THE UNIVERSITY OF MANITOBA

NEOPLASTIC PROGRESSION, CELLULAR DIFFERENTIATION AND RIBONUCLEOTIDE REDUCTASE: STUDIES WITH WILD TYPE AND HYDROXYUREA-RESISTANT MAMMALIAN CELL LINES

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DAVID CHARLES CREASEY

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DAVID CHARLES CREASEY

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TO

MALGORZATA

Who made me believe in myself

Together we paid the ultimate price

It was not worth it

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ABBREVIATIONS

α-MEM	alpha minimal essential medium
ADM	adriamycin
ADP	adenosine 5'-diphosphate
AdR	2'-deoxyadenosine
Ap^4A	diadenosine tetraphosphate
ara-A	1-β-D-arabinofuranosyl adenine
ara-C	1-β-D-arabinofuranosyl cytosine
ATP	adenosine 5'-triphosphate
AUFS	absorbance units full scale
5-azaC	5-azacytidine
BUDR	bromodeoxyuridine
cAMP	3',5'-cyclic adenosine monophosphate
CAT	catalase
CDP	cytidine 5'-diphosphate
CdR	2'-deoxycytidine
CHO	Chinese hamster ovary
conA	concanavalin A
CPK	creatine phosphokinase
CRABP	cellular retinoic acid binding protein
CS	calf serum
CTP	cytidine 5'-triphosphate
D_{10}	drug concentration which reduces RPE to 10%

dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
CdR or dC	2'-deoxycytidine
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
DEPC	diethylpyrocarbonate
DES	diethylstibestrol
DMBA	dimethylbenzanthracene
DMSO	dimethylsulfoxide
DNA POL	DNA polymerase alpha
dNTP	deoxynucleoside triphosphate
dpm	disintegrations per minute
DTT	dithiothreitol
EC	embryonal carcinoma
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
Fus+	fusion competent (wild type)
Fus-	fusion defective
FBS	foetal bovine serum
GDP	guanosine 5'-diphosphate
GdR	2'-deoxyguanosine
GSH	reduced glutathione or golden Syrian hamster
GSSG	oxidized glutathione
GTP	guanosine 5'-triphosphate
HBS	HEPES buffered saline

HDF	human diploid fibroblast
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	high pressure liquid chromatography
HPRT	hypoxanthine phosphoribosyl-transferase
H^R	hydroxyurea-resistant
HSV	herpes simplex virus
IAP	intracisternal particle
IBMX	1-isobutyl 3-methylxanthine
LTR	long terminal repeat
3-MC	3-methylcolanthrene
M1	effector-binding subunit of RRase
MMS	methylmethane sulphonate
MMTV	murine mammary tumor virus
MNNG	N-methyl-N'-nitro-N-nitroso-guanidine
$NADP^+$	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NC^R	N-carbamoyloxyurea-resistant
NCU	N-carbamoyloxyurea
ND	not determined
NDP	nucleoside diphosphate
NK	natural killer
OD	optical density
ODC	ornithine decarboxylase

OUA	ouabain
PBS	phosphate buffered saline
PCA	perchloric acid
PDL	population doubling level
PE	plating efficiency
PHA	phytohemmaglutinin
PMA	phorbol myristate acetate
RA	retinoic acid
RPE	relative plating efficiency
rpm	revolutions per minute
RRase	ribonucleotide reductase
S.C.	subcutaneous
SHE	Syrian hamster embryo
SOD	superoxide dismutase
$Temp^s$	temperature sensitive
TdR	thymidine
6-TG	6-thioguanine
thioredoxin $(SH)_2$	oxidized thioredoxin
TK	thymidine kinase
TnOA	tri-n-octylamine
TPA	12-O-tetradecanoylphorbol-13-acetate
UDP	uridine 5'-diphosphate
UTP	uridine 5'-triphosphate
UV	ultraviolet
V_{max}	maximum velocity

WGA	wheat germ agglutinin
WT	wild-type
ZTP	5-amino-4-imidazolecarboxamide riboside triphosphate

ABSTRACT

The progression of the neoplastic state may be considered as the negative diversion of the developmental pathway normally exhibited by mammalian cells. The interrelationship between transformation and differentiation is complex and poorly understood. Ribonucleotide reductase, whose activity is apparently rate-limiting for the replication of the hereditary material in mammalian organisms, has been described as a progression-linked enzyme in neoplasia and also as the transforming principle of herpes simplex virus 2. Elevations in this activity are closely linked with the expression of the neoplastic state. The results of the experiments described in this thesis demonstrate, for the first time, that alterations in ribonucleotide reductase activity are significantly associated with alterations in morphological and biochemical differentiation competence in mammalian cells. Hydroxyurea, an antineoplastic drug which targets this enzyme, has been used to select several drug-resistant, variant clones from differentiation competent, immortal muscle cell lines. The hydroxyurea-resistance phenotype of these variants was found to correlate strongly with defective myogenesis, elevated ribonucleotide reductase activity and elevated deoxyribonucleoside triphosphate pools. Concomitantly with these changes, the variants displayed statistically significant increases in anchorage-independent growth, increased tumor growth rates in nude mice and decreased tumor latencies. The examination of many myoblast cell lines has indicated that neither defective differentiation nor simple exposure to hydroxyurea were sufficient for the changes in the transformation associated characteristics observed with these cells. Studies with other rodent cell types revealed a statistically significant decrease in the tumor

latencies exhibited by hydroxyurea-resistant mouse cells with elevated ribonucleotide reductase activity. Another mouse cell type exhibited defective adipogenic-competence and ablation of tumorigenicity accompanying resistance to the drug and elevations in enzyme activity. Highly tumorigenic hamster cell lines exhibited no changes in tumorigenicity accompanying their altered ribonucleotide reductase activity and hydroxyurea-resistance. These combined results strongly suggest that acquisition of the hydroxyurea-resistance phenotype uncouples or misregulates growth and differentiation in a variety of cell systems through changes in the activity of ribonucleotide reductase. The acceleration of tumorigenicity exhibited by the hydroxyurea-resistant myoblast variants is strikingly similar to that observed with other immortal rodent cell lines expressing activated oncogenes. It appears that elevated ribonucleotide reductase activity is not simply linked with the transformed state as suggested by past studies, but may itself promote the state of neoplastic transformation in a manner characteristic of known oncogenes. Thus it appears that ribonucleotide reductase has transforming potential.

The discovery that hydroxyurea-resistant rodent cell lines with elevated ribonucleotide reductase activity are cross-resistant to the cytotoxic effects of the male antifertility drug gossypol is described for the first time in this thesis. This observation has recently been substantiated by the finding that gossypol is a potent inhibitor of the enzyme in rodent cell extracts. These results stongly suggest for the first time that the intracellular target of gossypol responsible for its antiproliferative and contraceptive effects in humans is the enzyme ribonucleotide reductase. This finding may have important implications for the routine use of this agent in humans.

HISTORICAL REVIEW

Ribonucleotide Reductase

Ribonucleotide reductase (EC 1.17.4.1) is a multisubunit, allosterically regulated enzyme common to all animal systems that reduce ribonucleotides to deoxyribonucleotides required for the duplication and preservation of genetic information. The reductive activities of this enzyme are absolutely required for growth and cell division, as elegantly demonstrated by Fuchs et al (1972) in temperature-sensitivie mutant E. coli cells defective in DNA synthesis by virtue of an alteration in the dnaF gene encoding the bacterial reductase. In addition to its obvious place of importance in DNA synthesis, ribonucleotide reductase is believed to play a role in other important biological processes, including immunodeficiency diseases (Ullman et al 1979), differentiation (Creasey and Wright 1984), neoplastic transformation (Takeda and Weber 1981, Huszar and Bachetti 1983, Galloway and McDougall 1983, Roguska and Gudas 1984) and aspects of cell cycle progression (Bjursell and Reichard 1973, Eriksson et al 1984a).

Classes of Ribonucleotide Reductase

Studies of the enzyme in partially purified preparations from a number of different organisms have revealed several different types of the enzyme, classified according to the substrate specificity and cofactor dependence of the preparations. The most commonly studied form of RRase is that of E. coli and mammalian systems, which although quite distinct from each other, share a number of properities in common, including the utilization of nucleoside diphosphate substrates, allosteric regulation by nucleoside and deoxynucleoside triphosphates, a

strict requirement for iron and separability into two components (B1 and B2 in E. coli, M1 and M2 in mammalian cells), one regulatory (B1, M1) and the other containing non-heme iron and a tyrosyl free radical when active (B2, M2). Both subunits are necessary for catalytic activity. Unlike the E. coli-type of RRase, the enzyme from Lactobacillus leichmannii (Beck and Hardy 1965) and Euglena gracillus (Gleason and Hogenkamp 1970, Hamilton 1974) utilizes a nucleoside triphosphate substrate and requires 5'-deoxyadenosylcobalamin for activity. A variety of cyanobacteria exhibit this type of RRase activity (Gleason and Wood 1976). A third type of ribonucleotide reductase is typified by nucleoside diphosphate substrate utilization and 5'-deoxyadenosylcobalamin dependence, as occurs in Bacillus megaterum and Corynebacterium nepheidii (Hogenkamp 1984).

Structure

A clear picture of the structure of RRase has been prevented by difficulties encountered in its purification and its inherent instability in vitro. An understanding of the physiologically significant structure of the enzyme is further complicated by its potential involvement in a multi-enzyme complex during DNA replication in vivo.

Involvement in the replitase

Recent evidence from Pardee's laboratory has suggested that ribonucleotide reductase is part of a macromolecular complex involved in the duplication of DNA (Reddy and Pardee 1980). The components of this multienzyme complex, DNA polymerase, ribonucleotide reductase, thymidine kinase, NDP kinase, dihydrofolate reductase and thymidylate synthase (termed the 'replitase') can be found cosedimenting with each other in

S-phase cells, but not in quiescent cells. These authors have even suggested that the assembly of this complex may signal the inititation of S-phase of the cell cycle. Further evidence for the existence of such a complex has been provided by these authors in a study in which the rate-limiting nature of RRase in DNA synthesis was recognized (Reddy and Pardee 1982). Lysolecithin-permeabilized Chinese hamster embryo cells effectively incorporated radio-labelled CDP (reduced to dCDP by RRase) into DNA in a manner refractile to dilution by added dCTP (the ultimate substrate of the polymerase), suggesting that the ribonucleoside diphosphates were channeled directly through RRase to DNA polymerase, in a tightly coupled complex reaction sequence. Similar results have been obtained with soluble extracts (Noguchi, Reddy and Pardee 1983). This 'channeling' hypothesis has recently been questioned, however. Spyrou and Reichard (1983) have presented evidence suggesting that radio-labelled CDP believed to be incorporated into DNA, was actually being incorporated into RNA ineffectively hydrolyzed during the purification of DNA by Pardee's group. Chiba, Bacon and Cory (1984) have shown that unlike the CHEF18 cells used in Pardee's laboratory, leukemia L1210 cells displayed decreased [^{14}C] cytidine incorporation into DNA in the presence of cold deoxycytidine.

Additional evidence in favor of the replitase model has been provided by the apparent restriction of the catalytic activity of thymidylate synthase to S-phase, and to association with the replitase complex (Reddy 1982). Furthermore, inhibition of DNA polymerase α, ribonucleotide reductase and/or topoisomerase with either aphidocolin, hydroxyurea or novobiocin, results in the simultaneous inhibition of thymidylate synthase in intact, S-phase cells, but not in soluble extracts. These results suggest a physical, allosteric interaction

between the components of the replitase, and are further supported by studies involving 3-aminobenzamide inhibition of MMS induced DNA repair, where both thymidylate synthase activity and DNA replication are blocked (Boorstein and Pardee 1983). Chiba et al (1984) however did not observe similar results in their L1210 cell system. Furthermore, the apparent indirect inhibition of thymidylate synthase by hydroxyurea or aphidocolin via RRase and DNA POLα may be due to perturbations in thymidine nucleotide pools (Rode et al 1980, Rode, Jastreboff and Bertino 1985). Although an ever increasing body of evidence appears to be casting doubt on the validity of the replitase and its ability to channel nucleotides to the replication fork, evidence is also accumulating in favor of such a complex in other cell systems such as human lymphoblastoid cells (Wickremasinghe et al 1983) and yeast (Jazwinski and Edelman 1984, Sclafani and Fangman 1984).

Studies with purified enzyme

Many different studies have examined the molecular weight of RRase and its individual components. Thelander's group has purified M1 to homogeneity and report a molecular weight of 84,000 (Thelander et al 1980). Mattaliano et al (1981) have reported the purification of M1 and M2 to homogeneity from calf thymus and report molecular weights of 84,000 and 58,000 respectively, by SDS-PAGE. These authors found that the native form of M1 is principally a monomer, whereas native M2 is found as a dimer (or trimer). Cory and Fleischer (1982a) have exmained the molecular weights of RRase and its components in Ehrlich tumor cells by sedimentation equilibruim and reported a weight of 304,000 and 254,000 for intact enzme reducing CDP and ADP respectively. Moreover, these authors suggest that the association of M1 and M2 is not equi-

molar, that the composition is alterable by nucleotide effectors and that different species of RRase catalyze the reduction of ADP and CDP. Youdale et al (1982) have reported that the RRase of rat liver shows a monomeric weight for M1 of 45,000 by HPLC and SDS-PAGE, whereas M2 was 120,000 by HPLC, but separable into 75,000 and 45,000 molecular components by SDS-PAGE.

Recently, Thelander et al (1985) have elegantly exploited the characteristic of hydroxyurea-resistant mammalian cells in culture to overproduce the M2 component of RRase, and the cross-reactivity of antitubulin antibody to this component, to purify M2 to homogeneity. The anti α-tubulin antibody YL 1/2 is able to bind M_2 by virtue of a common C-terminal amino acid sequence (Asp-Phe) present in both of these proteins (Standart et al 1985). They have reported that the pure protein is a dimer of molecular weight 88,000, containing stoichiometric amounts of non-heme iron and a tyrosyl free radical.

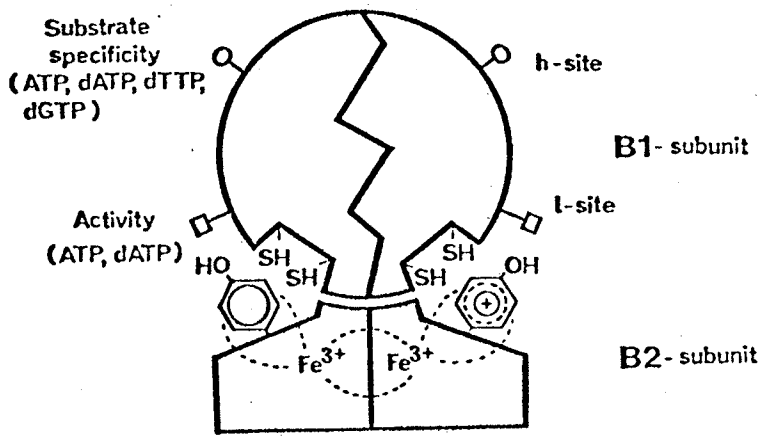
Studies with M1 have now been extended to the DNA level with the recent cloning of the M1 gene from S49 mouse T-lymphoma cells (Caras et al 1985, in press). The gene coding region (2.4 kilobases) predicts a protein of molecular weight 90,234 and 792 amino acid residues. Primary amino acid sequence comparison between this putative protein, bacterial and viral sequences has revealed an interesting pattern of conservation. The N-terminal domain is apparently common to the bacterial and mammalian enzymes, whereas the C-terminal domain is common to the mammalian and viral M1 proteins. All three species of M1 (viral, bacterial, mammalian) apparently share a common central domain.

Many studies have provided evidence for separate and distinct ribonucleotide reductase activities (Youdale and MacManus 1979). Morita et al (1984) have reported the separation of CDP reductase activity from

that of UDP, ADP and GDP. Collins et al (1972) have reported on a similar phenomenom in regenerating rat liver and have documented the preferential inhibition of CDP versus ADP reduction with actinomycin D.

Support for the distinctive natures of ribonucleotide reductase activities is also provided by a relative insensitivity of UDP reductase activity to hydroxyurea (Blocker and Roth 1977, Murayama et al 1985), indicating that separate enzymes may catalyze the reduction of purine and pyrimidine ribonucleotides. The observation of Cory and Mansell (1975) that unequal ratios of CDP and ADP reductase were obtained during purification procedures and that UDP did not inhibit CDP reduction in Ehrlich cell extracts, even when present in great excess, is also suggestive of separate nucleotide reducing activities. CDP and ADP reductase activities have also been shown to vary independently during the cell cycle (Peterson and Moore 1976). Lewis et al (1978) have also shown that GDP and CDP reductase activities increase noncoordinately after the release of CHO cells from isoleucine starvation. The rate of reduction of various ribonucleotides has also been shown to be unequal in CHO (Hards and Wright 1981) and mouse L cells (Kuzik and Wright 1979). Although the exact stoichiometry of active RRase is still controversial, most authors favor the tetrameric model of Thelander, composed of a dimer of B1 and a dimer of B2, as depicted in figure I for the E. coli enzyme (Thelander and Reichard 1979). The stoichiometry of the free radical of RRase is also poorly understood but it appears that there is only one tyrosyl free radical per molecule in the best preparations of B2 (Sjoberg et al 1977) and this radical is believed to have its spin density delocalized over the aromatic ring of tyrosine (Reichard and Ehrenberg 1983) and not localized to the β-carbon of the amino acid as first reported (Sjoberg et al 1977).

FIGURE I: Model of ribonucleotide reductase proposed for *E. coli* as described by Thelander and Reichard (1979). Diagram is from their review.


Substrate specificity (ATP, dATP, dTTP, dGTP)
h-site
B1- subunit
Activity (ATP, dATP)
l-site
HO
SH
SH
SH
SH
OH
Fe3+
Fe3+
B2- subunit

The Reduction Mechanism

A variety of evidence, from procaryotes and eucaryotes suggests that ribonucleotide reduction proceeds by a mechanism involving substrate-radical intermediates and the C-(3) hydrogen of the ribose moiety (Reichard and Ehrenberg 1983). Early work by these authors on the E. coli (B2) subunit demonstrated the apparent involvement of an organic free radical with enzyme activity (Ehrenberg and Reichard 1972) later identified with a tyrosine residue (Sjoberg et al 1977). Characteristics of the B2 electron spin resonance (ESR) spectrum (a peak at 410 nm and a shoulder at 390 nm) correlated with gain and loss of activity following removal of iron by chelators or treatment with either hydroxyurea or hydroxylamine (Atkin et al 1973). Interestingly, the hydroxyurea treatment does not remove iron from the subunit, but the drug inactivation is rapidly reversed by the iron replacement techniques used for reactivating the apoproteinacious form of B2.

In addition to an apparent role for iron and an organic free radical in the reaction, Thelander et al (1976) have indicated redox active dithiols of B1 are lost during irreversible inhibition by the nucleotide analog 2'-deoxy-2'-chlorocytidine 5'-diphosphate (Cl-CDP). Inactivation of the B1 subunit of E. coli RRase by Cl-CDP requires active B2 and results in release of halogen, pyrophosphate, ribose and free base.

Analysis of ribonucleotide reduction with 2'-deoxy-2'azido-nucleotides has been used to examine events and reaction intermediates more intimately involved with the B2 subunit. Specific inhibition of B2 by azido analogs occurs by selective destruction of the organic free radical and binding of the active site on B2 (Thelander et al 1976). Concomitant with the suicidal reaction and loss of the B2 radical is the

formation of a short-lived nucleotide radical (Sjoberg, Graslund and Eckstein 1982), suggesting that a transient nucleotide radical may be formed during the normal reaction sequence. Stubbe and Kozarich (1980) have also suggested the formation of a nucleotide radical through studies with 2'-deoxy-2'-fluoronucleotides and analogy to halogen elimination reactions with substituted alcohols and Fenton's reagent:

$$Fe^{2+} + H_2O_2 \longrightarrow \cdot OH + OH^- + Fe^{3+}$$

$$CH_2OHCH_2X + \cdot OH \longrightarrow \cdot CHOHCH_2 \overset{\frown}{\text{—}} X \longrightarrow \cdot CHOHCH_2 +$$

$$+CHOCH_2\cdot + H^+ + Fe^{++} \longrightarrow CH_3CHO + Fe^{3+}$$

The authors have noted that abstraction of the 1' or 3' hydrogen of a NDP by RRase may facilitate the reduction at the 2'-carbon of the nucleotide. Further evidence of 3' C-H bond cleavage is provided by work on the RRase from Lactobacillus leichmannii. Stubbe et al (1981) have demonstrated that the 3' C-H bond is cleaved without release of tritium-labelled hydrogen at the 3'-carbon into the solvent.

Taken together, a reaction scheme can be formulated (Reichard and Ehrenberg 1983) in which the tyrosyl radical transiently extracts a C(3') hydrogen, activating the dithiol dependent release of the C(2') hydroxyl group and its ultimate reduction.

The enzymic reduction of ribonucleotides by RRase is apparently specific for deoxyribonucleoside diphosphates in keeping with the remarkable specificity of virtually all known enzyme catalyzed reactions. The possibility exists however that RRase may also catalyze the reduction of hydroxyurea to urea. Colvin and Bono (1970) have documented the enzymic reduction of hydroxyurea to urea by mouse liver

homogenate, noting that much of the activity is present as a potassium cyanide inhibitable, mitochondrial fraction, suggesting the involvement of the electron transport chain. The NADPH (or NADH) stimulated reaction was further stimulated by FAD, but FAD alone had little effect. Interestingly, these authors also noted the chemical reduction of hydroxyurea *in vitro* by ferrous iron. The ubiquitous nature of the subcellular distribution of hydroxyurea reducing activity also suggested to these authors that other enzyme systems might exist in the cell which demonstrate this activity. Furthermore, Krakoff, Brown and Reichard (1968) have noted that hydroxyurea could possibly be broken down to hydroxylamine at the catalytic surface of RRase.

The role of iron

The exact role played by iron in the reaction mechanism is unclear, but is believed to involve stabilization of the tyrosyl radical. Resonance Raman spectroscopy suggests that the two iron atoms of B2 form a μ-oxo bridge (Sjoberg *et al* 1980). This binuclear iron centre is thought to be analogous to the met-forms of hemerythrine. Recent primary structure comparisons between B2, Epstein Barr and Herpes virus and the components of a marine mollusc RRase, have revealed that His-118, His-241, Glu-115, Glu-238 and Asp-237 are ligands of the iron centre of ribonucleotide reductase, aiding the stabilization of the tyrosyl radical, tentatively located at Tyr-122 (Sjoberg *et al* 1985). The availability of X-ray crystallographic quality preparations of B2 should greatly facilitate our understanding of the reductase, mechanistically and biologically, in the near future (Joelson *et al* 1984). Moreover, the similarity of the B2 and M2 proteins, including the nature of their tyrosyl radical (Graslund, Ehrenberg and Thelander 1982),

promises to allow extension of the observations of bacterial RRase dynamics to the mammalian enzyme. In this regard, similar experiments with the recently purified M2 subunit from hydroxyurea-resistant M2-overproducing mouse cells (Thelander, Graslund and Thelander 1985) will be most enlightening.

Recently, Thelander, Graslund and Thelander (1983) have implicated iron in a regulatory mechanism controlling mammalian ribonucleotide reduction. They found that the tyrosyl free radical of purified RRase could be regenerated by iron, oxygen and DTT. The short half-life of the radical ($\sim$ 10 minutes) demanded that the agents be present continuously. Thus, one way of controlling the overall activity of the enzyme may be through regulating the number of active M2 subunits with tyrosyl free radicals via limitations in oxygen or iron. Unlike the *E. coli* system, deprivation of oxygen or iron results in rapid loss of RRase activity.

Ferritin and transferrin

The availability of iron for mammalian RRase must ultimately depend on ferritin and transferrin. Iron is essential for the growth of eukaryotic cells and is rapidly absorbed from the gut by the gastro-intestinal mucosa, where it binds to apoferritin. Ferritin, the iron-apoferritin complex, is a large multisubunit protein which effectively binds and stores up to 4500 atoms of iron (in the ferric state) per complex (see Thomas *et al* 1985). Large deposits of ferritin are also found in the liver, spleen and bone marrow. Iron is released from ferritin through reduction to the ferrous state and is transported to the cells of the body as a diferric complex with apotransferase (transferrin).

Cellular uptake is mediated by the transferrin receptor, an integral membrane glycoprotein whose expression is strongly correlated with cell proliferation. Receptors for transferrin are unequally distributed over the cell surface, occurring predominantly in the leading edge of cells (Bretscher 1983), probably a direct result of its endocytic traverse through the cell. Diferric transferrin, as opposed to apotransferrin, binds tightly to the transferrin receptor and the bound complex is taken into the cell by endocytosis into an acidic nonliposomal vesicle (Klausner et al 1983). The acidic environment of the vesicle promotes the dissociatin of iron from the complex, releasing it into the cell, while the apoferritin-receptor complex is recycled to the surface of the cell where each component is released and used again (Dautry-Varsat et al 1983).

The exact mechanism by which iron is released intracellularly is poorly understood, as are the growth promoting effects of iron and transferrin. Iron has been found to stimulate purified guanylate cyclase, suggesting that cGMP may be involved in the process(es) (Basset et al 1985). Interestingly, these same authors found that chelation of iron in the culture medium of L1210 cells by disferrioxamine, blocked exponential phase cells at the G_1/S boundary. These results are compatable with a control mechanism for M2 tyrosyl radical formation via limitations in the amount of iron available for production of active RRase.

Increasing attention is being given to the role of iron and transferrin in cellular proliferation with the recent discoveries that ras proteins may affect cellular growth through the formation of complexes with transferrin receptor (Finkel and Cooper 1984, Cooper 1984) and the protein product of the Blym-1 oncogene which is partially homologous to

the aminoterminal end of transferrin (Goubin et al 1983). Further insight into this relationship will undoubtedly progress rapidly in view of the recent molecular cloning of the human transferrin receptor gene (Kuhn et al 1984).

The role of oxygen

Oxygen, like iron, is also receiving increased attention because of its effects on cellular proliferation. Ehrlich ascites tumor cells are arrested at the G_1/S boundary when cultured anaerobically (Loffler et al 1983). Concomitant with the anaerobic arrest is a marked drop in dCTP and dTTP pools, without a similar change in dATP pools. Remarkably, this anaerobic arrest is reversible by the addition of deoxycytidine, suggesting that pyrimidine nucleotide reduction by RRase is important in the cell cycle progression of mammalian cells, and underscoring the regulatary role of oxygen in cellular growth. Most interestingly, Ehrlich ascites cells undergoing active genome replication respond to deoxygenation with a decrease in the level of replicon initiation, while the elongation and maturation of nascent chains remains unaffected (Probst et al 1984). The hypoxic state in vivo may also be able to regulate DNA synthesis. Recently, the chemoreceptors for oxygen in the carotid body have been identified as the glomus cells, which apparently signal hypoxia by releasing dopamine (Fishman, Greene and Platika 1985). It is tempting to speculate that this hypoxia-stimulated catecholamine release may affect DNA replication in vivo, since dopamine and related antineoplastic agents have been shown to inhibit RRase and DNA polymerase in the reduced (quinol) and oxidized (quinone) forms, respectively (Fitzgerald and Wick 1985). These observations may have some bearing on the role of catecholaminergic

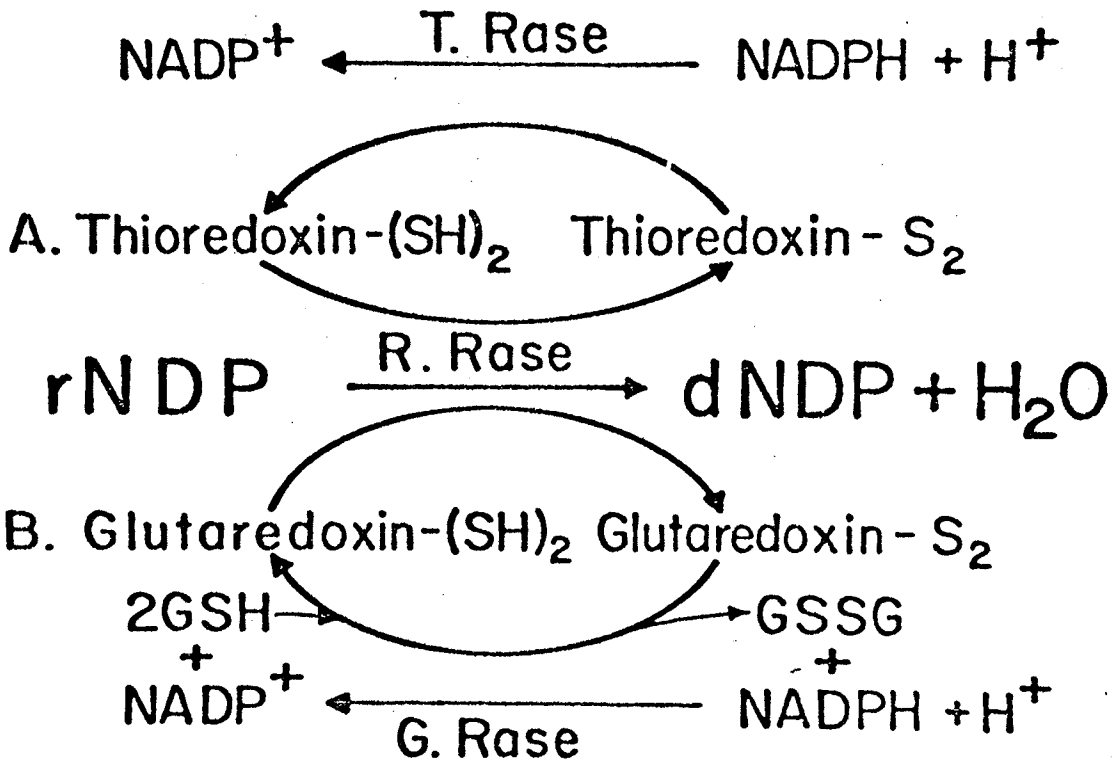
processes in carcinogenesis, as reported by Gurkalo and Zabezhinski (1984). Further evidence for the role of oxygen in proliferation has been documented by Pentland and Marcello (1983), where epidermal keratinocytes have been shown to be unusually sensitive to low oxygen tensions. The response of epidermal tissue to variations in oxygen tension is critical in the processes of wound healing.

Hydrogen donor systems

The *in vivo* hydrogen donor substrate for ribonucleotide reductase, necessary for maintenance of the active dithiol group of RRase, is still unknown, but reduced thioredoxin or glutathione can serve effectively in this capacity *in vitro* (Holmgren 1981). Both systems can be found in eucaryotes and procaryotes. The reactions involved in the *in vitro* maintenance of RRase reductase by these agents are shown in figure II. In both systems, NADPH is the ultimate hydrogen donor. Further similarities include the use of small ($\sim$12,000 molecular weight) heat-stable flavoproteins (thioredoxin and glutaredoxin) as carriers of the reducing power in active dithiols regenerated by specific reductases. The overall mechanism of reduction coupling to RRase is poorly understood although Hoog *et al* (1982) have suggested the presence of a glutathione binding site on *E. coli* RRase.

Much of the speculative relationship between these reducing systems and RRase stems from the isolation of bacterial variants deficient in glutathione synthesis (Fuchs and Warner 1975) or thioredoxin reductase (Fuchs 1977). In these deficient variants, the remaining alternate system of reduction may be used. Indeed, thioredoxin-negative mutants of *E. coli* were used in the discovery of glutaredoxin (Holmgren 1976). The primary enzymes of glutathione metabolism have also been shown to be

FIGURE II: Hydrogen donor systems for ribonucleotide reductase (from Wright 1983).


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

lacking in Entamoeba histolytica, a parasitic amoeba (Fahey et al 1984). Thus, eucaryotic systems can also exhibit independence from one of these two reducing systems.

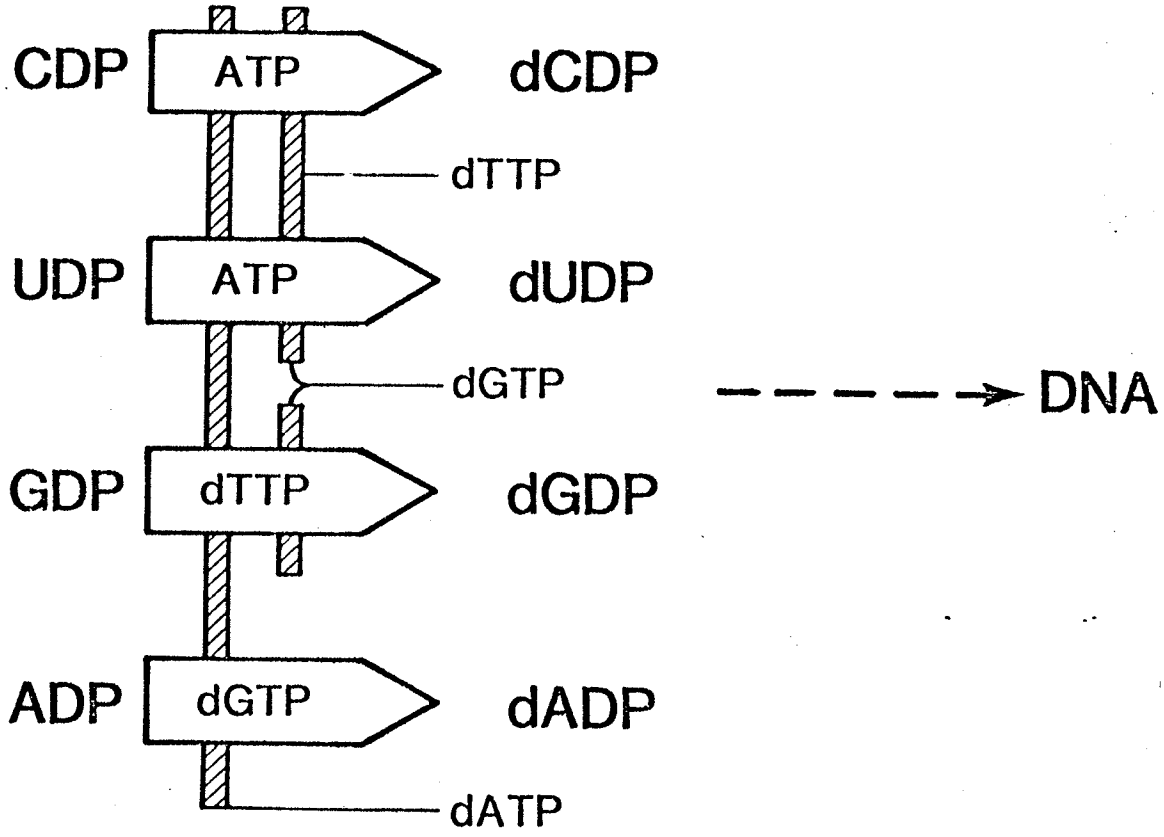
Regulation

Ribonucleotide reductase activity is regulated at many levels and by a variety of compounds and environments encountered in vivo. The review that follows focuses primarily on the allosteric regulation of RRase by various nucleotide effectors and is later accompanied by a brief survey of other regulatory considerations. The regulation of ribonucleotide reductase has been extensively reviewed (Reichard 1978, Thelander and Reichard 1979, Holmgren 1981, Hunting and Henderson 1982, Cory 1983, Cory and Sato 1983, Wright 1983, Hogenkamp 1984, Jackson 1984).

Allosteric regulation

The first model of ribonucleotide reduction summarizing its allosteric regulation by nucleotide effectors was published by Moore and Hurlbert (1966). A schematic representation of their model is presented in figure III. While many authors have presented similar models, with minor variations, this summary of allosteric regulation of a partially purified preparation from Novikoff rat hepatoma cells conveniently depicts the basic activation and inhibition patterns seen in many other systems. The general inhibitor of all four reductions is dATP which thus effectively serves as an overall regulator of activity. Pyrimidine reduction is stimulated in the presence of ATP and effectively inhibited not only by dATP and dGTP but by dTTP as well. This pattern serves to limit pyrimidine reduction while stimulating purine reduction through the effects of dTTP, thus helping to maintain a balance between purine

FIGURE III: Allosteric regulation of ribonucleotide reductase. Inhibitory effects are indicated by vertical bars. Nucleotide in the arrows are positive effectors (from Wright 1983).


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

and pyrimidine products. It should be noted that pyrimidine reduction showed an absolute requirement for ATP while purine reduction required dTTP.

The general pattern of apparent coordination between purine and pyrimidine reduction presented by Moore and Hurlbert (1966) is seen in a variety of cell systems that have been studied, including calf thymus and mouse T-lymphoma (Eriksson et al 1979, 1981), human diploid fibroblasts and MOLT-4F cells (Dick and Wright 1980, Chang and Cheng 1979a, 1979b) and Chinese hamster ovary cells (Lewis et al 1978). In this connexion however, it is important to realize that the majority of such studies have not addressed the dynamic complexity of RRase, usually examining a single parameter at a time in partially purified preparations. The physiological state of ribonucleotide reductase, including its association with other proteins involved in DNA replication, is undoubtedly more complex than suggested by such artificial constructs.

Protein synthesis and thymidylates

A thymidylate metabolite, possibly dTTP, has been implicated in the apparent stimulation of de novo RRase synthesis. Elford et al (1977) have treated Hela cells and partially hepatectomized rats with MTX and 5-FUDR, lowering the deoxythymidylate pool and increasing (3-4 fold in Hela) CDP RRase activity in a cycloheximide inhibitable manner. Thus, while the lowered dTTP pool and increased CDP reductase activity are consistent with the acknowledged role of dTTP as a negative feedback regulator, the dependence of the system on active protein synthesis suggested to these authors that components of the thymidylate pool may be repressors or corepressors of RRase activity.

Endogenous inhibitors

The demonstration of endogenous inhibitors of RRase has called attention to another set of agents that may regulate RRase activity in a physiologically relevant manner. High molecular weight or non-dialyzable, heat-labile factors have been shown to inhibit RRase and be inversely correlated with the proliferative state in normal rat liver (Elford 1972) and in many other transformed or normal cell systems (Cory and Sato 1983). These inhibitory factors are lacking detailed characterization; both phosphatases and proteases have been suggested as possible mediators of the effect, but remain without substantiation. Cory (1973) has reported the inhibition of Ehrlich ascites tumor RRase activity by rRNA, tRNA and polycytidylate, apparently in a competitive manner with CDP reduction. Demonstration of the analogous *in vivo* situation and its possible significance has proven difficult and remains undocumented.

Lewis *et al* (1976, 1977) have shown that a highly phosphorylated dinucleotide obtained from *Achlya* and Chinese hamster ovary cells that inhibits both the fungal and mammalian reductase activities. Like the high molecular weight inhibitors noted above, these dinucleotides are inversely correlated with the proliferative state (Goh *et al* 1977, Lewis *et al* 1976). They are also known to increase during glutamine deprivation-induced quiescence. Structurally, the polyphosphorylated dinucleotide of LeJohn's group appears to resemble the alarmone A_p4A (Varshavsky 1983), although they would obviously serve in an opposite manner, perhaps even complementary, since increases in A_p4A levels are correlated very tightly with DNA synthesis, serving to stimulate or perhaps initiate DNA replication (Rapaport and Zamecnik 1976, Grummt 1978, Grummt *et al* 1979, Probst *et al* 1983, Weinmann-Dorsch *et al* 1984a,

1984b). Some A_p4A binding proteins have been shown to copurify with DNA polymerase α (Grummt et al 1979, Baril et al 1983) and have a molecular weight of 57K or 92K (dimer of 47K chains) respectively, in calf thymus and Hela cells. Rapaport and Feldman (1984) have characterized the calf thymus protein in some detail, describing a 54K protein with A_p4A phosphohydrolase activity in the free, unbound form, but no such activity when copurified with DNA POL α. In addition, it should be noted that A_p4A is known to perturb poly (ADP) ribosylation (Surowy and Berger 1983), as well as inhibit the kinase activity of $pp60^{v\text{-}src}$ (Maness et al 1983) and that of the phorboid receptor (Shoyab 1985).

The ability of dinucleotides, such as those described above, to modulate DNA synthesis and its associated activities, underscores the need for careful consideration of the normal in vivo environment in the regulation of complex cellular activities, such as those of ribonucleotide reductase.

Fluctuations in subunit activity

A further consideration in the regulation of the overall activity of ribonucleotide reductase is the relative amount of each subunit available to form active RRase, at any given point in time. The two subunits have been shown to fluctuate independently during the cell cycle: M1 remains relatively constant while M2 decreased 60% in G1 mouse lymphoma cells (Eriksson and Martin 1981). Deuterated tyrosine pulse chase experiments with cells synchronized by isoleucine deprivation have indicated that de novo protein synthesis is responsible for the rise in the rate-limiting M2 activity near the G1/S boundary (Eriksson et al 1984a). In contrast to these results are the findings of Cory and Fleischer (1982b) in regenerating rat liver, where M1, the

effector binding subunit, was found to be limiting after the maximal increase in M2, the non-heme iron subunit, 24 hours after partial hepatectomy. Thus the nature of reductase subunit fluctuation in the cell cycle remains controversial and may vary according to the particular stress applied to the cellular environment (such as injury).

Nucleotide Metabolism and the Cell Cycle

Ribonucleotide redutase activity levels vary directly with DNA synthetic activity throughout the cell cycle of amethopterine synchronized L cells (Turner et al 1968), increasing at the G1/S boundary, rising through S-phase and continuing into G2 (Murphree et al 1969) in DON-C Chinese hamster fibroblasts synchronized with colcemid. Both studies confirmed the lability of the increase in activity to inhibition by cycloheximide. Interestingly, the latter authors reported an increase of more than two-fold in the RRase activity of partially synchronized cells treated with 1 mM hydroxyurea. This apparent anomaly was explained by postulating a decrease in deoxyribonucleotide repressor(s) or decreased feedback inhibition by deoxyribonucleotides. Consistent with this hypothesis is the finding of Walters, Tobey and Ratliff (1973), that hydroxyurea treatment of cells in G1 results in elevations of dTTP, dCTP and dGTP, but not dATP when DNA synthesis should have been (but was not) initiated. The apparent induction of ribonucleotide reductase by the inhibitor hydroxyurea has also been noted in some of the mouse L cells used in the present work (A. Chan and G. McClarty, personal communication). The nature of this effect is poorly understood, but similar observations have been made with 5-fluorouracil-enhanced RRase activity in Saccharomyces cerevisiae (Lowdon and Vitols 1973) and mammalian cells (Elford et al 1977). The

authors have also noted the correlation between increased ribonucleotide reductase and S-phase in this lower eucaryote. Kucera, Brown and Paulus (1983) have confirmed the continuance of RRase activity through S, G2 and M phases, with a rapid drop occuring late in mitosis to low undectable levels during G1. Youdale et al (1984) have also noted the continuance of significant RRase activity after the completion of DNA synthesis in normal and thyroparathyroidectomized rats. Interestingly, Neckers et al (1985) have reported significant non-S-phase DNA synthesis in human lymphoid cells, suggesting that DNA synthesis may be more dynamic than is generally believed. It should be noted that in contrast to these results, Turner et al (1968), Lewis et al (1978) as well as the work of Lowdon and Vitols (1973), indicate a drop in RRase activity at the S/G2 interface. Continuing work in this area has recently confirmed the presence of significant RRase activity in G2 phase mouse T-lymphoma cells synchronized with dibutyryl cAMP (Albert and Gudas 1985). Moreover, the S-phase specific increase in RRase activity appeared to be due to an increase in M2 activity, not M1, although there were no apparent increases in the amount of the protein subunit, as determined by examination of 2-D gels. The M2-dependent increase in RRase activity in the cell cycle has been confirmed by Engstrom et al (1985). These authors note that a nonallosterically mediated increase in RRase activity, apparently not involving any substantial increase in M2 protein, is consistent with the finding that permeabilized cells, freed of endogenous nucleotide effectors, exhibit cell-cycle dependent changes in RRase activity.

A candidate that must be considered in the increase of M2 activity and the accompanying increase in RRase activity at the G1/S boundary, is the generation and maintenance of the tyrosyl free radical associated

with active M2. The regeneration of this radical, its role in the catalytic mechanism and its reversible destruction by hydroxyurea, have already been presented in this review.

Accompanying the cell cycle correlated changes in RRase activity are corresponding changes in nucleotide pools, and many of the effects of RRase on cellular metabolism may be mediated through changes in endogenous pools. Nordenskjold et al (1970) demonstrated parallel increases in the rate of DNA synthesis and increases in the pools of dATP and dTTP in serum stimulated primary mouse embryo cells. Accompanying these increases were increases in RRase and thymidine kinase activity. While these authors found no increase in pool sizes prior to the beginning of DNA synthesis, Walters et al (1973) found that all four deoxyribonucleotide pools increase just prior to the DNA snythetic event, increasing throughout S-phase. A detailed study of synchronized mouse T-lymphoma cells by Albert and Gudas (1985) has revealed a pattern of dNTP pool fluctuations consistent with the general allosteric model presented previously in this review. These authors found that cells arrested in G1 with dibutyryl cAMP had decreased dTTP and dCTP pools but relatively stable dATP and dGTP pools. S-phase enriched populations displayed a slight decrease in dATP accompanied by large increases in dTTP and dCTP pools which persisted into G2/M enriched populations even when the dATP pool increased drastically in the same situation. The pool of dGTP remained relatively unchanged.

The pool of dCTP has received special attention as a possible regulator of DNA synthesis (Bjursell and Reichard 1973). Although increases in all four dNTP pools are generally noted during, or shortly after the G1 to S phase transition, the largest increase is seen in the dCTP pool, exhibiting the best correlation with the percentage of cells in S phase

(Eriksson et al 1984b) and with growth rate (Jackson 1984) and rate of DNA synthesis (Reichard 1978). Newman and Miller (1983) have suggested that the dTTP/dCTP ratio is critical in the regulation of DNA replication, with dCTP acting as a stimulator. In addition to the ability of deoxcytidylate to act an an antioxidant as mentioned previously (Loffler et al 1983), its role in other cellular processes is implicated in its apparent lack of effect, in comparison with other dNTPs, on the allosteric regulation of RRase (see figure III). Thus the apparent preferential increase in CDP reductase activity seen in hydroxyurea-resistant cell lines, may have special significance with regards to the nature of the selective pressure induced in the presence of hydroxyurea. In this light it is interesting to note that deoxycytidine supplementation of Chinese hamster cells treated with 5-fluorodeoxyuridine, allowing DNA synthesis in the presence of an imbalanced dTTP/dCTP ratio, has been implicated in a mechanism of potentiated resistance to this agent (Peterson, Peterson and Danenberg 1983). Methotrexate potentiation of ara-C activity is believed to be mediated through attenuation of the dCTP pool increase seen following exposure to the nucleoside analogue (Roberts and Peck 1981). These potentiating effects are believed to result from changes in the allosteric regulation of deoxycytidine kinase or deoxycytidylate deaminase.

Recently, Jackson (1984) has reviewed the literature on dNTP pool regulation and has formulated a kinetic model in which the net flux of the dNTP pool is regulated by the V_{MAX} of RRase, and not by the general allosteric inhibitor dATP. In this author's comprehensive survey of the literature it was concluded that the allosteric regulation of RRase by dNTP pools does not regulate the net reaction rate, but rather functions to maintain a balanced ratio of the dNTP pools.

Nucleotide pool imbalance

The genetic and non-genetic consequences of nucleotide pool imbalance are manifold. Much of our knowledge of the regulation of these pools stems from work with drug treated or resistant mammalian cells and some of these effects have already been mentioned. The pools most often studied are the deoxyribonucleoside 5'-triphosphate pools of whole cells, although some work has been done with pools from cytoplasmic and nuclear enriched preparations.

The genetic effects of nucleotide pool imbalance have been reviewed (Kunz 1982, Meuth 1984a, 1984b). In his extensive treatment of the subject, Kunz has noted that while excess dTTP, dCTP, dATP and dGTP have been associated with enhanced mutagenesis in many cell systems (though most notably with the pyrimidines), thymine nucleotide deprivation has overall been found to be non-mutagenic. All of these conditions however, display chromosome aberrations of many kinds including strand scission and recombinational events. Many of the drugs that affect nucleotide pool balance, as previously mentioned, produce similar effects and can induce the neoplastic state. Nucleotide pool imbalance and the accompanying replicative errors have been considered as contributors to carcinogenesis, mutagenesis, aging and evolution (Peterson, Landolph, Peterson and Heidelberger 1978).

The mutator phenotype

Ribonucleotide reductase has been recognized as a mutator gene product in many laboratories. Weinberg, Ullman and Martin (1981) have isolated mouse S-49 T-lymphoma cell lines resistant to deoxyguanosine by virtue of nucleotide binding alterations in the M1 component of RRase

(Thelander, Eriksson and Akerman 1980, Ullman et al 1980). These dG^R lines have elevated dNTP pools in the order of 1.5-10 fold. Fluctuation analysis revealed increased mutation rates to 6-thioguanine and dexamethasone resistance. Indeed, it appeared that the rate approximated the degree of pool perturbation. Manipulation of pools in wild type cells by increases in the exogenous supply, also elevated the mutation rate, though slightly, implicating the pool disturbance rather than the altered RRase per se, in the mutator effect. Further evidence for this was provided through exogenous manipulation of pools in a deoxycytidylate deaminase variant selected by these same authors (Weinberg et al 1981), where pharmacological expansion of the depressed dTTP pool actually decreased the enhanced mutation rate seen in these variants as well. Similar results have been observed by Maus et al (1984a).

Imbalanced deoxypyrimidine pools have been implicated in the mutator phenotype of CHO cells selected for resistance to 6(p-hydroxyphenylazo)-uracil (HPUra)(Arpaia, Ray and Siminovitch 1983b). These mutants have a structural alteration in RRase characterized by a 2-4 fold increase in K_m for the substrates UDP and CDP. The authors recognize, however, that HPUra may not necessarily target the reductase at the M1 component, as these results might suggest. They offer other explanations for this K_m effect, including allosteric changes, but clearly, perturbations affecting the nucleoside diphosphate substrate binding characterisitics of RRase are correlated with the mutator phenotype. Interestingly, these authors have noted that while the rate of mutation to 6-thioguanine resistance was increased, no effect was seen on the rate of mutation to emetine resistance, suggesting a degree of specificity between different genetic loci. This phenomenom is poorly understood, but has been documented in the dCMP deaminase mutator system of

Maus et al (1984a, 1984b), where Saccharomyces cerevisiae deficient in dCMP deaminase show increased rates of mutation to canavanine resistance and reversion to tryptophan prototrophy but not for reversion to lysine and arginine prototrophy. Indeed, the rate for the latter actually decreased to 60% of control.

Roguska and Gudas (1984) have selected variants of the S49 T-lymphoma cell line with altered RRase and an increased rate of spontaneous mutation to 6-thioguanine resistance. The CDP reductase activity of these variants is 70-90% decreased in comparison with wild type S49 cells, whereas ADP reductase activity is increased slightly (yet another display of discoordinate purine and pyrimidine reduction) and the overall dNTP pools are lowered to about half that of wild-type. Interestingly, these variants are 5-10 fold more sensitive to tunicamycin and compactin. The authors suggest that their evidence is consistent with a change in the M1 component of RRase. Furthermore, the uniform decrease in the dNTP pools of these variants, maintain the normal ratios between the four pools which are suggestive of a mechanism more complex than simple dNTP competition.

The level of ribonucleotide reductase activity present within a cell has also been implicated in a mutator phenotype. Aphidicolin resistant mouse FM3A mammary carcinoma cells have been isolated by Ayusawa, Iwata and Seno (1983) with increased ribonucleotide reductase activity and decreased thymidylate synthase. Two other classes of variants were also characterized, one selected for resistance to aphidicolin with no changes in RRase activity but decreased sensitivity to dATP inhibition and the other temperature-sensitive for thymidylate synthase activity (Ayusawa et al 1981). These variants were shown to have much in common, including modestly altered dNTP pools (the largest

being dATP at 2-6 fold), cross-resistance to ara-A, ara-C and hydroxyurea, a mutator phenotype (as seen in 10-100 fold increases in the frequency of 6-thioguanine resistance) and an interesting enhanced sensitivity of the cytotoxic action of bleomycin. Bleomycin sensitivity correlated well with altered CDP RRase activity (using the assay of Lewis and Wright 1978) in the conditional thymidine auxotroph mutants, as did resistance to hydroxyurea. The authors note that similar correlations between hydroxyurea-resistance and RRase activity have been found by Wright et al (1981) and Wright (1983). Thus, hydroxyurea-resistance and increased ribonucleotide reductase activity have been found to be correlated with a mutator phenotype in ara-A resistant mutants selected in a manner independent of hydroxyurea resistance.

Hydroxyurea-resistant cell lines and other drug-resistant strains with perturbations in known enzymes of nucleotide metabolism, have also contributed to our appreciation of the mutational effects of dNTP pool imbalance. Alterations in key enzymes affecting nucleotide pools have been noted with regards to their potential role in mechanisms of neoplastic transformation (see under 'Somatic Mutation Theory), yet no study so far (excluding the present work) has correlated changes in the nature or activity of a known mutator gene product in mammalian cells selected for such an alteration, with changes in transformation related aspects of the measurable phenotype. Creasey and Wright (1984a) have demonstrated a tight correlation between increased ribonucleotide reductase activity, hydroxyurea-resistance and decreased myogenic development in variant clones independently selected from wildtype, differentiation competent (myogenesis-positive) L_6 and L_8 rat myoblast clones.

The studies described above have suggested that disturbances in the level of RRase activity, in mechanisms involving either or both of its

components, M1 and M2, can contribute to deoxynucleotide pool imbalance and a mutator phenotype. The mutator phenotype in these examples is accompanied by resistance to agents believed to act at both the M1 (i.e. deoxyguanosine) and M2 (i.e. hydroxyurea) components. A further mechanism of enhanced mutagenesis may involve the reductase at the level of its association with the replitase. Decreased fork-displacement rates have been correlated with the altered ribonucleotide reductase (Gjerset, Weinberg and Kapp 1985) of the S49 dG^R mutants of Weinberg et al (1981) described above. A similar phenomenon was not seen with the dCMP deaminase mutant. A revertant of the dG^R phenotype was selected by long-term cultivation in hydroxyurea which also showed normal fork-displacement rates. Although the altered replication dynamics correlated with altered RRase activity, they did not correlate with the mutator phenotype or dNTP pool alterations as seen in the two cell lines with normal reductase. In this system, altered pools alone can apparently stimulate or abrogate the mutator phenotype, but as these authors note (Gjerset et al 1985) the association of RRase with the replitase may affect the fidelity of replication.

Pool imbalance, ribonucleotide reductase and neoplasia

The importance of ribonucleotide reductase and altered dNTP pools in the transformed state has been illustrated in a wide variety of studies. The activity of RRase has been shown to be correlated with the proliferative rate of numerous hematologic malignant cell lines (Takeda et al 1984) and most elegantly in series of rat hepatomas studied by Elford et al (1970). The 200-fold increase in CDP reductase activity between rapidly and slowly growing tumor populations was the largest increase in enzyme activity measured in these tumors. The 'growth rate'

referred to here is not that measured classically *in vitro* but rather was defined in a manner relating to tumor latency time required for the growth of a viable tumor. Thus, the elevated reductase activity seen in malignancy continues to be an important target in cancer chemotherapy. Elford *et al* (1970) have even suggested that RRase may be looked upon as a 'barometer of malignancy'.

In keeping with the increases in RRase activity in malignant tissue are the documented imbalanced increases in dNTP pools found in such cells (Tattersall *et al* 1980). Interestingly, with regards to the mutator phenotype, Bockstahler *et al* (1982) have shown that the intracellular environment of human tumor cells is more mutagenic than their normal counterparts. The resistance of HSV-1 infectivity to iododeoxycytidine in plaque assays was shown to increase when the virus was propagated in tumor cells as opposed to normal cells, in some cases using normal cells from the same patient as controls. Iododeoxycytidine resistance is a measure of forward mutation in the HSV genome, presumably the result of a defective viral TK gene product. These authors have noted that one of the factors mediating the fidelity of DNA synthesis is the balance of the cellular nucleotide pool.

The results of Elford's studies (Elford *et al* 1970) are consistent with the 'molecular-correlation concept' of Weber (Weber 1983). A lifetime of work in George Weber's laboratory on the enzymic and metabolic patterns of neoplastic cells has revealed that the neoplastic genome has an identifiable biochemical strategy which manifests itself in the pattern(s) of its gene expression (Weber *et al* 1981, 1983). The activities of 'key enzymes' such as ribonucleotide reductase, which regulate the flux of metabolic pathways often in a rate-limiting manner (Weber *et al* 1980) are tightly linked with neoplastic transformation and progres-

sion and display themselves in accordance with the proliferative rate and degrees of malignancy and differentiation exhibited by the system. The role of RRase in the neoplastic program has been examined by Takeda and Weber (1981). These authors have shown that dNTP pool elevations in newborn and regenerating rat liver, as well as hepatoma cells, are attributable to increases in ribonucleotide reductase activity (Jackson et al 1980, Weber et al 1980). The 18,000% increase in reductase activity seen in rapidly growing hepatomas compared to normal liver, similar to the 200-fold increase reported for hepatoma by Elford et al (1970), was the largest increase in any enzyme activity measured including most notably, DNA polymerase, thymidine kinase, dTMP kinase, dTMP synthase, deoxycytidine kinase, dCMP deaminase and CTP synthetase, which increases the most in hepatoma (Weber 1983). Five of these key enzymes (underlined above), including ribonucleotide reductase, have been described in conjunction with a mutator phenotype, as discussed above and in the section of this review dealing with the somatic mutation hypothesis of neoplastic transformation.

Viral Enzyme and Neoplastic Transformation

Evidence for the involvement of ribonucleotide reductase in cellular transformation has been provided in studies with herpes simplex virus (HSV) types 1 and 2 and with related virus. Jariwalla, Aurelian and Ts'o (1980) have demonstrated the immortalization and neoplastic transformation of primary GSH cells with a defined double digest restriction fragment (BglII/HpaI, CD_{s-1}) of HSV-2 S1 DNA, homologous to the BglII/HpaI CD fragment of strain 333 mapping between 0.43 and 0.58 m.u. on the physical map of the viral genome. The transfectants were shown to express ICP10, an immediate early viral protein now

identified as RRase, and immunologically identical to AG-4, the cervical tumor associated antigen. A similar transforming fragment has been described for human cytomegalovirus DNA (Clanton, Jariwalla, Kress and Rosenthal 1983). Interestingly, the transforming fragment of HSV-2 DNA was further dissected biologically into an immortalizing fragment (the left 64%, formed by the Hind III cleavage of the BglIII C fragment at 0.525 m.u.) and a fragment responsible for the conversion to tumorigenicity (right hand 36% of BglIIC, 0.525-0.585 m.u.). SHE cells transfected with the left 64% fragment of BglII C escaped senescence and were able to grow in reduced serum and in sloppy (0.3%) agarose at early passages, but were nontumorigenic after more than 50 passages in culture. Those cells transfected with BglII C (whole, left and right) acquired the tumorigenic phenotype after serial cultivation (Jariwalla, Aurelian and T'so 1983). Thus immortalization is seen as an early step in a multistep model of neoplastic transformation. Interestingly, these authors noted that the normal SHE cell DNA shared homology with the BglII C fragment.

The transforming regions of the HSV genome have received detailed study. A second site in the genome, associated with focus formation and altered growth properties, is contained within the BglII N (0.580-0.620 m.u.) fragment. Four separate mRNA transcripts have been identified in this region (Jenkins and Howett 1984) and the sythesis of 5 proteins (molecular weights of 140K, 61K, 56K, 35K and 23.5K) have been associated with the fragment in hybridization studies with selected mRNA species (Galloway et al 1982). Transcripts homologous to this region overlap with the adjacent BglII C fragment, complicating the analysis.

Some protein products of the transforming regions have been mapped precisely. The 35-38K protein maps to 0.582-0.596 m.u. on the HSV-2

genome (Suh et al 1983) and its message has recently been characterized in detail (Frost et al 1984). Galloway and Swain (1984) have examined this region in detail and have shown that the carboxy-terminal 163 amino acids of the 140 K protein overlap into the transcriptional regulation region of the sequence encoding the 38K protein. Although the role of the 38K protein remains obscure (Preston et al 1984), both it and the 140 K protein have been identified with the viral ribonucleotide reductase activity (Huszar and Bacchetti 1983) and apparently they share a common antigenic determinant (Galloway, Goldstein and Lewis 1982, Goldstein et al 1983) even though their DNA regions do not overlap.

Ribonucleotide reductases from HSV-1 and HSV-2 are similar in apparent molecular weight, composition and responsiveness to effectors (Averett et al 1983, 1984, Bacchetti et al 1984). Both enzymes are characterized by their refractility to inhibition by dTTP and dATP, and their inhibition by ATP and $MgCl_2$, unlike the mammalian enzyme. The 140 K protein is the major antigenic species recognized by polyvalent rabbit serum raised against the reductases, and is designated ICP6 and ICP10 in HSV-1 and HSV-2, respectively.

New evidence by Cohen's group suggests that the two components of the viral reductase, H_1 and H_2, may be analogous to the mammalian components, M1 and M2, though they are distinctly different in their heat inactivation profiles and sensitivity to hydroxyurea (Cohen et al 1985). As the causative agent in a number of human diseases, including infectious mononucleosis and genital herpes, as well as their association with neoplasia (i.e. Burkitt's lymphoma and Epstein-Barr virus, cervical carcinoma and HSV-2), antiviral chemotherapy has been used to attack the viral reductase with agents such as ara-A and acylolovir (Rapp 1984). Recently, however, the effectiveness of antiviral therapy

aimed at the reductase has been questioned by Nutter et al (1985), since it appears that 95% or more of the viral reductase activity is not required for virus replication.

The identification of RRase in the transforming region of HSV-2 DNA by Huszar and Bacchetti, and the known expression of the related antigens (140K: 1CP-10, 1CP-6, AG4 and 38K) in transformed cells, suggests that RRase may be involved in HSV-2 induced transformation. These authors have noted that HSV infection is known to cause dNTP pool imbalance (Cohen 1972, Jamieson and Bjursell 1976a, 1976b) and suggests that the ability of altered RRase to perturb dNTP pools and affect the fidelity of replication as a mutator supports the concept of a 'hit-and-run' mechanism for HSV-2 induced transformation. Galloway and McDougall (1983) have documented the retention of HSV DNA in transformed cells and human cervical tumors and have suggested a number of mechanisms whereby HSV might initiate the transformed state without apparent need for the continued presence of its coding sequences, including the ability of HSV to act as a mutagen and mediate aspects of gene amplification (Matz et al 1985).

Hydroxyurea

Hydroxyurea (hydroxycarbamide, carbaminohydroxamic acid) is a cytotoxic analogue of urea used in the chemotherapy of a wide range of solid tumors (Creasey et al 1970) and chronic leukemia (Rushing et al 1982). The antineoplastic acitivity of hydroxyurea (Pinedo and Chabner 1983) was not recognized for almost a century after its synthesis by Dresler and Stein in 1869 (see the review by Timson 1975). Since then it has also found application in the treatment of psoriasis (McDonald 1981) and polycythemia vera (Donovan et al 1984) and as a cell cycle

synchronizing agent in procaryotes and eukaryotes (Adams and Lindsay 1967, Hamlin and Pardee 1976).

Biological Effects

Many studies have examined the effects of hydroxyurea in its various forms on procaryotic and eucaryotic metabolism and development and have produced an extensive list of perturbations caused by this antineoplastic agent: induction of free-radical formation (DeSesso 1979, Przybyszewski et al 1982), the enhancement of tumor production after carcinogen treatment (Iverson 1981), interference with PMA-induced first-stage promotion (Kinzel et al 1984), various developmental abnormalities in amphibia (Brachet 1967), enhancement of metastatic capacity (McMillan et al 1985), suppression of phagocytosis by granulocytes (Malec et al 1984), inhibition of sea urchin embryo cleavage (Eisenberg et al 1965), induction of foetal hemoglobin synthesis (Letvin et al 1984, Kolata 1984, Linch et al 1984, Platt et al 1984), inhibition of epidermal growth factor degradation in Hela cells (Masuda et al 1982), alteration of nuclear membrane permeability (Adams et al 1971), reduction of histone mRNA levels and rates of transcription (Sittman et al 1983), inhibition of induction of ornithene decarboxylase (Cress et al 1979), inhibition meiotic events (Simchen et al 1976), potentiation of UV killing (Burg et al 1977), the enhancement of the lethality of X-rays (Phillips et al 1966), the shortening of G_1-phase in next generation CHO cells (Cress et al 1977), inhibition of DNA synthesis (Turner et al 1966, Young et al 1964), inhibition of DNA repair (Mullinger et al 1983), inhibition of ribonucleotide reductase (Frenkel et al 1964, Elford 1968), RNA synthesis (Rosenkranz et al 1968), thymidine kinase (Kaplay et al 1983), thymidylate synthase (Boehm et al

1982), suppression of DNA chain elongation (Wawra et al 1983), nicks in DNA (Jacobs et al 1970, Walker et al 1977), DNA fragments (Magnusson 1973a, 1973b, Martin et al 1977), loss of unstable amplified DHFR genes (Snapka et al 1983) and perhaps, interference in the ability of the components of the DNA synthesis replication complex (replitase) (Reddy and Pardee 1980) to assemble (Wawra et al 1983).

Hydroxyurea is a potent inhibitor of DNA synthesis in many organisms including bacteria (Rosenkranz and Levy 1965, Gale et al 1964, Elford 1968), insects (Swindlehurst et al 1971) and mammalian cells (Young et al 1967), and apparently it is exclusively cytotoxic to cells synthesizing DNA at the time of exposure (Sinclair 1965, Pfeiffer and Tolmach 1967). While some anti-mitotic activity has been reported, cells exposed to the drug progress to the G_1/S boundary and cease proliferation. In such cases, RNA synthesis and protein synthesis can continue, giving rise to enlarged metabolizing cells. This 'unbalanced growth' pattern is also seen as megaloblastosis in human patients treated with hydroxyurea (Frenkel and Arthur 1967, Spier et al 1973).

S-Phase Specificity and Inhibition of Ribonucleotide Reductase

S-phase specificity and disruption of the conversion of ribonucleotides to deoxyribonucleotides (Frenkel et al 1964, Rosenkranz and Levy 1965) by hydroxyurea is the result of direct inhibition of the enzyme ribonucleotide reductase. Hydroxyurea scavenges the tyrosyl free radical of mammalian and bacterial RRase, reversibly inactivating the enzyme activity (Atkin et al 1973, Sjoberg et al 1977, Akerblom et al 1981, Grasland et al 1982, Thelander et al 1985) with no apparent effect on the non-heme iron centre of the active reductase. The in vitro chemical regeneration of this radical in the E. coli system requires

extraction and reintroduction of the iron from the existing B_2 components (Atkin et al 1973). This is accomplished with a strong metal chelator under slightly denaturing conditions, resulting in an apoprotein, followed by exposure to iron (as Fe^{2+}) in the presence of ascorbic acid and oxygen. Thus, while the iron centre of RRase is believed to stabilize the tyrosyl radical, the observation by Moore (1969) that ferrous ions could partially reverse the inhibition of RRase by hydroxyurea, probably relates the metal chelating effects of hydroxyurea (Young et al 1967). Iron-chelating agents (i.e. EDTA, 8-hydroxyquinoline) are known to ameliorate the effects of hydroxyurea on RRase (Sato et al 1983). While such agents can be used to actually extract iron from RRase, at low concentrations they probably allow for a greater effective concentration of hydroxyurea. Cory, Lasater and Sato (1981) have suggested however, that hydroxyurea (and other agents like guanazole and IMPY) may complex RRase iron in situ in cellular systems where RRase activity is apparently independent of exogenous iron (i.e. Ehrlich tumor cells, Molt-4F cells).

Structure/activity studies of various urea analogs and related compounds have revealed that the hydroxylamine group (R-NOH) is required for inhibition of RRase (Young et al 1967, Rosenkranz et al 1971, Lea et al 1973). The carbonyl group is apparently not required for inhibition of RRase by the various compounds, but can affect the potency of the molecules (Young et al 1967). An hydroxylamine linked to its R group through a carbonyl carbon atom is known as a hydroxamic acid. The nature of the 'R' group in such molecules is also important to the effectiveness of hydroxamic acids (R-CONHOH). Interestingly, the inhibitory activity of substituted hydroxamic acid and hydroxylamines on ribonucleotide reductase correlates well with the antineoplastic

activity of these compounds (Adamson 1965). Such studies underscore the importance of RRase as a target for the chemotherapy of neoplasia and remind us of the ability of RRase to alter the growth of tumors.

Many of the effects of hydroxyurea may be attributable, at least in part, to its many potential breakdown products. The stability of this compound in dry and aqueous form is high, though gradual decomposition does occur. Chemotherapeutically, its use in humans must recognize its hydrolysis by urease produced by bacteria of the gastrointestinal tract (Fishbeine and Carbone 1963) and its conversion to urea by the liver, as demonstrated in rodents (Adamson et al 1965, Colvin and Bono 1970). The inhibition of urease by hydroxyurea and the accompanying enzymic hydrolysis of hydroxyurea to hydroxylamine, ammonia and carbon dioxide are well documented (Fishbeine et al 1965a, 1965b, Fishbeine and Carbone 1963). It has been noted that hydroxylamine is ineffective as an antineoplastic and thus the proposition that the breakdown of hydroxyurea to hydroxylamine is responsible for the antineoplastic behavior of hydroxyurea (Fishbeine and Carbone 1963) is in error (Adamson et al 1965). This type of analysis, however, does not detract from the realization that therapeutically administered hydroxyurea, catabolized in vivo can produce significant quantities of hydroxylamine, which can have diverse biological effects. Hydroxylamine cleaves thioesters and Fishbeine and Carbone (1963) have noted that patients treated with hydroxyurea have acteohydroxamic acid in the blood (one of the breakdown products of acetyl-coenzyme A and hydroxylamine). Hydroxylamine inhibits DNA, RNA and protein synthesis (Young et al 1964, 1967, Rosenkranz and Bendich 1964). Hydroxylamine is not toxic in micromolar quantities to CHO cells, yet chromosomal abnormalities may be seen at 10^{-5} M (Borenfreund et al 1964). The chromosomal anomalies were not

persistant, but presumably could give rise to advantageous and deleterious effects that could be maintained and fixed through selective pressure. Indeed, hydroxylamine is weakly mutagenic (Freese et al 1967). Hydroxylamine is oxidized to nitrate in vitro in rat liver preparations and may be an intermediate in the oxidation of ammonia (Saul and Archer 1984) in rat liver in vivo and by Fenton's reagent in vitro. Agents with a free hydroxylamine group (i.e. hydroxyurea and hydroxylamine) are known to produce hydrogen peroxide (H_2O_2) when exposed to oxygen (Freese et al 1967), yet Rosenkranz, Carr and Rosenkranz (1966) failed to decrease the bacteriocidal effects of hydroxylamine with exogenously applied catalase. These experiments, however, did not rule out an intracellular role for H_2O_2 in hydroxylamine cytotoxicity. Studies with bacterial and mammalian cells grown in vitro have indicated that hydroxyurea and hydroxylamine have different modes of action, as evidenced by the fact that the latter inhibits macromolecular sythesis in general, whereas hydroxyurea inhibits only DNA synthesis and at lower concentrations (Pollak and Rosenkranz 1967). Thus, the macromolecular evidence does not support the notion that hydroxylamine is produced in amounts significant enough to be responsible for all of the biological effects of hydroxyurea. The breakdown of these compounds to hydrogen peroxide, however, may be significant in situ in the local environment of the DNA molecule. H_2O_2 is a well recognized source of oxygen radicals and exhibits diverse biological effects. Radical production is self-propagating and immensely destructive. Oxygen radicals are known to be mutagenic to cells in culture (Lesko et al 1984,1985) and are thought to be involved in the aging process (Harman 1984). The role of the pro-oxidant state has recently been reviewed by Cerutti (1985). In this design it is

interesting to note that the genotoxicity of hydroxyurea is apparently mediated by a cytochrome P-450 dependent monooxygenase (Andrae 1984). Catalase abrogates hydroxyurea-induced repair in human lymphoblastoid cells incubated with a liver microsome activation system, NADPH and hydroxyurea (Andrae and Grem 1979). The damage of erythrocytes by hydroxyurea is partially prevented by radical scavengers (Malec et al 1984). Hydroxyurea induced cytotoxicity is also decreased by radical scavengers in L5178Y lymphoblasts (Przybyszewski and Malec 1982). The production of free radicals by hydroxyurea has also been implicated in the teratogenic effects of the drug and its ability to cause rapid cell death (DeSesso 1979). Many of the DNA strand scission effects of hydroxyurea may be due to free radical formation. It is tempting to speculate that the proposed formation of a hydroxyl radical during the reduction of ribonucleotides to deoxyribonucleotide by RRase (Reichard and Ehrenberg 1983) may be rendered detrimental to the cell when perturbed by hydroxyurea.

Phenotypically, hydroxyurea manifests its action on cells in two distinct ways, cytostasis and cytotoxicity, depending on the duration and severity of treatment. The inhibition of DNA synthesis by hydroxyurea is a very rapid and easily reversible phenomena. Cell death, however, takes much longer and is apparently restricted to cells actively synthesizing DNA semi-conservatively (Sinclair 1965, 1967, Pfieffer and Tolmach 1967, Kim et al 1967, Farber and Baserga 1969, Bachetti and Whitmore 1969).

Rosenkranz and coworkers (citations noted below) have studied the biological effects of hydroxyurea and its various derivations and breakdown products in detail using E. coli. The duality of cellular perturbations exhibited in hydroxyurea treated cells was recognized and

investigated. The working hypothesis was that a degradation product or activated form of hydroxyurea was responsible for cytotoxicity and the destruction of the DNA molecule (Jacobs and Rosenkranz 1970). It has been suggested that N-carbamoyloxyurea, an oxidation product of hydroxyurea, may be the reactive intermediate (Jacobs and Rosenkranz 1970, Cammeron and Jeter 1973). Hards and Wright (1981) using CHO cells have noted that N-carbamoyloxyurea is not as effective an inhibitor of RRase as hydroxyurea (by 12 fold) and that hydroxyurea is 1.5 times more potent as a cytotoxic agent. Thus it appears that hydroxyurea does not owe its cytotoxicity to conversion into NCU. These studies however, support the notion that both drugs have a similar cytotoxicity on a molar basis. Hards and Wright (1981) have noted that this is in contrast to the difference in their relative inhibitory effectiveness towards RRase. It appears that the cytotoxicity of these drugs may have a common or similar mechanistic basis. The production of free radicals may be such a common mechanism. The self- propagating nature of free radicals could explain why NCU and hydroxyurea exhibit similar cytotoxicities on a molar basis, while having different abilities in affecting RRase. If the radicals in question are produced during DNA synthesis, at or near the replicating DNA, a less efficient source of radical damage and less efficient inhibitor of RRase (i.e. NCU), would appear to have nearly the same cytotoxicity as a more efficient source of radicals. Thus the self propagating and rapidly destructive nature of free radicals may be masking the common mechanism of NCU and hydroxyurea induced cytotoxicity.

The cytotoxicity of hydroxyurea, unlike its cytostatic capabilities, has often been ascribed to its ability to damage and/or degrade DNA. Fresh solutions of hydroxyurea are ineffective on purified DNA,

but aged solutions, or solutions maintained at 37°C or higher, will degrade DNA *in vitro*. Such preparations have been shown to contain a number of breakdown products, including isohydroxyurea, carbamoyloxyurea and various nitrosoureas (Rosenkranz, Pollak and Schmidt 1969, Rosenkranz and Rosenkranz 1969, Jacobs and Rosenkranz 1970). Many of these compounds are mutagenic and carcinogenic. Compounds with a free-NOH group are mutagenic and carcinogenic and it is believed that these effects stem from the ability of such substances to form free radicals and peroxides (Freese *et al* 1967, 1968, Kotin and Falk 1963). It has been noted that such compounds predominantly induce inactivating DNA alterations rather than mutations (Freese *et al* 1967). Inactivating alterations can also be mutagenic, but are more frequently lethal and result in chromosomal breaks and gross abnormalities. Chromosomal aberrations induced by hydroxyurea and its derivatives include sister chromatid exchanges, chromatid fragmentation and breakage, translocations, chromosomal lag and changes in ploidy (Borenfreund *et al* 1964).

The Somatic Mutation Theory of Neoplastic Transformation

The somatic mutation theory and the aberrant cellular differentiation theory have arisen out of a desire to understand the mechanisms underlying neoplastic transformation by a wide variety of agents. While these hypotheses are not mutually exclusive, each makes specific, testable predictions (Monnat and Loeb 1983). A summary of the literature data in support of each model follows, but it should be realized at the outset that each model embraces a stable alteration in gene expression: while the somatic mutation theory model predicts an actual change in the structure of DNA, the aberrant differention theory predicts no change in DNA sequence or structure.

The mutational basis of neoplasia has received a tremendous amount of support in the literature since Boveri first suggested a relationship between chromosomal abnormalities and the heritable characteristics of malignancy. Siminovitch (1976) has defined

> "... a mutational event as that which involves any hereditable nucleotide base change, deletion, or rearrangement in the primary structure of DNA ..."

The recent discovery of oncogenes and the surge in interest for the cellular functions of the proteins encoded in such DNA were preceeded only recently by the first direct demonstration that perturbations in DNA could mediate neoplastic transformation. In studies by Barrett, Tsutsui and Ts'o (1978) Syrian hamster cells were treated with BUDR and examined for aspects of transformation after irradiation by near UV light. Populations which received BUDR followed by irradiation showed single strand DNA breaks and dose-dependent increases in mutation frequency for 6-TG and OUA resistance. Treated cultures also showed evidence of increased frequencies for morphological transformation and tumorigenicity in syngenic newborn hosts. Control cultures, treated with BUDR or near-UV irradiation alone, showed no increases in these transformation-linked properties. Since BUDR incorporates specifically into DNA, shifting its absorbance spectrum to longer wavelengths and thus increasing the number of photochemical lesions in BUDR-substituted DNA after irradiation, these studies demonstrated that changes in the DNA molecule could mediate neoplastic transformation in mammalian cells. Futher studies confirmed these observations in synchronized cells (TsuTsui, Barrett and Ts'o 1979) and revealed that transformation could only be induced in cultures treated in early to middle S-phase.

Continued investigation by Ts'o showed that DNA damage by [methyl-^{3}H] thymidine resulted in increased mutation frequency to 6-TG^{R} and neoplastic transformation (Lin, Takii and Ts'o 1982). Interestingly, the frequency of OUA^{R} transformants was not increased, suggesting that deletion was involved in the damaging effects. Liposome encapsulated DNAase I has been used as a third agent to specifically damage DNA and induce neoplastic transformation (Zajac-Kaye and Ts'o 1984). Treated populations of hamster cells exhibited increased saturation density, growth in semisolid medium and tumorigencity. DNAase I damaged cells also exhibited increased frequency of mutation to 6-TG^{R} but not OUA^{R} similar to tritiated thymidine treated cells suggesting gross damage as opposed to point mutation as the basis for genetic change.

Bouck and DiMayorca (1976) have demonstrated that mutational events are important in the progression of the transformed state. The spontaneously derived Syrian hamster cell line BHK21 (Stoker and Macpherson 1964) was assayed for the characteristics of mutation using the anchorage-independent phenotype as an indicator of transformation. The apparent spontaneous transformation frequency to anchorage independence was low ($8.8x10^{-7}$/viable cell), in keeping with the frequency with which spontaneous mutations appear to arise at defined loci in mammalian cells (Thompson and Baker 1973, Siminovitch et al 1972). The transformation frequency was increased with the mutagenic carcinogens nitrosomethylurea and 4-nitroquinoline-1-oxide and temperature-dependent transformants could be selected, suggesting that the more transformed phenotype was mediated by a protein. Ethylmethane sulphonate induced revertants for $temp^{s}$ anchorage independence were isolated, providing even further evidence of a mutational basis for aspects of the trans-

formed phenotype. The work of Bouck and DiMayorca is consistent with the somatic mutation theory but it should be emphasized that the hamster cell line chosen (BHK21) was already spontaneously transformed. Their studies employed a subclone with a low frequency of anchorage-independent growth. Thus while these studies provided evidence for a mutational basis of neoplastic progression, they are difficult to interpret in terms of initiation of transformation.

The progressive, multi-step nature of neoplastic transformation has been elegantly demonstrated by Barrett and Ts'o (1978a, 1978b). Mutations at the HPRT and Na^+/K^+ ATPase loci were scored after induction by benzo[a]pyrene and MNNG. These mutations were examined concomitantly with two indicators of transformation, morphological transformation and anchorage independent growth. Assays for morphologic transformation employed were those of Berwald and Sachs (1965) and DiPaolo et al (1969) and are based on the formation of foci and other altered patterns of growth by carcinogen treated cells. The morphologic transformation of golden Syrian hamster cells scored in this manner, shows a very high correlation with tumorigenicity in adult Syrian hamsters (Pienta et al 1977). Both of these measures of transformation indicated a basic difference between somatic mutation and phenotypic transformation. The frequency of morphologic transformation was 25-540 fold greater than that of somatic mutation, even though both appeared temporally related. Moreover, anchorage independence appeared at a frequency comparable to somatic mutation, but only after 32-75 doublings after treatment with carcinogen. These studies are consistent with the multistep nature of carcinogenesis in vivo and emphasize the progressive and dynamic character of neoplastic transformation in vitro.

Modification of chromosomal material by agents which interact with DNA provides a further line of evidence in support of the somatic mutation theory of neoplasia. Chemical carcinogens and radiation have been used extensively to study the relationship between the induction of neoplasia and perturbations occurring in the DNA molecule. Berwald and Sachs (1963) demonstrated the transformation of golden hamster and C57B1/6 mouse cells with 3,4-benzpyrene and 3-methylcolanthrene. Previous attempts with a variety of cell types had failed. The chemical transformants they obtained were similar to polyomavirus transformed lines of the same parental origin.

Virtually all carcinogens are mutagenic, though there are very important exceptions, and they tend to interact, covalently and noncovalently, with the DNA molecule. The covalent binding of dibenz [a,h] anthracine to DNA was first demonstrated by Heidelberger and Davenport (1961). Since then, a great many types of modifications to DNA have been characterized. The formation of adducts of deoxyadenosine by polycyclic aromatic hydrocarbons correlates with tumorigenesis in mouse skin (Digiovanni et al 1979). Eastman and Bresnick (1979) have demonstrated a correlation between persistent binding of 3-methylcholanthrene to mouse lung DNA and increased susceptibility to pulmonary neoplasia. The specific interaction of carcinogens with DNA and the subsequent formation of DNA-adducts does not approbo the mutational nature of chemically-induced neoplastic transformation, but does suggest a means whereby such agents may upset the cell. Indeed, an initial mutational event may describe the origin of transformation, but certainly, it alone cannot account for the tumorigenic phenotype (Barrett and Ts'o 1978a).

The initiation/promotion model of carcinogenesis in mouse and rabbit skin (Rous 1941, Berenblum 1941) is an artifical construct that

has developed as a conceptual framework upon which the multi-step nature of chemical carcinogenesis might be better understood (Trosko et al 1981). Thus while complete carcinogens can initiate neoplastic transformation apparently through mutation-selection mechanisms, the initiated state does not guarantee growth autonomy in vivo (Farber 1984a). Initiation does not always result in morphological transformation in vitro and morphologically transformed cells seen after carcinogen treatment are not always stable and tumorigenic. In vivo focal proliferations, such as polyps in the colon, nodules in the liver and papillomas in the skin, represent pre-cancerous lesions of initiated cells which often preceed cancer in these organs, but such lesions are not always seen before such disease (Farber 1984b). The lack of growth autonomy in such lesions is indicative of the need for further events, the need of a promotional environment, for the full expression of the disease. Although a new state of differentiation exists in such lesions, the cancerous phenotype is not expressed.

The effects of tumor promoters on DNA synthesis and structure are well documented. Birnboim (1982) has suggested that the action of PMA as a tumor promoter may be related to the DNA strand breakage seen in the PMA-induced respiratory burst of human leukocytes. The inflammatory response of skin exposed to PMA followed by the phagocytic respiratory burst could produce damage to the DNA of initiated cells through the production of active oxygen species. Promotion could thus be faciliated by the changes in gene expression and advantageous mutations that might result from such damage. TPA and teleocidine ellicit effects on macrophage in a manner similar to that of PMA (Keisari et al 1984). The oxidative burst is associated with the increased production of superoxide anions and hydrogen peroxide (Freeman 1984), resulting in a pro-

oxidant state. The prooxidant state can result in gross changes in the biology of the cell including chromosomal aberrations and mutations, and is thought to play an important role in tumor promotion (Cerutti 1985). Substantiating this view are the findings that catalase suppresses MMTV induction by PMA (Cerutti 1985) and antioxidants suppress ODC induction by PMA (Weeks et al 1982). Goldstein et al (1981) have shown that the rate of superoxide anion radical (O_2^-) production by human polymorphonuclear leukocytes correlated with the tumor promoting activity of PMA. These authors also note that retinoids inhibit both tumor promotion and phagocytic O_2^- production. Phagocytes themselves have been shown to be carcinogenic. C3H 10T 1/2 mouse fibroblasts show an increased frequency of transformation when treated with TPA and human neutrophils, or with neutrophils alone (Weitzman et al 1985). Simple exposure of the neutrophils to the mouse fibroblasts was found to increase the release of superoxide, without the need for the phorbol ester as an activator of the oxidative burst.

The exact molecular species mediating the effects seen with tumor promoters on radical formation are unknown, but the role of active oxygen species in aspects of tumor promotion is well documented. The abilities of superoxide in DNA strand scission (Lesko et al 1980), chromosome breakage and sister chromatid exchange (Emerit et al 1982) suggest a mutational basis for tumor promotion in some systems.

Radiation induced DNA strand scission and carcinogenesis also provides significant evidence in support of the somatic mutational theory of neoplasia and is another agent known to exert its effects via reactive oxygen species. Many reports have documented the ability of γ-irradiation to induce neoplastic transformation in primary diploid cells (Namba et al 1978, 1985). X-rays are also known to induce the

neoplastic state in such cell systems (Borek 1980) and Wolman et al (1985) have recently documented the association between the acquisition of tumor markers in metastatic foci after irradiation of the parental tumor. γ-irradiation of Syrian hamster cells is mutagenic (Lesko et al 1984, 1985) and activated oxygen species have been shown to be involved in the process.

Active oxygen species are capable of acting over different distances intracellularly, owing to their relative reactivity and the specificity of that activity. The hydroxyl radical is highly reactive and unselective in its attack on biomolecules and thus its potential damage will occur nearer its site of production than superoxide (O_2^-). Superoxide and hydrogen peroxide are less reactive and thus may be able to exert their effects over a greater area (Freeman 1984). Turrens et al (1982) have shown that H_2O_2 can cross mitochondrial membranes and other studies have indicated that the plasma membrane is equally permeable (Schroy et al 1981). Thus while active oxygen production may produce lesions in chromosomal DNA, a plethora of effects may be seen elsewhere in the cell. Indeed, some of the effects on DNA may be indirect, via other cellular biomolecules. Lipid peroxidation resulting from the prooxidant state is believed to play a role in carcinogenesis (Cerutti 1985, Slater et al 1984) and is stimulated by γ - and UV-irradiation, iron, NADPH and thiols. Lipid peroxidation and its byproducts, such as 4-hydroxy-alkenals, are known to inhibit DNA synthesis (Popov and Konev 1985, Slater et al 1984). Lipid peroxidation is also involved in the production of leukotrienes (Samuelsson 1983).

The production of DNA strand breaks by hydrogen peroxide is known to involve a transition metal in vivo. Observations by a number of laboratories has indicated the importance of iron in the production of

the hydroxyl radical from H_2O_2 (Lesko, Ts'o and Umans 1969, Umans, Lesko and T'so 1969, Filho and Meneghini 1984, Nagata et al 1973). Formation of the highly reactive hydroxyl radical is generally thought to occur via the Haber-Weiss reaction:

$$O_2^- + Fe^{3+} \longrightarrow O_2 + Fe^{2+} \qquad (1)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + \cdot OH \qquad (2)$$

$$O_2^- + H_2O_2 \longrightarrow O_2 + OH^- + \cdot OH \qquad (3)$$

The Fenton-like reaction shown above (reaction #2) is also involved in the in vitro activation of 3,4-benzopyrene (Nagata et al 1973) and the in vivo production of single-strand breaks in human fibroblasts (Filho et al 1984). Strong chelators of iron, o-phenanthroline and bipyridine, abolish H_2O_2 induced strand scissions. The authors have noted that since the intracellular diffusion of $\cdot OH$ is so low ($\sim$60) and its reactivity is so high, the production of strand scissions by H_2O_2 occurs through production of the reactive species at or very near the DNA molecule. EDTA abrogates the effect of H_2O_2 on strand scission and since EDTA-Fe^{2+} is known to be such a suitable reagent for the Fenton reaction, Filho et al (1984) have concluded that EDTA must be removing iron localized at the DNA molecule. It is interesting to note that the strand scission effect of hydrogen peroxide is mediated by a non-dialysable compound (Meneghini and Hoffman 1980). It is tempting to speculate that the iron-stabilized, radical-catalyzed reduction of ribonucleotides to deoxyribonucleotides near the replica-

tion fork by ribonucleotide reductase may be of special significance to the types of DNA strand scissions induced by hydroxyurea during DNA replication. The apparent need for DNA synthesis in the fixation of initiation (Cayama et al 1978) and the promotional effects of phorbol esters may have an iron based, perhaps RRase mediated basis for some of the structural damage they are known to produce in chromosomal DNA. Thomas et al (1985) have demonstrated that ferritin, the primary storehouse of intracellular iron, may provide the free-iron necessary for O_2^- dependent lipid peroxidation. Free iron is essentially nonexistant in the cell and the authors speculate that sequestration of iron intracellularly is a protective mechanism against oxidative cytolysis involving O_2^- . Such action by superoxide may thus increase the iron-oxygen oxidation-reduction reactions initiating lipid peroxidation.

The hereditary nature of neoplasia supports the somatic mutation hypothesis of cancer. DNA repair deficient disease states such as ataxia telangiectasia, xeroderma pigmentosum, Fanconi's anaemia and immune deficiency diseases have been shown to be associated with an increased incidence of malignancy (Knudson 1974). Some dominantly inherited disorders such as retinoblastoma and familial polyposis are also associated with specific cancers. The heritable nature of the transformed phenotype is also manifest in the transplantability of tumors and the tumorigenicity of permanent cell lines. Chromosomal anomalies are also found associated with neoplasia. The translocation of part of human chromosomes 22 and 9, the Philadelphia chromosome, serves as a marker of chronic granulocytic leukemia. Recently, Hesterberg and Barrett (1985) demonstrated the apparent direct interaction between asbestos fibres and chromosomes. The Syrian hamster embryo cells treated with asbestos showed a 20 fold increase in the

incidence of chromosomal anomalies. The authors have suggested that the induction of neoplastic transformation by mineral fibres may utilize a mechanism involving contact with the DNA molecule and numerical chromosomal changes due to asbestos-induced chromosomal lag.

The discovery of oncogenes and their subsequent investigation has underscored the importance of the DNA molecule and its mutation in relation to neoplastic transformation. The identification of oncogenes by transfection of DNA from tumor cell lines into susceptable cells (Shih et al 1979, Cooper et al 1980) or solid human tumors (Pulciani et al 1982) through RNA tumor viruses, has led to the discovery of specific proteins capable of altering the regulatory characteristics of cells. Both oncogenes and anti-oncogenes have now been described (Todaro 1984, Knudson 1985).

The presence of oncogenes in cellular systems was predicted by Huebner and Todaro (1969) in their virogene/oncogene hypothesis. The viral etiology of animal cancer had been well known for more than fifty years (Rous 1911) but the oncogene hypothesis predicted the presence of normal cellular genes homologous to the viral transforming genes. Comings (1973) proposed a similar idea and recognized the normal role such genes might play in cells and their special link with differentiation/development. Evidence for the presence of cellular proto-oncogenes homologous to viral transforming genes was provided through studies with the avian sarcoma virus src (v-src) gene (Stehelin et al 1976, Spector et al 1978). The cellular gene, c-src, has since been shown to exist in the genome of all vertebrates examined.

The transforming potential of tumor cell DNA was first recognized in the experiments of Shih et al (1979). Mouse and rat cells transformed by various agents (3-methylcolanthrene, 7,12-dimethylbenzan-

thracene, benzo[a]pyrene, UV- and X-rays) were used as sources of donor DNA in tranfection experiments with NIH 3T3 cells as host. Purified DNA from BALB/c 3T3 and C3H 10T 1/2 cells transformed by the chemical carcinogen 3-methylcolanthrene proved to be efficient in transfecting the NIH 3T3 cells, as measured by the production of foci in treated monolayer cultures of recipient cells. Subsequent work also revealed the tumorigenicity of these transformed populations. Thus, the transmissable nature of the transformed phenotype by purified DNA supports the role of DNA in transformation, yet a mutational basis for the phenomena cannot be assumed.

The discovery that a single base change in the structure of a proto-oncogene is sufficient for its activation is germane to the somatic mutation theory of neoplasia. Studies on the ras oncogene family have been particularly enlightening in this regard. The protein products of the ras family (Harvey:Ha, Kirstein:Ki) are 21 kDa (p 21) proteins found associated with the plasma membrane which have a GTP binding and GTPase activity. The ras^H gene product (and transforming principle) of the human bladder carcinoma cell line EJ (T24)(Parada et al 1982) is known to possess an amino acid change (gly-val) in the 12th position, resulting from a point mutation (GT transversion) in the 12th codon of the C-Ha- ras gene (Tabin et al 1982, Reddy et al 1982, Taparowsky et al 1982, Capon et al 1983). Mutations in codons 12, 59 and 61 are particularly important in the transforming and functional ability of c-Ha-ras (Varmus 1984). Indeed, both the GTPase and autophosphorylating activities of Ha-ras are affected by mutations in these positions (Gibbs et al 1984, McGrath et al 1984, Manne et al 1985). While a change in position 12 (which can be detected by virtue of the loss of a cleavage site for Hpa II and Msp I) in c-Ha- ras does

not appear to be involved in many human carcinomas, the activation of Ki-ras is fairly common (Cooper and Lane 1984) and also primarily involves the 12th codon. A third member of the ras family N-ras (Shimizu 1983a, 1983b) has been detected in a human neuroblastoma cell line, in three human T-cell leukemias (Souyri and Fleissner 1983) and in the bone marrow cells of a patient with acute myeloblastic leukemia (Gambke et al 1984). The lack of correlation between N-ras activation and level of expression suggests that mutations are also implicit in the transforming ability of this ras member. The importance of mutation in the twelth codon of ras sequences is underscored by the observations of Santos et al (1983) where a spontaneous GT transition occurred resulting in asp rather than gly. The authors have noted that a conformational change in the p21 product may be sufficient for acquisition of the transformed phenotype.

The activation of oncogenes has been shown to occur by a variety of mechanisms, some mutational and some involving changes in expression (Hunter 1984, Land et al 1983a, 1983b). Oncogene activation and expression can be acquired not only through altered structure from point mutation but from amplification, juxtaposition, the presence of enhancer sequences or novel transcriptional promoters and chemical carcinogens as well. The result of such activation is an altered or increased protein product whose aberrant expression can often be correlated with oncogenicity. The c-mos gene, homolog of the mos oncogene of Moloney sarcoma virus, can be activated by insertion of the LTR of an IAP particle, 5' to its coding sequence (Hunter 1984, Cooper and Lane 1984). Integration of the avian leukosis virus (ALV) transcriptional promoter in provirus form next to an oncogene can induce its expression. Activation of c-myc leading to its enhanced expression has been shown to be caused by the

proviral insertion of ALV next to the cellular oncogene in chick bursal lymphocytes (Hayward et al 1981). Rearranged and deregulated myc and myb in plasmacytomas and immunoglobulin gene regions of the chromosome have been well documented (Astrin and Rothberg 1983). Transcriptional levels of oncogenes can be modulated by enhancer sequences which can affect transcription upstream and downstream from promoters and exert their influence over a substantial distance of the DNA molecule.

The amplification of oncogenes as a mechanism of increased dosage of oncoprotein product, has received much attention. The Ki-ras gene is amplified $\sim$50 fold in mouse adrenocortical tumor cells (Schwab et al 1983) and 3-5 fold in human colon carcinoma cells (McCoy et al 1983). N-myc is amplified in many neuroblastomas (Stark and Wahl 1984, Kohl et al 1984), and N-myc amplification has been implicated in the progression of a neuroblastoma tumor in a human patient (Schwab et al 1984). The human myeloid leukemia cell line, HL60, contains multiple copies of myc-related DNA sequences (Collins and Groudine 1982), yet other human myeloid leukemic cell lines do not exhibit this amplification. The proto-oncogenes c-abl and c-myb are amplified in a chronic myeloid leukemia cell line and in acute myeloid leukemia (Varmus 1984).

Specific gene amplification has been demonstrated in a wide variety of cell systems and neoplastic diseases, but the extent of the role played by the amplification of oncogenes in carcinogenesis remains controversial (Schimke 1984). Certainly, the levels of oncogene products can affect the neoplastic state. The ALV mediated activation of c-myc, noted earlier, and subsequent transformation of NIH 3T3 cells seen by Hayward et al (1981) is an example. DeFeo et al (1981) and Chang et al (1982) have shown that increased levels of c-Ha-ras, produced by the ligation of the LTR of Ha-MuSV, were capable of inducing

neoplastic transformation of NIH 3T3 cells. Similar results were obtained by Blair et al (1981) with the LTR of Moloney sarcoma virus. Indeed, in the original bladder carcinoma line used as the DNA donor in the studies of Shih et al (1979), the oncogene being transferred was c-Ha-rasl (Parada et al 1982), from 3-methylcolanthrene treated cells.

Recently, Shalloway et al (1984) have demonstrated that increased expression of c-src does not fully transform NIH 3T3 cells. In a study designed to test whether v-src-induced transformation is mediated by overexpression of the gene or functional (perhaps structural) differences between c-src and v-src, these authors demonstrated that elevated levels alone could not induce the transformed state to the same degree as in v-src transformed cells. These results suggested that v-src induced transformation may result from the combined effects of overexpressed protein product and mutational differences between c-src and v-src.

Tarpley and Temin (1984) have also investigated src gene over-expression. Insertion of v-src at the 3' end of an avian retroviral selectable vector containing the herpes simples virus type I TK gene resulted in lower levels of expression and a reduced toxic effect, but an enhanced transforming effect. High expression of src seen when the gene was inserted at the 5' end of the retroviral genome resulted in an acute toxic effect and thus no transformation. The role of mutation and dosage in $pp60^{v\text{-}src}$ mediated transformation is complex and remains an active area of investigation.

The activation of oncogenes by many chemical carcinogens could support the somatic mutation theory, since many of these agents are mutagenic as well. The 3-methylcolanthrene induced transformation of mouse fibroblasts that Shih et al (1979) transfected into NIH 3T3 cells,

has already been mentioned. The nature of the alteration in DNA by 3-MC has been discussed (Weinberg 1982) and it appears that 3-MC activates the same oncogene in independently transformed C3H 10T 1/2 mouse fibroblast lines (Shilo and Weinberg 1981). *ras*^{K} activation has been demonstrated in 3-MC transformed mouse fibrosarcomas (Eva and Aaronson 1983). Fibrosarcomas induced with 20-methylcolanthrene in Japanese quails, however, indicate that the transcriptional activation of the c-*myc* gene may be due to hypomethylation (Saule *et al* 1984). Recently it has been demonstrated that DNA from epidermal mouse squamous cell carcinomas induced by the initiation/promoter pair DMBA/TPA, transfers an activated, apparently duplicated, c-Ha-*ras* that morphologically transforms NIH 3T3 cells (Balmain and Pragnell 1983).

The cellular transformation of NIH3T3 cells to the tumorigenic phenotype by various oncogenes should be studied in light of the fact that this cell line is immortal; it is a permanent cell line amenable to tumorigenic activation by a single oncogene (Levine 1984, Klein and Klein 1984, Land, Parada and Weinberg 1983b). The cell line was originally developed as a highly contact-inhibited, anchorage dependent system for the study of viral transformation (Jainchill *et al* 1969). The transfection of a single oncogene (i.e. *ras*) into an established cell line like NIH 3T3 can transform the recipients, but similar transfer into primary, senescing cell strains requires a second transfecting oncogene or a cotransfecting oncogene before the fully transformed, tumorigenic state is induced (Land, Parada and Weinberg 1983a, 1983b). Thus it was proposed that oncogenes may complement one another in bringing about the transformed state, perhaps in a manner similar to the initiation and promotion model of 2-stage skin carcinogenesis. While that model details the strict sequence of chemical carcinogenesis

in some systems, oncogenes from the two complementation groups that have been recognized can apparently induce the transformed state in any order of exposure (Levine 1984). These two complementation groups comprise the immortalizing myc-type oncogenes (c-myc, c-myb, adenovirus E1A, polyoma large T, plt gene) and the ras-like oncogenes (c-ras, c-src, adenovirus E1A, polyoma middle T, pmt gene) responsible for the induction of the tumorigenic phenotype. Evidence for multi-step models of carcinogenesis such as this one, also come from transformation studies utilizing the herpes simplex genome (Jariwalla, Aurelian and Ts'o 1980, 1983, Manak, Aurelian and Ts'o 1981) where immortalization and conversion to tumorigenicity can be conveyed by two separate restriction fragments of the HSV-2 genome. Recent analysis has indicated that the tumorigenic transformation of preneoplastic GSH cells (immortalized with the carcinogen diethylstilbestrol) by v-Ha-ras may require three or more steps in total to induce tumorigenicity (Thomassen, Bilmer, Annab and Barrett 1985). Studies such as these are doing much to advance our understanding of the progressive, multi-step nature of neoplastic transformation.

Oncogene activation is also implicated in the very late stages of carcinogenesis (Albino et al 1984, Schwab et al 1984) where the metastatic phenotype provides for the dissemination of the primary tumor. This is the stage of cancer that has progressed the furthest and is the most difficult to treat and prevent its often lethal consquences. Transfer of the metastatic phenotype by transfection with cellular DNA has recently been demonstrated (Thorgeirsson et al 1985, Bernstein and Weinberg 1985). NIH 3T3 cells transfected with acute lymphocytic leukemic cell DNA contain activated N-ras oncogenes and the metastatic phenotype, whereas metastatic tumor DNA, as donor, efficiently trans

ferred the metastatic phenotype, but ras and myc like sequences were not detected.

The two oncogene complementation groups apparently demonstrate site specificity intracellularly, with the myc-like proteins being nuclear and the ras-type proteins being plasma membrane-associated (Klein and Klein 1984, Cooper and Lane 1984). A distinct and integrative role for the nuclear and plasma membrane localization of immortalizing and establishing oncogenes and chemical agents is implied in the work of Connan et al (1985). Treatment of primary rat embryo fibroblasts with the membrane active tumor promoter TPA after transfection with polyoma virus plt or rearranged myc, two nuclear associated immortalizing genes, resulted in focus formation, whereas without the application of TPA, no foci were formed in the immortalized transfectants. The ras oncogene and polyomavirus pmt had the same effect as TPA. Thus the membrane-associated, group II oncogenes appear to behave like tumor promoters and the immortalizing, group I oncogenes seem to behave in a manner analogous to most physical and chemical carcinogens.

The mutator phenotype, a measurable change in the rate or frequency of mutation (usually drug resistance) at a specific genetic locus, between a variant and parental cell population, has been implicated in mutational mechanisms of neoplastic transformation (Loeb et al 1974, Nelson and Mason 1972). The fidelity with which DNA is replicated is alterable at many levels, including nucleotide precursor pool imbalance (Meuth 1984a), template structure (Hillebrand and Beattie 1985), metal ions (Kunkel and Loeb 1979) and alterations in the activity or structure of key enzymes like DNA polymerase (Conkell and Yanofsky 1970, Liu et al 1983), ribonucleotide reductase (Weinberg et al 1981, Arpaia et al 1983, Ayusawa et al 1983, Roguska and Gudas 1984, Gjerset et al 1985), dCMP

deaminase (Weinberg et al 1981, Maus and Haynes 1984a, Maus et al 1984b, Sargent and Mathews 1985), deoxycytidine kinase (Meuth 1983), CTP synthetase (Trudel et al 1984, Chu et al 1984) and ornithine decarboxylase (Kamatani et al 1984). Many anti-neoplastic agents which target important replicative enzymes and upset the nucleotide pool balance, are known to be effectors of replicative fidelity and to induce morphological transformation and neoplasia. Adriamycin induces transformation in vitro and in vivo in rats and mice (Bucciarelli et al 1982, Marquardt et al 1976). Hydroxyurea induces morphological transformation and tumorigenicity in mouse embryo cells (Chlopkiewicz and Koziorowska 1983) and tumorigenicity in hamster cells (Jones et al 1972). Transformation of hamster cells and mouse embryo cells by araC is well documented (Benedict et al 1975, Jones et al 1977, Benedict and Jones 1979). Similar results have been obtained with methotrexate and 5-fluorouracil. The increase in mutation frequency and neoplastic transformation properties of cells with a disturbed replicative apparatus induced by imbalanced nucleotides or by altered enzymes of DNA metabolism provides strong evidence for the mutational basis of neoplasia. Indeed the very chemotherapeutic agents used to effect such enzymes in neoplastic disease states can themselves induce transformation. Once again we are reminded of the importance of nucleotide metabolism and the DNA synthetic machinery in the process of the neoplastic state in vivo and transformation in vitro.

The Aberrant Differentiation Theory of Neoplastic Transformation

The abberrant differentiation hypothesis of neoplastic transformation encompasses all those phenomena of neoplasia not readily accounted

for by a mutational mechanism. The plethora of evidence linking differentiation and neoplasia implores us to simultaneously investigate their relationship, yet the strength of the observations in favor of a mutational basis for the initiation of neoplasia has clouded popular opinion regarding the role of non-mutational changes in neoplastic disease. Although there is a paucity of data in this regard, there is a body of literature which recognizes the epigenetic nature of neoplastic progression. Indeed, the literature tends to favor a mutational basis for initiation of the tranformed state, whereas progression appears alterable by both genetic and epigenetic events. The following discussion relates the evidence regarding non-mutational changes in neoplasia, but the reader is reminded that there is often no clear distinction between genetic and epigenetic phenomena and also that the actual demonstration of a mutational event is exceedingly difficult and its operational definition is still a controversial area (Siminovitch 1976, Ts'o 1979a, Davidson 1979).

Support for the aberrant differentiation hypothesis arises from work in many different areas. Some of the strongest evidence is based on 1) the reversibility of the transformed state in some cell systems, 2) the non-mutagenic nature of tumor promotors, 3) the expression of developmentally regulated genes, often of early (embryonic) stages, 4) the ability to induce differentiation in cultures of malignant cells, 5) changes in transformation attributable to alterations in DNA structure, but not sequence (methylation, helical form, etc.) and 6) the ability to modulate the transformed state by changes in the cytoskeleton and intercellular communication.

The reversibility of the transformed state has been documented both _in vivo_, and in a number of cell culture systems _in vitro_. Puck and

associates have pursued the dibutyryl-cAMP-induced reversion of transformation in Chinese hamster ovary cell lines for over a decade (Hsie and Puck 1971, Puck 1977, 1979, Rumsby and Puck 1982, Ashall and Puck 1984). The microtubular-microfilamentous network comprising the cytoskeleton has been implicated in the cAMP-mediated reversion, for a variety of reasons. Colcemid and vinblastine prevent the nucleotide-induced reversion to the fibroblastic state (Hsie and Puck 1971) and this reversion is rapidly reversible in the absence of cAMP (Johnson et al 1971). Thus the polymerization of tubulin and formation of microtubules is thought to be the essence of cAMP mediated reversion. Puck has noted that the action of proteases, lectins and antibodies directed at the cell surface, and the secretion of fibronectin by normal cells (while depressed in transformed cells) with its relation to the extracellular matrix, provide evidence that the cytoskeletal framework is intimately involved in the control of cell and nuclear shape. The reverse transformation phenomenon of cAMP has been shown by electron microscopy to be concomitant with changes in the cytoskeleton to a more ordered structure (Porter et al 1974).

Accompanying the morphological changes seen after cAMP treatment of CHO-K1 cells is a reversion to normal DNAse I sensitivity (transformed cells are more resistant than normal cells) and this effect is seen only with intact cells and not with isolated nuclei (Ashall and Puck 1984), thus further substantiating the intimate relationship between the nucleus and cytoskeletal components. These data support a mechanism of neoplastic transfromation based on perturbation of microfibrillar function, complex enough to encompass the aneuploid nature of malignancy (Puck 1979). Interestingly, Iype et al (1985) have suggested that methapyrilene, an apparently nongenotoxic carcinogen, disturbs elements

of the cytoskeleton in rat liver epithelium cell cultures and may produce aneuploidy when cells are exposed continuously to this agent.

Thyberg (1984) has reviewed the literature on the initiation of DNA replication and the microtubular cytoskeleton and has concluded that the integrity of the cytoskeleton can either inhibit or enhance passage into S-phase in different cell systems. The importance of microtubular structures in cell division is stressed in the CHO variants of Cabral's group, which require the presence of the microtubular stabilizing agent taxol for continued cell growth (Cabral 1983, Cabral et al 1983). Taxol is an antineoplastic known to inhibit the initiation of DNA synthesis by epidermal growth factor or thrombin (Crossin and Carney 1981) in a manner believed to underscore the ability of microtubular depolymerization to initiate DNA synthesis. Microtubular and microfilament disorganization has been documented in many laboratories as characteristic of the transformed state and is used as an indicator of morphological transformation. The importance of the cytoskeleton in cellular transformation has recently been reviewed (Scott 1984).

Ornithine decarboxylase induction in G_1 arrested normal human fibroblasts, Hela and CHO cells is readily seen after a change to fresh serum supplemented medium (Rumsby and Puck 1982), but whereas the response in the fibroblasts is essentially eliminated by the depolymerizing agent colchicine, Hela cells show little inhibition. Thus it would appear that the normal cell is more dependent upon a cytoskeletal system for reinitiation of growth than is the transformed cell and that ODC is critically associated with normal and transformed growth states and molecular aspects relating to the cytoskeleton. Studies in other laboratories have also indicated the importance of ODC activity in cell growth and the aberrant regulation of this activity has been associated

with the transformed state (O'Brien et al 1975, Weber 1983). The product(s) of ornithine decarboxylase, putrescine (and CO_2), is the precursor of spermidine. Putrescine stimulates S-adenosylmethionine decarboxylase, effectively removing S-adenosylmethionine from the pool used in transmethylation reactions (Pegg et al 1983). The high turnover rate exhibited by ODC and the apparent rate-limiting activity which it exhibits on spermidine biosynthesis underscore the importance of this activity to cell growth (O'Brien et al 1975, Tabor and Tabor 1984). While the biological functions of polyamines are poorly understood, research in this area continues to illuminate the molecular significance of ornithene decarboxylase in the growth response and in tumor formation.

The apparent ability of cytoskeletal disarray to affect the dynamic state of chromatin and the transformed properties of eukaryotic cells suggests the presence of a mechanochemical organization in the cell, linking the cell membrane and its components to the nuclear membrane and its matrix associated components, including specific chromosomal loci, via a cytoskeletal framework composed of the microtubular and microfibrillular systems (Ashall and Puck 1984). The biology of the nuclear envelope and the components of its matrix is complex and only recently have studies provided sufficient details for discussion regarding the function of the matrix and its components. A large body of evidence now indicates that the origins of DNA replication in chromatin are attached to the nuclear envelope matrix (van der Velden et al 1984) and contain components of the replicative machinery, including DNA polymerases (Brown et al 1981, Smith and Berezney 1983) and topoisomerase (Nishizawa et al 1984). Foster and Collins (1985) have suggested that rates of DNA synthesis may be determined by the binding equilibruim of DNA POL α with

the nuclear matrix. Ribonucleotide reductase is also apparently associated with the perinuclear region (Engstrom et al 1984) and has been isolated as a membrane bound complex with other DNA synthetic enzymes (Baril et al 1972). The nuclear envelope (or matrix) has been been shown to be the site of UV-induced repair (McCready and Cook 1984) and the active transcription of genes (Jackson et al 1981, Ciejek, Tsai and O'Malley 1983), including the synthesis of influenza virus RNA (Jackson et al 1982). Clawson and Smuckler (1980) have indicated that hnRNA (heterogenous nuclear RNA) may be processed while attached to envelope associated structures. Indeed the work of Jackson et al (1981) suggested that transcripts are attached to the nuclear cage as they are produced and they note that Cervera et al (1981) have shown the later attachment of such transcripts to the cytoskeleton in the cytoplasmic form, perhaps providing a structural framework upon which processing may occur. Interestingly, organelles are known to translocate bidirectionally on microtubules (Schroer and Kelly 1985).

Continuing work by Jazwinski's group implies that initiation of DNA synthesis may be regulated by a dynamic equilibrium between replicon loci attached and unattached to higher order structures in the cell (see Jazwinski and Edelman 1984). The decreased need for cytoskeletal integrity manifested by transformed cells may relate to the differences seen in nuclear matrix protein patterns seen between normal and transformed cells (Kuzmina et al 1984) and demands a closer examination of the structure/function relationship between the components of the nuclear matrix. Undoubtedly the most important structures identified with the envelope are the origins of DNA replication. Specific DNA sequences have been found associated with replication origins and the

nuclear matrix and are generally found to be enriched in repeat sequence elements (Goldberg et al 1983, Neuer and Werner 1985).

Further evidence for the involvement of the DNA replication origins with the nuclear matrix has been provided by the observation that supercoiled loop domains in DNA are of approximately the same length as replicons (Vogelstein, Pardoll and Coffey 1980, Buongiorno-Nardelli et al 1982). Given that the sites of replication are fixed, it has been proposed that replicating loops are motile, moving through the matrix complex during synthesis.

One fascinating possibility regarding cytoskeletal organization and gene expression has recently been noted by Crabbe (1985a, 1985b). He has noted that if oncogenes are capable of altering the cytoskeleton, then proto-oncogenes may interact directly with the cytoskeleton and perhaps exhibit similar physical properties if both act at a common site or if the proto-oncogene mimicks the cytoskeletal protein. Sequence comparison revealed homologies between cytoskeletal proteins and proto-oncogene products. Crabbe suggests that a relationship exists between mechanisms of neoplastic transformation, cytoskeletal changes, and differentiation that may involve direct interaction between oncogene products and the cytoskeleton or perturbation of nucleotide signal-transduction. In this respect it is interesting to note that tubulin, the major constituent of microtubules, binds GTP and hydrolyzes it to GDP during polymerization in vitro. The precise role of the guanine nucleotide in microtubule formation is poorly understood, but Wehland and Sandoval (1983) have shown that mouse and human cells injected with the non-hydrolyzable GTP analog guanosine 5'-[α,β-methylene] trisphosphate show profound changes in cytoskeletal structure, including the Golgi apparatus. Photoaffinity labelling of tubulin with GTP showed

preferential incorporation into the α-subunit exchangeable site, rather than the β-subunit and the free state of tubulin bound more GTP than the assembled state (Nath et al 1985). Recently Kadowaki et al (1985) have demonstrated that the substrate of the insulin receptor kinase may be tubulin. The purified kinase phosphorylates native tubulin on tyrosine residues. The authors have noted that microtubular involvement in transformation dictates the need for analysis of tyrosine-kinases in general and their relationship with microtubules. That alteration in the biology of tubulin may affect gene expression relating to transformation is supported by the recent observation that of 28 anticancer agents tested for Epstein-Barr virus early antigen (EBV-EA) activation in the human lymphoblastoid cell line Raji (including adriamycin, diethylstilbestrol, methotrexate, and 5-fluorouracil) only the antitubulin agents vinblastine and vincristine showed EBV-EA enhancing activity (Fujita et al 1985). Further analysis revealed that other antitubulin agents produced the same results. The authors noted that tumor promoters and antitubulin agents share properties in common regarding their effects on the cytoskeleton.

Transformation has been induced in the apparent absence of mutation using diethylstilbestrol. Syrian hamster embryo cells treated with DES show a frequency of morphological transformation equal to that of benzo [a] pyrene, but somatic mutation to OUA^R and $6TG^R$ showed no increase in incidence over untreated controls (Barrett, Wong and McLachlan 1981). The results suggest that transformation by DES may occur through non-mutational mechanisms. DES is a synthetic estrogen with demonstrated carcinogenic potential, yet fails to behave as a mutagen in many systems. Clinically, it has seen extensive use in the pharmacotherapy of pregnant women in distress during the 1940s, 50s and 60s. The

teratogenic and carcinogenic effects of this drug contraindicate its use in human patients at present and many lawsuits are still pending in the United States by victims of DES exposure (Lynch and Reich 1985). The mechanism of action of DES is poorly understood, but recent work suggests that DES may induce both single and double-strand breaks in murine L5178Y lymphoblast DNA (Goldenberg and Froese 1985). The agent is seeing a revival as an antineoplastic. While DES may be capable of strand breakage (in addition to its ability to cause nondisjunction), the work of Barrett et al (1981) suggests that this ability may not be necessary for its carcinogenic effects. Interestingly, Sawada and Ishidate (1978) have noted colchicine and diethylstilbestrol exhibit similar effects on the cytoskeleton.

L-ethionine is an apparently non-mutagenic agent that can induce the neoplastic state in a number of systems (Farber 1963, Bruce et al 1984). Golden Syrian hamster F13 normal diploid fibroblasts treated with L-ethionine showed a frequency of 6-thioguanine and ouabian resistance of less than 10^{-6} yet escaped senescence and acquired the ability to clone in low serum and soft agar, with passage in culture (Gyi 1982, Bruce et al 1984). The results are consistent with a non-point mutational mechanism for the induction of the transformed phenotype.

The structure of DNA may be altered without changing its sequence and such changes (i.e. methylation and helical character) may contribute to altered gene expression in the neoplastic state, thus providing epigenetic mechanisms for aberrant gene expression. Supercoiling effects may include the acquisition of the DNAase I and S1 nuclease hypersensitivity sites known to occur at the 5'-ends of genes (Selleck et al 1984, Lilley 1984) and to correlate with developmental gene

expression. Nordheim et al (1981) have suggested that Z DNA may be a transcriptional control in Drosophilia. Consistent with the suggestion are the findings of Brahms et al (1985) that low and moderate negative superhelical densities (< the natural form) enhance transcriptional activity of the plasmid pBR322, while increased supercoiling and the change from right (B) to left (Z)-handed form of DNA, significantly inhibited the activation of transcription. Spermidine was used to increase the superhelicity and nalidixic acid or novobiocin to decrease DNA supercoiling, taking advantage of their effects on procaryotic topoisomerase activity. The tumor promotor TPA has been examined for its effects on DNA supercoiling with regards to induced differentiation in chronic lymphocytic leukemia cells (Lipetz et al 1982). These authors have observed that TPA increases supercoiling of DNA in normal lymphocytes but decreases this process in chronic lymphocytic leukemic lymphocytes, suggesting that the state of differentiation and the pattern of supercoiling may be responsive to each other. Indeed, aflatoxin B1 carcinogenesis may involve its binding to DNA (Croy et al 1978) and Norheim et al (1983) have demonstrated that this carcinogen perturbs salt-induced B-DNA/Z-DNA conversion which could affect gene expression. Covalently bound benzpyrene-diol epoxides are intercalated in the DNA helix and produce distortions that may also be capable of altering gene expression (Hogan et al 1981). The ultimate carcinogen N-acetoxy-acetylaminofluorene has been shown to preferentially attack the control region for the origin of replication of DNA in the SV40 chromosome, apparently a function of the guanine residue content of the region (Beard, Kaneko and Cerutti 1981). The intercalative agents actinomycin D and proflavin alter the B to Z transition of poly (dG-dC) (Mirau and Kearns 1983). Carcinogen and mutagen binding at the C(8)

position of deoxyguanosine in DNA is facilitated by the Z helix, and AT-rich regions, unlike GC rich regions, do not undergo a salt-induced B-Z transition (Dickerson et al 1982). These observations and the demonstration that potential Z-DNA forming sequences (poly dCdA/dGdT) appear to be highly repeated in the human genome has prompted Hamada and Kakunaga (1982) to suggest that Z DNA may be important in aspects of mutagenesis and gene expression.

Evidence supporting a role for non-mutational changes in transformation is also provided by studies involving methylation. DNA methylation is an informational coding system quite separate and distinct from the better recognized triplet-based genetic code. Tissuespecific methylation patterns and the maintenance and resulting hereditability of such patterns (Wigler et al 1981, Pollack et al 1980) allow for a newly recognized level of gene control that may account for the non-mutagenic character of many carcinogens and the reversibility of many malignant states (Riggs and Jones 1983, Jones, Taylor and Wilson 1983).

Methylation of mammalian DNA occurs exclusively at the C(5) postion of dC, usually in the dinucleotide CG. S-adenosylmethionine is the methyl donor in a transmethylation reaction catalyzed by the DNA methylase apparently specific for the CG dinucleotide sequence in hemimethylated DNA (Gruenbaum et al 1982). Maintenance methylases quickly catalyze transmethylation of nascent DNA during DNA replication (only unmethylated dCTP is used by the polymerase), preserving the original pattern of methylation (Gruenbaum et al 1983).

The involvement of the state of DNA methylation with gene expression has been extensively examined. It appears that while there are notable exceptions, hypomethylation correlates very well with the increased gene expression, especially with regards to the 5'-flanking

regions containing gene control regions (Riggs and Jones 1983). Indeed, Jones' group has proposed a model for demethylation during the course of differentiation where embryonic cells begin with a methylation pattern that is demethylated specifically in different cell types during the maturation process (Riggs et al 1983, Jones et al 1983). Support for such a concept is provided by the involvement of DNA-methylation in differentiation and development, as well as neoplasia. Constantinides et al (1977) have demonstrated the induction of myogenesis in C3H 10T 1/2 mouse cells treated with 5-azacytidine. The incorporation of 5-azaC into DNA inhibits the maintenance methylase(s), resulting in an under-methylated state. The induction of differentiation by 5-azaC has been reported by many laboratories. The analog induces hemoglobin synthesis in humans (Ley et al 1982, Kolata 1982), adipocyte and chondrocyte formation as well as macrophage formation in mouse cells (Taylor and Jones 1979, Boyd and Schrader 1982). Analysis of this type is complicated however, by the finding that non-histone nuclear proteins other than DNA methylases can form stable complexes with 5-azaC substituted DNA and may be involved in the analog's effects (Adams, Fulton and Kirk 1982, Christman, Schneiderman and Acs 1985). Thus the pleiotropy seen with 5-azaC treatment may involve not only epigenetic mechanisms stemming from changes in methylation patterns, but also from non-mutational interactions with nuclear proteins, perhaps even involving changes in chromatin structure.

The importance of methylation in studies of transformation, and further support for non-mutational changes in neoplasia is provided by the work of Wilson and Jones (1983a) who have shown that various carcinogens can inhibit the maintenance of 5-methylcytosine patterns in mammalian cells through DNA damage. Moreover, 5-azaC potentiates

carcinogen-induced initiation in rats, suggesting that both mutagenic and non-mutagenic changes in DNA may be involved in initiation (Denda et al 1985). The hypomethylation of genes in cancer cells as compared to their normal counterparts has been documented from the normal and disease tissue of human patients (Feinberg and Vogelstein 1983). These authors have also noted even more extensive hypomethylation in a metastasis from one of these patients. Progressive hypomethylation is also known to occur during the senescence of primary human, mouse and hamster cells, in contrast to the more stable transformed (and thus immortal) lines (Wilson and Jones 1983b).

Intercellular communication is a non-mutational phenomenon readily perturbed under a variety of circumstances and important in many aspects of neoplastic transformation. Phorbol ester tumor promoters like TPA induce a pleiotropic response in treated cells and many of their effects may be attributable to their interactions with cell membranes, as noted in a previous section. Some of their effects on tumor promotion may result from its documented reversible inhibition of junctional communication in mammalian cells (Enomoto et al 1981). Junctional communication allows for metabolic cooperation between cells and is believed to play an important role in embryonic development (Goodhall and Johnson 1982, Warner et al 1984, Guthrie 1984). Cell surface components are suggested to play a role in the cell sorting seen in mixed mammalian cell cultures that demonstrate preferential homologous junctional communication in vitro (Pitts and Kam 1985). Analogous results have been obtained by Enomoto and Yamasaki (1984). The authors have shown that cells growing in 20-methylcolanthrene transformed BALB/c 3T3 cell foci, in contact with a surrounding monolayer of untransformed cells, fail to transfer the fluorescent dye Lucifer Yellow to untransformed

cells after microinjection. Both transformed and untransformed cells show significant dye transfer within their own populations, but not between each other. Thus, loss of communication between cells, perhaps such as in a preneoplastic nodule, may be one of the important criteria that must be met for the acquisition of autonomous growth. Yamasaki et al (1985) have noted that variants of BALB/c 3T3 cells with increased susceptability to UV and benzo[a]pyrene induced transformation also show a loss in intercellular communication at confluence unlike more resistant variants, again suggesting that loss of communication may be important in neoplastic transformation. Jone et al (1985) have noted that gap junctional mediated intercellular communication is alterable at several levels, including cell recognition and assembly of junctions. Perturbation in metabolic cooperation seen after a variety of physical and chemical treatments may thus allow initiated cells to proliferate within a cellular community otherwise refractory to autonomous growth. Interestingly, the junctional permeability of mammalian cells is known to increase with increases in cAMP attained through exogenous supply or hormonal induction (Wiener and Loewenstein 1983). It is tempting to speculate that the protein kinase activity associated with the cAMP acceptor may be responsible for some of the phenotypic reversion seen in the cyclic nucleotide treated CHO cells in the studies of Puck, mentioned previously.

A further body of evidence in support of the aberrant differentiation hypothesis of transformation is seen in consideration of the fact that many malignant states are characterized by the expression of developmentally regulated genes, often of embryonic character (Alpert and Hirai 1983). Furthermore, many tumor promoters and antineoplastics are capable of inducing differentiated states, perhaps through non-muta-

genic mechanisms. The relationship between differentiation and transformation is discussed in detail in the section entitled 'Model systems of cellular differentiation and neoplastic transformation'.

The phenotypic manifestation of the neoplastic state is alterable at many levels, both genetic and epigenetic, and many of these processes have already been discussed. Non-mutagenic promotion of the transformed state may, however, be mediated by many other processes. For instance, the tumor promoter PMA has been shown to rapidly induce polyADP-ribosylation, apparently via a mechanism that is kinetically distinct from the ability of PMA to cause DNA strand breakage (ADP-ribosylation is stimulated by DNA strand breaks) (Singh, Poirer and Cerutti 1985). Concommitant with the increase in ADP-ribosylation, these authors noted a drop in intracellular NAD and suggested that the prooxidant state induced by promoters like PMA may have a basis in the rapid induction of nucleotide ribosylation by PMA. The importance of this process to malignant transformation has been illuminated by Borek et al (1984). They have noted that golden hamster embryo cells and C3H 10T 1/2 cells are inhibited from achieving the malignant state after exposure to UV, x-rays and chemical carcinogens if they are treated with inhibitors of poly (ADP-ribose) formation. While such inhibitors (i.e. benzamide, 3-aminobenzamide) can affect a wide range of metabolic processes not all of which are attributable to changes in poly ADP-ribosylation (Milam and Cleaver 1984), Borek et al (1984) used low concentrations of these inhibitors, apparently in a range specific for inhibition of poly (ADP-ribose) formation.

The requirement for ADP-ribosylation in cellular metabolism and the role these reactions play in the cell are poorly understood. While ADP-ribosylation patterns in nucleosomal histones may play a role in

chromatin structure and gene expression (Huletsky *et al* 1985), poly (ADP-ribosylation of non-histone proteins such as DNA polymerase, DNA ligase and terminal deoxynucleotide transferase has been demonstrated *in vitro* to inhibit the reactions of these enzymes (Yoshihara *et al* 1985). The *in vivo* consequences of such reactions are obvious. The metabolism of ADP-ribose may also be involved in the expression of early neoplastic transformation markers in hepatocytes (Althaus *et al* 1982) and can mediate changes in chondrocytic differentiation in cultures of chick limb bud (Nishio *et al* 1983). Interestingly, Lim *et al* (1985) have shown that cholera toxin and pertussis toxin catalyze the ADP- ribosylation of purified brain tubulin. Cholera toxin increases intracellular cAMP levels through deregulation of adenylate kinase and stimulates the tumorigenicity of virally transformed cell lines, but not spontaneously transformed lines (Gottesman, Roth, Vlahakis and Pastan 1984). Apparently, the increased tumorigenicity correlates with increased phosphorylation and subsequent activity of $pp60^{v\text{-}src}$. Beckner (1984) has also shown that the adenylate cyclase responsiveness of transformed cell lines to cholera toxin is less than that of parental, untransformed lines. Thus, some of the effects of ADP-ribosylation on cellular metabolism and the transformed state may be similar to the effects of aberrant phosphorylation and adenine nucleotide metabolism.

The ionic environment within and surrounding the cell is receiving increasing attention as a modulator of cell cycle related events in normal and mitogen stimulated cells. Transient increase in free cytoplasmic Ca^{2+} preceeding a sustained rise in intracellular pH is now recognized as a common ionic response in many types of cells stimulated to divide by lectins (i.e. conA) and tumor promoters as well as other

agents (Hesketh et al 1985). As one might expect, extracellular pH can also regulate the cell cycle as demonstrated by the pH-dependent stimulation of cells to enter the cell cycle from a quiescent state (Taylor and Hodson 1984). Stress related effects on cells in cultures exposed to heat, anoxia and other physical treatments are diverse and include changes in differentiation (Wolfe and Tata 1984). Disproportionate evaporation of growth medium by in vitro culture vessels is known to contribute to the expression of cellular heterogeneity in 3T3 cells and a chronic high salt environment can apparently even mediate colony forming efficiency in soft agar and tumorigenicity in nude mice (Rubin and Chu 1984). Promoter mediated changes in intracellular pH and the intracellular ionic environment, as demonstrated by Balk et al (1984) and Grinstein et al (1985), might thus be able to affect aspects of the transformed state, in addition to their effects on proliferation and the initiation of DNA replication (Leister et al 1985).

The aberrant differentiation hypothesis of neoplastic transformation supports the concept of misregulated gene expression during development and differentiation as the underlying basis of the cancerous state. Fahmy and Fahmy (1970) have provided evidence in support of the aberrant differentiation theory through examination of carcinogen altered gene expression in Drosophila. These authors have found that no significant correlation exists between the carcinogenicity of a number of agents including nitroso compounds, hydrocarbons, alkylating agents and aromatic amines, and the induction of point mutations in carcinogen treated fruit flies. Instead, these authors found small chromosomal deletions to be a common mechanism of induction of the Minute phenotype by these agents. These 'gene eliminations' manifest themselves as dominant visible mutations (i.e. changes in bristling) and cytogeneti-

cally as deletions of band regions in Drosophila salivary chromosomes. Continued investigation has revealed that carcinogens of many kinds can perturb cellular differentiation through alterations in gene expression mediated by the stimulated transposition of mobile elements in the Drosophila genome (Fahmy and Fahmy 1980, 1983a, 1983b). Observations on genic instability at the white eye locus of Drosophila has revealed that the mutagenicity of carcinogens applied to larvae, as measured in the frequency of sex-linked recessive traits in the male progeny, bears no correlation to the frequency of altered gene expression at the white eye color locus. However, the timing of appearance of carcinogen-induced reversion to wild-type (white) eye color was dependent on the developmental state of the organism. The authors have suggested that many nonviral forms of cancer may involve processes analogous to retroviral evolution, not unlike the oncogene theory of Huebner and Todaro (1969) or the protovirus theory of Temin (1971). Whether genetic transposition truly plays an important role in carcinogenesis is still a matter of speculation and controversy (Cairns 1981, Klein 1981, Baltimore 1985), but the similarities between eukaryotic transposable elements and oncogenic retroviruses are intriguing and highly suggestive of an epigenetic mechanism whereby gene expression may be skewed toward the transformed state.

The induction of differentiation and apparent reversion of the transformed state in a variety of systems has provided much evidence on the relative permanence of the transformed phenotype. While some drug-induced reversions have already been mentioned, they are by no means complete. Examples like the methotrexate-induced differentiation of human choriocarcinoma cells (Friedman and Skehan 1979) and the in vitro differentiation of primary cultures of human myelogenous leukemia cells

by TPA, actinomycin D or retinoic acid (Honma et al 1985) serve to illustrate the ability of some transformed cells to exhibit differentiated functions. Studies with retinoic acid have been particularly interesting.

Retinol (vitamin A) is a fat-soluble vitamin known for its importance in growth and differentiation of epithelial tissue in vivo and for its role in vision as the aldehyde form, retinal (Pawson 1981). Equally well recognized is the ability of vitamin A to protect against carcinogen-induced tumor formation, both in animals and man (Bollag and Matter 1981). In addition to their protective effects, retinoids are potent inducers of fetal liver cell maturation (Chou and Ito 1984) and neural differentiation in embryonal carcinoma (EC) cells (Jones-Villeneuve, Rudnicki, Harris and McBurney 1983). Interestingly, predisposition to colon cancer is associated with abnormal resistance to vitamin A in fibroblasts from human patients (Kopelovich, Drozdoff and Gansler 1983). While aspects of the protective effect of retinoids against transformation may involve effective inhibition of oxygen radical formation (Witz et al 1980), recent studies have focussed on the intracellular retinoid-binding proteins. The levels of CRABP (cellular retinoic acid binding protein) have been measured in a number of EC cell lines. Although the interaction of CRABP with retinoic acid may be necessary for its effects on transformation and the differentiated state, no correlation could be found between CRABP levels and the disposition of EC cells to retinoic acid-induced alterations in differentiation (Matthaei et al 1983). Indeed, these authors have noted that this lack of correlation between CRABP and the tendency of EC cell lines toward differentiation indicates that EC cells may have alternative, retinoid- independent pathways for differentiation and apparent neoplastic reversion. Studies with murine

melanoma cells resistant to retinoic acid induced inhibition of proliferation have resulted in similar conclusions (Lotan et al 1983). Current work suggests that retinoic acid mediated changes in gene expression may involve the non-covalent binding of the vitamin to chromatin in situ, in orders of structure higher than the nucleosomal level (Liau, Ong and Chytil 1985).

Model Systems of Cellular Differentiation and Neoplastic Transformation

Permanent Cell Lines

L6 and L8 rat myoblasts

The L6 and L8 rat myoblast cell lines were isolated by Yaffe as spontaneously transformed clones of newborn rat skeletal thigh muscle explant cultures (Yaffe 1968, Richler and Yaffe 1970). Both strains have retained the capacity for myogenic development even after prolonged cultivation in vitro. The myogenic phenotype exhibited by L6 and L8 myoblasts manifests itself in a manner thought to be analogous to aspects of the in vivo phenomena. Mononucleated myoblasts will fuse in culture to form terminally differentiated multinucleated myotubes when monolayer cultures are allowed to reach confluence. Accompanying the cessation of proliferation and the morphological changes are the appearance and increases in a number of muscle-specific proteins, including creatine phosphokinase, myokinase and acetylcholine receptor (Yaffe 1971, Merlie et al 1977). Kinetically, the course of myogenesis exhibited by L6 and L8 rat myoblasts is less synchronous than in primary, senescing muscle cell cultures. Quantitative analysis of the in vitro phenomena often employs nutrient (serum) deprivation or induc-

tion by chemicals or hormones such as insulin, to partly synchronize the differentiation process and thus facilitate its analysis.

Many studies with L6 and L8 rat myoblasts have focussed attention on the cell cycle and the role/requirement for DNA synthesis in these models of cellular differentiation. Primary avian cell systems have been used extensively in this regard and while some investigators favor a stochastic model of myogenesis (O'Neill and Stockdale 1972, Stockdale and O'Neill 1972, Konigsberg et al 1978, Buckley et al 1974) others find a deterministic model better fits the available data (Bischoff and Holtzer 1969, Kligman and Maneroff 1980). Delain et al (1981) have provided evidence that DNA synthesis is required for differentiation in L6 rat myoblasts. Inhibition of replication by reducing the serum content of growth medium to zero prevented myoblast fusion. The readmission of serum to the medium allowed fusion to occur, but only after one or two cell cycles by the L6 cells. Wahrmann et al (1981) have also indicated that L6 cells prevented from undergoing DNA synthesis by ara-C and culturing on a positively-charged substratum fail to express the myogenic program even when treated at confluence. In contrast to these results, Nadal-Ginard (1978) has characterized a clone of L6 rat myoblasts (L6E9) refractile to disturbances in the myogenic program by inhibition of DNA synthesis. These authors have suggested that a latent period exists in every G_1 phase from which L6E9 cells may progress to another DNA synthetic period or commit themselves to differentiate. Pinset and Whalen (1985) have also provided evidence that L6 myoblasts do not require a round of DNA synthesis for the induction of myogenesis. These authors have taken advantage of the fact that L6 myoblasts will grow to confluence in Ham's F12 medium with 20% FCS, but will not differentiate. The L6 myoblasts become quiescent and

remain undifferentiated until fed Dulbecco's modified Eagle's medium containing insulin, but no other protein (Pinset and Whalen 1984). Insulin is an effective inducer of myogenesis in L6 myoblasts (Mandel and Pearson 1974) and effectivley induces the differentiation (or releases the myogenic block imposed by Ham's F12/20% FCS) in the myoblasts without the apparent need for a round of DNA synthesis. Thus the importance of the DNA synthetic period preceeding myogenesis remains obscure not only in the rat myoblast cell lines, but also in other myogenic models.

The L6 and L8 rat myoblast cell lines exhibit many properties characteristic of the transformed state, including tumorigenicity, providing an appropriate host animal is used. Fusion-competent, wild-type L8 cells are non-tumorigenic in female NIH Swiss nu/nu mice when $3x10^6$ cells are injected subcutaneously (Kaufman and Parks 1977, Kaufman et al 1980), but nonfusing variant cells (selected from the proliferating pool by repeated rescue from dense, fusing cultures) are highly tumorigenic under the same conditions. The fusing and nonfusing populations also differ in their ability to grow in agar (0.001% versus 49% colonies formed) and have markedly different saturation densities in culture concomitant with a greater adherance to the substratum by the WT cells. The nonfusing L8 cells also exhibit an increased rate of hexose uptake as classically measured with [^{3}H] 2-deoxyglucose. Moreover, while the L8 WT is nearly diploid, the nonfusing clones were found to be polyploid (pseudotetraploid). A detailed analysis of many nonfusing L8 clones have revealed that no single 'transformed characterisitic' is common to all nonfusing variants, but the loss of control of proliferation appears to prevent myogenic development and promote tumor formation (Kaufman et al 1980). These authors have concluded that neoplastic

transformation is the natural alternative to 'normal' myogenic development inthis system and that the failure to withdraw from the cell cycle is a manifestation of a defective commitment process which results in the continued growth of variant cells. Unfortunately the biochemical lesion(s) responsible for these changes are unknown due to the manner in which the nonfusing variants were selected.

Bignami et al (I982) have examined the transformed properties of $L_8$and L_6 clones and have concluded that a transformed phenotype is expressed by both populations, but to a different extent. They found that both fusing and nonfusing L_8 variants were tumorigenic when injected into neonatal Wistar rats ($I0^6$ cells/I00 1 S.C.). Fusion-competent L_6 cells however, were nontumorigenic in this host system. Hillion et al (I982,I984) have confirmed the results with fusion - competent L_6 cells in this system and have further shown that fusion-incompetent cells will form tumors in the suckling Wistar rats if injected at $3xI0^6$ cells per animal. In contrast to Kaufman's group, Bignami et al (I982) found that fusing and nonfusing (RSV-transformed) L_8 variants grew well in soft agar with a plating efficiency of about 30%. L_6 WT cells however, grew poorly in soft agar (P.E. = 0.2%) but fairly well in soft agarose (P.E. = I6%). Furthermore, L_6 WT exhibited a high degree of plasminogen activator synthesis as measured in casein hydrolysis assays, unlike fusing and nonfusing L8 populations. Thus, L6 WT cells exhibit a nontumorigenic phenotype even though they are immortal, anchorage independent and express other transformed properties, whereas L8 cells (Fus+) are tumorigenic as well. These authors suggest that many of the differences may be due to variation in clones possessed by different laboratories and may represent different degrees of attenuation of the transformed phenotype.

Lawrence and Coleman (1983), in an examination of myogenic competence and the suppression of transformed properties in cell x cell hybrids between fusion competent and incompetent L6 myoblasts, have shown that L6 (Fus^+) cells, selected for resistance to 6-TG ($HPRT^-$), are non-tumorigenic when injected (10^7 cells/100μl S.C.) into male outbred Swiss nu/nu mice, but a nonfusing, $HPRT^+$ clone isolated from this strain forms tumors with a latency period of only one week. The significance of this finding however, is obscured by the fact that only two mice were injected with the fusing L6 clone, one of which died after two weeks (without a tumor), the other being observed for only 8 weeks post-injection. These authors also noted that the fusing population had a cloning efficiency in soft agar much less than that of the nonfusing clones (<0.001% versus 8.20%).

It is apparent that the L6 and L8 rat myoblast cell lines exhibit many transformed properties. Myogenic competence can be present or absent along with properties associated with the transformed state, though generally the transformed state appears enhanced in differentiation incompetent myoblasts. While in some systems transformation and myogenesis appear to be mutually exclusive, such is in RSV-transformed chick muscle cells (Holtzer et al 1975), in other systems myogenic clones have been shown to be tumorigenic, such as in 5-azacytidine treated T984-15 murine myogenic cells (Walker et al 1984). Thus if a permissive host animal is chosen for tumorigenesis studies and a number of in vitro correlates of transformation are examined in independent clones of myogenic and nonmyogenic populations, insight could be gained into the relationship between differentiation and transformation. Moreover, if the biochemical defect responsible for the differentiation defective phenotype is known, understanding may be gained as to the

ability of such perturbations to mediate changes in the differentiation/transformation pathways of these cell lines. The L6 and L8 rat myoblast cell lines are thus amenable to studies concerning the relationship between differentiation and transformation.

A wide variety of treatments have been used to probe the myogenic process in L6 and L8 rat myoblasts, including the selection of drug resistant, lectin resistant and temperature senstive variants. A summary of these studies is presented in table I. Interestingly, not all drug resistant myoblast cell lines exhibit defective differentiation (i.e. OUA^R, 8-azaG^R, 6-TG^R), indicating that some but not all cellular processes can affect differentiation. Few studies have been performed on the degree of transformation exhibited by drug resistant, differentiation-defective L6 and L8 myoblast strains. Kaufman et al (1980) have reported on the isolation of conA resistant L8 myoblasts which unlike their lectin sensitive parental strain, will form tumors in NIH Swiss nude mice, grow with a 10-30% cloning efficiency in soft agar and take up 2-deoxyglucose at an increased rate (1.6-1.9 fold more than wild-type). Somers et al (1975a, 1975b) have noted that α-amanitin-resistant L6 rat myoblasts that are fusion-defective and show no increases in creatine phosphokinase activity at confluence, have an increased plating efficiency in soft agarose in comparison with α-amanitin sensitive cells (1.0 for the variant verses $<10^{-3}$ for the parental). Thus it appears that the myogenesis defective phenotype in drug resistant L6 cells is associated with the acquisition of characteristics typical of the transformed state. An important consideration however is that in the latter example the parental cells were mutagenized with EMS to increase the isolation frequency from 5×10^{-7} to 10^{-5} (20 fold). In addition, the selection protocol involved

TABLE I: Summary of fusion potential of myoblast cell lines resistant to, or treated with, a variety of agents

Drug or Process	Cell Lines	Fusion	Reference
Passage & Cloning after extensive fusion	L_8	-	Kaufman et al, 1977
	L_6	-	Hillion et al, 1982
α-Amanatin^R	L_6	-	Crerer et al, 1977,1982 Mandel et al, 1974
	L_6	-	Senechal et al, 1982a,1982b
	L_6	-	Somers et al, 1975a,1975b
5-AZAC^R	L_6	-	Whatley et al, 1976
	L_6	-	Rogers et al, 1978
	L_6	-	Ng et al, 1977
8-AZAG^R	L_6	+	Whatley et al, 1976
	L_6	+	Rogers et al, 1978 Luzzati, 1974
OUA^R	L_6	+	Luzzati, 1974
Serum	L_8	$\pm$	Yaffe et al, 1977
Temp^S	L_6	$\pm$	Loomis et al, 1973
	L_6		Luzzati et al, 1972
6-TG^R	L_6	+	Lawrence et al, 1983
conA^R	L_6	-	Parfett et al, 1981
	L_6	-	Cates et al, 1984
PHA^R	L_6	-	Gilfix et al, 1983
WGA^R	L_6	$\pm$	Gilfix et al, 1983
H^R	L_6, L_8	-	Creasey et al, 1984a
Infection with RSV	L_8	-	Bignami et al, 1982
Growth in Ham's F12/20% FCS	L_6	-	Pinset et al, 1984,1985
Growth on a positively charged surface	L_6	-	Wahrmann et al, 1981

+ : fusion competent (wild type) - : fusion defective

multiple exposures to the selective agent, as was the case with the conA resistant variants of Kaufman et al (1980). Such procedures complicate the genetic analysis of a pleiotropic change in phenotype since genetic damage at other loci may arise which could contribute to the observed phenotypic changes, as could alternate mechanisms of resistance to the selective agent when applied in high multiple doses. Furthermore, while the conA resistant variants produce tumors in the host system described, unlike the sensitive parentals, it should be noted that FUS^+ L6 wild-type cells will produce tumors in Balb/c nu/nu male mice and are capable of modest growth in soft agar or agarose (see results section of this thesis). Thus, such studies do not address the possibility that in a permissive host like the Balb/c nude mouse, the tumorigenicity of conA resistant cells may be virtually indentical to that of sensitive parental cells. Indeed, such has been seen to be the case with a conA resistant L6 myoblast variant isolated by Parfett et al (1981) (see results section of this thesis). It would be most revealing to conduct similar experiments with the α-amanitin resistant variants. While these cells might also form tumors in a normally non-permissive host system (perhaps for a variety of reasons), a permissive host system might reveal no in vivo growth advantage by the variant myoblasts. Interestingly, decreased tumor latency periods have been observed in hydroxyurea resistant L6 myoblasts compared to sensitive parental clones, when the Balb/c nude mouse is used as host (see results section of this thesis).

Other models utilizing immortal cell lines.

A number of permanent cell lines have found wide use in the study of differentiation and neoplastic transformation. Included in this

group are the Swiss 3T3 line of Todaro and Green (1963), the BALB/c 3T3 line of Aaronson and Todaro (1968a) and the C3H 10T 1/2 mouse embryo line (Reznikoff et al 1973). These fibroblastic mouse cell lines possess a number of features characteristic of the transformed state, including immortality and an aneuploid karyotype. However, the two properties most closely associated with the transformed state, anchorage-independent growth and the ability to form tumors in host animals, are not exhibited by these cell lines. Under some conditions though, such as prolonged cultivation without recloning or when attached to glass beads (Boone 1975), these cell lines will grow in vivo to form tumors (the detailed properties of these model systems have been reviewed Heidelberger et al 1983, Meyer 1983).

Recently, Grieg et al (1985) have shown that if a suitable host system is used, the NIH 3T3 cell line (commonly used to demonstrate the oncogenic potential of various DNA sequences, or oncogenes) is tumorigenic, in contrast to the widely-held belief that the WT cell line is non-tumorigenic. These authors found that BALB/c nu/nu mice from Life Sciences (the same animal model used in the work presented in this thesis) was a permissive host for the growth of wild-type NIH 3T3 cells in vivo. A transfectant clone derived from this cell line, containing an activated c-Ha-ras-1 oncogene, also produced tumors in this host system, but with a decreased latency time compared to the parental NIH 3T3 cells. Thus it appeared that ras was not conferring the tumorigenic phenotype on this cell line, as previously proposed, but rather, the activated oncogene was accelerating the tumorigenicity of NIH 3T3 cells.

Many mouse 3T3 cell lines have been found to differentiate into adipocytes when maintained as confluent monolayers (Green and Kehinde 1976). Adipogenic conversion is characterized by the slow (3-4 week) or

rapid drug-induced (1 week) formation of lipid in droplets which gradually coalesce. Liu (1982) has shown an increase in cAMP-dependent protein kinase concomitant with dexamethazone, insulin, IBMX-induced differentiation. Interestingly, agents which increase intracellular cAMP such as dibutyryl cAMP or prostaglandin E_1, fail to stimulate adipogenic conversion, yet prostaglandin F_2 effectively triggers the process (Russell and Ho 1976).

The growth arrest of 3T3 T proadipocytes and subsequent differentiation *in vitro*, occurs in a distinct G_1 phase state designated G_D (Scott *et al* 1982) topographically different than serum (G_S) or nutrient (G_N) deprivation-induced growth arrest states (Willie and Scott 1982). Cells normally arrested at G_D can differentiate without DNA synthesis whereas cells arrested in other states (i.e. G_S, G_N) must undergo a further DNA synthetic period. These observations have suggested that the order of the states is G_D:G_S:G_N proceeding towards S phase. It has been suggested by these authors that a G_D-like state may be physiologically relevant in view of the apparent *in vivo* coupling of differentiation and G_1 growth arrest believed to regulate growth rate (Lajtha *et al* 1978) and similar G_1 arrest and differentiation seen *in vitro*, such as in the L6 myoblasts (Nadal-Ginard 1978).

Based on their early observations on growth arrest, differentiation and the importance of strict control of cell proliferation, Scott's group has suggested that the uncoupling of G_D arrest and differentiation could result in neoplastic transformation. Recently, this aberrant growth control concept received support from the observation that differentiation-defective 3T3 T proadipocytes exhibit an increased rate of spontaneous transformation in focus-forming assays (Scott and

Maercklein 1985). The observation that UV irradiation, a known initiator of carcinogenesis, inhibits adipogenesis in this cell line without affecting the control of proliferation mediated by the G_S and G_N arrest states, suggests that proliferation and differentiation are mediated at separate states of the cell cycle. These observations are consistent with their concept of a complex process for the initiation of carcinogenesis involving defects in the control of cellular differentiation. Thus, aberrant commitment to proliferation and differentiation, like immortality in many transformed cells, may be an early event in carcinogenesis. Diamond et al (1977) have reported on the ability of tumor promoting phorbol esters to inhibit BALB/c 3T3 adipogenesis (and the formation of many other terminally differentiated states) which suggested to these authors that the promotional effects of these agents (seen after initiation) may be due to their ability to disturb the commitment process.

The regulation of growth arrest and differentiation have also been uncoupled in the HL-60 human promyelocytic leukemic cell line. Yen (1985) has shown that a brief exposure of HL-60 cells to DMSO results in little cellular differentiation but significant G_1/G_o-growth arrest. The demonstration that growth arrest can occur in the absence of concomitant differentiation, by careful manipulation of culture conditions, suggests that the two processes are under separate control. Recall that similar observations have been made with the L6 rat myoblasts maintained as confluent growth arrested populations cultured in Ham's F12/20% FCS (Pinsett and Whalen 1984). Hydroxyurea-resistant, differentiation-defective L6 myoblasts are also known to cease proliferation without committing to the myogenic pathway (Creasey et al 1982, Creasey and Wright 1984a), another case in which growth arrest and differentation

are apparently uncoupled. Interestingly, tumor virulence is thought to vary inversely with the degree of differentiation, in patients with medullary thyroid carcinomea (MTC), and the status of differentiation of MTC cells grown *in vitro* (TT cells) varies inversely with the cell growth rate (Berger *et al* 1984). The TT cell line may prove useful as a model of differentiation and tumor progression in humans.

Studies with Untransformed Cells

The golden Syrian hamster cell systems

The golden Syrian hamster (GSH; *Mesocricetus auratus*) is an excellent animal model for the study of the interrelationships among neoplastic transformation, cellular differentiation and *in vitro* senescence and *in vivo* aging. The GSH cell culture system utilizes cryopreserved pools of primary explant cultures from littermate fetuses and normal adult individuals. The various normal diploid cell strains available in this system exhibit a very low rate of spontaneous transformation and do not produce tumors when placed into syngenic animals. In addition to these normal characteristics, these strains exhibit senescence patterns very similar to that of normal human cell strains, including increases in cell volume, loss of proliferative potential and discrete morphological changes. The model system and its use in studies into the interrelationships between neoplastic transformation, differentiation and senescence has been reviewed (Ts'o 1979b, 1982, Heidelberger *et al* 1983, Bruce *et al* 1984).

The first use of the GSH cell system for the study of chemical carcinogenesis was reported by Berwald and Sachs (1963) and some of the contributions of this system to our present understanding of somatic

mutation and neoplastic transformation have already been presented in this review of the literature (see section of Mechanisms of Neoplastic Transformation). More recently, the GSH system has found use in the study of differentiation/senescence relationships. Bruce et al (1981a, 1981b, 1982) have shown that cultures derived from 13 day gestation fetuses (F13), and adults of 6 months (A6) and greater than 20 months of age ($A20^{+}$) exhibit an average maximum population doubling level (PDL) of 20.3, 17.0 and 10.8, respectively (not to be confused with 'passage' where lifespan is recorded by the number of culture splits occuring during serial cultivation). Interestingly, continous exposure of these cells to tumor promoters (i.e. TPA) protracts their proliferative lifespan (Bruce et al 1982). Treatment with carcinogens however (i.e. MNNG or benz[a]pyrene), results in morphologically transformed cells which proliferate beyond the senescent pattern exhibited by controls. Whereas embryonic (fetal) cultures progressed to anchorage independence and a neoplastically transformed phenotype, treated adult cell cultures attained anchorage independence less frequently and could not avoid a second crisis period (Bruce et al 1981a, 1981b, Ts'o 1982). These results suggest that the frequency of neoplastic transformation in vitro is inversely related to in vivo cellular age.

The GSH cell system has been used most elegantly in the illucidation of the progressive nature of neoplastic transformation. Neoplastic-associated phenotypes are acquired progressively and sequentially in GSH cells treated with [^{3}H]-thymidine (Lin, Takii and Ts'o 1982), DNAase I (Zajac-Kaye and Ts'o 1984) and benz[a]pyrene (Barrett and Ts'o 1978a, 1978b). Morphological transformation occurs frequently and early after treatment, followed by enchanced fibrinolysis, cloning in low serum, cloning in soft agar and tumorigenicity.

Studies in adipogenesis are also possible with embryonic (E9) cells of the GSH systems. Spontaneously dividing adipocytes arise at a frequency of 20% of colonies formed at passage 1 and 2, but at 50% or better by passage 4 (Bruce et al 1984). Like the adipogenic development described above for 3T3 cells, differentiation in this system required a senescent or nonproliferative (confluent) state. Thus, younger more proliferative E9 cells are apparently less committed to differentiate in vitro. The cellular activity of cells such as those described above, remains long after the individual members of the population have ceased division and been judged senescent. The expression of differented states of activity in terminally differentiated cells has often been viewed as an in vitro correlate of senescence in vitro. Classically, most studies in this regard have been performed on human primary explant cultures, as described below.

Normal human diploid fibroblasts

Primary explants of normal human diploid fibroblasts (i.e. Wl-38, MRC-5, HSC172) from skin biopsy or fetal lung, are available in cryopreserved pools as models of senescence or differentiation. When cultured in vitro, populations of these cell strains typically exhibit a gradual decline in proliferative potential and ultimately cease cell division. This pattern of senescence, similar to the one described for the GSH cell system, is believed to be similar to aging in vivo, since explants from older donors apparently have a decreased 'lifespan' in vitro compared with cultures of younger (or fetal) origin (Hayflick 1965).

Gross changes in morphology and proliferation accompany the senescent pathway in normal human diploid cells. The intimate relationships seen between senescence, differentiation and proliferation in a wide

variety of cell systems has prompted many detailed examinations of the senescent pathway in HDF. Following the establishment period (phase I) after explantation, human cell populations enter a long period of gradually decreasing proliferative potential (phase II) and increasing heterogeneity in the interdivision times of individuals in the population (Hayflick 1965, Macieira-Coelho et al 1966, Absher et al 1974, Smith and Whitney 1980). Accompanying these changes are increases in cell area and protein content (Rosen et al 1981). Cristofalo and Kritchevsky (1969) have noted that there is a disproportionate increase in RNA/mg protein in aged Wl-38 cells and have postulated that while the cells can no longer divide, they are capable of imbalanced growth, biochemically and morphologically. Senescing cells characteristically exhibit increases in cell volume (Schneider and Fowlkes 1976), especially during phase III where a more polymodal, broadened frequency distribution is seen in cell diameters (Simons 1967). Mitsui and Schneider (1976) have demonstrated an inverse relationship between the growth rates of Wl-38 fibroblasts and cell volume. The two to five fold increases in cell volume typically exhibited in senescing human cells are not restricted to this pattern of growth however, and have been shown to occur in certain environmental conditions such as decreased serum concentration, decreased temperature of incubation and inhibition of DNA synthesis by hydroxyurea (Mitsui and Schneider 1976). Interestingly, treated cells exhibit a cell volume change and proliferative state which mimicks that of their pattern of senescence. Morphological and proliferative changes can be used as indicators of the degree of senescence of cell populations in vitro and in vivo.

The increase in cell cycle transit times seen during the cultivation of normal human diploid cells in vitro has been examined exten-

sively, since changes in the cell cycle bear on the biochemical differences seen between young and old populations and because altered cell cycle control is implicated in many disease states. Some authors have suggested that the heterogeneity seen in cell cycle times may reflect a disturbance in the transition from an arrested state to a proliferative state (Kapp and Klevecz 1976) but most studies indicate that protraction of G1 and G2 phases coupled with an increased proportion of G1 phase cells and a decreased fraction of cells in S phase accounts for the apparent increases in division times seen in aged cultures (Macieira-Coelho et al 1966, Grove and Cristofalo 1976, Schneider and Fowlkes 1976, Hasegawa et al 1985). Griffiths and Carpenter (1980) also note that a decreased proportion of cells in S phase was primarily the cause of the decreased [^{3}H]TTP labelling seen in aged cultures, but have also presented evidence that a portion of the decrease may stem from an actual decrease in the rate of DNA synthesis or replicon initiation. Macieira-Coelho et al (1966) have noted that phase III W1-38 cultures have a slightly lengthened S phase (8h) compared with phase II populations (6h) and Petes et al (1974) have observed a slowing of DNA synthesis in senescing MRC-5 cells. These authors have suggested that the slowing of DNA chain growth seen in these cells may be due to smaller pools of DNA precursors. Continuing work by Griffiths however, has indicated no decrease in the rate of DNA chain elongation in W1-38 or MRC-5 cells (Griffiths et al 1983). Correction of [^{3}H]TTP uptake data for the percentage of cells in S phase however, still did not eliminate the decrease seen in aged populations. These authors suggest that slightly slower rates of DNA synthesis are consistent with their notion of altered rates of initiation, since the number of events per replicon cluster remains the same in young and old cells. The minimum

time required for completion of S phase apparently does not increase in senescent cultures and remains relatively constant at 6-8h, though the average S phase transit time varies between 10.5 and 12.5 h, in Wl-38 and MRC-5 cells (Griffiths 1984). These results suggest that the DNA of aged cultures is relatively intact in comparison with that of young cells and that the DNA replicative enzymes are kinetically normal. A detailed study by Hasegawa et al (1985) on IMR-90 primary human cells has also indicated that DNA replication as a whole remains virtually unchanged during cellular aging. Their study included an examination of dTTP pool fluctuation in young and old cells, since pyrimidine pools have been implicated in a regulatory role for entry into S phase (see section on dNTP pools under Ribonucleotide Reductase). In keeping with the presumed conservation of the replicative process, these authors found no change in dTTP pools.

A number of laboratories have suggested that DNA synthesis is qualitatively and quantitatively different in young and old cells, lending weight to certain theories of aging involving increased mutation rates and the accumulation of errors in DNA and protein synthesis capable of affecting the stochastic nature of the process(es). Changes in dNTP pool sizes have been documented in young and old HSC172 fibroblasts, apparently the result of aberrant or misregulated RRase activity (Dick and Wright 1985) and in log phase, quiescent and drug-treated human diploid foreskin fibroblasts (Snyder 1984). Hydroxyurea-treated cells show a pattern of dNTP change consistent with the observations of many other laboratories: a preferential decline in purine dNTP pools and a slight increase (or no change) in pyrimidine dNTP pools after brief treatment with drug. While the size of the cellular pools changed with exposure to drug, the changes in cellular concentration are difficult

to interpret since no allowance was made for the changes in cell volume known to occur with hydroxyurea treatment in human cells (Mitsui and Schneider 1976). Similarly, the dNTP pool changes reported by Dick and Wright (1985) in young and old HDF are difficult to interpret since on a cellular basis there is about a 2-3 fold drop in pool sizes overall, which is essentially negated by the corresponding decrease in the proportion of S-phase cells observed in senscent HDF (Macierа-Coelho et al 1966, Grove and Cristofalo 1976, Schneider and Fowlkes 1976, Griffiths and Carpenter 1980, Hasegawa et al 1985). Thus, the importance of these dNTP pool observations and the suggestion that such changes play a role in mechanisms of senscence in normal diploid cells remains ambiguous. Interestingly, increased mutation frequencies have been noted with age in human lymphocytes (Trainor et al 1984), in keeping with the postulated role of dNTP pool imbalance in a mutator phenotype, as noted previously. Such observations are not universal however, since the frequency of 6-TG resistance apparently showed no age-related increase in mice (Horn et al 1984).

Further evidence in support of changes in the DNA synthetic process during senescence in vitro has been provided by work on the replicative enzymes DNA polymerase and ribonucleotide reductase. Pendergrass et al (1982) have suggested that there is a drop in the activity of the polymerase with aging and Dick and Wright (1982) have suggested that a similar decrease occurs in the activity of ribonucleoside diphosphate reductase. The latter study has utilized young, mid-passage and old clonally related HSC172 fibroblasts grown in vitro to show a 2-3 fold drop in activity with age, in keeping with the apparent drop in dNTP pools seen by these authors. However, the significance of this decline in activity is obscured by the lack of suitable correction for

the decline in the proportion of S phase cells in old populations, since RRase activity is strictly associated with the S-phase of the cell cycle. Although Pendergrass et al (1982) have allowed for the change in S phase populations, the significance of this work is also clouded, since the young and old cells studied were not clonally related. In addition, the corrected activity values presented were 52 ± 18 (young) versus 30 (old; one determination); again the difficulty and apparent heterogeneity seen in studies with these primary cell systems prevents a clear interpretation of the results, but indicates that changes in proliferation and S phase-associated events may be important in cellular senescence. Continuing investigations into the nature of 'young and old' DNA polymerases have shed little light on this problem since it appears generally that while the fidelity with which polymerases copy DNA templates in vitro is apparently reduced when purified from cultured cells (Lin et al 1982), no changes in fidelity are seen when the sources of enzymes is tissue from aging animals (Fry, Loeb and Martin 1981, Silber et al 1985). Thus the differences in fidelity seen with in vitro and in vivo derived enzyme may be a manifestation of the environmental condition or stress. Remarkably, Krauss and Linn (1982) have reported decreased fidelity of polymerases apparently as a function of culture confluence in young fibroblasts.

Studies with drug-resistant and drug-treated primary cell models and animals has provided some interesting insights into the aging and senescence processes and some of these studies have already been mentioned. Mutants resistant to ouabain (Mankovitz, Buchwald and Baker 1974), 8-azaguanine (Albertini and DeMars 1970) and α-amanitin (Buchwald and Ingles 1976) exhibit no changes in their in vitro 'lifespan', but cells treated with/resistant to hydroxyurea (Dick and Wright 1984) or

other DNA damaging agents (including alkylating agents) exhibit accelerated aging *in vivo* or senescence *in vitro* (Gensler and Bernstein 1981).

Senescence of cultured HDF as it occurs upon subculturing *in vitro* may be an invalid model of aging (the *in vivo* process). Bell *et al* (1978, 1979) have observed that, as with untransformed GSH cells and many other primary cell types, human foreskin fibroblasts could be cultured and shown to be metabolically active for extended periods of time (more than a year) after ceasing cell division and terminally senescing. These authors have suggested that the differences reported in the literature between phase II and phase III cells, both biochemical and morphological, are actually differences in the state of differentiation. The ability of primary cells to enter and leave the division cycle may be analogous in some respects to the process already described in permanent differentiating cell lines. Martin *et al* (1974) have also arrived at similar conclusions with HDF. Bell's group has further suggested that immortality may be characteristic of 'senescent' diploid cells if properly cultured and maintained. Thus the transformed cell may differ only in its replicative ability and inability to terminally differentiate.

Significant advances are being made in the reversal of the transformed state in human patients. The induction of differentiation in many blood cell neoplasms promises to be a useful technique in the management of such disorders (Sachs 1982). Treatment of a variety of leukemias with low doses of ara-C are especially promising, and have been witnessed to have therapeutic effects (Baccarani *et al* 1983, Michalewicz *et al* 1984). Similar results are now being seen with phorbol esters in acute undifferentiated leukemia (Shkolnik *et al* 1985). These

concepts in cancer chemotherapy have in many ways spawned from work with cells in culture.

The ability of tumor promoters and various other agents to induce differentiation in a variety of cell systems, yet promote the transformed state in other circumstances, provides further evidence for the interrelationship between differentiation and transformation. Human promyelocytic leukemia cells (HL-60) can be induced to differentiate with PMA, TPA and other phorbol esters at extremely low ($<10^{-9}$M) concentrations (Huberman and Callahan 1979, Lotem and Sachs 1979). Interestingly, the regulatory ability of these agents parallels their tumor promoting activity. Myogenic development is inhibited by TPA in primary human myoblast cultures (Fisher et al 1983) and by PMA in primary chick embryo muscle (Cohen et al 1977).

A landmark study by Yuspa and Morgan (1981) has shown that the initiation of carcinogenesis is associated with resistance to terminal differentiation. An initiating dose of DMBA was given topically to BALB/c mice, followed by explantation of the treated skin cells 6 weeks later. Maintained as a proliferative population in low calcium containing medium, initiated cells resisted the proliferative block and induction of differentiation when later switched to high concentrations of Ca and formed foci effectively. Epidermal cell colonies were rarely seen in cultures from control animals. These and other data have suggested to these authors that perturbations in the control of normal differentiation, associated with the proliferative block in terminal differentiation, are early (possibly initiating) events in chemical carcinogenesis. Recently, infection of mouse basal keratinocytes with Kirsten or Harvey sarcoma virus has been suggested to block high Ca induction by altering the maturation state of the keratinocytes, holding them

at the basal cell stage (Yuspa et al 1985). While phorbol esters accelerate terminal differentiation in mature basal cells, the virus infected cells, expressing an activated ras gene, display phenotypic reversion to a lesser stage of maturation. The authors have proposed that activated ras, while unable to fully transform the cells to tumorigenicity, may mediate the necessary proliferative signal for tumor formation. The relationship between the initiation of skin carcinogenesis and the resistance to terminal differentiation induced by chemical carcinogens and other transforming agents has also received support by the findings of Kilkenny et al (1985). These authors have correlated the in vivo initiating potency of several chemical carcinogens and UV light with their ability to act as inducers of altered differentiation in vitro. Their work provides a further line of evidence that the cessation of proliferation seen in response to a differentiation signal can be uncoupled by chemical carcinogens and other environmental disturbances, supporting the concept that the cell differentiation program is altered as a result of the initiation of carcinogenesis.

Human prokeratinocytes also display Ca-responsive induction of differentiation. Wille et al (1984), noting that epithelial stem cells are targeted by many carcinogens in man, have examined the relationship between growth and differentiation in this system. The differential clonal growth and differentiation seen with Ca and EGF in this system has suggested to these authors that the growth and differentiation responses are inversely related. Thus, proliferation and differentiation appear to be regulated in an integrated fashion in this system.

It is not surprising to find that the interrelationships between neoplastic transformation and cellular differentiation/senescence are also documented in non-mammalian systems. Arresting larval development in the nematod Caenorhabditis elegans by complete starvation is reversable, resulting in cultures which mature at normal rates. Interestingly, lifespan is prolonged apparently linearly with the duration of starvation: for each day of arrest a further day is gained in lifespan (Johnson et al 1984). The first report of malignant growth in the regenerating limbs of newts has allowed a rare glimpse of amphibian neoplasia (Tsonis and Huang 1984). Amputation of the forelimbs of Cynops pyrrhogaster distal to the elbow allows the study of the limb bud regeneration process. In one animal which died shortly after treatment, regeneration was inhibited, as evidenced by the lack of usual dedifferentiation of muscle at the site of injury. Non-keratinizing (undifferentiated) epithelial cells were seen infiltrating nearby tissue, but lacked an increased, apparent mitotic index. Tsonis and Huang (1984) suggest that aberrant (neoplastic) proliferation may disturb the normal regeneration/ differentiation process in this system.

MATERIALS AND METHODS

SOURCES of MATERIAL

Most biochemicals were obtained from Sigma Chemical Co., St. Louis, MO., or Fisher Scientific Co. Ltd. and most radiochemicals from Amersham Corp. Table II lists those materials obtained from other suppliers. Sources of cell lines are dealt with under a separate heading in "Routine Procedures".

Table II: Sources of Material

Material	Source
agar (Bacto-)	Difco Labs
agarose	Bio-Rad Labs
AG1-X8 anion exchange resin (200-400 mesh)	Bio-Rad Labs
α-MEM	Flow Labs
amphotericin B	Gibco Ltd. and Calbiochem
calf serum	Gibco Ltd.
cryotubes	Nunc
culture dishes (35,60,100,150 mm;mammalian)	Lux Scientific Corp.
culture dishes (6 and 96 multi-well)	Linbro Chemical Co.
culture dishes (100 mm;bacterial)	Fisher Scientific Co.
diadenosine pentaphosphate	Boerhinger Mannheim and Sigma Chemical Co.
foetal bovine serum	Gibco Ltd.
gentamycin	Roussel Canada Inc.
glycerol	Matheson, Coleman and Bell
glycyl-glycine	British Drug Houses Ltd.
guanazole (NSC#1895)	B. Wood Jr. and L.H. Kedda, U.S. National Cancer Institute,
[^{14}C]-guanazole	Dr. W.H. Yanko, Monsanto Research Corp.
haematoxylin	Schmid and Co.
horse serum	Gibco Ltd.
nude mice	Life Sciences Inc.
partisil PXS 10/25-SAX anion exchange column	Whatman

Con't

Material	Source
photographic film and chemicals	Kodak and Ilford
PPLO broth and agar base	Becton Dickinson and Co.
rhamnose	Nutritional Biochemicals Corp.
syringes and needles	Becton Dickinson and Co.
tri-n-octylamine	ICN Pharmaceuticals
trypsin (Bacto-)	Difco Labs
Tween 80	J.T. Baker Co.
zinc chloride	Matheson, Coleman and Bell

Care and Handling of Animals

Nude mice were housed in sterile plastic cages fitted with sterile bedding, water bottles, food pellets and filter bonnets The animals were maintained with no more than 6 individuals per cage, in a stream of sterile air provided by a horizontal laminar flow cabinet (Canadian Cabinets model 3000 sterile animal module). Bedding was changed aseptically and food and water maintained by the University of Manitoba Animal Care Services personnel.

Individual mice were marked for identification by ear punch for long term experiments and tail inking for short term studies. Animals were terminated by etherization followed by cervical dislocation. Weak and unhealthy animals were allowed a short period in which to recover before a decision to sacrifice.

Cell Lines

The L_6 and L_8 rat myoblast cells were kindly provided by Dr. B.D. Sanwal (University of Western Ontario) as clones of the original

cell lines isolated by Yaffe (Yaffe 1968, Richler and Yaffe 1970). The BALB/c 3T3 wild type cell line was a gift of Dr. R. Shenin (University of Toronto). Dr. J. Wright brought the original CHO WT cells from the Department of Medical Biophysics at the University of Toronto to this laboratory in 1971. L60 is a wild-type mouse L cell line (Earle 1943) cloned from a population originally provided to us by Drs. Holloway and Cormack of the Manitoba Cancer Treatment and Research Foundation.

Routine Procedures

Cell culture

All cell lines were grown at 37°C in a humidified atmosphere of 5% CO_2, 95% air as monolayer cultures in Eagle's α-MEM (without ribosides and deoxyribosides) supplemented with either 10% (v/v) calf serum (L_6 rat myoblasts) or 10% (v/v) foetal bovine serum (L_8 rat myoblasts, CHO, mouse L and BALB/c 3T3 cell lines). Antibiotics were routinely added to the culture media (100 units penicillin G/ml and 0.0685 mg/ml streptomycin sulphate). Tumor explants of all cell lines were cultured in antibiotic supplemented 10% FBS/α-MEM to which fungizone (amphotericin B, 250 ng/ml) and gentamycin (80 I.U./ml) had been added.

L_6 and L_8 myoblast cell lines and 3T3 lines were maintained as subconfluent monolayers to avoid loss of differentiation competent cells, since wild-type clones will differentiate spontaneously as they approach and reach confluence. Such differentiated cells suffer irreversible loss of proliferative capacity. All L_6 and L_8 cell lines

were studied during the first twenty passages beyond the cloning event to ensure relative uniformity of populations. One passage is equal to approximately five cell doublings and represents the replating of about 100,000 cells onto a 100 mm culture dish and subsequent growth to about 2-4 million cells before the next subcultivation/trypsinization event.

Trypsinization

Cultures of cells for subcultivation or harvesting and experimentation were removed from the surfaces of tissue culture dishes and flasks by trypsinization. Growth medium was aspirated with a Pasteur pipet and replaced with about 1/5 volume of PBS. After a momentary wash, the saline solution was aspirated and 1/5 volume of 0.2% Bactotrypsin in PBS was added to the culture vessel for approximately 30 seconds. The trypsin solution was then removed and the culture allowed to stand for an additional 5-7 minutes (depending on the relative activity of the solution) at room temperature. Cells were then removed from the surface of the vessel with the aid of a stuffed pipet using an appropriate volume of either PBS or normal growth medium.

Cryopreservation

The long-term storage of cell lines was accomplished by pelleting harvested cells out of growth medium using a centrifuge with a fixed angle rotor. Five to ten milliliters of cell suspension was centrifuged in a sterile plastic test tube (Falcon) at about 800 g for 4-8 minutes depending on the depth of the cell suspension. The growth medium was then aspirated from above the cell pellet and the cells resuspended with normal growth medium supplemented with 7% DMSO. Cells were resuspended at 2-10 x 10^5 cells/ml of cryopreservative and stored as 1 ml aliquots

in sterile cryotubes (Nunc) at -70°C and -80°C or under liquid nitrogen.

Cell lines were resuscitated from the cryopreserved-state by rapid thawing under water at 37°C for approximately 90 seconds. The unfrozen cell suspension was then dispersed into a culture dish containing normal growth medium and incubated under standard conditions (37°C, 5% CO_2, 95% air) until the cells were firmly attached to the surface of the dish (generally 1-2 hours). The growth medium was then replaced with fresh medium to remove all but traces of the cryopreservative.

Cell counting

Enumeration of cell suspensions involved removing an aliquot (generally 0.1 ml) and diluting the sample 1:400 in normal saline. An electronic particle counter (Coulter Electronics model A) was then used to score the total number of particles in 0.5 ml aliquots. Each sample was counted three times and an average value was determined and used for estimation of the cell density in the original cell suspension.

Determination of cell volume

A model ZB1 Coulter particle counter (Coulter Electronic) was used for cell volume determinations using giant ragweed pollen as a standard. Cells to be measured were trypsinized in the normal way from actively growing near confluent cultures and diluted in PBS to approximately $5x10^4$ cells/ml. Size distributions were determined by stepwise increments in the minimum threshold signal needed for registration by the particle counter.

Testing for mycoplasma

The rat myoblast cell lines were tested for the presence of mycoplasma during the initial phase of this work. Spent medium and

scraped cells were mixed into PPL) broth or streaked onto PPLO-agar medium and blood-agar then incubated aerobically and anaerobically at 37°C. Test samples were examined after one week and fresh streaks were made from the incubated broths. Samples were incubated for another 2 weeks and re-examined. Samples of serum and α-MEM were tested for mycoplasma in a similar manner.

Determination of generation times

Cell growth kinetics were assayed by seeding $5x10^4$ cells in 5 ml of normal growth medium into 60 mm culture dishes and incubating under standard conditions. Periodically, duplicate dishes were removed from the incubator, the growth medium removed by aspiration and replaced with 2 ml of 0.2% Bactotrypsin in PBS. Trypsin solution was allowed to bathe the cells for approximately 5 minutes. The cells were then harvested in the trypsin solution and 1 ml of suspension was diluted 1:40 in normal saline and counted. Cell density was expressed as cells cm^{-2} and plotted on semi-log graph paper. Generation times were calculated for the exponential phase of growth using graphical estimates from the best-fit line and the following formulae:

$$g = \frac{\Delta t}{N} = \frac{t_2 - t_1}{N} = \text{generation time}$$

$$\text{where } N = \text{Ln} \frac{\text{cells } cm^{-2} @ t_2}{\text{cells } cm^{-2} @ t_1}$$

t_I=time initial, t_2=time final, N=coefficient of cell #

Protein determination

The protein content of cell extracts were determined using the Bio-Rad protein assay kit (technical bulletin #1051). Bovine serum albumin was used as a standard in the normal assay. Dilute samples were

assayed for protein using the 'microassay' procedural variation when necessary.

Photography

Unstained cell cultures and chromosome preparations were photographed with Carl Ziess and Nikon photomicroscopes on 35 mm Kodak Technical Pan Film 2415. Negative development was accomplished in a 300 ml 1:3 dilution of Ilford Microphen developer. Development proceeded with continual agitation for the first 30 seconds followed by agitation for 5 seconds every half minute for 8-10 minutes at 20-22°C. Negative development was stopped with continual agitation in 1% acetic acid and was followed by fixation in Kodafix for 5 minutes with intermittent agitation. The developed film was then treated with a 1:200 dilution of Kodak Photo-Flo and hung to dry in the presence of the wetting agent.

Stained colonies and agar or agarose colonies were photographed *in situ* using a Polaroid land camera equipped with a 500 mm lens and Polaroid type 55 positive/negative film.

Contact sheets and finished prints were prepared professionally upon request by the University of Manitoba Instructional Media Centre or the Health Sciences Centre of Winnipeg Department of Medical Photography.

Selection of Variant Cell Lines

Cloning of wild-type cells

Myogenic clones of L_6 and L_8 rat myoblasts were isolated by seeding 10 to 100 cells in 100 mm tissue culture dishes containing 10 ml of serum supplemented medium. After about 2 weeks incubation at standard growth conditions, the colonies which developed were cloned with glass cloning cylinders and trypsin solution. The harvested cells

from each colony were placed into 60 mm dishes containing 5 ml of complete growth medium and allowed to grow to near confluence ($\sim 10^6$ cells). Each clonal population was then transferred by trypsinization to 100 mm culture dishes. When these transferred populations reached near confluence they were passaged in the normal way and designated passage number one. Thus by P1 the WT clones had undergone about 20 cell doublings and 2 trypsinizations since cloning. Pools of these cells were cryopreserved and used for selection of variant cell lines, as shown in figure 1.

Selection of L_6 and L_8 variant cell lines

Hydroxyurea-resistant variant cell lines were selected from WT parental clones by sib-selection, as shown in figure 1. Typically, 1.5 to 2.5×10^6 cells were plated directly into 1.0 to 1.5 mM drug and incubated at 37°C for three to six weeks. In some experiments, the initial drug medium was removed three days later and a 2 to 3 fold lower concentration of drug was given to allow for the outgrowth of low resistant variants. In one case, a variant clone (L_6H^R-5) was isolated from a population exposed two times to 2.0 mM hydroxyurea. Cells able to proliferate in the presence of these cytotoxic hydroxyurea concentrations developed into colonies, which when initially discovered were refed fresh growth medium without drug and allowed to develop into larger colonies before cloning. Each surviving clone was then cryopreserved and tested in clonogenic assays for resistance to the selective agent. Each clone which exhibited drug resistance properties was designated H^R (hydroxyurea-resistant) and was studied in more detail in parallel experiments with its untreated passage-matched parental clone. Some of the L_6H^R cell lines were the gifts of Dr. J.A. Wright and Dr. C.L.J. Parfett.

The wild-type and drug-resistant cell lines of mouse and hamster origin were selected by several individuals in this candidate's doctoral laboratory during the last decade. The adipogenic line BALBc/3T3 WT-1 and its variant BALBc/3T3 H^R-1 were isolated by Dr. J. Wright in 0.2 mM hydroxyurea. The mouse L cell lines L_2Cl_3 and L_1H_2 were selected by Dr.B.Kuzik and Dr.S.Koropatnick, respectively, from WT L60 cells (clone L1). L_1H_2 was selected in a single step at 0.3 mM drug (Koropatnick and Wright 1980). L_2Cl_3 arose from a multiple step protocol involving increasing concentrations of hydroxyurea (Kuzik and Wright 1980).

A few years later, the L_2Cl_3 population was used in the isolation of a very H^R population, LHF. Dr. J. Wright and J. Morgan subjected L_2Cl_3 to multiple rounds of selection in increasing concentrations of hydroxyurea. During a period of several months, populations of cells were derived which were capable of proliferation in 1.5, 2.0, 3.0, 4.0 and finally 5.0 mM (LHF) hydroxyurea. The two drug-resistant CHO cell lines described in this work, CHO HU^R-2 and CHO NC^R-30A2, were isolated by Dr.W.H. Lewis and Dr. R.G. Hards, respectively (Lewis and Wright 1974, 1978, Hards and Wright 1983a). Both of these variant lines are derived from CHO WT C125.

Assays for Differentiation

Preparation of assay cultures and induction of differentiation

Cultures examined for differentiation competence were trypsinized and seeded at $5x10^4$ cells/60 mm dish/5ml of normal growth medium. Assay cultures were fed fresh medium every two days until confluence and then were induced to differentiate. L_6 and L_8 rat myoblasts and the

BALB/c 3T3 mouse cell lines were refed daily with 1% CS (L_6) or 1% FBS (L_8, 3T3) supplemented α-MEM to achieve a more synchronous onset of events. In some assays horse serum was used. Experiments were also performed in the absence of serum for some cell lines.

Quantification of cell fusion

L_6 and L_8 rat myoblast cell lines were scored daily for cell fusion by microscopic examination for progressive increases in multinucleation, beginning at day 0 (induction with 1% serum/α-MEM) and continuing for about 6-8 days. Some fusion assays lasted up to 3 weeks in length. Nuclei were made more discrete by brief plasmolysis in DMSO:10^{-3}M $ZnCl_2$ (1:4) as originally described by Parfett et al (1981), followed by fixation for 30 seconds in 50% methanol. Cultures were examined under glycerol by phase-contrast microscopy at a final magnification of 200x. One thousand or more nuclei were scored in randomly selected fields of each cell line, each day. The fusion index was calculated as the percentage of nuclei contained within multinucleated cells relative to mononucleated and binucleated cells.

Creatine phosphokinase assay

Creatine phosphokinase activity was measured in duplicate cultures of myoblast cell lines, in parallel with assays of fusion competence. The method used is that of Oliver (1955) as modified by Shainberg, Yagil and Yaffe (1971) with the exception that diadensosine tetraphosphate rather than AMP, was used to inhibit adenylate kinase activity. The assay is based on the accumulation of NADPH (as assayed spectrophotometrically at 340 nm) resulting from the coupled reactions of glucose-6-phosphate dehydrogenase and hexokinase, as driven by ATP during the

catabolism of creatine phosphate. One unit of activity corresponds to the formation of 1 nmole of NADPH min^{-1} (a change in O.D. of 6.22 min^{-1}) at 30°C, pH 6.75.

Determination of adipogenic development

Cultures of BALB/c 3T3 WT-1 and its variant, HR-1, were assayed microscopically for lipid droplet accumulation using cultures prepared and induced in an identical manner to the myoblast cell lines. Cultures were periodically stained and assayed over the course of several weeks. The medium was aspirated from above the induced cultures and the cells were fixed in formalin for about 10 minutes, then rinsed briefly with 70% ethanol. Fixed preparations were stained for 5 minutes in oil red 0 saturated ethanol:acetone:water (4:5:1) followed by fast washes in 70% ethanol and water. The stained preparations were then counterstained with modified Harris haematoxylin, washed with water and blued with ammonia water (Durfee 1968). Final preparations were washed and examined at 40-200x under dilute glycerol.

Assay of Ribonculeotode Reductase

RRase activity in permeabilized cells

Ribonucleotide reductase activity was measured in whole permeabilized cells using a modification of Tween-80 methods previously developed in Dr. Wright's laboratory for hamster (Lewis, Kuzik and Wright 1978, Hards and Wright 1983a, 1983b, 1984a, 1984b), mouse (Kuzik and Wright 1979, Koropatnick and Wright 1980) and human cells (Wright, Hards and Dick 1981). Exponentially growing cells were plated at a density of 1.0 to 2.5 x 10^6 cells per 150 mm culture dish containing 25 ml of normal growth medium, and incubated at 37°C. After 40-50 hours

cells were rapidly removed from the surface of the plate with trypsin solution. An aliquot was then removed for cell counting and the remainder of the cell harvest was centrifuged. The cells were then resuspended at 1 x 10^7 cells/ml in permeabilizing buffer consisting of 1% Tween-80, 0.25 M sucrose, 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes) buffer pH 7.2 and 2 mM dithiothreitol. Permeabilization was routinely performed at 30-32°C for 30 minutes. After permeabilization the cells were centrifuged and resuspended in fresh permeabilizing buffer at an appropriate density to give 2.5 to 5.0 x 10^6 cells/assay point/200μl aliquot.

Cytidine 5'-diphosphate (CDP) reduction was assayed by combining 200 μl of permeabilized cells with 100 μl of concentrated reaction buffer at 37°C and stirring with 2 x 7 mm magnetic bars for 5 minutes. The final reaction buffer consisted of 50 mM Hepes buffer pH 7.2, 6 mM DDT, 8 mM $MgCl_2$, 4 mM ATP, 0.4 mM ^{14}C-labelled CDP (5,000 cpm/nmole, Amersham), 0.67% Tween-80 and 0.167 M sucrose. The reaction was terminated by boiling for 4 minutes. The amount of product formed was determined as previously described (Wright, Hards and Dink 1981). Briefly, this involves conversion of deoxyribonucleotides with Crotalus atrox venom (Sigma) and separation of deoxycytidine on a Dowex-1-borate (Bio-Rad) ion-exchange resin column. Samples were counted using Scintiverse II (Fisher Scientific) and a Beckman LS9000 liquid scintillation counter. Adenosine 5'diphosphate (ADP) reduction was assayed in a similar manner, except that the final reaction buffer consisted of 50 mM Hepes buffer pH 6.8, 6 mM DDT, 0.5 mM 2'-deoxyguanosine 5'-triphosphate (dGTP), 0.4 mM ^{14}C-labelled ADP (5000 cpm/nmole), 0.67% Tween-80 and 0.167 M sucrose. Reactions were allowed to run for 30 minutes.

The concentration of the assay agents DTT, ATP and Mg, as well as the

final pH of the CDP reductase reaction mixture were 6, 4 and 8 mM respectively, at pH 7.2. These values were found to be optimal, or nearly optimal, in a variety of other cell systems, including CHO (DTT 6 mM, ATP 2-6 mM, Mg 8-10 mM, pH 7.2; Lewis and Wright 1978, Lewis et al 1978, Hards and Wright 1981), mouse L cells (DTT 6 mM, ATP 4 mM, Mg 5-8 mM, pH 7-7.2; Kuzik and Wright 1979, 1980, Koropatnick and Wright 1980) and human fibroblasts (DTT 6 mM, ATP 3-4 mM, Mg 8 mM, pH 7.2; Dick and Wright 1980, 1984) in assays employing whole cell extracts and/or permeabilized cells. A survey of the literature revealed the following mean values (± S.D.) for those reagents in CDP reductase assays using permeabilized cells or cell extracts: DTT 7.4±2.9 mM, ATP 4.0±1.5 mM, Mg 6.5±2.5 mM, pH 7.3±0.2 (Vitols et al 1970, Chang and Cheng 1979a, 1979b, Meuth and Green 1974, Tai et al 1983, van't Riet et al 1979, Fridland 1984, Tarr and Gardner 1971, Mattaliano et al 1981, Spector and Averett 1983, Hovemann and Follman 1977, Lewis and Srinivasan 1983, Bradley et al 1982, Eriksson and Martin 1981, Tyrstead and Gamulin 1979, Takeda et al 1984, Ayusawa et al 1981, Arpaia et al 1983a). The cell types assayed with these concentrations of reagents range from baker's yeast, salmon testes and beans to 1° and 2° human cell strains, mouse, rat, hamster and calf thymus. Thus, optimum assay conditions need not necessarily be worked out for every cell line if only a serviceable assay for CDP reductase is required, as in the present study.

RRase activity in whole cell extracts

CDP reductase was measured in cell extracts with cultures prepared as described above (section 7.1). Trypsinized cells were harvested by centrifugation and subjected to 60 strokes in a Potter Elvehjem tissue homogenizer at 4°C in PBS. The homogenate was centrifuged at 100,000 g for 60 minutes and the supernatant extract was used to measure CDP

reductase activity. Assays consisted of approximately 1 mg supernatant protein in 125 μl of 100 mM NaH_2PO_4 buffer to which 25 μl of concentrated reaction mixture was added to a final assay of 4 mM ATP, 8 mM $(CH_3COO)_2Mg.4H_2O$, 6 mM DTT, 0.05 mM [^{14}C]CDP (5000 cpm/nmole). Reactions were performed for 30 minutes at 37°C and terminated by boiling for 4 minutes. The amount of product formed was measured as described in the previous section for the assay with permeabilized cells

Determination of Nucleotide Pools

Extraction of nucleotides

Nucleotides were extracted with perchloric acid, neutralized with amine and preserved with carbonate using a variation of published methods (Garrett and Santi 1979, Pogolotti and Santi 1982, Khym 1975, Chen et al 1977, Reinhart and Koroly 1982). Cells were seeded at $5x10^5$ to $1x10^6$ per 150 mm culture dish in 25 ml of normal growth medium and grown for 40-50 hours at 37°C. Ten to twenty dishes were used in each extraction for a given cell line and WT and H^R pairs of lines were extracted in parallel. Cells were harvested in the cold in PBS (ice cold) using trypsin solution and counted. A final cell density of $5x10^7$ cells ml^{-1} was obtained by centrifuging the harvest in a swinging bucket rotor and resuspending the cells in ice cold PBS containing [^{3}H]-uridine or [^{14}C]-adenosine as a marker of dilution error. The suspensions were made 0.5 M with 70% $HClO_4$ and incubated on ice in the cold for 30 minutes. At the completion of the extraction, DEPC was added to 10% and 2 volumes of 0.5 M TnOA in trichlorotrifluoroethane were added followed by 2 minutes of vortexing. The neutralized extract was then centrifuged briefly and the aqueous layer collected and dispensed as 125μl aliquots into 1 ml cryotubes and frozen at -70°C or under liquid nitrogen until needed.

HPLC separation and analysis of ribonucleotides

Chromatographic separation of ribonucleotides was accomplished by high performance anion-exchange liquid chromatography. A Partisil PXS 10/25 SAX anion exchange column (25 cm x 4.6 mm bore) was equilibrated with the initial eluant, 7 mM KH_2PO_4 (pH 4.00 0.02) for two hours at a flow rate of 3 ml min^{-1} delivered by a Beckman model 110A pump. Cryopreserved acid extracts of actively growing cells were thawed rapidly and injected in 100 μl aliquots by partial loading in a 500 μl sample loop with a 500 μl Hamilton syringe. A five-minute isocratic flow of low salt solvent was followed by a 30 minute linear gradient to 250 mM KH_2PO_4, 250 mM KCl (pH 5.00 0.02) using a second pump coregulated by a Beckman model 339 system controller. The gradient was followed by 10 minutes of isocratic high salt buffer. Spectrophotometric analysis was performed at 254 nm with a Beckman model 160 absorbance detector equipped with an 8 μl flow cell. The column bed was readied for another separation by a 15 minute linear gradient back to low salt eluant and a 30 minute re-equilibration.

Pool size analyses were performed measuring peak heights from independent experiments.

HPLC separation and analysis of deoxyribonucleotides

Deoxyribonucleotides were freed of contaminating ribonucleotides using periodate distruction (Garrett and Santi 1979), then chromatographed isocratically on a Partisil 10/25 SAX anion exchange column equillibrated with 250 mM KH_2PO_4, 250 mM KCl, pH 5.00 0.02. A Beckman model 110A pump regulated by a model 332 controller provided solvent at a flow rate of 2 ml/min. Spectrophotometric analyses were performed at 254 nm with a Beckman model 160 absorbance detector. Samples volumes of each run were 75 μl. Pool size analyses were

performed by cutting and weighing peaks in triplicate from independent experiments.

In Vitro Assays of Transformation

[^{3}H]-deoxyglucose uptake

[^{3}H]-deoxyglucose uptake was measured by a modification of the technique of Kaufman et al (1980). Cells were seeded in 35 mm plastic dishes or 6-well multi-dishes at a density of $5x10^4$ cells/2 ml growth medium/60 mm culture dish and incubated 3-4 days. Growth medium was aspirated and the cells were exposed to 1 ml of prewarmed (37°C) [^{3}H]-deoxyglucose (1 Ci/ml; 16.2 Ci/mmole). Cells were allowed to take up the sugar over the course of 30 minutes, then the dishes were aspirated, washed 4x with 2 ml of ice-cold PBS and solubilized with 1 ml 1N NaOH for 20 minutes at 4°C. Samples (800 μl) from triplicate dishes were neutralized with HCl and counted with Scintiverse II.

Growth in semisolid media

Cells were plated in agar or agarose using a modification of the method of MacPherson and Montagnier (1964), at densities of 500, 10^3, 10^4 or 10^5 cells per 60 mm dish in 0.33% agar overlaid on 0.5% agar base. Colonies were allowed to develop for 10-30 days at 37°C then scored visually. A minimum 250 colonies were measured at random for each cell line, with the aid of a micrometer eyepiece at 200x. A colony was defined as a ball of cells with a diameter greater than 100 μm.

In Vivo Assays of Transformation

Tumor formation

Tumorigenicity testing was accomplished with athymic male nu/nu BALB/c mice as permissive hosts. Actively growing cells were harvested

by trypsinization (or by scraping with a rubber policeman in some experiments) and washed once with cold α-MEM in the absence of serum. The cells were resuspended in α-MEM at a density of 10^7 cells/ml and kept on ice until the moment of injection into the animals. Each animal was injected with 10^6 cells in 100 μl of α-MEM, subcutaneously in the back when 6-10 weeks old. In some experiments as many as $3x10^6$ or as few as 10 cells were injected but always in a volume of 100 μl.

Tumor growth was measured periodically (daily for rapidly growing tumors) with the aid of an outside micrometer caliper. Two distances were always measured at right angles to each other at the longest and widest aspects of each tumor. The product of these two values was taken as the 'tumor index'. Tumor incidence was defined as a tumor index greater than or equal to 0.40, a value approximately corresponding to a tumor volume of 100 μl. Thus, a tumor was defined as a palpable mass with a volume at least as great as the original inoculum.

Tumor explantation

Tumor cells were recovered from animals by surgical explantation. Tumor-bearing mice were sacrificed by etherization/cervical dislocation and the tumors were rapidly removed with scissors and forceps after cleansing the tumor area with 70% ethanol. The instruments were flame sterilized with alcohol and the entire procedure was carried out in a HEPA laminar flow hood. When tumors were to be weighed, the entire tumor was removed and freed of as much host dermis as possible, but when tumor cells were to be grown in culture, only a portion of the tumor was removed. These portions were placed into 100 mm plastic culture dishes and minced with scissors and forceps in the presence of normal serum supplemented medium to which gentamycin and amphotericin B had been added. Tumor cells were allowed to attach and grow on the surface of

the dish in this medium under normal incubation conditions for 2-7 days before the larger pieces of tumor and unattached cells were removed by aspiration. Tumor explant populations were maintained in this medium as uncloned populations and cryopreserved/subcultured in the normal manner.

Uptake of Hydroxy [^{14}C]-Urea and [^{14}C]-Guanazole

The cellular uptake of hydroxy [^{14}C]-urea (7.3 mCi/mmole) and [^{14}C]-guanazole (9.9 mCi/mmole) was determined in the same experimental protocol as [^{3}H]-deoxyglucose uptake (section 9.1). Experimental points were done in duplicate or triplicate using 10 to 100 μM labelled drug. In some experiments where drug concentrations exceeded 100μM, labelled drug was diluted with freshly prepared cold drug. All experiments were performed at 37°C, except where indicated, and drug uptake was normalized to the number of cells used, as determined in triplicate cultures grown in parallel.

Gene Transfer

Isolation of high molecular weight DNA

Total genomic DNA was isolated by a modified phenol/chloroform extraction using actively growing cells (Maniatis et al 1982). Four 150 mm culture dishes containing actively growing cells were washed once with 5 ml of PBS and given 3 ml of lysis buffer consisting of 500 g/ml proteinase K and 0.5% sodium lauryl sulphate in 500 mM Tris pH 8.0. Cells were lysed for 10-15 minutes at room temperature. The lysate was collected with a rubber policeman into a 50 ml glass centrifuge tube and incubated for 3 hours in a 65°C water bath. An equal volume of phenol:-

chloroform:isoamylalcohol (25:24:1) was added to the lysate and the mixture was inverted several times, then centrifuged at 800 g for 10 minutes. The aqueous phase was collected and the DNA precipated with an equal volume of 70% isopropyl alcohol overnight at 4°C. The DNA was pelleted by centrifugation at 10,000 g for 30 minutes at 4°C and the supernatant poured off. The pellet was washed with an equal volume of 100% isopropanol and centrifuged again. The supernatant was again poured off, followed by vacuum dessication for 10 minutes. Five volumes of TNE buffer (100 mM NaCl, 1 mM EDTA, 10 mM Tris pH 8.0) were used to resuspend the DNA. A 10μl sample was taken and diluted to 2 ml with TNE buffer, then assayed at 260 and 280 nm in a Beckman DU-8 spectrophotometer. The isolated DNA was used for transfection on the same day and was stored at -70°C.

Transfection with high molecular weight DNA

Transfection of wild-type cells with high molecular weight DNA was performed using the calcium phosphate coprecipitation technique of Graham and Van der Eb (1973) as modified by Gorman et al (1983). Wild type recipient cells were seeded at 1-5x10^5 cells per 100 mm culture dish 24 hours prior to transfection. Each culture received 10 μg DNA as 1 ml of a $CaPO_4$ coprecipitate. The precipitate was prepared by adding the DNA in TNE buffer to water made 124 mM with $CaCl_2$ from a 2 M stock solution. This mixture was added dropwise to an equal volume of double strength HEPES buffered saline (HBS 8.18 gm NaCl, 5.95 gm HEPES, 0.20 gm Na_2HPO_4 in 1.0 l of water, pH 7). Precipitation was facilitated by simultaneous bubbling of air through the mixture.

Cultures were incubated under standard conditions for 4 hours after receiving the DNA. Concentrated sodium butyrate was added to a final concentration of 6 mM and the cultures incubated an additional 13 hours.

The butyrate treatment was followed by a 2 minute wash with 15% glycerol in HBS at 37°C and a 24 hour recovery period in normal serum supplemented α-MEM. Transfectants were then selected in 0.75 mM hydroxyurea for one week and rescued in normal serum supplemented α-MEM without drug for 4 days. Surviving colonies were then cloned using glass cloning cylinders and trypsin solution.

Transfection experiments involved the use of both WT and H^R cellular DNA in parallel experiments. Only WT cells were transfected and cultures treated with DNA from WT or H^R cells were treated in an identical manner.

RESULTS

Isolation of Hydroxyurea-resistant Myoblast Cell Lines

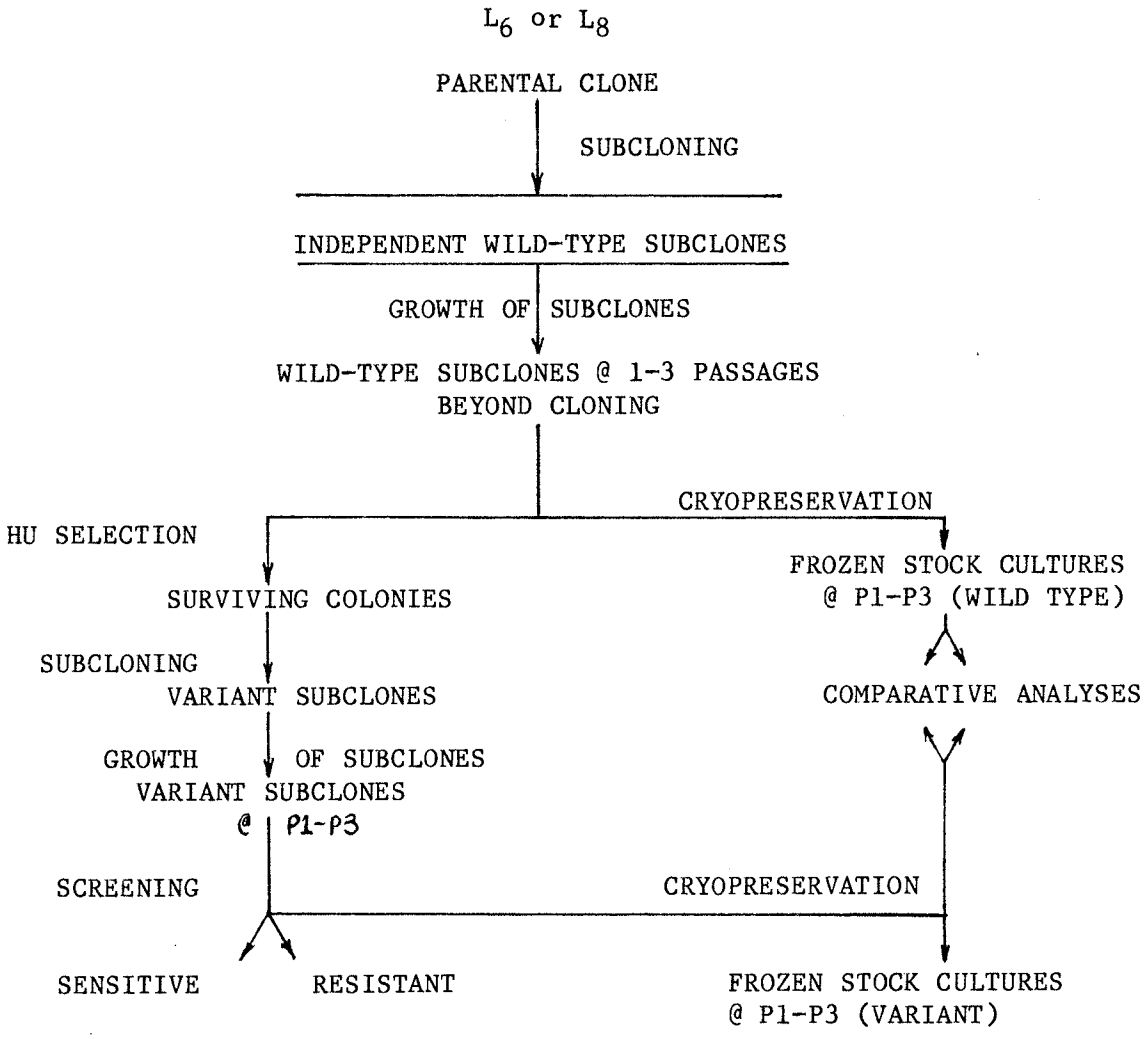
The establishment of the interrelationship between differentiation competence, progression of the transformed state and ribonucleotide reductase activity by an experimental approach employing the isolation of hydroxyurea resistant variants from myogenic clones of the L_6 and L_8 rat cell lines requires the use of freshly isolated, young passage clones of wild type cells. Correlations between changes in the competence of wild type and selected variant populations are possible only if the frequency of nonmyogenic variants is known for the unselected population, since Yaffe (1973) has shown that the apparent myogenic competence of L_6 myoblasts drops to less than 20% in old passage (50-60) clones. Previous studies in this candidate's doctoral laboratory by Dr. C.L.J. Parfett have estimated that the frequency of nonmyogenic clones arising in L_6 myoblast wild type cell lines is less than 0.013 (Parfett, Jamieson and Wright 1981). This low frequency of incompetent variants permits correlations to be observed between the loss of myogenic competence and the acquisition of altered traits, such as drug resistance, if several independent experiments are performed which exhibit similar results. For example, if 5 or 6 clones are isolated randomly from young passage L_6 cells in independent experiments, the possibility that all 5 or 6 clones might be nonmyogenic is less than 10^{-12} to 10^{-10}. Thus, in the present study only early passage, independent clones of wild-type populations were used in the isolation of drug resistant variants.

Several populations of L_6 and L_8 rat myoblasts were isolated from the original cell lines by seeding well dispersed cells at low cell density and allowing the growth and formation of discrete colonies.

Individual colonies were isolated with the aid of glass cloning cylinders and grown as clonal populations. Each 'wild type' colony was cloned without known selection bias. Since the frequency of nonmyogenic variants arising in the population was known, these individual clones were not pre-tested for differentiation competence prior to use in the selection of drug resistant variants. The wild type clones isolated in this manner were designated L_6WT-1,2,3,4,5,6 and L_8WT-1. The specific number assigned to each clone is not related to the order of use or selection; numbers were assigned randomly later in the study.

Each wild type clone was used shortly after isolation for the selection of hydroxyurea resistant variants. Several independent experiments were performed, each with a clonally independent parental population, as described in figure 1. Cryopreserved pools of early passage (P1-P3) WT clones were maintained at -70°C while their siblings were seeded at 1-5x10^5 cells per 100 mm culture dish and exposed to 1-2 mM hydroxyurea. Several weeks later, surviving colonies of cells were rescued in medium without drug and cloned shortly thereafter. These surviving populations of variant siblings were grown and maintained in a manner indentical to that of their parental populations before selection. Siblings of each variant clone were also cryopreserved at early passage then tested for resistance to the selective agent in colony forming assays employing passage matched parental populations (resuscitated from the cryopreserved pools) as controls. This sib selection technique insures that variant populations are exposed to drug only during selection (and usually only a single challenge). Variants which exhibited resistance to hydroxyurea were then examined further for changes in ribonucleotide reductase activity, differentiation and transformation, again using only cryopreserved, passage-matched

FIGURE 1: Scheme describing the selection and analysis of L6 and L8 rat myoblast clones sensitive and resistant to hydroxyurea. Note that each variant clone has a specific and clonally-independent parent population from which it was selected. Analyses were performed using cyropreserved pools of early, passage-matched wild-type and variant clones.


L_6 or L_8
PARENTAL CLONE
SUBCLONING
INDEPENDENT WILD-TYPE SUBCLONES
GROWTH OF SUBCLONES
WILD-TYPE SUBCLONES @ 1-3 PASSAGES BEYOND CLONING
CRYOPRESERVATION
HU SELECTION
FROZEN STOCK CULTURES @ P1-P3 (WILD TYPE)
SURVIVING COLONIES
SUBCLONING
VARIANT SUBCLONES
COMPARATIVE ANALYSES
GROWTH OF SUBCLONES
VARIANT SUBCLONES @ P1-P3
SCREENING
CRYOPRESERVATION
SENSITIVE
RESISTANT
FROZEN STOCK CULTURES @ P1-P3 (VARIANT)

populations. To insure that enough cells would be available for extensive analysis, each population was used up to 20 passages beyond its original cloning event and pools of these growing populations were routinely cryopreserved and resuscitated later for use during the experimental lifetime of 20 passages.

Colonies which were cloned from the sib selections were often not resistant. The overall frequency with which resistant colonies were isolated from all myoblast selection experiments was approximately 5.0×10^{-7} or about one per two million cells originally seeded from any WT myoblast clone. One of the non-resistant clones, isolated from the sib-selection protocol of L_6WT-4 (which resulted in the isolation of a resistant clone, L_6H^R-4), was used to replace its original WT parental clone and was designated L_6WT-4 (hereafter known simply as L_6WT-4). This clone exhibited normal WT sensitivity to hydroxyurea and behaved in a manner identical to the original WT parental clones used in the selection of H^R variants, even though it had gone through the sib-selection protocol. This clone was used in place of the original parental line such that any effects of the drug selection itself could be determined, serving essentially as a control population. The colony forming abilities of the variant clones in hydroxyurea are shown in figures 2 and 3. All WT clones exhibited a 90% reduction in colony forming ability at a concentration of drug (D_{10}) equal to 0.34 ± 0.02 mM and are shown in the figure as a single set of data points for simplicity. The six hydroxyurea resistant L6 myoblast clones, $L_6H^R-1,2,3,4,5,6$, displayed a wide range of relative resistance to the selective agent which was found to be stable upon repeated subcloning. Results from experiments using such subclones have been incorporated into figure 2. D_{10} values for each variant clone were as follows:

FIGURE 2: Effects of various concentrations of hydroxyurea on the colony forming ability of wild type and hydroxyurea-resistant L6 rat myoblast clones. ○ L_6WT-6, △ L_6H^R-1, ▲ L_6H^R-2, ● $L6H^R$-3, □ $L6H^R$-4, ○ $L6H^R$-5 and □ $L6H^R$-6. The L6 WT curve shown is the average of all values from parental clones. Bars depict the range of experimental values obtained in at least two independent experiments with each pair of drug-sensitive and resistant cell lines

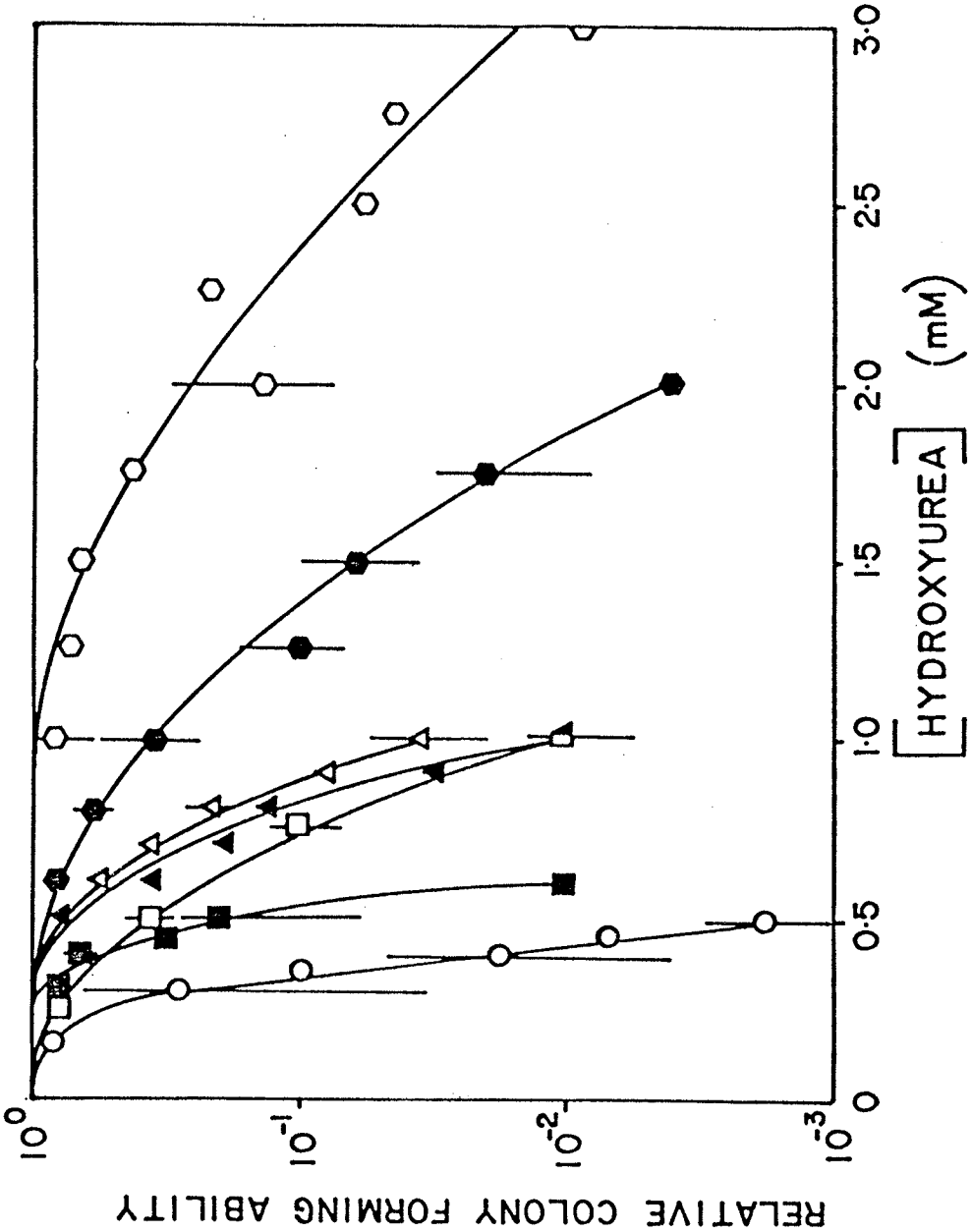
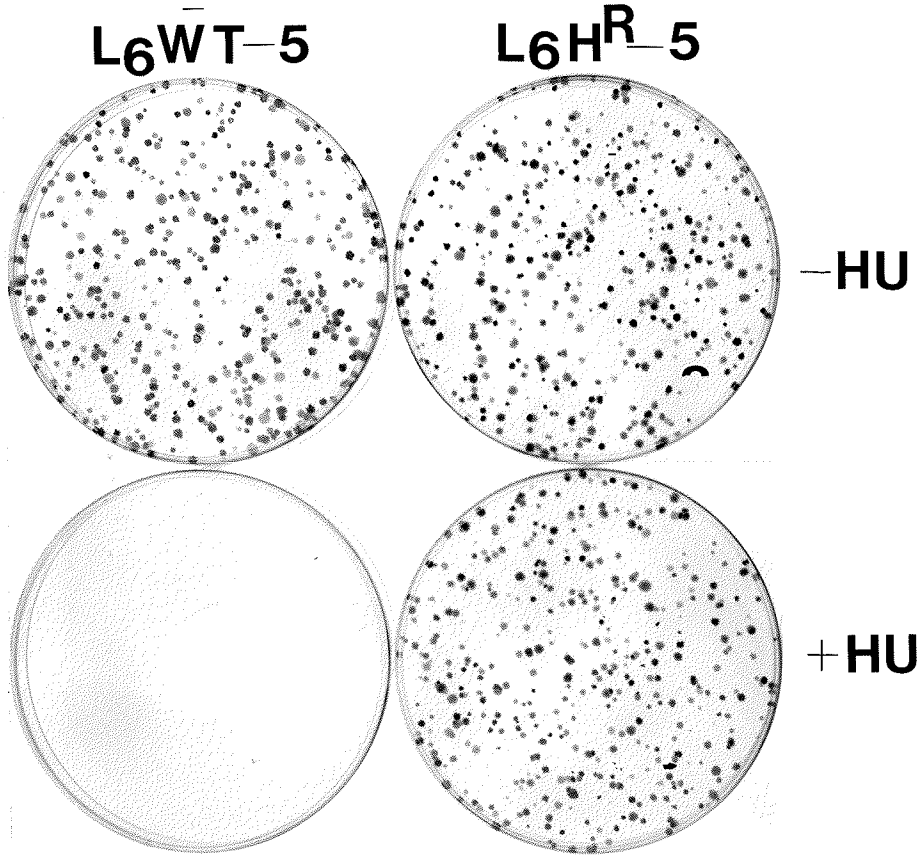

RELATIVE COLONY FORMING ABILITY
10^{0}
10^{-1}
10^{-2}
10^{-3}
0
0·5
1·0
1·5
2·0
2·5
3·0
[HYDROXYUREA] (mM)

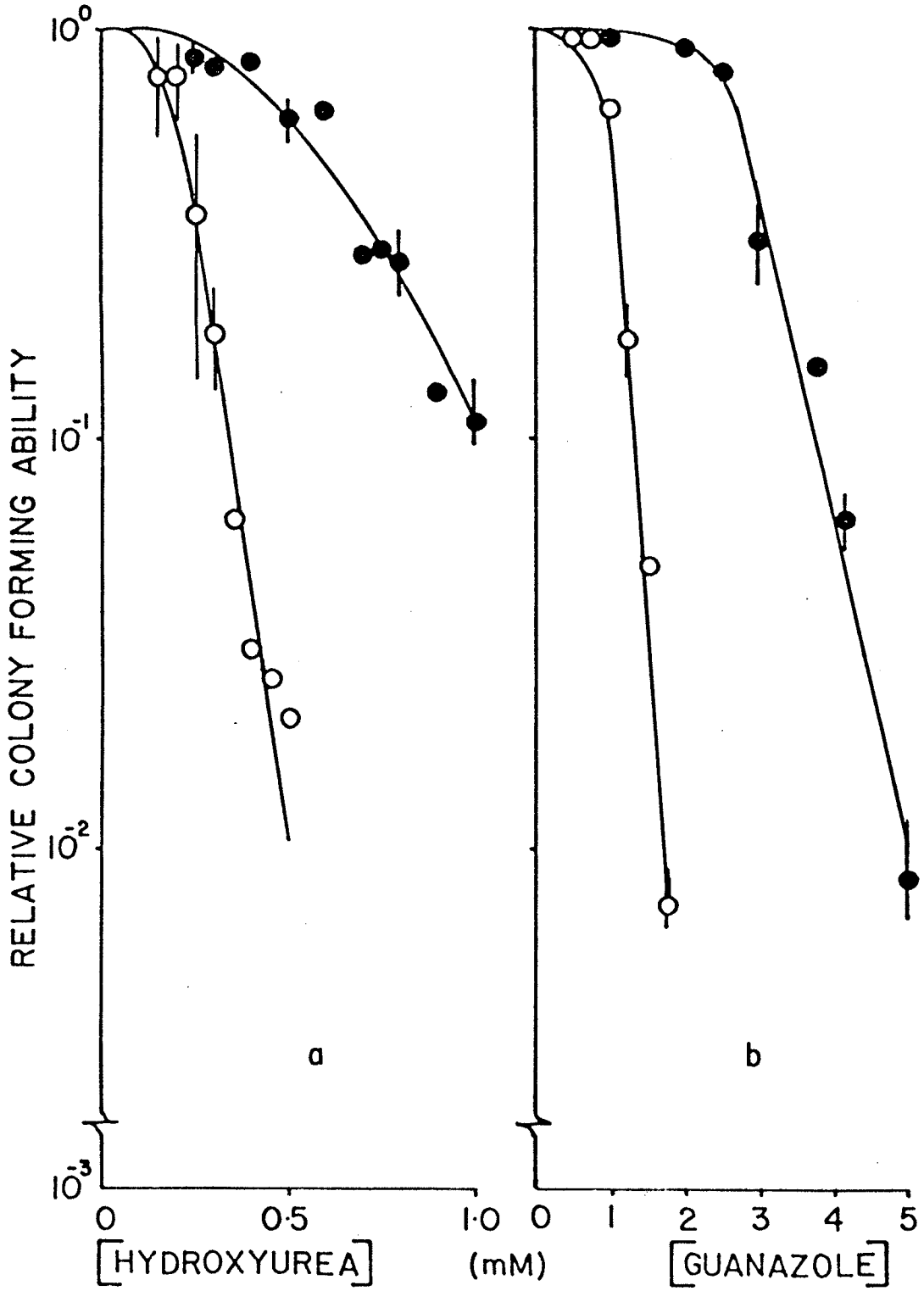
FIGURE 3: Photograph of the effect of hydroxyurea on the colony forming ability of L6WT-5 (left) and $L6H^R$-5 (right) myoblast clones. Plates were seeded with 500 cells and displayed a 70% plating efficiency by both clones after two weeks in the absence of drug (upper). In the presence of 0.5 mM hydroxyurea (lower) the plating efficiency of the variant remained the same while that of the sensitive parent clones was lowered 3 orders of magnitude.


L6WT-5
L6HR-5
-HU
+HU

L_6H^R-1, 0.9 mM; L_6H^R-2, 0.8 mM; L_6H^R-3, 1.3 mM; L_6H^R-4, 0.7 mM; L_6H^R-5, 2.0 mM; L_6H^R-6, 0.5 mM. The very high resistance of L_6H^R-5 and the very low resistance of L_6H^R-6 reflect the manner in which they were selected (see Materials and Methods section). Variant clones L_6H^R-3 and L_6H^R-5 were selected from cyropreserved pools of L_6WT-3 and L_6WT-5, which were used in earlier selections for resistance to the lectin conA (Parfett et al 1981). In the experiments of Parfett et al (1981, 1983) these two wild type clones were designated L_6Cl_6WT and L_6Cl_2WT, respectively. The use of common WT clones for the selection of $conA^R$ and H^R variants was exploited in later experiments examining the role of fusion incompetence and specific resistance in aspects of the transformed state.

A hydroxyurea resistant L_8 myoblast variant was also selected in a manner similar to the L_6H^R-1. The colony forming ability of this clone and its parental population in hydroxyurea and guanazole is shown in figures 4a and b. The D_{10} of L_8WT-1 is similar to that of the L_6WT clones, being 0.30 $\pm$ 0.03 mM. L_8H^R-1 displays a 3 fold increase in hydroxyurea resistance, with a D_{10} of 1.0 mM. Previous work in this candidate's doctoral laboratory has shown that cells resistant to hydroxyurea are also cross-resistant to another agent which inhibits ribonucleotide reductase activity, the triazole compound guanazole (Wright and Lewis 1974). As expected, L_8H^R-1 was also 3 fold more resistant to this agent in comparison to L_8WT-1, with each population displaying a D_{10} value of 3.9 and 1.3 mM, respectively. Similar cross-resistance was seen with the highly resistant clone L_6H^R-5, where the D_{10} value of its parental clone L_6WT-5 was also 1.3 mM (data not shown). Interestingly, L_6H^R-1 exhibited

FIGURE 4: Effect of various concentrations of hydroxyurea (a) and guanazole (b) on the colony forming ability of L8 rat myoblasts. ○ L8WT-1 and ● $L8H^R$-1. Bars represent the standard error of mean values from at least two independent experiments.


RELATIVE COLONY FORMING ABILITY
10⁰
10⁻¹
10⁻²
10⁻³
a
b
0
0·5
1·0
[HYDROXYUREA]
(mM)
0
1
2
3
4
5
[GUANAZOLE]

cross-resistance to the male sterility drug gossypol, in colony forming assays performed with L_6WT-1 as control. The apparent D_{10} values were 25 and 40 μM for L_6WT-1 and L_6H^R-1 respectively (Creasey et al 1985, submitted). Furthermore, L_6H^R-2 exhibited a greater than two fold increase in plating efficiency compared to L_6WT-2 after a 10 minute exposure to 100μM H_2O_2 at $1x10^6$ cells/10 ml.

The isolation of hydroxyurea-resistant L_6 and L_8 rat myoblast variants by sib selection from independent wild type parental clones allowed for the examination of other properties which might also be found in common to all these variants, but not their parental populations. Since the only difference between WT and H^R populations is exposure to drug during selection, similar properties exhibited by variant populations may be correlated with resistance to the selective agent and other properties found in common.

Growth and Differentiation of H^R Myoblast Clones and Their Parental Populations

Each hydroxyurea-resistant L_6 or L_8 myoblast clone and its respective WT parental population was examined for differentiation competence by two criteria: kinetics of fusion into multinucleated myotubes and the accompanying increase in creatine phosphokinase activity. Such experiments allowed the measurement of both morphological and biochemical aspects of differentiation for each pair of hydroxyurea-sensitive and resistant clones.

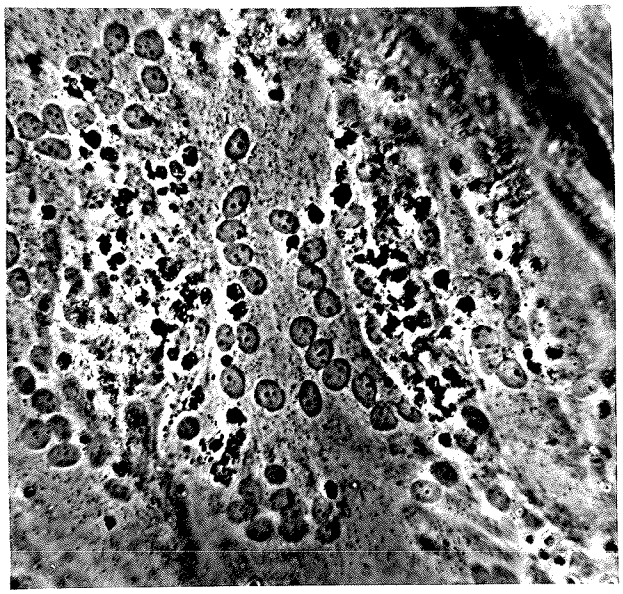
Without exception, every H^R clone was found to be grossly fusion incompetent under standard assay conditions. The differences in morphological differentiation between L_6WT and H^R clones are shown in

figures 5 and 6 and those of L_8WT-1 and L_8H^R-1 are shown in figure 7. L_6 and L_8 wild type clones typically exhibited 93 $\pm$ 6% and 86 $\pm$ 4% of all their nuclei in syncytia respectively, 4 to 6 days after reaching confluence, when partially synchronized to do so by lowering the serum content of their growth media to 1% or less (Creasey and Wright 1984a). This striking morphological change in WT populations is readily apparent in figure 5a, where L_6WT-1 has been used as a representative example, and in the accompanying figure 5b where no such change is evident in L_6H^R-1 after similar treatment. In a typical fusion assay, clones L_6H^R-2,4 and 5 would never exhibit any such morphological change, whereas clone L_6H^R-1 could rarely be observed to form a fusion element containing 3 or more nuclei. These events are rare enough that the effective fusion index of L_6H^R-1 remains at <1%. In contrast, when fusion was scored in colony-forming assays, 3% of L_6H^R-1 colonies exhibited limited fusion (table 1), whereas 90% of L_6WT-1 colonies show extensive fusion in such assays. Thus the inability to note significant fusion in L_6H^R-1 under standard assay conditions probably reflects the limitations inherent in the protocol. Like the L_6H^R-1 variant, L_6H^R-3 colony forming fusion assays correlated well with standard assay values (table 1).

Interestingly, myogenic competence was not completely extinguished in two other hydroxyurea-resistant cell lines, L_6H^R-3 and L_6H^R-6. Standard fusion assays repeatedly revealed that these two clones could exhibit partial fusion to levels of about 30% and 20% of their nuclei in syncytia, respectively (figure 6). The apparently attenuated morphological change did not appear to rise above these plateau levels even after prolonged maintenance of induced cultures of L_6H^R-3 and L_6H^R-6, nor did L_6H^R-1,2,4 and 5 exhibit any futher evidence of

FIGURE 5: Phase-contrast micrograph of wild type and hydroxyurea-resistant rat myoblasts during the course of a typical fusion assay, four days post-confluence. (a) L_6^{WT}-1 and (b) $L_6^{H^R}$-1.

a



b

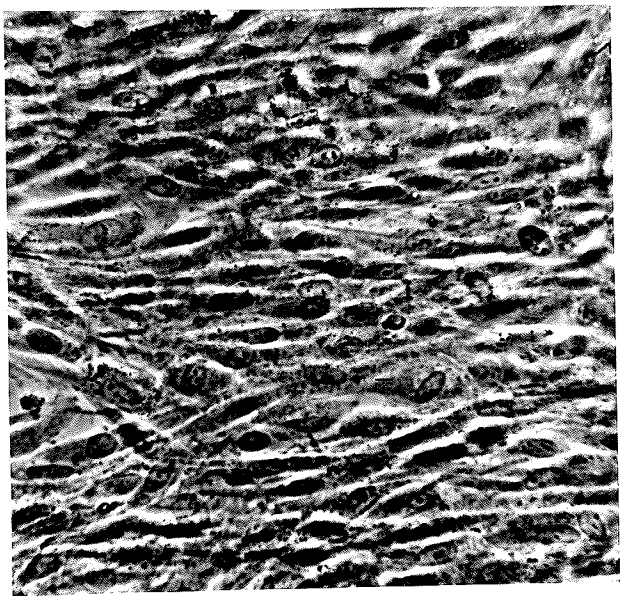


FIGURE 6: Fusion kinetics of L6 myoblast clones in 1% CS/α-MEM during the course of a normal fusion assay.
○ L_6^{WT1-6}, ● L_6H^R-1,-2,-4,-5, ◑ L_6H^R-3 and ◐ L_6H^R-6
The curve shown for parental clones is the average obtained for all assays with wild type cells. Variant clones 2,4 and 5 show no evidence of fusion in such assays, but a fusion element can be seen rarely (fusion index <1%) in assays with L_6H^R-1. Bars are the standard error of mean values of at least two independent experiments.

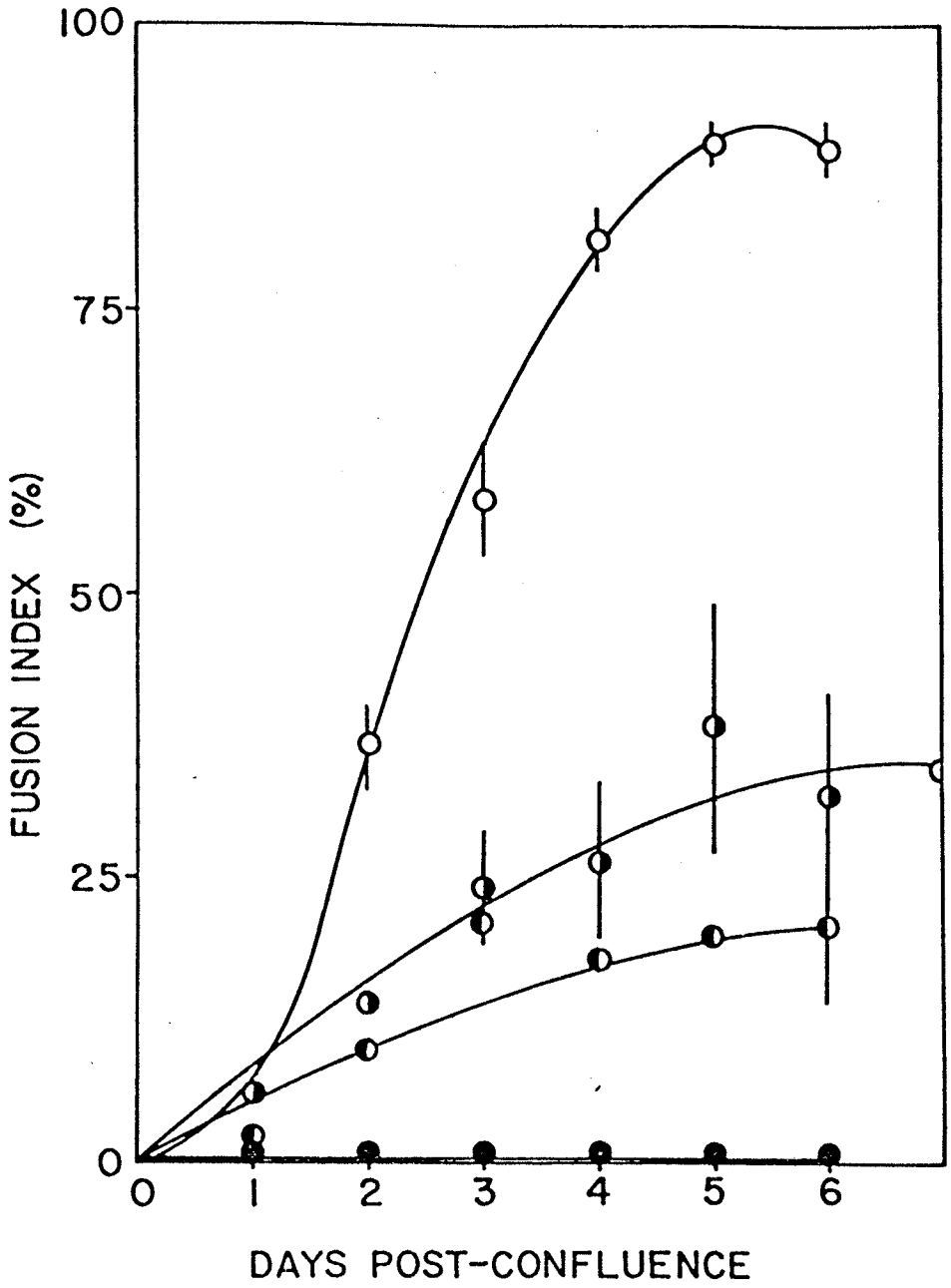

100
75
50
25
0
FUSION INDEX (%)
0
1
2
3
4
5
6
DAYS POST-CONFLUENCE

FIGURE 7: Fusion kinetics of L8 myoblast clones in 1% FCS/α-MEM during the course of a normal fusion assay. ○ L8WT-1 and ● $L8H^R$-1 Standard errors of the means are shown for two independent experiments.

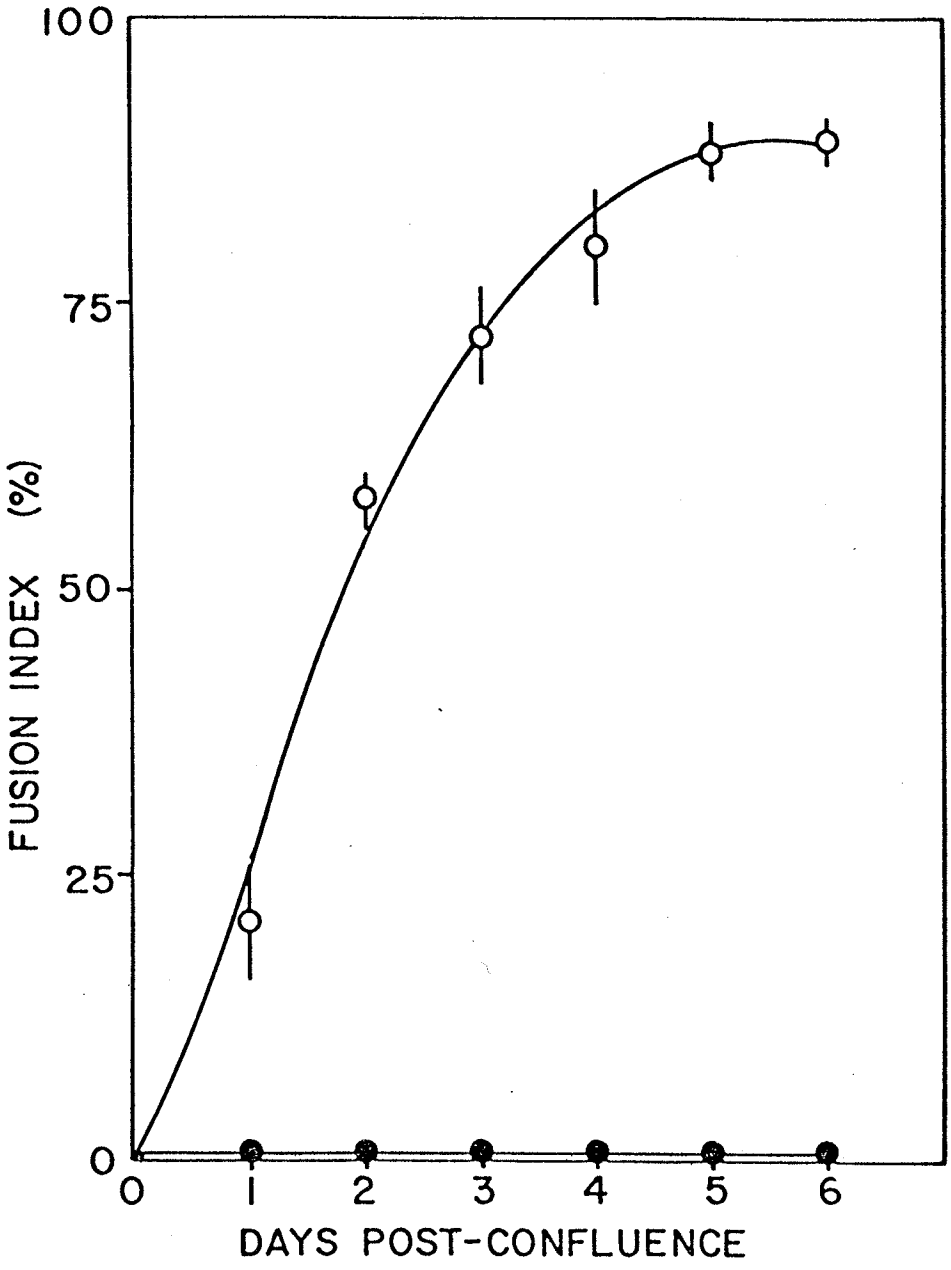

100
75
50
25
0
FUSION INDEX (%)
0
1
2
3
4
5
6
DAYS POST-CONFLUENCE

TABLE 1: Comparison of fusion index values obtained by standard assay and in colony forming assays

NUMBER OF COLONIES PER 100 mm PLATE[a]

L_6WT		L_6H^R-1		L_6H^R-3	
Fusing	Total	Fusing	Total	Fusing	Total
				2	7
20	22	2	22	1	5
				4	6
22	24	0	18	1	7
				4	4
24	27	0	26	1	9
				1	2
13	13	1	19	1	3
				2	4
27	28	0	27		
106	114	3	112	17	47

	L_6WT	L_6H^R-1	L_6H^R-3
Colony forming fusion index[b]	93	3	36
Confluent culture fusion index[c]	90	<1	35

[a] Colonies were formed by seeding 10-50 cells in 100 mm dishes and incubating under standard growth conditions for 10-14 days. Media was replaced with 1% CS/α-MEM and the colonies incubated a further week. The numbers of methylene blue stained colonies and the proportion which exhibited myotube formation were scored microscopically.

[b] Calculated as described under Materials and Methods. Values are those of figure 5 (day 6).

[c] Colony forming fusion index is the percentage of colonies with evidence of myotube formation in comparison to the total number of colonies formed.

fusion even after 3 weeks incubation under low serum and confluent conditions. Even in the absence of serum, these H^R variants could not be induced to fuse into multinucleated myotubes (Creasey and Wright 1984a). Retinoic acid (all trans), at concentrations of $5x10^{-8}$ M also failed to induce fusion in variant cells (L_6H^R-1) when added to cultures 2 days before reaching confluence in standard fusion assays. Similar results were obtained with 5-azacytidime at a concentration of 1 μg/ml (approximately the D_{50} value; data not shown) and with hydroxyurea at concentrations of 0.05 to 0.70 mM. Furthermore, hydroxyurea did not abrogate fusion in L_6WT-1 at these concentrations, though cell death prevented the differentiation from progressing fully. Addition of deoxynucleotides (CdR, TdR, AdR, GdR) at final concentrations of 0.1 to 0.5 mM also failed to abrogate fusion in L_6WT-1 and did not induce fusion in L_6H^R-1.

The abrogation or apparent attenuation of differentiation seen in the morphological assays of H^R variants described above also manifested itself as a similar change in the increases in creatine phosphokinase activity which normally accompany fusion. No significant increases in CPK activity could be detected in L_6H^R-1,2,4 and 5 and L_8H^R-1 in experiments performed with parallel cultures during the course of normal fusion assays (figures 8a, b,d,e and g respectively). In keeping with the partial fusion seen with cultures of L_6H^R-3 and L_6H^R-6, increases in CPK activity in these clones was correlated with their apparent fusion competence. The variation seen in the maximal activity of WT clones was in keeping with values obtained in a large number of such clones examined by Parfett (Ph.D. thesis 1982). The L_8WT-1 clone exhibited considerably higher CPK activity than the

FIGURE 8a: Creatine phosphokinase activity of wild type (○) L_6 rat myoblast clones and the respective hydroxyurea-resistant variants (●) selected from them. Values are the averages of duplicate determinations using cell sonicates collected in parallel during the course of normal fusion assays. (a) L_6WT/H^R-1, (b) L_6WT/H^R-2, (c) L_6WT/H^R-3, and (d) L_6WT/H^R-4.

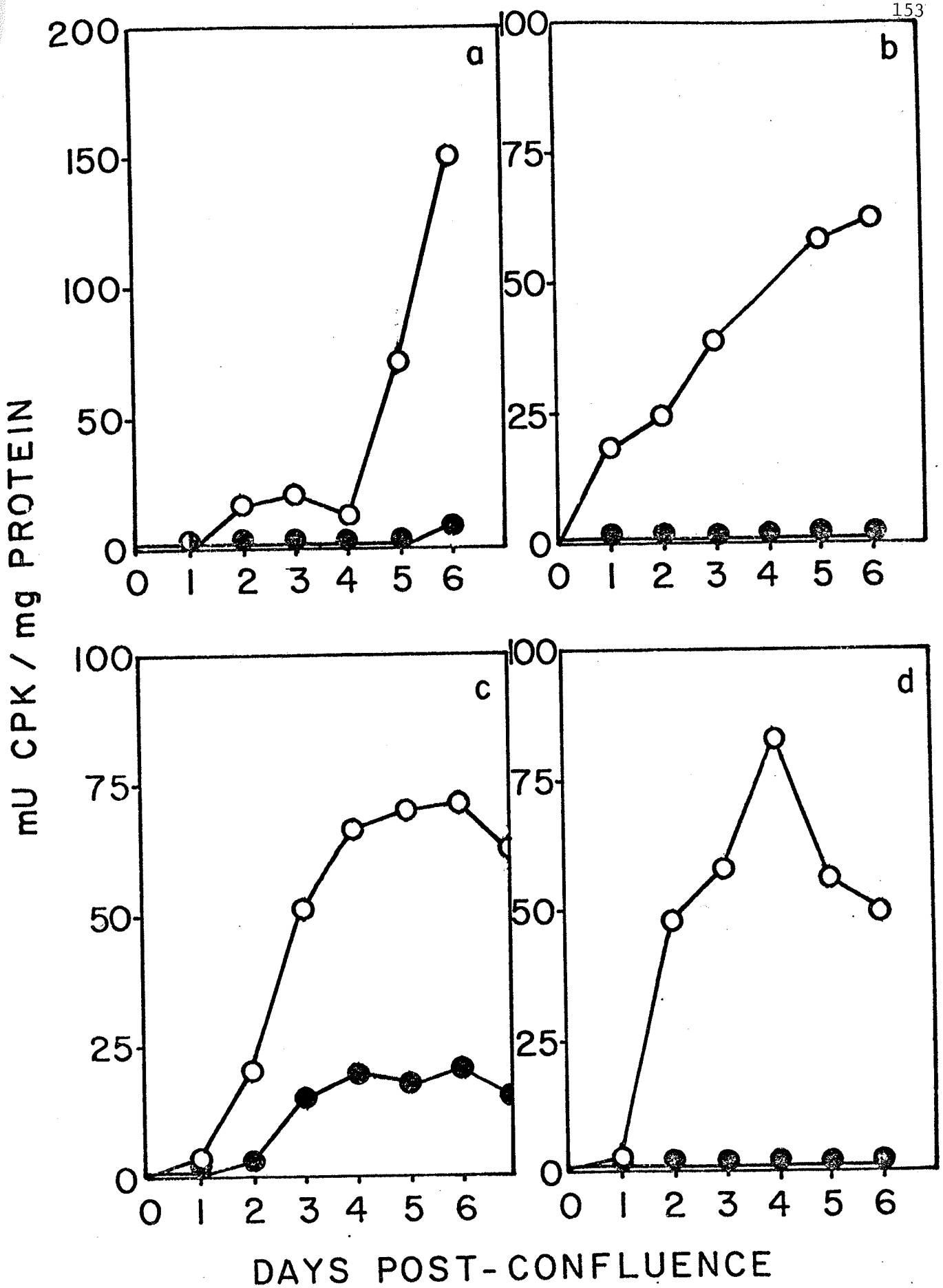
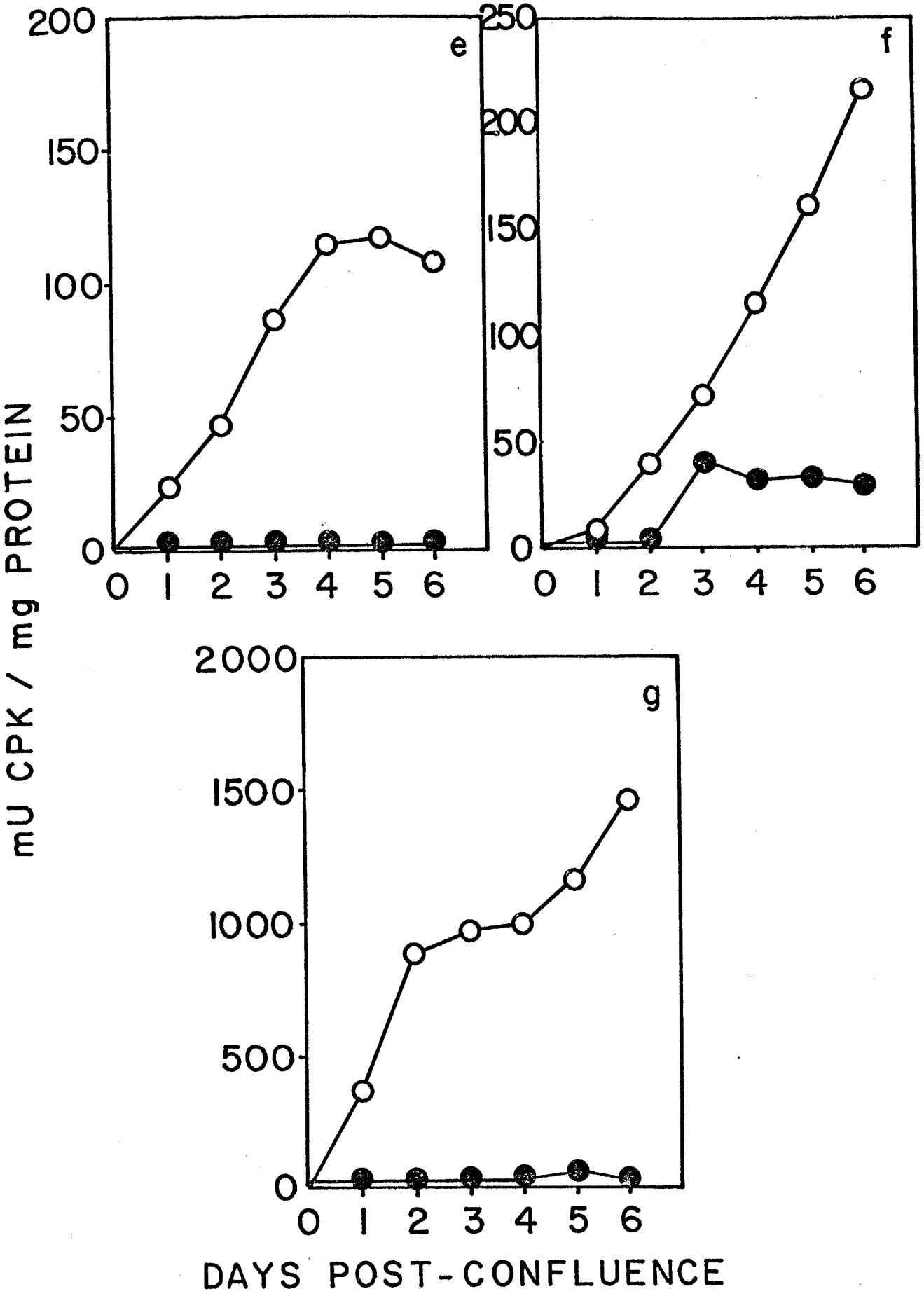

a
b
c
d
200
150
100
50
0
100
75
50
25
0
0 1 2 3 4 5 6
mU CPK / mg PROTEIN
DAYS POST-CONFLUENCE

Figure 8b: Creatine phosphokinase activity of wild type (○) L_6 and L_8 rat myoblast clones and the respective hydroxyurea-resistant variants (●) selected from them. Values are the averages of duplicate determinations using cell sonicates collected in parallel during the course of normal fusion assays. Data shown for L_6WT-5 is that of C.L.J. Parfett (with permission) (e) L_6WT/H^R-5, (f) L_6WT/H^R-6 and (g) L_8WT/H^R-1.


e
f
g
200
150
100
50
0
250
200
150
100
50
0
2000
1500
1000
500
0
0 1 2 3 4 5 6
mU CPK / mg PROTEIN
DAYS POST-CONFLUENCE

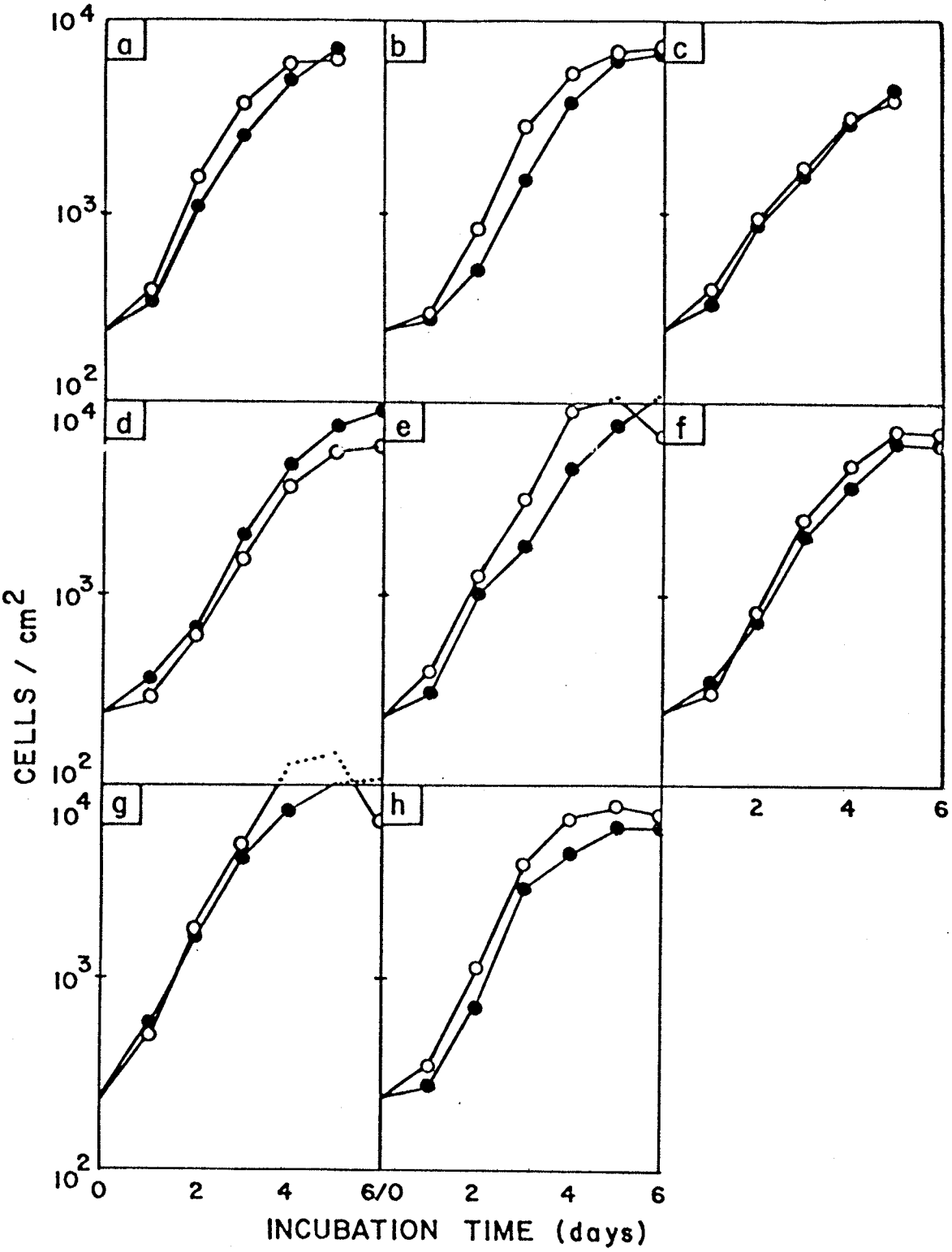
L_6 clones examined in this study, showing increases similar to those seen in many primary muscle systems (Shainberg et al 1971).

The apparent inability of the hydroxyurea-resistant myoblast clones to differentiate in a manner similar to their parental cell lines does not appear to be related to any unusual proliferative abilities exhibited by these variants. All H^R myoblasts proliferated in vitro at rates less than or nearly equal to that of their parental populations and attained similar saturation densities (figure 9a-g). Generation times estimated from these growth curves varied between about 12 to 20 hours, depending upon the clone being examined. As noted previously (Creasey and Wright 1984a). the non- fusing variants did not appear to proliferate rather than fuse during differentiation assays, even when under the severest growth-arrest conditions of complete serum starvation.

Cellular Drug Uptake by Variant Cell Lines

The cell membrane is intimately involved in the process(es) of cell fusion in the L_6 and L_8 myoblast cell lines. Previous work in this candidate's doctoral laboratory with $conA^R$ L_6 myoblast clones defective in myogenesis has shown that specific membrane changes are correlated with the fusion-negative phenotype (Parfett et al 1981, 1983). Thus, it was conceivable that the defective myogenic phenotype exhibited by H^R myoblasts clones was secondary to membrane alterations affecting cellular drug uptake and not the result of changes in RRase activity commonly associated with resistance to hydroxyurea. This possibility was examined using radio-labelled guanazole and hydroxyurea as probes for altered membrane function and drug uptake. [^{14}C]-

FIGURE 9: Growth curves of L6 and L8 rat myoblast clones and BALB/c 3T3 cells under standard growth conditions at 37°C. Wild type and hydroxyurea-resistant cells; (a) L_6WT/H^R-1, (b) L_6WT/H^R-2, (c) L_6WT/H^R-3, (d) L_6WT/H^R-4, (e) L_6WT/H^R-5, (f) L_6WT/H^R-6, (g) L_8WT/H^R-1 and (h) Balb/c 3T3 WT/H^R-1. Values are averages of duplicate determinations from a single experiment. The estimated generation times calculated from these plots are: L_6WT-1, 12 h; L_6H^R-1, 14 h; L_6WT-2, 13 h; L_6H^R-2, 15 h; L_6WT-3 17h; L_6H^R-3, 20 h; L_6WT-4, 14 h; L_6H^R-4, 14 h; L_6WT-5, 16 h; L_6H^R-5, 18h; L_6WT-6, 16 h; L_6H^R-6, 18 h; L_8WT-1, 16h; L_8H^R-1, 18 h; BALB/c 3T3 WT-1, 12 h; BALB/c 3T3 H^R-1, 12 h.


a
b
c
d
e
f
g
h
10^4
10^3
10^2
CELLS / cm^2
0
2
4
6/0
2
4
6
INCUBATION TIME (days)

guanazole was available for these studies whereas hydroxy[^{14}C]-urea became available much later in this study only after being synthesized by special order.

The cellular uptake of [^{14}C]-guanazole by L_6^{WT}-1 and L_6H^R-1 is shown in figures 10a and b (drug uptake by a revertant population is also shown in figure 10c; studies with this revertant population will be presented later, in the results dealing with myoblast tumor formation). Each of these cell lines took up the drug equally, suggesting that defective drug uptake was not responsible for the resistant phenotype. Extensive pretreatement of wild type myoblasts with the energy-inhibitor sodium azide did not abrogate drug uptake (figure 11a). These results suggest that guanazole uptake is a passive process in these cell lines. Uptake studies in all L_6^{WT} and L_6H^R clones examined, as well as CHO, have indicated that the apparent intracellular concentration of guanazole approaches but never exceeds that of the external medium. Maximal guanazole uptake occurs in about one to two hours in rat myoblast and Chinese hamster cells, reaching a cellular concentration of approximately three-quarters that of the external environment. The presence of a 250 fold excess of cold guanazole has no effect on the amount of label incorporated in such studies, up to the maximal concentration used (1 mM). Similarly, hydroxyurea has no effect on guanazole uptake in either of these cell systems, even when present in quantities greater than two orders of magnitude in excess of the labelled guanazole.

Hydroxyurea uptake was examined in greater detail in the cell lines used in this study. Cellular uptake of this drug, like that of guanazole, shows no apparent reduction when cells are extensively pretreated with sodium azide (figure 11b) or a variety of other energy inhibitors

FIGURE 10: Uptake of [^{14}C]-guanazole at 37°C by (a) L_6WT-1, (b) L_6H^R-1 and (c) L_6REV-1. The concentration of drug used was 12 μM, or 12 nmoles/ml/well. The final apparent concentration of guanazole after 30 minutes was 8.0 8.0 and 8.6 μM for L_6WT-1, L_6H^R-1 and L_6REV-1, respectively, when corrected for cell volume (L_6WT-1, 2.89±0.31 pl; L_6H^R-1, 2.84±0.27 pl; L_6REV-1 3.11±0.30 pl, mean ± S.D.)

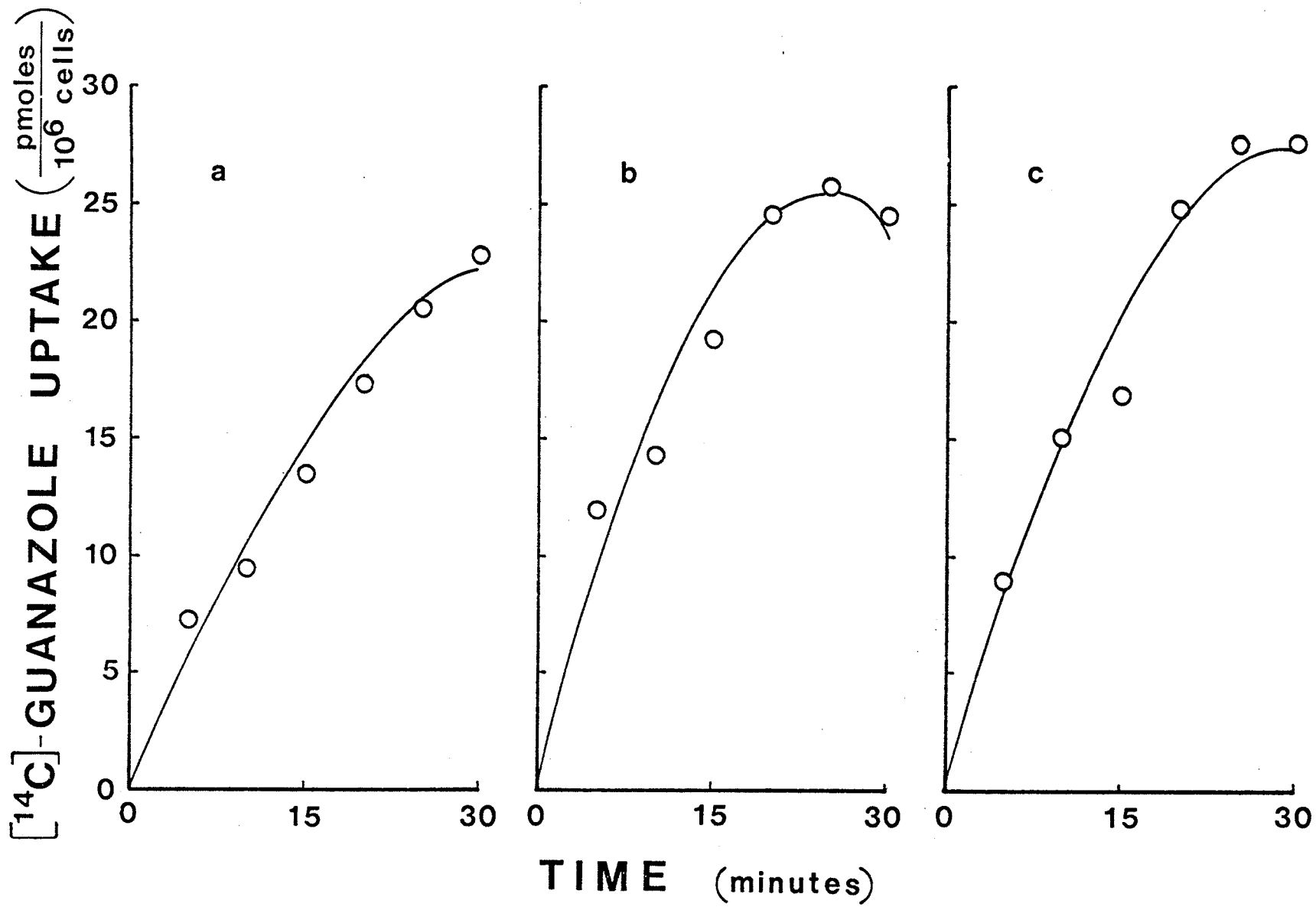
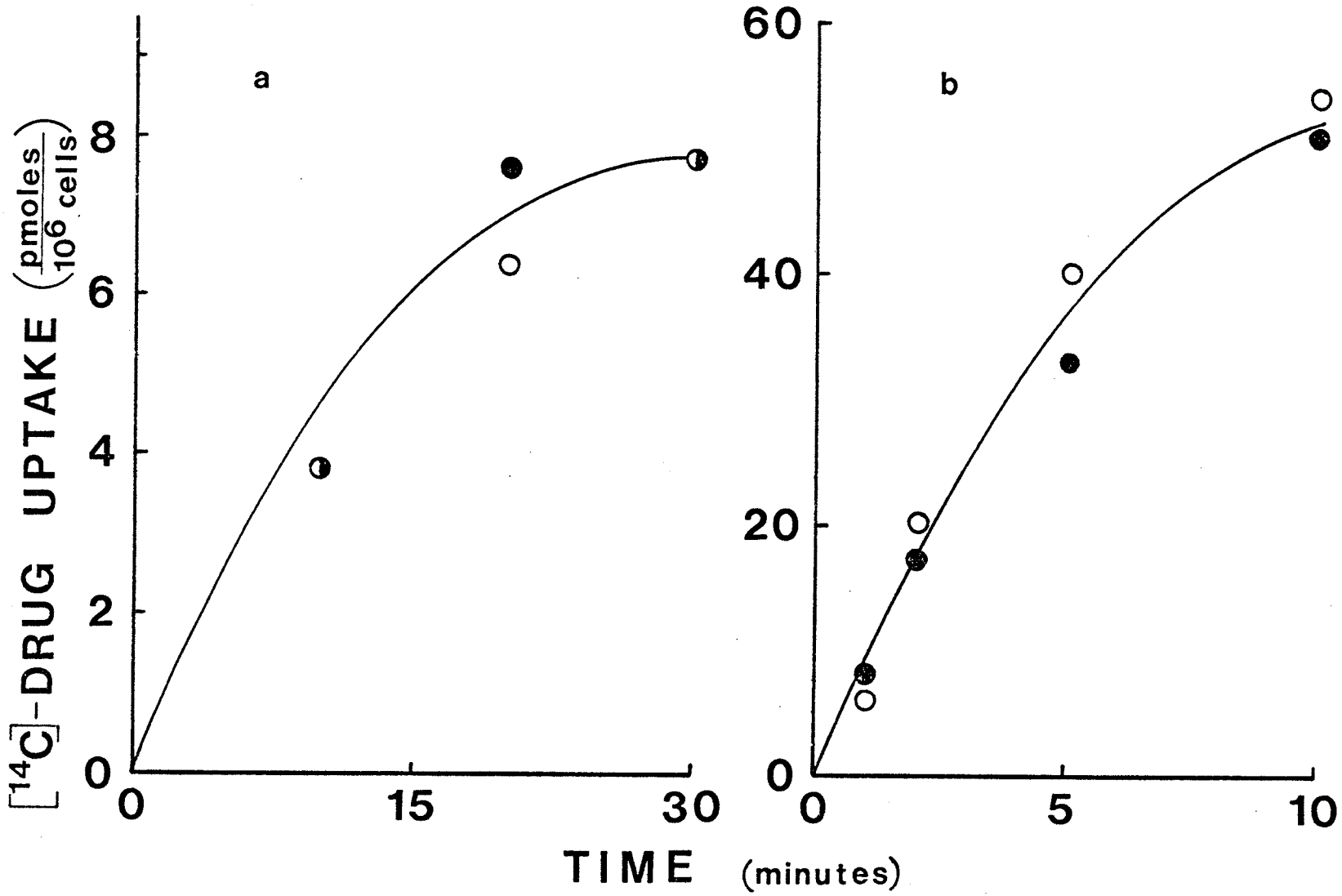

[14C]-GUANAZOLE UPTAKE (pmoles / 10^6 cells)
a
b
c
0
5
10
15
20
25
30
0
15
30
TIME (minutes)

FIGURE 11: Effect of 30 minute pretreatment (●) or no treatment (○) with 10 mM sodium azide on the uptake of [^{14}C]-guanazole at 37°C by (a) L_6WT myoblasts and (b) CHO WT C125.


a
b
[14C]-DRUG UPTAKE (pmoles / 10^6 cells)
0
2
4
6
8
0
15
30
0
20
40
60
0
5
10
TIME (minutes)

(i.e. potassium cyanide, 2,4-dinitrophenol). No significant differences in uptake were observed in the presence of a large excess of cold guanazole or hydroxyurea, as reported for the uptake of guanazole. Similarly, uptake was unaffected by the presence of hexoses.

The cellular uptake of hydroxyurea by L_6^{WT}-1 and $L_6^{H^R}$-1 appears to be virtually identical (figures 12a and b). All of the myoblast cell lines exhibited a similar pattern of drug uptake in such studies, except $L_6^{H^R}$-5 and $L_6^{H^R}$-6, the most and least resistant clones, respectively, and $L_8^{H^R}$-1. These lines showed the same amount of uptake as their parental clones after an hour, but lesser uptake after only 20 to 30 minutes. Thus all the myoblast cell lines exhibited the same maximal uptake of hydroxyurea after one hour in a manner very similar to that of guanazole. In addition, the Chinese hamster cell lines and mouse L cell lines used in this study also exhibit an identical pattern of drug uptake (figures 13 and 14). Interestingly, the apparent intracellular concentration of both hydroxyurea and guanazole reached the same levels in all these cell lines (rat, hamster, mouse) and with similar kinetics.

There are very few reports in the literature on the uptake of hydroxyurea. Since this drug is an important cancer chemotherapeutic agent, it was of interest to examine the properties of this process in some detail. The uptake of hydroxyurea is linear during the first few minutes in which cells in culture are exposed to the drug (figure 15). Kinetic analyses were performed in 2 minute assays as this provided for rapid, reproducible quantification of drug uptake under a variety of conditions. Chinese hamster ovary cells (WT C125) were chosen as the model system because of the ease with which this line could be propagated. Hydroxyurea, at concentrations up to 1 mM, which typified the

FIGURE 12: Uptake of hydroxy[^{14}C]-urea at 37°C by (a) L_6WT -1, (b) L_6H^R-1 and (c) L_6REV-1. Values are the averages of at least duplicate determinations from a single experiment. The concentration of drug used was 79 μM, or 79 nmoles/ml/well. The final apparent cellular concentration of hydroxyurea after 30 minutes was 34, 30 and 32 μM for L_6WT-1, L_6H^R and L_6REV-1, respectively, when corrected for cell volume (see legend to fig 10).

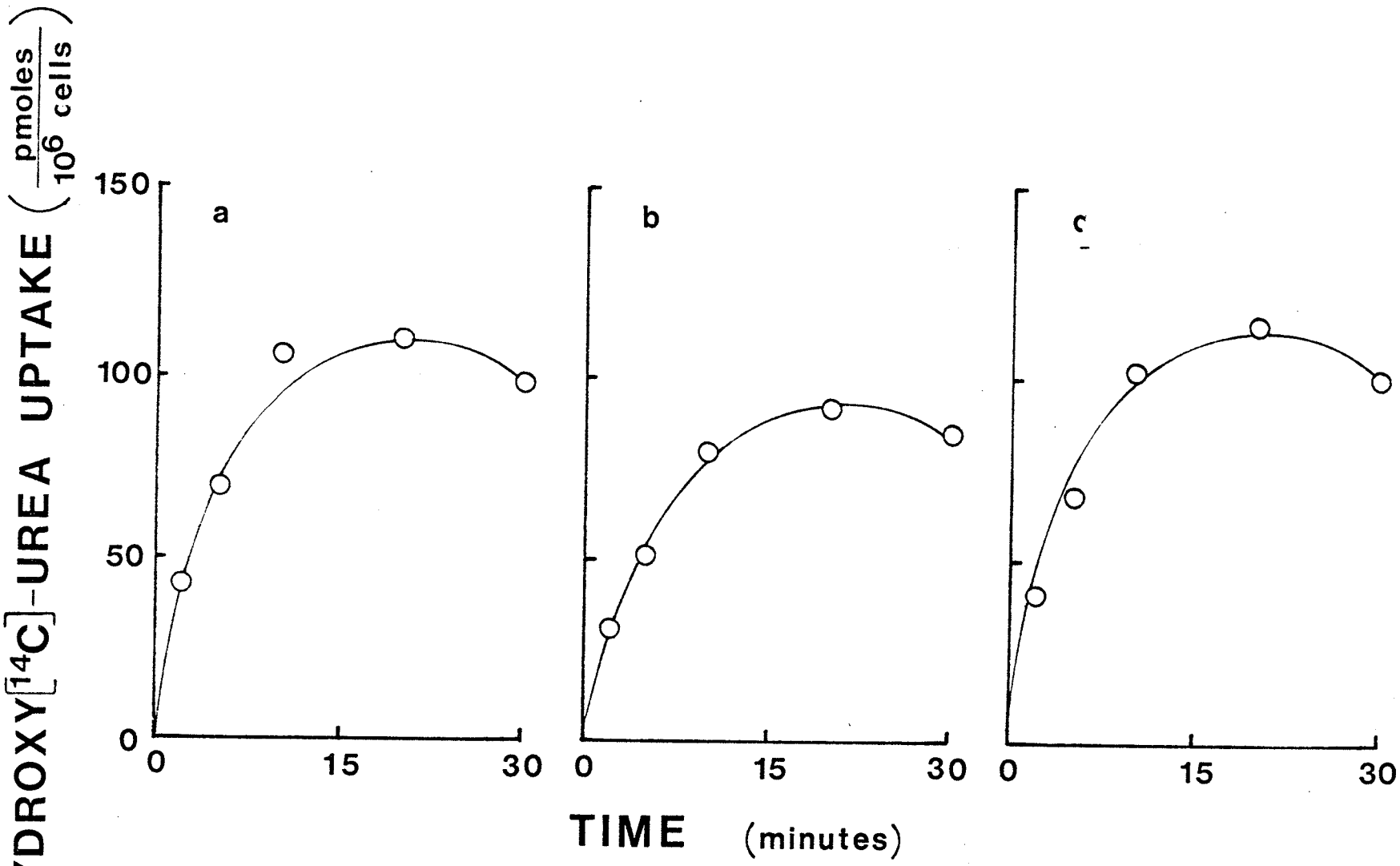

HYDROXY[14C]-UREA UPTAKE (pmoles / 10^6 cells)
150
100
50
0
a
b
c
0
15
30
TIME (minutes)

FIGURE 13: Uptake of hydroxy[^{14}C]-urea at 37°C by (a) CHO WT C125, (b) CHO HU^{R}-2 and (c) CHO NC^{R}-30A2. Values are the averages of at least duplicate determinations from a single experiment. The concentration of drug used was 81 μM, or 81 nmoles/ml/well. The final apparent cellular concentration of hydroxyurea after 30 minutes was 33, 29 and 41 μM for CHO WT C125, CHO Hu^{R}-2 and CHO NC^{R}-30A2, respectively, when corrected for cell volume (CHO WT C125, 2.03±0.19 pl; CHO Hu^{R}-2, 2.27±0.42 pl; CHO NC^{R}30A2, 2.51±0.36 pl; mean ± S.D.).

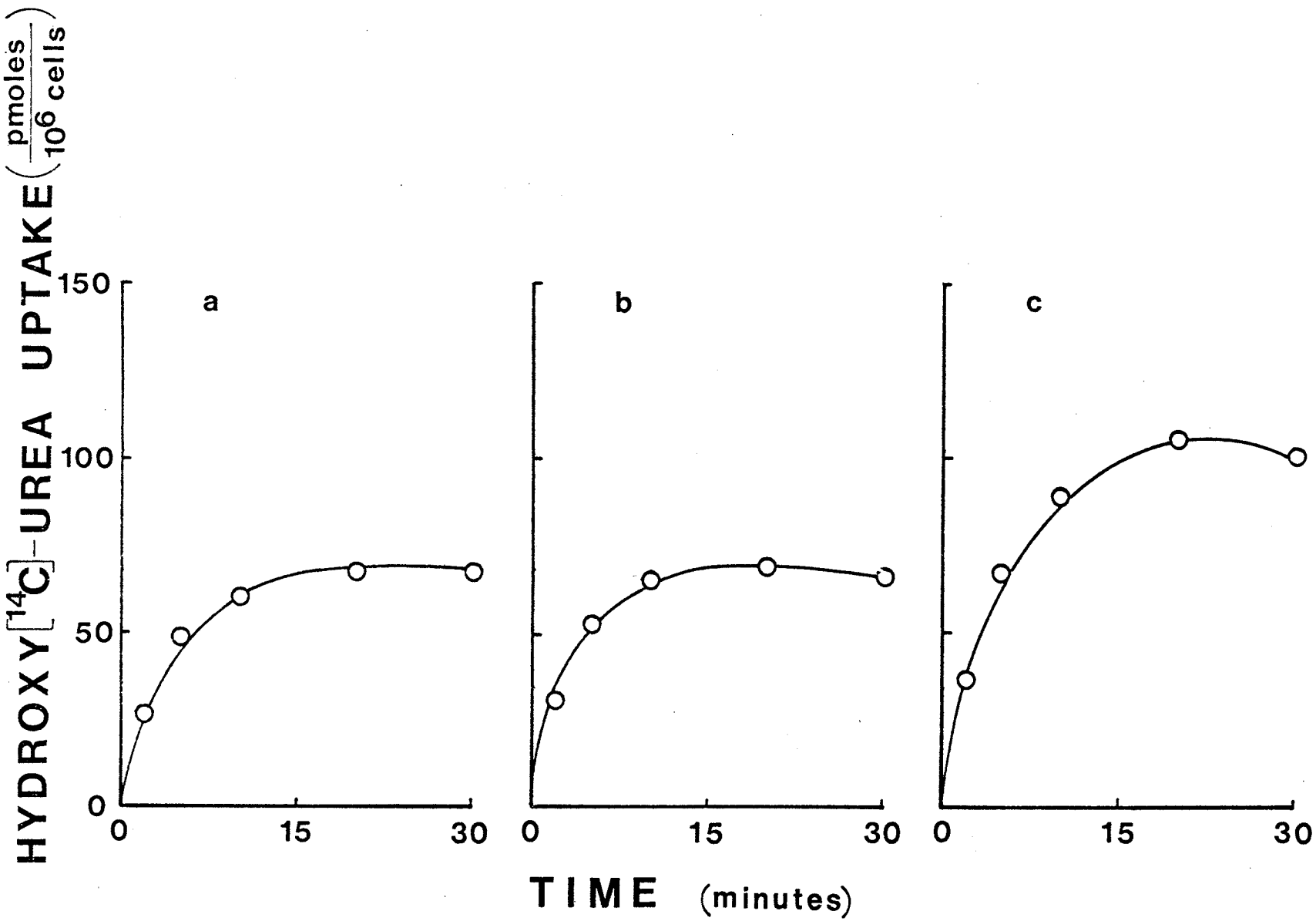
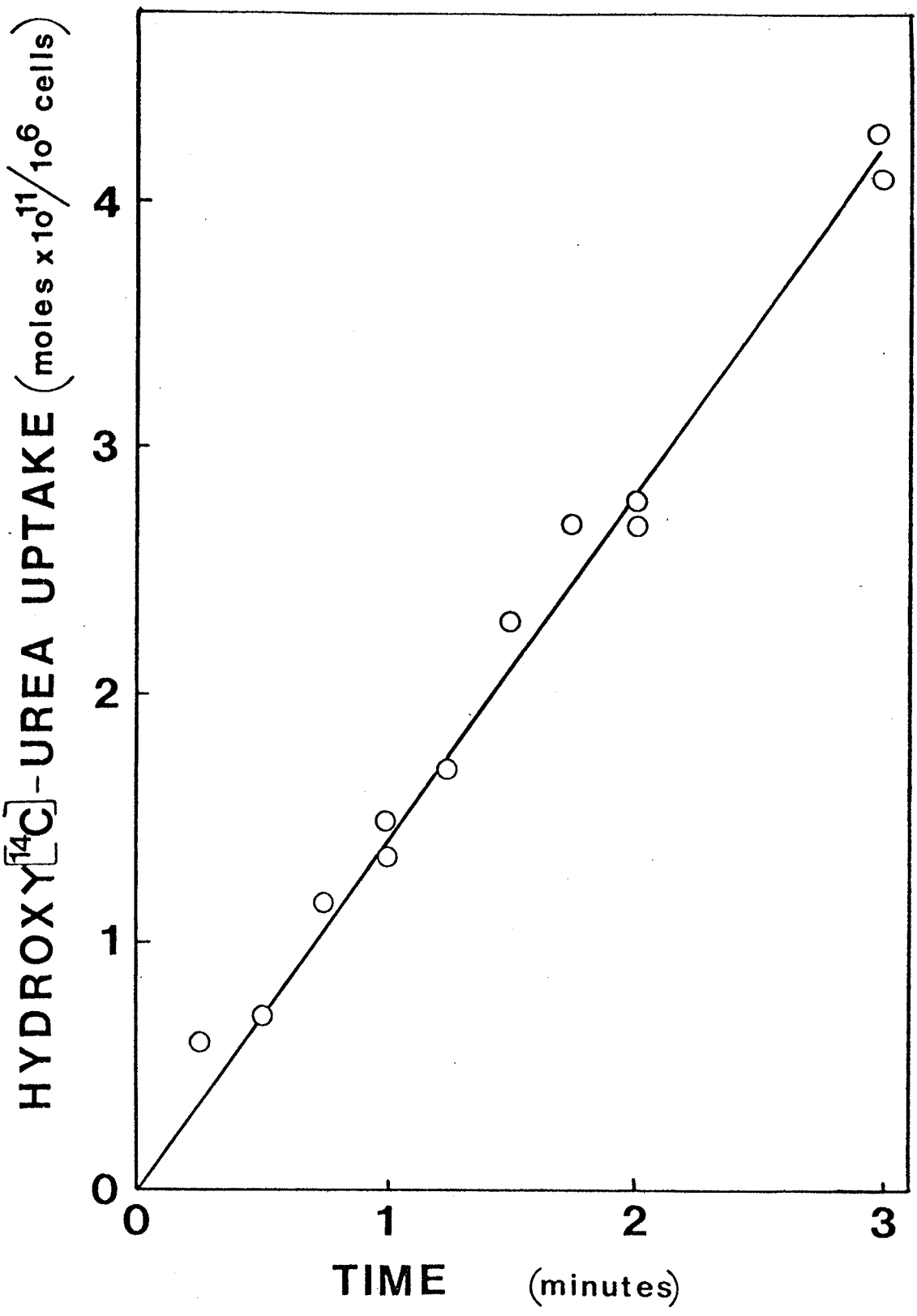

HYDROXY[14C]-UREA UPTAKE (pmoles / 10^6 cells)
150
100
50
0
a
b
c
0
15
30
TIME (minutes)

FIGURE 14: Uptake of hydroxy[^{14}C]-urea at 37°C by (a) $L_{60}WT$, (b) L_1H_2 and (c) L_2Cl_3. Values are the averages of at least duplicate determinations from a single experiment. The concentration of drug used was 63 M, or 63 nmoles/ml/well. The final apparent cellular concentration of hydroxyurea after 30 minutes was 43, 45 and 63 M for $L_{60}WT$, L_1H_2 and L_2Cl_3, respectively, when corrected for cell volume ($L_{60}WT$, 2.35±0.42 pl; L_1H_2, 2.29±0.12 pl; L_2Cl_3, 1.84±0.09 pl; mean ±S.D.).

HYDROXY [14C]-UREA UPTAKE (pmoles / 10^6 cells)
150
100
50
0
a
b
c
0
15
30
TIME (minutes)

FIGURE 15: Response of hydroxy[^{14}C]-urea uptake by CHO WT C125 to time allowed for drug uptake. Values are the averages of duplicate determinations from a single experiment.


HYDROXY[14C]-UREA UPTAKE (moles x 10^11/10^6 cells)
0
1
2
3
4
0
1
2
3
TIME (minutes)

amounts used normally with these mammalian cell lines in culture and with concentrations used in clinical situations with human patients, was taken up linearly with a velocity of approximately 140 pmoles/min/10^6 cells when monolayer cultures are exposed to 1 mM drug as a 1 ml solution in 35 mm diameter culture dishes (figure 16). At much higher concentrations of drug, up to 0.5 molar, the velocity of uptake also appears to be linear, both in CHO and normal human diploid fibroblasts in culture (figures 17a and b). Thus, the apparent K_M for hydroxyurea in these cell lines appeared to be zero and the V_{MAX} appeared to be effectively infinite. Drug uptake was examined at these very high concentrations (which are never normally encountered in the laboratory or in clinical practise) since there are conflicting reports in the literature on the nature of the uptake of the parent analog urea in various cell systems. One study has reported saturable self-exchange flux of urea with a half-maximal flux ($K_{1/2}$) of 396 mM in human red cells (Brahm 1983). While this report has indicated that urea uptake proceeds by a facilitated diffusion process, Hunter's group has reported that urea and thiourea uptake in the chicken apparently occurs in the absence of a carrier (Hunter et al 1965). The use of extremely high concentrations of compounds in uptake experiments is complicated, however, by osmotic volume changes which can yield apparent saturation kinetics if not taken into account (Wildbrandt and Fuhrmann 1977). In the present study, one experiment did indicate reduced uptake at 500 mM hydroxyurea, but was not in keeping with the majority of results obtained. Interestingly, the uptake of hydroxyurea has been reported to occur by simple diffusion in rat small intestine (Evered and Selhi 1970).

FIGURE 16: Hydroxy[^{14}C]-urea uptake velocity plot for CHO WT C125 at low concentrations of drug. Values are the averages of duplicate determinations from a single experiment.

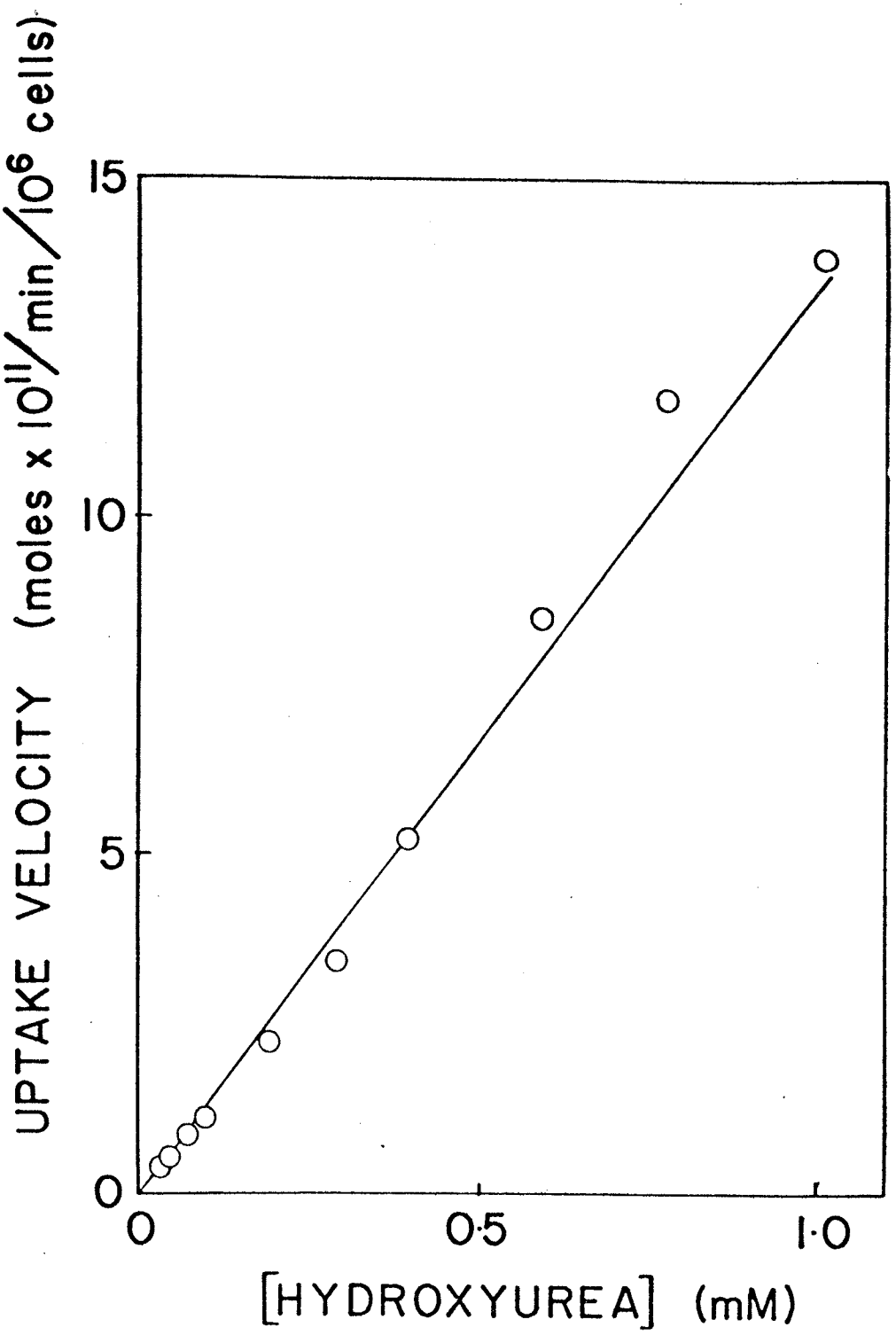
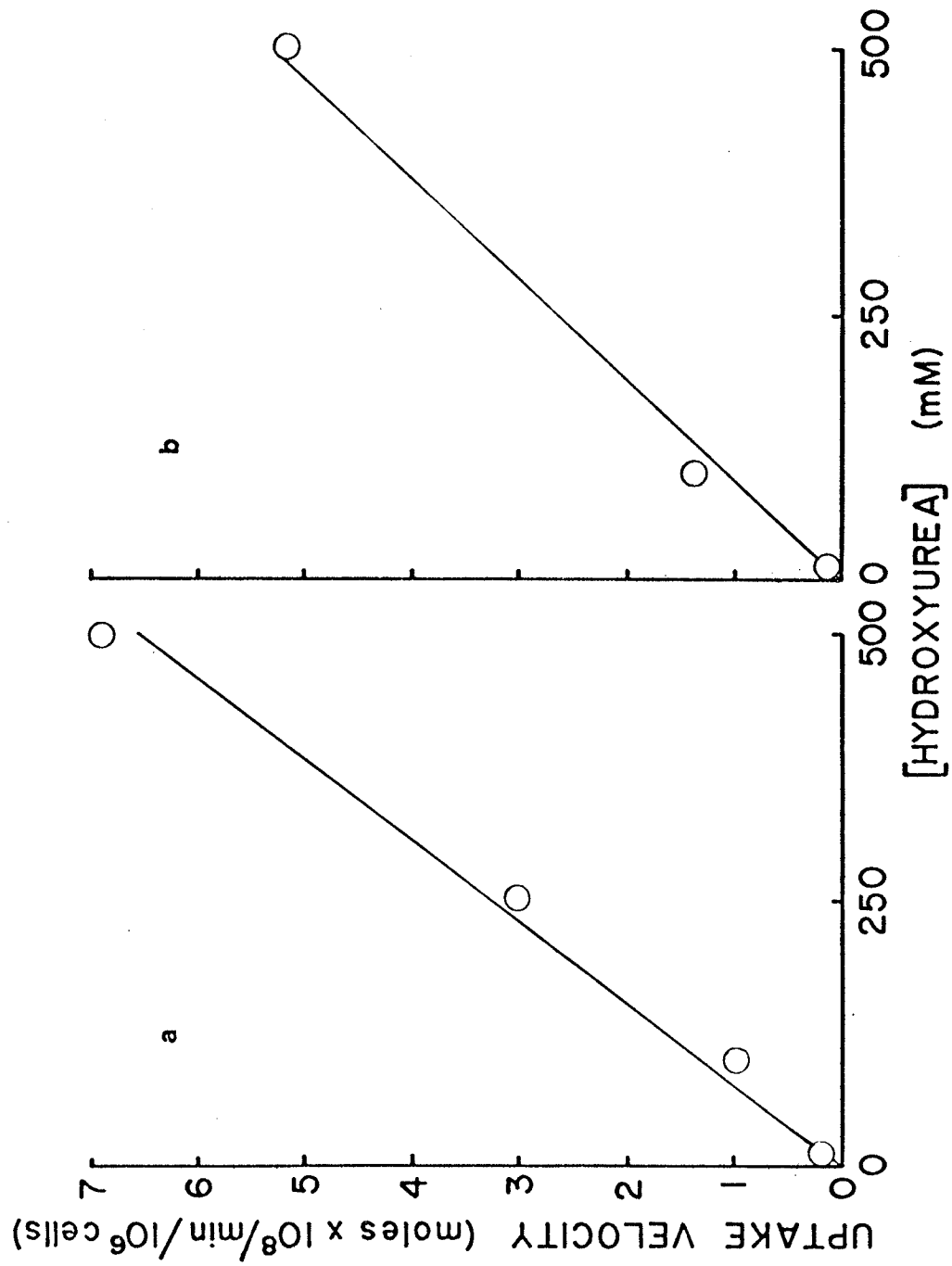

UPTAKE VELOCITY (moles x 10¹¹/min/10⁶ cells)
15
10
5
0
0
0·5
1·0
[HYDROXYUREA] (mM)

FIGURE 17: Hydroxy[^{14}C]-urea uptake velocity plots for (a) HSC172 normal human diploid fibroblasts and (b) CHO WT C125 at high concentrations of drug. Values are the averages of duplicate determinations from single experiments.


UPTAKE VELOCITY (moles x 10⁸/min/10⁶ cells)
0
1
2
3
4
5
6
7
a
b
0
250
500
0
250
500
[HYDROXYUREA] (mM)

Isolation of Hydroxyurea-resistant Differentiation-defective BALB/c 3T3 Cells

The study of hydroxyurea-resistance and its correlation with defective differentiation in the rat myoblasts was extended to include a second and distinctly different developmental cell system. A wild type, adipogenic BALB/c 3T3 population was used in the selection of a hydroxyurea-resistant clone, by a sib-selection protocol similar to that used for the L_6 and L_8 rat myoblast cell lines. A single, resistant line was isolated which displayed an eight fold increased colony forming ability (as measured at the D_{50} value; BALB/c 3T3 WT-1: 0.08 mM and BALB/c 3T3 H^R-1: 0.65 mM) in the presence of hydroxyurea (figure 18). The stability of this drug-resistant phenotype has not been examined in detail.

The differentiation competence of the wild type and variant 3T3 cell lines was examined in a manner similar to that used for the myoblasts. Cultures of the 3T3 cells were grown to confluence and then maintained in 1% serum supplemented α-MEM to induce adipogenic development. Periodic removal of cultures and staining for lipid content with oil red O revealed an apparent developmental defect in the H^R 3T3 cell line (figure 19). The variant population exhibited extensive fat droplets in 10% of its cells three weeks post-confluence compared to greater than 60% by the WT population. This differentation- defective phenotype, like that of the H^R myoblasts, is apparently not due to an unusual proliferative ability since the WT and H^R 3T3 cell lines reach similar saturation levels and have comparable growth rates *in vitro* (figure 9h).

FIGURE 18: Effect of various concentrations of hydroxyurea on BALB/c 3T3 cells. ○ BALB/c 3T3 WT-1 and ● BALB/c 3T3 H^R-1. Bars depict the range of experimental values obtained in at least two independent experiments.

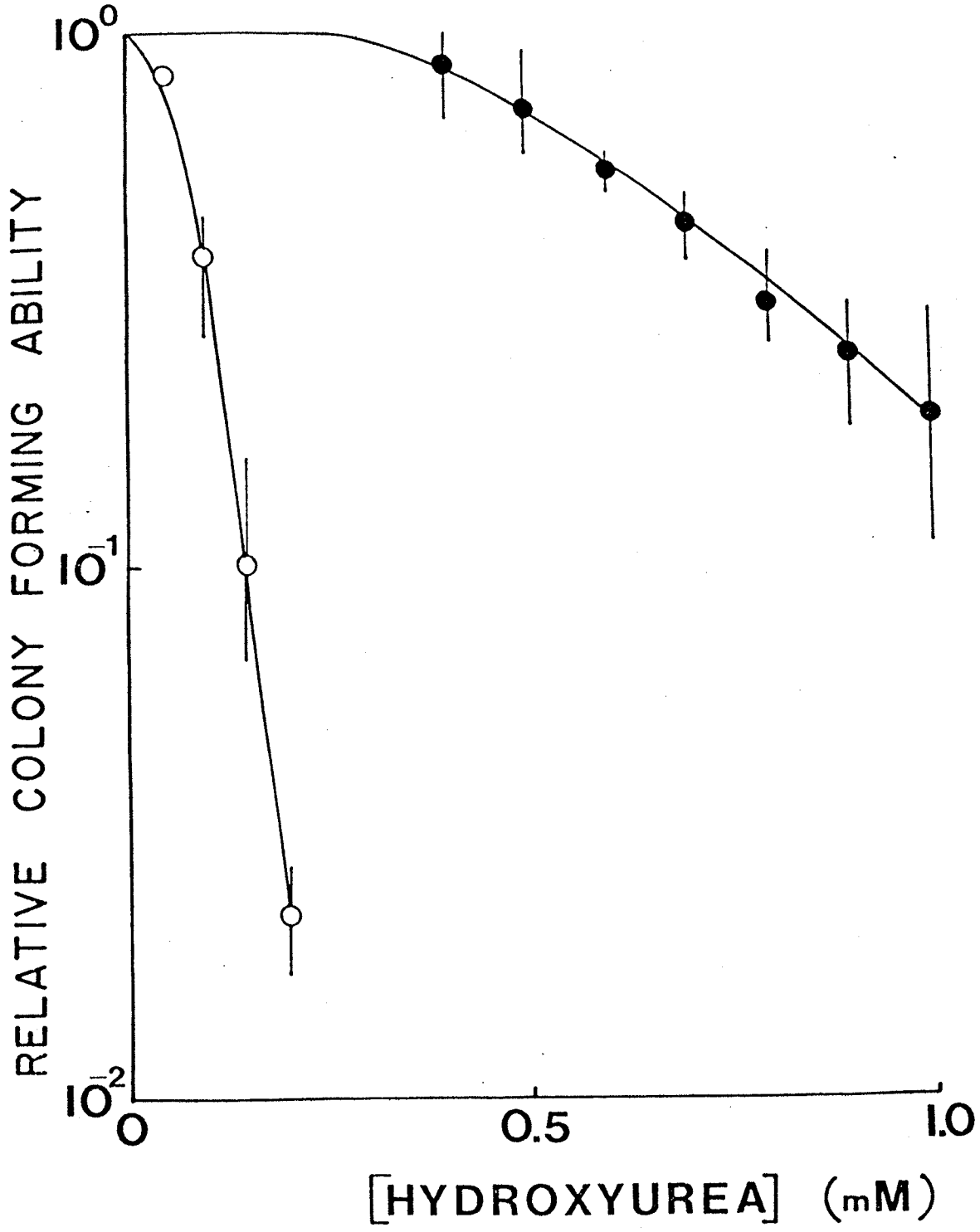
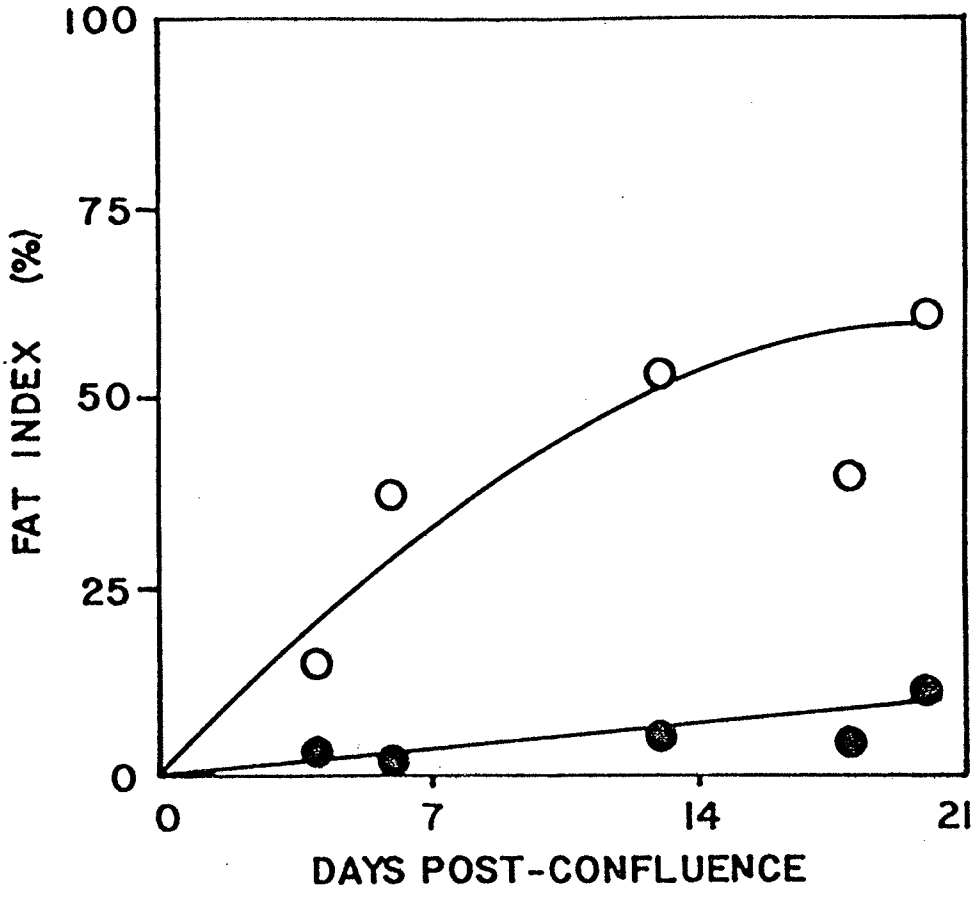

RELATIVE COLONY FORMING ABILITY
10^0
10^-1
10^-2
0
0.5
1.0
[HYDROXYUREA] (mM)

FIGURE 19: Adipogenic development in BALB/c 3T3 cells in 1% FBS/α-MEM ○ BALB/c 3T3 WT-1 and ● BALB/c 3T3 H^R-1. Data shown is from a single experiment.

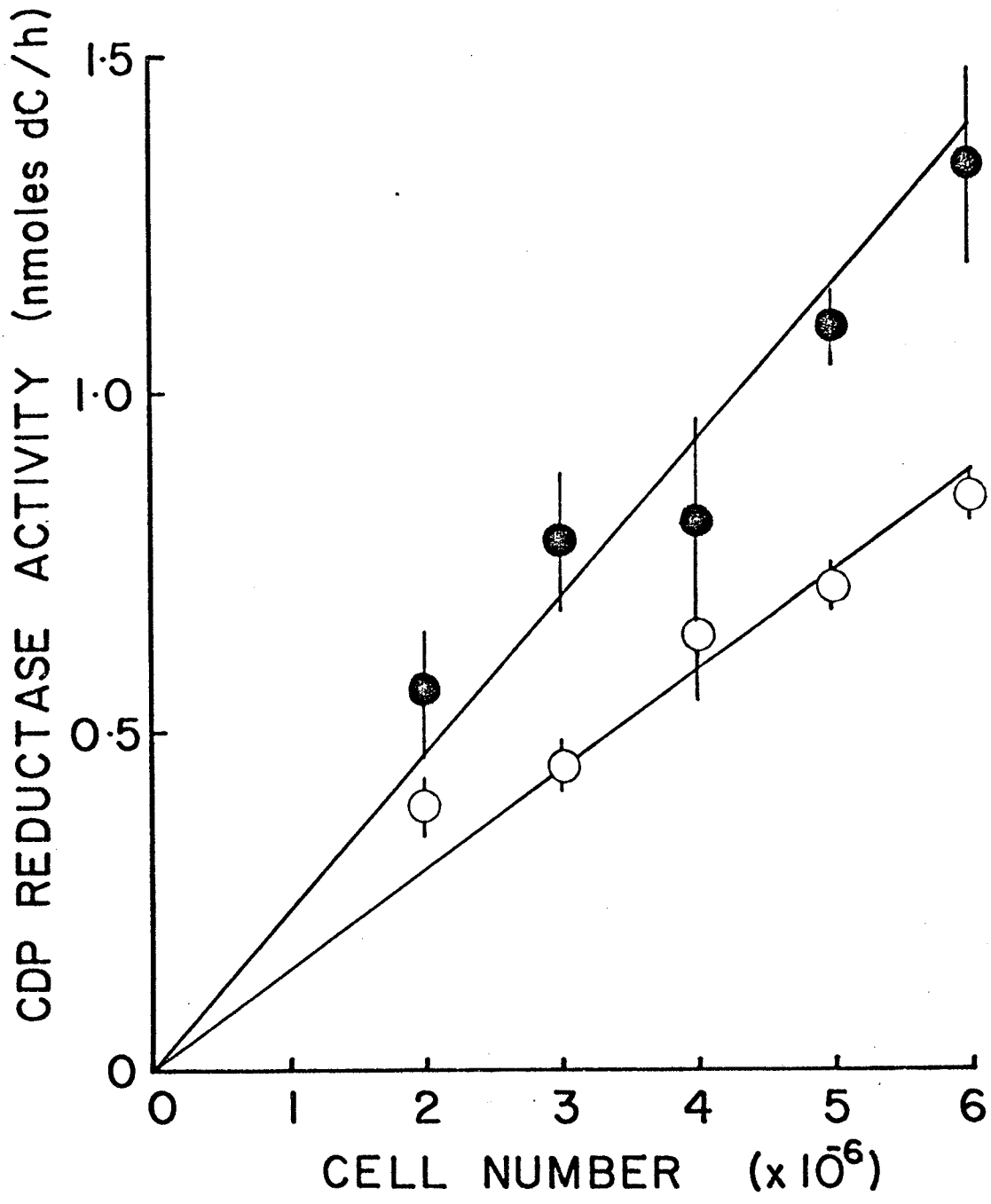

100
75
50
25
0
FAT INDEX (%)
0
7
14
21
DAYS POST-CONFLUENCE

Ribonucleotide Reductase Activity in Wild Type and Hydroxyurea-resistant Cell Lines

Previous studies in this candidate's doctoral laboratory have indicated that there is a close correlation between hydroxyurea-resistance and increased ribonucleotide reductase activity since hydroxyurea is known to inhibit the reductase (Wright 1983). An assay procedure for the quantification of RRase activity in permeabilized cells, which has been used extensively in this and other laboratories, was therefore employed to examine for changes in the reductase activity of the hydroxyurea-resistant cell lines isolated in this study. The activity of CDP reductase was studied primarily, since the assay for this activity is somewhat less expensive and more rapid than that for the other substrates. Changes in CDP reductase activity were used as a basic indicator of perturbations in RRase and more specifically as a measure of changes in pyrimidine reduction in the cell. A limited number of studies were also performed with an established assay for ADP reductase activity as a measure of change in purine reduction.

The more extensive analysis of CDP reductase activity in the H^R cell lines were made more convenient by the finding that the activity obtained experimentally was linear with cell number over the range of 2-6 million cells per assay point (figure 20). Thus while the assay was performed routinely at 2.5×10^6 cells/assay, the linear relationship between cell number and activity allowed for the use of more than twice as many cells when needed, such as when particular batches of [^{14}C]-CDP gave an unusually low signal to noise ratio experimentally. The high backgrounds encountered with some batches of radio-labelled substrate were apparently independent of the number of cells per assay.

FIGURE 20: Response of CDP reductase activity to cell number in permeabilized L6 rat myoblasts. Standard reaction conditions were employed as described under Materials and Methods. Values are the averages of duplicate determinations from a single experiment. ○ L6WT-1 and ● $L6H^R$-1.


CDP REDUCTASE ACTIVITY (nmoles dC/h)
1·5
1·0
0·5
0
0
1
2
3
4
5
6
CELL NUMBER (x 10⁻⁶)

Although problems with high background values could occasionally be circumvented by HPLC purification of the [^{14}C]-CDP in a manner similar to that used in the analysis of ribonucleotide pools (described in a later section), the occasional use of higher cell number/assay was often sufficient to alleviate the difficulty. High background values were never a problem with the ADP reductase assay and allowed the use of a constant number of cells per assay ($5x10^6$ cells).

CDP and ADP reductase activities in permeabilized cells were found to vary linearly with time over a period of 30 minutes in both wild type and variant cell lines. Examples of this linear relationship are shown in figures 21a and b for L_6WT/H^R-3. Although assays of ADP reductase activity were routinely and reproducably performed at 30 minutes, the assay for CDP reductase activity was found to vary apparently dependent on the quality of [^{14}C]-CDP available. Thus many assays were performed at 5 minutes rather than 30 minutes when the linear relationship between time and activity could not be extended appreciably beyond 5-10 minutes.

A survey of the CDP reductase activity exhibited by each of the wild type and H^R myoblast cell lines was conducted using the permeabilized assay as outlined above. The results of this survey are presented in figure 22. In addition, the results of a preliminary investigation with BALB/c 3T3 WT/H^R-1 are also presented. Each assay of the activity encountered in a WT/H^R pair of cell lines has been summed, averaged and compared in a relative manner to the parental activities for those assays. The data is presented as relative activity because of the variation often seen in the activities using the permeabilized assay, but each variant exhibited a mean CDP reductase activity significantly greater than its parental clone ($p<0.05$) when examined by paired

FIGURE 21: Response of (a) CDP and (b) ADP reductase activity to time in permeabilized L6 rat myoblasts. Standard reaction conditions were employed as described under Materials and Methods. Values in (a) are the averages of two independent experiments while values in (b) are from a single experiment. ○ L6WT-3 and ● $L6H^R$-3.

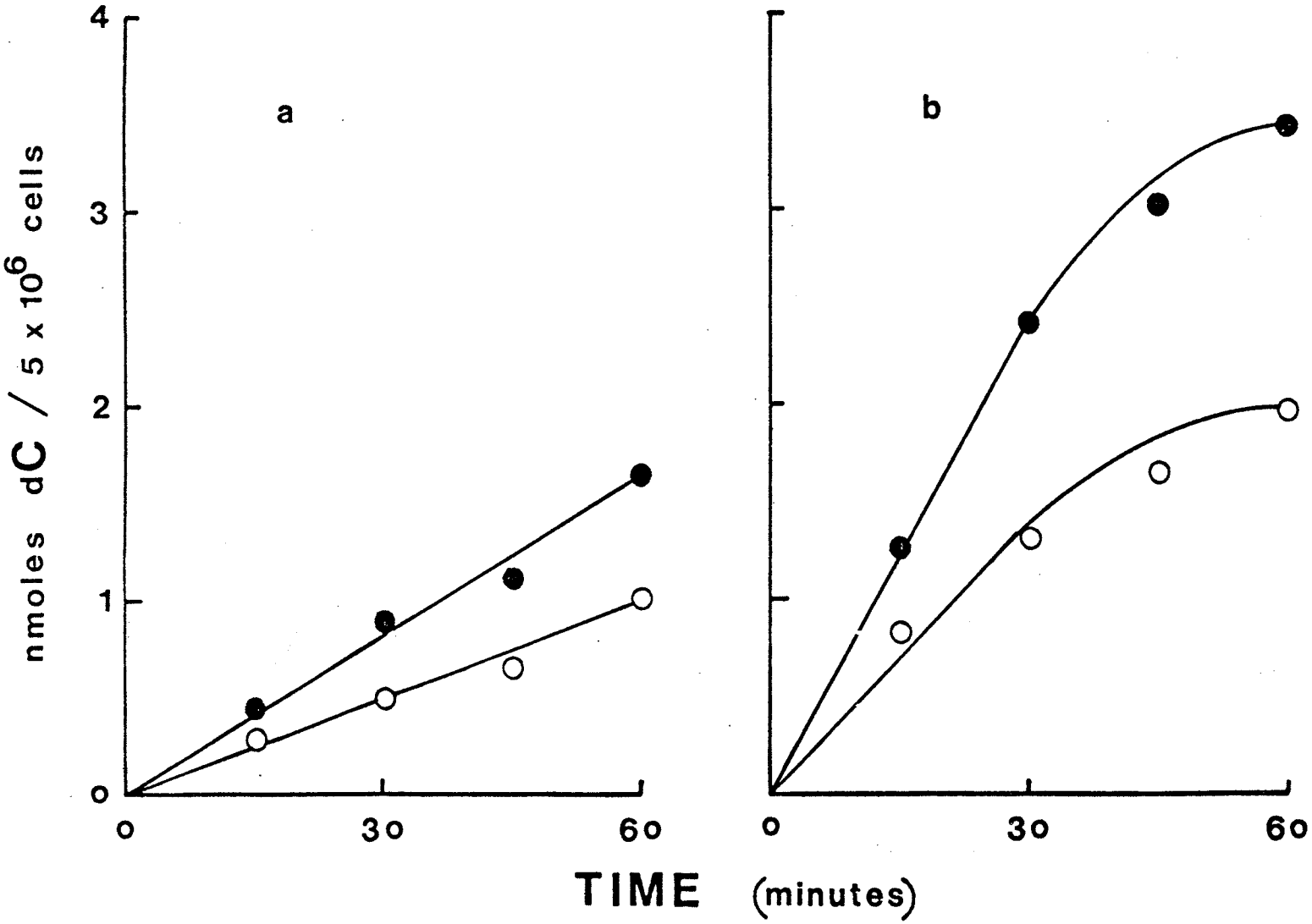
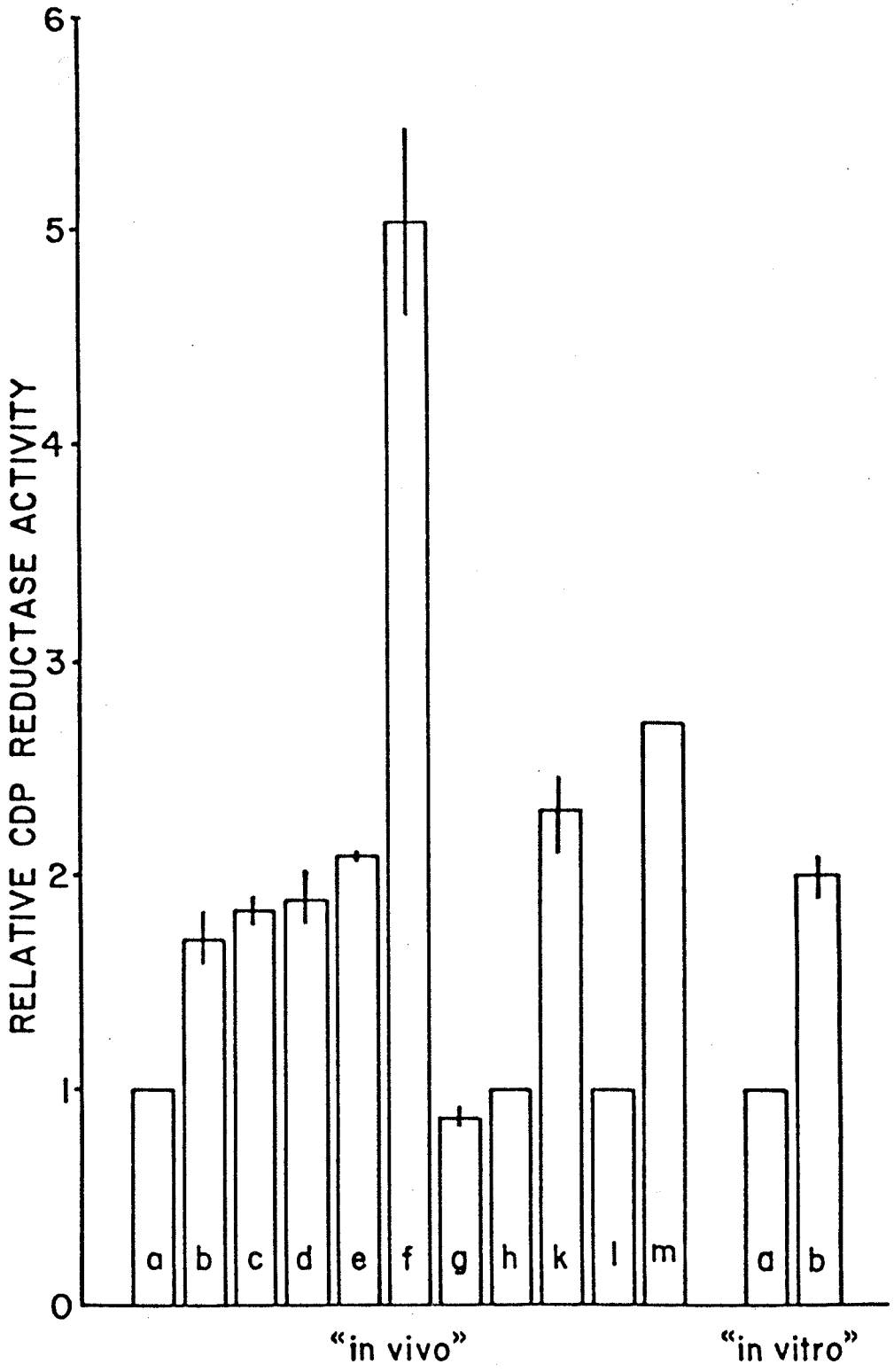

a
b
nmoles dC / 5 x 10^6 cells
0
1
2
3
4
0
30
60
0
30
60
TIME (minutes)

FIGURE 22: CDP reductase activities of hydroxyurea-resistant cell lines relative to their drug sensitive parental clones, in permeabilized (in vivo) cell assays and in cell extracts (in vitro). Wild type clones showed an average activity of 1.37 ± 0.70 (mean S.D.) nmole dC formed/h/5×10^6 cells. (a) L_6^{WT}1-6 (b) L_6H^R-1, (c) L_6H^R-2, (d) L_6H^R-3, (e) L_6H^R-4 (f) L_6H^R-5, (g) L_6H^R-6, (h) L_8^{WT}-1, (k) L_8H^R-1, (l) BALB/c 3T3 WT-1 and (m) BALB/c 3T3 H^R-1. All myoblast values are the averages of at least two independent experiments; standard error is represented by vertical bars. BALB/c 3T3 values are those of a single experiment by A. Tagger (with permission).


RELATIVE CDP REDUCTASE ACTIVITY
0
1
2
3
4
5
6
a
b
c
d
e
f
g
h
k
l
m
a
b
"in vivo"
"in vitro"

Student's t-tests. For example, L_6WT-1 and L_6H^R-1 displayed mean CDP reductase activities of 1.47 and 2.51, with standard deviations of 0.55 and 1.14. The overall approximate CDP reductase activity of L_6WT cell lines has been reported as one nanomole product (dC) per hour per $5x10^6$ (Creasey and Wright 1984a), but continued analysis has revealed the sample mean of all L_6WT clones to be 1.37 $\pm$ 0.70 (S.D.) nanomoles dC/h/$5x10^6$ cells. A comparable average value for all L_6H^R clones is 2.57 $\pm$ 1.95 (S.D.). Interestingly, the ratio of L_6H^R1-6 to L_6WT1-6 CDP reductase activities, 1.88, approximates the relative differences seen in the dNTP pools when the parental and variant values are summed and averaged (see table 4).

CDP reductase activity in L_6WT/H^R-1 was also examined in <u>in vitro</u> assays employing whole cell extracts. Two such assays were performed and gave results similar to those of the permeabilized <u>in vivo</u> cell assay (figure 22). Parental and variant activities were 0.045 $\pm$ 0.012 and 0.093 $\pm$ 0.030 nanomoles dC formed/hour/mg protein. These results, however, are not statistically significant when analysed by paired Student's t-test $p<0.05$, probably owing to the small sample size.

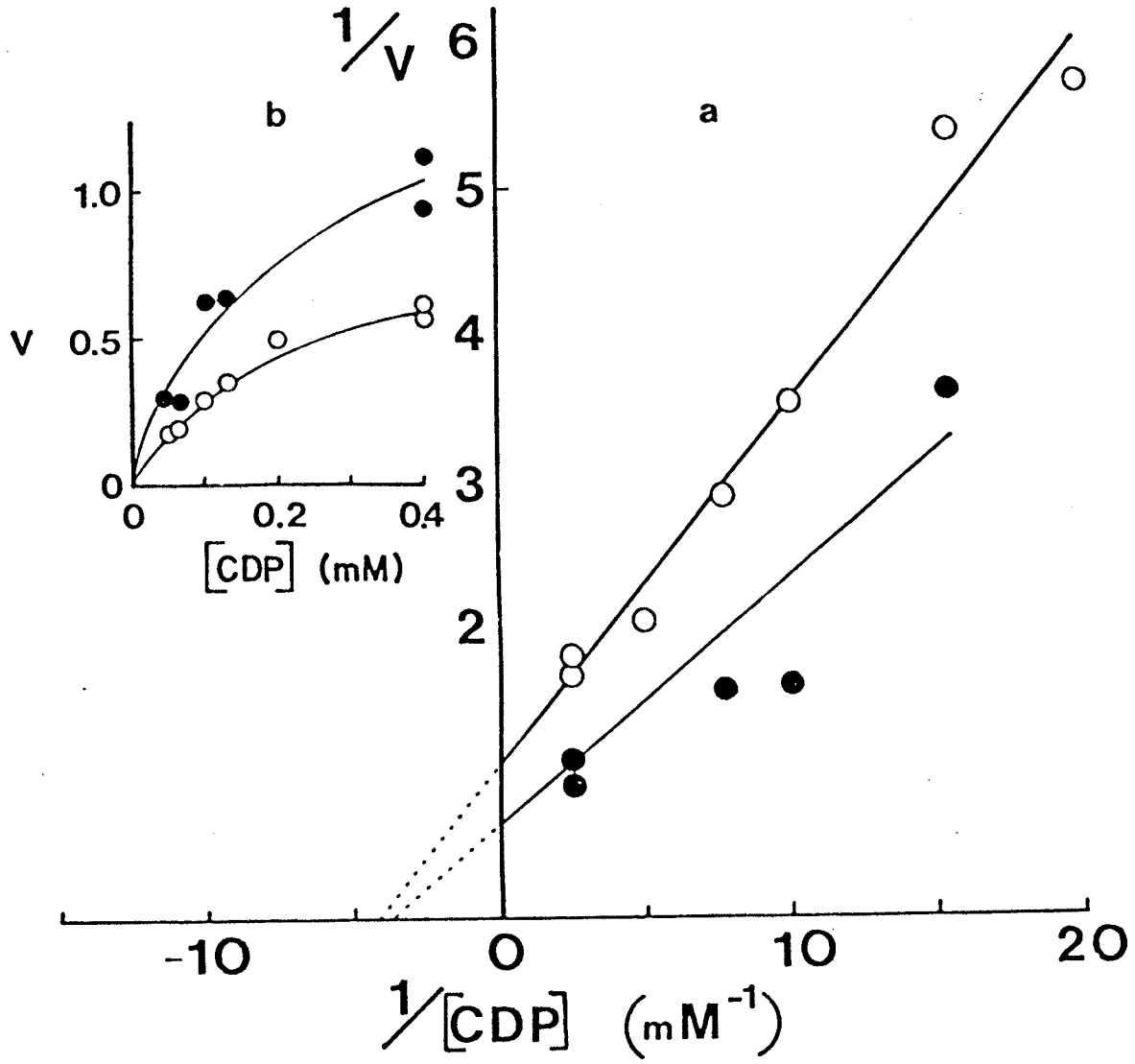
The changes seen in ADP reductase activities in H^R myoblasts are modest, in keeping with the small but significant increases seen in CDP reductase activities in these variants (table 2). The average L_6WT ADP reductase activity found in the experiments noted in table 2 was 2.06 $\pm$ 1.23 nanomoles dA/h/$5x10^6$ cells. The corresponding average variant activity was 3.03 $\pm$ 1.69 nanomoles dA/h/$5x10^6$ cells. These values were taken from a total of eleven independent experiments and are not statistically significant when subjected to a one-way analysis of variance. Paired Student's t-tests indicate that while the mean ADP

TABLE 2: ADP reductase activity in L_6 and L_8 rat myoblasts

CLONES	FOLD INCREASE IN ADP REDUCTASE ACTIVITY[a]
L_6H^R/WT-1	1.3 ± 0.3
L_6H^R/WT-2	1.5 ± 0.2
L_6H^R/WT-3	1.8 ± 0.1
L_6H^R/WT-6	1.1 ± 0.1

[a] The average parental wild type activity was 2.0 nmoles product formed/h/$5x10^6$ cells. Error values are the standard errors of the means.

FIGURE 23: CDP reductase substrate velocity plots and activity inhibition by hydroxyurea in L_6^{WT}-1 (○) and L_6^{HR}-1 (●). (a) Lineweaver-Burke plot, (b) Michaelis-Menton plot and (c) inhibition by hydroxyurea. Values shown are from a single experiment. Velocities are expressed as nmoles product formed/hr./$5x10^6$ cells.


1/V
6
5
4
3
2
a
b
1.0
0.5
V
0
0
0.2
0.4
[CDP] (mM)
-10
0
10
20
1/[CDP] (mM⁻¹)

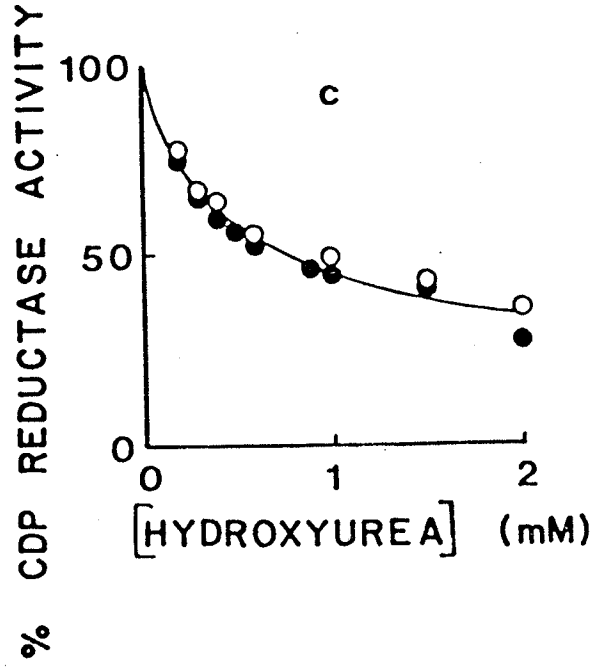
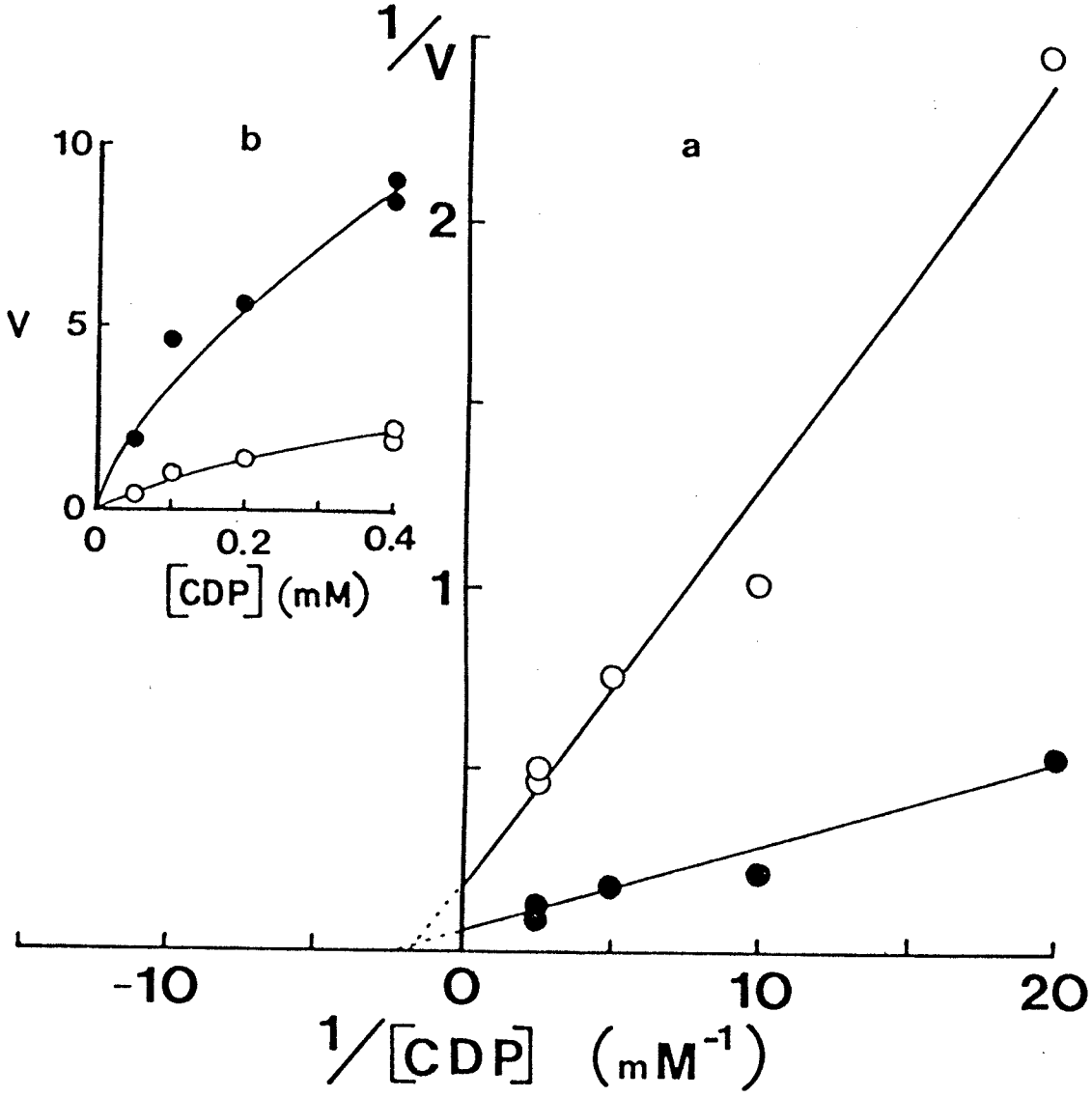

% CDP REDUCTASE ACTIVITY
100
50
0
c
0
1
2
[HYDROXYUREA] (mM)

FIGURE 24: CDP reductase substrate velocity plots and activity inhibition by hydroxyurea in L_6^{WT}-5 (○) and $L_6^{H^R}$-5 (●). (a) Lineweaver-Burke plot, (b) Michaelis-Menton plot and (c) inhibition by hydroxyurea. Values shown are from a single experiment. Velocities are expressed as nmoles product formed/hr./$5x10^6$ cells.


1/V
a
b
10
V 5
0
0
0.2
0.4
[CDP] (mM)
2
1
-10
0
10
20
1/[CDP] (mM⁻¹)

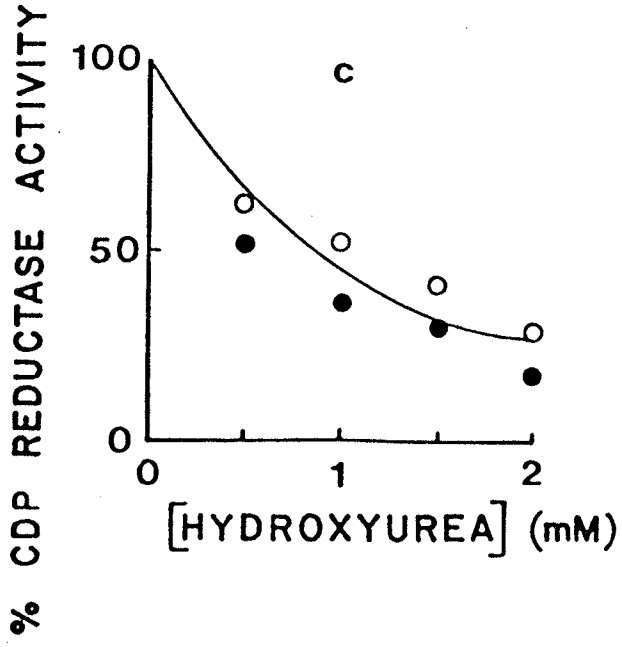
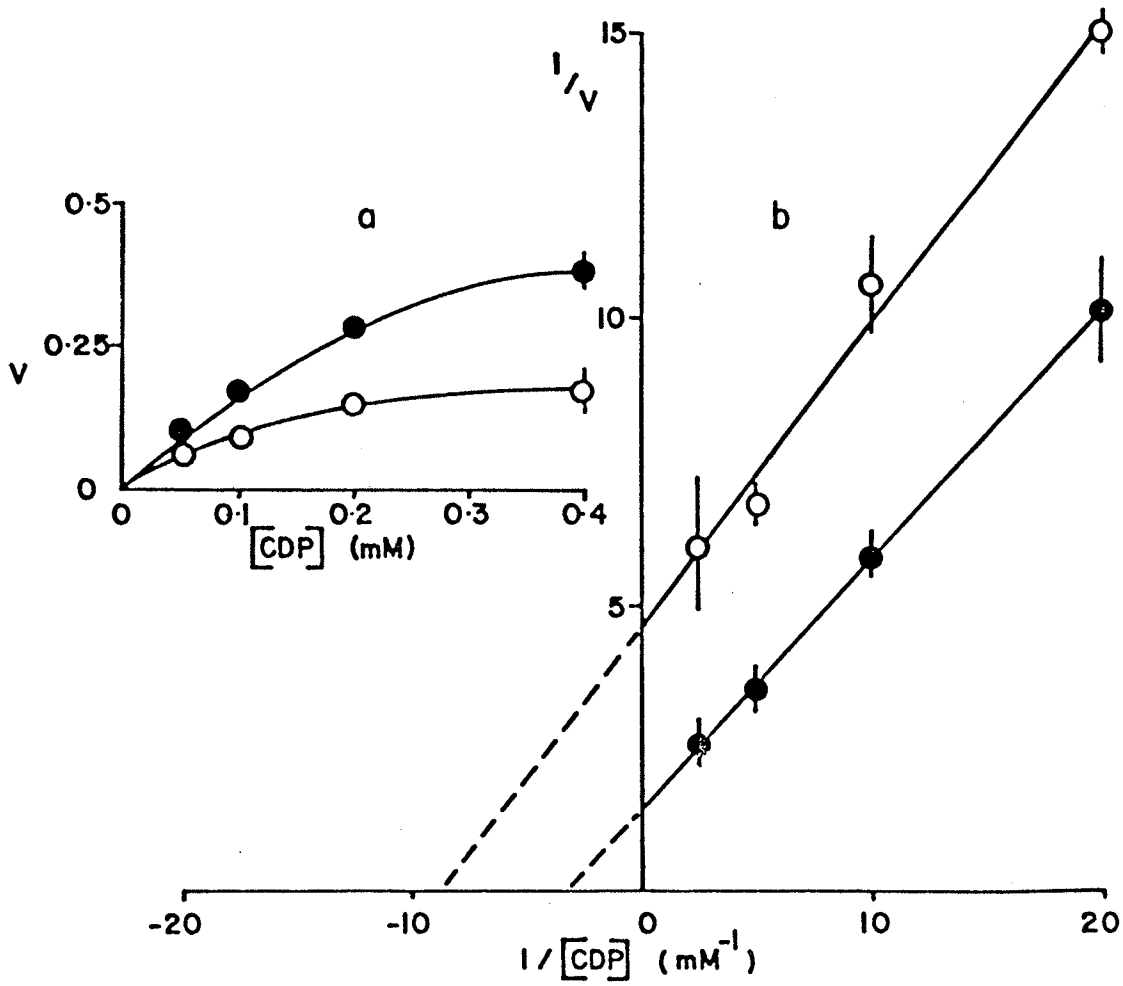

% CDP REDUCTASE ACTIVITY
100
50
0
c
0
1
2
[HYDROXYUREA] (mM)

FIGURE 25: CDP reductase substrate velocity plots for L_8WT-1 (○) and L_8H^R-1 (●). (a) Michaelis-Menton plot and (b) Lineweaver-Burke plot. Velocities are expressed as nmoles product formed/hr./$5x10^6$ cells. Values shown are the single experiment, with the range of the values indicated by the vertical bars.


a
b
V
0·5
0·25
0
0
0·1
0·2
0·3
0·4
[CDP] (mM)
1/v
15
10
5
-20
-10
0
10
20
1/[CDP] (mM⁻¹)

reductase activity in L_6WT/H^R-3 are significantly different ($p<0.01$) the other clonal pairs show no statistically significant increase in this enzyme activity. Interestingly, the fold increase in ADP reductase activity in L_6H^R-3 compared to its WT clone, approximates the fold increase in the CDP reductase activity of this clone. Furthermore, none of the variant clones listed in table 2 displayed an ADP reductase activity less sensitive to inhibition by hydroxyurea than their respective parental clones (data not shown).

Kinetic analysis of CDP reductase activity in L_6 and L_8 rat myoblast WT and H^R pairs of clones revealed no significant differences between parental and variant cells. No apparent difference in the Km for CDP was found in L_6H^R-1 compared to its parental population; the values for each being approximately 0.25 mM (figures 23a and b; Creasey and Wright 1984a). Similarly, no differences were seen in the hydroxyurea-sensitivity of this activity between L_6WT/H^R-1 (figure 23c), each being inhibited approximately 50% by 0.75 mM hydroxyurea. L_6WT/H^R-5 gave similar results and again, the only apparent differences in CDP reductase activity between WT and H^R clones was in reaction velocity, not affinity for substrate or drug-sensitivity (figures 24a,b and c).

Kinetic studies with L_8WT-1 and its hydroxyurea-resistance clone L_8H^R-1, paralleled the findings in the L_6 myoblast clones for changes in CDP reductase activity. Although the activity displayed by the variant population was significantly greater than that of the parental cells, no significant differences in the K_M for CDP were found (figures 25a and b). L_8H^R-1 showed a K_M for CDP of 0.3 mM, approximately that of the L_6 clones, whereas the parental clone, L_8WT-1, showed a somewhat lower K_M of 0.12 mM. Similar results

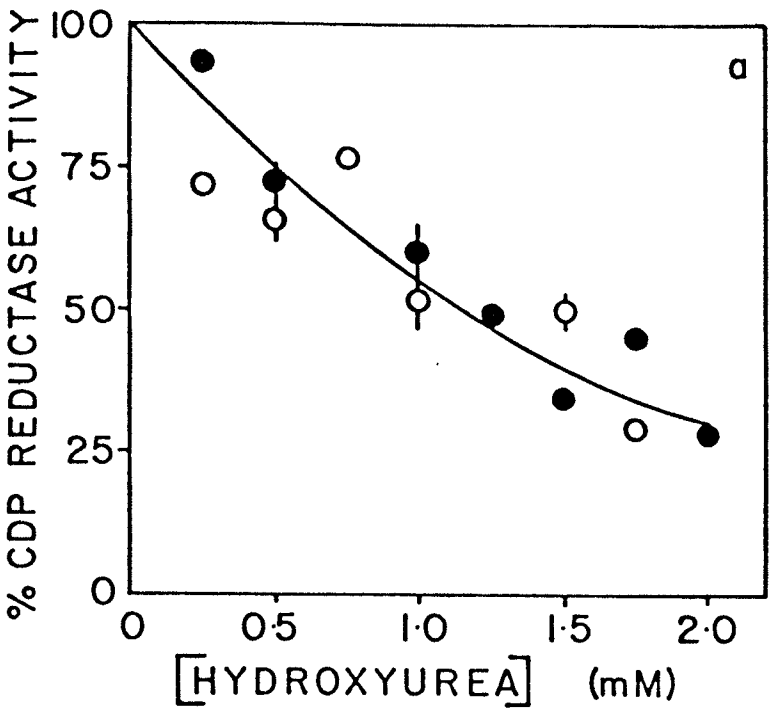
were obtained in a second experiment, where the K_M values for L_8WT-1 and L_8H^R-1 were found to be 0.11 and 0.40 mM, respectively. Paired Student's t-tests revealed no significant difference ($p<0.05$) between the L_8WT and H^R K_M values.

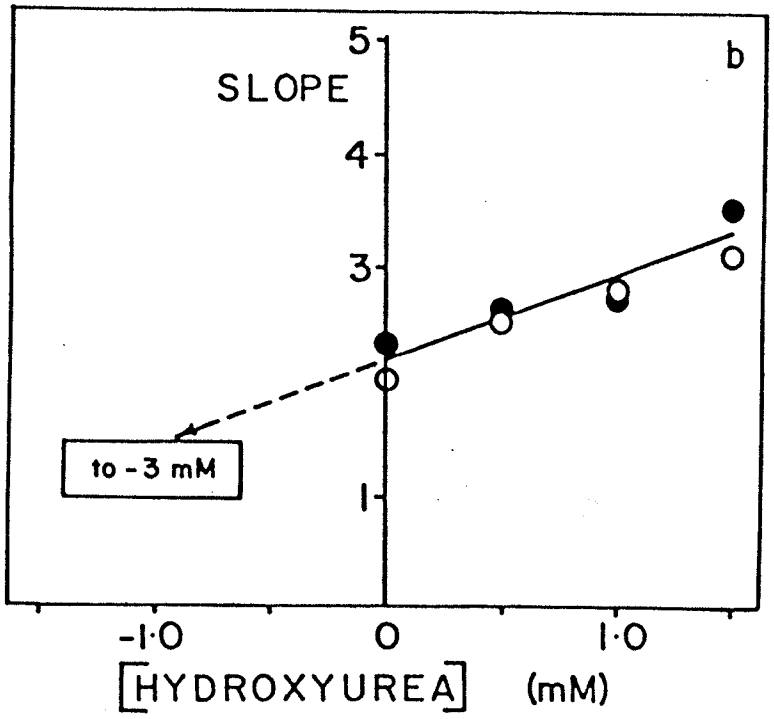
The inhibition of CDP reductase activity in L_8 myoblasts by hydroxyurea is approximately equal in WT and H^R cells, although apparently somewhat reduced in comparison with the L_6 myoblasts (figures 26a and b). The reductase activity was inhibited approximately 50% by 1.2 mM hydroxyurea and gave a slope Ki value of 3 mM.

Nucleotide Pools in Whole Cell Extracts of Hydroxyurea-sensitive and Resistant Cell Lines

The small but significant differences seen in CDP reductase activities between wild type and hydroxyurea-resistant cell lines isolated in this study suggested that similar changes may be present in the nucleotide pools of these cell lines. Since disturbances in the product pools of ribonucleotide reductase are known to be associated with increased mutation frequencies at a variety of genetic loci in mammalian cells (Weinberg et al 1981, Arpai et al 1983, Ayusawa et al 1983, Roguska and Gudas 1984, Gjerset et al 1985), the demonstration of such pool imbalances in the H^R myoblasts might offer a possible explanation for the correlation between resistance to hydroxyurea and the differentation-defective phenotype. Thus, deoxyribonucleotide pools, as well as the pools of some ribonucleotides, were examined by high performance liquid chromatography of acid extracts from actively dividing population of WT and H^R cell lines.

FIGURE 26: Effects of hydroxyurea on CDP reductase activity in L_8WT-1 (○) and L_8H^R-1 (●). (a) Inhibition of activity by various concentrations of drug and (b) slope Ki curves obtained from Lineweaver-Burke plots in which CDP concentrations were varied at several fixed levels of hydroxyurea. The data for assays without drug were taken from fig. 25.


% CDP REDUCTASE ACTIVITY
100
75
50
25
0
a
0
0·5
1·0
1·5
2·0
[HYDROXYUREA] (mM)


SLOPE
5
4
3
1
b
to -3 mM
-1·0
0
1·0
[HYDROXYUREA] (mM)

A tracing of a chromatogram representative of the separation of ribonucleotides achieved by the HPLC protocol used in this study is shown in figure 27 and that for the separation of deoxyribonucleoside triphosphates is shown in figure 28. A good separation of ribonucleoside triphosphates (UTP, CTP, ATP and GTP) and two ribonucleoside diphosphates (CDP and ADP) was achieved through the use of a linear gradient to a high salt concentration. The resolution of GDP was often decreased by the presence of a highly variable peak following within one minute of this nucleotide. UDP and TDP were poorly resolved from each other in this separation protocol. Furthermore, the separation of the various nucleotides steadily and irreversably decreased with the degeneration of the analytical column. Impurities present in the phosphate buffer system contribute to this problem but could be partially alleviated by periodic washings with EDTA and/or phosphoric acid (Rabel 1980). Even the most careful maintenance of the analytical columns did not prevent this gradual loss of resolution.

The resolution of deoxyribonucleoside triphosphates was considerably less than that of the corresponding ribonucleotides (Figure 28). Measurements of dGTP were near the limit of detection by the aforementioned protocol, partly owing to the small pools of this deoxyribonucleotide present in the rat myoblast cell lines. Moreover, the analysis of the deoxyribonucleotide pools in general was made difficult by the steadily falling baseline encountered in this separation technique. An unidentified peak which was found eluting between dCTP and dATP (see figure 28) often necessitated re-analysis since it frequently interferred with the satisfactory separation of dCTP. After the completion of this study it was learned that this peak could be eliminated by changing the order of addition of the reagents used to destroy

FIGURE 27: A representative chromatogram showing the separation of ribonucleoside di- and triphosphates in acid extracts of exponentially growing myoblasts. The figure is reduced to about 66% of actual size. Dashed lines within peaks represent one fifth of the peak height attained.

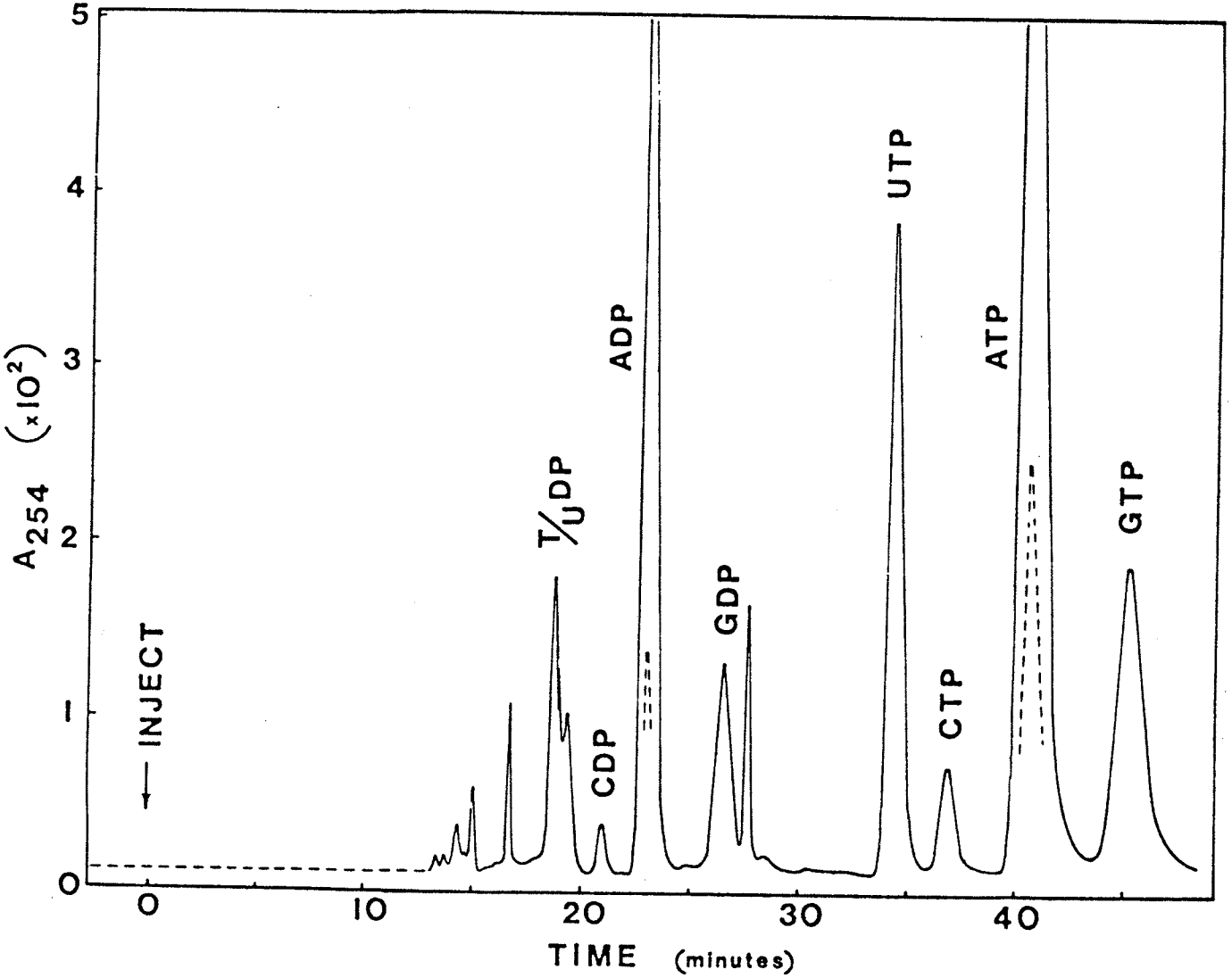
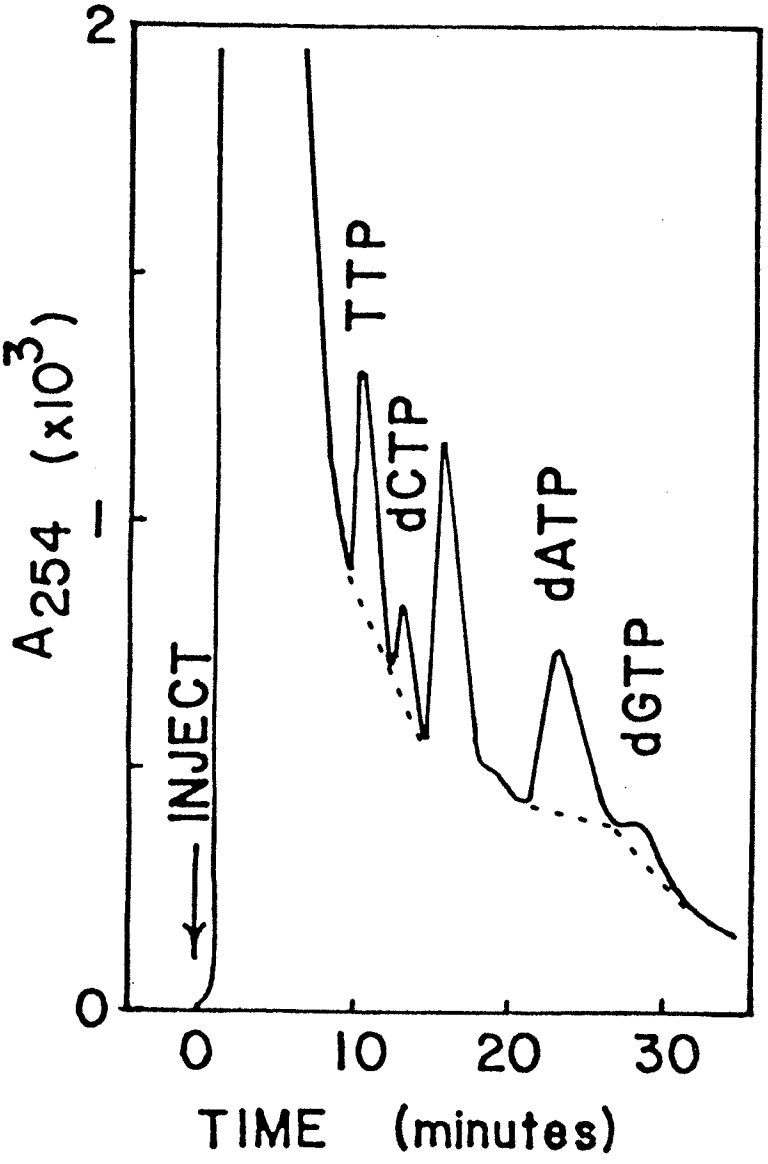

5
4
3
2
1
0
A_{254} ($\times 10^2$)
INJECT
T/UDP
CDP
ADP
GDP
UTP
CTP
ATP
GTP
0
10
20
30
40
TIME (minutes)

FIGURE 28: A representative chromatogram showing the separation of deoxyribonucleoside triphosphates in periodate treated acid extracts of exponentially growing myoblasts. Note the low resolution of dGTP. The figure is enlarged approximately 1.66 fold from actual size.


2
1
0
A_{254} ($\times 10^3$)
INJECT
TTP
dCTP
dATP
dGTP
0
10
20
30
TIME (minutes)

ribonucleotides prior to analysis (Tanaka et al 1984). Furthermore, these authors also found that addition of deoxyguanosine to the reaction mixture helped prevent the loss of dGTP by oxidation.

The changes in ribonucleotide and deoxyribonucleotide pool sizes between each H^R variant and its parental clone are diagrammed in figures 29,30,31 and 32. No analyses were performed with L_6WT/H^R-4 since they were isolated very late in the present study. Ribonucleotide pool analyses were not performed on L_6WT/H^R-5 nor L_8WT/H^R-1, but their deoxyribonucleotide pool sizes were measured and are presented in figure 33.

None of the ribonucleotide pool sizes measured in hydroxyurea-resistant myoblast clones 1,2,3 and 6 were significantly different from their parental values when a Students's paired t-test is used to analyze the mean peak heights from duplicate experiments with each pair of clones. An identical type of analysis for the differences in the mean dNTP pool sizes between WT and H^R myoblasts (L_6WT/H^R 1,2,3,5,6 and L_8WT/H^R-1), however, revealed a significant increase ($p<0.05$) in these pool sizes. The actual pool sizes determined for the deoxyribonucleoside triphosphates of these cell lines are presented in table 3 and the overall mean pool size values of all WT and all H^R clones are shown in table 4. It is interesting to note that the two partial fusing clones L_6H^R-3 and L_6H^R-6 exhibited elevated but apparently balanced dNTP pools, unlike all the other non-fusing H^R myoblast variants which exhibited elevated and imbalanced dNTP pools.

A one-way analysis of variance on the L_6 and L_8 parental dNTP pool sizes determined in twelve independent experiments indicated that the method of overall pool size comparison shown in table 4 is valid for dCTP, dATP and dGTP since the interclonal variation was low enough that

FIGURE 29: Ribonucleotide and deoxyribonucleotide pool sizes in L6H^R-1 relative to its parent L6WT-1. All determinations represent the average of two independent experiments. Ribonucleotide pool analysis was performed by comparing peak heights whereas deoxyribonucleotide pool analysis utilized the weights of peaks cut from triplicate copies of the original chromatograms. (a) ribonucleoside diphosphates, (b) ribonucleoside triphosphates, (c) deoxyribonucleoside triphosphates.

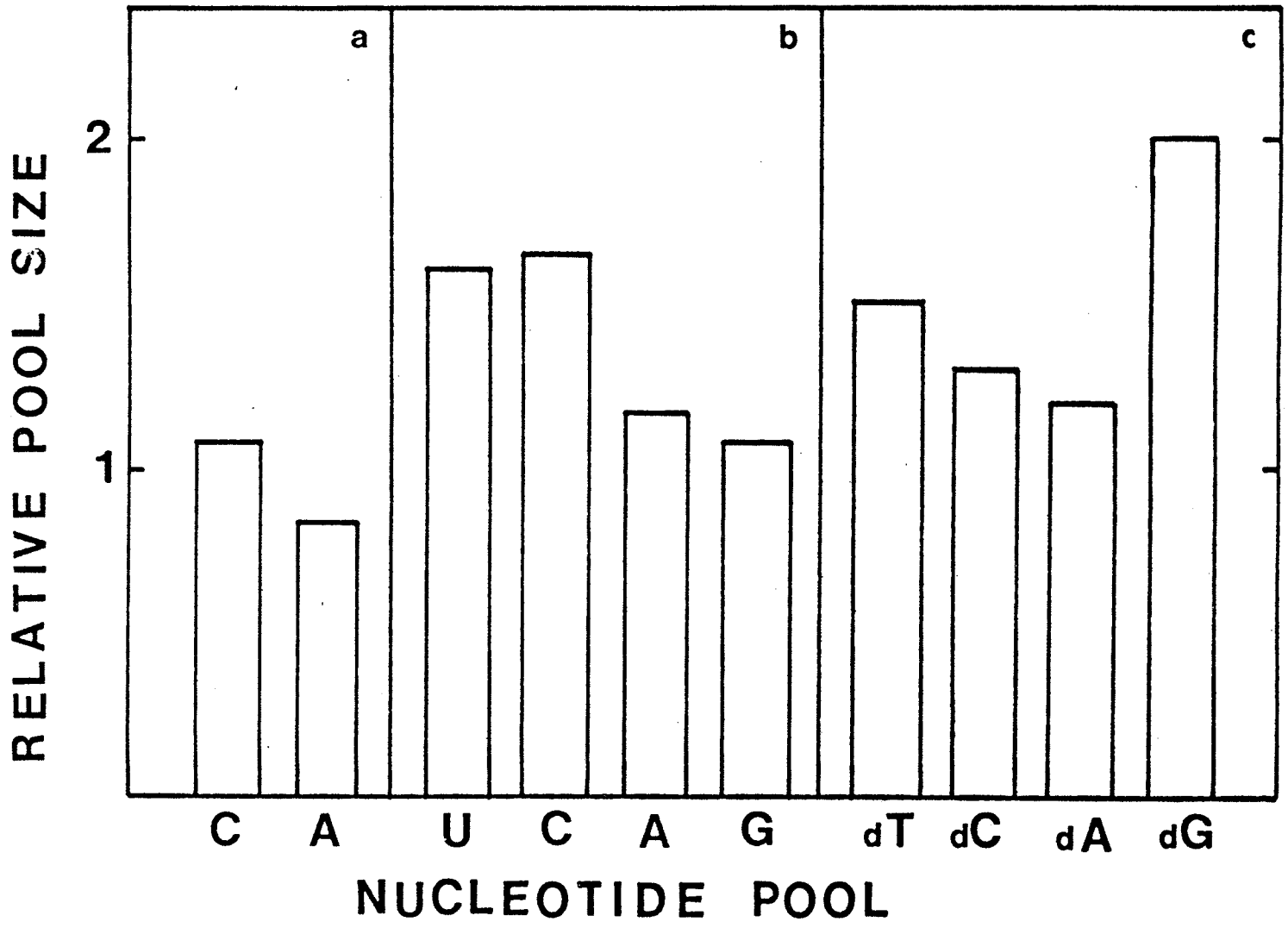

a
b
c
2
1
RELATIVE POOL SIZE
C
A
U
C
A
G
dT
dC
dA
dG
NUCLEOTIDE POOL

FIGURE 30: Ribonucleotide and deoxyribonucleotide pool sizes in L6H^R-2 relative to its parent L6WT-2. Analyses were performed as described in the legend to figure 29. (a) ribonucleoside diphosphates, (b) ribonucleoside triphosphates, (c) deoxyribonucleoside triphosphates.

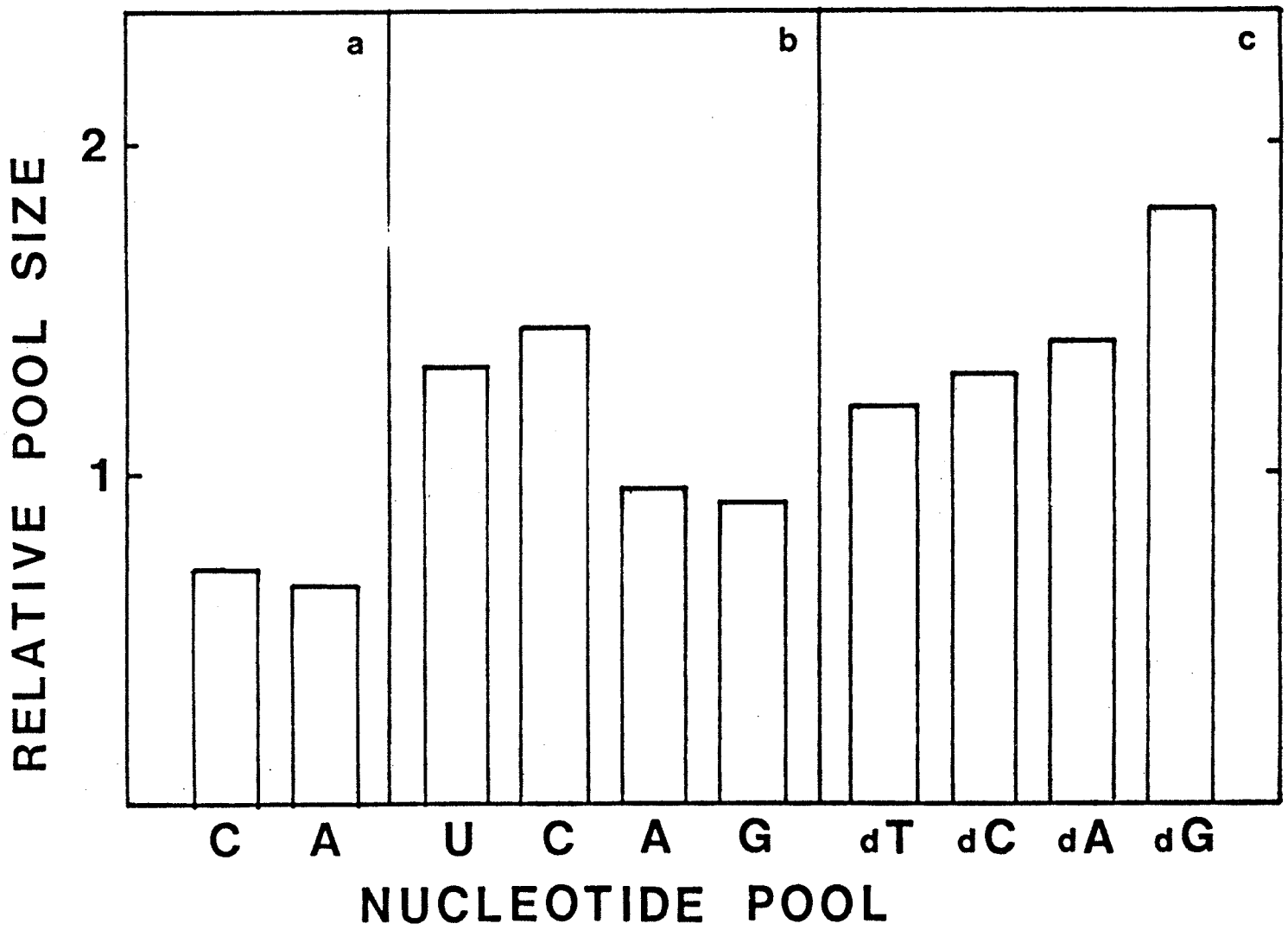

a
b
c
2
1
RELATIVE POOL SIZE
C
A
U
C
A
G
dT
dC
dA
dG
NUCLEOTIDE POOL

FIGURE 31: Ribonucleotide and deoxyribonucleotide pool sizes in $L6H^R$-3 relative to its parent L6WT-3. Analysis were performed as described in the legend to figure 29. (a) ribonucleoside diphosphates,(b) ribonucleoside triphosphates, (c) deoxyribonucleoside triphosphates.

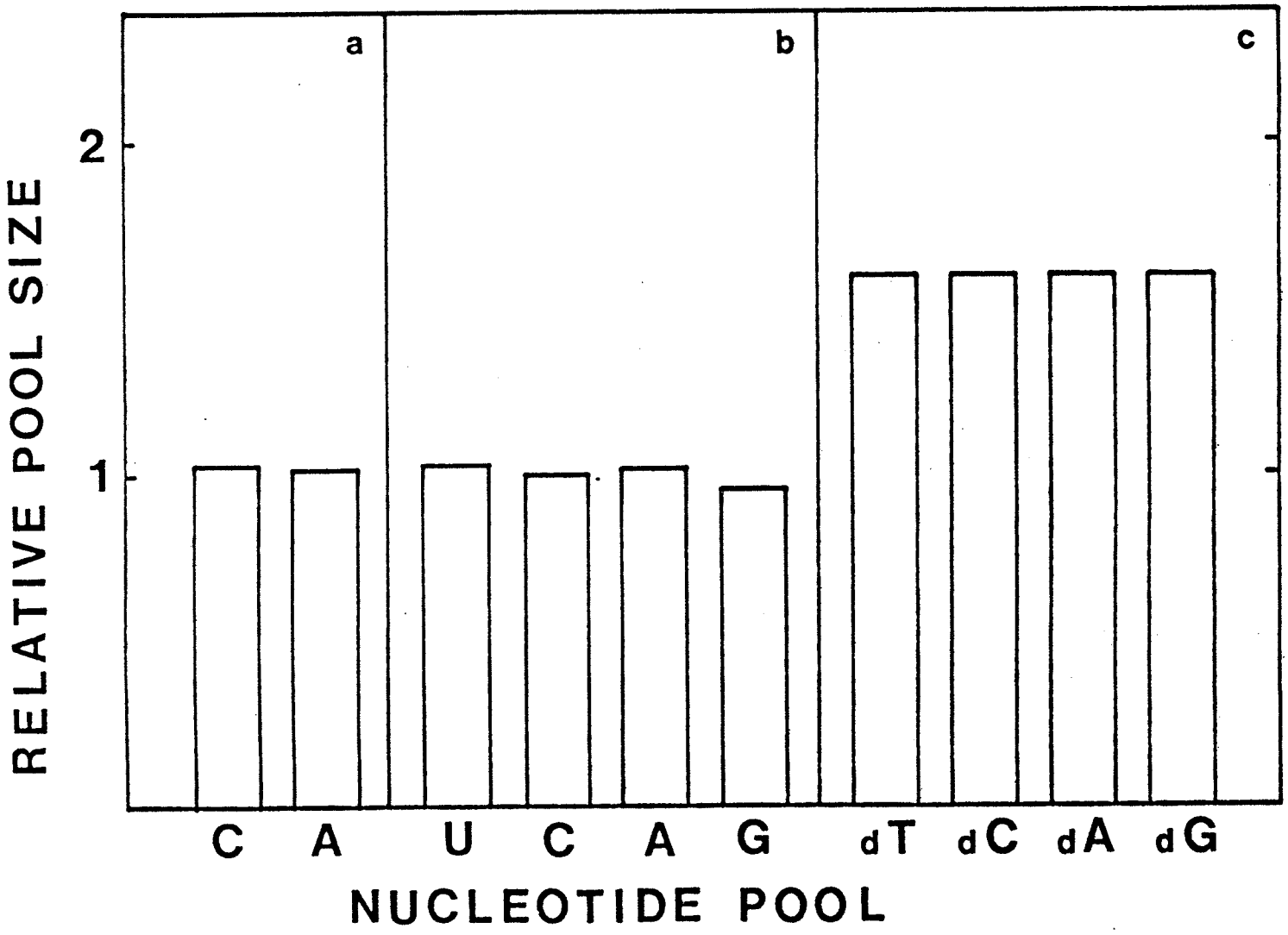

a
b
c
2
1
RELATIVE POOL SIZE
C
A
U
C
A
G
dT
dC
dA
dG
NUCLEOTIDE POOL

FIGURE 32: Ribonucleotide and deoxyribonucleotide pool sizes in L6H^{R}-6 relative to its parent L6WT-6. Analyses were performed as described in the legend to figure 29. (a) ribonucleoside diphosphates, (b) ribonucleoside triphosphates, (c) deoxyribonucleoside triphosphates.

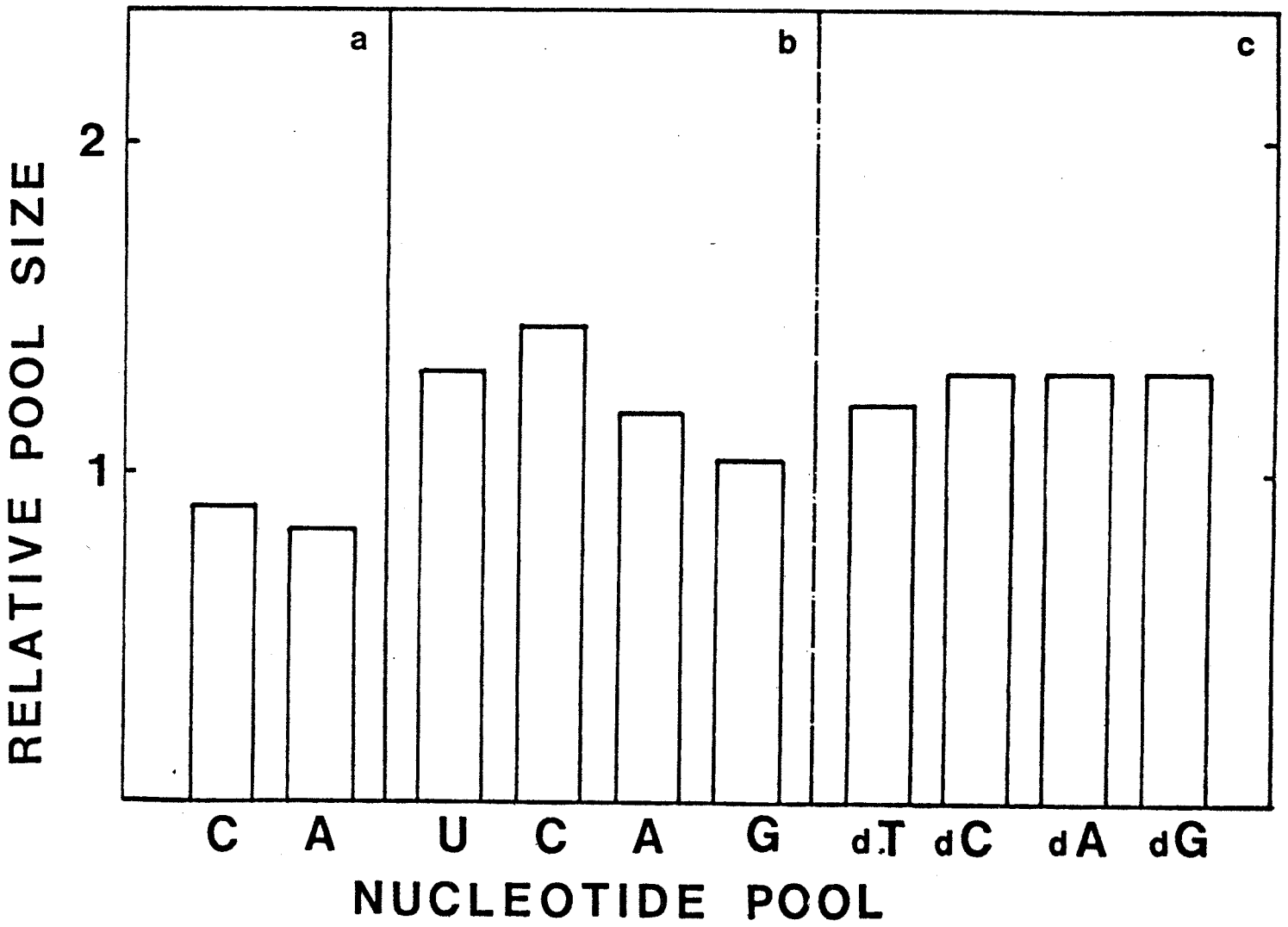

a
b
c
2
1
RELATIVE POOL SIZE
C
A
U
C
A
G
dT
dC
dA
dG
NUCLEOTIDE POOL

FIGURE 33: Deoxyribonucleotide pool sizes in hydroxyurea-resistant myoblasts relative to their sensitive parental clones. All determinations represent the average of two independent experiments. Analyses were performed by cutting and weighing triplicate copies of chromatogram peaks. (a) L_6WT/H^R-5 and (b) L_8WT/H^R-1.

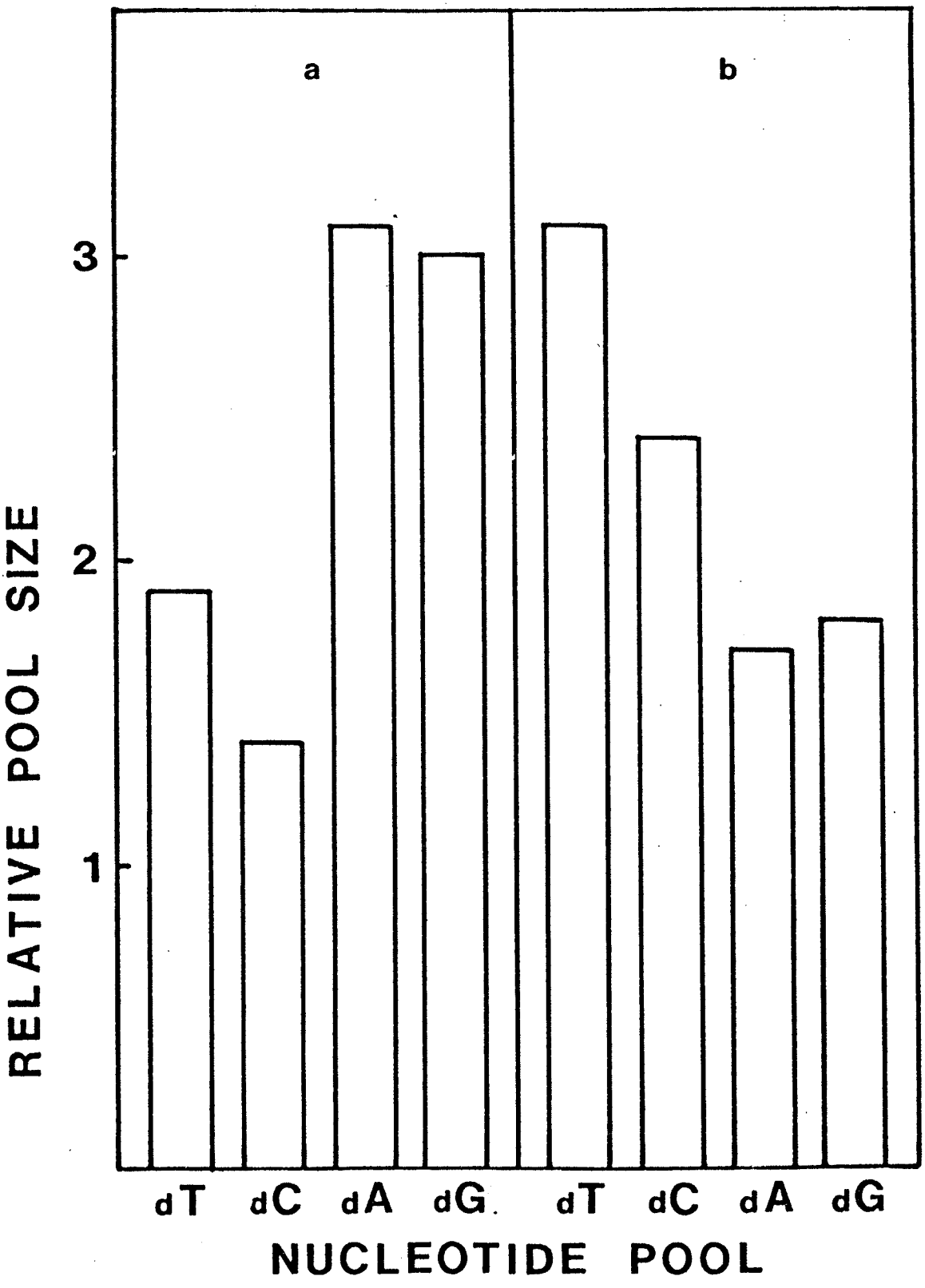

a
b
3
2
1
RELATIVE POOL SIZE
dT
dC
dA
dG
dT
dC
dA
dG
NUCLEOTIDE POOL

TABLE 3: Deoxyribonucleotide pool sizes in parental wild type and hydroxyurea-resistant cell lines.

	dNTP POOLS (pmoles/10^6 cells)[a]			
CLONE	dTTP	dCTP	dATP	dGTP
L_6^{WT}-1	40 ± 4	14 ± 2	38 ± 1	3 ± 1
L_6H^R-1	60 ± 7	18 ± 1	44 ± 3	6 ± 1
L_6^{WT}-2	41 ± 4	15 ± 1	40 ± 3	4 ± 1
L_6H^R-2	51 ± 3	20 ± 4	54 ± 3	7 ± 1
L_6^{WT}-3	75 ± 8	12 ± 2	35 ± 2	5 ± 1
L_6H^R-3	120 ± 39	19 ± 7	57 ± 15	8 ± 3
L_6^{WT}-5	51 ± 9	7 ± 2	26 ± 5	2 ± 1
L_6H^R-5	97 ± 10	10 ± 2	80 ± 7	6 ± 1
L_6^{WT}-6	31 ± 8	12 ± 3	30 ± 6	3 ± 1
L_6H^R-6	37 ± 1	15 ± 1	39 ± 5	4 ± 1
L_6^{WT}-1	27 ± 5	10 ± 3	26 ± 6	5 ± 1
L_8H^R-1	83 ± 9	24 ± 5	45 ± 4	9 ± 2
3T3WT-1	73	142	63	9
3T3H^R-1	99	78	64	23

[a] Values are means from at least two independent determinations, except in the case of the 3T3 lines where only a single experiment was performed.

TABLE 4: Clonal Variation and Deoxyribonucleotide Pool Sizes in L_6 and L_8 Myoblasts

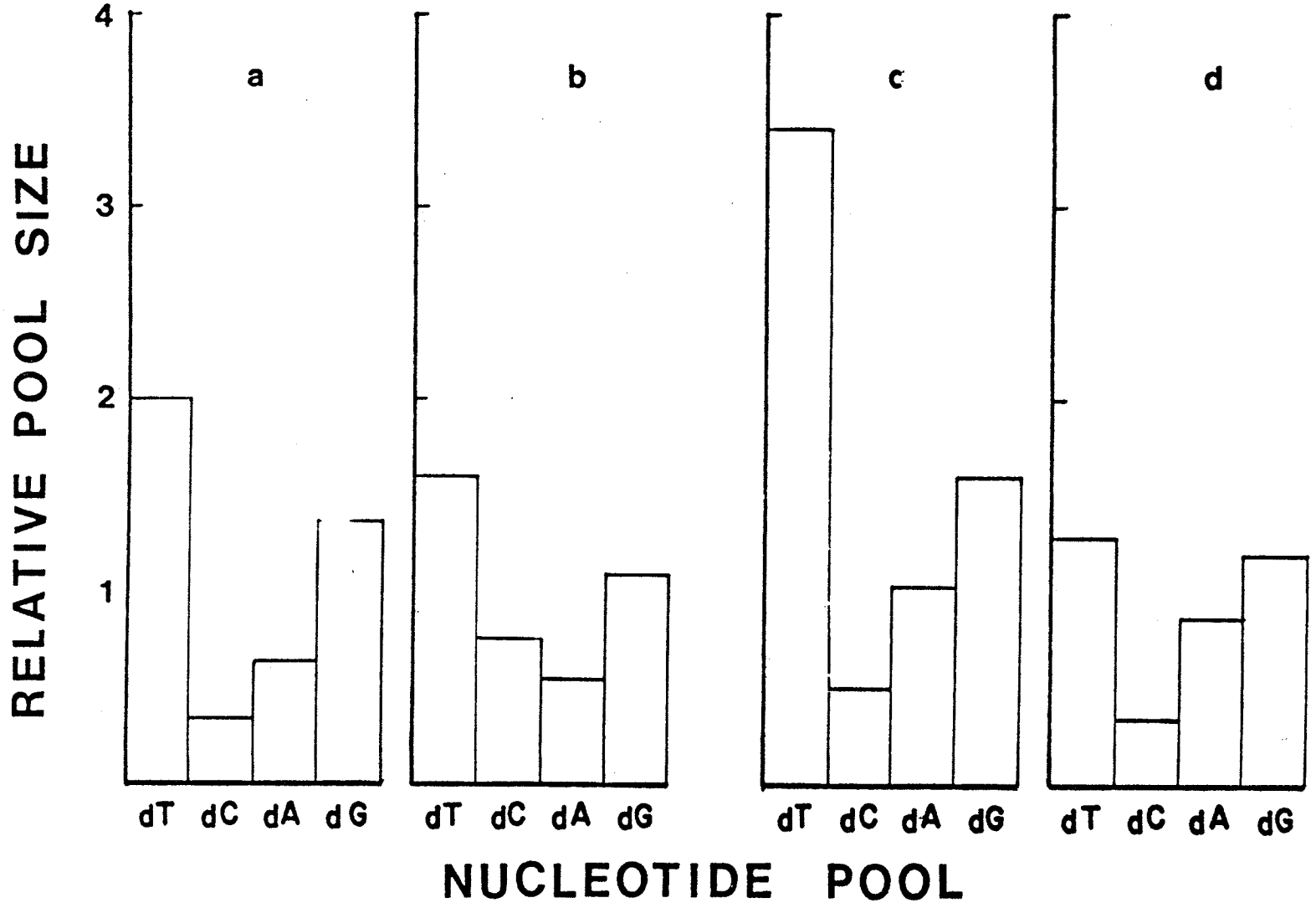
	DEOXYRIBONUCLEOTIDE POOL SIZES[a] (pmoles/10^6 cells)			
	dTTP	dCTP	dATP	dGTP
All L_6 and L_8^{WT} clones	44 ± 7	12 ± 1	33 ± 3	4 ± 1
All L_6 and $L_8^{H^R}$ clones	75 ± 13	18 ± 2	53 ± 6	7 ± 1
Ratio of all H^R/All WT	1.7	1.5	1.6	1.8

[a] Values shown are the means calculated when all myoblast parental clones or all myoblast drug resistant clones are considered. Standard errors for the means are shown. Data is from table 3.

all WT values may be considered as having been determined from the same statistical population. This was not the case for the dTTP pool however, where a significant amount of clonal variation existed in the WT populations. Thus, while the mean dTTP pool size in the hydroxyurea-resistant myoblasts was significantly different from that of the parental myoblasts when a paired analysis was performed, the latter analysis (1-way analysis of variance) indicated that the WT clones themselves exhibited significant clonal variation in this nucleotide pool. Interestingly, the overall dNTP pool increases in the H^R myoblasts approximated the overall increases in CDP reductase activity observed in these variants. The interdependence of the dNTP pool sizes because of the allosteric regulation of RRase, prevented a more detailed comparison of the relationship between the reductase activity associated with a specific nucleoside diphosphate and its corresponding deoxyribonucleotide pool.

Deoxyribonucleotide pools were also examined in parental and variant myoblasts grown in the presence of hydroxyurea. Previous studies on the inhibition of RRase by hydroxyurea in the various myoblast cell lines had indicated that while the H^R variants displayed increased rates of CDP reduction, the velocity of the reaction was inhibited equally by hydroxyurea in the WT and H^R cell lines. Thus a similar pattern of dNTP pool changes should be seen in the sensitive and resistant clones when grown in the presence of the drug. Indeed, this was found to be the case, as diagrammed in figure 34 for L_6WT/H^R-1 and L_8WT/H^R-1. While the extent of dNTP pool size change varied somewhat between these cell lines (statistically insignificant by a paired t-test), the overall pattern of change was approximately the same for all the clones: an increase in dTTP was accompanied by a reciprocol

FIGURE 34: Effect of growth in hydroxyurea on the dNTP pools of drug-resistant and sensitive L6 and L8 rat myoblasts. Values are the ratios of triplicate peak weights from single experiments. Cultures were prepared as for standard nucleotide extractions but were refed with 0.7 mM hydroxyurea in serum supplemented α-MEM 24 hr after seeding. Treated cultures were harvested and extracted 48 hr later. Note the general pattern of decreased dCTP and increased dTTP and the lesser changes in purine deoxynucleotide pools. (a) L_6WT-I, (b) L_6H^R-I, (c) L_8WT-I, (d) L_8H^R-I


RELATIVE POOL SIZE
4
3
2
1
a
b
c
d
dT dC dA dG
dT dC dA dG
dT dC dA dG
dT dC dA dG
NUCLEOTIDE POOL

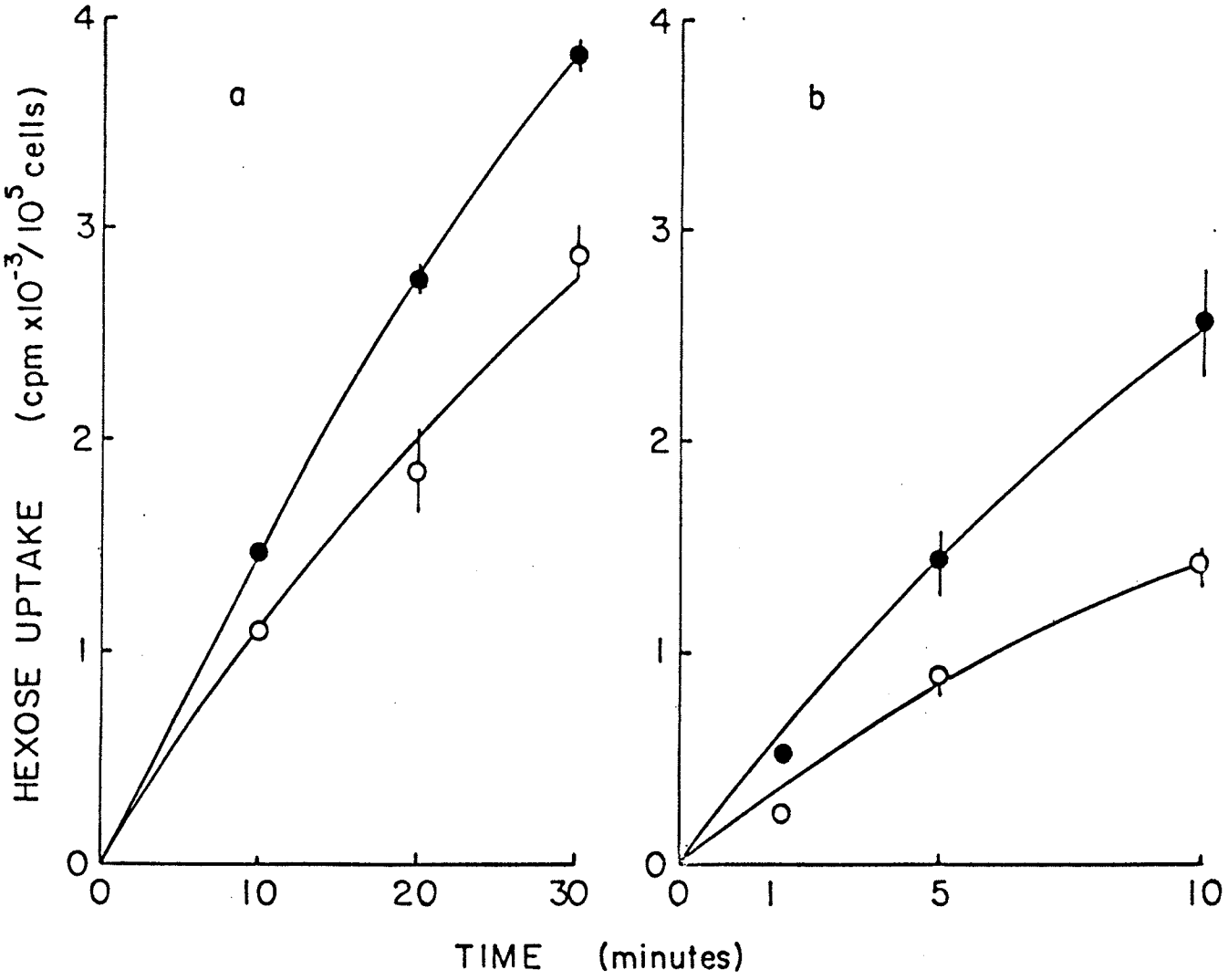
change in the dCTP pool while the pool of dATP decreased or remained unchanged and the dGTP pool increased slightly or remained unchanged. Thus, no significant changes in dNTP pool alterations were observed between these WT and H^R myoblast cell lines grown in the presence of hydroxyurea.

Studies on the In Vitro Transformation Characteristics of WT and H^R Cell Lines

The differentiation-defective phenotype of the hydroxyurea-resistant cell lines, coupled with their increased ribonucleotide reductase activity and accompanying deoxyribonucleoside triphosphate pool elevations and imbalance, suggested that these drug-resistant variants may exhibit an increased degree of neoplastic tranformation. Therefore, a preliminary examination was conducted on a transformation-associated property easily measured in vitro: hexose uptake.

The uptake of [^{3}H]-deoxyglucose was measured in actively dividing cultures of L_6 and L_8 myoblast cell lines. Two pairs of clones were studied, L_6WT/H^R-5 and L_8WT/H^R-1, and their hexose uptake profiles are shown in figures 35a and b, respectively. Each hydroxyurea-resistance clone exhibited a substantially increased rate of hexose uptake when compared to its respective parental cell line. While the differences were not large, the low degree of variation in the values obtained in these two experiments suggested that the H^R cell lines might exhibit other properties indicative of a more highly transformed state. It was known that the wild type L_6 and L_8 myoblast cell lines grew poorly, if at all, in sloppy agar and thus, a survey was

FIGURE 35: Hexose uptake by (a) L_6WT/H^R-5 and (b) L_8WT/H^R-1 rat myoblast cell lines. ○ Wild type parental clones and ● hydroxyurea-resistant clones. Values are the averages of triplicate determinations from a single experiment. Vertical bars represent the range of values obtained.


HEXOSE UPTAKE (cpm x10^{-3}/10^5 cells)
a
b
0
1
2
3
4
0
10
20
30
0
1
5
10
TIME (minutes)

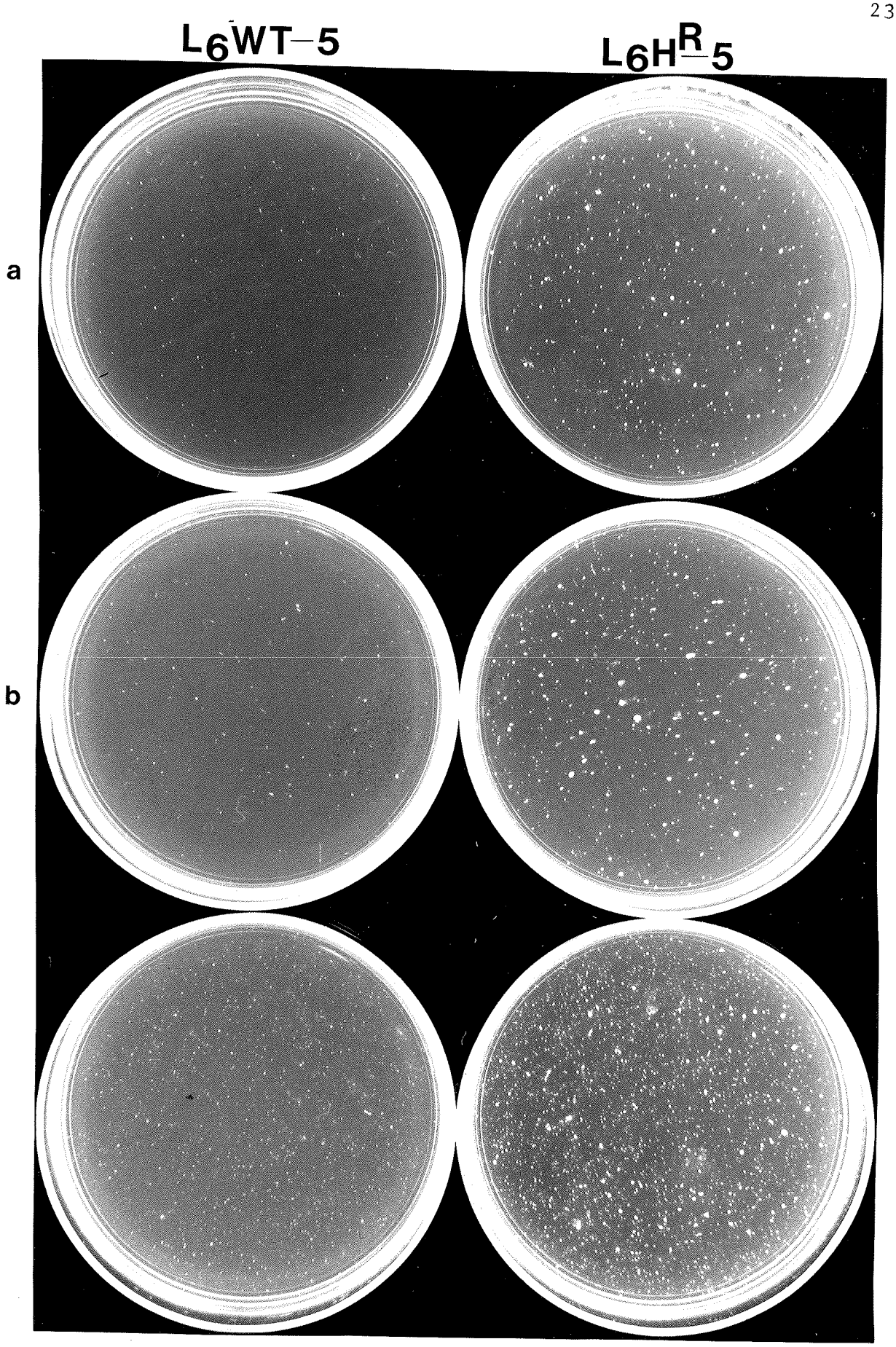
performed on the anchorage-independent growth potential of the WT and H^R myoblast clones.

The colony forming ability of all the L_6 and L_8 rat myoblast clones isolated in this study is shown in table 5, for growth in 0.3% agar and 0.3% agarose. In addition, the colony forming ability of two L_6 transfectants (T1-3 and T1-4) derived from L_6WT-5 using L_6H^R-5 DNA, as well as that of the BALB/c 3T3 cell lines and several mouse L cell lines are documented in this table. With only one exception (L_6WT-5), all L_6 and L_8 wild type clones formed colonies in sloppy agar with an efficiency of less than 1%. L_6WT-5 also grew poorly in sloppy agar, but exhibited a 2.8% colony forming ability. The growth of L_6WT-5 and L_6H^R-5 in sloppy agar is shown in the photographs of figure 36. Statistical analysis of the colony forming indices of all WT and H^R myoblasts by Student's paired t-test revealed a significant difference in the mean values obtained with parental and variant clones ($p<0.05$). An identical analysis using the sloppy agarose data indicated no significant difference at the same probability value, but was significant at $p<0.10$. BALB/c 3T3 WT/H^R-1 grew very poorly in sloppy agar (<1%) but showed modest growth in sloppy agarose. Interestingly, the drug-resistant clone BALB/c 3T3 H^R-1 failed to grow as well as its parental cell line in agarose. The significance of this finding, however, is unknown since only a single experiment was performed. Thus, while the findings with the 3T3 cell lines indicate little if any change in anchorage-independent growth, the myoblast cell lines exhibited a close correlation between hydroxyurea-resistance, increased ribonucleotide reductase activity, defective cellular differentiation and the degree of transformation, as seen by their capacity for anchorage- independent growth.

TABLE 5: Colony forming ability of wild type and hydroxyurea-resistant cell lines in soft agar and soft agarose

CELL LINE	COLONY FORMING INDEX AGAR	AGAROSE
L_6WT-1	0.004	ND
L_6H^R-1	0.250	ND
L_6WT-2	0.008	0.008
L_6H^R-2	0.076	0.008
L_6WT-3	0.001	0.001
L_6H^R-3	0.073	0.016
L_6WT-4	0	ND
L_6H^R-4	0.076	ND
L_6WT-5	0.028	0.120
L_6H^R-5	0.568	0.164
T1-3	0.104	ND
T1-4	0.020	ND
L_6WT-6	0.004	0.008
L_6h^-6	0.004	0.016
L_8WT-1	0	0.004
L_8H^R-1	0.220	0.060
3T3WT-1	0.002	0.105
$3T3H^R-1$	0.002	0.055
L_{60}	0.800	ND
L_1H_2	0.300	ND
L_2Cl_3	0.268	ND
LHF^-	0.232	ND
LHF^+	0.092	ND

FIGURE 36: Photograph of L_6WT-5 (left) and L_6H^R-5 (right) colony formation in soft agar after four weeks incubation. The numbers of cells originally plated were (a) 500, (b) 10^3 and (c) 10^4.


L6WT-5
L6HR5
a
b

Tumor Formation by L_6 and L_8 Rat Myoblasts in BALB/c nu/nu Mice

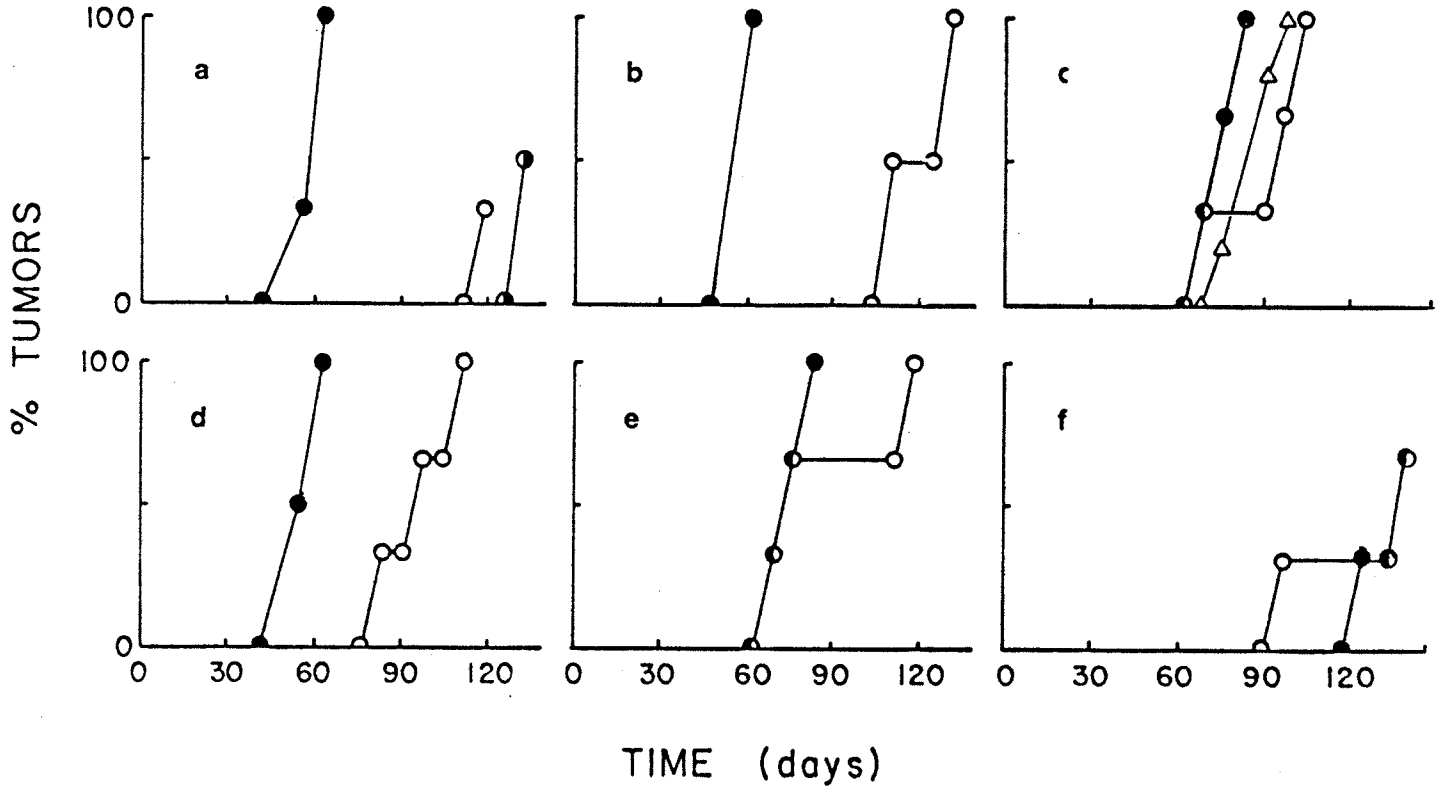
The statistically significant increase in the capacity for anchorage-independent growth exhibited by the hydroxyurea-resistant myoblast cell lines compared to their drug-sensitive WT clones suggested that the growth of these cell lines *in vivo* should be examined, as a further marker of their degree of neoplastic transformation. Studies conducted in other laboratories have shown that fusion-competent wild type clones of L_6 myoblasts are non-tumorigenic when injected into Swiss *nu/nu* mice or suckling Wistar rats. Fusion-competent L_8 myoblasts are tumorigenic in Wistar rats but non-tumorigenic in Swiss *nu/nu* mice. The examination of the *degree* of tumorigenicity in WT and H^R myoblasts demanded that a permissive host system be used, since fusion-incompetent myoblasts are tumorigenic in a number of hosts, unlike their differentiation-competent clones. Thus if L_6WT and H^R cells were injected into Swiss *nu/nu* mice, the fusion-incompetent clones would undoubtedly form tumors whereas the parentals would not. No significance could be ascribed to such observations in a system non-permissive for WT tumor formation, in terms of the relevance of the H^R and increased RRase activity phenotypes, since the accompanying differentiation-defective trait is known to result in a tumorigenic state. Thus a search was commenced for a permissive host system that would allow for the description of differences between tumors formed by H^R and WT L_6 and L_8 rat myoblasts.

The first host system examined for the allowance of tumor formation by wild type, fusion competent L_6 and L_8 rat myoblasts was the

BALB/c nu/nu mouse. Fortunately, this animal model possessed the environment necessary for the comparison of WT and H^R tumors. A total of eight mice were originally obtained. Two mice each were injected with either L_6WT-1, L_6H^R-1, L_8WT-1 or L_8H^R-1. Cells for injection were harvested from actively growing cultures by scraping with a rubber policeman, and were given to each aminal as 100μl aliquots of $1x10^6$ (L_8) or $1.5x10^6$ (L_6) cells, subcutaneously. The four mice injected with L_8 myoblasts developed tumors rapidly. Fifty-five days after injection the L_8WT-1 tumor indices averaged 1 cm^2 and those of the H^R variant averaged 2 cm^2. Explantation of the tumors at this time revealed that the L_8WT-1 tumor cells were still fusion competent whereas the variant tumor cells were not. The L_6 tumors which developed, grew much more slowly. Only two tumors developed, one each for the L_6WT-1 and L_6H^R-1 clones. One hundred and forty-seven days after injection the variant tumor index was 4.76 cm^2 and upon explantation, the tumor weighed 3.09 grams. The L_6WT-1 cell mass measured only 0.37 cm^2. As a consequence of these results, the BALB/c nu/nu mouse was chosen as the animal model with which more extensive studies would be performed on the WT and H^R myoblasts.

Parental and variant L_6 myoblasts were injected subcutaneously into the backs of BALB/c nu/nu mice. Three mice were used for each clone (L_6WT-6 and L_6H^R-6) for a total of 3 dozen animals. The latency period of each tumor and the total number of tumors formed in the L_6 study are shown in figures 37a-e. One wild type mouse and two variant mice died before developing tumors and of the mice remaining, 13 of 17 mice injected with L_6WT myoblast clones and 14 of 16 mice given L_6H^R myoblast clones, developed tumors within 139 days, the total period of the study. Although no significant difference was observed in

FIGURE 37: Tumor formation by L6 wild type (○) and hydroxyurea-resistant (●) myoblast clones. A total of 3 mice (BALB/c nu/nu) were injected with $3x10^6$ cells from exponentially growing cultures of each clone. A positive result was defined as a palpable mass with a calculated tumor index of ≥ 0.40, corresponding to a volume ($\geq$ 0.1 ml) greater than or equal to that of the original inoculum. Three mice died before developing tumors (L_6WT/H^R-2 and L_6H^R-4). (a) L_6WT/H^R-1, (b) L_6WT/H^R-2, (c) L_6WT/H^R-3 (d) L_6WT/H^R-4, (e) L_6WT/H^R-5, and (f) L_6WT/H^R-6.
Tumor formation by L_6REV-1 (◑) is shown in (a). Tumor formation by the conA resistant variant L_6C1_6 V5 (△) is shown in (c).


100
0
a
b
c
d
e
f
% TUMORS
0
30
60
90
120
TIME (days)

tumor incidence between those mice injected with WT or H^R myoblasts, a one-way analysis of variance in L_6 tumor latency revealed a statistically significant ($p<0.05$) difference in the WT and H^R mean tumor latencies. When a minimum latency time of 139 days was assigned to those mice which failed to develp tumors, the mean L_6WT1-6 tumor latency was 108 days (standard deviation 24 days) and the mean L_6H^R1-6 tumor latency was 81 days (standard deviation of 29 days). Interestingly, the low-resistant variant L_6H^R-6, formed only one tumor (like its parental clone), and this tumor had a latency period of 30 days longer than that of L_6WT-6. Even the inclusion of this clone did not abrogate the significance of the decreased latency exhibited by the tumors arising from the hydroxyurea-resistant cell lines. The highly-resistant clone L_6H^R-5 showed little difference in tumor latency in comparison with its sensitive parental clone which grew quite well in sloppy agar. Similarly, L_6H^R-3 tumor latency was only modestly different from that of its parent L_6WT-3. A conA resistant, fusion-incompetent clone (L_6Cl_6V5) selected from L_6WT-3 displayed no significant difference in tumor latency in comparison with its fusion-competent, lectin-sensitive parental population. A one-way analysis of variance between the tumor latencies of L_6WT or L_6H^R tumors and those of the conA resistant variant indicate that the variation in conA tumor latencies was not significantly different from that of either H^R or WT tumors. The mean tumor latency of L_6Cl_6V5 was 89 ± 9 days and is thus effectively intermediate between those of the H^R and WT clones. However, if the L_6H^R-6 tumor which arose at 128 days (and the two which did not, but were assigned values of 139 days each) were eliminated from the analysis because of their virtually WT drug resistance, RRase activity and dNTP pool-sizes, then the conA

resistant clone exhibited a significantly different (increased) mean tumor latency ($p>0.001$) in comparison to that of the fusion-incompetent, H^R clones which have a revised tumor latency of 68 ± 9 days.

A one-way analysis of the variation in L_6WT tumor latencies indicated that the WT tumors arose from the same statistical population. A similar analysis of L_6H^R tumor latencies indicated that these tumors also arose from the same statistical population, but different from that of the L_6WT tumors. This is true however, only if L_6H^R-6 is not included in the L_6H^R tumor latency analysis of variance and thus effectively indicated that L_6H^R-6 arose from a statistically different population than that of the other L_6H^R tumors. Such analyses permit the accumulation and comparison of the L_6 tumor latency data as shown in figure 38. This figure allows one to more readily appreciate the differences in L_6WT and L_6H^R tumor latencies. Indeed, from this figure it is clear that greater than 50% of the L_6H^R tumors (L_6H^R1-5) arise before even the first L_6WT tumor (L_6WT1-6).

The growth rates of L_6WT and L_6H^R tumors can also be accumulated and compared in a manner similar to the tumor latency data of figure 38. When L_6H^R-6 was excluded from the variant data on the basis that its various traits were virtually those of the parental cells, an unpaired Students's t-test indicated that there is a statistically significant difference in the mean tumor indices of L_6WT and L_6H^R tumors for each set of measurements taken up to and including 105 days after injection of cells into the host animals. The overall growth kinetics of L_6WT/H^R1-5 tumors are shown in figure 39. No data is shown beyond the 105th day, even though the study was continued to day 139, because beyond the 105th day individual animals began to be

FIGURE 38: Clonal variation and tumor formation in L6 wild type (○) and hydroxyurea-resistant (●) myoblasts. Values represent the accumulation of all wild type and parental L6 myoblast tumor latency data from figure 37 with the exception of L_6H^R-6, because of its atypically low resistance to hydroxyurea and other properties closely resembling the wild type clones (see text for full explanation).

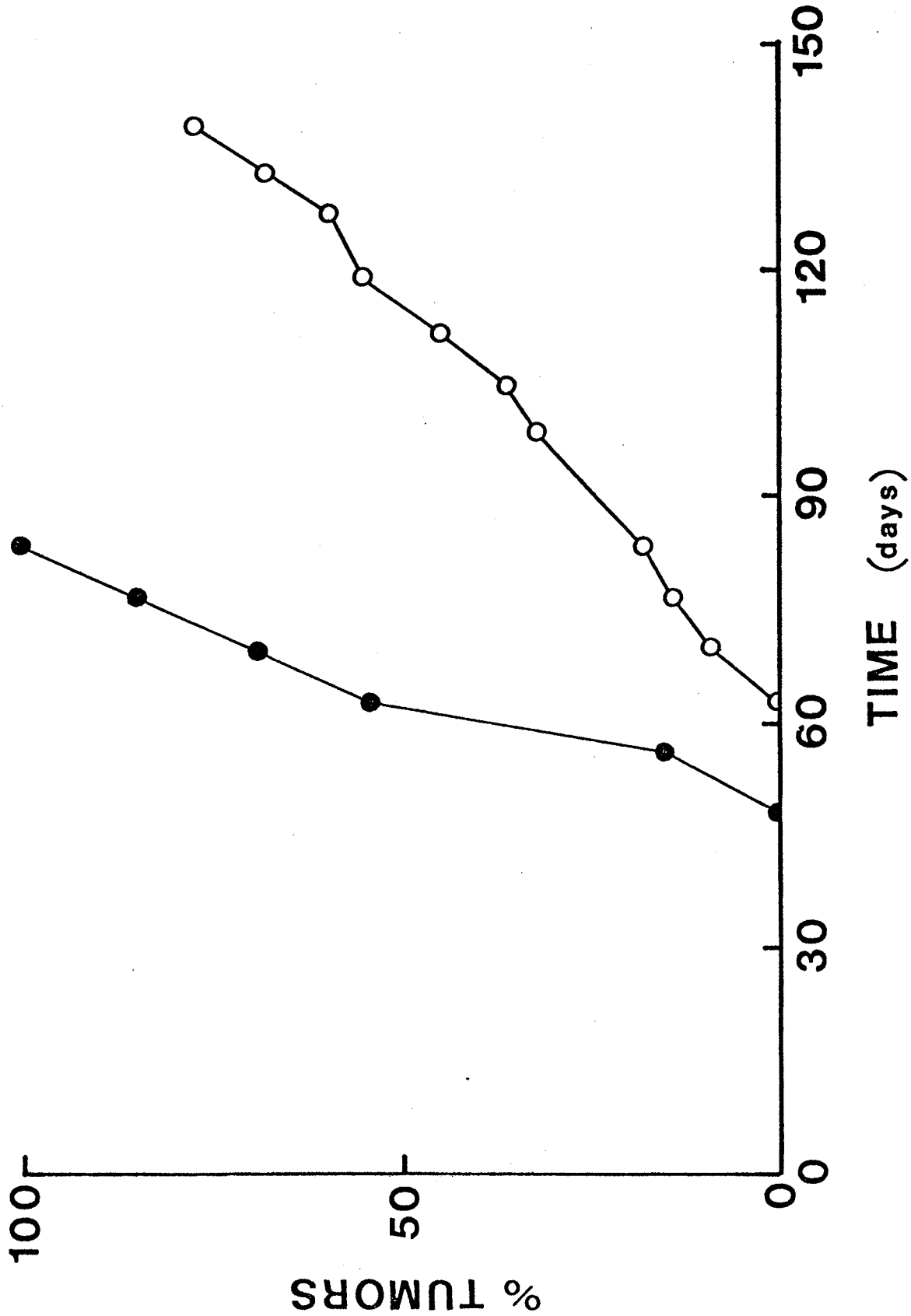
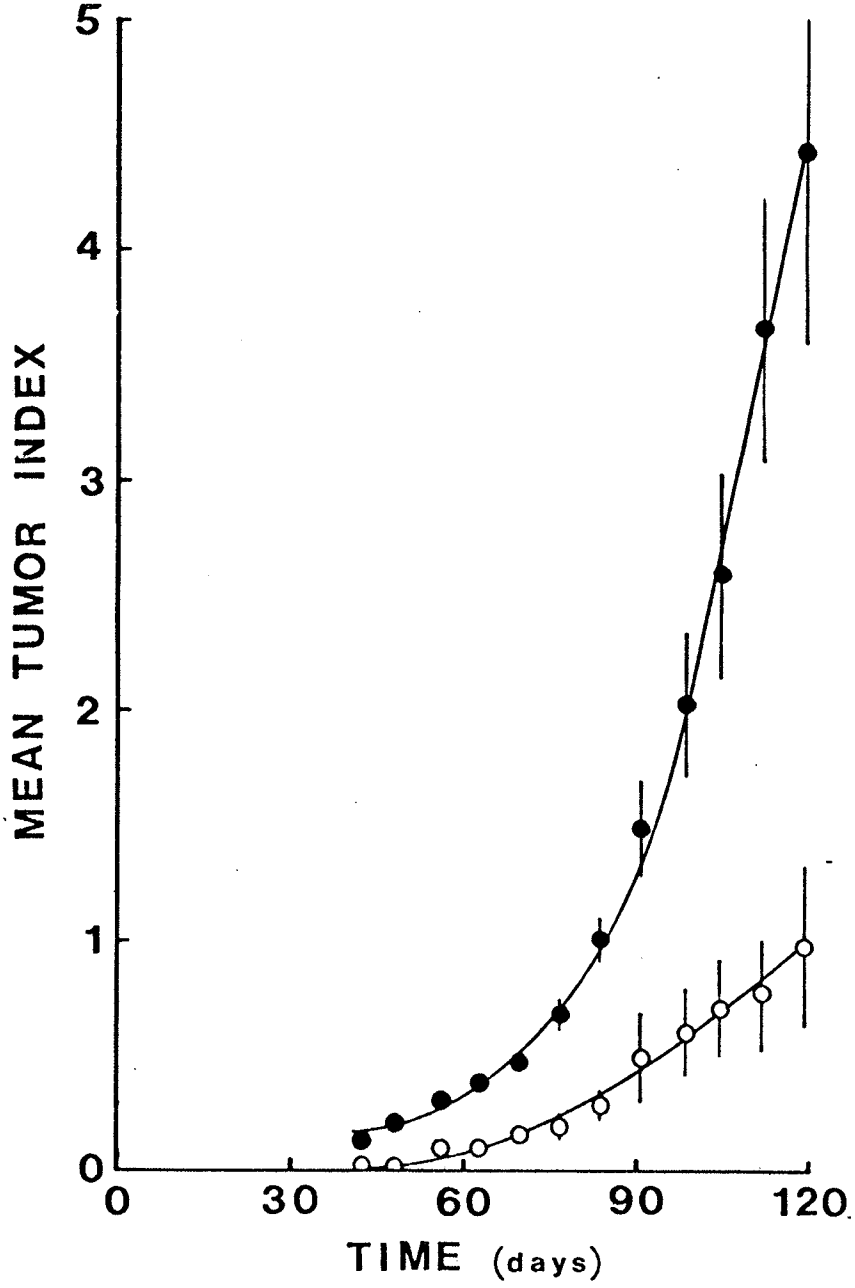

% TUMORS
100
50
0
0
30
60
90
120
150
TIME (days)

FIGURE 39: Mean tumor growth rates of hydroxyurea-resistant (●) and sensitive (○) L_6 myoblast clones in BALB/c nu/nu mice. Data was obtained from the same experiments described in the legend to fig. 37. Vertical bars represent the standard error in the mean values.


MEAN TUMOR INDEX
5
4
3
2
1
0
0
30
60
90
120
TIME (days)

sacrificed if their tumors were deemed to be placing an undue burden on the animal. Some tumor-bearing animals were sacrificed with tumors smaller than those of animals bearing larger tumors because of the excessively necrotic nature of some tumors. Other animals were also sacrificed if they possessed persistent local infections (often in the eyes) which appeared to cause them unnecessary distress.

Studies involving tumor formation by L_8WT-1 and its variant L_8H^R-1 have produced results very similar to those seen with L_6 myoblast cell lines. Statistical analyses however, indicated that no significant differences existed between the WT and drug-resistant variant, whereas significant differences were suggested by such analyses with the L_6 lines. The reasons for this apparent discrepancy are manifold, but certainly the lower number of samples and the availability of only one WT/H^R pair in the L_8 system hamper the appreciation of the apparent differences seen between L_8WT-1 and L_8H^R-1.

The tumor latency exhibited by L_8WT/H^R-1 is presented in figure 40. No statistically significant difference exists in the variation of the mean tumor latency times of these two cell lines, when examined by a one-way analysis of variance.

The mean tumor indices of the tumors formed by L_8WT/H^R-1 are shown in figure 41. Values for one tumor from L_8H^R-1 cells were not included in this figure since this tumor regressed completely after 7 weeks. A one-way analysis of variance on the individual tumor indices indicated that no statistically significant difference existed in the variation of the mean values obtained for the L_8WT-1 and L_8H^R-1 tumors. The plots of the L_8WT and L_8H^R tumor growth rates eventually cross about twelve weeks after injection. When a linear

FIGURE 40: Tumor formation by L8 rat myoblast clones. Data from four mice injected with L8WT-1 (○) and five mice injected with L_8H^R-1 (●) is shown. Each animal was injected with $3x10^6$ cells and tumors were scored as per legend to figure 37.

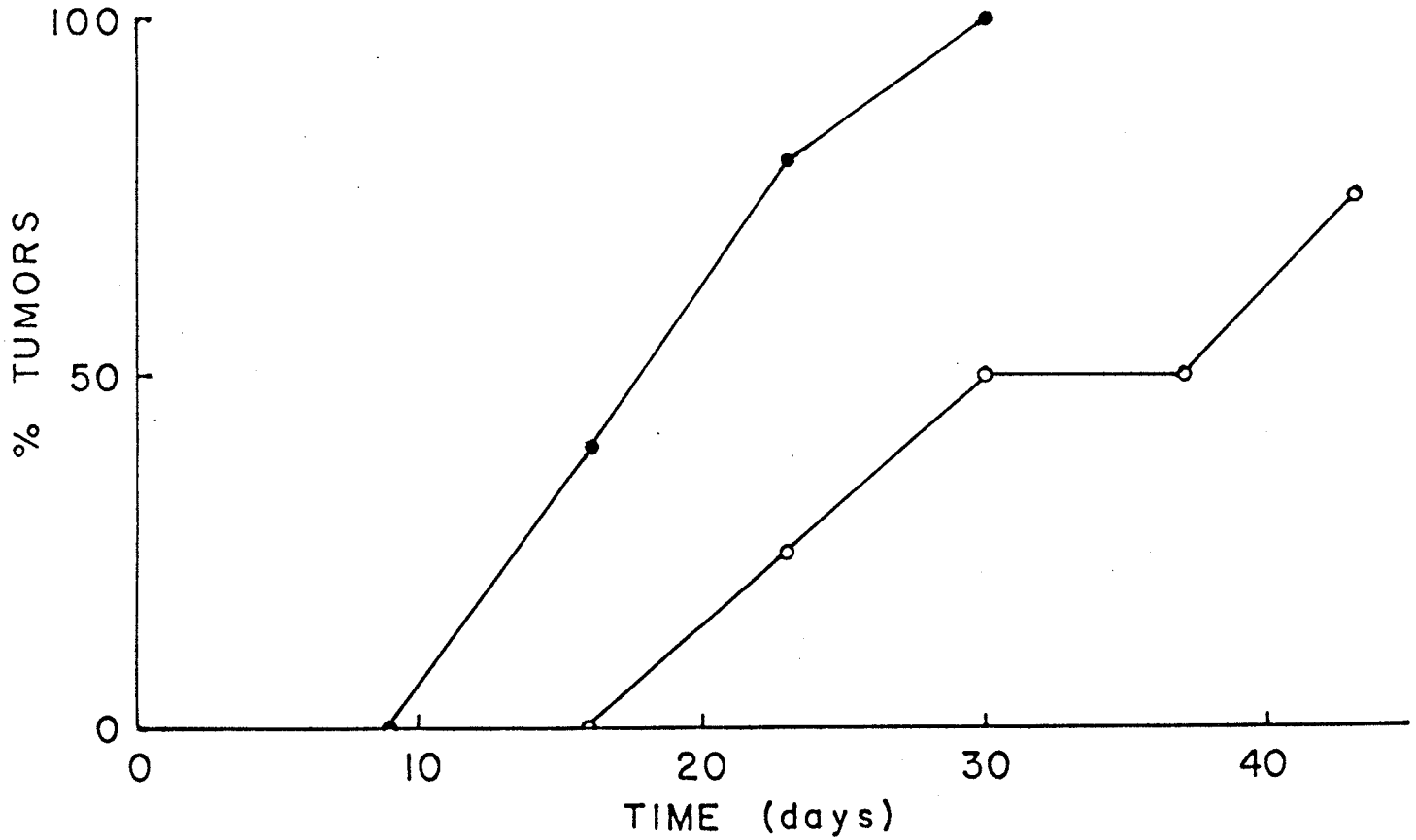
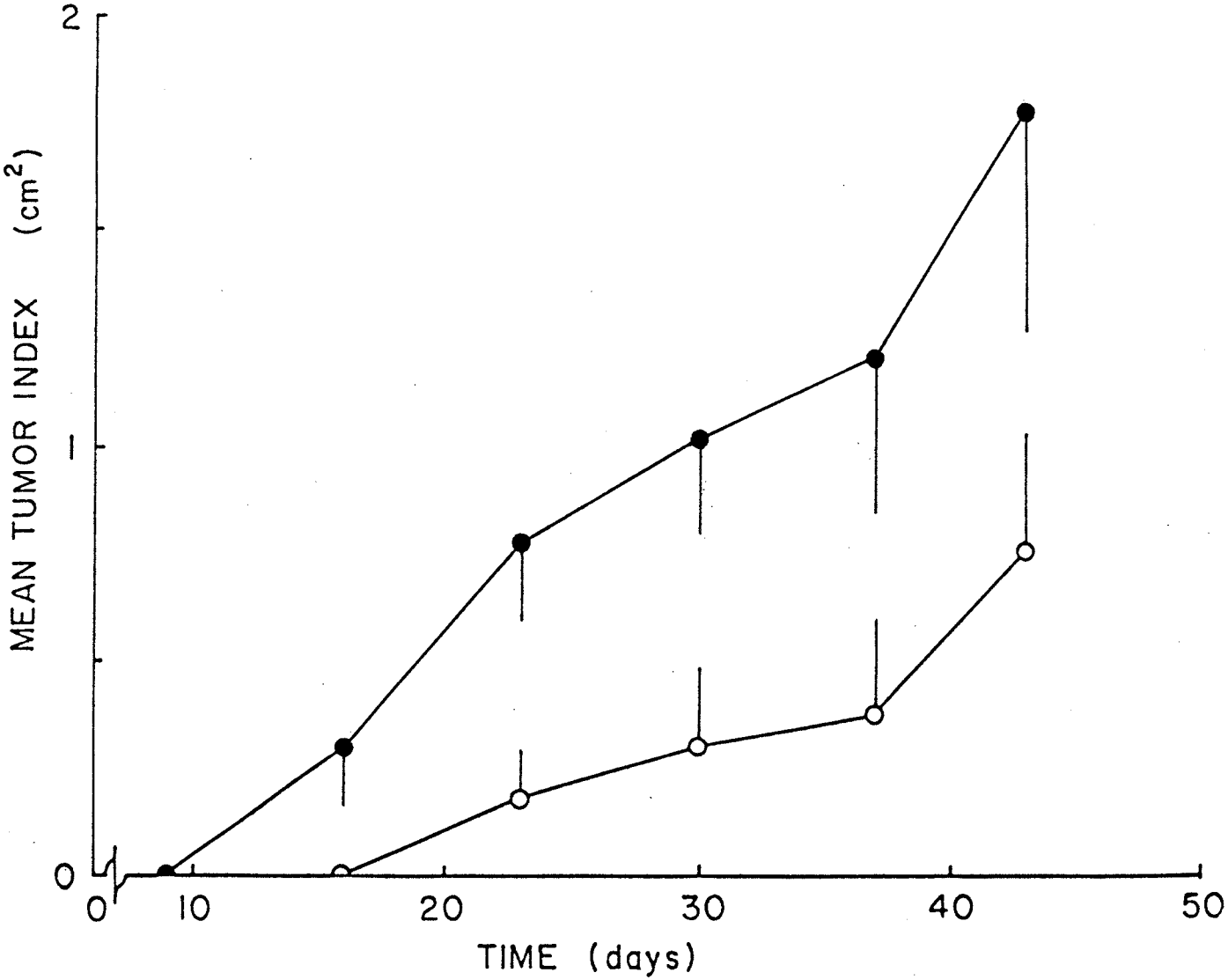

100
50
0
% TUMORS
0
10
20
30
40
TIME (days)

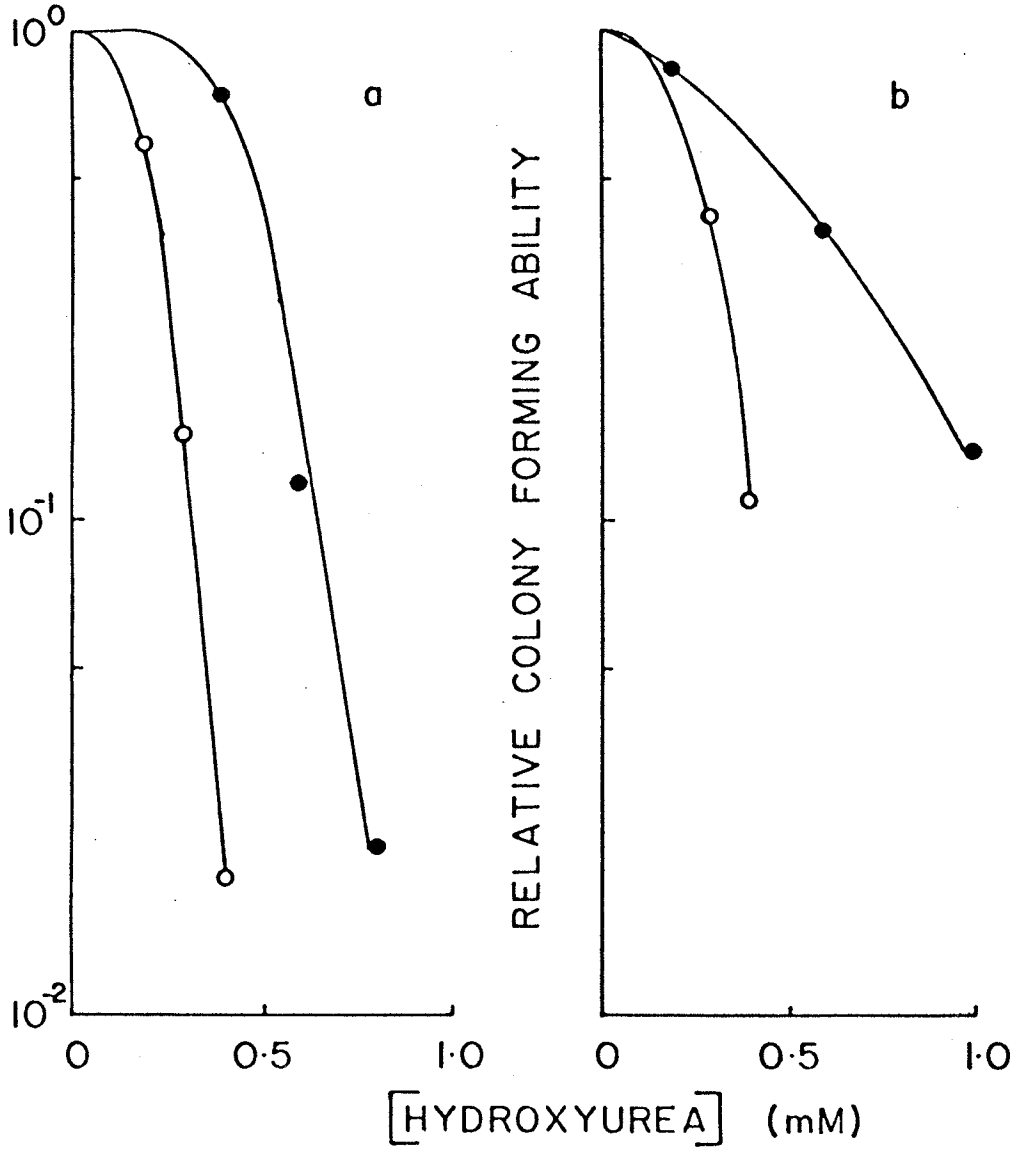
FIGURE 41: Growth rate of L_8^{WT}-1 (○) and $L_8^{H^R}$-1 (●) tumors in BALB/c nu/nu mice. Data was obtained from the same experiment described in the legend to figure 40. Values are the averages of five mice for each clone. The growth curves eventually cross by 11-12 weeks after injection because of variation in tumor shape and height. Vertical bars represent the standard error in the mean values.


MEAN TUMOR INDEX (cm²)
2
1
0
0
10
20
30
40
50
TIME (days)

regression was performed with the individual tumor indices, and a rate of tumor growth was obtained for each mouse, one-way analyses of variance indicated a statistically significant difference ($p<0.05$) in the variation of the sample means of L_8WT and L_8H^R tumor growth rates during the first 7 weeks of the study, but not when the data was analysed over the full duration of the experiment. The latter analyses employed rate values from linear regressions on all the data. During the first 7 weeks, the mean tumor growth rates and their standard deviations were 0.02 ± 0.02 and 0.07 ± 0.02 cm^2/day, a difference of 3.5 fold between L_8WT-1 and L_8H^R-1 respectively. These rates calculated over the entire length of the study were 0.08 ± 0.07 and 0.08 ± 0.02 cm^2/day for the wild type and drug-resistant tumors respectively.

It was noted earlier that the L_8 cells derived from tumors explanted in this study retained their respective myogenic phenotype: cells from L_8WT-1 tumor explants were still capable of fusion (>50% of nuclei in syncytia 4 days post confluence) whereas cells from L_8H^R-1 tumor explants could still not be induced to fuse. The drug-resistant properties of these cell lines were also maintained after passage through nude mice, as were those of L_6WT/H^R-1. The colony forming abilities of these explant cultures in the presence of hydroxyurea are shown in figures 42a and b. The cells explanted from tumors formed by L_6WT clones are still fusion competent, but not to the same extent as those of L_8WT. Explant cultures of the L_6WT tumors (L_6WT-1,3,4,5; L_6WT-2 and 6 tumors were not explanted) fused less than 10%, eight days after reaching confluence and maintained in 1% CS/α-MEM with amphotericin B and gentamycin. The fusion observed in these cultures was generally not evident for the first four to five days post

FIGURE 42: Effect of various concentrations of hydroxyurea on the colony forming ability of (a) L_6 and (b) L_8 myoblasts after passage through nude mice. Note that the explanted tumor populations exhibit similar resistance to the selective agent before and after growth as tumors in animals (compare with figures 2 and 4a). (○) explant of wild type tumor and (●) explant of variant tumor.

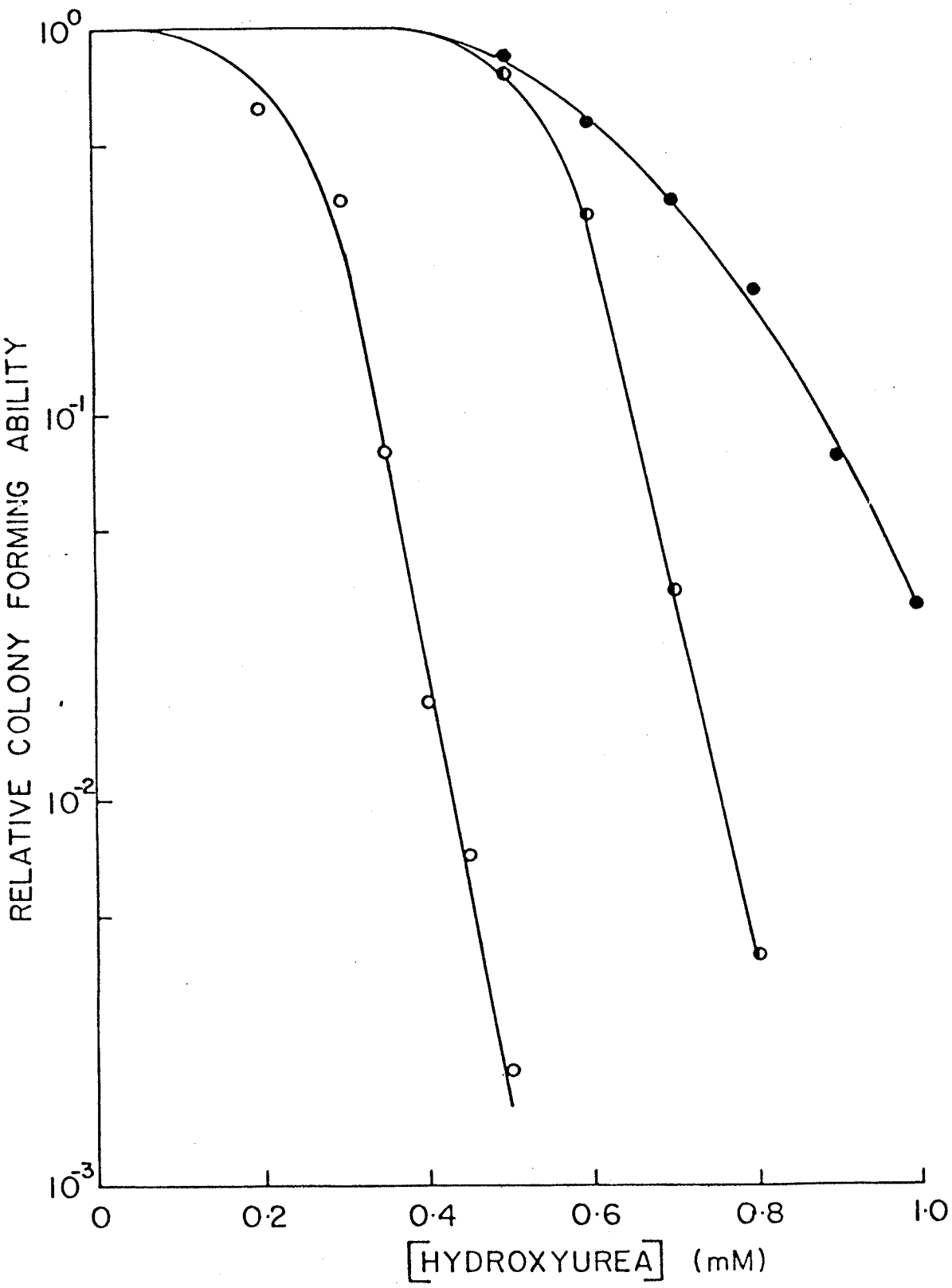

10⁰
10⁻¹
10⁻²
a
b
RELATIVE COLONY FORMING ABILITY
0
0·5
1·0
0
0·5
1·0
[HYDROXYUREA] (mM)

confluence, a period of time longer than that seen in freshly cloned WT populations (compare with data of figure 6). None of the corresponding tumor explant cultures of the L_6H^R variants could be induced to fuse.

Late in the study of the myoblast variants, a revertant population was isolated from L_6H^R-1. Several 150 mm culture dishes containing this drug-resistant variant were grown to confluence then refed with growth medium supplemented with 1% CS, the same serum supplementation used in normal fusion assays. The cultures were maintained in low serum for several days and examined for rare fusion elements (containing 3 or more nuclei) which were known to occur in induced cultures of this variant at a very low frequency. A small element was identified by light microscopy and an area around it was demarcated such that cells from within this region could be harvested by scraping with a Pasteur pipet. The harvested cells were transferred to a 100 mm culture containing normal growth medium and again grown to confluence. This population of cells was then subcloned by standard procedures. One of these subclones was then chosen at random for a second round of subcloning. Secondary subclones were isolated in this manner, designated L_6REV-1, and subjected to analyses of drug-resistance and differentiation competence.

The colony-forming ability of two L_6REV-1 clones in the presence of hydroxyurea are shown in figure 43. The range of values obtained with these two subclones approximated the size of the circles for the data points and are thus not apparent. These two clones were used interchangeably in subsequent experiments. The D_{10} value of L_6REV-1 in hydroxyurea is approximately 0.65 mM, intermediate between

FIGURE 43: Effect of various concentrations of hydroxyurea on the colony forming ability of L_6^{WT}-1 (○), L_6^{REV}-1 (◐), and $L_6^{H^R}$-1 (●). Values for L_6^{WT}-1 and L_6H_R-1 are from figure 2 and are included for ease of comparison. Values for the revertant are the averages of a single experiment employing two independent subclones of the revertant population. Vertical bars represent the range of values obtained.


RELATIVE COLONY FORMING ABILITY
10^0
10^-1
10^-2
10^-3
0
0·2
0·4
0·6
0·8
1·0
[HYDROXYUREA] (mM)

that of the original L_6WT-1 parental clone (0.34 mM) and the variant L_6H^R-1 (0.9 mM) from which it was selected.

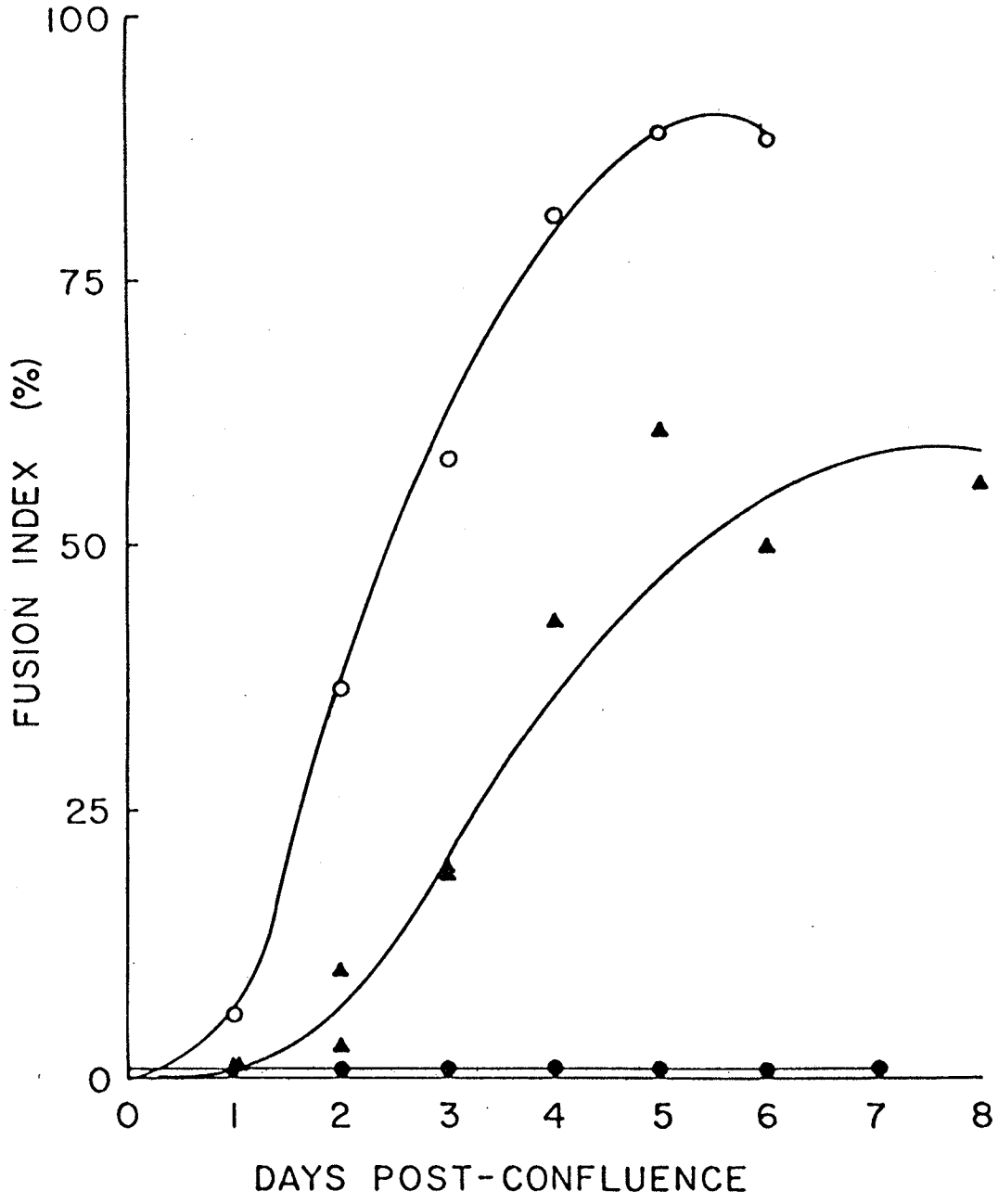
The intermediate character of L_6REV-1 also manifested itself in its apparent fusion-competence. Analysis of its myogenic potential in standard fusion assays revealed that greater than 50% of its nuclei could be found in syncytia after less than one week in low serum containing growth medium and confluent conditions (figure 44).

The CDP reductase activity of L_6REV-1 has been determined in a single parallel experiment with L_6WT-1. The WT parental activity was 1.66 nmoles dC formed/h/$5x10^6$ cells, whereas that of the revertant population was 1.56 nmoles dC formed/h/$5x10^6$ cells. In the same experiment, L_6REV-1 exhibited a K_M for CDP of 0.22 mM, essentially identical to that of L_6WT/H^R-1. Thus, L_6REV-1 exhibited a CDP reductase activity virtually identical to that of the original parental clone.

The uptake of guanazole and hydroxyurea has also been examined in L_6REV-1 cells. There was no apparent difference in the uptake of guanazole by L_6REV-1 in comparison to L_6WT-1 and L_6H^R-1 (figure 10c). Similarly, the revertant takes up hydroxyurea to the same extent as L_6WT/H^R-1 (figure 12c). In addition, the cell volume of L_6REV-1 was found to be virtually indentical to that of L_6WT-1 and L_6H^R-1 (L_6WT-1: 2.89 pl, L_6REV-1: 2.72 pl, L_6H^R-1: 2.84 pl). Also, no significnt difference in growth rates existed between the three cell lines.

Remarkably, L_6REV-1 exhibited a tumor latency very similar to that shown by L_6WT-1 and distinctly different than that of L_6H^R-1 (figure 37a). Three BALB/c nu/nu mice were injected subcutaneously with L_6REV-1 in the same experiment in which all other L_6 clones were

FIGURE 44: Fusion kinetics of L6WT-1 (○), L_6REV-1 (▲) and L_6H^R-1 (●) in 1% CS/α-MEM. Values for the wild type and H^R variant are from figure 6 and are included for ease of comparison. Values for the revertant were gathered in two independent experiments with early passage clones.


100
75
50
25
0
FUSION INDEX (%)
0
1
2
3
4
5
6
7
8
DAYS POST-CONFLUENCE

examined. One of these mice died shortly after injection, before developing a tumor. One of the two remaining mice failed to develop a tumor during the study and the other displayed a palpable mass with a tumor index of 0.50 on the last day (139) of the experiment. Interestingly, only 1 of 3 mice injected with L_6WT-1 developed a tumor, and not until the 120th day after injection. All three mice injected with the differentiation- defective L_6H^R-1 clone developed tumors by the 63rd day of the study.

Studies with Myoblast Transfectants

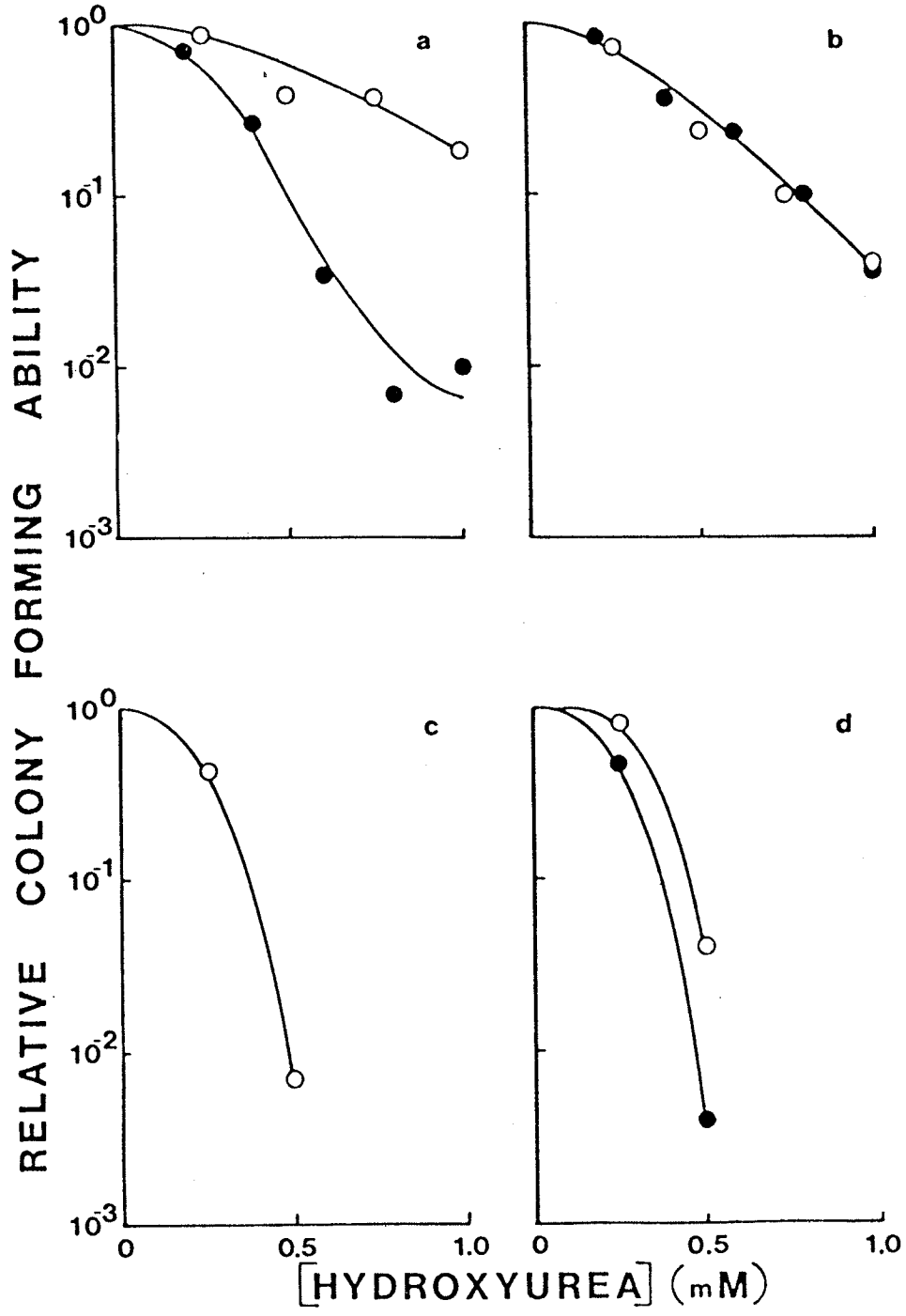
Early passage L_6WT-5 cells were used as recipients in the transfection of high molecular weight DNA extracted from L_6H^R-5. A parallel experiment employed DNA from L_6WT-5 itself, as a control. Using the calcium coprecipitation technique of Graham and van der Eb (1973), four cultures of L_6WT-5 (at $1x10^5$ cells/100 mm dish) were transfected with L_6H^R-5 DNA and three cultures of L_6WT-5 (at $1x10^5$ cells/100 mm dish) were transfected with L_6WT-5 DNA. The cultures were maintained in 0.75 mM hydroxyurea, a concentration of drug which would reduce the relative colony forming ability of the WT to less than $3x10^{-6}$, as estimated by linear regression analysis and extrapolation of known survival curve data. Ten days after transfection, no colonies were present on the 3 dishes given L_6WT-5 DNA, but 22 colonies were found on the dishes given L_6H^R-5 DNA (a relative colony forming ability of $5.5x10^{-5}$, or 20-fold increased over the colony forming value for normal WT cells estimated by regression analysis). All seven cultures were refed fresh medium without hydroxyurea and incubated a further week. A total of 28 colonies were cloned

from the cultures given L_6H^R-5 DNA, one week after rescue. Three colonies were discovered (after rescue) on the cultures given L_6WT-5 DNA, which were apparently not present before rescue. These colonies were also cloned and propagated. Three of the 28 L_6H^R-5 DNA clones were analysed with respect to drug-resistance, myogenic competence and tumor formation in nude mice.

The relative colony forming abilities of the transfectant clones in the presence of hydroxyurea are shown in figures 45 a-d. The clones designated L_6WT-5 T1-2, T1-3 and T1-4 are those which were transfected with variant (L_6H^R-5) DNA. The clone designated L_6WT-5 T5-1 is one of the clones originally exposed to L_6WT-5 DNA. Neither L_6WT-5 T1-4 or L_6WT-5 T5-1 displayed increased resistance to hydroxyurea at early or late passage after cloning (figures 45c and d). Both of these clones exhibited a D_{10} value approximating that of normal, parental L_6WT cells. L_6WT-5 T1-2 (Figure 45a) appeared quite resistant to hydroxyurea when first tested at passage number one, apparently with a D_{10} value greater than 1 mM. This resistance however, was unstable and was effectively lost by the seventh passage after cloning. L_6WT-5 T1-3 (Figure 45b) was the only transfectant studied which exhibited stable resistance to hydroxyurea. The D_{10} value of this clone has remained unchanged at approximately 0.8 mM, for up to 17 passages after isolation.

The differentiation competence of the transfectant clones is reduced in comparison to untreated parental myoblasts. L_6WT-5 T5-1 exhibited greater than 50% of its nuclei in syncytia 4-6 days post confluence in 1% serum supplemented growth medium. L_6WT-5 T1-2 showed no evidence of fusion in similar assays, whereas L_6WT-5 T1-3 and T1-4 exhibited limited (less than 10%) fusion under the same conditions.

FIGURE 45: Effect of various concentrations of hydroxyurea on the colony forming ability of myoblasts transfected with L_6H^R-5 DNA at early (○) and late (●) passage after selection and cloning. (a) L_6WT-5 T1-2 at P1 and P7, (b) L_6WT-5 T1-3 at P1 and P6, (c) L_6WT-5 T1-3 at P1 and (d) L_6WT-5 T5-1 at P1 and P4. The latter clone was transfected with L_6WT-5 DNA as a control.


a
b
c
d
10^0
10^{-1}
10^{-2}
10^{-3}
RELATIVE COLONY FORMING ABILITY
0
0.5
1.0
[HYDROXYUREA] (mM)

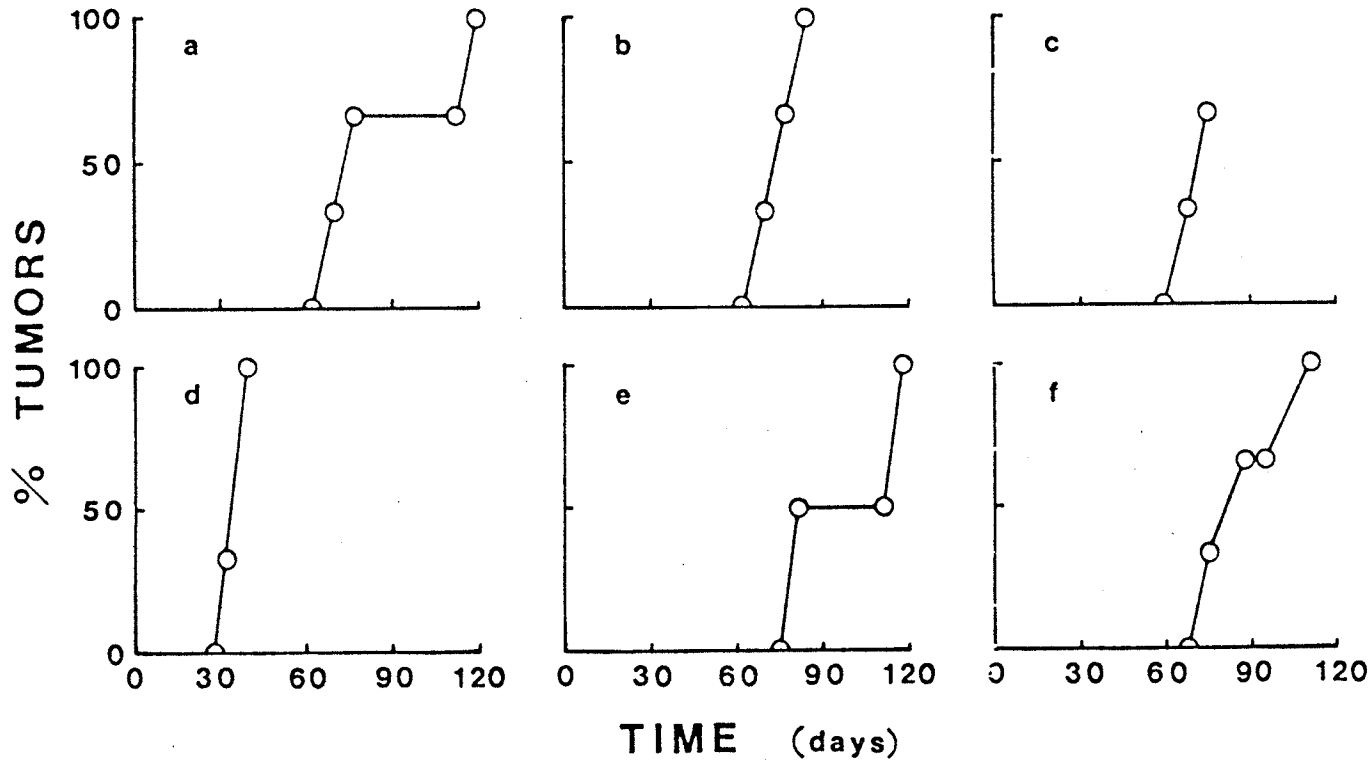
Studies on the tumor forming abilities of the transfectant clones have revealed that only L_6WT-5 T1-3, the stably drug-resistant isolate, exhibited decreased tumor latency (figure 46). Indeed, all 3 mice injected with L_6WT-5 T1-3 developed tumors within 39 days after implantation into BALB/c nu/nu mice. Two of these tumors had a measured tumor index greater than 8.5 cm^2 by the 81st day. In contrast, the other 3 transfectants developed tumors in a time frame similar to that of L_6WT/H^R-5. One of the 3 mice injected with L_6WT-5 T1-2 failed to develop a tumor during the entire time of the study (118 days). One mouse injected with L_6WT-5 T1-4 died shortly after injection, before developing a tumor.

L_6WT-5 T1-3 and T1-4 were examined for anchorage independent growth in sloppy agar. L_6WT-5 T1-4 exhibited a colony forming index of 0.020 (table 5), similar to that of L_6WT-5 (0.028). The hydroxyurea-resistant transfectant L_6WT-5 T1-3 exhibited a colony forming index in sloppy agar of 0.104, a value similar to that obtained with the L_6H^R variants.

Tumor Formation by Hamster and Mouse Cell Lines Sensitive and Resistant to Hydroxyurea

Several other cell lines selected by other researchers for resistance to hydroxyurea, were examined for changes in tumor forming ability, in a manner similar to that used with the L_6 and L_8 rat myoblast cell lines. The BALB/c 3T3 WT/H^R-1 cell lines were injected subcutaneously into the backs of BALB/c nu/nu mice. All animals injected with BALB/c 3T3 WT-1 developed tumors in 2-4 months whereas no tumors were seen in the animals injected with the corresponding hydroxy-

FIGURE 46: Tumor formation by L6 clone 5 myoblasts. Values for L6WT-5 and L_6H^R-5 are from figure 37 and are included for ease of comparison. Three mice were injected with $3x10^6$ cells of each clone for a total of 18 mice. Note that L_6WT-5 T1-3 is the only clone exhibiting substantially decreased tumor latency. Tumor formation was scored as shown in the legend to figure 37. (a) L_6WT-5, (b) L_6H^R-5, (c) L_6WT-5 T1-2, (d) L_6WT-5 T1-3, (e) L_6WT-5 T1-4 and (f) L_6WT-5 T5-1.


% TUMORS
100
50
0
a
b
c
d
e
f
0
30
60
90
120
TIME (days)

urea-resistant clone BALB/c 3T3 H^R-1 (figures 47 and 48). Similar results were seen in two independent experiments employing 3 mice per clone.

Two clones of Chinese hamster ovary cells, one selected for resistance to hydroxyurea (CHO HU^R-2), the other for resistance to N-carbamoyloxyurea (CHO NC^R-30A2), were compared for their tumor forming ability in nude mice, with reference to the parental wild type clone CHO WT C125. Each of these drug resistant clones has been shown to exhibit altered ribonucleotide reductase activity, though the statistical significance of these reductase activity changes has not been documented. No statistically significant differences in tumor latency were seen when these cell lines were injected into BALB/c nu/nu mice (figure 49). The first of two experiments employing 5 mice per clone suggested that differences might exist, but a subsequent experiment and an analysis of the acumulated data revealed no significant differences. Similarly, no significant differences were seen in the tumor growth rates (figure 50).

Work in other laboratories has indicated that Chinese hamster cells form tumors less rapidly in NIH nu/nu (Swiss) mice than that seen by this candidate in the BALB/c nu/nu mice. Ten mice of the former type were employed to investigate the possibility that differences in tumor formation by CHO WT C125 and CHO HU^R-2 might be apparent in a less permissive system. Five animals were injected with CHO WT C125 cells and five with CHO HU^R-2. Each mouse received 10^4 to 10^6 cells of either cell type. The results of this titering experiment are shown in figure 51. One NIH nu/nu mouse injected with CHO WT C125 died before developing a tumor (the mouse injected with $5x10^4$ cells). Each mouse injected with CHO HU^R-2 developed a tumor before its comparable

FIGURE 47: Tumor formation by BALB/c 3T3 wild type cells (○) and the hydroxyurea-resistant variants (●) selected from them. Two independent experiments were performed using 3×10^6 cells/100 l/animal, and 3 mice clone/experiment. One mouse injected with BALB/c 3T3 WT-1 died shortly after treatment and is not included in the values shown. Tumors were scored as shown in the legend to figure 37.

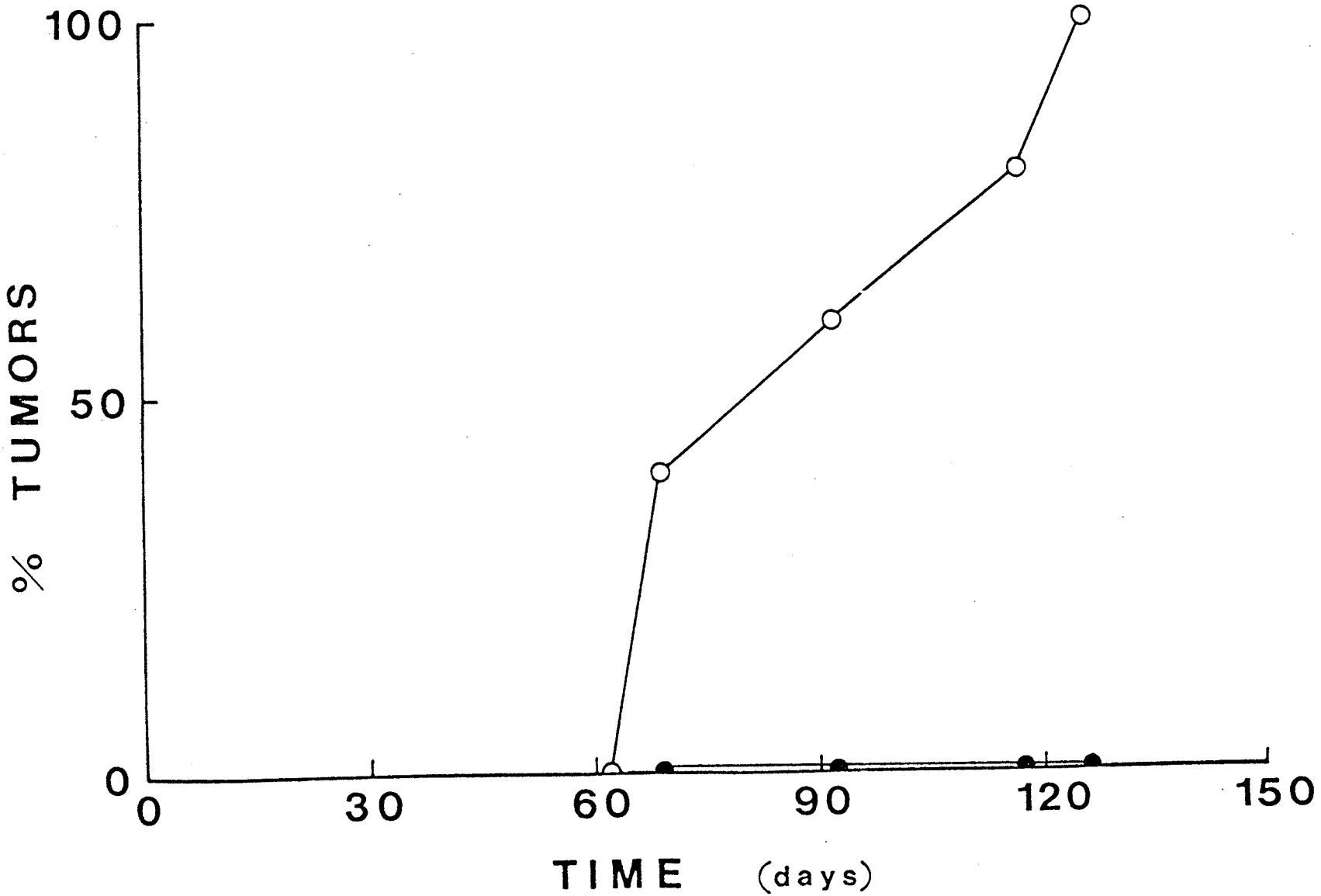

100
50
0
% TUMORS
0
30
60
90
120
150
TIME (days)

FIGURE 48: Photograph of a representative BALB/c 3T3 WT-1 tumor *in situ* (left) in a BALB/c *nu/nu* mouse and an aneoplastic mouse injected with BALB/c 3T3 H^R-1 (right). The mice were sacrificed and photographed five and a half months after treatment. Apart from mild cachexia, note the otherwise healthy appearance of the tumor-bearing animal. The tumors formed by all cell lines used in this study were typically non-invasive and could approach one third the total weight of the affected animal without disabling or severely imparing host function.


9
10
11
12
13
14
15
16
17
18
19
20
4
5
6
7
8
9

FIGURE 49: Tumor formation by Chinese hamster ovary cells in BALB/c nu/nu mice. Five nude mice were injected with $1x10^6$ cells each, for a total of fifteen mice. Two such independent experiments were performed. One mouse injected with CHO WT C125 did not form a tumor. Tumor formation was scored as shown in the legend to figure 37. ○ CHO WT C125, ■ CHO HU^R-2 and ● CHO NC^R-30A2.

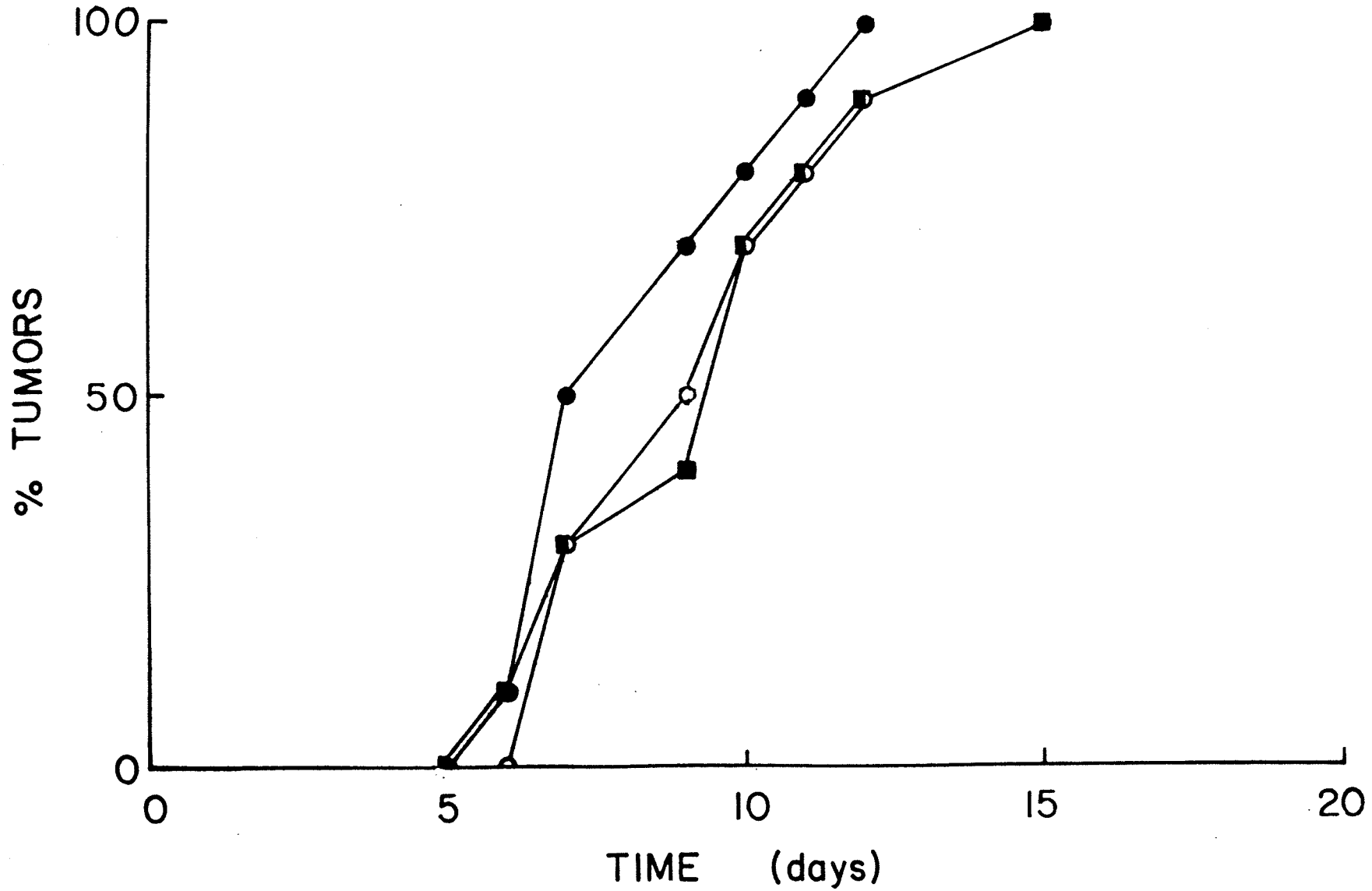

100
50
0
% TUMORS
0
5
10
15
20
TIME (days)

FIGURE 50: Growth rates of Chinese hamster ovary cell tumors in BALB/c nu/nu mice. Values are the averages of two independent experiments of five mice per clone, each injected with $1x10^6$ cells. Vertical bars represent the standard error in the mean values. ○ CHO WT C125, ■ CHO HU^R-2 and ● CHO NC^R-30A2.

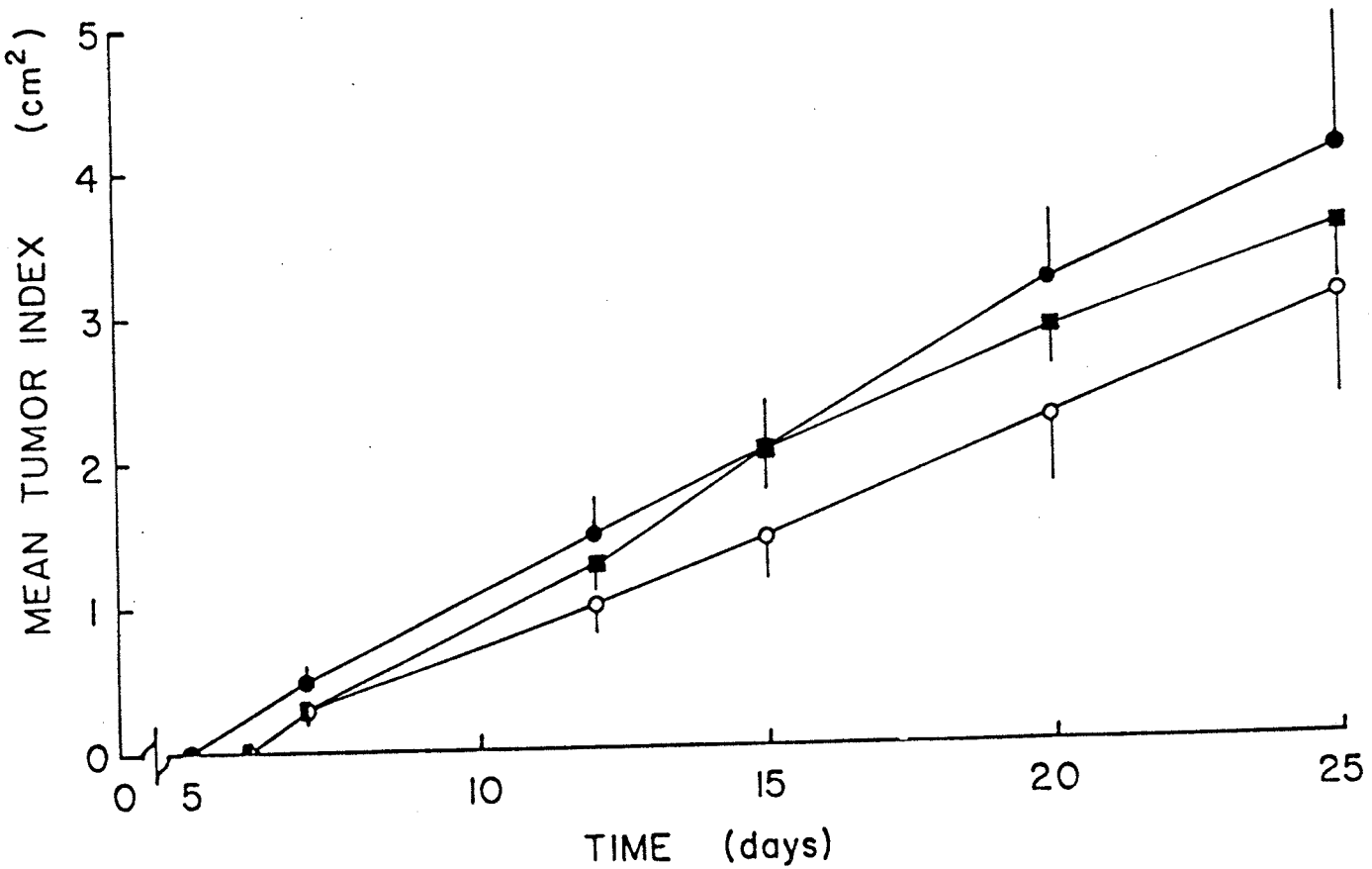
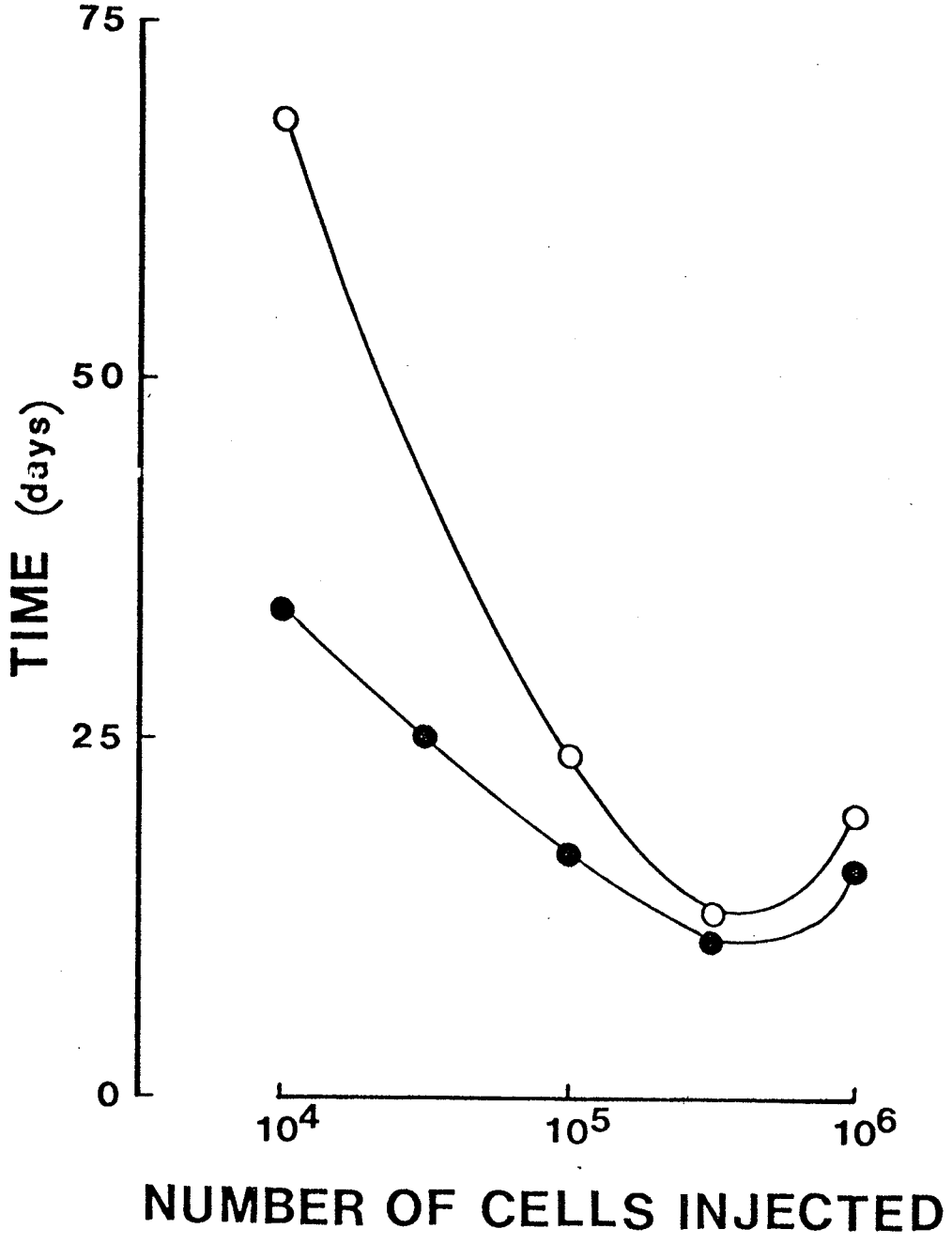

MEAN TUMOR INDEX (cm²)
0
1
2
3
4
5
0
5
10
15
20
25
TIME (days)

FIGURE 51: Tumor formation by Chinese hamster ovary cells in response to the number of cells injected into Swiss NIH nu/nu mice. Tumors were scored as shown in the legend in figure 37. Results are from a single experiment employing one animal for each determination. ○ CHO WT C125 and ● CHO HU^R-2.


75
50
25
0
TIME (days)
10^4
10^5
10^6
NUMBER OF CELLS INJECTED

partner injected with CHO WT C125 developed a tumor. While the tumor latency seen with mice injected with CHO HU^R-2 doubled when the number of cells injected was reduced from 10^5 to 10^4, the tumor latency nearly tripled in the mice injected with CHO WT C125 at the same cell numbers. The significance of this data is not readily apparent owing to the small number of mice employed in this experiment.

Several clones of mouse L cells, all with the same parental clone (L_1WT or $L_{60}WT$), and selected for resistance to hydroxyurea by a number of researchers, were also examined for differences in tumor formation in nude mice. Twenty-five BALB/c nu/nu mice were injected with either $L_{60}WT$ cells or one of the hydroxyurea-resistant variants selected from it: L_1H_2, L_2Cl_3, LHF^-, LHF^+. Five mice were employed for studies with each clone. All mice injected developed tumors and their latency times are plotted in figure 52. An unpaired Student's t-test of the mean tumor latency seen with the various cell lines indicated that the differences observed between $L_{60}WT$ and either of the two LHF populations (LHF^- and LHF^+; grown extensively in the absence or presence of hydroxyurea, respectively, prior to injection) were statistically significant ($p<0.025$), as are the differences between LHF^- and LHF^+. No significant difference existed between $L_{60}WT$ and L_1H_2 or L_2Cl_3 mean tumor latency. Similarly, a one-way analysis of the variation of the tumor indices recorded between days 14 and 23 indicated a statistically significant difference in the mean tumor growth rates of $L_{60}WT$ tumors and L_2Cl_3, LHF^- and LHF^+ ($p<0.05$), but not between $L_{60}WT$ and L_1H_2 (figure 53). Extensive statistical analysis beyond the 23rd day is not possible since the time of tumor measurements did not coincide between the cell lines after that day. Interestingly, the

FIGURE 52: Tumor formation by mouse L cells in BALB/c nu/nu mice. Five nude mice (for a total of 25 animals) were used for each clone and each was injected with $1x10^6$ cells. All mice injected formed tumors, scored as noted in the legend to figure 37. (a) $L_{60}WT$, (b) L_1H_2, (c) L_2Cl_3, (d) LHF^- and (e) LHF^+.

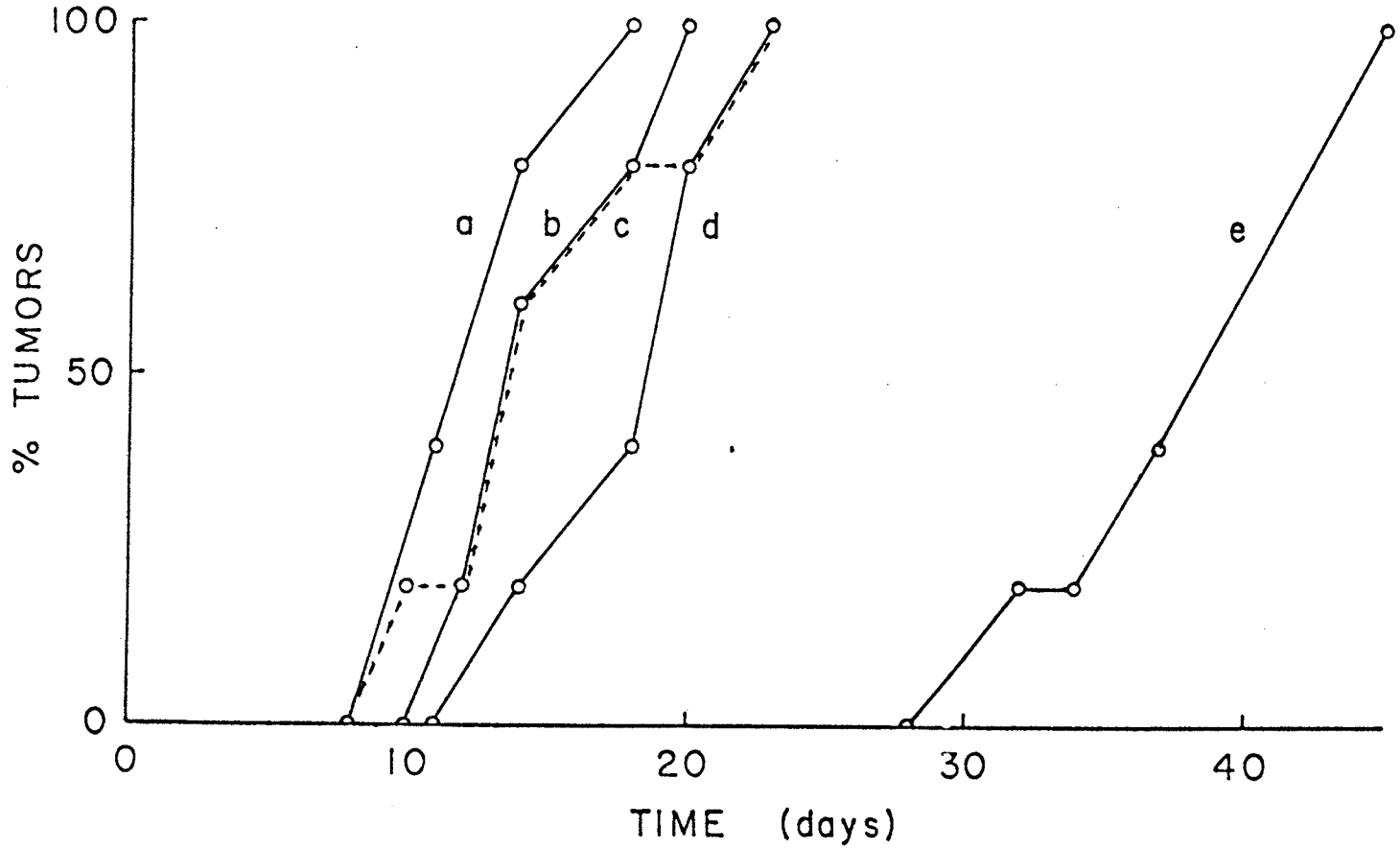
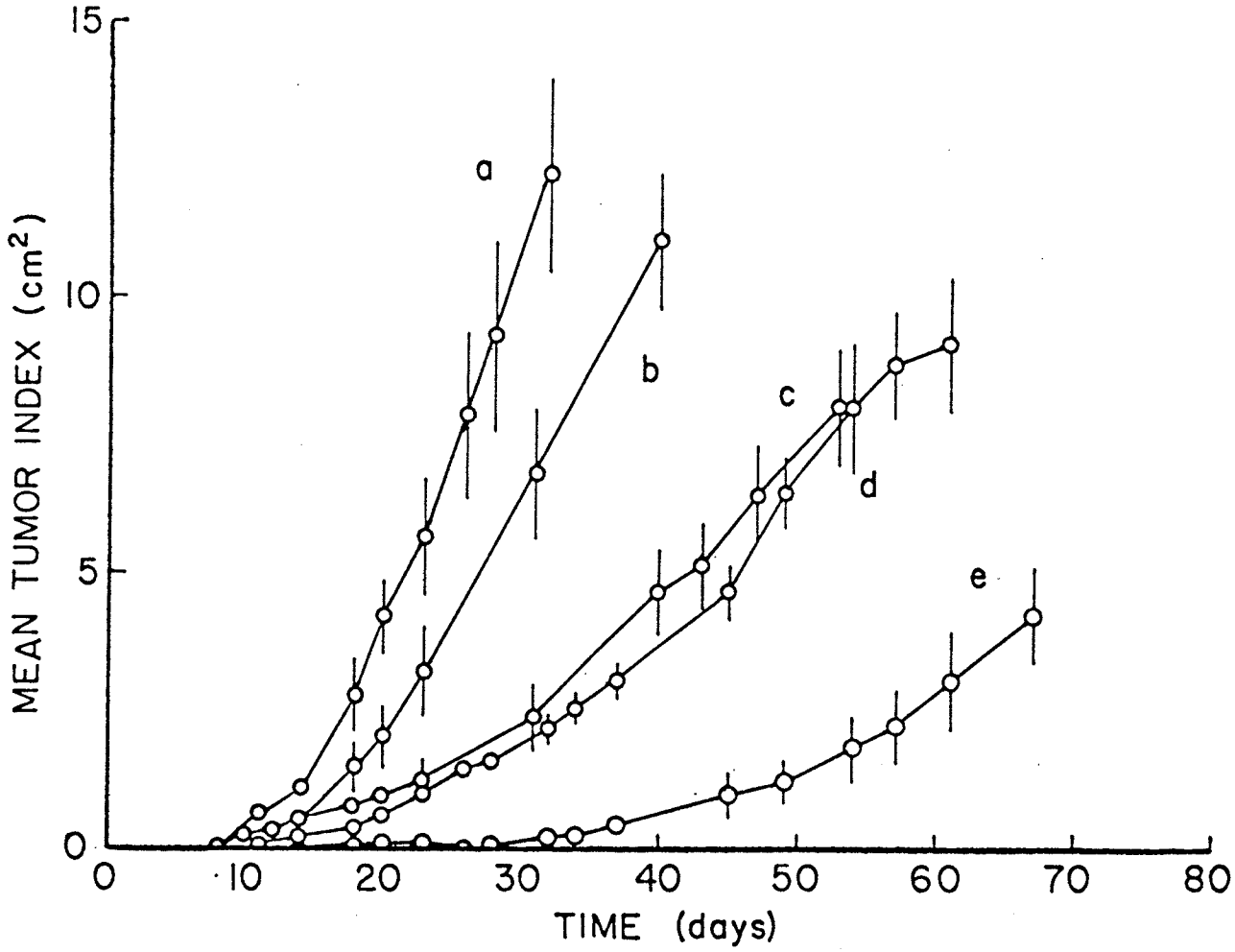

100
50
0
% TUMORS
a
b
c
d
e
0
10
20
30
40
TIME (days)

FIGURE 53: Growth of mouse L cell tumors in BALB/c nu/nu mice. Data is from the same set of mice as reported in figure 52. Vertical bars represent standard error for mean values. (a) L_{60}^{WT}, (b) L_1H_2, (c) L_2Cl_3, (d) LHF^- and (e) LHF^+


15
10
5
0
MEAN TUMOR INDEX (cm²)
0
10
20
30
40
50
60
70
80
TIME (days)
a
b
c
d
e

mouse L cell lines resistant to hydroxyurea exhibited substantially slower growth rates *in vitro* (figure 54), unlike the L_6 and L_8 myoblast cell lines. Indeed, the LHF^+ population grew nearly twiceas slow as $L_{60}WT$, when cultured in the presence of drug, but in the absence of hydroxyurea this cell line rapidly conformed to the growth rate seen with its counterpart (LHF^-) grown continuously in the absence of drug.

The mouse L cell lines have also been examined for anchorage independent growth in sloppy agar and for differences in hexose uptake. The parental clone $L_{60}WT$ grew very well in sloppy agar and exhibits a colony forming index of 0.800 (table 5). Without exception, as with the observed tumor latencies, the hydroxyurea-resistant mouse L cell clones exhibited a colony forming index in sloppy agar less than that of their parental clone $L_{60}WT$. Interestingly, LHF^+ exhibited the least ability to grow in sloppy agar. It should be noted that these agar studies were performed in the absence of hydroxyurea and thus the difference between the growth of LHF^- and LHF^+ (colony forming idices of 0.232 and 0.092, respectively) in sloppy agar is remarkably greater than the difference seen in their *in vitro* growth rates in liquid medium in the absence of drug (figure 54).

The hydroxyurea-resistant mouse L cell clones exhibited substantially reduced hexose uptake in comparison with $L_{60}WT$ (figure 55). The changes in the rates of hexose uptake by these cell lines were only apparent when their drastic differences in cell volume was taken into account.

FIGURE 54: Growth curves of mouse L cells under standard growth conditions at 37°C. (○) $L_{60}WT$, (●) L_1H_2, (■) L_2Cl_3 (□) LHF^-, (△) LHF^+ in the absence of hydroxyurea and (△) LHF^+ in the presence of 5 mM hydroxyurea. Values are the averages of duplicate determinations from a single experiment. The estimated generation times for the cell lines were: $L_{60}WT$, 15 h.; L_1H_2, 16 h; L_2Cl_3, 18 h; LHF^-, 19 h; LHF^+ (in the absence of drug), 21 h; LHF^+ (in the presence of drug), 30 h.

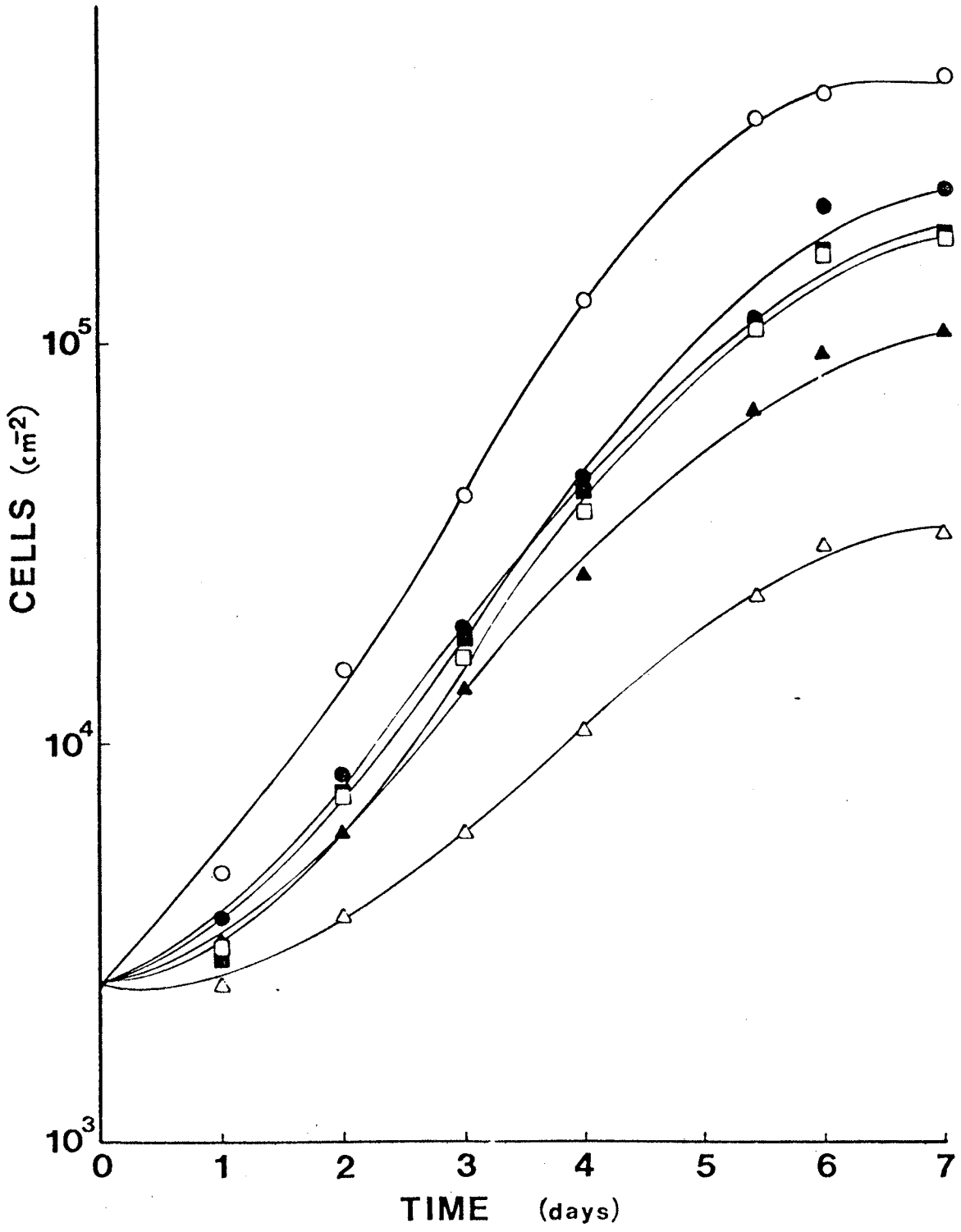
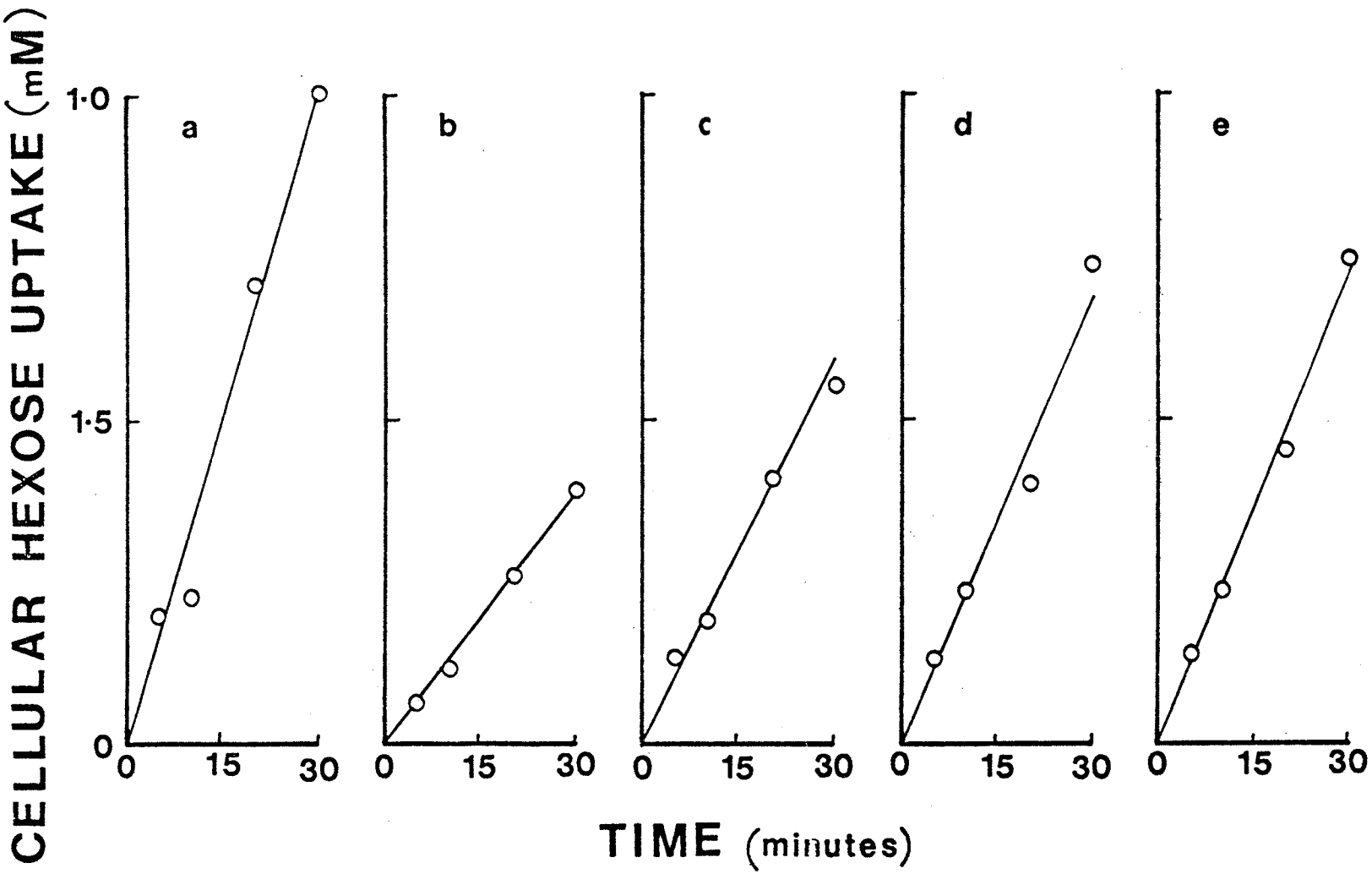

CELLS (cm⁻²)
10⁵
10⁴
10³
0
1
2
3
4
5
6
7
TIME (days)

FIGURE 55: Hexose uptake by mouse L cell lines sensitive and resistant to hydroxyurea. Values are corrected for cell volume differences and represent the averages of duplicate determinations from a single experiment. The cell volumes for $L_{60}WT$, L_1H_2 and L_2Cl_3 were noted in the legend to fig 14. The cell volume of LHF^- was 2.73±0.71 pl and that of LHF^+ was 4.15 ± 0.88 pl. (a) $L_{60}WT$, (b) L_1H_2, (c) L_2Cl_3, (d) LHF^+ and (e) LHF^-.


CELLULAR HEXOSE UPTAKE (mM)
1·0
1·5
0
a
b
c
d
e
0
15
30
TIME (minutes)

DISCUSSION

The elucidation of the cellular processes involved in the control of differentiation and transformation is germane to man's continuing efforts to improve the quality of human life and help alleviate the burdens borne by those individuals suffering from neoplasia and other diseases of differentiation. One of the most fruitful lines of research in this area of biology has been the recent identification of discrete DNA fragments (oncogenes) capable of transforming or accelerating the oncogenic properties of mammalian cells. Continuing work with these transforming sequences has begun to identify some of their functional properties in the cell. As outlined in the historical review section of this thesis, some transforming sequences have been found to code for proteins with nucleotide binding capabilities and/or tyrosine kinase activity and some have been identified with cellular growth factors. It has become increasingly clear that many of these transforming proteins are the abnormal counterparts of normal cellular proteins involved in growth regulation. The transformation-associated character of these proteins manifests itself either through actual mutation in their genes, leading to a structurally altered protein product or by the overexpression/misregulation of the amount of protein product present in the cell.

A growing body of evidence is implicating ribonucleotide reductase activity in aspects of the transformed state. The ubiquity of RRase in living systems, the rate-limiting nature of its activity in DNA synthesis, its propensity for affecting mutational rates even when modestly altered and its strong correlation with cellular proliferation, underscore the importance of this activity in living cells of every description. The re-patterning of gene expression observed in transformed cells (Weber 1983) manifests itself most notably through tremendous increases in RRase activity, obviating the protein as a key

target in cancer chemotherapy and suggesting that RRase is a progression-linked enzyme.

The interrelationship observed in many systems between cellular development and neoplastic transformation, together with the demonstrated involvement of ribonucleotide reductase in proliferation and the fidelity of DNA synthesis, suggested a somatic cell genetic approach to the elucidation of many of the transformation-associated properties of RRase and its importance in the phenotype of the cell. The isolation of independent somatic cell clones with mutations affecting differentiation and transformation was recognized as a practical approach to determine the transforming ability of altered RRase activity, since mutants with altered RRase activity are obtainable through selection with hydroxyurea, the agent used to inhibit RRase activity clinically. If, as predicted, the selection of cells resistant to hydroxyurea, by virtue of increased RRase activity, could repeatedly be shown to be concomitant with the acquisition of a more transformed, less differentiated phenotype by such cells, then correlations could be observed which could have clinical significance to the treatment of proliferative disorders and significantly advance the knowledge concerning the normal, functional properties of transforming proteins.

The L_6 and L_8 rat myoblast cell lines isolated by Yaffe are immortal and capable of terminal differentiation into syncytia, which express many properties observed in normal developing muscle tissue (Yaffe 1973). These cell lines, especially L_6, have been frequently used to examine questions in terminal differentiation and neoplastic transformation. In many respects, the L_6 myoblasts resemble the wild type NIH 3T3 cell line: immortalization, lack of tumorigenicity in hosts of parental origin (L_6 WT myoblasts fail to grow in Wistar rats,

and WT NIH 3T3 cells fail to grow in NIH mice), anchorage-dependent growth and aneuploidy. Clearly, these cell lines are transformed, yet neither is apparently tumorigenic. The acquisition of tumorigenicity has been correlated with the expression of active oncogenes in NIH 3T3 cells and the loss of differentiation-competence in L_6 myoblasts. Thus, each of these cell lines is amenable to the definition of conditions which promote the transformed state and may serve as appropriate models with which to examine the transforming abilities of ribonucleotide reductase.

Selection of Hydroxyurea-resistant Myoblasts and the Differentiation-defective Phenotype

Hydroxyurea is a useful selective agent for the isolation of cell lines with perturbations in RRase activity. Cellular resistance to hydroxyurea is strongly correlated with increased CDP reductase activity in many cell systems (Lewis and Srinivasan 1983, Wright 1983), especially when large increases are involved. Hydroxyurea, as noted in the historical review of this thesis, is also capable of transforming cells *in vitro* after brief exposure (Jones *et al* 1972, Chlopkiewicz and Kozioroski 1983). Thus, if correlations are to be made regarding the phenotypes exhibited by H^R cells and the acquisition of the H^R phenotype, the effects produced by simple exposure to the antineoplastic must be examined during the selection and characterization of such clones. A number of clones served in this regard (L_6WT-4, L_6H^R-6, L_6WT-5 T1-2, T1-4) and are discussed below in detail.

The biological properties of seven independent pairs of hydroxyurea sensitive and resistant myoblast cell lines have been investigated. In

addition, several apparently transfected L_6 myoblast cell lines have been isolated. Every hydroxyurea resistant myoblast clone isolated, without exception, possessed an apparently absent or reduced capacity for the developmental pathway expressed by its wild type clone. All hydroxyurea-resistant L_6 and L_8 rat myoblasts were selected from drug sensitive, wild type parental cell lines in a manner (sib selection) that permitted comparison of the resultant H^R clones with untreated, passage matched wild type clones from cryopreserved pools of each WT clone. The frequency with which H^R myoblasts were isolated ($5.0x10^{-7}$) approximates the minimum population size necessary, and the size of the WT clonal population used, for the successful isolation of H^R myoblast clones. Parental clones were used for selection at passage 1-3, which represent a minimum of $4x10^6$ cells produced from the original clone. The frequency of isolation of these variant cell lines corresponds well with the frequency of isolation of many somatic cell mutants selected for drug resistance in vitro (Parfett et al 1981, Thomson and Baker 1973). The apparent isolation frequency of 'transfectant' variants from L_6WT-5 cells treated with L_6H^R-5 DNA, as measured by the number of apparently H^R colonies noted before rescue from the selective pressure (hydroxyurea), was $0.6x10^{-4}$. Comparison with the apparent isolation frequency of $0.5x10^{-6}$ for spontaneously arising H^R variants, reveals an increase of two-orders of magnitude in the frequency of isolation when cells are treated with L_6H^R-5 DNA. This suggests that either the L_6H^R-5 DNA or another agent of the transfection procedure, mediated this increase in isolation frequency. All of the transfectant clones isolated exhibited either reduced or absent myogenic competence in a manner independent of stable resistance to hydroxyurea (see the Results of this thesis,

under the subheading: Studies with Myoblast Transfectants). This effect may be partly attributable to the transfection procedure, since sodium butyrate is known to reversibly inhibit the proliferation and differentiation of L_6 myoblasts (Leibovitch et al 1984). The pulse of sodium butyrate in the transfection protocol was used since it has been shown to increase the expression competence of cells transfected with DNA and to increase enhancer-dependent transcription (in some systems)(Gorman et al 1983). Recently however, sodium butyrate has been shown to inhibit the expression of transfected and microinjected cloned genes (Yuan et al 1985). The apparent discrepancy between the results of Gorman et al (1985) and Yuan et al (1985) has been recognized (by the latter authors) as probably the result of the manner in which the agent was used: pulse or permanent exposure. Since sodium butyrate inhibits cell proliferation (Wright 1973), DNA synthesis (Kawasaki et al 1981) and induces morphological changes and differentiation in a variety of mammalian cell types (Freshney 1985), it is conceivable that exposure of myoblasts to this agent may affect their apparent differentiation-competence for extended periods of time. Thus no correlation was observed between the H^R phenotype and defective differentiation in the 'transfectants', perhaps because of their exposure to butyrate.

Simple exposure of myoblasts to hydroxyurea during the sib-selections does not correlate with defective differentiation since L_6WT-4 exhibits typical WT parental properties, even though it is a subclone of the original WT clone used in the selection of L_6H^R-4, isolated as a rare survivor from the sib-selection itself. The culture history of this clonal pair thus permits a greater appreciation of the correlates of the H^R phenotype observed in the variant myoblasts.

The variant clone L_6H^R-3 exhibited a degree of myogenic competence which contrasted with the other highly drug resistant (2 fold or greater) myoblast variants. A similar pattern of defective differentiation was observed in a very low resistant (<1.5 fold) variant (L_6H^R-6). These observations indicated that acquisition of the H^R phenotype does not always completely prevent myogenic development. Further evidence of attenuation rather than abrogation of myogenic competence in H^R clones was provided by the observation of rare fusion elements in studies with L_6H^R-1. The apparently attenuated myogenic capacity of this clone was most evident in colony forming fusion assays, where 3% of the colonies formed were observed with evidence of myotube formation. The reason for the partial differentiation observed with L_6H^R-3 and L_6H^R-6 may involve the nature of the dNTP pool changes exhibited by these clones, since only these two cell lines were found to have slightly elevated but nearly balanced pools of the four deoxyribonucleoside triphosphates (figures 31 and 32). Interestingly, all H^R myoblasts isolated by sib-selection exhibited defective, or attenuated myogenic competence, whereas all the parental clones which were also selected without reference to their differentiation competence, exhibited a much higher level of competence as observed in assays of fusion kinetics and increases in creatine phosphokinase activity (figures 6,7,8a and 8b and table 1). Thus acquisition of the H^R phenotype appeared to be strongly correlated with defective or attenuated differentiation competence in the L_6 and L_8 rat myoblast cell lines.

Further evidence for the correlation between the H^R phenotype and attenuated myogenesis was provided by studies with a revertant population, L_6REV-1. The reversion of this clone to a less than 2 fold (from an almost 3 fold) increase in resistance to hydroxyurea appeared

concomitant with a reversion to near WT fusion competence (figures 43 and 44). This reversion of drug resistance and differentiation competence was apparently not the result of a change in the clone's ability to take up hydroxyurea intracellularly (figure 12), but was accompanied by a reversion to WT parental levels of CDP reductase activity, suggesting that the decrease in the activity of this enzyme was responsible for its reversion to a more drug sensitive state.

Resistance to hydroxyurea by the various cell lines studied herein was apparently not due to any significant difference in the ability of the drug resistant variants to accumulate the drug intracellularly. Every cell line described in this thesis was able to accumulate hydroxyurea to approximately the same extent as its parental clone within one hour of exposure to the drug. Furthermore, each cell line studied failed to concentrate the drug above the level present in the experimental environment and took up the drug in a manner not observably altered by extensive pretreatment with an energy inhibitor. Combined with the apparent insaturability of the rate of uptake of hydroxyurea observed with human and CHO cells, these observations strongly suggest that hydroxyurea uptake is a non-energy requiring process and are compatable with the simple diffusion mechanism of uptake. This interpretation of the results is consistent with observations of Evered and Selhi (1970) in studies with rat intestinal epithelia. Their report and the studies presented in this thesis are the only two such studies known to this doctoral candidate which have examined the uptake of hydroxyurea in mammalian cells and this is the first study to examine this phenomenon in normal human cells.

The cross resistance to guanazole exhibited by H^R myoblasts (figure 4 and text) and the ability of wild type, drug-resistant and revertant clones to take up guanazole in an equal manner (figure 10),

further suggest that an inability to take up a drug which inhibits RRase activity is not responsible for the drug resistant phenotype of these cells. These observations with guanazole further implicate an increase in RRase activity as the cause of cellular resistance to hydroxyurea, since guanazole resistance has been found to be associated with increased RRAse in other hydroxyurea-resistant rodent cell lines (Wright and Lewis 1974, Lewis and Wright 1974)) and is known to inhibit RRase activity (Brockman et al 1970).

The cross-resistance of L_6H^R-1 to the male antifertility drug gossypol provides the first suggestion known to this doctoral candidate of an enzymic intracellular target for this drug which might begin to explain its ability to inhibit DNA synthesis (Wang and Rao 1984), to inhibit Ehrlich ascites tumor cell proliferation (Tso 1984) and to attenuate spermatogenesis in human males (Soufir et al 1984, Lancet editorial 1984). Interestingly, gossypol is known to be very unstable in aqueous solutions or when exposed to white light and it has been suggested that these breakdown products may be responsible for the contraceptive effect of the drug (Yuan and Lee 1985). Some of the effects of gossypol include the ability to induce DNA strand breaks and sister chromatid exchange (Nordenskjold and Lambert 1984), but other evidence suggests that gossypol does not significantly increase chromosomal aberrations (Tsui, Creasy and Hulten 1983). Although gossypol appears to be relatively non-toxic in man its ability to cause irreversible azoospermia in humans and its capacity for genetic damage have contributed to the interest shown this agent by the World Health Organization in its global effort to define the mechanism of action of gossypol and other aspects of its effects on humans (World Health Organization 1982, 1983). The observations on gossypol noted in this

thesis contribute significantly to the further understanding of the mechanism of action of this drug and may be of clinical significance since prolonged human exposure could conceivably allow for the outgrowth of cells resistant to the drug by virtue of increased RRase activity, which may promote the neoplastic state and/or alter the state of differentiation in a manner similar to the H^R myoblast cell lines.

The increased survival of L_6H^R myoblasts in comparison to WT cells after brief treatment with hydrogen peroxide is the first observation of its kind concerning RRase and H_2O_2. The difference seen may reflect an increased antioxidant capability expressed by the variants as a result of increased intracellular GSH concentration, perhaps induced by the selection for cells with increased RRase activity. Depletion and enhancement in the levels of glutathione by buthionine sulfoximine and L-2-oxothiozolidine-4-carboxylate respectively, sensitizes or protects primary calf arterial endothelial cells from damage by H_2O_2 (Tsan et al 1985) and depletion of intracellular glutathione levels effectively sensitizes Chinese hamster cells to x-rays (Bump et al 1982, Russo et al 1984, Durrand 1984). The normal level of glutathione is not known to be rate-limiting for the activity of RRase in mammalian cells (Holmgren 1981) but increased levels may be observed in H^R cells with elevated RRase activity if the radical-forming abilities of hydroxyurea exert significant selective pressure on the treated cells during sib-selection for H^R. Further experimentation is necessary to resolve the mechanism underlying the apparent cross-resistance of H^R myoblasts to H_2O_2.

Attempts to induce myogenic development in the differentiation defective, H^R myoblast cell lines with various agents, including

hydroxyurea, retinoic acid, deoxyribonucleosides, 5-azacytidine and with various treatments, including prolonged cultivation in the absence or presence of low serum supplemented growth media (Creasey and Wright 1984a) has proven unsuccessful. The concomitant inability of hydroxyurea and deoxyribonucleosides, added to the cultures in combination or alone, to abrogate myogenic development in the differentiation competent, wild type myoblast cell line L_6WT-1 appears incongruent with the correlation between the H^R, differentiation-defective phenotype of the variant myoblast. The inability of exogenously supplied hydroxyurea to abolish myogenesis in WT cell lines may reflect its ability to arrest treated cells at or near the G_1/S-phase boundary of the cell cycle, well after the differentiation state of arrest observed in early to mid S phase in rodent cell lines (i.e. to G_0 state described by Scott *et al*, 1982 and Wille *et al*, 1982 for 3T3 T proadipocytes and Chen and Wang, 1984 for Syrian hamster cells). The importance of S-phase and its associated events in the process(es) of myogenesis, while underscored by the evidence presented in this thesis, remains controversial. The inability of exogenously applied deoxyribonucleosides to abolish myogenic competence may reflect in turn the complexity of the biology of nucleosides and nucleotides in cellular phenomena. The known compartmentation of deoxyribonucleotide pools in the nucleus and cytoplasm (Skoog and Bjursell 1974, Nicander and Reichard 1983, Leeds and Mathews 1985) and the relative activities of nucleoside metabolizing enzymes may be involved in their apparent lack of effect on myogenesis. More study is clearly required to fully understand the role (if any) of deoxyribonucleosides (or -tides) in myogenic development.

Ribonucleotide Reductase Activity and Nucleotide Pools

Every hydroxyurea-resistant myoblast cell line isolated and characterized in this study exhibited significantly increased CDP reductase activity in comparison with its specific WT parental clone. This difference was always manifested as an increase in RRase activity, except in the case of L_6H^R-6 which exhibited a statistically significant decrease in activity to about 80% of the parental level (figure 22). This decrease in activity is inconsistent with the strong correlation noted in many studies between the H^R phenotype and increased CDP reductase activity (Lewis and Srinivasan 1983, Wright 1983). Variants with decreased CDP reductase activity have been described in temperature sensitive (B1 subunit) E. coli (Fuchs et al 1972) and in thymidine-sensitive S49 mouse T-lymphoma cells (Roguska and Gudas 1984). In the latter study, the S49 mutant line exhibited only 10-30% of the CDP reductase activity and slightly higher ADP reductase activity than observed in WT cells. Accompanying the changes in activity were decreases in dNTP pools and a mutator phenotype. These authors have noted that the mutator phenotype was observed in these cells concomitant with balanced (although decreased) dNTP pools, unlike many of the other cell lines which have been described with a dNTP pool mediated mutator phenotype. That these types of changes might also have occurred in L_6H^R-6 is not completely known, but clearly it is possible that the pleiotropic changes seen in variants such as L_6H^R-6 could result from dNTP pool changes induced by perturbations in RRase activity or the activity of several other important enzymes involved in nucleotide metabolism. Small changes in RRase activity or resistance to hydroxyurea could be mediated by small dNTP pool changes

induced by the aberrant activity of an enzyme other than RRase. A rescue-selection technique such as that employed in the isolation of L_6H^R-6 may efficiently select for such very low-resistant variants.

Accompanying the increases in CDP reductase activity in the myoblast variants was a mean 1.5 fold increase in ADP reductase activity in the variants examined for this property. Clonally, this increase was only significant in one pair of cell lines, L_6WT/H^R-3. It was noted earlier in this discussion that L_6H^R-3, while quite resistant to hydroxyurea, is able to differentiate in culture to a level approximately 1/3 that of WT parental cells. The significant 1.8 ± 0.1 fold increase in ADP reductase activity by this particular clone and coordinate significant increase in CDP reductase activity is the only observation of its type in the cell lines studied in the present work and may reflect the balanced though elevated dNTP pool changes observed between L_6H^R-3 and L_6WT-3. The role of such changes in the differentiation defective phenotype of L_6H^R-3 is unknown, but conceivably could be similar to the example with the S49 mouse T-lymphoma mutants cited earlier (Roguska and Gudas 1984), where balanced changes in dNTP pools were observed with a complex cellular phenotype (i.e. increased sensitivity to tumicamysin and compactin and a mutator phenotype).

Kinetic analyses of the CDP reductase activities of several pairs of myoblast lines, sensitive and resistant to hydroxyurea, revealed no statistically significant changes in K_M values among the clones (figures 23,24 and 25). Similarly, no changes were observed in the relative sensitivities of parental and variant CDP or ADP reductase activities to inhibition by hydroxyurea nor did the variants display a significantly different pattern of dNTP change compared to parental cells, when grown in the presence of hydroxyurea. Apparently only the

levels of enzyme activity are altered in the myoblast variants.

The overall increase in the CDP reductase activities of the H^R myoblasts in comparison with their WT clones (1.9 fold) approximates the observed overall increase in dNTP pools (1.5-1.8 fold). The lack of significant changes in ribonucleotide pools (ADP, CDP, ATP, CTP, UTP, GTP), concomitant with significant changes in dNTP pools, which approximates the changes in RRase activity in these cell lines, substantiates the conclusion that the modest changes in RRase activity observed in these lines is real, that these changes are accompanied by modest changes in cellular dNTP pool sizes and that these changes probably account for the equally modest changes in hydroxyurea-resistance exhibited by the variant clones. The modest elevations and obvious imbalances in the dNTP pools seen in the more drug resistant and differentiation defective myoblast lines (L_6H^R-1,2,4,5, L_8H^R-1) are not unlike those seen in many mammalian cell lines documented as having increased rates of spontaneous mutation, (i.e. the FSthy-2Ts mouse FM3A cell mutant of Ayusawa et al (1983), the dGuo-L mouse S49 lymphosarcoma mutant cell line of Weinberg et al (1981) and Gjerset et al (1985) and the HPU^RB and HPU^RC mutant CHO cell lines of Arpaia et al (1983)). All of these cell lines, including the myoblast cell lines described in this thesis, have imbalanced dNTP pool changes of 3 fold or less in relative size. Since the hydroxyurea-resistant phenotype and the altered ribonucleotide reductase activity phenotype are both correlated with the mutator phenotype in a variety of cell systems (as outlined in the historical review of this thesis), it is very possible that the H^R myoblasts described in this thesis also have a mutator phenotype. This possibility is supported by the finding of pleiotropic changes in phenotype in these variant cell lines, including changes in differen-

tiation competence and neoplastic transformation. Nongenetic effects mediated by dNTP pool changes are equally possible and indeed both genetic and nongenetic perturbations may be mediated concomitantly by the nucleotide pool changes observed in the H^R myoblasts.

Interestingly, the unequal distribution of cellular constituents during cell division has been implicated in the variation observed in the generation times of individual cells in CHO cell populations (Kimmel et al 1984). These authors have demonstrated that cytokinesis can result in an unequal division of RNA to daughter cells and thus may provide a source of randomness in the cell cycle. They have noted that cellular constituents other than RNA could also be involved. This type of random event could be at least partly responsible for the normal distribution observed in the lengths of the G_1 phase in cycling mammalian cells (Hersh and Kitos 1980). Other nongenetic effects of dNTP pool imbalance could include changes in the activities or functional abilities of proteins which bind dNTP effectors, such as the ability of dATP or dCTP to substitute for ATP in the stimulation of the synthetic rate of calf thymus DNA polymerase (Wierowski et al 1983).

A further possible mechanism which may contribute to the defective-differentiation phenotype exhibited by the H^R myoblast cell lines would involve the unique role believed to be played by pyrimidine deoxynucleotides in DNA synthesis and cell cycle progression, as outlined in the historical review presented in this thesis. The apparent ability of treatment with hydroxyurea to preferentially select for cells with increased CDP reductase activity and increased cellular levels of dCTP, perhaps due to the ability of deoxycytidylate to act as a free-radical sink (as noted in the historical review presented in this thesis), may preferentialy select for cells with an increased propensity

for continued cell cycling if CdR actually is important as a signal for the progression of cells through G_1 to S phase. While such an effect would not necessarily lead to significant changes in generation times, it may be responsible for the apparent attenuation in the proportion of myoblast cells able to leave the log phase of growth for the state of arrest necessary for myogenic development in this system.

Studies on the Transforming Potential of Ribonucleotide Reductase Activity in H^R Myoblasts

The evidence presented in this thesis is consistent with the notion that ribonucleotide reductase is a progression-linked enzyme and suggests that rather than increases in the activities of this enzyme being only correlates of tumor progression, increases in ribonucleotide reductase activity may actually further the progression of the neoplastic phenotype in mammalian cells. A number of lines of evidence in this study are particularly important in this regard, including the isolation and characterization of a revertant clone and hydroxyurea-resistant 'transfectants' selected with hydroxyurea. In addition, the results with several independently-selected clones of myoblasts have established a correlation between the aquisition of the H^R phenotype and the apparently concomitant aquisition of accelerated tumorigenicity and increased anchorage-independent growth. These phenotypes have been found concomitantly with increased RRase activity, elevated, imbalanaced deoxyribonucleoside triphosphate pools and defective cellular differentiation.

Hydroxyurea-resistant myoblasts have been shown to exhibit a statistically significant increase in tumor growth rate and an equally

significant reduction in mean tumor latency in comparison to their specific drug-sensitive parental clones. A direct role for the increased RRase activity accompanying the hydroxyurea-resistance is implicated as the cause of this pleiotropic phenotype since a revertant clone (L_6REV-1) was isolated from a differentiation-defective H^R myoblast clone (L_6H^R-1), concomitant with a reduction in its two fold increase in hydroxyurea-resistance. Accompanying this drop in drug resistance was a drop in RRase activity to normal values and loss of the accelerated tumorigenicity exhibited by the original L_6H^R-1 cell line (figure 37a). Differentiation-competence returned to near normal levels in assays of myotube formation in this clone. The concomitant re-expression of these phenotypes by L_6REV-1 myoblasts demonstrates that acquisition of the individual characteristics making-up the variant phenotype is correlated with aquisition of hydroxyurea-resistance. Moreover, the reversibility of the many characterisitics comprising the H^R phenotype suggests that nonmutational changes mediate the pleiotropic phenotype contributed by H^R myoblasts. If each change observed in a H^R myoblast (i.e. reduced tumor latency, defective-fusion, increased anchorage independent growth) were to represent a mutation in a specific gene then the chance of isolating a revertant population with a similar number of conteracting mutations is extremely small. Furthermore, all the traits comprising the H^R phenotype were observed concomitantly. Any mutations(s) occurring in these cells must appear within the first few passages after cloning and isolation of the variant, since all phenotypic characteristics noted with the H^R myoblasts can be observed in early passage clones. Thus a reversion in the H^R phenotype may represent the reversion of many mutations if all such mutations were unusually unstable, but more likely represents the

reversion of a single mutation (H^R) which has the ability to affect a wide variety of other cellular characteristics. An alternative explanation may be that the demonstrated reversion is epigenetic in nature. Epigenetic changes in phenotype have been described which appear very stable. A third possibility is that an independent mutation has occurred whereby expression of the H^R phenotype is suppressed (i.e. a 'suppressor mutation'). Even this explanation however, implies that a common mechanism may be reversing the various characteristics, However, it may still be that a single, basic mechanism confers the pleiotropic phenotype upon H^R myoblasts.

The basic mechanism underlying the complex H^R phenotype observed *in vivo* is apparently independent of the differentiation competence of the specific clone. Both L_6 and L_8 WT myoblast cell lines were capable of growth *in vivo* if transplanted into BALB/c *nu/nu* mice, even though they exhibited myogenic competence in assays of cell fusion and creatine phosphokinase activity before and after passage through the animal host. In addition, the partly-fusing clones L_6H^R-1,3 and 6 and L_6WT-5 T1-3, T1-4 and T5-1 also formed tumors in nude mice. Kinetic studies of tumor formation by the myoblast cell lines have revealed that only H^R clones exhibited significant reduction in tumor latency. Differentiation-incompetent $conA^R$ cells isolated from one of the L_6WT parental clones by sib-selection (Parfett *et al* 1981) were found to belong to the same statistical population as differentiation-competent parental cells, when examined for changes in tumor latency, indicating that differentiation-incompetence *per se* is not always associated with decreased tumor latency. Further evidence in support of this observation is supplied by the nearly normal tumor latency exhibited by L_6WT-5 T1-2, a 'transfectant' clone. Apparently fusion-

incompetence is not correlated with accelerated tumorigenicity in all myoblast clones; only possession of the hydroxyurea-resistance and increased CDP reductase activity are strongly correlated with reduced tumor latency and increased tumor growth rate.

References to individual cell lines for the service of clarification of the interrelationships observed in the H^R phenotype offer useful insight into the potential significance of the differences in specific observations reported in this thesis, but such analyses should be interpreted carefully. The true significance of an observation can only be appreciated when the normal variability of that observation is addressed for the population being studied. Many independent myoblast clones must be examined in detail before significant correlations may be drawn between the various characteristics of each clone. Thus to single out any specific clone for the sake of example may lead to erroneous conclusions if many such examples cannot be presented from the collected observations. The example of L_6Cl_6V5, the $conA^R$ variant, was tested in such a way that a large enough number of mice were examined to appreciate the statistical identity of its mean tumor latency with the same population as that of the fusion competent parental clones.

The observation that L_6WT myoblasts are capable of growth *in vivo* is novel and may bear on the conclusions reported in studies by other investigators who have examined cellular differentiation and neoplastic transformation in these and other cell models. The report by Bignami *et al* (1982) in which fusion competent L_8 myoblasts were shown to be capable of growth *in vivo* if transplanted into neonatal Wistar rats (the strain of origin for L_6 and L_8 myoblasts), is equally unique in this regard. The reason for the contrasting results of this report and that of Bignami *et al* (1982) in comparison with the apparent nontumorigencity

of these cell lines as reported by other investigators (Kaufman and Parks 1977, Kaufman et al 1980, Lawrence and Coleman 1983, Hillion et al 1982) may reflect differences in the length of time that test animals were observed, the use of different stock cultures of myoblasts or the use of different test animals. Similar sites of transplantation were used in most of these studies (subcutaneous; dorsal). Furthermore, only Bignami's study has reported on the fate of L_8 cells transplanted into neonatal Wistar rates and only the present study has examined the growth of L_6 myoblasts in BALB/c nu/nu mice. Interestingly, Bignami et al (1982) reported that fusion competent and fusion incompetent L_8 myoblast clones isolated from colonies in agar exhibited no significant difference in tumor incidence or tumor latency. In fact, these tumors appeared slightly later than those of their non-agar derived, fusion competent L_8 parental cells. Only their Rous sarcoma virus infected L_8 cell line (RSV-L_8) exhibited reduced tumor latency (one-half that of L_8WT) in Wistar rats. These results are analogous to the observations reported in the present study with the L_6 and L_8 cell lines, where only H^R myoblasts exhibited reduced tumor latency and fusion competence which was apparently independent of this acceleration in tumorigenicity.

The enchanced state of neoplastic transformation observed with H^R myoblast cell lines was also manifested by increased anchorage-independent growth. This in vitro correlate of the transformed phenotype was found to be significantly increased in H^R myoblast cell lines in comparison with the more anchorage dependent growth of their respective parental clones. As outlined in the historical review presented in this thesis, many investigators have found that L_6 and L_8 WT cell lines grow poorly in sloppy agar in comparison to fusion-defective myoblast

cell lines (Kaufman and Parks 1977, 1980, Bignami et al 1982, Hillion et al 1982, Lawrence and Coleman 1983). Bignami et al (1982) however, have reported that their stock of L_8WT cells grows very well in sloppy agar and agarose. Their results are inconsistent with the findings presented in this study and with the results of others (Kaufman and Parks 1977, 1980). The reason for the discrepancy in their results is not readily apparent, but may involve genetic drift.

Since differentiation-defective L_6 and L_8 rat myoblasts universally appear to exhibit increased anchorage-independent growth in many studies it is difficult to interpret the role, if any, played by the drug-resistance and altered RRase activity components of the phenotype exhibited by the myoblast cell lines. However, the fact that L_6H^R-3 exhibits a large increase in its colony forming index in sloppy agar compared to its WT clone, whereas L_6H^R-6, which is also capable of 20-30% fusion in a manner similar to L_6H^R-3, exhibits no such difference in anchorage independent growth, implies that defective differentiation itself is not responsible for increased anchorage-independence. The only other differences noted between L_6H^R-3 and L_6H^R-6 are the relative resistance to hydroxyurea, the significant increase in the CDP and ADP reductase activities exhibited by L_6H^R-3 and tumor latency. Moreover, a conA resistant, fusion-incompetent clone selected from L_6WT-5 (and designated L_6Cl_2VI by Dr. C.L.J. Parfett) exhibited a plating efficiency in 0.3% agar of 0.006, similar to that of fusion-competent L_6 myoblasts (C.L.J. Parfett, Ph.D. thesis, U. of Manitoba 1982). These results seem to suggest that it may be the H^R phenotype, via an increase in RRase activity that may mediate the increase in anchorage-independent growth. This notion is further supported by the increase in anchorage-independent growth exhibited by L_6WT-5 TI-3 in comparison with a sensitive line L_6WT-5

T1-4, since both these cells were fusion-competent, but exhibited a significant reduction in the degree of differentiation-competence (<10% of media in myotubes under standard assay conditions).

Studies with Other H^R Rodent Cell Lines

The significant increases in tumor growth rate and anchorage-independent growth, coupled with the decreased tumor latency exhibited by H^R myoblast cell lines with increased RRase activity and altered dNTP pools prompted an examination for a similar uncoupling of growth and differentiation in other mammalian cell lines. Several interesting observations have been made regarding the transforming potential of the H^R phenotype in hamster and mouse cells which suggest that altered ribonucleotide reductase activity may mediate aspects of the transformed state.

A H^R BALB/c 3T3 clone was obtained, which was isolated from a WT adipogenic population. Like the L_6 and L_8 rat myoblasts, this cell line exhibited elevated RRase activity and an apparent decrease in differentiation competence in comparison to its WT, drug-sensitive parental population. Modest changes in dNTP pools were also noted and substantiate the single observation of increased CDP reductase activity. Unlike the L_6 and L_8 rat myoblasts selected for hydroxyurea resistance, BALB/c 3T3 H^R-1 exhibited no decrease in tumor latency. Indeed, it apparently was unable to grow *in vivo* in nude BALB/c mice unlike BALB/c 3T3 WT-1 cells (figure 47). No appreciable difference in anchorage-independent growth was noted between these two hamster cell populations, apart from a 2 fold reduction in colony forming index exhibited by BALB/c 3T3 H^R-1 growing in agarose.

The formation of tumors by the parental BALB/c 3T3 cells and the apparent abrogation of this ability in the H^R cells selected from them, appears anomalous since the H^R variant cells are not behaving in a manner similar to L_6H^R myoblasts and BALB/c 3T3 cells are reportedly nontumorigenic when transplanted into animals. Several important observations by other researchers bear on the results obtained with the 3T3 cells in this study. It has been noted in the literature that mouse and rat cells cultured *in vitro* often aquire the ability to grow *in vivo* (Yoakum *et al* 1985). Although the original line isolated by Aaronson and Todaro (1968a) was shown by these authors to be non-tumorigenic in syngenic animals (Aaronson and Todaro 1968b), failure to maintain BALB/c 3T3 cultures in exponential growth (i.e. allowing cultures to repeatedly reach confluence) has been associated with their spontaneous neoplastic transformation (Boone *et al* 1973). Furthermore, BALB/c 3T3 cells have been noted to be tumorigenic in syngeneic host animals if transplanted attached to a solid surface (Boone 1975). Since the full culture history details of BALB/c 3T3 WT-1 examined in this study are unavailable, further understanding of its tumorigenic phenotype is compromised by the available data. This cell system serves to demonstrate however, the simple aquisition of the H^R phenotype and the accompanying increase in CDP reductase activity does not itself initiate the tumorigenic state in all experimental cases. The correlation seen between hydroxyurea-resistance and altered adipogenic competence in BALB/c 3T3 H^R-1, apparently in support of the phenotype observed with the H^R myoblast cell lines, must also be interpreted with caution.

Several Chinese hamster ovary cell lines resistant to hydroxyurea (CHO Hu^R-2) or N-carbamoyloxyurea (CHO NC^R-30A2), with well characterizd alterations in ribonucleotide reductase activity, did not exhibit accelerated tumorigenicity when transplanted into BALB/c nude mice. No significant differences were observed in tumor incidence, latency or growth rates. CHO NC^R-30A2 exhibited an approximately 3 fold increase CDP reductase activity and about a 2 fold increase in ADP reductase activity in comparison to CHO WT cells (Hards and Wright 1983a). CHO NC^R-30A2 also exhibited an altered Ki for hydroxyurea, guanazole and N-carbamolyoxyurea inhibition. CHO HU^R-2 cells were not characterized for enzyme levels but did show altered hydroxyurea Ki values for CDP, ADP and GDP reduction (Lewis and Wright 1978). The decreased tumor latency exhibited by CHO HU^R-2 transplanted into NIH (Swiss) nude mice in comparison to CHO WT C125, especially when low numbers of cells are employed, is suggestive of the same effect exhibited by the H^R myoblasts. The observations with the NIH mice however, were not statistically significant.

The apparent inconsistency in the tumor phenotypes of H^R and H^S cells of L_6 and L_8 myoblasts in relation to the observations made with the Chinese hamster ovary cell lines may reflect the already highly tumorigenic phenotype of CHO WT C125 transplanted into BALB/c nude mice. The extremely short tumor latency exhibited by the WT cells may preclude an appreciable reduction in this period in a manner analogous to the H^R myoblast cell lines, if the minimum tumor latency for the system is nearly equal to that exhibited by the CHO WT cells.

The mouse L cell lines used in the present study have afforded many interesting observations concerning the role of ribonucleotide reductase activity in neoplastic progression. All of the hydroxyurea-resistant

mouse L cell lines (L_1H_2, L_2Cl_3 and LHF) exhibited an increase in mean tumor latency compared with their H^S parental cell line, $L_{60}WT$. The differences observed with LHF, whether cultured in the presence or absence of hydroxyurea just prior to transplantation, were statistically significant. These results are in marked contrast with similar studies employing hydroxyurea-resistant and sensitive L_6 and L_8 rat myoblast cell lines. The reason(s) for this discrepancy is not known, but a number of suggestions may be made based on other observations reported in this thesis and the culture history of these cell lines.

The H^R mouse L cell lines, unlike any of the other H^R cell lines reported in this study, exhibited substantially slower growth rates in vitro in comparison to $L_{60}Wt$ cells, when cultured as monolayers in liquid medium (figure 54). In a similar manner, the H^R mouse L cells exhibit a decreased ability for anchorage-independent growth when cultured in sloppy agar (table 5). The reduction in colony forming ability in agar observed with these clones parallels their decreased in vitro growth rates (i.e. L_{60} grows best in agar and is the fastest growing of the mouse lines in liquid culture and L_1H_2, L_2Cl_3, and LHF exhibit progressively decreasing colony forming ability in agar and progressively decreasing growth rates in liquid culture). Although a direct comparison of in vitro and in vivo growth rates of mammalian cells is not valid in most studies, the decreasing tumor growth rates and increasing tumor latencies exhibited by these H^R mouse L cell lines exactly parallels their differences in liquid culture growth rates in vitro and their anchorage-independent growth in sloppy agar. This suggests, but does not prove, that these cell lines possess a metabolic defect(s) affecting cellular growth that manifests

itself both *in vitro* and *in vivo* in a similar manner. Although other processes may be limiting the growth of these cells *in vivo*, which are not present in the *in vitro* situation, the striking parallelism between their *in vitro* and *in vivo* growth characteristics may truly reflect a comon mechanism for the defect(s).

Recent evidence gathered by Dr. D.A. Chow of the Manitoba Institute of Cell Biology suggests that one of the mouse L cell lines described in this thesis may have altered sensitivity to normal immune surveillance mechanisms. L_1H_2 was apparently more sensitive to natural resistance *in vivo* measured in an ^{131}I UdR labelled tumor elimination assay, than wild-type mouse L cells (Dr. D.A. Chow, personal communication). Nude mice are congenitally athymic and exhibit a deficiency in T-lymphocyte-related immunity but are still capable of producing immunoglobulins (Sharkey and Fogh 1984). The role of natural resistance in immunosurveillance and the mechanisms employed in this phenomenon have recently been reviewed (Greenberg *et al* 1983, Herberman 1984, Kimber and Moore 1985). Thus some of the reduction in apparent tumor growth rate by these H^R mouse L cells may have been due to an enhanced susceptability to component(s) of the natural immune system of the test animals. NK-cell reactivity *in vitro* and *in vivo*, employing a $conA^R$ CHO cell line, has recently been shown to be selectively enhanced over sensitivity to hypotonic lysis and cytotoxicity by human lymphokine-activated killer cells (Pohajdak, Wright and Greenberg 1984). These results suggested that a defect in oligosaccharide biosynthesis, which manifested as a change in membrane carbohydrate, was responsible for the enchanced NK-cell reactivity. The $conA^R$ CHO cell line exhibited a significantly *reduced* tumor growth rate and was markedly less tumorigenic in BALB/c *nu/nu* mice. Thus, one mechanism whereby the H^R mouse

L cells reported in this study may exhibit enhanced NK-cell reactivity may be through alteration in surface carbohydrate.

A further inconsistency observed between the H^R myoblast and mouse L cell lines involved the rates with which hexose uptake occurred in these cell. Consistent with the faster tumor growth rates of the H^R myoblasts, two cell lines taken as representative of H^R myoblasts (L_6H^R-5 and L_8H^R-1), exhibited increased rates of hexose uptake compared to their WT parental clones (figure 35). This common characteristic of enhanced cellular transformation was changed in the opposite direction in H^R mouse L cells (figure 55). The decreased hexose uptake exhibited by these cells is consistent with their observed decreased tumor latency and growth rates and also consistent with their decreased anchorage-independence and slower growth rates in vitro. A similar observation was not made with H^R myoblasts, where each H^R cell line was shown to exhibit an equal or decreased in vitro growth rate compared to its parental clone. Thus it appears that increased or decreased hexose uptake is correlated with increased or decreased tumor latency in these two cell systems (myoblast and mouse) but not necessarily with in vitro growth rate.

A further consideration regarding some of the contrasting results discussed herein, between H^R myoblasts and the other H^R rodent cell lines employed in this study is the culture history of the individual cell lines. The myoblast cell lines are well defined and each has its own clonally-independent parent population. All studies with these lines were performed with closely passage -matched WT and H^R pairs of myoblast clones routinely resuscitated from cryopreserved pools of early passage cells. All myoblast cell lines were maintained as subconfluent populations in culture and were not allowed to reach confluence except

in assays of differentiation. Old passage clones were discarded beyond 20 passages in vitro and routinely replaced. Most experiments were performed using cells within 10 passages of the isolation. In contrast, the other cell lines were originally isolated for other purposes (e. g. Wright 1983) in which maintaining a strict culture regime was not required. Therefore, these cell lines may have undergone a very large number of divisions from the time of isolation. Recloning of these populations does not guarantee that a population will be isolated with the characteristics of the original early passage clone. Cryptic changes and observable pleiotropy in the phenotype may contribute to the heterogeneity common in subclones of a cell line passaged extensively in vitro.

On the Interelations Between Hydroxyurea-Resistance, Increased Ribonucleotide Reductase Activity, Defective Cellular Differentiation and Neoplastic Progression: A Summary

The results of the present study demonstrate that resistance to hydroxyurea is strongly correlated with defective biochemical and morphological differentiation, increased CDP reductase activity, increased and imbalanced dNTP pools, increased anchorage independent growth, increased tumor growth rate and decreased tumor latency in the L_6 and L_8 rat myoblast cell lines of Yaffe (1968) and Richler and Yaffe (1970). The demonstration of a correlation between altered RRase activity and dNTP pools, drug-resistance and altered cellular differentiation has been previously published (Creasey and Wright 1984a)

and various aspects of this work have been presented elsewhere (Creasey, Parfett and Wright 1982, Creasey and Wright 1984b), including aspects of the study into the interrelationship between altered RRase and neoplastic transformation (Creasey and Wright 1984c).

Evidence has been presented which indicates that defective differentiation alone is not sufficient for the enhancement in the transformation-associated characteristics observed in the H^R myoblast cell lines. These observations, coupled with the independent manner in which these H^R variants were selected from clonal populations of WT cells and the isolation of a revertant subclone which exhibited an apparent reinstatement of WT characterisitics, suggest that increases in ribonucleotide reductase activity may enhance the degree of neoplastic transformation in some types of mammalian cells. This is the first demonstration that a defect in an enzymic activity with a known cellular function may be able to accelerate the transformed state of mammalian cells, to the best of this candidate's knowledge. This progression-enchancing alteration in enzyme activity can also apparently attenuate the commitment of mammalian cells towards a course of terminal differentiation.

Suggestions that ribonucleotide reductase may play an important role in neoplastic transformation have been published by several authors, as outlined in the historical review presented in the thesis. Elford et al (1970) and Takeda and Weber (1981) have provided correlative evidence in support of a role for ribonucleotide reductase in neoplastic transformation and Huszar and Bacchetti (1981) have provided evidence for a transformation-associated role for HSV ribonculeotide reductase. The results described in the present study substantiate and extend the observations and conclusions of these authors.

In addition, the results presented in this thesis are consistent with many of the studies presented earlier in the historical review and suggest many mechanisms whereby the H^R phenotype may enhance the transformed, less differentiated state of mammalian cells, while providing evidence against other possible mechanisms.

The enhanced tumor growth rate observed with H^R myoblasts expressing increased ribonucleotide reductase are consistent with the observation that this enzyme activity is the rate-limiting step of DNA synthesis and is strongly correlated with cellular proliferation. Cells which are already transformed and tumorigenic, such as the L_6 and L_8 rat myoblasts, may possess all the necessary requirements for enhanced growth but may be limited by the level of RRase activity they are able to express. Aquisition of increased RRase activity may therefore allow for an increased rate of growth in vivo. This effect may even be mediated through alterations in the association of the M_1 and M_2 components of the enzyme, which are known to be alterable by nucleotide effectors. The demonstrated pool imbalances reported for the myoblast variants would be capable of mediating this effect. Alterations in the subunit composition or activity of the enzyme may perturb the initiation of S phase if the assembly of the replitase complex actually plays a role in signalling this process. The special role that deoxycytidylate may play in the cell cycle is another possible mechanism whereby H^R myoblasts may be less able to arrest and differentiate. This concept has already been mentioned in the historical review presented in this thesis. The role of deoxycytidylate as an antioxidant and its ability to reverse G_1-arrest induced by oxygen depletion has also been mentioned in light of a mechanism for preferential increase in CDP reductase activity exhibited by H^R cells. A contributing factor in

this regard could be the speculated breakdown of hydroxyurea to hydroxylamine at the catalytic surface of RRase (Krakoff et al 1968).

The finding that H^R myoblasts are apparently less able to differentiate as readily as drug-sensitive WT myoblasts by leaving G_1 and entering a 'G_0' state suitable for myogenesis, coupled with the enhanced state of neoplastic transformation exhibited by these lines, is consistent with the observations of others which suggest that transformed cells are more difficult to block in the G_1-phase of the cell cycle and are more easily released from this state (Rumsby and Puck 1982). It should be noted however, that protraction of the length of time spent in G_1-phase by L_6 myoblasts is not sufficient in itself for myogenic development (Pinset and Whalen 1984) and that the requirement for DNA synthesis in the differentiation of this cell line remains an area of controversy, as described in the historical review presented in this thesis. The nature of the process(es) regulating cellular commitment to proliferation and differentiation are poorly understood but may involve the accumulation of specific cellular constituents, proteinacious or otherwise, necessary for progression from the G_1 to the S phase of the cell cycle (Pardee 1974, Rossow et al 1979, Svetina 1981). A number of highly labile proteins appearing late in the G_1 phase of the cell cycle have been implicated in this regard (Croy and Pardee 1983, Popolo and Alberghina 1984). One such candidate is cyclin, a nuclear protein whose enhanced synthesis is correlated with cellular proliferation (Celis et al 1984, Bravo et al 1985, Celis and Celis 1985). Recent studies however, have indicated that the synthesis of cyclin is not correlated with cell transformation nor is it coupled to DNA synthesis (Bravo and Graf 1985, Macdonald, Bravo and Bravo 1985), but may signal S-phase just prior to the synthesis of DNA. The results

of the present study as well as the results of others suggest that RRase or one of its components may also be capable of affecting the G_1/S-phase transition of mammalian cells, since the H^R myoblasts appear less able to leave G_1 towards a state of terminal differentiation and instead appear to have acquired a propensity for progressing through G_1 to S phase. Interestingly, Aizawa et al (1985) have recently characterized a strain of mouse teratocarcinoma cells which have a mutator phenotype by virtue of imbalanced dNTP pools (most notably a nearly 12 fold increase in the intracellular concentration of dCTP), but which retain their multipotency when injected into blastocysts. Thus, imbalanced dNTP pools and a mutator phenotype are not necessarily associated in a functional manner with defective-differentiation in mammalian cell lines.

The historical review presented at the beginning of this thesis outlined many observations regarding the biological effects of hydroxyurea that are relevant to aspects of the present study.

Thus it was very important to include internal controls in the experiments described in this thesis to monitor any effects of hydroxyurea itself, rather than resistance to hydroxyurea, that may affect changes in the phenotypic characteristics of the H^R variants. These internal controls have demonstrated that simple exposure to hydroxyurea was not correlated with defective-differentiation or tumor progression in the myoblast cell lines. The demonstration of a revertant population with near WT differentiation-competence, WT tumor latency and WT RRase activity suggests that the phenotypic changes observed in the variant lines were probably not caused by gross chromosomal aberrations or multiple mutations in the genetic material. Just as a single event is believed to have been responsible for the acquisition of hydroxyurea-

resistance, increased ribonucleotide reductase activity, defective cellular differentiation and a more progressed state of neoplastic transformation in the H^R variant lines, so is it believed that a single event was probably responsible for the reversion of these characteristics. The frequency with which the H^R phenotype was found is similar to the frequency with which somatic cell mutants have been isolated in vitro in many studies. These observations are consistent with a mutational mechanism for the acquisition of the altered myoblast phenotype. However, they are also consistent with an unilateral epigenetic change in expression that need not necessarily involve changes in the external environment. Changes in phenotype, even those associated with neoplastic transformation, are mediable by many epigenetic mechanisms, as outlined in the historical review (see "The Aberrant Differentiation Theory of Neoplastic Transformation"). Therefore, the evidence presented in this thesis is consistent with a single genetic or epigenetic event that is capable of concomitant alterations in RRase activity and progression of the transformed state. This event is selectable in vitro with hydroxyurea, suggesting that the single event in question is either exposure to hydroxyurea or aquisition of cellular resistance through increased CDP reductase activity. Each of these conditions (exposure and increased RRase activity) is known to be associated with aspects of tumor progression, as previously outlined. For reasons already given regarding hydroxyurea and the internal controls planned into the present study, it appears that acquisition of increased CDP reductase activity by mammalian cells can further the progression of neoplastic transformation and attenuate cellular differentiation in a more decisive manner than that previously indicated by the correlative studies of Elford et al (1979) and Takeda and Weber

(1981).

That an increase in RRase activity in cells selected for resistance to hydroxyurea may be capable of enhancing aspects of the transformed state is consistent with the demonstrations of others that cellular transformation is alterable (and inducible) by the expression of a normal cellular gene product at abnormal levels (Cooper et al 1980) and is associated with mutations that result in the overexpression of such normal cellular genes (Klein 1981). Numerous examples have been described in which altered or elevated protooncogene products have defined their cellular genes as oncogenic based on the ability of these gene products to coinitiate or enhance the neoplastic state. These types of disturbances in oncogene expression (as noted in the historical review) are mediable by both point mutation and epigenetic events and are transferable from one cell to another by DNA transfection techniques. The ability of oncogene products to induce neoplastic transformation in NIH 3T3 cells and various primary cells has been noted (see historical review). It may be however, that these oncogene products only enhance rather than initiate, the neoplastic state in NIH 3T3 cells since Greig et al (1985) have demonstrated the growth of wild type NIH 3T3 in vivo. These authors have demonstrated that BALB/c nu/nu mice present a permissive host environment for the formation of tumors by WT NIH 3T3 cells. Thus, this cell line does not appear to be nontumorigenic as previously reported. These authors have noted the use of different cell stocks, animal suppliers or experimental observation times alloted for the examination of tumor formation. Most interestingly, these authors noted that a transfectant cell line derived from NIH 3T3 containing an activated c-Ha-ras-1 oncogene, was also tumorigenic in BALB/c nu/nu mice but appeared to accelerate tumor formation as determined by a more than

two fold decrease in mean tumor latency. Cell transfected with ras reached a tumor diameter of 2-3 cm in 16-20 days whereas tumors formed by untransfected NIH 3T3 cells reached this size in 32-50 days. In addition, injection into the foot pads of the mice rather than S.C. in the back, revealed an apparent increase in the number of pulmonary metastasis, suggesting that transfection of an activated ras into mammalian cells can enhance tumor progression. The acceleration of tumorigenicity observed by these authors is very similar to the observed changes documented in this thesis for L_6 and L_8 rat myoblasts resistant to hydroxyurea. These observations indicate that increases in ribonucleotide reductase activity may mediate the same types of effects on neoplastic transformation as some of the known oncogenes and further suggest that acquisition of hydroxyurea-resistance is not simply a marker of neoplastic progression, but that the increases in ribonucleotide reductase activity may mediate aspects of each of these characteristics of tumor progression.

A strong correlative link between aberrant cellular differentiation and aspects of neoplastic transformation has been demonstrated by the results of the studies presented in this thesis. The establishment of a permissive test animal for the formation of tumors by L_6WT, differentiation-competent myoblasts, akin to the study of Greig et al (1985) with WT NIH 3T3 cells with some rodent host, in conjunction with the demonstrated ability of L_8WT myoblasts to also grow in vivo as demonstrated by Bignami et al (1982) and the results of the present report, indicate that differentiation and neoplastic transformation are not mutually exclusive and thus serves to clarify the relationship between differentiation and neoplastic transformation in these cell lines. Kaufman and Parks (1977) and Kaufman et al (1980) have examined

this relationship in L_8WT and variant, non-differentiating cells selected from the proliferative pool of cells remaining in differentiated cultures and have questioned whether the tumorigenicity of the non-fusing L_8 variants reflected absolute changes or changes in the degree of transformation. While their work, and the work of others (Hillion et al 1982, Lawrence and Coleman 1983) using L_6 myoblasts has suggested that transformation may be an alternative to myogenic development in these cell lines, evidence has now been presented which demonstrates that it is the degree of transformation that is altered in myoblasts selected from the proliferative pool by hydroxyurea and that a mechanism for this acceleration of tumorigenicity and decline in differentiation competence may be an increase in CDP reductase activity.

Suggestions for Further Experimentation

The relationships described between the acquisition of hydroxyurea-resistance, increased ribonucleotide reductase, defective cellular differentiation and the progression of the neoplastic state, including evidence which suggests that the mechanism of altered differentiation, transformation and drug-resistance is an increased ribonucleotide reductase activity in affected cells, suggest that experiments be performed to unequivocably define the importance of ribonucleotide reductase in these biological processes. The M_1 gene has been cloned recently (Caras et al 1985) and it may not be long before the M_2 gene is also reported to be cloned. This will inevitably allow for the widespread dissemination of these cloned sequences for use as molecular probes in many laboratories. When these probes become generally

available, more definitive experiments will be available to analyze this problem.

The most direct approach for examining the transforming potential of increased ribonucleotide reductase activity would involve the placement by transfection, of M_1 and M_2 genes into these cell lines (or other suitable models of differentiation and neoplastic transformation) in a manner such that their transfection would be selected for in a manner independent of their expression, which would be controllable by the manipulations of the experiments. Such an experiment might employ the contransfection of another selectable marker such that the enrichment of transfection-competent clones isolated by selection for the additional marker (Brown et al 1981) would allow for testing of sibling transfectants from cryopreserved populations of selected clones which had never been exposed to hydroxyurea. These siblings would be tested for hydroxyurea-resistance in the presence and absence of an externally controlled environment which would allow or disallow the expression of any M_1 and M_2 genes cotransfected into the competent clones. Resistant transfectants could then be examined directly for the influence of increased RRase activity on cellular differentiation and transformation.

Experiments should be conducted to examine the extent of the effect produced by elevated RRase activity, to show definitively that such an enzymic disturbance can ehance the transformed state. Defects in membrane structure or composition may be detectable if studies examining for altered sensitivity to lectin cytotoxicity or other membrane-active agents were performed. Resistance to conA has been correlated with differentiation incompetence, defective glycoprotein biosynthesis and altered surface glycoproteins in L_6 myoblast cell

lines (Parfett et al 1981, 1983). Similar observations have been made by other researchers (see references to table 1 in the historical review). Examination for these types of biochemical defects could contribute to the better understanding of the role played by such changes in aspects of differentiation, anchorage-independence and tumorigenicity. As noted earlier, $conA^R$ CHO cell lines, with well defined defects in glycoprotein metabolism (Wright et al 1979, Blaschuk et al 1980) and membrane properties (Ceri and Wright 1977) have been described and reviewed (Wright 1979). In addition, it was noted that such variants exhibit enhanced NK-cell reactivity (Pohajdak et al 1984). Experiments such as the ones proposed here would bear on the experimental and functional signficance of such alterations in cellular metabolism.

The definitive demonstration of a direct role for increased RRase activity in accelerated tumorigenesis and defective differentiation, as first suggested by the evidence of the present study, would obviate the search for a similar phenomenon in other cell systems, such as those used in the present study. Problems concerning culture history and phenotypic heterogeneity could be avoided by using cloned RRase genes in a controllable, expressable vector in cotransfection experiments with a second selectable marker, in the manner described above. With rigid conformity to strict culturing techniques employing the counting of cells at every passage as a measure of cell doublings, together with allowances for cell losses due to cryopreservation, subculturing and other environmental disturbances, these transfectant cell systems could be used to confirm and extend the understanding of the progressive nature of neoplastic transformation (Barrett and Ts'o 1978a, 1978b).

The role of imbalanced dNTP pools in the pleiotropic effect observed with elevated RRase activity could be investigated more fully as mediators of the oncogenic phenotype through detailed, repeated analyses of the counteracting effects that may be observable with certain combinations of exogenously supplied deoxyribonucleosides. As noted previously, reversions of the mutator phenotype, believed to be conferred upon cells by altered dNTP pool sizes, have been documented by several researchers (Gjirset et al 1985, Aizawa et al 1985). Experiments in which RRase activity and dNTP poolsare fluctuated in a controlled manner such as that described, would be useful in discerning the relative contribution of each perturbation (increased RRase compnent(s), or imbalanced dNTP pool(s)) to the progression of the neoplastic state.

Some of the suggestions presented in this thesis are based on the assumption that hydroxyurea itself has caused no serious effects on the phenotypic changes noted in the H^R variants. Since exposure to hydroxyurea has been documented as being associated with the induction of chromosomal aberrations, H^R cell lines to be examined for changes in differentiation and transformation should be screened for karyotypic abnormalities. Many cell systems are more amenable to such analysis than others and should be taken into consideration before extensive experiments are planned which involved exposure to hydroxyurea. The rat cell lines described in the present study are difficult to analyze in this regard owing to the large number of chromosomes possessed by them (L_6WT-3, 68; L_6H^R-3, 65; median number of chromosomes present in 30 metaphase spreads of each cell line) and their relatively small size. Meaningful cytogenetic analyses require an experienced observer expert in the karyotypes of the cell lines being examined.

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