THE RIBONUCLEOTIDE REDUCTASE PERMEABILIZATION ASSAY AS A METHOD OF SURVEYING FOR MAMMALIAN CELL MUTANTS CONTAINING ENZYME STRUCTURAL CHANGES AND/OR ALTERED LEVELS OF ENZYME ACTIVITY

by

SHAUN ELIZABETH KOROPATNICK

A Thesis Submitted to the Faculty of Graduate Studies in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

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"..... this one thing I do: forgetting what lies behind and reaching forward to what lies ahead, I press toward the goal for the prize of the upward call of God in Christ Jesus."

Philippians 3: 13 and 14.

ABSTRACT

Mouse L cells showing altered sensitivities to the cytotoxic effects of the anti-tumor agent hydroxyurea were isolated via single- and double-step selection procedures and cloned. The drug-resistant phenotype was stable after prolonged cultivation in the absence of hydroxyurea and resistances as much as 10-fold or greater than the wild type were maintained. The D_{10} value for killing in both L1 and L3 wild type cell lines was 0.14 mM hydroxyurea while resistant cells showed D_{10} values ranging from 0.32 to over 1.0 mM.

Ribonucleotide reductase activity was studied in detail in cells made permeable to nucleotides by treatment with the detergent Tween-80. In vivo levels of ribonucleotide reductase activity and estimates of hydroxyurea inhibition (apparent K_i's) were determined for both CDP and ADP reduction in each of 12 independently selected hydroxyurea-resistant mutant cell lines. Elevated levels of reductase activity and/or altered apparent K_i constants corresponded closely with increased cellular resistance to hydroxyurea in all 12 hydroxyurea-resistant clones surveyed. Drug-resistant clones contained 2 to 13 times the wild type level of CDP reductase while ADP reductase levels were 2 to 5 times higher than the wild type. In several resistant cell lines both CDP and ADP reductase showed a 2-to 3-fold increase in the apparent K_i for hydroxyurea. Apparent K_m values for both CDP and ADP reduction also varied 1.5 to 3 times from the wild type. These findings

suggest the presence of a structurally altered ribonucleotide reductase which has been rendered less sensitive to the inhibitory effects of hydroxyurea. Therefore, in mouse L cells with altered sensitivities to hydroxyurea, the resistant phenotype can be accounted for by increased levels of ribonucleotide reductase activity alone or in combination with the presence of a more resistant enzyme.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

With the advent of tissue culture as a tool for studying mammalian systems came the potential to examine phenotypic variation in mammalian cell lines and the exact nature thereof. The isolation and characterization of numerous variant cell lines as a means of studying biochemical processes and control mechanisms has precipitated the investigation into the origin of these phenotypic aberrations.

One source of much controversy in the past and the centre of intense exploration today is whether inheritable variation observed in cultured somatic cells is indeed a result of mutational events at the DNA level or merely the prolonged expression of a stably altered phenotype (Harris, 1971; Mezger-Freed, 1972). Much research revolves around attempts to correlate a modification in the physical parameters, structure and/or assembly of a specific protein directly with an observed change in the expression or control of a certain trait or biochemical process within the cell. Findings of this type are strongly supportive of a mutational rather than epigenetic basis for induced phenotypic change, and recently a number of well-characterized systems testify to this (Friedrich and Coffino, 1977; Capecchi et al., 1977; Lewis and Wright, 1978a,b; Lewis and Wright, 1979; Kuzik and Wright, 1979; Thompson et al., 1978; Ashman, 1978; Gupta and Siminovitch, 1978a,b; Nakano et al., 1978; Hochman et al., 1975).

The work reported in this thesis involves the isolation and characterization of a number of variant mouse L cell lines which show resistance to the cytotoxic effects of the anti-tumor agent hydroxyurea. As many drug-resistant cell lines are obtained by multi-step selection procedures, which result in the alteration of several parameters, a thorough understanding of the nature of the variation is not easily apprehended. The difficulty lies in determining which of the alterations occurs at which step in the selection process. The issue is further complicated by the fact that resistance to hydroxyurea arises by more than one mechanism including a structural alteration in the enzyme ribonucleotide reductase, the target protein, and/or changes in the intracellular level of the native drug-sensitive enzyme (Lewis and Wright, 1978b; Lewis and Wright, 1979; Kuzik and Wright, 1979). These mechanisms have been postulated for other types of drug-resistance in mammalian cells (Flintoff, Davidson and Siminovitch, 1976; Steinberg, O'Farrell, Friedrich and Coffino, 1977; Chang and Littlefield, 1976). In this thesis a comparison is drawn between cell lines developed via a single-step selection procedure and those selected via a double-step process. This involves the in vivo analysis of enzyme levels and kinetics in cells made permeable to nucleotides. An attempt is made to correlate a structural change in the target enzyme ribonucleotide reductase and/or a modification in enzyme levels with increased resistance to the cytotoxic effects of hydro-

xyurea. By this we can determine the nature of the alteration(s) giving rise to the drug-resistant phenotype and, possibly, at what point in the selection process each may have occurred.

HISTORY

Before one can attempt to understand the function and regulation of biochemical mechanisms within the cell one should first acquire an understanding of the genetics governing these processes. This involves the study of somatic mutation at the molecular level. Thus, somatic cell genetics has become an area of intense research owing to the potential it affords in analyzing mutational events, genetic expression and biochemical processes.

Previous to the advent of tissue culture, biochemical and genetical studies of cellular mechanisms in whole animals was difficult due to the very complex multicellular phenotypes and the fact that such analyses necessitated meiosis and the mating of individuals. For this reason, many of the advances in the field of molecular biology dealt with bacterial mechanisms. However, recent developments in tissue techniques have allowed mammalian cell biologists to culture pure lines in semi-defined media and molecular biology is no longer a field reserved for the bacteriologist.

The advancement of bacterial genetics and biochemistry through the isolation and study of mutant strains is clearly demonstrated by the publication of numerous works to this effect (for reviews see Harris, 1964; Siminovitch et al., 1972). Most recently, mammalian cell biologists have been made aware of the importance of mutants selected

from somatic cells in culture to the study of regulation and metabolism in animal tissue (for reviews see Clements, 1975; Siminovitch, 1976; Basilico, 1977; Wasmuth and Caskey, 1978; Wright, 1979). The selection, isolation and characterization of a multitude of mutants with stably altered phenotypes has greatly contributed to the understanding and elucidation of biochemical processes and molecular controls. Among these include mutation to auxotrophy, temperature-sensitive conditional lethality and drug-resistance.

Much controversy has arisen over the nature of inheritable variation in cultured somatic cells. For an excellent review of this subject, the reader is directed to the publication by Siminovitch (1976). Structural gene mutants which synthesize products that are biochemically distinguishable from the wild type are the strongest evidence for an altered genotype as the basis for a stably altered phenotype. The phenomenon of drug-resistance has played an important role in somatic cell genetics and efforts to verify the genetic control of phenotypic variation. Numerous variants of mammalian cells showing resistance to a variety of cytotoxic drugs have been isolated and characterized. Among these are cell lines resistant to 8-azaguanine and 6-thioguanine, methotrexate, α-amanitin, trichodermin, emetine, hydroxyurea, 5-bromodeoxyuridine and a variety of lectins. A number of molecular mechanisms responsible for resistance are postulated and include decreased membrane permeability (Till et al., 1973; Juliano and Ling, 1976;

Bosmann, 1971), modified drug metabolism (Gillin et al., 1972; Orkin and Littlefield, 1971; Clive et al., 1972; Ullman et al., 1979), altered molecular transport across membranes (Heiser and Englesberg, 1979; Taub and Englesberg, 1978; Goldman, 1971; Ullman et al., 1979), structural alterations of a target protein (Hochman et al., 1975; Steinberg et al., 1977; Boersma et al., 1979b; Gupta and Siminovitch, 1977; Gupta and Siminovitch, 1978a; Wright and Lewis, 1974; Lewis and Wright, 1974; Lewis and Wright, 1978b; Irwin, Oates and Patterson, 1979) and altered enzyme levels (Lewis and Wright, 1979; Clive et al., 1972; Nunberg et al., 1978; Meuth and Green, 1974; Wright, Jamieson and Ceri, 1979) including gene amplification (Flintoff et al., 1976; Chang and Littlefield, 1976; Atl et al., 1978; Shields, 1978). The isolation of such mutants has provided good evidence that cells with altered genotype could be obtained. In this historical treatment the discussion will be limited to only a few of the more interesting somatic cell mutants dealing with drug-resistance.

Examination of a mouse lymphoma cell line resistant to the cytotoxic effects of cyclic AMP suggests that the phenotype is produced by a mutation in the structural gene coding for the regulatory subunit of cAMP-dependent protein kinase (Hochman et al., 1975). In wild type S49 mouse lymphoma cells elevated levels of intracellular cAMP stop cell division in the G_1 phase of the cell cycle, induce

phosphodiesterase activity and within days cause cytolysis while mutants of the S49 lymphoma cell line continue to replicate with almost normal efficiency in the presence of cAMP concentrations toxic to the wild type. Reduced sensitivity of the mutant cell line to the biologic effects of cAMP directly reflects an alteration in the activity of the cAMP-dependent protein kinase; there is a ten-fold increase in the apparent cAMP activation constant of the mutant protein kinase compared to the wild type enzyme. Two-dimensional polyacrylamide gel electrophoresis of mutant S49 cell extracts reveal regulatory subunits of cAMP-dependent protein kinase with altered patterns of charge shifts consistent with single amino-acid substitutions (Steinberg et al., 1977).

The rarest of the kinase-defective mutants of S49 mouse lymphoma cells are those known as kinase-negative mutants (Kin^-) which are pleiotropically devoid in all known cAMP-mediated S49 cell responses (Steinberg, Van Daalen Wetters and Coffino, 1978). These Kin^- mutants are deficient in both cAMP-dependent protein kinase activity and cAMP-binding activity (Insel et al., 1975; Insel and Fenno, 1978). The absence of cAMP-dependent protein kinase activity in extracts of Kin^- cells is evidence for an alteration causing reduced expression of the regulatory subunits of cAMP-dependent protein kinase. A mutational basis for this alteration is suggested by the very low frequency of Kin^- variants (Friedrich and Coffino, 1977), the increased frequency of the Kin^- phenotype among cells mutagenized with ICR 191 compared with

unmutagenized cells (Friedrich and Coffino, 1977), and the stability of the phenotype upon repeated subcloning (Steinberg, Van Daalen Wetters and Coffino, 1978). The dominance of the Kin^- lesion in hybrids with wild type S49 cells rules out the simplest explanation of the mutant phenotype - that the lesion inactivates a single functional catalytic allele. Rather, the Kin^- phenotype is attributed to structural alterations in the protein kinase regulatory subunit genes leading to changes in the phosphorylation patterns and, therefore, expression of the regulatory subunits of the cAMP-dependent protein kinase.

A third class of kinase-defective mutants selected from the S49 mouse lymphoma cell line are specifically defective in cAMP-mediated cytolysis although they otherwise respond like the wild type cell to cAMP (Lemaire and Coffino, 1977). Unlike other cAMP-resistant mutants the "deathless" (D) mutant has a functionally normal cAMP-dependent protein kinase and retains normal ability to induce phosphdiesterase and arrest cell growth in G_1. The exact nature of the defect is not known, however, several human hereditable metabolic disorders have been described that illustrate possible models for D mutants (Hug, Schubert and Chuck, 1969; Huijing and Fernandes, 1969; Mommaerts et al., 1959). Since protein kinase activity is not affected, the possibility exists that the D mutant is deficient in a kinase substrate required for cytolysis or that the kinase has been changed in such a way so as to effect its enzymatic activity for some

endogenous substrates and not others. Nevertheless, cAMP-resistant mutants of the S49 mouse lymphoma cell line provide a model system which will facilitate studies of the mechanisms of cAMP-induced cytolysis and growth regulation.

Another isolate of cultured mammalian cells bearing the drug-resistant phenotype is the methotrexate-resistant cell line. Variants of this type have been derived from both murine (Alt, Kellems and Schimke, 1976; Alt et al., 1978; Schimke et al., 1978) and Chinese hamster cells (Nunberg et al., 1978) as well as baby hamster kidney (BHK) cells (Chang and Littlefield, 1976). The anti-tumor antifolate drug, methotrexate (amethopterin), is the 4-amino analogue of folic acid and inhibits folate metabolism at a variety of stages although the most pronounced effect is its interaction with dihydrofolate reductase (DHFR). Methotrexate is a specific, tight-binding inhibitor of DHFR and does so in an essentially stoichiometric and irreversible manner. A kinetic analysis of the behavior of the DHFR-methotrexate system in 4 cell lines of varying origin revealed that the intrinsic resistance of 70-fold to the toxic effects of methotrexate could at least be partially attributed to a 25-fold difference in K_i values from the most sensitive to the most resistant line (Jackson, Hart and Harrap, 1976). Albrecht et al. (1972) have reported the isolation of a methotrexate-resistant Chinese hamster cell line which contains a structurally

altered DHFR and Flintoff et al. (1976) describe a class of methotrexate-resistant CHO cells with an apparent structural alteration in the DHFR enzyme. It is possible, however, that other factors aid in determining resistance of the cell to methotrexate, for example, rate of drug uptake, total DHFR activity, rate of synthesis of new enzyme and nucleotide pool sizes (Tattersall and Harrap, 1973).

Baby hamster kidney (BHK) cells showing resistance to 10^4 times higher levels of methotrexate are found to contain 140 times more DHFR than is found in the parental line (Chang and Littlefield, 1976). Analysis of tryptic and chymotryptic digests yields identical fingerprints of methotrexate-resistant and wild type DHFR. The conclusion drawn is that there is no detectable difference, either structurally or chemically, between mutant and parental DHFR and that the high content of DHFR in methotrexate-resistant BHK cells of this type must be due to an alteration of mRNA levels. Evidence in favour of this is two-fold: (1) a 150-fold increase in gene copy number accompanying a 200-fold increase in specific activity of the reductase in a Chinese hamster ovary (CHO) cell line approximately 50,000 times more resistant to methotrexate than the wild type (Alt, Kellems and Schimke, 1976) and (2) the localization of amplified DHFR genes to a homogeneously staining region of a single chromosome in a methotrexate-resistant CHO cell line (Nunberg et al., 1978).

Perhaps the best characterized system dealing with the drug-resistant phenomenon is that concerning the HGPRT locus and resistance to purine analogues (for review see Caskey and Kruh, 1979). The purine analogs 6-thioguanine, 8-azahypoxanthine, 8-azaguanine and 6-mercaptopurine have been used to select for cultured somatic cells deficient or lacking in the enzyme hypoxanthine-guanine phosphoribosyl-transferase (HGPRT) which is a purine salvage enzyme and non-essential under normal culture conditions (Fujimoto et al., 1971; DeMars, 1974). These analogs in themselves are non-toxic to cells but are converted by HGPRT to toxic nucleotides. Therefore, when grown in a specific medium containing metabolic inhibitors of de novo purine synthesis, it becomes these processed purine analogues which are responsible for the inhibition of phosphorylation and synthesis of purines via the salvage pathway.

Siminovitch (1976) provides a review of the evidence suggesting that many phenotypic variants resistant to 6-thioguanine and other purine analogues may indeed have a genetic origin and arise from structural gene mutation. Cell lines resistant to purine analogues have been isolated by many independent investigators and most of these mutants show the $HGPRT^-$ phenotype. This phenotype is reported to be stable for long periods in the absence of selective agent and its occurrence in the wild type population is increased upon mutagenesis (Friedrich and Coffino, 1977; Huang and Lieberman, 1978; Burki and Aebersold, 1978; Tong and Williams, 1978). These mutants also appear phenotypically identical to cells

obtained from human males with the Lesch-Nyhan syndrome where the defect has been located on the X chromosome (Fujimoto et al., 1971).

Sharp et al. (1973) selected several variants of mouse L cells which contained gene products with altered physical properties. One alteration found among the resulting variant cell lines was a HGPRT enzyme with altered kinetic constants causing in vivo and in vitro resistance to 8-azaguanine. Chasin and Urlaub (1976) also describe the isolation and biochemical analysis of $HGPRT^-$ mutants producing enzyme activity altered in its kinetic and electrophoretic behaviour. Such changes can be explained in terms of altered amino acid sequences in the enzyme resulting from changed base sequences in the corresponding structural gene. Fenwick et al. (1977) report the isolation and characterization of Chinese hamster $HGPRT^-$ mutants with an altered enzyme of the same molecular weight as the wild type but with altered substrate binding properties. The suggestion is that this particular variant is the result of a missense mutation in the gene coding for HGPRT.

An important observation is that phenotypically $HGPRT^-$ mouse L cells have been found which produce an enzymatically inactive protein that cross-reacts immunologically (CRM^+) with antibodies to purified HGPRT (Wahl, Hughes and Capecchi, 1975; Beaudet, Roufa and Caskey, 1973). Recently, the isolation and characterization of nonsense mutants and nonsense suppressors in mammalian cells adds supporting evidence

regarding the genetic nature of the $HGPRT^-$ phenotype in cultured L cells. A nonsense mutation generates a polypeptide-chain-termination codon (UAA, UAG or UGA) in the interior portions of a structural gene. In the presence of a mutant tRNA, which allows the translation of the termination codon as an amino-acid codon, what would otherwise be an amino-terminal polypeptide fragment becomes the completed polypeptide product of a nonsense mutant. An active gene product may be restored if the substituted amino-acid at the site of the mutation does not significantly alter the protein structure. Capecchi et al. (1977) report the CRM^+ protein in $HGPRT^-$ mutant L cells as having an altered carboxy-terminal peptide. Chromatographic analysis strongly implies that the mutant peptide is shorter than the wild type HGPRT peptide. The contention that the $HGPRT^-$ phenotype results from a nonsense mutation in the HGPRT gene is substantiated by the finding that the mutant can be "phenotypically corrected" by microinjection of bacterial or yeast ochre-suppressor tRNA but not wild-type tRNA. From these properties it is inferred that this mutant contains a nonsense mutation in the HGPRT structural gene.

The demonstration of temperature-sensitive (ts) HGPRT in purine-analogue resistant clones also substantiates present evidence for the production of an altered enzyme in $HGPRT^-$ variants. Fenwick et al. (1977), Sharp et al. (1973) and Epstein et al. (1977) have identified ts mutants among their Chinese hamster, mouse and human mutants, respectively.

It has been suggested that the HGPRT mutation in purine analogue-resistant clones is pleiotropic and that it alters a regulatory effect of the gene for HGPRT on the expression of the phosphoribosyl pyrophosphate (PRPP) synthetase gene. A single clone isolated from mutagenized rat hepatoma cells selected for resistance to 6-thioguanine or 6-mercaptopurine overproduced purines de novo and contained an increased quantity of apparently normal PRPP synthetase activity. It also retained 40 percent of the normal HGPRT catalytic activity and immunologic studies revealed that the HGPRT molecules were genetically altered. By way of explanation, Graf et al. (1976) postulate that this particular clone of rat hepatoma cells possesses a single but pleiotropic mutation in the structural gene for HGPRT which regulates the intracellular levels of PRPP synthetase as well as codes for the catalytic function of HGPRT. Similar multiple genetic defects as a result of a single mutation are documented by Taylor et al. (1977) who have characterized adenine phosphoribosyltransferase (APRT)-deficient mutants in Chinese hamster cells.

Pyrimidine analogues have also been useful in selecting for cultured somatic cells with altered phenotypes. Mutations in thymidine kinase have been obtained in mouse lymphoblasts (Kit et al., 1963; Kit et al., 1966; Clive et al., 1972) and CHO cells (Roufa, Sadow and Caskey, 1973) by exposing sensitive clones to the thymidine analogue 5-bromodeoxyuridine (BrdU). By culturing wild type cells in the presence of BrdU,

resistant sublines with decreased thymidine kinase can be selected for; a loss of thymidine kinase activity prevents the lethal incorporation of BrdU into DNA. Since full-scale resistance to BrdU cannot be obtained in a single isolation procedure and cell populations generally acquire resistance only after long repeated exposure to BrdU, it has been suggested that the locus for thymidine kinase is present in two copies and that cells with intermediate levels of resistance are heterozygotes (Clive et al., 1972). Similar findings are reported for the APRT locus (Jones and Sargent, 1974).

Recently, Suttle and Stark (1979) report cells which overproduce a target enzyme complex in response to incubation in the presence of metabolic inhibitors of pyrimidine biosynthesis. A SV40-transformed Syrian hamster line and a Chinese hamster lung line were exposed to increasing concentrations of 6-azauridine and pyrazofurin. Cells selected for resistance to these inhibitors were found to overproduce coordinately both orotate phosphoribosyltransferase and orotidine-5'-phosphate decarboxylase; the amount of increase was up to 67 times that found in wild type cells. Levinson et al. (1979) describe the isolation and characterization of stable mouse T-lymphoma (S49) cell variants which also have altered levels of both orotate phosphoribosyltransferase and orotidine-5'-phosphate decarboxylase. The affinities of these two enzymes for autologous substrates, and their heat stabilities, isoelectric points and feedback inhibition by nucleotides

are unaltered.

Cells showing resistance to N-(phosphonacetyl)-L-aspartate (PALA), a transition state analogue inhibitor of aspartate transcarbamylase, have coordinately increased levels of the first three enzymes of the de novo pyrimidine pathway, carbamyl-P synthetase, aspartate transcarbamylase and dihydrooratase (CAD) (Kempe et al., 1976). Recently, Padgett et al. (1979) have shown that overproduction of the proteins is accompanied by a corresponding overproduction of a single, large mRNA which, when translated in vitro, gives a product indistinguishable from the purified polypeptide containing the CAD complex.

A Chinese hamster ovary cell line resistant to 1-β-D-arabinofuranosyl cytosine (ara C) and thymidine, as well as being auxotrophic for thymidine has been isolated and partially characterized (Meuth, Trudel and Siminovitch, 1979). Resistance to the antileukemic deoxyribonucleoside analogue, ara C, is attributed to 1) a deficiency of deoxycytidine kinase activity, hence the cells' inability to phosphorylate ara C to its toxic form ara CTP and 2) an expanded pool of deoxy CTP which confers resistance by diluting the ara CTP (De Saint Vincent and Buttin, 1979). Although not conclusive, the observation that revertants to thymidine prototrophy show a simultaneous reversion to ara C and thymidine sensitivity strongly suggests that this complex phenotype is the consequence of a single mutation. In one case it has been

shown that alterations of the enzyme ribonucleotide reductase appear to account for the expanded deoxy CTP pool (Meuth and Green, 1974; Meuth, Aufreiter and Reichard, 1976).

Hydroxyurea is a potent inhibitor of cellular DNA synthesis (Elford, 1968; Moore, 1969), and for this reason has been used clinically as a chemotherapeutic agent to treat a wide variety of tumors as well as leukemia (Kennedy and Yarbro, 1966). The target protein, ribonucleotide reductase, is the enzyme solely responsible for the conversion of the four ribonucleotides to the corresponding deoxyribonucleotides which are required for DNA synthesis and cell replication (Reichard, 1968; Larsson, 1969). Ribonucleotide reductase is allosterically controlled by a complex, intricate feedback mechanism involving several nucleoside triphosphates whereby a balanced supply of the nucleotide precursors for DNA synthesis is assured (Reichard, 1971). Intracellular levels of ribonucleotide reductase are strictly regulated during the cell cycle and a strict parallelism was found between the measured enzyme levels and fraction of cells forming DNA (Turner, Abrams and Lieberman, 1968).

Hydroxyurea has been used as a selective agent for obtaining drug-resistant CHO and mouse L cell lines (Wright and Lewis, 1974; Lewis and Wright, 1974; Lewis and Wright, 1978a,b; Lewis and Wright, 1979; Kuzik and Wright, 1979). The genetic properties of the CHO cell lines have been studied in detail. They maintain their resistant phenotype after extensive cultivation in the absence of the drug. Reconstruction experiments indicated that the expression of

hydroxyurea-resistance and the frequency of drug-resistant colonies were independent of cell densities up to 5×10^5 cells per 100 mm selection plate. Luria-Delbruck fluctuation analyses indicated that the appearance of hydroxyurea-resistant cells in wild type populations occurred spontaneously and at a rate of 4.8×10^{-6} per cell per generation in the presence of 0.33 mM drug. Studies with the mutagen, ethyl methane sulfonate indicated that it was capable of increasing the frequency of hydroxyurea-resistant cells by a factor of approximately 10. Also, cell-cell hybridization experiments showed that hydroxyurea-resistance behaves as a dominant or codominant trait and that hydroxyurea-resistance was a useful new genetic marker for selection of somatic cell hybrids. Furthermore, similar to many other drug-resistant cell lines hydroxyurea-resistant cells were found to exhibit an altered sensitivity to a number of non-selective agents (guanazole, N-carbamoyloxyurea, formamidoxime, and hydroxyurethane). Except for guanazole these compounds are structurally very similar to hydroxyurea and may be expected to have similar modes of action. The results support the view that hydroxyurea-resistance is expressed as a normal genetic trait and is a useful genetic marker for somatic cell genetic studies.

Rigorous biochemical characterization of the CHO drug-resistant mutants indicated that the mutant cells either contained a structurally altered ribonucleotide reductase whose activity was less sensitive to inhibition by hydroxyurea (Lewis and Wright, 1974; Lewis and Wright, 1978b) or contained

increased intracellular levels of drug-sensitive ribonucleotide reductase activity which correlated with the degree of cellular resistance to hydroxyurea (Lewis and Wright, 1979). Also it was possible to isolate revertant cell lines from the mutants which overproduced ribonucleotide reductase; these cell lines which were approximately as sensitive to drug as wild type, contained approximately normal levels of enzyme activity (Lewis, Kuzik and Wright, 1978; Lewis and Wright, 1979).

Recently, a mouse L cell line resistant to hydroxyurea has been characterized in detail (Kuzik and Wright, 1979). A detailed analysis of the target enzyme ribonucleotide reductase in both wild type and hydroxyurea-resistant enzyme preparations suggested that the drug-resistant cells form a ribonucleotide reductase enzyme which contains a structural alteration rendering it less sensitive to inhibition by hydroxyurea. Ki values for hydroxyurea inhibition of ribonucleotide reduction in enzyme preparations from hydroxyurea-resistant cells were significantly higher than corresponding values from preparations from wild type cells. The Km for CDP reduction in enzyme preparations of drug-resistant cells was approximately three-fold higher than the corresponding parental wild type value. In addition, in vivo enzyme assays detected a major difference between the temperature profiles of ribonucleotide reduction in nucleotide permeable drug-resistant and wild type cells. When levels of ribonucleotide reductase activity were measured in vivo it was found that the drug-resistant cells contained approximately three times the wild type level of CDP reductase activity, and twice the wild type level of GDP reductase activity.

This was the first indication that hydroxyurea-resistant cell lines can be isolated which contain a combination of enhanced enzyme levels plus an altered sensitivity to drug inhibition.

A variety of mammalian cell variants have been selected for their resistance to the protein synthesis inhibitors emetine (Boersma et al., 1979; Gupta and Siminovitch, 1978b; Gupta and Siminovitch, 1977), trichodermin (Gupta and Siminovitch, 1978a), blasticidin S (Kuwano et al., 1977), carbomycin (Bunn and Einsenstadt, 1977), and α-amanitin (Lobban, Siminovitch and Ingles, 1976; Ingles et al., 1976; Buchwald and Ingles, 1976; Somers et al., 1975; Amati et al., 1975). Of these, phenotypic variations in culture resistance to emetine (a potent alkaloid drug inhibitor of ribosome function) has probably been characterized the most definitively. Gupta and Siminovitch (1977) describe the isolation and biochemical characterization of CHO cells selected for resistance to emetine. Extracts of these cells showed relatively normal rates of protein synthesis and analysis of ribosomal components by two dimensional polyacrilamide gel electrophoresis indicated that resistance to emetine reflected an alteration in the 40S ribosomal subunit. In 1978, these same authors genetically and biochemically characterized several highly-resistant second-step emetine mutants which were derived from the population of emetine-resistant CHO cells selected in 1977. The data collected showed that high levels of resistance to emetine in CHO cells results only after two descrete mutational events in the structural gene coding for a particular family of ribosomal

proteins. Both mutations were found to be recessive and localized in the polyribosomal fraction based on the following observations: (1) the inability to obtain a mutant resistant to higher concentrations of emetine in a single-step, and (2) the segregation of mutations leading to emetine resistance from somatic cell hybrids derived in second-step mutants. Boersma et al. (1979) reported preparations of Chinese hamster cell 40S and 60S ribosomal subunits from several emetine-resistant clones. Those proteins were analyzed by two-dimensional electrophoresis and one clone (Em^r-2) was found to contain a single altered 40S ribosomal subunit protein, S20, whose electrophoretic migration was distinguished from the wild type. The fact that S20 in Em^r-2 apparently differs from wild type S20 only in its charge and not in its molecular weight indicates that S20 in Em^r-2 is the product of a point mutation. Boersma et al. (1979a) performed electrophoretic and genetic experiments designed to test the linkage between Em^r-2's genes that specify altered S20 protein and emetine resistance. Several tetraploid hybrid clones were selected from a derivative of Em^r-2 fused with emetine-sensitive Chinese hamster cells. In each of four segregants isolated which displayed the emetine-sensitive phenotype both the wild type S20 and Em^r-2 S20 40S ribosomal subunits were expressed. Five independent emetine-resistant segregants were isolated from the hybrid clones and all five expressed only the altered S20 protein characteristics of Em^r-2. The most direct interpretation of these segregation data supports the idea that the loci that encode mutant ribosomal protein

S20 and the emetine-resistant phenotype are linked to the same chromosome and that the structural gene which codes for S20 protein contains a mutation responsible for both altered 40S ribosomal subunit protein and resistance to emetine.

Characterized less well, but nevertheless significant to the discussion of drug resistance, is the isolation of mammalian clones resistant to the cytotoxic effects of α-amanitin, a bicyclic octapeptide produced by the mushroom Amanita phalloides. The target protein for the toxic effects of α-amanitin is recognized as RNA polymerase II, one of the DNA-dependent RNA polymerases responsible for the synthesis of different classes of cellular RNA. Mutant human diploid fibroblasts and rat myoblast L_6 cell lines resistant to the cytotoxicity of α-amanitin contain two forms of RNA polymerase II, one identical to the wild type enzyme and one showing a moderately increased resistance to α-amanitin inhibition (Buchwald and Ingles, 1976; Somers et al., 1975). This finding is strongly suggestive of a co-dominant expression of the resistant marker (Lobban and Siminovitch, 1975). In contrast to the rat myoblast and human fibroblast cell lines, the pseudodiploid CHO cell lines contain only an α-amanitin-resistant form of RNA polymerase II activity. CHO cell hybrids of wild type α-amanitin-sensitive cells fused with α-amanitin-resistant mutants contain both sensitive and resistant forms

of RNA polymerase II and in this respect resemble the rat myoblast mutant. When these hybrids were grown in medium containing α-amanitin the sensitive form of polymerase II was inactivated and the intracellular concentration of the α-amanitin resistant enzyme was found to increase proportionally (Guialis et al., 1977). Using immunoprecipitation to recover RNA polymerase II from CHO α-amanitin-resistant mutants, Guialis et al. (1979) have collected data which indicate that a coordinate increase in the synthesis of at least three polypeptides of RNA polymerase II is responsible for the compensatory increase in the activity of the α-amanitin resistant enzyme. This increased rate of synthesis of subunit polypeptides of RNA polymerase II is coupled with accelerated degradation of the α-amanitin-inactivated wild type polymerase II.

Lobban, Siminovitch and Ingles (1976) have isolated and purified RNA polymerase II from an α-amanitin-resistant mutant of the CHO cell line. When extracts were chromatographed on DEAE-cellulose the peak eluting where polymerase II was expected was not α-amanitin sensitive. This activity was found to be inhibited equally well by anti-RNA polymerase II serum as wild type enzyme activity confirming the RNA polymerase II origin of the purified protein. The mutant polymerase was reported to be 600 times less sensitive to α-amanitin and more thermolabile than the wild type enzyme consistent with its having an altered primary structure. Furthermore, Ingles et al. (1976) have shown that the polymerase II from

mutant CHO cells has a decreased affinity for binding α-amanitin and that the ability of polymerase II to bind α-amanitin is related to the degree of α-amanitin resistance of the enzyme activity. Together, these observations provide evidence that the mutation to α-amanitin resistance in animal cells involves an alteration in the structural gene coding for a polypeptide of RNA polymerase II. Isolation and characterization of α-amanitin resistant cell lines has proven, therefore, to be a valuable source of verification for the genetic basis of somatic cell mutants.

Yet another class of drug-resistant mammalian mutants are those with alterations in the transport function of the cell membrane. Several investigators have utilized the inhibitory effects of high concentrations of each of the thirteen essential amino acids present in Eagle's minimal essential medium to select for amino acid transport mutants of Chinese hamster and mouse cells (Heiser and Englesberg, 1979; Englesberg, Bass and Heiser, 1976). Isolation on the basis of resistance to membrane active drugs has proved to be a useful method for obtaining mutants with altered membranes. Ouabain, or strophanthin G, is a cardioactive steroid which is known to inhibit the (Na^+, K^+) activated ATPase of the plasma membrane, the enzyme responsible for the active transport of K^+ into the cell and extrusion of Na^+ from the cell. Mammalian cell lines selected for their resistance to ouabain are suggested to contain a lesion in a nuclear gene(s)

specifying a membrane protein(s). Mutants resistant to ouabain have been selected from HeLa cell lines (Rosenberg, 1975; Baker, 1976; Robbins and Baker, 1977), mouse fibroblasts and CHO cells (Till et al., 1973; Cole and Arlett, 1976), Ehrlich ascites cells (Mayhew and Levinson, 1968; Mayhew, 1972), murine plasmocytoma cells (Zachowski et al., 1977) and human diploid fibroblasts (Mankovitz, Buchwald and Baker, 1974; Corsaro and Migeon, 1978; Breslow et al., 1977; Corsaro and Migeon, 1977). The Na^+/K^+ transport system in such mutants is more resistant to inhibition by ouabain and the ATPase activity in membrane preparations from several clones of differing origin has been found to be more resistant to inhibition than that from corresponding wild type cells. Reported changes in the binding affinity constants of ATPase for ouabain is said to directly reflect a mutated sequence in the structural gene coding for the (Na^+, K^+) ATPase (Baker et al., 1974; Robbins and Baker, 1977).

From the data presented it is obvious that our knowledge of genotypic mutation in mammalian cells and the mechanisms governing phenotypic expression has greatly increased with the development of techniques for studying somatic cells in culture. In particular, the study of mutants holds amazing potential for the investigation of many aspects of biology, including those areas of metabolic regulation and cellular differentiation. As the overall state of an organism

depends on the normal functioning of regular cellular processes, it is easy to recognize the value in understanding them and how they compare with those processes reflected in genetic disorders common to man. The use of somatic cells *in vitro*, therefore, represents a new development in biology which provides researchers with an ideal tool for qualitative and quantitative analysis of genetic biochemical properties of the multicellular animal.

MATERIALS AND METHODS

1. Cell Lines and Culture Conditions

The permanent mouse L cell line isolated by Earle (1943) was used in this study. In our hands, L cells had a doubling time of approximately 18 hours at 37° and were routinely grown to monolayer on the surface of 16 ounce glass Brockway bottles or on 60 or 100 or 150 mm plastic tissue culture plates. The support medium used was alpha minimal essential medium (αMEM) supplemented with 10 percent (v/v) fetal calf serum (FCS) (complete medium). The antibiotics Penicillin G (100 units/ml) and streptomycin sulphate (100 µg/ml) were added to the medium prior to its sterilization by millipore filtration under pressure. As the pH of the medium is maintained by a carbonate buffer, 2.2 mg/ml sodium bicarbonate was also added to the medium prior to filtration. A tabulation of the constituents of αMEM has been published by Stanners et al. (1971) and did not contain ribonucleosides or deoxyribonucleosides. Cells were incubated at 37°C in a humidity-controlled incubator containing an atmosphere of 5 percent CO_2 in air.

2. Standard Procedures

(a) Removal from solid surfaces: A sterile solution of 0.05 percent trypsin in phosphate-buffered saline stored at 4°C and pre-warmed to room temperature was used to aid removal of cells from both glass and plastic culture vessels. The growth medium was aseptically poured off and replaced with a

sufficient volume of trypsin to cover the cells. After 3-5 minutes the cells were detached from the solid surface with gentle agitation. The cells were then collected in sterile plastic 15 mm test tubes and removed from suspension by centrifugation in an International Clinical Centrifuge model CL at a speed setting of 7 for 1-2 minutes. The cell pellet was resuspended in αMEM plus 10 percent FCS.

(b) Cell counting: Aliquots of cells previously trypsinized and resuspended in fresh complete medium were diluted 1:40 in phosphate-buffered saline. The cell density of this suspension was determined by a Coulter Particle Counter (Coulter Electronics Ltd., Florida).

(c) Storage of cell lines: At the first opportunity, cultures were frozen away in 7 percent dimethylsulphoxide (DMSO) in complete medium to ensure the availability of the cell lines should the original be lost to contamination or other unfortuitous events. A heavy monolayer of cells grown on a 16 ounce glass Brockway bottle was treated in the following manner. The cells were trypsinized, centrifuged to remove the trypsin and resuspended in 3 ml αMEM plus 10 percent FCS plus 7 percent (v/v) DMSO. The suspension was divided in half and aseptically dispensed into 2 12 x 75 mm plastic, capped tubes for storage at $-76^{\circ}C$.

3. Cloning Procedure

Colonies resistant to hydroxyurea were isolated from plates containing several colonies according to the following

protocol. An individual colony was isolated visually with the aid of a phase contrast microscope. The colony was scraped loose from the surface of the plate with a sterile pasteur pipette, transferred to a 60 x 15 mm plastic culture dish containing fresh complete medium and grown at 37°C to monolayer. At or near confluence the cells were trypsinized, counted and resuspended in fresh medium at a density of approximately 5 cells/ml. Approximately 0.2 ml of the suspension was placed in each well of a 96-well Linbro dish. After incubation at 37°C for approximately 10 days single colonies were aseptically transferred to 100 mm plates containing complete medium and eventually to glass Brockway bottles for regular maintenance.

The wild type cell lines were cloned in the same manner but not by the author personally.

4. Mutant Selection

Mutant cell lines were selected on the basis of the ability to form colonies while grown in the presence of a given concentration of drug (Wright and Lewis, 1974). The selective agent in this case was hydroxyurea and was administered in culture as a solution in αMEM plus 10 percent FCS. Parent cells were plated in complete medium containing no drug on 100 x 15 mm plastic culture plates and allowed to attach to the surface for 1-2 hours at 37°C. A given volume of a stock solution of hydroxyurea in αMEM plus 10 percent FCS was then added to give the desired concentration and the plates were returned to the incubator for a total

of 7 to 10 days. After approximately 5 days without disturbance the plates were surveyed microscopically for colony formation and at such time that surviving colonies were large enough to subculture, they were picked from the plate using a sterile pasteur pipette and transferred to 60 x 15 mm plastic culture dishes containing medium minus drug. After incubation at 37°C until confluent, the selected phenotypic variant was removed from the plate with trypsin and cloned according to the format outlined previously.

5. Drug Inhibition Studies

Cells were routinely maintained on glass Brockway bottles at 37°C in αMEM plus 10 percent FCS as described earlier. Exponentially growing cells were removed with trypsin, resuspended in a known volume of fresh medium and counted. A series of varying cell number was plated on 60 x 100 mm plastic culture plates in complete medium against a range of drug concentrations so that the number of surviving colonies was within the statistically countable range of 30 to 300. This number is arrived at only after a few trial plating efficiencies have been carried out and depends much on the cell lines being tested and its plating efficiency at the drug concentration employed. The plates were incubated at 37°C for 7 - 10 days at which time the medium was removed and a saturated solution of methylene blue in 50 percent ethanol was applied to the exposed surface. The

stain was poured off after 5 - 10 minutes at room temperature and the plates gently rinsed in lukewarm water. After allowing the plates to air dry, colonies of at least 30 cells were scored and plating efficiencies calculated. The plating efficiency (PE) is defined as the number of surviving colonies scored divided by the number of cells originally plated. When considering drug inhibition studies the relative plating efficiency (RPE) is defined as the plating efficiency of the cells at a given concentration of drug divided by the PE of the same cell line in the absence of the drug. For example, a cell line exhibiting a PE of 0.70 at 0 drug and 0.35 at a certain drug concentration is said to show a RPE of 0.50 at that drug concentration. As a means of comparing the effects of the drug tested on the various cell lines, D_{10} values were reported and defined as the drug concentration which reduces the RPE to a value of 0.10, i.e. 10 percent of the PE in the absence of drug.

6. Ribonucleotide Reductase Permeabilization Assay

Exponentially growing cells were plated at a density of 2×10^6 cells/100 x 15 mm plastic tissue culture plate in αMEM plus 10 percent FCS. After 46 - 50 hours incubation at $37^{\circ}C$ cells were harvested with trypsin, washed once in medium plus serum and counted. The cells were then resuspended at a density of 10^7 cells/ml of standard permeabilizing buffer consisting of 1 percent Tween-80, 0.25 M sucrose,

0.05 M HEPES (pH 7), 2 mM dithiothreitol (DTT) (Lewis, Kuzik and Wright, 1978). The permeabilizing process was routinely done at 24°C for 30 minutes after which the cells were centrifuged and resuspended at a concentration of 2.5×10^7 cells/ml of fresh standard permeabilizing buffer. 200 µl aliquots of this suspension were added to 100 µl of standard reaction buffer to give a final concentration of 0.05 M HEPES (pH 7), 6 mM DTT, 8 mM $MgCl_2$, 4 mM ATP and 0.4 mM ^{14}C-labelled CDP (5000 cpm/nmole) in the final reaction mixture. The cells were incubated shaking at 37°C for 30 minutes. The reaction was terminated with boiling for 4 minutes. To remove terminal phosphate groups from the nucleotides each assay was incubated shaking at 37°C for 2 hours with 2 µg Crotalux atrox venom dissolved in 0.1 M HEPES (pH 8) plus 10 mM $MgCl_2$. The reaction was again terminated with boiling for 4 minutes. Deoxycytidine was separated from cytidine according to the method of Steeper and Steuart (1970) as modified by Cory and Whitford (1972). Sufficient distilled water was added to each assay to bring the volume up to 1 ml. After centrifugation to remove heat precipitated material the supernatant was applied to a 5 x 80 mm Dowex-1-borate column. Due to the properties of the exchange resin the ribonucleosides complex with and remain attached to the borate ion in the column while the deoxyribonucleosides are eluted from the column with 4 ml distilled water. 10 ml of aqueous counting scintillant (ACS) were added to the eluant and shaken vigorously. The amount of

radioactivity in each sample was counted with a Beckman model LS230 liquid scintillation spectrophotometer. The spectrophotometer contained a ^{14}C channel set at a pulse height of 100 - 1000 divisions (4.5 percent gain) and counted a ^{14}C standard with an efficiency of 60 percent.

A similar protocol was followed for adenine diphosphate (ADP) reduction except for a few differences: the reaction buffer consisted of 0.05 M HEPES (pH 7.0), 6 mM DTT, 0.5 mM deoxyGTP and 0.4 mM ^{14}C-labelled ADP (5000 cpm/nmole) in the final reaction mixture. Three μmoles of carrier AMP were added with the snake venom to each assay and the assay volume was made up to 1 ml with 1 mM sodium borate. The borate Dowex was equilibrated with 10 ml 1 mM sodium borate before the supernantant was applied to the column and the sample was then followed by 4 ml 1 mM sodium borate. The deoxyadenine nucleosides were then eluted with 16 ml 1 mM sodium borate, 4 of which were added to ACS and counted in a liquid scintillation spectrophotometer as described above.

One unit of enzyme activity is defined as the amount of enzyme which will reduce 1 pmole of nucleotide in 30 minutes.

7. Source of Materials

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

8. Definition of the Term "Mutant" as Used in This Thesis

In the absence of suitable recombination and segregation methods to analyze the genetic properties of somatic cells exhibiting altered phenotypes, I have used the word "mutant" to describe a cell line which shows a stably altered phenotype when cultured in the absence of selective agent (Till et al., 1973; Siminovitch, 1976).

TABLE 1

Source of Materials

Material	Source
Aqueous Counting Scintillant	Amersham Corp., Illinois
Culture Plates (60x15 mm plastic)	Lux Scientific Corp., Newbury
Culture Plates (100x15 mm plastic)	"
Culture Plates (150x15 mm plastic)	"
Culture Bottles (16 oz glass)	Brockway Glass Co., New York
Dowex 1-X8 Anion Exhange Resin (200-400 mesh, chloride form)	Bio-Rad Laboratories, Richmond
Fetal Calf Serum	GIBCO Ltd., Grand Island
Linbro Cloning Dish (#1S-FS-96-TC)	Linbro Scientific Inc., Connecticut
α-Minimal Essential Medium	Flow Laboratories, Virginia
Penicillin G	GIBCO Ltd., Detroit
Streptomycin	GIBCO Ltd., Detroit
Trypsin Bacto (#0153-61)	Difco Laboratories, Detroit
Tween-80	J.T. Baker Chemical Co., Phillipsberg

RESULTS

1. Growth Rate of Wild Type L Cell Lines

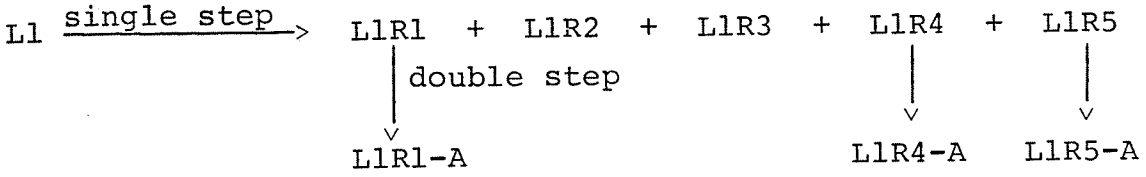
Figure 1 indicates the growth rate of L1 and L3 wild type cells under routine culture conditions. When grown on 60 mm plastic culture plates in complete medium, L1 and L3 cells increased exponentially in number from 10^5 cells/plate to over 10^6 cells/plate over a period of 96 hours. The doubling times at 37°C under routine culture conditions were approximately 22 and 18 hours for L1 and L3 cells, respectively. This growth rate is typical for mouse cells grown in culture (Todaro and Green, 1963; Kuzik and Wright, 1979).

2. Effect of Hydroxyurea on the Plating Efficiency of Wild Type L Cells

The colony producing ability of wild type L cells was examined in the presence of increasing amounts of hydroxyurea. In agreement with other investigations (Bacchetti and Whitmore, 1969; Skoog and Nordenskjold, 1971) Figures 2 and 4 show that both wild type L1 and L3 cells were sensitive to relatively low concentrations of hydroxyurea. At 0.70 mM the plating efficiencies (PE) of L1 and L3 cells were reduced to 50 percent or less of that in the absence of drug. Concentrations of drug greater than 0.70 mM caused an exponential decrease in the PE with 0.26 mM reducing the relative plating efficiencies (RPE) of L1 and L3 cells to 4.75×10^{-4} and 6.25×10^{-4}, respectively.

3. Growth Rate of Hydroxyurea-Resistant L Cell Lines

Single-step hydroxyurea-resistant cell lines were isolated according to the protocol for mutant selection described in the Materials and Methods section. The double-step mutants were obtained by subjecting the various single-step cloned isolates to a second, higher drug concentration and isolating a cell line capable of proliferating in the presence of very high drug concentrations (Wright and Lewis, 1974). Table 2 indicates the concentrations of hydroxyurea used to select the various single-and double-step drug-resistant cell lines. Each hydroxyurea-resistant cell line received a designation based on the wild type or single-step clone from which it was derived. For example, L3R1-A is a double-step mutant derived from the single-step mutant L3R1 which was derived from the L3 wild type clone. The selection of hydroxyurea-resistant of mutants can be schematically represented as such:



L3 single step → L3R1 double step → L3R1-A + L3R1-B + L3R1-C

The growth of both single- and double-step hydroxyurea-resistant cell lines was examined under routine culture conditions and the various doubling times were determined. The

TABLE 2

Drug concentrations for selection of single- and double-step hydroxyurea-resistant mutants

Cell Line	Single-Step HU* Concentration (mM)	Double-Step HU* Concentration (mM)
L1	-	-
L3	-	-
L1R1	0.3	-
L1R2	0.3	-
L1R3	0.3	-
L1R4	0.5	-
L1R5	0.5	-
L3R1	0.3	-
L1R1-A	0.3	0.9
L1R4-A	0.3	0.9
L1R5-A	0.3	0.9
L3R1-A	0.5	1.1
L3R1-B	0.5	1.1
L3R1-C	0.3	1.1

*HU - hydroxyurea

growth curves of four typical L cell drug resistant cell lines, two derived from L1 wild type and two derived from L3 wild type are plotted in Figure 1. Each cell line demonstrated a slightly longer doubling time than the corresponding parental wild type. The doubling times under normal growth conditions at 37°C were as follows: L1R1, L1R1-A, L3R1, L3R1-C showed doubling times of 24, 25, 23, and 20 hrs respectively. These growth rates are typical of the results obtained with all the drug-resistant cell lines shown in Table 2. It should be noted that similar findings have previously been reported for studies with other mammalian hydroxyurea-resistant lines (Lewis and Wright, 1978; Kuzik and Wright, 1979).

4. Effect of Hydroxyurea on the Plating Efficiency (PE) of Hydroxyurea-Resistant L Cells

Single- and double-step mutant cells were incubated at various cell number in the presence of increasing concentration of hydroxyurea. After 7 to 10 days at 37°C without disturbance, surviving colonies were stained and counted and PEs and RPEs were calculated. RPEs were plotted against hydroxyurea concentration and D_{10} values determined.

The single-step L cell lines were significantly more resistant to the cytotoxic effects of hydroxyurea as compared to parental wild type cells. Figures 2 through 4 demonstrate the effect of hydroxyurea on six independently selected single-step drug-resistant clones. L1R4 and L1R5 were selected

at 0.50 mM hydroxyurea while L1R1, L1R2, L1R3 and L3R1 were selected in the presence of 0.30 mM hydroxyurea. L1R1, L1R4, L1R5 and L3R1 showed similar patterns of killing in the presence of hydroxyurea; D_{10} values are reported as 0.32, 0.40, 0.38, and 0.34 mM hydroxyurea, respectively, compared to 0.14 for both L1 and L3 wild type cells. On the other hand, L1R2 and L1R3 exhibited even greater resistance to the drug than the previously mentioned cell lines and showed D_{10} values greater than 1.0 mM hydroxyurea.

Figures 2 through 4 also show the effect of hydroxyurea on the plating efficiencies of six independently selected double-step drug-resistant clones. D_{10} values among the double-step mutants were consistently higher than the single-step mutants, not including L1R2 and L1R3 which showed exceptionally high resistance compared to other single- and double-step mutants. D_{10} values for the double-step mutants L1R1-A, L1R4-A, L1R5-A, L3R1-A, L3R1-B and L3R1-C are reported as 0.92, 0.87, 0.78, 0.72, 0.82, and 0.84 mM hydroxyurea, respectively. Table 3 summarizes the D_{10} values for hydroxyurea of both single- and double-step mutants.

5. Ribonucleotide Reductase Activity as a Function of Time and Cell Number

When considering the kinetics of an enzyme system it is important that all conditions are maximal and that no single parameter biases the results to a significant degree. The

TABLE 3

D_{10} values for inhibition of cellular growth by hydroxyurea in wild type and hydroxyurea-resistant mutant cell lines.

Cell Line	D_{10} (mM hydroxyurea)
L1	0.14
L3	0.14
L1R1	0.32
L1R2	>1.0
L1R3	>1.0
L1R4	0.40
L1R5	0.38
L3R1	0.34
L1R1-A	0.92
L1R4-A	0.87
L1R5-A	0.78
L3R1-A	0.72
L3R1-B	0.82
L3R1-C	0.84

D_{10} values reported above are typical for those cell lines; similar results were observed in each of three experiments.

linearity of ribonucleotide reductase activity with respect to time and cell number was checked in representative wild type and single- and double-step drug resistant cell lines (L1, L1R1, L1R1-A, L3, L3R1, L3R1-C). Figures 5 through 16 show that at a substrate concentration of at least 0.4 mM all cell lines tested exhibited a linear increase in ribonucleotide reductase activity over the thirty minute assay period for both CDP and ADP reduction. Figures 5 through 16 also show that the increase in ribonucleotide reductase activity was linear with cell number to 1×10^7 cells per assay. These results are in agreement with other studies with L cells (Kuzik and Wright, 1979) and CHO cells (Lewis, Kuzik and Wright, 1978) which indicated linearity of ribonucleotide reducrase activity with time to at least 30 minutes and with cell number up to 9×10^6 cells per assay.

6. The Effect of Hydroxyurea on Ribonucleotide Reductase Enzyme Kinetics

(a) Changes in Michaelis Constants

Hydroxyurea is believed to be toxic to cultured mammalian cells through inhibition of the target enzyme ribonucleotide reductase (Lewis and Wright, 1974; Lewis and Wright, 1978b; Brockman et al., 1970). Using a novel permeabilization technique developed in this lab (Lewis, Kuzik and Wright, 1978) ribonucleotide reductase activity was studied in intact wild type and hydroxyurea-resistant cells

made permeable to nucleotides.

Table 4 is a summary of apparent K_m and V_{max} values for both CDP and ADP reduction in wild type and mutant cell lines surveyed. Each value reported is the average of three trials $\pm$5-12%. Kinetic studies were routinely done with exponentially growing cells treated in the manner described in Materials and Methods under Ribonucleotide Reductase Permeabilization Assay. Standard velocity (V) versus substrate (S) curves were obtained at substrate concentrations between 0.022 and 2 mM for both CDP and ADP reduction. Lineweaver-Burke double reciprocal plots of data from V versus S curves were linear and yielded apparent K_m and V_{max} values for each cell line. Best fitting double reciprocal plots were generated by linear regression. Please refer to Figures 17 through 44 for typical kinetic curves.

In vivo levels of ribonucleotide reductase activity concurred with levels of cellular resistance exhibited by wild type and hydroxyurea-resistant mouse L cell lines. Under standard assay conditions L1 and L3 wild type mouse cells showed maximum velocities of 80 and 104 pmoles CDP reduced 30 min/5 x 10^6 cells, respectively. Single-step mutants (L1R1, L1R4, L1R5 and L3R1) showing a 2.3- to 3-fold increase in resistance to hydroxyurea demonstrated a corresponding increase in the rate of CDP reduction from an average of 80 for parental wild type cells to 213, 276 and 176 pmoles CDP reduced/30 min/ 5 x 10^6 L1R1, L1R4, and L1R5 cells, respectively; and from 104 for parental wild type cells to 208 pmoles CDP reduced/

TABLE 4

Apparent Michaelis constants for ribonucleotide reductase activity in wild type and hydroxyurea-resistant mutant cell lines

Cell Line	CDP Reduction		ADP Reduction	
	K_m (mM)	Vmax (ρmoles/30'/5x10^6 cells)	K_m (mM)	Vmax (ρmoles/30'/5x10^6 cells)
L1	0.11	80	0.40	131
L3	0.16	104	0.42	148
L1R1	0.56	213	0.38	296
L1R2	0.39	1039	0.49	458
L1R3	0.36	1028	0.52	432
L1R4	0.48	276	0.64	124
L1R5	0.47	176	0.65	117
L3R1	0.56	208	0.40	310
L1R1-A	0.47	568	1.12	630
L1R4-A	0.40	652	1.28	700
L1R5-A	0.37	500	1.31	585
L3R1-A	0.49	475	1.41	611
L3R1-B	0.34	614	1.07	635
L3R1-C	0.44	537	1.67	647

Each value represents an average of 3 trials ± 5-12 percent.

30 min/5 x 10^6 L3R1 cells. A similar increase was demonstrated by ADP reductase activity in L1R1 and L3R1 cells where wild type apparent maximum velocities of 131 and 148 ρmoles ADP reduced/30 min/5 x 10^6 L1 and L3 cells, respectively, increased 2- to 2.3-fold to 296 and 310 ρmoles ADP reduced/30 min/5 x 10^6 L1R1 and L3R1 cells, respectively. However, two single-step mutants L1R4 and L1R5 showed no increase in ADP reduction. Two exceptionally resistant single-step mutants L1R2 and L1R3 exhibited quite a dramatic increase in the apparent maximum velocity of CDP reductase activity and a more conservative increase in the level of ADP reductase activity. Each showed an average apparent maximum velocity of 1034 ρmoles CDP reduced/30 min/5 x 10^6 cells which is a 13-fold increase in the wild type level of CDP reduction, and 445 ρmoles ADP reduced/30 min/5 x 10^6 cells which represents a 3.4-fold increase in wild type ADP reductase activity.

Apparent K_m values reported for CDP reductase in all six single-step drug-resistant clones were between 2.1 and 4.3 times higher than their respective wild type values. This is demonstrated by an increase in apparent K_m from 0.11 mM CDP in L1 wild type cells to 0.56, 0.39, 0.36, 0.48, and 0.47 in L1R1, L1R2, L1R3, L1R4, and L1R5 cells, respectively, and from 0.16 to 0.56 mM CDP in L3R1 cells. ADP reductase exhibited little or no change in apparent K_m from the wild type values of 0.40 and 0.42 mM ADP for L1 and L3 cells, respectively. Apparent K_m ADP values for L1R1, L1R2, L1R3,

L1R4, L1R5, and L3R1 were 0.38, 0.49, 0.53, 0.64, 0.65, and 0.40 mM ADP, respectively.

A similar pattern of changes was found in hydroxyurea-resistant clones isolated via a double-step selection process. As before, 4.7- to 8.5-fold and 4.1- to 4.7-fold elevations in the levels of CDP and ADP reductase activity, respectively, correlated well with 4.9- to 6-fold increases in cellular resistance to hydroxyurea. CDP reductase activity increased from 80 with parental wild type cells to 568, 652, and 500 ρmoles CDP reduced/30 min/5 x 10^6 L1R1-A, L1R4-A and L1R5-A cells, respectively; and from 104 with parental wild type cells to 475, 614, and 537 ρmoles CDP reduced/30 min/5 x 10^6 L3R1-A, L3R1-B and L3R1-C cells, respectively. ADP reductase activity increased from 131 with parental wild type cells to 630, 700, and 585 ρmoles ADP reduced/30 min/5 x 10^6 L1R1-A, L1R4-A and L1R5-A cells, respectively; and from 148 with parental wild type cells to 611, 635, and 647 ρmoles ADP reduced/30 min/5 x 10^6 L3R1-A, L3R1-B and L3R1-C cells, respectively. Apparent K_m values 2.6 to 4 times the wild type were observed for both CDP and ADP reductase in all six double-step drug-resistant clones. CDP reductase showed apparent K_m values of 0.47, 0.40 and 0.37 mM CDP in L1R1-A, L1R4-A, and L1R5-A, respectively, compared to the wild type value of 0.11 for L1 cells, and 0.49, 0.34 and 0.44 mM CDP in L3R1-A, L3R1-B, and L3R1-C, respectively, compared to the L3 wild type apparent K_m value of 0.16 mM CDP. Unlike the single-step mutants which did not show significant changes

from the wild type in apparent K_m's for ADP reduction, all six double-step mutants exhibited changes in apparent K_m for ADP reduction from the L1 wild type value of 0.40 mM ADP to 1.1, 1.3, and 1.3 for L1R1-A, L1R4-A, and L1R5-A, respectively, and from the L3 wild type value of 0.42 mM ADP to 1.4, 1.1, and 1.7 for L3R1-A, L3R1-B, and L3R-C, respectively.

(b) Changes in Inhibition of Enzyme Activity

Since hydroxyurea is known to be a potent inhibitor of ribonucleotide reductase in mammalian cells (Lewis and Wright, 1974; Young et al., 1967) and previously isolated hydroxyurea-resistant cell lines have been found to exhibit resistance to the drug of the enzyme lvel (Lewis and Wright, 1978b; Kuzik and Wright, 1979), the enzyme activities of wild type and hydroxyurea-resistant cell lines isolated in this study were examined in the presence of hydroxyurea.

Studies of the inhibition of ribonucleotide reductase activity by hydroxyurea were carried out using permeabiliized cells (Lewis, Kuzik and Wright, 1978) and an estimation of drug inhibition was determined. Using the standard assay procedure previously described in Methods and Materials, ribonucleotide reductase activity was determined in the presence of increasing amounts of hydroxyurea from 0.1 to 1.0 mM and expressed as a percent of the activity in the absence of drug. The percent activity was plotted as a function of hydroxyurea concentration and an estimation of the apparent K_i values were obtained by observing the amount of hydroxyurea which

reduced ribonucleotide reductase under standard assay conditions to 50 percent of that in the absence of drug. These values are summarized in Table 5 and represent the average of two trials $\pm$ 5 to 10 percent. Typical hydroxyurea inhibition curves are shown in Figures 45 through 54.

Ribonucleotide reductase activity from three of the six single-step hydroxyurea-resistant clones L1R1, L1R4, and L3R1 was as sensitive to hydroxyurea as the enzyme from parental wild type cells. No appreciable change from the wild type in the inhibition of enzyme activity by hydroxyurea was observed for CDP or ADP reductase in the single-step mutants mentioned. CDP reductase exhibited apparent K_i values of 0.35 and 0.30 mM hydroxyurea in L1 and L3 wild type cells, respectively, and 0.35, 0.49, 0.40 in L1R1, L1R4 and L3R1 cells, respectively; while ADP reductase showed apparent K_i values of 0.32 and 0.30 mM hydroxyurea in L1 and L3 wild type cells, respectively, and 0.30, 0.40, 0.34 in L1R1, L1R4 cells, respectively. On the other hand, both CDP and ADP reductase in L1R2 and L1R3 showed a 3- to 4-fold increase in the apparent K_i for hydroxyurea while L1R5 exhibited a 2.3-fold increase in the apparent K_i for CDP reductase and a 2.8-fold increase in the apparent K_i for ADP reductase. In L1R2 hydroxyurea apparent K_i values for CDP and ADP reductase increased from 0.35 mM in parental wild type cells to 1.3 mM and from 0.32 in parental wild type cells to 1.0 mM, respectively; and in L1R3 from 0.35 mM to 1.6 and from 0.32 mM to 1.4. Apparent K_i values for CDP and ADP

TABLE 5

Inhibition of ribonucleotide reductase activity by hydroxyurea in wild type and hydroxyurea-resistant mutant cell lines

Cell Line	Apparent K_i (mM hydroxyurea)	
	CDP reduction	ADP reduction
L1	0.35	0.32
L3	0.30	0.30
L1R1	0.35	0.30
L1R2	1.30	1.00
L1R3	1.60	1.40
L1R4	0.49	0.40
L1R5	0.80	0.88
L3R1	0.40	0.34
L1R1-A	0.35	0.30
L1R4-A	0.35	0.30
L1R5-A	0.36	0.33
L3R1-A	0.42	0.40
L3R1-B	0.40	0.36
L3R1-C	0.35	0.35

Each value represents an average of 2 trials ± 5-10 percent

reductase in L1R5 increased from 0.35 to 0.80 mM and from 0.32 to 0.88 mM hydroxyurea, respectively.

No significant changes in ribonucleotide reductase sensitivity to hydroxyurea for CDP or ADP reductase were detected in any of the six double-step drug-resistant clones as compared to their respective wild type. At this point it should be noted that all double-step mutants were selected by coincidence from single-step counterparts which also did not show changes from the wild type in inhibition of ribonucleotide reductase by hydroxyurea. Apparent K_i values for CDP reductase in L1R1-A, L1R4-A and L1R5-A remained unchanged from the L1 wild type apparent K_i of 0.35 at 0.35, 0.35, and 0.36 mM hydroxyurea, respectively, while L3R1-A, L3R1-B and L3R1-C apparent K_i values showed little change from the L3 wild type value of 0.30 at 0.42, 0.40 and 0.35 mM hydroxyurea, respectively. ADP reductase apparent K_i values for hydroxyurea also showed little deviation from the wild type values of 0.32 mM for L1 cells and 0.30 mM for L3 cells; L1R1-A, L1R4-A and L1R5-A showed apparent K_i values for inhibition of ADP reductase activity of 0.30, 0.30 and 0.33 mM hydroxyurea, respectively, while L3R1-A, L3R1-B and L3R1-C exhibited apparent K_i values of 0.40, 0.36, and 0.35 mM hydroxyurea, respectively.

FIGURES

FIGURE 1: Growth of wild type and hydroxyurea-resistant mouse L cell lines under routine culture conditions. Exponentially growing cells of L1 (Fig. 1A), L1R1 (Fig. 1B), L1R1-A (Fig. 1C), L3 (Fig. 1D), L3R1 (Fig. 1E), and L3R1-C (Fig. 1F) were removed from the surface of a Brockway bottle with a sterile trypsin solution. Aliquots of 5×10^5 cells were then plated onto 60 x 15 mm plastic culture dishes containing complete medium. At various times, cells were removed from the surface of the plate with trypsin and counted.

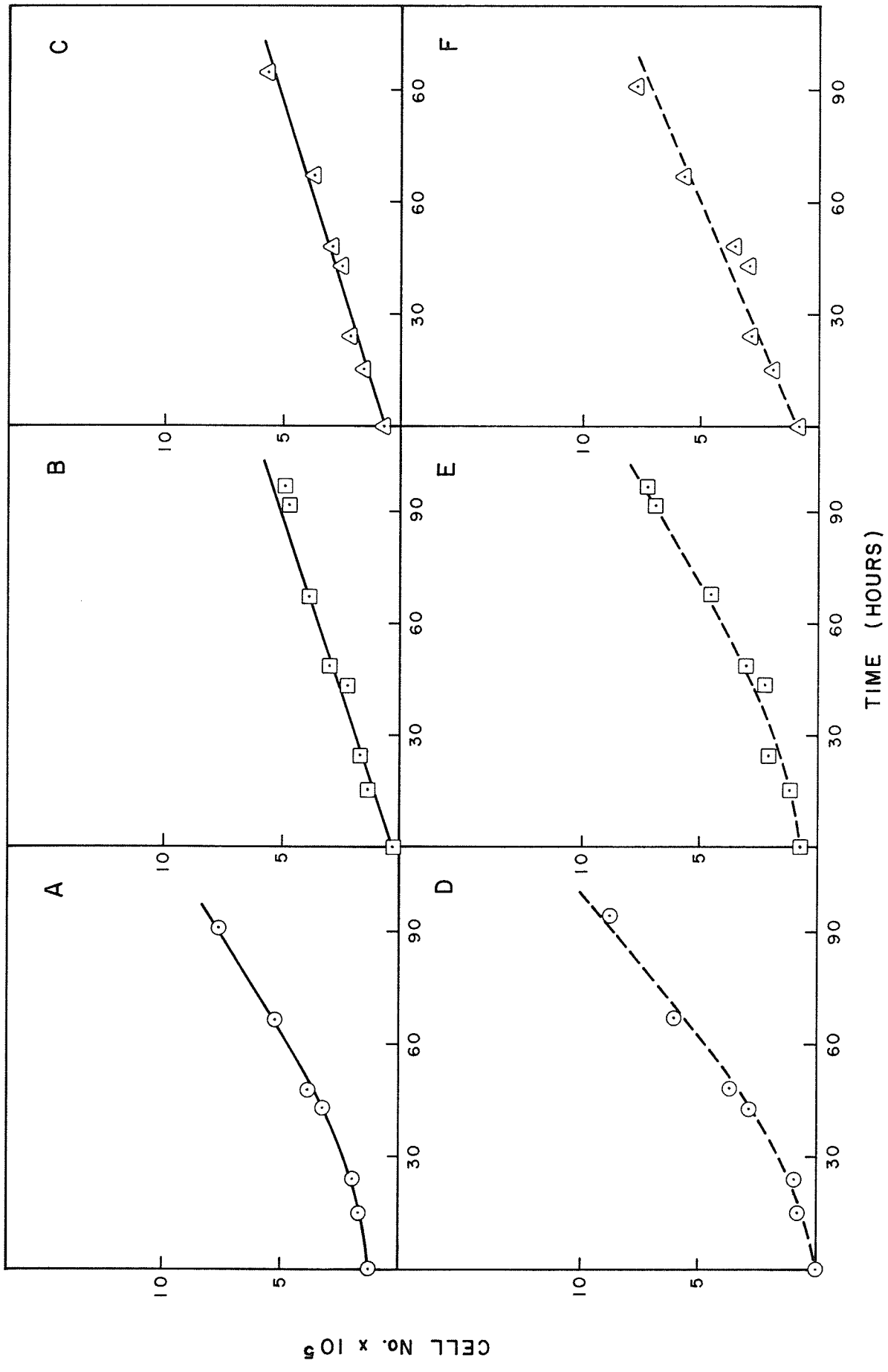

A
B
C
D
E
F
10
5
30
60
90
CELL No. x 10⁵
TIME (HOURS)

FIGURE 2: Effect of hydroxyurea on the relative plating efficiency of L1 (⊙), L1R1 (◇), L1R2 (△), L1R3 (⊡) and L1R1-A (⊙). Cells were plated onto 100 x 15 mm plastic culture dishes containing complete medium plus various concentrations of hydroxyurea. After approximately 10 days at 37°C the medium was poured off and the colonies were stained and counted.

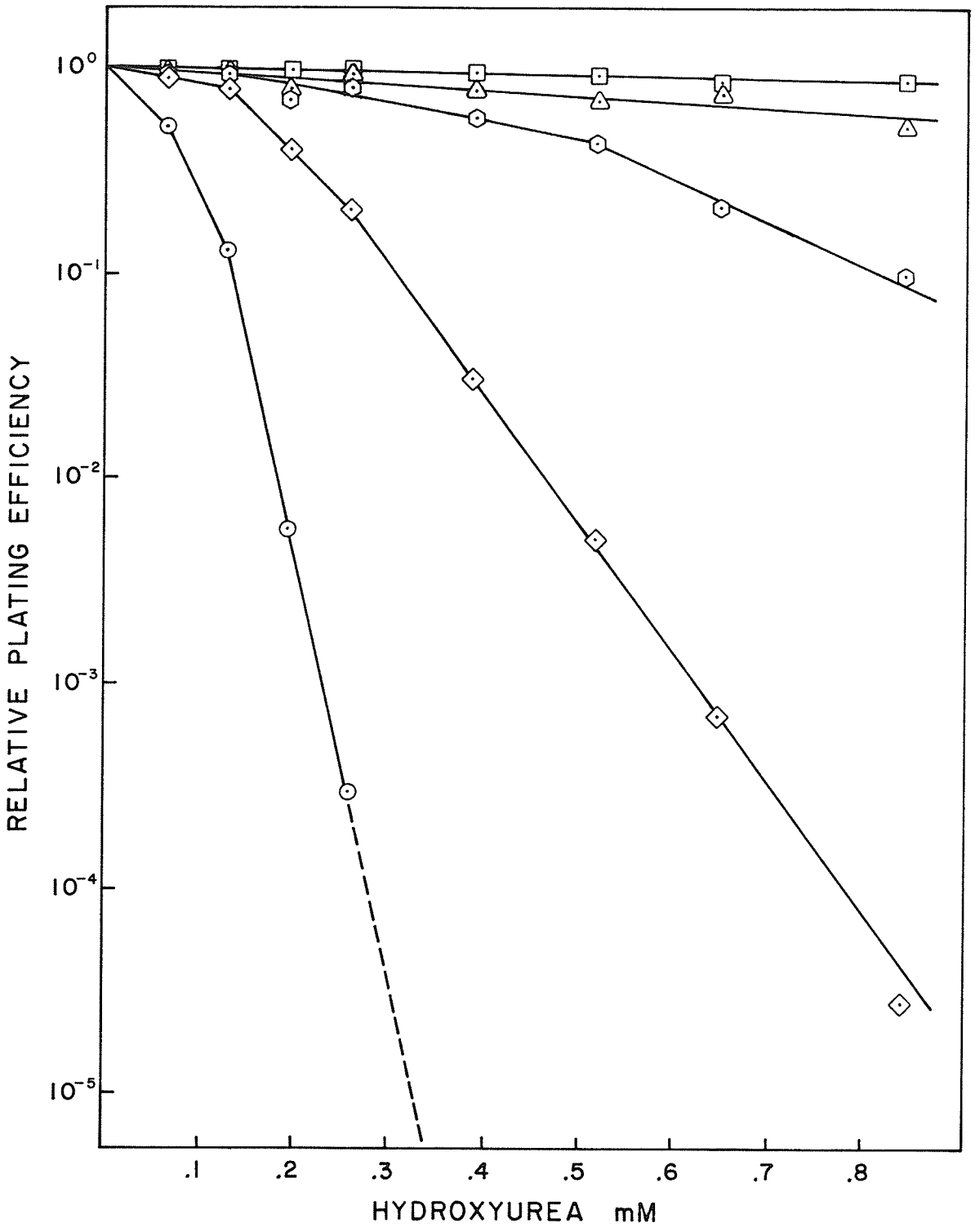

RELATIVE PLATING EFFICIENCY
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
.1
.2
.3
.4
.5
.6
.7
.8
HYDROXYUREA mM

FIGURE 3: Effect of hydroxyurea on the relative plating efficiency of L1 (⊙), L1R4 (△), L1R5 (◇), L1R4-A (⊡) and L1R5-A (⬡). Cells were plated onto 100 x 15 mm plastic culture dishes containing complete medium plus various concentrations of hydroxyurea. After approximately 10 days at 37°C the medium was poured off and the colonies were stained and counted.

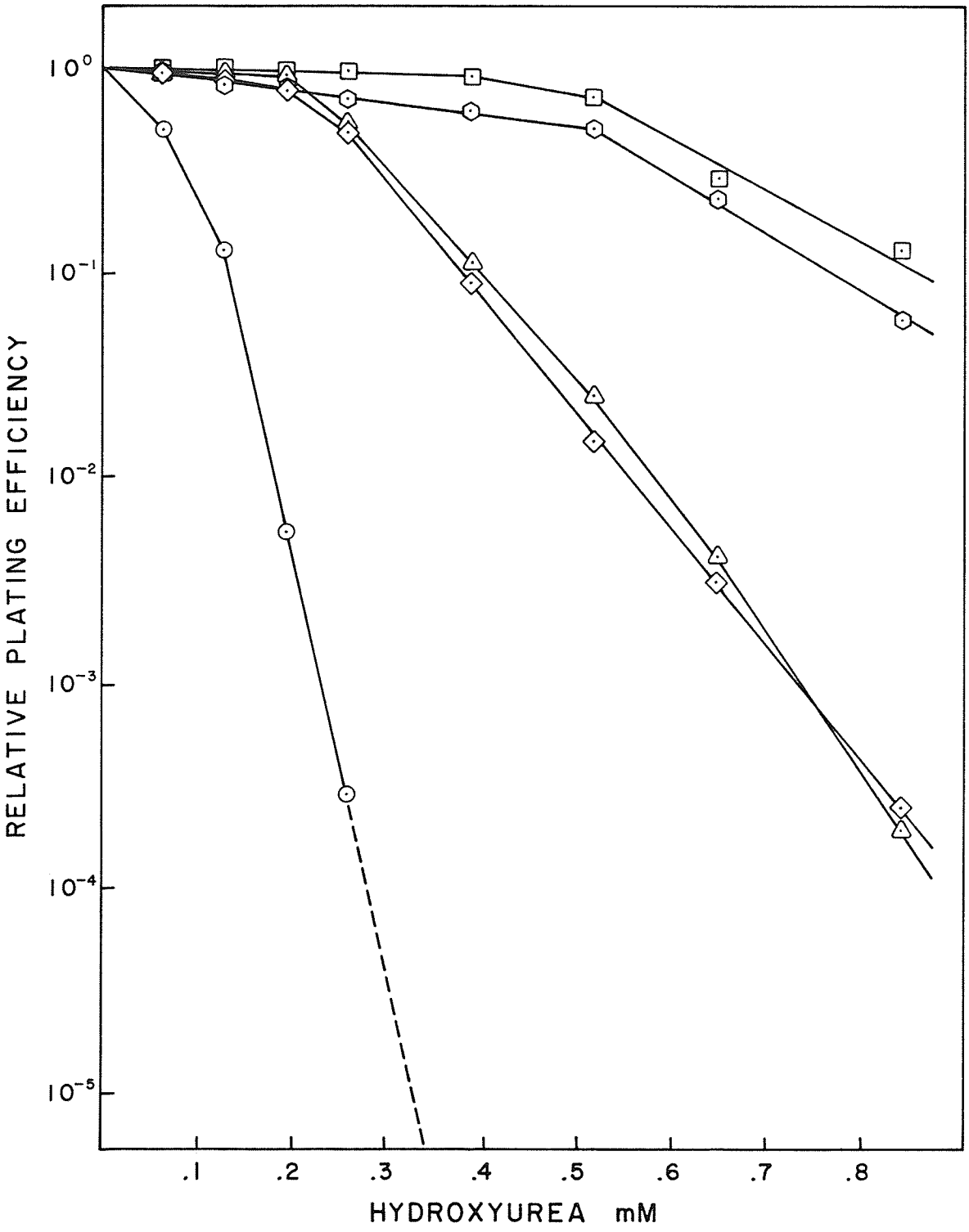

RELATIVE PLATING EFFICIENCY
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
.1
.2
.3
.4
.5
.6
.7
.8
HYDROXYUREA mM

FIGURE 4: Effect of hydroxyurea on the relative plating efficiency of L3 (⊙), L3R1 (⊡), L3R1-A (⊙), L3R1-B (◇) and L3R1-C (△). Cells were plated onto 100 x 15 mm plastic culture dishes containing complete medium plus various concentrations of hydroxyurea. After approximately 10 days at 37°C the medium was poured off and the colonies were stained and counted.

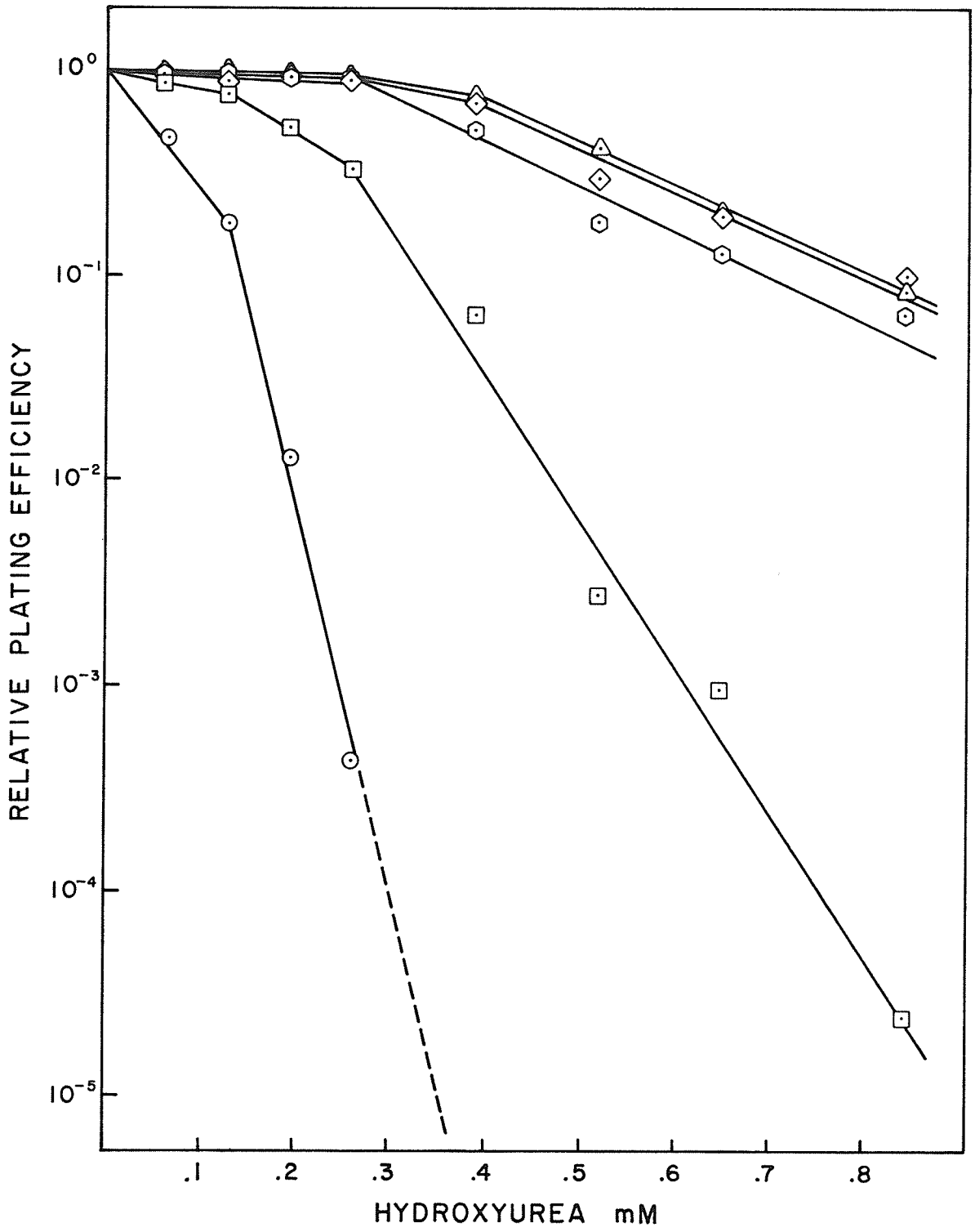

RELATIVE PLATING EFFICIENCY
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
.1
.2
.3
.4
.5
.6
.7
.8
HYDROXYUREA mM

FIGURE 5A: Effect of increasing cell number on L1 wild type CDP reduction. Various amounts of cells were added to standard CDP reductase assay reaction mixture. After incubation at 37°C for 30 minutes the amount of CDP reduced was determined. Velocity (as defined in Materials and Methods) is pmoles of ribonucleotide reduced per 30 minutes.

FIGURE 5B: Response of CDP reduction assay in L1 wild type cells with time of incubation. Standard aliquots of cells were added to complete reaction mixture and incubated at 37°C. At the times indicated, the reaction was terminated by boiling, and the amount of CDP reduced was determined. Velocity (as defined in Materials and Methods) is pmoles of ribonucleotide reduced per 5×10^6 cells.

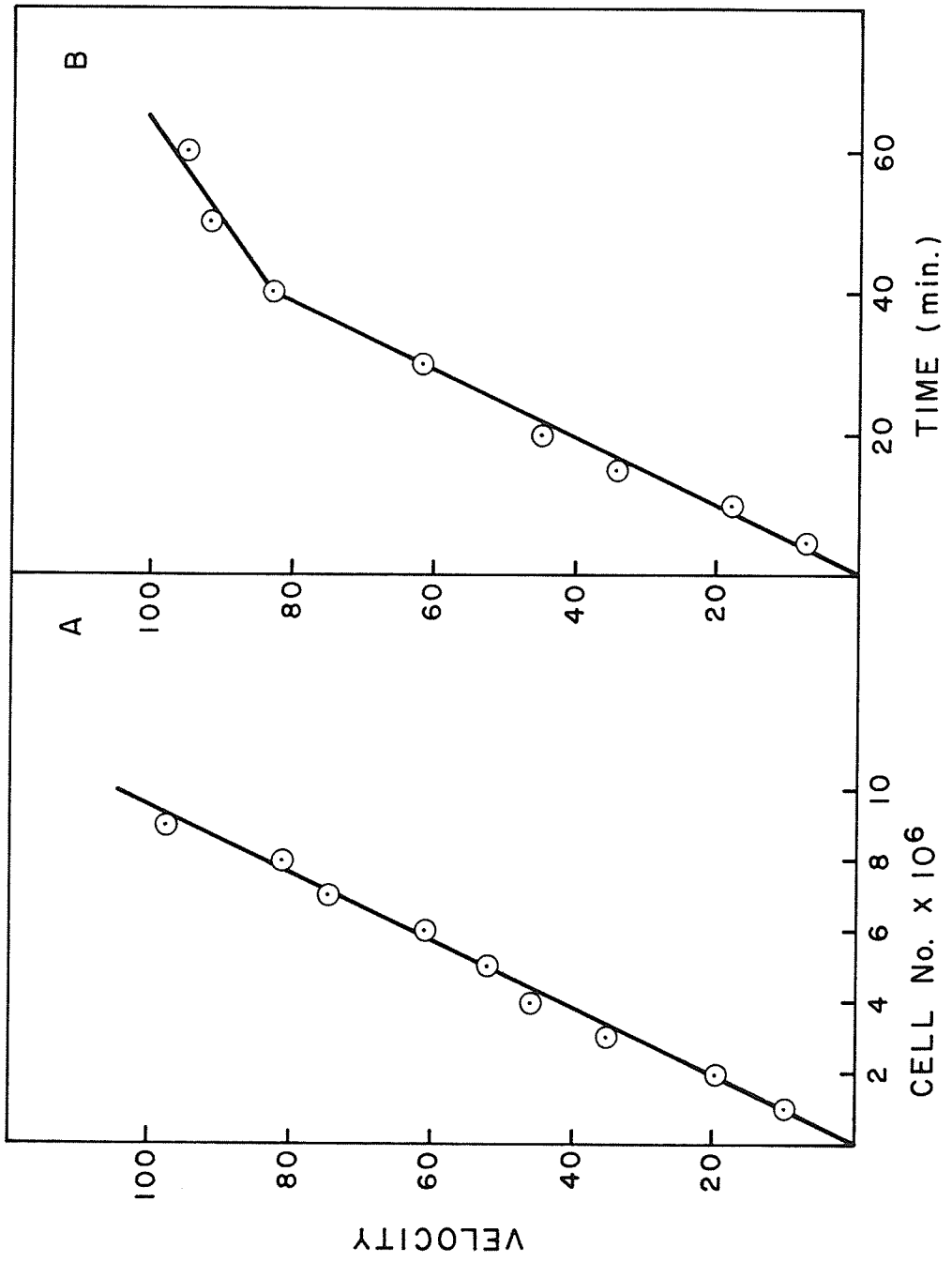

A
B
VELOCITY
100
80
60
40
20
2
4
6
8
10
CELL No. x 10⁶
20
40
60
TIME (min.)

FIGURE 6A: Effect of increasing cell number on L1 wild type ADP reduction. Various amounts of cells were added to standard ADP reductase assay reaction mixture. After incubation at 37°C for 10 minutes the amount of ADP reduced was determined.

FIGURE 6B: Response of ADP reduction in L1 wild type cells with time of incubation. Standard aliquots of cells were added to complete reaction mixture and incubated at 37°C. At the times indicated, the reaction was terminated by boiling, and the amount of ADP reduced was determined.

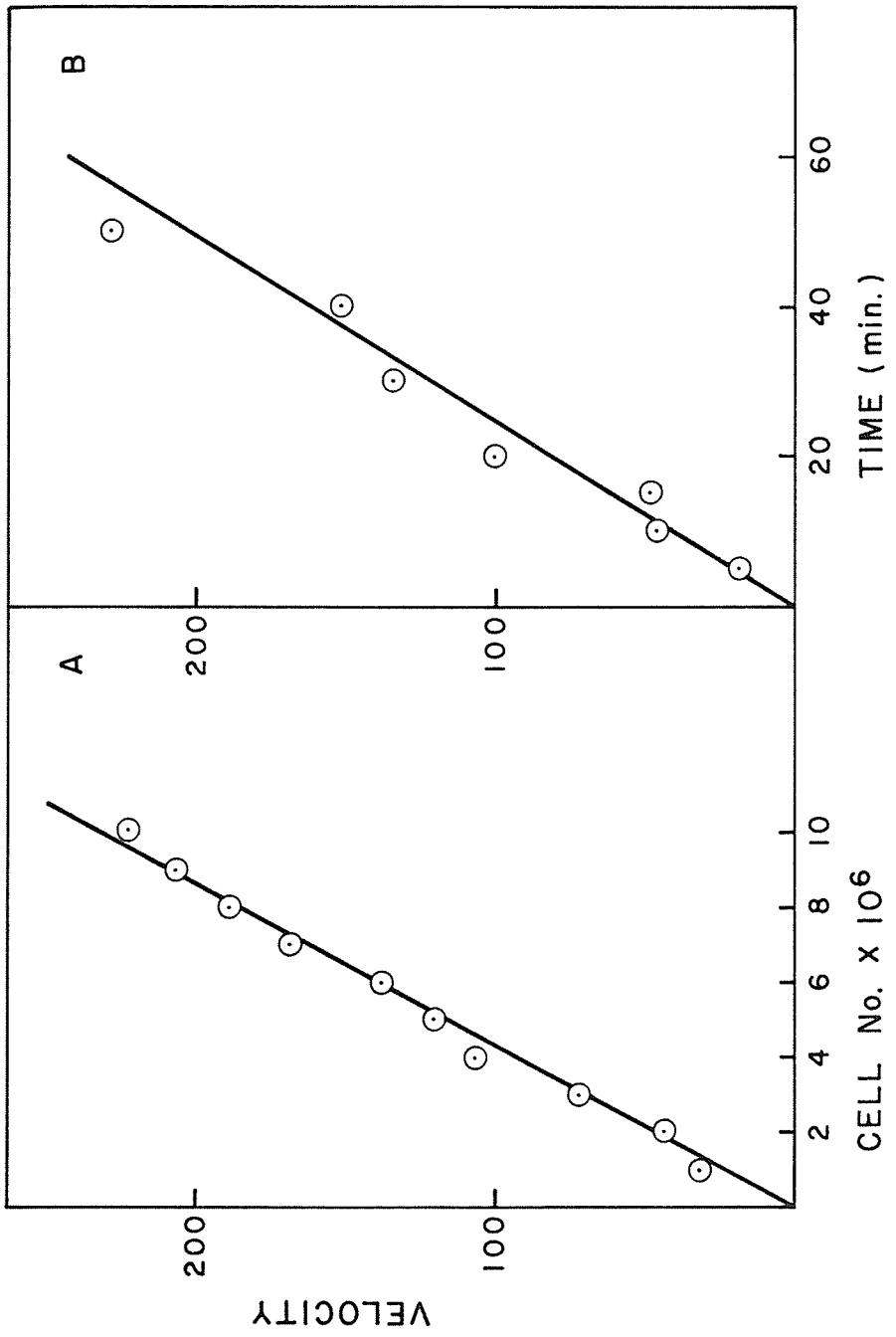

A
B
200
100
VELOCITY
2
4
6
8
10
CELL No. X 10⁶
20
40
60
TIME (min.)

FIGURE 7A: Effect of increasing cell number on L1R1 CDP reduction. Various amounts of cells were added to standard CDP reductase assay reaction mixture. After incubation at 37°C for 30 minutes the amount of CDP reduced was determined.

FIGURE 7B: Response of CDP reduction assay in L1R1 hydroxyurea-resistant cells with time of incubation. Standard aliquots of cells were added to complete reaction mixture and incubated at 37°C. At the times indicated, the reaction was terminated by boiling, and the amount of CDP reduced was determined.

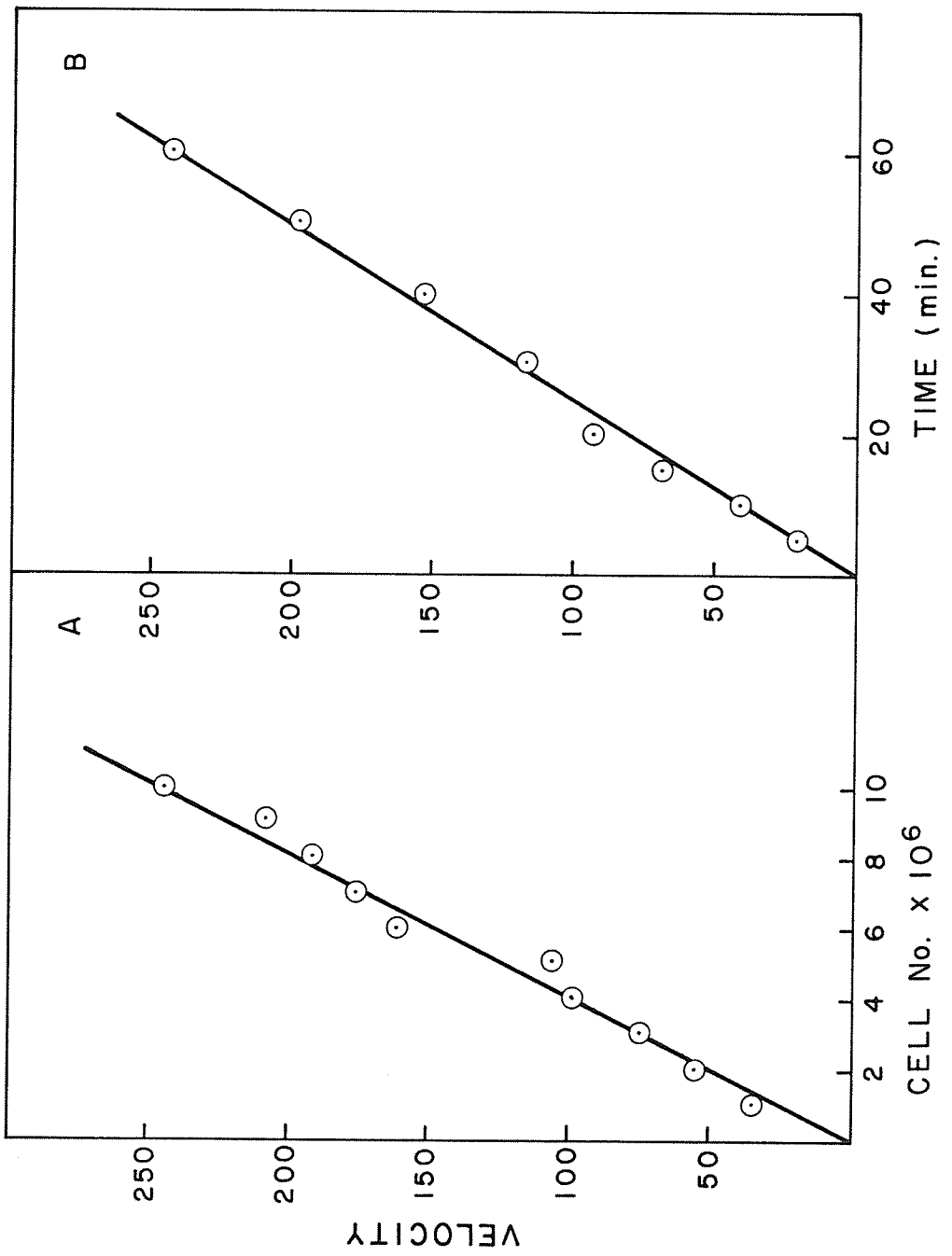

A
B
VELOCITY
250
200
150
100
50
2
4
6
8
10
CELL No. x 10⁶
20
40
60
TIME (min.)

FIGURE 8A: Effect of increasing cell number on L1R1 ADP reduction. Various amounts of cells were added to standard ADP reductase assay reaction mixture. After incubation at 37°C for 30 minutes the amount of ADP reduced was determined.

FIGURE 8B: Response of ADP reduction assay in L1R1 hydroxyurea-resistant cells with time of incubation. Standard aliquots of cells were added to complete reaction mixture and incubated at 37°C. At the times indicated, the reaction was terminated by boiling, and the amount of ADP reduced was determined.

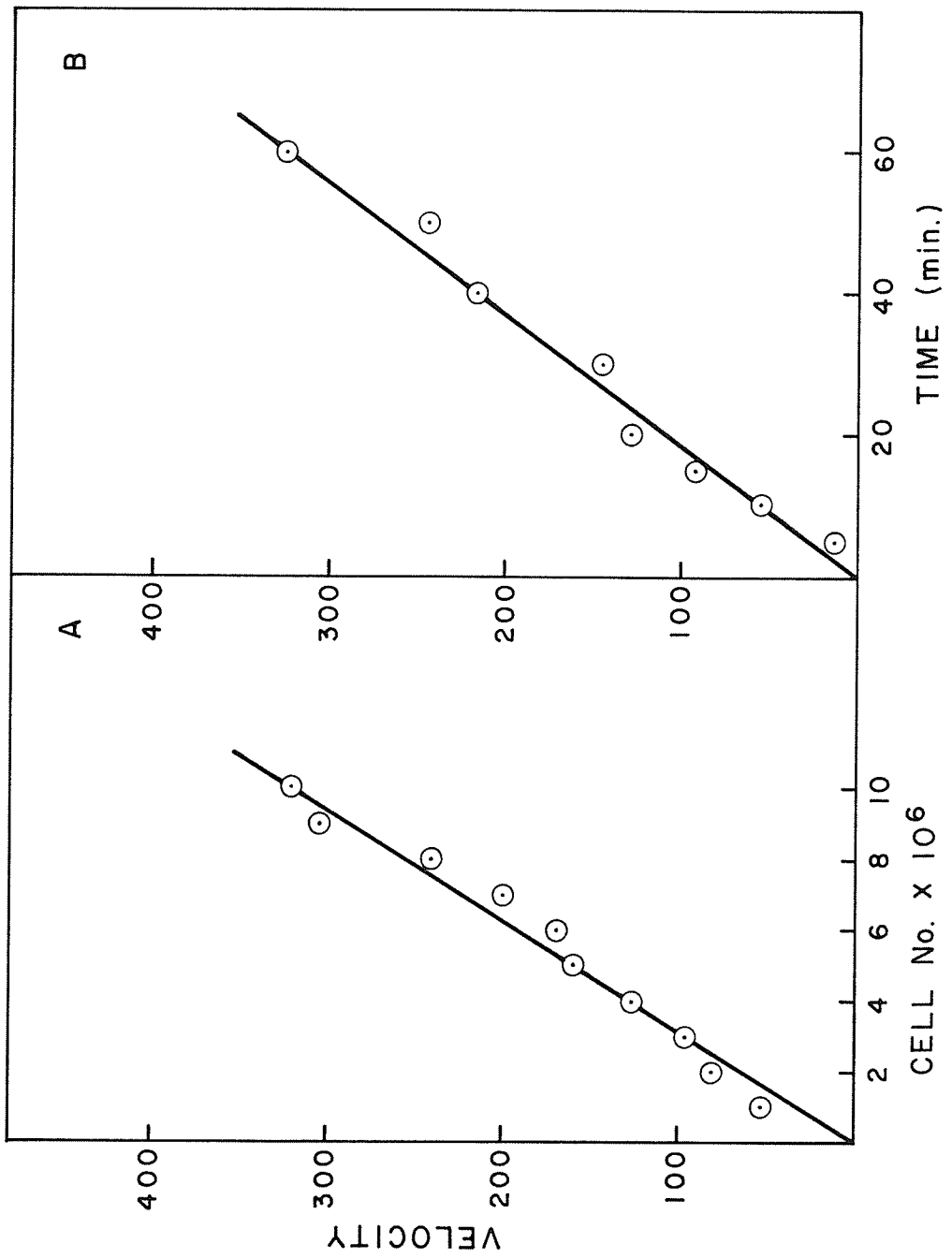

A
B
VELOCITY
100
200
300
400
2
4
6
8
10
CELL No. X 10⁶
20
40
60
TIME (min.)

FIGURE 9A: Effect of increasing cell number on L1R1-A CDP reduction. Various amounts of cells were added to standard CDP reductase assay reaction mixture. After incubation at 37°C for 30 minutes the amount of CDP reduced was determined.

FIGURE 9B: Response of CDP reduction assay in L1R1-A hydroxyurea-resistant cells with time of incubation. Standard aliquots of cells were added to complete reaction mixture and incubated at 37°C. At the times indicated, the reaction was terminated by boiling, and the amount of CDP reduced was determined.

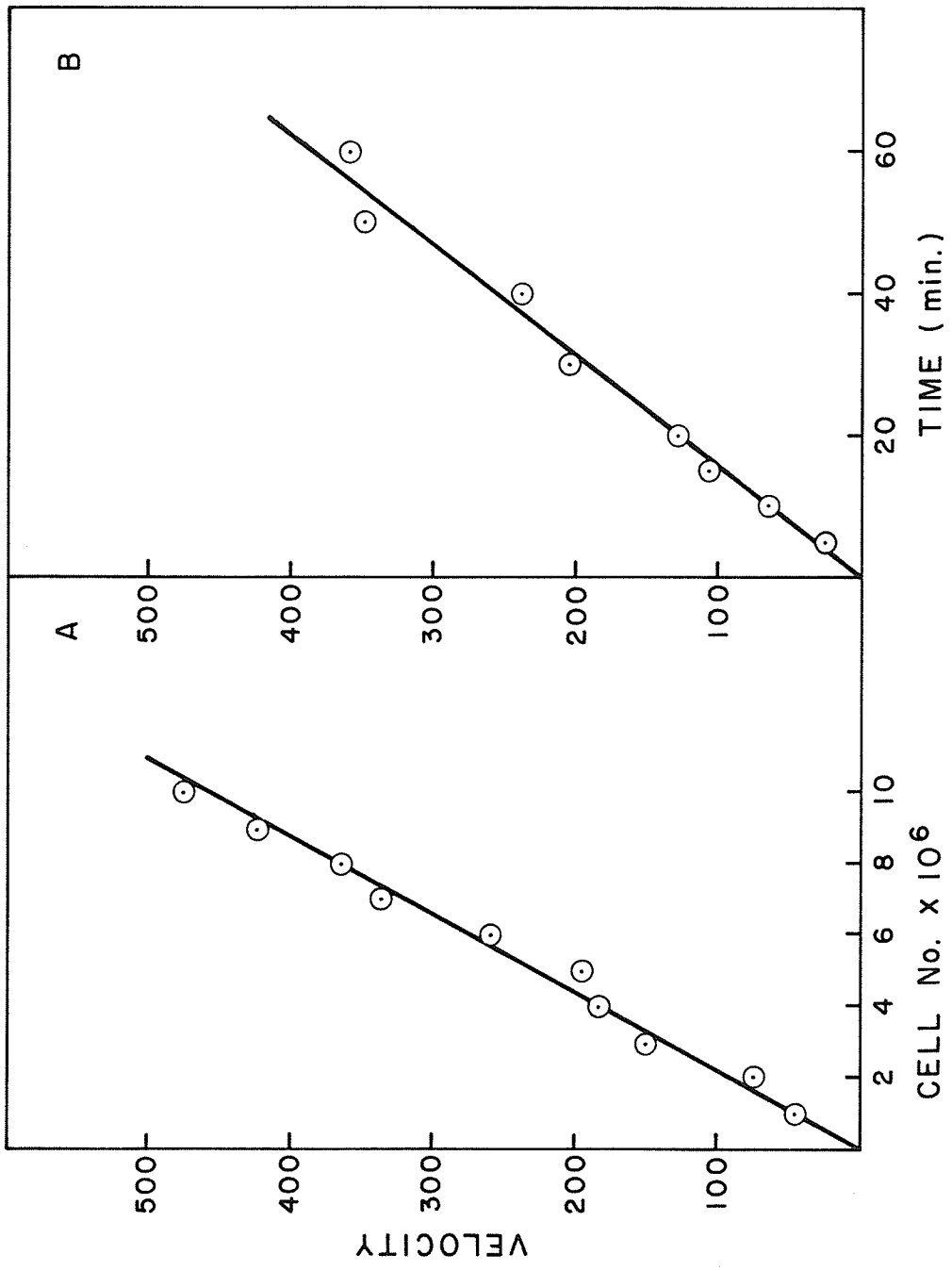

A
B
VELOCITY
500
400
300
200
100
2
4
6
8
10
CELL No. X 10⁶
20
40
60
TIME (min.)

FIGURE 10A: Effect of increasing cell number on L1R1-A ADP reduction. Various amounts of cells were added to standard ADP reductase assay reaction mixture. After incubation at 37°C for 30 minutes the amount of ADP reduced was determined.

FIGURE 10B: Response of ADP reduction assay in L1R1-A hydroxyurea-resistant cells with time of incubation. Standard aliquots of cells were added to complete reaction mixture and incubated at 37°C. At the times indicated, the reaction was terminated by boiling, and the amount of ADP reduced was determined.

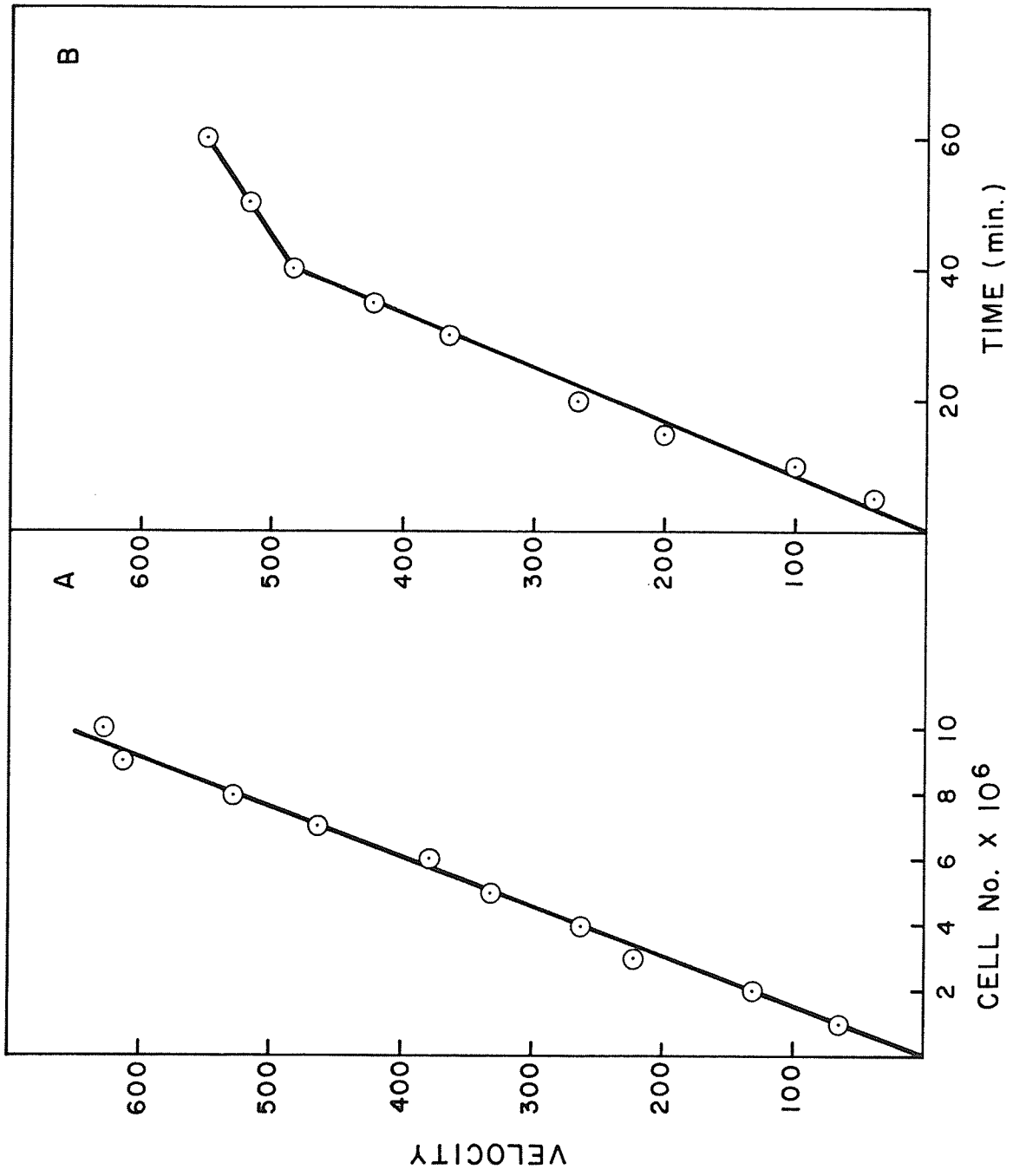

A
B
VELOCITY
100
200
300
400
500
600
2
4
6
8
10
CELL No. X 10⁶
20
40
60
TIME (min.)

FIGURE 11A: Effect of increasing cell number on L3 wild type CDP reduction. Various amounts of cells were added to standard CDP reductase assay reaction mixture. After incubation at 37°C for 30 minutes the amount of CDP reduced was determined.

FIGURE 11B: Response of CDP reduction assay in L3 wild type cells with time of incubation. Standard aliquots of cells were added to complete reaction mixture and incubated at 37°C. At the times indicated, the reaction was terminated by boiling, and the amount of CDP reduced was determined.

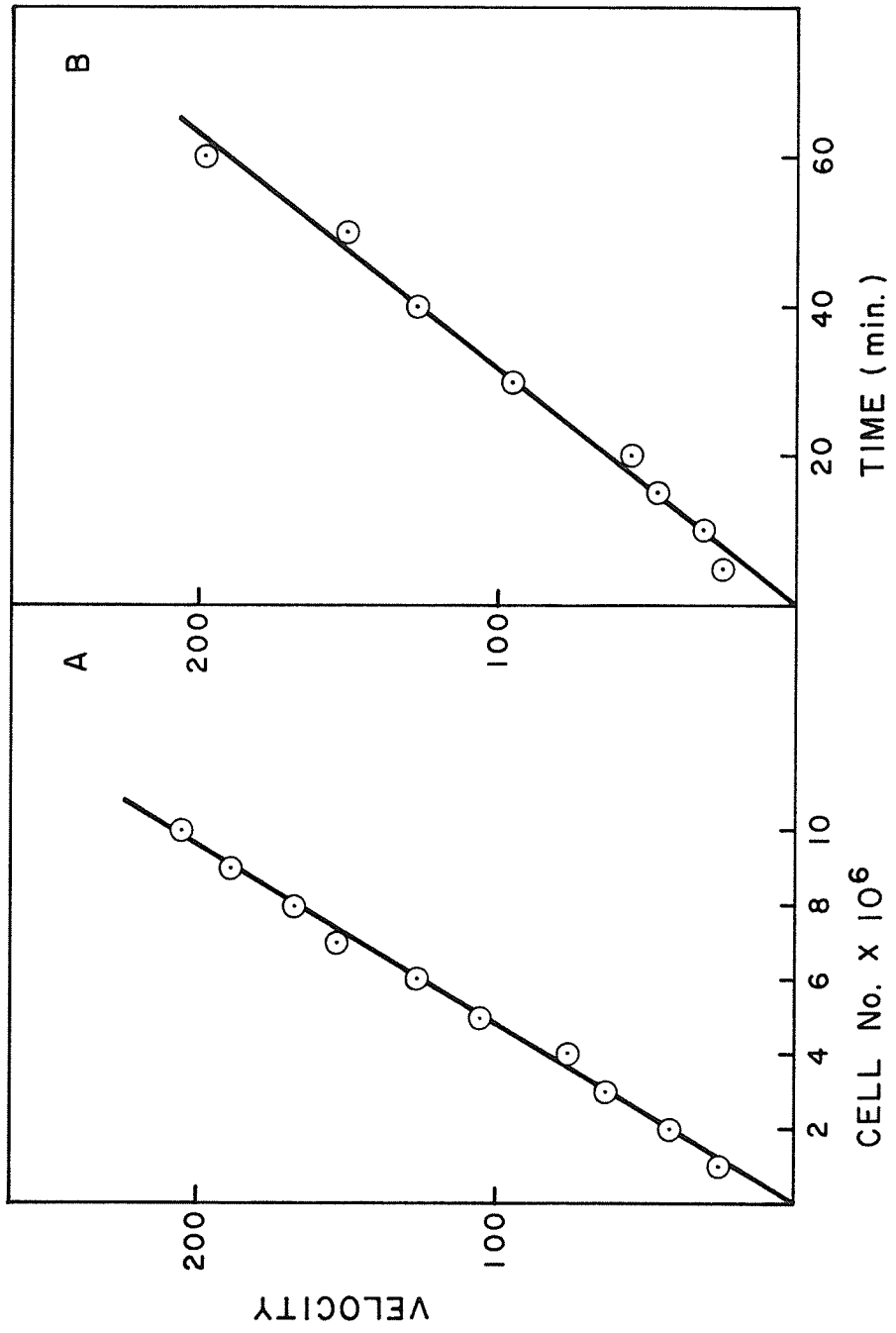

A
B
VELOCITY
200
100
200
100
2
4
6
8
10
CELL No. X 10^6
20
40
60
TIME (min.)

FIGURE 12A: Effect of increasing cell number on L3 wild type ADP reduction. Various amounts of cells were added to standard ADP reductase assay reaction mixture. After incubation at 37°C for 30 minutes the amount of ADP reduced was determined.

FIGURE 12B: Response of ADP reduction assay in L3 wild type cells with time of incubation. Standard aliquots of cells were added to complete reaction mixture and incubated at 37°C. At the times indicated, the reaction was terminated by boilding, and the amount of ADP reduced was determined.

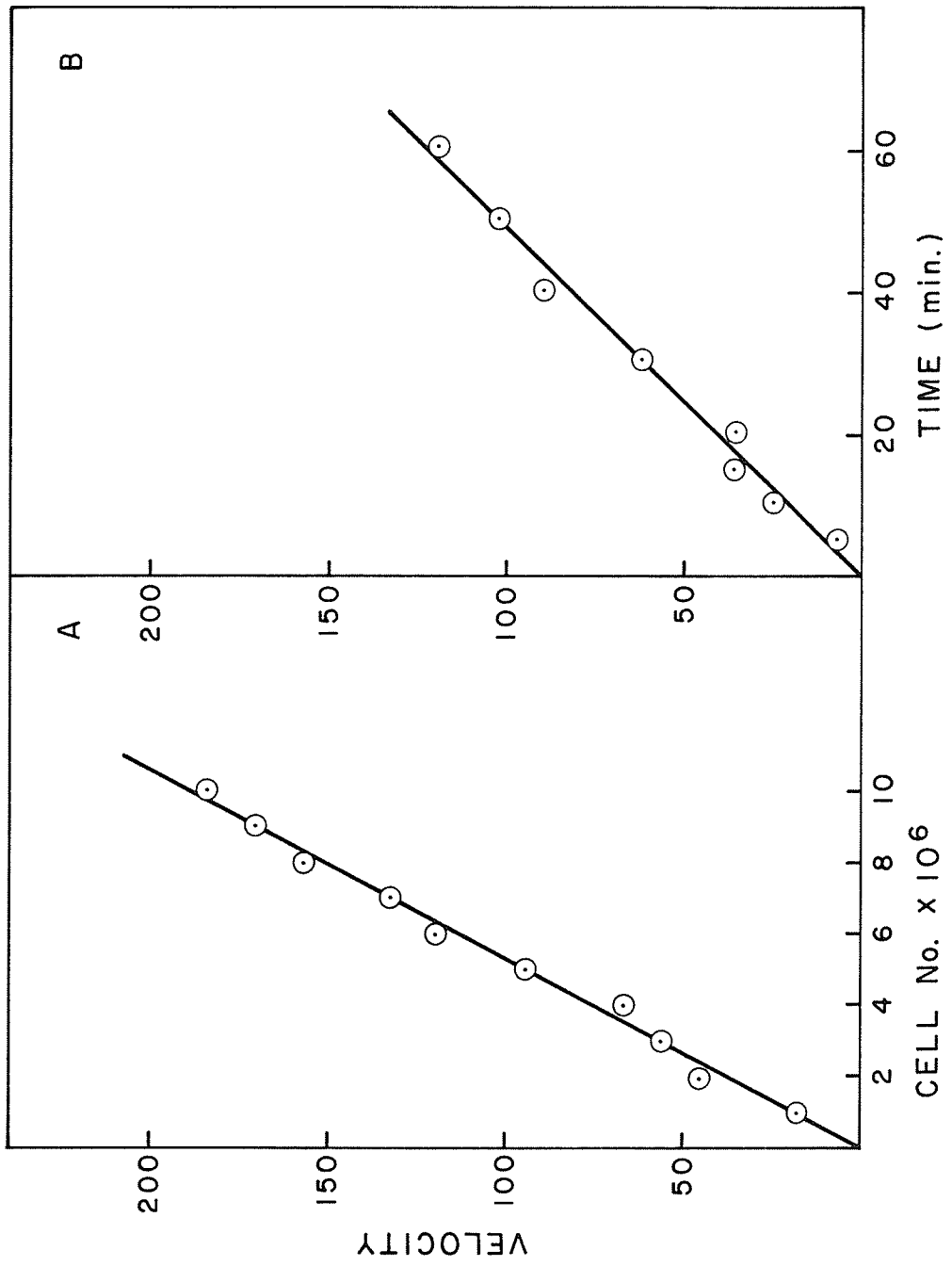

B
A
200
150
100
50
VELOCITY
2
4
6
8
10
CELL No. X 10⁶
20
40
60
TIME (min.)

FIGURE 13A: Effect of increasing cell number on L3R1 CDP reduction. Various amounts of cells were added to standard CDP reductase assay reaction mixture. After incubation at 37°C for 30 minutes the amount of CDP reduced was determined.

FIGURE 13B: Response of CDP reduction in L3R1 hydroxyurea-resistant cells with time of incubation. Standard aliquots of cells were added to complete reaction mixture and incubated at 37°C. At the times indicated, the reaction was terminated by boiling, and the amount of CDP reduced was determined.

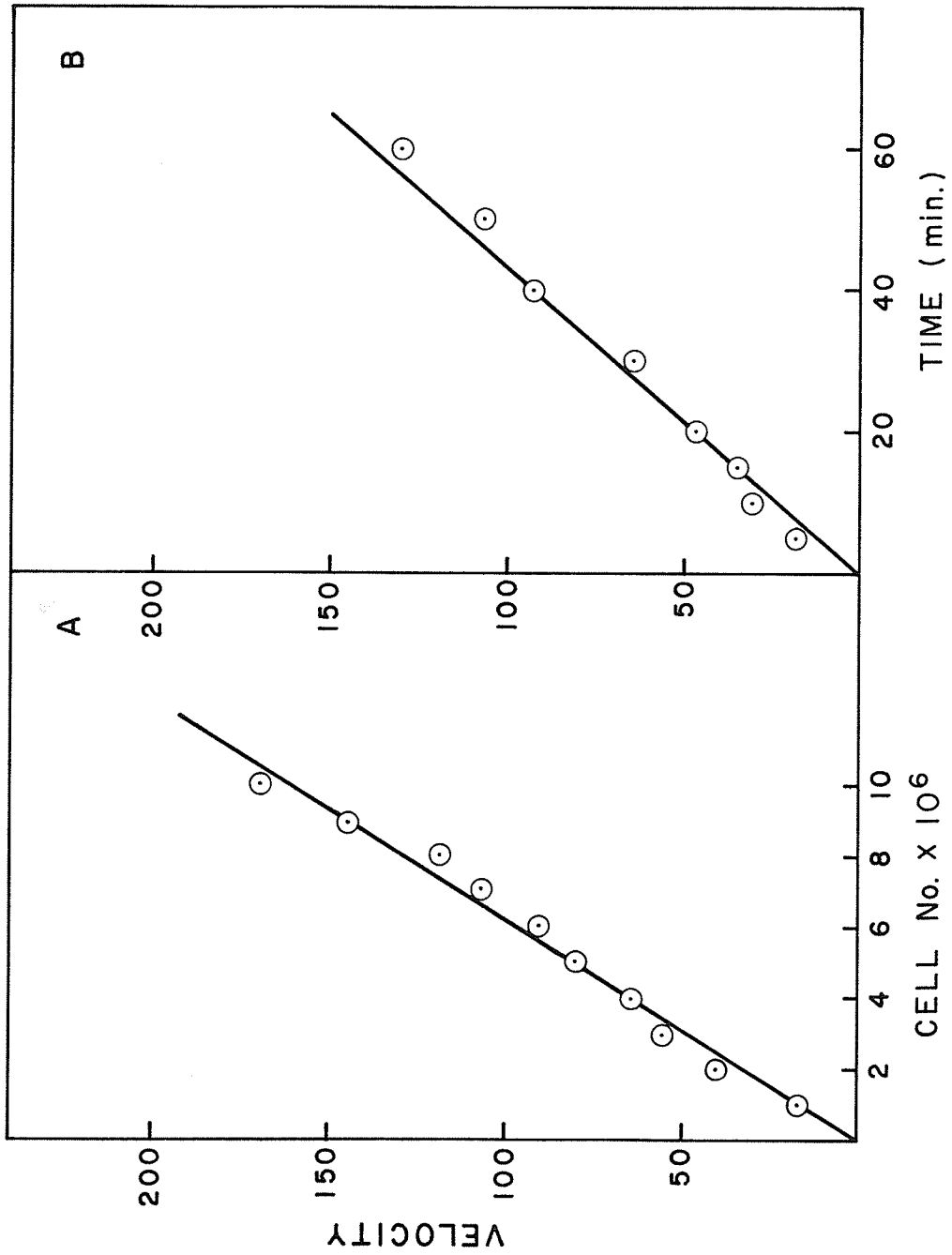

B
A
200
150
100
50
VELOCITY
2
4
6
8
10
CELL No. X 10⁶
20
40
60
TIME (min.)

FIGURE 14A: Effect of increasing cell number on L3R1 ADP reduction. Various amounts of cells were added to standard ADP reductase assay reaction mixture. After incubation at 37°C for 30 minutes the amount of ADP reduced was determined.

FIGURE 14B: Response of ADP reduction assay in L3R1 hydroxyurea-resistant cells with time of incubation. Standard aliquots of cells were added to complete reaction mixture and incubated at 37°C. At the times indicated, the reaction was terminated by boiling, and the amount of ADP reduced was determined.

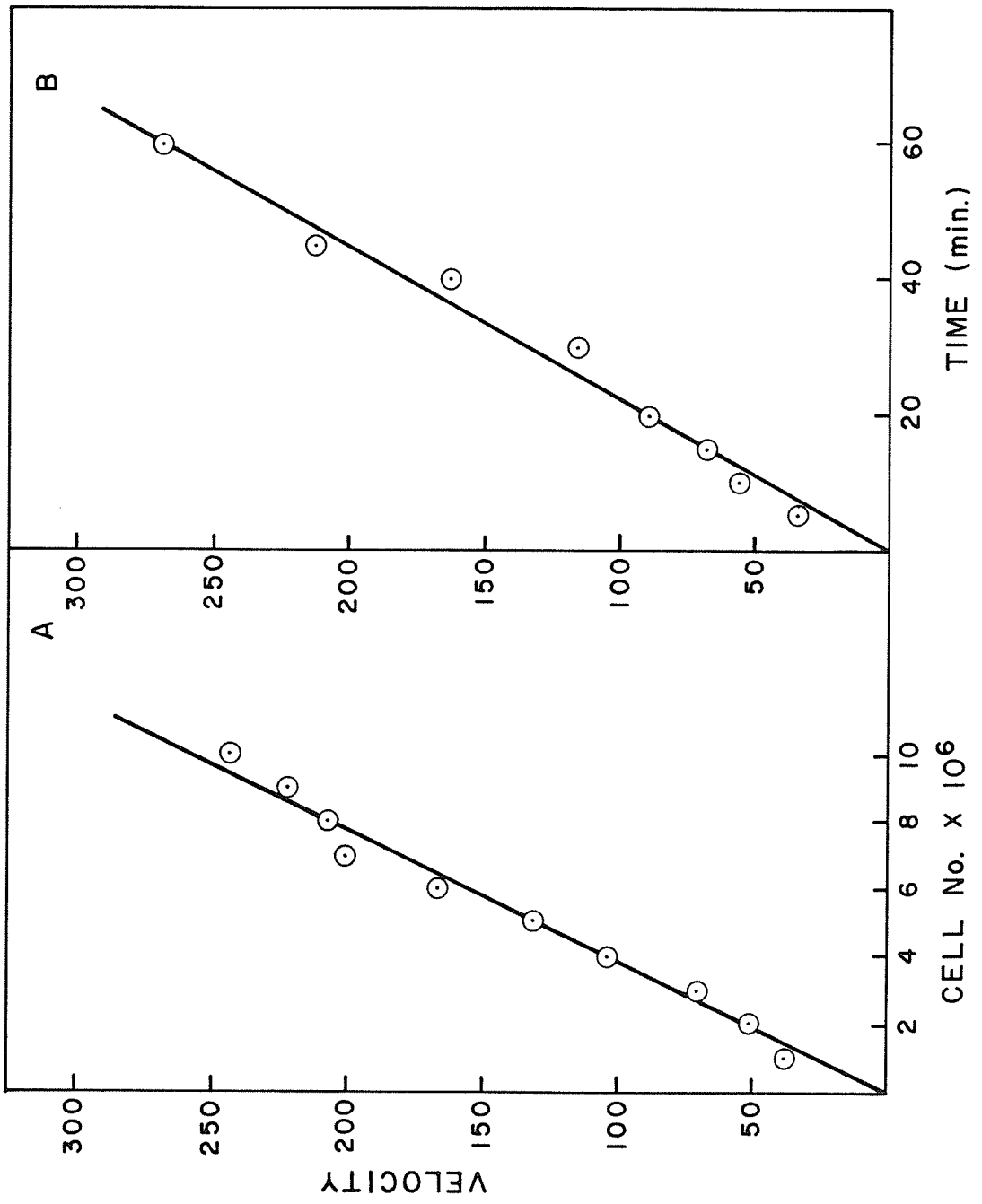

A
B
VELOCITY
300
250
200
150
100
50
CELL No. X 10⁶
2
4
6
8
10
TIME (min.)
20
40
60

FIGURE 15A: Effect of increasing cell number on L3R1-C CDP reduction. Various amounts of cells were added to standard CDP reductase assay reaction mixture. After incubation at 37°C for 30 minutes the amount of CDP reduced was determined.

FIGURE 15B: Response of CDP reduction assay in L3R1-C hydroxyurea-resistant cells with time of incubation. Standard aliquots of cells were added to complete reaction mixture and incubated at 37°C. At the times indicated, the reaction was terminated by boiling, and the amount of CDP reduced was determined.

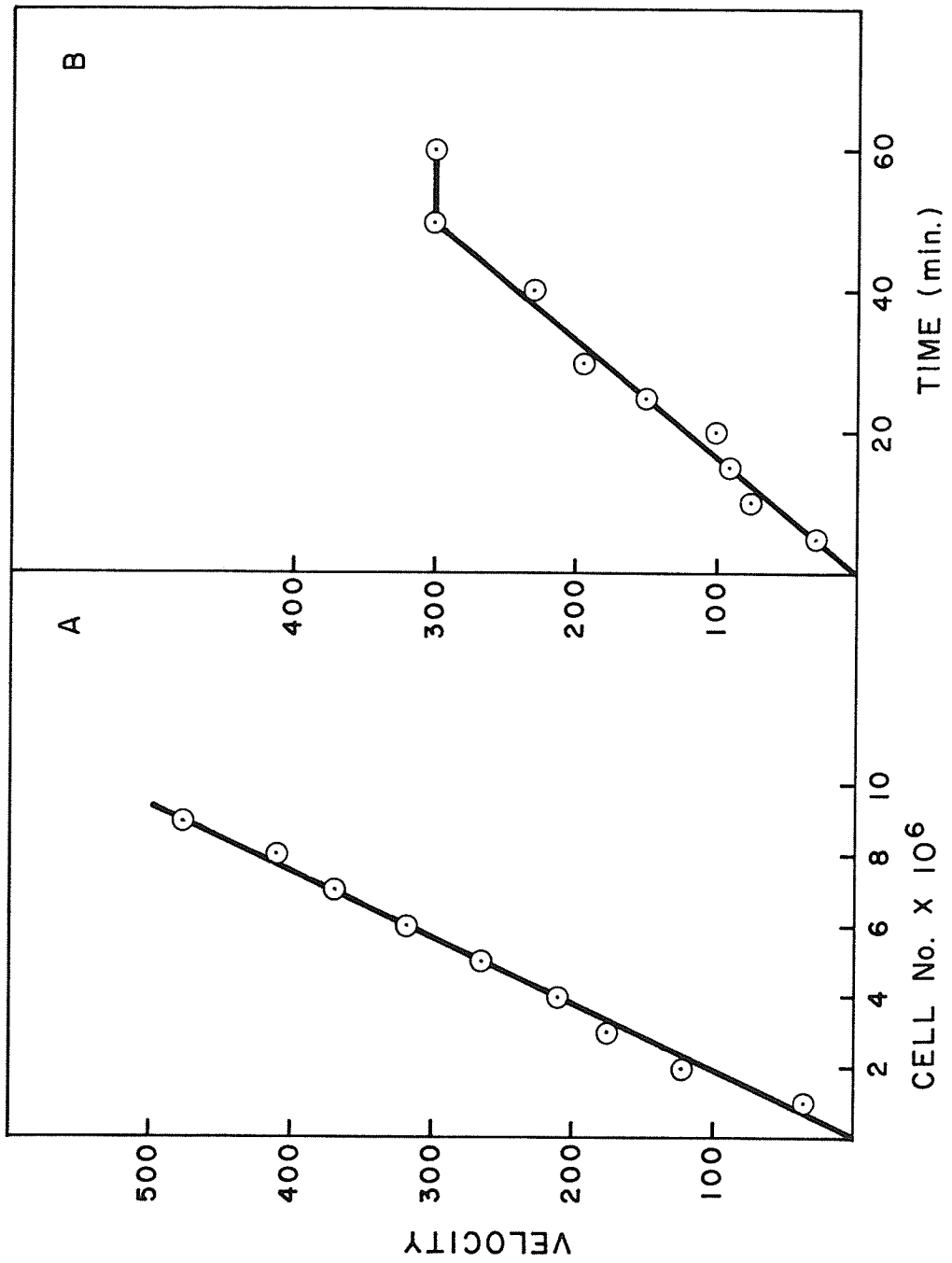

A
B
VELOCITY
500
400
300
200
100
2
4
6
8
10
CELL No. X 10⁶
20
40
60
TIME (min.)

FIGURE 16A: Effect of increasing cell number on L3R1-C ADP reduction. Various amounts of cells were added to standard ADP reductase assay reaction mixture. After incubation at 37°C for 30 minutes the amount of ADP reduced was determined.

FIGURE 16B: Response of ADP reduction assay in L3R1-C hydroxyurea-resistant cells with time of incubation. Standard aliquots of cells were added to complete reaction mixture and incubated at 37°C. At the times indicated, the reaction was terminated by boiling, and the amount of ADP reduced was determined.

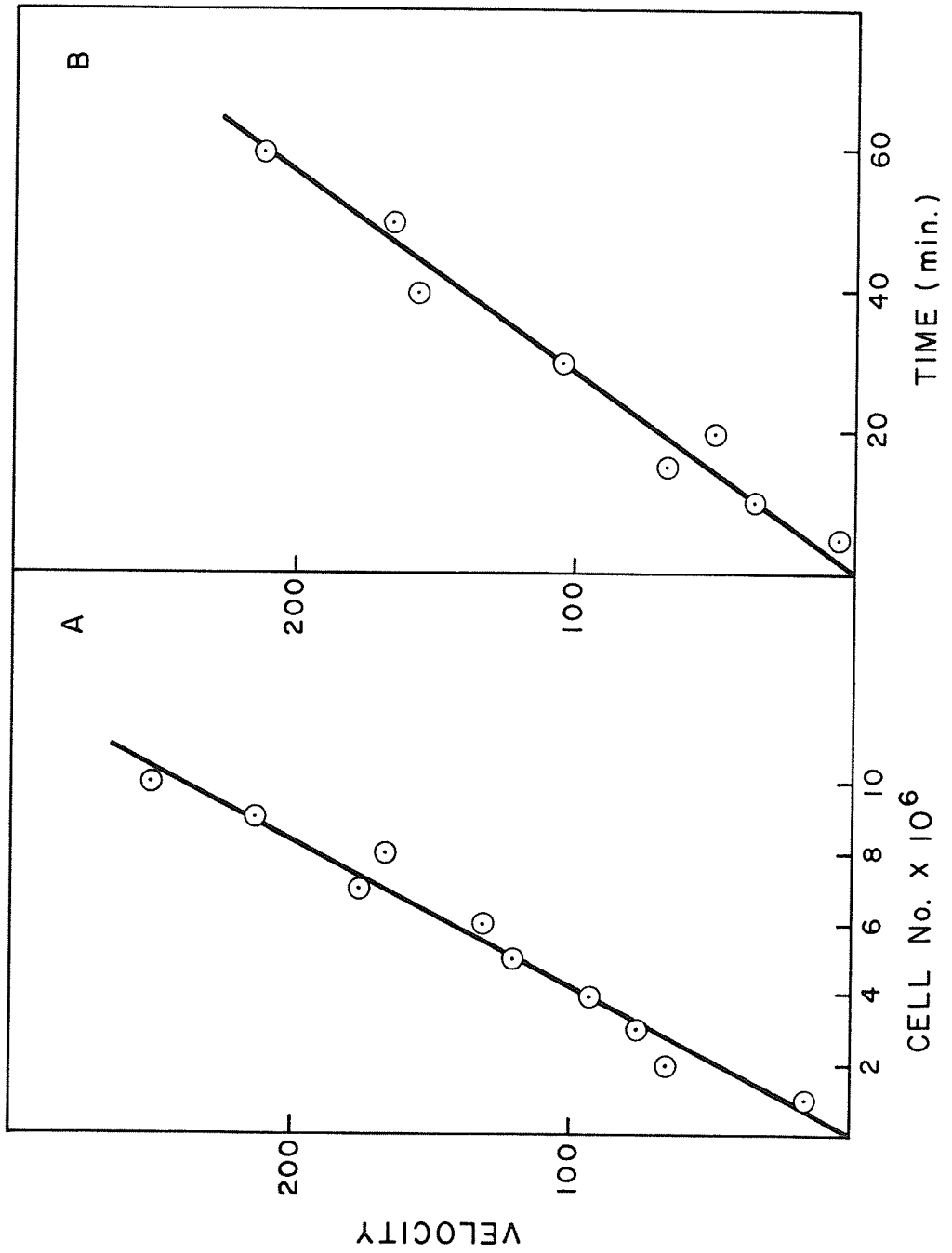

A
B
VELOCITY
200
100
200
100
2
4
6
8
10
CELL No. X 10^6
20
40
60
TIME (min.)

FIGURE 17: Double reciprocal plot of the variation in CDP reduction with CDP concentration in L1 wild type cells.

Insert: Velocity of CDP reduction as a function of various concentrations of CDP.

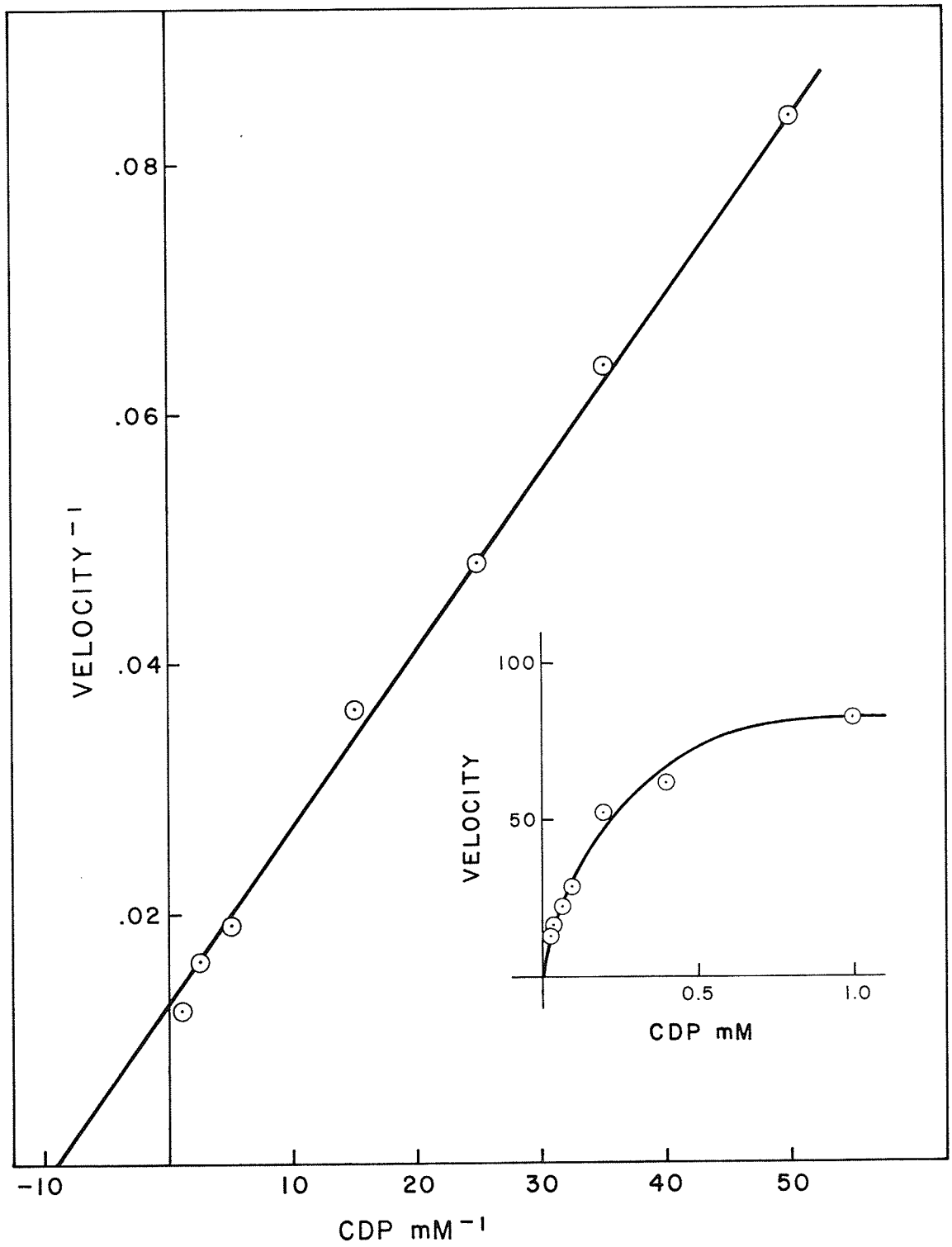

.08
.06
.04
.02
VELOCITY⁻¹
-10
10
20
30
40
50
CDP mM⁻¹
100
50
VELOCITY
0.5
1.0
CDP mM

FIGURE 18: Double reciprocal plot of the variation in ADP reduction with ADP concentration in L1 wild type cells.

Insert: Velocity of ADP reduction as a function of various concentrations of ADP.

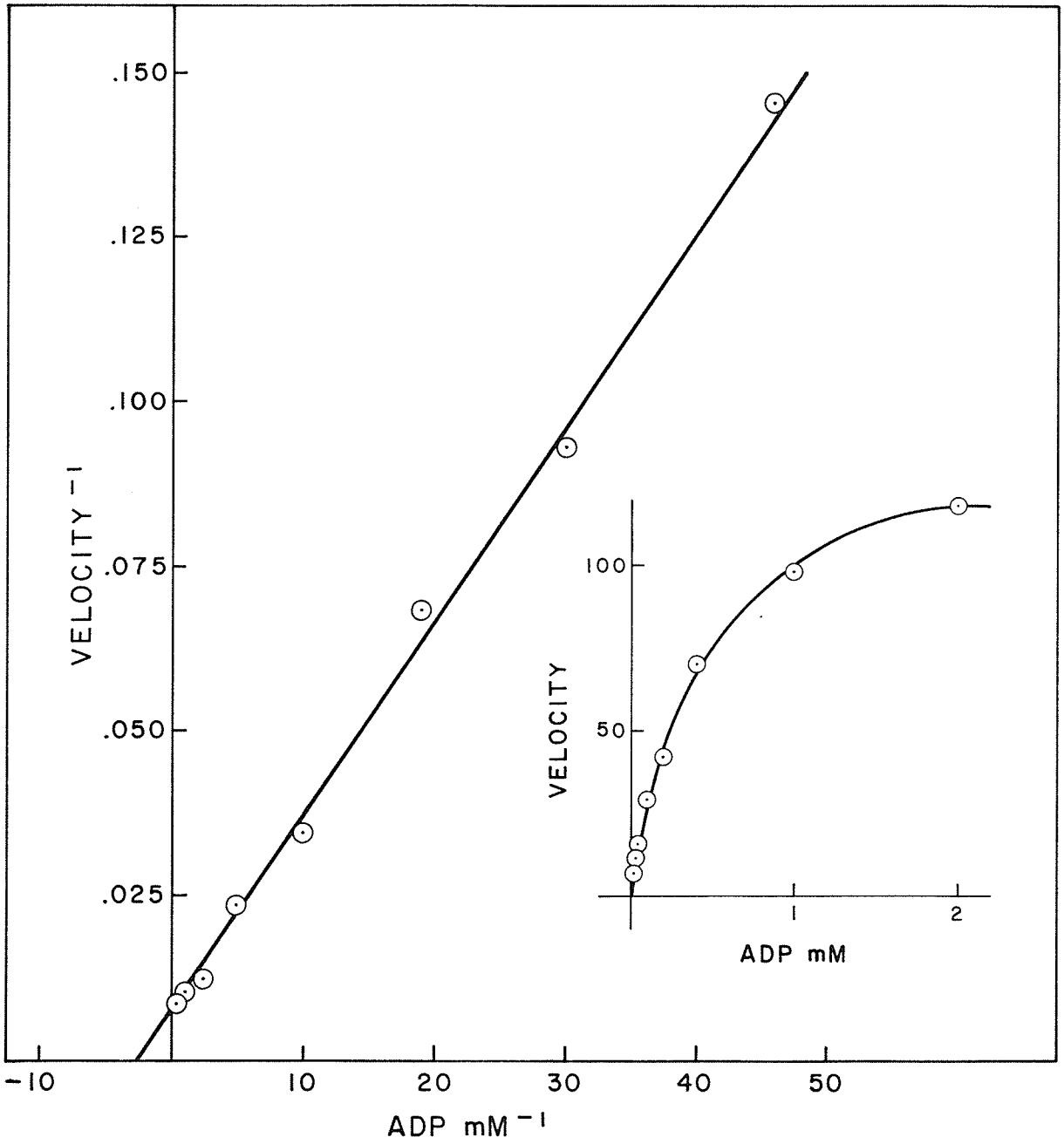

.150
.125
.100
.075
.050
.025
VELOCITY⁻¹
−10
10
20
30
40
50
ADP mM⁻¹
100
50
VELOCITY
1
2
ADP mM

FIGURE 19: Double reciprocal plot of the variation in CDP reduction with CDP concentration in L3 wild type cells (best fitting straight line was generated by linear regression as stated in Results).

Insert: Velocity of CDP reduction as a function of various concentrations of CDP.

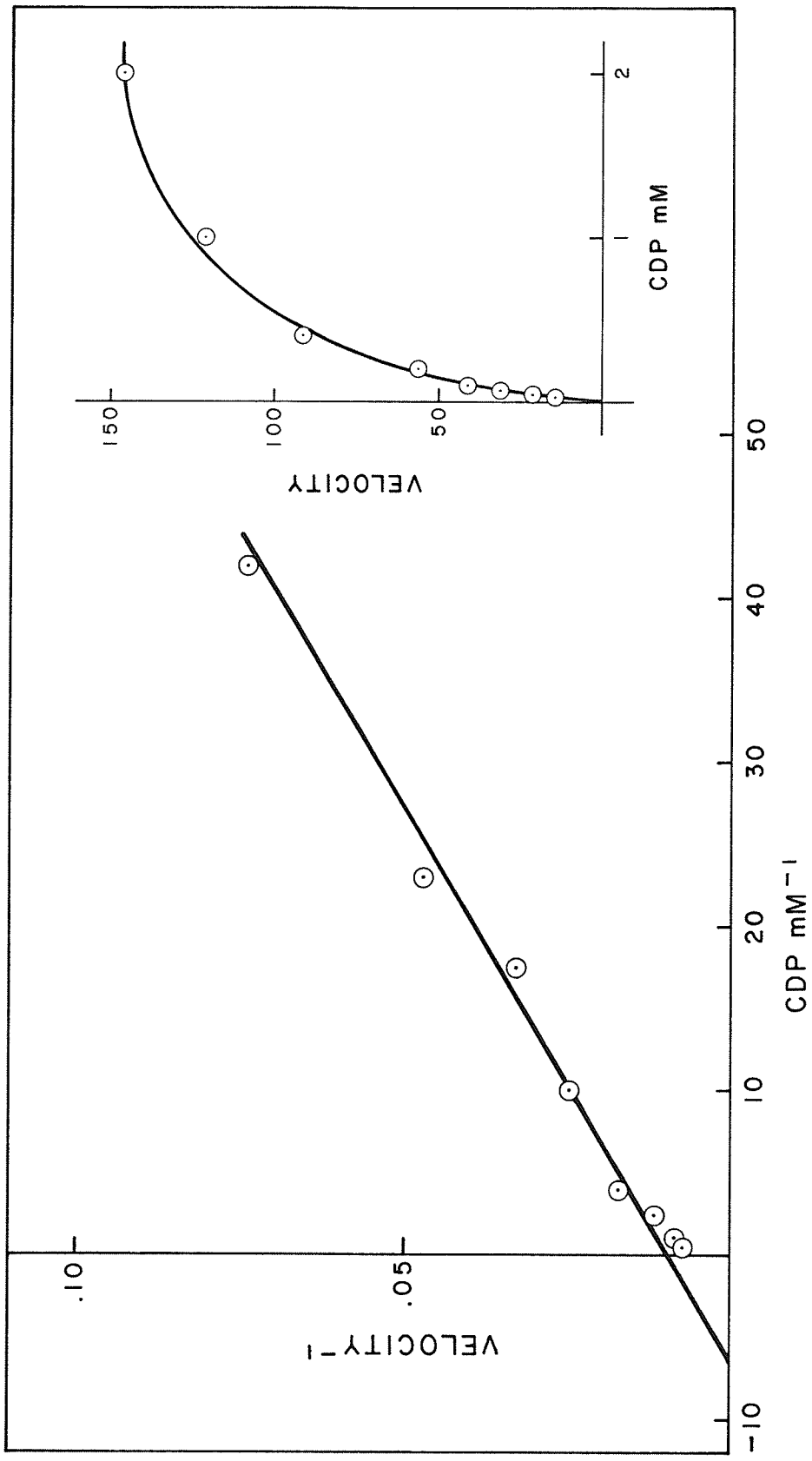

VELOCITY⁻¹
.10
.05
-10
10
20
30
40
50
CDP mM⁻¹
VELOCITY
150
100
50
1
2
CDP mM

FIGURE 20: Double reciprocal plot of the variation in ADP reduction with ADP concentration in L3 wild type cells.

Insert: Velocity of ADP reduction as a function of various concentrations of ADP.

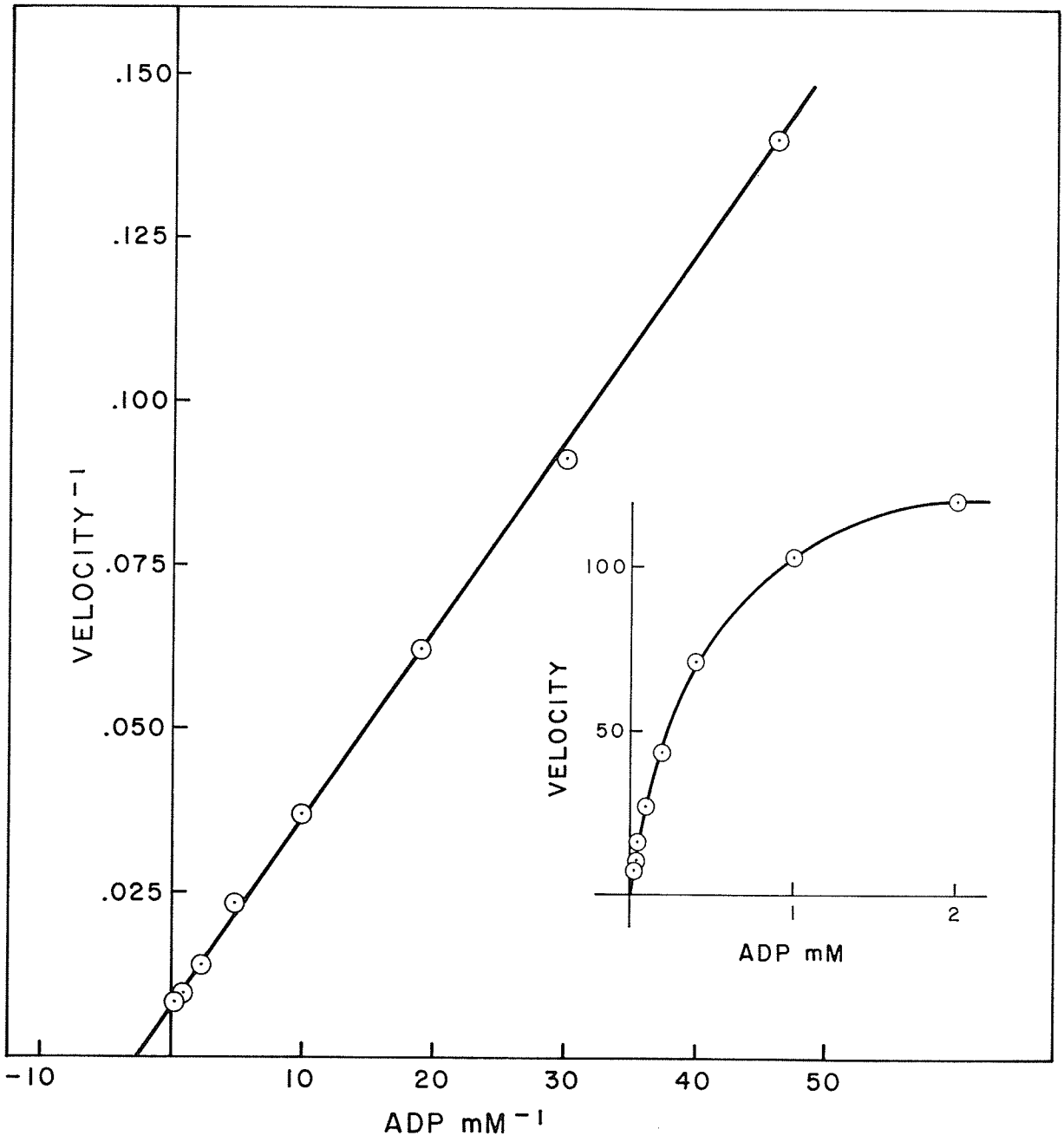

.150
.125
.100
.075
.050
.025
VELOCITY⁻¹
-10
10
20
30
40
50
ADP mM⁻¹
100
50
VELOCITY
1
2
ADP mM

FIGURE 21: Double reciprocal plot of the variation in CDP reduction with CDP concentration in L1R1 hydroxyurea-resistant cells.

Insert: Velocity of CDP reduction as a function of various concentrations of CDP.

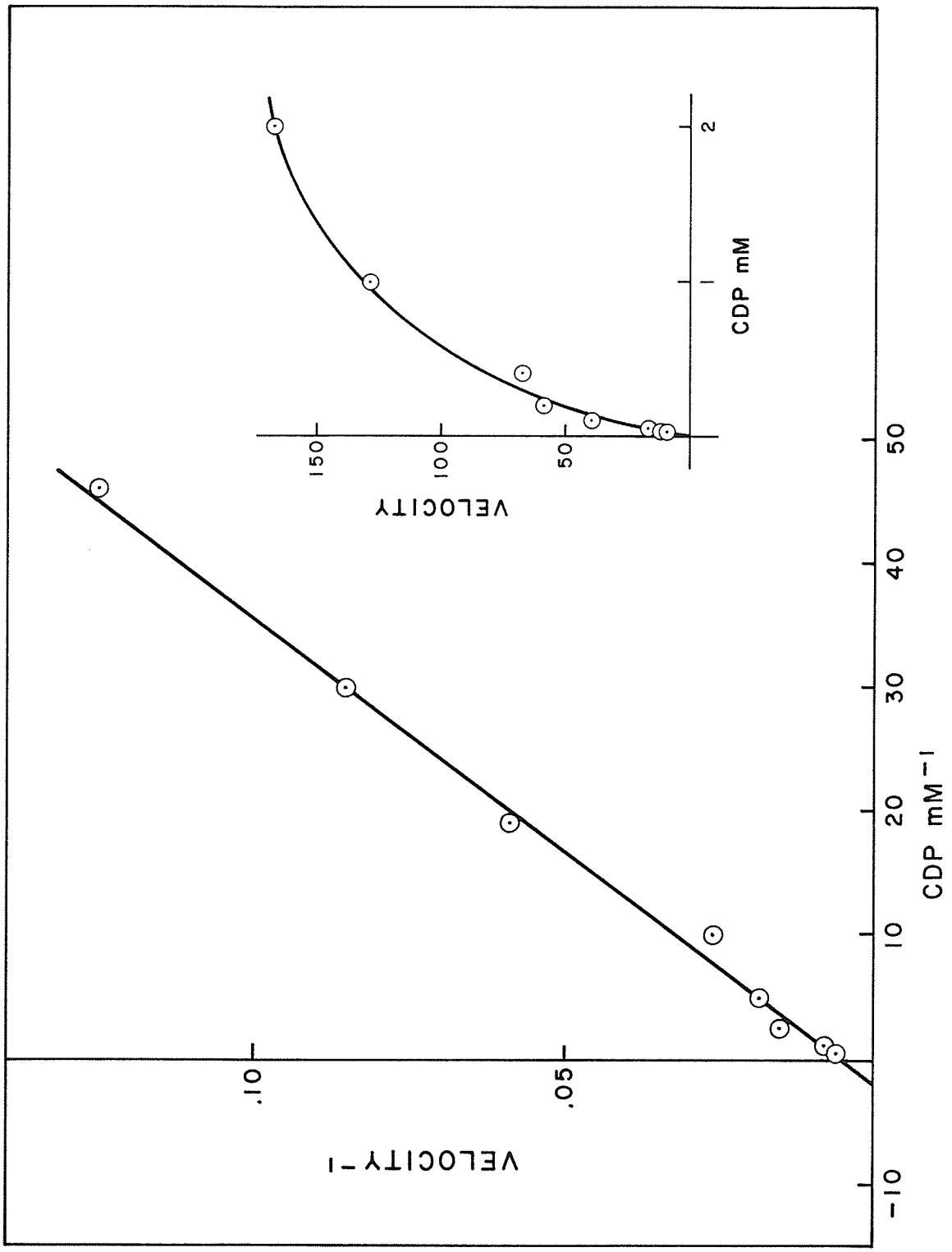

VELOCITY⁻¹
.10
.05
-10
10
20
30
40
50
CDP mM⁻¹
VELOCITY
150
100
50
1
2
CDP mM

FIGURE 22: Double reciprocal plot of the variation in ADP reduction with ADP concentration in L1R1 hydroxyurea-resistant cells.

Insert: Velocity of ADP reduction as a function of various concentrations of ADP.

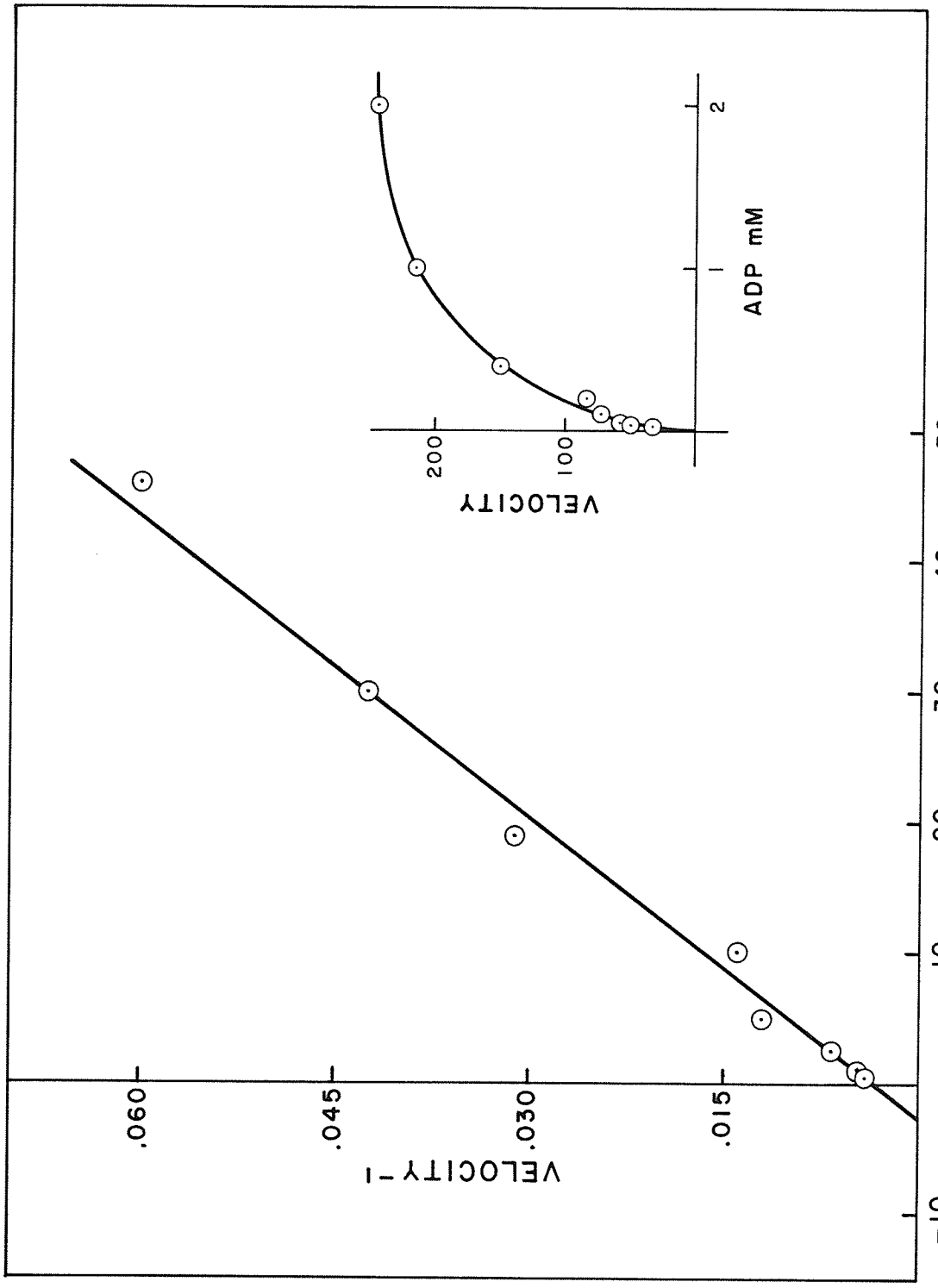

VELOCITY⁻¹
.060
.045
.030
.015
-10
10
20
30
40
50
ADP mM⁻¹
VELOCITY
200
100
1
2
ADP mM

FIGURE 23: Double reciprocal plot of the variation in CDP reduction with CDP concentration in L1R2 hydroxyurea-resistant cells.

Insert: Velocity of CDP reduction as a function of various concentrations of CDP.

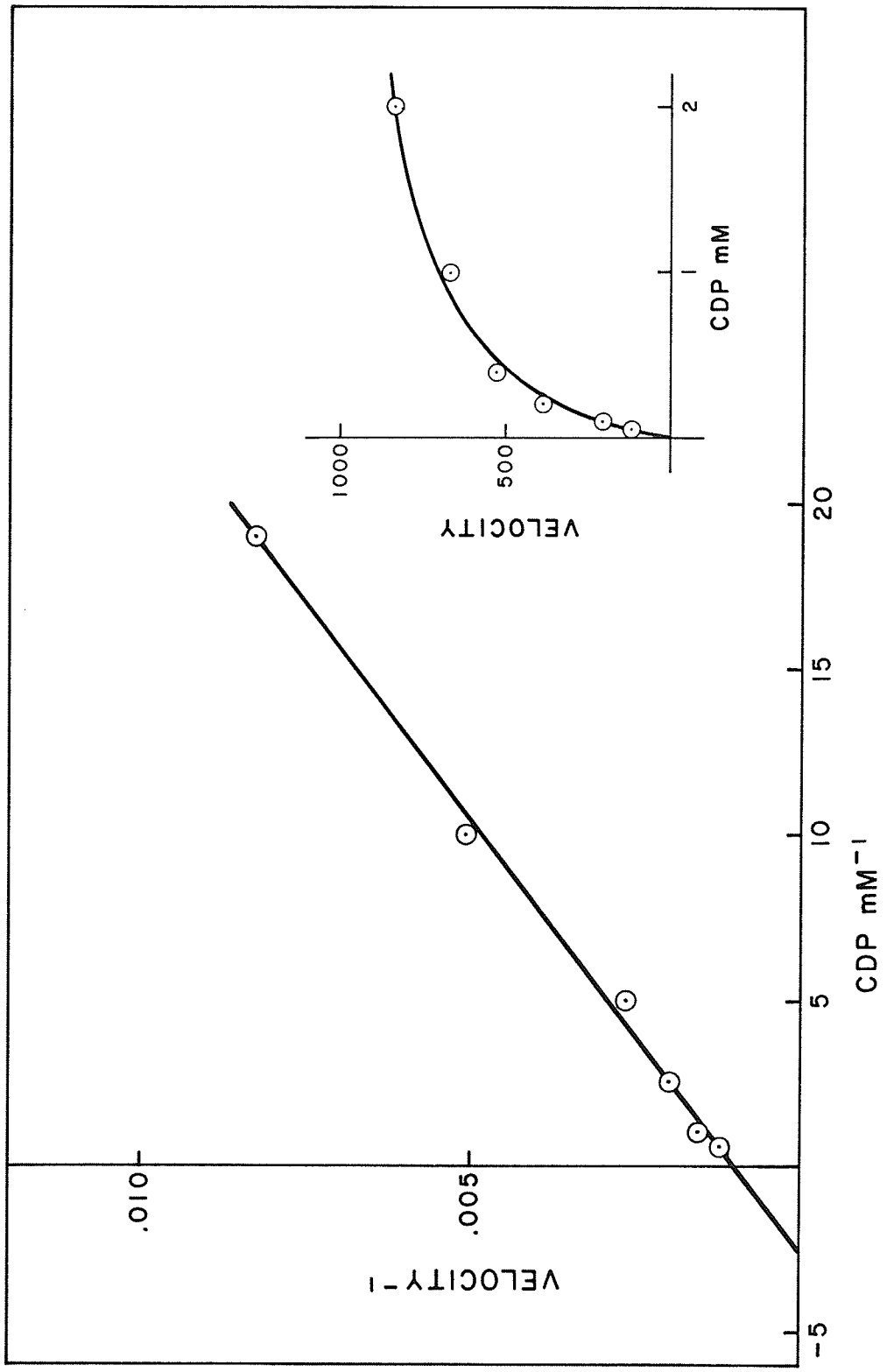

VELOCITY⁻¹
.010
.005
-5
5
10
15
20
CDP mM⁻¹
VELOCITY
1000
500
1
2
CDP mM

FIGURE 24: Double reciprocal plot of the variation in ADP reduction with ADP concentration in L1R2 hydroxyurea-resistant cells.

Insert: Velocity of ADP reduction as a function of various concentrations of ADP.

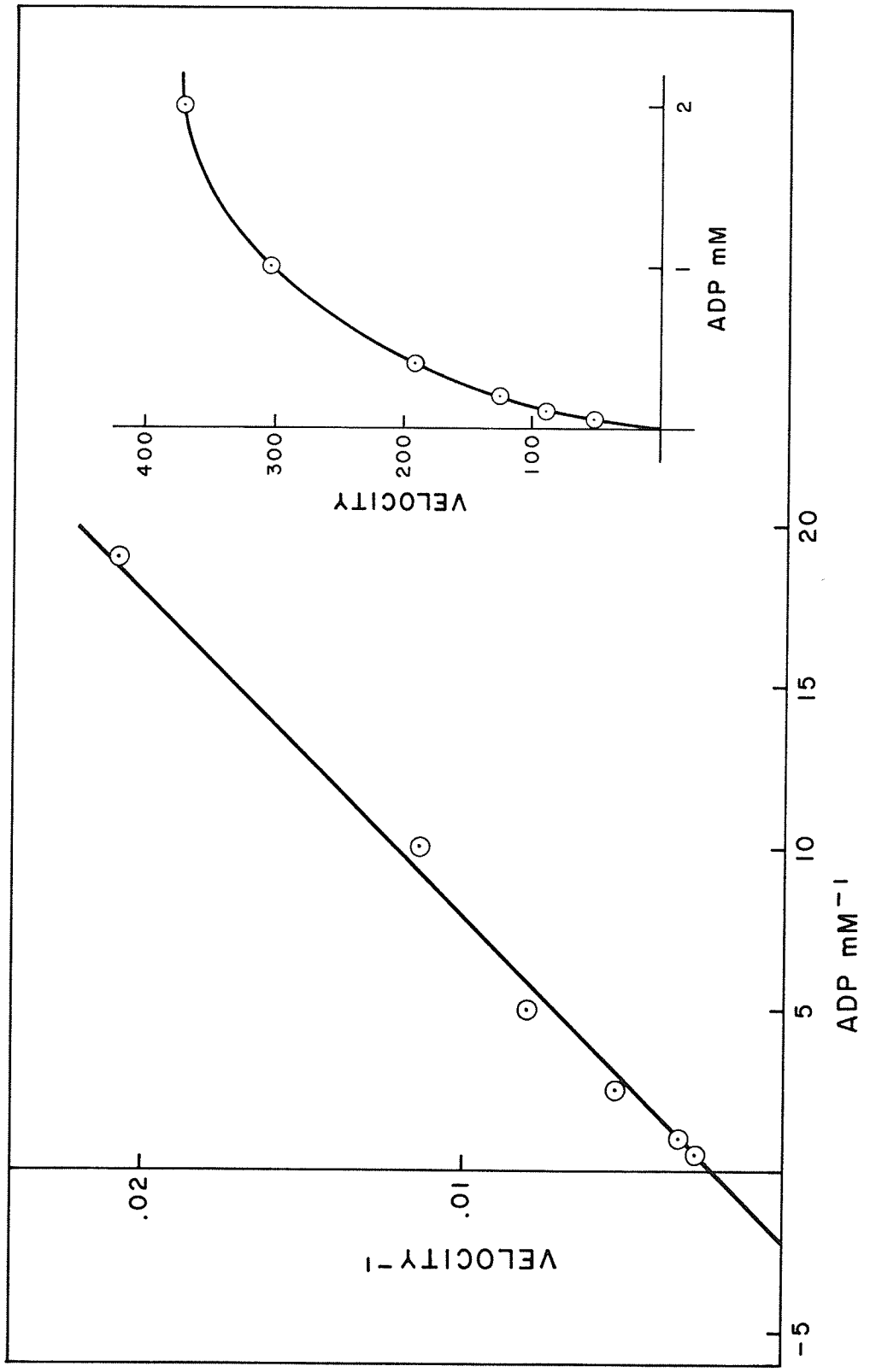

VELOCITY⁻¹
.02
.01
-5
5
10
15
20
ADP mM⁻¹
VELOCITY
400
300
200
100
1
2
ADP mM

FIGURE 25: Double reciprocal plot of the variation in CDP reduction with CDP concentration in L1R3 hydroxyurea-resistant cells.

Insert: Velocity of CDP reduction as a function of various concentrations of CDP.

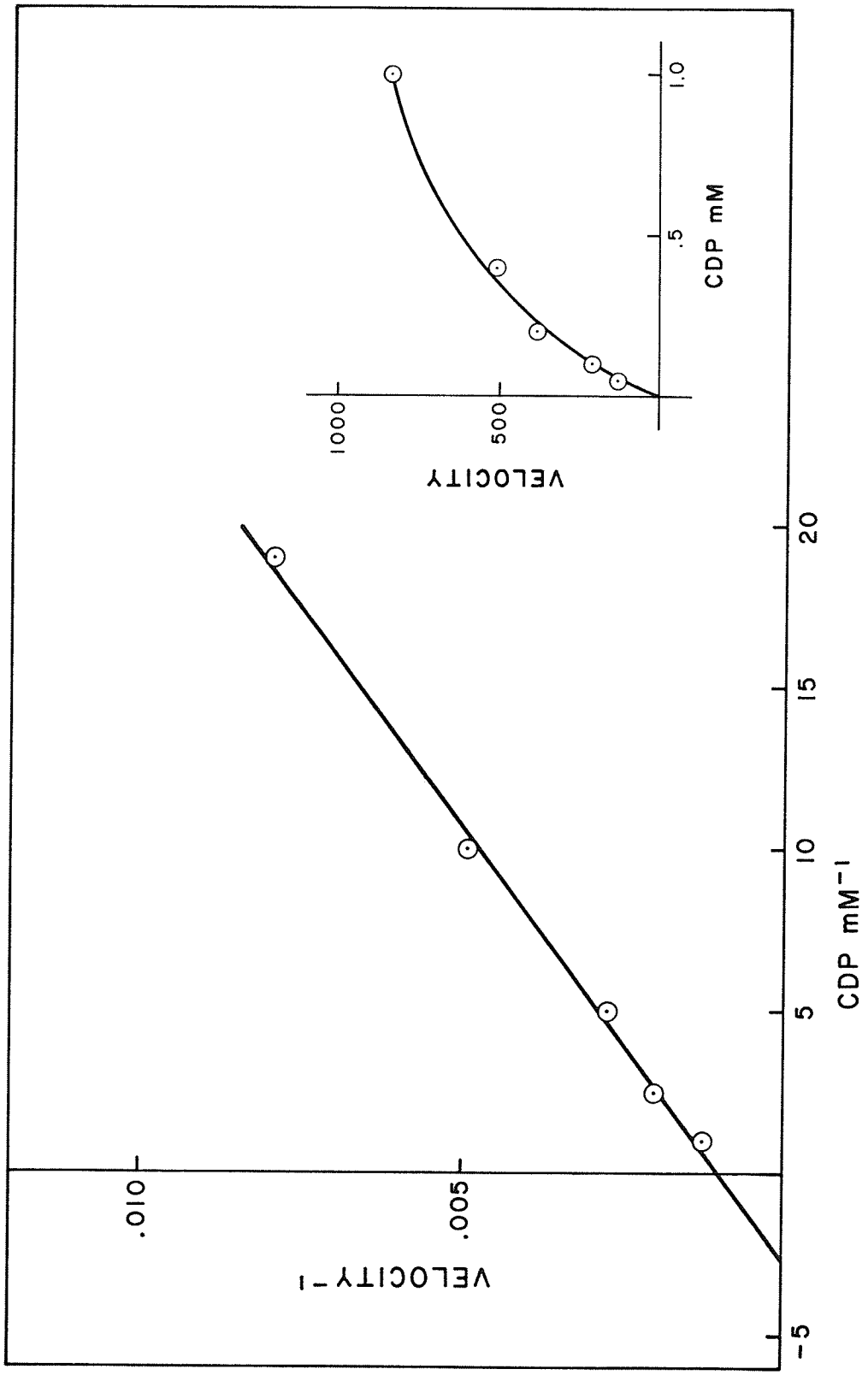

VELOCITY⁻¹
.010
.005
-5
5
10
15
20
CDP mM⁻¹
VELOCITY
1000
500
.5
1.0
CDP mM

FIGURE 26: Double reciprocal plot of the variation in ADP reduction with ADP concentration in L1R3 hydroxyurea-resistant cells.

Insert: Velocity of ADP reduction as a function of various concentrations of ADP.

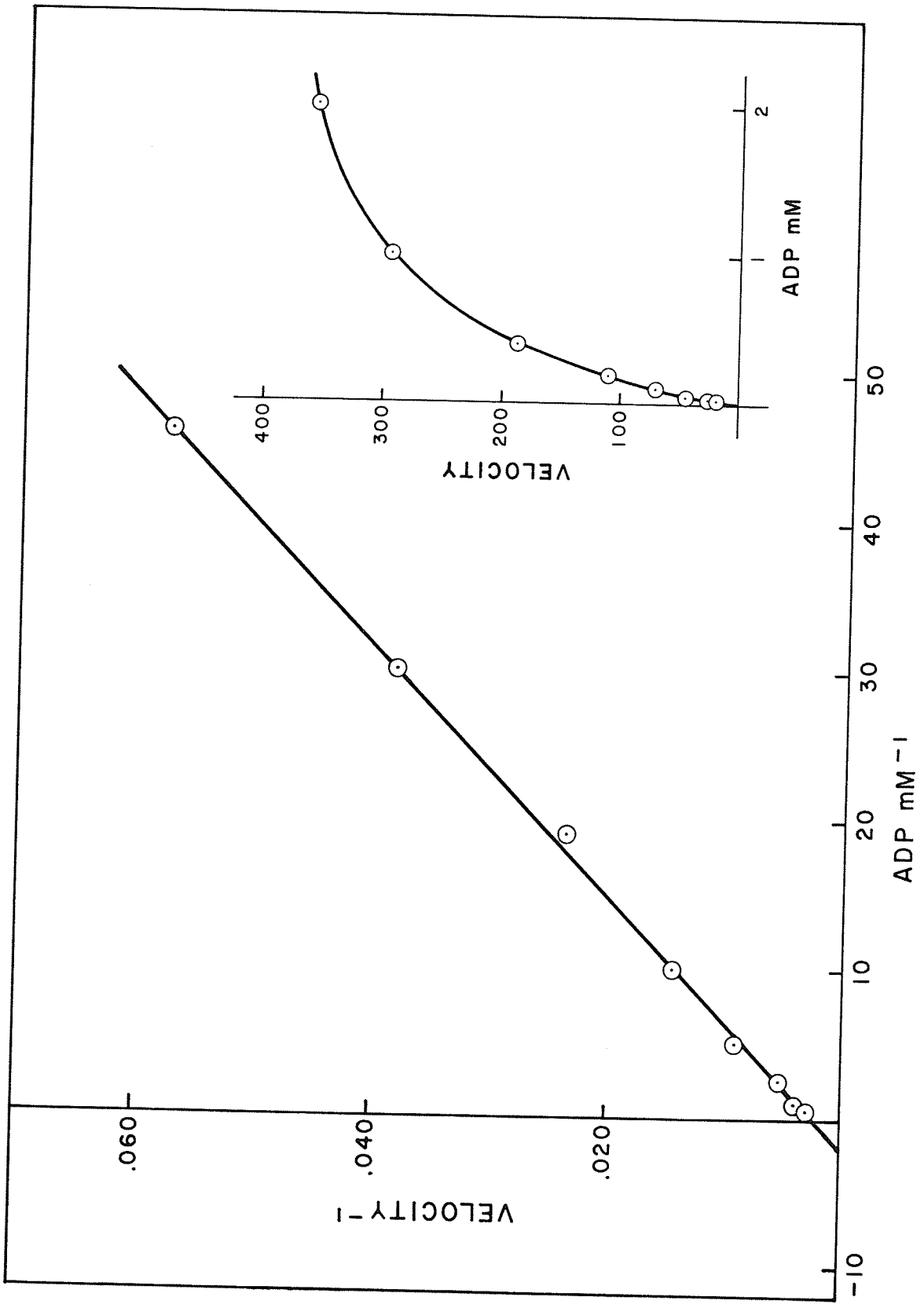

VELOCITY⁻¹
.060
.040
.020
-10
10
20
30
40
50
ADP mM⁻¹
VELOCITY
400
300
200
100
1
2
ADP mM

FIGURE 27: Double reciprocal plot of the variation in CDP reduction with CDP concentration in L1R4 hydroxyurea-resistant cells.

Insert: Velocity of CDP reduction as a function of various concentrations of CDP.

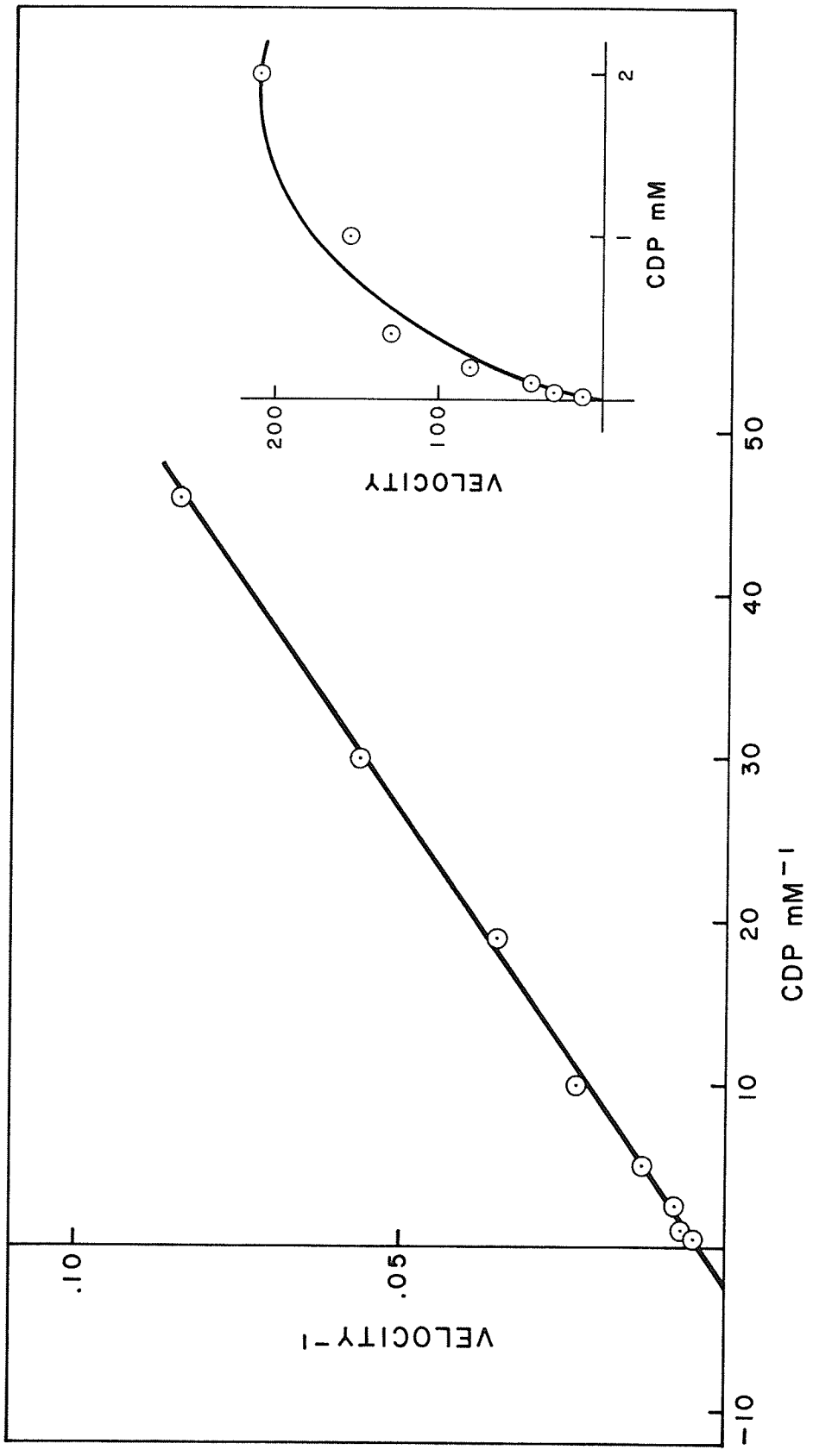

VELOCITY⁻¹
.10
.05
-10
10
20
30
40
50
CDP mM⁻¹
VELOCITY
200
100
1
2
CDP mM

FIGURE 28: Double reciprocal plot of the variation in ADP reduction with ADP concentration in L1R4 hydroxyurea-resistant cells.

Insert: Velocity of ADP reduction as a function of various concentrations of ADP.

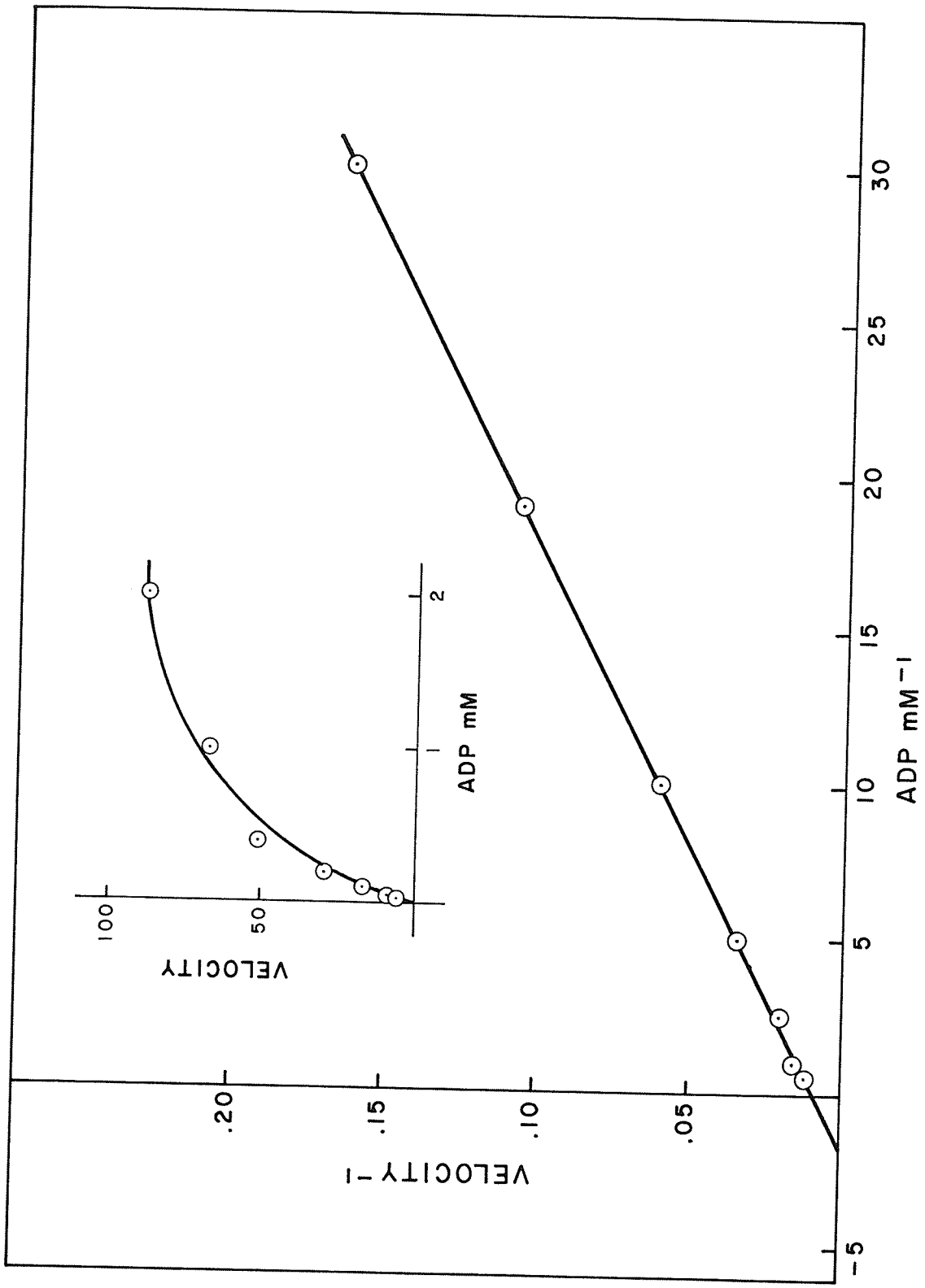

VELOCITY
100
50
ADP mM
1
2
VELOCITY-1
.20
.15
.10
.05
-5
5
10
15
20
25
30
ADP mM-1

FIGURE 29: Double reciprocal plot of the variation in CDP reduction with CDP concentration in L1R5 hydroxyurea-resistant cells.

Insert: Velocity of CDP reduction as a function of various concentrations of CDP.

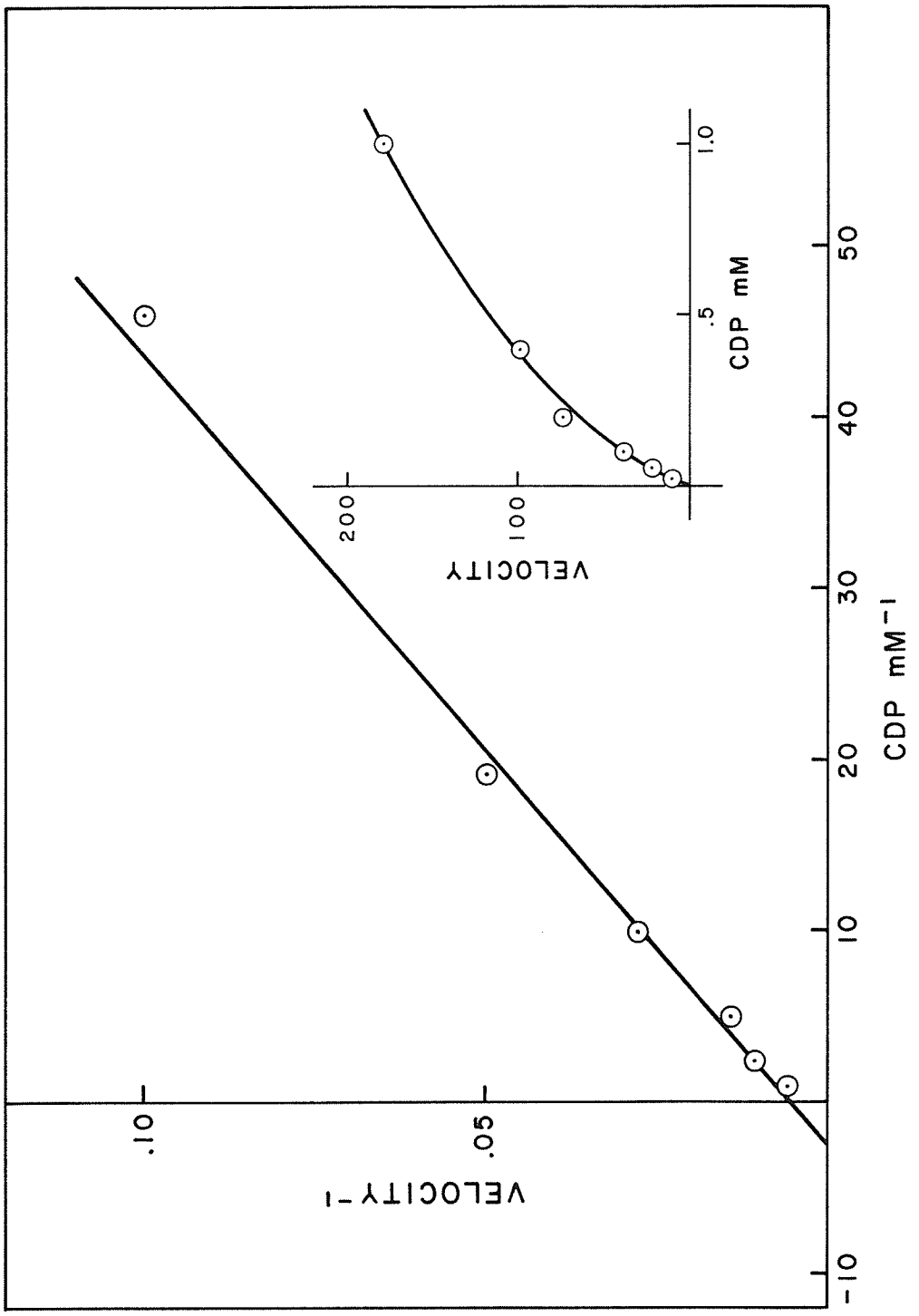

VELOCITY⁻¹
.10
.05
-10
10
20
30
40
50
CDP mM⁻¹
VELOCITY
200
100
.5
1.0
CDP mM

FIGURE 30: Double reciprocal plot of the variation in ADP reduction with ADP concentration in L1R5 hydroxyurea-resistant cells.

Insert: Velocity of ADP reduction as a function of various concentrations of ADP.

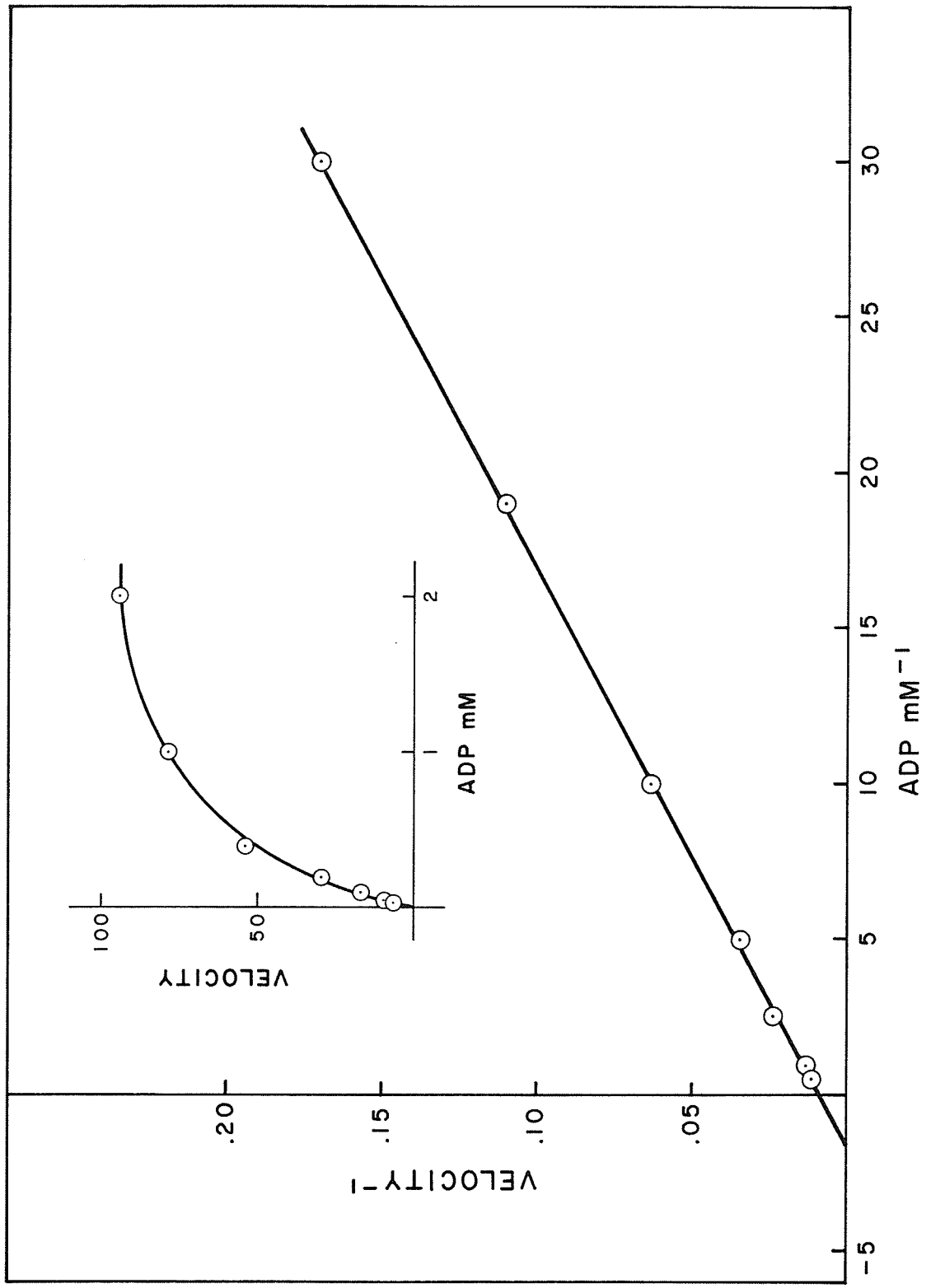

VELOCITY⁻¹
.20
.15
.10
.05
-5
5
10
15
20
25
30
ADP mM⁻¹
VELOCITY
100
50
1
2
ADP mM

FIGURE 31: Double reciprocal plot of the variation in CDP reduction with CDP concentration in L3R1 hydroxyurea-resistant cells.

Insert: Velocity of CDP reduction as a function of various concentrations of CDP.

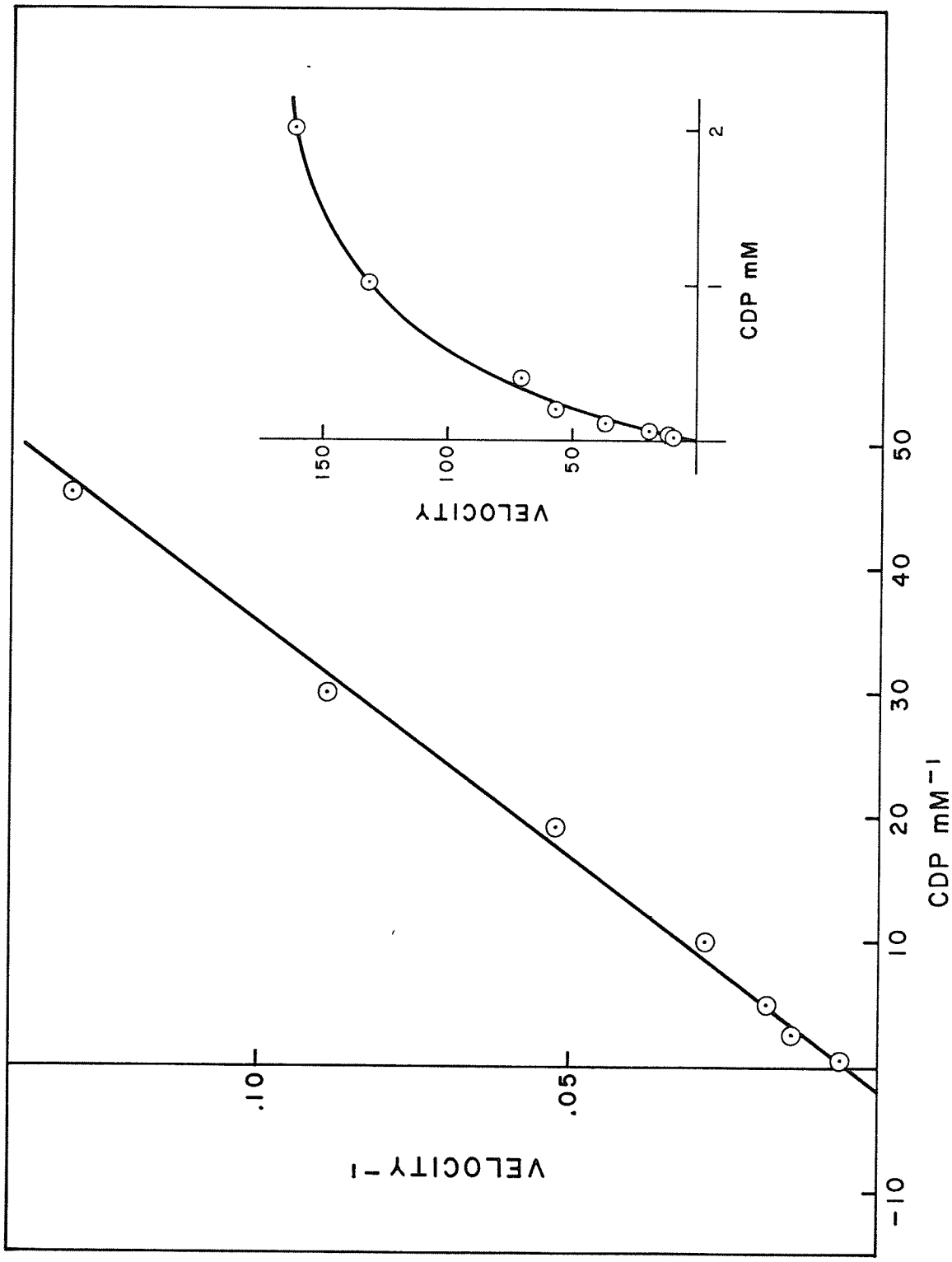

VELOCITY⁻¹
.10
.05
-10
10
20
30
40
50
CDP mM⁻¹
VELOCITY
150
100
50
1
2
CDP mM

FIGURE 32: Double reciprocal plot of the variation in ADP reduction with ADP concentration in L3R1 hydroxyurea-resistant cells.

Insert: Velocity of ADP reduction as a function of various concentrations of ADP.

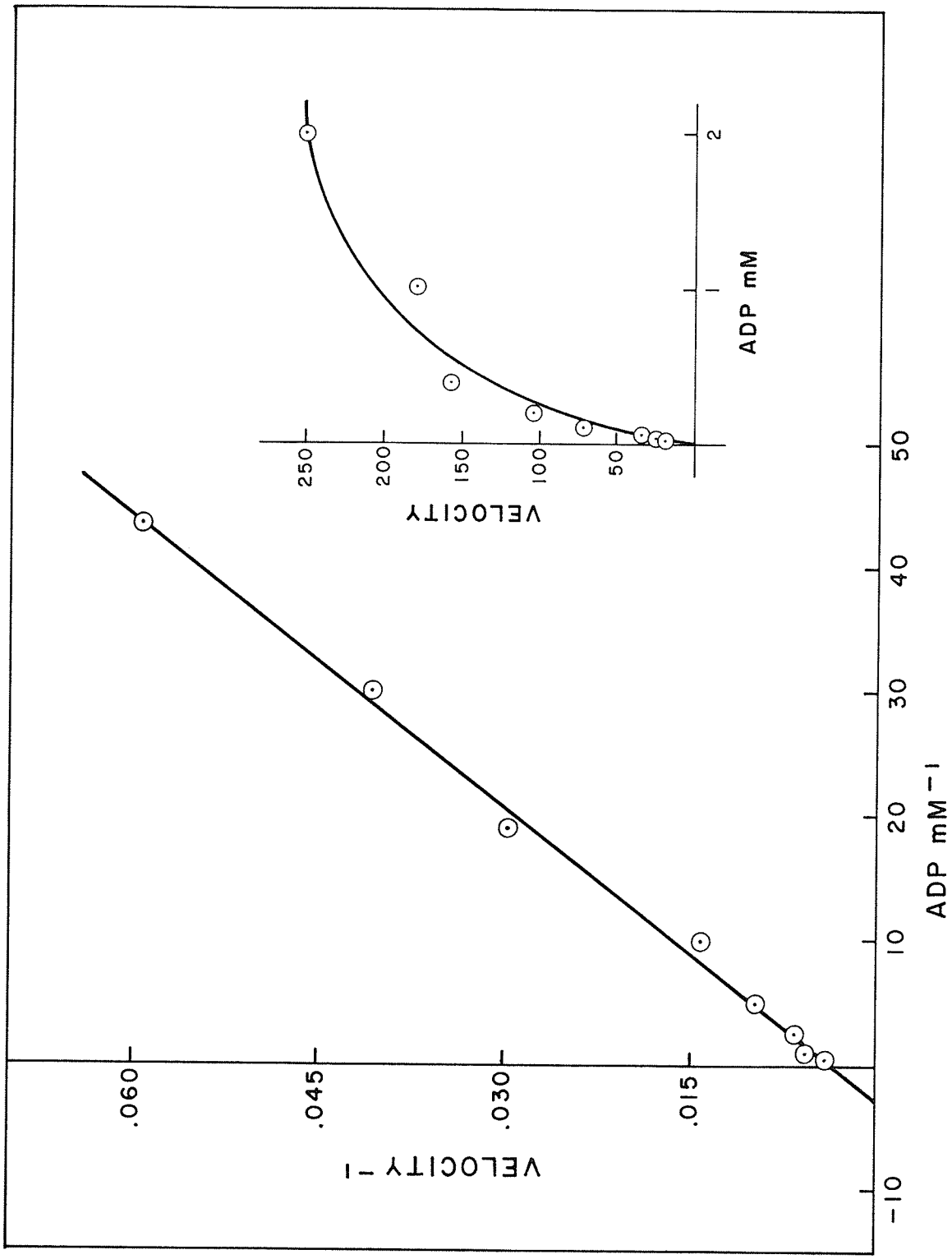

VELOCITY⁻¹
.060
.045
.030
.015
-10
10
20
30
40
50
ADP mM⁻¹
VELOCITY
250
200
150
100
50
1
2
ADP mM

FIGURE 33. Double reciprocal plot of the variation in CDP reduction with CDP concentration in L1R1-A hydroxyurea-resistant cells.

Insert: Velocity of CDP reduction as a function of various concentrations of CDP.

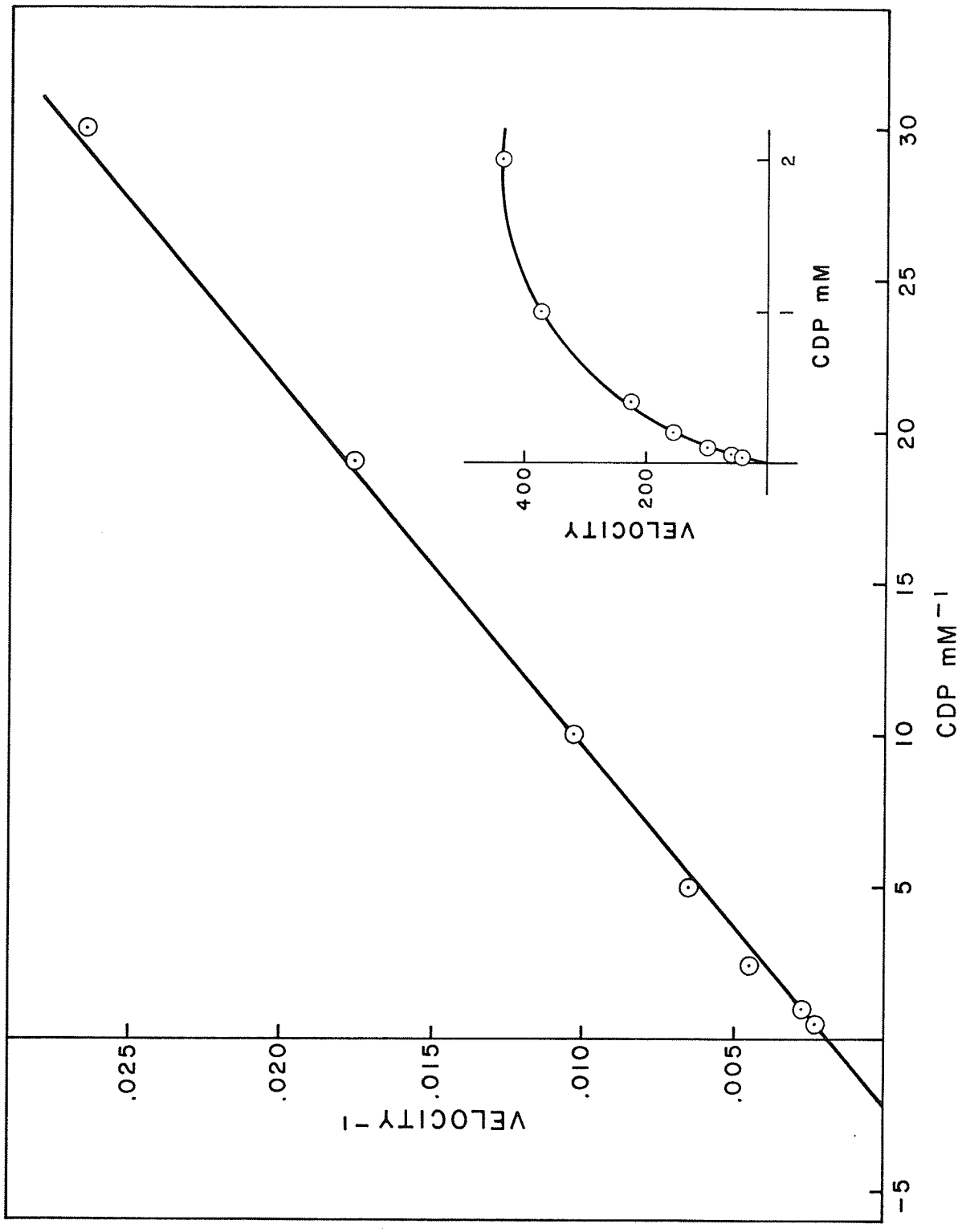

VELOCITY⁻¹
.025
.020
.015
.010
.005
-5
5
10
15
20
25
30
CDP mM⁻¹
VELOCITY
400
200
1
2
CDP mM

FIGURE 34: Double reciprocal plot of the variation in ADP reduction with ADP concentration in L1R1-A cells.

Insert: Velocity of ADP reduction as a function of various concentrations of ADP.

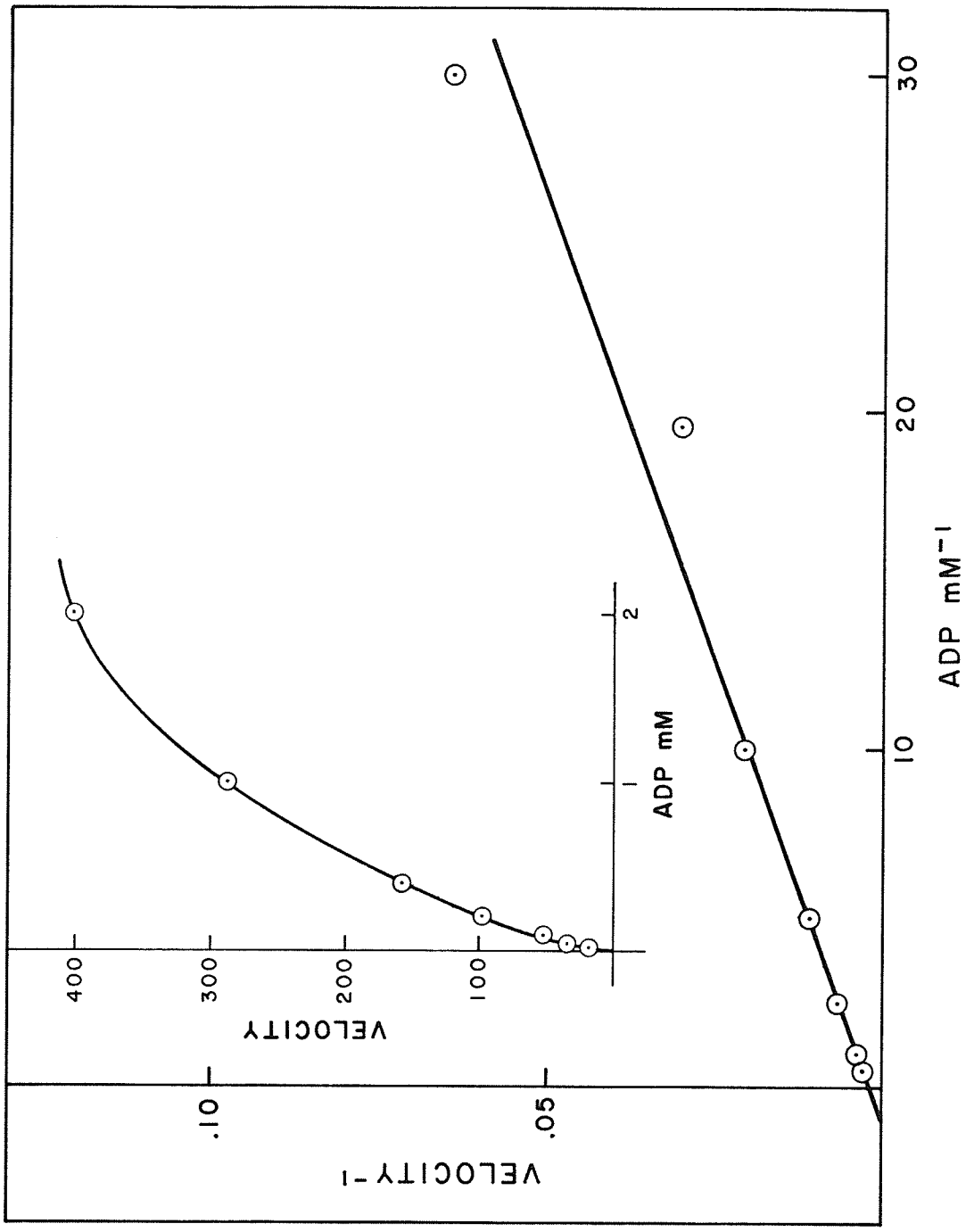

VELOCITY-1
.10
.05
10
20
30
ADP mM-1
VELOCITY
400
300
200
100
1
2
ADP mM

FIGURE 35: Double reciprocal plot of the variation in CDP reduction with CDP concentration in L1R4-A hydroxyurea-resistant cells.

Insert: Velocity of CDP reduction as a function of various concentrations of CDP.

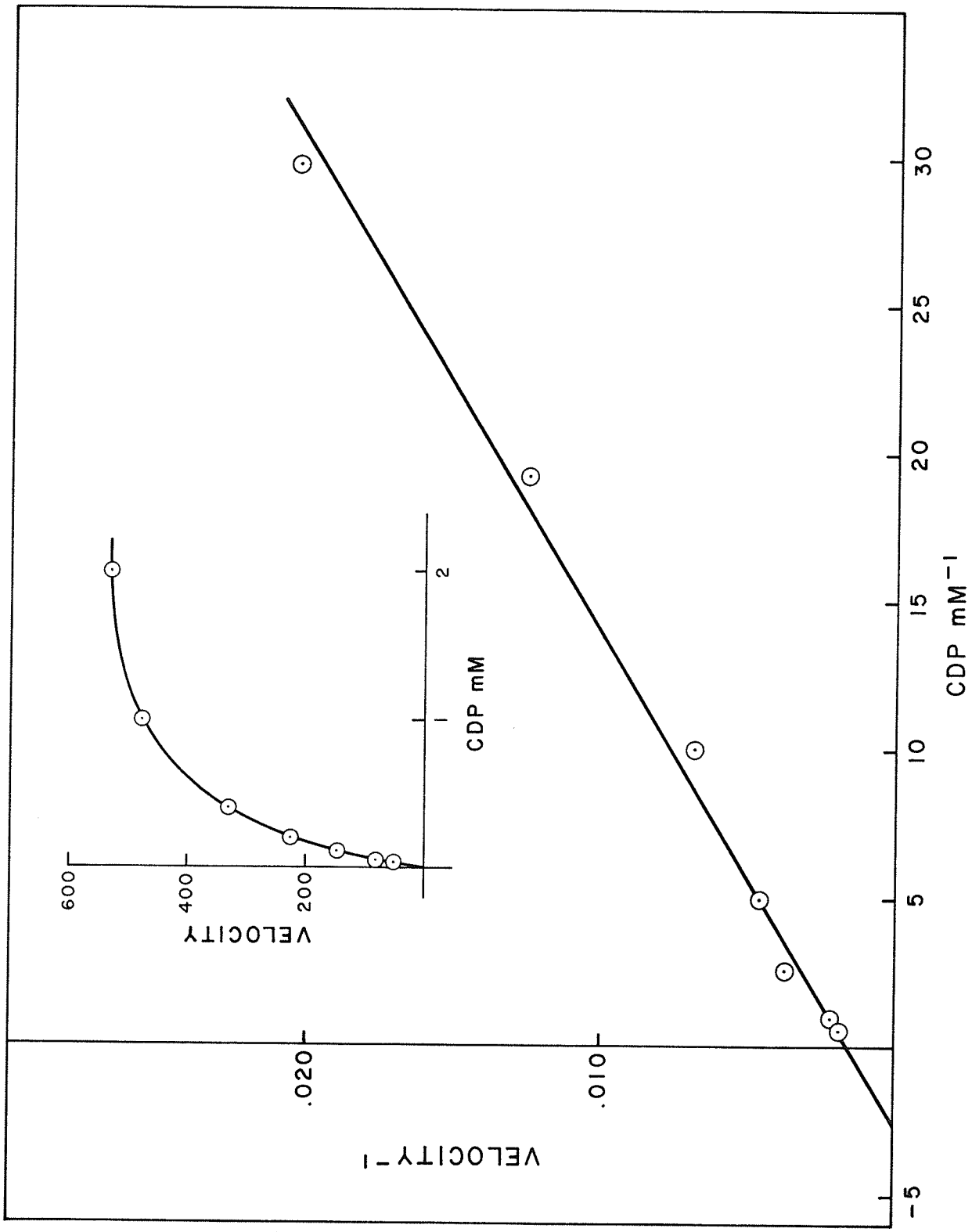

VELOCITY⁻¹
.020
.010
-5
5
10
15
20
25
30
CDP mM⁻¹
VELOCITY
600
400
200
1
2
CDP mM

FIGURE 36: Double reciprocal plot of the variation in ADP reduction with ADP concentration in L1R4-A hydroxyurea-resistant cells.

Insert: Velocity of ADP reduction as a function of various concentrations of ADP.

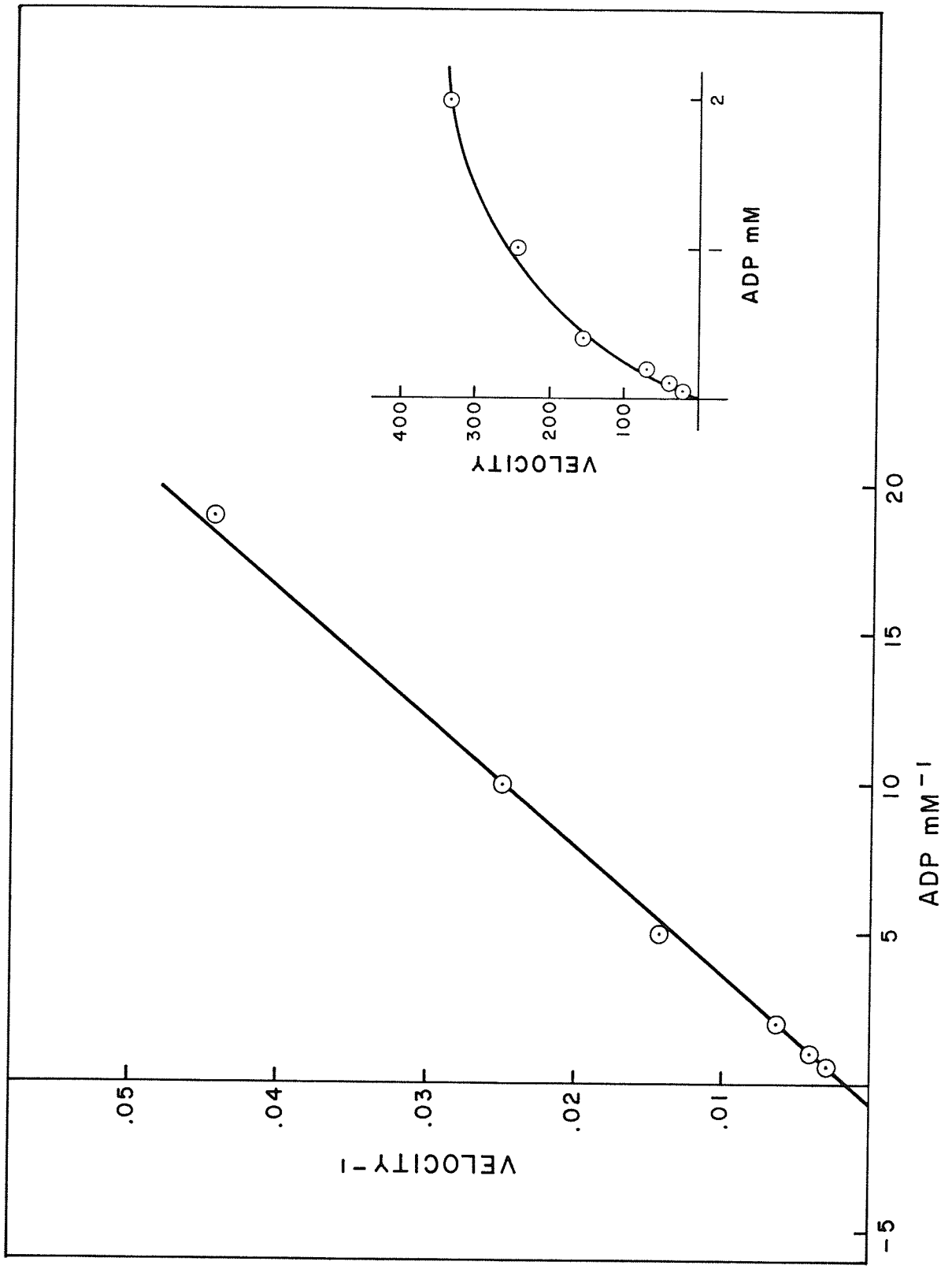

VELOCITY-1
.05
.04
.03
.02
.01
-5
5
10
15
20
ADP mM-1
VELOCITY
400
300
200
100
1
2
ADP mM

FIGURE 37. Double reciprocal plot of the variation in CDP reduction with CDP concentration in L1R5-A hydroxyurea-resistant cells.

Insert: Velocity of CDP reduction as a function of various concentrations of CDP.

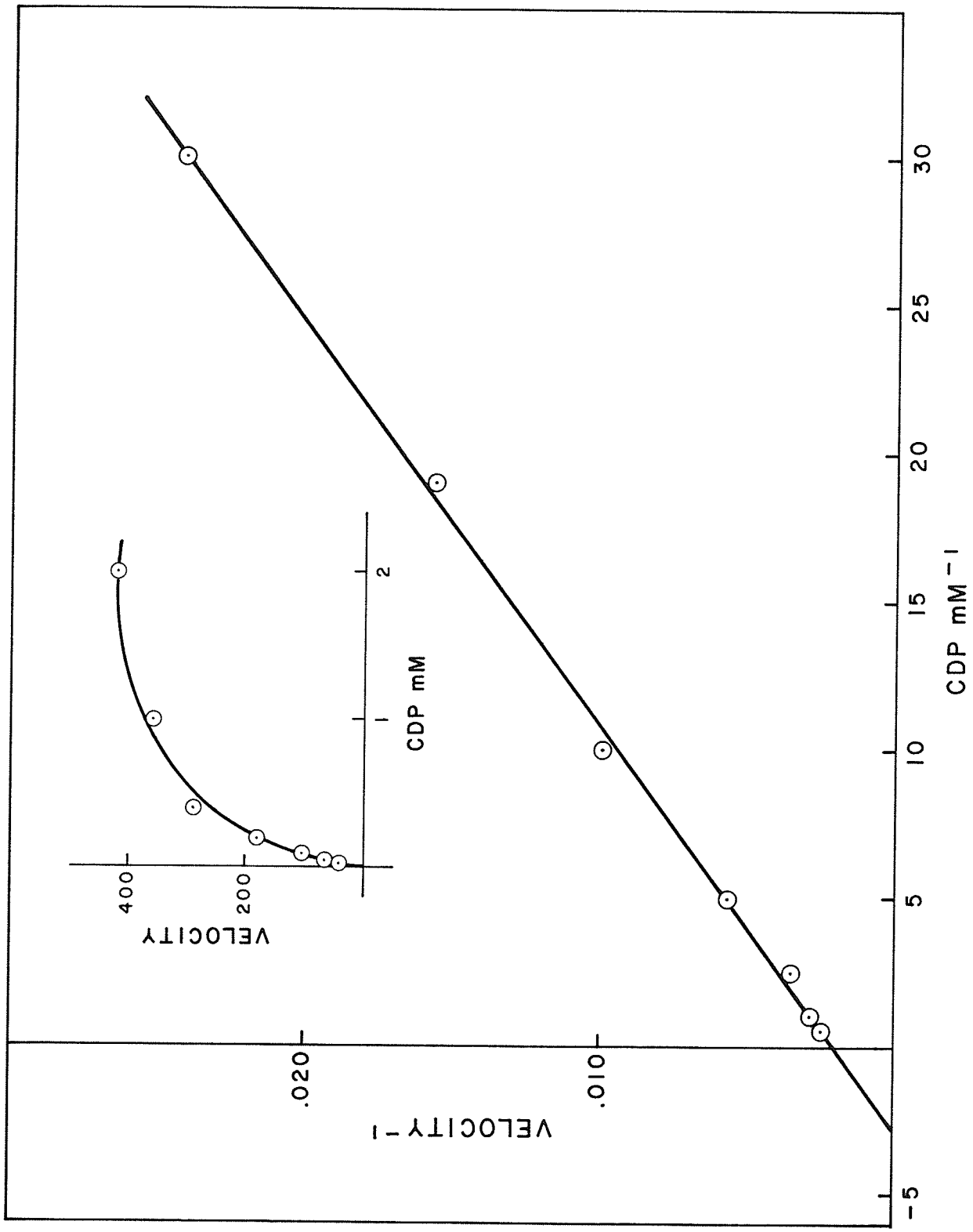

VELOCITY⁻¹
.020
.010
-5
5
10
15
20
25
30
CDP mM⁻¹
VELOCITY
400
200
1
2
CDP mM

FIGURE 38: Double reciprocal plot of the variation in ADP reduction with ADP concentration in L1R5-A hydroxyurea-resistant cells.

Insert: Velocity of ADP reduction as a function of various concentrations of ADP.

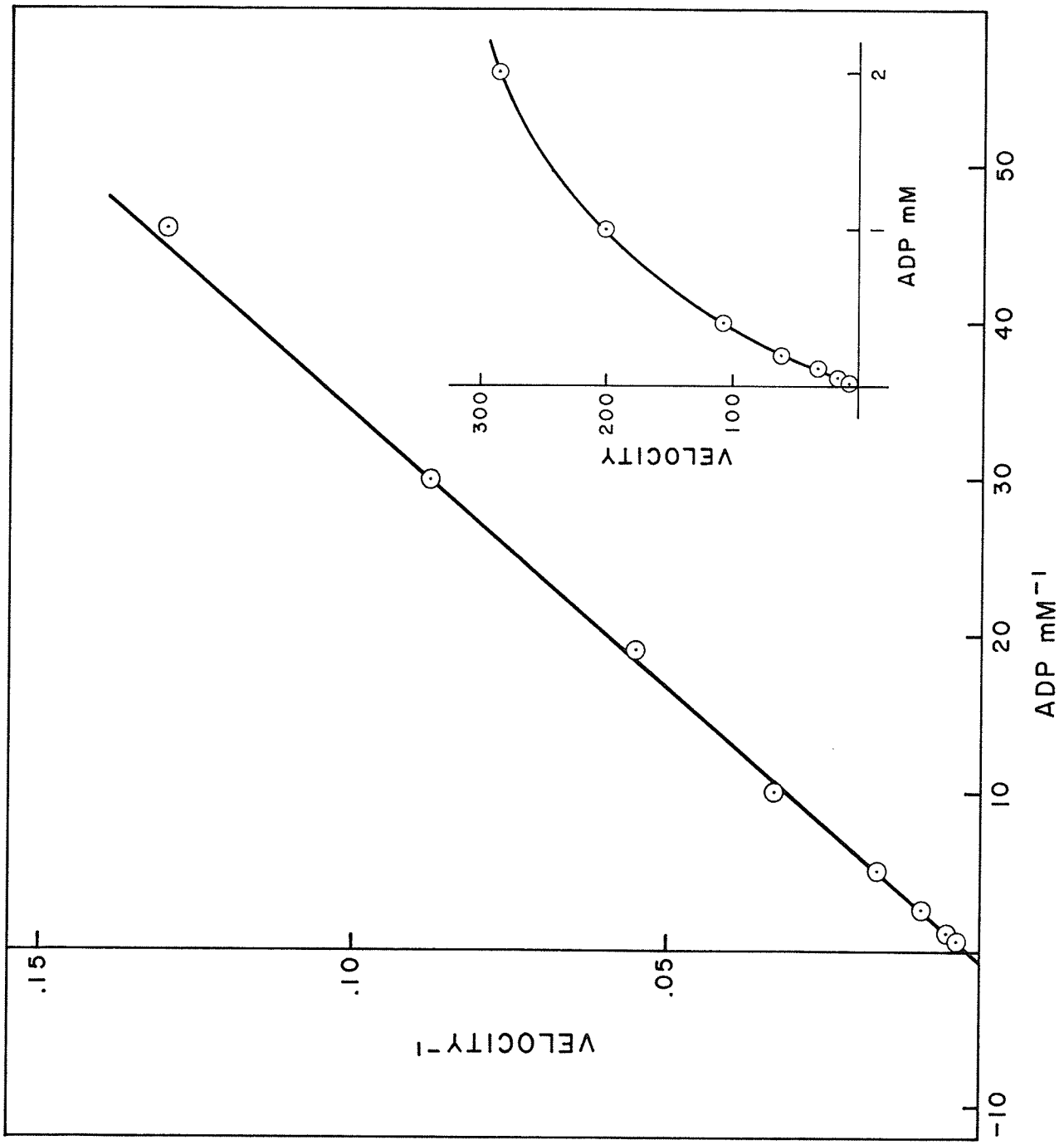

VELOCITY⁻¹
.15
.10
.05
-10
10
20
30
40
50
ADP mM⁻¹
VELOCITY
300
200
100
1
2
ADP mM

FIGURE 39: Double reciprocal plot of the variation in CDP reduction with CDP concentration in L3R1-A hydroxyurea-resistant cells.

Insert: Velocity of CDP reduction as a function of various concentrations of CDP.

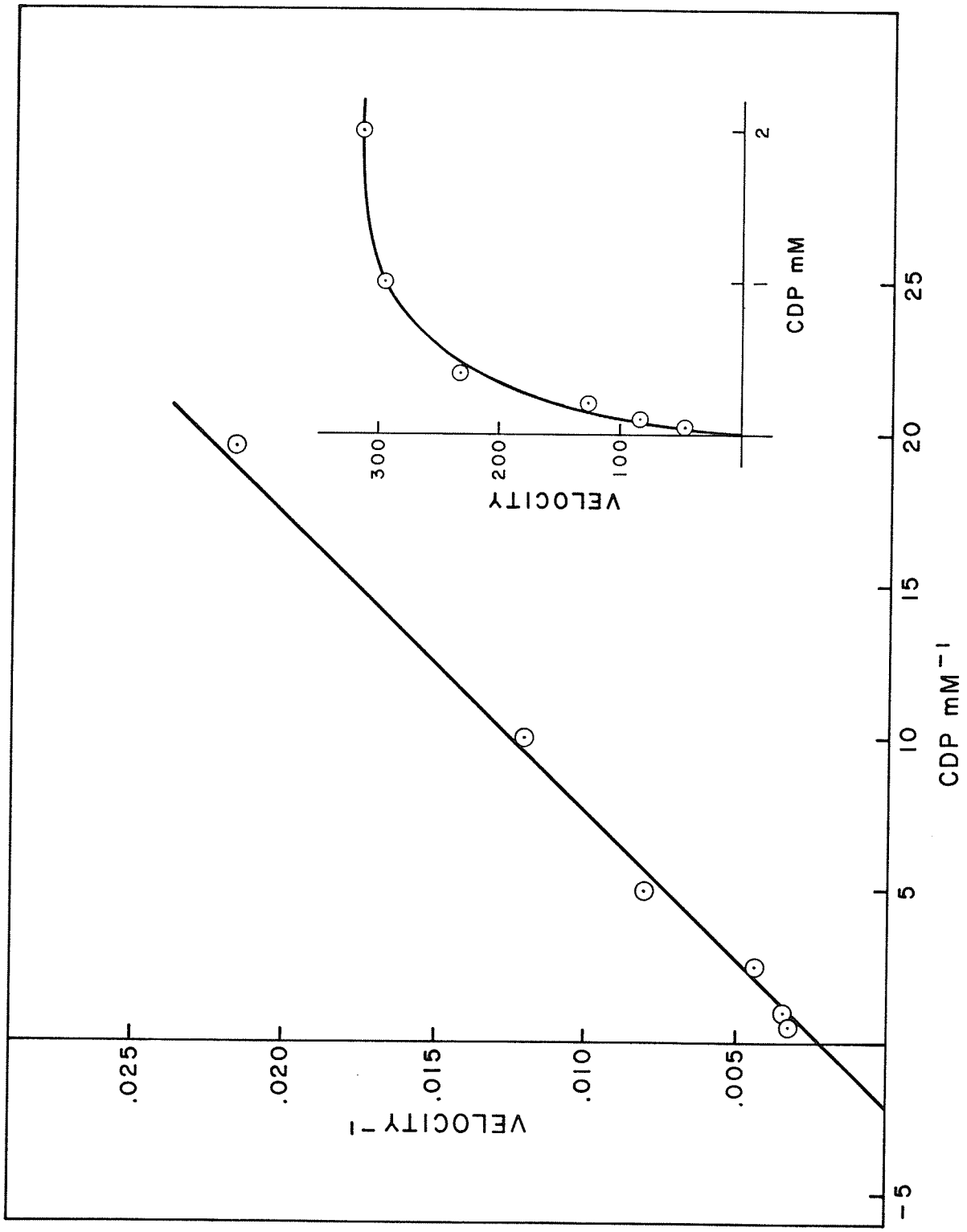

VELOCITY⁻¹
.025
.020
.015
.010
.005
-5
5
10
15
20
25
CDP mM⁻¹
VELOCITY
300
200
100
1
2
CDP mM

FIGURE 40: Double reciprocal plot of the variation in ADP reduction with ADP concentration in L3R1-A hydroxyurea-resistant cells.

Insert: Velocity of ADP reduction as a function of various concentrations of ADP.

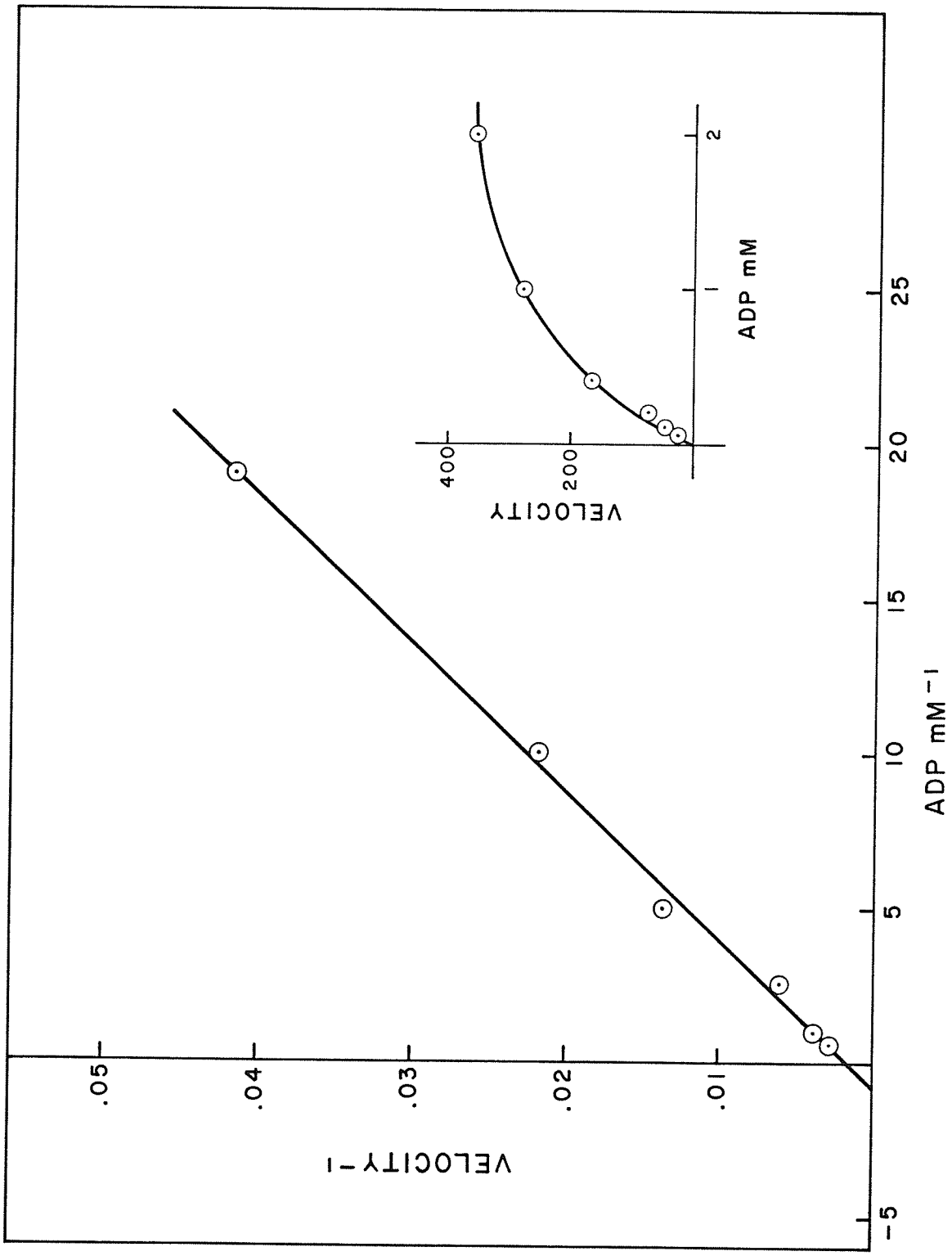

VELOCITY⁻¹
.05
.04
.03
.02
.01
-5
5
10
15
20
25
ADP mM⁻¹
VELOCITY
400
200
ADP mM
2
1

FIGURE 41: Double reciprocal plot of the variation in CDP reduction with CDP concentration in L3R1-B hydroxyurea-resistant cells.

Insert: Velocity of CDP reduction as a function of various concentrations of CDP.

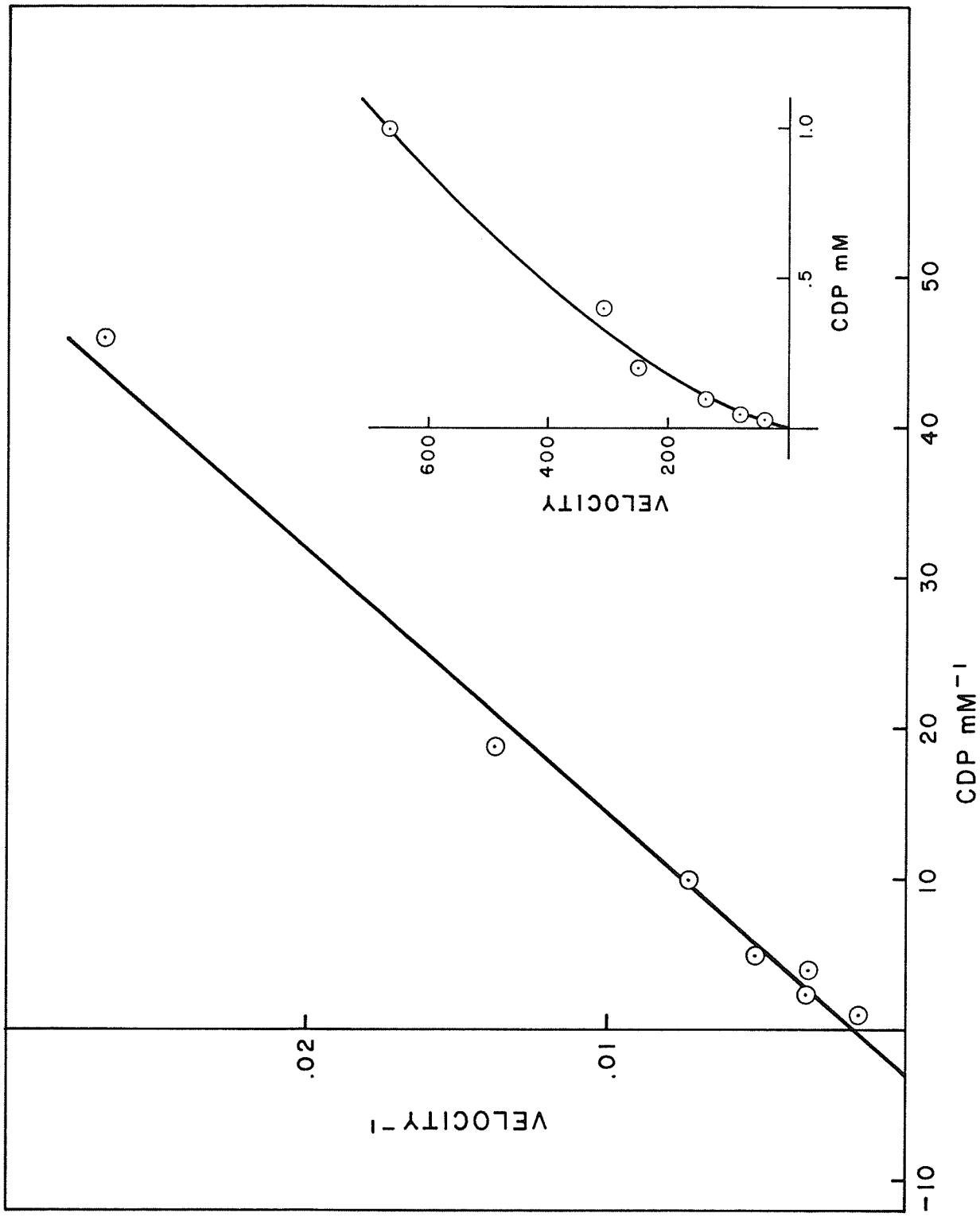

VELOCITY⁻¹
.02
.01
-10
10
20
30
40
50
CDP mM⁻¹
VELOCITY
600
400
200
.5
1.0
CDP mM

FIGURE 42: Double reciprocal plot of the variation in ADP reduction with ADP concentration in L3R1-B hydroxyurea-resistant cells.

Insert: Velocity of ADP reduction as a function of various concentrations of ADP.

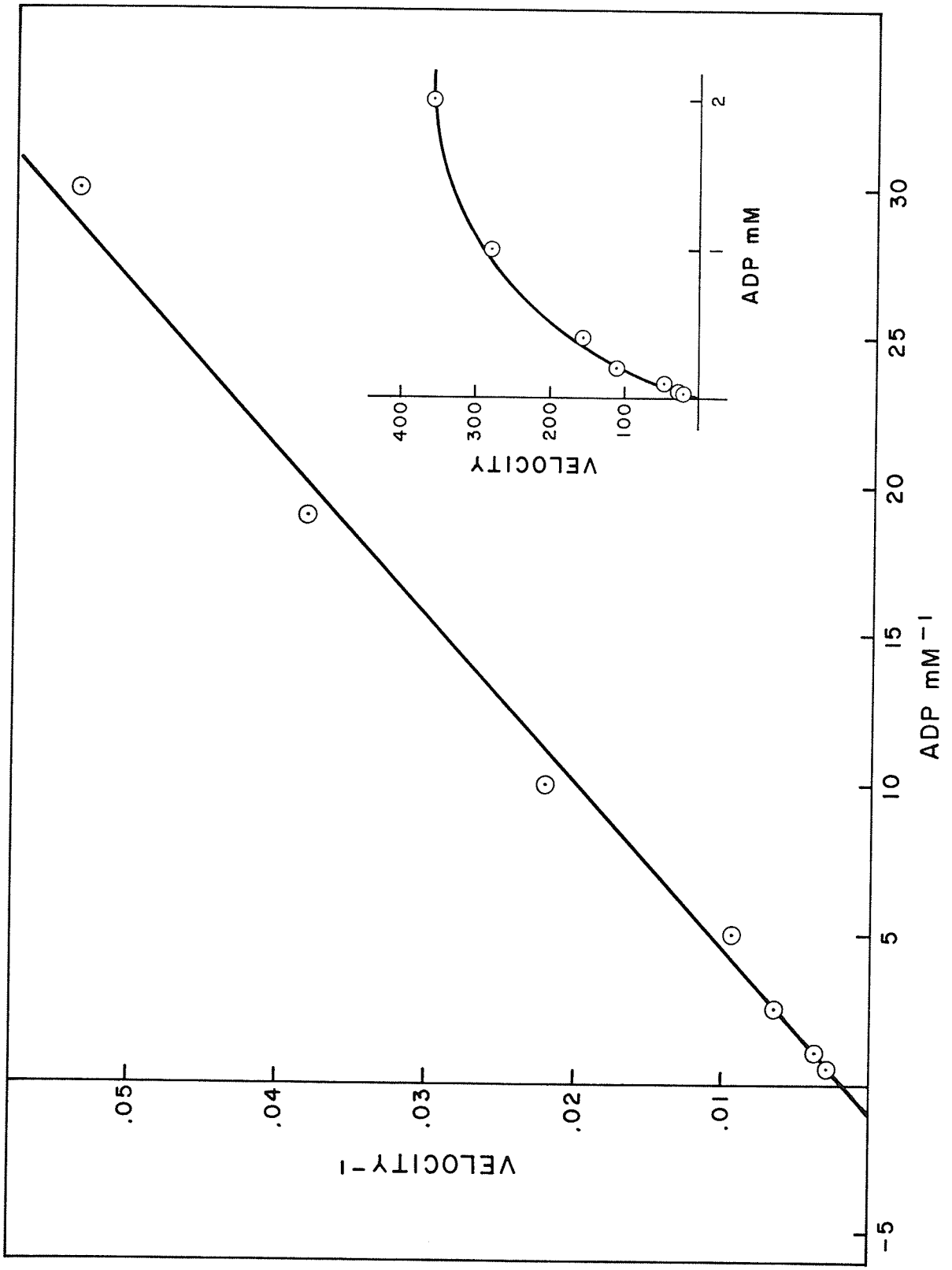

VELOCITY-1
.05
.04
.03
.02
.01
-5
5
10
15
20
25
30
ADP mM-1
VELOCITY
400
300
200
100
1
2
ADP mM

FIGURE 43: Double reciprocal plot of the variation in CDP reduction with CDP concentration in L3R1-C hydroxyurea-resistant cells.

Insert: Velocity of CDP reductuion as a function of various concentrations of CDP.

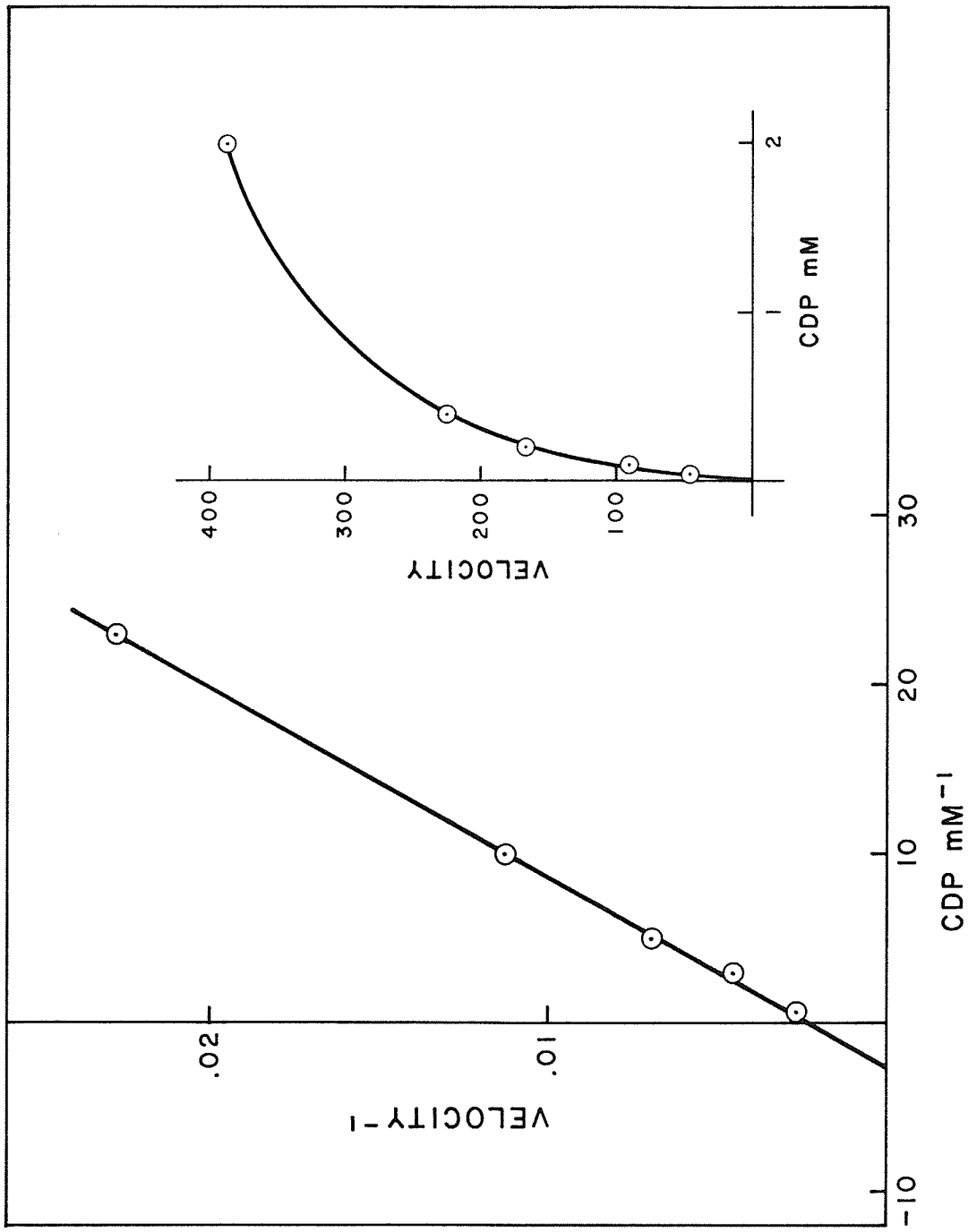

VELOCITY⁻¹
.02
.01
-10
10
20
30
CDP mM⁻¹
VELOCITY
400
300
200
100
1
2
CDP mM

FIGURE 44: Double reciprocal plot of the variation in ADP reduction with ADP concentration in L3R1-C hydroxyurea-resistant cells.

Insert: Velocity of ADP reduction as a function of various concentrations of ADP.

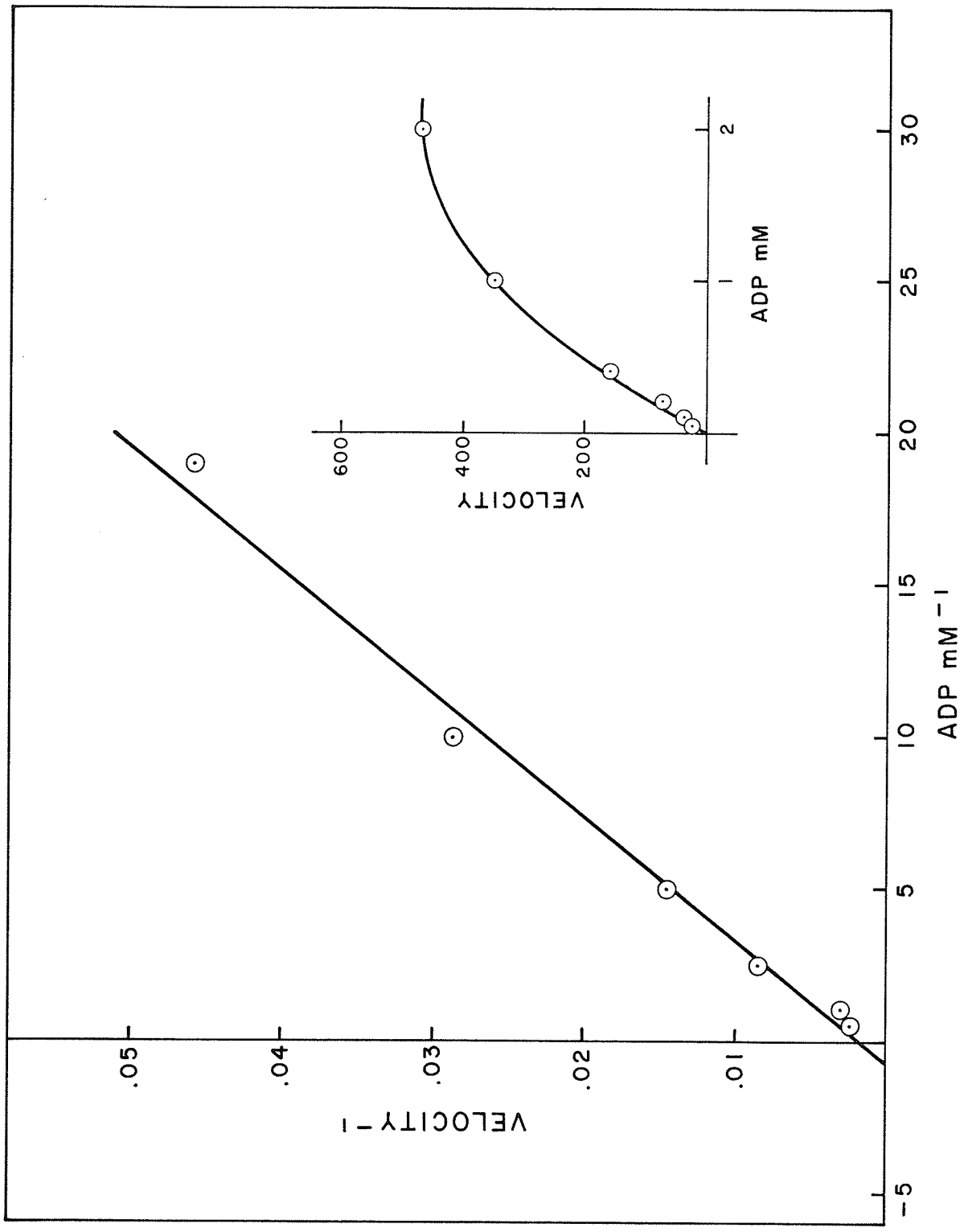

VELOCITY⁻¹
.05
.04
.03
.02
.01
-5
5
10
15
20
25
30
ADP mM⁻¹
VELOCITY
600
400
200
1
2
ADP mM

FIGURE 45: Effect of hydroxyurea on CDP reductase activity in L1 wild type (Fig. 45A) and L3 wild type (Fig. 45B) cells. Exponentially growing cells were incubated with standard CDP reaction mixture plus various concentrations of hydroxyurea at 37°C. After 30 minutes the reaction was terminated by boiling and the amount of CDP reduced was determined.

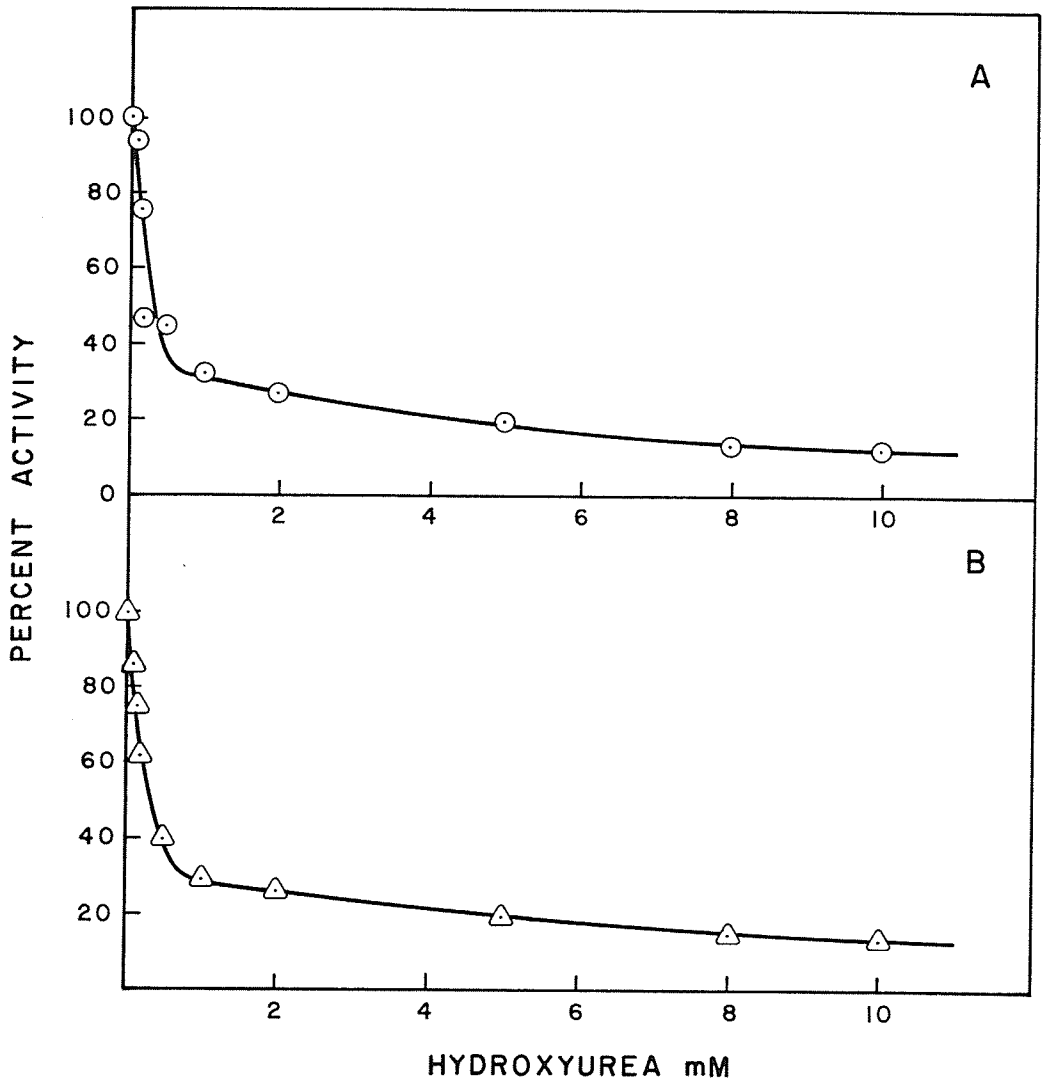

A
B
100
80
60
40
20
0
2
4
6
8
10
PERCENT ACTIVITY
HYDROXYUREA mM

FIGURE 46: Effect of hydroxyurea on ADP reductase activity in L1 wild type (Fig. 46A) and L3 wild type (Fig. 46B) cells. Exponentially growing cells were incubated with standard ADP reaction mixture plus various concentrations of hydroxyurea at 37°C. After 30 minutes the reaction was terminated by boiling and the amount of ADP reduced was determined.

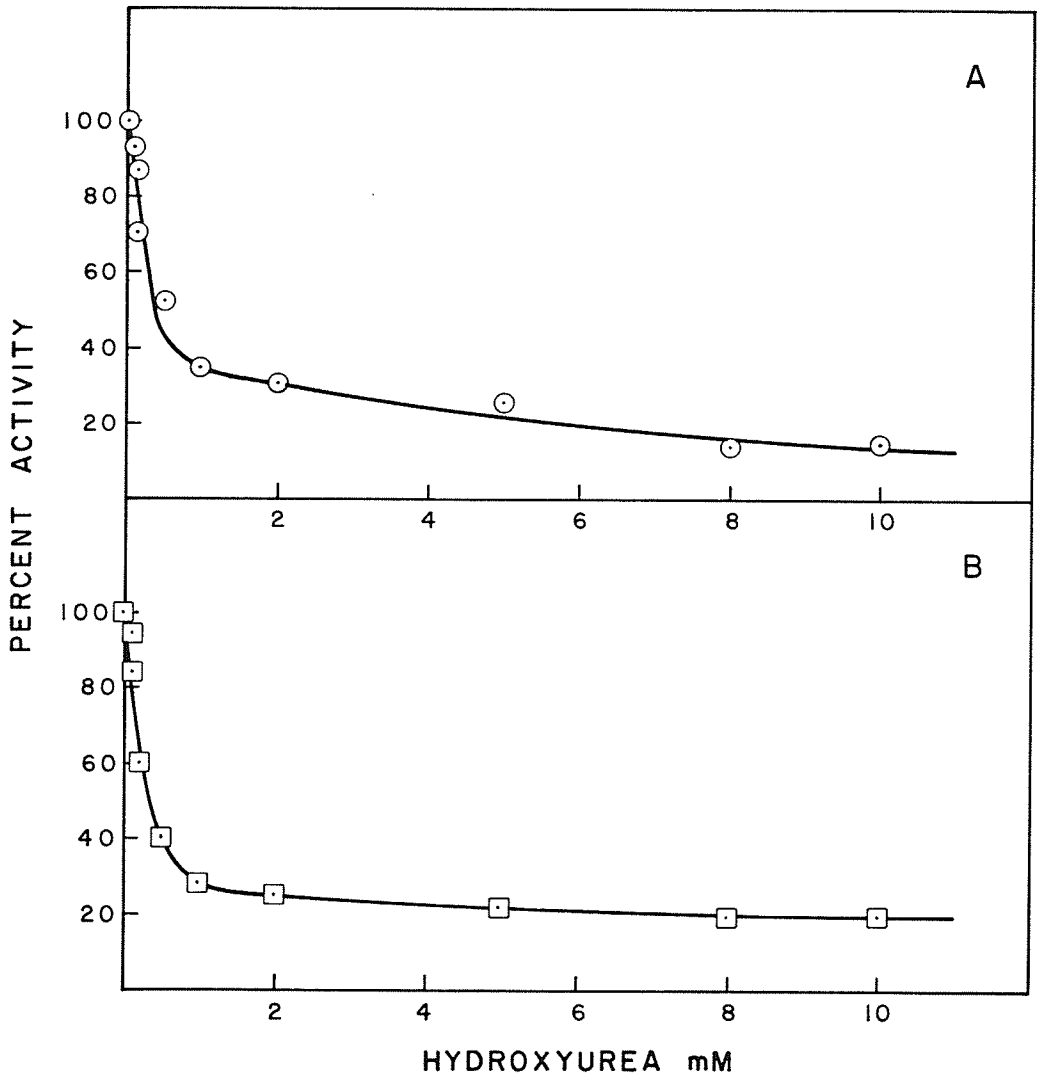

A
B
100
80
60
40
20
2
4
6
8
10
PERCENT ACTIVITY
HYDROXYUREA mM

FIGURE 47: Effect of hydroxyurea on CDP reductase activity in L1R1 hydroxyurea-resistant (Fig. 47A), L1R2 hydroxyurea-resistant (Fig. 47B), and L1R3 hydroxyurea-resistant (Fig. 47C) cells. Exponentially growing cells were incubated with standard CDP reaction mixture plus various concentrations of hydroxyurea at 37°C. After 30 minutes the reaction was terminated by boiling and the amount of CDP reduced was determined.

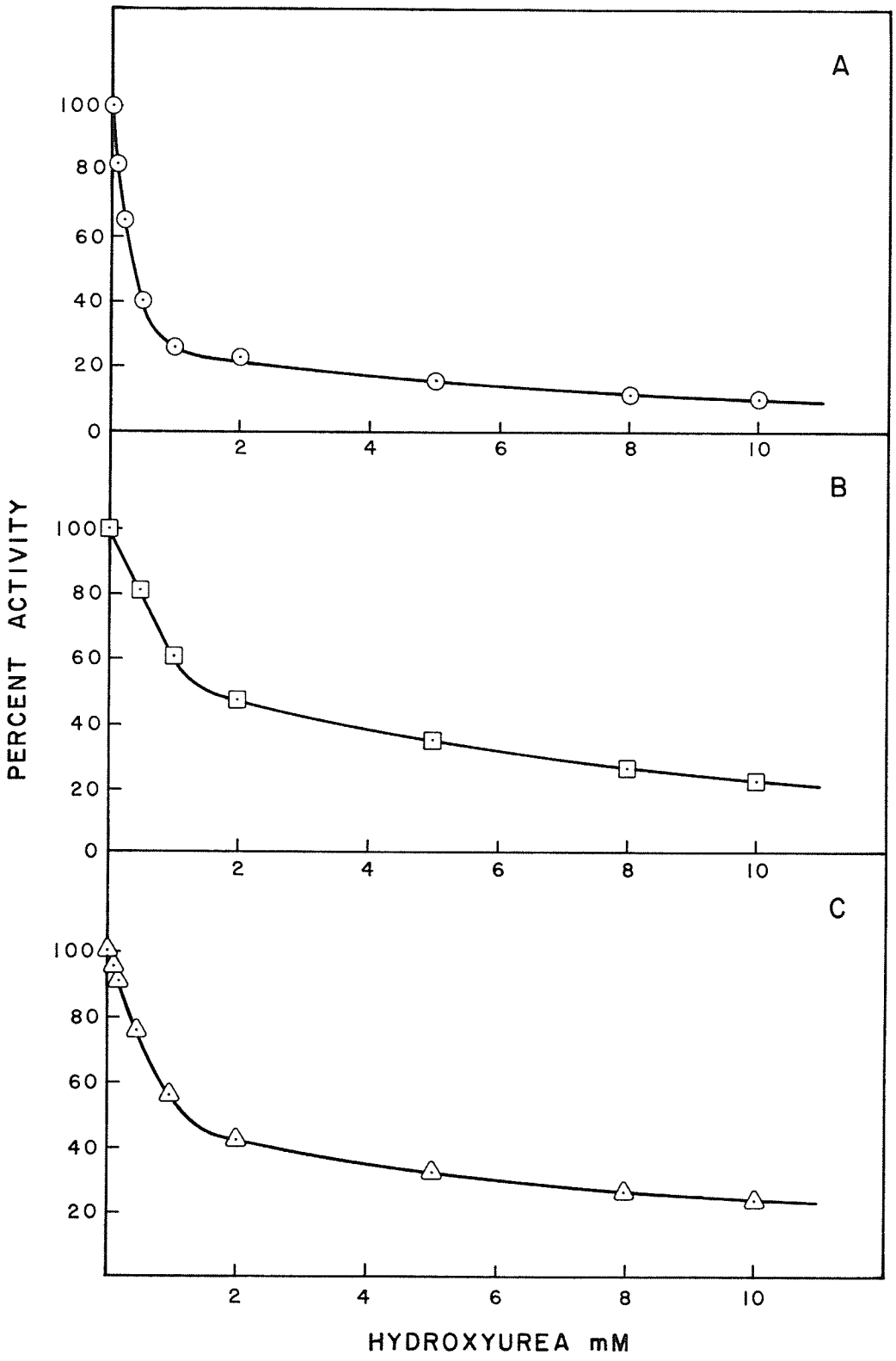

A
B
C
100
80
60
40
20
0
2
4
6
8
10
PERCENT ACTIVITY
HYDROXYUREA mM

FIGURE 48: Effect of hydroxyurea on ADP reductase activity in L1R1 hydroxyurea-resistant (Fig. 48A), L1R2 hydroxyurea-resistant (Fig. 48B), and L1R3 hydroxyurea-resistant (Fig. 48C) cells. Exponentially growing cells were incubated with standard ADP reaction mixture plus various concentrations of hydroxyurea at 37°C. After 30 minutes the reaction was terminated by boiling and the amount of ADP reduced was determined.

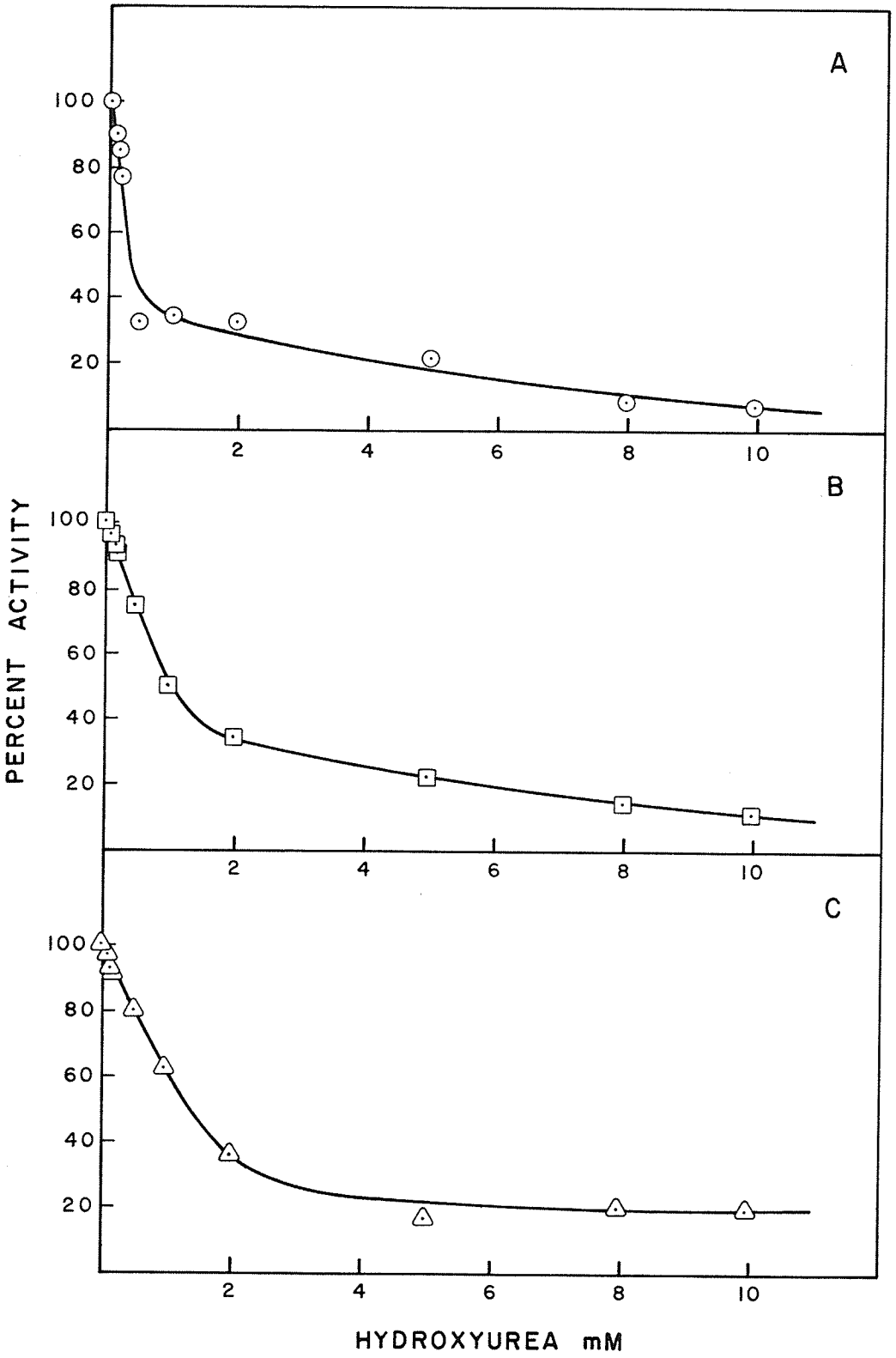

A
B
C
100
80
60
40
20
2
4
6
8
10
PERCENT ACTIVITY
HYDROXYUREA mM

FIGURE 49: Effect of hydroxyurea on CDP reductase activity in L3R1 hydroxyurea-resistant (Fig. 49A), L1R4 hydroxyurea-resistant (Fig. 49B), and L1R5 hydroxyurea-resistant (Fig. 49C) cells. Exponentially growing cells were incubated with standard CDP reaction mixture plus various concentrations of hydroxyurea at 37°C. After 30 minutes the reaction was terminated by boiling and the amount of CDP reduced was determined.

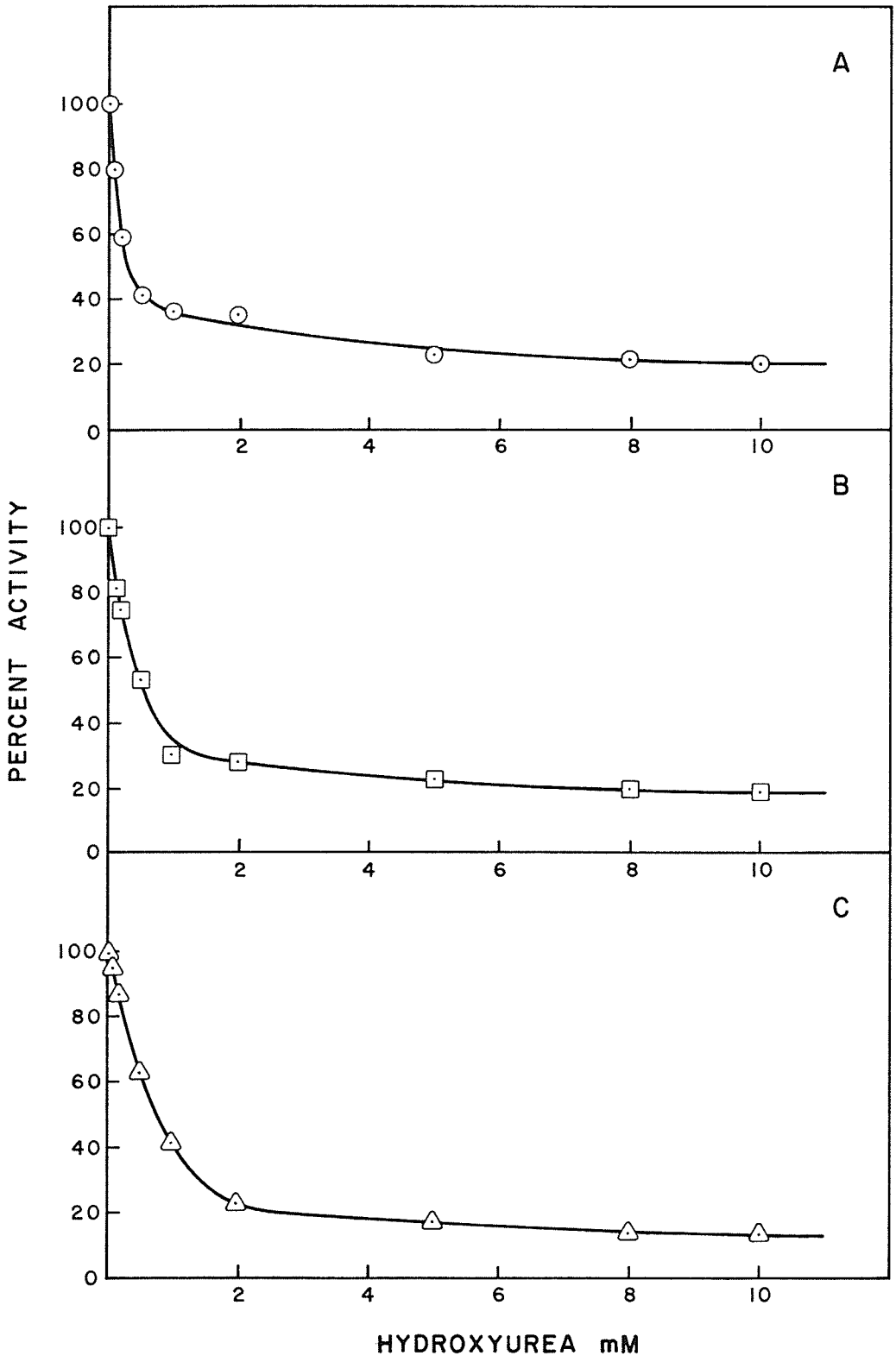

A
B
C
100
80
60
40
20
0
2
4
6
8
10
PERCENT ACTIVITY
HYDROXYUREA mM

FIGURE 50: Effect of hydroxyurea on ADP reductase activity in L3R1 hydroxyurea-resistant (Fig. 50A), L1R4 hydroxyurea-resistant (Fig. 50B), and L1R5 hydroxyurea-resistant (Fig. 50C) cells. Exponentially growing cells were incubated with standard ADP reaction mixture plus various concentrations of hydroxyurea at 37°C. After 30 minutes the reaction was terminated by boiling and the amount of ADP reduced was determined.

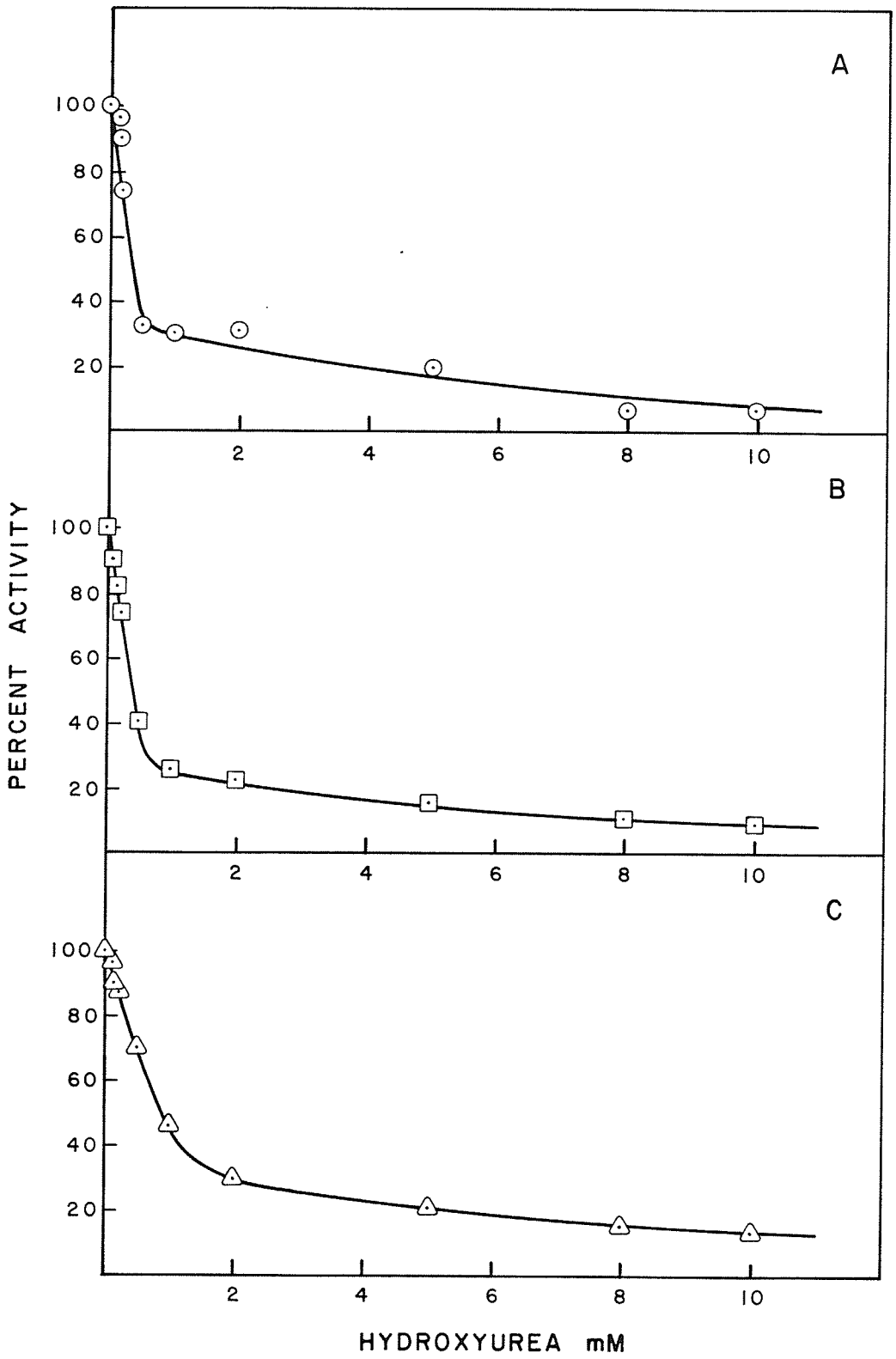

A
B
C
100
80
60
40
20
2
4
6
8
10
PERCENT ACTIVITY
HYDROXYUREA mM

FIGURE 51: Effect of hydroxyurea on CDP reductase activity in L1R1-A hydroxyurea-resistant (Fig. 51A), L1R4-A hydroxyurea-resistant (Fig. 51B), and L1R5-A hydroxyurea-resistant (Fig. 51C) cells. Exponentially growing cells were incubated with standard CDP reaction mixture plus various concentrations of hydroxyurea at 37°C. After 30 minutes the reaction was terminated by boiling and the amount of CDP reduced was determined.

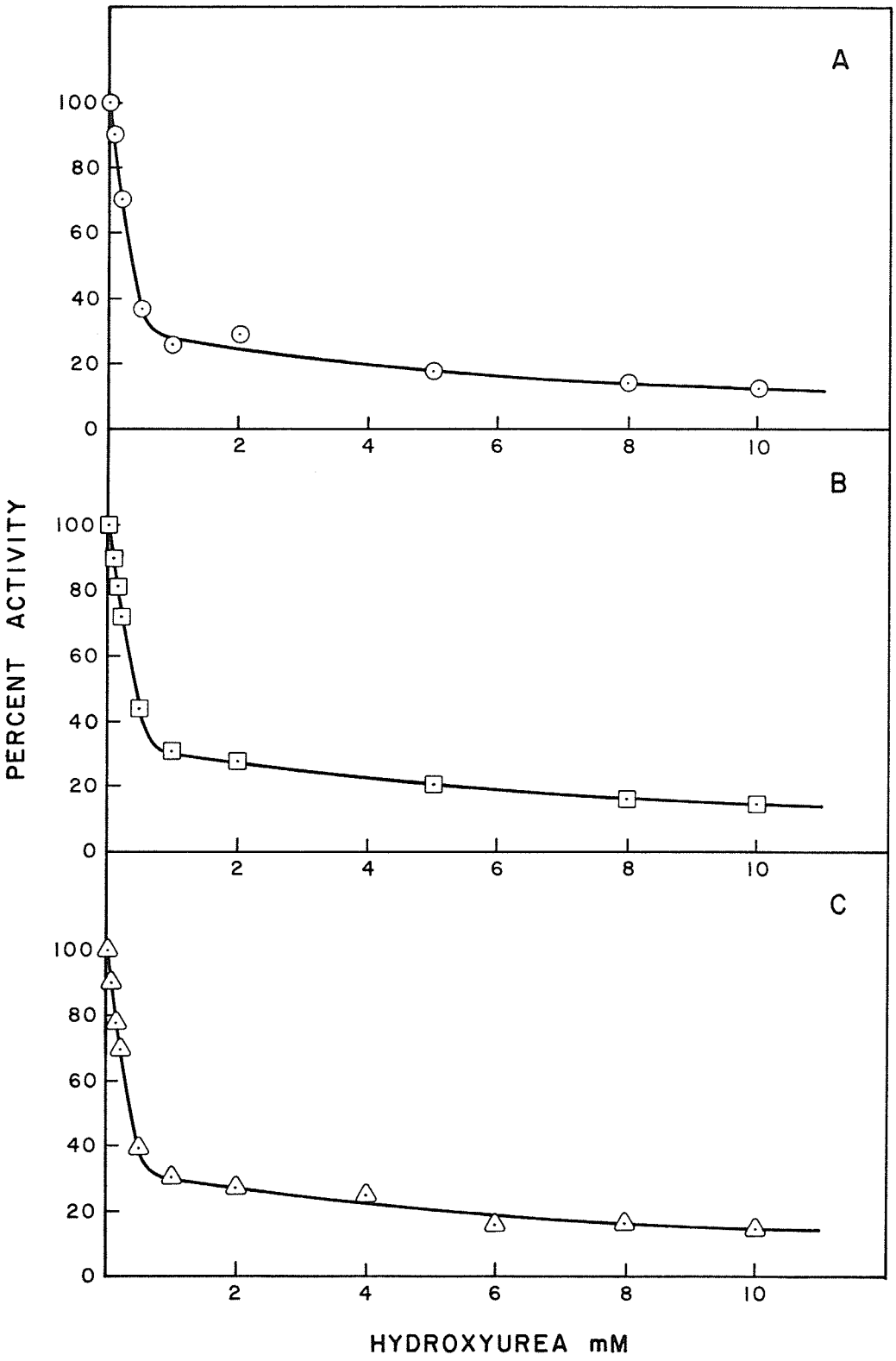

A
B
C
100
80
60
40
20
0
2
4
6
8
10
PERCENT ACTIVITY
HYDROXYUREA mM

FIGURE 52: Effect of hydroxyurea on ADP reductase activity in L1R1-A hydroxyurea-resistant (Fig. 52A), L1R4-A hydroxyurea-resistant (Fig. 52B), and L1R5-A hydroxyurea-resistant (Fig. 52C) cells. Exponentially growing cells were incubated with standard ADP reaction mixture plus various concentrations of hydroxyurea at 37°C. After 30 minutes the reaction was terminated by boiling and the amount of ADP reduced was determined.

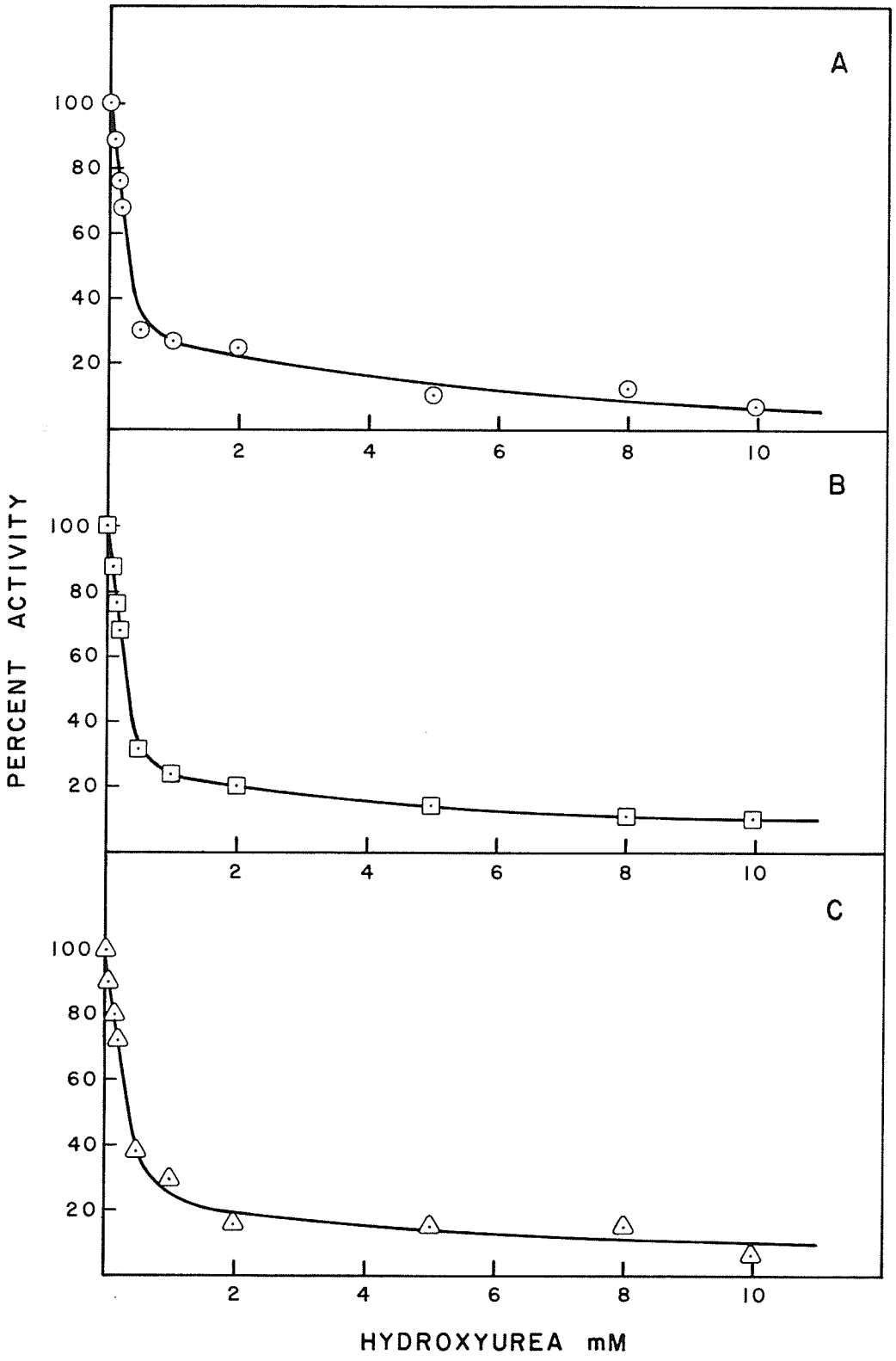

A
B
C
100
80
60
40
20
2
4
6
8
10
PERCENT ACTIVITY
HYDROXYUREA mM

FIGURE 53: Effect of hydroxyurea on CDP reductase activity in L3R1-A hydroxyurea-resistant (Fig. 53A), L3R1-B hydroxyurea-resistant (Fig. 53B), and L3R1-C hydroxyurea-resistant (Fig. 53C) cells. Exponentially growing cells were incubated with standard CDP reaction mixture plus various concentrations of hydroxyurea at 37°C. After 30 minutes the reaction was terminated by boiling and the amount of CDP reduced was determined.

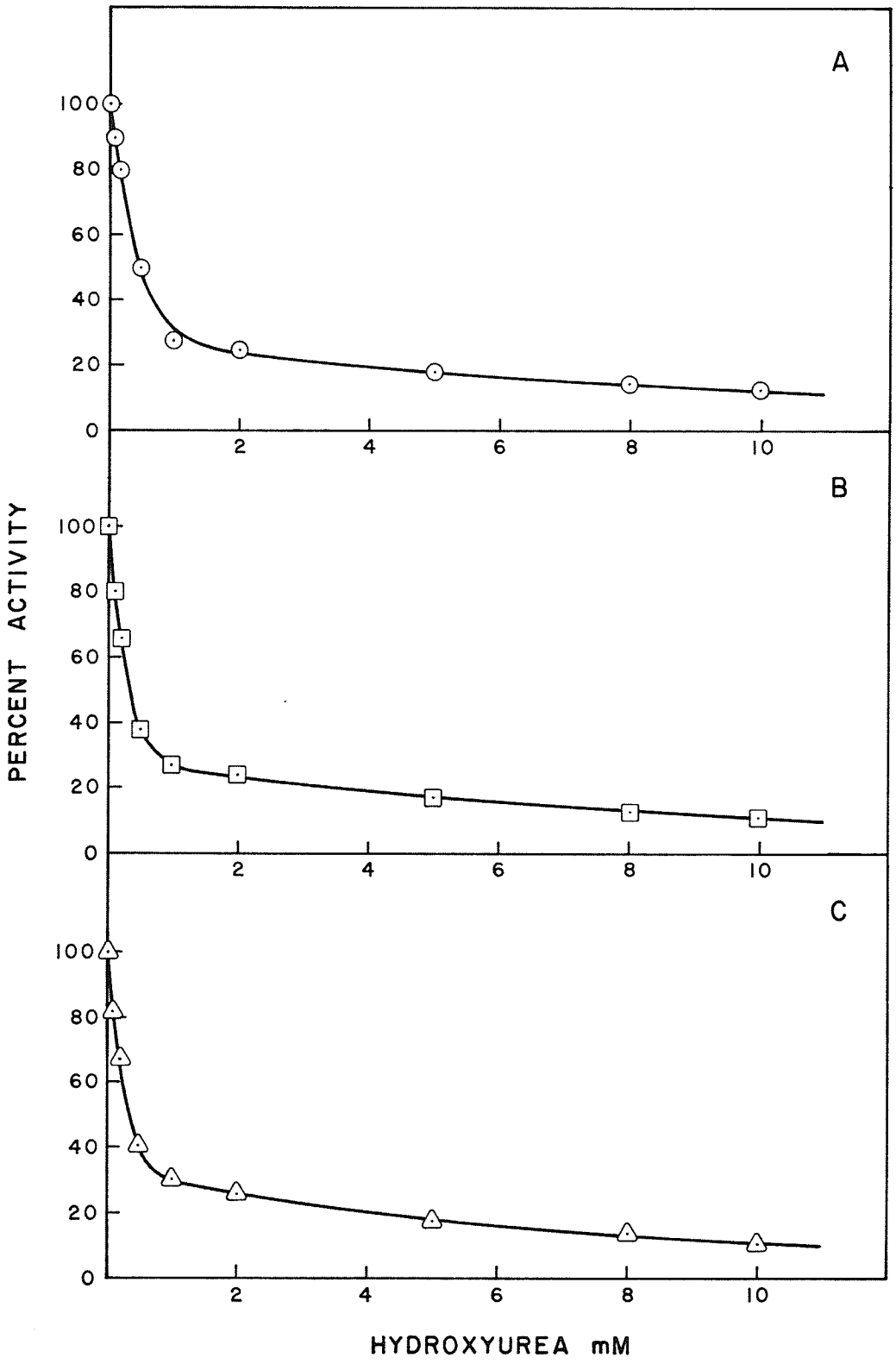
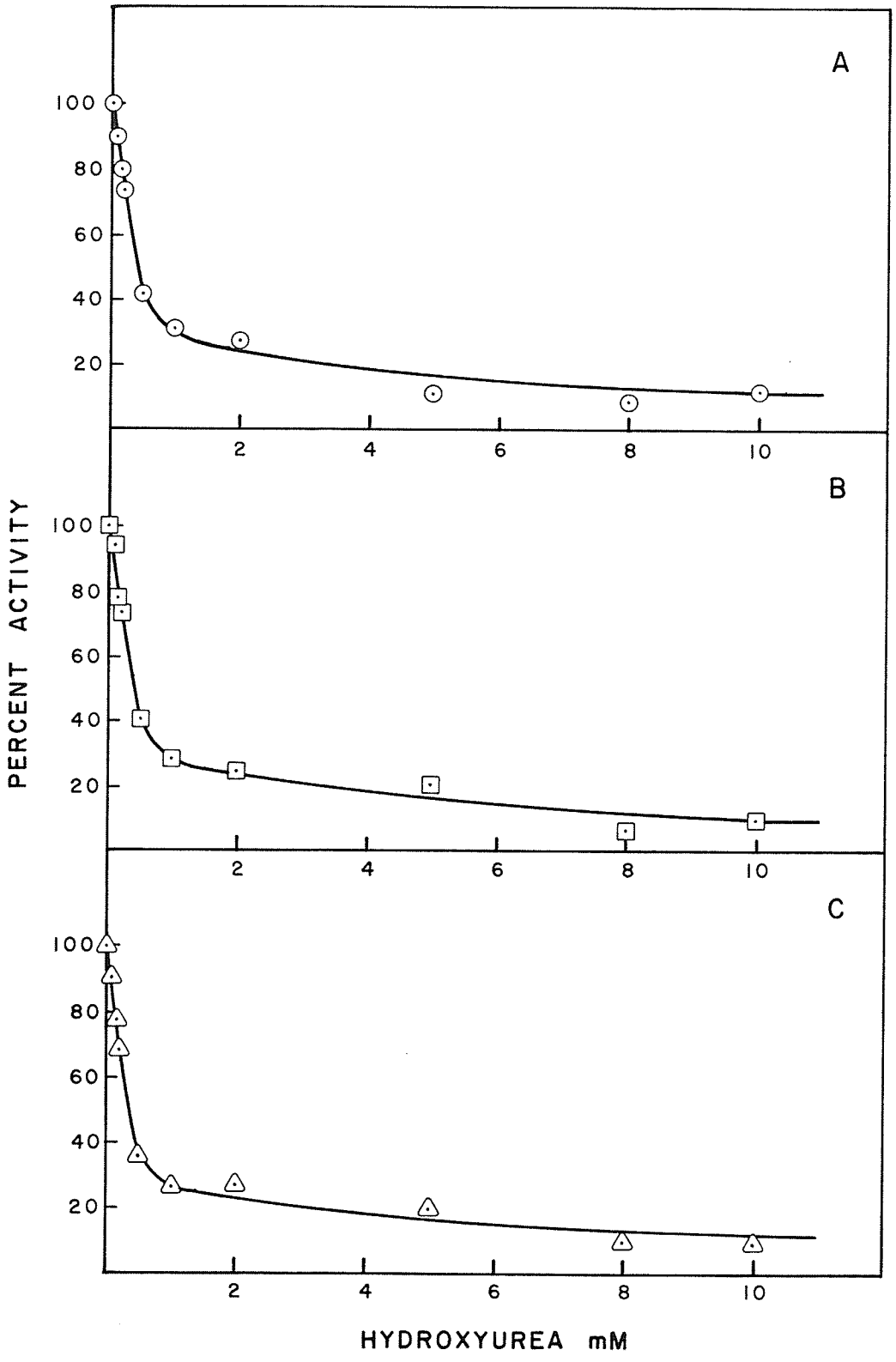

A
B
C
100
80
60
40
20
0
2
4
6
8
10
PERCENT ACTIVITY
HYDROXYUREA mM

FIGURE 54: Effect of hydroxyurea on ADP reductase activity in L3R1-A hydroxyurea-resistant (Fig. 54A), L3R1-B hydroxyurea-resistant (Fig. 54B), and L3R1-C hydroxyurea-resistant (Fig. 54C) cells. Exponentially growing cells were incubated with standard ADP reaction mixture plus various concentrations of hydroxyurea at 37°C. After 30 minutes the reaction was terminated by boiling and the amount of ADP reduced was determined.


A
B
C
100
80
60
40
20
2
4
6
8
10
PERCENT ACTIVITY
HYDROXYUREA mM

DISCUSSION

Hydroxyurea exerts a strong inhibitory effect on exponentially growing mouse L cells. The plating efficiency is severely reduced by 0.26 mM hydroxyurea to approximately 6×10^{-6} and proliferation of wild type mouse L cells ceases within 30 hours after addition of 0.35 mM hydroxyurea (Kuzik and Wright, 1979). The ability of the drug to quickly stop cell division makes it an ideal selective agent for isolating drug-resistant mammalian cell lines.

In agreement with previous reports on the inhibition of ribonucleotide reductase by hydroxyurea (Young and Hodas, 1964; Kuzik and Wright, 1979), the results of studies in this thesis show that ribonucleotide reductase activity in intact mouse cells made permeable to nucleotides by a recently developed technique (Lewis, Kuzik and Wright, 1978) is also drastically reduced in the presence of relatively low concentrations of hydroxyurea. In vivo levels of both CDP and ADP reductase activity were reduced by 0.30 mM hydroxyurea to 50 percent of that in the absence of drug and concentrations between 1 and 10 mM inhibited the enzyme almost entirely, reducing its activity to less than 10 percent of wild type levels.

Cells isolated and cloned for their resistance to hydroxyurea were selected after one or two exposures to the drug. Other investigators have used multiple-step selection procedures to isolate mutant cell lines resistant to high drug concentrations (e.g. Bunn and Eisenstadt, 1977; Gupta and Siminovitch, 1978a; Lewis and Wright, 1974; Kuzik and Wright,

1979). A similar approach was utilized to select for mouse L cells resistant to high concentrations of hydroxyurea. Cellular resistances of 2- to over 12-fold higher than the wild type were maintained after prolonged cultivation in the absence of the drug. Four of the six single-step hydroxyurea-resistant cell lines (L1R1, L1R4, L1R5 and L3R1) showed D_{10} values 2.3, 2.9, 2.8 and 2.3 times higher than the wild type. Unlike the others, two of the six clones isolated after a single exposure to hydroxyurea, L1R2 and L1R3, showed exceptionally high resistances to hydroxyurea and correspondingly high D_{10} values of greater than 1.0 mM which represents at least a 10-fold increase above the wild type D_{10} of 0.14 mM hydroxyurea.

All six clones selected after two exposures to hydroxyurea showed increased cellular resistance to the inhibitory effects of hydroxyurea. D_{10} values for the double-step drug-resistant clones L1R1-A, L1R4-A, L1R5-A, L3R1-A, L3R1-B, L3R1-C were 0.92, 0.87, 0.78, 0.72, 0.82 and 0.84 mM hydroxyurea, respectively. This represents a 6.7-, 6.3-, 5.6-, 4.9-, 5.6- and 5.8-fold increase in their corresponding wild type D_{10}. The majority of the double-step mutant cell lines exhibited resistances up to 3 times that achieved with a single exposure to hydroxyurea, not including the two exceptionally resistant single-step mutants L1R2 and L1R3.

A detailed *in vivo* study of ribonucleotide reductase in intact mouse L cells made permeable to nucleotides revealed

four kinds of drug-induced changes from the wild type; (1) a pronounced increase in levels of CDP and ADP reductase activity (over 13- and 5-fold, respectively) plus a 3-fold increase in the resistance of both CDP and ADP reductase to inhibition by hydroxyurea. These changes were reported in two exceptionally resistant single-step mutants, L1R2 and L1R3, which showed D_{10} values over 10 times higher than the wild type; (2) a moderate increase in the levels of both CDP and ADP reductase (2- to 2.7-fold and 2- to 2.1-fold, respectively) in single-step mutants showing no change in resistance to hydroxyurea at the enzyme level. This type of drug-induced change was demonstrated by L1R1 and L3R1, both of which showed D_{10} values 2.3 times the wild type value; (3) a substantial increase in the levels of both CDP and ADP reduction (4.6- to 8.1-fold and 4.1- to 7.9-fold, respectively in double-step mutants showing no change in resistance of ribonucleotide reductase activity to inhibition by hydroxyurea. This type of drug-induced change was detected in all six double-step hydroxyurea-resistant mutants, L1R1-A, L1R4-A, L1R5-A, L3R1-A, L3R1-B, and L3R1-C, which showed between 5.1- and 6.6-fold increases in the wild type D_{10}; and (4) a significant increase in CDP reductase only. L1R4 and L1R5 exhibited no change from the wild type with respect to ADP reductase. L1R4 showed a 3.5-fold increase over parental wild type in CDP reduction and no change in resistance to inhibition of enzyme activity by hydroxyurea, while L1R5 contained only 2.2 times the wild type levels of CDP reductase

in addition to 2.6-fold increase in the resistance of both CDP and ADP reductase to inhibition by hydroxyurea. The D_{10} values for L1R4 and L1R5 were both approximately 3-fold higher than the parental wild type value.

The isolation of hydroxyurea-resistant mutant cell lines which exhibited type 1 drug-induced changes supports other findings reported in this laboratory (Kuzik and Wright, 1979). It appears that elevated levels of ribonucleotide reductase activity in combination with the production of a less-sensitive enzyme are responsible for the hydroxyurea-resistant phenotype displayed in some hydroxyurea-resistant mouse L cells. Enhanced levels of ribonucleotide reductase activity alone, described in this thesis as type 3 changes, have also been reported to account for drug resistance in some mammalian cells. This mechanism of drug resistance has recently been described in CHO cells selected for resistance to hydroxyurea (Lewis and Wright, 1979). These findings suggest that increased rates of ribonucleotide reductase activity at the second stage of drug selection may reflect changes in the enzyme induced during the transition from single- to double-step which render the cell still further resistant to the cytotoxic effects of hydroxyurea.

A thorough understanding of ribonucleotide reductase from mammalian sources awaits purification of the enzyme.

Several attempts at purification of eucaryotic ribonucleotide reductase have been made (Moore, 1967; Larsson, 1973; Lewis and Wright, 1978b) but with only marginal success. However, it has come to our attention that a research group from Sweden has managed to obtain a substantial, although not homogeneous, preparation of ribonucleotide reductase from calf thymus. (This work has been recently submitted for publication, Engstrom et al., 1979). In the meantime, it is suspected that mammalian ribonucleotide reductase behaves much the same as that from E. coli and that the enzyme is composed of two non-identical subunits which combine to form the catalytically active complex (Brown, Larsson and Reichard, 1967). The substrate specificity as well as rate of catalytic activity is controlled in a complex, allosteric fashion by several nucleoside triphosphates (Brown and Reichard, 1969b; Moore and Hurlbert, 1966; Elford, 1971). More specifically, high and low affinity sites for nucleotide effector binding have been proposed by Brown and Reichard (1969a); low affinity sites involved in regulation of catalytic activity and high affinity sites which determine substrate specificity. The final product in the deoxynucleotide synthetic pathway, dATP, serves as the ultimate regulator of ribonucleotide reductase activity. When deoxynucleotides are no longer required for DNA synthesis and dATP begins to accumulate it immediately shuts down the synthesis of all deoxynucleotides. Substrate specificity is determined by the ratio of nucleotide effectors and their affinities for ribonuc-

leotide reductase. In the presence of ATP the enzyme is stimulated toward pyrimidine reduction. On the other hand, dTTP and dGTP inhibit the reduction of pyrimidines by altering the conformation of the active site to accomodate purines and not pyrimidines. The overall pattern of allosteric control proposed for the enzyme in Novikoff ascites rat tumor cells is schematically represented in Figure (i).

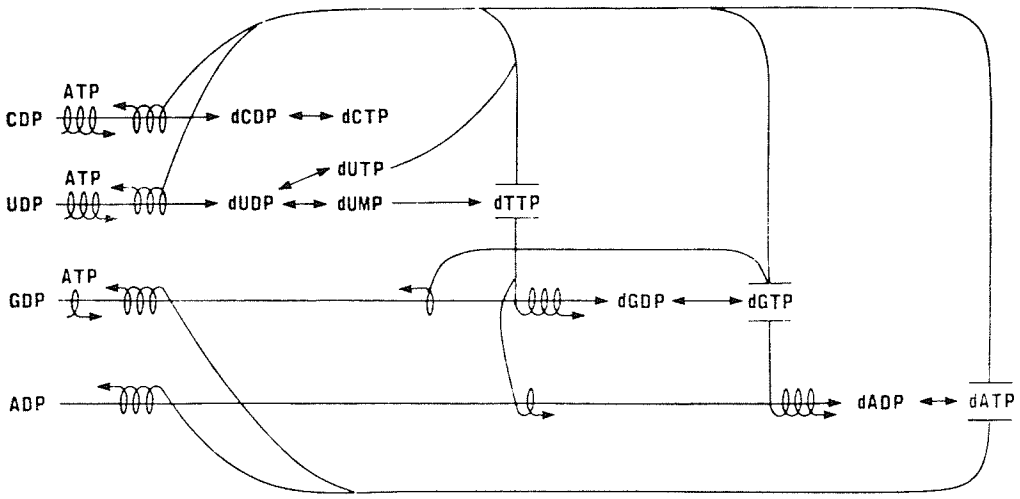


FIGURE (i): Allosteric regulation of mammalian ribonucleotide reductase by nucleotide triphosphate effectors. (Taken from Moore and Hurlbert, 1966). Swirled arrows to right signify activation; to the left, inhibition.

In view of what is known about the catalytic function and allosteric control of ribonucleotide reductase, it is interesting to speculate why ADP reductase in group 1 and

group 4 hydroxyurea-resistant mouse L cells did not demonstrate as dramatic a change in levels as CDP reductase and why ADP reduction did not correlate as well as that for CDP with the degree of cellular resistance to hydroxyurea. By way of explanation the following are suggested: (1) it is possible that there are two separate and descrete enzymes for the reduction of purines and pyrimidines (Peterson and Moore, 1976; Cory and Mansell, 1975) and that one but not the other is affected in hydroxyurea-resistant mutants which show differences in CDP relative to ADP reductase activity. However, this explanation must be treated with some caution since it seems more likely that there is only one enzyme which reduces both purines and pyrimidines as has been found in E. coli (Engstrom et al., 1979); (2) since the enzyme activity was measured in intact cells and the concentrations of nucleotides after permeabilization was not determined, it seems possible that any large variation in nucleotide effector pool sizes among the drug-resistant cell lines could influence the relative purine to pyrimidine reductase activities (Reichard, 1972). Therefore, a future study of the nucleotide pool sizes in these cell lines could prove interesting; (3) a change in the primary structure of ribonucleotide reductase may disturb the natural folding of the protein so that the substrate-binding site is disrupted, and this change in conformation might affect ADP binding in a different way than CDP binding. Therefore, the native

ribonucleotide reductase enzyme which is present in higher than wild type levels in mutant cells may have a less than normal affinity for ADP; (4) since the substrate specificity is controlled by several nucleotide effectors, the change in folding pattern may not affect the substrate-binding site directly but may alter the binding affinities of nucleotide effectors for the enzyme and thereby induce changes in the activity of ribonucleotide reductase toward purine and pyrimidine substrates; and (5) any combination of the above. It should be mentioned that neither CDP nor ADP reduction was fully characterized with respect to optimal assay conditions and nucleotide effector concentrations in the 14 cell lines surveyed as it was not within the scope of this study to thoroughly analyze the compelx catalytic properties of mammalian CDP and ADP reductase. In view of this, it is very possible that other changes may have occurred in the reductase activity of the various cell lines which were not detectable in this study. Also, as noted earlier, attempts to investigate some of the fundamental biochemical properties of the mammalian ribonucleotide reductase are also hindered by the lack of a highly purified enzyme preparation. Clearly, resolution of the catalytic and allosteric properties of mammalian ribonucleotide reductase in the various mutant and wild type cell lines will be greatly enhanced when a homogeneous enzyme preparation is eventually obtained.

CONCLUSIONS

Drug resistance can arise by a variety of mechanisms including production of a structurally altered enzyme which is less sensitive to inhibition by the drug. Numerous somatic cell mutants have been isolated in which production of an altered target protein has been offered as at least a partial explanation for drug resistance. Examples include mammalian cells resistant to emetine (Gupta and Siminovitch, 1978a), methotrexate (Flintoff et al., 1976), hydroxyurea (Lewis and Wright, 1978b; Kuzik and Wright, 1979), α-amanitin (Lobban et al., 1976), ouabain (Robbins and Baker, 1977), and fluorocitrate (Wright, 1975).

Drug resistance in cultured somatic cells can also arise from the production of enhanced levels of a native drug sensitive enzyme. For example, methotrexate-resistance in BHK cells (Chang and Littlefield, 1976), and CHO cells (Flintoff et al., 1976; Nunberg et al., 1978) is accompanied by enhanced levels of dihydrofolate reductase; and a hydroxyurea-resistant CHO cell line has recently been isolated which appears to owe its resistance to the production of 5 to 7 times the wild type amount of drug-sensitive ribonucleotide reductase (Lewis and Wright, 1979). Similarly, enhanced levels of ribonucleotide reductase activity are also characteristic of hydroxyurea-resistant mouse L cells. A highly resistant mouse L cell line previously isolated in this lab exhibited elevated levels of ribonucleotide reductase, but in addition, it was observed that the enzyme was less

sensitive to hydroxyurea and demonstrated altered Michaelis constants, inhibition constants, temperature profile and other physical parameters indicative of a structural change (Kuzik and Wright, 1979). It was concluded, therefore, that the drug-resistant phenomenon can arise by enhanced enzyme levels alone or in combination with the production of a structurally altered enzyme which is less sensitive to drug inhibition.

The work reported in this thesis supports these previous findings. Using a novel in vivo ribonucleotide reductase assay procedure recently developed for CHO cells (Lewis, Kuzik and Wright, 1978), it was possible to compare mean levels and some of the properties of CDP and ADP reductase activity in 2 wild type and 12 independently selected hydroxyurea-resistant mouse L cell lines. This detailed investigation revealed that 4 types of hydroxyurea-resistant mutants were isolated: (1) single-step mutants showing a 3-fold increase in resistance to hydroxyurea at the enzyme level in addition to a dramatic increase in both CDP and ADP reductase activity; (2) single-step mutants showing moderate increases in both CDP and ADP reductase activity; (3) double-step mutants showing a substantial increase in both CDP and ADP reductase activity; and (4) single-step mutants which exhibited changes in the levels of CDP reduction only.

In summary, it was found that hydroxyurea-resistant

mouse L cells could exhibit two important changes; enhanced levels of ribonucleotide reductase activity and an apparent structural alteration in the ribonucleotide reductase enzyme which renders it less sensitive to inhibition by hydroxyurea. The two changes in ribonucleotide reductase activity may arise from a single pleiotropic mutation (e.g. Wright, 1973; Bech-Hansen et al., 1976) which results in a structurally altered enzyme and enhanced levels of activity. This is suggested by the fact that single as well as double-step hydroxyurea-resistant clones were isolated which showed simultaneous changes in levels of reductase activity and Michaelis constants. However, the possibility that two independent mutational events give rise to both drug-induced changes in ribonucleotide reductase still exists. Studies designed to resolve this question are currently underway in our laboratory, but a definitive answer awaits final purification of the enzyme.

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