). Figure 59C shows that dATP is a potent inhibitor of the reaction, 20 μM resulting in almost 50% inhibition of CDP reduction. This agrees well with reported studies on dATP inhibition of CDP reductase in mammalian cell extracts (Meuth & Green 1974) and with the results in CHO cell extracts already described (Table 22).

Figure 60 shows a double reciprocal plot of the variation in the rate of CDP reduction with CDP concentration. The plot is linear and yields an apparent Km for CDP of 0.13 mM, a value identical to that obtained with the partially purified CDP reductase (Fig 39) and close to the value of 0.094 mM reported for DON hamster fibroblasts (Peterson & Moore 1976).

3. Characterization of GDP reduction in whole cells: In agreement with other mammalian systems reported, it was found that optimal activity of GDP reductase in permeabilized CHO cells required the presence of dTTP, DTT and $FeCl_3$ (Table 24, Fig 61). The reaction proceeded linearly for at least

Figure 60: Double reciprocal plot of the variation in CDP reduction (hour nmoles^{-1}) with CDP concentration (mM^{-1}), using 5 X 10^6 permeabilized CHO cells in the standard reaction mixture containing varying amounts of CDP.

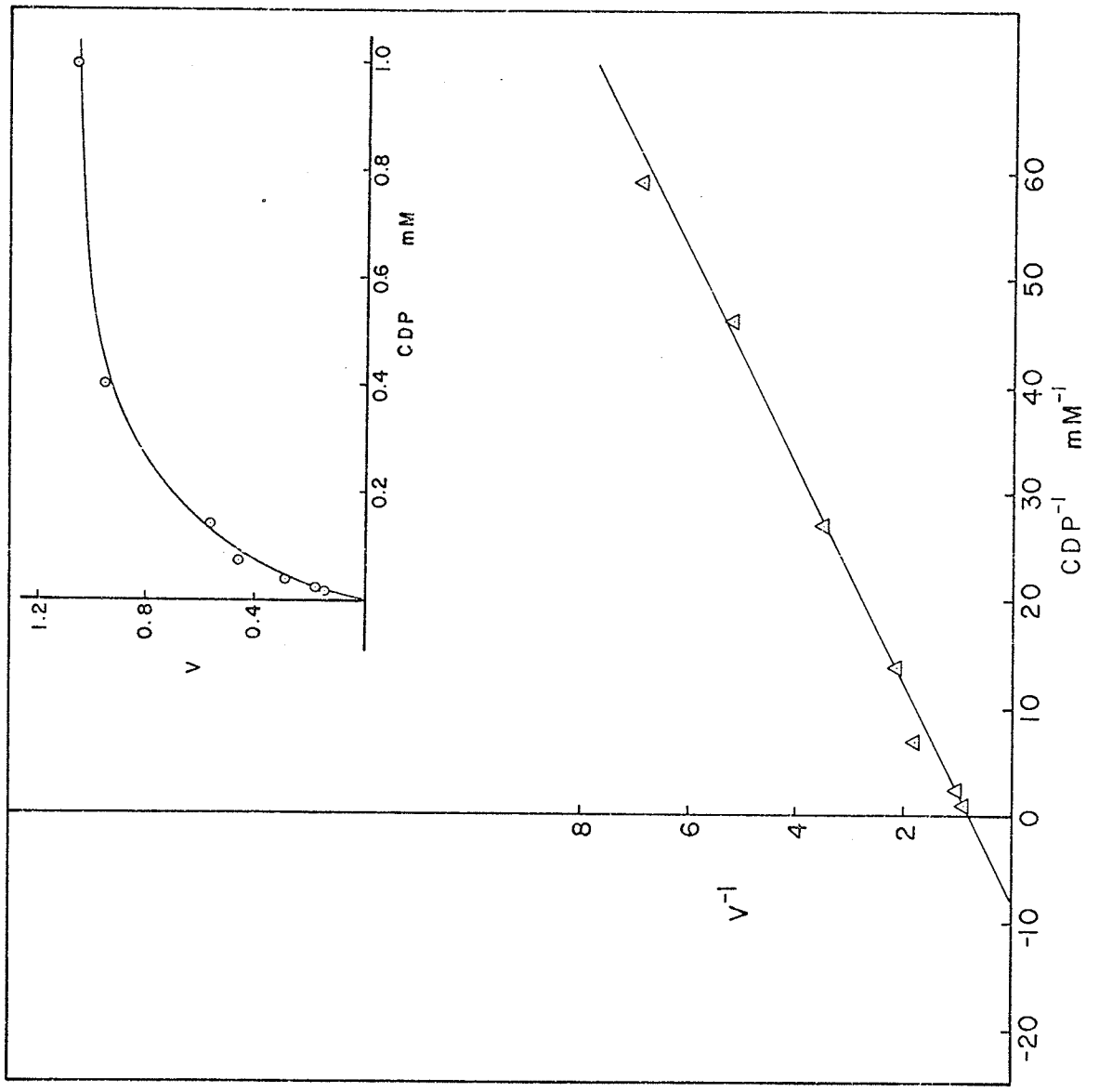
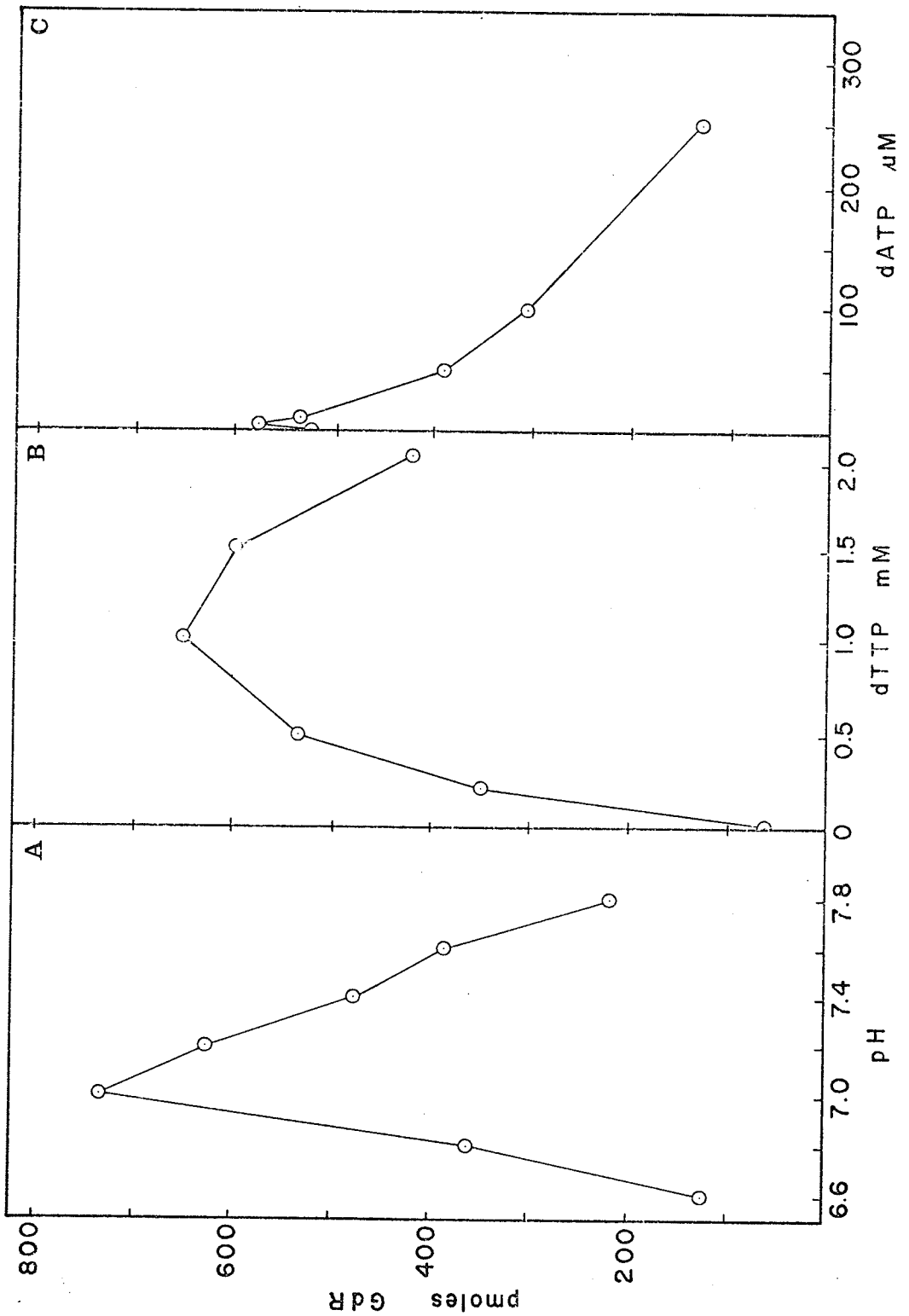

v
1.2
0.8
0.4
0.2
0.4
0.6
0.8
1.0
CDP mM
V^{-1}
8
6
4
2
-20
-10
0
10
20
30
40
50
60
CDP^{-1} mM^{-1}

Figure 61: GDP reduction in permeabilized CHO cells. Effect of pH (A), dTTP concentration (B), and dATP concentration (C) on reaction rate. GDP reduction was measured with the standard procedure using 5 X 10^6 permeabilized WT CHO cells incubated with the reaction mixture for 60 min at 37^{o}C as described in Materials & Methods.


A
B
C
pmoles GdR
800
600
400
200
6.6
7.0
7.4
7.8
pH
0
0.5
1.0
1.5
2.0
dTTP mM
100
200
300
dATP µM

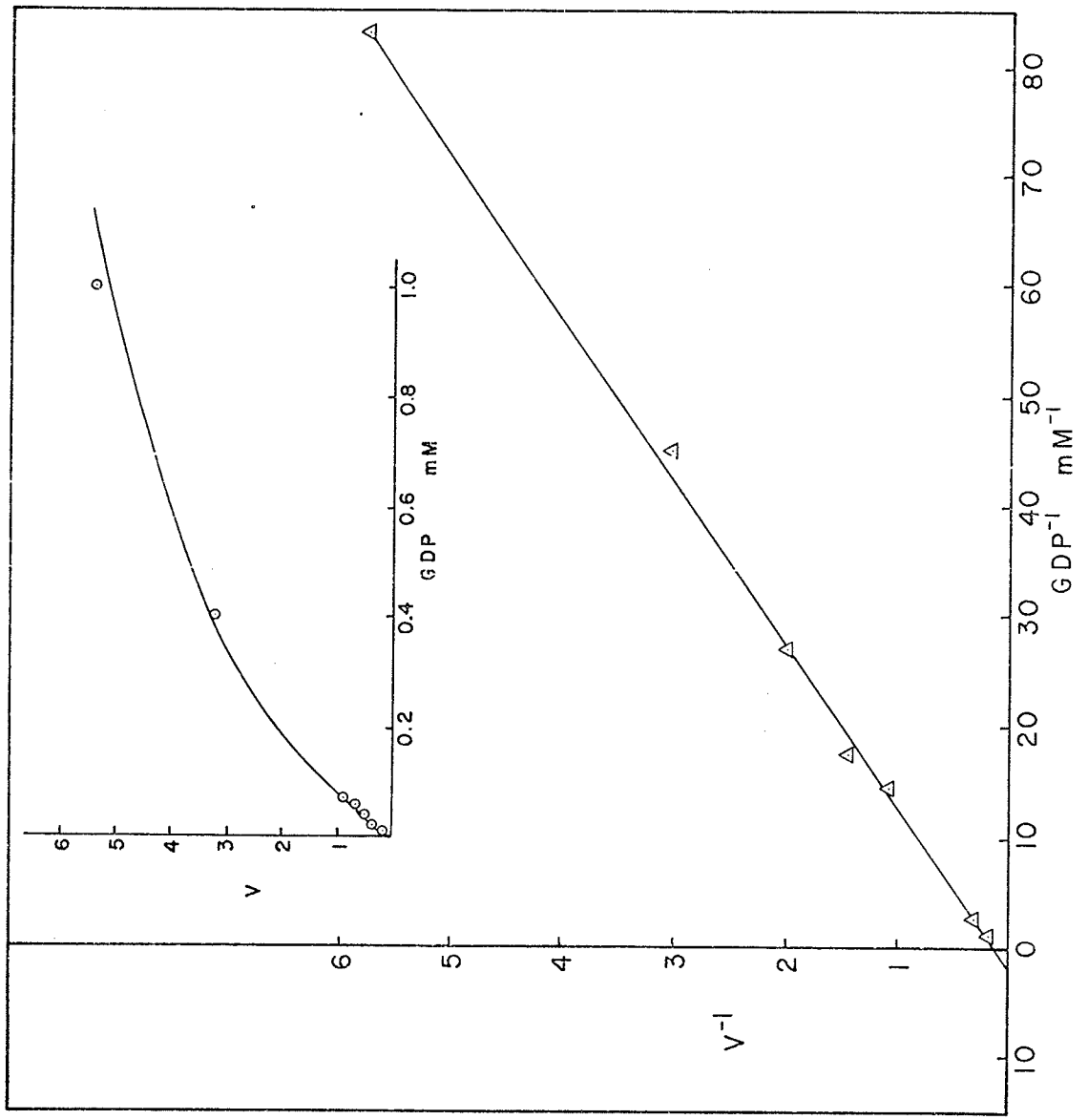
60 min at 37°C (Fig 58B). Unlike CDP reduction, GDP reduction was stimulated by added $FeCl_3$ and inhibited by 1 mM $MgCl_2$ (Table 24). Hydroxyurea at 1 mM inhibited GDP reduction by almost 80%.

Figure 61A shows the pH optimum of the reaction to be 7.0 which is close to the value obtained with partially purified enzyme (Fig 48A). At 1 mM dTTP, GDP reduction was maximally stimulated (Fig 61B). The 0.25 mM concentration required for half-maximal activation is somewhat lower than the 0.80 mM required with cell extracts (Fig 49). Figure 61C shows that dATP is a potent inhibitor of GDP reduction, as it was with CDP reduction.

Figure 62 shows a double reciprocal plot of the variation in the rate of GDP reduction with GDP concentration. As expected, the plot is linear and yields an apparent Km value for GDP of 0.5 mM. This value is about 10-fold higher than the apparent Km value found in partially purified CHO cell extracts (Fig 50). The assay conditions for the two systems were quite different, however. For example, the higher Km value was found in the presence of $FeCl_3$. Peterson and Moore (1976) reported a similarly high apparent Km value of 1.67 mM for the purine ribonucleotide substrate, ADP.

4. Effect of enzyme concentration on CDP and GDP reduction: Work with E.coli has shown that mechanical disruption of cells or dilution of cell extracts results in

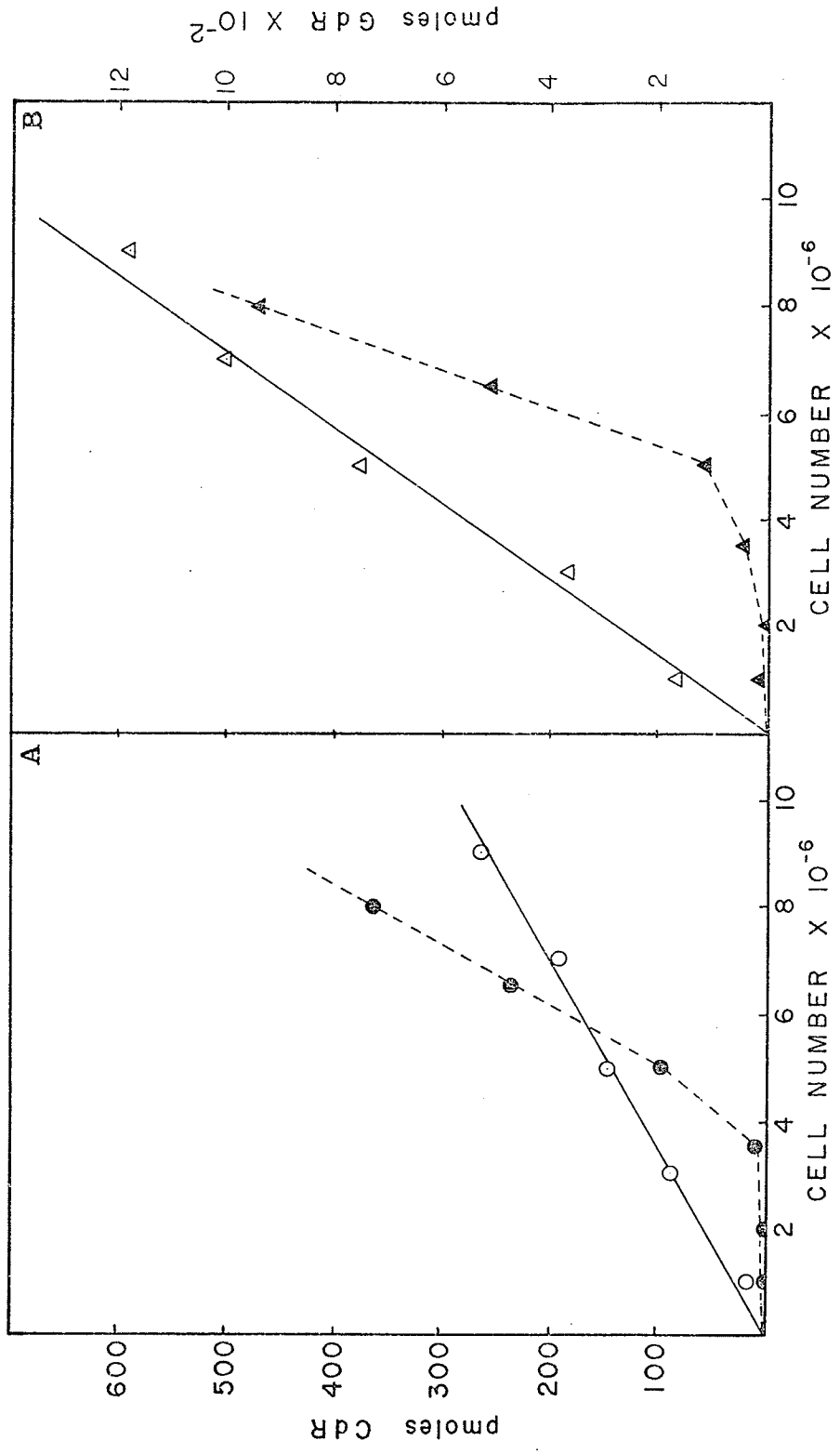
Figure 62: Double reciprocal plot of the variation in the rate of GDP reduction (0.5 hour/nmole) with GDP concentration (mM^{-1}), using 5 X 10^6 permeabilized CHO cells in the standard reaction mixture containing varying amounts of GDP.


V
GDP mM
0.2
0.4
0.6
0.8
1.0
1
2
3
4
5
6
V^{-1}
GDP^{-1} mM^{-1}
10
0
10
20
30
40
50
60
70
80

a loss of ribonucleotide reductase activity and also a change to non-linear response with increasing enzyme concentration (Warner 1973, Eriksson 1975). To determine whether a similar phenomenon could be demonstrated in mammalian cells, ribonucleotide reductase activity was measured in both whole cells and corresponding cell extracts (Fig 63). Samples of cell extract corresponding to 1 - 8 X 10^6 cells were assayed for CDP and GDP reductase activities. As with other mammalian cell extracts (Hopper 1972, Peterson & Moore 1976) CDP reductase activity did not increase linearly with low concentrations of protein (Fig 63A). This effect was absent when whole cells were used to assay CDP reduction. The amount of CDP reduced increased linearly up to at least 9 X 10^6 cell per assay (Fig 63A). As with E. coli, the activity at low levels with whole cells was far greater than the activity with the corresponding cell extract. The increase in CDP reductase activity in extracts became linear above 0.39 mg protein per assay, a value which compares well to that reported by Peterson & Moore (1976). At high protein levels the activity was greater in cell extracts than with whole cells (Fig 63A).

Figure 63B shows that GDP reductase responded to protein concentration as CDP reductase did. With cell extracts GDP reduction increased linearly with protein only above 0.39 mg protein per assay, whereas the activity with whole cells was linear at all cell concentrations tested.

Figure 63: Dependence of the rate of ribonucleotide reduction on enzyme concentration. CDP reduction was measured for 60 min with the standard reaction mixture containing varying numbers of permeabilized CHO cells (⊙) or varying amounts of cell-free extract (●). GDP reduction was measured for 60 min with the standard reaction mixture containing varying numbers of permeabilized CHO cells (△) or varying amounts of cell-free extract (▲). A 100,000g cell extract of WT CHO was prepared as described in Materials & Methods and then passed through a column of Sephadex G25 equilibrated with 50 mM HEPES pH 7.2, 1 mM DTT. The final extract preparation contained 3.8 mg protein per ml.


A
B
pmoles CdR
pmoles GdR X 10-2
CELL NUMBER X 10-6
CELL NUMBER X 10-6

At all points tested, the activity with whole cells was greater than that found in cell extracts (Fig 63B).

5. Ribonucleotide reductase levels in various cell lines: The standard permeabilizing and assay conditions were used to measure CDP and GDP reductase activities in a variety of mammalian cell lines. Table 25 shows the CDP and GDP reductase activities for a number of hydroxyurea-resistant cell lines derived from GAT^-CHO. The GAT^-CHO cell line consistently showed somewhat lower levels of both CDP and GDP reductase activities than WT CHO. This may be due to the presence of thymidine which is added to the GAT^-CHO growth medium, for a thymine derivative is thought to be responsible for the repression of ribonucleotide reductase synthesis in E. coli (Biswas et al 1965). From Table 25 it can be seen that the hydroxyurea-resistant GAT^-HU^R-12 SC8 cells have high CDP and GDP reductase activities. When compared to the parental GAT^-CHO cell line, SC8 had 4 to 5 times more CDP reductase activity and almost 7-fold more GDP reductase activity (Table 25). Furthermore, when SC8 is grown for several generations in the presence of 0.26 mM hydroxyurea, a concentration of drug which does not reduce the growth rate of the mutant (see Fig 23), the cells are capable of increasing the levels of both CDP and GDP reductases by a further 2-fold. The parental GAT^-CHO is incapable of growth at this concentration of hydroxyurea. As expected, the revertant subclone HU^R-12 SC17, which shows the same

Table 25. CDP and GDP reductase levels in various GAT$^-$CHO cell lines.

Cell Line	pmoles CdR	pmoles GdR
GAT$^-$ CHO	130 ± 70	220
GAT$^-$HU^R-12SC8	560 ± 90	1520
GAT$^-$HU^R-12SC8 + hydroxyurea	1030 ± 110	2530
GAT$^-$HU^R-12SC17	176 ± 65	550
(ts X SC8)	1400 ± 260	-

Ribonucleotide reductase was measured for 60 min with 5 X 10^6 permeabilized cells as detailed in Materials and Methods. The values for CDP reduction represent the average of three separate experiments, and the GDP reduction values are from single experiments. The value for CDP reduction by the hybrid (ts X SC8) represents the average of two experiments,

†GAT$^-$HU^R-12SC8 was grown in the presence of 0.26 mM hydroxyurea for at least 48 hours before permeabilization and enzyme assay.

sensitivity to hydroxyurea as $GAT^{-}CHO$ (Fig 22) has enzyme levels reduced to near the parental level (Table 25). These results with CDP reductase measured with permeabilized cells show the same pattern as that obtained with cell extracts of these cell lines (Fig 57).

When the hybrid cell line (ts X SC8) was tested for CDP reductase levels by the standard permeabilizing assay, it was found to contain a CDP reductase specific activity of 1400 pmoles CdR reduced per hour per 5 X 10^6 hybrid cells. This value is a little over twice the level found in the hydroxyurea-resistant SC8 parental cell line and suggests that the mechanism conferring elevated levels of ribonucleotide reductase may behave in a genetically dominant fashion in hybrid cells containing both wild-type and hydroxyurea-resistant genomes.

Table 26 shows the results of measurements of CDP and GDP reductase levels in the hydroxyurea-resistant HU^R-2 and HU^R-4 cell lines. Both have CDP reductase levels elevated approximately 3-fold over WT CHO enzyme levels. As well, HU^R-2 seems to have slightly higher GDP reductase levels when compared to WT CHO. As with the hydroxyurea-resistant SC8 (Table 25), if HU^R-2 is grown in the presence of 0.26 mM drug, both CDP and GDP reductase levels are further increased (Table 26).

The permeabilized cell assay was also used to measure CDP and GDP reductase levels in a variety of other mammalian

Table 26. CDP and GDP reductase levels in WT CHO cells and cell lines resistant to hydroxyurea.

Cell Line	CdR	GdR
WT CHO	195 ± 16 (10)*	644± 60 (5)
HU^R-2	568 ± 47 (4)	873 ± 63 (3)
HU^R-2 + hydroxyurea†	1532	1162
HU^R-4	690	-

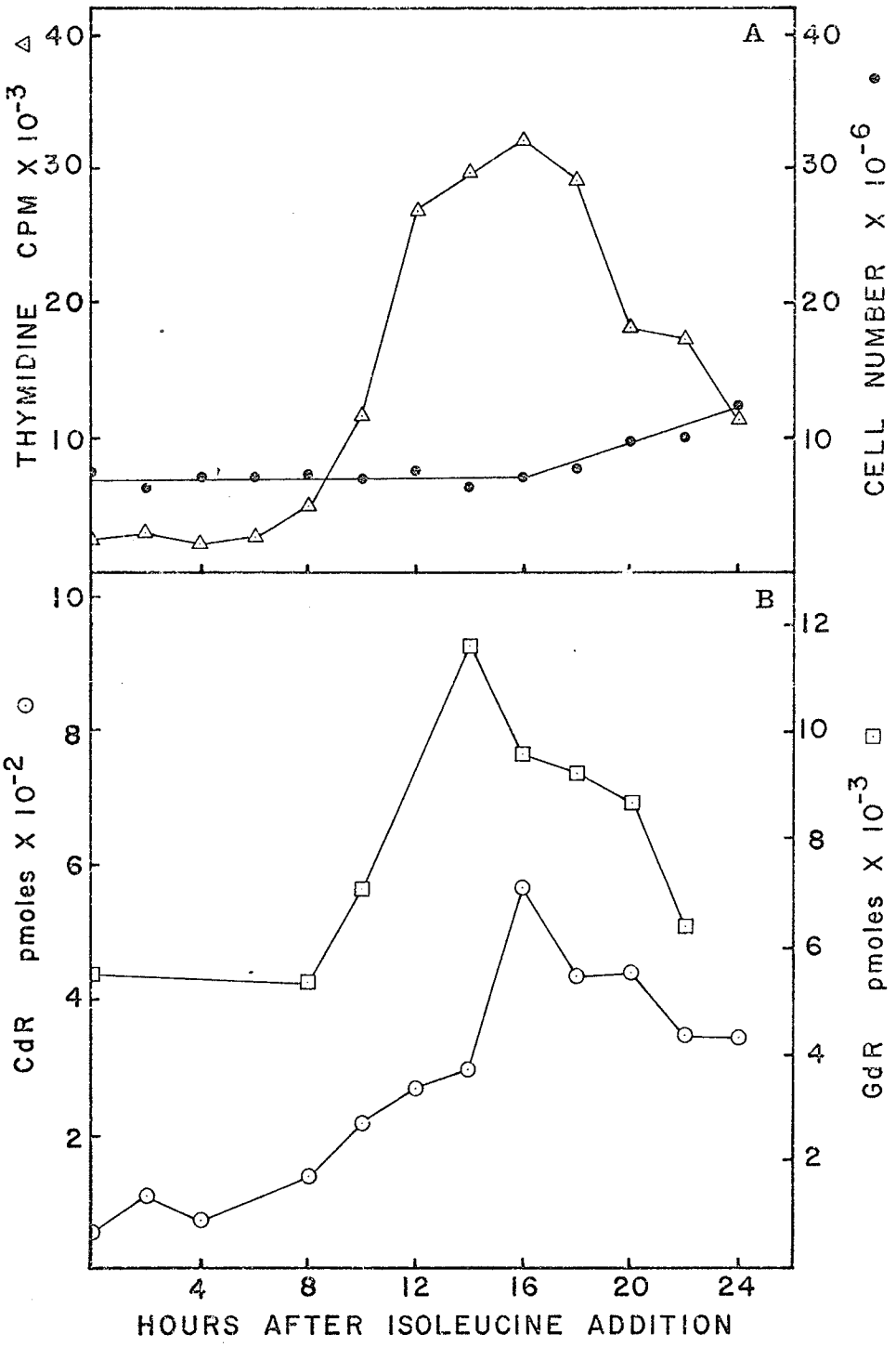
*CdR and GdR results are given in pmoles/hour/5 X 10^6 cells. Figures in parentheses indicate number of independent samples measured.

†HU^R-2 was grown in the presence of 0.26 mM hydroxyurea for at least 48 hours before cell permeabilization and enzyme assay.

cell lines. The permeabilizing and assay procedures were equally effective with CHO, mouse L cell, mouse 3T3 or SV40 transformed 3T3 (data not shown).

6. CDP and GDP reductase activities in synchronized CHO cells: Hamster cells deprived of the essential amino acid isoleucine gradually stop growing and accumulate in G1 or a G1-like phase of the cell cycle (Ley & Tobey 1970). Peterson and Moore (1976) studied fluctuations in ribonucleotide reductase levels in hamster fibroblasts synchronized by isoleucine deprivation. They found that CDP and ADP reductase activities were low in arrested cells. Upon readdition of isoleucine and the subsequent resumption of cellular DNA synthesis, however, both CDP and ADP reductases increased to much higher levels. I repeated their work using Tween-80 permeabilized CHO cells to measure CDP and GDP reductase levels during the CHO cell cycle. In Figure 64A it can be seen that, 8 hours after the addition of isoleucine to starved CHO cells, thymidine incorporation into acid precipitable material increased rapidly and reached a maximum rate at 16 hours. By 20 hours after isoleucine replacement, the rate of thymidine incorporation had declined and cell division had begun. This time course agrees well with published work on the percentage of cells synthesizing DNA as measured by autoradiography. Peterson and Moore (1976) for example, found that only 10% of hamster cells were synthesizing DNA at 9 hours after reversal of isoleucine starva-

Figure 64: Ribonucleotide reductase activities during the CHO cell cycle. At zero time, complete medium containing isoleucine was added to CHO cells arrested in the G1 phase of the cell cycle due to isoleucine starvation (see Materials & Methods). In A: At intervals, the amount of ^{3}H-thymidine incorporated into acid precipitable cell material in 10 min was measured (△), and the cells were trypsinized and counted (●). In B: 5 X 10^{6} Tween-80 permeabilized cells were used to measure the rate of CDP reduction (⊙) or GDP reduction (⊡) in 60 min. Near saturating levels of CDP (0.4 mM) or GDP (0.4 mM) were used in the standard reaction mixtures.


A
B
THYMIDINE CPM X 10-3 △
CELL NUMBER X 10-6 ●
CdR pmoles X 10-2 ⊙
GdR pmoles X 10-3 □
HOURS AFTER ISOLEUCINE ADDITION
4
8
12
16
20
24
10
20
30
40
2
4
6
8
10
12

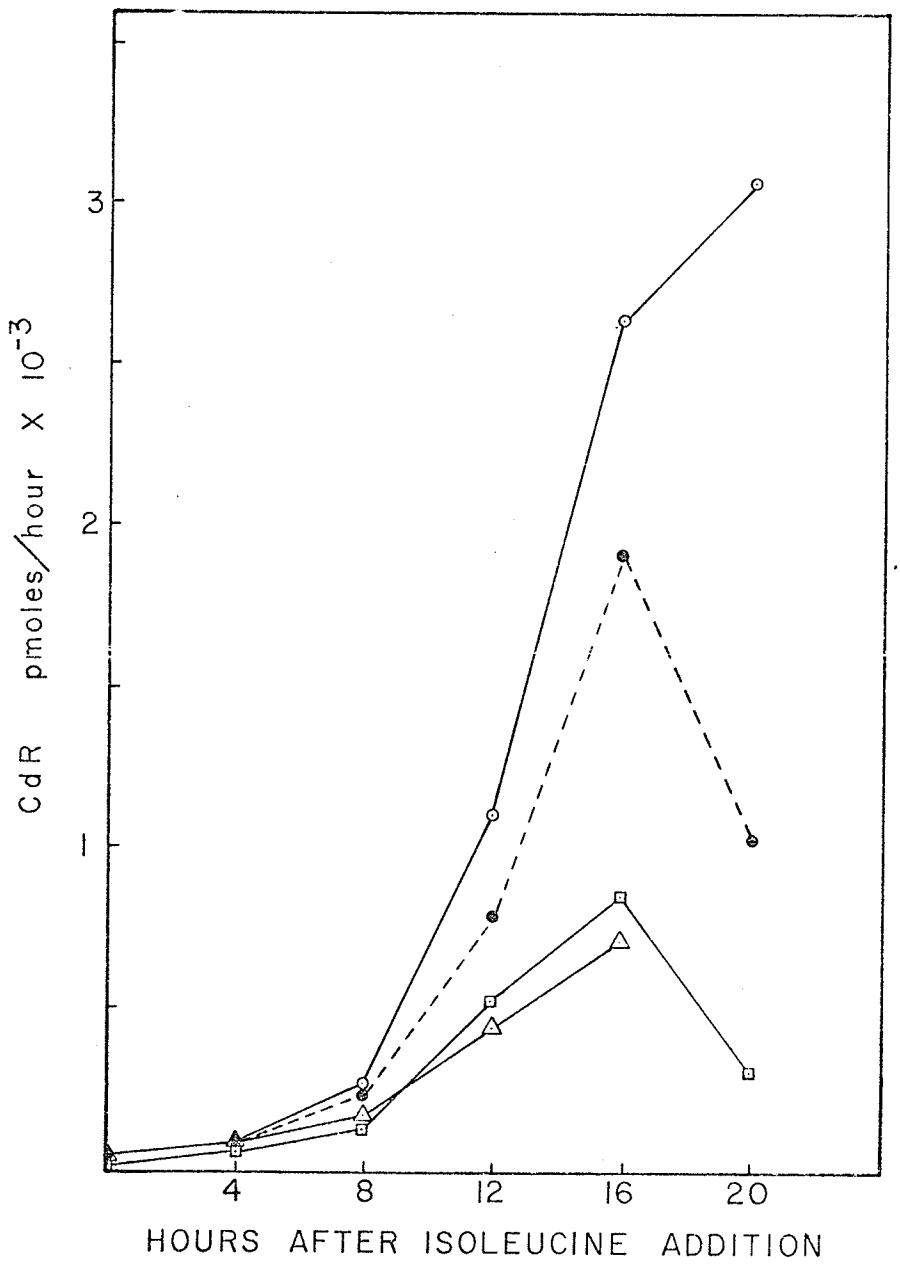
tion, but that by 15 hours essentially all the cells were engaged in DNA synthesis. When CDP reductase activity was measured in permeabilized CHO cells which were arrested in G1, only very low levels of the enzyme activity were found. However, upon the addition of isoleucine and the subsequent reinitiation of DNA synthesis, the level of CDP reductase greatly increased. In Figure 64B it can be seen that the CDP reductase level increased from 60 pmoles/hr in arrested cells to 560 pmoles/hr by 16 hours after isoleucine addition, and then gradually declined. This increase in CDP reductase activity paralleled the induction of DNA synthesis; both processes peaked at 16 hours after isoleucine addition (Fig 64).

The level of GDP reductase activity in isoleucine starved cells was some 100-fold greater than CDP reductase. The levels of GDP reductase, however, showed a pattern of induction similar to that of CDP reductase as the cells proceeded into the DNA synthetic phase. GDP reductase levels of 5500 pmoles/hr in arrested cells increased to 11,600 pmoles/hr 14 hours after isoleucine addition (Fig 64B). This is only a two-fold increase as compared to a ten-fold increase in CDP reductase activity during the same time period. Peterson and Moore (1976) reported a similar difference in degree of induction when CDP reductase activity was compared to ADP reductase activity in synchronized hamster fibroblasts.

It was of interest to determine whether the increase in ribonucleotide reductase levels seen in exponentially growing SC8 cells in the absence or presence of hydroxyurea (Table 25) occurred throughout the cell cycle or whether the increase occurred only during the DNA synthetic or S phase. To determine this, $GAT^{-}CHO$, SC8 and the revertant SC17 were all synchronized by isoleucine starvation and the levels of CDP reductase in the G1 arrested cells were followed as the cells resumed DNA synthesis after isoleucine addition. Figure 65 shows that all the cell lines had nearly identical levels of CDP reductase in the G1 arrested state and that during the period of maximum DNA synthesis,16 hours after isoleucine addition, the enzyme levels were significantly higher in SC8, both in the absence and presence of hydroxyurea, than in either the parental $GAT^{-}CHO$ or the revertant SC17. Sixteen hours after isoleucine addition, SC8 cells had almost three times higher CDP reductase levels in the absence and four times higher levels in the presence of hydroxyurea, than the parental $GAT^{-}CHO$.

7. Thymidine kinase levels in hydroxyurea-resistant cell lines: Since many enzymes involved in DNA synthesis (eg thymidine kinase, deoxycytidine deaminase, DNA polymerase) are induced at the same time as ribonucleotide reductase, the possibility existed that the mutation resulting in overproduction of ribonucleotide reductase in the S phase of SC8 cells, might also coordinately affect the levels of the other enzymes. In other words, the mutation may affect

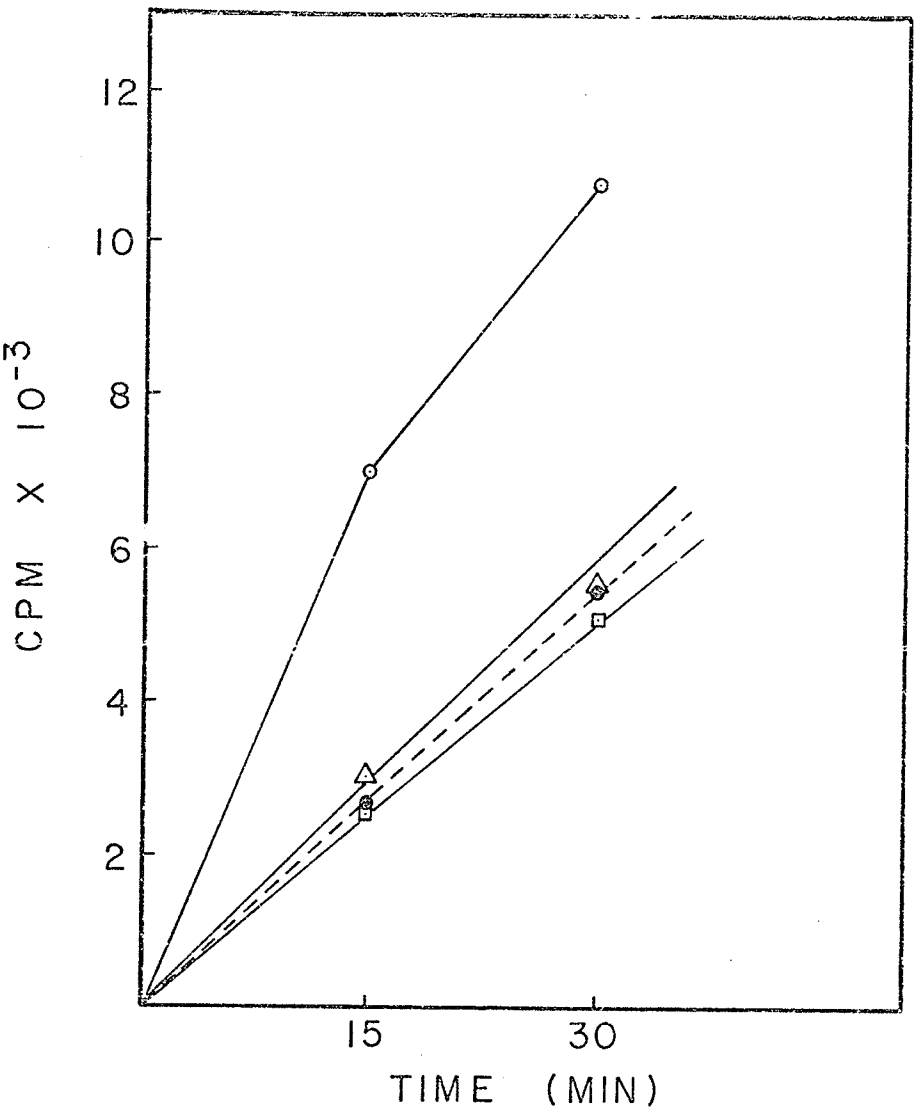
Figure 65: CDP reductase activity during the cell cycle of $GAT^{-}CHO$, $GAT^{-}HU^{R}$-12 SC17 and $GAT^{-}HU^{R}$-12 SC8. The cell lines were grown on 100 mm plates and arrested in the G1 phase of the cell cycle by isoleucine starvation as described in Materials & Methods. At zero time complete medium containing isoleucine was added to each plate of $GAT^{-}CHO$ (△), SC 17 (⊡), SC8 (●) and complete medium plus 0.26 mM hydroxyurea was added to additional plates of SC8 (⊙). At specific intervals, 5 X 10^{6} cells from plates of each culture were trypsinized, permeabilized and their CDP reductase level determined as described in Materials & Methods.


CdR pmoles/hour X 10^{-3}
3
2
1
4
8
12
16
20
HOURS AFTER ISOLEUCINE ADDITION

the levels of all the other enzymes if they were induced by the same cellular mechanism. To test this possibility, the hydroxyurea-resistant SC8 cell line was checked to see if it had elevated levels of thymidine kinase by assaying this enzyme in Tween-80 permeabilized cells. Figure 66 shows that exponentially growing GAT^{-}CHO, SC8 and the revertant SC17 cells all had identical levels of thymidine kinase. SC8 cells grown in the presence of 0.26 mM hydroxyurea, however, had almost twice the level of thymidine kinase when compared to the other cell lines growing in the absence of hydroxyurea (Fig 66).

Thus, it would appear that the mutation affecting levels of ribonucleotide reductase in SC8, does not coordinately affect the levels of thymidine kinase. The increase in thymidine kinase activity in the presence of hydroxyurea is similar to that seen with ribonucleotide reductase under the same conditions (Tables 25,26), however, and this observation suggests that a partially coordinated induction mechanism may exist for both enzymes.

Figure 66: Thymidine kinase levels in exponentially growing $GAT^{-}CHO$ (△), $GAT^{-}HU^{R}$-12 SC8 (●), $GAT^{-}HU^{R}$-12 SC17 (⊡) and $GAT^{-}HU^{R}$-12 SC8 which had been cultivated in the presence of 0.26 mM hydroxyurea (⊙). Cells were grown on 100 mm plates and permeabilized in the manner described for ribonucleotide reduction assays. Permeabilized cells (5×10^{6}) of each culture were incubated with the thymidine kinase assay mixture containing ^{3}H-thymidine substrate. Twenty µl samples of each reaction mixture were removed after 15 and 30 min incubation at $37^{o}C$ and the amount of radioactivity associated with phosphorylated thymidine compounds was determined (see Materials & Methods for details).


12
10
8
6
4
2
CPM X 10^{-3}
15
30
TIME (MIN)

F. INHIBITION OF RIBONUCLEOTIDE REDUCTASE BY NATURALLY OCCURRING DINUCLEOTIDE COMPOUNDS IN EUCARYOTIC CELLS

Endogenous inhibitors of ribonucleotide reduction have been found in a number of eucaryotic systems (Cory & Monley 1970, Vitols et al 1970, Elford 1972). The purification and physiology of these compounds have yet to be reported, however. Recently, LéJohn et al (1975) reported the isolation of highly phosphorylated dinucleoside compounds, designated HS2 and HS3, in the water mold Achlya. These compounds have been shown to interact with the various RNA polymerases in a complex manner (McNaughton et al 1975). Since little is known of the process by which eucaryotic organisms regulate the replication of their DNA, we wished to determine whether these unusual dinucleotide compounds might also affect DNA synthesis. Ribonucleotide reductase seemed a possible candidate for HS regulation both because it is known to be regulated by a large number of nucleotide compounds and because it is thought to represent the rate-limiting step in DNA synthesis and cell dividion (Elford et al 1970).

1. Ribonucleotide reductase activity in Achlya: When prepared as described in Materials and Methods, crude extracts of Achlya cells harvested 10 hours after spore germination contained at least 20 mg protein per ml. After passage through Sephadex G25 to remove endogenous inhibitors of low

molecular weight (Cory & Monley 1970), both purine and pyrimidine ribonucleotide reductions could be demonstrated. The activity increased linearly with protein concentration above 0.75 mg/assay. The reaction had an optimum temperature of 22°C and proceeded linearly for at least 60 min at this temperature.

The CDP and ADP reductase activities resembled those found in mammalian systems. Table 27 shows that CDP reduction in Achlya is dependent upon the presence of ATP. Similarly, ADP reduction requires dGTP. As expected, dATP, a general feedback inhibitor of this enzyme, inhibited both reactions. Also, hydroxyurea inhibited both CDP and ADP reduction to the same degree as it did the mammalian enzyme. Unlike the case with some mammalian systems (Moore 1969), ferrous ion did not stimulate the reaction.

2. Inhibition of ribonucleotide reductase by dinucleotide compounds: The unusual dinucleotides isolated from Achlya were tested for their effects on purine and pyrimidine ribonucleotide reductase activities in vitro. Both HS2 and HS3 significantly inhibited pyrimidine reduction at concentrations less than 0.1 mM. Figure 67A shows that both compounds decreased CDP reduction by 50% at 0.1 mM. Purine ribonucleotide reduction responded in a similar manner to HS3, being inhibited 50% by a concentration of 0.1 mM (Fig 67B). Figure 67B also shows that HS2 had no effect on ADP

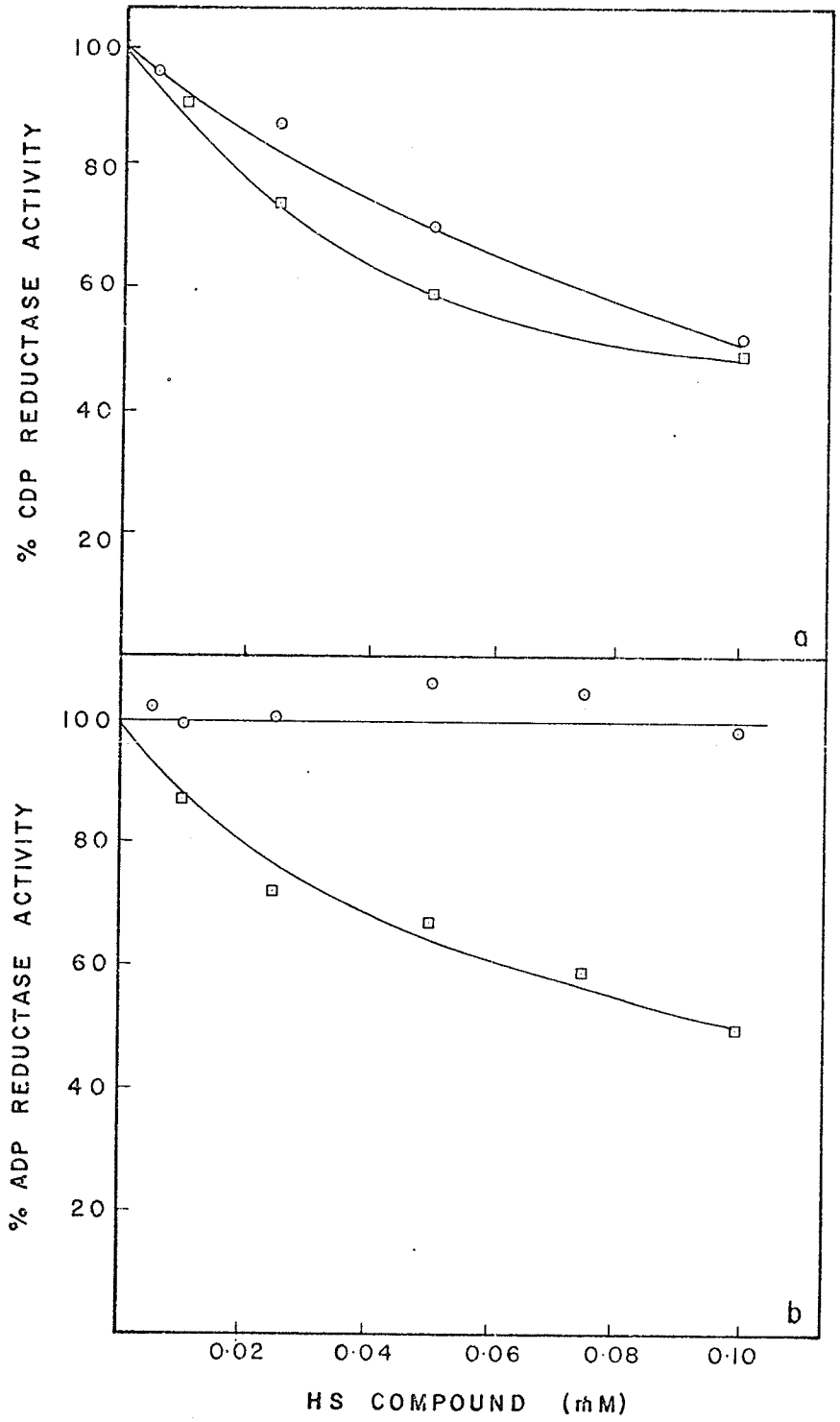
Table 27. Properties of ribonucleotide reductase from Achlya.

Omission or Addition	CDP Reductase Specific activity*	ADP Reductase Specific activity*
complete	1.92	0.77
- ATP	0.28	†
- dGTP	†	0.01
+ dATP (1 mM)	0.21	0.08
+ hydroxyurea (1 mM)	0.24	0.36
+ $Fe(NH_4)_2(SO_4)_2$ (0.1 mM)	1.95	0.63

*Specific activity is in nmoles deoxynucleoside formed per hour per mg extract protein.

†CDP reductase assay mixture contains no dGTP and ADP reductase assay mixture contains no ATP.

Figure 67: Inhibition of Achlya ribonucleotide reductase activity by HS2 and HS3 isolated from Achlya. CDP reductase (a) and ADP reductase (b) were assayed as described in Materials & Methods, with 0.80 mg extract protein per assay. Each point for HS3 (⊡) represents the average of duplicate assays from three separate experiments using two separate preparations of HS3 compound and cell extract. The points for HS2 (○) represent the average of duplicate assays from a single HS2 and enzyme preparation.


% CDP REDUCTASE ACTIVITY
100
80
60
40
20
a
% ADP REDUCTASE ACTIVITY
100
80
60
40
20
b
0·02
0·04
0·06
0·08
0·10
HS COMPOUND (mM)

reduction in the concentration range tested.

A number of ribonucleosides and their di- and triphosphate derivatives, as well as phosphate and pyrophosphate were tested for their effect on ribonucleotide reduction. Table 28 shows that, as expected, none of the compounds tested significantly inhibited CDP reduction at concentrations equivalent to or greater than those tested for the dinucleotide inhibitors.

3. Ribonucleotide reductase levels and HS3 pool sizes in vivo: The specific activity of CDP reductase was determined during the period from 8 to 15 hours after Achlya spore germination. As can be seen in Figure 68, CDP reductase activity peaked at 9 hours and then declined more than 10-fold within the next four hours. The rate of thymidine incorporation into acid precipitable material also peaked at 9 hours and subsequently declined along with ribonucleotide reductase levels. Also shown in Figure 68 is the rate of HS3 synthesis which began to increase at 9 hours and showed a dramatic peak at 11 hours (data courtesy of D. McNaughton). Cellular pool sizes of HS compounds are known to increase rapidly and are maintained after this time (McNaughton et al 1975). Thus, there is a very good correlation between the fall in both ribonucleotide reductase activity and thymidine incorporation into DNA and the increase in biosynthesis of HS3.

Table 28. Specificity of HS inhibition of Achlya CDP reductase.

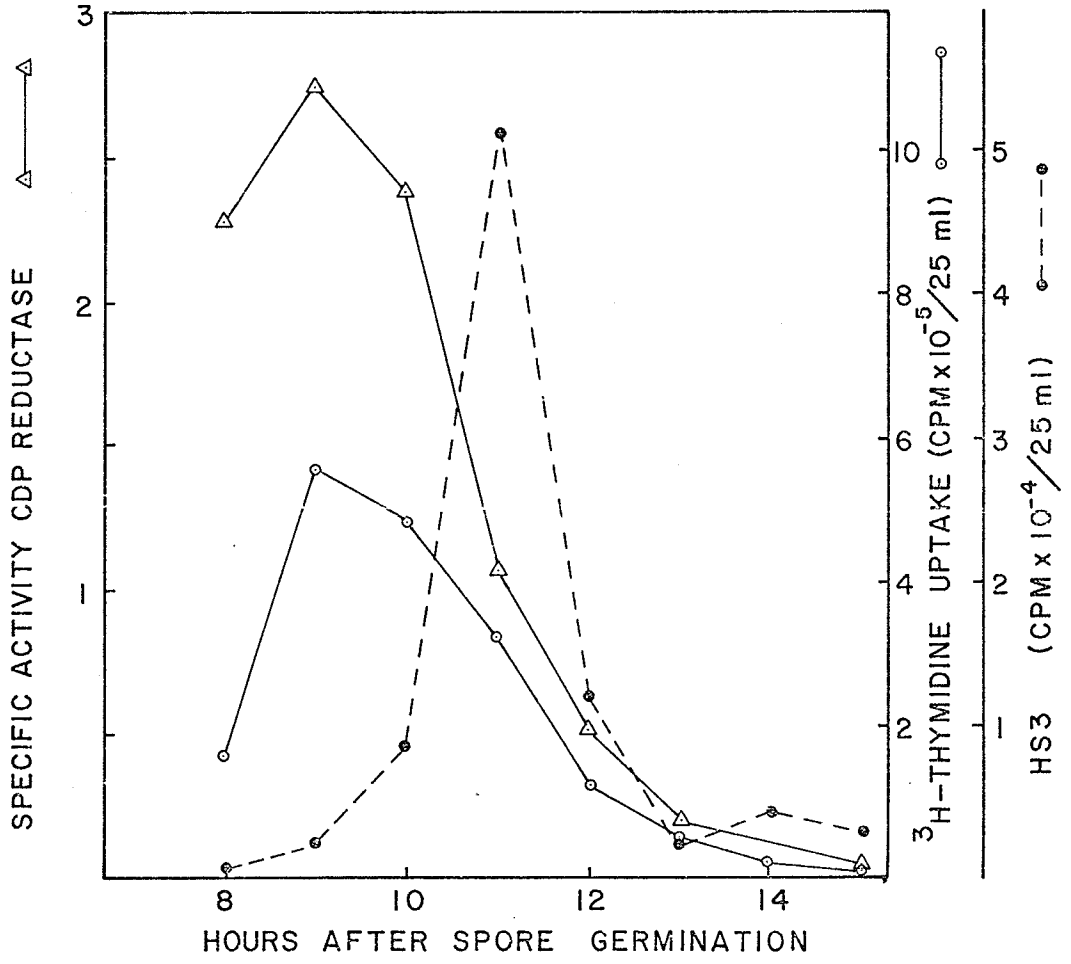
Addition*	% Activity	Addition*	% Activity
none	100% (2.0)†		
1. PO_4 (1 mM)	101	7. ADP	96%
2. P_2O_7 (5 mM)	102	8. GDP	96
3. Adenosine	99	9. UDP	101
4. Cytidine	92	10. ATP	104
5. Guanosine	98	11. CTP	91
6. Uridine	98	12. GTP	98
		13. UTP	96

*Unless otherwise stated, all additions were at 0.1 mM

†The control assay gave a rate of 2.0 nmoles deoxycytidine formed per hour.

Figure 68: Levels of ribonucleotide reductase, thymidine uptake and HS3 biosynthesis during the growth cycle of Achlya.

Fifteen litre carboys of glucose (5 g/l), yeast extract (0.5 g/l) medium were inoculated with 6×10^7 Achlya spores. The carboys were incubated at $28^{\circ}C$ with aeration and at hourly intervals, samples were filtered and cell extracts were prepared as described in Materials & Methods. Also, 25 ml volumes of the fungal culture were pulsed for 10 min with ^{3}H-thymidine or ^{32}P-ortho-phosphate as described in Materials & Methods. Specific activity of CDP reductase (△), thymidine uptake (⊙) and rate of HS3 bio-synthesis (●).

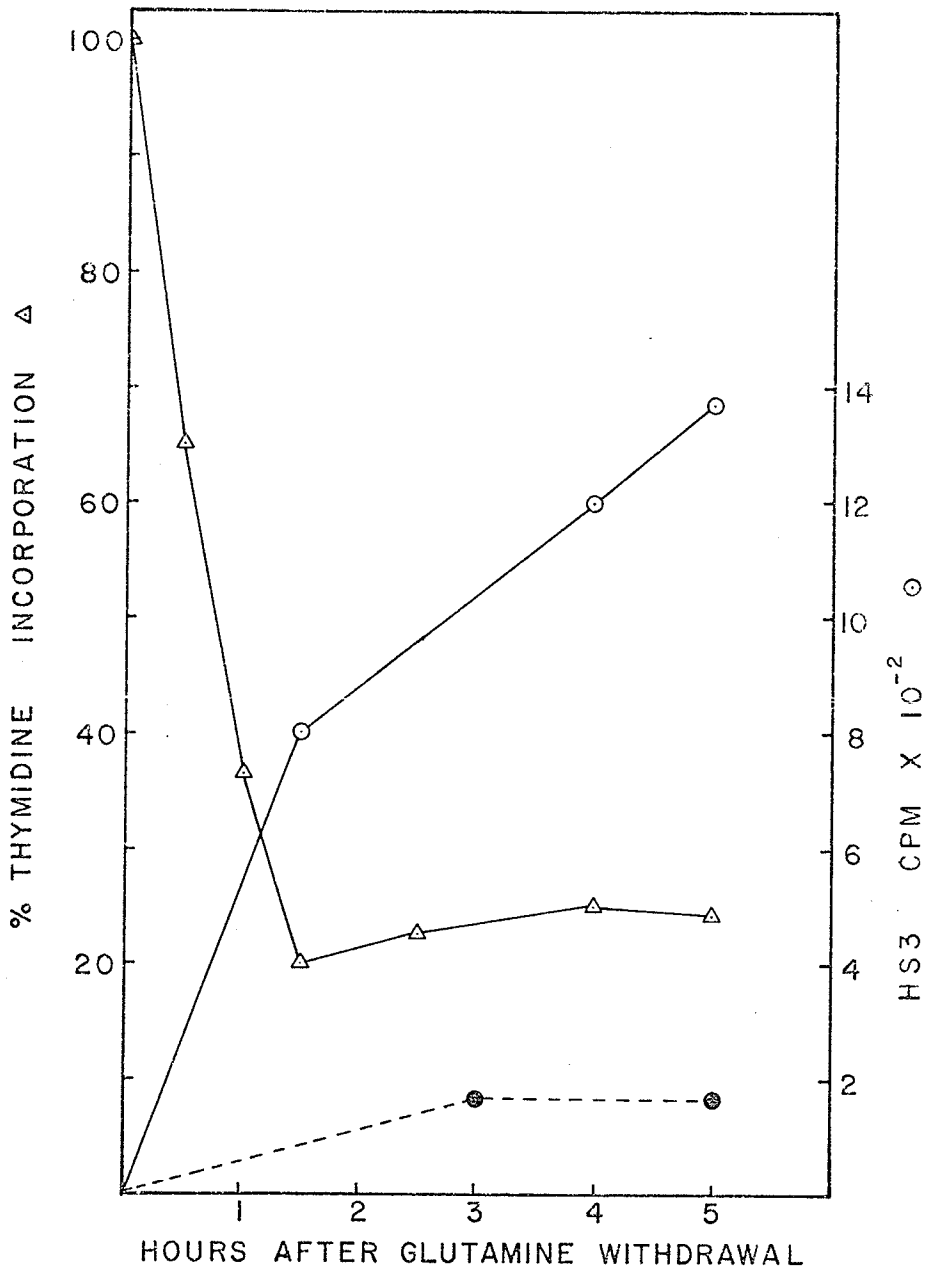

SPECIFIC ACTIVITY CDP REDUCTASE
3
2
1
8
10
12
14
HOURS AFTER SPORE GERMINATION
^{3}H-THYMIDINE UPTAKE (CPM x 10^{-5}/25 ml)
10
8
6
4
2
HS3 (CPM x 10^{-4}/25 ml)
5
4
3
2
1

4. Dinucleotide inhibitor in CHO cells: Goh et al (1977) have recently shown that a similar highly phosphorylated dinucleoside compound also exists in WT CHO cells. The HS3 compounds purified from either Achlya or CHO cells exhibited similar physical and chemical properties (Lewis et al 1977). Furthermore, the synthesis of the mammalian HS3 is greatly increased during glutamine starvation and may be connected with de novo and salvage pathways of nucleotide biosynthesis (Goh et al 1977).

Figure 69 shows that when glutamine is withdrawn from the CHO growth medium, HS3 pools dramatically increase within a few hours (HS3 data courtesy of S.H. Goh). During this increase in HS3 levels, DNA synthesis drastically declines. Sixty minutes after glutamine withdrawal, ^{3}H-thymidine uptake into acid precipitable material was inhibited by 65% (Fig 69). RNA synthesis on the other hand was inhibited only 7% in the same time period (Goh et al 1977) suggesting that the inhibition of DNA synthesis during this period was not due to a general lack of ribonucleotides caused by glutamine starvation. We wished to determine whether the inhibition of DNA synthesis could be attributed to an inhibition of CHO ribonucleotide reductase by the increased pool sizes of HS3.

5. HS3 inhibition of mammalian ribonucleotide reductase: CDP reductases from E. coli, Achlya and WT CHO cells were prepared and assayed as described in Materials and Methods.

Figure 69: Rate of DNA synthesis and pool sizes of HS3 during glutamine starvation in CHO cells. At zero time the medium was aspirated from 60 mm plates containing approximately 1 X 10^6 exponentially growing WT CHO cells each. Standard medium minus L-glutamine plus 10% dFCS was prewarmed and added to each plate. The amount of ^{32}P label incorporated into HS3 pools during continuous labelling with ^{32}P-orthophosphate (see Materials & Methods) was determined for glutamine starved cells (⊙) or control cells (●) incubated in medium containing L-glutamine. At intervals, plates were also pulsed for 10 min with ^{3}H-thymidine and the amount of ^{3}H label incorporated into acid precipitable (△) cell material measured as described in Materials & Methods. The amount of ^{3}H-thymidine incorporated into control cultures in the presence of glutamine was 25,000 cpm/10 min.


100
80
60
40
20
% THYMIDINE INCORPORATION Δ
14
12
10
8
6
4
2
HS3 CPM X 10⁻² ⊙
1
2
3
4
5
HOURS AFTER GLUTAMINE WITHDRAWAL

HS3 purified from either fungal or mammalian cells was used to inhibit the various CDP reductases (Table 29). At 50 μM of both CHO and Achlya HS3, there was only marginal inhibition of E. coli ribonucleotide reductase but significant inhibition of the fungal and mammalian enzymes. CHO CDP reductase was the most sensitive to inhibition by HS3 from either source, being inhibited 54% by CHO HS3 and 65% by Achlya HS3. It is not clear whether the lower inhibition seen with mammalian HS3 as compared to fungal compound is significant. Depending upon the preparation and length of storage, the exact amount of inhibition of CDP reduction by HS3 from either source varied somewhat. However, all preparations of HS3 from mammalian or fungal cells showed the same pattern of significant inhibition below 50 μM. Because of a far greater yield and ease of isolation from Achlya, HS3 from this source was used for the remaining kinetic inhibition studies.

Figure 70 shows the time course of CDP reduction in the presence and absence of 50 μM HS3. The reaction proceeded linearly in both cases for the standard incubation time of 60 min. HS3 inhibited the reaction by approximately 64%.

The effect of increasing HS3 concentrations on CDP reduction is shown in Figure 71. The reaction was inhibited by approximately 20% at 10 μM HS3, and the inhibition increased to about 75% at 60 μM. Significant inhibition can

Table 29. HS3 inhibition of CDP reductases from various sources.

Enzyme Source†	% Inhibition by 50 μM HS3 from: CHO	Achlya
E. coli	2	3
Achlya	20	40
CHO	54	65

†Extracts of the various cell types were prepared and chromatographed on Sephadex G25 as described in Materials and Methods. CDP reduction mas measured as described in Materials and Methods with the addition of 1 mg protein per assay.

Figure 70: CDP reduction in the presence of HS3. Partially purified WT CHO ribonucleotide reductase (250 µg protein) was added to the standard CDP reductase assay mixture. The reaction was allowed to proceed for various periods of time in the absence (⊙) or in the presence of 50 µM HS3 (⊡).

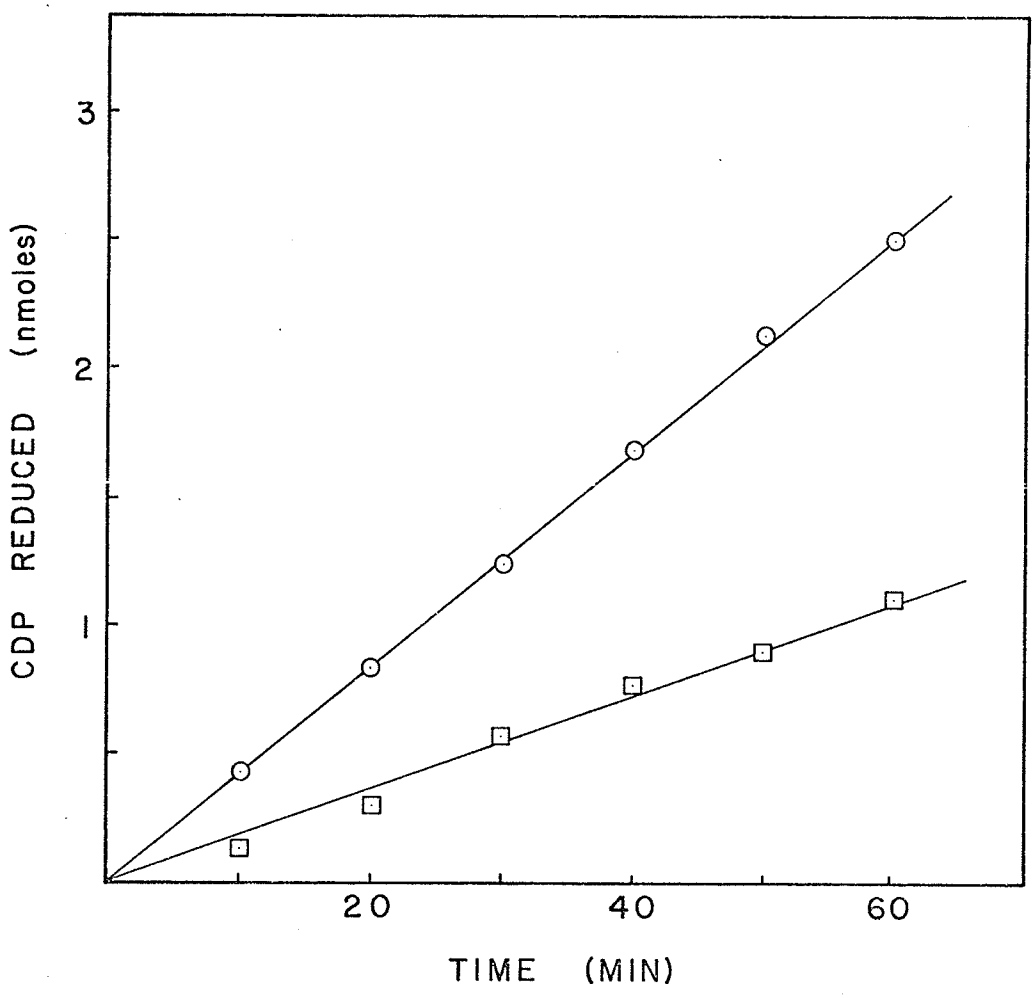
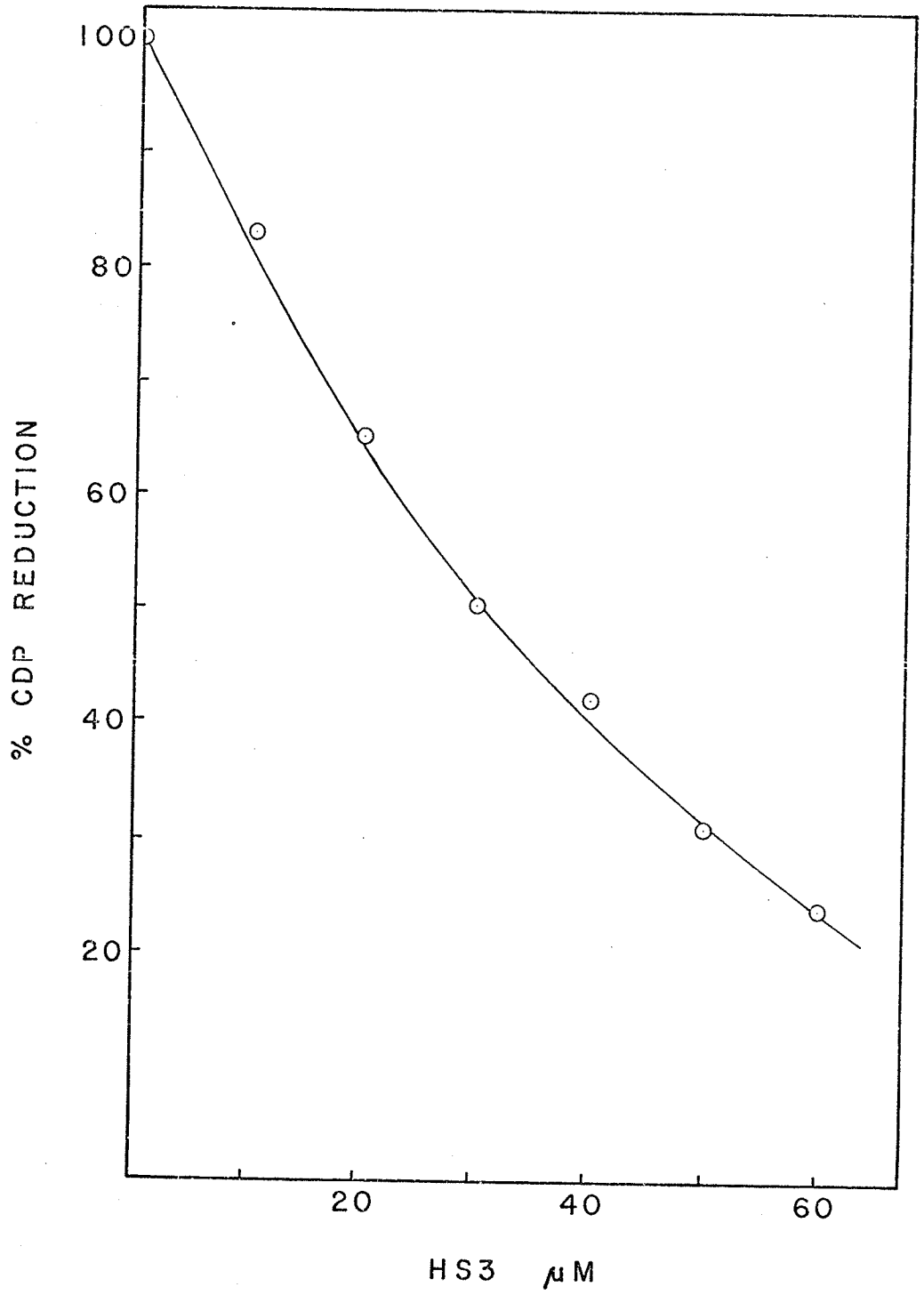

CDP REDUCED (nmoles)
3
2
1
20
40
60
TIME (MIN)

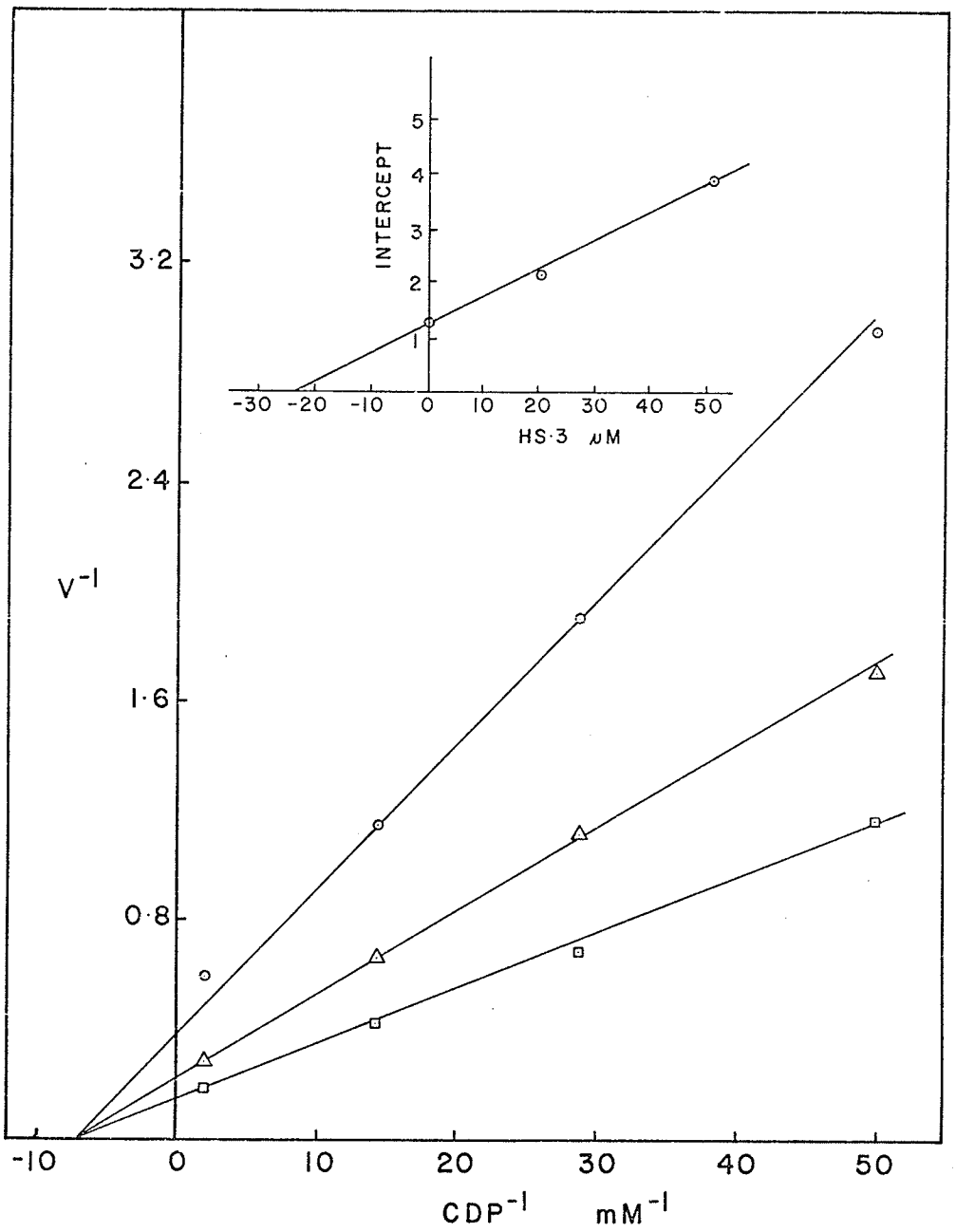
Figure 71: CDP reduction in the presence of varying concentrations of HS3. Partially purified CHO cell extract (250 µg protein) was added to the standard CDP reductase assay mixture containing varying amounts of HS3. The amount of CDP reduction is given as a percentage of the control reaction incubated in the absence of HS3 (1.9 nmoles/hour).


100
80
60
40
20
% CDP REDUCTION
20
40
60
HS3 μM

be seen with as little as 5 μM HS3. Clearly the HS3 compound is a potent inhibitor of CDP reduction in CHO extracts. Because of technical problems, it was not possible to determine whether the HS3 molecule was intact after the ribonucleotide reductase assay. However, a number of ribonucleosides and their di- and triphosphate derivatives as well as phosphate, pyrophosphate and glutamate all had no significant effect on CDP reduction when tested at concentrations equal to or far greater than those of HS3 causing significant inhibition (results similar to those in Table 28). Furthermore, the linearity of CDP reduction in the presence of HS3 (Fig 70) suggests that during the assay period the added HS3 was not being converted to a more or less inhibitory form by the CHO extract.

To determine more precisely the level of HS3 which significantly inhibited CDP reduction and also to determine whether the inhibition was due to a competition of HS3 with the ribonucleotide substrate, the rate of the reaction was measured with varying CDP concentrations. A Lineweaver-Burk plot (Fig 72) of the rate of CDP reduction versus CDP concentration was linear and yielded an apparent Km value of 0.13 mM for CDP in the presence or absence of HS3. Increasing HS3 concentrations noncompetitively inhibited the rate of CDP reduction without affecting the apparent Km for CDP. A replot of the ordinate intercepts versus HS3 concentration, gave an inhibitor constant (Ki) value of 23 μM

Figure 72: Double reciprocal plot of the variation in the rate of CDP reduction (hour/nmole) with CDP concentration (mM^{-1}) in the absence of HS3 (□), in the presence of 20 μM HS3 (△) or 50 μM HS3 (⊙). Inset shows a replot of the ordinate intercept versus HS3 concentration. A partially purified CHO cell extract (250 μg protein) was assayed in standard CDP reductase assay mixtures containing varying amounts of CDP and HS3.


INTERCEPT
5
4
3
2
1
-30 -20 -10 0 10 20 30 40 50
HS·3 μM
3·2
2·4
1·6
0·8
V⁻¹
-10 0 10 20 30 40 50
CDP⁻¹ mM⁻¹

for HS3 (inset Fig 72).

When the partially purified CHO cell preparation was tested for its reducing activity with other ribonucleoside diphosphate substrates, both ADP and GDP reduction could readily be demonstrated with the standard reaction mixtures. Figures 73 and 74 show Lineweaver-Burk double reciprocal plots of ADP and GDP reduction in the presence of increasing concentrations of HS3. As with CDP reduction, HS3 noncompetitively inhibited both reactions. The apparent Km for ADP was 0.2 mM, while the apparent Km for GDP was 0.03 mM. Replots of the intercepts yielded HS3 Ki values of 14 μM for ADP reduction and 16 μM for GDP reduction (insets Figs 73, 74).

Figure 73: Double reciprocal plot of the variation in the rate of ADP reduction (hour/nmole) with ADP concentration (mM^{-1}) in the absence of HS3 (■), or in the presence of 15 µM HS3 (⊡), 30 µM HS3 (●) and 50 µM HS3 (⊙). Inset shows a replot of the ordinate intercept versus HS3 concentration. A partially purified CHO cell extract (250 µg protein) was assayed in standard ADP reductase assay mixtures containing varying concentrations of ADP and HS3.

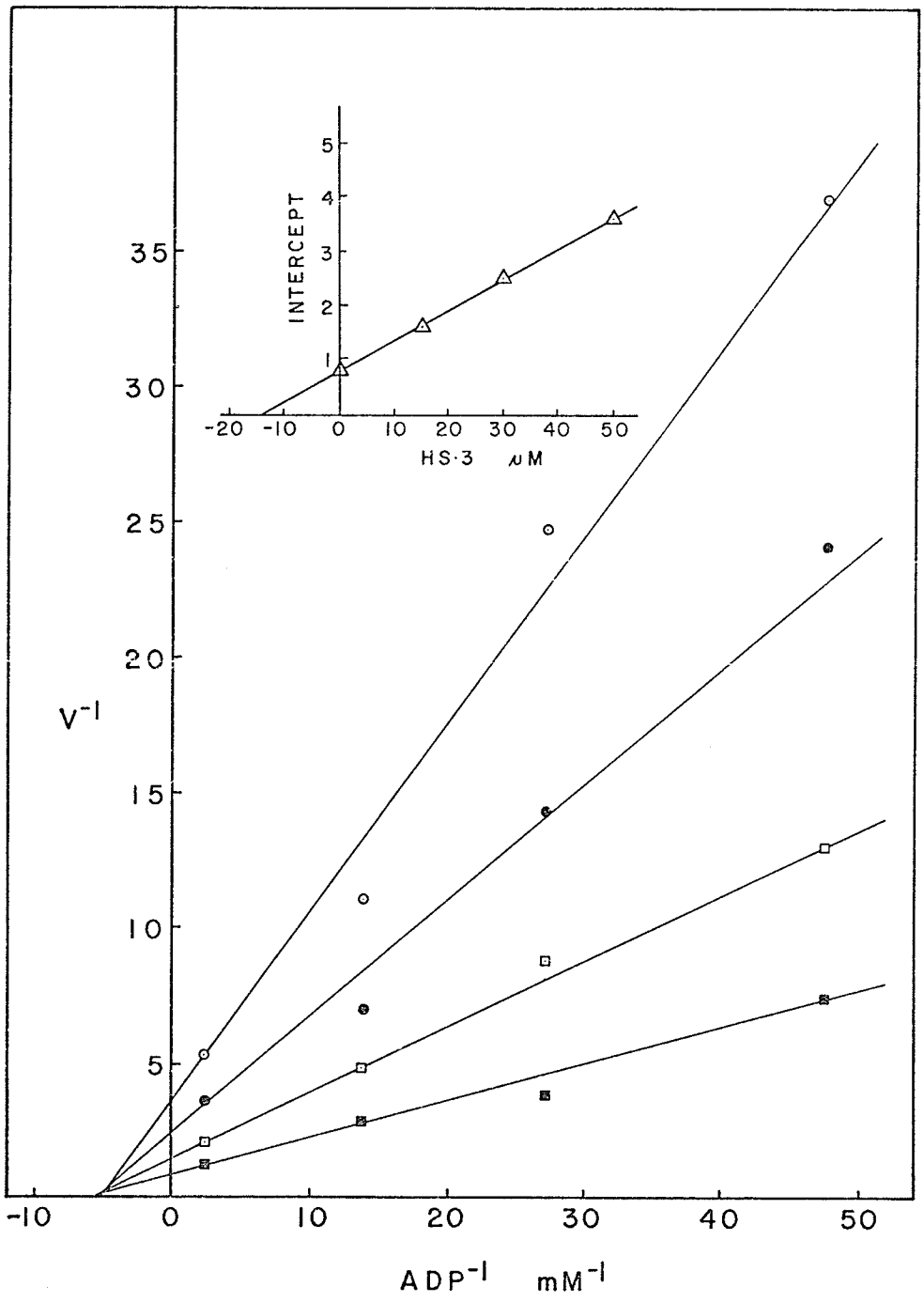
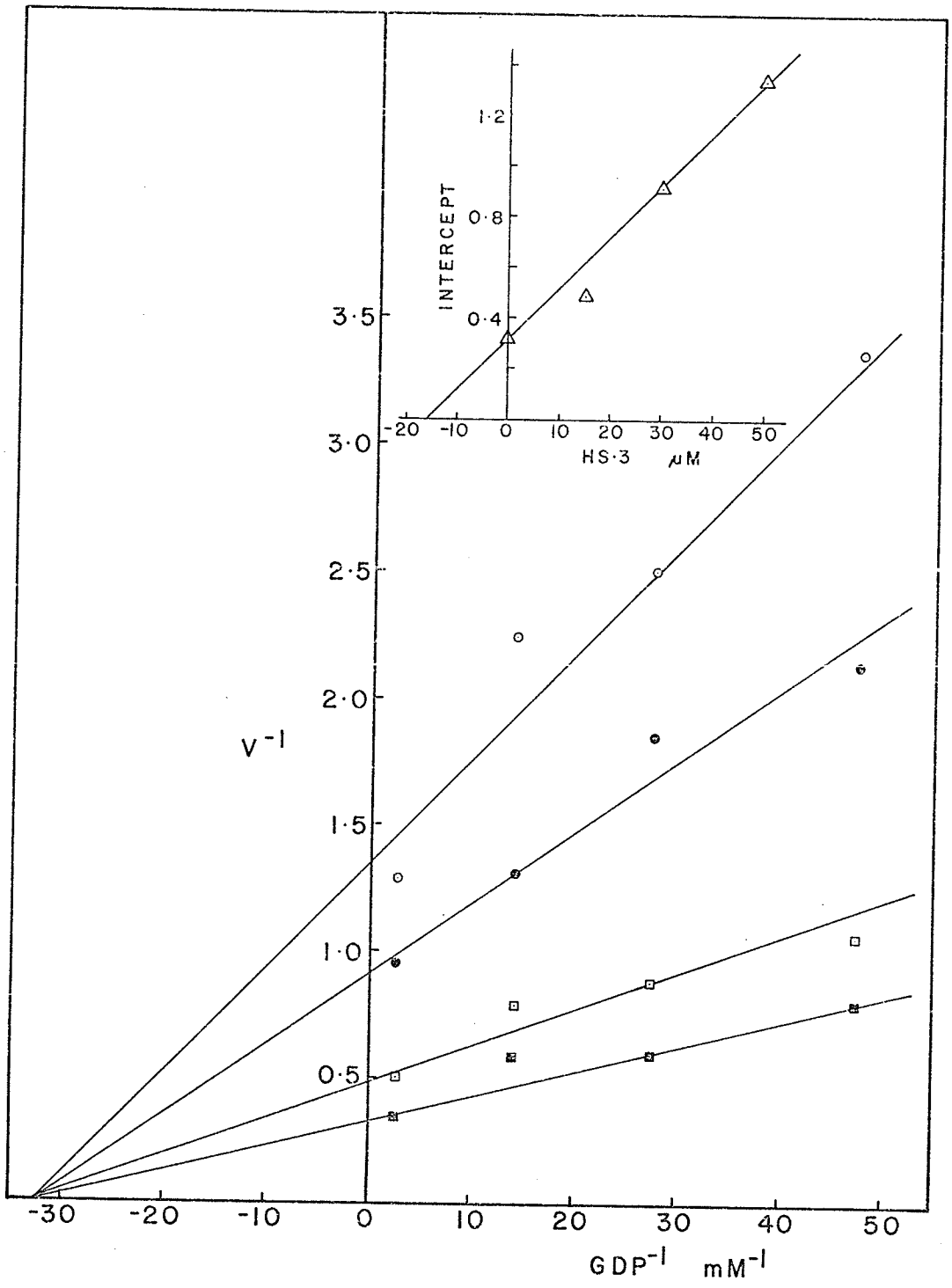

INTERCEPT
HS·3 μM
V⁻¹
ADP⁻¹ mM⁻¹

Figure 74: Double reciprocal plot of the variation in the rate of GDP reduction (hour/nmole) with GDP concentration (mM^{-1}) in the absence of HS3 (■), or in the presence of 15 µM HS3 (⊡), 30 µM HS3 (●) and 50 µM HS3 (⊙). Inset shows a replot of the ordinate intercept versus HS3 concentration. A partially purified CHO cell enzyme preparation (250 µg protein) was assayed in standard GDP reductase assay mixtures containing varying concentrations of GDP and HS3.


INTERCEPT
1·2
0·8
0·4
-20
-10
0
10
20
30
40
50
HS·3 μM
3·5
3·0
2·5
2·0
1·5
1·0
0·5
V⁻¹
-30
-20
-10
0
10
20
30
40
50
GDP⁻¹ mM⁻¹

DISCUSSION AND CONCLUSIONS

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A. Effect of Hydroxyurea on Wild-type CHO Cells

It is clear from the data presented that hydroxyurea has a potent physiological effect on CHO cells in culture. At a concentration of 0.33 mM, hydroxyurea halts CHO cell division within 24 hours (Fig 2) and reduces the number of cells able to form colonies by 10^{-5} (Fig. 5). The drug was found to almost completely inhibit DNA synthesis within 15 min after addition to an exponentially growing CHO cell population (Fig 3). These results are all consistent with the inhibitory effects of hydroxyurea previously reported for hamster cells (Mohler 1964, Sinclair 1967) as well as for other mammalian cells in culture (Kim *et al* 1967, Bacchetti & Whitmore 1969).

Hydroxyurea has been shown to inhibit the activity of CDP reductase *in vitro* (Elford 1968, Moore 1969) and the results with the partially purified CHO cell enzyme confirm this finding. Hydroxyurea inhibited CDP reduction potently, exhibiting a Ki value of 0.08 mM (Fig 40). Similarly, hydroxyurea inhibited both ADP and GDP reduction *in vitro* with Ki values of 0.13 mM and 0.07 mM respectively (Figs 46, 51).

Guanazole, which has also been reported to inhibit

cell division and DNA synthesis in mammalian systems (Brockman 1970), was found to act on CHO cells in an analagous fashion to hydroxyurea. Although it is less potent on a molar basis than hydroxyurea, guanazole had almost identical effects. At a concentration of 2 mM, guanazole halted DNA synthesis (Fig 3) and cell division (Fig 2) and reduced the relative plating efficiency of the CHO cells to 10^{-5} (Fig 6). Similarly guanazole inhibited the partially purified CHO cell CDP reductase in vitro, with a Ki value of 0.57 mM (Fig 41).

Although many molecular mechanisms have been proposed for the cytotoxicity of hydroxyurea, the majority of evidence supports an inhibition of ribonucleotide reduction as the primary lethal site of action. The results reported here for WT CHO are consistent with this hypothesis. The concentration of hydroxyurea required to reduce the cellular plating response by 50% was 0.1 mM (Fig 5). This value is almost identical to the Ki values of 0.08 mM, 0.13 mM and 0.07 mM found for 50% inhibition of CDP, ADP and GDP reductase activities in vitro. Furthermore, the concentration of guanazole required to reduce CHO cell plating efficiency by 50% (0.6 mM) is practically the same as that required to inhibit CDP reduction by 50% (0.57 mM) (Fig. 41).

In contrast, many other cellular effects attributed to hydroxyurea are observed only at much higher concentrations. Inhibition of glucosamine uptake, for example, occurred only at 10 mM (Hawtrey et al 1974); and degradation of bacterial DNA occurred at 200 mM (Jacobs & Rosenkranz 1970) and of Tetrahymena DNA only at 50 mM (Cameron & Jeeter 1973). On the other hand, the 1 mM concentrations of hydroxyurea routinely used for mammalian cell synchronization have been shown to be without effect on chromosome structure (Yu & Sinclair 1968).

The correspondence between cellular killing and inhibition of target enzyme has not often been observed with other drugs. For example, it requires 10 times more methotrexate to reduce the RPE of CHO cells to 0.5 than it does to reduce dihydrofolate reductase activity in vitro by 50% (Flintoff et al 1976a). A similar disparity has been reported for α-amanitin cytotoxicity and inhibition of RNA polymerase II in CHO cells (Chan et al 1972). It has been suggested that the concentrations of these drugs within the cells are much less than in the surrounding growth medium (Chan et al 1972). This could be because of a partial permeability barrier or to a degradation of the drug by the cells. The results with hydroxyurea suggest that the intra- and extracellular concentrations of the drug are equivalent. Hydroxyurea is a small molecule with

no charge at physiological pHs (the pKa of the hydroxyl group is 10.6) and thus it may be expected to diffuse freely into the cell. Furthermore, it has been shown that cell extracts do not significantly degrade hydroxyurea (Table 16). Thus the drug has inherent advantages as a selective agent for resistant cell mutants.

B. Selection and Characterization of Hydroxyurea-resistant Cell Lines

At a frequency of approximately 1 in 10^5, CHO cells can survive and form colonies in the presence of 0.33 mM hydroxyurea (Fig 5). Such clones were isolated in a single step and found to be viable in hydroxyurea at concentrations up to 20 times greater than the concentration causing inhibition of WT cell PE (Figs 8, 22, Table 11). The three independent clones characterized in detail——HU^R-2, HU^R-4 and GAT^-HU^R-12 SC8——when compared to WT CHO cells at 50% RPE exhibited 19-, 15- and 6-fold more resistance respectively. All three hydroxyurea-resistant cell lines were able to grow, albeit at a slightly reduced rate, in concentrations of hydroxyurea which reduced wild-type CHO plating efficiency 10^{-4} to 10^{-5} (Figs 9, 10, 23).

The majority (9 or 12) of hydroxyurea-resistant clones maintained their resistant phenotype upon extensive cultivation in the absence of hydroxyurea (Tables 2, 11).

HU^R-2 and HU^R-4, for example, maintained high drug-resistance for over two years in serial cultivation. The loss of resistance of one of the clones (GAT^-HU^R-12) was probably due to an accumulation of hydroxyurea-sensitive revertants which grow more quickly (Section B, Table 14).

Reconstruction experiments indicated that the expression of hydroxyurea-resistance and the frequency of hydroxyurea-resistant colonies was independent of cell densities up to 5×10^5 cells per 100 mm selection plate (Table 3, Fig 7 Plate 6). For example, 400 HU^R-2 cells plated in the presence of 0.4 mM hydroxyurea gave rise to almost the same number of colonies per plate whether or not 5×10^5 wild-type cells were included (Table 3). Above 5×10^5 cells per 100 mm plate, the number of hydroxyurea-resistant colonies declined rapidly (Fig 7). The reason for this is not known. Reduced mutant recovery at high cell densities has frequently been reported in drug-resistant cell selection and has been attributed to such events as metabolic cooperation, exhaustion of medium or an accumulation of toxic substances (Shapiro et al 1972, Clements 1975).

Luria-Delbrück fluctuation analyses (Luria & Delbrück 1943) indicated that spontaneous appearance of

hydroxyurea-resistant cells in wild-type CHO cell populations occurred randomly and at a rate of 4.8×10^{-6} per cell per generation at a hydroxyurea concentration of 0.33 mM (Tables 4, 5). The mutation rates reported for mammalian cell mutants vary considerably depending upon the selective conditions and cell lines employed (Clements1975). The rate of appearance of hydroxyurea-resistant cells is consistent with many other drug-resistant selection systems (Shapiro et al 1972, Harris 1971, Sibley and Tomkins 1973) and is well within the high rate of 1×10^{-3} per cell per generation reported for immunoglobulin variants of mouse P3 cells (Baumal et al 1973) and the low rate of 5×10^{-8} for ouabain-resistance in CHO cells (Baker et al 1974).

Studies with the mutagen EMS indicate that it is capable of increasing the frequency of hydroxyurea-resistant cells by a factor of approximately 10 (Fig 12). This is similar to the effect that EMS has on the frequency of methotrexate-resistant cells (Flintoff et al 1976a) and the frequency of colchicine-resistant cells (Till et al 1973) in CHO cultures.

The ribonucleotide reductase activities of the two highly hydroxyurea-resistant cell lines, HU^R-2 and HU^R-4, were examined for sensitivity to hydroxyurea. CDP reductase activities in crude extracts of both cell lines were found to be significantly more resistant to the drug when compared

to wild-type activity (Fig 32). Furthermore, as expected from the cellular responses, the CDP reductase activity was also cross-resistant to inhibition by guanazole (Fig 33). On the other hand, response to the unrelated inhibitor, dATP, was indistinguishable in wild-type and drug-resistant cell lines (Table 22).

The increase in resistance of CDP reductase to hydroxyurea was not due to an increased degradation of the drug. Hydroxyurea, incubated with cell extracts under ribonucleotide reductase assay conditions, gave the same colorimetric reaction and inhibitory effect as control solutions of hydroxyurea (Table 16). Furthermore, the cross-reacting compound guanazole was not converted to hydroxyurea by cell extracts (Table 16).

When ribonucleotide reductase was partially purified from HU^R-2 and compared to the activity purified from wild-type CHO cells, significant differences were seen in the hydroxyurea Ki values with all three ribonucleotide substrates tested. The HU^R-2 Ki values were approximately 5-fold higher for CDP reduction, 3-fold higher for ADP reduction and 6-fold higher for GDP reduction than the wild-type activities (Table 20). As expected, the HU^R-2 enzyme also exhibited cross-resistance to guanazole, HU^R-2 CDP reductase having a 6-fold higher Ki than the wild-type

enzyme (Table 20).

The most logical explanation for the increased Ki values observed is that the HU^R-2 cell line has a structurally altered ribonucleotide reductase whose activity is less sensitive to inhibition by hydroxyurea or guanazole. Similar alterations in target enzymes have been reported in a number of drug-resistant CHO cell lines including those resistant to α-amanitin (Chan et al 1972, Lobban et al 1976), ouabain (Baker et al 1974), fluorocitrate (Wright 1975), methotrexate (Flintoff et al 1976a), diphtheria toxin (Moehring & Moehring 1977) and emetine (Gupta & Siminovitch 1977).

Hydroxyurea-resistance at the cellular level is accompanied by an increased resistance of ribonucleotide reduction to hydroxyurea inhibition. This is strong evidence that, at least for the concentrations tested, the primary site of action of this drug is an inhibition of the enzyme, ribonucleotide reductase. Similarly, the cross-resistance observed at both the cellular and enzyme level to the inhibitory effects of guanazole would indicate that this drug also owes its cytoxicity to an inhibition of ribonucleotide reductase. Because it has not been possible to purify mammalian ribonucleotide reductase to homogeneity, it cannot be ruled out that the alteration leading

to increased Ki values has occurred with some other component of the enzyme preparation. However, such a component would still have to be a large molecule which copurifies with the ribonuceotide reductase enzyme and is intimately involved in ribonucleotide reduction.

The 5- or 6-fold increase in hydroxyurea Ki values of the HU^R-2 ribonucleotide reductase activity cannot explain the almost 20-fold increase in cellular PE resistance to hydroxyurea exhibited by this cell line (Fig 8). However, it is known that the levels of ribonucleotide reductase are under cellular control. E. coli cells when starved of thymine produce much higher levels of the enzyme, and this fact led Biswas et al (1965) to suggest that the ribonucleotide reductase levels are normally repressed by a thymine derivative. Similarly, the levels of ribonucleotide reductase are known to fluctuate during the course of the mammalian cell cycle (Turner et al 1968, Peterson & Moore 1976). Furthermore, hydroxyurea has been reported to increase the levels of CDP reductase during the S phase of synchronous hamster cells (Murphee et al 1969). The levels of ribonucleotide reductase were measured in HU^R-2 cells permeabilized by Tween-80 treatment. Significantly higher levels of CDP and GDP reductase were found in the presence or absence of hydroxyurea when

compared to the wild-type enzyme levels. When grown in the presence of 0.26 mM hydroxyurea, HU^R-2 had some 7-fold higher levels of CDP reductase and 2-fold higher levels of GDP reductase than the wild-type CHO cell line (Table 26). These increased levels combined with a 5- or 6-fold increased resistance of the enzyme would presumably be adequate to explain the 20-fold resistance of the HU^R-2 cellular plating response.

If the CHO genome is functionally diploid, then two alleles of ribonucleotide reductase may be expressed in wild-type cells. The resistant cell lines may contain structural alterations in only one of the alleles and thus only half of the total ribonucleotide reductase activity produced would have increased resistance to inhibition by hydroxyurea. An increase in total ribonucleotide reductase activity would result in higher levels of the resistant enzyme, the normal enzyme being preferentially inhibited by hydroxyurea. Growing the cells in the presence of hydroxyurea would then result in sufficient induction of total ribonucleotide reductase activity to allow DNA replication and cell division to proceed normally. It has been reported that both α-amanitin-resistant rat myoblast cells (Somers et al 1975) and human diploid fibroblasts (Buchwald & Ingles 1976) contain two forms of the target molecule, RNA polymerase II, one with wild-type sensitivity

and one with decreased sensitivity to the drug. Thus the α-amanitin-resistant phenotype is expressed in a codominant fashion with the wild-type phenotype. The results with the hydroxyurea-resistant CHO cell lines HU^R-2 and HU^R-4 are consistent with a similar mechanism.

The results with the GAT^-HU^R-12SC8 (SC8), cell line which shows only a 6-fold increase in RPE in the presence of hydroxyurea, suggest that an increase in ribonucleotide reductase levels alone is sufficient to account for this level of resistance. Unlike HU^R-2 and HU^R-4, SC8 ribonucleotide reductase shows no increase in resistance to hydroxyurea inhibition in vitro. However, extracts of SC8 contained almost 10-fold higher levels of CDP reductase than wild-type cell extracts (Fig 57). Furthermore, revertants and partial revertants isolated from SC8 show enzyme levels closer to wild-type levels (Fig 57). When SC8 was tested for ribonucleotide reductase levels utilizing permeabilized cells, exponentially growing cells were found to have 4-fold higher CDP reductase levels and 7-fold higher levels of GDP reductase than similarly grown wild-type cells. The revertant again had enzyme levels approaching those of the wild-type (Table 25). Furthermore, SC8 cells growing exponentially in the presence of hydroxyurea were able to increase their ribonucleotide reductase levels by an

additional factor of almost two. The resulting 10-fold higher levels of CDP and GDP reductase would be adequate to explain the 6-fold increase in PE when compared to wild-type CHO cells. These results with the $GAT^{-}HU^{R}$-12SC8 cell line suggest that this cell line owes its hydroxyurea-resistance solely to an elevation in ribonucleotide reductase activities. Such an increase may have resulted from a mutation in a regulatory gene for the enzyme. Alternatively, a more active enzyme may have resulted from an alteration in ribonucleotide reductase affecting its intrinsic specific activity. It is difficult to differentiate between these possibilities due to the lack of purity of the mammalian enzyme.

The increased levels of CDP reductase in SC8 appear to occur only during the DNA synthetic or S phase (Fig 65). This fact raised the possibility that the mutation affecting ribonucleotide reductase levels may also affect other DNA synthetic enzymes which are known to be coordinately induced (Elford et al 1970). The levels of one such enzyme, thymidine kinase,however, were shown to be equivalent in the drug-resistant SC8, the parental wild-type and revertant cell lines (Fig 66). SC8 cells grown in the presence of hydroxyurea exhibited a two-fold higher level of thymidine kinase than those grown in the absence

of the drug (Fig 66). This observation is similar to the 2-fold increase in ribonucleotide reductase seen under the same culture conditions (Table 25, Fig 65) and it suggests that the mechanism of hydroxyurea-induced elevation of both ribonucleotide reductase and thymidine kinase may be the same.

The $GAT^{-}HU^{R}$-12SC8 cell line appears to be similar to the Class III methotrexate-resistant CHO cell lines reported by Flintoff et al (1976a). The class III mutants contain approximately 10-fold more dihydrofolate reductase activity when compared to wild-type CHO cell lines. The methotrexate-resistant cell system appears to be similar to the hydroxyurea-resistant system in many respects. In both systems the altered phenotype is stable, the frequency of drug-resistant cells is increased 10-fold by mutagen treatment and cell lines with structurally altered enzymes (Class I) and increased enzyme activity (Class III) occur. Thus both methotrexate-resistance and hydroxyurea-resistance in CHO cells satisfy the majority of the criteria for classification as genetic mutants (Flintoff et al 1976a).

Methotrexate-resistant cells can also exhibit a decreased permeability of whole cells to the drug (Class II mutants). Although this is a common mechanism of resistance for many other drugs (Bosmann 1971, Till et al 1973, Ling

& Thompson 1974), such a class of permeability mutants has not yet been demonstrated for hydroxyurea-resistance.

The only other report of alterations induced in ribonucleotide reductase activity was by Meuth and Green (1974). They showed that 3T3 cells selected for resistance to ara-C had a CDP reductase activity in crude extracts which was desensitized to dATP inhibition. Since ara-C and its phosphorylated derivatives have been shown to have little effect on mammalian ribonucleotide reductase (Moore & Cohen 1967) it is not clear why altered ribonucleotide reductase would increase cell resistance to the drug. The authors have suggested several mechanisms whereby the increased production of deoxycytidine by the altered enzyme would counteract the inhibitory effect of ara-C (Meuth & Green 1974, Meuth et al 1976). The hydroxyurea-resistant cell line HU^R-2 shows no obvious cross-resistance to ara-C (Table 6) suggesting that the alteration in CDP reductase from the ara-C-resistant cell line is different from the alteration present in the HU^R-2 enzyme.

Meuth and Green (1974) also isolated cells resistant to deoxyadenosine from the ara-C resistant cell line. Such cell lines were found to have 4- to 10-fold elevated levels of CDP reductase activity, but there was no further increase in resistance of the enzyme activity to

dATP inhibition. Selection of cells resistant to deoxyguanosine resulted in neither increased CDP reductase levels nor altered enzyme response to any of the effectors dGTP, dATP or dTTP. Since this system has not been well characterized genetically and the variants isolated in multiple selection steps, it is difficult to compare this system with the hydroxyurea-resistant CHO cell lines reported here.

C. Cross-resistance of Hydroxyurea-resistant Cell Lines to Other Compounds

Table 6 shows that the hydroxyurea-resistant cell line HU^R-2 is cross-resistant to four other compounds tested: guanazole, N-carbamoyloxyurea, formamidoxime and hydroxyurethane. Except for guanazole, these compounds are structurally very similar to hydroxyurea (Table 8) and may be expected to have similar modes of action. That all these compounds owe their primary cytotoxicity to an inhibiition of ribonucleotide reductase is strongly suggested. Cell lines selected for resistance to any of the four compounds also exhbit some cross-resistance to all four compounds plus hydroxyurea (Table 7). Furthermore, when the CDP reductase activity of dialysed crude extracts from cell lines resistant to hydroxyurea, N-carbamoyloxyurea or guanazole were tested for their response to the drugs

in vitro, all the cell line extracts exhibited enzyme activity with increased Ki values for the selecting drug and in most cases increased Ki values for the other drugs (Table 22). In control experiments, the resistant cell line ribonucleotide reductase activities had similar Ki values for dATP inhibition (Table 22).

It has been suggested that hydroxyurea owes its cytotoxicity to an intracellular conversion to N-carbamoyloxyurea (Jacobs & Rosenkranz 1970, Cameron & Jeeter 1973). The results reported in this work do not support this suggestion. When compared to hydroxyurea on a molar basis, N-carbamoyloxyurea is less effective in inhibiting CHO plating efficiency (Table 7) and CHO CDP reductase (Table 22). Furthermore, hydroxyurea incubated with CHO cell extracts did not lose any colorimetric reactivity which would be expected if it were being converted to N-carbamoyloxyurea (Tables 8, 16, Fig 14).

D. Expression of Hydroxyurea-resistance in Hybrid Cells

Hybrids were formed from hydroxyurea-resistant and wild-type (hydroxyurea-sensitive) CHO cell lines under conditions non-selective for the hydroxyurea-resistant trait. These hybrids were examined to determine whether the hydroxyurea-resistant phenotype is expressed when the

wild-type allele is known to be present. In all cases tested, the hydroxyurea-resistant phenotype appeared to behave in a codominant fashion. The hybrid cell lines tested included hybrids between HU^R-2 and two different hydroxyurea-sensitive cell lines (tsH1 O^R-2A and GAT^-O^R-1), a hybrid between HU^R-4 and GAT^-O^R-1, and finally a hybrid between GAT^-HU^R-12SC8 and tsH1 (Table 9). In all four cases the hybrid cell lines exhibited a plating efficiency in the presence of hydroxyurea close to that exhibited by the hydroxyurea-resistant parental cell line (Figs 18, 20, 21, 24). The hybrid cell lines showed a substantially reduced growth rate at higher hydroxyurea concentrations, however, and the colonies formed were much smaller than those of the diploid parental lines. The one hybrid cell line tested also showed codominant expression of cross-resistance to guanazole (Fig 19). Control hybrids between the wild-type CHO and GAT^-O^R-1 or tsH1 O^R-2A exhibited hydroxyurea sensitivities close to, but significantly higher than those of the diploid parental lines. The reason for this is not known, but a similar effect was reported when hybrids between two ouabain-sensitive CHO cell lines were found to be slightly more resistant to ouabain inhibition than either parental line (Baker et al 1974).

The hydroxyurea-resistance marker can be used effectively for the selection of hybrids of hydroxyurea-sensitive and hydroxyurea-resistant cell lines. If a cell line contains both an additional recessive marker and the hydroxyurea-resistance marker, it can be used to hybridize with other cell line that need not carry any selective markers. The $GAT^{-}HU^{R}$-1 cell line, for example, can be hybridized with any cell line that is wild-type for hydroxyurea-sensitivity and folate metabolism (ie GAT^{+}). The resulting hybrids can be selected in the presence of hydroxyurea and the absence of glycine, adenosine or thymidine. Table 10 shows that using the hydroxyurea-resistance marker to select hybrid cells was as effective as using the temperature-sensitive marker present on the tsH1 cell line. Because of the ease of selection and codominant expression of hydroxyurea-resistance in CHO cells, this mutation should prove a valuable addition to the few codominant markers available at present for somatic cell genetic studies.

When ribonucleotide reductase was purified from the (H2 X GAT) hybrid cell line, it exhibited a Ki value for hydroxyurea which was intermediate between the Ki values of the HU^{R}-2 and GAT^{-} parental cell enzymes (Fig 56, Table 20). This result is consistent with a codominant expression of

the mutant ribonucleotide reductase allele in the hybrid cells, and closely resembles the codominant expression of both wild-type and methotrexate-resistant dihydrofolate reductases in wild-type X methotrexate-resistant cell hybrids (Flintoff et al 1976b).

It was possible to isolate hydroxyurea-resistant mouse L cells which seemed to have altered ribonucleotide reductase activity (Table 15). It should be possible to isolate hydroxyurea-resistant human diploid fibroblasts in a similar fashion to that reported here for the CHO and L resistant cell lines. Mutants of human diploid fibroblasts have been obtained with such drugs as 8-azaguanine (DeMars & Held 1972), ouabain (Buchwald 1977) and α-amanitin (Buchwald & Ingles 1976). Because of the codominant expression of the hydroxyurea-resistant phenotype, hybrids between such resistant human cells and sensitive CHO cells should express hydroxyurea-resistance. Human-hamster cell hybrids lose human chromosomes preferentially (Weiss & Green 1967), and it should be possible to select for those hybrid cells retaining the human chromosome determining hydroxyurea-resistance by cultivating the hybrids in the presence of hydroxyurea. Sensitive hybrids which had lost the human determinant for resistance presumably would not be able to grow in the presence of hydroxyurea. Thus it may be possible to assign the ribonucleotide reductase

gene(s) to a particular human chromosome utilizing existing techniques (eg Klebe et al 1970).

Hybrids between $GAT^{-}HU^{R}$-12SC8 (SC8), the cell line with elevated ribonucleotide reductase levels, and the tsH1 cell line exhibited ribonucleotide reductase levels approximately twice the levels found in the hydroxyurea-resistant diploid parental SC8 (Table 25). This suggests that the mechanism controlling the levels of ribonucleotide reductase behaves in a dominant fashion, inducing both the hydroxyurea-resistant and sensitive genomes to produce increased levels of the enzyme. Although other explanations are possible, if the above were the case, techniques analagous to the ones described could be used to isolate human diploid fibroblasts with increased levels of ribonucleotide reductase and to determine the human chromosome responsible for the altered levels. It would be of interest to see whether the structural determinants for ribonucleotide reductase are linked on a single human chromosome to the regulatory determinants.

E. Ribonucleotide Reductase

Extracts from CHO cells grown exponentially in suspension were found to contain higher levels of ribonucleotide reductase than any other eucaryotic source (Table 17).

Utilizing sequential affinity chromatography on ATP-agarose, blue dextran-Sepharose and GTP-agarose it was possible to obtain a preparation of ribonucleotide reductase with a higher specific activity than any other purified mammalian preparation reported to date (Table 18). Routinely, CHO cell ribonucleotide reductase was purified using ATP-agarose as the sole affinity chromatography step.

CDP reductase activity purified from CHO cells resembled the enzyme activity reported in other mammalian cell types (Moore 1967, Larsson 1969, Hopper 1972). The reaction had a pH optimum of 7.2 in HEPES buffer (Fig 36A), a dithiothreitol optimum concentration of 6 mM, and a $MgCl_2$ optimum concentration of 10 mM (Fig 37A, 37B). As with other systems, (Moore & Hurlbert 1966, Murphee et al 1968) CDP reduction required the presence of ATP (Fig 38). The reaction proceeded linearly for at least 60 min at 37°C and the reaction rate increased linearly only above certain protein concentrations (Fig 35B). A similar non-linear response to protein concentration has been reported in other systems (Hopper 1972, Peterson & Moore 1976). The apparent Km for CDP was found to be 0.13 mM (Fig 39), a value close to the 0.12 mM reported for Novikoff rat hepatoma cells (Moore & Hurlbert 1966) and the 0.094 mM reported for DON hamster fibroblasts

(Peterson & Moore 1976).

ADP reductase activity purified from CHO cells resembled the enzyme activity reported for other mammalian cell types (Moore 1967, Moore & Hurlbert 1966, Cory & Mansell 1975, Peterson & Moore 1976). The reaction had a pH optimum of 6.8 in PIPES buffer, and a dithiothreitol optimum of 6 mM (Fig 43A, 43B). Mg^{+2} ion stimulates ADP reduction in some mammalian systems (Cory & Mansell 1975), however, Mg^{+2} did not stimulate the CHO enzyme (Table 19). The reaction showed an almost 8-fold stimulation in the presence of the activator dGTP (Fig 44). ADP reduction proceeded linearly for at least 60 min and in agreement with other systems (Peterson & Moore 1976), the reaction also increased linearly with protein concentration, even at low protein levels (Fig 42A, 42B). The apparent Km was found to be 0.13 mM for ADP, which compares well to the value reported for rat hepatoma cells (Moore & Hurlbert) but is 10-fold higher than the value reported for DON hamster fibroblasts (Peterson & Moore 1976).

GDP reductase activity purified from CHO cells resembled the enzyme activity reported for rat hepatoma cells (Moore & Hurlbert 1966, Moore 1967). The reaction had a pH optimum of 6.8 in PIPES buffer and a dithio-threitol optimum concentration of approximately 6 mM (Fig

48A, 48B). $MgCl_2$ stimulated the reaction at low concentrations (Table 19), but the stimulation observed was variable depending on batch and degree of purity of the enzyme. Similar to the data reported for hepatoma cells (Moore & Hurlbert 1966), the presence of 2 mM dTTP stimulated the reduction of GDP by over 20-fold (Fig 49). GDP reduction proceeded linearly for at least 60 min at $37^{o}C$, and in a manner analogous to CDP reduction, the activity did not increase linearly at low protein concentrations (Fig 47A, 47B). The apparent Km for GDP was found to be 0.03 mM (Fig 50) and compares well to the 0.027 mM reported for rat hepatoma cells (Moore & Hurlbert 1966).

The CDP and ADP reductase activities found in extracts of the fungus Achlya resembled those of the CHO system (Table 27). A similar CDP reductase activity has been reported in Saccharomyces (Vitols et al 1970), but Pithomyces, the only other fungal source examined, was shown to require 5'deoxyadenosylcobalamin as a cofactor and thus contained a ribonucleotide reductase activity more closely resembling the type found in Lactobacillus (Stutzenberger 1974).

F. Levels of Ribonucleotide Reductase

Ribonucleotide reductase levels can be measured easily and reproducibly in low numbers of CHO cells made permeable to nucleotides by treatment with Tween-80. The permeabilizing treatment results in the removal of low molecular weight inhibitors of ribonucleotide reduction while preserving high enzyme activity and a linear response to enzyme concentration (Fig 63). Both CDP and GDP reductase activities in permeabilized cells closely resembled the corresponding activities in partially purified CHO cell extracts. The permeabilized cell assay allowed the accurate measurement of enzyme levels in as few as 5×10^6 cells, which could easily be grown on a single culture plate. In contrast, previous measurements of ribonucleotide reductase levels in crude cell extracts had not been reproducible and had required large volumes of suspension-grown cells in order to achieve sufficient extract with a high protein content. This had placed severe limitations on the types of experiments which could be carried out. The permeabilized cell assay allowed the assay of ribonucleotide reductase levels during the course of the normal CHO cell cycle (Figs 64, 65). Additionally, the assay appears to be useful in measuring other nucleotide dependent reactions. Measurements of

thymidine kinase levels for example, were easily and reproducibly performed with this technique (Fig 66).

The permeabilized cell assay was used to screen a number of hydroxyurea-resistant CHO cell lines to detect those lines containing altered levels of ribonucleotide reductase. $GAT^{-}HU^{R}$-12SC8 was found to have 4- to 10-fold higher levels of CDP and GDP reductase when compared to wild-type or revertant cell lines by this method (Table 25). This technique shows promise for monitoring ribonucleotide reductase levels under a variety of growth conditions. For example, it should be possible to assay ribonucleotide reductase levels of SC8 cells grown in the presence of various concentrations of hydroxyurea in order to find conditions under which optimal induction of the enzyme occurs. By growing the mutant cells under these conditions and applying routine purification procedures a far greater specific activity of ribonucleotide reductase should be achieved. The availability of this mammalian cell line with elevated levels of ribonucleotide reductase may prove invaluable in achieving a final purification of the enzyme to homogeneity - in the same way that derepressed and overproducing strains of E. coli have been indispensible for

purification and study of the bacterial enzyme (Biswas et al 1965, Sjöberg et al 1977).

In Escherchia coli, one enzyme is responsible for the conversion of all four ribonucleotides to the deoxyribonucleotides (Larsson & Reichard 1966a&b). However, several reports have suggested that more than one ribonucleotide reductase may be present in mammalian systems (Cory & Mansell 1975, Peterson & Moore 1976). Peterson and Moore (1976) reported that CDP and ADP reductase activities varied independently in synchronously and exponentially growing hamster cells. Similarly, Figure 64 shows that CDP and GDP reductase activities appear to vary independently in synchronized CHO cells. CDP reductase increased 10-fold during the induction of DNA synthesis. On the other hand, GDP reductase, which had a far higher specific activity than CDP reductase, only increased 2-fold during the same induction period. Caution must be exercised, however, in comparing the various ribonucleotide reductase levels for the optimal assay conditions bear little resemblance to intracellular conditions. Since the HU^R-2 cell line contains a similar alteration in the reducing activity with all three substrates tested (CDP, ADP & GDP), it suggests that at least the subunit of ribonucleotide reductase sensitive

to hydroxyurea is common to the three reducing systems. Similarly, cell lines with elevated levels of CDP reductase always exhibit elevated levels of GDP reductase, suggesting that a common control mechanism exists for all ribonucleotide reducing activities or that only one enzyme catalyzes all three activities. Clearly, the resolution of the various ribonucleotide reductase activities must await the final purification of the mammalian enzyme(s).

G. Inhibition of Ribonucleotide Reductase by Naturally Occurring Dinucleotide Compounds

The two unusual dinucleotides, HS2 and HS3, isolated from the fungus Achlya have been shown to inhibit purine and pyrimidine ribonucleotide reduction by 50% (Fig 67) at concentrations well below their estimated intracellular levels of 0.2 to 0.5 mM (McNaughton et al 1975). Thus the inhibition demonstrated is probably physiologically significant. HS2 inhibition of CDP reduction but not of ADP reduction (Fig 67) resembles the effect of several known allosteric regulators of ribonucleotide reductase known to preferentially affect either purine or pyrimidine reduction (Moore & Hurlbert 1966). The observation that ribonucleotide reductase

levels and DNA synthesis decline *in vivo* as HS3 synthesis increases (Fig 68) would again support the physiological significance of the inhibition observed *in vitro*.

When CHO cells are deprived of glutamine they rapidly accumulate high levels of the unusual dinucleotide HS3 (Goh & LéJohn 1977, Goh *et al* 1977). As HS3 accumulates, the rate of DNA synthesis drastically declines (Fig 69). This phenomenon is similar to that found in *Achlya*, where the levels of HS3 are also inversely related to the rate of DNA synthesis (Fig 68).

HS3 compounds have been purified from both *Achlya* and CHO cells and have been shown to be very similar, if not identical, in physical and chemical properties (Lewis *et al* 1977). The HS3 compounds from both *Achlya* and CHO cells are capable of potently inhibiting mammalian and fungal CDP reduction (Table 29) but have little effect on the enzyme activity from *E. coli*. HS3 inhibits ADP, CDP and GDP reduction by 50% at concentrations less than 50 μM (Figs 72, 73, 74). Glutamine starved CHO cells have been estimated to produce pool sizes of HS3 of at least 100 to 200 μM (Lewis *et al* 1977), a level sufficient to almost completely inhibit the various ribonucleotide reductase activities *in vitro*. Such an inhibition, if it occurs *in vivo* would drastically reduce the pool sizes of

the four deoxyribonucleoside triphosphates resulting in a rapid inhibition of DNA synthesis.

Mammalian ribonucleotide reductase is known to be responsive to a complex pattern of allosteric control by a variety of ribonucleoside triphosphates (Elford 1972). The only physiological compound with an inhibitory effect similar to HS3 on CDP, ADP and GDP reduction, however, is dATP which is thought to act as a general feedback inhibitor of the reaction in vivo. No deoxyribose can be detected in HS3 (H. LéJohn, personal communication), so its effect may be quite separate from dATP inhibition. Also HS3 seems to be slightly more potent than dATP under our assay conditions. Whether the effect of HS3 is mediated through a specific allosteric binding site or whether the compound interacts competitively at other nucleotide binding sites remains to be determined. It is clear from the kinetic data presented that HS3 does not compete with any of the ribonucleoside diphosphate substrates tested.

Much remains to be learned of the interaction between HS3 and eucaryotic ribonucleotide reductase, and of the effect of that inhibition on the rates of DNA synthesis in vivo.

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