THE UNIVERSITY OF MANITOBA

# STUDIES ON RIBONUCLEOTIDE REDUCTASE FROM NORMAL SENESCING HUMAN DIPLOID FIBROBLASTS

by

JOHN EDGAR DICK

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF

THE DOCTOR OF PHILOSOPHY DEGREE

Department of Microbiology

Winnipeg, Manitoba

January 1984

# STUDIES ON RIBONUCLEOTIDE REDUCTASE FROM NORMAL SENESCING HUMAN DIPLOID FIBROBLASTS

by

John Edgar Dick

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

DOCTOR OF PHILOSOPHY

To my wife, **Lorna**, whose unconditional love and unfailing support, even during the difficult times, allowed me the opportunity to pursue and achieve this milestone.

To my children, Jonathan and Kristy, who also paid the price of my pursuit.

Finally to my parents and parents-in-law, for their love and continual encouragement and support.

# ACKNOWLEDGMENTS

It is indeed a pleasure to acknowledge Jim Wright for providing the exciting atmosphere which enabled me to carry out and complete this project. His teaching skills, enthusiasm for science, and constant encouragement were largely responsible in making the last several years stimulating and enjoyable. Above all, I value his friendship and thoughtful advice.

My gratitude is also extended to Jessica Morgan, Aaron Tagger and John Blondel for assistance with some of the experiments reported here, and to David Creasey for many late night discussions and assistance with the dNTP determinations. Also, to Dr. M. Ray who generously taught me chromosome banding techniques.

In addition, I would like to thank those staff and colleagues who provided thoughtful guidance and who made this work most enjoyable. Specifically, I would like to thank Bob Hards, Thor Borgford, Craig Parfett, John Hickerson, Arthur Chan, and Rita Blosmanis.

Also, I wish to acknowledge support from the University of Mantioba for a student fellowship, the Natural Sciences and Engineering Research Council for a scholarship, and the Manitoba Health Research Council for a scholarship.

Finally, I would like to thank Lorna Dick, Shelley Penner and Karen Nedohin who went beyond the call of duty to draw graphs and type the manuscript of this thesis.

TABLE OF CONTENTS

Page

Page

# LIST OF FIGURES

# LIST OF TABLES

## SOURCES OF MATERIAL

Most biochemicals were purchased from Sigma Chemical Co., St. Louis or Fisher Scientific and all radioactive chemicals were from Amersham Corp.

| Material | Source |
| --- | --- |
| α-minimal essential medium | Flow |
| Fetal bovine serum | Gibco |
| Tissue culture plates | Lux |
| Tween 80 | Matheson Coleman & Bell and J. T. Baker |
| AG1-X8 anion exchange resin (200-400 mesh) | Bio-Rad |
| ACS (aqueous country scintillant) | Amersham |
| Glass fiber filters #9-730-62 | Fisher |
| Partisil 10 SAX HPLC column | Whatman |
| $KH_2PO_4$ (HPLC grade) | Fisher |
| Methanol (HPLC grade) | Fisher |
| NSC tissue solubilizer | Amersham |
| Cryotubes | Gibco |
| Scintiverse II | Fisher |

# LIST OF ABBREVIATIONS

| | |
|---|---|
| $\alpha$-MEM | - alpha minimal essential medium |
| ACS | - aqueous counting scintillant (Amersham) |
| ADP | - adenosine 5'-diphosphate |
| AdR | - 2'-deoxyadenosine |
| 9-Ag | - 8-azaquanine |
| ATP | - adenosine 5'-triphosphate |
| AUFS | - absorbance units full scale |
| CDP | - cytidine 5'-diphosphate |
| CdR | - 2'-deoxycytidine |
| CHO | - Chinese hamster ovary |
| con A | - concanavalin A |
| CTP | - cytidine 5'-triphosphate |
| $D_{10}$ | - concentration of drug which reduces RPE to 10% |
| dA | - 2'-deoxyadenosine |
| dC | - 2'-deoxycytidine |
| dATP | - 2'-deoxyadenosine 5'-triphosphate |
| dCTP | - 2'-deoxycytidine 5'-triphosphate |
| dGTP | - 2'-deoxyguanosine 5'-triphosphate |
| dTTP | - 2'-deoxythymidine 5'-triphosphate |
| dN | - deoynucleoside |
| DMSO | - dimethylsulfoxide |
| DNA | - deoxyribonucleic acid |
| dNTP | - all four of the deoxyribonucleoside triphosphates; TTP, dCTP, dATP, dGTP |
| dpm | - decays per minute |
| DTT | - dithiothreitol |
| EDTA | - ethylenediamine tetraacetic acid |

EMS - ethyl methane sulphonate

FCS - fetal calf serum

GAT - glycine, adenosine, thymidine

$GATO^R$-1 - GAT auxotroph, ouabain resistant

GDP - guanosine 5'-diphosphate

GTP - guanosine 5'-triphosphate

G6PD - glucose 6-phosphate dehydrogenase

HDF - human diploid fibroblast(s)

HEPES - N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HPLC - high performance liquid chromatography

HU - hydroxyurea

Ka - concentration of activator which increases enzyme activity by 50%

$K_i$ - concentration of drug which reduces enzyme activity by 50%

MPD - mean population doubling

$M_1$ - effector binding subunit of RRase

$M_2$ - iron containing subunit of RRase

mRNA - messenger ribonucleic acid

NDP - all four of the ribonucleoside diphosphates; UDP, CDP, GDP, ADP

NTP - all four of the ribonucleoside triphosphates UTP, CTP, ATP, GTP

OD - optical density

PBS - phosphate buffered saline

PCA - perchloric acid

PD - population doubling

PE - plating efficiency

| | |
|---|---|
| PEG | - polyethylene glycol |
| RPE | - relative plating efficiency |
| RRase | - ribonucleotide reductase |
| SDS | - sodium dodecyl sulfate |
| $SV_{40}$ | - simian virus strain 40 |
| TCA | - trichloroacetic acid |
| TdR | - deoxythymidine |
| 6-Tg | - 6-thioguanine |
| TnOA | - tri-n-octylamine |
| TRIS | - tris (hydroxymethyl) amino methane |
| tRNA | - transfer ribonucleic acid |
| TTP | - deoxythymidine 5'-triphosphate |
| UDP | - uridine 5'-diphosphate |
| UTP | - uridine 5'-triphosphate |
| UV | - ultra violet |
| VSV | - vesicular stomatitis virus |

# ABSTRACT

Human diploid fibroblasts grown in culture more closely resemble the *in vivo* state than cultures of permanent cell lines and therefore are an important model system in which to study complex regulatory processes. Ribonucleotide reductase is a key rate limiting enzyme in the regulation of DNA synthesis and is involved in regulating a variety of cellular functions. The experiments outlined in this thesis provide, for the first time, a general characterization of ribonucleotide reduction in normal human diploid fibroblasts. A novel permeabilized cell assay was developed and used to examine the regulation of ribonucleotide reductase in low and high passage cells. Several important changes in the regulation of reductase activity from high passage cells were observed. There was a marked decrease in the level of reductase activity in high passage cells. Furthermore, detailed kinetic studies indicated that the allosteric regulation of ribonucleotide reductase by dATP was altered with enzyme from high passage cells. In keeping with the age-related decline in reductase activity high passage cells were found to contain reduced deoxyribonucleotide pools. A variety of age-related alterations in the ribonucleoside di- and triphosphate pools were also observed. In particular, the GDP pool was greatly reduced. This marked reduction in the GDP pool appeared to affect the incorporation of mannose, via the sugar nucleotide GDP-mannose, into cell surface membranes of high passage cells. High passage cells incorporated one half as much mannose as low passage cells.

These observations suggested that changes in ribonucleotide reductase activity may alter the replicative lifespan of normal cells grown in culture. This idea was tested by developing a unique selection system for the isolation of hydroxyurea resistant human diploid fibroblasts. The lifespan of the variants which were isolated by this procedure was significantly reduced by 20 to 40%. It was found that the variant cells overproduced reductase activity and exhibited altered levels of deoxyribonucleotide pools. These studies provided evidence, for the first time, that there is a link between senescence, ribonucleotide reductase activity, and altered deoxynucleotide pools.

Somatic cell hybridization techniques were used to isolate hybrids between normal human fibroblasts and CHO cells. No intact human chromosomes were identified in the hybrid cell lines. However, a variety of chromosomal alterations were observed in the hybrid lines; these alterations may have resulted in the translocation of a piece of human DNA, carrying the gene(s) for reductase onto the hamster chromosomes. Such a small piece of human DNA present in the hamster genome would represent a large purification of the gene(s) for human reductase. These hybrid lines could, therefore, be useful in isolating the gene(s) for human ribonucleotide reductase.

# HISTORICAL REVIEW

# HISTORICAL REVIEW

## A. Senescence of Normal Human Diploid Fibroblasts in Culture

### Introduction

The temporal nature of man's existence on earth is one of the most perplexing concerns which has confronted mankind from the very beginning. This ultimate paradox, that we age from the moment of birth, is one that each of us must face at some point in our lifetime. The stark reality of the fact has caused more than one person to utter with the ecclesiastical Preacher:

> Yet when I surveyed all that my hands had done and what I had toiled to achieve everything was meaningless, a chasing after the wind; nothing was gained under the sun.
>
> Ecclesiastes 2:11

People have followed many different roads in the quest to find meaning and answers to their personal impermanence. The search is as old as mankind itself.

Our own "youth orientated" society shares many of the same aspirations which led Ponce de Leon the Spanish conquistator to search for the "Fountain of Youth". This is especially evident as the ranks of those people over 65 years of age continue to swell. In fact the fastest growing segment of the population of the United States and probably Canada is that group of individuals over 85 years of age (Moment, 1978). The rapid growth in the number of aged people has enormous implications for society in terms of allocation of resources, social security programs, health care, and future manpower shortages.

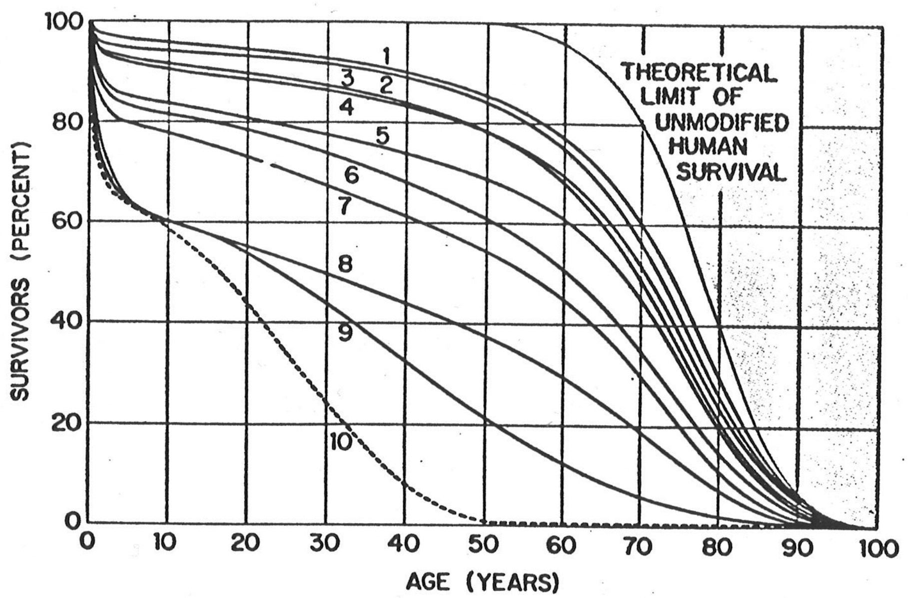
Despite the importance of understanding the aging process, only recently have the methods of scientific inquiry been applied to this puzzle. Science and technology have contributed greatly to the improvement of the human condition. This improvement is best reflected in the extensions of the average lifespan over the last one hundred years. These changes are illustrated in Figure I.

The most dramatic improvement in the last fifty years has been the reduction in infant mortality. The key feature of this figure is the fact that although lifespan has improved, largely due to reduction in disease, a definite upper limit still exists. The reason for this limit is that age related physiological changes in the body lead to increased susceptability to disease, such as cardiovascular disease or cancer. Medical advances have improved lifespan because of the striking advances in treating acute disease; however our lack of understanding basic mechanisms of aging and age-related diseases, indicates a limit in the improvement of lifespan is being reached.

Although immortality may be a tantalizing goal for some, most scientists and others alike would not regard this as feasible, albeit from philosophical or theological grounds rather than scientific reasoning. The goal, then, of aging reasearch would be to either alleviate some of the suffering that almost inevitably results at the end of one's lifetime; or to extend the length of our healthy lifespan in some appreciable fashion.

Aging research is being conducted on as many different fronts as the study of biology itself, because, after all aging is a biological phenomenon open to experiment; and not some mystical process (for reviews see Strehler, 1977; Finch et al., 1979; Finch & Hayflick, 1977; Hayflick, 1980). Aging research is broadly encompassed by work

Figure I. Historical changes in the human survival curve (taken from Comfort, 1979).




1 NEW ZEALAND, 1934-1938
2 U.S. (WHITES), 1939-1941
3 U.S. (WHITES), 1929-1931
4 ENGLAND AND WALES, 1930-1932
5 ITALY, 1930-1932
6 U.S. (WHITES), 1900-1902
7 JAPAN, 1926-1930
8 MEXICO, 1930
9 BRITISH INDIA, 1921-1930
10 STONE AGE MAN

in four major areas: whole animal studies; tissues and organs; cellular biology; and subcellular and molecular events. Each of these areas has its own unique contribution to make as well as deficiencies and problems.

Whole animal studies focus on the age-related changes which occur in the organism as a whole as the result of genetic and environmental influences (Finch & Hayflick, 1977). For instance, it has long been known there is an exponential increase in the probability of dying with advancing age; mathematically described as Gompertz's Law (1825). Such whole animal studies look at incidence of disease, effect of nutrition, role of body temperature, and a whole variety of age-related physiological changes that affect longevity. The emphasis of whole animal studies lies in determining the nature of the interactions between the many systems which comprise the whole organism; rather than individual changes in cells, tissues, and organs (Shock, 1977). These complex interactions are also the major limitations of these studies since any specific observations must be considered in light of the fact that a great many uncontrolled variables are present at any one time.

Likewise tissue and organ studies, while removing the complexity of the interorgan effects of the whole body, are constrained by the hetergenous nature of the cell types that make up the organ and tissue. Cell biological studies using cells in culture permit the investigation of homogenous populations of cells growing in a completely or almost completely controlled environment. Ironically this "reductionist" approach is also the very limition these studies inherently have. What is the relevance of studies on cells growing in a totally artificial environment to cellular aging and death in whole animals? Finally molecular approaches, which may ultimately provide mechanistic answers to this fundamental problem, are also limited by this question of relevance. What is the relation of an enzyme assay carried out on disrupted cells in a completely artificial environment of synthetic buffers and salts, to its normal function in a whole cell?

Validity of In Vitro Aging to In Vivo Aging

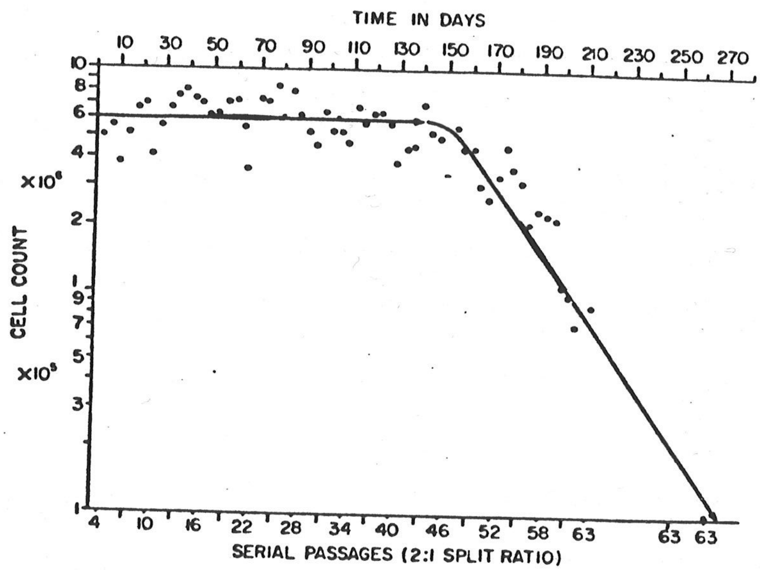
Limited Lifespan. Although aging research can be carried out using several different approaches mentioned earlier; it would however be advantageous to investigate this complex process in a simple model system under experimentally controlled conditions. The advent of tissue culture techniques around the turn of the century suggested this may be a useful method to study such complex biological phenomona (Harrison, 1907). The question was whether or not individual cells showed the same age-related decrements in function as the organism from which they were established. This question was first addressed by Carrel (1912; 1914; 1935) and Ebeling (1913) who grew chick heart fibroblasts in culture continuously for a period of some thirty-four years without any apparent loss of growth capability. The conclusion of these observations was that these cultures of cells were immortal, probably as a result of the loss of some control signal not found in the artificial environment.

These results were challenged by a series of experiments in human embryonic lung fibroblasts which were continuously subcultured *in vitro* (Hayflick & Moorhead, 1961; Hayflick, 1965). The experiments showed that when cells were subcultured according to a rigid protocol of trypsinization and dilution whenever cultures became confluent the cells stopped proliferating after several months of growth. In fact different populations of cells consistently stopped growing after 50 ± 10 passages. This result is illustrated in Figure II.

This figure shows that cells grew normally for about 45 passages (phase II) and then declined in their ability to proliferate to about passage 60 (phase III), after which the cells could not divide. Phase I, although not indicated in this figure, is the establishment period during the initial outgrowth from a primary explant. Similar results to these have subsequently been found using a great variety of cells from many different tissues and animals (Hayflick, 1977). In fact no cell cultures which are karyotypically normal have ever been shown to be able to grow indefinitely (Hayflick, 1977). The apparent discrepancy between Hayflick's and Carrel's results appearsto lie in the methodology of Carrel's culture system. The chick fibroblasts were grown in media supplemented with chick embryo extract; a supplement which has been shown to contain live fibroblasts when prepared according to Carrel's method. Moreover it has not been possible to reproduce their experiments; chick heart fibroblasts always senesce (Hayflick, 1977).

<u>Transformation</u>. Although it has been firmly established that some cell strains exhibit limited proliferative capacity, there are many unequivocal examples of cell lines established from normal tissue which in fact do have unlimited growth potential (Earle,

Figure II. Cell yield during aging in a human diploid cell culture (taken from Hayflick, 1965).



TIME IN DAYS
10 30 50 70 90 110 130 150 170 190 210 230 250 270
CELL COUNT
X10⁶
X10⁵
10
8
6
4
2
1
9
7
5
3
1
4 10 16 22 28 34 40 46 52 58 63 63 63
SERIAL PASSAGES (2:1 SPLIT RATIO)

1943). Upon close examination, these cell lines exhibited a variety of abnormal morphological and cytogenetic characteristics that distinguish them from normal cells. In fact many of these cell lines are tumorigenic. Such transformed cells can arise spontaneously during the subculture of normal cells obtained from a variety of animal and avian sources (Todaro & Green, 1963). Different frequencies of transformation are seen depending on the cell source, however it is usually a rare event seen most frequently in rodent cells and never seen in normal human fibroblasts (Namba et al., 1978). The conclusion of these studies was that these cell lines are genetically and biochemically abnormal ; therefore they have lost their normal control mechanisms which are involved in aging. These differences betwen normal and abnormal cells also suggest much caution ought to be exercised in extrapolating data, biochemical or genetic, obtained using transformed cell lines to similar processes in normal cell strains.

Culture Conditions. Environmental influences could still have been the cause of limited growth *in vitro*. This problem was addressed in a simple and ingenious fashion. High population doubling level (PDL) cells from male donors were co-cultured with low PDL female cells; the PDL at senescence was determined for each cell type and compared with controls cultured in parallel (Hayflick & Moorhead, 1961). The two different cell types could be distinguished on the basis of the Barr body found only in female cells. The results indicated the old male cells senesced at the same PDL as the control cells, and the young female cells grew vigorously until they also senesced, at the same PDL as their own control. These experiments established that culture conditions did not contribute to the senescence of normal cells in culture. They also ruled out the presence of a soluble mediator which induces senescence in a culture of cells. Furthermore, cells which were frozen in liquid nitrogen at a particular PDL "remembered" at what level they were frozen and resumed their growth from that point until senescence at passage 50.

As noted earlier cell age is usually expressed in terms of the number of doublings, passage numbers, or PDL accumulated. The question of how cells "mark time" is of great importance. There is still some controversy regarding this question, however it has been rather convincingly answered by Dell'Orco et al., (1973). They kept cells in a non-dividing state for varying periods of time, ranging from 21 to 177 days, by serum deprivation. After readdition of serum the cells began to grow again and senesced at the same PDL as controls which were not deprived of serum. The similarity in PDL at senescence between controls and experimental cultures, despite very marked differences in chronological time, indicates that chronological time is not as important as division events.

Hayflick's classical experiments indicated that the regulation of senescence was intrinsic to the cell and precisely timed.

In Vivo Transplantation. Despite an intrinsic timing mechanism which appears to exist in cells; the question of relevance to *in vivo* aging still remained. This question has been addressed using a variety of experimental approaches (Daniel, 1977), several of which will be discussed. One approach has been to follow the fate of a variety of cell types after they are allowed to proliferate in vivo. It is clear that most organs and tissues of the body do not have the capacity for continued self renewal, since such organs would then be able to function indefinitely and the host animal would also live indefinitely long. The somatic cells that comprise these tissues and organs obviously die when the animal dies, so following the fate of injected cells in one host may not be feasible. This problem can be circumvented by serially transplanting marked cells in a series of young animals, thereby creating the in vivo counterpart to the in vitro experiment. The prediction would be that these marked cells would also senesce after some definite period of transplantation. Such studies are difficult to carry out and interpret because of all the uncontrolled variables and the fact that usually the phenotype rather than a specific cell is followed.

Transplantation experiments have been performed using a variety of tissues such as mammary tissue, skin cells, bone marrow cells, and antibody producing cells (Daniel, 1977). The results of all these experiments, though controversial and subject to various interpretations, indicate that cells can not be serially passaged in vivo for indefinite periods of time. The length of time required to reach the limit varies with the cells used and the experimental protocol. Furthermore, since individual cells cannot be observed the actual number of doublings the various cells accumulate can not be measured. Harrison (1972; 1973; 1978) devised an elegant experiment in which marrow cells from young and old donors were transplanted into genetically anemic mice. The ability of the donor marrow cells to grow and produce erythrocytes and cure the anemia was followed for successive transfers of donor cells. The marrow cells were able to continue to cure anemia for as long as 73 months; with no apparent difference between young and old cells. This length of time is much longer than the lifespan of one mouse, and although it can not be determined precisely, many cell doublings must have occurred. Futhermore the numbers of colony forming units of erythroid cells were also found to decline with successive transplants; supporting the view that replicative capacity was being reduced. It must be emphasized that no cells were able to cure the anemia indefinitely. These studies were supported by the observation that the number of colony forming units of stem cells serially passaged in irradiated mice were also found to decline progressively (Siminovitch et al., 1964).

Williamson and Askonas (1972) followed the antibody production of a clone of spleen cells serially passaged in syngenic mice. The production of a highly specific antibody, as measured by isoelectric focusing, was found to decline progressively with time. The production of antibody was proportional to the number of producing cells, suggesting the loss of antibody was due to a decline in the proliferative capacity of secreting cells. In fact the proliferative capacity of this clone was calculated to be about 90 cell doublings.

These results using hemopoietic cells must be interpreted cautiously since a mass population of cells is being followed indirectly, and many assumptions must be made. For example, how many times are the stem cells actually dividing during transplantation? The apparent discrepancy in the lifespan of the cells curing anemia and those producing antibody may arise because Harrison allowed his transplants to remain in the host a long time; while Williamson and Askonas observed aging after several short interval transplants. The rapid transplants may have forced the stem cells to divide, whereas they may have become quiescent in the other experiments.

These experiments support the view that individual cells have a limited proliferative capacity even when passaged in vivo. Another feature of in vitro aging can also be seen in vivo; namely the inverse relationship between donor age and lifespan in culture. Krohn (1962) found that skin transplants from old mice deteriorated faster than skin transplants from young mice. This indicates the fibroblasts were not capable of repopulating this graft after tranplantation.

The other feature common to in vitro and in vivo studies is the spontaneous appearance of transformed cells capable of indefinite serial passage. Till et al. (1964) islolated several clones of cells which could be serially passaged indefinitely which along with other histological alterations indicated these cells had become transformed.

Relationship of In Vitro and In Vivo Aging. These in vivo experiments support the view that in vitro cellular senescence is not an artifact and does, in fact have some relevance to in vivo aging. A thorough discussion of this relationship is beyond the scope of this review; however several key points can be made. It is unlikely the tissues and organs of the body age because their cells run out of doubling potential (Hayflick, 1979). Although the individual cells do have a limited lifespan; this limit, seen in vitro, is probably never reached in vivo. Tissues are typically made up of stem cells resting or dividing, large numbers of mature and immature differentiated cells, old cells, and even dying cells. The physiological integrity of tissues in young animals is a reflection of the ability of the stem cells to replenish any cell loss. As time goes on, the growth rate of these stem cells declines, and they become less able to replenish the cell loss which is continually occurring (Walton, 1982). In fact the rate of cell loss may accelerate with age due to the accumulation of damaging agents. The result of these changes is the appearance of alterations, characteristic of old age, in the structure and function of tissues. This loss of mitotic activity in stem cells varies from tissue to tissue; as a result different tissues show different rates of decline and loss of function. The loss of function of key organs such as the thymus or various other endocrine tissues contributes greatly to the other age-related changes which are manifest in the function of many different organs, ultimately leading to death of the whole animal (Makinodan, 1977).

If in vitro aging is a reflection of in vivo aging it should be possible to show a decrement in proliferative potential in fibroblasts from old donors, as compared to fibroblasts from young donors. An early study showed that fetal fibroblasts were able to acheive a higher PDL than adult skin fibroblasts (Hayflick, 1965). Martin et al. (1970) in a sample of about one hundred people found that the in vitro proliferative potential of skin fibroblasts obtained from older donors was lower than fibroblasts obtained from younger people. Despite the large degree of variance in the data they found a statistically significant decline of 0.2 PD per year. Although some have argued against their conclusion (Strehler, 1977); their results have been confirmed in a larger study (Schneider and Mitsui, 1976). Several other parameters of replication have been determined and are summarized in Figure III.

Taken together these results indicate replication potential and lifespan are reduced in fibroblasts obtained from old subjects as compared to those taken from young subjects.

Accelerated Aging Syndromes. There are two diseases, progeria and Werner's syndrome, which are characterized as accelerated aging syndromes. Clinically affected individuals with progeria, who are less than ten years of age, show many of the physiological and physical changes that are normally seen in adults in their seventies. These include graying hair, atherosclerosis, calcification of blood vessels and decelerated growth (Hayflick, 1977). Likewise Werner's syndrome is also characterized by symptoms normally seen in older people, although these changes occur at a later stage in life than in progeria. Interestingly the *in vitro* lifespan of fibroblasts taken from patients with these syndromes showed very marked reductions in lifespan compared to normal controls grown under identical conditions (Martin et al., 1970; Goldstein et al., 1969).

The conclusion of these studies points to the fact that limited lifespan as expressed by cells grown in culture is not an artifact of growth in an artificial environment; rather it is related to aging and senescence *in vivo*. This suggests that the mechanisms and features of *in vitro* aging may be similar to, or at least bears some resemblance to the process which occurs *in vivo*. Therefore although not perfect,

Figure III. Determination of *in vitro* "aging" indices on skin fibroblast cultures derived from young and old human donors and W1-38 cell cultures at early and late *in vitro* passage (taken from Schneider & Mitsui, 1976).

| Replication parameter | Young subjects (20–35 years) | | | Old subjects (65+ years) | | |
|---|---|---|---|---|---|---|
| Onset of senescent phase (PD)[b] | 35.2 | ± 2.1 | (23)[c] | 22.5 | ± 1.7 | (21) |
| *In vitro* life span (PD) | 44.6 | ± 2.5 | (23) | 33.6 | ± 2.1 | (21) |
| Cell population replication rate (hours) | 20.8 | ± 0.8 | (18) | 24.3 | ± 0.9 | (18) |
| Percentge replicating cells[d] | 87.1 | ± 1.6 | (7) | 79.6 | ± 2.5 | (7) |
| Cell number at confluency ($\times 10^4$ cells/cm$^2$) | 7.31 | ± 0.42 | (18) | 5.06 | ± 0.52 | (18) |
| Percentage of cells able to form colony of 16 cells[e] | 69.0 | ± 3.3 | (9) | 48.0 | ± 4.4 | (8) |
| Sister chromatid exchanges/cell[f] | 67.9 | ± 1.6 | (7) | 56.1 | ± 1.4 | (6) |

[a] The results of these studies originally were published in Danner *et al.* (1978), Schneider and Gilman (1978), Schneider and Mitsui (1976) and Smith *et al.* (1978).

[b] PD, Population doublings.

[c] Numbers within parentheses indicate number of cell cultures examined; values are mean ± standard error of the mean.

[d] Determined by incubating cells for 24 hours with tritiated thymidine and then measuring the frequency of labeled nuclei by autoradiography.

[e] Two weeks after plating at low cell densities.

[f] Cell cultures were incubated for 48 hours with 7.5 ng/ml mitomycin C. Fifteen cells from each culture were analyzed for SCE.

this is a useful model system in which to study the complex phenomenon of cellular aging.

Characterization of In Vitro Aging

Enzyme Changes. As mentioned previously, it is unlikely that age-related changes in the tissues and organs of whole animals are due exclusively to the loss of replicative potential in the cells of these tissues and organs. However, many changes occur in the metabolic and physiological functions of normal cells in culture as they approach senescence and while they are still growing. These changes could lead to some of the major changes seen in cells and cell populations of aging animals. A great many recent studies on *in vitro* aging have focussed on the changes in cellular metabolism that occur as normal cells age in culture.

Hayflick (1980) provides an exhaustive list including some two hundred different properties that have been found to increase, decrease, or remain constant as normal cells approach senescence. These range from increases in enzyme activities, RNA content, lipids, cell size, glycogen content, total protein, and cell cycle time, to decreases in enzyme activities related to DNA and RNA synthesis, the number of proliferating cells, and numbers of mitochondria, and to properties which remain constant such as the level of respiratory enzymes, virus susceptibility, and levels of cAMP. What all these changes mean and how they relate to aging is not clear at this time. Most of these studies are still at the stage of phenomenology, merely describing changes; what is needed now is a mechanistic approach to determine if the changes are the result of senescence or the cause of it (Adelman, 1977).

Some very specific age-related changes in enzymes and proteins have been identified as fibroblasts age (Gershon et al., 1979). For instance, there is a loss of catalytic activity per antigenic unit in enzymes such as liver aldolase, superoxide dismutase, catalase, tyrosine aminotransferase, and lactic dehydrogenase. The losses in activity range from 30 - 70 % and do not appear to be the result of transcriptional or translational modification since no altered protein could be identified on the basis of charge differences. This suggests that some process of post-translational modification, such as sulfhydryl or methionine oxidation, leads to the production of inactive forms of these enzymes. Holliday and Tarrant (1972) showed an increase in the proportion of heat labile glucose-6 phosphate dehydrogenase (G6PD) in senescing normal human diploid MRC-5 fetal lung cells. Many abnormal proteins, including mutant human G6PD, are thermolabile. Others using more purified preparations have not detected any changes in heat stability (Khan et al., 1977; Pendergrass et al., 1976); moreover extracts from aging cells when added to purified G6PD also cause a difference in heat lability. This suggests that post translational modifications have altered the properties of the enzyme (Rothstein, 1975). Duncan et al., (1977) observed that differences in heat lability were due to shifts in the quaternary structure of G6PD from dimer to tetramer.

Population Heterogeneity. As we have discussed earlier a great many changes occur as normal diploid fibroblasts are grown in culture and approach senescence. Since senescence is a highly predictable and consistent phenomenon found in mass cultures of normal cells, it is important to determine whether all the cells in the population behave the same. That is, do they all age at the same rate and with the same mechanism? Conversely, such populations could be heterogenous; composed of cells with differing proliferative capacity some high and others low. The end result would be that the appearance of senescence in mass cultures reflects the average lifespan of each of the various cells of the population. The answer to this question should yield important clues as to the underlying mechanism(s) of aging. This problem has been addressed in several different ways.

Human diploid cell strains were cloned from mass populations at various PDL (Merz & Ross, 1969; Smith & Hayflick, 1974; Smith & Whitney, 1980). The PDL achieved was then determined for each of the several hundred clones isolated. Surprisingly, a large number of clones from mass cultures as low as PDL 8 were capable of only 8 or less further doublings. The fraction of clones capable of greater than 8 doublings fell from 50% at PDL 9 to only 5% at PDL 32. The mass culture grew to PDL 53. Interestingly, a high percentage of clones (about 20%) were capable of exceeding the number of population doublings observed in the mass culture, with the highest PDL observed some 30% greater than that of the mass culture. It has also been well-established that cloning efficiency varies dramatically in different populations of HDF. Moreover, there exists a great heterogeneity of colony size, suggesting that individual cells of a population have differing growth potential. In fact, colony size distribution is highly predictable and has been used as a measure of proliferative potential remaining (Smith et al., 1978). This high degree of heterogeneity must be taken into account when developing theories relating to mechanisms of aging. For instance, are the mechanisms which cause individual cells to stop dividing even in "young" cultures the same as those which cause the entire culture to die?

In an attempt to understand the basis for this heterogeneity, the fate of individual cells was followed using time-lapse cine-microphotography (Absher et al., 1974; Absher & Absher, 1976; Bell et al., 1978) and flow cytometry (Rabinovitch, 1983). These studies showed conclusively that a large degree of heterogeneity exists in the interdivisional times (IDT) of sister cells from young and old cell populations. On average IDT's were longer in cells from old populations. Moreover, non-cycling cells were seen even at early passages, with an increase in frequency in later passage cells. There also seemed to be a class of slowly-dividing cells with very long IDT (Bell et al., 1978); perhaps a class of cells analogous to those quiescent cells in $G_0$ which divide when restimulated with serum.

Since cell proliferation and cell cycle progression are intimately involved with DNA synthesis, several groups have used autoradiographic techniques to follow DNA synthesis in individual cells (Cristofalo & Sharf, 1973; Matsumuro et al., 1979). Cell cultures were incubated with $^3$H-thymidine (TdR) for various periods of time, resulting in the labelling of newly synthesized DNA. The cells were then fixed and exposed autoradiographically. The number of labelled nuclei was about 100% in early passage cells, and declined gradually with increasing passage number, indicating that a lower percentage of cells are in S phase in older cells. Moreover, the time course of $^3$H-TdR labelling in middle PDL fibroblasts identified at least two subpopulations; one which labels quickly and is actively proliferating, and another which labels more slowly and is probably proliferating slowly. Although some controversy exists, there may also be a population of non-dividing cells which do not label even with very long exposure to $^3$H-TdR (Maciera-Coelho, 1974). These DNA synthesis studies correlate very closely to the clonal studies, which when taken together, suggest a bimodal distribution of cells in a mass population; one slow growing with low growth potential and the other actively growing with a high growth potential (Smith & Whitney, 1980). As the mass population approaches senescence, the distribution becomes skewed toward the slowly proliferating pool, until finally there are no actively proliferating cells remaining.

Regulation of Cell Cycle. The animal cell cycle is classically divided into four phases: $G_1$, the time between mitosis and the beginning of DNA synthesis; S, DNA synthesis; $G_2$, the period between S and mitosis; and M, mitosis. The length of the cell cycle in mammalian cells is typically 10-30 hours. Much of the heterogeneity of cell cycle times in cells from various sources and growth environments appears to reside in $G_1$, with S, $G_2$, and M being relatively constant (Pardee et al., 1978). There is, in addition to these four phases, an additional phase, $G_0$, whose actual existence is still the subject of debate (Baserga, 1977). Animal cells can exist in a non-dividing state for very long periods of time by serum deprivation. These quiescent cells can be restimulated to divide by adding serum back to the culture media. For human diploid fibroblasts the length of time between restimulation and the onset of DNA synthesis increases the longer the cells are maintained in the non-dividing state (Augenlicht & Baserga, 1974).

There is a close relationship between DNA synthesis, cell cycle progression and cell proliferation (Pardee et al, 1978). Since the main feature of senescence is the loss of proliferative capacity, it may well be that the factors which control the cell cycle may also be involved in establishing the non-growing state characteristic of senescence.

One approach to understanding the mechanism which controls the cell cycle has been to fuse cells which are at different stages of their cell cycle. For instance, Hela cells in S phase can be fused to Hela cells in $G_1$. Such studies conclude that entry into S phase is positively controlled; since S phase cells can induce DNA synthesis in $G_1$ phase cells but not $G_2$. Cells in $G_2$ cannot stimulate DNA synthesis in $G_1$ cells (Rao & Johnson, 1970). Moreover, this activation is present throughout S phase. When early, middle, and late $G_1$ Hela cells were fused to one another in varying combinations, DNA synthesis was prematurely initiated, with late phase cells providing the most stimulation and early phase the least (Rao et al., 1978). This suggests that some inducer accumulates, as the cells traverse $G_1$, to some critical threshhold level prior to S phase. Recently, efforts have been made to charaterize the nature of such an inducer.

In an elegant series of experiments, Pardee's group has shown that a complex interaction between serum growth factors and nutrients and specific cellular receptors induces factors which are required for the initiation of DNA synthesis, resulting in commitment to undergo DNA synthesis (Pardee et al., 1978; Croy & Pardee, 1983). Once the cell has reached this commitment point called a restriction (R) point, the rest of the cell cycle is unaffected by growth condition (Pardee, 1974). Very recently, Croy and Pardee (1983) have provided evidence that commitment at R is dependent on a labile 68,000 MW protein whose synthesis can be blocked by serum deprivation or protein synthesis inhibitors. Transformed cells contain either a more stable protein or elevated levels of this protein, and are therefore not as sensitive to poor growth conditions or protein synthesis inhibitors. Interestingly, chemically transformed and RNA transformed mouse fibroblasts contained either elevated levels of the labile protein, or more stable protein, or both; while DNA virally transformed cells did not have higher levels of labile protein. This suggests the regulation of growth control in DNA virus transformed cells is different from other transformed cells, perhaps involving the expression of T antigen (Tijan et al., 1978).

Although the labile protein studies and cell fusion studies indicate that cell cycle regulation is positively controlled (Ord, 1979), the question of whether such regulation is positively or negatively controlled is still the matter of debate (De Pamphilis & Wasserman, 1980).

Regulation of Senescence. In an effort to learn the nature of the controlling elements in senescence, cell fusions between senescent and young replicating HDF were carried out (Norwood et al., 1974).

These studies showed that DNA synthesis was not initiated in the senescent nuclei; in fact, DNA synthesis was inhibited in the young nuclei. This suggests that an inhibitor was present in the senescent cells. Conversely, fusion between Hela or $SV_{40}$-transformed human cells and senescent cells did result in activation of the senescent nuclei (Norwood et al., 1975).

These studies have been extended to include fusion between senescent or quiescent cells and a variety of tumor derived, chemically transformed, radiation transformed, RNA virus transformed, and DNA virus transformed cell lines (Stein et al., 1982; Stein & Yanishevsky, 1979; 1980; 1981). Interestingly, only the DNA virus transformed cells and Hela cells were capable of reinitiating DNA synthesis in senescent cells. The nature of the transformation that resulted in Hela cells is unknown, but these studies imply that Hela cells may have been transformed by DNA viruses since all other tumor cell lines tested, including other cervical carcinoma cells, were incapable of inducing DNA synthesis in senescent and quiescent nuclei. Fusion studies between young and senescent HDF indicate that cells already in S phase can proceed through the cell cycle; only the initiation is prevented. It is also interesting to note that quiescent cells behave in exactly the same fashion as senescent cells in their ability to prevent DNA synthesis in heterokaryons between quiescent and young cells (Rabinovitch & Norwood, 1980) or quiescent cells, and the variety of neoplastic lines mentioned above (Stein & Yanishevsky, 1981).

Taken together, these results suggest that:

1. senescent and quiescent HDF contain a diffusible inhibitor which prevents entry into S phase;
2. cells transformed by chemicals, or radiation, or RNA virus are still sensitive to the inhibitor but have lost the ability to express it; and
3. cells transformed by DNA viruses can overcome the inhibitor with a dominant transforming factor (Stein et al., 1982).

The inhibition of DNA synthesis could also be overcome by: a) a protein which inactivates the inhibitor, or b) synthesis of an altered or new positive regulator acting in late $G_1$ which is insensitive to the inhibitor (Rabinovitch & Norwood, 1980). Stein et al. (1982) have proposed that this inhibitor would be synthesized or activated when normal cells experience poor growth conditions such as serum deprivation. This inhibitor would establish and maintain a state of viable quiescence in these cells. They also propose that HDF are less able to utilize growth factors as they progress toward senescence, a proposition supported by Cristofalo & Rosener (1979). At some critical point when the cells can no longer use the growth factors in the medium, they become physiologically serum deprived, even in the presence of serum; and according to the model the putative inhibitor induces a viable quiescent state. The difference is that normally, this cell cannot be rescued from this state as a true quiescent cell can.

These proposals, while explaining many features of senescence and quiescence, do not, in their present form, account for the heterogenous nature of senescing populations of cells. They assume the population is more or less homogenous with respect to growth potential. Moreover, the data does not rule out a model where an inducer of DNA synthesis is produced by the actively proliferating cells and is absent in senescent cells. Cell fusion would effectively dilute such an inducer below a critical threshold needed for DNA synthesis. The dominant cell lines capable of initiating DNA synthesis would presumably contain elevated levels of inducer (or some other dominant factor) and overcome the dilution upon fusion, thereby inducing DNA synthesis in the senescent nuclei. Pardee's labile protein (p68) could, in fact, be such as inducer, produced normally in proliferating cells. However, in senescent cells, the complex regulating signals and controlling elements are no longer responsive to growth conditions such as mitogens in the serum. It would follow, therefore, that the labile protein cannot be produced and no initiation of DNA synthesis would occur. Moreover, in hybrids, dilution due to larger cell volume would reduce the level of p68 below that needed for initiation. The dominant cell lines would contain some dominant transforming factor, or a p68 which is insensitive to poor growth conditions and dilution. Therefore, DNA synthesis could be initiated in hybrids.

The inhibitor hypothesis is consistent with previously observed growth similarities between normal cells, carcinogen transformed, and RNA virus transformed cells. Normal cell strains subjected to poor growth conditions such as serum deprivation, $Ca^{++}$ deprivation, high cell density, and inhibitors of protein synthesis with cycloheximide arrest in the $G_1$ phase of the cell cycle (Pardee et al., 1978). Carcinogen and RNA virus transformed cells also accumulate in $G_1$ when grown under the conditions listed above, although the block is not as stringent and the deprivation must be more extreme (Dubrow et al., 1979; Fernandez Pol et al., 1979; Paul & Ristow, 1979; Medrano & Pardee, 1980). This suggests these transformed cells retain some of the normal mechanisms which cause the cells to stop proliferating under restrictive growth conditions.

Cells transformed by DNA viruses apparently do not accumulate in $G_1$, under restrictive conditions; rather they cycle very slowly or die (Medrano & Pardee, 1980; Paul et al., 1974; Bartholmew et al., 1976; Schiaffonati & Baserga, 1977). Under very restrictive conditions DNA virus transformed lines were unable to enter a quiescent state because either no inhibitor was produced; or a positively controlled element was constitutively produced and therefore insensitive to restrictive conditions.

As noted earlier, many experiments suggest that some gene product(s) are required at some point R in late $G_1$ in order for the cell to initiate DNA synthesis and traverse S phase (Croy & Pardee, 1983). The nature of this gene product is not known, however, replication factors and enzymes are known to be assembled in late $G_1$. If the level of the replication enzymes or activators declined for some reason below a critical threshold level, either because of a change in the rate of synthesis or the presence of an inhibitor, then DNA synthesis would not begin. As will be discussed later in more detail, the DNA replication enzymes appear to exist in a multienzyme complex called a replitase, and may play a key role in the regulation of DNA synthesis (Reddy & Pardee, 1980; Kornberg, 1980). Recently, two of the enzymes of the replitase have been investigated with regard to senescence and transformation; DNA polymerase α (Pendergrass et al., 1982), and ribonucleotide reductase (Dick & Wright, 1982, 1983). The levels of both these enzymes decline in senescent cells to about one-half the amount of enzyme found in young proliferating normal HDF. Ribonucleotide reductase levels declined progressively with increased PDL, while polymerase α was only assayed in young and old cells. To further test the "threshold hypothesis" the levels of DNA polymerase α were measured in a variety of normal human cell strains at early and late PDL, tumor cell lines from various sources, and permanent rodent cell lines. The levels of DNA polymerase α were found to correlate very closely with the transformed cell line's ability to "rescue" or initiate DNA synthesis in senescent HDF. Cell lines containing less than a critical level of enzyme activity were unable to rescue senescent nuclei, while those cell lines with higher levels could initiate DNA synthesis. Interestingly, the level of the polymerase was found to be somewhat unstable since some recessive lines became dominant after a period of subculture. These derivative cell lines were subsequently found to contain higher levels of enzyme. Moreover, an estimate of the minimum level of DNA polymerase α needed to initiate DNA synthesis in heterokaryons was calculated and found to be very close to the level of polymerase activity in near senescent HDF. This low level of activity may be insufficient for initiation of DNA synthesis resulting in the characteristic non-dividing state of senescence. Interestingly, Burmer et al. (1983) found that cybrids between young HDF and cytoplasts from senescent cells were also incapable of entering S phase. Moreover, cybrids between young HDF and cytoplasts from young cells were able to enter S phase. Although these experiments support the inhibitor hypothesis, they do not rule out the threshold hypothesis since the level of replication enzymes in the senescent cytoplast may be very low compared to the levels in young cytoplasts.

The mechanism which causes the decline in DNA polymerase $\alpha$ or ribonucleotide reductase activity is not known. It may be that an inhibitor is produced by senescent cells which is subsequently diluted by the excess of replication factors in the dominant transformed cell lines. The level of replication factors in the recessive cells would not be high enough to dilute such an inhibitor. Naturally occurring inhibitors of both these enzymes can, in fact, be isolated in mammalian cells (Cory, 1983; Varshavsky, 1983).

There may not be any need to postulate the presence of an inhibitor since a decline in the rate of synthesis, at the level of gene expression would result in lower level of DNA polymerase $\alpha$ and ribonucleotide reductase. Therefore, any mechanism which involves regulation of expression of genes (activators, repressors, or methylation) could control quiescence and senescence; while a loss of regulation would result in unlimited growth.

Dominance of Senescence in Hybrids. The biochemical approach of following the ability to initiate DNA synthesis has been extended to determine the replicative potential of such hybrids. The use of somatic cell hybridization to determine the dominant or recessive nature of transformation and tumorgenicity has provided very controversial results. Some transformed phenotypes have been suppressed in some hybrids (Harris, 1979; Stanbridge et al., 1982) and expressed in others (Croce, 1980). It has been suggested these studies selected for rapidly proliferating cells and did not take into account the many hybrids which failed to proliferate (Pereira - Smith & Smith, 1983). Several groups have carefully followed many hybrid cells to determine the lifespan of individual clones and found that hybrids between immortal transformed cell lines and normal mortal cell strains yielded hybrids with a finite division potential (Muggleton - Harris & DeSimone, 1980; Pereira - Smith & Smith, 1981, 1983). Immortal variant cells appeared in the populations of non-proliferating senescent hybrid cells at a frequency of about $10^{-5}$ suggesting this is a rare event (Pereira - Smith & Smith, 1981). Since immmortality is therefore recessive, hybrids were formed between the tumor lines of different origins, to determine if complementation could occur. SV-40 derived cells and tumor-derived cells could complement each other, and produce hybrids with limited lifespan. This indicates at least two different alterations in the normal program of senescence can lead to unlimited growth, and these changes represent at least two independent complementation groups (Pereira - Smith & Smith, 1983).

As we will discuss later, these latter results strongly argue against the error or mutational hypothesis and favor models involving changes in gene expressions and regulation as the mechanism of senescence. The reason for this is that error and mutational theories would predict that fusions between immortal cells lines should only yield immortal cell lines. Furthermore the heterokaryon data discussed earlier in this review also strongly supports the view that the control of sencescence, quiescence, and unlimited growth is intimately associated with DNA synthesis and cell cycle regulation.

Aging Theories

Research directed specifically toward age-related problems has really arisen only in the last twenty years; gerontology is, therefore, a relatively young science. As a result there is still a real lack of fundamental data around which to form a unified theory of aging. Age-related biological changes can be seen in almost all systems from the molecular level up to the whole animal. The problem lies in determining whether these changes are causal or only secondary to another more fundamental alteration. There are as a result many different theories of aging, each based on a particular change with little evidence supporting the causal nature of such an alternative.

Although there are many variations and overlaps; aging theories can be divided into two general groupings: those based on extrinsic causes and those based on intrinsic causes. As we shall see these groupings are somewhat artificial since some theories encompass aspects common to both. It should be emphasized that there is probably no single cause of aging in the same way there is no single mechanism of differentiation or cancer. These are extremely complex processes which are likely to be manifest in a variety of different mechanisms. Most aging theories, but not all, center around changes in the stability of the genetic material as causes of aging.

An examination of the maximal lifespans of a great many species shows that the variation in the lifespan within the species is less than the variation between species (Comfort, 1979). For instance, the maximum recorded lifespan of a mouse is about 3 years, man about 100 years, and the tortoises greater than 150 years (Comfort, 1979). The influence of the genetic constitution on lifespan is also reflected in the positive correlation between the longevity of parents and their offspring (Sacher, 1977). The variation in the lifespan of monozygotic twins is less than the variation in lifespan between dizygotic twins (Moment, 1979). Furthermore, it has long been evident that the lifespan of males is markedly shorter than females; clearly a genetic trait. These observations all serve to strengthen the view that the genetic constitution plays an important role in determining the maximum lifespan of a particular species.

Extrinsic Theories of Aging. One of the earliest extrinsic theories of aging revolves around the premise that aging is the result of a progressive accumulation of errors in information containing molecules. This can be further divided into the somatic mutation hypothesis and the error catastrophe hypothesis. These two hypotheses, because of the testable nature of their predictions, are probably the most controversial and the most tested of all the various proposals which have been put forward (Hayflick, 1977).

Orgel (1963, 1970) hypothesized that the various components of the protein synthetic apparatus either in RNA synthesis, in tRNA charging, or in codon recognition are subject to a small degree of error. Therefore, at a certain frequency, proteins with some errors will occur. Errors could also occur in the proteins which comprise the protein synthetic machinery resulting in more proteins with errors, creating a cascade of errors that ultimately leads to complete loss of function, and death of the cell. The hypothesis assumes that errors would diverge in an exponential fashion to catastrophe; however this need not be the case, rather a steady state could also occur with no subsequent catastrophe. Orgel (1973), acknowledging this possibility, altered his proposal to include positive feedback in the accumulation of errors in any of the macromolecular constituents of the cell, including DNA. At this point the error catastrophe theory naturally dovetails into the somatic mutation theory (Medvedev, 1972; Burnet, 1974). This theory predicts that mutations accumulate in the DNA to the point where the cell cannot tolerate the load of errors in the DNA and the protein products, causing cell death. There could also be an increase in intrinsically produced mutations due to alterations in the proteins which synthesize DNA or those involved in DNA repair. This could lead to more errors in protein, then DNA, then protein and so on until the cell ceases to function. There are several major predictions of these error/mutation theories:

1. many abnormal proteins accumulate with age;
2. the number of mutations or the mutation rate should increase with age, ie., cells become more susceptable to mutation;
3. mutagens should reduce lifespan;
4. the fidelity of DNA synthesis should decline with age;
5. there should be alterations in DNA repair with age;
6. there should be alterations in scavenger proteases, activation enzymes for premutagens, and inactivation enzymes for mutagens.

One of the most tested predictions of these theories has been the matter of altered proteins. Holliday has been the chief supporter of the altered protein theories, basing many of his conclusions on changes in the level of heat labile G6PD (Holliday & Tarrant, 1972; Holliday, 1975), which is assumed to be a mutant form of G6PD. As previously indicated others have suggested these may not be mutational changes in G6PD, rather the result of post-translational modification (Morrow & Garner, 1979). Gershon and Gershon (1976) have characterized a variety of post-translational modifications which cause a decrease in the specific activity of purified enzymes. Moreover Pendergrass et al. (1976) failed to show any thermolabile changes in G6PD from their aging cell system. The error catastrophe theory predicts errors in many if not all proteins of the cell; however this is clearly not the case. In fact most proteins are unaltered. Proteins from senescent and proliferating cells have been analyzed using two-dimensional gel electrophoresis. No alterations were found in the protein separated in this way (Wilson et al., 1978).

Further compelling evidence against the error theory comes from experiments using viral probes of aging (Tomkins et al., 1974; Holland et al., 1973). Various viruses such as Herpes, VSV, and polio virus were used to infect early and late passage cells. There was no difference in viral yield between young and old cultures. Moreover, there was also no increase in the production of mutant viruses between these young and old cell strains. These viruses all use the host cellular machinery to synthesize their proteins and any error-prone or altered synthetic apparatus would result in inaccurate assembly of viral particles. Since mature viable viruses can not be assembled unless there is a high degree of accuracy in the proteins, reduced yield, or the appearance of mutant virus would be seen if the host was error-prone.

Cell-free or intact cell model systems have failed to reveal any difference in the fidelity of protein synthesis in low and high passage cells, or cells from old donors (Wojtyk & Goldstein, 1980; Goldstein et al., 1979). These studies were based on misincorporation of amino acids in a synthetic polyphenylalanine peptide, or infidelity of amino acid incorporation into actin using intact cells. The error frequency was in fact higher in $SV_{40}$ transformed cells in contradiction to the error hypothesis which would predict a decrease in errors in transformed cells (Wojtyk & Goldstein, 1980; 1982).

In a direct test of the error theory, cells were grown in the presence of nontoxic concentrations of amino acid analogues. No reduction in lifespan was observed, despite the presence of high levels of protein with altered amino acids, indicating that lifespan is not dependent on abnormal protein (Ryan et al., 1974). Using other non-mammalian systems, such as Drosophila, Harrison and Holliday (1967) fed amino acid analogues to Drosophila larvae and found a reduced lifespan in the resulting adults, although others found no effect feeding analogues to young adults ( Comfort, 1979 ).

). More studies will be needed to resolve these discrepancies.

At present, many apparently contradictory conclusions are found in the literature regarding altered proteins as a result of infidelity in protein synthesis or transcription. It is not possible at present to determine why this is so, except that much enzyme work is done in crude systems; interpretation regarding kinetics and activity must therefore be subject to caution. Moreover, much of the alteration seen in various proteins is undoubtedly the result of post-translational modification; and probably of a secondary nature vis a vis its role in aging. Although some evidence supports it, the weight of evidence bears against the error hypothesis.

The somatic mutation theory of aging has a certain intuitive appeal as a method of aging since aging, lifespan, and genetics are so closely linked. The havoc radiation plays with DNA is well-established, and its role in disease processes such as cancer is also well-known. Much work regarding this theory is, as previously mentioned, somewhat conflicting with the bulk of evidence against any simple version of this theory (Strehler, 1977; Martin, 1977; Morrow & Garner, 1979).

If mutations are responsible for aging, the mutation rate must change with time since aging is an exponential function. Moreover, the mutations, as they accumulate, must not retard the growth rate or the cells would be lost due to selection (Holliday, 1975). The mutation rate would also need to be very high, at least according to some models, approaching one per cell per generation (Holliday, 1975). The mutation rate has been estimated for several markers, recessive and dominant in human fibroblasts grown in culture (Fulder, 1979; Gupta, 1980). Fulder (1979) found that the frequency of G6PD mutants increased as cells aged, while Gupta found no difference in resistance to 6-thioguanine or diptheria toxin. It is important to note that the experiments by Gupta were based on the characteristics of well-established mutants with known mechanisms of alteration;

while there is some doubt as to the nature of the mutation in the experiments of Fulder (Martin, 1977).

The mutation rate in humans has recently been estimated using a novel approach to select for 6-thioguanine (6-Tg) or 8-azaguanine (8-Ag) resistant peripheral lymphocytes (Morley et al., 1982; Evans & Vijayolaxmi, 1981; Strauss & Abertini, 1979). The assay is based on the ability of cells, resistant to 6-Tg or 8-Ag because of an altered HGPRT enzyme locus, to enter DNA synthesis in the presence of the drug. DNA synthesis is measured autoradiographically by the incorporation of $^{3}$H-TdR into nuclei of resistant cells. The results seem to indicate a rise of about 3% per year in the frequency of 6-Tg resistant cells with age; however, one group did not find any age-related changes in frequency (Strauss & Albertini, 1979). Clearly, this could be a powerful technique to ultimately test whether the mutation rate in the cells of the intact organism changes with age.

If somatic mutations are playing a role in the aging process, mutagens should accelerate this process. Human diploid fibroblasts were repeatedly exposed to EMS or MNNG; both potent mutagens, with no shortening of lifespan (Gupta, 1980). In fact, one clone appeared to have its lifespan lengthened by about 10%. An <u>in vivo</u> counterpart to this experiment also did not result in a reduction in the lifespan of mice fed mutagens such as EMS (Curtis, 1966). The life shortening effects of radiation on mammals is well-documented (Comfort, 1979). The data are not as comprehensive in humans. Although there was no apparent alteration in the lifespan of radiologists exposed continuously to low levels of radiation (Comfort 1979), there was a shortening of lifespan of normal HDF grown in culture if exposed to low doses of ionizing radiation (Lima et al., 1972; Maciera-Coelho et al., 1978). Irradiation of high passage cells, however, had no effect on lifespan although extensive chromosomal damage was found (Little, 1976). The results of experiments using ionizing radiation must be interpreted with caution since a great deal of irreparable DNA damage occurs in treated cells. Chemical mutagens on the other hand produce relatively subtle changes of the kind predicted by error/mutational theories. There is much evidence which suggests that biological aging and irradiation life shortening in vivo are in fact, different processes making interpretation of in vitro studies difficult (Sacher, 1977).

When DNA is damaged in some way very efficient repair processes are induced (Lindahl, 1982). It follows that permanent damage to DNA can result if the repair processes decline or if the rate of damage would increase over the rate of repair. Hart and Setlow (1974) found a particularly strong correlation between the longevity of various species and their ability to repair UV induced damage. This excision repair is measured autoradiographically as unscheduled DNA synthesis (UDS) by blocking semi-conservative DNA synthesis with hydroxyurea. Repair has also been measured in HDF at various PDL. These studies, as many others, are inconclusive as some groups show a decrease in repair only in high PDL fibroblasts implying that repair was not a factor in controlling in vitro lifespan (Goldstein, 1971; Painter et al., 1973). Others found repair activity to decrease beginning during the proliferative phase as the cells approach senescence (Hart

& Setlow, 1976). In contrast, Smith and Hanawalt (1976) were unable to measure any differences in repair between growing and confluent, early and late passage, or normal and $SV_{40}$ transformed W138 cells. The rate of repair is reduced in post-mitotic muscle cells suggesting the decline in repair capability may be most detrimental to post-mitotic cells (Lampidis & Schaiberger, 1975). It would be very important to know whether inhibition of DNA repair would result in a decreased lifespan. In a recent preliminary report, caffeine, an inhibitor of repair, caused a marked reduction in the lifespan of normal mouse fibroblasts while having no effect on permanent fibroblasts (Icard & Beaupin, 1980).

Despite some inconsistency in different experiments, the ability to repair damaged DNA probably does diminish with increased PDL. While this may not be the cause of the loss of proliferative capacity; it may play a significant role in the loss of function in senescent post mitotic cells.

Alterations in the activity of enzymes capable of either activating or inactivating premutagens and mutagens, respectively, were inversely correlated to species lifespan (Schwartz, 1975; Schwartz & Moore, 1979). Dimethyl-benzanthracene (DMBA), a premutagen was added to irradiated feeder cells of a specific species. $V_{79}$ hamster cells were plated on the feeder layer as an indicator cell line which could not metabolize DMBA but could be assayed for mutation frequency to 8-azaguanine resistance. The ability of the various cell lines to activate DMBA and increase the mutation rate in $V_{79}$ was found to correlate inversely to the donor species lifespan. This result parallels the results with repair enzymes and supports the view that mutation and rate of repair of mutations contribute in some way to species lifespan.

A loss of fidelity in the enzymes and/or controlling factors of DNA synthesis can also result in an increase in the rate of somatic mutations. Two of the key DNA synthetic enzymes have recently been associated with mutator loci (Meuth, 1981; Chan et al., 1981; Weinberg et al., 1981; Liu et al., 1983). The fidelity of DNA replication is influenced by the relative concentration of "precursor" deoxyribonucleotide pools. Altered regulation of ribonucleotide reductase can result in a large increase in the spontaneous mutation rate at several other drug resistant loci (Chan et al., 1981; Weinberg et al., 1981). Although the increased mutation rate is seen in cell lines containing mutant reductase enzyme which is insensitive to regulation by dATP, the contribution of normal reductase to the spontaneous mutation rate under conditions where the effector pools are altered is not known. It is interesting that the levels of RRase do decline during senescence and may therefore contribute to any increase in mutation rate seen with aging (Dick & Wright, 1982, 1983).

Aphidicolin resistant cell lines containing altered DNA polymerase $\alpha$ have also been isolated. These cells have an elevated mutation rate for several drug resistant loci (Liu et al., 1983). The altered polymerase was shown to have an increase affinity of dCTP and was resistant to aphidicolin. These drug resistant cells in addition to the higher spontaneous mutation rate were very sensitive to UV-induced mutagenisis suggesting that DNA polymerase $\alpha$ may also be involved in error prone UV repair synthesis. Interestingly, DNA polymerase activity also declines dramatically in young and old HDF, supporting the idea that this enzyme may be involved in increasing the mutation rate as cells age (Pendergrass et al., 1982). Linn et al. (1976) found an age-related decrease in the fidelity of DNA polymerase α activity in crude extracts from senescent and low PDL cells. The assay was based on the ability to replicate synthetic templates. Others, however, have failed to see any age-related changes in the fidelity of DNA synthesis (Fry & Weisman-Shomer, 1976), although the activity per cell does decline in high passage cells (Pendergrass et al., 1982). Similarly, <u>in vivo</u> studies show declines in polymerase α activity in bone marrow cells from old mice as compared to those taken from young mice. This probably refects the lower growth rate of stem cells from old animals (Muller et al., 1980).

As we will discuss later, the involvement of DNA polymerase and ribonucleotide reductase in aging can also be explained in terms of a deterministic programmed senscence model.

There is a good deal of controversy surrounding the data which is used to either prove or disprove the error/mutation hypothesis, as seen in this discussion. At present, it is probably fair to say there is considerable information which is compatible with some version of the error hypothesis. There does exist, however, considerable evidence which is incompatible with such hypotheses (Martin, 1977).

Clonal variation experiments have conclusively indicated rapid development of intraclonal variability in doubling potential as the number of cell division increase (Smith & Whitney, 1980). In fact, the division potential of two cells from a single mitotic event can differ by several fold. If mutational mechanism were involved, a very high rate of mutation, approaching one per cell division would have to occur. Asynchronous division would also require unequal partitioning of proteins with errors if the predictions of the error catastrophe hypothesis are correct.

Any theory of senescence must also account for the appearance of cell lines which have escaped senescence and are immortal. These cell lines are subject to the same extrinsic environment as normal cells yet they do not senesce. Error theories predict that immortal cell lines must be relatively error free in protein synthesis; a prediction which has already been shown not to be the case (Goldstein et al., 1979). Furthermore, they must also have lower rates of mutation or more active repair enzymes, both of which are not seen.

The most compelling argument against the error/mutation hypotheses come from experiments where transformed and normal cells were hybridized together. As discussed earlier, such hybrids between HDF and Hela, $SV_{40}$ transformed HDF, or a variety of other transformed cell lines have clearly indicated that immortality or unlimited growth potential is recessive (Bunn & Tarrant, 1980; Muggleton-Harris & DeSimone, 1980; Pereira-Smith & Smith, 1983); hybrid clones all senesced after limited cell division. Variant cells which were immortal occurred in these populations at frequencies of about $10^{-5}$ indicating the appearance of immortality is a relatively rare event. Interestingly, at least two complementation groups could be identified in fusions between tumor cell lines which produced such hybrids which had a limited lifespan. The error/mutational theories predict that fusions between immortal cell lines should yield only immortal clones.

Intrinsic Theories of Aging. The complex changes that occur during embryogenesis and development are under the control of a variety of genetic signals which act at specific times in a specific order. The mechanism by which these developmental genes are turned on and off are programmed into the cellular pool of genetic information. It seems reasonable that changes which occur during aging could also be the result of a specific program being played out in an orderly sequence (Hayflick, 1975). In fact, cell death is a normal part of the developmental changes that occur during embryogenesis (Beaulaton & Lockshin, 1982). Death and resorption of cells are major contributors to the formation of the limbs and the general shape of animals.

A program for aging could involve the switching of specific genes at particular points in time resulting in the slowing down of essential physiological functions, at the end of the program a specific set of genes could be activated to cause the cessation of growth and death of the cell. An analogy would be the simple sequence of events whereby a tape which has run out shuts down the tape recorder. Hayflick (1975) has alternately proposed that senescence is the result of the accumulation of small functional failures which occur in vital systems once the genetic program has run out. The rate at which the failures accumulate determines the lapse in time until death occurs. This time interval, because it is specific and highly predictable, can be viewed as the "mean time to failure". This concept is common in mechanical systems; for instance, a car, where there is a certain degree of deterioration or "noise" in each vital part. As time goes on the noise increases to a threshold above which the system fails. Although the rate of deterioration is random for each component, the system has "mean time to failure" which for a car is about seven to eight years. The same concept can be applied to biological systems such as aging. All biological activities are not completely perfect and can tolerate a certain degree of noise. In this view, then, a cell simply runs out of program and "drifts" toward death, as a result of deterioration, in a highly predictable "mean time to failure". The form in which this theory is presented does not account for the asynchronous growth of cells even at low PDL, although it still may have some validity. This hypothesis implies some overlap between error and program theories since the "mean time to failure" would be influenced by low levels of error inherent in macromolecular synthesis as well as the fidelity of repair processes. Immortal cell lines would not be subject to the "mean time to failure" since their genetic program has been altered or circumvented so their physiologic status does not decline with age.

Population studies on mass cultures support the idea that a very precise timing mechanism exists in normal cells which controls the lifespan of that particular species of cell (Hayflick, 1979). However, the highly asynchronous growth of normal HDF argues against a precise counting mechanism as the sole cause of aging. The bimodal distribution of doubling potential and the great variation in interdivisional doubling times indicates there is a significant stochastic element in the growth of normal HDF (Smith & Lumpkin, 1980). Smith and Lumpkin (1980) have proposed a theory on the premise that some underlying stochastic element, whose exact nature is unknown, is responsible for _in vitro_ aging. The essential features of this theory are:

1. normal cells contain a gene for an inhibitor of DNA synthesis;
2. the expression of this gene is prevented by a repressor;
3. the genes for the repressor are present in multiple copies;
4. the repressor gene copies could be lost by imperfect partioning between sister cells or imperfect replication (the stochastic element);
5. the inhibitor gene is no longer repressed when the level of repressor gene falls below a threshold; and,
6. once the inhibitor is expressed at a sufficient level, positive feedback ensures continued production.

The two main elements of this proposal are:

1. the loss of repressor genes gradually with time resulting in a gradual loss of proliferation potential; and,
2. The sudden loss of repressor genes with a probability of occurence inversely proportional to the number of gene copies present.

The later case would result in a rapid loss of doubling potential, as seen in the bimodal distribution of intraclonal doubling potential.

The presence of an inhibitor of DNA synthesis is consistent with fusion experiments between mortal and immortal lines. The inhibitor could diffuse from the senescent cell to the immortal nuclei and begin the senescence process resulting in the limited growth potential seen in these hybrids (Smith & Whitney, 1983). The heterokaryon studies discussed earlier also predict the presence of an inhibitor which prevents the initiation of DNA synthesis in some transformed nuclei. The dominant cell lines which did initiate DNA synthesis may require a higher level of inhibitor or alternately breakdown the inhibitor. Furthermore, cytoplasts from senescent cells fused to young proliferating cells can prevent the initiation of DNA synthesis in young nuclei. In contrast when cytoplasts from young cells were fused to young cycling cells, no inhibition of DNA synthesis was measured (Burmer et al., 1983). Nuclei from young cells when fused to whole senescent cells result in hybrids that double a few times indicating dominant control of growth potential (Muggleton-Harris & Palumbo, 1979). These experiments are somewhat at variance with each other in that DNA synthesis must be initiated in order for the nuclei/whole cell hybrid to proliferate even a few times. The condition of the experiments are, however, different in that two cytoplasms and two nuclei were fused together in the former study; while in the latter study, the hybrid cell contained two nuclei and one cytoplasm. Nevertheless, these studies both support the idea that senescence is controlled by some inhibitor.

The gene(s) for the postulated inhibitor could be carried as an autosomal dominant or as multiple copies. In order for variant immortal cell lines to arise, both copies of the gene or all the multiple copies must be altered or lost. This would be a very rare event. Cell transformation is indeed a very rare event since there are no documented cases of spontaneous transformation in normal human diploid fibroblasts (Namba et al., 1978). Even with the use of potent carcinogens and mutagens, such as $\gamma$ radiation or 4-nitroquinoline oxide (4NQO), transformants are isolated at very low frequency (Kakunaga, 1978, Namba et al., 1978). It has also recently been shown that normal hamster fibroblasts can not be transformed by the Ha-ras oncogene, while fibroblasts immortalized by chemical carcinogens are readily transformed by this oncogene, indicating stringent growth regulation in normal cells (Newbold & Overell, 1983).

The repressor genes present in multiple copies are postulated to be lost during in vitro growth. Such loss of multiple gene copies with age has been documented for the tRNA genes (Shmookler-Reis & Goldstein, 1980; Johnson & Stehler, 1972). The loss of tandemly repeated genes could occur by unequal sister chromatid exchange, somatic recombination, or by deletion of hair pin loops (Smith & Lumpkin, 1980). The acquisition and loss of multiple copies of genes can best be seen in drug resistant cells which have amplified genes (Stark & Wahl, 1983). The mechanisms which allow gene amplification and loss in these resistant cell lines may be relevant to the processes which normally regulate repetative genes.

Pendergrass et al. (1982) have showed that it may not be necessary to invoke the presence of an inhibitor as the cause of senescence since lowering the level of a replication enzyme or a controlling protein below a critical threshold would also prevent initiation of DNA synthesis. The mechanism of this decline in enzyme activity is not known, but may occur as the result of some stochastic random process. Most cell cycle studies have indicated that regulation of DNA synthesis is under positive control (Ord, 1979; Croy & Pardee, 1983). The polymerase data would, however, support either positive or negative control processes.

The asynchronous growth of normal HDF appears to require a strong stochastic component while the narrow range of maximum population doublings imply that some deterministic mechanism must ultimately control the whole process. A purely stochastic model would predict that previous clonal history would have no effect on the maximum lifespan of subclones. The theory by Smith and Lumpkin (1980) and Shmookler-Reis et al. (1980) suggest aging is best represented by a deterministic model with stochastic elements.

A variation of the deterministic/stochastic model is the view that aging is a form of terminal differentiation (Hayflick, 1975; Martin, 1977; Bell et al., 1978). The process of differentiation could be modelled after the process which is seen in the hemopoietic system. The hemopoietic system consists of cells in three possible states. Firstly, the stem cells with a high proliferative potential; secondly, cells with limited proliferative potential that have begun to differentiate; thirdly, non-replicating fully differentiated cells. This process whereby stem cells enter the differentiated state or remain as proliferative stem cells has been explained in terms of a purely stochastic model (Till et al., 1964). Very heterogenous populations of cells are the result of this type of differentiation process, since the stem cells can divide in an asynchronous fashion (Kay, 1965). On the basis of cellular heterogeneity it can be argued that the loss of proliferative cells is a function of differentiation rather than some intrinsic aging process (Bell et al., 1978). Martin (1977) views the limited replicative lifespan of normal cultured cells as a two-step process. In step one, there is a shift in the proportion of cells from a high proliferative state to a low proliferative state (clonal attenuation) leading to a gradual slowing of growth asociated with differentiation, leading to non-proliferating cells. In step two, actual senescence occurs as a result of deleterious changes in the structure and function of key intracellular molecules. The key stochastic element lies in the change of cells from high to low proliferative states; when all the cells reside in the low state, the culture rapidly ceases to proliferate.

The key feature of differentiation is that its control lies not in irreversible changes in the genome, but at the level of regulation of genes. As such, differentiation can sometimes be thought of as a reversible process. If the correct stimuli are given, silent inactivated genes can be reactivated (Morrow, 1977; Bradley, 1979). The reinitiation of DNA synthesis in senescent nuclei by fusion to transformed cells, or the stimulation of senescent cells to divide with nuclei from actively growing cells are both examples which strongly suggest senescence is the result of a regulatory alteration in gene expression.

Differentiation hypotheses would predict that there should be changes in gene expression of major regulatory processes with aging.

One such system, the phosphorylation-dephosphorylation of proteins is a major regulator of cellular metabolism, protein synthesis and gene expression (Krebs & Beavo, 1979). The activity of a variety of protein kinases have recently been shown to change progressively during in vitro aging (Kahn et al., 1982). The levels of some histone/protamine kinases decreased with aging, while a new histone kinase progressively appeared. The appearance of the new kinase seemed to be due to a change in gene expression rather than posttranslational modification.

Furthermore, the progressive age-related decline in the level of DNA polymerase $\alpha$ (Pendergrass et al., 1982) and ribonucleotide reductase (Dick & Wright, 1982, 1983) may also be the result of changes in gene expression. Changes in these enzymes would directly affect the cells abiity to synthesize DNA.

The control of gene expression is generally a poorly understood area with many different mechanisms of control. Some of these include DNA methylation, histone phosphorylation/acetylation, changes in chromatin structure, and binding proteins to DNA. All these changes are known to occur in aging cells (Whatley & Hill, 1980).

Certain drugs can also affect the differentiation process. Glucocorticoids such as hydrocortisone when added to HDF significantly increase the lifespan of these cells (Cristofalo, 1970). Glucocorticoids altered the rate of entry of cells into DNA synthesis and increased the number of cells in the rapidly proliferating pool (Cristofalo & Rosener, 1979). Such steroids are known to cause changes in gene expression, although the specific mechanism which causes lifespan enhancement is not known.

Conclusion

*In vitro* aging is a complex process which involves many changes in the physiological status of the cell. Many of these changes have been quantified, however the difficulty lies in determining cause and effect. As a result, many of the observed changes are prematurely discussed in terms of that particular effect being causal in nature. This has led to a profusion of hypotheses and concepts regarding the overall mechanism of aging. It is likely, however, that many observed age-related changes accompany other more primary alterations, such as those which cause a slowing or cessation of growth. In order to understand a phenomenon as fundamental as senescence one must understand the very essence of how a cell functions. Clearly, such a process is unlikely to be due to a single change; rather senescence may be the end result of many fundamental alterations. The data which has accumulated appears to support several of the current theories being advocated, especially those involving changes in gene expression. The loss of proliferative capacity after a limited period of time in culture appears to be an intrinsic property of normal cells, the exact nature of which little is known.

*In vitro* aging has many features in common with aging in whole organisms. There is limited evidence that the loss of proliferative capacity of stem cells may contribute to the overall decline in aging in animals (Walton, 1982). Rosen (1978) has cogently argued that individual proliferating cells do not senesce in the normal sense. Senescence is interpreted to mean the increase in the probability of death per unit time with increasing age. He suggests these cells have a constant probability of senescence and only post-mitotic cells can be considered to truly senesce. This is similar to Martin's two stage hypothesis. However, the same phenomena which cause the loss of proliferative potential may in fact be the same as those which cause post-mitotic cells to senesce (Hayflick, 1979).

Aging *in vivo* is probably several orders of magnitude harder to understand than aging *in vitro* since one is dealing with complex populations of cells which interact with each other in a network. An understanding of the individual elements, although important, is not sufficient since very slight changes in the many network interactions, without significantly affecting the individual elements could lead to a system failure (Rosen, 1978). This type of deterioration is commonly seen in complex computer networks where the whole system can fail because of trivial changes in the connections between computers.

Burnet (1974) has hypothesized such network changes in the immune system which is a complex array of interactions where changes in one component could lead to many subtle changes in the way each of the elements recognize each other. This breakdown of surveillance could lead to a loss of recognition of self, with subsequent damage to the whole organism. It is very difficult to study networks since conventional approaches tend to destroy networks and study the individual components.

In summary, it is important to understand the age-related changes which occur in individual cells using model systems like *in vitro* aging of HDF. Such studies yield information on how the individual elements of a complex organism behave during aging. In order to understand the whole process of aging at the level of the whole organism, a systems approach must eventually be taken into consideration.

## B. Somatic Cell Genetics of Normal Human Diploid Fibroblasts

### Introduction

The study of genetics has progressed rapidly in the last forty years, from the discovery in 1943 by Avery, Macleod, and McCarty that DNA was the hereditary material; to the present when genes are characterized on the basis of their nucleotide sequence and structure (Kornberg, 1980). The ability to isolate and characterize mutants has been a powerful tool for studying complex regulatory biochemical process in prokaryotes. This is especially evident in viruses where the complete mucleotide sequence as well as the function of each gene is completely characterized (Kornberg, 1980). A process or pathway with many different components can be better understood if each of the components can be manipulated independantly so as to identify the function of that specific component. This genetic approach has proved to be very useful when applied to somatic mammalian cells grown in culture; particularly permanent cell lines such as CHO or L cells (for review see Wright et al., 1980). Very few genetic studies have been carried out using normal human diploid fibroblasts in spite of the importance of these normal lines. There are, as discussed in the previous section, some very marked differences between normal and permanent or transformed cell lines, especially with respect to control of cell cycle, cell proliferation, and response to mitogenic stimuli. Furthermore a large variety of different genetic markers are useful for hybridization studies, chromosome mapping, and complementation studies. The value of cell lines like CHO lies in the great variety of mutants which can be selected using drug resistance, temperature sensitivity, auxotrophy, and lectin resistance. These mutant cell lines also form the basis of DNA mediated gene transfer and other molecular biological experiments (Wright et al., 1980). Such a variety of mutants is also needed in human diploid fibroblasts in order to understand complex biochemical processes in these normal cells. Mutants are also needed in order to study mutagenesis mechanisms. There is currently a great need to develop test systems to determine the mutagenic nature of the large number of environmental agents that must routinely be screened. Since there arevariety of species specific determinants which are involved in mutagenesis, it is important to use a model system which most closely resembles the donor species; in our case this would be normal human diploid fibroblasts. Another important question which should be addressed using normal HDF is whether drug resistance mechanisms like gene amplification occur in normal cells, or is this phenomenon restricted to transformed cell lines.

There are several reasons why there have been so few genetic mutants characterized in HDF. These cells have a limited life span which, once cloning and selection have taken place, leaves a relatively short remaining lifespan. Normal HDF also show density-dependant and anchorage dependant growth which limits the amount of material available for detailed biochemical studies. The frequency of isolation of mutants may to be somewhat lower in HDF than in permanent cell lines (Mankovitz et al., 1974; Buchwald & Ingles, 1976; Dick & Wright, 1983).

During the early period of mutant selection a great deal of debate revolved around the question of whether altered phenotypes were the result of true mutations (alterations in DNA sequence), or the result of stable epigenetic changes in gene expression commonly seen during differentaion. Several key studies using permanent cell lines (Chasin & Urlaub, 1975; Siminovitch, 1976) and normal HDF (DeMars, 1974) showed convincingly that many altered phenotypes resulting from drug selection were due to true mutational mechanisms rather than epigenetic changes.

Drug resistant mutants have been isolated more often than any other type of mutant because of the availability of a variety of cytotoxic drugs with a specific site of action and the ease with which they can usually be obtained. There have been four dominant or codominant and two recessive different drug resistant mutants of normal HDF selected to date. These are briefly outlined below.

## Examples of Drug Resistance in Normal Human Diploid Fibroblasts

Resistance to Guanine Analogues; Altered HGPRT Activity. The first mutant HDF cells were resistant to the drug 8-azaguanine (Albertini & DeMars, 1970; 1973; DeMars & Held, 1972). This purine analogue was first used by Szybalski (1958) to isolate mutants in a human tumor line. 8-AG becomes toxic when it is transfered into the cell and converted to a nucleotide by the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT). Cell lines resistant to 8-AG either lack HGPRT or have an altered enzyme with reduced substrate affinity (Caskey and Kruh, 1979). Mutants of either class have also been isolated in HDF at a frequency of about $10^{-6}$ (DeMars, 1974). These mutants resemble cells obtained from individuals with Lesch-Nyhan syndrome (Seegmiller et al., 1976). Lesch-Nyhan fibroblasts contain altered HGPRT activity making guanine analogue mutants a useful model system in which to study biological and genetic aspects of human disease.

The mutation rate for 8-AG in HDF was estimated to be about 5 x $10^{-6}$ per cell per generation (Van Zeeland & Simons, 1976). The mutation rate could be increased by several orders of magnitude with the use of mutagens such as MNNG, EMS and radiation (DeMars, 1974). The problem with 8-AG as a selective agent is the cell density dependent killing, where metabolic co-operation between wild type and mutant cells allows the transfer of toxic drug from the wild type to the mutant cells. High cell densities inhibit the recovery of drug-resistant mutants. 6-Thioguanine, an analogue with higher affinity for HGPRT than 8-AG can to some extent overcome this problem (Cox & Masson, 1976).

Resistance to Diaminopurine; Altered APRT. Most cells contain another salvage enzyme called adenine phosphoribosyl transferase (APRT) which is specific for adenine nucleotides. Diaminopurine has been used to isolate human diploid mutants without APRT or with altered affinity for adenine nucleotides (Rappaport & DeMars, 1973).

Resistance to Ouabain; Altered Na/K ATPase. Ouabain, a plant alkaloid, is a potent inhibitor of Na/K ATPase found in the cell membrane. Normal HDF are about $10^2 - 10^3$ times more sensitive to ouabain than permanent cell lines such as CHO (Mankovitz et al., 1974). Ouabain, in contrast to 8-AG does not exhibit density dependent killing allowing large numbers of cells to be screened on a single plate. Mankowitz et al. (1974) reported the isolation of ouabain resistant human cell strains that were up to 180 times more resistant to drug than wild type cells. These mutants were isolated at a very low frequency of about $10^{-7}$ after mutagenesis. In a more detailed study the spontaneous mutation rate was later estimated to be about $7 \times 10^{-8}$ per cell per generation (Buchwald, 1977). These mutants contained an altered Na/K ATPase with reduced affinity for ouabain. This mutation rate is about two orders of magnitude lower than 8-AG resistant mutants; reflecting the fact Na/K ATPase is required for cell growth, any mutation significantly affecting the function should be lethal. Therefore, only a certain class of mutants could be selected resulting in the low mutation rate. This view is supported by the fact that only those mutagens which induce point mutations raised the mutation rate; others which cause more extensive mutations such as frameshift mutations have no effect on the mutation rate (Buchwald, 1977).

Resistance to α-Amanitin; Altered RNA Polymerase II. α Amanitin, a mushroom toxin, is a specific inhibitor of RNA polymerase II and can be used to isolate CHO (Chan et al., 1972), rat myoblast (Somers et al., 1975), and normal human diploid fibroblast (Buchwald & Ingles, 1976) drug resistant cells. All these mutants contain an

RNA polymerase II which is less sensitive to α-amanitin. The mutation is codominant in resistant HDF and rat myoblasts which contain both resistant and sensitive enzyme activty. In the presence of the drug, the sensitive enzyme is inactivated and the resistant form of the RNA polymerase becomes elevated. Interestingly, CHO mutants contain only resistant enzyme, indicating they are hemizygous for the RNA polmerase II gene (Ingles et al., 1976).

The frequency of isolation of α-amanitin resistance in HDF is about $5 \times 10^{-8}$ after mutagenesis; a frequency somewhat lower than ouabain resistance. These mutants are about 2 - 3 times more resistant that wild type cells, using comparison of $D_{10}$ values. In keeping with this low level of resistance, RNA polyermase from these mutants was about three fold more resistant to a α-amanitin than wild type.

<u>Resistance to Diphtheria Toxin; Altered Elongation Factor 2</u>. Diphtheria toxin is an inhibitor of protein synthesis probably by inactivating elongation factor 2 (Gupta & Siminovitch, 1978a). Normal HDF resistant to diphtheria toxin can be isolated at a relatively high frequency of about $1 - 8 \times 10^{-6}$ (Gupta & Siminovitch, 1978b). A mutation rate of $5 \times 10^{-7}$ per cell per generation was determined by fluctuation analysis; a value comparable to 8-AG resistance. These resistant cell strains contain an altered elongation factor 2 which is resistant to inactivation by diphtheria toxin.

This could be a very useful system for mutagenisis testing since there are no cross feeding or cell density effect and mutants can readily be isolated from a single plate (Gupta & Siminovitch, 1978).

Resistance to Hydroxyurea; Altered Ribonucleotide Reductase Activity. Recently, normal HDF resistant to the anti-tumor agent hydroxyurea have been isolated (Dick & Wright, 1983). Hydroxyurea is an inhibitor of ribonucleotide reduction. The role of riboncleotide reductase, a key enzyme in DNA synthesis, will be discussed in the next section. These mutants contain elevated levels of RRase as well as alterations in the accompanying deoxyribonucleotide pools. These drug-resistant cells resemble class II CHO mutants which overproduce normal enzyme (Lewis & Wright, 1979). In addition to class II mutants, our laboratory has characterized class I mutants which have a reduced affinity for hydroxyurea and Class III mutants which overproduce enzyme with reduced affinity to hydroxyurea (Wright et al., 1980; Wright, 1983). The level of RRase activity correlates very closely to cellular resistance.

Hydroxyurea resistant HDF cells are extremely rare, with a frequency of isolation of about $1 \times 10^{-8}$. This may be a reflection of the narrow range of alterations which are permitted in this enzyme; any large alterations may be lethal.

Interestingly these hydroxyurea resistant mutants showed reductions of between 20 and 40 percent in maximum lifespan. This result along with other data on enzymes levels (Dick & Wright, 1982) suggest that RRase may be a key determinant in *in vitro* senescence.

Other Selective Systems

There are several other poorly characterized mutants isolated using normal HDF. Duthu et al., (1982) using a long lived normal HDF cell strain have isolated, in two steps, a 6-TG and ouabain resistant cell strain. This double mutant should prove to be very useful in hybridization studies.

Cox and Masson (1974) isolated a normal HDF able to grow using fructose as a carbon and energy source. Although the nature of the mutation was not determined, UV radiations increased the frequency of isolation of these variants.

Hankinson and Jacoby (1975) reported the isolation of a HDF capable of growing in medium where homocysteine is substituted for cysteine. Very little genetic or biochemical analysis of these cultures was carried out. These variants grew slowly and apparently were unable to use homocysteine; instead they used the small amount of cysteine which is bound to serum proteins. It is not clear from this study whether these cells were in fact stable variants.

Conclusion

There are only about a half dozen mutant systems which have been characterized using normal human diploid fibroblasts. It is obvious a greater variety needs to be developed in order to more accurately compare the differences which occur in basic regulatory processes found in transformed permanent cell lines and normal human diploid cell strains. Furthermore normal cell strains could also be used to test the effects of anti-neoplastic agents used in cancer chemotherapy since these normal cells resemble the *in vivo* state.

C. Role of Ribonucleotide Reductase in Cellular Metabolism

Brief Description

DNA synthesis is a highly regulated and complex process which requires the functional integration of many diverse cellular components.

One of the basic requirements is a continuous and balanced supply of the four deoxyribonucleotides (dNTP) which are supplied by the reduction of the four corresponding ribonucleotides. This reduction occurs at the diphosphate level in mammalian cells via the action of ribonucleotide reductase (EC 1.17. 4.2) (Thelander & Reichard, 1979). The dNDP's are phosphorylated by nucleoside phosphate kinase, except for TTP which arises through the inter conversion of dUMP to dTMP, or dCMP to dUMP to dTMP by the enzymes thymidylate synthase or dCMP deaminase and thymidylate synthetase respectively (Weber, 1983).

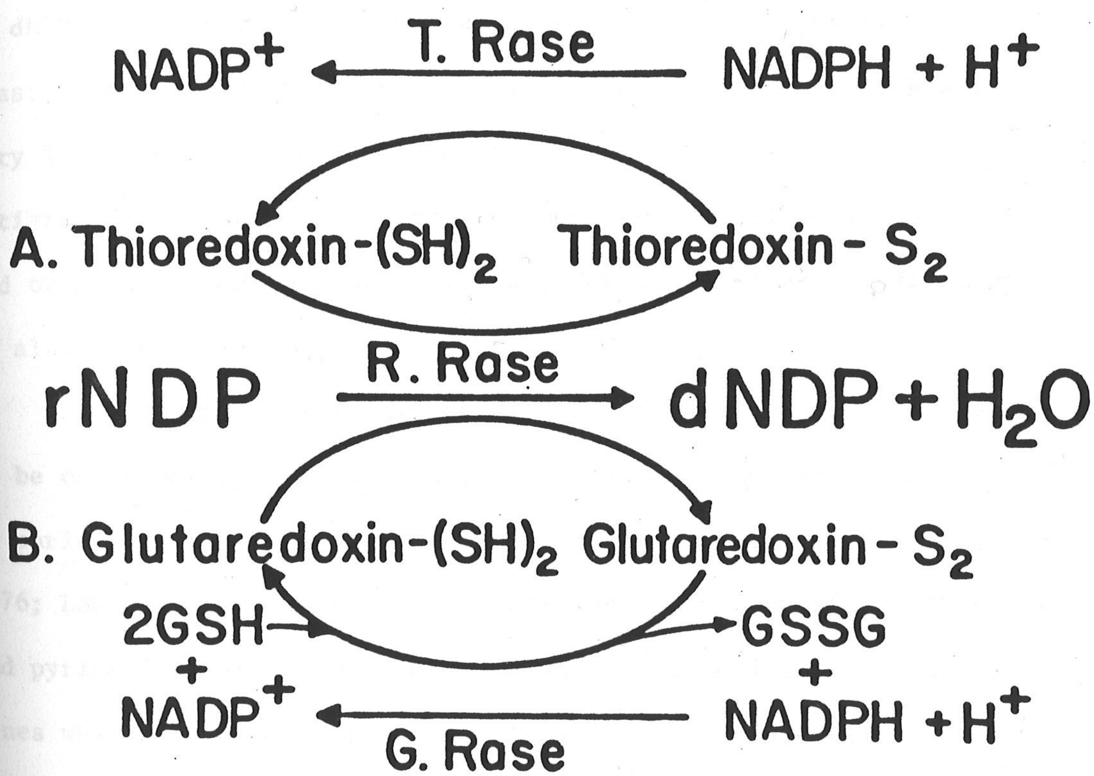
Reduction of NDP occurs at the 2' carbon atom of D-ribose, with the aid of one of the two small proteins, thioredoxin via the thioredoxin reductase system (TRase), or glutaredoxin via glutathione (GSH) and glutathione reductase (GR) serving as hydrogen carriers (Luthman et at., 1979). Reducing power ultimately comes from NADPH. The reaction is schematically represented in Figure IV.

It is important to note that RRase is the sole enzyme responsible for the conversion of ribonucleotides to their corresponding deoxyribonucleotides. The signficance of this fact is strengthened by the observation that the endogenous supply of dNTP's is only sufficient to maintain DNA synthesis for a very short period of time (Skoog et al., 1973).

The importance of RRase to the overall biology of the cell cannot be underestimated. For this reason a great deal of information has been gathered in the last twenty years regarding the function and regulation of RRase. Several current reviews have recently been published which summarize the current state of knowledge with respect

85

Figure IV. Reduction of ribonucleotides (rNDP) to deoxyribonucleotides (dNDP) by ribonucleoside diphosphate reductase (RRase), in the presence of (a) thioredoxin reductase (TRase) and thioredoxin, and (b) glutathione reductase (GRase), glutathione (GSH) and glutaredoxin (taken from Wright, 1983).


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

to RRase and its role in cellular metabolism (Wright et al., 1981; Wright, 1983; Cory, 1983; Hunting & Henderson, 1982; Reichard & Ehrenberg, 1983). This review will be restricted to a brief outline of the biological importance of RRase as well as some key regulatory and physical aspects.

## Biological Importance of Ribonucleotide Reductase

Cell Cycle and Cell Proliferation. Since the endogenous levels of dNTP's required for DNA synthesis are very low the activity of RRase must increase prior to DNA synthesis. The levels of RRase are very low throughout the cell cycle except for a dramatic rise in activity just prior to S phase, followed by a rapid decline at the end of S phase (Murphee et al., 1969; Peterson & Moore, 1976; Lewis et al., 1978).

Interestingly the levels of CDP and ADP reductase do not appear to be coordinately regulated during the cell cycle, since the ratio of purine and pyrimidine reductase fluctuates (Peterson & Moore, 1976; Lewis et al., 1978). This non-coordinate regulation of purine and pyrimidine reductase is also evident in a variety of mutant cell lines which overproduce RRase (Lewis & Wright, 1979; Kuzik & Wright, 1980; Koropatnick & Wright, 1980; Wright et al., 1980; Hards & Wright, 1983a). Furthermore there is evidence that the different subunits of RRase are also not coordinately synthesized or degraded. In Erlich tumor cells, regenerating rat liver, and CHO cells $M_1$ is the limiting subunit (Cory & Fleischer, 1982; Wright & Cory, 1983) while in S49 lymphoma, $M_1$ is relatively constant throughout the cell cycle, and $M_2$ is present at very low levels except during S phase (Eriksson &

Martin, 1981). More work is needed to outline the mechanisms responsible for the regulation of the levels of RRase.

There is a close correlation between enzyme activity and cell proliferation. Tissues with high rates of cell renewal such as thymus, spleen, bone marow, or intestine contain high levels of RRase activity, while those which are composed of mainly non-dividing cells such as brain contain low or undetectedable levels of RRase. (Millard, 1972; Elford, 1972; Takeda & Weber, 1981).

Evidence has been provided which suggests RRase is functionally and physically associated with several other enyzymes including DNA polymerase, NDP kinase and thymidylate synthetase, (Reddy & Pardee, 1980; 1982; 1983). This complex called replitase, appears to preferentially use NDP's as a substrate rather than the dNTP pools (Reddy & Pardee, 1980; Forsdyke & Scott, 1980; Wickremasinghe & Hoffbrand, 1983). This suggests that NDP's are channeled to the DNA polymerase to produce high level concentrations of dNTP's. RRase is the rate limiting enzyme of the replitase. Some doubt has very recently been cast on the conlusion of some of these experiments (Spyrou & Reichard, 1983); these authors suggest the NDP's are in fact found in RNA rather than DNA. Obviously, more work is needed to resolve this very important question.

A great many studies, therefore, support the view that RRase is closely linked to DNA synthesis and plays a key role in the regulation of cell division (Reichard, 1978; Wright et al., 1981; Elford, 1972; Wright, 1983; Cory, 1983). Many more studies are needed to understand how RRase mediates this control; whether through dNTP pools or directly at the level of the protein itself.

Neoplasia. As noted above there is a close correlation between RRase activity and DNA synthesis. In keeping with this fact, there is a remarkable correlation between the level of reductase activity and the growth rate of hepatomas, as measured by mean generation time (Elford et al., 1970; Takeda & Weber, 1981, Weber et al., 1981). Therefore RRase belongs to a relatively small class of proteins that are progression linked (Weber, 1983). Furthermore, tumor cell lines contain even higher levels of RRase activity than normal cells growing at the same rate. These increases of RRase activity in neoplastic cells are also reflected by increases in the concentration of the four dNTP pools (Weber et al., 1981). These results indicate that RRase is also a transformation-linked enzyme (Weber, 1977a,b; 1983). RRase correlates more closely to transformation and progression than has been observed with any other enzyme in the DNA synthetic pathway (Elford, 1972; Weber, 1983).

Recently viral RRase has been implicated as the transforming protein of Herpes virus transformed cells (Huszar & Bacchetti, 1983). The gene fragment from HSV which posesses transformation activity produces two polypeptides which can be immunoprecipitated with antibody against viral reductase (Galloway et al., 1982; Huszar & Bacchetti, 1983). This evidence although indirect suggests that viral RRase may play a key role in establishing transformation. Viral reductase and mamalian reductase appear to be quite distant from each other (Wright, 1983), however more studies need to be carried out to determine the relationship between them.

Anti-Tumor Drugs. RRase occupies a key rate limiting step in DNA synthesis and cell proliferation making it an attractive target against which chemotherapeutic drugs could be directed. Although many potentially useful drugs are being developed (Brinkley et al., 1978; Gale et al., 1979; Cory et al., 1981; Elford et al., 1981); hydroxyurea is the only inhibitor of RRase in general clinical use. Hydroxyurea can be used alone (Lerner et al., 1969), or in combination with other drugs to achieve a potentiated response (Gale et al., 1979). Hydroxyurea may be particularly useful when it is used to manipulate dNTP pools in such a way that other drugs can act more efficiently. For instance hydroxyurea has been used to decrease the dCTP pool, thereby enhancing the cytotoxicity of ara-C (Rauscher & Codman, 1983).

Hydroxyurea is used primarily to treat chronic granulocytic leukemia (Sinclair, 1981). The major disadvantage, however, is the need for administering increasing doses resulting in toxicity, as well as the development of resistance (Wan't Riet et al., 1979). A deeper understanding of the regulation of RRase is necesary for designing drugs and protocols for drug delivery directed to this site (Elford et al., 1981).

Mutator Locus. DNA synthesis requires a balanced and continuous supply of deoxyribonucleotides. The fidelity of this process is impaired if the relative concentrations of the dNTP pools, particularly the dCTP/TTP ratio, are changed (Meuth et al., 1979; Ashman & Davidson, 1981; Ashman et al., 1981; Weinberg et al., 1981). RRase, which plays a key role in maintaining the dNTP pools, has been shown to have a mutator function associated with it (Meuth, 1979, Weinberg et al., 1981; Chan et al., 1981). RRase with mutations affecting the regulation of activity produced abnormal dNTP pool sizes relative to one another; these cell lines were subsequently shown to have higher rates of mutation at specific loci (Meuth et al., 1979; Ashman et al., 1981; Weinberg et al., 1981; Chan et al., 1981). It is interesting to note that cell lines with reduced levels of dCMP deaminase also show increased mutation rates, presumably by altering the dCTP/dTTP ratio (Weinberg et al., 1981). The key in all these studies is the dCTP/TTP ratio which appear to cause elevated mutation rates at high ratios, perhaps, because of simple competition between correct and incorrect dNTP's at the site of base addition (Kunkel & Loeb, 1980; Meuth, 1981). Conversely dCTP, which appears to play a special role in the positive control of DNA synthesis (Thelander & Reichard, 1979) may induce some error prone synthesis or repair process (Weinberg et al., 1981). It is also clear that level of dCTP alone is not the only mediator of the mutator phenotype (Meuth, 1981).

It is interesting that a mutator phenotype is also associated with another key enzyme of the replication complex, DNA polymerase α Lui et al., 1983). The DNA polymerase from aphidicolin resistant cell lines were shown to have a ten fold increase in affinity for dCTP along with being resistant to aphidicolin. This cell line was hypersensitive to UV radiation suggesting the the DNA repair system, of which polymerase α is a component, was also impaired. Although the relationship between dCTP and semi-conservative replication or repair is not known, it is very interesting that mutator functions associated with altered dCMP deaminase, RRase, and DNA polymerase all involve dCTP in some way.

Immunodeficiency Diseases. There are a variety of immunodeficiency diseases in man which are caused by the inability of the T and B cells to proliferate when stimulated. The basic defects in these conditions are the result of deficiencies of two salvage enzymes, purine nucleoside phosphorylase and adenosine deaminase; as a result the normal catabolism of purines and/or pyrimidines is impaired leading to a build up of dGTP or dATP (Chan, 1978; Ullman et al., 1976; Martin & Gelfand, 1981). These nucleotide effectors inhibit CDP reductase via allosteric mechanisms, depleting the dCTP pool thereby preventing DNA synthesis and cell proliferation (Cohen et al., 1983).

Senescence and Differentiation. As discussed in section A, the levels of RRase activity correlate very closely to the decline in proliferative potential as normal HDF senesce in culture (Dick & Wright, 1982). Furthermore, HDF selected for resistance to hydroxyurea were found to senesce at an accelerated rate (Dick & Wright, 1983). These mutant cells contained elevated levels of RRase activity as well as altered dNTP pools providing further support for the involvement of ribonucleotide reductase in the senescence process.

Added evidence for the involvement of RRase in complex biological processes comes from recent experiments using the $L_6$ and $L_8$ myoblast differentiation systems (Creasey et al., 1982; Creasey & Wright, 1983). Myoblasts grown normally in culture can be induced to fuse and form multinucleated myotubes. Hydroxyurea resistant myoblasts which show elevated levels of RRase as well as dNTP pool alterations were unable to differentiate into myotubes (Creasey & Wright, 1983). Whether this alteration in the normal differentiation program was due directly to changes in RRase itself or the accompanying dNTP pools is not yet known. However these results clearly establish a close relationship betwen RRase and the initiation of differentiation. Studies are currently in progress to determine the exact nature of this relationship.

Regulation of RRase. It is not unusual that an enzyme which occupies such a key position in cellular metabolism would be highly regulated. Indeed, RRase is probably the most highly allosterically regulated enzyme found in the cell. The essential features of the allosteric regulation are present in Figure V.

Reduction of the pyrimidines requires ATP while reduction of GDP requires TTP and ADP reduction requires dGTP. This scheme implies that reduction begins with pyrimidines, and as they accumulate TTP activates GDP reductase, and the dGTP produced activates ADP reduction. As long as the dNTP's are being used in DNA synthesis the reduction of all four dNTP's would continue. When DNA synthesis slows down or stops, dATP would accumulate preventing further reduction of all four dNDP's by inhibiting all four reductases. dATP has a higher affinity for all four reductases than their respective activators, thereby displacing these activators. It should be mentioned there are several variations of this scheme depending on the cell line used and differing assay conditions. These models

Figure V. Allosteric regulation of ribonucleoside diphosphate reductase. The bars indicate inhibitory effects and the nucleotides shown in the arrows act as positive effectors (taken from Wright, 1983).

differ with the one described above with respect to the role that nucleotides such as GTP, and dCTP play in activation of RRase (Hunting & Henderson, 1982; Hards & Wright, 1983a,b). As well, some of these studies ascribe different roles to TTP, dGTP, and dATP than outlined above.

There are at least three other levels of regulatory processes which appear to control RRase. The involvement of RRase in a multienzyme complex with other DNA synthetic enzymes has already been discussed above. RRase carries out reductions by a mechanism using a free radical localized to a tyrosine residue in the $M_2$ protein (Reichard & Ehrenberg, 1983). Hydroxyurea inhibits RRase by destroying this free radical (Lankinen et al., 1982). The process whereby this radical is regenerated could provide another level of control of RRase activity. Using E. Coli a regeneration complex has been identified which used $O_2$ and iron to regenerate the tyrosine radical (Barlow et al., 1983). The mechanism of radical regeneraton is more complex in mammalian systems and although no such complex has conclusively been identified, there are some indications that some active process is involved in regeneration of the tyrosine free radical (Akerblom et al., 1981). Further studies are necessary to resolve the nature of this potentially very interesting control mechanism. Several groups have identified endogenous inhibitors of reductase which are present during periods when DNA synthetic activity is low, and are absent during periods of active DNA synthesis (Elford, 1972; Cory, 1979; Lewis et al., 1977). These inhibitors are poorly characterized. Elford (1972) reported the presence of a heat labile, non dialyzable factor from normal rat liver whose concentration was inversely proportional to RRase activity. Lewis et al., (1977) isolated a highly phosphorylated dinucleoside from CHO cells which was a very effective inhibitor of RRase. This compound was similar to one found in the fungus Achlya (Lewis et al., 1976; Goh et al., 1977). This inhibitor also accumulated during periods of no DNA synthesis. Although the physiological role of these compunds need to be elucidated they may play a role analogous to $Ap^4A$, an inhibitor of DNA polymerase (Varshavsky, 1983); providing another important mechanism of regulation of DNA synthesis.

Structural Features. Although the existence of RRase has been known for about 25 years, only recently has it been possible to obtain highly purified extracts of this enzyme (Engstrom et al., 1979; Yondale et al., 1982; Mattaliano et al., 1981). The degree of purity as well as the reproducibility differ in each of the reported systems making precise qualitative analysis difficult. For this reason there is still no consensus as the whether one enzyme catalyzes all four reactions or whether modified forms of the enzyme reduce the four different dNTP's (Wright, 1983; Cory, 1983). Clearly the problem can only be resolved using homogenous preparations of RRase and/or molecular biological techniques directed at the gene(s) for RRase.

## Summary

RRase is a complex and important enzyme in the cell, clearly much more work still needs to be done to clarify the nature of the enzyme as well as its many regulatory components. The recent developments regarding the multienzyme complex as well as the novel radical regenerating system indicate that the regulation and control of RRase may be even more complex than is presently understood. The solutions to these remaining questions can be approached using mutant cells lines with a variety of structural alterations in the $M_1$ or $M_2$ components of RRase, as well as alterations in the expression of these two components (Wright, 1983). Our understanding of RRase could also be improved if pure preparations were available to study the interactions between $M_1$ and $M_2$. Finally,molecular DNA probes to determine the nature of the regulation of expression at the level of the RNA and the DNA are clearly necessary to understand more fully how RRase is controlled.

# METHODS AND MATERIALS

# METHODS AND MATERIALS

## Cells and Growth Conditions

Human Cells. The fetal lung fibroblast strain, HSC 172, kindly provided by Dr. Buchwald, University of Toronto, was derived from a 12 week old female fetus (Buchwald & Ingles, 1976). The karyotype of this strain was normal 46, XX. These primary cells can be grown on the surface of glass bottles (16 oz. Brockway bottles) or plastic tissue culture plates. The cells were routinely maintained on 100 mm tissue culture plates, however when large quantities of cells were needed for enzyme assays 150 mm plates were used. The cells were grown in a α-minimal essential medium (α-MEM) supplemented with penicilin (100 units/ml), streptomycin sulfate (68 ug/ml) and 15% fetal bovine serum preselected for high PE characteristics.

CHO Cells. The Chinese hamster ovary (CHO) cell line is a permanent cell line established by Puck et al., (1958). This cell line grows well in culture and exhibits a stable karyotype (Worton et al., 1977). These cell lines can be maintained in monolayer or suspension culture. The $GATO^R$-1 cell line used in cell hybridization experiments is a double mutant CHO line, which is resistant to ouabain and auxotrophic for glycine, adenosine, and thymidine because of a defect in folic acid metabolism (McBurney and Whitmore, 1974). Ouabain resistant $GAT^-$ cell lines were developed by Lewis & Wright (1978a). $GATO^R$-1 grew in α-MEM supplemented with 10 μg/ml of adenosine and thymidine; glycine is present in α-MEM.

Assay for Ribonucleotide Reductase Activity in Intact Cells

CDP Reductase. Exponentially growing HSC 172 cells were plated at a density of $2 \times 10^6$ cells/150 mm plate and incubated at 37°C for approximately 40 hours. Cells were removed with 0.1% trypsin, centrifuged, resuspended in growth medium and counted using a particle counter (Coulter Electronics). The cell pellet was resuspended at $5 \times 10^6$ cells/ml in permeablizing buffer consisting of 1.0% Tween 80, 0.25 M sucrose, 0.05 M N-2-hydroxy-ethyl piperizine-n'2-ethanesulfonic acid (Hepes) buffer, pH 7.2 and 2 mM dithiothreitol (DTT). The cells were incubated at 30°C for 75 or 90 min., after which they were centrifuged and resuspended in fresh permeabilizing buffer as described above. The cell suspension was then dispensed at 200 µl/tube into reaction tubes containing 100 µl of reaction mixture. The final assay for CDP reduction consisted of 0.05M Hepes pH 7.2, 6 mM DTT, 8 mM $MgCl_2$, 4 mM ATP, 0.4 mM $^{14}C$-labelled CDP (5000 dpm/nmole, $6 \times 10^5$ dpm/assay), 0.67% Tween 80, 0.167 M sucrose, and $1\text{-}3 \times 10^6$ cells. The DTT, $MgCl_2$, ATP, and cold CDP were made up as concentrated stocks in Hepes and were diluted in a common tube prior to the assay to ensure all assays were treated in the same way. Purified $^{14}C$-CDP ($6 \times 10^5$ dpm/assay) was also added to the common tube prior to dispensing the assay mix in all assay tubes.

A 2 x 7 mm magnetic stirring bar was placed in each assay tube, and the reaction tubes were incubated at 37°C for 20 min. in a water bath on top of a magnetic stirrer (Belco Glass Co.) operating at high speed. The reactions were terminated by boiling for 4 min., after which 2 mg/assay of Crotalus atrox venom in 0.1 M Hepes buffer, pH 6.8 containing 10 mM $MgCl_2$ was added to each tube. The tubes were then incubated for a further 2 hours at 37°C. The reaction was terminated by boiling and the deoxycytidine formed was measured as described by Steeper and Steuart (20). The deoxycytidine was eluted from a Dowex 1-borate ion-exchange resin (Bio-Rad Laboratories) with 5 ml of water. Radioactivity was determined by adding Scintiverse II cocktail (Fisher) to the 5 ml of water containing deoxycytidine. Enzyme activity was expressed an nmoles product formed per hour per $3 \times 10^6$ cells (Dick & Wright, 1980).

ADP Reductase. Permeabilized cells were prepared in exactly the same manner as described for CDP reductase. The final assay for ADP reduction consisted of 0.5 M Hepes pH 7.2, 6 mM DTT, 1.0 mM dGTP, 0.4 mM $^{14}C$-labelled ADP (5000 dpm/nmole, $6 \times 10^5$ dpm/assay) 0.67% Tween -80, 0.167 M sucrose, and $1\text{-}3 \times 10^6$ cells. The ADP samples were then incubated at 37°C for 20 min in a reciprocating water bath or over top a magnetic stirrer when stir bars were added to the assay mix. The reaction was terminated by boiling after which 2 mg/assay Crotalus atrox venom in 0.1 M Hepes pH 6.8 containing 10 mM $MgCl_2$ was added to each tube. The cells were incubated a further 2 hours at 37°C. The deoxyadenosine formed was determined by the method of Cory et al., (1973). The deoxyadenosine was eluted with 20 mls of 1.0 mM sodium borate from Dowex 1-borate. Radioactivity was determined by counting 5 mls of the eluant added to Scintiverse II cocktail. Enzyme activity was expressed as nmoles product/hour/$3 \times 10^6$ cells (Dick & Wright, 1980).
All the enzyme assays reported in this thesis were carried out at least twice and usually greater than three times. The replicate experiments all showed the similar trends and values. The data presented in this thesis are representative of one such experiment except where

Preparation of Dowex 1-Borate. The ion exchange resin was purchased as Dowex 1-chloride x 8 or AG1-chloride x 8, 200-400 mesh and had to be converted to the borate form. One pound of resin was resuspended in 6 l of saturated sodium borate and stirred overnight at room temperature. The resin was then collected by the filtration on a large Buchner funnel and again resuspended in fresh saturated sodium borate. The next day the resin was again collected by filteration and washed with 16 l of deionized water. Finally the Dowex 1-borate was resuspended in a small amount of water to make a thick slurry and stored at 4°C.

Purification of $^{14}$C-CDP. During the course of this study many different lots of $^{14}$C-CDP were obtained for use in the reductase assay and unacceptable high background values were found in some lots. In order to determine the nature of the material contributing to the background and eliminate it from the CDP substrate to ensure more reproducible results it was necessary to repurify the labeled substrate. The purification was rapidly and reproducibly performed by ion exchange chromatography using the HPLC system. The sample (490 μl) was applied to a Partisil 10 SAX column (Whatman) and eluted with a buffer consisting of 125mM $KH_2PO_4$ and 125mM KCl pH 4.5 at a flow rate of 2 ml per minute. Fractions corresponding to CDP were collected and pooled. These pooled fractions were dried down to a volume of about 1 ml by placing the tubes in a 37°C waterbath and allowing a stream of dry $N_2$ gas to bubble through the buffer. This sample was then divided into aliquots and frozen at -70°C.

Assay of Ribonucleotide Reductase in Crude Extracts

Log phase cells were harvested from a large number of 150 mm tissue culture plates and the cell pellet was frozen for later homogenization. The cell pellet was thawed and resuspended in homogenization buffer consisting of 100 mM TRIS, pH 7.6, 250 mM sucrose, 5 mM magnesium acetate, 10 mM KCl, and 2 mM DTT. The cell suspension was homogenized using a motorized Teflon pestle and ice-cold glass tube. Approximately fifty strokes at high speed were required to disrupt the cells. This homogenate, approximately 4 ml, was centrifuged in a Beckman L2-65B using a Ti60 rotor with adaptors for 90 minutes at 30 K. After this time a thin layer of lipid formed at the surface of the supernatant. Thereafter a pipet was used to remove the supernatant underneath this lipid. This extract was used directly for titration of $M_1$ and $M_2$ subunits.

$M_1$ and $M_2$ components used for titration were obtained from the CHO cell line, GAT-SC8, which is resistant to hydroxyurea (Lewis & Wright, 1979). These components were kindly provided by Arthur Chan in our laboratory. The components were prepared according to previously described methods (Cory et al., 1978). Briefly a cell extract was prepared as described above and ammonium sulphate was added to the supernatant to 40% saturation and stirred at 4°C for 30 minutes. The precipitate was removed by centrifugation at 10,000 g for 10 minutes and dissolved in a buffer at pH 7.6 consisting of 100 mM Tris-HCl, 10 mM $MgCl_2$, and 2 mM dithiothreitol. The solution was then desalted by passing through a G-25 Sephadex column equilibrated with 20 mM Tris-HCl, pH 7.0 with 1 mM dithiothreitol. This desalted preparation was then chromatographed on blue dextran Sepharose. The non-heme-iron containing component (NHI) was eluted with 20 mM TRIS pH 7.0 and 1 mM

DTT. The effector-binding (EB) component was eluted with 20 mM Tris-HCl pH 7.0, 1.0 mM DTT and 250 mM NaCl. The EB fraction corresponds to the $M_1$ subunit while the NHI fraction contains the $M_2$ subunit. Neither of these subunits posessed activity unless the other component was present (Wright & Cory, 1983).

Determination of Deoxynucleotide Pools

The levels of intracellular deoxyribonucleotide pools in wild type and drug resistant cells were measured by HPLC using a modification of the procedures of Garrett and Santi (1979) and Hartwick and Brown (1975). Cells were plated at a density of 2 x $10^6$/150 mm tissue culture plate and grown for about 40 hours. Wild type and drug resistant cells were harvested and extractions of each were made at the same time at 4°C (Khym, 1974). The cells were removed with trypsin (0.2%), counted, and centrifuged in as short time as possible. The cell pellets were resuspended at 5 x $10^7$ cells/ml in PBS and a small aliquot of $^3$H-thymidine was added in order to later estimate the dilution factor. This resuspension was then transferred to glass test tubes, sufficient 70% perchloric acid (PCA) to achieve a final concentration of 0.5 M was added, and the tubes were placed on ice for 30 min. Diethyl pyrocarbamate (100 µl/ml) and 2.2% tri-n-octylamine (TnOA) in Freon (2 vol/1 vol of extract) were added to the extract and vortexed for 2 min. The tubes were then centrifuged at low speed for 2 min, and the supernatant was collected and immediately frozen at -70°C.

The separation and identification of the deoxyribonucleotides first required the destruction of ribonucleotides with periodate and methylamine (Garrett and Santi, 1979). Four microliters of sodium periodate was added to 100 μl of extract and incubated for 5 minutes at 37°C. Then 5 μl of 4 M methylamine pH 7.5 was added. After 55 minutes incubation at 37°C, 1 μl of 1 M Rhamnose was added to remove excess periodate and incubated for a further 5 minutes. The sample was placed on ice or frozen at -70°C. The extracts were then separated by HPLC on an anion exchange resin (Hunting and Henderson, 1982). A Beckman model 332 gradient liquid chromatograph consisting of 2 model 110A pumps coordinated by a model 420 system controller programmer was used. The nucleotides were detected with a Beckman model 160 detector at 254 nm, with maximum sensitivity of 0.001 AUFS. A Partisil 10/25 SAX (Whatman) strong anion exchange column (25 cm x 4.6 mm) preceded by a guard column (7.0 cm x 2.0 mm) was used in the separation process. Good resolution of the four deoxyribonucleotides was achieved using isocratic elution with 0.25 M $KH_2PO_4$ (Fisher, HPLC grade) and 0.25 M KCl (Fisher, ACS grade), pH 5.0 at a flow rate of 2 ml/min. All buffers were filtered through a 0.45 μm membrane filter and degassed prior to use. Identification was by retention times as compared to known standards. The peaks were quantitated on the basis of peak weight.

Determination of Ribonucleotide Pools

Exponentially growing cells were extracted with PCA and neutralized with TnOA/Freon according to the procedure outlined above. The separation of the mono-, di-, and triphosphates of the four different 5' ribonucleotides was based on a modification of the method of

Hartwick and Brown (1975) as described (Creasey, unpublished). The NTP's and NDP's were separated by HPLC using anion exchange chromatography. The Beckman HPLC system has been previously described. Separation of nucleotides was achieved using a rather complex protocol in order to separate the four NTP's and the four NDP's in a single run. Since the low concentration of dNTP's are undetectable at the detector sensitivity used, their contributions to the corresponding NTP peaks were ignored. The initial eluent, 7 mM $KH_2PO_4$ and 7 mM KCl adjusted to pH 4.0 with $H_3PO_4$, was run at 3.0 ml/min for 5 min. This isocratic elution was followed by linear gradient from 0% of the final eluent to 100% of the final eluent run over 30 min. The concentration of the final eluent was 250 mM $KH_2PO_4$ and 250 mM KCl adjusted to pH 5.0 with KOH. The final eluent was run isocratically for 10 min and complete separation was achieved by 48 min when the last peak, GTP, was eluted. The column was then re-equilibrated in low salt by running a linear gradient from 100% final eluent to 0% final eluent over 15 min and running the initial eluent for further 30 min after which the column was ready for another run.

The nucleotides were identified on the basis of retention time compared with known standards. Nucleotides were quantitated on the basis of peak height.

To prolong the life of the Partisil 10 SAX column a 25 cm x 4.6 mm precolumn packed with silica was placed in the solvent line between the pump and the injector. As well, a guard column (7.0 cm x 2.0 mm) was placed between the injector and the analytical column.

## Routine Procedures

Subculture. HSC 172 is a primary cell strain which senesces after several months in culture, therefore it was necessary to maintain an accurate record of the number of passages or subcultures that have been performed. In order to maintain this record, subculturing these cells must be performed according to a rigid protocol. The medium of confluent plates was removed by aspiration using a suction flask and sterile pastur pipet. Ice cold 0.1% purified trypsin (3 x crystalized, Sigma) in sterile phosphate buffered saline was added to the plates (3 ml/100 mm dish or 5 ml/100 mm dish). This purified trypsin was found to be much more active in the detachment of cells than crude trypsin and generally provided enhanced cell viability. After exactly two minutes this trypsin solution was aspirated leaving a residual film on the plate. The degree of cellular detachment was monitored visually by holding the plate up to the light (2-5 min) 4 ml of α-MEM and 15% FBS were added to the plate. A sterile pasteur pipet was used to flush the cells off the surface of the plate and make a single cell suspension. One ml of this cell suspension was then added to 3 additional plates plus the plate being trypsinized (i.e. 1:4 dilution); two passage numbers were then added. One passage number was added for a 1:2 dilution and 3 passage numbers were added for a 1:8 dilution (Hayflick, 1965). Under these circumstances one passage number was equivalent to one mean population doubling (MPD) (Harley & Goldstein, 1978; Smith & Hayflick, 1974).

Cell Counting. Freshly trypsinized cells suspended in medium were counted after dilution in PBS using an electronic particle counter (Coulter Electronics). The accuracy of the count was occasionally checked using other particle counters in the Institute or a hemocytometer.

Protein Determination. Protein concentration was estimated with the Bio-Rad protein determination kit (13, Technical Bulletin 1051) using bovine serum albumin as a standard. Using this procedure a linear relationship between absorbance and protein concentration was observed from 0 to 80 µg.

Colony Forming Ability in the Presence and Absence of Drug. Exponentially growing cells were harvested and counted. The colony forming ability was determined by seeding 500 to 1000 cells on a 100 mm dish and adding 10 ml of medium. After 10 - 14 days incubation at 37°C the plates were drained and saturated methylene blue in 50% ethanol was added to the plates. After several min the stain was decanted and the plate was gently rinsed in a beaker of flowing water. The plates were dried and examined under a dissecting microscope, any colonies with greater than 16 cells were scored. Human colonies are difficult to score for several reasons:

1. the colonies are large and diffuse because of the fibroblast's ability to migrate. If a large number of colonies are on the plate they grow into one another causing marked underestimation of actual colonies present.
2. If there are colonies growing against a background of non-dividing cells, again it is difficult to differentiate aggregations of cells from actual colonies. This is especially true if the colonies are slow growing and not too dense.

3. Human diploid fibroblasts have a marked tendency to clump in suspension especially if the culture is at confluence. This again results in an underestimation of P.E. since some larger colonies may have arisen from several clumped cells rather than a single cell.

4. There is a marked heterogeneity of colony size and morphology making it difficult to determine satellite colonies from original colonies.

The effects of various drugs on the growth of human cells can be determined by measuring the relative plating efficiency (RPE). The RPE is defined as the ability to form colonies in the presence of drug divided by this ability in the absence of drug. The RPE is generated by plating known numbers of cells in increasing concentrations of drug. The $D_{10}$ value is the concentration of drug which reduces the relative plating efficiency to 10%. Since HDF generally have a low plating efficiency (10 - 25%) large numbers of cells need to be plated to obtain a low RPE. For instance in CHO cells it is not difficult to obtain RPE values as low as $10^{-6}$ (i.e. 1 colony in $10^{6}$ cells seeded). However with HDF there was a non-linear response to the effects of hydroxyurea, therefore the same cell number was used in the plates without drug as the plates with drug. Since only 1000 - 2000 cells were seeded, a RPE in the range of $10^{-2}$ to $10^{-3}$ was the lowest that could technically be achieved by this procedure.

Low RPE values were obtained by performing the killing curve in a different way. Cells were plated at 8 x $10^{5}$ cells/100 mm plate and grown for 18 to 24 hours, at which time the growth medium was removed and replaced with medium containing 100 mM hydroxyurea. At varying lengths of time up to 96 hours plates were aspirated, trypsinized, and cell counts were taken. These cells were then seeded at varying dilutions in medium without drug, and the plates were incubated for 14 days. The RPE was determined by dividing the PE of cells exposed to drug for various lengths of time by the PE of cells not exposed to drug. This procedure resulted in RPE as low as $10^{-6}$.

Storage of Cell Lines. Human diploid fibroblasts were stored by suspending all the cells from a confluent plate in about 1.0 ml of MEM plus 15% FBS plus 7% DMSO. This cell suspension was placed in a small Cryotube (Nunc), tightly capped and placed at -70°C. Unless the culture would be required within one month these vials were then placed in liquid nitrogen at -192°C. For recovery, the vials were rapidly thawed with warm tap water, flooded with ethanol and the contents were added to tissue culture plates and medium. One passage number was added to compensate for any cell loss during storage.

Trypan Blue Staining. The procedure used to determine cell viability and cell permeabilization is essentially the same as described by Patterson, (1979). Cell viability was determined by trypsinizing monolayer cultures and resuspending cells in medium. The vital stain trypan blue was added to this cell suspension at a ratio of 1:9 and incubated for 5 minutes. A small portion of the suspension was then placed on a hemocytometer, and the percentage of stained to unstained cells was determined. The trypan blue stain was prepared by adding 0.4 g trypan blue, 0.81 g NaCl, and 0.06 g $K_2HPO_4$ to 100 ml $H_2O$. The mixture was brought to boiling, cooled and the pH adjusted to 7.2.

Mutant Selection

Low passage cells (passage numbers between 10 and 32) were plated at $8 \times 10^5$ cells/100 mm tissue culture plate and grown for 18 to 24 hours, at which time the growth medium was removed and replaced with medium containing 100 mM hydroxyurea. Cells (a total of $2 \times 10^6$ cells/experiment) were cultured in the presence of drug for 60 to 75 hours and then removed with trypsin solution and subcultured at 1:2 or 1:4 dilution. The cells were refed with growth medium without hydroxyurea and incubated at 37°C. This removal of cells with trypsin was necessary since a large fraction of cells, incapable of cell division would not lift off the plates after drug treatment, and would hinder the formation of colonies by cells capable of proliferation. Attempts were made to allow incubation with drug to the extent that only a few hundred cells remained on the plate, however no colonies grew up, although some non-dividing cells did remain. In addition it was also important to use subconfluent plates without any confluent areas, because killing was cell density dependent and the presence of confluent patches reduced the extent of killing by hydroxyurea. Three to four weeks later colonies were observed on some of the plates against a light background of non-dividing cells which may be acting as a feeder layer for proliferating cells. The largest colonies of about 1 cm in diameter were removed using glass cloning cylinders or were picked with a sterile Pasteur pipette and transferred to 60 mm tissue culture plates for incubation. When these plates contained confluent cultures, the cells were frozen in liquid nitrogen and tested for drug sensitivity (Dick & Wright, 1983).

In some experiments, colonies appearing after drug treatment were not picked; instead the culture was grown to a partial monolayer and then subjected to one or more additional rounds of drug treatment as described above, before isolating a colony for testing. When cultures were treated with ethyl methanesulfonate (EMS; Eastman) prior to selection for drug resistance, low passage HSC 172 cells were added at $4 \times 10^5$ cells/100 mm plate and cultured for 18 to 24 hours. EMS was then added to these cells at a final concentration between 300 and 400 μg/ml and incubated for about 24 hours. This treatment resulted in a reduction in colony forming ability of 50% to 90%. The viable cells were then allowed to grow to confluence in fresh growth medium, subcultured, and used in the selection procedure described above. The term mutant is used in the broadest sense to describe a stable change in phenotype even though conclusive evidence of a true mutational change (change in DNA) may be lacking (Wright et al., 1980).

Chromosome Staining

The techniques for slide preparation and chromosome banding are those described by Worton & Duff (1979) and Cox & Ray (1977). Colchicine (0.05 μg/ml) was added to actively growing cultures of cells in order to accumulate metaphase cells. After approximately three hours incubation at 37°C cell medium was collected and the cells were trypsinized, the cells and media were centrifuged using a clinical centrifuge. CHO cells were collected by gently shaking the monolayer shortly after trypsinization. This resulted in the collection of a large fraction of metaphase cells. The cell pellet was rapidly resuspended in prewarmed 75 mM KCl and incubated for ten to thirty minutes at 37°C. After incubation the cells were centrifuged gently ( 500 g for 5 to 8 minutes) and the supernatant was decanted allowing about 0.2 mls of 75 mM KCl to remain in the tube. The pellet was resuspended in the 75 mM KCl by swirling gently taking care to obtain an essentially clump-free suspension. Ice cold fixative (3:1 methanol:acetic acid) was added drop wise very slowly to the wall of the tube with constant mixing of the cell suspension. The cells were incubated on ice for about 15 min. This step was repeated twice. Slides were prepared by dropping the fixed cells onto wet slides from a height of about 8 inches. It was necessary to perform this step near boiling water as humidity appeared to play an important role in obtaining high quality slides. These slides were examined under phase contrast to ensure adequate chromosomal spreading had occurred. A bright halo around the metaphase spreads indicates that too much cytoplasm is still around the chromosomes and the spreading conditions needed to be adjusted.

G-Banding. G-banding was performed on slides that were several days old but not more than several weeks old. A 4% trypsin solution was prepared in saline and 4% Giemsa was prepared in Gurr's buffer pH 6.8. Slides were immersed in trypsin for about 15 seconds for hamster cells and about 5 seconds for human lines, rinsed rapidly in two changes of saline, and stained in Giemsa for about 8 min. Slides were then rinsed in distilled water, and examined under high power (1200x). If adequate banding had occurred slides were dried and coverslips were permanently mounted with permount. The following day micrographs were taken with Kodak Technical Pan film using a

Zeiss photomicroscope, and enlarged to 8 x 10 inches for analysis and karyotype.

Q-Banding. Slides were prepared as described above and rinsed in 100, 70, 50, and 20% ethanol for two min each. The slides were then placed in McIlvaine's buffer pH 4.5 for five min after which they were incubated in quinacridine dihydrochloride. After thirty minutes, the slides were placed in three changes of McIlvaine's buffer for two min, two min, and 10 min, respectively. A drop of water was placed on the slide and a large coverslip was mounted on the slide and sealed with nail polish. These slides were immediately viewed under UV illumination using a Zeiss UV photomicroscope. Micrographs were taken using Kodak Technical Pan film and enlarged to 8 x 10 inches for analysis.

Cell Fusion

Mammalian cells can be fused together using polyethylene glycol (PEG). The cell suspension fusion protocol of (Davidson et al., 1976) was used to hybridize CHO and human cells. Parental log phase cells were harvested, counted, mixed together (1-3 x $10^6$ cells of each line) and centrifuged. This pellet was quickly resuspended in 1 ml of 50% (w/v) PEG 1000 in $\alpha$-MEM without serum. The PEG was freshly prepared by autoclaving 10 g of PEG 1000 (Sigma) and adding 10 ml of $\alpha$-MEM to the cooled solution. It was usually necessary to adjust the pH back to neutrality with NaOH. After 1 min the cell suspension was diluted with 9 mls of $\alpha$-MEM and the cells are gently pelleted. The pellet was washed 2 times with $\alpha$-MEM and finally resuspended in 3 ml of $\alpha$-MEM and 15% FCS and incubated for 30 min at 37°C. After incubation cells are pelleted, resuspended and added to 60 mm plates at a 1/100 dilution. These plates are incubated overnight in non-selective medium. After 24 hours selective media containing 1.0 mM ouabain and 0.4 mM hydroxyurea was added. In some experiments the medium was changed after one week to remove cellular debris; colonies appeared after about 2 - 3 weeks. These colonies were picked, grown up, and tested for resistance to hydroxyurea. These clones were then frozen for later chromosome studies.

Incorporation of $^3$H-Thymidine Into DNA

DNA synthesis was measured by following the incorporation of $^3$H-thymidine into acid precipitable material as outlined by Hards and Wright (1983a). Exponentially growing cells, at a density of about $10^5$ cells per mm tissue culture plate, were incubated with 1 μCi/ml $^3$H-thymidine and 0.5 μg/ml thymidine in α-MEM plus 15% FCS for various lengths of time. For drug inhibition studies, hydroxyurea was added at the time of addition of thymidine. The enzyme reaction was stopped by the removal of medium and the addition of 10% ice cold trichloroacetic acid (TCA). After incubation on ice for 30 min the cells were scraped off the plate with a rubber policeman. This suspension was filtered through Whatman glass fiber filters which had been pre-soaked in ice cold 10% TCA. The filters were then washed with 2 x 10 ml aliquots each of 5% TCA, PBS, and 70% ethanol in succession. When multiple samples were assayed, a thirty sample Millipore manifold was used to rapidly wash the filters. After washing, the filters were placed in a glass scintillation vial; 1 ml of NCS tissue solubilizer was added, the caps tightened and the vials were heated at 60°C for 1 hour. The vials were allowed to cool, 10 ml of ACS cocktail and 75 μl of glacial acetic acid was added. The glacial acetic acid reduces the chemiluminescence which can be a problem when using tissue solubilizer. The vials were also often incubated in the dark prior to scintillation counting to reduce chemiluminescence.

## $^3$H-Mannose Incorporation into Cell Surface Membranes

Preparation of Cell Surface Membrane. Ten 150 mm plastic culture dishes (Lux Scientific Co.) containing 25 ml of αMEM supplemented with 15% fetal bovine serum, were seeded with $10^6$ cells, and incubated at 37°C until the monolayer approached confluence. One μCi/ml $^3$H-mannose was added to the plates and incubated for a further eight hours (Wright et al., 1979). The medium was aspirated and the cells rinsed once with 5 ml of PBS. Five ml of ethylene diamine tetraacetic acid (EDTA) solution in PBS (0.2 mg/ml) was added and the cells were scraped from dishes with a rubber policeman. Cells were harvested by centrifugation and washed once in 40 ml of PBS. Cell surface membranes were stabilized with 1 mM $ZnCl_2$ and disrupted by about 300 manual strokes of a Dounce homogenizer by the method of Brunette and Till (1971), except the polyethene-dextran two phase system was centrifuged only once at 100 x g for 10 min to ensure adequate amounts of protein were obtained for analysis by SDS gel electrophoresis.

SDS Slab Gel Electrophoresis. Slab gels (0.2 cm x 25 cm x 11.5 cm) were prepared and used according to the method of Laemmli (1970) and Parfett et al. (1983). For each track, 50 to 100 μg purified membrane protein in 10 μl of sample buffer was solubilized by heating to 100°C for three to five min. Electrophoresis was carried out at a constant current of 10 m Amperes, until the bromophenol blue tracking dye had reached the bottom of the separating gel (overnight). Proteins were fixed overnight in water:methanol:acetic acid (46:46:8). The gels were stained with Coomassie brilliant blue R and dried onto Whatman #1 filter paper. The slab gel was calibrated using proteins of known molecular weight.

The distribution of $^{3}H$-mannose labelled glycoproteins within each gel track was determined by simultaneously cutting 1 mm slices of the dried gel tracks to be compared, and estimating the radioactivity in each slice according to the method of Ames (1974) by scintillation counting.

# RESULTS

# RESULTS

## A. Selection of Normal Human Cell Strains

As discussed in the Historical section of this thesis, ribonucleotide reductase is critically associated with DNA synthesis and cell growth. Increased levels of RRase are characteristic of rapidly dividing tissues such as regenerating rat liver (Larsson, 1969), embryonic organs (Elford, 1972), and malignant growths (Elford et al., 1970). Ribonucleotide reduction is closely coupled to DNA synthesis and evidence suggests that the enzyme activity is a rate limiting step in this process and plays a key role in the regulation of cell division (Reddy & Pardee, 1980; Cory, 1983; Wright et al., 1981; Wright, 1983).

Much of the detailed analysis of RRase has been carried out using extracts from calf thymus (Eriksson et al., 1979), rabbit bone marrow (Hopper, 1972), regenerating rat liver (Cory, 1983), tumor derived cell lines (Takeda & Weber, 1981; Cory, 1983), and extracts or permeabilized permanent cell lines such as CHO, L, or 3T6 (Wright et al., 1981; Wright, 1983; Nicander and Reichard, 1981). Very few studies have been carried out with RRase from human tissue although detailed enzymological studies have been performed using abnormal transformed MOLT4F human lymphoblasts (Chang & Cheng, 1979a,b,c; 1980). The enzyme has been detected in phytohemagglutinin (PHA) stimulated human lymphocytes and bone marrow cells, however no detailed characterization has been reported (Tyrsted & Gamulin, 1978; Fujoka & Silber, 1971). A limiting factor in attempting a detailed investigation of the normal human enzyme is the difficulty in obtaining large quantities of material with high enzyme activity needed for enzyme purification. However it is important to examine the properties of the normal human reductase, especially in light of the close association between RRase and tumor progression (Elford et al., 1970,; Weber, 1983), transformation (Weber, 1983), somatic cell mutation rates (Weinberg et al., 1981; Chan et al., 1981; Meuth, 1979), the multi-enzyme DNA synthetic complex (Reddy & Pardee, 1980), control of differentiation (Creasey and Wright, 1983), and the imbalance of dNTP's which are involved in immunodeficiency diseases in man (Ullman et al., 1979).

Since RRase plays such a key role in cellular regulation, a convenient cell source which most closely resembles the normal _in vivo_ state was chosen. The source for these studies was the cell culture of normal human diploid fibroblasts. Human cell strains of fetal origin have a high rate of growth in culture and it was reasoned that they should contain high enough levels of RRase activity to carry out a general enzyme characterization study. It should be noted that a novel assay procedure had to be developed so conveniently obtainable quantities of cells could be used for the assay (Dick & Wright, 1980).

The particular cell strain of HDF which was eventually used was determined on the basis of three criteria. Firstly, since normal HDF have a limited lifetime _in vitro_ it was important to select a strain which was capable of many population doublings (PD) or passages. The cell strain had to be capable of maintaining a relatively high growth rate for a large portion of its _in vitro_ lifespan. Secondly the cell strain had to be able to grow as colonies at limiting dilution. This feature was necessary since genetic studies were planned to further characterize RRase regulation in normal HDF. Finally the cell yield per tissue culture plate had to be high in order to obtain as much material for enzyme assays as possible.

Table I shows several of the parameters which were measured for three different cell strains. El San is a skin fibroblast strain derived from a normal eight-year-old male. The main drawback to the use of cells obtained from non fetal tissues is the reduced lifespan (Schneider & Mitsui, 1976) as well as the low plating efficiency (PE). W1-38 is the most common HDF strain in present use and was isolated by Hayflick (1965) from female fetal lung tissue. This cell strain has a maximum lifespan of 50 $\pm$ 10 PD and showed a low plating efficiency and saturation density in α-MEM medium. HSC 172 was also derived from normal female fetal lung tissue (Buchwald & Ingles, 1976), however, the maximum lifespan of 95 $\pm$ 5 PD was almost twice as high as W1-38. This maximum lifespan determined in our laboratory for HSC 172 was identical to the value Gupta (1978) has reported for the same strain grown in similar medium. Other fetal cell strains have also recently been isolated which are capable of about the same number of doublings (Duthu et al., 1982). Furthermore the HSC 172 culture showed a higher saturation density and a higher PE than the other two cell strains (Table I). Therefore HSC 172 appeared to satisfy the requirements for a useful cell strain in which to study RRase, and also carry out somatic cell genetic experiments. It is worth noting that this strain has been used to isolate variants resistant to α-amanitin (Buchwald & Ingles, 1976) and diptheria toxin

Table I. Growth Characteristics of Three Normal Human Diploid Fibroblast Cell Strains

| Cell Line | Relative Colony Forming Ability (%) | Maximum Lifespan (Population Doubling) | Saturation Density Cells/100 mn Plate |
|---|---|---|---|
| EL SAN[a] | 2 | 15 - 35[a] | - |
| WI-38[b] | 1 | 50 $\pm$ 10[b] | 2-3 x $10^6$ |
| HSC 172 | 25 | 95 $\pm$ 5 | 5-6 x $10^6$ |

[a] from American Type Culture Collection catalogue.

[b] Hayflick, 1965.

(Gupta & Siminovitch, 1978b).

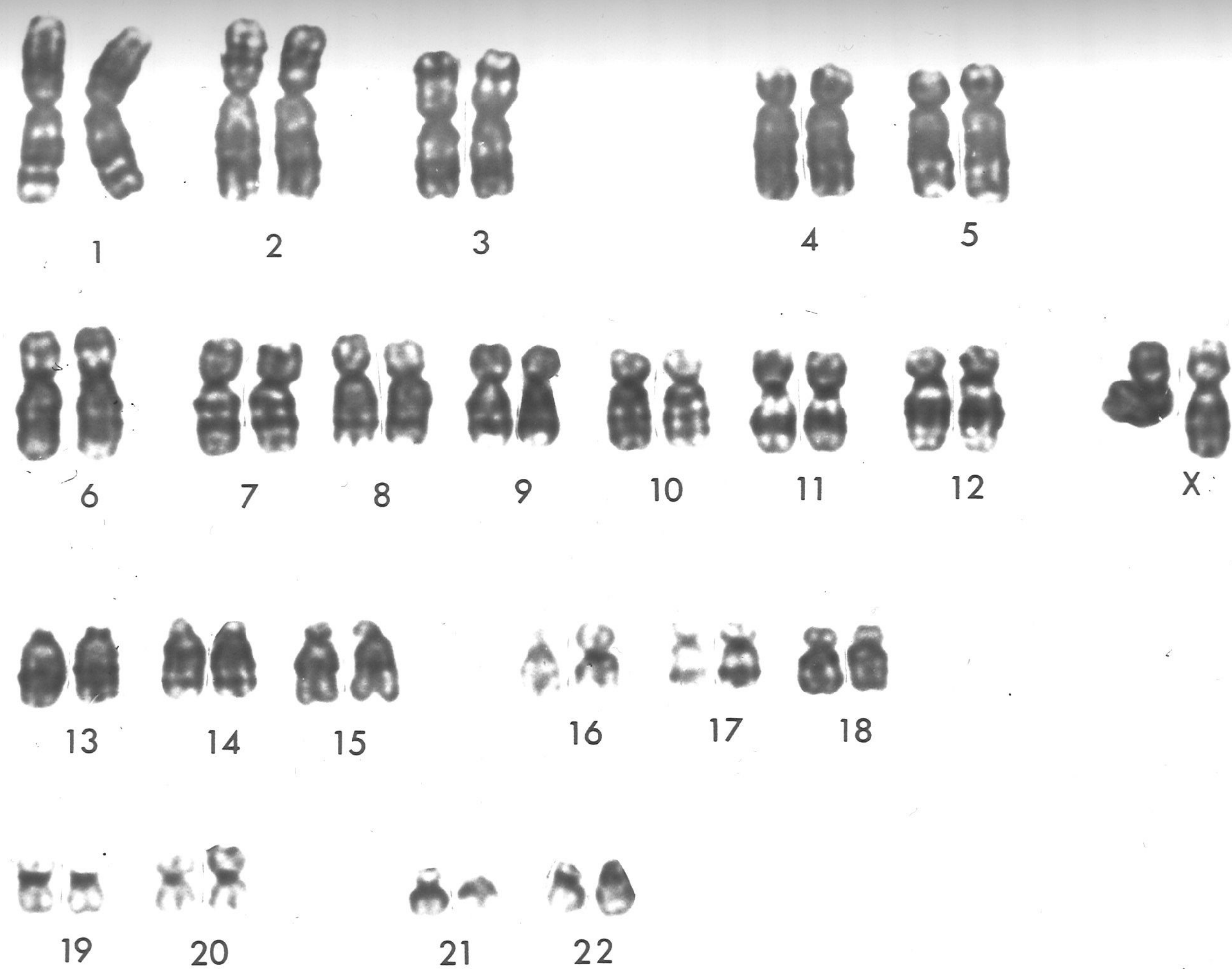
One of the key ways to examine for normality of a cell strain is to determine the karyotype. Many supposed normal cell strains have been found to be contaminated with Hela cells (Lavappa et al., 1976). Figure 1 shows a Giemsa trypsin banded karyotype of HSC 172. The karyotype was found to be 46, XX with no detectable abnormalities.

B. Ribonucleotide Reduction in Normal Human Diploid Fibroblasts

Development of RRase Assay in HDF

In spite of the long-lived nature of HSC 172, it was not possible to obtain enough material to attempt a large scale purification of RRase since HSC 172, as all normal HDF, showed anchorage and density dependant growth. It must be emphasized that mammalian RRase from any source has been extremely difficult to purify. To date there have been only three groups which report purification of at least one of the two subunits ($M_1$ or $M_2$), with only $M_1$ having been purified to apparent homogeneity (Engstrom et al., 1982). RRase is present at rather low intracellular levels and much activity is lost during purification. For instance Engstrom et al., (1979) started with 5 kg of calf thymus and ended up with 10 mg of highly purified enzyme. However this enzyme preparation consisted mainly of $M_1$ component with $M_2$ present in low non-stoichiometric amounts. Furthermore purification of an enzyme as complex as RRase may mask some key regulatory features normally found inside the cell. This is evident when a theoretical estimate of the amount of reductase activity required by the cell in order to maintain DNA synthesis is compared to the actual yield in extracts (Lewis et al., 1978); the specific activity is very low.

Figure 1. Karyotype of HSC 172 normal human diploid fibroblasts. Chromosomes were trypsin banded and stained with Giemsa according to the method of Seabright (1971) as described in Methods and Materials.


1
2
3
4
5
6
7
8
9
10
11
12
X
13
14
15
16
17
18
19
20
21
22

This problem of purification was addressed by the development of a novel permeabilization assay in our laboratory for CHO and L cells (Lewis et al., 1978; Kuzik & Wright, 1980; Koropatnick & Wright, 1980; Hards and Wright, 1983a). This procedure involves the permeabilization of cells with the detergent Tween 80 to allow endogenous inhibitors to escape from the cell as well as nucleotides to enter the cell since normally the cell membrane is impermeable to nucleotides. This technique, therefore, assays the intracellular activity of RRase in as near a normal environment as possible. In this way activities of RRase which are able to account for 50 - 100% of the predicted levels required for DNA synthesis have been measured (Lewis et al., 1978; Hards & Wright, 1983b).

Since this _in vivo_ permeabilization assay required relatively few cells compared to enzyme purification, attempts were made to modify the assay in order to measure purine (ADP) and pyrimidine (CDP) reduction in normal HDF (Dick & Wright, 1980). This novel procedure enabled the detection and characterization of RRase from normal HSC 172 human diploid fibroblasts (Dick & Wright, 1980; 1982; 1983; Wright et al., 1981). The assay was relatively convenient and able to measure the enzyme inas few as $10^6$ cells obtained from a single tissue culture plate.

The exact protocol used in the reductase assay for HDF is described in detail the Methods and Materials section. The assay is based on the conversion of $^{14}C$-NDP to $^{14}C$-dNDP. The $^{14}C$-dNDP was converted to $^{14}C$-dN with _Crotalus atrox_ phoshodiesterase, and run through a Dowex 1-borate anion exchange column to separate the product from the substrate (Steeper & Steuart, 1970; Cory et al., 1973).

Figure 2 shows the time course of snake venom phosphodisterase hydrolysis of dADP and dCDP to dA and dC respectively. The reaction was essentially complete by 60 minutes; however, samples were routinely incubated for two hours to ensure complete hydrolysis of the phosphates.

Dowex 1-borate binds cis-diols of the type seen in D-ribose; since 2' deoxyribose does not contain a cis-diol configuration, deoxynucleosides do not bind to the resin (Khym, 1966). Figure 3A shows that complete elution of dC was achieved by running 5 mls of distilled $H_2O$ through a small 0.5 cm x 7 cm column. Complete elution of dA was achieved after 20 mls of 1 mM sodium borate had been run through the column (Figure 3B). The ribonucleoside bases cytosine and adenine bind tightly to the column and can only be removed by eluting with saturated sodium borate.

During the course of these studies many different commercial batches of $^{14}$C-CDP label were used for enzyme assays. Radiolabeled CDP breaks down during storage so background values can fluctuate greatly. Normally these background values can be subtracted to give total net activity. However, some preparations of commercially available $^{14}$C-CDP were found to contain high levels of background counts; greater than 2000 dpm/assay, with some lots as high as 7000 dpm. Normal backgrounds were less than 1500 dpm. Since HDF normally have rather low levels of RRase activity as compared to permanent cell lines the ratio of dpm in assay/dpm in background (signal to noise) became unacceptably low. At such low ratios, counting errors and other random effects became exaggerated resulting in non-reproducble

Figure 2A. Time course of the conversion of dADP to dA by _Crotalus_ _Atrox_ snake venom phosphodiesterase under standard assay conditions. At each time point the assay mixture was passed through a Dowex 1-borate column with 20 mls of 1 mM Na Borate. The absorbance at 280 nm of 0.2 OD represented 100% conversion of dADP to dA.

Figure 2B. Time course of the conversion of dCDP to dC by _Crotalus_ _Atrox_ snake venom phosphodiesterase under standard assay conditions. At each time point the assay mixture was passed through a Dowex 1-borate column with 4 mls of distilled $H_2O$. The absorbance at 280 nm of 0.25 OD represented 100% conversion of dCDP to dC.

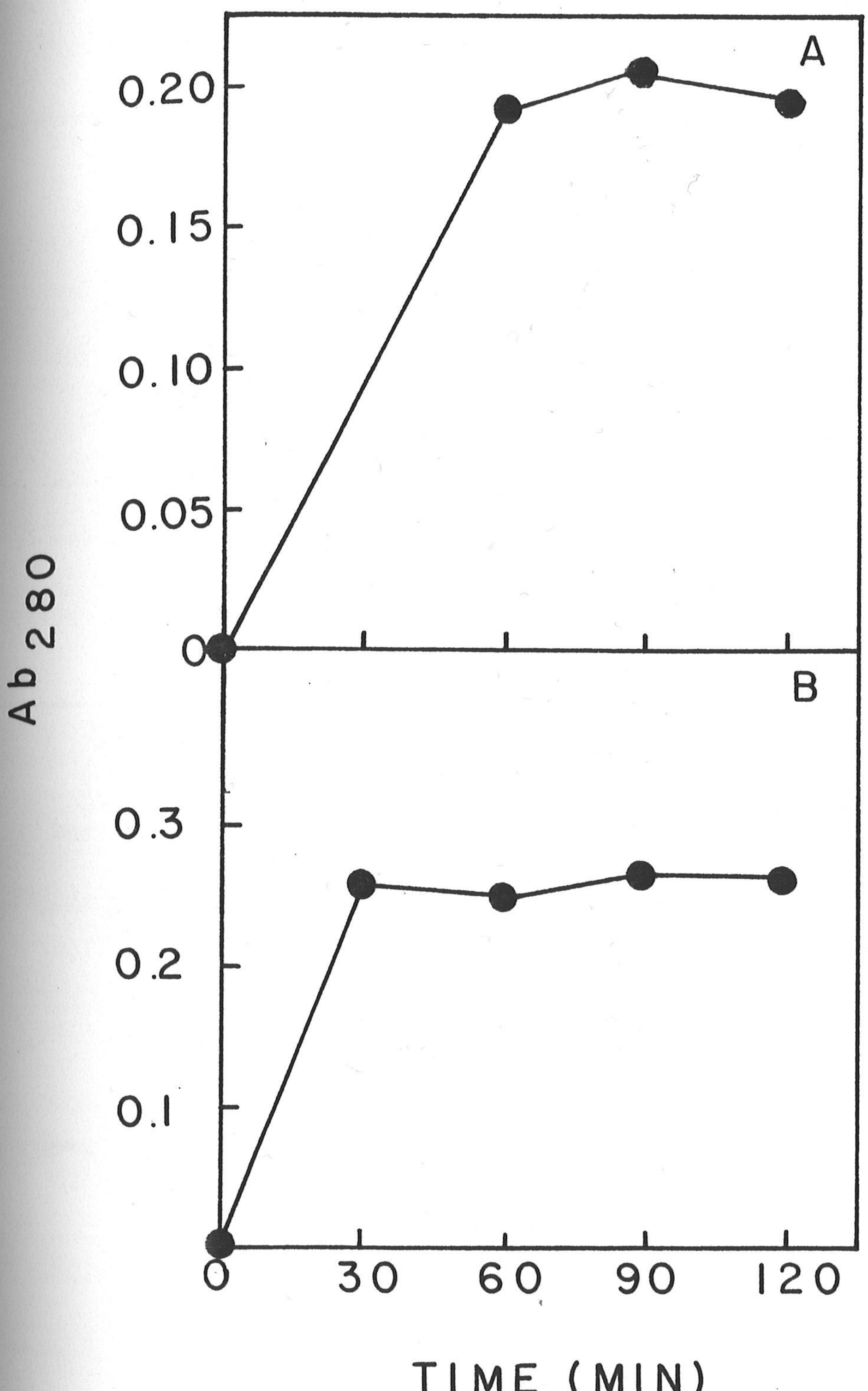
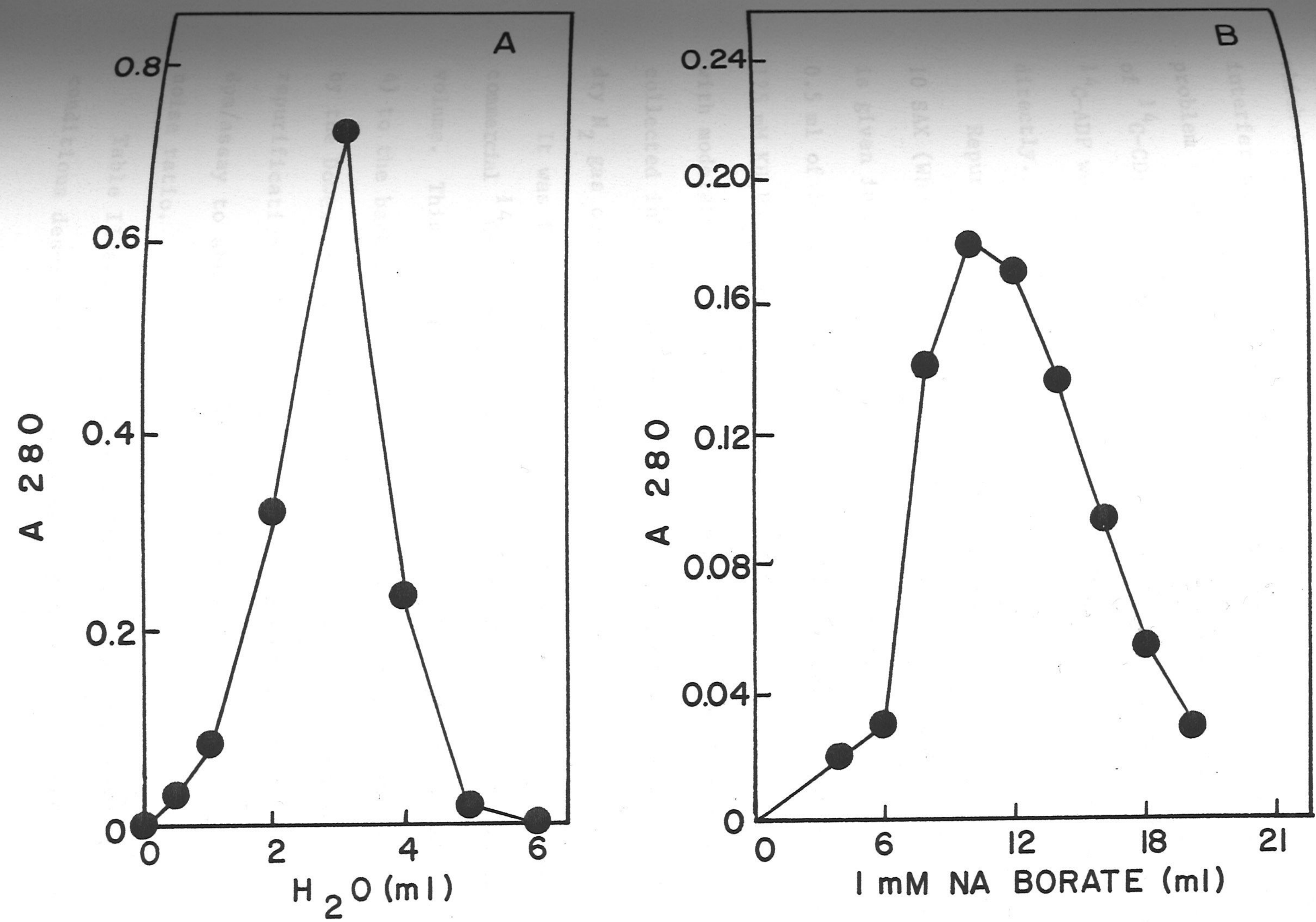

A
B
0.20
0.15
0.10
0.05
0
0.3
0.2
0.1
Ab 280
0
30
60
90
120
TIME (MIN)

Figure 3A. Elution profile of dC on a Dowex 1-borate anion exchange column (0.5 x 8 cm). dC was added to the column and washed with 1 ml aliquots of $H_2O$ which were collected and the absorbance at 280 nm was determined for each fraction.

Figure 3B. Elution profile of dA on a Dowex 1-borate anion exchange column (0.5 x 8 cm). dA was added to the column and washed with 2 ml aliquots of 1 mM Na borate which were collected and the absorbance at 280 nm was determined for each fraction.


A
0.8
0.6
0.4
0.2
0
A 280
0
2
4
6
H 2 O (ml)
B
0.24
0.20
0.16
0.12
0.08
0.04
0
A 280
0
6
12
18
21
1 mM NA BORATE (ml)

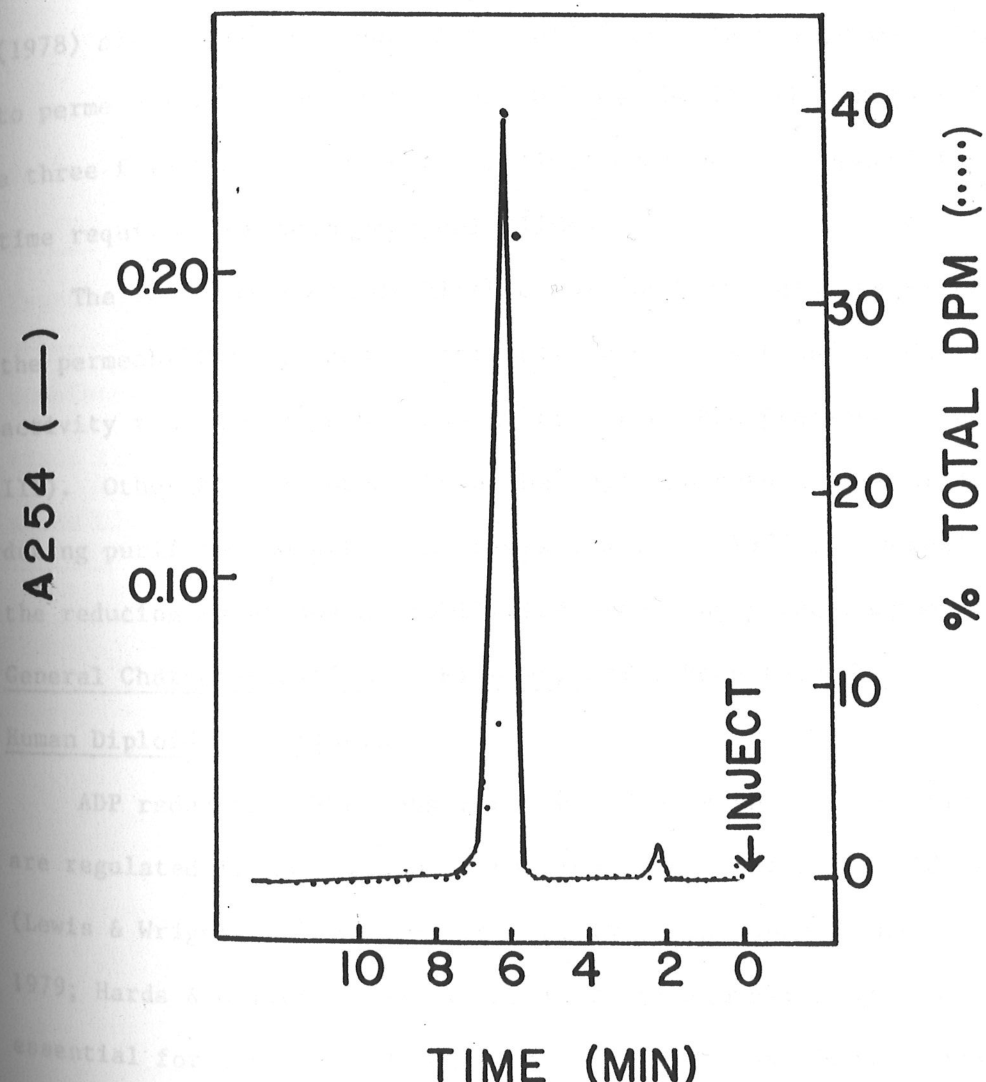
results. Often the enzyme activity dropped to very low levels suggesting that the impurity in the commercially labeled nucleotide which was contributing to the high background may also have been interfering with the reductase assay. In order to overcome this problem it became necessary to repurify the commercial preparations of $^{14}C$-CDP. The background counts in commercial preparations of $^{14}C$-ADP were routinely low, therefore these preparations were used directly.

Repurification of $^{14}C$-CDP was carried out by HPLC using a Partisil 10 SAX (Whatman) anion exchange column. A representative chromatogram is given in Figure 4. This figure shows the isocratic elution of 0.5 ml of 50 $\mu$Ci/ml of $^{14}C$-CDP (500 mCi/m mole) using 125 mM KCl and 125 mM $KH_2PO_4$ pH 4.5. The nucleotide was rapidly eluted in 7 minutes with moderate sample dilution. Generally the complete peak could be collected in 4 mls of buffer and concentrated by passing a stream of dry $N_2$ gas over the sample.

It was found that most of the background dpm in the impure commercial $^{14}C$-CDP originated in a compound which eluted in the void volume. This fraction contributed about 0.6% of the total dpm (Figure 4) to the background and over 50% of this fraction was not retained by the Dowex column resulting in high background counts. This repurification of $^{14}C$-CDP reduced the background from a high of 7000 dpm/assay to about 200 dpm/assay, greatly enhancing the signal to noise ratio.

Table II shows that permeabilizing normal HDF under the conditions described in Methods and Materials does enhance detection

Figure 4. Chromatographic separation of commercial preparations of $^{14}C$-CDP (Amersham) by HPLC. $^{14}C$-CDP (450 µl; 22.5 µCi) was injected into a Partisil 10 SAX anion exchange column (25 cm x 4.6 mm; Whatman) and eluted with 125 mM KCl, 125 mM $KH_2PO_4$ pH 4.5 running at 2 mls per minute. Absorbance at 254 nm was monitored with a Beckman model 160 detector and 2 ml fractions were collected with a LKB fraction collector. Radioactivity in each of the fractions was determined on a Beckman Model 9800 liquid scintillation counter programmed to integrate the radioactivity present in each peak.


A254 (—)
0.20
0.10
% TOTAL DPM (.....)
40
30
20
10
0
←INJECT
10 8 6 4 2 0
TIME (MIN)

of RRase activity for both ADP (70%) and CDP (80%) reductions, probably by allowing the entry of nucleotides and the removal of endogenous inhibitors (Billen and Olson, 1978; Lewis et al., 1978). The time of permeabilization was about three times longer than that required for CHO cells (Lewis et al., 1978). Interestingly, Billen and Olson (1978) also found that normal diploid fibroblasts were more difficult to permeabilize in order to assay DNA synthesis; they required about a three fold increase in permeabilization time as compared to the time required for permanent cell lines.

The reducing agent dithiothreitol (DTT) was also required during the permeabilization procedure; only about 8% ADP and 4% CDP reductase activity remained when DTT was omitted from the procedure (Table III). Other studies have shown that DTT protects enzyme activity during purification attempts (Lewis & Wright, 1978b), suggesting that the reducing agent has a stabilizing effect upon the enzyme.

## General Characterization of Ribonucleotide Reductase from Human Diploid Fibroblasts

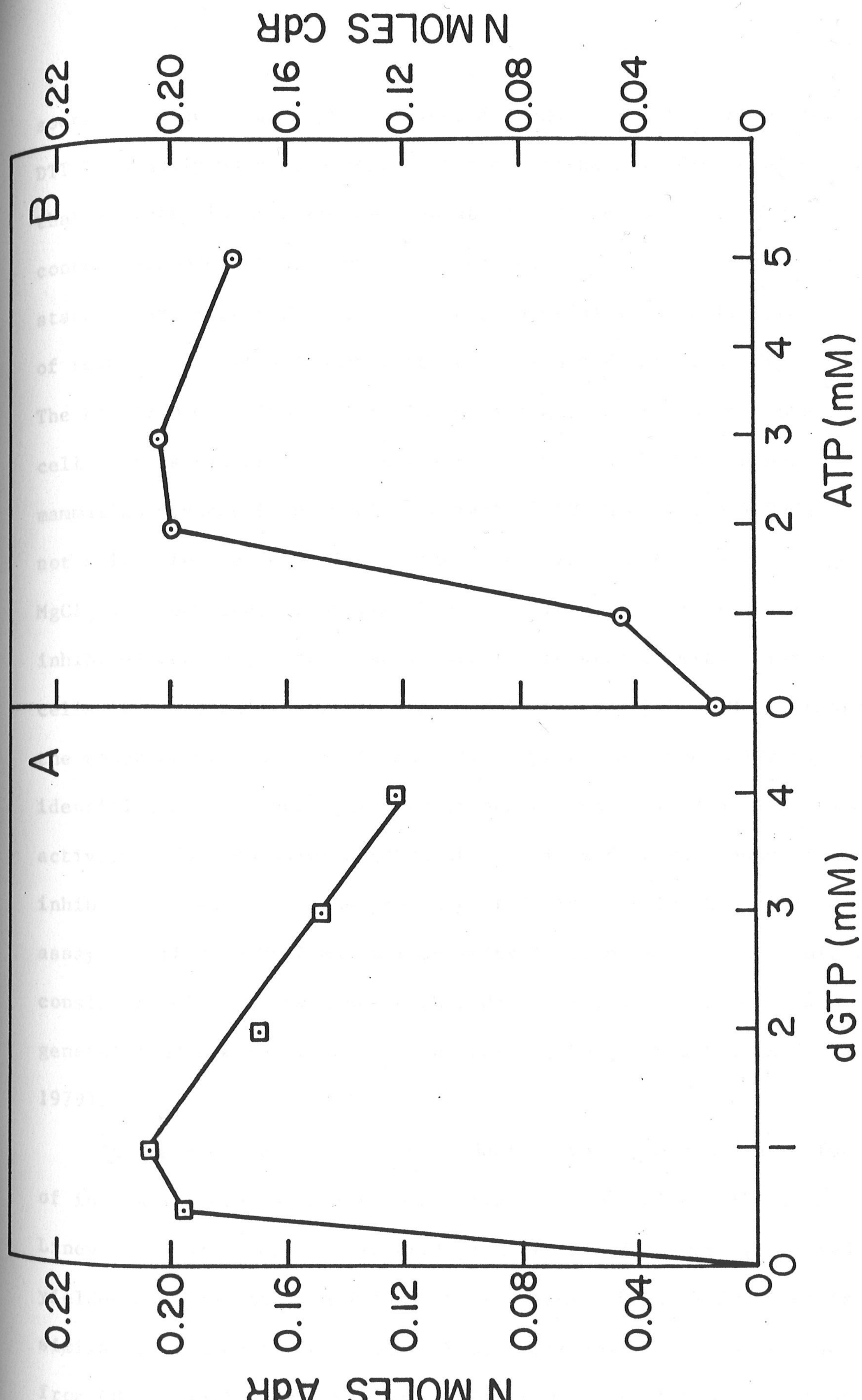
ADP reducton: The substrate specificity and the reaction rate are regulated in an allosteric fashion by several nucleotide triphosphates (Lewis & Wright, 1978b; Cory et al., 1975, Thelander & Reichard, 1979; Hards & Wright, 1983b). As in other mammalian systems dGTP was essential for maximum ADP reductase activity. As shown in Figure 5A a concentration of 0.5 mM to 1.0 mM dGTP resulted in maximum purine reduction. Thioredoxin, through the thioredoxin reductase system (Thelander, 1976) or glutaredoxin via glutathione and glutathione reductase (Luthman et al., 1979) can function as an endogeneous

Table II. Permeabilization Treatment and Ribonucleotide Reduction in Human Diploid Fibroblasts

| Treatment | % reductase ADP | activity CDP |
|---|---|---|
| complete | 100 | 100 |
| minus tween-80 | 30 | 20 |
| minus DTT | 8 | 4 |

Figure 5A. Response of ADP reduction in HSC 172 cells to varying concentrations of dGTP. Standard ADP reductase assay conditions were used except for varying concentrations of dGTP.

Figure 5B. Response of CDP reduction in HSC 172 cells to varying concentrations of ATP. Standard CDP reductase assay conditions were used except for varying concentrations of ATP.


A
B
N MOLES AdR
N MOLES CdR
dGTP (mM)
ATP (mM)
0.22
0.20
0.16
0.12
0.08
0.04
0
1
2
3
4
5

source of electrons for ribonucleotide reduction. In enzyme assays DTT is usually used as a source of reducing power. Figure 6A shows that activity is observed between about 1.0 and 16 mM DTT with a concentration of about 6 mM being optimal. Furthermore, using the standard assay procedure (Methods and Materials) the cells are capable of reducing ADP at a linear rate for at least 70 minutes (Figure 7A). The reaction was also linear with cell number over a wide range of cell numbers (Figure 8A). It was found that unlike some other mammalian systems (i.e. Kuzik & Wright, 1980; Moore, 1969) $FeCl_3$ did not stimulate ADP reduction in the human fibroblasts; and although $MgCl_2$ was required for optimal CDP reduction, it significantly inhibited the reduction of ADP. Similar to studies with hamster cells (Lewis et al., 1978b; Hards & Wright, 1983b) 8 mM $MgCl_2$ inhibited the reaction by 50%. In all mammalian systems examined dATP has been identified as a potent inhibitor of purine and pyrimidine reductase activity. In keeping with these observations dATP was found to markedly inhibit ADP reduction; the presence of 40 μM dATP in the standard assay resulted in half maximal activity (Figure 9A). This result is consistent with the hypothesis that dATP fulfills the role of a general feedback inhibitor of the enzyme (Thelander & Reichard, 1979).

The rate of reaction of ADP reduction was determined as a function of increasing concentration of substrate (inset, Figure 10A). A Lineweaver-Burk double reciprocal plot of this data was linear and yielded an apparent Km of $0.10 \pm .05$ mM ADP (Figure 10A). This value is similar to the Km for ADP observed with partially purified enzyme from CHO cells (Lewis & Wright, 1978b) and is about two times higher

Figure 6A. Response of ADP reduction in HSC 172 cells to varying concentrations of DTT. Standard ADP assay conditions were used except for varying concentrations of DTT.

Figure 6B. Response of CDP reduction in HSC 172 cells to varying concentrations of DTT. Standard assay conditions were used except for varying concentrations of DTT.

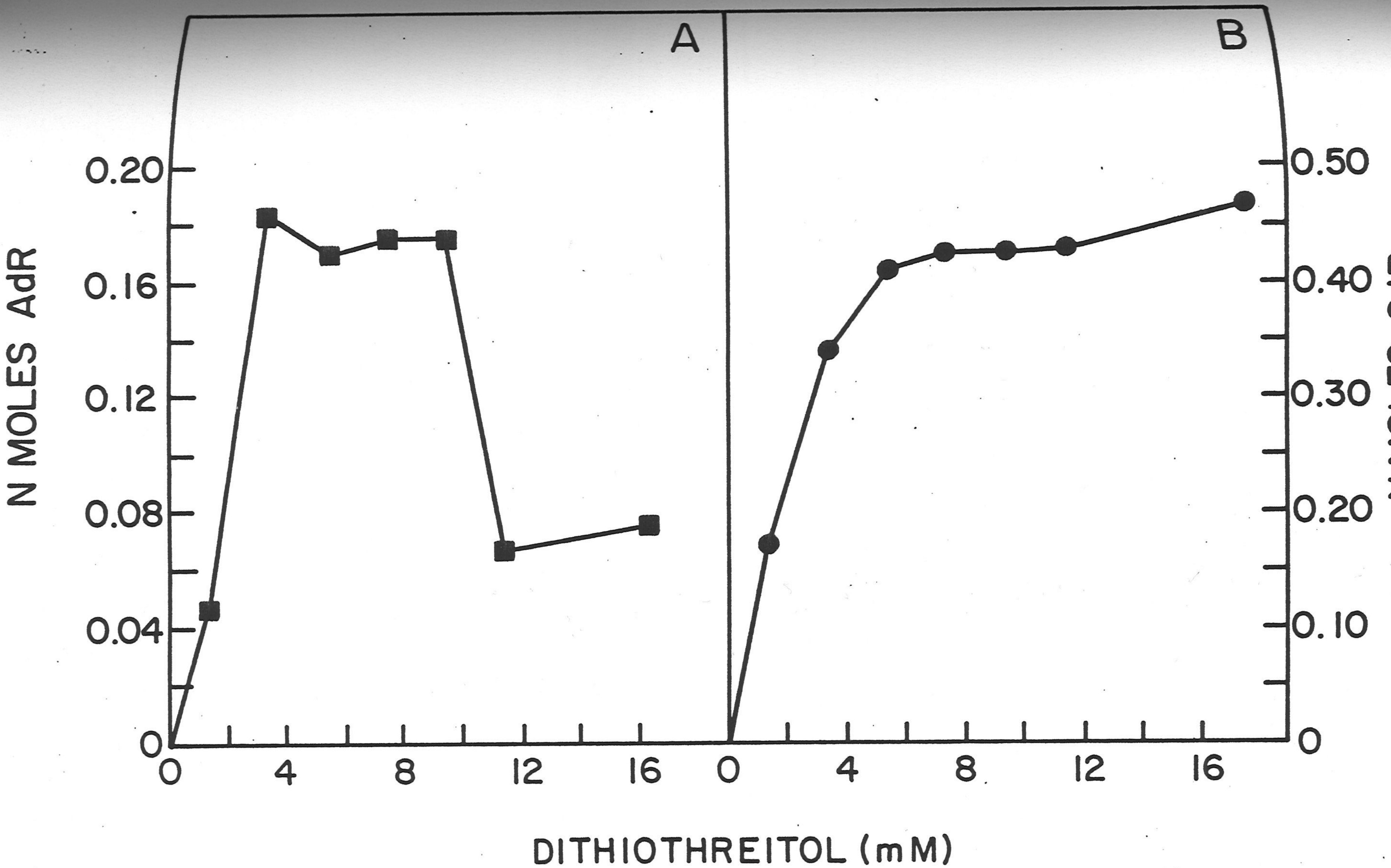

A
B
N MOLES AdR
N MOLES CdR
0.20
0.16
0.12
0.08
0.04
0
0.50
0.40
0.30
0.20
0.10
0
0
4
8
12
16
0
4
8
12
16
DITHIOTHREITOL (mM)

Figure 7A. Response of ADP reduction in HSC 172 cells to varying incubation times. Standard ADP reductase assay conditions were used except for the variation in incubation time.

Figure 7B. Response of CDP reduction in HSC 172 cells to varying incubation times. Standard CDP reductase assay conditions were used except for the variation in incubation time.

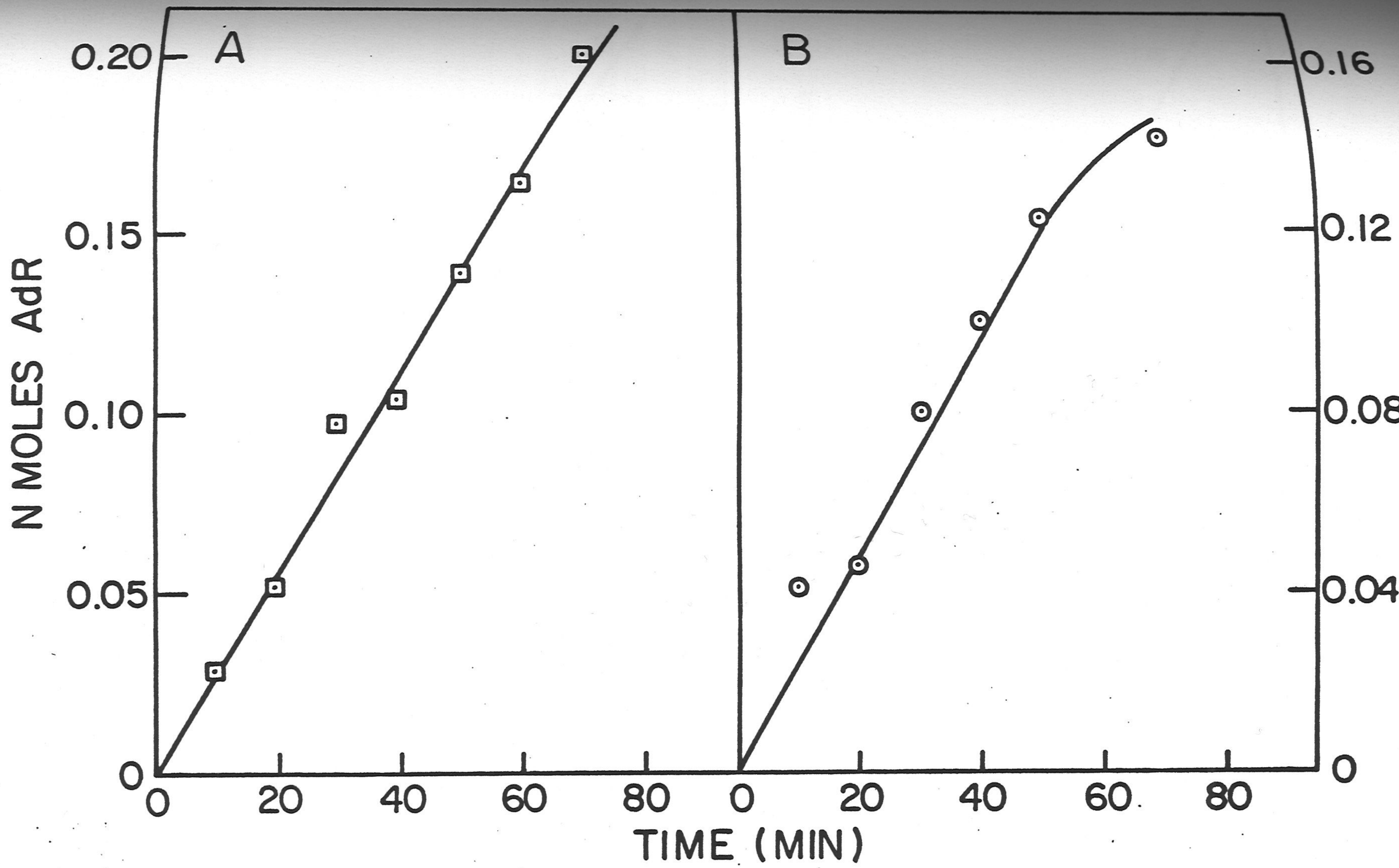

A
B
0.20
0.15
0.10
0.05
0
N MOLES AdR
0.16
0.12
0.08
0.04
0
N MOLES CdR
0
20
40
60
80
0
20
40
60
80
TIME (MIN)

Figure 8A. Response of ADP reduction in HSC 172 cells to varying cell numbers. Standard ADP reductase assay conditions were used except for the variations in cell number.

Figure 8B. Response of CDP reduction in HSC 172 cells to varying cell numbers. Standard CDP reductase assay conditions were used except for the variation in cell number.

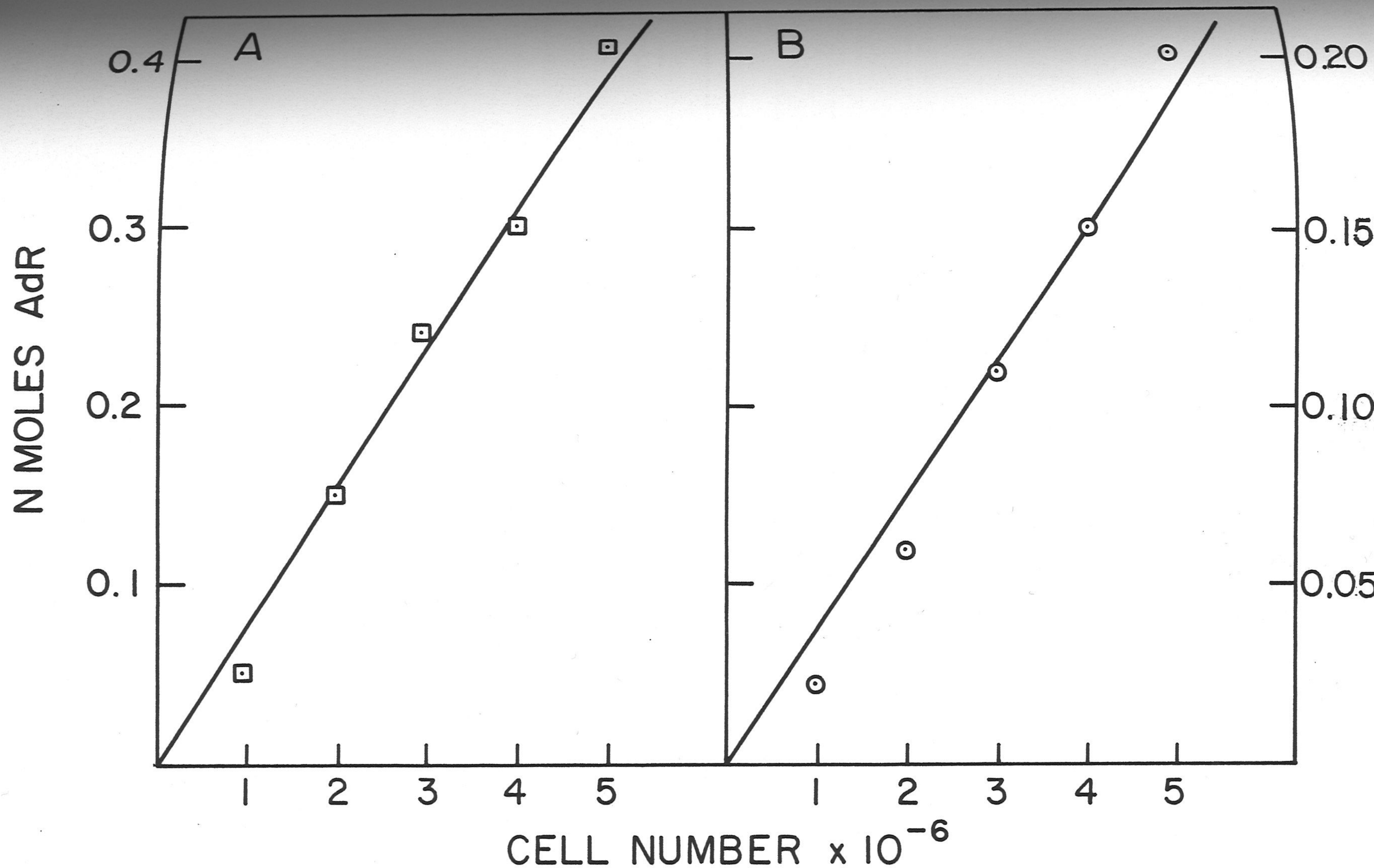

A
B
0.4
0.3
0.2
0.1
N MOLES AdR
0.20
0.15
0.10
0.05
N MOLES CdR
1
2
3
4
5
CELL NUMBER x 10^-6

Figure 9A. Response of ADP reduction in HSC 172 cells to varying concentrations of dATP. Standard ADP reductase assay conditions were used except for varying concentrations of dATP.

Figure 9B. Response of CDP reduction in HSC 172 cells to varying concentrations of dATP. Standard CDP reductase assay conditions were used except for varying concentrations of dATP.

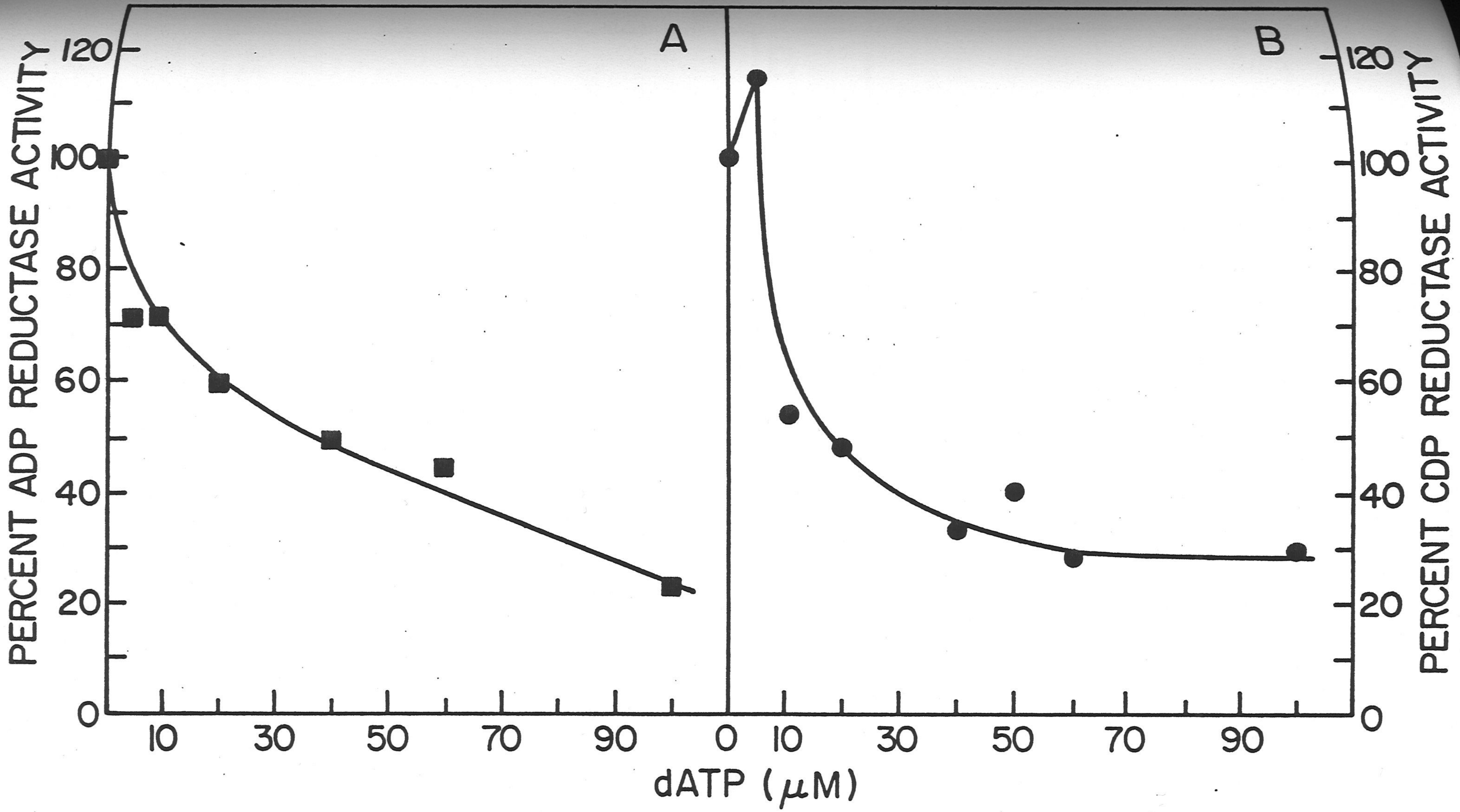
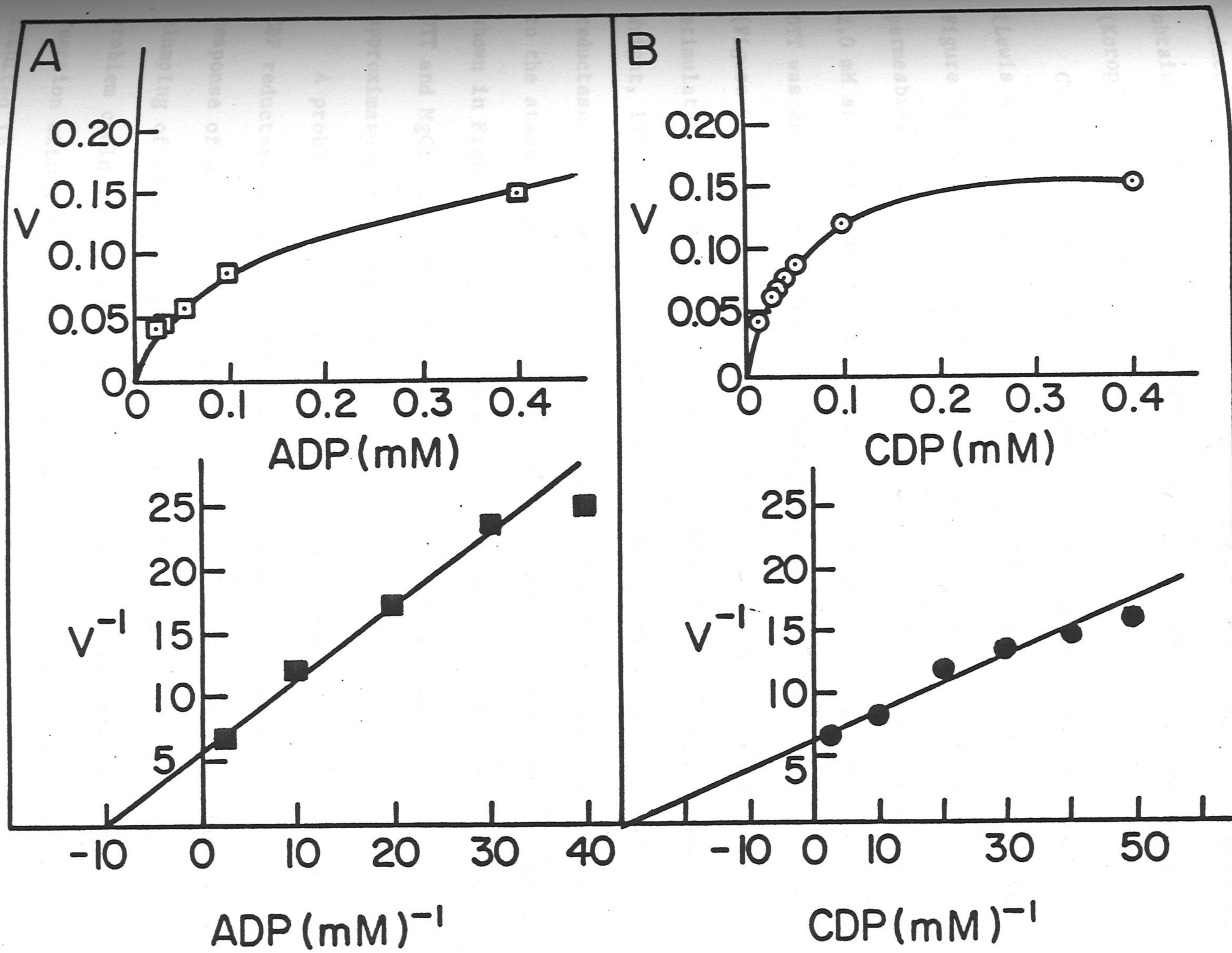

A
B
PERCENT ADP REDUCTASE ACTIVITY
PERCENT CDP REDUCTASE ACTIVITY
0
20
40
60
80
100
120
10
30
50
70
90
dATP (μM)

Figure 10A. Double reciprocal plot of the variation in the rate of ADP reduction with varying ADP concentrations using HSC 172 cells. Standard ADP reductase assay conditions were used except for varying concentrations of ADP.

Inset: Velocity of ADP reduction in the presence of varying concentrations of ADP.

Figure 10B. Double reciprocal plot of the variation in rate of CDP reduction with varying CDP concentration using HSC 172 cells. Standard CDP reductase assay conditions were used except for varying concentrations of CDP.

Inset: Velocity of CDP reduction in the presence of varying concentrations of CDP.

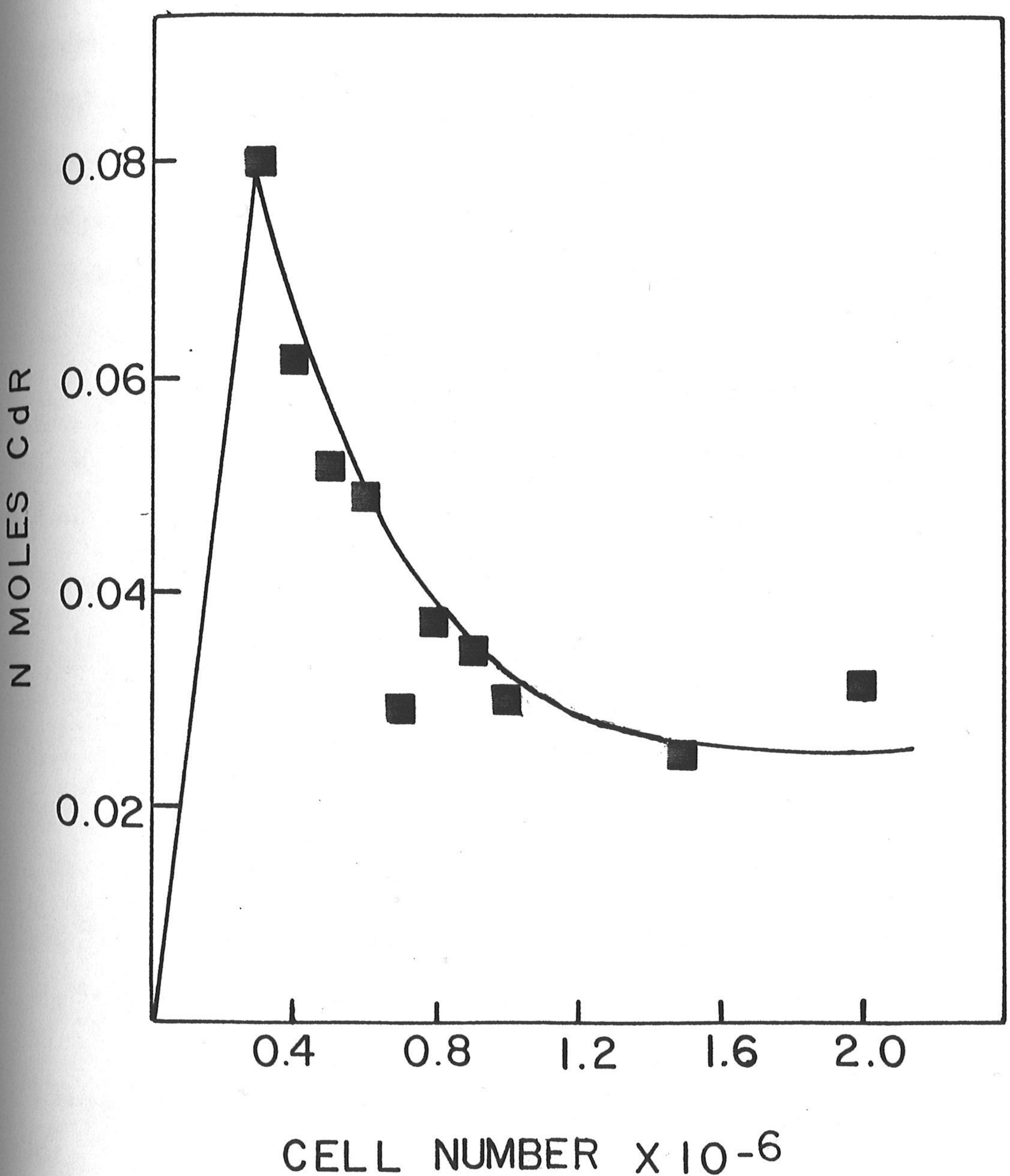

A
B
V
0.20
0.15
0.10
0.05
0
0.1
0.2
0.3
0.4
ADP (mM)
CDP (mM)
$V^{-1}$
25
20
15
10
5
-10
30
40
50
ADP $(mM)^{-1}$
CDP $(mM)^{-1}$

than the value recently reported for highly purified calf thymus ADP reductase (Eriksson et al., 1979). It also compares well with results obtained with permeabilized CHO (Lewis et al., 1978) and mouse cells (Koropatnick & Wright, 1980).

CDP reduction: ATP is a positive effector for CDP reductase (Lewis & Wright, 1978b; Eriksson et al., 1979; Hards & Wright, 1983b). Figure 5B shows that ATP is required for optimal activity in permeabilized fibroblasts with maximum stimulation occuring between 2.0 mM and 3.0 mM nucleotide. Also, as in the ADP reductase assay, DTT was an excellent source of reducing power for CDP reduction (Figure 6B). It has been shown in other systems that magnesium stimulates CDP reduction (Cory et al., 1975; Larsson, 1969; Kuzik & Wright, 1980). A concentration of 8 mM $MgCl_2$ stimulated the CDP reductase activity in fibroblasts five fold and was routinely added to the standard assay for CDP reduction in permeabilized cells. As shown in Figure 7B, in the presence of optimal concentrations of ATP, DTT and $MgCl_2$ the CDP reductase activity was linear with time to approximately fifty minutes.

A problem initially encountered during the development of the CDP reductase assay with human diploid fibroblasts was nonlinear response of activity with cell number (Figure 11). This was due to a clumping of cells during the reaction and it was found that the problem could be solved by spinning the cells during the reductase reaction with a 2 x 7 mm stirring bar, which prevented clumping and resulted in linear response of enzyme activity with cell number (Figure 8B). Interestingly cells in ADP reaction buffer did not

Figure 11. Response of CDP reduction in HSC 172 cells to variations in cell number with no mixing of the assay tube. Standard CDP reductase assay conditions were used except for the variation in cell number and no spin bars were added to reaction tubes.


N MOLES CdR
0.08
0.06
0.04
0.02
0.4
0.8
1.2
1.6
2.0
CELL NUMBER X 10-6

clump; since the only difference between the two buffers is the presence of $MgCl_2$, presumably $MgCl_2$ enhanced cell-cell interaction.

As noted earlier dATP is considered to be a general feedback inhibitor of purine and pyrimidine reductase activity (Lewis & Wright, 1978b; Thelander & Reichard, 1979). In the permeabilized fibroblast cells dATP was a potent inhibitor of CDP reduction at concentrations greater than 5 μM, with 20 μM dATP resulting in half maximal activity (Figure 9B). At very low concentrations of effector a slight stimulation of activity was often observed. Eriksson et al., (1979) have shown that in the presence of an excess of ATP the dATP inhibition is reduced, presumably by competition at the dATP binding site. However the nature of the stimulation in this case is not clear but may involve complex enzyme conformational changes in presence of the negative effector (Thelander & Reichard, 1979).

A double reciprocal Lineweaver-Burk plot of CDP reduction at various substrate concentrations is shown in Figure 10B. The plot is linear and gives an apparent Km value of $0.04 \pm .02$mM CDP. This estimate is very similar to the Km determination reported for highly purified calf thymus CDP reductase (Eriksson, et al., 1979) and about two fold lower than the values found with partially purified hamster (Lewis & Wright, 1978b) or mouse (Kuzik & Wright, 1979) enzyme.

Hydroxyurea inhibition of ribonucleotide reductase: Ribonucleotide reductase is found in one of two basic forms in a variety of organisms. The _Lactobacillus_ type reduces ribonucleoside triphosphates (Panagou et al., 1972), requires 5'-deoxyadenosylcobalamin for activity and is not inhibited by the antitumor drug, hydroxyurea. The _E. coli_ type which reduces substrates at the diphosphate level, contains iron and is inhibited by hydroxyurea (Lewis & Wright, 1974; 1978b) has been found in all mammalian and fungal sources studied to date (Eriksson et al., 1979; Thelander & Reichard, 1979; Lewis et al., 1977; Wright, 1983; Cory, 1983).

It is thought that hydroxyurea acts by destroying the tyrosine free radical of the $M_2$ subunit thereby inhibiting the ability of the enzyme to perform reductions (Thelander & Reichard, 1979). This free radical can be regenerated when hydroxyurea is removed from the assay, in contrast hydroxyurea inhibition of the E. coli enzyme is irreversible. Hydroxyurea inhibited the reduction of CDP in human cells with half maximal activity obtained at about 0.2 mM drug (Figure 12). Figure 13 shows a time course of ADP and CDP reduction in the presence and absence of hydroxyurea. In both cases the reaction was markedly inhibited by hydroxyurea and proceeded linearly in the presence and absence of drug. To examine the inhibitory effects of hydroxyurea further, the purine and pyrimidine substrates were individually varied in the presence of different fixed concentrations of drug. The results of the study are presented in Figures 14 A and B. When either of the two substrates was varied the double reciprocal plots were linear and indicated noncompetitive or mixed type inhibition. Replots of the intercepts against substrate concentrations were also linear and yielded apparent Ki values of hydroxyurea inhibition of $0.40 \pm .1$ and $0.41 \pm .1$ mM for ADP and CDP respectively. Interestingly, these Ki values are about four fold higher than the apparent Ki from CHO cells (Lewis & Wright, 1978; Hards & Wright, 1981) suggesting the

Figure 12. Response of CDP reduction in HSC 172 cells to varying concentrations of hydroxyurea. Standard CDP reductase assay conditions were used except for the varying concentrations of hydroxyurea.

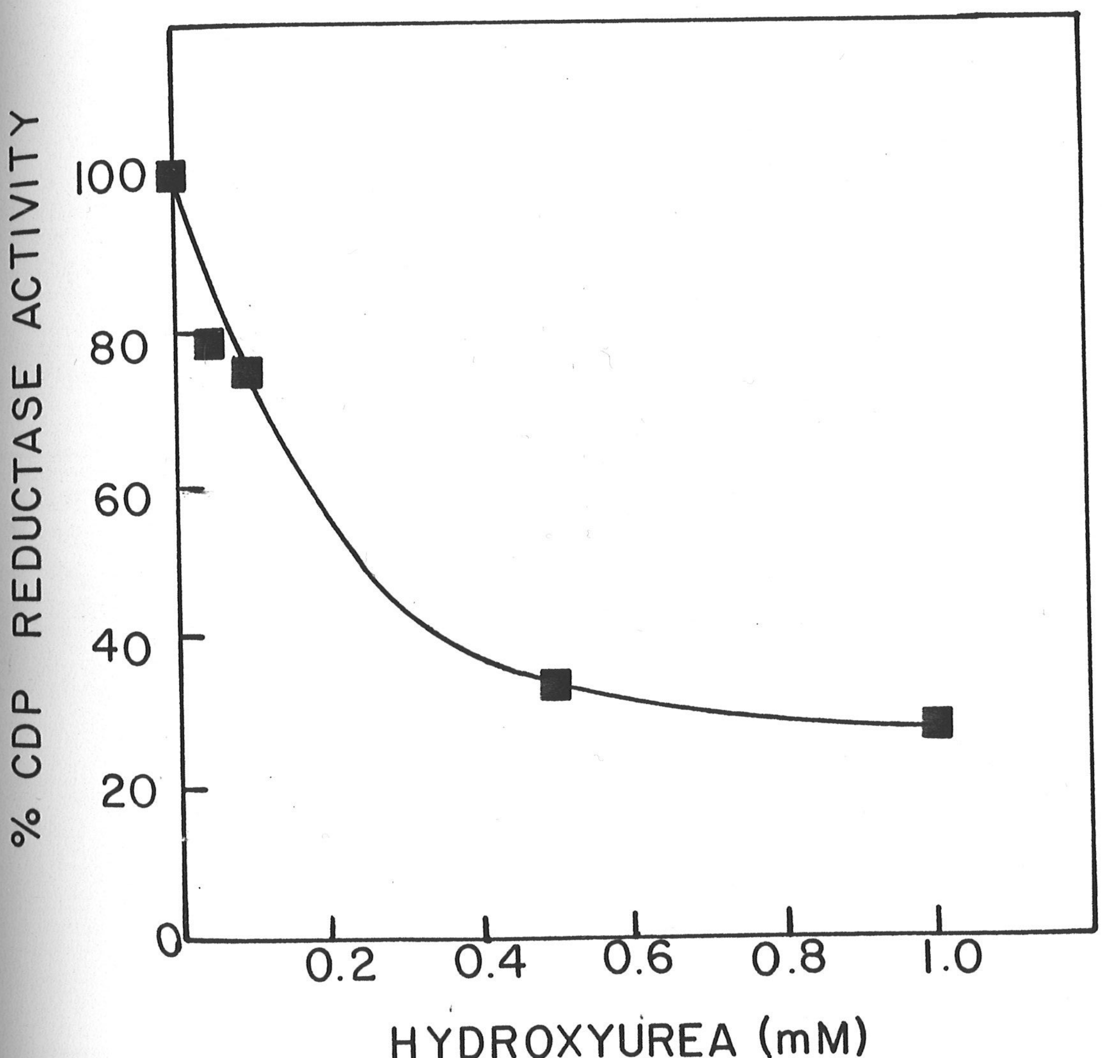

% CDP REDUCTASE ACTIVITY
100
80
60
40
20
0
0.2
0.4
0.6
0.8
1.0
HYDROXYUREA (mM)

Figure 13A. Response of ADP reduction in HSC 172 cells to variation in incubation time in the presence (□) or absence (■) of 1.0 mM hydroxyurea. Standard ADP reductase assay conditions were used except for the variation in incubation time and the addition of 1.0 mM hydroxyurea.

Figure 13B. Response of CDP reduction in HSC 172 cells to variations in incubation time in the presence (○) or absence (●) of 1.0 mM hydroxyurea. Standard CDP reductase assay conditions were used except for the variation in incubation time and the addition of 1.0 mM hydroxyurea.

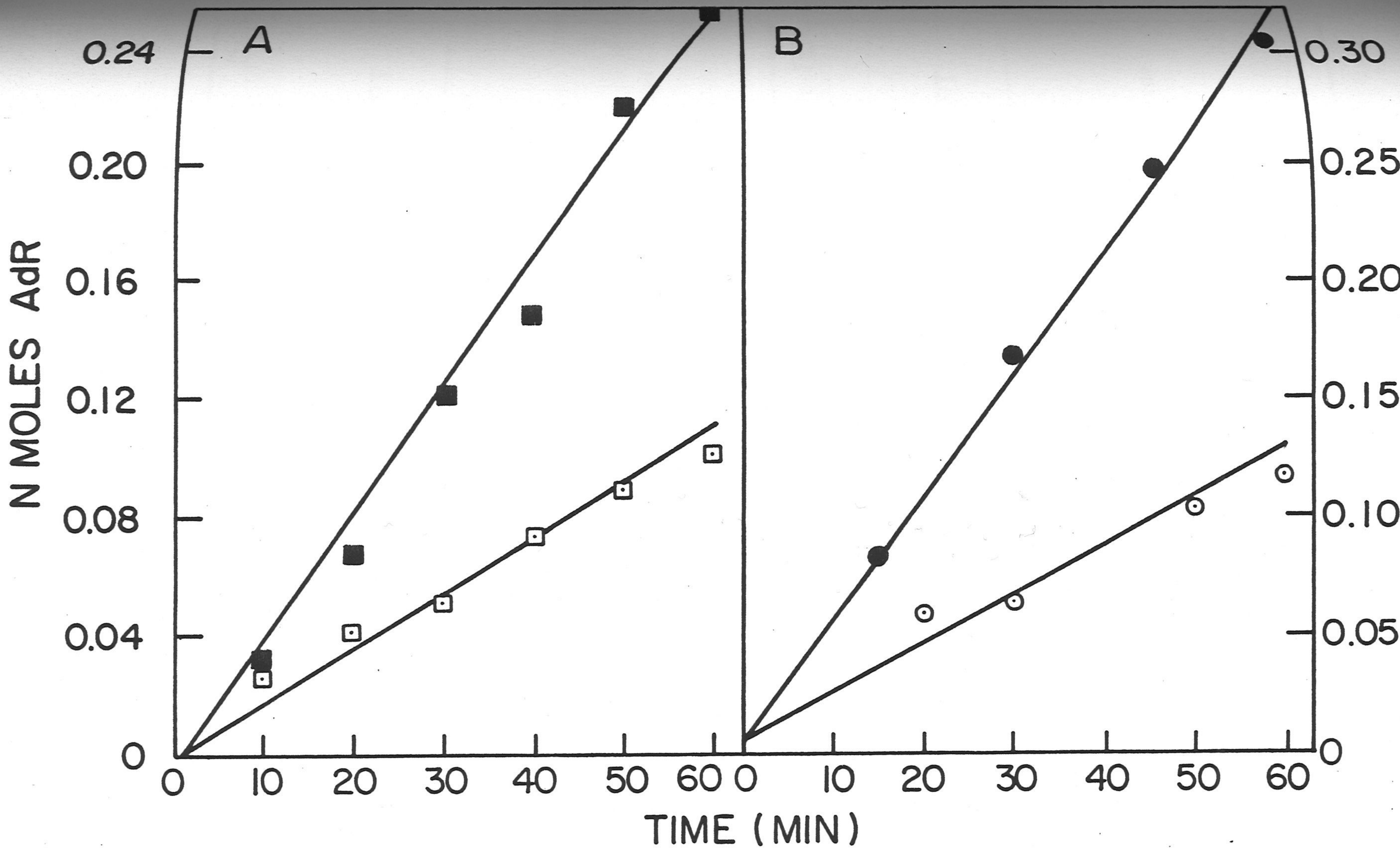
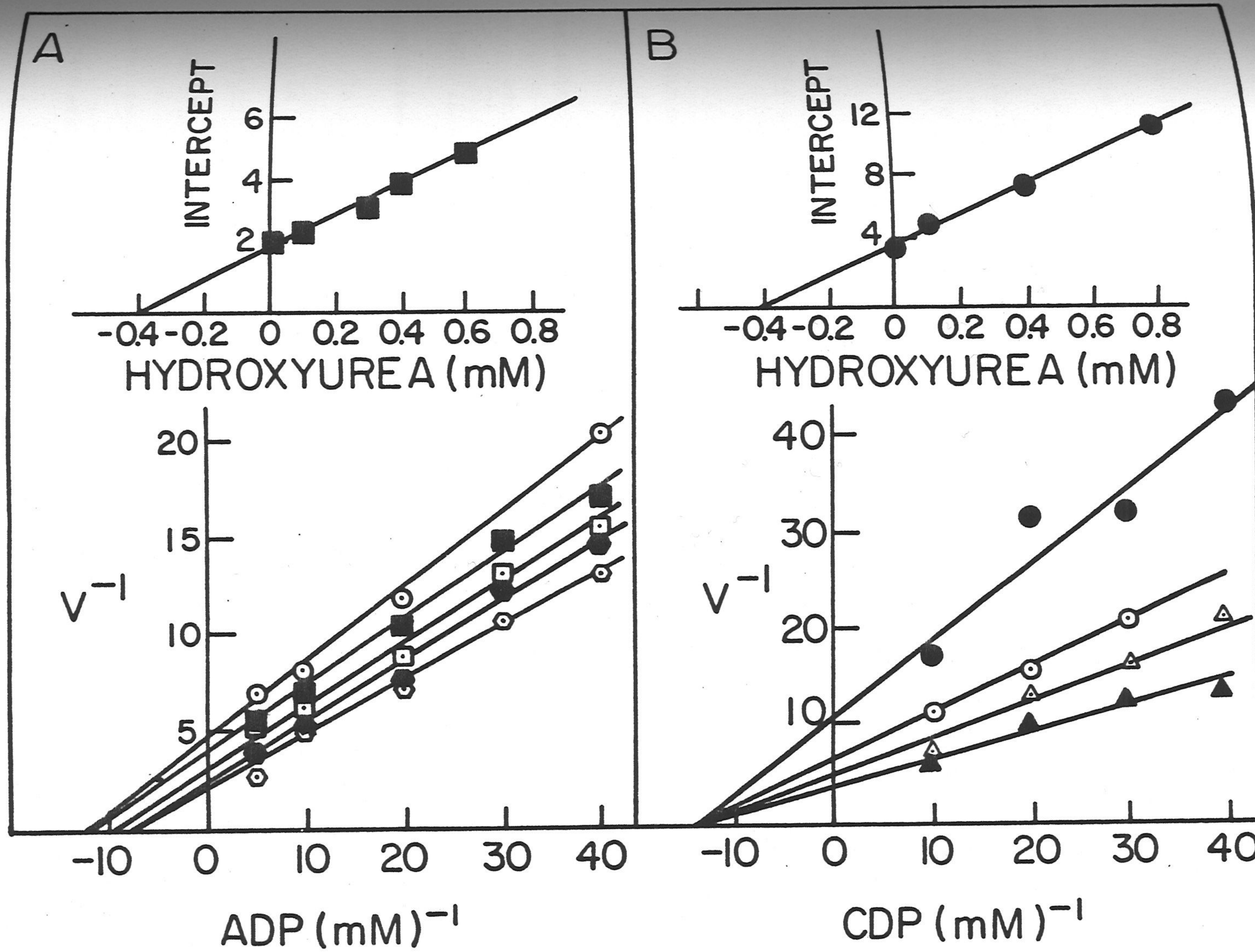

A
B
N MOLES AdR
N MOLES CdR
TIME (MIN)
0.24
0.20
0.16
0.12
0.08
0.04
0
0.30
0.25
0.20
0.15
0.10
0.05
0
0
10
20
30
40
50
60

Figure 14A. Double reciprocal plot of the effect of hydroxyurea on the rate of ADP reduction with varying ADP concentration. Standard assay conditions were used except for varying concentrations of ADP and the absence ( ⬡ ) or presence of 0.1 mM ( ⬢ ), 0.3 mM ( □ ), 0.4 mM ( ■ ), and 0.6 mM ( ○ ) hydroxyurea.

Inset: Replot of the velocity intercepts versus hydroxyurea concentration.

Figure 14B. Double reciprocal plot of the effect of hydroxyurea on the rate of CDP reduction with varying CDP concentration. Standard assay conditions were used except for varying concentrations of CDP and the absence ( ▲ ) or presence of 0.1 mM ( △ ), 0.4 mM ( ○ ), and 0.8 mM ( ● ) hydroxyurea.

Inset: Replot of the velocity intercepts versus hydroxyurea concentration.

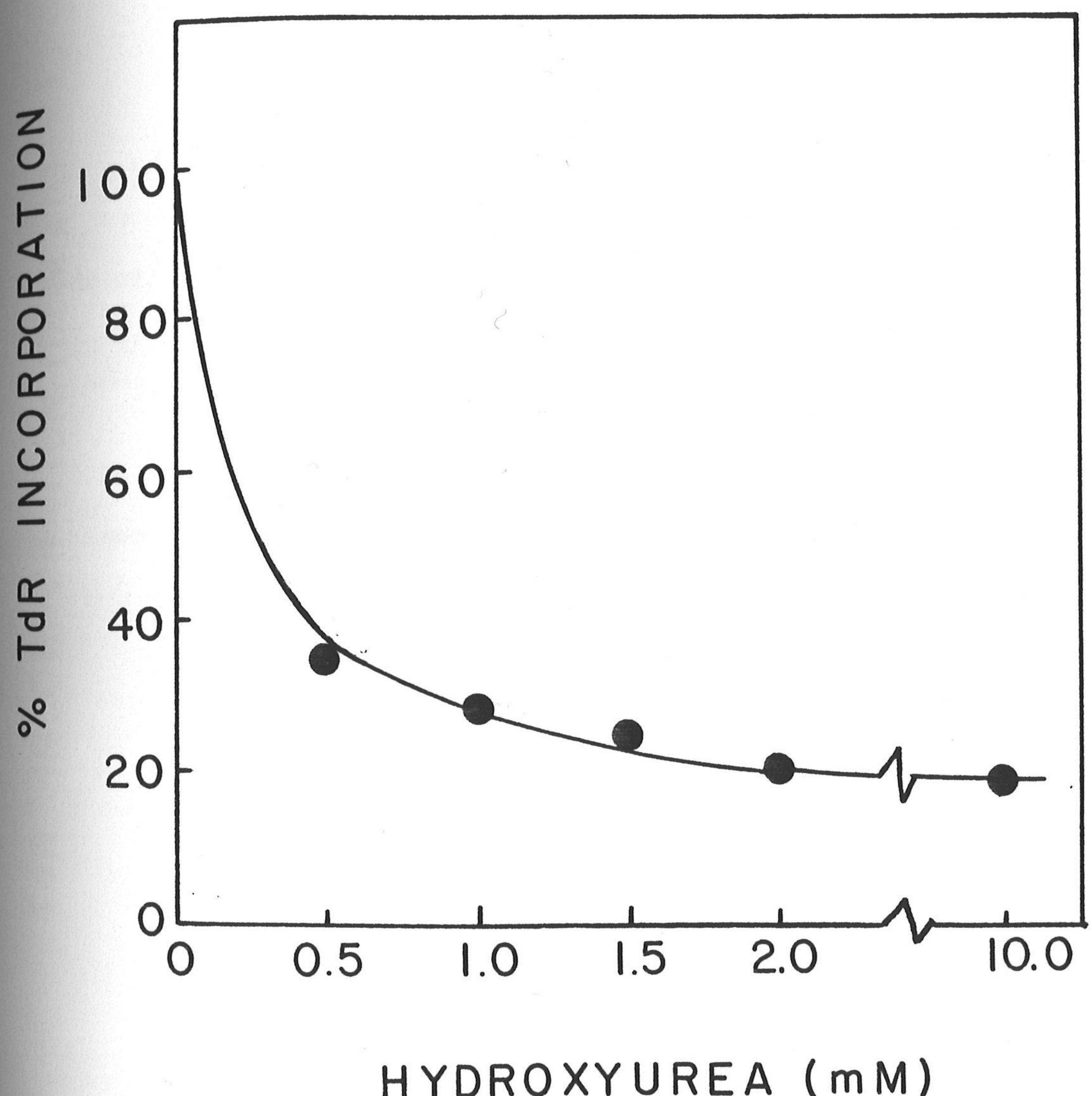

A
INTERCEPT
2
4
6
-0.4 -0.2 0 0.2 0.4 0.6 0.8
HYDROXYUREA (mM)
$V^{-1}$
5
10
15
20
-10 0 10 20 30 40
ADP (mM)$^{-1}$
B
INTERCEPT
4
8
12
-0.4 -0.2 0 0.2 0.4 0.6 0.8
HYDROXYUREA (mM)
$V^{-1}$
10
20
30
40
-10 0 10 20 30 40
CDP (mM)$^{-1}$

reductase in HDF may be structurally different than the enzyme in CHO cells.

Hydroxyurea has been investigated as a therapeutic agent in the treatment of some neoplasms (Moran & Straus, 1980; Engstrom et al., 1982) and it has been known for some time that it effectively inhibits DNA synthesis and the growth of animal cells in culture (Bacchetti & Whitmore, 1969; Wright & Lewis, 1974). Hydroxyurea was also found to inhibit DNA synthesis, as measured by $^{3}$H-TdR incorporation into DNA, in normal HDF (Figure 15). The concentration of hydroxurea which reduces DNA synthesis by 50% was about 0.25 mM, a value very similar to that which inhibited RRase activity by 50% (Figure 12). Interestingly, the rate of $^{3}$H-TdR incorporation could not be reduced to zero even at high concentrations of hydroxyurea. This low level of activity may represent repair synthesis since hydroxyurea apparently does not inhibit repair (Timson, 1975). Figure 16 shows a time course of $^{3}$H-TdR incorporation into TCA precipitable material. The rate of incorporation was inhibited by hydroxyurea and proceeded in a linear fashion after ten minutes in the presence and absence of drug.

Many studies (Lewis & Wright, 1974; 1978b; Castellot et al., 1978) have shown that cytotoxicity and inhibition of DNA synthesis by hydroxyurea are probably due to specific inhibition of RRase. The cytotoxicity of hydroxyurea was measured in two different ways using normal HDF. The growth of HDF in culture was inhibited by hydroxyurea as evidenced by the cessation of growth after forty hours of treatment (Figure 17). The cell number increased by about two fold in the first forty hours following addition of drug. This increase in cell

Figure 15. $^3$H-thymidine incorporation into TCA precipitable material of HSC 172 cells in the presence of varying concentrations of hydroxyurea. Five mls of α-MEM + 15% FCS + $^3$H-thymidine (1 μCi/ml, 0.5 μg/ml) + varying concentrations of hydroxyurea were added to logarithmically growing HSC 172 cells. After 20 minutes of incubation at 37°C the plates were rinsed and the reaction was stopped by the addition of 10% TCA. The amount of $^3$H-TdR incorporated into the acid precipitable material of cells in the presence of hydroxyurea was compared to incorporation in the absence of hydroxyurea using the procedure described in Methods and Materials.



100
80
60
40
20
0
% TdR INCORPORATION
0
0.5
1.0
1.5
2.0
10.0
HYDROXYUREA (mM)

Figure 16. Effect of incubation time on the incorporation of $^{3}$H-thymidine in TCA precipitable material of HSC 172 cells in the presence ( ■ ) and absence ( ● ) of hydroxyurea. The incorporation of $^{3}$H-thymidine was measured according to the procedure described in Methods and Materials except for the variation in incubation time.

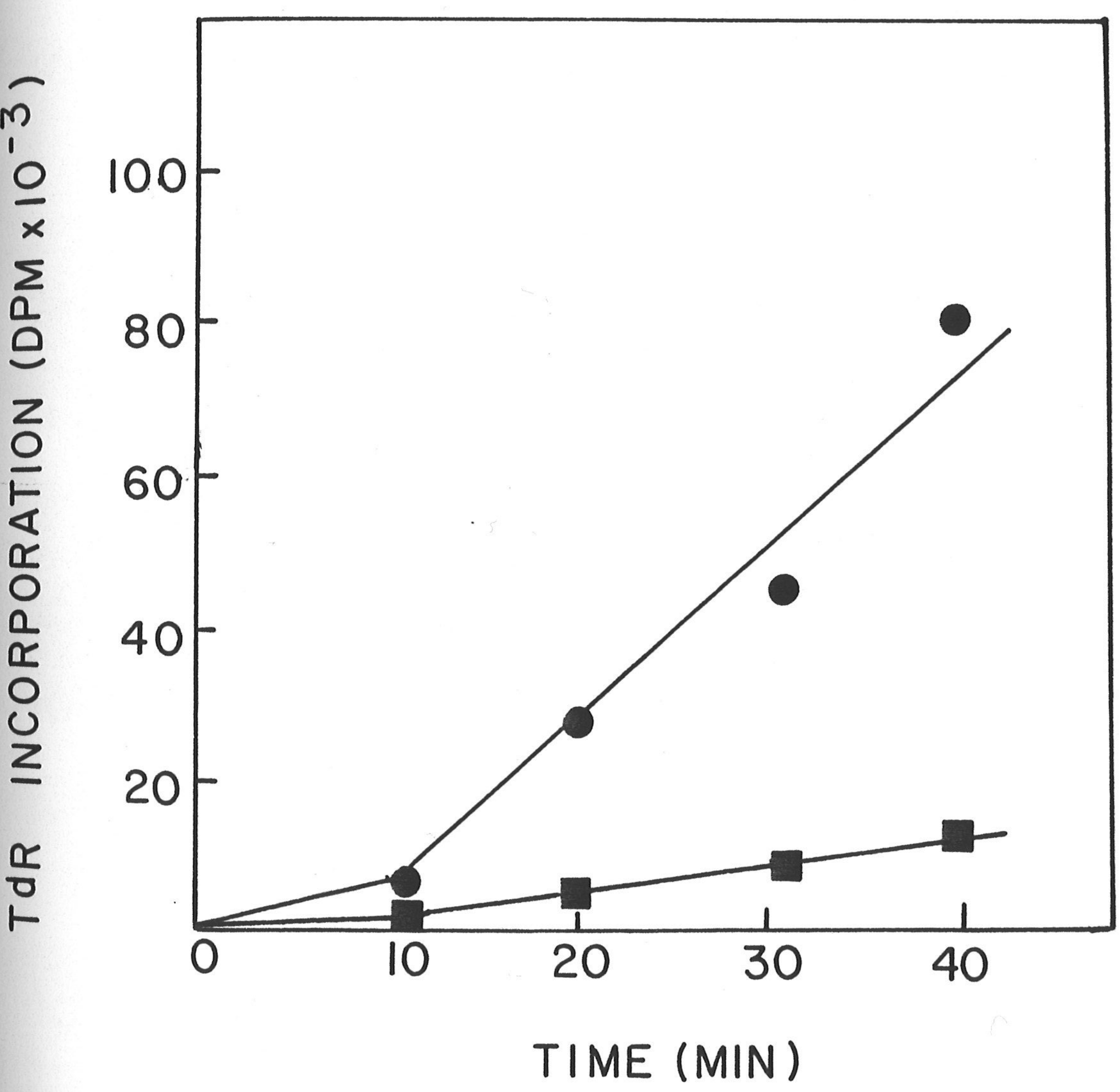
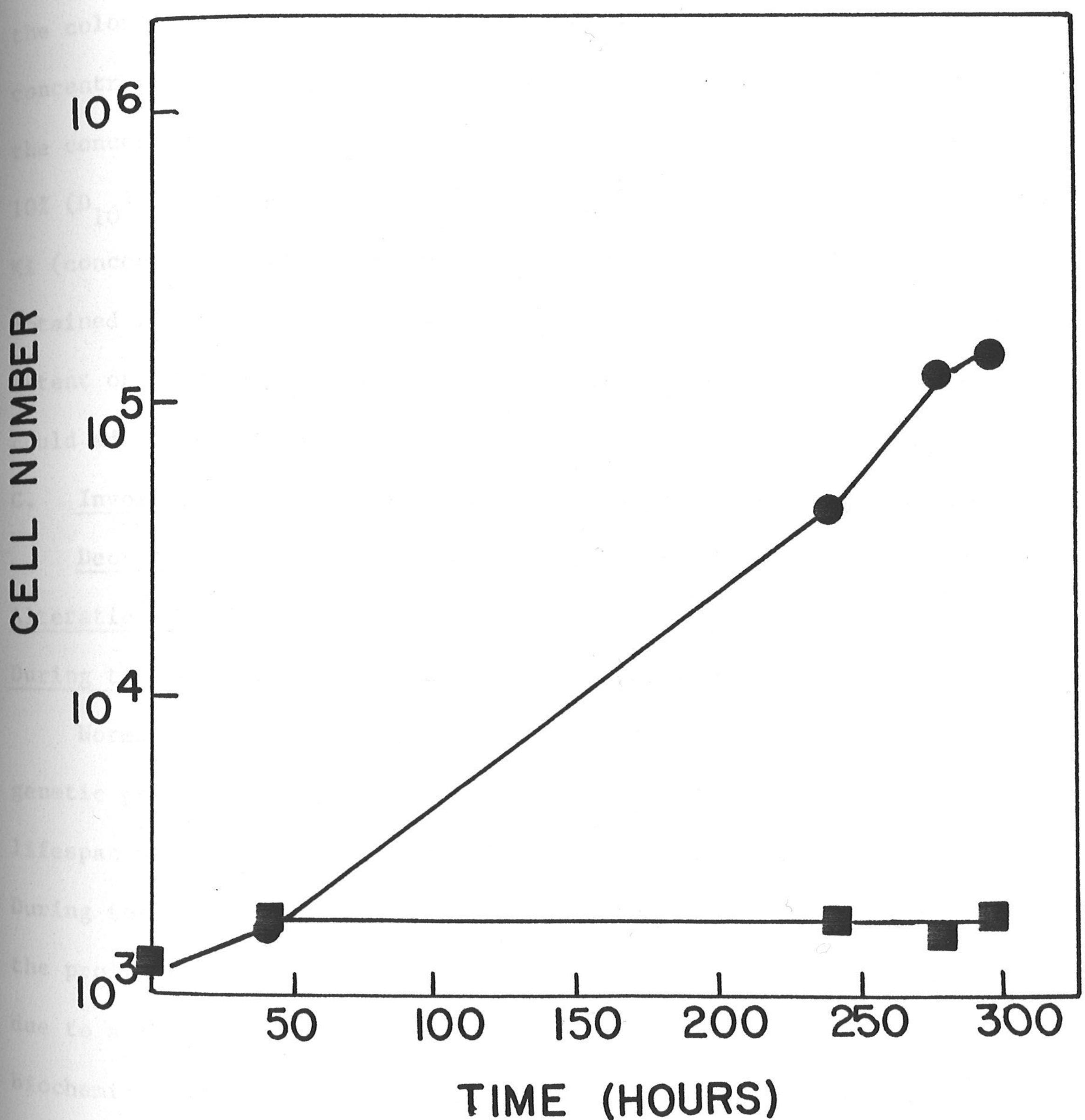

TdR INCORPORATION (DPM x 10^-3)
100
80
60
40
20
0
10
20
30
40
TIME (MIN)

Figure 17. Effect of 1.5 mM hydroxyurea on the growth of HSC 172 cells. Approximately $10^3$ HSC 172 cells were seeded onto 60 mm tissue culture plates containing α-MEM ( ● ) or α-MEM plus 1.5 mM hydroxyurea ( ■ ). At various times the cells were trypsinized and cell counts were taken using a particle counter.



CELL NUMBER
$10^6$
$10^5$
$10^4$
$10^3$
50
100
150
200
250
300
TIME (HOURS)

number is probably due to the synchronizing effect of hydroxyurea (Walters et al., 1973, 1976); that is, cells in various stages of the cell cycle proceeded until they accumulate at the $G_1$/S border.

The cytotoxicity of hydroxyurea was also measured by determining the colony forming ability of HDF in the presence of varying concentrations of hydroxyurea (Figure 18). This figure shows that the concentration of drug which reduces the colony forming ability to 10% ($D_{10}$) was about 0.55 mM. This value is very close to the apparent Ki (concentration which inhibits enzyme activity by 50%) estimates obtained for ADP and CDP reductase. These studies indicate that the extent of RRase inhibition as analyzed by the *in vivo* assay system could adequately account for the cytotoxic effect of the drug on HDF.

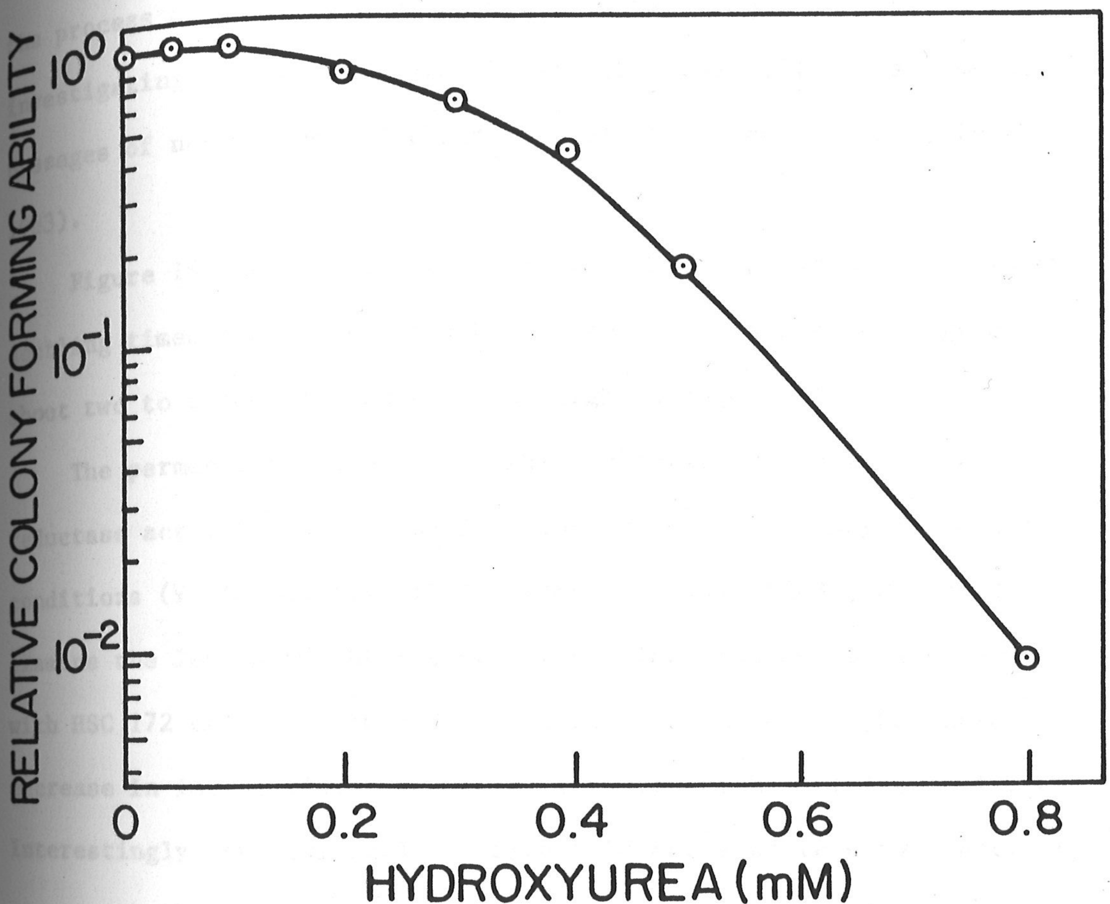
## C. Involvement of Ribonucleotide Reductase, Ribonucleotides, and Deoxyribonucleotides in the Senescence of Human Diploid Fibroblasts

### Alterations in the Level of Ribonucleotide Reductase Activity During the Senescence of Normal Diploid Fibroblasts

Normal human diploid fibroblasts appear to retain their normal genetic program for aging as evidenced by the limited replicative lifespan which all normal cells grown in culture show (Hayflick, 1980). During terminal senescence these cultures gradually lose cells from the proliferating pool, exhibit a prolonged cell cycle time primarily due to a lengthening in $G_1$, and show a variety of morphological and biochemical alterations, including a deficiency in the ability to synthesize DNA (Hayflick, 1980; Rabinovitch, 1983).

The key role of RRase in DNA synthesis and cell proliferation as well as the close association between RRase and transformation, mutation

Figure 18. Effect of increasing concentrations of hydroxyurea on the relative colony forming ability of HSC 172 cells. Approximately 1000 cells per 100 mm tissue culture plate were incubated at 37°C for 14 days in standard growth medium containing varying concentrations of hydroxyurea. The colonies which formed were scored using a dissecting microscope.


RELATIVE COLONY FORMING ABILITY
10^0
10^-1
10^-2
0
0.2
0.4
0.6
0.8
HYDROXYUREA (mM)

rates of cells in culture, and neoplastic growth (Wright, 1983) suggested that this key activity may also play a significant role in the process of cellular senescence. The idea was tested by investigating the intracellular levels of RRase activity at various passages of normal human diploid fibroblasts (Dick & Wright, 1982, 1983).

Figure 19 shows that HSC 172 cells exhibit progressively slower doubling times during serial subculture. Low passage cells grew about two to three fold faster than high passage cells.

The permeabilization assay, which determines the level of reductase activity in a situation closely resembling physiological conditions (Wright et al., 1981; Hards & Wright, 1983), was used to examine the levels of CDP reductase as a function of passage number with HSC 172 cells. Figure 20 shows that there was a substantial decrease in intracellular reductase activity with serial subculture. Interestingly, approximately a three fold decrease in enzyme activity occurred between passage 17 and passage 79, which closely matches the three fold decrease observed in doubling times between these two passages (Figure 19). It is important to stress that even with the permeabilization assay it was difficult to obtain enough material at very high passage to carry out detailed enzyme assays. Within the last 5 - 10 passages of senescence the saturation density usually declines by five to ten fold, greatly increasing the number plates needed for even a single assay point. As well the growth rate was much slower (Figure 19) therefore the number of assays that could be performed in a given period of time was also reduced.

Figure 19. Doubling time of HSC 172 cells at increasing passage numbers. The doubling times of HSC 172 cells at various passage numbers were determined from growth curves.

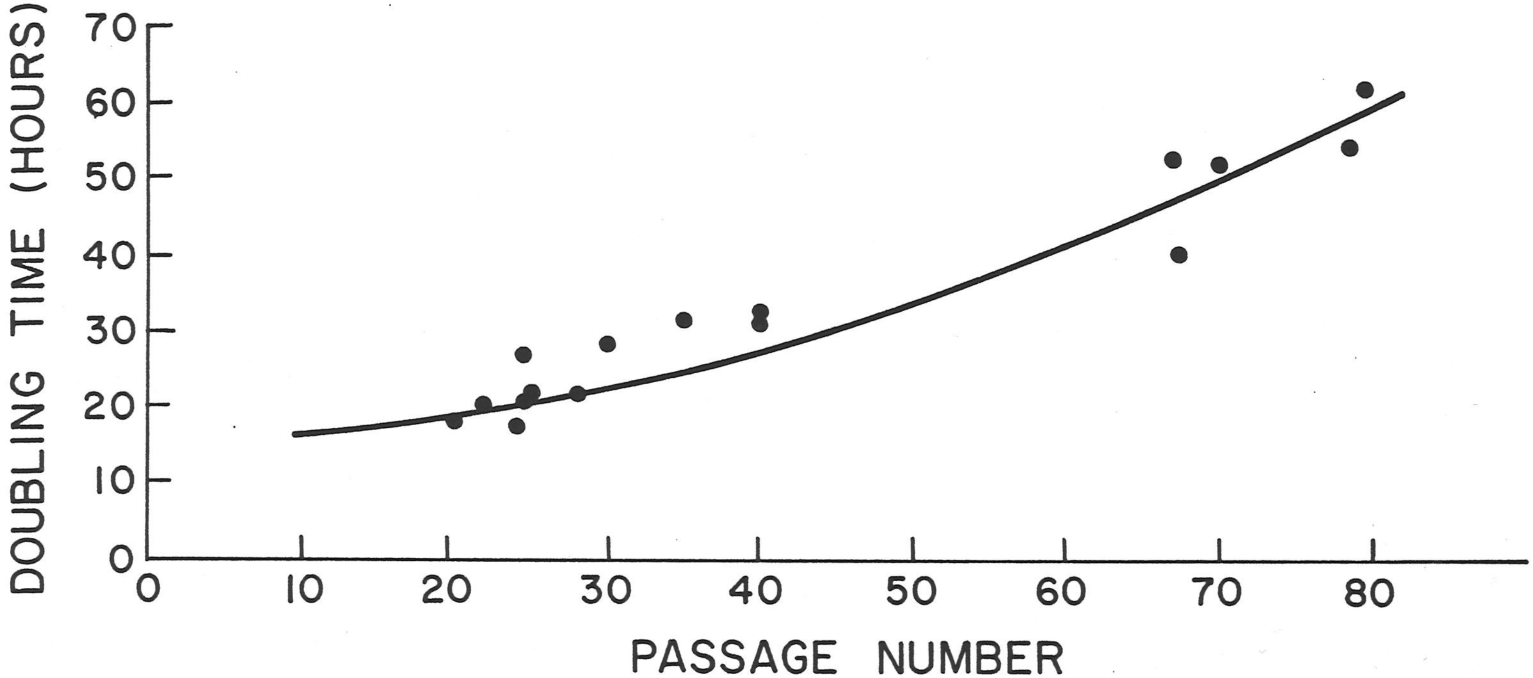
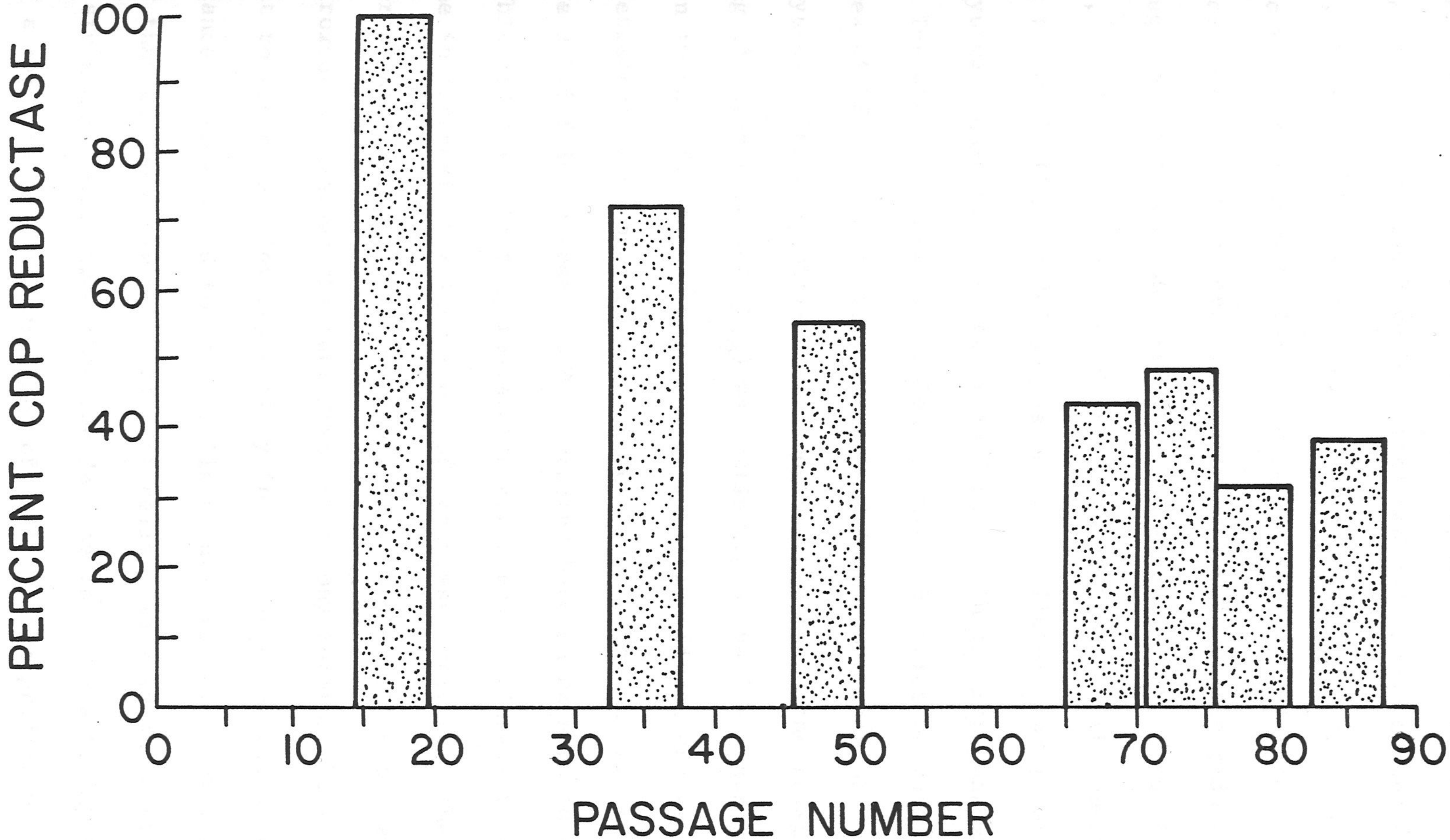
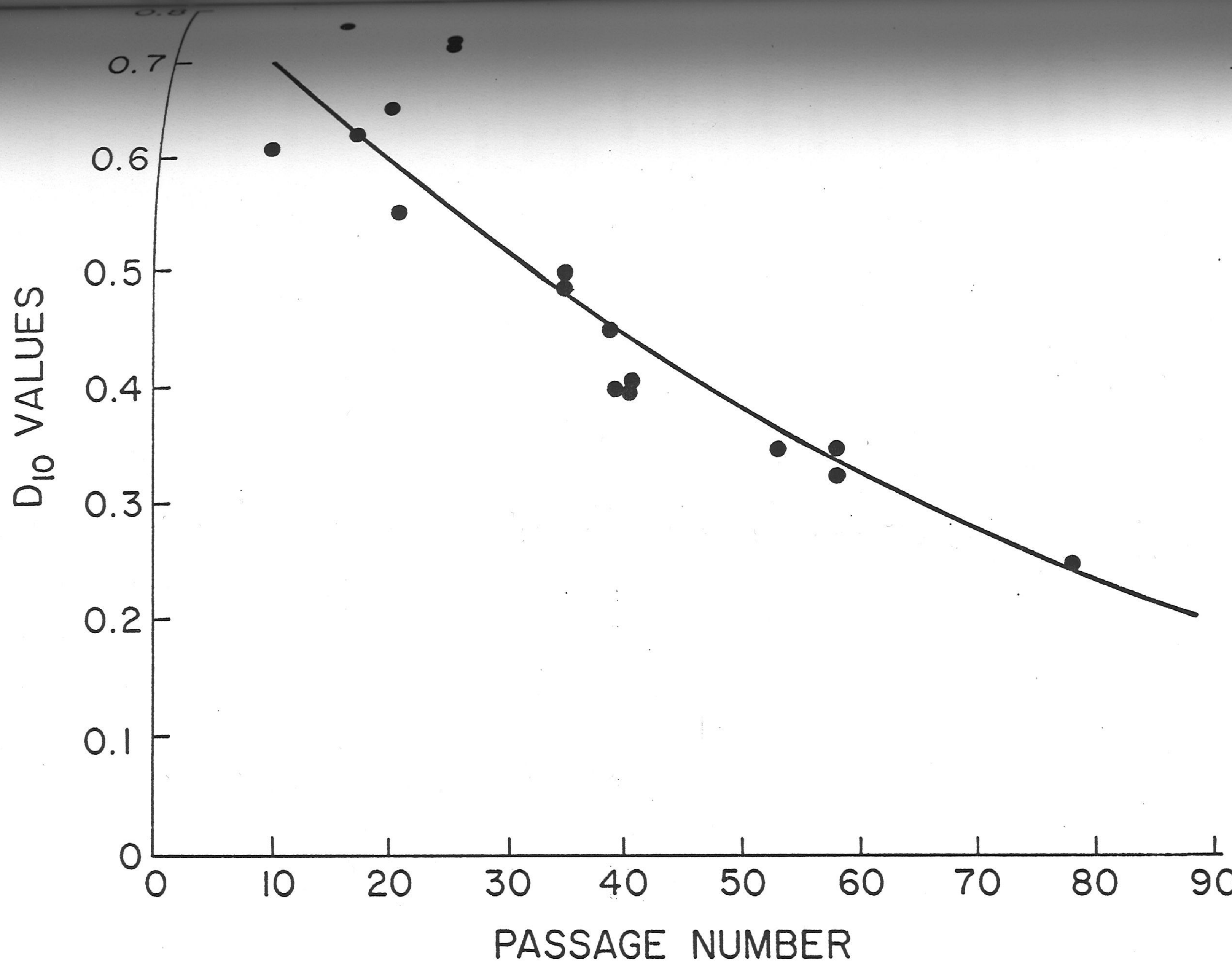

DOUBLING TIME (HOURS)
0
10
20
30
40
50
60
70
PASSAGE NUMBER
0
10
20
30
40
50
60
70
80

Figure 20. Relative levels of CDP reductase activity in HSC 172 cells at increasing passage numbers. CDP reductase activity at passage 17 is 0.230 nmoles CdR/hour/3 x $10^6$ cells.


PERCENT CDP REDUCTASE
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
PASSAGE NUMBER

Cell lines resistant to drugs which specifically inhibit ribonucleotide reductase have been isolated and characterized (Wright et al., 1980; Wright, 1983). One of these drugs, hydroxyurea, has been used to isolate cells with increased levels of ribonucleotide reductase. A strict correlation between intracellular levels of the reductase and drug resistance was observed (Lewis & Wright, 1979; Hards & Wright, 1981). The change in CDP reductase activity with senescence of HSC 172 cells (Figure 20) suggested that cellular resistance to hydroxyurea should decrease correspondingly with <u>in vitro</u> passage time. Therefore the relative colony-forming abilities of various passages of HSC 172 cells were determined in the presence of hydroxyurea. The concentration of drug which reduced the relative plating efficiency to 10% ($D_{10}$) was estimated at various passages between 10 and 79. As shown in Figure 21 a significant decrease in drug resistance was observed with increased passage number. For example a 2.6 fold decrease in $D_{10}$ occurred between passage 10 and 79. This reduction in drug resistance corresponded closely to the decline in intracellular levels of CDP reductase activity. There is another significant point to be noted. Since the measure of resistance to hydroxyurea involves the inhibition of colony forming ability with respect to the colony forming ability in the absence of drug, relative resistance is normalized to those cells capable of cell division, rather than to the whole population of cells. This is an important point since one could argue that the decline in RRase activity is merely a reflection of the decrease of cells in the proliferating pool with an accompanying increase in the non proliferating pool.

Figure 21. Resistance of HSC 172 cells at increasing passage numbers to the cytotoxic effects of hydroxyurea. Resistance ($D_{10}$) is expressed as the concentration of hydroxyurea (mM) required to reduce the relative colony forming ability to 10%.



0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
$D_{10}$ VALUES
0
10
20
30
40
50
60
70
80
90
PASSAGE NUMBER

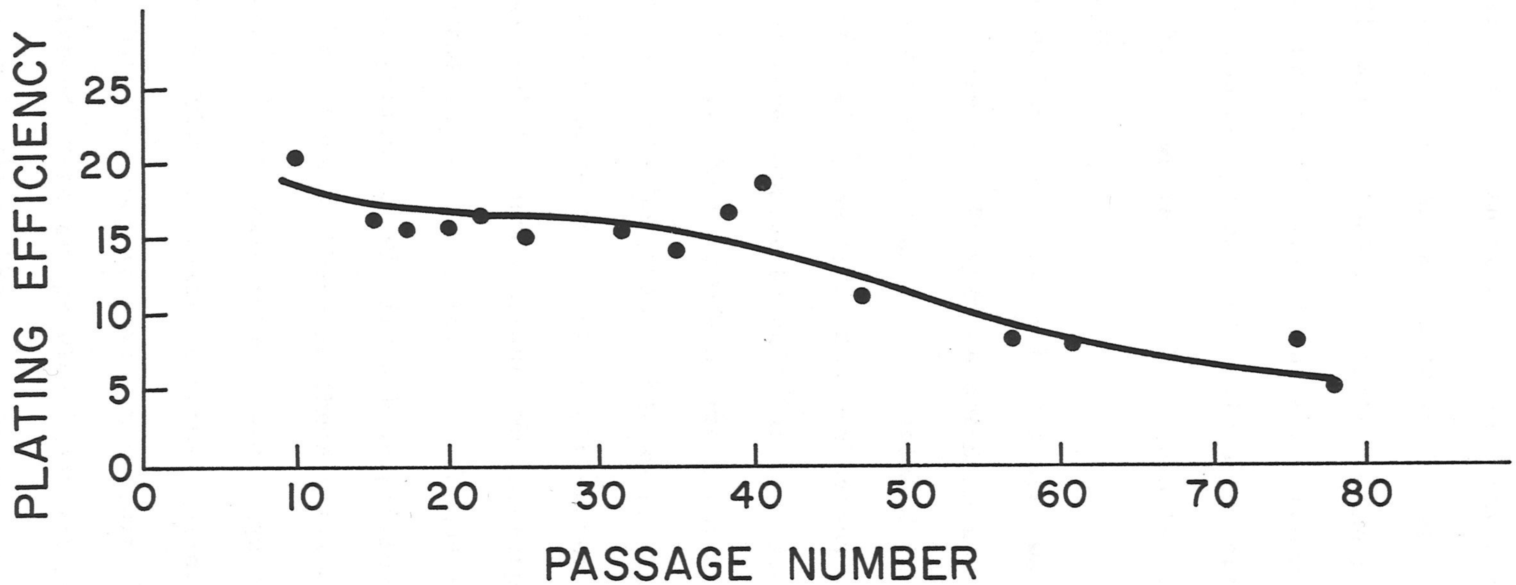
Therefore the age-related increase in sensitivity to hydroxyurea is a phenomenon related to those cells in the proliferating pool since resistance was measured by a clonogenic assay. By inference then the level of CDP reductase activity is lower even in those cells capable of proliferating. This conclusion is supported by the recent finding that another enzyme, DNA polymerase $\alpha$, which is intimately associated with RRase in the multi-enzyme DNA synthetic complex, also declines in proliferating cells at high passage numbers (Pendergrass et al., 1982).

The decline in the proliferating pool of cells is reflected by the reduction in PE of cells at increasing passage numbers (Figure 22). The decline in PE follows a pattern different from the decline in $D_{10}$, in that the PE is approximately constant to passage 40 and then declines with increasing passage numbers. The size of these colonies also declines during serial subculture of HDF cells. Smith et al. (1978) have studied this decline in colony size in detail and established a relationship between colony size and replicative potential remaining.

Isolation of Hydroxyurea-Resistant Human Diploid Fibroblasts with Altered Replicative Lifespan

Cytotoxic drugs whose intracellular target is ribonucleotide reductase have been used as selective agents in cell culture to isolate drug resistant cell lines with specific alterations in enzyme activity. The enzyme properties of these mutants have been studied, in a situation resembling physiological conditions, using the permeabilization assay as well as in purified extracts (Hards &

Figure 22. Colony forming ability of HSC 172 cells at various passage numbers. The colony forming ability is determined by seeding approximately 1000 cells per 100 mm tissue culture plate and incubating the plate at 37°C for 14 days. Colony forming ability is defined as the number of colonies divided by the number of cells plated.


PLATING EFFICIENCY
0
5
10
15
20
25
PASSAGE NUMBER
0
10
20
30
40
50
60
70
80

Wright, 1981, 1983; Lewis & Wright, 1978a, b, 1979; Kuzik & Wright, 1979; Koropatnick & Wright, 1980; Wright & Cory, 1983; Wright et al., 1981). These studies have identified at least three classes of drug resistant mutants in hamster and mouse cells in culture (Table III, Wright et al., 1981; Wright, 1983). Mutants of the first class apparently contain structural alterations which render the RRase less sensitive to inhibition by hydroxyurea. Class two mutants contain elevated levels of RRase, while class three mutants overproduce a drug resistant enzyme.

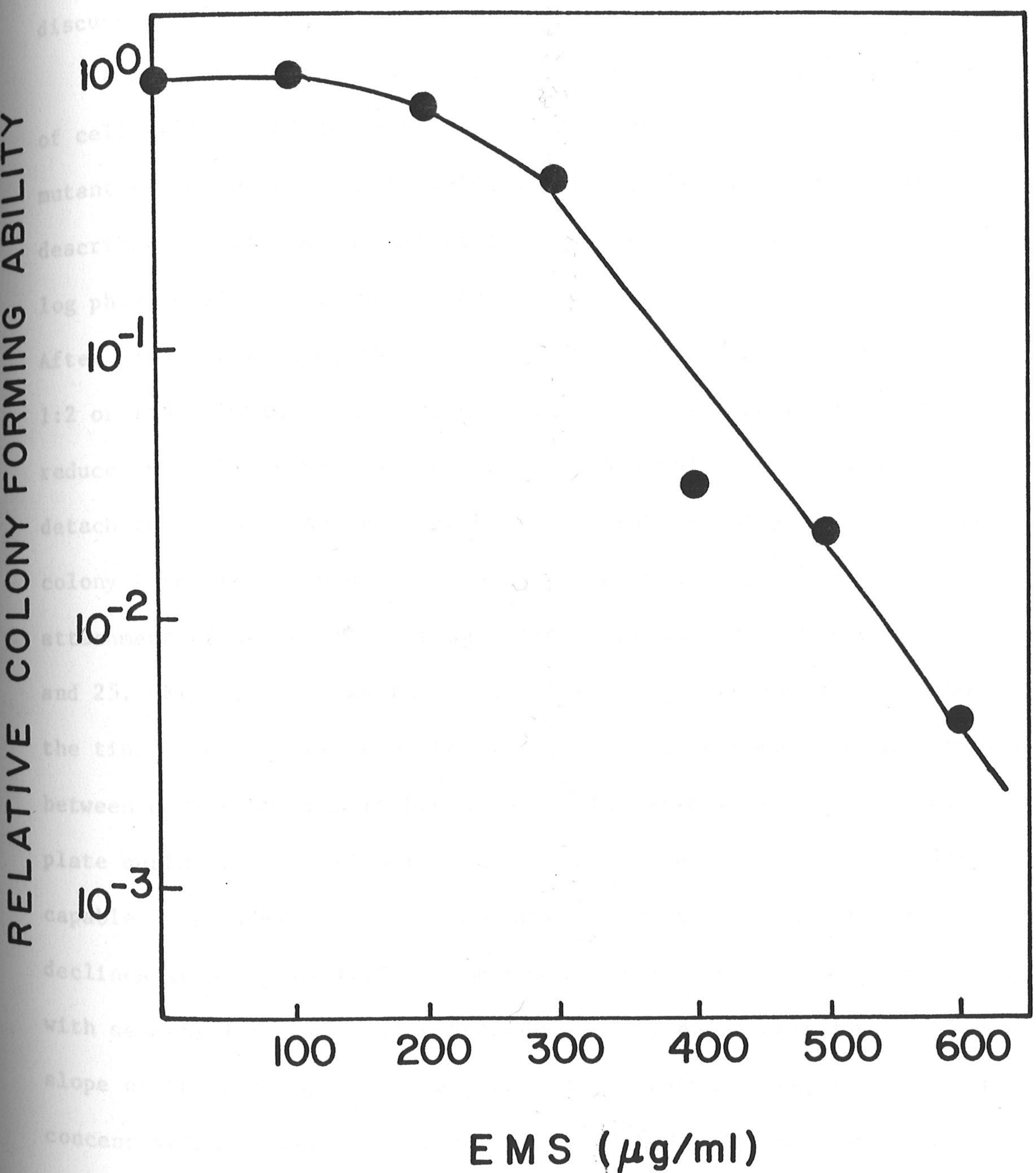
The isolation of HDF resistant to hydroxyurea was attempted primarily for three reasons. Firstly as noted in the Historical section very few mutant strains have been isolated in HDF. Such mutants are useful for studying rates and mechanisms of mutation and should be useful for gene mapping by cellular hybridization techniques (McKusik & Ruddle, 1977). Furthermore an important question in gene amplification studies is whether this mechanism of drug resistance is also possible in normal cells or is this a phenomenon restricted to abnormal transformed cells. Human mutants which overproduce RRase may be useful in examining this possibility. Secondly, hydroxyurea resistant mutants in permanent cell lines have provided much information about the nature of RRase in those cells (Wright, 1983). Hydroxyurea resistant HDF may also reveal novel regulatory and structural features of RRase relevant to normal cells. Thirdly, since it had been found that RRase was involved in cellular senescence, HDF with genetically altered RRase may provide important information regarding the biological role of RRase in the senescence process.

Table III. Summary of Ribonucleotide Reductase Changes in Drug Resistant Cell Lines.

| | |
|---|---|
| 1. | Altered sensitivity to drugs; drug $K_i$ values increased. |
| 2. | Increased intracellular activity; no other change detected. |
| 3. | Altered sensitivity to drugs and increased intracellular activity. |

Hydroxyurea resistant Chinese hamster ovary and mouse L cells can be isolated at a frequency of about $10^{-5}$ by adding $5 \times 10^{5}$ cells to 100 mm culture plates containing growth medium supplemented with drug (Wright & Lewis, 1974; Wright et al., 1980, 1981; Wright, 1983). Several attempts were made to try to isolate drug resistant HDF by a similar procedure. HDF were plated at a density of $10^{5}$ to $10^{6}$ cells per 150 mm plate on over 160 plates containing 1.0 mM or 2.0 mM hydroxyurea. These attempts were unsuccessful since killing of these cells by hydroxyurea was cell density dependent even at low concentrations. For instance when cells were plated at less than 1000 cells/100 mm culture plate in the presence of hydroxyurea, colonies formed in approximately two weeks, however, at greater cell concentrations distinct colonies were not observed, instead nearly all the cells remained attached to the plate and proliferated slowly to form a monolayer culture. Cultures could be maintained in this state up to several months, with little detachment of cells from the plate surface. This ability of human diploid fibroblasts to remain quiescent for months has been documented (Hayflick, 1965). Clearly, if rare drug resistant clones were to be isolated, a selection scheme would have to be used which involved large numbers of cells and reduced the attachment of nonproliferating cells. Furthermore ouabain resistant and α-amanitin resistant HDF could only by isolated by mutagenesis prior to selection since the spontaneous mutation rate for these loci was very low (Mankovitz et al., 1974; Buchwald & Ingles, 1976). Therefore in some selection experiments, cells were exposed to EMS prior to selection. Figure 23 shows the survival

Figure 23. Relative colony forming ability of HSC 172 cells exposed to varying concentrations of EMS. Logarithmically growing cells were exposed to various concentrations of EMS for 24 hours after which they were trypsinized, and replated at various cell numbers in growth medium without drug. After 14 days the relative colony forming ability was determined.


RELATIVE COLONY FORMING ABILITY
$10^0$
$10^{-1}$
$10^{-2}$
$10^{-3}$
100
200
300
400
500
600
EMS (µg/ml)

curve of HSC 172 cells after 30 hours of exposure to various concentrations of EMS. EMS concentrations of between 300 and 400 $\mu$g/ml were used to treat HDF prior to some selection experiments as discussed in Methods and Materials.

A novel selection scheme was developed to obtain a high degree of cell killing and detachment from the plate in order to allow the mutant cells to proliferate (Dick & Wright, 1983). This protocol, described in Methods and Materials, involved exposing about $2 \times 10^6$ log phase cells to 100 mM hydroxyurea for varying lengths of time. After exposure to drug the cells were trypsinized and replated with a 1:2 or 1:4 dilution. The trypsinization was necessary in order to reduce the cell density and to remove non-viable cells which did not detach from the tissue culture plate and subsequently interfered with colony formation. This phenomenon of a high degree of substrate attachment of non proliferating cells is illustrated in Figures 24 and 25. Figure 24 shows that most of the cells remain attached to the tissue culture plate up to eighty hours of exposure to hydroxyurea; between eighty and ninety hours the cells rapidly detach from the plate surface. In contrast Figure 25 shows that the loss of cells capable of proliferation, as measured by colony forming ability, declines steadily beginning from the initial exposure to hydroxyurea, with several logs of killing between seventy and eighty hours. The slope of the killing curve (Figure 25) was rather steep so that drug concentration, length of exposure, and cell density were critical for colony development and data reproducibility. This selection protocol was used to isolate, for the first time, hydroxyurea resistant

Figure 24. Change in cell number of HSC 172 cells exposed to 100 mM hydroxyurea for varying lengths of time. Passage 40 ( ■ ) and passage 86 ( ● ) HSC 172 cells, at a cell density of about $2 \times 10^6$ cells per 100 mm tissue culture plate, were exposed to 100 mM hydroxyurea in complete growth medium. At various lengths of time the cells were rinsed, trypsinized, and cell counts were determined using a particle counter. The arrow indicates the length of exposure to drug where no cells remained in the plate. It represents the upper limit of relative cell number at that length of exposure.

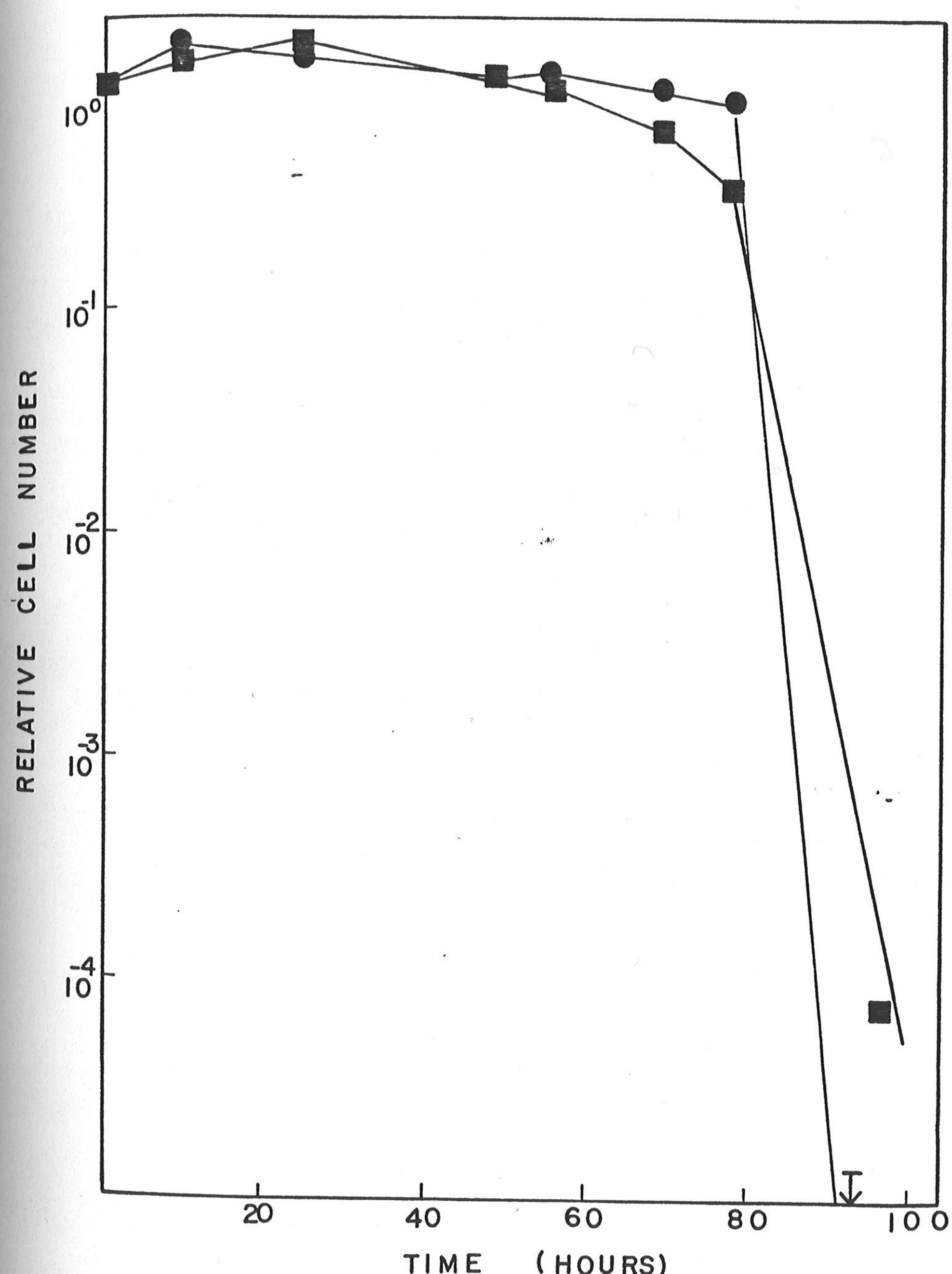
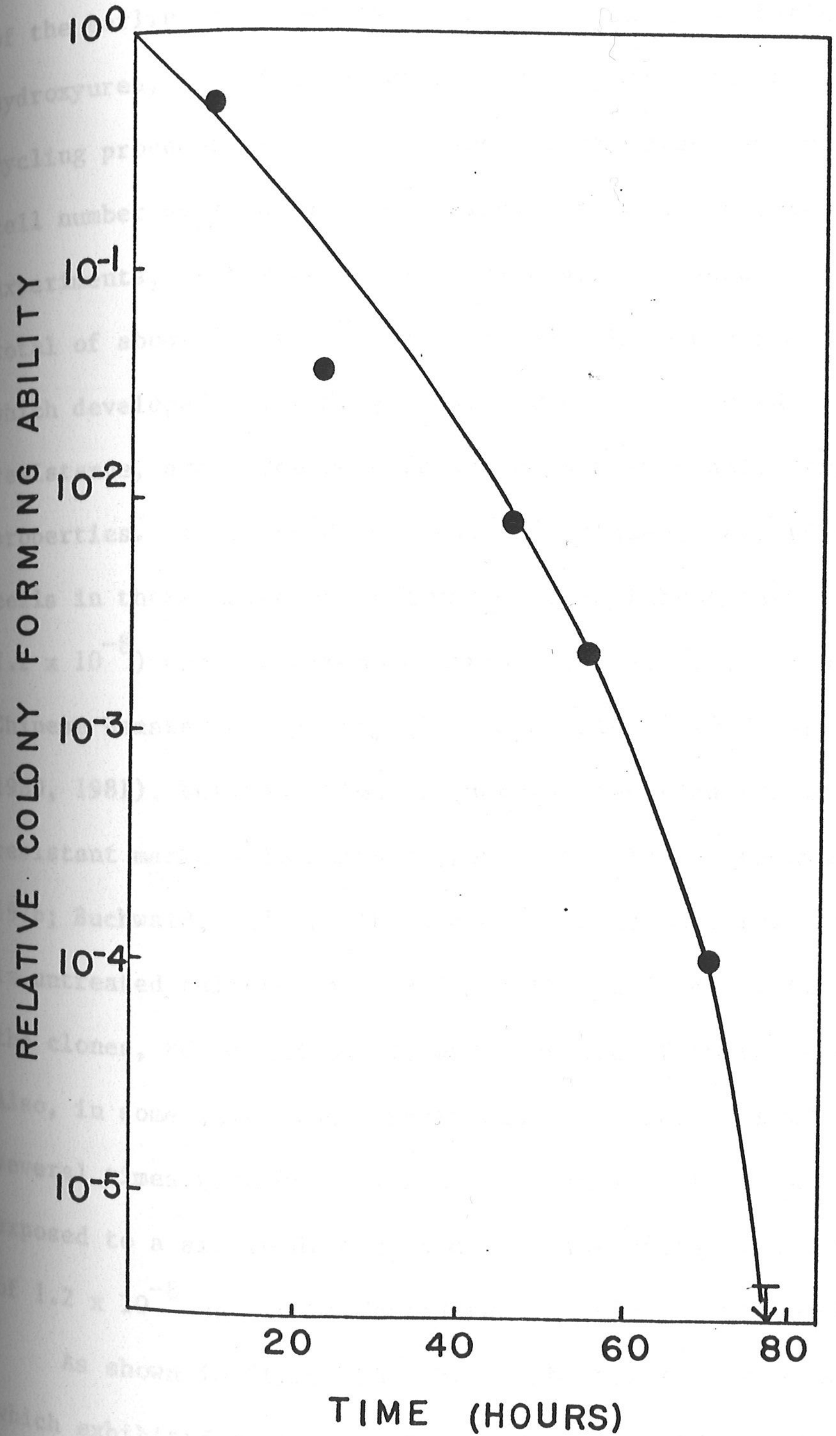

RELATIVE CELL NUMBER
$10^0$
$10^{-1}$
$10^{-2}$
$10^{-3}$
$10^{-4}$
20
40
60
80
100
TIME (HOURS)

Figure 25. Relative colony forming ability of HSC 172 cells exposed to 100 mM hydroxyurea for varying lengths of time. Passage 40 cells at a cell density of about $2 \times 10^6$ cells per 100 mm tissue culture plates were exposed to 100 mM hydroxyurea in complete growth medium. At various lengths of time the cells were rinsed, trypsinized, and replated in complete medium at various cell numbers. After fourteen days incubation at 37°C the number of colonies at each time point was determined. The relative colony forming ability represents the colony forming ability of cells exposed to drug for various lengths of time divided by the colony forming ability of cells which were not exposed to drug. The arrow indicates the length of exposure to drug where no colonies were observed. It represents the upper limit of colony forming ability at that length of exposure.


RELATIVE COLONY FORMING ABILITY
$10^0$
$10^{-1}$
$10^{-2}$
$10^{-3}$
$10^{-4}$
$10^{-5}$
20
40
60
80
TIME (HOURS)

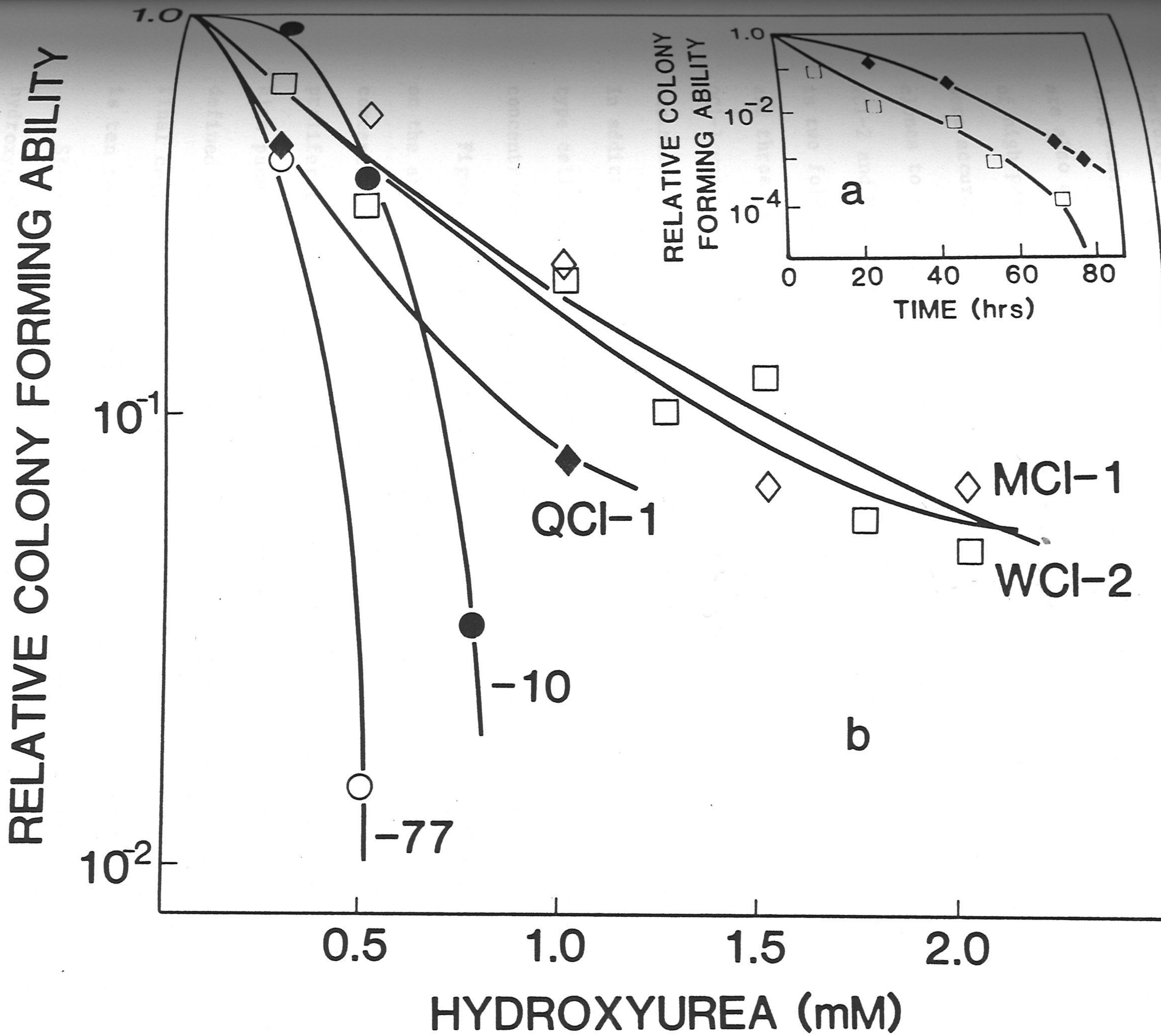
HDF cultures (Dick & Wright, 1983).

Over the course of about two years many attempts were made to isolate hydroxyurea resistant HDF using the outlined procedure. Most of the earlier experiments involved exposing populations of cells to hydroxyurea, allowing regrowth, then exposing the cells again. This cycling procedure was used in about fifty experiments with an initial cell number of about $1 \times 10^8$ cells. In later selections over 65 experiments, each with $2 \times 10^6$ cells were exposed to drug. From a total of about $2.5 \times 10^8$ cells, in all these experiments, 150 colonies which developed after drug treatment were re-tested for hydroxyurea resistance, and three were found to exhibit stable drug resistance properties. This indicates that the frequency of hydroxyurea resistant cells in these cultures of human diploid fibroblasts is very low (about $1.2 \times 10^{-8}$) when compared to permanent cell lines such as mouse L and Chinese hamster ovary cells (Lewis & Wright, 1978; Wright et al., 1980, 1981), but resembles frequencies reported for other drug resistant markers in human diploid fibroblasts (Buchwald & Ingles, 1976; Buchwald, 1977). It should be noted that the mutation frequency in untreated cultures may be lower than this estimate since two of the clones, MC1-1 and QC1-1, were isolated from EMS treated populations. Also, in some selection experiments, cell cultures were treated several times with hydroxyurea, whereas in others, the cells were exposed to a single drug treatment. Therefore, the mutation frequency of $1.2 \times 10^{-8}$ should be considered as only a very rough estimate.

As shown in Figure 26B three fibroblast clones were isolated which exhibited drug resistance properties as determined by colony

Figure 26A. Relative colony forming aility of HSC 172 passage 40 (□) and QC1-1 (◆) after exposure to 100 mM hydroxyurea for various lengths of time. The method for determining the relative coloy forming ability is described in Methods and Materials as well as the legend to figure 25.

Figure 26B. Relative colony forming ability of wild type and hydroxyurea resistant variants of HSC 172 cells in the presence of various concentrations of hydroxyurea. The relative colony forming ability of HSC 172 passage 10 (●) and passage 77 (○), WC1-2 (□), MC1-1 (◇) and QC1-1 (◆) cells were determined by growing 1000 and 2000 cells per 100 mm tissue culture plate in presence of various concentrations of hydroxyurea for fourteen days. Similar results have been obtained in at least three separate experiments.


RELATIVE COLONY FORMING ABILITY
1.0
10^-1
10^-2
0.5
1.0
1.5
2.0
HYDROXYUREA (mM)
QCl-1
MCl-1
WCl-2
-10
-77
b
RELATIVE COLONY FORMING ABILITY
1.0
10^-2
10^-4
a
0
20
40
60
80
TIME (hrs)

forming ability in the presence of various concentrations of hydroxyurea. For comparison, the colony forming abilities of two drug sensitive cultures at low and high passage numbers (10 and 77) are also shown. These killing curves reflect the increased sensitivity of high passage HDF to hydroxyurea. For this reason it is probably more accurate to compare the relative resistance of the three mutant clones to the age matched high passage wild type cells. The clones WCl-2 and MCl-1 showed a $D_{10}$ value of about 1.4 mM hydroxyurea which is two fold higher than the $D_{10}$ for the low passage wild type culture and three to four fold greater than the high passage culture. Clone QCl-1 was less resistant than WCl-2 and MCl-1 but exhibited drug resistance properties when compared to wild type fibroblast cultures. In addition, Figure 26B shows that QCl-1 is more resistant than wild type cells when resistance is determined using short exposure to high concentrations of drug, as was carried out in selection experiments.

Figure 27 shows a measurement of the resistance of MCl-1 based on the ability of MCl-1 to proliferate in the presence of varying concentrations of hydroxyurea. MCl-1 shows an increased ability to proliferate in growth medium supplemented with drug as compared to high passage wild type cells. For instance, the relative cell number, defined as the final cell number in plates with drug divided by the final cell number in plates without drug after eight days incubation, is ten fold lower in the wild type cells as compared to MCl-1.

Since these mutants were able to proliferate in the presence of hydroxyurea, the ability of one of the clones, MCl-1, to synthesize DNA was measured in the presence and absence of the drug. Table IV

Figure 27. Effect of hydroxyurea on the growth of wild type and hydroxyurea resistant HSC 172 cells. Wild type (■) and drug resistant MCl-1 ( ● ) cells were seeded at $2 \times 10^4$ cells per 60 mm plate and incubated in the presence of various concentrations of hydroxyurea at 37°C. After eight days the cells were rinsed, trypsinized, and cell counts were determined using a particle counter.

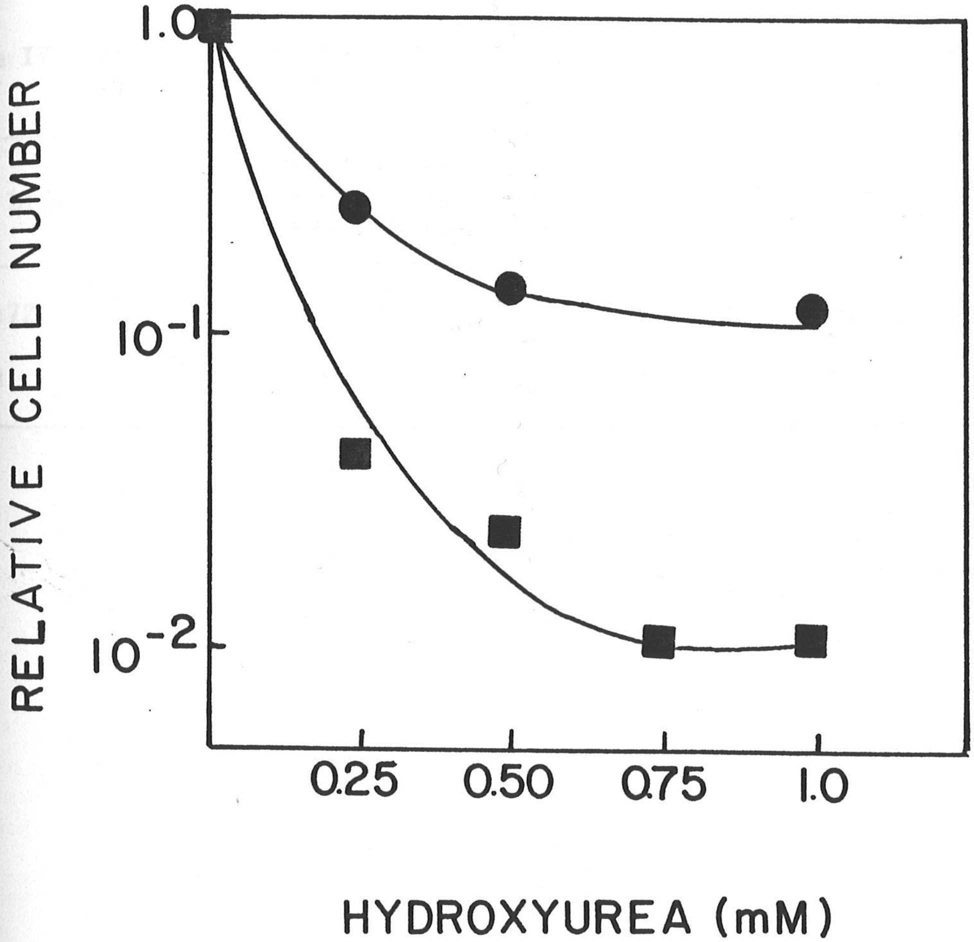

1.0
10-1
10-2
RELATIVE CELL NUMBER
0.25
0.50
0.75
1.0
HYDROXYUREA (mM)

Table IV. Resistance of DNA Synthesis and Cell Growth to Inhibition by Hydroxyurea.

| CELL LINE | Relative Cell Number in 1.0 mM Hydroxyurea After 5 days | % TdR Incorp. 0.5 mM Hydroxyurea |
|---|---|---|
| HSC 172 | 0.08 | 38 |
| MC1-1 | 0.27 | 63 |

shows that DNA synthesis in MCl-1 was more resistant to inhibition by 0.5 mM hydroxyurea than wild type cells. This table also supports the view that MCl-1 is better able to proliferate in 1.0 mM hydroxyurea than wild type cells.

Table V gives the details for the selection of the three clones characterized, as well as showing that each of the clones also exhibited a limited replicative lifespan in vitro. The total number of passages that accumulated during selection of drug resistant cultures was determined by actual cell counts except in the cloning steps where the number of cells per colony was estimated from colony size. These values closely matched the estimated number of generations accumulated during isolation of mutant cell lines in other studies (Thompson, 1979). Interestingly, these calculations indicated that there was a significant decrease in the lifespan of the three drug resistant clones as compared to the wild type fibroblasts. With two clones, MCl-1 and WCl-2, there were 25% and 20% reductions respectively in lifespan, while QCl-1 showed a 40% reduction. It is worth noting that none of the other mutants isolated for drug resistance, using normal HDF, have been reported to show reduced lifespan. In fact one double mutant isolated for resistance to ouabain and 6-Tg was reported to exhibit the same lifespan as the wild type HDF strain (Duthu et al., 1982).

Ribonucleotide Reductase Activity in Hydroxyurea Resistant HDF. Since drug resistant human diploid fibroblasts sencesce in culture at early passage numbers (Table V) only a limited amount of material was available for enzyme studies. However, the intact cell assay system developed for analyzing ribonucleotide reductase activity in

Table V. Lifespan of Wild-Type and Mutant Clones

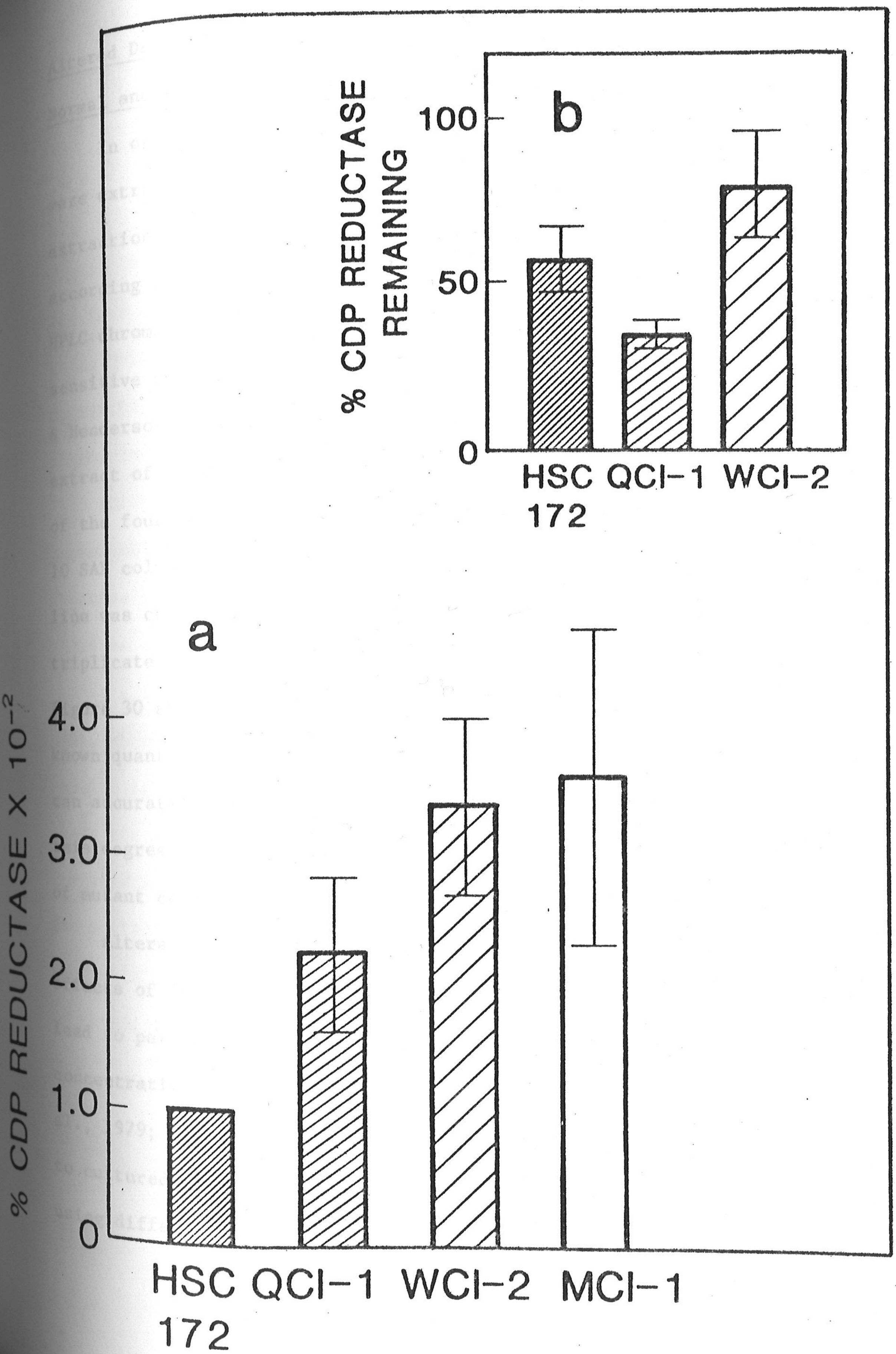
| Steps in Isolation of Mutants | Passages during Selection | | Passages after Cloning | Cummulative Total | % Decrease |
|---|---|---|---|---|---|
| MC1-1 | | | | | |
| Start | 15 | | | | |
| Mutagenesis | | 3 | | | |
| Selection | | 4 | | | |
| Regrowth | | 2 | | | |
| Selection | | 4 | | | |
| Regrowth | | 3 | | | |
| Selection | | 14 | | | |
| Cloning | | 9 | | | |
| | | 39 | $17 \pm 2$ | 71 | 22 |
| WC1-2 | | | | | |
| Start | 32 | | | | |
| Selection | | 15 | | | |
| Cloning | | 11 | | | |
| | | 26 | $18 \pm 2$ | 76 | 20 |
| QC1-1 | | | | | |
| Start | 10 | | | | |
| Mutagenesis | | 4 | | | |
| Selection | | 9 | | | |
| Cloning | | 14 | | | |
| | | 27 | $20 \pm 3$ | 57 | 42 |
| Wild-type HSC 172 | | | | $95 \pm 5$[a] | |

[a] an identical result has been reported by Gupta using HSC 172 (48).

permeabilized human diploid fibroblasts (Dick & Wright, 1980, 1982) was used to investigate the reductase in resistant and wild type cells. It has been shown that ribonucleotide reductase levels change during the lifespan of human diploid fibroblasts (Figure 20; Dick & Wright, 1982). In order to accurately compare enzyme activity in drug resistant and wild type cells it was necessary to match these cells with respect to passage numbers prior to senescence. Wild type HSC 172 cells, under these culture conditions, senesced after 95 $\pm$ 5 passages whereas the drug resistant cultures senesced within 20 passages after isolation (Table V). Therefore, wild type cells that were between passage 75 and 90 were used for comparative purposes in enzyme experiments. Figure 28 shows that the three drug resistant cultures contain elevated levels of ribonucleotide reductase activity when compared to wild type cells. The increased levels of enzyme activity, between 2 and 3.5 fold, correlate very closely to cellular resistance to hydroxyurea (Figure 26). To test whether or not drug resistant cells possess an enzyme with altered affinity for hydroxyurea, the activity of ribonucleotide reductase was examined in the presence of 0.5 mM hydroxyurea, a concentration which reduces the wild type activity by approximately 50%. There appear to be only slight differences in enzyme sensitivity to drug among the various cultures, suggesting that drug resistance in these human diploid fibroblasts is due to an overproduction of normal enzyme activity (Figure 28B). The human mutants therefore resemble class II mutants from rodent cells which overproduce normal enzyme (Wright et al., 1981; Wright, 1983).

Figure 28A. Relative CDP reductase activity in QCl-1, WCl-2, and MCl-1 compared to age-matched wild type HSC 172 cells. Results are from an average of eight determinations for QCl-1 and WCl-2, and three determinations for MCl-1.

Figure 28B. CDP reductase activity remaining in the presence of 0.5 mM hydroxyurea. The results are given as the percent enzyme activity in the presence of drug divided by enzyme activity in the absence of drug.


b
% CDP REDUCTASE REMAINING
100
50
0
HSC 172
QCI-1
WCI-2
a
% CDP REDUCTASE X $10^{-2}$
4.0
3.0
2.0
1.0
0
HSC 172
QCI-1
WCI-2
MCI-1

Altered Deoxyribonucleotide Pools in Senescing Normal and Drug-Resistant Human Diploid Fibroblasts

In order to characterize these mutants further the dNTP pools were extracted from age-matched wild type and WC1-1 cells. The extraction and HPLC were carried out under rigorous conditions according the protocol outlined in Methods and Materials. The use of HPLC chromatography to separate and detect dNTP's can be a very sensitive method providing a high sensitivity detector is used (Hunting & Henderson, 1982). Figure 29 shows a typical chromatograph of an extract of wild type HSC 172 cells at low pasage. Complete separation of the four dNTP's was achieved in thirty minutes using a Partisil 10 SAX column. Since the peaks came off the column when the base line was changing, they were quantitated by weighing each peak in triplicate and comparing this weight with known concentration standards. Figure 30 shows standard curves which were generated by running known quantities of the four dNTP's through the HPLC. This technique can accurately measure the dNTP's extracted from about $5 \times 10^6$ cells; this degree of sensitivity was important since very limited quantities of mutant cells were available for these measurements.

Alterations in ribonucleotide reductase activity due to the effects of inhibitors or mutations affecting enzyme regulation can lead to perturbations in intracellular deoxyribonucleotide concentrations (Skoog et al., 1973; Theiss & Fisher, 1976; Ullman et al., 1979; Ashman et al., 1981). The effects of adding hydroxyurea to cultured cells remain somewhat inconclusive as various studies using different analytical methods show a variety of pool changes,

Figure 29. Representative chromatogram of the separation of the four deoxyribonucleotide from extracts of HSC 172 cells using HPLC techniques. The technique of extracting the deoxyribonucleotides and the destruction of the interfering ribonucleotides are described in the Methods and Materials. This chromatogram was generated by applying 100 µl of extract, representing 5 x $10^6$ cells, to a Partisil 10 SAX anion exchange column. The dNTP's were eluted isocratically using a buffer consisting of 250 mM KCl and 250 mM $KH_2PO_4$ pH 5.0 run at a flow rate of 2.0 ml per minute. The large initial peak, which probably represents the destroyed ribonucleotides results in a rather steeply changing baseline in the region where the dNTP's elute. For this reason the peaks were quantitated on the basis of peak weight against known standards. The dotted line represents the baseline of each peak when they were cut out. The original chromatogram was photocopied in triplicate, cut out, and weighed on an analytical balance.

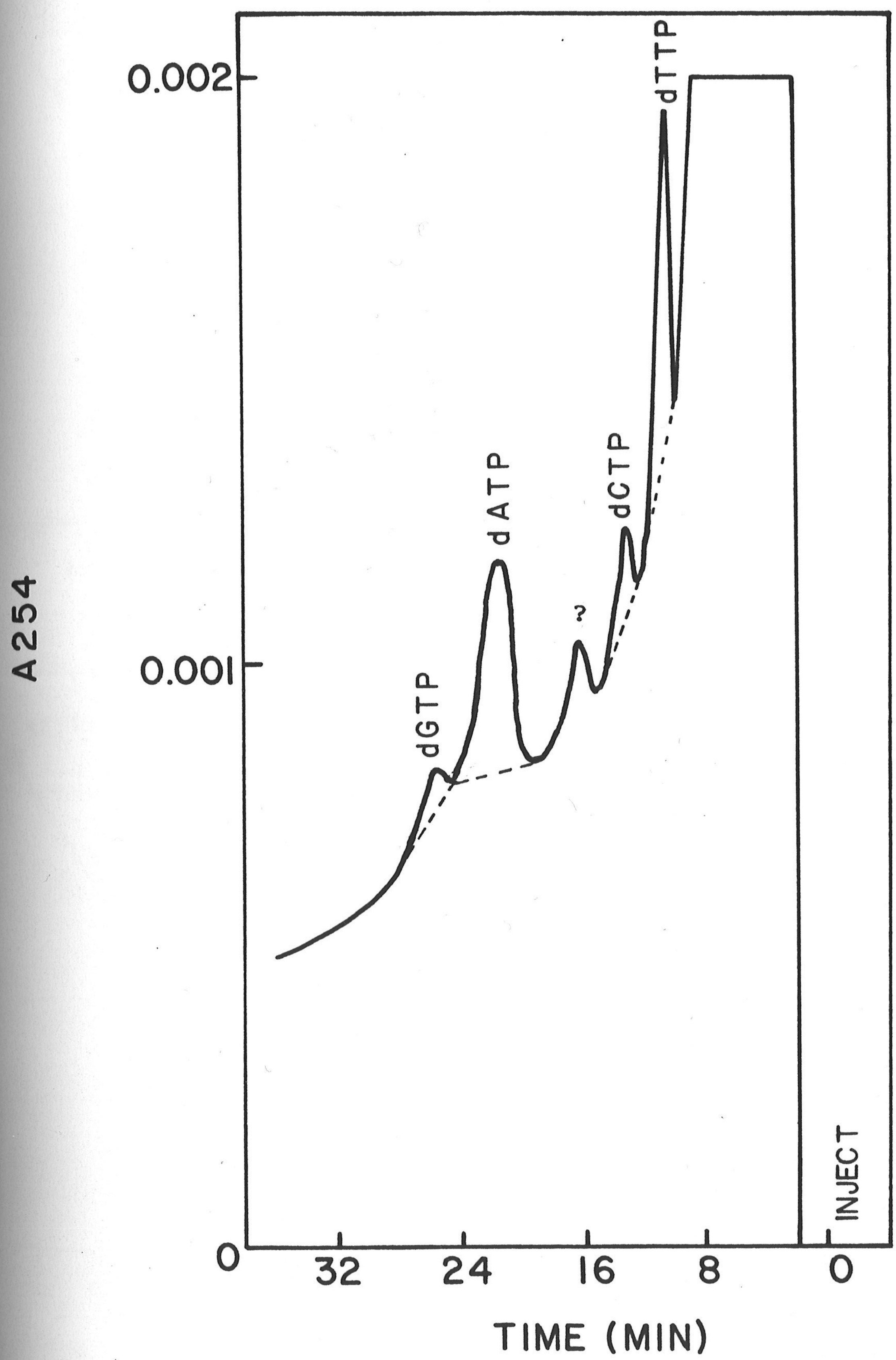
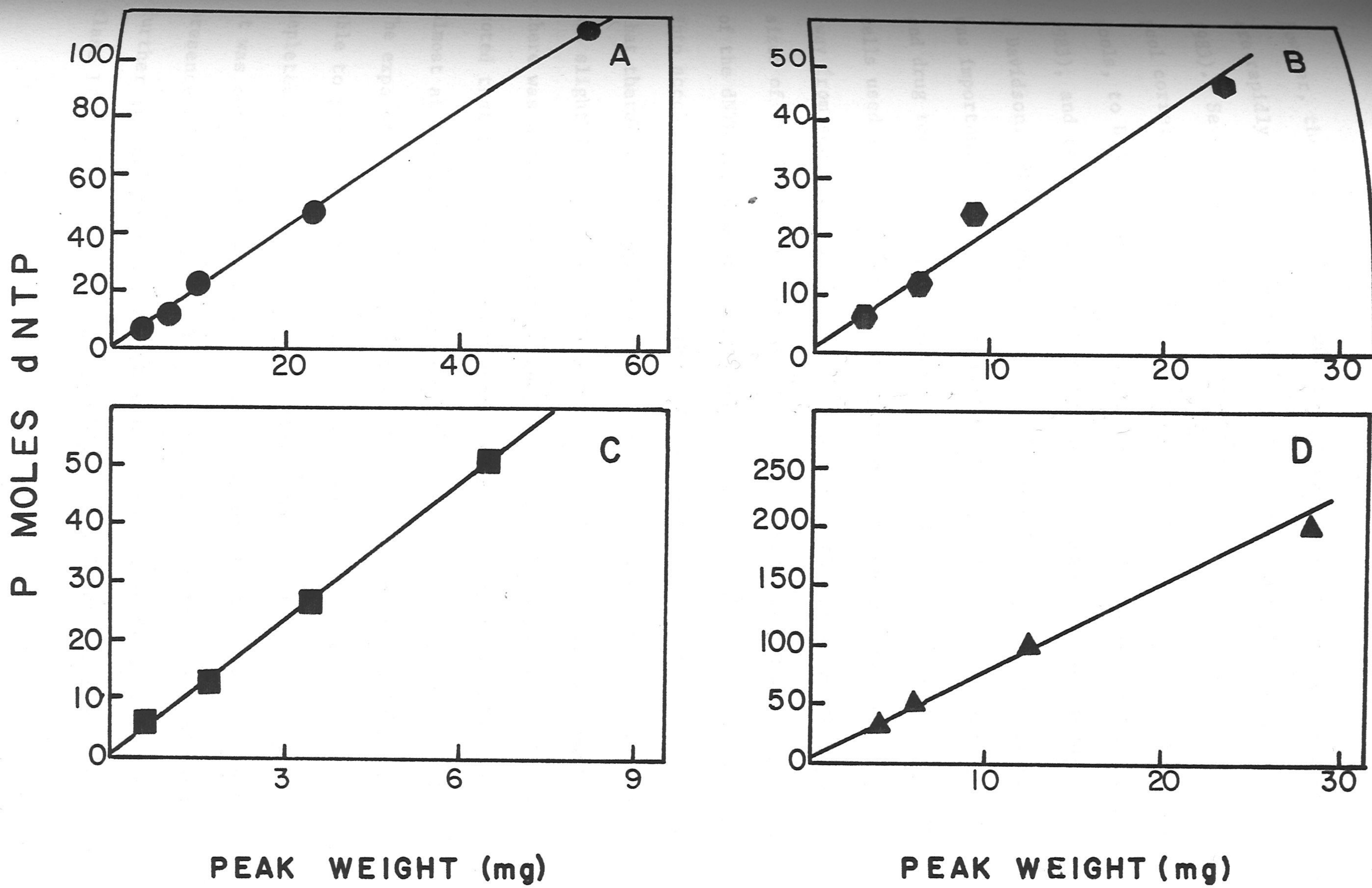

A254
0.002
0.001
0
dGTP
dATP
?
dCTP
dTTP
INJECT
32
24
16
8
0
TIME (MIN)

Figure 30. Standard lines of the variation in peak weight with known concentrations of deoxyribonucleotides. Deoxy NTP standards were prepared gravimetrically and run on the HPLC as described in the Methods and Materials. The peaks were quantitated on the basis of peak weight using the method described in the legend to Figure 29. These standard lines were prepared for each detector sensitivity scale used. These lines represent the concentrations of (A) dATP, (B) dGTP, (C) dCTP, and (D) TTP at a detector sensitivity of 0.002 AUFS.


P MOLES dNTP
PEAK WEIGHT (mg)
PEAK WEIGHT (mg)
A
B
C
D
0
20
40
60
80
100
0
10
20
30
40
50
0
50
100
150
200
250
3
6
9

however, there seems to be general agreement that pyrimidine pools are rapidly depleted (Thiess & Fisher, 1976; Rauscher & Cadman, 1983). Several studies have suggested that the level of the dCTP pool correlated more closely than the three other deoxyribonucleotide pools, to DNA synthesis (Reichard, 1978), cell proliferation (Weber, 1983), and the fidelity of DNA synthesis (Kunkel & Loeb, 1979; Asham & Davidson, 1981; Ashman et al., 1981; Meuth, 1981). Therefore, it was important to examine the dNTP pool sizes in age matched wild type and drug resistant HDF cultures. WC1-2 and passage 92 wild type HDF cells used in these experiments were both three to four doublings away from senescence. Figure 31 represents the relative dNTP pool sizes of WC1-2 with respect to the wild type cells. The actual sizes of the dNTP pools expressed as pmole dNTP/5 x $10^6$ cells or μM (taking into account cell volume) are included in Table VI. Figure 31 shows that there was a significant (1.8 fold) expansion of the dCTP pool and slight increases in TTP and dATP concentrations. In addition, there was a large decline in the dGTP pool, although it should be noted that the level of dGTP was very low in wild type cells as well, almost at the limit of detection (less than 25 pmole/5 x $10^6$ cells). The expansion of the dCTP pool may be the reason this cell strain was able to proliferate in the presence of hydroxyurea, which normally depletes this pool. With the limited amount of material available it was not possible to measure the dynamics of the dCTP pool in the presence of hydroxyurea. The dCTP pool may, in fact, expand even further in the variant since studies in CHO cells indicate that class II mutants overproduce even higher levels of CDP reductase when

Figure 31. Relative deoxribonucleotide pool sizes in WCl-2 and HSC 172 passage 92 cells. The cells were extracted at the same time and chromatographed on the HPLC. The procedures used are described in Methods and Materials. Similar results were obtained in two independant experiments. The actual concentrations of the individual pools are given in Table VI.

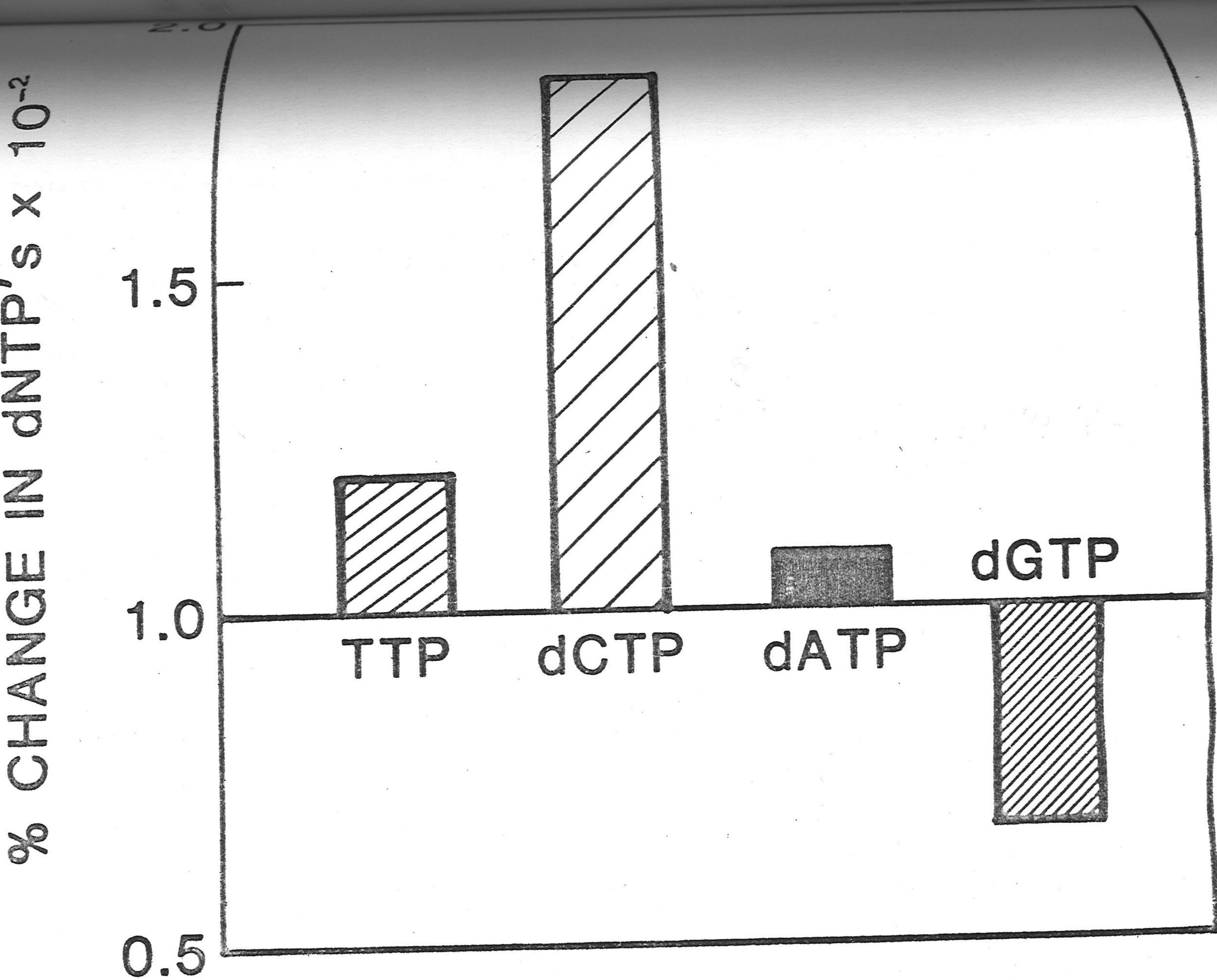

% CHANGE IN dNTP's x 10⁻²
2.0
1.5
1.0
0.5
TTP
dCTP
dATP
dGTP

Table VI. Deoxyribonucleotide Triphosphate Pool Sizes

| CELL STRAIN | TTP | | dCTP | | dATP | | dGTP | |
|---|---|---|---|---|---|---|---|---|
| | $pmoles/5x10^6$ | µM | $pmoles/5x10^6$ | µM | $pmoles/5x10^6$ | µM | $pmoles/5x10^6$ | µM |
| HSC 172-21[a] | 519 | 27 | 118 | 6 | 115 | 8 | 48 | 2.5 |
| HSC 172-86[b] | 494 | 5.3 | 84 | 0.9 | 247 | 2.6 | 44 | 0.5 |
| HSC 172-92[b] | 248 | 2.6 | 45 | 0.5 | 180 | 1.9 | 22 | 0.2 |
| WC1-2[b] | 282 | 3.0 | 95 | 1.0 | 223 | 2.4 | 12.5 | 0.1 |

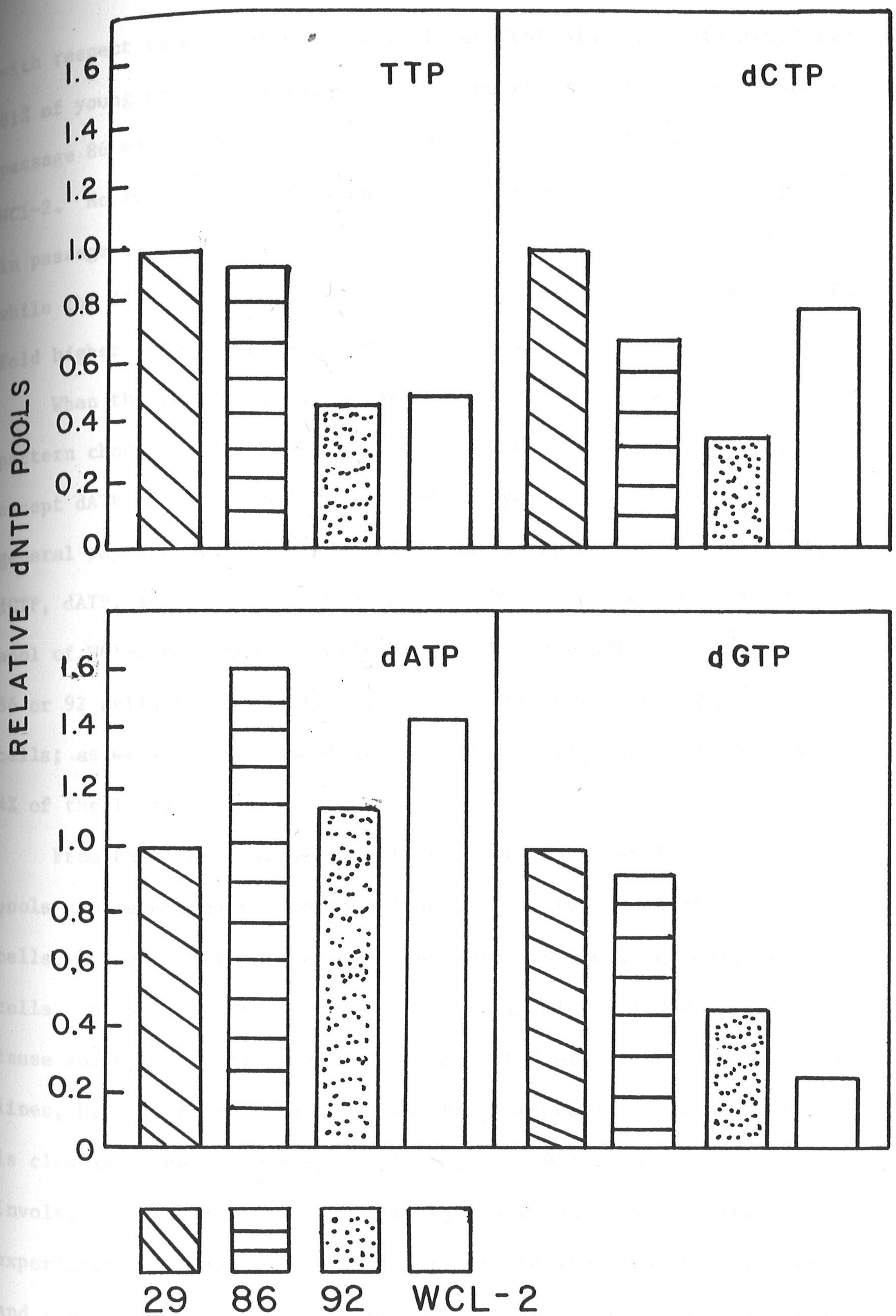
[a] cell volume 3.88 ± .05 pl

[b] cell volume 18.8 ± .2 pl

they are grown in the presence of hydroxyurea (Lewis & Wright, 1979; Hards & Wright, 1983a).

In order to examine the role that altered dNTP pools may play in the senescence of wild type and drug resistant HDF, the dNTP pools were measured in young (less than thirty passages), old (seventy to ninety passages), very old (greater than ninety), and drug resistant HDF. The results of these studies are summarized in Table VI and Figures 32 and 33. Data on dNTP pools can be presented in two different ways; either as pmoles/cell number (as was done in the previous experiments shown in Figure 31) or as concentration (μM). The latter measurement takes into account the marked differences in cell volume which occur during senescence (Simons, 1967). Low passage cells had a volume of 3.88 $\pm$ 0.05 pl/cell while high passage cells had a volume of 18.8 $\pm$ 0.2 pl/cell. These values were based on measurements of trypsinized cells under microscopy using a micrometer eyepiece (Simons, 1967). This 4.8 fold difference in cell volume drastically alters the relative relationship between dNTP pool sizes from young and old cells. Table VI gives the absolute values of the dNTP pools using both measurements. It is evident that some important changes occur in dNTP pool sizes as cells age. Figure 32 represents these relative changes with respect to passage 29 cells, expressing the pool sizes as pmoles/5 x $10^6$ cells. TTP remains relatively constant between passage 29 and 86, however, there is a 50% reduction in TTP in very old passage 92 cells and the mutant WC1-2 cells. The dCTP pool drops to 70% of low passage HDF culture in passage 86 cells and to 38% in passage 92 cells. The elevation of the dCTP pool of WC1-2

Figure 32. Relative deoxyribonucleotide pool sizes of WC1-2 and HSC 172 passage 92 and 86 cells with respect to HSC 172 passage 29 cells. The procedures used to extract and measure the dNTP pools are described in Methods and Materials. The sizes of the dNTP pools were expressed as pmoles/5 x $10^6$ cells and are given in Table VI.

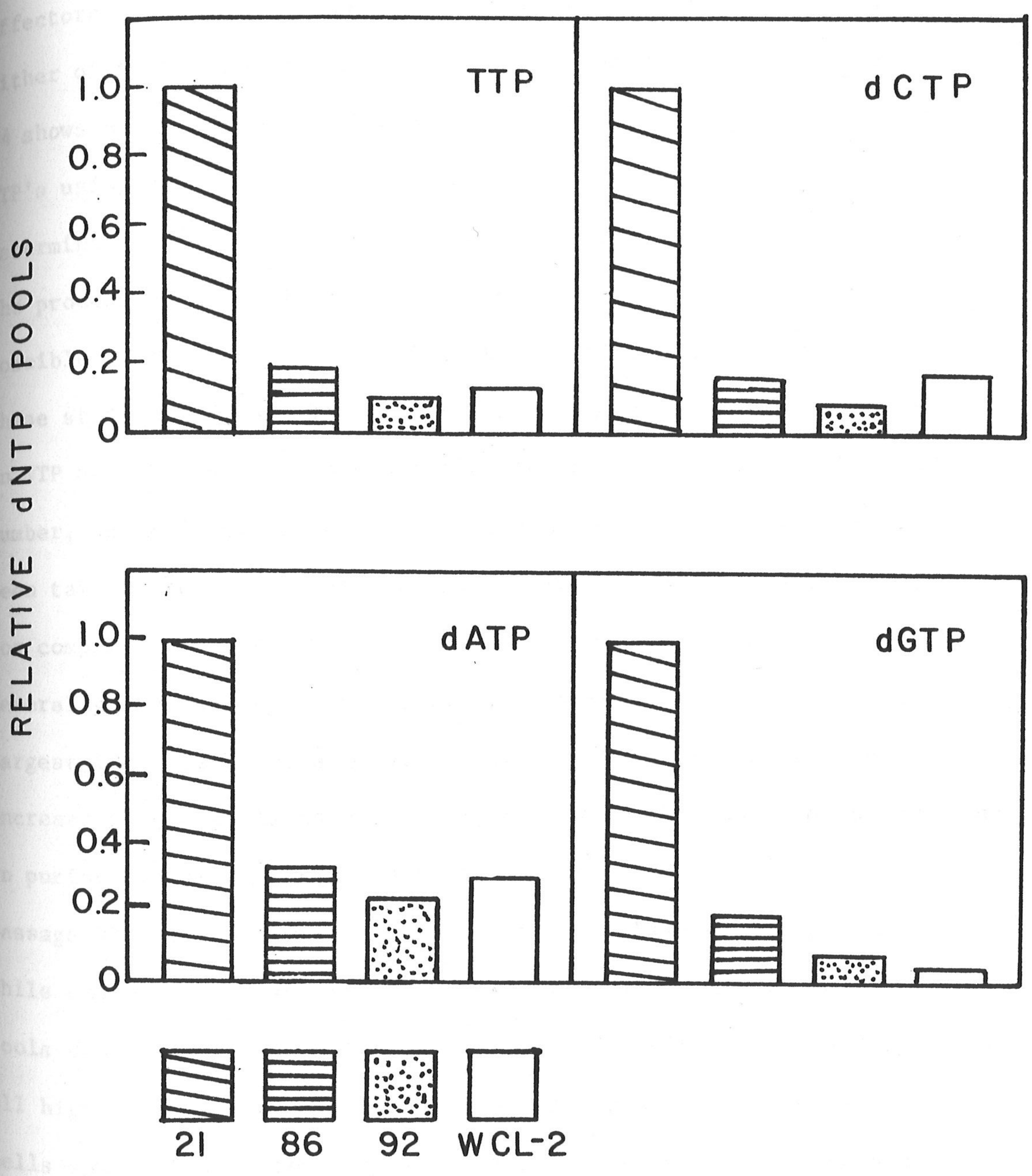

TTP
dCTP
dATP
dGTP
RELATIVE dNTP POOLS
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
29
86
92
WCL-2

with respect to high passage cells is evident since it only drops to 81% of young passage cells. Deoxy GTP remains relatively constant in passage 86 cells, while dropping to 45% in passage 92 and to 25% in WC1-2. No reductions were seen in the dATP pool, in fact this pool in passage 86 cells was 1.6 fold higher than young passage cells, while the dATP pool from passage 92 and WC1-2 cells was 1.16 and 1.44 fold higher than low passage cells, respectively.

When the changes in cell volume were taken into account the pattern changes somewhat (Figure 33). In this case all the pools except dATP declined to less than 20% of young passage cells. The general pattern of passage 86 cells containing slightly higher TTP, dCTP, dATP, and dGTP pools than passage 92 was conserved. The dCTP pool of WC1-2 remained slightly higher than the dCTP pool in passage 86 or 92 cells but markedly lower than this pool in young passage cells; as well the dGTP pool in the mutant cells was very low, only 4% of the level in young cells.

From these pool studies it is evident that overall the dNTP pools are regulated in the same fashion in aging and drug resistant cells, however this process is accelerated in the drug resistant cells. As with any dNTP pool studies it is difficult to determine cause and effect. As noted before many studies, using permanent cell lines, have supported the view that the intracellular level of dCTP is closely linked to DNA synthesis (Weber, 1983), and is intimately involved in the fidelity of DNA synthesis (Meuth, 1981). The experiments discussed above also support the view that CDP reductase and the dCTP pool may play a special role in hydroxyurea resistance (Hards & Wright, 1983a).

Figure 33. Relative deoxyribonucleotide pool sizes of WC1-2 and HSC 172 passage 92 and 86 cells with respect to HSC 172 passage 29 cells taking cell volume into account. The procedures used to extract and measure the dNPT pools are described in Methods and Materials. The sizes of the dNTP pools were expressed as concentration (μM) and are given in Table VI.


RELATIVE dNTP POOLS
TTP
dCTP
dATP
dGTP
1.0
0.8
0.6
0.4
0.2
0
21
86
92
WCL-2

Altered Ribonucleotide Pools in Senescing Normal and Drug-Resistant Human Diploid Fibroblasts

In addition to measuring the dNTP pools, the substrates and effectors of RRase were also analyzed since changes in the levels of either of these components could affect reductase activity. Figure 34 shows a typical separation and identification of the NDP's and NTP's using HPLC. Normal HDF at similar passages as for dNTP determinations were extracted and run directly on the HPLC. Using the procedure outlined in Methods and Materials it was not always possible to resolve UDP, therefore this nucleotide was left out of these studies. Figure 35 presents the data as the relative changes in NTP and NDP pools with respect to low pasage cells, based on cell number, while Figure 36 presents the same data except cell volume has been taken into account and the mutant WC1-2 has been included here for comparison. Certain trends can be seen in these extracts; generally the NTP pools are elevated in higher passage cells with the largest increase in passage 92 cells. While the reason for this increase in NTP pools is not clear, it may be the result of alterations in purine and pyrimidine metabolism as the cells near senescence; passage 92 cells are within three to four doublings of senescence while passage 82 cells are still rapidly proliferating. The NDP pools show a different pattern than for NTP pools since GDP drops in all high passage cells while ADP pools increase, with passage 92 cells again showing the largest increase. CDP pools drop in high passage cells and increase in very high passage 92 cells. When the data is analyzed, taking cell volume into account, a different pattern

Figure 34. Representative chromatogram of the separation of ribonucleoside diphosphates and triphosphates from extracts of HSC 172 cells using HPLC techniques. The procedures of extracting and chromatographing the ribonucleotides are described in Methods and Materials. This chromatogram was generated by applying 20µl of extract representing 1 x $10^6$ cells to a Partisil 10 SAX anion exchange column. Separation of the ribonucleotides was achieved by elution with two buffers: the initial eluent was 7 mM KCl and 7 mM $KH_2PO_4$; pH 4.0; and 250 mM KCl and 250 mM $KH_2PO_4$ pH 5.0. The flow rate was 3.0 mls per minute. The initial eluent was run isocratically for five minutes followed by a linear gradient from 0% of the final eluent to 100% of the final eluent run over thirty minutes. The final eluent was run isocraticaly for ten minutes after which the column was re-equilibrated in low salt by running a linear gradient from 100% final eluent to 0% final eluent over fifteen minutes and running the initial eluent for a further thirty minutes. The dashed line within the ATP peak represents the height of the peak at a detector sensitivity five fold lower. Peak heights were measured to quantitate the relative size of each pool.

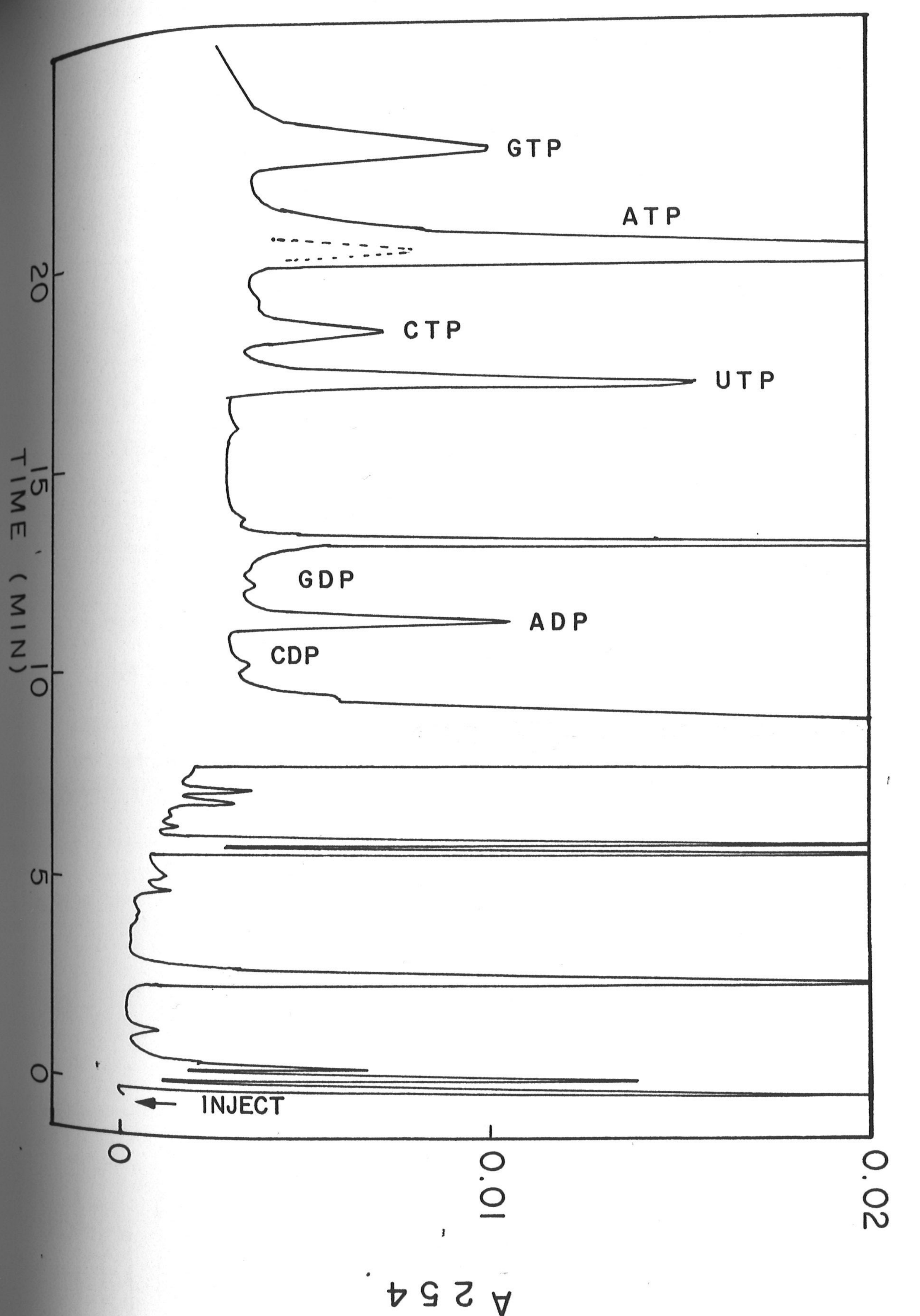
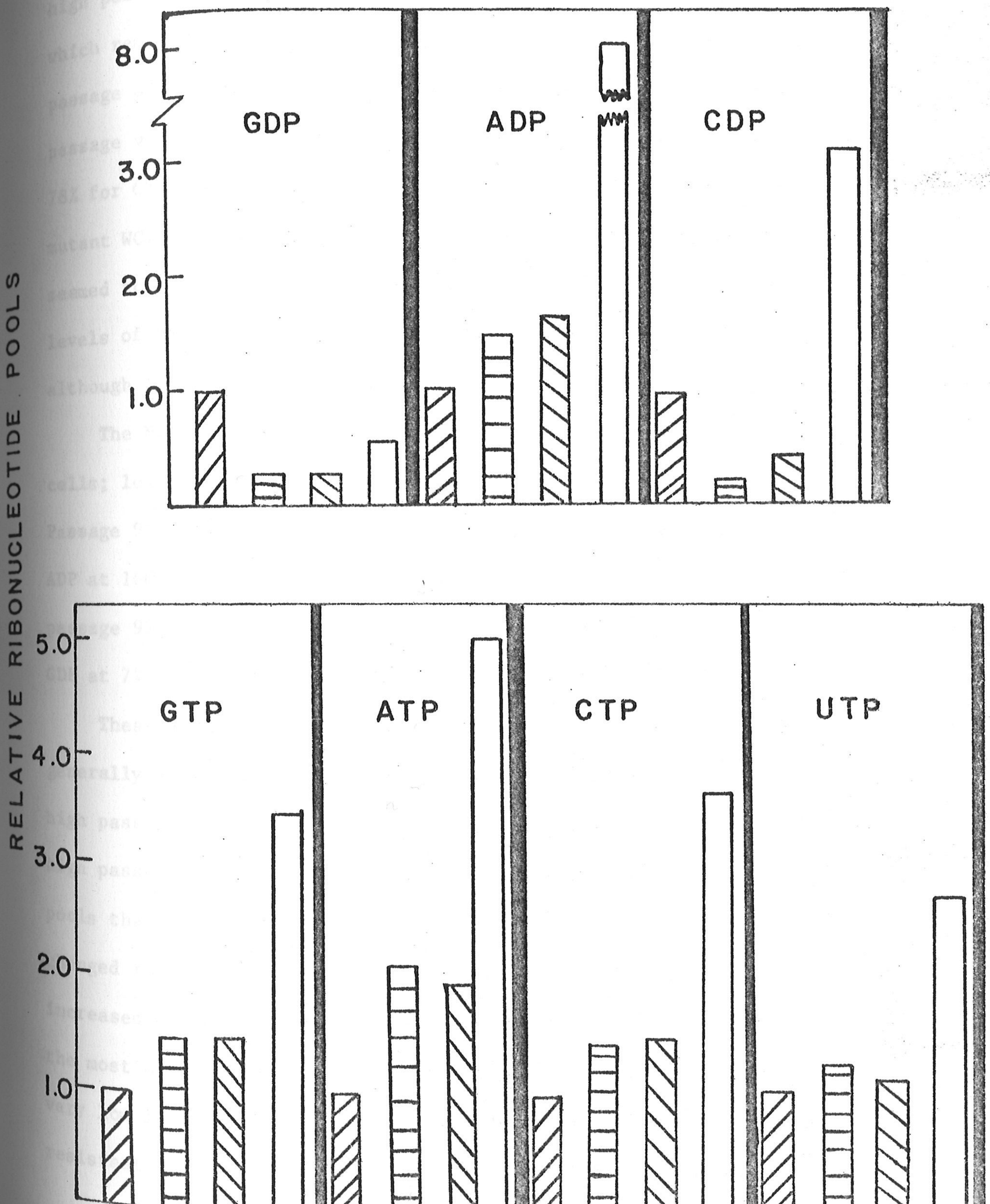

GTP
ATP
CTP
UTP
GDP
ADP
CDP
INJECT
TIME (MIN)
20
15
10
5
0
A 254
0
0.01
0.02

Figure 35. Relative ribonucleoside di- and triphosphate pool sizes of HSC 172 passage 92, 86, and 82 cells with respect to passage 29 cells. The procedures used to extract and measure the pools are described in Methods and Materials. These determinations do not take cell volume into account. The legend for the various cell types is shown in Figure 36.

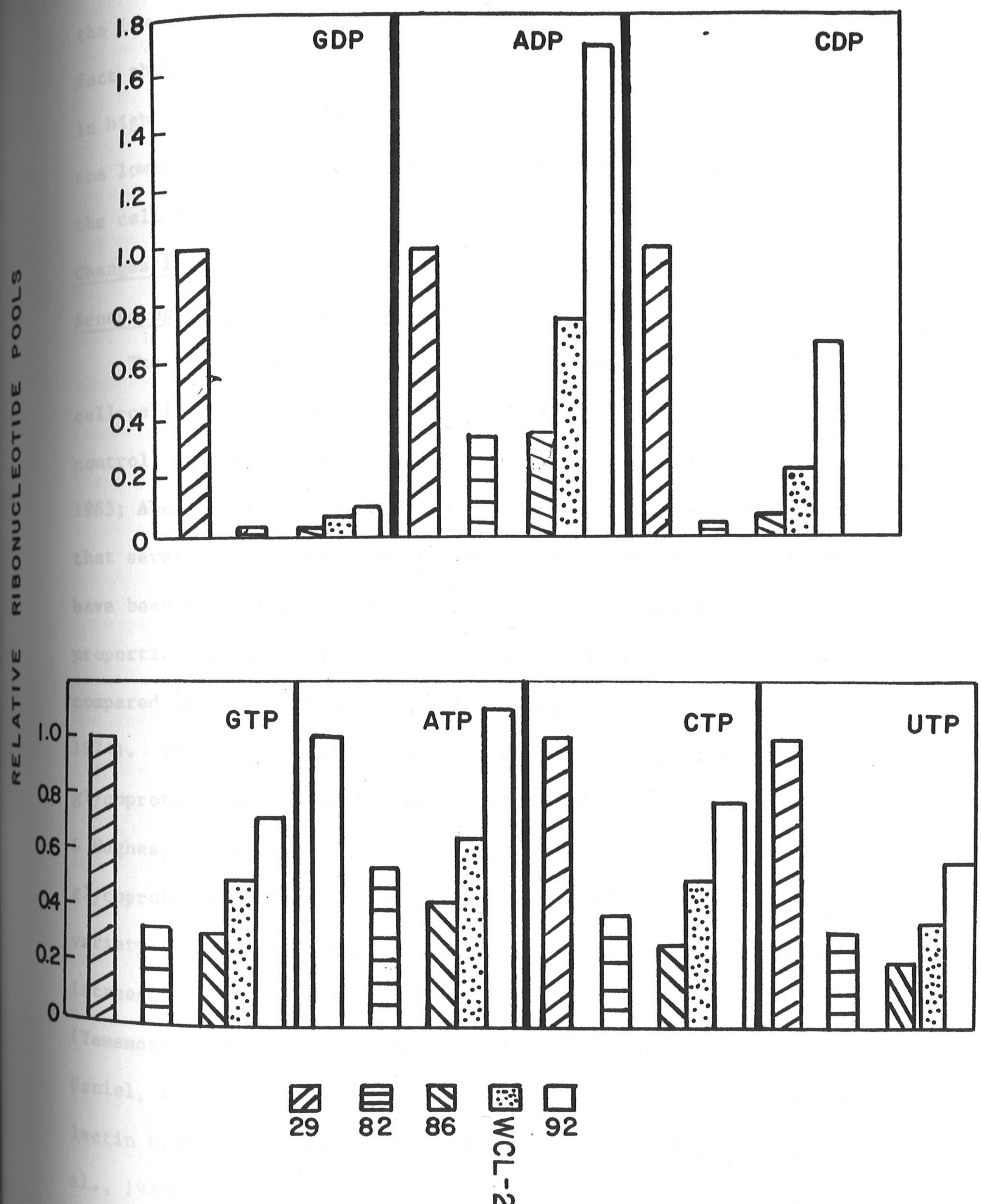

RELATIVE RIBONUCLEOTIDE POOLS
8.0
3.0
2.0
1.0
GDP
ADP
CDP
5.0
4.0
3.0
2.0
1.0
GTP
ATP
CTP
UTP

emerges (Figure 36). These histograms show that NTP pools drop in high passage cells and passage 92 cells, except for the ATP pool which remains about the same in passage 92 cells. The NTP pools for passage 82 and passage 86 cells drop to less than 40% of the low passage value while passage 92 cells show reductions of 70% for GTP, 78% for CTP, 58% for UTP, and an increase of 110% for ATP. The mutant WC1-2 culture which was also several passages from senescence seemed to follow the pattern for passage 92 cells in that the relative levels of NTP pools are higher than for passage 82 and 86 cells although they are slightly lower than passage 92.

The NDP pools decline to very low levels in passage 82 and 86 cells; less than 5% for GDP and CDP and less than 40% for ADP. Passage 92 cells have GDP pools which are 10% of low passage cells, with ADP at 160%, and CDP at 70%; again WC1-2 follows the pattern of passage 92 in that NDP pools are higher than passages 82 and 86 with GDP at 7%, CDP at 25%, and ADP at 80%.

These data indicate that the NTP and NDP pools of the mutant cells generally appear to be regulated in the same age-related way as normal high passage cells. Furthermore, the regulation of the pools in very high passage cells is altered in some way since they contained higher pools than cells which were ten passages younger. The most marked changed in passage 92 cells was in the ATP and ADP pools which are increased above the levels found in low passage cells. Interestingly, the most marked reduction occurred in the GDP pool which dropped to very low levels in all the high passage cells (as well as the drug resistant cells). The reason for this change is not known, however

Figure 36. Relative ribonucleoside di- and triphosphate pool sizes of WC1-2, and HSC 172 passage 92, 86 and 82 cells with respect to passage 29 cells taking cell volume into account. The procedures used to extract and measure the pools are described in Methods and Materials.



RELATIVE RIBONUCLEOTIDE POOLS
GDP
ADP
CDP
GTP
ATP
CTP
UTP
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
29
82
86
WCL-2
92

the low level of this substrate for GDP reductase may account for the fact that dGTP drops to very low levels (< 25 pmoles/5 x $10^6$ cells) in high passage cells (Figure 33), and generally dGTP is present at the lowest concentration of all the four deoxynucleotides found in the cell (Walters et al., 1976).

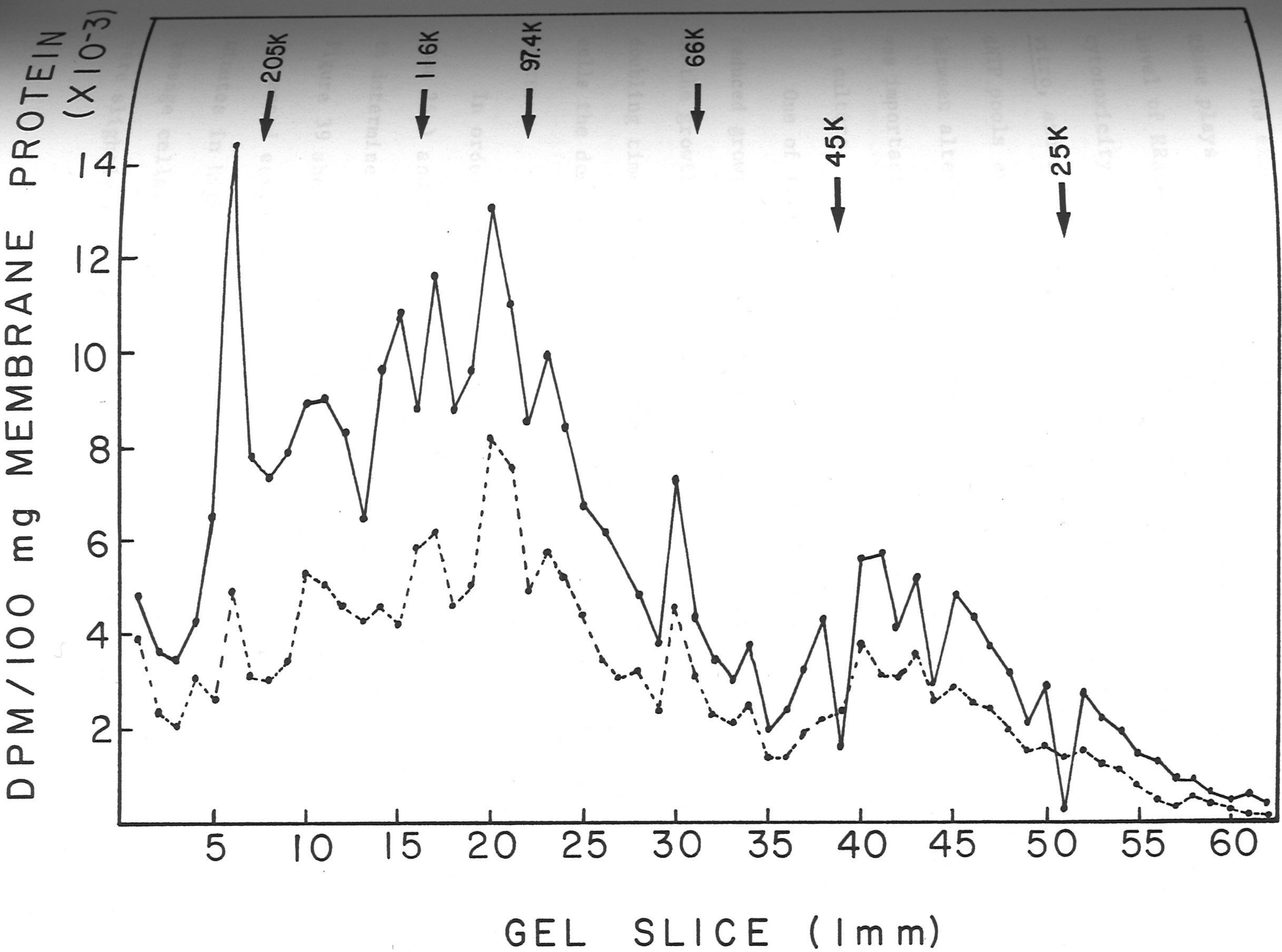
## Changes in Membrane Glycoproteins in Senescing Human Diploid Fibroblasts

The cell surface membrane is an important organelle involved in cell-cell recognition, antigenic recognition, differentiation, growth control, and cancer transformation (Wright, 1979; Parfett et al., 1983; Abercrombie, 1979; Wallach, 1975). It is interesting to note that several age-related changes associated with the cell surface have been reported. For instance there is an increase in the proportion of mucopolysaccharide on the surface of senescent HDF as compared to earlier passage proliferating cells (Courtois & Hughes, 1974). The level of glucosamine incorporation into membrane glycoprotein appears to be unaltered in high passage cells (Courtois & Hughes, 1974) while the level of sialic acid in membrane glycoproteins drops with <u>in vitro</u> passage (Milo & Hart, 1976). A variety of other age-related changes which have been reported include increased cell size (Simons, 1967), reduced Con A agglutination (Yamamoto et al., 1977), altered cell surface topography (Bowman & Daniel, 1975), cell adhesion (Azenocott et al., 1975), changes in lectin binding (Auberg et al., 1980; Yamamoto et al., 1977; Aizawa et al., 1980), and an increase in fibronectin (Courtois & Hughes, 1976).

Kaplan et al., (1984) have shown that the level of nucleotide sugars correlates with the level of glycoprotein biosynthesis. The low intracellular levels of GDP in high passage HDF (Figure 36) suggested the nucleotide sugar associated with GDP may be affected. In order to test whether high passage HDF cells have alterations involving the nucleotide sugar GDP mannose, the incorporation of $^{14}$C-mannose into the cellular membranes of high passage (86) and low passage (33) cells was determined. High passage cells incorporated 2065 dpm/$\mu$g membrane protein while low passage cells incorporated 4070 dpm/$\mu$g membrane protein. This constitutes a two fold reduction in the amount of mannose in membrane glycoprotein in low passage cells. The purified membrane protein was then dissolved in SDS and separated by molecular weight using SDS polyacrylamide electrophoresis as described by Parfett et al. (1983). The gel was dried, sliced into 1 mm sections, and the amount of radioactivity in each slice was determined. The results of these experiments are shown in Figure 37. The reduced incorporation of $^{14}$C-mannose into the membrane glycoprotein of high passage cells is clearly evident. This reduction was most evident in the high molecular weight proteins, however even the low molecular weight proteins contained decreased amounts of labelled sugar. There may also be some minor alterations in the positions of some labelled peaks, although the positions of most of the major peaks were unchanged.

Further studies must be done to explain why high passage cells incorporate less mannose. For instance the activity of mannosyl transferase may be reduced so less mannose is added to the glycoprotein or less mannose is available for the transferase because

Figure 37. Distibution of label into cell surface membrane preparations following $^{3}H$-mannose incorporation and SDS slab gel electrophoresis. HSC 172 passage ( 33) and ( 88) were incubated for eight hours with 1 μCi/ml of $^{3}H$-mannose. The cell membranes were then extracted, dissolved in SDS, and electrophoresed using the procedures described in Methods and Materials. Adjacent tracks of dried gel which contained low and high passage preparations were sliced into 1 mm portions and the radioctivity was determined by liquid scintillation counting. The solid line represents passage 33 preparations and the broken line shows passage 88 preparations. The molecular weights indicated by the arrows represent the relative position of myosin (205,000), β-galactosidase (116,000), phosphorylase b (97,400), bovine albumin (66,000), egg albumin (45,000), and carbonic anhydrase (25,000).



DPM/100 mg MEMBRANE PROTEIN (X10-3)
14
12
10
8
6
4
2
205K
116K
97.4K
66K
45K
25K
5
10
15
20
25
30
35
40
45
50
55
60
GEL SLICE (1mm)

the low GDP pool has become rate limiting in these high passage cells.

Characterization of Ribonucleotide Reductase in Low and High Passage Human Diploid Fibroblasts

The experiments which have been described above indicate that RRase plays an important role in the process of *in vitro* aging. The level of RRase activity and the cellular resistance to hydroxyurea cytotoxicity decreases as normal diploid fibroblasts are passaged *in vitro*, and mutant cell lines with altered reductase activity and dNTP pools appear to senesce prematurely. Because of this association between altered ribonucleotide reductase and cellular senescence it was important to examine the regulation of RRase as cells are passaged in culture.

One of the key features of normal diploid fibroblasts is the reduced growth rate in high passage cells. This phenomona is evident in the growth curves shown in Figure 38. The low passage cells had a doubling time of less than twenty-four hours while in high passage cells the doubling time was extended to greater than forty-eight hours.

In order to properly compare reductase activity in low (less than 25 ) and high (greater than 70) passage cells it was necessary to determine if assay conditions were altered in high passage cells. Figure 39 shows that permeabilization prior to assay results in increased enzyme activity, with optimal activity between 60 - 90 minutes in high passage cells and between 30 - 75 minutes in low passage cells. As mentioned earlier the conditions of these assays were slightly changed between these experiments and those discussed

Figure 38. Growth curve of HSC 172 passage 25 (●) and passage 78 (■) cells.

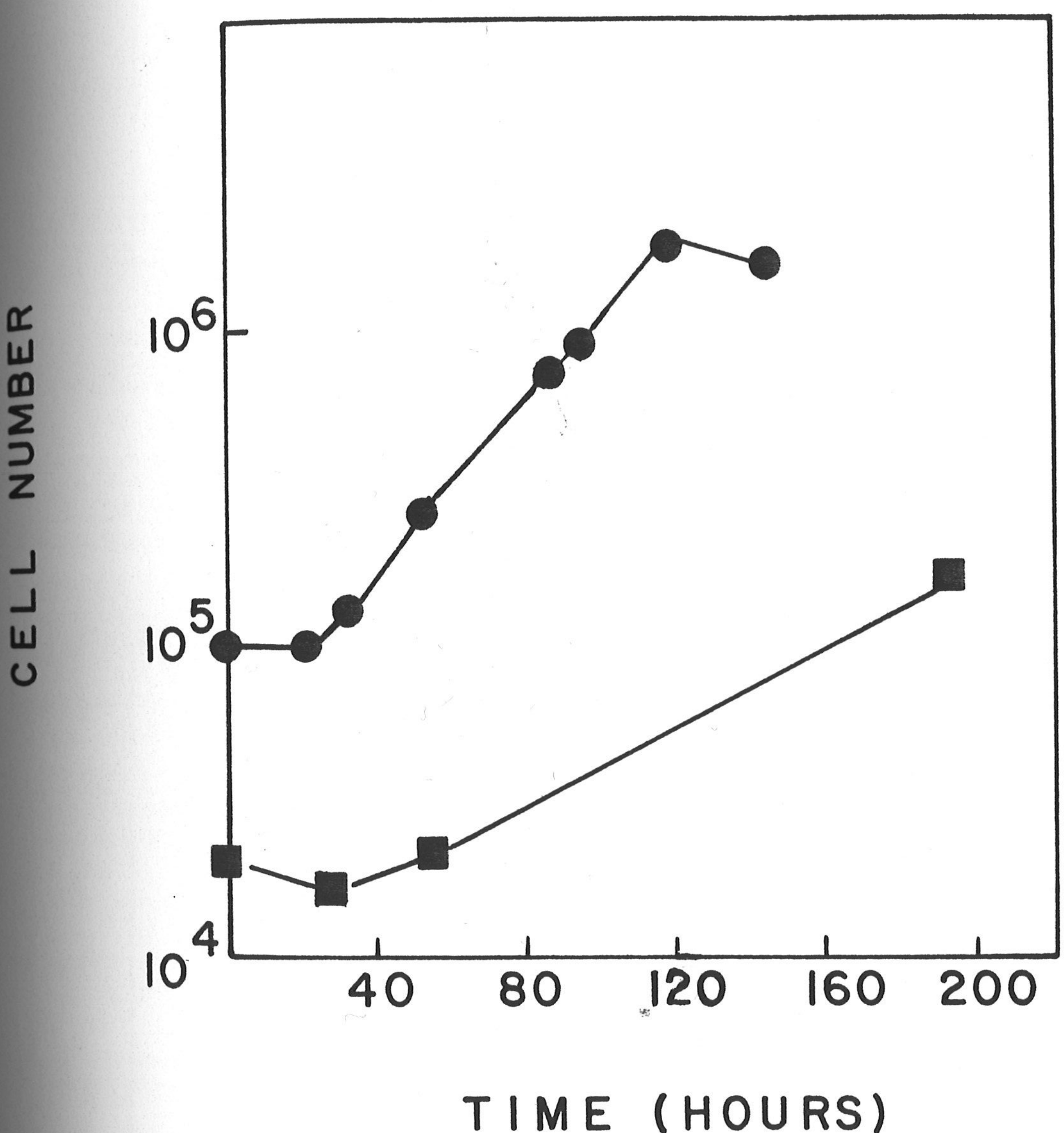
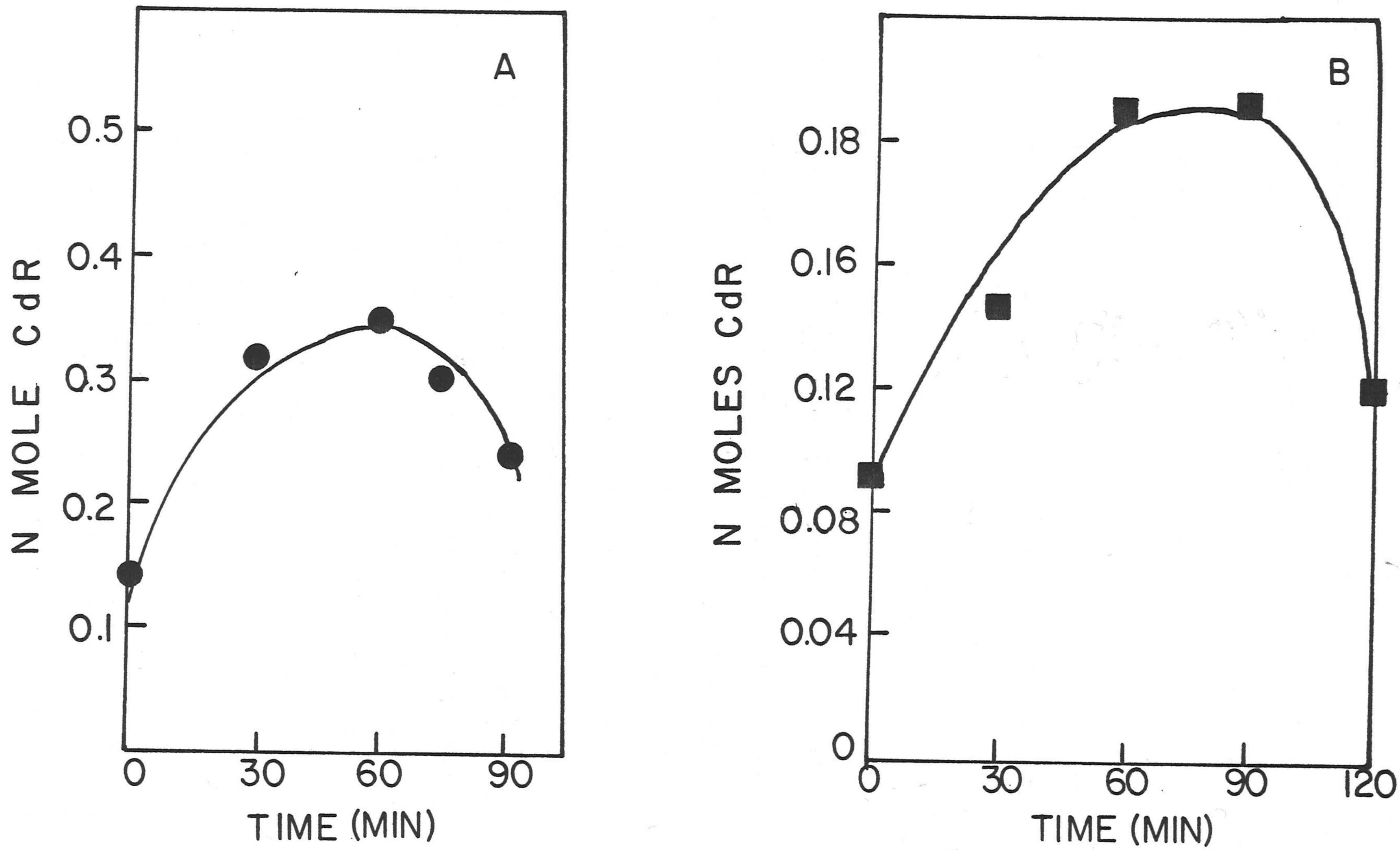

CELL NUMBER
10^6
10^5
10^4
40
80
120
160
200
TIME (HOURS)

Figure 39A. Response of CDP reduction in HSC 172 passage 22 cells to incubation in permeabilization buffer for various lengths of time.

Figure 39B. Response of CDP reduction in HSC 172 passage 78 cells to incubation in permeabilization buffer for various lengths of time.


A
N MOLE CdR
0.1
0.2
0.3
0.4
0.5
0
30
60
90
TIME (MIN)
B
N MOLES CdR
0
0.04
0.08
0.12
0.16
0.18
0
30
60
90
120
TIME (MIN)

earlier during the development of the assay. Different commercial lots of Tween 80 had to be used in the latter experiments resulting in a slight change in optimal permeabilization time. In addition, the assay was linear with time to only thirty minutes.

In order to confirm that these cells were indeed permeabilized, the ability of high and low passage cells to exclude trypan blue was measured. It is generally assumed that the vital dye trypan blue requires intact membranes for it to be excluded from cells (Patterson, 1979), therefore permeabilized cells should retain the dye and stain blue. It was found that low and high passage cells showed approximately equal permeabilization abilities in that 73 $\pm$ 1% high passage and 75 $\pm$ 0.5% low passage cells were stained blue after 75 minutes incubation in permeabilization buffer. Figure 40 indicates that optimal permeabilization occurs after 75 minutes of incubation. Two of the key features of the permeabilized cell reductase assay are that cells remain viable during permeabilization and the fact that permeabilization is reversible. For instance in CHO cells a high percentage of cells are able to replate in normal culture medium and form colonies after thirty minutes of permeabilization (Hards & Wright, 1983b). The PE of low and high passage cells was determined after 75 minutes of incubation in permeabilization buffer, and compared to the PE obtained after suspending the cells in tissue culture medium for a similar length of time. The RPE was 80% for low passage cells and 69% for high passage cells indicating that a relatively high proportion of cells remain viable during permeabilization.

The activation of CDP reductase requires ATP with optimal activity at 4 mM in low and high passage cells (Figure 41). In order to

Figure 40. Effect of incubating HSC 172 passage 21 cells in permeabilization buffer for various lengths of time on trypan blue staining. At various times cells were removed from the permeabilization buffer, spun down and resuspended in PBS. Four percent trypan blue stain was added to the resuspended cells at a ratio of 1:9. After five minutes the cells were added to a hemocytometer and the percent of blue stained cells was determined. At least 250 cells were counted for each determination.

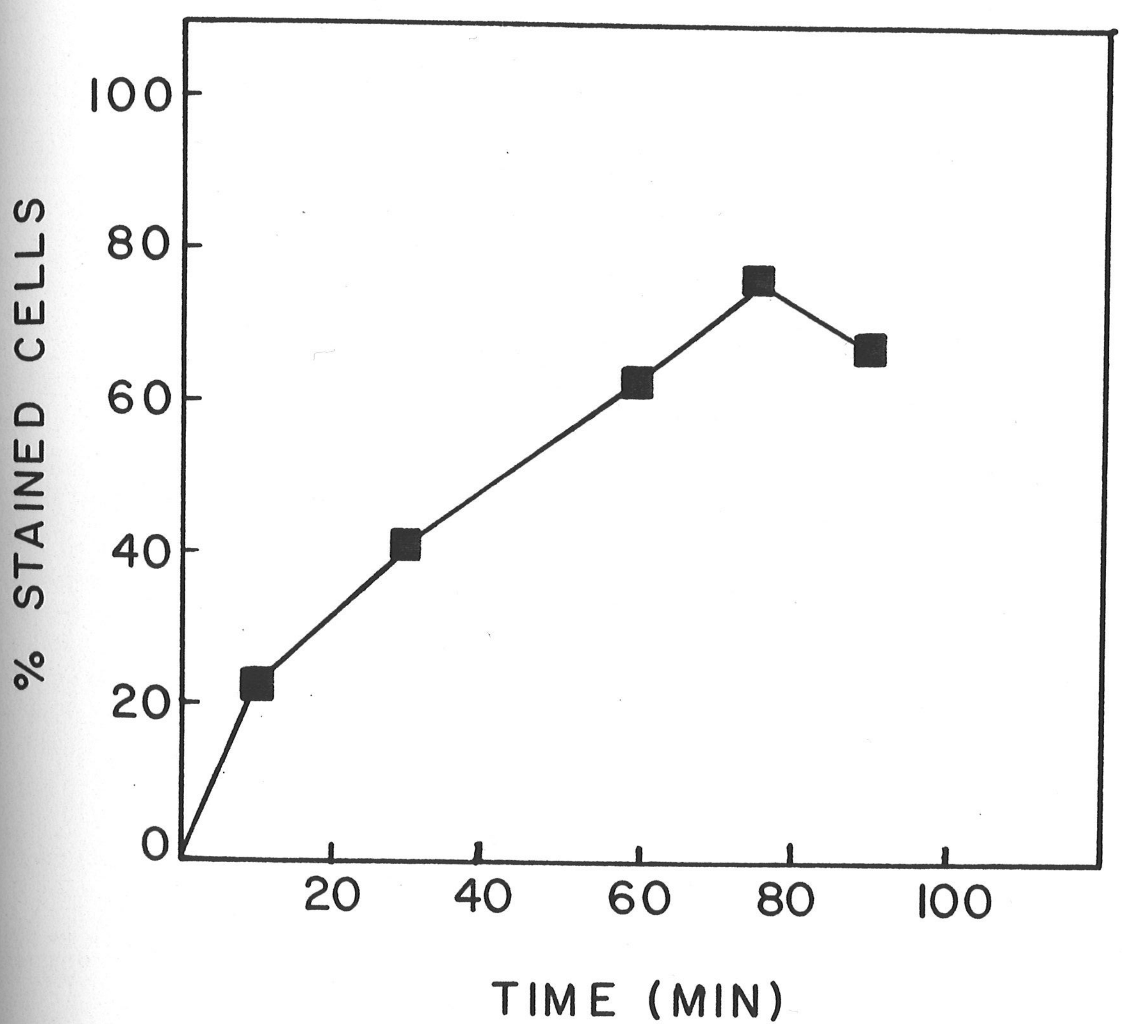
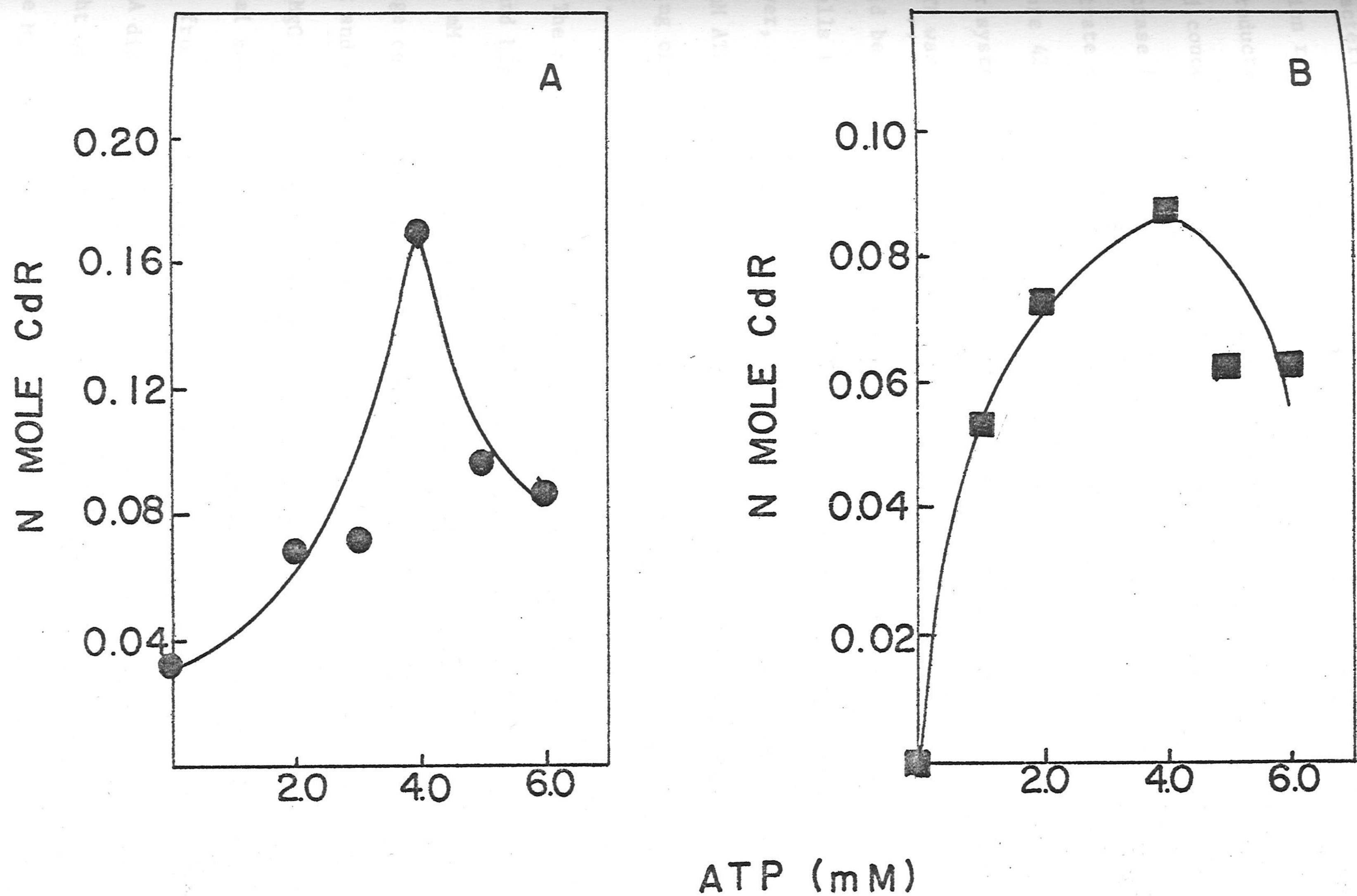

100
80
60
40
20
0
% STAINED CELLS
20
40
60
80
100
TIME (MIN)

Figure 41A. Response of CDP reduction in HSC 172 passage 12 cells to varying concentrations of ATP.

Figure 41B. Response of CDP reduction in HSC 172 passagae 73 cells to varying concentrations of ATP.


A
B
N MOLE CdR
0.20
0.16
0.12
0.08
0.04
0.10
0.08
0.06
0.04
0.02
2.0
4.0
6.0
ATP (mM)

characterize the activition of CDP reductase by ATP in a more detailed fashion reciprocal Lineweaver-Burk plots were generated by measuring CDP reductase activity at various CDP concentrations using several fixed concentrations of ATP. Figure 42 shows that ATP activates CDP reductase by increasing the velocity as well as the affinity for substrate using low passage cells. The Ka for ATP binding was 1.4 mM (Figure 42, inset). This value compares well to the Ka determined in other systems (Chang and Cheng, 1979). The activation of CDP reduction by ATP was also estimated in high passage cells (Figure 43). It should be noted that it was difficult obtaining large enough quantities of cells to carry out detailed studies at these high passage numbers, however, a similar pattern of activation was observed and a Ka of 2.0 mM ATP was calculated (Figure 43, inset) suggesting that the binding of ATP to the enzyme is similar in low and high passage cells.

The activation of ADP reduction by dGTP was also determined in low and high passage cells. The optimal concentration was between 1 and 2 mM dGTP in high passage cells, and between 0.5 and 2 mM in low passage cells again indicating no major changes in dGTP activation in young and old cells (Figure 44).

$MgCl_2$ activated CDP reduction in both young and old cells with optimal activity at about 4 to 8 mM in high passage cells and a broad range from 4 to 12 mM in low passage cells (Figure 45).

A discussed earlier hydroxyurea is a specific inhibitor of RRase (Wright et al., 1981) probably by destroying the tyrosine free radical in the $M_2$ subunit which is involved in reduction (Graslund et al., 1982).

Figure 42. Double reciprocal plot of the variation in the rate of CDP reduction with varying CDP concentrations at several fixed concentrations of ATP. Permeabilized cells were at passage 19. Standard assay conditions were used except for varying concentrations of CDP and the presence of 0.5 mM ( ● ), 1.0 mM ( ⬢ ), 2.0 mM ( ■ ), and 3.0 mM ( △ ) ATP.

Inset: Replot of the velocity intercepts versus the reciprocal of the ATP concentration.

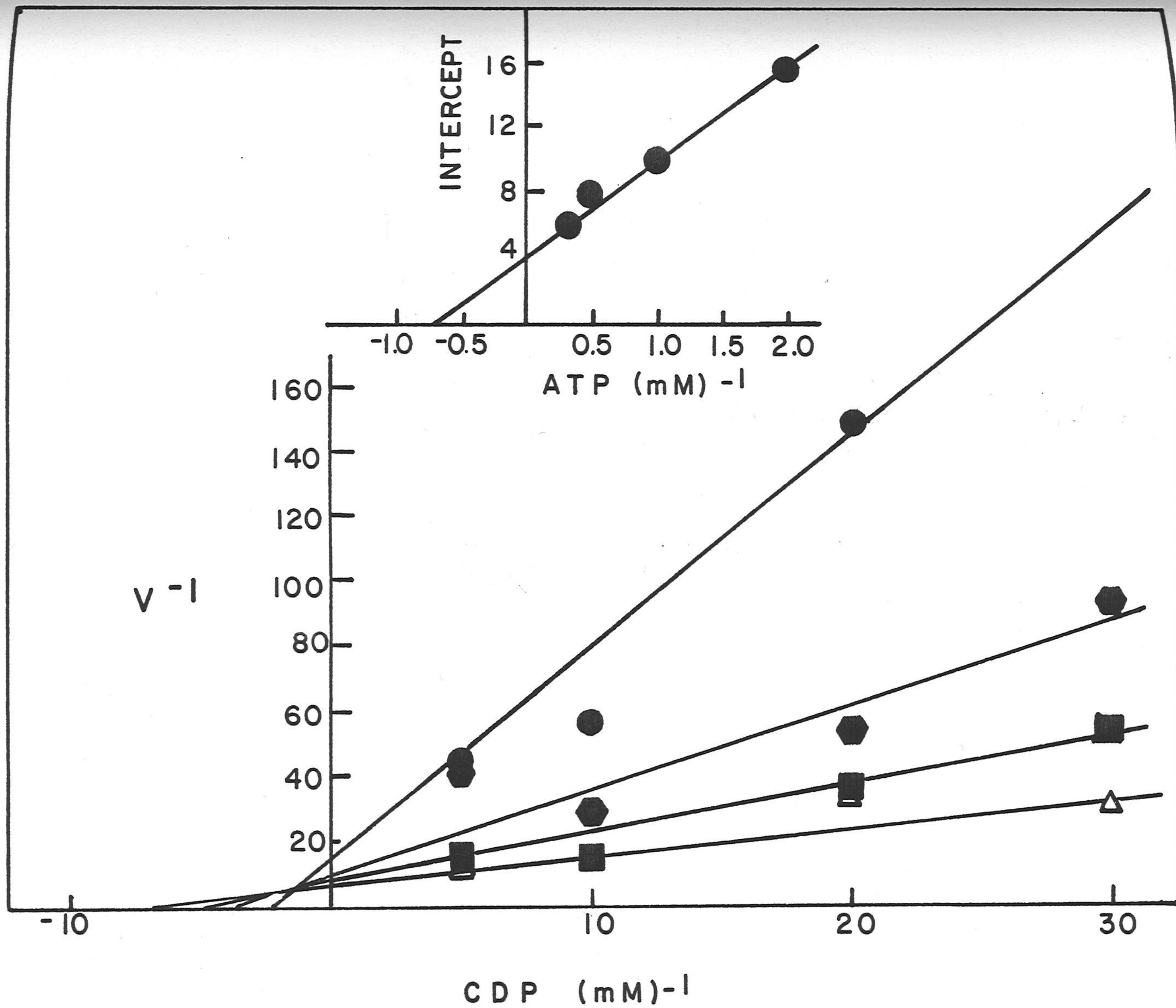
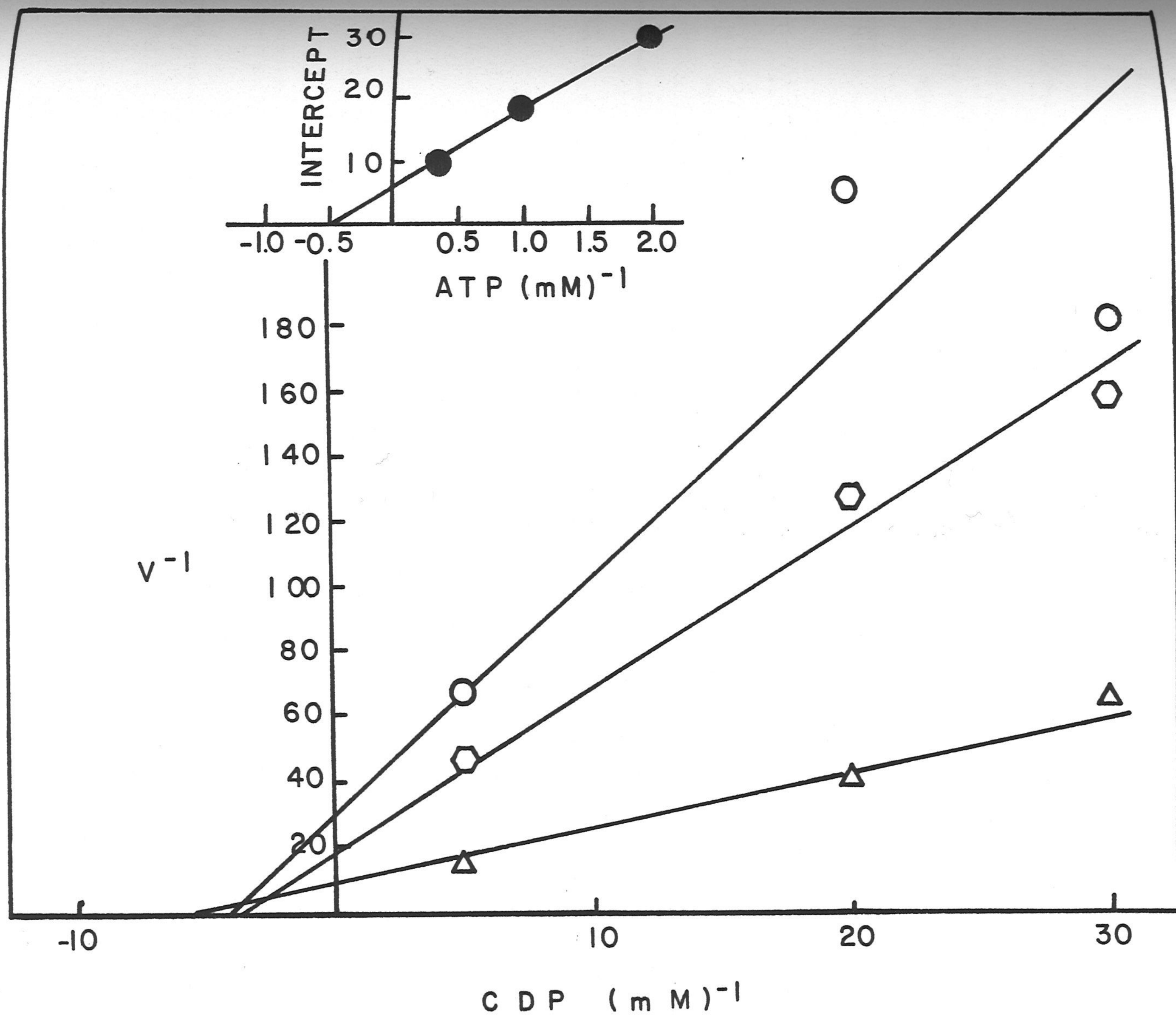

INTERCEPT
16
12
8
4
-1.0
-0.5
0.5
1.0
1.5
2.0
ATP (mM) -1
160
140
120
100
80
60
40
20
V -1
-10
10
20
30
CDP (mM)-1

Figure 43. Double reciprocal plot of the variation in the rate of CDP reduction with varying CDP concentration at several fixed concentrations of ATP. Permeabilized cells were at passage 78. Standard assay conditions were used except for varying concentrations of CDP and the presence of 0.5 mM ( △ ), 1.0 mM ( ⬡ ) and 3.0 mM ( △ ) ATP. Inset. Replot of the velocity intercepts versus the reciprocal of the ATP concentration.



INTERCEPT
30
20
10
-1.0 -0.5
0.5 1.0 1.5 2.0
ATP (mM)$^{-1}$
180
160
140
120
100
80
60
40
20
V$^{-1}$
-10
10
20
30
CDP (mM)$^{-1}$

Figure 44A. Response of ADP reduction in HSC 172 passage 15 cells to varying concentrations of dGTP.

Figure 44B. Response of ADP reduction in HSC 172 passage 75 cells to varying concentrations of dGTP.

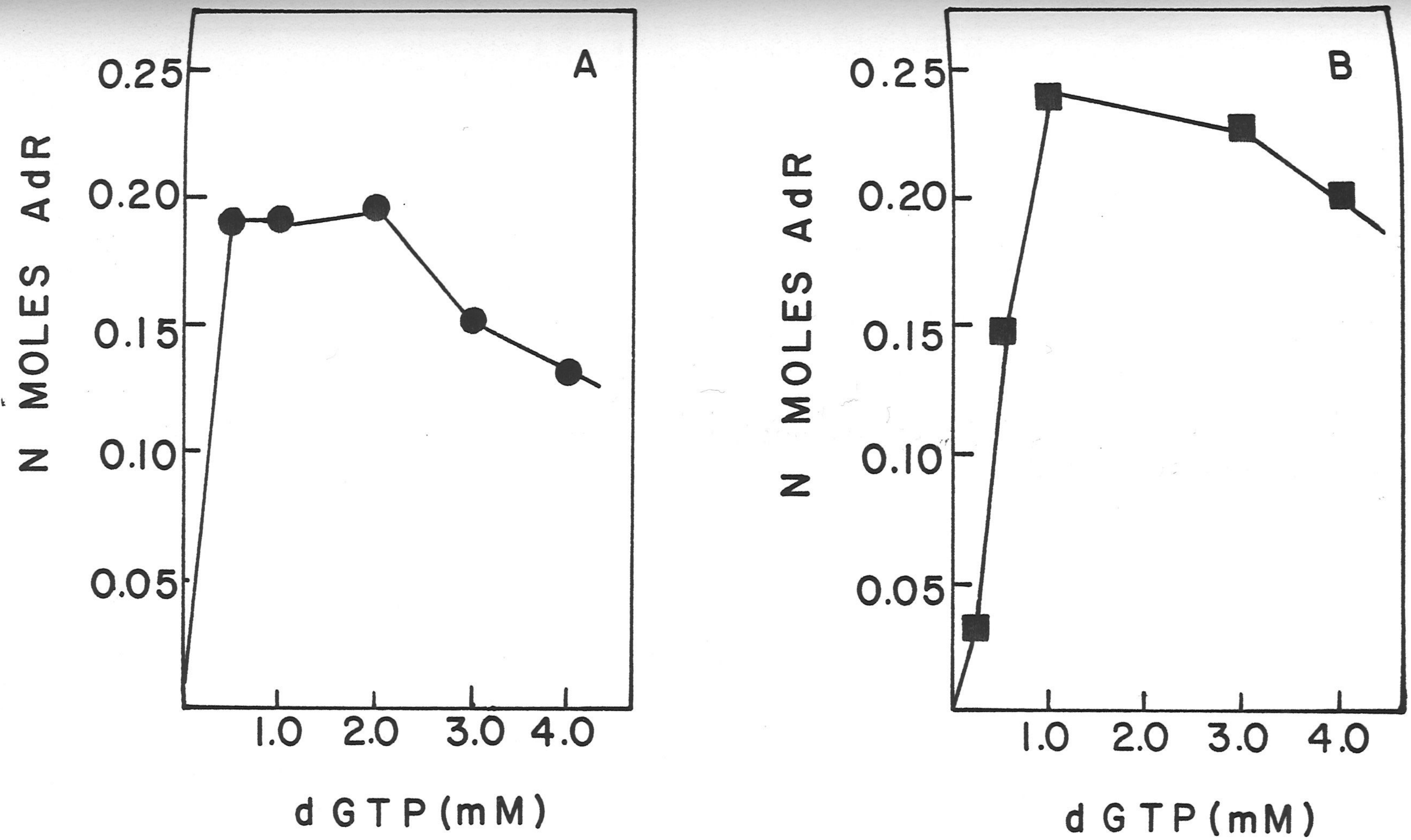
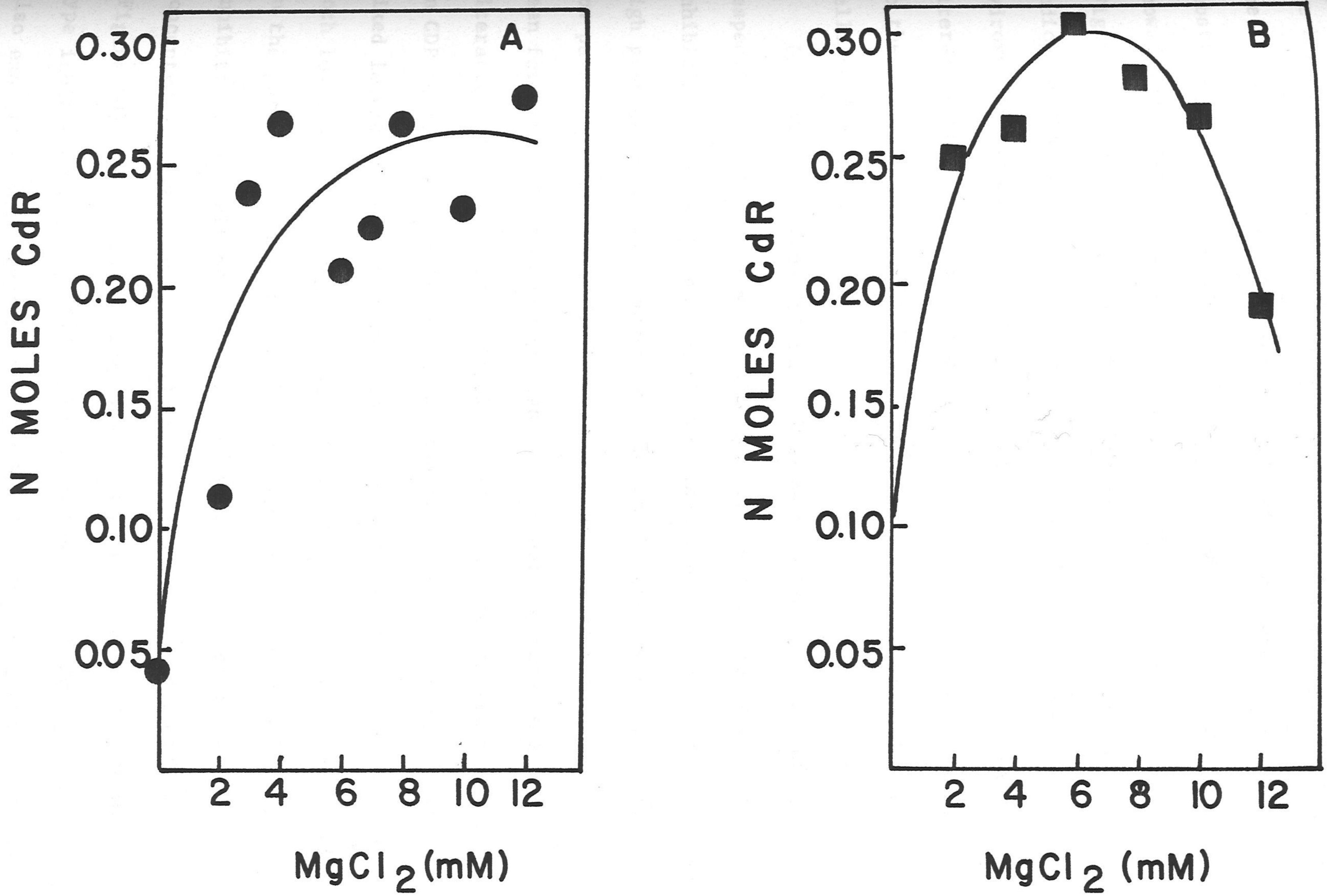

A
B
N MOLES AdR
0.25
0.20
0.15
0.10
0.05
1.0
2.0
3.0
4.0
d GTP (mM)

Figure 45A. Response of CDP reduction in HSC 172 passage 22 cells to varying concentrations of $MgCl_2$.

Figure 45B. Response of CDP reduction in HSC 172 passage 71 cells to varying concentrations of $MgCl_2$.


A
B
N MOLES CdR
0.30
0.25
0.20
0.15
0.10
0.05
2 4 6 8 10 12
MgCl2 (mM)

The binding of hydroxyurea was examined by double reciprocal plots of the variation in CDP reductase activity with the concentration of CDP substrate at several fixed levels of hydroxyurea. These experiments showed that both low passage (Figure 46) and high passage cells (Figure 47) had an apparent Ki for hydroxyurea of 0.5 mM. This indicates that the increased sensitivity of high passage cells to hydroxyurea cytotoxicity (Figure 21) is not due to a structural alteration in the $M_2$ subunit which reduces the $K_i$; rather it is due to the decreased levels of RRase activity found in these high passage cells (Figure 20).

DeoxyATP inhibited CDP and ADP reductase (Figures 48 and 49 respectively) in both low and high passage cells. The extent of inhibition of RRase by dATP differs markedly between young and old cells, high passage cells were more resistant to the effects of dATP than low passage cells. This increased resistance to dATP inhibition was seen for both ADP and CDP reductase. In order to characterize the alterations in more detail, double reciprocal plots of the variation in CDP reductase activity with CDP substrate concentration at several fixed levels of dATP were constructed. These plots, generated using both low and high passage cells, revealed some interesting alterations in the pattern of inhibition. Low passage cells showed mixed type inhibition; in addition the replot of y intercepts versus dATP concentrations was linear and yielded an apparent $K_i$ of 55 μM dATP (Figure 50). This type of inhibition is referred to as linear mixed type inhibition (Segal, 1975). The reductase from high passage cells also exhibited mixed type inhibition by dATP, however, the replot was

Figure 46. Double reciprocal plot of the effect of hydroxyurea on the rate of CDP reduction with varying CDP concentrations. Permeabilized cells were at passage 10. Standard assay conditions were used except for varying concentrations of CDP and the absence ( △ ) or the presence of 0.2 mM ( □ ), 0.5 mM ( ○ ), and 0.7 mM ( ⬡ ) hydroxyurea.

Inset: Replot of the velocity intercepts versus hydroxyurea concentrations.

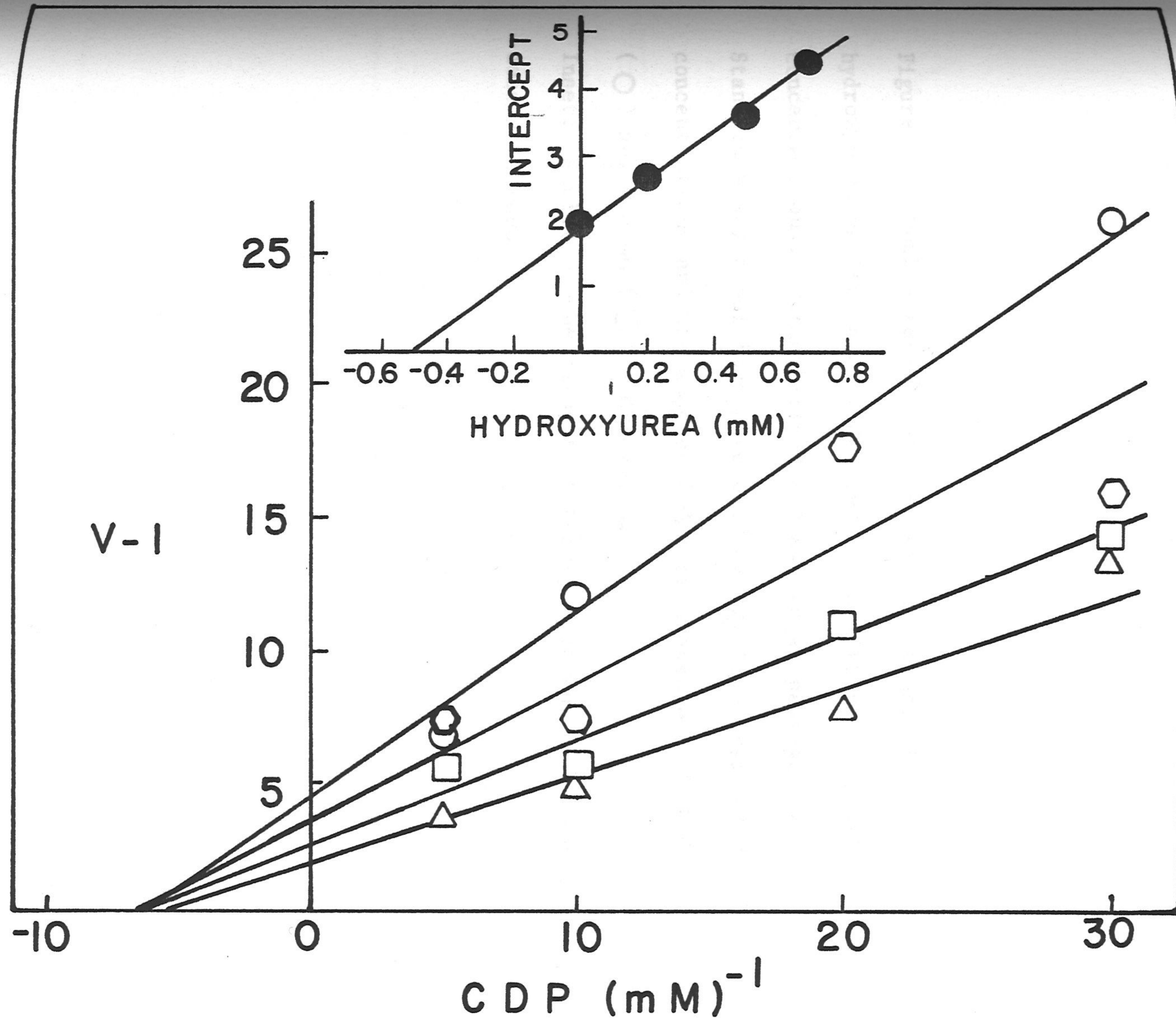

INTERCEPT
1
2
3
4
5
-0.6
-0.4
-0.2
0.2
0.4
0.6
0.8
HYDROXYUREA (mM)
V-1
5
10
15
20
25
-10
0
10
20
30
CDP (mM)-1

Figure 47. Double reciprocal plot of the effect of hydroxyurea on the rate of CDP reduction with varying CDP concentrations. Permeabilized cells were at passage 85. Standard assay conditions were used except for varying CDP concentrations and the absence ( △ ) or presence of 0.3 mM ( ○ ) and 0.8 mM ( □ ) hydroxyurea.

Inset: Replot of the velocity intercepts versus hydroxyurea concentrations.

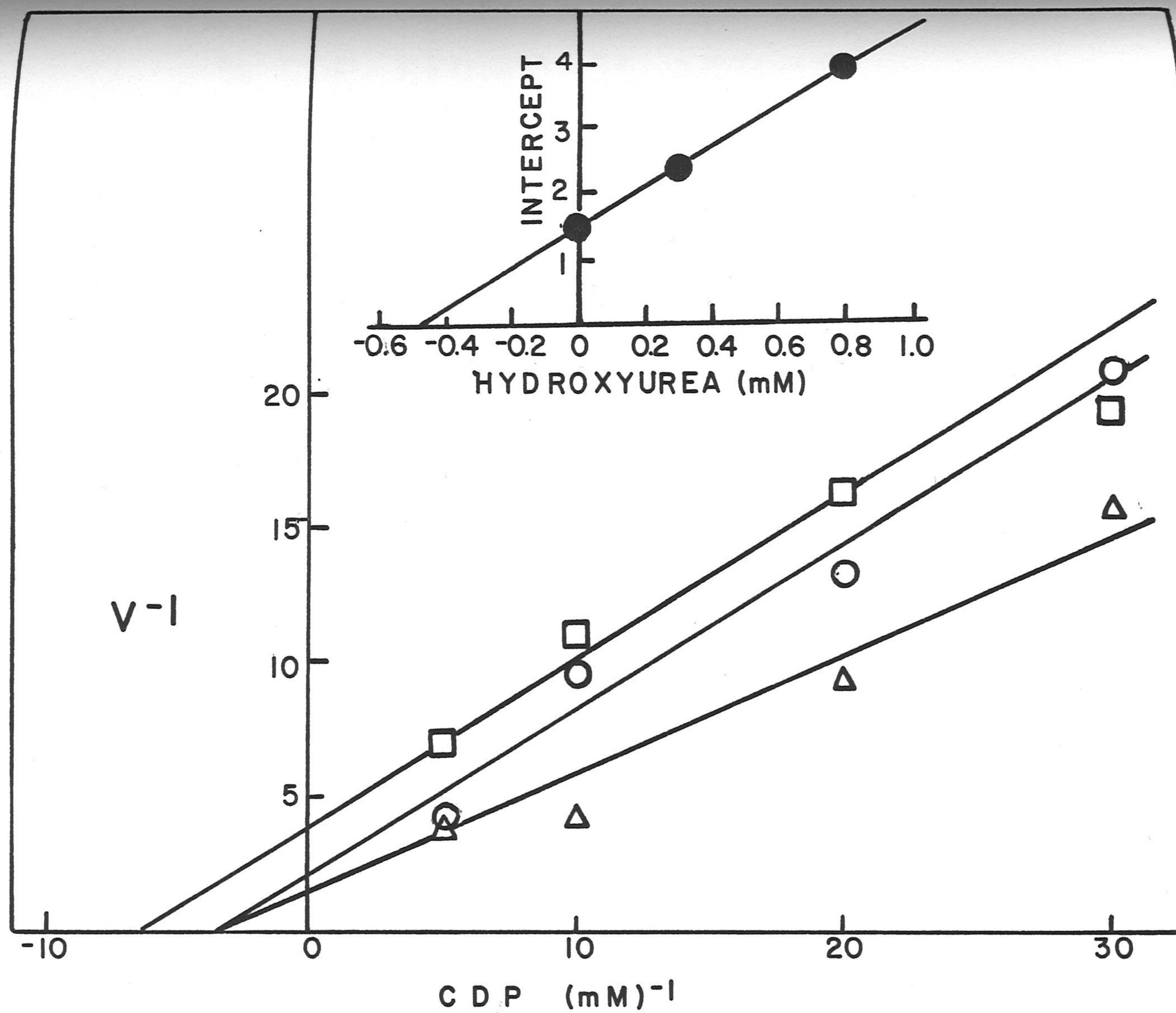

INTERCEPT
4
3
2
1
-0.6 -0.4 -0.2 0 0.2 0.4 0.6 0.8 1.0
HYDROXYUREA (mM)
20
15
10
5
V -1
-10
0
10
20
30
C D P (mM)-1

Figure 48. Response of CDP reduction in HSC 172 passage 24 ( ● ) and passage 74 ( ■ ) cells to varying concentrations of dATP. Each point represents the mean $\pm$ 1 standard deviation of four determinations.

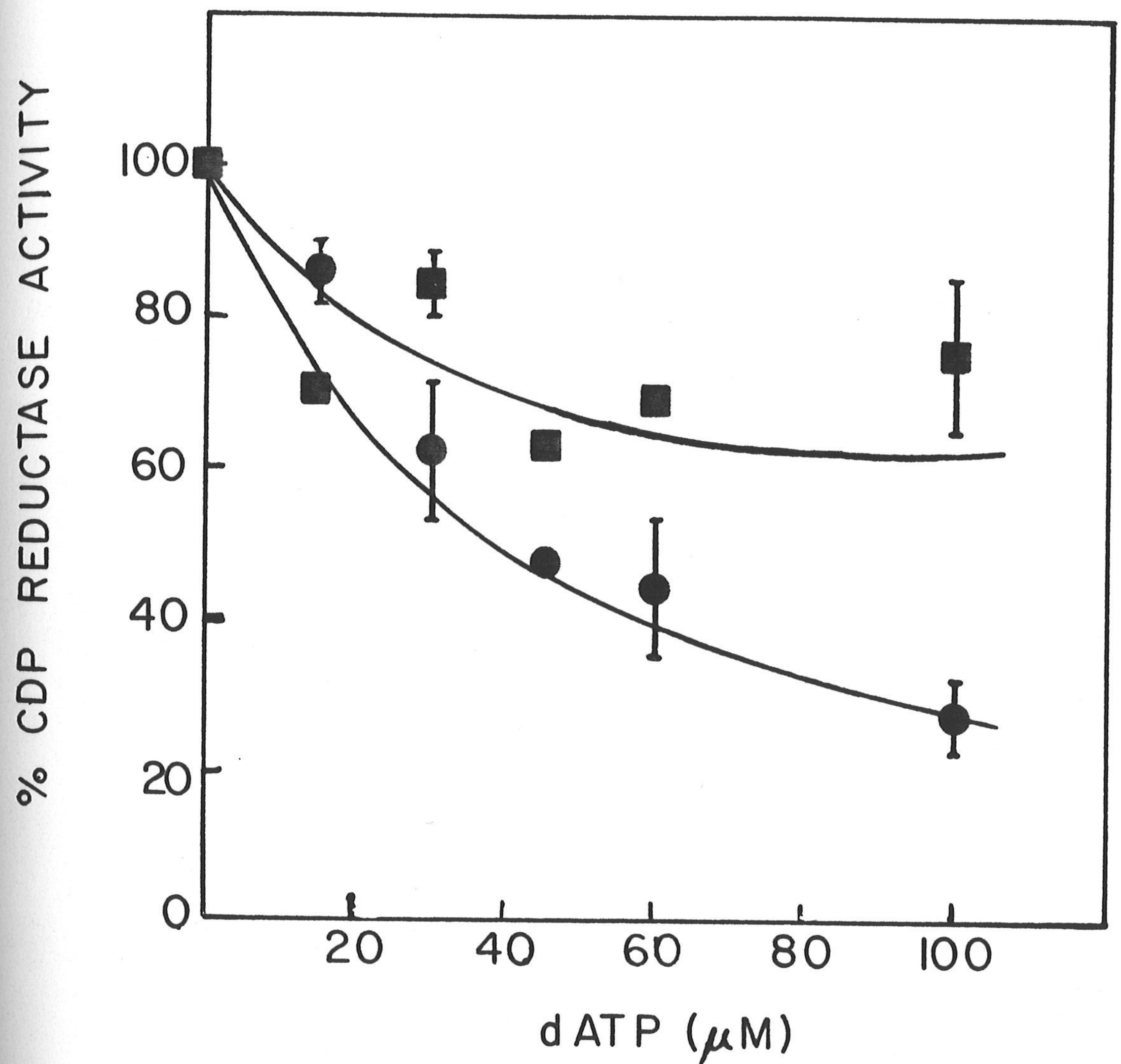

% CDP REDUCTASE ACTIVITY
100
80
60
40
20
0
20
40
60
80
100
dATP (μM)

Figure 49. Response of ADP reduction in HSC 172 passage 21 ( ● ) and passage 74 ( ■ ) cells to varying concentrations of dATP. Each point represents the mean $\pm$ 1 standard deviation of four determinations.

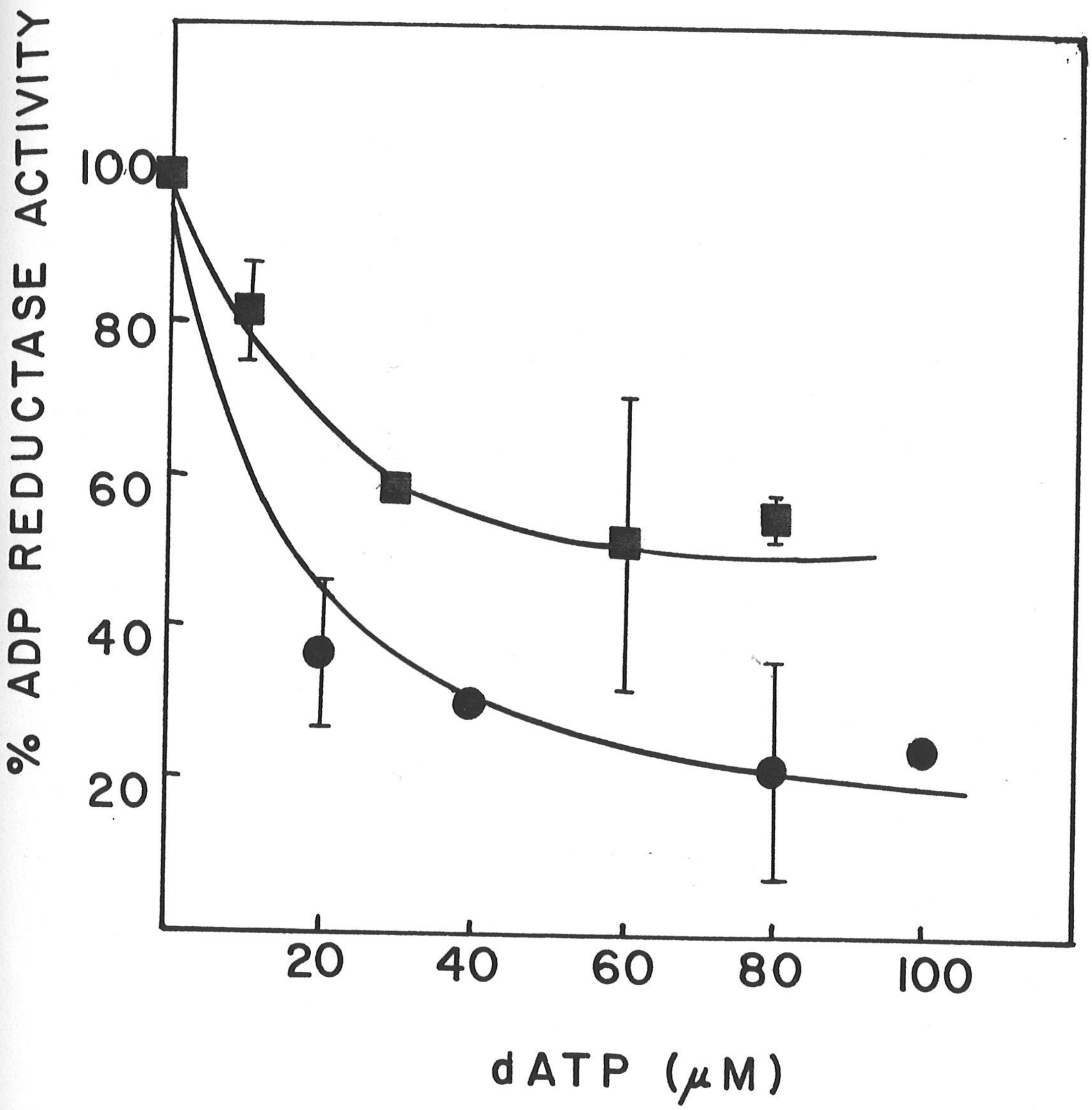
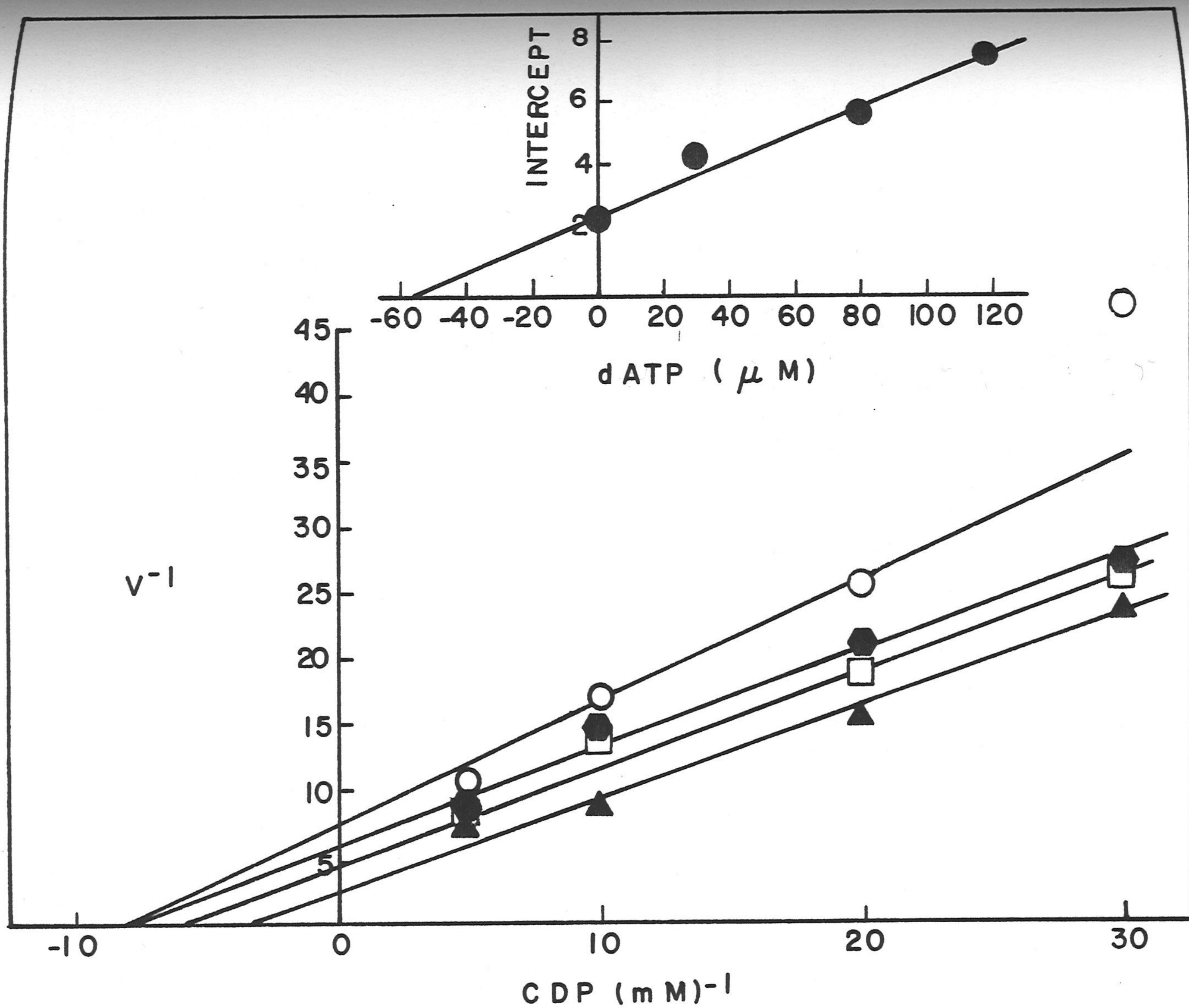

% ADP REDUCTASE ACTIVITY
100
80
60
40
20
20
40
60
80
100
dATP (μM)

Figure 50. Double reciprocal plot of the variation in the rate of CDP reduction with varying CDP concentration at several fixed concentrations of dATP. Permeabilized cells were at passage 22. Standard assay conditions were used except for varying conditions of CDP and the absence ( ▲ ) or presence of 30 μM ( □ ), 80 μM ( ⬢ ), and 120 μM ( ◑ ) dATP.


INTERCEPT
2
4
6
8
-60 -40 -20 0 20 40 60 80 100 120
dATP (μM)
45
40
35
30
25
20
15
10
5
V⁻¹
-10
0
10
20
30
CDP (mM)⁻¹

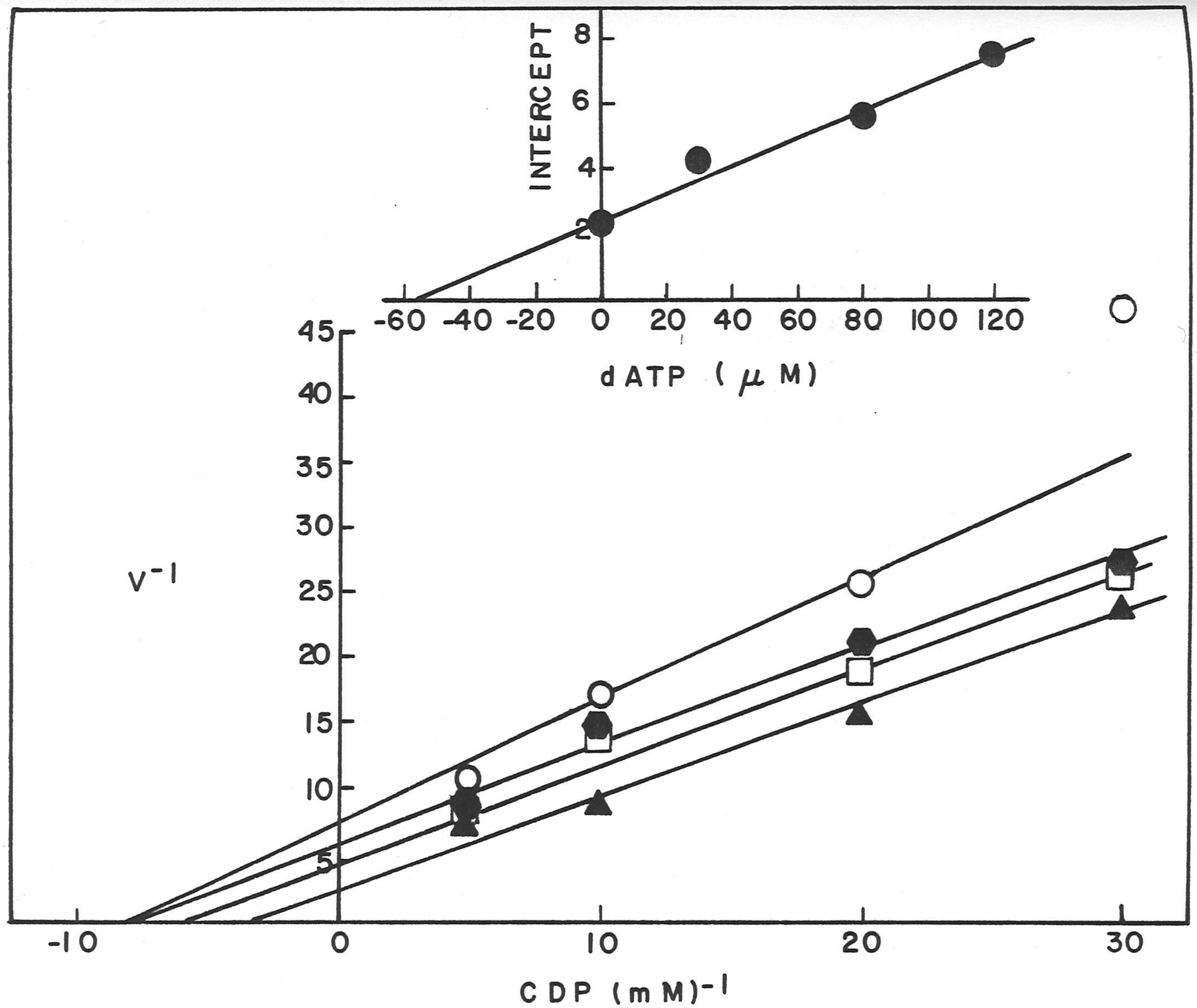
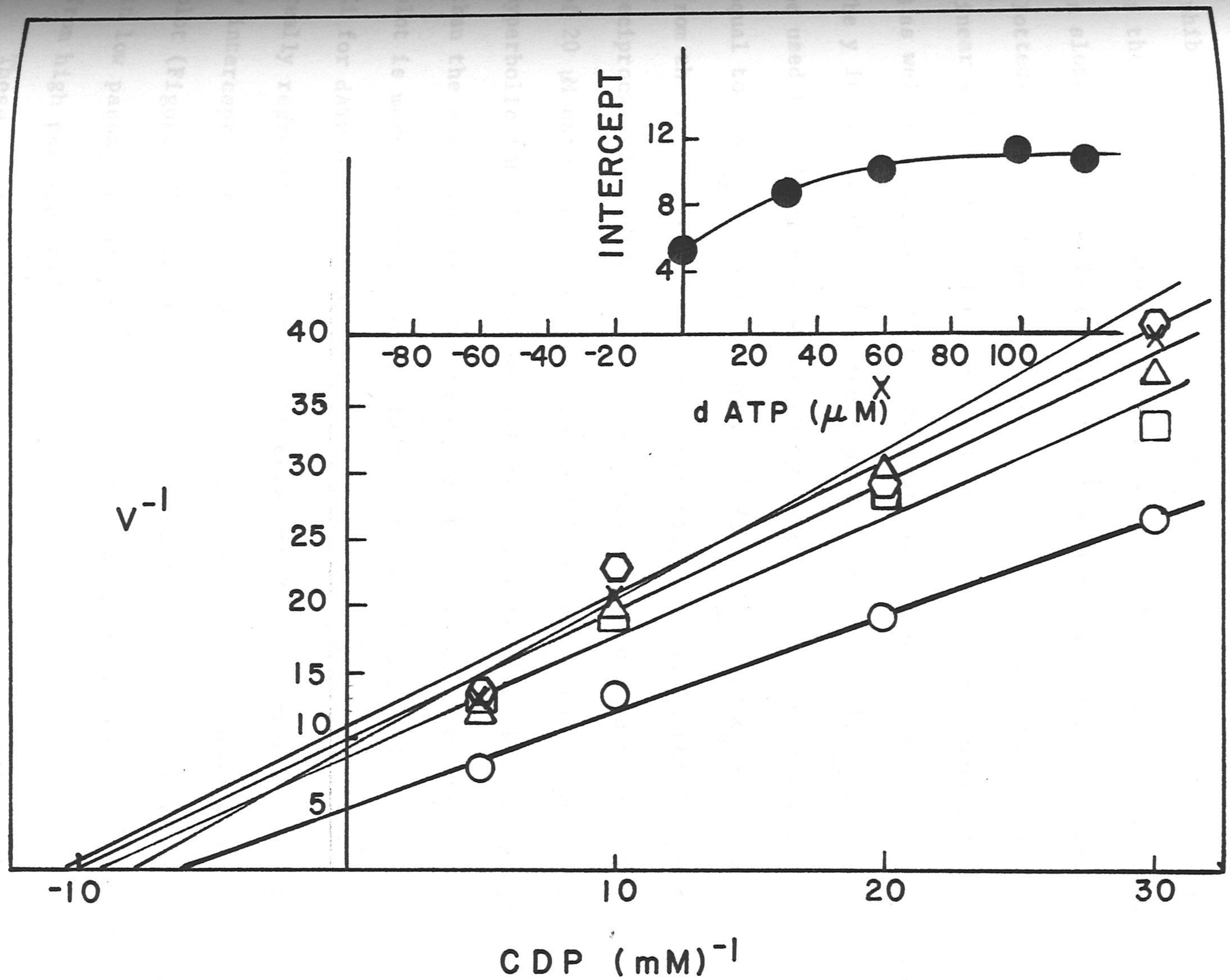

INTERCEPT
8
6
4
2
-60 -40 -20 0 20 40 60 80 100 120
dATP (μM)
45
40
35
30
25
20
15
10
5
V⁻¹
-10 0 10 20 30
CDP (mM)⁻¹

Figure 51. Double reciprocal plot of the variation is the rate of CDP reduction with varying CDP concentration at several fixed concentrations of dATP. Permeabilized cells were at passage 79. Standard assay conditions were used except for varying concentrations of CDP and the absence ( ○ ) or presence of 30 μM ( □ ), 60 μM ( △ ), 100 μM ( ⬡ ), and 120 M ( × ) dATP.

Inset: Replot of the velocity intercepts versus dATP concentrations.

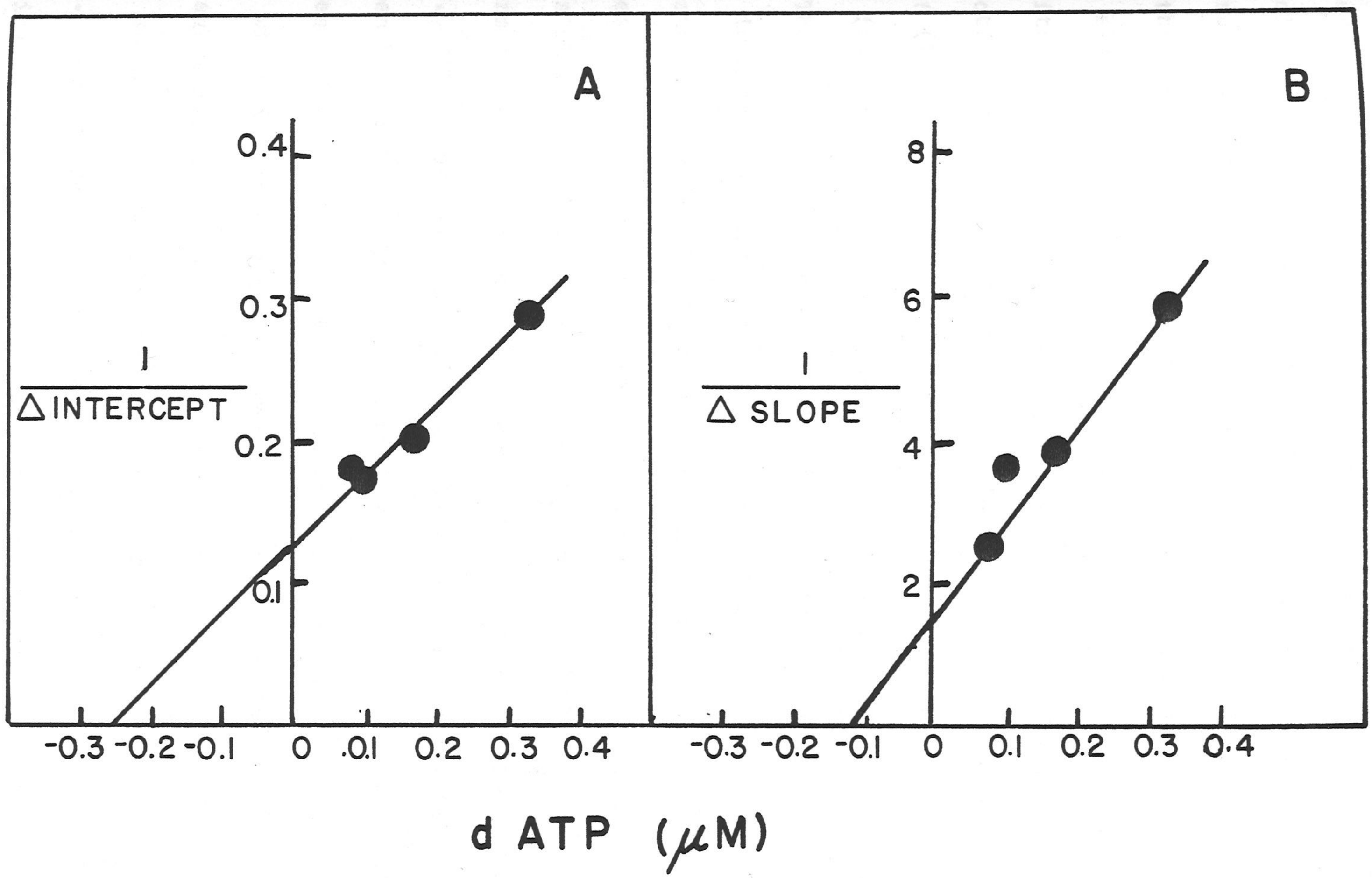

INTERCEPT
12
8
4
-80 -60 -40 -20 20 40 60 80 100
d ATP (μM)
40
35
30
25
20
15
10
5
V⁻¹
-10
10
20
30
CDP (mM)⁻¹

hyperbolic (Figure 51). The hyperbolic nature of the replots can be subjected to further analysis by plotting the inverse of the change in intercepts (defined as the intercept at a given concentration of inhibitor minus the intercept with no inhibitor) versus the inverse of the inhibitor concentration. Similarly the inverse of the change in slope versus the inverse of the inhibitor concentrations were also plotted. These two plots are shown in Figure 52. Both plots are linear and can be used to calculate the kinetic rate constants $\alpha$ and $\beta$ as well as the Ki. The x intercept in both plots is equal to $-\beta/\alpha Ki$. The y intercept of the intercept plot is equal to $\beta V_{max}/1-\beta$ and can be used to calculate $\beta$, while the y intercept of the slope plot is equal to $\beta V_{max}/K_s\ (\alpha - \beta)$ (Segal, 1975). $V_{max}$ and $K_s$ are determined from the y intercept and x intercept respectively in the double reciprocal plot (Figure 51). These calculations result in a Ki value of 20 μM using the intercept plot and 50 μM using the slope plot. In hyperbolic inhibition the contribution of intercept changes is greater than the slope changes and therefore the Ki value from the intercept plot is more accurate (Segal, 1975). As mentioned earlier the apparent Ki for dATP using low passage cells was 55 μM, however this value really represents $\alpha$Ki. The rate constant $\alpha$ can be calculated from the y intercept of the highest drug concentration in the double reciprocal plot (Figure 50); the constant was found to be 0.44. The actual Ki in low passage cells, then, is 24 μM, a value very similar to the Ki from high passage cells (20 μM).

These kinetic data indicate that the binding of dATP to RRase is about the same in high and low passage cells. The difference lies in the

Figure 52A. Replot of the reciprocal of the change in velocity intercepts versus the reciprocal of the dATP concentration. The data for this replot comes from Figure 51.

Figure 52B. Replot of the reciprocal of the change in slope intercepts versus the reciprocal of the dATP concentration. The data for this replot comes from Figure 51.


A
B
1/Δ INTERCEPT
1/Δ SLOPE
0.4
0.3
0.2
0.1
8
6
4
2
-0.3 -0.2 -0.1 0 .01 0.2 0.3 0.4
-0.3 -0.2 -0.1 0 0.1 0.2 0.3 0.4
d ATP (μM)

fact that reductase from low passage cells is inhibited according to a linear model, while the enzyme from high passage cells is inhibited according to a hyperbolic model. The linear model predicts that the substrate and inhibitor bind to different sites in the enzyme and once the inhibitor has bound no product can be formed. The hyperbolic model also predicts different binding sites, however the binding of inhibitor to the enzyme still allows, at a very low rate, the production of product. This results in the hyperbolic nature of the replot (Figure 51), as well as the levelling off of the inhibition curve (Figure 48); the reaction therefore can never be driven to zero.

The reason this change of dATP inhibition occurs in high passage cells is not clear, however, several mechanisms are possible. The accurate functioning of RRase requires that both $M_1$ and $M_2$ interact with each other in a precise fashion in order for reduction to occur (Caras et al., 1983). dATP binds to $M_1$ and presumably induces a conformational change such that reduction no longer occurs (Caras et al., 1983); perhaps as cells age the nature of the interaction between $M_1$ and $M_2$ is no longer as stringent and the inhibition by dATP becomes "leaky". It is interesting to note that it has been reported RRase with different molecular weights can be isolated, indicating that aggregation of subunits can occur (Cory & Fleischer, 1982). These subunit interactions may be altered in RRase from high passage cells. The age related alterations which cause a change in dATP inhibition could be the result of mutation, errors in protein synthesis, or post-translational modification of the enzyme subunits. It is important to note that each of these mechanisms has been reported to cause changes in enzymes during in vitro aging (Morrow & Garner, 1979).

One way to approach the question of the subunit composition of RRase during aging has been to determine if there are any major changes in the relative concentration of either the $M_1$ or $M_2$ subunit in aging cells. Several studies have shown that $M_1$ and $M_2$ are not regulated in the same fashion in mammalian cells (Eriksson & Martin, 1981; Cory & Fleischer, 1982). In some cell lines the $M_2$ component is limiting (Eriksson & Martin, 1981) while in others the $M_1$ component is limiting (Wright & Cory, 1983). The relative level of each can be determined without the use of probes, by titrating crude cell extracts with purified $M_1$ or $M_2$ and deteming which component increases the RRase activity in the extract. Presumably the added component is complementing any free subunit which is not bound to the other subunit (Wright & Cory, 1983). Table VII shows the effect of adding either $M_1$ or $M_2$ prepared from CHO cells to extracts from low and high passage human diploid fibroblasts. $M_2$ provided the greatest stimulation of activity in both cases indicating this subunit is limiting in low and high passage cells. There were no significant differences in the degree of stimulation in high and low passage cells. Interestingly, the level of reductase activity was lower in extracts from high passage cells as compared to extracts from low passage cells, paralleling the results described earlier using the permeabilization assay (Figure 19).

D. Isolation of Chinese Hamster Ovary X Human Diploid Fibroblast Somatic Cell Hybrids

Human diploid fibroblasts are at least three fold more resistant to the cytotoxic action of hydroxyurea than CHO cells. Figure 53

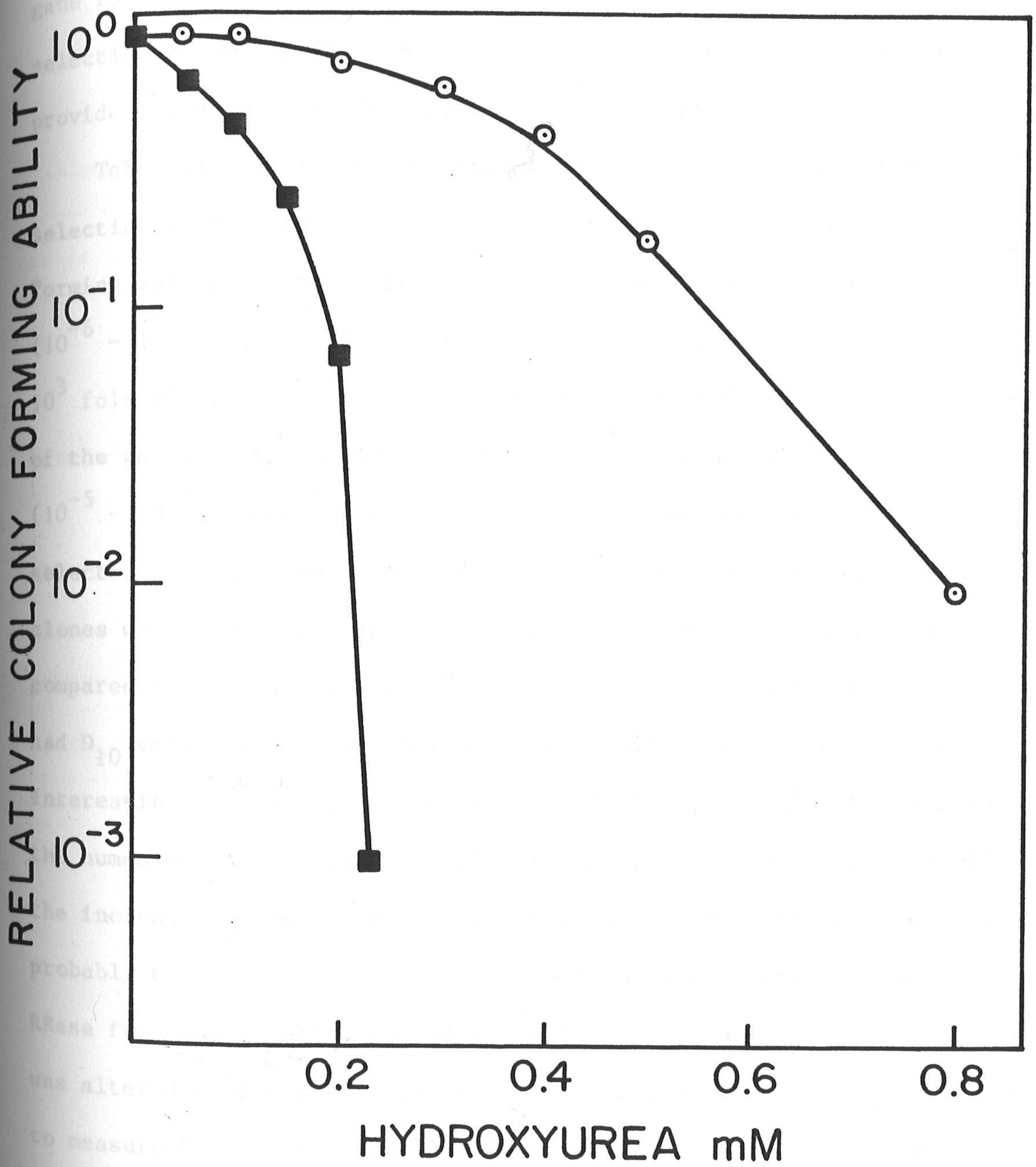
Table VII. Increase in CDP Reductase Activity with Exogenously added $M_1$ and $M_2$ Subunits in Low and High Passage Human Diploid Fibroblasts.

| | HSC 172 - 19 | Fold Increase | HSC 172 - 75 | Fold Increase |
|---|---|---|---|---|
| Extract | 0.053 ± .004 | 1.0 | 0.009 ± .005 | 1.0 |
| Extract + $M_1$ | 0.044 ± .005 | 1.2 | 0.010 ± .005 | 1.1 |
| Extract + $M_2$ | 0.059 ± .005 | 1.7 | 0.017 ± .001 | 1.9 |

No activity with $M_1$ or $M_2$ alone

Protein/assay -- old = 575 μg; young = 600 μg shows that at 0.4 mM hydroxyurea, less than $10^{-4}$ CHO cells survive in the drug while normal HDF still show greater than 50% survival. This native difference in cellular resistance to hydroxyurea is the result of differences in the sensitivity of RRase to inhibition by hydroxyurea from these two cultures. The apparent $K_i$ for hydroxyurea inhibition of RRase in human cells is 0.4 mM (Figure 14; Dick & Wright, 1980) while in CHO cells the $K_i$ is 0.1 mM (Lewis & Wright, 1978). The differences in $K_i$ as well as cellular resistance to hydroxyurea between CHO and normal HDF suggested that it may be possible to isolate hybrids between CHO cells and normal HDF by selection in hydroxyurea. The hybrid cells should retain only a small number of human chromosomes including the chromosome(s) which contain the gene(s) for RRase. Using conventional chromosomal banding techniques the human chromosomes could be identified, and the concordance of hydroxyurea resistance and a specific chromosome would be determined. The CHO cell line used was $GATO^R$-1, which carried a dominant marker for ouabain resistance, as well as being auxotrophic for growth, requiring additions of glycine, adenosine, and thymidine to the tissue culture medium (McBurney & Whitmore, 1974). This CHO cell line grows very well in the presence of $10^{-3}$ M ouabain, while HDF are rapidly killed by concentrations of ouabain as low as $10^{-6}$ M (Mankovitz et al., 1974). The selection protocol described in Methods and Materials consisted of fusing CHO and HDF cells together with 50% W/V PEG 1000 in α-MEM without serum, incubating the fused diluted cells in complete medium for twenty-four hours, and then adding selective medium containing 1 mM ouabain and 0.3 or 0.4 mM

Figure 53. Relative colony forming ability of HSC 172 ( ○ ) and CHO ( ■ ) cells in the presence of varying concentrations of hydroxyurea.


RELATIVE COLONY FORMING ABILITY
$10^0$
$10^{-1}$
$10^{-2}$
$10^{-3}$
0.2
0.4
0.6
0.8
HYDROXYUREA mM

hydroxyurea. Any hybrid cell which grows up in this selective medium should express the human form of RRase in order to overcome the effects of hydroxyurea, and it should also express the mutant hamster gene for Na-K ATPase, the intracellular target of ouabain. In some selection medium the GAT supplement was also left out in order to provide a double selection against the CHO cell line.

Table VIII outlines the conditions of cell fusion and hybrid selections. This table shows that in the absence of PEG the colony forming ability in the presence of selective medium is very low ($10^{-6}$ - $10^{-7}$) while with PEG the colony forming ability was about $10^{3}$ fold higher ($10^{-3}$ - $10^{-4}$). Furthermore the colony forming ability of the parental type cells in selective medium was also very low ($10^{-5}$ - $10^{-7}$). Five clones from four different experiments were selected and examined in more detail. Figure 54 shows that all these clones were more resistant to the cytotoxic effects of hydroxyurea compared to the parental GATO$^{R}$-1 cell line. The hybrid clones all had $D_{10}$ values which were three to four fold higher than GATO$^{R}$-1. Interestingly these $D_{10}$ values resembled the $D_{10}$ values obtained for the human parental strain HSC 172 (Figure 18). As noted previously the increased cellular resistance of human cells to hydroxyurea was probably the result of higher $K_i$ values for hydroxyurea inhibition of RRase from human cells. In order to determine whether RRase activity was altered in the hybrid clones, the permeabilization assay was used to measure RRase activity. Figure 55 A shows that the level of RRase activity was not elevated in any of the clones examined suggesting that enzyme overproduction was not the reason these clones were

Table VIII. Permeabilization Conditions.

| Cells and Growth Conditions | Relative Colony Forming Ability |
|---|---|
| GATO$^R$-1 x HSC 172 - PEG in selective medium | $10^{-6}$ - $10^{-7}$ |
| GATO$^R$-1 x HSC 172 + PEG in selective medium | $10^{-3}$ - $10^{-4}$ |
| HSC 172 in 1 mM ouabain | $10^{-6}$ - $10^{-7}$ |
| GATO$^R$-1 in 0.4 mM hydroxyurea | $10^{-5}$ - $10^{-6}$ |

Figure 54. Relative colony forming ability of parental CHO cells ( ● ) and hybrid clones GH-2 ( ⬢ ), HGAT-7 ( ● ), GOW-2 ( ◆ ), OH-1 ( ▲ ), and OH-5 ( ■ ) in the presence of varying concentrations of hydroxyurea.

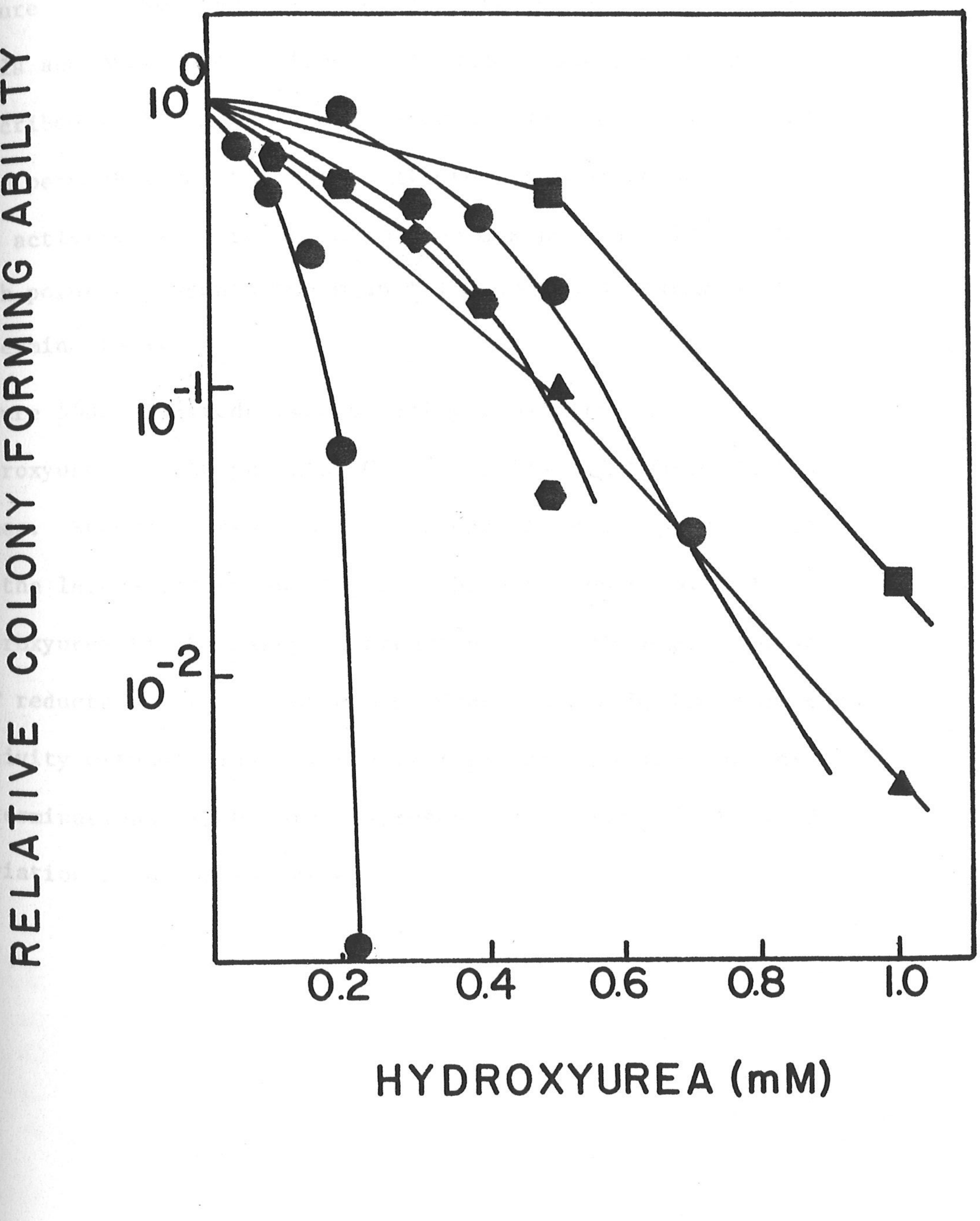
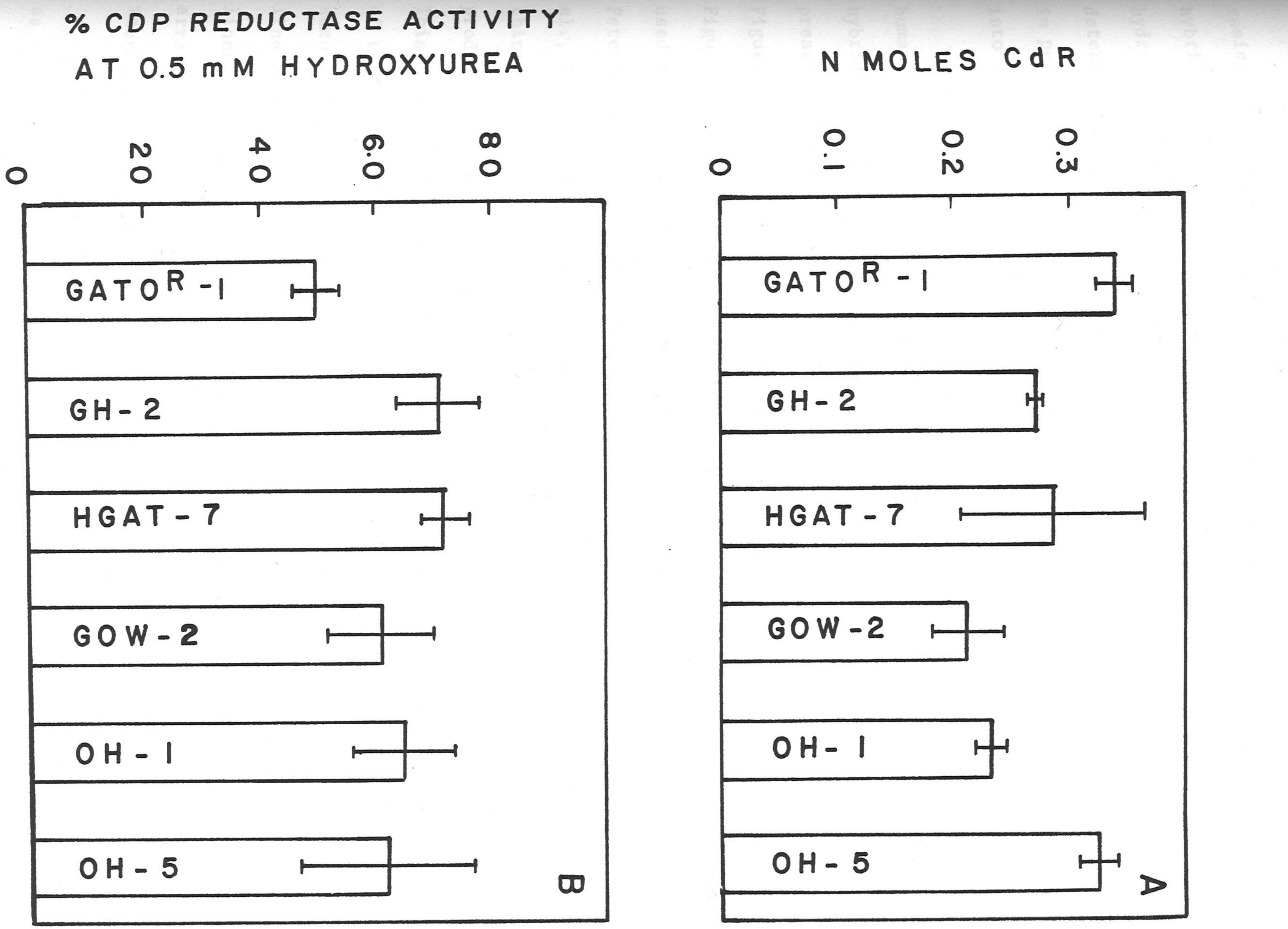

RELATIVE COLONY FORMING ABILITY
$10^0$
$10^{-1}$
$10^{-2}$
0.2
0.4
0.6
0.8
1.0
HYDROXYUREA (mM)

Figure 55A. CDP reductase activity in CHO parental $GATO^R$-1 cells and hybrid cell lines. Standard assay conditions described in the Methods and Materials were used except cells were permeabilized for thirty minutes at room temperature. The activity is expressed as nmoles CdR/hour/3 x $10^6$ cells. Each point represents the mean $\pm$ 1 standard deviation of 4 determinations.

Figure 55B. CDP reductase activity remaining at 0.5 mM hydroxyurea in CHO parental $GATO^R$-1 cells and hybrid cell lines. Standard assay conditions were used except as noted in the legend for Figure 55A and the addition of 0.5 mM hydroxyurea to the assay. Percent activity is expressed as CDP reductase activity at 0.5 mM drug divided by CDP reductase activity without drug. The bars represent the mean of three determinations. Each point represents the mean $\pm$ 1 standard de deviation of 4 determinations.


% CDP REDUCTASE ACTIVITY
AT 0.5 mM HYDROXYUREA
0
20
40
6.0
80
GATOR-1
GH-2
HGAT-7
GOW-2
OH-1
OH-5
B
N MOLES CdR
0
0.1
0.2
0.3
GATOR-1
GH-2
HGAT-7
GOW-2
OH-1
OH-5
A

resistant to hydroxyurea. Figure 55 B shows that RRase from the hybrid clones was more resistant to drug inhibition with 0.5 mM hydroxyurea than RRase from the parental CHO cells. This method of determining enzyme resistance is only approximate since small changes in percent activity at a particular drug concentration can translate into large changes in $K_i$ (Koropatrick, 1980).

Since these hybrid clones appeared to contain the more resistant human form of RRase, karyotypes of G-banded chromosomes of these hybrids were prepared to identify any human chromosome which were present. The karyotype of the $GATO^R$-1 parental cell line is shown in Figure 56. The karyotype of the human parental cell was shown in Figure 1 to be a normal female karyotype of 46, XX. The nomenclature used to identify the hamster chromosomes was that of Deaven and Peterson (1973) and karyotypes were arranged according to Worton et al., (1977). Hamster cell lines such as CHO contain either twelve or thirteen rearranged marker chromosomes, designated Z, which are the products of a variety of translocations, deletions and inversions. This study by Worton et al., (1977), examined the possible derivation of the marker chromosomes which were characteristic of the proline auxotrophic ($pro^-$) subline of CHO. It is worth noting that the cell line Worton studied was the progenitor of the GAT and $GATO^R$-1 cell lines used in these studies. The karyotypes presented here were also arranged in a similar fashion and any new marker chromosomes were identified as $M_1$, $M_2$, etc.

The double mutant $GATO^R$-1 was found to have the same karyotype as the wild type and $GAT^-$ cells characterized by Worton et al., (1977). The karyotypes of the five hybrid clones are shown in Figures 56 to 62 and the details are summarized in Table IX. Intensive analysis of these five clones as well as an additional 8 clones (data not shown) has failed to reveal any detectable intact human chromosomes, although some changes in the hamster chromosomes were seen. The changes in the hamster karyotypes include the apparent loss of a complete chromosome Z-8 in most of the hybrid clones. It is interesting to note that one of the marker chromosomes ($M_1$) in GH-2, GOW-2 and OH-1 appears to contain at least part of the p-arm of Z-8. In addition, one copy of chromosome 6 appears slightly altered in GH-2 (Figure 57), GOW-2 (Figure 59 & 60) and OH-1 (Figure 61). The significance of these changes is not known. However they do appear to be quite consistent in all the hybrid clones. The lack of any detectable human chromosome was a surprising result since the survival curves and enzyme data suggested the human form of RRase was present. Several explanations of these experiments are possible. For instance, it is possible that these lines are not true hybrids, rather they may be CHO hydroxyurea resistant variants which were able to grow in the selection medium. Several lines of evidence argue against this possibility. Firstly the frequency of occurance of hybrid colonies was 10 - 100 fold higher (Table VIII) than the mutation frequency to hydroxyurea resistance in CHO cells (Lewis & Wright, 1978a). Secondly the $GAT^-$ auxotrophic cell line is more sensitive to hydroxyurea then wild type CHO cells (Lewis & Wright, 1979) and no hydroxyurea resistant cells have been isolated in a single step using drug concentrations higher than 0.3 mM (unpublished observations in our laboratory). Thirdly, one of the clones HGAT-7

Figure 56. Giesma banded karyotype of $GATO^R$-1 CHO cells. The karyotype is arranged according to Worton et al. (1977). The letter Z designates rearranged hamster chromosomes which are characteristic of this CHO cell line.


1
2 Z-2
X Z-12
Z-4 Z-3
Z-7 Z-5a
6
Z-9 Z-8
8 Z-10
9
10 Z-13
11

Figure 57. Giemsa banded karyotype of hybrid clone GH-2. The karyotype is arranged according to Worton et al. (1977). The question mark indicates uncertain chromosomal identity and $M_1$ represents a rearranged chromosome not present in the parental cells.

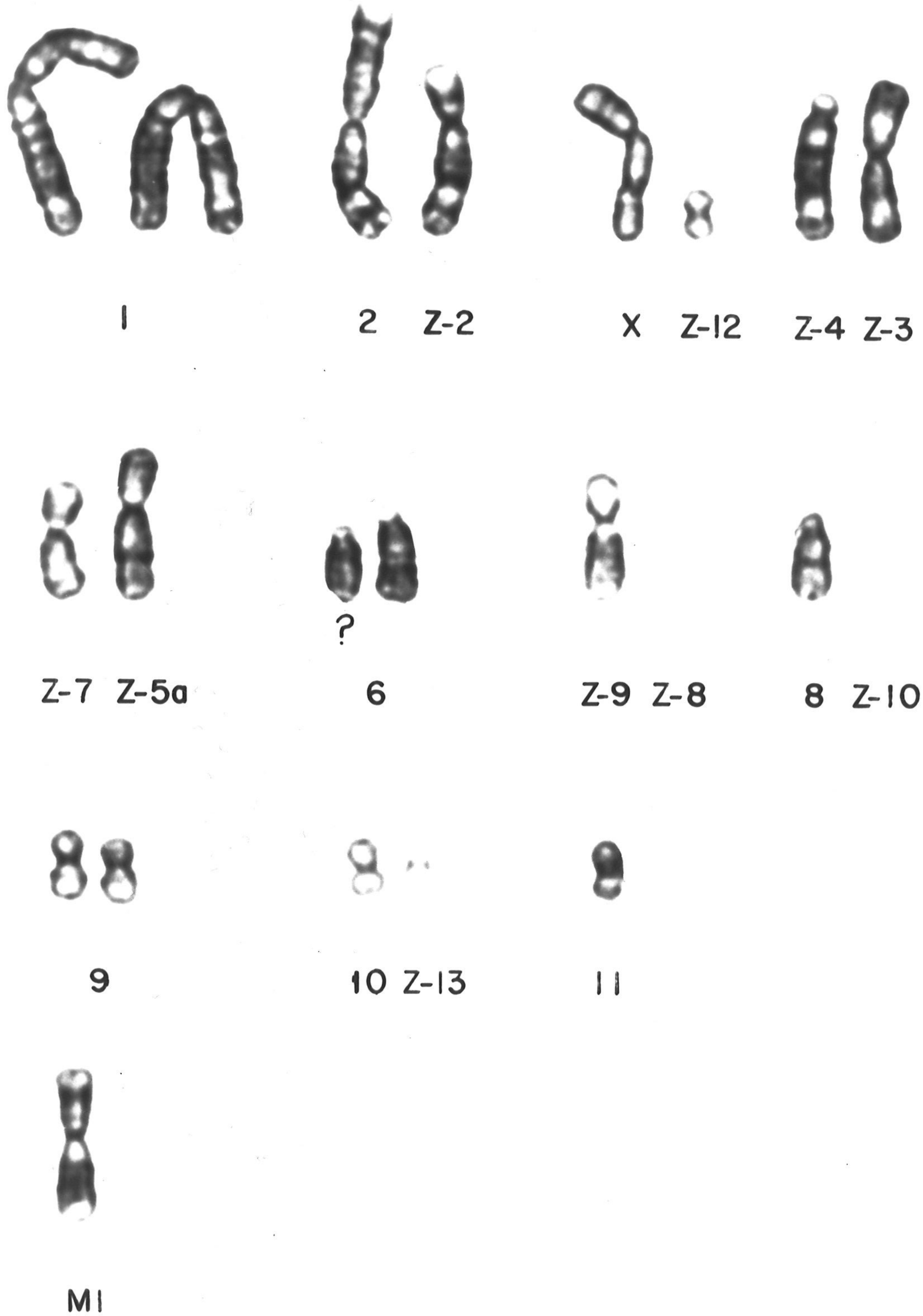

1
2 Z-2
X Z-12
Z-4 Z-3
Z-7 Z-5a
?
6
Z-9 Z-8
8 Z-10
9
10 Z-13
11
M1

Figure 58. Giemsa banded karyotype of hybrid clone HGAT-7. The karyotype is arranged according to Worton et al. (1977). The arrow indicates an overlapped chromosomes Z-9 and $M_1$ and $M_2$ represent rearranged chromosomes not present in the parental cells. The question mark indicates uncertain chromosomal identity.

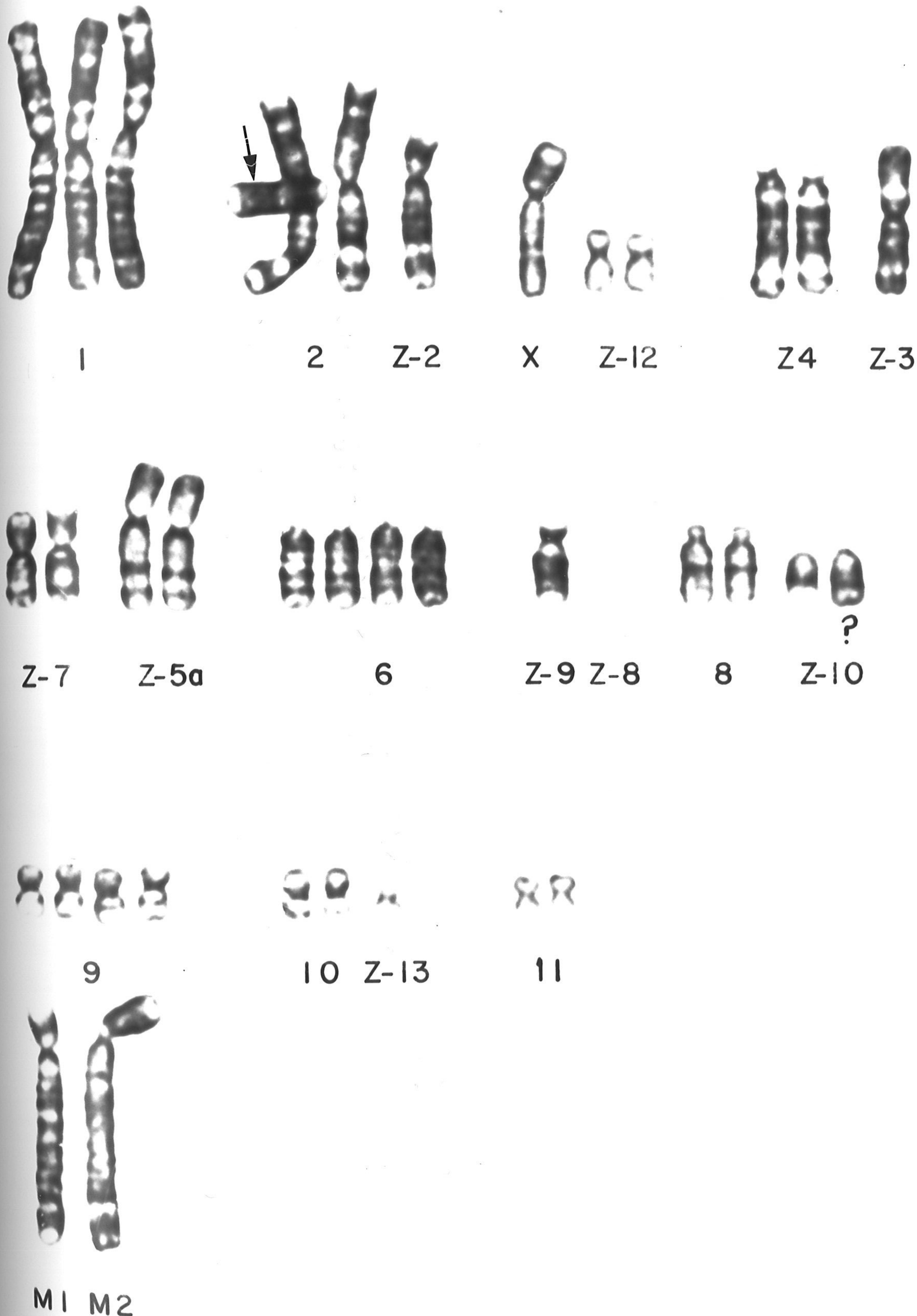

1
2
Z-2
X
Z-12
Z4
Z-3
Z-7
Z-5a
6
Z-9
Z-8
8
?
Z-10
9
10
Z-13
11
M1
M2

Figure 59. Giemsa banded karyotype of hybrid clone GOW-2. The karyotype is arranged according to Worton et al. (1977). $M_1$, $M_2$ and $M_3$ represent rearranged chromosomes not present in the parental cells.

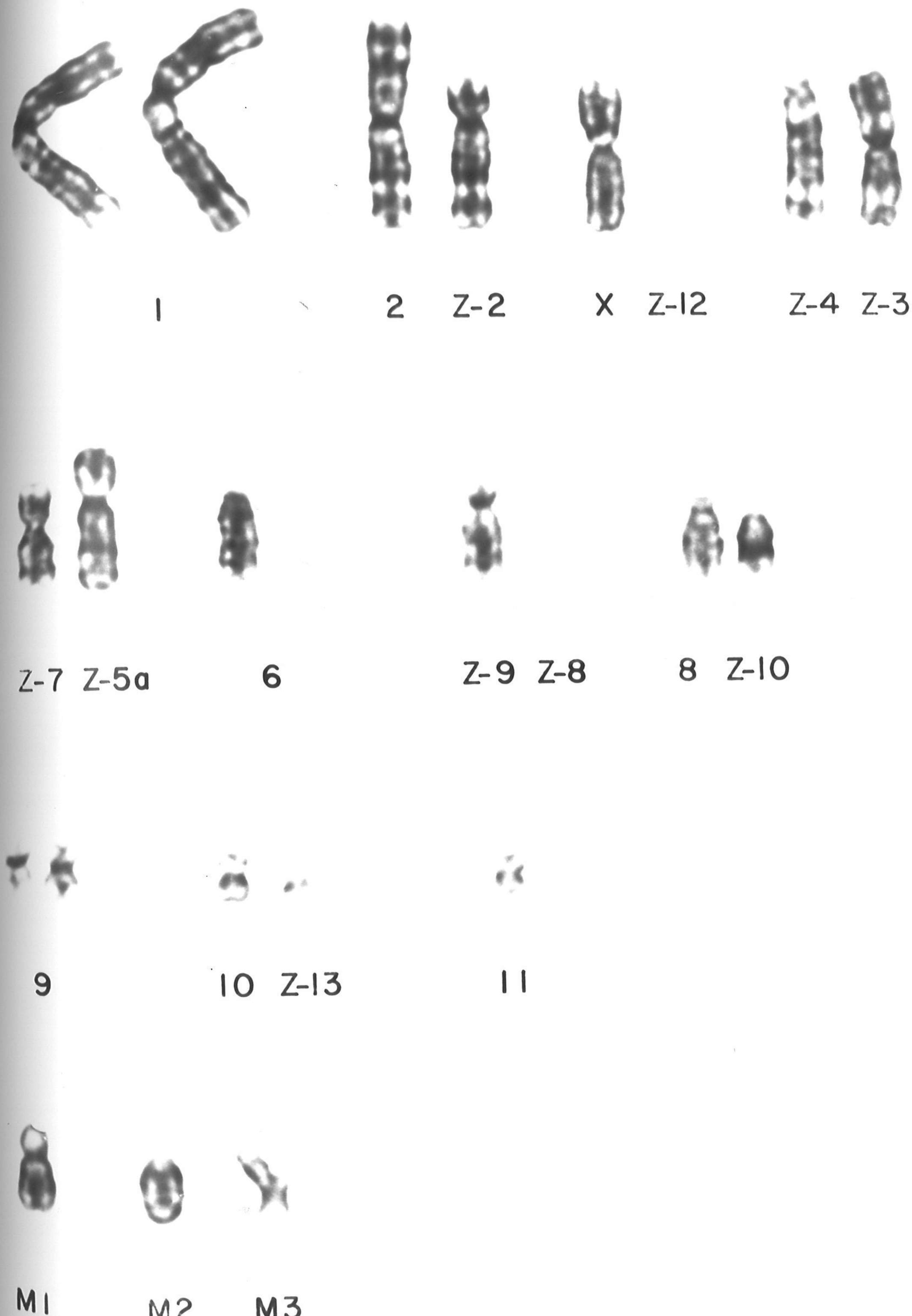

1
2 Z-2
X Z-12
Z-4 Z-3
Z-7 Z-5a
6
Z-9 Z-8
8 Z-10
9
10 Z-13
11
M1
M2
M3

Figure 60. Giemsa banded karyotype of hybrid clone GOW-2. The karyotype is arranged according to Worton et al. (1977). The question marks indicate uncertain chromosomal identity and $M_1$, $M_2$, $M_3$, $M_4$, $M_5$ and $M_6$ represent rearranged chromosomes not present in the parental cells. The arrow shows an overlapped chromosome Z-3.

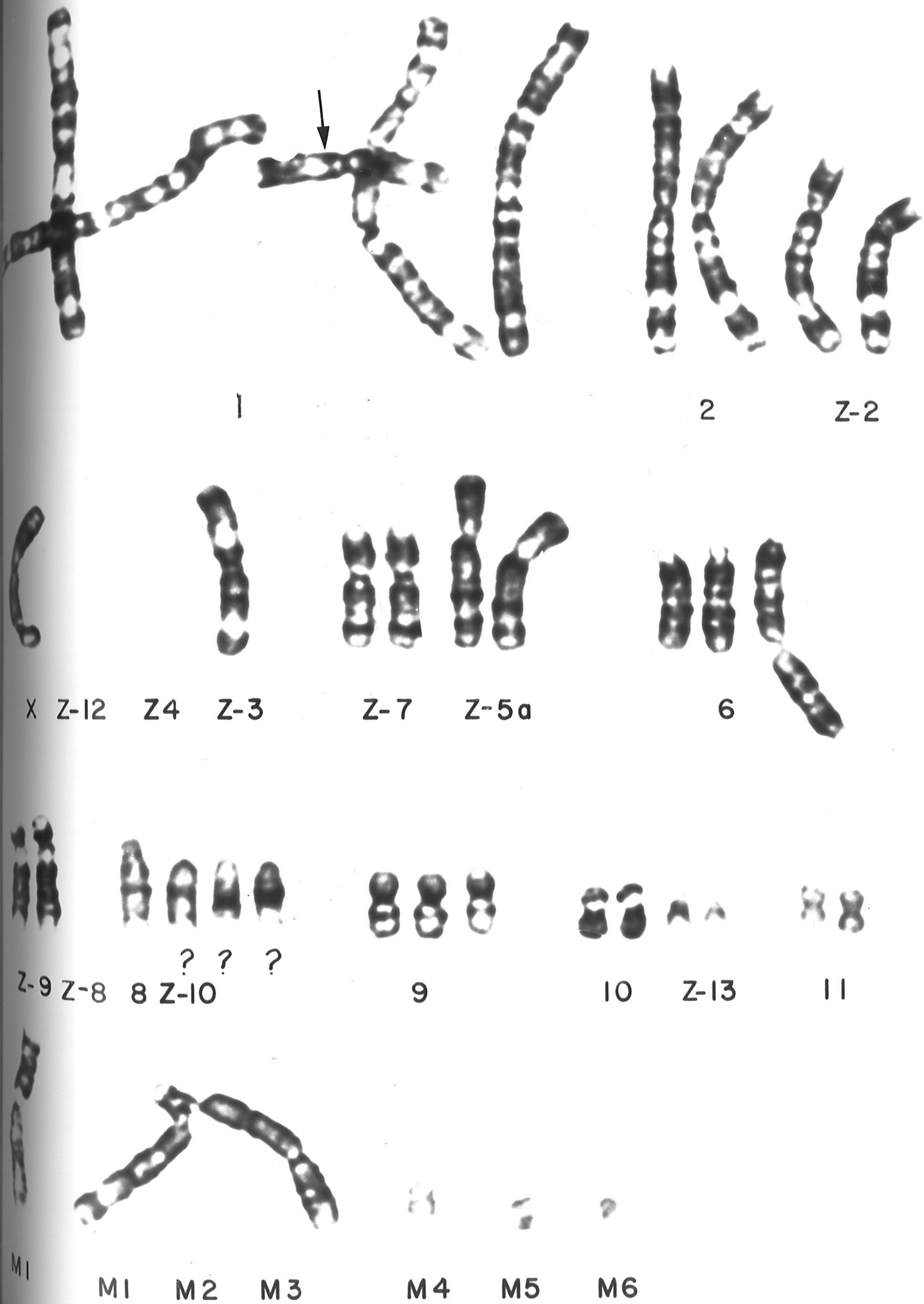

1
2
Z-2
X
Z-12
Z4
Z-3
Z-7
Z-5a
6
? ? ?
Z-9
Z-8
8
Z-10
9
10
Z-13
11
M1
M1
M2
M3
M4
M5
M6

Figure 61. Giemsa banded karyotype of hybrid clone OH-1. The karyotype is arranged according to Worton et al. (1977). The long arrow indicates an overlapped chromosome X and the short arrow an overlapped chromosome Z-5a. $M_1$ represents a rearranged chromosome not present in the parental cells. The question mark indicates uncertain chromosomal identity.

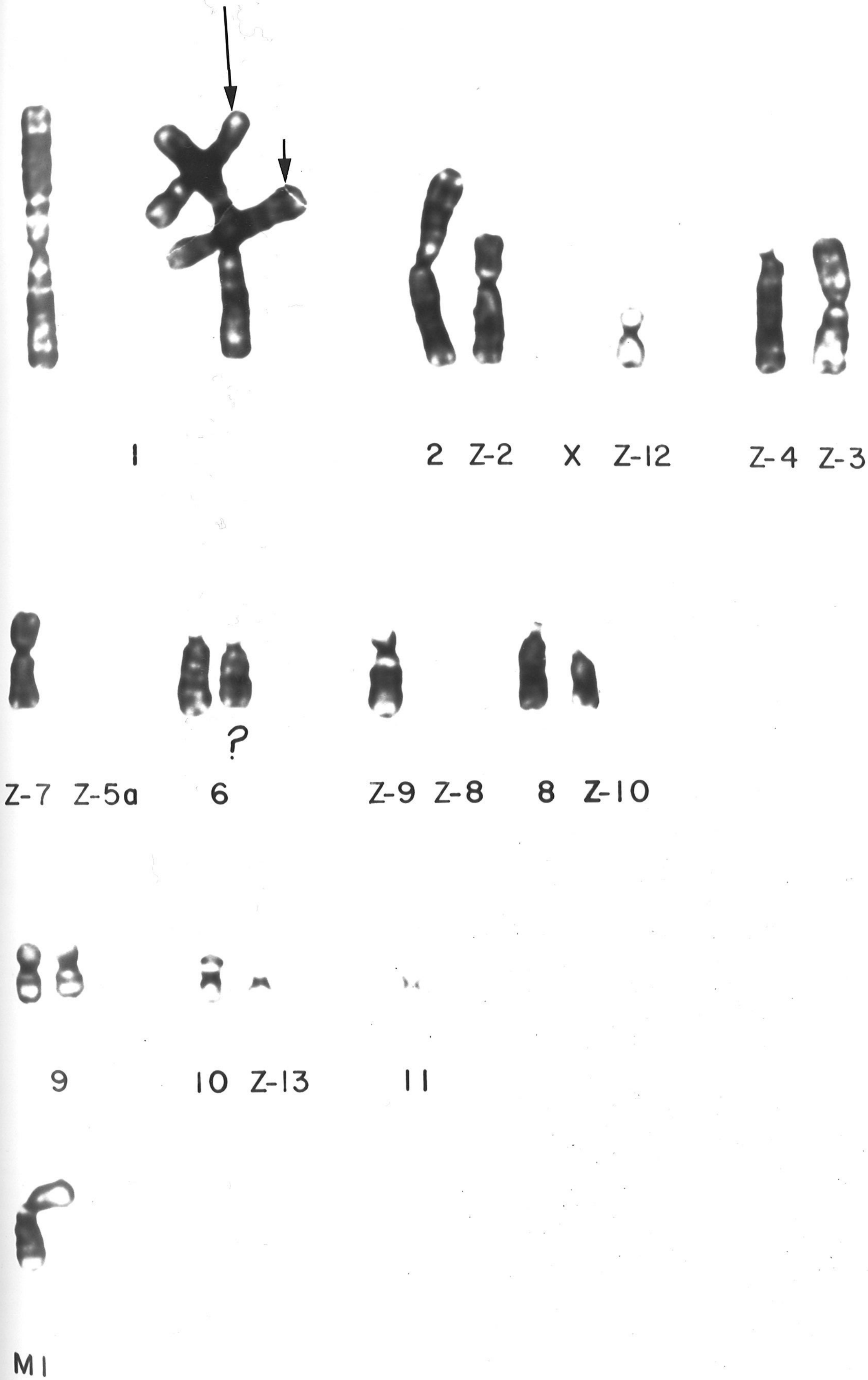

1
2 Z-2 X Z-12
Z-4 Z-3
?
Z-7 Z-5a 6
Z-9 Z-8 8 Z-10
9
10 Z-13
11
M1

Figure 62. Giemsa banded karyotype of hybrid clone OH-5. The karyotype is arranged according to Worton et al. (1977). $M_1$ and $M_2$ represent rearranged chromosomes not present in the parental cells.

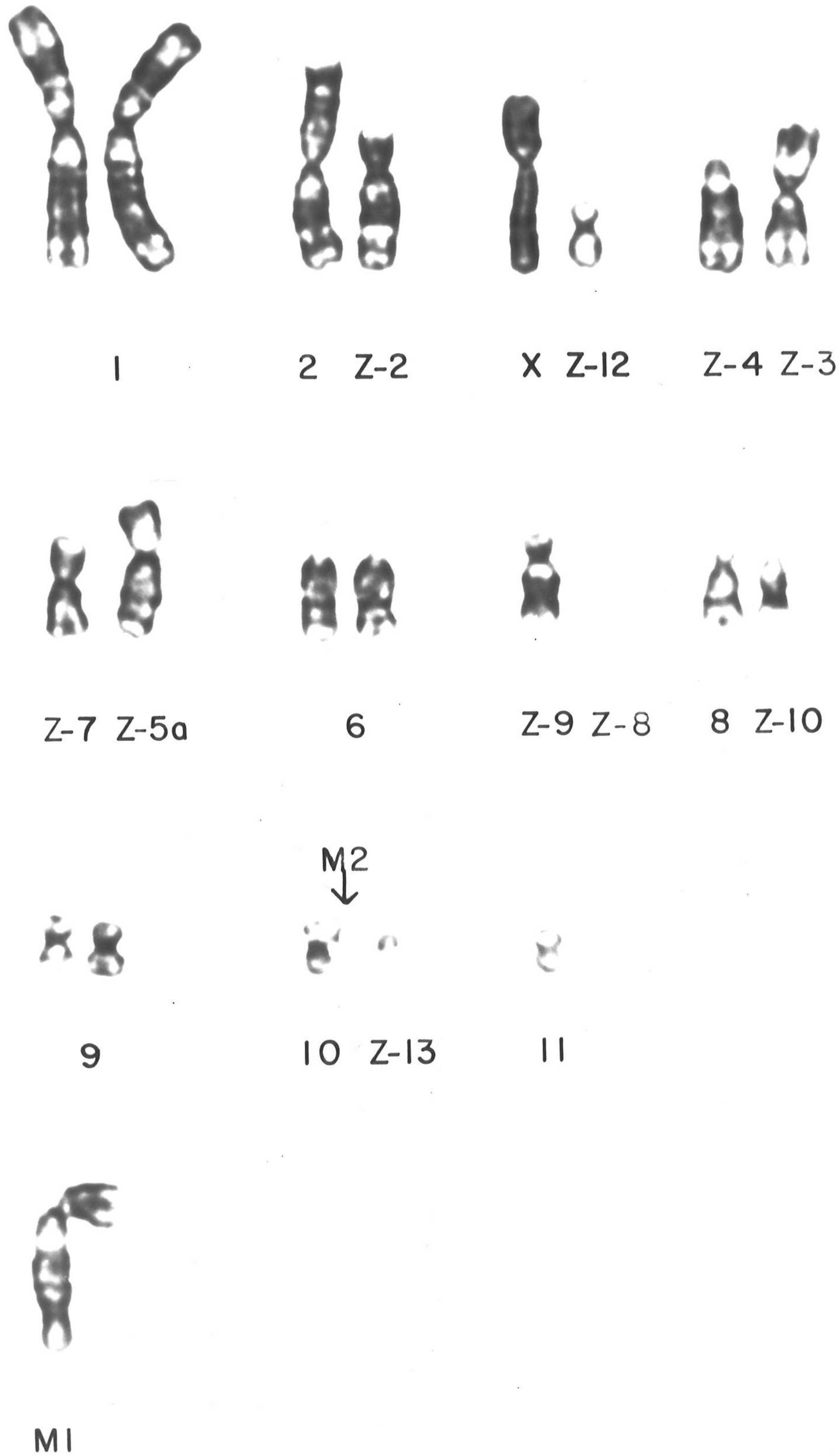

1
2 Z-2
X Z-12
Z-4 Z-3
Z-7 Z-5a
6
Z-9 Z-8
8 Z-10
M2
9
10 Z-13
11
M1

Table IX. Summary of Karyotype Analysis.

| Cell Line | Number of Cells Observed | Number of Chromosomes | Abnormal Chromosomes | Human Chromosomes |
|---|---|---|---|---|
| $GATO^R$-1 | 36 | 21 | − | − |
| GH-2 | 12 | 21 (20% tetraploid) | + | − |
| HGAT-7 | 15 | 21 (40% tetraploid) | + | − |
| GOW-2 | 31 | 21 (27% tetraploid) | + | − |
| OH-1 | 10 | 21 | + | − |
| OH-5 | 5 | 21 | + | − |

* Abnormalities include rearrangements and decentric chromosomes, deletions, pulverized chromosomes, tri-radial chromatid exchange, and strand breaks.

grew up in the double selection medium where the GAT supplement was omitted from the selective medium. This clone subsequently was able to proliferate in the absence of GAT supplement as well as being resistant to hydroxyurea. If this clone was a mutant, two independant mutations must have occurred; resistance to hydroxyurea and reversion of GAT auxotrophy. The chance of two such independant mutations being selected in a single step is very low (ie. about $10^{-5} \times 10^{-5}$).

Close examination of the chromosomes from these clones revealed a variety of chromosomal abnormalities which are indicative of chromosomal instability. For instance the G-banded karyotype of GOW-2 (Figures 59 and 60) indicate that pseudodiploid and pseudotetraploid cells are present in this culture of cells. Furthermore Figure 59 shows a typical tri-radial chromosomal configuration which is indicative of chromosomal instability (Cox & Ray, 1977). Interestingly, all of these clones contained one or more rearranged chromosome (designated $M_1$, $M_2$, etc.) which could not be conclusively identified. Q-banded chromosomal preparations from two clones GOW-2 and OH-1 demonstrated the existance of a variety of other chromosomal abnormalities. Examples of the abnormalities which were observed are shown in Figure 63 and 64. These examples include chromatid breaks, homogenously stained regions, tri- and quadra-radial configurations, and dicentric and pulverized chromosomes. It is important to note that these hybrid clones were grown in hydroxyurea to ensure retention of the drug resistant phenotype. Hydroxyurea has been reported to cause a number of severe chromosomal defects in cells grown in the presence of high concentrations of drug (Yu & Sinclair, 1968; Oppenheim

Figure 63. Quinacrine dihydrochloride banded chromosomes of hybrid cells showing a variety of chromosomal abnormalities. The arrows indicate chromatid breaks (A, D, E, F and G) dicentric chromosomes (B) and quadri-radial chromatid exchange (C).

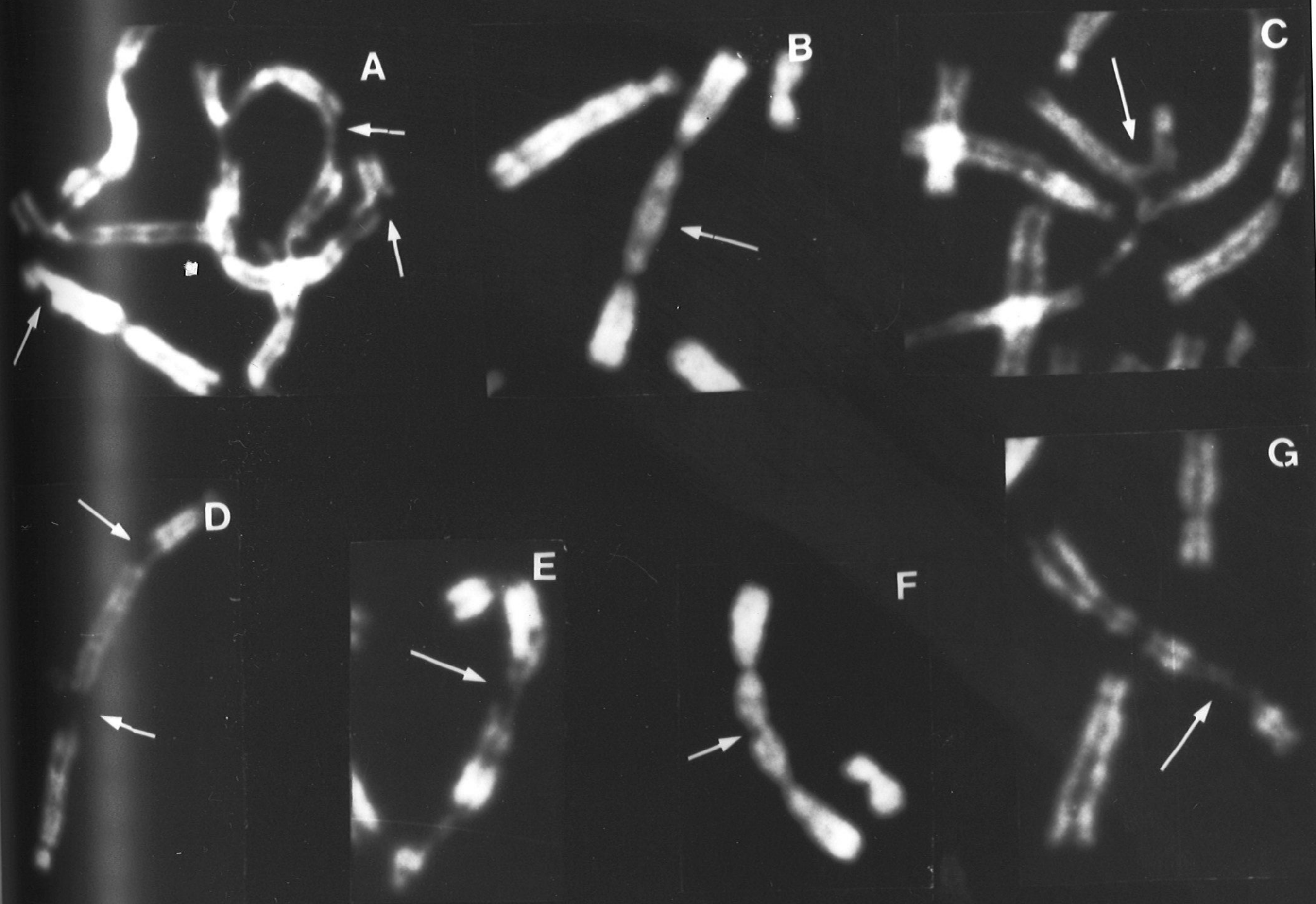
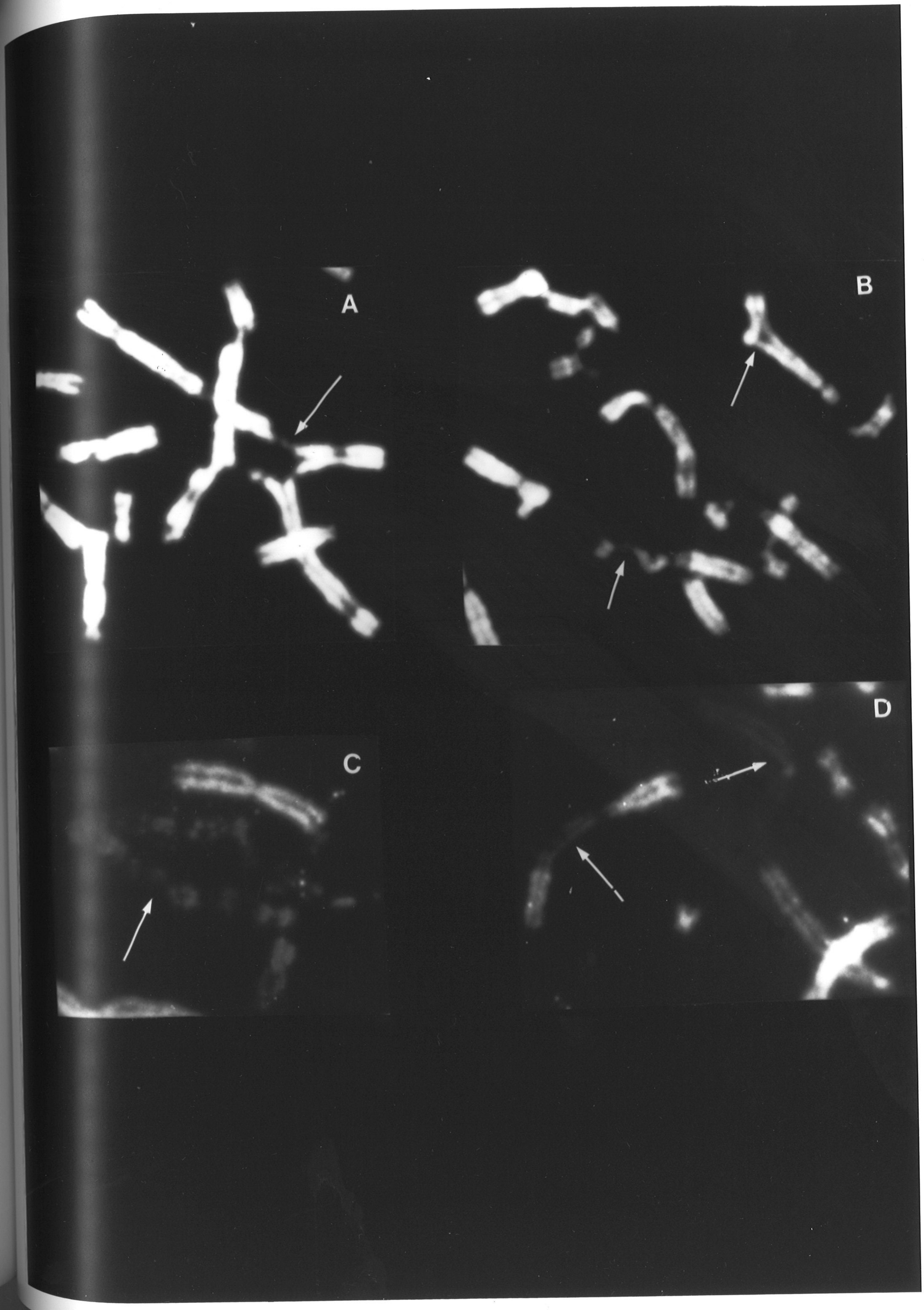

A
B
C
D
E
F
G

Figure 64. Quinacrine dihydrochloride banded chromosomes of hybrid cells showing a variety of chromosomal abnormalities. The arrows indicate chromatid breaks (B), tri-radial chromatid exchange (A), homogenously stained regions (D), and pulverized chromosomes (C).


A
B
C
D

& Fishbein, 1965). Therefore growing hybrid cells in hydroxyurea may have caused this large degree of chromosomal instability. It is known that chromosomal fragmentation enhances the frequency of insertions and translocations. For instance Cirullo et al., (1983) fused human leukocytes and temperature sensitive CHO cells. These hybrids which contained a limited number of human chromosomes were immediately $\gamma$ irradiated to fragment the chromosomes and hybridized with another temperature sensitive CHO cell and selected at the non permissive temperature. The resulting hybrid could only grow if the human gene was present. In this way they were able to select a cell line whose DNA contained only about 0.02% human DNA sequences inserted into the hamster chromosomes.

Therefore, it may be that the chromosomal instability and fragmentation seen in the hydroxyurea resistant hybrids has led to the incorporation of a small amount of human DNA, containing the gene for RRase, into hamster chromosomes. This human DNA would confer hydroxyurea resistance to the hybrid line.

More work needs to be done to resolve the problem of how these cells became resistant to hydroxyurea. The use of cDNA probes derived from middle repetitive sequences could be useful in determining whether human DNA is present in these clones (Cirullo et al., 1983; Shih & Weinberg, 1982). If as suspected, a small amount of human DNA carrying the gene for RRase has been incorporated into the hamster chromosomes, then these cell lines could be used to isolate the gene for human RRase using recently described methods (Gusella et al., 1980; Cirullo et al., 1983). These methods involve probing Southern blots of hybrid DNA with human middle repetative cDNA probes to detect human DNA fragments. These fragments are then isolated, restricted, and probed again with the human middle repetitive cDNA. The unique DNA fragments which are then isolated are expanded in cloning vectors and tested for their ability to produce the proteins in question by in vitro translation. Another method for detecting larger fragments of human chromatin which may have translocated onto the hamster DNA is the alkaline-Giemsa histochemical staining technique (Friend et al., 1976). This staining procedure differentially stains rodent chromatin purple and human chromatin blue.

# DISCUSSION

# DISCUSSION

## A. General Characterization of Ribonucleotide Reductase from Normal Human Cells

Human diploid fibroblasts grown in culture more closely resemble the *in vivo* state than cultures of permanent cell lines (Hayflick, 1980) and therefore are an important model system in which to study complex regulatory processes. Several important features of normal HDF grown in culture were discussed in the Historical section; the most striking being the inability of these cells to proliferate indefinitely. The experiments outlined in this thesis were designed to characterize RRase activity in normal HDF and examine what possible roles this key regulatory enzyme plays in the senescence of HDF grown *in vitro*. The strain of HDF, designated HSC172, used in these studies was derived from female fetal lung tissue and was relatively easy to grow, had a high colony forming ability, and the karyotype was normal 46, XX (Figure 1). As with all other human diploid cell strains, HSC172 was anchorage and density dependent, and had a limited lifespan of 95 $\pm$ 5 passages or mean population doublings. These properties limit the extent of biochemical and genetic studies which generally require large numbers of cells. In spite of these limitations it was important to investigate the nature of ribonucleotide reduction in these normal cultures, therefore, a permeabilization assay, similar to that developed for CHO cells (Lewis et al., 1978), was developed which required relatively low numbers of cells (Dick & Wright, 1980). This assay also had the advantage of measuring enzyme activity in a situation closely resembling the normal cellular environment (Hards & Wright, 1983b). This novel assay enabled, for the first time, a general examination of purine and pyrimidine reduction in normal human diploid fibroblasts (Dick & Wright, 1980).

The assay conditions are outlined in Figures 2 through 18. Briefly, ADP and CDP reduction was activated by the positive effectors dGTP and ATP respectively, with optimal concentrations of about 1mM dGTP and 3 to 4 mM ATP respectively (Figure 5). Kinetic analysis indicates that ATP served to increase the Vmax of CDP reduction and decrease the Km for CDP binding (Figure 42). The apparent Ka for ATP binding was found to be approximately 2 mM. Dithiothreitol could provide reducing power for the enzyme, as well DTT also appeared to protect enzyme activity during permeabilization (Table II).

Optimal enzyme activity resulted at DTT concentrations of 4 - 10 mM for ADP reduction and 5 - 16 mM for CDP reduction (Figure 6). Both CDP and ADP reduction reactions were linear with time up to 40 and 60 minutes respectively when assayed under optimal conditions (Figure 7). ADP reduction was linear with cell number over a wide range of cell concentrations (Figure 8), while CDP reduction produced a non-linear response which appeared to be due to clumping of the cells during the reactions (Figure 11). When the clumps were disrupted by adding micro spin bars to the assay tube; a linear response to cell number was achieved (Figure 8). Optimal activity was observed when cells were incubated in permeabilizing buffer for 75 to 90 minutes (Figure 39). This permeabilization time was about three fold longer than the time required to permeabilize CHO cells, as well the temperature of incubation was 30°C as opposed to 22° for CHO cells (Hards & Wright, 1983b). The increased difficulty in permeabilization of HDF was also found by Billen and Olson (1978) who were measuring DNA synthesis in permeabilized cells. They found that HDF required about three fold longer incubation times than permanent cells from a variety of sources. Although the reason for this effect is not known it may be related to differences in the cellular membrane of normal cells and transformed permanent cells (Abercrombie, 1979).

RRase is a highly allosterically regulated enzyme which is sensitive to various activators mentioned above, as well as a variety of nucleotide inhibitors, naturally occurring endogenous inhibitors, and anti-tumor drugs (Wright, 1983). Deoxy ATP is a potent feedback effector which inhibits the reduction of all four substrates (Thelander & Reichard, 1979). Deoxy ATP was also found to inhibit RRase from HDF, with half maximal activity at about 40 μM for ADP reduction and about 20μ M for CDP reduction (Figure 9). Deoxy ATP was a mixed type inhibitor of CDP reduction with a Ki of 20 μM (Figure 50). As will be discussed in the following section, the pattern of inhibition by dATP changed in enzyme from high passage HDF cells. Hydroxyurea is a specific inhibitor of ribonucleotide reductase from a variety of cell sources (Lewis & Wright, 1974; Thelander & Reichard, 1979). It is thought to inhibit the enzyme by destroying the tyrosine free radical which is involved in the reduction step (Graslund et al., 1982). Hydroxyurea was found to be a potent mixed type or non-competitive inhibitor of both CDP and ADP reduction (Figure 14). The apparent Ki for inhibition of ADP and CDP reduction was approximately 0.4 mM hydroxyurea. Interestingly this Ki was about four fold higher than the Ki reported for CHO cells (Lewis & Wright, 1978; Hards & Wright, 1981) suggesting there may be some structural differences between the enzyme from these two sources. The increased enzyme resistance to inhibition by hydroxyurea was reflected in the increased resistance of normal HDF to the cytotoxic action of hydroxyurea as compared to CHO cells (Figure 18).

In general, these studies indicate that RRase appears to be regulated in much the same fashion as enzyme from other mammalian sources. Many studies have demonstrated a close association between RRase and cancer transformation (Takeda & Weber, 1981; Elford et al., 1970; Wright, 1983), however, none of these studies used genetically related normal and transformed cells. The development of an assay for RRase as well as a general characterization of the enzyme from normal HDF provides a "baseline" or reference point against which RRase from a variety of human transformed cell lines can now be compared. It would be very interesting to isolate transformants from the particular cell strain used here and then determine if any alterations in the regulation of RRase activity could be identified. Such transformants could be induced using a variety of methods including virus, chemicals, or irradiation. However normal human cells are very difficult to transform, only two transformants have been isolated, in which actual transformation has been demonstrated (Kakunaga, 1978; Namba et al., 1978). Initial attempts to transform HSC 172 with benzo (a) pyrene metabolites, 3 methyl colanthrene, and $^{60}Co$ irradiation have proved to be unsuccessful. However further efforts in our laboratory are continuing in order to compare RRase in normal and $SV_{40}$ transformed Wl-38 cells and CT-l $\gamma$-radiation transformed Wl-38 cells (Tagger & Wright, unpublished). Furthermore normal mouse embryo fibroblasts which are easier to transform are being used to obtain transformants, thereby permitting the investigation of RRase in genetically related normal and transformed cells.

B. Involvement of Ribonucleotide Reductase in the Senescence of Normal Human Cells

Changes in Enzyme Activity. As discussed in the Historical section, the process of cellular senescence of normal HDF grown *in vitro* bears many similarities to the process of *in vivo* aging. A thorough understanding of the cellular mechanisms which underlie cellular senescence should greatly enhance our understanding of the complex aging phenomenon. The value of fundamental aging research to society can readily be appreciated because of the magnitude of age-related disease conditions.

RRase is an important enzyme which plays a key role in the regulation of cellular proliferation, in tumor progression, in transformation, in determining the spontaneous mutation rates of cells in culture, in aspects of cellular differentiation, and indirectly in certain immunodeficiency diseases in man (Wright, 1983). Since RRase plays such an important role in cellular regulation the question of whether it also plays a role in cellular senescence of HDF was addressed using a biochemical and somatic cell genetic approach. In order to determine if any change in the activity of RRase took place during senescence, the level of RRase activity was measured in HDF at a variety of passage levels (Dick & Wright, 1982). A significant drop in enzyme activity of approximately three fold was observed between low and high passage cells (Figure 20). Interestingly, enzyme activity decreased steadily in cultures as the passage numbers increase. The replicative potential of HDF grown in culture follows a different pattern as demonstrated in Figure I (Historical). Replicative potential remains constant throughout most of the lifespan, dropping off rapidly near the end of the replicative lifespan. Therefore the drop in RRase activity seems to precede the loss in replication potential.

Many studies have shown that during senescence mass population of cells gradually lose cells from the proliferating pool, and exhibit large variations in doubling times (Rabinovitch, 1983; Smith & Whitney, 1980). Therefore it was important to determine whether the reduction in the level of RRase was due to a reduction in the number of proliferating cells or whether the specific activity per replicating cell was reduced. Hydroxyurea kills cells by inhibiting RRase and many studies have shown that the level of cellular resistance to hydroxyurea correlates very closely to the level of RRase activity (Lewis & Wright, 1979; Hards & Wright, 1983a; Wright et al., 1981; Wright, 1983). Cytotoxicity of hydroxyurea was based on colony forming ability of cells in the presence of drug divided by colony forming ability in the absence of drug, thereby, normalizing the cellular resistance to those cells capable of proliferating and forming colonies in the absence of drug. The relative cellular resistance of HDF, at various passage levels, to hydroxyurea using the colony forming assay, was found to decline in exactly the same manner as the decrease in the levels of RRase (Figure 21). Since cellular resistance and the levels of RRase are closely associated, this decline in cellular resistance with increased passage numbers suggests that RRase activity decreases in cells capable of proliferating. It is worth noting that the colony forming ability of cells in the control plates with no drug also dropped with increasing passage number, reflecting the loss of cells from the proliferating pool (Figure 22). However this decrease in colony forming ability followed a different pattern than the age-related decline in RRase activity or cellular resistance to hydroxyurea.

These studies demonstrated for the first time an association between RRase activity and the senescence of normal fibroblasts grown *in vitro*. Recently another key enzyme of the DNA synthetic multienzyme complex, DNA polymerase α, has been reported to decline wtih increasing passage levels on the basis of activity per S phase cell, paralleling the decrease described in this study with RRase (Pendergrass et al., 1982). These observations suggest that the basic controls regulating DNA synthesis may be closely linked to mechanisms of senscence.

*Isolation of Hydroxyurea Resistant Human Diploid Fibroblasts with Altered Replicative Lifespan*. During the past fifteen years development of selection procedures for isolating a large variety of stability altered mammalian cell variants in culture has been extremely rapid. The list now includes temperature sensitive, auxotrophic, drug resistant and lectin resistant cell lines as well as cultures containing altered products of differentiation (Wright et al., 1980;

Siminovitch, 1976). Genetics has greatly contributed to our understanding of regulatory mechanisms in microorganisms and it is now clear that the field of somatic cell genetics is capable of making similar contributions to our understanding of mammalian cells (Wright et al., 1980). As a general class, the drug resistant mutants have been studied more often than any of the others because of the wide variety of specific mutants that can be isolated, and the relative ease with which they can usually be obtained. Moreover, there are many cytotoxic drugs which specifically inhibit essential cellular functions, and these can be used as selective agents to isolate drug resistant mutants (Wright et al., 1980).

Cytotoxic drugs whose intracellular target is ribonucleotide reductase have been used as selective agents in cell culture to isolate drug resistant cell lines with specific alterations in enzyme activity. The enzyme properties of these mutants has been studied, in a situation resembling physiological conditions, using the permeabilization assay as well as in purified extracts (Hards & Wright, 1981, 1983; Lewis & Wight, 1978a,b, 1979; Kuzik & Wright, 1979, Koropatnick & Wright, 1980; Wright & Cory, 1983; Wright et al., 1981). These studies have identifed at least three classes of drug resistant mutants in hamster and mouse cells in culture (Table III, Wright et al., 1981). Mutants of the first class apparently contain structural alterations in the $M_2$ subunit of RRase since the enzyme in these mutant lines is significantly less sensitive to drug inhibition than enzyme from wild type cells (Lewis & Wright, 1974; 1978a,b; Lewis et al., 1978; Hards & Wright, 1982b; Wright, 1983).

Hydroxyurea inhibits enzyme activity by interfering with tyrosine free radical formation required for the reduction process (Graslund et al., 1982). The most logical explanation of class I drug resistance is that these cells contain a structural gene mutation in the $M_2$ subunit which reduced the affinity of hydroxyurea for the protein. This suggestion needs to be tested further with purified $M_2$ components from drug resistant and sensitive cell lines.

The second class of drug resistant mutants overproduce reductase activity with normal sensitivity to drug inhibition (Lewis & Wright, 1979; Hards & Wright, 1981; 1982a; Cory & Wright, 1983; Dick & Wright, 1983; Wright, 1983; Wright et al., 1981; Akerblom et al., 1981; Graslund et al., 1982). Drug sensitive revertants with reduced enzyme activity have also been isolated from this class of mutants (Lewis & Wright, 1979). Furthermore, hydroxyurea resistant lines have been identified which overproduce either the $M_2$ component alone (Graslund et al., 1982) or both $M_1$ and $M_2$ components (Wright & Cory, 1983). The mechanism responsible for increased enzyme activity is not known, however, several possibilities exist. For example, mutants may possess an altered regulatory gene involved in controlling intracellular levels of the reductase components. It is also possible that overproducers possess a gene amplification and thus may resemble the methotrexate resistant cells which contain multiple copies of dihydrofolate reductase genes (Alt et al., 1978; Flintoff, et al., 1982) or N-(phosphonacetyl)-L-aspartate resistant lines which overproduce a multi-enzyme complex (CAD) associated with UMP biosynthesis through the amplification of the CAD gene (Wahl et al., 1979). Interestingly, gene amplification has now been characterized in a variety of different genes from a variety of cell types (Stark & Wahl, 1983). Mutants of this type are extremely useful for investigating regulatory mechanisms of gene expression in mammalian cells. It is worth noting that gene amplification models for carcinogenesis have been proposed (Pall, 1981; Lavi, 1982), in addition, some malignant tumor cell lines have been found to contain amplified genes (Alitalo et al., 1983).

The third class of drug resistant cell lines contain a combination of elevated RRase activity and an apparent structural alteration which makes the enzyme less sensitive to drug inhibition (Wright et al., 1981; Kuzik & Wright, 1980; Koropatnick & Wright, 1980; Hards & Wright, 1981; 1982a). It is interesting that these mutants exhibit unequal increases in purine and pyrimidine reduction. In addition to alterations in drug sensitivity, other changes such as in substrate $K_m$ were also observed providing further indication of a structural change in the enzyme. Whether these two changes in reductase activity are due to a single pleiotropic mutation, as observed in other mutant cell lines (Wright, 1973; Wright et al., 1979), or is due to two independent events is not known.

The isolation of normal diploid cells resistant to hydroxyurea was attempted primarily for three reasons. Firstly, very few mutant strains have been isolated in HDF. These mutants are useful for studying rates and mechanisms of mutation. Such information would be valuable in determining mutational mechanisms relevant to humans and also for the development of mutagenesis assays of environmental agents which accurately predict risk (Albertini, 1979). Secondly, hydroxyurea resistant mutants in permanent cell lines such as CHO or L cells have been very useful in elucidating the nature of RRase in these cells. Hydroxyurea resistant HDF would be useful to determine if any novel regulatory or structural features of RRase exist which are unique to normal cells. And thirdly, since the levels of RRase are reduced in aging cells, drug resistant HDF containing genetically altered RRase activity should be useful biological tools for investigating the role of RRase in the senescence process.

Hydroxyurea resistant HDF were isolated for the first time using a novel selection scheme which was developed in order to achieve a high degree of cell killing (Dick & Wright, 1983). The isolation of these drug resistant cells proved to be much more difficult than had been anticipated based on the experience of mutant selections with permanent rodent cell lines. The selection experiments which were carried out over the course of about two years involved approximately one thousand selection plates which had to be maintained, screened for colonies, and grown up again for reselection. Over 150 colonies were isolated, grown up, frozen in liquid nitrogen, and screened for drug resistance. Only three colonies were found to exhibit stable drug resistance properties upon retesting (Figure 26), indicating that the frequency of isolation of drug resistant cells was very low (approximately $1.2 \times 10^{-8}$) when compared to permanent cell lines such as CHO cells (Lewis & Wright, 1978; Wright et al., 1980, 1981). This frequency resembles those reported for the α-amanitin and ouabain drug resistance markers in human diploid cells (Buchwald & Ingles, 1976; Buchwald, 1977). The low frequency of isolation may reflect the critical role RRase plays in the cell. Those mutations which produce large alterations in enzyme activity may be lethal so only a very limited range of alterations can be selected. The fact that the hydroxyurea resistant cells were only three to four fold more resistant than wild type cells would also support this idea. It should be noted that the mutation frequency in untreated cultures may be lower than this estimate since two of the clones, MC1-1 and QC1-2 were isolated from EMS treated populations. Also in some selection experiments cell cultures were treated several times with hydroxyurea whereas in others, the cells were exposed to a single drug treatment. Therefore the mutation frequency of $1.2 \times 10^{-8}$ should be considered as only a very rough estimate. The difficulty in isolating hydroxyurea resistant HDF was due to :

1. The low mutation frequency; therefore large numbers of cells needed to be screened; and
2. the density dependent cell killing by hydroxyurea interfered with colony formation.

At high cell densities the fibroblasts were able to remain attached to the tissue culture plates. These fibroblasts could remain in this state for many weeks, and some cultures could continue to grow if the drug was removed. For this reason the usual method of selecting mutants by allowing colonys to form in the presence of drug was not successful. These problems were partially overcome by exposing the cells to high concentrations of drugs for short periods of time, then removing the cells with trypsin solutions and replating the cells at 1:4 dilution. This produced a killing curve with a rather steep slope so that drug concentration, length of drug exposure, and cell density were critical for colony development (Figure 25). These were especially important factors since the drug resistant cells were only three to four fold more resistant than wild type cells.

The drug resistant cell strains exhibited a limited replicative lifespan since they all senesced less than twenty passages after isolation (Table V). It readily became apparent that the senescence process had been accelerated in these variants since the number of cell doublings which had occurred during selection, in addition to the passage numbers of the wild type strain used for selection, indicated that reductions of 20%, 25%, and 40% had occurred in MC1-1, WC1-2, and QC1-1 respectively. A large degree of heterogeneity had been demonstrated in the replicative potential of individual cells from a mass population. Smith and Whitney (1980) have shown that the replicative potentials of the individual cells of a population are distributed in a bimodal fashion. The low replicative potential pool of cells produces small colonies while the high replicative potential pool of cells produces large colonies which are generally capable of accumulating as many or more population doublings as the mass population (Smith & Hayflick, 1974; Smith & Whitney, 1980). The mutant selection scheme outlined, inherently selects for cells with high growth potential since only large colonies (> 1cm in diameter) were isolated from drug selection plates. Furthermore, three independent clones all exhibited this phenomenon. It is worth noting that of the other examples of drug resistance using normal HDF (Albertini & DeMars, 1970; Rappaport & DeMars, 1973; Mankovitz et al., 1974; Buchwald & Ingles, 1976; Gupta & Siminovitch, 1978a) including one variant with two markers (Duthu et al., 1982), none were reported to have alteration in lifespan as compared with the mass population.

These data provide further support for the idea that RRase plays an important role in the senescence process, since variants selected for altered RRase appear to show accelerated senescence. In order to determine the nature of the alteration in RRase which led to drug resistance, the permeabilization assay was used to analyze the enzyme in these variants. Each of the three variants was subsequently found to contain elevated levels of RRase activity which correlated closely to the level of cellular resistance (Figure 28). The close association between drug resistance and CDP reductase activity has been reported for hydroxyurea resistant mutants in CHO and mouse cells (Hards & Wright 1983a; Lewis & Wright, 1979; Wright et al., 1981; Wright et al., 1981; Wright 1983). The sensitivity of RRase to hydroxyurea was measured in variant cells and found to be similar to enzyme from wild type cells, indicating that the affinity of the enzyme for hydroxyurea was probably not altered. The enzyme from these variants resembles class II mutants lines which overproduce normal enzyme activity (Wright et al., 1981; Wright, 1983).

It would be very interesting to determine if the overproduction of RRase was the result of gene amplification. This mechanism of drug resistance has been demonstrated in a variety of permanent cell lines (Stark & Wahl, 1983) however no examples of gene amplification have been identified using normal diploid cells. It would be important to determine whether gene amplification is a phenomenon restricted to abnormal permanent cell lines or are normal cells also capable of gene amplification. For this reason it would be particularly important to determine the cause of enzyme overproduction in these hydroxyurea resistant variants. DNA probes complementary to the genes which code for the components of RRase could be used to examine this problem.

Alterations in Deoxyribonucleotide Pools of Drug Resistant and Senescing Human Diploid Fibroblasts

Ribonucleotide reductase is responsible for providing a continuous and balanced supply of the deoxyribonucleotide precursors essential for DNA synthesis. The fidelity of DNA replication is influenced by the relative concentrations of the deoxyribonucleotide pools, and contributes to the characteristic mutation rate of the cell. The importance of this point is emphasized in the recent findings of a mutator gene associated with mammalian ribonucleotide reductase, which results in increased rates of spontaneous mutation due to an imbalance of endogenous deoxyribonucleoside triphosphate pools (Weinberg et al., 1981; Meuth et al., 1979). Alterations in RRase activity due to the effects of inhibitors or mutations affecting enzyme regulation can lead to perturbations in intracellular dNTP concentrations (Skoog et al., 1973; Theiss & Fischer, 1976; Ullman et al., 1979; Ashman et al., 1981). In order to more fully understand the regulation of RRase, the sizes of the dNTP pools were measured, using HPLC techniques, in drug resistant and wild type cells at various passage levels. These studies represent the first time the dNTP pools have been analyzed in normal HDF.

The effects of adding hydroxyurea to cultured cells remain somewhat inconclusive as various studies using different analytical methods show a variety of pool changes, however, there seems to be general agreement that pyrimidine pools especially dCTP are rapidly depleted when hydroxyurea is added (Theiss & Fischer, 1976; Rauscher & Cadman, 1983). Several studies have suggested that the level of the dCTP pool correlates more closely than the three other deoxyribonucleotide pools, to DNA synthesis (Reichard, 1978), cell proliferation (Weber et al., 1981), and the mutagenic nature of altered ribonucleotide reductase regulation (Ashman et al., 1981). One of the variants WC1-2, which was used in these studies, had significant expansion (1.8 fold) of the dCTP pool and slight increases in TTP and dATP pool sizes as compared to age matched controls (Figure 31). In addition there was a large decline in the dGTP pool, although it should be mentioned that this measurement was difficult since the pool was very small. These findings suggest that the elevated dCTP pool in resistant cells may play a special role in cellular resistance, and support previous observations that the levels of CDP reductase are particularly important for resistance mechanisms involving hydroxyurea and other drugs with a similar modes of action (Hards & Wright, 1983a). Since hydroxyurea normally depletes the dCTP pool in wild type cells, the expansion of this pool in variant cells may enable these cells to proliferate in the presence of drug. Class II CHO mutants which overproduce CDP reductase can be stimulated to produce even higher levels of enzyme activity by growing the cells in hydroxyurea (Wright et al., 1981; Hards & Wright, 1983a). Presumably this increase in CDP reductase activity would lead to a further expansion of the dCTP pool.

Unfortunately it was not possible to measure the dynamics of the dCTP pool in the presence of hydroxyurea because of the limited numbers of cells which were still actively proliferating.

In order to determine if the relative dNTP pool sizes change during senescence, cell extracts were prepared from HDF at low (< 30) and high (75 - 90) passages (Figures 32 & 33). In addition, the levels of the dNTP pools of the drug resistant variant WC1-2 were compared to the levels found in low passage cells to see if the variant pools showed the same changes as age matched wild type cells (very high passage cells; > 90). These measurements can be expressed as pmoles/cell number or as concentration (μM). The latter measurement takes into account the large increase in cell size which is seen in high passage cells. When the data was expressed as pmoles/5 x $10^6$ cells, TTP remained fairly constant between passage 29 and 86 cultures, however this pool was reduced to 50% of low passage values in very high passage 92 and WC1-2 cells. The dCTP pool in passage 86 cells dropped to 70% of the pool from low passage cells and to 38% in passage 92 cells. The elevation of the dCTP pool in WC1-2 cells with respect to very high passage age-matched cells, was evident in that it only dropped to 81% of low passage values. The dGTP pool remained fairly constant in passage 86 cells while dropping to 45% in passage 92 cells and to 25% in WC1-2. In contrast, the dATP pool was 1.6 fold higher in passage 86 cells while the pool from passage 92 and WC1-2 cells was 1.2 and 1.4 fold higher respectively than low passage cells.

When the sizes of the pools were expressed as concentration, the 4.88 fold increase in the cell volume of high passage cells dropped the pool size measurements accordingly (Figure 33). Very marked reductions of the dCTP, TTP and dGTP pool size were observed in all high passage cells. These pools fell to approximately 20% of the pools from low passage cells. Deoxy ATP dropped to about 30% of young passage cells in the high passaage and WC1-2 cells.

From these dNTP pool studies it is evident that there is a marked decline in the levels of the four dNTP's as wild type and drug resistant HDF senesce in vitro (Figure 33). It is also clear that overall, the pool size changes appear to be regulated in the same fashion in wild type and drug resistant cells, except that the process is accelerated in the drug resistant cells. As with any dNTP pool studies it is difficult to determine cause and effect. As noted before many studies, using permanent cell lines, have supported the view that the intracellular level of dCTP is closely linked to DNA synthesis (Weber, 1983), and is intimately involved in the fidelity DNA synthesis (Meuth, 1981). If this positive correlation were to hold true in HDF, one may suppose the variants should show increased rather than reduced lifespan. However, very little is known about the biological effects of perturbing the balance between the dNTP pools. For instance while dCTP was elevated compared to high passage age matched controls, the dGTP pool was very small (4%) compared to the low passage cells suggesting it may not be large enough to initiate DNA synthesis. In fact this depletion of dNTP's in very high passage cells may be one of the reasons these cells stop proliferating; the levels of the dNTP's may have declined below a critical threshold level required for the initiation of DNA synthesis (Walters et al., 1976). In the drug resistant clone, WCl-2, this depletion has occurred at an accelerated rate causing earlier senescence. While it is not yet possible to analyze in detail the properties of the $M_1$ and $M_2$ subunits of RRase in the variant, it may be that some alteration in the regulation or interaction of these subunits is contributing to the acceleration of the aging process. Furthermore, it must also be pointed out that pool size measurements were made using whole cell extracts and several workers have suggested that dNTP pools may not be equally distributed between the cytoplasm and nucleus so that the effective concentrations are different in the nucleus as compared to the cytoplasm (Forsdyke & Scott, 1980; Reddy & Pardee, 1980). Moreover, the local concentrations of dNTP's at the site of utilization may also be higher than the cytoplasmic or nuclear pools since the DNA synthetic multi-enzyme complex appears to channel dNTP's to the replication forks (Reddy & Pardee, 1982, 1983). In order to fully understand the relationship between the different dNTP pools, this problem must be resolved.

Alterations in the Ribonucleotide Pools of Senescing Human Diploid Fibroblasts

The four ribonucleoside diphosphate pools serve as the source of substrates for RRase while ATP and GTP (Hards & Wright, 1983b) are allosteric regulators of the enzyme. Since changes in the concentrations of these pools could also affect RRase activity, the relative sizes of these pools were measured. A complex pattern of changes in the ribonucleotide pools was observed in HDF at various passage levels (Figures 35 & 36). Generally, the levels of the triphosphate pools were approximately 50% lower in high passage cells as compared to low passage cells when the measurements are expressed as concentration. The levels of all four triphosphate pools were higher in passage 92 cells as compared with passage 82 and 86 cells. The level of ATP found in passage 92 cells was similar to passage 29 cells while the level of ADP was two-fold higher in passage 92 cells. Interestingly, the GDP pool was the lowest pool in all the high passage 82, 86, and 92 cells, dropping to about 5% to 10% of low passage values. This low level of concentration of substrate for GDP reduction may be the reason the dGTP pool is so low in high passage cells.

The low cellular concentration of GDP suggested that other cellular functions associated with GDP may also be altered in high passage cells. Some studies have reported that the level of nucleotide sugars can affect the rate of glycoprotein biosynthesis (Kaplan et al., 1984). Glycoproteins play an important role in cellular regulation, especially the glycoproteins found on the cell surface membrane. The cell membrane is intimately involved in cellular recognition and appears to play a role in cell proliferation (Hughes, 1976a,b). Therefore, the amount of incorporation of $^{14}C$-mannose, via the nucleotide sugar GDP-mannose, into membrane glycoproteins was measured in low and high passage cells. Three independent experiments showed that low passage cells incorporate approximately two-fold more mannose into their membrane glycoprotein than high passage cells. SDS polyacrylamide gel electrophoresis of the purified membrane protein revealed a large reduction in the amount of $^{14}C$-mannose found in high molecular weight glycoproteins in high passage cells (Figure 37). Smaller reductions were observed in the lower molecular weight species as well. It would be very interesting to determine why less mannose is incorporated into the surface membranes of high passage cells. For instance, the level of mannosyl transferase enzymes, which add the sugar to oligosaccharide chains, may be reduced in high passage cells, or the level of GDP may be rate limiting in the formation of GDP-mannose. These possibilities need to be tested further since alterations in the cell membrane of aging fibroblasts would significantly affect the normal regulatory properties of this organelle.

Regulation of Ribonucleotide Reductase in Young and Aging Human Diploid Fibroblasts

The experiments which have been outlined above all support the view that RRase plays an important role in senescence of HDF in culture, therefore it would be important to determine if any major changes in the allosteric regulation of RRase could be identified in high passage cells. These experiments are somewhat limited in scope since large quantities of cells at high passage are difficult to obtain.

The experiments showed that the conditions of the assay which yielded optimal activity were essentially unchanged in high passage cells as compared to low passage cells examined in parallel (Figures 39 - 47). Both strains of cells exhibited optimal activity after about 75 minutes of incubation in permeabilization buffer. In addition, about 75% of both low and high passage cells were permeabilized as measured by staining with trypan blue. The optimal concentrations of ATP (4 mM) and dGTP (1 mM), the positive effectors for CDP and ADP reduction respectively, were also unchanged (Figures 41 & 44). Kinetic analysis indicated that CDP reduction was activated by ATP in a mixed fashion in both cell types; with an apparent $K_a$ of 1.4 mM and 2.0 mM ATP for high passage (Figure 43) and low passage cells (Figure 42) respectively. The sensitivity of these cells to hydroxyurea was also unchanged, since estimates of the apparent $K_i$ were both 0.5 mM drug (Figures 46 & 47). Apparently, there were no major alterations in the affinity of the enzyme for hydroxyurea.

One significant change in the regulation of RRase was observed in high passage cells. The enzyme from high passage cells appeared to be more resistant to inhibition by the allosteric inhibitor, dATP (Figure 48 & 49). The nature of this difference was investigated in detail. Kinetic analysis of double reciprocal plots of the inhibition of CDP reductase at various fixed concentrations of dATP revealed a complex pattern of mixed type inhibition. Replots of these data gave linear lines for low passage cells and a hyperbolic curve for high passage cells. The apparent $K_i$ for dATP inhibition of enzyme from low passage cells was about 55 μM (Figure 50), but this kinetic parameter could not be estimated for the enzyme from high passage cells because of the nonlinear nature of the replot (Figure 51). Further kinetic analysis of the hyperbolic replot was carried out by plotting the inverse of the change in slope and the inverse of the change in intercepts versus dATP concentration (Figure 52). From the analysis, the $K_i$ was found to be approximately 20 μM. The kinetic constants were also calculated from the inhibition curve obtained with low passage cells; these constants were then used to calculate the actual $K_i$ which also turned out to be approximately 20 μM. This indicates that the affinity of dATP for the enzyme was similar in low and high passage cells. The key to understanding ribonucleotide reductase in high passage cells which are more resistant to dATP lies in determining the causes of the hyperbolic replot. Hyperbolic replots are the result of a particular kind of mixed type inhibition, where even under saturating concentrations of inhibitor a low amount of product is always formed (Segal, 1975). In other words, the inhibition by dATP is not complete, rather it is somewhat "leaky". The reason why enzyme inhibition becomes less complete in high passage cells is not known, however the answer may be in the interaction between the $M_1$ subunit to which dATP binds, and the $M_2$ subunit (which contains the free radical required for ribonucleotide reduction). Deoxy ATP binding to $M_1$ has been reported to induce conformational changes which alter the active site in enzyme from calf thymus (Caras et al., 1983). This conformational change prevents substrate binding to the active site. Some unknown age-related enzyme change could alter the conformation of the active site in such a way that a small amount of product is still produced even in the presence of dATP. A variety of changes in the subunits caused by mutation, post-translational modification, or errors in the protein synthesizing machinery could produce such an alteration. It is interesting to note that such age-related protein modifications have been reported in other enzymes. For instance, post-translational modification appears to alter the interaction between the subunits of G6PD in such a way that the enzyme becomes more heat labile (Morrow & Garner, 1979). It would also be very interesting to determine if the level of either $M_1$ or $M_2$ is altered as cells senescence. Crude measurements of the relative quantities of $M_1$ and $M_2$ in cell extracts from low and high passage cells failed to reveal any marked differences in the relative quantity of either subunit (Table VII). The $M_2$ subunit appeared to be limited in both cultures, similar to the situation reported in S49 mouse lymphoma cells (Eriksson & Martin, 1981). Clearly, more work needs to be done to determine if the interaction between $M_1$ and $M_2$ is changed in aging cells, and if so, what mechanism underlies this alteration.

Relationship to Aging Theories

The studies in this thesis provide the first indication that there is an important link between a key rate-limiting step in DNA synthesis; RRase, and the *in vitro* senescence of normal human diploid fibroblasts. Several of the major theories of aging have been discussed in the Historical section. The age-related changes in RRase activity provide support for the intrinsic or programmed theories of aging. These theories predict that several key proteins govern senescence in much the same way they are involved in the control of cellular differentiation or the control of cell cycle progression. It is appropriate to note that RRase appears to play a role in aspects of cellular differentiation (Creasey et al., 1982; Creasey & Wright, 1983) and it has been suggested that one of the subunits of the reductase may belong to a small group of proteins that are directly involved in regulating cell cycle progression (Eriksson & Martin, a way that the enzyme becomes more heat labile (Morrow & Garner, 1979). It would also be very interesting to determine if the level of either $M_1$ or $M_2$ is altered as cells senescence. Crude measurements of the relative quantities of $M_1$ and $M_2$ in cell extracts from low and high passage cells failed to reveal any marked differences in the relative quantity of either subunit (Table VII). The $M_2$ subunit appeared to be limited in both cultures, similar to the situation reported in S49 mouse lymphoma cells (Eriksson & Martin, 1981). Clearly, more work needs to be done to determine if the interaction between $M_1$ and $M_2$ is changed in aging cells, and if so, what mechanism underlies this alteration.

Relationship to Aging Theories

The studies in this thesis provide the first indication that there is an important link between a key rate-limiting step in DNA synthesis; RRase, and the *in vitro* senescence of normal human diploid fibroblasts. Several of the major theories of aging have been discussed in the Historical section. The age-related changes in RRase activity provide support for the intrinsic or programmed theories of aging. These theories predict that several key proteins govern senescence in much the same way they are involved in the control of cellular differentiation or the control of cell cycle progression. It is appropriate to note that RRase appears to play a role in aspects of cellular differentiation (Creasey et al., 1982; Creasey & Wright, 1983) and it has been suggested that one of the subunits of the reductase may belong to a small group of proteins that are directly involved in regulating cell cycle progression (Eriksson & Martin, 1981). Models to describe these phenomena generally fall into three classes: the deterministic models which predict that these processes are controlled by a tightly coordinated sequence of events which are the products of a programmed series of gene activations; the random models which generally predict that cells exist in proliferating and nonproliferating states and random events govern the probability of transition from nonproliferation to proliferation; and those models which contain elements of both deterministic and random models (Yanishevsky & Stein, 1980). Moreover, the control of cell cycle progression, which is closely related to the control of senescence, has also been described in terms of stochastic or random transition models (Smith & Martin, 1973; Yanishevsky & Stein, 1980; Rabinovitch, 1983). In these models the initiation of DNA synthesis is governed by the probability that a small number of initiation factors will rise above a critical threshold level and DNA synthesis will begin. If these factors do not rise above the threshold the probability of cell cycle progression remains very low. The experiments described in this thesis which establish the correlation between modified RRase activity and the establishment of the nonproliferative or senscent state can be explained in terms of such random models.

One of the key elements of senescence is the loss of proliferative ability of HDF at high passages. However, this nonproliferative state is partially reversible since hybridization of these cells to suitable transformed cell lines results in reinitiation of DNA synthesis in the senescent nuclei (Stein et al., 1982). Since the level of RRase is known to decrease as cells approach senescence, it may be that at some point the level of RRase falls below a critical threshold level needed to initiate DNA synthesis. At this point, the culture would no longer proliferate and all the other age-related features characteristic of senescense would come about. Fusion of such senescent cells to transformed cell lines, which contain higher levels of RRase activity would allow the reinitiation of DNA synthesis in the senescent nuclei. It is worth noting that such a hypothesis has also been proposed by Pendergrass et al. (1982) for DNA polymerase α, which also exhibits age related decreases in activity in normal HDF. Recently Pardee's group has postulated that the assembly of the multienzyme DNA synthesis complex and its entry into the nucleus may provide the signal for the initiation of DNA synthesis (Reddy & Pardee, 1980). Furthermore in light of the fact that RRase is the key rate limiting enzyme of the complex it would be interesting to know if the reductase or one of the components plays a key role in this assembly process. It is tempting to speculate that the age related change in RRase which leads to altered dATP inhibition may also alter the assembly of the multienzyme complex, changing the course of cell cycle progression and perhaps contributing to establishing the senescent state. These ideas could be tested if the multienzyme complex could be isolated in low and high passage cells. Then the kinetics of assembly of the complex from low and high passage cells could be examined _in vitro_. Furthermore it would also be interesting to characterize the _in vivo_ assembly and intracellular location of the complex.

The bimodal distribution of proliferative potential among the individual cells of a population as well as the apparent randomness or stochastic nature inherent in that distribution (Martin, 1977; Smith & Whitney, 1980; Smith & Lumpkin, 198 ) can also be explained in a model based on the RRase. Some models to describe these distributions such as those proposed by Smith and Lumpkin (1980), are based on the appearance of an inhibitor in a process with underlying stochastic elements. In this regard, a variety of naturally occurring endogenous inhibitors of RRase have been identified (Elford, 1982; Cory, 1979; Lewis et al., 1977; Goh et al., 1977). These inhibitors are poorly characterized although their expression appears to be inversely proportional to RRase activity (Cory, 1983). It would be interesting to know if such inhibitors exist in normal HDF and if so whether they accumulate as the culture senesces. The bimodal distributions of proliferative potentials as well as the heterogeneity in doubling times among individual cells of mass populations have also been explained using models based on transition probabilities as described above (Bell et al., 1978; Rabinovitch, 1983). The previous discussion of the role of RRase in the initiation of DNA synthesis and cell cycle progression would also be applicable to this problem of heterogeneity of doubling time and proliferation potential. That is, the level of RRase could be predicted to fluctuate randomnly so that even young populations of cells would contain a certain fraction of cells which had low levels of RRase and could not initiate DNA synthesis. In addition the longer doubling times would arise because the time needed to reach the threshold level of RRase would vary from cell to cell.

In order to determine how RRase is involved in the control of senescence and cell cycle progression it would be valuable to measure the levels of $M_1$ and $M_2$, as well as the level of mRNA for each of these subunits during the in vitro passage of normal HDF. Such measurements should be possible when cDNA probes and monoclonal antibodies are available. It would also be very interesting to determine if the expression of $M_1$ or $M_2$ is altered in the hydroxyurea resistant variants which senesce at an accelerated rate.

It should also be noted that the involvment of RRase in the senescence of human diploid cells does not contradict the error or mutational theory of aging. RRase and the associated dNTP pools have recently been reported to contribute to the spontaneous mutation rates of cultured cells (Weinberg et al., 1981; Meuth et al., 1979; Chan et al., 1981). Cell lines which contain a genetically altered RRase, whose allosteric regulation is modified, contain dNTP pools which show large alterations in relative sizes compared to wild type cell lines. The mutation rates at selected loci are significantly increased in these mutant cell lines. It is interesting to note that DNA polymerase $\alpha$ has also recently been shown to have mutator functions in aphidicolin resistant cell lines (Liu et al., 1983) indicating that perturbations in two enzymes associated in a multi enzyme-complex can have severe consequences for the cell. It may be that the age-related changes in RRase activity and regulation may enhance the mutation rates during senescence. The dNTP pools are somewhat altered as HDF senesce in culture (Figure 33), however there is little evidence that the mutation rates are increased in high passage cells (Gupta, 1978).

## C. Characterization of CHO X HSC172 Somatic Cell Hybrids

One of the main objectives of this thesis was to characterize RRase in normal senescing HDF. Knowledge of the chromosomal location of the gene(s) for RRase would provide valuable information regarding the enzyme and its control in normal cells. The selection protocol to isolate hybrid cells containing the human gene for RRase was based on the inherent difference in sensitivity of human cells and CHO cells to hydroxyurea (Figure 53). Five independent clones which were isolated from different experiments were all three to four fold more resistant the hydroxyurea than the parental GATO$^R$-1 cells (Figure 54). Interestingly, the HSC 172 human parental line was also three to four fold more resistant than the CHO cell line; therefore the hybrid clones resembled the human cells in this regard. One of the clones GOW-2 was selected from a fusion between GATO$^R$-1 and the human drug resistant variant WC1-2. The relative resistance of this clone did not resemble WC1-2; rather it resembled the wild type human cells. In order to determine the nature of drug resistance in the five hybrid clones, the permeabilization assay was used to measure RRase activity. None of the clones showed higher levels of RRase activity than the CHO wild type cells; in fact some were slightly lower (Figure 55) probably reflecting the lower doubling times of these cells. There was a difference in the sensitivity of RRase to inhibition by hydroxyurea in each of these clones. CDP reductase from each of the clones was more resistant to inhibition by hydroxyurea than the CHO parental cells (Figure 55). The hybrid clone GOW-2 which was derived by fusion with the human overproducing variant did not contain elevated levels of enzyme but did exhibit an increase in the resistance of its RRase to inhibition by hydroxyurea. This suggests that the regulatory alteration which caused RRase to be overproduced in the variant human cells was not expressed in the hybrid. These data support the view that the human structural gene(s) for RRase is expressed in the hybrids conferring hydroxyurea resistance upon the cell line. One hybrid cell line HGAT-7 was selected in growth medium which contained ouabain and hydroxyurea and did not contain the GAT supplement. This hybrid probably retained the human gene which complemented the defect in folic acid metabolism in the CHO parental line. Subsequent experiments indicated that HGAT-7 could grow in the absence of GAT supplement while the parental $GATO^R$-1 cell line could not. This indicates that at least two human genes were probably expressed in HGAT-7, one which conferred hydroxyurea resistance and another which allowed the cell to grow in the absence of GAT supplement.

It was very surprising not to detect intact human chromosomes in any of the hybrid lines which were observed. These cells were generally grown in the presence of hydroxyurea to guard against random chromosome loss. However growth in hydroxyurea has been known to cause many severe chromosomal abnormalities (Oppenheim & Fishbein, 1965). A great deal of chromosomal damage was observed in these hybrid clones (Figures 63 & 64) including tri- and quadra-radial chromatid exchange, chromatid breaks, homogenously stained regions, dicentric chromosomes, and pulverized chromosomes. Each of the hybrid clones contained at least one rearranged chromosome which was not present in the CHO parental cells. This large degree of chromosomal instability may have caused a fragment of human DNA, containing the gene(s) for RRase, to become incorporated into the hamster genome. If in fact this is the case then these could be very valuable cell lines from which the gene(s) for human RRase could be isolated. Gusella et al., (1980) reported a methodology for carrying out such a purification using human x rodent hybrid cells which contained a small amount of human DNA. Small amounts of human DNA can be detected within a large excess of rodent DNA because of the high degree of species specificity in the middle repetitive sequences (Schmid & Deininger, 1975; Gusella et al., 1979; 1980). The middle repetitive sequences are evenly distributed throughout the human genome every two or three kilobases (Schmid & Deininger, 1975). It is important to unequivocally establish that these hybrid clones truly contain human DNA. This could be done by probing Southern blots (Southern, 1975) with either total $^{32}$P-nick translated human DNA (Cirullo et al., 1983) or DNA probes specific for human middle and highly repetitive DNA (Perucho et al., 1981). Large chromosomal fragments of human DNA which have been translocated into hamster chromosomes could also be detected by the alkaline - Giemsa differential staining techniques (Friend et al., 1976).

D. Summary

The experiments presented here provide for the first time, a general characterization of RRase from normal human diploid fibroblasts. A novel assay was developed which involved measuring enzyme activity in permeabilized cells. The advantage of this assay was the relatively low numbers of cells which were required for each determination. The permeabilization assay was used to investigate RRase from both low and high passage cells. These studies revealed marked decreases in enzyme activity in high passage cells providing a link between senescence and the activity of RRase. Detailed kinetic studies also indicated that enzyme regulation was altered in high passage cells. The enzyme from high passage cells was not inhibited by dATP to the same extent as low passage cells. Although the cause of this alteration is unknown it may be the result of changes in the interaction of the two subunits of RRase, $M_1$ and $M_2$. Drug resistant HDF were selected in hydroxyurea and found to overproduce RRase. Interestingly, these drug resistant variants appeared to senesce at an accelerated rate providing further support for the view that RRase is closely linked to cellular senescence. In addition to containing elevated levels of RRase, these variants were also found to contain a larger pool of dCTP and apparently a smaller dGTP pool. High passage human cells contained very low levels of dATP, dGTP, dCTP, and TTP, probably reflecting the drop in RRase activity. Overall the dNTP pools in the drug resistant cells appeared to be regulated in much the same age-related fashion as high passage wild type cells. RRase is a key regulatory protein in the cell and a great deal of work has closely linked this enzyme with the control of DNA synthesis. In light of this regulatory role in cell proliferation, the association of RRase and cellular senescence provides support for the intrinsic programmed or differentiation models of senescence, although it does not exclude the error models.

In addition to the age-related decrease in the deoxyribonucleotide pools a variety of changes were also observed in the ribonucleoside di- and tri-phosphate pools of cells at high passage number. There was a marked drop in the GDP pool in high passage cells. This reduction in the GDP pool appeared to affect the incorporation of mannose, via GDP-mannose, into cell surface membrane glycoproteins. Low passage cells incorporated twice as much mannose as high passage cells. Although the reason for this reduction in mannose incorporation is not known it may be that the GDP pool has become rate limiting in the biosynthesis of mannose containing oligosaccharides. It was not possible to identify the human chromosomes carrying the gene(s) for RRase using somatic cell hybridization techniques since no intact human chromosomes could be detected. The hybrid cell lines did contain a variety of chromosomal alterations which may have resulted in the translocation of a small piece of human DNA, containing the gene(s) for RRase, into the hamster chromosomes. These hybrid cell lines could be very useful for isolating the gene(s) for RRase since only a small amount of human DNA must be present. This small amount of human DNA represents a large initial purification of the gene(s) for human RRase making it be practical to screen the remaining human DNA for sequences which code for RRase.

# BIBLIOGRAPHY

# BIBLIOGRAPHY


Abercrombie, M. (1979). Nature 281: 259-262.

Absher, P. & Absher, R. (1976). Exp. Cell Res. 103: 247-255.

Absher, P., Absher, R. & Barnes, W. (1974). Exp. Cell Res. 88: 94-104.

Adelman, R. (1977) In, Handbook of the Biology of Aging, (Ed. Finch, C. & Hayflick, L.) Van Nostrand Reinhold, New York, pp. 63-72.

Aizawa, S., Mitsui, Y, Kurimoto, F. (1980). Exp. Cell Res. 125: 287-296.

Albertini, R. (1979). Banbury Report 2, Mammalian Cell Mutagenesis, pp 359-337.

Albertini, R. & DeMars, R. (1970). Sci. 169: 482-485.

Albertini, R. & DeMars, R. (1973). Mutat. Res. 18: 199-224.

Alitalo, K., Schwab, M., Lin, C., Varmus, H. & Bishop, J. (1983). Proc. Natl. Acad. Sci. U.S.A. 80: 1707-1711.

Alt, F., Kellems, R., Bertino, J., Schimke, R. (1978). J. Biol. Chem. 253: 1357-1370.

Ashman, C. & Davidson, R. (1981). Mol. Cell. Biol. 1: 254-260.

Ashman, C. & Davidson, R. (1981). Somat. Cell. Genet. 7: 751-768.

Ashman, C., Reddy, G. & Davidson, R. (1981). Somat. Cell. Genet. 7: 751-768.

Aubery, M., Guillouzo, A., Bernard, R. & Font, J. (1980). Exp. Cell Res. 129: 273-280.

Augenlicht, C. & Baserga, R. (1974). Exp. Cell Res. 89: 255-262.

Azencott, R., Hughes, R. & Courtois, Y. (1975). Adv. Exp. Med. Biol. 53: 147-155.

Bacchetti, S. & Whitmore, G. (1969). Cell Tissue Kinet. 2: 193-211.

Barlow, T., Eliasson, R., Platz, P., Reichard, P. & Sjoberg, B. M. (1983). Proc. Natl. Acad. Sci. U.S.A. 80: 1492-1496.

Bartholomew, J., Yohota, H. & Ross, P. (1976). J. Cell. Physiol. 88: 277-286.

Baserga, R. (1977). In, Handbook of the Biology of Aging (Ed. Finch, C. & Hayflick, L.) Van Nostrand, Reinhold, New York, pp. 101-121.

Beaulaton, J. & Lockshin, R. (1982). Int. Rev. Cytol. 79: 215-235.

Bell, E., Marek, L., Levinstone, D., Merrill, C., Sher, S., Young, I., & Eden, M. (1978). Science 202: 1158-1163.

Billen, D. & Olson, A. (1978). In, Methods in Cell Biology (Ed. Prescott, D. M.) Academic Press, New York, 20: 315-324.

Bowman, P. & Daniel, C. (1975). Mech. Aging Dev. 4: 147-158.

Bradley, W. (1979). J. Cell. Physiol. 101: 325-340.

Buchwald, M. (1977). Mutat. Res. 44: 401-412.

Buchwald, M. & Ingles, C. (1976). Somat. Cell. Genet. 2: 225-233.

Bunn, E. & Tarrant, G. (1980). Exp. Cell Res. 1: 385-396.

Burmer, G., Motulsky, H., Ziegler, C. & Norwood, T. (1983). Exp. Cell Res. 145: 79-84.

Burnet, F. (1974). Intrinsic Mutagenesis: A Genetic Approach to Aging. John Wiley & Sons, New York.

Caras, I., Jones, T., Eriksson, S. & Martin, D. (1983). J. Biol. Chem. 258: 3064-3068.

Carrel, A. (1912). J. Exp. Med. 15: 516-528.

Carrel. A. (1914). J. Exp. Med. 20: 1-2.

Carrel, A. (1935). Man the Unknown, Harper, New York, p. 173.

Castelott, J., Miller, M. & Pardee, A. (1978). Proc. Natl. Acad. Sci. U.S.A. 75: 337-355.

Chan, T. (1978). Cell 1: 523-530.

Chan, V., Guttman, S. & Juranka, P. (1981). Mol. Cell. Biol. 1: 568-571.

Chang, C.-H. & Cheng, Y.-C. (1979a). Cancer Res. 39: 436-442.

Chang, C.-H. & Cheng, Y.-C. (1979b). Cancer Res. 39: 5081-5086.

Chang, C.-H. & Cheng, Y.-C. (1979c). Cancer Res. 39: 5087-5092.

Chang, C.-H. & Cheng, Y.-C. (1980). Cancer Res. 40: 3555-3558.

Cherington, P., Smith, B. & Pardee, A. (1979). Proc. Natl. Acad. Sci. U.S.A. 76: 3937-3941.

Cirullo, R., Dana, S. & Wasmuth, J. (1983). Mol. Cell. Biol. 3: 892-902.

Cohen, A., Barankiewiez, J., Lederman, H. & Gelfand, (1983). J. Biol. Chem. 258: 12334-12340.

Comfort, A. (1979). The Biology of Senescence, Elsevier, New York.

Corsaro, C. & Migeon, B. (1976). Exp. Cell Res. 95: 47-53.

Cory, J. (1979). Adv. Enzyme Reg. 17: 115-131.

Cory, J. (1983). Pharmac. Ther. 21: 265-276.

Cory, J. & Fleischer, A. (1982). J. Biol. Chem. 257: 1263-1266.

Cory, J., Fleischer, A. & Munro, J. (1978). J. Biol. Chem. 253: 2898-2901.

Cory, J., Mansell, M. & Whitford, T. (1975). Adv. Enzyme Reg 14: 45-61.

Cory, J., Russell, F. & Mansell, M. (1983). Anal. Biochem. 55: 449-456.

Courtois, Y. & Hughes, R. (1974). Eur. J. Biochem. 44: 131-138.

Courtois, Y. & Hughes, R. (1976) Geront. 22: 371-379.

Cox, R. & Masson, W. (1974). Nature 252: 308-310.

Cox, R. & Masson, W. (1976). Mutat. Res. 36: 93-104.

Cox, D. & Ray, M. (1977). In, Handbook of Clinical Neurology (Vivhen, P. & Buyn, G., eds.) North Holland, Amsterdam, pp. 341-366.

Creasey, D., Parfett, C. & Wright, J. (1982). Proc. Can. Fed. Biol. Soc. 25: 172.

Creasey, D. & Wright, J. (1983). Bioscience Rep. (in press).

Cristofalo, V. (1970). In, Aging in Cell and Tissue Culture (Ed. Holeckova, E. & Cristofalo, V.) Plenum, New York.

Cristofalo, V. & Rosener (1979). Fed. Proc. 38: 1851-1586.

Cristofalo, V. & Sharf, B. (1973). Exp. Cell Res. 76: 419-427.

Croy, R. & Pardee, A. (1983). Proc. Natl. Acad. Sci. U.S.A. 80: 4699-4703.

Curtis, H. (1966). Biological Mechanisms of Aging. Thomas, Charles, C., Springfield, Illinois.

Davidson, R., O'Malley, K. & Wheeler, T. (1976). Somat. Cell. Genet. 2: 271-280.

Daniel, C. (1977). In, Handbook of The Biology of Aging (Ed. Finch, C. & Hayflick, L.) Van Nostrand Reinhold, New York. pp. 122-158.

Deaven, L. & Peterson, D. (1973). Chromosoma 41: 129-144.

Dell'Orco, R., Mertens, J. & Kruse, P. (1973). Exp. Cell Res. 77: 356-360.

DeMars, R. (1974). Mutat. Res. 24: 335-364.

DeMars, R. & Held, K. (1972). Human Genetic 16: 87-110.

De Pamphilis, M. & Wassarman, P. (1980). Ann. Rev. Biochem. 49: 627-666.

Dick, J. & Wright, J. (1980). J. Cell. Physiol. 105: 63-72.

Dick, J. & Wright, J. (1982). Mech. Aging Dev. 20: 103-109.

Dick, J. & Wright, J. (1984). Mech. Aging Dev. In press.

Dubrow, R., Riddle, V. & Pardee, A. (1979). Cancer Res. 39: 2718-2726.

Duncan, M., Dell'Orco, R. & Guthrie, P. (1977). J. Cell. Comp. Physiol. 93: 59-56.

Duthu, G., Braunscheweiger, K., Pereira-Smith, O., Norwood, T. & Smith, J. (1982). Mech. Aging Dev. 20: 243-252.

Earle, W. (1943). J. Nat. Cancer. Inst. 4: 165-212.

Ebeling, A. (1913). J. Exp. Med. 17: 273-285.

Elford, H. (1972). Adv. Enzyme Reg. 10: 19-38.

Elford, H., Freese, M., Passamani, E. & Morris, H. (1970). J. Biol. Chem. 245: 5228-5233.

Elford, H., Van't Riet, B., Wampler, G., Lin, A. & Elford, R. (1981). Adv. Enzyme Reg. 19: 151-168.

Engstrom, P., MacIntyre, J., Douglass, H., Muggia, F. & Mittelman, A. (1982). Cancer 49: 1555-1560.

Engstrom, Y., Eriksson, S., Thelander, L. & Akerman, M. (1979). Biochem. 18: 2941-2952.

Eriksson, S., Thelander, L., & Akerman, M. (1979). Biochemistry 18: 2948-2952.

Eriksson, S. & Martin, D. (1981). J. Biol. Chem. 256: 9436-9440.

Evans, H.& Vijayalaxmi (1981). Nature 292: 601-605.

Fernandez-Pol, J., Bono, V. & Johnson, G. (1977). Proc. Natl. Acad. Sci. U.S.A. 74: 2889-2893.

Finch, C. & Hayflick, L. eds. (1977). Handbook of The Biology of Aging (Van Nostrand Reinhold, New York).

Flintoff, W., Weber, M., Nagaimis, C., Essani, A., Robertson, D. & Salser, W. (1982). Mol. Cell. Biol. 2: 275-285.

Forsdyke, D. & Scott, F. (1980). In, Cell Compartmentation and Metabolic Channelling. (Nover, L., Lynen, F., Mother, K. ed.) Elsevier, North Holland, pp. 177-184.

Friend, C., Chen, S. & Ruddle, F. (1976). Somatic Cell. Genet. 2: 183-188.

Fry, M., Weisman-Shomer, P. (1976). Biochem. 15: 4319-4329.

Fujioka, S. & Silber, R. (1971). J. Lab. Clin. Med. 77: 59-64.

Fulder, S. (1979). Mech. Aging Dev. 10: 101-115.

Gale, G., Atkins, L., Meischen, S. & Schwartz, P. (1970). Cancer Treat. Rep. 63: 449-456.

Galloway, D., Goldstein, L. & Lewis, J. (1982). J. Virol. 42: 530-537.

Garrett, C. & Santi, D. (1979). Anal. Biochem. 99: 268-273.

Gershon, D., Reznick, A. & Reiss, U. (1979). In, Physiology and Cell Biology of Aging (Ed. Cherkin, A., et al.). Raven Press, New York. 8: pp. 21-26.

Gershon, D. & Gershon, H. (1976). Gerontol. 22: 212-219.

Goh, S., Wright, J., LeJohn, H. (1977). J. Cell. Physiol. 43:353-362.

Goldstein, S. (1971). Proc. Soc. Exptl. Biol. Med. 137: 730-734.

Goldstein, S., Wojtyk, R., Harley, C., Pollard, J., Chamberlain, J. & Stanners, C. (1979). In, Aging - Its Chemistry (Ed. Deitz, A.) American Association for Clinical Chemists, pp. 248-258.

Goldstein, S., Littlefield, J., Lolldner, J. (1969). Proc. Natl. Acad. Sci. U.S.A. 64: 155-160.

Gompertz, B. (1825). Phil. Trans. Roy. Soc. London. 513-585.

Graslund, A., Ehrenberg, A., Thelander, L. (1982). J. Biol. Chem. 257: 5711-5715.

Gupta, R. (1980). J. Cell. Physiol. 103: 209-216.

Gupta, R., Siminovitch, L. (1978a). Somat. Cell. Genet. 4: 553-571.

Gupta, R. & Siminovits, L. (1978b). Proc. Natl. Acad. Sci. U.S.A. 75: 3337-3340.

Gusella, J., Keys, C., Varsanyi-Breiner, A., Kao, F., Jones, C., Puck, T. & Housman, D. (1980). Proc. Natl. Acad. Sci. U.S.A. 77: 2829-2833.

Hards, R. & Wright, J. (1981). J. Cell. Physiol. 106: 309-319.

Hards, R. & Wright, J. (1983a). Can. J. Biochem. Cell. Biol. 61: 1210-1229.

Hards, R. & Wright, J. (1983b). Arch. Biochem. Biophys. 220: 576-583.

Hards, R. & Wright, J. (1984a). Arch. Biochem. Biophys. In press.

Hards, R. & Wright, J. (1984b). Arch. Biochem. Biophys. In press.

Harley, C. & Goldstein, S. (1978). J. Cell Physiol. 97: 509-515.

Harris, H. (1979). Somat. Cell. Genet. 5: 923.

Harrison, B. & Holliday, R. (1967). Nature 213: 990-992.

Harrison, D. (1972). Nature 237: 220-221.

Harison, D. (1973). Proc. Natl. Acad. Sci. U.S.A. 70: 3184-3188.

Harrison, D. (1978). In, The Biology of Aging (Ed. Behnke, J. & Moment, G.) Plenum Press, New York. pp. 33-55.

Harrison, R. (1907). Proc. Soc. Exp. Biol. and Med. 4: 140-143.

Hart, R. & Setlow, R. (1974). Proc. Natl. Acad. Sci. U.S.A. 71: 2169-2173.

Hart, R. & Setlow, R. (1976). Mech. Aging Dev. 5: 67-77.

Hartwick, R. & Brown, R. (1975). J. Chromatogr. 112: 651-662.

Hayflick, C. (1965). Exp. Cell Res. 37: 614-636.

Hayflick, L. (1977). In, Handbook of Aging (Ed. Finch C. & Hayflick, L.) Van Nostrand Reinhold, New York. pp.159-186.

Hayflick, L. (1979). Mech. Aging Dev. 9: 393-408.

Hayflick, L. (1980). Ann. Rev. Geront. and Geriat. 1: 26-67.

Hayflick, L. & Moorhead, P. (1961). Exp. Cell Res. 25: 585-621.

Holland, J., Kohne, D. & Doyle, M. (1973). Nature 245: 316-318.

Holliday, R. (1975). Fed. Proc. 34: 51-55.

Holliday, R. & Tarrant, G. (1972). Nature 238: 26-30.

Hopper, S. (1972). J. Biol. Chem. 247: 3336-3340.

Hughes, R. (1976a). Membrane Glycoproteins Butterworth, London. pp. 269-284.

Hughes, R. (1976b). Membranes Glycoproteins. Butterworth, London. pp. 285-308.

Hunting, D. & Henderson, J. (1982). CRC Crit. Rev. Biochem. 13: 325-348.

Hunting, D. & Henderson, J. (1982). Meth. Cancer Res. 20: 245-284.

Huszar, D. & Bacchetti, S. (1983). Nature, 302: 76-79.

Icard, C. & Beaupain, R. (1980). Mech. Aging Dev. 14: 81-87.

Johnson, R. Strehler, B. (1972). Nature 240: 412-414.

Kahn, A., Guillouzo, A., Liebovitch, M., Cottreau, D., Bourel, M. & Dreyfus, J. (1977). Biochem. Biophys. Res. Comm. 77: 760-766.

Kahn, A., Meienhofer, M., Guillouzo, A., Cottreau, D., Baffet, G., Henry, J. & Dreyfers, J. C. (1982). Geront. 28: 360-370.

Kakunaga, T. (1978). Proc. Natl. Acad. Sci. U.S.A. 75: 1334-1338.

Kaplan, H., Woloski, B. & Jamieson, J. (1984). Comp. Biochem. Physiol. In press.

Kay, J. (1965). Lancet. 13: 418-419.

Khym, J. (1966). Meth. Enzymol. 12A: 93-101.

Kornberg, A. (1980). DNA Replication. W. H. Freeman & Co., San Francisco.

Koropatnick, S. (1980). MSc. Thesis, University of Manitoba.

Koropatnick, S. & Wright, J. (1980). Enzyme 25: 220-227.

Krebs, C. & Beavo, J. (1979). Ann.Rev. Biochem. 48: 923-959.

Krohn, P. (1962). Proc. Roy. Loc. (London) Ser. B., 157: 128-147.

Kunkel, T. & Loeb, L. (1979). J. Biol. Chem. 254: 5718-5725.

Kunkel, T. & Soeb, C. (1980). J. Biol. Chem. 255: 9961-9966.

Kuo, P. & Hogenkamp, P. (1980). J. Biol. Chem. 255: 1273-1278.

Kuzik, B. & Wright J. (1979). Enzyme 24: 285-293.

Kuzik, B. & Wright, J. (1980). Biochem. Genet. 18: 311-331.

Lajtha, L. G. (1963). J. Cellular Comp. Physiol. 62: suppl. 1, 143-145.

Lampidis, T. & Schaiberger, G. (1975). Exp. Cell Res. 96: 412-416.

Lavappa, K. Macy, M. & Shannon, J. (1976). Nature 259: 211-213.

Lavi, S. (1982). J. Cell. Biochem. 18: 149-156.

Lerner, H., Beckloff, G. & Godwin, M. (1969). Cancer Chemther. Rep. 53: 385-395.

Lewis, W., Kuzik, B. & Wright, J. (1978). J. Cell. Physiol. 94: 287-298.

Lewis, W. McNaughton, D., Goh, S., LeJohn, J. & Wright, J. (1977). J. Cell. Physiol. 93: 345-352.

Lewis, W., McNaughton, D., LeJohn, H., Wright, J. (1976). Biochem. Biophys. Res. Comm. 71: 128-135.

Lewis, W. & Wright, J. (1974). Biochem. Biophys. Res. Chomm. 60: 926-933.

Lewis, W. & Wright, J. (1978a). J. Cell. Physiol. 97: 73-86.

Lewis, W. & Wright, J. (1978b). J. Cell. Physiol. 97: 87-98.

Lewis, W. & Wright, J. (1979). Somat. Cell. Genet. 5: 83-96.

Lima, L., Malaise, C. & Macilira-Coelho, A. (1972). Exp. Cell Res. 73:345-350.

Lindahl, T. (1982). Ann. Rev. Biochem. 51: 61-87.

Linn, S., Kairis, M. & Holliday, R. (1976). Proc. Natl. Acad. Sci. U.S.A. 23: 2818-2822.

Lui, P. Chang, C. C., Trosko, J., Dube, D. Martin, G. & Loeb, L. (1983). Proc. Natl. Acad. Sci. U.S.A. 80: 797-801.

Luthman, M., Eriksson, S., Holmgren, A. & Thelander, L. (1979). Proc. Natl. Acad. Sci. U.S.A. 76: 2158-2162.

Maciera-Coelho, A. (1974). Nature 248: 421-422.

Maciera-Coelho, A., Diatloff, C., Billard, M., Fertil, B., Malaise, E. & Fries, D. (1978). J. Cell. Physiol. 95: 235-238.

Makinodan, T. (1977). In, The Handbook of the Biology of Aging. (Finch, C. & Hayflick, L., eds.) Van Nostrand Reinhold, New York pp 379-408.

Mankovitz, R., Buchwald, M. & Baker, R. (1974). Cell 3: 221-226.

Martin, G. (1977). Am. J. Pathol. 89: 484-512.

Martin, G., Sprague, C. & Epstein, C. (1970). Lab. Invest. 23: 86-92.

Martin, D. & Gelfand, E. (1981). Ann. Rev. Biochem. 50: 845-877.

Matsumura, T., Pferdt, E. & Hayflick, L. (1979). J. Gerontol. 34: 323-327.

Mattaliano, R., Sloan, A., Plumer, E. & Klippenstein, G. (1981). Biochem. Biophys. Res. Comm. 102: 667-674.

McBurney, M. & Whitmore, G. (1974). Cell 2: 173-182.

McKusick, V. & Ruddle, F. (1977). Science 196: 390-405.

Medrano, C. & Pardee, A. (1980). Proc. Natl. Acad. Sci. U.S.A. 77: 4123-4126.

Medvedev, Z. (1972). Exp. Gerontol. 7: 227-238.

Merz, G. & Ross, J. (1969). J. Cell. Physiol. 74: 219-222.

Meuth, M. (1981). Mol. Cell. Biol. 1: 652-660.

Meuth, M., L'Heureux-Huard, N. & Trudel, M. (1979). Proc. Natl. Acad. Sci. U.S.A. 76: 6505-6509.

Millard, S. (1972). J. Biol. Chem. 247: 2395-2400.

Milo, G. & Hart, R. (1976). Arch. Biochem. Biophys. 176: 324-333.

Moment, G. (1978). In, The Biology of Aging (Behnke, J., Finch, C. & Moment, G. eds.) Plenum Press, New York, pp. 1-17.

Moore, E. (1969). Cancer Res. 29: 291-295.

Moran, R. & Straus, M. (1980). Cancer Treat. Rep. 64: 81-86.

Morley, A., Cox, S., Holliday, R. (1982). Mech. Aging Dev. 19: 21-26.

Morrow, J. (1977). Mutat. Res. 44: 391-400.

Morrow, J. & Garner, C. (1979). Geront. 25: 136-144.

Moses, H., Proper, J., Volhenant, J., Wells, D. & Getz, M. (1978). Cancer Res. 38: 2807-2812.

Muggleton-Harris, A. & DeSimone, D. (1980). Somat. Cell. Genet. 6: 689-699.

Muggleton-Harris, A. & Hayflick, L. (1978). Exp. Cell Res. 103: 321-330.

Muggleton-Harris, A. & Palumbo, M. (1979). Somat. Cell. Genet. 5: 397-407.

Muller, W., Zahn, R., Guertsen, W. & Munsch, N. (1980). Mech. Aging Dev. 13: 119-126.

Murphee, S., Stubblefield, E. & Moore, E. (1969). Exp. Cell Res. 58: 118-124.

Namba, M., Nishitani, K. & Kimoto, T. (1978). Japan. J. Exp. Med. 48: 303-311.

Newbold, R. & Overall, R. (1983). Nature 304: 648-651.

Nicander, B. & Reichard, P. (1981). Biochem. Biophys. Res. Comm. 103: 148-155.

Norwood, T., Pendergrass, W. & Martin, G. (1975). J. Cell Biol. <u>64</u>: 551-556.

Norwood, T., Pendergrass, W., Sprague, C. & Martin, G. (1974). Proc. Natl. Acad. Sci. U.S.A. <u>71</u>: 2231-2235.

Oppenheim, J. & Fishbein, W. (1965). Cancer Res. <u>25</u>: 980-985.

Ord., M. (1979). Internat. Rev. Cytol. (Suppl.) <u>9</u>: 222-278.

Orgel, L. (1963). Proc. Natl. Acad. Sci. U.S.A. <u>49</u>: 517-521.

Orgel, L. (1970). Proc. Natl. Acad. Sci. U.S.A. <u>67</u>: 1476.

Orgel, L. (1973). Nature <u>243</u>: 441-445.

Painter, R., Clarkson, J. & Young, B. (1973). Radat. Res. <u>56</u>: 560-564.

Pall, M. (1981). Proc. Natl. Acad. Sci. U.S.A. <u>78</u>: 2465-2468.

Panagou, D. On, M., Dunstone, J. & Blakely, R. (1972). Biochemistry <u>11</u>: 2378-2388.

Pardee, A. (1974). Proc. Natl. Acad. Sci. U.S.A. <u>71</u>: 1286-1290.

Pardee, A., Dubrow, R., Hanlin, J. & Kletzien, R. (1978). Ann. Rev. Biochem. <u>47</u>: 715-50.

Parfett, C., Jamieson, J. & Wright, J. (1983). Exp. Cell Res. <u>144</u>: 405-415.

Patterson, M. (1979). Meth. Enzymol. <u>58</u>: 141-152.

Paul, D., Henahan, M. & Walter, S. (1974). J. Natl. Cancer Inst. <u>53</u>: 1499-1503.

Paul, D. & Ristow, H. (1979). J. Cell. Physiol. <u>98</u>: 31-40.

Pendergrass, W., Martin, G. & Borrstein, P. (1976). J. Cell. Physiol. <u>87</u>: 3-14.

Pendergrass, W., Saulewicz, A., Burmer, G., Rabinovitch, P., Norwood, T. & Martin, G. (1982). J. Cell. Physiol. <u>113</u>: 141-151.

Pereira-Smith, O. & Smith, J. (1983). Science <u>221</u>: 964-966.

Perucho, M., Goldbarb, M., Shimizu, K., Lamo, C., Fogh, J. & Wrigler, M. (1981). Cell <u>27</u>: 467-476.

Peterson, D. & Moore, E. (1976). Biochem. Biophys. Acta <u>432</u>: 80-91.

Puck, T., Cieciura, S. & Robinson, A. (1958). J. Exp. Med. <u>108</u>: 945-956.

Rabinovitch, P. (1983). Proc. Natl. Acad. Sci. U.S.A. <u>80</u>: 2951-2955.

Rabinovitch, P. & Norwood, T. (1980). Exp. Cell Res. <u>130</u>: 101-109.

Rao, R. and Johnson, R. (1970). Nature <u>225</u>: 159-164.

Rao, P., Wilson, B. & Sunkara, P. (1978). Proc. Natl. Acad. Sci. U.S.A. <u>75</u>: 5043-5047.

Rappaport, H. & DeMars, R. (1973). Genetics <u>75</u>: 335-345.

Rauscher III, R. & Cadman, E. (1983). Cancer Res. <u>43</u>: 2688-2693.

Reddy, G. & Pardee, A. (1980). Proc. Natl. Acad. Sci. U.S.A. <u>77</u>: 3312-3316.

Reddy, G. & Pardee, A. (1982). J. Biol. Chem. <u>257</u>: 12526-12531.

Reddy, G. & Pardee, A. (1983). Nature <u>304</u>: 86-88.

Reichard, P. (1978). Fed. Proc. <u>37</u>: 9-14.

Reichard, P. & Ehrenberg, A. (1983). Science <u>221</u>: 514-519.

Rosen, R. (1978). Internat. Rev. Cytol. <u>54</u>: 162-191.

Rothstein, M. (1975). Mech. Aging Dev. <u>4</u>: 325-338.

Ryan, J., Ouda, G. & Cristofalo, V. (1974). J. Geront. <u>29</u>: 616-621.

Sacher, G. (1977). In, <u>Handbook of the Biology of Aging</u>. (C. E. Finch & L. Hayflick, eds.) Van Nostrand Reinhold Co., New York, pp. 582-638.

Sato, A. & Cory, J. (1981). Bioscience Rep. 1: 627-633.

Schioffonati, L. & Baserga, R. (1977). Cancer Res. 37: 541-545.

Schmid, C. & Deininger, P. (1975). Cell 6: 345-358.

Schneider, E. & Matsui, Y. (1976). Proc. Natl. Acad. Sci. U.S.A. 73: 3584-3588.

Schwartz, A. (1975). Exp. Cell Res. 94: 445-447.

Schwartz, A. & Moore, C. (1979). Fed. Proc. 38: 1989-1992.

Segal, I. (1975). Enzyme Kinetics, John Wiley & Sons, Toronto.

Shih, C. & Weinberg, R. (1982). Cell 29: 161-169.

Shmookler Reis, R. & Goldstein, S. (1979). Cell 21: 739-749.

Shmookler Reis, R., Goldstein, S. & Harley, C. (1980). Mech. Aging Dev. 13: 393-395.

Shock, N. (1977). In, Handbook of the Biology of Aging, (C. Finch & L. Hayflick, eds.) Van Nostrand Reinhold, New York, p. 639-665.

Siminovitch, L. (1976). Cell 7: 1-11.

Siminovitch, L., Till, J. & McCulloch, E. (1964). J. Cellular Comp. Physiol. 64: 23-32.

Simons, J. (1967). Exp. Cell Res. 45: 336-350.

Sinclair, W. (1981). Int. J. Radiat. Oncol. Biol. Phys. 7: 631-637.

Singh, D., Tamao, Y. & Blakely, R. (1977). Adv. Enzyme Regul. 15: 81-100.

Skoog, L. Nordenskjold, B. & Bjursell, K. (1973). Eur. J. Biochem. 33: 428-432.

Smith, C. & Hanawalt, P. (1976). Biochem. Biophys. Acta, 447: 121-131.

Smith, J. & Hayflick, L. (1974). J. Cell Biol. 62: 48-53.

Smith, J. A. & Martin, L. (1973). Proc. Natl. Acad. Sci. U.S.A. 70: 1263-1267.

Smith, J. & Lumpkin, C. (1980). Mech. Aging Dev. 13: 387-392.

Smith, J., Pereira-Smith, O. & Schneider, E. (1978). Proc. Natl. Acad. Sci. U.S.A. 75: 1353-1357.

Smith, J. & Whitney, R. (1980). Science 207: 82-84.

Spyrou, G. & Reichard, P. (1983). Biochem. Biophys. Res. Comm. 115: 1022-1026.

Stanbridge, E. Der, C., Doerson, C. Nishim, R., Peehl, D., Weissman, B. & Wilkinson, J. (1982). Science, 215: 252-259.

Stanners, C. & Till, J. (1960). Biochem. Biophys. Acta 37: 406-419.

Stark, G. & Wahl, G., 1984. In press. Ann Rev. Biochem.

Steeper, J. & Steuart, C. (1970). Anal. Biochem. 34: 123-130.

Stein, G. & Yanishevsky, R. (1979). Exp. Cell Res. 120: 155-165.

Stein, G. & Yanishevsky, R. (1980). J. Supramol. Struct. Suppl. 4: 152.

Stein, G. & Yanishevsky, R. (1981). Proc. Natl. Acad. Sci. U.S.A. 78: 3025-3029.

Stein, G. Yanishevsky, R., Gordon, L. & Beeson, M. (1982). Proc. Natl. Acad. Sci. U.S.A. 79: 5287-5291.

Strauss, G. & Albertini, R. (1979). Mut. Res. 61: 353-379.

Stehler, B. (1977). Time, Cells, and Aging, (Academic Press, New York).

Takeda, E. & Weber, G. (1981). Life Sciences 28: 1007-1014.

Theiss, J. & Fischer, G. (1976). Biochem. Pharm. 25: 73-79.

Thelander, L. (1967). J. Biol. Chem. 242: 852-859.

Thelander, L. & Reichard, P. (1979). Ann. Rev. Biochem. 48: 133-158.

Thompson, L. (1979). Meth. Enzymol. 58: 308-322.

Tijan, R., Fey, G. & Graessmann, A. (1978). Proc. Natl. Acad. Sci. U.S.A. 75: 1279-1283.

Till, J., McCulloch, E. & Siminovitch, L. (1964). J. Natl. Cancer Inst. 33: 707-720.

Timson, J. (1975). Mutat. Res. 32: 115-132.

Todaro, G. & Green, H. (1963). J. Cell. Biol. 17: 299-313.

Tomkins, G., Stanbridge, E. & Hayflick, L. (1974). Proc. Soc. Exp. Biol. Med. 146: 385-390.

Tyrsted, G. & Gamulin, V. (1979). Nucleic Acids Res. 6: 305-319.

Ullman, B., Cohen, A. & Martin, D. (1976). Cell 9: 205-211.

Van't Riet, B., Wampler, G. & Elford, H. (1979). J. Med. Chem. 22: 589-592.

Van Zeeland, A. & Simons, J. (1976). Mutat. Res. 34: 149-158.

Varshavsky, A. (1983). Cell 34: 711-712.

Wahl, G., Padgett, R. & Stark, G. (1979). J. Biol. Chem. 254: 8679-8689.

Wallach, D. (1975). Membrane molecular biology of neoplastic cells. Elsevier, Amsterdam.

Walters, R., Tobey, R. & Hildebrand, C. (1976). Biochem. Biophys. Res. Comm. 69: 212-217.

Walters, R. Tobey, R. & Ratliff, R. (1973). Biochem. Biophys. Acta 319: 336-349.

Walton, J. (1982). Mech. Aging Dev. 19: 217-244.

Weber, G. (1977a). N. Engl. J. Med. 296: 486-493.

Weber, G. (1977b). N. Engl. J. Med. 296: 541-551.

Weber, G. (1983). Cancer Res. 43: 3466-3492.

Weber, G., Olah, E., Denton, J., Lui, M., Takeda, E., Tzeng, D. & Ban, J. (1981). Adv. Enzyme Reg. 19: 87-102.

Weinberg, G. Ullman, B. & Martin, D. (1981). Proc. Natl. Acad. Sci. U.S.A. 78: 2447-2451.

Whatley, S. & Hill, B. (1980). Geront. 26: 138-154.

Wickremasinghe, G. & Hoffbrand, A. (1983). Fed. Eur. Biol. Soc. 159: 175-179.

Williamson, A. & Askonas, B. (1972). Nature 238: 337-339.

Wojtyk, R. & Goldstein, S. (1980). J. Cell,Physiol. 103: 299-303.

Wojtyk, R. & Goldstein, S. (1982). J. Cell Biol. 95: 704-710.

Worton, R., Ho, C. & Duff, C. (1977). Somat. Cell. Genet. 3: 27-45.

Worton, R. & Duff, C. (1979). Meth. Enzymol. 58: 322-344.

Wright, J. (1979). Int. J. Biochem. 10: 951-956.

Wright, J. (1983). Pharmac. Ther. 22: 81-102.

Wright, J. & Cory, J. (1983). Bioscience Rep. 3: 741-748.

Wright, J., Hards, R. & Dick, J. (1981). Adv. Enzyme Regul. 19: 105-127.

Wright, J., Jamieson, J. & Ceri, H. (1979). Exp. Cell Res. 121: 1-8.

Wright, J., Lewis, W. & Parfett, C. (1980). Can. J. Genet. Cytol. 22: 443-496.

Yamamoto, K., Yamamoto, M. & Ooka, H. (1977). Exp. Cell Res. 108: 87-93.

Yanishevsky, R. & Stein, G. (1980). Internat. Rev. Cytol. 69: 223-259.

Youdale, T., MacManus, J. & Whitfield, J. (1982). Can. J. Biochem. 60: 463-470.

Yu, C. & Sinclair, W. (1968). J. Cell. Physiol. 72: 39-42.